

Detection of Transposable Elements in *Drosophila* Salivary Gland Polytene Chromosomes by *In Situ* Hybridization

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Summary

In situ hybridization is particularly appropriate for mapping specific DNA sequences on polytene chromosomes of *Drosophila* and other dipterans. This technique is based on the recognition and binding of one labeled sequence (the probe) to homologous sequences on chromosomes fixed on a microscope slide. The probes are labeled with biotin or other nonradioactive products, and the probe signal can be detected as a thin line on the chromosomes, following the shape of the classical Giemsa-stained chromosome bands, thus allowing the detection of TE insertions within the range of 50 to 200 kb. In our laboratory we work on many individuals from natural populations, and as a result we process high numbers of slides hybridized with various DNA probes of transposable elements every day. Therefore, the *in situ* hybridization technique we use is a simplification of earlier published protocols. This chapter presents our simplified standard *in situ* hybridization protocol for labeling polytene chromosomes of *Drosophila* with biotin and a fluorescence stain (FISH).

Key Words: Transposable elements; *in situ* hybridization; FISH; *Drosophila*; polytene chromosomes.

1. Introduction

In situ hybridization is a powerful technique for localizing specific DNA sequences on chromosomes. It has been used in many experiments since the 1970s, and it is particularly appropriate for mapping specific DNA sequences on polytene chromosomes of *Drosophila* and other dipterans. This technique is based on the recognition and binding of one labeled sequence (the probe) to homologous sequences on the chromosomes fixed on a microscope slide. Although radioactive probes were initially used, it is now more common to use probes labeled with biotin or other nonradioactive products. These advanced

labeling methods allow more precise localization of the probe on polytene chromosomes because the probe signal can be detected as a thin line on the chromosomes, following the shape of the classical Giemsa-stained chromosome bands, thus allowing the detection of TE insertions within the range of 50 to 200 kb. We process high numbers of slides hybridized with various DNA probes of transposable elements every day in our laboratory. Therefore, the *in situ* hybridization technique we use is a simplification of earlier published protocols (1–7). The present chapter presents our simplified standard *in situ* hybridization protocol for labeling polytene chromosomes of *Drosophila* with biotin (8–10) and a fluorescence stain (FISH) (11). *In situ* techniques used for mitotic chromosomes are discussed by P. Dimitri in the following chapter.

2. Materials

1. Giemsa solution (prepare immediately before use). Add 3 mL Giemsa (Merck), 3 mL phosphate buffer (buffer tablets, pH 6.8, GURR Merck) to 94 mL of water.
2. 10X PBS: 1.3M NaCl, 0.07M Na₂HPO₄, 0.03M NaH₂PO₄.
3. 20X SSC: 3.0M NaCl, 0.3M trisodium citrate 2H₂O, adjusted to pH 7.0 with NaOH.
4. 1X SSC: 0.15M NaCl, 0.015M sodium citrate.
5. Triton X100 in 1X PBS: add 1 mL of Triton X100 to 1 L of 1X PBS. Stir until completely dissolved.
6. 50% dextran sulfate (w/v): dissolve 1 g of dextran sulfate in 1.3 mL of distilled water for at least 6 hrs. Complete melting is essential for high-quality *in situ* hybridization. Do not hesitate to work with fresh product. Store at 4°C.
7. 10X BSA stock (bovine serum albumin): 10% BSA in 10X PBS. Store at 4°C. For preparation of the 1X BSA solution prewarm the 10X stock solution at 37°C before dilution.
8. Extravidin-horseradish peroxidase conjugate (we usually use Sigma, cat. no. E 2886). Mix 4 µL conjugate with 996 µL of 1X BSA solution.
9. DAB solution: Just before use dissolve 5 mg DAB (diaminobenzidine tetramine—Life Technology, ref. 15 972-011) in 10 mL of 1X PBS. **Caution:** This compound is a carcinogen. Use gloves and carry out all manipulations in the hood. Just before treating the slides with the DAB solution add 3.33 µL of a 30% H₂O₂ stock. DAB solutions are light sensitive, thus keep it in dark bottles, and also treat the slides in the dark.
10. Sodium Tris buffer (STB 5): For 1 mL add 200 µL 20X SSC, 50 mg BSA (stored at 4°C), 1 µL Triton X 100 (stored at room temperature), to 800 µL UHQ water.
11. Sodium Tris buffer (STB 1): For 1 mL add 200 µL 20X SSC, 10 mg BSA, and 1 µL Triton X 100, to 800 µL UHQ water.
12. Extravidin-FITC (50 µg/mL final).
13. Anti-DIG-rhodamine (4 µg/mL final).
14. Phosphate buffer albumine (PBA): add 398 mL 4X SSC, 1.6 mL 30% BSA (stored at 4°C), and 400 µL Triton X 100, store at room temperature).

15. Wash buffer (pH 7.2–7.3): add 40 mL 20X SSC, 200 μ L Triton X 100 to 160 mL of UHQ water.

3. Methods

We presently use biotinylated probes for *in situ* hybridizations without any troublesome effects on the quality of the chromosomes. This outcome may be due to our simplified method. In contrast to other *in situ* protocols we omit additional steps like acetylation (8) or RNase treatments. In addition, third-instar larvae are dissected directly in 45% acetic acid, and the salivary glands are placed in a clean drop of this acid before being squashed (*see Note 1*).

3.1. Slides and Coverslip Treatment

The slides and the coverslips are washed but not siliconized: First place the slides in chromic acid, then rinse them in 95% ethanol, and finally in distilled water. Slides can, however, be rinsed in ethanol only and wiped clean with thin paper, but this method depends on the slides and must be checked carefully. Clean the coverslips with lens paper or use them as they are. They are not siliconized because we found that siliconized coverslips might cause breakage of the spread chromosomes when the coverslip is removed after freezing in liquid nitrogen.

3.2. The Squash

1. Place one pair of salivary glands in a drop of 45% acetic acid, cover with a coverslip, and tap gently with an eraser without holding the coverslip. This dissociates the cells and makes the chromosomes flow in the liquid. Check the quality of the squash visually—the cells should be well spread out. If the squash does not appear good enough, the coverslip should be tapped again gently. Squashing is finished by slightly scratching the whole area of the coverslip in a zigzag motion with a blunt needle (or a pencil, as preferred). This improves the quality of the chromosome spread.
2. Place the slide and coverslip on blotting paper and crush firmly under the thumb, or in the jaws of a vise. We now use a vise because this avoids damage to fingers from acetic acid, and it is easy for young or less strong students to perform. This stage is essential because it completely flattens the chromosomes (*see Note 2*).

3.3. Squash Dehydration

1. Immerse the squashed slides in liquid nitrogen for at least ten minutes. Afterward, flip off the coverslips with a razor blade. Removal of the coverslip must be quick; otherwise, parts of the chromosomes will stick to the coverslip.
2. Immediately dehydrate the slides in ethanol at room temperature. Two baths of 70% ethanol followed by two baths of 95% ethanol can be used, but immersion in one bath of 95% ethanol for 10–15 min is usually sufficient.

3. Air-dry the slides and place them in boxes for later hybridization. At this step the slides should be reexamined under the microscope, and only good squashes should be selected for further analyses. They are stable for months at room temperature, but the best *in situ* hybridization results are generally obtained with 2- to 3-d-old slides. We have had successful results even with 2-yr-old preparations but only with long probes, such as those made from long retro-transposon sequences.

3.4. Preparation of DNA Probes

We currently use probes (1 μ g of DNA) labeled by nick translation (**12**) because it is a simple technique that does not require DNA denaturation or extraction of the insert from plasmids. We use the Bionick™ Labeling System kit from Life Technology based on biotin-14-dATP, which requires the mixing of only two vials. We have also worked with biotin-11-dUTP and biotin-16-dUTP, obtaining good results (*see Note 3*). Biotinylated DNA can be kept at 4°C or at -20°C for months. Random prime labeling techniques are appropriate for short DNA probes.

1. For homologous high-stringent probes prepare the hybridization mix in 50% formamide as following (*see Note 4*):

biotinylated DNA	10 μ L
sterile 20X SSC	10 μ L
50% dextran sulfate	10 μ L
formamide	30 μ L

For heterologous probes (to improve the detection of signals from homologous sequences that have diverged, or when working with species that have diverged from the probe species) prepare the hybridization mix in 35% formamide. The *in situ* hybridization is then said to be under heterologous conditions.

labeled DNA	10 μ L
sterile 20X SSC	8 μ L
50% dextran sulfate	8 μ L
formamide	14 μ L

2. Heat the vial containing the hybridization mix in boiling water for 8–10 min (do not forget to make a small hole in the cap), and cool it quickly in a mixture of ice and ethanol. The vial can be store at 4°C for months (*see Note 5*).

3.5. In Situ Hybridization

1. Warm the slides in a bath of 2X SSC at 70°C for 30 min, and dehydrate them in 95% ethanol. They can be kept in boxes at 4°C for months after this treatment.
2. Soak slides in 0.07N NaOH for 2–3 min to denature the chromosomes, wash them in 95% ethanol, and air-dry. These slides are ready for hybridization (*see Note 6*).



Fig. 1. *In situ* hybridization of *D. melanogaster* salivary gland chromosomes with a biotinylated DNA probe for a transposable element. The brown-labeled insertions are easily distinguished from the blue, Giemsa-stained bands of the chromosomes. The chromosome arms (X, 2L, 2R, 3L, 3R) are noted, as are the highly labeled chromocenters (C).

3. Add one drop per slide of biotinylated DNA probe (see **Subheading 3.4.**), cover with a cleaned coverslip, and place slides overnight in a humid chamber at 37°C.
4. The following morning, wash slides for 10 min in 2X SSC, followed by two quick washes for 3 s each in 0.1% Triton in 1X PBS, and then in 1X PBS (see **Note 7**). Add one drop per slide of the extravidin-horseradish peroxidase solution and cover with a cleaned coverslip. The reaction is allowed to proceed in a humid chamber at 37°C for 30 min.
5. Rinse slides in 0.1% Triton in 1X PBS for 3 s followed by a second wash for a few sec in 1X PBS. After these washes cover the slides with the DAB solution for 3–4 min and incubate them in the dark (see **Note 8**). Finally rinse the slides quickly in 1X PBS and stain them with freshly prepared Giemsa solution for 4–8 min.

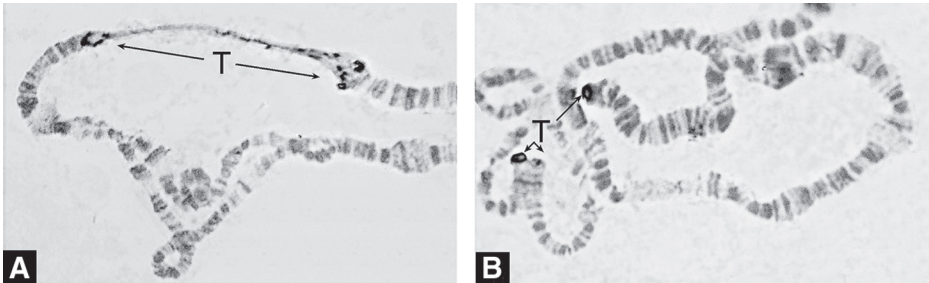


Fig. 2. *In situ* hybridization of salivary gland chromosomes of *D. melanogaster* with the telomeric *HeT-A* element. (A) *HeT-A* hybridizes across the length of the stretched sequences of thin DNA pulled out between the telomeres of the 2R and 2L chromosomes. (B) Hybridization follows the morphology of the extreme end of the chromosome, and the intensity of the labeling differs according to the chromatids, which are separated.

6. Mount the slides in EUKITT resin (Merck, ref. 82601) under a coverslip. The chromosome preparation must be completely dry before the resin is added. The slides can then be kept at room temperature for years without any significant alteration (see Figs. 1 and 2).

3.6. Fluorescence In Situ Hybridization on Polytene Chromosomes With Two Probes

The protocol presented below follows the procedure described by Muleris et al. (13) with some modifications. The hybridization mixture (70 μ L) using two different probes simultaneously under homologous conditions contains:

deionized formamide	30 μ L
sterile 20X SSC	10 μ L
50% dextran sulfate	10 μ L
probe 1: biotin-labeled DNA, about 500 ng	10 μ L
probe 2: digoxigenin-labeled DNA, 500 ng	10 μ L

The steps for preparation of the hybridization mixture and denaturation are identical to those described in Subheadings 3.4. and 3.5.

1. Add 10 μ L/slide of the hybridization mixture, cover with a cleaned coverslip, and place them overnight in a moist chamber at 37°C.
2. Remove the coverslips and incubate the slides in 2X SSC at 39°C for 2 \times 10 min.
3. Place 80 μ L of STB5 solution on the marked squash, cover with a large coverslip, and incubate slides in a humid chamber for 30 min at 37°C.
4. Remove the coverslip, pour off the STB5 solution, and add 80 μ L of STB1 detection solution. Add anti-DIG rhodamine and extravidin FITC just before use.

5. Incubate the slides for 30 min at 37°C in a humid chamber. Remove coverslips and wash the slides for 10 min at 37°C in the wash buffer.
6. Stain chromosomal DNA by adding 15 µL of DAPI (4'-6-diamidino-2-phenylindole)-Vectashield mounting solution (see **Note 9**).

4. Notes

1. Big salivary glands are obtained from well-fed larvae raised under uncrowded conditions. Adding a solution of fresh yeast on first-instar larvae improves the future quality of the polytene chromosomes.
2. We usually encircle good squashes by scratching the surface of the slide with a needle. It helps to limit the amount of liquid used.
3. There is no need to remove the TE probe from its plasmid for nick translation. A longer probe will always give rise to a stronger signal compared to a short one. Two labeled nucleotides can be used for nick translations of very short probes. If the DNA sequence of the probe is too rich in long stretches of the same nucleotide, try mixing the cold nucleotide with the labeled one at a 1 : 1 ratio. The purity of the probe DNA is essential.
4. We never use Denhardt's solution, but dextran is absolutely necessary, and the freshness of the dextran sulfate powder is important. Do not hesitate to use a fresh vial from time to time.
5. The nick translation kit, the extravidin, or the dextran sulfate should be checked if there is no hybridization signal or when the signal gets fainter and fainter with successive runs of hybridization. *In situ* hybridization must always be done with a previously tested DNA probe control.
6. It is often stated that *in situ* hybridization of *Drosophila* polytene chromosome squashes using biotin leads to deterioration of the chromosomes. Lakhotia et al. (14) even suggest treating the slides with gelatin to overcome this problem. We have never used subbed slides and our protocol does not cause chromosomes to deteriorate. The quality of the chromosomes after hybridizations is as it was when checked after squashing. Exact timing of the denaturation step is crucial. The treatment with Triton X is optional, although it helps to obtain clean preparations as the detergent removes unspecific hybridizations as well as dust.
7. The chromosomes and cytoplasm may come loose on the slide after hybridization. When the cytoplasm does not adhere well to the slides, try to be very gentle during the washes (SSC, PBS, Triton, etc.). Do not shake the slides, not even during the final Giemsa-staining step. Slow movements of the rack containing the slides are generally sufficient to insure efficient washing and homogeneous staining.
8. Because DAB is sensitive to light and humidity, the usually white powder can sometimes be yellow or even brown. Although this color change does not seem to be a vital problem, we prefer to use a new, fresh vial when the DAB color is too strong. The DAB solution can be aliquoted and kept frozen at -20°C, but we have had problems with this method because the solution was sometimes too colored. We now make up fresh DAB solution as required.

9. DAPI can be conserved as stock solution (100 µg/mL) at -20°C in the dark. Vectashield (Vector Laboratories, Burlingame, CA 94010) is a mounting medium added to DAPI to prevent fluorescence fading and also to favor a better conservation of the slides. Prepare DAPI-Vectashield (500 ng/mL) as follows: 2.5 µL DAPI (100 µg/mL) and 497.5 µL Vectashield. Store at -20°C. Use gloves for the manipulation of DAPI.

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