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[13] Nonisotopic *in Situ* Hybridization in Paraffin-Embedded Tissue Sections

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Introduction

Protocols for nonisotopic *in situ* hybridization (NISH) to tissue sections (tissue NISH) have been developed and applied to the study of the spatial organization of chromosomes (1–4), as well as numerical and structural chromosome aberrations in nuclei of tissue cells (5–15). Also, the behavior of host donor cell lines after transplantation may be studied in this way (16). In such a procedure, preservation of tissue morphology and the penetration of a given probe to its target DNA are considered the most critical parameters. Accessibility depends on the choice of the tissue sample, the fixative, including its application mode and time, and the thickness of tissue sections, as well as optimized pretreatment protocols. The protocols described in this chapter are performed with deparaffinized sections of 6- to 10-μm thickness firmly adherent to slides and have yielded a good preservation of tissue morphology, in particular nuclear structure, together with a high hybridization efficiency (for a schematic outline see Fig. 1).

Alternatively, Manuelidis and Borden (3) have performed nonisotopic *in situ* hybridization with nonadherent 25- to 40-μm-thick Vibratome sections bathed in the respective solutions for pretreatment, hybridization, and washing procedures and put the sections on glass slides only after signal detection. The latter approach has the advantage that probes can penetrate into the sections from both sides and has been applied for the three-dimensional (3D) investigation of intact nuclei with relatively large diameters. Thick sections however are less useful for evaluations in routine histopathology and show a high amount of autofluorescence in the case of fluorescence signal detection.

Tissue Fixation, Embedding, and Sectioning

Tissues should be obtained as fresh as possible and fixed immediately. Unsatisfactory results in NISH to routinely processed tissue sections are often due to the choice of an unsuitable fixative or overfixation of the tissues. We recommend 4% (v/v) formaldehyde in phosphate-buffered saline (PBS) with neutral pH. This fixative results in good preservation of tissue morphology and nuclear 3D structure and is compatible with NISH procedures (17), including fluorescence probe labeling or detection schemes. Note that deviations in the pH may lead to swelling artifacts. Fixatives with

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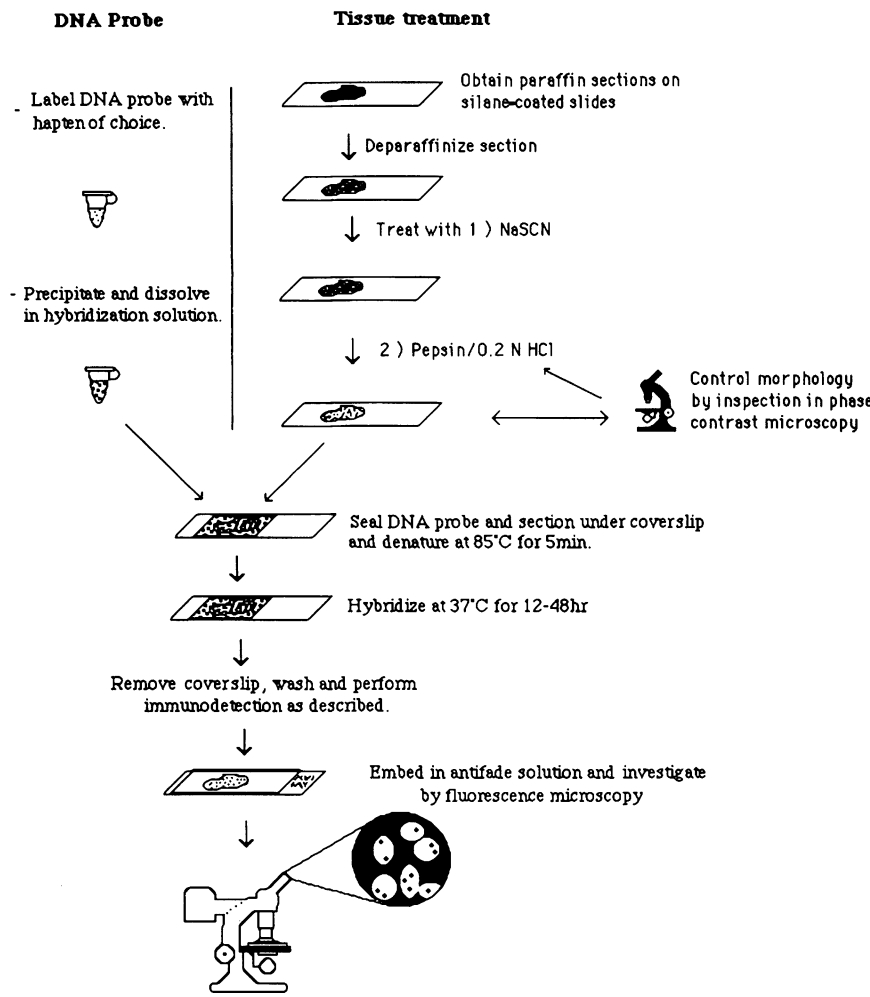

FIG. 1 Schematic outline of the processing of paraffin-embedded tissue sections for fluorescence *in situ* hybridization.

much stronger protein–protein and DNA–protein cross-linking properties than formaldehyde, such as glutaraldehyde and osmium tetroxide, will impair the accessibility of the probes to target DNAs. When using experimental animals, perfusion of the whole animal or the organ in question with 4% buffered formaldehyde (15 min), followed by an additional perfusion with PBS (15 min), is considered as the method of choice. In cases where perfusion is not possible, a fixation time of 4 to 6 hr should be sufficient for the complete fixation of tissue cubes with 4-mm lateral length; fixa-

tion times >16 hr should be strictly avoided. Overfixation has two disadvantages: (*a*) it favors cross-linking, resulting in both impaired probe penetration and increased autofluorescence, and (*b*) it leads to significant DNA degradation and DNA losses, in particular if nonbuffered formaldehyde is used for prolonged times, which favors the formation of formic acid. In stained sections, e.g., with hematoxylin–eosin, severe DNA losses are indicated by "pale" staining nuclei (18).

Fixed tissue samples can be embedded in paraffin following standard protocols to ensure a homogeneous rigidity and stability for thin and even sectioning. Prior to embedding, perfusion-fixed tissue may be cut into cubes a few millimeters in lateral length or larger. Embedding is preferably done with Paraplast (paraffin wax, mp 55–57° C), as it infiltrates tissue blocks four times as fast as standard paraffin wax (18). For example, a protocol that yielded good results for NISH of mouse, rat, and human testis is given below. For other tissues it may be necessary to change the dehydration times depending on the water content of each tissue. The paraffin wax should be remelted several times and degassed in a desiccator before use. This results in a more fine crystalline structure of the wax and gives better results in sectioning. Paraffin-embedded tissues may be stored for extended periods of time, even years, before they are sectioned and used for tissue NISH. Six- to 10-μm sections are cut from the paraffin blocks, floated on a clean water surface at 40° C, and mounted on glass slides.

Alternatively, Manuelidis and Borden (3) have applied formaldehyde–picric acid fixation and cut 25- to 40-μm brain tissue sections with a vibratome.

Protocol

1. Obtain fresh tissue sample.
2. Dissect tissue into small cubes of approximately 4 mm^3.
3. Submerse tissue cubes (4 hr–maximum 16 hr) in a solution of PBS containing 4% (v/v) formaldehyde (pH 7).
4. Wash cubes 3 × 5 min in PBS and once in 0.9% (w/v) NaCl.
5. Dehydrate cubes by incubation for 30 min each in 70, 80, and 90% ethanol and 3 × 10 min each in 100% ethanol. (Note: The presence of phosphate could lead to a milky precipitate.)
6. Submerse cubes for 20 min each in ethanol/toluene (1/1, v/v) and toluene.
7. Submerse cubes in Paraplast/toluene (1/1, w/v) for 20 min at 60° C and in Paraplast for 20 min at 60° C.
8. Place cubes in molds; add melted Paraplast. After solidifying, store cubes at room temperature.
9. Perform thin sectioning (6–10 μm) with a standard microtome. [Note: Putting cubes into the freezer (−20° C) for 30 min prior to sectioning facilitates the generation of sections with homogeneous thickness.]
10. Mount sections on aminoalkylsilane-coated slides (see below).

Aminoalkylsilane Coating of Glass Slides

Loss of sections during pretreatment, *in situ* hybridization, and detection procedures can be a severe problem. Coating of slides with aminoalkylsilane was deemed superior in our hands to other coating procedures, e.g., with egg white, gelatin, collagen or poly(L-lysine) (7, 19). Aminoalkylsilane-treated slides can be stored for several weeks at room temperature before use. In our experience, it is advisory to use freshly coated slides, in particular for tissues rich in fat and protein such as mammary and brain. Due to a maximum number of nonoxidized aminoalkyl groups present at the glass surface, these slides show maximum binding capacities for the ketone groups present in a tissue.

Protocol

1. Incubate slides in 70% EtOH and dry with a paper wipe.
2. Incubate slides 15 sec in freshly prepared 2% (v/v) 3-aminopropyltrimethoxysilane diluted in acetone.
3. Wash twice in acetone and once in distilled water (several seconds each).
4. Air dry at room temperature or in an incubator at 37° C.
5. Store dust-free at room temperature.

DNA Probes and Labeling Protocols

Several types of DNA probes, including probes for tandemly repetitive sequences, yeast artificial chromosome (YAC) clones, cosmid clones, and whole chromosome paint probes, have been successfully used for tissue FISH (4, 9, 13, 14) (T. Cremer, H. Scherthan, and M. Speicher, unpublished data). For DNA labeling, nick-translation and random priming protocols have found widespread applications. The reader is referred to step-by-step protocols published elsewhere (20). In our laboratory, nick-translation is routinely used. A critical point in tissue FISH is the fragment size of the labeled DNA probe. Fragment sizes of 100–300 bp have yielded good penetration and signal strength. We recommend monitoring the size of labeled probes routinely by running an aliquot (80 ng) through a 2% agarose minigel.

By virtue of their small size (<50 bp), oligonucleotides directed to highly repetitive targets have been particularly useful for tissue NISH (21). In addition to their efficient penetration, their single-stranded nature excludes self-annealing of the probe during *in situ* hybridization. Labeling of oligonucleotides can be performed by 3′ end-labeling using terminal deoxynucleotidyltransferase with nucleotides modified with haptens (e.g., biotin, digoxigenin) (22, 23) or directly with fluorochromes. In

the case of hapten-labeled nucleotides, an optimal spacing within the tail improves the access of detection agents [avidin–FITC (fluorescein isothiocyanate) or antibodies]. This can be achieved by adding dATP to the tailing reaction in addition to hapten or fluorochrome-labeled dUTP. Note that optimal spacing depends on the size of the hapten. For example, in the case of digoxigenin, a molar ratio of approximately 1:6 has been found optimal for digoxigenin-11–dUTP/dATP, whereas the optimal ratio was approximately 1:1.5 in case of biotin-11–dUTP/dATP (23).

Protocol

1. Combine 15 pmol oligonucleotides; 1 nmol biotin-11–dUTP and 1.5 nmol dATP (or 1 nmol digoxigenin-11–dUTP and 6 nmol dATP); 10 μl 5× cacodylate buffer; 0.5 μl $CoCl_2$; and 75 U terminal transferase; add distilled H_2O to a final volume of 50 μl.
2. Incubate at 37° C for >3 hr or overnight.
3. Add 20 μg of sheared salmon sperm DNA.
4. Add 3 vol of ethanol to precipitate tailed oligonucleotides along with salmon sperm DNA at −20° C for >10 min. (Note: Additional salt is not required for precipitation.)
5. Spin for 30 min.
6. Wash pellet with 70% EtOH.
7. Air dry and dissolve pellet in TE buffer (5 ng/μl)

Dot-Blot Test for Efficiency of Hapten Labeling

This assay is designed to test the successful incorporation of hapten moieties in the probe DNA. Commercial kits are available.

Protocol

1. Spot 6 drops of 9 μl 6× SSC each on Parafilm.
2. Add 1 μl of the labeled oligonucleotides (approx 0.3 pmol/μl) to the first drop, mix by pipetting the drop several times, then transfer 1 μl to the next drop.
3. Repeat step 2 for all 6 drops.
4. Remove 1 μl from the drop with lowest probe concentration and spot it onto a nitrocellulose membrane. Repeat this step for drops with increasing probe concentrations.

5. Incubate the membrane for 5 min at 80° C.

6. Place the membrane in a small plastic jar and cover with blocking buffer. Incubate at 50° C for 10 min.

7. Pour off blocking buffer, cover filter completely with AP1 buffer containing streptavidin alkaline-phosphatase conjugate at 0.5 μg/ml. Incubate for 15 min at room temperature (preferably with agitation of the jar during this and the following steps).

8. Remove solution and wash 3 × 3 min with AP1 and once with AP2.

9. Seal filter in a plastic bag with 1 ml of substrate solution and develop dots in the dark. The dot with the highest oligonucleotide amount (approx 3×10^{-3} pmol) should become clearly visible within 5–10 min, dots 4 and 5 (with 3×10^{-4} and 3×10^{-5} pmol) should be visible after 30 to 40 min, and the dot with the lowest amount (3×10^{-6} pmol) will remain invisible in most cases even after prolonged incubation times.

10. Remove the filter from the bag and stop the reaction by a brief wash in 70% ethanol. Air dry.

Pretreatment of Tissue Sections, *in Situ* Hybridization, and Posthybridization Stringency Washes

To facilitate access of a DNA probe to target sequences in tissue nuclei as well as the access of subsequent detection reagents, a number of protocols that make use of limited proteolytic treatment have been described. Proteinase K and pepsin can be efficiently used to remove cytoplasm and nuclear proteins (2, 5–15). Prolonged digestion times with proteinase K in conjunction with extensive denaturation steps often result in inferior morphology of the sections, including nuclear morphology. Such adverse effects are less apparent with pepsin (11). Pepsin digestion times (see below) can be further reduced if protein complexes are dissociated prior to the enzymatic treatment, by incubation of the sections with chaotrophic chemicals such as guanidinium thiocyanate or sodium thiocyanate (11). Optimal concentrations of pepsin and optimal digestion times have to be determined empirically in each laboratory for each tissue. Conditions given in Table I may be considered a rule of thumb. Table II describes the composition of *in situ* hybridization mixtures for various types of DNA probes. Probe DNAs and target DNAs are denatured simultaneously. Generally, the denaturation temperature and time have to be increased in formaldehyde-fixed tissue sections in comparison to standard protocols using methanol/acetic acid-fixed chromosome preparations (3, 4, 10, 11, 13, 14). However, to preserve optimally the nuclear morphology, the lowest temperature and shortest time possible without compromising the hybridization efficiency should be determined. After *in situ* hybridization, the preparations are routinely washed in a buffer containing formamide. However, the large scale use of formamide may be harmful and creates an environ-

TABLE I Digestion Conditions for Paraffin-Embedded Tissue Types[a]

Tissue type	Pepsin concentration (mg/ml)	Digestion time (min at 37°C)	Fixation with buffered formaldehyde
Rat testis	0.1	30–60	Organ perfusion; 20 min
Mouse testis	0.1	30–45	Organ perfusion; 20 min
Human testis	1	60	Immersion of tissue fragments; 5–12 hr
Human or rat brain	1.2	120	See above
Mouse skin	1.5	45	See above
Human mammary	1	90	See above

[a] Routinely applied in our laboratory. These conditions should be considered only as a rule of thumb to serve in initial experiments. Modifications may be necessary, since optimal conditions for different samples even from the same tissue may vary.

mental burden. Formamide can be avoided without compromising the stringency by using appropriately diluted buffers with monovalent cations (24). Although some investigators have worried about negative effects of such highly diluted buffers on nuclear morphology, we have not noticed any obvious effects (25, 26).

TABLE II Composition of Hybridization Solution

Component:	Repetitive DNA probes	Chromosome paint probes	40-mer oligonucleotide probes
SSC concentration	1×	2×	2×
Formamide	68%[a]	50%	30%
Dextran sulfate	—	10%	10%
Herring sperm DNA	1 μg/μl	1 μg/μl	1 μg/μl
Suppression DNA (Cot1 fraction)	—	0.25–2 μg/μl	—
Phosphate buffer (optional)	20 m*M*	20 m*M*	20 m*M*
DNA probe	1–10 ng/μl	50–100 ng/μl	0.1–0.5 ng/μl

[a] For optimal stringency, different alphoid DNA probes may require different formamide concentrations, which should be determined by FISH to chromosome preparations.

Protocol

1. To melt and drain off excess wax, place slides with paraffin sections in upright position on a paper wipe and incubate for several hours at 65° C.
2. Transfer the warm slides quickly into a Coplin jar containing xylene at room temperature. Incubate slides 2 × 10 min in xylene.
3. Wash slides 5 min each in 100, 86, and 70% ethanol.
4. Incubate slides for 30 min in 4× SSC/0.1% Triton X-100.
5. Drain off excess fluid and apply 100 μl 1 *M* NaSCN under a large coverslip. Incubate at 56° C for 30 min in a humid chamber.
6. Dip wash sections in H_2O.
7. Digest sections with 100 μg to 4 mg pepsin/ml H_2O (pH 2) at 37° C for times indicated in Table I. To monitor the progress of the digestion, cover the wet section with a coverslip and inspect the section at low magnification in a phase-contrast microscope. Sufficient protein digestion is achieved when most nuclei appear free from cytoplasm (Fig. 2).
8. Rinse sections with PBS. Postfix in 1% formaldehyde/PBS for 5 min and wash in PBS for another 5 min.

FIG. 2 Phase contrast micrograph of a human testis section taken during pepsin digestion. The presence of many nuclei free of cytoplasm indicates that this section is ready for further processing. Bar: 10 μm.

9. Drain excess fluid from the sections and dry the glass surface around the sections with a paper wipe.

10. Apply hybridization solution (Table II, 6 μl for an 18 $\times$ 18-mm coverslip) and seal under a coverslip with rubber cement.

11. Denature the preparation on a hot plate, e.g., at 85° C for 5 min. Incubate overnight (or longer) in a humid chamber at 37° C.

12. Peel off rubber cement with forceps.

13. In the case of whole chromosome composite probes, chromosome-specific satellite DNA probes, and cosmid probes, wash preparations 3 $\times$ 5 min in 0.03 $\times$ SSC at 42° C. In the case of oligonucleotide probes, wash 3 $\times$ 5 min in 0.3 $\times$ SSC at 42° C.

Signal Detection

Posthybridization washes are immediately followed by immunocytochemical (e.g., 5, 27, 28) or immunofluorescence detection procedures (e.g., 29). In the first approach, haptenized DNA probes are detected with enzyme-linked avidin in the case of biotinylated probes or antibodies directed to the respective hapten. Different commercial kits that use horseradish peroxidase or alkaline phosphatase are available. As substrates, diaminobenzidine (DAB) and NBT/BCIP are widely used (1–3, 5–12, 15, 27). The advantage of such a detection procedure is that the signals are permanent and can be contrasted with a permanent counterstain and embedded for long-term storage. Furthermore, DAB-stained sections offer sufficient contrast for transmission electron microscopy. If desirable, contrast can be enhanced by additional silver intensification [e.g., (3)]. The second approach offers the possibility to delineate several probes simultaneously with multiple colors (30–33). Two-color fluorescence detection has been applied in tissue sections (14). We expect that multiple-color tissue FISH will gain wide application in the future. Six- to 8-μm sections result in low autofluorescence but may lead to an underestimation of chromosome copy number in truncated nuclei. This problem can be overcome by serial sections (7) or by appropriate statistical analysis (11). Figures 3A and 3B show examples of human and mouse testis sections subjected to FISH with repetitive DNA probes (35). Figure 3C shows a section of a human seminoma subjected to FISH with chromosome 1- and 8-specific DNA libraries (34, 36, 37). A rat testis section hybridized with a rat satellite I oligomer and peroxidase/DAB detection is shown in Fig. 4A. For details see figure legends.

Protocols

Immunocytochemical Detection Procedure with DAB

DAB is a strong mutagen. Gloves must be worn when handling DAB solutions. DAB substrate solution that is not needed can be inactivated by adding a small volume of

30% H_2O_2. Some investigators have performed a methanol/peroxide incubation prior to immuno-enzymatic detection to block endogenous peroxidase activity [e.g., (11)]. However, after the pretreatment and denaturation steps described above, we have never observed endogenous peroxide activity in our specimens.

1. Equilibrate slides after posthybridization washes for 5 min in 0.5% bovine serum albumin (BSA)/bicarbonate tween (BT) buffer at 37° C.
2. Apply 100 μl BT buffer containing avidin peroxidase (1 μg/ml) and incubate for 45 min at 37° C in a humid chamber.
3. Wash slides 3 × 3 min at 42° C in BT buffer.
4. Apply 100 μl BT buffer containing biotinylated goat antiavidin antibody (5 μg/ml). Incubate 30 min at 37° C.
5. Wash 3 × 3 min in BT buffer at 42° C.
6. Apply 100 μl BT buffer containing avidin–peroxidase (1 μg/ml). Incubate for 30 min at 37° C.
7. Wash 3 × 3 min in BT at 42° C and once in PBS.
8. Apply 100 μl freshly prepared DAB substrate solution to the sections and cover with a 22 × 60-mm coverslip. Incubate the reaction in a dark box for 10 min or longer. (Note: Progress of the substrate precipitation can be monitored using a phase-contrast microscope at low magnification.)
9. Stop the reaction by extensive washes in tap water.
10. Rinse once in deionized water and dry in a stream of air.
11. Counterstain with methylene blue for 20 min.
12. Rinse in water, apply 20 μl of PBS buffer containing 90% glycerol, and seal with a 22 × 60-mm coverslip.

Two-Color Fluorescence Detection Protocol for Biotin- and Digoxigenin-Labeled Probes

1. Equilibrate slides for 5 min in 0.5% BSA/BT buffer at 37° C. (Note: Sections should never dry.)
2. Apply 100 μl BT buffer containing avidin–FITC (1 μg/ml) and mouse anti-digoxigenin (0.2 μg/ml) to each slide and incubate for 50 min at 37° C.
3. Wash slides 3 × 3 min at 42° C in BT buffer.
4. Apply BT buffer containing biotinylated goat antiavidin antibody (5 μg/ml) and rabbit anti-mouse TRITC IgG (1 μg/ml) and incubate for 30 min at 37° C.
5. Wash 3 × 3 min in BT buffer at 42° C.
6. Apply 100 μl BT buffer containing avidin–FITC (5 μg/ml) and goat anti-rabbit TRITC (1 μg/ml) and incubate for 30 min at 37° C.
7. Wash 3 × 3 min in BT buffer at 42° C.
8. Apply antifade solution (20 μl under a 22 × 60-mm coverslip).

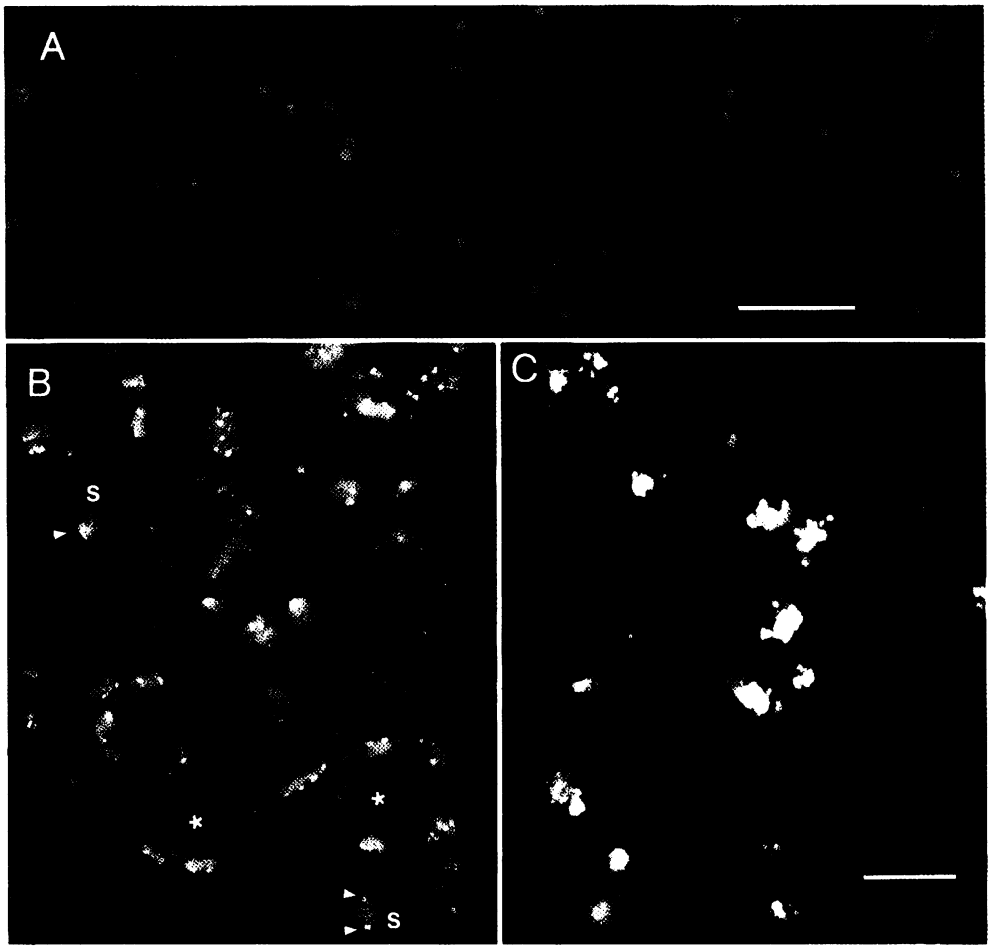

FIG. 3. (A) Tissue FISH of a biotin-labeled chromosome 1-specific satellite III DNA probe (pericentromeric heterochromatin) (35) to a 8-μm human ovary paraffin section showing high hybridization efficiency. Nuclear DNA is counterstained with propidium iodide (red). The probe was detected with avidin–FITC (yellow). Bar: 10 μm. (B) Two-color tissue FISH to a 10-μm mouse testis paraffin section showing part of the periphery of a testis tubule. Centromeric satellite DNA was hybridized with a digoxigenin-labeled oligomer homologous to the mouse major satellite and detected with rhodamine conjugated anti-digoxigenin antibodies (red). Telomeres were hybridized with biotinylated $(TTAGGG)_7$ and $(CCCTAA)_7$ oligomers homologous to the vertebrate telomere sequence (22) and detected with avidin–FITC (green). Microphotographs were taken using a double band pass filter set (Chroma, Battleboro, VT). The blue staining of the nuclei was obtained by double exposure of signals with the fluorescence of the DNA-specific counterstain DAPI. Several spermatocytes I at early meiotic prophase are seen (stage IX seminiferous tubule). Note the peripheral distribution of satellite and telomere sequences in spermatocytes I (asterisks). Sertoli cells (S) exhibit one or two satellite clusters with the proximal telomeres fused into one or two telocenters (arrow heads). DAPI stain of nuclear DNA is weak in these cells. Scale as in (A). (C) A 6-μm paraffin section of a human seminoma after two-color chromosome painting (36, 37) of chromosome 1 (FITC), whitish-green and chromosome 8 (TRITC, red) territories. Note the compactness of the territories in interphase. Chromosome-specific plasmid DNA libraries (34) were used as the probes. The blue staining of the nuclei was obtained by double exposure of signals together with DAPI. Bar: 5 μm.

Specimen Counterstaining

Nuclei in sections can be counterstained with various fluorochromes, such as DAPI (0.2 μg/ml) or propidium iodide (1 μg/ml) in the antifade solution. Antifade solutions containing *p*-phenylenediamine (38) may turn brown on oxidation during long-term storage, leading to increased background fluorescence. Such pitfalls can be avoided with antifade solutions containing diazabicyclooctane (13, 23) or commercially available antifade solutions such as Vectashield (Vector, Burlingame, CA).

Transmission Light Microscopy and Epifluorescence Microscopy

Sections are inspected in an epifluorescence microscope equipped with appropriate filter sets for the visualization of green, red, and blue fluorescence. Double bandpass and triple bandpass filter sets have become available that allow the simultaneous investigation of two and three different fluorochromes, such as fluorescein, rhodamine, and aminomethylcoumarin. Such filter sets avoid the problem of image shifts often observed when separate filter sets are used to discriminate two closely spaced, distinctly colored signals.

Transmission Electron Microscopy

Sections stained with electron-dense chromophores like DAB can be embedded in polymers (e.g., Epon) and after thin sectioning examined in the transmission electron microscope (TEM) (Fig. 4B). After embedding, the sections are removed from the glass slides by repeated freezing in liquid nitrogen and thawing and reblocked in gelatin capsules, and ultrathin sections are obtained on a conventional ultratome.

Protocol

1. After detection with peroxidase/DAB, cover section with 90% (v/v) glycerol/PBS; apply a coverslip and inspect sections in the phase-contrast microscope for positive staining.
2. Wash sections thoroughly in PB buffer.
3. Incubate sections for 15 min in 1 *M* OsO_4/0.1 *M* phosphate buffer (PB)/6.85% (w/v) sucrose.
4. Wash 5 × 2 min in PB buffer.
5. Immerse for 5 min in 50% EtOH.
6. Stain with uranyl acetate 5 min at room temperature.
7. Dehydrate in 90 and 96% ethanol (10 min each), and 2 × 10 min in 100% ethanol.

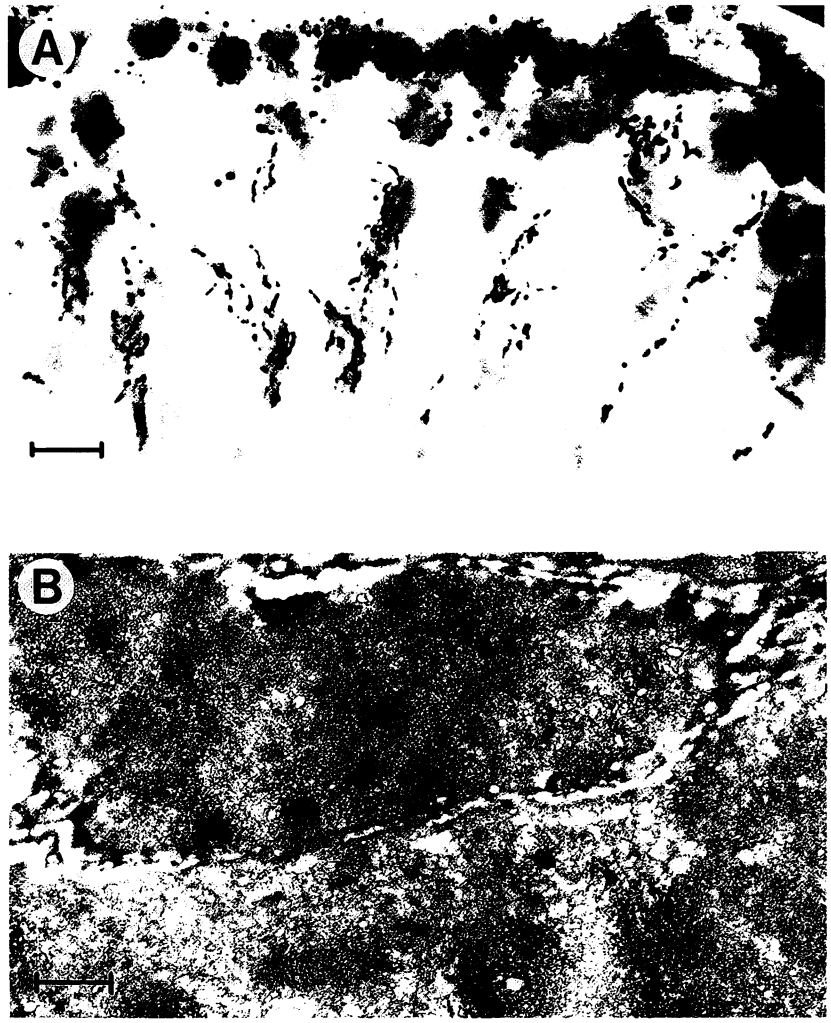

FIG. 4 (A) Rat testis section hybridized *in situ* with a 42-mer rat satellite I consensus oligonucleotide after immunoperoxidase detection and counterstaining with methylene blue. The microphotograph shows part of a tubule with numerous spermatogonia and spermatocytes I in the upper part and sperm heads in the lower part. Clusters of specifically stained heterochromatin are present in all nuclei. In contrast to mouse spermatocytes I (compare Fig. 3B) these heterochromatin clusters are observed both at the periphery and in the interior of the nuclei. Bar: 50 μm. (B) Transmission electron micrograph of an ultrathin section showing a mouse spermatogonium nucleus hybridized with a mouse major satellite consensus oligonucleotide detected with DAB (upper part). Various satellite clusters are delineated by electron-dense DAB precipitates. DAB staining is also present in neighboring nuclei (left and lower part). Although nuclear chromatin appears well preserved, the gap observed around parts of the nuclear edge indicates the removal of cytoplasm by acid protease and NaSCN pretreatments. Bar: 1 μm.

8. Immerse 2 × 10 min in propylene oxide.
9. Immerse (20 min each) in propylene oxide/Epon (3/1), in propylene oxide/Epon (1/1), and in propylene oxide/Epon (1/3) and two times in Epon.
10. Cover section with a thin layer of Epon and polymerize at 55° C for 48 hr. After polymerization, cut glass slide with a diamond pen and break slide in pieces. Submerse glass piece(s) with section or section fragments repeatedly in liquid nitrogen until the embedded sections float off.
11. Collect section or fragments and place on a slide. An Epon-filled gelatin capsule is then inverted over the section fragment and polymerized at 55° C for 48 hr.
12. Freeze slide on dry ice and remove the capsule.
13. Trim the block to the region of interest and perform ultrathin sectioning on an ultratome.
14. Collect ultrathin sections on Formvar-coated grids and examine in the TEM.

Materials, Solutions, Buffers

3-Aminopropyltrimethoxysilane (Merck, Darmstadt, Germany)
5-Bromo-4-chloro-3-indolyl phosphate (BCIP) (50 mg/ml in dimethylformamide)
Antifade solution: mix 245 mg Diazabicyclo[2.2.2]octane (Sigma, St. Louis, MO) + 200 μl 1 *M* $NaHCO_3$ (pH 8) + 800 μl distilled H_2O + 9 ml glycerol (86%). Dyes can be added in the following concentrations: DAPI 0.5 μg/ml; propidium iodide 1 μg/ml.
AP1 buffer (0.15 *M* NaCl, 50 m*M* Tris–HCl, pH 7.5)
AP2 buffer (0.15 *M* NaCl, 50 m*M* Tris–HCl, pH 9.5, 50 m*M* $MgCl_2$)
Avidin–CY_3 (Jackson-Immuno, West Grove, PA) (CY_3 is a red cyanine-based fluorescent label)
Avidin–FITC (ExtrAvidin-fluorescein, Sigma)
Avidin peroxidase (Sigma)
Biotin-11–dUTP, 0.4 m*M* (other biotin-linked nucleotides may also be used)
Blocking buffer (1% BSA or 1% nonfat dry milk in AP1).
Bovine serum albumin (fraction V, Serva, Heidelberg, Germany)
BT buffer (0.15 *M* $NaHCO_3$, 0.1% Tween 20, pH 8.3)
Cacodylate buffer 5× (1 *M* potassium cacodylate, 125 m*M* Tris–HCl, 1.25 mg/ml BSA, pH 6.6)
$CoCl_2$, 25 m*M*
DAB (BRL, Gaithersburg, MD). Dissolve in PBS at a final concentration of 10 mg/ml. Store aliquots protected from light at −20° C.
DAB substrate solution (0.5 mg/ml DAB/PBS, 0.01% H_2O_2). Always prepare immediately before use.
DAPI (4′,6-diamidino-2-phenylindole) (Sigma)

dATP, 1 m*M* (prepared from a 100 m*M* stock, Boehringer, Mannheim, Germany)
Digoxigenin-11-dUTP (1 m*M*, Boehringer)
Formaldehyde stock solution, 37% (Merck)
Goat antiavidin, biotinylated (Vector)
Goat anti-rabbit antibody TRITC-conjugated (Sigma)
H_2O_2, 30% (Merck)
Mouse antidigoxigenin antibody (Boehringer)
NaSCN (Merck)
Nitro blue tetrazolium (NBT) (75 mg/ml in 70% dimethylformamide)
Nitrocellulose membrane (Schleicher & Schuell, Keene, NH)
Oligonucleotides: The 42-mer deoxyoligonucleotide 3′-AAGAA AACTG AAAAT CATGG AAAAT GAGAA ACATC CACTT GA (MS1) homologous to the mouse major satellite was derived from the consensus sequence of the mouse major satellite DNA (39). The 42-mer rat satellite I-specific deoxyoligonucleotide (RS1) 3′-CTGAA ACACT GTTTC TTTGT GAATT CAGTT AGTTC CTTCT AG was derived from the conserved region of the *Rattus rattus* and *Rattus norvegicus* satellite I (40).
Paraffin wax (T_m 60° C)
Paraplast (T_m 55–57° C, Shandon, Pittsburgh, PA)
PB buffer (Mix 0.1 *M* NaH_2PO_4 and 0.1 *M* Na_2HPO_4 to obtain a pH of 7.4)
PBS buffer (130 m*M* NaCl, 7 m*M* Na_2HPO_4, 3 m*M* NaH_2PO_4, pH 7)
Pepsin (3200–4000 U/mg, Sigma)
Propidium iodide (Sigma)
Rabbit anti-mouse antibody TRITC-conjugated (Sigma)
Sheared salmon or herring sperm DNA (10 mg/ml, approx 300 bp average fragment length) (Sigma)
SSC 20× (3 *M* NaCl, 0.3 *M* Trisodium citrate, pH 7)
Streptavidin–alkaline phosphatase (BRL)
Substrate solution consisting of 4.4 μl of the NBT solution and 3.3 μl of the BCIP solution in 1 ml of AP2
Suppression DNA (Cot 1 fraction, BRL)
TE buffer (10 m*M* Tris–HCl, pH 7.4, 1 m*M* EDTA)
Terminal deoxynucleotidyltransferase (Boehringer)
Triton X-100 (Serva)

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