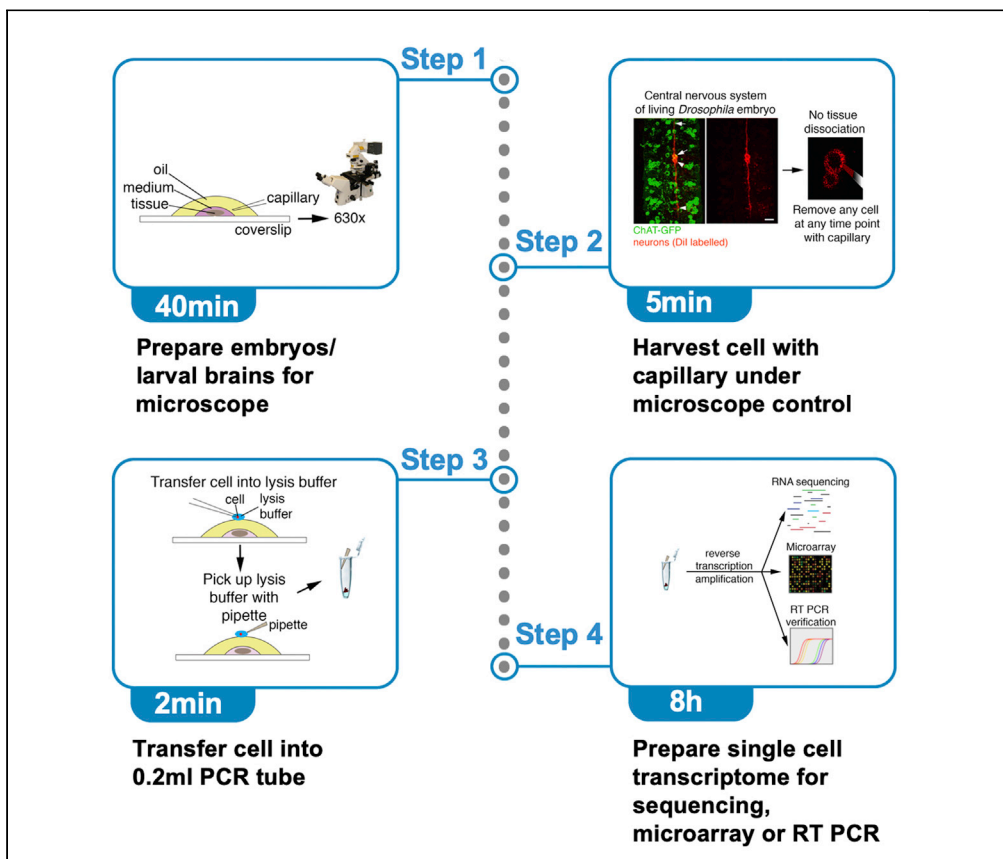


Protocol

Direct isolation of single cells from living brains of *Drosophila melanogaster* without dissociation for transcriptome analysis



Here, we describe a protocol to remove single identified cells directly from *Drosophila* living brains and analyze their transcriptome. We detail the steps to harvest fluorescent cells using a capillary under epifluorescence and transmitted light to avoid contamination. We then outline the procedure to obtain the transcriptome by reverse transcription and amplification. The process from cell harvesting to the initiation of reverse transcription only takes 2 min, thus avoiding transcriptional activation of cell damage response or cell death genes.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Claudia S. Barros,
Torsten Bossing

torsten.bossing@
plymouth.ac.uk

Highlights

Single-cell
transcriptome
analysis of cells
isolated from
Drosophila living
brains

Harvesting of
fluorescent cells
without dissociation
using a capillary

Cell identity is known
at isolation without
the need to designate
by software clustering

Time points of cell
harvest can be
precisely reproduced

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Protocol

Direct isolation of single cells from living brains of *Drosophila melanogaster* without dissociation for transcriptome analysisClaudia S. Barros¹ and Torsten Bossing^{1,2,3,*}¹Peninsula Medical School, University of Plymouth. John Bull Building, 16 Research Way, Plymouth PL6 8BU, UK²Technical contact³Lead contact*Correspondence: torsten.bossing@plymouth.ac.uk
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SUMMARY

Here, we describe a protocol to remove single identified cells directly from *Drosophila* living brains and analyze their transcriptome. We detail the steps to harvest fluorescent cells using a capillary under epifluorescence and transmitted light to avoid contamination. We then outline the procedure to obtain the transcriptome by reverse transcription and amplification. The process from cell harvesting to the initiation of reverse transcription only takes 2 min, thus avoiding transcriptional activation of cell damage response or cell death genes. For complete details on the use and execution of this protocol, please refer to Barros and Bossing (2021), Bossing et al. (2012), Gil-Ranedo et al. (2019), and Liu and Bossing (2016).

BEFORE YOU BEGIN

We developed and have used this protocol for the removal and transcriptional analysis of single cells from embryonic brains and larval brains of *Drosophila*. Because cell position and identity at harvesting is known, oversampling followed by clustering is not required, reducing the necessary sample number and costs significantly.

The protocol can also be applied for the harvesting and transcriptional analysis of single cell culture cells and cells from tissue samples. Only the capillary diameter, which should be equal to 2/3 of the diameter of the cells intended to be harvested, but not the capillary shape, needs to be adjusted.

Preparation 1: Capillaries

⌚ Timing: 2–3 h

1. Pull capillaries.

Pull borosilicate capillaries without inner filament (Outer diameter 1 mm, inner diameter 0.58 mm, Harvard Apparatus, Cat. No. 30-0017) using a Sutter P-97 puller with a 2 mm square box filament (Figure 1).

Note: We use the following settings: Heat: Ramp -20, Pull:35, Velocity:40, Time:130, Air:500.

Note: The shape of the capillaries should be comparable to patch clamp needles with a short shunt (Figure 2A).



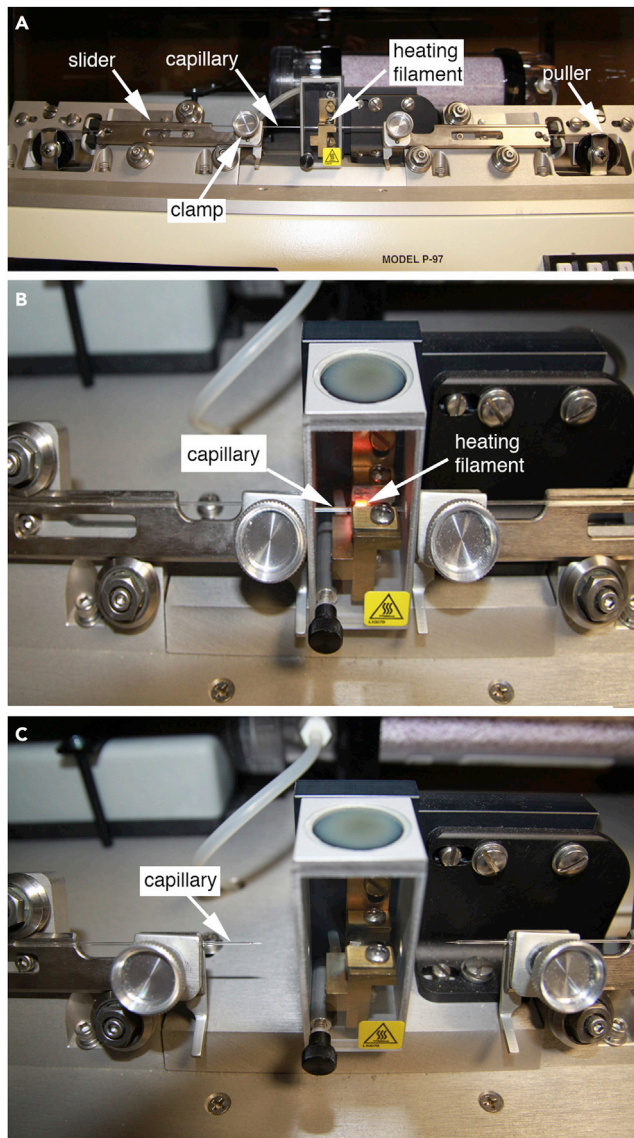


Figure 1. Pulling of capillaries

(A) At the start the capillary is inserted into the groove which connects both clamps. Alignment with the groove guides the capillary through the middle of the heating filament.

(B) Both clamps are pulled toward the filament, screws are tightened and the filament activated (red glow).

(C) After the pull is complete two identical capillaries with defined pointed tips have been created.

Note: The puller will loop once.

2. Bevel the capillaries.

- a. Bevel the capillaries to create a tip which allows a tight seal with the cells and an opening between 3 to 4 μm depending on cell size.

Note: Pressing the capillary harder onto the grinding plate will increase the capillary's opening.

- b. Capillary tips need to beveled under water to prevent blockage of the tip by glass splinters.

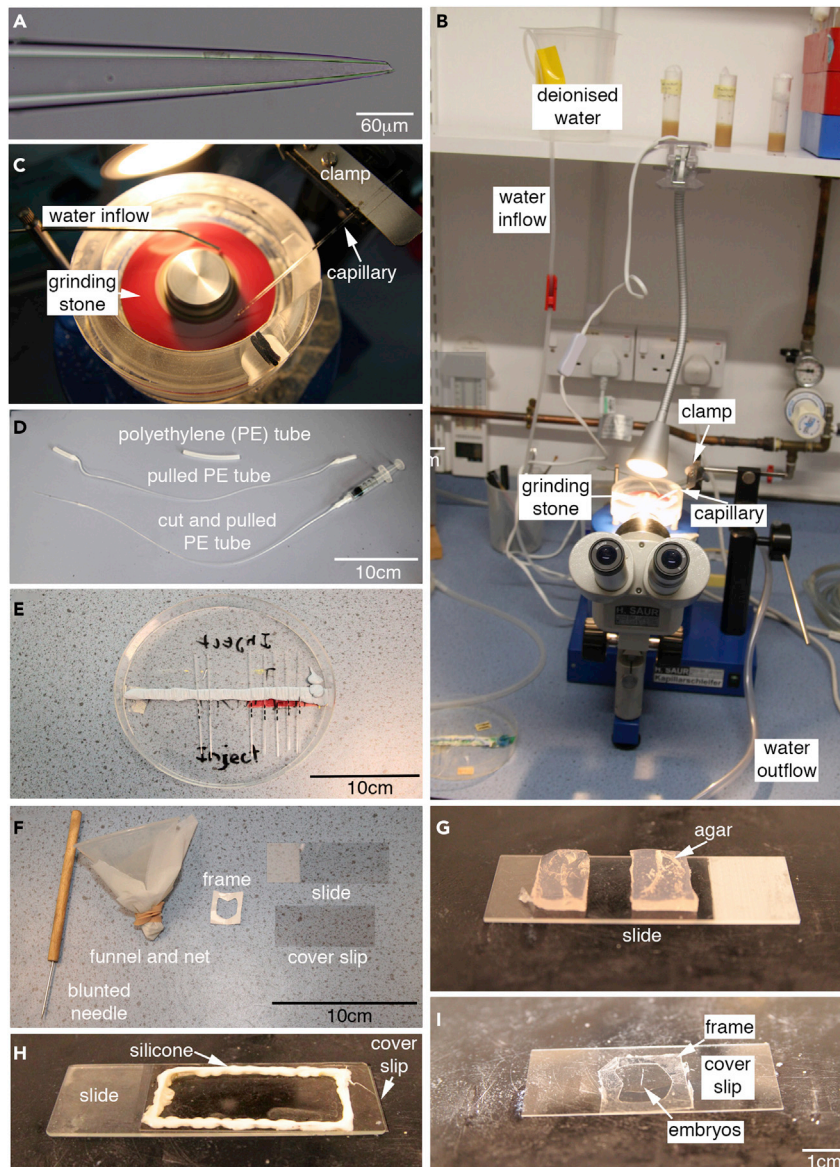


Figure 2. Preparation for single cell removal

(A) Shape of the capillary after beveling. The angle of the tip should be approximately 40° . The tip diameter should be $2/3$ of the diameter of the cell targeted for removal.

(B) Capillary tip beveller. The grinding stone needs to be constantly rinsed with de-ionized water. Water flow is regulated by gravity and a tube clamp (red). The capillary is inserted into a clamp with an adjustable angle. This allows to set the angle of the tip. Time of beveling and pressure onto the grinding stone defines the opening diameter of the tip.

(C) The grinding stone with water inflow. Black line labels observation window and is used to adjust the capillary tip before lowering onto the stone.

(D) The tube connecting the syringe needs to be pulled over a Bunsen burner. Unpulled PE tube (top) and finished PE tube (bottom).

(E) Capillaries can be re-used and stored in petri dishes pressed into a strip of plasticine. Tips cannot touch the dish to avoid breakage.

(F) Materials required to bleach embryonic chorion, to orientate embryos and for the transfer onto glue-coated cover slip.

(G) Setup for aligning and orientating embryos.

(H) Coverslip with silicone border used to align larval brains.

(I) Aligned embryos surrounded by frame to prevent oil run off.

- c. We use a microtip beveller (Figures 2B and 2C, Kapillarschleifer H.Saur Laborbedarf, Reutlingen, Germany).
- d. Adjust the capillary holder to a 30° angle.
- e. Insert the capillary into the middle groove of the holder.
- f. Grab the capillary just below the clamp of the holder and move forward until the tip is above the center of the observation window of the beveller.

Note: Avoid touching the end of the capillary.

- g. Lower the capillary into the observation window.
- h. Change the scope of the beveller to 2× magnification and focus on the tip of the capillary.

Note: If the tip is not visible, move the capillary forward.

- i. After focusing on the tip change the magnification of the dissecting scope attached to the beveller to 4×.
- j. Slowly lower the tip towards the grinding stone.

Note: The water will obscure the tip and any contact between tip and stone will not be visible.

- k. Observe the tip and stop lowering when water flows into the needle. Keep beveling the tip for approximately 10 s;
- l. Raise the capillary, remove it from the clamp and connect the pressure tube with syringe attached to the end of the needle (Figure 2D);
- m. Hold the midst of the capillary and increase the pressure in the tube by pressing the piston of the syringe.
- n. Hold piston and slowly lower the tip of the capillary into the watch glass with deionized water.
- o. Stop lowering when bubbles can be seen.
- p. Wash the capillary three times by pumping the syringe up and down.

Note: Seeing the liquid moving into the needle is usually not possible.

- q. Suck up the liquid for 5 s and blow out the water until bubbles can be seen.
- r. Repeat the procedure with acetone.
- s. Transfer the capillary onto a slide, securing it in place with a piece of Blu Tack.
- t. Check under a microscope (100× magnification) for shape and diameter to ensure the correct pressure was used during grinding.
- u. Store the capillaries in a 21 cm diameter petri dish, securing them into a line of plasticine or Blutack (Figure 2E).

Note: Capillary tips must not touch the bottom of the dish.

△ CRITICAL: After beveling the capillaries, the tips must be cleaned with water followed by acetone. After the first use, capillaries can be stored in petri dishes (Figure 2E). Used capillaries can be re-used but tips need to be filled with halocarbon oil during storage.

Preparation 2: Polyethylene (PE)/ pneumatic tubing

⌚ **Timing:** 10 min

3. Cut 10 cm pieces out of PE tubing (6 mm O.D., 4 mm I.D.).
4. Switch-on a Bunsen burner and adjust oxygen to get a straight and blue flame.
5. Hold both ends of the tubing piece and move the middle over the tip of the flame.

6. Slowly rotate the piece in the flame until the tube turns transparent.
7. Immediately move the tube out of the flame and begin to slowly pull until the length tripled (Figure 2D).

Note: Bunsen burner can be replaced with a heat gun.

△ **CRITICAL:** Only pull PE tubes in a well-ventilated area and out of a draft. The flame must not flicker. Tubes will break and melt if too long over the flame but never burn. To avoid melting remove tube out of the flame as soon as the tube becomes transparent. Pull slowly (with 'snail speed') and stop pulling when tube turns opaque again.

Preparation 3: Glue

⌚ **Timing:** 30 min

8. Cut double-sided Scotch 3 M tape (Size OSFA) into 5–10 cm long pieces.
9. Fill a 50 mL glass bottle with tape cuts. Push the tape down to compact the tape inside the bottle.

Note: The bottle should be filled with tape cuts tightly.

10. Fill the bottle with n-heptane. Close the lid and wrap with parafilm.
11. Place the bottle on a tube roller and let it roll overnight.
12. Next day remove most of the tape using forceps.

Note: Glue cannot be easily removed from the inside of the bottle making it unsuitable for the storage of any other liquids. Set one bottle aside for glue making and storage.

Preparation 4: Egg lays

⌚ **Timing:** 65 min

13. Bring to the boil 600 mL of tap water.
14. Add 23 g agar (Agar Agar fine powder, Amazon) and 200 mL Ocean Spray Classic Cranberry Juice (Amazon) into a 500 mL flask. Dissolve partially by swirling the flask.
15. At boiling point of the tap water add the partially dissolved agar solution and stir 2–3 times with wooden cooking spoon.
16. Wash out the flask twice with 100 mL juice and empty each time into the boiling solution.
17. Boil the agar for 10 min and stir continuously.
18. Take off the heat and cover the bottom of a small petri dish (5 cm, Fisher Scientific Cat. No. 16640272) with the liquid.
19. Place petri dish into freezer for 3 min. Test if agar solidifies.
20. If agar solidifies pour into small petri dishes.

Note: A cover of approximately 5 mm thickness is sufficient.

21. Add 100 mL of tap water into a 250 mL beaker.
22. Slowly sprinkle dried live yeast pellets (Allison Dried Active Baking Yeast, Amazon) into the water and stir with a spatula.
23. Continue adding yeast until you created a paste of the consistency of tooth paste.

Note: Keep paste in fridge and cover the beaker with tin foil to slow down evaporation. Yeast will last up to 4 weeks in the fridge. Discard if the paste turns dark brown.

△ **CRITICAL:** Before you expose the flies to the egg lays, add a pea size amount of yeast paste on the egg lay and warm them up for 30 min at 25°C.

Preparation 5: Sample preparation

⌚ **Timing:** 70 min (30 min for step 24, 30-40 min for step 25)

This protocol allows harvesting of single cells from embryos or larval brains.

24. Embryo preparation.

- a. Remove yeast from the egg lay plate.
- b. Cover the plate with commercial bleach (1% NaOCl/ H₂O).
- c. Coat the middle of a long coverslip (24 × 60 mm) with glue (see Material and Methods), spread the glue evenly between both long edges (Figure 2F) using a small coverslip (18 × 18 mm).
- d. After 5 min on the orbital shaker (60 rpm) wash the embryos into a funnel of which the end is closed off with a fine mesh (Figure 2F, www.flystuff.com, Cat. 57-102).
- e. Dry the tip of the net by touching it three times onto a tissue.
- f. Remove the net from the funnel and spread out on a piece of tissue.
- g. Cut out two pieces of agar from an empty egg lay and place them on a slide (Figure 2G).
- h. Pick up embryos from the mesh with a fine brush under a dissecting microscope (10× magnification) and deposit on one of the agar pieces.
- i. Pick each embryo up from the first agar piece with a blunt needle (Figure 2F) and transport to the second agar piece.
- j. Orientate the embryo with the needle so that the ventral side is pointing up.
- k. Repeat with 10 embryos; forming a line on the second agar piece.
- l. After the glue coated coverslip is touch dry, stick a cut out frame made from book binding foil (Figures 2F and 2I) over the glue area.

Note: Make sure to place the frame on the glue coated side of the coverslip.

- m. Turn the glue coated side of the coverslip towards the embryos and slowly let the coverslip fall onto the embryos.
- n. Under the dissecting scope (10× magnification), press with the blunt needle onto the coverslip along the embryo line.

Note: Observe under the dissecting scope that the embryos make contact with the coverslip.

- o. Use the blunt needle to lift the coverslip from one side off the agar.
- p. When the edge is freed from the agar grab the coverslip and turn it around (Figure 2I).
- q. Desiccate the embryos for about 6 min (21°C, 35% Relative Humidity).
- r. Cover the embryos with halocarbon oil.

△ **CRITICAL:** Embryos will only adhere strongly onto coverslips when glue is touch dry.

25. Larval brain preparation.

⌚ **Timing:** 30–40 min

- a. Place larvae into a drop of 1 × PBS.

- b. Using two sharp tweezers (Dumont No.5) take hold of the mouth hooks of the larvae and tear the larvae open. Please see <https://www.youtube.com/watch?v=GgrkPnfZnoo> (courtesy of Suzuki Laboratory, Tokyo Institute of Technology) for detail.

Note: The central nervous system (CNS) will be expelled together with the oesophagus.

- c. Fill a L-polylysine (Merck Cat. No. P8920) coated long coverslip (24 × 60 mm) of which the edges have been lined with silicone (Figure 2H) with Schneider's medium (Merck, Cat. No. 59895).
d. Use a blue pipette tip wetted in 1 × PBS 10% Fetal Bovine Serum (FBS, Fisher Scientific, Cat. No. 15818947) to transfer the dissected brain.
e. Use the side of a fine tungsten needle to gently maneuver the brain into one third of the coverslip, not into the center.

Note: Never touch the CNS with the needle tip.

- f. Immediately push the brain with the side of the needle onto the bottom of the coverslip.

Note: Press the hemispheres onto the coverslip if removal of cells from the hemispheres is intended. Turn the CNS on its side if removal of cells from the ventral nerve cord is intended.

- g. Mechanically disrupt the blood brain barrier with the fine tungsten needle under 10× magnification.
h. Aspirate most of the Schneider's medium leaving only a small drop around the brain.

Note: Do not let the brain fall dry.

- i. Fill up the whole coverslip with halocarbon oil.
j. Mark the position of the brain with a permanent marker line onto the silicone frame.

△ CRITICAL: To create the sticky polylysine coating, first attach the cover slip onto a slide with a drop of immersion oil. Wash the coverslip with 100% Ethanol followed by a quick scrub with a dishwasher-soaked sponge. Without washing off the dishwasher remnants, apply 0.1% Polylysine solution immediately, covering the entire surface for 10 min. Let the polylysine solution run into a tissue and keep the coverslip overnight upright. Since the polylysine coat tends to dissolve in Schneider's medium, attempt to attach the brain quickly. If the brain does not attach after two trials use a new coverslip.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Drosophila melanogaster embryo (stage 8–17)	Bloomington Drosophila Stock Center	RRID:BDSC_5
Drosophila melanogaster larvae (4 h, 24 h and 72 h after hatching)	Bloomington Drosophila Stock Center	N/A
Chemicals, peptides, and recombinant proteins		
Dithiothreitol (DTT)	Thermo Scientific	Cat.No. 10578170
DEPC treated water	Thermo Scientific	Cat. No. 10213130
Expand Long Template PCR System	Sigma	Cat. No. 11681834001
L-polylysine	Merck	Cat. No. P8920
NP-40 alternative	Merck	Cat. No. 492016

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Schneider's medium	Merck	Cat. No. 59895
SuperScript™ II Reverse Transcriptase	Invitrogen	Cat. No. 100004925
SUPERase In™ RNase Inhibitor	Invitrogen	Cat. No. AM2694
RNase H	Thermo Fisher Scientific	Cat. No. EN0201
RNase inhibitor	Merck	Cat. No. 556881
Halocarbon Oil 700	Sigma	Cat. No. H8898
Acetone	Fisher	Cat. No. 11374708
Oligonucleotides		
Primer: Anchored polyT	(Bossing et al., 2012)	AAGCAGTGGTATCAAC GCAGAGTACT ₍₂₆₎ VN
Primer: SM	(Bossing et al., 2012)	AAGCAGTGGTATCAACGC AGAGTACGCrGrGrG Bold: primer consensus for nested primer
Primer: Nested AAGCAGTGGTATCAACGCAGAGT	(Bossing et al., 2012)	AAGCAGTGGTATCAACGCAGAGT
Other		
Borosilicate capillaries	Harvard Apparatus	Cat. No. 30-0017
Capillary puller	Sutter Instrument	P-97
Dissect Needle	Fisher	Cat. No. 11845091
Dissecting scope for sample preparation	Nikon	SMZ645
Dissecting scope for drop pickup after cell harvest	Leica	Zoom 2000
Inverted microscope	ZEISS	Axiovert
Micromanipulator	Maerzhaeuser, Wetzlar, Germany	STM-3
Microtip beveller	Kapillarschleifer H.Saur Laborbedarf, Reutlingen, Germany	N/A
Pneumatic tubing (6 mm O.D., 4 mm I.D.)	Fisher Scientific	Cat. No. 10455951
Thermal cycler	Fisher Scientific	Cat. No. 12196530

MATERIALS AND EQUIPMENT

1× PBS buffer (for 1 L)	Final concentration	Amount
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na ₂ HPO ₄	10 mM	1.44 g
KH ₂ PO ₄	1.8 mM	0.24 g

Set buffer up 10×, adjust pH to 7.4 with HCl and store at room temperature. Storage time up to 1 year.

Lysis mix	Final concentration	Amount
10% NP40 in DEPC H ₂ O	0.04%	4 μL
0.1 M DTT	0.02 M	200 μL
DEPC H ₂ O	N/A	796 μL
Total	N/A	1 mL

Note on storage conditions.

△ **CRITICAL:** Lysis Mix has to be made fresh on day of cell harvest. It cannot be stored or frozen. Keep at 4°C.

Reverse transcription (RT) mix	Amount
10 μ M anchored polyT primer (see key resources table for sequence)	0.3 μ L
10 μ M SM primer (see key resources table for sequence)	0.3 μ L
RNase inhibitor (Superase, Ambion)	0.4 μ L
Lysis Mix	2 μ L
Total	3 μL

△ CRITICAL: Prepare a fresh mix on the day of use. Set up a master mix for 10 samples. Aliquot 3 μ L each into 10 0.2 mL thin walled PCR tubes. Store in a cold PCR cooling block until use. Do not freeze.

Superscript II/ RNase inhibitor (SR) mix	Amount
Superscript II reverse transcriptase	3 μ L
RNase Inhibitor (Merck)	0.5 μ L
Total	3.5 μL

△ CRITICAL: Prepare a fresh mix every day. Store in PCR cooling block until use. Do not freeze. Superscript II gave superior results to Superscript III or IV. Use both RNase inhibitors because although they have a significant overlap, Merck RNase inhibitor also inactivates RNase T2.

STEP-BY-STEP METHOD DETAILS

Harvesting the cells

⌚ **Timing:** 10 min workspace preparation and 30 min/ session

This section describes the steps required to remove single cells from living tissues. See [Figures 3A](#) and [3B](#) for setup.

1. Workspace preparation:
 - a. Install a dissecting scope next to the inverted microscope intended to use for micromanipulation. 10 \times magnification is sufficient.
 - b. Cool down the centrifuge to 4°C.
 - c. Fill a styrofoam box with dry ice.
 - d. Spray the work bench area that will be used with RNase decontamination solution (RNAaseZap, AM9780, Invitrogen).
2. Capillary adjustment and block.
 - a. Attach the capillary to the polyethylene (PE) tube.
 - b. Insert needle into capillary holder but do not tighten clamp at this point.
 - c. Install holder into the micromanipulator.
 - d. Place the coverslip with the embryos/ brain, but without the slide, into the universal sample holder and onto the microscope stage ([Figure 3B](#)).
 - e. Switch on the transmitted light and manually move the tip of the capillary into the light cone.
 - f. Clamp the capillary.
 - g. Using a 10 μ L pipette deposit 1 μ L of BSA (gamma irradiated, sterile filtered, Fisher Scientific Cat. No. 11580506) onto the halocarbon oil covering the sample.

Note: Deposit the drop away from the capillary tip.

- h. Using the stage controls move the drop under the capillary tip.

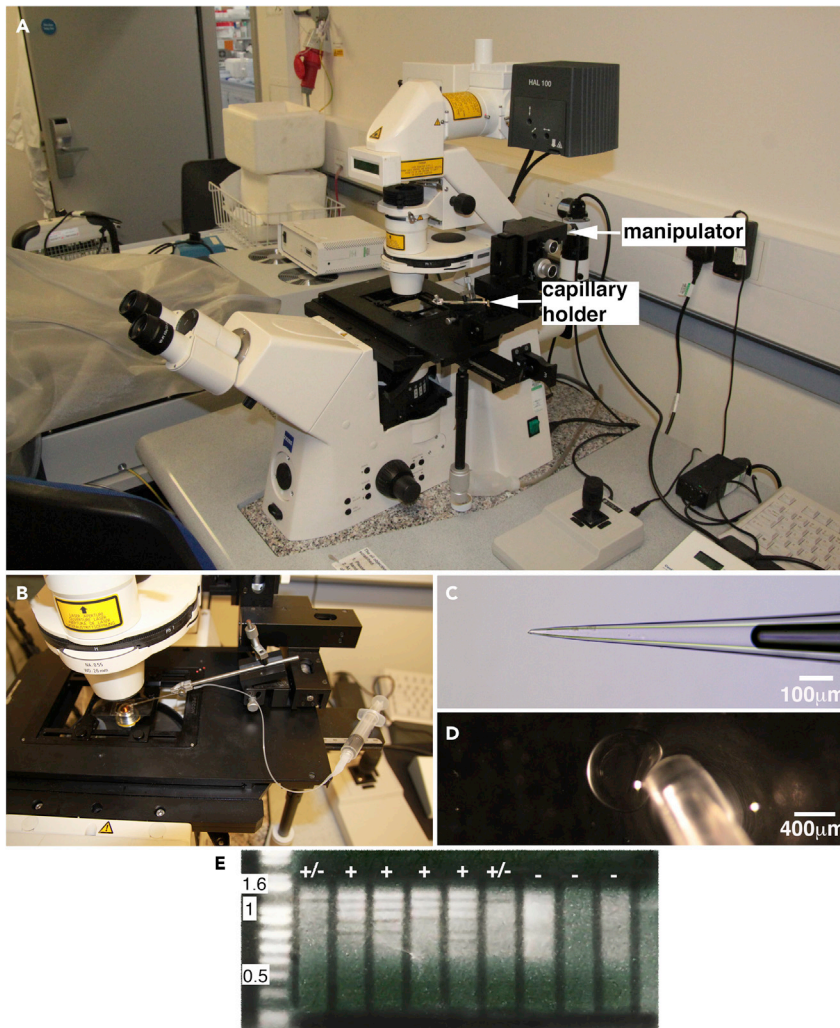


Figure 3. Harvest of single cells

(A) Inverted microscope with manipulator and capillary holder. The angle of the capillary holder should be as shallow as possible.

(B) The capillary holder.

(C) The tip of the capillary needs to be filled with halocarbon oil during penetration into the tissue and during storage.

(D) After release of the cell contents into a drop of reverse transcript mix, aspirate all of the drop including some halocarbon oil using a 0.5-2 μ L pipette under a dissecting microscope.

(E) Examples of successful (+) and failed (-) single cell transcriptome amplifications run in 1% Agarose gel. Samples with a clear cDNA banding pattern in the range between 0.5 and 1.6 kb show excellent results on microarrays. Samples with no signal, only smear or only low bands have failed. Samples with weak bands (+/-) can be used to confirm sequencing or microarray results by qPCR. Numbers mark kb.

- i. Using the micromanipulator controls lower the tip into the drop.
 - j. Attach the 5 mL syringe to the end of the PE tube to stop BSA flowing into the needle.
 - k. Expel the BSA by pressing the piston and fill the tip only with halocarbon oil (Figure 3C).
3. Cell harvest.
- a. Embryos:
 - i. Lift capillary and remove the slip.
 - ii. Deposit a drop of immersion oil onto the 63 \times objective, replace the coverslip and move up the 63 \times objective so that the immersion oil drop touches the under side of the coverslip.

Note: Most of the immersion oil will hang below the cover slip and adding or wiping oil while switching between 10× and 63× objective is no longer required.

- iii. Using the 10× objective and the micromanipulator controls, move the tip close the outer embryo membrane.
- iv. Focus on the rim created by the contact between embryo and glue to adjust the Z position of the tip close to cover slip.

Note: Move the capillary slightly back and forth to ensure it is not trapped in the glue.

- v. Switch to 63× objective and turn on epifluorescence to focus on cell targeted for removal.
- vi. Memorize cell position, close shutter and do not re-focus.
- vii. Move the tip of the capillary into focus.
- viii. Pierce through the vitelline membrane with the capillary tip but stay out of the embryo proper.
- ix. Move the stage in Y-position so that the capillary rips the membrane along approximately 25% of embryo length.
- x. Move the capillary up and down with the micromanipulator.

Note: These capillary movements will break the membrane and allow maneuvering inside the embryo.

- xi. Press the piston of the syringe lightly to allow the oil in the capillary to move to the tip.

Note: During the next step ensure that the oil stays at the tip. This will prevent the influx of any unwanted tissue whilst moving towards the cell of interest.

- xii. Using the stage and micromanipulator controls move towards the cell whilst constantly switching between epifluorescence and transmitted light.

Note: Avoid long epifluorescent exposure (more than 10 s).

- xiii. When reaching the cell of interest, make sure oil is still the only visible content in the tip.
- xiv. Switch on epifluorescence and using the micromanipulator move the tip tight against the cell.

Note: If cell contact is established, a deformation of the cell membrane will be visible.

- xv. Slowly increase suction using the syringe and continue when fluorescently labeled material is moving into the needle (see graphical abstract).

Note: If the suction shows no effect, adjust the needle again. The cell should be moving into the needle smoothly and with low suction. Avoid high suction since this results in the removal of unlabeled tissue. Leave approximately 5% of fluorescence behind to ensure that only one cell is removed,

- xvi. Hold piston of syringe in position and remove capillary from the embryo.

Note: Watch the cell at the tip of the capillary. If it moves up the capillary, lower piston and thereby decrease suction. If it moves out of the capillary increase suction. Switching between epifluorescence and transmitted light confirms that only one cell is in the capillary.

- xvii. Pull the tip out of the embryo into the surrounding halocarbon oil.
- xviii. Slowly increase suction to move the cell to the back of the capillary tip but keep it in the field of view.
- xix. Lift the capillary out of the oil and change to the 10× objective.
- xx. with a 2 μL pipette remove 0.6 μL of RT mix from the PCR tube and deposit it on top of the halocarbon oil close to the capillary tip.
- xxi. Move the stage to steer the RT drop under the capillary tip.
- xxii. Lower the tip into the RT mix and under ocular observation expel the capillary contents into the RT mix.

Note: Stop pressure when oil which fills the back of the capillary is forming drops in the RT mix.

- xxiii. Lift the needle and move the coverslip back on to the slide.
- xxiv. Set the pipette to 1 μL and under the dissecting scope (5× magnification) suck up the drop of RT mix from the top of the oil (Figure 3D).

Note: Remove also some halocarbon oil to ensure you pick up all of the mix.

- xxv. Deposit content of pipette tip into the same PCR tube from which you have removed the 0.6 μL previously (step xxi).

Note: Always keep tubes in cooling block.

- xxvi. Wipe 63× objective and add new oil.
- xxvii. Set timer to 20 min and repeat from step ii.
- xxviii. Stop picking cells after 20 min and proceed to reverse transcription.

b. Brains:

Removal of brain cells follows the protocol listed for embryos with few amendments.

- i. Schneider's and oil form a clear border. Avoid that the medium surrounding the brains is sucked into the capillary. When moving closer to the brain, minimally increase pressure within the capillary and make sure that the oil in the needle stays at the tip.
- ii. Due to the rupture of the blood brain barrier with the tungsten needle, the capillary should move freely along the brain border. Restrict the removal of cells to the ruptured barrier.
- iii. For inspection of the capillary content move the tip back into the halocarbon oil. To avoid clogging of the tip, do not leave the tip for longer periods in the Schneider's medium.

△ CRITICAL: Do not use Mercury or Xenon bulbs as epifluorescence source but LEDs. In particular, Mercury lamps tend to damage fluorescently labeled cells. Keep the angle of the capillary as flat as possible to avoid damage to non-fluorescent tissue. During any movement inside the tissue there is the need to press lightly and hold the piston of the syringe, keeping over-pressure inside the capillary to avoid contamination with unlabeled cells. The oil at the capillary tip will help to move cells out of the way. To re-use the capillary, the tip must always be stored filled with halocarbon oil. To avoid contamination with microorganisms and RNase, ice for cooling the PCR tubes should not be used. Wear gloves.

Reverse transcription and amplification

⌚ Timing: 8 h

The following steps lay out the procedure for reverse transcription and amplification of the harvested RNA.

4. Spin the PCR tubes with the harvested cells at 14k rpm (35 g) for 1 min at 4°C.
5. Set thermal cycler to 70°C for 3 min and pause when temperature has been reached.
6. After spin has ended, place tubes in thermal cycler, start cycler and wait for 2.5 min.
7. After 2.5 min, remove tubes from cycler and place immediately on dry ice.

Note: Do not wait until cycler cools down.

8. Keep tubes in dry ice and add 1 µL first strand buffer and 0.5 µL NTP (10 mM) to every tube on one side of their inside wall. Add on the opposite side 0.5 µL of SR mix.

Note: Do not add the ingredients directly into the RT mix.

9. Defrost the samples by spinning at 14k (35 g) for 3 min at 4°C.
10. Set thermal cycler to 42°C for 90 min followed by 65°C for 10 min to inactivate enzyme.

Optional: Add 0.5 µL RNase H and incubate for 20 min at 37°C followed by RNase inactivation at 65°C for 10 min.

Note: Always start amplification immediately after reverse transcription or RNase treatment.

PCR reaction master mix

Reagent	Amount
DNA template	5 µL first strand mix
DNA Polymerase (Expand Long Template System, Merck)	0.5 µL
Primer 1	2 µL nested primer
Primer 2	–
Buffer 1 (from Expand Long Template System, Merck)	5 µL
NTP 10 mM	2 µL
ddH ₂ O	35.5 µL

In the case of more than one sample, produce a master mix without the enzyme. Add the enzyme last.

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Annealing	50°C	5 min	1
Extension	68°C	15 min	1
Denaturation	95°C	20 s	30 cycles
Annealing	60°C	1 min	
Extension	68°C	7 min + 10 s per cycle	
Final extension	68°C	15 min	1
Hold	4°C	forever	

△ CRITICAL: Only use Expand Long Template kit. During protocol optimization we obtained poor cDNA yields with Taq or Phusion enzymes.

EXPECTED OUTCOMES

Load 5 µL of each sample on a 1% Agarose gel with 1 µL of SYBR-Green added (Fisher Scientific, Cat. No. 10328162). Keep the rest of the samples in the 4°C PCR block in fridge or at 4°C in the thermal cycler (see [problem 3](#)). Run gel for about 30 min at 80 V. Do not run the gel for longer since the bands will fade or completely disappear. Good samples will show a clear DNA banding pattern stretching between 500 bp to 1,600 bp. Failed samples only show one band, very low weight bands or a smear

(Figure 3E). We have not investigated the identity of the specific bands but they are cell type specific. Samples with only low bands or only smear did result in a very low number of spots on the microarrays (100–200 spots) or failed completely to hybridize.

LIMITATIONS

There are a few limitations to the removal of single cells from intact brains.

During the removal about 5% of the targeted cell should be left behind to guarantee that no adjacent non-fluorescent material contaminates the sample. This conservative approach may result in loss or reduction of asymmetrically membrane-bound RNA.

Due to their irregular shape, it may not be possible to harvest glia cells without contamination.

Harvesting neuronal cell bodies from mature, dense nerve cords needs practice because the elasticity of axons tends to pull the neuronal cell body back into position.

The method we describe here relies on either chemically or genetically fluorescently labeled cells. Without a fluorescent label it is not possible to identify the cell and to ensure that only one cell has been removed. This currently limits the approach to widely used model systems, such as *Drosophila*, mouse, zebrafish and chicken which have a large reservoir of tagged genes (www.flybase.com) or employ random activated expression techniques such as flybow (Hadjieconomou et al., 2011), Raeppli (Kanca et al., 2014) or brainbow (Richier and Salecker, 2015) to express fluorescent proteins in single cells.

Our low throughput method is designed to quickly identify transcripts controlling molecular processes by focusing on specific single cell types at precise time points. Functional analysis of the identified transcripts can then follow. This contrasts with high throughput single cell methods like 10× Genomics or Smart2-seq which are more suitable to analyze expression patterns of many single cells dissociated from whole tissues at many time points, allowing to construct expression patterns over whole tissues.

TROUBLESHOOTING

Problem 1

In a new capillary-tube-syringe system, when suction in the syringe is increased, an inflow of oil into the capillary should follow immediately. To re-use the capillary-tube-syringe system, test the suction before entering the tissue. If there is any delay between suction and inflow into the capillary tip, it is likely that the capillary is blocked, the polyethylene tube has taken up moisture or the syringe needs replacement. An immediate feedback between syringe and capillary suction is essential to pick up single cells efficiently (refer to cell harvesting).

Potential solution

First replace the syringe and PE tube and re-test suction by lowering the capillary into the oil. If suction is still compromised replace the capillary.

Problem 2

The protocol has been developed for transcriptome analysis using microarrays. Due to the increased sensitivity and low costs of whole genome sequencing, transcriptional analysis by microarrays has been replaced with Illumina sequencing. The cDNA pool obtained from PCR amplification is not Illumina ready and the blunt ended products do not allow Nanopore sequencing (refer to [reverse transcription and amplification](#)).

Potential solution

To prepare the cDNA pool for sequencing we recommend the Illumina DNA Prep (Cat. No. 20060060). The kit uses a mutated hyperactive Transposase 5 to fragment the cDNA and simultaneously add the Illumina P5 and P7 tags.

Problem 3

If after 30 PCR cycles all samples show no DNA on the Agarose gel.

Potential solution

Add 0.5 μ L DNA polymerase to each refrigerated sample and run an additional 5 cycles. Test again on an Agarose Gel. If there is still no signal, the sample preparation failed. Please note that you should only compare single cell samples prepared with same cycle number (refer to [expected outcomes](#)).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Torsten Bossing, torsten.bossing@plymouth.ac.uk.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/ analyze datasets or codes.

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AUTHOR CONTRIBUTIONS

Both authors contributed to writing and proof reading of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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