Video Article

Immunostaining Phospho-epitopes in Ciliated Organs of Whole Mount Zebrafish Embryos

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Abstract

The rapid proliferation of cells, the tissue-specific expression of genes and the emergence of signaling networks characterize early embryonic development of all vertebrates. The kinetics and location of signals - even within single cells - in the developing embryo complements the identification of important developmental genes. Immunostaining techniques are described that have been shown to define the kinetics of intracellular and whole animal signals in structures as small as primary cilia. The techniques for fixing, imaging and processing images using a laser-scanning confocal compound microscope can be completed in as few as 36 hr.

Zebrafish (*Danio rerio*) is a desirable organism for investigators who seek to conduct studies in a vertebrate species that is affordable and relevant to human disease. Genetic knockouts or knockdowns must be confirmed by the loss of the actual protein product. Such confirmation of protein loss can be achieved using the techniques described here. Clues into signaling pathways can also be deciphered by using antibodies that are reactive with proteins that have been post-translationally modified by phosphorylation. Preserving and optimizing the phosphorylated state of an epitope is therefore critical to this determination and is accomplished by this protocol.

This study describes techniques to fix embryos during the first 72 hr of development and co-localize a variety of relevant epitopes with cilia in the Kupffer's Vesicle (KV), the kidney and the inner ear. These techniques are straightforward, do not require dissection and can be completed in a relatively short period of time. Projecting confocal image stacks into a single image is a useful means of presenting these data.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53747/>

Introduction

The techniques described here are the outcome of studies that have sought to define downstream targets of Ca^{2+} signals during events that occur during early development, including fertilization, gastrulation, somitogenesis and trunk, eye, brain and organ formation.¹⁻³ The original discoveries of embryonic Ca^{2+} signaling were dependent on the use of natural and engineered Ca^{2+} indicators, such as aequorin⁴ and fura-2.⁵ Even with current technology, the detection of transient elevations of Ca^{2+} requires cumbersome analytical tools and does not reveal the targets of such Ca^{2+} signals.

This laboratory investigates Ca^{2+} signals that act through the Ca^{2+} /calmodulin-dependent (multifunctional) protein kinase known as CaMK-II, an enzyme that is enriched in the central nervous system and originally identified as a regulator of long-term potentiation.⁶ CaMK-II is not brain-specific, is widely expressed and highly conserved throughout the entire lifespan and bodies of species throughout the animal kingdom, including invertebrates.^{7,8} CaMK-II has the unique capability of sustaining its own activity even after Ca^{2+} levels have diminished due to its ability to autophosphorylate at Thr²⁸⁷. In this autophosphorylated state, CaMK-II remains active in a Ca^{2+} /CaM-independent manner, until dephosphorylated.⁹ Thus, the localization of phosphorylated CaMK-II (Thr²⁸⁷) can identify cells in which natural, relevant Ca^{2+} elevations have occurred.

An antibody against autophosphorylated (P-Thr²⁸⁷) mammalian CaMK-II has been well-characterized and was initially used to localize activated CaMK-II in brain tissue.⁹ Zebrafish (*Danio rerio*) have seven CaMK-II genes^{10,11} whose protein products contain a sequence of MHRQE[pT²⁸⁷]VECLK in this region.^{10,11} This sequence is very similar to the phosphopeptide antigen used to create this rabbit polyclonal antibody (MHRQE[pT]VDCLK; Upstate/Millipore) and therefore it was not a complete surprise that this antibody cross-reacted with zebrafish CaMK-II. This laboratory showed that this antibody reacts with zebrafish CaMK-II in proportion to autophosphorylation and Ca^{2+} /CaM-independent activity.¹² Additional pan-specific CaMK-II antibodies have also been shown to cross-react with zebrafish CaMK-II.¹³

This antibody has been used to demonstrate that zebrafish CaMK-II is preferentially activated in cells on one side of the zebrafish Kupffer's Vesicle (KV), the ciliated organ necessary for establishment of left/right asymmetry.¹² This antibody was used to demonstrate that CaMK-II

is transiently activated in four adjacent cells on the left side of the KV during the exact same developmental phase that organ positioning is determined.¹² In addition to the Kupffer's Vesicle (KV), autophosphorylated (P-T²⁸⁷) was also located in specific intracellular sites in other ciliated tissues including the kidney, neuromasts, and inner ear.^{12,13} In the zebrafish kidney, P-T²⁸⁷-CaMK-II is enriched along the apical border of ciliated ductal cells and within cloacal cilia where it influences their assembly.¹³ Finally, in the developing inner ear, P-T²⁸⁷-CaMK-II is intensely concentrated at the base of cilia and influences cell differentiation through the Delta-Notch signal pathway.¹⁴ In summary, the detection of activated CaMK-II has pinpointed sites of intracellular Ca²⁺ release and illuminated potential new signaling pathways.

These discoveries were completely dependent on developing a sensitive and accurate method to localize activated (P-T²⁸⁷-autophosphorylated) CaMK-II. The methods to fix and immunostain the zebrafish KV, kidney and inner ear are described. The limitations of this technique are also described. These techniques should be useful to any investigator who seeks to obtain high-resolution images in two fluorescent channels of not just phospho-epitopes, but any epitope, during early vertebrate development.

Protocol

The zebrafish procedures in this protocol have been approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University.

1. Preparation of Reagents

1. 4% PFA/PBS. Weigh 8 g of paraformaldehyde (PFA) in the fume hood. While still in the fume hood, dissolve the dry PFA in ~80 ml distilled H₂O with stirring and heating to 50 °C. While stirring, add 3 - 10 drops of fresh 1N NaOH until PFA is completely dissolved and solution clarifies. Remove from heat and add 100 ml 2x phosphate buffered saline (PBS). Bring volume to 200 ml with distilled H₂O. Cool to RT and confirm pH of 7.4 before use. Store in the dark at 4 °C but use within one week.
2. Use 100% methanol without any supplements. Use 95% ethanol without any supplements.
3. Prepare Phosphate-buffered Tween (PBT). Supplement Phosphate-buffered saline (PBS) with 0.1% Tween-20. Add 0.5 ml of a 20% Tween-20 stock solution to 100 ml 1x PBS. Store at RT.
4. Prepare Phosphate-buffered Triton-X (PBTx). Supplement PBS with 0.1% Triton X-100. Add 0.5 ml of a 20% Triton X-100 stock to 100 ml of PBS. Store at RT.
5. Prepare 10% NGS/PBTx. Normal goat serum (NGS) blocks non-specific binding sites. Store NGS in aliquots at -20 °C. To use, add 1 ml to 9 ml PBTx.
6. Prepare 50% glycerol/PBS. Mix thoroughly equal parts of 2x PBS and 100% glycerol. Store at RT.

2. Embryo Fixation

1. **Breeding.** Obtain wild type (AB and WIK) or transgenic (e.g., β -actin:CAAX-GFP) embryos through natural matings. If desired, inject embryos with constructs or morpholinos, as described.^{15,16}
2. **Raise embryos.** Collect embryos and incubate at 28.5 °C in the presence of 0.003% 1-phenyl-2-thiourea (PTU) to block pigmentation as described.¹⁷
3. **Fix.** When embryos have reached the desired developmental stage,¹⁸ anesthetize the embryos using MESAB, remove as much system water as possible and add fresh 4% PFA/PBS for 3 - 4 hr at RT. NOTE: At times less than 20 hr post fertilization (hpf), fix prior to dechorionation. At times after 20 hpf, dechorionate prior to fixation using two pairs of fine point forceps under a dissecting microscope.
4. **Changing solutions.** Transfer embryos using a wide bore pipet, as described.¹⁷ Change solutions in 1.5 ml capped microcentrifuge tubes, simply by allowing embryos to settle by gravity without centrifugation.
5. **Post-fixative.** After 3 - 4 hr, remove PFA/PBS, replace with 100% methanol and store at -20 °C for at least 48 hr. NOTE: For maximal P-CaMK-II immunoreactivity, limit total methanol storage time to one week.

3. Immunostaining Whole Embryos

1. Place a minimum of 10 embryos for each experimental condition in capped 1.5 ml microcentrifuge tube, and label them.
2. Allow the embryos to settle. Remove and discard methanol and rehydrate with progressive washes containing 0.5 ml of decreasing concentrations of ethanol, as indicated in the next step. Each step is a 5 min wash with rocking.
3. Progressively rehydrate with these 5 solutions: 66% ethanol/33% PBTx, 33% ethanol/66% PBTx, 100% PBTx, 100% PBTx (this is the first repeat of PBTx), 100% PBTx (this is the second repeat of PBTx).
4. Remove last PBTx wash. Add 0.5 ml 10% NGS/PBTx. Incubate for at least 1 hr at RT with gentle rocking and then remove and discard solution.
5. Prepare primary antibody solution by diluting in 10% NGS/PBTx. If co-immunostaining, use higher affinity antibody first. For this study, incubate with the mouse anti-acetylated tubulin monoclonal antibody diluted 1:500. For example, if there are 10 samples, combine 6 μ l of the stock antibody solution with 3 ml of 10% NGS/PBTx and then distribute 0.3 ml of this into each tube.
6. Add 0.2 - 0.5 ml of the diluted primary antibody to each tube to immerse all embryos. Incubate with gentle rocking O/N at RT.
7. In the morning, remove and discard antibody solution. Add 0.5 ml 2% NGS/PBTx to wash away excess primary antibody. Gently rock for 5 min. Remove and discard solution. Repeat twice.
8. Dim overhead lights and dilute the appropriate fluorescently-conjugated secondary antibody in 10% NGS/PBTx so that each condition contains at least 0.5 ml. With the mouse anti-acetylated tubulin primary antibody, use the green-fluorescent dye goat anti-mouse IgG at a 1:500 dilution.
9. Incubate secondary antibody for 4 hr with gentle rocking at RT in the dark. From now on, process the samples under dimmed lights and incubate in a dark container or by wrapping in aluminum foil.

10. At the end of the incubation, remove and discard the secondary antibody solution. Add 0.5 ml 2% NGS/PBTx to wash. Gently rock for 5 min. Remove and discard solution. Repeat twice.
11. If co-immunostaining, obtain the second primary antibody from a different species than the first primary antibody. In these studies, follow the rabbit anti-phospho CaMK-II antibody by the red-fluorescent dye-conjugated goat anti-rabbit IgG secondary antibody.
12. Dilute rabbit anti-phospho CaMK-II antibody 1:20. For each tube of embryos, suspend in 0.3 ml 10% NGS/PBTx, then add 15 µl of the stock antibody solution.
13. Ensure that embryos are immersed and lights are dimmed. Incubate with gentle rocking O/N at RT in the dark.
14. In the morning under dim lights, remove and discard antibody solution. Add 0.5 ml 2% NGS/PBTx. Gently rock for 5 min. Remove and discard solution. Repeat twice.
15. Keep overhead lights dim and dilute enough of the appropriate fluorescently-conjugated secondary antibody so that each condition contains at least 0.5 ml. In this study, dilute the red-fluorescent dye-conjugated goat anti-rabbit IgG secondary antibody (red channel) 1:500 in 10% NGS/PBTx.
16. Incubate second secondary antibody for 4 hr with gentle rocking at RT in the dark.
17. At the end of this incubation and under dim lights, remove and discard the secondary antibody solution. Add 0.5 ml PBTx. Gently rock for 5 min. Remove and discard solution. Repeat twice. Store in either PBTx or 50% glycerol/PBS depending on the imaging procedure.

4. Confocal Imaging and Processing

1. Mount embryos for imaging. Place 1 - 5 embryos on a glass slide. Create a chamber between the main coverslip and the slide using coverslip fragments on each side of the chamber. Typically, four #1 coverslips are used to make these spacer stacks without sealing.
NOTE: No mounting medium is necessary.
2. Use a 100X oil immersion objective to bring single embryo into focus using transmitted light. Turn off light. Turn on confocal microscope. Ensure that appropriate lasers (green and/or red) are turned on and remote focus accessory is engaged.
3. Turn on the confocal program. In the "Acquire bar," select the proper objective. In the "XY Basic" bar, set image size to 1,024 by clicking the 1,024 button.
4. In the "Laser and Detector" bar, click on the red 488 box and the green 568 box to turn on laser and detector for each channel. Set pinhole to medium and adjust the gain in the "Gain" bar of each channel to visualize.
5. In the "Acquire Settings" bar, click "Live" to begin acquiring images. In the "View Settings" bar, uncheck the "Force Integral Zoom" box to center in the "Live" window.
6. In the "Acquire Settings" bar, under the "Z" tab, step through the layers of the image. After selecting the number of optical sections to incorporate into the image, move to some point in the "center" of the z-plane.
7. Under the "Z" tab, click the small red "Reference" box. This will zero the RFA at the point chosen as the "center". In the box labeled "Step Size", select the thickness of the layers (between 0.25 µm and 2.0 µm is ideal). Pay attention to the "File Size" box and try to limit to 1GB.
NOTE: Typically, up to 40 optical sections of 0.5 - 1.0 µm are obtained, but the number of sections can be determined empirically.
8. To find the top extreme of the image, click the circle next to "Top" and move through the z layers. Now click the circle next to "Bottom", the computer will take the image back to the layer as the center. Again move through the layers to find the bottom extreme of the image.
9. Click on "Live" again in the "Acquire Settings" box to stop the laser acquisition. In this panel, click the red boxes labeled Average and Z-stack. These boxes will turn green.
10. In the "Acquire Settings" box, click "Single" to acquire the entire series of images. You can monitor the progress as it scans in both channels and through the entire z-stack.
11. To save, click on the volume window and click "save as" and name the file. Save as an ".ids" file. To volume render the file while the z-stack is still open, select the Data pull down menu and click on "volume render". Save the rendered file as a tiff file. This is the projected image that are shown in this publication.

Representative Results

Optimal Conditions for Visualizing Phospho-epitopes

Methods describing the immunolocalization of protein epitopes in zebrafish embryos have been relatively sparse compared to those for localizing mRNAs via *in situ* hybridization. Fixatives used in localizing protein epitopes in ciliated cells of zebrafish embryos have included 4% PFA/PBS and Dent's fixative, which is a mixture of methanol and DMSO.¹⁹ Localization of RNA by whole mount *in situ* hybridization (WISH) is typically achieved using PFA followed by storage in 100% methanol.^{20,21} Our studies revealed that the WISH fixative combination, when limiting the time in methanol to between two and seven days, was vastly superior to any other fixative for immunolocalization with the P-CaMK-II antibody. The signal achieved with this PFA/methanol combination was also significantly greater than when Dent's fixative, 4% PFA/PBS or methanol were used alone, depending on the developmental time and location within the embryo.

When optimizing the technique described here and for each fixative and developmental time used, a control sample was prepared in which all steps were followed, except that the primary antibody was omitted. Such control samples lacked a fluorescent signal when imaged under the same conditions described above. This control is recommended for other investigators replicating this approach with any antibody.

The PFA/methanol fixative combination yielded the strongest P-CaMK-II signal and was also compatible with immunostaining for acetylated tubulin, which is a standard marker for cilia. Developmentally, the first ciliated organ that emerges and has been imaged in these studies is the Kupffer's vesicle (KV). The KV is located at the posterior end of the notochord as shown at the 12-somite (15 hpf) stage (**Figure 1**). The KV is a transient organ and is responsible for left-right asymmetry. It emerges at the 2-somite stage (12 hpf) and disappears around the 18-somite stage. Beating primary cilia generate a circular flow of fluid, which leads to an elevation of Ca^{2+} in the ciliated cells lining the KV and the activation of CaMK-II in discrete locations (**Figure 1**). P-CaMK-II reactivity appears and disappears on one side of the KV as long as cilia are beating.¹² At this early stage, total embryonic CaMK-II levels are still only half the level as at 24 hpf and one tenth of the level at 3 days of development.¹¹ Perhaps because total CaMK-II expression is relatively low at 15 hpf, any improvement in the immunostaining technique at this stage is especially important.

Between 24 and 72 hpf of development, P-CaMK-II appears on the apical surface of ciliated cells lining specific regions of the pronephric ducts (**Figures 2,3**). The immunoreactivity of total CaMK-II along the entire pronephric duct and throughout adjacent somites (**Figure 2**) demonstrates that only a subset of embryonic CaMK-II is activated (P-CaMK-II).

Fixation Conditions are Compatible with Preserving GFP Fluorescence

These fixation techniques are also compatible with retaining green fluorescent protein (GFP) fluorescence without enhancement (**Figure 3**). In the GFP-tagged CaMK-II construct that is used in this figure, GFP retains sufficient fluorescence though PFA/methanol fixation and subsequent immunostaining. In a high magnification view of cells lining the pronephric duct (**Figure 3**), the mosaic expression of GFP-CaMK-II can be viewed in cells that have also been immunostained for P-CaMK-II.

The zebrafish embryonic ear is another tissue in which the elevation of Ca^{2+} , acting through CaMK-II, influences its development.¹⁴ Zebrafish ears normally appear at around one day of development. At this time, CaMK-II is intensely activated at the base of the kinocilia and at lower levels along the kinocilium (**Figure 4**). This set of images shows that this fixation and immunolocalization technique can detect staining within cilia, a structure whose cross-section is less than 1 μm . These cilia retain their structure throughout this fixation and immunostaining process.

An additional example of two-color counterstaining in the inner ear at a later time of development was achieved by staining with both Alexa⁴⁸⁸ phalloidin and anti-acetylated tubulin followed by an Alexa⁵⁶⁸ tagged secondary antibody (**Figure 5**). It is noteworthy that the PFA/methanol fixation method preserves both F-actin and tubulin, which is not true of all fixatives. This example is shown as a merged color image for the entire ear and then for sub-regions.¹⁴

A final representative example of two-color counterstaining was achieved with a strain of fish that produced embryos with membrane-targeted GFP (**Figure 6**). Membrane targeted GFP is not attached to any other protein and is lost when extracted with methanol, therefore this image was obtained from fish that were fixed with PFA alone. This result suggests that methanol treatment extracts membranes, so it is not recommended for proteins that may be exclusively membrane bound. The P-CaMK-II signal in this figure is diminished relative to that achieved if methanol were also used, but this demonstrates that at this stage and location (kidney) of the developing embryo, the signal is strong enough to be detected without methanol treatment. That is not true at the KV stage; *i.e.*, methanol is required to visualize P-CaMK-II immunostaining. This example is only shown as a merged image where the kidney's pronephric duct is adjacent to trunk muscle.

In summary, the PFA/methanol fixation method described here is compatible with the preservation of GFP and with staining for F-actin, acetylated tubulin, total CaMK-II and P-CaMK-II.

Figure 1. P-CaMK-II Counterstained for Acetylated Tubulin (cilia) in Zebrafish KV. Views of a single live 12 somite stage (12 ss) embryo reveals the posterior location of the KV by the arrowhead (lateral view, **A**) and the circle within the box (ventral view, **B**). Embryos at this stage were fixed using PFA/methanol. Embryos were immunostained for acetylated tubulin followed by an Alexa⁴⁸⁸-labelled secondary antibody (green channel). Next, the rabbit polyclonal antibody against P-CaMK-II was followed by an Alexa⁵⁶⁸-labelled secondary antibody (red channel). Two-channel fluorescent image projections were acquired as described at progressively higher magnifications (**C,D**). Scale bar = 10 μm . This figure is modified from a previous publication.¹² [Please click here to view a larger version of this figure.](#)

Figure 2. P-CaMK-II Counterstained for Total CaMK-II along Zebrafish Kidney. A single dechorionated embryo at 30 hpf was fixed using PFA/methanol. Embryos were then stained for total CaMK-II followed by the Alexa⁴⁸⁸ labeled secondary antibody (green). Next, the P-CaMK-II primary antibody was followed by the Alexa⁵⁶⁸ labeled secondary antibody (red). Staining reveals an enrichment of activated CaMK-II (in red) along the apical surface of cells that line the ducts of the pronephric zebrafish kidney whereas total CaMK-II is enriched at the somite boundaries of adjacent muscle tissue and throughout sarcomeres. Scale bar = 50 μ m. These images were acquired at a lower magnification than is described in the text in order to visualize the entire kidney. This figure is modified from a previous publication.¹³ [Please click here to view a larger version of this figure.](#)

Figure 3. P-CaMK-II Counterstained for GFP in Zebrafish Kidney Cells. This 30 hpf embryo was injected with a cDNA encoding a dominant negative GFP-CaMK-II.¹³ Such injections typically show mosaic expression. The embryo was fixed using PFA/methanol and then immunostained for P-CaMK-II using an Alexa⁵⁶⁸ labeled secondary antibody. Imaging reveals that GFP persists through PFA/methanol fixation, rehydration, blocking and immunostaining. Scale bar = 10 μ m. This figure is modified from a previous publication.¹³ [Please click here to view a larger version of this figure.](#)

Figure 4. P-CaMK-II Counterstained with Acetylated Tubulin in Zebrafish Inner Ear. This 30 hpf embryo was fixed using PFA/methanol. The anti-acetylated tubulin antibody was followed in order by the Alexa⁴⁸⁸-labeled secondary antibody, the P-CaMK-II antibody and the Alexa⁵⁶⁸ labeled secondary antibody. Staining reveals that P-CaMK-II is present along inner ear cilia and co-localizes with acetylated tubulin. Scale bar = 5 μ m. This figure is modified from a previous publication.¹⁴ [Please click here to view a larger version of this figure.](#)

Figure 5. Actin and Acetylated Tubulin in Zebrafish Inner Ear. A three day old (72 hpf) zebrafish embryo was fixed and immunostained for acetylated tubulin using Alexa⁵⁶⁸-labeled secondary antibodies, followed by actin visualization using Alexa⁴⁸⁸ phalloidin. Cilia as well as axonal innervation of the ear is revealed by acetylated tubulin (in red) and F-actin structures include muscle fibers (in green). Two ciliated sensory regions of the zebrafish ear are shown in more detail: the anterior macula (am) and posterior crista (pc). Scale bar = 10 μ m. This figure is modified from a previous publication.¹⁴ [Please click here to view a larger version of this figure.](#)

Figure 6. P-CaMK-II Counter Imaged for Membrane GFP in Zebrafish Kidney. This single merged image in which the β -actin:CAAX-GFP embryo (30 hpf) was fixed and stained for P-CaMK-II followed by the Alexa⁵⁶⁸ labeled secondary antibody. Staining reveals an enrichment of activated CaMK-II (in red) along the apical surface of cells that line the ducts of the pronephric zebrafish kidney. These kidney ductal cells are nestled just underneath muscle tissue and both are marked by the expression of membrane targeted GFP (in green). Scale bar = 50 μ m. [Please click here to view a larger version of this figure.](#)

Discussion

The PFA/methanol method was developed in this laboratory with the primary objective of optimizing the immunolocalization of the phospho-T²⁸⁷-CaMK-II epitope during zebrafish development. This method successfully localized P-CaMK-II during the formation of several ciliated organs including the zebrafish KV,¹² inner ear¹⁴ and kidney.¹³ Particularly at the KV stage, this technique was necessary. The success of this method is likely due to a combination of a) minimization of autofluorescence, b) preservation of the phospho-CaMK-II epitope and c) enhanced accessibility of the epitope to antibodies.

A primary limitation of this approach is the time of storage in methanol. While immunoreactivity of P-CaMK-II is maximal after two days in methanol at -20°C, the reactivity of this epitope is lost after one week of storage in methanol. It is not clear whether at this time the phosphate group is hydrolyzed or further denaturation occurs that decreases immunoreactivity. Methanol/EGTA at -20 °C is a traditionally rapid fixative for preserving tubulin-rich structures during the development of early embryos.²² However, methanol alone is not desirable for preserving morphology or even structures such as the actin cytoskeleton and must be preceded by PFA.

Additional advantages of this approach are that it also preserves other epitopes such as F-actin and acetylated tubulin and does not eliminate the native fluorescence of GFP. Other fixatives, such as Dent's fixative, are superior for other epitopes¹³ and remain compatible with P-CaMK-II staining, although P-CaMK-II staining is weaker in Dent's fixative than PFA/methanol. Since phospho-proteins are prevalent in signaling pathways that are active during early development, Dent's fixative and PFA/methanol are recommended as two fixatives for other phospho-epitopes in zebrafish embryos. In developing applications with this technique for other proteins and their antibodies, it has been helpful to have antibodies that are also reactive on immunoblots and to have cloned and overexpressed proteins with which antibodies can be validated.¹²

It is worth the effort to optimize techniques for immunolocalization because it a) can verify gene suppression at the protein level and b) enables the development of models of action. In this laboratory, the results of these assays have allowed the formulation of models through which CaMK-II functions. For example, its location in the KV is transient and asymmetric, like the role of this organ. In the kidney, its location on the apical side of pronephric ductal cells suggests roles that respond to signals from the duct. Finally, the activation of this enzyme in specific intense puncta at the base of ear kinocilia suggests a localized Ca²⁺ signal. The methods described here should also be useful to any investigator who seeks to obtain high-resolution images of any embryonic cell type in two fluorescent channels.

Disclosures

The authors declare that they have no competing financial interests.

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References

1. Webb, S. E., & Miller, A. L. Calcium signalling during embryonic development. *Nat Rev Mol Cell Biol.* **4**, 539-551, (2003).
2. Webb, S. E., & Miller, A. L. Ca^{2+} signalling and early embryonic patterning during zebrafish development. *Clin Exp Pharmacol Physiol.* **34**, 897-904, (2007).
3. Whitaker, M. Calcium at fertilization and in early development. *Physiol. Rev.* **86**, 25-88 (2006).
4. Yuen, M. Y. *et al.* Characterization of $\text{Ca}(2+)$ signaling in the external yolk syncytial layer during the late blastula and early gastrula periods of zebrafish development. *Biochim Biophys Acta.* **1833**, 1641-1656 (2013).
5. Tombes, R. M., & Borisy, G. G. Intracellular free calcium and mitosis in mammalian cells: anaphase onset is calcium modulated, but is not triggered by a brief transient. *J. Cell Biol.* **109**, 627-636, (1989).
6. Hudmon, A., & Schulman, H. Neuronal Ca^{2+} /Calmodulin-Dependent Protein Kinase II: The Role of Structure and Autoregulation in Cellular Function. *Annu. Rev. Biochem.* **71**, 473-510 (2002).
7. Tombes, R. M., Faison, M. O., & Turbeville, C. Organization and Evolution of Multifunctional Ca^{2+} /CaM-dependent Protein Kinase (CaMK-II) Genes. *Gene.* **322**, 17-31 (2003).
8. Braun, A. P., & Schulman, H. The Multifunctional Calcium/Calmodulin-Dependent Protein Kinase: From Form to Function. *Annu. Rev. Physiol.* **57**, 417-445 (1995).
9. Rich, R. C., & Schulman, H. Substrate-directed function of calmodulin in autophosphorylation of Ca^{2+} /calmodulin-dependent protein kinase II. *J Biol Chem.* **273**, 28424-28429 (1998).
10. Rothschild, S. C. *et al.* Tbx5-mediated expression of Ca^{2+} /calmodulin-dependent protein kinase II is necessary for zebrafish cardiac and pectoral fin morphogenesis. *Dev Biol.* **330**, 175-184, (2009).
11. Rothschild, S. C., Lister, J. A., & Tombes, R. M. Differential expression of CaMK-II genes during early zebrafish embryogenesis. *Dev Dyn.* **236**, 295-305 (2007).
12. Francescatto, L., Rothschild, S. C., Myers, A. L., & Tombes, R. M. The activation of membrane targeted CaMK-II in the zebrafish Kupffer's vesicle is required for left-right asymmetry. *Development.* **137**, 2753-2762, (2010).
13. Rothschild, S. C., Francescatto, L., Drummond, I. A., & Tombes, R. M. CaMK-II is a PKD2 target that promotes pronephric kidney development and stabilizes cilia. *Development.* **138**, 3387-3397, (2011).
14. Rothschild, S. C. *et al.* CaMK-II activation is essential for zebrafish inner ear development and acts through Delta-Notch signaling. *Dev Biol.* **381**, 179-188, (2013).
15. Yuan, S., & Sun, Z. Microinjection of mRNA and morpholino antisense oligonucleotides in zebrafish embryos. *J Vis Exp.* e1113 (2009).
16. Rosen, J. N., Sweeney, M. F., & Mably, J. D. Microinjection of zebrafish embryos to analyze gene function. *J Vis Exp.* e1115 (2009).
17. Westerfield, M. *The Zebrafish Book: A guide for the laboratory use of zebrafish (Brachydanio rerio)*. University of Oregon Press, (1993).
18. 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).
19. Obara, T. *et al.* Polycystin-2 immunolocalization and function in zebrafish. *J Am Soc Nephrol.* **17**, 2706-2718 (2006).
20. Chitramuthu, B. P., & Bennett, H. P. High resolution whole mount *in situ* hybridization within zebrafish embryos to study gene expression and function. *J Vis Exp.* e50644 (2013).
21. Thisse, C., & Thisse, B. High-resolution *in situ* hybridization to whole-mount zebrafish embryos. *Nature protocols.* **3**, 59-69 (2008).
22. Harris, P., Osborn, M., & Weber, K. Distribution of tubulin-containing structures in the egg of the sea urchin *Strongylocentrotus purpuratus* from fertilization through first cleavage. *J Cell Biol.* **84**, 668-679, (1980).