Video Article

Immunostaining of Dissected Zebrafish Embryonic Heart

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Abstract

Zebrafish embryo becomes a popular *in vivo* vertebrate model for studying cardiac development and human heart diseases due to its advantageous embryology and genetics [1,2]. About 100-200 embryos are readily available every week from a single pair of adult fish. The transparent embryos that develop *ex utero* make them ideal for assessing cardiac defects [3]. The expression of any gene can be manipulated via morpholino technology or RNA injection [4]. Moreover, forward genetic screens have already generated a list of mutants that affect different perspectives of cardiogenesis [5].

Whole mount immunostaining is an important technique in this animal model to reveal the expression pattern of the targeted protein to a particular tissue [6]. However, high resolution images that can reveal cellular or subcellular structures have been difficult, mainly due to the physical location of the heart and the poor penetration of the antibodies.

Here, we present a method to address these bottlenecks by dissecting heart first and then conducting the staining process on the surface of a microscope slide. To prevent the loss of small heart samples and to facilitate solution handling, we restricted the heart samples within a circle on the surface of the microscope slides drawn by an immEdge pen. After the staining, the fluorescence signals can be directly observed by a compound microscope.

Our new method significantly improves the penetration for antibodies, since a heart from an embryonic fish only consists of few cell layers. High quality images from intact hearts can be obtained within a much reduced procession time for zebrafish embryos aged from day 2 to day 6. Our method can be potentially extended to stain other organs dissected from either zebrafish or other small animals.

Video Link

The video component of this article can be found at http://www.jove.com/video/3510/

Protocol

1. Preparation of slides and humidified chamber

1. The humidified chamber can be made from a box such as an emptied tip box. Wrap both the chamber and the cover with aluminium foil to protect the samples from light.
2. Inside the chamber, put a pile of wet paper towels to maintain the humidity within the chamber, which will prevent the heart samples from drying. Set a small microplate on the top of paper towel as a rack for the slides.
3. Draw either lines or circles on the surface of a microscope slide using the immEdge pen to make wells for heart samples with a dimension about 5x5 mm. Let the slides dry.
4. Put the slides in the humidified chamber and add 50ul fresh 4% formaldehyde in each well.

2. Dissection of embryonic heart

1. Anesthetize the fish embryo in E3 egg water [7] containing 0.4% tricaine for about 1 minute until fish stop body movement but still have normal heart beating.
2. Put a concave microscope slide on the stage of a dissection microscope. Transfer the embryos to the concaved well.
3. Adjust the light combination of the dissecting microscope to clearly reveal the heart. Depending on personal preference, it could be either dark field or DIC-like polarized light condition.
4. Clean two insulin syringes with 75% ethanol, then dry.
5. Adjust the dissection microscope to higher magnification and focus on the heart. Physically fix an embryo by inserting one needle tip into the yolk-somite boundary close to the head. Insert another needle close to the heart region and pull the heart out quickly. If the heart is not totally separated from the embryo, cut off the remaining connected tissue between the heart and the yolk.
6. Adjust the microscope to lower magnification and focus on the separated heart. Use a 10µl pipetteman and set it to 1µl volume. Apply the tip of the pipetteman to either draw or touch the heart sample, so that the isolated heart will be either within or attached to the surface of the tip.
7. Dip the pipette tip in 4% formaldehyde solution on prepared slides and depress the pipette piston to release the heart into the solution. The tension of water surface also helps to release the heart from pipette tip to the solution. Put less than 3 hearts in each well.

3. Immunostaining

1. Close the chamber and put it on a shaker with a gentle rocking movement. Fix the heart sample for 20 minutes at room temperature.
2. Wipe off the water on the back of the slides and put the slides on the stage of a dissection microscope for buffer exchanging.
3. Dip the tip from a 10µl pipetteman into an empty region in each well and keep the tip as far as possible from heart samples. Take cautions to avoid the heart attaching to the tip, since hearts might move with the water flow when the solution is drawn into the tip. Change the tip localization, if needed.
4. Dispose the solution onto a tissue and avoid producing any bubbles in the tip since the heart sample is easily lost in the presence of air bubbles. Keeping the heart samples partially dry for a short time can help the hearts stay attached to the slides.
5. Add 50µl PBST to cover the heart sample and incubate in the humidified chamber in a slow rocking movement for 5 min. Repeat the rinse one more time. After this step, the heart samples can be stored in the humidified chamber up to 1 week at 4°C.
6. Block the heart sample with 10% sheep serum in PBST for 1 hour at room temperature.
7. Incubate the heart sample with primary antibodies, such as 1:200 dilution of β-catenin antibody and 1:200 dilution of mef2 specific antibody, in 10% sheep serum/PBST for 1 hour at room temperature.
8. Wash the heart sample with PBST three times (5 min. each) at room temperature.
9. Incubate the sample with secondary antibodies, such as 1:1000 dilution of Alexa-tagged anti-mouse and/or anti-rabbit antibodies, in PBST for 0.5 hour.
10. Wash the heart samples with PBST three times (5 min. each) at room temperature.

4. Imaging

1. Remove PBST and add 10 µl mounting medium to each well. Rock the chamber for several minutes till the solution becomes clear.
2. Remove most of the mounting medium and cover all of the wells in one slide with a microscope cover glass (24x50).
3. Image fish heart using Zeiss Axioplan 2 microscope equipped with ApoTome (Carl Zeiss).

5. Representative Results

An example of an image which reveals membrane and nuclei of all zebrafish cardiomyocytes is shown in Fig. 1. mef2c and β-catenin antibodies were used together to stain heart samples dissected from 52 hpf zebrafish embryo. While mef2c antibody stains the nuclei of cardiomyocytes, β-catenin antibody reveals the border of each cell [8-10]. By adjusting the stage of the compound microscope, images of the heart samples can be adjusted to the same orientation, which will allow the observation of outer curvature and inner curvature in the heart [11]. The total cardiomyocyte number, cardiomyocyte cell size, and circularity can then be measured.

Another example which reveals the sarcomeric structure of an embryonic heart is shown in Fig. 2. We performed immunostaining using F59, a myosin antibody, in a dissected embryonic heart. The myofibril network in a whole embryonic heart can be clearly revealed at lower magnification, while the striated band of thick filament can be revealed at higher magnification (Fig 2) [6].

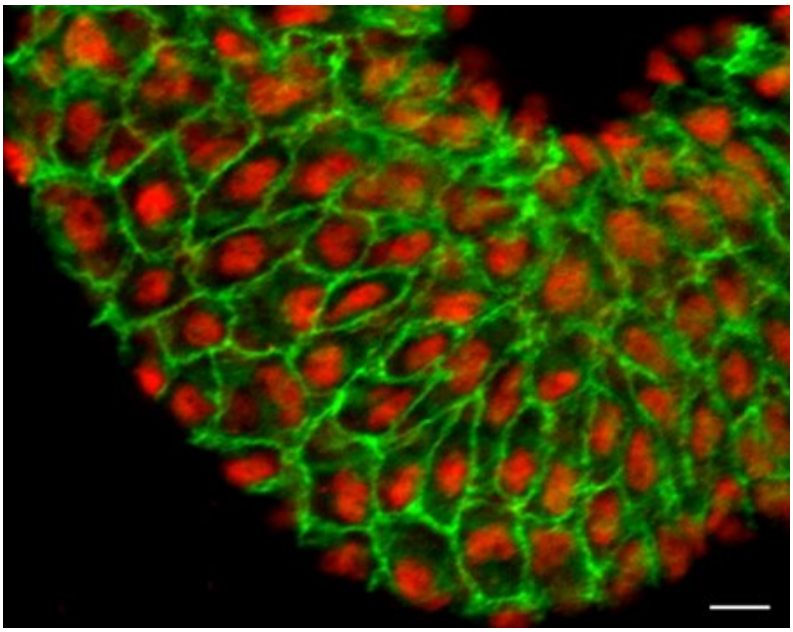

Figure 1. Immunostaining of mef2 (red) and β-catenin (green) to show the nuclei and outlines of cardiomyocytes in a zebrafish ventricle. Scale bar=10µm

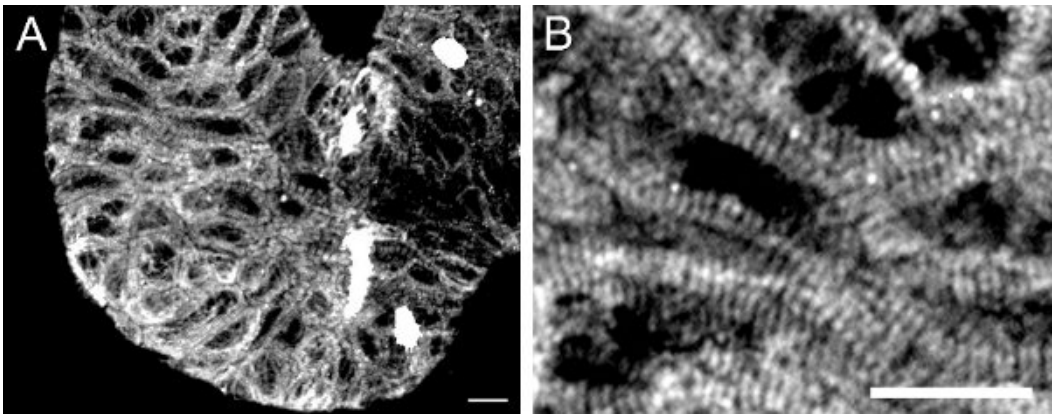

Figure 2. Immnostaining of myosin to show the thick filament in a zebrafish ventricle at either low magnification (A) or high magnification (B).scale bar=10μm

Discussion

Compared to classic whole mount immunostaining methods, our method has the following advantages. First, much stronger fluorescent signals can be consistently obtained due to improved penetration. In the whole mount immunostaining method, the dense skin tissue surrounding the heart significantly reduced the penetration of many antibodies, resulting in high background in the whole body. This problem is especially severe for embryos older than 3-day post-fertilization (dpf). In contrast, the dissected hearts only consist of a few cell layers, rendering much better penetration. Secondly, because of the much improved penetration, the whole process of immunostaining can be reduced from 1 day to just several hours. We have successfully reduced the incubation time from 1 hour to 30 minutes for some antibodies such as Actinin, Integin-linked kinase (Ilk), and β-catenin specific antibodies. Third, high-resolution images can be consistently obtained from intact hearts to reveal cellular/subcellular information. Because of the localization of the heart inside the pericardiac sac that is next to the yolk, higher magnification objective lenses cannot be used to image intact embryos. Therefore, the heart will need to be dissected if images of an intact heart are needed. However, detergents such as triton-x-100 that are used to improve penetration in the whole mount staining procedure weaken the embryos. As a consequence, hearts are easily broken during the dissection procedure. In contrast, a live heart is much stronger, which can be easily separated from its neighboring tissues. Therefore, intact morphology are more easily maintained using the proposed method. Although dissecting a small zebrafish heart might appear challenging, most people can easily conduct this procedure after several practices.

We have utilized this method to stain hearts aged from 2 dpf to 6 dpf. Because of its physical location, hearts from even earlier staged embryos are difficult to dissect. It remains to be determined whether this method can be adjusted for larva or adult hearts. It is worthwhile to point out that local damage to the heart might result during the dissection process, which involves physical force. This complication can be overcome by assessing several hearts, and then selecting images with consistent results and the best maintained morphology.

Because of much improved resolution, this method can be used to reveal both cellular and subcellular structures of a developing zebrafish heart. For example, we have already applied this method to count total number of cardiomyocytes, to quantify individual cardiomyocyte size, to assess proliferation and apoptosis, and to reveal the process of sarcomere assembly [6,12]. Together with unique genetic tools in zebrafish, the present method will facilitate the study of cardiovascular biology in this *in vivo* model.

Disclosures

No conflicts of interest declared.

Acknowledgements

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References

1. Shin, J.T. & Fishman, M.C. From Zebrafish to human: modular medical models. *Annu. Rev. Genomics Hum. Genet.* **3**, 311-40 (2002).
2. Beis, D. & Stainier, D.Y. *In vivo* cell biology: following the zebrafish trend. *Trends. Cell. Biol.* **16**, 105-12 (2006).
3. Schoenebeck, J.J. & Yelon, D. Illuminating cardiac development: Advances in imaging add new dimensions to the utility of zebrafish genetics. *Semin. Cell. Dev. Biol.* **18**, 27-35 (2007).
4. Nasevicius, A. & Ekker, S.C. Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* **26**, 216-20 (2000).
5. Dahme, T., Katus, H.A., & Rottbauer, W. Fishing for the genetic basis of cardiovascular disease. *Dis. Model Mech.* **2**, 18-22 (2009).
6. Huang, W., Zhang, R., & Xu, X. Myofibrillogenesis in the developing zebrafish heart: A functional study of tnnt2. *Dev. Biol.* **331**, 237-49 (2009).
7. Westerfield, M. *The Zebrafish Book.*, (2007).
8. Buckingham, M.E. Muscle: the regulation of myogenesis. *Curr. Opin. Genet. Dev.* **4**, 745-51 (1994).
9. Wheelock, M.J. & Knudsen, K.A. Cadherins and associated proteins. *In Vivo.* **5**, 505-13 (1991).
10. Sun, X., *et al.* Cardiac hypertrophy involves both myocyte hypertrophy and hyperplasia in anemic zebrafish. *PLoS One.* **4**, e6596 (2009).
11. Auman, H.J., *et al.* Functional modulation of cardiac form through regionally confined cell shape changes. *PLoS Biol.* **5**, e53 (2007).
12. Chen, Z., *et al.* Depletion of zebrafish essential and regulatory myosin light chains reduces cardiac function through distinct mechanisms. *Cardiovasc. Res.* **79**, 97-108 (2008).

