Whole mount *in situ* hybridization for the detection of mRNA in *Drosophila* embryos*

Diethard Tautz, Institut für Genetik, University Cologne

In situ hybridization for the detection of mRNA in tissues has traditionally been performed on sectioned material. This was necessary since the probes were usually labeled with ^{3}H or ^{35}S, which required the use of a photographic film emulsion covering the sections for the detection of the signal. The development of highly sensitive nonradio-actively labeled probes allows now *in situ* hybridizations to be performed directly in tissues, such as whole *Drosophila* embryos (Tautz D, Pfeifle C, 1989). This "whole mount" *in situ* hybridization procedure is highly sensitive and the resolution of details is unparalleled. Complex expression patterns in particular, can only be analyzed in whole embryos, since the reconstruction of a three-dimensional pattern from sections can be very cumbersome. The method has therefore by now been adapted to other types of embryos and tissues as well, both from invertebrates and vertebrates and has become a general procedure in research laboratories studying questions of embryology and developmental biology.

In the original method DNA fragments were used that were labeled by random priming with DIG-dUTP. While this is still the method of choice if one wants to have a quick overview over the expression profile of a newly cloned gene, it has by now become clear that RNA probes can provide much better results (Lehmann R, Tautz D, 1994). Higher sensitivity can be obtained, because labeling efficiency is high and because they are single stranded. Moreover, since RNA-RNA hybrids are more stable than RNA-DNA hybrids, elevated hybridization temperatures can be employed, which result in a higher specificity and less background.

The detection of the hybridization signal is usually done with chromogenic substrates which develop a color at the place where the probe has bound. Different substrates are available, which allow to use different colors for differently labeled probes, *e.g.* biotin or fluorescein as haptens. A triple-labeling and detection procedure has been described for *Drosophila* (Hauptman G, Gerster T, 1996). Signal detection has also been done with fluorescently labeled antibodies against the respective haptene. While this results in a reduced sensitivity of detection, it is particularly useful in conjunction with the use of laser scanning microscopy, which allows a much better resolution of internal hybridization signals (Hughes et al.1996).

The experimental procedures given below are optimized for *Drosophila*. However, they may also be applied to other types of embryos. Only the procedures for the collection of the embryos and for the removal of the extra-embryonic membrane (if one exists) will have to be modified.

* Reprinted from Kessler, C. [ED]: Nonradioactive Analysis of Biomolecules, 2nd. ed. 2000; 573-580, Springer Lab. Manual, ISBN 3-540-64901-9, with permission of Springer Verlag GmbH.

I. Embryo collection and fixation

1. Collect the embryos on an apple juice agar plate and transfer them into a little basket made from polyethylene tubing (or an reaction vial) and stainless steel mesh (Wieschaus et al., 1986).
2. Wash embryos with double dist. water and dechorionate in a solution of 50% commercial bleach (Klorix) for about 2–3 min.
   Control this step under the binocular microscope. Dechorionated embryos float to the surface of the solution because the vitellin membrane is hydrophobic.
3. Wash with 0.1% Triton X-100 and transfer the embryos into a glass scintillation vial containing 4 ml Fixation solution [0.1 M Hepes, pH 6.9; 2mM $MgSO_4$; 1 mM EGTA].
4. Add 0.5 ml 37% Formaldehyde solution and 8 ml heptane.
5. Shake the vial for 15-20 min.
6. Remove the lower phase as far as possible (the embryos should be at the interphase).
7. Add 10 ml methanol and shake vigorously for 10 s.
   This step causes the vitellin membranes to burst and the devitellinized embryos will sink to the bottom.
8. Transfer the embryos into an reaction vial and wash them with methanol.
   The embryos may be stored at this stage for several weeks or even longer in the refrigerator (at -15 to -25°C).

II. Prehybridization treatment

Unless otherwise indicated, all the following steps are done in reaction vials in a volume of 1 ml at room temperature and on a rotating wheel.

Potential sources of RNAse contamination should be avoided.

1. Wash embryos 3 × 5 min in PBT [130 mM NaCl; 10 mM sodium phosphate, pH 7.2; 0.1% (v/v) Tween 20].
2. Postfix the embryos in 1 ml PBT, 4% Formaldehyde for 15 min.
   This postfixation may not be necessary when working with DNA probes, but is advisable for RNA probes, as these are hybridized under more harsh conditions.
3. Wash embryos 5 × 5 min in PBT.
4. Incubate the embryos for 2–5 min in a solution of 15-30 mg/ml Proteinase K in PBT.
   The exact length of this incubation step should be optimized for each new batch of Proteinase K. Too short digestion times result in a loss of signal intensity, too long digestion times may cause the embryos to burst during the subsequent steps.
5. Stop the Proteinase K digestion by incubating for 2 min in 2 mg/ml glycine in PBT
   This step may be dispensible if proteinase activity is low.
6. Wash 2 × 5 min with PBT.
7. Refix the embryos in 1 ml PBT, 4% Formaldehyde for 20 min.
8. Wash 5 × 5 min in PBT.

III. Hybridization

The hybridization procedure is similar for both DNA and RNA probes, both DIG labeled. Only the incubation temperatures are higher for RNA probes and the Hybridization buffer is more acidic, since this stabilizes the embryos during the high temperature incubation steps.

DNA probes should not be used under such acidic conditions, since they would become depurinated.

1. Wash the embryos in Hybridization solution [Hybridization solution for DNA probes: 750 mM NaCl; 75 mM Na-citrate (= 5× SSC) pH 7.0; 50% (v/v) formamide; 0.1% (v/v) Tween 20; 50 µg/ml heparin; 50 µg/ml sonicated Salmon Sperm DNA.
   Hybridization solution for RNA probes: same as for DNA probes, but pH 5.0] diluted 1:1 with PBT for 10 min.
2. Wash 10 min in Hybridization solution.
   This stepwise transfer into the Hybridization solution is not strictly required, but embryos which are slightly overdigested with Proteinase K would burst, if they were brought directly into the formamide containing solution. The same considerations apply for the washing after the hybridization (see below).
3. Prehybridization
   - Prehybridize in Hybridization solution for 20–60 min in a waterbath at 45°C (55–65°C for RNA probes).
   - Remove most of the liquid, leaving about 2 mm of solution above the surface of the settled embryos.
     This corresponds usually to a hybridization volume of about 100 ml.
4. Probe preparation
   - Add 2 µl of the probe to 5 µl of a solution of 2 mg/ml sonicated salmon sperm DNA (this has to be scaled up appropriately if more then one hybridization is carried out).
   - Denature at 100°C for 3 min, cool shortly on ice and add directly to the embryos in Hybridization solution.
5. Hybridization
   - Mix thoroughly and incubate at 45°C (55-65°C for RNA probes) overnight.
     Slight agitation may be advantageous to avoid clumping of the embryos, but is not strictly necessary.

IV. Washing and detection

The following washing protocol is very extensive and is necessary if high background is encountered. However, fewer and shorter steps may be sufficient for many applications.

1 Wash steps
- Wash 2 × 30 min in 500 µl Hybridization solution at the hybridization temperature.
- Proceed with washes in serial dilutions (4:1, 3:2, 2:3, 1:4) of Hybridization solution in PBT for l0 min each at room temperature.
- Wash 2 × 10 min in PBT.

2 ***Optional:*** Block embryos with 1% serum of the species the antibody is derived.

3 Preparation of anti-DIG antibody conjugate

The anti-DIG antibody conjugate should be freshly preabsorbed for 1 h against fixed embryos in order to remove any unspecifically binding material. The final working dilution of the antibody conjugate is 1:2000 in PBT. The preabsorption step should be adjusted accordingly.

Example: *If ten reactions are processed in parallel, use about 200 µl embryos in 1 ml PBT with an antibody conjugate dilution of 1:200. This solution is then further diluted 1:10 in the next step.*

! *The diluted antibody solution may be reused for two or more stainings within a few days.*

4 Antibody incubation

Incubate the embryos for l h in 500 µl diluted and preabsorbed anti-DIG antibody complex.

5 Washing steps
- Wash 3 × 20 min in PBT.
- Wash 3 × 5 min in Staining buffer
[100 mM NaCl; 50 mM $MgC1_2$; l00mM Tris-HCl, pH 9.5].

6 Staining of embryos

Transfer embryos in a small dish with 1 ml Staining buffer containing 4.5 µl NBT solution and 3.5 µl BCIP solution.

! *Let the color develop in the dark with occasional inspection under the binocular microscope. Color develops usually within 1 h, but the reaction may also be left overnight.*

7 Stop the staining reaction by washing in PBT.

8 Transfer embryos into 70% glycerol, equilibrate for several hours and transfer them onto a microscope slide for inspection and photography.

! *For permanent mounting dehydrate the embryos in an alcohol series (70%, 90%, and 100%) and mount in Euparal.*

5

V. Results

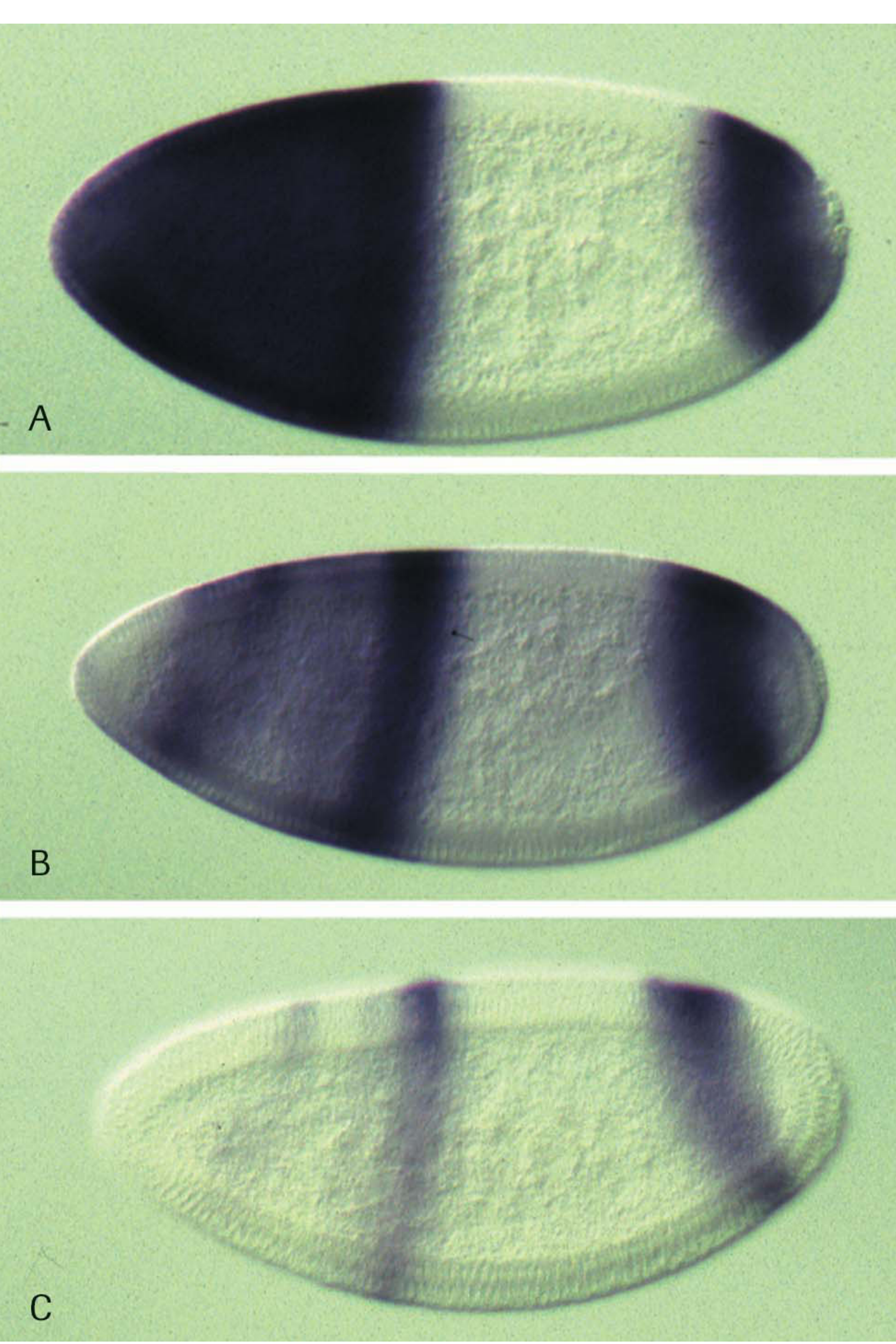

Figure 1: Expression of the segmentation gene hunchback in *Drosophila* in different stages of development. Whole mount *in situ* hybridization is particularly useful to follow dynamically developing spatially expressed patterns. The stages shown here are approximately 20 min part from each other in developmental time. For further information on hunchback expression see Tautz, Nature 332, 281.

VI. Troubleshooting

A. *If* a poor signal is observed, then:

1. Check whether the probe is correctly labeled (see Sect. 3.1).
2. Check Proteinase K digestion. Depending on the tissue and the Proteinase activity, it may be necessary to perform rather extensive Proteinase K digestions. Set up a series of digestion conditions and test these.
3. Check under the binocular microscope whether the vitellin membranes are fully removed after the methanol step.
4. Check for RNAse contamination of the solutions. Treat the solutions with diethylpyrocarbonate (Sigma) before use, in particular the PBT solution before addition of Tween 20.

B. *If* there is too much background, then:

1. Prolong the washing step, because of insufficient washing each step may be done for longer times.
2. Perform longer preabsorption of the anti-digoxigenin complex.
3. Include levamisole in the staining solution. Levamisole acts as a potent inhibitor for endogeneous lysosomal phosphatases. These are, however, usually not a problem in early *Drosophila* embryos.
4. Increase detergent concentration in the PBT. Tween 20 may also be replaced by SDS.
5. Include a xylene treatment step after the fixation.

References

Hauptman G, Gerster T (1996) Multicolour whole-mount *in situ* hybridization to *Drosophila* embryos. *Dev Gen Evol* **206**:292-295

Hughes SC, Saulier-Le Drean B, Livne-Bar I, Krause HM (1996) Fluorescence *in situ* hybridization in whole-mount *Drosophila* embryos. *BioTechniques* **20**:748-750

Lehmann R, Tautz D (1994) *in situ* hybridization to RNA. *Methods in Cell Biology* **44**: 575-598

Tautz D, Pfeifle C (1989) A nonradioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**:81-85

Wieschaus E, Nüsslein-Vollhard C (1986) Looking at embryos. In: Roberts DB (ed). *Drosophila* – A Practical Approach. IRL Press, Oxford, pp 199-228

5

Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
Triton X-100	Vicous, liquid	10 789 704 001	100 ml
Hepes	Purity: 98% (from N)	10 737 151 001 10 242 608 001	500 g 1 kg
DIG RNA Labeling Kit*	2× 10 labeling reaction	11 175 025 910	1 kit
DIG RNA Labeling Mix*	10× solution (20 reaction)	11 277 073 910	40 µl
DIG DNA Labeling and Detection Kit*	25 labeling reaction and 50 blots	11 093 657 910	1 kit
DIG-High Prime*,‡	Premixed solution for 40 random-primed DNA labeling reactions with DIG-11-dUTP	11 585 606 910	160 µl (40 labeling reactions)
Anti-Digoxigenin-AP*	750 units/ml Anti-Digoxigenin, Fab fragments conjungated to alkaline phosphatase	11 093 274 910	150 U (200 µl)
NBT solution	100 mg/ml nitroblue tetrazolium salt in 70% (v/v) dimethylformamide	11 383 213 001	3 ml (300 mg) (dilute priorto use)
BCIP solution	50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), toluidinium salt in 100% dimethylfo rmanide	11 383 221 001	3 ml (150 mg)
NBT/BCIP solution	Stock Solution	11 681 451 001	8 ml
Proteinase K, rec., PCR Grade	Lyophilizate	03 115 836 001 03 115 879 001 03 115 801 001 03 115 852 001	25 mg 100 mg 2 × 250 mg 4 × 250 mg
Tween 20		11 332 465 001	5 × 10 ml

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

‡ This product or the use of this product may be covered by one or more patents owned by Roche Diagnostics GmbH, including the following: US patent 5.814.502.