Video Article

Two-Photon-Based Photoactivation in Live Zebrafish Embryos

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Abstract

Photoactivation of target compounds in a living organism has proven a valuable approach to investigate various biological processes such as embryonic development, cellular signaling and adult physiology. In this respect, the use of multi-photon microscopy enables quantitative photoactivation of a given light responsive agent in deep tissues at a single cell resolution. As zebrafish embryos are optically transparent, their development can be monitored *in vivo*. These traits make the zebrafish a perfect model organism for controlling the activity of a variety of chemical agents and proteins by focused light. Here we describe the use of two-photon microscopy to induce the activation of chemically caged fluorescein, which in turn allows us to follow cell's destiny in live zebrafish embryos. We use embryos expressing a live genetic landmark (GFP) to locate and precisely target any cells of interest. This procedure can be similarly used for precise light induced activation of proteins, hormones, small molecules and other caged compounds.

Video Link

The video component of this article can be found at http://www.jove.com/video/1902/

Protocol

We describe a protocol of cell labeling using caged fluorescein, however, other photo-activatable dyes and proteins can be similarly used.

1. Injection of Caged Fluorescein

1. Prepare 5% stock solution (5mg caged-fluorescein / 100 µL 0.2M KCl) of Dextran-conjugated 4,5-dimethoxy-2-nitrobenzyl (DMNB) caged fluorescein (10,000 MW dextran, anionic, Invitrogen, molecular probes, Carlsbad, CA, cat. no. D-3310). Aliquot and store in -20°C. Note that DMNB-caged fluorescein is sensitive to light and should be kept in the dark.
2. Prepare an injection trough made of 1% agarose (Sigma, St. Louis, MO, cat. no. A9539) as described in "The zebrafish book"[1].
3. Prepare 2 liters of E3 medium by diluting a 100 x E3 stock solution.
4. On the evening before injection, prepare mating cages separating the male(s) from the female(s) of the desired transgenic line expressing a visible fluorescent landmark (GFP, RFP etc.) used to localize the target cells for photoactivation.
5. Prepare capillary injection needles (Thin Wall Glass Capillaries with filament, World Precision Instruments, Sarasota, FL, cat. no. TW100F-6) using a micropipette puller (Sutter Instrument, Novato, CA, P-97).
6. Thaw an aliquot of 5% caged fluorescein on ice.
7. Dilute the 5% caged fluorescein stock solution in 0.2M KCl to a final concentration of 1%, and keep on ice. Avoid light exposure.
8. Setup mating crosses prepared in step 4.
9. Collect the fertilized eggs as soon as they are laid. Orient one-cell stage embryos in the agarose injection mold overlaid with E3[1].
10. Using a microloader tip (Eppendorf, Hamburg, Germany, cat. no. 5242 956.003) backfill an injection needle with ~1 µL of 1% caged fluorescein solution.
11. Place loaded needle into a micromanipulator attached to a gas-powered microinjector (pneumatic picopump, World Precision Instruments, Sarasota, FL, PV820).
12. Adjust the injection volume to 2-3nl and inject the caged-fluorescein directly into the cell's cytoplasm. Inject a minimum of 50 embryos per experiment.
13. Incubate embryos in the dark at 28.5°C in E3 to the desired developmental stage. If embryos older than 24 hours post fertilization are analyzed, add 0.1% phenylthiourea (PTU, Sigma, St. Louis, MO, cat. no. 22290-9) to inhibit pigmentation.

2. Embryo Mounting

1. Prepare fresh E2 solution (see solutions part; can be stored at 4°C for a week).
2. Coat 60 mm Petri dishes with a thin layer of 1% agarose dissolved in E2 medium.

3. Prepare 2% low melting point agarose (Ultra Pure LMP agarose, Invitrogen, Carlsbad, CA, cat. no. 16520100) for embedding: dissolve the agarose in sterile double distilled water by microwave heating until boiling. Keep the tube's cap open during heating. Aliquot 1 mL into microcentrifuge tubes and keep in a heating block at 72°C.
4. Remove the chorions of the embryos by gently pulling the chorion apart with sharp forceps (No. 5). Keep embryos in E2 solution on an agar-coated dish (see step 2). Dechorionated embryos should be handled with fire-polished glass Pasteur pipette as they may stick to polypropylene pipettes. Avoid exposing the dechorionized embryo to air as these embryos might be damaged by the liquid surface tension.
5. Collect the selected embryo in a small volume of E2 using a fire-polished glass Pasteur pipette, and place the embryo on 60 mm Petri dish. Mount a drop (~150 µL) of 2% agarose (step 3) beside the embryo. Quickly mix the two drops to obtain a homogenous final ~1% agarose mixture and using a dissecting microscope orient the embryo with its dorsal side facing the objective lens.
6. Wait until the agarose has solidified and add E2 medium until the embedded embryo is submerged in liquid. It is preferred to obtain a single embryo per dish as it may be released from the agarose after the photoactivation process.

3. Photoactivation

1. We use a Zeiss LSM 510 META NLO two-photon microscope equipped with a broadband tunable (700-1020nm) Mai Tai- HP-femtosecond laser from Spectraphysics.
2. Place the mounted embryo on a plate holder under the microscope. The fluorescent landmark in the embryo is visualized using 40x/0.8W Achroplan water immersed objective. For the following steps we use non-descanned detector (NDD) with band pass filter (BP) of 500-550nm.
3. As a prerequisite to the photoactivation process, obtain spatial fluorescent information of the region of interest by acquiring image series from the most ventral to dorsal part of the detected fluorescent signal with two-photon laser wavelength of 860nm (for GFP detection). To this end, sufficient resolution can be obtained using a span of 6µm between each acquired focal plane.
4. Select the target z- plane for photoactivation and bring this region into focus. Depending on the purpose of the experiment, the region of interest can be either within or outside the GFP visible landmark. Take a single image in this z-plane. Save the image and keep it open in a separate window.
5. Set the two- photon laser wavelength to 720nm and when the laser is in mode-locked open the 'Edit bleach' menu. Define the region of interest (ROI) for photoactivation according to the image taken at 860nm (Step 4) using the 'Define Region' menu. It is crucial to select the ROI on this image after shifting the laser wavelength to 720nm as the latter action changes the x,y positions of the observed field.
6. Adjust the following parameters for photoactivation in the 'Edit bleach' menu (see step 9): a) relative laser power; b) number of iterations; c) scan speed.
7. Press 'Bleach' in the 'Edit bleach' window and wait until the laser stops.
8. Switch back to 860nm, acquire a single plane image to evaluate the intensity of the resulting photoactivated material and a second series of image acquisition to evaluate the Z-span (thickness) of the activated domain.
9. Consider the following parameters for photoactivation:
    a. One should empirically determine the optimal conditions for photoactivation by varying two parameters: laser intensity and duration of photoactivation. The relative laser power can be controlled by the Acousto-Optic Modulator (AOM) transmission. The duration of photoactivation is a function of the number of iterations and scan speed.
    b. Determine the linear range of fluorescence intensity in order to avoid saturation of the uncaged fluorescence.
    c. There is a linear correlation between the obtained fluorescence intensity of the uncaged molecules and the z- axis span of the photoactivated clone[2].

4. Detection of the Uncaged Fluorescein

1. After photoactivation, raise the embryos in the dark at 28.5°C in E2 medium containing 0.1% PTU to prevent the appearance of skin pigmentation.
2. At the desired developmental stage, remove the embryo from the agarose under a dissecting microscope. Using a pointed scalpel, create a "V" shaped incision in the agarose near the embryo so that the "V" is pointing towards the embryo's head. Place a closed forceps at the point of the "V" near the head of the embryo and gently push open the forceps separating the agarose along the length of the embryo and releasing the embryo into the medium.
3. Collect the embryos with a fire-polished Pasteur pipette and fix with paraformaldehyde (PFA; 4% in 1 x PBS, PH 7.2) for either 3 hours at room temperature or overnight at 4°C.
4. Remove the fixative solution and rinse 2-3 times in PBST for at least 5 minutes at room temperature.
5. Wash embryos with 100% methanol for 10 min, replenish with fresh methanol and incubate at -20°C for at least 2 hr.
6. Rehydrate embryos by successive incubations (5 min each) in a series of diluted methanol in PBSTr: 80%, 60%, 40% and 20%. Wash 2 x 10 minutes in PBSTr.
7. Incubate for 30 minutes at room temperature in 500 µL of Blocking solution.
8. Add an anti-Fluorescein- alkaline phosphatase (AP) Fab fragments (Roche, Palo Alto, CA, cat. no. 11426338910), diluted 1:500 in Blocking solution and incubate overnight at 4°C (place microcentrifuge tubes on their sides).
9. Wash 5 x 15 minutes in PBST.
10. Wash for 5 minutes in 0.2M NaCl in TNT.
11. Wash for 5 minutes in 0.4M NaCl in TNT.
12. Equilibrate 3 x 5 minutes in freshly prepared Prestain solution.
13. Dissolve one Fast Red tablet (Roche, Palo Alto, CA, cat. no. 11496549001) in 2 mL Prestain solution. It is recommended to filter the solution using Whatman No. 2 paper to omit un-dissolved particles. Alternatively, spin down solution and use the clear supernatant liquid.
14. Add 500 µL Fast Red solution to each tube, incubate in the dark at room temperature, and monitor color development until the desired signal-to-background ratio is reached (20 minutes to several hours). If background develops, transfer to 4°C. After one hour of incubation, replenish with a new Fast Red staining solution.
15. Stop the staining reaction by 3 x 5 minutes washes in PBST.
16. Fix for 20 minutes in 4% PFA at room temperature.

17. Wash 2 x 5 minutes in PBST.
18. Clear embryos through a series of 25%, 50% and 75% glycerol in PBS until embryos are settled at the bottom of the tube. Embryos can be stored at 4°C for a few days.

5. Fluorescent Visualization of the Uncaged Area

1. Prepare a microscope slide for mounting: using a 1 mL syringe apply four spots of clear silicone grease on the slide, placed at a distance of ~15mm from each other. Mount the embryo at the center in ~200 µL of 75% glycerol and place a coverslip (no. 0; 18x18mm) on top and gently push it against the silicone until the glycerol solution occupies the surface between the slides.
2. Place the mounted embryo onto the stage and bring it into focus using a 40x objective. Acquire a confocal Z-series of images using 488nm Argon and 543nm HeNe lasers as the excitation source.
3. The z-axis span of the photoactivatated domain can be analyzed using 3D reconstruction of the uncaged embryo using an image analysis software.

6. Solutions

100 x E3	174mM NaCl, 21mM KCl, 12mM $MgSO_4$, 18mM Ca(NO3), 15mM HEPES, pH=7.4. Sterilize by filtration and store at 4°C
20 x E2	0.3M NaCl, 10mM KCl, 20mM $CaCl_2$*2HOH, 20mM $MgSO_4$*7HOH, 3mM KH_2PO_4, 0.8mM Na_2HPO_4*2HOH, filtrate and store in room temperature
1 x E2	Dilute 20 x E2 in double distilled water and add freshly made $NaHCO_3$ (to final concentration of 0.7mM), Penicillin and Streptomycin (dilute pen strep solution to a final concentration of 1 x which contains 100U of penicillin and 100mg streptomycin per mL (Invitrogen, Carlsbad, CA, cat. no. 15140-122))
PBST	0.1% TWEEN-20 in PBS
PBSTr	0.3% Triton in PBS
Blocking solution	10% BSA, 0.3% Triton, 1% DMSO in PBS
TNT	100mM Tris-HCl pH-7.5, 150mM NaCl, 0.5% TWEEN-20 in double distilled water
Prestain solution	100mM Tris-HCl pH-8.2, 0.4M NaCl, 0.1% TWEEN-20 in double distilled water

7. Representative Results

An example of the use of two-photon microscopy to photoactivate agents in a living zebrafish embryo is presented. Using a similar approach we previously traced the lineage of the photoactivated labeled neural progenitors in the zebrafish brain[2,3]. Figure 1 shows photoactivation of a caged fluorescein conjugated tracer dye in anterior neural plate of zebrafish embryos carrying a *neurog1::gfp* reporter transgene, which served as a live intrinsic landmark.

Embryos were injected with caged fluorescein at the one cell stage and embedded in agarose at bud stage. At 3- 5-somite stage the spatial coordinates were measured in the *neurog1::gfp* transgenic embryo to determine the region of interest (ROI) for uncaging (Figure 1A, A'). We uncaged the fluorescein lineage tracer in a specific domain of the neural plate (Figure 1B, B'). Subsequently, the fate of this given labeled neural progenitor subdomain was determined at prim5 (24 hours post fertilization) stage by immunostaining of the uncaged fluorescein (Figure 1C, C').

The extent of two-photon laser uncaging, i.e. the intensity of the uncaged fluorescence and the thickness of the photoactivated domain (z-span), can be controlled by adjusting two parameters: laser intensity and duration. The latter can be controlled by adjustment of the iteration number and scan speed (see part 3, step 9; Russek-Blum, 2009). In the example shown in Figure 1, we used relative laser power of 12% AOM transmission, 20 iterations and scan speed of 25.6 µsec/ pixel. Using these settings we labeled a ROI containing 9-10 cells and a photoactivated z- span of 30µm (~1-2 cell rows).

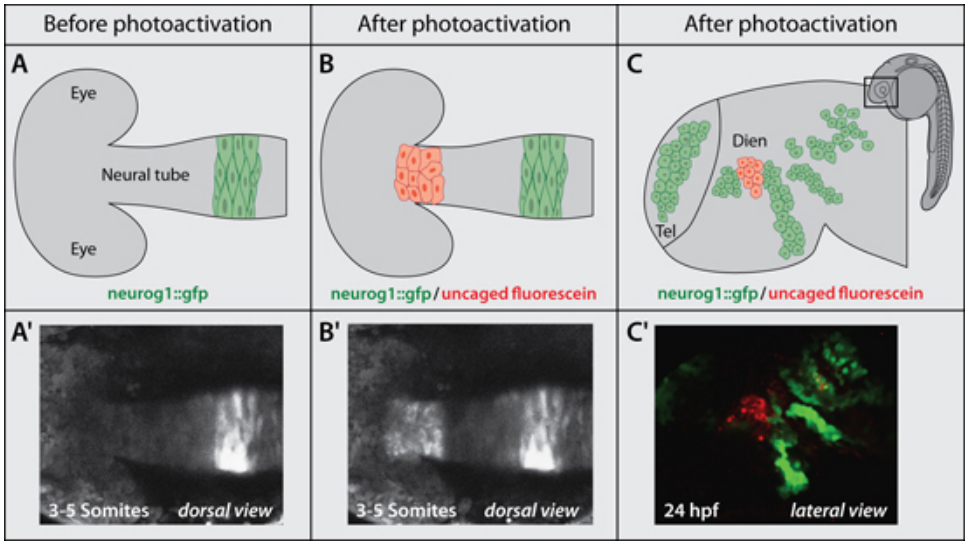

Figure 1. A Representative result of photoactivation of caged fluorescein in live zebrafish embryo using two-photon microscopy.

Schematic illustration (A-C) and representative images (A'-C') of the photoactivation process. Live zebrafish embryo (A, A') expressing GFP under the control of neurogenin-1 promoter (neurog1::gfp) that was injected with caged fluorescein tracer dye at the 1 cell stage. At the 3-5-somite stage, a discrete area of the forebrain primordium (diencephalon) was photoactivated and the uncaged fluorescein tracer dye could be detected (B, B'). Subsequent to the photoactivation procedure, the embryo was incubated at 28.5°C and brain cells containing the uncaged fluorescein were traced by anti-Fluorescein immunostaining at 24 hours post fertilization (hpf; C, C'). Dien., Diencephalon; Tel., Telencephalon.

Discussion

Photo-activatable compounds are molecules whose function is masked until they are illuminated with a specific wavelength (usually UV), inducing a photochemical reaction that converts the molecules into a biologically or chemically active state. These probes provide very powerful tools in cell biology research, since the activation can be precisely controlled temporally and spatially by limiting their exposure to light.

The significant advantage of multi-photon microscopy is its relatively deep optical penetration and reduced nonspecific phototoxicity. For the activation process, two photons of low energy must be absorbed by the photo-activatable compound at the same time. As the probability of such an event is dependent on the photon density, the activation remains restricted to the focal plane. The activation region can therefore be selectively manipulated in a defined volume within the tissue.

We present a protocol for photoactivation of caged fluorescein using two-photon microscopy in live zebrafish embryos. In the specific example shown herein we use two-photon microscopy to induce caged-fluorescein photolysis in order to mark cells at early embryonic stages and thereafter trace the lineage of the labeled cells in the developing zebrafish brain. In comparison to photoactivation by laser and flash-lamp, which results in the activation of the tracer dye in a few tens of cells and lack of resolution in the z axis[4,5], two-photon-based photoactivation can reach a spatial resolution of a few micrometers obtaining axial resolution of a proximally one to two cell rows [2,3].

The procedure reported herein can be utilized for the activation of a variety of compounds at a single cell resolution in live specimens. For example, the Kaede protein can be similarly used to label cells following its photoconversion from a green to red fluorescenct protein[6,7]. Other light-activated proteins that can be applied include the light-gated ion channel, Channelrhodopsin, which can modulate neuronal activity[8] and photosensitizers such as KillerRed, which induces cell death upon light irradiation[9].

A variety of caged molecules have been reported, allowing modulation of physiological processes by manipulation of extracellular and intracellular compounds[10]. These include, active neurotransmitters (e.g. glutamate[11,12]) and second messengers (e.g. calcium[13]) and steroid hormones (e.g. retinoic acid[14]). Lastly, photo-mediated control of gene activation and silencing in zebrafish has been introduced. Two-photon-based photoactivation of caged antisense oligonucleotides (morpholinos) and RNAs can be used for gene knockdown and gain-of-function in restricted cell population of a live zebrafish embryo[15,16].

In sum, two- photon- based photo-activation in live zebrafish embryos is: 1) Precise- one to two cell rows along x,y,z axes. 2) Quantitative- the extent of photoactivation can be controlled. 3) Versatile- may be applied for a variety of photo-convertible proteins.

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