Protocol

Single-Neuron Isolation for RNA Analysis Using Pipette Capture and Laser Capture Microdissection

Ditte Lovatt, Thomas Bell, and James Eberwine¹

Department of Pharmacology, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania 19104

The field of single-cell analysis has greatly benefitted from recent technological advances allowing scientists to study genomes, transcriptomes, proteomes, and metabolomes at the single-cell level. Transcriptomics allows a unique window into cell function and is especially useful for studying global variability among single cells of seemingly the same type. Generating transcriptome data from RNA samples has become increasingly easy and can be done using either microarray or RNA-Seq techniques. RNA isolation is the first step of transcriptomics. Numerous RNA isolation procedures exist and differ with respect to the type and number of cells from which they are capable of isolating RNA. Although it is trivial to isolate RNA from bulk tissue or culture plates, sophisticated methods are required to capture RNA from single cells in a pool of cells or in intact tissue. We describe here the protocols used for isolating the soma of single neurons in cultures and in tissue slices using the pipette capture and the PALM or laser capture microdissection (LCM) approaches, respectively. LCM was developed to isolate cells from tissue sections primarily for pathological tissue analysis. LCM can be used to isolate individual cells or groups of cells from ethanol or paraffin-embedded formalin-fixed tissue sections and dissociated tissue cultures. The soma isolates from either technique can subsequently be used for RNA amplification procedures and transcriptome analysis. These procedures can also be adapted to other cell types in cultures and tissue sections and can be used on neuronal subcellular structures, such as dendrites.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Collection tube buffer <R>

Collection tube buffer prepares the sample for reverse transcription using Superscript III (Invitrogen). It should be adapted for the desired downstream goal, but we recommend keeping the reaction volume small and always including freshly added RNase inhibitors for transcriptomics studies.

Cultured neurons from mouse hippocampus (7–10 d in vitro)

Ethanol (absolute)

¹Correspondence: eberwine@pharm.med.upenn.edu

© 2015 Cold Spring Harbor Laboratory Press

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot072439

Frozen tissue (e.g., from rat brain)

Snap-freeze tissue of interest and store at -80°C until needed.

HEPES-buffered cell medium <R>

Pipette solution <R>

RNase-free H_2O

RNaseZap

Equipment

Air table

Cell culture incubator

Cryostat

Forceps

Glass capillary tubes (borosilicate, size/cap $1.5\text{--}1.8 \times 100$ mm; Kimble Chase 34500 99)

New, unopened packs of glass tubing can be considered RNase-free. Used, opened packs of glass capillary tubes can be made RNase-free by heat treating at $\sim 200^{\circ}\text{C}$ for 8 h or overnight. When heat treatment is used, the glass tubing should be wrapped in aluminum foil at ~ 10 glass tubes per pack. A new RNase-free pack of glass capillary tubes can be used for each experiment.

Inverted microscope with 10 $\times$, 20 $\times$, and 40 $\times$ air immersion objectives

Microcentrifuge

Microcentrifuge tubes (RNase-free, 1.5 mL)

Micromanipulators

Micropipette holder

PALM collection tubes

PALM hardware and software system

PALM membrane-coated glass slides or RNase-free glass slides (see Step 6)

Petri dish (35 mm)

Pipette puller

Pipette storage container

Plastic tubing

Polylysine-coated glass coverslips with a 12-mm diameter

Recording chamber or lid of 35 mm Petri dish

Stopcock and suction line assembly

METHOD

To prepare primary cell cultures for single-cell collection by pipette capture, begin at Step 1. To capture single cells from fresh frozen ethanol-fixed rat brain tissue sections with laser capture microdissection, begin at Step 6. Clean gloves should always be worn when handling the glass pipettes, tubes, and reagents. If a collection chamber is used, and it is not designated for single-cell analysis only, it should be cleaned with RNaseZap and rinsed five times with RNase-free water before each use.

Pipette Capture

1. Plate neurons on polylysine-coated glass coverslips according to your culture protocol of choice. After plating, culture the cells for a minimum of 2 h to allow the cells to adhere to the coverslip.

We culture neurons for 7–10 d on the coverslip such that subcellular structures (e.g., dendrites) and synaptic connections are developed.

Functional analyses (e.g., electrophysiological recordings or pharmacological treatments) can be performed before cell collection to couple functional phenotypes with transcriptomics analysis.

See Troubleshooting.

2. Prepare single-cell collection glass pipettes.

- i. Insert the glass tubing into the pipette puller according to the manufacturer's instructions. Set the pipette puller parameters to make standard whole-cell recording pipettes.

We use a Flaming/Brown Micropipette Puller Model P-87 from Sutter Instruments with the following settings: heat 680, pull N/A, velocity 20, time 111.

- ii. Inspect the tips of the pipettes under the microscope (Fig. 1); the diameter of the collection pipette tip must be about the size of the cell of interest. If the tips of the pulled pipettes have diameters much smaller than those of the cells of interest, break each tip by carefully touching and dragging it across a clean Kimwipe (Mackler and Eberwine 1993).

Alternatively, if patch-clamp analysis is performed on the cell before collection, pull the recording pipette away from the cell after the electrophysiological analysis is completed, and then gently break the tip of the recording pipette against the coverslip using the micromanipulator on the fine control setting to approximately the same diameter as the cell. This step should be done in the same visual field, but in a location as far as possible from the cell.

Pulling pipettes immediately before starting each experiment prevents accumulation of contaminating agents over time. However, if you have an RNase-free pipette holder, you can make several sets of pipettes in advance.

3. Locate the target cell using the microscope.

- i. Prepare collection tubes with collection tube buffer, and store them on wet ice during the collection step. Also, if RNA will not be processed immediately after collection, prepare a separate container with dry ice for storing collected cells.
- ii. Before taking neurons out of the incubator, make sure that the microscope, pipette holder, stopcock, and suction line assembly are ready to use (Fig. 2).
- iii. Place 4 mL of prewarmed (37°C) HEPES-buffered cell medium in a 35-mm Petri dish, and place 3 mL in the inverted lid of a 35-mm Petri dish.
- iv. With a clean pair of forceps, gently rinse the coverslip to which the neurons are affixed by submerging it in the HEPES-buffered cell medium in the Petri dish. Immediately transfer the coverslip to the lid of the Petri dish. Gently press down on the coverslip with the forceps to ensure that the coverslip does not float around.
- v. Immediately proceed to the microscope. Focus on the cells using a 20× objective and scan the culture with the stage manipulators to find a target cell. Place the target cell in the center of the visual field. Visually check the integrity of the cell under higher magnification (e.g., using a 40× objective).

Make sure the target cell looks healthy and bright phased without large vacuoles or other unusual protrusions erupting from the cell membrane. Also, select cells that are firmly attached to the coverslip. To test the cell's attachment, gently tap the microscope stage and visually observe if the cell moves on the coverslip. Proper adhesion to the coverslip prevents the cells of interest from floating away from the collection pipette.

FIGURE 1. Visual inspection of pipette tip sizes. After pulling the glass pipette on the pipette puller, it is necessary to adjust the tip size by breaking it against the coverslip or by dragging it over a clean Kimwipe. (A) Before adjusting the tip size, the pipette tip size is too small for capturing cells. (B) An optimal result after adjusting the tip size. (C) After adjusting the tip size, the resulting tip is too large, and the pipette should be discarded.

FIGURE 2. Pipette holder and suction line assembly. (A) Disassembled materials: (1) pipette holder without pipette; (2) plastic tubing; (3) connected stopcock; and (4) 1-mL sterile syringe. (B) Properly assembled pipette holder and suction line. (C) Pipette holder with pipette and suction line assembly setup on the inverted microscope with a Petri dish lid containing buffer and the coverslip with cells.

vi. Once a cell is selected, change the objective to lower magnification (e.g., a 10× objective).

4. Capture single cells for mRNA reverse transcription.

- i. While wearing gloves, take one of the collection pipettes and fill the tip of the pipette by dipping it in a microcentrifuge tube that is filled with pipette solution for ~3 sec. Visually check the pipette for the fluid level: Solution should be present in the narrow area of the tip (i.e., in the first ~5 mm of the pipette).

Do not strike the tip of the pipette against the side of the microcentrifuge tube. If this occurs, the tip might now be broken and too large to use, so discard it.

See Troubleshooting.

- ii. Place the collection pipette in the pipette holder (Fig. 2C). Make sure the syringe is at 0.4 mL. Open the stopcock on the suction line. While carefully applying 0.1 mL positive pressure via the syringe on the suction line, lower the pipette into the bath using micromanipulator at the coarse control setting. Once the pipette is submerged in the bath, close the stopcock and gently place it down on the air table.
- iii. Under low magnification (e.g., using a 10× objective), find the tip of the pipette and place it in the center of the visual field.

Finding the tip of the pipette can be a challenging step.

See Troubleshooting.

- iv. After finding the tip, change the magnification to a 20× or 40× objective, and slowly lower the pipette close to the target cell. Switch the micromanipulator to the fine control setting, and place the pipette tip directly next to the cell (Fig. 3).

FIGURE 3. Pipette capturing of single neuron. (A) Before capturing a single cell in neuronal cultures. (B) During capture and after applying negative pressure to the suction assembly line. This step is only a few seconds in duration. Note that it is only the soma of the neuron that is collected. This should be taken into consideration when interpreting, for instance, transcriptome results because the dendrites also contain RNA. (C) After capture, validating that only a single cell was captured.

Placing the pipette very close to the cell reduces the amount of negative pressure that is needed to collect it. Be careful not to touch the cell or glass coverslip with the tip; this may damage the cell or break the tip.

- v. To collect the cell, open the stopcock and gently begin to apply negative pressure capacity. If the pipette is next to the cell, you will start to see the cell move with a small amount of pressure. Continue to gently increase the amount of negative pressure until the cell is pulled off the coverslip and goes completely into pipette. At this point, stop applying negative pressure and close the stopcock immediately to prevent an excess amount of buffer from filling the pipette. Immediately raise the pipette out of the bath. Using clean gloves, remove the pipette from the pipette holder, and proceed immediately to Step 5.

See Troubleshooting.

5. Transfer the collected cell into the collection tube.

- i. Immediately after removing the pipette from the pipette holder, take a collection tube with one hand, and with the other hand hold the pipette close to the tip just like you would hold a pen when writing.
- ii. Gently break the glass tip on the side of the tube under the 100- μ L mark. Ideally, most of the tapered region on the pipette should be broken away. If necessary, aspirate the volume of the pipette by inserting a syringe into the top of the pipette (see Mackler and Eberwine 1993).

See Troubleshooting.

- iii. Close the tube, centrifuge briefly in a microcentrifuge to force all contents to the bottom of the tube, and place the tube on dry ice.

*The cells can be stored at -80°C until needed, but best results are obtained if reverse transcribed immediately using the procedure for first-strand cDNA synthesis in Protocol: **Antisense RNA Amplification for Target Assessment of Total mRNA from a Single Cell** (Morris et al. 2014).*

Laser Capture Microdissection

6. Prepare frozen tissue sections for LCM.

- i. Cut 10- μm sections with a cryostat and mount on RNase-free glass slides.
- ii. Fix frozen sections via dehydration in ice-cold 70% ethanol for 3 min.

No modifications are necessary for preparing frozen sections, although mounting media should be omitted as it will interfere with the laser cutting and lifting steps. Usually, the cells can be microdissected from standard glass slides or specialized membrane-coated laser capture glass slides. Specialized PALM membrane-coated laser capture glass slides (Zeiss) are designed to improve sample collection. The membrane improves the sample collection by adding structural support underneath the sample, which helps lift the entire region of interest from the slide. In the absence of the support membrane, the entire sample is not always lifted from the slide.

Store slides at -80°C until needed. If desired, tissue sections can be stained with standard histological staining procedures before collecting single cells.

7. Collect single cells by LCM.

- i. Add 5 μ L of RNase-free water to the cap of the PALM collection tube. Place the PALM collection tube in the collection tube holder.

Alternatively, PALM collection tubes with adhesive caps can be used without any buffer solution in the cap. After the collection, briefly centrifuge the sample to the bottom and then add 5 μ L of RNase-free water.

- ii. Place the slide containing the tissue section on the inverted microscope. Focus on the cells with a 20 $\times$ objective. To find the appropriate laser energy to cut the sample, simultaneously start cutting the sample and adjusting the laser power until the laser cuts a fine line (e.g., pencil line).
- iii. Focus on the cells and scan the section with stage manipulators until a target cell is located. Place the target cell in the center of the visual field. Check the integrity of the cell under high magnification using a 40 $\times$ objective, and photograph the area with the cell of interest.
- iv. Select the target cell or area of interest by outlining the region with the manufacturer's collection software (e.g., PALM). Activate the laser via the software to catapult the sample into the collection tube. Confirm that the sample was dissected from the slide and photograph the area without with cell. Briefly centrifuge the sample and all contents to the bottom of the tube.

*Store the sample at -80°C or immediately reverse-transcribe immediately using the procedure for first-strand cDNA synthesis in Protocol: **Antisense RNA Amplification for Target Assessment of Total mRNA from a Single Cell** (Morris et al. 2014).*

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 1): Neurons are not plated at an optimal density.

Solution: Plating neuronal cultures at low density (60,000 cells/mL) allows easy access to individual neurons. Although high-density (400,000 cells/mL) cell cultures can also be used for single analysis, this makes the cell collection step more challenging.

Problem (Step 4.i): Pipette tip fills with too much or not enough buffer.

Solution: If the solution is not visible in the pipette, re-dip the pipette into the buffer for another ~ 3 sec. If it fails again, discard the pipette and start again with another. If the pipette substantially fills up with buffer after ~ 3 sec (>8 mm), the pipette tip is too big and should be discarded.

Problem (Step 4.iii): It is difficult to find the tip of the pipette in the field of view.

Solution: 1. After locating the target cell on high magnification ($\sim 40\times$), switch to the lowest magnification objective on the scope ($\sim 10\times$). 2. While applying positive pressure via the suction line into the pipette, lower the pipette into the bath. Once the pipette is submerged in bath, stop lowering the pipette. Close the stopcock and gently place it on the air table. 3. Next, use the pipette micromanipulators to move the pipette back and forth across the bath in the horizontal direction. The shadow of the pipette should be visible traveling across the bath. If not, move the focal plane from the cells toward the surface of the bath until the shadow of the pipette is visible. 4. Once the shadow of the pipette is visible, move the pipette until the tip of the pipette is visible. The shadow may start to disappear, as you move toward the tip. If this occurs, adjust the focus until the shadow reappears. 5. Once the tip is found and placed in the center of field of view, simultaneously start lowering the pipette and moving the focal plane down toward the cells. This approach will allow you to follow the tip down to level of the cells. 6. Once both the cells and tip are in the same focal plan, switch the micromanipulator to the fine-control setting to lower the pipette directly next to the target cell.

Problem (Step 4.v): It is difficult to collect cells from high-density cultures.

Solution: To collect cells from high-density cultures, the cells must be firmly attached to the coverslip. To prevent other cells from entering the pipette, limit negative pressure to 50% of your capacity. Place the pipette as close as possible to the cell. Start with low negative pressure ($\sim 10\%$ of capacity) on the target cell and slowly increase the negative pressure until the target cell pulls off of the coverslip. Once the cell completely enters the pipette, it will be pulled out of the visual field. At this point, stop applying negative pressure.

Problem (Step 4.v): The target cell will not come off the coverslip.

Solution: The two most common problems are (1) the pipette tip is too far away from the cell or (2) there is bath solution in the suction line. If the cell does not move by the time $\sim 50\%$ of the negative pressure capacity is reached, simultaneously move the pipette closer to cell and continue applying negative pressure until $\sim 75\%$ of the negative pressure capacity is reached. If bath solution is in the suction line, clean the suction line and restart the collection with a fresh pipette.

Problem (Step 5.ii): Difficulty is encountered in putting cell into the collection tube.

Solution: Once the cell is collected, the final step is to safely place the harvested cell into the collection tube. The approach that yields the best results in our laboratory is to gently break the glass tip on the side of the microfuge tube. Some investigators prefer to expel the cell contents from the collection pipette via positive pressure into the collection tube. One key negative to this approach is the large volume of bath solution that can be expelled into the collection tube. Most downstream reactions are salt-sensitive, so the additional bath solution can introduce unwanted variability in the subsequent reactions. Using the “tip breaking approach” only adds a small amount ($\sim 1\text{--}2\ \mu\text{L}$) of bath solution to the collection tube. With this approach our single-cell reverse transcription-polymerase chain reaction (RT-PCR) success rate is $\sim 90\%$ (Mackler and Eberwine, 1993).

Problem (Step 7.iv): The sample will not catapult off the slide.

Solution: Select a cell or area away from the area of interest. Repeat Step 8.ii, but increase the laser energy until the laser cuts a broader line (e.g., $\sim 2\times$ broader than original fine pencil line) and repeat the collection process. If the collection fails again, continue to increase thickness of the cutting line by $2\times$ increments until the sample lifts off. Alternatively, if another slide is available, try changing to a new slide.

Problem (Step 7.iv): Molecular analysis fails.

Solution: Sample collection and catapult efficiency can be verified by visualizing the sample in the collection device with the appropriate objective ($10\times$, $20\times$, or $63\times$) by using the software function “go to checkpoint.” This function moves the slide out of the light path so the contents in collection tube can be viewed. Most samples can be found in the cap. If using fresh frozen sections, the samples should be ethanol fixed and harvested/collected within $\sim 5\text{ d}$ of sectioning and freezing. Simultaneously process RNA control samples (Step 9) with the LCM samples to ensure that all the molecular reagents are working satisfactorily.

DISCUSSION

Sophisticated methods are required to capture RNA from single cells in a pool of cells or in intact tissue (Morris et al. 2011; Eberwine et al. 1992). The pipette approach provides an easy method to isolate RNA for single-cell transcriptomics for studies in which cultured cells provide an appropriate model system. This approach also allows the combination of functional electrophysiology or imaging with transcriptomics. The collection process can be recorded by video or by images to document the removal of only one cell from the coverslip, thus providing a comforting level of quality control postcapture. Furthermore, the protocol can also be adapted to collect subcellular structures, such as dendrites. Finally, pipette isolation of individual cells has been used successfully to harvest the mRNA from cells in the live tissue slice preparation. This allows analysis of the RNA from single cells that are

D. Lovatt et al.

REFERENCES

Eberwine J, Yeh H, Miyashiro K, Cao Y, Nair S, Finnell R, Zettel M, Coleman P. 1992. Analysis of gene expression in single live neurons. *Proc Natl Acad Sci* **89**: 3010–3014.

Mackler S, Eberwine J. 1993. Diversity of glutamate receptor subunit mRNA expression within live hippocampal CA1 neurons. *Mol Pharm* **44**: 308–315.

Morris J, Singh JM, Eberwine JH. 2011. Transcriptome analysis of single cells. *J Vis Exp*.

Morris J, Bell TJ, Buckley PT, Eberwine JH. 2014. Antisense RNA amplification for target assessment of total mRNA from a single cell. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot072454.

Cold Spring Harbor Protocols

Single-Neuron Isolation for RNA Analysis Using Pipette Capture and Laser Capture Microdissection

Ditte Lovatt, Thomas Bell and James Eberwine

Cold Spring Harb Protoc; doi: 10.1101/pdb.prot072439

Email Alerting Service

Receive free email alerts when new articles cite this article - [click here](#).

Subject Categories

Browse articles on similar topics from *Cold Spring Harbor Protocols*.

[Analysis of Gene Expression](#) (197 articles)
[Analysis of Gene Expression, general](#) (129 articles)
[Neuroscience, general](#) (357 articles)
[RNA](#) (317 articles)
[RNA, general](#) (269 articles)

To subscribe to *Cold Spring Harbor Protocols* go to:
<http://cshprotocols.cshlp.org/subscriptions>