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Streamlined Protocol for Polytene Chromosome In Situ Hybridization

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The ability to localize a gene sequence by polytene chromosome in situ hybridization (PISH) has long been a quintessential feature of *Drosophila* molecular genetics. Fittingly, numerous investigators have sought to optimize PISH, and a search of the FlyBase literature database (3) uncovers dozens of

articles describing modifications of the technique. The end result of these efforts is several useful protocols that produce excellent results, and these should be consulted by anyone attempting PISH for the first time (1,2,5-9,11). However, I have found that these procedures contain extraneous or protracted steps that can be avoided while still producing excellent results. In this report, I detail a streamlined protocol (Table 1) that gives results that should satisfy the demands of nearly every investigator (Figure 1). Items that I do not discuss, such as squashing and probe labeling, are covered in the references noted above. The remainder of this article presents additional observations that may be of assistance in carrying out the procedure.

Although nearly every protocol recommends it, I have found it unnecessary for the microscope slides or coverslips to be washed with ethanol or detergent or treated with acid before us-

ing them. Also, I have seen no differences in the final results by using any combination of untreated or gelatin-coated (subbed) slides and untreated or silanized coverslips. These choices have more effect on the ease with which the chromosomes can be spread than anything else. The chromosomes essentially always stick to the slide no matter which combination is used. I prefer to use Superfrost®/Plus slides (Fisher Scientific, Pittsburgh, PA, USA) and untreated coverslips.

To make the chromosome preparations, I dissect and squash the larvae directly in 45% acetic acid; no additional fixatives are necessary. Thumb squashing works well if a small number of squashes are to be prepared; for larger endeavors, the method of Hepperle (4) provides an inexpensive solution to thumb fatigue.

While several reports have described using ethanol baths chilled to -70°C to dehydrate the frozen squash prepara-

Figure 1. PISH used to localize the positions of transgenic P elements introduced into the genome by germ-line transformation. The digoxigenin-labeled probe specifically identifies the transgene. Arrowheads note the hybridization signal. For these photographs, the chromosomes were left unstained, and the plane of focus was centered on the hybridization signal. Orcein-stained chromosomes in the plane of focus would show significantly clearer detail. (A) Two nuclei, each showing signal at the two sites of transgene integration (polytene positions 21B and 42A). (B) A high magnification view of a different nucleus showing the signal at 21B.

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Table 1. PISH Protocol

A. Squashing

1. Dissect, fix and squash salivary glands directly in 45% acetic acid.
2. Freeze the squash on dry ice or in liquid nitrogen and flip off the coverslip with a razor blade.
3. Immerse the slide immediately in 95% ethanol at room temperature. The slide can be left in the ethanol for as little as 15 min or as long as overnight with no obvious effects on the final results.
4. Use a black permanent marker to demarcate (on the bottom of the slide) the position of the coverslip used during squashing. This mark will identify the site of the chromosomes for the rest of the procedure.

B. Probe Preparation

1. The DNA probe can be labeled with biotin or digoxigenin by polymerase chain reaction (PCR) or random priming using previously published procedures or commercially available kits (Life Technologies [Gaithersburg, MD, USA] or Boehringer Mannheim). When the reaction is complete, add 10 µg of tRNA carrier to the sample, bring the volume to 100 µL and purify through a Sephadex® G-50 spun column (10) developed in 1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA. Lyophilize the sample to dryness and resuspend the pellet with 10 µL water.
2. After quantitating the yield of labeled product (by gel analysis or spot test), make the probe concentration approximately 2 ng/µL in HYB solution (50% formamide, 4× sodium saline citrate phosphate [SSCP], 200 µg/mL sheared carrier DNA, 10% dextran sulfate). I routinely use SSCP (1× SSCP is 120 mM NaCl, 15 mM Na citrate, 20 mM Na phosphate, 1 mM EDTA, pH 7.0) for my experiments, although standard saline citrate (SSC) works just as well. The probe is denatured by incubating the sample at 80°C for 5–7 min and chilling it on ice until needed.

C. Slide Treatment

1. Treat squashes by heating the slides in 2× SSCP at 68°C for 30 min.
2. Dip the slides in water at room temperature for a few seconds and then denature the chromosomes by submerging the slides in 70 mM NaOH for 2 min.
3. Neutralize in 2× SSCP with 2 washes, 3 min each.
4. Dehydrate the samples: 2× 4 min in 70% ethanol, then 4 min in 95% ethanol.
5. Air dry.

D. Hybridization and Washes

1. Pipet 10 µL denatured probe onto a clean 25- × 25-mm coverslip. Lower the slide onto the coverslip, pick it up and invert so that the coverslip is on top. The viscosity of the HYB mixture will prevent the coverslip from moving. Place the slide into a plastic slide rack positioned on its side. The slide should be horizontal. Repeat for additional slides.
2. Put 10 mL of 20× SSCP into a 600- or 1000-mL Kimax beaker. Place the slide rack into the beaker and cover tightly with Saran® wrap. The SSCP will keep the chamber humidified, and there is no need to use rubber cement to seal the coverslips to the slides.
3. Place the beaker in a 37°C incubator and hybridize overnight.
4. The next day, submerge the rack of slides in 2× SSCP at room temperature to remove the coverslips. Gentle nudging with forceps is usually needed to assist their loosening.
5. Wash the slides 3× 20 min in 2× SSCP at 68°C.

E. Immunological Detection

1. Wash the slides for 1 min in buffer 1 (150 mM NaCl, 100 mM Tris-HCl, pH 7.5).
2. Incubate the slides in buffer 2 (1% blocking reagent in buffer 1) for 30 min at room temperature. (Reserve this solution for step 5.)
3. Dilute α-digoxigenin antibody 1:5000 with buffer 2.
4. Place the slides horizontally on a level surface and pipet 400 µL of antibody over squash.
5. After 10 min, shake off the antibody solution and dip each slide briefly in buffer 2 (reserved from step 2).
6. Wash 3× 3 min in buffer 1.
7. Wash 3 min in buffer 3 (100 mM NaCl, 50 mM MgCl₂, 100 mM Tris-HCl, pH 9.5).
8. Place 50 µL of staining solution (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate [NBT/BCIP] in buffer 3) on a clean 22- × 40-mm coverslip. Lower the slide onto the coverslip by picking it up. Quickly invert and reposition the coverslip over the squash. Place in a lighttight container and incubate for 15–60 min.
9. After 10 min, begin monitoring the progress of the reaction by using a microscope with phase contrast optics. Viewing the slide for several minutes at a time has no obvious effect on the reaction or background staining.
10. Stop the reaction by washing the coverslip off and immersing the slide in water for 5 min.
11. Optional: To better visualize the chromosomes, stain the squash for a few seconds with lacto-acetic acid orcein stain diluted 1:10 with (lactic acid:acetic acid:water) (1:1:1). Stop staining with a brief rinse of water.
12. Place a coverslip over the wet squash area and examine it with the microscope.
13. Remove the coverslip and allow the slide to air-dry. Slides can be stored at room temperature for years and can be viewed at any time by repeating step 12.

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tions (Table 1, step A3), ethanol at room temperature has worked well for all of my PISH applications.

For Table 1 (Sections C and D), I have found it convenient to use plastic slide racks and boxes (Catalog Nos. 195 and 196; Lipshaw, Detroit, MI, USA). Metal racks will also work well. These racks withstand high temperatures and are perfectly vertical when placed on their side. Glass racks have neither of these virtues. Also, I have found that acetylation of the slides before hybridization is completely unnecessary for biotinylated or digoxigenin-labeled probes.

The protocol in Table 1, Section E, is for the detection of digoxigenin-labeled probes. All detection reagents are from the Genius™ kit (Boehringer Mannheim, Indianapolis, IN, USA). This protocol also works well for biotinylated probes when minor changes are introduced to accommodate the dif-

ferences in the detection system.

Finally, the chromosomes are much easier to read after the detection steps if they are stained with orcein prepared in lactic and acetic acids (1). The blue precipitate of the histochemical reaction is easily distinguished from the dark red chromosome banding pattern produced by the orcein. However, this discrimination is lost during black and white photography and, if such images are needed, it is usually better to photograph the squash before staining. The orcein staining is reversible, and it can be removed with a brief rinse in lactic acid:acetic acid:water (1:1:1).

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