Journal of Visualized Experiments

Visualizing the Developing Brain in Living Zebrafish Using Brainbow and Time-Lapse Confocal Imaging

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60593R1
Full Title:	Visualizing the Developing Brain in Living Zebrafish Using Brainbow and Time-Lapse Confocal Imaging
Section/Category:	JoVE Developmental Biology
Keywords:	microscopy; Neuroscience; development; Brainbow; Zebrafish; lineage tracing; in vivo imaging; brain; hindbrain; cell division; Apoptosis; cell death
Corresponding Author:	Tamily Weissman Lewis and Clark College Portland, OR UNITED STATES
Corresponding Author's Institution:	Lewis and Clark College
Corresponding Author E-Mail:	weissman@Lclark.edu
Order of Authors:	Zoe T. Cook
	Nicole L. Brockway
	Tamily Weissman
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Portland, OR, USA

Lewis & Clark College

Department of Biology

0615 S.W. Palatine Hill Road
Portland, Oregon 97219-7899
Phone 503-768-7511
Fax 503-768-7658
E-mail biology@lclark.edu
www.lclark.edu

October 11, 2019

Dear Dr. Bajaj,

Thank you for you prompt review and invitation to revise our manuscript entitled: *"Using Brainbow and time-lapse confocal imaging to visualize the developing brain in living zebrafish"*.

We are grateful to the reviewers for their time in reading and evaluating our work. I have included a detailed response letter on the following pages. We have also addressed all of the editorial comments. Our revised version of the manuscript includes track changes that display all edits that have been made since the initial submission.

Thank you again for your review of our manuscript. I truly appreciate the time and attention you have given to our work.

Sincerely,

Tamily A. Weissman, Ph.D.
Associate Professor in Biology
Co-Chair, Neuroscience Program
Lewis & Clark College
Portland, OR 97219
(503) 768-7994

TITLE:

Visualizing the Developing Brain in Living Zebrafish Using Brainbow and Time-Lapse Confocal Imaging

AUTHORS AND AFFILIATIONS:

Zoe T. Cook[1], Nicole L. Brockway[1], Tamily A. Weissman[1]

[1]Lewis & Clark College, Biology Department, Portland, OR, USA

Corresponding Author:

Tamily A. Weissman (weissman@lclark.edu)

Email Addresses of Co-Authors:

Zoe T. Cook (zoecook@lclark.edu)

Nicole L. Brockway (nbrockway@lclark.edu)

KEYWORDS:

microscopy, neuroscience, development, Brainbow, zebrafish, lineage tracing, in vivo imaging, brain, hindbrain, cell division

SUMMARY:

In vivo imaging is a powerful tool that can be used to investigate the cellular mechanisms underlying nervous system development. Here we describe a technique for using time-lapse confocal microscopy to visualize large numbers of multicolor Brainbow-labeled cells in real time within the developing zebrafish nervous system.

ABSTRACT:

Development of the vertebrate nervous system requires a precise coordination of complex cellular behaviors and interactions. The use of high resolution in vivo imaging techniques can provide a clear window into these processes in the living organism. For example, dividing cells and their progeny can be followed in real time as the nervous system forms. In recent years, technical advances in multicolor techniques have expanded the types of questions that can be investigated. The multicolor Brainbow approach can be used to not only distinguish among like cells, but also to color-code multiple different clones of related cells that each derive from one progenitor cell. This allows for a multiplex lineage analysis of many different clones and their behaviors simultaneously during development. Here we describe a technique for using time-lapse confocal microscopy to visualize large numbers of multicolor Brainbow-labeled cells over real time within the developing zebrafish nervous system. This is particularly useful for following cellular interactions among like cells, which are difficult to label differentially using traditional promoter-driven colors. Our approach can be used for tracking lineage relationships among multiple different clones simultaneously. The large datasets generated using this technique provide rich information that can be compared quantitatively across genetic or pharmacological manipulations. Ultimately the results generated can help to answer systematic questions about how the nervous system develops.

INTRODUCTION:

In the early phases of development, pools of specialized progenitor cells divide repeatedly in proliferative zones, producing diverse arrays of daughter cells. The cells born during this developmental period will then differentiate and travel to form the nascent organs. In the nervous system, progenitors such as radial glia give rise to immature neurons in ventricular zones. As neurons migrate away from ventricles and mature, the expanding tissue eventually forms the highly complex structures of the brain[1–6]. The coordination between division of progenitors and differentiation and migration of neurons will determine the eventual size, shape, and thus function of the brain, directly impacting behavior[7–10]. While tight control over these processes is clearly crucial for normal brain development, the global mechanisms that regulate these dynamics are not well understood. Here we describe a tool to study nervous system development at a cellular resolution, allowing researchers to visualize progenitor cells and neurons in vivo in the developing zebrafish brain with Brainbow and track their behavior over time via time-lapse confocal microscopy[11]. The approach can also be adapted to visualize other parts of the developing embryo.

To observe and distinguish among cells in the developing zebrafish brain, we have adapted the Brainbow cell-labeling technique[11]. Brainbow utilizes the randomly determined, combinatorial expression of three distinct fluorescent proteins (FPs) to label a population of cells. While the default expression for Brainbow expression is the red FP dTomato, recombination by the enzyme Cre recombinase results in expression of mCerulean (cyan fluorescent protein, CFP) or yellow fluorescent protein (YFP)[12,13]. The combined amount of each FP expressed in a cell gives it a unique hue, allowing clear visual distinction from neighboring cells. Additionally, when a progenitor cell divides, each daughter cell will inherit the color from its mother cell, producing color-coded clones and allowing researchers to trace cell lineage[11,14]. While originally used to analyze neuronal circuitry in mice[12], Brainbow has since been expressed in a wide variety of model organisms, including zebrafish[15].

Our technique builds on previous multicolor labeling and imaging methods to directly image multiple color-coded clones over time in living zebrafish. Due to their optical transparency as embryos, zebrafish are well suited to imaging experiments[16], and previous studies have utilized Brainbow in zebrafish to study a variety of tissues, including the nervous system[11,15,17–27]. The ability to directly image the inside of the organism, along with their rapid ex utero development, make zebrafish a valuable model of vertebrate development. In contrast to the mammalian brain, the entire proliferative zone of the zebrafish hindbrain is readily available for imaging without disruption to its endogenous environment[6]. This allows experiments to be conducted in the living organism, rather than in in vitro or fixed tissue preparations. In contrast to fixed imaging experiments, in vivo studies allow for a longitudinal design, producing hours of data that can be analyzed for patterns, thus increasing the likelihood of observing relatively rare events. Depending upon the speed and length of the events of interest, researchers may choose to perform short (1–2 h) or long (up to ~16 h) time-lapse imaging experiments. By using the zebrafish heat shock promoter 70 (hsp70, hsp), Brainbow expression can be temporally controlled[28,29]. Additionally, the mosaic expression induced by this promoter is well suited for

labeling and tracking many clones[11].

The ability to visually identify multiple clones within the living brain is an advantage of this method. Important previous studies that investigated the role of clones within development of the nervous system utilized retroviral vectors to label a single progenitor cell and its progeny using a single FP or other readily visualized protein. Such labeling allows researchers to observe a single clone over time, either in vitro or in vivo[2,30–38]. In contrast to methods to track the behavior of cells within one clone, the distinct colors of Brainbow allow researchers to observe dynamics among clones. Additionally, by using Brainbow to label many clones within the brain, additional data on clonal behavior is collected relative to techniques that label a single clone[11]. Importantly, the approaches described here can be expanded to generate developmental comparisons between fish that have undergone different genetic or pharmacological manipulations[18]. Overall, these advantages make time-lapse in vivo confocal imaging of Brainbow-expressing zebrafish ideal for researchers exploring development of the vertebrate nervous system, particularly those interested in the role of clones.

PROTOCOL:

Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at Lewis & Clark College.

1. Microinjection of zebrafish embryos

1.1. Set up wild type, adult zebrafish in sex-segregated mating tanks the afternoon prior to performing microinjections[39,40].

1.2. Prepare the DNA solution in the morning of the microinjections. Dilute *hsp*:Zebrabow[11] plasmid DNA to a concentration of ~10 ng/µL in 0.1 mM KCl, along with 2.5% phenol red and 3.75U Cre recombinase enzyme.

1.3. Perform microinjections of DNA solution into one-cell zebrafish embryos within 45 min of fertilization[39,41]. Inject approximately 4.2 nL of this solution into each embryo, equivalent to ~42 pg of plasmid DNA.

NOTE: The microinjection step can be omitted if a transgenic, Brainbow-expressing zebrafish line is mated instead, such as *Tg(ubi:Zebrabow)*[15] and *Tg(neurod:Zebrabow)*[18]. Performing microinjections can be advantageous because it allows researchers to titrate the copy number and labeling density. Furthermore, a second DNA construct that tags or manipulates a specific gene product can be injected alongside Brainbow if desired (e.g., a far-red fluorescent protein that complements Brainbow[18]).

1.4. Maintain injected embryos in Petri dishes of E3 medium[39] in a 28 °C incubator for 24 h.

2. Heat shock to induce Brainbow expression

NOTE: If the plasmid DNA injected or the transgene expressed does not utilize the hsp70 promoter to drive the expression, the heat shock step can be skipped, and healthy embryos should be immediately transferred to phenylthiourea (PTU) at 24 h post fertilization (hpf).

2.1. At 24 hpf, cull dead and deformed embryos from the group of injected embryos. Then, transfer the healthy embryos to a 50 mL tube, with up to 20 embryos/tube.

2.2. Fill the tubes with 10 mL of E3. Place a cap on top of each tube but do not close tightly.

2.3. Place the tube rack containing the 50 mL tubes upright in a 37 °C water bath. Ensure that the water level in the bath is higher than the level of the E3 in the tubes and leave them for 80 to 90 min.

2.4. Remove the tube rack with the embryos from the water bath and return to the 28 °C incubator upright. Allow up to 1 h for the E3 to cool and the embryos to gradually re-acclimate to the temperature. Then, transfer the embryos to Petri dishes of 0.2 mM PTU in E3 warmed in the incubator to prevent pigmentation of embryos.

CAUTION: PTU is a toxic chemical that should be handled with care and disposed of appropriately. Wearing gloves is suggested.

3. Screening embryos for Brainbow expression

3.1. At 2 to 4 h after the heat shock, examine the embryos under a standard fluorescence dissection microscope for expression of CFP or YFP, which indicates successful Brainbow recombination (from default expression of dTomato).

NOTE: If multiple fluorescence filter options are available, screening for CFP is the most efficient way to identify well-recombined fish. If CFP and YFP filters are not available, YFP can also be dimly visualized under green fluorescent protein (GFP) filters due to the overlap in their spectral profiles.

3.2. Select embryos with robust FP expression throughout and transfer to a separate dish with PTU. Image embryos at 1 day post fertilization (dpf; 28 hpf or later) or maintain embryos in PTU and image at 2 or 3 dpf.

4. Mounting embryos for in vivo imaging

4.1. Prior to the day of the experiment, prepare the imaging chamber and embryo manipulation tool.

4.1.1. Prepare the imaging chamber by carefully supergluing a plastic ring to the center of a 60 mm Petri dish.

4.1.2. Optionally, prepare a custom-made embryo manipulator to move embryos within the dish and orient embryos while mounting. Construct the manipulator by supergluing a small length of nylon fishing line (~1/2 in, ~6 lb) to the end of a wooden cotton swab stick that has been cut to a ~4 in length.

4.2. If necessary (e.g., imaging earlier than 2 dpf), before mounting the embryos dechorionate using syringes under a dissection microscope.

4.3. Anesthetize zebrafish embryos.

4.3.1. Fill a 60 mm Petri dish halfway with E3 and add 5–6 drops of 4 mg/mL MS-222 Tricaine-S to a final concentration of ~0.2 mM. Swirl to mix.

CAUTION: Tricaine should be handled with care and disposed of appropriately.

4.3.2. Transfer embryos from the PTU to the tricaine solution, ensuring as little PTU is transferred as possible. While fish reflexes should cease after 1–2 min, leave the embryos in tricaine for up to 10 min to ensure thorough anesthetization for time-lapse imaging experiments.

4.3.3. Confirm that the embryos are properly anesthetized. Assess the startle reflex by touching the embryo tails gently with the embryo manipulator. If a reflex movement is observed, add an additional drop of tricaine to the dish as far from the embryos as possible and swirl to mix. Observe the embryo heart rate under a dissection scope. If it is abnormally slow, add additional drops of E3 to the dish to reduce the tricaine concentration.

4.4. Mount anesthetized embryos in agarose.

4.4.1. Transfer the fish to the center of plastic ring within the imaging chamber and remove as much excess E3 as possible using a fine-tipped transfer pipette.

4.4.2. Using a clean transfer pipette, cover the fish with 1.0% low-melt agarose (LMA) in E3 stored at 40 °C and fill the entire plastic ring with a thin layer of agarose. Use a transfer pipette to gently pull the embryos up into the pipette tip and back into the agarose without introducing air bubbles.

4.4.3. Before the agarose hardens, quickly use an embryo manipulator to orient the fish appropriately. If imaging with an upright microscope, position the embryos as close to the upper surface of the agarose as possible. Position the embryos parallel to the bottom of the imaging chamber with the tail straight.

NOTE: For hindbrain imaging, the embryos can be positioned in a dorsal or sagittal view. Care should be taken to ensure that the heads of the embryos do not sink while the agarose is still liquid. Other orientations may be appropriate to target other developing tissues for imaging. For

inverted microscopes, mounting would be done differently, typically positioning the fish close to the bottom of a glass-bottomed Petri dish.

4.5. Fill the imaging chamber with E3. Add as much E3 as possible to account for the evaporation over the course of imaging.

5. Time-lapse confocal imaging of developing zebrafish hindbrain

5.1. Place the imaging chamber on the confocal microscope in preparation for the imaging.

5.1.1. Place the imaging chamber with the fish and E3 on the confocal microscope.

5.1.2. Select an objective with a high numerical aperture and a long working distance, such as a 20x water immersion objective (1.0 NA).

5.1.3. Find a region with appropriately dense and bright labeling of the cell type(s) of interest.

NOTE: A temperature-controlled stage/apparatus on the microscope may also be used to regulate temperature and humidity during long imaging sessions. This can decrease evaporation.

5.2. Set up the acquisition parameters for Brainbow imaging.

5.2.1. Image each FP channel sequentially. If using a commercial software (e.g., Zen software), this is done by preparing three "tracks". Use an Argon laser to excite CFP at 458 nm and YFP at 514 nm. Use a DPSS 561 nm laser to excite dTomato. Collect emissions between 463–509 nm for CFP, 519–555 nm for YFP, and 566–691 nm for dTomato.

5.2.2. For on-screen display, code CFP as blue, YFP as yellow, and dTomato as red.

NOTE: These settings will vary depending upon which laser lines and other features are available on the confocal microscope. To increase the speed of imaging, CFP and dTomato channels can be imaged simultaneously without appreciable bleed-through. If a separate FP is also being imaged, such as a far-red FP, this can be imaged sequentially or simultaneously with YFP.

5.2.3. Set up the general imaging parameters to take 16-bit images with a resolution of at least 1024 x 1024 and averaging twice. Adjust the zoom depending on the region and cell type of interest (i.e., for hindbrain imaging with a 20x objective the zoom will range from 1.0–2.5). Maximize the field of view to allow for growth of the fish over time.

5.2.4. Viewing one track at a time (and turning off other lasers to prevent photobleaching), optimize the acquisition settings for each FP individually (e.g., laser power, pinhole size, photomultiplier tube gain, etc.).

5.2.5. Select Z-stack range to image.

NOTE: For long imaging sessions (>2 h), take into consideration that the fish will continue to grow throughout the course of imaging, thus both the XY field and the Z-stack range should account for this with extra room. If imaging the hindbrain, include space in the field for the tissue to move rostrally during the imaging period. Space also needs to be included for growth in the Z dimension. Include in the image approximately 10–20 µm above the region of interest, whether the fish is positioned for sagittal or dorsal view.

5.3. Set up parameters for time-lapse imaging.

5.3.1. Select a time interval. Interval length ranges between 10–30 min to track mitotic and apoptotic events in the developing hindbrain. Interval length will vary depending on the speed of the events of interest and the total time it takes to capture a single Z-stack.

5.3.2. Select the length of the imaging session. Time-lapse imaging sessions generally occur overnight and can last at least 16 h.

5.4. Run the experiment.

NOTE: The fish can be euthanized immediately following imaging or carefully dissected from the agarose using syringes and returned to E3 to recover. Recovered fish can then be imaged again at a later time point, though cell position and depth will shift during this period due to overall growth.

6. Time-lapse file management

6.1. Import the image file into the analysis software.

6.1.1. Save the file in .czi format once image acquisition is completed. Make sure to save raw data in a format compatible with Fiji[42] and/or other software.

6.1.2. Import into Fiji software using BioFormats Importer.

NOTE: Time-lapse files will be large and can require significant amounts of time and memory to open for analysis. Thus, it is helpful to be strategic in how they are saved and opened. It can be useful to save a lower-quality version that can be opened more easily to search for events of interest by eye.

6.2. If using Fiji, create smaller, more manageable subsets of time-lapse files. Generate subsets either by time (e.g., viewing full Z-stack at the first time point) or by depth (e.g., viewing the first 50 Z-sections at each time point) by clicking **Image**|**Stacks**|**Tools**|**Make Substack**.

7. Quantitative clonal color analysis of Brainbow images

7.1. Measure the mean red, green, and blue (RGB) intensity values for the cells of interest in Fiji.

NOTE: These instructions are specific to image analysis in Fiji. However, researchers may prefer to obtain mean RGB intensity values in an alternative software program.

7.1.1. Ensure that the file is correctly cropped so that there is no black space around the edges of the image. Black space will artificially lower the minimal intensity measurements needed for the analysis. If the file does need to be cropped, select the **Rectangle Selection Button** and draw a region of interest (ROI) around the field of view, excluding all black space. Click **Image**|**Crop**.

7.1.2. Set the measurement tool to take mean, minimum, and maximum gray value measurements. Click **Analyze**|**Set Measurements.** Ensure the checkboxes for **Min & Max Gray Value** and **Mean Gray Value** are both selected.

7.1.3. Viewing the raw data files or subsetted Z-stacks in Fiji, find cells of interest for clonal color analysis. In the hindbrain, putative clones can be visually identified by their shared hue as well as their radial orientation. Do not select cells that are in close contact with, and thus difficult to resolve from, neighboring cells of another color. It may be difficult to quantify their hue.

7.1.4. Find the center Z-plane of the cell of interest using the **Z-scrollbar**. Right-click the **Oval Selection Button** and select the **Elliptical Selection Button**. Other selection tools will be appropriate for different cell types. Draw an elliptical ROI around the central ~2/3 of the cell body.

7.1.5. Using the **C-scrollbar**, select the red (dTomato) channel. Take the mean intensity measurement for this channel by selecting **Analyze**|**Measure**. Repeat with the same ROI and focal plane for the green (YFP) and blue (CFP) channels and save all mean intensity values.

7.1.6. Click anywhere on the image to remove the elliptical ROI in the cell of interest.

7.1.7. To measure background levels for normalization, use the **C-scrollbar** and select the red (dTomato) channel. Take the minimum intensity measurement for the whole field in this channel by selecting **Analyze**|**Measure**. Repeat with the same focal plane for the green (YFP) and blue (CFP) channels and save all minimum intensity values.

7.2. Calculate relative RGB channel weights for the cells of interest.

NOTE: Calculation of relative RGB channel weights from these intensity values can be performed in a variety of software programs, such as Microsoft Excel or R[43].

7.2.1. Normalize the mean intensity measurement of the cell ROI for each channel by subtracting the minimal intensity from the entire field in that channel and focal plane.

7.2.2. Sum the normalized mean RGB intensity values from each channel to find the total

normalized RGB intensity for the cell.

7.2.3. Calculate the relative channel weight for each channel by dividing the normalized mean intensity value for that channel by the total normalized RGB intensity.

7.3. Display relative RGB channel weights as ternary plots using the Ternary package for R[43,44]. Ternary plots are useful to visualize the clustering of similar colors in 3D RGB space.

7.4. Calculate the color spread coefficient to quantify the difference between cell colors.

7.4.1. Calculate the Euclidean distance between the two colors in 3D RGB space using the following equation:

$$distance = \sqrt{(R_1 - R_2)^2 + (G_1 - G_2)^2 + (B_1 - B_2)^2}$$

where R, G, and B are the relative RGB channel weights calculated in 7.2.

7.4.2. For ease of comprehension, normalize the color distance by dividing by $\sqrt{2}$, the maximum possible distance between colors, to obtain a color spread coefficient between 0 and 1, where 0 indicates exactly the same color and 1 indicates completely different colors (e.g., pure red and pure blue).

REPRESENTATIVE RESULTS:

This section illustrates examples of results that can be obtained using the in vivo multicolor time-lapse imaging approach described here. We show that Brainbow color-coded clones of cells in the proliferative ventricular zone of the developing zebrafish hindbrain[14] (**Figure 1**).

Typically, when Brainbow-labeled cells were arranged along a particular radial fiber, they shared the same color (**Figure 1D**), which could be quantified as the relative RGB channel weights (**Figure 1E**). This suggests that these radial groups were clones of dividing cells and that their similar color could be used to identify them as being clonally related, as observed in previous studies in zebrafish, mouse, and chick[14,15]. When Brainbow labeling density is relatively sparse, this color-coding can be used to clearly visualize and track many distinct radial clones within proliferative regions of the living vertebrate brain.

A quantitative color analysis showed that daughter cells expressed the same color as their mother (progenitor) cell (**Figure 1F**), but that neighboring radial clusters of cells can be distinguished from one another[11] (**Figure 1E**). This means that numerous clones of related cells can be followed simultaneously over hours in vivo (**Figure 2**), allowing for a multiplex lineage analysis and comparison. Quantification of color expression in clones at 2 dpf and then again at 3 dpf (**Figure 2A-B**) showed that Brainbow expression also remained relatively constant from 2–3 days in vivo[11]. Individual cells can thus be identified and tracked in real time for relatively long periods of time during development.

Time-lapse imaging revealed numerous cells undergoing interkinetic nuclear migration and cell division during the period from 1–2 dpf (**Figure 2C–F**)[11], permitting study of the cell cycle. Using a time-lapse interval of 30 min, we did not always capture the mitotic figure during cell division. This introduced a potential error of up to 30 minutes to the assigned time of cell division. Additionally, we observed some clones appearing to contain two progenitor cells (e.g., **Figure 2F**). Within these constraints, the length of the cell cycle can be measured. By tracking individual Brainbow-labeled progenitors over time we calculated an average cell cycle of 8.4 ± 1.5 h, comparable to previous measurements in zebrafish[1,45,46]. Furthermore, using the Brainbow time-lapse imaging technique, we were also able to observe individual cells undergoing the stereotypical morphological changes associated with apoptosis (**Figure 3**)[11], such as membrane blebbing and cell fragmentation[47,48].

FIGURE AND TABLE LEGENDS:

Figure 1: Brainbow labeled clonally related clusters of dividing cells in the developing zebrafish hindbrain. (**A**) Schematic of hsp:Zebrabow (Brainbow) DNA transiently expressed to color-code clones. (**B**) In vivo transmitted light images of developing zebrafish at 1 and 2 dpf; arrows indicate the general hindbrain region targeted for imaging. Translucency of the embryos is demonstrated by a micrometer placed below each fish. Experimental timeline showing injections at one-cell stage, heat shock (HS) at 24 hpf, and imaging from 1–3 dpf. (**C**) Dorsal view of 29 hpf zebrafish hindbrain labeled with Brainbow. The transmitted light channel showed the morphology of the hindbrain and ventricle overlaid with maximum intensity projection of Brainbow-labeled clones representing 165 µm. Rostral is up. (**D**) In vivo Brainbow expression in the hindbrain, shown in maximum intensity projections representing 41 µm in sparsely labeled 51 hpf zebrafish (**D_1**), and 81 µm in 63.5 hpf zebrafish (**D_2**). In both panels, dorsal is up and rostral is to the left. (**E**) The color of cells in D_1 and D_2 was quantified as relative channel weights in corresponding ternary plots (n = 54 cells E_1; 461 cells E_2). (**F**) Cell color remained relatively constant in daughter cells following cell division. Series of time points showing in vivo mitotic events in the hindbrain of 29 hpf Brainbow-labeled zebrafish over 1 h (**F_1**, maximum intensity projections, 30 µm depth). The color of mother and daughter cells, indicated by the black boxes in F_1, is represented as relative channel weights in the ternary plot in **F_2**. The inset shows a zoom of same plot to show tightly clustered cell colors (M is mother; D_1 and D_2 are daughters at 30 min/squares, and 60 min/triangles). Scale bars represent 30 µm in C, 20 µm in D_1, 16 µm in D_2, and 5 µm in F_1. In C, D, and F, dTomato is coded as red, YFP is coded as green, and CFP is coded as blue. Images reprinted with permission from Brockway et al.[11].

Figure 2: Time-lapse in vivo imaging combined with Brainbow color-coding distinguished multiple neighboring clones of cells undergoing interkinetic nuclear migration and dividing. (**A**) Color-coded radial clusters labeled in the hindbrain of one fish expressing Brainbow at 2 dpf (55 hpf; A_1) and 3 dpf (71 hpf; A_2); maximum intensity projections representing 62 µm and 47 µm respectively. Three color-coded radial clusters are identified with arrowheads at each time point. (**B**) The color of all Brainbow-labeled cells in A_1 and A_2 was quantified as relative channel weights in the corresponding ternary plots in B_1 and B_2 (n = 106 cells, 119 cells). (**C**) Zebrafish hindbrain at 35 hpf labeled with Brainbow (maximum intensity projection showing 24 µm); inset indicated with dashed white box is the first time point displayed in D. (**D**) Series of time points taken at 30

min intervals in the hindbrain from C. A period of >9.5 h is shown. The labeled cells underwent interkinetic nuclear migration; white arrowheads indicate a cell at the apical surface. Rounding up of the cell soma at the apical surface and a corresponding increase in clone number (e.g., red cell at 5.5 h and then 8 h) was considered a mitotic event. (**E**) Dorsolateral view of zebrafish hindbrain at 30.5 hpf labeled with Brainbow, where the white dashed-and-dotted line shows the approximate boundary of the head, and the white dashed box indicates the inset shown in the first time point of F. (**F**) Series of time points taken at 30 min intervals in the hindbrain from E. Over a period of 1.5 h, two blue cells indicated by the white arrowheads could be observed moving to the apical surface and undergoing mitosis, suggesting a clone containing two progenitor cells. Scale bar represents 20 µm in A, 25 µm in C, and 20 µm in F. In A–D, dorsal is up and rostral is to the left. In E and F, rostral is to the left. In all images, dTomato is coded as red, YFP is coded as green, and CFP is coded as blue. Images reprinted with permission from Brockway et al.[11].

Figure 3: Cells in the ventricular zone undergo cell death in vivo. (**A**) Zebrafish hindbrain at 31 hpf labeled with Brainbow (maximum intensity projection representing 36 µm). White dashed box indicates inset shown in the first time point in B. (**B**) Series of time points showing hindbrain in A at 30 min intervals. White arrowhead in the first panel shows a lavender cell that underwent apoptosis as shown in subsequent panels, indicated by cell fragmentation followed by gradual clearance of apoptotic bodies. Neighboring labeled cells appeared healthy throughout. Dorsal is up and rostral is to the left. Scale bar represents 20 µm in B. In all images, dTomato is coded as red, YFP is coded as green, and CFP is coded as blue. Images reprinted with permission from Brockway et al.[11].

DISCUSSION:

This protocol describes a method to visualize clones of progenitor cells and neurons in the developing zebrafish hindbrain and follow them in vivo using Brainbow and time-lapse confocal microscopy[11]. The major advantage of this protocol in comparison to in vitro or ex vivo studies is the ability to directly observe the proliferative zone of the vertebrate brain in its natural milieu over time. This technique builds on previous studies, which labeled a single clone using retroviral vectors. In contrast, the use of the *hsp*:Zebrabow construct color-codes many clones simultaneously, allowing multiplex lineage tracing and a focus on dynamics among clones[11]. This protocol can be modified to focus on development in other systems or cell types. For example, different Brainbow constructs can be expressed in zebrafish embryos. Use of the zebrafish hsp70 promoter in *hsp*:Zebrabow will result in mosaic labeling of a variety of cell types following heat shock, including neural progenitors and neurons[11], and gives researchers temporal control over gene expression[29]. It should be noted that use of the heat shock promoter consistently labels color-coded clones, while other promoters may not clearly delineate cell lineage in this manner. When clonal identity is not a factor of interest, constructs utilizing other promoters may be used to label specific cell types. For example, the neuroD promoter can be used to label immature neurons[18,49]. Additionally, instead of performing microinjections, researchers may choose to utilize transgenic Brainbow zebrafish, including lines such as *Tg(ubi:Zebrabow)*[15] and *Tg(neurod:Zebrabow)*[18]. In this case, crosses to lines expressing Cre recombinase, such as *Tg(hsp:Cre^{a134})*[15], produce uninjected embryos that are collected and maintained in a similar

manner. While this protocol is designed to image fish between 1–3 dpf, in order to coincide with the major period of developmental neurogenesis[50], zebrafish may be maintained for longer depending on the developmental stage of interest. However, heat shock to induce Brainbow expression prior to 24 hpf may be more detrimental to embryos[51]. Depending upon the age and tissue of interest, different mounting strategies will be appropriate to have the targeted tissue oriented correctly.

There are a number of steps that may require troubleshooting, in particular if modifications are made to the protocol. The concentration of DNA, bolus size injected, and total amount of DNA delivered per embryo may all be adjusted if Brainbow expression is dim or sparse or if embryos do not appear healthy. Additionally, the length and timing of the heat shock step may be modified if the desired expression is not achieved. The acquisition parameters used in imaging will vary depending on the laser lines and filters available as well as the brightness of expression, mounting, and type of analysis desired. Additionally, the time-lapse parameters must be adjusted depending upon the speed of the events of interest and the time need to acquire a single Z-stack. One of the most critical steps in the protocol is the appropriate mounting of the fish in agarose: if the angle of the fish is inappropriate or if the fish is covered by too much agarose, optimal imaging will not be possible. Optimizing this technique may take some practice. Additionally, it is often useful to mount multiple fish prior to imaging as it can be difficult to ascertain whether mounting is appropriate before viewing FP expression on the confocal microscope itself. An additional critical step is the screening and selection of the specific fish to be imaged. If performing microinjections, brightness, labeling density, and Brainbow copy number can all vary significantly among fish. Images taken in fish with dim labeling, overly dense labeling, or a low copy number resulting in few colors may be more difficult to analyze.

With this protocol, we have been able to continuously image living zebrafish up to 16 h[11]. This length of time can be limited by E3 evaporation from the imaging chamber, growth of the fish out of the plane of imaging, death or movement of the fish, or user restraints on confocal microscope time. Evaporation could be decreased by the use of a temperature-controlled accessory on the microscope stage. It should be noted that the 5D datasets generated using this approach tend to be large files that require significant hard drive space (e.g., up to ~100 GB for one overnight time-lapse) and adequate computing power to analyze.

This protocol guides researchers to visualize color-coded clones within a population of neural progenitors and daughter neurons in the developing zebrafish brain and track their dynamics over time. One possible expansion is the combination of Brainbow time-lapse imaging with far-red FP tagging to assess the roles of specific genes in development[18]. In this way, clones expressing manipulated genes can be visualized in a distinct color, tracked over time, and compared to Brainbow-labeled, non-manipulated neighboring clones. In vivo multicolor imaging can be used to address important mechanistic questions about how the nervous system forms and functions.

ACKNOWLEDGMENTS:

We thank Y. A. Pan, J. Livet and Z. Tobias for technical and intellectual contributions. This work

was supported by the National Science Foundation (Award 1553764) and the M.J. Murdock Charitable Trust.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Lyons, D. A., Guy, A. T., Clarke, J. D. W. Monitoring neural progenitor fate through multiple rounds of division in an intact vertebrate brain. *Development*. **130**, 3427–36 (2003).
2. Noctor, S. C., Flint, A. C., Weissman, T. A., Dammerman, R. S., Kriegstein, A. R. Neurons derived from radial glial cells establish radial units in neocortex. *Nature*. **409** (6821), 714–720 (2001).
3. Kriegstein, A., Alvarez-Buylla, A. The glial nature of embryonic and adult neural stem cells. *Annual Review of Neuroscience*. **32**, 149–184 (2009).
4. Florio, M., Huttner, W. B. Neural progenitors, neurogenesis and the evolution of the neocortex. *Development*. **141**, 2182–2194 (2014).
5. Mcconnell, S. K. Constructing the Cerebral Cortex: Neurogenesis and Fate Determination Review. *Neuron*. **15**, 761–768 (1995).
6. Schmidt, R., Strähle, U., Scholpp, S. Neurogenesis in zebrafish – from embryo to adult. *Neural Development*. **8**, 3 (2013).
7. Chenn, A., Walsh, C. Regulation of Cerebral Cortical Size by Control of Cell Cycle Exit in Neural Precursors. *Science*. **297** (5580), 365–369 (2002).
8. Lui, J. H., Hansen, D. V., Kriegstein, A. R. Development and Evolution of the Human Neocortex. *Cell*. **146** (1), 18–36 (2011).
9. Nonaka-Kinoshita, M. et al. Regulation of cerebral cortex size and folding by expansion of basal progenitors. *The EMBO Journal*. **32**, 1817–1828 (2013).
10. Homem, C. C. F., Repic, M., Knoblich, J. A. Proliferation control in neural stem and progenitor cells. *Nature Reviews Neuroscience*. **16** (11), 647–659 (2015).
11. Brockway, N. L. et al. Multicolor lineage tracing using in vivo time-lapse imaging reveals coordinated death of clonally related cells in the developing vertebrate brain. *Developmental Biology*. **453** (2), 130–140 (2019).
12. Livet, J. et al. Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. *Nature*. **450** (7166), 56–62 (2007).
13. Weissman, T. A., Pan, Y. A. Brainbow: New resources and emerging biological applications for multicolor genetic labeling and analysis. *Genetics*. **199** (2), 293–306 (2015).
14. Loulier, K. et al. Multiplex Cell and Lineage Tracking with Combinatorial Labels. *Neuron*. **81** (3), 505–520 (2014).
15. Pan, Y. A. et al. Zebrabow: multispectral cell labeling for cell tracing and lineage analysis in zebrafish. *Development*. **140**, 2835–2846 (2013).
16. Ko, S. K., Chen, X., Yoon, J., Shin, I. Zebrafish as a good vertebrate model for molecular imaging using fluorescent probes. *Chemical Society Reviews*. **40**, 2120 (2011).
17. Kesavan, G., Hammer, J., Hans, S., Brand, M. Targeted knock-in of CreERT2 in zebrafish using CRISPR/Cas9. *Cell and Tissue Research*. **372**, 41–50 (2018).
18. Cook, Z. T. et al. Combining near-infrared fluorescence with Brainbow to visualize

expression of specific genes within a multicolor context. *Molecular Biology of the Cell*. **30** (4), 491–505 (2019).
19. Kuwata, M., Nikaido, M., Hatta, K. Local heat-shock mediated multi-color labeling visualizing behaviors of enteric neural crest cells associated with division and neurogenesis in zebrafish gut. *Developmental Dynamics*. **248**, 437–448 (2019).
20. Kinkhabwala, A. et al. A structural and functional ground plan for neurons in the hindbrain of zebrafish. *Proceedings of the National Academy of Sciences*. **108** (3), 1164–1169 (2011).
21. Heap, L. A., Goh, C. C., Kassahn, K. S., Scott, E. K. Cerebellar Output in Zebrafish: An Analysis of Spatial Patterns and Topography in Eurydendroid Cell Projections. *Frontiers in Neural Circuits*. **7**, 53 (2013).
22. Robles, E., Filosa, A., Baier, H. Precise Lamination of Retinal Axons Generates Multiple Parallel Input Pathways in the Tectum. *The Journal of Neuroscience*. **33** (11), 5027–5039 (2013).
23. Dirian, L. et al. Spatial Regionalization and Heterochrony in the Formation of Adult Pallial Neural Stem Cells. *Developmental Cell*. **30**, 123–136 (2014).
24. Chen, X. A. et al. Behavioral/Cognitive QRFP and Its Receptors Regulate Locomotor Activity and Sleep in Zebrafish. *The Journal of Neuroscience*. **36** (6), 1823–1840 (2016).
25. Albadri, S., De Santis, F., Di Donato, V., Del Bene, F. CRISPR/Cas9-Mediated Knockin and Knockout in Zebrafish. In *Genome Editing in Neurosciences*. Edited by Jaenisch, R., Zhang F., Gage, F., 41–49, Springer. Cham, Switzerland (2017).
26. Furlan, G. et al. Life-Long Neurogenic Activity of Individual Neural Stem Cells and Continuous Growth Establish an Outside-In Architecture in the Teleost Pallium. *Current Biology*. **27**, 3288–3301 (2017).
27. Herget, U., Arturo Gutierrez-Triana, J., Salazar Thula, O., Knerr, B., Ryu, S. Single-cell reconstruction of oxytocinergic neurons reveals separate hypophysiotropic and encephalotropic subtypes in larval zebrafish Brainbow-guided morphology of oxytocinergic cells. *eNeuro*. **4** (1), (2017).
28. Halloran, M. C. et al. Laser-targeted gene expression. *Development*. **127**, 1953–1960 (2000).
29. Shoji, W., Sato-Maeda, M. Application of heat shock promoter in transgenic zebrafish. *Development, Growth & Differentiation*. **50**, 401–406 (2008).
30. Luskin, M. B., Pearlman, A. L., Sanes, J. R. Cell lineage in the cerebral cortex of the mouse studied in vivo and in vitro with a Recombinant Retrovirus. *Neuron*. **1** (8), 635–647 (1988).
31. Price, J., Thurlow, L. Cell lineage in the rat cerebral cortex: a study using retroviral-mediated gene transfer. *Development*. **104**, 473–482 (1988).
32. Walsh, C., Cepko, C. L. Widespread dispersion of neuronal clones across functional regions of the cerebral cortex. *Science*. **255** (5043), 434–441 (1992).
33. Walsh, C., Cepko, C. L. Clonal dispersion in proliferative layers of developing cerebral cortex. *Nature*. **362** (6421), 632–635 (1993).
34. Cai, L., Hayes, N. L., Nowakowski, R. S. Synchrony of Clonal Cell Proliferation and Contiguity of Clonally Related Cells: Production of Mosaicism in the Ventricular Zone of Developing Mouse Neocortex. *The Journal of Neuroscience*. **17** (6), 2088–2100 (1997).
35. Reznikov, K., Acklin, S. E., Van Der Kooy, D. Clonal Heterogeneity in the Early Embryonic Rodent Cortical Germinal Zone and the Separation of Subventricular From Ventricular Zone Lineages. *Developmental Dynamics*. **210**, 328–343 (1997).

36. Qian, X., Goderie, S., Shen, Q., Stern, J., Temple, S. Intrinsic programs of patterned cell lineages in isolated vertebrate CNS ventricular zone cells. *Development*. **125**, 3143–3152 (1998).
37. McCarthy, M., Turnbull, D. H., Walsh, C. A., Fishell, G. Telencephalic Neural Progenitors Appear to Be Restricted to Regional and Glial Fates before the Onset of Neurogenesis. *The Journal of Neuroscience*. **21** (17), 6772–6781 (2001).
38. Yu, Y. C., Bultje, R. S., Wang, X., Shi, S. H. Specific synapses develop preferentially among sister excitatory neurons in the neocortex. *Nature*. **458** (7237), 501–504 (2009).
39. Nusslein-Volhard, C., Dahm, R. *Zebrafish*. Oxford University Press. Oxford, UK (2002).
40. Nasiadka, A., Clark, M. D. Zebrafish Breeding in the Laboratory Environment. *ILAR Journal*. **53** (2), 161–168 (2012).
41. Rosen, J. N., Sweeney, M. F., Mably, J. D. Microinjection of Zebrafish Embryos to Analyze Gene Function. *Journal of Visualized Experiments*. **25**, e1115 (2009).
42. Schindelin, J., et al. Fiji: An open-source platform for biological-image analysis. *Nature Methods*. **9** (7), 676-682 (2012)
43. R Core Team. R: A language and environment for statistical computing. (2017).
44. Smith, M. R. Ternary: an R package to generate ternary plots. (2017).
45. Baye, L. M., Link, B. A. Development/Plasticity/Repair Interkinetic Nuclear Migration and the Selection of Neurogenic Cell Divisions during Vertebrate Retinogenesis. *The Journal of Neuroscience*. **27** (38), 10143–10152 (2007).
46. Leung, L., Klopper, A. V, Grill, S. W., Harris, W. A., Norden, C. Apical migration of nuclei during G2 is a prerequisite for all nuclear motion in zebrafish neuroepithelia. *Development*. **139**, 2635 (2012).
47. Kerr, J. F. R., Wyllie, A. H., Curriet, A. R. Apoptosis: A Basic Biological Phenomenon with Wide-ranging Implications in Tissue Kinetics. *British Journal of Cancer*. **26**, 239 (1972).
48. Cole, L. K., Ross, L. S. Apoptosis in the Developing Zebrafish Embryo. *Developmental Biology*. **240**, 123–142 (2001).
49. Liao, J., He, J., Yan, T., Korzh, V., Gong, Z. A Class of NeuroD-Related Basic Helix-Loop-Helix Transcription Factors Expressed in Developing Central Nervous System in Zebrafish. *DNA and Cell Biology*. **18** (4), 333–344 (1999).
50. Trevarrow, B., Marks, D. L., Kimmel, C. B. Organization of hindbrain segments in the zebrafish embryo. *Neuron*. **4** (5), 669–679 (1990).
51. Yabu, T., Todoriki, S., Yamashita, M. Stress-induced apoptosis by heat shock, UV and gamma-ray irradiation in zebrafish embryos detected by increased caspase activity and whole-mount TUNEL staining. *Fisheries Science*. **67**, 333–340 (2001).

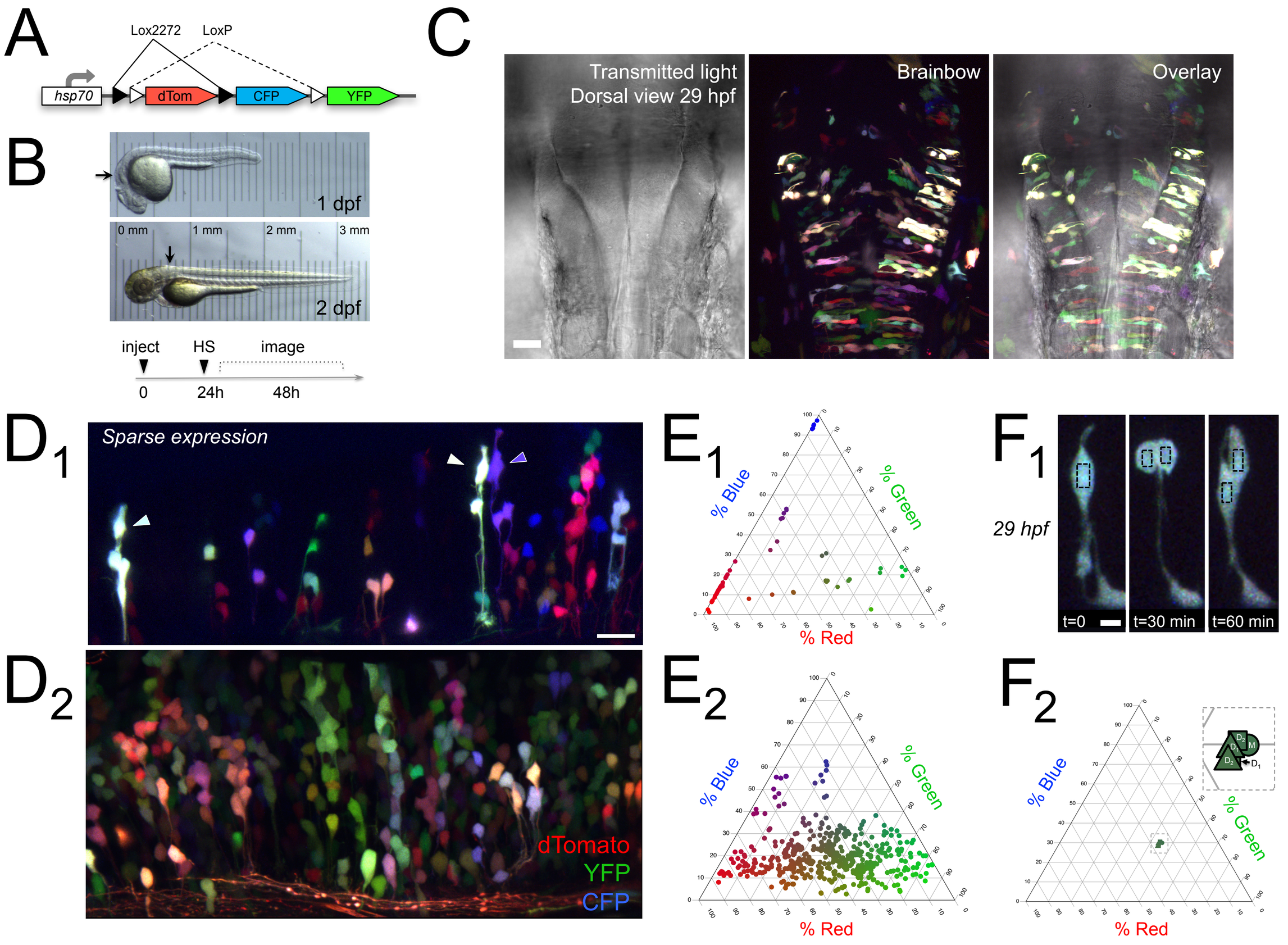
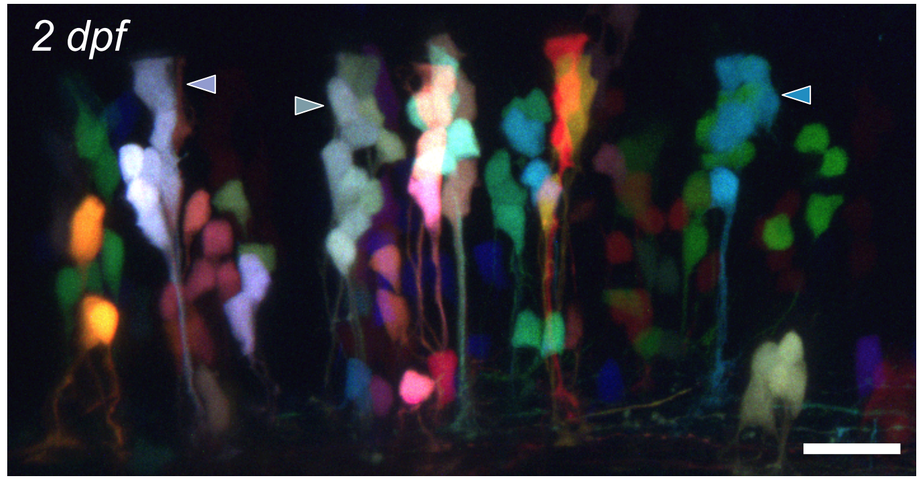
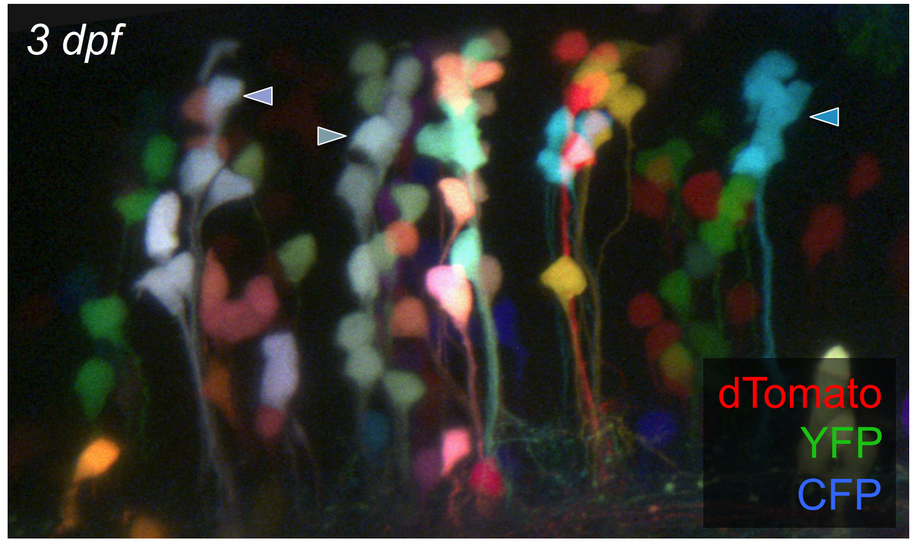
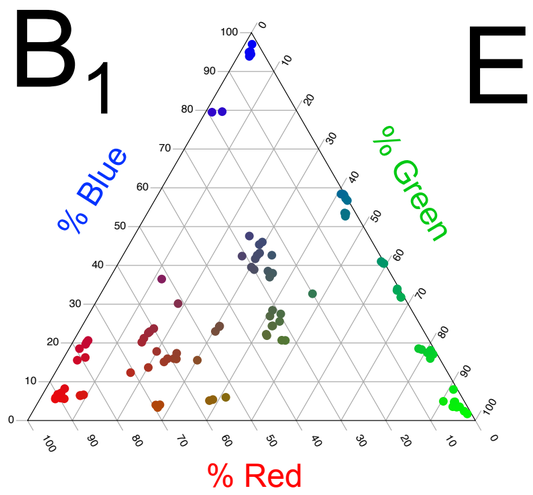
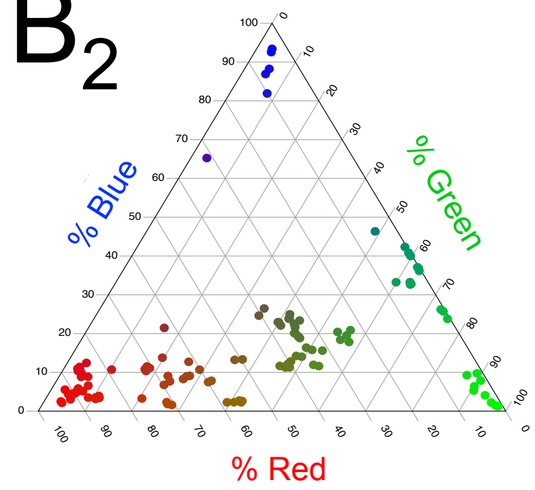


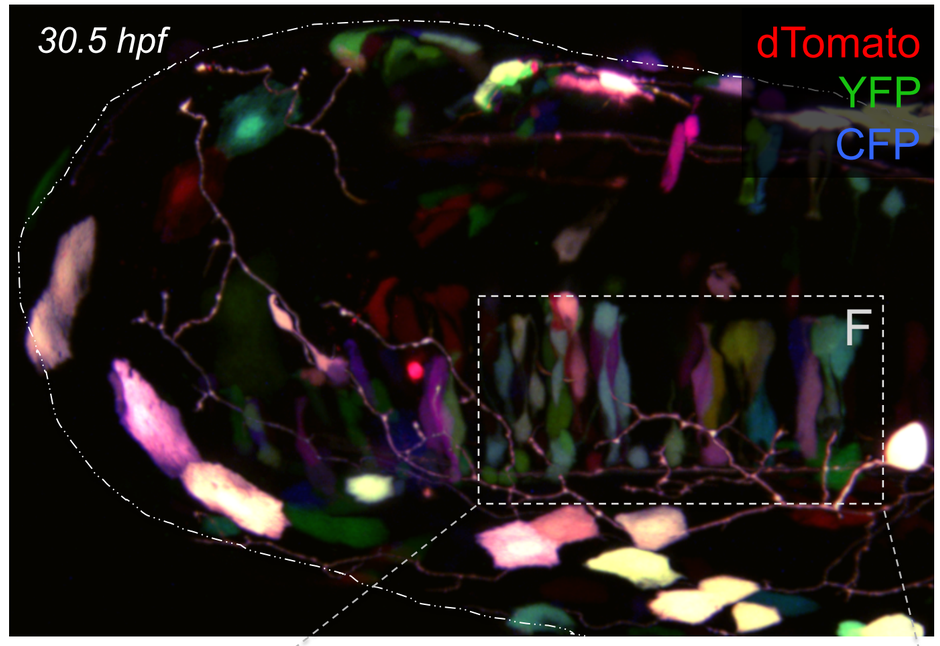
Figure 2

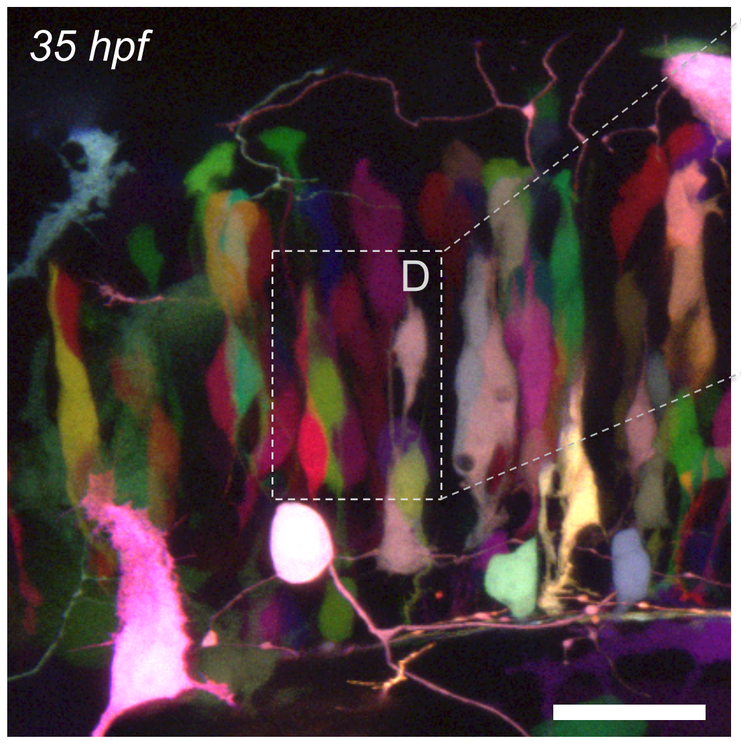


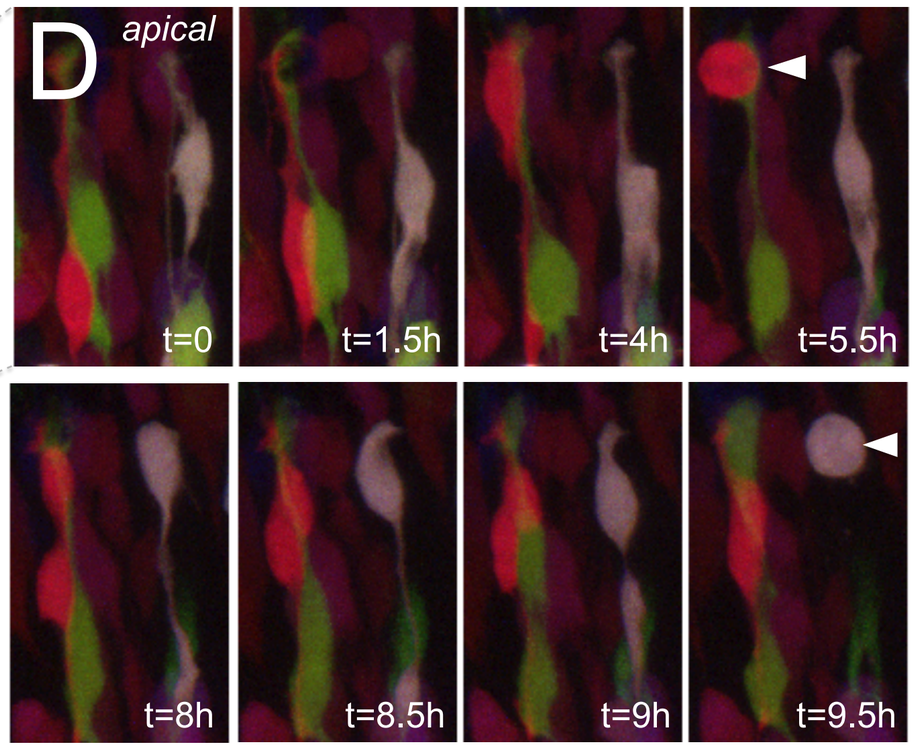












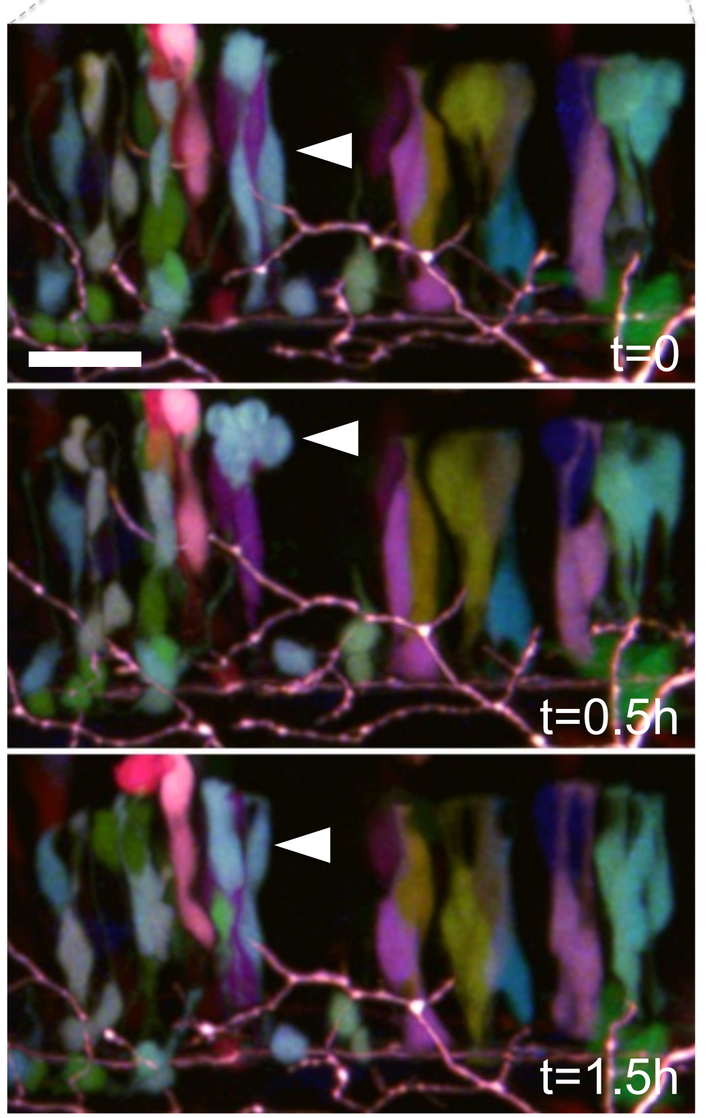
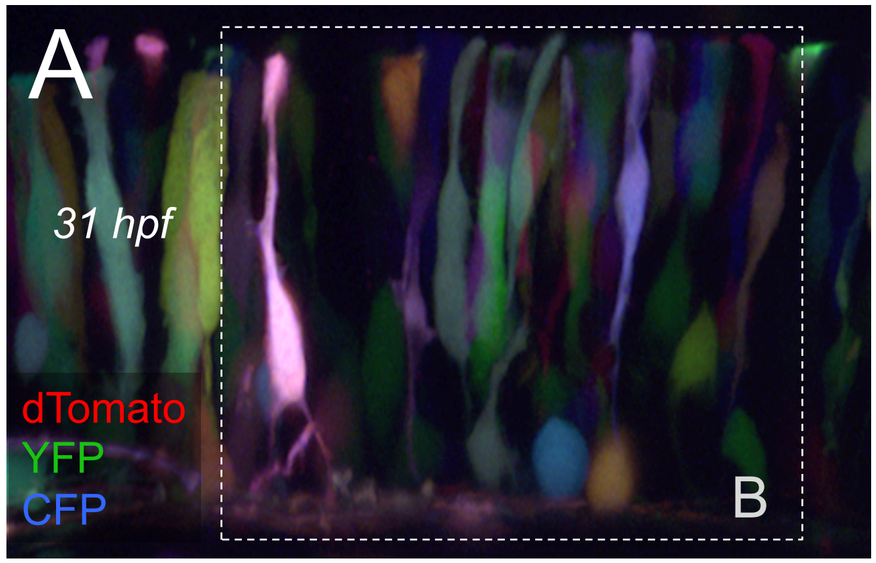
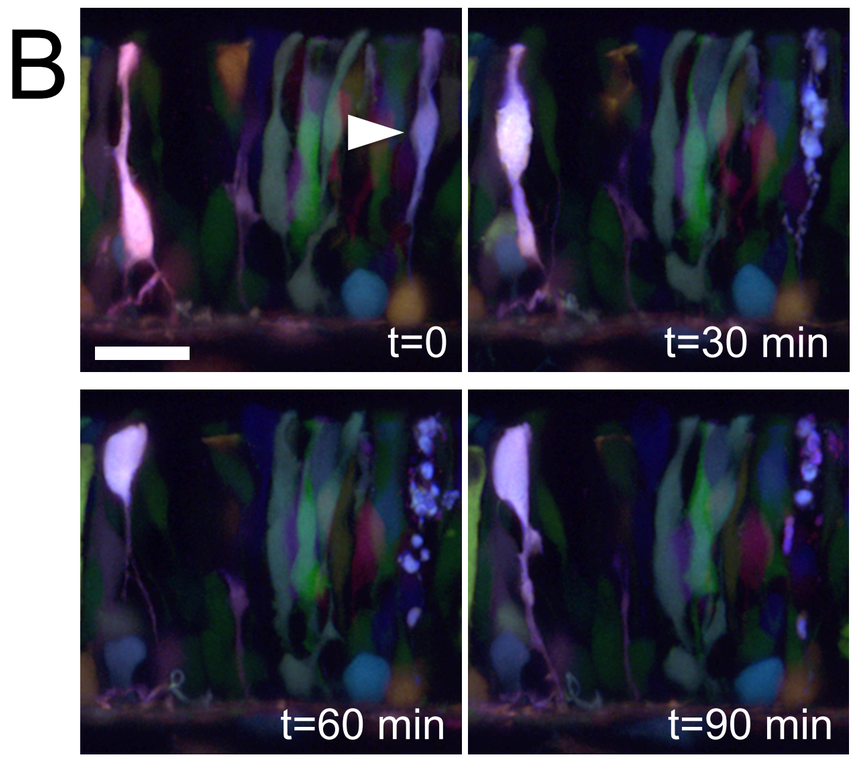


Figure 3





Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5mL transfer pipet	Globe Scientific, Inc.	134020	
1-phenyl-2-thiourea (PTU)	Alfa Aesar	L06690	Diluted to 0.2 mM in E3 to prevent embryo pigmentation
50ml conical tubes	Corning	352070	For heat shocking embryos
6 lb nylon fishing line	SecureLine	NMT250	For making embryo manipulators
7.5mL transfer pipet	Globe Scientific, Inc.	135010	
CaCl2	Sigma	C3881	For E3
Cotton swabs	Puritan	867-WC NO GLUE	For making embryo manipulators
Cre recombinase	New England Biolabs	M0298M	
Digital dry bath	Genemate	490016-616	Used to store LMA at 40°C
Epifluorescence dissection scope			
Glass capillary tubes	World Precision Instruments	TW100F-4	
Incubator	Forma Scientific	3158	To maintain embryos at 28°C
Injection plate molds	Adaptive Science Tools	TU-1	
Isotemp water bath	Fisher Scientific	2320	For heat shocking embryos
KCl	AMRESCO	0395	For E3 and for DNA solution for injections
Laser-scanning confocal microscope	Zeiss	LSM710	
LE agarose	Genemate	E3120	To create agarose injection plates
Low-melt agarose (LMA)	AMRESCO	J234	
Mating tanks	Aquaneering, Inc.	ZHCT100	
Methylene blue	Sigma	M9140	For E3
MgSO4	Sigma	9397	For E3
Micromanipulator	World Precision Instruments	M3301	
Micropipette Puller	Sutter Instrument Co.	P-97	
MS-222 Tricaine-S	Western Chemical, Inc.		Stock made at 4 mg/mL in reverse osmosis (RO) water, then added dropwise to E3 to final concentration of 0.2 mM to anesthetize embryos

NaCl	J.T. Baker	4058-01	For E3
Petri dishes (90 mm, 60 mm)	Genesee Scientific	32-107G	To house embryos and create imaging chamber (60 mm)
Phenol red	Sigma	P0290	
Soft stitch ring markers	Clover Needlecraft, Inc.	354	For creating imaging chamber with Petri dish
Super glue (Ultra gel control)	Loctite	1363589	For making embryo manipulators
Syringe needles	Beckton Dickinson	BD329412	For dechorionating embryos

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Brainbow & time-lapse confocal imaging…

Author(s): Cook, Brockway, & Weissman

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Tamily Weissman
Department:	Biology Dept.
Institution:	Lewis & Clark College, Portland, OR
Title:	Associate Professor of Biology
Signature:	
Date:	7/22/19

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Reviewer response letter, Cook et al.

We are grateful to the reviewers for the time they have taken in reviewing our work, and for their thoughtful summaries and evaluation. Below we respond to each of their requests point-by-point. Our revised version of the manuscript includes track changes that display all edits that have been made since the initial submission.

Reviewer #1 comments & responses:

Minor Concerns:

1) It is unclear to me whether the yellow highlighting in the submitted manuscript was intended, or was left over from an earlier draft.

We wish to thank this reviewer for the careful eye they used in going through our manuscript, and for their suggestions that have helped us to make key improvements.

The yellow highlighting is requested by JoVE to indicate the sections of the protocol that will be filmed.

2) In point 1.1, I think it would be useful to either say more about setting up fish, or at least provide a reference, so novices know where to find additional information.

We have added references.

3) In point 1.3 need to be clear that the "DNA solution" is the one from point 1.2 Calling it the "DNA solution" in 1.2, or calling it the "injection mix" in 1.3 would clarify this point.

We have clarified this ambiguity by using the reviewer's suggestion to call it "DNA solution" in both spots.

4) In 1.4 it might be helpful to say how long the embryos are maintained in E3.

Done.

5) In point 2.1, does the "group" refer to the embryos from 1.4?

Yes. We have added a clarification in the text for 2.1.

6) In point 2.3 it would be useful to reiterate the incubator temperature. Also, the word embryos is missing after the word transfer.

Thank you for this catch. Temperature has been added and misspelling corrected – in what is now numbered 2.4.

7) The Note at the bottom of section 2 is the first time the hsp70 promoter is mentioned. This should be brought up earlier. Also, it isn't clear what immediately refers to. After what step?

We have now added text about the heat shock promoter in the Introduction, and we have also moved this Note to the beginning of section 2.

8) In point 3.1, it isn't clear whether embryos can be examined two to four hours after the beginning of the heat shock, or after the end of the heat shock. It might also be useful to mention magnification here, although it is covered in more detail below.

We have clarified point 3.1 to indicate that imaging can begin 2-4 hours after the completion of the heat shock step.

9) What type of fishing line?

We have added more information about the fishing line to clarify this point. The specific brand information is listed in the table of materials.

10) I didn't understand what line refers to in 5.2.1

We have removed this detail for simplicity. Line referred to the type of sequential imaging that can be done.

11) The legend to Figure 1 needs to be proof read. For example, there are no B1, B2 or H in the figure.

Thank you - we have corrected the typos in the figure legend.

Also, the Ds and M in the inset are very difficult to see. The inset should be blown up and cropped so the cell representations are larger.

We have enlarged the inset as suggested.

12) I felt that some of the information in the Discussion should be redistributed to the Introduction and the Protocol. For example, the different brainbow constructs could be brought up much earlier. I also wonder whether it wouldn't be more useful to put some of the troubleshooting into the protocol at the appropriate step, rather than saving it all up for the end.

We have addressed the first suggestion by listing the different Brainbow constructs earlier in the manuscript. We have left several troubleshooting points in the Discussion, however, as guided by the JoVE protocol.

13) There is a table called Comments/Description at the end. It was not at all clear what this is for.

This was a hanging column from the required materials table, and has been removed.

Reviewer #2 comments & responses:

Major Concerns:

1) There is very little explanation about the post-imaging stages of time-lapse analysis. For example, the authors present quantification of relative channel weight in ternary plots but do not explain their value and how to generate them, but simply refer to the software. More information should be provided on analysis tools for the unique properties of Brainbow imaging.

We wish to thank this reviewer for their time in reviewing our manuscript carefully, and for their extremely useful suggestions that have helped us to improve the manuscript.

We have added a new section (Section 7) that describes our quantitative analysis of Brainbow images.

2) When it comes to long time lapse imaging, it is common to use a heated stage so that a fixed pre-determined temperature is maintained and in very long time lapse imaging even an apparatus that allows circulation of embryo medium. The authors should at least mention these considerations and if they think these are unnecessary explain why.

Since the room temperature of our imaging room (~75F) is within about one degree of 28C we do not use a heated stage and we have found that it is not necessary for fish survival. Some users may wish to use a heated stage to control temperature and avoid evaporation. We have now included a note about this in the manuscript.

Minor Concerns:

1) Please specify which kind of glue is used for preparing the imaging chamber (line 174).

We have added this information. The specific brand and catalog number are listed in the table of materials.

2) Figure legend 1 refers to B1 and B2 but in the figure only B is marked. Also, there are no G or H panels in the image but these are referred to in the legend text (lines 346, 347).

Thank you for this catch. The figure legend has been corrected.

ELSEVIER LICENSE
TERMS AND CONDITIONS

Nov 08, 2019

This Agreement between Tamily Weissman ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4704250542251
License date	Nov 08, 2019
Licensed Content Publisher	Elsevier
Licensed Content Publication	Developmental Biology
Licensed Content Title	Multicolor lineage tracing using in vivo time-lapse imaging reveals coordinated death of clonally related cells in the developing vertebrate brain
Licensed Content Author	Nicole L. Brockway,Zoe T. Cook,Maritte J. O'Gallagher,Zachary J.C. Tobias,Mako Gedi,Kristine M. Carey,Vivek K. Unni,Y. Albert Pan,Margaret R. Metz,Tamily A. Weissman

Licensed Content Date	Sep 15, 2019
Licensed Content Volume	453
Licensed Content Issue	2
Licensed Content Pages	11
Start Page	130
End Page	140
Type of Use	reuse in a journal/magazine
Requestor type	academic/educational institute
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	3
Format	electronic
Are you the author of this Elsevier article?	Yes

Will you be translating?	No
Title of new article	Using Brainbow and time-lapse confocal imaging to visualize the developing brain in living zebrafish
Lead author	Cook
Title of targeted journal	Journal of Visualized Experiments
Publisher	JoVE
Expected publication date	Mar 2020
Portions	Portions of figures 1, 2, and 3
Requestor Location	Tamily Weissman Lewis & Clark College - Biology Dept. 0615 SW Palatine Hill Road MSC-53 PORTLAND, OR 97219 United States Attn: Tamily Weissman
Publisher Tax ID	98-0397604
Total	0.00 USD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.

6. If the permission fee for the requested use of our material is waived in this instance,

please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment

terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
    - via their non-commercial person homepage or blog
    - by updating a preprint in arXiv or RePEc with the accepted manuscript
    - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
    - directly by providing copies to their students or to research collaborators for their personal use
    - for private scholarly sharing as part of an invitation-only work group on

commercial sites with which Elsevier has an agreement

- After the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

Terms & Conditions applicable to all Open Access articles published with Elsevier:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.

CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.

CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

v1.9

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.