Protocol

In Vitro Imaging of Retinal Whole Mounts

Philip R. Williams, Joshua L. Morgan, Daniel Kerschensteiner, and Rachel O.L. Wong

Neuronal circuits of the vertebrate retina are organized into stereotyped laminae. This orderly arrangement makes the retina an ideal model system for imaging studies aimed at understanding how circuits assemble during development. In particular, live-cell imaging techniques are readily applied to the developing retina to monitor dynamic changes over time in cell structure and connectivity. Such imaging studies have collectively revealed novel strategies by which retinal neurons contact their presynaptic and postsynaptic partners to establish synaptic connections. We describe here the procedures developed in our laboratory for confocal and multiphoton live-cell imaging of the developing retina using in vitro retinal explants. Retinas can be removed from the eye and kept in culture conditions for several days with limited disruption to the retinal circuit. The explanted retina is amenable to a variety of labeling techniques and provides a large, flat, unobstructed surface that is ideal for optical imaging experiments. This protocol describes procedures for mounting and imaging the isolated mouse retina. The same general procedure, with only minor modification (composition of culture medium), has been used to image retinas from a variety of vertebrates (e.g., chick, ferret, and rabbit).

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.

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

Reagents

Agarose, low-melting-point

aCSF for retina <R>

Mice or other vertebrate of interest

Penicillin-streptomycin, 100× (Sigma-Aldrich P0781)

Equipment

Brush, fine (size 00)

Dissecting microscope

Filter paper, black (Millipore)

Forceps, fine

Adapted from *Imaging in Developmental Biology* (ed. Sharpe and Wong). CSHL Press, Cold Spring Harbor, NY, USA, 2011.

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Image analysis software for viewing data, preprocessing, reconstruction, and morphological analysis:

- ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij/)

  ImageJ is free, excellent for exploration by scrolling through imaging stacks and basic image adjustments, and an increasing number of analysis tools are available.

- Amira (Visage Imaging, http://www.amiravis.com/)

  This software provides excellent three- and four-dimensional visualization, image segmentation, some analysis, and good interfacing with MATLAB.

- Imaris (Bitplane Scientific Software, http://www.bitplane.com/)

  Imaris provides good three- and four-dimensional visualization, excellent analysis, and interfacing with MATLAB.

- MetaMorph (Molecular Devices, http://www.moleculardevices.com)

  This software enables good image visualization by scrolling through image stacks; some analysis tools are available.

Imaging setup:

- Microscope configurations

  Live cell imaging of the developing retina can be performed using either microscopes with upright or inverted configurations. We focus here on using upright microscopes with water-dipping objectives. The advantage of using this setup is that small temperature fluctuations that cause tissue drift are more readily dealt with compared with the use of oil objectives in the inverted configuration. Also, water objectives have longer working distances (1–3 mm), which enable focusing deeper into the tissue, compared with more limited working distances of oil objectives (<0.5 mm). The major disadvantage of water objectives is their lower numerical aperture (NA) compared with oil objectives resulting in dimmer, lower resolution images.

- Lasers

  For confocal microscopy, it is useful to have a microscope system that has lasers suited for exciting fluorescent proteins (FP) of all colors (cyan [CFP], green [GFP], yellow [YFP], red). Lasers with excitation lines at 440 nm (CFP), 488 nm (GFP), 514 nm (YFP), and 559 nm or 568 nm (DsRed, td-Tomato) are suitable. For multiphoton microscopy, a tunable, pulsed, infrared laser (Broadband Mai Tai, Spectra-Physics or Chameleon, Coherent, Inc.) is needed.

- Objectives

  To optimize resolution and light collection, the highest-NA objective that has the required working distance should be used. For an Olympus FV1000 confocal microscope, a 60x water-immersion objective (1.1 NA, LUMFL, WD 1.5 mm; Olympus) provides a good compromise. Its working distance is large enough to image the entire depth of a flat-mounted retina, its field of view is large enough to encompass most cell types, and it has a relatively high NA. For multiphoton microscopy, sensitivity can be improved by using lower-magnification objectives that collect emission light scattered over a larger area. For example, 20 × or 25 × objectives with high NA have recently become available (e.g., Olympus, Carl Zeiss). The user should seek advice from the various microscope vendors for more information about their high-NA water objectives.

Incubation chamber (any chamber suitable for maintaining tissue in a humidified and oxygenated environment)

Indium tin oxide (ITO) glass, 50 × 50-mm, 0.6-mm-thick (Cell MicroControls)

Microwave oven

Needle, 30-gauge

Perfusion chamber, custom-designed (see Fig.1)

Imaging live retinal explants requires a temperature-controlled chamber that allows superfusing the tissue with oxygenated aCSF. Perfusion chambers typically used for electrophysiology are suitable for live imaging. Here we describe a custom perfusion chamber designed for imaging on an upright microscope using water-immersion objectives (Fig. 1). The principal design requirements of such a perfusion chamber are to (1) secure the tissue in place, (2) allow enough space for a wide dipping cone objective to access the entire retina, (3) prevent fluid vibrations or changes in fluid level that would distort images, and (4) prevent changes in temperature and thus focus shifts.

A flow rate of ~1 mL/min can be achieved using a gravity feed system (inlet) and vacuum (outlet) or perfusion pumps (MPP-100-220, CBS Scientific Company, Inc.). To achieve steady suction from the outflow pipette, the end of a glass pipette can be beveled with fine-grade sandpaper. Ideally, the central well of the perfusion chamber should be just large enough to hold the filter paper in place. Otherwise, the retina and filter paper can

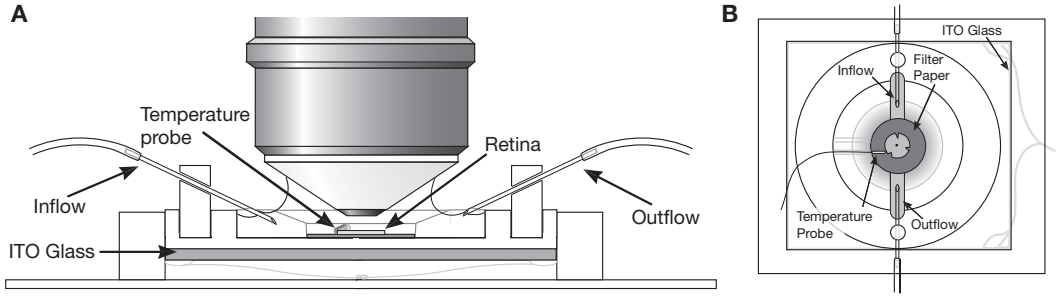

FIGURE 1. Custom-designed perfusion chamber used for imaging live retinal explants on an upright microscope. (*A*) Cross-section through the imaging chamber. (*B*) *Top-down* view of imaging chamber.

be held down with vacuum grease and/or a piece of flattened, 0.5-mm-diameter, platinum wire bent into a ring. Uniform heating can be provided with a Cell MicroControls temperature controller through the ITO glass base (Cell MicroControls, HI-25 or -55) of the chamber.

Pipettes

Polyethylene tubing (e.g., PE-10 [0.61 OD, 0.28 ID], PE-15; A-M Systems, Inc.)

Scalpel blade (#10)

Scissors, fine

Slides, glass

Water bath (37°C)

METHOD

Preparation of Retina Explant for Imaging

Flatmounting a retina on filter paper results in a large plane of intact cells that can be easily labeled and imaged. However, flattening the retina can distort some retinal structures. This distortion is most significant in the retinal periphery and in very young tissue, where there is a greater difference between the inner and outer diameter of the retina. If excessive buckling occurs, consider whole mounting in agarose as an alternative preparation.

Retinal Dissection (5–10 min)

1. Remove eyes from killed animals. Pierce the front of the cornea with 30-gauge needle and immerse the eye in cold (4°C) oxygenated aCSF. If tissue is to be maintained for more then 6 h, aCSF should contain penicillin/streptomycin.
2. Insert fine scissors into the hole created by the needle and remove the cornea by cutting along the retinal margin.
3. While holding the sclera with one pair of forceps, remove the lens, vitreous humor, and ciliary body with a second pair of forceps.
4. Carefully tear the sclera away from the retina in pieces.

Flatmounting on Filter Paper (20–30 min)

For access to the ganglion cell side of the retina, follow Step 5. For access to the photoreceptor side of the retina, follow Step 6.

5. For access to the ganglion cell side of the retina:
   i. Float the retina onto black filter paper, scleral side down.
   ii. Place the filter paper on a glass slide out of the aCSF. Make three radial cuts with the scalpel blade at the folded edge of the retina.

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iii. Use the surface tension between the folded edges of the retina and a fine brush to unfold the retina.

iv. Place the filter paper on a laboratory tissue to wick aCSF through, while replacing the fluid on top of the retina with a pipette. Repeat this step if the retina begins to detach from the filter paper.

v. Return the retina to aCSF and store in an oxygenated incubation chamber.

6. For access to the photoreceptor side of the retina:

i. Float the retinal tissue onto a clean glass slide, scleral side down. Drain excess fluid and make radial cuts around the outer third of the retina with a #10 scalpel blade to flatten the retina.

ii. Use the surface tension between the folded edges of the retina and a fine brush to unfold the retina.

iii. Drain the excess fluid. Gently place a piece of dry black filter paper onto the surface of the flattened retina.

iv. Moisten the filter paper with a few drops of aCSF.

v. Return the retina attached to the filter paper to aCSF and store in an oxygenated incubation chamber.

Mounting in Agarose (20–30 min)

7. Add low-melting-point agarose to aCSF to a final concentration of 0.5%–1% and microwave until the agarose dissolves.
8. Maintain the agarose in a 37°C water bath to delay solidification.
9. Suspend the retina in agarose. Using a glass transfer pipette, gently suck up the retina and a sufficient amount of agarose to cover the retina. Drop the retina and agarose onto black filter paper.
10. Place the filter paper onto an iced slide to rapidly solidify the drop of agarose coating the retina.
11. Return the retina to aCSF and store it in an oxygenated incubation chamber.

Labeling

12. Label flat-mounted retinas with vital dyes by immersion, filling with an intracellular patch pipette, or ballistic labeling with a gene gun (see **Shooting DNA, Dyes, or Indicators into Tissue Slices Using the Gene Gun** [Morgan and Kerschensteiner 2011]). In addition, explanted retinas transfected with DNA by electroporation or ballistic particle delivery can be maintained in culture long enough to express fluorescent proteins.

Agarose mounting can reduce access to the retina with some labeling techniques. This limitation can be overcome with minor modifications of the procedure such as mounting the retina on a coverslip that can be removed later.

Image Acquisition

13. Begin perfusion of the perfusion chamber with oxygenated aCSF and stabilize the temperature at 32°C–34°C and the flow rate at ~1 mL/min.
14. Transfer the retinal tissue to the perfusion chamber for screening with epifluorescence.
15. Adjust imaging parameters on a test cell. Keep the following points in mind:

- To take advantage of the optical resolution of the system, pixel size should be no more than half the diameter of the optical point spread function.
- To minimize bleaching and phototoxicity, multiple fast scans with averaging or Kalman filtering are preferable to single slow scans of the same total pixel dwell time.

- To optimize image contrast, avoid saturation of both excitation and emission detection. For excitation, laser power should be at a level where increasing power continues to increase the in-focus signal. For emission, a high/low color map with dedicated colors for 0 and saturated pixels can be used to determine appropriate photomultiplier tube (PMT) voltage, gain, and offset.
- To estimate the laser power that can be used in a time-lapse experiment, collect multiple stacks on a test cell and increase the laser power until bleaching between scans is observed.

16. Acquire image stacks.

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 16): The tissue shifts during imaging.
Solution: Consider the following:

- Ensure that perfusion inflow and outflow are continuous and at equal rates. Discontinuous or uneven perfusion can result in vibrations and changes in fluid depth, both of which can shift the tissue. Make sure that the inflow pipette is lower than the suction pipette.
- Sudden changes in temperature can result in changes in tissue position relative to the imaging plane. Setting temperature controllers to target a particular temperature can result in the frequent switching of voltage and therefore shifts in tissue position. This problem tends to increase as the thermal delay between the heating element and the temperature probe increases. By setting a temperature controller to provide a nearly constant voltage output, most focus shifts can be avoided.
- Filter paper movements can generally be avoided by using an imaging well of the proper size or by using vacuum grease or a platinum ring. However, if aCSF is oxygenated to the point of outgassing, bubbles can form under the filter paper and may need to be removed manually.

Problem (Step 16): The image quality is poor.
Solution: Consider the following:

- Check that the objective is clean and that no bubbles have formed between the tissue and the lens surface.
- Make sure that no vitreous or connective tissue has been left on the surface of the retina.
- The surface of the tissue can be evaluated by capturing reflected light during laser scanning.
- Mature photoreceptor outer segments can cause significant diffraction. Imaging through the ganglion cell layer may produce better images even when targeting the outer plexiform layer.
- Substitute the use of gain, which does not affect signal-to-noise ratios, with higher PMT voltages and more averaging.
- Median filtering can reduce PMT noise, but compromises resolution. Decreasing voxel size relative to the optical point spread function can compensate for this loss of resolution but will increase bleaching.
- If the tissue is bleaching, reduce laser power, dwell time (lower resolution), or stack size (if not imaging with two-photon).

Problem (Step 16): The explant is unhealthy.
Solution: Consider the following:

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- Improve dissection. Ideally, physical contact with the retina should be restricted to cutting the edges before flattening. Rough edges and uneven transmission of light are signs of physical damage.
- Check the tissue for bacteria. Bacteria can sometimes be seen using transmitted light or epifluorescence at the blue end of the spectrum. Fresh penicillin/streptomycin should prevent bacterial growth. Autoclave tools and follow sterile culture technique if problems persist.
- Make sure that the retina is receiving a steady supply of oxygen and that the temperature is not fluctuating above 35°C.

DISCUSSION

Much information can be gained from isolated retinal whole mounts, or explants, in which retinal neurons can be viewed without distortion by the optics of the eye. En face views of the retina are useful because subtypes of retinal neurons, such as various ganglion cell subtypes, are more readily identified (by their branching pattern) from this angle. In vitro imaging also enables easy manipulation of the composition of the extracellular milieu during pharmacological experiments. In addition, it is more straightforward to combine imaging studies with electrophysiology in the isolated retina (Lohmann et al. 2002) than in in vivo experiments. An obvious disadvantage of in vitro imaging is that the duration of recording is limited and the cellular environment is not the same as that of the intact animal, even though the intrinsic connectivity of the retina is maintained in wholemount preparations. Therefore, we generally perform our recordings within 36 h of the initial dissection. During this period, we have been able to image a wide variety of cell behaviors including spontaneous activity (Wong 1998; Stacy et al. 2005), cell migration (Huckfeldt et al. 2009), axonal targeting of synaptic lamina (Morgan et al. 2006), dendritic remodeling (Wong et al. 2000), and synaptogenesis (Kerschensteiner et al. 2009). Two examples of the quality of images acquired from live retinal explants are provided here.

Example 1

The neurites of retinal neurons such as horizontal cells can be imaged during development of their circuitry in the explanted retinas of the GAD67-GFP transgenic mouse (Fig. 2A; Chattopadhyaya et al. 2004). Two-photon imaging allowed us to obtain multiple large image stacks of these cells, capturing their detailed morphology, with minimal photobleaching.

Example 2

To study synapse formation between bipolar cells and retinal ganglion cells, we biolistically labeled retinal ganglion cells (RGC) with a dendritic fill (td-Tomato) and a marker of glutamatergic postsynaptic sites (PSD95) fused to fluorescent proteins. (See **Shooting DNA, Dyes, or Indicators into Tissue Slices Using the Gene Gun** [Morgan and Kerschensteiner 2011].) After transfecting RGCs in a postnatal day 21 (P21) transgenic mouse that expresses YFP in ON bipolar cells, fluorescently tagged PSD95 can clearly be seen to cluster at the sites of apposition with bipolar cell terminals (Fig. 2B). Time-lapse imaging of RGCs expressing PSD95-YFP at the onset of glutamatergic synaptogenesis (P5) demonstrates that PSD95-YFP clusters appear to be stable over a time course in which dendrites are remodeling (Fig. 2C). The biolistic transfection of the RGCs required a ganglion cell-side-up flat mount preparation and 12–24 h of incubation at 32°C–35°C.

RELATED INFORMATION

A protocol is also available for **In Vivo Imaging of Zebrafish Retina** (Williams et al. 2013).

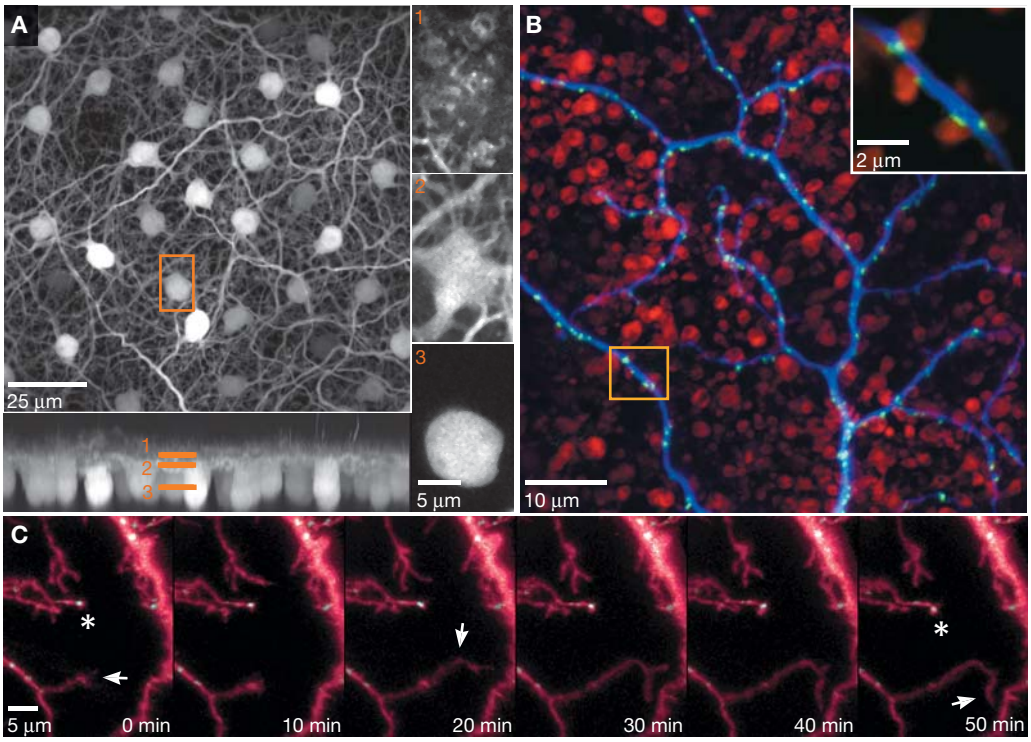

FIGURE 2. Three examples of live mouse retinal explant imaging. (*A*) Horizontal cell mosaic of a P7 GAD67-GFP transgenic mouse imaged with a custom-built two-photon microscope. (*Bottom*) Orthogonal view of the outer retina. (*Right* panels) Individual planes of region within the orange box at the depth indicated by the orange lines in the *bottom* panel. The retina was mounted in agarose to reduce distortion of the outer retina. Images obtained using a 60× 1.1 NA Olympus objective at 1.5× zoom. Voxel size is 0.134 × 0.134 × 0.5 µm. Image median filtered 3 × 3. (*B*) Live confocal imaging of YFP-expressing bipolar cell terminals (red) with PSD95-CFP puncta (green) in an ON retinal ganglion cell; dendrites are labeled by expression of td-Tomato (blue). (*Inset*) Single plane of the boxed region. Voxel size is 0.063 × 0.063 × 0.3 µm. Image was median filtered 5 × 5. (*C*) Confocal time-lapse of P5 retinal ganglion cell expressing td-Tomato (red) and PSD95-YFP (cyan). Images were acquired every 10 min. Asterisk, a stable PSD95-YFP punctum; arrow, a growing dendritic branch.

RECIPE

aCSF for Retina

	For mouse		For chick	
Reagent	g/L	mM	g/L	mM
NaCl	6.95	119	7.29	124
KCl	0.18	2.5	0.37	5
$MgCl_2 \cdot 6H_2O$	0.26	1.3	0.41	2
$CaCl_2 \cdot 2H_2O$	0.37	2.5	0.30	2
NaH_2PO_4	0.12	1.0	–	–
KH_2PO_4	–	–	0.17	1.25
Glucose	1.98	11	3.6	20
HEPES (free acid)	4.76	20	5.48	20

Add 800 mL of double-distilled H_2O to a 1-L flask, and then add the compounds above in the order listed. Titrate the pH to 7.3–7.4 using 5 M NaOH. Bring the volume up to 1 L with double-distilled H_2O. Store at 4°C for 1 d only.

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