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Plant Meiosis

Methods and Protocols

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Chapter 2

Analysis of Plant Meiotic Chromosomes by Chromosome Painting

Martin A. Lysak and Terezie Mandáková

Abstract

Chromosome painting (CP) refers to visualization of large chromosome regions, entire chromosome arms, or entire chromosomes via fluorescence in situ hybridization (FISH). For CP in plants, contigs of chromosome-specific bacterial artificial chromosomes (BAC) from the target species or from a closely related species (comparative chromosome painting, CCP) are typically applied as painting probes. Extended pachytene chromosomes provide the highest resolution of CP in plants. CP enables identification and tracing of particular chromosome regions and/or entire chromosomes throughout all meiotic stages as well as corresponding chromosome territories in premeiotic interphase nuclei. Meiotic pairing and structural chromosome rearrangements (typically inversions and translocations) can be identified by CP. Here, we describe step-by-step protocols of CP and CCP in plant species including chromosome preparation, BAC DNA labeling, and multicolor FISH (Fig. 1).

Keywords Chromosome painting, Fluorescence in situ hybridization, BAC FISH, Pachytene chromosomes, DNA labeling

1 Introduction

The chromosome painting (CP) technique in human and animal cytogenetics serves in situ identification of whole chromosomes and specific chromosome regions using flow sorted, Degenerate Oligonucleotide Primed PCR (DOP-PCR)-amplified, and fluorescently labeled chromosomes or chromosome segments. Due to abundant and diverse DNA repeats homogeneously distributed across chromosome complements (1), flow sorted or microdissected chromosomes are not suitable for preparation of chromosome-specific painting probes in plants. Instead, chromosome-specific high-capacity DNA vectors have become widely utilized in plant cytogenetics since the mid-1990. Specifically, individual bacterial artificial chromosome (BAC) clones and BAC contigs (continuous sets of BAC clones) are most frequently used

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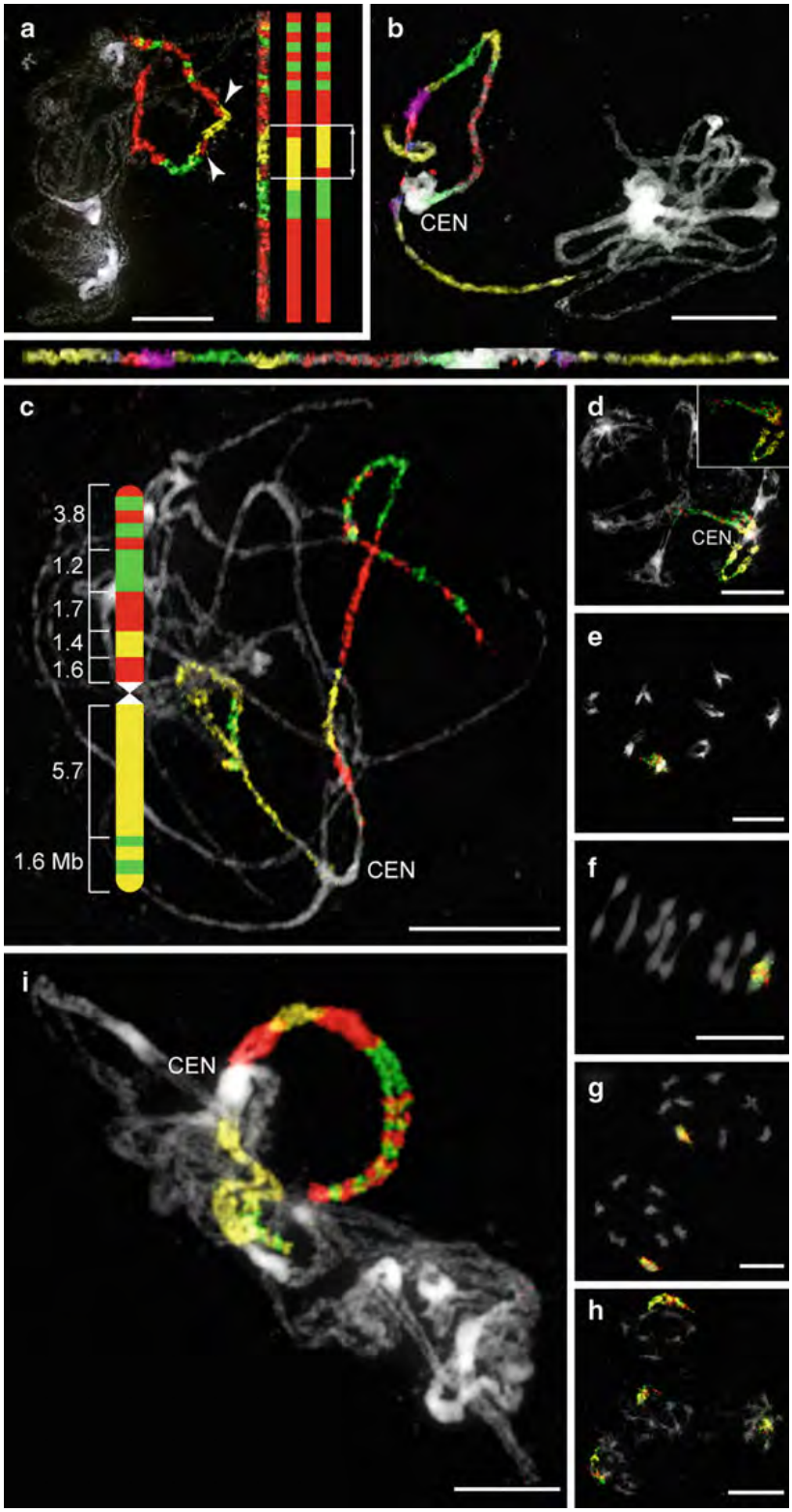

Fig. 1 Application of comparative multicolor chromosome painting for meiotic studies. (**a**) Chromosome painting in Shahdara ×Columbia (Sha ×Col) hybrid of *Arabidopsis thaliana* (*n* = 5, At1–At5). A 2.2 Mb paracentric inversion on the top arm of chromosome At3 is specific for Sha and absent in Col. Paired Sha and Col

as chromosome-specific probes. Fluorescence in situ hybridization (FISH) of single or several BAC clones is referred to as BAC FISH, whereas BAC painting or chromosome painting applies to in situ hybridization of BAC contigs covering larger chromosome regions (e.g., chromosome arms) or whole chromosomes (Fig. 1). Chromosome-specific BAC contigs are used as painting probes either in the same species (2–5) or in species with sufficient chromosome homeology (cross-species or comparative chromosome painting, CCP) (3, 6–8). Recently, reciprocal BAC painting and multi-species BAC painting (using painting probes of two or more species to chromosomes of another species) on pachytene chromosomes has been established (9).

Although CP enables identification of chromosome regions and whole chromosomes during all (pre)meiotic stages (2), pachytene bivalents and multivalents usually offer the highest resolution. Here, we provide a protocol to paint meiotic and mitotic chromosomes of plants using chromosome-specific BAC clones and BAC contigs. These procedures are essentially based on our long-term experience with CP and CCP in crucifer species (*Brassicaceae*) (2, 3, 6, 8, 9).

2 Materials

Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1 Collection of Floral Material

1. Freshly prepared Carnoy's I fixative: 3 parts ethanol, 1 part glacial acetic acid; or Carnoy's II fixative: 6 parts ethanol, 3 parts chloroform, 1 part glacial acetic acid (see Note 1).

Fig. 1 (continued) homologues were identified by differentially labeled *A. thaliana* BAC contigs (inversion marked by *arrowheads*).(**b–i**) Comparative chromosome painting in *Brassicaceae* species using chromosome-specific *A. thaliana* BAC contigs. (**b**) Pachytene chromosome Sn4 of *Stenopetalum nutans* ($n=4$, Sn1–Sn4) painted by 14 differentially labeled *A. thaliana* BAC contigs. Image of the same chromosome digitally straightened using the "straighten-curved-objects" plugin in the Image J software (12). (**c–h**) CCP of chromosome Ca1 in diploid *Cardamine amara* ($n=8$, Ca1–Ca8) at pachytene (**c**), diplotene (**d**), diakinesis (**e**), metaphase I (**f**), telophase I (**g**), and anaphase/telophase II (**h**). (**i**) CCP of a pachytene tetravalent of Ca1 in autotetraploid *C. amara* ($n=16$, Ca1–Ca16). Chromosome-specific *Arabidopsis* BAC contigs were labeled by biotin-dUTP (*red*) and digoxigenin-dUTP (*green*), and immuno-detected using antibodies coupled to Texas Red and Alexa Fluor 488, respectively. *Yellow* signals correspond to Cy3-dUTP-labeled contigs. In addition, BAC clones hybridized to chromosome Sn4 in (**b**) were also labeled by DEAC-dUTP (*blue*) and DNP-dUTP (Cy5, *magenta*). CENs refer to centromeres (not labeled by BAC clones). Size of BAC contigs in Mb according to http://www.arabidopsis.org. All meiotic chromosomes were isolated from anthers and counterstained with DAPI. *Bars* = 10 m

2. 70% ethanol.
3. Forceps.
4. Glass vials or microcentrifuge tubes.

2.2 Chromosome Preparation

1. 10× citrate buffer: 40 mL of 100 mM citric acid and 60 mL of 100 mM trisodium citrate, adjust pH to 4.8; store at 4°C.
2. Pectolytic enzyme mixture in 1× citrate buffer: 0.3% pectolyase, 0.3% cellulase, 0.3% cytohelicase (Sigma Aldrich, St. Louis, MO, USA) prepared from frozen 1% stock solutions in 1× citrate buffer (see Note 2).
3. 60% glacial acetic acid in distilled water (see Note 3).
4. Freshly prepared ice-cold Carnoy's I fixative.
5. 4% freshly prepared formaldehyde in distilled water.
6. "Assistant" staining blocks with glass cover (Karl Hecht Assistant, Sondheim/Rhön, Germany).
7. Humid box.
8. Incubator at 37°C.
9. Stereomicroscope, microscope with phase contrast.
10. SuperFrost microscope slides (Fisher Scientific, Suwanee, GA, USA).
11. Dissection needles.
12. Fine forceps.
13. Glass Pasteur pipette.
14. Heating block.
15. Hair dryer.

2.3 Chromosome Preparation Pretreatment

1. 20× Saline Sodium Citrate (SSC): 3 M sodium chloride, 300 mM trisodium citrate, pH 7.0.
2. RNase: 100 g/mL DNase-free ribonuclease A (AppliChem, St. Louis, MO, USA). Make stock of 1 mg/mL in distilled water. Store aliquots at –20°C.
3. Pepsin from porcine gastric mucosa (Sigma Aldrich, St. Louis, MO, USA) 0.1 mg/mL in 10 mM HCl. Prepared from 100 mg/mL stock in 10 mM HCl. Store aliquots at –20°C.
4. 4% freshly prepared formaldehyde in 2× SSC.
5. 70%, 80%, and 96% ethanol.
6. 4′, 6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, St. Louis, MO, USA): 2 g/mL in Vectashield antifade (Vector Laboratories, Burlingame, CA, USA). Store at 4°C.
7. Coverslips (24 ×24 mm and 24 ×50 mm).
8. Coplin or Hellendahl jar (see Note 4).

9. Humid box.
10. Incubator and water bath at 37°C.
11. Plastic tube rack.

2.4 Probe Labeling

1. 10× NT buffer: 500 mM Tris–HCl pH 7.5, 50 mM $MgCl_2$, 0.05% bovine serum albumin.
2. Nucleotide mixture: 2 mM dATP, dCTP, dGTP, and 400 mM dTTP (Roche Applied Science, Indianapolis, IN, USA).
3. 1 mM *x*-dUTP (*x* stands for biotin, digoxigenin, Cy3 or other hapten/fluorochrome; see Note 5).
4. 0.1 M β-mercaptoethanol.
5. DNase I (Roche Applied Science, Indianapolis, IN, USA). Use a 1:250 dilution of a 1 mg/mL DNase stock in 0.15 M NaCl in 50% glycerol.
6. DNA polymerase I (10 U/ L; Fermentas, Glen Burnie, MA, USA).
7. 0.5 M EDTA, pH 8.0.
8. 100 bp DNA ladder.
9. 0.5 mL microcentrifuge tubes.
10. Thermocycler for 15°C and 60°C.
11. Electrophoresis system.
12. 1% agarose gel.

2.5 Probe Preparation and In Situ Hybridization

1. 3 M sodium acetate, pH 5.2.
2. 70% ethanol, 96% ice-cold ethanol.
3. Hybridization buffer: 50% formamide, 10% dextran sulfate in 2× SSC.
4. 2 mL microcentrifuge tubes.
5. Refrigerated centrifuge.
6. Vacuum desiccator or SpeedVac.
7. Thermomixer (a heating block with exact temperature control) at 80°C.
8. 24 ×24 mm, 22 ×22 mm, or 24 ×32 mm coverslips.
9. Rubber cement.
10. Humid box.
11. Incubator at 37°C.

2.6 Fluorescence Detection of Hybridized Probes

1. 2× SSC.
2. 50% or 20% deionized formamide in 2× SSC, pH 7.0 (see Note 6).
3. 4T buffer: 4× SSC pH 7.0, 0.05% Tween-20.

4. Blocking solution: 5% bovine serum albumin, 0.2% Tween-20 in 4× SSC.
5. TNT buffer: 100 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20.
6. TNB buffer: 100 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% blocking reagent (Roche Applied Science, Indianapolis, IN, USA).
7. Antibodies: avidin–Texas Red (Vector Laboratories, Burlingame, CA, USA), goat anti-avidin–biotin (Vector Laboratories, Burlingame, CA, USA), mouse anti-digoxigenin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), goat anti-mouse–Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA).
8. DAPI (2 g/mL) in Vectashield antifade.
9. 70%, 80% and 96% ethanol.
10. Coplin or Hellendahl jars (see Note 2).
11. Water bath shaker at 42°C.
12. Coverslips (24 ×32 mm and 24 ×50 mm).
13. Humid box.
14. Incubator at 37°C.
15. Epifluorescence microscope equipped with optical filters for DAPI and other fluorochromes, a digital charge-coupled device (CCD) camera, and image acquisition software.

3 Methods

Carry out all procedures at room temperature unless otherwise specified. To minimize fluorochrome bleaching, avoid overexposure to light during procedures 3.4–3.6.

3.1 Collection of Floral Material

1. Fix entire inflorescences, individual flower buds, or anthers in Carnoy's fixative at room temperature or at 4°C overnight; change the fixative several times.
2. Exchange the fixative for 70% ethanol and store the fixed material at –20°C (see Note 7).

3.2 Chromosome Preparation

1. Rinse floral material with distilled water in a staining block or small petri dish for 10 min. Under a stereomicroscope, remove and discard unwanted parts (e.g., yellow anthers containing pollen).
2. Replace water with 1× citrate buffer and wash two times, 5 min each wash.

3. Incubate the material in ~1 mL of pectolytic enzyme mixture in a humid box at 37°C for 3 h (see Note 8).
4. Replace the enzymes with 1× citrate buffer and keep the material on ice or at 4°C until use (see Note 9).
5. Put a single flower bud/anther on a microscope slide using a Pasteur pipette, remove the excess fluid and add ~20 L of 60% acetic acid. Disintegrate the bud by dissection needles until a fine suspension is formed.
6. Place the slide on a heating block (50°C) and spread the suspension by careful circular stirring with a needle for ~30 s (see Note 10).
7. Fix the chromosomes by pipetting 100 L of Carnoy's I fixative around the suspension drop. Discard the fluid by tilting the slide and dry using a hair dryer.
8. Using a phase-contrast light microscope, examine the preparation for specific meiotic stages and the amount of cytoplasm.
9. Post-fix the slides in a Coplin or Hellendahl jar with 4% formaldehyde in distilled water for 10 min and leave to air-dry. Carry out this step in a fume hood.
10. Store dried slides in a dust-free box at 4°C (see Note 11).

3.3 Chromosome Preparation Pretreatment

1. Wash slides two times in 2× SSC in a Coplin jar, 5 min each wash.
2. Add 100 L of RNase solution, cover with 24 ×50 mm coverslip and incubate the slides in a humid box at 37°C for 1 h (see Note 12).
3. Tilt slides to let the coverslip fall off and wash slides as in step 1.
4. To remove cytoplasm, treat slides with pepsin at 37°C for 5 min in a Coplin jar placed in a water bath.
5. Wash slides as in step 1.
6. Dehydrate slides in an ethanol series (70%, 80%, and 96% ethanol, 3 min each) and leave them to air-dry.
7. Apply 15 L of Vectashield with DAPI to a slide and cover it with a 24 ×24 mm coverslip. Check the slide with a fluorescence microscope. Chromosomes should be undamaged and free of cytoplasm. When cytoplasm is persistent, remove the coverslip using running water and repeat steps 4–7.
8. Remove the coverslip using running water flow and wash slides as in step 1.
9. Post-fix slides in a Coplin or Hellendahl jar with 4% formaldehyde in 2× SSC for 10 min. Carry out this and the following step in a fume hood.
10. Wash slides as in step 1.

11. Dehydrate slides in an ethanol series (70%, 80%, and 96% ethanol, 3 min each) and leave them to air-dry.

3.4 Probe Labeling by Nick Translation (See Note 13)

1. Combine in an 0.5 mL microcentrifuge tube: 1 g of BAC DNA in 32 or 29 L distilled water, 5 L of 10× NT buffer, 5 L of nucleotide mixture, 1 L of 1 mM comercial x-dUTP or 4 L of 1 mM custom-made x-dUTP, 5 L of 0.1 M β-mercaptoethanol, 1 L of DNase I, and 1 L of DNA polymerase I. Vortex gently and spin briefly (see Note 14).
2. Incubate at 15°C for 90 min.
3. Transfer the tube on ice and load 5 L of the reaction volume on a 1% agarose gel along with a 100-bp DNA ladder and run the electrophoresis.
4. When the smear of labeled fragments is ~200–500 bp in size, stop the nick translation by adding 1 L of 0.5 M EDTA and heating at 60°C for 10 min. When fragments are longer than 500 bp, extend the incubation at 15°C for further 30 min and repeat steps 3 and 4.
5. Store the probe at –20°C until use.

3.5 Probe Preparation and In Situ Hybridization

1. Pool individually labeled BAC clones by pipetting 5 L (~100 ng of DNA) of each BAC into a 2 mL microcentrifuge tube.
2. To reduce the probe volume and remove unincorporated nucleotides, precipitate the DNA by adding 0.1 volume of 3 M sodium acetate and 2.5 volume of ice-cold 96% ethanol. Vortex and keep on ice or at –20°C for at least 30 min.
3. Centrifuge at 13,000 ×*g* at 4°C for 30 min.
4. Carefully discard the supernatant.
5. Add 500 L of 70% ethanol and centrifuge again for 5 min.
6. Repeat step 4.
7. Dry the pellet using a desiccator or SpeedVac.
8. Resuspend the pellet in 20 L of hybridization buffer and incubate at 37°C in a thermomixer (see Note 15).
9. Add 20 L of probe to the chromosome preparation (see Subheading 3.3), cover with a cover slip and seal with rubber cement around the edges.
10. Denature the probe and the chromosomal DNA by placing the slide on a heating block at 80°C for 2 min.
11. Incubate the slide in a humid box at 37°C overnight (see Note 16).

3.6 Fluorescence Detection of Hybridized Probes

Signal detection and amplification is described here for hapten-labeled probes (biotin- and digoxigenin-dUTP) visualized by indirect immunofluorescence via Texas Red and Alexa Fluor 488, respectively (dinitrophenyl (DNP)-dUTP can also be used as a third hapten). This protocol can be also used with fluorochrome-labeled probes (e.g., Cy3- or DEAC-dUTP) that require only post-hybridization washing prior to microscopic evaluation (steps 1–4, and 14). All washing steps are carried out at 42°C in Coplin or Hellendahl jars placed in a water bath shaker. All incubation steps are carried out at 37°C on microscope slides covered with 24 ×50 mm coverslips and placed in a humid box. To minimize unspecific background, do not let the slides dry during the entire procedure.

1. Remove the rubber cement frame with forceps and let the coverslip fall off.
2. Wash slides in 2× SSC for 2 min.
3. Wash slides three times in 50% or 20% formamide, 5 min each wash (see Note 6).
4. Wash slides in 2× SSC for 2 min. If fluorochrome-labeled probes are used, proceed with step 14.
5. Wash slides in 4T buffer for 5 min.
6. Incubate slides in 100 L of blocking solution for 30 min.
7. Wash slides in 4T buffer 2 ×5 min.
8. Incubate slides in 100 L of avidin–Texas Red in TNB buffer (1:1,000) for 30 min.
9. Wash slides in TNT buffer 2 ×5 min.
10. Incubate slides in 100 L of mixed goat anti-avidin–biotin antibody (1:200) and mouse anti-digoxigenin antibody (1:250) in TNB buffer for 30 min (see Note 17).
11. Repeat step 9.
12. Incubate slides in 100 L of mixed avidin–Texas Red (1:1,000) and goat anti-mouse Alexa Fluor 488-coupled antibody (1:200) in TNB buffer for 30 min.
13. Repeat step 9.
14. Dehydrate the slides in an ethanol series (70%, 80%, and 96% ethanol, 3 min each) and leave them to air-dry.
15. Apply 15 L of Vectashield with DAPI to each slide and cover it with a 24 ×32 mm coverslip.
16. Observe and photograph slides under a fluorescence microscope equipped with appropriate optical filters, CCD camera, and image acquisition software.

4 Notes

1. Carnoy's II is believed to be more suitable than the Carnoy's I fixative for fixation of floral material. However, we did not observe a significant difference between the two fixatives and use the 3:1 fixative routinely.
2. The enzyme mixture can be reused several times (store at –20°C). Digestion time might need to be increased after each use.
3. Glacial acetic acid can be used at concentrations from 45% to 60%.
4. A Coplin jar can hold up to nine slides, Hellendahl jar up to 15 slides.
5. Labeled nucleotides are available commercially or can be synthesized by coupling allylamine-dUTP to succinimidyl-ester derivatives of haptenes or fluorochromes (10).
6. 50% formamide should be used for stringent post-hybridization washing after same-species (homologous) in situ hybridization; 20% formamide should be used in case of cross-species (homeologous) in situ hybridization.
7. Fixed material can be used after several months or years of storage. However, best quality preparations are usually obtained from freshly fixed material.
8. The amount of enzyme mixture should be proportional to the amount of digested material (tissue should be submerged). The incubation time of 3 h may not be appropriate to all species/types of floral material. After the incubation, we make a test preparation following protocol (3.2, steps 5–8). If the tissue is hard to disintegrate and there is a large quantity of cytoplasm and tissue fragments, we extend enzyme incubation for another 30 min or longer.
9. Digested floral tissue can be stored in 1× citrate buffer at 4°C overnight or longer. Overnight storage further softens the digested material.
10. The amount of acetic acid can be increased to 40–60 L and the duration of suspension spreading can be prolonged. The duration of spreading can be shortened in case of very small flower buds or anthers. The needle should not touch the slide surface.
11. Post-fixed slides can be stored at 4°C for several weeks or months. However, freshly prepared or few-days old slides guarantee better results.
12. Omitting the RNase treatment may result in a longer pepsin treatment used in Subheading 3.4, step 4 and/or increased background.

13. As *Brassicaceae* possess very small genomes and low amount of repeats localized in pericentromeric heterochromatin, chromosome-specific BAC clones from euchromatic regions can be used without the need to block repetitive sequences from cross-hybridization. For chromosome painting in other plant taxa, BAC clones have to be screened for the presence of dispersed repeats or genomic/Cot DNA needs to be applied for blocking.
14. DNA of large-insert clones (usually BAC clones) can be isolated by use of a standard alkaline lysis protocol (11) or using a DNA isolation kit (e.g., the Qiagen Plasmid Midi Kit; Qiagen, Hilden, Germany). A larger amount (4 L) of custom-made x-dUTPs must be used as custom dUTPs label DNA less efficiently than commercial nucleotides (10). DNA of several probes/BAC clones can be pulled and labeled together in a single nick translation reaction. However, in our laboratory we label each probe individually.
15. Time needed to resuspend the probe is dependent on the initial amount of precipitated DNA. DNA of several BAC clones is well dissolved after several minutes or hours. Complex probes comprising several dozen or hundred clones should be incubated overnight.
16. Overnight incubation is usually sufficient for in situ hybridization of homologous (same species) sequences. Longer hybridization times (48–72 h) may be required to ensure hybridization of homeologous (cross-species) sequences.
17. If the first detection step for hapten-labeled probes (see Subheading 3.6, step 8) yields a sufficiently strong signal, further signal amplification (e.g., by goat anti-avidin–biotin antibody in this protocol) can be omitted.

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