Chapter 9

Live Imaging of ESCRT Proteins in Microfluidically Isolated Hippocampal Axons

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Abstract

Live imaging of microfluidically isolated axons permits study of the dynamic behavior of fluorescently tagged proteins and vesicles in these neuronal processes. We use this technique to study the motility and transport of ESCRT proteins in axons of primary hippocampal neurons. This chapter details the preparation of microfluidic chambers, as well as the seeding, fluidic isolation, and lentiviral transduction of hippocampal neurons in these chambers, optimized for the study of ESCRT protein dynamics.

Key words ESCRT, Microfluidic, Primary neuronal culture, Hippocampal neuron, Axonal transport, Axonal isolation, Live cell imaging

1 Introduction

The emerging roles of the ESCRT pathway in multiple aspects of nervous system function, from neuronal morphogenesis to synaptic plasticity and vesicle secretion [1, 2], have led to heightened interest in the study of this pathway in neurons. Such interest has only intensified as ESCRT pathway dysfunction is increasingly linked to neurodegenerative disease etiology. For instance, mutations in the ESCRT-III protein CHMP2B cause frontotemporal dementia and amyotrophic lateral sclerosis, and deletion of other ESCRT components (i.e., HRS, TSG101) in mouse models induces profound neurodegeneration [3–8]. It has been difficult to study the ESCRT pathway in axons and presynaptic terminals due to the low endogenous expression of ESCRT proteins in these compartments. However, expression of fluorescently tagged ESCRT proteins via lentiviral transduction, coupled with microfluidic isolation and live cell imaging in rat hippocampal neurons, has allowed us to study the localization and dynamic behavior of ESCRT proteins in axons of cultured neurons. Here we provide a detailed protocol for this process.

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1.1 Fabrication of Microfluidic Devices to Cultivate and Image Axons of Primary Hippocampal Cells

Microfluidic isolation of axons facilitates investigation of axonal ESCRT dynamics in three important ways. First, it allows for the easy identification of axons when fluorescently -tagged cargo exhibits low expression or axonal localization, enabling study of ESCRT proteins in this particular subcellular structure of the neuron. Second, it allows the experimenter to control which cells express lentivirus, and thus deduce the directionality of axonal transport. Third, it permits the environmental segmentation of axonal and somatodendritic compartments, allowing the experimenter to differentially manipulate these compartments. Preparing microfluidic devices to isolate axons of primary neurons is the first and most involved task of this protocol. Our methods are adapted from well-established protocols [9, 10] in order to maximize neuronal health and ensure high levels of tagged ESCRT protein expression. Our microfluidic chambers are fabricated using soft lithography in a poly (dimethylsiloxane) (PDMS)-based encapsulant, using silicon wafer molds that can be fabricated [9] or purchased commercially. PDMS-based chambers can also be purchased commercially from several different companies. Once chambers have been fabricated, they can be seeded with freshly isolated and dissociated primary neurons [11, 12] (Fig. 1), which can be cultured for 5–7 days before lentiviral transduction. Dissection and dissociation of embryonic rat hippocampal neurons will not be discussed in detail here, but a comprehensive protocol can be found in Pacifici and Peruzzi [13], and additional protocols in Kaech and Banker and in Waites et al. [11, 12].

1.2 Lentiviral Transfection and Imaging Axons

Lentivirus is produced from transfected HEK293T cells as described previously [14–16] with minor modifications. Given the large size of many ESCRT proteins, it is vital to produce virus of high titer to ensure optimal expression levels. After 5–7 days of viral expression, live imaging can be performed on isolated axons using an epifluorescence or confocal microscope with a high-magnification objective, and data analysis can be performed using image processing software such as ImageJ/FIJI. We have used these techniques to study the axonal dynamics of several ESCRT proteins, including HRS, STAM, and TSG101 (Fig. 2), and our preliminary data indicate that ESCRTs are bidirectionally motile in axons. Future studies will lead to a deeper understanding of the axonal dynamics of ESCRT proteins, and how this pathway is regulated both in axons and in the neuron as a whole.

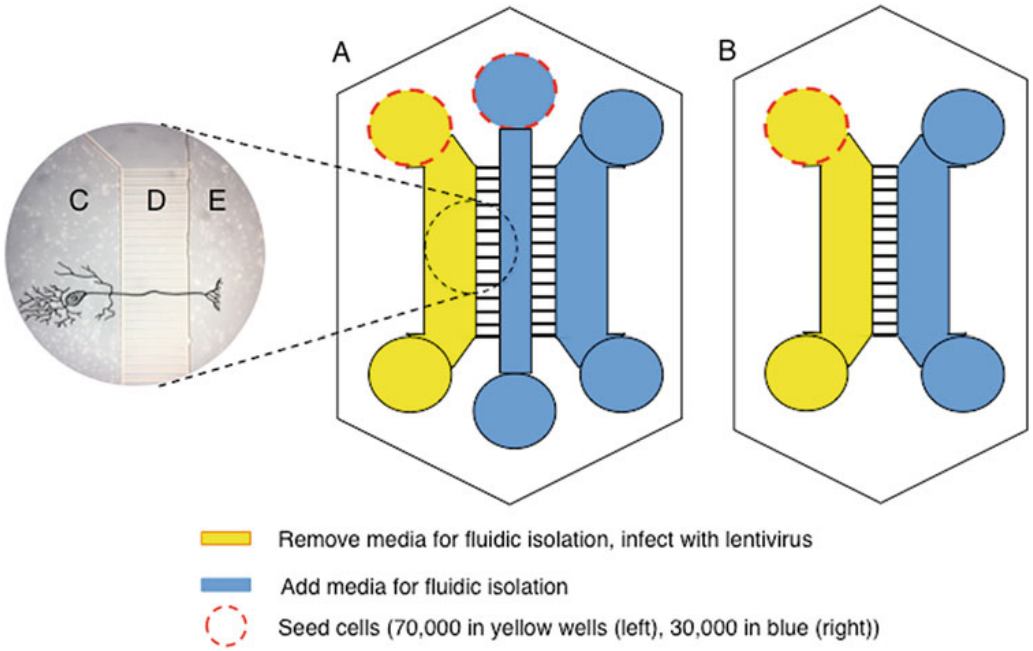

Fig. 1 Schematic diagram for seeding neurons and microfluidic isolation in tripartite (**a**) and bipartite (**b**) chambers. Medium must be removed from the side to be transduced, and placed into corresponding wells on the other side of the chamber to maintain a continuous flow across the microgrooves. This step is necessary to prevent diffusion of lentivirus into other compartments. The image to the left of (**a**) is a representative photograph of a tripartite chamber, showing the somatic compartment that is lentivirally transduced (**c**), the axonal compartment containing channels that are imaged (**d**), and the somatic compartment that is not transduced (**e**). The cartoon neuron illustrates how neurons grow in the chamber to isolate the different cellular domains

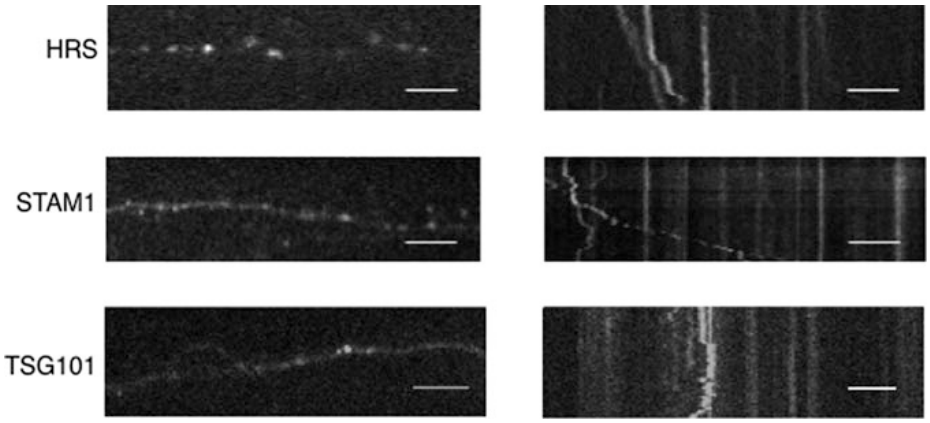

Fig. 2 Representative still images (left) and corresponding kymographs (right) of fluidically isolated axons from neurons expressing the indicated ESCRT proteins. Still images were taken before corresponding videos, and movement of each puncta over the course of the video is represented by the kymographs. HRS is very punctate, while STAM1 and TSG101 are punctate but also show low levels of diffuse protein. All three ESCRT proteins have motile and nonmotile pools in axons. Scale bar is 10 μm

2 Materials

2.1 Microfluidic Chamber Preparation, Reversible Bonding, and Seeding

1. Transparency mask.
2. Photoresists, SU-8 5 and SU-8 2050 (Microchem Corp., Westborough, MA).
3. Propylene glycol methyl ether acetate (PGMEA) photoresist developer.
4. Silicon wafer.
5. Isopropanol.
6. Deionized sterile water (dH_2O).
7. Pressurized nitrogen gas.
8. 100 mM boric acid solution: 6.2 g of boric acid in 1 L of dH_2O (pH 8.0). The pH is adjusted with NaOH.
9. Poly-L-lysine (PLL) solution: 0.25 mg/mL PLL in 100 mM boric acid (pH 8.0).
10. Commercially available two-component clear silicone encapsulant (PDMS based, e.g., Sylgard 184, Qsil216).
11. Dissociated hippocampal neurons (*see* Pacifici and Peruzzi [13] for protocol and additional reagents).
12. Neurobasal media.
13. B27 supplement.
14. 100× GlutaMAX.
15. 1× antibiotic-antimycotic: 10,000 units/mL of penicillin, 10,000 μg/mL of streptomycin, and 25 μg/mL of amphotericin B.
16. Neuronal culture media: 2% (v/v) B27, 1× GlutaMAX, 1× antibiotic-antimycotic in neurobasal media (all from Thermofisher).
17. Minimum essential media (MEM).
18. Plating media: 1 mM pyruvic acid, 0.6 mM glucose, 10% (v/v) horse serum (heat-inactivated), and 1× antibiotic-antimycotic in minimum essential media (MEM).
19. Disposable plastic cups for mixing PDMS solution.
20. Disposable stir rods (plastic knife).
21. Clean room tape.
22. 50 mm glass bottom dishes No. 1.5, uncoated and gamma irradiated (MatTek).
23. Razor blades.

24. Disposable biopsy punch, 5 mm diameter.
25. Spin coater.
26. Mask aligner.
27. Digital balance.
28. Hot plate.
29. Laboratory oven for curing PDMS polymer.
30. Vacuum desiccator for degassing PDMS polymer.
31. UV light box.
32. Dissection and tissue culture hood.

2.2 Lentiviral preparation

1. HEK293T cell line.
2. Dulbecco's modified Eagle medium (DMEM).
3. HEK293 cell media: 1× GlutaMAX, 10% (v/v) fetal bovine serum (heat inactivated), and 1× Pen/Strep in Dulbecco's modified Eagle medium (DMEM).
4. Lentiviral transfer plasmid (e.g., FUGW).
5. Packaging plasmid (e.g., delta P).
6. Envelope plasmid (e.g., VSV-G—plasmid information available from Addgene under "lentiviral guide").
7. Calfectin transfection reagent.
8. 10 cm cell culture-treated plates.
9. 0.45 μm syringe filter.
10. 10 mL syringe.
11. 2 mL cryotubes.
12. 1.5 mL Eppendorf tubes.
13. qPCR lentivirus titration kit.
14. Tissue culture hood.
15. Vortex mixer.
16. Microcentrifuge.

2.3 Imaging and Data Analysis

1. Computer with image acquisition software.
2. Epifluorescence or confocal microscope equipped for live cell imaging.
3. Stage warmer or incubation chamber capable of warming samples to 37 °C.
4. Image processing software, for example, ImageJ/FIJI.

3 Methods

3.1 Preparing Microfluidic Chambers

3.1.1 Fabrication of Master Mold (**Steps 1–19** To Be Performed in a Nanofabrication Facility) (See **Note 1**)

1. Dehydrate a 4 in. silicon wafer at 200°C for 5 min on a hot plate. Let it cool to room temperature before proceeding (*see* **Note 2**).
2. Clean wafer with pressurized nitrogen.
3. Flood SU-8 5 on the silicon wafer (about 4 mL or enough to cover the surface).
4. Place the silicon wafer on a spin coaster and hold vacuum.
5. Spin on the spin coater at 2500–3500 rpm for 60 s. Use a faster speed for shorter height of the grooves. For height specifications, *see* **Note 1**.
6. Bake the wafer for 2 min at 95 °C on a hot plate.
7. Transfer the wafer to a mask aligner equipped with a UV lamp.
8. Expose the wafer to UV light through the transparency mask containing the microgroove pattern (100 mJ/cm^2, constant dose).
9. Bake the wafer for 2 min at 95°C on a hot plate.
10. Immerse wafer in developer (propylene glycol methyl ether acetate) for 2–3 min or until fully developed.
11. Wash with isopropanol and dry with pressurized nitrogen/inert gas. If the isopropanol wash creates a white or yellow residue, then develop for longer.
12. Bake the wafer for 5 min at 95 °C to completely dry the wafer.
13. Flood SU-82050 on the wafer (about 4 mL or enough to cover the surface) and spin at 1500–2000 rpm for 60 s. Use a faster speed for shorter height of the compartments.
14. Bake for 3 min at 65 °C, and then for 9 min at 95 °C on a hot plate.
15. Align the chamber mask to the grooves pattern. Expose to UV light (250 mJ/cm^2, constant dose).
16. Bake for 1–2 min at 65 °C, and then for 6–7 min at 95 °C on a hot plate.
17. Immerse wafer in SU-8 developer for 10 min or until fully developed. Rinse once with fresh SU-8 developer. If the isopropanol wash creates a white or yellow residue, then develop for longer.
18. Wash with isopropanol, and then with deionized water. Dry with pressurized nitrogen/inert gas.
19. Bake for 5 min at 150 °C.
20. Prepare about 130 g PDMS polymer per master mold.

21. Cover the bottom of a glass Petri dish with ~30 g PDMS polymer and bake for 15 min at ~70 °C.
22. Place the master mold on top, cover with remaining PDMS polymer, and bake at ~70 °C for at least 3 h or overnight.
23. Cut out chambers, and the mold is now ready to use.

3.1.2 Fabrication of Poly (dimethylsiloxane) Polymer-Based Chamber

1. Weigh silicone polymer and curing agent in the ratio of 10:1 in a disposable plastic cup and mix thoroughly for 2–5 min. Follow the manufacturer's recommendation for making and mixing the polymer solution.
2. Vacuum mix PDMS polymer solution in a vacuum desiccator for 1 h or until bubbles are mostly gone.
3. Clean dust and particles from the master mold using tape.
4. Carefully pour solution into the mold, and let it sit at room temperature for 20 min.
5. Remove any remaining bubbles from the surface by lightly blowing.
6. Place the mold into a level laboratory oven and cure for at least 3 h at 70 °C. The polymer should solidify when fully cured (*see* **Note 3**).
7. Carefully cut around the inner edge of the mold using a razor blade and slowly remove the cured polymer piece (*see* **Note 4**).
8. Keeping the microgrooved side up, cut out individual chambers using a razor blade and punch out channels using a steel punch. Chambers can be kept in a sealed container at room temperature.

3.1.3 Preparation of Chambers and Seeding of Cells (All Steps Performed in Laminar Flow Hood)

1. Coat glass bottom dishes with PLL solution for >12 h at 37 °C.
2. Wash 3× with deionized sterile water (dH_2O), 2 min per wash, and then let dry.
3. Sterilize chambers by immersing briefly in 70% ethanol, and dry fully in laminar flow hood before use.
4. Assemble sterile, dried chambers and dishes by placing the chamber lightly on glass, grooved side down. Ensure full seal by lightly tapping the corners of the chamber (*see* **Note 5**).
5. After assembly, fill channels with warmed neuron culture media. Assemble the chambers 3 h before seeding and place them in a well-humidified incubator at 37 °C with 5% CO_2 (*see* **Notes 6** and 7).
6. Prepare E18 rat hippocampal dissociated cell suspension, according to Kaech and Banker or Pacifici and Peruzzi [11–13], with an approximate density of 10,000 cells/mL of plating media.

7. Aspirate media from chamber wells, being careful not to remove media from the channels (*see* **Note 8**).
8. Seed cells into the two top left wells of a tripartite chamber, 70,000 cells in the upper left well and 30,000 in the upper middle well (Fig. 1) (*see* **Note 9**).
9. Place chamber back in incubator for 30 min–1 h, then fill the remaining wells with plating media.
10. Remove plating media and replace with neurobasal containing antibiotic-antimycotic 24 h later.
11. To counteract media evaporation, add enough media to fill the wells every 3–4 days.

3.2 Lentiviral Preparation (All Steps Performed in Laminar Flow Hood)

1. Plate HEK293T cells in HEK cell media in a culture-treated 10 cm dish. Cells should be 60–70% confluent at transfection. (*see* **Note 10**).
2. Change media prior to transfection.
3. For each plate, set up transfection mixture in a 1.5 mL tube (*see* **Note 11**). To each tube, add: 1 mL DMEM (serum-free), 10 μg FUGW plasmid (containing the desired ESCRT construct), 7.5 μg delta P (packaging plasmid), and 5 μg VSV-G (envelope gene).
4. Vortex tubes to mix, then spin down briefly.
5. Add 35–40 μL of CalFectin reagent/tube and mix gently.
6. Incubate tubes for 15 min at room temperature.
7. Add the mixture drop-wise to a 10 cm plate of HEK cells; swirl gently to mix, and place cells back in the incubator.
8. After 12–18 h of transfection, remove HEK cell media and replace with 8 mL of Neurobasal media.
9. Collect virus 24 h after media change.
10. To collect, pipette media off the cells, filter into a 50 mL conical tube using a 10 mL syringe and a 0.45 μm filter.
11. Aliquot into labeled cryotubes and store at −80 °C.
12. Test viral titer before use (*see* **Note 12**).

3.3 Lentiviral Transduction (Microfluidic Isolation, Fig. 1)

1. Transduce neurons between day in vitro (DIV) 5–7.
2. Fill all four wells with the same volume (100 μL) of fresh media.
3. For a dipartite chamber, move 50 μL of media from a well on one side of the chamber (e.g., left) and add it to the well on the opposite side (right). For a tripartite chamber, add media to both wells across from the well to be transduced (*see* Fig. 1 for example).

4. Repeat for all wells of the chamber (remove from left well, add to corresponding right well).
5. Add 30–50 μL of thawed virus to the desired well(s) on the left side of the chamber (*see* **Note 13**).
6. Image chambers 5–7 days after viral transduction. Check for viral expression and fluidic isolation and adjust accordingly (*see* **Notes 14** and **15**).

3.4 Imaging and Data Analysis (see Note 16, Fig. 2)

1. Image fluidically isolated axons expressing fluorescently tagged ESCRT proteins using an epifluorescence or confocal microscope with 40× or 63× oil immersion objective (*see* **Note 17**).
2. Acquire images every 5 s for a total of 50 frames for imaging of early ESCRT proteins (e.g., HRS, Tsg101). Faster or slower image acquisition can be used based on the experimental question (*see* **Note 18**).
3. Analyze videos using the Manual Tracking ImageJ/FIJI plug in. Information about puncta movement and directionality (i.e., speed, anterograde/retrograde/bidirectional movement, moving puncta per axon) can be extracted from these raw data by writing a custom macro in ImageJ, or with custom Matlab code.

4 Notes

1. To determine proper spin conditions for desired thickness of mold, consult http://cleanroom.byu.edu/su8. The conditions described are what we generally use for 5-μm-tall microgrooves and 75–150-μm-tall compartments.
2. After all dehydration steps, the wafer should be allowed to cool to room temperature before proceeding. However, proceed to coating as soon as the wafer is cooled to avoid re-adsorption of water.
3. Chambers can be baked overnight. Make sure that the inside of the oven is clean and level.
4. Make sure to cut all the way around the mold, and that the tip of the razor blade goes all the way through the PDMS when cutting it out of the molds. If the PDMS is not separated completely from the mold, it can rip when pulled out.
5. The polymer will stick easily to the treated glass of the dishes; plasma bonding is not necessary for the protocol.
6. Warm up media before adding it to the chamber in order to prevent bubbles from being trapped in the channels. If bubbles do form, they can be removed by repeatedly pipetting media through the channels.

7. Given the small volume of liquid in the chambers, it is vital that they be stored in a well-humidified incubator after seeding to ensure cell survival. If media evaporation occurs, chambers can be placed in a container with a small amount of deionized sterile water (dH_2O), (chambers should be above the dH_2O, not in contact with it).
8. Leave a small amount of media in the wells to avoid introducing bubbles.
9. The use of a bipartite or tripartite chamber depends on the needs of the experiment to be performed.
10. Because open reading frames (ORFs) encoding ESCRTs tend to be long (~2.5 kb), the size of resulting FUGW plasmids can be >12 kb, often resulting in low-efficiency virus. Thus, it is essential to use healthy, low passage number (i.e., <15 passages) HEK293T cells for lentiviral production.
11. These are the specific transfer, packaging, and envelope plasmids that we use for our experiments, based on previous work [14]. Multiple lentiviral plasmids are available at Addgene (addgene.org) and may be optimal for expression of different proteins.
12. Viral titer can be tested directly using a qPCR Lentivirus Titration Kit (Applied Biological Materials, cat. # LV900; 10^6 virus/mL is excellent), and viral efficiency should be measured empirically by transducing test wells of neurons with varying amounts of lentivirus. On average, 10 μL of viral volume per 10 mm diameter coverslip should transduce ~20–30% of neurons.
13. Lentiviral transduction volume will depend on the quality and titer of the virus, the ESCRT protein being expressed, and the goals of the experiment. The ideal volume should be determined by test transductions, as described earlier.
14. Relatedly, as the volume of virus changes, the volume moved to ensure fluidic isolation will also have to be adjusted. The basic concept is that in order to isolate the virus in one side of the chamber (necessary for knowing the directionality of axon growth and puncta movement), it is important to maintain a hydrostatic pressure imbalance between the transduced and non-transduced chambers. This hydrostatic pressure difference is created by a small difference in volume between compartments. Determining the correct volume difference may take some trial and error to figure out, given the goals of the experiment, transduction volume, and size of the chamber, but a volume difference of ~50 μL is a good place to start.
15. Finally, the ideal DIV for imaging and transduction will also depend on the goals of the experiment. This protocol is

optimized to ensure that imaging is done in neurons that have formed synapses. Transducing and imaging as early as possible will make it easier to maintain neuronal health, as long-term culturing (i.e., longer than 12–14 DIV) in microfluidic chambers can be difficult.

16. ESCRT protein expression in neurons tends to be relatively sparse and punctate in axons (*see* Fig. 2). These features allow for the tracking of individual ESCRT-positive puncta.
17. The microscope should be equipped with a stage warmer or incubation chamber to allow for imaging at 37 °C.
18. Number of frames and exposure time must be calibrated to minimize bleaching of fluorescent tags. If a long exposure is necessary, videos may need to be shorter. Moreover, with a longer exposure time, it may not be possible to image a given axon for more than a few minutes.

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