Assessing the Chromosome Copy Number in Metaphase II Oocytes by Sequential Fluorescence in Situ Hybridization

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Purpose: Aneuploidy in oocytes is the main cause of failed embryo implantation and of miscarriage. At present, only limited data on the prevalence of aneuploidy in freshly collected human oocytes are available and all studies have been performed with conventional methods for karyotyping. In this feasibility study, multiple-hybridization fluorescence in situ hybridization (FISH) was evaluated as an alternative method to determine the number of chromosomes in oocytes.

Methods: Fifty-two spare oocytes were collected from 23 patients treated with gonadotropins for intrauterine insemination or intracytoplasmic sperm injection. A conventional dual color FISH approach using mixtures of chromosome-specific standard alpha-satellite probes was applied consecutively to the chromosomes of the same metaphase II oocyte. Mixtures of three to six probes were designed in order to allow chromosome identification based on signal color and centromeric index.

Results: One hybridization cycle was possible in 52 unispermated metaphase II oocytes, two hybridizations in 43 oocytes (82.7%), three hybridizations in 30 oocytes (57.6%), four hybridizations in 27 oocytes (51.9%), and five hybridizations in 15 oocytes (28.8%). Altogether, 591 chromosomes could be marked (47.4% of the entire chromosome complement, 11.4 chromosomes per oocyte). The most important single factor contributing to technical failure was loss of the oocyte from the slide.

Conclusion: This feasibility study demonstrates that multiple-hybridization FISH can be used for the assessment of

a larger proportion of the chromosome complement in oocyte as compared to previous studies based on FISH.

KEY WORDS: Alpha-satellite probes; aneuploidy; fluorescence in situ hybridization; metaphase II oocytes.

INTRODUCTION

Female meiosis is the single most important biological process involved in the origin of aneuploidy (1). While recent investigations have shed some light on possible mechanisms involved in the failure of the meiotic process in the oocyte, such as susceptible chiasma configurations (2) or premature separation of the bivalent at meiosis I (3), its causes remain largely unknown. Based on the findings that both granulosa cell apoptosis (4) and oocyte aneuploidy (5) are more prevalent in older women, a research project was initiated to study the possibility of a causal relationship between these two phenomena.

The aim for this study was to devise a simple and reliable method to determine the number of as many individual chromosomes as possible in human metaphase II oocytes. The conventional chromosome preparation from oocytes remains difficult and laborious (6) and the reported success rate varies between 52.6% (7) and 86.9% (8). Another drawback of most published studies is the use of inseminated but unfertilized oocytes from infertile women, aged during the attempted in vitro fertilization prior to their fixation for the cytogenetic analysis. It has been demonstrated that the ageing of animal oocytes is associated with increased rates of aneuploidy (9). Due to the scarcity of human oocytes for research purposes, few studies reported on the incidence of chromosomal abnormal-

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ities in freshly collected, noninseminated oocytes. Taking all published data together the chromosome complement of 295 nonaged oocytes have now been analyzed (10–14).

All previous karyotyping of human oocytes was performed with Giemsa (10) or with 6-diamino-2-phenylindole (DAPI) (15). Conventional fluorescence in situ hybridization (FISH) repeatedly has been used in oocytes (or first polar bodies) without prior chromosome preparation to determine the copy number of specific chromosomes (16–18). FISH using chromosome or locus specific probes has been applied successfully to obtain information on the chromosomal constitution of cells unsuitable for conventional cytogenetic approaches such as fetal cells in the maternal circulation (19), blastomeres (20–22), polar bodies (16,18), and sperm (23). However, in these studies only a limited number of chromosomes in the interphase was investigated. Meanwhile, technical limitations due to the restricted availability of fluorescence dyes have been overcome by advanced digital image analysis such as M-FISH (24) or spectral karyotyping (SKY) (25) allowing the differential staining of all chromosomes in metaphase spreads. M-FISH or SKY, apart from being unavailable, were considered too complex for a single cell approach.

In order to increase the number of detected chromosomes, we decided to combine traditional chromosome preparation with a simple and reliable FISH approach using chromosome-specific standard alpha-satellite probes in a dual color system. In a feasibility study we applied probe mixtures to chromosome spreads from metaphase II oocytes and repeated the hybridization cycle with different probe mixtures. It was calculated that five hybridization cycles would allow the identification of the entire chromosome complement.

MATERIAL AND METHODS

Collection of Spare Metaphase II Oocytes

Thirty-five oocytes were collected from 15 infertile women previously treated with recombinant follicle-stimulating hormone (FSH) (Gonal-F, Serono, Aubonne, Switzerland) and with human urinary chorionic gonadotropin (hCG) (10,000 IU, Pregnesin, Serono) for induction of ovulation followed by intrauterine insemination (IUI). Seventeen oocytes also were obtained from eight patients, in whom

scheduled intracytoplasmic sperm injection (ICSI) could not be carried out due to lack of spermatozoa. In all, 15 women treated for ovulation induction and insemination a gonadotropin-releasing hormone agonist (Decapeptyl, 0.5 mg, triptorelin acetate, Ferring, Wallisellen, Switzerland) was given following the ultrashort protocol. Those treated with ICSI had received one ampoule of Decapeptyl Retard (3.75 mg of triptorelin acetate, Ferring) during the luteal phase of the menstrual cycle preceding ovarian stimulation with gonadotropins. The median age of the patients was 32 years (range, 25–36 years). The median duration of infertility was 54 months (range, 23–91 months). The main indication for treatment was male infertility in 21 couples, polycystic ovary syndrome in one patient, and unexplained infertility in another.

If at the day of ovulation induction with hCG more than three ovarian follicles with a diameter of more than 14 mm were detected with vaginal sonography, the couple was informed about the risk of a high-grade multiple pregnancy. They were confronted with the option of cancellation of the treatment cycle or alternatively with the possibility of ultrasound-guided transvaginal puncture and aspiration of the supernumerary follicles. If they chose the latter option, they were asked whether the supernumerary oocytes could be fixed and processed for analysis of the chromosome complement. If they agreed to do so, informed and signed consent was obtained. The examination of the chromosome complement of noninseminated and nonfertilized human oocytes was presented to and approved by the Ethics Committee of the Medical Department at the University Hospital of Basel. The protocol for aspiration of supernumerary follicles for the prevention of multiple pregnancies was validated and described in detail previously (26). Briefly, the cumulus oophorus–oocyte–complexes were collected by transvaginal ultrasound-guided aspiration 33 to 35 hr after ovulation induction with 10,000 IU hCG and incubated in standard in vitro fertilization (IVF) culture medium (MediCult, Jyllinge, Denmark) for a few hours. Thereafter, the oocytes were freed of the surrounding cumulus cells enzymatically (hyaluronidase type IV-S, Sigma, St. Louis, MO) and mechanically by aspirating and repulsing the oocyte back and forth through a small-bore pipette. Under an inverted microscopy the polar body was identified (IRBM, Leica, Cologne, Germany) and only those oocytes with an extruded polar body (e.g., metaphase II) were used for further study.

Preparation of Metaphase II Oocytes

After triple wash with culture medium the oocytes were transferred for gradual fixation to a microscopic slide with three cavities (10). The first cavity contained a hypotonic solution consisting of 0.9% sodium citrate and the oocytes were incubated in it for 12 to 15 min at room temperature. Then the oocytes were transferred to the second cavity containing the first fixative solution, consisting of methanol (50% vol/vol), acetic acid (40% vol/vol), and H₂O (10% vol/vol). After 1 to 3 min of continuous observation under the stereomicroscope the oocytes were transferred to an encircled area of another microscope slide (Superfrost/Plus, Merck, Dietikon, Switzerland) and the oocyte was then covered with the second fixative solution now consisting of methanol (75% vol/vol) and acetic acid (25% vol/vol). The slide containing the fixed oocyte was dried on a warming plate. The coordinates of the oocyte's position on the slide were recorded using a light microscope. The slides were stored at 4°C until FISH.

Fluorescence in Situ Hybridization

For denaturation the slides were immersed in 70% formamide/2 × sodium chloride/sodium citrate (SSC) pH 7.0 at 72°C for 2 min and dehydrated in 70%, 85%, and 100% ice-cold ethanol for 2 min each. Chromosome-specific indirectly labeled alpha-satellite DNA probes were obtained from Oncor Appligene (Illkirch, France). These probes hybridize to highly repetitive alphoid DNA (171-bp-long tandem repeat monomers) located at the centromere regions of human chromosomes. However, the chromosome-9-specific probe was here an exception, because it hybridizes to shorter repeats related to an AATGG sequence in classic satellites located in the pericentric heterochromatin of the human chromosome 9. To the prewarmed slide (37°C for 5 min) Oncor hybridization buffer (Hybrisol VI, consisting of 65% formamide/2 × SCC and blocking reagents) was added. This hybridization mixture was applied to the denatured slide and the area was covered by a coverslip and sealed with rubber cement. Thereafter, the slides were incubated for 4 to 16 hr at 37°C in 0.25 × SCC for 5 min at room temperature, then in 0.25 × SCC for 5 min at 72°C and washed in phosphate buffered detergent (PBD) for 2 min.

For dual color fluorescence probe detection a mixture of fluorescein (green) labeled avidin and rhodamine (red) labeled antidigoxigenin antibodies were

used according to the protocol of the manufacturer (Oncor Appligene, Illkirch, France). The slides were covered with a plastic coverslip and incubated 10 min at 37°C. After removal of the coverslips the slides were rinsed three times in PBD for 2 min each time and counterstained with 6-diamino-2-phenylindole (DAPI, Vysis, Downers Grove). For the next hybridization cycle the coverslip was removed by immersing the slide in PBD at room temperature. The previous FISH probes were removed by denaturing the slide in 70% formamide/2 × SCC, at pH 7.0, at 72°C for 2 min, and dehydrated in 70%, 85%, 100% ice-cold ethanol for 2 min each. The subsequent steps were performed as described above. For visualization and image storage an Axioskop fluorescence microscope (Zeiss, Oberkochen, Germany) was used in conjunction with a CCD camera (Photometrix, Tucson, AZ) and the Spectrum software (IPLab, Cambridge, UK).

In order to identify each individual chromosome five consecutive hybridizations using mixtures of three to six probes were performed. The probes mixed were chosen carefully in order to enable an unequivocal identification of chromosomes based on signal color and centromeric index of the marked chromosome (Fig. 1).

Scoring Criteria

A normal metaphase II oocyte has a haploid chromosome set consisting of univalent chromosomes with two chromatids each. Signals of probes targeting the centromeric region should appear double dotted. The observation of a single dot may indicate premature division or the overlap of two chromatids.

Controls

Chromosome preparations from peripheral blood lymphocytes were used as controls in order to ensure a normal function of the DNA probes and general hybridization setup. With this control the normal function of DNA probes was assessed.

Statistics

For each chromosome the odds ratio was calculated based on the number of successful hybridizations both in oocytes and lymphocytes as related to the number of attempted hybridizations. The corresponding 95% confidence intervals were calculated based on the method described by Pearson, Mantel, and Haenszel.

Fig. 1. Schematic representation of the choice of dual color probe mixtures in order to allow the identification of all individual chromosomes based on color and centromere index.

RESULTS

All 15 patients treated with transvaginal ultrasound-guided aspiration of supernumerary follicles ovulated subsequently and were inseminated. Seven became pregnant as a result of the infertility treatment (pregnancy rate: 46.7%, five singleton pregnancies, two twin pregnancies). None of the pregnancies ended prematurely in miscarriage. Three of the eight patients treated with ICSI became pregnant (pregnancy rate: 37.5%). One carried an intact singleton pregnancy, whereas two pregnancies ended in miscarriage.

To prevent the ovulation of supernumerary follicles and high-grade multiple pregnancies 68 cumulus oophorus–oocyte–complexes were aspirated and examined microscopically (4.6 per patient). Among these 35 oocytes were found to be in metaphase II

and suitable for characterization of the chromosome complement (2.3 per patient). In all 52 oocytes one hybridization was performed permitting the identification of six chromosomes in each oocyte. Due to the loss of some oocytes during removal of the hybridization probes a second hybridization was possible only in 43 oocytes (82.7%), a third hybridization in 30 oocytes (57.6%), a fourth hybridization in 27 oocytes (51.9%) and a fifth hybridization in 15 oocytes (28.8%). Altogether, 591 chromosomes were marked and identified, which corresponds to 47.4% of the entire normal chromosome complement of the 52 oocytes studied (1248 chromosomes). A mean of 11.4 chromosomes was identified per oocyte. The lowest number of chromosomes identified in one oocyte was three chromosomes. In two oocytes 23 chromosomes were marked with multiple-hybridization FISH. A representative example is given in Fig. 2. The only reason for not completing five hybridization cycles was premature loss of the oocyte from the slide.

The number of failed hybridizations was considerably higher in metaphase II oocytes (174; 23.7%) than in lymphocytes (11; 1.4%). For most chromosomes the identification rate exceeded 50% (Table I). Chromosome X was identified in all oocytes. Chromosomes 9, 19, and 5 were the most difficult to identify with the probes used in this study.

Based on the evaluation of DAPI-staining only, 15 oocytes out of the 52 prepared displayed an abnormal chromosome complement (28.8%): 8 were hypohaploid (having 13 to 22 chromosomes) and 7 were hyperhaploid (having 24 chromosomes). However, in none of the oocytes examined in the present study FISH permitted the detection of a hyperhaploid or a diploid chromosome complement. Furthermore, no case of predivision was detected (3), in which the double-dot signal would have appeared as two single dots (16).

DISCUSSION

Few studies have been performed to study the aneuploidy rate in freshly collected human oocytes (10–14). All previous studies were performed with conventional karyotyping methods, which gives rise to good quality chromosomal preparations only in a small proportion of the available oocytes. The limited number of studies performed with FISH using aged oocytes after a failed IVF attempt have been performed with only few specific DNA probes considered to be the clinically most relevant ones.

The present study was performed to widen the

Fig. 2. (a) Metaphase II oocyte after the first hybridization cycle. (b) Same oocyte after the second hybridization cycle. (c) Same oocyte after third hybridization cycle. (d) Same oocyte after fourth hybridization cycle. Paired signals of chromosomes 1 and 5 were detected but not chromosome 19. The signals of chromosomes 17 and 7 were weak and only visualized when the image of the chromosomes were magnified. (e) Same oocyte after fifth hybridization cycle. (f) Software generated single image of the result of all five hybridization cycles.

number of chromosomes identified with FISH to as many as possible. The identification of all 24 chromosomes by a triple-color FISH approach would theoretically require eight subsequent hybridization cycles. Although this apparently can be achieved in single oocytes, we considered the risk of losing the oocyte during eight repeated hybridizations as too high. Therefore, we chose to apply FISH on chromosome spreads from metaphase II oocytes in order to be able to use additional criteria for chromosome identification, such as the size of the chromosomes and the centromere index. In contrast to conventional chromosome analysis, the quality of the chromosome preparation is not of major concern here, since individual chromosomes can be identified by the specific FISH signal. Based on two signal colors in each hybridization step and by careful selection of simultaneously hybridized probes (Fig. 1) it was considered that five subsequent hybridization cycles would allow

the detection of all 24 chromosomes in unfertilized metaphase II oocytes.

This feasibility study clearly demonstrates that the present FISH technique, modified by the use of consecutive hybridization cycles on chromosome preparations, is a suitable experimental method to increase considerably the number of identified chromosomes even in the difficult single-cell environment of metaphase II oocytes. However, two major technical problems were encountered, namely the loss of the oocytes from the slide and an insufficient hybridization efficiency with some of the chromosome specific probes used here (e.g., those for chromosomes 5 and 19). The choice of alternatives from the growing list of commercially available probes might solve the latter. A reliable fixation of the chromosome spread to the slide remains critical in cytogenetic analyses of single cells involving recurrent preparatory steps.

The removal of previously hybridized FISH probes

Table I. Efficacy of Identification of Individual Chromosomes with Multiple-Hybridization FISH

Chromosome	Attempted hybridization	Successful hybridization (%)	Odds ratio (95% CI)
13	52	47 (90.4)	1.11 (0.58–1.92)
21	52	42 (80.8)	1.24 (0.57–2.17)
X	49	49 (100)	1
14	52	43 (82.7)	1.21 (0.57–2.11)
22	52	39 (75.0)	1.33 (0.57–2.35)
10	49	32 (65.3)	1.53 (0.55–2.78)
6	43	42 (97.7)	1.02 (0.55–1.87)
16	43	34 (79.1)	1.26 (0.54–2.35)
18	43	28 (65.1)	1.50 (0.53–2.84)
8	42	24 (57.1)	1.75 (0.52–3.38)
20	29	18 (62.1)	1.50 (0.45–3.31)
11	30	29 (96.7)	1.03 (0.49–2.13)
2	30	25 (83.3)	1.20 (0.48–2.51)
12	30	17 (56.7)	1.76 (0.46–3.86)
1	27	25 (92.6)	1.08 (0.47–2.32)
5	22	10 (45.5)	2.00 (0.38–5.24)
19	17	6 (35.3)	1.83 (0.30–6.12)
17	27	24 (88.9)	1.13 (0.46–2.43)
7	27	23 (85.2)	1.17 (0.46–2.55)
4	15	11 (73.3)	1.36 (0.34–3.96)
15	15	12 (80.0)	1.25 (0.35–3.58)
3	15	8 (53.3)	1.88 (0.33–5.76)
9	14	3 (21.4)	4.67 (0.24–19.01)
All chromosomes	775	591 (76.3)	1.29 (0.86–1.50)

by denaturing conditions and subsequent rehybridization on metaphase chromosomes and interphase cells has been reported earlier (27–29). Repeated hybridization cycles of multiple probe assays have been systematically investigated on oocytes, single blastomeres, or interphase cells recently (30–33). Two rounds of triple-probe FISH were applied to unfertilized oocytes detecting five different chromosomes (30). Human blastomeres underwent three hybridization cycles with a total of six different chromosome-specific probes in another recent feasibility study (31,32). Zhen and co-workers (33) termed their interphase approach in another rare event situation, i.e., fetal cells in the maternal circulation, “poly-FISH” and claimed the theoretical possibility to detect all 24 chromosomes. Loss of nuclei has been of major concern also in some of these studies.

In the meantime, a first application of one of the new advanced FISH techniques (spectral karyotyping, SKY) to oocytes was reported (34). The method requires expensive specialized hard- and software as well as suitable chromosome spreads. In contrast to conventional cytogenetics and SKY, the technical quality of the chromosome spreads is not critical with the consecutive hybridization of centromere or locus specific probes. The latter, however, is not suitable

to detect structural changes. Further studies are required to determine the practical value of the different methods to assess the chromosome number in metaphase II oocytes. Advances in molecular cytogenetics have provided new approaches to study the chromosome complement of oocytes, which already are used for clinical diagnosis in preimplantation genetics. Their potential to diagnose more easily aneuploidy in single oocytes might help to define factors involved in the origin and causes of nondisjunction.

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REFERENCES

- Warburton D: Human female meiosis: New insights into error-prone process. *Am J Hum Genet* 1997;61:1–4
- Lamb NE, Freeman SB, Savage-Austin A, Pettay D, Taft L, Hersey J, Gu Y, Shen J, Saker D, May KM, Avramopoulos D, Petersen MB, Hallberg A, Mikkelsen M, Hassold TJ, Sherman SL: Susceptible chiasmate configurations of chromosome

- 21 predispose to nondisjunction in both maternal meiosis I and meiosis II. *Nat Genet* 1996;14:400–405
3. Angell R: First-meiotic-division nondisjunction in human oocytes. *Am J Hum Genet* 1997;61:23–32
4. Seifer DB, Gardiner AC, Ferreira KA, Peluso JJ: Apoptosis as a function of ovarian reserve in women undergoing in vitro fertilization. *Fertil Steril* 1996;66:593–598
5. Eichenlaub-Ritter U: Genetics of oocyte ageing. *Maturitas* 1998;30:143–169
6. Zenzes MT, Casper RF: Cytogenetics of human oocytes, zygotes, and human embryos after in vitro fertilization. *Hum Genet* 1992;88:367–375
7. Pieters MHEC, Geraedts JPM, Dumoulin JCM, Evers JLH, Bras M, Kornips FHAC, Menheere PPCA: Cytogenetic analysis of in vitro fertilization (IVF) failures. *Hum Genet* 1989;81:367–370
8. Racowsky C, Prather AL, Johnson MK, Olvera SP, Gelety TJ: Prematurely condensed chromosomes and meiotic abnormalities in unfertilized human oocytes after ovarian stimulation with and without gonadotropin-releasing hormone agonist. *Fertil Steril* 1997;67:932–938
9. Mailhes JB, Young D, London SN: Postovulatory ageing of mouse oocytes in vivo and premature centromere separation and aneuploidy. *Biol Reprod* 1998;58:1206–1210
10. Wramsby H, Liedholm P: A gradual fixation method for chromosomal preparations of human oocytes. *Fertil Steril* 1984;41:736–738
11. Martin RH, Mahadevan MM, Taylor PJ, Hildebrand K, Long-Simpson L, Peterson D, Yamamoto J, Fleetham J: Chromosomal analysis of unfertilized human oocytes. *J Reprod Fertil* 1986;78:673–678
12. Wramsby H, Fredga K, Liedholm P: Chromosome analysis of human oocytes recovered from preovulatory follicles in stimulated cycles. *N Engl J Med* 1987;316:121–124
13. Tarín JJ, Gómez E, Sampaio M, Ruiz M, Remohí J, Pellicer A: Cytogenetic analysis of human oocytes from fertile women. *Hum Reprod* 1991;6:1100–1103
14. Gras L, McBain J, Trounson A, Kola I: The incidence of chromosomal aneuploidy in stimulated and unstimulated (natural) uninseminated human oocytes. *Hum Reprod* 1992;7:1396–1401
15. Angell RR, Xian J, Keith J: Chromosome anomalies in human oocytes in relation to age. *Hum Reprod* 1993;8:1047–1054
16. Munné S, Dailey T, Sultan KM, Grifo J, Cohen J: The use of first polar bodies for preimplantation diagnosis of aneuploidy. *Hum Reprod* 1995;10:1014–1020
17. Verlinsky Y, Cieslak J, Freidline M, Ivakhnenko V, Wolf G, Kovalinskaya L, White M, Lifchez A, Kaplan B, Moise J: Pregnancies following pre-conception diagnosis of common aneuploidies by fluorescent in-situ hybridization. *Hum Reprod* 1995;10:1923–1927
18. Verlinsky Y, Cieslak J, Ivakhnenko V, Lifchez A, Strom C, Kuliev A: Birth of healthy children after preimplantation diagnosis of common aneuploidies by polar body fluorescent in situ hybridization analysis. Preimplantation Genetics Group. *Fertil Steril* 1996;66:126–129
19. Hahn S, Sant R, Holzgreve W: Fetal cells in maternal blood: Current and future perspectives. *Mol Hum Reprod* 1998;4:515–521
20. Munné S, Weier HU: Simultaneous enumeration of chromosomes 13, 18, 21, X, and Y in interphase cells for preimplantation genetic diagnosis of aneuploidy. *Cytogenet Cell Genet* 1996;75:263–270
21. Griffin DK, Wilton LJ, Handyside AH, Winston RM, Delhanty JD: Dual fluorescent in situ hybridisation for simultaneous detection of X and Y chromosome-specific probes for the sexing of human preimplantation embryonic nuclei. *Hum Genet* 1992;89:18–22
22. Delhanty JD, Griffin DK, Handyside AH, Harper J, Atkinson GH, Pieters MH, Winston RM: Detection of aneuploidy and chromosomal mosaicism in human embryos during preimplantation sex determination by fluorescent in situ hybridisation (FISH). *Hum Mol Genet* 1993;2:1183–1185
23. Miharū N, Best RG, Young SR: Numerical chromosome abnormalities in spermatozoa of fertile and infertile men detected by fluorescence in situ hybridization. *Hum Genet* 1994;93:502–506
24. Speicher MR, Ballard SG, Ward DC: Karyotyping human chromosomes by combinatorial multicolor FISH. *Nature Genet* 1996;12:368–375
25. Schröck E, du Manoir S, Veldman T, Schoell B, Wienberg J, Ferguson-Smith MA, Ning Y, Ledbetter DH, Bar-Am I, Soenksen D, Garini Y, Ried T: Multicolor spectral karyotyping of human chromosomes. *Science* 1996;273:494–497
26. De Geyter C, De Geyter M, Castro E, Bals-Pratsch M, Nieschlag E, Schneider HPG: Experience with transvaginal ultrasound-guided aspiration of supernumerary follicles for the prevention of multiple pregnancies after ovulation induction and intrauterine insemination. *Fertil Steril* 1996;65:1163–1168
27. Spathas DH, Divane A, Maniatis GM, Fergusonsmith ME, Fergusonsmith MA: Prenatal detection of trisomy 21 in uncultured amniocytes by fluorescence in situ hybridization: A prospective study. *Prenat Diagn* 1994;14:1049–1054
28. Wang MR, Perissel B, Malet P: Rehybridization on metaphases studied previously by FISH. An approach to analyze chromosome aberrations. *Cancer Genet Cytogenet* 1995;85:58–60
29. Epstein L, DeVries S, Waldman FM: Reutilization of previously hybridized slides for fluorescence in situ hybridization. *Cytometry* 1995;21:378–381
30. Martini E, Flaherty SP, Swann NJ, Payne D, Matthews CD: Analysis of unfertilized oocytes subjected to intracytoplasmic sperm injection using two rounds of fluorescence in-situ hybridization and probes to five chromosomes. *Hum Reprod* 1997;12:2011–2018
31. Liu J, Tsai YL, Zheng XZ, Yazigi RA, Baramki TA, Compton G, Katz E: Feasibility study of repeated fluorescent in-situ hybridization in the same human blastomeres for preimplantation genetic diagnosis. *Mol Hum Reprod* 1998;4:972–977
32. Liu J, Tsai YL, Zheng XZ, Baramki TA, Yazigi RA, Katz E: Potential use of repeated fluorescence in situ hybridization in the same human blastomeres for preimplantation genetic diagnosis. *Fertil Steril* 1998;70:729–733
33. Zhen DK, Wang JY, Falco VM, Weber W, Delli-Bovi L, Bianchi DW: Poly-FISH: A technique of repeated hybridizations that improves cytogenetic analysis of fetal cells in maternal blood. *Prenat Diagn* 1998;18:1181–1185
34. Marquez C, Cohen J, Munné S: Chromosome identification in human oocytes and polar bodies by spectral karyotyping. *Cytogenet Cell Genet* 1998;81:254–258