Chapter 33

Fluorescence In Situ Hybridization on Early Porcine Embryos

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Abstract

Insight into the normal and abnormal function of an interphase nucleus can be revealed by using fluorescence in situ hybridization (FISH) to determine chromosome copy number and/or the nuclear position of loci or chromosome territories. FISH has been used extensively in studies of mouse and human early embryos, however, translation of such methods to domestic species have been hindered by the presence of high levels of intracytoplasmic lipid in these embryos which can impede the efficiency of FISH. This chapter describes in detail a FISH protocol for overcoming this problem. Following extensive technical development, the protocol was derived and optimized for IVF porcine embryos to enable investigation of whole chromosome and subchromosomal regions by FISH during these early stages of development. Porcine embryos can be generated in-vitro using semen samples from commercial companies and oocytes retrieved from discarded abattoir material. According to our method, porcine embryos are lyzed and immobilized on slides using Hydrochloric acid and "Tween 20" detergent, prior to pretreatment with RNase A and pepsin before FISH. The method described has been optimized for subsequent analysis of FISH in two dimensions since organic solvents, which are necessary to remove the lipid, have the effect of flattening the nuclear structure. The work in this chapter has focussed on the pig; however, such methods could be applied to bovine, ovine, and canine embryos, all of which are rich in lipid.

Key words: Fluorescence in situ hybridisation, Porcine embryos, Genome organisation, Lipid

1. Introduction

The normal functioning, or "nuclear health" of an interphase nucleus, including appropriate regulation of gene expression is related both to chromosome copy number and the spatio–temporal organization of the chromatin and associated proteins (termed "nuclear organization" or "genome organization") (Foster and Bridger 2005). A critical stage of development where gene regulation

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needs to be tightly controlled temporally is in the preimplantation stages of early embryo development when master regulator genes are being switched on and off in a highly coordinated manner. By implication, genome organization may similarly be tightly regulated at this developmental timepoint, both temporally and in three-dimensional space. Study of genome organization is considered to be fundamental to our understanding of the earliest stages of development; moreover, analysis of chromosome copy number in preimplantation embryo nuclei can provide insight into the origin of mosaicism, a common phenomenon leading to pregnancy complications and specific disease phenotypes such as Prader-Willi syndrome (1). While access to the early embryos of oviparous animals is relatively straightforward, in mammals it is complicated by the fact that fertilization and subsequent development usually occur internally. Embryos from in vitro-fertilization (IVF) provide the opportunity to circumvent this problem, and some studies have been able to assess genome organization in preimplantation human development (2–4). However, due to ethical constraints surrounding the use of human IVF embryos for research, such work has thus far been limited to the analysis of FISH preparations previously designed to determine chromosome copy number. The loci examined are therefore commonly limited to centromeres used for detection of aneuplopidy and requiring three-dimensional extrapolations from FISH procedures performed on flattened (2D) nuclei. In model organisms, some studies have been able to determine directly the 3D organization of the interphase nucleus preimplantation embryos of mammalian model organisms such as mouse (5) and cattle (6), however, such studies are in their infancy.

In many areas of scientific enquiry, the pig has been reported as an important model organism for human disease and genomics (7). Unlike the mouse, it is similar to humans in size and physiology (7); for this reason, it is the primary candidate for xenotransplantation studies (8). Many traits studied in detail for agricultural reasons (e.g., fatness, disease resistance, and fertility) are common issues in human health and since pig meat is the most commonly eaten in the World (9), material from most porcine cell types is relatively easy to obtain from abattoirs. From both a chromosomal and sequence-based standpoint, pigs are much more closely related to humans than mice (10) and, for all the above reasons, the sequencing of the porcine genome is near completion at the time of writing:

http://www.pre.ensembl.org/Sus_Scrofa/Info/Index
http://www.animalgenome.org/pigs/, http://www.ncbi.nlm.nih.gov/projects/genome/guide/pig/, http://www.sanger.ac.uk/Projects/S_scrofa/, http://www.projects.roslin.ac.uk/pigmap/, http://pigenome.nabc.go.kr/ (11).

Although the study of chromosome copy number and genome organization of preimplantation mammalian embryos is impor-

tant, there remain certain technical issues, particularly when working with porcine embryos. With this in mind, the purpose of this chapter is to describe how IVF may be used to produce porcine preimplantation embryos, the nuclei from which may be fixed to glass slides in order to perform FISH.

2. Materials

2.1. In Vitro Porcine Embryo Production

Unless otherwise stated, all chemicals were sourced from Sigma-Aldrich UK

1. An 18.5-gauge needle.
2. A 10 mL disposable syringe.
3. Pig ovaries acquired from an abattoir (see Note 1).
4. Holding Medium – Tissue Culture Medium TCM199, supplemented with 5.0 mM $NaHCO_3$, 15.0 mM Hepes (Na salt/free acid) (Merck Biosciences Ltd), 0.05 g Kanamycin Sulphate/L 0.4 g Bovine Serum Albumin/L (Fraction V), and 0.04 g Heparin/L. This should be prewarmed to 39°C.
5. Oocyte maturation medium (TCM199 plus 0.1% (w/v) Poly (Vinyl alcohol), average mol wt 30,000–70,000 containing 0.5 μg porcine FSH mL/L, 0.5 μg porcine LH mL/L, 0.57 mmol cysteine, and 10 ng epidermal growth factor (EGF) mL/L).
6. Mineral oil, embryo culture tested.
7. 0.1% (w/v) hyaluronidase in 0.9% NaCl.
8. In vitro fertilization medium (IVF), medium – modified Tris-buffered medium (mTBM) (12); supplemented with 2.5 mM caffeine and 0.4% (w/v) BSA.
9. CO_2 Incubator.
10. Dissecting stereomicroscope.
11. Porcine Spermatozoa (see Note 2).
12. Bench top centrifuge.
13. 45%: 90% Percoll gradient (GE Healthcare Bio-Sciences AB (Uppsala)) – (see Note 3).
14. NCSU23 + 0.4% Bovine Serum Albumin (essentially fatty acid free) medium for embryo culture (13).
15. Bench-top Vortex.

2.2. Fixation and Preparation of Early Porcine Embryos for FISH

1. Hydrochloric Acid (HCl).
2. Tween-20.
3. Phosphate-buffered saline tablets.
4. Pulled glass pipettes – (see Note 4).
5. Poly-L-lysine slides.

6. Dissecting microscope (Olympus SZX 7 stereo microscope system) with a 39°C heated stage.
7. Diamond pen.
8. Ethanol series (70%, 90%, and 100%).
9. Pepsin.
10. RNAse A.
11. Sodium saline citrate (SSC).
12. Spreading solution (0.1% Tween 20, 0.01 N HCl).

2.3. Fluorescence In Situ Hybridization of Porcine Chromosomes in Nuclei of Early Porcine Embryos

1. Flow sorted porcine chromosome paints – (see Note 5).
2. Porcine genomic DNA – (see Note 6).
3. Herring sperm DNA.
4. 3 M Sodium Acetate.
5. High quality ethanol.
6. Hybridization mixture (50% formamide, 10% dextran sulphate, 2× Sodium Saline Citrate (recipe), and 1% Tween-20).
7. Water baths.
8. Hot block.
9. Glass coverslips 18 × 18 mm.
10. Rubber cement.
11. Humidified container.
12. Formamide.
13. 2×SSC (0.15 M Sodium citrate, 0.1 M NaCl).
14. Tween 20.
15. Bovine serum albumin (BSA).
16. Distilled H_2O.
17. Strepavidin-cyanine 3.
18. Vectashield containing 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Bethesda).

3. Methods

Porcine embryos contain large amounts of lipid, mainly in the form of triglyceride (14) meaning that FISH on this material can be particularly challenging if not addressed. It is important therefore to remove sufficient amounts of the lipid to avoid impairing the experimental regime. We have pursued a number of methods to improve the visualization of FISH signals in porcine embryos. The method presented in this chapter is adapted from Rooney

and Czepulkowski (15), which gives the best results, but has the effect of flattening the nucleus.

3.1. In Vitro Embryo Porcine Embryo Production

1. Aspirate oocytes from nonatretic antral follicles that are 3–6 mm in diameter using an 18.5-gauge needle attached to a disposable syringe containing 2 mL of prewarmed Holding Medium (see Fig. 1). The needle should puncture the follicle and the contents are removed by filling the syringe.
2. Pass the contents of the aspirated fluid through a 70 μm cell strainer (Falcon), emptied into a 9 cm Petri-dish and oocyte-cumulus complexes selected. Select OCCs (see Note 7), with an intact, evenly granulated ooplasm and a minimum of two layers of cumulus cells. Wash the OCCs twice in Holding Medium without heparin and a further three times in oocyte maturation medium (16).
3. Culture groups of 50 OCCs in 100 μL droplets of oocyte maturation medium previously covered in mineral oil and preequilibrated in air with 5% CO_2 in air for 40–44 h.
4. Prior to IVF, add 2 μL 0.1% (w/v) hyaluronidase to the droplets and incubate at 30°C; this will loosen of the expanded cumulus oophorus.
5. Wash the OCCs in IVF medium three times.
6. Place groups of 35 OCCs in 50 μL droplets of IVF medium, previously overlaid with mineral oil and preequilibrated in a 5% CO_2 incubator at 39°C.
7. Prepare the 45%: 90% Percoll gradient (see Note 3).
8. Thaw frozen spermatozoa in a 45°C water bath for 10 s. Motile spermatozoa are isolated via centrifugation at 1,000 × *g* in a 45%: 90% Percoll gradient for 30 min (17).

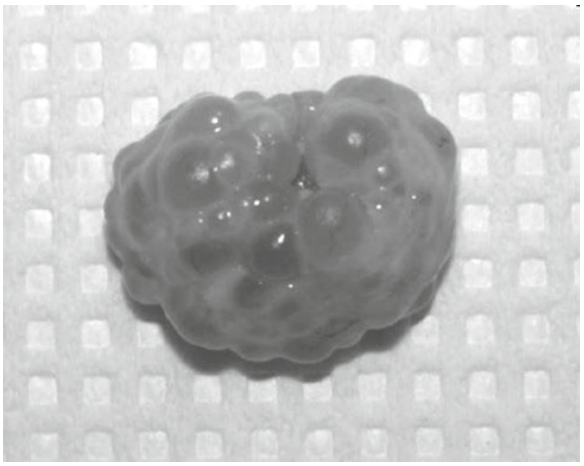

Fig. 1. Abattoir-derived porcine ovary, removed from the reproductive tract.

9. To remove all traces of Percoll, the motile spermatozoa are resuspended in 4 mL pregassed mTBM and centrifuged at $500 \times g$ for 10 min.
10. Resuspend the pellet in mTBM and add 1.5×10^6 sperm to each of the IVF droplets containing the oocytes. Incubate the gametes for 6 h to allow fertilization to occur.
11. Collect presumptive zygotes and place in 1 mL of preequilibrated NCSU23 medium (18), supplemented with 0.4% (w/v) BSA, and vortex for 2 min to detach the spermatozoa and cumulus cells.
12. Wash the putative zygotes twice in preequilibrated NCSU23 + 0.4% (w/v) BSA, prior to culturing in 20 μL droplets of the same medium for 144 h (blastocyst) or 168 h (expanded blastocyst).

3.2. Preparation of Embryos for FISH

The following protocol was developed for porcine embryos involving using acid/Tween 20 prior to pretreatment with RNase A and pepsin for subsequent FISH procedures. It is based on previously published work (2, 4, 15, 19–22).

1. Wash the porcine embryos in 1× PBS prior to treatment.
2. Transfer the embryos using a finely drawn glass Pasteur pipette primed with spreading solution to a drop of spreading solution onto a poly-L-lysine coated slide (see Note 8). Allow the embryos to lyse completely and the lipid contents disperse over a relatively large area of the slide, thus freeing the nuclei from the cytoplasm. Lipid droplets from the lysed embryos clearly appear as black flecks within the spreading solution. This should be observed using a dissecting microscope (e.g., we used an Olympus SZX 7 stereo microscope system using 10× eyepieces and a 40× objective) with a 39°C heated stage. Add fresh spreading solution gently until the embryos are completely lysed (see Note 8).
3. Once embryo lysis occurs, immediately air-dry the embryos at 39°C on the heated microscope stage. The area of embryo lysis should be marked on the slide using a diamond pen.
4. Incubate the slides in PBS for 5 min, then through a 70%, 90%, and 100% ethanol series (5 min each).
5. The slides and adhered embryos can be stored for up to 2 weeks at room temperature in a sealed box containing silica crystals to provide a desiccated environment, or stored at −20°C for up to 1 year.

3.3. FISH on Early Porcine Embryos

The following pretreatment was adapted from van Minnen and van Kesteren, (1999) (23).

1. Incubate slides in 100 μg RNase A, 2× SSC for 1 h at 37°C, and rinse three times in 1× PBS.

2. Incubate the slides in 0.005% pepsin, 0.01 M HCl at 37°C for 15 min.
3. Rinse the slides three times in 1×PBS, dehydrate through a 70%, 90%, and 100% ethanol series, and air-dry.
4. Place 300 ng biotin-16-dUTP-labeled chromosome paint, 50 μg sheared porcine genomic DNA, and 3 μg herring sperm into an 1.5 mL Eppendorf tube and add one-tenth volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol.
5. Incubate at –80°C for at least 1 h.
6. Centrifuge at 15,000×*g* for 30 min in a microfuge and remove supernatant.
7. Wash pellet in ice-cold 70% ethanol and centrifuge for a further 15 min at 15,000×*g*.
8. Remove supernatant and repeat step 7 one more time.
9. Dry the pellet on a hot-block set at 37°C (see Note 9).
10. Dissolve the probe in hybridization mixture at 50°C for a minimum of 2 h before performing FISH.
11. Denature the probe for 3.5 min at 75°C before application to the embryo slides.
12. Seal the probe on the slide with an 18×18 mm coverslip and rubber cement.
13. Place the sealed slide on a hot-block set at 75°C for 3 min 15 s.
14. Incubate the slides at 37°C overnight in a humidified container.

3.4. Posthybridization Washes and Visualization of Probe

1. Remove the coverslips carefully and wash the slides three times for 5 min each in 50% formamide, 2× SSC, pH 7.0, at 45°C.
2. Wash the slides with 0.1× SSC prewarmed to 60°C but placed in a 45°C water bath, three times, for 5 min each.
3. Transfer the slides to 4× SSC, 0.05% Tween 20 for 15 min at room temperature.
4. Apply the blocking solution to the slide – 150 μl 4× SSC, 0.05% Tween 20 containing 3% bovine serum albumin (w/v), and incubate for 20 min at room temperature.
5. Apply 150 μL of 120 mg/mL streptavidin cyanine 3, 4× SSC, 3% BSA, and 0.05% Tween 20 to each slide and incubate at 37°C for 30 min in darkness.
6. Wash the slides three times in 4× SSC, 0.05% Tween 20 in darkness at 42°C for 5 min each, before a brief wash in fresh deionized water.
7. Air-dry the samples and mount in Vectashield antifade mountant containing 2 μg DAPI as a counterstain.
8. View and image on an epifluorescence microscope. (Fig. 2).

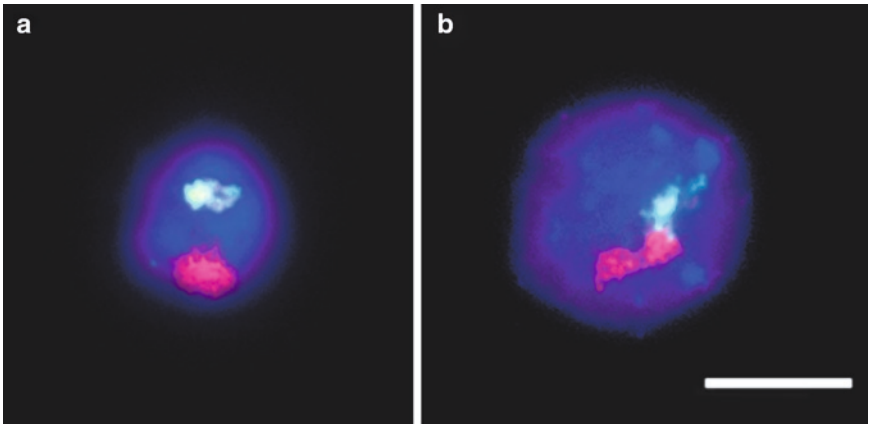

Fig. 2. Nuclei derived from the fixed in vitro constructed porcine embryos (**a**). A nucleus from a 2-cell embryo and (**b**). A nucleus from a blastocyst. DAPI staining is shown in *blue* and porcine chromosome X paint in *red* and porcine chromosome Y in *green*. Scale bar = 10 μm.

4. Notes

1. Many slaughterhouses or abbatoirs will allow collection of sows' ovaries not required by the meat trade to be used for scientific research. Ovaries should be transported back to the laboratory under temperature-controlled conditions. It is sufficient to use a thermos-style flask (for example Dilvac Dewar, UK) containing PBS prewarmed to 39°C supplemented with 400 μl of antibiotic–antimicotic solution (10,000 U penicillin G per mL, 10,000 μg streptomycin sulphate per mL; Invitrogen Life Technologies).
2. "Cryopreserved spermatozoa from a boar of proven fertility can be purchased from commercial genetic companies." Examples would include (but not exclusively) Genus Breeding, ACMC, JSR.
3. The Percoll™ gradient (45%/90%) wash is performed on sperm prior to IVF to remove dead sperm and extenders. The 90% Percoll™ is made by adding 0.6 mL of "Percoll additives" to 4.5 mL of Percoll. Percoll additives comprise 209 mmol $CaCl_2 \cdot 2H_2O$/L, 40 mmol $MgCl_2 \cdot 6H_2O$/L, 806 mmol NaCl/L, 31.1 mM KCl, 2.88 mM $NaH_2PO_4 \cdot 2H_2O$, 10 mg/mL Gentamycin. 42.1 mM Hepes Free Acid, 49.8 mM Hepes Na salt, and 0.64 mM Na–Lactate made up in sterile water. The 45% Percoll is made by adding 2 mL of "SPTL" to 2 mL of 90% Percoll. SPTL comprises 100 mM NaCl, 3.78 mM KCl, 0.38 mM NaH_2PO_4, 10 mg Gentamycin mL, 5.28 mM Hepes free acid, 6.24 mM Hepes Na Salt, 0.2 mM Na Lactate, 1.67 mM $CaCl_2 \cdot 2H_2O$, 0.32 mM $MgCl_2 \cdot 7H_2O$,

and 0.5% (v/v) phenol red solution, made up in water. To make the gradient, layer 2 mL of 45% Percoll on top of 2 mL of 90% Percoll in a 12 mL centrifuge tube and warm to 39°C. Thawed spermatozoa should be layered on top of the gradient and centrifuge-washed as detailed above.

4. Construction of hand-pulled Pasteur pipettes: The neck of the Pasteur pipette is gently heated over a burner until the glass begins to soften. It is then removed from the flame and pulled/stretched producing a narrow diameter pipette for aspirating oocytes and embryos.
5. The flow-sorted porcine chromosome material in our hands was obtained from Prof Malcolm Ferguson-Smith, Department of Clinical and Veterinary Medicine, University of Cambridge. The chromosomes were amplified by DOP-PCR to create to template stocks, primary and secondary. Labeled painting probes were obtained from the secondary template. At the time of writing, porcine chromosome paints are also available from Cambio (Cambridge).
6. To make porcine genomic DNA for suppression of repetitive sequences, we used pig liver that had been freshly removed from a culled pig and frozen at –20°C, and standard kit-based extraction protocols.
7. OCCs are oocytes surrounded by cumulus cells. The OCCs with two layers of cells should be collected as they are optimal for IVF.
8. The spreading solution is used to dissolve the embryo's zona pellucida and cytoplasm. However, due to the high lipid content of porcine embryos (14), blastomeres appear dark, therefore, it can be difficult to detect nuclei from the embryos at this stage.
9. Do not over-dry the pellet – dry it until it is just transparent.

References

1. Hanel ML, Wevrick R (2001). The role of genomic imprinting in human developmental disorders: lessons from Prader-Willi syndrome. *Clin Genet*, **59**, 156–164
2. McKenzie LJ, Carson SA, Marcelli S, Rooney E, Cisneros P, Torskey S, Buster J, Simpson JL, Bischoff FZ. (2004). Nuclear chromosomal localization in human preimplantation embryos: correlation with aneuploidy and embryo morphology. *Hum Reprod*, **19**, 2231–2237.
3. Diblík J, Macek M Sr, Magli MC, Krejcí R, Gianaroli L. (2007) Chromosome topology in normal and aneuploid blastomeres from human embryos. Prenat Diagn, **27**, 1091–1099.
4. Finch KA, Fonseka G, Ioannou D, Hickson N, Barclay Z, Chatzimeletiou K, Mantzouratou A, Handyside A, Delhanty J, Griffin DK. (2008). Nuclear organisation in totipotent human nuclei and its relationship to chromosomal abnormality. *J Cell Sci*, **121**, 655–663.
5. Lanctôt C, Kaspar C, Cremer T. (2007). Positioning of the mouse Hox gene clusters in the nuclei of developing embryos and differentiating embryoid bodies. *Exp Cell Res*, **313**, 1449–1459.
6. Koehler D, Zakhartchenko V, Froenicke L, Stone G, Stanyon R, Wolf E, Cremer T, Brero A. (2009). Changes of higher order chromatin

arrangements during major genome activation in bovine preimplantation embryos. *Exp Cell Res*, **315**, 2053–2063.

7. Lunney JK. (2007) Advances in swine biomedical model genomics. *Int J Biolol Sci*, **3**, 179.
8. Weiss RA. (1998) Transgenic pigs and virus adaptation. *Nature*, **391**; 327–327.
9. Guan TY, Holley RA (2003) Pathogen Survival in Swine Manure Environments and Transmission of Human Enteric Illness-A Review Sponsoring organizations: Manitoba Livestock Manure Management Initiative and Manitoba Rural Adaptation Council. In.: *Am Soc Agronom*, 383–392.
10. Rettenberger G, Klett C, Zechner U, Kunz J, Vogel W, Hameister H. (1995) Visualization of the conservation of synteny between humans and pigs by heterologous chromosomal painting. *Genomics*, **26**, 372–378.
11. Lim D, Cho YM, Lee KT, Kang Y, Sung S, Nam J, Park EW, Oh SJ, Im SK, Kim H. (2009). The Pig Genome Database (PiGenome): an integrated database for pig genome research. *Mamm Genome*, **20**, 60–66.
12. Abeydeera LR, Day BN. (1997) Fertilization and subsequent development in vitro of pig oocytes inseminated in a modified tris-buffered medium with frozen-thawed ejaculated spermatozoa. *Biol Reprod*, **57**, 729–734.
13. Petters RM, Reed ML. (1991) Addition of taurine or hypotaurine to culture medium improves development of one and two cell pig embryos in vitro. *Theriogenology*, **35**, 253.
14. Sturmey RG, Leese HJ. (2003) Energy metabolism in pig oocytes and early embryos. *Reproduction*, **126**, 197–204.
15. Rooney DE, Czepulkowski BH. (1986) Human Cytogenetics: A Practical Approach. Oxford, IRL Press.
16. Abeydeera LR, Wang WH, Cantley TC, Rieke A, Murphy CN, Prather RS, Day BN. (2000) Development and viability of pig oocytes matured in a protein-free medium containing epidermal growth factor. *Theriogenology*, **54**, 787–797.
17. Jeong BS, Yang X. (2001) Cysteine, glutathione, and Percoll treatments improve porcine oocyte maturation and fertilization in vitro. *Mol Reprod Dev*, **59**, 330–335.
18. Petters RM, Johnson BH, Reed ML, Archibong AE. (1990) Glucose, glutamine and inorganic phosphate in early development of the pig embryo in vitro. *J Reprod Fertil*, **89**, 269–275.
19. Coonen E, Dumoulin JC, Ramaekers FC, Hopman AH. (1994) Optimal preparation of preimplantation embryo interphase nuclei for analysis by fluorescence in-situ hybridisation. *Hum Reprod*, **9**, 533–537.
20. Harper JC, Dawson K, Delhanty JD, Winston RM. (1995) The use of fluorescent in-situ hybridisation (FISH) for the analysis of in-vitro fertilization embryos: a diagnostic tool for the infertile couple. *Hum Reprod*, **10**, 3255–3258.
21. Xu K, Huang T, Liu T, Shi Z, Rosenwaks Z. (1998) Improving the fixation method for preimplantation genetic diagnosis by fluorescent in situ hybridisation. *J Assist Reprod Genet*, **15**, 570–574.
22. Daphnis DD, Delhanty JD, Jerkovic S, Geyer J, Craft I, Harper JC. (2005) Detailed FISH analysis of day 5 human embryos reveals the mechanisms leading to mosaic aneuploidy. *Hum Reprod*, **20**, 129–137.
23. van Minnen J, van Kesteren RE. (1999) Methods towards detection of protein synthesis in dendrites and axons. In: Modern Techniques in Neuroscience Research (Windhorst U, Johansson H, eds.), Chapter 3, pp. 57–88. Springer-Verlag, Heidelberg, Germany.