Two-color fluorescence in situ hybridization using chromogenic substrates in *Xenopus*

Zachary Agricola¹ and Sang-Wook Cha²

¹Division of Neonatology and Pulmonary Biology, Cincinnati Children's Hospital Medical Center, OH and ²Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, OH

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Xenopus embryo yolk proteins present a serious barrier to fluorescence microscopy. Previously, *in situ* assays of gene transcripts in whole embryos was limited to the use of chromogenic alkaline phosphatase substrates, which restricted researchers' ability to gauge coexpression of transcripts. Here, we describe a modified *in situ* hybridization (ISH) protocol that uses fluorescent substrates and a novel yolk-clearing technique for simultaneous visualization of the expression of two genes in whole *Xenopus* embryos with high resolution. This protocol employs two well-known fluorescent substrates, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) and Vector Red, in a sequential dual *in situ* hybridization procedure. Subsequent clearing of the samples with refractive-index-matching solution (RIMS) renders the samples amenable to confocal microscopy, allowing imaging at sufficiently high resolution to discern coexpression of transcripts within individual cells.

Assessment of spatiotemporal expression domains of gene transcripts is effectively conducted through *in situ* hybridization (ISH) of endogenous transcripts with synthetic labeled antisense riboprobes. While conventional chromogenic substrates of alkaline phosphatase (AP), such as

BM Purple and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP), yield robust signals, they don't allow single-cell colocalization of multiple transcripts through a double-ISH procedure. This is due to the fact that their dark hue obscures the signal from lighter substrates such

as Vector Red, Fast Red, and NBT/BCIP. Furthermore, the opacity of yolk platelets, which accumulate in endodermal cells of *Xenopus* embryos, limits the resolution of whole-mount ISH with bright-field imaging (1). ISH with a fluorescent substrate and ISH with thin sections are conventional methods for producing high-resolution images with limited depth (2–4). The use of fluorescent AP substrates in combination with confocal fluorescence imaging is intended to circumvent these obstacles and optimize the ISH protocol.

In zebrafish, Vector Red and NBT/BCIP make an effective duo of fluorescent AP substrates (5). They are excited by red and far-red light, respectively, and they are chromogenic, so signal development can be monitored by eye. To address the opacity of the yolk proteins in *Xenopus* embryonic endoderm, the embryos were treated with refractive-index-matching solution (RIMS) (6) as a clearing agent, in addition to the customary bleaching procedure. The clearing procedure rendered the embryos transparent enough for laser excitation of Vector Red and NBT/BCIP, enabling the acquisition of high-resolution fluorescence images in deep tissue. Through the use of this novel *Xenopus* protocol, researchers can verify the colocalization of transcripts within distinct cells.

Xenopus laevis embryos at Nieuwkoop and Faber stage (NF) 24 were fixed in MEMFA [1× MEM (100 mM MOPS, 2 mM EGTA, 1 mM magnesium sulfate, pH 7.4), 3.7% formaldehyde] as previously described (2). The embryos were then dehydrated in 100% ethanol and stored at -20°C. Samples were serially rehydrated in 70% ethanol, 40% ethanol, 25% ethanol/75% PBSTw, and 100% PBSTw [1× PBS (137 mM NaCl, 2.68 mM KCl, 10.1 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4), 0.1% Tween 20] via single 5-min washes. Embryos were washed in PBSTw for 3 5-min washes and then digested for 8 min with a

METHOD SUMMARY

Here, we describe an improved fluorescence *in situ* hybridization (FISH) protocol for single-cell resolution imaging of two different probes in *Xenopus* embryos using the highly sensitive chromogenic substrates nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) and Vector Red in combination with the refractive-index-matching solution (RIMS) clearing reagent.

proteinase K (Thermo Fisher Scientific, Waltham, MA) solution (2 $\mu\text{g}/\text{mL}$ in PBSTw). Subsequently, the embryos were washed in PBSTw for 2 5-min washes and then refixed in 3.7% formaldehyde/PBSTw via a 20-min incubation. For the penultimate pre-hybridization phase, the embryos underwent 5 5-min PBSTw washes. Embryos were then incubated in hybridization buffer [50% formamide, 5 $\times$ SSC (750 mM NaCl, 75 mM sodium citrate, pH 7.0), 1 mg/mL torula type IX yeast RNA (R3629-25G; Sigma-Aldrich, St. Louis, MO), 1 $\times$ Denhardt's solution [0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone (PVP), 10 mM EDTA, 0.1% SDS, 100 $\mu\text{g}/\text{mL}$ heparin, 0.1% Tween 20] for 3 h at 65°C.

Riboprobes for the gene *spib.a*, which encodes an ETS transcription factor expressed in primitive myeloid cells in the anterior ventral blood island (aVBI), and the myeloperoxidase gene (*mpo*), which encodes a peroxidase produced by neutrophilic granulocytes arising in the aVBI, were synthesized with digoxigenin-11-UTP (DIG) and fluorescein-12-UTP (FL) labels (Roche Diagnostics, Indianapolis, IN), respectively (2). While *spib.a* and *mpo* are known to be expressed in primitive myeloid cells (7), it is unclear whether these genes are expressed uniformly in the myeloid population or if there are subpopulations with different expression profiles. The more strongly expressed *mpo* probe was used with FL and the Vector Red AP substrate (Vector Laboratories, Burlingame, CA) to compensate for the lower intensity of the Vector Red fluorescence signal compared with the other substrate used in this study, NBT/BCIP (Roche Diagnostics).

After pre-hybridization, embryos were incubated with *spib.a*-DIG and *mpo*-FL probes simultaneously at concentrations of 2 $\mu\text{g}/\text{mL}$ and 1 $\mu\text{g}/\text{mL}$, respectively, in hybridization buffer. Hybridization was conducted at 65°C for 16 h. After hybridization, embryos were removed from the riboprobe solution and transferred to 2 $\times$ SSC. Embryos were washed in 2 $\times$ SSC for 3 30-min washes. The embryos were incubated with 1 $\mu\text{g}/\text{mL}$ RNaseA in 2 $\times$ SSC for 20 min to eliminate remaining unhybridized riboprobe. Embryos were then treated with 3 30-min 0.2 $\times$ SSC

Figure 1. Fluorescence imaging of nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) and Vector Red signals from whole-mount in situ hybridization (WMISH). (A) Schematic diagram of the superior resolution offered by fluorescence in situ hybridization (FISH). Dual FISH could distinguish the phenomenon of multiple transcripts expressed in a single cell from the phenomenon of multiple proximal cells uniquely expressing transcripts that collectively mimic coexpression. (B) *Xenopus* embryos after two alkaline phosphatase (AP) reactions and bleaching. After refractive-index-matching solution (RIMS) treatment, the front end of the foregut was clearly visible (white arrowhead). (C) Volume reconstruction of the anterior ventral blood island region (aVBI). Signals for *spib.a* (green), *mpo* (red), and the nucleus (blue) are shown. Scale bar: 200 μm . (D) Higher magnification of the inset from the upper left panel of (C). The *spib.a* and *mpo* genes had distinct expression patterns. Scale bar: 60 μm . (E) Volume reconstruction of transverse section with *Nr1h5* staining in a thick section (300 μm) of NF35 embryos. The Z-stacks are shown in Supplementary Movie S1.

washes followed by 3 30-min 1× MAB (maleic acid buffer: 100 mM maleic acid, 150 mM NaCl, pH 7.5) washes. Subsequently the embryos were incubated in blocking buffer [1× MAB, 20% heat-inactivated lamb serum, 2% Blocking Reagent (Roche Diagnostics)] for 2 h. Embryos were then incubated with anti-DIG-AP (Roche Diagnostics) diluted to 150 mU/mL in blocking buffer for 12 h at 4°C. Subsequently, embryos were subjected to 12 30-min 1× MAB washes.

In preparation for signal development, embryos were treated with AP buffer (100 mM Tris pH 9.5, 50 mM MgCl₂, 100 mM NaCl, 0.1% Tween 20, 5 mM levamisole [(–)-tetramisole hydrochloride, L9756-10G; Sigma-Aldrich]) for 2 5-min washes. Riboprobe detection was done sequentially, with the weaker *spib.a*-DIG being detected first. NBT/BCIP was diluted to 0.35% in AP buffer; 1 mL of this development solution was used per sample of embryos. Samples were developed over a period of multiple days, initially at room temperature but ultimately at 4°C as they approached desired signal levels. Upon the culmi-

nation of signal development with the *spib.a*-DIG riboprobe, embryos were treated with 1× MAB/10 mM EDTA for 2 5-min washes and then serially dehydrated into ethanol to reduce background fluorescence. Embryos were washed in methanol overnight; therefore, it was imperative to develop the NBT/BCIP colorimetric signal first because it is not soluble in organic solvents, whereas Vector Red is.

After the overnight methanol wash, embryos were serially rehydrated in 1× MAB. Upon complete rehydration, embryos were treated with blocking buffer and MAB washes as described before, except with anti-FL-AP (Roche Diagnostics) instead of anti-DIG-AP. To develop the signal from the *mpo*-FL probe, Vector Red AP substrate was used according to the manufacturer's instructions. Given the limited longevity (~45 min) of freshly prepared Vector Red solution, 2 consecutive 1-h incubations with fresh Vector Red solution were carried out. Earlier experiments had indicated that two or three Vector Red washes were optimal for devel-

opment of *mpo*-FL and many other riboprobes (data not shown). Development was ceased at the desired time by replacement of the chromogenic substrate with 1× MAB/10 mM EDTA. Embryos were then washed in Pseudo Bouin's solution (9.25% formaldehyde, 5% acetic acid) overnight to preserve the NBT/BCIP and Vector Red stains.

Bleaching of the embryos' pigment was accomplished by 4 30-min washes of bleaching solution (5% formamide, 1% hydrogen peroxide, 0.5× SSC) under a fluorescent light (Figure 1B). Prior to clearing, embryos were washed in 1× PBS for 5 10-min washes and then incubated in 1× PBS with 1:10000 4',6-diamidino-2-phenylindole (DAPI) (D1306; Thermo Fisher Scientific) for 2 h. Embryos were then rinsed with 1× PBS and transferred to RIMS [88% Histodenz (iohexol, D2158-100G; Sigma-Aldrich), 20 mM phosphate buffer, 0.01% sodium azide, 0.1% Tween 20, 2.5% 1,4-diazabicyclo[2.2.2] octane (DABCO) (D27802-100G; Sigma-Aldrich)] optimized for confocal imaging (1 mL per sample of 6 to 12

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embryos). Yolk proteins, which render the embryonic *Xenopus* endoderm opaque and autofluorescent, necessitate the application of a clearing agent to permit fluorescent imaging. An overnight treatment with RIMS was sufficient to fully clear the embryos (Figure 1B). Despite the conspicuous lack of transparency in white light, this level of clearing was sufficient to allow for confocal imaging. Embryos were stored in RIMS at room temperature, while protected from light, for >4 weeks. Multiple images were taken over a span of 2 weeks and there was no noticeable signal reduction over that time (data not shown). RIMS solution was used since a conventional clearing solution such as BABB (also known as Murray's clear) (8) causes the diffusion of Vector Red precipitate into solution, and since RIMS is non-corrosive when used with most of the metal/coating parts of a microscope. Moreover, RIMS preserves the fluorescence signal from a protein such as GFP or mCherry.

Whole embryos were mounted in RIMS on a chamber slide in prepa-

ration for image acquisition. A Nikon (Tokyo, Japan) A1R LUN-V inverted microscope equipped with 405, 445, 488, 514, 561, and 647 nm solid state diode lasers was used to image the embryos. Other microscopes with similar equipment can be used. Plan Apo λ (10 $\times$) and Plan Apo VC (20 $\times$) DIC objective lenses were used. NBT/BCIP fluorescence was excited using the 647 nm diode laser, detected with a 740 nm long pass filter, and then imaged with a high-quantum efficiency (QE) bi-alkali photomultiplier tube (Nikon) with 10% QE at 800 nm. Vector Red fluorescence was imaged with a 561 nm diode laser and detected with a 595/50 nm emission filter. Nuclear counterstaining with DAPI was acquired using the 405 nm diode laser with a 450/50 nm emission filter.

Volume reconstructions of confocal Z-stacks with single-cell resolution of *spib.a* and *mpo* expression were produced using the Nikon NIS-Elements Advanced Research software. The images demonstrate colocalization of *spib.a* and *mpo* transcripts within

individual cells (Figure 1, C and D). Even *spib.a*- and *mpo*-coexpressing cells that have migrated out of the aVBI and infiltrated some distance into the endoderm can be detected. *Spib.a* and *mpo* are almost always coexpressed, but a small number of *mpo*-expressing cells appear to be devoid of *spib.a* transcripts (Figure 1D).

To evaluate our FISH technique in deeper tissue, *nr1h5* expression in liver progenitor cells was assessed. NF35 embryos were harvested and cut into 300 μ m transverse sections before being subjected to the ISH procedure described above. An *Nr1h5*-DIG riboprobe was used at a concentration of 1 μ g/mL, and signal was developed with NBT/BCIP. Embryos were bleached, stained with DAPI, and cleared with RIMS as described above, and NBT/BCIP and DAPI were detected using a Nikon A1R LUN-V inverted microscope. *Nr1h5*-positive cells were visible up to approximately 100 μ m deep in the section, depicting a large portion of the liver anlage (Figure 1E).

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In conclusion, our FISH protocol for *Xenopus* allows researchers to examine the expression of two genes at single-cell resolution using the highly sensitive AP substrates NBT/BCIP and Vector Red. In combination with bleaching and clearing, this method can improve the ability to visualize weak or variably expressed genes in both superficial and deep tissues.

Author contributions

Z.A. performed experiments. Z.A. and S.C. analyzed the data. Z.A. and S.C. prepared the manuscript.

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Competing interests

The authors declare no competing interests.

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Address correspondence to Sang-Wook Cha, Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH, 45229. E-mail: sang-wook.cha@cchmc.org.

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