Video Article

Single Cell Fate Mapping in Zebrafish

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Abstract

The ability to differentially label single cells has important implications in developmental biology. For instance, determining how hematopoietic, lymphatic, and blood vessel lineages arise in developing embryos requires fate mapping and lineage tracing of undifferentiated precursor cells. Recently, photoactivatable proteins which include: Eos[1, 2], PAmCherry[3], Kaede[4-7], pKindling[8], and KikGR[9, 10] have received wide interest as cell tracing probes. The fluorescence spectrum of these photosensitive proteins can be easily converted with UV excitation, allowing a population of cells to be distinguished from adjacent ones. However, the photoefficiency of the activated protein may limit long-term cell tracking[11]. As an alternative to photoactivatable proteins, caged fluorescein-dextran has been widely used in embryo model systems[7, 12-14]. Traditionally, to uncage fluorescein-dextran, UV excitation from a fluorescence lamp house or a single photon UV laser has been used; however, such sources limit the spatial resolution of photoactivation. Here we report a protocol to fate map, lineage trace, and detect single labeled cells. Single cells in embryos injected with caged fluorescein-dextran are photoactivated with near-infrared laser pulses produced from a titanium sapphire femtosecond laser. This laser is customary in all two-photon confocal microscopes such as the LSM 510 META NLO microscope used in this paper. Since biological tissue is transparent to near-infrared irradiation[15], the laser pulses can be focused deep within the embryo without uncaging cells above or below the selected focal plane. Therefore, non-linear two-photon absorption is induced only at the geometric focus to uncage fluorescein-dextran in a single cell. To detect the cell containing uncaged fluorescein-dextran, we describe a simple immunohistochemistry protocol[16] to rapidly visualize the activated cell. The activation and detection protocol presented in this paper is versatile and can be applied to any model system.

Note: The reagents used in this protocol can be found in the table appended at the end of the article.

Video Link

The video component of this article can be found at http://www.jove.com/video/3172/

Protocol

1. Synthesis of caged fluorescein-dextran (adapted from Ref. 14)

1. Prepare a 0.1 M solution of sodium borate diluted in ddH_2O.
2. Measure 4 mg of 10 kDa aminodextran and add it to the CMNB-caged fluorescein tube.
3. Add 500 µL of the prepared sodium borate solution (step 1.1) to the CMNB-caged fluorescein tube. Cap the tube and vortex for 30 seconds to dissolve the mixture.
4. Allow the mixture to react overnight on a nutator at room temperature. Cover the tube with metal foil to prevent exposure of the mixture to ambient light.
5. Obtain a Zeba desalt spin column and twist off the bottom cap. Mark a dot on the column, which will be used to orient the column when centrifuging. Place the column in a 15 mL conical Falcon tube. Note: The solution in the spin column is column buffer.
6. Loosen the top cap on the desalt spin column. Place the 15 mL conical Falcon tube that houses the desalt spin column in a centrifuge. Orient the spin column with the marked dot (step 1.4) facing the center of the centrifuge rotor. Centrifuge the tube at 1000 x g for 2 minutes at room temperature.
7. After centrifugation, remove the desalt spin column from the Falcon tube. Discard both the collected flow through and the 15 mL conical tube. Obtain a new 15 mL conical Falcon tube, and place the desalt spin column in the new Falcon tube. Note: After centrifuging, the column resin bed should appear compacted. Use the column immediately.
8. Obtain the reacted mixture (caged fluorescein-dextran) in step 1.3. Remove the cap from the spin column and aspirate the reacted mixture and apply it slowly to the center of the desalt spin column. After applying the reacted mixture, replace the cap on the spin column.
9. Place the Falcon tube that houses the desalt spin column in a centrifuge. As done in step 1.5, orient the spin column with the marked dot facing the center of the centrifuge rotor. Centrifuge the tube at 1000 x g for 2 minutes at room temperature.
10. After centrifugation, a yellow mixture (caged fluorescein-dextran) will be collected (approximately 400 µL) in the 15 mL Falcon tube. Transfer the caged fluorescein-dextran mixture to a 1.5 mL microcentrifuge tube.
11. Immediately cover the tube with metal foil to prevent exposure of the mixture to ambient light. Lyophilize the caged fluorescein-dextran mixture in a speedvac. Lyophilization of 350 µL should take approximately 4 hours.

12. After complete lyophilization, resuspend the powder (about 3 mg) in ddH_2O to a final concentration of approximately 1 % w/v. Mix by briefly vortexing to suspend the powder.
13. Aliquot the caged fluorescein-dextran into separate metal foil covered tubes, and store them at -20 °C.

2. Harvesting of embryos and microinjection of caged fluorescein-dextran

* This procedure uses the zebrafish as the animal model system.

1. On the day before injections, set up zebrafish crosses either 1:1 male-to-female or 2:1 male-to-female in breeding tanks with dividers.
2. The next morning change the breeding tank water and remove the dividers. Immediately prepare a 5 µL 1/10 diluted working solution of caged fluorescein-dextran in either 1 X Danieau media (see Preparation of Solutions) or ddH_2O. Cover the working solution tube with metal foil to prevent exposure to light.
3. Pipette the prepared caged fluorescein-dextran solution (step 2.2) into the needle. Before cutting the needle tip to an appropriate size under the dissection microscope, place a piece of transparent yellow filter in front of the white light source. The yellow filter will prevent spontaneous uncaging of the fluorescein-dextran during injections. When looking through the ocular eye piece, the light source should appear yellow.
4. Collect embryos and pipette them into a microinjection mold tray. Set the microinjection time to deliver 3 to 4 nL of caged fluorescein-dextran. Under the dissection microscope, orient the embryos with forceps and inject (3 to 4 nL) caged fluorescein-dextran into the base of the blastomere cells (blastomere-yolk interface). The embryo stage for injection should be 1- to 2-cell stage.
5. After injection, remove the embryos from the microinjection mold tray and place them in a clean petri dish containing fresh fish water. Methylene blue may be added to the fish water to prevent fungal growth at a final concentration of at 0.02 % w/v. Cover the petri dish with metal foil to prevent uncaging of the injected caged fluorescein-dextran.
6. Raise the injected embryos to a stage when the cells of interest need to be photoactivated. For photoactivation, prepare a 0.6 % low melting agarose solution in ddH_2O. Depending on the embryo stage, Tricaine (MS222) can be added to the low melting agarose. To do this, dilute 0.02% Tricaine in low melting agarose to a final concentration of 0.0014 %.
7. As soon as the embryos reach the appropriate developmental stage, dechorionate the embryos in 1 X Danieau media with forceps. When dechorionating, make sure that a transparent yellow filter is placed in the path of the white light source (see step 2.3).
8. Heat the 0.6 % low melting agarose solution to 37 °C. Pipette 1 mL of low melting agarose into the well of a LabTek chambered coverglass slide. Carefully mount 3 to 4 embryos in low melting agarose and orient them with forceps with the cells to be photoactivated facing downward against the bottom coverslip.
9. Allow the agarose to solidify. After solidification, pipette 1 mL of 1 X Danieau media over the agarose.
10. Repeat steps 2.8 to 2.9 for more embryos.

3. Two-photon activation of caged fluorescein-dextran

* This procedure assumes that the embryo expresses reporter GFP. The embryo is viewed using the argon ion (488 nm) laser source attached to the LSM 510 META NLO microscope. A single GFP positive cell is selected and photoactivated using the Mai Tai laser (femtosecond laser) coupled to the LSM 510. The procedure is the same for non-fluorescent embryos. Alternatively, white light can be used to find the cell to be activated, followed by activation using the Mai Tai laser source.

1. Load the LSM 510 software. Place a transparent yellow filter in the white light beam path.
2. Select Scan New Image and click Start Expert Mode.
3. Select the Laser tab. Turn on the argon ion (488 nm) and Mai Tai (femtosecond) laser sources.
4. Set the Mai Tai wavelength to 740 nm.
5. Select the config tab. Set the optical path configuration for the argon ion and Mai Tai laser.
6. Select the Scan tab. Change the objective to 20X/0.8.
7. Set the max scan speed by clicking Max.
8. Set Mode, Method, and Number to Line, Mean, and 1.
9. Under the Channels tab deselect all laser sources except for the Mai Tai.
10. Place a power meter in the optical beam path.
11. Select the Cont button to activate continuous scanning.
12. Adjust the transmission % until the power meter reads an average laser power of 47 mW.
13. Stop the scan by selecting the Stop button.
14. Under the Channels tab deselect the Mai Tai laser source and select the argon ion (488 nm).
15. Select the Fast xy button. Under the Channels tab adjust the pinhole, detector gain, amplifier offset, and amplifier gain for the argon ion laser to achieve the best image quality.
16. Using the stage controller, find and focus on the cell to activate.
17. Click the Stop button.
18. Using the zoom tool, zoom in on the activated cell until the zoom factor under the Mode tab displays 50 to 70.
19. Stop the scan by selecting the Stop button.
20. Under the Channels tab deselect the argon ion laser (488 nm) and select the Mai Tai laser.
21. Under the Mode tab set the scan speed to 31.46 sec.
22. Select the Single button to start the scan for two-photon activation of caged fluorescein-dextran of the selected cell.
23. After activation, deselect the Mai Tai laser under the Channels tab, and reselect the argon ion laser (488 nm).
24. Set the zoom factor under Mode to 1, and click the Max button under the Speed heading to set the fastest scan speed.
25. Select Fast xy to review the photoactivated region. Check that the photoactivated region is not laser ablated. **For more information about laser ablation, please refer to the discussion section.**
26. Repeat the above steps for other embryos.

4. Immunodetection of uncaged fluorescein-dextran (adapted from Ref. 16)

1. After photoativation, place a transparent yellow filter in front of the white light source and manually remove each embryo from low melting agarose using sharp forceps. Place all removed embryos in a new petri dish containing fresh 1 X Danieau media. Cover the petri dish with metal foil to prevent exposure of embryos to light.

2. If needed, rear the embryos to the desired developmental stage. In the meantime, prepare a 4 % paraformaldehyde solution (see Preparation of Solutions). Aliquot 1.5 mL of prepared paraformaldehyde into a microcentrifuge tube.
3. After the embryos have reached the developmental stage of interest, pipette the embryos into the microcentrifuge tube (from step 4.2). Cover the tube with metal foil to prevent exposure to light.
4. Nutate the tube overnight at 4°C. For the next day prepare graded ethanol (EtOH) solutions in phosphate buffer saline (PBS) and ddH_2O; 30 % EtOH:PBS, 50 % EtOH:PBS, 70 % EtOH:ddH_2O, and 100 % EtOH.
5. The next day, remove the embryos from 4°C. Remove the metal foil covering the tube and quickly aspirate off paraformaldehye (leave behind a small volume that is enough to cover the embryos). Pipette 1.5 mL of 30 % EtOH:PBS into the tube. Re-cover the tube with metal foil. Nutate the tube at room temperature for 10 mins.
6. Repeat step 4.5 using 50, 70, and 100 % graded EtOH solutions. After the 100 % EtOH wash, rinse the embryos again in 100 % EtOH for 10 mins.
7. After the washes, store the embryos overnight at -20 °C. For the next day prepare phosphate buffered tween (PBT; see Preparation of Solutions), 5 X blocking buffer (see manufacturer protocol for preparation), maleic acid (protocol for preparation can be found in the blocking buffer protocol), 10 mg/ml proteinase K, and a 10 X antibody solution (Anti-fluorescein-AP; see Preparation of Solutions).
8. Also prepare 2 % lamb serum (LS; see Preparation of Solutions) diluted in PBT. Store the antibody at 4 °C overnight shaking on a nutator. The 2 % LS can be kept at 4 °C.
9. The next day remove the embryos from -20 °C. Remove the metal foil covering the tube and quickly aspirate off 100 % EtOH. Add 1.5 mL of 70 % EtOH:ddH_2O to the tube. Re-cover the tube with metal foil. Nutate the tube at room temperature for 10 mins.
10. Repeat step 4.8 using 50 and 30 % graded EtOH solutions, and 100 % PBT. After the 100 % PBT wash, rinse the embryos 3 more times in 100 % PBT for 10 mins.
11. If the embryos are older than 24 hours, proteinase K treat them. If not, proceed to step 4.13. To do this, dilute the prepared proteinase K (step 4.7) 1:1000 in PBT. Incubate the embryos in proteinase K for 10 mins, or longer if the embryos are older. Keep the embryos covered with metal foil to prevent exposure to light.
12. After proteinase K treatment, wash the embryos 5 times in PBT for 10 mins. After washing, fix the embryos in 4 % paraformaldehyde for 30 mins at room temperature on a nutator. Afterward, immediately wash the embryos 5 times in PBT for 10 mins. Keep the embryos covered with metal foil to prevent exposure to light.
13. Make blocking buffer by diluting 5 X blocking buffer in maleic acid buffer to 1 X. Remove the embryos from PBT and place them in 1 X blocking buffer. Cover the embryos with metal foil and nutate them at room temperature for 5 to 6 hours.
14. After 5 to 6 hours of blocking, heat shock the embryos at 65 °C for 15 to 20 mins.
15. Obtain the antibody solution and the 2 % LS prepared the previous day. Centrifuge the antibody solution at max rpm. Transfer the aqueous phase to the 2 % LS tube. Invert the tube to mix.
16. Transfer the embryos from blocking buffer to the LS-antibody mixture. Keep the embryos covered with metal foil to prevent exposure to light. Nutate the embryos at 4 °C overnight.
17. The next day wash the embryos at room temperature 6 times for 15 mins in PBT. Prepare AP buffer (see Preparation of Solutions).
18. Wash the embryos at room temperature 2 times for 10 mins in AP buffer. Prepare NBT/BCIP developing solution (see Preparation of Solutions).
19. Transfer the embryos to NBT/BCIP. Allow the stain to develop in the dark.
20. Stop the development by washing the embryos 3 times in PBT for 5 mins each.
21. For image acquisition mount the embryos in 0.6 % low melting agarose or 3 % methylcellulose, and acquire images using an inverted or upright compound microscope.
22. Photoactivated samples can be stored for future analysis (staining remains stable) by dehydrating them in a graded EtOH wash. Follow steps 4.4 through 4.7 for dehydrating the samples.

5. Representative Results:

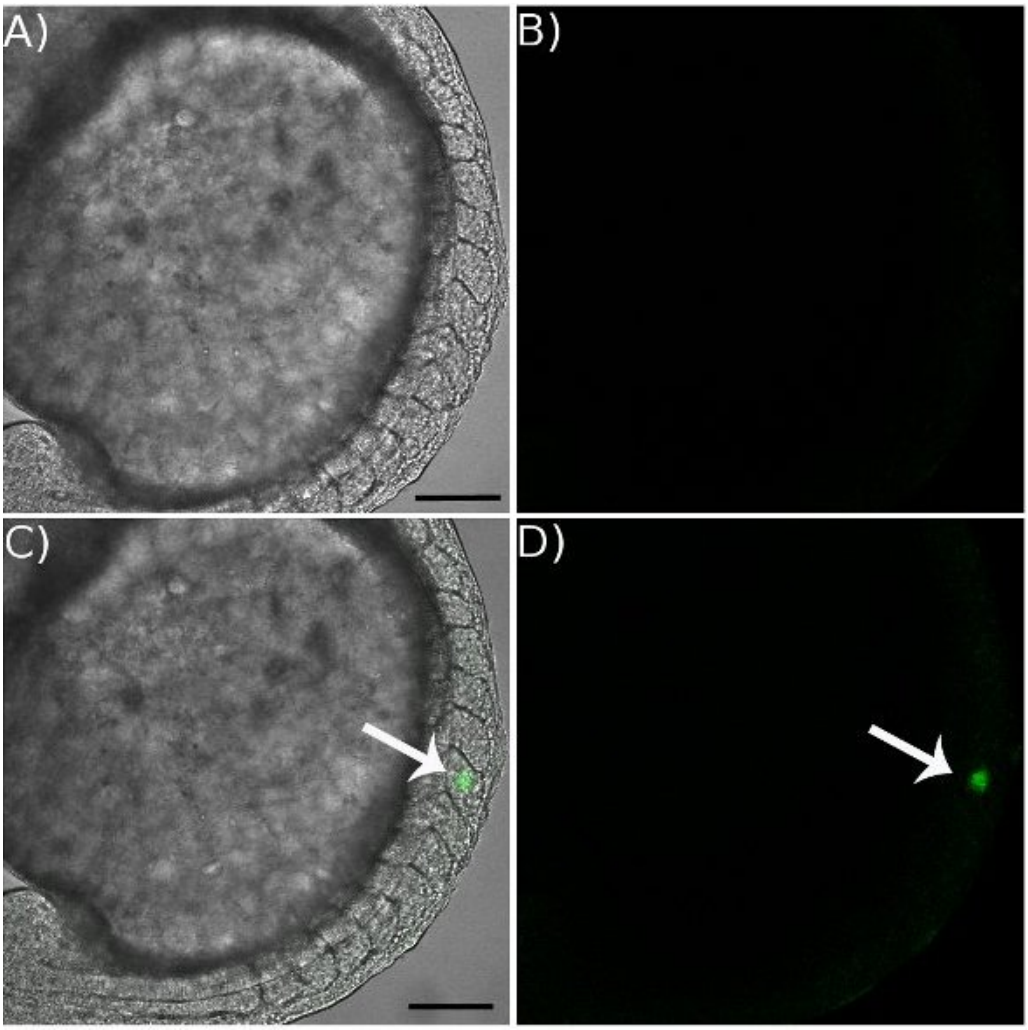

Figure 1. Photoactivation of caged fluorescein-dextran. (A,B) Brightfield and fluorescence image of a lateral view zebrafish embryo at the 13/14-somite stage before photoactivation. (C,D) The embryo was photoactivated at the13/14-somite stage in one of the somites (arrow) with a wavelength of 740 nm, scan time of 31 secs, and an average laser power of 47 mW. Post-activation, (d) fluorescence from uncaged fluorescein-dextran is observed. Orientation: Dorsal (right), Ventral (left). Scale bar 100 µm.

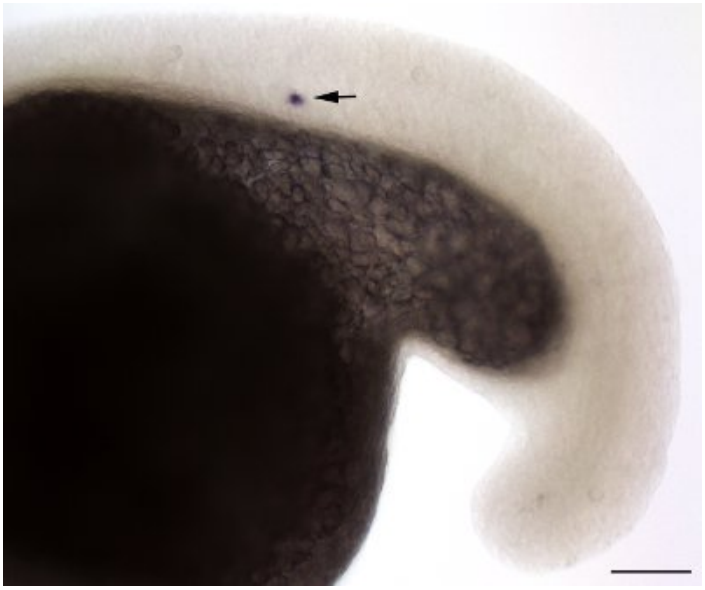

Figure 2. Immunodetection of caged fluorescein-dextran. Figure 2 depicts the immunostaining of uncaged fluorescein-dextran in an 18/19 hpf zebrafish embryo. At the 10-somite stage a small region of the embryo was activated in the lateral plate mesoderm using the same laser parameters stated in Figure 1. Using the immunodetection procedure, the arrow identifies the activated region with minimal background staining. Orientation: Dorsal (top), Ventral (bottom). Scale bar 100 µm.

Discussion

This paper describes a versatile method for single cell fate mapping based on the photoactivation of caged fluorescein-dextran. Uncaging of caged fluorescein-dextran in single cells is achieved using two-photon absorption from a Mai Tai femtosecond laser coupled to the Zeiss LSM 510 META confocal microscope. Uncaged fluorescein-dextran in photoactivated cells is rapidly visualized using a simple immunohistochemistry procedure.

In this protocol the two-photon absorption wavelength for photoactivation of caged fluorescein-dextran is 740 nm. This wavelength was experimentally determined by photoactivating caged fluorescein-dextran injected wildtype embryos. The laser excitation wavelength was varied from 600 to 800 nm, and the presence or absence of fluorescein fluorescence post-activation was qualitatively determined. We found that 740 nm produced the strongest fluorescein signal following activation. Ideally, 740 nm should work for all model systems; however, we advise testing a range of wavelengths to determine the optimal two-photon excitation wavelength for your sample.

In caged fluorescein-dextran injected wildtype embryos, for each two-photon excitation wavelength tested we also varied the average laser power. For an excitation wavelength of 740 nm, an average laser power of 47 mW produced a stronger fluorescein signal at the activated region in comparison to lower average laser powers. Higher average laser powers did not produce stronger fluorescein signals, but induced ablation of the tissue. Since femtosecond laser pulses are efficient at ablating biological tissue[15], we recommend determining the threshold average laser power for photoactivation that avoids tissue ablation.

Two-photon absorption occurs within a small interaction volume. To ensure proper activation of caged fluorescein-dextran, the cell must be brought into proper focus. In the above protocol, GFP positive cells were simultaneously imaged under white light to confirm cell focus. Therefore, white light acquisition in addition to other channels is advisable. After confirming cell focus, our protocol suggests magnifying the activated cell. A zoom factor of 50 to 70 is suggested, however, this value depends on the size of the chosen cell. Increasing the zoom isolates the activated cell from adjacent cells, and limits the scan area for two-photon activation. Ideally, the scan area should cover a large area of the cell.

Detection of single activated cells requires that the background staining be minimal. In the above immunodetection protocol it is important that the sample is blocked in blocking buffer for 5 to 6 hours (step 4.13). When developing with NBT/BCIP, uncaged fluorescein-dextran should become visible in 10 to 20 mins. If background staining is strong, we suggest a longer blocking time and additional washes to remove the antibody (step 4.17).

Figures 1 and 2 provide representative results of photoactivation and immunodetection. In Figure 1, early 1- to 2-cell stage wildtype embryos were injected with caged fluorescein-dextran. The embryos were raised to mid somitogenesis and mounted in low melting agarose in the lateral orientation. Figure 1(a,b) depict brightfield and fluorescent images of the embryo before photoactivation. Evidence that the embryo remained uncaged is demonstrated by the absence of fluorescein fluorescence in Figure 1(b). A small region within a somite was activated with a wavelength of 740 nm for 31 secs using an average laser power of 47 mW, Figure 1(c; arrow). Post-activation, two-photon uncaging of fluorescein-dextran occurred as shown by the fluorescence from the activated area, Figure 1(d; arrow). Activation was not accompanied by laser ablation, as the somitic tissue remained intact, Figure 1(c). Figure 2 demonstrates the immunodetection of uncaged fluorescein-dextran. A small region in the lateral plate mesoderm of a 10-somite stage embryo was photoactivated using the same laser parameters as mentioned in Figure 1. The embryo was raised and processed using steps 4.1 through 4.20. The arrow in Figure 2 locates the region activated, as indicated by the accumulation of blue precipitate. Only the activated location is clearly detected without interfering background staining.

Preparation of Solutions:

1 X PBT (1 L)

100 mL 10 X PBS

2 g BSA

10 mL 20 % Tween

10 X PBS (1 L)

80 g NaCl

2 g KCl

6.1 g Na_2HPO_4

1.9 g KH_2PO_4

ddH_2O to 1 L

pH to 7.3

4 % Paraformaldehyde (160 mL)

32 % Paraformaldehyde (two 10 mL vials)

8 mL 20 X PBS

132 mL ddH_2O

AP Buffer (50 mL)

1 mL 5 M NaCl

2.5 mL 1 M $MgCl_2$

5 mL 1 M Tris pH 9.5

250 µL 20 % Tween

41.25 mL ddH_2O

Antibody solution (for 1 sample)

5.5 µL Acetone powder

40 µL PBT

0.8 µL LS

0.1 µL Anti-fluorescein-AP antibody

2 % LS (360 µL for 1 sample)

7.2 µL 100 % LS

352.8 µL PBT

1 X Danieau media (1 L)‡

11.6 mL 5 M NaCl

700 µL 1 M KCl

400 µL 1 M $MgSO_4$

600 µL 1 M $Ca(NO_3)_2$

5 mL 1 M HEPES

ddH_2O to 1 L

pH adjust to 7.6

‡ Danieau is an ideal salt solution for rearing dechorionated zebrafish embryos. Other media may be used, provided they are isotonic solutions with properly osmolarity (~ 300 mOsm for zebrafish)

NBT stock (1 mL)

50 mg Nitro Blue Tetrazolium

0.7 mL dimethyl formamide anhydride

0.3 mL ddH_2O

BCIP stock (1 mL)

50 mg 5-bromo-4-chloro-3-indolyl phosphate

1 mL dimethyl formamide anhydride

NBT/BCIP developing solution

1 mL AP buffer

4.5 µL NBT stock

3.5 µL BCIP stock

Acetone powder

1. Select 20 adult fish and freeze them in liquid nitrogen.
2. Grind the frozen fish with a mortar and pestle to a powder.
3. Transfer the fish powder into a 50 mL conical tube.
4. Fill the conical tube with 100 % acetone. Vortex the tube.
5. Spin the tube at max rpm for 10 mins. Discard the supernatant.
6. Wash the pellet another 3 times in 100 % acetone and spin the tube at max rpm for 10 mins. By the last wash the supernatant should appear clear.

7. Add 100 % acetone to the pellet again and let the tube stand at room temperature. The more dense particles will settle, while the finer particles will remain in suspension.
8. Decant the fine particles into a new tube. Aliquot the powder solution into separate tubes and store them at -20 °C.

Disclosures

No conflicts of interest declared.

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