

Video Article

Dissection, Culture, and Analysis of *Xenopus laevis* Embryonic Retinal Tissue

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Abstract

The process by which the anterior region of the neural plate gives rise to the vertebrate retina continues to be a major focus of both clinical and basic research. In addition to the obvious medical relevance for understanding and treating retinal disease, the development of the vertebrate retina continues to serve as an important and elegant model system for understanding neuronal cell type determination and differentiation¹⁻¹⁶. The neural retina consists of six discrete cell types (ganglion, amacrine, horizontal, photoreceptors, bipolar cells, and Müller glial cells) arranged in stereotypical layers, a pattern that is largely conserved among all vertebrates^{12,14-18}.

While studying the retina in the intact developing embryo is clearly required for understanding how this complex organ develops from a protrusion of the forebrain into a layered structure, there are many questions that benefit from employing approaches using primary cell culture of presumptive retinal cells^{7,19-23}. For example, analyzing cells from tissues removed and dissociated at different stages allows one to discern the state of specification of individual cells at different developmental stages, that is, the fate of the cells in the absence of interactions with neighboring tissues^{8,19-22,24-33}. Primary cell culture also allows the investigator to treat the culture with specific reagents and analyze the results on a single cell level^{5,8,21,24,27-30,33-39}. *Xenopus laevis*, a classic model system for the study of early neural development^{19,27,29,31-32,40-42}, serves as a particularly suitable system for retinal primary cell culture^{10,38,43-45}.

Presumptive retinal tissue is accessible from the earliest stages of development, immediately following neural induction^{25,38,43}. In addition, given that each cell in the embryo contains a supply of yolk, retinal cells can be cultured in a very simple defined media consisting of a buffered salt solution, thus removing the confounding effects of incubation or other sera-based products^{10,24,44-45}.

However, the isolation of the retinal tissue from surrounding tissues and the subsequent processing is challenging. Here, we present a method for the dissection and dissociation of retinal cells in *Xenopus laevis* that will be used to prepare primary cell cultures that will, in turn, be analyzed for calcium activity and gene expression at the resolution of single cells. While the topic presented in this paper is the analysis of spontaneous calcium transients, the technique is broadly applicable to a wide array of research questions and approaches (**Figure 1**).

Video Link

The video component of this article can be found at <https://www.jove.com/video/4377/>

Protocol

All experiments are performed following protocols approved by the Institutional Animal Care and Use Committee at the College of William and Mary. Developmental stages referenced in this protocol are according to Nieuwkoop and Faber⁴⁶.

1. Dissection

1. Allow the Cell Culture Medium and Calcium Magnesium Free Medium (CMF) to equilibrate to room temperature. You will also need 0.1X Marc's Modified Ringer's (MMR) pH 7.4-7.6.
2. Sterilize the following items by applying a UV light for 30 min in a cell culture laminar flow hood. (Spray each item with 70% ethanol before placing in the hood.)
 - 35 mm plastic Petri dishes.
 - 60 mm plastic Petri dishes.
 - 35 mm Nunclon surface dishes.
 - 1.5 ml microcentrifuge tubes.
 - p1000 micropipette (1,000 µl micropipette).
 - Aerosol resistant p1000 tips (1,000 µl micropipette aerosol resistant tips).
 - Alcohol resistant pen.

- Fine dissecting forceps.
 - *15 ml conical tubes (only for imaging calcium activity).
 - *Only for imaging of calcium activity.
3. Once the hood has been UV sterilized and solutions have equilibrated to room temperature, turn on the laminar air flow in the hood, spray down gloves and media bottles with 70% ethanol and place media bottles in the hood.
 4. Inside the hood prepare the following with a p1000 micropipette for each **plate of cells**, ensuring appropriate labeling.
 - One 60 mm plastic Petri dish containing 10 ml Cell Culture Medium - **Dissection Plate**.
 - One 35 mm Nunclon surface dish containing 2 ml Cell Culture Medium - **Culture Plate**. (Nunclon plastic dishes are not further treated with any adhesives.)
 - One empty 1.5 ml microcentrifuge tube- **Explant Dissociation Tube**.
 - *One 15 ml conical falcon tube containing 15 ml Cell Culture Medium. For washing out excess Fluo4-AM when imaging calcium activity.
 5. Inside the hood, prepare the following with a p1000 micropipette for each **dissection session** (more than one retina can be dissected in one session).
 - One 35 mm plastic Petri dish containing 2 ml CMF - **Explant Rinse Plate**.
 - One 35 mm plastic Petri dish containing 2 ml CMF - **Explant Dissociation Plate**.
 6. Outside the hood prepare the following:
 - One 60 mm plastic Petri dish per plate of cells containing 10 ml 0.1X MMR - **Holding Plate**.
 - One 60 mm plastic Petri dish per plate of cells containing 10 ml 0.1X MMR - **Sibling Plate**.
 - One 60 mm plastic Petri dish per dissection session containing 10 ml 0.1X MMR + 0.5 mg/ml MS-222 (tricaine) - **Anesthetization Plate**.
 7. Add 40 μ l 5% trypsin in CMF to the **Explant Dissociation Plate** (resulting in a 0.1% trypsin solution), swirl to mix, and set aside.
 8. If dissecting an embryo that is younger than stage 30, add 10 mg Collagenase B to the **Dissection Plate** (resulting in a 1 mg/ml Collagenase B solution), swirl to dissolve and set aside.
 9. Select an embryo of the desired stage, transfer to the **Holding Plate** with a non-sterile transfer pipette, and remove the vitelline membrane (if the embryo has not yet hatched) using fine forceps.
 10. Transfer several identically staged embryos to the **Sibling Plate** with a non-sterile transfer pipette.
 11. If the embryo is stage 25 or earlier, proceed directly with dissection, otherwise transfer the embryo to the **Anesthetization Plate** with a non-sterile transfer pipette and allow the embryo to sit until movement and response to stimuli ceases. This could take up to a minute.
 12. Proceed with dissection (**Figure 2**) under a dissecting scope (4X objective, 10X eyepiece).

For embryos **stage 25 or younger**:

13. Transfer embryo to the **Dissection Plate** with a non-sterile transfer pipette.
14. Using two pairs of fine forceps, make a midsagittal cut on the ventral side of the embryo, beginning on the posterior end and continuing through the cement gland in the anterior portion of the embryo.
15. Carefully open the embryo by grasping either edge of the cut and spreading left and right. This results in an embryo with the dorsal side facing into the **Dissection Plate**, and with the ventral ectoderm spread open to reveal the endoderm and mesoderm within.
16. Begin removing the endoderm and mesoderm (**Figure 2A and 2B**). The first and largest layer of this tissue is very "fluffy" in appearance with large cells and little obvious organization.
17. After removing this layer, the somites and notochord will become visible. For better contrast, move the embryo to a separate 60 mm plastic Petri dish containing 10 ml Cell Culture Medium and 100 μ l of 1% solution of Nile Blue Sulfate (in water) for 2-3 min before transferring back to the **Dissection Plate**. This will stain the ectoderm, somites, and notochord for easier identification and orientation.
18. Focusing on the anterior portion of the embryo, carefully remove the notochord and any remaining mesoderm exposing the brain and optic vesicles (**Figure 2C**).
19. Once the brain and optic vesicles are completely exposed, use the forceps to sever the neural tube and underlying ectoderm just posterior to the brain.
20. Turn this portion (the brain and optic vesicles) over so that the ectoderm is on top.
21. Taking care not to damage the underlying optic vesicles, carefully remove the ectoderm (**Figure 2D**).
22. Finally, using forceps, separate the optic vesicles from the brain (**Figure 2D and 2E**).

For embryos **older than stage 25**:

23. While the embryo is in the **Anesthetization Plate**, remove the ventral portion of the embryo with forceps.
24. For better contrast, move the embryo to a 60 mm plastic Petri dish containing 10 ml 0.1X MMR and 100 μ l of 1% Nile Blue Sulfate for 2-3 min before transferring to the **Dissection Plate**. This will stain the ectoderm blue.
25. Once in the **Dissection Plate**, pull back the ectoderm overlying the retina (or optic vesicle for the embryos stage 26-30) (**Figure 2F and 2G**). If Nile Blue Sulfate was used, the ectoderm will be stained blue but the underlying retinal tissue will not.
26. Remove the retina or optic vesicle carefully with forceps (**Figure 2H, 2I, and 2J**). It is helpful to hold the embryo in place with one pair of forceps and remove the retina or optic vesicle with the other.

2. Dissociation of Tissue and Plating of Cells

Important Note - When transferring tissues, do not allow the retina or optic vesicle to touch any air-liquid boundaries; if this occurs the cells will lyse.

1. Once the optic vesicle or retina has been dissected, carefully transfer to the **Explant Rinse Plate** with a p100 micropipette using aerosol resistant tips and allow to sit for 30 sec.
2. Transfer 80 μ l of 0.1% trypsin in CMF from the **Explant Dissociation Plate** to the **Explant Dissociation Tube**.
3. Using a p100 micropipette and aerosol resistant tips, transfer the retina or optic vesicle from the **Explant Rinse Plate** to the **Explant Dissociation Tube**, avoiding transfer of the explants rinse solution.
4. Allow the tissue to dissociate in the **Explant Dissociation Tube** for 1 hr at room temperature. One retina was used per plate, however we note that it is possible to use more than one per plate if so desired to increase the number of cells per plate.
5. To plate the cells, first slowly remove 40 μ l of the solution from the **Explant Dissociation Tube** and discard using a p100 micropipette and aerosol resistant tips. Using a p100 set to 40 μ l and aerosol resistant tips, transfer the cells (now that the tissue has dissociated) to the **Culture Plate**. When aspirating the cells onto the plate, pipette slowly and keep the cells in a small area in the center of the plate.

Note: If multiple images of the cells are to be taken throughout the experiment, attach a grid (Cellattice) to the underside of the **Culture Plate** with a small drop of superglue. This will allow images of identical cells in identical orientations to be taken at different points in the procedure.

6. Allow the cells to sit undisturbed for at least 1 hr to adhere to the Nunclon plate. After this time, they must be treated very gently or they could become detached.
7. Culture cells to desired stage by observing the sibling embryos, which are reared at the same temperature as the cells. If live cells are not being used for further manipulation, they can be fixed in MEMFA solution at this time.

Important Note: Most of our experiments are fixed within 6 hr of plating the cells. The longevity of the cells in culture is dependent upon the stage at which the tissue was dissected and the amount of yolk still present in the cells; cells dissected from younger (neurula stages) remain healthy in culture for 5-6 days while cells acquired from older embryos (swimming tadpole stages) will remain viable in culture for 2-3 days.

3. Calcium Imaging⁴⁷⁻⁴⁹

This protocol utilizes calcium-sensitive Fluo4-AM and confocal microscopy to quantify calcium transients in individual cells. All steps using Fluo4-AM should be performed away from light, as Fluo4-AM is light sensitive. All washes should be added or removed using a p1000 micropipette and aerosol-resistant tips. Pipetting should be done slowly and towards the edge of the plate to avoid disturbing the cells. Media is not changed because the cultures are generally fixed within 6 hr of being dissected. For longer-term cultures, the media should be changed daily.

1. Fluo4-AM is stored at -20 °C, and we suggest storing in 5 μ l aliquots. Before using, thaw a 5 μ l aliquot of Fluo4-AM away from light and add 2 μ l of Pluronic F-127 directly to this aliquot.
2. After culturing the cells for the desired amount of time (at least 1 hr), remove 1 ml of the cell culture medium from the **Culture Plate**. Add this to the Fluo4-AM and Pluronic, mixing by gentle pipetting.
3. Slowly transfer all of this solution back to the edge of the **Culture Plate** of the cells to be imaged and leave the cells in this solution undisturbed for 1 hr to absorb the Fluo4-AM.
4. To wash out excess Fluo4-AM from the cell culture medium, complete the following wash series:
 - Remove 1 ml of media from the plate.
 - Add 3 ml of fresh cell culture media (from the 15 ml conical tube) to the plate.
 - Perform two additional washes, each time slowly removing 3 ml of solution from the plate and adding 3 ml of fresh cell culture media.
5. The cells should now be in 4 ml of cell culture medium and contain Fluo4-AM. Fluo4-AM is enzymatically cleaved once inside the cell, preventing it from diffusing out of the cell or into any membrane bound compartments.
6. Before loading the plate onto the stage of the confocal microscope for imaging, draw a vertical red line with permanent marker on the dish while keeping cover on to prevent marker particles from contaminating the culture. The vertical line is drawn down the side of the Petri dish (the base of the Petri dish, not the lid). Its purpose is to indicate the orientation of the culture plate with respect to the field of view so that the precise orientation and alignment of the individual cells could be re-established at a later point.
7. The plate is now ready for loading onto the stage of the confocal microscope for imaging. Visualize the cells using the bright field settings on a laser scanning confocal microscope (objective 20X). Find an area of dense cells that does not contain clumps, preferably with a field of view that contains the large grid numbers on the Cellattice coverslip to make finding the same field of view easier later on when imaging the culture after *in situ* hybridization. Prior to imaging, focus down through the culture and take a bright field image of the grid below the cells to record the location and orientation of the cell culture. This allows for realignment of the cells for subsequent analysis.
8. Raise the focal plane and capture a bright field image of the cells prior to beginning calcium imaging (**Figure 3A**).
9. Once the center plane of the cells is in focus, the plate is ready for imaging of calcium activity. Settings will vary depending on the microscope and the application. We image the cells using an Argon 488 nm laser for 1, 2, or 12 hr with the following parameters:
 - **1 hr images:**
 - Argon 488 nm laser scans at 4% of its maximum 30 mW power.
 - Scan once every 4 sec (900 scans per hr).
 - **2 hr images:**
 - Argon 488 nm laser scans at 4% of its maximum 30 mW power.
 - Scan once every 8 sec (450 scans per hr).
 - **12 hr images:**
 - Argon 488 nm laser scans at 2% of its maximum 30 mW power.
 - Scan once every 48 sec (75 scans per hr).

These parameters will result in a set of 900 still frame images for each time frame. Calcium activity can then be recorded by analyzing fluorescence levels over the time course images (**Figure 3B**) with the use of an image processing application such as ImageJ⁵⁰.

4. Fixing Cells

1. Following imaging, cells can be fixed for 30 min by removing 3 ml of solution from the plate and adding 1 ml 2X MEMFA to the remaining 1 ml of culture media.
2. After fixation, remove MEMFA and dehydrate cells with a series of 5 min washes, each a volume of 1 ml:
 - 25% ethanol in 1X phosphate-buffered saline (PBS).
 - 50% ethanol in 1X PBS.
 - 75% ethanol in sdd H₂O (sterile deionized distilled water).
 - Replace the last wash with 1 ml 100% ethanol and store plate at -20 °C.

Note: Methanol can also be used for these washes and for storage.

5. Fluorescent *in situ* Hybridization (FISH)

Cultures can be assayed using the standard FISH protocol for *Xenopus* as described⁵¹ with modifications for cell culture as outlined by the Anderson Lab⁵². Modifications to the Anderson Lab protocol are listed below. All washes are conducted on the 35 mm Nunclon plates in volumes of approximately 1 ml, unless otherwise noted. Solutions are as defined in Sive *et al.*⁵³ unless otherwise noted.

Important Note: Do not use fluorescein-labeled RNA probes when performing *in situ* hybridization on cells imaged for calcium activity. Anti-fluorescein antibodies will bind to residual Fluo4-AM in the cells and may cause positive signal in all cells in the culture.

Day 1: Permeabilization and Hybridization

1. Rehydration washes should be graded to the dehydration solvent used for storage. For our experiments, washes were graded to ethanol in 1X PBS.
2. Following rehydration, wash the cells three additional times in 1X PBS for 5 min each at room temperature.
3. Mix 25 ml of 0.1 M triethanolamine (pH 8.0) with 62.5 µl of acetic anhydride in a Falcon tube and quickly mix until thoroughly dispersed as discussed in the Anderson Lab⁵² protocol. Incubate cultures in this mixture for 10 min at room temperature. Following acetic anhydride treatment, wash cells in 1X saline sodium citrate (SSC) for 5 min at room temperature.
4. Remove the last SSC wash and replace with 0.2 M HCl (in sdd H₂O) for 10 min to permeabilize cells.
5. Wash out HCl with two washes in 1X PBS for 5 min.
6. Prehybridize in ISH buffer at 60 °C in a shaking water bath for a minimum of 6 hr, but do not prehybridize overnight as this severely diminishes signal.
7. Hybridize overnight at 60 °C for 8-14 hr in 750 µl of diluted probe (1:250 dilution from a purified probe). The purified probe concentration ranges from 1.0-1.5 µg/µl.

Day 2: Removal of Unbound Probe and Antibody Incubation

8. Remove the probe and rinse cultures for 5 min in 0.2X SSC at 60 °C. Wash in fresh 0.2X SSC for 1 hr at 60 °C.
9. Remove cultures from the water bath and allow them to adjust to room temperature for 5 min.
10. Rinse in 0.2X SSC at room temperature for 5 min.
11. Wash for 15 min in 1X PBS with 0.1% Triton-X-100 (PBT).
12. To inactivate endogenous peroxidases, wash for 1 hr in a 2% H₂O₂ solution in PBT.
13. Rinse with a 15 min wash in 1X Tris Buffered Saline with 0.1% Tween 20 (TBST).
14. Block in 2% Blocking Reagent in Maleic Acid Buffer (MAB) for 1 hr at room temperature. We have found that this blocking method increases sensitivity in comparison to previous methods of blocking in 20% sheep serum in PBT.
15. Incubate cultures in 1 ml of 2% Blocking Reagent in MAB containing a 1:1,000 dilution of a POD-conjugated antibody overnight at 4 °C.

Day 3: Removal of Unbound Antibody and Fluorescence Development

16. Remove antibody solution and rinse 3 times in TBST for 5 min at room temperature.
17. Wash 4 times in TBST for 15 min at room temperature while continuously rocking at a slow speed.
18. Wash twice in PBT at room temperature for 10 min.
19. Apply 750 µl of 1:200 fluorescein-tyramide or 1:25 Cy3 tyramide diluted in PBT. Incubate for 5 min at room temperature and add 2.5 µl 0.3% H₂O₂. Rock for 40 min to allow signal to develop.
20. Wash at least 4 times for 15 min each in TBST at room temperature with rocking.
21. Once signal has fully developed, wash for 5 min in 1X PBT.
22. Fix in 1X MEMFA for 1 hr at room temperature and store in 1X PBS at 4 °C.

6. Image FISH Results and Co-register with Calcium Imaging Data

1. Upon completion of FISH, cell culture plates are imaged to determine which cells display a positive fluorescence signal for a given probe. Use the Cellattice coverslip to find the exact field of view studied during calcium imaging and align the plate to the orientation of the calcium image frames.

2. Focus to the center plane of the cells and take a single high-resolution image using the Helium-Neon HeNe 543 nm laser at 15% of its maximum 1 mW power (**Figure 3C**).
3. In the original 900 frame set of images taken from the Calcium Imaging protocol, identify individual cells as regions of interest (ROIs) through the use of an image processing program (such as ImageJ⁵⁰). Extract ROI fluorescence data as a set of data points representing time-fluorescence pair values (**Figure 4**).
4. Overlay the FISH results image with the identified ROIs from the calcium images (**Figure 3D**).
5. Register each ROI as positive for the probe, negative for the probe, or unknown - in the case that the cell corresponding to the ROI can no longer be found within the field of view of the FISH image.
6. Process ROI fluorescence data using statistical analysis scripts (as designed through MATLAB or other programming language) to compare calcium activity levels among different dissected stages (**Figure 5A and 5C**) or cells positive for different FISH probes (**Figure 5B and 5D**). All scripts used in our lab are freely available upon request.

Representative Results

Examples of successfully dissected optic vesicles (stage 25) and retinae (stage 35) are shown in **Figures 2E and 2J**. While this protocol can be used at various stages of development, it is critical to obtain only retinal tissue to ensure accuracy for further experiments. Carefully remove the epidermis at all stages and ensure that your forceps do not puncture the retinal tissue. In stage 35 or older, the lens can be seen as a clear layer on top of the retina and can be removed by cautious scraping using forceps.

A successfully plated primary cell culture is shown in **Figure 3A**. When dissociating tissue, it is important not to leave the retinal explant in the rinse solution for longer than the recommended 30 sec incubation time to prevent the tissue from dissociating in the rinse solution. Additionally, ensure that the tissue is permitted to dissociate in the trypsin solution for a full hour (older embryonic and larval stages may require even longer) to allow an even distribution of cells in the culture plate. Once the cells are plated, exercise caution when moving the plate and changing solutions to avoid washing the cells from the plate.

Figures 3B and 3C demonstrate images from calcium activity scanning and fluorescent *in situ* hybridization experiments (FISH), respectively. Cells that appear bright green in the calcium image are cells that are undergoing a transient spike in intracellular calcium levels as a result of the release of internal calcium stores. Cells can then be assayed for gene expression using FISH, and gene expression patterns can be correlated to patterns of calcium spiking activity by overlaying the images (**Figure 3D**). Using a combination of Python and MATLAB scripts (freely available upon request), data is obtained by analyzing the fluorescence activity of each individual region of interest (ROI) through the course of the imaging. An example of the calcium activity for an individual cell (ROI) over 1 hr of imaging is shown in **Figure 4**; a spike is visible at approximately 57 min of imaging. In **Table 1**, representative data for the percentage of cells positive for various probes are shown; while there are no data specific to this experiment reported in the literature (the point of our current experiments is to determine these percentages), our findings are consistent with data from similar types of experiments relating to calcium activity in the spinal cord^{17,55,56}.

Parameters utilized for analysis include the number, frequency, and amplitude of spikes. **Figure 5** provides representative results of calcium imaging and FISH experiments in embryonic retinal cells using probes for xVglut1, Ptf1a, and xGAD67. Using MATLAB scripts, the number of spikes for each ROI is determined and the number of spikes is averaged for all ROIs in the experiment. The average number of spikes from each experiment can then be compiled with other experiments, resulting in the average for a group of experiments. Cumulative distribution plots can be created to display the distribution of the average number of spikes for all experiments within a group. MATLAB scripts are then used for statistical analysis of the data.

Figure 5 shows representative data following MATLAB analysis. Briefly, our results show that calcium activity is developmentally regulated, with spiking peaking at stage 35 ($p < 0.002$) (**Figures 5A and 5C**). In terms of correlating calcium activity with cells positive for a specific probe, while there were no statistically significant differences, however there was a trend ($p=0.08$) for cells positive for xGAD67 (a marker for differentiated GABAergic cells) to display higher levels of calcium spiking activity than cells positive for a glutamatergic marker or for Ptf1a, a gene encoding a transcription factor gene correlated with promoting the GABAergic phenotype. Although preliminary, these data suggest that there may be GABAergic cells, which develop in a manner independent of Ptf1a or that activity-dependent neurotransmitter specification is not acting at the level of Ptf1a.

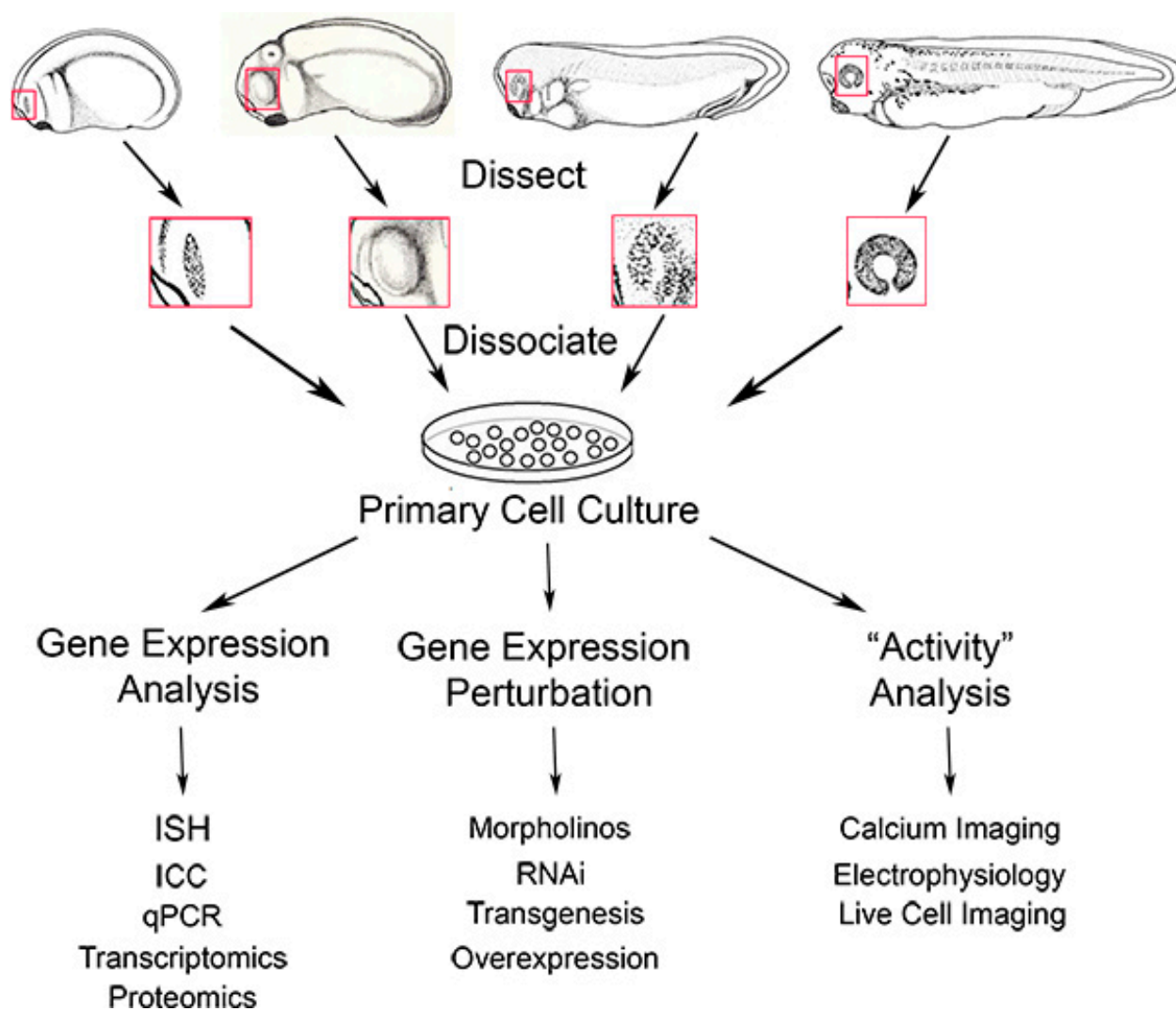


Figure 1. Procedural schematic. Once retinal tissue is dissected and dissociated, primary cell culture can be used for a wide variety of applications.

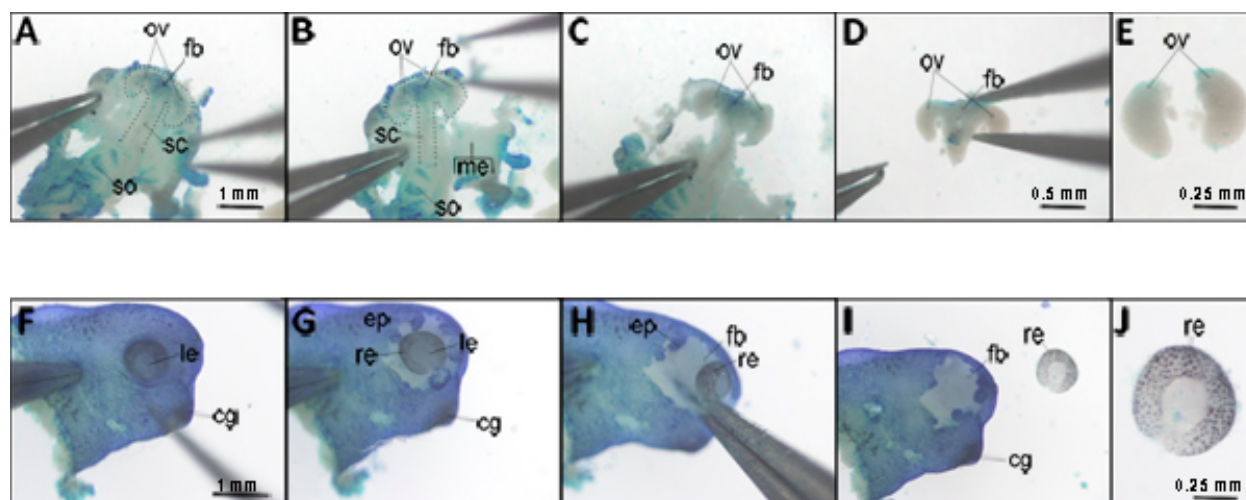


Figure 2. Dissection photographs. Embryos were stained in Nile Blue Sulfate for increased contrast. A-E: Stage 24. F-J: Stage 35. Abbreviations: ov, optic vesicle; fb, forebrain; sc, spinal cord; me, mesoderm; so, somites; le, lens; cg, cement gland; re, retina; ep, epithelium. [Click here for larger figure.](#)

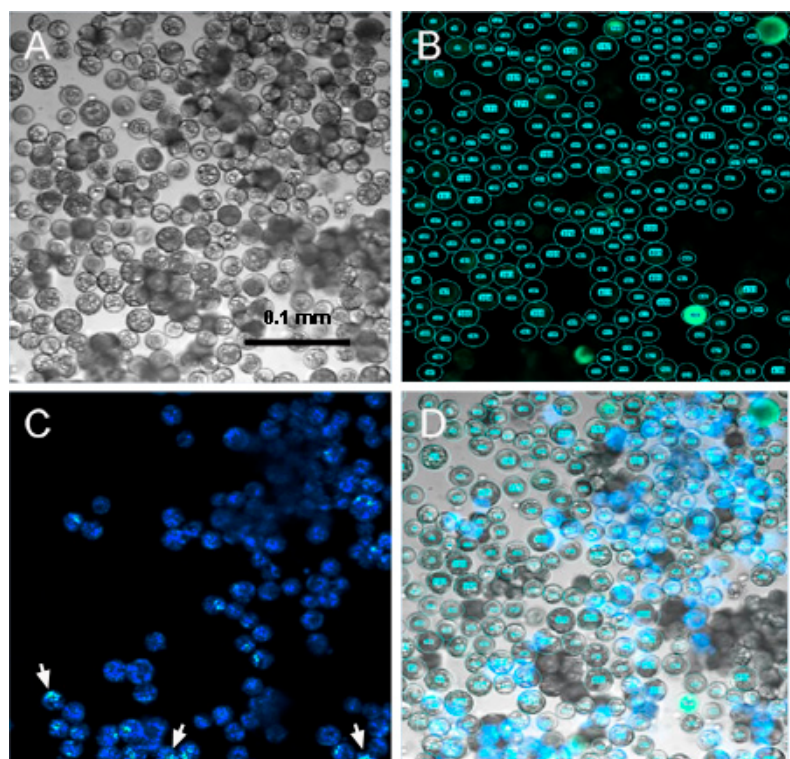


Figure 3. Images of cell culture with ROIs circled. A. Bright field. B. Image following Fluo-4 (AM) treatment with ROIs circled. C. FISH image (arrows indicate examples of cells positive for the alpha subunit of the voltage gated calcium channel CaV2.1). D. Overlay of bright field, Fluo-4, and FISH images.

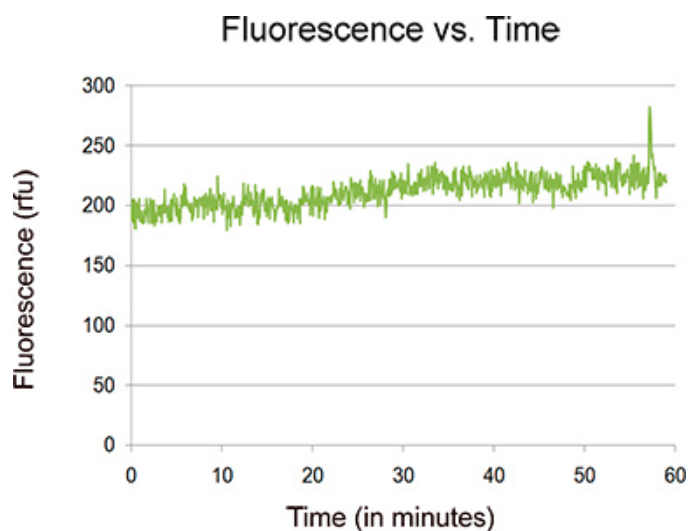


Figure 4. Example of calcium activity for a single cell (ROI). Plot of the fluorescence (in relative fluorescence units, rfu) vs. time (in minutes) from a Fluo4 calcium fluorescence image.

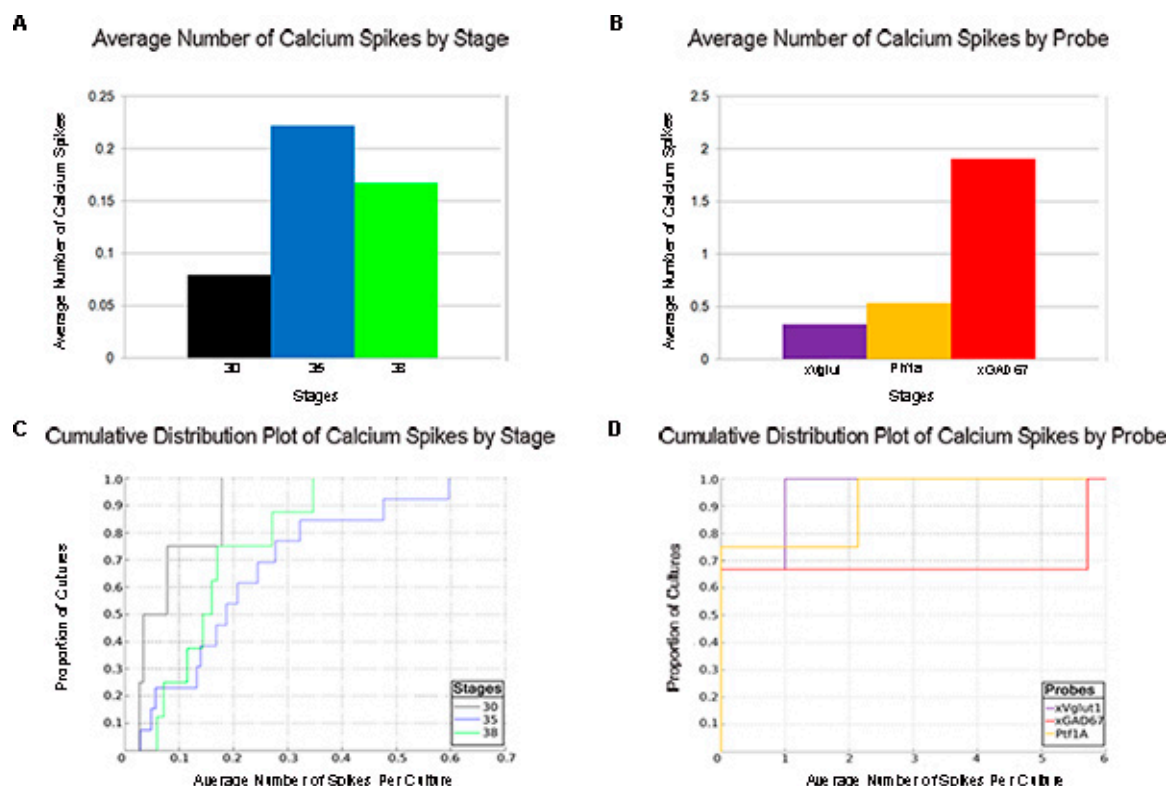


Figure 5. Results displaying calcium spiking by stage and probe. A. Average number of calcium spikes at stages 30 (n=4), 35 (n=8), and 38 (n=13). B. Average number of calcium spikes per culture among different probes used for *in situ* hybridization at stage 35 xVglut1 (n=3), xGAD67 (n=3), and Ptf1a (n=4). C. Cumulative distribution plot of the average number of spikes per culture at stages 30, 35, 38. D. Cumulative distribution plot of the average number of spikes per culture for cells identified as having positive signal for the *in situ* hybridization probes at stage 35; xVglut1, xGAD67, and Ptf1a. [Click here for larger figure.](#)

Probe	Stage	n (plates)	n (cells)	Percent Positive
xGAD67	35	4	1042	2%
	38	4	690	2%
Ptf1a	30	3	352	1%
	35	6	1856	1%
xVglut1	30	1	47	57%
	35	3	692	2%
	38	3	364	4%

Table 1. Percent positive cells.

Discussion

With its well-characterized cell types that are conserved across all vertebrates, the retina provides a useful model for studying the molecular-cellular processes governing cell type specification and differentiation. Primary cell culture affords a powerful method for investigating a wide range of processes including gene expression, protein dynamics, and calcium and electrical activity at the level of single cell resolution. Here we present a straightforward technique for primary cell culture from dissected presumptive retinal tissue in *Xenopus laevis*, a particularly amenable organism for such studies given that the presumptive retina is easily accessible from the very earliest stages of development and that primary cell culture can occur in a defined media consisting of a simple saline solution.

Although easily adaptable to most laboratory settings, there are several steps that require particularly close attention. Dissections should be performed with freshly sharpened forceps. Younger stages (< st. 30) benefit from treatment with Collagenase B mixed with the Cell Culture Medium. All steps involving the transfer of tissue or cells from one media to another must be performed with special care to prevent the tissue or cells from coming into proximity of the air-solution interface. The pipette containing the tissue should be fully submersed in plenty of fluid and the cells or tissue expelled very slowly. After the cells have been plated, all fluid transfers including, Fluo4-AM addition, and Cell Culture Medium, or fixation washes, must be carefully performed given that cells can be easily dislodged. As with all cell culture, sterility is concern, so care should be taken to sterilize all materials. Finally, letting the dissociated retinal explants sit for the designated timeframe in the dissociation medium is critical for successful cell attachment and to avoid clumping.

During the dissection and culture condition described in this protocol, both necrosis and apoptosis are extremely limited (less than 5-10% of the cells). Loss of cells on the plate (apoptosis) is rarely noticed during pre and post confocal imaging sessions. Handling cell cultures carefully is key to cell viability. Solution changes must be performed very slowly and steadily to preclude cell necrosis and apoptosis. While experiments to delineate the precise ratio of retinal cell types are currently ongoing, we do note that a variety of retinal cell types appear to be represented in the cultures and that Muller glial cells (which have a distinct morphology) are not dominant in the cultures.

A potential concern when analyzing plates after *in situ* hybridization experiments is that some of the cells may have moved, but this may be addressed with the use of cell tracking programs. Also, inherent in the technique of primary cell culture, a major limitation is the obvious loss of spatial patterning that is present in the intact tissue. The identity of individual cells must be established using molecular assays rather than by position in the tissue. However, this feature also provides the opportunity to analyze the state of specification of cells very precisely and in the absence of continuing cell-cell interactions. It also allows the investigator to selectively treat the cells with growth factors or other compounds and precisely analyze the effects without the influence of signals from neighboring cells. While we have employed this retinal dissection and primary cell culture technique for correlating calcium imaging with specific neurotransmitter phenotype markers, this technique is adaptable to a wide range of other downstream applications including electrophysiological recordings and single cell gene expression assays using RT-PCR or "next-gen" transcriptome analysis (**Figure 1**).

Disclosures

No conflicts of interest declared.

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