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Visualizing mRNA by *in situ* hybridization using 'high resolution' and sensitive tyramide signal amplification

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▼Methods for analyzing the spatial distribution of mRNA by *in situ* hybridization have played a key role in studying development in *Drosophila* and other systems. The first methods for *in situ* hybridization made use of radioactive probes on wax sections (Ref. 1) or in whole mount (Ref. 2) followed by autoradiographic detection. This labour-intensive technique was slow and suffered from poor resolution. A substantially improved method involves the use of non-radioactive digoxigenin (DIG)-labelled probes in whole mount *in situ* hybridizations with highly sensitive alkaline phosphatase-based histochemical detection (Ref. 3, 4). However, the resulting dark stains are incompatible with fluorescent labelling and are visualized with much poorer spatial resolution than fluorescent markers.

One improvement in the resolution of histochemical detection has been to embed the whole-mount specimens in resin and section them using a microtome (Ref. 5). However, this variation is too labour intensive for general use and does not improve resolution sufficiently. Another solution has been to use alkaline phosphatase substrates that yield a red stain that is also fluorescent (Ref. 5). Unfortunately, the resulting stain slowly diffuses away from the site of enzyme activity, leading to poor spatial resolution and the need to analyze the samples immediately. In addition, the red stain emits mainly in the rhodamine channel but also partly in the fluorescein (FITC) channel, making it unsuitable for accurate double-fluorescence labelling. Some of these problems were recently solved by visualizing *Drosophila* mRNA with fluorescein-12-UTP labelled probes that are only barely detectable over background. The signal was improved by multiple antibody layers culminating in FITC-coupled antibodies (Ref. 6) or Cyanine-5 (Cy5)-coupled antibodies (G.S. Wilkie and I. Davis, unpublished). However, the fluorescent signal produced is fairly weak, making the technique practicable only for detecting a single highly abundant mRNA.

We have developed a new high-resolution fluorescence *in situ* hybridization protocol that overcomes the problems associated with previously described methods by using tyramide signal amplification (TSA). The technique depends on peroxidase-mediated deposition of fluorochrome-labelled tyramides at the location of the probe (Ref. 7). The detection is highly sensitive as it relies on enzymatic amplification of signal. High resolution is obtained because the reaction produces tyramide radicals that react covalently with proteins at the site of HRP activity, preventing appreciable diffusion of the signal (Ref. 8). Furthermore, the use of fluorescence allows quantitative high-resolution detection as well as double and triple labelling of different transcripts and other cell components.

Here, we use Cyanine-3 (Cy3) TSA to co-visualize *runt (run)* mRNA with the nuclear envelope at high resolution. *run*mRNA is exclusively localized to the apical cytoplasm, mainly in punctate dots (Fig. 1a). Nascent transcripts can be seen as two very intense foci of fluorescence in the nucleus, as previously reported in the case of other *Drosophila* transcripts using histochemical stains (Ref. 9, 10). In a separate experiment, we used the TSA technique to co-visualize the pair-rule transcripts *run* and *fushi tarazu* (*ftz*) with two different tyramide fluorochromes. *run* and *ftz* are expressed in a partially overlapping pattern in the embryo (Fig. 1b).

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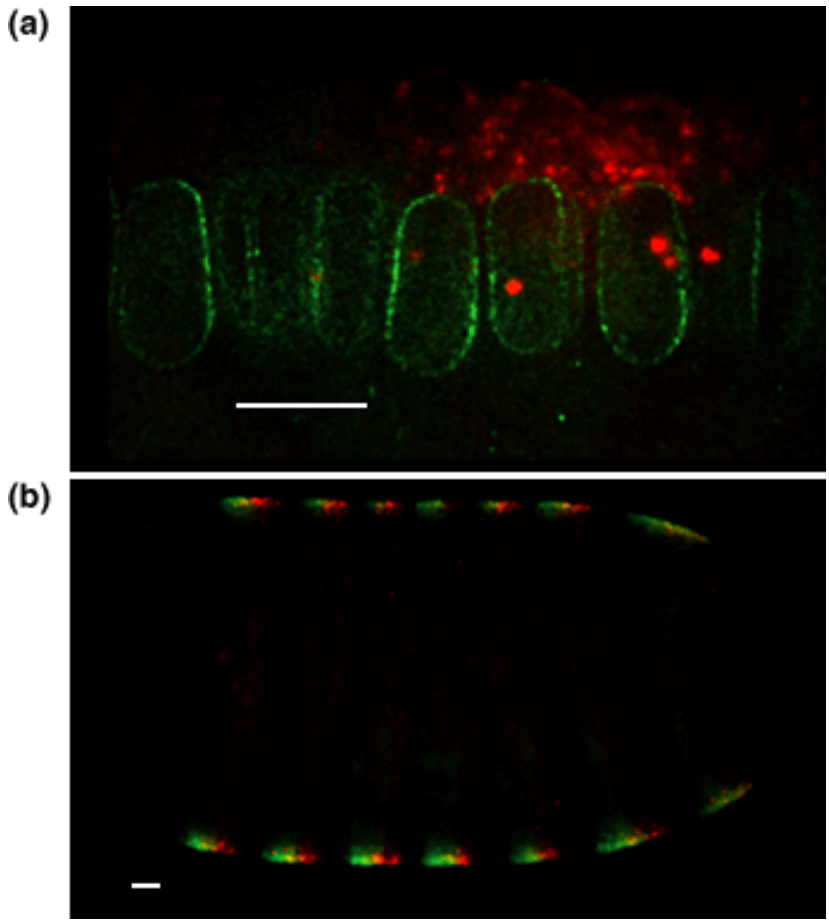

FIGURE 1. Co-visualising *ftz* and *run* transcripts. (a) A high power (× 100 objective) view of *run* mRNA (Cy3-tyramide in red) and the nuclear envelope (WGA-FITC in green) in cross-section through a stage 5 blastoderm embryo. The edge of the embryo is oriented uppermost. The image represents 1 μm of focal depth, produced by projecting (superimposing) the maximum intensity of 5 adjacent 0.2 μm deconvolved Z sections into one image. *run* mRNA is visible in the apical cytoplasm as very bright red fluorescent dots and bright nascent transcripts within the nucleus. The scale bar represents 10 μm. (b) A low power (× 20 objective) co-visualisation of *run* mRNA (FITC-tyramide in green) and *ftz* mRNA (Cy3 tyramide in red) in cross-section through a stage 5 blastoderm embryo. The scale bar represents 20 μm.

We have found that TSA has improved resolution compared with histochemical detection and is at least an order of magnitude more sensitive than fluorescent detection with secondary antibodies (Ref. 6). We anticipate that this technique could replace the existing methods for routine use in *Drosophila* and many other systems.

Protocol

Fixation

Wild type (Oregon R) *Drosophila melanogaster* embryos were dechorionated in 7% hypochlorite solution for two minutes, rinsed thoroughly with water and fixed in a two-phase mixture of 37% formaldehyde (Sigma) and heptane for five minutes with very gentle mixing. In addition to being faster than fixation in buffered 4% formaldehyde, this technique provides better preservation of microtubules in the embryo (Ref. 11). Fixed embryos were devitellinised in methanol, washed twice in methanol and stored at −20°C.

Prehybridization

Embryos were rehydrated for two minutes in 50% methanol: 50% PBT (1 × PBS + 0.1% Tween20), washed twice in PBT for two minutes and then post fixed for ten minutes in 4% formaldehyde in 1 × PBS. Embryos were then washed five times for five minutes in PBT, followed by a five-minute wash in 50% PBT : 50% hybridization solution (HYB) and a further five-minute wash in HYB. Embryos were then prehybridized for at least one hour in HYB at 70°C. HYB consisted of 50% formamide, 5 × SSC, 50 μg/ml heparin, 100 μg/ml *Escherichia coli* tRNA and 0.1% Tween20, and was adjusted to pH 6.5 with concentrated HCl.

Probe synthesis and hybridization

Antisense RNA probes, labelled with digoxygenin (Boehringer Mannheim) or FITC (Boehringer Mannheim), were synthesized as directed by the manufacturer by run-off transcription from restriction digested plasmids with SP6, T7 or T3 polymerases. *ftz*and *run* probes were 2 kb and 3 kb in length, respectively. Probes were used at a concentration of 0.5 ng/μl for hybridization overnight at 70°C.

Post-hybridization washes and antibody incubation

All washes (20 minutes each) were carried out at 70°C in a heat block with no agitation. Embryos were washed once in HYB, once in 50% HYB: 50% PBT, and four times in PBT. The embryos were then incubated for one hour in HRP-conjugated anti-digoxygenin IgG Sheep Fab fragment (Boehringer Mannheim), diluted 1/1000 in PBT, followed by three washes in PBT. When detecting very abundant transcripts we found that preabsorption of antibodies and blocking were not necessary. However, when detecting rarer transcripts, we found that non-specific background could be reduced by preabsorbing the antibody at a dilution of 1: 100 overnight at 4°C with fixed and rehydrated (four ten-minute washes with PBT) embryos in PBT. The background can be reduced further by using a blocking step (0.5% non-fat milk powder or 2% bovine serum albumin) before antibody incubation and by adding 5% normal sheep serum to the antibody incubation step.

TSA Detection

The labelling was checked before TSA detection by developing a small sample of embryos with diaminobenzidine (Vector) (DAB) under standard conditions. DAB staining (brown/black) was visible within a few minutes under a dissecting stereo-microscope. Cyanine-3 (Cy3) tyramides (NEN Life Sciences, UK) or FITC tyramides (NEN Life Sciences, UK) from stock solutions were then diluted 1/50 in amplification diluent [TSA-Direct FISH (NEN Life Sciences, UK)] and added to embryos. Alternatively, the reagents could be used according to previously described methods of synthesising and using fluorescent tyramides (Ref. 7). The reaction was allowed to proceed for 2–10 minutes before washing three times for five minutes in PBT to remove unreacted substrate. The fluorescent tyramides were not visible in bright field microscopy, but the optimal reaction times were approximately the same with DAB and fluorescent tyramides. We were able to shorten the entire *in situ* hybridization procedure to a single day, at least in the case of abundant transcripts, by shortening the hybridization time to four hours and reducing each 20-minute wash to 10 minutes.

Co-visualization of two transcripts and of other cell components

To demonstrate the utility of the technique, we co-visualized two different mRNAs using Cy3-tyramine TSA to detect an FITC-labelled probe and FITC-tyramine TSA to detect a DIG-labelled probe. The first HRP-coupled antibody [anti-Fluorescein IgG Sheep Fab fragment (Boehringer Mannheim)] can be inactivated by a ten-minute incubation in 0.01M HCl (Ref. 8) or by a 15-minute incubation at 70°C (G.S. Wilkie and I. Davis, unpublished) before adding the second HRP-coupled antibody (anti-digoxygenin IgG Sheep Fab fragment).

Following TSA staining, the nuclear envelope was labelled by incubation in FITC -conjugated Wheat Germ Agglutinin (WGA) (Molecular Probes) at a concentration of 5 μg/ml in PBT for 1–4 hours. This decorates the nuclear pore complexes (NPCs). In other experiments (data not shown) we also co-visualised mRNA with microtubules by labelling with monoclonal anti β-tubulin antibody (Amersham) diluted 1/100 in PBT and then detected with Cy-5-conjugated F(ab')2 Donkey anti mouse IgG (H + L) antibodies (Jackson) at a concentration of 3 μg/ml (G.S. Wilkie and I. Davis, unpublished).

Mounting and imaging

Embryos were mounted in vectashield (Vector Laboratories) and images were captured with a PXL-cooled CCD (Photometrics) camera on a Sedat/Agard widefield microscope (Applied Precision) based on an Olympus IX70 inverted microscope. Out-of-focus light was reassigned using Sedat/Agard 3-D deconvolution algorithms (Delta Vision software). Very similar results were obtained using a Leica laser scanning confocal microscope (data not shown).

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Products Used

FITC: FITC from Institut Pasteur
FITC: FITC from Jackson ImmunoResearch Laboratories Inc
FITC: FITC from Boehringer Mannheim
FITC: FITC from Molecular Probes
HRP-conjugated anti-Digoxygenin IgG Sheep Fab frag: HRP-conjugated anti-Digoxygenin IgG Sheep Fab frag from Boehringer Mannheim
Cyanine-3 (Cy3) tyramides: Cyanine-3 (Cy3) tyramides from NEN Life Science Products
FITC: FITC from Institut Pasteur
FITC: FITC from Jackson ImmunoResearch Laboratories Inc
FITC: FITC from Boehringer Mannheim
FITC: FITC from Molecular Probes
TSA-Direct FISH: TSA-Direct FISH from NEN Life Science Products
anti-Fluorescein IgG Sheep Fab fragment: anti-Fluorescein IgG Sheep Fab fragment from Boehringer Mannheim
FITC: FITC from Institut Pasteur
FITC: FITC from Jackson ImmunoResearch Laboratories Inc
FITC: FITC from Boehringer Mannheim
FITC: FITC from Molecular Probes
antibodies: antibodies from Sigma
antibodies: antibodies from Amresco Inc
vectashield: vectashield from Vector Laboratories Inc
microscope (Stemi 2000): microscope (Stemi 2000) from Leica Microscopy & Scientific Instruments Group
microscope: microscope from Carl Zeiss
agar: agar from Difco