

ABSTRACT

In this chapter we describe how to use photoconvertible fluorescent proteins for fate mapping, and fluorescent protein fusions to study intracellular processes. For photoactivation we will use a modified protocol originally described by Hatta et al. 2006 Nat Protoc. 2006;1(2):960-7.

INTRODUCTION

Fluorescent proteins (FP) are a powerful tool to study a protein or process of interest. GFP was the first transgenic fluorescent protein expressed in an organism and fused to a protein-of-interest. A battery of other FPs have since been discovered and engineered, including proteins with different spectra, proteins with spectra that are altered by intracellular conditions, and proteins whose spectra can be altered by exposure to high energy light. These fluorescent proteins make it possible to monitor multiple proteins simultaneously, to monitor real time changes within intracellular environments, and to monitor cell fate and organelle turnover. Because zebrafish larvae are optically transparent, fluorescent proteins are particularly useful in the study of zebrafish development. Development involves cells getting to where they need to go, and differentiating to carry out cell-type specific processes. With this in mind, here we explore (1) using photoactivatable fluorescent protein fusions to study the fate of a cell-type of interest, and (2) using fluorescent protein fusions to study intracellular processes in a cell-type of interest.

(1) Using Photoactivatable Proteins

Fluorescent proteins, like fluorescent dyes, absorb light of a defined wavelength (the excitation spectra) and emit lower energy light (the emission spectra). The chemical structure responsible for absorbing and emitting light (the chromophore) is stable in most fluorescent proteins. However, a number of fluorescent proteins are now available that can undergo changes in chromophore structure. These changes are induced by exposure to high-energy (low wavelength) light. Following this exposure, the chemical structure of the chromophore and its spectral properties are altered. Once photoactivated, the spectra of the targeted fluorescent protein population are distinct from the unactivated population. Therefore, a set of proteins can be highlighted. Proteins outside of the photoactivated region as well as proteins folded and/or translated after the photoactivation will be spectrally distinct (effectively a different color). This makes it possible to pulse label entire cells (for fate mapping) or components within cells (for studies of protein and organelle turnover, etc.)

MATERIALS AND METHODS

Lines used:

Several convertible color-switching fluorescent proteins are now used for photoactivation studies, including Kaede, Dendra2, and Eos. In the photoactivation experiments below, we will study cell fate in four different transgenic lines: (1) a Sox10:Eos transgenic line that expresses Eos in migrating neural crest cells, derivatives of the neural crest, and a variety of glial cells; a related line Sox10:NLS-Eos that expresses nuclear localized Eos in these cell types; a NeuroD:Eos line that expresses Eos in a diverse set of neurons including the lateral line system, central nervous system, and brain, and a NeuroD:NLS-Eos line that expresses nuclear localized Eos in these cell types. These lines were created by Andrew Prendergast in the Raible lab.

EXPERIMENTAL PROTOCOL:

Sample preparation:

1. Two days before carrying out the experiment, set the male and female fish as a pair or as a group in a spawning tank to keep the fish from eating their eggs. (A tank with marbles or a net at the bottom will work for this purpose.) **Embryos must be kept in the dark as much as possible, as fluorescent light in the lab (and sun light) will convert many photoactivatable proteins including Eos.**
2. Collect eggs using a siphon and net and clean them by transfer with a pipette. (Zebrafish will lay eggs at dawn; typically within 1 h after the light is turned on).
3. Raise the embryos in the dark in a 28.5 °C incubator to obtain a standard developmental speed (or at 23 °C to obtain an approximately 1.6-times slower developmental speed). Remove dead embryos with a Pasteur pipette to ensure it is kept clean.
4. Monitor the expression of green Eos in the embryos using a dissection microscope equipped with a GFP filter set. **(Be careful not to expose the embryos to light through a low wavelength filter like DAPI; this will photoconvert Eos.)**
5. Transfer embryos expected to have pigmentation during observation (beyond 24 hpf) to a solution of EM containing 0.003% PTU before 24 hpf to inhibit pigmentation.

Mounting embryos for imaging and photoconversion:

A range of options exist for mounting embryos, depending on their age and purpose: Option A (for embryos under 10 hpf), Option B (for embryos under 10 hpf to avoid unintended rotation during observation), or Option C (for embryos older than 10 hpf).

Note1: Embryos that have already started moving (after 16 hpf) should be anesthetized with 0.2 mg/ml Tricaine in EM, both prior to mounting and during the entire period of observation. When using LMP agarose, Tricaine should be added to the agar solution.

Note2: Check on the configuration of the microscope (inverted or upright) before mounting your embryos, as this will affect how you want to embed the embryos.

- A. Embryos that have not reached 10 hpf:
 - i. Set the dechorinated embryo free in a small hole made in a layer of 1% LMP agarose using a 0.5-ml PCR tube as a mold on a cover-glass-bottomed culture dish filled with (HEPES-buffered) EM.
 - ii. Carefully rotate the embryo with a hair loop so that the desired aspect of the embryo faces the bottom.
- B. Preventing rotation of embryos under 10 hpf:

Place the embryo in melted 0.1% agar in EM cooled to <37C, transfer it into the hole together with the agar, and quickly rotate the embryo with a hair loop to fix its orientation. This prevents the embryo from undergoing any unexpected rotation without disturbing epiboly and gastrulation.
- C. Embryos older than 10 hpf:

Place the embryo into melted and cooled 1% LMP agarose in EM and transfer the embryo directly to a cover-glass-bottomed culture dish in a drop of LMP agarose

before quickly rotating the embryo with a hair loop to fix its orientation so that the area of interest faces the bottom. Wait until the agar is solidified (~10 minutes). Carefully fill the dish with (HEPES-buffered) EM.

Photoconversion:

Eos (like Kaede and Dendra2) converts from green to red when exposed to UV or violet light (light around 400nm). Two types of light sources can be used. DAPI filters on fluorescent microscopes with a mercury or LED light source will pass appropriate low wavelength light. This light can be used to activate an exposed field of cells; the field size can be restricted by reducing the aperture. Alternatively, low wavelength lasers (e.g. 405nm diode lasers) are now available on many confocal microscope systems. These lasers will photoactivate Eos, and can often be directed to particular cells of interest by specifying a target region of interest through the software interface.

- A. Photoactivation using a fluorescence microscope and DAPI filter:
 - i. First, use the GFP filter set to observe green fluorescence. Confirm at this point that Eos-expressing cells do not show significant fluorescence in the red (Rhodamine or RFP) channel. **(Again, be careful not to expose the embryos to light through a low wavelength (e.g. DAPI) filter.)** Use the GFP filter to focus on the cells of interest. Close the aperture for the excitation light and make a small circular window so that the cells to be labeled are within that spot. Then switch to the filter set for DAPI.
 - ii. Photoconvert green Eos to red Eos by irradiating with UV light passed through the DAPI filter set. The exposure time required for photoconversion varies (from a few seconds to a few minutes) depending on the light intensity and magnification of the objective lens.
 - iii. To observe photoconverted Eos, use the filter set for Rhodamine or RFP. Switch to the rhodamine filter set to monitor the appearance of red color, and then switch back to the GFP filter set to see whether the green color has diminished.
 - iv. Repeat UV irradiation until and the brightness of red fluorescence reaches a plateau.

- B. Photoactivation using a confocal microscope and ~400nm laser.
 - i. Scan embryos expressing Eos with a configuration appropriate for imaging GFP (typically a ~490-nm laser and ~505-530 bandpass filter).
 - ii. To photoconvert Eos, target the 400-nm range laser to a region of interest. (Often, software packages have a FRAP module that makes it relatively simple to select a region and target it with a specified laser for a user-defined time and power. The time and power needed for photoactivation vary from a fraction of a second to a few minutes depending on the strength of the laser, the size and complexity of selected region, and the objective lens magnification.
 - iii. Image the red signal of (photoconverted Eos) using a configuration appropriate for rhodamine or RFP (typically a ~560-nm laser and 570nm long pass filter).
 - iv. Repeat targeting with ~400 nm range laser until the brightness of red fluorescence reaches a plateau.

These procedures should allow you to identify a cell type of interest, uniquely label this cell type, and subsequently follow its fate. It is possible to follow labeled cells and their mitotic progeny for days following photoactivation.

Issues to keep in mind:

- As cells divide, they decrease the amount of photoactivated Eos. Rapidly dividing cells will lose signal more rapidly. It is possible to identify photoactivated cells and reactivate them, since more unactivated Eos will be synthesized in these cells.
- Exposure to low wavelength light can cause phototoxicity. You may need to photoactivate at a level below the maximum conversion you can achieve to avoid damaging the cells.

(2) Using Fluorescent Protein Fusions to study intracellular processes.

Because fluorescent proteins are relatively inert, they can be fused to a protein-of-interest to follow its behavior. Fusing a fluorescent protein to a protein-of-interest can affect its function. However, very often these fusions recapitulate the activity of the native protein. When they do so, they make it possible to dynamically follow a protein-of-interest in a living cell. Additionally, FP fusions can be used to monitor intracellular compartments, organelles, and structures. Targeting and binding motifs can be used to deliver fluorescent proteins to endosomes, the endoplasmic reticulum, the Golgi apparatus, mitochondria, actin filaments, microtubules, junctional complexes, and many other intracellular structures. Because development involves tissue formation, the conversion of non-differentiated cells into cells with specific architecture, and rearrangements in intracellular structures, being able to monitor these structures can shed light on developmental processes.

MATERIALS AND METHODS

Approaches to transiently express markers:

To survey some of the tools available for monitoring intracellular structures, we will transiently express fluorescent protein fusions. There are two approaches to do so—injecting mRNA and injecting DNA. Because fluorescent protein fusions are directly transcribed from mRNA, levels of expression can be controlled by adjusting the amount that is injected. However, mRNA expression occurs throughout the entire embryo, and signal is rapidly lost as the embryo develops. In our experience, fluorescent protein fusions transcribed from mRNA are often difficult to detect beyond 24 hours post injection. Fluorescent proteins expressed from injected DNA are regulated by the promoter element in the injected construct. Therefore, fusions can be expressed in particular cell types at particular developmental stages. DNA is more stable than mRNA, and therefore, expression can be seen in older larvae. In our experience, DNA constructs injected at the one cell stage often continue to express beyond 5 days post injection. However, it is difficult to control expression levels, and expression is restricted to a small number of cells. DNA constructs can be integrated into the genome by using recombination sites flanking the promoter and open reading frame. Even if a transgenic line is not needed, this can help increase expression in cell types of interest.

Microinjecting mRNA or DNA:

1. The evening before injecting, set the male and female fish as a pair in a spawning tank and place a divider between the fish. When the morning lights are turned on, pull

the divider and replace the water with fresh water. Monitor the pair and collect the embryos soon after the fish spawn. It is generally best to inject at the one cell stage.

2. Load a microinjection needle with DNA or mRNA and phenol red. Attach the needle to the micropipette holder connected to the microinjector. Place embryos in agar wells and focus on an embryo. Move the needle until you can see the tip in the same field as the embryo. Using a fine pair of forceps, break the tip of needle. Use a manipulator to move the injection needle to penetrate the egg. Subsequently, inject DNA or mRNA into the cytoplasm of one- to four-cell-stage embryos. The pressure and duration of injection will vary considerably depending on the micropipette. Adjust them so that the diameter of the injected solution is approximately one-fourth the diameter of the egg.

3. Maintain the embryos and mount them as described above under sample preparation.

Imaging:

Imaging intracellular structures is more demanding than imaging cells. To clearly resolve structures within cells, you often need to deconvolve widefield images, or capture confocal images. At high magnification, it is easy to bleach or damage your sample with light exposure, and high magnification lenses generally have shorter working distances which may require more careful sample mounting.

A number of parameters can significantly help with these challenges. Gain settings on detectors—either photomultiplier tubes or CCD cameras—dramatically affect how much laser excitation you need in order to detect a signal. Binning on CCD cameras (effectively combining the signal from adjacent pixels) also enhances signal. On confocal systems with adjustable pinholes, changing the pinhole size dramatically affects signal for a given amount of laser power. And the particular lens you use (whether it is an oil, glycerol, or water lens; whether it is high NA or low NA; whether it color corrected or not) also affects signal. It is worth spending the time to optimize imaging parameters. A good initial test is to determine how many images you are likely to want over a time course, and then capture them continuously to get a sense of whether the parameters you are using are appropriate. If fewer images are needed, you can optimize parameters for low noise and high resolution. The more images you require, the more you will likely need to tolerate more noise and poorer resolution.

Using fluorescent protein markers, you can identify organelles or structures-of-interest, and investigate how these structures behave and how to optimize imaging to follow their behavior.

REFERENCES

Andersen E., Asuri N., Clay M., Halloran M. 2010. Live imaging of cell motility and actin cytoskeleton of individual neurons and neural crest cells in zebrafish embryos. *J Vis Exp* 3;(36): 1726.

Caron, S.J., Prober, D., Choy, M., and Schier, A.F. 2008. In vivo birthdating by BAPTISM reveals that trigeminal sensory neuron diversity depends on early neurogenesis. *Development* 135(19): 3259-3269.

Chudakov, D.M., Lukyanov, S., and Lukyanov, K.A. 2007. Tracking intracellular protein movements using photoswitchable fluorescent proteins PS-CFP2 and Dendra2. *Nat Protoc* 2(8): 2024-2032.

Hatta, K., Tsujii, H., and Omura, T. 2006. Cell tracking using a photoconvertible fluorescent protein. *Nat Protoc* 1(2): 960-967.

Megason, S.G., Fraser, S. E., 2003., Digitizing life at the level of the cell: high-performance laser-scanning microscopy and image analysis for in toto imaging of development. *Mech of Dev* 120(11): 1407-1420.

Tsutsui, H., Karasawa, S., Shimizu, H., Nukina, N., and Miyawaki, A. 2005. Semi-rational engineering of a coral fluorescent protein into an efficient highlighter. *EMBO Rep* 6(3): 233-238.

Wiedenmann, J., Oswald, F., Nienhaus, G.U. 2009. Fluorescent proteins for live cell imaging: opportunities, limitations, and challenges. *IUBMB Life* 61(11): 1029-42.