Published in final edited form as:
J Vis Exp. ; (82): e51018. doi:10.3791/51018.

Optimized Protocol for Retinal Wholemount Preparation for Imaging and Immunohistochemistry

Elena Ivanova[1], **Abduqodir H Toychiev**[1], **Christopher W Yee**[1], and **Botir T Sagdullaev**[1]
[1]Ophthalmology, Weill Medical College of Cornell University

Abstract

Working with delicate tissue can be a complicating factor when performing immunohistochemical assessment. Here, we present a method that utilizes a ring-supported hydrophilized PTFE membrane to provide structural support to both living and fixed tissue during immunohistochemical processing, which allows for the use of a variety of protocols that would otherwise cause damage to the tissue. First, this is demonstrated with bolus loading of fluorescent markers into living retinal tissue. This method allows for quick visualization of targeted structures, while the membrane support maintains tissue integrity during the injection and allows for easy transfer of the preparation for further imaging or processing.

Second, a procedure for antibody staining in tissue fixed with carbodiimide is described. Though paraformaldehyde fixation is more common, carbodiimide fixation provides a superior substrate for the visualization of synaptic proteins. A limitation of carbodiimide is that the resulting fixed tissue is relatively fragile; however, this is overcome with the use of the supporting membrane. Retinal tissue is used to demonstrate these techniques, but they may be applied to any fragile tissue.

Keywords

Basic Protocol; Issue 82; hydrophilized PTFE membrane; retina; bolus loading; carbodiimide fixation; immunohistochemistry; antibody staining; microscopy

Introduction

Performing immunohistochemistry in delicate intact tissues runs the risk of damage during handling and transfer. This can occur either in brain slices or other thin tissue, such as degenerated retina. Additionally, there are certain methods of tissue fixation that can be advantageous for immunostaining of neuronal structures, but result in compromised structural stability, precluding their use. A particular example of this is carbodiimide-based

Correspondence to: Botir T Sagdullaev at bos2005@med.cornell.edu.

Disclosures
The authors have no competing financial interests.

Video Link
The video component of this article can be found at http://www.jove.com/video/51018/

fixation, which is superior for staining receptors and hormones[1–7] but is commonly avoided due to the instability of the fixed tissue.

Here, we describe a procedure which utilizes a hydrophilized PTFE membrane to structurally support delicate tissue, either fixed or unfixed, for a variety of staining techniques. The supporting membrane allows for the manipulation of delicate tissue before and after fixation, allowing for several steps of processing while minimizing the risk of tissue damage. Overall, this simple method to preserve tissue integrity provides the opportunity to use techniques that might otherwise be avoided. As such, this approach could also be successfully used for preparation of wide variety of tissues such as brain slices that become fragile following slicing procedures.

Protocol

In all experimental procedures, animals were treated according to the regulations in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with protocols approved by Weill Cornell Medical College. Animals were euthanized by carbon dioxide and subsequent cervical dislocation.

1. Dissection of Wholemount Retina

1. After the mouse is euthanized, enucleate its eyes and place them into oxygenated HEPES-buffered Ringer's solution (Table 1).
2. Remove the cornea by cutting in a circular path along the *ora serrata* with small scissors, holding the eye at the limbus with forceps. Pull out the lens with forceps.
3. Remove as much vitreous as possible, using blunted-edge forceps. This step is important for bolus injections and electrophysiological recordings; it is not necessary for when performing immunohistochemistry alone.
4. Detach the retina from the eyecup by positioning forceps between the retina and the eyecup, moving the forceps slowly around the circumference. Stabilize the eye cup by holding the sclera firmly with a second set of forceps. Cut the optic nerve at the location between the retina and the eyecup to ease the separation of the retina from the eyecup.
5. The retina can be dissected into pieces (*e.g.* quarters) or used as a whole. To cut into pieces, use scissors to make incisions from the retinal periphery all the way down to the optic nerve. Alternatively, to use the entire retina, make incisions from the periphery halfway to the optic nerve. The incisions will help to make a flat preparation.

2. Mounting onto Hydrophilized PTFE Membrane Insert

1. Prepare the membrane as described below. When physiological recordings or dye injection experiments are performed, shorten the holder by using either a lathe or a handsaw so that the remaining ring has a height of 1 mm.

Note: The culture plate inserts may be either unmodified or modified (Figure 1A). The choice of the configuration is dictated by the purpose of the experiment. For immunostaining, an unmodified insert is suitable.

2. Polish down rough surfaces with fine (300 grit or higher) sandpaper.
3. (Optional) Remove the feet underneath the ring with scissors.
4. To remove any debris, rinse or sonicate the membrane in distilled water for 2–3 min.
5. Mount the tissue onto the membrane (Figure 1A). Place the tissue with some solution on top of the membrane with a plastic transfer pipette. Cut off the narrow tip of the pipette if necessary, to avoid having the tissue sample adhere to the pipette during transfer.
6. Attach the retina with ganglion cells (GCs) up: on the membrane, gradually remove solution while spreading the retina and unfolding the folds with forceps.
7. On the opposite side of the membrane, position an empty syringe (1 ml) directly underneath the tissue. Apply suction by pulling back on the plunger to a volume of 0.2–0.3 ml. Increase the surface area of suction by cutting the holding tip of the syringe prior to use. The tissue does not require further treatment for adhesion, as it is physically enmeshed within the membrane.

3. Bolus Injection of Isolectin Alexa 488

1. Prepare patch pipettes and staining solution. Pull patch pipettes from borosilicate capillary glass with a Flaming/Brown puller, with a resistance of ~1 MΩ. Gently break the tip of the pipette.
2. Make staining solution by adding 0.3 μl of isolectin Alexa 488 stock solution to 10 μl of the HEPES-buffered Ringer's solution described in step 1.2.
3. Transfer sample to the patch-clamp setup for injection (Figure 1C). Place the membrane with attached tissue into the recording chamber perfused with warmed (37 °C) and oxygenated HEPES-buffered Ringer's solution.
4. Place the pipette containing dye against the inner limiting membrane (ILM, Figure 2A). Using micromanipulator, further advance the pipette against the ILM until it penetrates it. Inject the staining solution with a pressure 10–20 psi for 1 sec using a picospritzer. Single or multiple locations may be targeted for injection (Figure 2B). If a picospritzer is not available, a 10 ml syringe can be used to deliver a positive air pressure for injection.
5. Incubate injected tissue for 10 min. During incubation, the injected tissue can be viewed under epifluorescence illumination.
6. After labeling, the preparation may be taken to a microscope for imaging. Transfer the insert to a glass-bottom culture dish, placing retina side down (Figure 1C).
7. Add a drop of HEPES solution to keep the specimen moist.

8. Place 2–3 spots of vacuum grease around the perimeter of the plastic ring to firmly secure the insert to the bottom of the culture dish.

9. Transfer the dish to the microscope stage.

4. Paraformaldehyde and Carbodiimide Fixation

1. Fix the sample by transferring the insert into fixative for 15 min. Longer or shorter fixation times may be used, dependent on application. Both fixatives can be effectively used to fix GFP, isolectin, or fluorescent Alexa hydrazides (*e.g.* used for filling the cells in patch-clamp recordings).

   Note: For fixation, either 4% carbodiimide or 4% paraformaldehyde solutions may be used. To maintain adherence, fixation should be done after the tissue has already been attached to the membrane. The tissue can be fixed immediately after mounting, or after physiological recording and/or bolus injection.

2. Rinse the tissue in phosphate buffer (PB) for 30 min. After this, the tissue may be processed either as a wholemount (step 5) or cut into vertical sections and then processed (steps 6 and 7).

5. Immunostaining of Retinal Wholemounts

1. In a 24-well dish (Figure 1D) block retinal pieces on the membrane for 1 hr in a staining solution.

2. Dilute mouse anti-PSD-95 primary antibodies 1:300 in the staining solution and apply for 48 hr at room temperature in dark. Add ~100–150 μl antibody solution in each well with the tissue. To prevent drying out, fill unused wells with distilled water.

3. Rinse for 20 min in PB, 3x.

4. Incubate for 24 hr in donkey anti-mouse Alexa 568 secondary antibody dissolved 1:500 in the staining solution.

5. Rinse for 20 min in PB, 3x.

6. Place the insert with the stained tissue into a standard 3 cm Petri dish filled with PB solution. With small scissors, cut out the portion of the membrane containing the retina from the ring.

7. Transfer the retina on the membrane onto a slide retina up. Remove PB, add mounting medium. Place small pieces of a broken cover slip glass in between the slide and the coverslip glasses to avoid extensive squeezing and damage to the retina. Place coverslip.

8. Seal the coverslip in place with nail polish. Keep slides in the dark, in a refrigerator.

6. Vertical Sectioning of Membrane-mounted Tissue

1. After fixation, submerse the tissue on the membrane into the sucrose gradient solutions of 10%, 20%, and 30% sucrose for 30 min, 1 hr, and overnight, respectively. The overnight step is performed at 4 °C in refrigerator, all other steps at room temperature.
2. Using scissors, remove the membrane with the tissue from the plastic insert ring.
3. Place the cut off membrane onto Parafilm with retina up and remove as much solution as possible with Kimwipes.
4. Put the Parafilm with the tissue into the cryostat chamber and add a drop of OCT medium. Use the quick freezer device to accelerate the freezing.
5. Detach the frozen sample and flip it over.
6. Cut excess frozen OCT. Leave approximately 2 mm around the sample on three sides, and leave 5–6 mm from the side which will be attached to the holder.
7. Cover exposed retinal side (membrane side) with a drop of OCT. Leave the attaching area free.
8. Freeze the membrane with retinal tissue. Now the sample is ready for placing onto a cutting holder.
9. Place a drop of OCT onto a cold holder ring. Immediately insert the frozen sample into the drop.
10. Cut sections 10–20 μm thick, setting cryostat temperature at −19 °C. Use warm (room temperature) polysine adhesion slides. Leave the slides with the attached sections onto a heating platform for ~2–3 min (41 °C) in dark to ensure firm adhesion of sections.
11. Put the slides into a storage box and keep at −20 °C until use.

7. Immunostaining of Retinal Sections

1. Take the slides with the sections out of the freezer. Let them stay at room temperature for 1 min to remove the condensate.
2. Encircle the sections with a liquid blocker to create a barrier for further incubation.
3. Place the slides into the incubation box. Cover the bottom of the box with water to prevent evaporation of the antibody solution.
4. Rinse with PB for 5 min.
5. Prepare the same staining solutions and follow the steps as in step 5. The incubation with the primary antibody is overnight and with the secondary 1 hr. Do not use broken glass between the coverslip and the slide.

Representative Results

Demonstrated here are two representative experiments that benefit from the use of a hydrophilized PTFE membrane to support wholemount retina during staining procedures. The first experiment, illustrates the method for fast and simple characterization of retinal vasculature, an elaborate network of blood vessels that span across multiple retinal layers (Figure 2). This approach combines bolus loading of isolectin with immersion labeling with SRH. This is an efficient method of labeling living tissue that can be visualized and scanned almost immediately. In the retina, blood vessels can be labeled from superficial to the deep layer (Figure 2E). In contrast to relatively well diffusible SRH, isolectin does not permeate well across the inner limiting membrane (ILM). This limitation is overcome by injecting the solution containing isolectin underneath the ILM with a glass pipette. For SRH blood vessels labeling, it is better to stain the tissue before mounting on the insert. The SRH stains only living tissue and the labeling disappears following fixation with either paraformaldehyde or carbodiimide.

The second experiment (Figure 3), shows the advantages of fixation with carbodiimide over conventional fixation with paraformaldehyde when labeling synaptic proteins. In general, the use of carbodiimide fixative is avoided because it results in relatively fragile tissue. However, mounting the tissue on a membrane ensures sufficient structural support, mitigating this issue. With a short period of xation with carbodiimide, followed by staining for the synaptic marker PSD-95 immuno uorescence in the IPL had a bright punctate appearance, suggesting that individual synapses were distinguished (Figure 3B). In contrast, when using conventional paraformaldehyde fixative, identification of the synaptic components is less clear (Figure 3E). Similar results were obtained in the retinal cryostat sections (Figures 3C and F).

Discussion

The hydrophilized PTFE membrane's high biocompatibility and transparency in solutions is advantageous when working with living tissue. This preparation has been successfully used in earlier work for patch-clamp recordings of light responses[8–10]. Here, we show how this approach can be adopted for structural analysis of the wholemount retina.

The bolus loading technique can be effectively used for labeling living tissue. This technique is aided by the structural support of the membrane insert. Applications include loading of calcium indicator[9], staining of the tissue with the fluorescent markers[11], or labeled ligands[12].

Fixed tissue processing can also benefit from the use of a supporting membrane. Differences in the appearance of postsynaptic density proteins depend on fixation time and the nature of the fixative[5]. Longer fixation times lead to excellent tissue preservation but poor antibody recognition of synaptic proteins that are cross-linked by the fixative. Shorter fixation times result in poor tissue preservation but bright punctate labeling. Similar results have been obtained for many other synaptic structural proteins and ion channels by different groups[1–7]. Using the membrane for structural support enables the use of carbodiimide to

simultaneously achieve short fixation times, well-defined bright labeling of synaptic structures and adequate overall tissue quality for both wholemount and cryostat section preparations. The preparations are stable and easy to handle, with reproducible results.

Several critical steps should be carefully performed to ensure high quality and reproducible results. For bolus injections and electrophysiological recordings, it is important to remove as much of the vitreous as possible when dissecting the retina. The absence of the vitreous will enable easy access of the patch pipette to the cells. The vitreous does not interfere with the described immunohistochemistry techniques. To maintain firm adherence, fixation should be done after the tissue has already been attached to the membrane. For cryostat sectioning, the sucrose solutions must be completely removed prior to adding the embedding OCT medium. Failure to remove the liquid may result in splitting the cut section along the frozen tissue and damaging it. The slides with the attached sections should be kept for 2–3 min on a heating platform. If this step is not performed, the tissue may detach from the slide during the staining procedure.

There are several limitations of this technique. First, once the tissue adheres to the membrane, it cannot be removed without incurring damage, as the tissue is enmeshed within the membrane. Second, tissue cannot be attached to the membrane after it has been fixed. Third, while the membrane is fully transparent in water-based mediums, the membrane may look slightly opaque when in mounting medium, compromising light transmission. This is mitigated by sectioning, since this removes the membrane from the path of light transmitted to the tissue.

Acknowledgments

This work was supported by NIH grant R01-EY020535 (B.T.S), International Retinal Research Foundation and Karl Kirchgessner Foundation (B.T.S).

References

1. Csaba G, Kovacs P, Pallinger E. EDAC fixation increases the demonstrability of biogenic amines in the unicellular Tetrahymena: a flow cytometric and confocal microscopic comparative analysis. Cell Biol Int. 2006; 30:345–348. [PubMed: 16530432]
2. Fletcher EL, Hack I, Brandstatter JH, Wassle H. Synaptic localization of NMDA receptor subunits in the rat retina. J Comp Neurol. 2000; 420:98–112. [PubMed: 10745222]
3. Gastinger MJ, O'Brien JJ, Larsen NB, Marshak DW. Histamine immunoreactive axons in the macaque retina. Invest Ophthalmol Vis Sci. 1999; 40:487–495. [PubMed: 9950609]
4. Haverkamp S, Wassle H. Immunocytochemical analysis of the mouse retina. J Comp Neurol. 2000; 424:1–23. [PubMed: 10888735]
5. Koulen P, Fletcher EL, Craven SE, Bredt DS, Wassle H. Immunocytochemical localization of the postsynaptic density protein PSD-95 in the mammalian retina. J Neurosci. 1998; 18:10136–10149. [PubMed: 9822767]
6. Pena JT, et al. miRNA *in situ* hybridization in formaldehyde and EDC-fixed tissues. Nat Methods. 2009; 6:139–141. [PubMed: 19137005]
7. Tymianski M, et al. A novel use for a carbodiimide compound for the fixation of fluorescent and non-fluorescent calcium indicators *in situ* following physiological experiments. Cell Calcium. 1997; 21:175–183. [PubMed: 9105727]

8. Sagdullaev BT, Eggers ED, Purgert R, Lukasiewicz PD. Nonlinear interactions between excitatory and inhibitory retinal synapses control visual output. J Neurosci. 2011; 31:15102–15112. [PubMed: 22016544]

9. Toychiev AH, Sagdullaev B, Yee CW, Ivanova E, Sagdullaev BT. A time and cost efficient approach to functional and structural assessment of living neuronal tissue. J Neurosci Methods. 2013; 214:105–112. [PubMed: 23370309]

10. Yee CW, Toychiev AH, Sagdullaev BT. Network deficiency exacerbates impairment in a mouse model of retinal degeneration. Front Syst Neurosci. 2012; 6:8. [PubMed: 22383900]

11. Ivanova E, Muller U, Wassle H. Characterization of the glycinergic input to bipolar cells of the mouse retina. Eur J Neurosci. 2006; 23:350–364. [PubMed: 16420443]

12. Dmitrieva NA, Strang CE, Keyser KT. Expression of alpha 7 nicotinic acetylcholine receptors by bipolar, amacrine, and ganglion cells of the rabbit retina. J Histochem Cytochem. 2007; 55:461–476. [PubMed: 17189521]

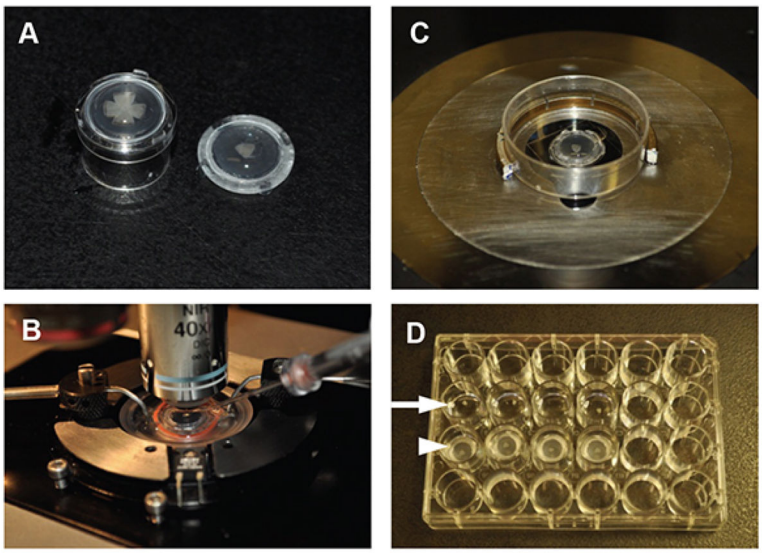

Figure 1. Versatile use of hydrophilized PTFE membranes

(A) Living retinal wholemount attached to an unmodified 12 mm membrane insert (left) and a quarter of retina attached to an insert with the holder portion removed. See video demonstrating mounting procedure. **(B)** Living retina on a stage of an upright microscope within a patch-clamp setup. **(C)** Living retina on a stage of an inverted confocal microscope. **(D)** Free floating retinal tissue (arrow) and the tissue mounted on membrane insert (arrowhead). Click here to view larger image.

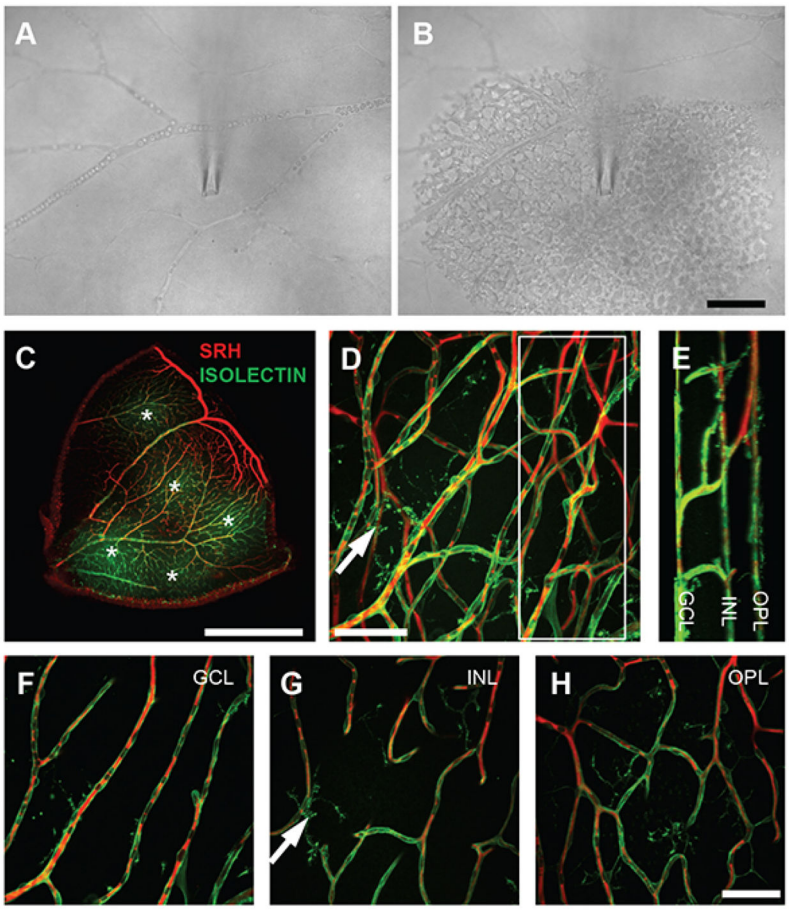

Figure 2. Focal and global labeling of the vasculature in the living retinal wholemount after injection of isolectin Alexa 488

(A) DIC image of the retina with a pipette filled with isolectin above the inner limiting membrane (ILM) prior to injection. **(B)** The same area as in A after penetration of the pipette beneath the ILM and pressure injection of the pipette solution. The spot with the clearly visible ganglion cells indicate that the solution has been injected between the ILM and the ganglion cell layer. **(C–H)** Confocal images of a live retinal wholemount labeled with sulforhodamine (SRH, red) and isolectin (green). **(C)** Isolectin applied with multiple bolus injections (asterisks). The size of the labeled area varies according to the pressure of the injection. SRH labels the entire vasculature. **(D)** Projection of a z-stack under high magnification shows all layers of vasculature and brightly labeled microglia (arrow). **(E)** Z-stack from the area highlighted in D rotated 90°. **(F–H)** A view of the individual layers of blood vessels corresponding to labels in **E**. OPL-outer plexiform layer, INL-inner nuclear layer, GCL-ganglion cell layer. Scale bars: 50 μm for **A–B** and **D–H**, 1 mm for **C**. Click here to view larger image.

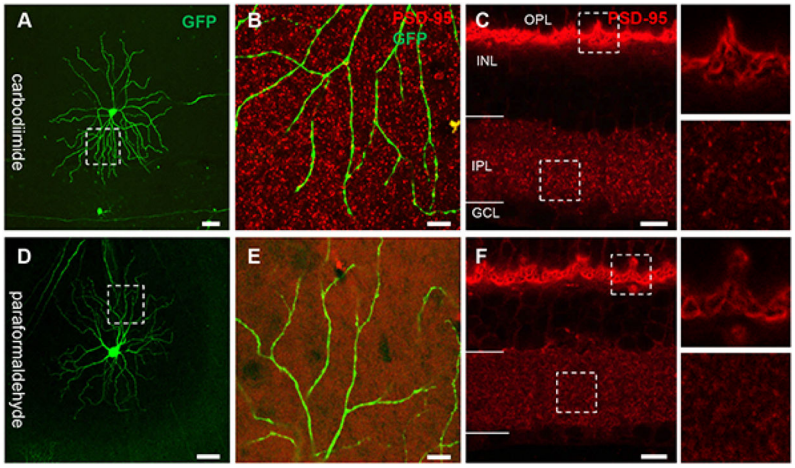

Figure 3. Role of the fixative in the antibody labeling of synaptic structures

Retinal wholemounts fixed with either carbodiimide **(A–C)** or paraformaldehyde **(D–F). (A,D)** Ganglion cells expressing green fluorescent proteinin B6.Cg-Tg(Thy1-YFPH)2Jrs/J mice (Jackson Lab. Stock #0033548). Squares indicate areas of high magnification shown in B and E. **(B,E)** Staining for the synaptic marker PSD-95 (red). Projections are within a narrow focal plane (two confocal images spaced 0.3 μm apart). **(C,F)** Single confocal image of vertical section stained for PSD-95. Note that the axon terminals of photoreceptors within the OPL are overexposed to show weaker staining for synapses in the IPL (left panels). Inserts (right panels) show high magnifications of the areas highlighted on the left panels. OPL-outer plexiform layer, INL-inner nuclear layer, IPL-inner plexiform layer, GCL-ganglion cell layer. Scale bars = 50 μm in **A** and **D**; 10 μm in **B**, **C**, **E**, and **F**. Click here to view larger image.

Table 1

Name	Company	Catalog Number	Comments
Millicell Cell Culture Insert, 12mm, hydrophilic PTFE (Biopore), 0.4 um	Millipore	PICM01250	
Insulin syringe, 1 ml	Beckton Dickinson	309659	
Scissors	Fine Science Tools	15003-08	dissection
Forceps, Dumont #55, inox	Fine Science Tools	11255-20	dissection
Cryostat	Leica	various	
Confocal Microscope System	Nikon	various	
Capillary glass	World Precision Instruments	1B150F-4	
P-97 Flaming/Brown Micropipette Puller	Sutter Instrument Co.		
Picospritzer III	Parker Hannifin		
Glass Bottom Culture Dishes	MatTek Corporation	P35G-0-14-C	
Petri dish	Falcon	1008	
Disposable Graduated Transfer Pipettes	VWR	16001-180	
Multiwell plates, 24 well	Beckton Dickinson	351147	
Cover glass, #1	Electron Microscopy Sciences	72200-30	
Polysine adhesion slides	Electron Microscopy Sciences	63412-01	
Microscope slides	Globe	1301	
Liquid Blocker	Electron Microscopy Sciences	71312	
Vectashield mounting medium	Vector Laboratories	H-1000	
OCT medium	Sakura	4583	
Parafilm laboratory film	Fisher	13-374-10	
Dow Corning high vacuum grease	Sigma-Aldrich	Z273554	
Mouse anti-psd95	Millipore	MABN68	antibody
Donkey anti-mouse Alexa 568	Invitrogen	A10037	antibody
Isolectin Alexa 488	Invitrogen	I21411	
Chemiblocker	Chemicon	2170S	
Triton X-100	Sigma-Aldrich	T9284	
Sulforhodamine	Sigma-Aldrich	341738	
Carbodiimide	Thermo Scientific	22980	EDC
Paraformaldehyde	Sigma-Aldrich	P6148	