Video Article

An Assay for Permeability of the Zebrafish Embryonic Neuroepithelium

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Abstract

The brain ventricular system is conserved among vertebrates and is composed of a series of interconnected cavities called brain ventricles, which form during the earliest stages of brain development and are maintained throughout the animal's life. The brain ventricular system is found in vertebrates, and the ventricles develop after neural tube formation, when the central lumen fills with cerebrospinal fluid (CSF) [1,2]. CSF is a protein rich fluid that is essential for normal brain development and function[3-6].

In zebrafish, brain ventricle inflation begins at approximately 18 hr post fertilization (hpf), after the neural tube is closed. Multiple processes are associated with brain ventricle formation, including formation of a neuroepithelium, tight junction formation that regulates permeability and CSF production. We showed that the Na,K-ATPase is required for brain ventricle inflation, impacting all these processes [7,8], while *claudin 5a* is necessary for tight junction formation [9]. Additionally, we showed that "relaxation" of the embryonic neuroepithelium, via inhibition of myosin, is associated with brain ventricle inflation.

To investigate the regulation of permeability during zebrafish brain ventricle inflation, we developed a ventricular dye retention assay. This method uses brain ventricle injection in a living zebrafish embryo, a technique previously developed in our lab[10], to fluorescently label the cerebrospinal fluid. Embryos are then imaged over time as the fluorescent dye moves through the brain ventricles and neuroepithelium. The distance the dye front moves away from the basal (non-luminal) side of the neuroepithelium over time is quantified and is a measure of neuroepithelial permeability (**Figure 1**). We observe that dyes 70 kDa and smaller will move through the neuroepithelium and can be detected outside the embryonic zebrafish brain at 24 hpf (**Figure 2**).

This dye retention assay can be used to analyze neuroepithelial permeability in a variety of different genetic backgrounds, at different times during development, and after environmental perturbations. It may also be useful in examining pathological accumulation of CSF. Overall, this technique allows investigators to analyze the role and regulation of permeability during development and disease.

Video Link

The video component of this article can be found at http://www.jove.com/video/4242/

Protocol

1. Preparing for Microinjection

1. Prepare microinjection needles by pulling capillary tubes using Sutter instruments needle puller.
2. Load microinjection needle with fluorescent dye (FITC-Dextran).
3. Mount needle on micromanipulator and microinjection apparatus.
4. Carefully break microinjection needle using forceps to roughly 2 µm in width, however, this will vary depending on your microinjector setup. For our microinjection needles, this corresponds to the first region of the needle from the tip that does not bend.
5. Measure drop size in oil, adjusting injection time and pressure, so that each injection delivers 1 nl. Example settings for Harvard Apparatus microinjector are: P balance = 1.4 psi, P out = 1.4 psi, P inject = 22.9 psi, P clear = 67.8 psi with an injection time of .4 to .7 sec. The diameter of our needle using these settings is roughly 2 µm. However, settings will be microinjector specific, and vary according to needle diameter.

2. Preparing the Embryos

1. Coat 2 dishes with 1% agarose in water for each condition, poke holes into agarose with a 1-200 µl pipette tip, and remove agarose plugs. Fill dishes with embryo media.
2. Using forceps, dechorionate embryos that are 18 hpf or older under a stereomicroscope. Embryos are staged according to Kimmel *et al.*[11] .
3. Transfer dechorionated embryos into first agarose coated dish.

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4. To anesthetize embryos, add tricaine (0.1 mg/ml) to the dish until embryos stop moving (made according to Westerfield[12]).

3. Injecting the Brain Ventricles

1. Orient embryos so you are looking at their dorsal side by putting the tail of the embryo into the hole. If your micromanipulator is on the right, then move the embryo so that the forebrain is to the left and hindbrain to the right.
2. Position needle at widest point of hindbrain ventricle.
3. Carefully pierce roof plate of hindbrain ventricle being sure not to go through the depth of the brain into the yolk (**Figure 1A**).
4. Inject 1-2 nl of fluorescent dye into the ventricles making sure the dye fills the whole length of the brain ventricles.
5. Transfer embryos to the second agarose coated dish filled with embryo media and re-anesthetize as described in 2.4.
6. IMMEDIATELY start imaging, as described in section 4 in order to get a time zero image.

4. Imaging

1. Orient embryos with their tail in the hole as described in 3.1.
2. Use a dissecting microscope with both transmitted and fluorescent light to take a brightfield dorsal image. Keep the magnification constant between imaging of different embryos. This allows for direct comparison of analyses performed using Image J (5.2-6).
3. WITHOUT MOVING the embryo, microscope or dish, take a corresponding fluorescent image.
4. Repeat for each embryo at desired time points.

5. Quantification of Dye Movement

1. Merge brightfield and fluorescent images in Photoshop as previously described by Gutzman and Sive[10].
2. Measure the distance the dye front moves in Image J software available at http://rsbweb.nih.gov/ij/.
3. Open merged file in Image J and use line tool to draw a line from the forebrain hinge-point to dye front at a 10-20 ° angle from neuroepithelium (**Figure 1A**). This region was chosen because it is the first and most noticeable site of dye leaking out of the wild type neuroepithelium.
4. Select measurement tool to calculate length of line.
5. Repeat for each time-point.
6. Calculate net distance the dye front moved over time by subtracting distance at t=0 from other time points.
7. Plot on graph.

6. Representative Results

An example of results obtained in a neuroepithelial permeability assay using wild type embryos is shown in **Figure 1B-D**. To accurately differentiate permeability, it is useful to test dyes with different molecular weightsto identify a size that is only slightly leaky in wild type or control embryos (**Figure 2**). This allows for identification of genetic mutants or environmental conditions that either increase or decrease permeability (**Figure 1D**, green and red lines respectively). For the 24 hpf zebrafish neuroepithelium, 70 kDa FITC Dextran leaks slowly over 2 hr, whereas 2,000 kDa does not and 10 kDa almost immediately leaks out. Therefore 70 kDa is the ideal molecular weight to identify conditions that both increase and decrease neuroepithelial permeability.

If the needle misses the ventricular lumen, fluorescence will appear outside the brain at t=0 (for an example see Gutzman and Sive, 2009[10]). These embryos should be discarded since the injected dye was not initially contained within the brain and no clear conclusion regarding movement of the dye and permeability of the neuropeithelium can be made.

Finally, if embryos have small ventricles or un-inflated brain ventricles, pre-injection of ventricles with a saline solution can be done prior to injection of the fluorescent dye. This inflates the ventricles making subsequent visualization of the ventricles easier when injecting with the fluorescent dye. Proper controls must be performed to determine whether injection of saline disrupts normal neural tube development.

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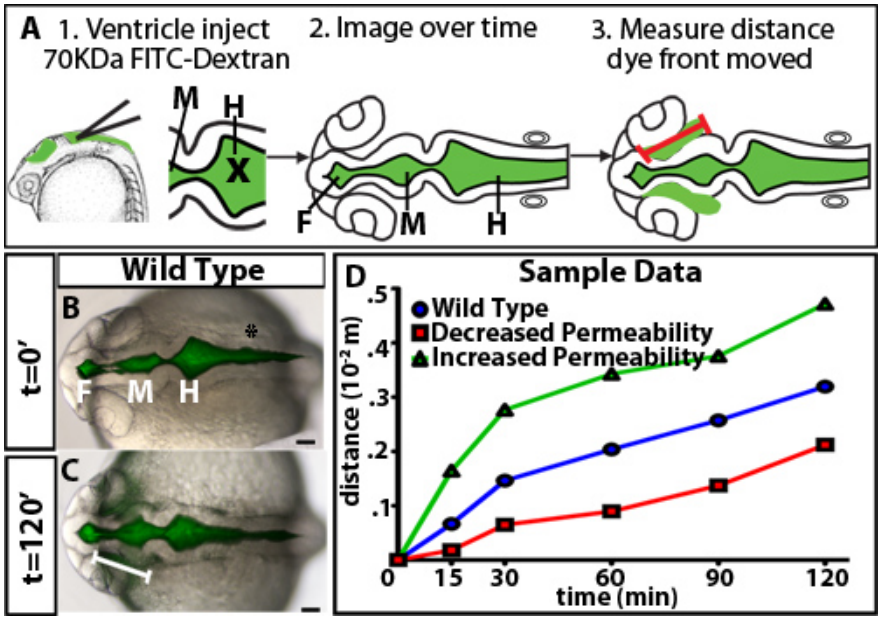

Figure 1. Time course of different molecular weight dyes. (A) Experimental Diagram. First, fluorescent dye is injected into the ventricles. X = position of needle for injection. Next dorsal images are captured over time. Finally, the distance moved by the dye front from the forebrain hinge-point is measured (represented by a red line). (B-C) Merged brightfield and fluorescent dorsal images at 22 hpf (t = 0 min, B) and 24 hpf (t =120 min, C). White line indicates distance of the dye front from forebrain ventricle. (D) Hypothetical sample permeability data. Blue = wild type or controls, red = sample with decreased permeability relative to control, and green = sample with increased permeability relative to control.

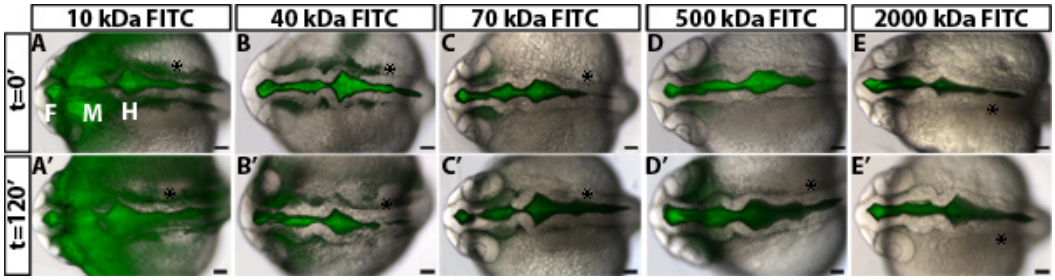

Figure 2. Measurement of neuroepithelial permeability to different molecular weight dyes. (A-E) Dorsal merged brightfield and fluorescent images of 22 hpf wild type embryos at t=0 min after injection with FITC-dextran of the following molecular weights: 10 kDa (A), 40 kDa (B), 70 kDa (C), 500 kDa (D) and 2,000 kDa (E). (A'-E') Same embryo as in (A-E) at t=120 min at 24 hpf. Anterior to left. F= forebrain, M= midbrain, H= hindbrain. Asterisk = ear.

Discussion

We demonstrate the ability to quantify permeability of the living embryonic zebrafish brain as determined for an injected dye of a given molecular weight. Our observation that the embryonic zebrafish neuroepithelium is differentially permeable to dyes of differing molecular weights suggests that the dye is moving via paracellular permeability. However, we cannot rule out the possibility of a transcellular contribution to the observed permeability. This technique could be applied to any other tubular structure as long as both the inside and outside of the tube can be seen and the lumen can be injected. However, identification of the ideal molecular weight for other organs will need to be determined as this may differ between tissues and developmental stages .

This assay will enable further investigation into the role of epithelial permeability during lumen inflation and the regulation of lumen size. In addition, this technique will help characterize alterations in epithelial permeability associated with disorders such as hydrocephalus and polycystic kidney disease.

Disclosures

No conflicts of interest declared.

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References

1. Harrington, M.J., Hong, E., & Brewster, R. Comparative analysis of neurulation: first impressions do not count. *Mol. Reprod. Dev.* **76**, 954-965 (2009).
2. Lowery, L.A. & Sive, H. Strategies of vertebrate neurulation and a re-evaluation of teleost neural tube formation. *Mech. Dev.* **121**, 1189-1197 (2004).
3. Salehi, Z. & Mashayekhi, F. The role of cerebrospinal fluid on neural cell survival in the developing chick cerebral cortex: an *in vivo* study. *Eur. J. Neurol.* **13**, 760-764 (2006).
4. Martin, C., *et al.* Early embryonic brain development in rats requires the trophic influence of cerebrospinal fluid. *Int. J. Dev. Neurosci.* **27**, 733-740 (2009).
5. Lehtinen, M.K., *et al.* The cerebrospinal fluid provides a proliferative niche for neural progenitor cells. *Neuron.* **69**, 893-905 (2011).
6. Gato, A., *et al.* Embryonic cerebrospinal fluid regulates neuroepithelial survival, proliferation, and neurogenesis in chick embryos. *Anat. Rec. A. Discov. Mol. Cell Evol. Biol.* **284**, 475-484 (2005).
7. Lowery, L.A. & Sive, H. Totally tubular: the mystery behind function and origin of the brain ventricular system. *Bioessays.* **31**, 446-458 (2009).
8. Lowery, L.A. & Sive, H. Initial formation of zebrafish brain ventricles occurs independently of circulation and requires the nagie oko and snakehead/atp1a1a.1 gene products. *Development.* **132**, 2057-2067 (2005).
9. Zhang, J., *et al.* Establishment of a neuroepithelial barrier by Claudin5a is essential for zebrafish brain ventricular lumen expansion. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 1425-1430 (2010).
10. Gutzman, J.H. & Sive, H. Zebrafish brain ventricle injection. *J. Vis. Exp.* (2009).
11. Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., & Schilling, T.F. Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203,** 253-310 (1995).
12. Westerfield, M., Sprague, J., Doerry, E., Douglas, S., & Grp, Z. The Zebrafish Information Network (ZFIN): a resource for genetic, genomic and developmental research. *Nucleic Acids Research.* **29**, 87-90 (2001).

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