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Antibody Uptake Assay in the Embryonic Zebrafish Forebrain to Study Notch Signaling Dynamics in Neural Progenitor Cells In Vivo

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Abstract

Stem cells can generate cell fate heterogeneity through asymmetric cell division (ACD). ACD derives from the asymmetric segregation of fate-determining molecules and/or organelles in the dividing cell. Radial glia in the embryonic zebrafish forebrain are an excellent model for studying the molecular mechanisms regulating ACD of stem cells in vertebrates, especially for live imaging concerning in vivo molecular and cellular dynamics. Due to the current difficulty in expressing fluorescent reporter-tagged proteins at physiological levels in zebrafish for live imaging, we have developed an antibody uptake assay to label proteins in live embryonic zebrafish forebrain with high specificity. DeltaD is a transmembrane ligand in Notch signaling pathway in the context of ACD of radial glia in zebrafish. By using this assay, we have successfully observed the in vivo dynamics of DeltaD for studying ACD of radial glia in the embryonic zebrafish forebrain.

Keywords

Antibody uptake assay; Asymmetric cell division; Live imaging; Notch signaling; Radial glia; Stem cell; Zebrafish

1 Introduction

Stem cells have the amazing ability to maintain a stem cell population while producing differentiating progeny, which critically underscores the generation of cell fate diversity during development. One important approach to achieve this is through asymmetric cell division (ACD), which produces one self-renewing daughter cell and the other committed to differentiation. How ACD of stem/progenitor cells is regulated has been extensively studied in invertebrates. Typically, cell fate asymmetry can be mediated by the asymmetric segregation of fate-determining molecules/organelles between the two daughter cells [1–4]. In contrast, the molecular details underlying ACD of vertebrate stem cells are only beginning to be characterized. Radial glia, multi-potent neural progenitor cells in the developing vertebrate central nervous system, predominantly undergo ACD during the peak phase of neurogenesis in mammals [5–8] and zebrafish [9, 10]. This feature, combined with the accessibility and transparency of zebrafish embryos, makes radial glia in zebrafish an excellent model for addressing questions regarding ACD of vertebrate stem cells, especially when concerning in vivo molecular or cellular dynamics [11].

To observe the in vivo dynamics of molecular and cellular processes in zebrafish, exogenous fluorescent fusion proteins are often transiently (e.g., by microinjecting the mRNA) or stably (e.g., by establishing the transgenic line) introduced. However, dosage sensitivity of certain proteins (in particular, those with important signaling activity) precludes such approach, as introducing exogenous copies of DNA or RNA molecules can lead to overexpression and disruption of normal phenotypes. Knock-in of the fluorescent protein sequences into the endogenous genomic loci is ideal, but remains challenging for zebrafish despite a few published successes [12–15]. Given the requirement of observing protein dynamics at its physiological level in zebrafish, antibody uptake assay can be a suitable approach, under the circumstances that the protein is transmembrane with an extracellular region and frequently undergoes endocytosis. Briefly, the complexes of primary antibodies and fluorescently labeled secondary antibodies are formed in vitro and then applied into the extracellular space. The antibody complex specifically binds to the extracellular region of the protein and is uptaken into the cell upon endocytosis of the labeled protein, which allows the tracking of in vivo dynamics of this protein with fluorescent confocal imaging. Previously, the antibody uptake assay was used to study the intracellular trafficking and asymmetric segregation of Delta and/or Notch, in the context of ACD of sensory organ precursor (SOP) cells in *Drosophila* [16–21] and neural precursor cells in zebrafish spinal cord [22]. Here, we describe how the antibody uptake assay is performed to study the in vivo dynamics of DeltaD in radial glia of embryonic zebrafish forebrain, as well as demonstrate its broad applicability to various developmental stages and brain regions.

2 Materials

1. Collect and raise zebrafish embryos: mating cages, dividers, plastic Petri dishes, 28.5 °C incubator.
2. Embryonic medium: 0.12 g $CaSO_4$, 0.2 g “Instant Ocean” Sea Salts, and 0.3 g Methylene Blue added to 1 l distilled water.
3. 0.3% Phenylthiourea (PTU).
4. Glass pipette.
5. Fine forceps (Inox 5, Dumont Electronic, Switzerland).
6. Stereo dissection microscope (Zeiss Stemi 2000 with a maximum magnification of 50×).
7. Tricaine.
8. Monoclonal mouse IgG1 anti-DeltaD primary antibody (zdd2, Abcam ab73331, 1 μg/μl) directed against the extracellular region of DeltaD.
9. Zenon® Alexa Fluor® Mouse IgG1 labeling reagent (Molecular Probes, Z-25008 for Alexa Fluor 647, 0.2 μg/μl).
10. PBS 1×.
11. Low-melting point agarose (Shelton Scientific, Inc. catalog #IB70050).
12. Heat block.

13. Glass capillaries (1.2 mm O.D., 0.94 mm I.D., with filament).
14. Flaming-Brown P897 puller (Sutter Instruments, Novato, CA, USA).
15. Eppendorf Microloader Tips for filling Femtotips and other glass microcapillaries (0.5–20 μl, Catalog No. 930001007).
16. Micromanipulator (WPI M3301R, World Precision Instruments, Sarasota, FL, USA).
17. Air pressure microinjector (Narishige IM 300 microinjector) with a nitrogen cylinder.
18. Halocarbon oil.
19. Stage micrometer.
20. Microsurgical knife.
21. 35 mm glass-bottom culture dish (MatTek corporation, Part# P35GC-1.5-10-C, www.glass-bottom-dishes.com).
22. Confocal microscope with a temperature controller.

3 Methods

All steps are performed at room temperature unless otherwise specified.

3.1 Preparation of Zebrafish Embryos

1. Two days before the antibody uptake assay, set up mating cages of zebrafish (*see* Note 1) with dividers to separate males and females.
2. One day before the antibody uptake assay, pull the dividers in mating cages.
3. Collect zebrafish embryos and incubate 50 fertilized embryos in a plastic Petri dish with 30 ml of embryonic medium containing 0.003% phenylthiourea (PTU) in an incubator at 28.5 °C.
4. On the day of the antibody uptake assay, when the embryos reach the developmental stage of 29 hpf (*see* Note 2), dechorionate the embryos manually with fine forceps under a stereo dissection microscope.
5. Add 1.2 ml tricaine stock (4 mg/ml, 25 ×) to the embryonic medium to anesthetize the embryos.

3.2 Preparation for Antibody Injection

1. Mix 0.5 μl of mouse IgG1 anti-DeltaD primary antibody (1 μg/μl) and 2.5 (μl of Alexa-labeled Zenon mouse IgG1 labeling reagent (0.2 μg/μl). This labeling reagent is Alexa-labeled goat Fab fragments targeted against the Fc tail of mouse IgG1 primary antibodies. Incubate the mixture at room temperature for 30 min, protected from light, and then dilute with PBS 1× to 5 (μl. The antibody complex

of primary and secondary antibodies is thus ready for the antibody uptake assay (*see* Note 3).

2. Prepare 1% (w/v) low-melting point agarose in embryonic medium. Aliquot the agarose solution in 2 ml microcentrifuge tubes and add 80 (μl of tricaine stock (4 mg/ml, 25 ×) into each tube after the agarose solution cools down a bit. Keep the aliquots in a heat block at ~37 °C.

3. Glass injection needles are pulled from capillaries on a puller. The needle tip is broken off with fine forceps to get a sharp end, whose diameter should be around 10 (μm (can be checked with a stage micrometer) and whose angle should be around 30°. The appropriate diameter and angle of the tip are very important for inserting the needle into the forebrain ventricle and injecting a suitable volume of antibody complex solution.

3.3 Antibody Uptake Assay

1. To mount the embryos, transfer two dechorionated embryos into 1% low-melting point agarose with a glass pipette. After a brief immersion, immediately remove the embryos along with small amount of agarose. Under a stereo dissection microscope, place each embryo on an inverted plastic Petri dish lid in an individual drop of agarose.

2. Orientate the embryos gently with a fiber probe (can be cut from an Eppendorf microloader tip) to a dorsal-up position before the agarose solidifies, so that the forebrain is accessible to the microinjection needle (*see* Note 4).

3. To prevent the Alexa Fluor® fluorescent tag in the antibody complex from photobleaching, from the next step, turn off the light in the room and turn down the light source of the stereo dissection microscope.

4. Backload the microinjection needle with 2 μl of antibody complex solution using an Eppendorf microloader tip. Make sure no air bubble is in the solution.

5. In order to adjust the volume of one single injection to 4 nl, manipulate the position of the microinjection needle with a micromanipulator to inject one drop of solution with an air pressure microinjector into the halocarbon oil (or other similar oil) placed on a stage micrometer. A drop with a diameter of 0.2 mm is about 4 nl in volume. Manipulate the injection parameters (pressure and/or time) with the air pressure microinjector to achieve the expected injection volume.

6. Insert the microinjection needle into the forebrain ventricle of the embryo (Fig. 1) (*see* Note 5) and inject 8 nl of antibody complex solution by injecting 4 nl twice (*see* Note 6), and the swelling of the forebrain ventricle can be easily observed (*see* Note 7).

7. Add 5 ml of embryonic medium containing 0.16 mg/ml tricaine to cover the agarose drops and carefully peel the agarose with a microsurgical knife to release the embryos.

8. To mount the embryos for imaging, transfer one embryo into 1% low-melting point agarose with a glass pipette. After a brief immersion, immediately remove the embryo from the agarose. Place the embryo on the center of a small glass-bottom culture dish in a single agarose drop (*see* Note 8).

9. Orientate the embryo gently with a fiber probe to a dorsal-down position (or a dorsal-up position if an up-right objective is used) before the agarose solidifies.

10. Place the dish properly on the temperature controlled stage of the confocal microscope with an inverted microscope. Adjust the temperature to 28.5 °C.

11. Add 3 ml 28.5 °C preheated embryonic medium containing 0.003% PTU and 0.16 mg/ml tricaine to cover the embryo.

12. Perform in vivo time-lapse confocal imaging with a fixed interval, and the embryo should be ~30 hpf now (*see* Note 9) (Fig. 2).

4 Notes

1. In order to better visualize the intracellular dynamics of DeltaD, a transgenic zebrafish line with a cell membrane marker (e.g., EF1α:myr-tdTomato) is often used. Under such circumstance, the fluorescent color of the secondary antibody for forming the antibody complex should not overlap with that of the cell membrane marker.

2. Antibody uptake assay can also be applied to other developmental stages for the embryonic zebrafish forebrain, at least from 24 to 32 hpf

3. The antibody complex solution can be stored at 4 °C, protected from light, for at least a month. In addition to labeling the primary antibody to form the in vitro primary-secondary antibody complexes by the Zenon labeling technology, the directly labeled antibody is presumed to be also workable in such antibody uptake assay.

4. It would be better to orientate the brains of all embryos on a plastic Petri dish lid towards the same direction, so that the subsequent microinjection can be more conveniently carried out, without the need to reorient the Petri dish lid every time before injecting the next embryo. Moreover, the process from mounting the embryos to releasing the embryos from the agarose should be done within half an hour; otherwise a large extent of the water in the agarose may evaporate, causing the agarose to shrink and the embryos to be crushed.

5. Antibody uptake assay can also be applied to other brain regions, like midbrain and hindbrain, by microinjecting into the mid-brain ventricle and hindbrain ventricle, respectively (the injection volume can be adjusted according to the injection position). At this developmental stage (~29.5 hpf), the ventricles of fore-brain, midbrain, and hindbrain are interconnected, and the antibody complexes microinjected into the forebrain ventricle can diffuse through the ventricles and thus can be uptaken into cells not only in the forebrain but also in the midbrain and hindbrain, if the protein endocytosis does happen there.

6. Sometimes inserting the needle into the forebrain ventricle can block the needle to some extent and thus affect the injection volume. Under such concern, it would be better to check the injection volume of each injection. A simple way to do this is to, after determining the injection volume by the stage micrometer, touch the tip of the needle onto the surface of an agarose drop used to mount the embryo, inject once and keep in mind the size of the solution drop formed on the surface of the agarose drop. Then after each injection into an embryo, inject on the surface of the agarose drop to ensure a solution drop is formed with a similar size. A significantly smaller size of the drop would indicate that the last injection volume is likely smaller than it should be and the needle is likely blocked. When this happens, the embryo just injected should be discarded, and a larger injection pressure or a "Clear" function of the microinjector should be used to clear the needle tip while a certain amount of solution remains in the needle, and then adjust the injection volume to 4 nl again by the stage micrometer.

7. If the ventricle swelling is not observed but the injection of the solution is indeed observed, then the solution may not be injected into the forebrain ventricle but probably some deeper tissues or even the yolk. The embryo should then be discarded.

8. Sometimes in a later step when covering the solidified agarose drop with the embryonic medium, the agarose can float up. To prevent this, one can apply the agarose across the whole concave glass area of the glass-bottom culture dish.

9. Clear intracellular fluorescent signals inside the forebrain should be observed within 15 min after injecting the antibody complex (15 min is roughly the minimal time required for releasing and remounting embryos followed by setting the microscope), and if the microscope settings are well controlled to minimize photobleaching (and phototoxicity), the fluorescent signals can be observed for at least 2 h. Clear fluorescent signals should also be observed in the forebrain ventricle, and their absence indicates the failure in the injection of the antibody complex solution, its preparation/storage, or the prevention of photobleaching during the experiment. In order to ensure the intracellular fluorescent signals are not the result of any nonspecific uptake of the antibody complex, necessary controls like injecting only the fluorescently labeled secondary antibody should be performed.

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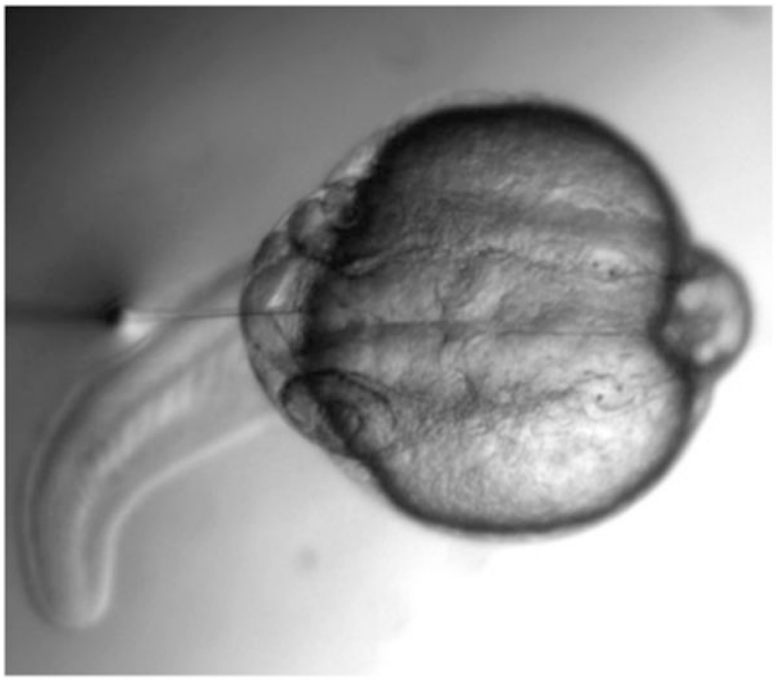

Fig. 1.

One zebrafish embryo at 29.5 hpf is mounted in an agarose drop, with a dorsal-up orientation and its forebrain ventricle readily approachable by the microinjection needle. The microinjection needle backloaded with the antibody complex solution is inserted into the forebrain ventricle, followed by the injection of 8 nl of the antibody complex solution using the air pressure microinjector

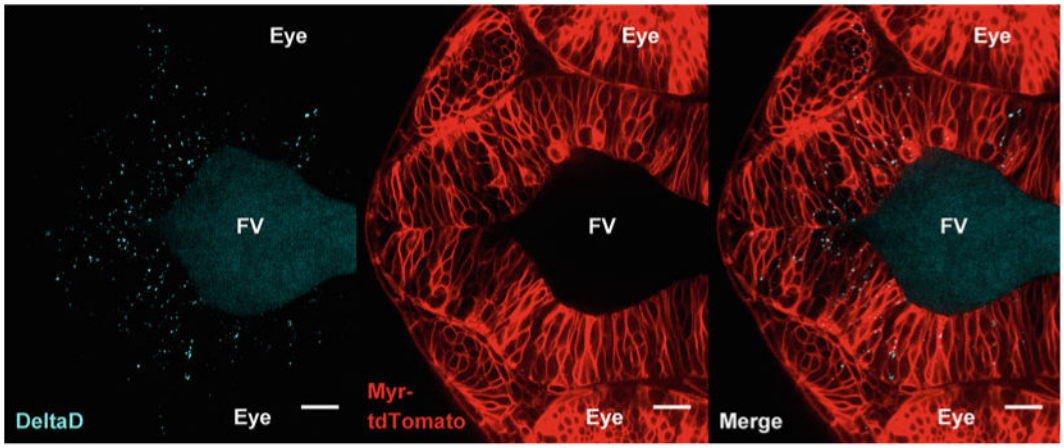

Fig. 2.

After injecting the anti-Delta D antibody complex solution (the secondary antibody in antibody complex is conjugated with Alexa Fluor 647) into the forebrain ventricle of the zebrafish embryo of EF1α:myr-tdTomato transgenic line (myr-td Tomato is a red cell membrane marker), the far-red fluorescent signals can be observed both inside the radial glia cells of the forebrain and on their cell membranes (FV, forebrain ventricle; Scale bar, 20 μm), indicating that anti-DeltaD antibody complex binds to DeltaD and undergoes uptake into the cell. Time-lapse confocal imaging can thus be performed to observe the dynamics of DeltaD