**Video Article**

**Multicolor Time-lapse Imaging of Transgenic Zebrafish: Visualizing Retinal Stem Cells Activated by Targeted Neuronal Cell Ablation**

Junko Ariga*, Steven L. Walker†, Jeff S. Mumm

Department of Cellular Biology and Anatomy, Medical College of Georgia

*These authors contributed equally

Correspondence to: Jeff S. Mumm at jmumm@mcc.edu

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**Abstract**

High-resolution time-lapse imaging of living zebrafish larvae can be utilized to visualize how biological processes unfold (for review see 1). Compound transgenic fish which express different fluorescent reporters in neighboring cell types provide a means of following cellular interactions 2 and/or tissue-level responses to experimental manipulations over time. In this video, we demonstrate methods that can be used for imaging multiple transgenically labeled cell types serially in individual fish over time courses that can span from minutes to several days. The techniques described are applicable to any study seeking to correlate the "behavior" of neighboring cells types over time, including: 1) serial 'catch and release' methods for imaging a large number of fish over successive days, 2) simplified approaches for separating fluorophores with overlapping excitation/emission profiles (e.g., GFP and YFP), 3) use of hypopigmented mutant lines to extend the time window available for high-resolution imaging into late larval stages of development, 4) use of membrane targeted fluorescent reporters to reveal fine morphological detail of individual cells as well as cellular details in larger populations of cells, and 5) a previously described method for chemically-induced ablation of transgenically targeted cell types; i.e., nitroreductase (NTR) mediated conversion of prodrug substrates, such as metronidazole (MTZ), to cytotoxic derivatives 3,5.

As an example of these approaches, we will visualize the ablation and regeneration of a subtype of retinal bipolar neuron within individual fish over several days. Simultaneously we will monitor other retinal cell types, including neighboring non-targeted bipolar cells and potential degeneration-stimulated retinal stem cells (i.e., Müller glia). This strategy is being applied in our lab to characterize cell- and tissue-level (e.g., stem cell niche) responses to the selective loss and regeneration of targeted neuronal cell types.

**Protocol**

1. **Transgenic and Mutant Lines**

   1. Establish/acquire transgenic zebrafish lines that have cell types of interest differentially labeled with fluorescent reporter variants. Optimally, the excitation/emission profile of the selected reporters should have minimal overlap (e.g., ECFP and EYFP), however this is not absolutely required. For our purposes, the use of membrane tethered fluorescent reporters greatly facilitates imaging of fine cellular details, such as neuritic processes, to resolve individual cell numbers and/or morphologies in large comingled groups and to 'highlight' regions of high membrane content, such as synaptic neuropils.

   2. For imaging at late larval stages, it is helpful to derive/cross transgenic lines into mutant backgrounds which reduce pigmentation [e.g., roy orbison (roy) or roy orbison; albino (roy;alb); 4]. In our experience, reduction of iridophores (e.g., roy) is the most critical issue, melanogenesis can be addressed chemically using 1-phenyl 2-thiourea (PTU) or with "albino" mutants which lack melanophores. Note however that Ren et al. 4 have demonstrated visual deficits and modest morphological changes in retinas of fish lacking melanophores, and that intense light can induce photoreceptor death in "albino" mutants. Thus, these issues need to be taken into consideration when designing imaging experiments and/or interpreting data.

   3. For this demonstration, the transgenic and mutant fish lines used were:

      a. Tg(nx:ný:Gal4-VP16)16a†; Tg(UAS-gap43-YFP)16b†; Tg(UAS-E1b:NfsB-mCherry)16d†; Tg(pax6-DF4:gap43-CFP)16e†; roy0/a9

      b. Tg(nx:ný:Gal4-VP16)16a†; Tg(UAS-gap43-YFP)16b†; Tg(UAS-E1b:NfsB-mCherry)16d†; Tg(gfapt:GFP)29/a9; roy0/a9

      c. Note: In fish "1", a subpopulation of ný promoter defined retinal bipolar cells express a membrane-tagged YFP and/or a NTR-mCherry fusion reporter (most express both), and nearly all retinal cells express a membrane-tagged CFP. Fish "2" are as above except Müller glia cells express cytosolic GFP and global retinal CFP expression has been eliminated. Expression of the NTR-mCherry fusion renders ný defined bipolar cells susceptible to prodrug-induced ablation 3,5.

2. **Selecting and Preparing Embryos/larvae for Live Imaging**

   1. Mate transgenic/mutant lines of interest, collect eggs and incubate/maintain at 28.5°C in 0.3X Danieau's solution (or 'embryo medium' of choice).

   2. If not in an "albino" background, PTU should be added to inhibit tyrosinase activity prior to any evidence of pigmentation in the tissue of interest (e.g., ~16 hpf for the eye, 200 μM final). Note that treatment with PTU at concentrations exceeding 75 μM may cause toxicity and/or teratogenic effects. However, because the eye is highly pigmented, treatments below 200 μM are not adequate for high-resolution confocal imaging; under these conditions it is important to select healthy looking fish for imaging. For imaging other tissues, 75 μM PTU may be preferable. Also, as noted above, use of "albino" mutant lines obviates the need to treat embryos with PTU and is a preferred strategy for imaging at late larval stages.

   3. Once fluorescent reporters are evident, sort fish according to transgene expression and general health using a fluorescence stereo zoom microscope or similar.
3. "Multicolor" in vivo Confocal Imaging

Note: We have tried to generalize the protocol below such that it provides relevant information for other microscope configurations. Specific references to Olympus and ImageJ software applications are in quotes. Our imaging system is an Olympus FV1000 upright confocal microscope equipped with 405, 440, 488, 515, 559, and 635 nm lasers, two variable barrier filter detection channels (allowing emission wavelength settings to be adjusted in 1 nm increments), one standard barrier filter channel (for red and far red imaging), and one transmitted light channel.

1. Open the imaging software ("FV10-ASW").
2. If collecting transmitted light images, focus the field diaphragm for Kohler illumination.
3. Choose appropriate fluorophores from the "Dye List" or load acquisition parameters from a previously saved image file. Make adjustments to emission ranges ("Spectral Setting") necessary for clean separation of reporter signals and/or maximal signal to noise ratios. These settings will need to be empirically defined for each experiment to minimize crosstalk but maximize signal; settings for the examples provided here are shown below:

| Fluorophores | Lasers (nm) | Barrier Settings / Emission Filter (nm) |
|--------------|------------|----------------------------------------|
| 1) ECFP, EYFP, mCherry* | 440, 515, 559* | 460-500, 530-545, 575-675 |
| 2) EGFP**, EYFP**, mCherry | 488, 515, 559 | 500-515, 530-545, 575-675 |

*Imaging of mCherry could be improved using a higher wavelength laser (e.g., 568 or 594 nm)
**A sequential imaging mode allowing dichroic mirror and barrier filter changes ("Virtual Channels") is required due to emission/excitation overlap between GFP and YFP. GFP and mCherry can be assigned to one channel, YFP to a separate channel. Note: crosstalk between GFP and YFP images will be evident prior to Image Subtraction (Part 6).

4. Make sure the objective being used is selected in the drop down menu to ensure any software defined presets are adjusted properly.

5. Focus lasers and set power levels, select a look up table (LUT) that reveals pixel saturation ("Hi-lo") for each channel. Set detector sensitivity ("HV", PMT voltage) to a value consistent with desired image quality (defined empirically) and gradually raise laser output until image intensity is acceptable avoid pixel saturation.

6. Check for crosstalk between channels; ensure that each laser line produces detectable signal only in the appropriate channel by manually turning lasers on and off while monitoring all imaging channels. To eliminate crosstalk reduce laser power. If no crosstalk is evident, all channels can be acquired simultaneously providing significant time savings.

7. In the event of unavoidable crosstalk, use a "Sequential" imaging mode that switches between lasers/channels individually. Extreme cases (e.g., imaging GFP and YFP) require the use of sequential modes that also switch dichroic mirrors and/or barrier filters ("Virtual Channels"). Note: this option introduces significant temporal delays into the image acquisition process and may not be appropriate for capturing highly dynamic processes.

8. "Zoom" and "Rotation" functions can be used to further frame the area of interest. Zoom can also be used to improve image resolution, up to the limits of the objective and scan format used (e.g., 512 x 512; for information about these issues see, http://corefacilities.systemsbiology.net/imaging/documents/app_note_image_res.pdf).

9. For 3D imaging in general, and of particular importance for time-lapse imaging, techniques which minimize phototoxicity issues (i.e., accumulation of free radicals) need to be emphasized; fast scan speeds (e.g., 2 μs/pixel) and low laser output levels (e.g., 1%) should be used whenever possible, as well as the lowest resolution scan format adequate for capturing salient information.

10. To improve image resolution, scan averaging ("Kalman") can be used during acquisition. Slower scan speeds also improve resolution but increase laser dwell time, which will exacerbate phototoxicity and bleaching issues. Note that both of these approaches are not ideal for time-lapse due to potential for inability to capture rapid changes across a z-series, a phenomenon we call "time blur". Note that averaging by line versus by frame can help to eliminate "time blur" issues within a single plane but not across z-depths.

11. If signal intensities are low and maximum image resolution is not required (e.g., simply counting cell numbers), increasing the confocal aperture (pinhole diameter) can be used to boost signal. This serves to keep laser intensities low, reducing phototoxicity and bleaching, however adjusting the aperture to a value greater than 1 airy unit leads to rapid losses in image quality.

12. Define z-depth dimensions using a rapid scan mode ("Focus x4"). Because signal intensity generally decreases with z-depth it is best to start z-stack acquisitions at the lowest depth and end at the most superficial, this allows more difficult signals to be acquired before significant bleaching occurs.

13. Check for adequate signal detection throughout the depth of the image. If desired, a z-dimension brightness correction function ("Bright Z") can be used to adjust image acquisition parameters at specified depths.
1. A 'catch and release' protocol is used to minimize the amount of time fish are immobilized and to increase the number of fish imaged per experiment. The usefulness of this technique is limited to biological processes which unfold over days (e.g., Mumm et al. 2006). One advantage of this approach is that it allows any observed changes to be internally controlled; i.e., comparisons between different states of individuals rather than across populations. For instance, tracking changes in the number of labeled cells present in a given tissue can be performed accurately even if the number of cells initially labeled varies greatly between individual fish. Methods for using this protocol within the context of a prodrug-induced cell ablation assay are provided here.

2. To visualize the ablation and regeneration of targeted cell types in individual fish over time, confocal images are acquired prior to prodrug administration (Pre-treatment), immediately following prodrug removal (Post-treatment) and during the recovery period (Recovery images).

3. Pre-treatment imaging: Fish are mounted and imaged as above. Once fish have been imaged they are released into embryo medium (+ prodrug) and incubated at 28.5°C until the Post-treatment imaging session. Working in teams of two, one mounting and releasing fish, the other imaging is a good way to maximize the number of fish that can be imaged per day.

4. To release fish, use jewelers forceps to cut through agarose on both sides of the larvae and then gently tease agarose away until fish floats freely in embryo medium.

5. Prodrug-induced ablation: Transfer larvae into an individual well of a multwell plate containing embryo medium (+/- PTU) + prodrug (e.g., 10mM metronidazole, MTZ). To ensure accurate final prodrug concentrations we typically aliquot a defined volume of embryo medium (+/- PTU) containing a 2X concentration of prodrug (e.g., 20 mM) into each well. Fish are then added to wells in an equal volume of embryo medium (+/- PTU) to bring the final prodrug concentration to 1X.

6. Incubate at 28.5°C until targeted cell ablation can be verified. Note: Prodrug treatment regimens required to achieve successful ablation vary according to the cell type targeted; prodrug concentration and treatment duration will need to be determined empirically, be aware that high concentrations cause general toxicity, e.g., >10 mM MTZ. In addition, perdurance of fragmented reporter signals can complicate assessments of ablation success, in some instances confocal microscopy is required to adequately visualize cell loss.

7. Post-treatment imaging: Fish are mounted such that the same tissue is optimally oriented and imaged as above. Once all fish on a given plate have been imaged they are released into embryo medium (+/- PTU) into individual wells (as above) and incubated at 28.5°C to allow regeneration of the ablated cell type.

8. Depending on the age of the fish and time required for recovery it may be necessary to feed fish during this stage of the assay. We use live food such as paramecia or rotifers to ensure optimal survival. It is also advisable to provide fresh media daily, particularly if fish are maintained in low volumes (e.g., 96 well plates).

9. Recovery imaging: To document the kinetics of cell replacement, fish can be imaged on a daily basis during the recovery period. Once the kinetics have been established the assay can be simplified by acquiring recovery images only at time points of interest (e.g., full recovery). Figure 1 provides an example of this approach from our studies of retinal cell regeneration.

10. At the conclusion of the experiment fish should be euthanized by immersion in 20X tricaine (15.3 μM) solution on ice.

11. 5. Rapid Time-lapse Imaging.

1. For rapid time-lapse imaging (i.e., ‘movies’) of multiple fluorophores, techniques which minimize phototoxicity issues are critical (see above). As stated above, this technique is best suited to fluorophores allowing simultaneous, rather than sequential, imaging.

2. Maintain fish at 28.5°C using a heated stage, ITO glass heating element, or similar.

3. Use methods which reduce evaporation to help stabilize temperature and osmolarity (e.g., o-ring sealed coverslips for inverted microscopes).

4. If using automated acquisition over extended times be sure to set the stack size to allow for growth and/or drift in the z-dimension.

5. Set time intervals and number of scans ("TimeScan" or "TimeController"). Acquisition rates will need to be established empirically according to the dynamics of the biological process imaged and/or adjusted according the phototoxic sensitivity properties of the cell population of interest.

6. Acquire images ("XZLZt" button) and save.

7. To construct ‘movies’, areas of interest will need to be defined and aligned in the x, y, and z dimensions. The full process is beyond the scope of this protocol review but several software programs are available which also facilitate zoom, rotation, and panning functions during 4D image assembly (Volocity, Amira, etc.).

6. Spectral Separation Image Processing using ImageJ Freeware

1. Optimal, when performing a multi-reporter imaging experiment it is best to use fluorophores that are well separated spectrally. However, this is not always practical and/or possible. Here we provide a simplified method for separating fluorophores that have overlapping excitation and emission profiles, in this case GFP and YFP. In the example provided, we have used the time-lapse imaging techniques described above to track the behavior of GFP-labeled retinal ‘stem cells’, Müller glia, during the targeted ablation and regeneration of retinal bipolar cells labeled with YFP and NTR-mCherry (Figure 2).

2. Collect images using sequential imaging modes and variable barrier filters that allow overlapping fluorophores to be acquired independently and partially separated, respectively (see above for GFP/YFP settings example). To remove crosstalk we use a simple image subtraction strategy which removes one channels signal from another.

3. Image Subtraction Process: Install the latest version of ImageJ (http://rsb.info.nih.gov/ij/notes.html), Loci Bio-Formats tool (http://www.locl.wisc.edu/bio-formats/imagej), and align stacks plug-in (http://www.med.harvard.edu/JPNM/IJ/plugins/Align3TP.html).
7. Representative Results

Figure 1. A time-lapse series of confocal images demonstrating targeted loss and regeneration of NTR-mCherry expressing retinal bipolar cells (red cells). A & A') Prior to treatment there is mosaic expression of membrane-tagged YFP reporter in "control" bipolar cells, shown in yellow, and nitroreductase-Cherry fusion protein in "targeted" bipolars shown in red. B & B') Following treatment with metronidazole, the red nitroreductase-expressing bipolar cells, are lost, while the yellow "control" bipolar cells, are spared. This demonstrates the highly specific nature of this ablation methodology. C & C') When metronidazole is removed, NTR-expressing (red) cells return.
Discussion

In this video, an overview of techniques we use for multicolor confocal time-lapse imaging and targeted cell ablation is provided. We employ time-lapse imaging to monitor the behavior of multiple cell types of interest in vivo, while targeted cell ablation is used to study neural circuit function and cell-specific neuronal regeneration paradigms. The examples shown highlight several advantages that can be gleaned from these approaches. Perhaps most significantly, time-lapse imaging provides an internally controlled paradigm; any and all phenomena observed can be related back to previous states. This allows direct rather than inferred comparisons to be made across experimental time points, making relative changes easier to quantify and reducing experimen trial "noise" associated with variations within a population. An advantage of multicolor imaging is the ability to visualize changes in interacting and/or neighboring cell types. For instance, using a transgenic line which labels Müller glia, we are now characterizing the response of these potential injury-induced stem cells to the loss of discrete retinal cell subpopulations. We have also used this approach to define novel mechanisms of neural circuit formation 1. Finally, in addition to studies of cellular regeneration, we have recently begun using the NTR-based ablation system to investigate the functional role of neuronal subtypes thus discrete neural circuits using various behavioral paradigms and electrophysiological assays. The unique advantage of employing the zebrafish for such studies is that, due to their regenerative capacity, induced deficits are temporary. Thus, by correlating with time-lapse imaging assays we can typify reinnervation mechanisms that lead to, as well as the extent of reinnervation required for, functional recovery.

The methods provided can be adapted to many different questions. For instance, similar analyses of ablation and regeneration can be applied to any transgenically definable cellular subtype. Collectively, such studies have potential to expand our understanding of regeneration at the level of individual cell types and within discrete stem cell niches.

Disclosures

J.S.M. has been awarded a patent on the use of the nitroreductase-based system of cell ablation in zebrafish (Mumm J.S., and Schroeter, E.H. Targeted and Regional Ablation Cellular Ablation in Zebrafish: US Patent 7514595. 7 Apr. 2009). This technique is used as the general platform for a tools and services biotechnology company, Luminomics, Inc., that J.S.M. founded and remains a stakeholder in.

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