

Improvements in Cytogenetic Slide Preparation: Controlled Chromosome Spreading, Chemical Aging and Gradual Denaturing

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Background: Metaphase spreading is an essential technique for clinical and molecular cytogenetics. Results of classical banding techniques as well as complex fluorescent in situ hybridization (FISH) applications, such as comparative genomic hybridization (CGH) or multiplex FISH (M-FISH), are greatly influenced by the quality of chromosome spreading and pretreatment of the slide prior to hybridization.

Materials and Methods: Using hot steam and a metal plate with a temperature gradient across its surface, a reproducible protocol for slide preparation, aging, and hybridization was developed.

Results: This protocol yields good chromosome spreads from even the most difficult cell suspensions and is unaffected by the environmental conditions. Chromosome spreads were suitable for both banding and FISH techniques common to the cytogenetic laboratory. Chemical

aging is a rapid slide pretreatment procedure for FISH applications, which allows freshly prepared cytogenetic slides to be used for in situ hybridization within 30 min, thus increasing analytical throughput and reducing benchwork. Furthermore, the gradually denaturing process described allows the use of fresh biologic material with optimal FISH results while protecting chromosomal integrity during denaturing.

Conclusion: The slide preparation and slide pretreatment protocols can be performed in any laboratory, do not require specialized equipment, and provide robust results. *Cytometry* 43:101-109, 2001.

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Key terms: protocol; metaphase spreading; slide aging; alcohol; hybridization; cytogenetics; G-banding; FISH technique; CGH; M-FISH

Cytogenetic slide preparation is essential for both classical banding procedures and in situ protocols, such as fluorescence in situ hybridization (FISH). Slide preparation techniques were described in numerous publications (1-7). Almost every cytogenetic laboratory inherited a procedure, which was then refined to yield good results. Different protocols were specifically designed for use with particular types of cells (amniocytes, lymphocytes, solid tumors, leukemias; 8,9) or for particular purposes (G-, C-, Q-, R-banding, FISH; 10-12). Instruments to aid in the making of slides have been designed (13) and developed (Thermotron Cytogenetic Drying Chamber CDS-5, Thermotron Industries, Holland, MD). FISH protocols are also quite diverse, but little details in the protocol greatly influence hybridization results (14). This report discusses several improvements in metaphase spreading, aging, and denaturing procedures. These modifications can be performed in any laboratory, on all types of cell suspensions, and do not require specialized equipment. The simple setting and experimental observations described in this

study allow customization of the degree of spreading of chromosomes, regardless of the cell type. Slides can be used for classical banding procedures or for FISH. Furthermore, chemical aging and gradual denaturing were developed to shorten the FISH protocol and maximize hybridization results. These procedures yield identical or better results when any other protocol used in our laboratories and decrease the time required for technologists to spend at the bench. FISH hybridization times are shortened as follows: 15 min for repetitive probes, 4-5 h for single-copy probes (cosmids, PACs, YACs), and overnight hybridization for complex DNA probes, as in CGH and M-FISH.

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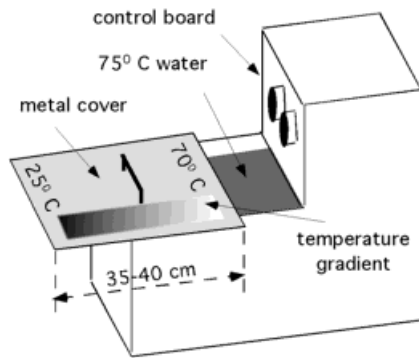


Fig. 1. Waterbath image showing metal plate position, allowing both the temperature gradient formation and access to the hot steam.

MATERIALS AND METHODS

Equipment

The equipment (Figs. 1,2) required for the metaphase spreading protocol includes (1) a source of hot steam (waterbath at 75–80°C); (2) a heated metal plate, ideally with a temperature gradient across its surface. To achieve this, the metal plate/lid covers the waterbath only partially (Fig. 1) and carries a temperature gradient between the hot end and a cold end. A 2–3-mm thick metal plate is ideal. We tested plates made of stainless steel (3 and 1.5-mm thick) and copper (1-mm thick). When the plate rests about 2 cm from the hot water surface, the part of the plate in contact with the hot steam shows roughly 70°C, whereas the other end of the plate (10–15 cm long) is at room temperature. If such a metal plate is not available, a minimum of two surfaces, one at 65–75°C (a simple heat block), and one at room temperature are required; and (3) good quality slides. Two brands of pre-cleaned slides, Gold Seal (Becton Dickinson, Portsmouth, NH) and Superfrost (Erie Scientific, Portsmouth, NH), were commonly used. They did not require any extra cleaning steps or washes prior to use. Cell suspensions were dropped on slides taken directly out of their original boxes. Lower quality glass slides can be passed through successive washes in acetone, HCl/ethanol, and triple distilled water and their quality compared.

Chromosome Preparation

Cell culture and fixation. Cell suspensions from peripheral blood, fibroblasts, bone marrow, lymphoblastoid cell lines, and germ cell tumors were used to prepare metaphases for G-banding and FISH. Cell culture was performed according to standard protocols (1). For harvesting, the hypotonic buffer used was 0.075 M KCl, at 37°C for 10–20 min (incubations were shorter for peripheral blood cultures, longer for tumors). Hypotonic treatment increases cell volume and disrupts the cell membrane of the red blood cells (allowing their removal).

After hypotonic treatment, usually in 15-ml centrifuge tubes, cells were pelleted by centrifugation 10 min at 1,000 rpm, resuspended in 1–1.5-ml fixative (3:1 metha-

nol-to-acetic acid concentration), and transferred into 1.5-ml vials. All subsequent fixative washes were done in these small vials, with centrifugations performed in a tabletop microfuge for 1–2 min at 6,000–7,000 rpm.

Chromosome spreading and G-banding. Cells in fixative were diluted at the appropriate density, empirically determined in any cytogenetic laboratory, to produce a reasonable number of well-spread metaphases in each microscopic field. With an automatic pipette, 25–35 μ l of cell suspension was evenly distributed on several locations on the slide and the liquid was spread by gently moving the pipette tip parallel to the surface. As the fixative gradually evaporated, the surface of the slide became grainy (cells visible). At that moment, the slide was placed face down into the steam of the hot water bath (75°C or more) for 1–3 s, then dried by placing the slide on the metal plate (carrying a gradient of temperature across its surface; Fig. 1). The degree of spreading is adjusted using the different temperatures of the heated plate, with higher temperatures increasing chromosome spreading. For difficult-to-spread cells, after the surface became grainy, the slide was passed briefly through the water vapors, then four to six droplets of acetic acid were placed on the slide. After the acetic acid slowly spread and covered the surface, the slide was held 3–5 s in the steam of the waterbath, then quickly dried on the hottest area of the metal plate or the metal block (65°C). After overnight incubation at 65°C (aging), G-banding was performed according to a standard laboratory procedure (1).

FISH: Chemical Aging and Slide Denaturing

Chemical aging. A freshly prepared slide was placed on the metal block of a thermocycler (a polymerase chain reaction [PCR] machine; Fig. 2). Ethanol (150–200 μ l) was pipetted on the slide, covered with a coverslip, and ethanol-soaked gauze was placed on top to prevent ethanol evaporation. A plastic lid (for example, from a box of pipette tips) was used to cover the slide-gauze assembly. The block was programmed to increase the temperature to 94°C, to keep it for 2–20 s, and to cool it to room temperature. Depending on the machine, the heating and cooling speed was 1–2°C/s. Alternatively, the slide can be incubated 10–15 s each, in jars with ethanol at 50, 75, 94, 75, and 50°C, followed by drying at room temperature.

After aging, the slides were subjected to brief (30–60 s) pepsin pretreatment, using 0.005% pepsin in 0.01 N HCl. This was followed by rinsing in PBS, ethanol series and air drying.

Gradual denaturing. In (a) simultaneous protocol (Fig. 6c), 11–12 μ l labeled DNA probe in hybridization buffer was pipetted on the slide, covered with a 22 \times 22-mm coverslip, and sealed with rubber cement. The slide was placed on the metal block of a thermocycler, which was programmed to gradually (within 90 s) heat the slide to 75°C, to keep that temperature for 90–120 s, and to gradually (90 s) cool to room temperature.

In (b) the separate protocol (Fig. 6f,g), labeled DNA probe was denatured 5 min at 75°C in a waterbath. For slide denaturing, 150 μ l of 70% formamide/2 \times SSC was

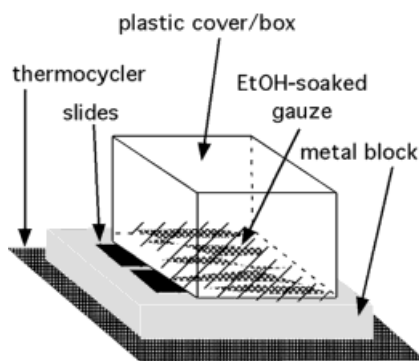


Fig. 2. Chemical aging setting. A few layers of gauze are placed in a plastic box (e.g., a box used to store slides). Two to three strips of tape are placed across the opening to hold the gauze in place when the box is turned upside down. Prior to use, the gauze is soaked in ethanol. Two to three freshly prepared slides are placed on the metal block of a thermocycler. Ethanol (200 μ l) is pipetted on each slide and covered with a coverslip. The plastic box is placed upside down on the slides so that the wet gauze comes in contact with the slides and covers them (the gauze will prevent ethanol evaporation during the subsequent heating step). The slides are heated according to the sequence described in the text. After heating, the plastic box/cover and the coverslips are removed and the slides are air dried.

pipetted on the slide, covered with a coverslip, and the assembly gradually heated on the metal block of the thermocycler as described above. The coverslip was discarded and the slide rinsed through an ethanol series, 3 min each in 70% and 100% ethanol at room temperature, then air dried. For laboratories not equipped with a thermocycler, an alternative separate denaturing protocol uses three jars with 70% FA/2 \times SSC solution at 45, 60, and 75°C. The slide was dipped 5–10 s each in the 45 and 60°C solutions,

then incubated 90–120 s at 75°C and dipped again 5–10 s each in the 60 and 45°C solutions, followed by brief rinsing in 70% and 100% ethanol and air drying.

Probe labeling and detection. Probes (centromeric repeats, chromosome paint probes, cosmids) were either purchased or were prepared and labeled by nick translation or PCR labeling, using commercially available (Boehringer Mannheim, Indianapolis, IN) or custom-made (15) FITC-, biotin-, or digoxigenin-dUTP. After overnight hybridization in a moist chamber at 37°C, the slides were subjected to one layer of detection for biotin (avidin-Cy3) and digoxigenin (anti-digoxigenin-FITC). Slides were examined with a Leica Aristoplan or an Olympus Provis AX70 microscope. Images were taken using cooled-CCD cameras (Photometrics, Tucson, AZ) and commercial software (PSI or Vysis, UK).

RESULTS AND DISCUSSION

We performed numerous tests aimed at improving the efficiency of cytogenetic slide preparation and increasing FISH signals. Several modifications of the general protocol resulted in better chromosome spreading, better chromosome morphology, and shorter hybridization times, and yielded brighter FISH signals. Although the procedures presented use mostly known reagents or conditions (ethanol, fixative, acetic acid, heat, water vapors), these are used in a more “logical” way, based on step-by-step observations made at the microscope. The various observations or improvements are discussed below.

Improved Cell Recovery by Microcentrifugation

The fixative solution (3:1 methanol-to-acetic acid concentration) preserves the cells in their “swollen” state

Table 1
Result Comparisons Between the Standard and the Proposed Spreading Protocols*

	Peripheral blood	BM1 (GCT1)	BM1 (GCT2)	BM1 (EB)	BM2 (GCT)	BM2 (EB)
Standard laboratory protocol						
No. metaphases	57	50	53	42	45	38
Average diameter (μ m)	32.83	17.76	20.56	18.48	16.97	16.89
SD	6.55	3.85	7.28	3.58	3.89	2.7
No. crossovers	242	NA	NA	NA	NA	NA
Crossovers/metaphases	4.3	NA	NA	NA	NA	NA
Proposed spreading protocol						
No. metaphases	85	58	45	47	35	47
Average diameter	39.41	22.31	24.21	22.95	22.26	21.37
SD	4.5	5.64	8.04	6.49	4.06	4.88
No. crossovers	115	NA	NA	NA	NA	NA
Crossovers/metaphases	1.4	NA	NA	NA	NA	NA

*Cells from a peripheral blood culture and two or three separate flasks from two bone marrow cultures were subjected to both the standard and the proposed slide preparation technique. Each slide was scanned from left to right and top to bottom using the 20 \times objective. All metaphases found (all fields containing at least one metaphase) were captured, to a final number of 36 microscopic fields on each slide (45 fields for the peripheral blood slide obtained with the proposed protocol). The diameter of every captured metaphase was measured (in microns). The number of crossovers for the longer, peripheral blood culture chromosomes was also recorded. As chromosomes from bone marrow cultures were usually short, the number of crossovers was reduced and not so informative, thus it was not included in the study. No. metaphases indicates the number of metaphases captured and measured. Average diameter indicates the arithmetic mean of all diameters measured. SD is the standard deviation of the values measured. No. crossovers is the number of crossovers found in all metaphases captured. Crossovers/metaphases indicates the average number of crossovers per metaphase. BM1 and BM2 are the two bone marrow cultures analyzed. GCT or EB are the different culture flasks used for the same BM culture. NA, not available/not performed.

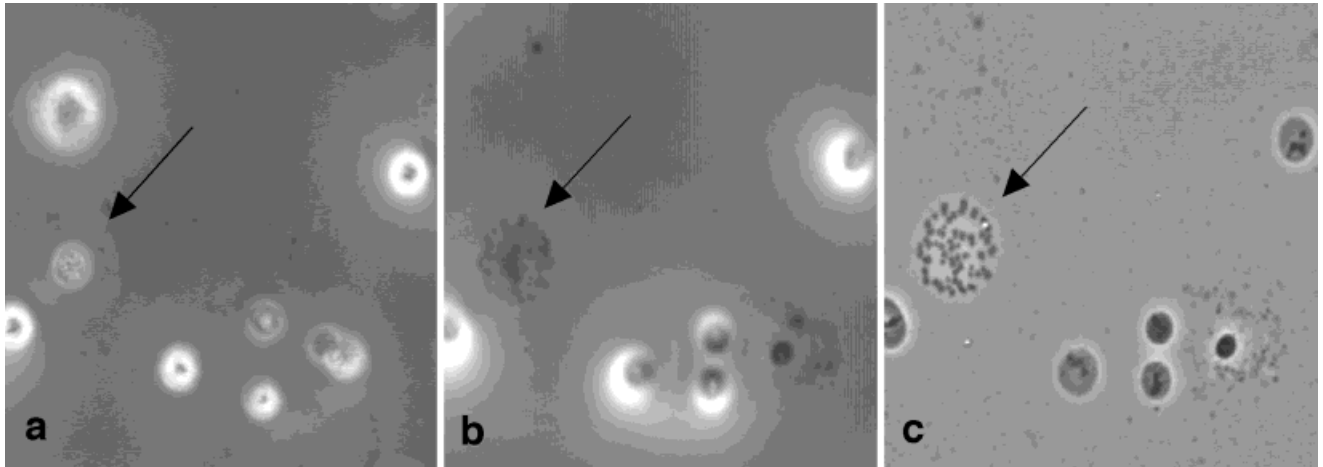


FIG. 3. Illustration of the arbitrary steps 1, 3, and 5 described in the chromosome spreading section. Cells were placed directly on the slide. As long as there is sufficient fixative, nuclei/chromosomes do not spread (a). As the fixative gradually evaporates and its surface touches the surface of the cells on the slide, cells in metaphase spread first. The other cells acquire a halo of light because of their spherical shape (b). As the fixative dries completely, the nonmitotic cells flatten as well, whereas the diameter of the metaphases increases slightly (c).

after hypotonic treatment, removes lipids, and denatures proteins. This makes the cell membrane very fragile, which helps chromosome spreading (4). Cells pelleted after hypotonic buffer treatment can be resuspended in 1 ml fixative and transferred to 1.5-ml microfuge tubes. All subsequent fixative washes can be performed in these small vials. The use of microfuge tubes increases the speed of cell processing, as various centrifugation steps can be performed in a microfuge from 2 min per 6,000 rpm to 10 s per 14,000 rpm. When compared with a regular protocol, which uses 15-ml tubes and 1,000 rpm centrifugation steps (1), there was no difference in cell or chromosome morphology (data not shown). Importantly, high-speed centrifugation reduces or eliminates cell loss during harvesting. Cell suspensions can be stored in fixative in the same 1.5-ml vials for years at -20°C .

Slide Storage

A common procedure in many clinical cytogenetic laboratories is to store the slides at room temperature, usually in a dessicator. We found that the quality of the biologic material on the slides is preserved better, especially for future FISH studies, if slides with metaphase spreads are stored at -20°C in jars with 100% ethanol. Slides stored at least 1–2 days in ethanol do not require aging prior to FISH. Slides used solely for G-banding can be stored dry at room temperature, but 18–24-month-old slides did not yield FISH results. By comparison, 6-year-old slides stored in ethanol yielded good quality FISH results.

Controlled Chromosome Spreading

In a standard laboratory procedure, to which our proposed protocol was compared, glass slides are washed and rinsed in triple distilled water, then kept in ice-cold water prior to preparing the chromosome spreads. Using a glass pipette, a few drops of cell suspension are placed on the

wet, ice-cold slide from a 30 to 40-cm distance. Excess liquid is drained on a paper towel and the slide is placed on a $60\text{--}65^{\circ}\text{C}$ hot plate to dry. By comparison, our proposed procedure uses the same factors (water vapors, hot plate, acetic acid) in a more precise, step-by-step fashion, based on the intimate processes taking place at the microscopic level. Chromosome spreading is superior to that obtained with the standard protocol. In our new procedure, precise timing is important. Initial slide exposure to hot water vapors provides a uniform layer of moisture on the slide immediately prior to adding the cells. After the cell suspension is spread evenly on the slide, it is important to allow the fixative to partially evaporate, until the surface becomes grainy in aspect. Immediately, the slide is exposed again to the hot water vapors, so water is evenly distributed on the surface. Water arrives on the slide when most of the methanol from the fixative has evaporated and there is a resulting brief increase in acetic acid concentration. The mix of acid and water at this moment, followed by quick drying on a hot surface, provides good chromosome spreading. To maximize spreading, after the slide surface becomes grainy, three to five drops of glacial acetic acid are placed on the slide and allowed to cover the surface. Immediately, the slide is exposed 4–5 s to the water vapors, then quickly dried on a hot metal surface. With good quality cell suspensions (e.g., peripheral blood), if overspreading occurs or metaphases break, slides should be dried on the colder areas of the metal plate containing the heat gradient (Fig. 1). Thus, adjusting the drying temperature allows optimal spreading of any cell suspension.

Results of comparisons between the standard and the proposed slide preparation protocol are summarized in Table 1. We calculated the metaphase diameter of metaphases obtained from one peripheral blood culture and two to three different culture flasks (slightly modified

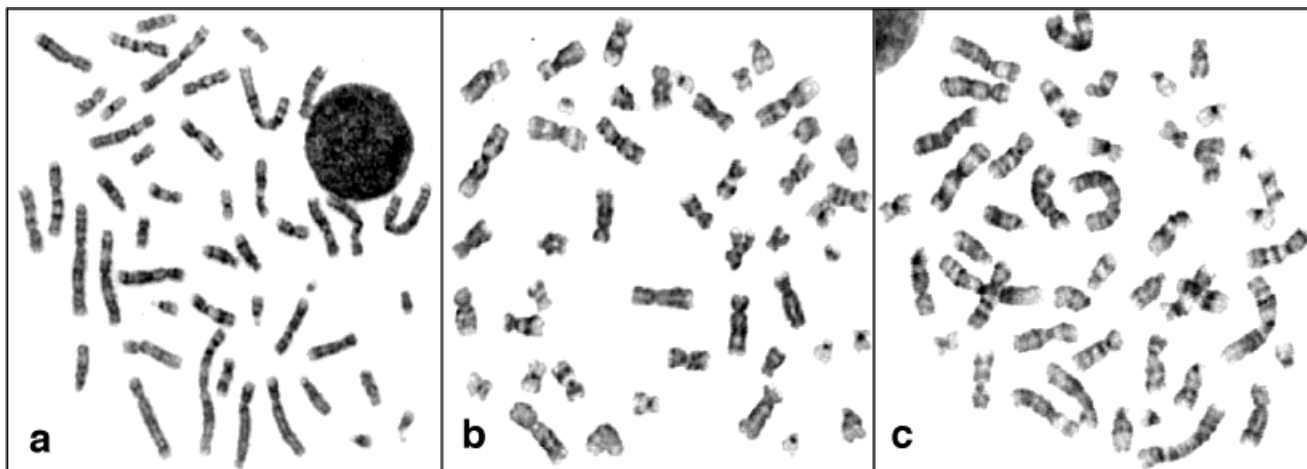


Fig. 4 a: G-banding analysis of a metaphase in a peripheral blood culture. The metaphase was prepared using the proposed protocol, which allows better spreading of chromosomes. b,c: G-banding analysis of a bone marrow culture showing a metaphase prepared with the standard protocol (b) and with the proposed protocol (c). G-banding sharpness is similar but the number of readable metaphases on the slide is higher with the proposed protocol.

culture conditions) of two bone marrow cultures (BM1 and BM2). In general, metaphase diameters were 20–33% larger using the procedure proposed in this study. This translated itself in metaphases with 45–70% increased surface. For the blood culture, where the chromosomes are longer, the number of all crossovers was also calculated. There were much fewer crossovers/metaphases when using the proposed procedure (1.4) versus the standard laboratory procedure (4.3). The standard deviation (SD) provided some interesting clues as well. For peripheral blood cells, our proposed protocol resulted in a lower SD, meaning that most metaphases had similar sizes and spreading was more homogenous. As peripheral blood metaphases usually spread better and easier than bone marrow metaphases, this result showed that our protocol increased metaphase spreading uniformly on the entire slide surface. With bone marrow cultures, our protocol resulted in a higher SD compared with the standard protocol. Bone marrow metaphases are generally more difficult to spread. Therefore, there will be a mixture of spread and nonspread metaphases on any slide. Using our protocol, more metaphases spread and acquire larger diameters compared with the standard protocol. However, because our slides still carried nonspread cells, the SD increased.

Another interesting phenomenon is the consistency of the results. Data in Table 1 show that, with both slide preparation protocols, the average diameters and SDs were consistent for the same cell suspension tested. For example, the BM1 culture showed better spread metaphases (larger average diameter) and higher SDs than the BM2 culture. There was also consistency when the various culture flasks of the same tumor were compared. For example, regardless of the spreading procedure, GCT2 culture of BM1 yielded better spread metaphases than the GCT1 and the EB culture. This indicates that spreading is influenced by the way the culture was handled (hypotonic treatment and fixation). Nevertheless, when compared

with the standard protocol, our spreading procedure resulted in the most improved spreading of BM2. As the difference in metaphase diameter between BM1 and BM2 is smaller with our protocol and higher with the standard laboratory procedure, it appears that the technician working with BM1 was more successful than the technician working with BM2. In other words, human errors influence the degree of metaphase spreading, even when, theoretically, the same standard protocol is in use in the laboratory.

Dropping Cells From a Height Does Not Improve Spreading

If the drying process is observed at the phase microscope (16), five arbitrary steps can be described: (1) When the cell suspension spreads on the slide, cells float wildly in all directions but ultimately touch the glass surface and immediately become immobile. No chromosome spreading takes place. Cells look like small gray spheres (Fig. 3a). (2) Fixative starts to dry. As its surface touches the cell surfaces, cells reflect the light and acquire a bright halo. Chromosomes do not spread at all. Macroscopically, the surface of the slide becomes grainy. (3) The mitotic cells lose their halo and flatten (chromosome spreading) faster than the nonmitotic cells. The chromosomes become dark and visible; most of the chromosome spreading is achieved at this step (Fig. 3b). Nonmitotic cells still show the halo of light, indicating that the nuclear membrane/content is much more resistant to the flattening force of the drying fixative. (4) As the fixative is drying, nonmitotic cells continue to flatten, whereas chromosome spreading increases just a little more (less than 7% change in the diameter of various metaphase spreads). (5) Tiny puddles of fixative surround each cell but quickly evaporate. Visible chromosome spreading stops, but minor changes may still take place (Fig. 3c). The drying process is the same, regardless of the height from which the cell suspension

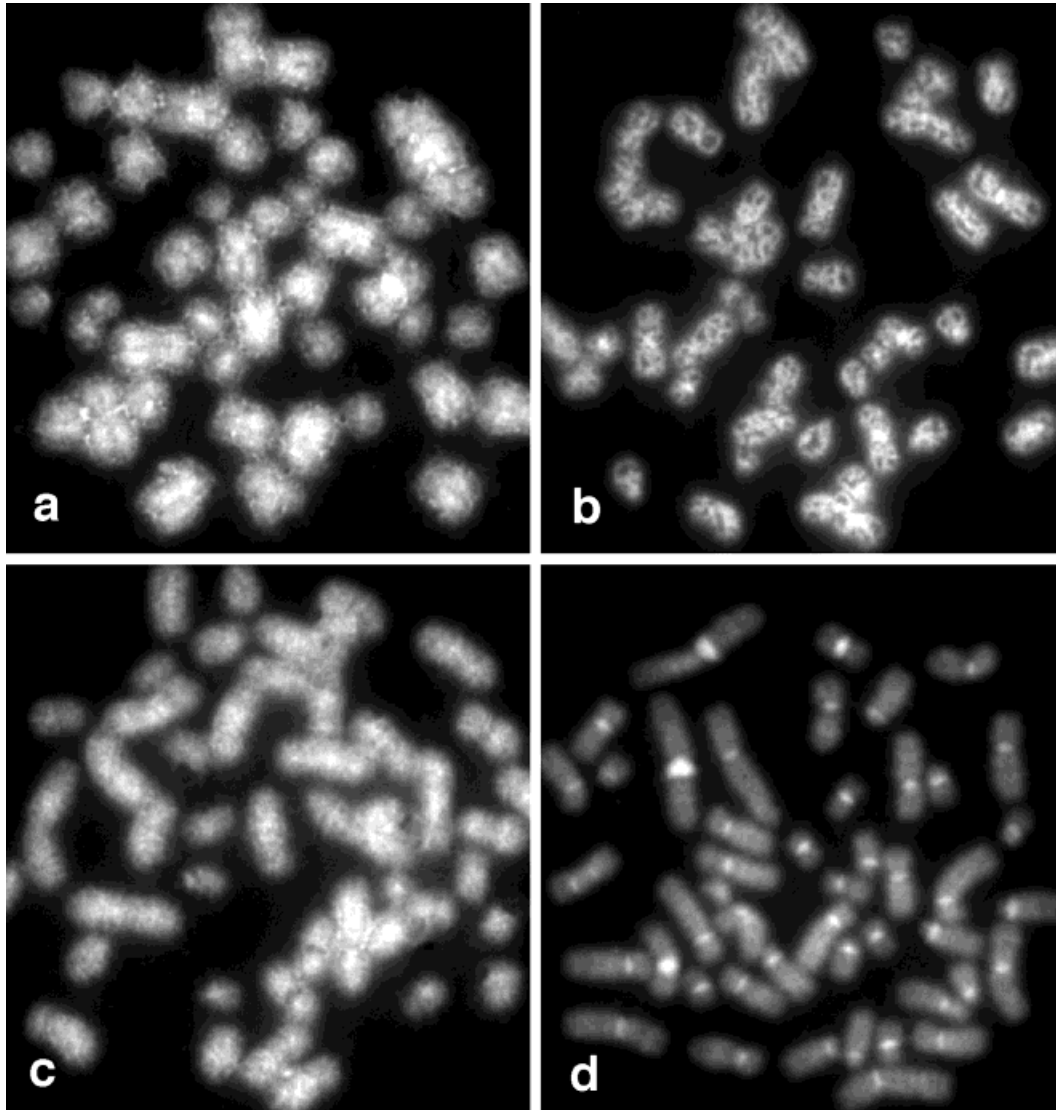


FIG. 5. **a–c:** Simultaneous denaturing using hybridization buffer containing 12–15% dextran sulfate and different brands of formamide. Slides were chemically aged 10 s at 94°C on a thermocycler block and stained with DAPI. Lymphocytes are depicted (peripheral blood culture). **d:** Another slide from the same lot as above was subjected to separate denaturing for 2 min in a Coplin jar containing 70% FA/2 × SSC at 75°C (same formamide as in Fig. 2c). The absence of dextran sulfate results in less distorted chromosomes. However, the brightly stained centromeres indicate overdenaturing of the chromosomes, yielding a pseudo C-banding aspect. Also compare with Figure 6c, where separate denaturing using the same formamide brand was done gradually, on the metal block of a thermocycler.

was dropped, indicating that height does not influence chromosome spreading. It may only help to distribute the cells more evenly on the slide surface. Actual spreading takes place later, when the surface of the slide becomes grainy. This is the critical step in which spreading can be helped by hot steam and acetic acid.

GTG-Banding

GTG-banding on a variety of cell types tested worked well on slides prepared with our new procedure. Our slides had an increased number of analyzable metaphases, with larger diameters, and fewer chromosome crossovers (Table 1). The more efficient spreading was accompanied

by reduced cytoplasmic residua, which contributed to the good quality of G-banded preparations (Fig. 4) on an increased number of metaphases. The brief acetic acid exposure did not appear to alter the sharpness of the Giemsa bands. On any slides, trypsin time can be varied. As a rule, longer trypsin incubation increases the thickness of the chromosomes and requires longer Giemsa-stain incubations in order for the chromosomes to become banded.

FISH: Chemical Aging

Aging of cytogenetic preparations denatures the proteins, removes water and fixative, and enhances the ad-

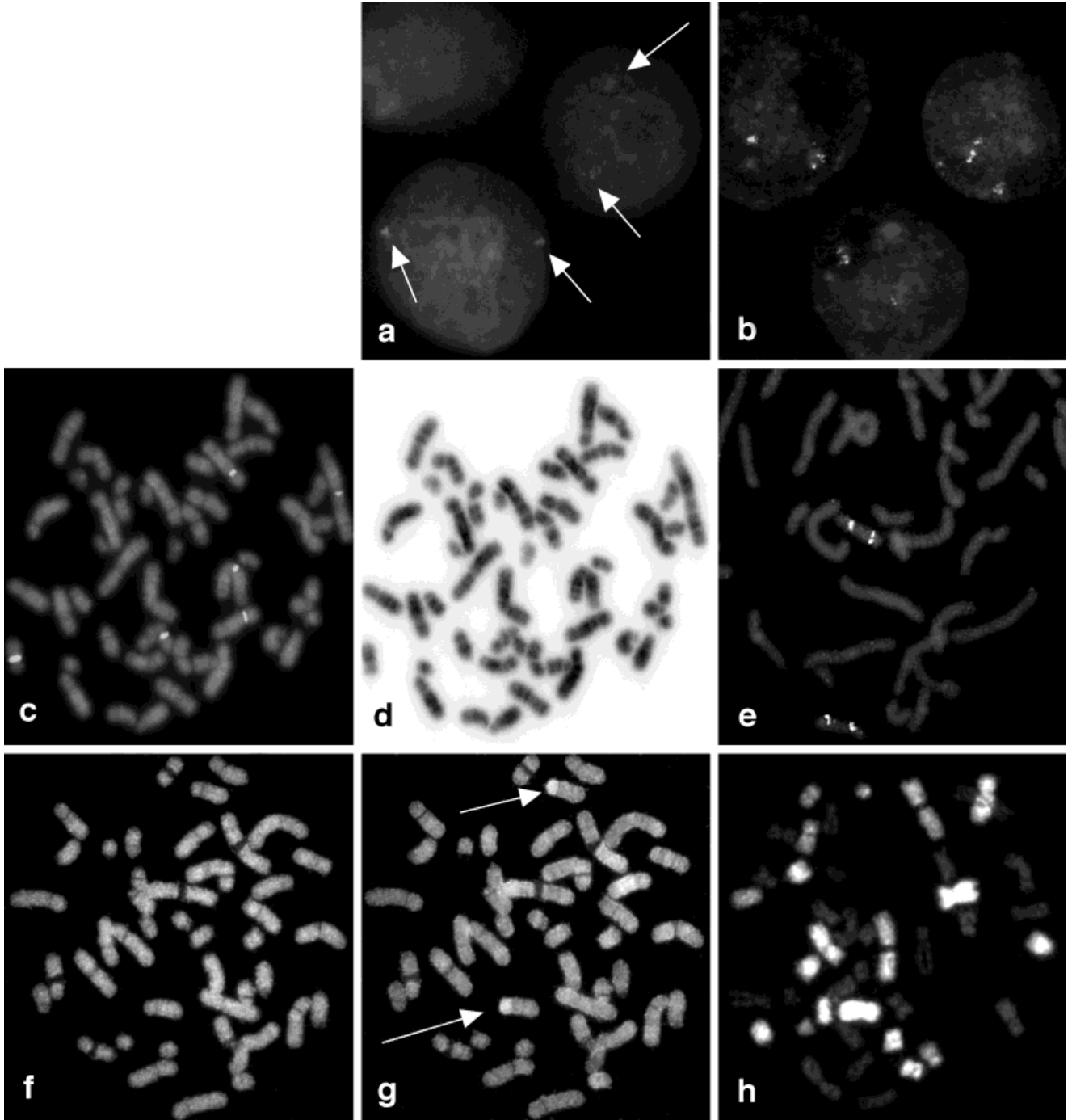


FIG. 6. **a,b:** Ten-minute hybridization of a probe for the alpha satellite repeat of chromosome 1, on cells chemically aged for 2 min. Arrows indicate weaker hybridization signals (a, no pepsin treatment) than in Figure 6b (pepsin treated). **c,d:** Twenty-minute hybridization of a biotinylated commercial probe for chromosome 1 (Oncor), detected with avidin FITC (c). The slide was subjected to 10 s chemical aging and separate denaturing on the metal block of a thermocycler. The gray-scale DAPI image of the metaphase shown in Figure 6c was inverted using Adobe Photoshop (d). Chromosomes 1, 5, and 19 (carrying hybridization signals) are identifiable. **e:** Ten-second chemical aging and separate slide denaturing on the metal block of a thermocycler followed by 5 h of hybridization using a commercial diagnosis probe (Oncor, Gaithersburg, MD) for Prader-Willi syndrome. **f,g:** Ten-second chemical aging and simultaneous denaturing of a CGH analysis on the metal block of a thermocycler followed by 18 h of hybridization. Normal DNA was labeled with digoxigenin and detected by antidigoxigenin-FITC antibody (f). Tumor DNA (testicular germ cell tumor) was biotin labeled and detected with Avidin Cy3 (g). Arrows show the typical 12p amplification characteristic for this tumors. Although the hybridization was even and of good quality, note the thickness of the chromosomes. This was mostly due to the presence of the dextran sulfate in the hybridization buffer during the direct denaturing phase. **h:** Ten-second chemical aging and separate slide denaturing on a metal block of a thermocycler followed by 18 h of hybridization of the FITC-labeled painting probes of an M-FISH analysis.

herence of the material to the glass. When fresh, nonaged slides are heat denatured, they either lose most of the material or chromosomes become distorted (puffy). If slides are extensively aged, hybridization efficiency decreases because the chromosomes are too hard. Chemical aging was developed as an alternative to dry-heat aging, in order to shorten the FISH protocol and preserve cell freshness (i.e., increases hybridization efficiency) and chromosome architecture (i.e., allows DAPI staining). Chemical reagents (usually alcohols) were used to achieve fixation. In a chemical test, identical slides were subjected to heat pretreatment with methanol, ethanol, isopropanol, butanol, fixative (3:1 methanol-to-acetic acid), or 1% formaldehyde at 37, 65, or 94°C for various amounts of time (from 1 s to 10 min, data not shown). Hybridization results and DAPI staining on all slides indicated that heat treatment in fixative or ethanol at 94°C for 2–20 s worked best. Ethanol was preferred because it was less toxic. The better quality of slides treated by chemical aging translates itself into shorter hybridization times: 15–20 min for centromeres (Fig. 6a,b), 4–5 h for single-copy probes such as cosmids (Fig. 6e), and overnight hybridization for complex probes, as for CGH (Fig. 6f,g) or M-FISH (Fig. 6h).

FISH: Pepsin Pretreatment

Regardless of the aging procedure, slides subjected to protease pretreatment prior to denaturing always showed improved hybridization results (Fig. 5). Pepsin (0.005%) in 0.01 N HCl is convenient because its action is pH dependent and can be immediately stopped by rinsing the slide in a buffer, pH 7–8. Incubation time in pepsin depends on the aging process. Slides chemically aged for 2 min require longer pepsin treatment (1–2 min) than slides chemically aged 10 s (30-s pepsin incubation) to yield similar hybridization results and DAPI banding. By comparison, slides dry-heated overnight at 65°C (as for G-banding) require 10–15 min pepsin treatment. Although DAPI bands are sharper, hybridization quality decreases.

FISH: Simultaneous Versus Separate Denaturing

Numerous experiments with plasmids, cosmids, and painting probes showed that both procedures yielded similar FISH results. The simultaneous-denaturing protocol is shorter and requires a larger amount of competitor DNA (two to three times more) to block repetitive sequences, as there is no preannealing step. The chromosomes become somewhat thicker (puffier), primarily because of the increased viscosity of the hybridization buffer, which usually contains dextran sulfate. To prevent chromosome distortion, the final dextran sulfate concentration should be between 5 and 8%. The shape of the chromosomes also depends on the brand of formamide used (Fig. 5a–c). Depending on formamide quality, the chromosomes may become either excessively thick (Fig. 5a) or with distorted, uneven surfaces (Fig. 5b). When comparing the three images, the best brand of formamide was the one used to denature the slide depicted in Fig. 5c, in which the chromosomes were not as thick as in Fig. 5a and were more evenly stained than in Fig. 5b. In our study,

the best formamide for simultaneous denaturing procedure was obtained from Fluka (Milwaukee, WI). The slide/cover slip assembly can be denatured directly on a simple, heated metal block for 2 min at 75°C. However, gradual heating and cooling using a thermocycler was gentler and better preserved chromosome architecture (superior DAPI banding). In the more common, separate-denaturing protocol, slides are denatured separately from the labeled DNA probe, using a similar gradual heating/cooling scheme as described. Viscosity of the 70% formamide solution prevents water from evaporating, so there is no need to cover the slide/cover slip assembly. Note that for the separate denaturing protocol, there were no visible differences among the brands of formamide tested. Regardless of the denaturing protocol, ethanol rinse solutions should be kept at room temperature to prevent exposure of the slides to large thermal shocks, which are detrimental to the final chromosomal shape.

DAPI Banding

Preservation of chromosome morphology depends on the correct balance among three factors: type of aging, pepsin treatment, and denaturing technique. Dry heat aging provides sharper DAPI bands but decreases hybridization signals compared with chemical aging. Gradual denaturing helps correct this problem, by allowing fresh, “soft” chromosomes to be denatured while preserving their architecture well (Fig. 6c,d). Pepsin treatment, while improving the hybridization results, always alters chromosome architecture. Therefore, the exposure time to the enzyme needs to be adjusted according to the aging and denaturing techniques. A useful trick for better DAPI banding on chemically aged slides is to incubate them for a few seconds in 1% formaldehyde or paraformaldehyde, immediately after pepsin treatment. Even 5–10 s are sufficient. Longer incubations, 30–60 s, yield better bands but visibly alter hybridization efficiency.

CONCLUSION

Appropriate chromosome spreading technique, followed by the use of a short aging process, appropriate protease pretreatment, and gradual denaturing yielded the best hybridization results.

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