Video Article

Lineage Labeling of Zebrafish Cells with Laser Uncagable Fluorescein Dextran

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Abstract

A central problem in developmental biology is to deduce the origin of the myriad cell types present in vertebrates as they arise from undifferentiated precursors. Researchers have employed various methods of lineage labeling, such as DiI labeling[1] and pressure injection of traceable enzymes[2] to ascertain cell fate at later stages of development in model systems. The first fate maps in zebrafish (*Danio rerio*) were assembled by iontophoretic injection of fluorescent dyes, such as rhodamine dextran, into single cells in discrete regions of the embryo and tracing the labeled cell's fate over time[3-5]. While effective, these methods are technically demanding and require specialized equipment not commonly found in zebrafish labs. Recently, photoconvertable fluorescent proteins, such as Eos and Kaede, which irreversibly switch from green to red fluorescence when exposed to ultraviolet light, are seeing increased use in zebrafish[6-8]. The optical clarity of the zebrafish embryo and the relative ease of transgenesis have made these particularly attractive tools for lineage labeling and to observe the migration of cells *in vivo*[7]. Despite their utility, these proteins have some disadvantages compared to dye-mediated lineage labeling methods. The most crucial is the difficulty we have found in obtaining high 3-D resolution during photoconversion of these proteins. In this light, perhaps the best combination of resolution and ease of use for lineage labeling in zebrafish makes use of caged fluorescein dextran, a fluorescent dye that is bound to a quenching group that masks its fluorescence[9]. The dye can then be "uncaged" (released from the quenching group) within a specific cell using UV light from a laser or mercury lamp, allowing visualization of its fluorescence or immunodetection. Unlike iontophoretic methods, caged fluorescein can be injected with standard injection apparatuses and uncaged with an epifluorescence microscope equipped with a pinhole[10]. In addition, antibodies against fluorescein detect only the uncaged form, and the epitope survives fixation well[11]. Finally, caged fluorescein can be activated with very high 3-D resolution, especially if two-photon microscopy is employed [12,13]. This protocol describes a method of lineage labeling by caged fluorescein and laser uncaging. Subsequenctly, uncaged fluorescein is detected simultaneously with other epitopes such as GFP by labeling with antibodies.

Video Link

The video component of this article can be found at https://www.jove.com/video/2672/

Protocol

1. Synthesis of Caged Fluorescein Dextran

1. Measure out 3.5 - 4 mg of aminodextran and add into the Invitrogen-supplied tinted tube containing 1 mg of CMNB-caged fluorescein SE. In our hands this ratio gives an average loading of ~ 2.5 dye molecules per dextran.
2. Add 500 µL of 0.1 M $Na_2B_4O_7$ (sodium borate) buffer to the tube.
3. Cap and vortex for 30 seconds to dissolve the aminodextran and caged fluorescein.
4. Let react overnight on a vortexing mixer.
5. Twist off the bottom closure on a Zeba spin column and loosen the cap. Mark a dot on the column with a felt-tip pen. Place column in a 15-mL conical tube.
6. Centrifuge at 1000 x g for 2 min with the dot facing the center of the rotor. Use column immediately after compacting.
7. Place compacted column in a new 15-mL conical tube.
8. Pool reaction mixture and transfer to the center of the compacted column resin bed. Replace cap.
9. Centrifuge at 1000 x g for 2 min with the dot facing the center of the rotor. After the spin, the eluent and the top of the column will be roughly the same shade of yellow in color.
10. Transfer the yellow eluent (~350 µL) to a 1.5-mL microcentrifuge tube.
11. Lyophilize to dryness in a speedvac. Cover the speedvac with foil.
12. Re-suspend the resulting ~ 2-3 mg of pale yellow foam in water to a final concentration of 1% w/v.
13. Store this solution at -20 °C in foil-covered tubes. It is a good idea to aliquot the dextran solution to prevent repetitive freeze-thawing.

2. Injection of Caged Fluorescein Dextran Into Zebrafish Embryos

1. Dilute 1% w/v stock of caged fluorescein dextran 1:5 or 1:10 in 0.2M KCl.
2. Inject 0.5 - 1.0nL of caged fluorescein solution into the yolk of one cell stage embryos using a pressure injector. Once injected, clutches of embryos must be kept in the dark at 28.5°C to reduce non-specific uncaging.
3. Manually remove chorions from embryos.
4. Anesthetize embryos in 4% tricaine.
5. Embryos will be mounted in 0.8% agarose in 35mmx10mm petri dish, then covered with egg water.
6. Equilibrate melted agarose in 50°C heat block for at least 15 minutes.
7. Using a glass pipette, draw up one embryo in a minimal amount of water and release it into the agarose. **Note:** It is important to cool the tube of agarose in your hand for approximately 30 seconds prior to putting the embryo into the tube to avoid killing the embryo.
8. Pipette the embryo from the tube, along with approximately 50µL of agarose and eject the contents onto the center of the petri dish.
9. Quickly orient the embryo using a plastic thread with the cells to be uncaged facing up. **Note:** The agarose with cool quickly so be hasty with orienting the embryo. Embryos can be damaged if manipulated after the agarose becomes rigid.
10. Allow the agarose to completely solidify (this takes a few minutes) and fill the dish two thirds full with egg water containing 4% tricaine and 0.003% PTU.

3. Laser Uncaging of Fluorescein Dextran

1. The microscope used for uncaging must have a 40X water immersion objective.
2. We use a Photonics Instruments laser with the 365 nm dye cell and the 70%/30% split dichroic mirror.
3. Prior to uncaging, the laser must be made parfocal with the objective and attenuated to the appropriate power.
4. To focus the laser, place a mirrored glass slide under the objective and focus the objective until scratches in the slide are visible.
5. Adjust laser attenuator until the laser can produce a clearly visible scratch in the mirror with a single pulse.
6. Adjust the objective focus up and down. If the laser can produce a scratch when the objective is out of focus, adjust the laser focus ¼ turn up or down. Repeat until the laser can scratch the mirror only when the objective is focused.
7. Place mounted embryo under the objective and focus on the area to be uncaged.
8. To uncage, focus on a cell of interest and pulse it 10-20 times at 2-3 Hz.
9. After uncaging, free the embryo by peeling agarose away with forceps and putting them in egg water containing 0.003% PTU. Let embryos develop in a light-tight box until they are fixed.

4. Antibody Labeling and Detection of Uncaged Fluorescein Dextran

1. Fix embryos in Ab fixative (4% paraformaldehyde, 0.3mM $CaCl_2$, 8% sucrose, 1X Phosphate Buffered Saline, pH 7.3) for two to four hours at room temperature (RT) or overnight (O/N) at 4°C. Keep embryos in a light tight box or foil-wrapped tube for all labeling steps.
2. Remove fixative and replace with 100% MeOH, leave for 10 minutes at RT, remove MeOH and replace with fresh 100% MeOH.
3. Store in MeOH at -20°C for at least 30 minutes. **Note:** You can store in MeOH for up to two weeks before epitopes begin to degrade.
4. Rehydrate embryos with 5 minute washes at RT of 75% MeOH/1X Phosphate buffered saline with 0.1% Tween 20 (1X PBSTw), then 50% MeOH/50% 1XPBTw, then 25% MeOH/75% 1X PBSTw.
5. Wash three times for 5 minutes in 1XPBSTw.
6. If embryos are >24 hours old, permiabilize embryos with 10 µg/mL proteinase K in 1XPBSTw. Permiabilization time is dependent on stage. Five minutes in proteinase K is sufficient if the embryos are between 24 and 48 hours post-fertilization (hpf). More time may be required if for older larvae. Re-fix in Ab fixative for 20 minutes at RT
7. Wash five times for 5 minutes in 1XPBSTw at RT.
8. Incubate in block solution (1XPBS, 0.1% Triton x100, 10% sheep serum, 10% Bovine serum albumen, 1% Dimethyl sulfoxide) for 1-2 hours at RT.
9. Dilute primary antibodies (anti-fluorescein plus antibodies against cell-type specific markers) in blocking solution.
10. Incubate embryos O/N at 4°C in solution containing primary antibodies.
11. Wash four times for 20 minutes in 1XPhosphate Buffered Saline with 0.1% Triton x100 (PBSTx) at RT.
12. Dilute fluorescently-labeled secondary antibodies (usually 1:300) in blocking solution.
13. Incubate O/N at 4°C in solution containing secondary antibodies.
14. Wash four times for 20 minutes in 1XPBSTx at RT.
15. Clear embryos in 50% glycerol/50% 1XPBS for at least two hours at RT, then remove glycerol/PBS mixture and add 100% glycerol and let stand O/N at 4°C.

5. Representative Results:

After immunolabeling, there should be an enriched area of staining in the cells that were uncaged (Figure.1). It is not unusual to see a relatively high signal to noise ratio in >48 hour old zebrafish, due to spontaneous uncaging of the fluorescein.

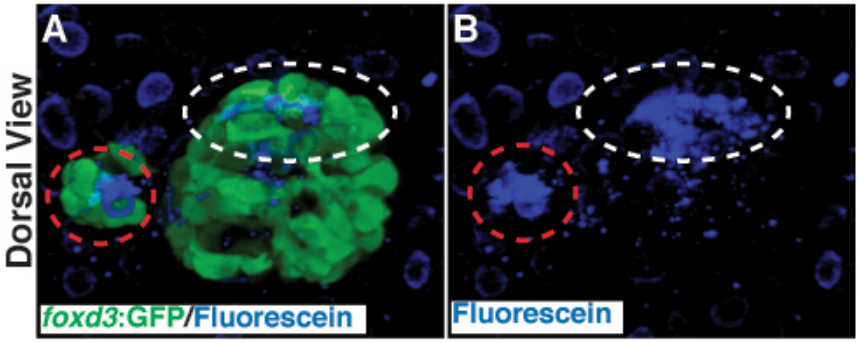

Figure 1. Lineage labeling of zebrafish pineal complex. At 24 hpf, a portion of the anterior pineal anlage, indicated by *foxd3*:GFP expression, was uncaged using UV laser pulses. **A)** By 48hpf, a left-sided cluster of cells called the parapineal (red circle) has emerged from the uncaged domain (white circle). **B)** Uncaged fluorescein signal is enriched in the parapineal (red circle) and the pineal organ (white circle).

Discussion

As described, this protocol provides a relatively quick lineage labeling method in zebrafish that is built upon the commonly used techniques of microinjection, microscopy, and immunofluorescence. We have found that laser uncaging to be the most efficient and cost effective way to uncage fluorescein in a localized fashion. This method could be used to lineage label with experimental endpoints as late as 4 dpf. However, as cells divide, the caged-fluorescein concentration per cell eventually decreases beyond detectable levels. In addition, there have been reports of spontaneous, non-specific uncaging of fluorescein in zebrafish larvae [11]. As such, detection of uncaged fluorescein is most effective in early larval stage (48-72 hpf) or earlier.

Our preferred uncaging method is by use of a pulsed nitrogen laser. These are commonly used for cell ablations experiments and are sufficiently accurate to uncage singe cells or small groups of cells[11-12]. However, uncaging can also be accomplished using a confocal microscope; two-photon confocal microscopes have been used to achieve very precise uncaging[12]. On the other end of the spectrum, if precision is not required then a laser is dispensable. An epifluorescent microscope equipped with a DAPI filter set can be used to uncage through a small pinhole[11].

We have found that detection of uncaged fluorescein using a primary antibody and fluorescent secondary antibody that emits at a red or far-red wavelength (e.g. Alexa 633) best suits our needs. This allows us to distinguish uncaged fluorescein from GFP expressed by a transgene (Figure 1).

Disclosures

No conflicts of interest declared.

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