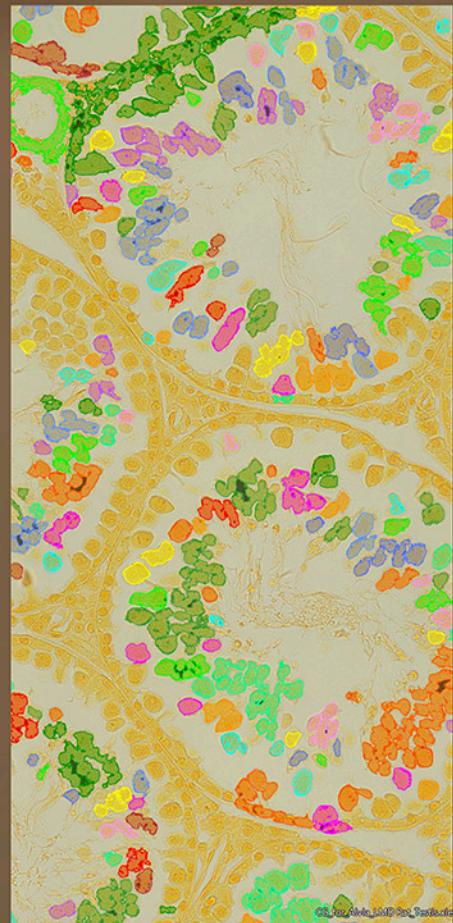
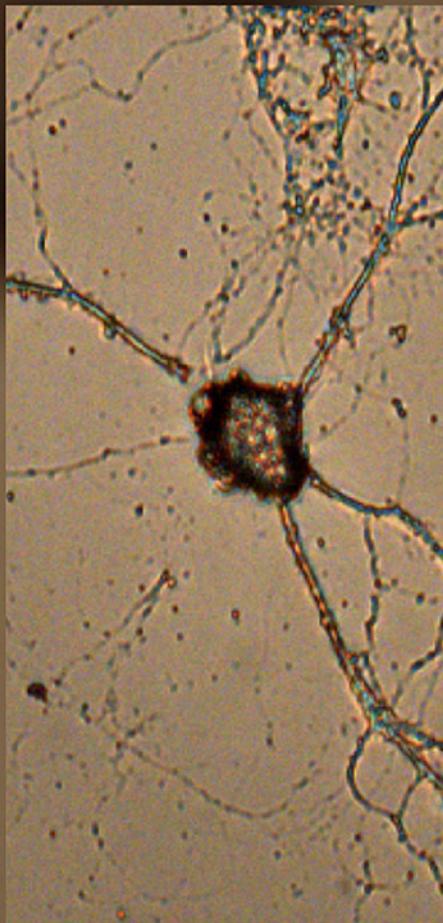
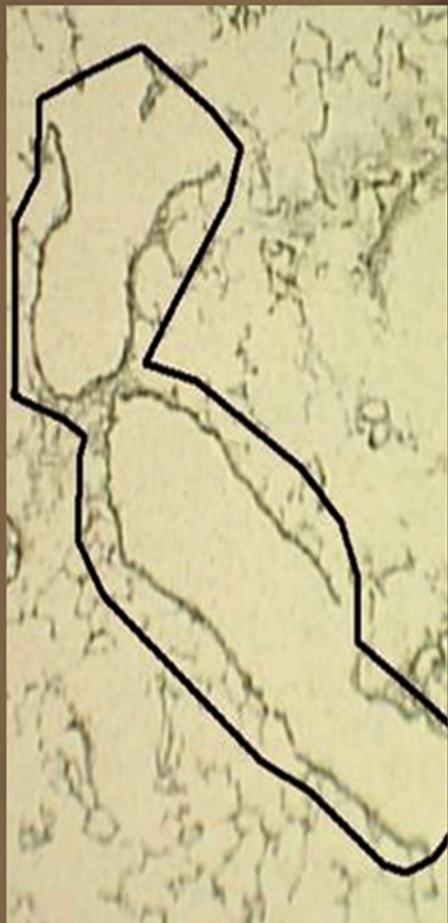
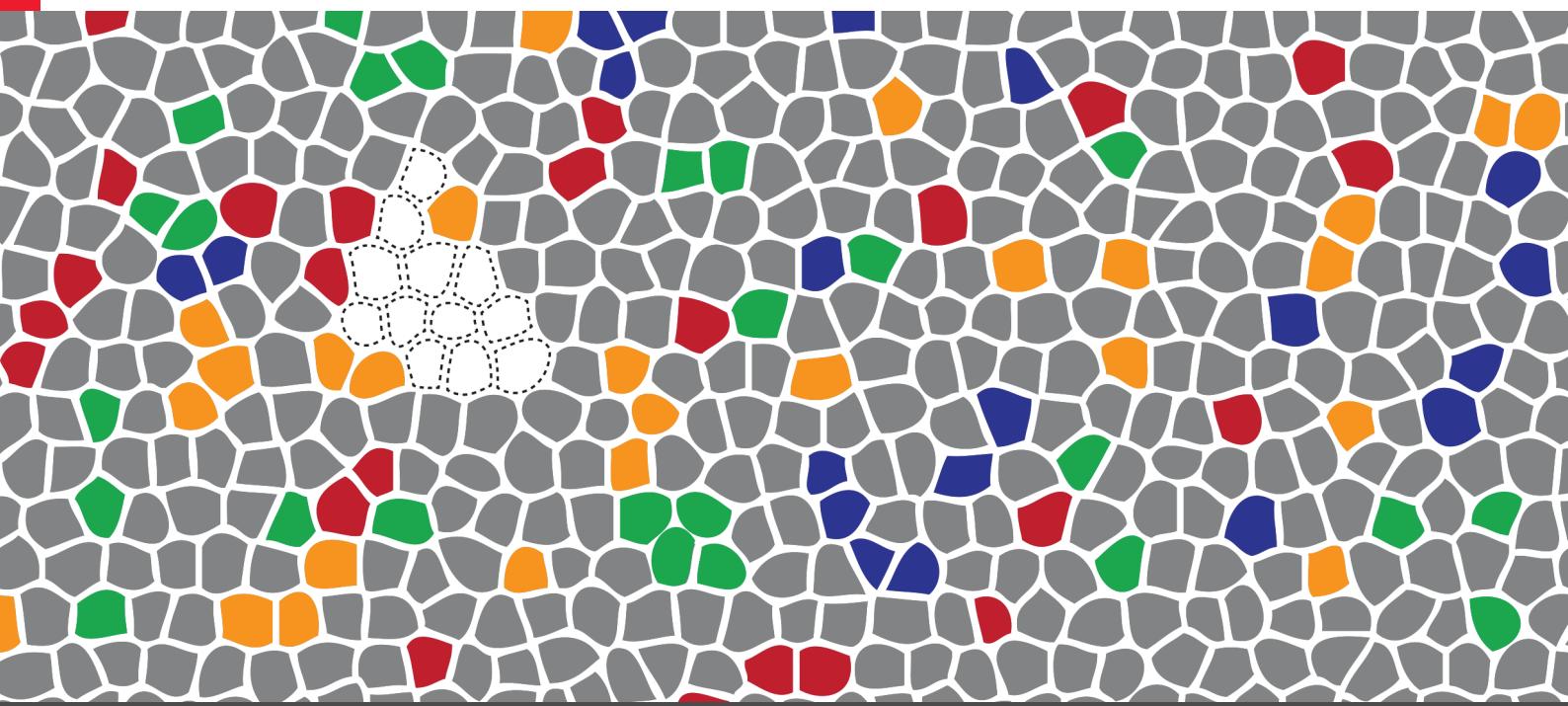


Bio-protocol Selections

Laser Microdissection Protocols for Tissue and Cell Isolation

Applications of LMD from complex tissues to single cells





Laser Microdissection

Specific areas of a sample often need to be isolated for downstream analysis or to decipher the details within a specific region, free of contamination. Laser microdissection (LMD), or laser capture microdissection (LCM), is a method designed to isolate and dissect target cells or entire areas of tissue from a wide variety of samples.

While tissue samples dissected using traditional LMD microscopes can be prone to contamination from surrounding cells, LMD platforms from Leica Microsystems collect the sample using gravity. By moving the laser rather than the sample, we can provide contact- and contamination-free isolation of specific target cells for downstream analysis.

Our LMD solutions are the systems of choice for the microscopic isolation of samples of interest as large as thick tissue volumes or as small as single living cells.

Learn more at <https://go.leica-ms.com/lmd-na>



Foreword

We are pleased to release the reprint collection “*Laser Microdissection Protocols for Tissue and Cell Isolation*,” which showcases various applications of laser microdissection (LMD) that span cancer research, neuroscience, immunology, and plant biology.

Since it was developed in 1976, LMD technology has been an important tool, enabling researchers to precisely extract specific cell populations from heterogeneous tissues. This precision became increasingly crucial with the development of molecular analysis techniques such as PCR, facilitating in-depth genomic, transcriptomic, and proteomic studies at the cellular level.

A key feature of this collection is the emphasis on detailed, step-by-step protocols that are the focus of *Bio-protocol* journal and that significantly enhance experimental reproducibility and reliability. In our annual user surveys, approximately 90% of users who apply the protocols published in *Bio-protocol* report that they are able to successfully reproduce them.

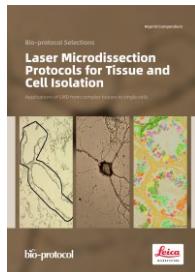
We extend our gratitude to Leica Microsystems for their generous sponsorship, which made this collection possible. The introduction of the Leica AS LMD system two decades ago is just one example of Leica Microsystems’ dedication to advancing research with innovative technology.

We hope this collection not only serves as a valuable resource but also inspires further research. You can explore our full protocol archive at www.bio-protocol.org. We hope you will consider contributing your own protocols to *Bio-protocol* in the future.

For more information, please contact:
Fanglian He
Publisher, *Bio-protocol*
fhe@bio-protocol.org
April 2024

Table of Contents

Circadian Gene Profiling in Laser Capture Microdissected Mouse Club Cells.....	1
Laser Capture Micro-dissection (LCM) of Neonatal Mouse Forebrain for RNA Isolation.....	8
Total RNA Isolation after Laser-capture Microdissection of Human Cervical Squamous Epithelial Cells from Fresh Frozen Tissue	24
Laser Capture Microdissection (LCM) of Human Skin Sample for Spatial Proteomics Research	28
Protocol to Isolate Germinal Centers by Laser Microdissection	36
Subcellular RNA-seq for the Analysis of the Dendritic and Somatic Transcriptomes of Single Neurons	47
Isolation of Rice Embryo Single Cell Type using Laser Capture Microdissection (LCM)	70



On the Cover:

Featured is a cross-section of images (from left to right) from the protocols “Circadian Gene Profiling in Laser Capture Microdissected Mouse Club Cells” (DOI: 10.21769/BioProtoc.3590), “Subcellular RNA-seq for the Analysis of the Dendritic and Somatic Transcriptomes of Single Neurons” (DOI: 10.21769/BioProtoc.4278), and an image of rat testis stained with Acridine Orange, with cells segmented using Aivia software provided by Leica Microsystems.

Circadian Gene Profiling in Laser Capture Microdissected Mouse Club Cells

Zhenguang Zhang^{§,*} and Andrew Loudon^{*}

School of Medical Science, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK;

[§]Present address: Hutchison/MRC Research Building, Department of Oncology, University of Cambridge, Cambridge, UK

*For correspondence: zz370@cam.ac.uk, Andrew.louodon@manchester.ac.uk

[Abstract] Cell heterogeneity is high in tissues like lung. Research conducted on pure population of cells usually offers more insights than bulk tissues, such as circadian clock work. In this protocol, we provide a detailed work flow on how to do circadian clock study by RNA seq in laser capture micro-dissected mouse lung club cells. The method uses frozen tissues and is highly reproducible.

Keywords: Club cell, Lung, Circadian clock, RNA seq, Laser capture micro-dissection

[Background] Circadian clock is found all over the body and in the lung it is shown to strongly oscillate in Club cells (Gibbs *et al.*, 2009 and 2014). Whole lung circadian gene profiling is carried before in mice and rat (Sukumaran *et al.*, 2011; Zhang *et al.*, 2014). However, at least 15% of those circadian genes are coming from leukocytes (Haspel *et al.*, 2014). It is useful but difficult to get cell type specific circadian gene profiling, given the high heterogeneity of cell population in tissues. Tissue digestion followed by flow cytometry sorting is usually to solve this problem but it is not clearly whether gene expression will change during this process. An alternative way is to use laser capture microdissection to take target cells from fixed tissues. Note that this could lead to comprised cell population purity. Specific cell population can be identified by antibody staining or anatomic information. In terms of Club cells in lung, they are known to be enriched in distal bronchiolar epithelium. A protocol showing how to do microarray using RNA from Club cells by laser capture (PixCell II Laser Capture Microdissection system) was published more than 10 years before and there are some advances in the technique (Betsuyaku and Senior, 2004; Cummings *et al.*, 2011). Here I present a detailed protocol on circadian gene profiling in Club cell RNA by laser capture microdissection in Leica LMD6500 system. It covers from mouse tissue collection to get RNA samples ready for RNA seq library construction. Generally, to get good quality RNA, all tools and working areas should keep RNase contamination to the minimum (like using RNaseZAP solution to clean and keep sections dry in microdissection). For all the downstream steps, please refer to the published protocol by Jiajia Li, *etc.* (Li *et al.*, 2015).

Materials and Reagents

1. Smiths Medical™ Portex™ Fine-Bore Polyethylene Tubing (Ref: 800/100/200, 0.58 mm ID and 0.96 mm OD)
2. 23 G needles and 1 ml syringe

3. PEN (polyethylene naphthalate) membrane slide (Leica, 11505158; Applied Biosystem, catalog number: LCM0522) and slide mail box
4. Thin wall 0.5 ml Eppendorf tubes (Invitrogen, catalog number: Q32856)
5. Mice (C57BL or others)
6. Phenobarbitone solution (for example, Pentoject, Animalcare limited, UK)
7. 70% ethanol
8. Dry ice or liquid nitrogen
9. PBS tablet (Sigma, catalog number: P4417-100TAB)
10. Sucrose
11. RNaseZAP solution (Thermo Fisher, catalog number: AM9780)
12. Ethanol solutions (100%, 95%, 75%, 50%)
13. Lysis buffer (supplied in kit) (Applied Biosystems, catalog number: KIT0204)
14. Picopure RNA isolation kit (Applied Biosystems, catalog number: KIT0204)
15. Qubit® RNA HS (High Sensitivity) assay kit (Invitrogen, catalog number: Q32852)
16. Agilent RNA 6000 Pico Kit (Agilent, catalog number: 5067-1511)
17. OCT (Optimal cutting temperature) compound solution (any brand, for example, Fisher Scientific, catalog number: 12678646) and PBS (see Recipes)

Equipment

1. Qubit machine
2. Light dark chamber
3. Cryostat
4. Forceps and scissors, fine string
5. Leica Laser Microdissection Scope LMD 6500
6. Tape station or Agilent 2100 Bioanalyzer
7. -80 °C freezers

Procedure

- A. Lung tissue collection
 1. Entrained mice [7 day L/D (12 h light/12 h dark)) followed by 1 day D/D (12 h dark/12 h dark)] are culled in D/D by either i.p. injection of phenobarbitone (200 µl/mouse) or neck dislocation. One mouse per time point is used and samples are collected every 4 h for 48 h. Once they are dead, take the bodies to the procedure area.

Notes:

- a. *In previous lung circadian gene profiling studies, the condition varies from L/D, D/D and L/L (12 h light/12 h light).*
- b. *The former method is preferred as sometimes tracheas can be damaged by neck dislocation.*

- c. *Though samples pooled from several mice (like n = 3) is used often in literature for this kind of study, the data from our study suggest that this may not be necessary. Sometimes, when mouse stock is limited, mice can be culled at different experiments (for example, only CT0-32 samples are collected in one experiment and the remaining is collected from another experiment). We don't find big effect on the result; even tissues are collected almost 6 months apart.*
2. Spray mice with 70% ethanol and make a vertical cut in skin from abdomen to chin. Open the pleural cavity by piercing the pleural membrane and remove ribs above the lung. Lift and cut off thymus to expose trachea. The connective tissues surrounding trachea can then be peeled apart by blunt forceps.
3. Use spring scissors to make a nick cut in the upper part of the trachea and insert the BAL lavage tube fitted with a 23 G needle to the trachea. Strings can be used to make a tie to hold the insert.
Note: It is not absolute necessary to do this as forceps can be used instead. But it helps in later steps during lung removal.
4. Fill about 0.8 ml OCT solutions [50% (v/v) OCT in PBS solution (made with DEPC H₂O) with 5% sucrose] in a 1 ml syringe. And slowly inject the solution to inflate the lung through trachea.
5. Remove the lung with the trachea closed with forceps or a string tie. Trim off the heart and thymus before freezing lungs in either dry ice or liquid nitrogen. For storage, the lungs covered with foil paper are put in -80 °C freezers.
Note: Samples frozen in liquid nitrogen are difficult to cut as the texture becomes very crisp. RNA quality from dry ice and liquid nitrogen frozen samples is comparable, without clear change.

B. Lung sectioning

1. Take out the lungs and place them in the cryostat for 30 min before cutting.
2. Mount the lung in vertical position and coronal sections are cut in 10 µm thickness, 3-4 sections per PEN membrane slide.
Note: Either RNase free or normal PEN slide can be used. For the latter, PEN membrane can be cleaned by UV light exposure in cell culture hood for 30 min or with RNaseZap.
3. It is all right to directly place the tissue sections into a slide from room temperature but once a section is on the slide, leave the slide in the cryostat. Examples of dissection areas are shown in the picture.
4. Usually, 10 slides/mouse are cut for RNA sequence study (150-450 ng total RNA yield after microdissection).
Note: The amount depends on chosen method to construct library for sequencing. For Illumina Truseq kit, 100 ng total RNA is the minimum amount requirement. When an amplification step can be used, much less material is required, with total RNA even in picogram range.
5. Yield from 2-3 slides is enough for getting RNA in qPCR study.
6. Place the slides in a closed slide mail box and store the slides in -80 °C freezers. It is recommended to finish the slides microdissection within 2 weeks.

C. Laser capture microdissection

1. Specimen slides were taken out of dry ice and quickly place into alcoholic solutions as below:

100% ethanol for 1 min, 75% ethanol 3 dips, 50% ethanol 3 dips, 95% ethanol 30 s, 100% ethanol 30 s, 100% ethanol 2-5 min. For ethanol solutions, avoid leaving open too long to prevent water coming in from air and change new solutions after prolonged use. Chemicals can be used to further dry the ethanol. It is essential to keep last ethanol solution 100%, as RNase can not work without water.

Notes:

- a. *Cresyl violet solution in ethanol can be used to stain sections but not necessary here. The bronchiolar structure is easy to recognise under light microscope without any staining.*
- b. *The aim of this process is to dissolve OCT in water and make the sections dry in the end to prevent RNase activity.*
2. Leica LMD 6500 laser capture microdissection machine is used. After slides being dried down, they are placed in the slide holder. For tissue collection, 0.5 ml thin wall PCR tubes are used in the tissue collector, with 30 µl lysis buffer added (add DTT before use according to the kit protocol, Picopure RNA isolation kit, Life Technologies).
3. Inspect the specimen to look for the bronchiolar airways under the microscope with 6.7x or 10x magnifications. Bronchiolar epithelium was cut off from junction between terminal bronchiole and alveolar ducts, proximally ~200 µm along the bronchiole. The laser setting should be tested beforehand. The microdissection view and laser setting used in our lab is shown in the picture below (Figure 1).

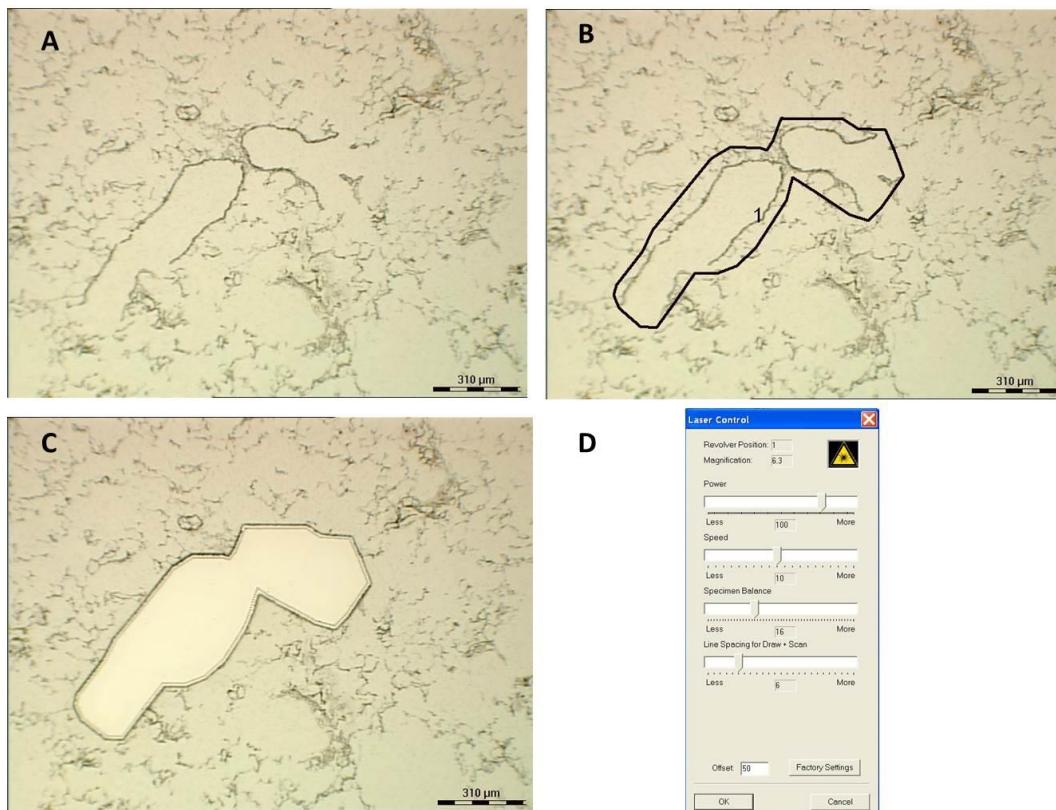


Figure 1. Illustration of laser capture of distal bronchiolar epithelial cells. The view is under 6.7x magnifications with 2 bronchiolar airways shown in the middle in A. B. shows how the cutting line is drawn and C. shows what the tissue ie like after cutting. Laser setting is shown in D.

4. Check the collection solution about every 1 h and add another 30 µl of lysis buffer or DEPC H₂O before the solution on the cap running dry. It is recommended to use 2 or 3 collection tubes for 1 sample to avoid the solution becoming too thick after long microdissection process. These tubes for the same sample can be pooled when it comes to RNA purification.

Note: For Picopure RNA extraction kit, it is recommended to incubate samples at 42 °C for 30 min first to fully lysis the tissue. This step can be done either after immediate dissection or just before RNA extraction.

5. It usually takes 5-8 h to manually dissect 10 slides. In the end, put the tube onto dry ice and the samples can be stored for long term in -80 °C.

Notes:

- a. *There is an option of automatic cutting after selecting multiple target areas. However, it is not uncommon to find target sections ‘hanging’ on the slide instead of falling off after closed cutting. In this case, a manual point shooting method is used to cut off target areas.*
- b. *Each slide takes 30-45 min to microdissect. Both coarse and fine stage movement can be used during microdissection. It is recommended to inspect the sections again with eyes under microscope after dissection of each slide to avoid missing target areas.*

D. RNA extraction and study

Picopure RNA extraction kit from Life Technologies to extract the RNA is used according to enclosed protocol. Among the extraction process, DNA on column digestion is performed as described in the protocol. Final RNA is eluted in 20 µl volume and low RNA binding Eppendorf tube is recommend to hold the samples.

Note: RNeasy micro kit from Qigen and EZNA microelute total RNA kit from VWR can also be used. It is also possible to do the extraction glycogen precipitation without any kit but it could take much longer time to do.

For quantification, it is recommended to take 1 µl of RNA and dilute 1:5 in H₂O. From this, use 1 µl to quantify RNA amount in Qubit® RNA HS (High Sensitivity) assay. The typical concentration is above 10 ng/µl. The remaining can be saved for qPCR validation of final RNA seq data. For quality assessment of RNA intactness, use Agilent RNA 6000 Pico Kit or Tape station. The RIN number is usually around 6-8. If the value is too low, new mouse lung samples should be collected to start the process from beginning again.

Notes:

- a. *When the concentration is measured in a normal NanoDrop machine, the concentration can be very inaccurate. Successful qPCR application is done with laser capture microdissected materials from just 2 slides when the concentration is in lower than 0 ng/µl range by NanoDrop.*
- b. *If the amount is too low, more slides can be used to microdissect and use this RNA solution to elute or pool the RNA together in the end. Also check Step A3 about an amplification step. But it is recommended to avoid this situation by collecting as much materials as possible in first place.*
- c. *Lung is known to have high levels of RNase. In other tissues, like brain, RIN number can be as high as 8-9.*

Typical RNA quality, qPCR result showing enrichment of Club cells and RNA seq result showing circadian gene expression are shown in figure below (Figure 2).

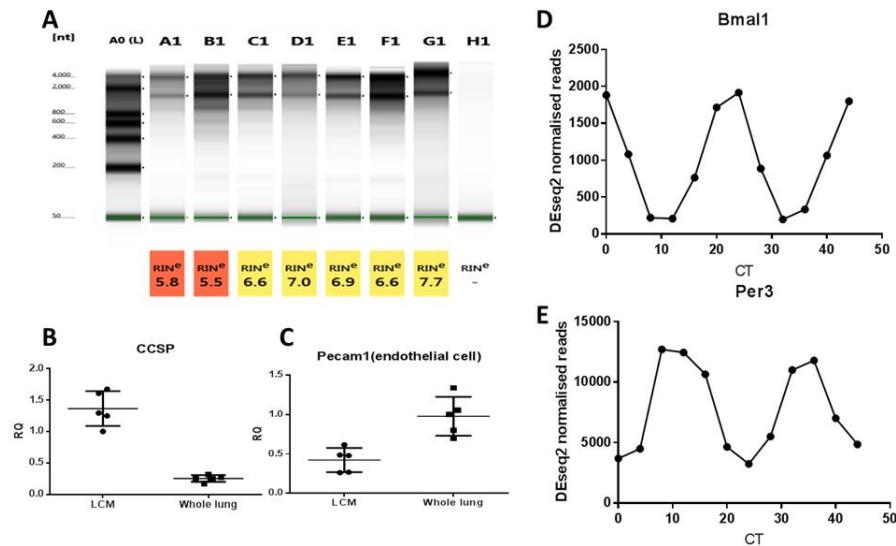


Figure 2. Typical RNA quality, qPCR and RNA seq result. A. Tape station showing RNA quality. B. and C. Enrichment of Club cell marker and little endothelial cell contamination shown by qPCR. D. and E. Bmal1 and Per3 gene expression profile from RNA seq results on laser capture microdissected Club cells.

Data analysis

Study results using this method is published on FASEB journal (Zhang *et al.*, 2019).

Recipes

1. OCT (Optimal cutting temperature) compound solution and PBS
 - a. Solutions (50% (v/v) OCT) in 1x PBS solution (made with PBS tablet in DEPC H₂O) with 5% sucrose
 - b. Dissolve sucrose first before adding OCT and this will make sucrose easier to dissolve

Acknowledgments

The authors thank BBSRC, UK for funding of this work and the method is based on Betsuyaku and Senior (2004) and Cummings *et al.* (2011).

Competing interests

The authors don't have any competing interests.

Ethics

All experiments on animals were carried out in accordance with the UK Home Office Animals (Scientific Procedures) Act of 1986 and European Directive 2010/63/EU, following approval by the University of Manchester Animal Welfare and Ethical Review Body.

References

1. Betsuyaku, T. and Senior, R. M. (2004). [Laser capture microdissection and mRNA characterization of mouse airway epithelium: methodological considerations.](#) *Micron* 35(4): 229-234.
2. Cummings, M., McGinley, C. V., Wilkinson, N., Field, S. L., Duffy, S. R. and Orsi, N. M. (2011). [A robust RNA integrity-preserving staining protocol for laser capture microdissection of endometrial cancer tissue.](#) *Anal Biochem* 416(1): 123-125.
3. Gibbs, J. E., Beesley, S., Plumb, J., Singh, D., Farrow, S., Ray, D. W. and Loudon, A. S. (2009). [Circadian timing in the lung; a specific role for bronchiolar epithelial cells.](#) *Endocrinology* 150(1): 268-276.
4. Gibbs, J., Ince, L., Matthews, L., Mei, J., Bell, T., Yang, N., Saer, B., Begley, N., Poolman, T., Pariollaud, M., Farrow, S., DeMayo, F., Hussell, T., Worthen, G. S., Ray, D. and Loudon, A. (2014). [An epithelial circadian clock controls pulmonary inflammation and glucocorticoid action.](#) *Nat Med* 20(8): 919-926.
5. Haspel, J. A., Chettimada, S., Shaik, R. S., Chu, J. H., Raby, B. A., Cernadas, M., Carey, V., Process, V., Hunninghake, G. M., Ifedigbo, E., Lederer, J. A., Englert, J., Pelton, A., Coronata, A., Fredenburgh, L. E. and Choi, A. M. (2014). [Circadian rhythm reprogramming during lung inflammation.](#) *Nat Commun* 5: 4753.
6. Li, J., Grant, G. R., Hogenesch, J. B. and Hughes, M. E. (2015). [Considerations for RNA-seq analysis of circadian rhythms.](#) *Methods Enzymol* 551: 349-367.
7. Sukumaran, S., Jusko, W. J., Dubois, D. C. and Almon, R. R. (2011). [Light-dark oscillations in the lung transcriptome: implications for lung homeostasis, repair, metabolism, disease, and drug action.](#) *J Appl Physiol (1985)* 110(6): 1732-1747.
8. Zhang, R., Lahens, N. F., Ballance, H. I., Hughes, M. E. and Hogenesch, J. B. (2014). [A circadian gene expression atlas in mammals: implications for biology and medicine.](#) *Proc Natl Acad Sci U S A* 111(45): 16219-16224.

Laser Capture Micro-dissection (LCM) of Neonatal Mouse Forebrain for RNA IsolationAchira Roy^{1,*}, Mei Deng², Kimberly A. Aldinger^{1,2}, Ian A. Glass^{1,2} and Kathleen J. Millen^{1,2,*}

¹Center for Integrative Brain Research, Seattle Children's Research Institute, Seattle, United States; ²Division of Genetic Medicine, Department of Pediatrics, University of Washington, Seattle, United States

*For correspondence: achira.roy@seattlechildrens.org; kathleen.millen@seattlechildrens.org

[Abstract] Precise and reproducible isolation of desired cell types or layers from heterogeneous tissues is crucial to analyze specific gene profiles and molecular interactions *in vivo*. Forebrain is the core site of higher functions, like cognition and memory consolidation. It is composed of heterogeneous and distinct cell types, interconnected to form functional neural circuits. Any alteration in the development or function often leads to brain disorders with profound consequences. Thus, precise molecular understanding of forebrain development in normal and diseased scenarios is important. For quantitative studies, most traditional analytical methods require pooling of large cell populations, that results in loss of *in vivo* tissue integrity and of spatial, molecular and cellular resolution. Laser capture microdissection (LCM) is a fast and extremely precise method of obtaining uncontaminated, homogeneous sets of specific cell types and layers. Our current procedure involves cryo-sectioning and laser microdissection of fresh-frozen mouse forebrains, that are genetically modified and treated with small-molecule therapeutics. Using LCM, specific regions of interest, such as neural layers, cells from adjacent yet distinct subregions within a tissue layer, are obtained under RNase-free conditions. These small cellular cohorts are further used for downstream, high-throughput genomic or transcriptomic assays. Here, we have introduced break-points at multiple stages throughout our protocol. This makes our method simpler and more user-friendly to follow, without compromising on the quality. The current protocol can easily be adapted for different brain regions, as well as for other model organisms/human tissue.

Keywords: Mouse, Forebrain, Laser capture microdissection, RNA isolation, Neural progenitors, Microscopy, RNA sequencing, Hippocampus

[Background] To understand the molecular mechanisms underlying normal and diseased neural processes, it is critical to analyze the interactions *in vivo*, within a specific tissue microenvironment. It is quite challenging to precisely access different areas of the developing brain, especially in deep areas like hippocampus. The level of complication increases further due to the existence of cellular heterogeneity even within a single neural layer. Traditional quantitative methods like microarray *etc.*, using gross tissue lysates or various primary and organotypic culture systems, rely heavily on pooling gene expression data from thousands of cells and reporting a population-wide average (Levsky and Singer, 2003). Thus, these procedures fail to capture subtle differences within distinct cell subsets as well as to maintain *in vivo* tissue/cell integrity. Moreover, there remains a high risk of tissue contamination from neighboring areas during sample collection. Laser-capture microdissection (LCM) combines the benefits of light microscopy and precision of laser beam to procure homogeneous subpopulations of cells from heterogeneous tissue under direct microscopic visualization (Emmert-Buck *et al.*, 1996). The laser cutting is usually less than 1 μm wide. This allows target cells to remain unaffected by the laser beam. LCM technology can

selectively and routinely harvest regions of interest, ranging from specific tissue layers to single cells and chromosomes (<https://www.leica-microsystems.com/products/light-microscopes/details/product/leica-lmd7/>). This enables the user to obtain reproducible, targeted starting material for biologically relevant data analyses.

Data using the described LCM protocol was published in (Roy *et al.*, 2019). In this study, we found that activating mutations in the phosphoinositide 3-kinase (PI3K) pathway results in focal disruption of cell-cell adhesion, proliferation and apical tissue integrity, leading to cortical gyration (in both neocortex and hippocampus) and developmental hydrocephalus in mice. Conventional quantitative analytical methods were not precise enough to distinguish cellular/molecular heterogeneity within a neural layer, between adjacent gyral and sulcal zones in the mutant mouse brain. Hence, we used LCM to specifically dissect selective portions of the developing ventricular zone from P0 control and mutant littermates, followed by RNA sequencing. This data was then analyzed to study the effect of the *PI3K* mutation on neural progenitors. Further, we performed the same experiment after administering a small-molecule drug *in utero*, to compare the effect of drug treatment on the gene expression profile between controls and mutants.

Here, we present detailed step-wise instructions including experimental ‘do’s and don’ts’, and also illustrate key components and steps using photographs, to aid new researchers in setting up this useful procedure. We have introduced a number of stopping steps throughout the protocol. This makes our method simple and more user-friendly, without making any compromise on the quality, quantity and integrity of the end product (RNA, DNA *etc.*). This method can be used with fresh-frozen and fixed tissues.

Some other applications of this protocol are as follows:

1. Absolute identification of possible mosaic mutations with cells of interest, in mouse models or human patients.
2. Downstream analyses like selective quantitative Real-Time PCR, protein assays like Western blot analysis, chromatin immuno-precipitation, genomics (DNA assays), as well as metabolomics.
3. Live cell culture, cloning and genetic manipulations.
4. Isolation of small quantity of specific tissue material, highly useful for plant biology, forensic science and prenatal diagnostics.

Materials and Reagents

1. Sterile gloves
2. DNase-free, RNase-free micropipette tips
3. Fisherbrand Permanent fine tip marking pen (Fisher Scientific, catalog number: 13-379-4), to label embedding molds, and tubes for genotyping, if required
4. Fisherbrand Petri Dish with Clear Lid 100 mm x 15 mm, sterile (Fisher Scientific, catalog number: FB0875713)
5. Bucket of Ice
6. Embedding molds (Thermo Scientific, catalog number: 70182)
7. Cryogenic Fiberboard Freezer Box, 2” (VWR International, catalog number: 89214-750)
8. Regular duty single edge blades (Personna, catalog number: 94-115-71)

9. Low Profile Microtome Blades (Sakura, Accu-Edge, catalog number: 4689)
10. Fisherbrand Superfrost Plus microscope slides, precleaned (Fisher Scientific, catalog number: 12-550-15)
11. MembraneSlides, nuclease and human nucleic acid-free polyethylene naphthalate (PEN)-Membrane 2 μm (PEN slides) (Leica Microsystems, catalog number: 11505189)
12. Pencil, to label slides
13. New plastic 5-slide mailer—open top (Electron Microscopy Sciences, catalog number: 71548-01), for storage of PEN slides
14. Sterile 50 ml Polypropylene conical tube (Falcon, catalog number: 352070) for staining or storage of PEN slides
15. Kimwipes (Kimberly-Clark Professional, catalog number: 34155 or 34120)
16. SamcoTM Graduated Transfer Pipettes, sterile individually wrapped (Thermo Fisher Scientific, catalog number: 274-1S)
17. Tissue collection tubes: 0.5 ml Tubes with Flat Cap, certified DNase/RNase-free (Thermowell Tubes, catalog number: 6530)
18. High quality bags from VacUpack (for example: 4 Mil Vacuum Bags, size: 8"X10", U.S. Vacuum Packaging and Wrapping LLC., catalog number: SS-VB4-0810)
19. Small biohazard bags, to dispose of mouse carcasses (Medline, catalog number: DYND30261)
20. RNase-free 1.7 ml tubes (Olympus Plastics, Genesee Scientific, catalog number: 24-282A)
21. Aqui-Pad Laboratory Mat (Therapak, catalog number: 10324G)
22. **Animal:** Mouse, neonatal pups (P0-P6)

Notes:

- a. *Male and female mice are mated, and embryos are staged considering the morning a vaginal plug is detected as embryonic day (E)0.5. In utero drug treatment can be done using this staging. Specifically, the types of mating used in (Roy et al., 2019) are described in detail in the article's Methods section.*
- b. *The day the pups are born is considered as P0. For FVB strain, the gestation period is E18-E19.*
- c. *No alterations to the protocol are required if different mouse strains are used.*
23. RNasin Plus RNase Inhibitor (Promega, catalog number: 5067-1513)
24. *Desiccants:* Indicator blue DrieriteTM desiccant (W.A. Hammond Drierite Company Ltd., mesh size: 8, stock number: 23005); regular DrieriteTM desiccant (W.A. Hammond Drierite Company Ltd., mesh size: 8, stock number: 12001)
25. RNaseZap RNase Decontamination Solution (Invitrogen by Thermo Fisher Scientific, catalog number: AM9780)
26. Phosphate Buffer Saline PBS pH 7.4 (1x) (Gibco, catalog number: 10010-023)
27. Dry Ice
28. Optimum Cutting Temperature (OCT) Embedding Compound (Electron Microscopy Sciences, TissueTek #4583, catalog number: 62550-01)
29. Cresyl violet powder (Sigma-Aldrich, Harleco, catalog number: 190-12), storage: +15 °C to +30 °C
Note: Keep the bottle RNase-free.
30. RNeasy Plus Micro Kit (50) (Qiagen, catalog number: 74034, storage: RNeasy MinElute Spin Columns

at 2–8 °C, remaining components at room temperature (15–25 °C)

31. Agilent RNA 6000 Pico Kit (Agilent Technologies, catalog number: 5067-1513), storage: 4 °C, RNA ladder at -80 °C
32. Molecular Biology Grade Water (Corning, catalog number: 46-000-CM)
33. Ethanol 200 Proof Anhydrous (Decon Labs, Inc., catalog number: 2716)
34. 2-Mercaptoethanol (β -mercaptoethanol) (Bio-Rad, catalog number: 161-0710)
35. 70% ethanol (100 ml) (see Recipes)
36. 95% ethanol (100 ml) (see Recipes)
37. RNaseZap/70% ethanol mixture (100 ml) (see Recipes)
38. 75% ethanol (freshly-prepared) (see Recipes)
39. Nissl stain for RNA research (50 ml) (see Recipes)

Note: If the experiment requires comparison of mouse tissue between control and genetically modified mutant models and/or \pm drug treatment, reagents to perform mouse tissue genotyping are required as briefly mentioned in (Roy et al., 2015 and 2019).

Equipment

1. Clean micropipettes
2. 16oz Rubber Mallet with Wood Handle, to crush dry ice (Olympia Tools, catalog number: 60661116)
3. Dissection instruments:
 - a. Dumont fine forceps #5 (Fine Science Tools, catalog number: 11252-20)
 - b. Dumont fine forceps #55 (Fine Science Tools, catalog number: 11255-20)
 - c. Dumont coarse forceps AA (Fine Science Tools, catalog number: 11210-20)
 - d. Fine Scissors–Sharp (Fine Science Tools, catalog number: 14060-10)
4. FisherbrandTM Stainless Steel Micro Chattaway Spatulas (Fisher Scientific, catalog numbers: 11543482 and 14-373)
5. Autoclave
6. Cryostat (Leica Microsystems, model: CM1850)
7. Brightfield Leica EZ4 Stereo Microscope (Leica Microsystems, catalog number: 10447197)
8. -80 °C freezer
9. -20 °C freezer
10. 4 °C refrigerator
11. Polyethylene ethanol wide-mouth wash bottle with polypropylene cap; 500 ml (Bel-Art SP Scienceware, catalog number: F11816-0019)
12. Laser Microdissection Microscope (Leica Microsystems, model: LMD7)
13. Agilent 2100 Bioanalyzer (Agilent Technologies, catalog number: G2939BA)
14. Chip priming station (Agilent Technologies, catalog number: 5065-4401)
15. IKA MS3 vortex mixer for Agilent 2100 Bioanalyzer
16. NanoDrop 2000 microvolume spectrophotometer (Thermo Scientific, catalog number: ND-2000)

17. Vacuum Desiccator (Fisher Scientific, catalog number: 08-648-107)
Optional: Desiccator, home-made using stacked mixture of regular Drierite and Indicator blue Drierite desiccant, in a large glass bottle.
18. Microcentrifuge (Eppendorf, catalog number: 5417R)
19. Vortex (Scientific Industries, model: Vortex Genie-2, catalog number: SI-T236)
20. Analytical electronic balance (Reshy, catalog number: JM-B)
21. UV transilluminator (one used here is from Fotodyne, catalog number: FD33500; should preferably be used only RNase-free and without any chemical contamination)
22. Rocking shaker (Reliable Scientific Inc., model 55), for solution preparation and staining of slides.
23. Vacuum Sealing System with Starter Kit (FoodSaver, catalog number: V2244)

Note: If the experiment requires mouse tissue genotyping, appropriate set of equipment should be available.

Software

1. Laser Microdissection System (Leica Microsystems, version: 8.2.0.6739)
2. Agilent 2100 Expert Software (Agilent Technologies, version: B.02.08.SI648(SR2))
3. NanoDrop software (Thermo Scientific, version used: 1.6.198)
4. *Optional:* Microsoft PowerPoint

Note: For genotyping, Image LabTM Software (Bio-Rad, 1708265) is required for gel documentation.

Procedure

Note: Prior to starting laser microdissection, the user should be well trained in cryo-sectioning and have sufficient knowledge of microscopy and histopathology to sample the correct tissue or cells. To minimize quality damage, the whole procedure of tissue handling should be carried out quickly, yet carefully.

A. Preparation of solutions and set-up for brain dissection

Notes:

- a. Wear sterile disposable gloves and change frequently at every step, from preparing reagents till RNA analysis etc.
- b. Use clean, RNase-free instruments. Before handling the next specimen, spray the instruments with RNaseZap, rinse with RNase-free water and wipe with clean Kimwipes.
- c. Use RNase-free or Nuclease-free solutions, glassware and plastic ware.
- d. Use RNaseZap or similar product to clean the equipment or the surface of the working area.
- e. Freeze tissues immediately after surgery to obtain high-quality RNA. This minimizes gradual RNA degradation by ubiquitous RNases or heat.

1. Prepare working solutions, as mentioned in the Recipes section
2. Label the cryomolds at room temperature using the Fisherbrand permanent marker, prior to the tissue

dissection experiment, preferably on the side-wall opposite to the notched side (for easier orientation)

3. Autoclave the dissection instruments and spatula for sterilization, prior to the experiment.

Note: The dissection tools should have never seen any fixative etc.; wipe them with RNaseZap, then RNase-free DNase-free water and dry with clean Kimwipes before starting the experiment.

4. Wipe the tissue dissection area thoroughly with 100% RNaseZAP solution, let the area dry before placing the laboratory mat for dissection.
5. Assemble all reagents and equipment before tissue dissection, as shown in Figure 1.
6. Crush some dry-ice using the mallet to generate a soft powdered area for future placement of the embedding mold (Figure 2A).
7. Pour OCT slowly and carefully along one edge of the sterile embedding mold till it is half-filled (Figures 2B and 2F).

Note: Avoid air bubbles in the OCT while pouring. Air bubbles disrupt the tissue integrity.

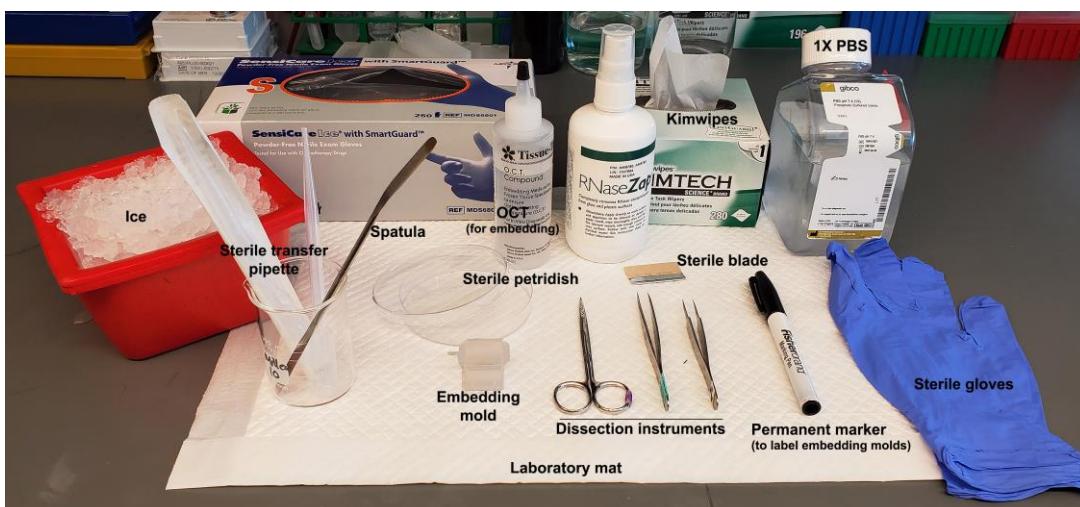


Figure 1. Materials needed for dissection of neonatal mouse forebrain. Reagents and equipment needed for mouse brain dissection are labeled. 1x PBS—Phosphate buffer saline, working solution.

B. Neonatal mouse brain dissection

1. Euthanize neonatal mouse pups of the desired age (P0-P6) using procedures permitted by your Institutional guidelines. In Roy *et al.* (2019), as per the approved animal protocol (see Ethics statement), euthanasia was performed by inducing hypothermia anesthesia, followed by decapitation. For this procedure, place the newborn pups on the lid of a Petri dish and set on top of crushed ice for 2-3 min, leading to their unconsciousness. Confirm anesthesia in pups by lack of response to firm pinch, once they stop moving. Decapitate using sharp scissors.
2. Transfer the heads to a sterile 100 mm Petri dish containing sterile 1x PBS.
3. At this stage, if genotyping of the pups is required, take a tiny piece of the tail (≤ 2 mm) of each pup and place them in labeled 1.7 ml microcentrifuge tubes for further processing.
4. Dispose of the carcasses using labeled biohazard bags.

5. Under a dissection microscope, dissect out the brains using fine and coarse forceps (Figure 2C). Dispose of the remaining carcasses using the biohazard bags.

Note: If the Petri dish becomes murky with blood, you can transfer the brains at this stage to another Petri dish containing sterile 1x PBS.

6. After removing the brain, sever the hindbrain and midbrain, retaining only the forebrain including the olfactory bulbs (Figures 2D-2E). The presence of the olfactory bulb helps to position the telencephalic hemisphere in the correct orientation in the embedding molds.

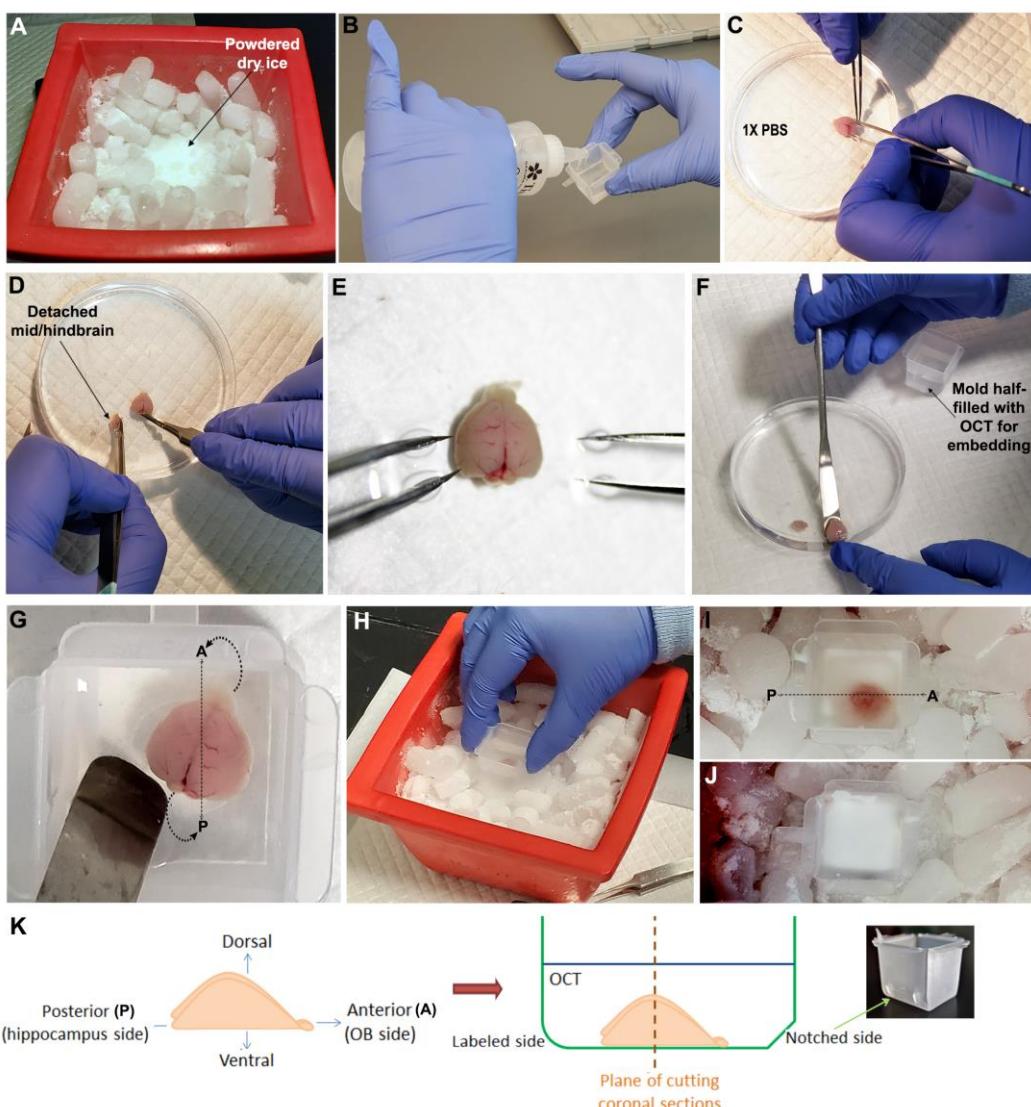


Figure 2. Steps of dissecting and embedding a neonatal mouse forebrain. A. Crushed dry ice prepared for freezing the embedding mold. B. OCT poured along an edge of the embedding mold to avoid air bubbles. C-E. Dissection of neonatal mouse forebrain, in sterile 1x PBS. F-G. Forebrain carefully transferred to and then oriented in the embedding mold filled with OCT. H-J. Mold with OCT-covered oriented mouse forebrain placed in dry ice, until OCT freezes to opaque form. K. Schematic of how forebrain was oriented, to be used for coronal sectioning (Roy *et al.*, 2019).

C. RNase-free brain mounting

- With the help of the spatula and coarse forceps (if needed), take the brain out of the buffer and transfer to the pre-labeled embedding mold half-filled with OCT (Figures 2F-2G).

Note: Try to remove liquid from the brain surface as much as possible without damaging the brain (you may carefully use a finely rolled Kimwipe if needed), before immersing it in the OCT.

- Orient the mouse forebrain such that it is parallel to or on the floor of the embedding mold.
- For coronal sectioning (perpendicular to the antero-posterior axis of the forebrain), slowly orient the anterior side of the brain (olfactory bulb side) directly perpendicular to the notched side of the mold. Avoid air bubbles in the process (Figures 2G and 2K).
- Once the brain is properly oriented, slowly pour OCT along one edge to fill the mold (or at least enough to cover the entire brain). Take precaution that the brain orientation remains intact and air bubbles do not develop in between OCT layers.
- Carefully place the mold within the powdered dry ice and support the mold from the sides by pieces of dry ice (Figures 2H-2I).
- Once the mold, with brain embedded, becomes completely opaque (Figure 2J), transfer it to a new, clean freezer box and immediately store at -80 °C freezer.

D. Preparation before tissue sectioning

- Clean cryostat, microtome blade and tools with a 1:1 mixture of RNaseZap and 70% ethanol (RNaseZap/70% ethanol) and then with sterile 95% ethanol (Recipes 1-3). Wipe dry with Kimwipes.
- Incubate PEN membrane slides for 30 min under UV light (on the UV transilluminator) to enhance the binding of the tissue to the membrane slide.
- Get a bucket of dry ice for tissue/embedded mold transportation.
- Prepare solutions with RNase-free water and high-quality ethanol:
 - 75% ethanol (freshly prepared, Recipe 4)
 - Nissl stain (Recipe 5, prepare at least 3 days before use and store at 4 °C). Aliquot the amount needed for current use, by roughly estimating the total number of slides to be stained (10-15 drops of the solution is adequate to rapid stain each slide).
- Set temperature of cryostat in the range of -15 °C to -18 °C
- Label PEN slides with brain code/genotype using a pencil.

E. Cryo-sectioning of brain tissue

- Wear new sterile disposable RNase-free gloves. Change gloves frequently between steps to maintain the RNase-free status.
- Transfer an embedded brain mold, placed securely on dry ice, from -80 °C freezer to the cryostat.
- Use new sterile, single-edge blade to tear the plastic embedding mold inside the cryostat. Trim the OCT-embedded block as needed.
- For coronal staining, attach the block on the cryostat chuck (specimen clamp) such that the anterior side of the brain (notched side of mold, Figure 2K) faces perpendicular to the microtome blade inside the

cryostat.

5. Place embedded brain inside the cryostat at least 20 min prior to sectioning, in order to allow it to come to the cryostat temperature.
6. Use a new sterile microtome blade for each experiment (Figure 3A).
7. Slice a few sections from the surface of the mold to avoid any sort of contamination, prior to the collection of actual sections to be mounted on slides.
8. Cut 8-10 μm sections and mount on regular charged plus slides (on the positively-charged coated side). To identify your region of interest, rapid stain with Nissl staining solution or other dye for about 1 min, swivel gently, and observe slides under stereo microscope.

Note: Staining procedure is explained in Step E14b.

9. Once you start getting sections of your region of interest, change the slicing thickness to 16-18 μm and mount on the PEN membrane slides. You can mount multiple sections on one slide. For example, ~12-14 sections were mounted on each PEN slide in Roy *et al.* (2019).

Note: Mount the sections within the extent on the PEN-coated membrane (Figure 3B). Please take care that the coating does not get any scratches or leave the RNase-free area.

10. Optimally, place the PEN slides on the desiccant during the section collection.
11. Clean the blade and cryo-sectioning stage with brush or Kimwipe in between sections.
12. Collect one section of every 5-10 sections and mount on the regular charged slide for histologic staining.
13. Air-dry the PEN slides at room temperature for about 2 min on desiccant. Make sure the collected sections are completely dry before staining.
14. After air-drying, PEN slides with collected sections can be:
 - a. Stored in plastic slide mailer in a vacuumed bag, containing desiccant, at -80 °C, for a few months or longer, until user is ready to perform LCM.
 - b. Immediately transferred to 100% ethanol at room temperature for dehydration, followed by Nissl staining.

The Nissl staining protocol is as follows [a quicker staining method, modified from (Grundemann *et al.*, 2008) and Leica LMD Protocol Guide (<https://www.leica-microsystems.com/products/light-microscopes/details/product/leica-lmd7/>)]:

- i. Stain slides one at a time using 50 ml tube or simultaneously using a big container.
- ii. Immerse the slides in fresh 100% ethanol, 5 min x 4 times, and place on the rocking shaker to remove any OCT attached.
- iii. Immerse slides in freshly-prepared 75% ethanol (Recipe 4) for ~1 min.
- iv. Place drops of Nissl stain (Recipe 5) directly onto the sections, using plastic transfer pipette, and incubate for 1 min. Swivel the slides gently. On average, 10-15 drops of the solution are adequate per slide.
- v. Quickly rinse the slides with fresh 100% ethanol, 5 s x 3 times.
- vi. Finally, immerse the slides in fresh 100% ethanol for 1 min, to allow the stain to bind onto the sections.
- vii. Observe sections under a brightfield dissecting microscope.

viii. Allow the slides to stand for at least 30 min in order to make them completely dry before storage or use.

Note: Optimally, finish staining of sections strictly within 10 min in total.

15. Store completely dried PEN slides with mounted sections at -80 °C, either before or after Nissl staining. Neither procedure will negatively affect the RNA quality. Keep the slides at -80 °C until you are ready to start LCM.

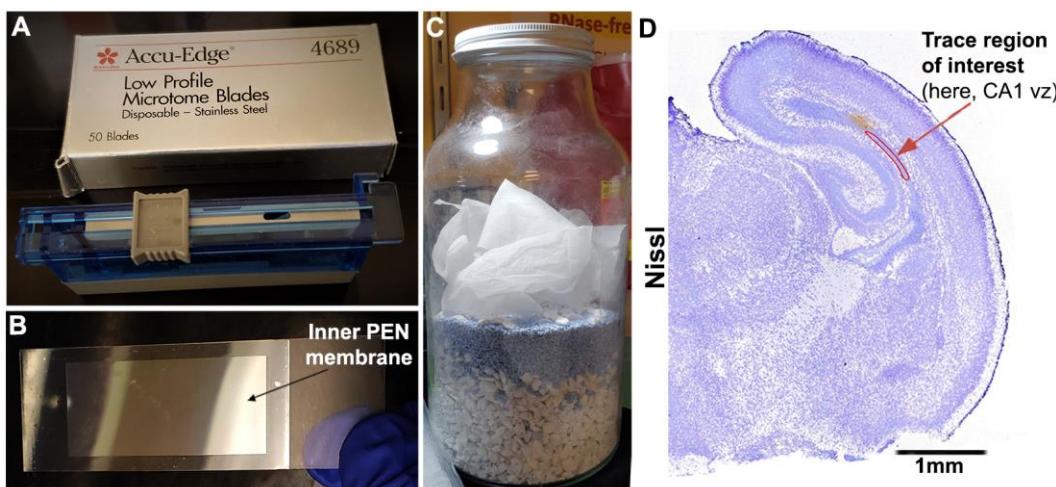


Figure 3. Preparation for tissue sectioning and laser microdissection. A. New sterile microtome blades used for brain sectioning. B. PEN slides: mount sections onto the inner film, which finally gets laser-cut. C. Home-made desiccator using Drierite to dry slides prior to LCM. D. Representative image of a Nissl-stained coronal hemi-section of P0 mouse forebrain, used in microdissection and data analysis (Roy *et al.*, 2019), showing how region of interest is traced. In this context, ventricular zone of P0 mouse hippocampal CA1 region (CA1 vz) was traced.

F. Laser microdissection

Notes:

1. All steps of laser microdissection should be performed in a designated RNase-free zone, in a clean, steady air-conditioned room.
2. Be cautious to slowly adjust frozen slides to room temperature before laser microdissection. This is critical for RNA quality since water (moisture) activates RNases.
3. Wipe the work area with Kimwipes dipped in RNase-free water, followed by 100% ethanol.
4. Clean the stage, substage and UV shield of the LMD machine sequentially with 95% and 100% ethanol.

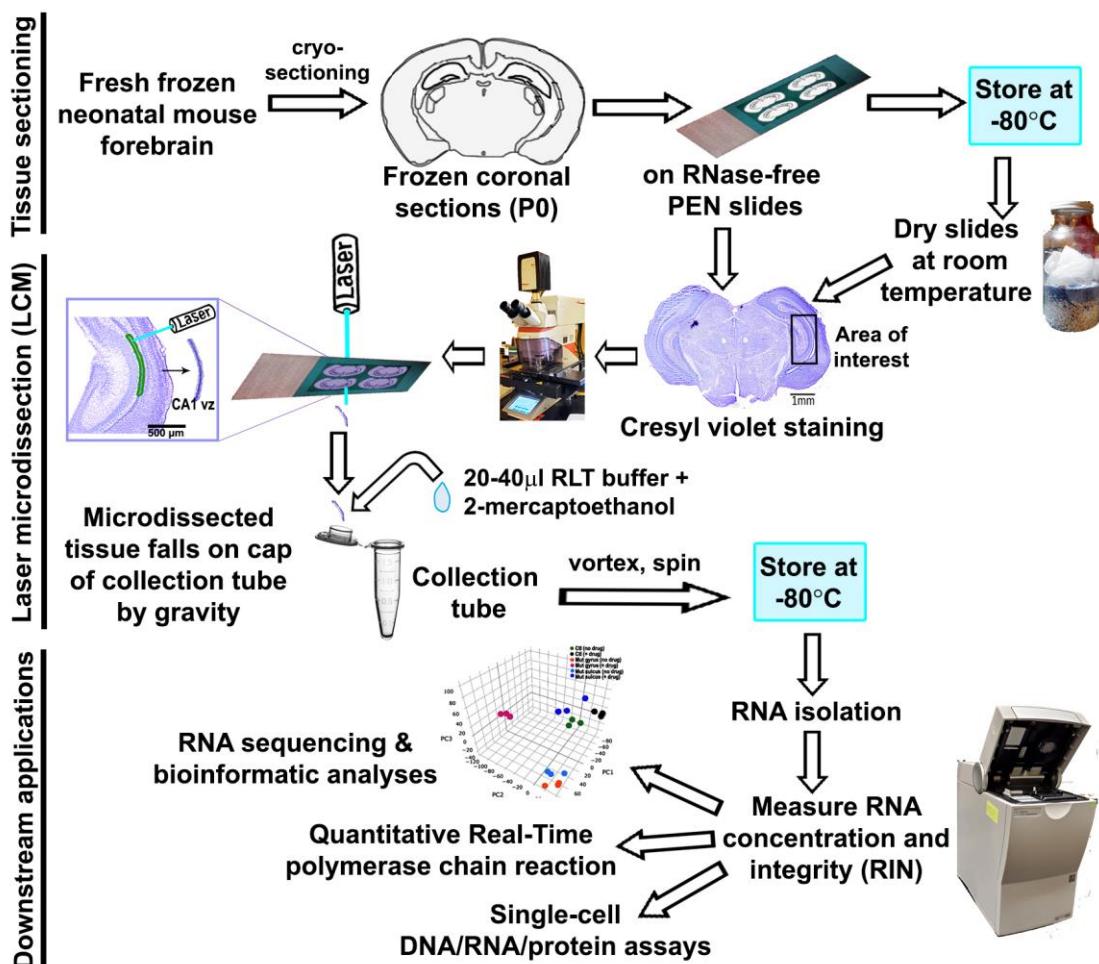


Figure 4. Flowchart showing different steps of laser microdissection of neonatal mouse forebrain tissue. Different steps—tissue sectioning, laser-capture microdissection, downstream applications. Some images are obtained from Roy *et al.* (2019), where we compared the genetic profiles of the hippocampal CA1 ventricular zone (CA1 vz) between P0 control and activating *Pik3ca* mutant ± drug, using RNA sequencing.

1. Take the PEN slides (in the vacuumed bag) out of -80 °C freezer and allow it to slowly reach room temperature, for at least 60 min, before opening the bag. This is to avoid formation of water condensation on the sections. This step is critical for preserving RNA quality. Another method of drying the slide is to use the vacuum desiccator or a home-made RNase-free desiccator (Figure 3C) and dry the slides on the same day.
2. Switch the power of the Leica LMD equipment on.
3. Clean the collect tube caps (0.5 ml) with RNaseZap, followed by a rinse with RNase-free water.
4. Stain the section collected on regular slide (see Step E12) to confirm the area of interest, before performing LCM on serial sections mounted on PEN slides.

Alternate method: If one studying the scientific question and one executing the laser microdissection are separate personnel, working at separate places/time zones, a crude method to designate the region of interest can be followed:

- a. *Image the Nissl-stained forebrain sections mounted on the regular plus slides, 1 for every 10 sections mounted on PEN slides.*
- b. *Copy and paste each image as a separate slide in Microsoft PowerPoint.*
- c. *Carefully trace the region of interest, using PowerPoint tools, as shown in Figure 3D.*
- d. *Using these representative tracings, successfully perform LCM on the related PEN slides having the respective serial sections.*
5. If not already done, stain sections on the PEN slides with Nissl stain, as described in the previous section, just prior to microdissection.
6. Dry the slides post-staining, in the desiccator.
7. Place PEN slides upside down in the stack-slide holder.
8. Create a sample overview under 1.25x lens, using LED/halogen illumination.
9. Mount 0.5 ml RNase-free, DNase-free collection tubes in the LMD tube holder. Add 20-40 µl of a mixture of RLT buffer and 2-mercaptoethanol to the cap of each tube (10 µl 2-mercaptoethanol in 1 ml RLT buffer from Qiagen RNeasy Plus Micro Kit).
10. Set the LMD software to Transmitted light-brightfield (TL-BF) mode.
11. Adjust software parameters to get suitable cutting including more accurate and less damage of tissue. Software parameters used in Roy *et al.* (2019): Draw shapes using “close line”. Use the “Move + Cut” mode for cutting respected tissue zones/cells. Use “Fine” X-Y-Z precision mode.
12. Guide the laser beam with mouse or touchscreen over your specific region of interest. The dissected tissue drops into the caps of collection tube by force of gravity without contamination. In Roy *et al.* (2019), ventricular zone of P0 hippocampal CA1 field (CA1 vz) was collected from each coronal hemisphere (Figure 4).

Note: Please load a new collection tube every 2 h if longer cutting time needed.

13. Once all sections of the first slide are laser-cut, move to the next slide and repeat Steps F9-F12. You can store the used slides for future inspection and documentation.

*Note: Depending on the size and density of the laser-dissected samples, you can decide whether to pool together samples obtained from each brain for RNA isolation and further processing. In Roy *et al.* (2019), our region of interest (ventricular zone of P0 mouse CA1 region) was both low in volume and in density. Hence, total RNA was isolated from LCM-enriched samples pooled across ~6 slides per genotype.*

14. After microdissection, carefully retrieve the collect tubes, close the cap upside down and vortex for 1 min.
15. Give the tubes a brief spin in the centrifuge in order to collect sample at the bottom of the tube. Store tubes at -80 °C for further analysis.

Note: If more than one cell type or cell population is needed from one slide, collect one type of tissue from all sections at the same time and make sure that the cut tissue had been detached from the section before starting to collect others. Load the collection tube for the next cell/tissue type in a different hole to avoid possible tissue contamination.

16. Store unfinished PEN slides (where further LCM is possible) in the vacuum desiccator with desiccant at room temperature up to 5 days.

G. RNA isolation

1. Pool all the collected RLT buffer (with micro-dissected samples of each cell/tissue type of each biological replicate) into a new, RNase-free 1.7 ml tube.
2. Vortex the tubes for 30 s.
3. Add an equal amount of 70% ethanol (RNase-free).
4. If the total volume is > 700 µl, transfer ~700 µl mixture each time to an RNeasy MinElute spin column and repeat this step using the same column, until the entire volume of mixture is used.
5. Follow the instruction given in the manual of RNeasy Micro Kit to isolate RNA from the LCM-ed samples.
6. Elute the RNA with 15 µl RNase-free water contained RNase Inhibitor (1 U/µl).
7. Repeat Step H6. If necessary, use the same tube for RNA collection.
8. Take 2 µl RNA elution into 2-3 µl RNase-free water for NanoDrop and RNA bioanalysis.
9. Proceed with measuring the concentration and purity of obtained RNA, as part of the data analysis.

Note: You may distribute each tube/genotype/sample into 3 technical replicates for further processing (for example, RNA sequencing).

Data analysis

Measure RNA concentration and RIN score

1. After RNA is isolated by the RNAeasy kit, measure the concentration of RNA per area of interest, per genotype, per biological replicate, using the NanoDrop.
2. Calculate RNA quality by measuring the RNA Integrity Number (RIN) as well as RNA concentration (more precise than NanoDrop) for each RNA sample, using the Agilent Bioanalyzer 6000 Pico Kit.

Note: RNA sample of RIN > 5.0 is considered usable (where maximum score can be 10.0).

3. Follow the manual available with the Agilent Kit for the consecutive steps. Briefly, (https://www.agilent.com/cs/library/usermanuals/Public/G2938-0049_RNA6000Pico_QSG.pdf):
 - a. Thaw RNA ladder on ice (avoid excessive warmth).
 - b. Prepare RNA gel matrix prior to the experiment (maximum: 4 weeks) and store at 4 °C.
 - c. Prepare the gel-dye mixture (components available within the kit) such that it is used within a day.
 - d. Load the gel-dye mixture carefully into each well of a new, sterile RNA Pico chip, with the help of the priming station (Figures 5A-5B).
 - e. Load RNA conditioning solution and RNA marker.
 - f. Load the thawed RNA ladder and samples. Vortex the Chip using the IKA vortex mixer (Figure 5C) before placing the former in the Agilent Bioanalyzer equipment for analysis.
 - g. Analyze RNA concentration and integrity using the Agilent software (Figure 5D).

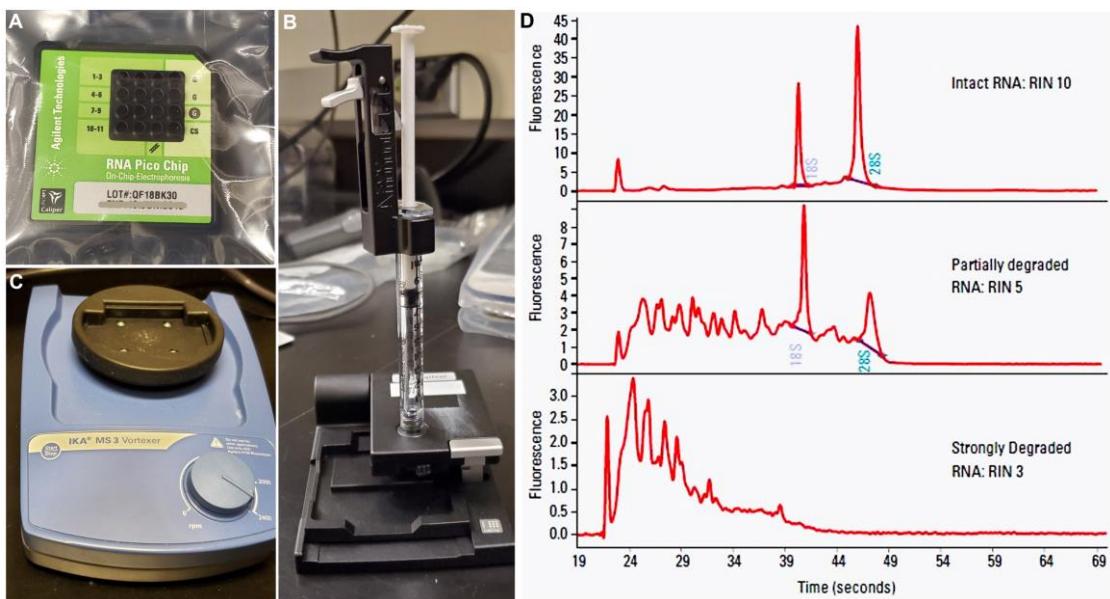


Figure 5. Components used to analyze isolated RNA. A. RNA Pico Chip, where the small quantity of RNA samples is loaded. B. Chip priming station, to be obtained separately to load the gel-dye mix into each sample-well of the Pico Chip. C. IKA vortex mixer is used to vortex the sample-loaded Chip before running it in the Agilent BioAnalyzer instrument. D. Representative image demonstrating different types of RNA integrity (modified from <https://www.agilent.com/cs/library/applications/5989-1165EN.pdf>).

- With good quality of RNA thus obtained, proceed for different downstream analyses, as needed. These can include RNA sequencing (tissue, single cell), quantitative Real-Time polymerase chain reaction (RT-PCR), protein assays (Figure 4).

Note: In our experience, RNA concentration of 1.5-7 ng/ μ l and integrity of > 7.0 was sufficient to proceed to successful RNA sequencing (see Methods, Figure 4 and Figure supplements 1-3, Source files 1-2 in Roy et al., 2019).

Recipes

Note: Use 100% RNase-free molecular grade water in the following recipes.

- 70% ethanol (100 ml)
 - Add 70 ml RNase-free absolute (100%) ethanol and 30 ml water to make 100 ml of 70% ethanol
 - Store at -20 °C
- 95% ethanol (100 ml)
 - Add 95 ml RNase-free 100% ethanol and 5 ml water to make 100 ml of 95% ethanol
 - Store at -20 °C
- RNaseZap/70% ethanol (100 ml)
 - Mix RNaseZap and 70% ethanol (Recipe 1) in 1:1 ratio. For 100 ml, measure 50 ml of RNaseZap and 50 ml of 70% ethanol into a clean, RNase-free wash bottle

- b. Mix the two liquids by rocking the bottle sideways for 4-5 min
Clean the cryostat, microtome blade and other tools with this mixture.
4. 75% ethanol (freshly-prepared)
 - a. Add 75 ml RNase-free 100% ethanol and 25 ml water to make 100 ml of 75% ethanol
 - b. Store at -20 °C
5. Nissl stain for RNA research (50 ml)
 - a. Measure 0.5 g cresyl violet powder in a 50 ml RNase-free tube
 - b. Add 100% ethanol (RNase-free), final concentration: 1% solution (weight/volume)
 - c. Shake the solution for a few hours and store at 4 °C, sealed air-tight and dark for up to 6 months.
This solution should be prepared at least 3 days prior to usage. Aliquot as required and discard the used solution.

Acknowledgments

This work was performed using the funding sources National Institutes of Health 1R01NS099027 (KJM), Seattle Children's Hydrocephalus Research Guild (KJM) and Hydrocephalus Association Innovator Award 2018 (KJM). This protocol was derived from the original research paper (Roy *et al.*, 2019).

Competing interests

The authors declare that no competing interests exist.

Ethics

Animal experimentation: All animal experimentation was conducted in accordance with the guidelines laid down by the Institutional Animal Care and Use Committees (IACUC) of Seattle Children's Research Institute, Seattle, WA, USA (protocol ID: IACUC00006; last day of triennial approval period: 11th July 2021).

References

1. Emmert-Buck, M. R., Bonner, R. F., Smith, P. D., Chuaqui, R. F., Zhuang, Z., Goldstein, S. R., Weiss, R. A. and Liotta, L. A. (1996). [Laser capture microdissection](#). *Science* 274(5289): 998-1001.
2. Grundemann, J., Schlaudraff, F., Haeckel, O. and Liss, B. (2008). [Elevated alpha-synuclein mRNA levels in individual UV-laser-microdissected dopaminergic substantia nigra neurons in idiopathic Parkinson's disease](#). *Nucleic Acids Res* 36(7): e38.
3. Levsky, J. M. and Singer, R. H. (2003). [Gene expression and the myth of the average cell](#). *Trends Cell Biol* 13(1): 4-6.
4. Roy, A., Murphy, R. M., Deng, M., MacDonald, J. W., Bammler, T. K., Aldinger, K. A., Glass, I. A. and Millen, K. J. (2019). [PI3K-Yap activity drives cortical gyration and hydrocephalus in mice](#). *eLife* 8:

e45961.

5. Roy, A., Skibo, J., Kalume, F., Ni, J., Rankin, S., Lu, Y., Dobyns, W. B., Mills, G. B., Zhao, J. J., Baker, S. J. and Millen, K. J. (2015). [Mouse models of human PIK3CA-related brain overgrowth have acutely treatable epilepsy](#). *eLife* 4: e12703.

Total RNA Isolation after Laser-capture Microdissection of Human Cervical Squamous Epithelial Cells from Fresh Frozen Tissue

Saskia M Wilting* and Renske DM Steenbergen

Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands

*For correspondence: s.wilting@vumc.nl

[Abstract] As most tissue specimens contain a mixture of different cell types including epithelial cells, stromal cells and immune cells, selection of the cells of interest is of utmost importance for the accurate determination of gene/microRNA expression. Laser capture microdissection enables the researcher to obtain homogeneous ultrapure cell selections from heterogeneous starting material. The following protocol was optimized for the isolation of total RNA from cervical (premalignant) squamous epithelial cells from fresh frozen biopsy specimens.

Materials and Reagents

1. Fresh frozen tissue specimens stored in liquid nitrogen
2. Tissue-Tek O.C.T. (Sakura Finetek Europe B.V., catalog number: 4583)
3. Mayers' Haematoxylin
4. Ethanol
5. Xylene
6. Liquid nitrogen or dry ice
7. TRIzol (Life Technologies, catalog number: 15596-026) or a preferred alternative RNA isolation method
8. Glycogen (F. Hoffmann-La Roche, catalog number: 10901393001)
9. Chloroform
10. Isopropanol (Isopropyl alcohol)
11. RNase-free water

Equipment

1. 21-26 gauge needle
2. PEN foil 2.0 UM covered slides (Leica, catalog number: 11505158)
3. 50 ml tubes with screw cap (Greiner Bio-one, catalog number: 210261)
4. Cryotome (rotary microtome in a frozen section environment)
5. Leica Laser Microdissection system (AS LMD, model: LMD6500 or LMD7000)

6. Centrifuge
7. -80 °C freezer for sample storage

Procedure

1. Take all necessary precautions for RNA handling throughout the whole procedure
 - a. Wear gloves at all times.
 - b. Only use RNase free equipment, plastics and reagents.
2. Pretreat the PEN foil slides with UV light for 15 min to reduce static electricity.
Note: We use a UV source of 50 Hz and 26 Watt.
3. Cut 8-10 µm thick sections from the frozen tissue specimens (embedded in Tissue-Tek) and apply sections directly to the PEN foil covered side of the slide.
Notes:
 - a. More than one section can be applied to the same slide depending on the size of the specimen.
 - b. Make sure that all sections are within the rectangle where the foil is not glued to the slide.
 - c. Keep sections dry and cold at all times (store in a closed 50 ml tube at -80 °C and transport in liquid nitrogen or on dry ice).
 - d. In addition a 4 µm section for standard H&E staining can be made to allow for demarcation of the tissue area/cells of interest by a pathologist or other expert.
4. Slides containing tissue sections should be stored in a closed 50 ml tube and kept cold at all times (store at -80 °C and transport in liquid nitrogen or on dry ice).
Note: The use of 50 ml tubes avoids condensation on the slide thereby keeping the tissue dry which reduces RNA degradation.
5. Stain sections with Mayers' Haematoxylin for 1 min at room temperature (RT).
Notes:
 - a. Before staining let slides come to RT while still in the closed 50 ml tube.
 - b. Be careful not to lose the tissue section. Staining is best done by pipetting the haematoxylin directly on the slide.
6. Carefully rinse slide in sterile (RNase free) water.
7. Dehydrate tissue sections by subsequent rinsing for 1 min each in 50%, 70% and 100% ethanol (can be done in 50 ml tubes) and an 8 min incubation in Xylene (use a glass container).
8. Store slides in a closed 50 ml tube at -80 °C or continue with the microdissection.
9. Add a little (10-20 µl) TRIzol to the tube cap in which the microdissected tissue will fall.
Notes:
 - a. Let slides come to room temperature while in the 50 ml tube right before starting the microdissection procedure for that slide.

- b. If an alternative RNA isolation method is preferred, TRIzol should be substituted by the first reagent used in that method.
10. Microdissect all areas/cells of interest from the slide using at a 10x magnification (see Figure 1) using the following settings:
- Intensity: 46
Speed: 5 (or lower)
Offset: 5
Aperture diff: 6

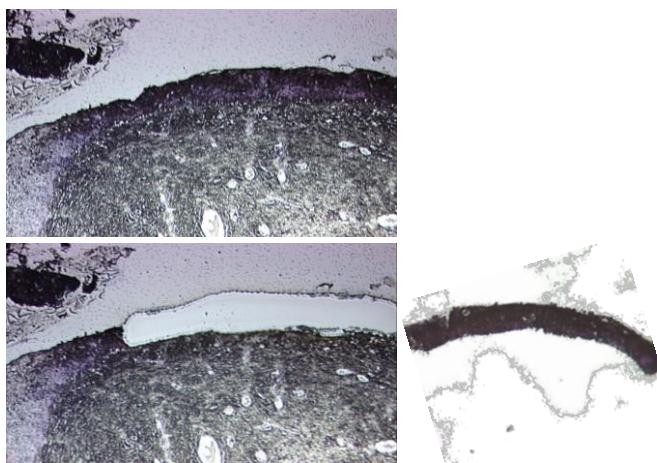


Figure 1. Example picture of a cervical tissue section before (upper panel) and after (lower panel) laser capture microdissection of the dysplastic epithelium. The microdissected tissue in the cap is shown in the left panel.

11. Add another 80-90 µl of TRIzol (or alternative isolation reagent) to the 0.2 ml tube containing the microdissected tissue and transfer tissue and TRIzol/alternative isolation reagent to a 1.5 ml tube.
Note: If preferred by the researcher an alternative RNA isolation method can be used. However, this protocol was optimized using TRIzol.
12. Add TRIzol to the tube to obtain a total volume of 1 ml.
Note: Store at -80 °C or continue with RNA isolation.
13. Add 1 µl glycogen and mix well (vortex for 10 seconds).
14. Disrupt the tissue/cells and shear the genomic DNA with 10 passes through a 21-26 gauge needle.
15. Add 0.2 ml of chloroform and vortex for 30 sec.
16. Spin down for 5 min at full speed (12,000 x g) at RT.
17. Transfer the aqueous phase to a clean tube.
18. Add 0.5 ml of isopropanol to the aqueous phase and mix well.
19. Incubate samples for at least 1 h at -20 °C for optimal precipitation of the RNA.
20. Spin down for 15 min at full speed (12,000 x g) at RT.

21. Discard supernatant
-
22. Wash pellet with 200 µl of 70% ethanol.
23. Spin down at full speed for 10 min at RT.
24. Discard supernatant.
25. Repeat steps 20-22.

Note: Careful not to disturb the pellet containing your RNA.

26. Airdry the pellet.
-
27. Resuspend the pellet in 10-30 µl RNase free water.
28. Store at -80 °C.

Note: This time it is important to completely remove the supernatant.

Note: If all supernatant is removed in step 23 this should only take a few minutes.

Acknowledgments

This protocol was optimised with the help of Elza de Bruin (de Bruin *et al.*, 2005) and Muriel Verkijten. This work was supported by the Centre for Medical Systems Biology (CMSB) in the framework of the Netherlands Genomic Initiative, Royal Netherlands Academy of Arts and Sciences, the VUMC-CCA institute of the VU University Medical Center, Amsterdam, The Netherlands (grant number CCA20085-04) and the Dutch Cancer Society (KWF, grant number VU2010-4668).

References

1. de Bruin, E. C., van de Pas, S., Lips, E. H., van Eijk, R., van der Zee, M. M., Lombaerts, M., van Wezel, T., Marijnen, C. A., van Krieken, J. H., Medema, J. P., van de Velde, C. J., Eilers, P. H. and Peltenburg, L. T. (2005). [Macrodisection versus microdissection of rectal carcinoma: minor influence of stroma cells to tumor cell gene expression profiles](#). *BMC Genomics* 6: 142.
2. Wilting, S. M., de Wilde, J., Meijer, C. J., Berkhof, J., Yi, Y., van Wieringen, W. N., Braakhuis, B. J., Meijer, G. A., Ylstra, B., Snijders, P. J. and Steenbergen, R. D. (2008). [Integrated genomic and transcriptional profiling identifies chromosomal loci with altered gene expression in cervical cancer](#). *Genes Chromosomes Cancer* 47(10): 890-905.
3. Wilting, S. M., Snijders, P. J., Verlaat, W., Jaspers, A., van de Wiel, M. A., van Wieringen, W. N., Meijer, G. A., Kenter, G. G., Yi, Y., le Sage, C., Agami, R., Meijer, C. J. and Steenbergen, R. D. (2013). [Altered microRNA expression associated with chromosomal changes contributes to cervical carcinogenesis](#). *Oncogene* 32(1): 106-116.

Laser Capture Microdissection (LCM) of Human Skin Sample for Spatial Proteomics Research

Qiyu Zhang^{1,3, #}, Huizi Gong^{1,3, #}, Jie Ma², Jun Li¹, and Ling Leng^{3, *}

¹Department of Dermatology and Venereology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

²State Key Laboratory of Proteomics, Beijing Proteome Research Center, National Center for Protein Sciences (Beijing), Beijing Institute of Lifeomics, Beijing, China

³Stem Cell and Regenerative Medicine Lab, State Key Laboratory of Complex Severe and Rare Diseases, Translational Medicine Center, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

*For correspondence: lengling@pumch.cn

#Contributed equally to this work

Abstract

In mammals, the skin comprises several distinct cell populations that are organized into the following layers: epidermis (stratum corneum, stratum granulosum, stratum spinosum, and basal layer), basement membrane, dermis, and hypodermal (subcutaneous fat) layers. It is vital to identify the exact location and function of proteins in different skin layers. Laser capture microdissection (LCM) is an effective technique for obtaining pure cell populations from complex tissue sections for disease-specific genomic and proteomic analysis. In this study, we used LCM to isolate different skin layers, constructed a stratified developmental lineage proteome map of human skin that incorporates spatial protein distribution, and obtained new insights into the role of extracellular matrix (ECM) on stem cell regulation.

Keywords: Skin, Laser capture microdissection (LCM), Spatial proteomics, Stem cell, Extracellular matrix (ECM)

This protocol was validated in: Nat Commun (2022), DOI: 10.1038/s41467-022-31659-9

Background

Skin, the largest barrier organ in the body, has a tough and pliable structure to adapt to external conditions by quickly repairing mechanical, chemical, and biological injuries. In mammals, the skin consists of several distinct layers: the epidermal, dermal, and subcutaneous fat layers. The epidermis is the outermost layer, with stratified cell layers maintained by keratinocytes including stem cells and an abundance of mature cells (Gonzales and Fuchs, 2017). The basal layers of the epidermis, which express the keratins KRT5 and KRT14, are the location of undifferentiated proliferative epidermal stem cells (Blanpain and Fuchs, 2006). The progenitor cells replenish basal layers and differentiate into mature keratinocytes in the granulosum and spinosum layers, which express KRT1, KRT10, and involucrin. Stratum corneum, the outermost layer, consisting of terminally differentiated and dead cells, acts as a scaffold for the lipid bilayers that comprise the epidermal barrier on the skin surface (Fuchs, 2007; Koster and Roop, 2007). Keratins, which constitute the cytoskeleton of epithelial cells, are highly expressed in the epidermis, especially in the stratum corneum (Li et al., 2022). In the epidermis, the transition of keratinocytes from the proliferative basal to the supra-basal cell layer during terminal differentiation and keratinization is characterized by keratin expression transition from basal cell keratins (KRT5, KRT14, and KRT15) to supra-basal cell keratins (KRT1 and KRT10) (Moll et al., 2008). The dermis provides nourishment and support for the skin and contains an abundance of extracellular matrix (ECM) proteins including collagens, glycosaminoglycans, hyaluronic acid, fibronectin, elastin, and laminin (Nyström and Bruckner-Tuderman, 2019). Basement membrane, a specialized layer of ECM proteins connecting the dermis and epidermis, serves as an important microenvironment for basal stem cells; understanding its composition is crucial to the study of basal stem cell fate and function. However, there is a lack of detailed information about the molecular composition and regulatory function of specialized proteins localized in different skin layers that are difficult to separate, especially the basement membrane zone, during the dynamic processes of skin development, homeostasis, and regeneration for wound healing (Dengjel et al., 2020; Dyring-Andersen et al., 2020).

As described above, the skin is a complex tissue composed of heterogeneous cell types with different spatial distributions. Studying the unique physiological and pathological functions of different spatially distributed cell types is essential for understanding the molecular characteristics of human skin. Laser capture microdissection (LCM) is a powerful technique for separating target cell populations with extremely high microscopic precision, thus providing a perfect solution to the problem of skin tissue heterogeneity. Here, we describe a protocol for the isolation of different skin layers by LCM for spatial proteomics research (Li et al., 2022). Using a combination of previously developed tissue engineering decellularization methods, dedicated to the removal of epidermis and separation of basement membrane (Leng et al., 2020, Liu et al., 2020), LCM, and mass spectrometry (MS), we isolated proteins from six skin layers (stratum corneum, stratum granular-spinous, basal layer, basement membrane, superficial dermis, and deep dermis) and constructed a stratified developmental lineage proteome map of human skin. By obtaining different skin samples, the protocol enables the analysis of spatial protein expression in normal and disease-specific skin tissues, which is important for understanding the pathogenesis of different skin diseases and discovering potential therapeutic targets.

Materials and Reagents

1. MMI MembraneSlides™ (MMI GmbH, catalog number: 50102)
2. MMI IsolationCaps transparent, 0.5 mL (MMI GmbH, catalog number: 50204)
3. Phospholipase A2 (Sigma-Aldrich, catalog number: P6534)
4. Sodium deoxycholate (Sigma-Aldrich, catalog number: V900388)
5. PBS (TBD Science, catalog number: PB2004Y)
6. EDTA (Sigma-Aldrich, catalog number: E9884)
7. NaCl (Sigma-Aldrich, catalog number: S9888)
8. DNase (Sigma-Aldrich, catalog number: 11284932001)
9. RNase (Sigma-Aldrich, catalog number: 10109134001)
10. OCT compound (SAKURA, catalog number: 4583)

Cite as: Zhang, Q. et al. (2023). Laser Capture Microdissection (LCM) of Human Skin Sample for Spatial Proteomics Research. Bio-protocol 13(05): e4623. DOI: 10.21769/BioProtoc.4623.

11. 4% paraformaldehyde (BOSTER Biological Technology, catalog number: AR1068)
12. Sucrose (Sigma-Aldrich, catalog number: V900116)
13. Delipidation solution (see Recipes)
14. DNase-RNase solution (see Recipes)
15. 3.4 M NaCl solution (see Recipes)
16. 30% sucrose solution (see Recipes)

Equipment

1. Laser microdissection system (MMI, cellcut plus)
2. Freezing microtome (Leica, model: CM1950)
3. Shaker (tcsysb, THZ-C)
4. Scalpel (BELEVOR MEDICAL, catalog number: 03.0030.01)

Procedure

A. Obtain native frozen skin tissue sections

1. Rinse skin tissue three times with cold PBS.
2. Fix tissue with 4% paraformaldehyde at room temperature for 24 h.
3. Perform dehydration with 10 mL of 30% sucrose solution at 4 °C until the tissue sinks to the bottom.
4. Freeze at -80 °C and then embed the skin tissues. Apply a layer of OCT compound in the groove of the embedding mold and place the tissue section down into the groove. Fill with embedding agent (OCT compound) and put the sample on the freezer until the embedding agent solidifies.
5. Cut 20-μm-thick sections of native skin tissues. Place the MembraneSlides™ (with the *MMI* logo face up) close to the slice, which will stick to it. Reverse the MembraneSlides™, gently press them at the corresponding position below the slice with your fingers, and the sample will completely adhere to the MembraneSlides™.
6. Mount sections on MMI MembraneSlides™ and store at -20 °C.

B. Obtain decellularized frozen skin tissue sections

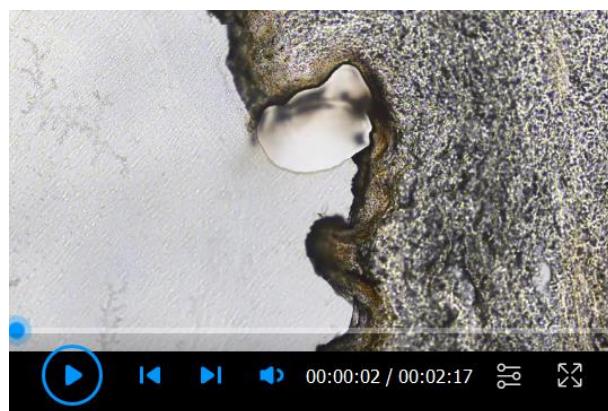
1. Rinse skin tissue three times with cold PBS containing 0.1% EDTA.
2. Perform delipidation by treating tissue with 25 mL of delipidation solution (see Recipes) for 4 h in a shaker at 37 °C until the tissue segments become oyster white.
3. Gently scrap the surface of the skin with the back of the scalpel to remove the epidermis.
4. Rinse the decellularized dermal scaffolds with 3.4 M NaCl (see Recipes) at 37 °C for 1 h.
5. Wash the samples with DNase-RNase solution (see Recipes) at 37 °C for 1 h.
6. Rinse skin tissue three times with cold PBS.
7. Fix tissue with 4% paraformaldehyde at room temperature for 24 h.
8. Perform dehydration with 10 mL of 30% sucrose solution in a 15 mL centrifuge tube at 4 °C until the tissue sinks to the bottom.
9. Freeze and embed the skin tissues as in step A2.
10. Cut 20-μm-thick sections of decellularized skin tissues as in step A3.
11. Mount sections on MMI MembraneSlides™ and store slides at -20 °C.

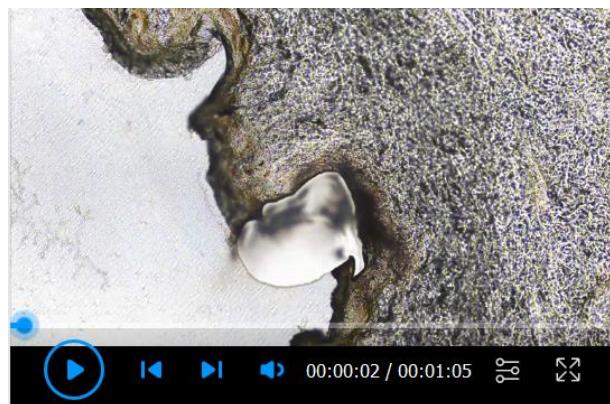
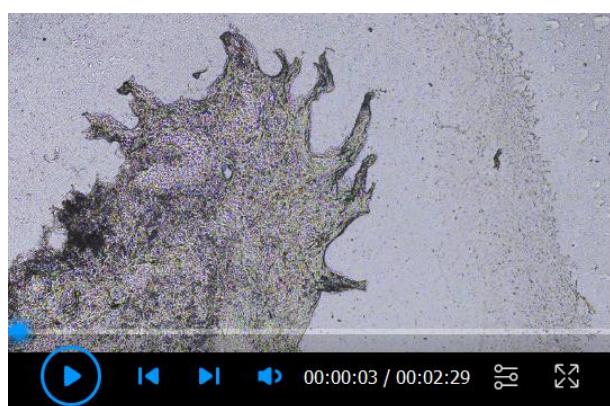
C. Laser capture microdissection (LCM) of frozen skin sections

1. Open LCM system. Install MembraneSlides and IsolationCaps transparent.
2. Switch to 4× objective and start slide scan and navigation.
3. Switch to 10× objective for subsequent dissection.
4. Set laser position indicator. Click the button to fire the laser and check whether the laser position matches the green cross. If not, click “CellCut” – “Laser” – “Set Laser Position” and click at the location where the laser is emitted.
5. Calibrate the parameters for the laser. Select the freehand tool, draw a long line on the empty area without tissues on the slides, and cut. Adjust “Cut velocity,” “Laser focus,” and “Laser power” to make the cutting line clear and precise.
6. Move to the target position, draw cut lines, and start cutting. Increase the cut velocity, cut power, and number of cuts appropriately to get the best cutting efficiency.
7. Pick up the sample under manual mode. Click the collecting button to attach the cutout to the EP tube cover and check if the tissue was successfully collected. If not, repeat collection or try to adjust the position of the EP tube and even recut.
8. Repeat steps C6 and C7 to isolate stratum corneum, granulosum-spinosum, basal layer, superficial dermis, and deep dermis on native sections and basement membrane on decellularized sections successively.
9. Check the entire slice to ensure that the target area is cut and collected.
10. Label the samples and store them at -20 °C for subsequent MS analysis.

Notes

1. To obtain a clearer view of tissue structure when cutting, hematoxylin-eosin staining can be performed on the sections.
2. The LCM systems allow simultaneous cutting of the same structure for up to 3–4 slices and collection of multiple structures with multiple tubes by using the group function.
3. When the sample is difficult to be dissected or collected by the EP tube cover, first check if the objective magnification is correct. Then, optimize laser parameters, such as increasing laser power or slowing down the cutting speed, and repeat the cuts. Finally, move the position of the EP tube cover to an area with less adherent samples or replace the collection tube with a new one.
4. The cutting circle should not be too small, or the tissue will be easily repelled away by the laser. Also, the cutting line should not be too long, or the tissue will break during collection.
5. For laser parameters calibration, first decrease “Cut velocity” and “Laser power” to a low value (as 5 µm/s and 5%) and cut. Then, change “Laser focus” until the cutting line becomes clear and spacious. Finally, decrease the “Laser power” to get a fine line and change the “Laser focus” again to find the finest line. After calibration, increase the “Cut velocity” to a high value (as 70 µm/s) and increase the “Laser power” accordingly to get a fast and precise cutting line. For best cutting efficiency, change “Cut velocity” and “Laser power” according to different tissue locations and thickness.
6. Videos 1–5 show the continuous process of cutting the stratum corneum, stratum granulosum-spinosum, basal layer, superficial dermis, and deep dermis of the native skin tissue sections from outside to inside. Video 6 shows the process of cutting the basement membrane of the decellularized skin tissue sections.

**Video 1. Isolation of stratum corneum****Video 2. Isolation of stratum granulosum-spinosum****Video 3. Isolation of basal layer**

**Video 4. Isolation of superficial dermis****Video 5. Isolation of deep dermis****Video 6. Isolation of basement membrane**

Recipes

1. Delipidation solution

- a. Dissolve 10 g of sodium deoxycholate powder in 1 L of deionized water.
- b. Add phospholipase A2 to above solution before delipidation treatment to make a final concentration of 2,000 U/L.

2. DNase-RNase solution

Dissolve 1 mg of DNase powder and 0.5 mg of RNase powder in 100 mL of PBS buffer before use.

3. 3.4 M NaCl solution

- a. Dissolve 198.9 g of NaCl powder with 800 mL of deionized water.
- b. After dissolving, fill with deionized water to 1 L.

4. 30% sucrose solution

- a. Dissolve 300 g of NaCl powder with 800 mL of deionized water.
- b. After dissolving, fill with deionized water to 1 L.

Acknowledgments

This work was supported by Beijing Municipal Science and Technology Commission (Z191100006619011), Capital's Funds for Health Improvement and Research (2020-2-4016), CAMS Innovation Fund for Medical Sciences (CIFMS 2020-I2M-C&T-B-048), and National Science and Technology Major Project (2021YFA1301603).

This protocol was adapted from Li et al. (2022).

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics

The authors declare no conflict with respect to ethical grounds.

References

- Blanpain, C. and Fuchs, E. (2006). [Epidermal stem cells of the skin](#). *Annu Rev Cell Dev Biol* 22: 339-373.
- Dengjel, J., Bruckner-Tuderman, L. and Nystrom, A. (2020). [Skin proteomics - analysis of the extracellular matrix in health and disease](#). *Expert Rev Proteomics* 17(5): 377-391.
- Dyring-Andersen, B., Lovendorf, M. B., Coscia, F., Santos, A., Moller, L. B. P., Colaco, A. R., Niu, L., Bzorek, M., Doll, S., Andersen, J. L., et al. (2020). [Spatially and cell-type resolved quantitative proteomic atlas of healthy human skin](#). *Nat Commun* 11(1): 5587.
- Fuchs, E. (2007). [Scratching the surface of skin development](#). *Nature* 445(7130): 834-842.

- Li, J., Ma, J., Zhang, Q., Gong, H., Gao, D., Wang, Y., Li, B., Li, X., Zheng, H., Wu, Z., et al. (2022). [Spatially resolved proteomic map shows that extracellular matrix regulates epidermal growth](#). *Nat Commun* 13(1): 4012.
- Moll, R., Divo, M. and Langbein, L. (2008). The human keratins: biology and pathology. *Histochem Cell Biol* 129(6): 705-733.
- Gonzales, K. A. U. and Fuchs, E. (2017). [Skin and Its Regenerative Powers: An Alliance between Stem Cells and Their Niche](#). *Dev Cell* 43(4): 387-401.
- Koster, M. I. and Roop, D. R. (2007). [Mechanisms regulating epithelial stratification](#). *Annu Rev Cell Dev Biol* 23: 93-113.
- Leng, L., Ma, J., Sun, X., Guo, B., Li, F., Zhang, W., Chang, M., Diao, J., Wang, Y., Wang, W., Wang, S., Zhu, Y., He, F., Reid, L. M. and Wang, Y. (2020). [Comprehensive proteomic atlas of skin biomatrix scaffolds reveals a supportive microenvironment for epidermal development](#). *J Tissue Eng* 11: 2041731420972310.
- Liu, B., Zhang, S., Wang, W., Yun, Z., Lv, L., Chai, M., Wu, Z., Zhu, Y., Ma, J. and Leng, L. (2020). [Matrisome Provides a Supportive Microenvironment for Skin Functions of Diverse Species](#). *ACS Biomaterials Science & Engineering* 6(10): 5720-5733.
- Nyström, A. and Bruckner-Tuderman, L. (2019). [Matrix molecules and skin biology](#). *Semin Cell Dev Biol* 89: 136-146.

Protocol to Isolate Germinal Centers by Laser Microdissection

Farbod Bahreini, Markus Niebuhr, Julia Belde, Jürgen Westermann and Kathrin Kalies*

Institute of Anatomy, University of Luebeck, Luebeck, Germany

*For correspondence: kathrin.kalies@uni-luebeck.de

[Abstract] During adaptive immune responses, germinal centers (GC) appear as transient microstructures, in which antigen-specific B and T cells interact with each other. Because only the antigen-activated B and T cells, such as Plasmablasts or follicular T helper (Tfh) cells, are present in GC, the in depth-analysis of GC is of great interest. To identify the cells that reside within GC, the majority of studies use the expression of specific surface molecules for analysis by flow cytometry. To do so, the tissue has to be disrupted for the preparation of single-cell suspensions. Thereby, the local information regarding neighborhoods of B cells and T cells and their potential interaction is lost. To study GC *in vivo* within their original microenvironment, we established a protocol for the isolation of GC by laser microdissection. To enable the identification of GC for subsequent transcriptomic analysis, the degradation of mRNA was diminished by using frozen tissues and by establishing a rapid staining protocol. This procedure enables histological and transcriptomic analysis of individual GC even within one lymphoid organ.

Keywords: Secondary lymphoid organs, Germinal centers, Follicular T helper cells, Laser microdissection, Cryo-preserved lymphoid structures, *In vivo* analysis

[Background] One hallmark of secondary lymphoid organs is their strict compartmentalization into B and T cell zones. In an inactive stage, B cells and T cells are separated from each other; while B cells populate in B cell follicles, T cells accumulate in T cell zones (Gasteiger *et al.*, 2016). This spatial segregation breaks down upon antigen encounter. Especially those B and T cells that are specific for the given antigen initiate the formation of GC within the B cell follicles (Qi, 2016). GCs are the predominant site of antibody gene somatic hypermutation and are therefore crucially important for human health by enabling the production of high-affinity antibodies against pathogens. They are the result of a well-controlled sequence of events during T dependent humoral immune responses, in which initially rare antigen-engaged B cells encounter rare cognate antigen-specific T cells to eventually generate antibody-producing long-lived plasma cells and memory B cells. GC are histologically visible, transient microstructures that contain a dark zone, a light zone, and a surrounding mantle zone (Allen *et al.*, 2007). Numerous GC emerge upon antigen exposure within secondary lymphoid organs. They are formed mainly from proliferating B cells, which undergo somatic hypermutations and class-switches in the dark zone. B cells bearing a newly formed B cell receptor migrate from the dark zone to the light zone, where they compete with other B cell clones for antigen and T cell help (Cyster and Allen, 2019). T cell help is provided by Tfh cells, which are licensed to enter GC by expression of the chemokine receptor CXCR5 (Vinuesa *et al.*, 2016).

Here, we describe a protocol for the isolation of GC-B cells and GC-Tfh cells within individual GC by laser microdissection (Niebuhr *et al.*, 2021). By keeping the microarchitecture intact and by using a short nuclear staining protocol that preserves the RNA at a high quality, this protocol has the advantage that it enables the analysis of gene expression *in vivo* and the usage of conserved tissues (especially cryopreserved) from former

experiments. Of note, gene expression and immune receptor repertoires from each individual GC within one lymph node can be analyzed and compared. Until today, it is not known how diverse the T-and B cell responses within individual GC of one lymph node are and whether potential high or low diversities between GC would impact the affinity of the antibodies produced. Of note, this protocol is not restricted to GC and can be used to isolate GC-adjacent and/or non-adjacent T and B cell zones in addition (Kalies *et al.*, 2008).

Materials and Reagents

1. Reaction tubes, 1.5 mL, safe seal, (Sarstedt, catalog number: 72.706.400)
2. Coverslips thickness 1.24 × 60 mm Gerhard Menzel GmbH (Carl Roth, catalog number: H878.2)
3. Superfrost Plus Microscope Glass Slides (Thermo Scientific, catalog number: J1800AMNZ), store at room temperature (RT)
4. Membranslide 1.0 PEN (Carl Zeiss AG, catalog number: 415190-9041-000), store at RT
5. Microtube 500 (D) (Carl Zeiss AG, catalog number: 415190-9221-000)
6. Mineral oil (Trinity Biotech, catalog number: 400-5), store at RT
7. 0.2 µm syringe filter (Macherey-Nagel, catalog number: 729022), store at RT
8. Acetone 99.8% (Carl Roth, catalog number: 9372.5), store at RT in a ventilated place
9. Aquatex (Merck, catalog number: 1.08562.0050), store at 15–25°C
10. Biotin hamster anti-mouse TCRβ chain (clone H57-597) (BD Biosciences, catalog number: 553169), store undiluted at 4°C
11. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A2153), store at 2–8°C
12. Chloroform 99% (Carl Roth, catalog number: Y015.1), store at RT in a ventilated place
13. di-Sodiumhydrogenphosphate dodecahydrate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) (Merck, catalog number: 1.06579.1000), store at RT
14. Ethanol 99.8% (Carl Roth, catalog number: 9065.4), store at RT
15. ExtrAvidin Alkaline Phosphatase (Sigma-Aldrich, catalog number: E2636), store in the dark at 2–8°C
16. ExtrAvidin Peroxidase (Sigma, catalog number: E2886), store in the dark at 2–8°C
17. Fast Blue BB salt (Sigma-Aldrich, catalog number: F3378), store at -20°C
18. Fast Red TR salt (Sigma-Aldrich, catalog number: 368881), store at RT
19. Liquid DAB+ Substrate Chromogen System (Agilent Technologies, catalog number: K3468), store in the dark at 2–8°C
20. Methanol 99.9% (Carl Roth, catalog number: 4627.5), store at RT in a ventilated place
21. Normal mouse serum (Invitrogen, catalog number: 10410), store at -20°C
22. Paraformaldehyde (Applichem, catalog number: A3813,1000), store at 4°C
23. Purified Rat anti-mouse Ki-67 (clone 16A8) (BioLegend, catalog number: 652402), store at 2–8°C
24. Purified Rat Anti-Mouse CD45R/B220 (clone RA3-6B2) (BD Biosciences, catalog number: 553084), store in the dark at 2–8°C
25. Rabbit Anti-Rat IgG (H+L), Human ads-BIOT (Southern Biotech, catalog number: 6185-08), store at 2–8°C
26. Biotinylated Hamster anti-mouse TCRβ antibody (clone H57-597) (BD Biosciences, catalog number: 553169), store undiluted at 4°C

store undiluted at 4°C

27. Sodium azide (NaN₃) (Sigma-Aldrich, catalog number: S2002S8032), store at RT in a dark and well-ventilated place
28. Sodium Chloride (NaCl) (Carl Roth, catalog number: 3957.1), store at RT
29. Sodium dihydrogen phosphate monohydrate (NaH₂PO₄·H₂O) (Merck, catalog number: 1.06346.1000), store at RT
30. Naphthol AS-MX phosphate (Sigma-Aldrich, catalog number: N4875), store at -20°C
31. Dimethylformamide (SERVA, catalog number: 20270), store at room temperature
32. (-)-Tetramisole hydrochloride (Levamisole) (Sigma-Aldrich, catalog number: L9756), store at 2–8°C
33. Tissue freezing medium (Leica, catalog number: 14020108926), store at RT
34. Toluidine blue (Fluka, catalog number: 89640), store at RT
35. Tris base (Sigma-Aldrich, catalog number: T1503), store at RT
36. Tween 20 (Merck, catalog number: 8.22184.0500), store at RT
37. Dako Pen (DAKO, catalog number: 5200230-2)
38. Antibody solution (see Recipes)
39. Tris Buffer (0.1 M) (see Recipes)
40. Alkaline Phosphatase - Anti-Alkaline Phosphatase (APAAP) substrate (see Recipes)
41. Fast Blue solution (see Recipes)
42. Fast red solution (see Recipes)
43. PBS (phosphate-buffered saline) (see Recipes)
44. PFA 4% (paraformaldehyde) (see Recipes)
45. TBS-Tween (Tris-buffered Saline-Tween) (see Recipes)
46. Toluidine blue 1% solution (see Recipes)

Equipment

1. Laser microdissection system (Carl Zeiss AG, model: PALM Microbeam Laser microdissection system).
2. Transmitted light microscope (Leitz, model: Laborlux 11)
3. pH211 Microprocessor pH Meter (HANNA Instruments)
4. Refrigerator and freezer
5. Stand-alone UV light lamp holder for ultraviolet germicidal lamp G30W T8 (Sylvania, catalog number: 0000518)
6. Vortex (Scientific Industries, model: Vortex-2 Genie)
7. LEICA® manual microtome (LEICA, model: CM3050S)
8. Eppendorf® microcentrifuge (Eppendorf, model: 5417R)
9. Heating and Magnetic Stirrer (Roth, model: MH 15)

Software

1. Palm Robo software, Version 4.8.0.1 (Carl Zeiss AG)

Procedure

A. Preparation of lymphoid organs for isolation of GC by laser microdissection

To enable transcriptional analysis, it is required to isolate GC while preserving mRNA at a high quality. Therefore, activated lymph nodes were fixed by snap-freezing in liquid nitrogen immediately after harvest and removal of adipose tissues (Figure 1A). Subsequently, tissues are stored in a -80°C refrigerator. Cryosections are prepared at -20°C using a cryomicrotome without allowing the tissue to thaw. To minimize the loss of RNA, two stainings are required to accurately identify GC: a short staining protocol with toluidine blue that preserves the mRNA and an immunohistochemical staining that allows the identification of GC and Tfh cells. To compare both stainings, serial cryosections were prepared on two slides: (i) a membrane slide for toluidine blue-staining and for laser microdissection; and (ii) a glass slide for immunohistochemical staining to visualize GC, T cells, and B cells (Figure 1B–1C and Figure 2). Of note, to preserve the mRNA, the incubation of the tissue sections in aqueous solutions should be as short as possible, such as the 2-2-2 min washing-toluidine blue-washing incubation described here (steps e–g). Because RNases lose most of their activity in dehydrated dried tissues (step h), the slides can be stored before further processing (step i).

For troubleshooting, to optimize the staining protocol and to test for RNA integrity, it is recommended to cut one whole section from the membrane slide before and after staining and compare RNA concentrations (either by OD measurements or by PCRs).

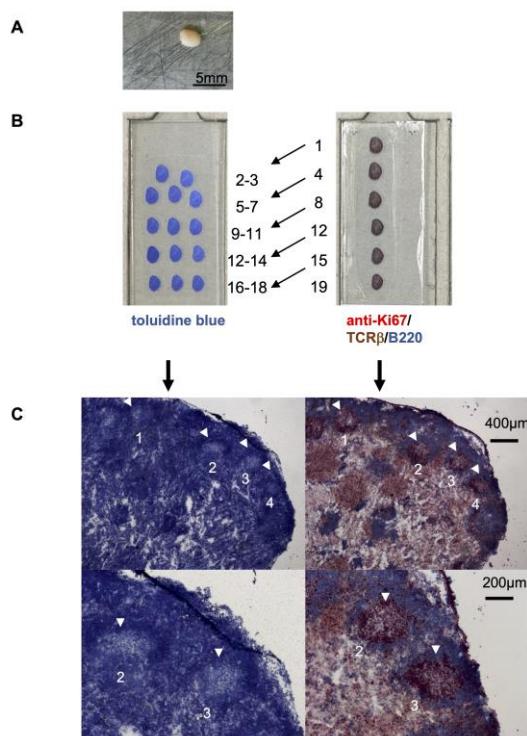


Figure 1. Preparation of lymph nodes for isolation of GC by laser microdissection. A. Activated murine

popliteal lymph node. The surrounding adipose tissue was removed carefully before snap freezing. B. Serial cryosections of lymph nodes distributed on two slides, which were either stained with toluidine blue (left slide) or with anti-Ki67 (red) for proliferating cells, with anti-TCR β for T cells (brown) and with anti-B220 for B cells (blue) (right side). The numbers indicate the order of serial sections. C. Example of two adjacent sections shown at higher magnifications. Toluidine blue-stained lymph node sections are shown on the left. Four GC are marked by white arrowheads (upper panel). Two GC are shown at a higher magnification on the lower panel. The immunohistochemical staining of the adjacent section on the right side confirms the identified GC (see Figure 2).

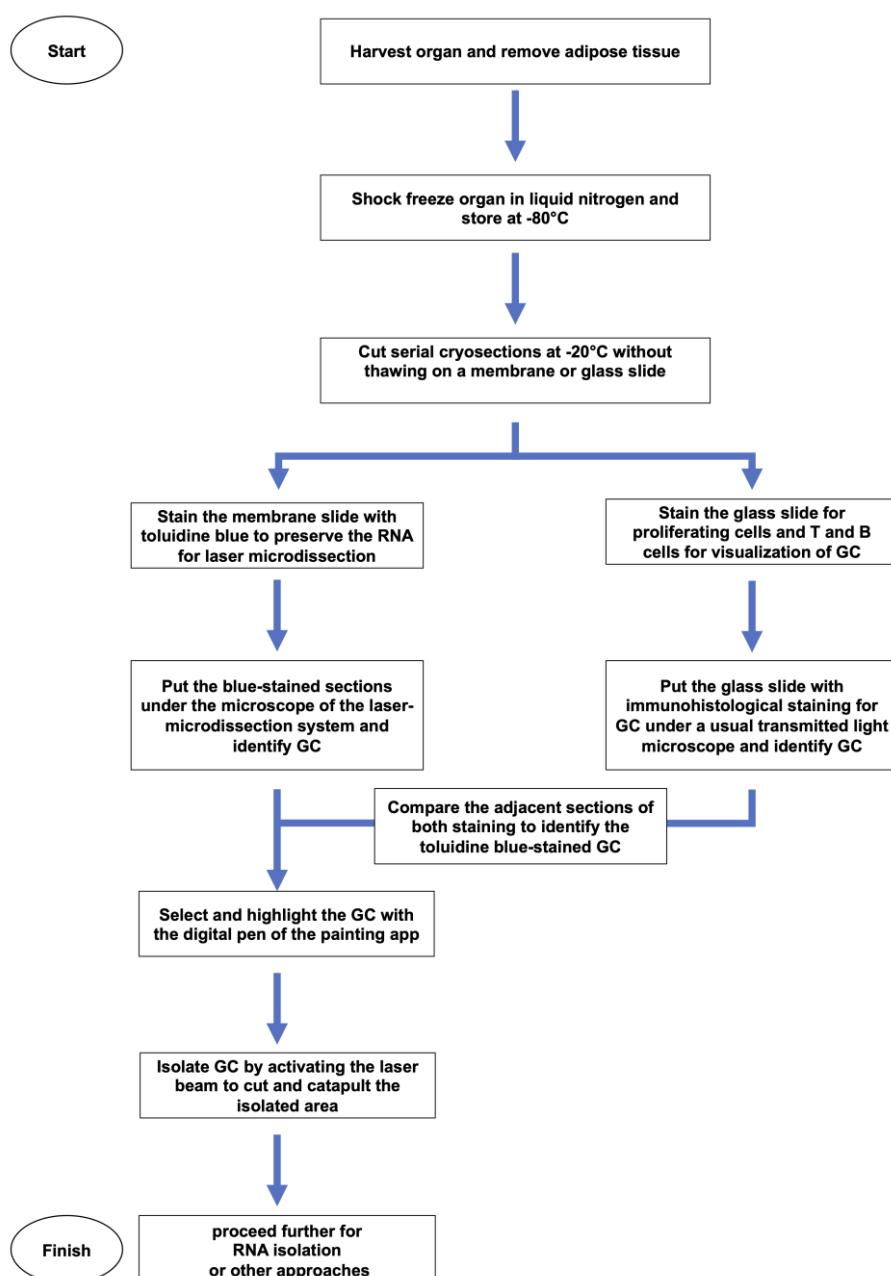


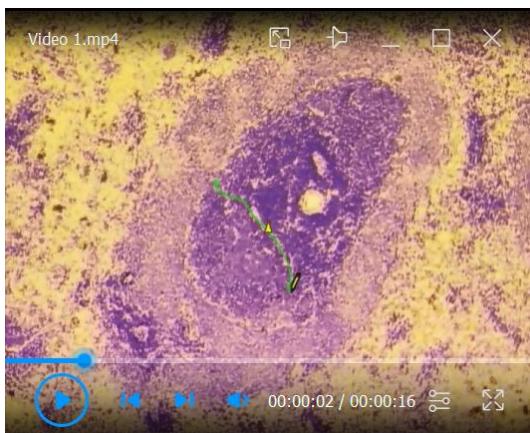
Figure 2. Workflow showing how the tissue is treated and processed in detail.

1. Staining of cryosections on membrane slides with toluidine blue for laser microdissection
 - a. Optional: Expose membrane slides to UV light for 30 min at a distance of 400 mm for disinfection and to improve stickiness for tissue sections.
 - b. Embed collected frozen lymph nodes in tissue freezing medium and cut until a plain cut-level is present.
 - c. Cut 12 μm -sections of lymph nodes using a cryomicrotome and mount several individual sections on UV-treated membrane slides and on glass slides (Figure 1).
 - d. Fix tissues in 75% ethanol for 10 min.
 - e. Rinse membrane slides in laboratory-grade H₂O for 2 min.
 - f. Stain tissue by applying 0.1% toluidine blue for 2 min.
 - g. Rinse two times in laboratory-grade H₂O for 2 min.
 - h. Dehydrate tissues by incubating in 96% ethanol for 15 s.
 - i. Use slides immediately or store them at -80°C until further processing.
2. Staining of cryosections for GC, T, and B cells on glass slides
 - a. Take the serial sections that have been mounted on glass slides.
 - b. Dry slides at RT for at least 1 h.
 - c. To fix tissues, incubate glass slides in chloroform for 10 min and acetone for 10 min. Afterwards, rinse tissues in TBS-Tween for 15 min and cover the lymph node section with 4% PFA and incubate them at 4°C for 45 min.
 - d. Rinse glass slides in TBS-Tween for 10 min and draw a circle around each tissue section with a Dako Pen to confine the liquids during staining.
 - e. Incubate sections with the **first** primary antibody anti-mouse Ki-67 at ambient temperature overnight (1:100 dilution in antibody solution).
 - f. Wash unbound antibodies away by incubation with TBS-Tween for 10 min and add the first secondary biotinylated anti-rat IgG antibody (1:500 dilution in PBS with 5% normal mouse serum) for 30 min.
 - g. Rinse with TBS-Tween for 30 min and cover tissue sections with ExtrAvidin Alkaline Phosphatase (1:100 in TBS-Tween) for 30 min.
 - h. Rinse with TBS-Tween for 10 min and apply Fast Red staining solution for 25 min.
 - i. Rinse with TBS-Tween for 10 min for removal of leftover staining solution.
 - j. Incubate sections with the **second** primary biotinylated anti-mouse TCR β antibody (1:50 in TBS-Tween) for 1 h and rinse glass slides in TBS-Tween for 10 min to remove the unbound antibody.
 - k. Add ExtrAvidin Peroxidase (1:100 in TBS-Tween) for 30 min, wash slides in TBS-Tween for 30 min, and rinse glass slides in TBS-Tween for 10 min.
 - l. Incubate sections with Liquid DAB+ Substrate for 5 min to visualize T cells and wash with TBS-Tween for 10 min.
 - m. Add the **third** primary anti-mouse B220 antibody (1:100 in antibody solution) for 1 h and rinse with TBS-Tween for 10 min.

- n. Incubate sections with the second secondary biotinylated anti-rat IgG antibody (1:500 dilution in PBS with 5% normal mouse serum) for 30 min and wash in TBS-Tween for 10 min.
- o. Apply ExtrAvidin Alkaline Phosphatase for 30 min and rinse slides with TBS-Tween for 15 min.
- p. Add Fast Blue staining solution 10 min to stain B cells. Subsequently, rinse sections again in TBS-Tween for 15 min and mount them with Aquatex and coverslips.

B. Isolation of GC by laser microdissection

1. Comparison of toluidine blue- and immunohistochemical-stained slides
 - a. Place a usual light transmitted microscope close to the PALM MicroBeam microscope (Figure 3A).
 - b. Take the toluidine blue-stained slides and dry them at RT for 10 min.
 - c. Switch on the PALM MicroBeam microscope and put the slide with the toluidine blue-stained sections onto the slide holder of the object table.
 - d. Open the Palm Robo software.
 - e. Take the immunohistochemical-stained slide and put it on the object table of a usual light microscope that should stand close to the PALM MicroBeam microscope.
 - f. Carefully compare the two slides for the identification of GC. Note that samples that were closer in cryosections must be compared together (*e.g.*, identify the GC by comparing cryosections 1 to 2 and 3).
2. Isolation of GC by laser microdissection
 - a. Before activating the laser beam, prepare the cap of the microtube and moisten it carefully with a very thin layer of mineral oil.
 - b. Put the cap and the microtube into the provided holder (Figure 3B).
 - c. Following the instruction of the manufacturer's protocol, adjust the laser beam to the digital pen of the painting app by using the Palm Robo software. In addition, use a spot on the slide that is not covered with tissue to select the thickness of the laser beam. This can be done by fine-tuning the laser energy and the speed of the microbeam mover.
 - d. Cautiously surround the identified GC by using the digital pen of the painting app. It is required to carefully close the circled area with the digital pencil (Video 1).



Video 1. Laser capturing of lymphoid tissue compartments. A toluidine blue-stained section of a rat

spleen is used as an example for the microdissection of a B cell zone from lymphoid tissues.

1. The T- and B-cell zone can be clearly distinguished. 2. The area of interest, in this case, is a primary B cell follicle, which is circled using the drawing tool from the Palm Robo software. 3. The command “RoboLPC” from the Palm Robo software activates the laser beam to cut the marked tissue and the underlying membrane. An additional UV laser pulse catapults the isolated area into the cap of the micropipette.

- e. Place the cap holder directly over the slide to collect these predefined areas (Figure 3C).
- f. Activate the laser beam to cut and catapult the isolated area by using the command “RoboLPC”. The UV-laser cuts the marked line and catapults the GC into the cap of the micropipette (Video 1 and Figure 3D).
- g. After collection of GC tissue (here: 2×10^6 – $10 \times 10^6 \mu\text{m}^2$ per sample), remove the cap and micropipette. Pool all microdissected and catapulted tissue pieces by closing the micropipette with the cap and spinning down with a centrifuge. Collected tissues can be used for subsequent analysis.

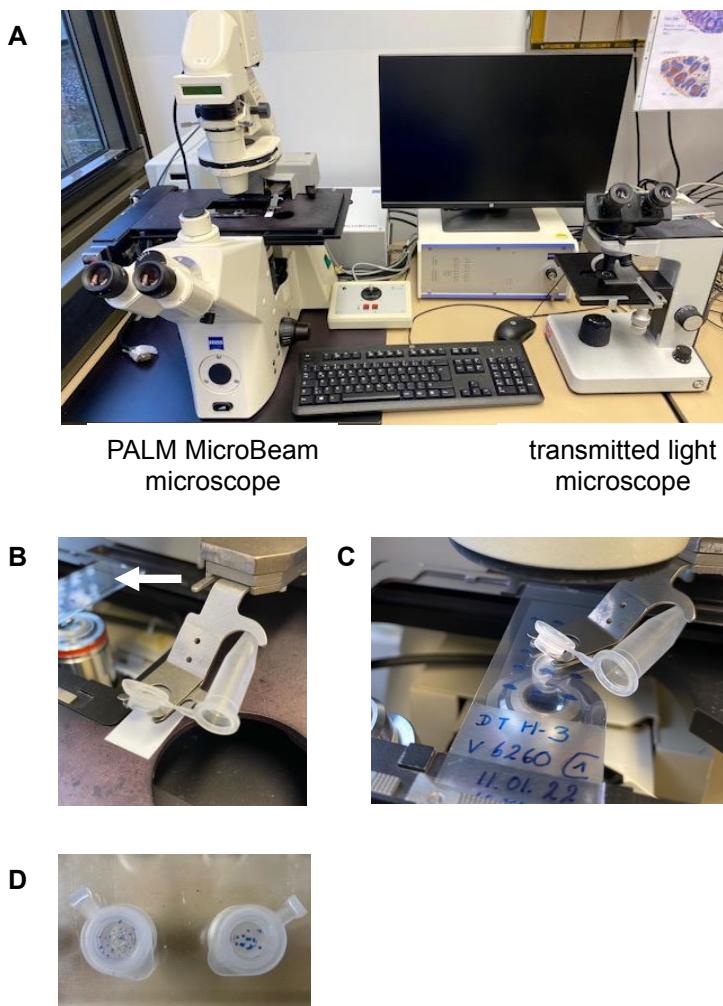


Figure 3. Equipment to isolate GC by laser microdissection. A. The workplace shows the PALM MicroBeam microscope for laser capturing the tissues (left side) and the transmitted light microscope for

immunohistochemical-stained sections to ensure the correct identification of GC (right side). B. A microtube is positioned into the holder. The white arrow indicates the membrane slide that contains the toluidine blue-stained sections in the slide holder. C. The cap- and microtube-holder must be placed directly over the slide to collect the catapulted tissues. D. The microdissected pieces of tissues can be clearly seen as toluidine blue-stained spots at the bottom of the microtube caps.

Recipes

1. Antibody solution

PBS + 1% BSA + 0.1% NaN₃.

The solution can be stored at 4°C for six months.

2. Tris Buffer (0.1 M)

Dilute 12.1 g of Tris base in laboratory grade H₂O to a final volume of 1 L. Adjust the pH to 8.2 with HCl.

The Tris buffer is stable at 4°C for six months.

3. Alkaline Phosphatase - Anti-Alkaline Phosphatase (APAAP) substrate

Dilute 20 mg of Naphthol AS-MX phosphate in 2 mL of N,N-Dimethylformamide, 100 μL of Levamisole (0.24 g/mL), and 98 mL of Tris-buffer 0.1 M.

The APAAP substrate is stable maximum for 30 days at 4°C.

4. Fast Blue staining solution

Dilute 0.002 g of Fast Blue BB salt in 4 mL of APAAP-substrate. Subject the solution to gentle shaking for 10 min. Prior to staining samples with the Fast Blue staining solution, filter with a 0.2 μm syringe filter. After treating the sample with the filtered solution, subject it to gentle shaking for 10 min. The solution should be used fresh.

Caution! The waste is hazardous, as the Fast Blue BB salt used in this solution is classified as a hazard when swallowed.

5. Fast Red staining solution

Dilute 0.01 g of Fast Red Salt in 3 mL of APAAP-substrate. After the dilution, subject the solution to gentle shaking for 5 min and then keep standing still for an additional 5 min. When added to the samples, subject the Fast Red staining solution to gentle shaking for 25 min.

Caution! Prepare the solution on a well-ventilated bench. The solution should be used fresh.

The waste is hazardous due to its last longing effect on aquatic life. Fast Red TR salt is hazardous as it causes severe skin burns and eye damage. The salt is also hazardous when swallowed.

6. PBS (phosphate-buffered saline) 5×

Dilute 90 g of NaCl, 2.704 g of NaH₂PO₄ monohydrate, and 28.794 g of Na₂HPO₄·12 H₂O in laboratory-graded H₂O with the final volume of 2 L. Adjust the pH to 7.4.

PBS 5× can be stored at 4°C for approximately six months.

PBS 1× should be used fresh after diluting 200 mL of PBS 5× in 800 mL of laboratory-graded H₂O.

7. PFA 4% (paraformaldehyde) solution

Prepare the PFA 4% solution by dissolving 8 g of paraformaldehyde in 200 mL of PBS 1× solution by stirring

(at 500 rpm) and heating (at 60°C) using a heating and magnetic stirrer.

Caution! Prepare the solution in a ventilated hood and use it fresh.

8. TBS-Tween (Tris-buffered Saline-Tween)

Prepare the TBS-Tween using a 10× TBS stock and a Tween-20 (5%) stock. Prepare the TBS 10× by diluting 242.28 g of Tris (hydroxymethyl aminomethane) and 344.40 g of NaCl in laboratory grade H₂O with a final volume of 4 L; adjust the pH to 7.6 and keep at 4°C for up to six months.

Prepare the Tween-20 (5%) by adding 190 mL of laboratory-grade H₂O to 10 mL of Tween-20. Prepare 1 L of TBS-Tween 1× by adding 890 mL of laboratory-grade H₂O to 100 mL of TBS 10× and 10 mL of Tween-20 (5%). The TBS-tween solution can be stored for up to six months.

9. Toluidine blue 1% solution

Dilute 0.5 g of toluidine blue in 1 mL of methanol, 15 mL of ethanol, and 50 mL of DEPC-treated water.

The solution can be kept in the dark at RT for one year.

Acknowledgments

This work was supported by grants from the German Research Foundation (DFG) within the framework of the Schleswig-Holstein Excellence Cluster I and I (EXC 306, Inflammation at Interfaces, project XTP4), the graduate school GRK 1727/2, GRK2633/1, and the TR-SFB654 project C4 at the University of Luebeck to KK and JW. Part of the figures is adapted and modified from the studies of Kalies *et al.* (2008) and Niebuhr *et al.* (2020 and 2021).

Competing interests

The authors indicate no potential conflicts of interest.

Ethics

All experiments were approved by the Animal Care and Use Committee of the state Schleswig-Holstein (Ministerium fuer Energiewende, Landwirtschaft, Umwelt, Natur und Digitalisierung), proposals: V 252-72241.122-1 (24/-3/02), V 312-72241.122-1 (32-3/06), V242-35471/2016 (68-6/16) and 23/A11/05. All animal experiments were conducted by certified personnel.

References

1. Allen, C. D., Okada, T. and Cyster, J. G. (2007). [Germinal-center organization and cellular dynamics](#). *Immunity* 27(2): 190-202.
2. Cyster, J. G. and Allen, C. D. C. (2019). [B Cell Responses: Cell Interaction Dynamics and Decisions](#). *Cell* 177(3): 524-540.
3. Gasteiger, G., Ataide, M. and Kastenmüller, W. (2016). [Lymph node - an organ for T-cell activation and](#)

[pathogen defense](#). *Immunol Rev* 271(1): 200-220.

4. Kalies, K., Konig, P., Zhang, Y. M., Deierling, M., Barthelmann, J., Stamm, C. and Westermann, J. (2008). [Nonoverlapping expression of IL10, IL12p40, and IFNgamma mRNA in the marginal zone and T cell zone of the spleen after antigenic stimulation](#). *J Immunol* 180(8): 5457-5465.
5. Niebuhr, M., Belde, J., Fahnrich, A., Serge, A., Irla, M., Ellebrecht, C. T., Hammers, C. M., Bieber, K., Westermann, J. and Kalies, K. (2021). [Receptor repertoires of murine follicular T helper cells reveal a high clonal overlap in separate lymph nodes in autoimmunity](#). *Elife* 10: e70053.
6. Qi, H. (2016). [T follicular helper cells in space-time](#). *Nat Rev Immunol* 16(10): 612-625.
7. Vinuesa, C. G., Linterman, M. A., Yu, D. and MacLennan, I. C. (2016). [Follicular Helper T Cells](#). *Annu Rev Immunol* 34: 335-368.

Subcellular RNA-seq for the Analysis of the Dendritic and Somatic Transcriptomes of Single Neurons

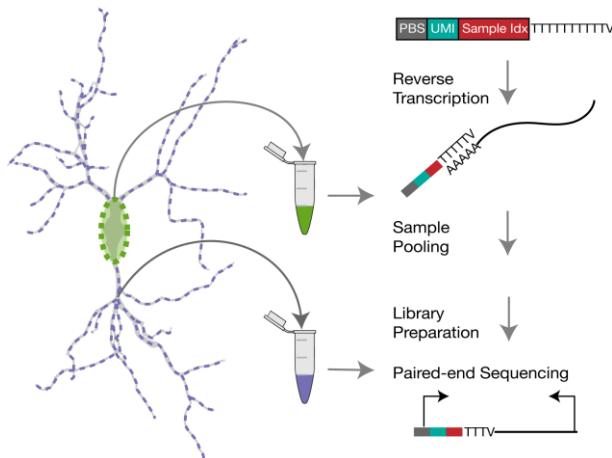
Julio D. Perez* and Erin M. Schuman

Department of Synaptic Plasticity, Max Planck Institute for Brain Research, Frankfurt, Germany

*For correspondence: jdperez101@gmail.com

[Abstract] In neurons, local translation in dendritic and axonal compartments allows for the fast and on-demand modification of the local proteome. As the last few years have witnessed dramatic advancements in our appreciation of the brain's neuronal diversity, it is increasingly relevant to understand how local translation is regulated according to cell type. To this end, both sequencing-based and imaging-based techniques have recently been reported. Here, we present a subcellular single cell RNA sequencing protocol that allows molecular quantification from the soma and dendrites of single neurons, and which can be scaled up for the characterization of several hundreds to thousands of neurons. Somata and dendrites of cultured neurons are dissected using laser capture microdissection, followed by cell lysis to release mRNA content. Reverse transcription is then conducted using an indexed primer that allows the downstream pooling of samples. The pooled cDNA library is prepared for and sequenced in an Illumina platform. Finally, the data generated are processed and converted into a gene vs. cells digital expression table. This protocol provides detailed instructions for both wet lab and bioinformatic steps, as well as insights into controls, data analysis, interpretations, and ways to achieve robust and reproducible results.

Graphic abstract:



Subcellular Single Cell RNA-seq in Neurons.

Keywords: Local translation, Local protein synthesis, Local transcriptome, Subcellular transcriptomics, Single cell RNA-seq, Neuron, Dendrites, Neuronal compartments

[Background] Within their highly polarized and complex structure, neurons create subcellular compartments that optimize operations requiring spatial and temporal isolation. The functional specialization of these compartments

is, in part, accomplished via the selective transport and local translation of mRNAs (Holt *et al.*, 2019). To characterize the neuronal local transcriptome, many studies have performed bulk RNA profiles in brain regions enriched in dendrites and axons (Zhong *et al.*, 2006; Cajigas *et al.*, 2012; Glock *et al.*, 2020), on synaptic particles isolated from tissue (Hafner *et al.*, 2019), or from neuronal cultures in chambers that separate cell bodies and neurites (Gumy *et al.*, 2011; Poon *et al.*, 2006). These studies have revealed that protein functions such as synaptic transmission, cytoskeletal regulation, and translation itself (among others) are encoded in the local transcriptome (Holt *et al.*, 2019). However, as recent advancements in single cell transcriptomics have revealed, the brain contains a complex array of neuronal types, raising the question of how variable the local transcriptome is across diverse cell types (Wang *et al.*, 2020; Perez *et al.*, 2021).

To address this question, single cell resolution of the local transcriptome is needed. There are three main challenges to implementing such an approach: (1) the isolation of mRNAs from distinct subcellular compartments of a single neuron, (2) the unbiased characterization of the local mRNAs, and (3) the characterization of hundreds to thousands of samples for robust cell type classification. Two pioneering studies used single-cell nanobiopsies or micropipettes to isolate material from the soma and dendrites of single neurons in culture, and RNA-seq to profile hundreds and thousands local mRNAs, respectively (Tóth *et al.*, 2018; Middleton *et al.*, 2019). However, both studies contained a relatively low number of samples (a few dozen), and neither investigated cell type effects on the local transcriptome. Recent breakthroughs in spatial transcriptomics have provided unprecedented *in situ* single molecule resolution of the local transcriptome, allowing for the characterization of mRNAs across and within subdomains of dendritic and axonal compartments. Using multiplexed error-robust fluorescence *in situ* hybridization, Wang *et al.* (2020) profiled the spatial location of hundreds of mRNAs in hundreds of cultured neurons, resulting in the identification of dendritic and axonal transcripts in glutamatergic and GABAergic neurons. However, this technique requires the experimenter to select *a priori* which mRNAs to target, and therefore, cannot provide an unbiased view of the local transcriptome. To circumvent this limitation, Alon *et al.* (2021) recently developed expansion sequencing (ExSeq), which combines expansion microscopy with fluorescent *in situ* sequencing. This technique allows the unbiased characterization of mRNAs anywhere within a neuron, in either cultured cells or tissue samples. However, so far untargeted ExSeq resolves only dozens of mRNA species per cell, and thus has not been used to profile cell type-specific variation in the local transcriptome.

Recently, we developed a method that allows for the separate isolation of dendritic and somatic mRNAs, as well as the unbiased characterization and molecular tallying of local mRNAs, in hundreds to thousands of single neurons (Perez *et al.*, 2021). Using this method, we profiled the dendritic transcriptome of glutamatergic and various GABAergic interneurons in culture, and identified dozens of mRNAs whose dendritic localization is regulated according to cell-type. Cell-type specific differences are substantially more common among somata as expected, since besides its own transcriptome, the soma also harbors the transcriptome of dendritic and axonal compartments. Similar observations were made by Wang *et al.* (2020) using an alternative approach. Our method combines laser capture microdissection (LCM) for the isolation of dendritic and somatic compartments (micron resolution; Figure 1A), with a sensitive scRNA-seq protocol (adapted from Picelli *et al.*, 2014; Macosko *et al.*, 2015), which tags mRNAs with a unique molecular identifier (UMI) and an index (Figure 1B). This index allows iterative pooling steps during library preparation, enabling the sequencing of 384 samples per run. The protocol can be executed from beginning-to-end in two weeks, and, if repeated 3 times or more, thousands of subcellular

samples can be accumulated. Since every cell is imaged before collection, this method can also be used to investigate correlations between the transcriptome and neuronal morphology. Nonetheless, several drawbacks of the protocol should be considered before starting. First, the method (as calculated using ERCC RNA standards) captures one of every 4 molecules present after LCM collection. This, however, likely overestimates the sensitivity to the actual number of molecules present inside the cell, as not all LCM catapulted pieces of cellular material land in the collection cap, an issue that appears more severe for dendrites. Thus, the more abundant an mRNA is, the more likely it is to be detected, and lower abundance mRNAs are more frequently missed. To compensate for this, we recommend increasing the number of samples until the number of detected mRNAs in dendrites reaches saturation. Second, the protocol requires the selection of neurons with little-to-no overlapping cellular processes in most of its dendritic arbor. This selection can introduce biases for some cell types over others. Indeed, we observed that, on average, GABAergic neurons have more accessible somata and processes, while glutamatergic processes are often heavily entangled with those of other cells. To estimate this potential bias, we suggest collecting somata-only samples from less accessible neurons in the same dish. The inclusion of such samples also improves unsupervised clustering, and thus enables more accurate cell-type determination of those neurons for which both soma and dendrites are collected.

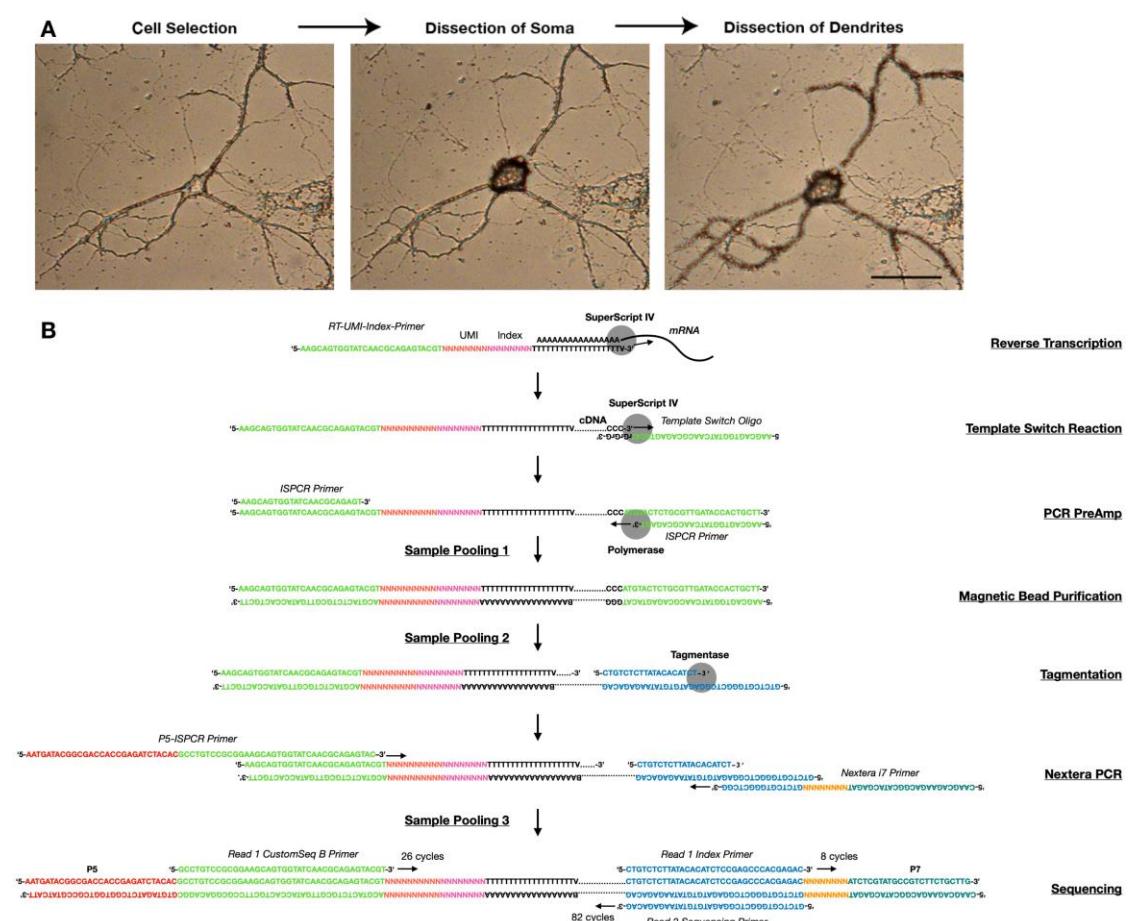


Figure 1. Subcellular scRNA-seq method.

A. Images showing the dissection of the soma and dendrites of a neuron using LCM. B. Library preparation workflow, showing the sequences, primers, and key enzymes used at every step.

Altogether, this method can serve as a powerful tool to achieve an unbiased investigation of cell type effects in the local transcriptome, and can be implemented across cells derived from different brain regions, developmental stages, or species. Additionally, it may be used to study single cell responses to pharmacological treatments, or other manipulations that induce changes in cell states (*e.g.*, paradigms of synaptic plasticity). It may also be useful to study the local transcriptome of other polarized cell types, such as astrocytes (Sakers *et al.*, 2017; Mazaré *et al.*, 2021), or epithelial cells (Moor *et al.*, 2017). Finally, it should be possible to adjust this protocol to profile non-coding RNAs, such as small RNAs (Hagemann-Jensen *et al.*, 2018).

Materials and Reagents

1. Glass-bottom culture 35 mm dishes, 14 mm glass diameter (MatTek, catalog number: P35G-1.5-14-C)
2. Qubit Assay Tubes (ThermoFisher, catalog number: Q32856)
3. Reagent Reservoir 25 mL (VWR, catalog number: 89094-662)
4. 96-well DNA LoBind Plate (Eppendorf, catalog number: 30129504)
5. AdhesiveCap-200 Clear (Zeiss, catalog number: 415190-9191-000)
6. Agencourt AMPure XP Magnetic Beads (Beckman Coulter, catalog number: A63881)
7. Betaine (ThermoFisher, catalog number: J77507AE)
8. Bioanalyzer HS-DNA Kit (Agilent, catalog number: 5067-4626)
9. Cultured neurons. We use Rat hippocampus primary neuronal cultures (prepared as described in Aakalu *et al.*, 2001).
10. CustomSeqB Primer (IDT, see [Supplementary File 1](#) with oligo information)
11. Dithiothreitol (DTT) (Bio-Rad, catalog number: 1610611)
12. dNTP mix (ThermoFisher, catalog number: R0192)
13. Elution Buffer (Qiagen, catalog number: 1014609)
14. ERCC RNA Spike-Ins (ThermoFisher, catalog number: 4456740)
15. ISPCR Primer (IDT, see [Supplementary File 1](#) with oligo information)
16. KAPA HiFi HotStart Mix (Roche, catalog number: KK2602)
17. Magnesium Chloride (ThermoFisher, catalog number: AM9530G)
18. Microseal B adhesive film (Bio-Rad, catalog number: MSB1001)
19. Molecular Biology Grade Ethanol (Sigma, catalog number: BP2818-100)
20. Nextera XT DNA Library Prep Kit (96 samples) (Illumina, catalog number: FC-131-1096)
21. Nextera XT Index Kit v2 Set A (Illumina, catalog number: FC-131-2001)
22. Nextera XT Index Kit v2 Set B (Illumina, catalog number: FC-131-2002)
23. NextSeq 1000/2000 P2 reagents (100 cycles) (Illumina, catalog number: 20046811)
24. Nuclease-free H₂O (ThermoFisher, catalog number: 10977-035)
25. P5-ISPCR Primer (IDT, see [Supplementary File 1](#) with oligo information)
26. Parafilm (Sigma, catalog number: PM-992)
27. Phosphate Buffer Saline (Sigma, catalog number: P5493-1L)

28. Poly-D-lysine (Corning, catalog number: 354210)
29. Qiagen Protease (Qiagen, catalog number: 19155)
30. Qubit dsDNA BR kit (ThermoFisher, catalog number: Q32853)
31. Qubit dsDNA HS kit (ThermoFisher, catalog number: Q32851)
32. RNase Inhibitor (Takara, catalog number: 2313A)
33. RNaseZap (ThermoFisher, catalog number: AM9780)
34. RT-UMI-Index Primer (16 variants) (IDT, see [Supplementary File 1](#) with oligo information)
35. SingleShot Cell Lysis Kit (Bio-Rad, catalog number: 1725080)
36. SuperScript IV (ThermoFisher, catalog number: 18090200)
37. Template Switch Oligo (IDT, see [Supplementary File 1](#) with oligo information)
38. Cell Lysis Mix (see Recipes)
39. RT Mix (see Recipes)
40. PreAmp PCR Mix (see Recipes)
41. Final PCR Amplification Mix (see Recipes)

Equipment

1. 2-20, μ L 12-channel, Multichannel pipette (*e.g.*, Ranin, catalog number: 17013808)
2. 20-200 μ L, 12-channel, Multichannel pipette (*e.g.*, Ranin, catalog number: 17013810)
3. 2100 Bioanalyzer Instrument (Agilent, catalog number: G2939BA)
4. Cell culture incubator (*e.g.*, ThermoFisher)
5. Curved, cover glass forceps (VWR, catalog number: HAMMHSC817-13)
6. DISH 35 CC Adapter (Zeiss, catalog number: 415101-2000-835)
7. Magnetic Stand for 96-well plates (ThermoFisher, catalog number: AM10027)
8. Minicentrifuge (*e.g.*, ThermoFisher, catalog number: 75004061)
9. Illumina DNA Sequencer (*e.g.*, NextSeq 2000)
10. PALM MicroBeam Axio Observer Laser Capture Microdissection Microscope (Zeiss)
11. PCR Plate Spinner (VWR, catalog number: 89184-610) or 96-well plate-compatible centrifuge
12. Qubit Fluorometer (ThermoFisher, catalog number: Q33238)
13. SingleCap Collector II 200 RM (Zeiss, catalog number: 415101-2000-951)
14. Thermal cycler (*e.g.*, Bio-Rad, C1000, catalog number: 1851197)
15. Tissue culture hood (*e.g.*, ThermoFisher)
16. Vortex (*e.g.*, VWR, catalog number: 10153-840)

Software

1. PALM RoboSoftware v3 or above (Zeiss, <https://www.zeiss.com/microscopy/us/products/microscope-software/palm-robosoftware.html/palm46>)
2. bcl2fastq v2.20.0.422 (Illumina,

- https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html)
3. FastQC v.0.11.9 (Babraham Bioinformatics/FastQC,
<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>)
 4. Picard Tools v.2.20.2 or above (broadinstitute/picard,
<https://github.com/broadinstitute/picard/releases/tag/2.20.2>)
 5. Drop-seq Tools v.2.3.0 or above (broadinstitute/Drop-seq, <https://github.com/broadinstitute/Drop-seq/releases/tag/v2.3.0>)
 6. STAR v.2.7.2b or above (alexdobin/STAR, <https://github.com/alexdobin/STAR/releases/tag/2.7.2b>)
 7. Fasta file containing the genome of the species being used. Genome fasta files of various species can be found in <https://hgdownload.soe.ucsc.edu/downloads.html> or <http://ftp.ensembl.org/pub/release-99/fasta/>
 8. GTF file containing transcript coordinates in the genome of the species being used. GTF files of various species can be found in <https://hgdownload.soe.ucsc.edu/downloads.html> or <http://ftp.ensembl.org/pub/release-99/gtf/>
 9. Seurat 3 or above (satijalab/seurat, <https://satijalab.org/seurat/articles/install.html>)

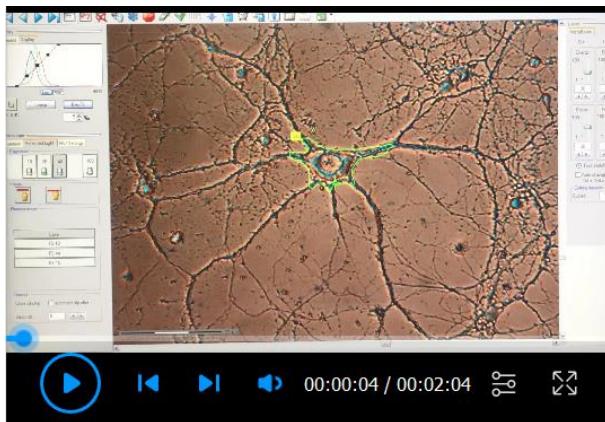
Procedure

- A. Determine the desired number of samples per sequencing run (see Note 1 for limitations and considerations). This procedure is designed for the sequencing analysis of 384 samples, including both positive and negative controls (see Note 2), which are indexed, and subsequently pooled in three separate steps (see Note 3).
- B. Before starting, clean pipettes, racks, centrifuges, and surfaces in the LCM microscope and bench space with RNaseZap, to avoid RNA degradation.
- C. Primary neuronal cultures
 1. Prepare mammalian neuronal cultures according to protocol of choice. In our case, we culture neurons from the hippocampus or cortex of newborn rats (P1), as previously described (Aakalu *et al.*, 2001).
 2. Plate cells on coverslips of MatTek 14mm diameter glass bottom dishes coated with a 0.1 mg/mL solution of poly-D-lysine, at a density of 20,000 cells/coverslip.
 3. Maintain plated cells in the cell medium of choice for at least 2 weeks and no longer than 5 weeks in culture for best confluence of neuronal processes. Unless cell age is a variable of interest, we strongly recommend that all neurons in an experiment share the same age, as the transcriptome may vary significantly over the lifespan of cultured neurons.
- D. Ethanol fixation
 1. Discard cell medium.
 2. Wash cultures by adding 5 mL of 1× PBS and immediately discard it. Repeat once, for a total of 2 washes.
 3. Add 5 mL of cold 70% ethanol (kept at -20°C). Wait 5 min and discard ethanol.

4. Seal the dish with parafilm and store at -80°C. RNA in cells will remain stable for at least 3 months.

E. Laser capture microdissection

1. Make 4 separate cell lysis master mixes, differing in which of the 16 RT-UMI-Index (1-16) Primer is used (see Recipe 1). Keep on ice.
2. Remove parafilm and cap from dish and place it on the DISH 35 CC adapter of a Zeiss PALM LCM microscope. Cells should be allowed to thaw for 5-10 min before dissection. Proceed to the next step in the meantime.
3. Open the PALMRobo software. Using the 20× objective, identify and register locations for microdissection. We recommend the collection of no more than 48 samples per plate (see Note 1): the somata and dendritic arbors of 16 neurons amenable for dissection, 12 somata whose dendritic arbors are not amenable for collection, and 4 empty cuts (see Note 2). Thus, at this point the location of 28 neurons and 4 empty regions should be saved. Neurons amenable for collection have isolated somata free of processes, AND isolated dendritic arbors in which most processes can be unambiguously assigned to the same neuron (See example in Figure 1A and [Supplementary File 2](#)). Avoid neurons at the edges of the coverslip, as these are inefficiently catapulted.
4. Switch to the 40× objective, go to the first registered location, and take a picture.
5. For somata or empty regions, select AutoLPC from the Cut Tools menu, and the circle drawing tool to delineate the soma or region of interest. For dendrites, select LineAutoLPC from the Cut Tools menu, and the free-hand drawing tool to delineate processes. Because of the continuous nature of neuronal compartments, there is no obvious point where the soma ends and dendrites begin, and thus what we consider the border between the two compartments is ultimately arbitrary. In our experiments, we delineate the soma as the cellular area containing and surrounding the nucleus, which dilates out until drastic decrements in width suddenly occur. The processes that continue are considered dendrites. However, processes that are qualitatively thinner than the rest are excluded as these might be axons (see example in Figure 1A, [Supplementary File 2](#), and Video 1).



Video 1. Delineation and laser capture microdissection of soma and dendritic processes of five single neurons.

6. Place cap of an AdhesiveCap-200 clear tube in the microscope's RoboMover containing the SingleCap Collector II 200 RM. Cut the stretch of the tube linking the cap to the tube, and discard the tube.
7. Click on Capture Device icon, go to the Adjust tab, and set working height to -12,500. Go to Operation, and double-click on top of the cap icon. This will bring the cap in the collection position above the culture dish. Adjust focus if necessary, to obtain a clear image of the neuron.
8. Set the following parameters for Laser Pressure Catapulting (LPC): Energy = 40, focus = 70%, and speed = 15%.
9. Go to the Colors icon on the Graphic toolbar, and on the LPC Distances tab set the Distance of AutoLPC shots to 2. This determines the density of the LPC punches.
10. Go to Element List, and select only the element delineating the soma. Click on the Start Cutting Laser icon. The area delineating the soma will be catapulted to the collection cap in many individual punches.
11. Click on Capture Device icon, go to the Operation tab, and click on the Home icon. This will bring the cap back to the loading position.
12. Using curved forceps, carefully remove the cap from the collection arm and place it on a surface, with the side where the material was catapulted into facing up.
13. Add 3 µL of the respective Cell lysis master mix to the cap containing the catapulted material.
14. Using curved forceps, carefully place the cap in a well of a 96-well DNA LoBind plate, with the side where the material was catapulted into facing down.
15. Repeat steps 5 to 7.
16. Go to Element List and select all of the elements delineating the dendrites of that particular neuron. Click on the Start Cutting Laser icon. The area delineating each dendrite will be catapulted to the collection cap in many individual punches.
17. Repeat steps 11 to 14.
18. Repeat steps 5 to 17 for each location registered in step 3. We recommend organizing samples shown in Figure 4A in each 96-well plate.
19. Proceed immediately to the next step.

F. Cell lysis

1. Seal the plate containing collection caps tightly with Microseal B adhesive film, turn it upside down, and vortex the side containing caps for 15 s.
2. Centrifuge plate for 1 min in plate spinner, or centrifuge for 1min at 1,000 × g, to bring volumes from the cap to the bottom of the well. Discard caps and reseal plate with Microseal B adhesive film.
3. Place plate in thermal cycler and run the following program (lid set to 105°C):
Step 1 (Protein Digestion): 50°C for 10 min
Step 2 (Protease Inactivation): 75°C for 10 min
Step 3: 4°C hold
4. Proceed immediately to the next step.

G. Reverse Transcription

1. Prepare RT master mix (see Recipe 2).
2. Split RT master mix into 12 PCR tubes, each containing 15.3 µL.
3. Using a multichannel pipette, pipette 3.4 µL of RT master mix out of the 12 PCR tubes prepared in the previous step, and add it to each reaction.
4. Seal plate with Microseal B adhesive film.
5. Mix, by quick vortex and 1 min centrifugation in plate spinner, or in centrifuge for 1 min at 1,000 × g.
6. Place plate in thermal cycler and run the following program (lid set to 105°C):
Step 1 (Reverse Transcription): 55°C for 10 min
Step 2 (Enzyme Inactivation): 80°C for 10 min
Step 3: 12°C hold

H. PCR pre-amplification

1. Prepare PCR PreAmp master mix (see Recipe 3).
2. Split PCR PreAmp master mix into 12 PCR tubes each containing 34.2 µL.
3. Using a multichannel pipette, pipette 7.6 µL of PCR PreAmp master mix out of the 12 PCR tubes prepared in the previous step, and add it to each reaction.
4. Seal plate with Microseal B adhesive film.
5. Mix by quick vortex and 1min centrifugation in plate spinner, or centrifuge for 1 min at 1,000 × g.
6. Place plate in thermal cycler and run the following program (lid set to 105°C):
Step 1 (Taq Activation): 98°C for 3 min
Step 2 (Denaturation): 98°C for 20 s
Step 3 (Annealing): 67°C for 15 s
Step 4 (Extension): 72°C for 6 min
(Repeat steps 2-4 for a total of 21 cycles*)
Step 5 (Final Extension): 72°C for 5 min
Step 6: 12°C hold

*Cycle number may need to be optimized, as samples with high starting amounts of RNA will require less cycles, and those with low starting amounts will require more.

7. It's safe to stop and store PCR reactions at -20°C for at least 3 months.
8. Proceed to the next step once all 384 samples have been accumulated (~8 culture dishes).

I. Sample pooling and PCR purification

1. Let AMPure XP DNA Magnetic Beads stand at room temperature at least 30 min (see Note 3 for best practices when performing DNA purification using magnetic beads).
2. Pool 8 reactions derived from the same source (*i.e.*, somata, dendrites, or empty cuts), but which were obtained from different RT index primers, into a single well in a DNA LoBind 96-well plate (pooling logic is described in detail in Note 4). This should result in a plate with 4 rows in which each well contains ~112 µL of pooled sample.
3. Add 112 µL of AMPure Magnetic Beads to each well. Seal plate. Mix well by vortexing.

4. Let plate stand at room temperature for 5 min.
5. Place plate in magnetic stand. Let stand for 5 min.
6. Prepare fresh 80% ethanol, considering that 48 pooled samples require ~22 mL of 80% ethanol.
7. Place 80% ethanol solution in 25 mL reservoir.
8. Discard supernatant from every well containing pooled samples, being careful not to disrupt the magnetic bead pellet.
9. Add 200 µL of freshly-made 80% ethanol to each well. Wait 30 s and remove.
10. Repeat previous step one more time, making sure to discard all ethanol at the end (may require additional pipetting out).
11. Let plate stand in magnetic stand for 5 min, or until the bead pellet looks dry. If cracks begin to appear in the pellet, proceed immediately to next step.
12. Remove plate from magnetic stand, and add 17.5 µL of Elution Buffer (EB) on top of pellet.
13. Resuspend pellet by vortexing and, if necessary, by repeatedly pipetting elution volume on top of pellet.
14. Wait 5 min.
15. Place tube back on magnetic stand and wait 2 min.
16. Transfer 15 µL of supernatant to wells in a new 96-well DNA LoBind plate.
17. Combine supernatant from 2 pooled samples derived from the same source (*i.e.*, somata, dendrites, or empty cuts) that contained no overlap in the 8 indexes pooled in step 2 (see Figure 4). Thus, this mix will contain 16 different indexes, each representing a different sample.

J. Quality metrics of cDNA libraries

1. Check concentration of the 24 pooled samples using Qubit dsDNA BR Kit according to manufacturer's instructions. Concentrations between 10-150 ng/µL per pooled sample are expected.
2. Check size distribution of the 24 pooled samples using the Agilent Bioanalyzer HS-DNA kit, according to manufacturer's instructions. Libraries are expected to have few or no peaks below 500bp and a large peak between ~800 bp and ~5,000 bp, centered at ~2,000 bp (Figure 2A).

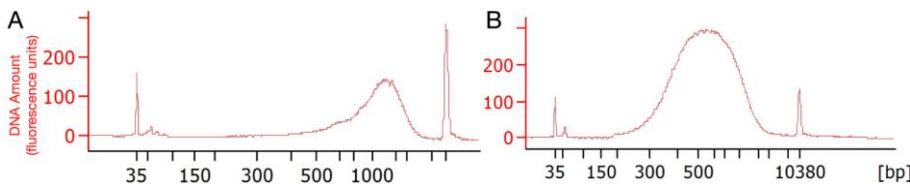


Figure 2. Expected size distribution of cDNA libraries.

A. Bioanalyzer electropherogram from a pooled of dendritic samples after section J, step 2. B. Bioanalyzer electropherogram from same sample after section N, step 2.

K. Tagmentation

1. In a new 96-well DNA LoBind plate, make 0.1 ng/µL dilutions for each pooled sample.
2. Add 10 µL of Nextera Tagment DNA Buffer to each well.
3. Add 5 µL of Nextera Amplicon Tagment Mix to each well.

4. Seal plate and centrifuge in plate spinner for 1 min.
5. Incubate samples for 5 min in a thermal cycler set to 55°C (heated lid 105°C).
6. Stop Tgmentation by adding 5 µL of Nextera Neutralize Tgment Buffer to each well.
7. Seal plate and centrifuge in plate spinner for 1 min.
8. Incubate plate at room temperature for 5 min.

L. Amplification of sequencing-compatible fragments

1. Prepare Final PCR master mix (see Recipe 4).
2. Add 24 µL of Final PCR Amplification mix to each reaction.
3. To each reaction, add 1 of the 24 different Nextera i7 primers (N7XX).
4. Seal plate and centrifuge in plate spinner for 1 min.
5. Place plate in thermal cycler and run the following program (lid set to 105°C):
Step 1 (Pre-PCR Incubation): 98°C for 3 min
Step 2 (Denaturation): 95°C for 30 s
Step 3 (Denaturation): 95°C for 10 s
Step 4 (Annealing): 55°C for 30 s
Step 5 (Extension): 72°C for 30s
(Repeat steps 2-4 for a total of 12 cycles)
Step 6 (Final Extension): 72°C for 5 min
Step 6: 12°C hold

M. PCR cleanup and purification

1. Let AMPure XP DNA Magnetic Beads stand at room temperature at least 30 min.
2. Add 30 µL of AMPure XP DNA Magnetic Beads to each reaction. Mix well by vortexing.
3. Repeat steps 4-15 of section I to purify amplified DNA.
4. It's safe to stop and store PCR reactions at -20°C. Sequence samples within 2 weeks.

N. Quality metrics

1. Check concentration of each sample using Qubit dsDNA HS Kit according to manufacturer's instructions. Concentrations between 1-10 ng/µL per sample are expected.
2. Check size distribution of each sample using the Agilent Bioanalyzer HS-DNA kit, according to manufacturer's instructions. Libraries are expected to show weak a large peak between ~300 bp and ~1,000 bp, centered at ~500 bp (Figure 2B).

O. Generation of 2 nM multiplexed library

1. Normalize the concentration of each library to 2 nM.
2. Combine 2 µL of each normalized library for a final volume of 48 µL. Mix well.
3. Confirm concentration of final multiplexed library using Qubit dsDNA HS Kit according to manufacturer's instructions. Adjust concentration to 2 nM if necessary.

P. Paired-end sequencing

1. Perform paired-sequencing according to manufacturer's protocol. Here, we describe the process for NextSeq 2000 using Nextseq 1000/2000 P2 reagents (100 cycles) v3.
2. Combine 12 µL of multiplexed sample with 12 µL of NextSeq RSB with Tween buffer. Vortex briefly and centrifuge for 1 min.
3. Combine 1.8 µL of Read1 CustomSeqB primer with 600 µL of HT1 buffer. Vortex and centrifuge.
4. Add 20 µL of diluted library to the bottom of the library well of the sequencing cartridge.
5. Load 550 µL of Read1 CustomSeqB primer dilution into well #1 of sequencing cartridge.
6. Follow manufacturer's instructions to start sequencing run.
7. Select custom 1 for Read 1.
8. Setup the following sequencing parameters and run: Read 1: 26 bp, Read 2: 82 bp, Read 1 Index: 8 bp.

Q. Generation of files needed for Drop-seq core computational protocol (see Note 5).

1. Generate sequence dictionary using the following command:

```
java -jar /path/to/picard/picard.jar CreateSequenceDictionary \
REFERENCE=my.fasta \
OUTPUT= my.dict \
SPECIES=species_name
```

2. Generate refFlat annotation file using the following command:

```
/path/to/Drop-seq_tools/ConvertToRefFlat \
ANNOTATIONS_FILE=my.gtf \
SEQUENCE_DICTIONARY=my.dict \
OUTPUT=my.refFlat
```

3. Generate reduced GTF file using the following command:

```
/path/to/Drop-seq_tools/ReduceGtf \
GTF=my.gtf \
SEQUENCE_DICTIONARY=my.dict \
OUTPUT=my.reduced.gtf
```

4. Generate intervals files using the following command:

```
/path/to/Drop-seq_tools/CreateIntervalsFiles \
REDUCED_GTF=my.reduced.gtf \
```

```
SEQUENCE_DICTIONARY=my.dict \
PREFIX=my \
OUTPUT=/path/to/output/files \
MT_SEQUENCE=chrM
```

5. Generate genome directory for alignment process using the following command:

```
/path/to/STAR/STAR \
--runMode genomeGenerate \
--runThreadN 8 \
--genomeDir path/to/output/files\
--genomeFastaFiles path/to/FASTA/file \
--sjdbGTFfile path/to/GTF/file \
--sjdbOverhang 81
```

R. Data processing pipeline for the generation of digital gene expression tables.

1. Demultiplex i7 indexes using the following command:

```
bcl2fastq -runfolder-dir /path/to/rawdata/folder/ \
-output-dir /path/to/output/folder/ \
--no-lane-splitting \
--loading-threads 8 \
--writing-threads 8 \
--minimum-trimmed-read-length 0 \
--mask-short-adapter-reads 0 \
--sample-sheet /path/to/sample/sheet/
```

2. Evaluate quality of sequencing data for all files using the following command:

```
/path/to/fastqc *.fastq.gz
```

(go to <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/Help/3%20Analysis%20Modules/>, for information on how to interpret results of FastQC quality metrics)

3. For each i7-index sample, convert Fastq file to Sam file while merging R1 and R2 files using the following command:

```
java -jar /path/to/picard/picard.jar CreateSequenceDictionary \
F1= SampleX_R1.fastq.gz \
F2= SampleX_R2.fastq.gz \
O= SampleX.bam
```

SM=SampleX

4. Extract the RT index sequence of each read using the following command:

```
/path/to/Drop-seq_tools/TagBamWithReadSequenceExtended \
INPUT=SampleX.bam \
OUTPUT=Indexed_SampleX.bam \
SUMMARY= Indexed_SampleX.summary \
BASE_RANGE= 9-16 \
BASE_QUALITY=10 \
DISCARD_READ=False \
TAG_NAME=XC \
NUM_BASES_BELOW_QUALITY=1
```

5. Extract the molecular barcode sequence of each read using the following command:

```
/path/to/Drop-seq_tools/TagBamWithReadSequenceExtended \
INPUT=Indexed_SampleX.bam \
OUTPUT=UMIed_SampleX.bam \
SUMMARY= UMIed_SampleX.summary \
BASE_RANGE= 1-8 \
BASE_QUALITY=10 \
DISCARD_READ=True \
TAG_NAME=XM \
NUM_BASES_BELOW_QUALITY=1
```

6. Remove reads with low quality RT index or molecular barcode sequences using the following command:

```
/path/to/Drop-seq_tools/FilterBam \
TAG_REJECT=XQ \
INPUT=UMIed_SampleX.bam \
OUTPUT= Filtered_SampleX.bam
```

7. Trim reads containing part of the template switch oligo using the following command:

```
/path/to/Drop-seq_tools/TrimStartingSequence \
INPUT=Filtered_SampleX.bam \
OUTPUT= Trimmed_SampleX.bam \
OUTPUT_SUMMARY= Trimmed_SampleX.summary \
SEQUENCE= AAGCAGTGGTATCAACGCAGAGTGAATGGG \
```

```
MISMATCHES=0 \
NUM_BASES=5
```

8. Trim polyA tails within reads using the following command:

```
/path/to/Drop-seq_tools/PolyATrimmer \
INPUT=Trimmed_SampleX.bam \
OUTPUT= PolyATrimmed_SampleX.bam \
OUTPUT_SUMMARY= PolyATrimmed_SampleX.summary \
SEQUENCE= AAGCAGTGGTATCAACGCAGAGTGAATGGG \
MISMATCHES=0 \
NUM_BASES=6 \
USE_NEW_TRIMMER =true
```

9. Convert bam files back to Fastq format using the following command:

```
java jar /path/to/picard/picard.jar SamToFastq \
INPUT=PolyATrimmed_SampleX.bam \
FASTQ= PolyATrimmed_SampleX.fastq
```

10. Align reads to genome using the following command:

```
/path/to/STAR/STAR \
--runMode alignReads \
--runThreadN 8 \
--genomeDir path/to/genome/folder/ \
--readFilesIn PolyATrimmed_SampleX.fastq \
--outSAMtype BAM \
--SortedByCoordinate \
--alignSoftClipAtReferenceEnds No \
--outFilterScoreMinOverLread 0.66 \
--outFilterMatchNminOverLread 0.66
```

11. Calculate quality metrics for RNA-sequencing using the following command:

```
java jar /path/to/picard/picard.jar CollectRNASEqMetrics \
I=Aligned_SampleX.bam \
O= SampleX.RNA_Metrics \
REF_FLAT = my.refFlat \
STRAND=FIRST_READ_TRANSCRIPTION_STRAND \
```

```
CHART_OUTPUT=SampleX_Metagene.plot \
RRNA_FRAGMENT_PERCENTAGE=0.8 \
MINIMUM_LENGTH=500 \
RIBOSOMAL_INTERVALS=/path/to/my.intervals/rRNA.intervals
```

(we expect the overwhelming majority of bases to map to mRNAs, and the metagene plot should show a strong 3' bias as seen in Figure 3.)

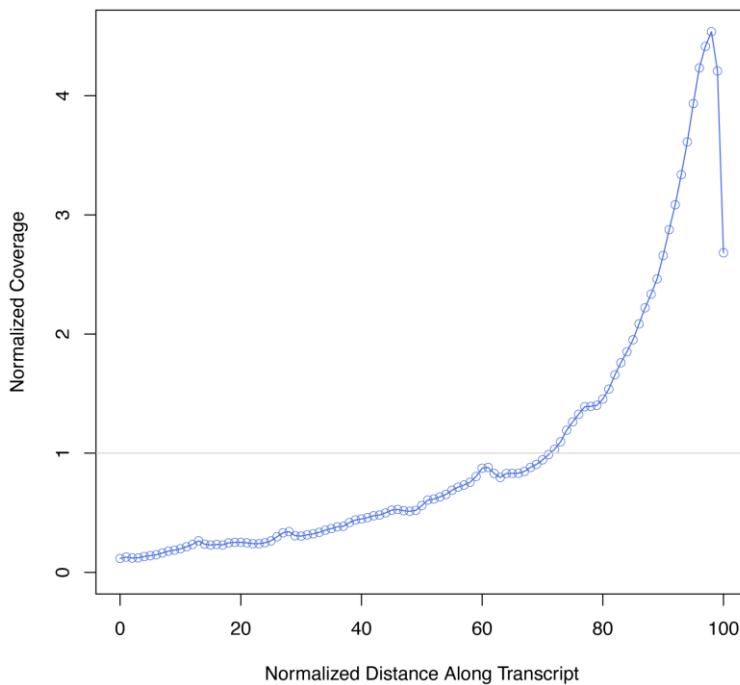


Figure 3. scRNA-seq data 3' bias.

Metagene plot showing the expected distribution of reads (normalized coverage) across the length of an mRNA, where 0 represents the 5' most region and 100 the 3' most region.

12. Merge aligned bam file with the Indexed and UMIed bam file using the following command:

```
java jar /path/to/picard/picard.jar MergeBamAlignment \
REFERENCE_SEQUENCE= /path/to/Genome/fasta \
UNMAPPED_BAM=UMIed_SampleX.bam \
ALIGNED_BAM= Aligned_SampleX.bam \
OUTPUT= Merged_SampleX.bam \
INCLUDE_SECONDARY_ALIGNMENTS=false \
PAIRED_RUN =false
```

13. Tag reads with gene name using the following command:

```
/path/to/Drop-seq_tools/TagReadWithGeneFunction \
I=Merged_SampleX.bam \
O= GeneTagged_SampleX.bam \
ANNOTATIONS_FILE= my.refFlat
```

14. Generate digital gene expression table using the following command:

```
/path/to/Drop-seq_tools/DigitalExpression \
I=GeneTagged_SampleX.bam \
O= SampleX.DGE.gz \
STRAND_STRATEGY=SENSE \
SUMMARY=SampleX.DGE.summary.txt \
CELL_BC_FILE=RT_Indexes*
```

*This is a text file listing line-by-line the 8nt sequences of all indexes used.

Data analysis

1. A dataset generated using this method on rat primary hippocampal neurons can be found in the NCBI Gene Expression Omnibus under the accession code GSE157204. A digital expression table generated from the data and a metadata file describing the experimental design can be found in [Supplementary File 1](#) and [Supplementary File 2](#) of our publication (Perez et al., 2021). The R source code used in analyses of the data can be found in our GitHub repository [DOI: 10.5281/zenodo.4384479](#).
2. The ERCCs Spike-Ins included in the protocol can be used to quantify the accuracy of the experiment, by comparing the input number of individual ERCCs with their average number in the sequenced results. A Pearson correlation >0.8 is expected. Also, the sensitivity of the experiment (how many of the mRNAs present in the lysis reaction were detected) can be calculated by determining the detection probability of ERCCs with different input values. On average, we detected 1 out of every 4 molecules present.
3. Before analyzing the digital gene expression table, additional quality evaluations and data cleaning are necessary. First, ERCCs should be used to evaluate the quality of library preparation and sequencing: outliers with little to no ERCCs sequenced should be discarded. Second, somatic or dendritic samples with a number of RNA molecules comparable to that of empty cuts, should also be discarded. Finally, as described below, unsupervised dimensionality methods can reveal cell types present in the dataset. In our experience, this may reveal samples enriched in markers for apoptotic or glial cells, both of which should be discarded.
4. To classify samples into types, use unsupervised dimensionality reduction methods such as UMAP or tSNE, and nearest neighbor approaches such as k-clustering, all of which are available in the Seurat package (Stuart et al., 2019). As dendritic transcriptomes are usually shallower than somatic ones, we perform cell type classifications based only on the somatic samples, and later extrapolate this information

to their corresponding dendrites.

5. Differential expression analyses can be performed both between the somata or dendrites of different cell types, or between the soma and dendrites of single neurons. For these, we recommend test based on logistic regression (Ntranos *et al.*, 2019), or a Poisson generalized linear model (Stuart *et al.*, 2019). When comparing soma versus dendrites, we recommend a paired-differential expression analysis using cell of origin as a latent variable.

Notes

1. During the experimental design phase, several factors should be considered when choosing an appropriate N. Power analyses should be perform considering the types of tests and analyses that will be eventually performed with the data, as the required N to achieve statistical power varies from test to test. For differential expression analyses between cell types or between subcellular compartments, we suggest tools optimized for single cell datasets such as powsimR (Vieth *et al.*, 2017) or scPower (Schmid *et al.*, 2020). The number of samples that can be profiled in a single sequencing run depends on the multiplexing capacity of the experimental setup. In this protocol, the RT primer carries 1 of 16 different indexes while the i7 Nextera PCR step adds 1 of 24 indexes to each of the previous ones, allowing a maximum of (16×24) 384 samples to be simultaneously profiled. For us, this number provided sufficient sequencing depth per sample. However, it is certainly possible to increase the number of indexes and therefore the multiplexing capacity per run. In principle, all samples could be derived from a single plate but this is not recommended. As LCM occurs at room temperature, and only one sample is collected at a time, RNA integrity will significantly decrease overtime. We observed a trend to less RNA molecules detected per sample (suggestive of lower RNA integrity) after 3 h of collection. Thus, we suggest to limit the number of samples collected per dish to an amount that can be safely collected in 3 h (in our case, 48). We recommend using dishes from multiple primary neurons preparations, since variability between preparations occurs, and thus dishes from the same preparation do not yield fully independent samples.
2. In addition to paired somatic and dendritic samples derived from the same single neurons, we recommend including two types of controls. To have a sense of the potential bias introduced by the selection of cells amenable for laser capture, we suggest collecting somata-only samples from less accessible neurons in the same dish. Secondly, as a negative control, we include samples in which Laser Pressure Catapulting is applied to regions in the dish that are devoid of soma and/or dendrites. The size of these cuts should be comparable to the area occupied by somata and/or dendrites. These samples serve to control for potentially contaminating extracellular RNAs, and can help set an expression cutoff to include a sample.
3. When using magnetic beads to purify DNA, it is important to thoroughly resuspend beads by vortexing, before mixing them with the samples. It is also important to accurately pipette the desired volume in section I-step 3, as inaccuracies in volume can increase or decrease the presence of smaller DNA fragments. If possible, use low-binding tips at this step. It is also important to monitor that the pellet does not become too dry and begins to show cracks in section I-step 11, as this will result in reduced DNA concentrations after resuspension.

4. To go from 384 collected samples to sequencing a single multiplexed sample, we incrementally pool at key steps of the protocol, namely steps 2 and 17 of section I, and step 2 of section N. To avoid pooling samples with the same index combination, and to perform the process rapidly and efficiently, we suggest the organization of samples based on the 96-well plate format illustrated in Figure 4.

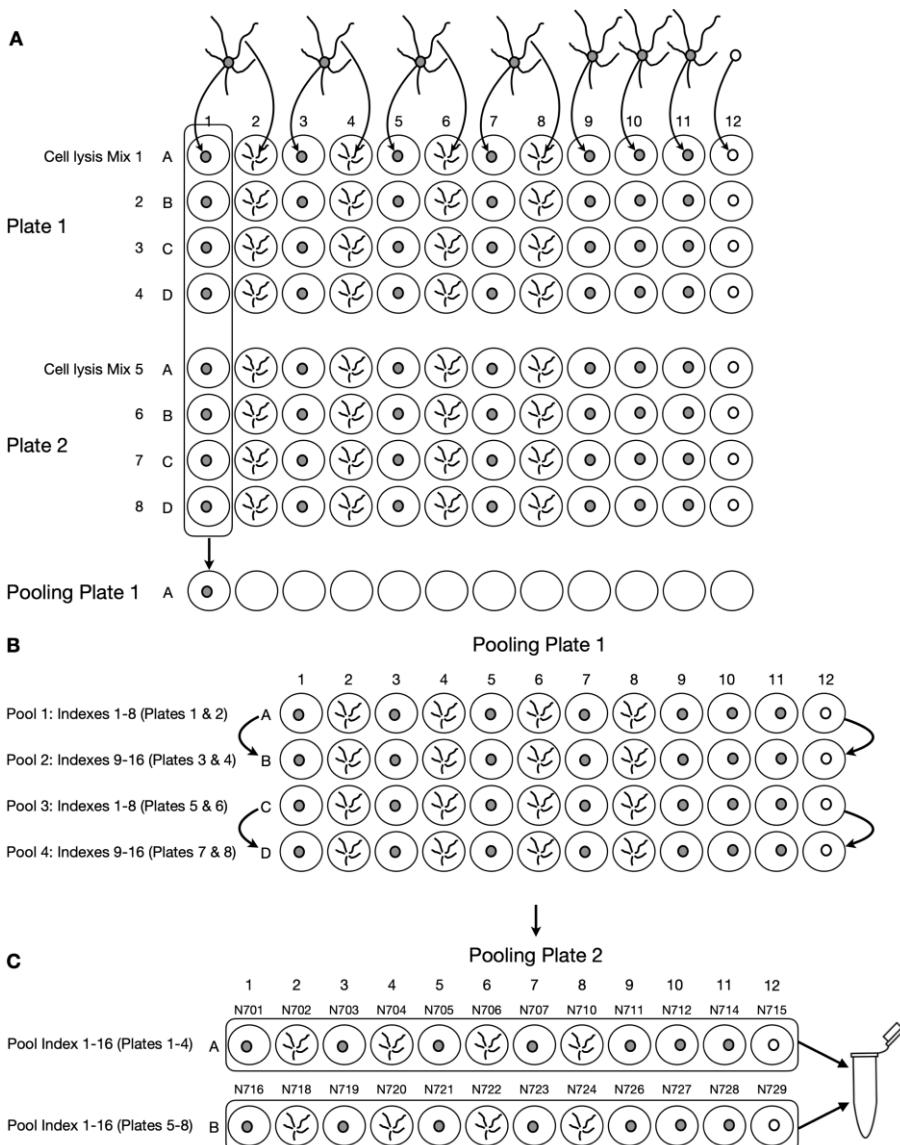


Figure 4. Sample pooling workflow.

A. Pooling performed in section I, step 2. Two 96-well plates containing 48 samples each (only rows A-D are shown, as rows E-H are unoccupied). Somatic samples are placed in columns 1, 3, 5, and 7, and their respective dendrites are placed in columns 2, 4, 6 and 8. Control somata from less accessible areas are collected in columns 9, 10, and 11. Column 12 contains empty cuts (negative controls). Each row of the plate contains 1 of 16 different RT-UMI-Index primers. In section I step 2, all samples within the same columns of two complementary plates (containing a non-overlapping set of indexes) are pooled together.

B. Pooling performed in section I, step 17. Two complementary samples within the same column are pooled together to reduce sample number to 24. C. Pooling performed in section N, step 2. After adding

Nextera indexes, all samples are pooled together to reduce sample number to 1.

5. Commands in sections P and Q are to be used in a command-line-interface in the directory where the analysis is performed.

Recipes

1. Cell Lysis Master Mix (Table 1)

Table 1. Cell Lysis Master Mix

Cell Lysis Master Mix	[Units]	[Stock]	[Final]	1 Rx (μl)	12Rx Master Mix (μl) ¹
Nuclease-free H ₂ O	-	-	-	1.602	21.1
SingleShot Lysis Buffer	X	6.25	1	0.48	6.3
Qiagen Protease	mAU/ml	900	45	0.15	2
dNTPs	mM	10	1	0.64	8.4
RT-UMI-Index(1-16) Primer	μM	100	1	0.064	0.8
Diluted ERCCs ²	X	100	1	0.064	0.8
-	--	-	Total	3	39.4

2. RT Master Mix (Table 2)

Table 2. RT Master Mix

RT Master Mix	[Units]	[Stock]	[Final] ²	1 Rx (μl)	48Rx Master Mix (μl) ¹
Nuclease-free H ₂ O	-	-	-	0.15	8.5
SSIV buffer	X	5	1	1.28	72
MgCl ₂	mM	1000	6	0.04	2.2
DTT	mM	300	5	0.11	6
Betaine	M	5	1	1.28	72
Template Switch Oligo	μM	100	1	0.06	3.6
RNase Inhibitor	U/μl	40	1	0.16	9
SuperScript IV	U/μl	200	10	0.32	18
-	-	-	Total	3.4	191.3

3. PCR PreAmp Master Mix (Table 3)

Table 3. PCR PreAmp Master Mix

PCR PreAmp Master Mix	[Units]	[Stock]	[Final] ⁴	1 Rx (μl)	48Rx Master Mix (μl) ¹
Nuclease-free H ₂ O	-	-	-	0.59	33
KAPA HiFi HotStart Mix	X	2	1	7	393.8
ISPCR Primer	μM	100	0.1	0.014	0.8

-	-	-	<i>Totals</i>	7.6	427.5
---	---	---	---------------	-----	-------

4. Final PCR Master Mix (Table 4)

Table 4. Final PCR Master Mix

Final PCR Master Mix	[Units]	[Stock]	[Final] ⁵	1 Rx (μl)	24Rx Master Mix (μl) ¹
Nuclease-free H ₂ O	-	-	-	8	250
Nextera PCR Master Mix	X	3.3333	1	15	468.75
P5-ISPCR Primer	μM	10	0.2	1	31.25
-	-	-	<i>Totals</i>	24	750

1. Master mix volume is calculated to account for pipetting errors.
2. To make 100× ERCC solution, dilute ERCC RNA Spike-In Mix 1, 1:200,000.
3. Final concentrations for RT master mix reagents are calculated based on a final volume of 6.4 μL (3 μL of cell lysis mix + 3.4 μL RT mix).
4. Final concentrations for PCR PreAmp master mix reagents are calculated based on a final volume of 14 μL (6.4 μL of RT reaction + 7.6 μL of PCR PreAmp mix).
5. Final concentrations for Final PCR master mix reagents are calculated based on a final volume of 50 μL (26 μL of Tagationtation reaction + 24 μL of Final PCR mix).

Acknowledgments

This work was supported by the Max Planck Society, and the Advanced Investigator award from the European Research Council (grant 743216), DFG CRC 1080: Molecular and Cellular Mechanisms of Neural Homeostasis, and DFG CRC 902: Molecular Principles of RNA-based Regulation. We thank Dr. Susanne tom Dieck, Ivy CW. Chan and current and past members of the Schuman lab for helpful discussions and advice. This protocol is derived from our previous work (Perez *et al.*, 2021; DOI: 10.7554/eLife.63092).

Competing interests

The authors declare no conflict of interest.

Ethics

The procedures involving animal care are conducted in conformity with the institutional guidelines that are in compliance with the national and international laws and policies (DIRECTIVE2010/63/EU; German animal welfare law, FELASA guidelines) and approved by and reported to the local governmental supervising authorities (Regierungspräsident Darmstadt). The animals were euthanized according to annex 2 of 2 Abs. 2 Tierschutz-Versuchstier-Verordnung.

References

1. Aakalu, G., Smith, W. B., Nguyen, N., Jiang, C. and Schuman, E. M. (2001). [Dynamic visualization of local protein synthesis in hippocampal neurons](#). *Neuron* 30(2): 489-502.
2. Alon, S., Goodwin, D. R., Sinha, A., Wassie, A. T., Chen, F., Daugherty, E. R., Bando, Y., Kajita, A., Xue, A. G., Marrett, K., et al. (2021). [Expansion sequencing: Spatially precise *in situ* transcriptomics in intact biological systems](#). *Science* 371(6528).
3. Cajigas, I. J., Tushev, G., Will, T. J., tom Dieck, S., Fuerst, N. and Schuman, E. M. (2012). [The local transcriptome in the synaptic neuropil revealed by deep sequencing and high-resolution imaging](#). *Neuron* 74(3): 453-466.
4. Glock, C., Biever, A., Tushev, G. and Bartnik, I. (2020). [The mRNA translation landscape in the synaptic neuropil](#). *bioRxiv*. doi:10.1101/2020.06.09.141960.
5. Gumi, L. F., Yeo, G. S., Tung, Y. C., Zivraj, K. H., Willis, D., Coppola, G., Lam, B. Y., Twiss, J. L., Holt, C. E. and Fawcett, J. W. (2011). [Transcriptome analysis of embryonic and adult sensory axons reveals changes in mRNA repertoire localization](#). *RNA* 17(1): 85-98.
6. Hafner, A. S., Donlin-Asp, P. G., Leitch, B., Herzog, E. and Schuman, E. M. (2019). [Local protein synthesis is a ubiquitous feature of neuronal pre- and postsynaptic compartments](#). *Science* 364(6441): eaau3644.
7. Hagemann-Jensen, M., Abdullayev, I., Sandberg, R. and Faridani, O. R. (2018). [Small-seq for single-cell small-RNA sequencing](#). *Nat Protoc* 13(10): 2407-2424.
8. Holt, C. E., Martin, K. C. and Schuman, E. M. (2019). [Local translation in neurons: visualization and function](#). *Nat Struct Mol Biol* 26(7): 557-566.
9. Macosko, E. Z., Basu, A., Satija, R., Nemesh, J., Shekhar, K., Goldman, M., Tirosh, I., Bialas, A. R., Kamitaki, N., Martersteck, E. M., et al. (2015). [Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets](#). *Cell* 161(5): 1202-1214.
10. Mazaré, N., Oudart, M. and Cohen-Salmon, M. (2021). [Local translation in perisynaptic and perivascular astrocytic processes - a means to ensure astrocyte molecular and functional polarity?](#) *J Cell Sci* 134(2): jcs251629-11. doi:10.1242/jcs.251629.
11. Middleton, S. A., Eberwine, J. and Kim, J. (2019). [Comprehensive catalog of dendritically localized mRNA isoforms from sub-cellular sequencing of single mouse neurons](#). *BMC Biol* 17(1): 5.
12. Moor, A. E., Golan, M., Massasa, E. E., Lemze, D., Weizman, T., Shenhav, R., Baydatch, S., Mizrahi, O., Winkler, R., Golani, O., et al. (2017). [Global mRNA polarization regulates translation efficiency in the intestinal epithelium](#). *Science* 357(6357): 1299-1303.
13. Ntranos, V., Yi, L., Melsted, P. and Pachter, L. (2019). [A discriminative learning approach to differential expression analysis for single-cell RNA-seq](#). *Nat Methods* 16(2): 163-166.
14. Perez, J. D., Dieck, S. T., Alvarez-Castelao, B., Tushev, G., Chan, I. C. and Schuman, E. M. (2021). [Subcellular sequencing of single neurons reveals the dendritic transcriptome of GABAergic interneurons](#). *Elife* 10: e63092.
15. Picelli, S., Faridani, O. R., Bjorklund, A. K., Winberg, G., Sagasser, S. and Sandberg, R. (2014). [Full-](#)

- [length RNA-seq from single cells using Smart-seq2](#). *Nat Protoc* 9(1): 171-181.
16. Poon, M. M., Choi, S. H., Jamieson, C. A., Geschwind, D. H. and Martin, K. C. (2006). [Identification of process-localized mRNAs from cultured rodent hippocampal neurons](#). *J Neurosci* 26(51): 13390-13399.
 17. Sakers, K., Lake, A. M., Khazanchi, R., Ouwenga, R., Vasek, M. J., Dani, A. and Dougherty, J. D. (2017). [Astrocytes locally translate transcripts in their peripheral processes](#). *Proc Natl Acad Sci U S A* 114(19): E3830-E3838.
 18. Schmid, K. T., Cruceanu, C., Böttcher, A., Lickert, H., Binder, E. B., Theis, F. J. and Heinig, M. (2020). [Design and power analysis for multi-sample single cell genomics experiments](#). *bioRxiv* 2020.04.01.019851. doi:10.1101/2020.04.01.019851.
 19. Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W. M., 3rd, Hao, Y., Stoeckius, M., Smibert, P. and Satija, R. (2019). [Comprehensive Integration of Single-Cell Data](#). *Cell* 177(7): 1888-1902 e1821.
 20. Tóth, E. N., Lohith, A., Mondal, M., Guo, J., Fukamizu, A. and Pourmand, N. (2018). [Single-cell nanobiopsy reveals compartmentalization of mRNAs within neuronal cells](#). *J Biol Chem* 293(13): 4940-4951.
 21. Vieth, B., Ziegenhain, C., Parekh, S., Enard, W. and Hellmann, I. (2017). [powsimR: power analysis for bulk and single cell RNA-seq experiments](#). *Bioinformatics* 33(21): 3486-3488.
 22. Wang, G., Ang, C. E., Fan, J., Wang, A., Moffitt, J. R. and Zhuang, X. (2020). [Spatial organization of the transcriptome in individual neurons](#). *bioRxiv* 101:123-45. doi:10.1101/2020.12.07.414060.
 23. Zhong, J., Zhang, T. and Bloch, L. M. (2006). [Dendritic mRNAs encode diversified functionalities in hippocampal pyramidal neurons](#). *BMC Neurosci* 7: 17.

Isolation of Rice Embryo Single Cell Type using Laser Capture Microdissection (LCM)Tie Liu^{1,2*}¹Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT, USA²Department of Plant Biology, Carnegie Institution for Science at Stanford University, Stanford, CA, USA*For correspondence: tieliu@stanford.edu

[Abstract] A lot of transcriptional profiling in plant and animals has used RNAs samples from many different cell types. The laser-capture microdissection (LCM) can identify and harvest pure cellular populations directly from heterogenous tissues based on histological identification. The molecules or protein isolated from LCM-captured cells can be suitable for single cell type analysis by using chip expression profiling or sequencing.

Materials and Reagents

1. Ethanol
2. Acetic acid
3. HistoClear (also named CitriSolv) (Thermo Fisher Scientific, catalog number: 5989-27-5)
4. DEPC H₂O
5. 75% (v/v) ethanol and 25% (v/v) acetic acid (see Recipes)
6. Gradient series of ethanol solutions in H₂O or histoClear (see Recipes)

Equipment

1. Microscope
2. Microtome (Waldorf, model: HM310)
3. Pix-Cell IIe LCM system (Arcturus)
4. RNase-free glass slides
5. 15 μm laser beam

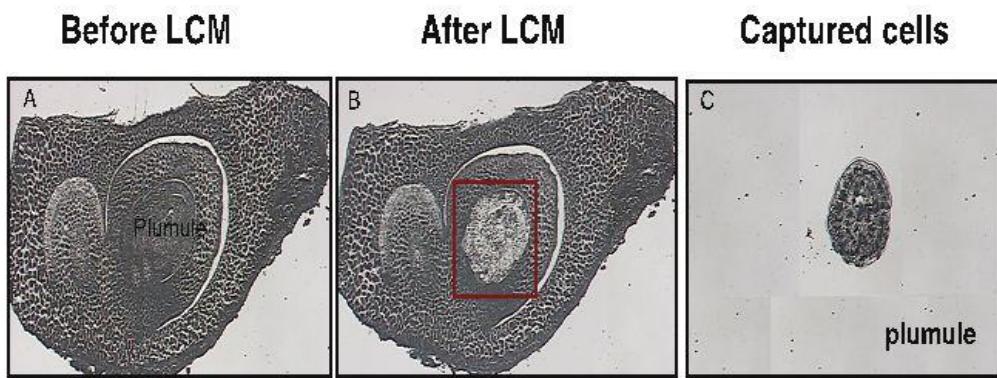
Procedure

- A. Cryosectioning, fixation and dehydration of rice embryos
 1. Dissect rice embryo from the seeds under the microscope.
 2. Fix samples immediately on ice in fixation solution containing 75% (v/v) ethanol and 25% (v/v) acetic acid. The samples were left in the vials at 4 °C over night.

3. Dehydrate the tissue in a series of ethanol concentrations (v/v) in order, 70%, 85%, 95%, 100%, each for 1 h at room temperature, followed by ethanol: histoclear (3:1, v/v), followed by ethanol: histoclear (1:1), ethanol: histoclear (1:3) and 100% histoclear treatment each for 1 h.
4. Incubate the dehydrated samples in histoclear over night at 60 degree and then infiltrate with paraffin at 60 degree over 2 days, replacing histoclear with paraffin every 12 h.
5. After embedding in paraffin, cut the embryo in 7 μm thick sections with a rotary microtome and place on RNase-free glass slides and store in darkness at 4 degree under dehydrating conditions with drierites.
6. Deparaffinize sections in histoclear at room temperature for two changes of 10 min and air-dried before LCM.

B. LCM

1. Perform laser-capture microdissection using the Pix-Cell LCM system. After deparaffinizing and drying the tissues, laser-capture microdissect the interesting cells according to the manufacturer's instructions.
2. Based on the cell size diameters, the embryo organ cell types were isolated using 15 μm laser beam, laser power settings were 100 mW, and laser pulse durations were 2.5 ms. Embryo organ cell types were successfully identified and removed from heterogenous tissue by comparison of the difference among the images of the tissue before captured, the images of the tissue after removal of the harvested cells and the images of the cells captured on the cap. Generally, between 5 to 8 slides were processed each LCM caps and non-specific tissue were removed from the LCM cap using a Post-It note. See figure below.



Recipes

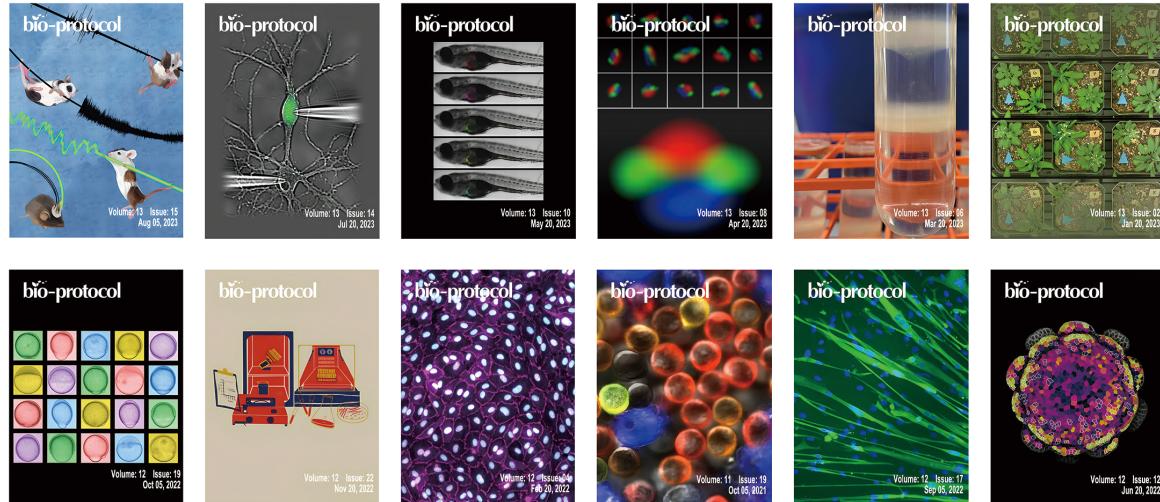
1. 75% (v/v) ethanol and 25% (v/v) acetic acid
2. Gradient series of ethanol solutions in H₂O or histoclear

Acknowledgments

This protocol is adapted from Kerk *et al.* (2003) and Jiao *et al.* (2009).

References

1. Jiao, Y., Tausta, S. L., Gandotra, N., Sun, N., Liu, T., Clay, N. K., Ceserani, T., Chen, M., Ma, L., Holford, M., Zhang, H. Y., Zhao, H., Deng, X. W. and Nelson, T. (2009). [A transcriptome atlas of rice cell types uncovers cellular, functional and developmental hierarchies](#). *Nat Genet* 41(2): 258-263.
2. Kerk, N. M., Ceserani, T., Tausta, S. L., Sussex, I. M. and Nelson, T. M. (2003). [Laser capture microdissection of cells from plant tissues](#). *Plant Physiol* 132(1): 27-35.
3. Nelson, T., Tausta, S. L., Gandotra, N., and Liu, T. (2006). [Laser microdissection of plant tissue: what you see is what you get](#). *Annu Rev Plant Biol* 57: 181-201.



A peer-reviewed, open access protocol journal

>4,800 high quality, validated, step-by-step protocols

- Contributed by **20,000+** scientists
- >91%** reproducibility (Bio-protocol user surveys)
- 1000+** videos of key procedural steps

Publish and share your protocols with us!



Bio-protocol Journal



Bio-protocol Preprint

NEW!

**Bio-protocol Preprint
Repository**

A preprint repository
for life sciences
protocols

