Video Article

Visualizing the Developing Brain in Living Zebrafish using Brainbow and Time-lapse Confocal Imaging

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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.

Video Link

The video component of this article can be found at https://www.jove.com/video/60593/

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[1,2,3,4,5,6]. 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[7,8,9,10]. 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[11,15,17,18,19,20,21,22,23,24,25,26,27]. The ability to directly image into the living 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[2,30,31,32,33,34,35,36,37,38]. 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. Set up wild type, adult zebrafish in sex-segregated mating tanks the afternoon prior to performing microinjections[39,40].
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.
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]).
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).

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

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.
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

1. **Prior to the day of the experiment, prepare the imaging chamber and embryo manipulation tool.**
   1. Prepare the imaging chamber by carefully supergluing a plastic ring to the center of a 60 mm Petri dish.
   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.
2. If necessary (i.e., imaging at 2 dpf or earlier), before mounting the embryos dechorionate using syringes under a dissection microscope.
3. **Anesthetize zebrafish embryos.**

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.
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.
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. **Mount anesthetized embryos in agarose.**
    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.
    2. Using a clean transfer pipette, cover the fish with 1.0% low-melt agarose (LMA) in E3 stored at 42 °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.
    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.

5. Wait for the agarose to harden, then 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

1. **Place the imaging chamber on the confocal microscope in preparation for the imaging.**
    1. Place the imaging chamber with the fish and E3 on the confocal microscope.
    2. Select an objective with a high numerical aperture and a long working distance, such as a 20x water immersion objective (1.0 NA).
    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.

2. **Set up the acquisition parameters for Brainbow imaging.**
    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.
    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.
    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.
    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. 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.

3. **Set up parameters for time-lapse imaging.**
    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.
    2. Select the length of the imaging session. Time-lapse imaging sessions generally occur overnight and can last at least 16 h.

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

1. **Import the image file into the analysis software.**

1. If using Zen software, 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.
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.

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

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

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].
   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.
   2. Sum the normalized mean RGB intensity values from each channel to find the total normalized RGB intensity for the cell.
   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.

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.
4. **Calculate the color spread coefficient to quantify the difference between cell colors.**
   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.
   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 min 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].
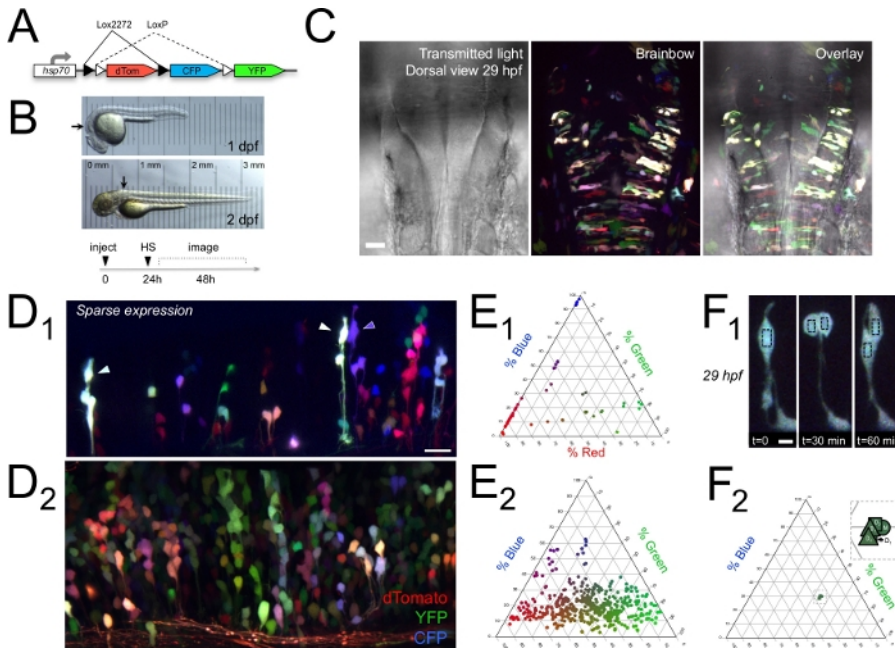

Figure 1: Brainbow labeled clonally related clusters of dividing cells in the developing zebrafish hindbrain. (**A**) Schematic of hsp:Zebrabow (Brainbow) DNA transiently expressed to color-code clones. (**B**) In vivo transmitted light images of developing zebrafish at 1 and 2 dpf; arrows indicate the 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 = 30 μm (C), 20 μm (D_1), 16 μm (D_2), and 5 μm (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]. Please click here to view a larger version of this figure.
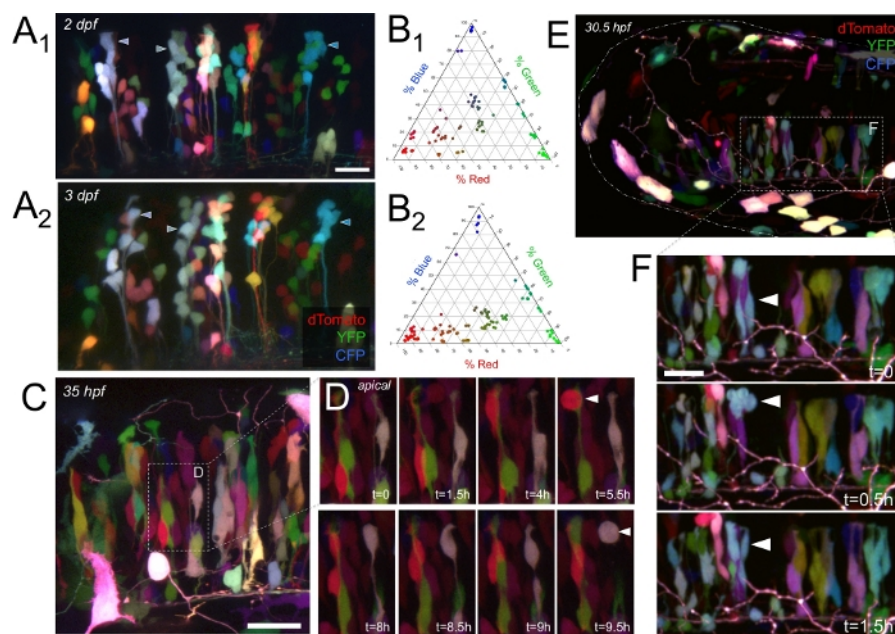
Figure 2: Time-lapse in vivo imaging combined with Brainbow color-coding distinguished multiple neighboring clones of cells undergoing interkinetic nuclear migration and dividing. (**A**) Color-coded radial clusters labeled in the hindbrain of one fish expressing Brainbow at 2 dpf (55 hpf; A$_1$) and 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 = 20 μm (A), 25 μm (C), and 20 μm (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]. Please click here to view a larger version of this figure.
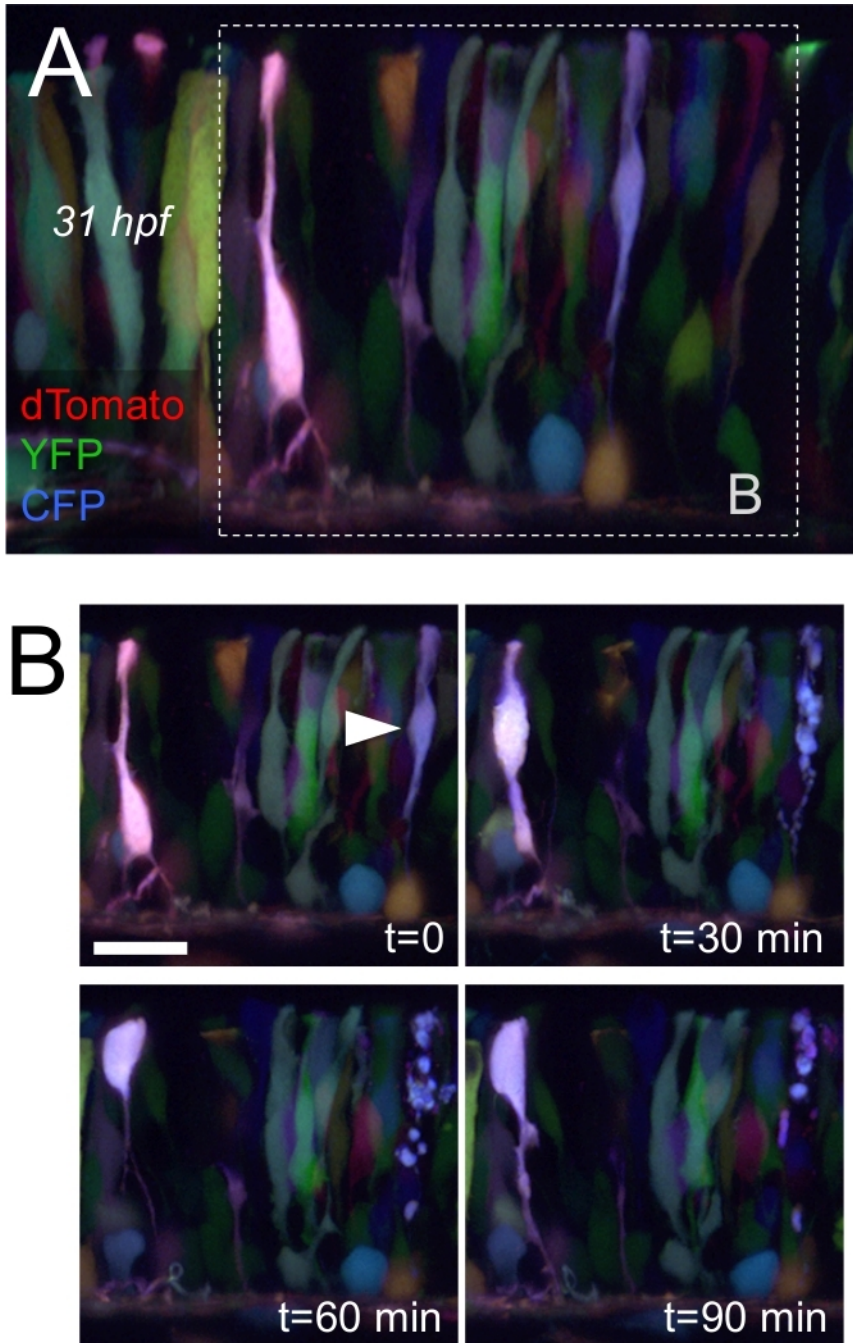
Figure 3: Time-lapse Brainbow imaging shows cells in the ventricular zone undergo cell death in vivo. (**A**) Zebrafish hindbrain at 31 hpf labeled with Brainbow (maximum intensity projection representing 36 μm). White dashed box indicates inset shown in the first time point in B. (**B**) Series of time 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 = 20 μm (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]. Please click here to view a larger version of this figure.

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 that 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.

Disclosures

The authors have nothing to disclose.

Acknowledgments

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).

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