

Improvements of preimplantation diagnosis of aneuploidy by using microwave hybridization, cell recycling and monocolour labelling of probes

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Recent studies indicate that preimplantation genetic diagnosis of aneuploidy significantly reduces spontaneous abortions and increases pregnancy rates in women of advanced maternal age undergoing in-vitro fertilization. A new protocol was developed involving cell recycling (sequential hybridizations) and microwave hybridization of repetitive probes in order to obtain in a few hours enumeration results for chromosomes X, Y, 1, 13, 15, 16, 18, 21 and 22 in single blastomeres. The error rate using this protocol was significantly lower than in previous protocols and the analysis of nine chromosomes can be achieved in a shorter time than before.

Key words: monosomy/mosaicism/pregnancy loss/spontaneous abortions/trisomy

Introduction

The causes of the implantation decline observed with increasing maternal age are still under debate, but studies on oocyte donation in women of advanced maternal age strongly suggest that the major defect is in the oocyte, not the uterus (Navot *et al.*, 1994). Ooplasmic components may be a factor involved (Keefe *et al.*, 1995; Brenner *et al.*, 1998; Cohen *et al.*, 1998), but the only clear link between maternal age and embryo competence is aneuploidy. Aneuploidy is the major cause of inherited diseases and the only risk factor known is maternal age, with trisomies increasing from 2% in recognized pregnancies of women 25 years old, up to 19% in women aged ≥ 40 years (Warburton *et al.*, 1986). The increase in aneuploidy with maternal age in spontaneous abortuses and live offspring has also been found in both cleavage-stage embryos (Munné *et al.*, 1995a) and unfertilized oocytes (Dailey *et al.*, 1996; Angell, 1997). The rate of chromosomal abnormalities in embryos was found to be higher than the one reported in spontaneous abortions, suggesting that a sizeable proportion of chromosomally abnormal embryos are eliminated before prenatal diagnosis can be performed. Such embryo loss could account for the decline in implantation with maternal age (reviewed by Munné and Cohen, 1998).

Because of the correlation between aneuploidy and declining implantation rates with maternal age, it was postulated that negative selection of chromosomally abnormal embryos through preimplantation genetic diagnosis (PGD) using fluorescence in-situ hybridization (FISH) could reverse this effect (Munné *et al.*, 1993). Two recent studies have compared cases of PGD of aneuploidy with matched controls. In one, implantation rates increased (Gianaroli *et al.*, 1999b), while in the other they did not increase significantly, although spontaneous abortions were reduced and the rates of ongoing and delivered babies improved after PGD (Munné *et al.*, 1999).

In the latter paper, we argued that several factors could be responsible for the unexpectedly modest increase in implantation rates after PGD found in that study. One of these factors was perhaps that embryos with abnormalities in the chromosomes targeted, such as X, Y, 13, 16, 18 and 21, would still implant in substantial proportions even if abnormal. Bahçe *et al.* (1999) demonstrated that other chromosomes, such as 1, 15, 17 and 22, produce more chromosomal abnormalities than some other chromosomes whose abnormalities, such as trisomy 13, XY and 18, can persist to term.

Clearly, it would be advantageous to analyse each cell for a larger number of chromosome aneuploidies. The number of chromosomes that can be analysed, however, is limited by the availability of different spectrally-distinct fluorescent labels that perform well in FISH experiments. In monocolour FISH labelling strategies, a distinctly different fluorescent label is required for each chromosome target to be detected. The number of different chromosomes detected per label can be increased by resorting to coded hybridization label combinations (reviewed by Morrison and Legator, 1999), in which two or more fluorescent labels are used as well as the single labels to identify different chromosome targets. This approach has been used previously in PGD to identify as many as six different chromosomes in a single hybridization using only three different fluorescent labels (Munné *et al.*, 1998a,b). Coded hybridizations, however, are subject to ambiguity resulting from overlapping targets sharing one or more colours. Although monocolour FISH requires more labels, overlapping targets can easily be distinguished.

In the present study we describe a protocol that involves two rounds of hybridization, using monocolour FISH labelling in order to both increase the number of chromosome targets detected (X, Y, 1, 13, 15, 16, 18, 21 and 22) and improve accuracy of enumeration. To minimize total assay time, micro-

wave technology (Bourinbairar *et al.*, 1991; Drury *et al.*, 1997) was used in the second round of hybridization, completing both rounds of hybridization in under 3 h. Although there is a shift in the IVF field towards day 5 replacement in the hope of minimizing multiple gestations while improving implantation, recent studies suggest that culture of certain types of embryos to day 5 may not be beneficial (M.Alikani *et al.*, personal communication). Since the majority of IVF transfers are replaced on day 3, the time taken for PGD analysis is still of extreme importance.

Materials and methods

PGD cases

Patients undergoing PGD of aneuploidy were women either of advanced maternal age, who had a history of repeated spontaneous abortions or repeated IVF failure. Each embryo had one or two cells biopsied on day 3 of development, as described previously (Grifo *et al.*, 1992), after which the embryo was returned to culture. Most of the embryos classified by PGD as normal were transferred to the patient on the same day as analysis. Some non-transferred embryos were reanalysed with all or most of their cells fixed individually as described previously (Munné *et al.*, 1996). Some grossly abnormal embryos were not reanalysed, therefore the rate of chromosome abnormalities in this group of embryos is not representative of the whole population of embryos analysed. The embryos were analysed at The Institute for Reproductive Medicine and Science at Saint Barnabas Medical Center, in accordance with guidelines approved by the internal review board of Saint Barnabas Medical Center, including written consent from the patients in each case.

FISH procedure

In pilot experiments we found that microwave denaturation and hybridization work properly with repetitive probes but not with locus-specific ones. For this reason, a FISH protocol was designed to use locus specific probes in the first round of hybridization, which involved hotplate denaturation and hybridization at 37°C, while only repetitive probes were used in a second round of microwave denaturation and hybridization. Fixed cells were analysed by two rounds of FISH; the first hybridization was performed with probes for chromosomes 13, 16, 18, 21 and 22 and the second round of hybridization using X, Y, 1 and 15 chromosome-specific probes. The first hybridization used the MultiVysion™ PGT multicolour probe panel hybridization mixture commercialized by Vysis (Downers Grove, IL, USA) with the probe for chromosomes as follows: 13 labelled with SpectrumRed™, 16 with SpectrumAqua™, 18 with SpectrumBlue™, 21 with SpectrumGreen™, and 22 with SpectrumGold™. The second hybridization mixture was prepared at Vysis and consisted of probes for chromosomes X (CEP X alpha satellite, Xp11.1–q11.1), Y (CEP Y alpha satellite, Yp11.1–q11.1), 1 (CEP 1 satellite II/III, 1q12), and 15 (CEP 15 satellite III, 15q11.2) labelled respectively in SpectrumGreen, SpectrumGold, SpectrumRed and SpectrumAqua, and ranging in concentration between 25 and 100 ng/assay.

Ten microlitres of the first hybridization solution were applied to the glass slide containing fixed blastomeres and covered with a 18 mm×18 mm coverslip. The slide was then placed for 5 min on a slide warmer preheated to 73°C, sealed with rubber cement, and placed in a dark moist chamber at 37°C for at least 2 h. After the hybridization, the slides were washed individually at 71°C in 0.7×SSC for 4 min. The slides were then mounted with 10 µl of antifade solution and observed with a fluorescent microscope (Olympus BX70)

equipped with a mechanized turret containing a triple-band pass filter set to simultaneously observe the SpectrumRed, SpectrumGreen and SpectrumBlue/Aqua fluorescence, and single-band pass filter sets for DAPI, SpectrumAqua, SpectrumBlue, SpectrumRed, SpectrumGold and SpectrumGreen. Images of each fluorochrome were captured with an IMAC-CCD S30 black and white video camera and the final image was composed with the ISIS3 v1.00 Metasystem software. This software did not permit the capture of five different colours — only red, green and blue; therefore, to record SpectrumGold and SpectrumBlue, they were captured in the following way: SpectrumGold was recorded twice, once with the green and once with the red filter (giving in the prints a yellow colour, Figure 1), and SpectrumAqua/Blue was captured in the same way but using filters red and blue (giving in the prints a pink colour, Figure 1).

After the analysis of the first set of probes, the slides were washed in 0.7×SSC at room temperature until the coverslips fell off, dipped in distilled water at 71°C for 10 s, and then dehydrated (70%, 85%, 100% ethanol, 2 min each). Ten microlitres of the second hybridization solution were applied per slide. Slides and probes were co-denatured either on a hotplate at 75°C for 5 min or in a commercial microwave (Spacemaker II, General Electric Co., Louisville, NY, USA; 1300 W and 2450 MHz) at power 6 (of a 1–10 range) for 1 min. Subsequently the slides were allowed to hybridize in the same microwave at power 1 for 5 min, and finally washed for 30 s in 0.4×SSC at 73°C. The washed slides were then mounted with DAPI in antifade (Oncor, FL, USA) and analysed. The signal quality in the second round was not compromised using the microwave.

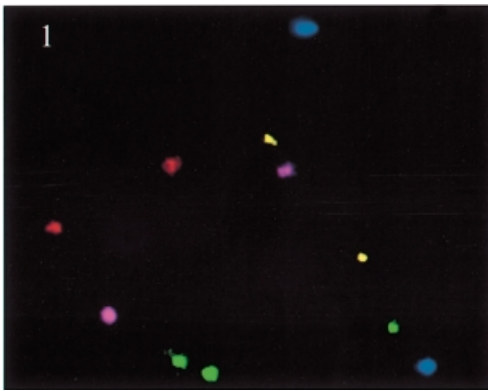
The scoring criteria to differentiate false positives and negatives from mosaicism have been described previously (Munné and Weier, 1996). To classify chromosomal abnormality types by FISH, we used the same criteria described by Munné *et al.* (1998a) but with one exception. Mosaics with <25% abnormal cells were considered normal and the abnormal cells were considered FISH errors because it was difficult to differentiate mosaics from FISH errors in embryos with six or fewer cells.

Results

Error rate

Of the non-transferred embryos from PGD cases, 89 were re-biopsied and all or most cells were analysed by FISH using the same protocol used for PGD (Table I). Of these, 81 had previous PGD results and eight did not. Three types of error were found. The most serious error either classified an embryo as normal by PGD, when in fact it was abnormal (false normal), or classified the embryo as abnormal by PGD when in fact it was normal (false abnormal). Of the 81 reanalysed embryos with previous PGD results, one was a false normal and five were false abnormal. A less serious error was when an embryo was classified as having a specific abnormality by PGD but was found to have a different abnormality after reanalysis (incorrect abnormality). Five of the embryo diagnoses were found to be incorrect abnormalities. Therefore the total FISH error was 11 (13.6%) although only the six (7.4%) false normal and abnormal results could have an impact on offspring and pregnancy outcomes.

Specifically, the false normal error was in fact a mosaic embryo with 40% of the cells abnormal for chromosome 16. Three of the false abnormal embryos were false monosomies (monosomy X, 16 and 22). Another false abnormal embryo was classified by PGD as monosomy 22, but was found to be



Figures 1. Blastomere hybridized with probes for chromosomes 13 (red), 16 (blue), 18 (pink), 21 (green), and 22 (yellow). The blastomere has a trisomy for chromosome 21.

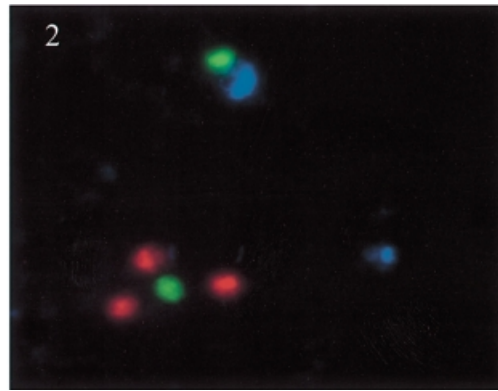


Figure 2. Blastomere rehybridized with probes for chromosomes X (green), Y (yellow), 1 (red) and 15 (blue). The blastomere is from a female trisomy 1 embryo.

a mosaic embryo with only 21% cells abnormal for chromosome 22 (almost normal), and the remaining one was a false chaotic embryo in which a multinucleated cell was biopsied. In addition, three abnormal diagnoses were ‘incorrect abnormality’ errors. One of them was an embryo classified as trisomy 13, when in fact it was a trisomy 13 plus monosomy 16 and 22. A second embryo was classified as monosomy 16 but later found to be monosomy 16 and trisomy 18. A third embryo was classified as monosomy 16 and later found to be trisomy 21. The other two ‘incorrect abnormality’ diagnoses were two embryos classified as complex abnormal, which after reanalysis showed one to be a monosomy 22, and the other a mosaic for chromosome 13 only. Complex abnormal were diagnoses in which at least three chromosomes were abnormal in the cell analysed by PGD.

The 7.4% error rate of false normal and abnormal PGD results obtained using this protocol is significantly lower ($P < 0.05$, χ^2 -test) than the one obtained from previous protocols (Munné *et al.*, 1998a,b). Those protocols used at least X, Y, 13, 16 and 21 probes but used combinatorial coding of the fluorescent labels, as opposed to the uncoded labelling scheme used here. The previous work using coding combination probes resulted in an 18.8% (30/160) rate of false normal and abnormal PGD results. Of those errors, 8.1% (13/160) were false monosomies compared to 3.7% (3/81) in the present study. The rate of erroneous abnormalities was similar in the present study and the two previous studies, 6.2% (5/81) and 6.9% (11/160) respectively.

Chromosome abnormalities

A total of 219 embryos were analysed by PGD (Table I). Of those, 77 were classified as aneuploid, 66 as normal, 43 as complex abnormal with the abnormality involving at least three chromosomes, nine as polyploid, seven as haploid, and 17 without result.

Table II shows the result of combining the data on the 89 embryos not replaced and reanalysed (Table I) with data from the embryos with only one cell analysed by PGD and not reanalysed. Of the 210 embryos with FISH results, 75 (36%) were aneuploid (Figures 1 and 2), 64 (30%) normal, 62 (30%)

mosaics, five (2%) haploid, and four (2%) polyploid. Nine did not have results after PGD and were not reanalysed. The PGD and overall result differed by little.

Pregnancy outcome

Women undergoing PGD and included in this study ($n = 18$) had an average maternal age of $38.8 (\pm 2.7)$, a mean number of retrieved oocytes of $17.7 (\pm 8.7)$ and a total of 60 (average 3 ± 1.5) embryos were transferred to these patients. Of those, 13 (21.7%) embryos implanted, but one of the test implantation sites was ectopic. Of the remaining implantation sacs with heartbeat, three spontaneously aborted, two delivered normal babies, and seven are ongoing on their third trimester of pregnancy. None of the babies born or prenatal diagnoses showed chromosome abnormalities. No chromosomal information on the spontaneous abortions could be obtained.

Discussion

The first goal of this study was to minimize the FISH error rate while increasing the number of chromosomes analysed, by using only one spectrally distinct fluorescent label per chromosome analysed and two rounds of hybridization. Microwave denaturation was used in the second round of hybridization, employing repetitive sequence probes, in order to achieve a total assay time compatible with embryo replacement on the same day of the biopsy.

Several groups have used two rounds of hybridization for the analysis of blastomeres and oocytes (Benadiva *et al.*, 1996; Martini *et al.*, 1997; Bahçe *et al.*, 1999). In two of these studies one of the chromosomes analysed in the first round was also analysed in the second, to give an internal control for the efficiency of the second round, and resulted in very low error rates (Martini *et al.*, 1997; Bahçe *et al.*, 1999). Because of those results, we chose not to repeat the experiments, and to analyse as many different chromosomes as possible with the limited number of fluorochromes available. But in contrast, we determined error rates by analysing most cells of non-transferred embryos.

We found that most errors can be attributed to two factors.

Table I. Preimplantation genetic diagnosis (PGD) results and reanalysis of non-replaced embryos

PGD result	No. of PGD	No. reanalysed	Reanalysis result
Aneuploid:			
Monosomy 1	2	1	Monosomy 1
Trisomy 1	1	0	
Trisomy 13	6	1	Trisomy 13
		1	Trisomy 13, monosomy 16, 22 ^a
		1	Mosaic aneuploidy for 13 (100%)
Monosomy 15	3	1	Mosaic chaotic
Trisomy 15	4	2	Trisomy 15
		1	Mosaic aneuploidy for 15 (63%)
		1	Mosaic chaotic
Monosomy 16	7	1	Monosomy 16, trisomy 18 ^a
		1	Trisomy 21 ^a
		1	Mosaic 2n/4n ^b
Trisomy 16	6	2	Trisomy 16
		1	Mosaic chaotic
		1	Mosaic aneuploidy for 16 (50%)
Monosomy 18	4	1	Monosomy 18
Trisomy 18	1	1	Trisomy 18
Monosomy 21	3	1	Mosaic chaotic
Trisomy 21	2	0	
Monosomy 22	3	1	Mosaic 2n/4n ^b
		2	Monosomy 22
Trisomy 22	8	4	Trisomy 22
		1	Mosaic aneuploidy for 22 (21%) ^b
Monosomy X	4	1	Mosaic 2n/4n ^b
		1	Monosomy X
Trisomy X	1	0	
Double aneuploidies	22	4	Mosaic chaotic
		2	Trisomy 16, 22
		1	Monosomy 16, 22
		1	Trisomy 15, 16
		1	Nullisomy 16 and mosaic
Complex abnormal ^c	43	15	Mosaic chaotic
		4	Monosomy 22 and mosaic
		1	Trisomy 13 and mosaic
		1	Trisomy 18 and mosaic
		1	Trisomy 22 and mosaic
		1	Normal ^b
		1	Mosaic aneuploidy for 13 ^a
		1	Monosomy 22 ^a
		1	Monosomy 16, trisomy 18, mosaic
		1	Trisomy 16, monosomy 18, mosaic
Haploid	7	3	Haploid
		2	Mosaic chaotic
Normal	66	2	Normal
		5	Mosaicism 2n/4n (<12%)
		1	Mosaic aneuploidy for 16 (40%) ^d
Polyploid:			
Complex	4	1	Mosaic chaotic
Triploid	4	1	Triploid
		1	Mosaic chaotic
Tetraploid	1	0	
No result:			
	17	3	Normal
		2	Mosaic 2n/4n
		1	Trisomy 16
		1	Trisomy 22
		1	Trisomy 16, monosomy 13
Total analysed	219	89	
Total confirmed		70	
Total error false normal ^d or abnormal ^b		6 (7.4%)	
Total error incorrect abnormality ^a		5	

^aError incorrect abnormality.

^bThree or more chromosomes involved in the abnormality.

^cError false abnormal.

^dError false normal.

Table II. Fluorescence in-situ hybridization results after preimplantation genetic diagnosis (PGD) and reanalysis

Result	No. by PGD	No. by reanalysis	Total
Normal	58	6	64
Monosomy 1	1	1	2
Trisomy 1	1	0	1
Trisomy 13	3	2 (1 with mosaicism)	5
Monosomy 15	2	0	2
Trisomy 15	0	2	2
Monosomy 16	4	0	4
Trisomy 16	2	3	5
Monosomy 18	3	1	4
Trisomy 18	0	2 (1 with mosaicism)	2
Monosomy 21	2	0	2
Trisomy 21	2	1	3
Monosomy 22	0	7 (4 with mosaicism)	7
Trisomy 22	3	6 (1 with mosaicism)	9
Monosomy X	2	1	3
Trisomy X	1	0	1
Double aneuploidy	13	10 (3 with mosaicism)	23
Mosaic chaotic	19	27	46
Mosaicism 2n/4n	0	10	10
Mosaic aneuploid for chromosome 22	0	1	1
Mosaic aneuploid for chromosome 16	0	2	2
Mosaic aneuploid for chromosome 13	0	2	2
Mosaic aneuploid for chromosome 15	0	1	1
Haploid	2	3	5
Triploid	2	1	3
Tetraploid	1	0	1
No result	9	0	9
Total	130	89	219

^aThree or more chromosomes involved in the abnormality.

The first is mosaicism, which may account for the false normal diagnosis of a mosaic for chromosome 16, a false abnormal in which a multinucleated cell was biopsied, and an incorrect abnormality error, which was a mosaic for chromosome 22. Mosaicism is very common in cleavage-stage embryos (Harper *et al.*, 1995; Munné *et al.*, 1995; Delhanty *et al.*, 1997), but it decreases with improving morphology and embryo development (Munné *et al.*, 1995; Munné and Cohen, 1998). Furthermore, only those mosaics that have at least some normal cells may produce misdiagnosis, while embryos with two or more abnormal and no normal cell lines at all will be classified as abnormal. We have calculated that ~5% of embryos will be misdiagnosed because of mosaicism (Munné, 1999).

The second factor producing errors is related to technique, and results in false monosomies. False monosomies could be caused by loss of micronuclei during fixation. When drops of fixative are added after the cytoplasm has been broken, the risk of losing micronuclei is increased. In a previous study we found that if we did not add more fixative after cell breakage, we were able to reduce the loss of micronuclei, and thereby the incidence of misdiagnosis (Munné *et al.*, 1998b). False monosomies may also be caused by overlaps of homologous chromosomes (Munné *et al.*, 1996) or of different chromosomes when combinatorial labelling is used. The use of the present protocol has resulted in a significant reduction in the error rate of false normal and abnormal PGD results when compared to the previous protocols in which similar probes were used (Munné *et al.*, 1998a,b). This is most likely because we used a unique fluorescent label for each chromosome analysed. In previous studies, some chromosomes were labelled with a

mixture of two or three fluorescent labels for lack of more colours. With combinatorial labelling, if two chromosome signals overlapped, the resulting signal would have the sum of the colours for both probes; which could correspond to a colour combination for a different chromosome, or to one of the two overlapping chromosomes, producing in the latter case a false monosomy in disomic embryos or false disomy in trisomic embryos. As we found, the rate of false monosomies is reduced here to <4% from 8% in those previous studies.

Very few errors could be attributed to false trisomies caused by signal splitting, as reported previously (Munné *et al.*, 1998b). Although this system has minimized the risk of errors by modifying the fluorochromes used, errors can still occur because of the similarity between SpectrumAqua and SpectrumBlue. In most instances, the single-band pass filters for these two fluorochromes do not perfectly block the other colour; overlaps between blue and aqua may still be misdiagnosed when the signals are faint. To further minimize false errors, at least false abnormal PGD results, a second cell could be biopsied and analysed. This is not a problem when the transfer is done on day 4, as already demonstrated (Grifo *et al.*, 1998; Gianaroli *et al.*, 1999a). However, the biopsy of two cells in all the embryos is discouraged because of circumstantial evidence of a negative effect on implantation (Munné *et al.*, 1999).

Provided that the second hybridization is done in the microwave, denaturation can be performed on the hotplate. When we used a different microwave oven for denaturation in pilot experiments (Crosley Group Inc., Winston-Salem, NC, USA; 1450 W, 2450 MHz) the coverslip exploded in a few

experiments damaging the fixed nuclei. However, hybridization was never a problem with any microwave oven tested. Clearly, a microwave oven, and perhaps special slides and coverslips, could be designed to improve microwave denaturation and hybridization conditions.

A second objective of our study was to obtain as much information as possible from a single cell using FISH. Specifically, we attempted to enumerate simultaneously chromosomes X, Y, 13, 15, 16, 18, 21 and 22 from a single blastomere, and this would cover 70% of the aneuploidies detected in spontaneous abortions. Recently, Liu *et al.* (1998) have performed cell recycling (sequential hybridizations) on the same cell three times, although they studied only a total of six chromosomes, and with a final efficiency of 78%. With microwave hybridization, a fourth round of cell recycling, and four sets of chromosome probes labelled with six different fluorophores (green, red, blue, aqua, gold, Cy5), the 24 chromosome types could be analysed in a single cell in a time frame compatible with IVF. Alternatively, methods to bring any cell into the metaphase stage for its further karyotype analysis have been recently published (Verlinsky and Evsikov 1999; Willadsen *et al.*, 1999).

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