Video Article

Preparing Fresh Retinal Slices from Adult Zebrafish for *Ex Vivo* Imaging Experiments

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Abstract

The retina is a complex tissue that initiates and integrates the first steps of vision. Dysfunction of retinal cells is a hallmark of many blinding diseases, and future therapies hinge on fundamental understandings about how different retinal cells function normally. Gaining such information with biochemical methods has proven difficult because contributions of particular cell types are diminished in the retinal cell milieu. Live retinal imaging can provide a view of numerous biological processes on a subcellular level, thanks to a growing number of genetically encoded fluorescent biosensors. However, this technique has thus far been limited to tadpoles and zebrafish larvae, the outermost retinal layers of isolated retinas, or lower resolution imaging of retinas in live animals. Here we present a method for generating live *ex vivo* retinal slices from adult zebrafish for live imaging via confocal microscopy. This preparation yields transverse slices with all retinal layers and most cell types visible for performing confocal imaging experiments using perfusion. Transgenic zebrafish expressing fluorescent proteins or biosensors in specific retinal cell types or organelles are used to extract single-cell information from an intact retina. Additionally, retinal slices can be loaded with fluorescent indicator dyes, adding to the method's versatility. This protocol was developed for imaging Ca^{2+} within zebrafish cone photoreceptors, but with proper markers it could be adapted to measure Ca^{2+} or metabolites in Müller cells, bipolar and horizontal cells, microglia, amacrine cells, or retinal ganglion cells. The retinal pigment epithelium is removed from slices so this method is not suitable for studying that cell type. With practice, it is possible to generate serial slices from one animal for multiple experiments. This adaptable technique provides a powerful tool for answering many questions about retinal cell biology, Ca^{2+} , and energy homeostasis.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56977/>

Introduction

The zebrafish (*Danio rerio*) has become widely used in medical and basic scientific research¹, owing to its small size, rapid development and vertebrate organ systems. The natural transparency of zebrafish larvae combined with established methods for transgenesis have enabled detailed visualization of cellular processes in a living animal. A number of genetically encoded fluorescent biosensors have been targeted to specific zebrafish cells to detect Ca^{2+} ², hydrogen peroxide³, apoptotic activation⁴ and ATP⁵.

In vivo imaging of zebrafish larvae has led to breakthroughs in the field of neuroscience, including mapping of brain circuitry⁶ and drug development for central nervous system disorders⁷. Zebrafish are well suited for vision research because their retinas feature the laminar structure and neuron types of higher vertebrates, and they display robust visual behaviors^{8,9}. Several types of retinal degenerations analogous to human disease have been modeled successfully and studied in zebrafish^{10,11}, including live imaging of individual photoreceptors degenerating within a retina^{2,12}.

While *in vivo* larval zebrafish imaging is a valuable tool, it becomes more challenging as fish grow and develop pigmentation, and some pharmacological treatments cannot permeate an entire animal. Further, certain cellular processes change with development and age, making later time points critical for understanding function and the progression of disease in adult animals. Biochemical methods such as immunoblot, quantitative PCR, O_2 consumption, and metabolomic analyses can provide important clues about biology of the retina as a whole, but it is difficult to discern contributions of individual cell types affected by disease. Imaging isolated retinal tissue *ex vivo* bypasses these issues, and while imaging flat mounted retinas affords a view of the outer retina¹³, deeper inner retinal features are obscured. Transverse retinal slices, such as those presented in fixed immunohistochemical analyses, enable a clear view of all layers and cell types but only offer a single snapshot of the dynamic processes involved in normal function and disease.

Here, we present a method for generating *ex vivo* transverse retinal slices from adult zebrafish for imaging. It is similar to methods for preparing amphibian and zebrafish retinal slices for electrophysiological and morphological studies^{14,15}, with important modifications for time lapse imaging *ex vivo* using confocal microscopy. Fluorescence responses of biosensors or dyes in slices are monitored in real time with a confocal microscope

while delivering pharmacological agents using perfusion. While the method was developed for imaging photoreceptors, it may be feasible to use it for visualizing Müller cells, bipolar cells, horizontal cells, amacrine cells, or retinal ganglion cells with appropriate fluorescent markers. Additionally, slices can be loaded with fluorescent cell-permeable dyes to report cell viability, vesicular transport, mitochondrial function, or redox state. This versatile preparation allows visualization of a wide range of subcellular processes throughout the retina, including Ca^{2+} dynamics, signal transduction and metabolic state.

Protocol

All animal experiments were approved by the University of Washington Institutional Animal Care and Use Committee.

1. Preparing Animals and Equipment

NOTE: The retinal pigment epithelium (RPE) is a dark sheet of tissue surrounding the outside of the retina whose pigmentation can obscure retinal features and damage the tissue when confocal imaging *ex vivo*. In darkness, the RPE of zebrafish is retracted away from the retina; dark adapt fish to facilitate future removal of the RPE from the retina before slicing and imaging.

1. Transfer fish to a spawning tank filled with fish water, and then wrap the spawning tank with dark fabric or place it in a dark cabinet.
 1. Dark adapt zebrafish for at least 1 h prior to euthanasia to allow near complete separation of the RPE from the retina. 30 min dark adaptation is sufficient to remove most RPE, though pieces of it may remain intercalated between photoreceptors.
2. Melt ~ 15 mL of petroleum jelly in a 50-mL beaker on a hot plate and then draw 3 mL of the liquid into a 3-mL slip tip syringe. Invert syringe, place in a test tube rack, and allow petroleum jelly to cool.
3. Make a reusable slicing chamber on a plain 7 cm X 2.5 cm microscope slide.
 1. Using clear nail polish, paint narrow lines to create a 3 cm X 2.5 cm rectangle in the center of the slide, allow it to dry, and then add another layer of nail polish to the lines.
4. Prepare imaging ladders on cover slips to hold slices during imaging. The slices will form the "rungs" of the ladder.
 1. For static imaging or injection experiments with minimal solution flow, make petroleum jelly ladders consisting of two flat wide parallel strips of petroleum jelly on 18 mm square glass cover slips. Use the syringe to apply two flattened ~ 1 cm long smears of cooled petroleum jelly 0.5 cm apart on the cover slip.
5. Ready the tissue slicer.
 1. Clean a double edge razor blade with ethanol and allow it to air dry. Cut it into quarters with scissors, first lengthwise into halves then across each blade.
 2. Place the slicing chamber on the stage of the tissue slicer, center it horizontally on the stage and mark a long edge with permanent marker for alignment.
 3. Load a blade section onto the tissue slicer arm, ensure the blade lies flat and centered on the slide without touching the nail polish, then gently tighten the blade apparatus. Lower the blade arm by adjusting the knob $\frac{1}{4}$ turn, then place a scrap of filter paper in the center of the imaging chamber and test cut it. If the paper is not cut fully through, remount the blade.
6. Using the syringe, place a single small dot of cooled petroleum jelly in a 10-cm Petri dish ~ 1.5 cm to the right of the center. Press an imaging ladder into it using forceps with the petroleum jelly facing up. Make another small petroleum jelly dot ~ 1 cm from the inlet edge of the imaging chamber.
7. Fit the petroleum jelly syringe with a 20g needle and uncap it. Hold the needle onto the syringe and use it to make two 1 cm long thin parallel strips of petroleum jelly lengthwise in the center of the slicing chamber. Space the strips ~ 1 cm apart.
8. Make a reusable wire eye loop tool by wrapping the center of a ~ 4 cm segment of 30 g tungsten wire around a pair of closed forceps once tightly. Adjust the diameter of the loop by sliding the wire up or down the forceps until it is slightly larger than a zebrafish eye, typically ~ 2-3 mm. Twist the wire ends and secure them to the end of a 6-cm wooden stick using laboratory tape.
9. Prepare Ringer's solution.
 1. Thaw 50X supplement stock solution (**Table 1**) and add it fresh to HEPES-buffered, non-bicarbonate Ringer's solution (**Table 2**) the morning of the experiment; dilute 200 μL of supplement stock solution in every 10 mL of Ringer's solution in a conical centrifuge tube or sterile glass bottle. For static imaging experiments, prepare at least 30 mL of Ringer's solution per experiment. The volume of Ringer's solution needed for perfusion experiments will depend on the flow rate and total experiment time.
 2. Check that the pH of the supplemented solution is 7.4 using a digital pH probe or pH paper, and adjust accordingly with dilute NaOH or HCl.
 3. Oxygenate supplemented Ringer's solution on ice by bubbling with 100% oxygen gas for at least 5 min using a standard medical oxygen tank and regulator fitted with a hose, or the optional gas bubbler manifold used for perfusion. Store oxygenated Ringer's solution on ice in a sealed conical centrifuge tube or sterile glass bottle near the dissection microscope; use this solution for dissection, imaging, and to dilute dyes or pharmacological agents.
 4. If other solutions are being used in the experiment, such as Na^+ -free Ringer's solution (**Table 3**), repeat steps 1.9.1.-1.9.3.
10. Gather Petri dishes, forceps, micro-scissors and other tools near the dissection microscope, and prepare a fish water ice bath for zebrafish euthanasia.

2. Preparing retinal slices (see Figure 1)

1. Working under red ambient light to minimize light adaptation (which can make the RPE stick more tightly to the retina), euthanize zebrafish by immersion in the ice bath until touch response is lost, typically 1-2 min. Transfer the fish to a Petri dish and use a scalpel to cervically dislocate but not decapitate the fish.

2. Use the wire loop to loosen connective tissue around one eye, and then pull the eye forward gently with the loop in one hand. Using micro-scissors in the other hand, cut the white optic nerve under the eye, taking care not to cut the back of the eye.
3. Transfer the eye using forceps to a Petri dish of cold Ringer's solution on ice, and repeat for the second eye. Keep the eyes in darkness or under red light until the RPE is removed from the retina.
4. Dissect the eyecups under a low power dissection microscope in a drop of cold Ringer's solution on a plain glass slide in a Petri dish.
 1. Pierce the cornea with fine forceps, then gently remove pieces of clear, brittle cornea and silvery sclera with forceps or scissors. Remove and discard the lens and most of the sclera (see **Figure 1A**), and handle the isolated eyecup minimally.
 2. Pieces of fat, small bits of sclera, and black RPE can remain attached to the eyecup and removed from the retina later. If the retina separates from the RPE in step 2.4.1, proceed with the same steps using extra caution not to damage the delicate isolated retina.
 3. Position eyecup open side down (RPE up) on the slide, and cut into thirds or quarters with a fresh single edge razor blade in one motion (see **Figure 1B**). Discard pieces of tissue that are highly curved.
5. Flat mount retina on filter paper.
 1. Wet a piece of filter paper with Ringer's solution and place it on the slide next to the eyecup pieces. Use flat forceps when handling the intact wet filter paper to avoid puncturing it. Add cold Ringer's solution to cover both the filter paper and tissue.
 2. Using forceps in each hand, carefully drag the filter paper underneath each eyecup piece with the RPE and photoreceptors facing up, i.e. with the back of the eye facing up. Position the eyecup pieces in a single line along the center of the filter paper (see **Figure 1C**). Handle the eyecup pieces gently with fine forceps only near an edge or corner.
 3. To help retinas adhere to the filter paper, place the wet filter paper on a dry paper towel for 3 s to wick moisture downward, but don't let the tissue become dry. Repeat until the eyecup pieces lie flat on the filter paper. Applying gentle suction to the underside of the filter paper helps flatten the retina, but this step is not essential.
6. If black sheets of RPE remain on the eyecup pieces, use fine forceps to gently peel it away starting from one corner (see **Figure 1D**) while the tissue is sitting in a drop of Ringer's solution. Should the retina lift off the filter paper, repeat the wicking step in 2.5.3. The retina may appear pink due to unbleached visual pigments.
7. Repeat retina dissection and flat mounting steps in 2.4-2.6 for the second eye, if desired, keeping the first flat-mounted retina immersed in cold Ringer's solution. To streamline the slicing procedure, pieces of both retinas may be placed on one filter paper. Once the RPE has been removed, the protocol can be carried out under normal room light unless experiments necessitate darkness.
8. Place the filter paper on a slide and trim it into a rectangle with a single edge razor blade, leaving ~ 0.5 cm of filter paper on either side of the line of retinas. Move the filter paper to the prepared slicing chamber, push the long filter paper edges into the thin petroleum jelly lines using forceps, and immerse the retinas in 3-4 drops of cold Ringer's solution.
NOTE: Some dyes, such as lipophilic dyes, are best loaded into flat mounts at this stage prior to slicing. These can be loaded, wicked away with a tissue, and washed in the slicing chamber. For instance, C¹² 558/568 BODIPY intensely stains retinal cell membranes and photoreceptor outer segments (see **Figure 4A**) when loaded at ~ 5 µg/mL for 15 min at room temperature (typically 23-27 °C), followed by a wash in excess Ringer's solution.
9. Transfer the slicing chamber to the tissue slicer stage, position the long edge along the marked line, and secure the chamber ends to the stage with laboratory tape. Starting at one end, cut the retina and filter paper using firm, gentle pressure on the slicing arm. Check that the first slice was cut fully, then use the micrometer to cut ~ 400 µm slices.
10. Assemble the imaging ladder.
 1. Place the slicing chamber with sliced retinal sections in the Petri dish from step 1.7 adjacent to the imaging ladder (see **Figure 1E**). Fill the dish with cold Ringer's solution to submerge its contents.
 2. Using forceps and keeping slices submerged, gently transfer strips of filter paper and retina to the ladder by sliding the Petri dish from left to right. Take care not to touch retinas directly. Rotate the slices 90° and bury the filter paper edges in petroleum jelly.
 3. Finely position retinal slices in the ladder with forceps so that retinal layers are clearly visible under the dissection microscope (see **Figure 1G**). For slices on each end of the ladder, ensure that the tissue is facing inward toward the other slices to minimize motion of the tissue during injection or flow. Discard any retinal slices that are not well adhered to the filter paper (see **Figure 2A**).
11. If desired, load dyes into retinal slices at this stage and wash in excess Ringer's solution prior to imaging.
NOTE: Propidium iodide (PI) and Hoechst 33342 robustly stain nuclei of dead and all cells, respectively (see **Figure 2B**), when incubated with retinal slices at 5 µg/mL for 20 min at room temperature. Tetramethylrhodamine (TMRM) accumulates in actively respiring mitochondria throughout the retina when incubated at 1 nM for 30 min at room temperature.
12. While the slices are staining, prepare the imaging chamber and injection apparatus. Use the syringe to flush the tubing with Ringer's solution and purge bubbles, attach the open end of the tubing to the imaging chamber inlet, and close the stopcock. Remove the syringe and fill it with reagent(s) for injection, then reattach it to the tubing.
13. Use forceps to transfer the coverslip with retinal slices to the imaging chamber, pressing the coverslip into the dot of petroleum jelly near the inlet edge of the imaging chamber (see **Figure 1H**). Fill the imaging chamber with Ringer's solution to cover slices.

3. Imaging retinal slices

1. Place the filled imaging chamber with the connected injection apparatus on the stage of an upright confocal microscope equipped with a 20 or 40X water dipping lens. Secure the imaging chamber with stage clips.
2. Lower the dipping lens over the ladder, and focus on a slice at one end of the ladder under dim trans-illuminated light. Examine each slice for transverse orientation, presence of photoreceptor outer segments, and secure adhesion to the filter paper.
3. Select the best slice for time lapse imaging and configure the microscope software for time lapse image acquisition. **Table 4** outlines typical imaging settings for various fluorescent markers and dyes.
NOTE: The rate of image acquisition will vary depending on the microscope, fluorescent marker(s), and biological process being studied. For instance, use 800x800 pixel resolution, 2 µs/pixel scan speed, and a 10-s frame rate for imaging calcium dynamics in photoreceptors with GCaMP3 and a red dye.

1. If available, use the software to trace physical landmarks in the slice (photoreceptor outer segments, cell bodies, nuclei) to aid potential reorientation during the time lapse. Also set up the software to monitor real-time fluorescence across the slice.
4. Begin imaging and monitor baseline fluorescence. When fluorescence across the slice stabilizes (typically within 2-5 min), proceed with the experiment. For injection, open the syringe stopcock, then slowly depress and draw back on the plunger twice to aid mixing.
NOTE: For instance, abolish mitochondrial function by injecting a concentrated solution of the protonophore CCCP to reach a final concentration of 1 μM in the imaging chamber. This induces a robust Ca^{2+} burst in photoreceptor cytosol reported by GCaMP, then a steady decrease in mitochondrial membrane potential reported by TMRM.
5. Closely monitor slices for drift during the experiment, and use the software to make micro-adjustments according to physical landmarks. Typically drift in the Z-direction during injection or perfusion is $< 5 \mu\text{m}$.

4. Imaging retinal slices during perfusion experiments where solutions are changed or flowed continuously

NOTE: Imaging retinal slices during perfusion experiments where solutions are changed or flowed continuously is similar to setup for static imaging or injection experiments, with the following modifications.

1. Instead of petroleum jelly ladders described in step 1.4, use sturdier wax ladders to hold retinal slices steady during solution flow.
 1. Place two small parallel cylinders of unflavored dental wax $\sim 0.5 \text{ cm}$ apart on a coverslip. On a flat surface, use a thumb to press each cylinder down and out toward the parallel edge of the coverslip. Score both flattened cylinders horizontally with a #1 coverslip (see **Figure 1E**, right side) then smear a thin layer of petroleum jelly between the wax strips with a spatula.
 2. When assembling the imaging ladder in step 2.10.2, press the sliced filter paper edges into the wax scores using fine forceps (**Figure 1G**).
2. To set up for perfusion in step 2.12, fill syringe reservoirs with preoxygenated solutions, or use the optional gas manifold to oxygenate solutions in each reservoir. Flush all tubing with Ringer's solution, ensure all lines flow when opened and purge large bubbles.
3. Before filling the imaging chamber in step 2.13, use the syringe to place another dot of petroleum jelly over each exposed corner of the coverslip to prevent it from sliding laterally during flow.
4. When the imaging chamber is filled and mounted on the microscope stage, turn on the aspirator and connect the aspirator tubing. Test the flow of the Ringer's solution and use the micropositioner to situate the aspirator tube over the outflow chamber so that small amounts of liquid are drawn off before the chamber overflows (typically $\sim 1 \text{ mm}$ above the solution surface for a 2 mL/min flow rate). Keep Ringer's solution flowing while selecting slices in step 3.4.
5. To conduct the experiment in step 3.4 for perfusion, switch flow of solutions by closing the stopcock of the first solution while opening that of the second solution.
 1. For instance, to deplete extracellular Na^+ from the imaging chamber, use two syringe reservoirs filled with Ringer's solution or Na^+ -free solution (**Table 3**). Flow Ringer's solution to establish baseline, then switch to Na^+ -free solution. This results in large cytosolic Ca^{2+} increases in photoreceptor outer segments and cell bodies (see **Figure 4**).
6. For gravity-fed perfusion systems, monitor the level of solution in each reservoir so the flow rate is constant during imaging. Top off reservoirs as needed with oxygenated solutions, or maintain continuous oxygen bubbling with the gas manifold.

Representative Results

Stable positioning and transverse orientation of slices are key to successful imaging with injection or perfusion of pharmacological agents. Carefully examine and reposition slices prior to confocal imaging as needed to ensure all retinal layers are visible (**Figure 2A**, slice ii). If a slice is rotated slightly forward (**Figure 2A**, slice iii), bundles of outer segments will be visible and small adjustments can be made with forceps to bring the desired retinal layers into focus. Slices poorly adhered to the filter paper (**Figure 2A**, slice i) or retaining RPE (**Figure 2A**, slice iv) should not be used for time lapse imaging.

Cell viability is paramount to observing physiological processes *ex vivo*; a cell viability stain such as propidium iodide (PI) is recommended to assay cell health while practicing retina slicing. Dead cells near the cut edge of all slices will accumulate PI in their nuclei (**Figure 2B**, left panels), while 5-10 μm deeper into the slice, healthy cells with normal morphology and no PI staining can be imaged. For example, in photoreceptors, PI negative cells below the cut edge commonly display a stereotypical polarized, elongated morphology (**Figure 2B**, right panels). Photoreceptors remain viable in retinal slices for at least 4 h when stored in oxygenated Ringer's solution.

Many retinal cell structures can be visualized using combinations of transgenic markers and dyes; example confocal images of fresh retinal slices with double and triple fluorescent labeling are presented in **Figure 3**. To visualize dynamics of cone photoreceptor endoplasmic reticulum (ER) relative to the nucleus, retinal slices from transgenic fish expressing GFP targeted to cone ER¹⁷ can be counterstained with a nuclear dye (**Figure 3A**). A similar strategy can be employed to view the actin cytoskeleton using retinal slices from transgenic zebrafish expressing GFP-fused actin¹⁸ (**Figure 3B**). With another cell-specific promoter, Müller cells can be labeled with the red fluorescent protein tdTomato¹⁹ and visualized in retinal slices (**Figure 3C**, top panel). Glucose uptake into the retina can be assayed *in vivo* by orally gavaging adult zebrafish with a fluorescent glucose analog (NBDG), which becomes incorporated into cells visible in a retinal slice²¹ (**Figure 3C**, bottom panel). Double transgenic zebrafish can be employed to monitor multiple cell processes or cell types in tandem, such as Ca^{2+} dynamics in a subtype of cones. **Figure 3D** shows a triple labeling scheme for this type of experiment, with tdTomato expressed in long-wavelength cones² together with mitochondrially-targeted Ca^{2+} sensor (mito-GCaMP) expressed in all cones²² and a nuclear stain.

Time lapse imaging of retinal cells is a key advantage of this slice prep. For instance, Ca^{2+} fluctuations in cone photoreceptor cytosol can be observed and later quantified during pharmacological manipulation, an experiment depicted in **Figure 4**. **Figure 4A** shows retinal slices expressing the Ca^{2+} sensor GCaMP² (top) or control eGFP¹⁶ (bottom) in cone photoreceptors counterstained with a red lipophilic dye. In this experiment Na^+ is isotonically depleted from the imaging chamber using perfusion, evoking large increases in cytosolic Ca^{2+} for the outer segment and cell body as reflected by GCaMP (**Figure 4A**, top panel). Fluorescence of retinal slices expressing Ca^{2+} -insensitive eGFP is unaffected by this treatment (**Figure 4A**, bottom panel). Time lapse movies can be analyzed using ImageJ software to isolate and quantify fluorescence responses from single cone outer segments, cell bodies, and synapses (**Figure 4B**).

Supplement Solution		
	50X stock* concentration (mM)	working concentration (mM)
D-glucose	500	10
sodium lactate	50	1
sodium pyruvate	25	0.5
L-glutamine	25	0.5
reduced glutathione	25	0.5
ascorbic acid	15	0.3

* store aliquots at -20 °C < 6 months; add fresh to Ringer's solution

Table 1. Components of 50X supplement stock solution and final working concentrations.

Ringer's Solution	
	concentration (mM)
NaCl	133
KCl	2.5
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	2
NaH_2PO_4	1.5
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	1.5
HEPES	10
pH to 7.4 using NaOH	
store at 4 °C in sterile bottle < 1 month	

Table 2. Components of standard Ringer's solution.

Na^+ -Free Ringer's Solution	
	concentration (mM)
TRIS	147
HCl	120
KCl	1
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	2
KH_2PO_4	1.5
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	1.5
HEPES	10
pH to 7.4 using HCl	
store at 4 °C in sterile bottle < 1 month	

Table 3. Components of Na^+ -free Ringer's solution.

Confocal Imaging Settings			
Fluorophore	Excitation		Emission Filter
	Wavelength	Laser Intensity	
GFP (including GCaMP)	488 nm	2 - 5%	eGFP or AlexaFluor 488
tdTomato	559 nm	5%	AlexaFluor 594
PI	559 nm	2%	PI
Hoechst 33342	405 nm	1%	DAPI
NBDG	488 nm	10%	eGFP or AlexaFluor 488
C ¹² 558/568 BODIPY	559 nm	1%	AlexaFluor 594
TMRM	559 nm	3%	RFP

Table 4. Imaging conditions for fluorescent markers and dyes presented in Figures 2-4.

Figure 1. Schematic for preparing fresh zebrafish retinal slices. (A) Dissect away the eyecup, and discard lens and sclera (step 2.4.1.). (B) Cut the eyecup into three pieces; discard small edge piece (step 2.4.2.). (C) Drag filter paper under eyecup pieces with the inner retina facing toward the filter paper (step 2.5.1.-2.5.2.). (D) Flatten retina by using a paper towel to wick Ringer's solution downward through the filter paper (step 2.5.3.), then gently peel away remaining RPE with forceps (step 2.6). Move the filter paper to the slicing chamber on the tissue slicer stage and cut 400 μ m slices (steps 2.8., 2.9.). (E) Transfer slicing chamber with slices and a wax or petroleum jelly ladder to a Petri dish of Ringer's solution (step 2.10.1.). (F) Use fine forceps to slide single slices from the slicing chamber to the ladder while keeping slices submerged. (G) Rotate filter paper strips (black) 90° and bury the edges of the filter paper in wax or petroleum jelly (steps 2.10.2.-2.10.3.). (H) Schematic of the final imaging chamber loaded with a ladder and slices. [Please click here to view a larger version of this figure.](#)

Figure 2. Examples of fresh zebrafish retinal slices displaying proper adhesion to the filter paper and cell viability. (A) Brightfield image of fresh retinal slices in a petroleum jelly ladder. Slices ii and iii display good adhesion and transverse retinal layers. Slices i and iv have retained substantial RPE, or are highly curved and not well-adhered to the filter paper, and should not be imaged. Scale bar = 200 μ m. **(B)** Top-down Z montage of a fresh retinal slice from transgenic zebrafish expressing eGFP in cone photoreceptors (*Tg(gnat2:eGFP)¹⁶*). Hoechst dye labels all nuclei; propidium iodide (PI) counterstaining labels nuclei of dead cells, which appear near the cut edge of the slice (left). Z-stack step size = 1 μ m; scale bar = 20 μ m. Fluorescent imaging conditions are outlined in Table 4. [Please click here to view a larger version of this figure.](#)

Figure 3. Sample confocal images of double- and triple-labeled ex vivo retinal slices from transgenic adult zebrafish. (A) Two-color imaging scheme employing transgenic GFP tagged endoplasmic reticulum in cones (*Tg(gnat2:calr-GFP)*¹⁷, top) with Hoechst nuclear counterstain (blue, bottom). (B) GFP tagged actin in cones (*Tg(gnat2:LifeAct-GFP)*¹⁸, top) with Hoechst nuclear counterstain (blue, bottom). (C) RFP labeled Müller cells (*Tg(GFAP:tdTomato)*¹⁹, top) from a zebrafish fed fluorescent glucose (NBDG) demonstrating glucose uptake into cones^{20,21} (green, bottom). (This image is from **Figure 2B** of Kanow, *et al.*²¹) (D) Example of three-color imaging using double transgenic zebrafish. Left, RFP targeted to long-wavelength cone photoreceptors (*Tg(trβ2:tdTomato)*²). Center, calcium biosensor GCaMP targeted to cone photoreceptor mitochondria (*Tg(gnat2:mito-GCaMP3)*²²). Right, overlaid images of tdTomato (magenta), mito-GCaMP (green), and Hoechst nuclear counterstain (blue). Images are maximum intensity Z-projections of 9 frames over a 7 μm tissue depth; scale bars represent 10 μm. Fluorescent imaging conditions are outlined in **Table 4**. [Please click here to view a larger version of this figure.](#)

Figure 4. Ca^{2+} imaging with GCaMP and control eGFP. **(A)** Representative images of fresh retinal slices expressing the fluorescent Ca^{2+} biosensor GCaMP (*gnat2:GCaMP3²*, top) or eGFP (bottom) in cone photoreceptors. Left, slices at baseline; right, slices 2 min after Na^+ was isotopically depleted from the imaging chamber using perfusion of a Tris-based Ringer's solution, which traps Ca^{2+} in photoreceptors. Scale bars = 10 μm . Fluorescent imaging conditions are outlined in **Table 4**. **(B)** Mean fluorescence changes of single photoreceptor compartments (outer segments, cell bodies, and synapses) during time lapse imaging of Na^+ depletion. Slices were imaged every 10 s, stacks were processed using ImageJ, and fluorescence of GCaMP or eGFP was normalized to signal from the membrane dye. Solid lines, GCaMP (n for outer segments = 15, cell bodies = 24, synapses = 26); dashed lines, eGFP (n for outer segments = 26, cell bodies = 20, synapses = 31). Error bars represent standard error of the mean. [Please click here to view a larger version of this figure.](#)

Discussion

Ex vivo imaging of fresh zebrafish retinal slices has proven to be a versatile tool for studying photoreceptor biology^{20,21,22}, and is unique in that it enables analysis of single cells in a mature, fully differentiated retina. With practice, it is possible to conduct multiple experiments with tissue from a single fish, even using serial slices from the same part of the retina. In addition to the challenges and suggestions regarding preparation of amphibian retinal slices for electrophysiology studies¹⁴, there are important considerations for imaging experiments.

For photoreceptors, cell viability generally correlates with cell morphology, so it is important to handle the delicate retina minimally, particularly after the RPE has been removed. After slicing, use fine forceps to carefully slide slices horizontally away from the slicing chamber (**Figure 1F**) to transfer them to the ladder rather than lifting slices straight up, and always keep slices submerged in Ringer's solution. If possible, assemble the ladder of retinal slices near the confocal microscope to minimize damage to slices from shaking during transport.

Strong adhesion of retinal tissue to the filter paper is critical for creating slices that will remain stable during injection or perfusion experiments. It is necessary to carefully inspect each slice for morphology and stability just prior to imaging (**Figure 2A**); gently tapping the microscope table while viewing slice movement through the confocal ocular lens reveals stability of a particular slice. Despite precautions, drift in the Z-direction during injection or perfusion can present challenges for analysis, so it is useful to load a control dye, such as lipophilic dyes for membranes and mitochondria, to stably label an identifiable cell structure for normalization (**Figure 4A**, magenta). If slices drift > 10 μm in the Z-direction it may not be possible to extract usable single-cell data. Moderate drift in the X-Y direction can be corrected in post-processing using registration software such as MultiStackReg for ImageJ (RRID:SCR_002285).

Under static conditions, fluorescence of markers in retinal slices should remain stable, although photobleaching can occur during imaging. Care should be taken to minimize laser exposure during time lapses by refining parameters such as laser intensity, scan speed, and frame rate. Imaging controls are recommended for each fluorescent marker used. Controls include injecting or perfusing Ringer's solution without pharmacological agents in separate experiments, or conducting imaging experiments with non-biosensor markers that fluoresce constitutively.

Depending on the confocal excitation laser being used, pigments in the RPE can contribute to autofluorescence and even generate heat during imaging, so it is important to remove most of this tissue prior to slicing. Dark-adapting animals aids in removal of the RPE while minimizing damage to the underlying retina. Inability to image the RPE is a limitation of this slice preparation, though this may be overcome by using albino animals with a transparent RPE. Another limitation is that retinal ganglion cells and end feet of Müller cells may become obscured from view by the filter paper; as an alternative preparation retinas may be flat mounted with the photoreceptor side down on the filter paper and then sliced to provide a clearer view of the innermost retina. It is also important to note that long horizontal projections of some cells, such as wide field amacrine cells, may be severed during dissection or slicing. A final limitation is that many fluorescent dyes accumulate nonspecifically in photoreceptor outer segments, so for this part of the retina it is advisable to use genetically encoded biosensors rather than indicator dyes.

Given the wide range of available fluorescent cell reporter dyes^{23,24} for tissue and genetically encoded fluorescent biosensors used successfully in zebrafish^{2,3,4,5}, this slice preparation could be used to study numerous biological processes in several retinal cell types (see examples in **Figure 3, Figure 4**). Imaging fresh retinal slices using live cell super-resolution microscopy could also provide exciting new insights to retinal function and health on a subcellular level. Further, this method can be adapted for *ex vivo* imaging of mouse retinal slices²⁵. While successful preparation of fresh retinal slices requires practice, it is a powerful tool that is useful for addressing a wide range of cell-specific biological questions in a mature retina.

Disclosures

The authors declare that they have no competing financial interests.

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