Video Article

Culture of Adult Transgenic Zebrafish Retinal Explants for Live-cell Imaging by Multiphoton Microscopy

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Abstract

An endogenous regeneration program is initiated by Müller glia in the adult zebrafish *(Danio rerio*) retina following neuronal damage and death. The Müller glia re-enter the cell cycle and produce neuronal progenitor cells that undergo subsequent rounds of cell divisions and differentiate into the lost neuronal cell types. Both Müller glia and neuronal progenitor cell nuclei replicate their DNA and undergo mitosis in distinct locations of the retina, *i.e.* they migrate between the basal Inner Nuclear Layer (INL) and the Outer Nuclear Layer (ONL), respectively, in a process described as Interkinetic Nuclear Migration (INM). INM has predominantly been studied in the developing retina. To examine the dynamics of INM in the adult regenerating zebrafish retina in detail, live-cell imaging of fluorescently-labeled Müller glia/neuronal progenitor cells is required. Here, we provide the conditions to isolate and culture dorsal retinas from *Tg[gfap:nGFP]*^{mi2004} zebrafish that were exposed to constant intense light for 35 h. We also show that these retinal cultures are viable to perform live-cell imaging experiments, continuously acquiring z-stack images throughout the thickness of the retinal explant for up to 8 h using multiphoton microscopy to monitor the migratory behavior of *gfap:nGFP*-positive cells. In addition, we describe the details to perform post-imaging analysis to determine the velocity of apical and basal INM. To summarize, we established conditions to study the dynamics of INM in an adult model of neuronal regeneration. This will advance our understanding of this crucial cellular process and allow us to determine the mechanisms that control INM.

Video Link

The video component of this article can be found at https://www.jove.com/video/55335/

Introduction

Unlike humans, zebrafish (*Danio rerio*) exhibit a robust regeneration response upon cell death of retinal neurons[1,2,3,4]. Tumor necrosis factor α, a signaling molecule that is released from dying retinal neurons induces Müller glia residing in the basal Inner Nuclear Layer (INL) of the retina, to proliferate[5] and produce neuronal progenitor cells that continue to proliferate before differentiating into the neuronal cell types that died[2,3,4]. During the proliferative phase of the regeneration response, the nuclei of Müller glia and their derived neuronal progenitor cells undergo a repetitive migratory pattern in phase with the cell cycle (Interkinetic Nuclear Migration, INM)[6,7]. Nuclei positioned in the basal INL replicate their DNA before migrating to the Outer Nuclear Layer (ONL) where they divide before the arising nuclei return basally to the INL. This process was first described during neuroepithelial development using histological methods, while live-cell imaging approaches later confirmed the interpretation by Sauer[8,9,10,11,12]. Both histochemical and live-cell imaging approaches have been used to determine mechanisms underlying INM and its function in developing neuroepithelia including the retina[9,11,12,13]. However, the mechanisms governing INM in the adult regenerating retina have not been studied in much detail[6,7]. Live-cell imaging will be an invaluable approach to advance our knowledge of the signaling pathways that control INM in the adult regenerating retina.

Until recently, live-cell imaging of INM in the retina was limited to either live zebrafish embryos or to embryonic chick or postnatal mouse retinal explants[9,10,11,12,14,15,16]. While retinal explants from adult animals of a variety of species including mouse, rat and zebrafish have been utilized for different cell biological approaches[17,18,19,20], live-cell imaging experiments using retinal explants have been restricted to brief periods of time and have not been executed continuously over several hours[21,22]. Here, we describe a detailed protocol to culture light-damaged adult zebrafish retinas to perform live-cell imaging experiments monitoring INM using multi-photon microscopy[6]. Live-cell imaging approaches are advantageous over immunohistochemical methods when investigating the mechanisms controlling INM, as the dynamics of INM, *e.g.,* velocities might be affected rather than the location of mitosis, which would potentially not be detected using immunocytochemistry.

In the future, this method has also the potential to be modified to study other dynamic processes during retinal regeneration, such as phagocytosis of dying photoreceptors by Müller glia or the behavior of microglia.

Protocol

Note: Zebrafish were raised and maintained in the Notre Dame Zebrafish facility in the Freimann Life Sciences Center. The methods described in this manuscript are approved by the University of Notre Dame Animal Care and Use Committee and are in compliance with the statement for the use of animals in vision research by the Association for Research in Vision and Ophthalmology.

1. Solutions

1. Prepare 70% ethanol to sterilize the tissue culture hood and any equipment/reagents that are transferred into the tissue culture hood.
2. Add 2 mL of 2-phenoxyethanol to 1 L of system water (1:500 2-phenoxyethanol).
3. Prepare 0.1 mM $NaHCO_3$, pH 8.0. Use a 10 mL syringe and a 0.2 µm pore-size syringe filter to sterilize the solution in a sterilized tissue culture hood.
   NOTE: $NaHCO_3$ is used to efficiently spread the cell and tissue adhesive across the coverslip surface of fluorodishes (see step 3.2).
4. Prepare 1.0 M $CaCl_2$ and 1.0 M $MgCl_2$. Sterilize with a 10 mL syringe and 0.2 µm pore-size syringe filter in a sterilized tissue culture hood.
5. To prepare Hank's-balanced Salt Solution (HBSS), add sterile $CaCl_2$ and sterile $MgCl_2$ to 1x HBSS without Ca^{2+}/Mg^{2+}, without phenol red at a final concentration of 1 mM each. Work in a sterile environment.
6. To prepare culture medium, mix 50% 1x Minimum Essential Medium (MEM) without phenol red, 25% HBSS containing $CaCl_2$ and $MgCl_2$ (see section 1.4), 25% Horse Serum (HS), 10 units/mL penicillin, and 10 µg/mL streptomycin. Work in a sterile environment.
   NOTE: Avoid medium containing phenol red as it autofluoresces, thereby affecting signal to noise ratios.[23]
7. Prepare 10 mL of 1% low melting point agarose in 1x MEM without phenol red. Melt agarose/1x MEM using a microwave. Prepare small volumes of agarose (*e.g.,* 10 mL) as repetitive reheating will change ion concentrations due to fluid evaporation.
8. Prior to culturing, sterilize 1.5 mL microcentrifuge tubes by autoclaving.

2. Light-damage Paradigm

1. Dark-adaptation
   1. Place 2 - 3 *Tg[gfap:nGFP]*^{mi2004} zebrafish (or other transgenic zebrafish of interest) at 6 - 14 months of age into an environment devoid of light for 14 d. For further detail see reference[2, 6, 24, 25].

2. Position the tank containing 2 - 3 dark-adapted transgenic zebrafish in system water between two fluorescent lamps that emit light of 2,800 lux[2, 6, 24, 25].
3. Expose zebrafish to constant intense light for 35 h. During the light exposure, assure that the water temperature is maintained between 31 - 33 °C.

3. Preparation for Culturing (On the Day of Retinal Isolation)

1. Using 70% ethanol, sterilize the tissue culture hood and the components/tools that are transferred into the tissue culture hood (*e.g.,* flasks containing MEM and HBSS, pipettes, sterile pipette tips, *etc.*).
2. Preparation of fluorodishes
   1. Coat one fluorodish for each retina with cell and tissue adhesive (see **Table of Materials**).
   2. Dilute the cell and tissue adhesive in 0.1 mM $NaHCO_3$ to a final concentration of 70 µg/mL, add 50 µL to each fluorodish and spread the solution across the center of the fluorodish with a 200 µL pipette tip (approximately 1 - 1.2 cm diameter).
   3. Incubate the coated fluorodishes for 1 - 3 h at RT (alternatively, incubate O/N at 4 °C).
   4. Remove the solution and rinse 3x with 500 µL of 1x MEM for each wash. To avoid the coated fluorodishes from drying out, maintain them in MEM until retinal explants are mounted.

3. Prepare culture medium as described in step 1.6. The culture medium can be stored for up to 1 week at 4 °C.

4. Isolation and Culturing of Retinal Explants

NOTE: The protocol outlined below is for the isolation of the dorsal retina, which is the retinal region that is predominantly lesioned by the described light-damage paradigm. Therefore, damage-induced proliferation and the associated event of interkinetic nuclear migration occur in the dorsal retina. However, the isolation procedure can be adjusted to yield retinal regions according to the specific requirements of the researcher/research question.

1. Euthanize one light-damaged transgenic zebrafish at a time in 1:500 2-phenoxyethanol.
2. Remove MEM from one fluorodish with a 1,000 µL pipette so that only a thin film of fluid remains.
3. Transfer the zebrafish onto a dry paper towel, remove the eye with a curved pair of Dumont forceps (forceps #5, 45° angle) and transfer it onto the fluorodish.
4. Using a stereomicroscope, orient the eye with the pupil onto the cover slip of the fluorodish so that the back of the eye with the optic nerve is visible (**Figure 1D**).
5. With a pair of McPherson-Vannas scissors remove the optic nerve, cutting close to the back of the eye. In addition, remove connective tissue lining the outside of the eye.
6. Hold the eye between its nasal and temporal side with a pair of #5 forceps while making an incision at the optic stalk by piercing with one scissor blade through the lamina cribrosa and cutting along both the nasal and temporal sides of the eye (see **Figure 1E**, segmented line).

7. Using two pairs of #5 forceps, one for holding the dorsal side of the retina and the other pair to separate the ventral from the dorsal retina, pull the tissue.
   NOTE: It is advisable to orient the dorsal retina so that the lens and the ganglion cell layer face the coverslip while the sclera is facing upwards.
8. Remove the sclera from the dorsal retina with one pair of #5 forceps, while holding the lens that is connected to the retina with a second pair of #5 forceps (**Figure 1H, I**).
9. To remove the lens, use McPherson-Vannas scissors and cut behind the lens without damaging the retina (**Figure 1I, J**). Sometimes, the lens separates in step 4.7. In this case, remove the sclera carefully with forceps by holding the retina at a cut edge with a second pair of #5 forceps.
10. Remove the vitreous while removing the lens without damaging the retina. Flatten the retina with the ganglion cell layer facing the cover slip of the fluorodish (**Figure 1L, M**).
11. Surround the retina with 10 µL of 1% low melting point agarose and let the agarose solidify.
12. Watch that the liquid agarose does not lift the retina as even a slight elevation might affect the ability to focus deeply into the tissue. If lifting is observed, use a pipette to remove agarose. Let residual agarose set before attempting to add more.
13. Repeat step 4.12 several times before adding 1% low melting point agarose to cover the entire fluorodish. Once the agarose has solidified, add 1.5 mL culture medium.
14. Maintain the retinal explant culture in a 5% CO_2/air environment set at 32 °C for approximately 12 h to allow the retina to recover from the stress incurred by the isolation procedure. Set the temperature to 32 °C to maintain retinas at the same temperature as light-treated zebrafish (see step 2.3).

5. Multiphoton Microscopy

NOTE: The experiments performed in this manuscript were optimized for a multiphoton microscope equipped with an infrared laser (see **Table of Materials**), a 40X Apo long distance water immersion objective (N.A. 1.15), a galvanometer scanner and an environmental chamber that contains an insert for four 35 mm Petri dishes. The images were acquired with a non-descanned detector (R-NDD).

1. Prior to imaging, equilibrate the environmental chamber to achieve a 5% CO_2/air atmosphere. Make sure that empty Petri dishes are inserted into the holder to avoid leakage of gas into the room.
2. Turn on the microscopy system.
3. Once the environmental chamber is equilibrated, add refractive index liquid onto the 40X Apo long distance water immersion objective (N.A. 1.15).
   NOTE: The refractive index liquid with optical properties similar to water is used to avoid evaporation of water during long-term imaging.
4. Place fluorodishes with retinal explants into the chamber. Using brightfield light, position the specimen into the light path and bring the midregion of the dorsal retina into the plane of focus.
5. Use GFP epifluorescent light to focus on *gfap:nGFP*-positive Müller glia nuclei (**Figure 2A, C**).
   NOTE: If explants are not mounted flat or agarose accumulated under the explant, it will be difficult to focus onto the *gfap:nGFP*-positive nuclei or they will fluoresce dimly.
   1. Check whether moving to a different region within the same retinal explant will overcome the focusing issue. Otherwise move to a different retinal explant.

6. In the image acquisition software, open the 'A1 MP GUI', the 'TiPad', the 'A1 Compact GUI' and the 'ND acquisition' windows. For multiphoton imaging, ensure that the 'IR NDD' option is chosen in the 'A1 Compact GUI'.
7. In the 'setting' field, select IR-DM for the 1st dichroic mirror and choose the band pass filter 525/50 to acquire GFP fluorescence.
8. Switch on the IR laser in the window labeled 'A1MP GUI'. It will take a few minutes for the laser to be ready. Set the wavelength to 910 nm to excite GFP fluorescence and align the laser by clicking the 'Auto alignment' button in the 'A1 MP GUI' window.
9. Ensure that room and equipment lights are switched off or covered before opening the shutter in the 'A1 MP GUI' to avoid overexposure of the photomultiplier tube. To reduce noise levels, house the microscope in a darkened environment.
10. Acquire images of a field of view of 300 x 300 pixels, at a zoom of two, and a pixel dwelling time of 4.8 µs/pixel. Roughly set up the laser power by changing the 'acquisition area' in the 'A1MP GUI' and the gain in the 'A1 Compact GUI' window.
11. Setting up the z-stack
    1. Focus on the ganglion cell layer to set the top focal plane of the z-stack in the 'z'-subwindow within the 'ND acquisition' window. Some *gfap:nGFP*-positive cells are typically located in the ganglion cell layer, which helps to identify the basal limit of the retina (**Figure 2A, D**).
    2. Move the focal plane through the level of the ONL (**Figure 2A, B**), which is characterized by the presence of dimly labeled *gfap:nGFP*-positive cells that are round and enlarged relative to their counterparts in the INL (**Figure 2A, C**).
    3. Set this plane as the bottom of the z-stack. Ensure that the entire ONL will be imaged (**Figure 2A, B**).
    4. When experiencing focal plane shifts that require re-adjusting during the imaging period, double-click on the middle position in the 'z' subwindow to assign it as the 'home' position. Change to 'symmetric mode defined' and click 'relative'.
    5. Set the z-step size between 0.7 to 1 µm.

12. Z-intensity correction:
    1. Apply z-intensity corrections to compensate for loss of pixel intensity due to light scattering when imaging in deep layers of the tissue.
    2. To set up the correction, open the 'z-intensity correction' window. To set the 'z-stack range', choose 'From ND'.
    3. Click on the bottom focal plane in the 'z-intensity correction' window (in this case, corresponds to the ganglion cell layer) and set the laser intensity ('acquisition area' in the 'A1MP GUI') and gain (A1 Compact GUI).
    4. Click the arrow next to the 'z-values' in the 'z-intensity correction' window to confirm the settings that are subsequently shown under 'device settings' in the 'z-intensity correction' window for the chosen focal plane.
    5. Repeat the process for the middle and top planes, increasing the laser power and gain. Additional focal planes can be added if necessary. See **Table 1** for specific laser and gain settings for experiments in **Figures 2 - 4**.

6. Set the 'acquisition area' in the 'A1 MP GUI' window. Avoid selecting an acquisition area larger than 15 and a gain higher than 126 at the start of imaging to circumvent photobleaching and increased noise levels.
   NOTE: As lasers and photomultiplier tubes differ between microscopy systems, test laser and gain settings to obtain optimal imaging conditions for the microscope set up while avoiding photobleaching.
7. Choose 'relative intensity correction' in the 'z-intensity correction' window.

13. In the 'timeseries' subwindow in the 'ND acquisition' window, set the duration to 8 h and the interval to 'no delay'. Then, make sure to click the 'Run z-correction' in the 'z-stack' sub window in the 'ND acquisition' window button to acquire the 3-D timeseries.
14. Maintain retinal explants at a temperature of 27 - 29 °C throughout the duration of image acquisition.
15. Throughout the image acquisition period, if necessary, readjust the power level and gain in order to maintain image quality for post-imaging analysis. For adjustments perform steps 5.11.2 - 5.11.4.
16. If the focal plane shifts, pause or stop the run and perform step 5.11 again.
    NOTE: If 'relative z-correction' was chosen in step 5.12.7 and step 5.11.4 was performed it should not be necessary to readjust power and gain levels, unless extensive photobleaching occurred.

6. Analysis of Velocity

1. Extract the time, setting a 'region of interest' on the image. Use the 'time measurement' tool and export the time values to a spreadsheet.
2. Crop a region that contains a dividing *gfap:nGFP*-positive nucleus.
3. Choose the cropped region so that it contains at least one nucleus that does not undergo INM in order to set a reference point to subsequently measure the distances that the dividing nucleus migrated in relation to the basal INL. NOTE: To choose a nucleus that remains in the basal INL, it helps to prepare a 3-D reconstruction of the timeseries.
4. Alternatively, if a *gfap:nGFP*-positive cell is observed in the ONL throughout the acquisition period, use a vertical line of fixed length spanning from the ONL nucleus to the basal INL in order to identify a reference point.
5. Using the 'show slices view' function, generate orthogonal projections. Subsequently, change the mode from 'slice' to 'maximum intensity projection'.
6. Turn off the 'xy' view of the orthogonal maximum projection. Depending on the orientation at which the migrating/dividing nucleus is best visible, also turn off either the 'xz'- or the 'yz'-view.
7. Click on the remaining image with the right mouse button and extract the 'xz' or 'yz'-image series with the 'Create new document from this view' function. If necessary, rotate the image.
8. Using the 'manual measurement' function, draw a horizontal line across the image at the bottom level of the nucleus that remains in the basal INL and does not undergo INM (= reference point; **Figure 4A - E**, red horizontal line).
9. Measure the distance between the reference line and the basal point of the migrating nucleus for the timeseries using the 'line measurement' tool in the analysis software (see **Figure 4A - E**).
10. Once the nuclear envelope breaks down, measure the basal position of the soma if it is identifiable following the diffusion of GFP into the entire cell.
11. Using a spreadsheet software, plot the distance a nucleus migrated against the time passed (**Figure 4F**).
    1. To determine the apical migration velocity (v_a), graph the distances traveled for the period before nuclear envelope breakdown occurs (**Figure 4G**).
    2. Select the data series within the chart, right-click the mouse and insert a linear regression curve including the corresponding function 'y=mx+c'. The slope 'm' in the function represents the velocity, v_a (**Figure 4G**).
    3. Repeat steps 6.11.1 and 6.11.2 for the first phase of rapid basal migration to determine the basal migration velocity, v_b (**Figure 4H**).

Representative Results

The isolation of the retina according to the procedure outlined in the schematic in **Figure 1** allows the culturing of a flattened dorsal retina from light-damaged adult *Tg[gfap:nGFP]*^{mi2004} zebrafish over a period of at least 24 h in a 5% CO_2/air environment. These flat-mounted retinal explants can be used to image focal planes at deep tissue levels. An example is Müller glia/neuronal progenitor cell nuclei labeled with GFP from the Müller glia-specific promoter *gfap* (glial fibrillary acidic protein) that are located in the ONL during mitosis in the light-damaged retina (**Figures 2A - D, 3**). To further confirm that this approach is applicable to image the various retinal cell layers, acutely isolated retinal explants were prepared from undamaged *Tg[rho:Eco.NfsB-EGFP]*^{nt19} zebrafish eyes that express EGFP in rod photoreceptors under the *rhodopsin* promotor. **Figure 2E - H** shows that it is possible to acquire images of GFP-positive rod photoreceptor and their inner segments. As rod inner segments extend between cone photoreceptors towards the retinal pigment epithelium, these data would imply that it is also possible to image cone photoreceptors. This was however not further investigated.

Having observed Müller glia/neuronal progenitor cell nuclei in the ONL in the light-damaged retina, we characterized their migratory behavior in detail (**Figure 3** and **Movie 1**). Müller glia/neuronal progenitor cell nuclei labeled by GFP migrate bi-directionally between the basal position of the INL where they typically reside and the ONL, the site of photoreceptor loss. Typically, *gfap:nGFP*-positive nuclei migrated a short distance in the INL (**Figure 3A - C**) before they underwent nuclear envelope breakdown, while still positioned in the INL, based on the redistribution of GFP into the cytoplasm of the entire Müller glia/neuronal progenitor cell (**Figure 3D**). Following cell division in the ONL, two *gfap:nGFP*-positive nuclei became visible in the ONL (**Figure 3F, G**) that returned to the basal INL (**Figure 3H - J**). Upon cell division, the newly arising nuclei were initially very dim and therefore difficult to identify (**Figure 3G**). However, within one to two time frames after mitosis, nuclear GFP fluorescence became bright, which enabled the visualization of nuclear migration (**Figure 3H - J**). Live-cell imaging also allowed the visualization of the division plane (**Figure 3F, G**), which occurred horizontally to the apical surface of the retina for the cell shown in **Figure 3**. It is also feasible to use other transgenic zebrafish lines such as the *Tg[gfap:EGFP]*^{nt11} zebrafish that express GFP in the cytoplasm (data not shown). Although it is possible to reliably determine apical movement and horizontal cell divisions, it is often difficult to exactly identify the position of the basally migrating daughter nuclei and vertical cell divisions in the *Tg[gfap:EGFP]*^{nt11} zebrafish retina (data not shown).

To determine the velocity, a *gfap:nGFP*-positive nucleus that did not migrate throughout the recording period was chosen as reference point (star, **Figure 4A - E**). The distance of the migrating nucleus (vertical red line, **Figure 4A - E**) was determined in relation to the reference *gfap:nGFP*-positive nucleus (horizontal red line, **Figure 4A - E**) at each timepoint of the image series on maximum xz or yz-projections (depending at which angle the nuclei could be visualized most efficiently). The distance that the nucleus migrated was plotted against the time in a spreadsheet (**Figure 4F - H**). Nuclear membrane breakdown was often observed in the graph as an initial basalward movement due to the re-distribution of GFP within the entire cell (basal most part of the cell body in this instance). The apical velocity was determined before nuclear envelope breakdown, fitting a linear regression curve (grey dotted line, **Figure 4G**) to the distance plotted against the time for the period before nuclear envelope breakdown (red diamond, **Figure 4G**). The slope 'm' of the linear regression curve (y = mx+c) represents the velocity 'dx/dt'. The apical velocity for the cell presented in **Figure 4** was 10.89 µm/h. The same approach was applied to calculate the velocity of the initial rapid basal movement (v_b) of the newly formed daughter nuclei (**Figure 4F, H**). The basal migration velocities v_b of -67.71 and 33.51 µm/h of daughter cells D1 and D2, respectively, were comparably faster than the apical velocity. Unfortunately, it was not possible to determine the apical velocity after nuclear envelope breakdown as the GFP diffused throughout the entire cell. Instead, the instantaneous velocity was calculated based on the timing and position of the nucleus before nuclear envelope breakdown and the first visualization of the newly formed daughter nuclei. The instantaneous velocity of 7.9 µm/h for the cell shown in **Figure 4** is likely an underestimate as the chromatin reaches the ONL prior to telophase of mitosis, when the daughter nuclei become visible.

To summarize, the culture of retinal explants allows live-cell imaging of INM in the adult regenerating zebrafish retina and the determination of migration and cell division parameters of individual nuclei.

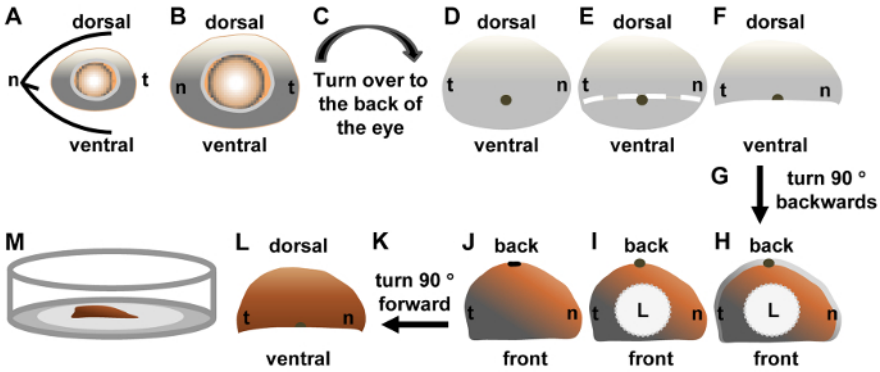


Figure 1: Retinal Isolation Procedure. A) Schematic illustrating a zebrafish head with its eye. **B**) The eye is removed from the zebrafish and oriented to show the front of the eye with the pupil. **C**) Turn the eye 180 ° so that the back of the eye with the optic stalk (filled black circle) becomes visible in (**D**) and the pupil faces downwards. The grey color indicates the presence of the sclera of the eye. **E**) One blade of a pair of McPherson-Vannas scissors is inserted into the optic stalk of the eye (filled black circle) to cut it into its dorsal and ventral sides which is indicated by the white segmented line. **F**) Dorsal half of the eye after the ventral half was removed. The black half circle indicates the residual part of the optic stalk. **G**) Turn the eye 90 ° backward to reveal (**H**) the inside of the eye with the retina (brown), vitreous (not shown) and the lens (**L**, light grey circle) that is still encased by the sclera (grey line). **I**) The sclera was detached (note, grey line is missing) and **J**) the lens and vitreous removed giving rise to only the retina (brown). **K**) Retina turned 90 ° forward giving rise to a flat-mount view of the dorsal retina (**L**). **M**) Dorsal retinal explant flat-mounted on a glass-bottom fluorodish. Please click here to view a larger version of this figure.

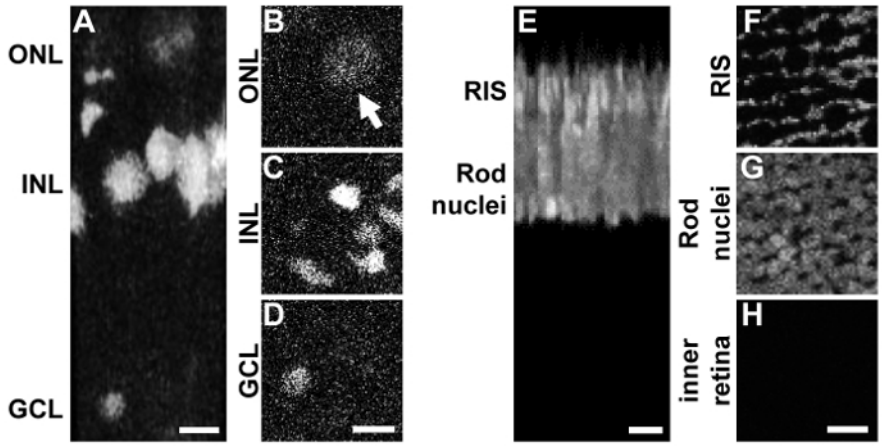


Figure 2: 3-D Reconstruction of Retinal Multiphoton Z-Stacks. A) 3-D reconstruction of a multiphoton z-stack image series of *gfap:nGFP*-positive cells in retinal whole-mount cultures from zebrafish after 48 h of light-damage. **B - D**) Single multiphoton xy-images of *gfap:nGFP*-positive cells displayed in the 3D-reconstruction in (**A**) at the level of the ONL (**B**), the INL (**C**), which corresponds to the layer that contains Müller glia soma and the GCL (**D**). Arrow in (**B**) indicates an enlarged round Müller glia in the ONL after nuclear envelope breakdown. **E**) 3-D reconstruction of a multiphoton z-stack image series of a retinal explant from an undamaged *Tg[rho:Eco.NfsB-EGFP]*^{nt19} zebrafish. **F - H**) Single multiphoton xy-images at the level of the rod inner segments (RIS, **F**), rod nuclear layer (**G**) and at the level of the inner retina (**H**). GCL, Ganglion Cell Layer; INL, Inner Nuclear Layer; ONL, Outer Nuclear Layer; RIS, Rod Inner Segment. Scale bar in A, D, E and H, 10 µm. Please click here to view a larger version of this figure.

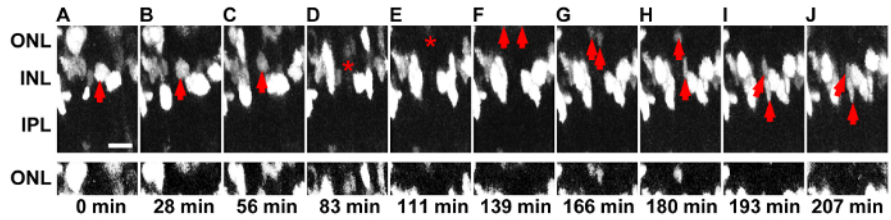


Figure 3: Timelapse Image Series of INM. A - J) Image series of 3D-reconstruction of z-stack timelapse series displaying *Tg[gfap:nGFP]*^{mi2004}-positive nuclei that undergo INM to divide in the ONL in retinal explants (top row). The position of one apically migrating nucleus and its basally migrating daughter nuclei is indicated by red arrows. Red star indicates nuclear envelope breakdown during mitosis. **A - J**, bottom row) The same image series as in the top row was oversaturated and cropped to focus on the dividing cells in the ONL. INL, Inner Nuclear Layer; IPL, Inner Plexiform Layer; ONL, Outer Nuclear Layer. Scale bar in A, 10 µm and is the same for B - J. Please click here to view a larger version of this figure.

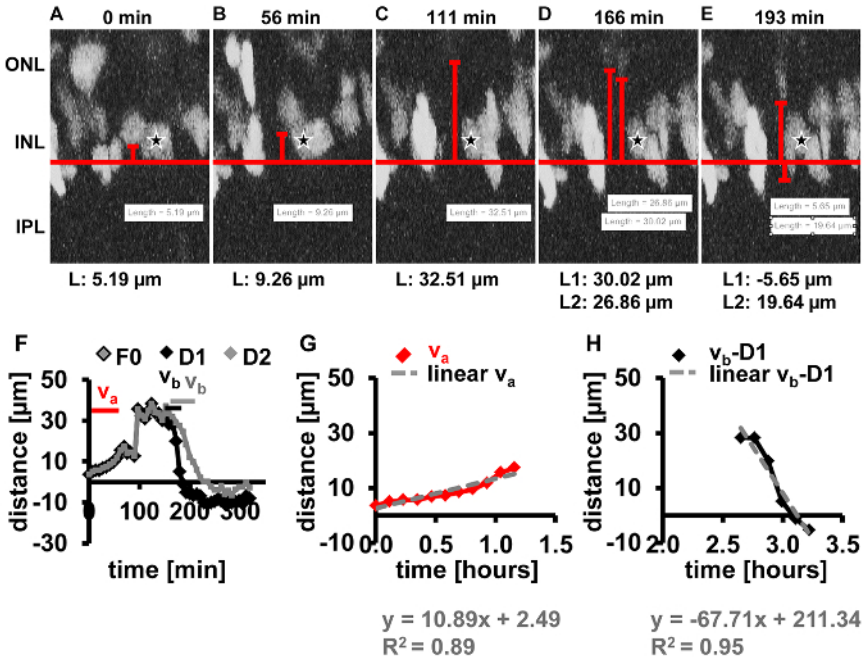
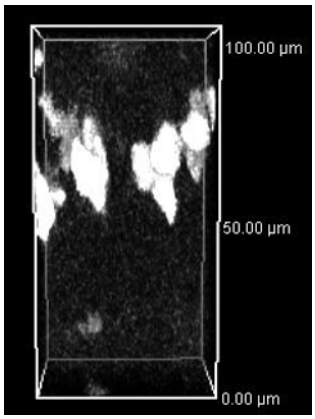


Figure 4: Analysis of Apical and Basal Migration Velocities. A - E) Maximum yz-projection of a z-stack image series of *Tg[gfap:nGFP]*^{mi2004} retinal explants at different timepoints of the timelapse recording acquired by multi-photon microscopy. Using the line tool under the 'manual measurement' function, a horizontal line was placed at the level of the reference cell, indicated by a star. A vertical measurement line was positioned starting at the horizontal line and extending to the basal position of the migrating *gfap:nGFP*-positive nucleus. The length of the measurement line is given in the white boxes on the images and for readability under the images. L1 = length that daughter cell 1 migrated; L2 = length that daughter cell 2 migrated. **F**) Distance that the cell (F0) measured in (**A - E**) migrated apically and that the arising daughter cells D1 and D2 migrated basally in the multiphoton timelapse recording. The red line indicates the measurements for which the apical migration velocity (v_a) was calculated while the black and grey lines indicate the measurements used to calculate the basal migration velocities (v_b) for D1 and D2, respectively. **G, H**) The distance was plotted against the time in Excel for apical migration before nuclear envelope breakdown (**G**) and the phase of rapid basal migration for daughter cell D1 (**H**). Linear regression curves were fitted and the functions are given below the graphs with the slope representing the velocity. INL, Inner Nuclear Layer; IPL, Inner Plexiform layer; ONL, Outer Nuclear Layer. Please click here to view a larger version of this figure.

	Tg[gfap:EGFP]^{nt11}		*Tg[rho:Eco.NfsB-EGFP]*^{nt19}	
	laser power	**gain**	**laser power**	**gain**
Top (rod photoreceptor)	13	126	3.5	118
Middle (Müller glia)	10	126	2.8	118
Bottom (GCL)	8	126	2.1	118

Table 1: Laser and Gain Levels Used for Z-Intensity Corrections at the Different Planes for Experiments Displayed in Figures 2 - 4.



Movie 1: Live-cell Imaging of Retinal Explants by Multiphoton Microscopy. (Right click to download).

Discussion

Studies investigating the mechanisms governing regeneration of the damaged adult zebrafish retina predominantly used immunocytochemical methods[5,25,26,27,28,29,30]. Establishing conditions to culture retinal explants and to perform live-cell imaging on phenomena, such as INM, provide us a technique to obtain in depth spatial and temporal information. This technique allows determining the migration velocities, the timing and position of nuclear envelope breakdown during prophase of mitosis and the length and position of cell division. Moreover, it is possible to establish the division plane and the fate of the arising daughter cells in regard to their subsequent location. Ultimately, this powerful approach will determine whether Müller glia and neuronal progenitor cells behave differently in regard to INM during retinal regeneration. The use of transgenic zebrafish lines that identify proliferating cells in the regenerating retina at distinct stages of the regeneration response will aid deciphering differential behavior[6]. In case of the multiphoton microscope used here, GFP reporter zebrafish lines are preferred as excitation of Red Fluorescent Protein (RFP) is not very efficient; however, other multiphoton systems may allow efficient excitation of RFP or equivalent fluorophores.

One of the most critical steps in the retinal isolation procedure is mounting of the retina. Retinas have to be mounted as flat as possible for the multiphoton light to be able to pass to the deeper z-levels of the ONL. Curving of the retina especially in the marginal region, residual vitreous, and/or accumulation of agarose under the retinal culture, which is used to immobilize the tissue, most commonly introduce extra space between the cover slip and the retinal culture. In addition, increased laser power and gain have to be used to acquire images if the retina is not flat and this will consequently cause increased photobleaching and reduced viability of the retinal culture. Incorrect mounting often also results in poorer image quality due to reduced light penetration and will affect the ability to measure the distances that nuclei had migrated and therefore the calculation of migration velocities.

The velocities of apical migration before nuclear envelope breakdown and of basal migration can be reliably determined in an image series from *Tg[gfap:nGFP]*^{mi2004} zebrafish retinal explants. However, it is currently not possible to determine the velocity of apical migration of the chromatin after nuclear envelope breakdown in this transgenic zebrafish line as the GFP re-distributes throughout the entire cytoplasm. Labeling of retinal explants with the nuclear dye Hoechst resulted in dim uptake into Müller glia nuclei that required the change in wavelength to 830 nm (Lahne & Hyde, unpublished data), which affected the viability of the tissue and caused stalling of the cell cycle (Lahne & Hyde, unpublished data). During retinal development, *Tg[h2afva:h2afva-GFP]*^{kca6} zebrafish that express GFP fused to histone 2 have been successfully used to monitor INM and determine migration velocities at the different stages of the cell cycle[12,31]. In the future, retinal explants from *Tg[h2afva:h2afva-GFP]*^{kca6} zebrafish will also enable the visualization of chromatin throughout the cell cycle and will allow the analysis of the apical migration velocity after nuclear membrane breakdown in the adult regenerating zebrafish retina.

Having established the conditions to monitor INM by live-cell imaging in retinal explants from light-damaged adult zebrafish and the corresponding analysis of velocities will form the basis to investigate the mechanisms governing INM. Some studies began examining the mechanisms of INM in the adult regenerating retina using immunocytochemistry to determine the position of mitotic phospho-histone 3-positive nuclei[6,7]. However, interfering with signaling pathways may not render the position of mitosis, but might affect other parameters such as velocity, timing of mitosis and division that would not be possible/very difficult to discern by immunocytochemistry. Previously, it was shown that phospho-histone 3-positive mitotic nuclei mislocalized to more basal positions in the developing retina in dynactin mutants and morphants[11]. However, subsequent live-cell imaging revealed that nuclear migration prior to cell division was delayed in dynactin-compromised cells, while an increased apical migration velocity enabled nuclei to reach and to undergo cell division at the apical surface[11,12]. This example exemplifies the power of live-cell imaging. Similarly, when unraveling the mechanisms that facilitate INM in the adult regenerating zebrafish retina, live-cell imaging studies will complement, and in various instances will be advantageous over, immunocytochemical approaches.

As discussed above, live-cell imaging offers many advantages over immunohistochemical/static methods; however, it has to be kept in mind that retinal explants are an *ex vivo* model. As such, damage incurred during the isolation procedure and/or culture conditions might affect cellular processes. In addition, the imaging procedure itself may exert toxic effects that may influence cellular behavior[23,32]. While there are disadvantages to live-cell imaging of retinal cultures, it is currently our best method to obtain dynamic information of INM. Importantly, the live-cell imaging procedure of retinal explants will be applicable to other biological questions during retinal regeneration. Based on immunocytochemical data, it was previously suggested that Müller glia phagocytose dying photoreceptors/debris following light-induced photoreceptor damage[33]. Adjusting parameters to image this process live will verify if phagocytosis of dying photoreceptors occurs in the regenerating zebrafish retina and can be used to study the underlying mechanisms. Having shown that images can be acquired at the level of rod nuclei and rod inner segments in *Tg[rho:Eco.NfsB-EGFP]*^{nt19} zebrafish, it should be feasible to adjust the conditions to image photoreceptor phagocytosis. Similarly, knowledge is limited regarding the dynamic behavior of microglia and the mechanisms governing their function in the degenerating and regenerating retina[34,35]. Exploiting microglia-specific transgenic zebrafish in conjunction with live-cell imaging will also increase our understanding of the function of microglia in the adult regenerating zebrafish retina [36, 37]. To summarize, live-cell imaging of retinal explants is a powerful tool to gain dynamic information of cellular processes and to determine the behavior and function of different cell types in the regenerating retina.

Disclosures

The authors have nothing to disclose.

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