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Gal Haspel · Anne C. Hart  
*Editors*



# *C. elegans*

Methods and Applications

*Third Edition*

 Humana Press

# METHODS IN MOLECULAR BIOLOGY

*Series Editor*

John M. Walker

School of Life and Medical Sciences

University of Hertfordshire

Hatfield, Hertfordshire, UK

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# *C. elegans*

## **Methods and Applications**

**Third Edition**

Edited by

**Gal Haspel**

*Department of Biological Sciences, New Jersey Institute of Technology, Newark, NJ, USA*

**Anne C. Hart**

*Department of Neuroscience, Brown University, Providence, RI, USA*

 **Humana Press**

*Editors*

Gal Haspel  
Department of Biological Sciences  
New Jersey Institute of Technology  
Newark, NJ, USA

Anne C. Hart  
Department of Neuroscience  
Brown University  
Providence, RI, USA

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## Preface

“Worms are not model animals; they are pioneer organisms and a discovery mechanism.” (paraphrasing Dr. M. Chalfie #Worm21)

*C. elegans*, and the community of researchers using this pioneer organism, have proven to be a discovery engine for the rules of life, from molecules and genes, through cellular physiology, metabolism and aging, neurobiology to behavior, individuality and interaction with the environment. This nematode was also used to pioneer methods that became common practice for diverse researchers, from fluorescent proteins and optogenetic tools, to genomic sequencing and editing, confocal microscopy, expansion microscopy, and 3D reconstruction of neurons, among many others.

We believe that the collegial spirit of the *C. elegans* community, explicitly envisioned and cultivated as the community expanded, has promoted these innovations and discoveries. We aim to abide by this spirit by editing this collection, which shares experience, knowledge, and perhaps wisdom.

The first two chapters provide practical advice for setting up research programs with *C. elegans*, either for a new research group or for a short-term project such as a teaching laboratory. The following chapters are written in a familiar format that, if successful, will resemble shadowing an experienced researcher as they run their favorite, well-honed experimental protocol, with a list of materials and equipment to prepare, step-by-step instructions, and useful notes. Some chapters from the last edition were updated and included here, while others that did not require updates can be found in that collection.

We thank our mentors, mentees, and collaborators for teaching us everything we know.

*Newark, NJ, USA*  
*Providence, RI, USA*

*Gal Haspel*  
*Anne C. Hart*

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## Contributors

- DIRK R. ALBRECHT • *Department of Biomedical Engineering, Worcester Polytechnic Institute, Worcester, MA, USA; Department of Biology and Biotechnology, Worcester Polytechnic Institute, Worcester, MA, USA*
- HEATHER L. BENNETT • *Department of Biology, Trinity College, Hartford, CT, USA*
- AMELIE BERGS • *Buchmann Institute for Molecular Life Sciences, Goethe University, Frankfurt, Germany; Institute of Biophysical Chemistry, Goethe University, Frankfurt, Germany*
- EDUARD BOKMAN • *Department of Genetics, Silberman Institute of Life Science, Edmond J. Safra Campus, The Hebrew University of Jerusalem, Jerusalem, Israel*
- EDWARD S. BOYDEN • *McGovern Institute for Brain Research and Koch Institute, MIT, Cambridge, MA, USA; Department of Biological Engineering, MIT, Cambridge, MA, USA; Department of Brain and Cognitive Sciences, MIT, Cambridge, MA, USA; Howard Hughes Medical Institute, Cambridge, MA, USA; Department of Media Arts and Sciences, MIT, Cambridge, MA, USA; K Lisa Yang Center for Bionics, and Center for Neurobiological Engineering, MIT, Cambridge, MA, USA*
- CHRISTIAN BRAENDLE • *Université Côte d'Azur, CNRS, Inserm, IBV, Nice, France*
- JOHN A. CALARCO • *Department of Cell and Systems Biology, University of Toronto, Toronto, ON, Canada*
- MATTHEW A. CHURGIN • *Department of Bioengineering, School of Engineering and Applied Science, University of Pennsylvania, Philadelphia, PA, USA*
- SONIA EL MOURIDI • *Biological and Environmental Science and Engineering Division (BESE), KAUST Environmental Epigenetics Program (KEEP), King Abdullah University of Science and Technology (KAUST), Thuwal, Saudi Arabia*
- YIFAT ELIEZER • *Department of Genetics, Silberman Institute of Life Science, Edmond J. Safra Campus, The Hebrew University of Jerusalem, Jerusalem, Israel*
- CHRISTOPHER FANG-YEN • *Department of Bioengineering, School of Engineering and Applied Science, University of Pennsylvania, Philadelphia, PA, USA; Department of Neuroscience, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA*
- STEVEN W. FLAVELL • *Department of Brain & Cognitive Sciences, Picower Institute for Learning & Memory, Massachusetts Institute of Technology, Cambridge, MA, USA*
- CHRISTIAN FRØKJÆR-JENSEN • *Biological and Environmental Science and Engineering Division (BESE), KAUST Environmental Epigenetics Program (KEEP), King Abdullah University of Science and Technology (KAUST), Thuwal, Saudi Arabia*
- CLOTILDE GIMOND • *Université Côte d'Azur, CNRS, Inserm, IBV, Nice, France*
- ELIZABETH E. GLATER • *Pomona College, Claremont, CA, USA*
- CASPAR GLOCK • *Buchmann Institute for Molecular Life Sciences, Goethe University, Frankfurt, Germany; Institute of Biophysical Chemistry, Goethe University, Frankfurt, Germany; Max-Planck-Institute for Brain Research, Frankfurt, Germany*
- ALEXANDER GOTTSCHALK • *Buchmann Institute for Molecular Life Sciences, Goethe University, Frankfurt, Germany; Institute of Biophysical Chemistry, Goethe University, Frankfurt, Germany*

- MARIA B. HARREGUY • *Federated Department of Biological Sciences, New Jersey Institute of Technology and Rutgers University, Newark, NJ, USA*
- HENRY H. HARRISON • *School of Molecular Biosciences, Washington State University, Pullman, WA, USA*
- ANNE C. HART • *Department of Neuroscience and Robert J. and Nancy D. Carney Institute for Brain Science, Brown University, Providence, RI, USA*
- GAL HASPEL • *Federated Department of Biological Sciences, New Jersey Institute of Technology and Rutgers University, Newark, NJ, USA*
- MICHAEL HENDRICKS • *Department of Biology, McGill University, Montreal, QC, Canada*
- THILO HENSS • *Buchmann Institute for Molecular Life Sciences, Goethe University, Frankfurt, Germany; Institute of Biophysical Chemistry, Goethe University, Frankfurt, Germany*
- KILEY J. HUGHES • *School of Biological Sciences, Illinois State University, Normal, IL, USA*
- MENACHEM KATZ • *Department of Biology, Technion-Israel Institute of Technology, Haifa, Israel*
- ROSS C. LAGOY • *Department of Biomedical Engineering, Worcester Polytechnic Institute, Worcester, MA, USA*
- ERIC LARSEN • *Department of Biomedical Engineering, Worcester Polytechnic Institute, Worcester, MA, USA*
- DAN LAWLER • *Department of Biomedical Engineering, Worcester Polytechnic Institute, Worcester, MA, USA*
- EREL LEVINE • *Department of Bioengineering, Northeastern University, Boston, MA, USA*
- CURTIS M. LOER • *Fletcher Jones Professor of Biology, Department of Biology, University of San Diego, San Diego, CA, USA*
- CHARLOTTE J. MARTIN • *Department of Cell and Systems Biology, University of Toronto, Toronto, ON, Canada*
- JATIN NAGPAL • *Buchmann Institute for Molecular Life Sciences, Goethe University, Frankfurt, Germany; Institute of Biophysical Chemistry, Goethe University, Frankfurt, Germany; APC Microbiome Ireland, University College Cork, Cork, Ireland*
- KEVIN F. O'CONNELL • *Laboratory of Biochemistry and Genetics, National Institute of Diabetes & Digestive and Kidney Diseases, Bethesda, MD, USA*
- DANIELLE M. OROZCO COSIO • *McGovern Institute for Brain Research and Koch Institute, MIT, Cambridge, MA, USA; Department of Brain and Cognitive Sciences, MIT, Cambridge, MA, USA*
- NAVIN POKALA • *Department of Biological and Chemical Sciences, Theobald Science Center, New York Institute of Technology, Old Westbury, NY, USA*
- NAUSICAA POULLET • *Université Côte d'Azur, CNRS, Inserm, IBV, Nice, France; URZ, INRAE, Petit-Bourg (Guadeloupe), France*
- SREEPARNA PRADHAN • *Picower Institute for Learning and Memory, Massachusetts Institute of Technology, Cambridge, MA, USA*
- AYUSH RANAWADE • *Department of Bioengineering, Northeastern University, Boston, MA, USA*
- HAROLD E. SMITH • *National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, USA*
- TRACY S. TRAN • *Federated Department of Biological Sciences, New Jersey Institute of Technology and Rutgers University, Newark, NJ, USA*

- NICHOLAS F. TROJANOWSKI • *Department of Bioengineering, School of Engineering and Applied Science, University of Pennsylvania, Philadelphia, PA, USA; Department of Neuroscience, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA; Department of Biology, Brandeis University, Waltham, MA, USA*
- ANDRÉS G. VIDAL-GADEA • *School of Biological Sciences, Illinois State University, Normal, IL, USA*
- JENNIFER L. WATTS • *School of Molecular Biosciences, Washington State University, Pullman, WA, USA*
- HAMILTON WHITE • *Department of Biomedical Engineering, Worcester Polytechnic Institute, Worcester, MA, USA; Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA, USA*
- CHIH-CHIEH (JAY) YU • *McGovern Institute for Brain Research and Koch Institute, MIT, Cambridge, MA, USA; Department of Biological Engineering, MIT, Cambridge, MA, USA*
- ALON ZASLAVER • *Department of Genetics, Silberman Institute of Life Science, Edmond J. Safra Campus, The Hebrew University of Jerusalem, Jerusalem, Israel*



# Chapter 1

## Starting a *C. elegans* Research Laboratory: Practical Advice

Anne C. Hart and Heather L. Bennett

### Abstract

This chapter provides practical guidance for scientists starting or reorganizing a *C. elegans* research group. This includes advice on joining the *C. elegans* community, on setting up the laboratory for *C. elegans* work, and on putting into place effective strategies for running a productive and inclusive research group. Also discussed are strategies for managing the group, standard practices in the *C. elegans* field, lists of resources, and several sample handouts for new research group members.

**Key words** Setting up, Organization, Management, Mentoring

---

## 1 Introduction

### 1.1 Who we Are Writing this for

This chapter is focused on providing advice to people setting up a *C. elegans* lab for the first time. Admittedly, most readers of this book have worked in a *C. elegans* laboratory or have related experience. But, we aim to also provide advice and information that is helpful for those who are new to *C. elegans*. Our approach is to write this chapter as if we were your mentors, based on what we wish we had known. Advice in this chapter is not prescriptive. There are many paths to success in a research career and other scientists would provide additional or alternative invaluable advice. We also believe that the advice we provide herein will be helpful to those working on other *Caenorhabditis* or other nematode species.

### 1.2 We Hope to Provide

This chapter is divided into several sections. The initial sections are very practical and discuss established norms or groups in the *C. elegans* community. Later sections of this chapter are more reflective of our personal experience in establishing *C. elegans* research groups. This includes reflections, advice, and information that we consider useful for on-boarding personnel to your *C. elegans* research groups. Finally, we provide thoughts about life management for the head of a *C. elegans* research group.

Admittedly, this last section is not specific to *C. elegans*, but it does contain some nuggets of information that we wish we had considered in the early days of running a research group.

### **1.3 We Are Not Providing**

This chapter does not contain equipment lists, recipes, or experimental protocols; these may differ between labs or these are available elsewhere (including in other chapters of this book). Also, in general, we are not providing generic advice that is applicable to any scientist starting their research career. There are other more comprehensive sources of information in that genera (e.g. “At the Helm”, online resources). Finally, we are not providing a comprehensive list of the numerous resources available for *C. elegans* research. These are constantly increasing and discussed widely within the community.

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## **2 Joining the Community**

### **2.1 Spend Time in an Existing *C. elegans* Lab**

You may have worked within an established *C. elegans* research group for years, but there is still enormous utility to spending a week embedded in a different *C. elegans* group. At a minimum, you will learn alternative strategies for organizing a laboratory, and new ways to approach problems. No one person or research group can be expert on all of the techniques and approaches used in *C. elegans* research. Interacting closely with another group of *C. elegans* researchers will expand your knowledge of the myriad ways our community uses this versatile experimental system. Additionally, visiting another *C. elegans* group provides you with additional people to consult in your journey to establishing your own research group. If you haven’t worked previously in an established *C. elegans* research group, then joining one for a few weeks will dramatically accelerate your picking up techniques and practical tips that might not be obvious from reading the literature. You do not need to travel far to find new ideas, but you should likely visit a research group that has a different “*C. elegans* lineage” (i.e. not run by a previous lab mate of your thesis or post-doctoral advisor). The group you visit will also benefit from this cross-pollination of ideas and protocols; it’s a win-win.

### **2.2 Community Resources and Organizations You Should Know About**

*Caenorhabditis elegans* Genetics Consortium (CGC, [cgc.umn.edu](http://cgc.umn.edu)) is an online repository where researchers can archive and purchase *C. elegans* strains, a small set of useful bacterial strains, and published transgenic lines. Additional *C. elegans* knock-out and deletion strains are available through the Japanese Knockout Consortium (<https://shigen.nig.ac.jp/c.elegans/>). You must register your research group with the CGC, WormBase, or Japanese Knockout Consortium to purchase available strains. More details on obtaining strains are provided below. Be sure to acknowledge

CGC, any lab from which you received strains or reagents from, as well as other resources when you publish your results. Instructions for acknowledgment are available on their websites.

When you establish a new research group, you should email the CGC to obtain unique lab codes for naming reagents and strains. These lab codes are essential as they provide a unique identifier for each strain, plasmid, transgene, and allele; these are usually required for publication in most peer-reviewed journals.

WormBase (<https://wormbase.org/>) is currently the primary online database for *C. elegans* genomics, genetics, publications, correct nomenclature, researchers, and many other topics. The database also includes the latest information on upcoming meetings, updates on gene names, and news within the worm community. WormBase is working hand-in-hand with the Alliance of Genome Resources, whose online database provides some of the same information, including cross-species orthology and information from other species. These sites are extremely useful to you and the community.

Each *C. elegans* researcher has a unique identifier and history page at WormBase. This is a key source of information for finding other *C. elegans* researchers and is used by journal editors, WormBase, and trainees looking for mentorship opportunities (e.g. students considering post-doctoral research positions). Be sure to keep your contact information up-to-date (email address, website, ORCID ([orcid.org/](http://orcid.org/))).

WormBook ([wormbook.org](http://wormbook.org)) is an open access, online book that provides detailed information on the biology of *C. elegans* and other nematodes. Within WormBook is WormMethods, which contains detailed protocols and techniques, as well as essential strategies needed for maintaining *C. elegans* and undertaking a wide range of studies.

WormAtlas ([wormatlas.org](http://wormatlas.org)) is an online database that provides additional information on *C. elegans* and nematode structural anatomy, neuronal circuits, and some behaviors.

WormBoard is an elected group of representatives from different constituencies within the *C. elegans* community. WormBoard members work together with the Genetics Society of America and meeting organizers to coordinate large-scale *C. elegans* meetings. Additionally, the board discusses initiatives to promote scientific advancement, techniques, and researchers within the *C. elegans* community.

## 2.3 Meetings and Conferences

Why are *C. elegans* scientific meetings important? Networking and development of collaborations are critical for accelerating your scientific career. These meetings help raise your profile in the scientific community and help you stay up-to-date with the newest innovations in the field. *C. elegans* meetings are complementary to meetings you will attend in your larger scientific field of research.

Within the *C. elegans* scientific community, there are several types of meetings; these range from topical meetings to large conferences. Below we provide a list of the larger conferences and examples of local meetings.

The International *C. elegans* meeting (IWM) usually takes place every 2 years. This meeting highlights cutting edge research in the *C. elegans* community. The meeting is diverse in topic and attracts researchers from around the world. Traditionally, most of the oral presentations are made by graduate students, post-doctoral fellows, and very early stage faculty. And, these talks and posters predominantly report unpublished results. Posters sessions are a key opportunity for students and fellows to practice presentation and to get feedback from the community. Indeed, poster sessions at *C. elegans* meetings are enormously popular and extremely well-attended, as an impressive depth and breadth of information is available.

There are also smaller *C. elegans* meetings, which are either topical or regional. These meetings are usually biannual, and held in years lacking an IWM. Usually, the community has several topical meetings. Currently, these include the Development, Cell Biology and Gene Expression meeting; the Neuronal Development, Synaptic Function, and Behavior meeting; the Aging, Metabolism, Stress, Pathogenesis and Small RNAs Meeting, and the virtual Evolution Meeting. Additionally, there are usually large regional meetings that focus on *C. elegans* research. These include the European Worm Meeting, the Asia-Pacific Meeting, the Latin American Worm Meeting, the Indian *C. elegans* meeting, and others that will likely form as our community expands.

There are many less formal and more local *C. elegans* meetings. Examples of this include the New York Area Worm Meetings (NYAWM) and Boston Area Worm Meeting (BAWM). These meetings typically occur once a month in the academic year and research in progress is presented. These meetings allow researchers to keep up with the latest techniques and methods. And, these meetings are an excellent environment for trainees to learn how information is communicated within the field, as well as a place to practice communicating science. If your local area doesn't already have a regular joint research meeting for *C. elegans* labs, suggest setting one up!

Student-focused conferences or meetings are also valuable, even when they do not highlight *C. elegans* research. SACNAS, Leadership Alliance, and ABRCMS are excellent examples of large meetings that are designed for students, with a particular emphasis on students from historically under-represented groups. And, there are many regional student-focused meetings that offer graduate students and/or undergraduates the opportunity to present their research. These meetings are excellent opportunities for students to join supportive scientific sub-communities, practice

communication of their scientific results, and to learn more about graduate schools and careers.

## 2.4 Communication

Beyond in-person meetings, Twitter, Slack, and Facebook are common platforms used by scientists to communicate and network. There are *C. elegans* specific social media groups on these platforms that are great for connecting with other *C. elegans* researchers, finding potential collaborators, highlighting the scientific interests or accomplishments of your research group, as well as promoting specific opportunities or positions available in your group. There are also smaller, topically-focused groups within the *C. elegans* online community (e.g. *C. elegans* at primarily undergraduate institutions). We suggest that you reach out and join groups that match your interests.

## 2.5 Connecting with your Local *C. elegans* Community

When you arrive at your new institution, we recommend initiating one-on-one contact with the heads of other *C. elegans* research groups at your new institution and in other institutions in the immediate area. Many of these people won't know that you are joining their local community. Offer to visit their research group for a few hours and offer to give a presentation about your work to their group or their department. Local *C. elegans* labs are helpful to each other as strain and reagent resources, as well as possible sources for equipment advice or technical expertise. Figure out what your overlapping and complementary areas of strengths and knowledge, which will help you determine how best you can help each other. Your new *C. elegans* neighbors may want to collaborate or coordinate with you, but this can't happen if they don't know that you have arrived.

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## 3 Setting up your *C. elegans* Lab

### 3.1 Organizing

Before beginning your position, develop your draft budget. This should include everything: lab personnel (salary, benefits, tuition, and healthcare), equipment, reagents (consumables and non-consumables), and travel (conferences, collaborations). We suggest that you ask *C. elegans* faculty who established their laboratories in the last few years for their advice. These individuals have recently gone through the same set up process that you are embarking upon. You should be able to get detailed specific advice on what they purchased. Buying microscopes, microinjection set ups, heating or cooling incubators, plate pouring equipment and supplies, as well as materials to store frozen *C. elegans* strains means that you are buying expensive specialized items. Get advice on these decisions from people who have recently worked this out for themselves. They will have advice on what equipment you really need to buy new versus which items you might borrow or buy used. And, they can also give you information about costs and how to best negotiate a good deal from vendors.

In addition, ask established faculty inside and outside of your institution for tips and advice. Mentors at your new institution will certainly provide good advice on general lab set up issues, even if they can't help with *C. elegans* specific issues. Each institution has a unique approach in how they handle justification of purchases, purchase orders, invoices, direct/indirect costs, student costs, moving, and reimbursements. Also, have a discussion with your new department about shared equipment and resources. Don't assume; ask questions early.

### **3.2 Equipment**

The minimal equipment specific to a *C. elegans* laboratory include the following: dissection microscopes with reflected visible light trans-illumination (from underneath), at least one with fluorescent illumination, incubators at various temperatures, a refrigerator ( $4^{\circ}$ ), and a freezer ( $-20^{\circ}$ ), ideally connected to emergency power outlets, access to a  $-70^{\circ}$  freezer, liquid nitrogen storage or both, an autoclave, and a plate pourer (ideally automated). You will likely need a simple camera to be mounted on a dissecting microscope; this can be complemented with simple, open-source behavioral analysis software. Alternatively, you can purchase a "worm tracker" system. These come in many different styles and vary enormously in price. Standard molecular biology lab equipment is also essential (e.g. pipettes, vortex, scales, stirrer, and PCR machine), but are not enumerated here. There are additional items that are very helpful, but not obvious. We suggest buying a tool box and high quality tools including a large variety of allen wrenches, screwdrivers, hammers, rubber mallet, vise grips, needle nose pliers, and a large adjustable wrench. Also consider purchasing flashlights and high quality extension cords for emergency use in power outages. Your lab will also need a place to store and organize the small items and supplies essential for minor adjustments, resupply or repair. We suggest an organizer unit with many small drawers, which store batteries, bulbs, fuses, velcro, utility knives, blades, and electrical tape in neatly organized and labeled compartments. Finally, we recommend that you prominently label all of your tools and storage units with your last name; else, those who borrow items won't know where to return them.

### **3.3 *C. elegans* Husbandry**

We recommend storing *C. elegans* cultures in plastic boxes. Boxes of diverse dimensions can be found at hobby shops, kitchen supply stores, and hardware stores. Scrapbooking boxes can work well; these and other boxes can be found at craft stores. Measure the dimensions of your shelves and the internal dimensions of incubators before you purchase boxes. You want to purchase boxes that fit efficiently with stacks of plates and into your incubator. Also, you want to purchase both large and small plastic boxes. Empty pipette tip boxes are also an option for storing small sets of plates in the incubator door racks. You might want a different set of boxes to

store NGM plates before they are used. In general, we avoid long-term use of cardboard boxes for storing *C. elegans*; these are tough to clean and provide hiding places for mites or cockroaches. You may find it useful to use commercially available “Insecticidal Shelf & Drawer Liner” paper in your 15 °C long term storage boxes. This paper is embedded with o-isopropoxyphenyl methylcarbamate and can kill mites as they move between plates.

As a biologist, you know that *C. elegans* are susceptible to spontaneous mutations. Combining this with serial passaging of small numbers of animals leads to genetic drift with altered phenotypes and experimental results. In addition, long-term starvation on plates may result in selection for specific traits or alleles. To minimize this, we recommend establishing a regular schedule for thawing *C. elegans* strains that are serially passaged in your lab. For example, thawing the wild type reference strain (N2) every 4 months is wise, if possible. Set a calendar reminder for when it is time to thaw the strain. Consider combining this approach to minimizing drift with the added precaution of passaging 8 to 10 animals per generation.

When starting a *C. elegans* lab, we recommend that you order basic strains, like N2 or *him-5* directly from CGC. Freeze *C. elegans* strains as soon as they arrive in your lab and freeze new strains immediately after construction or backcross. We suggest that you freeze several vials. If possible, keep one copy of each strain in a liquid nitrogen dewar and another copy in a –70 °C freezer. Adopt a scalable strategy and use a database for recordkeeping. And, make sure that your database is backed up automatically. Any strain or reagent that is included in a publication needs an appropriate and unique name. Insist on labeling each tube in your frozen strain collection with the correct *C. elegans* nomenclature. Your strain collection will last decades, allow you to share valuable resources with the community, and help you adroitly handle unexpected crises.

There are several protocols on how to pour and store NGM plates as well as maintaining *C. elegans* strains. Please see the Worm-Book Chapter entitled *Maintaining C. elegans* for details. *C. elegans* are usually fed with *E. coli* bacteria spread on NGM plates. The commonly used food-strains OP50 and HB101 are available from the CGC. OP50 is also subject to genetic drift, resulting in inconveniently thick lawns. We suggest freezing your bacterial strains and thawing them regularly.

### 3.4 Organizing

The ideal time to establish a good organizational strategy is when your lab is first set up. Your strategy needs to be scalable and sustainable over decades of employees, students, and fellows coming and going from your group. If the research groups(s) you trained in had excellent organizational strategies, go ahead and adopt them. If not, then try something else. This is a perfect time

to use your network; contact both experienced and new scientists who are running *C. elegans* research groups and get their advice. At a minimum, you need a good database program to keep track of your *C. elegans* strains and DNA constructs (e.g. plasmids). Ask others what programs they have used successfully.

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## 4 *C. elegans* Nomenclature, Data Management, Dissemination of Results and Reagents

### 4.1 Nomenclature

Establishing a *C. elegans* research laboratory means that you are joining a community of scientists with established norms for naming and describing *C. elegans* and other reagents. Ambiguity is evil. Adopting this carefully planned nomenclature strategy increases uniformity, decreases confusion, and increases accessibility of your work for other researchers. Following these standards also helps you organize and helps you generate the reagent and strain lists with the unique identifiers needed for publication. Indeed, adopting and strictly adhering to these norms enhance your standing in the community. A detailed explanation of nomenclature standards can be found in WormBook or in WormBase sections titled “Nomenclature.”

### 4.2 Distribution of *C. elegans* Strains and Reagents

*C. elegans* strains should be requested from the following sources, in the order listed. First, the primary sources for strains are the aforementioned *C. elegans* Genetics Center and the Japanese Knockout Consortium. Their mission includes both distributing and archiving *Caenorhabditis* strains, as well as related biological reagents (e.g. OP50 and other bacterial strains). Commonly used *C. elegans* strains, as well as many others are available for a modest fee or shipping costs.

For *C. elegans* strains not available from these consortia or for other reagents, it is appropriate to ask another *C. elegans* research group to share. Indeed, the *C. elegans* community is renowned for their willingness to share reagents, both pre- and post-publication. When possible, you should always ask the originating laboratory (that generated the strain or reagent) to send it to you. It is their responsibility to share published reagents. Alternatively, you can ask the originating laboratory if another group holding the reagent can provide it to you. Of course, you should not distribute materials created by others without permission. When asking for strains or reagents, if you ask for express shipping, then you should pay for that shipping. When sending *C. elegans* strains, it is polite to let the recipient know if you have recently seen mites in your lab.

#### **4.3 Data Management for your Lab**

1. Develop guidelines and expectations for your research group around maintaining notebooks and recording data in lab notebooks. Similarly, electronic data files and results should be backed up to external hard drives or online repositories. It is also important to set boundaries and expectations for archiving and backup of data files. Moreover, expectations for who has access to lab notebooks, data files, and results should be communicated to your lab.
2. For common lab documents, we suggest organizing lab access to shared materials. One effective strategy is creating a shared online drive with appropriate folders and editing access.
3. You may also consider using shared online calendars for your research lab. This will allow students to see when you are available and to suggest appropriate times to meet with you. We recommend that you keep your research and personal calendars separate.

#### **4.4 Publication of Results**

Peer-reviewed journals as well as online manuscript and preprint archives are the usual venue to publish research results. You might consider looking where *C. elegans* studies have already been published as you decide where to submit your work. The length of your manuscript may also help determine where you submit (e.g. G3 for smaller manuscripts versus Genetics for larger manuscripts). In addition to these standard venues, micropublications are an increasingly common mechanism to publish smaller projects, like the undergraduate thesis results and single figure results. These tiny manuscripts are peer-reviewed and indexed in PubMed (<https://www.micropublication.org/journals/biology/species/c-elegans/>).

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### **5 What I Wish Knew When I Started: Advice to New Colleagues**

#### **5.1 Onboarding**

When onboarding new people into your research program, it is important to be clear about expectations. This will provide clarity for trainees before joining your group. We have provided a few examples in the Subheading 6 of this chapter; please modify these for your specific needs and situation. In addition, we suggest providing background readings to help trainees acclimate to the lab, focused on both scientific topics of interest and *C. elegans* background. We recommended readings from Corsi et al., 2015 (DOI: <https://doi.org/10.1534/genetics.115.176099>) and At the Bench: A Laboratory Navigator (ISBN-13: 978-0879697082), as well as, a *C. elegans* nomenclature guide and relevant chapters of WormBook or WormAtlas.

### **5.2 Develop Communication Policies**

Running a research group means staying in communication with your personnel. However, reflexively immediately sending or responding to an email can be distracting and consuming. It is tempting to be glued to your mobile phone or other device that allows for constant access to your email and texts. However, this approach is not sustainable; a research career is a marathon, and not a sprint. You must adopt a work/life balance that is healthy and sustainable for you; everyone is different in how they handle this. One approach is to set boundaries for when you will check and respond to emails. If you decide to share your personal phone number with your research group, make sure to establish ground rules for situations when students should call or text you. Explaining your policies for email, texts, phone calls, and other communication with your students will provide group members with guidelines for when it is appropriate for them to contact you, as well as when they can expect to get a response. Your expectations for your mentees should be reflected in how you behave and communicate with them. Remember, you are modeling healthy boundaries surrounding email, text, and phone communication, as well as healthy work/life balance for your mentees and group members.

Establish regularly scheduled one-on-one meetings with each trainee in your group to discuss their research. Of course, you will have unscheduled informal interactions with them, but scheduled meetings ensure parity, establish priorities, and allow trainees to prepare for serious discussion and mentoring. Make it clear to the student you expect them to show up with their notebook ready to show and discuss their data and their plans for the coming week. You can use these sessions for mentoring as well as discussing that week's work. These meetings also allow you to plan long term and how you will manage progress. But, best practice might also be to set up an annual or semi-annual evaluation meeting with each lab member to discuss academic or career goals, as well as short term and long term goals. Additionally, lab members can complete and submit an Individual Development Plan (IDP) that can facilitate conversations. Setting aside time for weekly and annual meetings allows you to be fully present and focused on the training of your mentee.

### **5.3 Turn off Notifications**

Running a research group can be stressful. The buzz or ding from your mobile phone indicating a new email has arrived increases the pressure to check your phone and immediately respond; this makes concentrating or taking time off difficult. We recommend turning off notifications for emails; this alleviates the pressure to respond immediately. Consider turning off other notifications, especially when you choose to be offline or choose to focus on a specific task or discussion.

#### **5.4 Schedule Working Meetings**

You will have many demands on your time as the head of a research group; it will likely be challenging to find uninterrupted blocks of time to concentrate on projects. For collaborative projects, we suggest scheduling “silent working meetings” with your collaborators or with your research group members. This strategy is useful as you can block off specific times for concentrated productive work, with the ability to immediately ask a clarifying question about the project.

Ultimately, you will need to manage your time effectively to achieve your goals. There are many strategies for good time management skills, but you will need to prioritize tasks and allocate your time accordingly. One school of thought is to prioritize tasks aligned with how you will be evaluated for tenure or promotion at your institution.

#### **5.5 Supporting Students and Mentees**

As a mentor, it is critical that you support your students and trainees. You want to be available, be kind, and be respectful. But, some situations may be beyond your scope of expertise or require additional support mechanisms. Your institution should have a robust system for helping employees and trainees, but here we provide possibilities for external help in the USA: National Suicide Prevention Hotline: (800) 273-8255; Crisis Text Line: Text HELLO to 741,741; if you would like to speak directly with a counselor of color, you can text STEVE to 741,741; En español: (888) 628-9454; Veterans Crisis Line: (800) 273-8255; Trans Lifeline: (877) 565-8860; The Trevor Project: (866) 488-7386. This is not a comprehensive and complete list and some resources will be different depending on your institution or country. Check what resources are available to you in advance; this allows you to swiftly respond and immediately recommend resources during moments of crisis.

It should also be noted that setting boundaries in your relationship with trainees is important. This can be a difficult adjustment; when you start your research group, you may not be much older than your trainees. As their mentor, you should be friendly and supportive; but you are not their comrade, you are their supervisor and mentor.

#### **5.6 Stay in Contact with your Mentors**

It is important to stay in contact with your mentors and with individuals who can guide or inform your research and your career. We suggest setting up regular meetings with mentors and colleagues. If your institution doesn’t have a formal system for assigning senior mentors and scheduling meetings, we suggest you simply set up such a system yourself. Identify two or three senior colleagues and ask them to be your mentors, with the understanding that you will meet together on a regular schedule. Even if you only meet every few months over lunch, you can ask them for valuable advice. Just put these meetings on the calendar and commit!

Complementary to mentoring from senior faculty is peer mentoring. You can also establish a group of peers who can get together regularly for breakfast or lunch—once a week, once a month, whatever works—for networking and mutual advice, aid, and support. (e.g. [https://www.cell.com/molecular-cell/fulltext/S1097-2765\(09\)00912-5](https://www.cell.com/molecular-cell/fulltext/S1097-2765(09)00912-5)) Young lab heads are going through the same trials; you can help each other! Also, note that it can be very helpful to include a few members who have run their labs for up to 5 years. The experience of starting out is fresh in their memory, they are usually motivated to share their expertise with others, and their advice may be more relevant to what you are going through than the advice from more senior colleagues.

### **5.7 Inclusive Environment**

The richness and diversity of life experience, motivation, and preparation that you, your employees, and your trainees bring to your research group can be an enormous strength and source of inspiration. However, as head of the group, it is your responsibility to create an inclusive environment. Before opening your lab doors, it is critical that you educate yourself about strategies and practices that can help create a group ethos that is simultaneously inclusive and supportive, while being scientifically rigorous and productive. If you have not already done so, we suggest that you seek out training in culturally-aware mentoring, unconscious bias, and best strategies for mentoring. If you have already undergone such training, then we suggest that you review those materials and establish both clear expectations and practices for your group. Being an effective and responsive mentor requires a lifelong commitment and a thoughtful start is the best way to begin.

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## **6 Distributables for New Lab Members: Three Sample Handouts**

It is convenient and effective to have written guidelines for your lab members; this is especially useful for new folks. Clarity prevents misunderstandings. This last section contains three different sample handouts that you may want to adapt and distribute to those joining your research group. The first sample handout contains guidance for lab members in *C. elegans* husbandry and other practical aspects of working in the lab. This contains our accumulated advice, guidelines, and recommended practices for researchers, based on years of working with *C. elegans*. The next two sample handouts are intended for potential or new lab members. Your specific laboratory, institution, and research experience will certainly require different advice than we provide below. But, your documents should provide clear guidance for your trainees. Pay attention to what is working, what is not working, and make changes accordingly.

## 6.1 Sample Handout

1

### 6.1.1 Accumulated Wisdom: Guidelines for Successful *C. elegans* Studies

1. Mite infestations are not uncommon, but you can prevent them by quarantining strains when they arrive in our lab. Take the newly arrived *C. elegans* plate out of the envelope, but leave the envelope and paperwork on your desk. Check the newly arrived plate for mites or their eggs at the scope. Pick a few worms off the original plate to a new plate. Don't store the newly arrived plate or the envelope anywhere near your worm strains... a loosely closed plastic bag in a drawer is the best choice. If you see mites, immediately ask for help in clean-up and more rigorous quarantine. Every *C. elegans* or *Drosophila* lab is suspected of having mites (except the CGC).
2. Only lift the lid off a *C. elegans* culture plate when necessary. Really. Every minute gives mold an opportunity to waft onto your plate.
3. Use a laminar flow hood or the cleanest location you can find for spreading bacteria or drying NGM plates. Let the hood run for 10 min before using it. Remove your plates when you are done! Put the spread plates in boxes, but leave the box lid off so the plates will dry. You can use a large Kimwipe to cover your box or spread plates until they are dry, or move the plates to your bench and cover them with a cardboard box or large Kimwipes. Mold is the enemy!
4. Ideally, let freshly-poured NGM plates age ~5 days before you spread them with OP50. Then, you can tell if they are contaminated with mold. Don't forget that mold is the enemy.
5. Next, let the plates that you spread age ~5 more days before you use them for your *C. elegans* strains. Then, you can tell if the bacterial stock was contaminated. Label your box of plates with your name and the date spread so you can keep track.
6. When you passage strains:
  - (a) Label your box of worms with your name and the date before you put it in the incubator. Otherwise, it may magically move to the office of your faculty advisor.
  - (b) Label and date the **bottom** of each plate before you pick animals to it. Be legible, accurate, and unambiguous—else, you will regret it later.
  - (c) Rubber band plates containing strains together in boxes (or use tape).
  - (d) Do not reuse rubber bands. You can reuse boxes if no moldy plates were in the box. To clean boxes: hand wash the lids with Alconox/soap, rinse with water, 70% ethanol, and leave on pegs over the sink to dry. Treat box bottoms similarly or send them to dishwashing.
  - (e) Generally, to passage your strains, move 5–10 young adult hermaphrodites to a new plate and ignore eggs. Some

strains require selecting for a specific phenotype or no eggs. Ask if you aren't sure about your strain. Strains from CGC might have helpful instructions in their database.

- (f) Consider how to organize your strains. You can keep new plates of *C. elegans* in a different box from older plates. You can keep worms of the same genotype in the same rubber-banded stack. Consider if you should use different colors when labeling plates to avoid confusion about dates or genotypes.
  - (g) Keep a 15 degree box labeled "long-term storage" containing starved plates that are sealed with Parafilm. This should be kept in the 15 degree incubator and should also contain mite/cockroach repellent paper.
  - (h) When you passage, handle your moldy strains last. Avoid spreading mold by not inverting the lids, making sure to not reuse/mix up new versus old lids, and minimizing time the moldy plate is open.
  - (i) Tape stacks of plates closed when discarding (to prevent mold from growing and spores from wafting about in the room air).
  - (j) Use small boxes in the door of the incubators if you have a small number of plates.
  - (k) Do not regularly passage your strains by "chunking," which is cutting out a piece of agar and flipping it to a new plate. Chunking can obscure contamination. Use chunking only to revive from "long-term storage."
  - (l) Picks made of pricey platinum wire and personal. Put your name on yours. You can tape-wrap the pick to decrease breakage when it inevitably falls to the floor. Some rolls-and-falls can be prevented by creative taping or rubber pencil grips. Sometimes you can rescue a broken pick tip by melting it into a new Pasteur pipette.
7. If you open a box of unspread plates, spread them all. If you open a box of unspread plates to remove a subset, then make it obvious that you did so by moving the tape with the "date poured" on it to a horizontal position or some other obvious strategy.
  8. If you see a mite, let everyone know immediately by email. Write a note on the white board of the incubator where the box/plate came from. Wipe down the microscope and surrounding bench. Isolate and clean the strain. If you must keep the contaminated plate, put it in a plastic bag. Ask me for advice.

9. If you go away for a short trip, passage your strains with young animals to prevent starvation or consider asking a lab mate for a favor. When you leave for an extended time: draw down the number of strains you are maintaining and freeze your strains.
10. All strains must be frozen and named with appropriate *C. elegans* nomenclature before you submit your manuscript or thesis. All strains that we obtain or generate must be frozen. Ask the person in charge of strains for details.
11. It is critical that you use an alcohol-resistant marker (i.e. Lab Marker or Freezer Marker) to label your frozen strain tubes. Sharpies and other markers rub off. Stickers and tape fall off tubes in -70 or in liquid nitrogen. You have been warned....
12. Matings can be done at 15 or 22 °C, for 48 or 24 h respectively, with a ~1 cm diameter bacterial lawn. Ten males and 3 hermaphrodites is common, but not required.
  - (a) Always write out your cross strategy before you begin. Include generations, chromosome designation, map location, and genotypes. Do ask for help here!
  - (b) Make sure you can differentiate between self-progeny and cross-progeny.
  - (c) Unc hermaphrodites are slightly easier to mate into.
  - (d) If you will use PCR genotyping, then create a document with the requisite info (primers, temps, expected fragment lengths, restriction enzymes) You will thank yourself for this about 2 weeks later (and I want a copy in the lab Google Drive).
  - (e) L4 males and hermaphrodites are best for setting up a mating, but adults will do.
  - (f) Beware of picking eggs and L1s with your males, when setting up a mating.
  - (g) Note that transgenes or alleles often result in slow growth. This means that the homozygous (or heterozygous) animals you want from your cross/single/mating may grow slowly or reach adulthood days after the other animals on the plate. Pick your putatives on 2 consecutive days to provide yourself with a temporal window.
13. We are impatient. For maximum speed in crosses, move mating animals to a new plate after 48 h. Label this plate #2 and the old plate #1. You can now move #1 to 25 degrees (if your strain permits). Or, if necessary, you can single mated hermaphrodites after 48 h of mating... and assess mating efficiency by the % males in the progeny.
14. Clean strains have reproducible behavior, don't penetrate the agar, and are often more fecund. Clean strains are essential for

RNAi feeding experiments and for successful matings. Mold is problematic, but various bacterial/yeast infections (which we generically call slime) are a disaster. You should be concerned when smoothly spread “OP50” lawns on plates develop internal hills/bumps of bacteria, or when unstressed animals dig into the agar. The best way to keep strains clean is “Constant Vigilance.”

- (a) If a strain is moldy, isolate it from your other strains.
  - (b) If a strain is contaminated, get animals from an older clean plate or clean it.
  - (c) Bleaching is the only effective way to clean most strains. Pick 5 to 10 gravid adults to a drop of freshly prepared 1 part bleach, 1 part 10 N NaOH, 8 parts M9 which is placed off to the side of a bacterial lawn on a standard NGM plate. The hermaphrodites will die and decompose, the eggs inside will hatch, the L1s will crawl to the OP50. Pick at least three L1 animals 12 to 24 h later, as far as possible from the hermaphrodite carcasses. The L1 animals MUST be picked the next day to clean the strain (or they re-contaminate themselves).
  - (d) Expect every thawed strain to be contaminated.
15. Trust, but verify. Double check that strains and plasmids you receive are of the correct genotype and sequences, respectively. You don’t want to discover months later that the reagents you received aren’t what you thought it was. (This also applies to other reagents.)
  16. OP50 and other bacterial strains used for routine *C. elegans* maintenance should be rethawed every 6 months; streak out to a new plate every month. Start bacterial liquid cultures from individual colonies. Depending on the number of people in the lab, someone’s lab chore may be to create sterile single-use aliquots of OP50 for everyone to use. These can be stored in the refrigerator for up to 3 weeks.
  17. Genetic drift is a problem and there are obvious deleterious consequences of serial passaging. At minimum, N2 should be rethawed every 4 to 6 months. The lab member in charge of strains will do this and distribute N2 to everyone. Other essential and common strains likely also require regular thaw schedules. Some mutant strains have defects that are severe and under strong selection for accumulating suppressor mutations; these strains may need to be thawed for each use and can only be passaged a few times.
  18. Maintaining stable temperature in the *C. elegans* incubators requires air flow. The top halves of the top and bottom shelves must be empty of boxes to allow air flow. The tape on the inside

of the incubators is your guide for this. Also, boxes cannot be too close to the rear on the bottom shelf. Do not shift or move the wire guard at the rear inside the incubators or you will prevent critical air flow.

19. If you have only a few worm strains in an incubator box, be considerate and use a smaller box, placed in the incubator door shelves.
20. When shipping *C. elegans* overseas, label package contents as “non-toxic, non-infectious, biological sample”... not “live nematodes.” It is best to send starved plates. Put plates in individual plastic bags and try to cushion each plate individually; postal employees may smash on your envelope. Small plates are more resistant to postal employees crashing attempts (*C. elegans* are tough; some survived the Columbia Space Shuttle reentry disaster.) Warn the receiving lab if we have seen mites in the last 6 months.
21. When you passage your strains, keep the old plates in another box until the new plates have progeny. The old plate boxes need not be in an incubator (unless your strains require 15 degrees.)

#### 6.1.2 Lab Rules of the Road

1. Please make sure you (1) remove all liquid from beakers before putting them on the cart to be washed and (2) put reusable glass pipette in the water-filled pipet can. You will be grateful for this rule when you are in charge of dishes.
2. Do not leave agar in flasks, bottles or beakers that are left for washing. They cannot be put into the dishwasher with agar inside. Again, you will be grateful for this rule when you are in charge of dishes. Also, do not pour agar down the drain; it belongs in the biological waste bags (cut out of beaker or melt in microwave if needed).
3. If you plan to use a large number of *C. elegans* culture plates, then talk to the person in charge of plate pouring. They may be able to increase the plates being poured. If not, they will advise you to pour the plates yourself.
4. Please be considerate of your fellow lab members. Do not monopolize microscopes, especially the GFP dissection scopes. Sign up to use the injection microscope and no more than 2 h per person per day. (Personally, I find that >2 h injecting yields diminishing returns or catastrophes).
5. For UV/fluorescent microscopes, place the wristband atop the scope on your wrist when you turn on the UV/fluorescent light source. When you turn the light source off, you can return the wristband to the top of the scope. Hand the wristband to someone else to pass on this responsibility (The UV bulbs are expensive).

6. Whenever anything we ordered arrives in the lab, put all the paperwork on the technician desk or whoever is in charge of purchasing. This is critical for paying bills. For example, if you order strains from the Japanese Knockout Consortium, we need the enclosed paperwork.
7. Any plasmid, allele, transgene or new *C. elegans* strain you create and archive/keep needs an appropriate formal designation/name. Nomenclature should follow *C. elegans* guidelines ([wormbase.org//about/userguide/nomenclature](http://wormbase.org//about/userguide/nomenclature)). Someone in the lab is in charge of handing out sequential strain/plasmid/allele numbers; ask me who is currently in charge of this. Our lab strains start with - -; alleles start with - -, and plasmids start with p - - #. These prefixes are followed by a unique number (i.e., - -3461, - -345, or p- -#698.) All *C. elegans* strains that will be part of a published paper or thesis must be properly named, frozen and successfully thawed before submission. All plasmids and arrays must be similarly properly named and stored. I'm repeating this because it's important!

## 6.2 Sample Handout 2

### 6.2.1 Welcome to Working at the Bench in our Lab

#### Development of your Project

**Diversity, Equity, and Inclusion:** It is a shared responsibility for everyone in the group to make the laboratory an **inclusive**, pleasant place to learn and do science. I will work toward this goal, and am always open to new ideas and strategies. Our research group should be a respectful and collaborative place for all of us to work together.

#### Teach Others What You Have Learned

Students are expected to work to develop independence with the goal of working on their own project. All student projects are part of the lab's larger research questions. Communication with me and other lab members is key to a successful and positive experience in the lab. If you have an idea- share it. If you like or do not like how your project is going, come and talk to me. If you have too much or too little to do, come and talk to me. If you do not understand something or have a question, come and talk to me.

#### Laboratory Maintenance: Laboratory Chores and Duties

I do not expect students to come to the lab proficient in laboratory techniques. But, I do expect students to ask questions, and put in the necessary time to learn techniques and become independent. We operate by the philosophy “see one, do one, teach one.” That is, observe the experiment or how to do the technique. Then, do the experiment and become proficient. Lastly, once you are proficient in a technique or task, you can teach new lab members what you know.

#### Research Meetings

Mites, dust, and mold are the enemy!

Being in a group also means helping with upkeep. Everyone should pitch in with whatever tasks that need to be done to keep the lab functional. Lab chores and duties must be done by everyone, not just one person.

#### Data Management

Getting feedback on your progress and data is essential for the development of the research. Everyone will be expected to participate in group meetings, this includes attendance and presentation of results. Presentations may be project theses, preliminary data, or a scientific paper that informs the direction of your project. If you are not presenting, then it is expected that you make an effort to attend meetings, listen to your peers' presentations, and ask questions.

All members of the lab are required to keep a detailed record of what you have done in a lab notebook. You should include the date, the objective of the experiment, genotypes, strains, methods used, as well as any and all results. As members of the lab, I will provide hard copy paper bound notebooks. **Other forms of data management:** Electronic “lab notebook,” is really just a set of Word documents or Google Sheets for each project you work on. You may keep electronic notes, but you should make sure to share notes to

Google Drive, an external hard drive, Drop box, and with me. Please make sure that any microscopic images, figures, and raw data files (including calculations) are saved with meaningful file names to the lab external hard drive and to the cloud (Google Drive, Dropbox, and backup server). Maintaining your notebook (written or electronic) and saving your data files **must be done regularly and often!**

#### From Data Collection to Manuscripts

For our work to have impact we must publish our findings. To achieve our goal, we must move beyond data collection, to analyze our results and interpret the meaning of these results. Therefore, as soon as there is sufficient data, you should compose a preliminary figure and write a few sentences about the goals of the experiments, the methods used, and conclusions from the results or figures. Make sure to share your data, figures, and record your progress in your notebook. This activity will help us think critically about the work and better navigate the direction of the project to publish our work.

#### Authorship and Publications

Authorship is given to lab members and collaborators who make **substantial contributions** to conception and design, acquisition of data, or analysis and interpretation of data, and drafting the article or revising it critically for important intellectual content, and have approved the final version of manuscript to be published; and need to be accountable for all aspects of the work. *Policy and wording based on the International Committee of Medical Journal Editors (ICMJE)*. Discussing authorship sooner is better.

#### Conference Attendance and Travel

Conference travel support will be given to students who have made **significant contributions** toward a project, at least two data figure panels. Students attending conferences must be presenting their work as a poster or oral presentation at the conference. Travel fellowships are available from our institution and outside sources. Talk to me before you apply for a travel fellowship.

#### Lab Communication and Time Spent in Lab

When you join the lab, please update your contact details (email address, cell phone number, emergency contact name, and number) on our Google Doc contact table. If I need to communicate with you, I will usually send you an email to your email account or I will use our Slack workspace. If you need to communicate with me, please feel free to email me. Please use my cell phone to call me only if there is a serious lab emergency. You can text me if you are running late for a meeting.

**Time spent in lab:** Undergraduate and high school research students should plan on spending at least 8–10 h a week in the lab (if you are taking lab for course credit or this is a summer research project, you should expect to spend at least 20 h a week in lab). It is impossible to accomplish anything in less time than that.

Mentorship, Assessing  
Your Progress,  
Recommendation Letters  
and Additional Expectations

We will discuss your progress, data, and future plans during our weekly meetings and revisit it each semester to ensure we keep your program on the right track.

1. Create a timeline for what you expect to complete each semester and when you expect to complete it and email it to me at the beginning of the semester.
2. I am invested in your success as a student, a developing scientist, and in your career. Let me know your plans well ahead of time so that I can keep my eyes open and let you know of opportunities that will help you achieve those goals.

*I have received a copy of the lab expectations and have carefully read the guidelines and expectations. I agree to follow the listed guidelines and follow the outlined policies.*

|                        |      |
|------------------------|------|
| Student Printed Name   | Date |
| Student Signature      | Date |
| Name of faculty mentor | Date |

**6.3 Sample Handout****3****6.3.1 Expectations for all Laboratory Undergraduates**

Undergraduates work one-on-one with full-time researchers in our lab, who mentor them in the laboratory. All undergraduates in the laboratory are expected to become independent researchers running their own project and to write a senior honors thesis or a report on that project. Undergraduate projects are usually a part of and substantially related to the research project of their mentor. Substantial contribution to a research project takes time and commitment, but can result in exciting finding or authorship on an abstract or a manuscript.

**6.3.2 Before you Join the Laboratory**

1. Attend lab meetings and journal clubs regularly for several months to become familiar with research projects and lab members. After the first month, you should be an active participant in the discussion—at least asking a question or participating in discussion at each meeting.
2. Read at least 2 recommended review articles in the topical area of interest. Also, read the Accumulated Wisdom GoogleDoc, read the WormMethods introduction to the chapter on Behavior ([http://www.wormbook.org/chapters/www\\_behavior/behavior.html](http://www.wormbook.org/chapters/www_behavior/behavior.html)), and read recommended *C. elegans* reviews (e.g. [http://www.wormbook.org/chapters/www\\_celegansintro/celegansintro.html](http://www.wormbook.org/chapters/www_celegansintro/celegansintro.html) and <http://dev.biologists.org/content/130/20/4761.long>). You will meet with me to discuss these.
3. Meet with at least one full-time researcher in the lab to discuss their projects and how you might be able to undertake research with them. I can help guide you in finding a mentor in the lab.

**After You Have a Research Project and Join the Laboratory****Not taking research for credit, but undertaking volunteer research during academic year**

1. Continue to attend lab meetings/journal clubs at least once per week. Be an active participant in the discussion—contributing to intellectual discussion and asking questions at each meeting.
2. Pour *C. elegans* plates without contamination every other week. Everyone actively working in the lab contributes to plate pouring and other chores.
3. Your research project is your responsibility. On average, you should spend 10 h per week working on your research project. Each semester, you should discuss when you can be in the lab with your mentor. Advanced undergraduate students often spend more than 20 h in the lab each week, including work at the bench on their projects and at lab meetings. Time in the lab is driven by the science you are doing; sometimes evenings or weekends are required.

### Taking Research for Credit, During Academic Year

1. Continue to attend lab meetings/journal clubs at least once per week. Be an active participant in the discussion—contributing to intellectual discussion and asking questions at each meeting.
2. Pour *C. elegans* plates without contamination every other week. Everyone actively working in the lab contributes to plate pouring and other chores.
3. Your research project is still your responsibility and time commitment is the same as other classes. Students taking research for credit are expected to present their results in a formal lab meeting at the end of the semester. Seniors taking research for credit are also encouraged to present their research results at conferences.
4. Commit to writing a senior honors thesis.

### Undertaking Summer Research

Students are required to spend at least one summer in the laboratory. This involves working at least full time in the laboratory for at least 10 weeks and students are provided with stipend/support.

1. In the summer we have one lab meeting each week. Be an active participant in these meetings—contributing to intellectual discussion and asking questions at each meeting.
2. Continue pouring *C. elegans* plates and other chores.
3. Apply for summer research support, if possible. Obtaining support is not required, but applying is encouraged.
4. Working full time on your research project. Other commitments during the summer (i.e., shadowing, teaching, and classes) should be discussed with me in advance.
5. Students must present their results in poster format at our annual retreat and the University Summer Undergraduate Research Symposium or comparable poster session.

Flexibility: Joining our laboratory does not preclude a semester abroad or a summer off-campus. Also, we understand that required courses in specific semesters can interfere with lab meeting attendance and hours available for research. This should be discussed in advance of each semester.

Diversity, Equity, and Inclusion: It is our shared responsibility within the group to make our laboratory an inclusive, professional, and pleasant place to learn and do science. I will work toward this goal, and I am open to new ideas and strategies. Our research group should be a respectful and collaborative place for all of us to work together.

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## Acknowledgments

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# Chapter 2

## The Basics of Setting up Successful Teaching Labs and Short-Term Projects with *C. elegans*

Elizabeth E. Glater

### Abstract

*Caenorhabditis elegans* is an excellent organism for teaching or doing short-term research projects because of the many freely-available comprehensive resources describing its genome and biology, its short-generation time and the ease of working with it in the lab. However, it can be daunting to begin a short-term project or a teaching lab with an organism with which one has little or no experience. Therefore, in this article, we will discuss the minimal equipment, reagents, and protocols needed to begin doing experiments with *C. elegans* as well as inclusive teaching practices. We will also describe a teaching laboratory for introductory or advanced neuroscience undergraduate courses where students learn to understand the relationship between genetic mutations and behavior through using chemotaxis assays. In addition, instructions for students for the lab module are provided in Subheading “Example of Teaching Lab Module for Students”.

**Key words** *C. elegans*, Neuroscience, Inclusive teaching, Chemotaxis, Genetics

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### 1 Introduction

*C. elegans* is an excellent organism to use in short-term projects and in teaching undergraduates because one can examine the connections from genetic mutations to nervous system function to behavior or other phenotypes in a living organism during a single lab period. In addition, *C. elegans* are ideal for inquiry-based labs where students generate their own questions and experiments to address these questions because *C. elegans* are inexpensive and easy to maintain in the lab, have a fast generation time of three days, and much published information exists about them. Inquiry-based labs are more likely to engage students in science than doing a “cook-book” lab where students can easily predict the outcome [1]. There are many resources, including WormBase ([www.wormbase.org](http://www.wormbase.org)) and WormBook ([www.wormbook.org](http://www.wormbook.org)), providing a wealth of well-curated information about *C. elegans* biology and protocols

that students and instructors can easily access. In addition, the worm connectome which describes the synaptic connections among all 302 neurons that make up the *C. elegans* nervous system is available at WormAtlas ([www.wormatlas.org](http://www.wormatlas.org)).

As a practical matter, it is easy to obtain *C. elegans* strains and to maintain them in the laboratory. Thousands of *C. elegans* strains are kept at the *Caenorhabditis* Genetics Center (CGC) and can be ordered from CGC for a small fee. There is no need to purchase incubators because *C. elegans* thrives at room temperature, 20–25 °C. In addition, *C. elegans* can be stored frozen in –80 °C freezer for several years or in liquid nitrogen tanks indefinitely. *C. elegans* can be thawed and used for a specific teaching laboratory or experiment, but do not need to be maintained throughout the year. A further advantage is that there are many well-established protocols for working with *C. elegans* that can be learned relatively quickly. For some techniques that can take longer to learn, such as picking worms, we have included some alternative methods in the protocol below. Moreover, *C. elegans* researchers form a collaborative community that is open to sharing *C. elegans* strains, resources, techniques, and expertise.

In this chapter, we describe how to maintain *C. elegans* as well as provide an example of a teaching laboratory that can be used with undergraduates in an introductory or advanced neuroscience class. The overall learning objective of this lab is for students to learn to connect logically the functions of genes to behavioral phenotypes. In addition, the laboratory exercise aims to create an authentic research experience for students. Just as researchers try to understand the function of a novel gene, students will examine behavior of genetic mutant strains without knowing their identities and will try to figure out the functions of the mutated genes. Specifically, students will study the chemotaxis behavior of two genetic mutant strains of *C. elegans*, develop hypotheses about the functions of the genes that are mutated in the strains and then will use the scientific literature to research their hypotheses. The basic lab can be completed in one lab period; suggestions for an additional follow-up laboratory are included.

The laboratory would begin with an introduction about chemosensory behavior of *C. elegans*. Chemosensation, the detection of chemicals and volatile molecules, is one of the major ways that *C. elegans* gathers information about its environment. For example, *C. elegans* uses chemosensation to discriminate among volatile chemicals released by different kinds of bacteria, which are its major food source. The chemosensory system of *C. elegans* consists of 32 chemosensory neurons, about 10% of its entire nervous system. Most *C. elegans* chemosensory neurons express several different chemoreceptors. This is different from the mammalian system in which each olfactory neuron expresses only one type of olfactory receptor. *C. elegans* has two primary chemosensory

neuron classes that sense attractive volatile molecules, AWC and AWA, and three chemosensory neuron classes that sense repulsive odors, AWB, ASH, and ADL [2].

In the laboratory exercise, students will use *C. elegans* chemotaxis assays to examine quantitatively the attraction or repulsion of *C. elegans* for specific odors. In chemotaxis assays, *C. elegans* are placed in the center of an agar plate with the odor on one side and the diluent (the chemical that the odor is diluted in) on the other side of the plate [3]. Then *C. elegans* move around the plate for 1 h. The worms will move toward attractive odors and away from repulsive odors. On each spot with odor or diluent is also 1 µL of sodium azide. Sodium azide is a toxic chemical that causes worms to stop moving by inhibiting the electron transport chain in the mitochondria. Therefore, once worms reach the odor or diluent side, they stop moving and cannot “change their minds.”

Students are given wild type *C. elegans*, named N2, and two mutant strains: *odr-10* which encodes the olfactory receptor for diacetyl [4] and *odr-7* which encodes a transcription factor required for specifying AWA cell identity [5]. However, the students are not given the names of the strains, but instead are given plates of worms labeled “WT,” “Mutant A,” and “Mutant B.” The students test these strains in chemotaxis assays and then generate hypotheses about the function of the mutated genes. The expected results are that *odr-10* strain is defective in chemotaxis to diacetyl (AWA-sensed odor), but exhibits wild type chemotaxis to pyrazine (AWA-sensed odor) and isoamyl alcohol (AWC-sensed odor). The *odr-7* mutant which lacks functional AWA neurons is defective to chemotaxis to both odors sensed by AWA, diacetyl, and pyrazine, but exhibits wild type chemotaxis to isoamyl alcohol.

Students then generate hypotheses about the functions of Mutant A and Mutant B and propose experiments to test them. The instructor then can disclose the results of students’ proposed experiments by telling the students about experiments that have been reported in the published literature. After students receive this information, they can revise their hypotheses based on this new information. In the final step, instructors provide the names of the mutant strain and direct students to “test” their hypotheses based on information that they obtain from research articles or WormBase.

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## 2 Materials

Prepare all solutions using deionized water and store at room temperature unless noted. All *C. elegans* and bacterial waste should be collected in biohazard bags, autoclaved and then disposed.

## 2.1 C. elegans Maintenance

1. *C. elegans* strains: N2, CX32 *odr-10(ky32)*, and CX4 *odr-7(ky4)* (available from *Caenorhabditis* Genetics Center, (see Note 1)).
2. *E. coli* OP50 (available from *Caenorhabditis* Genetics Center).
3. Worm picks, purchase or make with glass Pasteur pipets and ~1-in. 32-gauge platinum wire (see Note 2). Break the end of Pasteur pipet, insert platinum wire into pipet end, heat glass in Bunsen burner flame so that glass melts around platinum wire. Tip of platinum wire may be flattened with a hammer and/or cut with razor blade to preferred shape (see Note 3) [6].
4. Nematode growth media plates: Make 5 mg/mL stock solution of cholesterol in ethanol and do not autoclave. Make stock solutions of 1 M MgSO<sub>4</sub>, 1 M CaCl<sub>2</sub>, 1 M KPO<sub>4</sub> buffer pH 6.0 (108.3 g KH<sub>2</sub>PO<sub>4</sub>, 35.6 g K<sub>2</sub>HPO<sub>4</sub>, H<sub>2</sub>O to 1 L) and autoclave all. Add 3 g NaCl, 22 g agar, and 2.5 g peptone in a 2 L Erlenmeyer flask. Add 975 mL H<sub>2</sub>O and large (~2 in.) magnetic stir bar. Use aluminum foil to cover mouth of flask tightly. Autoclave for 50 min. Cool flask to 55 °C while stirring on a stir plate or in a 55 °C water bath (see Note 4). Add 1 mL 1 M CaCl<sub>2</sub>, 1 mL 1 M MgSO<sub>4</sub>, 1 mL 5 mg/mL cholesterol in ethanol, and 25 mL 1 M KPO<sub>4</sub> buffer. Continue to stir on stir plate. Using sterile technique pour petri plates by pipetting or using a peristaltic pump (10 mL media per 60 mm petri plate; 24 mL media per 100 mm petri plate). Allow plates to cool at room temperature for 1–2 days and then store in air-tight container at room temperature for use over the next few weeks or at 4 °C for longer-term storage over months [6].
5. Luria Broth (LB): 10 g Bacto-tryptone, 5 g Bacto-yeast, 5 g NaCl, H<sub>2</sub>O to 1 L. Put 100 mL into 125 mL screw-cap bottles and autoclave [6].
6. LB agar plates: 10 g Bacto-tryptone, 5 g Bacto-yeast, 5 g NaCl, 15 g agar, H<sub>2</sub>O to 1 L. Autoclave and pour into petri plates [6].
7. *E. coli* OP50 liquid culture: streak bacterial stock out on LB agar plate and incubate at 30–37 °C overnight, inoculate bacteria from plate into 100 mL LB for 2 days at room temperature. Store bacterial plate sealed with parafilm and liquid culture at 4 °C.
8. Bacterial frozen stock: for long-term storage, make glycerol frozen stock in cryostat tubes: 500 µL bacterial liquid culture and 500 µL 50% sterile glycerol. Store at –80 °C.
9. NGM plates seeded with *E. coli* OP50 bacteria: using sterile technique pipet approximately 200 µL *E. coli* OP50 liquid culture to each 60 mm plate.
10. *C. elegans* freezing solution: first make S-buffer, 129 mL 0.05 M K<sub>2</sub>HPO<sub>4</sub>, 871 mL 0.05 M KH<sub>2</sub>PO<sub>4</sub>, 5.85 g NaCl.

Then make freezing solution, S-buffer + 30% glycerin (v/v). Autoclave both [6].

11. *C. elegans* bleach solution: mix immediately before use 0.5 mL 5 N NaOH with 1 mL household bleach or 5% sodium hypochlorite [6].
12. Cryotubes 1.8 mL (e.g., Nunc Cryotube Vials).
13. Dissecting stereomicroscopes with transilluminated base, total magnification 6×–50×.
14. Bunsen burner or alcohol burner.
15. Metal spatula.
16. Incubator holds temperature 13 °C to 30 °C (optional).

## 2.2 *C. elegans* Assays

1. Pasteur pipets and rubber bulbs.
2. Square petri dish with gridlines 100 × 100 × 15 mm.
3. Chemotaxis plates: Add 20 g agar, 975 mL H<sub>2</sub>O and large (~2 in.) magnetic stir bar to 2 L Erlenmeyer flask. Cover mouth of flask tightly with aluminum foil. Autoclave for 50 min. Cool flask to 55 °C while stirring on a stir plate or in a 55 °C water bath. Add 1 mL 1 M CaCl<sub>2</sub>, 1 mL 1 M MgSO<sub>4</sub> and 5 mL 1 M KPO<sub>4</sub> buffer. Continue to stir on stir plate. Using sterile technique by pipetting or using a peristaltic pump, pour 20 mL per square petri plate (see Note 5). Allow plates to cool at room temperature overnight and then use for chemotaxis. It is best to use plates the day after making. Older plates become dryer and will affect chemotaxis results [3, 7].
4. S-basal buffer: 1 g K<sub>2</sub>HPO<sub>4</sub>, 6 g KH<sub>2</sub>PO<sub>4</sub>, 5.85 g NaCl, H<sub>2</sub>O to 1 liter, autoclave [6].
5. Sterile water: 100 mL water in 125–250 mL glass bottles and autoclave.
6. Diacetyl: 1:1000 dilution, add 1 µL to 1 mL ethanol, make in chemical hood immediately before use.
7. Pyrazine: 10 mg/mL ethanol, make in chemical hood immediately before use.
8. Isoamyl alcohol: 1:100 dilution add 10 µL to 990 µL ethanol, make in chemical hood immediately before use.
9. Sodium azide: 1 M in sterile water.
10. Cell counters.
11. Kimwipes.
12. Microfuge tubes (1.5–2 mL).

### 3 Methods

All procedures should be performed at room temperature. Instructions for students for the lab module is in Subheading 3.7.

#### 3.1 Maintaining *C. elegans*: “Picking Worms” to Transfer *C. elegans*

1. Picking worms to transfer *C. elegans* from one plate to another. Gather stereomicroscope, one NGM plate with *C. elegans*, one seeded NGM plate without *C. elegans*, a worm pick and a Bunsen burner.
2. First heat up the wire end of the worm pick by placing it in the flame of a Bunsen burner or alcohol burner for a few seconds, then touch the pick to bacteria on seeded NGM plate and coat tip of pick with bacteria. While looking through the microscope, touch the pick with bacteria to a worm and the worm will stick to the pick. Move the pick to a fresh seeded NGM plate and patiently wait for worm to crawl off (see Note 6) [6].
3. Label the bottom of the NGM plate with the date and strain name.
4. If worms are maintained at room temperature, four worms should be transferred to a NGM plate every 3–4 days to prevent the worms from starving on the plates (see Note 7). Ideally, worms should be transferred at larval stage 4 (L4), the stage immediately before the young adult reproductive stage. A few differences between L4’s and young adult hermaphrodites: L4 *C. elegans* are slightly smaller than young adult hermaphrodites; the vulva in the middle of the L4 worm appears clear; and young adult hermaphrodites contain eggs whereas L4’s do not (see Note 8) [6].

#### 3.2 Maintaining *C. elegans*: “Chunking” to Transfer *C. elegans*

1. “Chunking” to transfer *C. elegans* from one plate to another is an easier technique than picking worms. Dip a metal spatula in ethanol and then hold in flame of Bunsen burner until ethanol burns off [6].
2. Use the spatula to cut out a small piece of agar from a plate with worms and transfer the agar chunk to a seeded NGM plate.
3. Chunking is fine for maintaining homozygous strains, but should not be used when mating strains. Chunking is also useful way to recover worms from a starved plate because it is very challenging to pick worms from a starved plate.

#### 3.3 Maintaining *C. elegans*: Making and Thawing *C. elegans* Frozen Stocks

1. Use a 100 mm plate that worms have recently exhausted the *E. coli* bacteria on the plate and has mostly L1 and L2 larvae on the plate [6].

2. Add 2.5 mL S-buffer and 2.5 mL S-buffer + glycerin to the worm plate. Mix by pipetting up and down 10 times, and then add 1 mL to each of four cryotubes.
3. Transfer tubes to a styrofoam box with holes for cryotubes and put box in  $-80^{\circ}\text{C}$  freezer so that the vials freeze slowly. After 1–2 days, move the frozen stocks to a permanent storage location in  $-80^{\circ}\text{C}$  freezer or in liquid nitrogen tank.
4. To thaw frozen stocks, place the frozen cryotube in room temperature water. When the stock is fully-thawed and is all liquid, pipet or pour the contents on to a seeded NGM plate. After 2–3 days, transfer worms to a new seeded NGM plate.

### **3.4 Maintaining *C. elegans*: Removing Contamination by Bleaching *C. elegans***

1. Occasionally, *C. elegans* strains become contaminated with mold or bacteria other than *E. coli*. The plate can be decontaminated by bleaching (see Note 9) [6].
2. To decontaminate the strain, use a 60 mm plate with gravid adults and/or eggs, then add 1 mL sterile water to the plate and transfer the water and worm solution to a microfuge tube.
3. Allow the worms to settle to the bottom, remove the supernatant, and then add 1 mL bleach solution. Place tube on rotating platform or vortex every 2 min for 10 min.
4. Then spin in centrifuge at approximately  $1000 \times g$ , to pellet the eggs. Remove the supernatant, add 1 mL sterile water, and invert tube seven times. Repeat wash.
5. Use a glass Pasteur pipet to transfer clean eggs to a fresh seeded NGM plate.

### **3.5 Instructor Preparation for Chemotaxis Teaching Lab**

1. Make NGM 100 mm plates, seed NGM plates with *E. coli* OP50 bacteria, and prepare 1 M sodium azide, a week or more before the lab.
2. Prepare *C. elegans* plates with N2, *odr-7*, and *odr-10* mutant strains. Four days before the lab, pick four L4 worms to an NGM plate for each strain. Or, 2 days before the lab, transfer a small chunk of a starved plate to an NGM plate (ideally transferring about 100–200 starved worms).
3. Label plates “N2,” “Mutant A,” or “Mutant B,” and write down the code. One NGM plate of adult worms provides enough worms for approximately two chemotaxis assay plates.
4. Prepare chemotaxis plates the day before the lab. Each condition (worm strain and odorant) should be tested on at least three separate chemotaxis plates. We suggest that each lab group tests all three strains (N2, Mutant A, and Mutant B) with one odorant. The whole class can discuss the data from all three strains with all three odorants.

5. Prepare 100  $\mu\text{L}$  aliquots of odors (pyrazine, diacetyl, and isoamyl alcohol), 1 M sodium azide, and ethanol immediately before use.
6. At each student workstation, place stereomicroscope, Pasteur pipets and bulbs (see Note 10), cell counter, P20 or P10 pipettor (see Note 11), Kimwipes, s-basal buffer, sterile water, microfuge tubes in rack, chemotaxis plates, worm plates, odorants, ethanol, sodium azide, waste beaker, and waste bag for pipet tips used with diluted odors and sodium azide.

### ***3.6 Making the Classroom Laboratory an Inclusive Environment for all Students***

1. It is important to create a classroom laboratory that is an inclusive environment. In inclusive teaching, the instructor actively works to make sure all students know that they belong in the course and that they can succeed in the laboratory module and in the course overall. Summarized below are three strategies for creating an inclusive classroom, largely based on “Implementing inclusive practices in an active learning STEM classroom” by Kathryn M. S. Johnson [8].
2. Guide students to think of their strengths, called an asset-based approach. Ask students to list the characteristics or skills that they think successful scientists have. It is likely that students will list characteristics such as persistent, creative, smart, good at working with others, performing careful laboratory work, excellent observation skills, and good note-taking. Then ask the students to think about which of these skills they already have, and to recall times when they have used these skills, and to consider how they will use them in this class. The instructor emphasizes that as each student has different sets of skills, they will succeed in the class in different ways and that is appropriate [8].
3. The instructor should tell students that this laboratory module is challenging, and they should expect to feel frustrated. However, this does not mean that students should feel like they have failed or are lacking in some way. Emphasize that ALL scientists regularly experience frustration when trying to learn a new protocol or make sense of their data. It is also true that scientists feel great joy and reward when they figure something out or achieve better understanding. Ideally, this lab module will bring students this satisfaction as well [8].
4. During discussions, it is helpful for instructors to use “Think, pair, share” often. The instructor poses a question to the class, asks students to think about an answer, and to confer with a lab partner or another student in the class about an answer. The last step is to share with the larger group what they have discussed. This method usually turns a quiet classroom into a room buzzing with discussion because students feel much more confident and comfortable about speaking after having talked over their ideas with a classmate [9].

### 3.7 Example of Teaching Lab Module for Students

#### 3.7.1 Introduction

Understanding the Connection from Genes to Behavior using *C. elegans* Chemotaxis.

1. ***C. elegans* and neuroscience.** *Caenorhabditis elegans* (*C. elegans*) is a small free-living nematode primarily found on rotten fruit and in compost heaps where it feeds primarily on bacteria. *C. elegans* is a widely used organism for neuroscience research for several reasons. First, it has a small nervous system with only 302 neurons. This nervous system is much smaller than the human brain, which is estimated to have about 86 billion neurons. Second, all of the synapses among the 302 neurons in *C. elegans* are known. To date, this is the only organism for which the complete connectivity among all neurons is known. Therefore, *C. elegans* is an excellent way to begin to understand how neurons function together to produce behavior. Third, *C. elegans* is an excellent genetic system with a sequenced and annotated genome. This means researchers can easily examine functions of genes in the whole organism or in specific cells in order to study the effects of genes on behavior. Fourth, many genes involved in nervous system function are conserved from *C. elegans* to humans. For example, *C. elegans* and human have similar serotonin, glutamate, and dopamine receptors and have homologous genes involved in synaptic vesicle recycling and release. As a result, research about *C. elegans* genes is often relevant for understanding human disease and behavior.
2. ***C. elegans* chemosensory behavior.** Chemosensation, the detection of chemicals and volatile molecules, is one of the major ways that *C. elegans* gathers information about its environment. For example, *C. elegans* uses chemosensation to discriminate among volatile chemicals released by different kinds of bacteria, which are its major food source. The chemosensory system of *C. elegans* consists of 32 chemosensory neurons, about 10% of its entire nervous system. Most *C. elegans* chemosensory neurons express several different chemoreceptors. This is different from the mammalian system in which each olfactory neuron expresses only one type of olfactory receptor. *C. elegans* has two primary chemosensory neuron classes that sense attractive volatile molecules, AWC and AWA, and three chemosensory neuron classes that sense repulsive odors, AWB, ASH, and ADL.
3. **The chemotaxis lab module.** Chemotaxis means movement (“taxis”) toward or away from chemicals (“chemo”). In chemotaxis assays, *C. elegans* are placed in the center of an agar plate with the odor on one side and the diluent (the chemical that the odor is diluted in) on the other side of the plate. Then

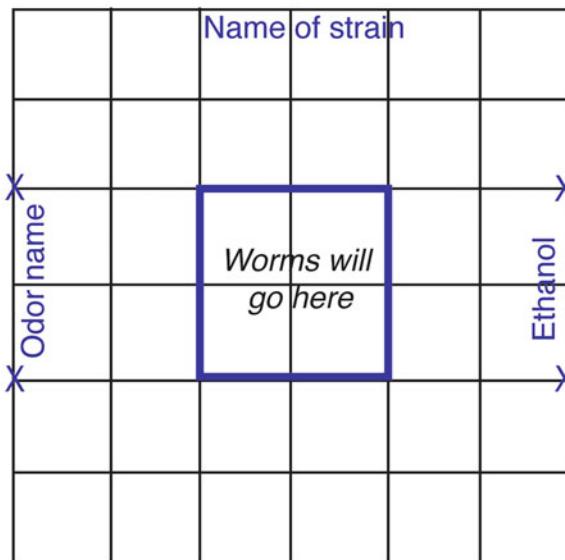
*C. elegans* move around the plate for 1 h. The worms will move toward attractive odors and away from repulsive odors. On each spot with odor or diluent is also 1  $\mu$ L of sodium azide. Sodium azide is a toxic chemical that causes worms to stop moving by inhibiting the electron transport chain in the mitochondria. Therefore, once worms reach the odor or diluent side, they stop moving and cannot “change their minds.”

4. In this lab, you will be given a wild type strain (N2) and two genetic mutant strains, labeled Mutant A and Mutant B. These strains were generated in a forward genetic screen for worms that are defective in chemotaxis behavior. In a forward genetic screen, animals are treated with a mutagen to mutate their DNA. Then their offspring are tested for a specific behavior phenotype, for example, defective chemotaxis to a specific odor. The resulting mutant strains are then studied to discover the function of the gene in the behavior being studied.
5. In this lab, your first task is to figure out the specific defects of Mutant A and Mutant B in chemotaxis behavior. The class will test each of these strains for chemotaxis to three different attractive odors: diacetyl (AWA-sensed odor), pyrazine (AWA-sensed odor), and isoamyl alcohol (AWC-sensed odor). Each lab group will test three strains with one odor. For example, one group will assay N2, Mutant A, and Mutant B for diacetyl chemotaxis. Please follow the chemotaxis protocol below.

### 3.7.2 Chemotaxis

#### Materials

1. N2 wild type (2–3 large plates).
2. Mutant Strain A (2–3 large plates).
3. Mutant Strain B (2–3 large plates).
4. Odors (diacetyl, pyrazine, isoamyl alcohol).
5. Ethanol (diluent).
6. Pasteur pipets and bulbs.
7. Stereomicroscope.
8. Cell counter.
9. P20 or P10 pipettor.
10. Kimwipes.
11. S-basal buffer.
12. Sterile water.
13. Microfuge tubes in rack.
14. Chemotaxis plates.
15. Waste beaker.
16. Waste bag for pipet tips used with odors and sodium azide.



**Fig. 1** Labeling chemotaxis plates. Label bottom (the side with the agar) of chemotaxis plates with name of strain on top, name of odor on one side, and ethanol on other side. Label an “X” as indicated. Pipet 1  $\mu\text{L}$  sodium azide on all four “X” spots; pipet 1  $\mu\text{L}$  ethanol on each of two “X” spots on ethanol side; and pipet 1  $\mu\text{L}$  odor on each of two “X” spots on odor side

### 3.7.3 Chemotaxis Methods

1. Before beginning chemotaxis assays, practice viewing *C. elegans* with stereomicroscope. Adjust brightness of light, focus, and distance between the eyepieces until you can easily observe worms without eyestrain. Compare the WT and mutant strains and write down observations about similarities and differences between the worm strains.
2. Label BOTTOM (agar side) of plates with a  $6 \times 6$  grid pattern (Fig. 1). Do 3 assay plates for each genotype and odor. For example: 3 N2 plates with diacetyl, 3 Mutant A plates with diacetyl, and 3 Mutant B plates with diacetyl.
3. Prepare chemotaxis plates. Wear GLOVES because sodium azide and some odorants are TOXIC. Pipet 1  $\mu\text{L}$  1 M sodium azide (POISON) on each spot marked with an “X.” You may reuse the same tip for sodium azide. Discard tip in clear plastic waste bags.
4. Pipet 1  $\mu\text{L}$  ethanol on the two labeled “X” spots on the ethanol side of the plate.
5. Mix odor by inverting tube 10 times. For each plate, pipet 1  $\mu\text{L}$  of odor on the two labeled odor spots. Place lid on plates and do not disturb until you put worms on the plate. Discard tip in clear plastic waste bags.

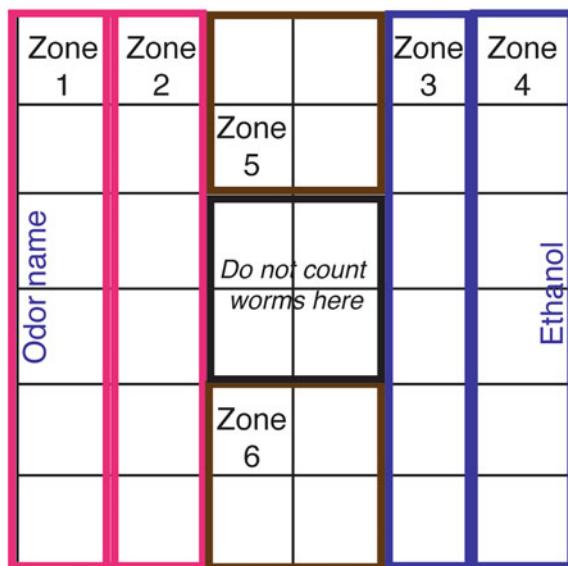
#### Prepare worms

6. Wash worms off plate with S-basal. Pipet 2 mL of S-basal buffer on each plate. Use glass Pasteur pipet to transfer worms from agar plate to 1.5 mL tubes. It is important to use glass pipets to transfer worms because worms stick to plastic. Each strain of worms should go in a separate tube.
7. Wait for the worms to settle to the bottom of the tubes. *Work quickly in doing the next steps because the worms will be sluggish in the chemotaxis assay if they remain in the wash buffer too long.* Then remove supernatant (liquid above the worms) from worms.
8. Do **first wash**: add 1 mL of S-basal buffer to each tube of worms. Allow worms to settle to the bottom, then remove the supernatant.
9. Do **second wash** of worms: add 1 mL of S-basal to each tube of worms. Allow worms to settle to the bottom, then remove the supernatant.
10. For the **last wash**: add 1 mL **sterile water** to each tube of worms. Allow worms to settle to the bottom of the tube, then remove supernatant from worms. Leave approximately 100  $\mu$ L of water with the worms.
11. Ask your instructor or TA for help with this step and do it under the microscope. With glass Pasteur pipet transfer approximately 50–200 worms in as little liquid as possible to the center of the chemotaxis plate. Use a Kimwipe to remove the buffer around the worms on the plate. When majority of worms are crawling rather than swimming, enough buffer has been removed.
12. Place the chemotaxis plates in a location where they will be not disturbed. Allow the worms to chemotax for 1 h. Plates then can be scored immediately, or stored in the fridge for 2–3 days and then scored.

### Scoring chemotaxis plates

13. Score the plates under the microscope. It is helpful to use a marker to outline the different “zones” of the  $6 \times 6$  grid (Fig. 2). For each plate, count the number of worms in each zone numbered above (1, 2, 3, 4, 5, and 6). The odor is zones 1 and 2, the diluent (ethanol) is zones 3 and 4, and the number of worms in neither is zones 5 and 6. Do not count the worms in the center four squares where you originally placed worms and blotted them.
14. Calculate the chemotaxis index for each plate:

$$\frac{\text{Number of worms on odor(zones 1, 2)} - \text{number of worms on diluent(zones 3, 4)}}{\text{Total number of worms(zones 1, 2, 3, 4, 5, 6)}}$$



**Fig. 2** Scoring chemotaxis plates. For each plate, count the number of worms in each zone numbered above (1, 2, 3, 4, 5, and 6). The odor is zones 1 and 2, the diluent (ethanol) zone is zones 3 and 4, and the number of worms in neither zone is zones 5 and 6. Do not count the worms in the center four squares where worms were placed at the start of the assay. It is helpful to use a marker to outline the different “zones” as in figure. The chemotaxis index for each plate is:

$$\frac{\text{Number of worms on odor(zones 1, 2)} - \text{number of worms on diluent(zones 3, 4)}}{\text{Total number of worms(zones 1, 2, 3, 4, 5, 6)}}$$

A chemotaxis of 1.0 indicates 100% attraction to odor, –1.0 indicates 100% repulsion to odor, and 0.0 indicates no preference.

15. Analyze your results in an Excel spreadsheet. Summarize the chemotaxis behavior of mutants A and B in comparison to wild type (N2) worms.
16. Your second task is to hypothesize about the functions of the genes that are mutated in Mutant A and Mutant B. The strains you are examining resulted from a published forward genetic screen for mutant strains that were defective in diacetyl chemotaxis. Researchers performed a series of experiments to identify the function of the mutated genes in diacetyl chemotaxis behavior. Imagine that you are these researchers, and you are trying to figure out the functions of these genes. Based on your data, what would you hypothesize are the functions of genes A and B? What experiments could be done to test your ideas?
17. After you discuss your hypotheses and experimental ideas, your instructor will reveal to you some more data from the published literature and eventually the identities of genes A

and B. You can then look up the functions of the genes in WormBase ([www.wormbase.org](http://www.wormbase.org)), a database with summary information about *C. elegans* genes. On WormBase website, in the search box in the upper right, select gene from the dropdown menu and type in the name of the gene. You can also use PubMed (<https://pubmed.ncbi.nlm.nih.gov/>) to find published articles about these genes.

18. Additional *C. elegans* resources:

WormBase ([www.wormbase.org](http://www.wormbase.org)).

WormBook ([www.wormbook.org](http://www.wormbook.org)).

WormAtlas ([www.wormatlas.org](http://www.wormatlas.org)).

### **3.8 Discussion of Chemotaxis Data**

1. The time while the worms are chemotaxing can be used for a discussion about how chemotaxis plates will be scored, a lecture about *C. elegans* olfaction and forward genetic screens, and/or lab clean up because only microscopes and cell counters will be needed for the rest of the lab. This is also a good time to ask students about their observations about physical similarities and differences between the worm strains. The *odr-7* and *odr-10* mutants have not been described to have any movement defects, but it is good scientific practice for students to write observations about strains. For example, students may notice that a plate is contaminated with mold or there is a higher density of worms on one plate than another.
2. Students analyze their data either per lab group or pooled data from the class. The expected results are that *odr-7* mutant will be defective in chemotaxis for diacetyl and pyrazine compared to N2, but normal for isoamyl alcohol. The *odr-10* mutant will be defective in chemotaxis only for diacetyl.
3. Remind students that the strains they are examining resulted from a forward genetic screen for mutant strains that had defective diacetyl chemotaxis. Researchers performed a series of experiments to figure out the function of the mutated genes in diacetyl chemotaxis behavior. Invite students to imagine themselves as those researchers who have just begun characterizing these strains. Ask students to generate hypotheses about the functions of Mutant A and Mutant B and propose experiments to test them.
4. It is hard to know what students will suggest, ask, and think. Encourage students to explain their logic and reasoning and to think of experiments to test their ideas. For example, students may hypothesize that Mutant A (*odr-10*) is the receptor for diacetyl and Mutant B (*odr-7*) is a receptor for both diacetyl and pyrazine. How could this hypothesis be tested? Students may suggest tagging the polypeptide that the mutated gene expresses with GFP. If both genes encode a receptor, they

would both be localized to the dendritic cilia of AWA neurons. The instructor then can disclose the results of their proposed experiments. For example, Fig. 5 in Sengupta et al., 1996 [4] shows that *odr-10::GFP* fusion localizes to AWA cilia and Fig. 5 in Sengupta et al., 1994 [5] shows that *odr-7::GFP* fusion localizes to nucleus.

- After students receive this information, students can revise their hypotheses based on this new information. For example, students can discuss the functions of proteins typically found in the nucleus of cells, such as transcription factors, RNA polymerase, or histones. Remind students that the genetic mutation affects DNA in every cell in the animal. If an animal had defective RNA polymerase in all its cells, the animal would not be viable. However, because the chemotaxis phenotype of Mutant B seems to be specific to AWA-sensed odors, it would make sense that the gene mutated in Mutant B encodes a gene that functions specifically in the AWA neurons rather than in all cells. In the final step, the instructor provides the names of the mutant strains and directs students to “test” their hypotheses based on information that they obtained from research articles or from WormBase ([www.wormbase.org](http://www.wormbase.org)).

### **3.9 Chemotaxis**

**Extension: Testing Novel Odorants in *C. elegans* Chemotaxis**

- An extension to this lab would ask students to test chemotaxis to odorants of their choice. Although *C. elegans* chemotaxis has been well-studied, it is likely that there are still odorants to be identified that are attractive or repulsive to *C. elegans*. It is possible that *C. elegans* could be used as a biosensor to detect environmental toxins or biomarkers of human disease [10]. To see a list of many of the odorants that have already been tested for *C. elegans* chemotaxis please see this reference 7.
- Possible projects: Can *C. elegans* distinguish between enantiomers? Does *C. elegans* prefer the smell of banana or apple? Is *C. elegans* attracted to household products, such as perfume, scented candles, or cleaning supplies?

## **4 Notes**

- N2 is the name of the wild type *C. elegans* strain. Genes in *C. elegans* are named either based on the mutant phenotype or the predicted protein product of the gene. The genes *odr-4* and *odr-7* are named “odr” (ODoRant response abnormal) because they were identified in a forward genetic screen for odorant response defective mutants [5]. Strain names and allele names are based on the code assigned to each head of a *C. elegans* laboratory.

2. Platinum wire is used to make worm picks because platinum wire heats quickly which sterilizes it and then cools quickly so when it is touched to a worm, it does not overheat it and injure it.
3. We have found that the bought worm picks to be more durable and easier for students to learn how to use than homemade picks.
4. It is important to cool the NGM media to 55 °C. If the other solutions are added to NGM when it is too warm, the media will turn cloudy and should not be used to make plates.
5. It is possible to fill plates with as little as 10 mL chemotaxis media, but we find it is easier to make more consistent plates if a larger volume is used.
6. It is very important to heat worm pick between each worm transfer to prevent cross-contamination between worm strains and to prevent bacterial contamination. If plates do get contaminated with mold or non-*E. coli* bacteria, then contamination can be removed from worms by bleaching, *see* Subheading 3.4.
7. If *C. elegans* are maintained at lower temperatures, they need to be transferred less frequently. If *C. elegans* are maintained at 13 °C, they only need to be transferred about once a week.
8. *C. elegans* are hermaphrodites and males. In wild type strains males arise spontaneously at a very low frequency, about 0.1% frequency [6]. Therefore, it is common practice to maintain most strains as hermaphrodites only. Adult males can be distinguished from adult hermaphrodites because adult males lack eggs and have a “club” tail (contains copulatory apparatus) rather than a tapered tail. It is difficult to distinguish males and hermaphrodites in larval stages.
9. Bleaching a contaminated plate with *C. elegans* eggs is an effective way to remove contamination because bleach kills most bacteria and mold. *C. elegans* eggs are resistant to bleach, but larval and adult worms are not [6].
10. It is best to use glass Pasteur pipets to transfer worms because worms tend to stick to plastic pipet tips.
11. If pipettors are not available to pipet 1 µL accurately, an option is to reduce the concentration by half and pipet 2 µL of each chemical. For example, instead of 1 µL of 1 M sodium azide, students pipet 2 µL of 500 mM sodium azide.

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# Chapter 3

## Cryopreservation of *C. elegans* and Other Nematodes with Dimethyl Sulfoxide and Trehalose

Kevin F. O'Connell

### Abstract

One of the key attributes that has contributed to the popularity of *Caenorhabditis elegans* as a model system is its ability to survive freezing. By preserving stocks at ultralow temperature, researchers have been able to generate an unlimited number of strains without the burden of constantly maintaining them. This has facilitated the use of large-scale forward genetic screens and CRISPR-mediated genome editing where large numbers of novel and informative mutants can be generated. Traditionally, *C. elegans* and other nematodes were frozen using glycerol as a cryoprotectant. While effective, a large majority of animals do not survive a typical freeze–thaw cycle. Here I describe an alternative method based on the popular combination of DMSO and trehalose as a cryoprotectant. This method allows the survival of large numbers of worms and effectively protects most developmental stages including adults.

**Key words** *C. elegans*, Nematode, Cryopreservation, Cryoprotectant, Dimethyl sulfoxide, Trehalose

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### 1 Introduction

One of the main advantages in working with *C. elegans* and related nematodes is their ability to be frozen and stored at low temperature. Thus, the long-term culturing of valuable strains, which is laborious and leads to the accumulation of genetic alterations that alter the original properties of the strain, is unnecessary. Accordingly, the ability to store strains as frozen stocks has facilitated the use of large-scale forward genetic screens where many useful mutants are generated in a relatively short period of time. Upon isolation, each new mutant line can be frozen immediately thereby preserving the strain in its original state and allowing researchers to analyze these mutants at the time of their choosing. Likewise, the advent of robust transgenesis [1] and genome editing protocols [2] have allowed researchers to generate new worm strains at an unprecedented pace; without the ability to cryopreserve worms,

researchers would face an ever-increasing burden of maintaining these new stocks.

For many organisms, environmental freezing is a death sentence as ice crystal formation both within and outside of cells can lead to cell membrane disruption, protein denaturation, and osmotic shock [3–6]. During freezing, ice crystals form in the extracellular environment. As solutes are excluded from the crystal lattice, ice crystal growth is accompanied by a progressive increase in the osmolarity of the shrinking liquid fraction [6–8]. At sufficiently slow cooling rates, this leads to an efflux of water from the cell. This suppresses intracellular ice formation that is known to be associated with a loss of cell viability [9, 10]. Excessive water loss, however, can lead to osmotic shock, loss of membrane integrity, and death [5]. Thus, controlled dehydration is a key parameter in maintaining the viability of frozen cells. The same appears to apply to whole organisms as it has been shown that nematodes with low-water content survive freezing better than nematodes with a high-water content [11].

Certain substances called cryoprotective agents or CPAs [12] can retard ice crystal nucleation and/or growth allowing bacteria, embryos, sperm, tissue culture cells, and even worms to retain viability after a freeze-thaw cycle. Among the known cryoprotectants, glycerol was one of the earliest to be identified and is broadly employed in cryobiology [13, 14]. Indeed, John Sulston, a member of Sydney Brenner's group that pioneered the use of *C. elegans* as a model genetic system found that glycerol could be used as an effective cryoprotectant to freeze *C. elegans* stocks [15]. The same basic protocol developed by Sulston is still in widespread use today [16].

Like glycerol, dimethyl sulfoxide (DMSO) is a low molecular weight cell-penetrating CPA that is commonly employed for the cryopreservation of a broad range of cell types [12, 17]. DMSO is known to stabilize membranes during freezing [18] and depress the ice-nucleation temperature [7, 19]. While effective as a CPA for cell suspensions, DMSO can also be used to freeze tissues but requires a prolonged exposure time in order to achieve deep penetration [20–22]. However, because DMSO is known to be cytotoxic [23–26] it is often used with a variety of additives. One such additive is trehalose, a non-reducing disaccharide known to naturally protect certain organisms against dehydration [27–29]. Trehalose has been shown to stabilize membranes against dehydration [30, 31] and is thought to hydrogen bond to water disrupting ice crystal formation and growth [32–34]. When added to aqueous solutions with DMSO, trehalose modulates ice crystal formation leading to increased cell viability post-thaw [35].

Here, I describe an alternative to the glycerol-based method for freezing worms. The new method uses DMSO and trehalose as a compound cryoprotectant and in my hands consistently results in a

significant improvement in survival relative to the traditional method. The new method has also been used to successfully cryopreserve evolutionary distant nematodes such as the extremophile *Auanema* sp. [36].

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## 2 Materials

1. M9 Buffer: Dissolve 3 g KH<sub>2</sub>PO<sub>4</sub>, 6 g Na<sub>2</sub>HPO<sub>4</sub>, and 5 g NaCl in 900 ml distilled deionized water. Adjust to 1 l and autoclave. After cooling, add 1 ml sterile 1 M MgSO<sub>4</sub> and mix thoroughly.
2. TDMSO Freezing buffer: 0.08 M Trehalose, 0.5 M DMSO in M9 buffer. Add 15.1 g trehalose and 17.7 ml DMSO (14.13 M) to 450 ml M9 buffer and mix until trehalose dissolves completely. Adjust volume to 500 ml with M9 buffer (*see Note 1*). Mix again and aliquot as desired. TDMSO is good for six months if stored at room temperature. It may last longer if shielded from light as DMSO is light sensitive (*see Note 2*).
3. Conical 15 ml centrifuge tubes.
4. Sterile Pasteur pipets.
5. Cryovials (e.g. Nalgene 1.5 ml cryogenic tubes).
6. Cell freezing container (commercially available or improvised device (*see Note 3*)).

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## 3 Methods

1. Strains that are to be frozen are grown on four NGM or MYOB (*see Note 4*) 100 mm plates seeded with OP50 bacteria until starved.
2. Wash worms off plate with sterile M9 buffer at room temperature and transfer to a 15 ml conical tube. Typically, I wash the plates twice; once with 10 ml and a second time with 5 ml.
3. Centrifuge at room temperature for 5 min at 2,000 × *g* and remove supernatant with a Pasteur pipet.
4. Wash the worms once in 4 ml TDMSO buffer and resuspend in 4 ml fresh TDMSO.
5. Dispense 1 ml of worms (in TDMSO) into each of four freezing tubes (*see Note 5*).
6. The four tubes are transferred to a freezing container engineered to achieve an optimal cooling rate of  $-1\text{ }^{\circ}\text{C min}^{-1}$ . The chamber is transferred to a  $-80\text{ }^{\circ}\text{C}$  freezer overnight after which the tubes are removed and placed in temporarily storage (*see Note 6*).

7. Worms can be retrieved from storage by thawing an entire tube and pouring the contents onto an NGM or MYOB plate. As the worms warm up, they will begin to move. Alternatively, it might be possible to recover a sufficient number of viable worms using a sterile spatula to scrape the top of the frozen stock (*see Note 7*). This would allow a single tube of frozen stock to be used multiple times.

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## 4 Notes

1. A more commonly used cryoprotectant is a solution of 30% glycerol in S buffer [129 ml 0.05 M K<sub>2</sub>HPO<sub>4</sub>, 871 ml 0.05 M KH<sub>2</sub>PO<sub>4</sub>, 5.85 g NaCl]. This is used as a 2× solution and is mixed 1:1 with a suspension of worms [15, 16]. While the glycerol-based cryoprotectant has been reported to be as effective as trehalose-DMSO [37], in my hands the latter is routinely far more effective.
2. I store buffer in 50 ml conical centrifuge tubes wrapped in tin foil.
3. A freezing chamber can also be fashioned from a polystyrene foam shipping container in which wells have made to accommodate the freezing tubes. Alternatively, the polystyrene foam racks supplied with some 15 ml conical tubes can be used. Place the freezing tubes in the wells of one rack and place the second rack face down over the first. The racks can be held together with lab tape.
4. While most labs use the traditional nematode growth media [16], I use the far simpler to prepare MYOB (Modified Youngren's Only Bacto-peptone) media developed by Eric Lambie. Per liter: 2.0 g NaCl, 0.55 g Tris-HCl, 0.24 g Tris-OH, 4.6 g bacto-peptone, 8 mg cholesterol, 20 g agar. Mix in deionized distilled water and autoclave.
5. I often leave the tubes at room temperature for 15–30 min to allow the worms to ingest the cryoprotectant so that it might better penetrate all tissues. However, DMSO is toxic [23–26], so prolonged exposure prior to freezing might result in lower viability.
6. I generally leave the tubes at –80 °C for at least a week before test thawing one vial. After confirming the viability of the test thaw, the other tubes are moved to permanent storage. Two are kept in the –80 °C freezer and the other stored in liquid nitrogen.
7. Because worms are slightly more dense than water [38], they will gradually settle to the bottom of the tube, and thus you may have to thaw the entire tube in order to recover viable

worms. There are, however, ways to mitigate this problem. One strategy that could be employed would be to freeze the tubes upside down or on their side. Another strategy not yet tried, would be to include a thickening agent in the buffer, similar to how agar is sometimes employed in the glycerol-based freezing buffer [16].

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# Chapter 4

## Genetic Methods for Cellular Manipulation in *C. elegans*

Menachem Katz

### Abstract

Neuron manipulation in vivo by ablation, activation or inactivation, and regulation of gene expression is essential for dissecting nervous system function. Here we describe genetic means for neuron manipulation in the nematode *C. elegans*, and provide protocols for generating transgenic animals containing these genetic tools.

**Key words** *C. elegans*, Microinjection, Transgenic worms, Cell ablation, Optogenetic, Neuronal silencing, Neuronal activation

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### 1 Introduction

How neuronal circuits form and function are central questions in neuroscience. *C. elegans* is an excellent model for addressing these questions. An adult hermaphrodite has a compact nervous system of 302 neurons, each of which develops in an invariant manner. The animal is transparent, enabling observation and manipulation of neurons in vivo, and exhibits diverse behaviors. These properties, together with a completely reconstructed neuronal connectome, allow neuronal functions within circuits to be dissected at high resolution.

Cell ablation is frequently used to analyze the roles of specific neurons within a network. In *C. elegans*, directed neuron elimination by laser microsurgery was initially used to dissect the neuronal circuits of sensory responses and feeding [1–4]. This method allows the elimination of almost any neuron in the first larval stage (L1) or of neuronal progenitor cells in the developing embryo. However, it is labor intensive and generates a limited number of ablated animals [5], making it unsuitable for studies of behavior, which involve scoring a large number of animals. Advances in genetic engineering now allow individual neurons to be killed by cell-specific expression of genes that induce apoptosis [6–10], degeneration [11, 12], or

necrosis [13–15]. Such genetic ablations are less laborious than laser ablation, and result in reproducible cell killing in transgenic animals that can be propagated indefinitely. Genetic ablations targeted to specific neurons can also be controlled in time, either by light-induced enzymatic activity [13–15] or by heat-induced gene expression [6] (for a description of these tools *see* Table 1). Cell-specific gene knockouts [16–18] or targeted protein degradation [19, 20] can serve not only to define endogenous gene function (*see* Table 1), but also to selectively control neuronal activities [21]. Moreover, transgenic methods can be used to rewire neuronal circuits, through the formation of ectopic electrical synapses [22].

Although effective, cell and gene ablation often cannot be used to study subtle and/or complex aspects of neuronal activity. A number of genetic methods for finer manipulation of intact neurons in living and behaving animals have been developed. These are summarized in Table 1, and include chronic or acute inhibition of neuronal activity by membrane hyperpolarization [23–33] or by blocking neurotransmitter release [34, 35]. Genetic methods for acute or chronic activation of neurons also exist [34, 36–53]. Temporal manipulation of neuronal activity over a wide range of time scales, from slow developmental processes to rapid behavioral responses, can be accomplished using tools with varying activation kinetics. Moreover, several neurons can be differentially manipulated in the same animal by combining chemical and optogenetic regulation or by using a combination of light-induced enzymes with non-overlapping activation wavelengths. Optogenetic tools, however, have some limitations. Strong blue-violet light is toxic to *C. elegans*, thus proteins that are activated at longer wavelengths are preferred. Limiting the intensity and duration of illumination or using pulsed light can also help to overcome toxicity. Importantly, *C. elegans* avoid blue-violet light, complicating behavioral analysis. While this can be overcome using animals in which the light receptor (LITE-1) is mutated [39], such *lite-1* mutants have locomotory defects, limiting their use in various behavioral assays.

Genetic manipulations often require cell-specific promoters, which may not be available for a given neuron at a given developmental time. A number of methods have been developed to overcome this constraint. For example, cell killing by caspase reconstitution allows each of two caspase fragments to be expressed using promoters with broad cell specificity. Cell death, however, would only ensue in cells expressing both promoters [6]. A similar strategy employs the combinatorial action of recombinases such as FLP or Cre [34, 54, 55]. Here, one promoter is used to drive expression of a transgene preceded by a transcription termination site flanked by FRT or loxP recombination sites. Expression of the appropriate recombinase using a second partially overlapping promoter recombines out the termination site allowing specific gene expression. Using optogenetic tools, cell specificity can be achieved

**Table 1**  
**Genetic tools for neuronal manipulation**

| Gene                                      | Cellular process | Comments   | References |
|---|------------------|--|------------|
| 1. Cell elimination                       |                  |  |            |
| Chronic:                                  |                  |  |            |
| <i>ced-3/4</i>                            | Apoptosis        | <i>ced-3</i> and <i>ced-4</i> are apoptotic genes that can induce cell death upon ectopic expression if they are integrated to the genome and homozygous<br><br>Cons: The efficiency of cell killing is moderate, where some cells are more resistant than others.<br>Efficiency can be increased by using <i>ced-9</i> mutant animals   | [8]        |
| recCaspase<br>( <i>ced-3</i> ; caspase-3) | Apoptosis        | <i>ced-3/caspase-3</i> are cloned in two separate fragments that mimic the proteolytically-cleaved subunits. Each fragment is fused to an antiparallel leucine-zipper dimerization motif<br><br>Co-expression of the subunits generates constitutively active caspase that leads to cell death<br><br>Integration to the genome and homozygous expression can increase efficiency<br><br>Since each fragment is expressed under a separate promoter, it can be used to achieve cell-specific ablation when a cell-specific promoter is unavailable. Moreover, this system can be used for inducible cell killing, if a heat-shock promoter regulates expression of one of the subunits | [6]        |
| <i>egl-1</i>                              | Apoptosis        | <i>egl-1</i> is a BH3-containing activator of the apoptotic pathway.<br>Ectopic expression of EGL-1 can induce cell death  | [9, 10]    |
| Diphtheria toxin                          | Apoptosis        | A catalytic fragment of the diphtheria toxin (DT-A), which induces cell death by blocking protein synthesis  | [7, 63]    |
| <i>mec-4</i>                              | Degeneration     | A dominant mutant of <i>mec-4</i> , a mechanotransduction channel, which can lead to cell swelling   | [11]       |

(continued)

**Table 1**  
**(continued)**

| Gene                                       | Cellular process                          | Comments   | References |
|--|---|--|------------|
|  |   | and degeneration when ectopically expressed  |            |
| <i>deg-3</i>                               |   |  |            |
|  | Degeneration                              | A dominant hyperactive mutant of <i>deg-3</i> , a subunit of the acetylcholine receptor, which can lead to channel opening, cell swelling and degeneration<br>Cons: Effective in only a limited subset of neurons that endogenously express <i>des-2</i> , which is required to form a functional acetylcholine receptor   | [12]       |
| Inducible:                                 |   |  |            |
| KillerRed                                  | Necrosis                                  | A type II photosensitizer that produces superoxide anion radicals following induction by green light, which results in neuronal death<br>Plasma membrane targeting of KillerRed increases its efficiency<br>Cons: Some neurons are resistant to KillerRed. Efficiency can be increased by using animals with mutated <i>sod-1</i> , which normally protects from cytoplasmic reactive oxygen species (ROS) | [13, 15]   |
| MiniSog                                    | Necrosis                                  | Mini singlet oxygen generator (miniSog) is a small monomeric fluorescent flavoprotein that upon blue light illumination induces ROS production and cell death. Effective cell death is achieved by mitochondrial-targeting of miniSog  | [14]       |
| 2. Cell-specific gene/Protein inactivation |   |  |            |
| <i>rde-1</i>                               | Cell-specific rescue of the RNAi response | <i>rde-1</i> mutant animals are resistant to RNAi. Cell specific expression of RDE-1 can rescue the RNAi response cell autonomously. This allows the use of feeding-RNAi method, which is easy to use and applicable for high-throughput screens   | [18]       |

(continued)

**Table 1**  
(continued)

| Gene  | Cellular process                                       | Comments  | References   |
|---|--|---|--|
|   |  | Cons: Feeding-RNAi is inefficient in neurons. Using sensitized mutant such as <i>rrf-3</i> , <i>lin-15b</i> , <i>eri-1</i> , or <i>nre-1</i> can increase efficiency  |  |
| Sense and antisense sequences of gene of interest |  | Expression, under cell-specific promoters, of sense and antisense RNA corresponding to a gene of interest. This method works well in neurons without the need of using RNAi-sensitized mutants<br>Cons: Not efficient for high-throughput screens   | [17]   |
| CRISPR/Cas-9 genome editing                       |  | The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated nuclease (Cas-9) can be used to knock in loxP sites within a gene of interest. Cre expression under a cell-specific promoter will then induce a directed deletion mutation, resulting in cell-specific gene inactivation. Alternatively, the loxP insertion can be designed in such a way that it will result in overall gene inactivation, whereas Cre expression in the cells of interest will specifically rescue the gene function | [16] and described in details in a chapter by Martin and Calarco, in this volume |
| AID (auxin-inducible degradation)                 | Protein degradation by the ubiquitin-proteasome system | This system allows for cell-specific conditional protein depletion by adopting the auxin-inducible degradation (AID) system from plants. For this, the gene of interest is tagged with a degron-motif (for example, by using the CRISPR method), which is recognized by the plant-specific E3 ubiquitin-ligase F-box protein, TIR1. TIR1 is not endogenously expressed in <i>C. elegans</i> , hence protein degradation by the proteasome   | [20]   |

(continued)

**Table 1**  
(continued)

| Gene                                  | Cellular process                                       | Comments  | References |
|---------------------------------------|--|---|------------|
|                                       |  | will ensue only in those cells that ectopically express TIR1. Moreover, TIR1 activity requires the small molecule auxin, thus auxin treatment allows for temporal control of protein depletion  |            |
| ZF1-mediated protein degradation      | Protein degradation by the ubiquitin-proteasome system | This system utilizes the endogenous E3 ubiquitin-ligase substrate-recognition subunit, ZIF-1, which is expressed in somatic tissues only in early embryonic stages. ZIF-1 recognizes and promotes the degradation of proteins that contain the small zinc-finger degron domain, ZF1. Hence, ZF1 addition to a gene of interest will result in specific protein depletion only in those cells that ectopically express ZIF-1 | [64]       |
| <b>3. Neuronal inhibition</b>         |  |   |            |
| Chronic:                              |  |   |            |
| <i>unc-103</i> (gf)                   | Hyperpolarization                                      | Gain-of-function mutation of ERG-like potassium channel (UNC-103) that induces membrane hyperpolarization   | [31]       |
| <i>twk-18</i> (gf)                    | Hyperpolarization                                      | A constitutively active mutation in a two-P domain K <sup>+</sup> channel (TWK-18) that induces membrane hyperpolarization  | [27, 28]   |
| Tetanus toxin (TeTx)                  | Blocks synaptic transmission                           | Expression of the light chain of TeTx blocks synaptic vesicle release through synaptobrevin cleavage  | [34, 35]   |
| Inducible:                            |  |   |            |
| Histamine/<br>Cl <sup>-</sup> channel | Hyperpolarization                                      | <i>C. elegans</i> does not use histamine as a neurotransmitter, and is insensitive to this molecule at low concentrations. Ectopic expression of the inhibitory <i>Drosophila</i> histamine-gated chloride channel (HisCl1)   | [30]       |

(continued)

**Table 1**  
(continued)

| Gene                   | Cellular process   | Comments  | References                                |
|------------------------|--|---|---|
|                        |  | together with histamine treatment results in graded neuronal silencing  |   |
| NpHR/Halo              | Hyperpolarization  | Halorhodopsin is a light-gated $\text{Cl}^-$ pump that is activated with yellow light   | [24, 33] and Bergs et al., in this volume |
| Arch                   | Hyperpolarization  | Archaerhodopsin-3 (Arch) is a green-yellow light-activated, outward-directed proton pump with higher inward currents compared to NpHR   | [23, 25, 29]                              |
| Mac                    | Hyperpolarization  | Mac is a blue-green light-activated outward-directed proton pump with higher inward currents compared to NpHR   | [23, 25, 32]                              |
| KR2                    | Hyperpolarization  | KR2 is a green-light-driven $\text{Na}^+$ pump  | [26]                                      |
| UVLamP                 | Activation of $\text{G}\alpha_{i/o}$ intracellular signaling | UVLamP is a $\text{G}\alpha_i$ -coupled bistable opsin, which can be switched on by UV light and off by blue light. By activating $\text{G}\alpha_{i/o}$ signaling, it is predicted to promote neuronal inhibition. However, its effect on <i>C. elegans</i> neuronal activities has not been confirmed. The narrow excitation spectrum in the UV range enables the use of UVLamP in combination with other optogenetic tools, with minimal overlap in the excitation spectra<br>Cons: UV and blue light are toxic to <i>C. elegans</i> | [65]                                      |
| 4. Neuronal activation |  |   |   |
| Chronic:               |  |   |   |
| <i>pkc-1/ttx-4(gf)</i> | Increases dense core vesicle (DCV) release                   | A gain-of-function mutation in <i>pkc-1/ttx-4</i> that induces neuronal activity by induction of DCV transmission and neuropeptide release  | [34, 47, 51]                              |

(continued)

**Table 1**  
**(continued)**

| Gene          | Cellular process | Comments   | References                                    |
|---------------|------------------|--|---|
| Inducible:    |                  |  |   |
| <i>lite-1</i> | Depolarization   | LITE-1 is a novel gustatory receptor (Gr) family-related ultraviolet light receptor<br>Upon UV illumination it induces activation of guanylate cyclase and (cGMP)-sensitive cyclic nucleotide-gated (CNG) channels, which results in neuronal depolarization. LITE-1 activation does not require the addition of all-trans retinol (ATR)<br>Cons: LITE-1 activity requires the expression of DAF-11, TAX-2, and TAX-4, thus it can only work in a subset of neurons. It requires strong violet light illumination for activation | [39, 44]                                      |
| ChR2          | Depolarization   | Channelrhodopsin-2 (ChR2) is a blue-light-activated cation channel from <i>Chlamydomonas reinhardtii</i> . ChR2 response is fast, inducing depolarization within the millisecond timescale. Slower variants for long-term manipulation (step function opsins, SFOs) are also available<br>ChR2 requires the addition of the cofactor all- <i>trans</i> retinal (ATR), which is provided to the worms by feeding with bacteria soaked in ATR  | [38, 45, 46] and Bergs et al., in this volume |
| VChR1         | Depolarization   | VChR1 is a yellow-light-activated channelrhodopsin from <i>Volvox carteri</i>  | [53]  |
| C1V1          | Depolarization   | C1V1 is a green-light-activated chimeric channelrhodopsin from <i>Chlamydomonas</i> and <i>Volvox</i>  | [40]  |
| Chrimson      | Depolarization   | Chrimson is a red-light-activated channelrhodopsin from <i>Chlamydomonas noctigama</i>   | [43, 50]                                      |

(continued)

**Table 1**  
(continued)

| Gene                  | Cellular process                                   | Comments  | References |
|-----------------------|--|---|------------|
| BeCyclOp/<br>RhGC     | Induction of cGMP-mediated intracellular signaling | BeCyclOp is a fusion of rhodopsin and guanylyl cyclase catalytic domain from <i>Blastocladia emersonii</i> . Upon light activation it catalyzes cyclic guanosine monophosphate (cGMP) synthesis<br><br>In neurons expressing the cyclic nucleotide-gated (CNG) channels (TAX-2/4), light activation of BeCyclOp promotes depolarization   | [37, 41]   |
| ceGAR-3Dq<br>(DREADD) | Activation of G $\alpha_q$ intracellular signaling | Designer receptors exclusively activated by designer drugs (DREADDs) are modified G-protein coupled receptors (GPCR) that are activated by the inert compound clozapine-N-oxide (CNO) to promote selective cellular signaling (i.e., G $\alpha_q$ , G $\alpha_s$ or G $\alpha_{i/o}$ signaling pathways)<br><br>ceGAR-3Dq is a <i>C. elegans</i> -specific DREADD, which activates the G $\alpha_q$ signaling pathway. Treatment of animals with CNO can drives intracellular calcium induction and neuronal activation specifically in DREADD-expressing neurons | [48, 49]   |
| optoXRs               | Activation of GPCR signaling pathways              | OptoXRs are light-activated chimeric GPCR, in which the intracellular loops of a rhodopsin are replaced by the corresponding loops of a ligand-activated GPCR. Like DREADDs optoXRs allow specific activation of the G $\alpha_q$ , G $\alpha_s$ , and G $\alpha_{i/o}$ signaling pathways  | [36]       |
| PAC                   | Induces synaptic vesicle release                   | Blue-light-activated adenylyl cyclase increases intracellular concentrations of the second messenger cAMP, leading to the induction of synaptic vesicle release   | [52]       |

(continued)

**Table 1**  
**(continued)**

| Gene                         | Cellular process                            | Comments   | References |
|------------------------------|---|--|------------|
|                              |   | Cons: Since cAMP serves as second messenger in various cellular processes cAMP induction might have pleiotropic effects  |            |
| Sonogenetic ( <i>trp-4</i> ) | Activation of a mechanotransduction channel | Low-pressure ultrasound is used to activate neurons that ectopically express the mechanotransduction channel <i>trp-4</i><br>Cons: The response is not highly specific. <i>trp-4</i> is endogenously expressed in several neurons, thus ultrasound stimulation triggers a basal behavioral response, which is not completely omitted even in <i>trp-4</i> mutant animals | [42]       |

by restricting illumination to the cell and time of interest. Methods for automatic cell-specific illumination in freely moving animals have been developed [32, 56, 57], but are low throughput.

There are many ways to manipulate neurons in a living organism. The approaches described above are based on transgenic techniques. Below is a detailed protocol for generation of transgenic animals by DNA microinjection.

## 2 Materials

### 2.1 Equipment

1. Inverted microscope with a gliding stage and 10× and 40× Plan-Neofluar lenses with a DIC or Hoffman Modulation Contrast.
2. Needle holder, connected to a coarse manipulator and a Three-axis Oil Hydraulic Micromanipulator.
3. Electronic pressurized microinjector systems.
4. Needle puller: laser-based or filament heating-based micropipette puller.
5. Standard dissecting stereomicroscope.

### 2.2 Buffers

1. M9 buffer (1 l): 3 g KH<sub>2</sub>PO<sub>4</sub>, 6 g Na<sub>2</sub>HPO<sub>4</sub>, 5 g NaCl, and 1 ml of 1 M MgSO<sub>4</sub>. Add H<sub>2</sub>O to 1 l, and autoclave.

2. NGM agar (1 l): 3 g NaCl, 17 g agar, 2.5 g peptone. Add 975 ml of H<sub>2</sub>O, and autoclave. Allow cooling to ~50 °C, and then add: 1 ml of 1 M CaCl<sub>2</sub>, 1 ml of 1 M MgSO<sub>4</sub>, 25 ml of 1 M potassium phosphate (pH 6), and 1 ml of 5 mg/ml cholesterol (in EtOH).
3. TE buffer: For 100 ml, mix 200 µl of 0.5 M EDTA (pH 8), 1 ml of 1 M Tris–HCl (pH 8), and 98.8 ml H<sub>2</sub>O.

### 2.3 Other

1. Agarose.
2. Cover slips #1.5, 24 × 50 mm.
3. Halocarbon 700 oil.
4. A worm pick: A platinum wire connected to a Pasture pipette. The end of the pick should be slightly wide and flat.
5. NGM plates seeded with OP50 *Escherichia coli* bacteria. Dispense 10 ml of NGM agar into 6 cm petri dishes; allow solidifying overnight. Inoculate OP50 *E. coli* bacteria in 500 ml LB, and incubate at 37 °C overnight. Seed NGM agar plates with several drops of OP50 bacteria (~200 µl). Allow the bacteria to dry and form a lawn by incubating the plates at room temperature for 2 days.
6. Worms: L4 hermaphrodites (around 50) picked a day before injection onto a fresh OP50-seeded plate, so that on the day of injection there will be many well-fed young adult animals, with a single row of eggs (see Note 1).
7. Capillaries: Borosilicate glass capillaries with filament (4 in., 1/0.58 OD/ID (mm), World Precision Inst., Sarasota, FL, USA) (see Note 2).
8. Plasmids: The specific genetically encoded tool (Table 1) depends on the planned experimental manipulation. Plasmids should be obtained from other researchers or depositories, such as AddGene, and sub-cloned as needed.

## 3 Methods

To generate transgenic lines for neuronal manipulations, animals are transformed with DNA encoding the appropriate genetic tool (Table 1). The most common method of transformation is DNA microinjection. In brief, DNA is injected into a distal gonadal arm, which contains a central core cytoplasm shared by many germ cell nuclei. Injected DNA can be taken up into the nuclei of mature oocytes as high-copy extrachromosomal arrays, which segregate randomly and transmit to subsequent generations with variable frequency, creating transgenic lines. The following protocol is based on [58] (see Notes 3 and 4).

The following steps should be done before starting the injection procedure:

**3.1 Preparing  
Injection Pads (See  
Note 5)**

1. Dissolve 2% agarose in water by heating it in a microwave until boiling, and mix well.
2. Using a Pasteur pipette, place a drop of hot agarose on a cover slip.
3. Immediately place another cover slip on top to flatten it. The size of the agarose pad should be approximately 2 cm in diameter.
4. Once the agarose has solidified, slide off the top coverslip.
5. Place the cover slips with the agarose pads in an open box, and cover loosely with an aluminum foil (which serves to prevent dust from sticking to the agar).
6. Allow to dry for several days.
7. Injection pads can be stored indefinitely; thus it is advised to prepare many at once.

**3.2 Preparing  
Microinjection Needles**

(According to Needle Puller P-2000 (Sutter Instruments) User Manual)

1. Turn the machine power switch on. Allow the machine to warm up with the lid closed for about 15 min.
2. Load the desired program. Parameters for needle pulling are varied, and should be defined individually (*see Note 6*). We are using the following parameters: HEAT, 275; FIL, 2; VEL, 45; DEL, 250; and PUL, 150.
3. Loosen clamping knob on the puller bar.
4. Place the capillary in the V-groove of the puller bar, slide it about 2 cm beyond the clamp, and tighten the knob. Be careful to hold the capillaries only at the edges.
5. Depress the spring stop on each puller bar to release them from their catch position.
6. Pull both bars toward each other using the finger bars. Hold bars in position using the thumb and index finger from one hand.
7. Using your free hand, loosen both clamping knobs and carefully slide the capillary through the holes in the side of the shroud and into V-groove of the opposite puller bar.
8. Tighten down clamping knobs.
9. Close the lid and press “PULL.” Needle pulling will take a few seconds.
10. Loosen the clamping knobs and remove the pipettes from the puller bars.

11. Place the pulled needles in an empty box with a strip of modeling clay in the bottom. Push the needle slightly into the clay such that the tip of the needle is hanging free in space (*see Note 7*).

On the day of injection

### **3.3 Preparing DNA for Injection**

1. Prepare 20 µl DNA mixture in TE buffer containing the desired plasmid DNA (according to Table 1), and a co-injection marker (*see Notes 8 and 9*). Total DNA concentration is 100–150 ng/µl (*see Note 10*). If the total concentration is less than 100 ng/µl, add an empty pBlueScript vector to the mixture.
2. Spin at maximal speed for 30 min using a bench-top microcentrifuge at 4 °C.
3. To prevent dust and other small particles from clogging the needle, use only the upper portion of the solution to load needles.
4. The DNA mixture could be kept at 4 °C for several days, or in –20 °C for a few months, but will need to be respun for a few minutes before use.

### **3.4 Loading the Needle with DNA**

1. Make a ring of adhesive tape, where the adhesive side is facing out, and stick it to the side of a table or a shelf. This will be used to hold the needle.
2. Pipette around 0.5 µl of DNA to the back (open) side of the needle, adhere the needle vertically on the adhesive tape, and allow it to stand for about 5 min. By capillary action, the DNA mixture will flow to the tip of the needle (*see Note 2*).
3. Verify that the mixture flowed down to the tip of the needle by holding the needle up to the light. Look at the needle under a dissecting microscope to make sure there are no large air bubbles at the tip. If there are, discard the needle and load a new one.

### **3.5 Mounting the Needle on the Microscope and Breaking the Needle**

1. Before you start, make sure the three knobs of the micromanipulator are set in the middle [5] and the needle holder is at about a 30° angle to the microscope stage.
2. Unscrew the end cup of the needle holder that is mounted to the microscope.
3. Insert the needle to the end cup from the back (open) end, until a few millimeters of the back end of the needle protrudes from the end cup.
4. Screw the end cup tightly back on to the needle holder.
5. With the coarse manipulator make sure that the needle is placed high enough to prevent it from colliding with the stage.

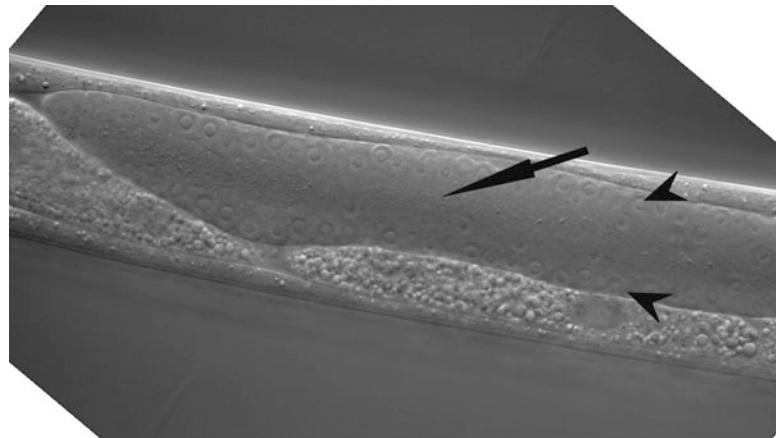
6. Turn on the microinjector and wait for the pressure to build up (injection pressure should be 1700 hPa and compensation pressure 50 hPa).
7. To center the needle in the field of view, close the transmitted light iris and turn the light intensity to maximum. Using the knobs of the coarse manipulator bring the needle to a position such that only the very tip of the needle is strongly illuminated. Make sure the needle is not too low on the z-axis. Reopen the transmitted light iris and dim the light.
8. Place a capillary on a coverslip and apply a drop of microinjection oil on it in the center. This will stabilize the capillary.
9. Put this capillary on the microscope, perpendicular to the needle. Using a 10× objective focus on the middle of the capillary in the z-axis. You should see clear dark lines on both sides of the capillary.
10. Carefully lower the needle into the oil, and use the micromanipulator to bring it close to the capillary. Make sure to keep the tip of the needle always in focus to prevent accidental breakage.
11. While pressing the injection foot-paddle, use the micromanipulator to move the needle until it slightly touches the capillary, and immediately retract it back. A constant flow of liquid at a moderate speed should be released from the tip of the needle into the oil. This step is tricky and might require some practice before getting the right size opening.
12. Raise the needle using the micromanipulator. The needle is loaded, aligned, open, and ready for injection.

### **3.6 Mounting the Worm on the Injection Pad**

1. Place a small drop of oil on the coverslip next to the agarose pad.
2. Flame to clean the edge of a pick. Dip it into the oil and then use a dissecting microscope to pick a worm that is outside of the bacterial lawn. Drop the worm into the oil droplet on the injection pad. Allow the worm to swim in the oil for a few seconds (*see Note 11*).
3. Scoop the worm from the oil and place it on the agarose pad. Allow the worm to adhere to the agarose by gently spreading out the oil on either side of the worm. To avoid dehydration, you may need to add more oil once the worm is immobilized, and the following steps should be performed quickly (*see Note 12*).

### **3.7 Injection**

1. Place the injection pad on the stage and use the 10× objective to orient the worm in a way that the dorsal side (where the distal part of the gonad is located) is facing the needle. By



**Fig. 1** Microinjection into the distal gonad arm. For microinjection, the needle is inserted into the core syncytium of the gonad (an arrow). The cytoplasmic core is at the correct focal plane for injection, when two rows of germ cell nuclei are visible (pointed arrow heads)

moving the stage, position the worm at about 15°–45° to the needle.

2. Lower the needle carefully until it is in the field of view.
3. Move to the 40× objective and focus on the core of one of the two distal gonad syncytia. When the syncytial gonad arm is in the right focal plane, it should be visualized as two rows of nuclei (on either sides of the gonad wall) with a clear cytoplasm in the middle (*see Fig. 1*). If a continuous sheet of nuclei is observed, you are probably at the surface of the gonad, and need to lower the plane of focus.
4. Carefully lower the tip of the needle into focus using the micromanipulator. Using the *x*-*y*-axis knobs place the needle near the center of this syncytium, gently touching the worm cuticle.
5. Very gently move the worm by moving the stage. First, push it up so that the needle will create a little curving in the worm cuticle, and then move it to the right, so that the needle penetrates the body wall and enters the gonad. Once the needle is inside the gonad, pull the worm away slightly to make sure that the tip of the needle is floating at the center of the cytoplasmic core.
6. To inject the DNA, press the food pedal for few seconds. You should see the gonad expanding and a flow of liquid in both the distal and proximal directions. You want to fill the gonad with as much solution as possible, but without letting the DNA solution spill out of the needle hole.

7. While still applying the pressure (to prevent the needle from clogging) pull the worm away from the needle by moving the stage to the left.
8. Raise the needle, and move the injection pad to the dissecting microscope (*see Note 13*).

### **3.8 Recovery of the Worm**

1. Under the dissecting microscope, pipette 0.5  $\mu$ l of M9 buffer on the worm. A healthy worm will be released from the agar pad and will start swimming in the buffer.
2. Use a bacteria-coated pick to transfer the worm to a seeded NGM plate.
3. Allow the worm to recover for around 30 min.

### **3.9 Selection for Transgenic Worms**

1. Pick two to three injected worms onto a seeded NGM plates and incubate at 20–25 °C.
2. Three days after injection, score the first generation (F1) for the transgenic phenotype under a standard or fluorescence-dissecting microscope, depending on the co-injection marker used. Pick transgenic F1s individually onto separate plates (*see Note 14*).
3. Three to four days later, score the F2 generation for stable transgenic lines. A line is defined if several transgenic animals are identified on the same plate, indicating a germ line transmission of the array.
4. Maintain several of these lines by selecting for the transgenic worms (*see Note 15*). Alternatively, the extrachromosomal array can be integrated into the genome [59] (*see Note 16*). This step is essential for proper expression of some of the genes that were reviewed here.

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## **4 Notes**

1. The DNA mixture is injected into the cytoplasmic syncytium at the distal gonad arm, which can be easily identified in young adult hermaphrodites. Older animals tend to accumulate a large amount of eggs in the proximal side of their gonads, reducing the volume of the syncytium in the distal part, which complicates the identification and needle insertion into the syncytium.
2. These capillaries have a fine filament adhered to their inner wall, which allows efficient backfilling of the DNA mixture to the tip of the needle by capillary force.

3. An informative video describing the process of microinjection can be found here: <http://www.jove.com/video/833/generation-of-stable-transgenic-c-elegans-using-microinjection>
4. An alternative method to injection is gene bombardment, which is based on coating micro-gold particles with DNA and using high pressure to bombard the worms. Despite the low rate of transformation, which requires starting with a large number of animals, transgenes are usually integrated into the genome at low copy number, generating stable transgenic lines [60].
5. The agarose on the injection pads is used to immobilize the worms during the injection procedure. Worms stick to pad due to absorption of water from the worm into the agarose; therefore, it is essential that the pad is completely dry.
6. Good needles are essential for successful injection. The parameters for needle pulling will vary between different needle puller models, different types of capillaries, and personal preferences. In general, the tip of the needle should be sharp (whereupon breaking the opening is narrow, ~1  $\mu\text{m}$ ), and rigid. This allows for easy insertion of the needle into the gonad, and a moderate liquid flow. If the tip of a capillary tapers too gradually, it will result in an ending that is very sharp but too flexible. Conversely, if the tip tapers too quickly the needle will be rigid but too wide. Thus, a good needle is a fine balance between the two.
7. Pulled needles can be stored for only a few weeks due to the liquid properties of the glass.
8. To identify transgenic progeny of an injected animal a visible or selectable marker should be included in the injection mix. Dominant or recessive markers can be used. The advantage of dominant markers is that they allow transformation of any *C. elegans* strain. A popular dominant marker is the pRF4 plasmid, which encodes a mutated *rol-6(su1006)* gene that results in a “roller” phenotype. This phenotype is easy to score under a standard dissecting microscope. However, since it severely affects the pattern of worm locomotion, it is not suitable for behavior analysis. Markers that drive strong expression of a fluorescence protein in a subset of cells are ideal for behavioral analysis, as they usually do not affect neuronal or muscular activity. This includes expression of fluorescence proteins under *myo-2*, *elt-2*, or *unc-122* promoters (which will drive expression in pharyngeal muscle, gut, and coelomocytes, respectively). The drawback of fluorescence markers is that selection of transgenic animals under a dissecting microscope equipped with fluorescent optics is required. For recessive markers, a rescue construct is added to the DNA mix to rescue

a mutant phenotype (such as *unc-119*, *lin-15*, and *pha-1*). This require working with mutant strains that might have an affect on animal development or behavior and thus might complicate neurobiology studies.

9. An alternative approach to working with plasmids is to generate a fusion between the desired regulatory promoter and a gene of interest using PCR and injecting the PCR product directly to the worms [61]. Such a method is described in details in a chapter by Bokman et al., in this volume.
10. If the working concentration for a plasmid is unknown, 30–50 ng/μl is a standard starting concentration. Some plasmid can be toxic or have pleotropic functions at high concentration. In these cases, concretions should be titrated down to as low as 1–5 ng/μl. On the other hand, some promoters drive very low expression levels, and hence injection of plasmids at higher concentration is required.
11. Allowing worms to swim in the oil will minimize the transfer of bacteria and liquids to the agar pad, thus facilitating the immobilization of the worm and preventing clogging of the needle with bacteria.
12. Experience users can mount and inject more than one worm at a time (up to ten worms if very skilled), If injecting more than one worm, it is important to use the pick to align the worms in the same orientation to minimize the need to change the orientation of the pad relative to the needle between injections.
13. Once you are confident with injection you can inject both gonad arms of the same worm to increase the rate of success.
14. Each transgenic F1 is considered an independent transformant, even if several come from the same injected P0.
15. DNA injection results in the formation of a large extrachromosomal array that is formed by homologous and nonhomologous recombination of plasmids (or linear DNA) injected. Those arrays that are transmitted to subsequent generations differ in the efficiency of transmission (5–95%) and in the levels of gene expression. Therefore it is advised to keep more than one transgenic line.
16. Bombardment [60] and the MosSCI [62] (described in details in a chapter by El Mouridi and Froekjaer Jensen, in this volume) techniques can be used to generate low or single-copy transgene integration to the genome, respectively. Thus if integrated lines with low to moderate transgene expression levels are desired, it is advised to consider these methods before starting the above protocol for generating transgenic worms.

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# Chapter 5

## Observing and Quantifying Fluorescent Reporters

Sreeparna Pradhan and Michael Hendricks

### Abstract

Genetically encoded fluorescent reporters take advantage of *C. elegans*' transparency to allow non-invasive, *in vivo* observation, and recording of physiological processes in intact animals. Here, we discuss the basic microscope components required to observe, image, and measure fluorescent proteins in live animals for students and researchers who work with *C. elegans* but have limited experience with fluorescence imaging and analysis.

**Key words** Microscopy, Fluorescent proteins, Digital image analysis

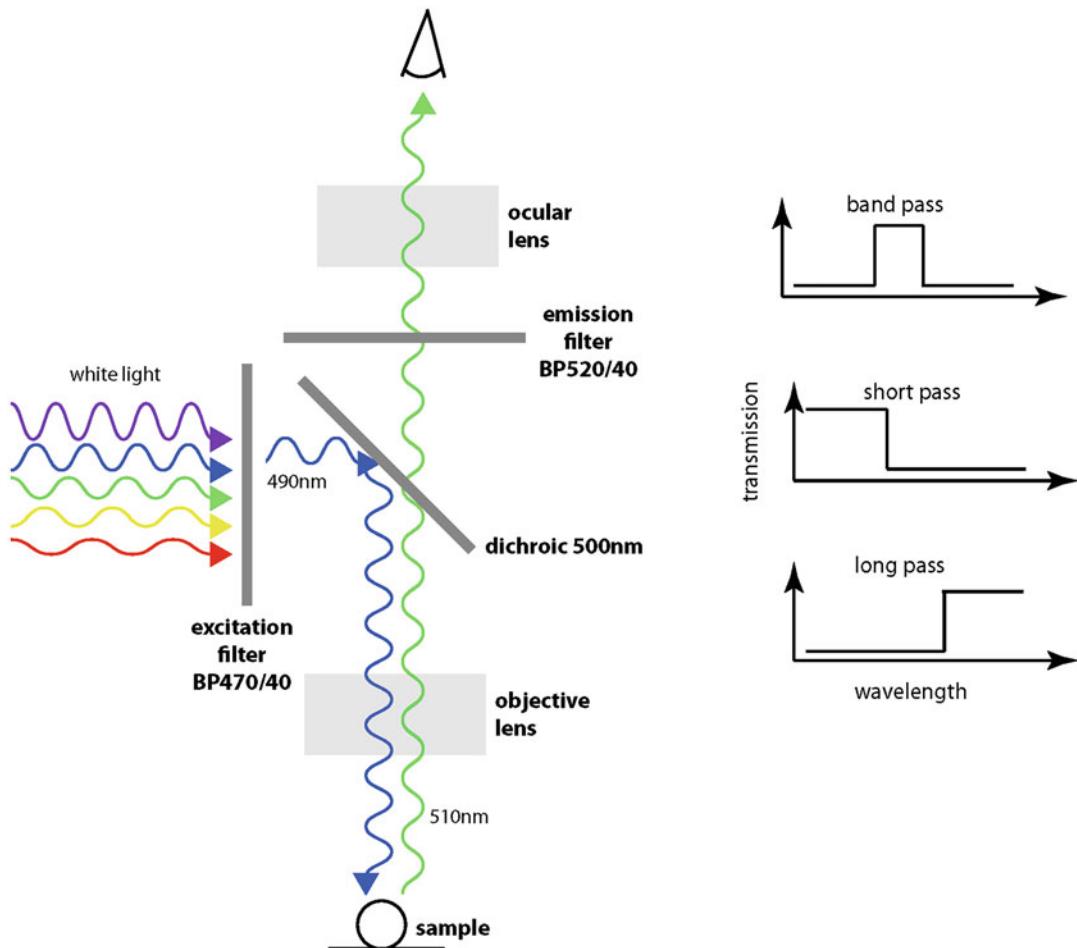
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## 1 Introduction

### 1.1 Fluorescent Molecules and Microscopy

Fluorescence occurs when an electron returns to its ground state after excitation to a higher energy state, emitting a photon. Fluorescent proteins commonly used in biology contain a fluorophore—the chemical moiety that absorbs and emits light—that is excited by a shorter wavelength of light and emits at a longer wavelength. Each fluorescent protein has characteristic ranges of wavelengths over which it is excited by and emits light, referred to as its excitation (or absorption) spectrum and emission spectrum, respectively. By using appropriate light sources and optical filters, this difference in the excitation and emission spectra allows the fluorescently labeled molecules or structure of interest to be observed with minimal background from the excitation light source.

In a simplified schematic of a fluorescence microscope (Fig. 1), a white light is passed through a filter that transmits light only in a specific range of wavelengths. “Band pass” filters such as this are usually specified by the middle wavelength and range over which they transmit light. For example, “BP470/40” is a band pass filter that transmits light ranging from 450 to 490 nm (nanometers) in wavelength. In contrast, a dichroic filter reflects light below a



**Fig. 1** Schematic of a light path in a simple fluorescence microscope. (Right) Graphical representations of common filter types encountered in fluorescence microscopy. See main text for details

certain wavelength but allows light to pass above this wavelength. In this example, the dichroic filter reflects anything below 500 nm through the objective lens and onto the sample, including the blue (490 nm) light coming through the excitation filter. Green fluorescence (510 nm) from the sample, in contrast, passes through the dichroic because of its longer wavelength.

Most fluorescence microscopes share this basic organization. Combinations of dichroic, excitation, and emission filters are usually sold assembled in “filter cubes” appropriate for particular fluorophores and fitting specific brands of microscope.

## 1.2 Fluorescent Proteins in Biology

While the use of green fluorescent protein (GFP) and its many variants is now ubiquitous in biology, *C. elegans* has the distinction of being the first multicellular organism in which GFP was demonstrated to be a useful *in vivo* marker for labeling cells [1]. Several

common ways in which fluorescent markers are used in *C. elegans* are described below.

1. *As a marker for a specific cell type or tissue.* Endogenous DNA regulatory sequences cloned upstream of GFP (or other fluorescent protein) coding sequences are used to drive expression in a subset of cells. This is most commonly used for comparative studies of the number, morphology, presence, or development of the same cells or tissues in different animals. A large number of regulatory sequence-cell type relationships have been determined for *C. elegans*.
2. *To estimate endogenous gene expression patterns and dynamics.* Upstream genomic sequences from a gene of interest are used to drive expression of a visible marker. This allows inference of normal expression patterns or regulation of expression under different conditions. A major caveat is that the results of such an experiment are not determinative of an endogenous expression pattern, which requires supplemental evidence from *in situ* mRNA hybridization or immunohistochemistry. One can never be sure that all positive and negative regulatory elements have been included, so the observed GFP transgene expression pattern may misrepresent the true expression pattern. With the advent of CRISPR/Cas9 based genome editing strategies, endogenous loci can be tagged with reporters that can reveal the true expression pattern with higher fidelity.
3. *To monitor the subcellular localization of a protein of interest.* In most cases, GFP can be expressed as a fusion protein with a gene product of interest without preventing proper protein localization or function (the ability of the fusion transgene to rescue a mutant phenotype is often a good test of this assumption). This allows the observation of the subcellular localization and dynamics of the tagged protein, though one must bear in mind the possibility of artifacts caused by excessive or ectopic expression.
4. *As a physiological reporter.* The most recently developed types of genetically encoded fluorescent probes are those that indicate changes in ion concentrations, neurotransmitters, pH, voltage, or other physiochemical processes. While the molecular mechanisms vary, these fall into two general classes:
  - (a) *Reporters based on change in intensity of a single fluorophore.* A reporter protein consists of a fluorescent fusion protein that binds a molecule of interest. Binding induces a conformational change that alters the amount of fluorescence emission, usually by altering solvent access to the fluorophore, thus changing its protonation state and fluorescence. Fluorescence intensity thus provides an indirect measurement of changes in concentration. This type of

reporter is simpler to image and analyze, but is more susceptible to motion artifacts.

- (b) *Reporters that rely on interactions between two fluorophores.* For the latter, the most common type is based on Förster resonance energy transfer (FRET). FRET occurs when two complementary fluorophores are in close proximity and dipole-dipole coupling occurs between a “donor” in its excited state and an “acceptor.” The most commonly used FRET pair in biological imaging consists of a CFP (cyan) donor and YFP (yellow) acceptor. When the reporter is in a tertiary conformation that allows the FRET interaction to occur, excitation of CFP will cause indirect emission from YFP. The ratio of CFP:YFP fluorescence is thus a measure of the relative amount of ligand-bound reporter. Because it is ratiometric, FRET measurements are less sensitive to motion artifacts.

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## 2 Materials

Because of the huge spectrum of possible reporters, protocols for handling and mounting animals, and microscope and camera styles and models, what follows will attempt to describe general principles and for observing and recording fluorescent signals in live *C. elegans*. Engineered transgenes are available as plasmid DNA. Many commonly used transgenes are available through Addgene ([www.addgene.org](http://www.addgene.org)). Published constructs not available in public repositories should be provided by authors on request.

### 2.1 Fluorescent Reporters Used in *C. elegans*

#### 2.1.1 Static Fluorescent Reporters

Static fluorescent reporters are commonly used to act as markers for cells or specific tissues, and are commonly used in cell and developmental biology. Dynamic reporters are more commonly used to observe physiological processes like neural dynamics *in vivo*.

Static reporters may include dyes and fluorescent proteins (Table 1), such as variants of GFP (EGFP, CFP, mNeonGreen) and DsRED (mCherry, mRuby3, mScarlet). Fluorescent proteins can be targeted through the addition of trafficking signals, for example to the nucleus or plasma membrane. Fluorescent proteins are also often fused to proteins of interest. Some common ways of using static fluorescent reporters are stated below:

1. *To identify subcellular localization:* DAF-16:GFP is normally localized to the cytosol, but upon environmental stress is nuclear localized [2], providing a real-time readout of the animal’s physiological state.
2. *Dyes to identify sensory structures:* Some fluorescent dyes (FITC and lipophilic dyes such as DiI and DiO) are preferably taken

**Table 1**  
**Commonly used fluorescent proteins**

| Fluorescent protein    | Peak Emission | References   |
|------------------------|---------------|--------------|
| mTagBFP2               | 456 nm        | [43]         |
| mCerulean3             | 474 nm        | [44]         |
| mTFP                   | 495 nm        | [44]         |
| mNG                    | 506 nm        | [45]         |
| mNeonGreen             | 506 nm        | [46]         |
| mEGFP                  | 509 nm        | [46]         |
| GFPnovo2               | 509 nm        | [47, 48]     |
| YFP                    | 526 nm        | [49]         |
| mECitrine              | 527 nm        | [44]         |
| mYPet                  | 530 nm        | [45, 50, 51] |
| CemOrange2             | 562 nm        | [49]         |
| mKO2                   | 566 nm        | [44]         |
| Dendra2                | 553 nm/573 nm | [52]         |
| TagRFP-T               | 589 nm        | [45]         |
| cyOFP1                 | 589 nm        | [9, 53]      |
| mScarlet               | 592 nm        | [44]         |
| mRuby, mRuby2          | 605 nm        | [54, 55]     |
| mKate2                 | 605 nm        | [49]         |
| mCherry                | 610 nm        | [56, 57]     |
| mNeptune, mNeptune 2.5 | 634 nm        | [44, 58]     |
| CemCardinal2           | 642 nm        | [49]         |

up by amphid and phasmid neurons, and abnormal or absent “dye-filling” has been used to screen for development defects in sensory structures [3].

3. *Interaction between cellular partners:* Self-associating split fluorescent proteins are used to label potentially interacting cellular partners which when come together form a functional fluorophore. These are useful to label synaptic partners and in identifying protein assemblies. Split-GFP fused to synaptic proteins (GFP Reconstitution Across Synaptic Partners- GRASP) has been a powerful tool for screening for mutations in synaptic growth and morphology [4]. More recently, this system has been expanded to include red fluorophores like sfCherry2, sfCherry3 (sfCherry3 Linker Across Synaptic Partners-

CLASP) which will enable multicolor imaging of complex interactions across synapses [5].

4. *To observe degradation of fusion proteins:* Differently colored fluorophores can be combined with the auxin-inducible degron system to observe effects of depleting a particular protein with respect to another tagged with a different fluorophore [6, 7].
5. *To resolve neuronal identities in volumetric imaging:* Whole-brain imaging methods often use a static fluorophore of a different color (for example, red-shifted fluorophores) while imaging dynamics of GCaMP fluorescence. These cellular markers are often nuclear localized and help to identify cells and in motion correction [8]. More recently, a strain called Neuro-PAL (Neuronal Polychromatic Atlas of Landmarks) has been developed by combining four different fluorophores which uniquely identifies neurons with a distinct colored label [9].

### 2.1.2 Calcium

Genetically encoded calcium indicators (GECIs) are widely used to measure neural activity in *C. elegans* in vivo.

1. One of the earliest in vivo uses of the FRET-based calcium indicator Cameleon was in *C. elegans* muscles and neurons [10, 11]. Ratiometric indicators are useful to account for motion artifacts, but there are other methods for motion correction more commonly used now.
2. The non-ratiometric  $\text{Ca}^{2+}$  indicator GCaMP (and its variants) has become the standard calcium imaging reagent in *C. elegans* [12, 13]. The latest versions of GCaMP (GCaMP6, GCaMP7, GCaMP8) exhibit improved signal-to-noise ratios, higher dynamic ranges and have versions for fast and slow kinetics. The website of Loren Looger's lab is a good way to keep up to date with GCaMP technology, and plasmids encoding these sensors are available through Addgene.
3. RCaMP is structurally similar to GCaMP, but is based on mRuby instead of GFP. It is excited by green light and emits red [14].
4. G-GECOs are a family of genetically encoded calcium indicators that vary in their excitation/emission wavelengths [15]. They have not been extensively used in *C. elegans*, and some early attempts suggest they may not perform as well as GCaMPs in nematode neurons. However, the possibility of a wide range of multi-spectral calcium imaging tools is intriguing.

### 2.1.3 Voltage

The use of genetically encoded voltage reporters in *C. elegans* is in its infancy, but has enormous promise. Early-generation reporters suffered from low signal-to-noise and suboptimal kinetics.

Genetically encoded voltage indicators (GEVIs) used in *C. elegans* are usually either derived from microbial opsins or are voltage sensitive proteins tagged with a fluorophore like GFP [16].

1. Arclight and its variants range in fluorescence amplitude from ~8% to 35%, with an associated trade-off in speed [17].
2. Two new variants of the Archaeorhodopsin-3 proton pump, Archer1 and Archer2, have been validated in *C. elegans* sensory neurons [18]. Stimulus conditions that give maximal calcium responses in the AWC sensory neuron showed 40% increases in Archer1 fluorescence. Archers can be used as voltage sensory when excited by red (655 nm) light; under green light they play the more familiar Archaeorhodopsin role of hyperpolarizing pumps.
3. A FRET-based voltage sensor class called VSFP-Butterflies has been recently developed [19] and has reportedly been successfully optimized for *C. elegans*.
4. The opsin-based fluorescent voltage reporter Archon1 has been used to image voltage transients in the AVA reversal command neuron in *C. elegans* [20]. The absence of blue-light crosstalk makes this sensor suitable to be coupled with optogenetics, and shows no photobleaching over behaviorally relevant timescales. Archon1 fluorescence was observed in AVA when an upstream nociceptive neuron ASH was optogenetically activated.
5. A recent study tested a range of microbial opsin-based voltage sensors such as Arch(D95N), QuasAr, Archon and eFRET based sensors like MacQ-mCitrine and QuasAr-mOrange in pharyngeal muscles and specific locomotory neurons of *C. elegans* and were able to robustly detect action potentials with significant changes in fluorescence [21].

#### 2.1.4 Physiological Reporters

A variety of other physiological reporters have been used in *C. elegans* or show significant promise for future use.

1. Clomeleons are FRET-based **chloride** reporters [22, 23], which potentially provide a crucial means of interrogating inhibitory synaptic connections and may be of particular interest to *C. elegans* researchers due to the prevalence of transmitter-gated chloride channels. All current chloride sensors suffer from a high degree of pH dependence.
2. **Redox** state can be measured through expression of genetically encoded redox sensors [24, 25].
3. The difference between vesicular and extracellular **pH** has been exploited to observe synaptic vesicle fusion using the pH-sensitive GFP variant pHluorin fused to synaptobrevin (SNB-1) to create synaptopHluorin [26, 27].

4. Neurotransmitter sensors like the **glutamate** (iGluSnFR) sensor [28] are now being commonly used in *C. elegans* to directly visualize glutamate release [29, 30]. A number of other sensors to visualize serotonin release (iSeroSnFR, GRAB<sub>5-HT</sub>), acetylcholine release (iAchSnFR) [31–33], etc. have been made available more recently.

## **2.2 Transgenic Worms**

The design of plasmid DNA constructs for creating transgenic *C. elegans* is beyond the scope of this chapter. For guides to generating transgenic worms by microinjection, see Chapters 10–12. Many transgenic strains are available from the *C. elegans* Genetics Stock Center (CGC) at the University of Minnesota, and published strains can usually be obtained by contacting the authors directly.

## **2.3 Microscope Equipped with Fluorescence Components**

A fluorescence microscope with a digital camera is essential. Again, there are too many variations available to address the use of specific equipment, and what follows are general guidelines. Many markers can be observed under relatively low-magnification fluorescence stereomicroscopes. Detailed morphology or quantitative measurement often requires increased magnification and the use of high numerical aperture objective lenses found on compound microscopes. Your microscope must be equipped with a light source and filter sets that are appropriate to the properties of the fluorophore you wish to observe.

## **3 Methods**

### **3.1 Mounting and Immobilization**

Live animals must be mounted in a way that permits observation and either allows or restricts movement according to experimental design. Observing and recording from immobilized animals is much simpler, and several methods can be used as follows.

1. *Cyanoacrylate glue*. Gluing worms to agar pads is the preferred method for electrophysiological recordings and is commonly used for imaging experiments as well [34].
2. *Microfluidic devices*. A huge number of designs have proliferated for small fluid channel chips for restraining, sorting, observing, and stimulating live worms. These have the advantage of avoiding the use of drugs or harsh chemicals, but require some specialized materials and preparation, reviewed in Chapter 16 of this book.
3. *Nanoparticle immobilization*. A simple method of immobilizing worms on agar using sub-micron polystyrene beads was recently reported [35]. Friction between the beads and the agar and cover slip prevents movement and allows for long-term imaging without the use of drugs and simple recovery of animals after imaging.

4. *Hydrogels.* Pluronic (PF127), a copolymer that undergoes reversible thermogelling at specific temperatures is also used in combination with polystyrene bead spacers to mount and immobilize animals on slides [36]. Vertical compression of animals between the slide and the cover slip maintained by appropriate dimensions of the microbeads together with the viscosity of the hydrogel restrict motility.
5. *Anesthetics.* Worms can be immobilized with anesthetics such as levamisole or low levels of sodium azide on agar pads and observed through cover slips. This is often not desirable as these compounds may interfere with the physiological processes in question [37]. They are usually appropriate for observing cellular expression patterns or morphology. However, when imaging circuit-wide neural dynamics, it is often necessary to immobilize animals with low doses of levamisole in addition to other methods of physical restraint like microfluidic devices or beads [38–40].

### 3.2 Acquiring Images

Documenting your fluorescent reporter requires a camera. Once again, a huge range exists, from inexpensive point-and-shoot equivalents that are useful for routine documentation, to highly specialized cameras with enormous sensitivity and speed. Some general principles for digital imaging are useful to bear in mind. These parameters are commonly found as adjustable settings in digital imaging software.

1. A grayscale digital picture consists of an array of pixels, each with a single numerical value assigned (*see Note 1*), ideally saved in an uncompressed file format (*see Note 2*). Most imaging software allows you to view a histogram of the distribution of pixel values in your image. It is usually important to make sure this entire distribution lies within the dynamic range of the camera for the current setting. Both minimum and maximum (also called a saturated pixel) values should be avoided for any pixel in a structure of interest, as this will distort measurements made later. In addition, some cameras can be damaged by long-term saturation.
2. Pixel values are a function of illumination intensity, exposure time, and both intrinsic and adjustable properties of the camera sensor. The light intensities used for fluorescence microscopy can be noxious or harmful to *C. elegans* over time. Lower intensity illumination and longer exposure times are less harmful, but increase noise and motion artifacts and decrease sensitivity. Shorter exposure times with higher illumination address these problems, but can be damaging or aversive to the organism and cause photobleaching of the fluorophore. Optimizing

these competing parameters is essential for each sample and experimental condition.

3. For time lapse acquisition, a frame rate must be set as well. Determining an optimal frame rate depends on the exposure time (the sampling interval must be greater than the exposure time, *see Note 3*) and on the temporal features of the signal you wish to measure (*see Note 4*).

### 3.3 Image Analysis and Measurement

Digital image analysis requires specialized software, which are either sold commercially or are custom-built. Acquisition software often include some analysis tools. Excellent free resources also exist.

For relatively short-term imaging of a limited number of regions of interest, by far the best choice for straightforward analysis is ImageJ, in particular the Fiji ImageJ distribution (<http://fiji.sc/Fiji>) [41, 42]. A huge number of free ImageJ plugins exist that perform functions that once required software costing thousands of dollars.

1. *Image segmentation.* Segmentation refers to the process of defining or selecting objects or regions of interest (ROI) within an image.
  - (a) Perhaps the simplest form of segmentation is based on pixel value. This can be done by direct thresholding by selecting all pixel values over a minimum threshold or using Otsu thresholding algorithm that classifies objects based on multiple intensity thresholds. The “Threshold” function in Fiji allows users to select pixels that are within a selected range of values.
  - (b) Alternatively, ROIs can be manually defined using drawing tools. Multiple ROIs can be defined in the ROI Manager.
2. *Motion correction.* In time lapse images, some amount of movement is inevitable. There are two primary approaches to dealing with objects of interest moving between frames:
  - (a) Frames can be automatically aligned using stack registration algorithms included in Fiji or available as ImageJ plugins. This is preferred when the image contains minor motion artifacts, and allows selection of a single ROI that is appropriate across all frames.
  - (b) Algorithms that segment and track moving objects across multiple frames are available, though these are more complex. These can include steps like repeated clustering, straightening, and registration, or custom machine vision approaches to train neural networks to recognize ROIs. This approach is required when multiple objects of interest move relative to one another throughout the time series.

3. *Measurement.* Fiji can measure a number of attributes of the pixels contained within an ROI as part of a defined object. For intensity, normally the mean pixel value of the object is measured.
4. *Normalization and analysis.* Raw pixel values are not informative with respect to the absolute amounts of a fluorescent reporter. In time lapse, it is common practice to select a baseline value corresponding and to measure intensity changes relative to this baseline. Differences in raw pixel values can arise from many sources, including expression level, illumination intensity, mounting method, and camera acquisition settings. For this reason, comparing intensities across animals is difficult; however, for a given set of carefully controlled imaging conditions, fluorescence intensity of a static reporter can, for example, be used as a proxy for expression level.

For measuring fluorescence intensity of calcium indicators to measure neural activity, the change in fluorescence is usually measured as  $\Delta F/F_0$ , where  $F_0$  is the baseline average intensity value of a defined time period before exposure to the stimulus.

For long-term imaging, photobleaching is common. This must be corrected by fitting the raw intensity data to a decaying exponential.

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## 4 Notes

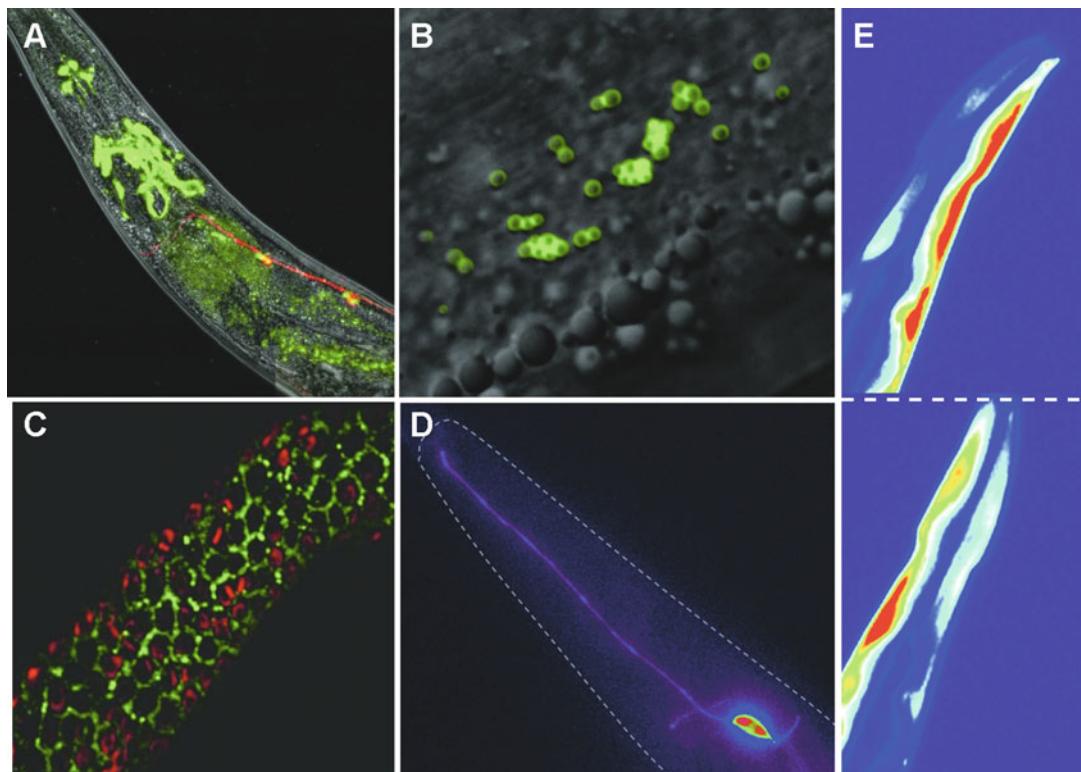
1. A simple way to think of a digital camera is as a sensor array that assigns values based on the number of photons collected over a given time period (the exposure time). “Bit depth” refers to the range of possible values for each pixel. An n-bit image has  $2^n$  values for each pixel. Common image formats include 8-bit (0–255), 12-bit (0–4095), and 14-bit (0–16,383). Larger bit depths increase file size, but provide a greater dynamic range.
2. Generally, compressed file formats should not be used for quantitative imaging. The most commonly found uncompressed file formats are TIFFs ([tagged image file format](#), which can be single or multi-layer) and AVI ([audio video interleave](#), for uncompressed movies).
3. *Optimal frame rates.* Undersampling occurs when the frame rate is too low to accurately measure events at a frequency of interest, introducing aliasing artifacts. The Nyquist-Shannon Theorem gives a simple metric for determining frame rate: the sampling rate must be at least  $2 \times$  the frequency of the lowest bandwidth events you wish to measure. For example, if you wish to measure a signal at 5 Hz, you must image at a minimum of 10 frames per second.
4. *Motion artifacts.* Long exposure times and inadequate immobilization contribute to motion artifacts. The most common

artifact consists of “smearing” of a fluorescent object over the course of an exposure. The major effects are to increase the apparent size of the object and decrease its mean fluorescence intensity. A second common motion artifact is caused by shifts in focal plane caused by muscle contractions. Depending on the source of the problem, suspected motion artifacts can be controlled for in several ways:

- (a) Decreased exposure times.
- (b) Improved immobilization.
- (c) Comparison or ratio measurement with a static fluorescent reporter.

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**Fig. 2** Example of in vivo fluorescent reporters. (a) The promoter of *acc-1* driving expression of GFP in neurons of the head. (b) A GFP fusion to DGAT-2 labels lipid droplets in muscles. (c) Actin filaments (GFP, green) and nuclei (DAPI, red) in the adult germline. (d) The genetically-encoded calcium indicator GCaMP3 (pseudocolored) expressed in the AWC sensory neuron. (e) GCaMP3 (pseudocolored) expressed in muscles under the *myo-3* promoter during a ventral (top) and dorsal (bottom) anterior body bend

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# Chapter 6

## Microbial Rhodopsin Optogenetic Tools: Application for Analyses of Synaptic Transmission and of Neuronal Network Activity in Behavior

Amelie Bergs, Thilo Henss, Caspar Glock, Jatin Nagpal,  
and Alexander Gottschalk

### Abstract

Over the past 15 years, optogenetic methods have revolutionized neuroscientific and cell biological research, also in the nematode *Caenorhabditis elegans*. In this chapter, we give an update about current optogenetic tools and methods to address neuronal activity and inhibition, as well as second messenger signaling, based on microbial rhodopsins. We address channelrhodopsins and variants thereof, which conduct cations or anions, for depolarization and hyperpolarization of the membrane potential. Also, we cover ion pumping rhodopsins, like halorhodopsin, Mac, and Arch. A recent addition to rhodopsin-based optogenetics is voltage imaging tools that allow fluorescent readout of membrane voltage (directly, via fluorescence of the rhodopsin chromophore retinal, or indirectly, via electrochromic FRET). Last, we report on a new addition to the optogenetic toolbox, which is rhodopsin guanylyl cyclases, as well as mutated variants with specificity for cyclic AMP. These can be used to regulate intracellular levels of cGMP and cAMP, which are important second messengers in sensory and other neurons. We further show how they can be combined with cyclic nucleotide-gated channels in two-component optogenetics, for depolarization or hyperpolarization of membrane potential. For all tools, we present protocols for straightforward experimentation to address neuronal activation and inhibition, particularly at the neuromuscular junction, and for combined optogenetic actuation and  $\text{Ca}^{2+}$  imaging. We also provide protocols for usage of rhodopsin guanylyl and adenylyl cyclases. Finally, we list a number of points to consider when designing and conducting rhodopsin-based optogenetic experiments.

**Key words** Optogenetics, Channelrhodopsin-2, Halorhodopsin, Archaerhodopsin, Mac, Calcium imaging, RCaMP, Electrochromic FRET sensor, Guanylyl cyclase rhodopsin CyclOp, Two-component optogenetics, TAX-2/TAX-4 CNG channel

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Amelie Bergs and Thilo Henss contributed equally to this work.

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## 1 Introduction

Optogenetics allows analyzing genetic determinants of synaptic transmission at the neuromuscular junction, as well as the causative role of single neurons in the generation of behavior, or their effects on signaling within neuronal circuits. This involves activators and inhibitors, which can be used as single tools, or in combination in different nodes of a neuronal network. Rhodopsin optogenetic tools are most commonly used. Opsins consist of seven transmembrane helices and require the vitamin A-related organic molecule retinal as a cofactor. When retinal is bound, the functional opsins are called rhodopsins. Retinal isomerizes upon the absorption of a photon, thus triggering a conformational change of the protein, which then enters a distinct photocycle [31–33]. Whereas type I rhodopsins combine the two tasks of light-sensation and (passive or active) ion flux across the plasma membrane, type II rhodopsins are G-protein coupled proteins that activate intracellular signal transduction pathways. Channelrhodopsin-2 (ChR2), a blue light-gated cation channel [34, 35], was the first microbial rhodopsin to be used for depolarizing neurons [2] and further to modulate behavior in a living organism [3]. In later studies, the yellow light activated chloride pump Halorhodopsin (NpHR) was used to hyperpolarize neurons [36]. Besides NpHR, archaerhodopsin (Arch) and the fungal rhodopsin Mac [37, 38] have emerged as further inhibitory optogenetic tools. More lately, anion channelrhodopsins, conducting chloride ions, were discovered or engineered in the lab [15, 39–41]. These constitute the most potent inhibitory rhodopsins, though in contrast to NpHR, they enable only shunting inhibition, as they cannot transport  $\text{Cl}^-$  against a gradient. Also, in neurons or neuronal compartments with inverse  $\text{Cl}^-$  gradient, these ACRs can be depolarizing [42]. Nowadays, the optogenetic toolbox comprises a wide variety of rhodopsins and variants thereof that are used to directly activate, directly inhibit or to indirectly modulate neurons [5, 15, 43]. We here included a chapter on the more recent additions to the *C. elegans* rhodopsin-based optogenetic toolbox. In addition, we describe the rhodopsin cyclases, which can be used as membrane-bound light-activated enzymes for the generation of cGMP, or, after mutation of the specificity-determining aminoacids, cAMP.

The experimental outcomes of optogenetic manipulations are evident by behavioral, electrical, or optical readouts [8, 44]. Combination of electrical recording and optical stimulation constitutes a powerful way to analyze the functionality of distinct neuronal connections [45, 46], but is less easily applied in *C. elegans*. Here, this methodology is feasible to investigate different mutants with pre- or post-synaptic defects to understand and/or determine their impact on synaptic transmission [47–50]. Optical readouts of

neuronal or network function are mostly obtained by calcium imaging. Genetically encoded calcium indicators (GECIs) permit a cell type specific observation of neuronal activity [51, 52]. However, the spectral overlap between the action spectrum of the optogenetic tool and the excitation spectrum of the GECI complicate this approach [53]. Yet, color-shifted variants of both sensor (like RCaMP; [44, 54, 55]) and actuator (like Chrimson; [56, 57]), enable more all-optical experiments nowadays. A recent addition in this context are the rhodopsin-based voltage imaging tools, which enable imaging of electrical events and can be combined with optogenetic actuation to some extent [27, 28, 30]. These proteins include variants of archaerhodopsin, which show a linearly voltage-dependent dim fluorescence of the retinal chromophore [58, 59] as well as the so-called electrochromic Foerster resonance energy transfer (eFRET) sensors [60–62], which combine archaerhodopsin and a fluorescent protein fused to it, that can transfer energy to the rhodopsin upon depolarization, and thus drops in its own fluorescence (which is more readily observable). Still, many applications of rhodopsins for functional circuit analyses in *C. elegans* lie in the combination of optogenetic actuation and simultaneous analysis of animal behavior [3, 47, 49, 54, 63–73].

## 2 Materials

### 2.1 Optogenetic Activation: Depolarizing Membrane Potential

#### 2.1.1 Channelrhodopsin-2

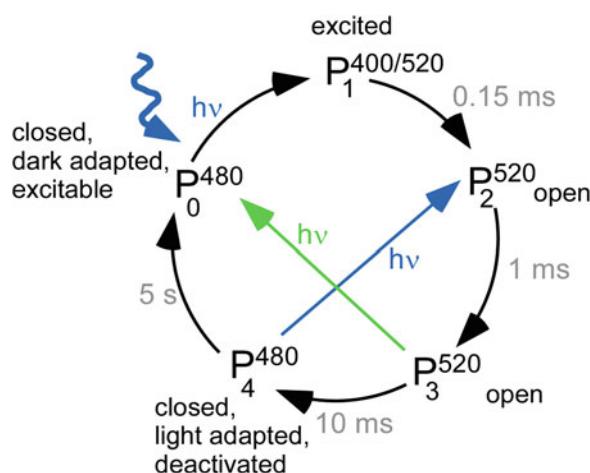
Channelrhodopsin-2 and other microbial rhodopsin-based optogenetic activators enable a spatio-temporally precise control of genetically targeted neurons. This optogenetic activation allows us to study their causative role in generating diverse behaviors.

Channelrhodopsin-2 (ChR2) is a directly light-gated cation channel endogenous to the green alga *Chlamydomonas reinhardtii*, where it is enriched in the so-called eye spot region, mediating phototaxis and photophobic responses [35, 74]. ChR2 requires all-*trans*-retinal (ATR) as a cofactor [75]. This light-absorbing chromophore is embedded within the hydrophobic core of the seven transmembrane (TM) helices, where retinal binds covalently to a conserved lysine residue of TM domain seven by forming a Schiff base [76]. A protonation of the Schiff base shifts the absorption of light into the blue range of the spectrum (around 470 nm). Upon absorption of a photon, retinal isomerizes from the all-*trans* to the 13-*cis* configuration in less than 50 ns and triggers a conformational change of the protein that causes the opening of the channel [34, 35]. This induces an inward rectifying current for different monovalent (e.g.  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{H}^+$ ) and some divalent cations, including (minute amounts of)  $\text{Ca}^{2+}$ , and in the native

context of a cell, particularly a neuron, thus depolarizes its plasma membrane [2, 3, 35].

The extent of current mediated by ChR2, as compared to other ion channels, is rather small (ca. 40 fS in contrast to ca. 40–60 pS in the case of a nAChR) [77]. In non-spiking neurons of *C. elegans*, all of the current required to activate a neuron has to be provided by ChR2, while in (mammalian) neurons firing action potentials, one “just” needs to reach threshold [11]. This implies that ChR2 expression levels need to be high, such that “strong” promoters should be used. Yet, there are more light-sensitive, conductive / better expressing variants of ChR2 available, that can be used as alternatives (see below).

The light-induced conductive state of ChR2 is part of a photocycle (Fig. 1) that underlies the mechanism of the channel [33]. In the dark, ChR2 is in the ground state D470. After absorption of a blue photon (maximal activation is reached with light of ca. 460 nm; however, as the rhodopsin spectrum is rather broad, light between ca. 350 and ca. 530 nm can activate ChR2), the intermediate state P500 is formed within nanoseconds by the light-induced isomerization of retinal. The following deprotonation of the Schiff base to the extracellular side converts the protein to the P350 state within ca. 25 µs. Reprotonation of the Schiff base from the cytoplasmic side leads to the conducting state P520 [32, 77, 78], which is in a pH-dependent equilibrium with the deprotonated form P390. Upon closing, the channel changes to the intermediate states P480<sub>a</sub> and P480<sub>b</sub> before it eventually recovers to the ground state after ca. 45 s. In a flash-photolysis (i.e. a “single-photon”) experiment, the latter is the rate-limiting step of the photocycle, in which hydrogen bonds of amino acids



**Fig. 1** Photocycle of Channelrhodopsin. Adapted from [32]. Blue light induces the conducting state, but can also short-circuit the light-adapted dark state to the open state. Green light can lead to channel closure

change and backbone rearrangements occur. However, during continuous blue light, P480<sub>b</sub> absorbs a photon to be directly converted into the early P500 intermediate, thereby circumventing the ground state D470. Thus, after a brief peak current, involving all available channels, steady state currents occur, as a distinct proportion of channels in the dynamic equilibrium can be converted again to open state after channel closure, i.e. the stationary-state level results from the desensitization of a fraction of channels. The larger the proportion of channels in the light-sensitive state, the more can be opened by light. The shorter the time required for channel closure and reactivation, the more channels can regain a light-sensitive state. From these effects, a higher steady state current will result. This has been achieved by certain mutations, e.g. in the “high efficiency” E123T variant [79]. Upon repeated stimulation, ChR2 exhibits a desensitized state, which leads to reduced initial peak currents in successive trials. Desensitized channels recover in the dark only slowly, with a typical time constant around 5 s, influenced by the extracellular pH and the membrane potential [35, 47]. Once the channel is open, it closes by spontaneous progression through the photocycle. However, channel closure may also be achieved by green light illumination of the P520 state [78]. In sum, size and the kinetic properties of the ChR2 photocurrent are a result of the described photocycle, the ion selectivity and the time constants of activation, deactivation, and inactivation [31].

In comparison to rhodopsins of higher eukaryotes, retinal does not dissociate from its opsin molecule after activation, but thermally reisomerizes to the initial all-*trans* state [80]. Because nematodes do not generate ATR, exogenous ATR has to be added to the bacteria lawn in order to render the channel functional [3]. However, transgenic animals grown without ATR can serve as convenient negative controls for ChR2 experiments in *C. elegans*.

### 2.1.2 ChR2 Variants with Distinct Functional Properties for Specific Applications

ChR2 is a powerful tool, but there is also room for improvement. Thus, ChR2 variants that enable specific optogenetic applications were engineered by site-directed mutagenesis, yielding channels with altered ion selectivity and conductance, spectral properties or photocycle kinetics. The mutated residues are often near the retinal-binding pocket or in close proximity of the putative pore of the channel. ChR2 with histidine 134 mutated to arginine, ChR2(H134R), shows a two-fold extended open state life time, a two-fold higher Na<sup>+</sup> conductance and a two-fold lower degree of inactivation [3, 81]. Conversely, the mutation of glutamate 123 to threonine, ChR2(E123T), shortens closing kinetics significantly and is thus capable to elicit firing frequencies in neurons with more than 200 Hz [79]. This has also been used to elicit very fast depolarization of neurons for electron microscopy analyses of the

stimulated neuromuscular junction [82]. Mutation of threonine 159 to cysteine, ChR2(T159C), displays up to tenfold enhanced photocurrents, due to higher conductance and/or better plasma membrane trafficking, as well as faster kinetics [83, 84]. The mutation leucine 132 to cysteine, ChR2(L132C), exhibits an enhanced calcium conductance by sixfold [85]. ChR2 was also altered to allow long-term manipulation at low light intensities [86–88]. Here, mutations within the so-called DC-gate (D156 and C128 (e.g. mutated to serine, C128S), step-function-opsins, SFOs) significantly delay the closing of the channel in the dark to several minutes, thus a large proportion of the channels accumulates in the open state. Similar effects were found for the D156A and D156C (ChR2-XXL) mutant as well as the C128S; D156A double mutant [89, 90], while a quintuple ChR2 variant (“QUINT,” ChR2(C128S;L132C;H134R;D156A;T159C)) showed the longest persistent depolarization observed in the worm so far (8–10 h following a single blue light pulse) [15]. For low light applications, ChR2-XXL is the reagent of choice, demonstrating significant activation (light-evoked contraction in muscle) at intensities as low as  $0.26 \mu\text{W/mm}^2$  [15, 88]. This effectively reduces the light intensity required to achieve activation, and both effects allow stimulating cells in the long term (even for hours) without the need for continuous intense blue light exposure that would be harmful. Alike wild type ChR2, this variant can be turned off by yellow or green light [86, 87]. While handling SFO expressing animals, especially QUINT and ChR2-XXL transgenics, working light should be reduced to a minimum, since even red filters for transmission light are insufficient to circumvent at least minor pre-activation of animals. Hence, after transferring SFO animals to experimental plates, a subsequent recovery period of several minutes in the dark is advisable before starting the experiment.

In general, we find that combining the H134R mutation with the above mentioned mutations improves the function in *C. elegans*, for unknown reasons. Last, variants of ChR have been generated that have altered activation spectral peaks. The chimeric protein C1V1, composed from *Chlamydomonas reinhardtii* ChR1 and *Volvox carteri* ChR1, with two additional mutations, is activated with green light instead of blue light [89, 91]. However, due to the naturally broad absorption band of rhodopsins, it can still respond to blue light, and is furthermore quite light-sensitive. Thus, in order to allow for independent blue and green activation of two distinct neuron populations, with ChR2 and C1V1-ETET, respectively, the highly efficient ChR2(H134R; T159C) double mutant should be used (to allow efficient activation with blue light while not co-activating C1V1), and the relative expression levels of the two proteins need to be carefully titrated [84].

## 2.2 Optogenetic Inhibition: Hyperpolarizing Membrane Potential

### 2.2.1 Halorhodopsin

The ability to silence the activity of genetically defined neurons in a temporally precise fashion allows investigating the causative role of the defined neuron cell type in driving the behavior. When used in combination with channelrhodopsins, optogenetic inhibitors enable multimodal optical interrogation of neural circuits [36, 38].

Optical inhibition of neural activity was first achieved using the yellow light-gated chloride pump Halorhodopsin (NpHR), from the archaeon *Natronomonas pharaonis* [36]. When expressed in *Xenopus* oocytes, it produced vectorial, hyperpolarizing inward currents associated with chloride influx into the cell, with a peak photocurrent observed at 590 nm excitation. Illumination with yellow light potently inhibited action potential firing in cultured hippocampal neurons expressing NpHR. Activation of NpHR in *C. elegans* body wall muscle cells resulted in the extension of the worm's body due to hyperpolarization and relaxation of muscle cells. In contrast to ChR2, which is a channel that is opened by absorption of a photon to allow passage of many ions, NpHR as a pump allows for only one ion to be transported per photocycle. Thus, as is the case for ChR2, though for different reasons, also NpHR requires robust plasma membrane expression. Unfortunately, the protein traffics inefficiently to the plasma membrane in eukaryotes. Hence, molecular engineering of NpHR, comprising of incorporation of ER export and trafficking motifs from mammalian membrane proteins, was performed [92, 93]. However, incorporating these plasma membrane targeting signals in NpHR did not enhance NpHR's ability to photo-inhibit excitable cells in worms as these signals are not conserved in *C. elegans* [38]. Thus, for a strong and robust inhibition, massive over-expression of NpHR is required in worms, such that enough protein makes it into the plasma membrane. This is not always possible, unless a strong promoter is used to drive its expression, which limits the neuronal networks that can be investigated in this ideal model system for optogenetics.

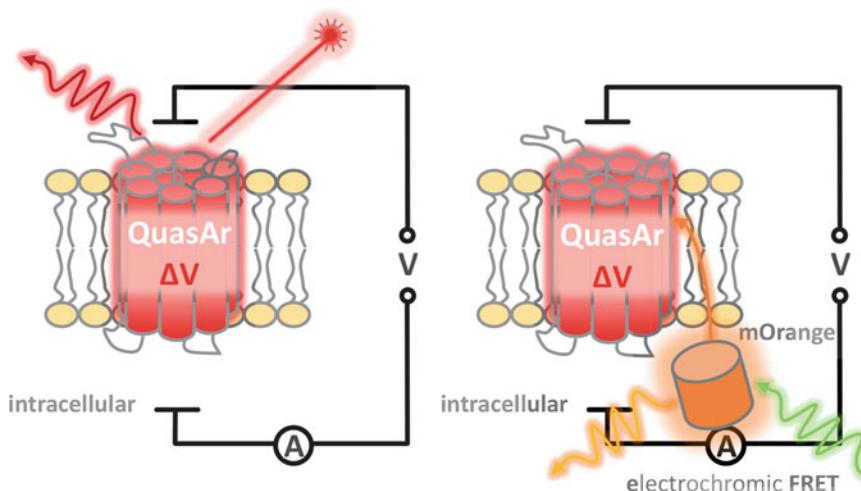
### 2.2.2 Archaerhodopsin and Mac

As alternative to NpHR for neuronal photoinhibition, light-driven outward-directed proton pumps were discovered or characterized for utilization in optogenetics in an extensive screen of microbial opsins carried out by Chow and colleagues [37]. Archaerhodopsin-3, known as Arch, from *Halorubrum sodomense*, and Mac, from the fungus *Leptosphaeria maculans*, are activated by yellow-green and blue-green wavelengths of light, respectively, and express well on the neural plasma membrane. The magnitude of photocurrents mediated by outward proton flux through Arch and Mac are higher than those with NpHR's chloride influx, making them more potent photo-inhibitors. Both Mac and Arch have been effectively utilized to inhibit excitable cells in *C. elegans* [38, 63]. Using behavioral assays and current recordings from dissected muscle cells expressing

NpHR, Mac or Arch, Husson and colleagues showed that Arch is the most potent hyperpolarizer when illuminated with yellow-green light. Also, the action spectrum of Mac is blue-shifted as compared to NpHR and Arch (primary peaks of their action spectra are 550 nm for Mac, 566 nm for Arch, and 590 nm for NpHR). Using the nociceptive ASH network, they also demonstrated that Mac and Arch can be used to interfere with downstream signaling in networks in which upstream neurons are stimulated by ChR2, using multimodal illumination strategies. A concern with the usage of the proton pumps is the gradual elevation of proton concentration in the extracellular space, which might have deleterious or non-specific effects on the activity of neurons. As the pseudocoelom is well buffered, this may not be a problem.

### 2.2.3 Voltage Imaging Tools

Since  $\text{Ca}^{2+}$ -imaging often fails to display fast neuronal dynamics, such as action potentials, the dim, but linear, voltage-dependent fluorescence of Archaerhodopsin (Arch) mutants is employed in the emerging field of rhodopsin-based voltage imaging [29, 58, 94]. In addition, voltage imaging also allows to reflect hyperpolarization, which is not the case for  $\text{Ca}^{2+}$ -imaging. Molecular engineering of the light-driven proton-pump Arch resulted in various mutants that are pump-dead and show an improved brightness and voltage sensitivity [30, 59]. Moreover, eFRET sensors were established, by fusing a fluorescent protein to the sensor (Fig. 2). Here, the bright fluorescence of the fluorescent protein is imaged, which becomes quenched upon depolarization of the membrane voltage,



**Fig. 2** Genetically encoded voltage sensors based on microbial rhodopsins. (a) QuasAr, a mutated version of Archaerhodopsin, can be used as a directly fluorescent voltage sensor, excited by strong 637 nm laser light, however, its fluorescence is dim. (b) As a combination (fusion) with fluorescent proteins (like mOrange), sensors like QuasAr (or Arch(D95N) or MacQ) can be used as indirect sensor, that quenches the fluorescence of the XFP during depolarized states, due to electrochromic Foerster Resonance Energy Transfer (eFRET)

thus enhancing the brightness of the imaging tools [28, 62]. As for GECIs, rhodopsin-based voltage indicators can be combined with other, mostly blue light activatable optogenetic actuators such as ChR2 or ACR2 [27, 59, 95].

In a *C. elegans* side-by-side study, the Arch mutant QuasAr [59] performed best and could reliably demonstrate ChR2- and ACR2-evoked de- and hyperpolarization, as well as recording of spontaneous action potentials in muscle cells ( $\sim 78\% \Delta F/F$  per 100 mV) [27].

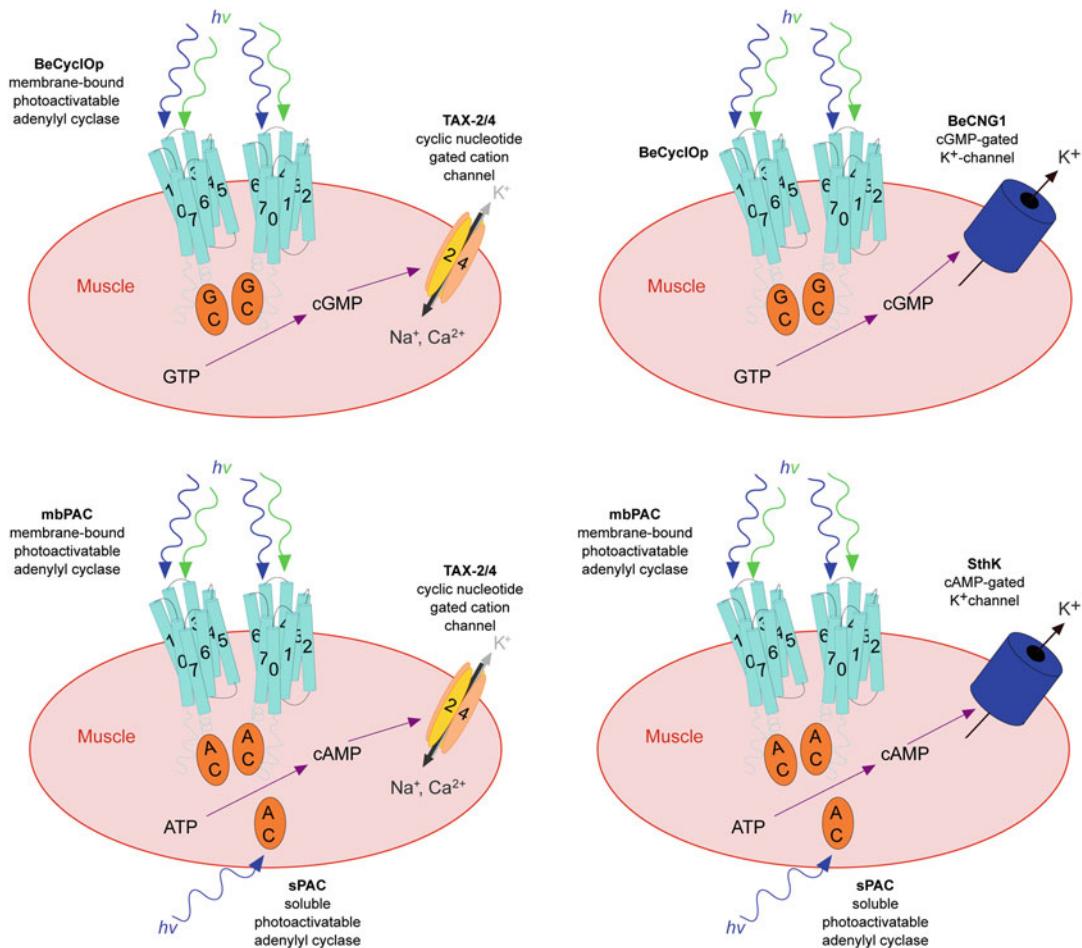
#### 2.2.4 Anion Channelrhodopsins (ACRs)

Next to commonly used optogenetic tools like ChR2 and NpHR, there has been great progress in extending the spectrum of available optogenetic actuators, by either molecular engineering of existing or discovery of novel natural rhodopsin variants. For the increasingly complex requirements of applications, new tools differ in conductivity, kinetics, spectral properties, and ion selectivity, whereas the latter defines the tool being either a de- or hyperpolarizer [81, 86–88, 96]. The gap of equally efficient hyperpolarizing tools was closed by the natural  $\text{Cl}^-$ -conducting anion channel rhodopsins (ACR1 and ACR2, derived from *Guillardia theta*) [39]. Prior to that, light-driven ion pumps as NpHR were the agents of choice, however being restricted by active transport, since only one ion is moved per photocycle [36, 38].

In *C. elegans* behavioral experiments, investigating the body length upon light stimulation, expression of ACRs in muscle (p $\text{myo-3}$  promoter) led to the strongest relaxation effects recorded to date (up to  $6.1 \pm 0.4\%$  body elongation), while requiring only very low light intensities (down to  $3.6 \mu\text{W/mm}^2$ ) [15]. Despite their high operational light-sensitivity, handling of ACR-expressing animals proved to be possible, as long as red filter foil was used for transferring of the transgenic, ATR-supplied worms (ATR incubation and storage of ATR-supplied animals in aluminum foil). In addition to their high efficiency, ACR1 is about 45 nm red-shifted compared to ACR2, which facilitates a selective stimulation by green light (peak absorption: 515 nm) and a combination with blue light-activated optogenetic tools such as ChR2 (peak absorption: 470 nm). In contrast to that, blue light activatable ACR2 (peak absorption: 470 nm) can be combined with red-shifted ChR variants, like ChRimson.

### 2.3 Guanylyl Cyclase Rhodopsin (CyclOp)

In 2014, a novel rhodopsin was described in the sweet water alga *Blastocladiella emersonii* [97]. This protein turned out to be a rhodopsin fused to a guanylyl cyclase domain that forms dimers and is activated by blue-green light to catalyze the conversion of GTP to cGMP at high efficiency and very high dynamic range [25, 98]. As could be shown, BeCyclOp is a robust tool in *C. elegans* excitable cells, i.e. muscles and neurons (Fig. 3). In muscles, in combination with the cGMP-gated cation channel



**Fig. 3** Cyclase rhodopsin-based two-component optogenetic systems for de- and hyperpolarization. **(a)** Co-expression of BeCyclOp (or CaCyclOp) with the cGMP-activated TAX-2/-4 cation channel effectively causes depolarization by blue-green illumination. **(b)** Co-expression of BeCyclOp with the BeCNG1 K<sup>+</sup>-channel allows driving mild hyperpolarizing currents. **(c)** Co-expression of BeCyclOp(A-2x) (or CaCyclOp(A-2x)), i.e. membrane-bound photoactivated adenylyl cyclases (mbPACs), as well as soluble PAC, also allows cAMP-driven gating of the TAX-2/-4 cation channel (even though the channel has much lower affinity for cAMP). **(d)** mbPACs as well as soluble PAC can also be used to trigger outward K<sup>+</sup>-currents via the cAMP-gated K<sup>+</sup>-channel SthK

encoded by TAX-2 and TAX-4 subunits, which have to be co-expressed, as they are not intrinsically present in muscle cells, BeCyclOp robustly causes depolarization upon photoactivation, that is also quite long-lasting, likely due to the low expression of phosphodiesterases in this tissue [25]. In sensory neurons (specifically, BAG neurons that detect oxygen), BeCyclOp photostimulation led to behavioral responses that mimicked photodepolarization by ChR2, i.e. increased locomotion speed. Thus, this rhodopsin is a valuable tool for research in sensory neurons, which often use cGMP as signaling molecule.

Recently [26], we implemented another CyclOp, from *Catenaria anguillulae* (CaCyclOp), as an additional tool for cGMP generation. CaCyclOp exhibits a slower cGMP production rate, but comparably high substrate specificity as BeCyclOp. Thus CaCyclOp may be used for the fine-tuning of cGMP levels in respective cell types. In addition, since no membrane-bound photoactivatable tool for cAMP generation existed, we mutated the specificity-determining amino acids of the cyclase domains, and implemented YFP-BeCyclOp(A-2x) and YFP-CaCyclOp(A-2x). These tools are characterized by high substrate specificity and magnitude of cAMP production.

In cholinergic neurons (*punc-17* promoter), the membrane-bound PACs and the soluble bPAC evoke similar increases in crawling speed and number of swimming cycles during illumination, however, the membrane-bound PACs induce longer-lasting behavioral changes.

Besides their application as single optogenetic tools, CyclOps can be combined with cyclic nucleotide-gated (CNG) channels, obtaining two-component optogenetic systems for de- and hyperpolarization of excitable cells. To this end, we co-expressed respective CyclOps for cGMP or cAMP creation with the CNG channels TAX-2/-4 ( $\text{Na}^+$  and  $\text{Ca}^{2+}$  conductive), and the cAMP- and cGMP-gated  $\text{K}^+$ -channels from *Spirochaeta thermophila* (SthK) and *Blastocladidella emersonii* (BeCNG1), respectively, displaying variable levels of activity, kinetics, and long- or short-lasting effects (see Note 1). Though these systems for de- and hyperpolarization require expression of 2–3 genes, they represent valuable tools in comparison to single rhodopsin tools due to the amplification of the primary signal (meaning reduced light budget) and  $\text{K}^+$ -conductance.

### 3 Methods

#### **3.1 Specific Application of Optogenetics to Investigate Synaptic Function**

*C. elegans* is particularly suited to study mechanisms of chemical synaptic transmission. The basic synaptic machinery involved in neurotransmission is conserved from *C. elegans* to mammals. The distinct advantage of working in worms is that most mutants affecting pre- or post-synaptic function are viable, and can be analyzed in adult animals, unlike in mammals. However, the physiological tools used to study synaptic function in worms—pharmacological and behavioral assays, as well as electrophysiological recording of neuronal activity [99–103]—are either slow and tedious or technically challenging. Here, optogenetics offers advantages as it allows non-invasive stimulation in intact moving animals, is repeatable, is specific for stimulation of a neuron type, and causes the release of the neurotransmitter at the synaptic sites only. In 2008, Liewald et al. introduced optogenetic investigation of neurotransmission

(OptIoN), which uses photo-induced electrical activity to affect behavior, or to drive transmission at dissected neuromuscular junctions (NMJ), in a quantitative and time-resolved manner. Selective and repetitive photostimulation of cholinergic or GABAergic neurons can be used to examine mutants for defects in various aspects of synaptic function [47, 49, 50]. Following on, Liu et al. also studied graded synaptic transmission at the NMJ using ChR2-mediated photostimulation [48]. Optogenetic stimulation followed by electron microscopic analysis of the synaptic ultrastructure has also yielded new insights into the maintenance of synaptic vesicle pools and synaptic activity [82, 104]. The basics of conducting an optogenetics experiment in worms are described below.

### 3.1.1 Expression of Optogenetic Proteins

Optogenetic activators and inhibitors are expressed using appropriate promoters. There are promoters known which can drive expression specifically in many if not all tissues and cell types in *C. elegans*. However, there are a very few promoters that drive expression specifically in only single (pairs of) neuron(s), which is particularly important for applications in this small nervous system, as every single neuron is expected to have a distinct role or even several roles. Thus, for optogenetic manipulation of a single cell or cell type, either combinatorial genetic approaches using intersectional promoter strategies with Cre or FLP recombinases should be used [105–108]. Alternatively, or in combination, patterned illumination with digital micromirror devices (DMDs), LCD projectors, etc. have been employed [53, 63, 64, 109]. The *myo-3* promoter, driving expression in body wall muscle cells, is commonly used to express and assess the efficacy of new optogenetic proteins [3, 36, 38, 84]. This is convenient, as photoactivation or inhibition of muscle cells lead to a straightforward behavioral readout (body contraction or elongation, respectively). Also, muscle cells are relatively more accessible than neurons to electrophysiological characterization of the optogenetic proteins [47, 110]. For dos and don'ts regarding the usage of the different optogenetic tools (*see Notes 2 and 3*).

### 3.1.2 Worm Rearing

Transgenic worms expressing the desired optogenetic protein are cultivated on standard NGM plates and fed with *E. coli* OP50. The microbial rhodopsins require all-*trans*-retinal (ATR) as a cofactor, which has to be exogenously supplied in the OP50 that is fed to the worms [3, 47]. Animals for optogenetic analyses are cultivated on NGM plates with OP50 supplemented with 2.5–5 µM ATR (calculated with respect to the volume of NGM in the culture dish) for one generation at 20 °C in the dark. Typically, 1 µl 100 mM ATR stock solution in EtOH is mixed with 1000 µl of OP50 culture, and 200–300 µl of this mixture are spread onto the top of the NGM plates. Thus, ATR is not mixed into the NGM medium itself. As a control, animals of the same genotype are grown on NGM plates

without ATR. Ca. 18 h prior to experiments, L4 larvae, grown on ATR plates, are placed on fresh ATR plates.

### 3.1.3 Illumination

1. An epi-fluorescence microscope with a mercury lamp as light source and standard bandpass filters for the desired excitation wavelength can be used to induce a behavioral response in transgenic animals. Optogenetic proteins expressed in *C. elegans* are commonly activated with light intensities in the 0.2–5 mW/mm<sup>2</sup> range.
2. The precise light intensity needs to be measured, e.g. with a light-meter, where the sensor is placed under the microscope instead of the culture dish, at the focal plane that would be used for worm imaging during the experiment. The diameter of the spot of light can be measured when focused on a piece of paper through a “Neubauer” chamber (used for cell counting, with a  $\mu\text{m}$  grating). Of course, it can also be calculated from the specifications of the microscope and objective used.
3. Duration of the light stimulus is set with a computer-controlled shutter. For a typical behavioral response, e.g. from mechanosensory neurons, a 500 ms light pulse is sufficient. For full muscle contraction to be achieved, a 2 s or longer light stimulus should be used.
4. LEDs with the desired wavelength are also commonly used as excitation light sources. Patterned single cell illumination [64, 109], and see above, is another means to provide illumination, even of single neurons in freely moving animals.

### 3.1.4 Video Analysis

1. The locomotion behavior of the worms on a solid NGM substrate can be quantified and several locomotion parameters like the worm body length, velocity, body bending angles, frequency of turns, etc. can be obtained (for a review of different tracking and behavior analysis systems available for *C. elegans*, see [111]).
2. For quantitative analysis of worm body length, consecutive frames (e.g. 30 frames per second) from the captured videos are extracted as individual images. Individual images are converted into binary images (black and white), background subtracted and skeletonized into a single pixel thick backbone. This image processing can be automatized with custom written ImageJ or Matlab scripts [47, 84, 109, 112]. The body length of the worm is recorded as the length of the backbone obtained after skeletonization.
3. Worm length is normalized by the mean length before the light stimulation and followed over hundreds of consecutive video frames. Length chronograms of multiple worms are then averaged and the length profiles of test vs. control strain can be

plotted [47]. For single time points, body length can also be measured “by hand,” by drawing a segmented line along the backbone of the animal, e.g. using ImageJ, and determining the length in pixels.

4. For precise annotation of the different aspects of locomotion, different worm-tracking packages based on machine vision tools have been developed by the worm research community [111]. These tracker systems can be combined with optogenetic experiments that require spatially and temporally precise illumination of the worm with various colors of light [63, 64, 109].

### **3.2 Combining Optogenetic Actuation with $\text{Ca}^{2+}$ Imaging**

$\text{Ca}^{2+}$  imaging provides a non-invasive way to monitor the activity of muscles and neurons in *C. elegans* in close to real time. This can be achieved in fully intact worms, due to the optical transparency of the animal. Different genetically encoded calcium indicators (GECIs) have been designed to visualize and quantify intracellular calcium levels [51, 52, 113–116]. For chemically synthesized  $\text{Ca}^{2+}$  sensitive dyes, we refer the reader to other reviews; application of such dyes in *C. elegans* is challenging due to the difficulty to get them across the cuticle. Instead, GECIs are preferred to capitalize on the genetic tractability of *C. elegans*. Since GECIs are built entirely from proteins, they may be expressed in targeted cells under a tissue-specific promoter. There are two major classes of GECIs: The FRET-based indicators such as cameleons [51] and Tn-XXL [116], as well as the single fluorophore indicators, such as the GCaMPs and GECOs [52, 113–115, 117].

Cameleons have been extensively used in *C. elegans* to monitor neural activity [118–121]. These GECIs use Förster resonance energy transfer (FRET) to indicate changes in  $\text{Ca}^{2+}$  concentration [51, 122]. FRET is a distance- and orientation-dependent, radiation-free transfer of energy from donor to acceptor chromophore (e.g. CFP and YFP), which results in the loss and gain of fluorescence of donor and acceptor molecule, respectively. In Cameleons CFP and YFP are connected via calmodulin and the calmodulin-binding peptide M13. When calmodulin binds  $\text{Ca}^{2+}$  ions, it undergoes a conformational change, binds M13, and thus affects CFP/YFP FRET efficiency. The ratio between YFP and CFP fluorescence serves as a measure for the  $\text{Ca}^{2+}$  concentration [51, 118], and the ratiometric nature allows to control for focus artifacts of the fluorescent signals, particularly in experiments with freely behaving animals.

The non-FRET  $\text{Ca}^{2+}$  indicators consist of a single circularly permuted (cp) GFP-variant that is fused to calmodulin at its C-terminus and to M13 at its N-terminus. Binding of  $\text{Ca}^{2+}$  causes the M13 and calmodulin domains to interact and to alter the environment of the chromophore (exclude water), which changes

the brightness of the indicator [113, 123]. Examples for single fluorophore GECIs are the GCaMPs, originally introduced by Nakai et al. in 2001 [52], and the GECOs [117]. Since then, GCaMPs have been improved continuously by using structure-based mutagenesis, with GCaMP6 as the most recent version [114, 115]. These  $\text{Ca}^{2+}$  indicators are prone to movement and focus artifacts. Thus, they are usually co-expressed with a reference fluorophore with a non-overlapping fluorescence, which must not alter with calcium concentration [124], to allow distinction of  $\text{Ca}^{2+}$ -induced vs. focus-dependent fluorescence intensity changes.

The combination of optogenetic modulators with calcium indicators constitutes a powerful approach to study neuronal functionality. However, the overlap of spectral channels is often problematic. ChR2 and CFP, GFP or YFP excitation spectra largely overlap, thus it is virtually impossible to image Cameleons or GCaMPs without concomitantly activating the ChR2-containing cell. One, however, “dirty” approach is to compare stimulating one cell with ChR2 and imaging a linked cell with Cameleon or GCaMP, and to perform this experiment twice, once with, and a second time without ATR. In the case of the ATR-experiment, one will not achieve a stable “baseline”  $\text{Ca}^{2+}$  trace, however. Another approach is to spatially restrict the actuation and the imaging light, provided that the cells to be imaged and the cell to be activated are spatially distinct from one another. This has recently been achieved by patterned illumination using a DMD-based illumination system in combination with a tracking system [53, 64]. A much less demanding approach is provided by using a spectrally shifted (relative to ChR2)  $\text{Ca}^{2+}$  indicator, namely RCaMP [44]. RCaMP is based on the red fluorescent protein mRuby. In 2012, Husson and colleagues used RCaMP as an optical readout for the excitation of the forward command neuron PVC by upstream photoactivation of the sensory PVD neuron [54]. Thus, the combination of ChR2 and RCaMP is feasible for the analysis of neuron to neuron and neuron to muscle communications. A similar, actually more potent red fluorescent  $\text{Ca}^{2+}$  indicator is R-GECO, which is based on the mApple protein [117]. However, R-GECO is not suited for optogenetic applications, as it shows photoconversion/photoactivation in response to blue illumination for concomitant ChR2 stimulation, which leads to a large increase in the red fluorescent signal, irrespective of the  $\text{Ca}^{2+}$  concentration [44]. A typical experiment combining optogenetic activation with *in vivo*  $\text{Ca}^{2+}$  imaging of the induced activity in body wall muscle cells is described below.

1. Animals expressing RCaMP in body wall muscle cells and ChR2 in the neuron of interest (for example, in locomotion command neurons) may be investigated to assess muscular calcium changes during photostimulation of the command neurons (mediated via evoked or inhibited activity of the

motor neurons). One day prior to the experiment, L4 larvae are transferred to either NGM plates seeded with bacteria, and supplemented with 2.5–5 µM ATR (from a 100 mM EtOH stock solution), calculated with respect to the amount of NGM in the culture dish; control animals are transferred to plates without ATR.

2. On the day of the experiment, up to three animals are picked onto a 10% agar pad (w/v, in M9 buffer) and are fully immobilized by using 0.1 µm polystyrene beads [125].
3. RCaMP fluorescence is monitored with a 10× air objective lens on an inverted fluorescence microscope. Two high power light-emitting diodes (LEDs) with the respective wavelengths of 470 nm for ChR2 excitation and 590 nm for RCaMP excitation are used as light sources. A beam splitter couples both LEDs to enable a simultaneous illumination of the specimen, thus allowing the excitation of RCaMP and the photostimulation of ChR2 in parallel. A double bandpass excitation filter permits wavelengths of 479 nm and 585 nm from the illuminator to pass onto a 605 nm beam splitter that reflects the light towards the sample. Eventually, the emitted light is filtered by a 647/57 bandpass barrier filter that allows only wavelengths about 647 nm to pass towards the eye or the detector. In order to run different photostimulation protocols, a Lambda SC Smart Shutter controller (Sutter Instrument Company) can be used to switch the power supply of the 470 nm LED through TTL pulses. Videos are obtained with a CCD camera or a sCMOS camera at a frame rate of 20 fps. Micro-Manager freeware (<https://www.micro-manager.org/>) can be used to adjust video settings. The exposure time is set to 30 ms and frames are binned to 4 × 4 pixels. The light power is adjusted to 1 mW/mm<sup>2</sup> at 470 nm.
4. For analysis, the image sequences can be analyzed using ImageJ (<http://imagej.nih.gov/ij/>). Animals are segmented into regions of interest (ROI) by only circumscribing the RCaMP expressing areas. The mean fluorescence intensity values are calculated for every ROI at every frame. A region outside the animal is chosen as background and subtracted from the mean fluorescence values. The traces for each ROI are normalized:

$$F_{\text{all}} = \frac{(F - F_0)}{F_0}$$

$F_0$  is the mean fluorescence of the first 100 video frames. The increase of the signal due to autofluorescence caused by the photostimulation is circumvented by normalizing the fluorescence values of the illumination period, with  $F_0$  as the first frame of the illumination.

### 3.3 Voltage Imaging Using Rhodopsins

Methodically, voltage imaging experiments are conducted similar to RCaMP measurements described above. Due to its dim fluorescence, direct imaging of the (non-eFRET) Archaeorhodopsin mutant QuasAr requires activation with a 637 nm laser at 1800 mW/mm<sup>2</sup> and the usage of highly sensitive EMCCD cameras is advisable. Using this configuration, QuasAr enables to monitor voltage dynamics at high frame rates (ca. 150 frames per second (fps), 2-ms exposure) in various tissues, ranging from muscle cells to individual neurons, such as the locomotion interneuron RIM [27]. For combination with optogenetic control (e.g. ChR2 or ACR2), blue light crosstalk by undesired fluorescence of free retinal can be reduced with low ATR concentrations (as low as 0.01 mM) and comparably low excitation intensities of around 300 µW/mm<sup>2</sup>. For the use in the pharynx, pumping can be induced by incubation of transgenic animals in 3 µl serotonin (20 mM, in M9 buffer) right before the experiment. Here, automated data analysis (KNIME [126] workflow, available from the authors on request) allows to synchronize pumping events on the behavioral level (identified by the opening of the grinder lumen) with preceding voltage signals recorded via the voltage sensor's fluorescence. To measure the relative change in fluorescence over time ( $\Delta F/F_0$ ), ROIs should cover the entire pharynx, while a smaller ROI can be set onto the grinder structure to track pumping motion (compare Subheading 3.2, step 4). Extracted parameters like pump- and voltage signal duration and kinetics enable conclusions about mutant phenotypes compared to the wild type N2. By calculating difference videos, it is even possible to spatially resolve compartmentalized voltage activity in the pharynx, where outcomes are in line with electropharyngeograms (extracellular recordings of pharyngeal electrical activity) [27].

Alternatively, much brighter eFRET sensors can be used to visualize voltage dynamics, e.g. for applications where high intensity laser light is undesired or costly camera equipment is not available. These kind of voltage indicators require conventional excitation light sources like LEDs or HBO or Xenon lamps, with appropriate filter sets (e.g. MacQ-mCitrine: excitation at 472/30 nm with 10 mW/mm<sup>2</sup> and emission at 520/35 nm. QuasAr-mOrange: excitation at 545/30 nm with 30 mW/mm<sup>2</sup> and emission at 610/75 nm). The selection of the indicator therefore depends on the spectral requirements in terms of combination with other actuators as well as hardware configuration and photosensitivity of the experiment.

### 3.4 Cyclase Rhodopsins and Two-Component Optogenetics

In general, application of CyclOp proteins for cyclic nucleotide monophosphate (cNMP) signaling studies work well [26], if one considers the following experimental details:

1. Usage of a red filter foil for the transfer of transgenic (ATR-cultivated) animals on an experimental plate.
2. Acclimation of these animals for several minutes in dark before starting the experiment.
3. Storage of the ATR-cultivated animals in aluminum foil to avoid pre-exposure to (ambient) light.
4. Application of a red bandpass filter for the transmission light of the microscope.
5. Depending on the cell type in which the respective CyclOp is used, photoactivation may trigger long-lasting effects, depending on the expression levels of phosphodiesterases (PDEs) in the respective tissue.

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## 4 Notes

1. Dos and Don'ts when using CyclOps and CNG channels: To reduce basal activation of CNG channels due to intrinsic cNMP, we recommend to inject <5.5 ng/μl of the respective channel DNA (TAX-2,-4 or SthK) to obtain a low expression level of the channel. In contrast, for BeCNG1 we suggest to work with a high expression level, i.e. the amount of injected DNA should exceed 40 ng/μl, to obtain quantifiable behavioral effects. Also, for the application of the BeCyclOp; BeCNG1 system, a long light pulse >15 s is advised. For use of SthK and the co-expressed PAC in (cholinergic) neurons, the expression levels had to be adjusted, and we recommend to inject <15 ng/μl of the respective PAC DNA (for PACs with low light-induced cAMP production, we suggest to inject >15 ng/μl DNA) and < 5.5 ng/μl of the SthK channel DNA. Here, the amount of injected DNA had to be individually adjusted for the respective systems, since in some cases, it was difficult to get transgenic strains (we occasionally observed transgenic larvae arresting at L1-L2 larvae stages, that did not develop into adults).
2. Dos and Don'ts when using ChR2: As ChR2 is a low-efficiency Na<sup>+</sup> channel, high expression levels need to be achieved. Thus, multicopy extrachromosomal arrays are required, that should be integrated into the genome. In some cases, we observed robust effects only after integration, e.g. for activation of PVD neurons, only 5% of the transgenic animals showed PVD-evoked escape behavior, while after integration, 100% of the animals responded [54]. Single-copy genomic integration, e.g. by MosSCI [127], is thus not suitable to generate optogenetic strains (at least not with ChR2(H134R)).

Of course, as *C. elegans* neurons produce graded potentials, higher blue light intensity also increases the ChR2-mediated effects [48, 49]. However, as *C. elegans* has an intrinsic light response to avoid UV and blue light, mediated by the *lite-1* receptor [128], any optogenetic experiment using ChR2 runs the risk of activating the photoavoidance response in addition to the ChR2-evoked behavioral response. This is particularly true when prolonged (>300–500 ms) photostimulation of the neuron of interest is required to evoke an observable behavioral response. There are two possibilities to distinguish the specific from the non-specific photoresponse: (1) Run the experiment with two groups of animals, one cultivated with, the other without ATR, and compare the behavioral response. This is problematic, however, if the behavior is actually modulated by the photoavoidance. (2) Perform the experiment in a *lite-1(ce314)* mutant background, which largely lacks the photophobic responses. This is to be done with caution, however, as *lite-1* mutants are not wild type, and for example exhibit slowed locomotion, particularly in swimming assays. Generally, as little blue light as necessary to evoke the full response should be used (both with respect to duration and intensity), and more light-sensitive variants of ChR2 can be used (C128S or H134R; T159C, see above), that mediate effects at light intensities that evoke almost no photophobic responses.

All-trans retinal itself has some non-specific effects on *C. elegans*, (e.g. animals show slightly deeper body bending with ATR), and too much ATR actually becomes toxic. Thus the concentration of ATR should be kept as low as possible, while still evoking maximal activity. This can be titrated for the specific application. Keep in mind that ATR is prone to oxidation and photo damage, so keep the plates in the dark and do not use them if they are older than 4–5 days.

3. Dos and Don'ts when using inhibitory rhodopsins: As a word of caution, Arch and Mac, possibly due to their enhanced membrane insertion in *C. elegans*, actually can cause toxicity when overexpressed, unlike NpHR. Our observations are that expressing lots of NpHR in body wall muscle (80 ng/μl injected DNA) had no negative effects on viability or muscle physiology, despite the presence of large intracellular protein aggregates. In contrast, similar amounts of Arch and Mac caused animals to be sick and muscle cells to show aberrant membrane protrusions (“blebbing”). This could be avoided by expressing much less (<10 ng/μl injected DNA), while still maintaining more potent inhibition than with NpHR [38].

Also, while yellow light required for NpHR activation does not evoke photophobic responses, and thus can be applied in

wild type animals, the blue-green light wavelengths needed for Arch activation do evoke photophobia. Thus, such experiments should be done in *lite-1* mutants, particularly if prolonged inhibition is required. Also for photoinhibition experiments, always include controls like the same transgenic animals cultivated without ATR, and perform experiments in wild type, with and without ATR, but with the same light exposure.

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# Chapter 7

## Optogenetic Perturbation of Individual *C. elegans* Pharyngeal Neurons While Monitoring Feeding Behavior

Nicholas F. Trojanowski and Christopher Fang-Yen

### Abstract

Optogenetic approaches have proven to be powerful for examining the roles of specific neurons in generating behaviors, especially in systems where electrophysiological manipulation is not possible. Here we describe a method for optogenetically manipulating single pharyngeal neurons in intact *C. elegans* while monitoring pharyngeal behavior. This approach provides bidirectional and dynamic control of pharyngeal neural activity while quantitatively assessing behavior and has allowed us to test hypotheses about the roles of individual pharyngeal neurons in feeding behavior.

**Key words** *C. elegans*, Optogenetics, Feeding behavior, Small circuits, Pharynx

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### 1 Introduction

The nematode *C. elegans* is a powerful tool for studying the function of neural circuits, in large part due to its genetic manipulability, optical transparency, and well mapped synaptic connectivity [1, 2]. Since it is not currently possible to electrophysiologically manipulate the activity of the *C. elegans* nervous system in intact animals [3], the primary method for studying the roles of specific neurons in behavior has been ablation of these neurons in young larvae using a laser beam, and assessment of behavior in adults [4]. However, laser ablation is a permanent, unidirectional manipulation of a neural circuit, and interpretations of these experiments can be complicated by functional redundancy or developmental compensation [5]. It is therefore suboptimal for understanding dynamic phenomena like neural activity.

Optogenetic techniques, which involve using light-sensitive ion channels and pumps to manipulate neural activity, have been important tools for neurobiology, as they enable bidirectional and dynamic manipulation in behaving animals [6]. Most optogenetic

experiments in *C. elegans* have been performed by illuminating the entire worm after expressing opsins in a desired subset of neurons [7, 8]. However, the cellular specificity of this approach—and therefore its utility for functionally dissecting neural circuits—has been limited by the difficulty of finding a promoter that drives gene expression only in the desired neurons. Genetic intersection approaches have been used [9–12], but even these are not guaranteed to provide the desired overlap. To address this problem, multiple groups have developed different methods for using patterned light to illuminate arbitrary parts of the worm corresponding to neurons of interest. One study was done using immobilized worms and optically monitoring calcium levels in neurons of interest [13]. Other work has performed targeted illumination in freely moving worms using a closed-loop imaging and illumination system [14–16]. Optogenetic manipulation and behavioral imaging have also been combined with calcium imaging in freely moving worms [17].

Here, we describe a method for using spatially restricted optogenetic illumination to investigate the behavioral effects of manipulating individual neurons in the pharynx (feeding organ). The pharyngeal neural circuit is one of the simplest in the worm, containing 20 neurons of 14 classes. We have used our targeted optogenetic approach to conduct a series of studies of the pharyngeal circuit and behavior. First, we elucidated the excitatory network for pharyngeal pumping, revealing genetic and neural degeneracy in the circuit [18]. Next, we identified mechanisms of pumping cessation during two types of *C. elegans* sleep [19]. Finally, we investigated the roles of the nervous system and pharyngeal muscle in generating the pharyngeal pumping rhythm [20].

In these experiments we use a digital micromirror device (DMD) to focus light in defined patterns on worms that express light-sensitive excitatory (e.g. blue-light-sensitive ion channel Channelrhodopsin-2 (ChR2)) [21] or inhibitory microbial opsins (e.g. blue-light-powered proton pump (Mac) from *Leptosphaeria maculans*) [22]. To stimulate single neurons, we immobilize worms on agar pads using polystyrene nanoparticles [23]. Despite immobilization of the body, the pharynx remains capable of pumping. This affords optogenetic perturbation with micron-scale spatial resolution without pharmacological manipulation. We use particle image velocimetry to automatically track movement of pharyngeal muscles during optogenetic manipulations. This approach improves on both the lack of specificity present in typical optogenetic experiments and the intra-observer variability inherent in manual observation [24].

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## 2 Materials

1. Optical table or breadboard (at least 1.5 m × 1.2 m surface).
2. 473 nm laser or other wavelengths appropriate to the opsins being used.
3. Inverted microscope and associated filter cubes.
4. Plan Apo 63× oil immersion objective lens.
5. Multichannel image splitter, configured for GFP/RFP imaging.
6. Cooled CCD or CMOS camera with software capable of imaging at 30 frames per second.
7. 1024 by 768 pixel digital micromirror device (DMD) with evaluation board interface and software (such as Discovery 4100 DLP, Texas Instruments/Digital Light Innovations).
8. Power meter.
9. MATLAB software.
10. Project box for mounting DMD control board (approx. 15 cm × 20 cm).
11. Optic parts:
  - (a) 6.24 mm focal length aspheric lens.
  - (b) 75 mm focal length plano-convex lens.
  - (c) 100 mm focal length, 2-in. diameter achromatic lens.
  - (d) 200 mm focal length, 2-in. diameter achromatic lenses, qty. 2.
  - (e) Mirrors, qty. 7–8.
  - (f) Adjustable irises, qty. 2.
  - (g) Long pass dichroic filter.
  - (h) Red filter for microscope transillumination.
12. Hardware for mounting each optic:
  - (a) Post-holders.
  - (b) Posts.
  - (c) Lens and mirror mounts.
  - (d) Post-clamps.
  - (e) 1/4"-20 cap screws.
  - (f) 8–32 cap screws (e.g. Thorlabs HW-KIT3).
13. Chemicals:
  - (a) Sodium fluorescein.
  - (b) NGM buffer (NGM from [25] but without agar, cholesterol, or peptone).

- (c) Agarose.
  - (d) Serotonin HCl.
  - (e) 2.5% (v/v) suspension of 50 nm or 100 nm diameter polystyrene beads.
14. Glass slides and coverslips.
  15. 0.5 mm thick plastic shim stock.
  16. 250 mL beaker.
  17. 50 mL centrifuge tube.
  18. Disposable spatulas.
  19. Nematode strains expressing opsins in cells of interest.

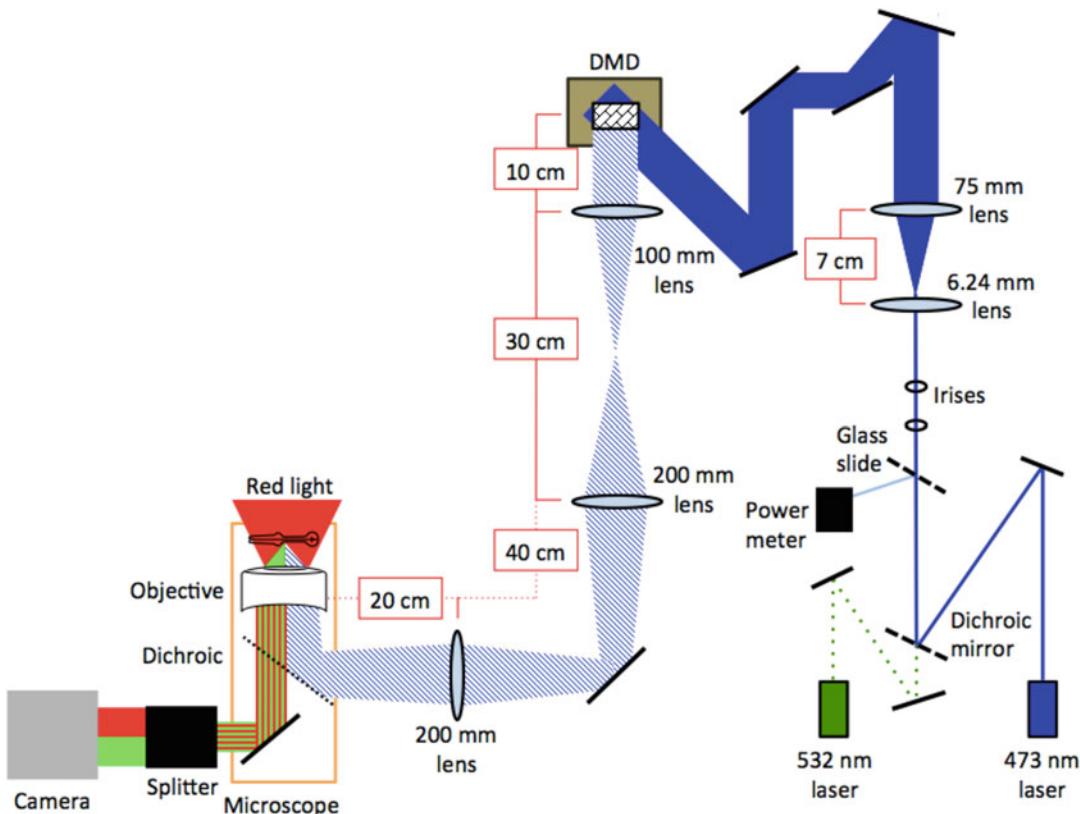
### 3 Methods

#### 3.1 Building the Rig

In the optical setup, a laser beam is first expanded in diameter by about 10 times by a telescope consisting of two lenses (Fig. 1). This laser beam is routed via mirrors to the DMD, which restricts the beam to a set of pixels selected by computer. Next, the image on the DMD is relayed to an intermediate image plane of the microscope with  $2\times$  magnification using lenses with focal lengths 100 mm and 200 mm. Finally, the image is relayed again to the worm via a relay lens system composed of a 200 mm focal length lens and the objective lens. The steps for constructing this setup are described below, and a schematic is depicted in Fig. 1.

Before starting work, consult your institutional laser safety protection office to determine what is needed to safely work with lasers. This may include training, obtaining the appropriate laser protection eyewear, laser curtains, or other safety measures.

1. Place the microscope and laser on an optical table and secure them so that they will not inadvertently move in relationship to the rest of the table (*see Note 1*).
2. Mount each mirror or lens in an appropriate mount and attach each mount to a post and post-holder. Fasten post-holders to the table in an out of the way location so that they are secured while you are performing the other steps.
3. Mount the DMD by attaching the DMD to a mirror mount and attaching the control board to the mounting case using screws and board standoffs. Ensure that the DMD power cable can be plugged in to the control board after mounting.
4. Place two irises in the path of the laser and adjust them and the laser so that the laser beam is horizontal and passes directly through the center of the irises.
5. Place the 6.24 mm focal length lens and the 75 mm focal length lens so they are about 81 mm apart (the sum of the



**Fig. 1** Setup for single neuron stimulation of pharyngeal neurons. Light leaves the blue laser and is reflected off a mirror (black line) and dichroic beam splitter. A small percentage of the light is then reflected to a power meter to monitor laser output. The light then passes through two irises before being expanded through a telescope composed of two lenses. The beam is then, through a series of mirrors, reflected onto the DMD (brown box) such that it covers the entire grid of mirrors. This beam then passes through a series of lenses along the light path. It then enters the microscope through an auxiliary port, where it is reflected by a dichroic on a custom filter cube into the back of the objective. The stage of the microscope is illuminated with red light. This red light and the green light emitted by fluorescent proteins then passes through a beam splitter, which separates the red and green channels, which are then recorded by a camera

focal lengths), beyond the irises. The lenses should be oriented according to their design specifications, with the sides designed for short conjugate distance facing each other.

6. Align these lenses so the beam passes through the center of the lenses and is collimated (i.e. neither converging nor diverging) after exiting the 75 mm focal length lens (*see Note 2*).
7. Install a filter cube and dichroic filter that reflects light from the desired port through the back of the objective. For GFP optics, use a longpass dichroic filter that reflects wavelengths shorter than 500 nm and transmits wavelengths longer than 500 nm (*see Note 3*) or as appropriate for your opsin(s) of interest.

8. Place one of the 200 mm lenses 200 mm away from the back of the objective, along the light path, with the rounded side facing the microscope (*see Note 4*).
9. Place the other 200 mm lens 400 mm beyond the first 200 mm lens, along the light path. The curved side should face away from the microscope (*see Note 5*).
10. Place the 100 mm lens 300 mm beyond the furthest 200 mm lens. The curved side should face the 200 mm lens.
11. Place the DMD 100 mm beyond the 100 mm lens. Screw down loosely, as the position will likely need to be adjusted later (*see Note 6*).
12. Plug in the DMD and use the software to set it so that all the mirrors are in the ON position.
13. Use mirrors as necessary to project the laser beam onto the DMD (*see Note 7*).
14. Adjust the angle of the DMD so that with the mirrors in the ON position, the laser beam is reflected along the reverse optical path of the microscope, through the center of the 100 and 200 mm lenses (*see Note 8*).
15. Place a glass slide at an angle in the light path shortly after the beam leaves the laser by taping the slide to an optical post (*see Note 9*).
16. Set up the power meter so the laser light reflected off the glass slide hits the center of the sensor.
17. Place a red filter in the transillumination light path to enable behavioral observation during the optogenetics experiments without off-target stimulation effects.
18. To allow independent analysis of the green (GFP/targeting) and red (brightfield/behavior) signals, attach the image splitter and camera to the imaging port of the microscope.
19. Align the image splitter according to the instructions that come with the device (*see Note 10*).
20. Register the DMD coordinates to the field of view of the camera. Create a series of images that contain a square moving in a grid across the DMD (*see Note 11*).
21. Prepare a slide with a thin layer of 1 M sodium fluorescein in water or glycerol between two shims under a coverslip and place it on the microscope stage.
22. Pass the series of images to the DMD (**step 20**) and record where (if at all) a fluorescent spot appears on the camera.
23. Using MATLAB, compute the correspondence matrix of the coordinates passed to the DMD and coordinates where fluorescence was detected.

24. Use this matrix to define an image transformation between the DMD and the camera.

### 3.2 Creating Worm Strains

1. In general, the same strains that are used for whole worm optogenetics can be used for this method (*see Note 12*).
2. These worms should be grown in the dark following standard procedures. When needed, add 2  $\mu$ L of 100 mM all-*trans* retinal (ATR) in ethanol to the bacterial suspension immediately before seeding (*see Note 13*).
3. Laser ablations, if desired, can be performed as previously described [4, 5].

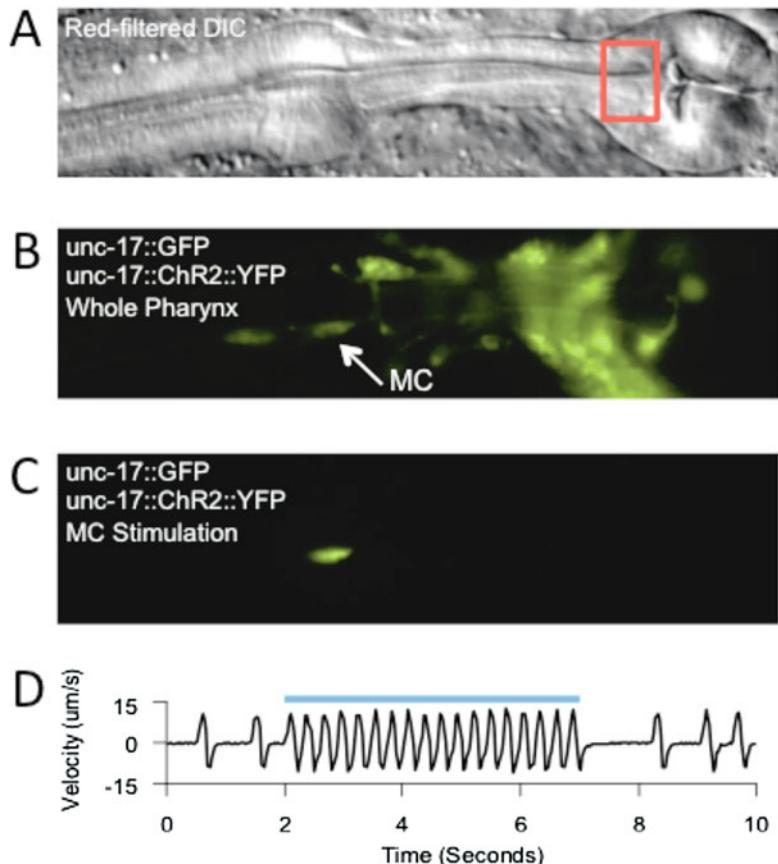
### 3.3 Preparing Agarose Pads

1. Prepare 6 slides by placing 0.5 mm thick shims, approximately 1 cm  $\times$  2 cm, on each end of each slide.
2. Prepare agarose pads by mixing 0.20 g agarose and 4 mg serotonin into 2 mL of NGM (10% agarose, 10 mM serotonin) in a 50 mL centrifuge tube (*see Note 14*).
3. Swirl the mixture gently so that the agarose is evenly dispersed in the liquid. Avoid getting too much of the mixture on the sides of the tube.
4. Rest the lid on top of the centrifuge tube, but do not screw it on.
5. Place the centrifuge tube in a 250 mL beaker.
6. Fill the beaker to ~50 mL with tap water, just above the level of liquid in the centrifuge tube. The water moderates the rate of heating and prevents spattering. It also keeps the agarose warm while pads are being made.
7. Microwave the beaker and centrifuge tube at high power for 50 s, at which point the agarose mixture should be clear, indicating that the agarose has dissolved, and may have bubbles.
8. Using a disposable plastic spatula, place a drop of molten agarose mixture about 1 cm in diameter onto the middle of a slide prepared with shims and immediately cover this slide with a second slide to form an agarose pad (*see Note 15*).
9. Make slides until all the agarose has been used (*see Note 16*). Once the agarose is allowed to solidify, it is difficult to reuse.
10. Allow slides to set for at least 1 min before adding worms.  
Steps 11–16 should be performed quickly for optimal immobilization.
11. Load 1.5  $\mu$ L of a 2.5% (v/v) suspension of 50 or 100 nm diameter polystyrene beads into a 20  $\mu$ L pipettor [23] (*see Note 17*).

12. Remove top slide from one of the pads and place the slide with the pad onto a plate lid on the microscope stage.
13. Use bacteria to stick up to 10 worms onto the bottom of a worm pick, but do not put them on the pad yet.
14. Expel the 1.5  $\mu\text{L}$  of beads onto the pad.
15. Gently, and as quickly as possible, place the pick into the beads and allow the worms to transfer into the beads on the pad (*see Note 18*).
16. Quickly place a coverslip on top of the pad to immobilize the worms. If possible, keep worms separate since worms that are immediately next to others may be poorly immobilized.
17. Wait 5–10 min before performing experiments to allow the worms to habituate to the pad.

### **3.4 Performing the Experiments**

1. Set the frame rate of the camera and the laser power (*see Note 19*).
2. Place a slide on the microscope stage and find a worm at 10 $\times$  magnification on the microscope under bright field illumination (*see Note 20*).
3. Switch to 63 $\times$  and move the pharynx of the worm into the center of the field of view under bright field illumination (*see Note 21*).
4. Manually record a z-stack of the pharyngeal neurons by illuminating the entire field using the DMD and focusing through the relevant regions. This illumination period should be less than 1 s.
5. If desired, use MATLAB to convert the images into JPEG files. MATLAB scripts for this and all other steps are available upon request (*see Note 22*).
6. Identify the images in the z-stack that most clearly show the neurons of interest.
7. Use the `roipoly` function in MATLAB to select the regions of these images you would like to use to stimulate each neuron (*see Note 23* and Fig. 2b, c).
8. Use MATLAB to create an image mask that contains the value 1 for the region you outlined in the previous step and the value 0 elsewhere and transform this file into DMD coordinates using the transformation computed in **step 23** of Subheading 3.1 (*see Note 24*).
9. Create a DMD script that specifies which images to display on the DMD and at which times they should be displayed (*see Note 25*).
10. Acquire a baseline recording of behavior without any illumination (*see Note 26*).



**Fig. 2** Stimulation of single pharyngeal neurons. (a) DIC image of the pharynx. Red box denotes region used for velocity calculations. (b) GFP fluorescence image of the same field of view as in A while illuminating the full field of view. The arrow points to an MC soma. (c) GFP fluorescence image of the same field of view as in a and b, during selective illumination of an MC soma. (d) Velocity from PIV algorithm during ChR2-mediated stimulation of the MC neurons. Each peak in the velocity represents a pump. The blue bar represents timing of laser illumination. (Modified from [18] with permission from American Physiological Society)

11. Run each script while recording from both the red and green channels (see Note 27).

### 3.5 Analyzing the Data

The following steps should be repeated for each experiment.

1. If desired, convert the images into JPEG files (see Note 22).
2. Use the `roipoly` function in MATLAB to draw a ROI around the brightest part of the neuron that was stimulated during the experiment (see Note 28).
3. Use MATLAB to track the pixel intensity of this region over the course of experiment.

4. Use MATLAB to identify the times at which the intensity of this region sharply increased or decreased. These are the times at which the stimulus was turned on and off, respectively.
5. Use MATLAB to select the rectangular region of the terminal bulb just anterior to the grinder (*see Note 29* and Fig. 2a).
6. Using the freely available package PIVlab, a Time-Resolved Digital Particle Image Velocimetry Tool for MATLAB, track the velocity this region over time (*see Note 30* and Fig. 2d).
7. Use this trace of velocity over time to identify the times at which a pump occurred by drawing a threshold and recording the times at which a positive-going threshold crossing occurs (*see Note 31*).
8. Determine the number of pumps that occur during each interval in which the stimulus is on or off (*see Note 32*).
9. Divide the number of pumps in a stimulus interval by the length of that stimulus interval to find the average pump rate (*see Note 33*).

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## 4 Notes

1. Our setup uses a Leica DMI3000B inverted microscope with a Leica Plan Apo 63 $\times$  oil immersion objective lens with N. A. = 1.4. Any microscope with a port that allows direct access to the back of the objective should suffice. An inverted microscope may be easier to set up due to the lower height of the fluorescence illumination path, but an upright microscope could be used as well.
2. The distance between the 6.24 mm lens and the 75 mm lens must be about 81 mm, but the distances from 75 mm lens to the irises, between the irises, and between the irises and the laser, can be arbitrary. Our rig is partially designed to work with a 532 nm laser as well as a 473 nm laser, so we use a system of mirrors and a dichroic beam splitter to allow us to use these two lasers through the same optical path. If only one laser is being used, it can be aimed directly through the irises, but we find it useful to have the beam reflect off at least one mirror and ideally two mirrors before entering the irises, because this makes it easier to adjust the alignment of the laser beam.
3. For the Leica DMI3000B, the fluorescent light port in the rear of the microscope contained optics of unknown parameters, so we used a side auxiliary port and custom filter cube (NUKLA-SER K type laser cube, Nuhsbaum Inc.) that allowed direct access to the back of the objective.

4. You should adjust this position so that a sharp back-scattered image through the objective forms 200 mm away beyond the lens. It is difficult to measure the 200 mm distance from the back of the objective to this lens precisely, so you may need to adjust this lens empirically later. If this lens needs to be adjusted after all the other lenses are set up, it may be easier to adjust the position of the microscope rather than move all the optics.
5. Due to spatial constraints, we put a mirror between the 200 mm lenses. This is fine so long as the total distance along the light path between the lenses is 400 mm. In general, any path can be folded by use of one or more mirrors in order to fit better on the optical substrate.
6. The 100 mm distance here should be adjusted empirically so that a crisp back-scattered image from the microscope appears on the DMD and is centered on the grid of mirrors, covering it completely.
7. The laser beam should just barely cover the DMD. If this is not the case, then you likely need to adjust the two smaller lenses so that the beam is not changing in size after it exits the 75 mm lens.
8. To do this, the light must be incident onto the DMD from the lower right, if you are facing the DMD. We find that is easiest to use multiple mirrors to reflect the laser beam at the correct angle, as this provides multiple degrees of freedom for adjustment.
9. This will allow a large amount of the light to pass through but will reflect a small fraction (about 8%) without disrupting the direction of the laser beam. You can measure the light power reflected and the light power at the objective to determine the percentage of light that is reflected and use this relationship to determine the light power at the objective at any time.
10. Alternatively, an image splitter could be built on the optical table with similar optics to those in the DV2, using dichroic mirrors and band pass filters.
11. In order to target a specific region of the microscope stage, you must first identify the DMD coordinates that correspond to this region. This process will need to be repeated if any of the optics are inadvertently moved, so make sure that all of the optics and mounts are securely fastened before beginning this step.
12. It is essential that the opsin of interest is tagged with an appropriate fluorescent protein (we used blue light to excite ChR2 and Mac, so we used opsins tagged with YFP or GFP). However, we found that for some transgenes the ChR2::YFP was not bright enough to resolve neuron processes (for

example, *zxIs6[unc-17p::ChR2(H134R)::YFP + lin-15(+)]*), likely because the ChR2 is membrane bound. After confirming expression in the relevant cell bodies, we crossed strains containing dim transgenes into a strain that expressed cytoplasmic GFP under the same promoter (we used *vsIs48[unc-17::GFP]*). We found this particularly useful for looking at off-target effects during ablation experiments, where we wanted to determine the effect of stimulating processes near a cell body after killing the cell body.

13. We stored ATR-seeded plates at 4 °C for up to 1 week before use.
14. We make the agarose mixture fresh for each experiment. Serotonin is necessary for inducing a basal pumping rate during immobilization. Other drugs can be added at this step as desired, though we found that adding too many ionic salts (more than 10 mM) caused the polystyrene beads to clump and made immobilization difficult.
15. We have found that the flexibility of the plastic spatulas makes it easier to get all the agarose out of the centrifuge tube, compared to stainless steel spatulas, and their low thermal mass may prevent premature cooling of the agarose.
16. We are usually able to make 6 slides from 2 mL of agarose solution.
17. We store 100 µL aliquots of the polystyrene bead suspension at 4 °C between uses.
18. When the pads contain serotonin, the beads clump quickly after they are added to the pad, which reduces the quality of immobilization. Thus, we try to have the beads on the pad for as little time as possible before we add the cover slip.
19. We use an exposure time of 30 ms and a frame rate of 32.7 fps. We set the laser power so that the irradiance of the laser at the objective is approximately 37 mW/mm<sup>2</sup>. These settings can be adjusted to increase the visibility of the fluorescent signal, if necessary.
20. Because of the way the DV2 splits the camera field of view, it is important that the head of the worm is close to aligned with the long direction of each channel's field of view (within ~30°). If the head is not aligned in this manner, rotate the slide or select a different worm on the slide. Do not rotate the camera or you will have to re-register the DMD and camera images.
21. It is important that the grinder is visible in the camera's field of view, since its motion will later be used to quantify feeding rate. It is also important that the cell bodies of the neurons of interest are visible in the camera. For pharyngeal neurons,

this should not be an issue as long as the worm is aligned as described in **Note 20**.

22. Some acquisition software produces images as multipage TIFF files. We find it easier to work with JPEG files than multipage TIFF files, but JPEG is a lossy compression method and may increase noise. Other image file formats may also be used.
23. This should be a region slightly larger than the cell body—about 2–3  $\mu\text{m}$  in diameter—to allow constant stimulation as the neural cell bodies move during the pump. It is important that the neuron of interest remains in this region during the entire experiment, because the fluorescent signal from this neuron will be used to determine the times of stimulation *post-hoc*. Counterintuitively, we found it difficult to immobilize some nearly paralyzed mutants, in which case we found it necessary to use a substantially larger stimulus region to ensure the neuron of interest was stimulated during the entire experiment.
24. The DMD mirrors can be set in either the ON or OFF position, so we pass an image that contains 1 s (representing ON) in locations corresponding to the area of the stage we wish to illuminate and 0 s (representing OFF) elsewhere.
25. The DMD works by reading in images that represent the pattern it should display and displaying this pattern, then pausing for a defined period, then reading in the next file, and so on. For our experiments, we begin with an all-off image for 5 s, followed by 5 s of illumination of the neuron of interest, followed by an all-off image for 5 s, repeating up to 10 times and ending with an all-off image.
26. We record the baseline for 30 s.
27. We wait 2–3 min between running each script, i.e., between each neuron we stimulate. We found that the behavior was most robust when experiments were performed within 90 min of immobilization.
28. Here, assuming there is minimal cell body movement during the experiment, we find it better to use a ROI smaller than that used for the experiment, because it is easier to detect changes in brightness when just looking at the brightest parts of the neuron.
29. We found that using a rectangle that extends from the anterior edge of the terminal bulb to the grinder, and is just slightly wider than the grinder, provides the best signal.
30. Tracking the velocity of this region over time will show a series of peaks, each representing a pump: a positive change in velocity represents the movement of the grinder toward the

posterior, and a negative change in velocity represents anterior movement.

31. We found that using a threshold of half of the maximum velocity provided high sensitivity and specificity, though a wide range of threshold values will give similar results on a good recording.
32. We did this for each stimulus interval by finding the number of pumps for which the value [pump time – stimulus time] was greater than 0 but less than the time between when the stimulus turned on and when it turned off.
33. The pumping rate during the stimulus-off windows is sometimes lower than the initial baseline, likely due to post-excitatory inhibition, so we typically do not use these values for looking at the effect of neuron stimulation. Rather, we use the pumping rate that we get from **step 10** of Subheading **3.4** as the baseline.

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# Chapter 8

## Antibody Staining for Nematodes with Heat-induced Antigen Retrieval (HIAR)

Curtis M. Loer

### Abstract

Immunocytochemistry remains a valuable and necessary tool for biologists working with nematodes, even those nematode model organisms with advanced molecular genetic tools and transgenics. Because of the highly idiosyncratic nature of successful immunostaining procedures, innovations can still be found for this long-established technique. Heat-induced antigen retrieval (HIAR) is well known from other systems, but seems not to have been applied to antibody staining in nematodes. For some antigens, adding HIAR to an established antibody staining protocol for nematodes can reveal strong and reliable staining that without HIAR is poor or completely absent.

**Key words** Immunocytochemistry, Immunofluorescence, Immunostaining, Heat-induced epitope retrieval (HIER), CRISPR-Cas9, Epitope tagging, Nuclear antigens, *Pristionchus pacificus*, *Caenorhabditis elegans*

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### 1 Introduction

Despite the extraordinary molecular genetic tools available to *C. elegans* biologists for studying gene expression and function—joining rapidly advancing CRISPR-Cas9 techniques with foreign protein sequences (varied fluorescent proteins, voltage and calcium detectors, degrons, etc.), there remains a place, however reluctantly employed, for traditional immunocytochemistry. This long-established tool may especially be needed in satellite model organism nematodes (and aspiring satellites) in which the molecular tools are not as well developed, or as yet entirely absent. Antibody staining may still be required even when CRISPR techniques are moderately successful; for example, if insertion of large sequences is still out of reach. That is currently the case, for example, with the nematode *Pristionchus pacificus*, which is otherwise a highly-developed satellite model organism. Transgenics and CRISPR are relatively new technologies in *P. pacificus* [1–4]. While CRISPR has

been used to make mutants and to knock-in short sequences, inserting larger sequences such as those of fluorescent protein genes is not yet possible: sequence insertion to date is limited to ~150 base pairs. Although this could certainly change in the near future, for the time being, following gene expression from native loci depends on epitope tagging a gene of interest via CRISPR and template-dependent repair, followed by immunofluorescence.

In the course of examining CRISPR-Cas9 generated epitope-tagged *P. pacificus* strains using an “Improved Finney–Ruvkun”-style fixation, permeabilization, and antibody incubation method (similar to [5, 6] and “Peroxide tube fixation” [7]), we found that four 2×-FLAG-tagged strains in one transcription factor gene (*Ppa-unc-3*) showed highly variable nuclear staining. A second set of three *Ppa-unc-86* 1×-FLAG-tagged strains showed no nuclear staining at all, despite testing several variations on the immunostaining protocol. We finally decided to try adding heat-induced antigen recovery (HIAR) to the protocol, and found that this made the *Ppa-unc-3::2xFLAG* staining stronger and much more reliable, and for the first time revealed nuclear staining in the *Ppa-unc-86::1xFLAG* strains [8]. If only to emphasize the highly idiosyncratic nature of such immunostaining, I have recently found that HIAR greatly *reduced* nuclear staining in two *P. pacificus* strains FLAG-tagged in a third transcription factor gene.

Heat-induced antigen recovery (HIAR), also known as heat-induced epitope retrieval (HIER), is a long-known and widely-used technique applied to formalin-fixed and paraffin-embedded tissues in the field of pathology ([9], reviewed in [10]), but apparently neglected in nematode studies. In this method, after fixation, tissue is heated to boiling temperature (or higher, under pressure), typically for a short time (~60 min or less) using boiling water, microwave oven, autoclave, pressure cooker, etc. Likely, the main mechanism of HIAR is breaking covalent bonds between proteins and other components formed during fixation, making antigens accessible by unmasking and changing their conformation, and perhaps also enhancing overall permeability of the tissue. HIAR is highly pH-dependent, working in low (<4) or high (>8) pH solutions, with high pH generally preferred for better tissue preservation [10]. In one study, HIAR was found to be essential for antibody staining against all 9 tested nuclear antigens, and 7 of 26 cytoplasmic and cell membrane antigens in ethanol-fixed human tissues [11].

Using an “improved Finney–Ruvkun” style fixation [5, 6] and permeabilization protocol for batch immunostaining in 1.5 ml microfuge tubes, HIAR just prior to the blocking step and primary antibody incubation, can result in greatly improved staining for some antigens.

## 2 Materials

Unless otherwise indicated, all solutions are kept at room temperature.

### 2.1 “Improved Finney–Ruvkun” Method Fixation Recipes (See Note 1)

1. 2× Ruvkun-Finney Buffer (RFB) [No PIPES]: final concentration – 160 mM KCl, 40 mM NaCl, 20 mM EGTA, 10 mM spermidine, 50% methanol. To make 10 ml, use 1.6 ml 1 M KCl, 80 µl 5 M NaCl, 0.4 ml 0.5 M EGTA solution, 0.1 ml 1 M spermidine (kept at –20 °C, –80 °C long term) with 2.82 ml deionized water.
2. Tris-TritonX100-EDTA (TTE): final concentration 100 mM Tris (pH 7.4), 1% Triton X-100, 1 mM EDTA. To make 100 ml, use 10 ml 1 M Tris (pH 7.4), 10 ml 10% Triton X-100, 0.2 ml 0.5 M EDTA (pH 8), and make up to 100 ml with deionized water.
3. Borate ( $\text{BO}_3$ ) Buffer with Triton X-100 (BBTx): final concentration 10 mM  $\text{H}_3\text{BO}_3$  (Boric acid, FW 61.8), 0.01 M NaOH, 1% Triton X-100, pH is ~9. To make 100 ml, use 2 ml 0.5 M  $\text{H}_3\text{BO}_3$ , 1 ml 1 M NaOH, 10 ml 10% Triton X-100 and make up to 100 ml with deionized water (see Note 2).
4. 1× Phosphate buffered saline (PBS), pH 7.4: final concentration 137 mM NaCl, 2.7 mM KCl, 8 mM  $\text{Na}_2\text{HPO}_4$ , and 2 mM  $\text{KH}_2\text{PO}_4$  (e.g., made from 10× PBS, Thermo-Fisher AM9625).
5. Antibody buffer A: final concentration 1% bovine serum albumin (BSA), 0.5% Triton X-100, 0.05%  $\text{NaN}_3$ , 1 mM EDTA in 1× PBS (pH 7.4); stored at 4 °C.
6. Antibody buffer B: final concentration 0.1% bovine serum albumin (BSA), 0.5% Triton X-100, 0.05%  $\text{NaN}_3$ , 1 mM EDTA in 1× PBS (pH 7.4); stored at 4 °C.
7. 32% Paraformaldehyde (PFA) solution—Electron Microscopy Services 15714-S), stored at 4 °C.
8. 100% 2-Mercaptoethanol (Beta-mercaptoethanol).
9. 1 M Dithiothreitol (DTT) solution in water; stored at 4 °C.
10. 30% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) solution with stabilizer in water; stored at 4 °C.
11. 100× DAPI (4',6-diamidino-2-phenylindole): 10 µg/ml in water; stored at 4 °C in the dark.

### 2.2 Cultivation Reagents

1. Nematode cultivation plates.
2. M9 Buffer: To make 1 L use 3 g  $\text{KH}_2\text{PO}_4$ , 6 g  $\text{Na}_2\text{HPO}_4$ , 5 g NaCl, 1 ml 1 M  $\text{MgSO}_4$ , and make up to 1 L with deionized water. Sterilize by autoclaving.

### 2.3 Equipment

1. Transfer pipettes.
2. Pipettors that cover the range of 1–1000 µl.
3. 1.5 ml microcentrifuge tubes.
4. Centrifuge (capable of 1000–3000 ×  $\text{g}$  for 1.5 ml tubes).
5. Microscope slides with agarose pads and coverslips.

## 3 Methods

Unless otherwise indicated, all procedures are performed at room temperature (RT).

1. Grow sufficient worms (synchronized, if desired) on 60 mm or 100 mm plates. A densely populated 60 mm plate has sufficient worms for a few different staining conditions performed in 1.5 ml microcentrifuge tubes. All procedures are more easily done in 1.5 ml tubes. Combine and use more 60 mm plates (or larger plates) to test more conditions.
2. Wash worms off plates with M9 buffer into 1.5 ml microfuge tube (if using a 60 mm plate) or 15 ml centrifuge tube (if using large plates). Wash repeatedly (5×) over 15–30 min (with centrifugation at 1000–3000 ×  $\text{g}$  and removal of supernatent), with the final wash step using distilled or deionized water. (All remaining steps are described for staining in 1.5 ml tubes. All “washes” indicate adding 1.5 ml of solution to the tube, followed by centrifugation and removal of supernatent.)

#### **Fixation.**

3. If using a 1.5 ml tube, adjust total volume to 450 µl with deionized water. Add 500 µl ice-cold freshly-made 2× RFB (Ruvkun–Finney buffer) followed immediately by adding 55 µl 32% paraformaldehyde solution (*see Note 3*). Mix thoroughly by vortexing or inversion.
4. Snap freeze in liquid nitrogen. Tubes may now be stored at –80 °C (*see Note 4*).
5. Thaw tube(s) under warm water or briefly in 37°C water bath (only until just thawed). Mix by inversion, and incubate for 3.5 h on ice with occasional mixing. (Invert tubes a few times every 15–30 min.)
6. Remove fix (centrifuge and remove supernatant), wash 1× with TTE.

#### **Permeabilization.**

7. Add 1 ml 1% 2-mercaptoethanol in TTE (freshly-made 10 µl 100% 2-ME +990 µl TTE), incubate 4 h at 37 °C with gentle mixing, i.e., on Nutator or rocker (*see Note 5*).

8. Wash 1× with BBTx.
9. Add 1 ml 10 mM DTT in BBTx (freshly-made 10 µl 1 M DTT + 990 µl BBTx), incubate 15 min 37 °C with gentle mixing.
10. Add 1 ml 0.3% hydrogen peroxide ( $H_2O_2$ ) in BBTx (freshly-made 10 µl 30%  $H_2O_2$  + 990 µl BBTx), incubate 15 min at RT with gentle mixing.
11. Wash 2× in BBTx.

#### **Heat-induced Antigen Retrieval (HIAR).**

12. Heat in BBTx (a high pH solution, ~pH 9), 15–30 min at 99–100 °C (*see Note 6*).

#### **Blocking.**

13. After removing BBtx and cooling to RT, add 200 µl Antibody Buffer B, and incubate for 30 min (or longer) at RT. Remove and add 200 µl Antibody Buffer A (*see Note 7*).

#### **Staining/Antibody Incubations.**

14. Incubate worms in antibody buffer A with appropriate antibody dilution, 4 °C ON (*see Note 8*).
15. Wash worms 4–8 × over 1–2 h in antibody buffer B at RT with gentle mixing.
16. Incubate with fluorescent secondary antibody at appropriate dilution in antibody buffer A at 4 °C overnight (*see Note 8*).
17. Wash 8× over 2 h with antibody buffer B at RT.
18. After final antibody buffer B wash, just before viewing, add DAPI to 1.5 ml tube (1 µl of 100× DAPI; since final volume of worms in tube usually ~50–100 µl, the final DAPI concentration is ~0.1 µg/ml); mount 3–5 µl worms on agarose pad, coverslip, and view immediately by fluorescence microscopy (*see Note 9*).

## **4 Notes**

1. One fixative recipe excludes the buffer PIPES, found in other versions of the Finney–Ruvkun protocol. This was likely an accidental omission; however, these experiments were performed using this recipe, so PIPES is not essential. A “corrected” version with PIPES is also included here: 2× Ruvkun–Finney Buffer (RFB) [With PIPES]: final concentration – 160 mM KCl, 40 mM NaCl, 20 mM EGTA, 30 mM PIPES, 10 mM spermidine, 50% methanol. To make 10 ml, use 1.6 ml 1 M KCl, 80 µl 5 M NaCl, 0.4 ml 0.5 M Na-EGTA solution,

- 0.6 ml 0.5 M PIPES (pH 7.4), 0.1 ml 1 M spermidine (kept at –20 °C, –80 °C long term) with 2.22 ml deionized water.
2. Triton X-100 is not included in the Borate buffer solution in the original F-R and Bettinger protocols, but is important to prevent loss of worms from sticking to plastic tube surfaces.
  3. The final concentration of PFA here is 1.75%; the original F-R or Bettinger protocols [5, 6] use a 37% PFA stock solution to yield 2% final concentration. The PFA concentration (and/or the length of fixation on ice) is likely an important parameter to vary when optimizing a protocol.
  4. It may be possible to store worms in 1× RFB fixative indefinitely at –80 °C; however, some experiments suggest that more than a few days to a week of fixation at –80 °C is detrimental. We usually proceed after overnight at –80 °C or at most a few days later.
  5. The permeabilization step with 2-ME is also likely an appropriate step to try variations when optimizing a protocol (especially by increasing the concentration and/or time of the incubation for greater permeabilization), particularly if one has done a ‘harder’ fixation (higher PFA concentration and/or longer). For example, a nematode anti-serotonin protocol that includes a 4% PFA fixation overnight at 4 °C, subsequently includes an overnight incubation with 5% 2-ME at 37 °C [12].
  6. The heat treatment can be carried out in boiling water (e.g., putting 1.5 ml tubes in water in a hot temperature block); we have also used an Eppendorf “Thermomixer” block at 99 °C (maximal setting). Note that the BBTx solution may become cloudy when heated to this temperature, but will clear upon cooling. When first testing antibody staining, it is important here to split a preparation into tubes for testing with and without heat since the treatment improves staining for some antigens, but reduces or destroys it with others. Also, heat variably destroys DAPI staining (nuclear, DNA stain), or significantly reduces it. This is notable since the standard F-R staining procedure *without HIAR* usually results in uniformly excellent DAPI staining. (DAPI is typically added at the very end of the staining protocol, just before viewing.) Experiments suggest that shorter HIAR (i.e., 15 min) preserves DAPI staining better than 30 min; however, a 5 min heat treatment appears inadequate for the improved antibody staining seen for some nuclear-FLAG-tagged antigens. An interesting phenomenon that could be useful and warrants additional attention is selective loss of DAPI staining following HIAR: bright, compact nuclei such as those of neurons and sperm seem more resistant to loss of staining. If staining nuclei in preparations is needed, one may want to try a histone antibody (made in a

different animal from your anti-epitope antibody), using a different color secondary antibody.

7. Once in Antibody buffer A, worms can likely be stored at 4 °C for weeks or even months before proceeding further; however, for testing new preparations, antibody incubations should begin immediately or at most a few days later. Note also that it is likely one can proceed directly into Antibody buffer A, skipping the antibody buffer B incubation, although this has not been tested rigorously. This is the protocol as found in the Bettinger F-R version [5].
8. Both primary and secondary antibody incubations can likely be carried out at RT overnight or for shorter periods of time at 37 °C. Incubations with fluorescent secondary antibodies should be done in the dark.
9. DAPI staining is useful to see all nuclei and to assess the quality of permeabilization. Poor or uneven DAPI staining indicates insufficient tissue permeabilization for good antibody staining—although good DAPI staining is not a guarantee of sufficient permeabilization for antibody staining. As noted above, however, DAPI staining may be reduced or completely eliminated (with high background) following HIAR.

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# Chapter 9

## ExCel: Super-Resolution Imaging of *C. elegans* with Expansion Microscopy

Chih-Chieh (Jay) Yu, Danielle M. Orozco Cosio, and Edward S. Boyden

### Abstract

Studies of *C. elegans* will benefit from a powerful method for super-resolution imaging of proteins and mRNAs at any 3-D locations throughout the entire animal. Conventional methods of super-resolution imaging in *C. elegans*, such as STORM, PALM, SR-SIM and STED, are limited by imaging depths that are insufficient to map the entire depth of adult worms, and involve hardware that may not be accessible to all labs. We recently developed expansion of *C. elegans* (ExCel), a method for physically magnifying fixed whole animals of *C. elegans* with high isotropy, which provides effective resolutions finer than the diffraction limit, across the entire animal, on conventional confocal microscopes. In this chapter, we present a family of three detailed ExCel protocols. The standard ExCel protocol features simultaneous readout of diverse molecules (fluorescent proteins, RNA, DNA, and general anatomy), all at ~70 nm resolution (~3.5 $\times$  linear expansion). The epitope-preserving ExCel protocol enables imaging of endogenous proteins with off-the-shelf antibodies, at a ~100 nm resolution (~2.8 $\times$  linear expansion). The iterative ExCel protocol allows readout of fluorescent proteins at ~25 nm resolution (~20 $\times$  linear expansion). The protocols described here comprise a versatile toolbox for super-resolution imaging of *C. elegans*.

**Key words** Super-resolution microscopy, Expansion microscopy, *C. elegans*, Immunohistochemistry, In situ hybridization, RNA expression analysis, Molecular mapping

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### 1 Introduction

Super-resolution microscopy enables observation of biological structures that are smaller than the diffraction limit of light (~250 nm [1]). Many super-resolution imaging methods have been adapted to *C. elegans*, including STORM [2, 3], PALM [3, 4], SR-SIM [5], STED [6], and expansion of *C. elegans* (ExCel) [7]. Among these methods, ExCel offers multiple unique advantages. Notably, ExCel enables imaging depths that are sufficient to map the entire depth of worms at any developmental stage. While worm diameter ranges from ~15 to ~60  $\mu$ m, from L1 to

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Chih-Chieh (Jay) Yu and Danielle M. Orozco Cosio contributed equally to this work.

adulthood, STORM, PALM, SR-SIM, and STED have imaging depths restricted to 1–20  $\mu\text{m}$ ; the ExCel imaging depth is >100  $\mu\text{m}$  and ExCel has no requirement for any specialized imaging hardware other than a standard confocal microscope. ExCel also provides demonstrated capability to perform simultaneous imaging of multiple molecular types (protein, RNA, and DNA) in the context of anatomical structures marked by an amine-reactive stain, including multiple endogenous proteins detected with high sensitivity. Finally, ExCel enables all of the above in the context of intact animals.

ExCel is based on previously established molecular strategies of expansion microscopy (ExM) [8–17], a family of protocols for physically enlarging biological specimens with high isotropy and nanoscale precision. To perform standard ExCel, intact, cuticle-enclosed animals are first fixed and treated with small molecular weight (<1 kDa), cuticle-permeable reagents that add a hydrogel-anchorable moiety to general proteins [12] and nucleic acids [9]. Animals are then embedded into an expandable hydrogel, a process during which anchor-equipped proteins and nucleic acids are covalently linked to the hydrogel network, thus preserving their relative positions throughout the downstream expansion procedures. Next, a protease treatment, with Proteinase K, is applied to the gelled samples, which both mechanically softens the tissue for uniform expansion, and permeabilizes the cuticle for antibody access. Antibodies and *in situ* hybridization probes are applied at this cuticle-permeabilized state, to label fluorescent proteins (which survive the protease treatment) and nucleic acids, respectively. The stained sample is then expanded to a linear expansion factor of ~3.3–3.8 $\times$ , and imaged on a standard confocal microscope at a sub-diffraction-limit effective spatial resolution of ~65–75 nm. With the standard ExCel protocol, fluorescent proteins, mRNA molecules, DNA, and general anatomical structures can be simultaneously visualized, all at sub-diffraction-limit resolutions, at any location throughout the entire anatomy of the worm.

A major limitation with the standard ExCel protocol is that fluorescent proteins are the only ones that can be detected, because their structure allows them to withstand the protease digestion, which in turn enables them to be detected by immunostaining. Under this limitation, studies of endogenous protein targets require the users to transgenically fuse the protein of interest to a fluorescent reporter. This limitation is resolved by an alternative version of ExCel, epitope-preserving ExCel, which replaces the epitope-disrupting Proteinase K treatment with an epitope-friendly combination treatment based on cuticle-disrupting collagenase and heat-mediated protein denaturation. After this combination of treatments, the majority (~70% [7]) of endogenous proteins assessed could be labeled with off-the-shelf antibodies. This protocol has slightly worse expansion isotropy (8–25% length

measurement error vs. 1–6% error from the standard ExCel protocol), a level that is still sufficient for many biological analyses that do not require precise length measurements, such as examining co-localization of two biomolecules, or the relative organization of biomolecules. Because this combination of treatments incompletely homogenizes worm tissue, a second round of hydrogel expansion is applied to the stained samples to compensate for the reduced expansion factor. Epitope-preserving ExCel achieves a final expansion factor of  $\sim 2.8 \times$ , allowing multiplexed imaging of endogenous protein targets at spatial resolutions of  $\sim 70$ – $100$  nm on a conventional confocal microscope.

A third protocol, iterative ExCel (iExCel), was developed for applications that would benefit from further sample expansion and finer spatial resolution ( $\sim 25$  nm). In iExCel, samples are first processed as in the standard ExCel protocol, including the strong, non-specific Proteinase K digestion, until immunostaining. An oligo-nucleotide-conjugated antibody is applied to stain for a target fluorescent protein. Then, a total of two rounds of tissue expansion are performed, where the oligonucleotide serves to both transfer stained signals across different hydrogels and also to amplify signal. Although iExCel enables finer spatial resolution, the protocol only supports the readout of fluorescent proteins in its current state. In addition, iExCel is more technically demanding, requiring longer processing time, and yielding fluorescent signals (over the same voxel size) weaker than standard ExCel. We therefore recommend that all users first confirm that the fluorescent protein of interest, expressed within the strain of interest, can be brightly observed under the standard ExCel protocol prior to applying iExCel.

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## 2 Materials

The default storage condition, for all reagents listed in this section unless specified otherwise, is at room temperature (RT) for up to 6 months. Any storage condition that differs from the default is noted after the reagent recipe. Refer to [expansionmicroscopy.org](http://expansionmicroscopy.org) for a list of vendors and product numbers that the authors have used and had successful results with. Pay extra attention to the quality of sodium acrylate (a major component of all monomer solutions used in this protocol), whose stock quality can vary significantly across batches and between vendors (*see Note 1*).

### 2.1 Common for all ExCel Protocols

#### 2.1.1 Fixation

1. PFA Fixative: 1 mL 10× PBS (final 1×), 2.5 mL 16% paraformaldehyde (final 4%), 6.5 mL ddH<sub>2</sub>O (*see Note 2*).
2. 40× Borate Buffer Stock: 3.1 g boric acid (final 1 M), 1.0 g NaOH (final 0.5 M), add 45 mL of ddH<sub>2</sub>O, and vortex to fully

dissolve the chemicals. Then, add ddH<sub>2</sub>O until the volume reaches 50 mL.

3. BT: 1 mL 40× Borate Buffer Stock (final 1×), 1 mL 20% (v/v) Triton X-100 (final 0.5%), 38 mL ddH<sub>2</sub>O.
4. BTB: 1 mL 40× Borate Buffer Stock (final 1×), 1 mL 20% (v/v) Triton X-100 (final 0.5%), 0.8 mL 2-mercaptoethanol (final 2%), 37.2 mL ddH<sub>2</sub>O (*see Note 3*).
5. PBST-0.5%: 1× PBS, 0.5% (v/v) Triton X-100.

### *2.1.2 RNA and Protein Anchoring*

1. AcX Stock: 10 mg/mL AcX (Acryloyl-X, SE; re-suspended in anhydrous DMSO) (*see Note 4*).
2. LabelX Stock: 100 μL of 1 mg/mL Label-IT Amine Modifying Reagent (re-suspended in the vendor-provided Reconstitution Solution), 10 μL of AcX Stock; incubate overnight at RT for the chemical reaction to complete, then store at -20 °C until needed (*see Note 5*).
3. RNA Anchoring Buffer: 20 mM MOPS pH 7.7, 0.1% (v/v) Triton X-100.
4. Protein Anchoring Buffer: 100 mM MES pH 6.0, 150 mM NaCl, 0.1% (v/v) Triton X-100.
5. MOPST-0.1%: 100 mM MOPS pH 7.0, 150 mM NaCl, 0.1% (v/v) Triton X-100.

### *2.1.3 Gelation and Expansion*

1. Monomer Solution Stock: 11.36 mL 33% (w/w) sodium acrylate, 2.5 mL 50% (w/w) acrylamide, 5 mL 5% (w/w) *N,N'*-diallyl-tartardiamide (DATD crosslinker), 20 mL 5 M NaCl, 2.5 mL 1 M MOPS pH 7.0, 5.64 mL ddH<sub>2</sub>O (*see Note 6*).
2. Non-activated Monomer Solution: 925 μL Monomer Solution Stock, 5 μL 20% (v/v) Triton X-100, 70 μL ddH<sub>2</sub>O (*see Note 7*).
3. Activated Monomer Solution: 925 μL Monomer Solution Stock, 5 μL 20% (v/v) Triton X-100, 30 μL 0.5% (w/w) 4-Hydroxy-TEMPO (4-HT), 20 μL 10% (w/w) *N,N,N',N'*-Tetramethylethylenediamine (TEMED), 20 μL 10% (w/w) Ammonium persulfate (APS; activator; add last) (*see Note 8*).
4. Non-expanding Digestion Buffer: 50 mM Tris pH 8.0, 0.5 M NaCl, 40 mM CaCl<sub>2</sub>, 0.1% (v/v) Triton X-100 (*see Note 9*).
5. TNT Buffer: 50 mM Tris pH 8.0, 1 M NaCl, 0.1% (v/v) Triton X-100.

### *2.1.4 General Purpose Buffer*

1. 20× SSC: 3.0 M NaCl, 0.3 M sodium citrate, pH to 7.0.
2. 5× SSCT: 5× SSC, 0.1% (v/v) Tween 20.
3. PBST-0.1%: 1× PBS, 0.1% (v/v) Triton X-100.

### 2.1.5 Antibody Staining

## 2.2 Standard Expansion of *C. elegans*

### 2.2.1 ExFISH-HCR (for RNA Readout)

1. Antibodies against the target fluorescent protein (for standard ExCel and iterative ExCel protocols) or other antigens (for the epitope-preserving ExCel protocol) (*see Notes 10–12*).

1. Re-embedding Monomer Solution: 1 mL 40% Acrylamide/Bis-acrylamide 19:1 40% (w/v) solution (commercially available), 50 µL 1 M Tris base, 8.85 mL ddH<sub>2</sub>O, 50 µL 10% (w/w) *N,N,N',N'*-Tetramethylethylenediamine (TEMED), 50 µL 10% (w/w) Ammonium persulfate (APS; activator; add last) (*see Note 13*).
2. Probe Set Stock Solution (*see Note 14*).
3. HCR Amplifier Stock Solution (*see Note 15*).
4. Probe Hybridization Buffer (*see Note 16*).
5. Probe Wash Buffer (*see Note 17*).
6. Probe Amplification Buffer (*see Note 18*).

## 2.3 Epitope-Preserving Expansion of *C. elegans*

1. Collagenase VII Stock Buffer: 2.5 mL 1 M Tris pH 8.0, 1.5 mL 5 M NaCl, 50 µL 1 M CaCl<sub>2</sub>, 45.95 mL ddH<sub>2</sub>O.
2. Collagenase VII Dilution Buffer: 7.5 mL 1 M Tris pH 8.0, 8.5 mL 5 M NaCl, 4 mL 1 M CaCl<sub>2</sub>, 30 mL ddH<sub>2</sub>O.
3. Collagenase VII Stock Solution: Add Collagenase VII Stock Buffer to Collagenase VII powder, to a final concentration of 1 kU/mL (*see Note 19*).
4. TNC-40020 Buffer: 2.5 mL 1 M Tris pH 8.0, 4 mL 5 M NaCl, 1 mL 1 M CaCl<sub>2</sub>, 42.5 mL ddH<sub>2</sub>O.
5. Protein Denaturation Buffer: Added in the following order, 25 mL ddH<sub>2</sub>O, 2.5 mL 1 M Tris base, 4 mL 5 M NaCl, 1 mL 1 M CaCl<sub>2</sub>, adjust pH to 9.0 with 5 M HCl, add ddH<sub>2</sub>O until the volume reaches 35.7 mL, vortex the solution to mix before SDS addition, 14.3 mL 20% (w/w) sodium dodecyl sulfate (SDS), vortex to mix, and store solution at 37 °C. Prolonged storage (>1 h) at RT causes SDS to precipitate, which can be reversed by heating the solution to 70–95 °C (*see Note 20*).
6. Epitope-preserving ExCel G2 Monomer Solution Stock: 11.36 mL 33% (w/w) sodium acrylate, 2.5 mL 50% (w/w) acrylamide, 3.75 mL 2% (w/w) *N,N'*-Methylenebisacrylamide (Bis crosslinker), 2.5 mL 1 M MOPS pH 7.0, 1.5 mL 5 M NaCl, 2 mL 1 M CaCl<sub>2</sub>, 23.39 mL ddH<sub>2</sub>O (*see Note 21*).
7. Non-activated G2 Monomer Solution: 930 µL Epitope-preserving ExCel G2 Monomer Solution Stock, 70 µL ddH<sub>2</sub>O (*see Note 22*).
8. Activated G2 Monomer Solution: 930 µL Epitope-preserving ExCel G2 Monomer Solution Stock, 30 µL 0.5% (w/w)

4-Hydroxy-TEMPO (4-HT), 20  $\mu$ L 10% (w/w) *N,N,N',N'*-Tetramethylethylenediamine (TEMED), 20  $\mu$ L 10% (w/w) Ammonium persulfate (APS; activator; add last) (*see Note 23*).

9. DATD-cleaving solution: 3.8 mL ddH<sub>2</sub>O, 200  $\mu$ L of 500 mM sodium meta-periodate, 500  $\mu$ L of 10× PBS, Adjust pH to 5.5 with 5 M HCl, add ddH<sub>2</sub>O until the volume reaches 5.0 mL (*see Note 24*).

## **2.4 Iterative Expansion of *C. elegans* (iExCel)**

1. DNA-conjugated Antibody Staining Buffer: 4 g of 10% (w/v) Dextran sulfate, 2 mL of 20× SSC, 1 mL of 20 mg/mL baker's yeast tRNA, 1 mL of 100% normal donkey serum, 11.9 mL ddH<sub>2</sub>O, 100  $\mu$ L of 20% Triton X-100 (*see Note 25*).
2. iExCel G2 Monomer Solution: 2 mL 50% (w/w) acrylamide, 1 mL 5% (w/w) *N,N'-diallyl-tartardiamide* (DATD crosslinker), 6.9 mL of ddH<sub>2</sub>O, 50  $\mu$ L 10% (w/w) *N,N,N',N'*-Tetramethylethylenediamine (TEMED), 50  $\mu$ L 10% (w/w) Ammonium persulfate (APS; activator; add last) (*see Note 26*).
3. iExCel hybridization buffer: 4× SSC, 20% (v/v) formamide.
4. iExCel G3 Monomer Solution Stock: 11.36 mL 33% (w/w) sodium acrylate, 2.5 mL 50% (w/w) acrylamide, 3.75 mL 2% (w/w) *N,N'-methylenebisacrylamide* (Bis crosslinker), 20 mL 5 M NaCl, 5 mL 10× PBS, 4.39 mL ddH<sub>2</sub>O (*see Note 27*).
5. Activated iExCel G3 Monomer Solution: 930  $\mu$ L Bis-crosslinked Expanding G3 Monomer Solution, 30  $\mu$ L 0.5% (w/w) 4-Hydroxy-TEMPO (4-HT), 20  $\mu$ L 10% (w/w) *N,N,N',N'*-Tetramethylethylenediamine (TEMED), 20  $\mu$ L 10% (w/w) Ammonium persulfate (APS; activator; add last) (*see Note 28*).
6. DATD-cleaving solution: Exactly the same recipe as the “DATD-cleaving solution” in Subheading 2.3.
7. Buffer A: 150 mM NaCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, pH to 7.4.
8. Buffer C: 150 mM NaCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, pH to 6.0.
9. Unconjugated whole IgG secondary antibody, which matches the host species of the primary antibody against the fluorescent protein (*see Note 29*).
10. DNA oligo-nucleotides to be conjugated with secondary antibodies. *See Table 4* for sequences. For readout of a single fluorescent protein target, use only the B1–B2 system. For readout of two targets, additionally use the A2–A1 system.
11. LNA oligo-nucleotides. *See Table 4* for sequences. Order at a synthesis scale of at least 250 nmol.

### 3 Methods

All three ExCel protocols involve at least 10 days of sample processing (from the beginning of fixation to final imaging), with daily tasks that takes ~3–6 h total, within which ~50% or more are incubation time (during which the user does not need to perform physical tasks). To facilitate planning of experiments, we have added the marker “\*\*\*” at the end of certain steps throughout these protocols, to symbolize steps that we typically end a day with (as many of these steps correspond to overnight incubations). We have also added sample schedules for each of the three protocols (*see* Tables 1, 2, 3).

#### **3.1 Standard Expansion of *C. elegans* (ExCel)**

##### **3.1.1 Overview**

1.  $3.3\text{--}3.8 \times$  linear expansion factor.
2. Supports readout of fluorescent proteins, RNAs, DNA, anatomical structures.
3. High isotropy (1–6% error over length scales between 0 and 100  $\mu\text{m}$ ).
4. Protocol duration, from fixation to imaging:
  - (a) For readout of fluorescent proteins only: 10 days.
  - (b) For readout of fluorescent proteins and RNAs: 14 days.
  - (c) To add the readout of anatomical structures via an amine-reactive dye: add 1 day to the estimates above.

*See* Fig. 1 for the workflow of the standard ExCel protocol, and Table 1 for a sample schedule. For all experiments involving RNA readout, all reagents and samples must be kept constantly RNase-free, as RNase contamination can lead to degradation of RNA molecules, and can result in both reduced signal intensity and count of the fluorescent puncta during the ExFISH-HCR readout (*see* Note 30).

##### **3.1.2 Fixation and Cuticle Reduction**

The default centrifugation setting is  $1000 \times g$  for 2 min for live worms, and  $400 \times g$  for 2 min for worms after fixation, unless stated otherwise. Washes are performed at room temperature (RT), unless stated otherwise.

1. Prepare PFA Fixative no more than 30 min before use, and chill to 4 °C.
2. Wash worms from plates into a 15-mL conical tube by pipetting 1–3 mL M9 buffer onto the plate and gently swirling to remove worms from the worm media surface and bacterial lawn. Pipette the loosened worms into the conical tube. Repeat this process until as many worms as possible have been transferred from the plate to the tube. Fill the tube with M9 buffer to 15 mL.

**Table 1**  
**Sample schedule for the standard ExCel protocol**

| Day | Approx. total time                | Steps   |
|-----|-----------------------------------|---|
| 1   | 5 h, 45 m                         | 3.1.2 Fixation and Cuticle Reduction, steps 1–9   |
| 2   | 30 m                              | 3.1.2 Fixation and Cuticle Reduction, steps 10–12   |
| 3   | 1.25–2 h                          | 3.1.3 Sample Allocation, steps 1–4<br>3.1.4 RNA Anchoring, steps 1 and 2  |
| 4   | 3.5 h                             | 3.1.4 RNA Anchoring, step 3<br>3.1.5 Protein Anchoring, steps 1 and 2   |
| 5   | 2 h                               | 3.1.5 Protein Anchoring, step 3<br>3.1.6 Selecting a method to track hydrogel orientation<br>3.1.7 Gelation, step 1   |
| 6   | 5–6 h                             | 3.1.7 Gelation, steps 2–14<br>3.1.8 Digestion, steps 1–3  |
| 7   | 30 m                              | 3.1.8 Digestion, step 4   |
| 8   | 3 h                               | 3.1.8 Digestion, steps 5–9  |
| 9   | 5 h, 30 m                         | 3.1.9 ExFISH-HCR: Re-embedding, steps 1–9   |
| 10  | 1 h, 30 m                         | 3.1.10 ExFISH-HCR: Probe hybridization and HCR amplification, steps 1–4   |
| 11  | 5 h                               | 3.1.10 ExFISH-HCR: Probe hybridization and HCR amplification, steps 5–10  |
| 12  | 3 h                               | 3.1.10 ExFISH-HCR: Probe hybridization and HCR amplification, step 11<br>3.1.11 Antibody Staining, steps 1 and 2  |
| 13  | 3 h, 30 m                         | 3.1.11 Antibody Staining, steps 3 and 4   |
| 14  | 3 h, 30 m                         | 3.1.11 Antibody Staining, step 5<br>3.1.12 NHS-ester Staining, step 1   |
| 15  | 4 h, 30 m (not including imaging) | 3.1.12 NHS-ester Staining, step 2<br>3.1.13 DAPI Staining steps 1 and 2<br>3.1.14 Preliminary Imaging at a Partially Expanded State, steps 1–4<br>3.1.15 Final Imaging at Fully Expanded State, steps 1–7 |

To speed up the protocol, days 2 and 3, 8 and 9, and/or 9 and 10 may be combined for a total minimum experiment length of 12 days (if fluorescent protein, RNA, and anatomical readout are all done). If anatomical readout is not performed, days 14 and 15 may also be combined for a total experiment length of 11 days.

3. Spin down (at  $1000 \times g$  for 2 min) and carefully replace the supernatant by pipetting 15 mL of fresh M9 buffer. Repeat the M9 wash until bacteria is gone and solution is clear. (It is typically sufficient to perform two washes total.)
4. Remove the clear M9 supernatant with a pipette. Re-suspend the worm pellet in 1–2 mL of M9 buffer and transfer worms to 1.5 mL tubes. For fixing large amounts of worms, split worms

**Table 2**  
**Sample schedule for the epitope-preserving ExCel protocol**

| Day | Approx. total time                       | Steps   |
|-----|--|---|
| 1   | 5 h, 45 m                                | 3.1.2 Fixation and Cuticle Reduction, <b>steps 1–9</b>  |
| 2   | 30 m                                     | 3.1.2 Fixation and Cuticle Reduction, <b>steps 10–12</b>  |
| 3   | 1–1.5 h                                  | 3.1.3 Sample Allocation, <b>steps 1–4</b><br>3.1.5 Protein Anchoring, <b>steps 1–2</b>  |
| 4   | 2 h                                      | 3.1.5 Protein Anchoring, <b>step 3</b><br>3.1.6 Selecting a Method to Track Hydrogel Orientation<br>3.2.2 Gelation, <b>step 1</b> |
| 5   | 5–6 h                                    | 3.2.2 Gelation, <b>steps 2–14</b><br>3.2.3 Collagenase VII-mediated Cuticle Digestion, <b>steps 1–3</b>                           |
| 6   | 3.5 h                                    | 3.2.3 Collagenase VII-mediated Cuticle Digestion, <b>steps 4</b><br>3.2.4 Protein Denaturation, <b>steps 1 and 2</b>              |
| 7   | 10 min                                   | 3.2.4 Protein Denaturation, <b>step 3</b>   |
| 8   | 6 h                                      | 3.2.4 Protein Denaturation, <b>steps 4–7</b>  |
| 9   | 2 h                                      | 3.2.4 Protein Denaturation, <b>steps 8 and 9</b>  |
| 10  | 2–3 h                                    | 3.2.4 Protein Denaturation, <b>steps 10–12</b><br>3.2.5 Antibody Staining, <b>step 1</b>  |
| 11  | 3.5–4 h                                  | 3.2.5 Antibody Staining, <b>steps 2 and 3</b>   |
| 12  | 6–7 h                                    | 3.2.5 Antibody Staining, <b>step 4</b><br>3.2.6 Preliminary Imaging to Check Antibody Staining, <b>steps 1–5</b>                  |
| 13  | 3 h, 30 m                                | 3.2.7 Antibody Anchoring, <b>step 1</b>   |
| 14  | 3.5 h                                    | 3.2.7 Antibody Anchoring, <b>step 2</b><br>3.2.8 Re-Embedding into an Expandable Second Gel, <b>steps 1 and 2</b>                 |
| 15  | 5–6 h                                    | 3.2.8 Re-Embedding into an Expandable Second Gel, <b>steps 3–14</b><br>3.2.9 Proteinase K Digestion, <b>steps 1–3</b>             |
| 16  | 6 h                                      | 3.2.9 Proteinase K Digestion, <b>steps 4–8</b>  |
| 17  | 4 h                                      | 3.2.10 Cleave DATD-crosslinked Gels #1, <b>steps 1–5</b>  |
| 18  | 30 min                                   | 3.2.11 NHS-ester Staining, <b>step 1</b>  |
| 19  | 3.5–4 h ( <i>not including imaging</i> ) | 3.2.11 NHS-ester Staining, <b>step 2</b><br>3.2.12 Expansion and Imaging, <b>steps 1–7</b>  |

To speed up the protocol, days 2 and 3, and/or 12 and 13 may be combined for a total experiment length of 17 days (if anatomical readout by NHS-ester staining is performed). If anatomical readout is not performed, days 18 and 19 may also be combined for a total minimum experiment length of 16 days.

into multiple tubes such that the pellet size in each tube is no more than ~50 µL per 1.5 mL tube. Spin down (at 1000 × g for 2 min) and remove as much supernatant as possible with a pipette without disturbing the worm pellet.

**Table 3**  
**Sample schedule for the iterative ExCel protocol**

| Day | Approx. total time                       | Steps   |
|-----|--|---|
| 1   | 5 h, 45 m                                | 3.1.2 Fixation and Cuticle Reduction, <b>steps 1–9</b>  |
| 2   | 30 m                                     | 3.1.2 Fixation and Cuticle Reduction, <b>steps 10–12</b>  |
| 3   | 1–1.5 h                                  | 3.1.3 Sample Allocation, <b>steps 1–4</b><br>3.1.5 Protein Anchoring, <b>steps 1–2</b>  |
| 4   | 2 h                                      | 3.1.5 Protein Anchoring, <b>step 3</b><br>3.1.6 Selecting a method to track hydrogel orientation<br>3.1.7 Gelation, <b>step 1</b> |
| 5   | 5–6 h                                    | 3.1.7 Gelation, <b>steps 2–14</b><br>3.1.8 Digestion, <b>steps 1–3</b>  |
| 6   | 30 m                                     | 3.1.8 Digestion, <b>step 4</b>  |
| 7   | 4 h                                      | 3.1.8 Digestion, <b>steps 5–9</b><br>3.3.4 Antibody Staining, <b>steps 1 and 2</b>  |
| 8   | 3.5–4 h                                  | 3.3.4 Antibody Staining, <b>steps 3 and 4</b>   |
| 9   | 3.5–4 h                                  | 3.3.4 Antibody Staining, <b>steps 5</b>   |
| 10  | 5 h, 30 m                                | 3.3.5 Expansion and Re-embedding into Non-Expanding Gel #2  |
| 11  | 1 h                                      | 3.3.6 Linker Hybridization, <b>steps 1–3</b>  |
| 12  | 3.5 h                                    | 3.3.6 Linker Hybridization, <b>steps 4</b>  |
| 13  | 3.5 h                                    | 3.3.6 Linker Hybridization, <b>step 5</b>   |
| 14  | 4–4.5 h                                  | 3.3.7 Re-embedding into Expanding Gel #3, <b>steps 1–7</b>  |
| 15  | 3.5–4 h                                  | 3.3.8 Cleave DATD-crosslinked Gels #1 and #2, <b>steps 1–4</b>  |
| 16  | 1 h                                      | 3.3.9 LNA Hybridization, <b>steps 1 and 2</b>   |
| 17  | 3.5 h                                    | 3.3.9 LNA Hybridization steps 3   |
| 18  | 3.5 h                                    | 3.3.9 LNA Hybridization, <b>steps 4</b>   |
| 19  | 3.5–4 h ( <i>not including imaging</i> ) | 3.3.10 Expansion and Imaging <b>steps 1–4</b>   |

To speed up the protocol, days 2 and 3, 10 and 11, and/or 13 and 14 may be combined for a total minimum experiment length of 16 days

5. Place worm pellets and PFA Fixative on ice for 5 min.
6. Add 1 mL of PFA Fixative to the worm pellet. Place sample on a tube rotator to mix vigorously at RT for 30 min.
7. Incubate sample at 4 °C for 4 h without mixing.
8. Wash sample (spin down, remove supernatant, add buffer and mix thoroughly by inverting tubes) with 1 mL of BTB three times with no extended incubation time. Use the centrifugation setting of 400 × g over 2 min, for all centrifugation steps from this point on.

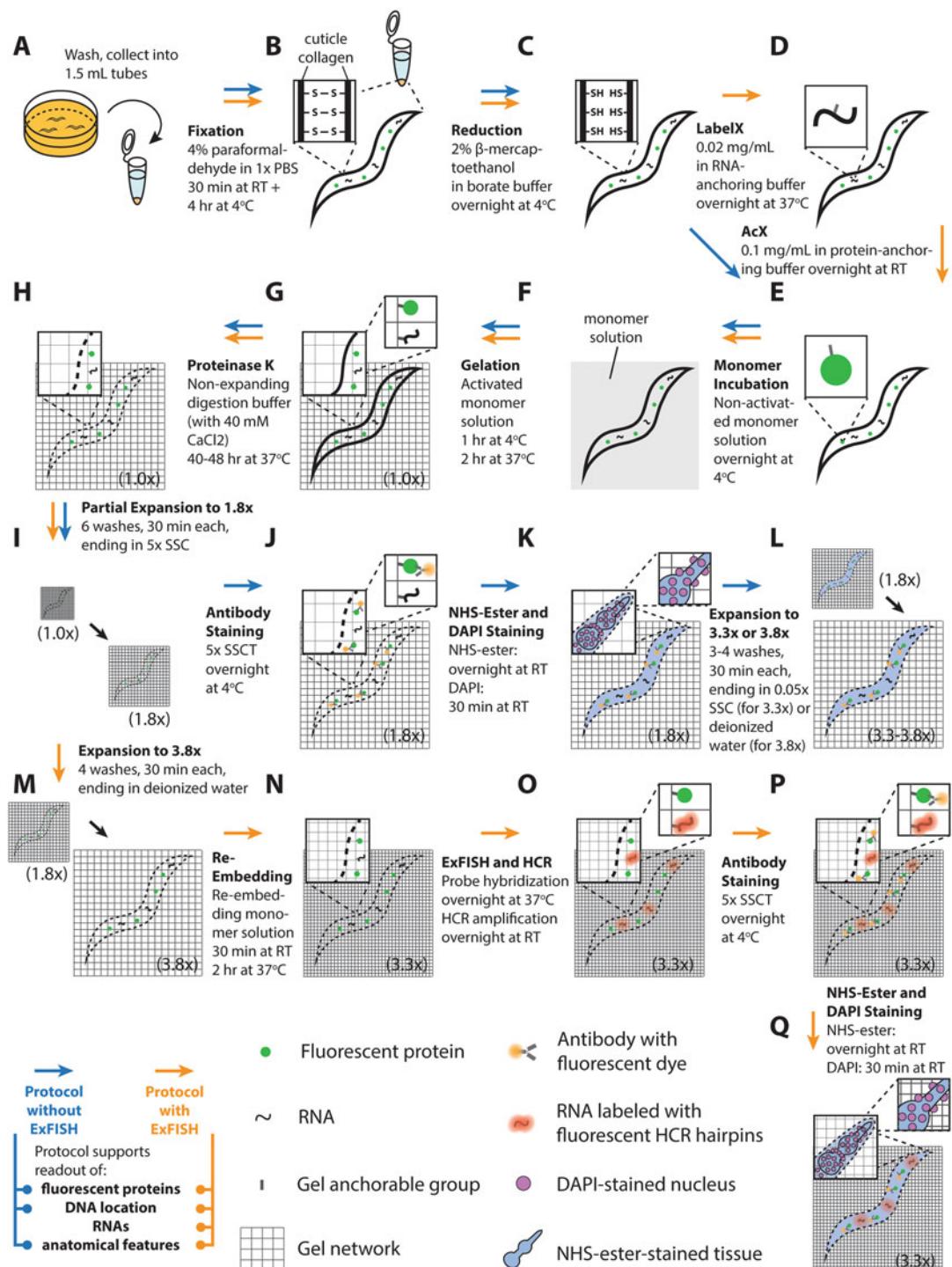
9. After the 3 quick washes with BTB, spin down, remove supernatant, and incubate sample in 1 mL of fresh BTB at 4 °C overnight. \*\*.
10. Wash with 1 mL of BT two times with no extended incubation time.
11. Wash with 1 mL of PBST-0.5% two times with no extended incubation time.
12. Wash with 1 mL of PBS. The fixed sample can be stored in this PBS at 4 °C for up to 2 weeks. \*\*.

### 3.1.3 Sample Allocation

Typically, a lot more worms are fixed compared to the quantity needed to be expanded for a given experiment. Processing too many more worms than needed can both waste reagents, and increase the labor and difficulty required to get all samples through the expansion pipeline. Before proceeding to the expansion protocol, perform the following steps to allocate the quantity of fixed worms that will be processed in the current experiment.

It is important to realize that the end product of this section is \*not\* individual tubes carrying an amount of worms that correspond to a single hydrogel. That splitting procedure will be performed in Subheading 3.1.7 (Gelation), but before then, it saves a lot of time and reagent to process all the to-be-gelled worms in larger batches (e.g. within a single or a few tubes). Thus, for example, if a user would like to make 20 hydrogel samples, the end product of this section will not be 20 individual tubes, but less than 5 tubes.

1. Determine the number of worm-embedded gels needed at the end, in order to achieve the experimental goal. If you follow through the next steps specified in this protocol, each gel will contain ~30–50 worms, and thus each gel may contain a sufficient number of biological replicates for statistical purposes (depending on your scientific question). However, each gel can only go through a single set of staining solutions, so animals in the same gel cannot be stained differently. Therefore, associate experimental conditions (i.e. type of antibody to apply; type of hybridization probes to apply; which spectral channels will be used for which signals) to individual gels, ideally in an organized spreadsheet. If you are trying this protocol for the first time, or are starting a new type of experiment that differs from what you have done in the past, it is recommended that you add at least 1–2 gels to the list, that correspond to the positive and/or negative control conditions (i.e. you can apply the same experimental condition as what has been previously done, which would give known outcomes, e.g. the antibodies or hybridization probes that have been demonstrated to work under ExCel [7]). Finally, for new



**Fig. 1** Workflow for the standard ExCel protocol. A method for expanding cuticle-enclosed intact *C. elegans*. Depending on whether the user intends to visualize RNAs or not, the protocol branches into two forms. The protocol without ExFISH, which supports the readout of fluorescent proteins, DNA location (in the form of DAPI staining), and anatomical features, is indicated with blue arrows, ending in Panel I. The protocol with ExFISH,

**Fig. 1** (continued) which additionally supports readout of RNAs, is indicated with orange arrows, ending in panel **q**. For all steps after hydrogel formation (Panels **g–q**), the linear expansion factor of the hydrogel-specimen composite is shown in parentheses. **(a–q)** Steps of the protocol, with the bold text indicating the title of the step. **(a)** Live animals are collected into 1.5-mL Eppendorf tubes. **(b)** Animals are fixed in 4% paraformaldehyde in 1× PBS, for 30 min at room temperature, and then 4 h at 4 °C. **(c)** Fixed specimens are incubated with BTB overnight at 4 °C. This step chemically reduces the disulfide bonds between collagen fibers in the cuticle, which could enhance diffusion of chemical reagents. After this step, specimens proceed to either a sequential treatment of LabelX and AcX (Panel **d** and **e**) for the protocol with ExFISH (orange arrows), or solely with a treatment of AcX (Panel **e**) for the protocol without ExFISH (blue arrow). **(d)** Specimens are incubated with LabelX overnight at 37 °C. This step equips nucleic acids with a polymer-anchorable moiety. **(e)** Specimens are incubated with AcX at a concentration of 0.1 mg/mL in protein anchoring buffer overnight at RT. This step equips proteins with a polymer-anchorable moiety. **(f)** Specimens are incubated in non-activated monomer solution overnight at 4 °C. **(g)** Hydrogel polymerization by incubating the specimens in activated monomer solution for 30 min at 4 °C, transferring the specimens into a gelling chamber, and incubating the chamber for 2 h at 37 °C. During polymerization, AcX-modified proteins and LabelX-modified nucleic acids are covalently anchored to the hydrogel network. **(h)** Specimens are treated with Proteinase K at 8 U/mL, in non-expanding digestion buffer for 2 days (40–48 h) at 37 °C. The CaCl<sub>2</sub> in the buffer keeps the hydrogel at the unexpanded state, to avoid partial expansion of the internal tissue before the cuticle is thoroughly digested. **(i)** Specimens are partially expanded from a linear expansion factor of 1.0× to 1.8×, with 6 serial washes lasting 30 min each, incrementally reducing salt concentrations each time (i.e. lowering Tris, NaCl, and CaCl<sub>2</sub> concentrations bit by bit throughout each of the six washes, ending with 5× SSC). We use this serial washing procedure to lower the speeds of expansion, which we reasoned could potentially result in more isotropic expansion after Proteinase K digestion, by permitting more time for the gel network to uniformly stretch out the embedded tissue. After this step, specimens proceed to either further expansion (Panel **m**) for the protocol with ExFISH (orange arrow) or antibody staining (Panel **j**) for the protocol without ExFISH (blue arrow). **(j)** To visualize fluorescent proteins, specimens are stained with fluorescent antibodies against fluorescent proteins in 5× SSCT overnight at 4 °C, to amplify their fluorescent signal. **(k)** To visualize anatomical features and DNA, specimens are stained with an NHS-conjugated dye and DAPI, respectively. NHS staining is performed at 2 μM in NHS staining buffer overnight at RT. DAPI staining is performed at 5 μg/mL in 5x SSCT for 30 min at RT. **(l)** Specimens are expanded from a linear expansion factor of 1.8× to 3.3× or 3.8×, with 3–4 serial washes lasting 30 min each, reducing the amount of SSC each time, until the final specimen is immersed in 0.05× SSC (for 3.3×) or deionized water (for 3.8×). As characterized previously [7], 3.8× expansion provides better effective resolution (~65 nm), but has faster decay of immunostained signal (dropped by ~35% over 3 h); 3.3× expansion provides ~75 nm effective resolution, with a signal decay of <10% over 3 h. After expansion, specimens were ready for imaging. **(m)** Specimens are expanded from a linear expansion factor of 1.8× to 3.8×, with 4 serial washes lasting 30 min each, reducing the amount of SSC each time, until the final specimen is immersed in deionized water. **(n)** Specimens are re-embedded into another non-expandable hydrogel to lock up its size at the expanded state. The re-embedding monomer solution contains charged molecules. Therefore, the linear expansion factor slightly drops from 3.8× to 3.3× during this step. After re-embedding, the linear expansion factor remains at ~3.3×, regardless of the immersing environment. **(o)** To visualize RNA molecules, we first perform *in situ* hybridization (ExFISH) of RNA-detection probes to the target RNA, overnight at 37 °C, and then perform hybridization-chain-reaction (HCR) to amplify the hybridized signal, overnight at RT. **(p)** To visualize fluorescent proteins, specimens are stained with fluorescent antibodies against fluorescent proteins in 5× SSCT overnight at 4 °C, to amplify their fluorescent signal. **(q)** To visualize anatomical features and DNA, specimens are stained with an NHS-conjugated dye and DAPI, respectively (conditions described in step **k** above). After staining, specimens were ready for imaging. (Figure is modified from Yu et al. 2020 [7], with permission granted by the Creative Commons Attribution license)

users who are not fully familiar with handling hydrogel samples, and who thus have a greater chance to lose samples due to unexpected failures (e.g. breaking gels, failing to embed worms or gels due to pre-mature gelation, etc.), it is recommended to add another ~2 gels to the list, as backup samples.

2. Once you determine the number of gels needed (from **step 1**), multiply that number by ~30–50 worms/gel (new users can aim for ~50–70 worms/gel, as there could be some loss during the gelation step, which is reduced as technique improves), to get the total number of worms that need to be processed in the current experiment.
3. Transfer the total number of worms, determined in **step 2**, from the stock tube of fixed worms, into a single new 1.5 mL tube. If you need to transfer hundreds of worms, it would be difficult to count accurately with a single pipetting attempt, so use the following approach instead. First, mix the contents in the stock tube of fixed worms well, with a pipettor (such that worms are evenly distributed throughout the solution). Then, pipette out 100  $\mu$ L of the well-mixed content into a separate, new 1.5 mL tube. Estimate how many worms were transferred, by holding the new tube against a lamp or light source to facilitate visualization of worms via naked eye. Then, back-calculate the volume of well-mixed content from the stock tube, that is needed to achieve the total number of worms calculated in **step 2**. Transfer additional volume of well-mixed content from the stock tube into the new tube, until the volume in the new tube reaches the calculated volume, i.e. until the total number of worms in the new tube reaches the desired number.
4. Store the stock tube back into 4 °C. Spin down the tube of allocated worms. Estimate the size of the worm pellet. If the worm pellet appears clearly larger than ~30  $\mu$ L, re-suspend the pellet back into the supernatant with a pipettor, and then split the evenly mixed content into multiple tubes, with the goal of having each tube to contain  $\leq$ 30  $\mu$ L of worm pellet after spin down. Repeat this distribution step until the goal specified in the previous sentence is achieved. This distribution step ensures that the reagents applied in the next several steps are always at molar excess compared to their biochemical substrates (i.e. total biomolecules in the total mass of worm) within each tube, and make final results more consistent.

Reaction volumes in the following sections are specified for a single 1.5 mL tube of worms (whose pellet size has been distributed to be  $<30 \mu$ L/tube). If you end up with multiple tubes after the worm pellet distribution, you can process all of them in parallel, but be sure that each tube receives the full volume specified in the next

sections (e.g. each tube gets a full 1 mL of Protein Anchoring Buffer for **step 1** in the “Protein Anchoring” section, and so forth).

#### 3.1.4 RNA Anchoring (Optional, for RNA Readout)

1. Spin down and remove PBS supernatant. Pre-incubate worms with 1 mL of RNA Anchoring Buffer at RT on a tube rotator for 1 h.
2. Spin down and remove buffer, then incubate worms with LabelX at 0.01 mg/mL (1:100 dilution of LabelX stock) in 1 mL of RNA Anchoring Buffer, overnight at RT on a tube rotator. \*\*.
3. Wash worms with 1 mL of PBST-0.1% three times, 30 min each at RT on a tube rotator.

#### 3.1.5 Protein Anchoring (for Fluorescent Protein Readout)

1. Spin down and remove PBS supernatant. Pre-incubate worms with 1 mL of Protein Anchoring Buffer at RT on a tube rotator for 1 h.
2. Spin down and remove buffer, then incubate worms with AcX at 0.1 mg/mL (1:100 dilution of AcX stock) in 1 mL of Protein Anchoring Buffer, overnight at RT on a tube rotator. \*\*.
3. Wash worms with 1 mL of MOPST-0.1% three times, 30 min each at RT on a tube rotator.

#### 3.1.6 Selecting a Method to Track Hydrogel Orientation

During the initial gelation step of any ExCel protocol (to be described in Subheading 3.1.7), the worms, which have diameters in the range of ~10–60 µm, settle to the bottom surface of the casted gel, which has a thickness of ~180 µm (set by the #1.5 cover glass, which serves as the spacer of the gelling chamber). Then, during the repeated washes throughout the ExCel protocols, the hydrogel sample could get flipped, which results in worms located on the top surface of the hydrogel. If the hydrogel is in such an upside-down orientation during a re-embedding process, or during the final imaging step, a short-working-distance objective (<1–1.5 mm; most high NA and magnification objectives are in this category) might not be able to reach parts or all of the expanded animals. Thus, it is necessary to ensure that the hydrogel sample is in the correct orientation (i.e. worms are on the bottom surface), at the following time points: (a) Prior to any expanding step; (b) Prior to any re-embedding step; (c) Prior to the final imaging step.

If the re-embedding or the final imaging step is preceded by hydrogel expansion, an orientation check needs to be performed prior to the expansion process (we will remind the users again at these later time points in the protocol), because if the gel is on the wrong side, expanded gels are typically too fragile to get flipped. If you have found that the gel is on the wrong side after expansion,

perform the expansion wash steps in reverse to shrink before flipping the gel. Do not try to flip an expanded gel, as it is likely to rip apart.

To track the orientation of the hydrogel (i.e. the surface of the gel the animals are located on), use either of the following two procedures after gelation, and before the gel is lifted off from the chamber. (a) Take a low-magnification image of the gelled sample **before** opening the chamber, to record the orientation of worms in the gel (to be described in **step 12** in Subheading 3.1.7). The image can be based on transillumination or fluorescence (if the fluorescent protein(s) are expressed in a way that allows delineation of the border of the worm). Later, to determine whether the gelled sample has been flipped or not, take another image of the gel, and compare this image to the initial image. (b) During gel trimming (to be described in **step 14** in Subheading 3.1.7), trim the gel into a shape that makes it feasible to identify the side of the gel. For example, a clearly-trimmed 4-edge shape (with inner angles 90°, 90°, 135°, 45°, consecutively) allows one to distinguish whether the gel has been flipped or not, because if flipped, the gel can never resume its original shape by rotation.

Both methods work well when executed properly (and are also mutually compatible; i.e. it is possible to do both on the same sample, if desired). The first method requires microscopy access whenever gel orientation needs to be checked, and might also require more experience and practice with microscopy (when checking the gel orientation, one has to be able to find expanded worms by transillumination and/or dim residual fluorescence). Select a method (or both methods) before proceeding to the next section.

### 3.1.7 Gelation

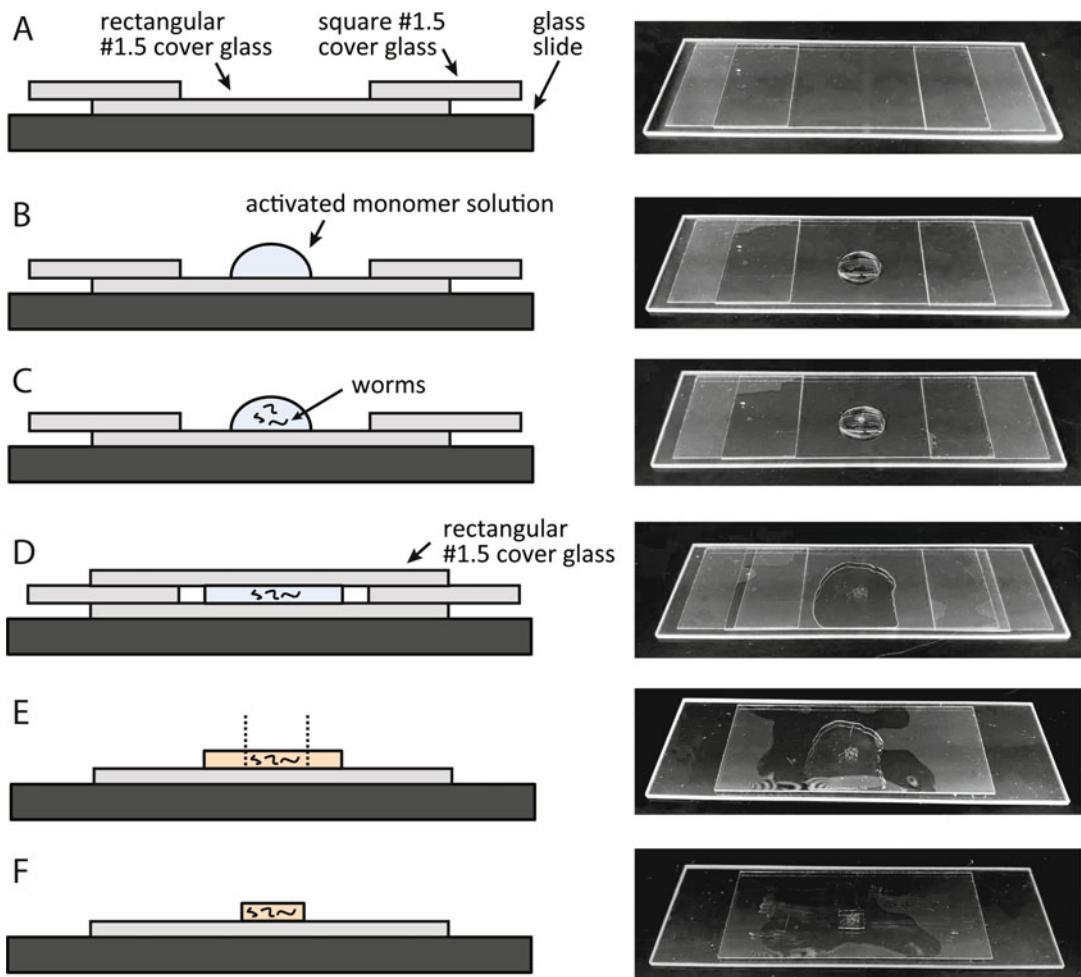
To ensure that the final state expanded worm could be captured within the working distance of a high-NA lens, perform either or both of the two methods for tracking hydrogel orientation (review Subheading 3.1.6). This is critical when performing epitope-preserving ExCel and Iterative ExCel (where failure to track the hydrogel orientation at various stages might result in no image at all at the end, because the worms could end up at a center region [on the z-axis] within the final hydrogel sample, instead of regions closest to the sample surface. In those cases, the user will be unable to optically access the worms via a short-working-distance lens.), and also recommended for standard ExCel, for optimal chance of successful imaging.

Due to variability in sodium acrylate qualities, it is recommended that all users perform a pilot gelation procedure (which takes <1 h) whenever a new batch of Monomer Solution Stock is made from a previously un-validated bottle of sodium acrylate powder (*see Note 1*). This procedure allows the user to determine

whether the 4 °C incubation time (for **step 6** in this section) in this gelation step needs to be reduced, to accommodate for a faster gelation timing for a specific sodium acrylate stock. Without doing this, the user might face the undesirable situation where pre-mature gelation occurs during the 4 °C incubation, which can trap the worms in hydrogel pre-maturely, in a tube rather than on a slide, and prevent them from being processed further with the ExCel protocols.

For users who are less experienced with this protocol, it is important to note that this section involves a time-sensitive procedure that, if not performed quickly and correctly, will destroy the sample (again, due to the hydrogel solidifying before the user has time to load them into chambers). The chance of such failure is much reduced if the user only make a few hydrogel (e.g. up to ~5) at a time, and is further reduced if the user pre-splits the entire batch of to-be-fixed worms, into individual tubes, each of which carries the amount of worms that correspond to a single hydrogel. This pre-splitting step is specified as the optional **step 2** in the following protocol.

1. Spin down and remove MOPST-0.1%, then incubate worms in 1 mL of Non-activated Monomer Solution overnight at 4 °C. \*\*.
2. (Optional; this step is a pre-splitting step that takes a longer time to perform, but reduces the chance of experimental failure during the time-sensitive gelation procedure later). If worms were split into several tubes in Subheading [3.1.3](#), **step 4**, regroup them into a single container (e.g. a 15 mL tube). Then, mix the content well with a pipette, and evenly distribute the content into  $N$  individual 1.5-mL tubes, where  $N$  is the number of hydrogel samples determined in Subheading [3.1.3](#), **step 1**. The number of worms inside each tube should be 30–50 worms (or 50–70 worms, whichever value used in Subheading [3.1.3](#), **step 2**). All following steps apply to each individual tube.
3. Prepare 1 mL of Activated Monomer Solution with all reagents except for APS (Monomer Solution Stock + Triton X-100 + 4-HT + TEMED; do not add the activator APS yet), and thoroughly mix the solution by a vortex. Chill the solution to 4 °C on a cold block. It is important to ensure that the solution is fully chilled to 4 °C before proceeding, because starting with a warmer solution will lead to faster gelation, which might cause sample loss (*see step 8*).
4. Spin down worms. Remove supernatant.
5. Activate the Monomer Solution from **step 3** by adding 20 µL of 10% APS, and thoroughly mix the solution by a vortex. Start



**Fig. 2** Gelation procedure for ExCel protocols. (a) To construct a gelling chamber, a #1.5 cover glass is adhered to a glass slide, with a drop of water pipetted onto the glass slide as adhesive. Then, two #1.5 cover glasses are adhered to opposite ends of a glass slide to serve as spacers. (b) Activated monomer solution is added to the center of the chamber. (c) Worms are loaded to the center of the droplet. (d) A rectangular cover glass is placed on top of the spacers. (e) The gelling chamber is incubated at 37 °C for 2 h to accelerate the formation of the hydrogel polymer. Afterwards, the top and side cover glasses are removed to facilitate trimming of the gelled sample. (f) Trimmed samples proceed to downstream digestion, staining, and expansion procedures. (Figure is modified from Yu et al. 2020 [7], with permission granted by the Creative Commons Attribution license)

a timer right after the APS is added. Perform these steps immediately before adding solution to worms.

6. Add 1 mL of Activated Monomer Solution to worms. Incubate sample at 4 °C for 30 min, on a pre-chilled cold block.
7. Near the end of incubation, construct gelling chambers (Fig. 2a). Adhere a rectangular cover glass (e.g. 22 × 50 mm) to a glass slide with a drop of water. Then adhere two pieces of

#1.5 square cover glasses (e.g. 22 × 22 mm) at separate ends of the rectangular cover glass, again using drops of water as adhesive. These square cover glasses function as spacers of the gelling chamber. If gelling chambers are constructed more than 5 min before the incubation will be finished, cover them to prevent dust accumulation.

8. Immediately after the 30-min incubation, spin down worms. Without disturbing the pellet, transfer ~30 µL of the supernatant as a droplet on the rectangular cover glass, between the spacers (Fig. 2b). Remove the rest of the supernatant and leave only ~30 µL along with the worm pellet. Note that the monomer solution should be completely liquid at this stage. If not, pre-mature gelation has occurred, and we do not recommend proceeding with the samples anymore. If pre-mature gelation occurs constantly, reducing the incubation time in **step 6** to 20 min should resolve the problem (*see Note 1*).
9. Carefully re-suspend the pellet in the leftover volume with a pipette, without creating bubbles. Load all the volume of the tube into a pipette. Push out any air in the tip of the pipette. Insert the pipette tip to the center of the droplet on the rectangular cover glass. Slowly push out the content so that the worms are highly concentrated at the center of the droplet (Fig. 2c). We find that the ideal number of worms in a gel is ~30–50. If more worms are in the droplet, they may be added to another gel on another gelling chamber.
10. Allow 30–60 s for worms to settle near the bottom of the chamber. If splitting a large pellet into multiple samples at this step, the time it takes to distribute worms across ~5 chambers should be enough to allow worms to settle. Close the chamber by carefully and slowly placing a rectangular cover glass (e.g. 22 × 50 mm) on top of the spacers, minimizing bubble formation (Fig. 2d). One way to achieve this is to use a tweezer to clip on one end of a rectangular cover glass, hold the cover glass above the chamber by ~5 mm (in parallel to the glass slide; no contact yet), press down the un-clipped end of the rectangular cover glass until it contacts the spacer, release the clipping force so that the cover glass just rests on the lower branch of the tweezer, and then slowly lower the tweezer until the cover glass contacts the other spacer. It is desirable to avoid worms from overlapping with one another (which can increase the difficulty with imaging), so if worms appear too concentrated, it can be useful to gently spread out the worms by repeatedly lifting and lowering the cover glass slowly, BEFORE closing it completely. If you have already closed the chamber and the worms appear concentrated, we recommend continuing without attempting to open and redistribute worms, as this is likely to introduce bubbles.

11. Incubate the gelling chamber at 37 °C for 2 h. Maintain high humidity with one of the two following methods: (1) in the incubator, keep a small open beaker of warm water near your gelling chambers; (2) place the gelling chambers in a humidified chamber (e.g. a 0.75-quart storage container, such as [Amazon.com](#) #B01M2BTKYB), with the gelling chamber sitting on top of a stack of two lids of 6-, 24-, or 96-well plates, with a small amount (10–20 mL) of deionized water at the bottom of the sealed container.
12. At the end of the 2-h incubation at 37 °C, remove samples from 37 °C, and perform a round of pre-expansion imaging. There are two goals of this imaging session: (A) to record the initial orientation of the hydrogel, which enables tracking of the hydrogel orientation at later stages; the importance of this procedure is described in Subheading 3.1.6; (B) to take pre-expansion images of a few (e.g. ~3) animals, which can later become useful for validation purposes, or in case troubleshooting becomes necessary at a later stage.

To perform (A), take large (i.e. across multiple fields of view as a grid, and ideally automatically stitched by the imaging software) images (bright field or fluorescence; the latter of which is typically more useful [if the strain contains reasonably bright and/or widespread expression of fluorescent protein(s)], because the fluorescent signals persist to much later stages in the protocol than brightfield outlines [with immunostaining-based signal amplification], whose scattering becomes increasingly dim after sample expansion, but still can be useful if the strain does not contain strong native fluorescence) of your gels at low magnification (e.g. via a 4× objective). Record whether the sample is imaged through the glass slide (i.e. right-side-up) or through the coverslip, which is necessary for correctly getting the gels to be flipped into the same orientation, during later stages.

To perform (B), take additional images at a higher magnification (e.g. via a 10×–40× objective), over areas of interest (e.g. fluorescent-protein-expressing regions) on individual animals. The working distances of some of these high-magnification lenses might be too small to go through the glass slide, which requires you to flip the sample upside-down, and image through the cover slip side. If possible, record where the imaged animals are located, on the low-magnification map that you have acquired in task (A). Typically, acquiring images for ~three animals are sufficient for general purposes of validation and troubleshooting (e.g. to confirm whether the anti-fluorescent protein staining, which is applied at a later stage, works as expected).

13. After pre-expansion imaging, prepare the digestion solution (*see step 1* of the next section). The digestion solution should be applied to the samples immediately after **step 14** (gel trimming) to avoid gel drying out.
14. With a tweezer or a razor blade, remove the top cover glass and spacers and leave the gel on the bottom cover glass (Fig. 2e). The gel can occasionally come off with the top cover glass. In that case, leave the gel on the cover glass and dispose the rest of the chamber. In the case that one part of the gel comes off with the top glass while another part of the gel remains on the bottom cover glass, place the top cover glass back, and try opening from the other side of the chamber (i.e. if you approached from the right spacer, now approach from the left spacer). Carefully try to remove one side of the chamber without ripping or stretching the gel. If necessary, a paint brush may be used to gently coax the gel off of the surface with less area stuck to it.

With a razor blade, trim away excessive gel (Fig. 2f). The size of the trimmed gel should be such that, when fully expanded, the gel can fit flatly into the imaging container (a good size is ~7 mm × 7 mm, which can fit into a 6-well plate when expanded). If the worms were successfully kept concentrated at the center of the gel, it should be relatively easy to retain most worms in the trimmed gel. If the worms were more spread out, you may want to trim into two gels near to, or smaller than, the suggested 7 × 7 mm size. To enable tracking of the hydrogel orientation, the user can trim the gel into a 4-edge parallelogram shape (with inner angles 90°, 90°, 135°, 45°, consecutively), which allows the user to later distinguish whether the gel has been flipped or not (review Subheading 3.1.6).

### 3.1.8 Digestion

Based on our experience, the extent of digestion seems to be quite sensitive to the quality of Proteinase K. Use Proteinase K ordered within 2 months. Reaction volumes in the following sections are specified for a single hydrogel. Scale up if you are processing multiple gels.

1. After the 2-h gelation incubation and before opening the gelling chambers, prepare 1 mL of digestion solution per gel by adding Proteinase K to Non-expanding Digestion Buffer to a final concentration of 8 U/mL (1:100 dilution).
2. Add ~50 µL of digestion solution to the trimmed gel immediately after trimming (the trimmed gel should still be on the coverslip). Let the gel sit immersed in the digestion solution for 5 min at RT.

3. Add ~950  $\mu$ L of digestion solution to a well in a 24-well plate for each gel. Prepare a flat-tip paint brush (if performing RNA readout with ExFISH-HCR, decontaminate the paintbrush from RNase by rinsing with RNase Zap and deionized water; *see Note 30*), then slowly insert the brush under the gel until most of the bristles are covered by the gel, lift the gel with the brush, keeping the gel horizontal and level with the slide, and transfer the gel into the well by lowering it into the Digestion Buffer. Allow the gel to float off of the brush, and gently wave it off in the liquid only if necessary. Try to avoid flipping the gel over, as you will want to keep track of which side the worms are closer to, for ease of imaging after expansion. Incubate sample overnight at 37 °C. \*\*.
4. Replace solution with 1 mL of **freshly prepared** digestion solution. Incubate sample overnight at 37 °C. \*\*.
5. Now that the worms have been embedded, we use “wash” to mean simply exchange the immersing solution in the 24-well plate, without the centrifuging; the solution exchange is typically performed by a P1000 pipettor; whenever solution is being removed from the well that contains the gelled sample, always confirm the exact location of the hydrogel inside the well first, by identifying the slightly scattering boundary or body of the trimmed gel, before inserting the pipette into the well and start retrieving solution; without this careful procedure, the gel can get sucked into the pipettor, which creates a typically very obvious obstruction to the fluid flow into the pipette tip, and which can damage the gelled sample. We recommend tilting the plate under bright light to identify the edge of the gel, placing the pipette tip in a corner of the well where the gel is not present, then slowly removing solution so as not to suck up the gel. If the gel is sucked up, it can be pushed back out and you may continue with the protocol, but your sample may be damaged (worms may break or come out of their gelled places). Maintain this careful process throughout the rest of the protocol. Wash gels once with 1 mL of TNT-20 Buffer (TNT Buffer + 20 mM CaCl<sub>2</sub>) for 30 min at RT.
6. Wash once with 1 mL of TNT-10 Buffer (TNT Buffer + 10 mM CaCl<sub>2</sub>) for 30 min at RT.
7. Wash twice with 1 mL of TNT Buffer for 30 min each at RT.
8. Wash once with 1 mL of 5× SSC-NaCl Buffer (5× SSC + 500 mM NaCl) for 30 min at RT.
9. Wash once with 1 mL of 5× SSC for 30 min at RT. \*\*.

### 3.1.9 ExFISH-HCR (for RNA Readout): Re-Embedding

ExFISH-HCR requires hybridization between DNA probes and RNA targets, which have reduced affinity in low ionic strength environment (e.g. deionized water, which is necessary to fully

expand the gelled sample). This trade-off can be resolved by first casting the expanded sample into a non-expanding acrylamide gel, which locks up the expansion factor of the first gel. The re-embedded gel can then be processed and imaged in higher-salt environments that favor hybridization and maximize HCR amplification stability.

1. Place a rectangular glass slide into a 4-well rectangular dish. Transfer each sample onto the glass slide with a paint brush, the same way you transferred it from the gelling chamber to the 24-well plate. Briefly image each gel to be sure it matches earlier images taken immediately after gelation (as instructed in Subheading 3.1.6, and step 12 in Subheading 3.1.7). This will ensure that worms, which were at the bottom of the gel during gelation, remain at the bottom of the gel (i.e. the gel is in its original orientation, instead of being up-side-down) for easier imaging of the entire volume once it is expanded.  
If gels are flipped, flip back over gently with an RNase-decontaminated paintbrush (washed with RNase Zap and deionized water; *see Note 30*). It is essential to perform this step now, as gels become more delicate as they are expanded.
2. Expand sample by washing serially in  $2.5 \times$  SSC,  $0.5 \times$  SSC,  $0.05 \times$  SSC and ddH<sub>2</sub>O using the same careful pipetting as in the 24-well plate, for 30 min each at RT. Use 3 mL for each washing step. Note that as the gel expands it becomes more delicate and easier to accidentally pipette up when removing washing buffers. Because of this we recommend you continue to use a P1000 pipettor, instead of a Pipet-Aid-type pipettor, to remove liquids despite the higher wash volume. Also take care to keep the expanded gel on top of the glass slide when removing liquids, and avoid letting it hang off of an edge of the slide between washes. This is made easier by following our recommended gel size of 7 × 7 mm during the trimming step after initial gelation.
3. Incubate the sample in 3 mL of re-embedding monomer solution for 30 min at RT with gentle circular shaking. The amount of re-embedding monomer solution should be just sufficient to barely reach the top surface of the gel (80–100% of gel height; even if the solution does not cover the gel at a still state, as long as the solution washes over the sample during the circular shaking, there will be sufficient diffusion of the monomers into the sample), but not much more than that. Oxygen in the ambient environment slows down the gelation reaction to allow complete diffusion of the gel monomers over the 30 min incubation; too much monomer solution can cause pre-mature gelation at the bottom of the well, due to reduced access to ambient oxygen. This is the reason we recommend that shaking

is gentle and circular and does not result in the gel being exposed to air, or the solution to become pooled at one end of the well (if it is tilted, for example).

4. During the incubation, prepare two spacers per gel of three times the height of initial gel spacers. For example, if #1.5 cover glass was used during the initial gelation step, use a stack of three #1.5 cover glasses adhered to each other with a small drop of ultrapure water between the coverslips. It is advisable to prepare these in advance to minimize oxygen exposure of your gels between pre-incubation and re-embedding.
5. After 30 min of incubation, transfer all monomer solution from the well to a separate 50-mL tube and set aside at RT for later use (in the next step). During the solution transfer, position the expanded gel on the center of the glass slide, by tilting the 4-well plate and/or gently moving the gel with a flow of monomer solution (using a pipette). The edges of the rectangular gel should be parallel to the edges of the slide and not be close to, or hanging off from, the edge of the glass slide. Use a blade or forceps to carefully transfer the glass slide from the 4-well plate onto a sheet of Kimwipe, to remove droplets on the bottom of the glass slide. Trim away excessive gel if it is large enough that it is at risk of hanging off the slide.
6. Place spacers prepared in **step 4** on both ends of the glass slide, with the gel in the center. Add a small amount (~50 µL) of the saved monomer solution onto the gel, and slowly place a rectangular cover slip on the gel, to enclose the gelling chamber in the same style as performed in the initial gelation. Avoid bubbles. Use a pipette aimed in the space created by your spacers to completely fill the chamber with extra monomer solution, such that the gel is fully enclosed by monomer solution, with minimal contact to ambient oxygen.
7. Incubate the gelling chamber at 37 °C for 2 h, in a humidified chamber (e.g. a 0.75-quart storage container, such as [Amazon.com](#) #B01M2BTKYB, with the gelling chamber sitting on top of a stack of two lids of 6-, 24-, or 96-well plates, and with a small amount (10–20 mL) of deionized water at the bottom of the sealed container).
8. Remove top cover glass, following the same guidelines as in the original gelation step for removing the cover glass, and trim away excessive gel. You should be able to see the border of the initial expanded gel to use as a guide. We recommend splitting large gels into smaller ones with no more than 1.5 cm per dimension, which simplifies downstream processing.
9. Transfer trimmed gels into individual wells in a 24-well plate containing 1 mL of 5× SSC using a paintbrush as described in previous steps of this protocol. Wash three times with 5× SSC, 10 min each. \*\*.

### 3.1.10 ExFISH-HCR (for RNA Readout): Probe Hybridization and HCR Amplification

ExFISH-HCR readout is performed with HCR v3.0 kits from Molecular Instruments (<https://www.molecularinstruments.com/>). It requires five items: (1) Probe Set Stock Solution (a.k.a. “HCR Probe Set” on the website) against the target mRNA; (2) HCR Amplifier Stock Solution (a.k.a. “HCR amplifier” on the website); (3) Probe Hybridization Buffer; (4) Probe Wash Buffer; (5) Probe Amplification Buffer. Thaw HCR Probe Set Stock and HCR Amplifier Stock Solutions at RT before adding to buffers and samples. For steps conducted at RT and 37 °C, bring the kit buffers to the working temperature before adding to your samples. Refer to Subheading 2.2.1 for instructions and notes of ordering and storing these reagents.

1. Use a spreadsheet to organize which gel samples will be stained with which probe sets, and then with which HCR amplifiers (according to the HCR initiators that are associated with the to-be-applied probe sets; the initiator associated with the probe set is also printed on the manufacturer’s packaging [marked as B1 to B5]).
2. Pre-incubate sample in 500 µL of the Probe Hybridization Buffer for 1 h at 37 °C. Note that buffers included in the HCR protocol are viscous, so take your time pipetting to ensure that you’ve picked up the correct volume.
3. Prepare 500 µL of probe solution by adding the HCR Probe Set Stock Solution to the Probe Hybridization Buffer for a final concentration of 4 nM. Mix the contents well with vortexing.
4. Incubate sample with 500 µL of the probe solution overnight at 37 °C. \*\*.
5. Wash sample with 1 mL of the Probe Wash Buffer four times, 30 min each at 37 °C.
6. Wash sample with 1 mL of 5× SSCT three times, 30 min each at RT.
7. Pre-incubate sample in 500 µL of the Probe Amplification Buffer for 30 min at RT.
8. During the incubation period in step 7, aliquot 2.5 µL of each HCR Amplifier Stock Solution (provided at 3 µM; for each HCR initiator, there are two separate tubes of HCR Amplifier Stock Solution—H1 and H2; both solutions need to be separately aliquoted; for example, if a gel sample is stained with 3 different probe sets, whose corresponding HCR initiators are B1, B2, and B4, then a total of 6 aliquots of HCR Amplifier Stock Solution – B1H1, B1H2, B2H1, B2H2, B4H1, B4H2—should be aliquoted at 2.5 µL each) into separate PCR tubes. Incubate these aliquots at 95 °C for 90 s, on a pre-warmed PCR machine. Let these aliquots sit in a dark environment at RT for 30 min.

9. Prepare HCR amplifier solution by adding 2.5  $\mu$ L of each 95 °C-treated HCR Amplifier Stock Solution to 500  $\mu$ L of amplification buffer. Mix the contents well with vortexing.
10. Incubate sample in 500  $\mu$ L of HCR amplifier solution in a dark environment overnight at RT. To avoid photobleaching the fluorophores on the HCR hairpin, the sample should be kept in a dark environment from this point on. This can be achieved by keeping the sample-containing plate in aluminum foil, throughout all downstream reactions and washes, and only temporarily remove the foil during sample handling (e.g. pipetting the immersion fluid in and out of the wells) or imaging. Unless otherwise noted, all incubations and washes in the following steps are performed in a dark environment, e.g. inside aluminum foil. \*\*.
11. Wash sample with 1 mL of 5 $\times$  SSCT for two times, 1 h each at RT.

**3.1.11 Antibody Staining  
(for Fluorescent Protein  
Readout)**

1. Pre-incubate sample in 1 mL of 5 $\times$  SSCT for 30 min at RT.
2. Incubate sample with primary antibody against fluorescent protein, at desired concentration (to determine this, the user can follow manufacturer's instruction, earlier literature, or use a default concentration of 10  $\mu$ g/mL) in 500  $\mu$ L of 5 $\times$ SSCT overnight at 4 °C. Depending on the resulting level of background and true signal, adjustments to the primary and secondary antibody concentrations may be helpful for optimizing image quality, with repeated trials of this protocol. \*\*.
3. Wash sample with 1 mL of 5 $\times$  SSCT three times, 1 h each at RT.
4. Incubate sample with secondary antibody with a fluorophore, at desired concentration (a good default concentration is 10  $\mu$ g/mL; this concentration can be adjusted) in 500  $\mu$ L of 5 $\times$  SSCT in a dark environment overnight at 4 °C. To avoid photobleaching the fluorophores on the secondary antibody, the sample should be protected from light from this point on (if not already in such state, as instructed in **step 10** of Sub-heading **3.1.10**, i.e. the ExFISH-HCR: Probe hybridization and HCR amplification section). This can be achieved by keeping the sample-containing plate in aluminum foil, throughout all downstream reactions and washes, and only temporarily remove the foil during sample handling (e.g. pipetting the immersion fluid in and out of the wells) or imaging. Unless otherwise noted, all incubations and washes in the following steps are performed in a dark environment, e.g. inside aluminum foil. \*\*.
5. Wash sample with 1 mL of 5 $\times$  SSCT three times, 1 h each at RT. \*\*.

### 3.1.12 NHS-Ester Staining (Optional, for Morphology Readout)

1. Incubate sample with 2  $\mu\text{M}$  of the NHS ester of a fluorescent dye (e.g. Alexa Fluor 546 NHS Ester or Alexa Fluor 647 NHS Ester) in 1 mL of NHS-ester staining buffer ( $5\times$  SSCT, pH 6.0), in a dark environment overnight at RT. To avoid photobleaching the fluorophores, the sample should be protected from light from this point on (if not already in such state). Unless otherwise noted, all incubations and washes in the following steps are performed in a dark environment, e.g. inside aluminum foil. \*\*.
2. Wash sample with 1 mL of  $5\times$  SSCT three times, 30 min each at RT.

### 3.1.13 DAPI Staining (Optional, for DNA Readout)

1. Incubate sample with 5  $\mu\text{g}/\text{mL}$  DAPI in 1 mL of  $5\times$  SSCT for 30 min in a dark environment at RT. To avoid photobleaching the fluorophores, the sample should be protected from light from this point on (if not already in such state). Unless otherwise noted, all incubations and washes in the following steps are performed in a dark environment, e.g. inside aluminum foil.
2. Wash sample with 1 mL of  $5\times$  SSCT three times, 10 min each at RT.

### 3.1.14 Preliminary Imaging at a Partially Expanded State (Only for Samples that Have Not Been Re-Embedded)

For samples that have not been re-embedded, they are currently at a  $\sim 2\times$  expanded state after the immersion in  $5\times$  SSCT. Although a round of preliminary imaging at this point is not necessary for the final imaging, we highly recommend doing this, in order to (1) confirm that the staining performed in the sections above (Subheadings 3.1.9–3.1.13) works as expected (e.g. positive and/or negative control conditions produce a pattern of staining consistent to past results), (2) for experimental conditions with no past results to compare to, get a sense of the localization of the stains, because the user will later need to find these signals at an expanded state, which yields dimmer per-pixel fluorescent intensity and larger physical space to search through, and which could thus make imaging quite challenging without some prior knowledge on where the stains are located, and (3) if pre-expansion images were acquired at the post-gelation stage (in step 12 of Subheading 3.1.7, i.e. the Gelation section), it is a good time to check whether the hydrogel is on its original orientation (i.e. with worms at the bottom surface of the hydrogel) or upside-down, by taking another low-magnification map of the hydrogel sample at this stage, and compare how the animals spatially distribute across the hydrogel sample, between the pre-expansion image and the current image (refer to Subheading 3.1.5 to review the importance of doing this).

1. Transfer sample to a container suitable for imaging, e.g. a glass-bottom 6-well plate. For samples that have not been re-embedded, expand sample by serial washes with reducing

salt concentration as follows. Note that as the gel expands it becomes more fragile, and use the methods described in previous steps to avoid sucking the gel into the pipettor when removing liquid during wash steps.

2. Remove as much liquid from the well as possible. The liquid should be removed to the extent that no more solution that can be taken up by a P1000 or a P200 pipettor, and the gel does not appear to be sliding around when the plate is slightly tilted (e.g.  $\sim 20^\circ$ ).
3. Perform imaging. The standard imaging workflow that we recommend is to first use a low-magnification objective (e.g.  $4\times$ ) to acquire a single  $z$ -plane map of the gel sample (ideally by automated acquisition and stitching of multiple evenly spaced fields of view, which is available in most imaging software), which helps to identify and locate all animals that can be imaged. Next, use the low-magnification map of the gel sample to navigate (via automated stage control, or manually) to the animals and regions of interest. Then, use a high-magnification objective (e.g.  $20\times$  or  $40\times$ ) to acquire  $z$ -stacks through the regions of interest.
4. After imaging, add 2 mL of  $5\times$  SSC to the well, to re-hydrate the sample. Keep sample at  $4^\circ\text{C}$  until ready for final imaging. \*\*.

### *3.1.15 Final Imaging at Fully Expanded State*

1. If a short working distance lens will be used, ensure that the hydrogel sample is in the same orientation as casted during the gelation step, so that worms are located at the bottom of the hydrogel (*see* Subheading 3.1.6 to review this concept). If a hydrogel sample is upside-down, use a flat-tip paintbrush to flip the hydrogel sample. This can be achieved by fully inserting the paintbrush to the bottom of the gel (which is floating in the  $5\times$  SSC in the well), horizontally lifting the paintbrush along with the gel, inverting the paintbrush, lowering the paintbrush until the gel makes contact with the buffer in the well, and finally retrieving the paintbrush without causing the gel orientation to flip back. If unsure about whether a flip was successful, re-image the sample until the sample is confirmed to be on the original orientation (i.e. worms at the bottom of the gel).

For samples that have not been re-embedded, proceed to **step 2**. Otherwise, proceed to **step 5**.

2. Wash sample with 2 mL of  $2.5\times$  SSC for 30 min at RT.
3. Wash sample with 2 mL of  $0.5\times$  SSC for 30 min at RT.
4. Wash sample with 2 mL of  $0.05\times$  SSC for 30 min at RT.
5. Remove as much liquid around the expanded gel as possible. The liquid should be removed to the extent that no more solution that can be taken up by a P1000 or a P200 pipettor,

and the gel does not appear to be sliding around when the plate is slightly tilted (e.g.  $\sim 20^\circ$ ).

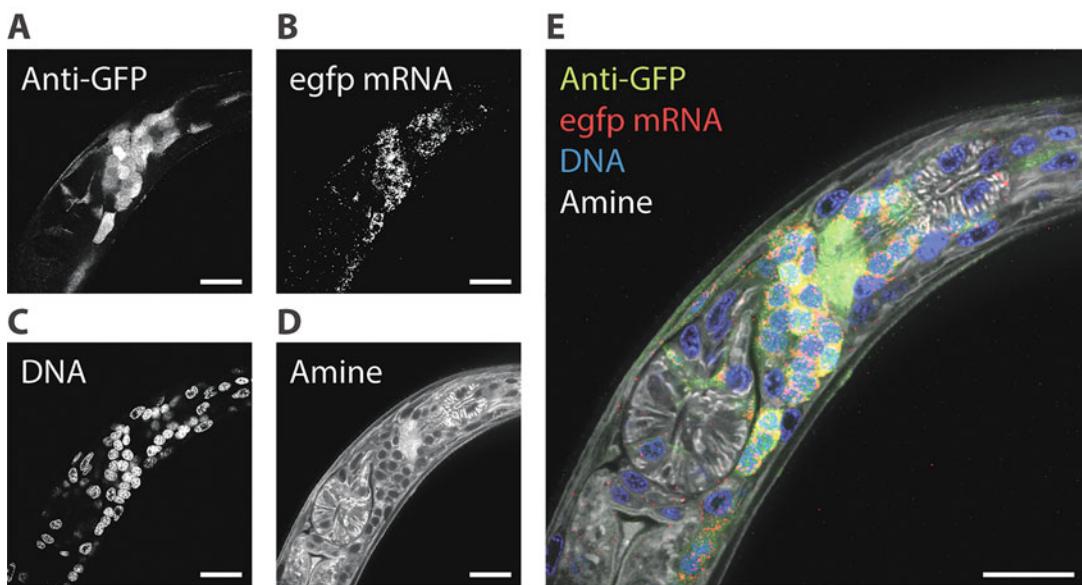
6. Perform imaging. The standard imaging workflow that we recommend is to first use a low-magnification objective (e.g.  $4\times$ ) to acquire a single z-plane map of the gel sample (ideally by automated acquisition and stitching of multiple evenly spaced fields of view, which is available in most imaging software), which helps to identify and locate all animals that can be imaged. Next, use the low-magnification map of the gel sample to navigate (via automated stage control, or manually) to the animals and regions of interest. Then, use a high-magnification objective (e.g.  $20\times$  or  $40\times$ ) to acquire z-stacks through the regions of interest.
7. To store the sample, re-immerse the sample in 2 mL of  $5\times$  SSC at  $4^\circ\text{C}$  after imaging. If the sample was just expanded for imaging, shrink the sample by serial 30 min washes with increasing salt concentration (the steps you used to expand, in reverse order). If the sample was re-embedded (HCR performed for RNA readout), you can apply  $5\times$  SSC directly after imaging. HCR amplicons and IHC staining are stable for up to a week.

### *3.1.16 Agar Immobilization (Optional, for Vibration-Free Imaging)*

If too much sample vibration or drifting is observed under a high-resolution objective (e.g.  $\geq 40\times$ ), affecting image quality, immobilize the expanded gel with 2% (w/w) low-melt agarose solution prepared by dissolving 2 g of agarose in 98 mL of ddH<sub>2</sub>O, heating to a near-boil in a microwave, and mixing by vortex until the agarose powder completely dissolves. Wait 5 min for the solution to cool and for the bubbles to settle to the top of the solution, then add the agarose solution (retrieved from the lower fraction of the solution, with no or little bubbles) to the edges of the expanded gel (after water is completely removed from the well, leaving only the gel at the center of the well) drop by drop (allow  $\sim 3$  s in between drops), starting at opposite corners, then proceeding to opposite sides. After  $\sim 2$  min, slowly add more low-melt agarose solution, again in a dropwise manner, to cover the entire gel. Wait for  $\sim 2$  more minutes for the agarose to solidify, and proceed to imaging.

### *3.1.17 Example of Final Images*

An example of confocal images acquired after the standard ExCel protocol is shown in Fig. 3. In this example, a transgenic L2 hermaphrodite animal expressing *tag-168p::GFP* is sequentially stained with ExFISH-HCR against the egfp mRNA, antibody against GFP, NHS ester of a fluorescent dye (Atto 647N NHS ester; against amines; for anatomical features) and DAPI (for DNA location). The sample is then expanded to  $3.3\times$  linear expansion factor, and imaged under confocal microscopy under a  $40\times$  objective (water immersion, with a numerical aperture of 1.15).



**Fig. 3** Example of confocal images acquired after the standard ExCel protocol. The pharyngeal region of a representative, standard-ExCel-processed L2 hermaphrodite animal, stained sequentially with ExFISH-HCR against the egfp mRNA, antibody against GFP, NHS ester of a fluorescent dye (Atto 647N NHS ester; against amines; for anatomical features) and DAPI (for DNA location), as schematized in Fig. 1a–i, m–q. (a–d) Single-channel images of each staining modality. (e) Merged composite image from combining (a–d). Strain expressed tag-168p::GFP. Images are single-z-plane confocal micrographs acquired under a 40 $\times$  objective (water immersion, with a numerical aperture of 1.15). Brightness and contrast settings: each channel was first set by the automatic adjustment function in Fiji, and then manually adjusted (raising the minimum-intensity threshold and lowering the maximum-intensity threshold) to improve contrast. Linear expansion factor: 3.3 $\times$ . Scale bars: 10  $\mu$ m (in biological units, i.e. post-expansion lengths are divided by the expansion factor of the worm). (Figure is modified from Yu et al. 2020 [7], with permission granted by the Creative Commons Attribution license)

### 3.2 Epitope-Preserving Expansion of *C. elegans*

#### 3.2.1 Overview

1. 2.5–3.5 $\times$  linear expansion factor.
2. Supports readout of a majority of endogenous and exogenous epitopes tested (~70% of epitopes that can be detected by non-IgM-class antibodies).
3. Moderate isotropy (8–25% error over length scales between 0 and 100  $\mu$ m).
4. Protocol duration, from fixation to imaging: 18 days.

See Fig. 4 for the workflow of the epitope-preserving ExCel protocol, and Table 2 for a sample schedule. To start the Epitope-Preserving ExCel protocol, first follow the standard ExCel protocol in Subheading 3.1 for Fixation and Cuticle Reduction (Subheading 3.1.2), Sample Allocation (Subheading 3.1.3) and Protein Anchoring (Subheading 3.1.5). Then, proceed to Subheading 3.2.2.

### 3.2.2 Gelation

Same as in gelation procedure in the standard ExCel protocol (Subheading 3.1.7), but in **step 13**, prepare Collagenase VII Solution instead of digestion solution. Also, to ensure that the final state expanded worm could be captured within the working distance of a high-NA lens, perform either or both of the two methods for tracking hydrogel orientation (*see* Subheading 3.1.6).

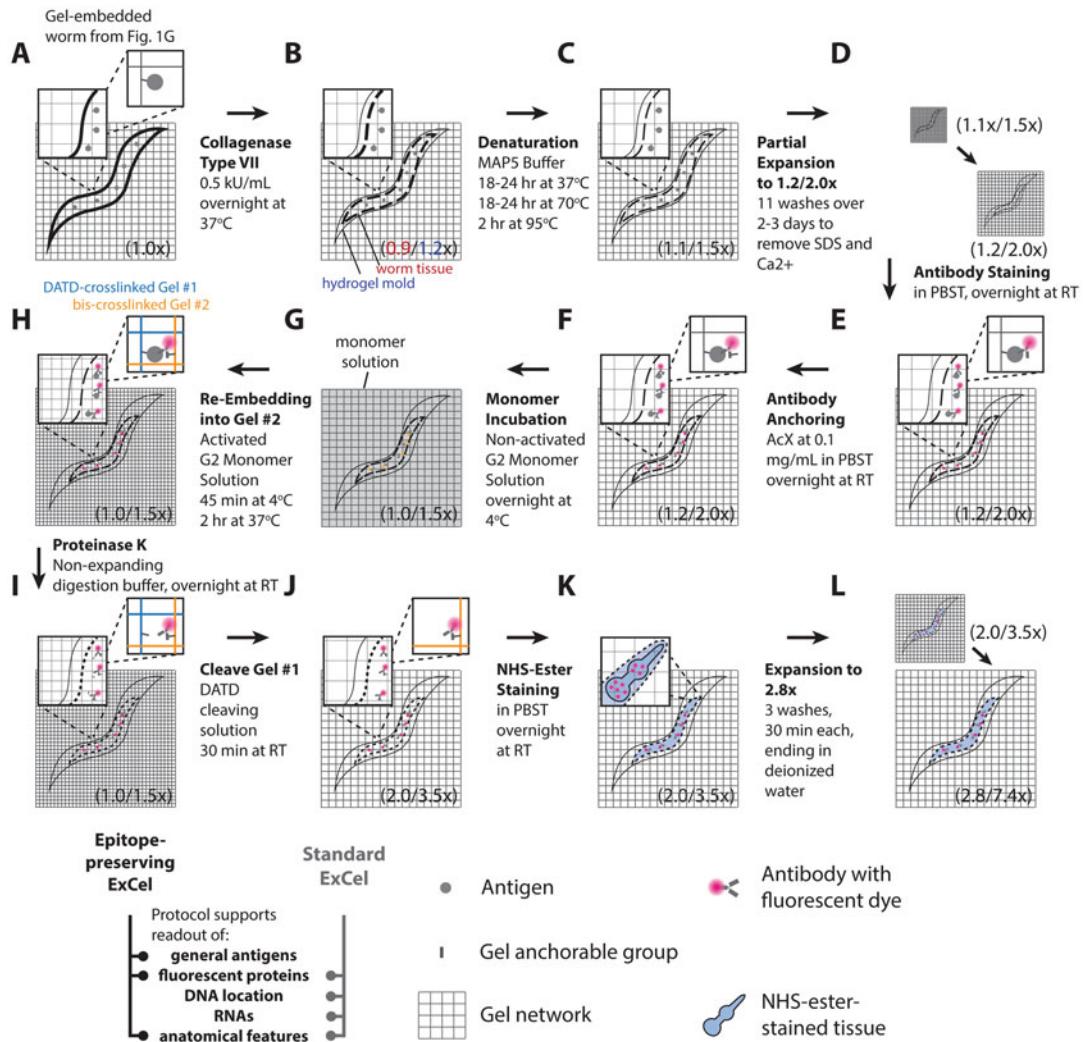
Reaction volumes in the following sections are specified for a single hydrogel. Scale up if you are processing multiple gels.

### 3.2.3 Collagenase VII-Mediated Cuticle Digestion

- Right after the samples are transferred to 37 °C for the 2-h incubation, thaw a frozen aliquot of Collagenase VII Stock Solution (500 µL per aliquot; at 1 kU/mL) by incubating the frozen aliquot at 4 °C until the 2-h gelation incubation is complete.
- After the 2-h gelation incubation and before opening the gelling chambers, prepare 1 mL of Collagenase VII Solution by adding 500 µL of Collagenase VII Stock Solution to 500 µL of Collagenase VII Dilution Buffer.
- Add ~50 µL of Collagenase VII Solution to the trimmed gel immediately after trimming (the trimmed gel should still be on the coverslip). Let the gel sit immersed in the solution for 5 min at RT.
- Add ~950 µL of Collagenase VII Solution to a 2-mL Eppendorf tube (which has a flatter bottom compared to the 1.5-mL tubes; e.g. VWR 20170-170; if not available, a 1.5-mL tube can work too). With a flat-tip paint brush, slowly insert the brush under the gel until most of the brush is inserted, vertically lift the gel with the brush, and transfer the gel into the tube. Incubate sample overnight at 37 °C. \*\*.
- Wash sample (i.e. replace the incubating solution) three times with 1 mL of TNC-40020 Buffer for 1 h each at RT.

### 3.2.4 Protein Denaturation

In this section, the hydrogel sample will be immersed in the Protein Denaturation Buffer, which contains a near-saturating concentration of SDS. As a result, at various steps of this section (especially during the washing steps after the 95 °C incubation of **step 4**), white precipitates of SDS can form inside the hydrogel. As long as you perform the extensive washes thoroughly, as specified in the steps below, the SDS precipitates should be completely removed from the hydrogel samples at the end of this section, and have never been observed to affect the final results. (But of course, if not completely washed away by the specified procedure, SDS can in principle disrupt antibody-antigen recognition, and result in reduced fluorescent signals from the immunostaining performed in the next section).



**Fig. 4** Workflow for the epitope-preserving ExCel protocol. A method for expanding cuticle-enclosed intact *C. elegans*, while permitting readout of a majority of antigens that are detectable through non-IgM-class antibodies (~70%; estimated from the immunostaining results from a panel of non-IgM antibodies [7]). Sample processing prior to Panel a is identical to the workflow for the standard ExCel protocol without ExFISH (as shown in blue arrows in Fig. 1) until, and including, the gelation step (Fig. 1a–c, e–g). The linear expansion factor of the hydrogel-specimen composite is shown in parentheses. For stages in which the worm tissue expands to a less extent than the surrounding hydrogel, which occurs due to incomplete homogenization of mechanical strength of the fixed worm tissue, the expansion factors of the worm and of the hydrogel are shown in front of and after a slash sign, respectively. (a–l) Steps of the protocol, with the bold text indicating the title of the step. (a) Hydrogel polymerization is performed on the specimen, by first incubating the specimens in activated monomer solution for 30 min at 4 °C, transferring the specimens into a gelling chamber, and incubating the chamber for 2 h at 37 °C. During polymerization, AcX-modified proteins are covalently anchored to the hydrogel network. (b) Specimens are treated with chromatography-purified collagenase type VII at 0.5 kU/mL, in a calcium-containing tris-buffered saline overnight (18–24 h) at 37 °C. During this treatment, the hydrogel expands by ~1.2× linearly, whereas the worm slightly reduces in size to ~0.9× linearly. Due to the mismatch in expansion factor between the worm and the gel, the worm

1. If sample is not already in a capable or sealable tube, transfer samples into one (e.g. 2-mL tube, VWR 20170-170), because the sample will be incubated in Protein Denaturation Buffer at elevated temperatures (37 °C, 70 °C, 95 °C) in the next few steps. Without sealing the content, evaporation of water from the buffer will cause extensive precipitation of SDS, and also change the concentrations of other ingredients of the denaturation buffer.
2. Incubate sample with 1 mL of Protein Denaturation Buffer (stored at 37 °C) overnight at 37 °C. \*\*.



**Fig. 4** (continued) tissue detaches from the surrounding hydrogel, but physically remains in the hydrogel mold that was made of its own shape during the gelation step in **a**. **(c)** Specimens are processed with a denaturation treatment, in which they are incubated in a minimally-expanding protein-denaturing buffer overnight (18–24 h) at 37 °C, overnight (18–24 h) at 70 °C, and 2 h at 95 °C. Reduced calcium and NaCl concentrations are used in this buffer, compared to other non-expanding buffers designed in this paper, due to their incompatible solubility with SDS at higher concentrations. **(d)** Specimens are washed four times in a tris-buffered saline to remove SDS from the hydrogel sample. Specimens are then washed four times in tris-buffered saline with reducing calcium concentration (once with TNT Buffer + 10 mM CaCl<sub>2</sub>, and then three times with TNT Buffer) to remove calcium ions from the hydrogel sample. Finally, specimens are washed with phosphate-buffer saline with reducing NaCl concentration (once with PBST-0.1% + 500 mM NaCl, twice with PBST-0.1%). **(e)** Specimens are immunostained with fluorescent antibodies against the target antigens. **(f)** Specimens are incubated with AcX at a concentration of 0.1 mg/mL in PBST-0.1% overnight at RT. This step equips proteins, including the fluorescent antibodies introduced in **e**, with a polymer-anchorable moiety. **(g)** Specimens are incubated in Non-Activated G2 Monomer Solution overnight at 4 °C, to ensure complete diffusion of the monomer solution throughout the specimen, prior to the gelation reaction. **(h)** Specimens are re-embedded into a second expandable hydrogel, by incubating the specimens in activated monomer solution for 30 min at 4 °C, transferring the specimens into a gelling chamber, and incubating the chamber for 2 h at 37 °C. During polymerization, AcX-modified fluorescent antibodies are covalently anchored to the hydrogel network of the second hydrogel (orange grids). We use blue grids to represent the hydrogel network of the first, DATD-crosslinked hydrogel (i.e. the network synthesized in Panel **a**), to differentiate it from the network of the re-embedding second hydrogel. **(i)** Specimens are treated with Proteinase K at 8 U/mL, in Non-expanding Digestion Buffer overnight (18–24 h) at RT, to further reduce the mechanical strength of the original worm tissue and permit greater expansion. During this proteolytic treatment, most proteins lose antigenicity, but some of the fluorescent signals from AcX-anchored fluorescent proteins are retained. **(j)** Specimens are treated with DATD-cleaving solution for 30 min at RT, to chemically disintegrate the first hydrogel, which contains the periodate-cleavable crosslinker *N,N*-diallyl-tartardiamide (DATD), while sparing the second hydrogel, which contains a periodate-resistant crosslinker, *N,N*-methylene-bis-acrylamide (bis). **(k)** To visualize anatomical features, specimens can be stained with an *N*-hydroxysuccinimide ester (NHS ester) of fluorescent dye. NHS-ester staining is performed at 5 mM in PBST-0.1% overnight at RT. **(l)** Specimens are expanded with one wash in 0.1× PBS and two washes in deionized water. At this stage, the hydrogel expands by ~7.4× linearly, whereas the worm tissue expands by ~2.8× linearly, within a range from 2.5 to 3.5× (median, 2.78×; mean, 2.83×;  $n = 10$  independently processed hydrogels from 2 sets of experiments [7]). After expansion, specimens are ready for imaging. (Figure is modified from Yu et al. 2020 [7], with permission granted by the Creative Commons Attribution license)

3. Replace solution with another 1 mL of Protein Denaturation Buffer (at 37 °C). Incubate overnight at 70 °C. \*\*.
4. Pre-warm 1 mL of Protein Denaturation Buffer to 70 °C.
5. Replace solution with 1 mL of Protein Denaturation Buffer (pre-warmed to 70 °C in **step 3**). Incubate for 2 h at 95 °C.
6. After the 95 °C incubation, incubate samples at 37 °C for 30 min. At this time, pre-warm at least 4 mL of TNC-40020 Buffer to 37 °C.
7. Wash sample three times with 1 mL of TNC-40020 (pre-warmed to 37 °C in **step 6**) for 1 h each at 37 °C. Then, incubate sample in TNC-40020 overnight at RT. \*\*.
8. Wash sample once with TNT-10 Buffer (TNT Buffer +10 mM CaCl<sub>2</sub>) for 30 min at RT.
9. Wash sample twice with TNT Buffer for 30 min each at RT. Then, incubate sample in TNT Buffer overnight or at least 6 h, at RT. The rationale here is to completely wash out remaining calcium ions from the gel, so when a phosphate-based buffer (PBS) is added in the next step to prepare for immunostaining, phosphate ions will not precipitate with calcium ions to form a nearly insoluble product, which can cause a cloudy appearance in the gel and affect image quality. \*\*.
10. Transfer gel sample to a 24-well plate by directing pouring the content in the 2-mL tube into a well. Alternatively, use a flat-tip paint brush to transfer the gel.
11. Wash sample once with PNT-500 (1× PBS + 0.1% Triton X-100 + 500 mM NaCl) for 30 min at RT.
12. Wash sample twice with PBST-0.1% (1× PBS + 0.1% Triton X-100) for 30 min each at RT.

### 3.2.5 Antibody Staining

1. Incubate sample with primary antibody, at desired concentration (to determine this, the user can follow manufacturer's instruction, earlier literature, or use a default concentration of 5 µg/mL; this concentration can be later adjusted to optimize for signal to background ratios) in 500 µL of PBST-0.1% overnight at RT. \*\*.
2. Wash sample with 1 mL of PBST-0.1% for three times, 1 h each at RT.
3. Incubate sample with secondary antibody with a fluorophore, at desired concentration (to determine this, the user can follow manufacturer's instruction, earlier literature, or use a default default concentration of 10 µg/mL; this concentration can be later adjusted to optimize for signal and background ratios) in 500 µL of PBST-0.1% overnight at RT. \*\*.

4. Wash sample with 1 mL of PBST-0.1% for three times, 1 h each at RT.

Linear expansion factor of the worm is  $\sim 1.2 \times$  at this stage. (Hydrogel expansion factor is  $\sim 2.0 \times$ , so worms will detach from the surrounding hydrogel, but stay inside the hydrogel mold. With reasonably gentle washes, retention of worms inside the hydrogel sample is typically  $>95\%$  despite of this detachment due to the expansion factor mismatch between the worm and the gel.)

### *3.2.6 Preliminary Imaging to Check Antibody Staining*

The samples are currently at a  $\sim 1.2 \times$  expanded state. Although a round of preliminary imaging at this point is not necessary for the final imaging, we highly recommend doing this, in order to confirm that the antibody staining works as expected (e.g. positive and/or negative control conditions produce a pattern of staining consistent to past results). The fluorescent intensity at the stained sites is maximal at the current stage, and will only reduce during the following procedures. Thus, if a particular stain is not showing a clear signal at this state, there is very little possibility that it will result in a clear image after expansion.

1. Transfer sample to a container suitable for imaging, e.g. a 24-well plate (glass-bottom ones are recommended, but plastic ones are also sufficient for this preliminary imaging), if samples are not already in such a container.
2. Remove most liquid (e.g. 95–99%) from the well as possible. The liquid should be removed to the extent that no more solution that can be taken up by a P1000, and the gel does not appear to be sliding around when the plate is slightly tilted (e.g.  $\sim 20^\circ$ ).
3. Perform imaging. The standard imaging workflow that we recommend is to first use a low-magnification objective (e.g.  $4 \times$ ) to acquire a single z-plane map of the gel sample (ideally by automated acquisition and stitching of multiple evenly spaced fields of view, which is available in most imaging software), which helps to identify and locate all animals that can be imaged. Next, use the low-magnification map of the gel sample to navigate (via automated stage control, or manually) to the animals and regions of interest. Then, use a high-magnification objective (e.g.  $20 \times$  or  $40 \times$ ) to acquire z-stacks through the regions of interest.
4. After imaging, add 1 mL of PBST-0.1% to the well, to re-immersing the sample for storage. The immunostained samples can be stored at  $4^\circ\text{C}$  for up to 3 days. \*\*.
5. Based on the imaging result (e.g. whether samples have the expected staining patterns and reasonable levels of signals), determine which samples will be processed further for the

final imaging at  $2.8\times$  linear expansion factor. Only proceed those samples through the next sections.

### *3.2.7 Antibody Anchoring*

1. Incubate sample with AcX at 0.1 mg/mL (1:100 dilution of AcX stock) in 1 mL of PBST-0.1%, overnight at RT. \*\*.

2. Wash sample with 1 mL of MOPST-0.1% for three times, 1 h each at RT.

### *3.2.8 Re-Embedding into an Expandable Second Gel*

1. Flip each gel to the same side as when they were casted during the initial gelation (refer to Subheading 3.1.6 to review this concept). This ensures that the worms remain on the bottom surface of the gels, so the working distance of the objective can maximally cover the depth of the worm at the final imaging stage.

2. Incubate sample in 1 mL of Non-activated G2 Monomer Solution overnight at 4 °C. \*\*.

3. Prepare 1 mL of Activated G2 Monomer Solution with all reagents except for APS (Epitope-preserving ExCel G2 Monomer Solution Stock + 4-HT + TEMED; do not add the activator APS yet), and thoroughly mix the solution by a vortex. Chill the solution to 4 °C on a cold block. It is important to ensure that the solution is fully chilled to 4 °C before proceeding.

4. Remove liquids from the sample.

5. Activate the monomer solution from step 3 by adding 20 µL of 10% APS, and thoroughly mix the solution by a vortex. Start a timer right after the APS is added.

6. Add 1 mL of Activated G2 Monomer Solution to the sample. Incubate the sample at 4 °C for 30 min, on a pre-chilled cold block.

7. During incubation, construct gelling chambers (based on the same architecture as shown in Fig. 2 and the Gelation section of the standard ExCel protocol above). Use a stack of one #0 cover glass and one #1 cover glass (total height ~250 µm) for each spacer.

8. Immediately after the 30-min incubation, transfer 65 µL of the monomer solution from the well that contains the gel sample to the center of the gelling chamber, as a single droplet.

9. With a flat-tip paint brush, transfer the gel sample into the droplet of monomer solution. If the gel orientation has been confirmed in step 1, ensure that the hydrogel is placed in the correct orientation (i.e. with worms located at the bottom surface of the gel). Then, use a pipettor to temporarily remove the monomer solution from the gel, to ensure that the gel is completely flat and not folded. If the gel is folded, use a paint

brush to unfold the gel. Then, add the monomer solution back on top of the gel to immerse the gel.

10. Close the chamber by carefully and slowly placing a rectangular cover glass (e.g. 22 × 50 mm) on top of the spacers, minimizing bubble formation. One way to achieve this is to use a tweezer to clip on one end of a rectangular cover glass, hold the cover glass right above the chamber by ~5 mm (in parallel to the glass slide; no contact yet), press down the un-clipped end of the rectangular cover glass until it contacts the spacer, release the clipping force so that the cover glass just rests on the lower branch of the tweezer, and then slowly lower the tweezer until the cover glass contacts the other spacer.
11. Incubate the gelling chamber at 37 °C for 2 h, in a humidified chamber (e.g. a 0.75-quart storage container, such as [Amazon.com](#) #B01M2BTKYB, with the gelling chamber sitting on top of a stack of two lids of the 6-, 24-, or 96-well plate, and with a small amount (10–20 mL) of deionized water at the bottom of the sealed container).
12. After the 2-h incubation and before proceeding to **step 13** (chamber opening), prepare the digestion solution (*see step 1* of the next section).
13. With a tweezer or a razor blade, remove the top cover glass and spacer and leave the gel on the glass slide. The gel can occasionally come off with the top cover glass. In that case, leave the gel on the cover glass and dispose the rest of the chamber.
14. At this point, the boundary of the re-embedded gel sample should be easily visible under sufficient room light. With a razor blade, trim away the excessive gel outside of the original gel sample.

### 3.2.9 Proteinase K Digestion

Based on our experience, the extent of digestion seems to be quite sensitive to the quality of Proteinase K. Use Proteinase K ordered within 2 months.

1. Prepare 1 mL of digestion solution by adding Proteinase K to Non-expanding Digestion Buffer to a final concentration of 8 U/mL (1:100 dilution).
2. Add ~50 µL of digestion solution to the trimmed gel. Let the gel sit immersed in digestion solution for 2 min at RT.
3. Add ~950 µL of digestion solution to a well in a 24-well plate. With a flat-tip paint brush to transfer the gel into the well. Incubate sample overnight (18–24 h) at room temperature. \*\*.
4. Wash once with 1 mL of TNT-20 Buffer (TNT Buffer + 20 mM CaCl<sub>2</sub>) for 30 min at RT.

5. Wash once with 1 mL of TNT-10 Buffer (TNT Buffer + 10 mM CaCl<sub>2</sub>) for 30 min at RT.
6. Wash three times with 1 mL of TNT Buffer for 1 h each at RT.
7. Wash sample once with PNT-500 (1× PBS + 0.1% Triton X-100 + 500 mM NaCl) for 30 min at RT.
8. Wash sample once with 1× PBS for 30 min at RT. \*\*.

**3.2.10 Cleave DATD-Crosslinked Gels #1**

1. Transfer sample into a 6-well plate with a flat-tip paint brush.
2. Make DATD-cleaving solution from freshly prepared sodium periodate stock solution.
3. Incubate sample in 2 mL of DATD-cleaving solution (or volumes sufficient to cover the entire gel) at RT for **exactly 30 min** with gentle shaking. Longer incubation periods can degrade the structural integrity of the bis-crosslinked Gel #2, which we aim to preserve here.
4. Wash sample with 5 mL of 1× PBS for 20 min at RT with gentle shaking.
5. Repeat **step 4** five times (six washes total), and then leave in 5 mL of 1× PBS at RT overnight with gentle shaking. It is crucial to completely wash away sodium periodate from the gels, since residual amounts of periodate ion can disintegrate the bis-crosslinked Gel #2 over a time scale of ~2–24 h. \*\*.

**3.2.11 NHS-Ester Staining (Optional, for Morphology Readout)**

1. Incubate sample with 5 µM of the NHS ester of a fluorescent dye in 2 mL of PBST, overnight at RT with gentle shaking. \*\*.
2. Wash sample with 2 mL of PBST three times, 30 min each at RT.

**3.2.12 Expansion and Imaging**

1. To ensure that the expanded sample will fit flatly into the final imaging container, the sample needs to be trimmed into sizes that, when multiplied by ~2× in each dimension (as the sample will expand by another ~2× from the current state), will completely fit in the imaging container. For example, if the final imaging container is a glass-bottom 6-well plate (*see step 2*), and if sample is larger than 1 cm in any dimension, cut the sample into pieces that are each within 1 cm in any dimension. One way to achieve this is by performing two perpendicular cuts, in the shape of a plus sign, at the center of the sample, which will result in 4 adequately-sized pieces that can each be expanded and imaged separately. The cutting can be performed with a #1.5 rectangular coverslip (we do not recommend a thinner thickness, as the coverslip can break easily) directly inside the 6-well plate that currently holds the samples.
2. Transfer sample to a container suitable for imaging, e.g. a glass-bottom 6-well plate. For high-magnification objectives with

limited working distance, ensure that the gel is flipped to the same orientation as when it was casted during the initial gelation, so that the animals will be located at the bottom surface of the gel. (Refer to Subheading 3.1.6 to review this concept.)

3. Wash sample with 2 mL of 0.1× PBS for 30 min at RT.
4. Wash sample with 2 mL of deionized water for 30 min at RT.
5. Repeat step 3 for one more time (two washes with deionized water total).
6. Remove as much liquid around the expanded gel as possible.
7. Perform imaging.
8. To store the sample, re-immerse the sample in 2 mL of 1× PBS and incubate at 4 °C after imaging. Due to the covalent linkage between the antibody and the hydrogel, performed in the “Antibody Anchoring” section above, fluorescent signal in the sample is stable for at least 1 week.

*See* Subheading 3.1.16 for agar immobilization protocol to prevent sample vibration or drift during imaging.

### 3.2.13 Example of Final Images

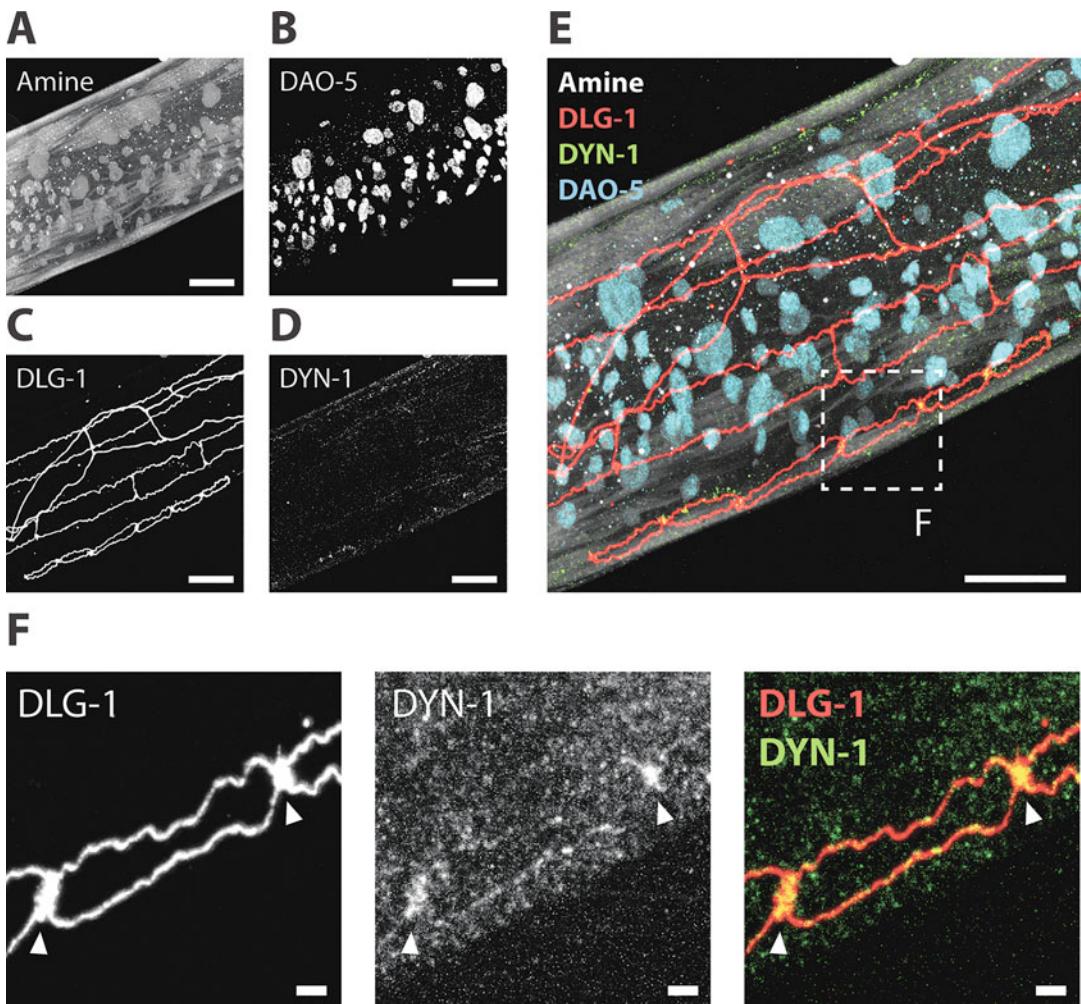
An example of images acquired after the epitope-preserving ExCel protocol is shown in Fig. 5.

## 3.3 Iterative Expansion of *C. elegans* (iExCel)

### 3.3.1 Overview

1. ~20.0× linear expansion factor.
2. Supports readout of fluorescent proteins; requires oligo-conjugated secondary antibodies (*see* Subheading 3.3.3 for the conjugation protocol; protocol duration: ~3 days).
3. Similar level of isotropy as ExCel (1.5–4.5% error over length scales between 0 and 100 μm).
4. Protocol duration, from fixation to imaging: 19 days.
5. It is highly recommended that the user first confirms successful staining and imaging of the fluorescent protein target with standard ExCel, prior to attempting the iExCel protocol on the same strain(s). This is because iExCel is both more technically demanding, requires longer processing times, and results in dimmer absolute signal (i.e. fluorescent intensity under a microscope) compared to the standard ExCel protocol. If a bright signal cannot be observed from the standard ExCel protocol for a given fluorescent protein on a given strain, the chance is very low that the signal will be successfully detected under the current iExCel protocol.

*See* Fig. 6 for the workflow of the iterative ExCel protocol, and Table 3 for a sample schedule. The iterative ExCel protocol described here only supports readout of fluorescent proteins, i.e. same as the standard ExCel protocol shown above. This protocol is currently not compatible with RNA readout. The sensitivity



**Fig. 5** Example of confocal images acquired after the epitope-preserving ExCel protocol. A representative epitope-preserving-ExCel-processed L2 hermaphrodite animal, stained with antibodies against DLG-1 (disc large; a scaffolding protein that localizes to adherens junctions), DYN-1 (dynamin; localizes to clathrin-mediated endocytic sites), DAO-5 (a nuclear protein) [18], and an NHS ester of a fluorescent dye (Alexa 405 NHS ester; against amines; for anatomical features). (**a–d**) Single-channel images of each staining modality, centered nearby the developing vulva (lower boxed region in **a**). (**e**) Merged composite image from combining **a–d**. Boxed region marks one of the six vulval progenitor cells, as delineated by the adherens junction marker DLG-1, and is shown in magnified views in Panel **f**. (**f**) Magnified view of the boxed region in **e**, as single-channel images of DLG-1 (left) or DYN-1 (middle) staining, or merged composite image between these two channels (right). Arrows, sites of contact between vulval progenitor cells, which shows co-localized signals of DLG-1 and DYN-1. Images are max-intensity projection of a confocal stack acquired through the entire animal (for Panels **a–e**), or only through the DLG-1 marked structure (for Panel **f**; to reduce the noise coming from planes outside of the structure of interest; i.e. the DLG-1 marked adherens junctions of the developing vulva), under a 40 $\times$  objective (water immersion, with a numerical aperture of 1.15). Brightness and contrast settings: each channel was first set by the automatic adjustment function in Fiji, and then manually adjusted (raising the minimum-intensity threshold and lowering the maximum-intensity threshold) to improve contrast. Linear expansion factor: worm, 3.2 $\times$ ; surrounding hydrogel, 7.9 $\times$ . Scale bars: Panel **a–e**, 10  $\mu$ m; Panel **e**, 1  $\mu$ m (in biological units, i.e. post-expansion lengths are divided by the expansion factor of the worm). The pharyngeal region of a representative, standard-ExCel-processed L2 hermaphrodite animal,

to read out target signal is lower than the standard ExCel protocol (due to a greater volumetric dilution associated with the expansion process), and therefore it works best on fluorescent proteins that are strongly expressed (ideally, with clearly observable signal at the pre-expansion state, when imaged under a high NA ( $>\sim 0.8$ ) objective under typical illumination settings on a confocal microscope).

Prior to starting the iterative ExCel protocol, read through Subheading 3.3.2, and synthesize the DNA-conjugated secondary antibody (or antibodies) for the upcoming experiment, unless you have enough stock from a previous conjugation. A protocol for conjugating the DNA oligo-nucleotides to secondary antibodies is shown in Subheading 3.3.3.

To start the iterative ExCel protocol, first follow the standard ExCel protocol in Subheading 3.1 for Fixation and Cuticle Reduction (Subheading 3.1.2), Sample Allocation (Subheading 3.1.3), Protein Anchoring (Subheading 3.1.5), Gelation (Subheading 3.1.7), and Digestion (Subheading 3.1.8). Then, proceed to Subheading 3.3.4.

### 3.3.2 Pre-iterative ExCel Notes and Protocols

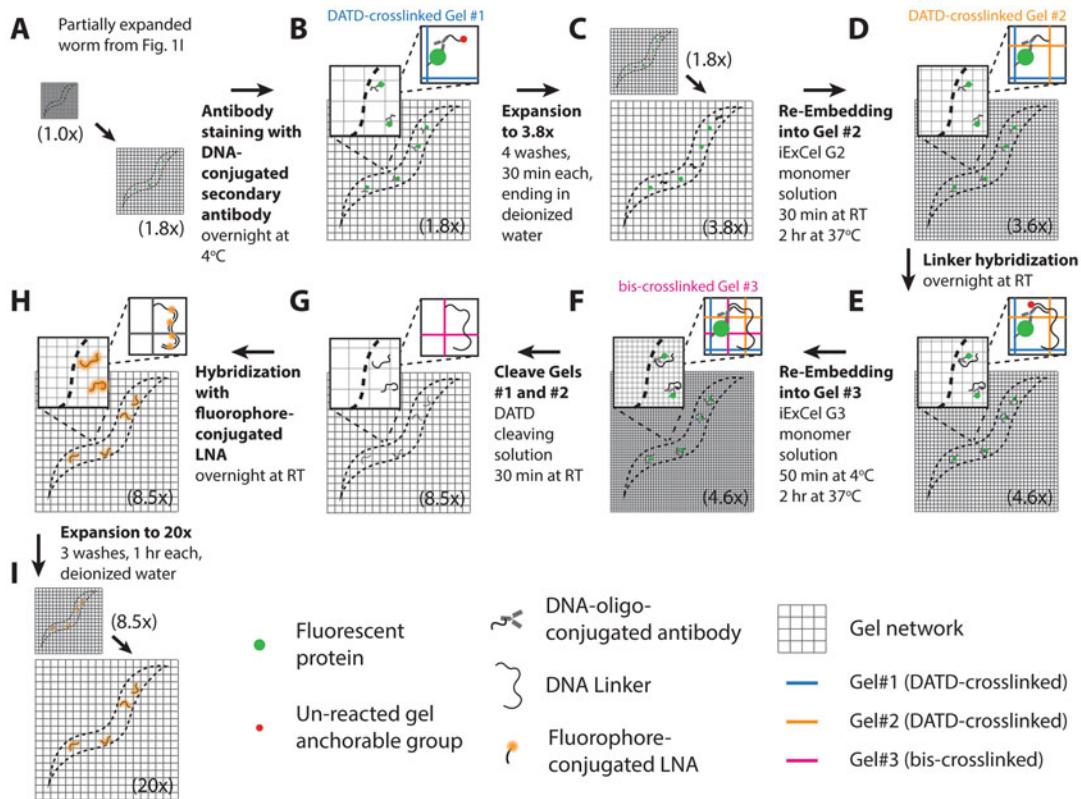
A number of oligo-nucleotides are used in this protocol. They serve several purposes: (1) acting as spatial anchors for the immunostained locations, (2) signal transfer between gels, and (3) signal amplification and fluorescent readout. Their sequences and ordering information can be found in Table 4.

It is also necessary to synthesize DNA-conjugated secondary antibody prior to starting the Iterative ExCel protocol. The procedure for this is shown in Subheading 3.3.3.

Finally, since the hydrogel sample will become  $\sim 20\times$  larger in the  $z$ -dimension at the end of this protocol, if it is desired to image with a short-working-distance objective (e.g.  $<1$  mm; most high NA magnification objectives are in this category) at the fully expanded state, it is necessary to ensure that the gel is flipped back to the same side as the Gel#1 casted state, at two time points: (1) when the gel is re-embedded into Gel #2, and (2) before the expansion process prior to the final imaging. This way, the worms (which settle to the lower  $\sim 50$   $\mu\text{m}$  during Gel #1 gelation, out of the full  $\sim 180$ - $\mu\text{m}$  thickness of the gel) will remain at the bottom

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**Fig. 5** (continued) stained sequentially with ExFISH-HCR against the egfp mRNA, antibody against GFP, NHS ester of a fluorescent dye (Atto 647N NHS ester; against amines; for anatomical features) and DAPI (for DNA location), as schematized in Fig. 1a–i, m–q. (a–d) Single-channel images of each staining modality. (e) Merged composite image from combining a–d. Strain expressed tag-168p::GFP. Images are single-z-plane confocal micrographs. Brightness and contrast settings: each channel was first set by the automatic adjustment function in Fiji, and then manually adjusted (raising the minimum-intensity threshold and lowering the maximum-intensity threshold) to improve contrast. Linear expansion factor:  $3.3\times$ . Scale bars: 10  $\mu\text{m}$ . (Figure is modified from Yu et al. 2020 [7], with permission granted by the Creative Commons Attribution license)



**Fig. 6** Workflow for the iterative ExCel (iExCel) protocol. A method for iteratively expanding cuticle-enclosed intact *C. elegans*, for a final linear expansion factor of ~20×. Sample processing prior to Panel A is identical to the workflow for the standard ExCel protocol without ExFISH (as shown in blue arrows in Fig. 1) until, and including, the post-Proteinase-K partial expansion step (Fig. 1a–c, e–g). The linear expansion factor of the hydrogel-specimen composite is shown in parentheses. (a–i) Steps of the protocol, with the bold text indicating the title of the step. (a) Specimens are partially expanded from a linear expansion factor of 1.0× to 1.8×, with the same protocol as shown in Fig. 1I. (b) Specimens are immunostained first with primary antibodies against fluorescent proteins in 5×SSCT overnight at 4 °C, and then with secondary antibodies that have been conjugated to a 24-base DNA oligonucleotide, in DNA-conjugated Antibody Staining Buffer overnight at 4 °C. The DNA oligo is conjugated to the antibody at the 3' end, and contains a gel-anchorable group at the 5' end. (c) Specimens are expanded from a linear expansion factor of 1.8× to 3.8×, with the same protocol as shown in Fig. 1M. (d) Specimens are re-embedded into another non-expandable hydrogel (“Gel #2”) to lock up its size at the expanded state, as shown in Fig. 1N, except that the monomer solution is replaced by DATD-crosslinked Re-embedding Monomer Solution, which results in a hydrogel that can be later disintegrated via crosslinker cleavage, to allow full expansion of the final expandable gel. The DATD-crosslinked re-embedding monomer solution contains a charged molecule APS. Therefore, the linear expansion factor slightly drops from 3.8× to 3.6× during this step. During hydrogel polymerization, the DNA oligo on the antibody, which contains a gel-anchorable group, is covalently anchored to the second hydrogel network (orange grids). (e) Specimens are incubated with a 100-base DNA oligonucleotide (“Linker”), which hybridizes to the 24-base DNA oligo on the secondary antibodies, and which contains a gel-anchorable group on its 5' end, in iExCel hybridization buffer overnight at RT. (f) Specimens are re-embedded into another expandable hydrogel (“Gel #3”), by incubating the specimens in Activated iExCel G3 Monomer Solution for 30 min at 4 °C, transferring the specimens into a gelling chamber, and incubating the chamber for 2 h at 37 °C. During polymerization, the linker DNA oligo, which contains a gel-anchorable group, is covalently anchored to the hydrogel network of the third hydrogel (magenta grids). (g) Specimens are treated with DATD-cleaving solution

surface of the gel at the end of this protocol, ensuring maximal coverage of the worm tissue by the working distance of the objective during the final imaging. In order to provide a way to check the orientation (side) of the gel throughout the protocol, at least one of the two additional procedures is necessary at the end of the Gelation step. Refer to Subheading 3.1.6 to review these concepts.

### 3.3.3 Protocol for Synthesizing DNA-Conjugated Secondary Antibody for iExCel

This protocol is essentially identical to the conjugation protocol in the original ExM manuscript (available on [www.expansionmicroscopy.org](http://www.expansionmicroscopy.org)), except for 1 modification. For the reaction between unconjugated secondary antibody and S-HyNic, three times of the S-HyNic concentration is used in this protocol compared to the original one (6:100 dilution of the S-HyNic stock solution, instead of 2:100 dilution in the original protocol).

#### Prepare the DNA for Conjugation

In this part, the process can be paused after any of these steps: **steps 10, 12, 15, 19, 29**. If pausing is desired, store the reagent at 4 °C, after the specified steps are complete.

1. Order from IDT the DNA oligo “5’Ac-AA-B1-AA-3’Amine” (see Table 4 for sequence) at a synthesis scale of 1 μmole. If two-color readout is desired, additionally order the oligo “5’Ac-AA-A2-AA-3’Amine.” Be sure to add the specified modifications at the 5’ and 3’ ends. Choose standard desalting as the purification method.

The instruction below applies to a single tube of DNA oligo from IDT. If two-color readout is desired, perform process to both tubes of oligos.

2. Add 100 μL of deionized water to the DNA oligo (shipped dry). Vortex for 1 min to dissolve. Spin with a tabletop centrifuge for 5 s to collect solution. Transfer the solution to a separate 1.5 mL tube. Keep the original tube from IDT, as its label contains DNA dry weight information that is useful later.
3. Add 100 μL of chloroform to the tube in a chemical hood. (Chloroform is volatile; handle only in the hood.) Vortex the tube to mix, and then spin down with a tabletop centrifuge to separate the aqueous and chloroform fractions from each other. (A clear line separating the two fractions should be visible.) Use

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**Fig. 6** (continued) for 30 min at RT, to chemically disintegrate the first and the second hydrogels, which contain a periodate-cleavable crosslinker *N,N*-diallyl-tartardiamide (DATD), while sparing the third hydrogel, which contains a periodate-resistant crosslinker *N,N*- methylene-bis-acrylamide (bis). **(h)** Specimens are incubated with a fluorophore-conjugated 15-base locked nucleic acid (LNA) oligonucleotide, which hybridizes to the 100-base linker DNA oligo at four locations, in iExCel hybridization buffer overnight at RT. **(i)** Specimens are expanded to a linear expansion factor of ~20×, with three washes in deionized water. After expansion, specimens are ready for imaging. (Figure is modified from Yu et al. 2020 [7], with permission granted by the Creative Commons Attribution license)

**Table 4**  
**Sequences and synthesis parameters of the oligo-nucleotides used in the iExCel protocol**

| DNA oligo name                     | Purpose  | Sequence   | Required modification | Purification       | Recommended synthesis scale |
|------------------------------------|--|--|-----------------------|--------------------|-----------------------------|
| B1-B2 system (for 1-color readout) |  |  |                       |                    |                             |
| 5'Ac-AA-B1-AA-3'Amine              | Conjugation to secondary antibody  | /5ACryd/AAG TTC GGA TTC TTA<br>GGG CGT AAA/3AmMO/  | 5'acrydite<br>3'amine | Standard desalting | 1 μmole                     |
| 5'Ac-B1'-4xB2'                     | Post-Gel#2 Linker to transfer signal location between gels; amplifies signal by 4x via branched DNA scheme | /5ACryd/AT ACG CCC TAA GAA<br>TCC GAA ATA GCA TTA CAG TCC<br>TCA TAA TAG CAT TAC AGT CCT<br>CAT AAT AGC ATT ACA GTC CTC<br>ATA ATA GCA TTA CAG TCC TCA<br>TA | 5'acrydite            | PAGE               | 1 μmole                     |
| LNA_B2-Atto647N                    |  |  |                       |                    |                             |
|                                    | Post-Gel#3 final readout   | T+GA +G+G+G C+T+G +T+A+A +T+GC<br>/3ATTO647NN/(+ denotes that the next base is a locked nucleic acid)  |                       | HPLC               | 1 μmole                     |
| A2-A1 system (for 2-color readout) |  |  |                       |                    |                             |
| 5'Ac-AA-A2-AA-3'Amine              | Conjugation to secondary antibody  | /5ACryd/AAA GAT TGA GAT GCC<br>TGT CAC CAA/3AmMO/  | 5'acrydite<br>3'amine | Standard desalting | 1 μmole                     |
| 5'Ac-A2'-4xA1'                     | Post-Gel#2 Linker to transfer signal location between gels; amplifies signal by 4x via branched DNA scheme | /5ACryd/GGT GAC AGG CAT CTC<br>AA TCT ATT ACA AAG CAT CAA<br>CGA TTA CAA AGC ATC AAC GAT<br>TAC AAA GCA TCA ACG ATT ACA<br>AAG CAT CAA CG                    | 5'acrydite            | PAGE               | 1 μmole                     |
| LNA_A1-Atto647N                    | Post-Gel#3 final readout   | C+GT +T+GA +TG+C +T+T+T G+T+A<br>/3ATTO565NN/(+ denotes that the next base is a locked nucleic acid)   | 3' Atto 565           | HPLC               | 1 μmole                     |

a pipette to take the top fraction (i.e. the aqueous fraction, where DNA is dissolved in) and transfer to a separate 1.5 mL tube. This process removes the impurities from the shipped oligo, as they dissolve in the chloroform.

4. Repeat **step 3** two more times (three chloroform washes total).
5. Add 10  $\mu$ L of 3 M NaCl to the DNA solution. Vortex for 5 s, and spin down for 5 s.
6. Add 250  $\mu$ L of ice cold 100% ethanol. White precipitates (DNA) should form immediately. Do not mix the content. Directly incubate the tube at  $-20^{\circ}\text{C}$  for 30 min.
7. Centrifuge the tube in a refrigerated centrifuge at  $4^{\circ}\text{C}$ , for 30 min, at  $18,000 \times g$  (or the maximum speed of the centrifuge if lower than  $18,000 \times g$ ). A firm white pellet (that consists of the DNA) should form after this step.
8. Remove supernatant (ethanol) from pellet. Gently add 1 mL of ice cold 70% ethanol to the inner wall of the tube, without disturbing the pellet, and then remove as much supernatant as possible.
9. Uncap the tube, and let it sit at RT for  $\sim 1$  h, to briefly dry pellet.
10. After 1 h, add Buffer A to make a DNA solution at 25  $\mu\text{g}/\mu\text{L}$ . The amount of Buffer A needed =  $(\text{DNA weight}/(25 \mu\text{g}/\mu\text{L}))$ . The DNA weight is available on the IDT data sheet, and also on the label of the shipped tube. Use a combination of vortexing and pipetting up and down to mix the content, until the pellet completely dissolves and is no longer visible.
11. To measure the DNA concentration in the tube (cannot use the information on the shipped tube, since there are losses during purification steps), mix 1  $\mu\text{L}$  of the DNA oligo stock solution with 99  $\mu\text{L}$  of Buffer A in a separate 1.5 mL tube.
12. Measure the absorbance at 260 nm for the  $100\times$  diluted DNA oligo solution with a Nanodrop (or equivalent spectrometer) using Buffer A as the blank solution. Use the extinction coefficient of the DNA (shown on the IDT datasheet) to convert the A260 to DNA concentration of the diluted sample. Multiply the value by 100 to obtain the concentration for the stock tube. Multiply the stock concentration by the volume inside the stock tube, to obtain the number of nanomoles of DNA in the stock tube.
13. Calculate the amount of Sulfo-S4FB solution needed:  

$$(\mu\text{L} \text{ of Sulfo-S4FB to use}) = (\text{nmol of DNA}) \times 349 \times 40 \times 15/10^6.$$
Explanation: 349 g/mol is the molecular weight of Sulfo-S4FB; (1 mg/40  $\mu\text{L}$ ) is the concentration of Sulfo-S4FB

solution. 15 is the target molar ratio between Sulfo-S4FB and DNA.  $10^6$  adjusts the unit to the correct order of magnitude.

14. Add 40  $\mu$ L of anhydrous DMSO (Thermo D12345) to 1 mg of Sulfo-S4FB (TriLink S-1008). Vortex for at least 1 min, spin down, and check if the entire dried pellet has dissolved. If more than 40  $\mu$ L of Sulfo-S4FB solution is needed, do this to multiple tubes and collect fully dissolved solutions into a single tube.
15. Add the calculated amount of Sulfo-S4FB to the DNA oligo stock tube. Vortex to mix, and spin down to collect the liquid. Incubate the tube at RT overnight.
16. Purify DNA from un-reacted S4FB with a Vivaspin 500 centrifugal filter with 5 kDa molecular weight cutoff (Vivaproducts VS0101). To do this, add Buffer C to the tube until the total volume is 1000  $\mu$ L. Transfer 500  $\mu$ L each to two centrifugal filters. Centrifuge the tube at  $13,000 \times g$  for 10 min. Discard the flow-through (the portion that comes out from the bottom of the filter).
17. Add Buffer C into each filter until the volume is ~500  $\mu$ L. Thoroughly mix the content, by pipetting up and down ~5 times. Centrifuge the tube at  $13,000 \times g$  for 10 min. Discard the flow-through (the portion that comes out from the bottom of the filter).
18. Repeat **step 17** three more times (5 spins total, from **steps 16–18**).
19. Transfer the concentrate from the filter to a separate 1.5 mL tube. Calculate the amount of Buffer C to add, in order to bring the total volume to 150  $\mu$ L. Add this amount of Buffer C to the centrifugal filter and pipette up and down to mix (to collect residual DNA oligo solution from the filter), and then transfer the solution to the tube. The 4FB-DNA stock solution can now be stored at 4 °C for at least 2 years.
20. Weigh out 5–10 mg of 2-hydrazinopyridine·2HCl (abbreviated as 2-HP from this point on, Millipore Sigma H17104). Dissolve the powder in ultrapure H<sub>2</sub>O, to a concentration of 50 mg/mL.
21. Add 91  $\mu$ L of the 2-HP solution to a tube containing 50 mL of 100 mM MES Buffer, pH 5.0. This 2-HP working solution remains stable for up to 30 days at 4 °C.
22. Prepare 2-HP blank solution by adding 2  $\mu$ L of water to 18  $\mu$ L of 2-HP working solution.
23. Prepare 4FB-DNA MSR solution by adding 2  $\mu$ L of the 4FB-DNA stock (mix stock well by vortexing before use) to 18  $\mu$ L of 2-HP working solution.

24. Vortex and spin down solutions made in **steps 22** and **23**. Incubate solutions at 37 °C for 60 min.
25. After incubation, spin down the tubes for 15 s to collect condensation to the bottom of the tube. Vortex for 5 s, and spin down once more for 5 s.
26. Measure the absorbance at 360 nm and 260 nm of the 4FB-DNA MSR solution on a Nanodrop (or equivalent spectrometer) using the solution made in **step 22** as the blank solution, under the UV-Vis function of the spectrometer. Read absorbance values from the 1 mm pathlength. However, if the A260 reading is much greater than 1, read from the 0.1 mm pathlength to get an accurate reading. Make sure to use A360 and A260 that came from the same path length setting (either use values that both come from the 1 mm setting or both from the 0.1 mm setting).
27. Calculate the molar substitution ratio (MSR) with the following formula:

$$\text{MSR} = \text{A360}/\text{A260} \times (\text{DNA extinction coefficient}/24,500)$$

The MSR should be close to 1.00 (0.90–1.20 is the acceptable range). If the MSR is greater than 1.20, repeat **steps 16–19** to further purify un-reacted S4FB from the 4FB-DNA stock solution, using new centrifugal filters, until the MSR is below 1.20.

28. Calculate the amount of 4FB-DNA stock solution needed for 100 µg of antibody with the following formula:

$$\begin{aligned} &(\mu\text{L of 4FB - DNA for } 100 \mu\text{g of antibody}) \\ &= 7.5 \times 100/150,000 \times 1000/(\text{MSR}) \\ &\quad \times [\text{4FB - DNA stock concentration in mM}]) \end{aligned}$$

Explanations: 7.5 is the molar ratio between 4FB-DNA and antibody. 100 µg is the antibody mass that we are calculating for. 150,000 g/mol is the molecular weight of an IgG antibody. 1000 adjusts to the correct order of magnitude. 4FB-DNA concentration is calculated from the A260 reading from UV-Vis and the extinction coefficient of the DNA oligo—be sure to account for the 1:10 dilution performed in **step 23**, and the pathlength setting used. For example, if the A260 is measured from the 4FB-DNA MSR solution at the 0.1 mm wavelength, multiply the A260 first by a factor of 10 (accounts for 1:10 dilution), and then by a factor of 100 (accounts for the 0.1 mm pathlength used, as the IDT extinction coefficient is given in the unit of  $(M \times \text{cm})^{-1}$ , where  $1 \text{ cm}/0.1 \text{ mm} = 100$ ). Divide this post-adjusted A260 by the extinction factor, to get the 4FB-DNA stock concentration in  $M$ . Finally, multiple by 1000 to get the quantity in mM.

29. Record the numbers from **steps 27** and **28** for each 4FB-DNA stock solution synthesized. The analyzed 4FB-DNA stock solution is ready for conjugation with antibodies, and can be stored at 4 °C for at least 2 years.

### Prepare the Antibody for Conjugation

The following protocol specifies quantities needed for a single conjugation between DNA oligo and antibody. If multiple combinations of DNA and antibody are desired, such as for the 2-color readout (e.g. anti-chicken conjugated to B1, anti-rabbit conjugated to A2), scale up by processing multiple batches in parallel. For this part, it is recommended to execute the entire protocol within a session (takes ~3–4 h) without pausing.

Buffer exchange 100 µL of the unconjugated secondary antibody into Buffer A using a spin columns with 40 kDa molecular weight cutoff. To do this, use the follow manufacturer's instructions (**steps 30–38**):

30. Remove the bottom closure (the plastic sealing material below the column) of the spin column, and loosen the cap (do not remove cap).
31. Place column into a 1.5 mL tube, and centrifuge at  $1500 \times g$  for 1 min.
32. Discard the flow-through and put the column back to the 1.5 mL tube.
33. Use a marker to place a mark on the side of the column where the compacted resin is slanted upward.
34. Add 300 µL of the equilibration buffer (i.e. the buffer to exchange the antibody into; in this case, use Buffer A) to the column. Place the cap back to the column (again, do not cap tightly). Centrifuge at  $1500 \times g$  for 1 min, with the mark facing outward from the center of the centrifuge. (This preserves the shape of the compacted resin bed across rounds of centrifugation, which yields better protein retention.).
35. Repeat **step 34** 2 more times (for a total of 3 Buffer A washes). For the third (final) wash, centrifuge at  $1500 \times g$  for 2 min, to completely remove buffer from the resin bed.
36. Move the column to a new 1.5 mL tube. Discard the original one.
37. Apply the antibody solution to the column. Centrifuge at  $1500 \times g$  for 2 min, again with the mark facing outward from the center of the centrifuge.
38. Discard the column, and save the flow-through, which is the antibody now buffer-exchanged into Buffer A.
39. Check antibody concentration in mg/mL with a Nanodrop (or equivalent spectrometer), using Buffer A as the blank

solution. Be sure to select the setting for IgG antibody (otherwise, the reading will be off by ~30%, because the mass extinction coefficient, i.e. A<sub>280</sub> of a 10% (w/w) solution, is 13.7 for IgG, but 10.0 for general proteins). Then, dilute the antibody to the concentration of ~1.0 mg/mL with Buffer A.

40. Add 350  $\mu$ L of DMSO to 1 mg of S-HyNic. Pipette up and down for 60 s, and then vortex until the dried pellet completely dissolves. This solution is stable if stored in anhydrous conditions at  $-20^{\circ}\text{C}$ , for up to 2 weeks.
41. Transfer 100  $\mu$ L of the ~1.0 mg/mL antibody solution into a separate 1.5 mL tube. Add 6  $\mu$ L of the S-HyNic solution. Mix thoroughly by pipetting up and down for 10 s. Incubate solution at RT for 2 h.
42. After 1 h and 50 min of incubation (i.e. 10 min before the incubation period ends), prepare a Zeba spin column using exactly the same protocol shown in **steps 30–36**, except that Buffer C should be used instead of Buffer A as the equilibration buffer, in order to exchange the antibodies into Buffer C this time.
43. After 2-h incubation, apply the S-HyNic-reacted antibody solution to the spin column, and complete the rest of the buffer exchange (same as **steps 37–38**). This S-HyNic-reacted antibody solution is not stable for more than a few hours, and needs to react with 4FB-DNA immediately (within 1 h), as instructed in **steps 44–46** in the next section.

#### Conjugate 4FB-DNA with S-HyNic-reacted antibody

44. Add 4FB-DNA to the S-HyNic-reacted secondary antibody, by the amount calculated in **step 28** in Part A. (If the antibody tube started from 100  $\mu$ L of the ~1 mg/mL stock in Buffer A, as instructed in **step 5** in Part B, then the antibody in solution should be around  $1 \text{ mg/mL} \times 100 \mu\text{L} = 100 \mu\text{g}$ . The amount calculated in **step 28** was for 100  $\mu\text{g}$  of antibody.)
45. Measure the total volume of the antibody-DNA mixture. Divide the total volume by 9, and then add this amount of 10 $\times$  Turbolink Catalyst Buffer to the reaction tube.
46. Mix thoroughly by pipetting up and down for 10 s. Incubate the tube at RT overnight.
47. Purify un-reacted 4FB-DNA from the DNA-conjugated antibodies, by using 0.5 mL centrifugal filters with 100 kDa molecular weight. To do this, transfer all content of the reaction tube into the spin column.
48. Add 1 $\times$  PBS to the column until volume is at 500  $\mu$ L. Thoroughly mix the content, by pipetting up and down ~5 times.

Centrifuge the filter for 5 min at  $14,000 \times g$ . Discard the flow-through.

49. Repeat **step 48** two more times, for a total of 3 spins.
50. Transfer the concentrate from the filter to a separate 1.5 mL tube. Calculate the amount of  $1\times$  PBS needed to bring the volume to the starting volume at the ~1 mg/mL stage (100  $\mu$ L). Add this amount of  $1\times$  PBS to the filter to collect residual DNA-conjugated antibodies, pipette up and down to mix, and then transfer the entire content into the 1.5 mL tube. The DNA-conjugated antibody is now ready for use, and is stable at 4 °C for at least 1 year.

### *3.3.4 Antibody Staining (for Fluorescent Protein Readout)*

1. Pre-incubate sample in 1 mL of  $5\times$  SSCT for 30 min at RT.
2. Incubate sample with primary antibody against fluorescent protein, at desired concentration (a recommended default concentration is 10  $\mu$ g/mL; this concentration can be later adjusted to optimize for signal to background ratios) in 500  $\mu$ L of  $5\times$  SSCT overnight at 4 °C. \*\*.
3. Wash sample with 1 mL of  $5\times$  SSCT three times, 1 h each at RT.
4. Incubate sample with secondary antibody conjugated to an acrydite-bearing DNA oligo (synthesis instruction is shown in the earlier section “Synthesis of DNA-conjugated secondary antibody”), at desired concentration (a recommended default concentration is 10  $\mu$ g/mL; this concentration can be later adjusted to optimize for signal to background ratios) in 500  $\mu$ L of DNA-conjugated Antibody Staining Buffer overnight at 4 °C. \*\*.
5. Wash sample with 1 mL of  $5\times$  SSCT for three times, 1 h each at RT.

### *3.3.5 Expansion and Re-Embedding into Non-Expanding Gel #2*

Same as in the standard ExCel protocol, in sub-section “*Part A: Maintain expansion factor of the gelled sample with re-embedding*” of the section “ExFISH-HCR”, except for 2 points:

1. Prior to the re-embedding protocol, flip the gels to the same side as when it was casted in Gel#1 gelation, to ensure that the worms are at the bottom of the gel. (Refer to Subheading 3.1.6 to review this concept.)
2. For re-embedding, replace the Re-embedding monomer solution with iExCel G2 monomer solution.

After Gel#2 gelation, it is recommended to trim the gels, to avoid samples too large to handle and image at the fully expanded final state. Trimming can be facilitated by observing where worms are located inside the gel, with naked eye, or on an upright

microscope, if it is desired to not dissect certain animals. Ideally, gels are trimmed into less than ~6 mm on each side, and have at least several worms ( $\geq 3$ ) or regions of interest in the trimmed gel. Also, not all trimmed gels need to proceed with the protocol (which might be laborious to handle), and can be saved at this point, in  $5 \times$  SSC at 4 °C for at least 2 months. For the first several attempts of this protocol, it is recommended to pick at most ~6 trimmed gels to proceed.

### 3.3.6 Linker

#### *Hybridization (to Amplify and Transfer Signals from Gel#1 to Gel#3)*

1. Transfer trimmed gels into a 24-well plate, if not already there.
2. Pre-incubate each trimmed gel in 1 mL of iExCel hybridization buffer for 30 min at RT.
3. Incubate gel with Post-Gel#2 acrydite-bearing linkers (refer to the second entry of the readout systems shown in Table 4) corresponding to the oligo sequence on the secondary antibody applied in the section “Antibody Staining,” at the concentration of 100 nM, in 1 mL of iExCel hybridization buffer overnight at RT with gentle shaking. \*\*.
4. Wash gel with 1 mL of iExCel hybridization buffer four times, for 1 h each for the first three washes and overnight for the final wash, at RT with gentle shaking. \*\*.
5. Wash gel with 1 mL of 1× PBS three times, 1 h each at RT.

### 3.3.7 Re-Embedding into Expanding Gel #3

1. Flip each trimmed gel to the same side as when they were casted during Gel#1 and #2 gelation. This ensures that the worms remain on the bottom surface of the gels, so the working distance of the objective can maximally cover the depth of the worm at the final imaging stage. (Refer to Subheading 3.1.6 to review this concept.)
2. Prepare 1 mL of Activated iExCel G3 Monomer Solution with all reagents except for APS (iExCel G3 Monomer Solution Stock + 4-HT + TEMED; do not add the activator APS yet), and thoroughly mix the solution by a vortex. Chill the solution to 4 °C on a cold block. It is important to ensure that the solution is fully chilled to 4 °C before proceeding.
3. Remove liquids from the sample.
4. Activate the monomer solution from step 3 by adding 20 µL of 10% APS, and thoroughly mix the solution by a vortex. Start a timer right after the APS is added.
5. Add 1 mL of Activated iExCel G3 Monomer Solution to worms. Incubate sample at 4 °C for 30 min, on a pre-chilled cold block.
6. During the incubation, construct a gelling chamber with the same architecture as in Gel#1 (step 6 in Subheading 3.1.7) but with 3× thicker spacer (i.e. the same thickness as used in Gel

- #2; e.g. if a #1.5 coverslip was used for Gel #1, use a stack of 3 here for each spacer). Do not close the chamber with the top coverslip yet.
7. Immediately after 30 min of incubation, transfer 105 µL of the monomer solution from the well to the center of the gelling chamber. Remove bubbles if any. Then, transfer the gel from the 24-well plate into the monomer solution droplet on the gelling chamber, using a flat-tip paint brush. Be sure that the gel is on the side where the worms are at the bottom surface of the gel, as confirmed in **step 1** (i.e. if the gel is already correctly sided in the 24-well plate, do not flip the gel during this transfer.). Slowly place a rectangular top cover slip on the gel, to enclose the gelling chamber in the same style as performed in previous rounds of gelation, avoiding any bubbles. If the distance between the gel to the border of the gelation solution is less than ~2 mm in any direction, add more monomer solution to the chamber until the solution border is sufficiently away from the gel. Alternatively, fill the entire chamber with monomer solution. (As explained previously, ambient oxygen inhibits gelation, and so the gel right at the solution-air interface is not well-formed.)
  8. Incubate the gelling chamber at 37 °C for 2 h, in a humidified chamber.
  9. Remove top cover glass. There should be a visible interface around the re-embedded gel. Trim away the excessive gel around the re-embedded gel.
  10. Transfer trimmed gels into a 6-well plate. Wash three times with 5× SSC, 10 min each. \*\*.

### *3.3.8 Cleave DATD-Crosslinked Gels #1 and #2*

1. Make DATD-cleaving solution from sodium periodate powder.
2. Incubate trimmed gels in 3 mL of DATD-cleaving solution (or volumes sufficient to cover the entire gel) at RT for 30 min with gentle shaking.
3. Wash gels with 5 mL of 1× PBS at RT for 15 min with gentle shaking.
4. Repeat **step 3** seven times (eight washes total), and then leave in 5 mL of 1× PBS at RT overnight with gentle shaking. It is crucial to completely wash away sodium periodate from the gels, since residual amounts of periodate ion will also disintegrate Gel #3 over a time scale of ~2–24 h. \*\*.

### *3.3.9 LNA Hybridization (to Attach Fluorophores to the Stained Locations for Final Readout)*

1. Pre-incubate each trimmed gel in 1 mL of iExCel hybridization buffer for 30 min at RT.
2. Incubate gel with fluorophore-conjugated LNA oligos corresponding to the DNA linker applied in the section

“Linker Hybridization,” at the concentration of 100 nM, in 1 mL of iExCel hybridization buffer overnight at RT with gentle shaking. \*\*.

3. Wash gel with 1 mL of iExCel hybridization buffer four times, for 1 h each for the first three washes and overnight for the final wash, at RT with gentle shaking. \*\*.
4. Wash gel with 1 mL of 5× SSC three times, 1 h each at RT.

To ensure that the sample (currently at ~8.5× expansion factor) can be fully expanded (to ~20× expansion factor) in the upcoming section, and still stay flat and fully contained inside the container, it might be necessary to trim the hydrogel sample at this point. First, identify the dimensions of the container where the expanded sample will be imaged in. Determine the maximum gel size allowed at the current stage, by evaluating (container size)/(20×/8.5×). For a 6-well plate, the maximum gel size at the current stage is ~9 mm × ~9 mm. Trim the samples into sizes below the maximum gel size, by first transferring the sample into a plastic 6-well plate, or on a glass slide, with a paintbrush. Then, use a #1.5 or thicker cover glass, or a razor blade, to cut the gel into smaller portions; perform this on an upright fluorescent microscope, to confirm the locations of animals, if it is desired to not dissect certain animals. Not all trimmed portions need to be expanded and imaged within the current round, and can be stored at this point, in 5× SSC at 4 °C for at least 2 months.

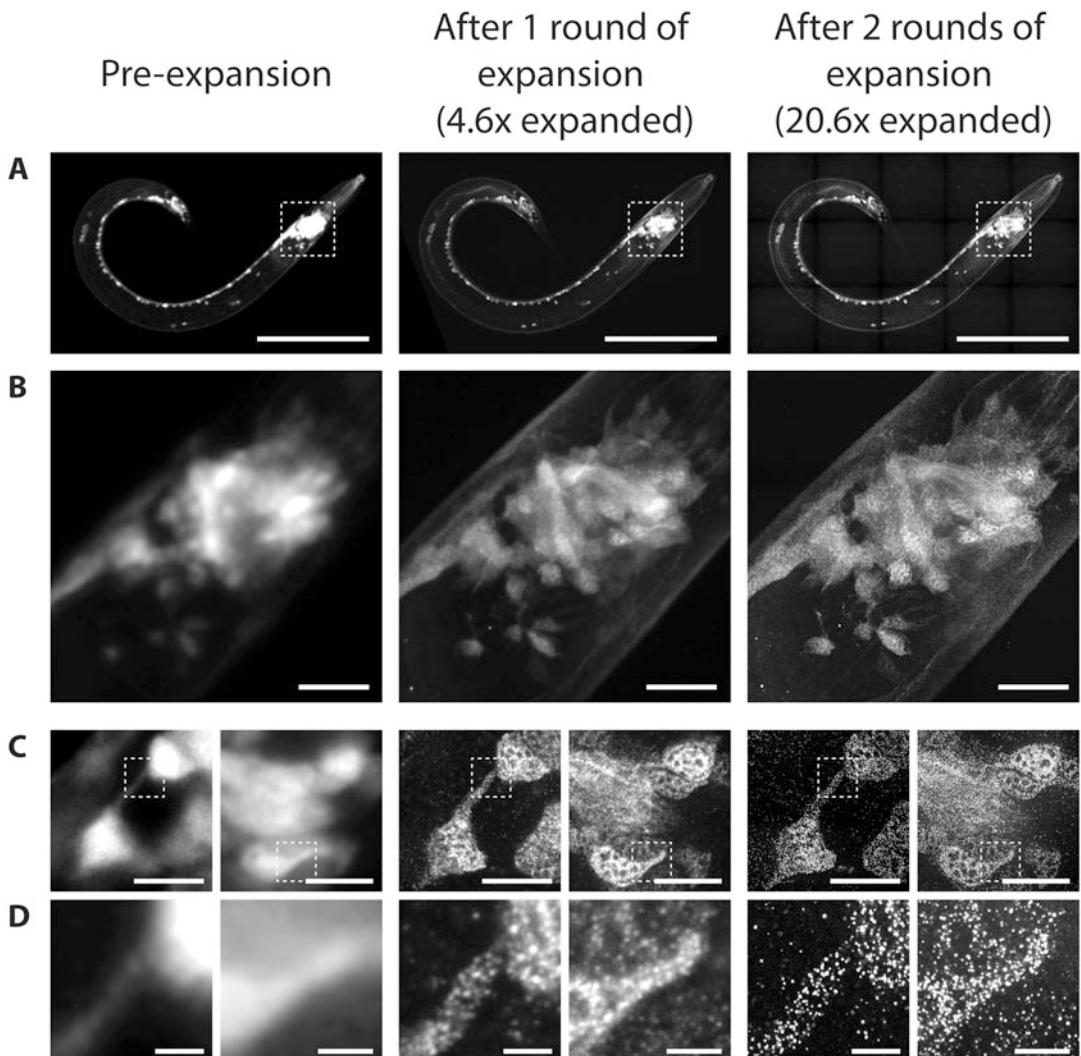
### *3.3.10 Expansion and Imaging*

1. Transfer sample to a container suitable for imaging, e.g. a glass-bottom 6-well plate. For high-magnification objectives with limited working distance, ensure that the gel is flipped to the same side as when it was first casted in Gel #1, so that the animals will be at the bottom of the gel. (Refer to Subheading 3.1.6 to review this concept.)
2. Wash sample with 5 mL of deionized water three times, for 1 h each at RT.
3. Remove as much liquid around the expanded gel as possible.
4. Perform imaging.

See Subheading 3.1.16 for agar immobilization protocol to prevent sample vibration or drift during imaging.

### *3.3.11 Example of Final Images*

An example of confocal images acquired after the iterative ExCel protocol is shown in Fig. 7. In this example, a transgenic L3 hermaphrodite animal expressing *tag-168p::GFP* is processed by the protocol for detecting the localization of GFP molecules. For the purpose of demonstrating the resolution after each round of expansion, we included additional steps, which are not incorporated into the routine protocol shown above (in Fig. 6),



**Fig. 7** Example of confocal images acquired after the iterative ExCel protocol. A representative iExCel-processed L3 hermaphrodite animal at various stages along the iExCel protocol. These stages include: (left column) right after first hydrogel embedding, (middle column) after re-embedding into the second hydrogel, and (right column) after full expansion with the third hydrogel. The strain used had pan-neuronal cytosolic expression of GFP, under tag-168p::GFP. Pre-expansion images were acquired from native GFP fluorescence. Post-1-round-expansion images were acquired after linker hybridization and before re-embedding into the third gel (as in Fig. 6e), accompanied by the following additional steps (not included in the routine protocol shown in Fig. 6, because we performed this intermediate readout only for the purpose of method validation): specimens were incubated with a fluorophore-conjugated 15-base DNA oligo that hybridizes to the 100-base linker, imaged, incubated in de-hybridization buffer (80% formamide, 0.1% Triton X-100) at 37 °C for 6 h to remove the fluorophore-conjugated-DNA-bound linker, and re-hybridized with a fresh set of linker, using the same hybridization protocol shown in Fig. 6e. This linker refreshment ensures that the linkers have completely unoccupied binding sites for the downstream LNA hybridization. Post-2-round-expansion images were acquired after full iExCel protocol (as in Fig. 6i). (a–d) The animal at various optical and digital magnifications. (a) Entire worm. White dotted box marks the pharyngeal region of the worm, which is shown in greater magnification in b. (b) Pharyngeal region of the worm, as marked by the white dotted box in a. (c) Two regions within the pharyngeal region of the worm, as shown in b. Corresponding regions were not marked in b,

to enable detection of GFP after the first round of expansion [7]. Briefly, these steps include incubation of the linker-hybridized samples (at the state of Fig. 6e) with a fluorescent-dye-conjugated 15-based DNA oligo, which hybridizes to the linker, and which fluorescently report the stained positions at the post-1-round-expansion state (with an expansion factor of  $4.6\times$ ). Additional details of this non-routine process can be found in the original publication [7]. Afterwards, the sample is de-hybridized in a de-hybridization buffer (80% formamide +0.1% Triton X-100), to remove the bound linker and fluorescent oligo molecules. The sample is then re-hybridized with the linker, and proceed through the rest of the routine protocol (as in Fig. 6d-i), to yield an expansion factor of  $20.6\times$ . All images were acquired under confocal microscopy under a  $40\times$  objective (water immersion, with a numerical aperture of 1.15).

#### 4 Notes

1. The quality of sodium acrylate can differ substantially from vendor to vendor, and even from batch to batch from the same vendor. Their differences include the following:
  - (a) Solubility: fully dissolved at the stock concentration 33% (w/w) [high quality], or having insoluble, string-like precipitates [poor quality].
  - (b) Color of the 33%(w/w) stock solution: close to clear [high quality], or moderately yellow [poor quality].

**Fig. 7** (continued) because the single-confocal-plane images shown in c do not clearly register to regions in b, which is a maximum-intensity projection acquired through the entire thickness of the animal. White dotted box marks subcellular features that are shown in greater magnification in d. (d) Subcellular features of neurons, such as a neuronal process (right) and a portion of the neuronal soma (left), as marked by the white dotted box in c. Objective used: (a, b)  $10\times$ , NA 0.50; (c, d)  $40\times$ , NA 1.15. Image depth: (a, b) max-intensity projections of confocal stacks acquired through the entire thickness of the animal. (c, d) single z-position confocal images, except for post-2-round-expansion images (right column), which are max-intensity projections of 2 consecutive images within the confocal stack, because the expansion-mediated improvement in the axial resolution causes each z-plane image to capture a reduced tissue thickness. Thus, a combination of features captured across two consecutive z-planes was required to register to all perceptible, z-distributed features in the pre-expansion and the post-1-round-expansion images. Brightness and contrast settings: each panel was first set by the automatic adjustment function in Fiji, and then manually adjusted (raising the minimum-intensity threshold and lowering the maximum-intensity threshold) to improve contrast. Linear expansion factor: post-1-round expansion,  $4.6\times$ ; post-2-round expansion,  $20.6\times$ . Scale bars: (a) 100  $\mu\text{m}$ ; (b) 10  $\mu\text{m}$ ; (c) 5  $\mu\text{m}$ ; (d) 1  $\mu\text{m}$  (in biological units, i.e. post-expansion lengths are divided by the expansion factor of the worm). (Figure is modified from Yu et al. 2020 [7], with permission granted by the Creative Commons Attribution license)

- (c) Timing of hydrogel formation at 4 °C, using the recipe of “Activated Monomer Solution” in Materials: >60 min [high quality], 30–60 min [moderate quality], <30 min [poor quality].

We screened through sodium acrylates from various vendors, and compiled the solubility of their lots (see results in the ExCel protocol on [expansionmicroscopy.org](http://expansionmicroscopy.org)). We recommend the following practice for ExCel users:

- Order sodium acrylate from multiple vendors. Dissolve ~5 g of each powder to make a 33% (w/w) stock solution. Reject powders that do not fully dissolve after ~1–2 min of vortexing. Among the stock solutions in which the sodium acrylate fully dissolves, select the one that appears the least yellow (the most colorless).
  - Immediately re-order multiple bottles of sodium acrylate from the same vendor, with the same lot number. Store the quality-screened lots of sodium acrylate powder at –20 °C in anhydrous conditions, which should remain stable for at least 1–2 years.
  - Never store 33% (w/w) stock solutions of sodium acrylate. Always use freshly made stock to produce a large batch of the Monomer Solution Stock (we typically make ~50 mL per batch), which can be in turn stored at –20 °C as 1 mL aliquots for 6 months, as the reduced final concentration of sodium acrylate (7.5% (w/w)) is more stable for storage.
  - Prior to using a newly made batch of Monomer Solution Stock, do a pilot run of gelation with the stock without any worm samples. To do this, follow the recipe “Activated Monomer Solution,” mix the content well, place the solution at 4 °C, and incubate for 45 min. Immediately after 45 min of incubation, use a pipettor to determine whether the solution is still completely liquid or whether hydrogel has partially formed. If the hydrogel has partially formed, repeat the test with gradually reduced (e.g. by 10 min each round) incubation times, until the timepoint where the solution remains completely liquid at 4 °C is determined. Apply this timing for the 4 °C incubation, during the actual gelation step for the worm samples. This procedure avoids the situation where a new batch of Monomer Solution Stock gels too fast (due to variations in the sodium acrylate quality), such that a precious worm sample is trapped inside the pre-maturely formed hydrogel, which would render the worm sample no longer usable.
2. PFA Fixative: Do not store; make this solution from brand new ampules of 16% paraformaldehyde, within 30 min before

- usage. After the solution is made, keep solution on ice or at 4 °C until usage.
3. BTB: Do not store; make this solution from 2-mercaptoethanol within 30 min before usage; store opened bottles of 2-mercaptoethanol at 4 °C for <6 months.
  4. AcX Stock: Store as ~50- $\mu$ L aliquots, in a desiccated environment (e.g. in a capped 50-mL tube that contains ~10 mL of Drierite desiccant particles), at -20 °C, for <6 months.
  5. LabelX Stock: Store either as aliquots or as a single tube, in a desiccated environment (e.g. in a capped 50-mL tube that contains ~10 mL of Drierite dessicant particles), at -20 °C, for <3 months.
  6. Monomer Solution Stock: Store as 1-mL single-use aliquots to avoid multiple freeze-thaw cycles at -20 °C for <6 months; stock solutions of sodium acrylate, acrylamide and DATD crosslinker should be prepared freshly from powder, within ~3 h before all the monomer solution stock ingredients are mixed, aliquoted into 1-mL portions, and stored at -20 °C.
  7. Non-activated Monomer Solution: Make at RT immediately before usage. Do not store.
  8. Activated Monomer Solution: Make at RT immediately before usage (do not store); stock solutions of 4-HT, TEMED and APS can be stored as ~100–200- $\mu$ L aliquots at -20 °C for <3 months; aliquots of these stock solutions can withstand freeze-thaw cycles at least three times.
  9. Non-expanding Digestion Buffer: Add Proteinase K right before use; store the buffer itself (i.e. without Proteinase K) at RT for up to 2 years.
  10. We recommend the following practices for receiving and storing antibodies: Upon receipt of an antibody, follow the storage condition specified by the manufacturer. In general, antibodies specified to be stored only at 4 °C (e.g. secondary antibodies from Thermo Fisher Scientific) can be stored accordingly without aliquoting. All other antibodies should be aliquoted into volumes that can be used up within a single experiment, or within a 4 °C storage period of ~1–2 weeks, and then frozen at the specified temperature (-20 °C or -80 °C). Avoid freeze-thaw cycles. Thaw frozen aliquots of antibodies at 4 °C, for at least 3 h prior to usage. A thawed aliquot can be stored at 4 °C for <2 weeks.
  11. The following final concentrations are recommended (for the first experiment; after observing the signal to background properties associated with a particular concentration, the user can adjust this parameter to optimize for image quality). For standard ExCel, 10  $\mu$ g/mL is used for staining fluorescent

proteins that are known to localize to large (e.g. >1- $\mu$ m scale), non-punctate, continuous structures, such as FP-filled cytosol of cells, FP-labeled cytoskeleton, etc.; 2  $\mu$ g/mL is used for staining targets that are known to be smaller (e.g. <500-nm scale), sparse, and/or can appear as punctate, such as synapses or vesicles. This lower concentration is recommended, because some antibodies (e.g. Abcam ab13970, an anti-GFP that the authors commonly used) can result in non-specific background puncta in the worm tissue, and can obscure the FP-tagged structure of interest; the reduced concentration significantly reduces the amount of this non-specific puncta. For epitope-preserving ExCel, 5  $\mu$ g/mL is used for all targets. For iterative ExCel, 10  $\mu$ g/mL is used for staining all fluorescent proteins.

12. Refer to [expansionmicroscopy.org](https://expansionmicroscopy.org) for a list of antibodies against fluorescent proteins, which the authors have validated and repeatedly used.
13. Re-embedding Monomer Solution: Make at RT immediately before usage (do not store); stock solutions of 4-HT and TEMED can be stored as ~100–200- $\mu$ L aliquots at –20 °C for <3 months; aliquots of these stock solutions can withstand freeze-thaw cycles at least three times.
14. Probe Set Stock Solution: Store in manufacturer’s packaging at –20 °C for  $\leq$ 5 years.
  - (a) Order from Molecular Instruments (<https://www.molecularinstruments.com/>) with the following instruction and parameters.
  - (b) On the ordering page, input the sequence of your mRNA target. For endogenous mRNA targets, you can find the sequence under the “Sequences” tag in the page of its corresponding gene on Wormbase (<https://wormbase.org/>) and/or from RefSeq (<https://www.ncbi.nlm.nih.gov/refseq/>).
  - (c) Select the probe set scale (used at 2 pmol per sample) based on the projected number of samples that will be stained with this probe.
  - (d) Select the probe set size (this is the number of 52-bp regions on the input mRNA sequence that will be detected by the hybridization; larger number of probes improves specificity, but also the cost; if budget-limited, a reasonable strategy is to use the minimal probe set size of 20 probes for screening across large numbers of mRNA targets, and the higher probe set sizes for getting the highest-quality images for a fewer number of selected targets).

- (e) Select the HCR initiator (B1-B5) associated with the probe, such that all the targets that you plan to simultaneously visualize within a single sample (by eventually attaching them with different colors of fluorophores) receive a different initiator sequence.
15. HCR Amplifier Stock Solution: Store in manufacturer's packaging at  $-20^{\circ}\text{C}$  for  $\leq 5$  years.
- (a) Order from Molecular Instruments (<https://www.molecularinstruments.com/>) with the following instruction and parameters.
  - (b) Check which fluorophores are compatible with your microscope, and assign fluorophores to the HCR initiator sequences that you will use (i.e. the HCR initiators that you selected to associate with the HCR v3.0 Probe Set Stock Solution; see final step in the ordering information for the previous reagent). If multiple fluorophores are available, we recommend Alexa 488 and 546, which are particularly photostable amongst the provided list of fluorophores.
  - (c) Select the amplifier scale (used at 7.5 pmol per sample).
16. Probe Hybridization Buffer: Store in manufacturer's packaging at  $-20^{\circ}\text{C}$  for  $\leq 6$  months. Order an appropriate volume (used at 1 mL per sample) from Molecular Instruments (<https://www.molecularinstruments.com/>).
17. Probe Wash Buffer: Store in manufacturer's packaging at  $-20^{\circ}\text{C}$  for  $\leq 2$  years. Order an appropriate volume (used at 4 mL per sample) from Molecular Instruments (<https://www.molecularinstruments.com/>).
18. Probe Amplification Buffer: Store in manufacturer's packaging at  $4^{\circ}\text{C}$  for  $\leq 2$  years. Order an appropriate volume (used at 1 mL per sample) from Molecular Instruments (<https://www.molecularinstruments.com/>).
19. Collagenase VII Stock Solution: Store as 500- $\mu\text{L}$  single-use aliquots to avoid multiple freeze-thaw cycles at  $-20^{\circ}\text{C}$  for  $< 6$  months; do not re-use aliquots after thawing.
- (a) Refer to [expansionmicroscopy.org](http://expansionmicroscopy.org) for the recommended vendor, as enzyme quality and product purity can vary substantially across vendors. In addition, collagenase VII products can exhibit a noticeable level of batch to batch variability; to control for its quality over multiple experiments (which is helpful in case the protocol needs to be debugged), it is recommended to order a single large batch with at least 7.5 kU, instead of ordering multiple smaller batches for each individual experiment.

20. Protein Denaturation Buffer: Add reagents in the order indicated, to prevent SDS precipitation; store at 37 °C for <1 month; if precipitation occurs during storage at 37 °C (which could be due to slight variations in incubation temperature, and/or increase in reagent concentration due to buffer evaporation, because the formulation is nearly saturating for SDS at 37 °C), the buffer is re-usable by first fully dissolving the SDS back into solution via heating the solution to 70–95 °C until the solution is clear, and then storing the solution at a temperature slightly greater than 37 °C, e.g. 40–45 °C, at which there should not be SDS precipitates at the steady state.
21. Epitope-preserving ExCel G2 Monomer Solution Stock: Store as 1-mL single-use aliquots (to avoid multiple freeze-thaw cycles) at –20 °C for <6 months; stock solutions of sodium acrylate, acrylamide and Bis crosslinker should be prepared freshly from powder, within ~3 h before all the epitope-preserving ExCel G2 monomer solution stock ingredients are mixed, aliquoted into 1-mL portions, and stored at –20 °C.
22. Non-activated G2 Monomer Solution: Make at RT immediately before usage (do not store).
23. Activated G2 Monomer Solution: Make at RT immediately before usage (do not store); stock solutions of 4-HT, TEMED, and APS can be stored as ~100–200-µL aliquots at –20 °C for <3 months; aliquots of these stock solutions can withstand freeze–thaw cycles at least three times.
24. DATD-cleaving solution: Make at RT immediately before usage (do not store); make the 500 mM sodium meta-periodate solution from powder right before the mixing of all solution ingredients; do not use if the sodium meta-periodate powder has been dissolved for more than 3 h. We note that the 500 mM stock solutions could either look completely clear or moderately cloudy, depending on the quality of the sodium meta-periodate powder. Even in the case of cloudy stock solution, good outcomes could be obtained by ensuring that the stock solution is well mixed (via vortexing) before its addition into the final cleaving solution, as it eventually dissolves to reach a final concentration of 20 mM.
25. DNA-conjugated Antibody Staining Buffer: Store at 4 °C for <6 months; store the stock solutions of 20 mg/mL baker's yeast tRNA and 100% normal donkey serum as 1-mL single-use aliquots at –20 °C for up to 2 years.
26. iExCel G2 Monomer Solution: The components without TEMED and APS can be pre-mixed and can be stored for up to 3 months at 4 °C.

27. iExCel G3 Monomer Solution Stock: Store as 1-mL single-use aliquots (to avoid multiple freeze-thaw cycles) at  $-20^{\circ}\text{C}$  for <6 months; stock solutions of sodium acrylate, acrylamide, and Bis crosslinker should be prepared freshly from powder, within ~3 h before all the iExCel G3 Monomer Solution Stock ingredients are mixed, aliquoted into 1-mL portions, and stored at  $-20^{\circ}\text{C}$ .
28. Activated iExCel G3 Monomer Solution: Make at RT immediately before usage (do not store); stock solutions of 4-HT, TEMED, and APS can be stored as ~100–200- $\mu\text{L}$  aliquots at  $-20^{\circ}\text{C}$  for <3 months; aliquots of these stock solutions can withstand freeze-thaw cycles at least three times.
29. Determine the primary antibody that will be used against the target fluorescent protein. A list of primary antibodies that we have successfully used, against GFP, mCherry, and tagRFP, are available on [www.expansionmicroscopy.org](http://www.expansionmicroscopy.org). Then, order unconjugated whole IgG secondary antibody to match the host species type of the primary antibody.
30. RNA molecules, which are the targets of ExFISH-HCR readout in the standard ExCel protocol, are highly sensitive to degradation by naturally occurring RNases found in biological debris (skin, hair) and microorganisms (bacteria, fungi), both of which can contaminate specimens. RNase-contaminated samples can in turn result in reduced fluorescent intensity and counts of ExFISH-HCR puncta. We recommend applying the following procedures at all times, to prepare the reagents for, and to execute, any ExFISH-HCR experiments (i.e. standard ExCel protocol with RNA readout):
  - (a) Use RNase decontamination solutions (e.g. RNase Zap; Thermo Fisher Scientific #AM9780) to frequently clean bench and work surfaces, and any tool that comes into close proximity to or direct contact with the samples, e.g. pipettors and paintbrushes. To decontaminate a paintbrush, first spray the decontamination solution on the tip of the paintbrush, until the tip is completely wet. Then, rinse the paintbrush with deionized water, to remove the decontamination solution.
  - (b) Always use filtered pipette tips, for making RNase-free solutions, and for handling hydrogel samples.
  - (c) Minimize the duration in which containers that carry RNase-free solutions and hydrogel samples are open (i.e. without a lid). Always place the lids back right after handling. Small biological debris, e.g. dust, hair, flaked skin, and tiny droplets of bodily fluids expelled during speaking or sneezing, can all contain RNases, so it is

beneficial to minimize the chance that they fall onto the solutions or samples.

- (d) Exchange gloves frequently; constantly track the surfaces that the gloves have come into contact (e.g. door of a refrigerator, light switches of the room, screen of a PCR machine); recognize ones that are not RNase-free, and could thus transmit RNase into your sample through gloves.
- (e) Use Ultrapure water (e.g. Thermo Fisher Scientific #10977015) for making any solution that will be used in an ExFISH-HCR experiment, including the fixative solution.

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# Chapter 10

## A Fusion PCR Method for Expressing Genetic Tools in *C. elegans*

Eduard Bokman, Yifat Eliezer, and Alon Zaslaver

### Abstract

*C. elegans* offer a unique opportunity for understanding computation in neural networks. This is largely due to their relatively compact neural network for which a wiring diagram is available. Recent advances in genetic tools for interrogating neural activity (e.g., optogenetics) make *C. elegans* particularly compelling as they can be expressed in many different combinations in target individual neurons. Thus, the prospect to decipher principles underlying functionality in neural networks largely depends on the ease by which transgenic animals can be generated. Traditionally, to generate transgenic animals one would inject a plasmid containing the gene of interest under the regulation of the cell- or lineage-specific promoter. This often requires laborious cloning steps of both the gene and the promoter. The Hobert lab has developed a simpler protocol in which linear PCR fragments can be injected to generate transgenic animals. Relying on this PCR fusion-based method, here we provide a detailed protocol that we have optimized for expressing various genetically encoded calcium indicators and optogenetic tools in individual or sets of neurons. We use these simple procedures to generate multiple constructs within a very short time frame (typically 1–2 days).

**Key words** *C. elegans*, Transgenics, Fusion PCR, Fluorescent reporters, Optogenetics

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### 1 Introduction

*Caenorhabditis elegans* nematodes provide an excellent model system for a wide range of biological research fields [1]. Neuroscience is one appealing field for which *C. elegans* confer a key advantage: their neural network is relatively simple and compact (302 neurons in total) and a full neuronal wiring diagram is available [2, 3]. In addition, *C. elegans* are compatible with a wide variety of molecular and genetic manipulations. In particular, it is possible to generate transgenic worms that carry DNA of choice [4].

Since first introducing the DNA injection method to *C. elegans*, many studies were performed by tagging either individual or sets of cells with many different reporter genes [5–7].

The DNA, whether injected as a supercoiled plasmid or in a linearized form, is organized into extrachromosomal, high molecular weight arrays and is usually transmissible and stable for many generations [8], although transmission rates are variable and can run between 20% and 90%. Additionally, these arrays can be integrated into the genome using UV irradiation [9], resulting in multi-copy, non-mosaic transgenic animals, with a 100% transmission rate, at the cost of a random insertion site.

More recent methods, such as the CRISPR-Cas9 system [10–12], allow for precise targeted editing of genomic sequences. These can be used for deletion of specific sequences or insertions of single-copy genes into a location of choice. Such methods are advantageous when the site of insertion is critical or when a single copy of the inserted gene is sufficient. These methods for genome editing in *C. elegans* also utilize injection of plasmids or linear DNA fragments, but do not form extrachromosomal arrays.

In the recent years, we witness major advances in the molecular tools developed for interrogating neural activity. These include various genetically encoded calcium indicators (GECIs) [13–15] and optogenetic tools [16, 17]. Thus, the prospect of expressing these optogenetic and GECI tools in single, or sets, of neurons makes *C. elegans* a powerful system for studying dynamics and computation in neural networks with single-neuron resolution.

Much of the success of interrogating neural circuit dynamics depends on the ease by which we can generate transgenic worms. Cloning the gene of interest downstream to a desired promoter can be a tedious process and often requires many steps of DNA purification and cloning. To minimize time and labor, the Hobert laboratory (University of Columbia) has developed a convenient and fast method where linear PCR products are injected to produce transgenic lines [18–20]. In this method, the gene of interest is fused to the promoter region using a single fusion PCR reaction. Moreover, injection of vector-free DNA fragments was shown to improve transgene expression [21]. We find this method to be particularly useful as we aim to express various proteins (e.g., calcium indicators or optogenetic tools) fused to many different neuron-specific promoters.

In our lab, we use this established technique for large-scale and rapid production of promoter-gene fusions. These fusion PCR products are then injected into animals to successfully generate transgenic lines. Such lines have been successfully employed by us and others in various fields of study, including behavior [22], neurodegeneration [23], metabolism [24], longevity [25, 26], and more. Here we provide a detailed protocol that is based on the method developed originally by the Hobert lab for fusing two overlapping PCR products [19, 20]. We added guidelines and notes that helped us to improve this technique and customize it for expressing GECIs in target neurons. This method involves one

step of cloning the reporter gene into the Fire lab vector pPD95.77 [27] (chosen for its robust expression of genes and its restriction sites; *see Note 1*), followed by three simple PCR reactions. All three PCR reactions can be performed in 1 day such that the fusion PCR product can be ready for microinjection within a very short time frame.

## 2 Materials

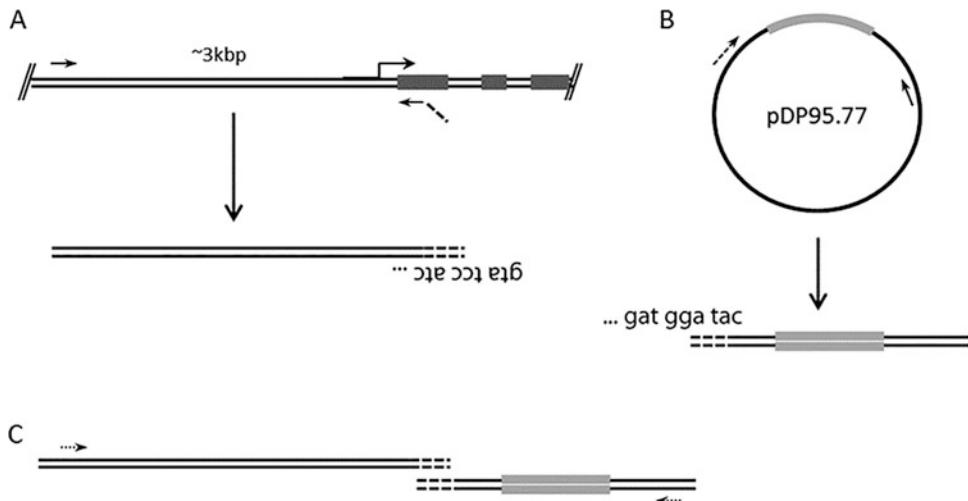
### 2.1 Promoter Cloning

1. Worm lysis buffer: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl<sub>2</sub>, 0.45% NP-40 (IGEPAL), 0.45% Tween-20, 0.01% gelatin, 2 mg/mL proteinase K.
2. Wild-type worms: N2 is the wild-type strain. It could be obtained from CGC.
3. Primers: According to the desired promoter, reverse primer should contain a 5' overhang (cca agt tgt tag cgt atc cat cgt tgt gag tg). This overhang is complementary to the 5' end of the gene of interest PCR product amplified from the pPD95.77 vector (Fig. 1A).
4. PCR reagents: High-fidelity DNA polymerase, polymerase buffer, dNTPs.
5. 1% agarose gel.
6. DNA cleaning column.

### 2.2 Cloning Reporter Gene

1. pPD95.77 plasmid: Can be acquired from Addgene or other worm laboratories. This plasmid contains an artificial intron for enhanced expression of the gene and an unc-54 3' UTR for the stabilization of the RNA transcript. For insertion of the gene, the pPD95.77 harbors an AgeI and EcoRI restriction sites.
2. Reporter plasmid: Could also be obtained from Addgene or colleagues. If a plasmid is not available but the sequence of the transgene is known, it could be cloned from transgenic animals as described here for promoter cloning.
3. Primers for amplification of the gene: Should be ordered with restriction sites.
4. PCR reagents: High-fidelity DNA polymerase, polymerase buffer, dNTPs.
5. AgeI and EcoRI restriction enzymes, ligase, gel purification kit.
6. 1% agarose gel.
7. DNA cleaning column.

Primers for amplification of the gene from the pPD95.77: pPD95.77 fwd (cac tca caa cga tgg ata c), pPD95.77 rev (cgc tta cag aca agc tgt). The fwd primer is the reverse complement of the



**Fig. 1** Schematic representation of the three PCR reactions needed for fusion PCR. **(A)** PCR amplification of the required promoter from genomic DNA. Bent arrow represents the transcription start site. Primers are the short arrows. Dashed lines, including the primer extension, represent the region of sequence homology between PCR product no. 1 and PCR product no. 2. **(B)** PCR amplification of the gene from the pPD95.77 vector. In gray is the gene of interest. Primers are the short arrows. Dashed lines represent the region of sequence homology between PCR product no. 1 and PCR product no. 2. **(C)** Fusion PCR reaction between PCR products no. 1 and 2. Dashed arrows denote the nested primers used in the reaction

aforementioned overhang. Therefore, the resulting PCR product will complement the overhang from the promoter amplification (Fig. 1B) and enable the following fusion PCR reaction.

### 2.3 Fusion PCR

1. PCR reagents: High-fidelity DNA polymerase, polymerase buffer, dNTPs.
2. Primers: The forward primer is a nested primer about 100 bp downstream from the beginning of the promoter PCR product. Therefore, it should be designed together with the primers for cloning the promoter (see left dashed arrow in Fig. 1C). The reverse primer is pPD95.77 rev nested (atc acc gaa acg cgc gag acg) (see right dashed arrow in Fig. 1C), upstream of the reverse primer of the reporter. If a different vector than pPD95.77 is used as a carrier for the reporter gene, then both of the reporter primers and the fusion reverse primer need to be designed accordingly.
3. 1% agarose gel.

### 3 Methods

Generation of a promoter-gene fusion requires two PCR products; the first is the promoter, which is amplified from genomic DNA of wild-type (N2) worms, and the second is the gene of interest, which in our case is cloned into the Fire lab vector pPD95.77 and amplified from there. This is followed by a third reaction in which we use nested primers in order to amplify the fused, partially overlapping, PCR products.

The fusion reaction is depicted in Fig. 1. The first PCR fragment is the required promoter amplified from genomic DNA (Fig. 2A). The second fragment is the gene of interest and flanking regions from the pPD95.77 plasmid (Fig. 2B). The reverse primer of the promoter includes an overhang, which corresponds to the 5' sequence of the second PCR fragment (*see Subheading 2*).

In the first step of the third reaction (Fig. 3A), following denaturation of the two fragments, the 3'-end of the promoter is annealed to the 5' of the gene, and this overlapping region functions as a primer, allowing the DNA polymerase to elongate these sequences and create a primary promoter-gene fusion.

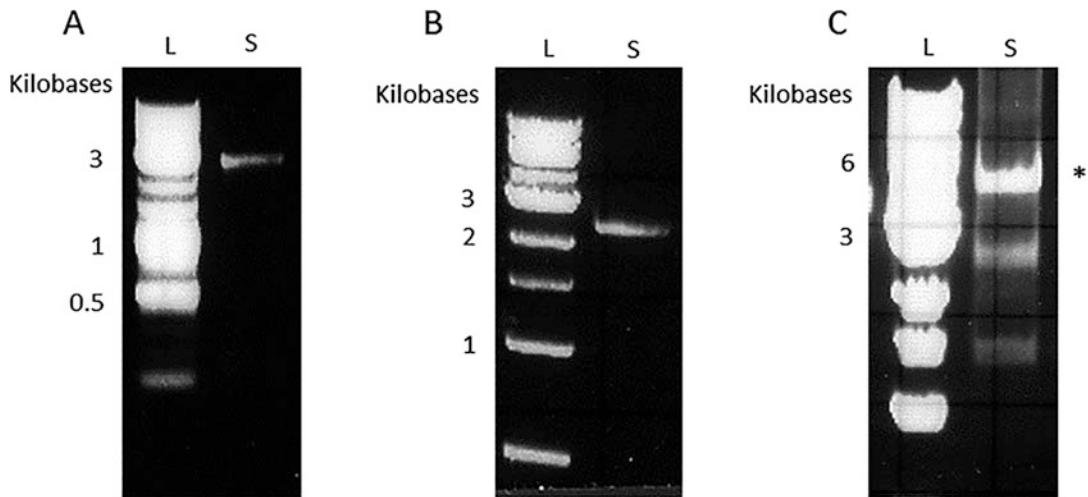
In the second step of this reaction (Fig. 3B), two nested primers are added to the reaction tube. These primers amplify the promoter-gene fusions that were generated in the first part of the reaction (Fig. 2C).

#### 3.1 Amplifying the Promoter from Genomic DNA

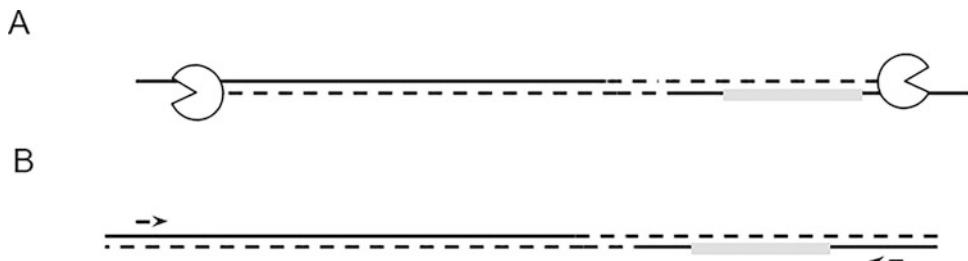
1. Preparation of genomic DNA from N2 worms: Ten young adult worms (*see Note 2*) are dissolved in 50 µL of worm lysis buffer. The tube is transferred to –80 °C for 30 min, then to 65 °C for 60 min, and 95 °C for 10 min (for the inactivation of the proteinase K). Genomic DNA can then be stored at –20 °C for a few months.
2. Primer design for promoter amplification: *C. elegans* promoters are typically positioned in the 3 kbp stretch of DNA upstream of the gene. Choose sequences of 20–24 bp that flank this region (*see Notes 3–5*).

The reverse primer should have a 5' overhang (*see Subheading 2*) that corresponds to the sequence of the pPD95.77—the 5' of the PCR product amplified from the plasmid (*see Note 6*).

3. Use the designed primers for promoter amplification. The PCR volume is 25 µL. Run 2.5 µL on 1% agarose gel and verify that the band is of the right size (*see Note 7*). Following the verification, the PCR product should be cleaned on a column, and DNA concentration can then be measured (*see Note 8*).



**Fig. 2** Representative images of the three PCR products on gels. **(A)** *sra-6* gene promoter amplified from N2 worm genomic DNA. **(B)** GCaMP6m calcium indicator amplified from pPD95.77 (including the vectors' flanking regions). **(C)** Fusion PCR between the *sra-6* promoter and GCaMP6m. Asterisk marks the whole fusion product. “L” is the ladder and “S” is the sample lane



**Fig. 3** Schematic representation of the fusion PCR reaction. **(A)** The first step of the reaction where the region of sequence homology acts as primers for elongation by DNA polymerase (“pacman” shapes). **(B)** The second step of the reaction depicts the amplification of the entire fusion fragment following the addition of the nested primers (dashed arrows)

### 3.2 Cloning and Amplifying the Gene of Interest

1. To clone the gene of interest into the pPD95.77 plasmid we amplify the gene from any given source by using primers which have restriction site extensions. We use the *Age*I restriction site for the forward (fwd) primer, and *Eco*RI restriction site for the reverse (rev) primer. There is no need to insert bases between the primer sequence and the restriction site sequence (*see Note 9*).
2. Insertion of the gene into the pPD95.77 is by standard techniques of restriction, gel purification, and ligation (*see Note 10*). One needs to verify the proper insertion of the gene by sequencing. Additionally, as mentioned before, this plasmid contains an *unc-54* 3' UTR. One should also verify that this fragment is still intact in the plasmid. To boost expression, the fusion PCR product will contain the *unc-54* 3' UTR and the synthetic intron that precedes it on the vector.

3. Fusion PCR reactions can be performed using the pPD95.77 vector containing the gene of interest. The primers used are the primers designated “pPD95.77 fwd” and “pPD95.77 rev” in the previous section. The annealing temperature used is 60 °C and denaturation and elongation temperatures are as recommended by the polymerase manufacturer. The duration of denaturation, annealing, and elongation are typically recommended by the polymerase manufacturer as well. The final volume of the PCR is 25 µL.
4. To verify that the PCR reaction succeeded, run 2.5 µL from the reaction on 1% agarose gel. The product should be the size of the gene plus ~0.5 kbp from the flanking vector sequences. This amplicon contains the artificial intron followed by the gene and the unc-54 3' UTR. Following the verification, the PCR product should be cleaned on a column and the concentration of the DNA should be determined using standard spectrophotometry methods (*see Note 11*).

### 3.3 Fusion PCR of the Promoter and Gene

1. Primary elongation to produce the full fusion: Transfer a similar amount of the gene and promoter PCR products (between 10 and 50 ng) to a PCR tube. Add dNTPs, buffer, and enzyme, and adjust the volume to 50 µL with DDW. Primers are not added to this reaction (*see Note 12*). Perform a short PCR of ten cycles with an annealing temperature of 50–58 °C.
2. Nested amplification of the fusion product: Add 1 µL of each of the two nested primers to the PCR tube; the fwd primer is the promoter-specific nested primer, and the rev primer is the primer designated pPD95.77 rev nested in Subheading 2 (*see Fig. 1*). Perform an additional PCR, this time with 30 cycles and an annealing temperature ranging from 55 to 68 °C, depending on the Tm of the promoters’ nested primer (*see Note 13*).
3. Run 5 µL from the PCR on gel. Verify that the band is of the right size (*see Notes 14 and 15*). No need for additional cleanup; the fusion PCR is ready for microinjection. Use an estimation of the concentration according to the band intensity compared to the DNA ladder bands, and inject between 20 and 40 ng/µL of the PCR product (*see Notes 16 and 17; [28, 29]*).

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## 4 Notes

1. One can use any other vectors for cloning the gene of interest provided that the primers for the first PCR reaction (gene amplification) and the rev nested primer for the third reaction (fusion PCR) are redesigned accordingly.

2. When preparing genomic DNA, picked worms should be young and gravid; this yields more DNA.
3. It is recommended to check the literature before deciding on the size of the promoter. Many gene promoters have already been characterized. Some promoters lead to different expression patterns of the protein when their length is changed.
4. The primers for amplifying the promoter should have approximately 50% GC content, and the annealing temperature should be similar for both primers (between 50 and 65 °C).
5. Run BLAST analysis on the promoter primers you choose in order to validate that you have a unique sequence and avoid nonspecific products.
6. When calculating the Tm of the promoter reverse primer, do not take the 5' overhang into consideration.
7. If more than one band appears on the gel when verifying the promoter PCR product, try to use a more stringent annealing temperature.
8. The cleanup of the two first PCR products is not mandatory. It may even be preferable to leave them not cleaned [18]. When doing so, use an estimation of the DNA concentration based on their band intensity compared to the DNA ladder band intensity.
9. It is highly recommended to add between four and six bases before the restriction site when designing the primers for cloning genes into the pPD95.77 plasmid; this improves the restriction reaction.
10. An alternative and faster method that can be used for cloning the gene of interest into the pPD95.77 vector is the restriction-free cloning, which also relies on PCR. A detailed description of this method can be found in [30].
11. When PCR amplifying the reporter gene from the pPD95.77 vector results in low yield, try to adjust the annealing temperature to the enzyme you are working with. Alternatively, try to extend the elongation time.
12. Note that you do not add primers to the first step of the fusion PCR.
13. If you are having difficulties fusing a specific promoter, either altering the primers or performing a gradient PCR usually solves the issue.
14. Sometimes additional nonspecific bands appear in the gel verifying the fusion PCR. If the desired band is the most intense one, there should be no problem injecting it as is (*see* Fig. 2C). However, if the desired band is very weak compared to other nonspecific ones, try to change the annealing temperature used in the second reaction of the fusion PCR.

15. Sometimes the fusion PCR band is surrounded by a DNA smear; this also should not interfere with the successful injection of the fusion PCR.
16. The fusion PCR product can be injected concurrently with other fusion PCR products or with other plasmids (linearized or not).
17. When fusion PCR yield is low, injections are still possible. We have successfully generated transgenic worms even when injecting as low as 2 ng/ $\mu$ L. This is not ideal, but nevertheless works.

## Acknowledgments

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# Chapter 11

## Approaches for CRISPR/Cas9 Genome Editing in *C. elegans*

Charlotte J. Martin and John A. Calarco

### Abstract

The clustered, regularly interspaced, short, palindromic repeat (CRISPR)-associated (CAS) nuclease Cas9 has been used in many organisms to generate specific mutations and transgene insertions. Here we describe our most up-to-date protocols using the *S. pyogenes* Cas9 in *C. elegans* that provides a convenient and effective approach for making heritable changes to the worm genome. We present several considerations when deciding which strategy best suits the needs of the experiment.

**Key words** *C. elegans*, CRISPR, Cas9, Genome editing, Genome engineering

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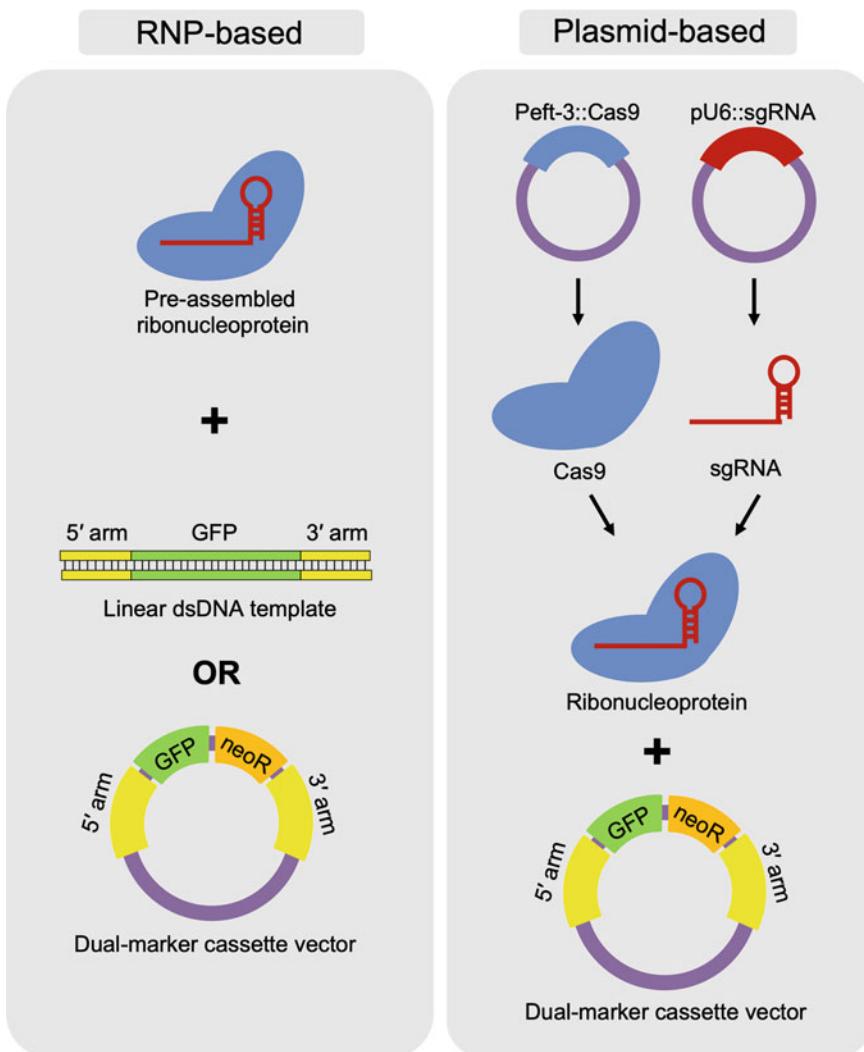
### 1 Introduction

The innovation of CRISPR-based genome editing has allowed for a revolution in our ability to engineer customized DNA modifications in organisms through its ease and simplicity [1]. The CRISPR/Cas9 editing strategy was adapted from an existing defense system that evolved in bacteria to protect them from invading viruses [1, 2]. Studies have revealed that a number of different CRISPR/Cas systems have emerged in nature, including the type II-A CRISPR/Cas9 system from *Streptococcus pyogenes* [1–3]. In this system, the Cas9 endonuclease is guided to DNA of interest by associating with both Clustered Regularly Interspaced Palindromic Repeat (CRISPR) RNA (crRNA) and trans-activating crRNA (tracrRNA), where the crRNA is complementary to the target DNA sequence of interest [1–3]. Early studies adopting this system discovered that the crRNA and tracrRNA can be fused as a single transcribed sequence called a single guide RNA (sgRNA) [1]. Thus, by simply using short 20 nucleotide RNA sequences, one can design sgRNAs that target specific sections of DNA [1, 2]. The only constraint for sequence recognition by the Cas9 endonuclease is that the 20 nucleotide region of DNA needs to be adjacent to a

NGG sequence (where N is any of the four bases), known as the protospacer-adjacent motif (PAM) [1, 2].

Once both strands of the DNA are cut by the Cas9 ribonucleoprotein complex a number of endogenous cellular mechanisms will repair double-strand breaks, including non-homologous end joining and homology-directed repair [4, 5]. Non-homologous end joining can lead to gene disruptions by causing insertions or deletions, likely resulting in loss-of-function mutations. In homology-directed repair, sections of DNA that are homologous to the cleaved DNA are used as a template to repair the double-strand break [4, 5]. Importantly, if a synthetic template is introduced into the cell for homology-directed repair, customized insertions, substitutions, and deletions can be added in the vicinity of the cleavage site [5]. Since the generation of precise genome edits requires only three components (Cas9, sgRNA, and some type of repair template), CRISPR/Cas9 genome editing has emerged as a widely used approach in a variety of cells and many of the commonly used multicellular animal model organisms, including *Caenorhabditis elegans*, fruit fly, zebrafish, and mouse [2, 5, 6].

In *C. elegans*, there have been a number of different techniques established making use of the CRISPR/Cas9 system for genome editing [6]. These approaches range from using entirely plasmid-based strategies to simply using purified protein, RNA, and short oligonucleotide components directly [6]. Depending on the nature of the sequences being substituted, inserted, or deleted, some of these approaches are equally suited or better suited over other approaches. The reader is encouraged to consult with a number of recent reviews which cover this specific topic in more detail [6, 7]. In this chapter we describe our preferred protocols for using CRISPR/Cas9 genome editing in *C. elegans*. One approach makes use of purified Cas9 and sgRNA, pre-assembled into a ribonucleoprotein (RNP) complex (Fig. 1 and Table 1). Another approach employs plasmids that express Cas9 and the sgRNA when after injection into the germline of adult hermaphrodites (Fig. 1 and Table 1). Additionally, the template used for homology-directed repair can either be a linear double-stranded DNA molecule with short homology arms or a plasmid with longer homology arms and a dual-marker selection cassette developed in our lab (Figs. 1 and 2) [8]. We provide different options and guidelines for the user to strike a balance between cost-effectiveness, time investment in the protocol, and efficiency of editing, allowing one to tailor the design to meet budget and time constraints.



**Fig. 1** Variety of approaches for CRISPR/Cas9 genome editing described in this chapter. (**Left panel**) Schematic of utilizing a pre-assembled Cas9/sgRNA ribonucleoprotein (RNP) complex. In this strategy, purified Cas9 protein and in vitro synthesized single guide RNA (sgRNA) is co-incubated prior to addition of the repair templates containing sequences with homology upstream and downstream of the target genomic DNA region of interest that is being edited (5' and 3' arms, respectively). Both linear double-stranded DNA (dsDNA) with short 5' and 3' homology arms or a dual-marker cassette vector with longer homology arms can be utilized with this approach. (**Right panel**) Alternative strategy that uses plasmid DNA to provide all of the components required for editing. In this approach, plasmids expressing Cas9 (from strong *eft-3* promoter) and sgRNA (from U6 RNA polymerase III promoter) are injected into worms along with our dual-marker cassette plasmid containing flanking homology arms. Cas9 mRNA is transcribed and then Cas9 protein is translated in the germline, and the sgRNA is transcribed in situ as well. Upon assembly of the RNP complex, editing proceeds and the homology-directed repair template provided is incorporated by the endogenous repair machinery.

**Table 1**

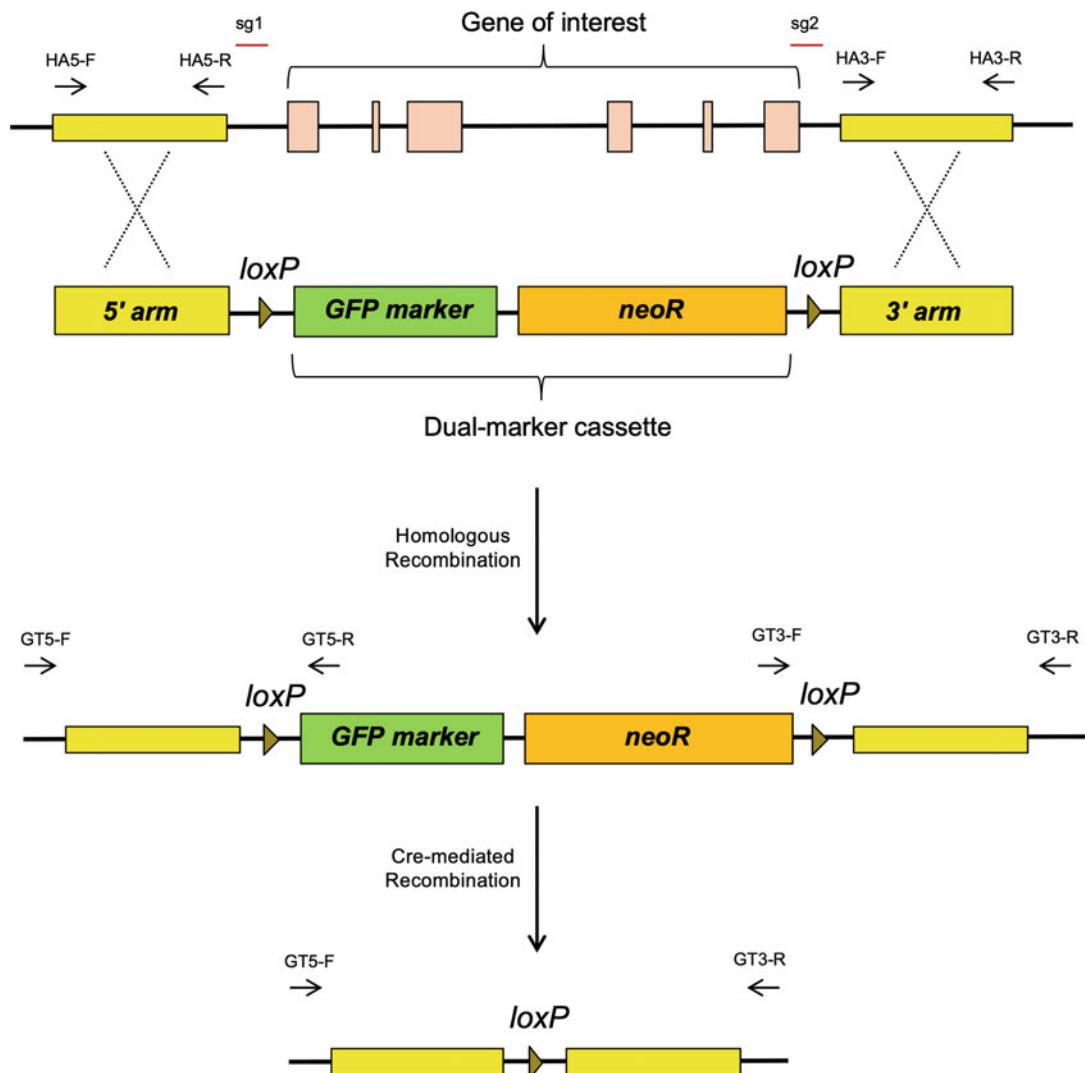
**A comparison of the different reagents/steps in the various approaches described. In this table, we present the different reagents (sgRNAs, Cas9, and repair templates) required for the approaches described in Subheading 3 of the chapter. We reference the specific sections that describe how to create these reagents, and in which version of the protocol they are used**

|   | <b>RNP methods</b>   | <b>All-plasmid method</b>  |
|---|--|--|
| Source of Cas9                              | • Purified Cas9 protein  | • Peft-3::Cas9 expression plasmid  |
| Source of sgRNA                             | • Direct purchase (Subheading 3.4.1)                                 | • pU6::sgRNA expression plasmid (Subheading 3.4.3)   |
|   | • In vitro transcription (Subheading 3.4.2)                          |  |
| Source of homology-directed repair template | • Linear dsDNA (direct purchase or PCR synthesis) (Subheading 3.5.1) | • Dual-marker cassette plasmid (Subheading 3.5.2)  |
|   | • Dual-marker cassette plasmid (Subheading 3.5.2)                    |  |
| Screening for successful editing events     | • PCR genotyping and sanger sequencing (Subheading 3.8.1)            | • Antibiotic selection with G418 and limited PCR genotyping and sanger sequencing (Subheading 3.8.2) |

## 2 Materials

### 2.1 sgRNA, Template DNA Generation, and Cloning of Homology Arm Vectors

1. Standard molecular biology equipment (PCR machine, pipettes, tips, tubes, agarose gel setup, incubators) are required.
2. Oligonucleotides from a preferred provider.
3. The following vectors: pU6::klp-12\_sgRNA and the dual-marker selection vectors Pmyo-2::GFP neoR loxP (disruption/deletion vector), GFP-Pmyo-2::GFP neoR loxP (GFP tagging vector) primers, RFP-Pmyo-2::GFP neoR loxP (RFP tagging vector), 3xHA-Pmyo-2::GFP neoR loxP (3xHA tagging vector), GFP-3xHA-Pmyo-2::GFP neoR loxP (GFP-3xHA tagging vector).
4. High Fidelity DNA Polymerase.
5. High Yield RNA Synthesis Kit.
6. PCR Master Mix (2×).
7. PCR cleanup and gel extraction kits.
8. T4 Polynucleotide kinase (PNK) and T4 DNA ligase.
9. RNA cleanup and purification kit.
10. Plasmid miniprep kits used during cloning steps.
11. DNA assembly master mix for Gibson Assembly.



**Fig. 2** Dual-marker insertion and genotyping example. A schematic showing the proposed insertion of our dual-marker cassette and simultaneous deletion of a gene of interest after homologous recombination has occurred. sgRNAs are typically designed to be close to the ends of the gene boundary, and homology arms are chosen to be in the vicinity of these sgRNA target sites. Homology arms can be amplified by PCR using primers HA5-F/R (for upstream arm) or primers HA3-F/R (for downstream arm). After the insertion, positive selection (resistance to antibiotic) and screening by fluorescence microscopy (to detect pharyngeal GFP signal) is used to identify candidate edited animals. After identification of candidates, genotyping PCRs are performed using the suggested primer configuration in the diagram. For example, the GT5-F and GT5-R primers detect successful recombination on the 5' side of the edit, while the GT3-F and GT3-R primers detect seamless repair on the 3' side of the edit. After genotyping confirms proper editing, Cre recombinase can be injected into animals to excise the dual-marker cassette, leaving behind only a single *loxP* site in the genome

12. LB agar plates supplemented with 100 µg/mL ampicillin.
13. Competent *E. coli* (e.g., DH5-alpha).
14. SacII, NotI, SpeI restriction enzymes.

## **2.2 Microinjection of *C. elegans* for CRISPR/Cas9 Genome Editing and Screening Animals**

1. The following plasmids are needed for microinjection, along with cloned sgRNA vectors and homology arm repair vectors for the gene of interest:  
Cas9 expression plasmid (Addgene plasmid #46168 - Peft-3::cas9-SV40\_NLS::tbb-2 3'UTR); co-injection marker plasmid #1 (Addgene plasmid #19327 - pCFJ90 - Pmyo-2::mCherry::unc-54utr); co-injection plasmid #2 (Addgene plasmid #19328 - pCFJ104 - Pmyo-3::mCherry::unc-54).
2. High-quality plasmid DNA purification kits. Several options available. We have used midiprep kits for purifying the Cas9 and co-injection marker plasmids. For sgRNA and homology arm repair plasmids, since these are used often only once or twice, we typically use a miniprep kit followed by a concentration kit.
3. Purified recombinant Cas9 (if using instead of plasmid expressed Cas9).
4. Wild-type *C. elegans* nematodes (N2 strain—obtain from *Cae-norhabditis* Genetics Center, CGC).
5. Nematode growth media (NGM) agar plates seeded with OP50 *E. coli* strain (CGC).
6. Microinjection apparatus. Several options are available.
7. Capillaries and needle puller for pulling needles for microinjection. Several options are available.
8. 20 × 60 mm glass coverslips coated with a drop of 2% agarose (dried overnight or longer). These are injection pads used to immobilize the animals.
9. Halocarbon oil to be used for immobilization and preventing worms from desiccating while on injection pad.
10. G418 sulfate for positive selection of transgenic animals.
11. Fluorescent stereomicroscope with excitation and emission filters to view GFP and mCherry expression.
12. Standard dissecting stereomicroscope for routine maintenance of animals.
13. 21 °C and 25 °C incubators for raising animals.
14. PCR Master Mix (2×).
15. Restriction enzymes appropriate to your gene of interest, if needed for screening/genotyping.

16. Single worm lysis buffer (Final concentrations: 50 mM Tris-HCl, 50 mM KCl, 2.5 mM, MgCl<sub>2</sub>, 0.45% NP40, 0.45% Tween 20, ddH<sub>2</sub>O).
17. Proteinase K, recombinant, PCR grade.

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### 3 Methods

#### 3.1 Considerations before Starting Editing Experiments

In this protocol, we describe three strategies for creating deletion mutations, inserting small epitopes or fluorescent protein tags, or custom nucleotide substitutions (Figs. 1 and 2). The different approaches have strengths and weaknesses that should be considered and one approach might be better suited for a particular desired editing outcome. For example, the use of the all plasmid strategy is economical and once plasmids are made experiments can be repeated many times without the need to re-purchase reagents. However, the use of purified Cas9 protein and synthesized sgRNA, although more expensive, is time-saving and has been demonstrated to be more efficient than using our Cas9 and sgRNA plasmids [9]. When using the dual-marker selection vector for homology-directed repair, the ability to select animals with insertions reduces the need to PCR genotype a large number of animals. Using a linear double-stranded repair template will require some type of extra screening strategy, including PCR genotyping, looking for restriction fragment length polymorphisms, Sanger sequencing, or direct visualization of the inserted tag by microscopy (in the case of fluorescent protein tag insertion). Finally, with the dual-marker selection cassette strategy, the dual-marker generally needs to be recombined out of the genome. This leaves behind a single loxP site, which we usually bury within a strong intronic sequence that will be spliced out of the final transcript of a protein coding gene. However, the presence of the loxP site and intron may be less desirable for small point mutations and substitutions, and as such the direct insertion approach with linear double-stranded DNA may be more desirable.

#### 3.2 Selecting Guide RNAs for Performing CRISPR Editing in *C. elegans*

We routinely use the IDT Custom Alt-R® CRISPR-Cas9 guide RNA website ([https://www.idtdna.com/site/order/designtool/index/CRISPR\\_CUSTOM](https://www.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM)) and the Simon Fraser University CRISPR guide RNA selection tool (<http://genome.sfu.ca/crispr/>) to identify sgRNAs targeting your gene of interest. The sgRNA should target within 35 bp upstream or downstream of your intended site of insertion when using linear double-stranded DNA with 120 bp homology arms, but we have found that sgRNA targeting as far as 150 bp away is fine when using 500 bp homology arms in our dual-marker cassette plasmid repair strategy. These websites use different filters and scoring methods to provide

information regarding the choice of the sgRNAs. We recommend consulting the documentation and guidelines listed on these websites to help with the choice of sgRNAs to use.

### **3.3 Selecting Homology Arms Flanking Your Sequence Being Inserted**

1. *Recombination using linear double-stranded DNA:* We have used homology arms of 120 bp upstream and downstream of the site of insertion. However, as reported recently [10–12], it is possible to have successful insertions with only 35 bp homology arm lengths.
2. *Recombination using dual selection marker plasmid method:* Choose homology arms that include 500 bp of upstream and downstream sequence from the desired site of insertion.

### **3.4 Synthesizing sgRNAs for Genome Editing**

#### **3.4.1 Directly Ordering sgRNA or crRNA/tracrRNA from a Company**

There are three options for synthesizing sgRNAs: direct ordering from a company, in vitro transcription from a DNA template, and construction of an sgRNA expression plasmid that will express the sgRNA in the worm. Below we outline the steps required for each option.

#### **3.4.2 Synthesizing sgRNAs from a DNA Template Using In Vitro Transcription**

1. Several sources are available to order sgRNA. We find that 2 nmol scale is sufficient. Alternatively, we have also used separated crRNA/tracrRNA complexes.

1. A new sgRNA DNA template for in vitro transcription is created by performing a polymerase chain reaction (PCR) using our pU6::klp-12\_sgRNA plasmid as a template and the following pair of primers (*see Note 1*):

sgRNA Forward primer: 5'- TTCTAATACGACTCAC  
TATA-G-(N<sub>20</sub>)-GTTTAGAGCTAGAAATAG - 3'.

sgRNA Universal Reverse primer: 5'- AAAAGCACC  
GACTCG-3'.

where N<sub>20</sub> is the target sequence of the gene of interest.

2. Set up a PCR with the components and thermal cycler settings below using the pU6::klp-12\_sgRNA plasmid as a template:

*Reaction (50 μL final volume)*

31.5 μL ddH<sub>2</sub>O.

10 μL 5× HF Buffer.

1 μL dNTPs (10 mM).

2.5 μL sgRNA Forward primer (10 μM).

2.5 μL sgRNA Universal Reverse primer (10 μM).

2 μL pU6::klp-12 sgRNA plasmid (10 ng/μL).

0.5 μL Phusion polymerase.

*PCR settings*

Step 1: 98 °C 30 s.  
Step 2: 98 °C 30 s.  
Step 3: 51 °C 30 s.  
Step 4: 72 °C 30 s.  
Step 5: go to step 2, 34 times.  
Step 6: 72 °C 5 min.  
Step 7: 4 °C hold  $\infty$ .

3. Set up a 2% agarose gel and load 5  $\mu$ L of the PCR product along with a DNA ladder. There should be a single band at  $\sim$ 100 bp.
4. If the PCR product looks good from **step 3**, purify the PCR product with the concentration kit, as recommended by the manufacturer, eluting with 10  $\mu$ L of nuclease-free water.
5. Quantify the DNA product with a spectrophotometer. The protocol can be stopped safely at this point and the DNA template can be stored at  $-20$  °C, or the protocol can be continued.
6. Set up an in vitro transcription reaction using the T7 High Yield RNA Synthesis Kit as recommended by the manufacturer.

*Reaction (30  $\mu$ L final volume)*

10  $\mu$ L NTP buffer mix.  
 $x$   $\mu$ L 75 ng of template DNA.  
2  $\mu$ L T7 RNA polymerase mix.  
(18 –  $x$ )  $\mu$ L ddH<sub>2</sub>O.

*Thermal cycler conditions*

- Step 1: 37 °C for 4–16 h (*see Note 2*).  
Step 2: 4 °C hold  $\infty$ .
7. After the in vitro transcription reaction is complete, treat the samples with DNase as indicated by the manufacturer.

*Reaction (52  $\mu$ L final volume)*

30  $\mu$ L IVT reaction.  
20  $\mu$ L ddH<sub>2</sub>O.  
2  $\mu$ L DNase.

*Thermal cycler conditions*

- Step 1: 37 °C for 15 min.  
Step 2: 4 °C hold  $\infty$ .
8. Purify the sgRNA product with the RNA concentration kit, according to the recommendations of the manufacturer, eluting with 15  $\mu$ L of nuclease-free water.
  9. Quantify the RNA product with a spectrophotometer. The sgRNA can be stored at  $-20$  to  $-80$  °C at this point.

**3.4.3 Creating a New Plasmid Expressing Your sgRNA of Interest in the Worm**

10. (Optional) Run 1  $\mu$ L of the RNA product on a 2% agarose gel in order to inspect if the in vitro transcription led to discrete transcription products. (*see Note 3*).

1. This procedure is adapted from our previous protocol described in [8]. Credit goes to Arneet Saltzman for development of this time-saving protocol.
2. First, primers are designed to be used with our pU6::klp-12\_sgRNA plasmid and will amplify the entire plasmid while adding the new sgRNA target sequence. The two primers are designed as follows:

sgRNA Forward: 5'-G(N<sub>20</sub>) GTTTTAGAGCTAGAAA TAGCAAG-3'.

sgRNA Universal Reverse: 5'- AAACATTAGATTG CAATTCAATTA-3'.

where N<sub>20</sub> is the 20 nucleotide target sequence specific to the gene of interest.

3. Starting with existing pU6::klp-12::sgRNA plasmid as a template, set up the following polymerase chain reaction (PCR) and settings:

*Reaction*

32.5  $\mu$ L molecular biology grade water.

10  $\mu$ L 5xHF Buffer.

1  $\mu$ L dNTPs (10 mM).

2.5  $\mu$ L sgRNA Forward primer (10uM).

2.5  $\mu$ L sgRNA Universal Reverse primer (10uM).

1  $\mu$ L klp-12 sgRNA vector (20 pg/ $\mu$ L).

0.5  $\mu$ L Phusion polymerase.

*PCR settings*

Step 1: 98 °C 30 s.

Step 2: 98 °C 30 s.

Step 3: 56 °C 10–30 s.

Step 4: 72 °C 1 min 45 s.

Step 5: go to step 2, 9 times.

Step 6: 98 °C 30 s.

Step 7: 61 °C 30 s.

Step 8: 72 °C 1 min 45 s.

Step 9: go to step 6, 14 times.

Step 10: 72 °C 5 min.

Step 11: 4 °C hold  $\infty$ .

4. Run 5  $\mu$ L of PCR reaction out on a 1% agarose gel. A band should be visible that is ~3.5 kilobases in length (*see Note 4*).
5. Purify reaction using a purification column according to manufacturer's guidelines, eluting DNA with 26  $\mu$ L of water or elution buffer with a 2 min incubation of the column at room temperature.
6. After DNA is purified, add 3  $\mu$ L of 10 $\times$  T4 DNA Ligase buffer (*see Note 5*) and add 0.5  $\mu$ L T4 PNK. Mix well.
7. Incubate sample at 37 °C for 20 min.
8. Remove 10  $\mu$ L of sample and transfer to new tube for a "No ligase" control in a subsequent step.
9. To remainder of reaction (~19  $\mu$ L), add 1  $\mu$ L T4 DNA ligase. Mix well.
10. Incubate sample with ligase and no ligase control tube at room temperature for 1 h.
11. Transform competent *E. coli* with 1  $\mu$ L of reaction and no ligase control according to manufacturer's guidelines.
12. Plate bacteria onto LB agar plates supplemented with 100  $\mu$ g/mL ampicillin, and grow overnight at 37 °C.
13. If ligation worked well, there should be many colonies on reaction plates and very few colonies on no ligase control plates.
14. Pick 3 single colonies to miniprep and validate insertion of new protospacer directly by sequencing (*see Note 6*).

### **3.5 Generating Homology-Directed Repair Templates**

#### **3.5.1 Suggestions for Preparing Linear Double-Stranded Templates with 35 or 120 bp Homology Arms**

There are two options for homology-directed repair templates: linear double-stranded templates and plasmid-derived templates. Below we outline the steps required for each option.

1. Depending on the size of the edit or insertion trying to be introduced into the genome, several methods can be used to generate linear double-stranded DNA templates. First, the double-stranded DNA can either be synthesized directly by a company or long oligonucleotides can be synthesized by a company and then annealed. Second, a PCR can be performed using oligonucleotides containing 35–120 bases of homology to the gene of interest that also anneal to a template containing an epitope or tag (such as GFP) of interest. Since this step involves fairly standard and well-established approaches and depends on the specific nature of the genome editing strategy, the choice is up to the user on how to proceed (*see Note 7*).
1. The 500 bp homology arms that will be cloned into one of the dual-marker cassette vectors can be generated by direct synthesis of double-stranded DNA from a company (such as gBlocks

**3.5.2 Generating Homology-Directed Repair Templates Using our Dual-Marker Selection Cassette Toolkit**

from IDT). Alternatively, primers can be designed to amplify homology arms from N2 genomic DNA by PCR. If synthetic DNA is ordered directly, proceed to Gibson Assembly step below. If performing PCR, proceed to **step 2** (*see Note 7*).

- Design primers for homologous regions of interest. Four primers need to be designed—a forward and reverse primer for the upstream and downstream homology arms. We have five different deletion or tagging vectors currently available. The specific primers to be designed are below.

*Pmyo-2::GFP neoR loxP (disruption/deletion vector) primers*

Upstream F: AACGACGGCCAGTGAATTCCCGCGG(Nx).

Upstream R: TTATAGGCCGCCTGATGCGCCGCGG (Nx-RC).

Downstream F: ATAGTTGCAGGACCCTGCGGCCGC (Nx).

Downstream R: ATGATTACGCCAAGCTTGCGGCCGC (Nx-RC).

where Nx and Nx-RC are homology arm specific sense and reverse complementary sequences, respectively.

*GFP-Pmyo-2::GFP neoR loxP (GFP tagging vector) primers*

Upstream F: AACGACGGCCAGTGAATTCACTAGT(Nx).

Upstream R: GTTCTTCTCCTTACTCATACTAGT (Nx-RC).

Downstream F: TGGATGAACTATAACAAAGCGGCCGC (Nx).

Downstream R: ATGATTACGCCAAGCTTGCGGCCGC (Nx-RC).

*RFP-Pmyo-2::GFP neoR loxP (RFP tagging vector) primers*

Upstream F: AACGACGGCCAGTGAATTCACTAGT(Nx).

Upstream R: CTTCACCCCTTGAGACCATACTAGT (Nx-RC).

Downstream F: TGGATGAATTGTATAAGCGGCCGC (Nx).

Downstream R: ATGATTACGCCAAGCTTGCGGCCGC (Nx-RC).

*3xHA-Pmyo-2::GFP neoR loxP (3xHA tagging vector) primers*

Upstream F: AACGACGGCCAGTGAATTCACTAGT(Nx).

Upstream R: CTGGAACGTCGTATGGGTAACTAGT (Nx-RC).

Downstream F: ACGTCCCAGATTACGCTGC GGCCGC  
(Nx).

Downstream R: ATGATTACGCCAAGCTTGC GGCCGC  
(Nx-RC).

*GFP-3xHA-Pmyo-2::GFP neoR loxP (GFP-3xHA tagging vector) primers*

Upstream F: AACGACGGCCAGTGAATTCACTAGT(Nx).

Upstream R: GTTCTTCTCCTTACTCATACTAGT  
(Nx-RC).

Downstream F: ACGTCCCAGATTACGCTGC GGCCGC  
(Nx).

Downstream R: ATGATTACGCCAAGCTTGC GGCCGC  
(Nx-RC) (*see Note 8*).

3. Once primers are ordered, set up a PCR with the components and thermal cycler settings below using N2 genomic DNA as a template. This is a suggested starting point, but some optimization might be required to obtain good PCR products.

*Reaction*

31.5  $\mu$ L ddH<sub>2</sub>O.

10  $\mu$ L 5XHF Buffer.

1  $\mu$ L dNTPs (10 mM).

2.5  $\mu$ L Forward primer (10  $\mu$ M).

2.5  $\mu$ L Reverse primer (10  $\mu$ M).

2  $\mu$ L genomic DNA (50 ng/ $\mu$ L).

0.5  $\mu$ L Phusion polymerase.

*PCR settings*

Step 1: 98 °C 30 s.

Step 2: 98 °C 30 s.

Step 3: Tm of specific primers 30 s.

Step 4: 72 °C 30 s.

Step 5: go to step 2, 34 times.

Step 6: 72 °C 5 min.

Step 7: 4 °C hold  $\infty$ .

4. Set up a 1% agarose gel and load 5  $\mu$ L of the PCR product and perform gel electrophoresis. There should be a single band at ~560 bp.
5. If a single clean band is observed, purify the remaining PCR product with the concentration Kit, eluting with 10  $\mu$ L of nuclease-free water. Alternatively, the remaining DNA can also be run on a gel, excised from the gel and purified.

6. Quantify the DNA with a spectrophotometer. Homology arms are now ready to be used in Gibson Assembly once the dual-marker cassette vector is digested.

7. In parallel with steps 2–6, set up a preparative restriction enzyme digest of the appropriate dual-marker cassette vector.

*Reaction (40 µL final volume)*

4 µL 10× CutSmart buffer.

1 µL Restriction Enzyme A (SacII for deletion vector, and SpeI for tagging vectors).

1 µL NotI.

x µL vector (total of 3 µg).

(40 - x) µL ddH<sub>2</sub>O.

8. Incubate the reaction in a thermal cycler at 37 °C for 3 h.

9. Run 5 µL of the reaction on a 1% agarose gel to confirm that the vector was digested correctly.

10. Purify remaining reaction with the concentration kit, eluting with 20 µL of nuclease-free water.

11. Set up a Gibson Assembly reaction of the digested vector and homology arms in a PCR tube on ice. Include a control reaction of the digested vector and assembly mix:

*Reaction (10 µL final volume)*

x µL digested vector and cassette (50 ng).

y µL 5' homology arm (proportional to digested vector).

z µL 3' homology arm (proportional to digested vector).

5 µL 2x Gibson Assembly mix.

(5 - x - y - z) µL ddH<sub>2</sub>O.

*Control reaction (10 µL final volume)*

x µL digested vector and cassette (50 ng).

5 µL 2× Gibson Assembly mix.

(5 - x) µL ddH<sub>2</sub>O.

12. Incubate the reaction at 50 °C for 1 h using a thermal cycler.

13. Transform the reaction into competent bacteria following the guidelines from the manufacturer.

14. Plate the transformed bacteria on to LB agar supplemented with 100 µg/mL ampicillin and incubate overnight at 37 °C.

15. If the assembly was successful, there will be a high enrichment of bacterial colonies on the plates compared to the digested vector-only control transformation.

16. Pick several individual colonies and grow overnight in liquid cultures of LB supplemented with 100 µg/mL ampicillin.

17. The vectors can then be purified with the miniprep kit and then confirmed through diagnostic restriction enzyme digestion and Sanger sequencing.

### **3.6 Preparing Injection Mixes for Microinjection**

#### **3.6.1 Purified Ribonucleoprotein with Linear Double-Stranded DNA**

Homology-Directed Repair Template

Depending on the three approaches used, the injection mixes are prepared slightly differently. In this section we describe how each solution is prepared.

This approach has been adapted in our lab inspired by a recent study from the Mello Lab [10].

1. Set up the following CRISPR/Cas9 injection mixture on ice.  
*Mixture (5 μL volume)*

2.5 μg Cas9.  
500 ng sgRNA1.  
500 ng sgRNA2 (*see Note 9*).

2. Use a thermal cycler to incubate the first three components of the injection mixture (Cas9 and sgRNA) at 37 °C for 10 min.
3. While ribonucleoprotein mixture is incubating, prepare donor DNA at a concentration of 100 ng/μL. Heat and cool the DNA in a thermal cycler using the settings below.

*Heating and cooling thermal cycler settings*

95 °C for 2 min.  
Ramp down at 1 °C/s.  
85 °C for 10 s.  
Ramp down at 1 °C/s.  
75 °C for 10 s.  
Ramp down at 1 °C/s.  
65 °C for 10 s.  
Ramp down at 1 °C/s.  
55 °C for 1 min.  
Ramp down at 10 °C/s.  
45 °C for 30 s.  
Ramp down at 1 °C/s.  
35 °C for 10 s.  
Ramp down at 1 °C/s.  
25 °C for 10 s.  
Ramp down at 1 °C/s.  
4 °C forever.

4. Add the final components of the injection mixture to the pre-incubated Cas9 and sgRNA.

**3.6.2 Purified Ribonucleoprotein with Plasmid Homology-Directed Repair Template**

*Injection mix (20 µL final volume):*

- 5 µL Pre-incubated Cas9/sgRNA mixture.
- 5 µL of heated and cooled donor DNA.
- 1 µL pCFJ90 co-injection marker (50 ng/µL).
- 9 µL nuclease-free water.

1. Set up the following CRISPR/Cas9 injection mixture on ice.
- Mixture (5 µL volume):*

- 2.5 µg Cas9.
- 500 ng sgRNA1.
- 500 ng sgRNA2 (*see Note 9*).

2. Use a thermal cycler to incubate the first three components of the injection mixture (Cas9 and sgRNA) at 37 °C for 10 min.
3. Add the final components of the injection mixture to the pre-incubated Cas9 and sgRNA.

*Injection mix (10 µL final volume):*

- 5 µL Pre-incubated Cas9/sgRNA mixture.
- 2 µL of plasmid DNA (250 ng/µL).
- 0.5 µL pCFJ104 co-injection marker (100 ng/µL).
- 0.5 µL pCFJ90 co-injection marker (50 ng/µL).
- 2 µL nuclease-free water.

**3.6.3 All Plasmid Injection Mix**

1. Set up the following injection mixture on ice:
- Injection mix (10 µL final volume)*

- 0.5 µL Peft-3::Cas9\_SV40\_NLS::tbb-2\_UTR (1 µg/µL).
- 0.5 µL pCFJ90 (50 ng/µL).
- 0.5 µL pCFJ104 (100 ng/µL).
- x µL repair template (500 ng total).
- y µL sgRNA1 (500 ng total).
- z µL sgRNA2 (500 ng total).
- (8.5 - x - y - z) µL molecular biology grade water.

**3.7 Performing Microinjections**

1. Regardless of which injection mix is prepared, spin mixes for 5 min at high speed in a microcentrifuge. Collect top 6 µL of solution to a new microfuge tube.
2. Load microinjection needle with 0.5–2 µL of injection solution.
3. Place needle into pressure regulated needle holder. Break needle open using a breaking needle and clear out any air bubbles with forced air pressure. The needle should have no sign of

clogging, with liquid flowing easily upon application of positive air pressure.

4. Mount adults with a single row of eggs in their uterus (*see Note 10*) on a 2% dry agarose pad coated with halocarbon oil. Worms should be mounted with dorsal gonad arms in an accessible position for microinjection. Make sure to cover animals completely in oil after mounting to avoid desiccation.
5. Working quickly, proceed to microinject DNA solution into each gonad arm of the animals. After withdrawing the injection needle, apply positive pressure and flood the animal with the injection solution. This will help release the animal from the agarose pad to enable recovery. With practice, one should be able to mount and inject several animals in one cycle.
6. Recover animals to a recovery plate. When executed properly, injected animals should be crawling immediately or within a few minutes after being deposited on the recovery plate.
7. Repeat this procedure until roughly 10 animals are injected (for the ribonucleoprotein with linear double-stranded DNA template injection mix) or 32 animals are injected (for the ribonucleoprotein with plasmid or all plasmid injection mixes).
8. When finished injecting, transfer a single animal to a plate across a total of 10 plates (for ribonucleoprotein with linear double-stranded DNA injection mix) or transfer 4 animals to a plate across a total of 8 × 35 mm NGM plates (for the ribonucleoprotein with plasmid or all plasmid injection mixes). Place animals in a 25 °C incubator.

### **3.8 Screening of Transgenic Progeny for Genome Edits**

#### *3.8.1 Screening Directly for the Editing Event by Fluorescence Microscopy or by PCR Genotyping (for Homology-Directed Repair Without the Dual-Marker Cassette)*

Depending on the editing approach used, slightly different steps are employed post-injection to screen for proper insertions. This section highlights the different options.

1. Approximately 24 h post-injection, observe the injected animals and their offspring under a fluorescent microscope. Observe the health of the injected animal and how many of its progeny are positive for the pCFJ90 (*P myo-2::mCherry*) co-injection marker. Select the two-three best looking injections based on the number of offspring with mCherry fluorescence, which will be the most likely to give rise to edited offspring. Return the plates to the 25 °C incubator.
2. Approximately 48–72 h post-injection observe the animals under a fluorescent microscope. At this time progeny that are positive for the insertion are starting to emerge. If inserting a fluorescent tag, survey the entire population for potential fluorescent F1 offspring (*see Note 11*). We have found successfully inserted animals among progeny both expressing or not expressing the mCherry co-injection marker transgene.

3. If no positive animals are observed 72 h post-injection proceed with genotyping the animals using a PCR-based strategy (*see Note 12*).
4. Pick 20–40 F1 progeny from the one-two plates with the highest number of progeny expressing the *Pmyo-2::mCherry* co-injection marker. Both mCherry positive and negative animals can be selected. Place a single F1 animal to each plate and incubate at 25 °C for a few days.
5. Once the F1s have had F2 progeny, proceed to lyse the F1 mothers. Set up on ice the lysis buffer solution below:

*Lysis buffer master mix for 24 reactions:*

- 180 µL Single worm lysis buffer.
- 0.6 µL Proteinase K (20 mg/mL).
- 6. Aliquot 6 µL to of the lysis buffer to PCR tubes.
- 7. Add the single F1 worm to each tube.
- 8. Place the PCR tubes at –80°C for at least 15 min.
- 9. Use a thermal cycler to incubate the lysates using the following settings:

*Thermal cycler settings for lysis:*

- 65 °C for 65 min.
- 95 °C for 15 min.
- 4 °C for ∞.
- 10. Set up a PCR reaction for each individual worm lysate using the lysate as the template:

*Reaction (20 µL final volume)*

- 8 µL ddH<sub>2</sub>O.
- 10 µL 2× PCR Mix.
- 0.5 µL Forward primer (10 µM).
- 0.5 µL Reverse primer (10 µM).
- 1 µL F1 lysate.

*PCR settings*

- Step 1: 95 °C 2 min.
- Step 2: 95 °C 30 s.
- Step 3: T<sub>m</sub> – 5 °C 30 s.
- Step 4: 72 °C 1 min (up to 2 kb).
- Step 5: go to step 2, 34×.
- Step 6: 72 °C 10 min.
- Step 7: 10 °C hold.

11. If using restriction enzyme fragment length polymorphism, complete **steps 12–14**. Otherwise, run the products of the PCR on a 2% agarose gel by electrophoresis. Screen for a PCR product of the correct size to confirm if the animal is carrying an insertion.
12. (Optional) If using a restriction enzyme fragment length polymorphism to identify successfully edited animals, immediately set up a restriction enzyme digest on ice for each PCR reaction and transfer a portion of each lysate PCR product to the reaction (*see Note 13*):

*Reaction (24 µL Final Volume)*

13.9 µL ddH<sub>2</sub>O.

1.6 µL 10× Restriction Buffer.

0.5 µL Restriction Enzyme.

8 µL PCR product of the F1 lysates.
13. Incubate the restriction enzyme digest for 1 h at the appropriate temperature of the restriction enzyme.
14. Run the 24 µL of the restriction enzyme digest and the remaining 12 µL PCR product on a 2% agarose gel. Visualize the DNA fragments and check if the expected restriction digestion pattern is observed, confirming if any animals have been successfully edited.
15. It is recommended to further confirm the precise nature of the editing event by Sanger Sequencing and additional follow-up experiments.

### 3.8.2 Antibiotic Selection and Screening of Animals with the Dual-Marker Cassette

1. After ~24 h post-injection and recovery, plates can be quickly screened under a fluorescent stereoscope to assess quality of injection. Should see many eggs on the plate, and a number of GFP positive embryos (more than 30) should already be observed at this time for successful microinjections.
2. For plates that look good, apply 125 µL of 25 mg/mL G418 solution to the lawn of each plate (for 35 mm plates, *see Note 14*), and rotate plate in a circular motion to spread antibiotic across entire surface of plate. Allow plates to air dry for a few minutes with the lids off to allow for complete absorption of solution. Return plates to 25 °C incubator.
3. After 5–6 days post-injection, plates can be screened for successful recombination and insertion of the dual-marker selection cassette into the genome. Candidate animals will be expressing dim, uniform GFP signal in the entirety of the pharynx and will also have completely lost mCherry co-injection marker expression. In contrast, extrachromosomal

array animals will retain mCherry expression and often display patchy, bright, and mosaic GFP expression.

4. Single several candidate animals from each plate containing putative genomic insertions to new plates and allow these animals to grow to adulthood and have offspring. Bona fide insertions will at least throw 75% offspring that are also GFP positive and again express dim, uniform GFP pharyngeal expression. At this point, additional confirmatory assays should be performed to genotype animals, such as performing PCR genotyping and Sanger sequencing (*see Note 12*).

### **3.9 Excision of Dual-Marker Cassette by Cre Recombinase**

This section of the protocol is implemented when using the dual-marker cassette vector for homology-directed repair, which can be excised after recombination with Cre recombinase (Fig. 2).

1. Prepare injection mix to inject Cre recombinase into homozygous animals carrying two copies of dual-marker cassette.

*Injection mixture*

1 μL Peft-3::Cre (pDD104) (500 ng/μL).  
 1 μL Pmyo-2::mCherry (pCFJ90) (25 ng/μL)  
 8 μL of nuclease-free water.

2. Follow the procedure described in Subheading 3.7 to perform microinjection of DNA solution. Typically injecting ten animals is sufficient to generate offspring with excised dual-marker cassettes.
3. After injection, let animals recover for a half hour to one hour at room temp. Then move –34 adults per plate and place in 25 °C incubator.
4. Two days after injection, pick 25–30 F1 progeny that are expressing the Pmyo-2::mCherry marker. Transfer 4–5 worms per plate and return for growth at 25 °C.
5. Two-three days after picking F1 animals, screen plates for F2 progeny that have completely lost pharyngeal GFP expression and have also lost mCherry expression. These animals should now be homozygous for the excised dual-marker cassette.
6. Propagate animals for appropriate downstream applications.

## **4 Notes**

1. The sgRNA primers are designed to be used with the pU6::klp-12 sgRNA plasmid and amplify the sgRNA. The primers are designed based on the specifications of a T7 Quick High Yield RNA Synthesis Kit. The forward primer contains the T7 promoter for the *in vitro* transcription, an extra Gs, the target

sequence of your gene of interest and the region complementary to the sgRNA scaffold sequence found in the pU6::klp-12 sgRNA vector. The kit manufacturer strongly suggests adding two GGs between the T7 promoter and the 20 nucleotides of your target sequence; however, we have found that one G is sufficient. The reverse primer anneals to the universal sgRNA scaffold found on the pU6::klp-12 sgRNA vector.

2. The 4 h incubation time is not always sufficient to create a high yield of RNA product, however, we consistently recover high yields with a longer overnight incubation. Therefore, the 16 h incubation is recommended.
3. It is more appropriate to run a denaturing formaldehyde-agarose gel to analyze RNA products. However, for convenience we run a native agarose gel stained with ethidium bromide to simply get a quick idea if the sgRNA is intact and not degraded. We typically see one to two bands with a smear in between 75–200 bp in size. In our experience, the IVT and cleanup steps are highly reliable and after several successful attempts at these reactions, this step can likely be skipped.
4. Sometimes a PCR product is not visible on the gel. However, proceeding with the subsequent phosphorylation, ligation and transformation steps can still result in colonies on the plates. Alternatively, increasing the PCR amplification cycle number to 30 or 35 generally increases yield, although this could risk increasing chance of point mutations.
5. The T4 ligase buffer is identical to the PNK buffer, with the exception that 1 mM ATP is already included in the 1× buffer, thus serving as buffer for both the phosphorylation and ligation steps, eliminating the need to perform a cleanup in between the two reactions.
6. For Sanger sequencing, the standard M13 forward primer available at most sequencing facilities can be used for verification of new target sequence insertion.
7. If the homology arms contain the target region of the sgRNA, then care must be taken to introduce nucleotide substitutions to prevent re-cleavage by Cas9 after genome editing has occurred. The simplest way to prevent re-cleavage is to mutate one of the G nucleotides in the PAM sequence. Alternatively, you can introduce several nucleotide substitutions in the seed region of the sgRNA (10–12 nucleotides immediately upstream of PAM). If the target sequence is in the coding region of the gene, you must ensure that any mutations made to mutate the target sequence of the sgRNA maintain the coding sequence. You also need to add on both the 5' and 3' ends of the homology arm 30 nucleotides of homology to the vector that the 500 bp homology arm is being assembled into

through Gibson Assembly. Finally, we also design the 500 bp homology arms to contain flanking restriction enzyme sites that match the restriction enzyme used to digest the vector they are being assembled into. This will enable the 500 bp homology arm when assembled into the vector to be excised from the vector if needed.

8. Our GFP, RFP, and GFP-3xHA ORFs have start codons built into the vector but no stop codons. Our 3xHA ORF has no start or stop codons. These ORFs have been designed to allow both N-terminal or C-terminal tagging. Care must be taken to ensure that reading frame is maintained or initiation depending on the editing strategy.
9. Alternatively, crRNA/tracrRNA could be used instead of sgRNAs.
10. Typically, we pick late L4 stage animals the day before an injection, and incubate these animals for ~18–24 h at 21 °C. This usually yields adult animals with a single row of eggs in their uterus, which in our hands work very well for microinjection.
11. Some fluorescently tagged endogenous proteins are not expressed at a strong enough level to be visualized with most standard fluorescence microscopes. In some cases, it may be better to screen for transgenic progeny using a compound fluorescence microscope or using confocal microscopy. If no fluorescence is observed, it is still worthwhile to screen progeny by PCR-based genotyping.
12. We generally use PCR primers that will lead to the unique amplification of a product that is specific to the genome-edited animals. For example, when inserting epitope or fluorescent protein tags, we design one primer to anneal in the tag or dual-marker cassette and another primer to anneal outside of the homology arm used in the double-stranded DNA template (*see Fig. 2* for example). Generally, primers are designed to test proper insertion of the tag on each side of the insertion site. We also recommend Sanger sequencing these amplicons to confirm integrity of homology arms and predicted deletion breakpoints. Finally, we also recommend back-crossing confirmed deletion strains to N2 several times to clean up any potential background mutations. For smaller substitutions, it is often desirable to identify or introduce restriction enzyme polymorphisms that can be detected by a combined PCR/Restriction enzyme digestion. Alternatively, samples can be screened directly by Sanger sequencing of amplified PCR products surrounding the edited region, although this is a bit more time-consuming.

13. When using a restriction digestion step for genotyping, we chose a polymerase and restriction enzyme systems with buffers that are compatible with both reactions. Therefore, there is no need to perform a cleanup step in between the PCR and restriction digestion reactions.
14. This volume and concentration of G418 has been determined empirically for reasonable positive selection on 35 mm plates. The volume would need to be adjusted for larger plates. For 60 mm plates, we have used anywhere from 250 to 500 µL of 25 mg/mL G418 solution. Note that G418 is not 100% effective, but does kill off a significant number of animals not carrying any transgenes. Screening needs to be performed by microscopy to identify recombinant animals.

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# Chapter 12

## Targeted and Random Transposon-Assisted Single-Copy Transgene Insertion in *C. elegans*

Sonia El Mouridi and Christian Frøkjær-Jensen

### Abstract

Transgenesis in model organisms is an essential tool for determining the function of protein-coding genes and non-coding regulatory regions. In *Caenorhabditis elegans*, injected DNA can be propagated as multi-copy extra-chromosomal arrays, but transgenes in arrays are frequently mosaic, over-expressed in some tissues, and silenced in the germline. Here, we describe methods to insert single-copy transgenes into specific genomic locations (MosSCI) or random locations (miniMos) using Mos1 transposons. Single-copy insertions allow expression at endogenous levels, expression in the germline, and identification of active and repressed regions of the genome.

**Key words** Mos1 transposon, Transgenesis, Minimal Mos1 transposon (miniMos), Mos1 single-copy insertion (MosSCI), Universal insertion sites, Germline expression, Endogenous levels of gene expression

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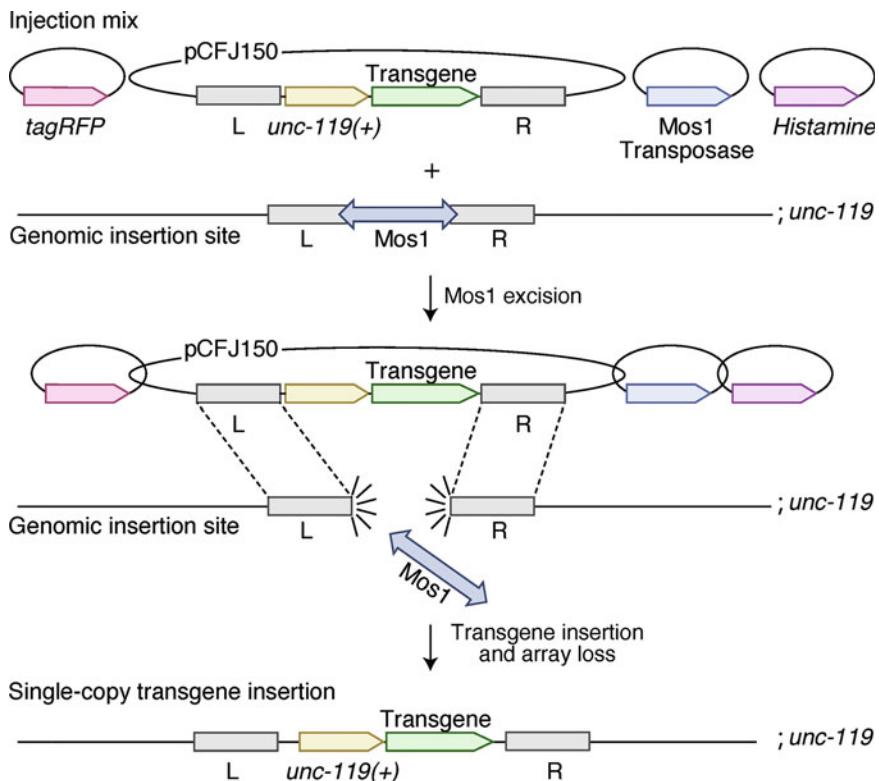
### 1 Introduction

Transgenic *C. elegans* are most often generated by injection of DNA into the gonad of adult hermaphrodites [1]. Injected plasmids form repetitive extra-chromosomal arrays that contain between 100 and 200 transgene copies. In each cell division, extra-chromosomal arrays are stochastically lost, and the DNA is, therefore, transmitted at a variable frequency to progeny. Arrays have the advantage that they are easy to generate, and transgene expression is often high, which is beneficial for fluorescence microscopy or the expression of optogenetic sensors [2]. The disadvantage of arrays is that expression often varies between different strains, and transgenes are frequently over-expressed relative to endogenous expression levels. Furthermore, expression from extra-chromosomal arrays in the germline [3] is frequently silenced through small RNA pathways [4–6]. Several alternatives to extra-chromosomal arrays have been developed to overcome these

limitations: biolistic transformation [7], low-copy transgene insertion by combined ultraviolet and trimethylpsoralen (TMP) mutagenesis [8], and Cas9-mediated genome engineering [9–12].

Here, we describe two distinct strategies that allow the insertion of single-copy transgenes using a *Mos1* transposon: *Mos1*-mediated Single-Copy Insertion (MosSCI) [13] and minimal *Mos1* transposon insertion (miniMos) [14]. The methods are based on the observation that the *Mos1* DNA transposon from *Drosophila mauritania* is active in the *C. elegans* germline [15]. Transgenic DNA can be inserted into the genome following *Mos1* excision (Fig. 1) [16, 17] or by transposition into the genome carried by a transposon (Fig. 2) [14]. For both MosSCI and miniMos, transgene insertions are generated by transient expression of the *Mos1* transposase by injection, and insertions are identified based on positive and negative selection markers [13, 14, 18, 19]. Recent MosSCI adaptations include a toolkit to generate MosSCI targeting vectors using Golden-Gate-based SapI cloning [20], selection markers that can be excised after transgene insertion [21], and novel selection markers [19].

Here, we cover the experimental steps for MosSCI and mini-Mos in a single protocol. The steps are similar and differ mainly in the use of different injection strains and cloning vectors. There are, however, important experimental considerations that favor the use of one or the other strategy (Table 1). MosSCI allows transgene insertion into a few well-defined “safe-harbor” genomic locations allowing comparison with other transgene insertions. However, transgenes can only be injected into a small set of insertion strains. In contrast, miniMos insertions can be generated in any genetic background, but different insertions are expressed at variable levels and are generally not comparable. Furthermore, miniMos insertions require a set of PCR reactions (“inverse PCR”) to map their location [22]. MosSCI relies on inserting transgenes by “copy and paste” based homologous repair, limiting insertions to approximately 10 kb and resulting in a relatively high frequency (15%) of damaged transgenes containing indels [13]. miniMos relies on “cut and paste” repair, which allows insertion of larger transgenes (at least 45 kb), and indels are very infrequently observed [14]. Both techniques are efficient, but only miniMos allows the recovery of several independent insertions from a single injection. We recommend using MosSCI when comparing different transgenes (e.g., regulatory elements), and when a stable genomic environment is required. We suggest using miniMos to generate large numbers of different insertions (e.g., fluorescent marker strains) or for the insertion of large transgenes where fidelity is crucial.



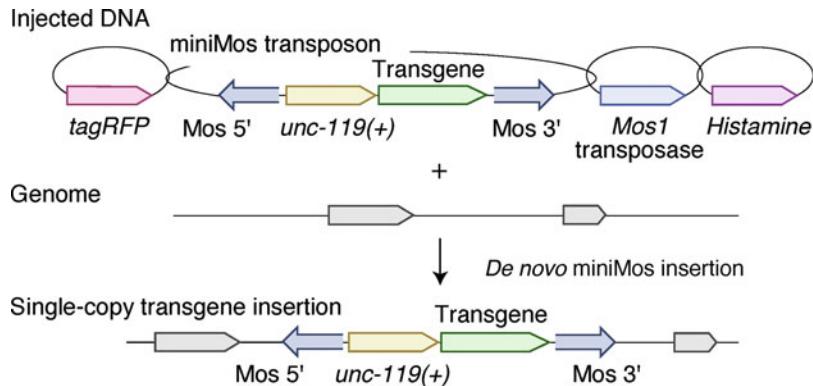
**Fig. 1** Targeted Mos1-mediated single-copy insertions (MosSCI). Targeted transgene insertion relies on generating a double-strand break (DSB) at a specific chromosomal location and a repair template that contains homology to the DSB. Mos1 mobilization by transposase injection creates the DSB. The targeting vector (here pCFJ150) contains homology to the DSB. DNA repair copies the transgene and the selection marker (*Cbr-unc-119*) into the genome. The fluorescent (*Pmlc-1::tagRFP*) and histamine selection markers (*Psnt-1::HisCl::ppl-3*) are used for visual and drug-induced selections against the array. L = left homology, R = right homology

## 2 Materials

To generate MosSCI and miniMos insertions, it is necessary to have access to and familiarity with standard *C. elegans* equipment and reagents, including NGM plates, a microinjection setup, a pipette puller to make injection needles, and a fluorescence dissection microscope. Injection strains are healthiest if maintained at 15–20 °C on NGM plates seeded with HB101 bacteria (see Note 1). Additional information about strains, plasmids, and protocols can be found at the website [www.wormbuilder.org](http://www.wormbuilder.org).

### 2.1 MosSCI Insertion Strains

- Injections into the standard MosSCI strains require matching targeting vectors with the appropriate injection strain carrying a Mos1 transposon. All strains and insertion sites are listed in Table 2 (see Note 2).



**Fig. 2 Random single-copy transgene insertions using miniMos.** The miniMos transposon is a shortened version of a Mos1 transposon, which can “hop” in the *C. elegans* germline while carrying a transgene [14]. The transgene is cloned into a miniMos transposon vector with a positive selection marker (here *cbr-unc-119(+)*). Co-injection with a plasmid expressing the Mos transposase mobilizes the transposon from extrachromosomal arrays for genome insertion at random locations. Vectors encoding *tagRFP* and a histamine-gated chloride channel are used to distinguish between rescued transgenic animals carrying extrachromosomal arrays and single-copy insertions

**Table 1**  
**MosSCI and miniMos comparison**

| Transgene            | MosSCI  | miniMos   |
|----------------------|---|---|
| Insertion site       | Validated safe harbor                                 | Random  |
| Validation           | PCR to determine transgene integrity                  | Inverted PCR to determine insertion site                  |
| Expression           | Consistent and comparable between insertions          | Variable due to position effects                          |
| Injection strain     | Strains with individual or universal Mos1 transposons | Any strain compatible with selection markers              |
| Insertion efficiency | 60%   | Several insertions possible from a single injected animal |
| Size limit           | Up to 10 kb   | Up to 45 kb   |

2. Injection into strains with universal landing sites (Table 3) is compatible with a single targeting vector (Table 4). The universal insertion strains contain a Mos1 transposon flanked by two selection markers (*NeoR* and *unc-18(+)* or *Pmyo-2::gfp::his-58*) (Fig. 3) (see Note 3). These selection markers are convenient for moving a transgene insertion into other genetic backgrounds by following neomycin resistance or pharyngeal GFP fluorescence.
3. Choose the insertion site based on whether germline expression is required and what experiments are planned (see Note 4). For example, do not choose an insertion site genetically linked

**Table 2**  
**MosSCI insertion strains**

| Locus                    | Genetic position | Chromosomal position | Strain | Germline expression |
|--------------------------|------------------|----------------------|--------|---------------------|
| <i>unc-119</i> selection |                  |                      |        |                     |
| <i>ttTi4348</i>          | I:-5.32          | I: 2.85 MB           | EG6701 | Yes                 |
| <i>ttTi4391</i>          | I:7.93           | I: 11.27 MB          | EG6702 | No                  |
| <i>ttTi5605</i>          | II:0.77          | II: 8.42 MB          | EG6699 | Yes                 |
| <i>cxTi10816</i>         | IV:1.41          | IV: 5.01 MB          | EG6703 | Yes                 |
| <i>cxTi10882</i>         | IV:-0.05         | IV: 4.24 MB          | EG6700 | Variable            |
| <i>ttTi14024</i>         | X:22.84          | X: 15.57 MB          | EG6705 | Late stages only    |
| <i>unc-18</i> selection  |                  |                      |        |                     |
| <i>ttTi4348</i>          | I:-5.32          | I: 2.85 MB           | EG6032 | Yes                 |

**Table 3**  
**Universal insertion sites**

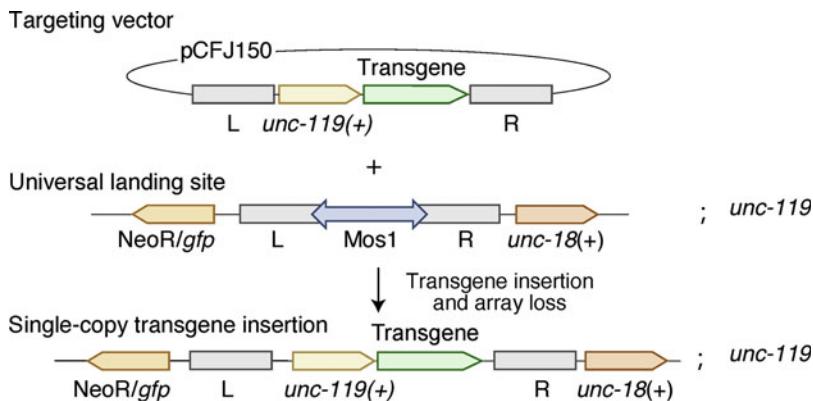
| Locus  | Genetic position | Chromosomal position | Strain | Genomic environment | Germline expression |
|--|------------------|----------------------|--------|---------------------|---------------------|
| <i>NeoR</i> and <i>unc-18</i> landing site             |                  |                      |        |                     |                     |
| <i>oxTi185</i>   | I:1.17           | I: 6.50 MB           | EG8078 | Intergenic          | Yes                 |
| <i>oxTi179</i>   | II:1.73          | II: 9.83 MB          | EG8079 | In <i>ZK938.3</i>   | Yes                 |
| <i>oxTi444</i>   | III:-0.85        | III: 7.01 MB         | EG8080 | In <i>lgc-38</i>    | Yes                 |
| <i>oxTi177</i>   | IV:7.43          | IV: 13.05 MB         | EG8081 | In <i>scl-10</i>    | Yes                 |
| <i>oxTi365</i>   | V:1.52           | V: 8.64 MB           | EG8082 | In <i>asp-13</i>    | Yes                 |
| <i>Pmyo-2::GFP::H2B</i> and <i>unc-18</i> landing site |                  |                      |        |                     |                     |
| <i>oxTi354</i>   | V:5.59           | V: 13.78 MB          | EG8083 | In <i>F53C11.3</i>  | Yes                 |

to any mutant backgrounds you plan to use with the transgene insertion.

- Request injection strains from the *Caenorhabditis elegans* Genetics Center (CGC, [www.cbs.umn.edu/research/resources/cgc](http://www.cbs.umn.edu/research/resources/cgc)).
- 2.2 MosSCI Targeting Vectors**
- Generate a targeting vector (Table 4) corresponding to the chosen insertion site. MosSCI insertions are generated by homology-based repair and each insertion site, therefore, requires a specific targeting vector carrying “left” and “right” flanking genomic DNA. The targeting plasmids (pCFJ150 and pCFJ350) are compatible with all universal insertion sites in

**Table 4**  
**MosSCI targeting vectors**

| Locus                        | Selection marker   | Three-fragment Gateway™ vector | Multiple Cloning Site vector |
|------------------------------|--------------------|--------------------------------|------------------------------|
| <i>ttTi4348</i>              | <i>Cbr-unc-119</i> | pCFJ210                        | pCFJ352                      |
| <i>ttTi4348</i>              | <i>unc-18</i>      | pCFJ448                        | pCFJ676                      |
| <i>ttTi4391</i>              | <i>Cbr-unc-119</i> | pCFJ604                        | pCFJ353                      |
| <i>ttTi5605 or Universal</i> | <i>Cbr-unc-119</i> | pCFJ150                        | pCFJ350                      |
| <i>cxTi10816</i>             | <i>Cbr-unc-119</i> | pCFJ212                        | pCFJ356                      |
| <i>cxTi10882</i>             | <i>Cbr-unc-119</i> | pCFJ201                        | pCFJ351                      |
| <i>ttTi14024</i>             | <i>Cbr-unc-119</i> | pCFJ606                        | pCFJ355                      |



**Fig. 3 Schematic of Universal MosSCI sites.** The universal MosSCI system relies on a set of landing sites that were introduced into locations across the genome. Because the sequence flanking the Mos1 transposon is identical for all insertion sites, the same targeting vectors (pCFJ150 and pCFJ350) can be used for insertion. The universal landing sites furthermore contain a selection marker adjacent to the insertion sites (NeoR or *Pmyo-2::gfp*) that facilitates following a MosSCI insertion in genetic crosses. L = left homology, R = right homology

addition to the *ttTi5605* (Chr. II) insertion site (Table 3 and Table 4). Vectors for three-fragment Gateway multisite cloning™ (Life Technologies) or standard restriction fragment cloning are available from Addgene ([www.addgene.org/Erik\\_Jorgensen/](http://www.addgene.org/Erik_Jorgensen/)). Use standard molecular biology protocols to insert the transgene of choice into the targeting vector.

2. Make transfection grade DNA preparations of the targeting vector(s) (see Note 5).

### 2.3 miniMos Targeting Vectors

1. Generate a targeting vector corresponding to the genetic or antibiotic selection marker of choice (Table 5). Vectors

**Table 5**  
**miniMos transposition vectors**

| Selection marker | Doublet counterselection | Three-fragment Gateway™ vector | Multiple Cloning Site vector |
|------------------|--------------------------|--------------------------------|------------------------------|
| <i>unc-119</i>   | None                     | pCFJ906                        | pCFJ909                      |
| <i>unc-119</i>   | hs:: <i>peel-1</i>       | pCFJ1001                       | pCFJ1201                     |
| <i>NeoR</i>      | None                     | pCFJ907                        | pCFJ910                      |
| <i>NeoR</i>      | hs:: <i>peel-1</i>       | pCFJ1002                       | pCFJ1202                     |
| <i>PuroR</i>     | None                     | pCFJ908                        | pCFJ1666                     |
| <i>PuroR</i>     | hs:: <i>peel-1</i>       | pCFJ1000                       | pCFJ1200                     |
| <i>HygroR</i>    | None                     | pCFJ1655                       | pCFJ1662                     |
| <i>HygroR</i>    | hs:: <i>peel-1</i>       | pCFJ1656                       | pCFJ1663                     |

containing the negative selection marker *peel-1* flanking the miniMos transposon can be used to select against dual inserts (see Note 6). Request vectors from Addgene ([www.addgene.org/Erik\\_Jorgensen/](http://www.addgene.org/Erik_Jorgensen/)) and use standard molecular biology protocols to insert transgenes into the targeting vector.

#### 2.4 Co-injection Markers

- Co-injection markers are required to induce transient expression of Mos1 transposase and to distinguish animals with extra-chromosomal arrays from strains with single-copy insertions. We use either red or green fluorescent markers for identifying animals with arrays (Table 6). Request vectors from Addgene ([https://www.addgene.org/Christian\\_Frockjaer-Jensen/](https://www.addgene.org/Christian_Frockjaer-Jensen/)).
- Make transfection grade DNA preparations of all co-injection markers.
- Make a 2× red co-injection mix: 20 ng/μL pCFJ1532 (*Psmu-1::mos1* transposase) (see Note 7), 10 ng/μL pSEM233 (*Pmlc-1::tagRFP-T*) (see Note 8), 10 ng/μL pSEM238 (*Psnt-1::HisCl::rpl-3*) (see Note 9). Make 2× stock solution and store at –20 °C.
- Make a 2× green co-injection mix: 20 ng/μL pCFJ1532 (*Psmu-1::mos1* transposase), 10 ng/μL pSEM231 (*Pmlc-1::GFP*), 10 ng/μL pSEM238 (*Psnt-1::HisCl::rpl-3*). Make 2× stock solution and store at –20 °C.

**Table 6**  
**Co-injection markers**

| Plasmid                          | Description  | Transgene  | Addgene ID |
|----------------------------------|--|--|------------|
| pCFJ1532                         | Mobilizes Mos1 transposon  | <i>Psmu-1::</i><br>Mos1 <sub>PATC</sub><br>transposase | #159807    |
| pSEM238                          | Negative selection, histamine-induced paralysis,<br>pan-neuronal expression  | <i>Psnt-1::histamine</i>                               | #161515    |
| <i>Red fluorescent markers</i>   |  |  |            |
| pSEM233                          | Visual array marker: Red, pharyngeal muscles, body wall<br>muscle, vulval muscles, stomato-intestinal muscles, anal<br>depressor   | <i>Pmlc-1::TagRFP-</i><br><i>T</i>                     | #159899    |
| <i>Green fluorescent markers</i> |  |  |            |
| pSEM231                          | Visual array marker: Green, pharyngeal muscles, body wall<br>muscle, vulval muscles, stomato-intestinal muscles, anal<br>depressor | <i>Pmlc-1::GFP</i>                                     | #159897    |

### 3 Methods

#### 3.1 MosSCI and miniMos Injections

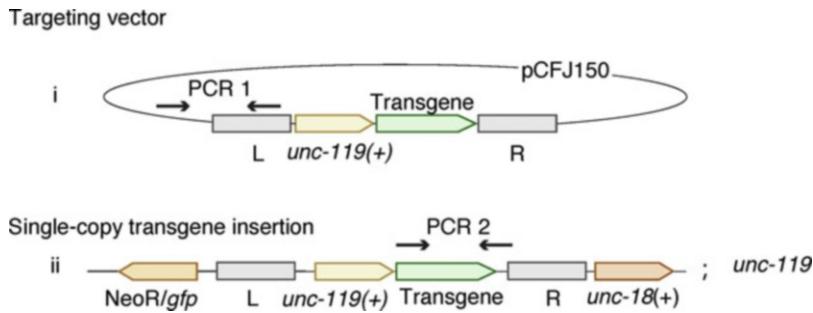
1. Centrifuge the 2× co-injection mix and the targeting vector at the highest speed on a tabletop microcentrifuge for 2 min.
2. Make 20 µL injection solution by mixing 10 µL of the 2× - co-injection mix (*see Note 10*) with the targeting vector to a final concentration of 10–50 ng/µL. Bring the final concentration of DNA up to 100 ng/µL with a DNA ladder without dye or another inert stuffer plasmid. Add molecular grade purified water to a final volume of 20 µL.
3. Immediately before injection, centrifuge the injection mix at the highest speed on a tabletop microcentrifuge for 2 min to prevent clogging injection needles.
4. Pick relatively healthy, unstarved, and young adult hermaphrodites for injection from strains maintained at 15–20 °C (*see Note 11*, if injecting into *unc-119* animals). Pick animals from the strain corresponding to the MosSCI targeting vector (Tables 2 and 3) or any strains compatible with the selection marker for miniMos.
5. Inject animals with the injection mix by following standard injection protocols [1] (*see Note 12*).
6. Place one to three injected animals on individual NGM plates seeded with OP50 or HB101. Place NGM plates with injected animals in a 25 °C incubator (*see Note 13*).

### 3.2 Isolation of Transgenic Animal with Single-Copy Insertion

- After approximately 1 week, the injected animals will have exhausted the food on the plate and can be screened for insertions (*see Note 14*).
- Add 500 µL of histamine (500 mM) to 6 cm NGM plates with starved animals and leave with the lid off to dry for 15 min. Animals with extra-chromosomal arrays are paralyzed by histamine, whereas animals with transgene insertions move normally.
- Screen plates for transgene insertions within 2 h of adding histamine with a fluorescence dissection microscope (*see Note 15*). Moving animals with transgene insertions are rescued for the mutant phenotype (*unc-119* or *unc-18*) or survive on antibiotic selection (e.g., hygromycin or G418) but do not contain the fluorescent co-injection marker.
- Pick 3–4 animals from each plate with a transgene insertion to a single NGM plate seeded with OP50 (*see Note 16*).
- Two to three days later, verify that the selected animals do not express the fluorescent co-injection marker and pick a single, clonal animal with eggs to a new plate from each plate. Only pick one insertion from each plate to ensure that all insertions are independent and treat each insertion as an independent allele (*see Note 17*).
- In the following generation, pick 4–8 clonal animals to individual plates to establish a homozygous strain for the insertion (*see Note 18*).

### 3.3 Validation of Single-Copy Transgene Insertion

- If the inserted transgene contains a fluorescent marker (e.g., *gfp*), check each independent strain for fluorescence (*see Note 19*).
- Some insertions contain two copies of the transgene (at the same location). Single-copy insertions can be validated by PCR amplification on genomic DNA. Oligos designed to amplify the junction between the plasmid backbone and the homology regions that flank the transgene can be used to identify dual inserts (Fig. 4) (*see Note 20*). Use the targeting vector as a positive control and N2 genomic DNA as a negative control for the PCR reaction. Single-copy insertions do not contain the plasmid backbone and should, therefore, not result in a visible amplification band.
- Validate intact, full-length transgene insertions by PCR amplification across the entire transgene, either as a single amplicon or as separate amplicons using oligos that do not anneal in non-transgenic animals (*see Note 21*). Use N2 genomic DNA as a negative control for PCR reactions.



**Fig. 4 Validation of MosSCI insertions.** Approximately 15% of MosSCI insertions contain one of two errors, either dual insertion of the transgene or indels within the transgene. Full-length, single-copy insertions are validated using two strategies: (i) A PCR designed to detect the junction between the targeting vector's bacterial backbone and the homology region. This DNA product should not be present in the final strain, and the presence of a PCR band, therefore, indicates a dual insertion. (ii) PCR amplification of the inserted transgene. Indels are typically large enough (approx. 1 kb [13]) to be detected by gel electrophoresis. For large transgenes, splitting the PCR into several smaller amplifications will facilitate the detection of aberrant insertions. L = left homology, R = right homology

4. Give each validated MosSCI or miniMos strain an allele name using the “Si” nomenclature corresponding to the lab allele designation (*see Note 22*).
- 3.4 Identification of miniMos Insertion Site**
1. miniMos insertions are random, and the transgene insertion site can be identified by inverse PCR (Fig. 5) [22], if necessary (*see Note 23*). Use filtered pipette tips for all steps to avoid cross-contamination.
  2. Isolate genomic DNA from a strain carrying a miniMos insertion using a standard kit for isolating genomic DNA (*see Note 24*).
  3. Digest 150 ng of genomic DNA with the DpnII restriction enzyme for at least 3 h at 37 °C (*see Note 25*).

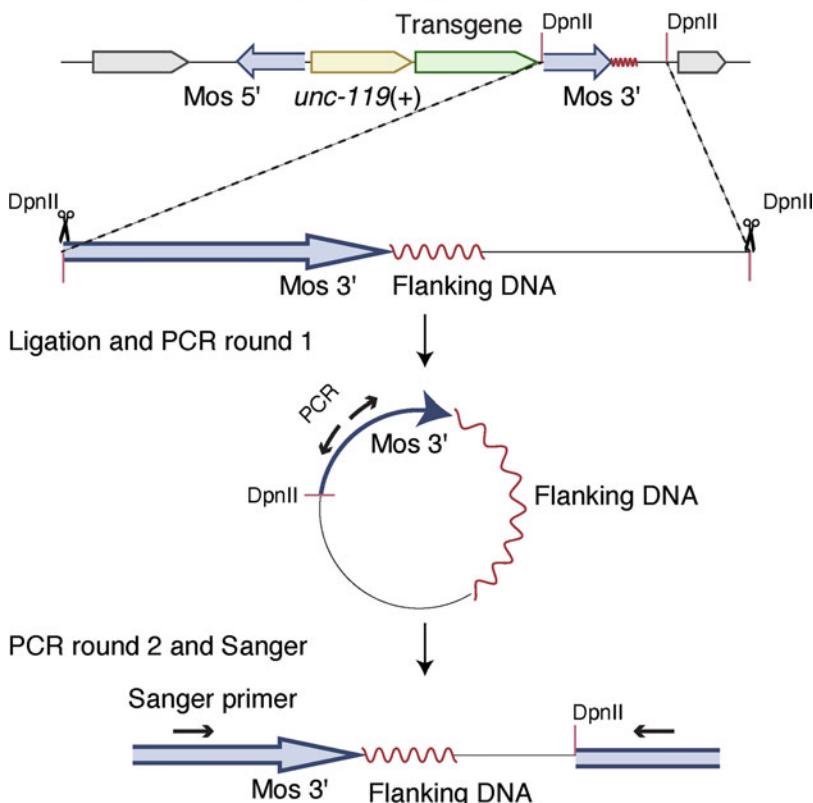
*Reaction mix:*

|                                 |                                  |
|---------------------------------|----------------------------------|
| Genomic DNA                     | 150 ng                           |
| Restriction enzyme buffer (10×) | 2.5 µL                           |
| DpnII (10 U/µL)                 | 1.0 µL                           |
| Deionized water                 | Add for a total volume of 25 µL. |

Heat-inactivate the reaction mix at 80 °C for 20 min.

4. Ligate 2.5 µL of the digested DNA in a 25 µL reaction with T4 ligase for 2 h at room temperature (20 °C) (*see Note 26*).

## Genomic DNA isolation and DpnII digest



**Fig. 5** Identifying miniMos insertion sites by inverse PCR. Random miniMos insertions can be mapped by molecular methods. Although mapping is not strictly necessary, it is often convenient to know where the transposon is inserted and to assess effects on nearby endogenous genes. The approach (“inverse PCR”) takes advantage of primers that anneal in the known transposon sequence to identify the flanking genomic sequence. This strategy relies on two rounds of PCR on circular DNA generated from genomic DNA from an insertion strain. A PCR template is generated by digesting DNA with a restriction enzyme (here, DpnII) that cuts in the transposon and at the nearest (unknown) location in the flanking genomic, and ligating the fragment to form a circle. Two rounds of nested PCR are required to amplify a PCR fragment that can be Sanger sequenced to identify the genomic DNA flanking the transposon insertion. The exact insertion site can be determined by a BLAST search against the *C. elegans* genome

Reaction mix:

|                          |         |
|--------------------------|---------|
| Digested genomic DNA     | 2.5 µL  |
| T4 ligation buffer (10×) | 2.5 µL  |
| T4 ligase (2000 U/µL)    | 1.0 µL  |
| Deionized water          | 19.0 µL |

The inverse PCR reactions can be paused at this step and stored at -20 °C.

5. Perform the first PCR reaction to amplify the Mos1 transposon and flanking DNA (*see Note 27*).

Reaction mix (10 µL total):

|                                       |        |
|---------------------------------------|--------|
| Ligation mix                          | 2.0 µL |
| Primer oCF1587 (10 µM)                | 1.0 µL |
| Primer oCF1588 (10 µM)                | 1.0 µL |
| dNTPs (10 mM)                         | 0.2 µL |
| DNA polymerase 5× GC buffer           | 2.0 µL |
| High-Fidelity DNA polymerase (2 U/µL) | 0.1 µL |
| Deionized water                       | 3.7 µL |

PCR conditions: 98 °C for 2 min, 30 amplification cycles (98 °C for 30 s, 64 °C for 20 s, 72 °C for 1 min).

6. Perform a second PCR reaction to amplify the Mos1 transposon and flanking DNA (*see Note 28*). Dilute the PCR reaction from the previous step by 100-fold.

Reaction mix (25 µL total):

|                                       |         |
|---------------------------------------|---------|
| PCR reaction 1 (100× diluted)         | 1.0 µL  |
| Primer oCF1589 (10 µM)                | 2.5 µL  |
| Primer oCF1590 (10 µM)                | 2.5 µL  |
| dNTPs (10 mM)                         | 0.5 µL  |
| DNA polymerase 5× GC buffer           | 5.0 µL  |
| High-Fidelity DNA polymerase (2 U/µL) | 0.2 µL  |
| Deionized water                       | 13.3 µL |

PCR conditions: 98 °C for 2 min, 30 amplification cycles (98 °C for 30 s, 64 °C for 20 s, 72 °C for 1 min).

7. Perform gel electrophoresis of the PCR product on a 1% agarose gel. Gel purify a single band, and Sanger sequence the band with the oligo oCF1590 (*see Note 29*).
8. Determine the genomic insertion site of the miniMos transposon. Align the Sanger sequencing read with the *C. elegans* genome using Blast ([https://wormbase.org/tools/blast\\_blat](https://wormbase.org/tools/blast_blat)). The miniMos transposon inserts into a TA dinucleotide, and the genomic insertion site will be flanked by the miniMos inverted terminal repeat sequence (*see Note 30*).

---

## 4 Notes

1. *unc-119* and *unc-18* animals propagated on HB101 bacteria are healthier than mutant animals propagated on standard OP50 bacteria. HB101 bacteria are available from the *Caenorhabditis elegans* Genetics Center (CGC). Injections into relatively healthy animals are much more efficient. Therefore, when using mutant strains, we take care to maintain relatively healthy animals by picking three individual adult animals to three different spots on a recently seeded HB101 plate daily. Propagating mutant animals at 15–18 °C further increases their health. The progeny from these picked animals are ready for injection after approx. 3–5 days.
2. We recommend the *ttTi5605* (Chr. II) and *ttTi4348* (Chr. I) as these are the most commonly used insertion sites.
3. We recommend using the universal insertion sites when an experiment requires several insertions at different locations due to the ease of using a single targeting vector. However, it is slightly more challenging to generate insertions into the universal landing sites, and the additional transgenes in the strains (NeoR and *unc-18(+)*) are not always desirable.
4. Expression in the germline was tested with the ubiquitous *dpy-30* promoter driving a *gfp::his-58* transgene.
5. Preparation of injection plasmids (co-injection plasmids as well as targeting vectors) with a plasmid purification method that yields transfection grade plasmids significantly improves the number of F1 progeny generated from each injection [14]. We routinely use the kit PureLink HQ miniPlasmid DNA Purification kit (Life Technologies).
6. Approximately 10% of miniMos insertions contain two transgenes inserted into the same genomic locus (“doublets”). If necessary, the negative selection marker *peel-1* can be used to select against animals with doublets by killing animals with dual inserts using a heat-shock protocol (1 h at 34 °C). In other cases, higher expression from two copies may be desirable.
7. Transgenes with non-coding Periodic A<sub>n</sub>/T<sub>n</sub> Clusters (PATCs) in introns are resistant to germline silencing [23] and generate MosSCI insertions at higher frequency [24].
8. We previously used a mix of three different fluorescent co-injection markers but have recently developed single co-injection markers based on the strong pan-muscular promoter *Pmlc-1*. These new co-injection markers are bright, non-toxic, and effectively avoid selecting false-positive insertions [19].

9. We recommend using a negative selection marker based on the addition of histamine [25, 26] instead of the previously described *peel-1* negative selection marker [18]. *peel-1* selection has variable penetrance and results in frequent false-positives that can be distinguished by the presence of the fluorescent co-injection marker(s). With histamine selection, we rarely observe false-positives, and these insertions can be detected without the use of a fluorescent dissection microscope.
10. We recommend using co-injection markers that are not homologous to the transgene; for example, for a *gfp*-tagged transgene, we recommend using the *tagRFP-T* marker. The repair process, which copies the transgene into the genome, is likely to be inhibited by the presence of several different homologous templates in the injection mix.
11. To facilitate freezing and handling, the injection strains contain an extra-chromosomal array with an *unc-119* rescue marker. This array needs to be lost before MosSCI injections and injections should be performed into *unc-119* mutant animals.
12. The number of injected animals necessary to generate a single MosSCI insertion will depend on the transgene (for example, larger transgenes >10 kb are more challenging to insert) and how experienced the researcher is at injecting. In our experience, insertions are generated in 10–50% of injected animals, but for researchers with less experience, the insertion frequency is often closer to 1–5%. Adjust the number of injections depending on transgene size and experience with injections.
13. The MosSCI insertion frequency is not temperature-dependent [14], but there are two advantages to placing animals at 25 °C. First, the animals develop faster and can be screened for insertions faster. Second, a higher percentage of MosSCI insertions are expressed in the germline when the injected animals are placed at 25 °C [23]. Even genotypically identical insertions do not necessarily express the transgene in the same way in the germline because of stochastic silencing by small RNAs [27].
14. It is possible to screen plates for insertions before the food is exhausted, but it is much more challenging to identify inserts by visual inspection. Premature addition of the negative selection (histamine) will arrest young F1 animals that may otherwise give insertions in the F2 generation. We, therefore, advise against early screening.
15. We generated a plasmid that allows negative selection using histamine-induced paralysis. Histamine selection is based on neuronal inactivation using a histamine-gated chloride channel HisCl1 from *Drosophila* [25]. For negative selection, we generated a selection plasmid with no fluorescent markers

(pSEM238, *Psnt-1::HisCl::rpl-3*). Worms with arrays containing pSEM238 are paralyzed within 15 min after exposure to 25 mM histamine with very few false-positives.

16. Following injection and propagation at 25 °C, animals show a relatively high incidence of sterility. Picking a single L1 animal with a transgene insertion is, therefore, sometimes not enough to establish a line. Instead, we pick several L1 animals from the same plate and only select a clonal animal when they have become adults, and the absence or presence of eggs in the animal is obvious.
17. When the Mos1 element is excised, the transposon sometimes inserts into another genomic location. So even if each strain contains the same transgene insertion, it is possible that the genetic backgrounds are different.
18. There is a strong selection towards homozygosity of the transgene insertion, so the longer a mixed population is propagated, the higher the proportion of homozygous animals will be. If possible, propagate animals for several generations before picking individual animals to minimize the effort and reagents used.
19. Single-copy insertions are often dimmer than extra-chromosomal arrays, and the strains should therefore be checked for fluorescence on a microscope with high magnification.
20. For a detailed protocol with oligos and examples, please see <https://sites.wustl.edu/nonetlab/protocols-2/>.
21. Approximately 15% of MosSCI insertions are either dual insertions or imperfect insertions that contain indels. In the absence of phenotypic rescue or consistent GFP fluorescence between several independent alleles, it is therefore important to validate every MosSCI insertion.
22. Alleles are designated by the lab-specific designation followed by “*Si*.” For example, our laboratory’s allele designation is “*kst*,” and all MosSCI alleles are therefore named *kstSixx*, where xx is the allele number. For detailed naming rules, please see <https://wormbase.org//about/userguide/nomenclature>.
23. It is not always necessary to identify the miniMos insertion sites. However, it is often convenient for genetic crosses to know what chromosome the insertion is on and to verify that the insertion does not impair a gene involved in a genetic process that may impact experiments.
24. We grow animals on large NGM plates and use one or two plates per genotype. Harvest animals for genomic DNA isolation shortly before the food is exhausted. We use a genomic DNA isolation kit with beads to disrupt worms for medium-

throughput DNA isolation, but any commercial kit can be used. Crude DNA from lysates generated by repeated freeze/thaw cycles and proteinase K treatment gives very variable results in our hands and is not recommended.

25. The DpnII restriction enzyme recognizes a four basepair sequence, which is a common enough DNA sequence to cut the miniMos transgene and at a short distance in the nearby flanking genomic region. The protocol can also be performed using the HpaII restriction enzyme.
26. The ligation reaction is performed at high dilution to favor intramolecular ligation instead of ligation between different restriction fragments.
27. Order oligos with standard desalting. oCF1587 (5' to 3'): AT AGTTGGCGCGAATTGAG and oCF1588: GGTGGTTC GACAGTCAAGGT.
28. oCF1589 (5' to 3'): AGAGCAAACGCGGACAGTAT and oCF1590: CGATAAAATATTACGTTGCGAGAC.
29. Only excise one band from each reaction and do not excise bands that are not distinct or when the gel shows a smear. Only excise bands that are larger than 100 bp, otherwise the flanking sequence will not be long enough to identify the insertion site. If the only band is shorter than 100 bp, then redo the reaction with HpaII.
30. Search the Sanger sequence read for the following sequence: 5' - ACATTCATACTTGTACACCTGA - 3' (miniMos sequence). Allow for two mismatches to accommodate poor sequence calls. The sequence adjacent to the 3' end specifies the flanking genomic region and can be identified by a Blast search against the *C. elegans* genome.

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# Chapter 13

## Mutation Mapping and Identification by Whole-Genome Sequencing

Harold E. Smith

### Abstract

Geneticists approach biology with a simple question: which genes are required for the pathway or process of interest? Classical genetic screens (aka forward genetics) in model organisms such as *Caenorhabditis elegans* have been the method of choice for answering that question. Next-generation sequencing provides the means to generate a comprehensive list of sequence variants, including the mutation of interest. Herein is described a workflow for sample preparation and data analysis to allow the simultaneous mapping and identification of candidate mutations by whole-genome sequencing in *Caenorhabditis elegans*.

**Key words** Sequence variant, Polymorphism mapping, NGS library construction, Whole-genome sequencing, Mutation analysis

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### 1 Introduction

For more than a century, researchers have used classical genetics (random mutagenesis and phenotypic screening) in model species to identify the genes involved in biological pathways [1, 2]. The availability of both self and outcross modes of reproduction makes *Caenorhabditis elegans* ideally suited for such genetic screens. Historically, the ability to generate interesting mutations in worms has greatly exceeded the ability to identify causative sequence variants: dozens or even hundreds of alleles might be recovered in a single mutant screen (e.g., [3, 4]). However, the application of next-generation sequencing technology (NGS) to mutation identification in *C. elegans* has largely eliminated this bottleneck [5–7]. Whole-genome sequencing provides a catalog of all sequence variants within the strain of interest. By crossing the strain into a highly polymorphic genetic background, both positional information and novel mutations can be obtained [8, 9]. Candidate genes identified in this manner can be validated easily by secondary

screening such as RNAi phenocopy [10] or CRISPR-mediated genome engineering [11].

The workflow for sample preparation for whole-genome sequencing consists of the following steps: (1) crossing the mutation-bearing strain to a polymorphic strain and picking homozygous F2 progeny; (2) isolating and shearing the genomic DNA (gDNA); and (3) constructing a sequencing library from the sheared gDNA sample. The indicated protocol produces sequence-ready libraries and is designed to accommodate phenotypes (e.g., nonconditional lethality or sterility) that are typically difficult to analyze. The only limitation is that the allele of interest be recessive, so that homozygous and heterozygous segregants can be distinguished. Sequencing per se is not described, as this service is typically performed by a core facility.

Each step in this workflow is subject to considerable variation. The protocol is designed for monogenic recessive alleles, but can be adapted for more complex variants [12]. Hawaiian strain CB4856 is the polymorphic strain used for the mapping cross, but other strains can be employed [e.g., 13]. The picking of F2 progeny (step 1) will be determined by the mutant phenotype in question. The gDNA isolation (step 2) is intended for small numbers of worms, but can be used for bulk samples as well. The library construction protocol (step 3) is specific to short-read ( $\sim 10^2$  bases) sequencing on an Illumina instrument (the most common and cost-effective platform for this application); the use of other sequencers will necessitate platform-specific sample preparation. In all cases, the reader is referred to the Notes section for relevant parameters to consider when using alternative methods.

The workflow for data analysis consists of the following steps: filtering to remove low-quality and contaminating data; alignment to the reference genome; post-processing of the aligned data; variant calling; extraction of Hawaiian SNPs for mapping; and annotation of novel variants to identify candidate mutations. The software pipeline described here performs well for *C. elegans* mutation mapping [14], but requires a computer running the Linux operating system plus some familiarity with command-line instructions. For users who prefer a web-based environment, the public Galaxy platform [15] provides a variety of software tools for building a custom workflow. It also offers MiModD [16], a beginning-to-end software pipeline for variant detection.

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## 2 Materials

### 2.1 Worm Growth and Mating

1. Dissecting microscope.
2. Worm pick (*see Note 1*).
3. NGM plates: In 2 L flask, mix 3 g NaCl, 17 g agar, 2.5 g peptone, and 975 mL H<sub>2</sub>O. Autoclave to sterilize. Cool to

55 °C. Add 1 mL cholesterol (5 mg/mL in ethanol; do not autoclave), 1 mL 1 M CaCl<sub>2</sub>, 1 mL 1 M MgSO<sub>4</sub>, and 25 mL 1 M KPO<sub>4</sub>, pH 6.0 (108.3 g KH<sub>2</sub>PO<sub>4</sub>, 35.6 g K<sub>2</sub>HPO<sub>4</sub>·H<sub>2</sub>O, 1 L H<sub>2</sub>O) (*see Note 2*). Dispense 14 mL into 6 cm petri plates. Cool plates to room temperature. Seed plates with ~50 µL OP50 culture (grown overnight in LB medium at 37 °C). Incubate seeded plates overnight at room temperature (*see Note 3*).

4. *E. coli* strain OP50 (*see Note 4*).
5. *C. elegans* strain CB4856 (*see Note 4*).

## **2.2 gDNA Isolation**

1. M9 buffer: Mix 3 g KH<sub>2</sub>PO<sub>4</sub>, 6 g Na<sub>2</sub>HPO<sub>4</sub>, 5 g NaCl, and 1 mL 1 M MgSO<sub>4</sub> in 1 L H<sub>2</sub>O; autoclave to sterilize.
2. TE buffer: Mix 10 mL 1 M Tris–HCl, pH 8.0 and 2 mL 0.5 M EDTA, pH 8.0 in 988 mL H<sub>2</sub>O; autoclave to sterilize.
3. Worm lysis buffer: Mix 100 µL 1 M Tris–HCl (pH 8.0), 20 µL 5 M NaCl, 100 µL 0.5 M EDTA, 125 µL 10% (v/v) SDS, and 655 µL H<sub>2</sub>O.
4. Proteinase K, 10 mg/mL concentration.
5. RNase A, 10 mg/mL concentration.
6. Sonicating water bath.
7. Low elution volume PCR purification kit.

## **2.3 Library Construction**

1. DNA library prep kit for NGS.
2. Multiplex oligos for NGS.
3. EB: 10 mM Tris–HCl, pH 8.0 or 8.5.
4. Paramagnetic beads for high-throughput purification.
5. Magnetic tube stand.
6. 80% (v/v) ethanol; prepare fresh immediately before use.

## **2.4 Data Analysis**

1. Computer workstation with (minimum) eight cores, 16 Gb RAM, 1 Tb hard drive storage.
2. Linux-type operating system (popular options include CentOS, Ubuntu, or the commercial distribution Redhat Enterprise Linux).

## **3 Methods**

### **3.1 Mating and F2 Selection (See Note 5)**

1. Using a dissecting microscope and worm pick, transfer two to three young adult hermaphrodites containing the mutation of interest to a NGM plate seeded with OP50. Pick 10–12 adult males of strain CB4856 to the same plate. Allow to mate overnight at room temperature (*see Note 6*).

2. Transfer each mated hermaphrodite to an individual fresh-seeded NGM plate. Incubate at room temperature until the F1 progeny begin to reach the L4/young adult stages (~4 days) (*see Note 7*).
3. Pick 10–12 F1 L4/young adult hermaphrodites to a fresh-seeded NGM plate. Incubate at room temperature until F2 progeny can be scored for the mutant phenotype (*see Note 8*).

### **3.2 gDNA Isolation**

1. Pick homozygous F2 progeny into a 1.5 mL centrifuge tube containing 500 µL M9 (*see Note 9*).
2. Vortex briefly (3–5 s), then spin 60 s at 1300 RCF.
3. Remove most of the M9 by pipette, taking care to avoid the pellet.
4. Resuspend in 500 µL M9 and repeat the wash at least four times (*see Note 10*).
5. Perform a final wash with 500 µL TE; spin 1 min at top speed; remove TE, leaving ~100 µL (*see Note 11*).
6. Add 400 µL worm lysis buffer to the worm sample; mix briefly.
7. Sonicate with the BioRuptor using the following settings: high power; 30 s on/30 s off; 2 × 15 min sonication time (*see Note 12*).
8. Add 50 µL proteinase K; mix well; incubate 1 h at 65 °C, vortexing briefly at 10–15 min intervals to maintain suspension (*see Note 13*).
9. Add 20 µL RNase A; incubate 30 min at 37 °C.
10. Purify sheared gDNA using a low elution volume purification column per the manufacturer’s protocol; the final elution volume is 10 µL (*see Notes 14 and 15*).

### **3.3 Library Construction**

#### **3.3.1 End Prep Reaction**

1. Mix in 0.5 mL tube (*see Note 16*): 10 µL sheared gDNA input, 40 µL EB or water, 3 µL 10× End Prep Enzyme Mix, 7 µL End Prep Reaction Buffer.
2. Incubate in a thermal cycler: 30 min at 20 °C; 30 min at 65 °C; Hold at 4 °C.

#### **3.3.2 Adapter Ligation Reaction**

1. Dilute the adapter immediately before use (*see Note 17*).
2. Add the following to the End Prep reaction and mix (*see Note 16*): 30 µL Ligation Master Mix, 1 µL Ligation Enhancer, 2.5 µL diluted adapter.
3. Incubate in a thermal cycler (the heated lid should be off): 15 min at 20 °C.
4. Add 3 µL USER enzyme and mix well.

5. Incubate in a thermal cycler (the heated lid should be on): 15 min at 37 °C; Hold at 4 °C (*see Note 18*).

**3.3.3 Paramagnetic-Bead Clean-up (See Note 19)**

1. Remove the reaction tube from the thermal cycler; add 87 µL beads; mix well by vortexing (*see Note 20*).
2. Incubate the reaction tube 5 min on the benchtop.
3. Place the reaction tube in the magnetic stand; incubate 5 min; remove and discard the supernatant (*see Note 21*).
4. Leave the reaction tube in the magnetic stand; add 200 µL freshly prepared 80% (v/v) ethanol; incubate 30 s; carefully remove and discard the supernatant.
5. Repeat **step 4** once, for a total of two washes. If necessary, spin briefly, return to the magnetic stand, and remove residual ethanol with a 10 µL pipet.
6. Air-dry the beads in the open tube for 5 min on the magnetic stand.
7. Add 17 µL EB to elute the DNA; pipette or vortex to resuspend the bead pellet; incubate for 2 min on the benchtop; return to the magnetic stand for 5 min; transfer 15 µL of supernatant (containing the DNA) to a new 0.5 mL tube (*see Note 18*).

**3.3.4 PCR Amplification**

1. Add the following to the DNA sample and mix: 25 µL PCR Master Mix, 5 µL index primer, 5 µL universal PCR primer.
2. Incubate in a thermal cycler. One cycle of: 30 s at 98 °C; Multiple cycles (*see Note 22*) of: 10 s at 98 °C, 75 s at 65 °C; One cycle of: 5 min at 65 °C; Hold at 4 °C.

**3.3.5 Final Paramagnetic Bead Clean-up (See Note 23)**

1. Follow the protocol above (Subheading 3.3.3) with the following differences: In **step 1**, add 45 µL beads; in **step 7**, add 35 µL EB; transfer 30 µL to a new tube. This purified library can be stored indefinitely at –20 °C (*see Note 24*).

**3.4 Data Analysis**

**3.4.1 Installation and Configuration (See Note 25)**

1. Download and install the following software tools from the indicated links (*see Notes 26 and 27*): BBMap: <https://sourceforge.net/projects/bbmap/>; SAMtools and BCFtools: <http://www.htslib.org/download/>; FreeBayes: <https://github.com/ekg/freebayes>; BEDtools: <https://bedtools.readthedocs.io/en/latest/>; ANNOVAR: <https://annovar.openbioinformatics.org/en/latest/user-guide/download/>; gtfToGenePred: [http://hgdownload.cse.ucsc.edu/admin/exe/linux.x86\\_64/](http://hgdownload.cse.ucsc.edu/admin/exe/linux.x86_64/)
2. Download copies of *C. elegans* datasets at <https://www.wormbase.org>. Select “Downloads,” then “Public FTP Site,”

connect as “Guest” if prompted, and follow the directory path “releases/WS278/species/c\_elegans/PRJNA13758”.

For the reference genome (*see Note 28*) in FASTA format, select:

“c\_elegans.PRJNA13758.WS278.genomic.fa.gz”.

For the gene list in GTF format, select:

“c\_elegans.PRJNA13758.WS278.canonical\_geneset.gtf.gz”.

For the list of common gene names, select:

“annotation/c\_elegans.PRJNA13758.WS278.geneIDs.txt.gz”.

3. Download a copy of the *E. coli* reference genome (used to remove contaminating sequences derived from the OP50 food source) at [https://bacteria.ensembl.org/Escherichia\\_coli\\_str\\_k\\_12\\_substr\\_mg1655/Info/Index](https://bacteria.ensembl.org/Escherichia_coli_str_k_12_substr_mg1655/Info/Index). Select “Download DNA Sequence (FASTA),” connect as “Guest” if prompted, and then select “Escherichia\_coli\_str\_k\_12\_substr\_mg1655.ASM584v2.dna.chromosome.Chromosome.fa.gz”.
4. Download a copy of the Hawaiian SNPs in VCF format at <https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0174446.s009&type=supplementary>
5. Create a directory for the downloaded reference files, then extract and move/rename them (*see Note 29*).

```
$ mkdir /PATH/TO/reference_genomes
$ mkdir /PATH/TO/reference_genomes/WS278_ref
$ gunzip c_elegans.PRJNA13758.WS278.genomic.fa.gz
$ mv c_elegans.PRJNA13758.WS278.genomic.fa \
/PATH/TO/reference_genomes/WS278_ref/Celegans.fa
$ gunzip c_elegans.PRJNA13758.WS278.canonical_geneset.
gtf.gz
$ mv c_elegans.PRJNA13758.WS278.canonical_geneset.gtf \
/PATH/TO/reference_genomes/WS278_ref/Celegans_geneset.
gtf
$ gunzip c_elegans.PRJNA13758.WS278.geneIDs.txt.gz
$ mv c_elegans.PRJNA13758.WS278.geneIDs.txt \
/PATH/TO/reference_genomes/WS278_ref/Celegans_geneIDs.
txt
$ gunzip Escherichia_coli_str_k_12_substr_mg1655.
ASM584v2.dna.chromosome.Chromosome.fa.gz
$ mv Escherichia_coli_str_k_12_substr_mg1655.ASM584v2.
dna.chromosome.Chromosome.fa \
/PATH/TO/reference_genomes/Ecoli.fa
$ mv journal.pone.0174446.s009.txt /PATH/TO/reference_-
genomes/Hawaiian_snps.vcf
```

6. Index the *C. elegans* reference genome for use with SAMtools.

```
$ cd /PATH/TO/reference_genomes/WS278_ref
$ /PATH/TO/SAMTOOLS/samtools faidx Celegans.fa
```

7. Convert/generate supplementary files for ANNOVAR.

```
$ /PATH/TO/gtfToGenePred -genePredExt Celegans_geneset.gtf WS278_refGene.txt
$ perl /PATH/TO/ANNOVAR/retrieve_seq_from_fasta.pl --format refGene \
--seqfile Celegans.fa WS278_refGene.txt --out WS278_refGeneMrna.fa
$ grep 'protein_coding_gene' Celegans_geneIDs.txt | sed
's/,/,/g' \
| awk -F "," 'BEGIN{OFS="\t";} {print $2,$3}' > WS278_gene_xref.txt
```

### 3.4.2 Pre-processing of Sequence Data

1. Download the sequence data file (*see Note 30*), create a directory named “data,” extract the sequence data if compressed, move it to the “data” directory, and change your working directory to “data”.

```
$ mkdir /PATH/TO/data
$ gunzip SEQUENCE_DATA.FQ.GZ (optional: only if file is compressed)
$ mv SEQUENCE_DATA.FQ /PATH/TO/data/
$ cd /PATH/TO/data/
```

2. Remove contaminating *E. coli* sequences (*see Note 31*).

```
$ /PATH/TO/BBMAP/bbduk.sh in=SEQUENCE_DATA.FQ out=FILTERED_DATA.FQ \
ref=/PATH/TO/reference_genomes/Ecoli.fa k=31 hdist=1
```

3. Remove contaminating adapter sequences (*see Note 32*).

```
$ /PATH/TO/BBMAP/bbduk.sh in=FILTERED_DATA.FQ out=FILTERED_2_DATA.FQ \
ref=/PATH/TO/ADAPTERS.FA ktrim=r k=23 hdist=1 mink=11 minlen=25 tpe tbo
```

4. Remove low-quality sequences.

```
$ /PATH/TO/BBMAP/bbduk.sh in=FILTERED_2_DATA.FQ out=TRIMMED_DATA.FQ \
qtrim=rl trimq=10 minlen=25
```

### 3.4.3 Alignment of Pre-processed Data to the C. elegans Reference Genome

```
$ /PATH/TO/BBMAP/bbmap.sh ref=/PATH/TO/reference_genomes/
WS278_ref/Celegans.fa \
sam=1.3 ambiguous=toss in=TRIMMED_DATA.FQ out=DATA.BAM
```

### 3.4.4 Post-processing of the Aligned Data for Variant Calling (Sort, Remove Duplicates, and Index)

```
$ /PATH/TO/SAMTOOLS/samtools sort -O bam -o SORTED_DATA.
BAM -T TEMP \
@ 8 DATA.BAM
$ /PATH/TO/SAMTOOLS/samtools rmdup -s SORTED_DATA.BAM DE-
DUP_DATA.BAM
$ /PATH/TO/SAMTOOLS/samtools index DEDUP_DATA.BAM
```

### 3.4.5 Hawaiian SNP Calling for Mapping (See Note 33)

```
$ /PATH/TO/FREEBAYES/freebayes -f > /PATH/TO/reference_
genomes/WS278_ref/Celegans.fa \
-F 0.01 -C 1 --pooled-continuous DEDUP_DATA.BAM > MAP-
PING_DATA.VCF
$ bedtools intersect -a MAPPING_DATA.VCF -b /PATH/TO/
reference_genomes/Hawaiian_snps.vcf > \
SEQUENCE_DATA_HAW.VCF
$ /PATH/TO/BCFTOOLS/bcftools query -f '%CHROM %POS %AB\n' \
SEQUENCE_DATA_HAW.VCF > HAW_FREQUENCY.TXT
```

### 3.4.6 Variant Calling for Candidate Mutations (See Note 34)

```
$ /PATH/TO/FREEBAYES/freebayes -f /PATH/TO/reference_gen-
omes/WS278_ref/Celegans.fa \
DEDUP_DATA.BAM > VARIANTS.VCF
$ /PATH/TO/FREEBAYES/vcfflib/vcffilter 'f "DP > 2" -g "GT =
1/1" VARIANTS.VCF > CANDIDATES.VCF
```

### 3.4.7 Annotation of Candidate Mutations (See Note 35)

```
$ perl /PATH/TO/ANNOVAR/table_annovar.pl FILE.VCF /PATH/
TO/reference_genomes/WS278_ref/ \
--buildver WS278 --out myanno --polish --remove --proto-
col refGene --operation g \
--vcfinput --xref WS278_ref/WS278.gene_xref.txt
```

## 4 Notes

- Instructions for making worm picks are available online and picks are also available commercially. (e.g., [http://openwetware.org/wiki/BISC\\_219/F10:\\_Gene\\_Linkage#Making\\_a\\_Worm\\_Pick](http://openwetware.org/wiki/BISC_219/F10:_Gene_Linkage#Making_a_Worm_Pick) or <http://www.wormbook.org/wbg/articles/volume-19-number-1/a-better-worm-pick-handle>).
- NGM medium lacks antibiotics, so sterile technique is essential to prevent contamination. The cholesterol solution is flammable and does not require sterilization. The remaining solutions

can be sterilized by autoclave or filtration. Airborne contaminants can be avoided by working in a laminar flow hood.

3. Plates can be stored seeded or unseeded in airtight containers at 4 °C for several weeks.
4. Strains are available from the *Caenorhabditis* Genetics Center (<http://www.cgc.cbs.umn.edu>).
5. For strains that do not require mapping, begin with gDNA isolation.
6. The mutation-bearing strain may be homozygous (preferable) or heterozygous (for phenotypes that preclude mating, such as nonconditional sterility or lethality). Conditional alleles that require maintenance at 15 °C should be allowed to mate for 24 h. Mutations that impair mating efficiency may require more hermaphrodites to ensure success.
7. Successful mating produces equal numbers of male and hermaphrodite outcross progeny, which are first distinguishable at the L4 stage. Use only those plates with successful mating, especially when the starting strain is heterozygous.
8. For heterozygous starting strains, only half of the F1 progeny will contain the mutation; therefore, the number of picked F1s should be doubled. Temperature-sensitive alleles should be incubated at the nonconditional temperature to allow discrimination of homozygous F2 mutants.
9. *This is a very important note:* Mapping and variant calling are dependent on homozygosity at the mutant locus. The number of animals to pick is determined by the stage of development: 200 mid-stage embryos or 50 adult hermaphrodites yield an adequate amount of gDNA for library prep. The F2 animals contain the recombinant chromosomes used for polymorphism mapping; 50 animals is the recommended minimum for optimal mapping resolution. For bulk samples, wash with  $\geq 10\times$  volumes of TE per volume of packed worms.
10. Washing is critical to remove as much of the OP50 bacteria as possible and minimize the amount of contaminating DNA in the sequencing library. Viable animals contain OP50 in the intestine. To remove, incubate the animals in M9 for 30 min with gentle shaking followed by multiple washes.
11. Samples can be frozen at –80 °C at this point if desired. To continue, thaw in ice bath and proceed with **step 6**.
12. Sonication disrupts the sample and shears the genomic DNA. If using a different instrument, parameters should be optimized to produce sheared gDNA in the 200–300 bp size range. Care should be taken to minimize sample heating and foaming.

13. The suspension should be clear by the end of incubation, indicating complete digestion.
14. Column purification kits are available from multiple vendors. Be sure that the protocol is compatible with samples containing ~1% SDS.
15. The amount of sheared gDNA should be 2–10 ng, which can be quantified by dye fluorometry or qPCR. Remember to increase the elution volume accordingly, leaving a final volume of 10 µL after quantification.
16. Some components of the library prep kit are viscous and require thorough mixing. After combining the reaction components, set a pipet (100 µL or 200 µL) to ~80% of the total reaction volume and pipet up and down multiple times. If necessary, spin the tube briefly before incubation to collect the liquid at the bottom of the tube. The presence of small bubbles will not adversely affect the reaction.
17. The proper dilution ratio is determined by the amount of sheared gDNA input. For  $\geq 5$  ng, dilute 1:10 (= 1.5 µM concentration); for  $< 5$  ng, dilute 1:25 (= 0.6 µM concentration).
18. The samples can be stored overnight at  $-20^{\circ}\text{C}$ .
19. Resuspend beads by vortexing immediately before pipetting; perform all incubations at room temperature.
20. Depending upon your magnetic stand, it may be necessary to transfer the sample to a tube of different size (e.g., 1.5 mL).
21. The solution will clear as beads adhere to the side of the tube adjacent to the magnet. When removing the supernatant, pipette slowly and carefully to avoid the bead pellet and bound gDNA.
22. For  $\geq 5$  ng of input gDNA, use eight cycles of amplification; for  $< 5$  ng of input gDNA, use 10 cycles of amplification.
23. Alternatively, a low elution volume purification column can be used for the final clean-up.
24. The library is now ready for quantification, using a method suitable for DNA concentrations in the low ng/µL range (e.g., dye fluorometry or qPCR). UV absorbance is relatively insensitive and therefore not recommended. Library quality can be assessed using the Agilent Bioanalyzer with a high-sensitivity DNA chip to detect the size distribution. If adapter dimer contamination (a discrete band of ~120 bp) is observed, it can be removed by size fractionation via gel isolation or AMPure bead size selection. Libraries constructed with different index primers can be pooled (aka multiplexed). The recommended minimum sequencing depth for mutation

identification is 20-fold genome coverage, or  $2 \times 10^9$  bp. The amount of data one obtains is determined by the sequencing capacity of the instrument and the degree of multiplexing; consult your sequencing service provider for guidance.

25. These steps are performed only once prior to running the analysis pipeline.
26. Follow the developers' instructions for the installation of software, including dependencies.
27. The default directory for downloads is typically located in your home directory (`/home/USERNAME/Downloads`). Directories and files can be relocated to a different directory via the “mv” (move) command and specifying the path to the new location. A common convention is to create a directory named “Sequencing” in your home directory (`$ mkdir /home/USERNAME/Sequencing`) and then creating subdirectories (e.g., software, reference\_genomes, data) there.
28. *This is a very important note:* Use the identical version of the reference genome for the entire workflow, including any downstream applications. For example, if you intend to use the UCSC Genome Browser for visualization, then download the reference genome from that source. The current WormBase version at the time of publication was WS278; different versions can be obtained by changing the number after “WS” in the link.
29. Command-line instructions use the following conventions:
  - (a) Each command is preceded by a dollar sign (\$);
  - (b) User-specific variables are shown in *UPPER\_CASE\_ITALICS*;
  - (c) Single-line commands that are too long to fit the page are split by backslash (\), do not include the backslash in the command;
  - (d) These instructions provide explicit paths to the software for operation; alternatively, the software directory can be added to the \$PATH variable.
30. Your sequencing service provider will provide instructions for downloading your data. The data should be provided in unaligned FASTQ format (suffix “.fq” or “.fastq”). It may also be compressed (typically in gzip format; suffix “.gz”).
31. The commands in this example assume single-end sequencing (typically annotated as “R1”); paired-end data (typically annotated as “R1” and “R2”) will require modification of the commands. Command usage and flag descriptions can be found at <https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/>.
32. Common adapter sequences are supplied with BBMap in the “resources” directory.

33. The final file *HAW\_FREQUENCY.TXT* contains chromosome, position, and Hawaiian SNP frequency information. The data for each chromosome can be plotted using Excel or R to determine the mapping interval (a gap in the Hawaiian SNP plot).
34. Candidate mutations are defined as homozygous variants ( $GT = 1/1$ ) with a minimum coverage of three reads ( $DP > 2$ ) to avoid spurious variant calls arising from low coverage.
35. The output file contains all categories of sequence variants. The overwhelming majority of causative mutations alter the coding potential of genes, so prioritize non-synonymous and splicing variants for subsequent validation.

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# Chapter 14

## Lipid Extraction and Analysis

Henry H. Harrison and Jennifer L. Watts

### Abstract

Lipids are major components of cellular membranes and energy stores. Lipids contribute vital structural, energetic, and signaling functions. We have optimized methods to extract and analyze lipids from the nematode *Caenorhabditis elegans* based on standard methods. Here we describe a method to extract total lipids from *C. elegans* larvae, adults, or embryos. We describe a thin-layer chromatography method to separate major lipid classes and a gas chromatography method to analyze fatty acid composition from lipid extracts, lipid fractions, or directly from nematode larvae, adults, or embryos.

**Key words** Thin-layer chromatography, Lipid extraction, Phospholipids, Neutral lipids, Fatty acid methyl esters, Gas chromatography

---

### 1 Introduction

Changes in lipid composition and metabolism are associated with many biological processes in *Caenorhabditis elegans*, including neuronal function, aging, innate immunity, cell death, and embryonic development [1–5]. The small size of *C. elegans* has traditionally been a barrier to biochemical analysis. While single-worm or single-tissue lipid analysis is not possible, mass spectrometry based methods are sensitive enough to analyze fatty acid composition of populations of several hundred adult-staged *C. elegans* [6, 7].

The combination of chloroform and methanol is widely used for lipid extraction across many species of microorganisms, plants, and animals. The Folch method uses chloroform/methanol in a ratio of 2:1 [8], while the Bligh and Dyer method uses chloroform/methanol in a ratio of 1:1, with water provided in various amounts due to samples in aqueous solutions, such as nematodes washed off of growth plates [9]. More recently, the methyl tert-butyl ether extraction method describes an alternative extraction solvent that does not require the use of chloroform [10, 11]. Others have demonstrated that various lipid extraction methods are appropriate for *C. elegans* [7]. Our method, based on Bligh and Dyer,

works well to extract a variety of phospholipids and neutral lipids from *C. elegans*.

Thin-layer chromatography of lipids uses a silica gel stationary phase and a mobile phase consisting of various organic solvents [12]. TLC is a somewhat “old fashioned” lipid separation technique, widely replaced by high performance liquid chromatography (HPLC) and mass spectrometry (MS) based lipidomics. However, using TLC to separate lipid classes prior to gas chromatography (GC) analysis offers several advantages, including low cost, convenience, and simplicity. Other lipid profiling techniques, such as “shotgun” lipidomics, in which raw lipid extracts are separated and analyzed using ion trap MS-MS and quadrupole-time of flight (Q-Tof) instruments, have been reviewed elsewhere and compared for their advantages and disadvantages for the analysis of *C. elegans* lipids [7].

For fatty acid composition analysis of whole worms, or for analysis of fatty acid composition in TLC-separated phospholipids, GC offers a sensitive and precise detection of a range of fatty acids, including cis/trans conformations and separation of isomers such as cis-vaccenic and oleic acid (18:1n-7 vs. 18:1n-9). Before injection on the GC, fatty acids associated with phospholipids and neutral lipids are converted to fatty acid methyl esters (FAMEs) which remain in the gas phase upon injection. GC coupled with a flame ionization detector can be used to separate and identify all the fatty acids in *C. elegans*. GC coupled with a single quad mass spectrometry detector (GC-MS) provides more certain species identification as well as the possibility for stable-isotope labeling experiments and 4,4-dimethyloxazoline (DMOX) derivatization to identify the double bond location [13–16].

The procedures described below allow for the separation of specific lipid types by polarity using TLC and GC. Depending on the solvent used, the TLC method separates various neutral lipid classes from polar lipids or separates individual phospholipids for further analysis.

---

## 2 Materials

### 2.1 Lipid Extraction

1. Chloroform:methanol (1:1): In a glass bottle, add 50 mL Chloroform (Approx. 0.75% Ethanol as Preservative/HPLC) and 50 mL methanol (OmniSolv for HPLC Gradient Analysis, Spectrophotometry, and Gas Chromatography). Mix well and store at –20 °C.
2. Hajra’s solution: 0.2 M H<sub>3</sub>PO<sub>4</sub>, 1 M KCl.
3. Glass screw top tubes, large (16 mm × 125 mm).
4. Glass screw tops, to fit large tubes.

5. 9-inch Pasteur pipette.
6. Glass screw top tubes, small (13 mm × 100 mm).
7. Glass screw tops, to fit small tubes.
8. Argon or Nitrogen gas.

## **2.2 Thin-Layer Chromatography**

1. TLC plates (Silica gel HL, 20 × 20 cm, 250 µm).
2. TLC tank (27.0 cm × 26.5 cm × 7.0 cm).
3. Cellulose filter paper (3MM CHR sheets, 15 cm × 17.5 cm).
4. Developing solvent for TLC (neutral lipids): 80 mL hexane (95% n-hexane, HPLC-grade), 20 mL diethyl ether (anhydrous with BHT as inhibitor), 2 mL glacial acetic acid (aldehyde-free/sequencing).
5. Developing solvent for TLC (phospholipids): 65 mL chloroform, 43 mL methanol, 3 mL water, 2.5 mL glacial acetic acid.
6. Hamilton syringe 25 µL.
7. Primuline spray: Dissolve 5 mg primuline in 100 mL of acetone/water (80/20, v/v).
8. Glass sprayer.
9. Internal standard. Pentadecanoic Acid, C15:0 (Nu-Chek Prep, N-15-A). Make stock solution of 2 mg/mL in hexane and dilute the solution to 0.1 M with hexane for the working stock. Store 2 mg/mL stock solution in a tightly sealed glass tube at -20 °C.
10. Sulfuric acid methanol solution: 2.5% sulfuric acid in methanol. In a fume hood, using a glass pipette, slowly pipette 25 mL sulfuric acid into 1000 mL of methanol (*see Note 1*).

## **2.3 FAMEs and Gas Chromatography**

1. Glass screw top tubes, small (13 mm × 100 mm).
2. Glass screw tops, to fit small tubes.
3. Internal standard. Pentadecanoic Acid, C15:0. Make stock solution of 2 mg/mL in hexane and dilute solution to 0.1 mg/mL with hexane. Store stock solution tightly sealed at -20 °C.
4. Sulfuric acid methanol solution (2.5% sulfuric acid in methanol). In a fume hood, using a glass pipette, slowly pipette 25 mL sulfuric acid into 1000 mL of methanol (*see Note 1*).
5. Hexane (95% n-hexane, HPLC-grade).
6. GC vials (2 mL crimp top vial).
7. GC vial inserts.
8. GC vial caps (11 mm aluminum caps with a PTFE/Silicone septa).
9. 11 mm E-Z Crimper.

10. GC-MS system equipped with a polar GC column (20 m × 0.25 mm).

#### **2.4 NGM Agar Plates for Growth of *C.***

**elegans**

1. Prepare 1 M stock solution of potassium phosphate buffer for use in Step 5 by mixing 132 mL of K<sub>2</sub>HPO<sub>4</sub> (1 M) with 868 mL of KH<sub>2</sub>PO<sub>4</sub> (1 M). Filter-sterilize.
2. Dissolve 3 g/L NaCl and 2.5 g/L bacto peptone in H<sub>2</sub>O.
3. Add 17 g/L agar (for 6- and 10-cm plates) or 10 g/L agar and 10 g/L agarose (for 14.5-cm plates).
4. Autoclave; then cool the solution to 50 °C.
5. Add the following components: 25 mL potassium phosphate buffer (prepared in step 1), 1 mL MgSO<sub>4</sub> (1 M), 1 mL CaCl<sub>2</sub> (1 M), 1 mL cholesterol (5 mg/mL in ethanol).
6. Mix the solution well and pour into Petri dishes.

#### **2.5 Alkaline Hypochlorite Method to Isolate *C. elegans* Embryos**

1. Prepare alkaline hypochlorite solution: 1.25 mL bleach, 0.25 mL 5 N NaOH, 3.5 mL water.
2. Harvest gravid adult *C. elegans* from NGM agar plates by rinsing plates with sterile water or M9 buffer (3 g KH<sub>2</sub>PO<sub>4</sub>, 6 g Na<sub>2</sub>HPO<sub>4</sub>, 5 g NaCl, 1 mL 1 M MgSO<sub>4</sub>, H<sub>2</sub>O to 1 L) and collecting the worm suspension into a 15 mL conical bottom tube.
3. Pellet the worms via centrifugation for 1 min at 1300 × *g*.
4. Remove the supernatant and suspend worm pellet in 5 mL of alkaline hypochlorite solution.
5. Mix the worms slowly so that they remain in suspension. Monitor worms under a stereomicroscope for dissolution of adults and release of embryos.
6. After adult worm carcasses have dissolved, pellet embryos via centrifugation for 1 min at 1300 × *g*.
7. Resuspend embryos in 10 mL sterile M9 buffer and centrifuge for 1 min at 1300 × *g*. Repeat M9 wash. Resuspend embryo pellet in 30–50 µL of water for FAMEs analysis.

### **3 Methods**

#### **3.1 Lipid Extraction from *C. elegans***

1. Wash worms grown on 20–30 10 cm NGM agar plates (see Subheading 2.4), approximately 1000 worms/plate, with water into a large glass screw top test tube. Let worms settle on ice (adults), centrifuge gently to form a pellet (larvae), or isolate embryos from gravid adult worms using the alkaline hypochlorite method (see Subheading 2.5). Remove water with a glass Pasteur pipette and wash with water, letting worms settle on ice or centrifuging gently for larvae or

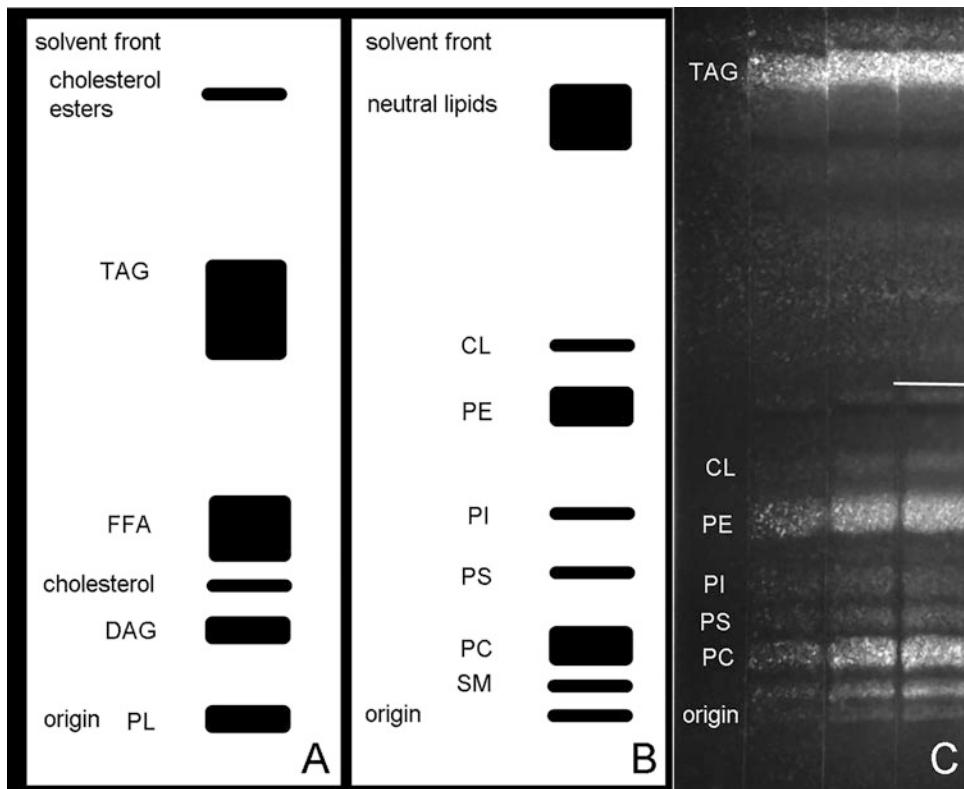
embryos. Repeat wash if necessary. The wash solution should not be cloudy with suspended *E. coli*. Worms can be lyophilized if desired, but it is not necessary.

2. Remove most of water from the worm pellet using a glass Pasteur pipet. The pellet should be 300–400 µL volume for worms, smaller for embryos. Quick freeze the pellet using liquid nitrogen and store the tube at –80 °C. Glass tubes with frozen worms or embryos can be stored at –80 °C for several weeks.
3. Using a glass pipette, add 5 mL of ice cold (–20 °C) chloroform:methanol ( $\text{CHCl}_3:\text{CH}_3\text{OH}$ )(1:1) directly to the frozen pellet (see Note 2). Do not let the pellet thaw out before adding the cold  $\text{CHCl}_3:\text{CH}_3\text{OH}$ . Shake tube vigorously and incubate the tube overnight in the –20 °C freezer. If too much water is present in the sample, two phases will form. If this occurs, add another 5 mL of cold  $\text{CHCl}_3:\text{CH}_3\text{OH}$  (1:1). This should lead to a single-phase solution. Let worm pellets extract at –20 °C overnight.
4. Use a 5 mL glass pipette to add 2.2 mL Hajra's solution to each sample tube and shake vigorously. If 10 mL of  $\text{CHCl}_3:\text{CH}_3\text{OH}$  was used for the overnight extraction, then add 4.4 mL Hajra's solution. Shake well and centrifuge for 1 min. at  $12,000 \times g$  using a clinical centrifuge. Solution will form two phases with the worm carcass pellet at the interface.
5. The lipids will be in the lower phase, the chloroform layer. Remove the lower phase carefully using a glass Pasteur pipette to transfer most of the lower phase into a new glass tube.
6. Re-extract the top phase with 0.5 mL chloroform (see Note 3). Centrifuge for 1 min at  $12,000 \times g$ . Remove the lower phase with a glass Pasteur pipette, combine this in the tube with the lower phase from the first extraction.
7. Using an evaporator dryer with a heat block, set heat at 50 °C and evaporate under a stream of nitrogen or argon until only 100 µL of chloroform remains. Wash the sides of the tube with 300 µL chloroform and continue evaporation until 100 µL remains. Do not let the lipids evaporate to dryness.
8. Bring the volume up to 200 µL or desired volume with chloroform. Flush tube with argon or nitrogen to remove air and store at –20 °C while preparing the TLC tank and the GC tubes.
9. If lipids are to be separated using TLC with subsequent GC-MS analysis, remove three 10 µL aliquots into 3 glass tubes and place tubes at –20 °C. These will be used to determine the fatty acid composition of the “total lipid” samples by GC.

10. The remaining lipid extract can be divided into 3 portions to separate on a TLC plate. Lipid extracts can be flushed with argon or nitrogen and stored at -20 °C for several weeks.

### **3.2 Thin-Layer Chromatography of *C. elegans* Lipid Extracts**

1. Preparation of TLC plates. For most consistent separation of lipids, bake the TLC plate in 110 °C oven for 1 h 15 min ( $\pm 15$  min max). This step is not typically necessary for neutral lipid separation in *C. elegans* but it improves the consistency of phospholipid separation.
2. Mark the TLC plate using a dull pencil with as many lanes needed for samples, plus two 1 cm lanes for standards on both edges of plate. Make a mark for loading the samples several cm from the bottom. If desired, use a razor blade to scrape a straight line between lanes.
3. Cut several sheets of cellulose filter paper to the size of the tank and place the paper in the tank.
4. Add TLC solvent mixture to the TLC tank (see Note 4).
5. Secure the lid to the TLC tank tightly. Let the solvent soak into the cellulose filter paper and allow the solvent to equilibrate the atmosphere in the tank for 30 min.
6. Load the samples onto TLC plate using a Hamilton syringe. Load 30–40  $\mu$ L of total lipid extract per lane (see Note 5).
7. Gently and quickly, lower the plate into the TLC tank, ensuring that the samples are above the solvent level. Secure the lid tightly with tape or a heavy brick (see Note 6). Allow the solvent to run up the TLC plate until the solvent front is within 1–2 cm of the top of plate. When finished, remove the plate and allow the solvent to evaporate in the hood or dry the plate with a nitrogen or argon stream. Dispose of TLC solvent in a proper waste container.
8. Instead of a one-solvent development, we routinely use a two-solvent development scheme to separate the major phospholipids and the major neutral lipid components of *C. elegans* on one plate. The TLC plate is first developed approximately 60% up the plate with chloroform:methanol:H<sub>2</sub>O: acetic acid (65:43:3:2.5) to separate phospholipids. The plate is removed from the TLC tank, air dried, and then developed in hexane: diethyl ether:acetic acid (80:20:2) to within 1–2 cm of the top of the plate (Fig. 1c).
9. In a fume hood equipped with a pressurized airline, attach the glass sprayer to the air line and spray the plate lightly with primuline solution, ensuring to not saturate the plate (see Note 7).
10. Visualize using a UV transilluminator (340 nm); lipids will appear as bright spots against the white silica of the plate



**Fig. 1** Separation of lipids from *C. elegans* using TLC. **(a)** Schematic of TLC plate developed with hexane: diethyl ether:acetic acid (80:20:2) to separate various neutral lipid species. **(b)** Schematic of TLC plate developed with chloroform:methanol:H<sub>2</sub>O: acetic acid (65:43:3:2.5) to separate phospholipids. **(c)** Photograph of TLC separation of *C. elegans* lipids developed in a two-solvent system. The plate was developed with chloroform:methanol:H<sub>2</sub>O: acetic acid (65:43:3:2.5) until solvent reached the white line. The plate was removed from the TLC tank, air dried, and then developed to the 1 cm below the top with hexane:diethyl ether:acetic acid (80:20:2). Lipid species were identified by comigration with known standards. PL: phospholipids; DAG: diacylglycerols; FFA: free fatty acids; TAG: triacylglycerols; SM: sphingomyelin; PC: phosphatidylcholine; PS: phosphatidylserine; PI: phosphatidylinositol; PE: phosphatidylethanolamine; CL: cardiolipin

(Fig. 1). Photograph the plate. Carefully mark the lipids of interest with a dull pencil, ensuring to not scrape the silica or look directly into UV light. Once bands of interest are identified, use a clean razorblade to carefully scrape the marked silica spot marked into labeled glass tubes for each lane, scraping and discarding extraneous silica between bands of interest. The blade should be held at a 45° angle and move toward you, allowing for precise scraping.

11. Use weigh paper and a funnel to add the scraped silica to a glass screw top tube (13 × 100 mm) with screw cap.
12. Prepare FAMEs from scraped silica samples as described below.

**3.3 Preparation of Nematode Samples for Fatty Acid Methyl Esters (FAMEs)**

1. Harvest 200–1000 well-fed *C. elegans* from several 6 cm NGM plates, avoiding the bacterial lawn.
2. Wash well-fed worms with 1–3 mL water into a glass tube (13 × 100 mm) with screw cap. Glass is preferred because plastic tubes can shed contaminants that will interfere with the FAMEs analysis (*see Note 8*).
3. Place tubes in a bucket of ice and let worms settle for at least 5 min. Once the worms have settled to the bottom of the tube, carefully remove as much of the water and bacteria as possible (without disturbing the pellet) using a 9-inch Pasteur pipette. Wash again with water if visible bacteria are still present. During the final wash, remove as much water from the loose worm pellet as possible (*see Note 9*). Tubes may be stored at –20 °C for several days.

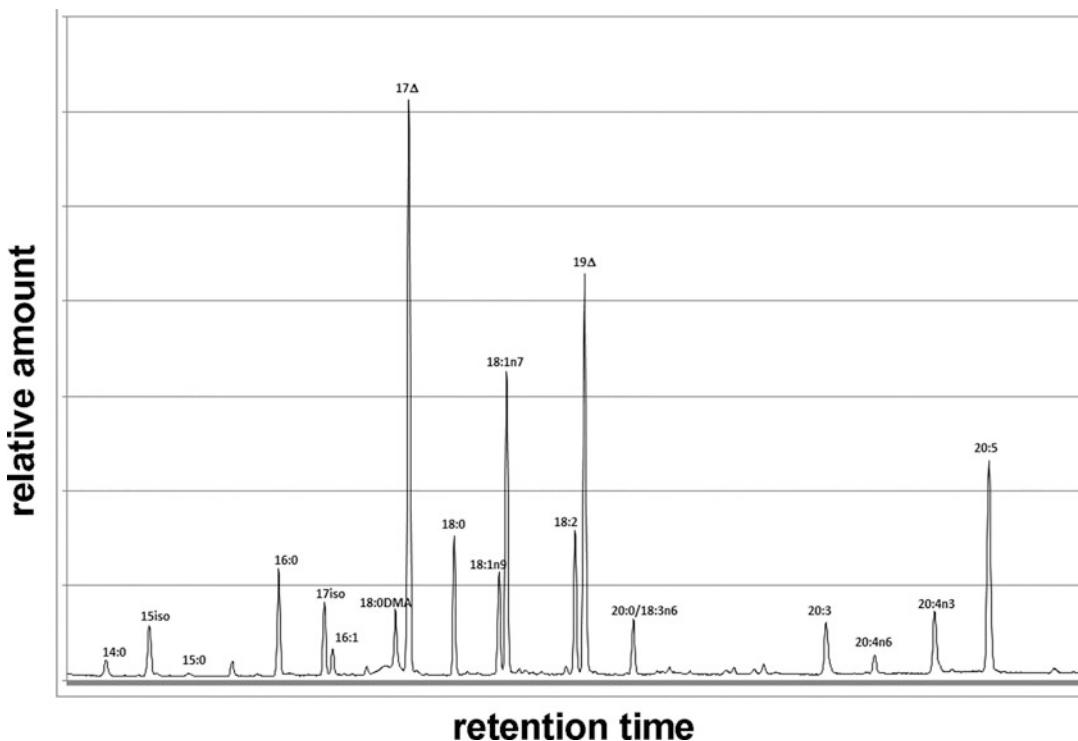
**3.4 Preparation of Fatty Acid Methyl Esters (FAMEs)**

This method to prepare fatty acid methyl esters (FAMEs) can be used to generate FAMEs directly from frozen or freshly harvested worm pellets, from lipid extracts, or from silica fractions scraped from TLC plates.

1. For quantification of lipids separated from TLC plates, add 30 µL of 0.1 mg/mL C15:0 fatty acid internal standard to each sample tube, including the three total lipid samples that have been set aside.
2. Add 1 mL of MeOH, 2.5% H<sub>2</sub>SO<sub>4</sub> to each tube, cap tubes tightly with screw caps, and heat for 60 min in a 70 °C water bath (*see Note 10*).
3. During incubation, check tubes periodically to make sure the methanol solution is not bubbling. Bubbling indicates a leak in the screw top and samples will eventually evaporate if tubes are not sealed properly.
4. After 60 min, remove tubes and allow to cool at room temperature for 5 min.
5. Add 0.2 mL hexane and 1.5 mL H<sub>2</sub>O to each tube. Higher volumes of hexane can be used for more concentrated lipid samples.
6. Shake tubes vigorously.
7. Centrifuge tubes in clinical centrifuge at 12,000 × *g* for 1 min.
8. Carefully remove 80 µL of the upper hexane phase by pipetting it into a GC vial fitted with a glass GC vial insert (*see Note 11*).
9. Cap the GC vials tightly using screw caps with Teflon liner or crimp caps.

**3.5 GC-MS Separation of FAMEs**

1. Separate FAMEs on a SP-2380 or other polar column using a GC system equipped with an inert mass spectrometry detector



**Fig. 2** Typical GC chromatogram of *C. elegans* FAMEs separated on a SP-2380 column showing the major fatty acid methyl ester peaks. Fatty acid nomenclature,  $x:y(nz)$ ,  $x = \#$  of carbons,  $y = \#$  of double bonds,  $nz =$  location of terminal double bond,  $z$  carbons from the methyl end of the fatty acid. 15:iso, 13-methyltetradecanoic acid; 17:iso, 15-methylhexanoic acid; 17 $\Delta$ , cis-9,10-methylenehexadecanoic acid; DMA, dimethylacetal; 19 $\Delta$ , cis-11-12-methylene octadecanoic acid, 20:0–18:3, sum of 20:0 and 18:3n6, peaks which do not resolve on our GC column under these running conditions

and an autosampler. A flame ionizing detector also works well for FAMEs analysis, as long as peak retention times can be compared to authentic standards.

2. Program the autosampler to inject 2  $\mu\text{L}$  of sample.
3. Program the GC oven. Initial temperature of 120°, hold for 1 min followed by an increase of 10°/min to 190° followed by an increase of 2°/min to 200°. This program takes 14 min per sample.
4. The relative amounts of each fatty acid in the sample are calculated by dividing the corrected area of each peak by the total area of all peaks (Fig. 2).

#### 4 Notes

1. The mixing of sulfuric acid and methanol is highly exothermic. In a fume hood, using a glass pipette, add the sulfuric acid very

slowly to the methanol with constant stirring. Wear protective lab coat, gloves, and eye protection.

2. From this point forward, always use glass tubes, pipettes, and bottles. Avoid plastic when working with lipids.
3. If the bottom phase is cloudy, add 0.5 mL ethanol:benzene (9:1).
4. To separate neutral lipids, use 80 mL hexane, 20 mL diethyl ether, 2 mL glacial acetic acid solvent mix (Fig. 1a). To separate phospholipids, use 65 mL chloroform, 43 mL methanol, 3 mL water, and 2.5 mL glacial acetic acid (Fig. 1b).
5. To avoid saturating the silica with chloroform, use a Hamilton syringe to apply 10  $\mu$ L at a time, drop by drop, evenly across the sample line and allow the chloroform to evaporate before applying the next 10  $\mu$ L aliquot. Load at least three TLC lanes per lipid sample, these are technical replicates.
6. A secure lid will maintain saturation of the atmosphere in the tank and prevent solvent evaporation.
7. Other visualization methods to preserve lipids are also be used, including iodine crystals, dichlorofluoresceine, rhodamine G. Alternatively, it is common to use radioactive labeling of lipids and visualization using a phosphorimager [12].
8. For consistency, harvest well-fed worms that have not yet cleared the *E. coli* lawn, as food runs out the fatty acid composition of nematodes changes.
9. Water competes with the methylation reaction, leading to hydrolysis, rather than methylation of fatty acids, leading to reduced peak size or random noise in the chromatograph readout obscuring the fatty acid peaks of interest.
10. This can be added directly to total lipid extracts, scraped silica from TLC plate, or freshly harvested worm pellets or frozen pellets. If worm samples were frozen, do not let samples in the tubes thaw, this leads to immediate destruction of lipids. Instead, add the 1 mL of 2.5%  $H_2SO_4$  in MeOH, directly to the frozen pellet.
11. Take care not to remove any of the aqueous phase; if this phase is injected into the GC-MS, it will destroy the GC column.

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# Chapter 15

## Isolating *Caenorhabditis elegans* from the Natural Habitat

Clotilde Gimond, Nausicaa Poulet, and Christian Braendle

### Abstract

Wild populations of the model organism *C. elegans* represent a valuable resource, allowing for genetic characterization underlying natural phenotypic variation. Here we provide a simple protocol on how to sample and rapidly identify *C. elegans* wild isolates. We outline how to find suitable habitats and organic substrates, followed by describing isolation and identification of *C. elegans* live cultures based on easily recognizable morphological characteristics, molecular barcodes, and mating tests. This protocol uses standard laboratory equipment and requires little prior knowledge of *C. elegans* biology.

**Key words** *Caenorhabditis elegans*, Natural genetic variation, Natural populations, Wild isolates, Natural habitat, Ecology

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### 1 Introduction

Insights into *C. elegans* biology are almost exclusively based on the analysis of a single reference strain (N2). Yet, research focusing on natural genetic and phenotypic variation within the *C. elegans* species has greatly enriched our understanding of this model organism, now also widely used to address questions in evolutionary biology [1–6]. A key discovery was that the reference strain N2 shows extensive laboratory adaptation, exhibiting multiple mutations with strong phenotypic effects that are absent in natural populations [7]. Genomic analysis of *C. elegans* indicated relatively little genetic differentiation of populations across the globe [5]. However, genetic divergence varies across the genome and recent observations suggest a much more complex picture, with the occurrence of extremely divergent genotypes in Hawaii [6]. Characterization of *C. elegans* natural variation, e.g., through characterization of the molecular basis underlying specific natural variants (e.g., [8–12]), has generated novel insights into *C. elegans* biology and further places the wealth of mechanistic knowledge of this model organism into a much-needed ecological and evolutionary context.



**Fig. 1** Geographic distribution of *C. elegans* wild isolates. Currently known distribution of *C. elegans*, based on data from CeNDR (<http://elegansvariation.org/>), our own unpublished data, and various past records [13–17]. Lists of currently available, cryopreserved *C. elegans* wild isolates can be consulted at <http://elegansvariation.org/> and <https://justbio.com/worldwideworms/>. For information on other *Caenorhabditis* species, see [18, 19] and for updates on species discovery, see <http://evolution.wormbase.org/>

Hundreds of *C. elegans* isolates have been collected worldwide (Fig. 1) and most of their genomes have been sequenced, which are available through the *C. elegans* Natural Diversity Resource (CeNDR) (<http://elegansvariation.org/>) [5, 6]. *C. elegans* is clearly more abundant in temperate than in tropical regions, and *C. elegans* isolates found in the tropics occurred mostly at high altitude [6, 18, 20]. This indicates, consistent with experimental determination of thermal optima in the laboratory [21, 22], that *C. elegans* does not tolerate prolonged exposure to temperatures above 25 °C.

Current insights into the global distribution of *C. elegans* are limited due to strong sampling bias, with some regions (e.g., France) having been frequently sampled, whereas others (e.g., Eastern Europe) have never or only rarely been sampled. Although likely to colonize highly diverse habitats, *C. elegans* has primarily been isolated from anthropogenic habitats, such as gardens or agricultural lands [13, 14, 23–27], and only rarely from more unperturbed sites (e.g., forests) [6, 25, 28].

*C. elegans* and other *Caenorhabditis* species have been isolated from a wide variety of organic substrates, mainly decomposing vegetal matter, such as compost, fruits, plant stems, or leaf litter [6, 18, 19, 29–31]. Despite commonly being referred to as a soil-

nematode, *C. elegans* has virtually never been isolated from soil samples but mostly from decaying vegetal matter on or above the soil layer. *C. elegans* has also been isolated from a range of live or dead invertebrates (e.g., beetles, isopods, millipedes, snails, and slugs) [3, 15, 23, 25, 32–34]. In contrast to other nematode species, there is no strong evidence for taxon-specific phoretic or necromenic invertebrate associations of *C. elegans* [3, 34, 35].

In this chapter, we provide a simple protocol for sampling and isolation of *C. elegans* wild isolates, chiefly aimed at researchers having minimal working knowledge using *C. elegans* as a study organism. Nevertheless, acquiring basic knowledge of *C. elegans* handling and maintenance [36] is highly recommended. Several alternative, yet usually more time-consuming nematode isolation protocols are available [33].

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## 2 Materials

### 2.1 Collection and Storage of Substrate Samples

1. Sampling containers, e.g., resistant plastic zip-lock bags.
2. Disposable plastic gloves.

### 2.2 Nematode Isolation in the Laboratory

1. Dissecting stereomicroscope with transmitted light source (5–50× magnification).
2. Nematode growth medium (NGM) [36]: 1 L: 3 g NaCl, 25 g agar, 2.5 g bacto-peptone, 975 mL H<sub>2</sub>O. (The increased agar concentration of 2.5% instead of the usual 1.7% helps preventing burrowing of nematodes into the culture plates.) After autoclaving, add 1 mL 1 M CaCl<sub>2</sub>, 1 mL 5 mg/mL cholesterol in ethanol, 1 mL 1 M MgSO<sub>4</sub>, and 25 mL 1 M KPO<sub>4</sub>. Mix well and dispense solution in Petri dishes (e.g., 90 mm and 55 mm diameter). Store at room temperature for 2–4 days.
3. Seed NGM plates with a central spot of *E. coli* OP50 (100–200 µL) and store at room temperature for 2–4 days. OP50 is available from the *Caenorhabditis* Genetics Center (CGC): <https://cgc.umn.edu/>.
4. Disposable plastic gloves.
5. Clean water or M9 buffer (1 L): 3 g KH<sub>2</sub>PO<sub>4</sub>, 6 g Na<sub>2</sub>HPO<sub>4</sub>, 5 g NaCl; autoclave; add 1 mL 1 M MgSO<sub>4</sub> before use [36].
6. Platinum worm pick [36].
7. Parafilm.

### 2.3 Morphological and Molecular Identification of *C. elegans*

1. Dissecting stereomicroscope with transmitted light source (50–100× magnification).
2. Standard PCR reagents and equipment and access to sequencing service.

**2.4 Genetic Identification Through Crosses with Established *C. elegans* Strains**

1. Dissecting stereomicroscope with transmitted light source (5–50× magnification).
2. Identified *C. elegans* strain, e.g., N2 (available from the CGC: <https://cgc.umn.edu/>).
3. NGM plates (55 mm diameter) (see Subheading 2.2, item 2).

**2.5 Establishment and Cryopreservation of *C. elegans* Wild Isolate Stocks**

1. NGM plates (55 mm diameter) (see Subheading 2.2, item 2).
2. Freezing solution [36]: 300 mL: Start with 100 mL of dH<sub>2</sub>O and add 1.76 g NaCl, 2.04 g KH<sub>2</sub>PO<sub>4</sub>, 1.7 ml of 1 M NaOH, 1.2 g agar, 71.6 ml glycerol. Heat to boiling until the agar is dissolved. Bring to 300 ml with dH<sub>2</sub>O. Distribute 50 ml per 100 ml bottles; autoclave. After autoclaving or when first using, add 1.5 ml 0.1 M MgSO<sub>4</sub> and 100 µL 1 M CaCl<sub>2</sub> per 50 ml bottle [33, 36].
3. Cryovials (2 ml).
4. –80 °C freezer and liquid nitrogen storage facilities.

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### 3 Methods

**3.1 Finding Suitable Habitats and Substrate Samples**

Potentially, *C. elegans* may be found in diverse, anthropogenic as well as unperturbed natural sites. In temperate regions of Europe (e.g., France, Switzerland), temporal sampling of natural populations uncovered *C. elegans* throughout all seasons (personal observations). However, *C. elegans* seems less abundant during summer months of temperate regions [25]. Population fluctuations further depend on the specific habitat and substrate sampled, given that certain habitats, e.g., composts heaps in gardens [24], may represent more stable habitats than others, such as seasonally occurring fruits. As a general rule, hot, dry, or very cold habitats are very unlikely to yield *C. elegans* although individuals in the dauer stage may be uncovered at low densities.

Substrates potentially containing *C. elegans* encompass any organic sample that contains or contained microbial organisms serving as possible *C. elegans* food source, including fruits, plant stems and leaves, compost, animal carcasses, and animal feces. Fruits in advanced stages of decomposition on the ground represent ideal target samples due to their high microbial content and likely also because they attract diverse invertebrates that might carry *Caenorhabditis* nematodes [2, 3, 37]. Direct sampling of such invertebrate carriers (arthropods, including insects, isopods as well as mollusks, such as snails or slugs) has yielded *C. elegans* and other *Caenorhabditis* species [3, 15, 24, 30, 33, 37, 38]. Sampling success is highest for rotting plant material that is not dry and not directly exposed to sunlight.

### 3.2 Collection and Storage of Substrate Samples

1. Using disposable plastic gloves, collect samples from suitable habitat (see above) and place into container, ideally transparent plastic zip-lock bags for plant substrates (for invertebrates, use hard plastic containers, e.g., Falcon tubes). Leave ample air in the bag and add some paper towel if the sample is very liquid, to avoid fermentation.
2. Note date of collection, locality (e.g., GPS coordinates), habitat, and substrate type (e.g., take photographs).
3. Store samples in the dark, and at temperatures of 15–25 °C (above 25 °C may lead to sterility in *C. elegans*). Before processing in the laboratory, substrate samples may be stored for several weeks in plastic bags but samples should be regularly aerated. For high success in nematode recovery, analyze substrates within 1 week after sampling.

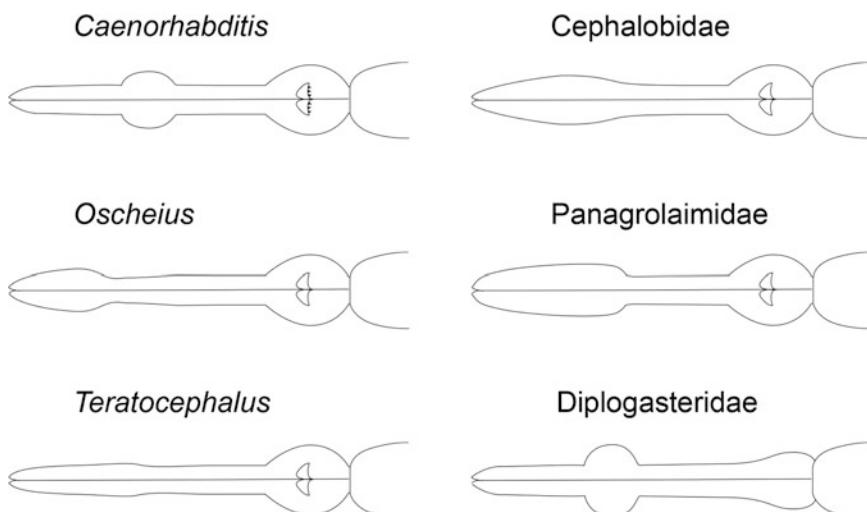
### 3.3 Nematode Isolation in the Laboratory

1. Place substrate samples on large (e.g., 100 mm diameter) NGM plates seeded with a spot of *E. coli* OP50 in the center of the plate. Tear up or cut large samples into smaller pieces and place them around the *E. coli* spot (sacrifice live invertebrates). Add 1–3 ml of clean water or M9 buffer onto the substrate. Avoid damaging the agar plates as this will facilitate burrowing of nematodes into the culture plates, making subsequent visual observation difficult. Cover the plate with lid. Samples, such as rotting fruit, usually contain diverse invertebrates (e.g., mites, insect larvae, flies): to avoid their spread and cross-contamination among plates, wrap plates with Parafilm. Keep samples at 15–20 °C.
2. Bactivorous nematodes, such as *C. elegans*, will crawl onto the *E. coli* lawn and can be easily observed using a standard dissecting stereomicroscope equipped with a transmitted light source. Nematodes may colonize the *E. coli* lawn within minutes to hours after placing the sample on the culture plate, but for samples with low densities (or individual dauer individuals) it may take several days to detect the nematodes.
3. Check for the presence of nematodes on the plate using a dissecting scope. Diverse bacteria and fungi stemming from the sample will also grow on the NGM plate, making observation difficult over time (see Note 1). If this is the case, cut out a piece of agar containing nematodes and transfer to a new plate to facilitate visual observation.
4. Isolate multiple individual nematodes to fresh NGM plates (55 mm diameter) using a platinum worm pick and amplify population (see Note 2).

### 3.4 Morphological and Molecular Identification of *C. elegans*

The following protocol for rapid and easy identification of *C. elegans* uses minimal morphological knowledge of *C. elegans* allowing an efficient initial elimination of nematodes that do not represent members of the genus *Caenorhabditis*. In a second step, only nematodes with a hermaphroditic mode of reproduction are selected. After these two initial steps of selection, analysis using species-specific DNA sequence-tag is performed to distinguish between different hermaphroditic *Caenorhabditis* species [18], which are morphologically very similar. Currently, only three androdioecious (hermaphrodite-male) *Caenorhabditis* species have been described: *C. elegans*, *C. briggsae*, and *C. tropicalis*; all other species (>60) show a gonochoristic (female-male) mode of reproduction [18, 19, 39]. For a detailed overview of *Caenorhabditis* identification methods, see previously published protocols [33].

1. Diverse nematode taxa may be uncovered in substrate samples, but a given sample often contains only one or two different nematode species. To check whether picked nematodes may represent *C. elegans*, use the following two criteria:
  - (a) The pharyngeal head region contains two distinct bulbs: the middle and the basal pharyngeal bulbs, the latter bulb exhibits a grinder (Fig. 2). This morphology is typical for all *Caenorhabditis* species [33] and can be examined using a dissecting scope at 50× or higher magnification (or using light microscopy).



**Fig. 2** Morphological characteristics of the *C. elegans* pharynx compared to a selection of other nematode taxa. The presence of two circular pharyngeal bulbs is a distinctive feature of *Caenorhabditis* species and can be observed using a dissecting stereomicroscope at >50× magnification and good illumination. Commonly found in the same habitat or substrate are nematodes of the genus *Oscheius*, which superficially show an overall morphology and size similar to *C. elegans*, yet present a clearly different pharyngeal structure (Adapted from [33, 40]).

- (b) Test whether reproduction occurs through self-fertilizing hermaphrodites: isolate juvenile stages onto individual NGM plates and determine whether fertile progeny is produced after a 2–5 days of growth at 20 °C (For isolation of gonochoristic *Caenorhabditis* species, see Note 3).
  - (c) Several additional simple morphological criteria (e.g., long and pointy tail, central vulva, oval embryos) are characteristic for *Caenorhabditis* and may also be used for identification [33]; for detailed morphological characterization of *Caenorhabditis* species, see [18, 41, 42].
2. Molecular identification of *C. elegans*: Kiontke et al. [18] have established a *Caenorhabditis* species barcoding method using sequencing of the ITS2 (Internally Transcribed Spacer) region, localized between the 5.8S and 28S rDNA genes.
- (a) To amplify the region of approximately 2 kb, use the following primers:  
 5.8S-1: (5'- CTGCGTTACTTACCAACGAATTG CARAC)  
 KK28S-4: (5'- GCGGTATTTGCTACTACCAYYAM GATCTGC).
  - (b) Sequence the amplified fragment with the sequencing primer:  
 KK-28S-22 (5'-CACTTTCAAGCAACCCGAC) [18].
  - (c) Use NCBI Blast to check for species identity of sequence. *Caenorhabditis* ITS2 sequences are highly species-specific and isolates of a given species differ by a maximum of 2 nucleotide polymorphisms [18].

### **3.5 Genetic Identification Through Crosses with Established *C. elegans* Strains**

In combination with ITS2 sequence analysis (3.4), or as an alternative approach, presumptive *C. elegans* species identity of a new isolate can be verified through crosses with known *C. elegans* strains, e.g., the reference strain, N2 (available from the *Caenorhabditis* Genetics Center: <https://cgc.umn.edu/>). Place three to four L4 hermaphrodites of the new isolate together with five to ten males of the identified *C. elegans* strains, and vice versa. The presence of a high proportion of males (>30%) in the F1 progeny indicates successful crossing, thus confirming *C. elegans* species identity (see Note 4).

### **3.6 Establishment and Cryopreservation of *C. elegans* Wild Isolate Stocks**

1. Strain establishment: Derive any new *C. elegans* isolate (i.e., strain) from an isolated single L4 stage hermaphrodite and amplify resulting populations over 4–5 generations, so that isolates can be considered nearly isogenic [33]. Unless individuals are isolated within hours after sampling, it is advisable to derive only a single isolate from a given sampling bag/unit. Given that *C. elegans* proliferates very rapidly, also within the

sample after collection, this procedure limits strain establishment of isolates with identical genotypes. Each new *C. elegans* isolate should have a unique name, ideally following *C. elegans* strain naming procedures, i.e., Lab name abbreviation followed by number ID [36].

2. Cryopreservation: All *Caenorhabditis* species can be cryopreserved [33] using standard *C. elegans* protocols, and stored at  $-80^{\circ}\text{C}$  and liquid nitrogen [36]. For a given isolate to be frozen, grow 5–10 NGM plates (55 mm diameter) until starvation when cultures contain a maximum of freshly starved L1 animals. Collect individuals from plates using M9 buffer and place solution into sterile test tube (e.g., 15 ml Falcon tube) and place on ice for 15 min. Centrifuge tube and keep 3 ml of solution containing worms, then add an equal volume of Freezing Solution to tube and mix well. Aliquot mixture to three cryovials, labeled with strain name. Place cryovials in a small styrofoam box with slots for holding microtubes (to ensure slow freezing of the worms) and place box in a  $-80^{\circ}\text{C}$  freezer overnight. Transfer tubes to stock collections at  $-80^{\circ}\text{C}$  or liquid nitrogen.
3. Database entry: Add strain information of newly discovered *C. elegans* wild isolates to Wormbase using the following form:  
[http://tazendra.caltech.edu/~azurebrd/cgi-bin/forms/wild\\_isolate.cgi](http://tazendra.caltech.edu/~azurebrd/cgi-bin/forms/wild_isolate.cgi)

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#### 4 Notes

1. To limit fungal contaminations on NGM plates, add 0.01% thimerosal to water or M9 solution used to moisten the substrate samples.
2. Substrates may harbor diverse microbes that are pathogenic to *C. elegans* [3, 37]. *C. elegans* and other nematode species therefore may show pathologies preventing development or growth, and may also complicate morphological analysis. Moreover, many bacterial contaminants generate thick films on NGM plates rendering observations difficult. To generate clean plates and *C. elegans* cultures, use NaOH and sodium hypochlorite mixture to decontaminate [36].
3. To isolate gonochoristic *Caenorhabditis* species, place either a single mated female (either gravid or with a mating plug) or a female plus male to a fresh plate. For detailed isolation methods of gonochoristic *Caenorhabditis* species, see [33].
4. Note that *C. elegans* does not produce any cross progeny with any other known *Caenorhabditis* species.

## Acknowledgments

This protocol makes use of diverse contributions from the worm community and is primarily based on *Caenorhabditis* isolation methods established by Antoine Barrière and Marie-Anne Félix. Our research is financed by the Centre National de la Recherche Scientifique (CNRS) and Université Côte d’Azur, Nice, France.

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# Chapter 16

## Microfluidic Devices for Behavioral Analysis, Microscopy, and Neuronal Imaging in *Caenorhabditis elegans*

Ross C. Lagoy, Eric Larsen, Dan Lawler, Hamilton White, and Dirk R. Albrecht

### Abstract

Microfluidic devices offer several advantages for *C. elegans* research, particularly for presenting precise physical and chemical environments, immobilizing animals during imaging, quantifying behavior, and automating screens. However, challenges to their widespread adoption in the field include increased complexity over conventional methods, operational problems (such as clogging, leaks, and bubbles), difficulty in obtaining or fabricating devices, and the need to characterize biological results obtained from new assay formats. Here we describe the preparation and operation of simple, reusable microfluidic devices for quantifying behavioral responses to chemical patterns, and single-use devices to arrange animals for time-lapse microscopy and to measure neuronal activity. We focus on details that eliminate or reduce the frustrations commonly experienced by new users of microfluidic devices.

**Key words** Microfluidics, *Caenorhabditis elegans*, Quantitative behavior, Locomotion, Time-lapse microscopy, Neuronal imaging, PDMS, Chemical stimulation

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### 1 Introduction

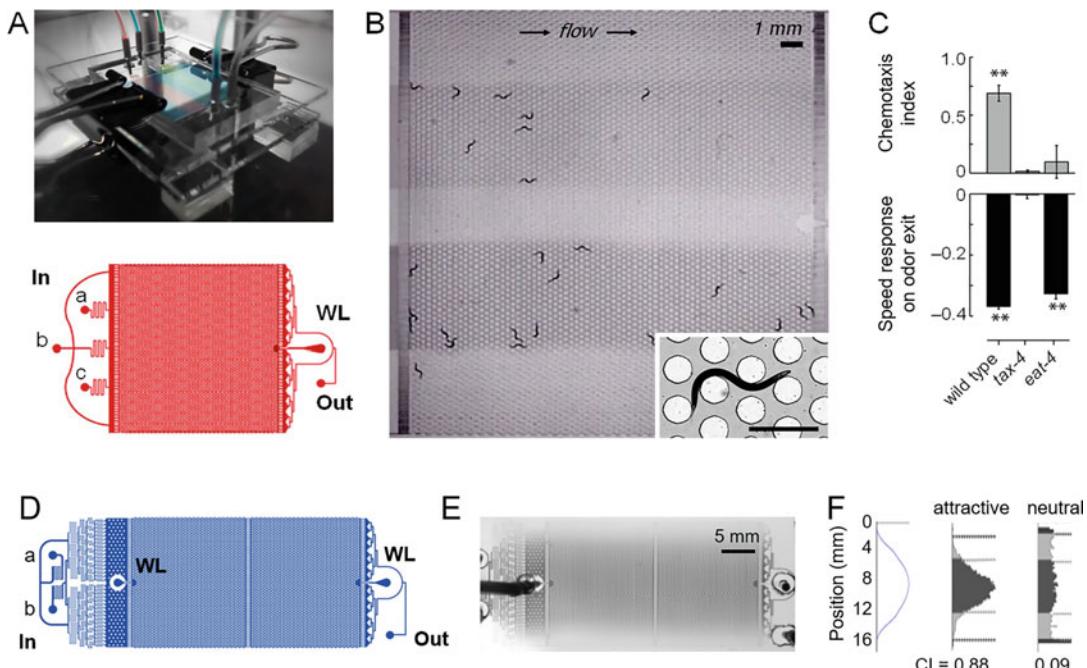
Microfluidics refers to plumbing at a small, submillimeter scale. Microfluidic-based technologies have found numerous applications in biology over the past decade, from miniaturization of molecular biology reactions to high-throughput analysis of cellular functions. Microfluidic devices offer the advantage of increased experimental productivity by collecting more data (conditions or repeats) in less time with fewer resources and lower costs. Further, predictable fluid physics at this length scale enables dynamic and reproducible control over the fluidic environment surrounding cells or small organisms, often increasing experimental reliability.

In *C. elegans* research, microfluidic devices have been particularly useful for presenting precise physical and chemical environments, immobilizing animals during imaging, quantifying

behavior, and automating phenotypic screens. A wide variety of specific *C. elegans* microfluidic devices have been surveyed in several recent reviews (1–8). Microfluidic devices are generally described as “easy, fast, and inexpensive,” especially when fabricated from elastomeric materials such as polydimethylsiloxane (PDMS). This description is technically true, but only if the user has prior experience with microfluidic devices, already has the equipment necessary for fabrication, and uses microfluidics often enough to reduce per-device costs by purchasing raw materials in bulk.

Microfluidic designs can be as simple as a single channel with one inlet and one outlet, or as complicated as a multilayered device containing dozens of fluidic ports and integrated, computer-controlled pneumatic valves. A simple design can be operated with a syringe reservoir and thin tubing, with fluid flow driven by hydrostatic pressure. A complicated system may require precisely balanced fluid and pneumatic line pressures and computer-actuated valves or syringe pumps. Simple and elegant systems that focus on ease of use are preferred, not just to reduce user frustration, but to improve reliability and reproducibility of an assay.

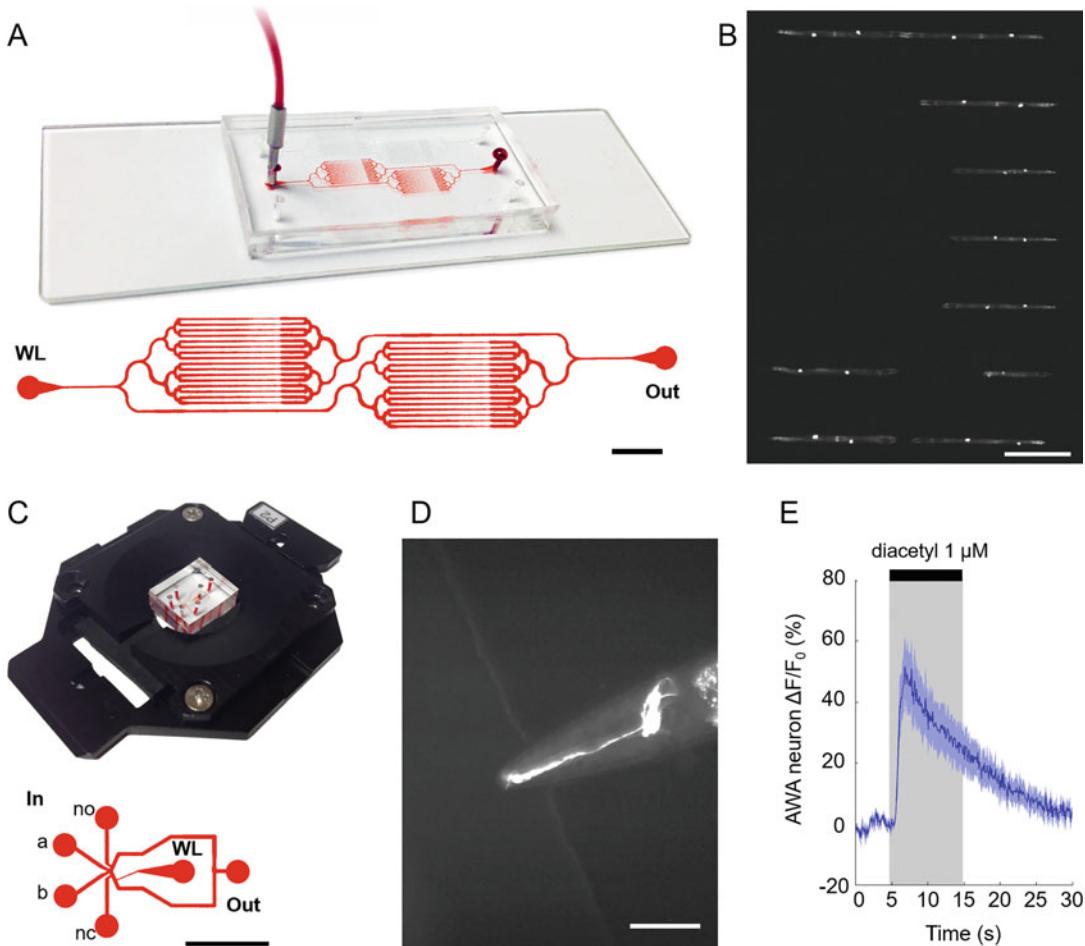
Here we describe the preparation and operation of several simple microfluidic *C. elegans* devices that are currently in use by many research labs. The first two devices are microfluidic arenas for observing locomotory behavior in response to precise and stable spatial patterns of chemical stimuli, either sharp or shallow chemical gradients. These are similar to plate-based chemotaxis assays, but with greater reproducibility and data content (9) (Fig. 1). These devices can be cleaned and reused dozens of times. The third and fourth devices are microfluidic worm traps for microscopy (10) and for high-resolution imaging of neural activity (11) using a fluorescent, genetically encoded calcium sensor (12) (Fig. 2). These devices are permanently bonded to a glass substrate, and most microfluidic systems are of this “single use” variety. All of these are fabricated from microfluidic molds prepared by photolithography in a cleanroom. Alternatively, lower-resolution devices can be created by cutting adhesive tape (xurography) with a consumer-grade craft cutter (13, 14), or even by hand (15), which can be useful for prototyping and educational applications. Two examples are included for monitoring individual animals in channels or in microwell arrays (Fig. 3). The following protocols are aimed at the user unfamiliar with microfluidic systems as well as the user who has experienced some difficulty in practice. While many details for preparing and operating microfluidic systems differ among published reports, here we highlight our preferred materials and methods and note alternatives when appropriate.



**Fig. 1** Microfluidic devices for quantifying behavioral responses to spatial stimulus patterns. **(a)** The “stripe” device presents two stable chemical stripes that flow from three inlet (In) ports to an outflow port (Out). Animals are loaded through a worm loading (WL) port. A completed device is shown clamped and mounted to the video capture stage (above). **(b)** A video frame showing 25 wild-type *C. elegans* animals responding to two horizontal stripes of the attractant isoamyl alcohol (IAA, 0.92  $\mu$ M top, 1.84  $\mu$ M bottom) (9). Inset shows a young adult animal moving around microposts. Scale bar, 500  $\mu$ m. **(c)** Quantitative behavioral data for chemotaxis index (the relative time spent inside versus outside the odor stripe, a measure of attraction to the stimulus) and for the change in speed upon exiting the odor stripe. Wild-type animals are attracted to the IAA odor and slow down upon exiting. Sensory mutant *tax-4*(*ks28*) shows no attraction or speed response to the odor, whereas glutamate-deficient *eat-4*(*ky5*) animals slow normally but do not reside in the odor due to the inability to turn appropriately (9). **(d)** The “gradient” device presents a symmetrical linear gradient from the midline to the outside edges from the inlet (In) ports to the outflow port (Out). This device contains two isolated arenas, separated by a worm barrier, to assess two animal populations at once. Animals are loaded through one of two worm loading (WL) ports. **(e)** A video frame showing a linear gradient across the device shown in **d**. Scale bar, 5 mm. **(f)** Chemical concentration across the vertical axis and corresponding histogram of animal position in response to attractive and neutral stimuli. Chemotaxis index (CI) calculated as the relative time spent inside the middle 50% band at higher concentration (dark gray) versus the outer regions at lower concentration (light gray)

## 2 Materials

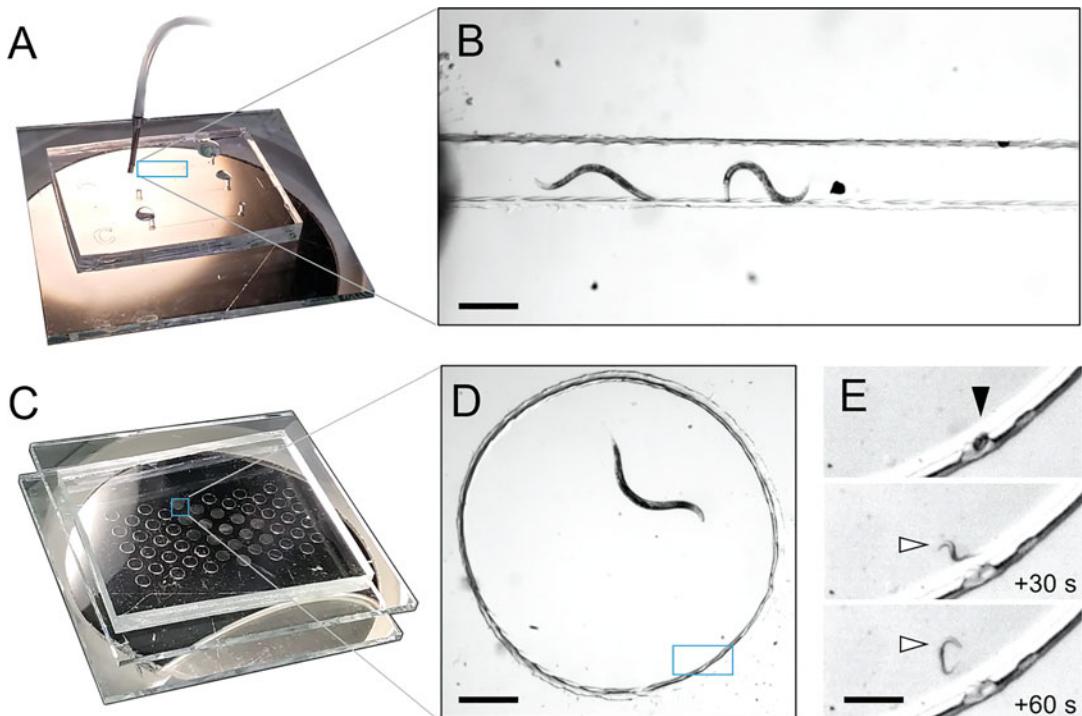
Microfluidic devices and accessories should be handled with gloves and pre-cleaned by washing in water, then 95% ethanol, then water again, and dried in an air or N<sub>2</sub> stream (see Note 1). All solutions are prepared in sterile worm buffer at room temperature, unless noted, using aseptic technique.



**Fig. 2** Microfluidic devices for animal trapping and alignment, time-lapse microscopy, and neural imaging. (a) A parallel array of 32 tapering animal traps (10) is loaded via a single worm loading (WL) port. Scale bar, 2 mm. (b) Animals are aligned and remain in a fixed position during time-lapse imaging. Two distal tip cells are shown labeled with GFP (strain JK2868). Scale bar, 0.25 mm. (c) A neural imaging device (11) mounted in a microscope slide holder. Fluid streams from inlet (a) or (b) pass over the animal's nose depending on which control channel (no) or (nc) is open. Scale bar, 2 mm. (d) AWA chemosensory neurons are shown expressing the calcium sensor GCaMP2.2b (strain CX14887). Scale bar, 50  $\mu$ m. (e) AWA neurons respond to addition of the odorant diacetyl (1  $\mu$ M) with increased relative fluorescence intensity ( $\Delta F/F_0$ ), indicating an “on” neural response (16)

## 2.1 Equipment

1. Vacuum desiccator.
2. Weigh balance (150 g range).
3. Magnetic stir plate.
4. Air or N<sub>2</sub> gun.
5. Micropipettes (10 or 20  $\mu$ L, 200  $\mu$ L, and 1000  $\mu$ L).
6. Serological pipettes (25 mL).
7. Lab oven (65 °C) with level shelves (check with bubble level).



**Fig. 3** Example microfluidic devices fabricated from stencil-cut adhesive tape on glass. **(a)** Three parallel 500  $\mu\text{m}$  wide channels are loaded with animals by tubing or by surface droplet. **(b)** Young adult animals exhibit swimming locomotion in 100  $\mu\text{m}$  tall channels shown, whereas <50  $\mu\text{m}$  tall channels enable crawling locomotion (not shown). Scale bar, 0.5 mm. **(c)** An array of 2 mm diameter chambers filled with a pipette and sealed with a hydrophobic glass slide. **(d)** Individual young adult animals can be imaged over time and analyzed for behavior, fluorescent markers, developmental events, or other responses. Scale bar, 0.3 mm. **(e)** At hour 2 of a 15 h time-lapse, an egg (filled arrowhead) hatched releasing the L1 animal (open arrowheads) in three images taken 30 s apart. Scale bar, 0.1 mm

8. Plasma bonding system; optional, if permanently bonding the microfluidic device to a glass substrate (*see Note 2*).
9. For rapid prototyping of adhesive tape mold masters, *see Subheading 2.7* for additional equipment.

## 2.2 Microfluidic Device Casting and Punching

1. Microfluidic master mold fabricated from plastic or a silicon wafer (*see Subheading 3* for sourcing) or adhesive tape (*see Subheading 3.8*).
2. PDMS: polydimethylsiloxane kit; e.g., Sylgard 184.
3. Nitrile or powder-free latex gloves (powder may inhibit PDMS curing).
4. 150 mm Petri Dish (*see Note 3*).
5. Large weigh boat.
6. Plastic transfer pipettes.

7. Stainless steel scalpel holder and No. 10 blade.
8. Single-edge heavy-duty razor blade.
9. Scotch Magic Tape (3 M cat. 810).
10. Dermal punch, 1 mm.
11. Dark fine-tip marker.
12. Self-healing cutting mat.

### **2.3 Microfluidic Device Accessories**

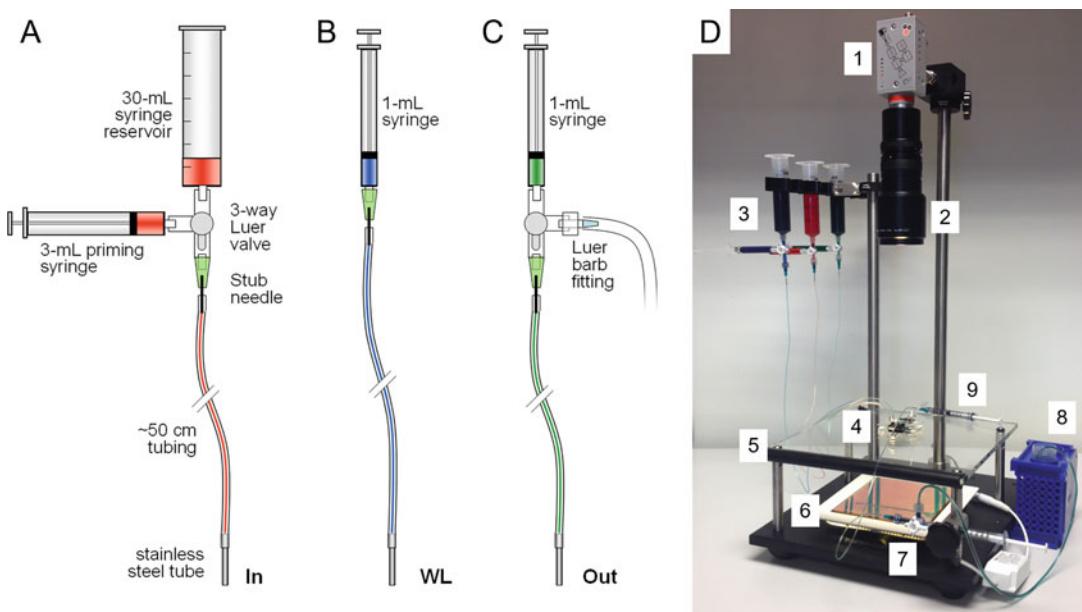
1. Glass slides, 1–1.5 mm thick, cut to desired dimensions if necessary (*see Note 4*) using a diamond-tipped scribe. Use cover slips (#2 or #1.5 thickness) for high-resolution microscopy applications.
2. TFOCS: (Tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane for preparation of hydrophobic glass. *CAUTION: TFOCS is corrosive and toxic and must be used in a fume hood.*
3. Dremel rotary tool with workstation stand and 5/64" diamond taper wheel point.
4. Metal clamp assembly, either: (a) Small 5/16" capacity binder clips, (b) Warner Instruments platform P-2 for small devices less than 20 × 20 mm<sup>2</sup>, or (c) a custom-fabricated device holder (*see Note 5*).

### **2.4 Solutions**

1. Worm buffer (*see Notes 6 and 7*): Dissolve 5.85 g NaCl, 1.0 g K<sub>2</sub>HPO<sub>4</sub>, and 6.0 g KH<sub>2</sub>PO<sub>4</sub> in 1 L deionized water. Check that pH is about 6.0 and adjust if necessary. Sterilize by autoclave.
2. Dye solution (100×): 10 mg/mL xylene cyanol in worm buffer (*see Note 8*). Dissolve 1.0 g xylene cyanol in 100 mL worm buffer and sterilize by autoclave or syringe-tip filter.
3. Loading buffer: 5% w/v Pluronic F127 with 0.5× dye solution. Prepare 1 mL aliquots and store at 4 °C.
4. Stimulus buffer(s): For example, a 1:10<sup>7</sup> dilution of isoamyl alcohol (IAA, 3-methylbutanol >99.8% purity) produces a robust attraction response (*see Note 9*).

### **2.5 Experimental Setup**

1. Tubing set (5 total): Insert the metal end of a blunt 23 gauge Luer stub adapter into one end of a 50 cm length of 0.020" inner diameter (ID) Tygon tubing (*see Note 10*). Insert a 1/2" (13 mm) long 19 gauge stainless steel tube about one-third of the way (4–5 mm) into the other end of the Tygon tubing as in Fig. 4a (*see Note 11*).
2. Solution reservoirs (3 total): Connect a 30 mL syringe and 3 mL syringe to a 3-way Luer stopcock valve as in Fig. 4a (*see Note 12*). Remove the plunger from the 30 mL syringe. Connect a tubing set (from item 1) to the valve.



**Fig. 4** Experimental setup for microfluidic flow via hydrostatic pressure. Tubing, syringes, and valves are shown for solution reservoirs (**a**), worm loading syringe (**b**), and outflow tubing (**c**). The complete video capture system (**d**) contains the (1) video camera, (2) zoom lens, (3) stimulus reservoirs, (4) microfluidic device, (5) glass stage, (6) LED backlight, (7) outflow syringe, (8) waste reservoir, and (9) worm loading syringe

3. Worm loading syringe: Connect a 1 mL syringe to one tubing set as in Fig. 4**b**.
4. Outflow tubing: Connect a 1 mL syringe, a tubing set, and a male Luer barbed fitting with ~50 cm of Tygon tubing (1/16" ID, 1/8" OD) as in Fig. 4**c**.
5. Reservoir rack and stand.
6. Solid 1/2" long 19 gauge stainless steel blocking pins (*see Note 13*).
7. Video capture system as in Fig. 4**d** (*see Note 14*).

## 2.6 Alternative Materials

1. *Tubing:* Tygon and other lab tubing can leach chemicals such as plasticizers that elicit a weakly attractive behavioral response in *C. elegans*. We have found this effect to be negligible when testing robust stimuli or when all inlet fluids flow at an equal rate. Alternatively, high-purity Teflon PFA tubing (e.g., Upchurch IDEX, 0.020" ID, 1/16" OD) does not elicit any behavioral response in our experience, although it is substantially more expensive. This tubing is rigid and can be inserted directly, without a metal tube, into a larger PDMS inlet port: in Subheading 3.1 step 10, use a 1.5 mm dermal punch. This tubing also requires specific fittings (IDEX LuerTight, P-835) to connect with syringe reservoirs.

2. *Automated valves*: Automated valves are useful for precise temporal delivery of stimuli, such as a sudden application or removal of a stimulus. We prefer electrically actuated, computer-controlled microvalves with low swept volume and an inert wetted path.

## 2.7 Materials for Rapid Prototyping of Adhesive Tape Molds

1. Electronic cutting tool (such as Silhouette Cameo) with computer and cutting software installed.
2. Cutting tool accessories: Premium ratchet blade (such as SILH-BLADE-PREM) and 12" × 12" adhesive cutting mat.
3. Glass slides, 1–1.5 mm thick (e.g., 25 × 75 mm, 50 × 75 mm, or 50 × 50 mm).
4. Adhesive tape of desired thickness (e.g., Scotch 311+, 50 µm thick).
5. Tweezers.
6. Metal ruler or other straight edge tool.

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## 3 Methods

This protocol assumes that the user has a microfluidic mold master (begin at Subheading 3.1) or pre-cast microfluidic devices (begin at Subheading 3.2). To obtain a photolithographic mold master, we recommend contacting the corresponding author of a publication presenting a microfluidic device of interest to request either a PDMS casting, a mold master, or the computer-aided design (CAD) file from which the master can be microfabricated. We encourage microfluidics-oriented labs to provide these resources openly or at moderate cost. There are a few publicly available resources for outsourcing the microfabrication of mold masters, including university-based and commercial vendors, although they tend to have fluctuating capabilities (for a list, see [albrechtlab.github.io](https://albrechtlab.github.io)). Most universities with engineering departments house a microfabrication facility, and many will either produce a mold master for nominal cost or provide training to users interested in learning fabrication methods. In-house photolithography may be feasible for some labs, requiring approximately \$25,000 in hardware and a cleanroom or hood. Fabrication steps are relatively quick (about 2–3 h) for simple device masters and are described in detail elsewhere [14–16].

Alternatively, microfluidic mold masters can be created by cutting adhesive tape on glass with a consumer-grade craft cutter (see Subheading 3.8). While these molds have lower geometric precision and stability than photolithographic molds, their rapid production (minutes versus hours) and far lower cost are useful for prototyping and educational applications.

### **3.1 Device Fabrication: PDMS Casting from a Mold Master**

1. Weigh a 10:1 (w/w) ratio of Sylgard 184 PDMS base to curing agent into a large weigh boat (*see Note 15*).
2. Mix PDMS base and curing agent thoroughly (*see Note 16*).
3. Degas the mixture in the vacuum desiccator for 30 min to 1 h to eliminate bubbles (*see Note 17*).
4. Clean the mold master, Fig. 5a, in an air stream if dust is visible (*see Note 18*).
5. Pour the PDMS mixture onto the mold master in a large (150 mm diameter) Petri dish (*see Note 3*), and fill to the desired device thickness, approximately 4–5 mm (*see Note 19*).
6. Inspect the poured PDMS and remove bubbles or dust (*see Note 20*).
7. Bake at 65 °C on a level shelf for between 3 h to overnight.
8. Remove the PDMS casting: Cut along the mold master perimeter with a metal-handled scalpel (*see Note 21*), gently peel up, and remove as in Fig. 5b–d.
9. Cut individual devices from the PDMS casting with a single-edge razor blade as in Fig. 5e (*see Note 22*).
10. Punch inlet and outlet holes (*see Note 23*) using a 1 mm diameter dermal punch as in Fig. 5f–h (*see Note 24*). Clean any debris remaining in the holes (*see Note 1*).

### **3.2 Device Fabrication: Glass Substrates**

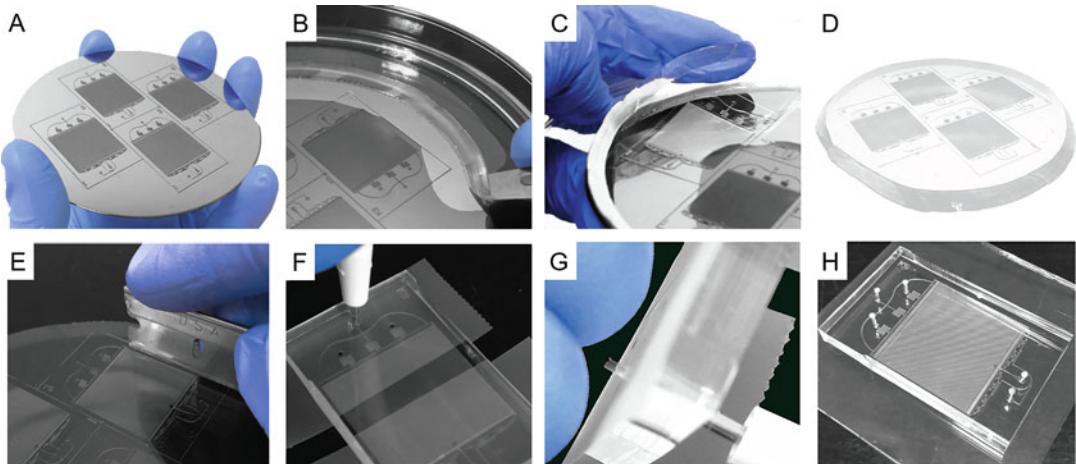
Microfluidic channels are formed upon sealing the PDMS casting to a flat substrate, usually a glass slide or cover slip. A reversible leak-resistant seal can be made by compressing the PDMS casting between a hydrophobic glass substrate and a second glass slide with holes drilled for tubing connections (Fig. 6a). This configuration is considered “reusable” as it can be opened after an experiment for cleaning and recovery of animals. However, it may leak under excessive fluidic pressure; thus, flow is best driven by hydrostatic pressure.

An irreversible leak-proof seal is prepared by plasma bonding a glass slide or cover slip to the PDMS casting as in Fig. 6b. Flow in bonded devices may be driven by pressurized reservoirs or syringe pumps without risk of leakage, and they are preferred for long-duration experiments and for high-resolution microscopy requiring a thin cover slip as the substrate. However, bonded devices are usually considered to be single use, as cleaning and animal recovery are difficult.

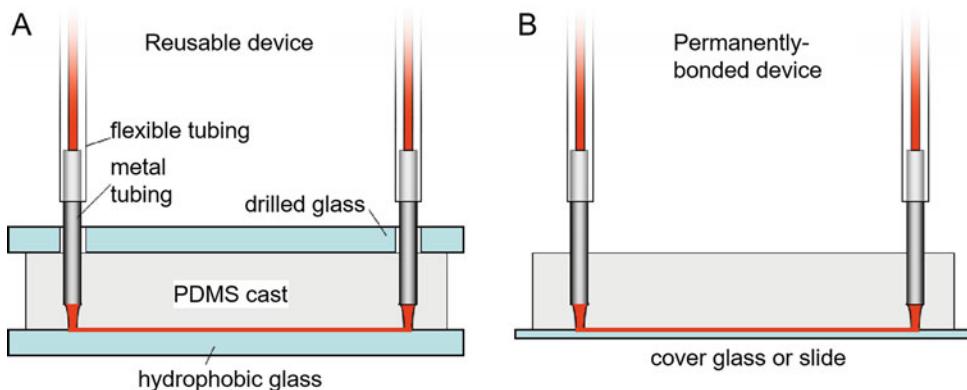
#### **3.2.1 Glass Plates for Reusable Devices**

Perform these steps only for reusable, reversibly sealed microfluidic devices as in Fig. 6a, then proceed to Subheading 3.3.

1. Prepare a hydrophobic glass slide slightly larger than the PDMS device (*see Note 4*) by TFOCS vapor deposition (*see Note 25*).



**Fig. 5** Device fabrication steps. PDMS polymer is cast over a mold master such as a micropatterned silicon wafer (**a**). The cast PDMS is cut out with a scalpel (**b**), peeled from the mold master (**c**, **d**), and trimmed into individual microfluidic devices with a razor blade (**e**). Inlet and outlet ports are punched (**f**), removing excess punched material (**g**). The microfluidic device is cleaned and ready for assembly or for permanent bonding to a glass substrate (**h**)



**Fig. 6** Cross-sectional view of a reusable microfluidic device (**a**) and a permanently bonded microfluidic device (**b**)

2. Drill inlet holes into a second glass slide of equal dimensions: Align the PDMS device on the glass and mark hole locations (*see Note 26*). Drill holes under constant water lubrication (*see Note 27*), and clean thoroughly (*see Note 1*).

### 3.2.2 Plasma Bonding

Perform these steps only for permanently bonded microfluidic devices (Fig. 6b).

1. Seal a clean, dry, dust-free PDMS device (*see Note 1*) onto a 2" × 3" glass carrier slide such that the micropatterned features face up.

2. Clean the glass substrate (cover slip or slide) with ethanol while rubbing with a lint-free wipe (e.g., Kimwipe). Dry with the air/N<sub>2</sub> gun and remove dust with tape. Transfer to the glass carrier slide and insert into the plasma vacuum chamber (*see Note 2*).
3. Expose to air plasma for 60 s (*see Note 28*).
4. Invert the slide or cover glass onto the PDMS device (*see Note 29*) and briefly apply gentle pressure (*see Note 30*).

### **3.3 Device Preparation and Assembly**

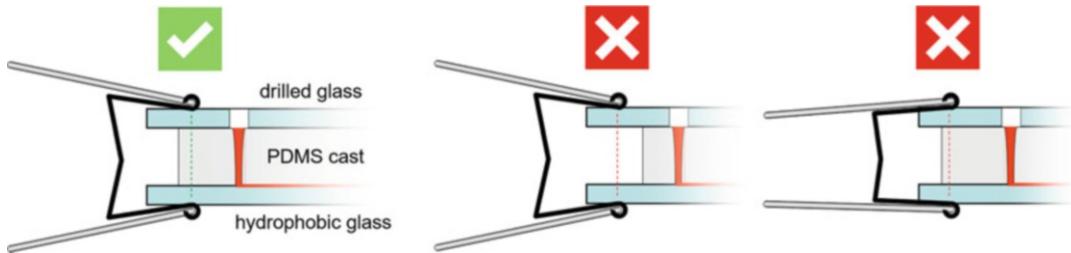
1. Before use, microfluidic devices should be cleaned by soaking in ethanol for several hours, rinsed with water (*see Note 1*), and baked for at least 1 h to evaporate residual ethanol (*see Note 31*).

*For permanently bonded devices, skip to step 5.*

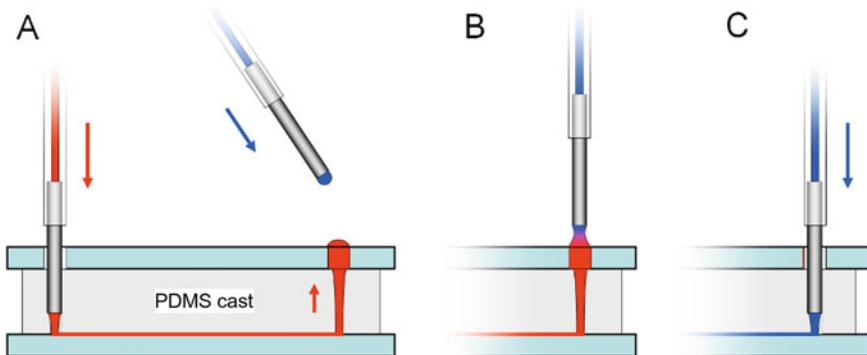
2. Seal a clean, dust-free microfluidic device against a clean, dust-free drilled top glass slide (*see Note 1*), aligning the drilled holes with the punched inlets. Remove any remaining dust with tape.
3. Seal a clean, dust-free hydrophobic glass slide against the micropatterned side of the device.
4. Clamp the device with binder clips as in Fig. 7 (or in a clamp apparatus, *see Note 5*). Holding the glass-PDMS-glass assembly firmly in the center, apply a binder clip to one side. Without releasing pressure, clip the opposite side. Test for proper compression force (*see Note 32*).
5. Block the worm loading port by inserting a solid blocking pin until it reaches ~2/3 (about 3 mm) through the thickness of the PDMS (*see Note 33*).
6. Degas the assembled microfluidic device in the vacuum desiccator for 10–30 min (*see Note 34*).

### **3.4 Device Filling**

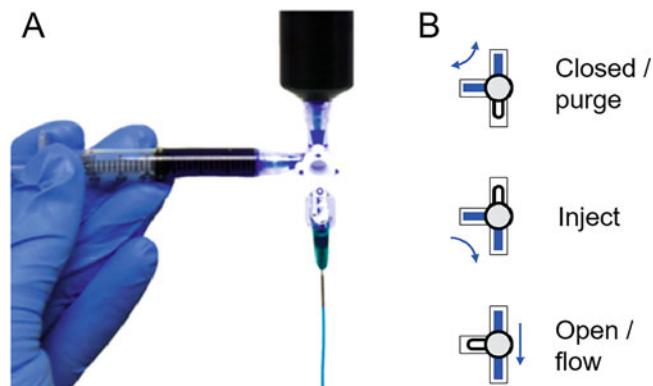
1. Prepare fluid reservoirs and tubing (as noted in Subheading 2.5). Position each reservoir on the rack and fill with stimulus or buffer solutions. Remove air bubbles (*see Note 35*) and fill the tubing (*see Note 36*).
2. Prepare outflow tubing. Fill with buffer, then draw 1 mL loading buffer into outflow syringe.
3. Remove the device from vacuum and connect the outflow syringe to the outflow port (*see Note 34*).
4. Gently inject loading buffer until it has completely filled the arena and begins to emerge as a droplet at an inlet.
5. Connect the buffer tubing to the inlet hole using a “drop-to-drop” connection as in Fig. 8 and aspirate any excess liquid (*see Note 37*).



**Fig. 7** Proper positioning of binder clip clamps (left). Avoid clamping where the glass slides are unsupported (center) or with a binder clip that is too small (right), as excess stress may crack the glass slide

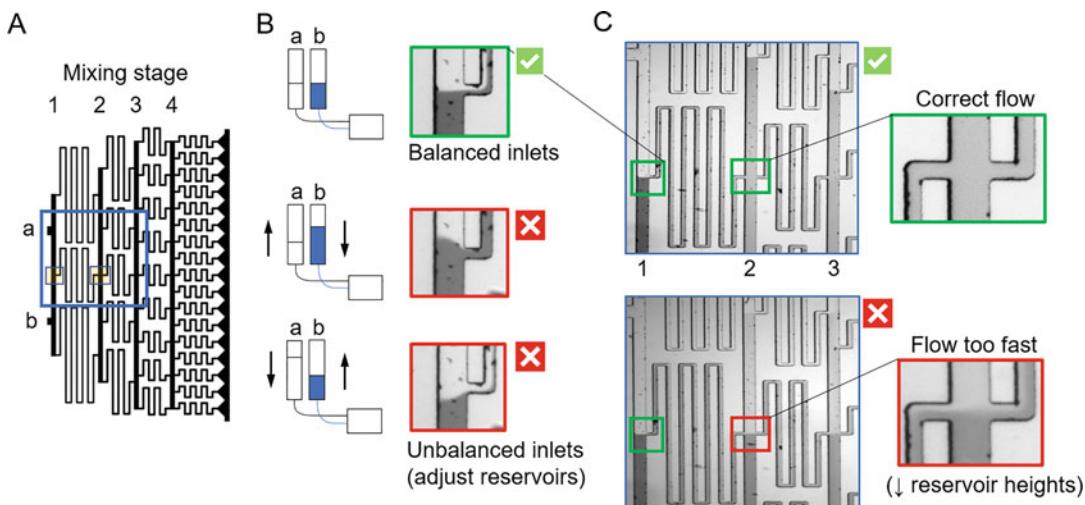


**Fig. 8** Bubble-free, “drop-to-drop” insertion of tubing into a port on microfluidic device. Allow liquid to flow in the device (red) until a drop appears at the port, and inject liquid in the tubing to be inserted (blue) until a droplet hangs from the tubing pin (a). Bring the droplets into contact (b) and insert pin about 2/3 into the port (c)



**Fig. 9** (a) Solution reservoir filled with dye and valve in the Closed/Purge position. (b) Valve positions used to control fluid flow during operation of the microfluidic device. Arrows indicate fluid flow direction

6. Open the buffer valve briefly (turn to “Flow” position, Fig. 9) until another inlet hole fills, then insert the next stimulus tubing with a “drop-to-drop” connection (*see Note 38*). Repeat for all inlet reservoirs.



**Fig. 10** Flow adjustments to achieve a linear chemical gradient with the “gradient” microfluidic device (Fig. 1d). **(a)** Subsection of upstream channels showing four numbered mixing stages. Fluids entering at inlets “a” and “b” converge at the center of stage 1 (left box) and should fully mix by the end of the serpentine channel (right box). **(b)** Balance inlet flows at the stage 1 center channel (left box in **a**). Lower the reservoir of inlet fluid that dominates in an unbalanced flow condition (red; middle and bottom) to achieve balanced flow (green, top). **(c)** Ensure complete mixing in the serpentine channel by checking at the center of stage 2 (right boxes in **a** and **c**). No gradient should be visible when flow is sufficiently slow (green box, top). Lower both reservoir heights if a gradient is visible due to fast flow and insufficient mixing (red box, bottom)

7. Once all ports are connected to tubing (or blocked), open a buffer valve and the outflow valve to flush the arena with worm buffer and displace the loading buffer (*see Note 39*).
8. Position the microfluidic device on the observation stage (*see Note 40*).
9. Adjust the relative heights of the stimulus reservoirs to establish the desired spatial pattern. Fluid flowrate is proportional to the height difference between fluid levels of the inlet reservoirs and outlet waste reservoir (*see Note 41*). For a stripe pattern device (Fig. 1a–c), raising a reservoir increases the width of the corresponding fluidic stripe. For a gradient pattern device (Fig. 1d–f), a balance of reservoir heights is necessary to maintain correct mixing for a linear spatial gradient (Fig. 10). Establish proper fluid flow prior to animal loading to avoid premature stimulus exposure.

### 3.5 Worm Loading and Device Operation

1. Pick 1–50 young adult *C. elegans* (*see Note 42*) onto an unseeded 60 mm agar plate (*see Note 43*).
2. Pour ~5 mL worm buffer onto the plate.
3. Fill the 1 mL worm loading syringe with worm buffer, purge any bubbles, and draw animals into the tubing (*see Note 44*) using a minimal volume of buffer (*see Note 45*).

4. Close the outflow valve, remove the blocking pin from the worm loading port, and briefly open the buffer valve to fill it with a buffer drop. Insert the worm loading tubing with a “drop-to-drop” connection (*see* Fig. 8).
5. Open the buffer and outflow valves (Fig. 9, “Flow” position) and gently inject animals into the arena (*see Note 46*).
6. Once all animals have entered the arena, pinch the worm loading tubing and clamp it with a mini binder clip to prevent flow into this channel.
7. Allow animals to disperse and adapt to the micropost environment for several minutes before initiating a recording and opening the stimulus valves (*see Note 47*).
8. Record animal behavior for 30–120 min (*see Note 48*) and analyze as desired, for example using “ArenaWormTracker” software (*see Note 49*) (9).

### *3.5.1 Troubleshooting Device Operation, Common Problems, and Recommended Solutions*

1. *Bubbles in a microfluidic channel or arena.* If the device was recently degassed, small bubbles will absorb into the PDMS (*see Note 34*). Wait 5–10 min and see whether the bubble has gotten smaller. If not, open the device, clean it, and set it up again as in Subheadings 3.3 and 3.4.
2. *Bubbles in inlet tubing.* Halt flow immediately by closing the outflow valve and pull out the tubing containing bubbles. Purge the bubbles by forcing them out with the syringe (valve position “Inject,” Fig. 9), then reinserst using drop-to-drop contact (*see* Fig. 8).
3. *Bubbles or air in outflow tubing.* Small bubbles in the outflow tubing are ok and useful to estimate fluid flowrate (*see Note 41*), but large air-filled regions may slow gravity-driven flow. Be sure to fill the tubing completely to the waste beaker using the outflow syringe.
4. *Leakage at the fluorinated glass slide.* A poor channel seal may be caused by insufficient clamp pressure (*see Note 32*), insufficient flatness of the PDMS device due to ethanol swelling (*see Note 31*), or a fabrication defect. Increase the compression force, evaporate longer in the oven, or use a new, flat device.
5. *Leakage at an inlet.* A poor seal at an inlet port may be due to insufficient tube insertion depth (ideal is ~2/3 of the port depth as in Fig. 6) or to poor punch quality. The inlet port should be punched straight with smooth walls. Damaged punches may cut a notch along the hole causing a slow fluid leak (*see Note 24*).
6. *Dust or particles in the microfluidic channels.* Clean devices thoroughly (*see Note 1*) and assemble quickly or in a clean

hood. Wash inlet holes after punching. Filter solutions if necessary.

7. *Warped stimulus pattern.* Ensure no debris or bubbles disturb the flow path. If binder clips clamp too strongly and reduce channel depth at the edges, horizontal fluid stripes will bow toward the center.
8. *Fluid flow is too fast or too slow.* Adjust the height difference between reservoirs and waste beaker. For a height difference of 50 cm, flowrate in the behavioral arena is typically  $\sim$ 0.8  $\mu$ L/s. Flowrate can be estimated as described in **Note 41**.

### **3.6 Device Cleanup and Storage**

1. Shut off all valves (Fig. 9, “Closed” position) and remove tubing from all ports.
2. Rinse all syringe reservoirs and tubing with water and blow dry with an air or N<sub>2</sub> gun (*see Note 50*). Eject remaining liquid in worm loading tubing, rinse with water, and dry.
3. For reusable devices, open the microfluidic assembly, separate the PDMS from glass slides, and optionally recover animals to a seeded agar plate by rinsing the PDMS and glass with worm buffer.
4. Rinse the PDMS with water (*see Note 1*) and soak it overnight in ethanol to remove any adsorbed stimulus (*see Note 51*), then clean (*see Note 1*) and bake for at least 1 h to evaporate residual ethanol (*see Note 31*).
5. Devices may be stored at room temperature or in an oven at <65 °C, and reused dozens of times.

### **3.7 Microfluidic Devices for Microscopy and Neural Imaging**

#### **3.7.1 Parallel Trap Array Device**

The methods described above for fabricating, cleaning, assembly, filling, loading, and operating microfluidic devices are general and can be used with most designs. Below, we describe two simple devices for arraying many animals in parallel for time-lapse microscopy (10) (Fig. 2a, b) and for high-resolution imaging of neural activity (11) (Fig. 2c–e).

This device aligns many animals within tapering channels for ease of imaging by microscopy. It is conveniently loaded through a single worm loading port and is often used for time-lapse microscopy in which individual animals can be observed over many hours.

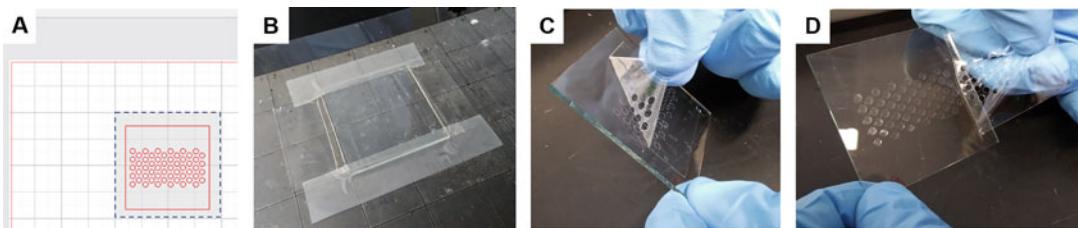
1. Prepare the trap array device (Fig. 2a) as described in Subheadings 3.1–3.4 and fill with worm buffer from the outflow port (*see Note 52*).
2. Draw animals into worm loading tubing and inject them into the channels as in Subheading 3.5. Ensure the outflow valve is open during worm loading.

3. Once animal traps are filled, close the outflow valve, remove the worm loading tubing, and replace it with a buffer reservoir (*see Note 53*). Open the outflow valve to initiate buffer flow.
4. Monitor changes in fluorescent markers (such as position or intensity) over time (Fig. 2b).

### 3.7.2 Neuronal Imaging Device

Calcium imaging studies determine a neuron's response to a sudden presentation or removal of a chemical stimulus. This device traps a single animal such that its nose protrudes into a channel that is rapidly switched between two fluidic streams. Recent modifications of this design increase the number of animals and stimuli tested per experiment (16, 17) or enable neural imaging in freely moving animals to correlate neural and behavioral responses (16).

1. Prepare the imaging device (Fig. 2c) as described in Subheadings 3.1–3.3 and fill with worm buffer from the outflow port (*see Note 52*).
2. Prepare two stimulus reservoirs and tubing as in Subheading 3.4. Typically, one reservoir contains worm buffer (a), and the other a chemical stimulus (b).
3. Prepare a control fluid reservoir connected to the common port of a 3-way actuated microvalve. Connect tubing sets to the normally open (no) and normally closed (nc) ports on the microvalve. Fill the reservoir with worm buffer (*see Note 54*) and prime the tubing (*see Notes 35 and 36*).
4. Connect all four inlet tubes to the inlet ports via drop-to-drop connections (*see Note 55*).
5. Inject one animal into the worm loading port. Observe the animal under the microscope, providing pressure from the worm loading syringe as necessary until it reaches the tapered clamp with its nose just protruding into the fluidic channel as in Fig. 2d.
6. Focus the objective on the neuron of interest under fluorescent excitation (*see Note 56*) and set up an acquisition stream (*see Note 57*).
7. Acquire video for 30 s to 3 min, actuating the microvalve (*see Note 55*) at the desired time and duration to apply a stimulus pulse, as in Fig. 2e.
8. Analyze neuronal fluorescence over time, for example using NeuroTracker (16) software (*see Note 49*). Display results as relative fluorescence change ( $\Delta F/F_0$ ) where  $F_0$  is the baseline fluorescence and  $\Delta F(t) = F(t) - F_0$ .



**Fig. 11** Preparation of an adhesive tape mold by xurography using an electronic craft cutter. **(a)** Design of a microwell array as it appears in the cutter software. Dotted line indicates the  $50 \times 50$  mm glass slide and cut lines are shown in red. **(b)** Taped glass slide secured to the cutting mat in the position indicated in software (panel a). Note the upper left corner is positioned 2" from the left edge and 1" from the upper edge, as in the software image. **(c)** Removal of excess tape from the glass slide by slowly peeling with tweezers or fingers. **(d)** Ensure the excess tape lifts cleanly free of the cut mold features

### 3.8 Rapid Prototyping of Microfluidic Mold Masters by Xurography

1. Clean hydrophobic TFOCS-treated glass slides (*see Note 25*) with 70% ethanol, a lint-free wipe, and compressed clean dry air.
2. Apply one or more layers of adhesive tape to the cleaned slides to build up the desired feature height (*see Note 58*). Use a metal ruler or straight edge to apply tape under tension to avoid trapping bubbles.
3. Align the taped glass on the adhesive cutting mat according to the grid marks (Fig. 11a, b). Secure using additional tape around the perimeter.
4. Import a vector graphics file to the craft cutter software or draw the pattern outline using the software tools. Scale the cutting design to desired dimensions and ensure it is located within the grid region that corresponds to the taped glass slide (*see Note 59*). Channels 500  $\mu\text{m}$  wide can be reliably fabricated (Fig. 3a).
5. Set the cutting parameters according to the cutting tool model and tape thickness (*see Note 60*).
6. Initiate cutting and monitor blade progress. Optionally, perform an initial test cut to check that tape has been cleanly cut, and adjust settings as needed (*see Note 61*). Once complete, unload the mat from the rollers and remove the glass substrate.
7. Use tweezers to remove excess tape regions leaving behind the raised microfluidic features (Fig. 11c, d). Tape should separate easily at cut lines with a sharp blade and correct settings (*see Note 61*).
8. Place the patterned tape-on-glass master at the bottom of a plastic petri dish and cast PDMS as described in Subheading 3.1 (*see Note 62*).
9. Assemble device as in Subheading 3.3 and perform experiment (Fig. 3).

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## 4 Notes

1. All components should be stored clean, dry, and dust-free in closed containers. Rinse microfluidic devices and glass substrates first with deionized water to prevent crystallization of salts within microchannels or tubing. Flush inlet ports using squirt bottles, ensuring the fluid stream passes through each inlet. Next, clean with ethanol, wiping away any debris and smudges with gloved fingers. Finally, rinse with water and quickly blow dry with an air or N<sub>2</sub> gun. This step prevents evaporation which may leave a residual film that is difficult to remove, especially from microfluidic channels. Remove surface dust using tape.
2. Alternative and less expensive PDMS plasma bonding methods have been reported, although they are generally less reliable and more sensitive to exact treatment conditions. These include a hand-held corona discharge wand ([18](#)) and an evacuation chamber placed in a standard microwave oven ([19](#)).
3. A disposable PDMS casting dish can be made from aluminum foil. We prefer a large 150 mm Petri dish as it has a flat and level bottom, comes with a lid, and protects the wafer from damage during handling and storage.
4. Trim glass slides as necessary to maintain about a 2–5 mm border around the PDMS microfluidic device. Use a 1.5 mm thick glass slide for devices larger than ~25 mm × 25 mm. Align a ruler along the desired cut line, firmly score once with a sharp diamond scribe, and snap the glass by applying evenly distributed pressure on each half. For best results, cut at least 12 mm away from any edge. With proper pressure, the score line should be thin and barely visible.
5. We custom machined a clamp from 1/4" thick aluminum bar, four 1/4"-20 bolts, and four springs. Two plates each contain a central imaging window and four holes in the corners; the bottom plate holes are tapped with 1/4"-20 threads while the top holes allow free movement of the bolts. The microfluidic device is held between the top and bottom plates by the bolts that compress springs. To assemble, place and center the microfluidic assembly into the device holder, then evenly tighten the four screws in a cross-diagonal manner to prevent uneven point stresses.
6. Our preferred worm buffer is “S Basal” without cholesterol, which sustains healthy animal behavior for long durations and is easy to prepare. A common alternative is “M9 Buffer”: Combine 5.0 g NaCl, 3.0 g KH<sub>2</sub>PO<sub>4</sub>, 6.0 g Na<sub>2</sub>HPO<sub>4</sub>, and 0.25 g MgSO<sub>4</sub>·7H<sub>2</sub>O in a beaker and add water to 1 L. Sterilize by autoclave.

7. Some chemicals may react with phosphate buffer, such as copper chloride. Choose an alternate buffered saline solution if necessary.
8. Wild-type worms may be weakly attracted to  $1\times$  xylene cyanol in the absence of other stimuli, but the dye usually does not affect behavior to a moderate stimulus. Nonetheless, it is beneficial to repeat experiments without dye to verify minimal contribution of the dye under the conditions tested.
9. Odor dilutions should be freshly prepared each day. IAA is hydrophobic, low density, and weakly soluble in aqueous buffers. Therefore, it rises to the liquid surface and adsorbs to the vial walls and to the pipette tip. We prefer the following dilution procedure: First prepare a  $1:10^3$  dilution by adding 30  $\mu\text{L}$  IAA to 30 mL worm buffer in a pre-cleaned 40 mL amber vial (ESS Vial). Pipette repeatedly to rinse the pipette tip, then quickly seal and vortex for 30 s. Transfer 3  $\mu\text{L}$  diluted IAA to a second vial containing 30 mL worm buffer for a  $1:10^7$  final dilution; if desired, include 0.5–1 $\times$  Dye solution for visualization.
10. Be careful not to gouge the inner surface of the tubing, which may result in a clog or poor seal.
11. Using gloves for improved grip, angle the metal tube into the Tygon tubing until it stretches over the metal tube. Next, push the Tygon tubing down along the metal tube while angling it back and forth. Then grip the Tygon tubing tightly at the metal tube and push firmly down on a cutting mat until it stretches about 1/3 of the way down the 1/2" long metal tube (4–5 mm).
12. For longer duration experiments, 60 mL syringes or larger reservoirs with Luer fittings may be used.
13. Blocking pins can also be created from stainless steel tubes (NE-1027-12) by applying a droplet of epoxy to one end to seal.
14. Video capture can be performed with a camera attached to the trinocular port of a microscope with a transmitted light base. Alternatively, a less expensive setup can be assembled from a camera, zoom lens, and support stand, for example as described at [albrechtlab.github.io](https://albrechtlab.github.io).
15. This is a messy process. Line the work area with disposable paper, use clean gloves, and be careful to prevent spills, as it is difficult to clean off the highly viscous and oily PDMS materials. We prefer to use a large disposable weigh boat containing 100–120 g PDMS for mixing by hand, as it is easier to ensure complete mixing compared with a cup with tall sides. It is convenient to weigh the viscous base PDMS on a balance,

then tare the balance and pour the curing agent to 1/10 (for 10:1 ratio) of the base weight. An exact 10:1 ratio is not critical, as PDMS will cure properly with a range of base to curing agent from 5:1 to 20:1.

16. We use a transfer pipette or plastic fork to mix PDMS for at least 1 min, using a folding motion to combine the viscous base component with the low viscosity curing agent. Many air bubbles should be generated with proper mixing vigor. Insufficient mixing may result in partially uncured PDMS, an unusable device, and/or a messy mold master.
17. To accelerate this process, briefly vent the chamber and reapply vacuum once or twice.
18. Remove any surface smudges with an isopropanol wash, then water, then air dry. A release agent should be applied to new, untreated mold masters. For silicon wafers, apply TFOCS as described in **Note 25**.
19. To determine the correct PDMS fill volume, first fill with water to the desired depth and note the weight of added water. After drying the mold, add the same weight of PDMS (density 0.97 g/mL is nearly equal to water). For 4–5 mm depth, we fill 85 g PDMS in a 150 mm Petri Dish and 45 g in a 4" diameter foil boat. To reduce bubble formation, pour quickly with the weigh boat about 1–2 cm above the mold surface while moving linearly across the mold to prevent the PDMS stream from folding upon itself and forming air bubbles.
20. Dust can be carefully removed with a transfer pipette and bubbles on the master surface can be dislodged by gently and slightly tilting the dish back and forth, allowing for viscous shear forces to release the attached bubbles. Bubbles that have risen can be removed by blowing lightly on the PDMS surface.
21. To cleanly cut out the casting, firmly grip the scalpel and insert into the PDMS at an outward angle near the edge of the mold master wafer. Then, making contact between the blade and the wafer, rotate the dish such that the blade follows the perimeter of the wafer making one continuous circumferential cut.
22. If individual devices are outlined with microchannels, it is convenient to align the single-edge razor by feeling for when it falls into the groove. Then, ensure the razor is vertical and press down firmly through to the cutting mat.
23. The punched inlet channel will taper in the direction of punching. Alignment of the inlet hole and the microfluidic features is easier when punching *from* the micropatterned side of the PDMS cast. However, we prefer punching *toward* the micro-patterned side for ease of tubing insertions. Tape and mark each inlet with a fine-tip marker on the micropatterned side,

then insert the dermal punch from the opposite side, keeping it straight and vertical. After completely punching through the device, remove the excess PDMS from the punch before pulling it out of the device (Fig. 5g).

24. Holes punched with a damaged or dull dermal punch are prone to leakage. It is advisable to punch a test hole and observe under magnification for smooth round edges free of nicks. Use a new punch if necessary.

25. Render glass and silicon wafer surfaces hydrophobic by vapor deposition of TFOCS. Place cleaned substrates in a vacuum desiccator and add 40  $\mu\text{L}$  of TFOCS to a small aluminum foil dish in the center. Treat for 1 h, then clean with isopropanol, then water, then dry in an air or  $\text{N}_2$  stream.

*CAUTION: TFOCS is corrosive and toxic.* Perform all steps in a fume hood.

26. Mark the drill locations on tape that is applied to the glass, to prevent the ink from washing off during drilling.

27. Hold the glass slide securely on a plastic multi-well (e.g., 96-well) plate, centering the drill hole over a well. This provides support beneath the glass during drilling. Align the bit to the mark, apply water as lubricant and coolant, and apply gentle pressure to grind the glass at 15,000 rpm. Ensure that the bit and glass are wet at all times, or the bit will very quickly become hot and damaged. Continue grinding, releasing pressure every 3–5 s, until the hole is complete.

28. Turn on the vacuum pump and plasma power. After 10–30 s, a plasma should be visible in the vacuum chamber (purple/orange glow). Adjust the gas needle valve until the plasma appears bright red-orange due to the presence of oxygen and nitrogen gases. If no plasma is visible or it appears dim purple, pressure may be too low (open the needle valve slightly to introduce air) or too high (close the needle valve and wait for pressure to decline).

29. Plasma-activated PDMS and glass will form a covalent bond on contact, with no opportunity to reposition them. Carefully align the glass over the microchannel surface, and gently release the glass substrate upon initial contact to prevent internal stresses.

30. Gentle compression for about 10 s ensures a good bond and forces trapped air bubbles out of the device. Test for a successful bond by gently peeling up at each corner of the PDMS; the PDMS device should remain sealed against the glass substrate.

31. Ethanol causes PDMS to swell by 4% (20). Ensure that all ethanol is evaporated by baking at 65 °C for at least 1 h or at room temperature overnight before proceeding with assembly.

Insufficient baking will result in swelling in the center of the device and a poor fluidic seal. Ethanol remaining inside PDMS can be visualized as a cloudy white haze after soaking in water or buffer; if this is observed, additional evaporation time is required.

32. Binder clips should be expanded by bending them open until a 3 mm gap remains at rest (such as by clipping a wide pen barrel and forcing the clip spine toward the barrel). Binder clips should be positioned over a supported PDMS edge without blocking the imaging area, as in Fig. 7. Avoid clamping at an unsupported region of glass or with a clamp that is too small, as stresses may cause the glass to break. The binder clips should grip firmly enough to remain in place but loose enough to be repositioned by sliding along the glass.
33. Fluid will initially flow to the port of least fluidic resistance, often the closest port. It is therefore helpful to block any open ports near the filling port with blocking pins (*see Note 13*) to ensure rapid filling of the entire fluidic network.
34. Air bubbles that form upon initial filling of a microfluidic device are absorbed into degassed PDMS. The rate and capacity of bubble absorption increases with time under vacuum and decreases with time outside the vacuum desiccator. Therefore, initial filling of the microchannels with liquid should occur quickly after removing the PDMS device from vacuum, ideally within a few minutes.
35. Remove bubbles from the 3 mL priming syringe by setting the valve to the “Purge” position (Fig. 9), slowly drawing liquid from the 30 mL reservoir, then quickly injecting it back. After 2–3 cycles, all bubbles should be purged.
36. To avoid bubbles trapped in the Luer stub, first ensure that the Luer stub and tubing are dry. Then, rotate the valve to the “Inject” position (Fig. 9) and slowly inject fluid filling the Luer fitting and tubing. If any bubbles are seen in the Luer fitting, flick the fitting while injecting to dislodge and purge them. It is important to remove these air bubbles to avoid their entry into the microfluidic channels during an experiment.
37. It is helpful to set up a vacuum line to aspirate excess liquid on the microfluidic device. Set up a vacuum trap (side-arm flask) and tubing terminated with a male Luer barb and a Luer stub needle (or a 200  $\mu$ L micropipette tip, trimmed to fit).
38. It is best to flush out loading buffer from the inlet ports, as the Pluronic surfactant reduces the grip between the inlet hole and the metal tube. Flushing will reduce the chance of an inlet tube popping out during an experiment.

39. We advise monitoring the tubing connections and glass substrate for droplet formation indicating a potential leak, especially for a new device. Adjust tubing or clamp tension as needed, or reassemble the device, if a leak occurs.
40. It is convenient to support the microfluidic device on the glass observation stage with four clean scrap PDMS pieces, each about  $5 \times 10 \times 10 \text{ mm}^3$ . These supports seal to the stage and to the bottom of the microfluidic device assembly and hold it securely during an experiment (*see* Fig. 1a). Ensure that the scrap PDMS supports are all equal height and do not obscure the arena.
41. To measure total flowrate while all valves are open, briefly remove and reinser the outflow tubing from the outflow port to introduce a small bubble. Measure the time for the bubble to move 1 cm down the outflow tubing to calculate flowrate (0.02" ID tubing contains 2  $\mu\text{L}/\text{cm}$  of length; a bubble moving 1 cm in 5 s indicates a flowrate of  $2 \mu\text{L}/5 \text{ s}$  or  $0.4 \mu\text{L}/\text{s}$ ).
42. Wild-type animals are conveniently picked at L4 larval stage 16–24 h prior to loading as young adult animals.
43. To avoid damaging animals, it is helpful to identify the focal plane of the agar surface with a small mark or bacteria using a worm pick. It is unnecessary to wash animals before transfer to this plate, as any residual bacteria will be washed away in the microfluidic device.
44. Avoid drawing up air bubbles by keeping the metal tube submerged. Ensure that no animals enter into the syringe barrel by drawing up less volume than the tubing (~100  $\mu\text{L}$  for a 50 cm length).
45. After collecting animals into the worm loading tube, deposit them slowly into a small region of the plate and draw them up again into a minimal volume. This process speeds loading of animals into the arena. Draw up enough buffer to observe the last animal in the clear tubing, to ensure that a “drop-to-drop” contact can be made at the worm loading port without losing any animals.
46. Observe animal loading into the arena during gentle injection to ensure they are not damaged. Animals that remain stationary after fast injection may have sustained physical damage.
47. Wild-type animals move slowly for the first 10–15 min in the device. It is beneficial to flush the arena with buffer during this time to wash away any residual bacteria. Verify correct valve positions before initiating the experiment.
48. We typically record at 2 frames per second at a camera resolution of about 40 pixels/mm. Adjust arena position and lens

zoom such that the recorded image is level and fully contained within the video frame. Adjust illumination brightness, camera gain, and/or acquisition time such that animals have good contrast and no pixels are saturated.

49. Commercial and open-source software packages are available for analysis of microfluidic experiment data. For example, ArenaWormTracker quantifies locomotion behavior and Neuro-Tracker quantifies neural responses, available at [albrechtlab.github.io](https://albrechtlab.github.io)
50. Buffer salts that crystallize in tubing may be difficult to remove. Flush with water and air dry by blowing with compressed air after experimentation.
51. Hydrophobic stimuli can leach into PDMS. We submerge devices in a stirred ethanol bath overnight, especially after experiments using high concentrations of hydrophobic stimuli.
52. A surfactant such as Pluronic F127 is not necessary in the loading buffer if the microfluidic channels are narrow. However, Pluronic F127 also prevents protein and bacteria adsorption to the channel surfaces, and should be included if animals are fed bacterial food in the device.
53. Slow buffer flow helps to keep animals in the tapered clamps during time-lapse recordings. For long-term culture in traps, animals may be fed with bacteria.
54. It is useful to add a dye to the control fluid such as 1 µg/mL fluorescein to visualize and confirm proper fluid flow and operation of the valve.
55. When inlets are configured as in Fig. 2c, the animal is subjected to stimulus “a” when the valve is at rest and to stimulus “b” when the valve is energized (11).
56. Minimize photodamage and photobleaching by limiting exposure to excitation light and reducing intensity with a neutral density filter.
57. Typical GCaMP recordings are made at ten frames per second. Use a reduced region of interest (ROI) and/or image binning to reduce the acquisition file size.
58. To apply adhesive tape without bubbles, it is convenient to place the clean glass slide(s) on a benchtop, then affix about 5 cm tape directly onto the benchtop, and guide the tape downward onto the glass slide with a ruler or straightedge. Use a blade to free the taped glass from the bench, leaving tabs on the taped glass to help secure it to the cutting mat.
59. Ensure the blade does not travel over the edge of the slide during cutting, otherwise it may become dull.

60. Example parameters for cutting one 50  $\mu\text{m}$  tape layer using the Silhouette Cameo are: force setting 6; speed 3; passes 4; blade depth 6. For two layers (100  $\mu\text{m}$  total), increase force to 9 and passes to 5.
61. If the blade setting is too deep or the force is too high, the blade can dull and scratch the glass. When experimenting with different tape thicknesses and layers, begin with lower settings and iteratively increase the depth and force until a clean cut is produced and cut tape regions separate cleanly.
62. After PDMS has cured and devices are peeled from the mold, check if any tape features came off the mold. Remove any tape in the PDMS channels with tweezers, and prepare new molds as necessary.

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# Chapter 17

## Neuronal Microsurgery with an Yb-Doped Fiber Femtosecond Laser

Maria B. Harreguy, Tracy S. Tran, and Gal Haspel

### Abstract

Laser microsurgery allows the user to ablate cell bodies or disconnect nerve fibers by using a laser microbeam focused through a microscope. This technique was pioneered in *C. elegans* where it led to exciting discoveries in the fields of development and neurobiology. All neurons studied so far in *C. elegans* can regenerate and regrow axons and dendrites after injury, allowing studies of the molecular and cellular basis of neuroregeneration. In this chapter, we describe how to assemble and operate a platform for Yb-doped fiber laser microsurgery. The novel laser setup described here is a more robust, lower cost, and user-friendly alternative to other femtosecond-pulsed laser systems.

**Key words** Laser, Microsurgery, Axotomy, Dendrotomy, Regeneration, Injury, Microscopy

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### 1 Introduction

Ablation of cells or cell parts such as nerve fibers with a laser microbeam has proven to be a useful tool to address questions of necessity and regeneration in multiple fields. Laser microsurgery has been widely used in *C. elegans* since it was first described by John White [1] in 1980 and was instrumental in multiple discoveries such as details of cell–cell interactions [1], the elucidation of the touch sensitivity circuit [2], and the role of GABA in *C. elegans* nervous system [3]. More recently, in 2004, Yanik [4] showed that some *C. elegans* axons are capable of regenerating, which established *C. elegans* as a model for regeneration studies. Further, the technique can also help identify specific behaviors associated with injury in addition to providing insight about factors that affect regeneration [5, 6].

Laser microsurgery platforms have evolved significantly since the 1980s, most modern laser ablation platforms are one of two kinds, either nanosecond or femtosecond lasers [7]. Nanosecond

lasers are more suited for cell ablation experiments while femtosecond lasers can be used for both cell ablation and axotomy as they can be adjusted to generate large areas of damage or to be precise and dissect subcellular structures as the laser beam can be focused onto a point in the sample with minimal damage to surrounding tissues [8]. This difference is mainly due to the million-fold decrease in pulse width increasing the required energy per pulse by the same ratio. While femtosecond laser pulses are typically in the order of tens of nanojoules (nJ), nanosecond pulses are usually in the order of tens of millijoules (mJ). Because excess energy diffuses away from the injury spot, nanosecond-long pulses generate more damage surrounding the injury [6].

Most commonly used femtosecond lasers in the field are Ti:Sapphire lasers which produce near-infrared (NIR) pulses with energies up to 50 nJ, a center wavelength of approximately 800 nm, pulse duration of 100–200 femtoseconds, and repetition rates of 80 MHz [9]. However, Ti:Sapphire lasers need constant maintenance, are very susceptible to room environmental conditions such as high humidity and tend to have a very high cost. In this chapter, we describe how to assemble and operate a laser microsurgery platform using an Yb-doped fiber laser. The advantages of this type of laser are higher possible power, lower maintenance, smaller footprint, and air cooling [10].

The specific Yb-fiber system described here (BlueCut, Menlo Systems GmbH, Germany) which generates ~400 fs pulses in the infrared (1030 nm) includes an internal pulse picker which simplifies setup and lowers the overall cost compared to Ti:Sapphire systems that require an external pulse picker. Further, Yb-fiber system can ablate with user-defined repetition rate of single shot to 50 MHz and pulse energies of nJ to  $\mu$ J. We have recently demonstrated that our novel setup shows comparable microsurgery results to those obtained with the Ti: Sapphire systems [10] without its disadvantages.

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## 2 Materials

### 2.1 Microscope Assembly

1. Yb-fiber system (BlueCut, Menlo Systems GmbH, Germany).
2. 4 circular mirrors: 2 for alignment and 2 for periscope.
3. Optical rails and base.
4. 10 $\times$  Achromatic Galilean Beam Expander, AR Coated: 650–1050 nm (can be replaced by a Galilean pair and optical rails).
5. Microscope (e.g., RAMM configured by Applied Scientific Instrumentation).

6. High NA Objective with high IR transmission (e.g., Olympus UAPO 40×, 1.35 NA).
7. Immersion oil.
8. 750 nm long-pass dichroic mirror.
9. Dichroic mirror cube holder.
10. sCMOS Camera (e.g., Flash4.0, Hamamatsu).
11. IR cut-off filter (that prevents 1030 nm from hitting camera, e.g., Chroma ET750sp-2p8).
12. LED light source (e.g., LED 120, X-cite).
13. 4 × 8 passive optical table.
14. Power and Energy Meter Console with S170C Microscope Slide Power Sensor.
15. NIR detector card (VRC4, Thorlabs).
16. Alignment plate.
17. Laser safety goggles for 1030 nm.
18. Beam blockers.

## 2.2 Laser Microsurgery

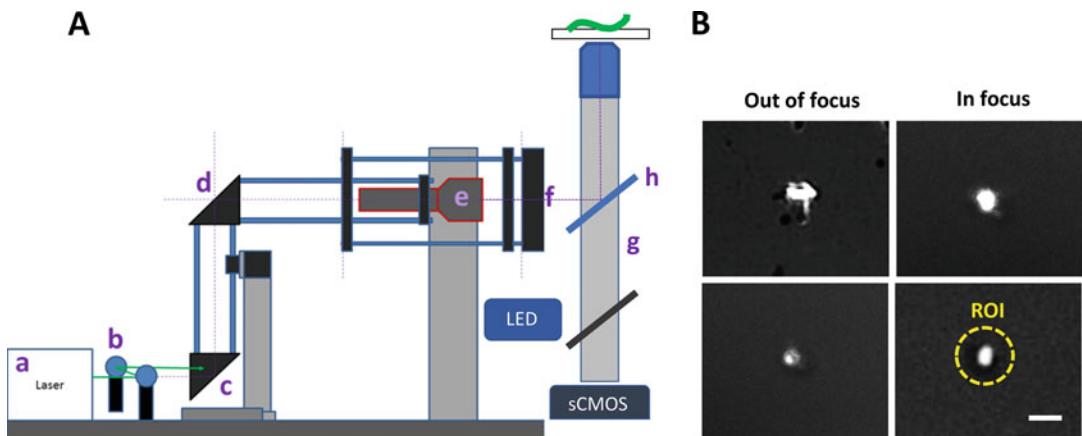
1. Immobilization Solution: Pluronic F127 36% (*see Note 1*), Tetramisole 1 μM (or similar anesthetic).
2. #1 Rectangular Microscopy Coverslips.
3. Dissection Microscope.
4. 60 mm petri dishes for strain maintenance.
5. NGM-Agar: [11] 3 g of NaCl, 2.5 g of peptone, 20 g of agar, 1 mL of cholesterol (5 mg/mL in ethanol), 1 mL of 1 M CaCl<sub>2</sub>, 1 mL of 1 M MgSO<sub>4</sub>, and 25 mL of 1 M (pH 6.0) KPO<sub>4</sub> to prepare 1 L.
6. OP-50-1 bacteria (GCG).
7. Transgenic *C. elegans* strain (e.g., NW1229 pan-neuronal GFP expression, CGC).

## 3 Methods

### 3.1 Laser Safety

Lasers are dangerous and should always be operated with caution. This laser is a class 4 laser which means it is hazardous for eye exposure and that it can burn through skin and some materials at close range and should be handled with extreme care. Always use the minimal energy and number of pulses that produce the required lesion. Before using the laser, it is very important to receive laser safety training. The laser manufacturer can provide further guidance.

In addition, beam blockers should always be in place even when the laser is off and only removed when performing experiments.



**Fig. 1** Laser setup and alignment. **(A)** Laser setup. BlueCut laser source (a) is fastened to an optical table. The beam is directed in free space to aligning mirrors (b), a pair of periscope mirrors (c and d), and a beam expander (e), to fill the back focal aperture (f) of the microscope (g) and onto the dichroic mirror (h). **(B)** Black Slide test. The laser damage spot should be circular and the focus of the microscope is adjusted in order to get defined and crisp edges. A circular region of interest is selected with the white lesion in its center. Scale bar = 5  $\mu\text{m}$

When the laser is on, safety goggles should be worn at all times and the user's hands should be free of any reflective surfaces such as jewelry or watches. “Laser in use” signs should be displayed on the door outside the room where the laser is set up to prevent people without appropriate protection from coming in.

### 3.2 Microscope and Laser Setup (Fig. 1A)

1. Set up optical table and secure the laser system so that the beam is aligned with the center of the table.
2. Secure all connection between the laser console, the AOM seed, and the computer, and make sure that no cables are in the path of the laser beam.
3. Set up the microscope, the LED fluorescent light source, and the camera at the other end of the table (*see Note 2*).
4. Install the IR cut-off filter to avoid damage to the camera.
5. Assemble the periscope and align it to direct the laser through the beam expander into the back focal plane of the microscope at the side of the filter cube.
6. Turn the laser on at a very low power setting and in continuous mode and use the NIR Detector Card to observe the path of the laser beam. Secure the small circular mirrors to the table so that their configuration allows to direct the beam (hitting the center of each mirror) to the center of the lower periscope mirror (*see Note 3*).
7. Adjust the angle of the lower periscope mirror to center the laser beam on the upper periscope mirror.

8. Set up the dichroic mirror in the dichroic mirror cube holder (*see Note 4*).
9. Attach the dichroic mirror to the top of the microscope's fluorescent filter turret.
10. Using the optical rails from the periscope attach the beam expander so that the beam expander optics are close to the back focal opening of the microscope.
11. Turn the laser in a low power setting again and using the NIR card adjust the iris on the beam expander so that the entire back focal aperture of the microscope is illuminated.
12. Proceed with alignment procedures.

### **3.3 Laser Alignment**

#### *3.3.1 Alignment Process*

Alignment is probably the most dangerous process when working with a laser as the user is adjusting, moving mirrors and introducing objects in the laser beam path. Please read Subheading 3.1 before starting alignment (*see Notes 5 and 6*).

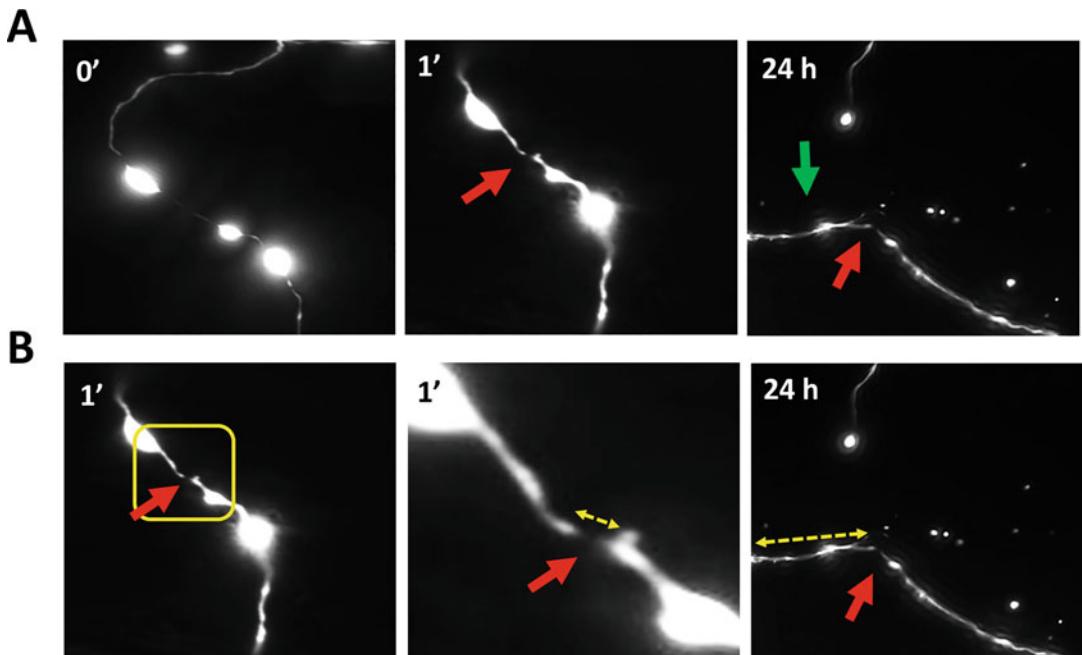
1. Turn on the laser in continuous mode at a low power setting and using the NIR card adjust the circular mirrors to hit the center of each mirror, starting from the one closest to the laser and up to the upper periscope mirror.
2. Use the alignment knobs and the alignment plate, placed on the rails between the periscope mirror and the beam expander, to adjust the mirrors further so that the laser beam is exactly at the center of the optical frame before the beam expander. Move the beam with the lower mirror when the plate is farthest from the microscope, and with the upper mirror when it is closer to the microscope.
3. You might need to repeat steps 1 and 2 several times as adjusting one mirror might alter where the laser hits other mirrors.
4. Adjust the beam expander so that the back focal aperture of the microscope is fully illuminated. The expanded beam should be as collimated as possible. You can test this by removing the microscope and verifying that the shape and size of the expanded beam does not change over some distance.
5. Proceed to fine alignment using the "Black ink slide" test (*see Subheading 3.3.2*) with the laser to pulse mode.
6. After optimal alignment has been reached, measure laser power with the power meter and record the number. Measuring power before every experiment is a good practice and can also help determine if the laser is in need of alignment.

### 3.3.2 Fine Alignment Test: "Black Ink Slide"

- Cover a #1 rectangular coverslip in black ink using a black permanent marker and allow the ink to dry for a few minutes.
- Place the coverslip under the microscope with the ink side facing away from the objective and focus on it. Use immersion oil for the high NA objective and use the microscope in Köhler-aligned brightfield mode (*see Note 7*).
- Starting with a low power setting fire the laser and keep increasing power until a white spot is visible on the slide.
- Keep doing this and adjust the microscope focus and the setting of the beam expander until the white spot is the smallest possible size, looks circular, and has defined and crisp edges (Fig. 1B).
- To be able to aim the laser for microsurgery, use ImageJ to draw a circular Region of Interest (ROI), with the laser spot in the center of the circle and save the ROI so it is easily available (Fig. 1B; *see Note 8*).

### 3.4 Laser Microsurgery

- On the day before performing the laser microsurgery, pick animals of the desired age and transfer them onto a new, seeded NGM plate (*see Note 9*).
- Before starting the experiment turn the laser on, make sure everything is working appropriately, perform the black ink slide test, open the saved ROI, and adjust it if needed (*see Subheading 3.3.2*).
- Fill a bucket with ice and place an empty tip-box lid on top of the ice making sure the flat surface is level.
- Mix Pluronic F127 36% solution with enough concentrated Tetramisole (1 mM solution to dilute to 1  $\mu$ M Tetramisole) in a 1.5 ml tube and keep the tube in ice (*see Note 10*).
- Place #1 rectangular coverslip on top of the tip box and wait a minute until the surface of the coverslip is cold, add 25  $\mu$ L of the Pluronic + Tetramisole mixture to the coverslip surface (*see Note 11*).
- With a pick transfer the animals onto the drop of Pluronic + Tetramisole mixture and gently press another coverslip on top until the liquid between the coverslips is evenly spread (*see Note 12*).
- Remove the coverslip from the ice and wait until the liquid mixture becomes solid or the glass does not feel cold to the touch anymore.
- Using a dissection microscope make sure that the animals are present between the coverslips and that the animals are healthy (*see Note 13*).



**Fig. 2** *C. elegans* neurites regrow following microsurgery with an Yb-doped fiber laser. **(A)** *C. elegans* axotomy. Commissures of GABAergic (D type) motoneurons injured in immobilized animals were found again on the next day to assess regeneration. Arrows indicate the sites of axotomy (red arrow) and regenerating branch (green arrow). **(B)** Example of measuring the sizes of the injury size and outgrowth. Size of injury is measured between the two retracting branches. Outgrowth is measured from the site of injury to the tip of the regenerated branch. Scale = 10  $\mu$ m

9. Place the coverslip in the microscope and secure it as if it were a slide (*see Note 14*).
10. Using the microscope software and adjusting the focus, find the target neuron or cell (*see Note 15*).
11. Once you locate the target cell, select the injury spot by placing it in the middle of the ROI.
12. Put on the laser safety goggles, remove the beam blocker from the laser opening, and press the pulse key to fire the laser. The injury should be immediately visible. If the injury is not visible, increase laser power slightly and try again (*see Note 16*).
13. Take before and after images of the injury site (Fig. 2A).
14. After all the desired cells have been injured, remove the coverslip from under the microscope and move it back on the ice.
15. After 5 min on the ice slowly separate both coverslips being careful not to break them. Once both sides are separated, place them under the dissection microscope to find the animals.

16. With a pick carefully remove the animals from the Pluronic + Tetramisole mixture which should have the consistency of a gel and place them into individual NGM plates.
17. After 24 h or the desired time for your experiment, repeat steps 2–9 to mount the animals between two coverslips again and image the injury site (Fig. 2A).

### 3.5 Data Analysis

1. The specific assay and statistical test depends on your scientific question. For example, you can score the number of neurons that regenerated or cells that survived by looking at images taken 24 or 48 h after injury. In that case, a Fisher's Exact test is appropriate.
2. For continuous values, such as extent of neurite regeneration, measure the initial injury size as well as outgrowth with a software such as FIJI (is just ImageJ v.1.53c [12, 13]; Fig. 2B; see Note 17). In this case, we suggest to visualize the data in shared-control Gardner–Altman plots, and to calculate the p-value by two-sided permutation t-test (Estimation Statistics; [14]), or to compare among treatment groups with an ANOVA test.

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## 4 Notes

1. Mix Pluronic F127 to 36% in deionized water by gradually adding the powder to a solution kept at 4 °C and stirred continuously. This can take a few hours. Do not let the solution warm up because it will solidify. Aliquot and keep at 4 °C. Prepare a concentrated Tetramisole 1 mM solution and use the Pluronic F127 36% solution to dilute to 1 µM.
2. We prefer a custom-made microscope without eyepieces for the obvious safety advantage. The suggested platform can be set up with any epifluorescence microscope that can accommodate a second dichroic mirror in its light path.
3. The laser beam should always hit the center of every mirror.
4. The dichroic mounting adapter directs the laser beam into the objective lens without interfering with the normal optical paths of the microscope.
5. For safety and efficiency reasons, we recommend that two people align the laser.
6. It is not reasonable to try and comprehensively cover laser alignment in this chapter. Your institution might have laboratories that are proficient in laser alignment or you could ask the vendor for advice.

7. Scoring a line or a grid on the ink with a sharp object might help with focusing.
8. The location of the ROI might need to be adjusted or at least checked with a black ink slide before every experiment.
9. For adult axotomy pick L4s the day before, it is easier to perform axotomy when the animal has few or no eggs.
10. It is very important to make and keep the Pluronic F127 36% solution at 4 °C or below as it will become solid at room temperature.
11. Try to avoid bubbles.
12. Four to five animals per coverslip works best.
13. You can use a thin permanent marker to draw a circle around each animal to make it easier to find under the microscope. You will use immersion oil so mark the coverslip that will be away from the objective and draw large circles.
14. If needed, you can place the coverslips on a glass slide. Mounting the animals between two coverslips allows optical access from either side of the animal more easily. We routinely lesion commissure neurites on the far side of the animal but they can end up closer to one coverslip or the other.
15. If epifluorescence is necessary to find the target cell, keep the shutter open as little as possible to avoid bleaching.
16. Always begin with a low laser power and slowly increase it until the injury is visible.
17. In case there is more than one branch in the regenerated neurite, sum the values.

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# Chapter 18

## An Imaging System for Monitoring *C. elegans* Behavior and Aging

Matthew A. Churgin and Christopher Fang-Yen

### Abstract

Many experiments in *C. elegans* neurobiology rely on imaging its behavior. Here we describe procedures for building a flexible and inexpensive imaging system using standard optical and mechanical components.

**Key words** *C. elegans*, Imaging, Behavior, Aging

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### 1 Introduction

The nematode *C. elegans* is a powerful model for investigating the neural and genetic bases of behavior, owing to its easily manipulable genome and well-mapped nervous system [1]. Much of our understanding of *C. elegans* neurobiology including synaptic function [2], sensory systems [3, 4], motor systems [5], and feeding [6] have been enabled in part from well-designed behavioral assays [7].

Many behavioral assays can be conducted by visual observation [7]. Others, such as those requiring observations over long periods (hours to weeks), or those involving subtle details of behavior are only practical with tools for automated imaging [8].

The simplest method for digital imaging is to add a digital camera to the trinocular port on an existing stereomicroscope. However, this approach may not be desirable when long-term imaging and/or simultaneous imaging on multiple setups are required.

We have developed a simple table-top imaging system using commercially available cameras, optics, light sources, and mechanical components. As the system is relatively modest in cost (USD 500–800 not including the computer), we have been able to construct several of them for various uses in our laboratory.

We have used these systems to record *C. elegans* behavior in studies of developmentally timed sleep [9], stress-induced quiescence [10–13], food-dependent behavioral states [12, 14], and aging [15].

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## 2 Materials

Part dimensions are specified in imperial units (inches, feet). Metric system parts with similar dimensions can be substituted.

1. Aluminum bottom plate 12" × 12" × ½".
2. Acrylic top plate 12" × 12" × ¼".
3. 1.5" post clamp, qty 2.
4. 1.5" diameter stainless steel mounting post, 14" long.
5. 1.5" diameter stainless steel mounting post, 6" long.
6. 0.5" diameter stainless steel post.
7. Post holder.
8. Post holder base.
9. Flexible red LED strip, 4.7" long, qty. 4.
10. 3–12 V power supply.
11. CMOS or CCD camera (at least 2592 × 1944 pixel sensor, up to 15 images/s).
12. Power supply for camera, if needed.
13. Data cable for camera.
14. Imaging software.
15. C-mount camera lens (2/3", 12.5 mm focal length, f/1.4).
16. C-mount extension rings.
17. Borosilicate glass sheet 8" × 8" × ¼".
18. Aluminum 2-hole inside corner bracket (80/20).
19. 5-min epoxy adhesive.
20. ¼"-20 stainless steel cap head screws (qty 5), set screw (qty 1), and 1/4" washer (qty 4).
21. Black gaffer's tape, 1" wide.
22. Binder clips.
23. Blackout fabric.
24. Self-adhesive bumpers (polyurethane, 7/8" Wide, 13/32" high), qty. 4.
25. 3/16" hex screwdriver.
26. *Tools required if machining top and bottom plates yourself:*

- (a) Drill press or handheld electric drill.
- (b) 1/8" diameter drill bit.
- (c) 5/16" diameter drill bit.

### 3 Methods

#### **3.1 Designing the Imaging System**

1. Decide what field of view (FOV) and resolution are needed for your experiment. The FOV is related to the sensor size SS, working distance WD (distance between the lens and object being imaged), and lens focal length f by the following formula (*see Notes 1 and 2*):

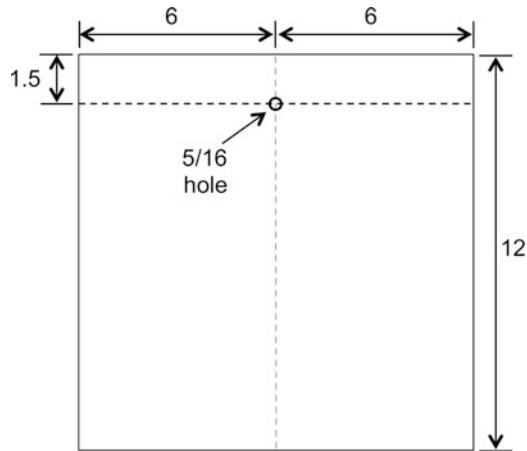
$$\text{FOV} = \text{SS} \times \text{WD}/f.$$

The field of view can be adjusted by moving the camera up or down. The setup detailed in Materials will accommodate a field of view ranging from approximately 2.7 cm × 2.0 cm to 11.1 cm × 8.3 cm.

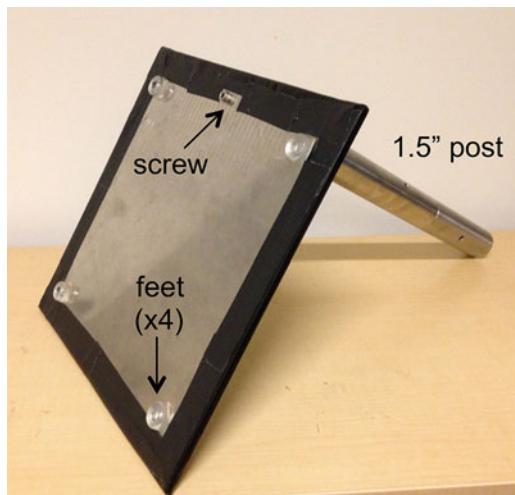
2. Select the lens. Considerations include the focal length, aperture size, and compatibility with the camera sensor size. We have used only fixed focal length lenses, but a zoom or varifocal lens may also be suitable.
3. Select the camera. Considerations include the desired pixel resolution, sensor size, and maximum frame rate. In our experience, data transfer using Gigabit Ethernet cameras is more reliable over the long term than with USB cameras.

#### **3.2 Constructing the Mechanical Components**

1. Drill 5/16" diameter holes at the points indicated on the top (acrylic) and bottom (aluminum) plates as indicated in Fig. 1. A tolerance of ±1/8" is acceptable (*see Notes 3 and 4*).
2. Wrap a 14" × 14" piece of blackout fabric tightly around base plate and tape to bottom of plate using gaffer's tape (Fig. 2). Alternatively, black spray paint can be used.
3. Feel for the hole on the top of the plate previously made in the base plate. Using a razor blade, cut the blackout fabric at an approximately 1" square around this hole and tape the fabric to the plate, leaving the hole clear (Fig. 2).
4. Attach 4 self-adhesive rubber feet to the bottom of the aluminum plate near the corners (Fig. 2).
5. Use a 1/4"-20 hex head cap screw and 3/16" hex screwdriver to attach the 1.5" diameter × 14" long stainless steel rod to the base plate (Fig. 2).
6. Using a 1/4"-20 set screw, connect the two 1.5" diameter stainless steel rods.

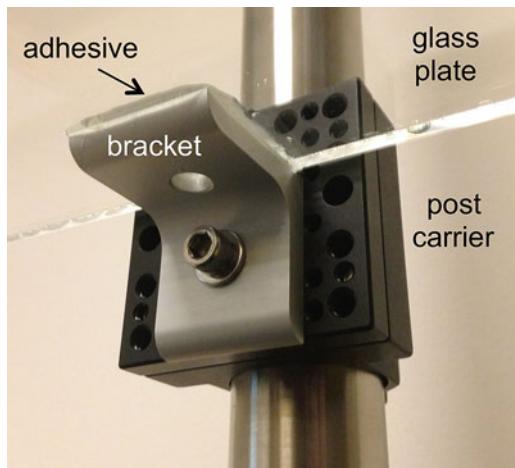


**Fig. 1** Location of holes in top and bottom plates for mounting to support rod. All dimensions in inches



**Fig. 2** Bottom view of bottom plate with blackout fabric, mounted 1.5" diameter support rod, and feet

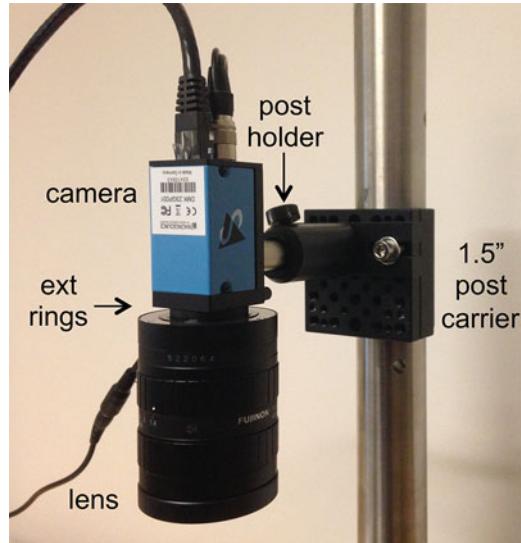
7. Place the first and second mounting carriers on the 1.5" diameter rod such that the side with the tapped holes faces the front of the apparatus. To set the height, tighten the clamp using a 3/16" hex screwdriver. When loosening either 1.5" post carrier clamp, support the carrier with your other hand, otherwise the carrier may fall and cause damage.
8. Attach the L-bracket to the edge of the glass plate using epoxy adhesive (Fig. 3). The adhesive will harden within minutes. Allow to cure overnight for full strength.
9. Mount the glass stage onto the lower post carrier using a 1/4"-20 screw with washer. Adjust so that the plate is level (Fig. 3).



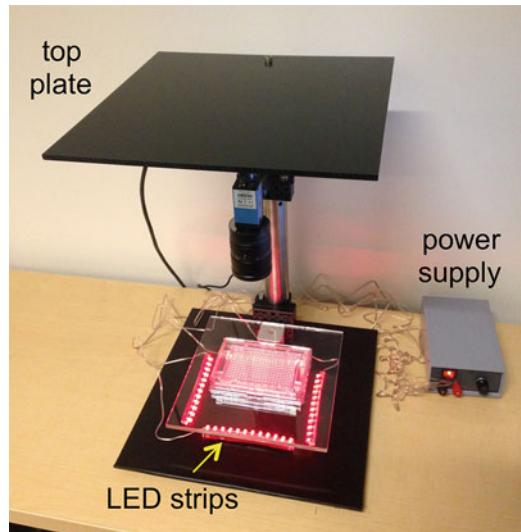
**Fig. 3** View from below of post carrier mounted on 1.5" post, 2-hole bracket, and glass platform

### 3.3 Assembling the Camera, Lens, and Light Source

10. Attach the top plate (black acrylic) to the top of the 1.5" diameter post using a 1/4"-20 screw and washer. Rotate the plate so that it is positioned directly above the base plate, then tighten the screw using the 3/16" hex screwdriver.
11. To minimize stray light from outside the imaging system, cut a piece of blackout cloth to cover the left, right, and back sides of the imaging system, and tape to the top plate. Add another piece to cover the front of the system.
1. Screw the lens onto the camera, separated by 5 mm and 1 mm extension rings. These rings allow the imaging system to focus on objects located closer than the normal minimum distance. Depending on your application, the extension distance may need to be increased or decreased (*see Note 5*).
2. Mount a base plate to a post holder using a 1/4"-20 socket head cap screw. Attach a 1/2" diameter post (Thorlabs TR2) to the camera tripod mount using a 1/4"-20 set screw. Mount the post to the post holder by loosening the adjustment knob (Fig. 4).
3. Mount the camera with post and base onto the 1.5" post carrier using at least two 1/4-20 cap screws (Fig. 4).
4. Attach the power supply and Ethernet cable to the camera. Install the camera software according to the manufacturer's directions.
5. Use a wire cutter to strip 1/2" of the insulation off the LED wires, if needed. Connect the bare leads of the LED to the positive and negative terminals of the power supply by



**Fig. 4** Camera assembly containing camera, lens, cables,  $\frac{1}{2}$ " post, post holder, base, 1.5" post carrier, and 1.5" post



**Fig. 5** Completed imaging system including LED strips, power supply, and sample to be imaged. During data acquisition, system should be completely covered by blackout curtains (not shown)

loosening the terminal screws, placing the bare wire behind the terminals, and retightening the terminals.

6. Place the LED strip in a ring on the base plate. Alternatively, place four shorter LED strips in a square configuration with LEDs facing inward (Fig. 5). The LEDs should be wired in

parallel (each pair of leads connected to the terminals of the power supply), not in series (*see Notes 6 and 7*).

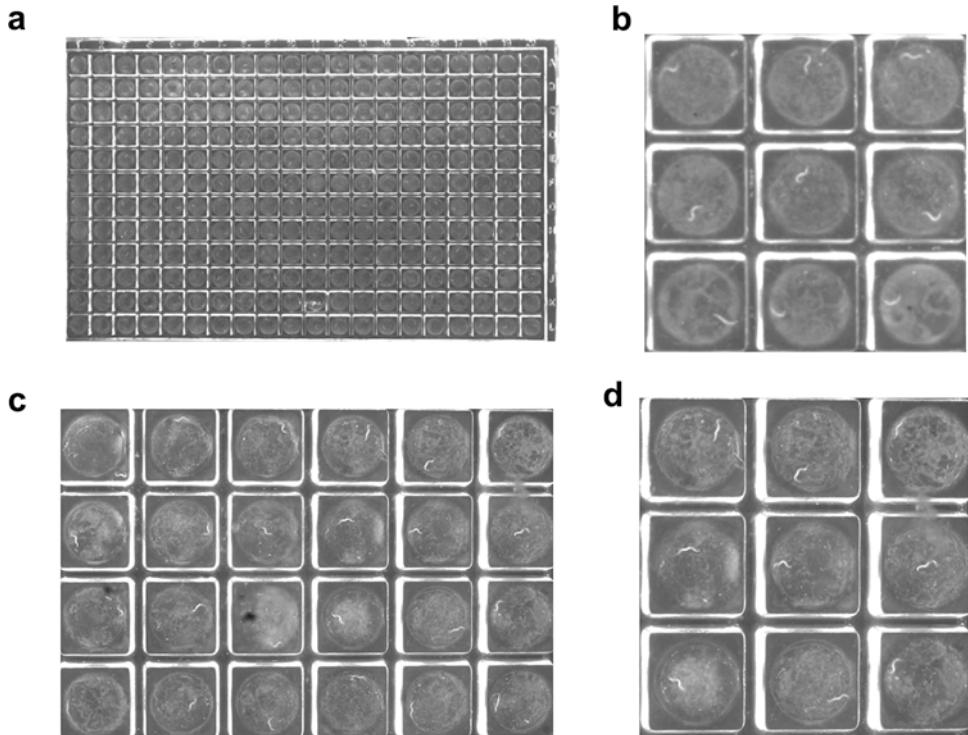
7. Test the LEDs by turning on the power supply. The brightness can be adjusted by varying the voltage (*see Note 8*).

### **3.4 Alignment and Optimization**

1. The lens has rings for adjustment of focus and aperture size. Set the lens focus near the middle of its range. Set the aperture size near the middle of its range.
2. Turn on the power supply for the LEDs. Open the camera software, select the camera, and set to preview mode. You should see an image, which may be out of focus or uniformly black or gray.
3. Place a worm plate, or any sample with height equivalent to what you will use during experiments, onto the glass stage. Adjust the height of the upper carrier to bring the object into focus (Fig. 6). Make fine adjustments using the lens focus. Familiarize yourself with the gain and exposure time settings. To minimize noise, set the gain to the minimum value and provide more light if necessary.
4. Set the lens aperture size. Opening the aperture will increase the amount of light collected and decrease the depth of field. The sharpness of the image is usually poor at the extremes of aperture (wide open or nearly closed) and best somewhere in the middle.
5. Set exposure time. Exposure should be set to collect an adequate amount of light without blurring due to movement. The optimum exposure is usually such that the brightest spots in the important part of the image represent about 75% of the dynamic range of the camera. If the exposure is too low, the signal-to-noise ratio will be poor. If the exposure is too high, parts of the image will be saturated (i.e., reach the maximum pixel value). The pixel values can be assayed within the IC Capture application by moving the cursor over the image.

### **3.5 Acquiring Image Data**

1. Acquire time lapse image sequences in IC Capture using the Sequence Timer dialog (Fig. 6). Save images in BMP format in a new folder devoted to the image sequence. Using JPEG format results in smaller files, but the lossy compression algorithm increases image noise.
2. To block all ambient light, close openings in the black curtains using binder clips.
3. The temperature inside the rig can be monitored by a thermometer. We use a RT100 thermistor probe and handheld meter (Omega). Note the LEDs may increase the temperature inside the rig; the voltage can be lowered to reduce LED heating (*see Note 9*).



**Fig. 6** (a) Image of a 240-well microfabricated WorMotel device containing one worm per well [15]. (b) Detail of 9 wells from a. (c) Image of 24 wells. (d) Detail of 9 wells. In all images, center-to-center spacing between wells is 4.5 mm. This imaging system also works well with concave glass wells [9], hanging droplets [16], and standard NGM plates [17]

4. Fogging of the plate lid can occur, particularly when the temperature drops. We prepare the lids with an anti-fog coating as follows: We pour a sterile-filtered solution of 20% Tween 20 in water onto the inner surface of the lid, pour off the excess, and allow the lid to dry in a sterile hood. Alternatively, a small amount of pure Tween 20 can be wiped onto the lid using a piece of sterile, lint-free tissue and allowed to dry.
5. Preparing plates in a clean hood minimizes the chances of media contamination.
6. Data can be acquired from multiple cameras using a single computer. To use multiple Gigabit Ethernet cameras, first install an Ethernet hub. Next, run a separate instance of IC Capture for each camera and configure them individually.
7. We analyze data using custom MATLAB scripts modified for each experiment. Image data acquired using this imaging system can be analyzed using worm tracking codes described elsewhere [8].

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## 4 Notes

1. This formula is approximately valid for WD much larger than  $f$ .
2. The sensor size can be found in the camera specifications. The sensor of the Imaging Source DMK 23GP031 camera is 5.70 mm(H)  $\times$  4.28 mm(V).
3. When drilling a 1/8" pilot hole in aluminum, use of a cutting lubricant will make machining easier. While a drill press is preferred, a hand drill can also be used. Be sure to properly secure the workpiece during drilling and include backing material if needed to avoid damaging the surface underneath.
4. For any use of power tools, wear eye protection and consult personnel trained in their use.
5. A CS-mount camera may come supplied with one 5 mm extension ring for C-mount compatibility; an additional ring should be added.
6. For bright field imaging, a single LED source can be placed under the plate, with a diffuser made from a round piece of paper added to improve image quality.
7. The use of red LEDs reduces the behavioral effects of the illumination compared with green, blue, or white light. Infrared LEDs can also be used.
8. If you accidentally wire the LEDs in reverse, no light will come out, but nothing else bad will happen.
9. In one of our setups, we placed multiple imaging rigs inside a 4'  $\times$  4'  $\times$  4' temperature-controlled chamber built using 80/20 framing and covered with black curtains. We installed a small, digital window-style air conditioner (Frigidaire Energy Star) to control the temperature inside the chamber to about  $\pm 1$  °C. Similarly, an electric heater with a temperature controller can be used to maintain temperature at a fixed value above the ambient.

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# Chapter 19

## Methods for Modulating and Measuring Neuromuscular Exertion in *C. elegans*

Kiley J. Hughes and Andrés G. Vidal-Gadea

### Abstract

The nematode *C. elegans* has been used widely to study the genetic and cellular basis of behavior. Yet the laboratory conditions under which it is typically studied offer only a narrow glimpse into the richness of natural behaviors this remarkable animal evolved over 500 million years of evolution. For example, burrowing behavior naturally occurs in the wild, but it remains understudied. Our group studies burrowing in an attempt to expand our understanding of the natural behavioral repertoire of *C. elegans*. Aside from being an interesting and tractable behavior, burrowing is experimentally useful and permits the titration of the muscular output exerted by *C. elegans*. Here we describe several burrowing assays that allow the modulation of muscular exertion. We used these to study both adaptive and pathological muscular processes such as muscle hypertrophy and dystrophy, respectively. We believe these assays will be of use for researchers studying the production of locomotion under normal and disease-challenged conditions.

**Key words** Muscle, Burrowing, *C. elegans*, Muscular exertion, Muscular hypertrophy, Muscular dystrophy

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### 1 Introduction

Due to its experimental amenability and high degree of genetic homology, the nematode *C. elegans* is used extensively to study many human disorders [1]. For example, worms have been successfully used to study many neurological and muscular disorders such as muscular dystrophies, Parkinson's disease, Alzheimer's, ALS, and more [2–5]. This is possible because the molecular components of neurons and muscles are largely conserved between worms and humans. Traditionally, modeling disorders is done by studying strains that mimic the underlying genetic insult in the disorder targeted. These animals are typically tested on an array of behaviors worms readily perform on the agar plates they are cultured. These behaviors include analysis of crawling locomotion, pharyngeal pumping, egg-laying, etc. Assays like these continue to provide a

wealth of insights both on basic biology, and our understanding of disease [6–8].

Ever since the original *C. elegans* screen by Sydney Brenner revealed a host of genes involved in locomotion, crawling worms on agar plates have proved reliably fruitful [9]. However, this approach is not without limitations. Worms raised under standard laboratory conditions grow on homogeneous bacterial lawns seeded on agar plates, where they easily move with minimal resistance. Compared to what worms likely experience in the wild, the laboratory environment represents a narrow environmental range. Perhaps not surprisingly then many of the phenotypes observed for animals modeling disease do not always recapitulate the severity characteristic of the disease in humans. For example, when observed on agar plates, worms mimicking Parkinson's disease through loss of dopaminergic neurons fail to show locomotor phenotypes common in patients. However, by expanding the tested behaviors to include transitions between swimming and crawling, the same animals more closely recapitulated the disorder and showed the characteristic failure to initiate motor programs associated with Parkinson's [10]. A similar situation occurs for worms modeling Duchenne muscular dystrophy through mutations on the dystrophin gene (*dys-1*). Our understanding of muscular dystrophy has greatly benefited from the use of *dys-1* mutants. However, on agar plates worms lacking dystrophin display only mild phenotypes compared to humans with similar mutations. Consequently, researchers in this field often use sensitizing mutations in order to produce salient (experimentally amenable) phenotypes [11].

Our lab is interested in the study of natural behaviors in part because we believe it has the potential to provide a wealth of untapped and useful insights that can be harnessed to study disease. We hypothesize that worms in their natural environment routinely experience both richer, and more demanding circumstances (at the neural and muscular levels) than what they experience under standard laboratory conditions. In the wild, *C. elegans* likely spends much of its time burrowing through dense organic matter or through soil while searching for food or avoiding predators or unsuitable environments [12].

For vermiform animals, burrowing is one of the most complex forms of locomotion. This is the case because of the heterogeneous nature of the environment [13, 14]. Only few studies have investigated burrowing in *C. elegans*. The Pomerai and the Williamson groups assessed worms burrowing through Pluronic gel to test their response to surfactants or to their hosts, respectively [15, 16]. More recently, the Arata group used agarose-filled microfluidic devices to study chemotaxis [17]. Pluronic gels are liquid at 25 °C but solid at 20 °C, which means that they can be safely cast onto living animals without causing thermal damage. This feature continues to make them attractive and useful [18]. The use of

Pluronic gel is however not without caveats. Pluronic gel ( $(C_3H_6O \cdot C_2H_4O)_x$ ) is chemically distinct from the NMG agar ( $(C_{12}H_{18}O_9)_n$ ) that is used to raise worms, and it is commonly used for its ability to dissolve water-insoluble compounds. How this difference affects *C. elegans* physiology and behavior is not presently understood. Because burrowing worms are completely surrounded by media, special care needs to be taken to ensure that the gel's pH, osmolarity, and other important variables mimic those in culture plates. Another potential caveat of using Pluronic gels derives from the very reason they are used. The transition between the liquid and solid phase of Pluronic gels depends on their concentration [19]. This precludes the use of different gel densities to modulate neural and muscular exertion. Furthermore, across the temperature range where *C. elegans* are typically cultured and tested (20–22 °C), gel viscosity is variable and unpredictable, making this media not ideal for the accurate assessment of animal behavior [19].

Instead of Pluronic gels, we use nematode growth media agar. We found that worms readily burrowed through various densities (0.5–9%) of medium, and that they used kinematics that were distinct from those during crawling or swimming [20]. Using this approach, the physical exertion required to achieve locomotion can be readily modulated by altering the density of the agar. This makes this assay uniquely suited to the study of neural and muscular disorders that are predicted to affect motor output.

With respect to Duchenne muscular dystrophy, our results recapitulated many of the most salient phenotypes of Duchenne muscular dystrophy to an extent not previously achieved using worms or other animal models [20]. This in turn allowed us to conduct a suppressor screen using dystrophic animals, and to study the pathophysiology of the disease [2]. The ability to modulate the muscular exertion required for locomotion is not just useful when studying disease. Using healthy animals, we can also study adaptive changes that take place as a consequence of changed environmental demands, such as muscle hypertrophy resulting from increased muscular exertion [2]. Over the past few years, we have continuously adapted our burrowing assays to improve effectiveness and reliability. Here, we present an overview of the burrowing assays we find most useful, and illustrate their deployment.

### 1.1 Single Worm: For the Study of Locomotion and Associated Behaviors

The study of behavior using *C. elegans* benefits greatly from the use of large number of animals. However, there are situations that necessitate the in-depth analysis of individuals, for example when pharyngeal pumping or other non-locomotor behaviors need to be measured. We found that at all but the lowest of agar densities (<1%) worms burrow predominantly by waves that propagate along their dorsoventral plane [20]. Because of this, studying single burrowing animals is best done using glass Pasteur pipettes. In this assay, a pipette controller is used to draw agar into a glass Pasteur

pipette. After filling the pipette with agar, we draw an additional 50 µl of OP50 *E. coli* which remains at the tapered tip of the pipette and acts as an attractant to stimulate worms to burrow through the pipette. After filling, we use Parafilm™ strips to carefully seal both ends of the pipette and allow the agar to solidify in the 4 °C refrigerator. Attractants diffuse much slower through agar than over it, therefore attractant gradients must be allowed to develop overnight. Worms are introduced at the top of the pipette by first scoring a hole in the agar with a worm pick, and then either injecting the worms with a glass capillary, or by placing the worms on the surface of the agar near the holes made in the agar. As the worms begin to burrow, they adopt a typical burrowing W-shape with bends that decrease in amplitude, as the agar density is increased [20]. The diameter of the pipettes allows a clear view of the animals in a wide range of agar densities, even when worms burrow close to the center of the pipette. This type of assay is most suited for the measurement of pharyngeal pumping, egg-laying, defecation, and some body kinematics (body bend frequency and amplitude). Because either pipette or camera must be moved to maintain the animal in the field of view, this approach is not optimal for measuring positional information (e.g., velocity of locomotion). Glass pipettes can be used for a few days but eventually the agar within them starts to dry, shrink, make air pockets where worms can crawl (rather than burrow). It is therefore advisable to discard them after 3 days.

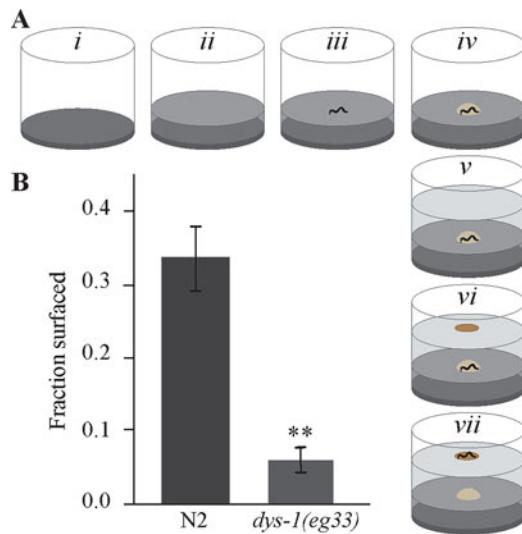
## 1.2 Long Distance Assay: Screening by Burrowing Ability

In situations where unique individuals need to be separated from larger populations of burrowing worms (e.g., genetic screen), we prefer to use long burrowing assays within 5 ml plastic serological pipettes. For example, we used these 30 cm pipettes to isolate animals with mutations suppressing the burrowing impairment of worms modeling Duchenne muscular dystrophy [20]. In this assay, chemotaxis agar of the desired density is used. Once solidified, the ends of the pipettes are cut using a heated razor blade (held with pliers) in order to ensure the agar completely fills the pipette end to end (no air pockets). Parafilm is then used to seal both ends of the pipette. Concentrated OP50 *E.coli* (or another attractant) is then injected into one end of the pipette and allowed to establish a gradient overnight in the 4 °C refrigerator. After removing the pipette from the refrigerator and allowing them to temperature-equilibrate, animals can be injected in liquid NGM (*see Note 1*) into the end of the pipette opposite to the attractant with the help of a glass capillary transfer pipette. With some practice, worms can be injected into the agar without much liquid. To do this, the worms must be expelled into the agar as the capillary is being drawn from it. This creates a vacuum that sucks the worms into the agar without the liquid they are in. One clear caveat of this assay is that it requires practice and dexterity to deposit the worms into

the agar free of liquid. If worms are left swimming in a liquid droplet, they appear unable to exit the liquid and enter the agar to start burrowing. However, with some practice, the assay allows screening of thousands of animals, and it has been used by our lab and others to perform screens and to study behavior [21, 22].

### **1.3 Surfacing Assay: Comparing Muscular Strength in Different Populations**

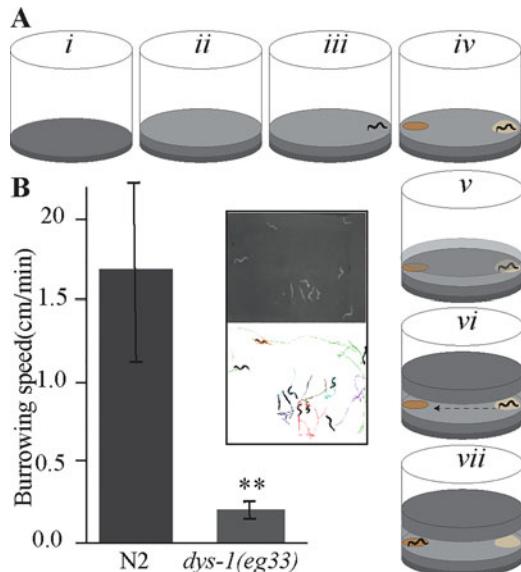
We previously showed that burrowing velocity depends on agar density and the neuromuscular integrity of the animals [20]. To challenge the muscular ability of animals, we developed a surfacing assay where worms in multiple-well plates perform chemotaxis through a layer of agar in order to reach the attractant on the surface of the well. Animals can then be tallied manually or automatically. By comparing time to surface between different populations of animals, differences in the muscular output profiles of distinct strains or treatments can be measured and compared. The assay geometry resembles a layer cake, with different agar layers serving different purposes (Fig. 1a). First, a thin layer of the chemotaxis agar is placed in the bottom of the well. Worms are transferred onto this layer using 1  $\mu$ l of liquid NGM (*see Note 1*). We allow the liquid NGM to be absorbed by the chemotaxis agar before proceeding. Next, to thermally insulate (protect) the worms from the hot (~55 °C) chemotaxis agar that will be poured on top of them, we first transfer a small volume (~2  $\mu$ l) of fish gelatin. This fish gelatin is standard NGM agar where agar powder is replaced with cooking gelatin powder (*see Note 2*). Like Pluronic gel, this fish gelatin is liquid at 25 °C but solidifies around 20 °C. It is also inexpensive. Once the fish gelatin solidifies, we add the final layer of chemotaxis agar. This layer occupies the majority of the well's volume and is the intended medium through which worms must burrow in order to reach the surface of the wells. The density of this agar can be modulated by altering the amount of agar used. We have used densities ranging between 0.5% and 9%, but most N2-derived strains are unable to enter agar with densities above 8%. We have found that dystrophic worms are significantly impaired when challenged with 2.5% agar (Fig. 1b). After the wells have solidified, 1  $\mu$ l of OP50 *E. coli* (or of 0.01% diacetyl solution) is added to serve as an attractant. Over a couple of hours, worms will burrow through the agar, surface, and enter the OP50 lawn provided. Here they can be tallied by hand at regular intervals, or automatically with the help of machine vision algorithms [23]. Examples of useful variables we obtain from this assay include: first worm to surface, average time to surface, and fraction of population to surface. One limitation of this assay is its reliance on chemotaxis to guide worm behavior. We have also successfully performed assays using blue light as an aversive stimulus to induce burrowing.



**Fig. 1** Preparation of surfacing assay. **(a)** (i) We prepare our surfacing assays using multiple-well plates. (ii) Chemotaxis agar of the desired density is added to the well. (iii) After this agar dries, worms are transferred in 1  $\mu$ l liquid NGM using a glass capillary. (iv) A 2  $\mu$ l drop of liquid (25 °C) fish gelatin is added above the worms. (v) Additional chemotaxis agar is then added above the fish gelatin agar. (vi) A 2  $\mu$ l drop of OP50 *E. coli* is added as an attractant. (vii) Worms burrow to the food on the surface and are tallied at regular intervals. **(b)** Using this assay, dystrophic worms showed significant impairment reaching the surface of the well compared to wild-type animals. Analysis two-tailed *t*-test,  $N = 17$  assays, \*\* $p < 0.001$

#### 1.4 Restricted Burrowing Assay: To Study Kinematics and Cell Activity

The surfacing assay is useful to obtain a sensitive evaluation of the neuromuscular capabilities of a population. However, this assay is less suited to monitor animal kinematics. To analyze the kinematics of a group of burrowing animals, it is necessary to restrict their locomotion to a thin horizontal volume where multiple animals can be filmed and analyzed simultaneously. We therefore modified our surfacing assay to achieve this goal by reducing the thickness of the burrowing layer, and by covering the burrowing layer with high-density agar the worms are unable to penetrate. Worms are thus trapped along a thin horizontal layer of burrowing agar where they can be imaged as they freely behave (Fig. 2a). To achieve this, a layer of high-density agar is placed on the bottom of the well. Worms are then transferred onto this layer in 1  $\mu$ l of liquid NGM (*see Note 1*), taking care to place them to one side of the well. As before, the liquid NGM is allowed to be absorbed by the bottom agar layer, and 1  $\mu$ l of fish gelatin (*see Note 2*) is placed above the worms to create a heat shield. As the fish gelatin is solidifying on one side of the well, 1  $\mu$ l of OP50 *E. coli* (or another attractant) is placed on the opposite side of the well to induce animals to burrow across the field of view. Next, a thin layer of burrowing agar is



**Fig. 2** Preparation of restricted burrowing assay. **(a)** *(i)* We prepare our restricted burrowing assays using multiple-well plates. *(ii)* A layer of high-density NGM agar is placed in the wells. *(iii)* After this agar dries, worms are transferred in 1  $\mu$ l liquid NGM using a glass capillary to one side of the well. *(iv)* A 2  $\mu$ l drop of liquid (25 °C) fish gelatin is added above the worms and a 2  $\mu$ l drop of OP50 *E. coli* is placed on the agar at the opposite end of the well from the worms. *(v)* A thin layer of chemotaxis agar of the desired (burrowing) density is placed above the worms. *(vi)* High-density NGM agar is placed above the burrowing layer to prevent animals from surfacing. *(vii)* Worms burrow across the chemotaxis agar to the opposite end of the well to arrive at the food source. **(b)** Burrowing worms move horizontally and can be filmed in order to be characterized kinematically (inset). Using this approach, we show that dystrophic worms burrow significantly slower than wild types. Analysis two-tailed *t*-test,  $N = 17$  assays,  $N = 3$  assays, 10 animals each,  $^{**}p < 0.001$

placed above the well; typically just enough to cover the well's surface (1 ml). Once this layer cools, a second layer of high-density agar is placed above it which prevents worms from exiting the burrowing layer. Under this configuration, animals are able to burrow within the less dense (burrowing) layer where they are easily filmed and analyzed (Fig. 2b inset). While thin enough to cause most animals to burrow parallel to the plate's surface, the burrowing layer is still thick enough to allow worms to freely burrow with their dorsoventral undulations parallel or perpendicular to the plate. Therefore, without the use of an orthogonal camera system, or other means to deduce their 3-dimensional motion [24], any kinematics obtained are limited to centroid-based measurements (e.g., velocity and reversals) unless when animals are burrowing with their dorsoventral plane parallel to the camera. Even without body curvature measurements, if the burrowing layer is

placed close to the camera many useful measurements can be obtained, such as calcium transients from the musculature of burrowing animals [2] (Fig. 2b).

An advantage of the previous two assays is that with a little practice these plates can be produced in large numbers, allowing the experimenter to screen many treatments at once.

### **1.5 Long-Term Burrowing Assays: Assessing Changes Associated with Exertion**

In the previous assays described worms are placed in wells and tested shortly after (within minutes to a couple of hours). In some circumstances, it might be desirable to allow worms to burrow for extended periods of time. For these long-term assays, special considerations are required to prevent starvation and to keep animals in one prescribed location where they can be repeatedly assessed. To prevent animals from starving, we mix the burrowing agar with a small volume of concentrated OP50 *E. coli*. We pellet 1.5 ml of OP50 *E. coli* by centrifugation, remove all supernatant, and re-solubilize the pellet in 100 µl of deionized water. This concentrate is then mixed with the burrowing agar prior to adding it to the well. Worms are able to ingest these bacteria and to develop and grow normally.

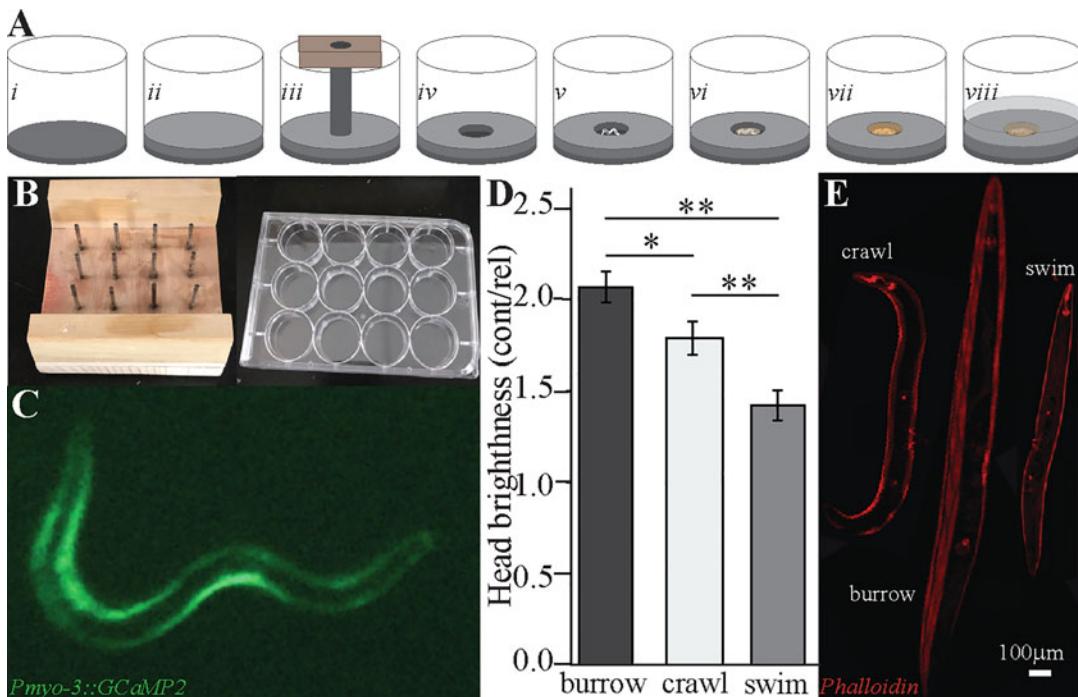
To maintain animals within a small agar volume so they can be repeatedly located and filmed (Fig. 3a), we use a mold to cast depressions in the initial high-density agar layer (see Fig. 3b). The worms and low-melting agar are then placed in these depressions, and the rest of the depressions' volume is filled using the burrowing layer (agar plus OP50 *E. coli*). A final layer of high-density agar is then used to completely seal the burrowing layer and the worms within it. Animals under these conditions are effectively trapped in a 3D cage of high-density agar they are unable to penetrate. They can be raised here from egg to adulthood and remain viable for many days. If the objective of the experiment is to test gene function, the burrowing agar plus OP50 bacteria layer can be replaced with RNAi (plus IPTG) agar and bacteria [2]. In some circumstances, adult animals need to be allowed to burrow for longer than 3 days (the time it takes their eggs to grow to adulthood). In these situations, care must be taken to prevent confusing different generations. To prevent these animals can be transferred to new wells every few days (see Note 3). Alternatively, worms can be chemically prevented from producing offspring using FuDR [25].

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## **2 Materials**

### **2.1 Single Worm Assay**

1. Glass Pasteur pipettes.
2. Pipette controller.
3. Worm pick.



**Fig. 3** Preparation of long-term burrowing assay. (a) (i) We prepare our long-term assays using multiple-well plates. (ii) A layer of high-density NGM agar is placed in the wells. (iii) A mold with metal pegs of the desired diameter (0.5–1.0 cm) is placed above the wells in order to cast a depression in the high-density agar. (iv) When the agar solidifies, the mold is carefully removed exposing the depression. (v) Using a glass capillary worms are transferred into this depression in 1  $\mu$ l liquid NGM. (vi) A 2  $\mu$ l drop of liquid (25 °C) fish gelatin is added above the worms. (vii) NGM agar of the desired density mixed with concentrated *E. coli* is added above the worms until the depression is filled. (viii) A second layer of high-density agar is placed above to completely encase the worms and prevent them from exiting the burrowing layer. (b) Wooden mold with metal pegs used to manufacture 12-well burrowing plates. (c) Worms can be imaged through the agar at regular intervals. Here calcium measurements are obtained from the musculature of a burrowing worm. (d) Using this assay we were shown that head muscles have greater calcium transients during burrowing than during crawling or swimming. Brightness is reported as the ratio of contracting over relaxing neck muscles. One-way ANOVA,  $N = x$  assays. (e) Long-term burrowing assays are suitable for studying adaptive phenomena such as hypertrophy. Here hypertrophic burrowing N2 animals are compared side to side to age-matched crawling or swimming animals. Red staining of f-actin using phalloidin. Analysis One-way ANOVA, Holm-Sidak multiple pairwise comparisons.  $N = 18$  animals, \* $p < 0.05$ , \*\* $p < 0.001$

4. Liquid transfer capillary pipette, preferably one that has a rotating volume control that enables precise single hand operation.
5. Parafilm strips ( $1 \times 4$  cm) to tightly seal open ends of the pipettes.
6. Capillary glass.
7. Dissecting microscope. Illumination is key during filming, having an oblique mirror beneath the plates is a great advantage.
8. Video camera mounted to microscope.

9. Attractant (e.g., OP50 *E. coli* or diacetyl). We prefer OP50 because worms are grown in these bacteria and are attracted to the many compounds they produce.
10. Liquid NGM [26], 100 ml: 97.5 ml deionized water, 0.3 g NaCl, 50  $\mu$ l CaCl<sub>2</sub>, 50  $\mu$ l MgSO<sub>4</sub>, 1.25 ml KPO<sub>4</sub>, and 50  $\mu$ l of cholesterol (*see Note 1*).
11. Chemotaxis agar (~2.5%) (*see Note 4*), 250 ml: Add 4.25 g agar (change to alter final density) to 243.5 ml deionized water. Heat until dissolved. After the agar cools enough to allow touching the beaker for a few seconds (~55 °C), the additional solutions can be added. Add 6.25 ml KH<sub>2</sub>PO<sub>4</sub>, 250  $\mu$ l MgSO<sub>4</sub>, and 250  $\mu$ l CaCl<sub>2</sub>. Add calcium chloride last to prevent its precipitation.

## **2.2 Long Distance Assay**

1. Glass Pasteur pipettes.
2. Pipette controller.
3. Worm pick.
4. Liquid transfer capillary pipette, preferably one that has a rotating volume control that enables precise single hand operation.
5. Capillary glass.
6. Dissecting microscope. Illumination is key during filming, having an oblique mirror beneath the plates is a great advantage.
7. 5 ml plastic serological pipettes.
8. Bunsen burner.
9. Razor blade.
10. Pliers.
11. Parafilm strips (1 × 4 cm) to tightly seal open ends of the pipettes.
12. Attractant (e.g., OP50 *E.coli* or diacetyl). We prefer OP50 because worms are grown in these bacteria and are attracted to the many compounds they produce.
13. Chemotaxis agar (~2.5%) (*see Note 4*), 250 ml: Add 4.25 g agar (change to alter final density) to 243.5 ml deionized water. Heat until dissolved. After the agar cools enough to allow touching the beaker for a few seconds (~55 °C), the additional solutions can be added. Add 6.25 ml KH<sub>2</sub>PO<sub>4</sub>, 250  $\mu$ l MgSO<sub>4</sub>, and 250  $\mu$ l CaCl<sub>2</sub>. Add calcium chloride last to prevent its precipitation.
14. Liquid NGM, 100 ml: 97.5 ml deionized water, 0.3 g NaCl, 50  $\mu$ l CaCl<sub>2</sub>, 50  $\mu$ l MgSO<sub>4</sub>, 1.25 ml KPO<sub>4</sub>, and 50  $\mu$ l of cholesterol (*see Note 1*).

**2.3 Surfacing Assay**

1. 12-well culture plates.
2. Worm pick.
3. Dissecting microscope.
4. Liquid transfer capillary pipette, preferably one that has a rotating volume control that enables precise single hand operation.
5. Capillary glass.
6. Attractant (e.g., OP50 *E.coli* or diacetyl).
7. Liquid NGM, 100 ml: 97.5 ml deionized water, 0.3 g NaCl, 50 µl CaCl<sub>2</sub>, 50 µl MgSO<sub>4</sub>, 1.25 ml KPO<sub>4</sub>, and 50 µl of cholesterol (*see Note 1*).
8. High-density agar (8%) (*see Notes 4 and 5*), 250 ml: 11.36 g agar, 243.75 ml deionized water. Heat until dissolved in a hot water bath with a stirrer. Once completely dissolved, allow to cool to 55 °C (cool enough to touch for 5 s). Add 6.25 ml KH<sub>2</sub>PO<sub>4</sub>, 250 µl MgSO<sub>4</sub>, and 250 µl CaCl<sub>2</sub>. Mix well and store in a water bath at 55 °C until used.
9. Fish gelatin (*see Note 2*), 100 ml: 97.5 ml of deionized water, 7 g fish gelatin, 2.5 ml KH<sub>2</sub>PO<sub>4</sub>, 100 µl MgSO<sub>4</sub>, 100 µl CaCl<sub>2</sub>.
10. Chemotaxis agar (~2.5%) (*see Note 4*), 250 ml: Add 4.25 g agar (change to alter final density) to 243.5 ml deionized water. Heat until dissolved. After the agar cools enough to allow touching the beaker for a few seconds (~55 °C), the additional solutions can be added. Add 6.25 ml KH<sub>2</sub>PO<sub>4</sub>, 250 µl MgSO<sub>4</sub>, and 250 µl CaCl<sub>2</sub>. Add calcium chloride last to prevent its precipitation.

**2.4 Restricted Burrowing Assay**

1. 12-well culture plates.
2. Worm pick.
3. High-density agar (8%) (*see Notes 4 and 5*), 250 ml: 11.36 g agar, 243.75 ml deionized water. Heat until dissolved in a hot water bath with a stirrer. Once completely dissolved, allow to cool to 55 °C (cool enough to touch for 5 s). Add 6.25 ml KH<sub>2</sub>PO<sub>4</sub>, 250 µl MgSO<sub>4</sub>, and 250 µl CaCl<sub>2</sub>. Mix well and store in a water bath at 55 °C until used.
4. Standard density NGM agar (~2.5%) (*see Note 4*), 250 ml: Add 4.25 g agar to 243.5 ml deionized water. Heat until dissolved. Allow agar to cool to 55 °C (cool enough to touch for 5 s). Add 6.25 ml KH<sub>2</sub>PO<sub>4</sub>, 250 µl MgSO<sub>4</sub>, and 250 µl CaCl<sub>2</sub>, 250 µl 5 mg/ml cholesterol in ethanol. Mix well and store in a water bath at 55 °C until used.
5. Fish gelatin (*see Note 2*), 100 ml: 97.5 ml deionized water, 7 g fish gelatin, 2.5 ml KH<sub>2</sub>PO<sub>4</sub>, 100 µl MgSO<sub>4</sub>, 100 µl CaCl<sub>2</sub>.
6. Liquid transfer capillary pipette.

7. Capillary glass.
8. Liquid NGM, 100 ml: 97.5 ml deionized water, 0.3 g NaCl, 50  $\mu$ l CaCl<sub>2</sub>, 50  $\mu$ l MgSO<sub>4</sub>, 1.25 ml KPO<sub>4</sub>, and 50  $\mu$ l of cholesterol (*see Note 1*).
9. Freshly cultured OP50 *E. coli*.

## **2.5 Long-Term Burrowing Assay**

1. 12-well culture plates.
2. Worm pick.
3. High-density agar (*see Notes 4 and 5*), 250 ml: 11.36 g agar (for 8% agar), 243.75 ml deionized water. Heat until dissolved in a hot water bath with a stirrer. Once completely dissolved, allow to cool to 55 °C (cool enough to touch for 5 s). Add 6.25 ml KH<sub>2</sub>PO<sub>4</sub>, 250  $\mu$ l MgSO<sub>4</sub>, and 250  $\mu$ l CaCl<sub>2</sub>. Mix well and store in a water bath at 55 °C until used.
4. Standard density NGM agar (2.5%) (*see Note 4*), 250 ml: Add 4.25 g agar to 243.5 ml deionized water. Heat until dissolved. Allow agar to cool to 55 °C (cool enough to touch for 5 s). Add 6.25 ml KH<sub>2</sub>PO<sub>4</sub>, 250  $\mu$ l MgSO<sub>4</sub>, and 250  $\mu$ l CaCl<sub>2</sub>, 250  $\mu$ l 5 mg/ml cholesterol in ethanol. Mix well and store in a water bath at 55 °C until used.
5. Fish gelatin (*see Note 2*), 100 ml: 97.5 ml of deionized water, 7 g fish gelatin, 2.5 ml KH<sub>2</sub>PO<sub>4</sub>, 100  $\mu$ l MgSO<sub>4</sub>, 100  $\mu$ l CaCl<sub>2</sub>.
6. Liquid transfer capillary pipette.
7. Capillary glass.
8. Liquid NGM, 100 ml: 97.5 m deionized water, 0.3 g NaCl, 50  $\mu$ l CaCl<sub>2</sub>, 50  $\mu$ l MgSO<sub>4</sub>, 1.25 ml KPO<sub>4</sub>, and 50  $\mu$ l of cholesterol (*see Note 1*).
9. Burrowing plate mold (Fig. 3b). We use a wooden block with metal pegs of the desired diameter and length to produce depressions of the desired diameter and at the desired depth in the freshly poured high-density agar.
10. Freshly cultured OP50 *E. coli*.

## **3 Methods**

### **3.1 Single Worm Assay**

1. Use a pipette controller to fill a long glass Pasteur pipette with chemotaxis agar leaving about 50  $\mu$ l of space.
2. With the controller still in place, move the pipette to a container with OP50 *E.coli* and draw 50  $\mu$ l of bacteria into the tip of the pipette.
3. Press the tip of the pipette against a padded surface to prevent the liquid from escaping the pipette while you remove the controller.

4. Use Parafilm strips to seal the top of the pipette, invert the pipette, and repeat with the tapered end containing the OP50.
5. Place the pipette in the 4 °C refrigerator overnight to allow a gradient to establish across the pipette.
6. Remove the pipette from the fridge when ready to use and allow it to reach room temperature.
7. Place a 2 µl drop of NGM on a parafilm strip and pick 10–20 worms into it (*see Note 1*).
8. Draw the worms into a glass capillary using a liquid transfer pipette.
9. Pierce the agar at the top of the pipette using the glass capillary to a depth of 3 cm and begin to slowly pull the capillary back while at the same time pushing the liquid with the worms into the agar. If done correctly the vacuum created in the agar will draw the worms in, allowing them to begin burrowing towards the attractant immediately. If done incorrectly, liquid droplets with swimming worms will be observed in the pipette. These animals are unable to enter the agar and burrow.

### **3.2 Long Distance Assay**

1. Use a pipette controller to fill a 5 ml plastic serological pipette with chemotaxis agar.
2. Press the tip of the pipette against a padded surface to prevent the liquid from escaping the pipette while you remove the controller.
3. Maintaining pressure, use Parafilm strips to seal the top of the pipette, invert the pipette, and repeat with the tapered end.
4. After the agar solidifies, use a Bunsen burner to heat a razor blade held safely with a pair of insulated pliers.
5. Use the hot blade to score the edge of the plastic pipettes making sure that no air pockets remain at the tips of the pipette (e.g., the agar reaches each end).
6. Inject 50 µl of OP50 bacteria 2 cm into one end of the pipette and reseal both ends with Parafilm.
7. Place the pipette in the 4 °C refrigerator overnight to allow a gradient to establish across the pipette.
8. Remove the pipette from the fridge when ready to use and allow it to reach room temperature.
9. Use 1 ml of liquid NGM (*see Note 1*) to transfer worms from a culture plate into a 1.5 ml centrifuge tube and allow animals to sink to the bottom of the tube. This should take about 15 min for adults but longer for larva. Alternatively, gently centrifuge them for a minute at 1000 RPM.

10. Use a pipette to draw as much liquid as possible without disrupting the worm pellet. It is easier to do this than to attempt to pipette the worms directly.
11. Once there is a concentrate of worms in ~50 µl of NGM, use a Drummond pipette to draw the worms into a glass capillary.
12. Pierce the agar at the top of the pipette using the glass capillary to a depth of 3 cm and begin to slowly pull the capillary back while at the same time pushing the liquid with the worms into the agar. If done correctly, the vacuum created in the agar will suck the worms allowing them to begin burrowing towards the attractant immediately. If done incorrectly, liquid droplets with swimming worms will be observed in the pipette. These animals are unable to enter the agar and burrow.
13. With the controller still in place, move the pipette to a container with OP50 *E.coli* and draw 50 µl of bacteria into the tip of the pipette.

### **3.3 Surfacing Assay**

1. Place 1.5 ml of chemotaxis agar of the desired density in the bottom of each well in a 12-well culture plate.
2. Place 20–30 day one adult worms into 2 µl of NGM liquid (*see Note 1*) in the center of the well. Let the NGM liquid be absorbed, the worms will go from swimming to crawling.
3. Place 15 µl of fish agar on top of the worms in each well. Let this agar solidify.
4. Place 1.5 ml of 3% or other density chemotaxis agar on top of the fish agar in each well in a 12-well culture plate. Allow agar to solidify in the 4 °C refrigerator for 2 min to help the fish gelatin solidify ahead of the next step.
5. Place 2 µl of condensed OP50 on top of the wells with 1 µl 1:1000 diacetyl.
6. Score when animals reach the surface of each well. We use the Wormbot from the Kaeberlein lab to score how many animals reach the surface every 10 min [23] (*see Note 6*).

### **3.4 Restricted Burrowing: Making Wells**

1. Place 1 ml of high-density (>8%) chemotaxis agar in the bottom of each well in a 12-well culture plate and let solidify.
2. Place 20–30 worms into a 2 µl drop of NGM liquid (*see Note 1*) to one side of each well. Let NGM liquid absorb, and dab worms with a kimwipe if necessary to prevent them from swimming in the assay. Having the worms to the side will ensure that the animals are burrowing, as they need to make it to the center of the well.
3. Cover worms with fish agar, about 15 µl. Let this agar solidify, it will insulate the worms from heat.

4. Place 1 ml of 3% or other density NGM agar mixed with condensed OP50 on top of the fish agar in each well in a 12-well culture plate. This will be the agar the worms will live and burrow in. Cool at 4 degrees for 2 min to rapidly solidify and protect animals.
5. The well is then capped with 1 ml of 8 chemotaxis agar on top of the burrowing layer in each well in a 12-well culture plate. Cool at four degrees for 2 min to rapidly solidify. This layer ensures the animals will not escape the arena, so be sure it covers the entirety of the top. Animals can then be filmed, and kinematics analyzed (*see Note 7*).

### **3.5 Long-Term Burrowing**

1. Place 1.5 ml of high-density chemotaxis agar in the bottom of each well in a 12-well culture plate.
2. Insert a mold with pegs into the center of each well. Let agar solidify.
3. Remove molds. In each hole, place 20–30 day one adult worms into 2  $\mu$ l of NGM liquid (*see Note 1*) in the center of the well. Let the NGM liquid be absorbed, the worms will go from swimming to crawling.
4. Place enough fish agar on top of the worms to cover them, about 10  $\mu$ l, in each well. Let this agar solidify.
5. Place 50  $\mu$ l of 3% or other density chemotaxis agar mixed with concentrated OP50 on top of the fish agar in each well in a 12-well culture plate. This is the agar the animals will live in. Allow agar to solidify in 4 °C fridge for 2 min, to prevent heat shocking the worms.
6. Cap the well with 1.5 ml of high-density chemotaxis agar. This agar should completely surround the burrowing layer, so that the worms cannot escape. Worms can later be retrieved for behavioral analysis (*see Note 3*).

## **4 Notes**

1. Many groups use M9 buffer for transferring animals. We use liquid NGM for transferring animals because this solution is chemically identical to the agar plates where worms are grown.
2. For fish gelatin, we replace agar with cooking fish gelatin powder available at cooking stores or through [Amazon.com](https://www.amazon.com). Like Pluronic gel, this agar melts around 25 °C and solidifies around 22 °C. We use it sparingly and only to provide thermal insulation for the worms in the assay. Typically, we add a couple of microliters directly above the worms and allow it to solidify. Once cooled, the hotter (~55 °C) burrowing agar will be poured above the worms. The specific heat of the fish gelatin

allows it to absorb the heat from the hot burrowing agar without hurting the animals. Worms do not like the fish gelatin and tend to escape it into the burrowing agar as soon as it has cooled enough for them to burrow.

3. During agar preparation bubbles must be avoided because worms might become trapped within them, and they make filming animals challenging. We use plastic wrap and a rubber band to tightly seal the top of the agar bottle while the agar is still hot. This provides a mechanism for removing unwanted gas dissolved in the agar media: as the agar cools so does the air within the container, shrinking in volume (following  $PV = nRT$ ) and creating a vacuum that successfully draws gas (bubbles) from the agar solution.
4. Making agar of high density requires slow mixing in a water bath.
5. Retrieving worms from agar:
  - (a) When having worms burrow for an extended period of time, you will want to remove the adult worms from the agar and put them into new wells after 2 or 3 days. This helps distinguish the day 5 adults that have been burrowing from their offspring. The plate is made identical to before.
  - (b) To collect the worms, each agar cube is removed from the wells and taken apart on a large 10 cm chemotaxis plate. This can be done using spatulas or razor blades or any other instrument.
  - (c) Once the agar is broken up, flood the plate with NGM (*see Note 1*), causing the worms to swim and dismantling them from the agar, which the worms blend into really well. Pick the worms from the NGM and place them into a seeded plate until you put them to burrow again.
  - (d) When the animals have been burrowing for the chosen length of time, the worms can be retrieved from the agar as described above for analysis.
6. Our Wormbot was built using information available on [wormbot.org](http://wormbot.org) and associated publication [23]. Briefly, Wormbot is a camera mounted on an automated mobile stage that can be programmed to take images or movies of the surfaces of up to 12 multi-well plates at regular intervals. The associated software detects worms on the surface of the well and has several useful features. Using this setup we are able to run 144 simultaneous wells (12 × 12-well plates) but it is scalable to use with plates with higher well count.
7. We use a custom Tierpsy setup [27] including to mount our Basler acA4024-29um camera. This camera allows for high

enough resolution so that each worm is at least 100 pixels in length. Tierpsy takes the video file generated and produces skeletons of each animal once parameters are set. The analysis then allows you to select “single worms,” “bad,” and “worm clusters” from the video, allowing you to choose which animals you analyze (i.e., we do not analyze worms that are not burrowing). Tierpsy then produces an excel sheet providing *summary* information for the entire plate including metrics of animal locomotion (speed, angular velocity) and animal posture (length, quirkiness). In addition, Tierpsy produces these metrics in percentiles so that you know the worst, best, and average performance as well as gives many metrics depending on body segment [27].

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# Chapter 20

## Recording and Quantifying *C. elegans* Behavior

Navin Pokala and Steven W. Flavell

### Abstract

Studies of *C. elegans* behavior have been crucial in identifying genetic pathways that control nervous system development and function, as well as basic principles of neural circuit function. Modern analysis of *C. elegans* behavior commonly relies on video recordings of animals, followed by automated image analysis and behavior quantification. Here, we describe two methods for recording and quantifying *C. elegans* behavior: a single-worm tracking approach that provides high-resolution behavioral data for individual animals and a multi-worm tracking approach that allows for quantification of the behavior of many animals in parallel. These approaches should be useful to a wide range of researchers studying the nervous system and behavior of *C. elegans*.

**Key words** *C. elegans*, Behavior, Tracking software, Behavioral states, Quantitative behavioral analysis

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### 1 Introduction

The quantitative analysis of animal behavior is a widely used approach in neuroethology, neuroscience, and neurogenetics [1]. For decades, researchers have quantified the behavior of small invertebrate animals to gain insights into basic principles of nervous system development and function. For example, genetic analysis of circadian rhythms in *D. melanogaster* revealed evolutionarily ancient mechanisms that underlie the molecular clock [2]. Behavioral studies of *C. elegans* date back to the 1970s [3, 4] and have been extended and refined over time. Quantitative analysis of *C. elegans* behavior has been a “workhorse” method for genetic studies [5, 6], ethological studies [7, 8], and studies of neural circuits [9] and behaviors [10, 11]. Automated behavior tracking methods pioneered in *C. elegans* [12] have inspired the development of automated methods for other animals [13].

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Navin Pokala and Steven W. Flavell contributed equally to this work.

We describe here two platforms for tracking and quantifying *C. elegans* behavior: a single-worm tracker and a multi-worm tracker. These methods contain the same basic elements—video recording, image analysis, and behavior quantification—but the protocols and resulting datasets differ considerably. The single-worm tracker uses the openAutoScope microscope platform [14] that builds upon previous single-worm tracking approaches [15, 16]. This method provides high-resolution information about *C. elegans* behavior, quantifying locomotion, posture, defecation, egg-laying, and pharyngeal pumping. Because this method allows for the simultaneous quantification of multiple distinct motor programs, it can be useful for studies of many different behavioral outputs. For example, this recently described approach enabled the identification of a dopaminergic circuit that couples locomotion and egg-laying as animals switch behavioral states [14].

The MultiWormTracker, an offshoot of the Parallel Worm Tracker program [17], permits recording and analysis of several animals simultaneously. The MultiWormTracker is best suited for quantifying locomotion parameters such as speed, reversals, and turns, and measuring how their distributions in populations evolve over time [9, 11]. It is highly flexible, and can function under a variety of different experimental conditions including uniform food lawns [18], finite food lawns [19, 20], off-food plates [21], chemotaxis [22], chemo- and optogenetics [23, 24], and microfluidics [25].

For both methods, we present required materials, standard recording protocols, and required data analysis procedures.

## 2 Materials

### 2.1 *C. elegans* *Growth and Maintenance*

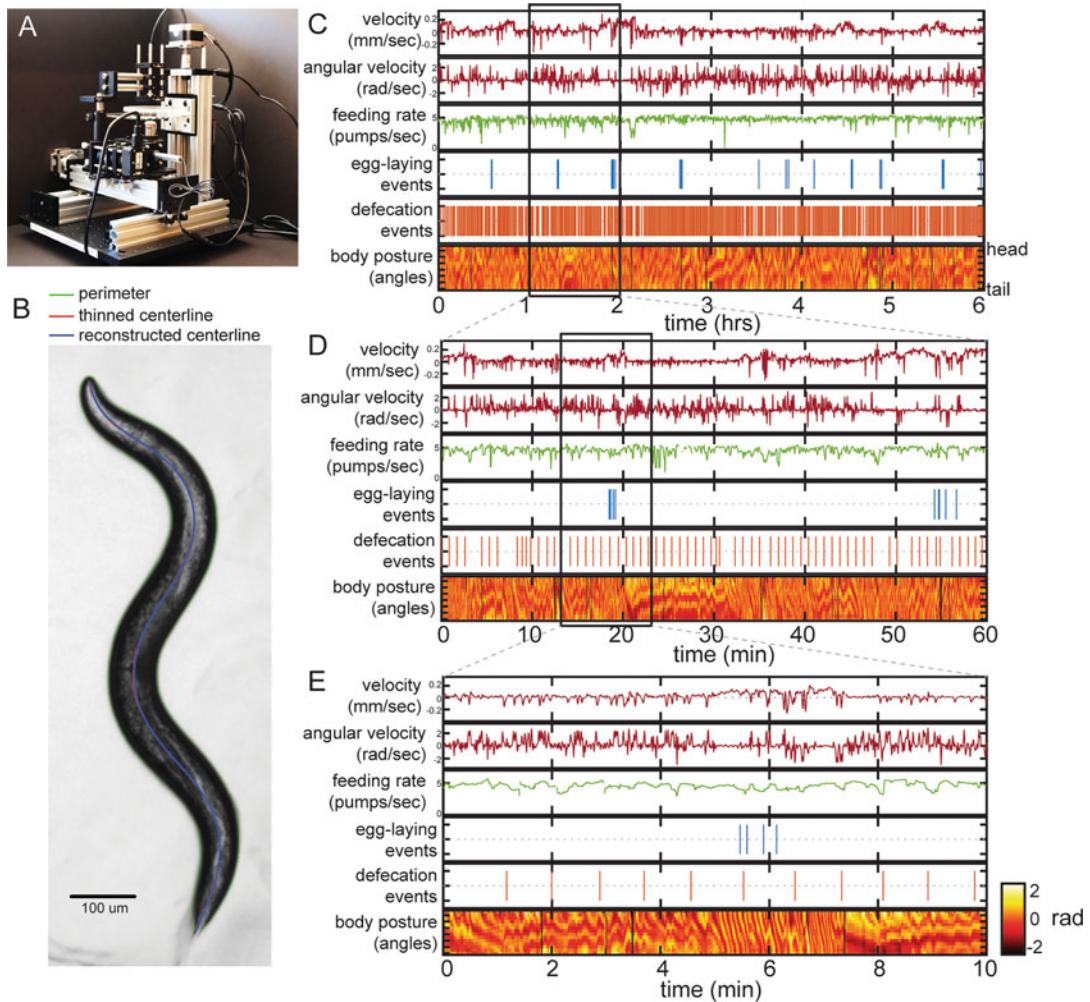
1. Nematode Growth Medium (NGM) plates: Mix 3 g NaCl, 2.5 g peptone, 17 g granulated agar, in 975 mL double-distilled water (deionized to 18 mega-ohm). Autoclave for 30 min and cool to 60 °C while stirring on a hot plate. Add 1 mL of 5 mg/mL cholesterol dissolved in ethanol. Add the following autoclaved salts and buffers in order: 1 mL 1 M CaCl<sub>2</sub>, 1 mL 1 M MgSO<sub>4</sub>, and 25 mL 1 M potassium phosphate pH 6.0. Pour into sterile petri dishes. Let dry overnight and store at 4 °C in plastic storage boxes for up to 6 months [26].
2. *Escherichia coli* OP50 suspension: OP50 bacteria can be obtained from the *Caenorhabditis* Genetics Center. Streak out OP50 from frozen glycerol stock onto a LB or NGM plate and grow overnight at 37 °C. Inoculate a single colony into one bottle of sterile LB (50–500 mL). Leave growing at room temperature without shaking for 2 days with cap slightly ajar

(though on top of bottle). Store at 4 °C for up to 2 months. Practice sterile technique during all steps of bacterial growth since there is no antibiotic resistance marker (*see Note 1*).

3. Copper chloride solution: 20 mM CuCl<sub>2</sub> in double-distilled water.
4. Filter paper: Whatman Grade 1 Qualitative Filter Paper.
5. Staged animals: Both tracking methods work best with young adults. Three days before behavioral recordings, the “staging” procedure to grow animals of a reliable age begins. Pick ~10–15 adult animals to a standard OP50 growth plate and permit them to lay eggs on the plate for 1 h. After the hour, remove all adults from the plate. Allow the plate to sit at room temperature for 72 h to obtain 1-day-old adult animals for recordings. Alternatively, pick L4 animals the day before the assay (*see Note 2*).
6. All-trans retinal: 50 mM all-trans-retinal in 100% ethanol. Store 1 µL aliquots at –80 °C.
7. OP50 bacteria concentrate for optogenetics: Grow 50 mL OP50 as described in **item 2**. Centrifuge the bacteria, and remove all but 10 mL of supernatant. Mix by vortexing. Store at 4 °C for up to 2 months.
8. Retinal feeding for optogenetics: The day before the optogenetics experiment, add 1 mL of OP50 bacteria concentrate to a freshly thawed 1 µL 50 mM all-trans-retinal aliquot (50 µM final retinal concentration). Immediately vortex and spread 200 µL on each unseeded NGM plate (*see Note 3*). Let the liquid absorb before adding animals. Transfer transgenic L4 animals to the plate. Pick a similar number of animals to a non-retinal plate. Store all of these plates in the dark overnight.

## **2.2 Single-Worm Tracking on the openAutoScope**

1. Low-peptone NGM plates: Follow recipe for NGM plates in Subheading **2.1**, **item 1**, but only add 0.2 g of peptone. All other components and aspects of preparation are identical. Use 10 cm petri dishes (*see Note 4*).
2. Computer hardware, software, and scripts: Use a computer running Windows 10. Download and install Arduino IDE (<https://www.arduino.cc/en/Main/Software>), teensyduino (<https://www.pjrc.com/teensy/teensyduino.html>), and R and R Studio (<https://rstudio.com/products/rstudio/>). Purchase and install National Instruments (NI) Labview (full 2017 or later), as well as NI Vision Acquisition Software and NI Vision Development Module. All custom software and scripts relevant to this protocol are available at <https://bitbucket.org/natecermak/openautoscope/src/master/>.



**Fig. 1** (a) Image of the openAutoScope tracking microscope. (b) Example image of a *C. elegans* animal from the tracking microscope. Green line denotes detected outline of worm; red line indicates worm's centerline, obtained by thinning the thresholded image; blue line is the centerline as reconstructed from a spline-based 14-parameter representation. (c–e) Example dataset from the tracking microscope, showing the main *C. elegans* motor programs over 6 h (c), 1 h (d), or 10 min (e). (Images are re-printed with permission from Cermak et al., 2020)

3. Microscope: Construction of the openAutoScope consists of several steps. A build tutorial with step-by-step instructions, including detailed images and a full parts list, is available at <https://bitbucket.org/natecermak/openautoscope/src/master/>. Here, we describe the components that are assembled (Fig. 1a):

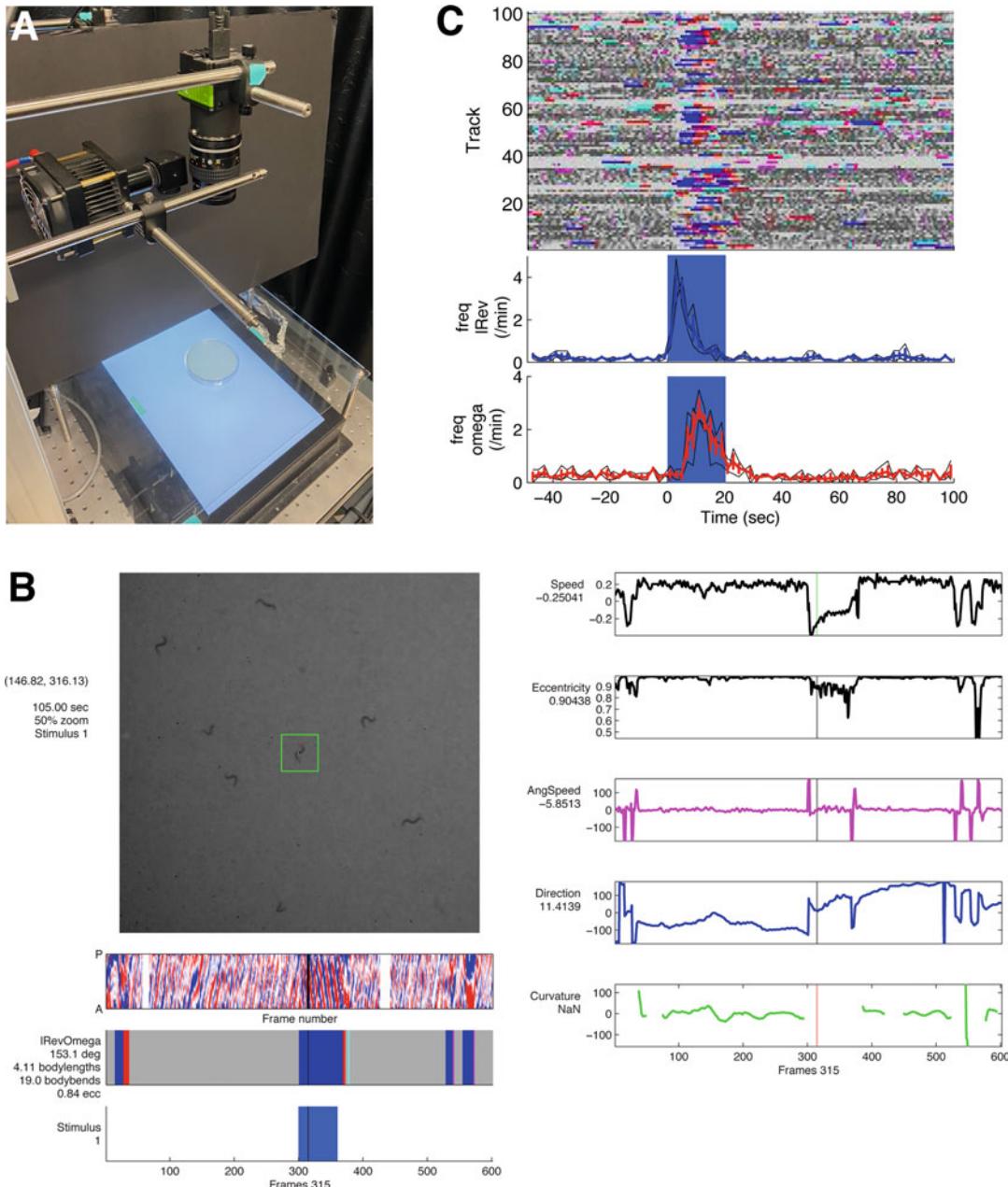
- (a) Optical components: The optical path of the microscope consists of a  $4\times/0.1\text{NA}$  Olympus PLAN objective coupled to a 150 mm tube lens, resulting in  $3.33\times$

magnification. The image is projected onto a monochrome FLIR Chameleon 3 camera with a 0.5 inch sensor, yielding a  $1.84 \times 1.47$  mm field of view at  $1.4 \mu\text{m}/\text{pixel}$  resolution. A 780 nm, 18 mW infrared LED is used for illumination in a transillumination configuration.

- (b) Laser for optogenetic stimulation: A 4.5 mW, 532 nm laser diode module is used for optogenetic stimulation. The laser is combined with the main optical path via a 550 nm dichroic and illuminates a 1.6 mm diameter region in the sample plane. Laser intensity is set to  $150 \mu\text{W}/\text{mm}^2$ .
- (c) Linear axes: Stage movement is controlled by motorized C-beam linear actuators with  $\sim 150$  mm of travel distance in each direction. The optical components of the microscope are mounted on the X and Y actuators so that the microscope moves (instead of the worm stage). The worm stage is mounted on the Z actuator. The stepper motors that move the actuators have a resolution of  $1.25 \mu\text{m}$  in each linear axis. The DRB8825 stepper motor controller ICs that control these motors are controlled with a Teensy 3.2 microcontroller.
- (d) Printed circuit board (PCB): The components of the microscope are connected on a PCB. The Teensy on the PCB is connected to a computer via a USB cable. The Teensy in turn controls the infrared LED, green laser for optogenetics, and the three linear actuators for microscope movement.
- (e) For computer control of the Teensy: Program the Teensy by opening the custom “scopeController.ino” file in the Arduino IDE.
- (f) Linking camera acquisition to microscope control for closed-loop tracking: Run the custom Labview code “scopeController.vi”.
- (g) Align the laser for optogenetic control and the LED light source. This should be done periodically though it is not required daily. For the laser, place a fluorescent slide on the stage. Turn the laser on in the Stage Control tab of the Labview software, set the display mode to Page 1 (Raw-Image), and adjust the right-angle mirror (using the knobs on it) until the brightest point is in the center of the field of view. After aligning the laser, turn off the laser and turn on the LED. To align the LED, manually move the overhead light source until the brightest spot is in the center of the field of view.

### 2.3 Multi-Worm Tracking

4. Data analysis: Open the custom R project woRmtools.rproj and install it locally by clicking Build/Install and restarting software. Open the custom analyzeWorms.R file, which has the full data analysis suite.
1. Thin-lawn plates: Thin-lawn plates are prepared by flooding standard NGM plates with a saturated OP50 culture, removing excess liquid, and incubating at room temperature for 16 h.
2. Copper ring: Copper (II) chloride is a potent worm repellent and can be used to prevent animals from leaving an area. Cut a Whatman filter paper frame of the desired size. The filter paper frame size and shape can be customized. For a standard 60 mm plate, we use a 1.125 inch (28 mm) square punch to create a hole. Trim a ~0.25 cm wide frame around the hole. Using forceps, submerge the frame in a petri dish containing 20 mM CuCl<sub>2</sub> for a few seconds. Remove the filter paper, and tap on the lid of the dish to remove non-absorbed drops of liquid. Gently place the frame on the assay plate agar. Use the forceps to gently flatten any folds, and ensure that the entire frame is flushed with the agar. Use within 1 h.
3. Worm pick: To transfer animals without food, flatten the ~5 mm of the tip of a platinum worm pick with needle-nose pliers.
4. Camera and recordings: We have used a FLIR Grasshopper 3 camera (FLIR GS3-U3-50S5M-C), controlled by Flycapture software from the manufacturer. We have also used a Pixelink PL-D7715 camera, controlled by Stremapix software (Norpix). The camera can be attached to an 18 mm fixed focal length lens, or to a stereomicroscope equipped with a wide-angle lens (Fig. 2a). Video files recorded at 3 Hz and saved as 8-bit grayscale avi files with MPEG-4 video compression work well. The resolution should be 15–40 pixels/mm. If a copper ring is used, plates and zoom should be positioned and adjusted such that the inner edge of the ring is clearly visible. The tracking analysis censors reversal and turn events that occur within 2.5 mm of the ring, and all behavior 10 s after a ring-induced event [27]. Before beginning actual recordings, adjust the zoom, focus, and lighting with a mock assay plate containing one or two animals.
5. Optogenetic stimulation: We use LED spotlights (for ChR2, 455 nm Mightex PLS-0455-030-S) driven by a computer-controlled power supply (Mightex SLC-SA02-US). The LEDs may be held by standard ring-stand clamps, or by dedicated optics hardware. Adjust the position and orientation of



**Fig. 2 (a)** Image of a multi-worm tracking microscope. **(b)** WormPlayer GUI displaying part of a track of an animal expressing ChR2 in the AVA command neurons. These animals reverse continually during photo-stimulation, then make an omega turn. This animal is in the midst of an AVA:ChR2-elicited reversal. The green box indicates the selected worm, with blue, green, and red dots indicating the head, mid-body, and tail, respectively. The vertical black line in the strip-charts indicates the current frame. Displayed charts include a body posture kymograph, ethogram, photostimulation state, and instantaneous speed, angular speed, direction, and other parameters. **(c)** Ethograms and examples of stimulus-onset-averaged event frequency time-courses for animals expressing ChR2 in the ASH sensory neurons. These animals make a long reversal (dark blue) immediately followed by an omega turn (red). The frequency time-courses are averaged over three movies; the black lines indicate the average trace from each movie

the LED and slide the attached lens to ensure that the entire worm arena is illuminated. Using the control software, adjust the LED current to achieve the desired light intensity as measured by a laser power meter. Retinal-fed ChR2-expressing animals respond robustly to a 455 nm LED projecting at  $25 \mu\text{W}/\text{mm}^2$ , while non-retinal-fed control animals show no response. To reduce excitation glare in recordings, the lens can be inserted into a paper cup whose bottom is replaced with a glued filter sheet (with appropriate wavelength filter to prevent transmission of optogenetic light source). Alternatively, a glass filter can be directly fit onto the camera lens. To minimize photoactivation by the transmitted white light illumination, a filter sheet can be placed directly between the transmitted white light source and the plate. For ChR2 activation, we use Rosco-lux #312 filter sheets.

## 6. MultiWormTracker Software.

- (a) The MultiWormTracker software runs in MATLAB (Mathworks) and requires the MATLAB Image Processing and Statistics toolboxes. While it works in newer versions (up to MATLAB 2020a), it was developed and tested most extensively using MATLAB 2011b on PCs running Microsoft Windows 10. The MultiWormTracker software can be downloaded from Github (<https://github.com/navinpokala/MultiWormTracker2020>).
- (b) Using Windows, copy the file put\_into\_C\_windows\_system32\pdftk.exe in the MultiWormTracker2020 directory to C:\Windows\System32.
- (c) Launch MATLAB and change to the MultiWormTracker2020 directory. Before you use the program for the first time, edit MultiWormTracker\_setup.m line 8 where MultiWormTracker\_CODE\_PATH is defined to where you put the MultiWormTracker2020 source code directory.
- (d) Run MultiWormTracker\_setup in MATLAB each time you want to use the code.
- (e) This will temporarily set the program path to the MultiWormTracker\_CODE\_PATH directory and temporarily remove any other non-core MATLAB paths. This is necessary to avoid conflicts between function names, etc. These path changes are NOT saved; you will need to run MultiWormTracker\_setup each time unless you manually save the path.

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### 3 Methods

The single-worm tracker and multi-worm tracker have different hardware, protocols, and analysis software, so they are described separately.

#### 3.1 Single-Worm Tracking on the openAutoScope

##### 3.1.1 Preparation

1. One day before recordings, seed the assay plates with OP50 bacteria. Take the desired number of 10 cm low-peptone NGM plates from the fridge, add 200  $\mu$ L OP50, and spread the bacteria into a roughly circular lawn whose edges are >1 cm from the edges of the petri dish. Let plates sit at room temperature overnight. There should be a dry, thin lawn the next morning. (*see Note 5*).
2. On the day of the recording, transfer one animal to a seeded assay plate. Be careful not to transfer large amounts of bacteria with the animal. Move the plate onto the microscope stage face down. If long recordings are desired, remove the lid to prevent condensation. The plate should be placed on  $\frac{1}{4}$ " spacers in this case. Use tape to attach plate to the stage so that it does not move as a result of microscope movement.
3. Open LabView software and open the scopeController.vi program. Set the Control COM port to the appropriate number. Click the white arrow in the upper left to initiate the program. Go to the Saving tab and uncheck all boxes. Go to the Tracking tab and uncheck all of the indicators to turn the tracking function off. Under the Stage Control tab, turn the LED on and click Motors Enabled on. The field of view should now be visible, with the stage function on and closed-loop tracking off. No data will be saved under further specified (*see Note 6*).

##### 3.1.2 Collect the Lawn Boundary Information

1. Set the filename of the lawn boundary file. This file stores the coordinates of the lawn boundary for later analysis. The filename should be specified as a .csv file in the Saving tab under "file tracking path."
2. Under the Stage Control tab, use the Up/Down/Left/Right controls to move the stage to the lawn boundary. Under the Camera tab, adjust the exposure time so that the lawn edge is easily visible. Focus up/down if necessary.
3. Under the Saving tab, check the "saving tracking data" indicator next to "file tracking path" to begin saving. Then Stop and Start the LabView program one more time using the red stop and white arrow icons in the upper left (required for running the program free of bugs). Then use the cursor controls to move the field of view along the lawn perimeter. The objective is to keep the lawn boundary in the center of field of view and

go all the way around the perimeter. Progress can be visualized in the Page 6 tab “worm trajectory” panel.

4. After you have completed the circle around the perimeter, go to the Saving tab and uncheck the indicator next to “file tracking path.” This completes the lawn boundary file. From this point forwards, do not adjust the plate position or else the lawn boundary will need to be re-recorded.
- 3.1.3 *Collect Worm Data*
  1. Rename all three file names with the names you would like to use for saving the worm data. The .avi file is not actually used. But the other file paths specify the directory for saving worm images and the file name for storing all of the tracking info (stage position, etc.).
  2. Find the worm on the plate and use the cursors to get the animal in the field of view.
  3. Once the animal is in view, go to the Tracking tab and check all 3 indicators, Process images, Tracking and Periodic autofocus, in the tab to initiate tracking. After this, the outline of the animal should be indicated and the microscope should automatically track the animal.
  4. Adjust the exposure time again so that the outline of the worm’s perimeter is precisely correct, including the head. Typically, the ideal brightness results in the head appearing brighter than the tail.
  5. If you are using optogenetics in your experiment, use the Laser Control tab to set the parameters of your stimulation protocol. Note that the stimulation protocol only begins when you manually click on “start simulation program,” so you may need to click on this when you start the video or at a later time if your stimulation should begin later in the video (the exact time points when the laser is on/off are logged, so the precise timing will be available during data analysis).
  6. Stop/Start the Labview program again and then click “start timed recording” under the Saving tab with the duration of the recording set as desired. All files will be fully saved after the specified time is elapsed.
- 3.1.4 *Analyzing the Data*
  1. The important files for data analysis are the lawnboundary.csv file, the trackingdata.csv file (which has stage movement information and more), and the worm images, which are stored as jpeg files in the folder that you specified.
  2. In the analyzeWorms.R file, specify the paths of the lawn boundary file, worm tracking file, and the image directory.
  3. Run the R code from top to bottom of the analyzeWorms script. The script is largely automated, but there are two points

that will require user input. First, after the majority of the analysis has completed, a matrix will appear in the plots window, showing the intensity of the worm's body along its centerline (as y-axis) over time (x-axis). It will ask whether the head was defined correctly. Since the head is brighter, you should answer yes if the bottom of the image is brighter and no if it isn't. If you enter no, it will invert the image and re-display, asking the same question again. Second, candidate egg-laying events need to be annotated by the user. During this stage, two consecutive images will appear in the plots window and the user will answer questions about whether an egg was laid during the time interval between the two images. First the user is asked to determine whether there is an actual egg-laying event until a true event is detected (enter "v" for a real event; enter "u" to proceed to next candidate event). This step is used to identify the ventral side of the body. After this, the script will display candidate egg-laying events on the ventral side of the body. The user is asked to input how many eggs were laid between these two consecutive frames. Enter 0 if there was no egg laid, or 1 (or higher) if eggs were laid.

4. The R code will save a CSV file at the end with the analyzed data. This file can be opened in any software for further analysis and inspection of behavioral data (Fig. 1b–c; *see Notes 7 and 8*). Time-dependent parameters in the CSV file include: body area and length; body posture (bodyangles and radius of curvature quantified from 14 evenly spaced segments along the anterior-posterior axis); head and mid-body coordinates; head and mid-body distances to food lawn edge; speed; behavior indicators (omega turn, defecation, egg-laying, pharyngeal pumping rate); optogenetic laser status; microscope autofocus status.

## **3.2 Multi-Worm Tracking**

### **3.2.1 Preparation**

1. The day before the experiment, pick L4 animals to a day-old thin-lawn plate. Each assay will require 20–30 animals. For optogenetic experiments, pick animals to retinal plates as described in Subheading 2.1, item 8, and pick a similar number of animals to a non-retinal plate.
2. Prepare thin-lawn food assay plates as described in Subheading 2.3, item 1, and incubate at room temperature overnight. Covering the entire plate eliminates lawn edge effects on exploration [19].
3. Food-free assay plates should be allowed to sit at room temperature overnight.
4. The day of the experiment, let food-free and seeded NGM assay plates dry for an hour, face down, tilted on their lids. Wipe off any condensation on the lids.

5. Prior to transferring animals to an assay plate, place a copper ring. Use within 1 h.
6. Using a mock assay plate containing a few worms, adjust the zoom, focus, and lighting of the recording arena.

### 3.2.2 Exploration Assay

On food lawns, *C. elegans* animals switch between bouts of roaming and dwelling [18]. When transferred to a food-free plate, these animals explore a local area by increasing their frequency of spontaneous reversals and turns for 10–20 min, then transition to a dispersal state with long forward runs with few reorientations [21].

1. Transfer 20–30 young adult animals to a thin-lawn food assay plate. Let the animals equilibrate for 30 min (*see Note 9*).
2. Gently place the plate under the camera and record for 10 min. These videos will allow quantification of on-food behavior.
3. Set the camera software to record for 1 h, so that minimal action (a single mouse click or keystroke) is required to trigger recording.
4. Place a copper ring on a food-free assay plate.
5. Start a timer for 2 min ( $t = 0$ ). Using a lump of food, transfer the animals from the food assay plate to a food-free plate (rinse plate). Flame the worm pick. Let the tip cool by touching it to the rinse plate agar. Scoop animals that have crawled away from the transfer drop point by sliding the flat pick between the animals and the agar. Quickly transfer the picked animals to the center of the food-free assay plate. Repeat the scoop and transfer until all the animals have been transferred to the assay plate, or when the 2 min are up ( $t = 2$  min).
6. Position the plate face down under the camera. At  $t = 3$  min, trigger recording.
7. Create four directories ('mystrain\_food', 'mystrain\_nofood', 'control\_food', 'control\_nofood'), and save the movies to the relevant directories.
8. Run MultiWormTracker({'mystrain\_food', 'mystrain\_nofood', 'control\_food', 'control\_nofood'}).

### 3.2.3 Multi-Worm Optogenetics

1. Place a copper ring frame on an assay plate relevant to the experiment, and transfer retinal-fed animals. If assaying animals off-food, transfer them as described in Subheading 3.2.2, step 5.
2. Depending on the experiment, you may wish to let the animals acclimatize to the plate prior to recording.
3. Wipe any condensation off the lid with a Kimwipe 10 min before recording.
4. Gently place the plate under the camera, with the lid facing up.

5. Start the recording and the photostimulation programs.
6. Create directories for the retinal-fed and non-retinal-fed controls, and save the movies to the relevant directories.
7. Launch MATLAB and change to the MultiWormTracker2020 directory. Run MultiWormTracker\_setup to set the program path. Go to the parent directory of the experiments.
8. Create a stimulus text file that describes when the light (blue, amber, green, or red) is on. An example is given in the README.txt file.
9. Process the movies as described below, but include the stimulus file as an argument. For example, MultiWormTracker({'retinal\_fed','controls'},'stimulus','my\_stimulus.txt'). The linked-Tracks and BinData files have fields that identify frames and times that are photostimulated. Plots that highlight behavior during stimulation times will be automatically generated as .pdf files. Peristimulus time histogram (psthistogram) psthistogram, psthistogram, and pdf plot files with tracks aligned at the start of stimuli are also produced.

### 3.2.4 Tracking and Analysis

1. Launch MATLAB and change to the MultiWormTracker2020 directory. Run MultiWormTracker\_setup to set the program path. Go to the parent directory of the experiments.
2. To track a single movie that includes a standard 28 mm square copper ring, run MultiWormTracker('my\_movie.avi'). To track a directory of movies that all include a standard 28 mm square copper ring, run MultiWormTracker('my\_directory').
3. To track multiple directories of movies that all include a standard 28 mm square copper ring, run MultiWormTracker({'directory1','directory2','directory3'}).
4. Prior to starting the fully automated tracking, the program will automatically find the all rings and attempt to find 20–30 animals for each movie. If it cannot find a ring, or cannot find 20–30 animals, a graphical user interface (GUI) will pop up with instructions for the user to follow. The program will then launch the fully automated tracking processes.
5. If you do not use the standard 1.125 inch (28 mm) square copper ring, take a short 10 s movie of an object of known size, and save it as “scale\_movie.avi.” Include this as an additional “scale” argument when tracking. For example, MultiWormTracker('mymovie.avi','scale','my\_scale\_movie.avi'). A GUI will guide the user to define the length of the scale object contained in 'my\_scale\_movie.avi'.
6. If your framerate is NOT 3 Hz, include “framerate,” your\_framerate\_in\_Hz as an argument. For example

`MultiWormTracker('mymovie.avi','scale','my_scale_movie.avi','framerate',5)` would be used to analyze a movie recorded at 5 Hz.

7. If number of worms <20 or >30, then you should include 'numworms', #worms as an argument. For example, `MultiWormTracker('my_movie.avi', 'numworms', 10)` will analyze a movie with only 10 animals.
8. A number of files and plots are produced for each movie. The two most useful .mat files are the `linkedTracks.mat` (tracks of individual animals with descriptors for each frame including instantaneous speed, coordinates, body posture and shape, pirouette parameters such as reversal length and turning angle, and behavior state) and `BinData.mat` (averaged time-course data).
9. The `README.txt` file in the downloaded software directory describes the `linkedTracks` and `BinData` files in more detail. MATLAB functions useful for analyzing `linkedTracks` and `BinData` files are also described.
10. `linkedTracks` is a structure array with an element for each animal. Fields within `linkedTracks` are arrays whose elements describe the behavior of the animal in each frame. Fields in `linkedTracks` encode information such as: time and movie frame; X and Y coordinates for the animal in each frame; heading direction in each frame; instantaneous speed and angular speed; instantaneous path curvature; posture information including body eccentricity, body curvature, head and body angles; parameters including reversal length and change in direction due to a turn for each reorientation event; instantaneous locomotion state of an animal such as forward, pause, reversal, omega turn, and epsilon turn; presence or absence of a stimulus (i.e., optogenetic light) at each frame.
11. `WormPlayer('mymovie.linkedTracks.mat')` is a GUI-based viewer for examining the tracks of individual animals overlaid onto the movie. The animal in the current track is boxed, with dots indicating the head, center, and tail. The "T" button toggles the track, which is colored by behavioral state. The "Z" button is used to zoom. When the movie is played, indicators on strip-charts show instantaneous behavioral parameters of the animal such as its speed, angular speed, eccentricity, behavioral state, path curvature, and body posture kymograph (Fig. 2b).
12. `BinData` is a structure whose fields contain averaged behavior time-courses, along with standard deviation and standard error of the mean. Instantaneous parameters (speed, posture, fraction of animals in a given locomotion state) are averaged over 1 s, while event frequencies are averaged over 10 s. Fields in

BinData encode information such as time; forward speed; path curvature; posture (body eccentricity, head and body angles); frequencies of events such as pauses, reversals, and turns; fractions of animals in locomotion states such as forward, pause, reversal, and turn; locomotion state transition probabilities.

13. The freqs.txt and non\_freqs.txt text files contain the data from BinData.mat in a tab-delimited text format which can be opened by other programs for further analysis. The pdf files display plots for a selection of these averaged time-courses data (Fig. 2c).
14. If you analyze a directory, BinData.mat, freqs.txt, and non\_freqs.txt files will be created that contain weighted-average time-courses from all the movies in that directory, along with pdf files that display plots of these data.

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## 4 Notes

1. We describe protocols here using OP50 bacteria, but many different strains and species of bacteria can be fed to *C. elegans*. This is a critical aspect of experimental design, since the food source of the animal profoundly impacts its behavior [28].
2. The developmental stage of the animal during the recording is important for two reasons. First, animals of different ages display different behaviors, so uniform staging is important for reproducible results. Second, both programs track animals based on their size, and default to young adults. This would need to be adjusted for younger animals that are smaller and more transparent. For single-worm tracking, in openAutoScope under Tracking tab, “criteria” would need to be adjusted. For multi-worm tracking, the minWorm and maxWorm parameters in define\_preferences.m would need to be adjusted.
3. Since 50 µM retinal can retard bacterial growth, a concentrated bacteria slurry is used for feeding animals retinal.
4. The use of low-peptone plates is important to prevent the bacterial lawn from growing too thick. Thick bacterial lawns degrade tracking quality, since they can mask the worm and the worm can leave behind high-contrast tracks that can confuse the tracker.
5. When animals reach the perimeter of the plate, they will be lost by the tracker and the recording will be functionally over. To prevent this, it is helpful to leave >1 cm between the lawn boundary and the plate perimeter. For recordings in the

absence of food, use filter paper with 20 mM CuCl<sub>2</sub> as is described in the multi-worm tracker protocol.

6. There is a green dot in the center of the field of view that indicates the target center position. Beware that if you click in the field of view, this will move this target (indicated by the green dot moving).
7. A small fraction of recordings can yield low-quality data. The main indicator of a low-quality recording is that the worm's posture was not identified in a large fraction of frames. For each recording, it is useful to check the number of "NA" entries in the bodyAngles data. If >10% of the entries are "NA," the video can be rejected on the basis of low-quality data.
8. The body angles in the saved .csv file are not adjusted for dorsal/ventral alignment. The source of this issue is that some animals lie on their left sides during movement and others lie on their right sides. The eggLoc variable can allow the user to align animals, though, since it indicates the body segment where the eggs were laid, and eggs are always laid on the ventral side. From the tip of the nose, segments 1–6 (arbitrarily numbered) are the left side of the animal as it appears on the screen, while the segments 7–12 are the right side. Thus, if one animal has its eggs laid along segments 3–4, but another has its eggs laid along segments 9–10, these animals were lying on opposite sides during recording. Body angles can then be inverted for select animals to then correct for dorsal/ventral alignment.
9. Transferring the worm by picking or other means can impact its behavior, typically stimulating it to move at increased speeds for 15–30 min afterwards. This should be taken into account when designing experimental protocols.

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# Chapter 21

## Primer on Mathematical Modeling in *C. elegans*

Ayush Ranawade and Erel Levine

### Abstract

Recently, applications of mathematical and computational models to biological processes have helped investigators to systematically interpret data, test hypotheses built on experimental data, generate new hypotheses, and guide the design of new experiments, protocols, and synthetic biological systems. Availability of diverse quantitative data is a prerequisite for successful mathematical modeling. The ability to acquire high-quality quantitative data for a broad range of biological processes and perform precise perturbation makes *C. elegans* an ideal model system for such studies. In this primer, we examine the general procedure of modeling biological systems and demonstrate this process using the heat-shock response in *C. elegans* as a case study. Our goal is to facilitate the initial discussion between worm biologists and their potential collaborators from quantitative disciplines.

**Key words** Mathematical modeling, *C. elegans*, Heat-shock response, Quantitative biology

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### 1 Introduction

Recent technological advances in the quantitative experimental tools have enabled system biologists to study the functionality, behavior, and dynamical properties of inherently complex biological systems. The resulting data, at an unprecedented scale and level of quantitative accuracy, drives the development of statistical and mathematical models. While statistical models are necessary to extract knowledge from complex data, mathematical models are used to formulate and test hypotheses about mechanistic roles of constituents and interactions. Additionally, math models are used to identify universal properties and design principles of biological systems. Such characteristics are often difficult to establish by the reductionist approach taken during the experimental dissection of individual aspects of complex systems.

Several animal models, including *C. elegans*, have been successfully used for mathematical modeling. Since its first use as an animal model nearly 50 years ago, *C. elegans* has come a long way to

facilitate our understanding of a wide range of biological questions [1]. The properties that make *C. elegans* a powerful experimental system also make it suitable for acquiring high-throughput quantitative data for building mathematical models. These properties include the transparency of the worm, which allows quantitative spatiotemporal measurements of gene reporter expression, in vivo molecular dynamics, and in vivo tracking of cell lineages. The compact and small nervous system and the advent of optogenetic tools in the worm enable observation and precise perturbation of neural activity. The worm's small body size allows the monitoring of individual and group behavior. Moreover, its relatively short life cycle (~3.5 days), fast reproduction cycle with a high progeny number (~300), and short lifespan (~2 weeks) permit quantitative characterization of aging and evolutionary phenotypes. Worms can be grown quickly at large numbers, which allows a wide array of high-throughput assays [2]. In addition, recent technological innovations in automated imaging of *C. elegans*, especially the adoption of microfluidics approaches, have made the acquisition of large-scale quantitative data possible [3].

Mathematical modeling approaches take advantage of these quantitative measurements by providing a set of analytical and numerical tools that bridge the gap between the observable phenotype and the mechanistic principles of the underlying biological system. Biological processes in *C. elegans* that has been subject to mathematical modeling include, for example, the vulval precursor cell development [4], neural control of behavior [5], calcium dynamics in the neuron [6], feeding dynamics [7], aggregation [8], foraging [9], hypoxia [10], chemotaxis [11], and locomotion [12]. These modeling studies have contributed towards improving our knowledge of these biological processes. However, despite the suitability of *C. elegans* for mathematical modeling, this approach has not been widespread.

The major hurdle for developing useful mathematical models is the requirement for in-depth knowledge of two different domains: mathematics and biology. This problem is compounded by the communication challenges characteristic of any interdisciplinary collaboration. This chapter attempts to address this issue by providing a useful guide for *C. elegans* experimental biologists interested in integrating mathematical modeling into their scientific toolkit. For this, we provide an introduction to the fundamental concepts needed for the construction, development, and investigation of mathematical models.

We organized this chapter around describing the general workflow for building a mathematical model and illustrate this process using a case study of modeling heat-shock response in *C. elegans*. Our goal is to lower the barriers and enable interdisciplinary communication and research collaborations between worm biologists and mathematicians.

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## 2 How Mathematical Models Are Built

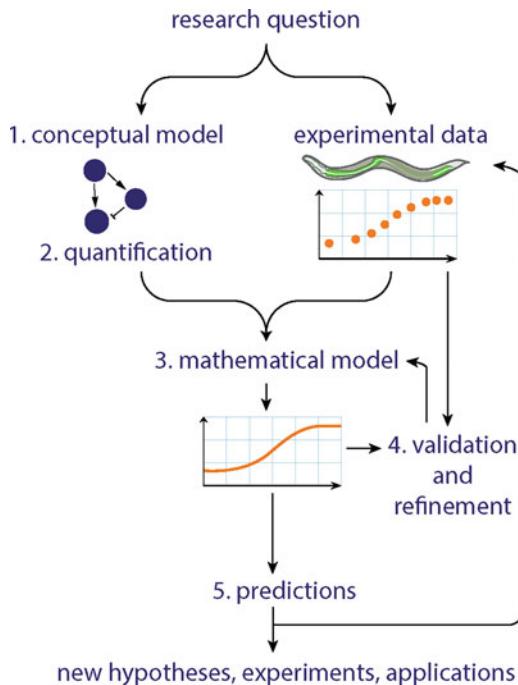
The purpose of models is not to fit the data but to sharpen the questions.—  
Samuel Karlin.

Like any scientific investigation, building a model starts with a well-defined question or hypothesis that relies on a concrete understanding of the system. Before describing the modeling process, it is worthwhile to distinguish between three commonly used terms: “biological system,” “model,” and “mathematical model.”

A “*biological system*” is a subset of biochemical species and physical entities that are thought to be at the core of some biological functionality, and the interactions among them. In most cases, the constituents of the system also interact in many ways with other entities and signals. The decision to include which components in the model and which are taken as external influences is an important modeling choice that must be carefully considered.

A “*model*”(or “conceptual model”) is the most straightforward representation (often in a pictorial form) of the biological system, which can be used to develop hypotheses, design experiments, and make sense of experimental results. For example, a conceptual model of a signal transduction pathway in a cell involves a systematic representation of different proteins’ activities and regulatory attributes. A conceptual model summarizes our knowledge about the biological system and represents our current understanding of how it works and is critical for guiding the next steps in the investigation of a biological system, but it has a couple of shortcomings: it does not provide a systematic way for exploring and evaluating these steps, and it cannot take full advantage of quantitative data when these are available.

A “*mathematical model*” is built on top of the conceptual model using the quantitative representation of static properties of the system, such as biochemical properties of the components and the interactions among them, and dynamic properties such as abundances and localizations. However, the name “mathematical model” can be misleading: for a given conceptual model, one can develop multiple mathematical models using different mathematical frameworks. The choice of a framework—discussed briefly below and in more detail in the previous edition of this book [13]—depends on the scientific question, the available data, the existing experimental tools for perturbing the system, and more. As with a conceptual model, the investigation of a mathematical model goes hand in hand with experimental work, as summarized in Fig. 1. We can describe a typical mathematical model-building process in five steps.



**Fig. 1** Overview of mathematic modeling and the interplay between experiments and modeling. (1 and 2) Existing knowledge and literature are used to develop a conceptual model, estimate parameters, and develop hypotheses and research questions. (3) A mathematical model is developed to best address the research questions given experimental tools for acquiring relevant quantitative data. (4) Results of simulations or solutions are compared with experimental data to validate the model, refine it, and determine its limitations. (5) A working model is used to formulate and test new hypotheses, simulate and design experiments, and guide the development of interventions and synthetic circuits

## 2.1 A Conceptual Model

The first step involves building a conceptual model that specifies the core molecular players and delineates all possible interactions among them to capture each relevant aspect of the biological system. This step entails compiling information from experimental studies and the common views in the field to establish what is currently known about the system of interest. Here the level of details depends both on the available data and knowledge, as well as on the nature of the question under investigation.

## 2.2 Developing a Quantitative Model

The second step involves decorating the conceptual picture with numbers. Here it is critical to distinguish variables from parameters. *Variable*—such as concentrations of molecules, fluxes of metabolites, and electrochemical currents—are dependent quantities that are evaluated by the model. Parameters—including reaction rates, diffusion and transport coefficients, binding affinities, and external signals—need to be assigned with values as part of the model definition. Often it is challenging to measure these parameters

in vivo in the relevant system; in such cases, the parameter values can be estimated from in vitro or similar systems, assuming they share a similar order of magnitude or maintain similar ratios irrespective of the absolute values. Some parameters cannot be determined independently. Those are left as so-called “free parameters” and can be estimated, if necessary, by selecting most likely values obtained from the experimental results. Interpreting the estimated values of these parameters can be challenging, especially if the number of free parameters is comparable with the number of observations, if observational noise skews the estimates, or if single parameters encompass multiple microscopic processes.

### **2.3 Formulating a Tractable Mathematical Model**

Finally, in the third step, quantitative interactions are transformed into a mathematical framework. These transformations could involve applying the laws of physics and chemistry to biochemical species, which renders the resulting models mechanistic. Alternatively, models can be more abstract, for example, using tools from control theory, information theory, or dynamical systems to evaluate the strengths and limitations of the biological system.

To be useful, a mathematical model needs to be tractable and understandable, which inevitably means that the model must be limited in scope. This fact often draws criticism from biologists who are painfully aware that biological systems vary across many scales of time and space; they contain large numbers of components whose functions and properties can fluctuate and can be significantly different across closely related species and even conspecifics. In this sense, every model can be disregarded for being “too simplistic.” However, such criticism could be suspended whenever model construction is done carefully, giving full attention to the assumptions and approximations involved, and when the model generates useful and testable predictions.

There are many ways in which a biological system can be carefully reduced into a simplified mathematical model. The most straightforward way is to limit the number of species that the model considers explicitly. For example, a model may represent multiple phosphorylated states of a protein complex by a single species, if this does not have a significant impact on model predictions. Similarly, in many models of gene expression, one may consider transcription as a single step and not distinguish between mRNA intermediates. Such simplifications can be harmless for some systems or biological question, but unacceptable for others.

Every biological system involves a wide range of time scales. For example, significant changes in the expression of a gene in response to a heat shock occur over tens of minutes, but molecular events at the promoters of these genes occur many orders of magnitude times faster. Changes in neuronal activity due to the introduction of an attractant occur on time scales of 100 ms, but changes in the behavior of worms on the plate occur between 100 and 1000 times

slower. Including processes at such different time scales in a single model is very challenging. Instead, one often identifies the time scales relevant to the question the model needs to address. Processes that are significantly slower are treated as if they are frozen, while processes that occur much faster are treated as if they maintain their average values.

It is well appreciated that all biological systems are subject to multiple sources of randomness, from the inherent (“intrinsic”) noise coming from processes driven by a small number of molecules to external (“extrinsic”) noise that comes from the fact that individuals (different cells in the same tissue, different animals in the same population) are naturally different. The way a model treats this randomness depends again on the question of interest. Models aiming to address typical or average behaviors may ignore them completely. Other models, which focus on individuality or attempt to explain variability in a population, may address these fluctuations more in detail, typically at the expense of other details of the system.

With all this in mind, one is prepared to choose a mathematical framework for constructing the model. Questions focused on the dynamics of a biological system are often addressed by a model constructed as a set of differential equations, where the variables are the entities that change in time. Models that aim to explain how a biological system responds to different signals may be structured as static models, where different processes balance each other to achieve different states in response to different signals. Questions related to individuality or inherently random processes, such as population genetics, aging, or propagation of a disease, may use the mathematical toolbox of stochastic processes. Other questions could be addressed within the theory of dynamical systems, control theory, information theory, and more. Some more details about these possibilities appeared in the previous edition of this book [13].

## 2.4 Testing and Validation

With a workable model in hand, the next step is to demonstrate that the model behaves in a manner consistent with the current knowledge of the system. Preferably, model validation should be done by comparing predictions of the model with experimental data that was not used during its development. The confidence in the model depends on the size and diversity of this test data set. A broad test set can not only uncover inconsistencies but also suggest possible remedies.

Possible refinements of a model include the addition of steps or molecular species that were neglected in the earlier version of the model, adding details to processes that were modeled crudely, or reworking the mathematical approach. Validation of the refined model requires additional data, which may call for additional experiments. This cycle of modeling and experiments is key to a

successful model and necessitates a joint effort of modelers and experimentalists.

Once validated, the model can be used to make new predictions and formulate novel hypotheses, as described below. Before describing the utilities of a working model, it is necessary to point out the importance of a failed model. An important power of a mathematical model is to check that a conceptual model makes quantitative sense, and the failure of a well-crafted model to capture the experimental observation may indicate such a discrepancy. For example, a mathematical model may reveal that the current assumptions about the working of the biological system can only hold true if certain parameters take values well outside their range of physiologically possible values, therefore challenging those assumptions. Alternatively, one may conclude that in order for the model to work, some unknown interaction between key components must exist, or that some assumed interactions cannot be in effect. These are important results—the first could lead to a shift in the view of the biological system, and therefore to new lines of investigations. The second could lead to new testable hypotheses about the wiring of the biological system.

## **2.5 Using the Model to Address Research Questions and Guide Experiments and Applications**

Once a model has been validated, it can be probed to reveal features that would be challenging or impossible to examine experimentally. For example, a model could be used to simulate the system's behaviors under different conditions (such as external signals, specific mutations, perturbations in signaling, and more). These simulations, also referred to as *in silico* experiments, can provide information at a spatial and temporal resolution, as well as at a combinatorial richness of conditions, that are unfeasible to achieve with wet experiments. Many open-source or otherwise inexpensive software packages are available for formulating, implementing, and executing these simulations.

From a rich set of simulations, researchers can propose mechanisms behind the function of the biological system and identify an optimal set of experiments to validate such hypotheses; they can identify combinations of mutations that give rise to informative phenotypes; and they can suggest genes or molecules that could serve as faithful reporters for the state of the system and could be used as clinical markers. Moreover, model simulations can identify points of weakness in the system, which could become targets for therapeutic interventions.

Model predictions can be used to formulate hypotheses and design experimental studies to test them. The new data resulting from these novel experiments could serve as the basis for broader and better models.

### 3 Example: Modeling the Heat-Shock Response

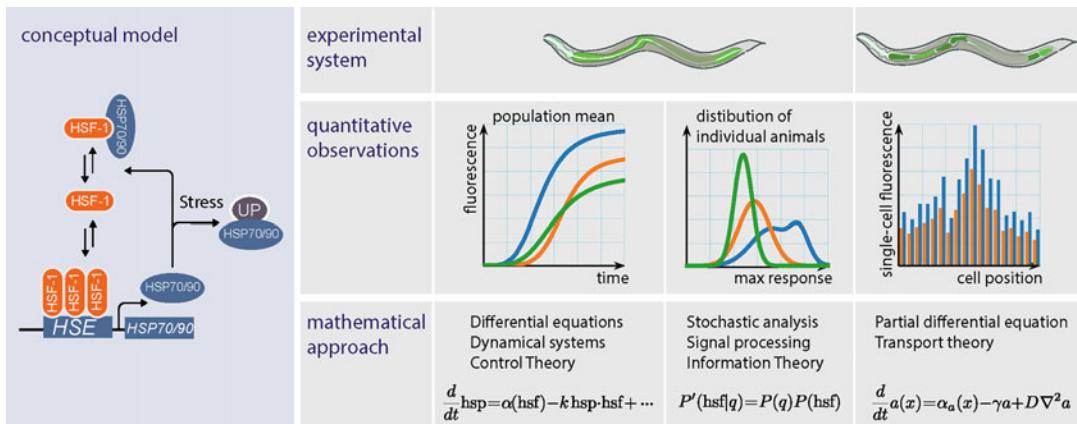
In the previous section, we discussed the steps of constructing a mathematical model for a biological system. Let us now demonstrate these steps by looking at a particular system, the heat-shock response (HSR) in *C. elegans*. The HSR is an evolutionarily conserved, ubiquitously homeostatic transcriptional program that maintains proteome homeostasis in response to unfavorable stress conditions, including elevated temperature [14]. These functions make the HSR an indispensable process for the adaptation and survival of the stressed organism. In this process, under unfavorable conditions (e.g., heat shock), heat-shock factor 1 (HSF-1), the master regulator of this response, induces the production of heat-shock proteins (HSPs) [15].

Efficient control of the HSR is essential for the organism's health, and its dysregulation often results in metabolic disorders, degenerative diseases, and cancer [16]. This well-defined and physiologically important system, which employs a small number of molecular species and responds to a distinct and controllable stress signal, is an attractive system for quantitative modeling. Over the last decades, the HSR has been investigated through quantitative modeling in multiple organisms, including bacteria, yeast, algae, worms, and mammalian cells [17–22] using different modeling approaches.

As described above, the first step towards modeling the HSR in *C. elegans* is to develop a conceptual model which identifies the critical molecular species and their interactions (Fig. 2, left). This model includes four such components: a heat-shock transcription factor (HSF-1), heat-shock proteins (HSPs) that act as molecular chaperones, a sub-group of HSPs that can also bind monomeric HSF-1 and deactivate it as a transcription factor (presumably HSP-70 and/or HSP-90), and a diverse group of unfolded proteins [17].

The chaperone activity of HSPs can restore proteostasis by facilitating stabilization of misfolded proteins to their native folded state, assisting their aggregation, or directing them to proteolysis. Regulation of the HSR is based on titration of HSF-1 away from the nucleus by unused HSPs, signaling that synthesis of additional HSPs is not required. Under proteomic stress, these HSPs are occupied by misfolded proteins, freeing HSF-1 to form homotrimers that bind Heat-Shock Elements (HSE) at promoter sites to enhance transcription of additional HSPs. This regulation mechanism is commonly referred to as the chaperone-titration feedback loop and appears in the HSR pathway in all kingdoms of life, from bacteria to animals [15].

The second step described involves the translation of the conceptual model into a quantitative one. Unfortunately, the current



**Fig. 2** Case study: mathematical modeling of the heat-shock response in *C. elegans*. Blue: A conceptual diagram illustrating the key biochemical species important in the system along with their interactions (UP: Unfolded Proteins). Gray: Three types of research questions are met with the appropriate experimental data and mathematical framework. Left: Questions about the dynamics of HSR are addressed by measuring the population average of a fluorescent reporter at different time points under different heat shocks. Mathematical models suitable for this question are often written in the form of multiple differential equations. Middle: Questions about the variability in heat-shock response among individuals in the population are addressed by measuring distributions of responses across multiple animals. Mathematical models are based on approaches that incorporate variability and noise. Right: Questions about spatial aspects of the response, including cell-specific response and cell-to-cell signaling, use measurements with single-cell resolution. Here mathematical models are focused on individual cells or continuous spatial coordinates

*C. elegans* literature is not rich in quantitative measurements of molecular-level characteristics, such as biochemical rate constants or concentrations of molecules and signals. Indeed, most of the *C. elegans* entries in BioNumbers [23], an online depository that tabulates known biological quantities, are related to its genome, anatomy, or behavior. Some parameters, such as the affinity of HSPs to HSF-1, appear to be similar in several other organisms where they were measured, from *Drosophila* to human cells, and one can assume that they are also similar in *C. elegans* [17]. Other quantities, such as the relative abundance of different HSP species under heat challenges, can be inferred from previous studies of the heat-shock response, as well as from “-omic” studies.

The next step—the formulation of a mathematical model—depends on the biological question and the available data. Consider the following three examples of different mathematical approaches that aim to gain insight into the heat-shock response system from different perspectives (Fig. 2). For each example, we provide the motivating question or perspective, the applicable modeling approach, and some examples of its utility once validated.

In example A, one asks how the properties of the heat shock affect the dynamics of heat-shock response. Addressing this question requires longitudinal measurements of the response in

individual animals to heat shocks of different properties (intensity, duration, etc.). Such measurements can be achieved, for example, by tagging some HSPs (and perhaps HSF-1) with fluorescent proteins, such that their abundance and localization (in and out of nuclei) can be monitored in real-time. A microfluidic device, which places individual animals in their own chambers, can assist in obtaining multiple time points from individual animal for several hours, from the initiation of the stress to the relaxation of the response [17].

Models that address questions of dynamics are written down as a set of differential equations (Fig. 2). Each equation describes how the rate of change in one of the components of the system depends on the level of all other components that interact with it. With a given set of parameters, this model can be simulated, giving the dynamics of each component starting from some initial conditions. These dynamics can then be directly compared with the measured dynamics of the fluorescently labeled proteins in order to support the model and to assign values to missing parameters. Once this step is complete, the model can be used to simulate situations where some of the components, some of the interactions, or some environmental conditions are perturbed, when the system interacts with other systems, and more. This type of model can also be analyzed within the framework of dynamical systems to identify mechanisms that determine the system dynamics and stability; within the framework of control theory, to characterize the controllability and robustness of the HSR; and more.

Example B considers questions about variability among different worms in the population. For example, we observe that at a lower temperature, the overall expression level of HSP-16.2 in different worms is relatively similar (within 20%), compared with higher temperatures where the variability is much larger (100% and more), raising the question of what parts of the HSR circuit are responsible for this temperature-dependent variability [17]. The experimental data required in this case should come from a large number of measurements from individual worms, such that one can faithfully estimate not only population averages but also population variances.

Multiple mathematical frameworks are available for building a model that incorporates variability and noise. A common example is stochastic differential equations, which are an extension of the models described above that includes random components. Alternative approaches that have gained recent popularity include statistical learning [24, 25] and information theory [7], which allows asking questions about how a worm interprets the information it collects from the environment and translates it into action.

Finally, in example C, one asks questions about the synchronized response of different cells in the worm intestine. Addressing this question requires data where the expression of heat-shock

proteins is monitored in individual cells over time [26–28]. The corresponding model can then be built around variables that describe the expression in individual cells, variables that describe the expression in each one of the rings that compose the intestine, or as a function that describes the expression along a continuous coordinate along the intestine [29]. This model can then test different mechanisms for synchronization, such as diffusive molecules, cell-cell communications, neuronal signaling, etc.

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## 4 Is My Question Suitable for Mathematical Modeling?

It is an exciting time for building bridges between experimental and mathematical scientists. Although interdisciplinary communication is still challenging, implementing mathematical models in biological systems offers potential benefits to formulate novel hypotheses and produce new understandings. Moreover, recent advancements in data acquisitions and user-friendly software have enabled all researchers to develop and evaluate their own mathematical models.

What makes a particular question suitable for mathematical modeling? As described above, mathematical modeling can address a wide range of topics, including questions about the mechanisms behind an observed phenotype; questions about the relationship between external and internal signals and the worm's genetic, neuronal, and behavioral response; questions about control, information, and dynamics; and many more. A prerequisite for mathematical modeling is a clear conceptual model, which integrates the existing knowledge about the system's components and their interactions. Enough information should be available to turn these conceptual understandings into a quantitative model. In addition, one should have tools that allow some perturbations of the system, which would be essential for testing the mathematical model and its predictions.

*C. elegans* is a great place to meet all these requirements. Many neuronal and genetic circuits in the worm are well characterized and are of manageable size. The worms have numerous advantages for acquiring highly quantitative data, and a substantial toolbox for neuronal, genetic, and environmental perturbations. These can be augmented with many large-scale approaches available in the worm, such as metabolomics, tissue-specific transcriptomics and proteomics, and more.

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