**Onish Lab: *C. elegans* Microinjection Protocol**

**EQUIPMENT**

Microinjection needles

* Pull needles fresh or keep from accumulating dust.

Injection pads

* Make 2% w/v agar pads with Nanopore water.
  + Heat in microwave. Dispense while hot using Pasteur Pipette. Technique is to use 1 drop per coverslip and let another coverslip drop on top. Then let cool ~5 min and uncover. Dry pads overnight.
  + Alternatively, can make same day and dry in 37C incubator.
* Use the 22x50 glass coverslips (Fisher, cat: 12-548-5E) to mount the agar pad on and to flatten the agar drop.

Injection oil

* Use Halocarbon oil.

Worm pick

Paint brush

M9 buffer for recovery

Worms

* Want worms to be late L4 to YA stage. Want about 1 – 3 or 1 – 5 embryos present in the worm.
* Wormbook: Well fed, young to middle aged (> 1 day old) gravid hermaphrodites with a full but single row of eggs.

NGM/OP50 plates

* Want 1 per injected worm.

Microinjection needle loading pipettes

* Use a P2 pipette to load the microinjection needle.

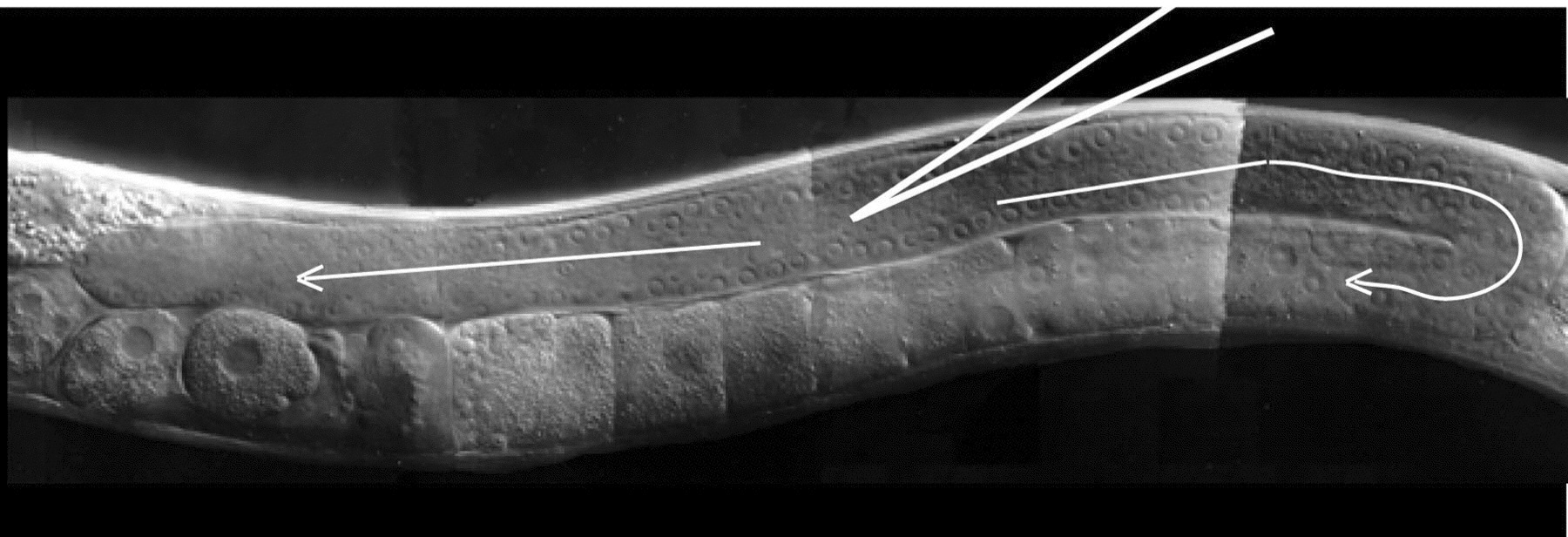
DNA for injection (either plasmid or linear fragments)

* For isolation of plasmids, use the PureLink HQ Mini Plasmid DNA Purification Kit (Invitrogen; K2100-01). The optional wash step in this protocol is NOT optional.
  + Isolated plasmids should be kept/used for about 1 month in -20C. Then need to be renewed.

**PROTOCOL**

Injection

1. Prepare DNA mix for injection (20 uL). Once prepared, centrifuge (30 min, 12,000g, 4C) and remove 10 – 15 uL off the top and transfer to a clean tube. Use this for injecting. Store on ice.
   1. Keep DNA mix in the fridge for further use, but don’t keep/use for more than 1 week.
   2. DNA Mix:
      1. Aim for a total of 80 – 90 ng/uL DNA. Higher concentrations of 100 – 150 ng/uL are okay but can lead to needle clogging.
      2. Adjust plasmid(s) of interest concentration based on desired expression level (i.e., if want high expression then aim for ~30 ng/uL of construct, if want low expression then aim for ~10 – 15 ng/uL).
      3. Add filler DNA (Bluescript or genomic DNA) to reach desired total DNA concentration (i.e. 80 – 150 ng/uL) for the mix.
         1. Salmon sperm DNA (15632-011; Invitrogen)
2. Load microinjection needles with ~1 uL of DNA using capillary action. Could do up to 4 uL into one needle, but don’t want to do in case the needle clogs. DNA can also evaporate if it takes too long to load.
3. Turn on the nitrogen gas. Want output PSI to be ~80.
4. Turn on the injection control board. Then set the duration to 0.2 sec. Then adjust the output pressure nob (turn to adjust the DNA droplet size); the target PSI varies based on the needle.
5. Mount the needle.
   1. The injection system/scope has a course micromanipulator on the needle setup and a fine micromanipulator on the microscope table. We have X, Y, and Z motion.
   2. OPTIONAL: Get ‘breaker’ slide and add a drop of halocarbon oil. Put the slide on the stage and switch to the 5X objective lens. Put microinjection needle in the oil and get close to the edge of the coverslip. Now switch to the 40X objective lens and use the fine micromanipulator to move the needle for breaking.
6. Put halocarbon oil on the corner of the agar pad.
7. Under the dissecting scope, pick the worm(s) into the oil on the agar pad, and smear the worm(s) keeping parallel to the needle.
   1. If the worms don’t adhere to the agar pad, can re-bake/dry the pads or use a thicker or higher concentration of agarose pads (i.e., 2%).
   2. Worms stick to the agar pads due to the absorption of water from the worm into the dry agarose. If this goes on too long the worms will desiccate and die.
8. Put the worm slide onto the injection scope. Using the 5X objective, position worm slide and the needle. Then switch over to the 40X objective.
9. Get the worm perpendicular to the needle. Line the needle up to the gonad.
10. Insert the needle into the distal gonad where embryos are in a syncytium. We want to get the embryos before they become cellularized.
    1. A single worm gets around nano – pico liter worth of DNA total.



1. Check to make sure the needle is in the gonad by doing a test injection. If in the gonad, will see fluid in the germline. If not in the gonad, will see fluid and movement elsewhere (worms will look bloated).
2. Now inject the worm(s). Can do more than 1 injection to fill up the gonad (i.e., test injection and real injection).
3. Once injected, take the worm slide over to the dissecting scope. Add about 1 – 5 uL of M9 to the worm directly, to pull the worm from the oil and rehydrate the worm.
4. Prepare a fresh NGM/OP50 plate for the injected worm, by spotting 10 uL of M9 onto it.
5. Now pick the injected worm into the M9 spot on the plate.
6. Check for transgenic worms over the next few days.

Integration

*Adapted for dTomato containing extrachromosomal arrays into mtl-2::GFP(Is) background. Adapted from Hoerndli Lab Transgene Integration protocol, which was adapted from Adapted from Mariol et al., 2013*

1. Prepare culture plates:
   1. Prepare about 500 small NGM plates
   2. Seed each plate with OP50
2. Evaluate the transmission rate of the transgenic line to be integrated:
   1. Determine the percentage of dTomato- vs. dTomato+ progeny for each strain that is being considered for integration
3. Choose a transgenic line for transgene integration:
   1. Select the transgenic line with the highest transmission rate as possible (ideally ≥80%).
4. Obtain a population of transgenic animals synchronized at the L4 larval stage for integration:
   1. Pick 30 dTomato+ adults onto five culture plates (six animals by plate)
   2. Let the worms lay eggs for 4-5 hrs at 20 °C.
   3. Eliminate the adults from the plates after checking for the presence of a few dozen eggs per plate – wait longer to remove the adults if not enough eggs have been laid.
   4. Culture the animals in a worm incubator at 20 °C until the progeny reaches the L4 larval stage (~48 hrs at 20 °C).
5. UV Irradiation and Recovery of Transgenic Worms
   1. Pick 100 fluorescent transgenic L4 animals onto separate culture plates (15-20 animals by plate).
   2. Place the plates, with the lids removed, upside-down in the UV gel transilluminator. Irradiate the plates for 15 seconds with the time starting when the UV light has come on.
6. Worm recovery after irradiation:
   1. Place plates with irradiated worms overnight at 15 °C for recovery.
   2. Check for the number animals that are alive - a survival rate of around 80-90% suggests efficient irradiation.
   3. Grow the irradiated animals at 20 °C until the progeny has reached adulthood
7. Selection of F1 animals:
   1. Pick single 150-200 dTomato+ F1 animals onto separate culture plates. For highly transmitting lines (≥80%), 100 F1 animals are enough.
   2. Keep these F1 plates at 20 °C until the progeny reaches adulthood
   3. Discard all F1 plates exhibiting either i) no progeny, indicating that the F1 animal was sterile or died, or ii) no or only few dTomato+ F2 animals, indicating that the F1 animal did not transmit the transgene at the expected rate.
8. Selection of F2 animals:
   1. Pick dTomato+ F2 animals from each selected F1 plate. If a fluorescent transgene is being integrated (ex. mCherry or gfp), you should pick F2 worms with a high level of fluorescence as this could indicate that these animals are homozygous for the integrated array.

*\*Note: when picking F2 animals it is critical not to carry along eggs or larvae in order to avoid false negatives in the next step.*

* 1. Grow F2 animals at 20 °C.

1. Isolation and validation of integrated transgenic strains:
   1. Screen F2 plates for 100% dTomato+ F3 worms. The screen is quite rapid as the presence of a single dTomato- worm indicates that the plate should be thrown away.
   2. Pick four dTomato+ F3 animals from selected plates to confirm the 100% inheritance of the transgene.
   3. If possible, keep several independent integrated transgenic strains, i.e. strains recovered from different F1 animals.
2. Outcross integrated strain
   1. Cross integrated L4’s with wild-type males
   2. Find an F2 isolate that is homozygous for the integration (usually done by finding a plate with 100% fluorescent progeny)
   3. Repeat steps 10.1 and 10.2 two more times

Hoerndli Lab Transilluminator info:

BioDoc-It 210 Imaging System

LM-20 Transilluminator

P/N 97-0166-01

100-115V~60Hz

**NOTES**

Selectable genetic markers for nematode transgenesis

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**C. elegans transgenesis: how to?**

Stable transformation of C. elegans was first achieved by microinjection of the DNA of interest into the syncytial gonad of the hermaphrodite [3, 28]. Exogenous DNA (plasmid or PCR product) is directly introduced into the cytoplasm of the germline. Then, as individual oocytes separate from the syncytium, it is incorporated into fertilized eggs. The exogenous DNA undergoes intermolecular ligation leading to the formation of tandem repeats of about 80 to 300 plasmid copies, also referred to as multicopy extrachromosomal arrays [3, 28]. These arrays are semi-stable, thus displaying non-Mendelian segregation and leading to mosaic animals. An array that is transmitted to the F2 generation is generally inherited by the following generations with a characteristic transmission rate ranging from 10-90% [28] (Figure 1).

Transformation and microinjection\* Thomas C. Evans, ed.§, Department of Cell and Developmental Biology, University of Colorado School of Medicine, Aurora, CO 80045 USA

Pointers from Rachel Doser

* Start with new DNA (purified within last 6 months old if kept at 4 degrees)
* Adjust DNA concentrations based on desired expression levels (i.e. if I want high expression of a tool, then the construct for that tool is at ~30ng/uL in my DNA mix. For low expression, I decrease the concentration to ~10-15ng/uL)
  + Note: This doesn't guarantee high/low expression, so you still need to screen isolates to find a strain with your desired expression.
* I shoot for a total DNA concentration of about 80-90 ng/uL in my DNA mix. DNA mixes with a concentration of 100-110ng/uL are okay, but they tend to lead to more needle clogging which you can prevent in advance by pulling a needle with a larger opening.
* Get comfortable with adjusting needle pulling protocols on the pipette puller for your specific injection - injections go much more smoothly and are more efficient if your needles are optimized. This means I wouldn't rely on breaking needle tips since this leads to a jagged needle tips which I've noticed cause more death of injected worms than a smooth tip. Additionally, you can't control the opening size of the needle you break so then you must guess what pressure and duration to set the picospritzer to.
* When starting out, inject **a lot.** Like two or three days in a row for a couple of weeks. I have seen that it will take people 6 months or longer (sometimes even never) to get the hang of injecting if they only attempt it once every couple of weeks.

Pointers from Kaz Knight

* Place all injected worms onto the same plate.
* For new plasmids, start at a known [] and go up or down from there (i.e., 5 – 30 ng)
* Inject 15 – 20 worms a day
* The 0.3 sec/psi is what Kaz uses but need to adjust for the developmental stage of worm (i.e., L4 vs YA)
* Recover worms from the slide/oil using either M9 or PBST. Then transfer worms to about 10 uL of same media on an NGM plate.
* DNA mix: make fresh, can keep for a couple of days but no more than a week
* Plasmid freshness: isolate and keep DNA for a month or so

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| **4. DNA transformation: Creating repetitive extrachromosomal arrays by microinjection** |
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| **4.1. Introduction** |
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| The easiest approach to make transgenic strains is to co-inject two or more DNAs into the distal gonad syncytium; one or more DNAs carrying the intended transgenes and a plasmid carrying a transformation marker. Transgene DNAs can be plasmids, cosmids, phage, YACs, or PCR products. Injected DNAs undergo homologous recombination with each other quite efficiently, so it is not necessary to physically link them before injection (Mello et al., 1991). For this reason, it works best if the injected DNAs share sequence homology in their backbones, although non-homologous recombination also occurs (Mello and Fire, 1995). It is feasible to inject several DNAs and recover animals carrying all injected molecules. Whether the injected DNA is circular or linear does not seem to influence results. |
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| Transgenic animals produced by injection typically carry large extrachromosomal arrays that contain many copies of the co-injected DNAs. These repetitive arrays are usually unstable to cell division but can become inheritable; a fraction of first generation progeny (F1) that contain the transgenes will transmit the array through many subsequent generations often without changes in heritability or expression (for somatic promoters). These heritable arrays still have varying degrees of mitotic instability and incomplete inheritance, although it is possible to integrate transgenic arrays into chromosomes (see Protocols 4-6). |
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| **Advantages.**This technique is relatively fast and efficient for genes expressed in somatic tissues; an experienced person injecting simple plasmids can generate 3-6 independent transgenic lines in 7-10 days from as few as 15-40 injected gonads. (If the transgene causes a deleterious phenotype or if large genomic fragments are used, the success rate can be lower). All that is needed is a plate of well-fed hermaphrodites and a few microliters of purified plasmids, and multiple constructs can be analyzed in a reasonable time frame. In addition, the mitotic instability of these arrays can actually be used to create genetic mosaics, which can then be analyzed to determine the lineages in which a gene functions (Herman, 1995), see Genetic mosaics. |
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| **Disadvantages.**(1) The transgene expression pattern may not mimic the endogenous gene, especially for germline-expressed genes. Transgenes in repetitive arrays are strongly silenced in germ cell nuclei (Kelly et al., 1997). In addition, suppressed or ectopic transgene expression is sometimes observed in somatic tissues (Mello and Fire, 1995). Rescue of a null mutant does not assure proper expression since even suppressed or ectopically expressed genes can give rescue. (2) It is difficult to predict and control the level of expression among different arrays. (3) Transgene expression can be variable among siblings of a single strain. Some variability probably relates to mitotic instability of arrays, but even integrated arrays can show expression variability for unknown reasons (Mello and Fire, 1995). (4) Arrays sometimes induce RNAi-like effects that suppress endogenous gene function (Dernburg et al., 2000). This could be due to DNA rearrangements during array formation that cause production of gene product fragments or antisense RNA (T. Evans and J. Kimble, unpublished). DNA rearrangements can also reposition gene regulatory elements leading to mis-regulated transgenes. Rearrangements may also occur during long term passage of some transgenic strains, since sometimes the properties of transgenic animals changes over time (T. Evans, unpublished). In spite of these pitfalls, many well-behaved transgenic strains have been created by this technique. Integration of arrays can minimize problems associated with transmission instability (Protocols 4-6). Therefore, as long as caution is exercised this remains a very important approach to nematode transformation. |
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| **4.2. Protocol 2. Formation of repetitive arrays by microinjection** |
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| [Modified from Mello and Fire (1995).](http://wormbook.org/chapters/www_transformationmicroinjection/transformationmicroinjection.html#bib20) |
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| Purify DNAs for injection: Standard alkaline lysis protocols, including commercial kits, are sufficient for plasmids and cosmids although residual contaminants can prevent transformation. To generate clean DNA preps, do extra washes in the commercial column-based procedures and/or further purify DNA by phenol choloroform extraction, G-50 spin column, chloroform extraction, and ethanol precipitation. Miniprep procedures that use LiCl or CTAB precipitation steps are also effective for transgene DNAs (Fire et al., 1990; Mello and Fire, 1995). Protocols for preparing YACs and phage for injection can be found in Mello and Fire (1995). DNAs can be stored in standard Tris/EDTA (TE) buffer. |
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| Mix transgene DNA (to 1-100 ug/ml final concentration) with pRF4 (or other marker; to 100 ug/ml). Dilute DNAs with sterile water or TE. Use lower transgene concentrations if the gene is toxic or if multiple plasmids/cosmids are used. Strive for a total DNA concentration of 100-200 ug/ml. An empty vector (e.g., Bluescript) can be used to reach this goal if desired. |
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| Inject 15-50 gonads for each DNA mix as described in Protocol 1. |
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| Transfer each injected worm to a separate, seeded NGM plate. Grow at 20-25°C. |
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| When F1 progeny reach L3 to L4 stages, pick rollers to new plates, 2-3 rollers per plate. Continue to pick more rollers over the next day or two if necessary. Make sure to label plates to identify the parent for each plate. |
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| Look for plates that produce F2 rollers and clone several F2 rollers from those plates. Typically, ~2-15% of F1 rollers will transmit the array to the F2 generation. Most F2 rollers will generate lines that continue to transmit their array at a consistent frequency, which can be 5-95% (30-60% is common). Keep only one strain from each injected parent, since lines from the same parent may not be independent transformants. |