Cellular resolution expression profiling using confocal detection of NBT/BCIP precipitate by reflection microscopy

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The determination of gene expression patterns in three dimensions with cellular resolution is an important goal in developmental biology. However, the most sensitive, efficient, and widely used staining technique for whole-mount in situ hybridization (WMISH), nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) precipitation by alkaline phosphatase, could not yet be combined with the most precise, high-resolution detection technique, confocal laser-scanning microscopy (CLSM). Here we report the efficient visualization of the NBT/BCIP precipitate using confocal reflection microscopy for WMISH samples of Drosophila, zebrafish, and the marine annelid worm, Platynereis dumerilii. In our simple WMISH protocol for reflection CLSM, NBT/BCIP staining can be combined with fluorescent WMISH, immunostainings, or transgenic green fluorescent protein (GFP) marker lines, allowing double labeling of cell types or of embryological structures of interest. Whole-mount reflection CLSM will thus greatly facilitate large-scale cellular resolution expression profiling in vertebrate and invertebrate model organisms.

INTRODUCTION

The most widely used method to determine gene expression patterns in developmental biology is in situ hybridization. Conventional in situ hybridization protocols use digoxigenin (DIG)-11-UTP-labeled RNA probes and detection with an alkaline phosphatase-coupled anti-DIG antibody (1–5). Alkaline phosphatase activity is visualized with the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and color development is enhanced by nitroblue tetrazolium (NBT) yielding an insoluble black-purple precipitate. The sensitivity and precision of alkaline phosphatase-NBT/BCIP staining in whole-mount in situ hybridization (WMISH) is very high, and it often surpasses that of fluorescent staining techniques, especially in the case of weakly expressed genes. However, to date, such samples have been documented only by bright-field microscopy. Being able to image NBT/BCIP samples by confocal laser-scanning microscopy (CLSM) would bring several advantages that greatly facilitate expression profiling in whole embryos, such as enhanced depth of focus and the ability to observe multiple fluorescent signals in isolation or in combination. Here we report a method that allows the detection of conventional WMISH samples by confocal microscopy in zebrafish, Drosophila, and the marine annelid Platynereis dumerilii. The method is based on reflection microscopy that has already been used to detect NBT/BCIP samples and other types of precipitates [e.g., diaminobenzidine (DAB)] in tissue sections and fixed cells (6–9), but not in whole-mount embryo samples. Our method will greatly facilitate the determination of the extent of gene expression in embryos in three-dimensional (3-D) volume rendering and also allow fluorescent co-staining and co-localization studies.

MATERIALS AND METHODS

Microscopy

Bright-field images were taken on a Zeiss Axiosphot microscope (Carl Zeiss, Jena, Germany) using differential interference contrast (DIC) optics. Confocal images were taken either with a Leica TCS SP2 or a Leica TCS SPE confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) with a 40× oil-immersion objective with a pinhole of 2 airy units. For whole-mount reflection CLSM, a 633 nm gas laser or a 635 nm diode laser were used, and the detection window was set to 630–640 nm. It was combined either with fluorescent antibody staining or fluorescent tyramide WMISH and confocal detection of fluorescence using appropriate laser lines. Z-projections and 3-D projections of confocal stacks and quantifications of average pixel intensities were done using ImageJ and Imaris 5.5.

Whole-Mount In Situ Hybridization

Platynereis and zebrafish embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) plus 0.1% Tween® 20 (PTW) for 2 h. Drosophila embryos were dechorionated in 50% bleach, fixed in a biphase mixture of heptane, PBS, and 40% formaldehyde as 1:0.3;0.03:0.2 for 20 min and devitellinized in 1:1 heptane, MeOH. Embryos were stored in methanol at -20°C and rehydrated in a 75%/50%/25%/methanol/PTW series, 5 min each, rinsed two times for 5 min in PTW and digested for 1 min in 100 μg/mL proteinase K. Embryos were rinsed twice in 2 mg/mL glycine/PTW, post-fixed for 20 min in 4% paraformaldehyde/PTW, and rinsed five times for 5 min in PTW. After prehybridization for 1 h in Hyb-Mix [50% formamide, 5× standard saline citrate (SSC), 50 μg/mL heparin, 0.1% Tween 20, 5 mg/mL torula RNA] at 65°C, DIG-11-UTP-labeled RNA probe, denatured at 80°C for 10 min in Hyb-Mix, was added to the prehybridized embryos. Hybridization was carried out overnight at 65°C. Embryos were washed twice in 50% formamide/2× SSC/0.1% Tween 20, twice in 2× SSC/0.1% Tween 20, and twice in 0.2× SSC/0.1% Tween 20 for 30 min at 65°C each. Embryos were blocked in 5% sheep serum in PTW for 1 h at room temperature and incubated in 1:2000 anti-DIG antibody (Roche, Indianapolis, IN, USA) in PTW/5% sheep serum overnight at 4°C. Optionally, mouse anti-acetyl-ated tubulin (Sigma, St. Louis, MO,
USA) and rabbit anti-green fluorescent protein (GFP) antibodies were also added at this step at 1:500. After six PTW washes, 10 min each, embryos were rinsed in staining buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 0.1% Tween 20) and stained for several hours in 337.5 μg/mL NBT and 175 μg/mL BCIP in staining buffer. Staining was stopped in pH 7.5 staining buffer, without NBT/BCIP, and embryos were mounted in glycerol. For the immunostained samples, fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) coupled anti-mouse and rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA), diluted as 1:250, were used in PTW/5% sheep serum following the NBT/BCIP staining step. Embryos were incubated with secondary antibodies overnight at 4°C, washed in PTW six times for 10 min, and mounted in glycerol with 2.5 mg/mL DABCO.

RESULTS AND DISCUSSION

We found that the NBT/BCIP precipitate of whole-mounted embryo samples of the polychaete Platynereis dumerilii (Lophotrochozoa, Annelida) generated with our standard WMISH protocol (6) can be detected using CLSM without any apparent loss in sensitivity (Figure 1, A–E). The signal comes from the black-purple precipitate as is evident by the comparison of bright-field and confocal images (compare Figure 1 panels, A with B, and D with E). We can routinely visualize the whole 3-D

![Figure 1. Reflection CLSM on Platynereis dumerilii, Danio rerio, and Drosophila melanogaster WMISH samples.](image_url)

(A and B) Bright-field and confocal images of Platynereis WMISH samples labeled with an r-opsin probe specific for rhabdomeric photoreceptors. (C) The embryo was co-stained with an anti-acetylated tubulin antibody to label the neurites and cilia of the larval brain (cyan). (D and E) Confocal detection of NBT/BCIP precipitate in axonal projections (arrowheads) of Platynereis rhabdomeric photoreceptors. (F) Intensity of the reflection signal from the NBT/BCIP precipitate in a Platynereis embryo illuminated with six different lasers and measured with a sliding 5 nm detection window. Measurements with the six lasers are shown in one graph, each peak corresponds to the signal obtained when the sample was illuminated with the indicated laser line. There was no reflection signal outside this sharp peak. (G and H) Bright-field and confocal images of a zebrafish WMISH sample labeled with a lateral line primordium specific probe. (I) GFP expressed in the lateral line primordium (ClaudinB:GFP) was detected using an anti-GFP antibody (cyan). The embryo was also co-stained with an anti-acetylated tubulin antibody to label trunk axons (green). (J and K) Bright-field and confocal images of a Drosophila WMISH sample stained with a probe for the pair-rule gene odd-skipped. (L) Close-up of one stripe from the same embryo showing reflection from NBT/BCIP particles. (M and N) Detection of cellular coexpression in Platynereis using a DIG-labeled probe for Pax6 and NBT/BCIP (red) and a fluorescein-labeled probe for r-opsin and fluorescein-syramide amplification (cyan). (O and P) Detection of cellular co-expression in zebrafish stained for a lateral line specific WMISH probe with NBT/BCIP (red) and an anti-GFP antibody (cyan). All brightfield images were taken on a Zeiss Axiohot microscope using DIC optics. Confocal images are z-projections of 1 μm spaced image stacks going 20–40 μm deep into the embryos. CLSM, confocal laser-scanning microscopy; WMISH, whole-mount in situ hybridization; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; GFP, green fluorescent protein; DIG, digoxigenin; DIC, differential interference contrast.
volume of a gene’s expression pattern, both in the case of genes expressed in single cells or genes expanding to broader domains in the embryo (see the Supplementary Movies S1 and S2 available online at www.BioTechniques.com). Importantly, such 3-D volume rendering would not be possible using conventional bright-field microscopy. We can also visualize subcellular structures with localized RNA, such as axonal projections, without a loss in sensitivity (compare Figure 1 panels, D with E). The signal-to-noise ratio is also generally higher on reflection images compared with bright-field images (for a quantification of pixel intensities see Supplementary Figure S1).

To test the spectral properties of the signal, we illuminated WMISH samples with six different laser lines and measured the spectral properties of light emitted by the sample. We used the spectral detector of a Leica TCS SP2 confocal microscope and a 5-nm-wide sliding window (the smallest possible detector width) moved at 1-nm intervals. When we illuminated the sample with any of the six laser lines, we detected the maximum intensity of the signal at the wavelength corresponding to the wavelength of illumination (Figure 1F). This indicates that we detect reflection, not fluorescence, from the NBT/BCIP precipitate. In other words, the laser lines normally used to excite a fluorophore are reflected from the NBT/BCIP precipitate (without a change in wavelength) and can be detected if the spectral detector is set to cover the wavelength of the illuminating laser line.

In our WMISH samples, high-magnification confocal images revealed the presence of distinct particles in the NBT/BCIP precipitate (Figure 1, E and L) responsible for light reflection. We also noted a weaker but reliable fluorescent signal at $>700$ nm, with a maximum at approximately 772 nm, when samples were excited with a strong 635 nm diode laser (Figure 2, A–C and J). The spatial extent of this signal also corresponds to the NBT/BCIP staining, and it most likely comes from the fluorescence of the indigo dye generated by the hydrolysis and dimerization of BCIP. However, this signal cannot be detected in weakly stained embryos or with microscopes equipped with a weaker gas laser or if the detector cannot be used at this end of the visual spectrum.

To help in the reproduction of our results, we gave detailed guidelines on how to set up a confocal microscope to get optimal reflection signal. We took the confocal images either with a Leica TCS SP2 or a TCS SPE confocal microscope with a 40× oil-immersion objective, with a pinhole of 200 μm corresponding to an airy disc unit of
2. Using this opening we could detect strong reflection signal, but lower opening can also be used if a better z-axis resolution is desired. The opening does not influence signal-to-noise ratios, since there is practically no background (Supplementary Figure S1 and data not shown). It only changes signal intensity and z-axis resolution. For reflection, a 633 nm gas laser or a 635 nm diode laser was used, and the detection window was set to 630–640 nm. The width of the detection window is not critical, since the signal has a very sharp peak corresponding to the wavelength of illumination (Figure 1F) [the detector can also be set, for example, to a 5 nm width (631–636), the smallest detector width on the Leica TCS systems]. To detect reflection with other lasers, the 5- to 10-nm-wide detection window has to be set accordingly. We obtained the best reflection images with the 633/635 nm lasers, as lower wavelength lasers produce more background reflection. We never observed bleaching of the reflection signal, even at high magnification and continuous scanning with maximum laser intensity (data not shown). This is because we measure an insoluble precipitate that is apparently not modified by laser light unlike a fluorophore. We therefore suggest the use of a 633/635 nm laser at maximum intensity, a 5–10 nm detection window covering the wavelength of the laser line, and a 1–2 air unit pinhole opening for an optimal reflection image. These parameters can be easily set up on microscopes that have a spectral detector and should give an optimal reflection image [besides the Leica machines, this includes the Olympus FluoView (Olympus, Center Valley, PA, USA) and the Zeiss LSM 510 Meta systems (Carl Zeiss)]. We also tested a Zeiss LSM 510 system that has no spectral detector. We could obtain high-quality reflection images using the following setup: a 633 nm excitation laser, a HauptFarbTeiler (HFT) UV/488/568/633 main dichroic beam splitter, and no emission filter, or a long-pass (LP) emission filter with a wavelength block below 633 nm (e.g., LP 585). Using this setup, we could detect reflection from the NBT/BCIP precipitate, even though the detection window is not so precise (e.g., >585, depending on the emission filter; data not shown). Using such a setup, one may not be able to combine reflection with certain fluorophores that are excited by the 633 laser and have an overlapping emission signal (e.g., Cy™5), but it is not a major problem since the lower end of the spectrum covered by the LP filter can be used for fluorescent detection.

To test whether the reflection method can be used in WMISH samples of other species, we used zebrafish and *Drosophila* embryos and standard protocols available for these organisms. In these preparations, we observed no NBT/BCIP particles and no or very poor reflection. However, when we tested the *Platynereis* WMISH protocol (6) on fish and fly embryos, we obtained clear and specific reflection signal (Figure 1, G–L). We found that the fast proteinase K digestion and post-fixation steps in our protocol are critical to obtain large NBT/BCIP particles Figure 1L that allow high-quality reflection CLSM.

Next we tested whether confocal detection of NBT/BCIP can be combined with the detection of other fluorescent markers to allow cellular resolution co-localization of gene products and other cellular markers (e.g., cilia). To this end, we performed double labeling experiments in *Platynereis* larvae and in zebrafish embryos. In *Platynereis*, we combined NBT/BCIP staining for one gene with anti-acetylated tubulin immunostaining (Figure 1C) or with fluorescent in situ staining for another gene (10). Using anti-acetylated tubulin immunostaining, we could co-localize the expression of a cell type-specific rhabdomic opsin (11) with axons and the apical dendrite of photoreceptor cells (Figure 1C and Supplementary Movie S1). Such double labeling, confocal imaging, and 3-D rendering of gene expression patterns and subcellular structures would be impossible or very difficult using bright-field imaging [e.g., on samples co-labeled by NBT/BCIP in situ and horseradish peroxidase (HRP)-DAB immunostaining]. In zebrafish, we used NBT/BCIP staining and anti-GFP antibody staining in a GFP line expressed in the lateral line primordium (12). By combining reflection and fluorescent detection, we could, therefore, in both *Platynereis* and zebrafish, detect cellular co-localization (Figure 1, N and P), with no or minimal shadowing of the fluorescent signal by the granular NBT/BCIP precipitate. Such co-expression studies have so far only been possible using fluorescent WMISH or double-fluorescent WMISH (10). Our method greatly enhances the sensitivity and ease of performing such experiments. For example, we can now perform co-expression studies with weakly expressed genes that, according to our experience, do not work or hardly work in fluorescent WMISH.

There are some possible sources of artifact when using the reflection method. We noted that light could be reflected not only by the NBT/BCIP particles but also by some embryological structures giving a background signal. In *Platynereis* embryos, we see reflection from the developing parapodial chaetae and spinning glands, and in zebrafish embryos, the yolk sac is reflecting. Such reflection is usually not disturbing, since either the reflecting tissue can be removed (such as fish yolk) or it is localized to a known anatomical structure. Glass coverslips are also strongly reflecting, and when doing z-sectioning, one has to define the beginning of the z-stack below the coverslip reflection signal. Another potential problem is that a strong NBT/BCIP precipitate from a broadly expressed gene can partly shadow the exciting light and the emitted fluorescence deeper in the sample. Shadowing is worse at lower wavelengths [e.g., 4′,6-diamidino-2-phenylindole (DAPI); for a quantification see Figure 2K] but is usually not a problem with green or red lasers. Even when looking at broadly expressed genes, we can scan 40–60 μm deep into *Platynereis* embryos (Figure 2, A–F and K) and can detect the whole volume of expression (see also Supplementary Movie S2).

Whole-mount reflection CLSM is a powerful alternative to fluorescent techniques and can be implemented in any confocal microscope with a spectral detector and also other...
systems where a reflection setup is possible (e.g., Zeiss LSM 510). The hybridization protocol used here with success for three species should work in a wide range of organisms to allow cellular resolution expression profiling, fluorescent counter-stainings, and 3-D volume rendering of gene expression patterns.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES


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