Protocol

In Vivo Imaging of Zebrafish Retina

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 vivo preparations. Zebrafish larvae are an ideal specimen for in vivo imaging experiments as they can be made to remain transparent throughout development. Isolated retinal cells can be readily labeled by DNA injection into the one-cell staged embryo, or via transplantation of fluorescently labeled cells from stable transgenics.

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 (0.5%)

Add 250 mg of low-gelling temperature agarose (Sigma-Aldrich A4018) to 50 mL of 0.3× Danieau's solution. Microwave for ~30 sec until the agarose is in solution. Aliquot into tubes or small jars that can be kept at 40°C on a heating block. Allow the agarose to cool before adding embryos.

Danieau's solution (30×) <R>

Prepare in reverse osmosis-purified water (RO water). Dilute 10 mL into 1 L of RO water for 0.3× Danieau's working solution. Store at room temperature or at 28°C–29°C.

PTU (1-phenyl-2-thiourea; 50×)

Dissolve 76 mg PTU into 50 mL of RO water. Stir or shake overnight; make sure the PTU is in solution. Store frozen in 1- to 2-mL aliquots. 50-mL dilutions in 0.3× Danieau's solution can be maintained at room temperature.

Tricaine stock (20×)

Dissolve 200 mg of Tricaine (Sigma-Aldrich A5040) and add 2 mL of 1 M Tris base (pH 9) into 48 mL of RO water. Adjust the pH to 7.0. Protect from light with aluminum foil. Store frozen in 1- to 2-mL aliquots. 50-mL dilutions in 0.3× Danieau's solution can be maintained at room temperature.

Zebrafish embryos

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

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Equipment

Center-well organ culture dishes, 60 × 15-mm (BD Falcon)

Fluorescence dissecting microscope

Forceps, fine

Heating block

Image analysis software

A variety of both open-source and commercial software packages are available for viewing data, preprocessing, reconstruction, and morphological analysis. We have experience with the following.

- 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 chamber, custom-designed

We designed a simple imaging chamber for imaging zebrafish embryos, shown in Figure 1. Petri dishes with a center well are held in place by a Plexiglas block with a hole as wide as the dish (6 cm). An indium tin oxide (ITO) glass (50 × 50-mm, 0.6-mm-thick [Cell MicroControls]) is mounted to the bottom of the holder and an indent in the Plexiglas holder prevents the ITO glass from making contact with the surface of the microscope stage. The ITO glass is held in place by silicon caulk. For an upright microscope, evaporation of the medium is reduced by placing a custom-machined Plexiglas cover with a hole 2–3 mm larger than the objective diameter. There is also a small hole on the surface of the cover to allow for insertion of a thermistor probe.

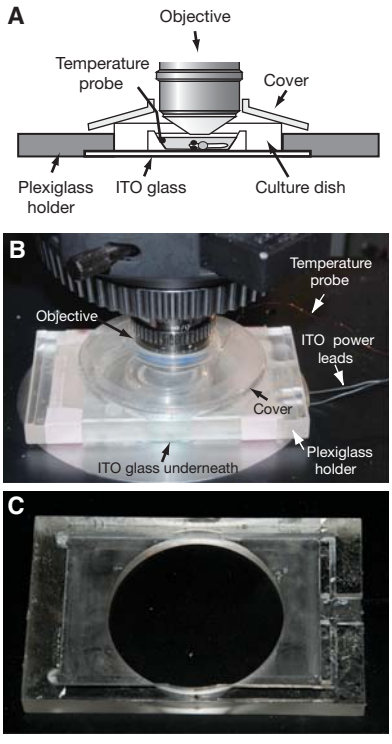

FIGURE 1. Schematic (*A*) and photographs (*B*,*C*) of a custom zebrafish imaging chamber. The zebrafish embryo sits in a center well of the culture dish that rests on a heated ITO glass. The ITO glass is held to a Plexiglas holder with silicone caulk to prevent heating of the microscope stage. A custom cover is also used to prevent evaporation of the media. There is a large center hole to custom fit the proper objective and a small hole on the side for inserting the temperature probe. The Plexiglas holder has a 6-cm hole drilled through the center to fit the culture dish and a rectangular relief for placement of the ITO glass.

Imaging setup:

Color separation, high image acquisition rates, and depth of the cells within the eye should be considered when deciding whether confocal (better color separation and spatial resolution) or multiphoton (less photobleaching and greater imaging depth) microscopy is appropriate.

- Microscope

  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 60× 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.

Temperature controller (TC2BIP, Cell MicroControls)

Transfer pipettes

METHOD

The protocol presented here has been optimized for long-term time-lapse imaging of multiple zebrafish embryos. For general methodology of mounting zebrafish for live-cell imaging, see ***Live Imaging of Zebrafish Development (Godinho 2011)****.*

Screening Zebrafish (20–40 min)

1. Between 10 and 16 h postfertilization (hpf), transfer the embryos into 0.3× Danieau's solution containing 1× PTU.
2. At the appropriate developmental stage, screen the embryos using a fluorescence dissecting microscope for the desired expression pattern.

   See Troubleshooting.

Mounting Samples (30 min)

3. If necessary, dechorionate the embryos with fine forceps.
4. Immobilize embryos in molten 40°C, 0.5% agarose (low melting point) with freshly added 1× PTU and 1× Tricaine.
5. Before the agarose sets, transfer the embryos to a center-well organ culture dish with a transfer pipette and array them as desired with fine forceps.

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6. After letting the agarose set for at least 15 min, immerse the embryos in 3–5 mL of 0.3× Danieau's solution with 1× PTU and 1× Tricaine.
7. Place the culture dish onto the Plexiglas holder and flood the outer well of the culture dish with Danieau's solution containing 1× PTU and 1× Tricaine.

Image Acquisition (20 min per sample)

8. Allow ~15 min for the temperature to equilibrate before imaging.

   Heating the samples during image acquisition is not necessary should they be removed from the microscope stage in between time points.

9. Adjust the correction collar on the objective (if using the 60× 1.1 NA Olympus water dipping objective) by looking through the epiflourescence and maximizing signal intensity.
10. For multiphoton imaging, optimize the wavelength and emission filter cubes for the fluorophore(s) in the sample.

    See Troubleshooting.

11. Identify the cells of interest and set the upper and lower boundaries of the *z*-stack using as little laser power as possible by decreasing pixel resolution to 512 × 512 and increasing the confocal aperture, gain, and/or photomultiplier tube (PMT) voltage. This will prevent unnecessary bleaching while setting up the scan.

    See Troubleshooting.

12. Set the desired pixel resolution for the final image (1024 × 1024, and 2× zoom for a 60×, 1.1 NA objective).
13. Clip a region containing the cell or cells of interest to prevent exciting a larger area than necessary.
14. Optimize the laser power, gain, PMT voltage, and offset using a grayscale high-to-low color map with unique colors for 0 and saturated pixels.
15. Acquire a sample using fast scan with a Kalman filter or averaging to reduce pixel dwell time and thus photobleaching.
16. If performing time-lapse imaging with intervals <1 h apart, leave the samples on the stage in the heated chamber. For longer intervals, it is better to remove the samples and place them in an oxygenated incubation chamber or to remove them from the agarose completely.

    See Troubleshooting.

TROUBLESHOOTING

Problem (Step 2): Samples are dim and difficult to screen.

Solution: If fluorescence is difficult to visualize, first be sure that the embryos are clear. If pigmentation is seen, it is likely that the batch of PTU is bad because PTU can be difficult to dissolve in high concentrations. A fresh batch of PTU stock should be made. If embryos are still in their chorion, dechorionation can improve visualization.

Problem (Step 10): Fluorescent signals are bleeding into both channels.

Solution: When performing two-color multiphoton imaging, it can be very difficult to separate fluorescent emission signals because the excitation curves for most fluorescent proteins are quite broad. With many color combinations (such as CFP/YFP and CFP/GFP) overlap is unavoidable, and with others it may occur if one fluorophore is significantly brighter than the other (such as YFP/td-Tomato). The optimal wavelength for balancing the signal from both

flurophores can change from sample to sample, depending on the intensity of each fluorophore. If necessary, linear unmixing can be used to isolate signals or samples can be analyzed with both channels displayed.

Problem (Step 11): The *z*-plane is drifting.
Solution: Consider the following:

1. Check to be sure the temperature is stable. Rapid temperature fluctuations can causes expansion and contraction of the agarose leading to *z*-plane distortions.
2. If the agarose does not properly adhere to the imaging dish, the sample will float and the *z*-plane will not step correctly. If so, remount the embryos and let the agarose set longer.

Problem (Step 11): The PMTs are saturating with a standard sample.
Solution: Reflected laser light can easily saturate PMTs, making imaging difficult and even causing damage to the detectors. Thus, it is very important to be sure that the samples being imaged have as little pigment as possible. If performing experiments beyond 36 hpf, it is critical to use a mutant that lacks iridophores, like *roy orbison* (Ren et al. 2002). Even in these iridophore-lacking lines treated with PTU, pigment can still develop, especially at ages 5 d postfertilization (dpf) and beyond. In our experience, the ventrotemporal regions of the eye are most resistant to pigment formation and are thus ideal for experiments beyond 5 dpf.

Problem (Step 16, multiphoton imaging): Some cells in the imaging field are dying.
Solution: With multiphoton imaging, some samples may show cell damage even without photobleaching. This is likely owing to heat generation within the region of interest. Photoreceptors seem to be especially susceptible to this damage regardless of fluorescent labeling. If patches of cells are routinely dying within your regions of interest, it is best to decrease the laser power until this is no longer a problem. Alternatively, it is possible to decrease the amount of laser exposure per scan by reducing the dwell time, or to decrease the frequency of exposure by taking less frequent time points. A final consideration is that longer wavelengths of light generate more heat in a sample, an effect that increases significantly past 900 nm. Maintaining the excitation laser at $\leq$890 nm will efficiently excite most fluorescent proteins and should somewhat decrease the likelihood of cell death.

Problem (Step 16): Some fish are dying.
Solution: If there are multiple fish mounted in your culture dish and the ones in the center are dying, the density is too high. For imaging over extended durations, the optimal density of samples per dish is highly dependent on age. Young embryos (24–48 hpf) can be maintained for extended periods with four to six embryos per dish, at 3 dpf, three to four embryos per dish, at 4 dpf, no more than three embryos can be maintained for extended periods, and at time points thereafter, one fish per dish. Bubbling samples with oxygen periodically can increase the number and duration of samples maintained over a time course. Imaging embryos older than 4 dpf for extended periods also requires occasional (approximately once every 12 h) removal of the embryo from agarose and imaging solution to allow more efficient gas exchange and for the embryos to recover from anesthesia. Safely removing embryos from agarose is easily performed with two pairs of fine forceps under darkfield illumination.

DISCUSSION

In vivo imaging is the ideal approach for studying retinal development under natural conditions. The challenge in performing in vivo imaging is finding ways to label cells of interest in the living organism. Visualization of cells in the zebrafish is attained either by transient expression of FPs or by generating

TABLE 1. Transgenic fish lines with expression in retina

Cell type	Reporter(s)	Promoter	Reference(s)
Rod photoreceptors (pan)	GFP	rhodopsin	Hamaoka et al. (2002)
Rod photoreceptors (pan)	GFP	*Xenopus* rhodopsin	Fadool (2003)
Cone photoreceptors (pan)	GFP	TransducinαC	Kennedy et al. (2007)
UV cone photoreceptors (pan)	GFP	SWS1	Takechi et al. (2003)
Blue Cone photoreceptors (pan)	GFP	SWS2	Takechi et al.(2008)
Green Cone photoreceptors (four subpopulation lines)	GFP	RH2-1, RH2-2, RH2-3, RH2-4	Tsujimura et al. (2007)
ON bipolar cells (dense mosaic)	gap43-YFP	nyctalopin:Gal4VP16; UAS	Schroeter et al. (2006)
Bipolar cells (S4 subset) Muller glia (small subset)	GFP	vsx2	Vitorino et al. (2009)
Bipolar cells (most except S4 subset)	GFP	vsx1	Vitorino et al. (2009)
Amacrine and horizontal cells (pan)	GFP	ptf1a	Godinho et al. (2005, 2007)
Amacrine cells (subset)	gap43-GFP gap43-CFP gap43-YFP	*Xenopus* EF1 α and Pax6 enhancer (quail)	Kay et al. (2004) and Godinho et al. (2005)
Amacrine cells (subset)	GFP	Tyrosine hydroxylase	Meng et al. (2008)
Retinal ganglion cells (subset)	gap43-GFP	brn3c	Xiao et al. (2005)
Retinal ganglion cells (pan?)	mCherry	islet2b	Pittman et al. (2008)
Muller glia (pan) ubiquitous	GFP	gfap	Bernardos and Raymond (2006)
	gap43-CFP	*Xenopus* EF1α and Pax6 enhancer (quail)	Godinho et al. (2005)

SWS, short wavelength sensitive; ptf1a, pancreas transcription factor 1a; gfap, glial fibrillary acidic protein; EF1a, elongation factor 1-α.

stable transgenic lines. Transient expression of FPs most often results in mosaic expression, allowing imaging of individual or small clusters of cells. Stable lines can also be created in which specific subtypes of retinal neurons express different FPs. Crossing lines in which retinal cells express different color FPs potentially enables simultaneous visualization of presynaptic and postsynaptic cells during development. Promoter elements and transgenic lines exist to label all major cell types in the zebrafish retina. Table 1 provides examples of transgenic lines well suited for imaging retinal cell populations in the zebrafish retina. Zebrafish genetics are also expanding at a high rate, making the identification of new genetic regulatory elements for cell subtype labeling an easier task. Mutant lines are rapidly accumulating, and although these are lacking, morpholinos can be used for the first few days of development to knock down the expression of a specific gene.

But however versatile the in vivo zebrafish retina is as a model system for time-lapse imaging studies, it does have drawbacks relative to in vitro flat mount preparations. Because images are acquired through the cornea, there is always some distortion that can make imaging of small cellular substructures difficult. This is particularly noticeable in the *z* dimension. The lens also makes imaging of the central regions of the retina very difficult, limiting observations to the peripheral areas. Maintaining the surface of the eye perfectly transparent is not always possible. Even in transgenic lines lacking iridiophores (i.e., *roy orbison*) treated with PTU, some pigment will form, particularly at later ages. A protocol for **In Vitro Imaging of Retinal Whole Mounts** (Williams et al. 2013) describes a procedure for mouse retina which can be applied to other vertebrates.

Nevertheless, using the cell-labeling approaches and imaging parameters outlined here, we have followed the development of axons (Schroeter et al. 2006), dendrites (Mumm et al. 2006) and neurites (Kay et al. 2004; Godinho et al. 2005) of all classes of retinal cells during the period of circuit assembly. Figure 2 shows examples of the labeling of the major classes of retinal cells and combinations of labeled cells in an individual zebrafish. In the future, experiments aimed at elucidating the mechanisms underlying synapse formation, maintenance, or elimination are likely to be more routinely performed.

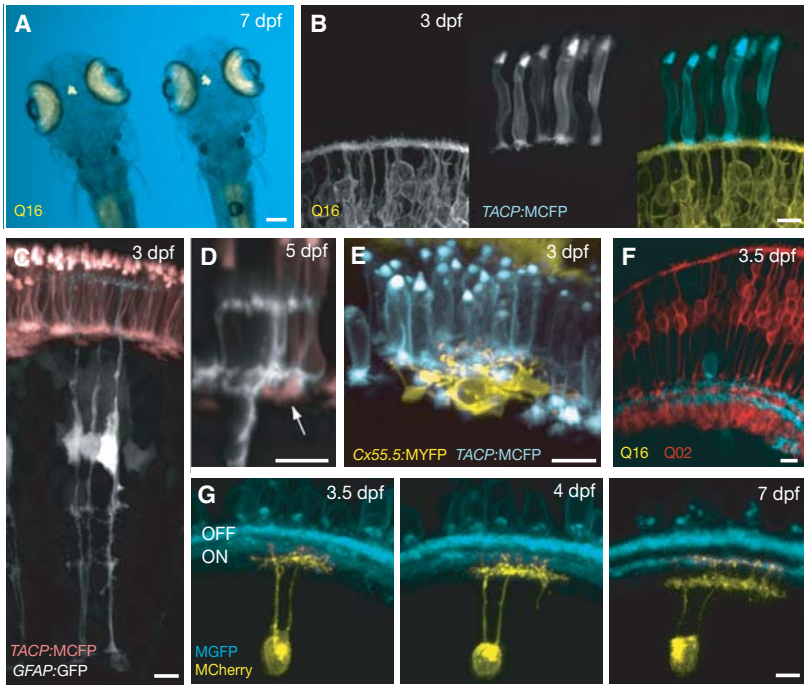

FIGURE 2. Examples of transgenic and transient cell labeling in the developing zebrafish retina. (*A*) Specific labeling of retinal ON bipolar cells using nyctalopin (nyx) promoter elements to drive gap43-YFP expression (Tg(nyx:Gal4;UAS-MYFP)Q16). (Reprinted, with permission of Cambridge University Press, from Schroeter et al. 2006.) (*B*) Presynaptic and postsynaptic partners in the outer retina such as cone photoreceptors and bipolar cells can be simultaneously visualized by injecting the plasmid, TaCP:MCFP into Q16. (DNA constructs modified from Kennedy et al. 2007.) (*C,D*) The relationship between Müller glial cell processes and cone photoreceptor terminals is also visualized by co-injecting TaCP:MCFP and GFAP:GFP plasmids. Shown here (arrow) is a glial process (grayscale) ensheathing cone photoreceptor terminals (pink). (*E*) Horizontal cells are labeled by coinjecting Cx55.5:Gal4VP16 and UAS:MYFP plasmids. Horizontal cell labeling as in Godinho et al. 2007. (*F*) In the inner retina, amacrine cell processes and bipolar cell axons are simultaneously visualized when the Q02 transgenic line is crossed with the Q16 transgenic line. (*G*) Time-lapse imaging of retinal ganglion cells, labeled by transient expression of MCherry driven by the brn3c promoter in Line 220. (Reprinted, with permission of Elsevier, from Mumm et al. 2006.) Scale bars, 500 µm (*A*); 5 µm (*B–G*).

RECIPE

Danieau's Solution (30×)

Reagent	Amount per 1 L	Concentration
NaCl	101.7 g	1740 mM
KCl	1.56 g	21 mM
$MgSO_4 \cdot 7H_2O$	2.96 g	12 mM
$Ca(NO_3)_2$	4.25 g	18 mM
HEPES buffer	35.75 g	150 mM

Add water to 1 L and stir until dissolved. Store at 4°C. The pH is 7.6.

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