Preface

In 1996, we organized a workshop, *inter alia*, at the National Research Council in Milan under the generous sponsorship of the European Science Foundation. On that occasion, a small group of investigators convened from many countries and presented early evidence of the possibility of assembling basic units of mammalian chromosomes into artificial constructs (or, indeed, reducing the relevant components to more manageable dimensions and defined constitution).

Progress in the following years has been slow but steady. Many scientists who took part in the workshop have since been engaged in active and productive research. It goes to the credit of Humana Press to have realized the need for a book on artificial chromosomes that aims to provide better tools to all scientists committed to this field who are confronted with very difficult technical problems.

We have strived to cover in *Mammalian Artificial Chromosomes: Methods and Protocols* all relevant areas of artificial chromosome research, from basic genetics to daring attempts to build new tools for genetic therapy. We are of course grateful to the authors who have accepted the task of describing the technical steps and pitfalls that can be encountered in their research. Rarely has a very delicate methodology been presented with such meticulous care.

We have been helped in this enterprise by the excellent librarian of the LITA Institute in Segrate, Italy, Ms. Claudia Piergigli, whom we thank warmly. Ms. Francesca Tarchi, ITB-CNR secretary, was also helpful. Ivo Castagna and Alberto Ribolla, provided useful technical support. Lastly, we thank Professor John Walker and Craig Adams of Humana Press for their patience and understanding during the preparation of this book.

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Methodologies Applied to Domestic Animal Chromosomes

Leopoldo Iannuzzi

1. Introduction

Chromosomes of domestic animals have attracted the attention of both scientists and breeders because chromosomal abnormalities have been strictly correlated with the reduced fertility in cattle carrying rob(1;29) ***(1)***. Domestic animal cytogenetics has expanded noticeably, extending its interest not only to clinical cytogenetics but also to evolutionary and, more recently, molecular cytogenetics (gene mapping). Chromosomes of domestic animals, especially those of bovids, are very difficult to study because all autosomes of cattle, goats, and dogs, most of them from sheep and river buffalo, and many of them from horses are acrocentric with a decreasing, but similar, size.

Chromosome banding techniques have been largely applied in domestic animals. International chromosome nomenclatures have established standard banded karyotypes for cattle, sheep, goat, pig, horse, river buffalo, and rabbit ***(2–7)***, although problems concern-

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ing some chromosomes, especially for cattle, goat, and sheep, have only recently been solved. Indeed, only when molecular markers were assigned to each cattle and sheep chromosomes ***(8)*** and the same markers were applied on both Q/G- and R-banded cattle chromosome preparations ***(9)*** were Q-, G-, and R-banded standard karyotypes of cattle, sheep, and goat arranged using only one common chromosome nomenclature ***(10)***.

This represents an important point of reference for further studies on domestic bovid chromosomes.

The recent development of molecular cytogenetics also in domestic animals offers another important tool to the cytogeneticists. The use of specific molecular markers, or of chromosome painting probes, and the fluorescence *in situ* hybridization (FISH) technique permit considerable advances in our knowledge of chromosome homologies among related and unrelated species and the straightforward identification of chromosome abnormalities (mainly reciprocal translocations and paracentric inversions) that normally escape the cytogenetic analyses, especially when acrocentric chromosomes are involved.

In this chapter, protocols for blood cell cultures, CBA-, RBA-, RBG-, and GBG-banding techniques, the *in situ* hybridization technique, and signal detection will be described for their easy use on domestic animal chromosomes.

2. Materials

1. Peripheral blood samples are collected by sterile tubes containing sodium heparin (vacutainer system).
2. Mitogen for blood lymphocyte cultures: Concanavalin A (Sigma, C-2010). Dissolve 50 mg Concanavalin A in 50 mL Puck's solution, pH 7.0, then filter with sterile 0.2-micron filter, aliquot in 5-mL sterile tubes or glass flash, and store at –20°C.
3. Physiological solution: Puck's solution 8.0 g/L NaCl, 0.4 g/L KCl, 1.0 g/L glucose, 0.35 g/L NaHCO. Bring to pH 7.0 with 1 *N* HCl.
4. Colcemid for cell cycle block at the metaphase. KaryoMax Colcemid solution (Gibco-BRL, cat. no. 15210-040).

5. BrdU (5-bromodeoxyuridine, thymidine base analog for replicating G and R banding; Sigma B-5002): Dissolve 20 mg BrdU in 20 mL Puck's solution, then filter with a 0.2-micron sterile filter and aliquot in 5-mL sterile tubes. Use 0.2 mL of this solution on 10 mL cell culture to obtain a WS at 20 μg/mL.
6. Methotrexate (MTX; Ametopterine, Sigma A-6770) for cell cycle synchronization. Dissolve 10 mg MTX in 10 mL distilled water (SS1 = 1 mg/mL), then dilute 0.5 mL SS1 in 19.5 mL Puck's solution (pH 7.0), filter with a 0.2-micron sterile filter and aliquot in 5-mL sterile tubes or glass flasks (SS2 = 25 μg/mL). Use 0.2 mL of SS2 in 10 mL cell culture to arrive at a final WS of 0.5 μg/mL. Store both SS1 and SS2 at –20°C.
7. Ethidium bromide (EB, Sigma E-8751) for more elongated G-banded chromosomes. Dissolve 20 mg EB in 20 mL distilled water (SS = 1 mg/mL), then filter with a sterile 0.2-micron filter and aliquot in 5-mL sterile tubes. Use 50 μL of SS in 10 mL cell culture to obtain a final concentration of 5 μg/mL.
8. 2X SSC (g/L). NaCl 17.53, 3-sodium citrate 8.82. Bring to pH 7.0 with 1 *N* HCl.
9. Phosphate buffer (P-buffer). Mix 39.0 mL solution **A** (6.95 g of NaH_2PO_4 in 250 mL distilled water) with 61.0 mL solution **B** (35.8 g$Na_2HPO_4.12H_2O$ in 500 mL distilled water).
10. Hoechst 33258 (Bisbenzimide, Sigma B-2883) for staining. Dissolve 10 mg Hoechst 33258 in 20 mL distilled water (SS = 0.5 mg/mL), aliquot in a 1-mL tube and store at –20°C until use. Dilute 1 mL of this solution in 20 mL distilled water for staining (WS = 25 μg/mL) and store at 4°C.
11. Hoechst 33258 (Bisbenzimide, Sigma B-2883) for cell cultures (R banding). Dissolve 20 mg Hoechst 33258 in 10 mL distilled water (SS = 2 mg/mL), then filter with a 0.2-micron sterile filter and aliquot in 5-mL sterile tubes. Use 0.2 mL of SS in 10 mL cell culture to reach 40 μg/mL as WS.
12. Biotin incorporation. BioNick labeling system kit (Gibco-BRL/Life technology, cat. no. 18247-015).
13. Hybridization solution (HS): 5 mL formamide (J. T. Baker, cat. no. 7042), 1 mL 20X SSC, and 2 mL dextran sulphate (Sigma, cat. no. D-8906 at 50%) = 8 mL HS. Mix the solution very well, and divide it in aliquots (1 mL each) and store at –20°C until use.

14. Bovine COT-1 DNA for *in situ* suppression of repetitive sequences present in the genomic probes (bovids) (Applied Genetic Laboratory [AGL], Inc., Melbourne, FL).
15. FISH detection kit (FITC–avidin): Oncor, Biotin-FITC kit S1333-BF.
16. FISH detection kit (anti-avidin): Oncor, same kit as for FITC-Avidin.
17. PN buffer for posthybridization washing buffer: 13.8 g/L NaH_2PO_4 (0.1 *M*), 35.8 g/L Na_2HPO_4 (0.1 *M*), Nonidet P-40 (0.1%). Bring the solution to pH 8.0 with 5 *N* NaOH.
18. Antifade (100 mL): 0.1 g 1,4-phenylendiamin (Sigma, cat. no. P-6001), PBS [NaH_2PO_4 (0.2 *M*) + Na_2HPO_4 (0.2 *M*) + NaCl (0.15 *M*)] 10 mL, glycerol (Rudi Pont, cat. no. 17500-11) 90 mL. Aliquot in 10-mL tubes and store at –20°C.
19. Antifade/Hoechst 33258 (2 µg/mL) solution: Antifade 50 mL, H-33258 0.2 mL from SS at 0.5 mg/mL (2 µg/mL, final concentration). The Oncor kit Biotin-FITC S1333 also contains antifade and antifade/propidium iodide solution.

3. Methods

3.1. Normal Cell Cultures

1. Add 0.8–1.0 mL peripheral blood sample to a 15-mL sterile tube or a 50-mL sterile flash (the same as that used for fibroblast cell cultures) containing 8.0 mL of TC medium (McCoy's 5A modified or RPMI 1640, Gibco), 1.0 mL of inactivated (at 56°C for 30 min) fetal or bovine calf serum, Concanavalin A (15 µg/mL, final concentration), penicillin/streptomycin (0.1 mL), L-glutamine (0.05 mL when present in the medium, 0.1 mL when not), and one drop of sterile sodium heparin (this prevents coagulation problems). Other mitogens, such as the Pokeweed or the PHA, can be used instead of Concanavalin A. However, the latter offers the best results as mitogen and is cheaper than Pokeweed and PHA. Only for horse and donkey cell cultures, Pokeweed mitogen must be preferred to the Concanavalin A.
2. Store cell cultures at the 37.8°C in a normal incubator or at 37.5°C in a CO_2 incubator (with CO_2 at the 4.5%). When using tubes, keep them with the highest inclination to improve cell growth.
3. Gently agitate cell cultures once a day.
4. Add 20–50 µL Colcemid (depending on species and expected chromosome contraction) 1 h before the harvesting (*see* **Note 1**).

5. Top spin at 1200*g* for 8 min, remove the supernatant, and add KCl 0.75 *M* (0.56 g %) drop by drop to arrive at 2 mL by shaking the tube gently. Mix cells thoroughly by using Pasteur pipet, and then add more solution to arrive at 14 mL. Mix cells with a Pasteur pipet and store the cell suspension at 37°C for 20 min. Then, add 1 mL of fix solution (FS) (acetic acid/methanol 1:3) and mix (*see* **Note 2**).
6. Top spin at 1000*g* for 10 min, remove the supernatant, and add (drop by drop) 2 mL FS. Then, mix thoroughly with a Pasteur pipet (be sure to break down cell clusters when present) and add more fix solution to arrive at 10 mL. Mix with a Pasteur pipet and store at room temperature for 20 min (*see* **Note 2**).
7. Top spin at 1000*g* and remove the supernatant. Add 5 mL of FS, mix with a Pasteur pipet, and store at room temperature for 10 min.
8. Repeat as in **step 7** and store at 4°C overnight.
9. Repeat as in **step 7**.
10. Repeat as in **step 7** by adding 0.5–1.0 mL fresh FS (the quantity depends on pellet size).
11. Spread two drops of cell suspension on slides previously cleaned with ethanol and immerse in cold distilled water.
12. Air-dry the slides and check cell density with a microscope by using phase-contrast.

3.2. BrdU-Treated Cell Cultures

Follow the protocol as for normal cultures with a few differences.

3.2.1. Late BrdU Incorporation (R Banding)

1. Add BrdU (20 μg/mL final concentration) and Hoechst 33258 (40 μg/mL final concentration) to cell cultures 6 h before harvesting.
2. Add 20–40 μL Colcemid 30–60 min before harvesting (*see* **Notes 1, 3, 4, 5, 6, 7**).

*3.2.2. Early BrdU Incorporation (G Banding) for Cattle, River Buffalo, Horse, and Donkey **(11,12)***

1. Add BrdU (20 μg/mL, final concentration) and MTX (0.5 μg/mL, final concentration) to the cell cultures 20–22 h before harvesting (afternoon).

2. Top spin cell suspension at 1200*g* after 16–18 h (early morning) and eliminate the supernatant.
3. Wash cells once with 15 mL Puck's solution or with the same medium, then spin at 1200*g* for 8 min and remove the supernatant.
4. Add fresh TC medium as in normal cultures containing also thymidine (10 μg/mL, final concentration) and store at 37.5°C (normal incubator) or 37.7°C (CO_2 incubator) for 5.5 h.
5. Add 20 μL of Colcemid 30 min before harvesting (*see* **Notes 8–11**).

3.2.3. Early BrdU Incorporation (G Banding) for Sheep, Goat, Pig, Dog, Rabbit, and Chicken

1. Add BrdU (20 μg/mL, final concentration) to cell cultures 8 h before harvesting (early morning);
2. After 2.5 h top spin at 1200*g*, remove the supernatant and follow the same protocol described above (**steps 3–5**) *(see* **Notes 8–11**).

3.3. Banding Techniques

Several banding techniques are available. I will refer only to those routinely used in my laboratory because they offer high-resolution banding patterns and the protocols are successfully repeatable.

3.3.1. CBA Banding

Use slides obtained from both normal and BrdU-treated cell cultures and stored at room temperature for at least 1 wk. This protocol is a modification of the original Sumner ***(13)*** protocol.

1. Immerse slides in HCl 0.1 *N* for 30 min at room temperature, then wash them with distilled water and air-dry.
2. Immerse slides completely in $Ba(OH)_2$ (5% filtered solution) at 50°C for 20–30 min. We normally use two slides per animal and two different treatment times (20 and 30 min) with $Ba(OH)_2$.
3. Because the slides are covered by $Ba(OH)_2$ solution, aspirate the white coat before to removing the slides or wash the slides directly in the same Coplin jar with tap water, then with distilled water.
4. Air-dry slides at 40°C for 5 min and immerse them in 2X SSC at 60°C for 30 min and then for 15 s in 2X SSC at room temperature.

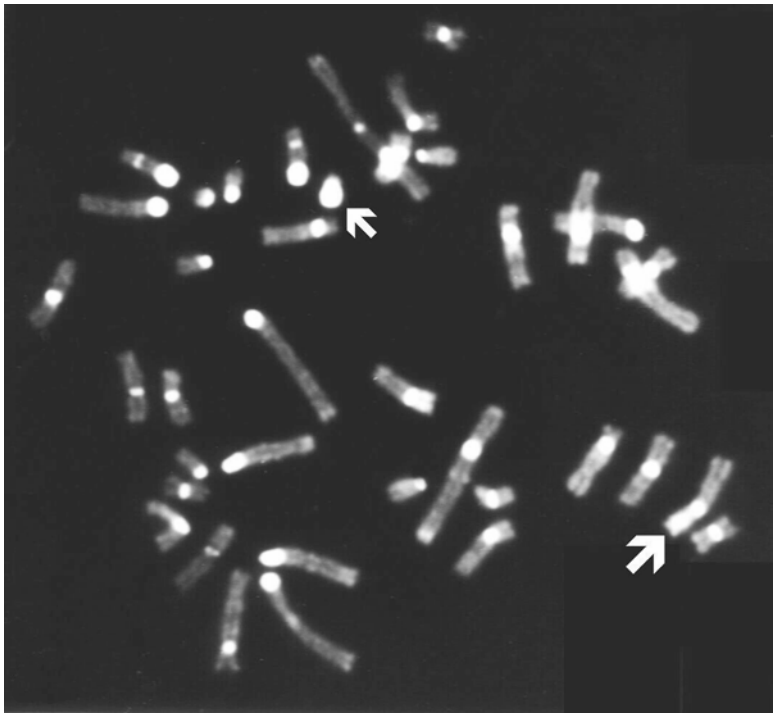

Fig. 1. CBA-banding in a male pig metaphase plate (2n = 38, XY). X (large arrow) and Y (small arrow) chromosomes are indicated. Notice the strong fluorescence (C band positive) in the entire Y chromosome.

5. Dehydrate slides in 75% and 95% alcohol series (3 min each) and air-dry.
6. Stain with acridine orange (0.1% in P buffer, pH 7.0) for 1 h. Then wash in tap and distilled water and air-dry.
7. Mount slides in P buffer with glass coverslip, press coverslip with paper to eliminate the excess of buffer, and seal with rubber cement.
8. Microscope observation a day later with appropriate filters (excitation filters at the 450–490 nm) **(Fig. 1**) *(see also* **Notes 3** and **4**).

3.3.2. RBA Banding

Stain slides obtained from late BrdU-incorporation cultures with acridine orange (0.1% in P buffer, pH 7.0) for 10 min and continue

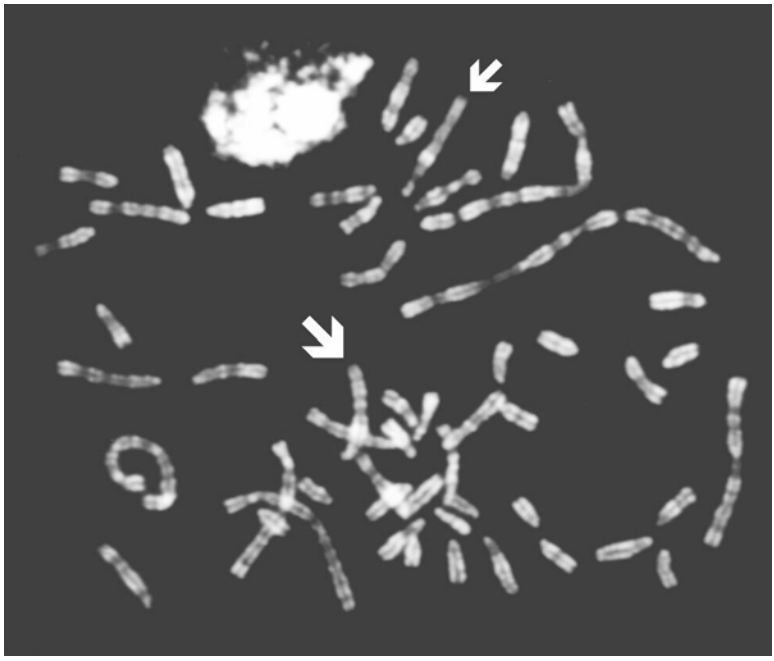

Fig. 2. RBA-banding in a female sheep early-metaphase plate (2n = 54, XX). Early (large arrow) and late (small arrow) replicating X chromosomes are indicated.

as for CBA banding (**steps 6–8**): fluorescence R banding will be performed (**Fig. 2**) *(see* **Notes 5–7**).

3.3.3. RBG Banding

1. Stain 1-wk-old (or more) slides with Hoechst 33258 (25 µg/mL in distilled water) for 20 min. Then, wash slides with distilled water and air-dry at 40°C for 10 min.
2. Mount slides with 1 mL 2X SSC (pH 7.0) using coverslip without pressure, then expose slides under UV light for 1 h at the distance of 4–5 cm from the lamp (30-W UV lamp). Wash slides with distilled water and air-dry at 40°C for 10 min.
3. Immerse slides in 2X SSC (pH 7.0) at 60°C for 30 min, then in 2X SSC at room temperature for 15 s.

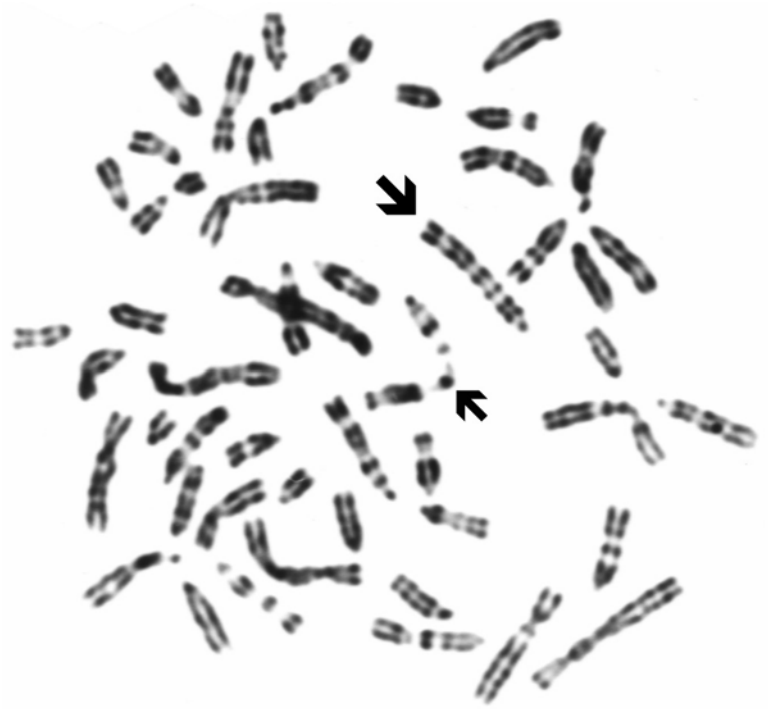

Fig. 3. RBG banding in a female river buffalo early-metaphase plate (2n = 50, XX). Early (large arrow) and late (small arrow) replicating X chromosomes are indicated.

4. Wash slides with tap and distilled water and air-dry. Then stain with Giemsa (8% in P-buffer, pH 7.0) for 30 min.
5. Microscope observation 1 d later without coverslip when slides are used for other banding techniques (C banding or Ag-NORs) or with coverslip by using Eukit as mounting slides (**Fig. 3**) *(see* **Notes 5–7**).

3.3.4. GBG Banding

Use slides from cultured treated with early BrdU incorporation and follow the same protocol used for RBG banding. Replicating G-banding patterns will be obtained (**Fig. 4**) *(see* **Notes 8–11**).

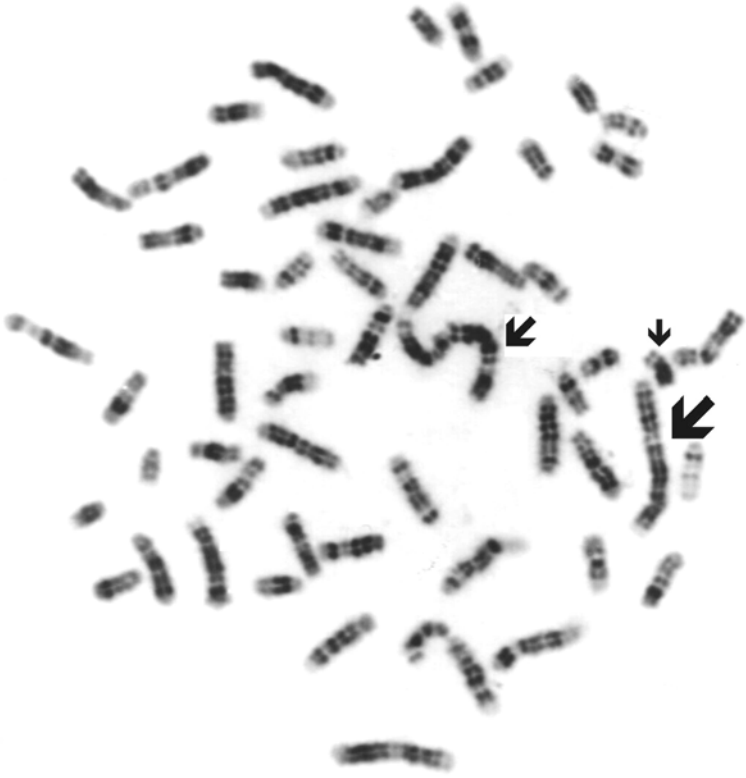

Fig. 4. GBG banding in a male cattle early metaphase plate [2n = 59, XY, rob(1;29)]. The translocated chromosome (large arrow), as well as X (medium arrow) and Y (small arrow) chromosomes are indicated.

3.4. Fluorescence In Situ *Hybridization (FISH)*

3.4.1. Biotin Incorporation and Probe Precipitation

Biotin-14-dATP is incorporated into 1 µg probe DNA (generally cosmids or BAC-clones) by Nick translation. Pipet the following components into a sterile 1.5-mL microcentrifuge tube on ice.

1. A quantity (µL) of probe DNA to arrive at 1 µg probe DNA, 5 µL of 10X dNTP mix, and sterile water to arrive to 45 µL and 5 µL enzyme mix (DNA Polymerase I and DNase I).

2. Close the tube, mix well, centrifuge for a few seconds at 12,000*g*, and incubate at 16°C for 1 h, then add 5 µL of stop buffer.
3. Add 100 µL of sonicated salmon sperm and then 15 µL of sodium acetate (2.5 *M*).
4. Mix well and add 300 µL cold –20°C ethanol (95%), mix well, and top spin (13,000*g*) for a few seconds.
5. Store at –20°C for at least 30 min and top spin at 13,000*g* in a cold centrifuge for 20 min.
6. Eliminate the supernatant and add 500 µL 70% cold ethanol.
7. Wash the pellet and top spin at 13,000*g* for 10 min.
8. Eliminate the supernatant and carefully air-dry the pellet (a vacuum pump system may be useful).
9. Add 33 µL hybridization solution (HB) to obtain a probe concentration at 30 ng/µL, and dissolve the pellet very well by using vortex.
10. Top spin at 13,000*g* for a few seconds and store the probe at –20°C until use (can be stored for up to 1 yr or more).

3.4.2. Probe Denaturation

1. For each *in situ* hybridization, prepare 11 µL probe DNA containing 4 µL probe stock (about 120 ng probe DNA), 6 µL HS and 1 µL bovine COT-1 DNA (for bovids only) or 1–2 µL total genomic DNA of species from which the genomic probe was prepared (*see* **Note 12**).
2. Mix with vortex, then centrifuge for a few seconds at 13,000*g*.
3. Immerse the tube containing the probe in water at 72°C for 15 min.
4. Immerse the tube containing probe DNA in water at 37.5°C for 1 h (annealing step) to suppress the repetitive sequences with bovine COT-1 DNA (or with total genomic DNA).

3.4.3. Chromosomal (Slide) Denaturation

1. Select good slides (good mitotic index and chromosome contraction) and the best slide area by recording the data (approx 2 × 2 cm) with the phase-contrast microscope.
2. Stain with Hoechst 33258 by following the same procedure as reported in **Subheading 3.3.**, **steps 1** and **2** (RBG banding).
3. Immerse slides in 70% formamide/2X SSC solution (pH 7.0) for 2.5 min at 72°C (we use two different glass Coplin jars, alternatively, to maintain the temperature at 72°C).

4. Immerse slides in an alcohol series (70%, 80%, and 96%) for 2 min each at 4°C (we keep the Coplin jar in ice) and quickly air-dry with air flush.
5. Mark the slide area (selected during **step 1**) of the *in situ* with an indelible pen (front side).

3.4.4. In Situ *Hybridization*

1. Apply the denaturated probe DNA mixture on the marked area of slides. It is preferable to achieve this step by combining probe DNA denaturation (and annealing step) with slide denaturation.
2. Mount slides with a glass coverslip (2 × 2 cm) without pressure, seal them with rubber cement and allocate slides in a moist chamber (Petri dish) and store at 37°C for 3 d (we generally hybridize during the weekend).

3.4.5. Signal Detection and R Banding

1. Remove coverslip and wash slides in three Coplin jars containing formamide/2X SSC (1:1), pH 7.0, at 42°C for 5 min each. Generally, the first washing is necessary to remove the coverslips only (let the coverslips be removed by themselves by gently washing the slides), and the following two washings are used to remove the nonspecific binding probe DNA.
2. Wash slides in 2X SSC, pH 7.0, at 39°C and in 2X SSC at room temperature (2 min each).
3. Wash slides in three PN buffer series (2 min each).
4. Add 20 µL FITC–avidin on the marked slide area (2 × 2 cm) and mount with plastic coverslip (2.5 × 2 cm) (pieces of parafilm can also be used) without pressure.
5. Incubate slides at 37.5°C in a moist chamber for 30 min, then repeat **step 3**.
6. Add 20 µL of anti-avidin antibody and repeat as in **step 4**.
7. Incubate slides at 37.5°C in a moist chamber for 30 min, then repeat **step 3**.
8. Add a second layer of FITC-avidin as in **step 4**.
9. Repeat **step 3** and mount slides with glass coverslips (24 × 56 cm) by using one large drop of Antifade/Hoechst 33258 solution.

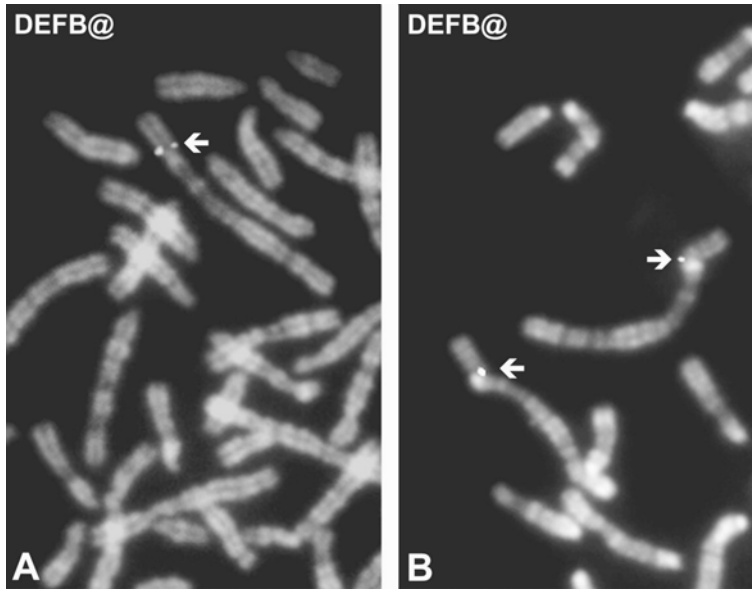

Fig. 5. Details of river buffalo prometaphase plates treated for FISH technique with a bovine BAC clone containing DEFB@. Simultaneous visualizations of the hybridization FITC signals (arrows) with RBH banding **(A)** or with RBA banding **(B)** are shown.

10. Press coverslip with paper to eliminate an excess of mounting solution.
11. Microscope observation with appropriate filter combinations with excitation filters at 340–380 nm for RBH banding (R banding by late BrdU incorporation and Hoechst 33258 staining) and at 450–490 nm for FITC signals (**Fig. 5A**).

When RBH banding is poor, slides can be counterstained with acridine orange (RBA banding) to enhance the R banding (**Fig. 5B**) by following this protocol:

1. Gently remove the coverslip and wash slides in a series of three PN buffers stored at 42°C (5 min each) and in PN buffer at room temperature (5 min), then wash slides in tap and distilled water.
2. Dehydrate slides in alcohol series (70%, 80%, and 96%) (5 min each) and air-dry.

3. Stain with acridine orange (0.1% in P buffer, pH 7.0) for 30 min, then wash slides in tap and distilled water and air-dry.
4. Mount slides in P buffer and then follow as for CBA banding (**steps 7** and **8**).
5. Microscope observation 1 d later with the same filter combination used for FITC signals (*see* **Notes 13–15**).

4. Notes

1. Colcemid treatment needs careful attention. Indeed, domestic animal chromosomes are very sensitive to this treatment. The reduction in time and quantity allow more elongated chromosome preparations to be obtained but give a lower mitotic index. Each laboratory should get the appropriate dose and time of treatment on the basis of its own needs.
2. The first fixation of cells is very important and needs special attention. When cell clusters are present, they must be broken by using a Pasteur pipet against the bottom of the tube. However, the adding of 1 mL fix solution after the hypothonic treatment not only blocks the KCl action but prevents the formation of cell clusters.
3. When observing the slides in the microscope, the C bands (CBA banding) must be very strong fluorescence, whereas the remaining part of the chromosomes must be dull fluorescence (**Fig. 1**). When other chromosome regions appear stained (fluorescent), the time of treatment in $Ba(OH)_2$ must be increased. The opposite when the C bands are very small and the chromosomes appear overtreated. When the slide treatment is good, also the nuclei show clear strong fluorescence heterochromatic regions.
4. Giemsa staining in C-banding technique (CBG banding) can be used instead of acridine orange by following the same protocol, although acridine orange is more effective than Giemsa staining. Indeed, it is possible to detect very small C bands (biarmed autosomes) or intercalar C bands, which are generally C-band negative when using Giemsa staining ***(14,15)***. Furthermore, CBA-banding technique is more repeatable than CBG banding. C banding is the best banding technique to identify sex chromosomes very easily in domestic animals because their C-banding patterns differ completely from those of the autosomes ***(14,15)***. Therefore, this technique is very useful to detect sex chromosome abnormalities ***(16,17)***.

5. RBA banding (**Fig. 2**) offers the advantage of simplicity and its use on fresh slides. Standard RBA-banded karyotypes for cattle, sheep, goat, and river buffalo are available ***(3,4,10)***.
6. RBG banding (**Fig. 3**) offers (1) higher banding pattern resolution than RBA banding (more bands compared with those achieved with RBA banding), (2) the possibility of treating slides and working on them later, and (3) keeping slides for years after staining.
7. R banding is the best banding technique to be applied routinely on domestic animal chromosomes in clinical, evolutionary, and molecular (FISH) cytogenetics ***(9,16–23)***. Standard R-banded karyotypes are available for cattle, sheep, goat, river buffalo, horse, and pig ***(3,4,6,7,10)***.
8. GBG banding (**Fig. 4**) is very useful when chromosomes of species must be characterized by banding techniques. Indeed, because GBG-banding patterns are exactly complementary to those obtained by R banding and are very similar to those obtained by GTG banding, this technique is a point of reference when structural G bands (GTG banding) must be compared with R-banding patterns.
9. The comparison between GBG banding with other banding techniques (GTG, RBA, and RBG banding) allowed better characterization of domestic bovid chromosome so as to obtain clear and detailed G- and R-banded ideograms following only one common banding nomenclature ***(24–26)***. The only problem with this technique is that it requires early BrdU incorporation, which takes time during cell cultures, especially when BrdU is added to the cell cultures in the morning and removed later.
10. In both normal cultures and early BrdU-treated cell cultures (GBG banding), longer chromosomes can be obtained by adding ethidium bromide (5 μg/mL) 2 h before harvesting.
11. When BrdU and MTX are simultaneously added to cattle, river buffalo, horse, and donkey cell cultures to obtain GBG banding, the partial block of the cell cycle (synchronization), because of MTX, occurs during the S phase ***(11,12)***. Standard GBG-banded karyotypes for cattle, sheep, goat, and river buffalo are available ***(4,10)***.
12. When observing FITC signal backgrounds during FISH technique, the quantity of COT-1 DNA (or the total genomic DNA) should be increased during the *in situ* procedure (annealing step).
13. For FITC signal acquisition, the use of appropriate charge-coupled device (CCD) cameras connected with the microscope is preferred

to microphotography. Indeed, metaphases can be captured and later processed with appropriate software. Several CCD cameras are currently available. These cameras capture the images in black/white or directly in color. Generally, the former are more sensitive and pseudo-color can be given after image capturing.

14. Generally, two images of the same metaphase (with FITC signals and with RBH banding) are separately captured after the detection step and later processed by superimposing the hybridization signals on R-banded chromosomes (**Fig. 5**). Several software programs for FISH technique (image acquiring and processing) are available.
15. After the FISH-detection step, antifade/propidium iodide can be used, instead of antifade/Hoechst 33258, to obtain FITC signals against the red chromatids (propidium iodide). In R-banded preparations, the use of antifade/propidium iodide allows FITC signals to be obtained with the filter at 450–490 nm (excitation) and RBPI banding with excitation filters at 515–560 nm. However, modification of this procedure (antifade/propidium iodide at pH 11) allows simultaneous visualization of FITC signals and R-banding patterns with the combination filters at the 450–490 nm (excitation) ***(9,21)***.
16. The FISH technique is a powerful tool to physically map specific loci on single chromosome bands. When two or more loci are clustered in the same bands, dual or multicolor FISH may be used ***(27)***.
17. Comparative FISH mapping among species may reveal small autosomal mutations, such as that found between Bovinae chromosome 9 and Caprinae chromosome 14 ***(28)***. A greater understanding may also be gained of chromosome evolution among related species by following the gene order within homologous chromosomes ***(23,29,30)***, as well as comparing unrelated genomes such as those of human and bovids by establishing the chromosomal rearrangements that differentiated bovids from primates ***(27,28,31–33)***. The use of specific molecular markers is also a potential tool to easily identify chromosomes involved in both numerical ***(34)*** and structural chromosome abnormalities ***(35–37)***.

5. Conclusions

Domestic animal cytogenetics should receive special attention, especially now that several molecular markers are available and can be used in both clinical and evolutionary cytogenetics. However,

more work must be done to prepare chromosome-specific painting probes and make them commercially available as for humans. This will facilitate comparisons among unrelated species (Zoo-FISH) and will accelerate the genetic improvement in domestic species because chromosomal abnormalities, especially reciprocal translocations and paracentric inversion, can easily be identified and eliminated from the animal populations. Sound collaboration among breeders, veterinary practitioners, and cytogeneticists is also essential if our domestic animal populations are to be genetically improved.

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