Video Article

Single-cell Photoconversion in Living Intact Zebrafish

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URL: https://www.jove.com/video/57024
DOI: doi:10.3791/57024

Keywords: Developmental Biology, Issue 133, zebrafish, photoconversion, Eos, confocal, microscopy

Date Published: 3/19/2018

Citation: Green, L., Smith, C.J. Single-cell Photoconversion in Living Intact Zebrafish. *J. Vis. Exp.* (133), e57024, doi:10.3791/57024 (2018).

Abstract

Animal and plant tissue is composed of distinct populations of cells. These cells interact over time to build and maintain the tissue and can cause disease when disrupted. Scientists have developed clever techniques to investigate characteristics and natural dynamics of these cells within intact tissue by expressing fluorescent proteins in subsets of cells. However, at times, experiments require more selected visualization of cells within the tissue, sometimes at the single-cell or population-of-cells manner. To achieve this and visualize single cells within a population of cells, scientists have utilized single-cell photoconversion of fluorescent proteins. To demonstrate this technique, we show here how to direct UV light to an Eos-expressing cell of interest in an intact, living zebrafish. We then image those photoconverted Eos^{+} cells 24 h later to determine how they changed in the tissue. We describe two techniques: single cell photoconversion and photoconversions of populations of cell. These techniques can be used to visualize cell-cell interactions, cell-fate and differentiation, and cell migrations, making it a technique that is applicable in numerous biological questions.

Video Link

The video component of this article can be found at https://www.jove.com/video/57024/

Introduction

Multiple distinct cells interact to build and maintain complex animal and plant tissues. These cells are often intercalated and difficult to distinguish from neighbors at a single cell level without high resolution microscopy that require fixation of tissue. However, to understand how these tissues form, are maintained, and become diseased, it has been essential to investigate how single cells within the tissue are interacting over time. Ideally, these experiments require the labeling of single cells within a tissue in a non-invasive manner without the requirement of fixation. Scientists have now developed numerous techniques to accomplish this task[1,2,3,4].

The discovery and implementation of the jellyfish green fluorescent protein (GFP) was one exciting approach that allowed for labeling of distinct cells in a tissue environment[1]. Using cell-specific promoters, it is possible to genetically select a subset of cells that are labeled[1]. Alternatively, viral induced expression of GFP can be utilized for user-selected expression of GFP[3,4]. Although quite useful, genetic mediated expression of GFP does not allow user-selected expression within a subset of cells in the tissue; and viral expression of GFP, although advantageous, can be invasive. With the advent of GFP derivatives and clever techniques like Brainbow to express distinct fluorescent proteins more sparsely within tissues, it has become possible to visualize single cells and the interactions among them in complex tissue[2,5]. However, these approaches label cells in a random fashion. If the desired experiment requires visualization of a single cell or population of cells that is defined by the experimenter, they are therefore limited. With such experiments, it would be advantageous to have a genetically expressed fluorescent protein that can be manipulated to distinguish, in a single cell fashion, it from other fluorescent and non-fluorescent cells.

To achieve this goal and visualize the cell biology of single cells within a complex living tissue, the scientific community uses single cell photoconversion of distinct fluorescent proteins[6,7,8]. Using genetically controlled expression of a photoconvertible protein (*i.e., eos, kaede, etc.*) that transitions from a green to red fluorescent state when exposed to UV (488 nm) light, we can distinguish a single cell from its fluorescently labeled neighbors[6,7,8]. This approach utilizes an apparatus attached to our confocal microscope which can direct light from a laser stack to a diffraction-limited region of interest. With this technique, we can either label single cells or larger populations in a user-defined manner[9,10,11]. The technique is minimally invasive compared to single cell injections of viral GFP. As a proof of concept, we show that we can photoconvert single cells within a ganglion in the peripheral nervous system and photoconvert larger populations like cells located on the ventral side of the spinal cord[9,10,11,12]. We then can visualize these photoconverted cell populations 24 h later to gain insight into their movement and differentiation during development.

Protocol

All animal studies were approved by the University of Notre Dame Institutional Animal Care and Use Committee.

1. Preparation of Zebrafish Specimen

1. Place one adult male and one adult female Tg(convertible protein) into a mating chamber per standard procedures[13]. In this manuscript, use Tg(sox10:eos) fish[9] because of access but other transgenic lines with photoconvertible protein can be equally used. Set up more than one chamber in case fish do not lay. Allow the fish to remain in the chamber overnight.
2. The next morning, collect eggs in 100 mm Petri dishes. Allow eggs to mature to 24 h post-fertilization (hpf) before screening.
3. If animals above were heterozygotes for the transgene, screen 24 hpf dishes for Tg(sox10:eos)+ embryos with a 488 nm light source and GFP filter sets on a dissecting microscope. Isolate Tg(sox10:eos)+ embryos and allow to mature to 48 hpf.
4. Dechorionate embryos manually with a needle or tweezers.
5. Prepare and microwave 5 mL of 0.8% low-melting point agarose solution.
    1. Once agarose is cool to the touch, place 3-4 anesthetized Tg(sox10:eos)+48 hpf fish in the center of a 10 mm glass-coverslip bottom Petri dish.
    2. Add enough agarose to cover the surface of the coverslip, approximately 1 mL. Use a probe needle to arrange fish on their sides. Allow agarose to solidify to ensure mounting of animals. It may be necessary to continually re-arrange the zebrafish until the agar solidifies[14]. Solidification takes approximately 2 min.
6. After the agarose has solidified for 2 min, slowly add embryo medium containing 0.02% aminobenzoic acid ester (Tricaine) to dish until the bottom surface of the agar and dish is submerged. This should be approximately 3mL.

2. Microscope Mounting and Pre-conversion Imaging

1. Open confocal software and select the capture and focus windows [**Figure 1**]. Under the capture window, select the lab-specific conversion imaging setting under the capture setting drop down tab [**Figure 1**]. Here, the lab-specific setting is called "**Fish Imaging**."
2. Place specimen on confocal scope and bring into focus using course and fine adjustment knobs.
3. Open the focus window and locate the desired region of interest (i.e. dorsal root ganglia).
4. Select the c488 laser under the Filter Set menu. Set the exposure to 300 ms, laser power to 5, and intensify to 75 [**Figure 2**].
5. Check the 3D box under the **capture type** section. In the **3D Capture** section, select **use current position** and check **range around current**. In the same section, set the range to 35, the number of planes to 36, and the step size to 1. The range can be increased or decreased to accommodate for the depth of the imaging area. For the spinal cord a range value between 35-40 stacks typically is sufficient [**Figure 5**].
6. Select current location [**Figure 5**].
7. Click start at the bottom of the capture window to acquire image.

3. Single-cell Photoconversion

1. Open confocal software and select the capture and focus windows [**Figure 1**]. Under the capture window, select the lab-specific conversion imaging setting under the capture setting drop down tab [**Figure 1**]. Here, the lab-specific setting is called "Fish ablate full chip."
2. Select the c488 and c541 laser under the Filter Set menu. If not using the same microscope software, find the menu to select different lasers and select the 488 nm and 541 nm lasers. Set the exposures to 300 ms, laser power to 5, and intensify to 75 [**Figure 2**]. These laser settings are selected based on producing enough fluorescent signal without causing photobleaching or toxicity. If toxicity or photobleaching is visualized, reduce laser power or exposure.
3. Open the focus window and click on the photomanipulation tab in the focus window. Adjust laser parameters accordingly. Change the laser stack power to 2, and then click **Go**. Change the Raster block size to 1 and click **Set**. Change the Double-click size to 4. Change the laser line to v405 [**Figure 3**].
4. Open the advanced capture settings in the capture window. Select the photomanipulation tab and change the Double-click repetitions to 2. Click OK [**Figure 4**].
5. Select the XY tab in the focus window. Double check the laser parameters from step 2 in the photomanipulation tab [**Figure 3**, **Figure 4**]. Set laser settings to photoconvert the cell of interest without photoconversion of surrounding cells.
    1. If photoconversion of adjacent cells is present, reduce laser power. If photoconversion of cells does not occur, laser powers can be increased. Optimally, set laser power to photoconvert only the region of interest and not surrounding areas.
6. Check the timelapse box under **capture type**. Then, click **Start** [**Figure 6A**].
7. Once the live timelapse window opens, select the circle tool on the top toolbar [**Figure 7**]
8. Draw a circle in the centermost region of the cell. Right click the drawn circle, select **FRAP region**, and wait 3 seconds. The selected area should become dimmer [**Figure 8**]. Click **stop capture**.
9. If imaging more than one animal, go back to the XY tab in the focus menu and select position 2. Then, repeat steps 4.1-4.6.
10. To convert a population of cells, follow the protocol and laser parameters for steps 4.1-4.4 except instead of drawing a circle to FRAP the region of interest, use the line tool. Draw a line on the region of interest and FRAP the region using the same parameters listed above.

4. Post-photoconversion Imaging

1. Once all points are photoconverted. Select the lab-specific standard image stack setting described in the precoversion imaging step 3. This is found under the capture setting drop down tab in the capture window [**Figure 5**].
2. Select the c488 laser and set the exposure to 300ms, laser power to 5, and intensify to 75 [**Figure 2**].
3. Select the c541 laser found under the same menu as the c488 laser. Set the exposure to 500 ms, laser power to 10, and intensify to 75 [**Figure 9**].

4. Check the 3D box under the **capture type** section. In the **3D Capture** section, select **use current position** and check range around current. In the same section, set the range to 35, the number of planes to 36, and the step size to 1. The range number may increase or decrease to accommodate the desired imaging depth [**Figure 5**].
5. If there are multiple points in the XY focus tab, select the multipoint list option in the capture window. If not, select current location.
6. Click start at the bottom of the capture window to acquire image.

Representative Results

Photoconversion of fluorescent proteins can be used to label distinct cells within a tissue[6]. To demonstrate this, *Tg(sox10:eos)* fish[9] were used to express the photoconvertible protein Eos under the regulatory sequences of *sox10*. The *Tg(sox10:eos)* animals at 48 hpf were first mounted, and then imaged to detect any non-specific photoconversion that may have occurred. The Eos unconverted fluorescent signal with little Eos photoconverted fluorescent signal was then visualized (**Figure 10**). Animals were kept in the dark to ensure non-specific photoconversion is minimal. Then, the regions of interest were exposed to UV light using the confocal microscope set-up. To confirm that single cells were photoconverted as a result of the UV exposure, we took z-stacks of the area spanning the photoconverted region. Consistent with successful photoconversion, there was little detection of non-converted Eos outside of the region of interest and photoconverted Eos was distinctly present within the region of interest. The resultant image shows a single cell within a ganglion that is labeled definitely from its neighbors (**Figure 10**).

To demonstrate the utility of this photoconversion technique, a population of cells was also exposed to UV light. In this paradigm, pre-photoconversion images were taken and no un-photoconverted Eos signal was present. Next, the ventral side of the spinal cord was exposed to UV using a line (**Figure 11**). To confirm successful photoconversion, post-photoconversion images were taken and photoconverted Eos in the ventral spinal cord cells at the location of the line of interest that we drew were visualized (**Figure 11**). Non-photoconverted Eos signal was visible in all other areas. To extend this technique we then took identical images 24 hours after the photoconversion. In these images, photoconverted Eos^{+} cells were seen scattered throughout the spinal cord region in both dorsal and ventral locations (**Figure 12**). These data are consistent with the hypothesis that ventral spinal cells relocated to dorsal locations as previously described[10].Together these two techniques demonstrate that photoconversion of Eos can be utilized to visualize single cells or populations of cells within a tissue region in a user-defined fashion. This has contributed to a better understanding of cellular characteristics such as cell migration, communication, and dynamics.

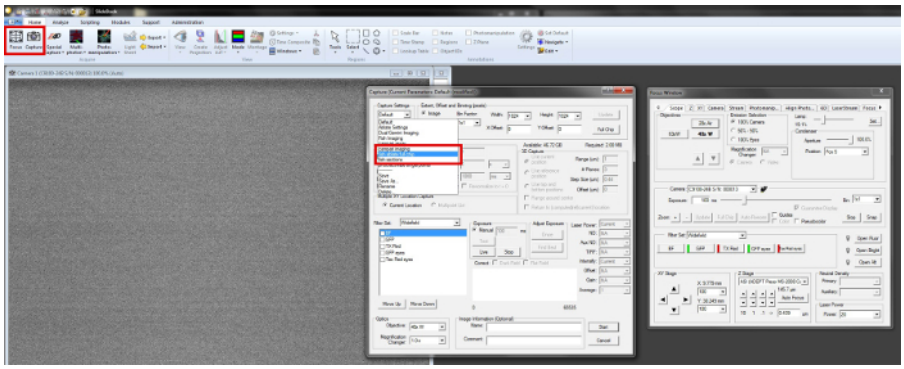

Figure 1: Capture and Focus Windows. Screenshot of the software depicting location of capture and focus windows and the lab specific "Fish ablate full chip" drop down tab. See red boxes. Please click here to view a larger version of this figure.

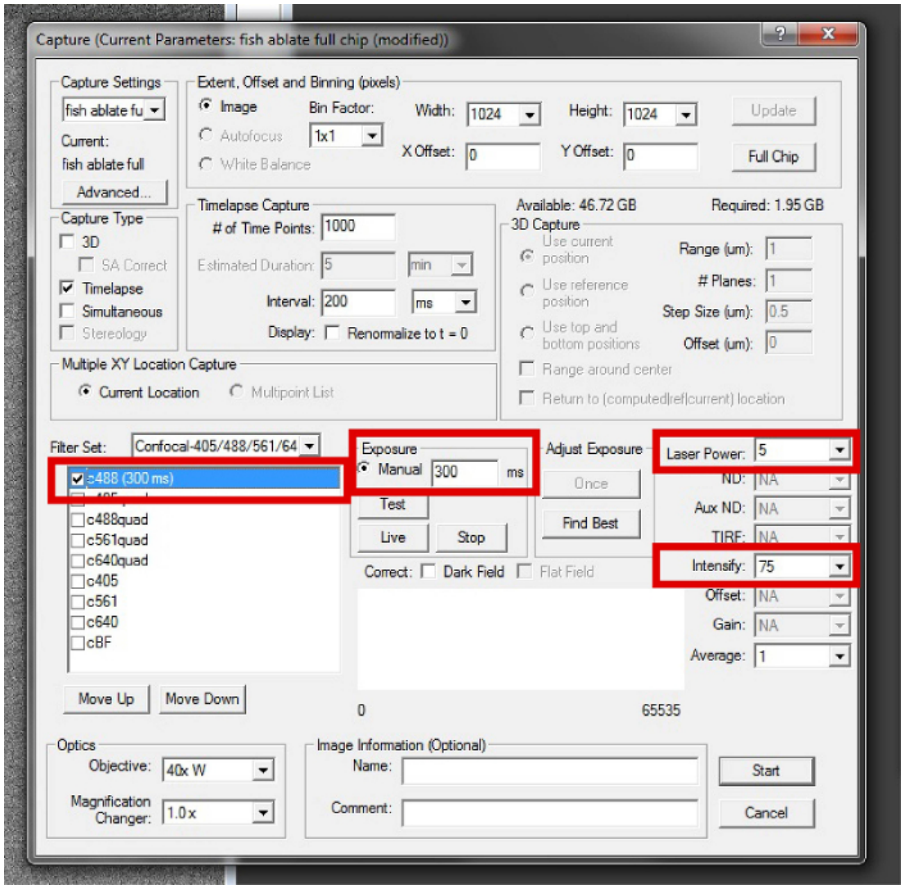

Figure 2: c488 Laser Parameters. Screenshot of the capture window showing the specific parameters for the c488 laser. Exposure is 300 ms. Laser power is 5. Intensify is 75. See red boxes. Please click here to view a larger version of this figure.

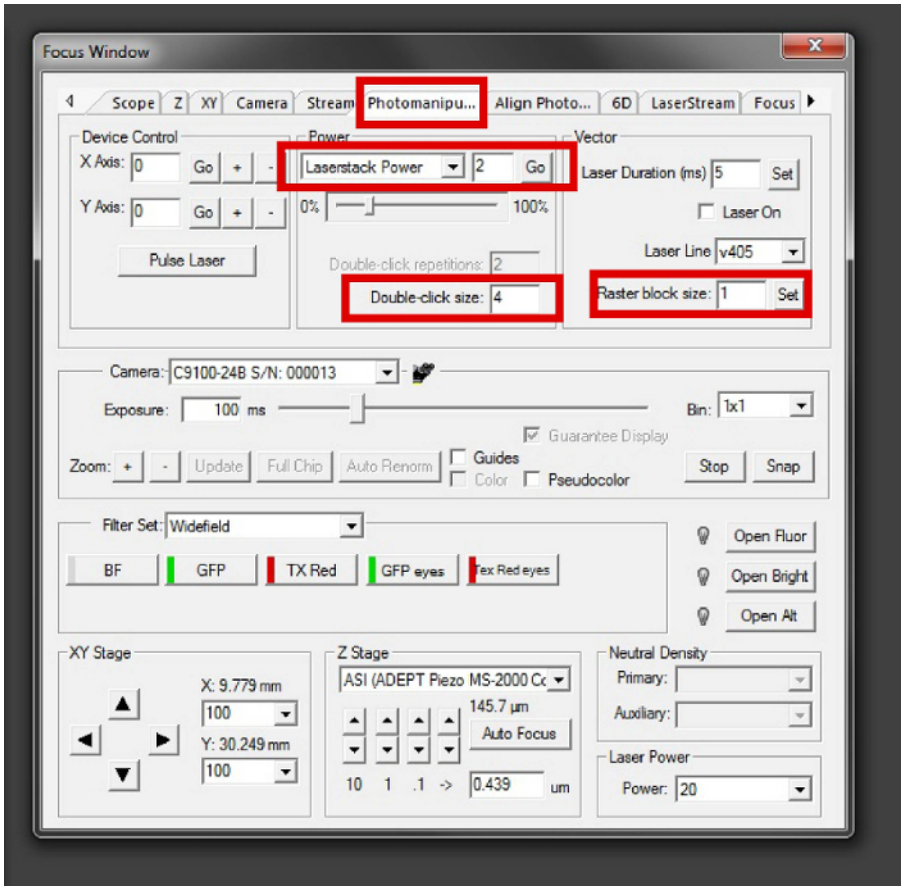

Figure 3: Photoconversion Laser Parameters. Screenshot of the photomanipulation tab in the focus window outlining the specific laser settings needed for photoconversion. Laserstack Power is 2. Double-click size is 4. Raster block size is 1. See red boxes. Please click here to view a larger version of this figure.

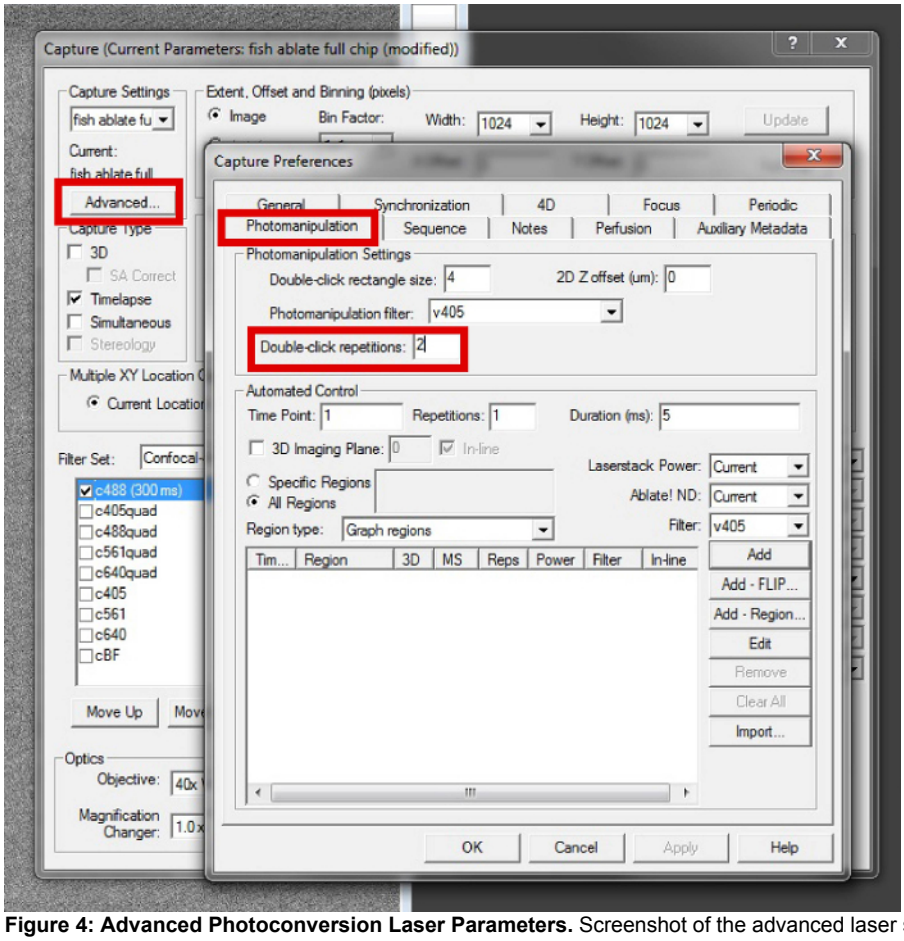

Figure 4: Advanced Photoconversion Laser Parameters. Screenshot of the advanced laser settings in the capture window. This opens to the capture preferences window with the photomanipulation tab. The Double-click repetitions is set to 2. See red boxes. Please click here to view a larger version of this figure.

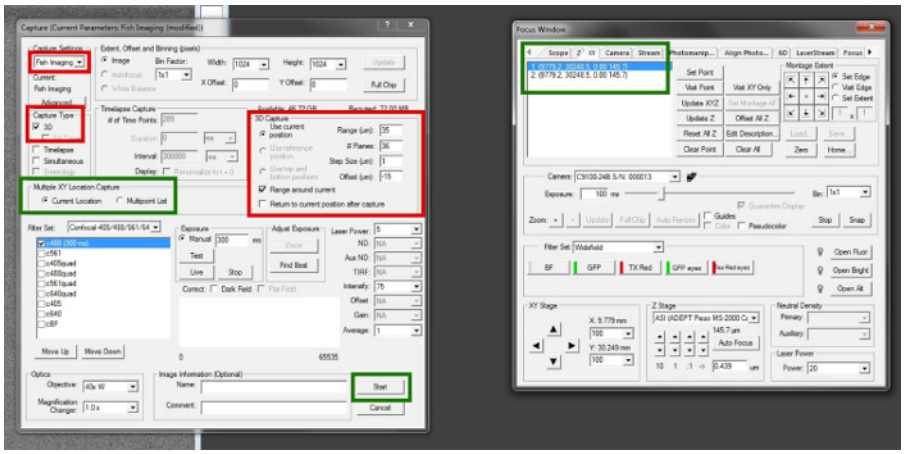

Figure 5: Pre-Conversion Imaging Parameters. Screenshots of the focus and capture windows. Highlights the capture window specific parameters needed for pre-conversion imaging. The capture setting is "Fish Imaging." The 3D box is checked beneath the capture type. And the 3D capture settings are specified as; Range is 35, Number of Planes is 36, Step Size is 1, and Offset is -15. The "current position" and "range around current" boxes are also selected. See red boxes. Depicts how to select single or multiple points on the capture window (left) and how to select individual points on the focus window (right). See green boxes. Please click here to view a larger version of this figure.

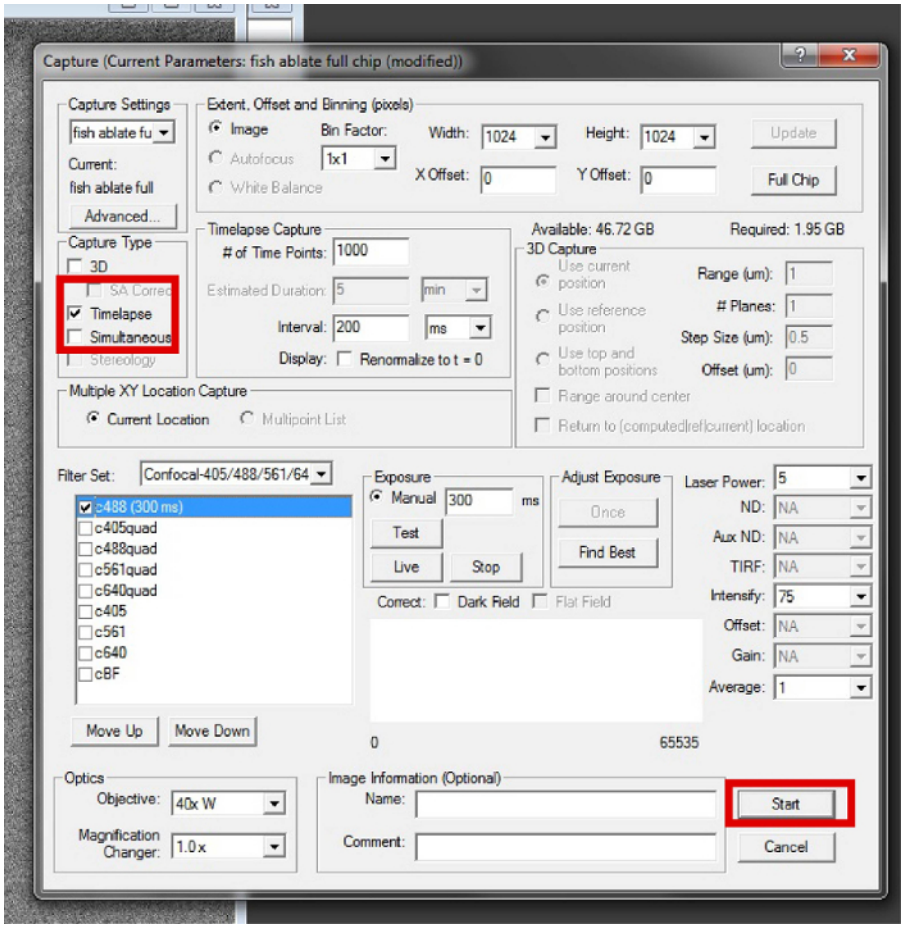

Figure 6: Opening the Photoconversion Window. Screenshot depicting the timelapse capture type is selected in accordance with previously set parameters. See red boxes. Please click here to view a larger version of this figure.

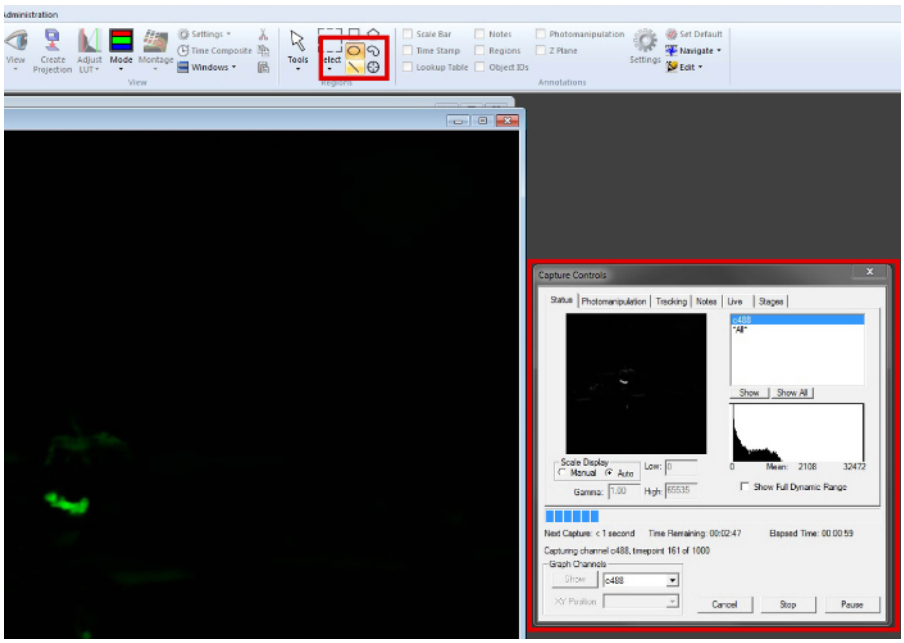

Figure 7: Setting up the Photoconversion. Screenshot showing the location of the circle tool (top) and the capture control window that appears (right). See red boxes. Please click here to view a larger version of this figure.

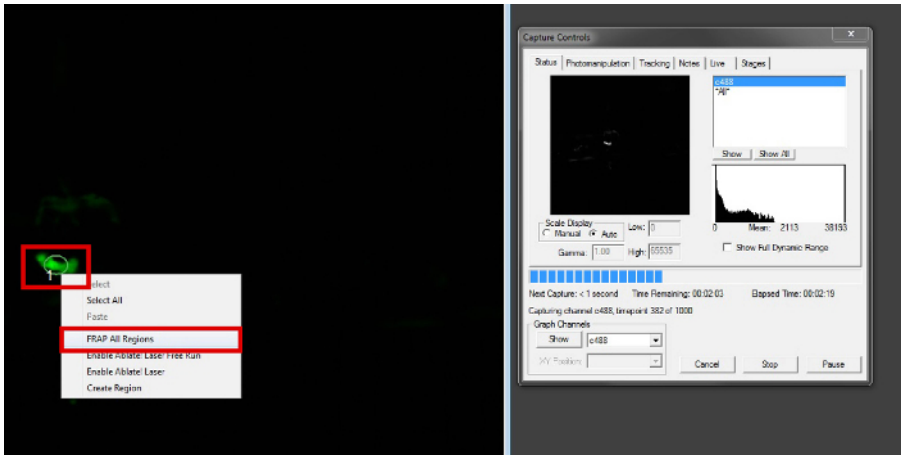

Figure 8: Single-Cell Photoconversion. Screenshot depicting the use of the circle photoconversion tool and the right click drop down menu. The "FRAP all Regions" conversion action is located within the right click drop down menu. See red boxes. Please click here to view a larger version of this figure.

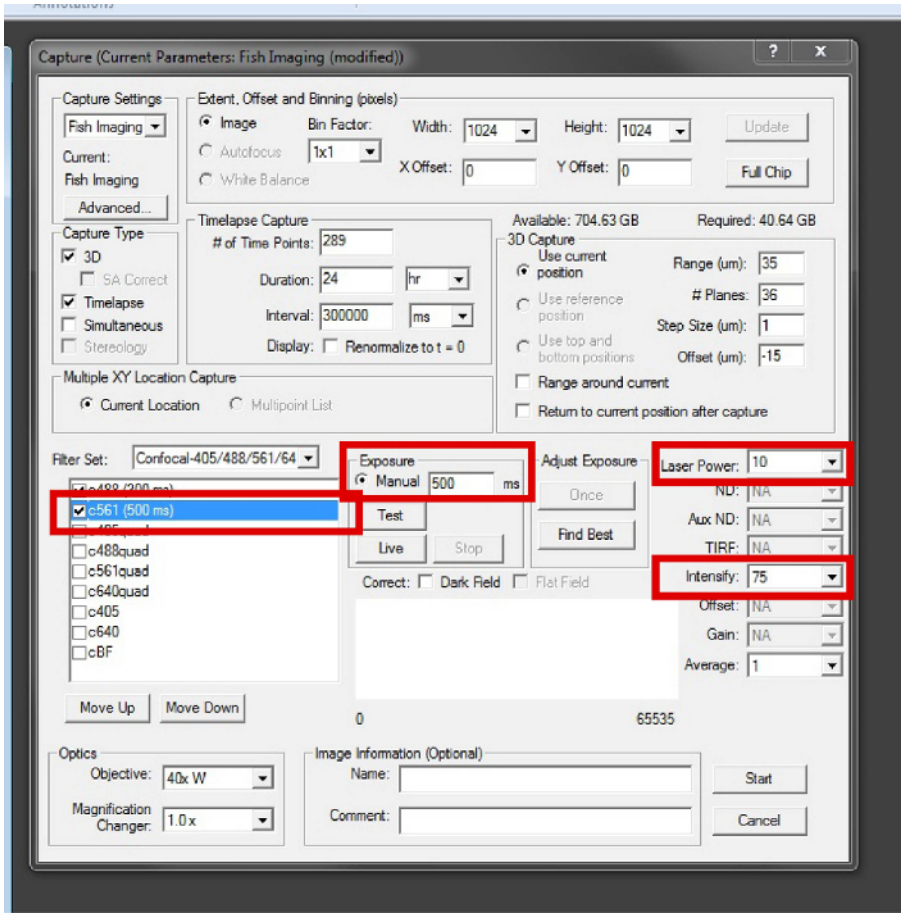

Figure 9: Post-Photoconversion c561Laser Parameters. Screenshot of the capture window showing the specific parameters for the c561 laser. Exposure is 500 ms. Laser power is 10. Intensify is 75. See red boxes. Please click here to view a larger version of this figure.

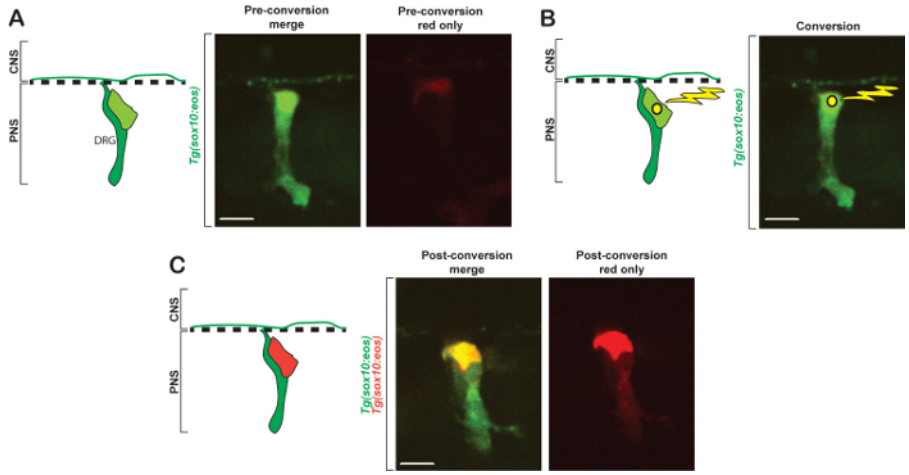

Figure 10: Single-cell Photoconversion. (A). Schematic and confocal z-projections of *Tg(sox10:eos)* zebrafish at 48 hpf showing dorsal root ganglia in the peripheral nervous system before photoconversion. Dashed line indicates approximate location of DRG spinal cord entry.. (B). Schematic and confocal z-projections of *Tg(sox10:eos)* zebrafish at 48 hpf showing dorsal root ganglia in the peripheral nervous system during photoconversion. Dashed line indicates DRG spinal cord entry. Yellow circle indicates conversion site and lighting bolt indicates UV light exposure. (C). Schematic and confocal z-projections of *Tg(sox10:eos)* zebrafish at 48 hpf showing dorsal root ganglia in the peripheral nervous system post-photoconversion. Dashed line indicates DRG spinal cord entry. CNS is an abbreviation for central nervous system and PNS is an abbreviation for peripheral nervous system (A-C). Scale bar equals 10 um. Please click here to view a larger version of this figure.

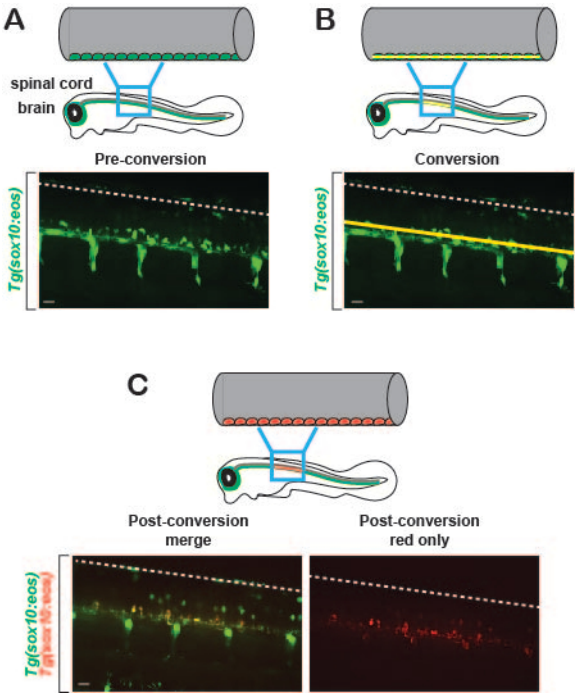

Figure 11: Photoconversion procedure in a larger spinal cord region. (A). Schematic and confocal z-projections of *Tg(sox10:eos)* zebrafish at 48 hpf showing OPCs and dorsal root ganglia in the ventral region of the spinal cord before photoconversion. Dashed line indicates dorsal region of spinal cord. (B). Schematic and confocal z-projections of *Tg(sox10:eos)* zebrafish at 48 hpf showing OPCs and dorsal root ganglia in the ventral region of the spinal cord during photoconversion. Solid yellow line indicates selected area for photoconversion. Dashed line indicates dorsal region of spinal cord. (C). Schematic and confocal z-projections of *Tg(sox10:eos)* zebrafish at 48 hpf showing OPCs and dorsal root ganglia in the ventral region of the spinal cord after photoconversion. Dashed lines indicate dorsal region of spinal cord. Scale bar equals 10 um.

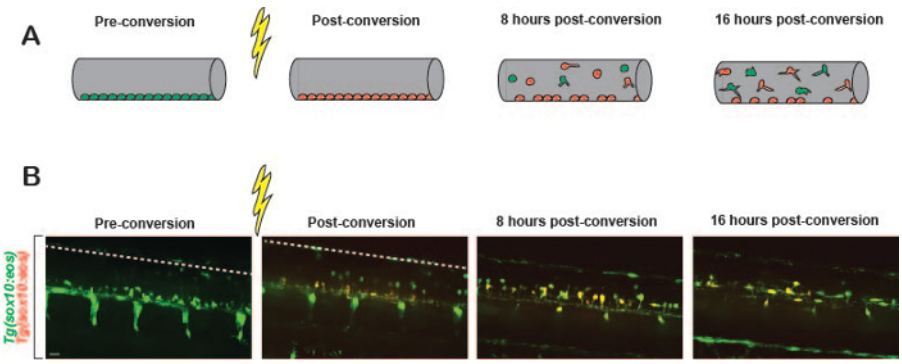

Figure 12: Cellular tracking and visualization post-photoconversion. (A). Schematic of *Tg(sox10:eos)* zebrafish spinal cord at 48 hpf showing OPCs and dorsal root ganglia in the ventral region of the spinal cord after photoconversion. Lightning bolt indicates UV light conversion. (B). Confocal z-projections of *Tg(sox10:eos)* zebrafish spinal cord starting at 48 hpf showing OPCs and dorsal root ganglia in the ventral region of the spinal cord after photoconversion. Lightning bolt indicates UV light conversion. Scale bar equals 10 um.

Discussion

In complex tissues, distinct cell-types organize into specific domains. Recently techniques have been utilized to label individual cells within these large tissue structures[1,2,3]. Here we demonstrate two techniques that can similarly be utilized to visualize both single cell interactions and cell population interactions within complex tissues. The advantage of the photoconversion technique is the spatial control the scientist has to distinctly label a cell. With a microscope that has the ability to expose smaller imaging areas within the larger imaging window, areas as small as single cells or areas of single cells can be labeled differently than other cells. Multiple photoconvertible proteins have now been introduced to the community making this a more widespread technique[7]. With the ability to photoconvert proteins within cells, scientists have utilized this technique or similar approaches to investigate cell migration, cell differentiation and neural circuit analysis.

Although the software and specific laser parameters are specific to our lab, this article demonstrates the universal ability to use the photoconvertable protein Eos in distinguishing single cells within a ganglion. Unfortunately, most genetic regulatory regions that can be used to drive fluorescent proteins express widely within ganglia at early developmental phases. This is likely because these cells are generated from similar progenitor pools and the markers identify those progenitor states[9]. It is therefore difficult to visualize the interactions of single cells within the larger ganglia during these earlier developmental stages. The approaches shown here represents one technique that can label individual cells within a ganglion and thus could be used to dissect scientific questions about ganglia development.

However, this photoconversion technique is not limited to studies of ganglia development. For example, in studies of regeneration, photoconversion of pre-injury conditions can be utilized to determine how specific cells within a ganglion respond following injury. These studies can help to elucidate the progenitor-like potential of specific cells in a ganglion. Similarly, photoconversion of specific tumor cells and tracking of those cells over time could reveal properties of single cells within complex tumors. This application of single cell photoconversion therefore expands the potential of the technique to make meaningful discoveries in the biomedical community.

This approach can also selectively label cells within an area of the spinal cord. In development, cells often migrate from precursor areas to produce differentiated cells in mature locations[11,14]. Long-term fate mapping with Cre has efficiently been utilized to dissect such processes. With Cre techniques, spatial resolution is limited to the genomic regulatory regions that drive Cre transcription[15]. With photoconversion, this spatial resolution can be achieved along with the ability to choose the time when the photoconversion occurs and the temporal resolution. Therefore, for shorter term fate-mapping, this photoconversion technique has many advantages over Cre fate-mapping. However, in samples where UV light cannot be easily achieved, like in an intact mouse, this photoconversion approach is unlikely to work[15]. Additionally, Cre fate-mapping permanently labels cells allowing for more long-term fate mapping than photoconversion that loses its label as the photoconverted version of the protein dissipates and new unconverted protein is produced by the cell. Nonetheless, depending on the scientific question, photoconversion can be a useful tool for fate-mapping of cell populations.

Although only two examples of the application of photoconversion are provided, numerous studies have demonstrated its efficacy. Depending on the spatial precision required, photoconversion can be performed with access to a UV light source. If the desire is to label single cells more advanced imaging like confocal or two-photon microscopy may be necessary. Nonetheless, the ability to select both the spatial and temporal area of labeled cells distinctly from other cells within an intact tissue has made UV-induced photoconversion a powerful technique to dissect biological questions.

Disclosures

The authors have nothing to disclose.

Acknowledgements

We thank Bernard Kulemaka and members of the Smith lab for their helpful comments and reagent guidance, Sam Connell and Brent Redford of 3i for fielding imaging questions and Deborah Bang, Karen Heed and Kay Stewart for zebrafish care. This work was supported by the University of Notre Dame, the Elizabeth and Michael Gallagher Family, the Alfred P. Sloan Foundation, Center for Zebrafish Research at the University of

Notre and Center of Stem Cells and Regenerative Medicine at the University of Notre Dame. All animal studies were done in compliance with University of Notre Dame IACUC to Dr. Cody Smith.

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