Molecular cytogenetic analysis of human blastocysts and cytotrophoblasts by multi-color FISH and Spectral Imaging analyses

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ABSTRACT

Numerical chromosome aberrations in gametes typically lead to failed fertilization, spontaneous abortion or a chromosomally abnormal fetus. By means of preimplantation genetic diagnosis (PGD), we now can screen human embryos *in vitro* for aneuploidy before transferring the embryos to the uterus. PGD allows us to select unaffected embryos for transfer and increases the implantation rate in *in vitro* fertilization programs. Molecular cytogenetic analyses using multi-color fluorescence *in situ* hybridization (FISH) of blastomeres have become the major tool for preimplantation genetic screening of aneuploidy. However, current FISH technology can test for only a small number of chromosome abnormalities and hitherto failed to increase the pregnancy rates as expected. ~~**Therefore, we developed technologies to fully karyotype single blastomeres using Spectral Imaging**~~. **We are in the process of developing technologies to score all 24 chromosomes in single cells within a 3 day time limit, which we believe is vital to the clinical setting**. **Also**, human placental cytotrophoblasts (CTBs) at the fetal-maternal interface acquire aneuploidies as they differentiate to an invasive phenotype. About 20-50% of invasive CTB cells from uncomplicated pregnancies were **found** aneuploidy, suggesting that the acquisition of aneuploidy is an important component of normal placentation, perhaps limiting the proliferative and invasive potential of CTBs. Since most invasive CTBs are interphase cells and possess extreme heterogeneity, we applied multi-color FISH **and repeated hybridizations** to investigate individual CTBs. ~~**In summary, this study demonstrates the strength of Spectral Imaging analysis and repeated hybridizations, which will allow us to fully karyotype single interphase cells.**~~ **In summary, this study demonstrates the strength of Spectral Imaging analysis and repeated hybridizations, which provides a basis for full karyotype analysis of single interphase cells.**

Keywords: Interphase cell, PGD, blastomeres, cytotrophoblast, aneuploidy, fluorescence *in situ* hybridization, Spectral Imaging.

INTRODUCTION

Numerical chromosome abnormalities have long been recognized as the major cause of reproductive failure, with an incidence of 21% in spontaneous abortions (Hassold et al., 1980, Warburton et al., 1980, 1986). Of these, trisomies involving chromosomes 21, 18, 16 and 13 and gonosomal abnormalities account for 50% of chromosomally abnormal abortions. In contrast to single gene defects, numerical chromosome abnormalities frequently occur *de novo*. The only known risk factor is maternal age, with the incidence of trisomy detected by amniocentesis increasing from 0.6% to 2.2% from age 35 to age 40 (Hook et al., 1992). Thus, analysis of embryos from older *in vitro* fertilization (IVF) patients using preimplantation genetic diagnosis (PGD) should significantly reduce the incidence of trisomic conceptuses and spontaneous abortions as well as the chances of delivering a trisomic offspring. Ploidy assessment of single blastomeres by fluorescence *in situ* hybridization (FISH) was first achieved in a time frame compatible with IVF in 1993 (Munné et al., 1993). **Based on the hypothesis that PGD of numerical chromosome abnormalities in embryos increases the pregnancy rates in women of advanced maternal age undergoing IVF, we are continuing with the goal of analyzing an increasing number of chromosomes.**

Currently, negative selection of aneuploid gametes or IVF embryos can only be done through PGD either by polar body or blastomere analysis (Sermon et al., 2004). Low metaphase yield and less than 30% of karyotypable metaphases together with the requirement of overnight culture in antimitotics (Santaló et al., 1995) make karyotype analysis by Giemsa-banding unsuitable for PGD. FISH, on the other hand, allows chromosome enumeration to be performed on interphase cell nuclei, without a need for culturing cells or preparing metaphase spreads. FISH has been applied to PGD of common aneuploidies using either human blastomeres (cells from **6** to 16-cell stage embryos) or oocyte polar bodies (Munné and Weier, 1996; Munné et al., 1998, 1999; Verlinsky et al., 1998a,b). Currently, commercially available kits allow only 5 chromosomes (X,Y,13,18,21 or 13,16,18,21,22) (Vysis, Inc., Downers Grove,

IL) to be detected simultaneously in interphase cells by filter-based fluorescence microscope systems. Repeated hybridizations on single interphase cells must be applied in order to score additional chromosomes. Some PGD centers screen chromosomes X, Y, 13, 14, 15, 16, 18, 21 and 22 in blastomeres using 2 rounds of hybridizations (Bahçe et al., 2000), with the potential of detecting 70% of the aneuploidies involved in spontaneous abortions. Ideally, one would like to detect aneuploidy involving any of the 24 human chromosomes in one to two blastomeres biopsied for PGD. With most cells available for analysis likely to be in interphase, we recognized a need to develop a rapid and innovative chromosome enumeration protocol based on the hybridization of a larger set of chromosome-specific probes.

Our studies of aneuploidy in early human development are not limited to fetal cells, but also extend to placental cells and tissues. We found that human cytotrophoblasts (CTBs) at the fetal-maternal interface become aneuploid as they begin to differentiate and change their gene expression repertoire and phenotype from that of a pluripotent progenitor CTBs to a more committed, invasive cell type (Weier et al., 2005). During the development of the placenta, CTB invasion is limited to the decidualized endometrium and the inner third of the myometrium, making this process more akin to tumorigenesis than to organogenesis (Fisher et al., 1989; Librach et al., 1991). In most pregnancies, the fetus and the placenta have the same chromosomal complement because both structures are descendants of the same zygote. But in 1-2% of viable pregnancies, chorionic villus sampling (CVS) at 10 to 12 weeks of gestation revealed a cytogenetic abnormality (most often trisomy) confined to the placenta (Ledbetter et al., 1992; Jenkins and Wapner, 1999). Confined placental mosaicism (CPM) (Lestou and Kalousek, 1998) can occur due to postzygotic errors in mitotic duplication, in which case the conceptus has a normal karyotype. Alternatively, a trisomic blastocyst may be rescued by loss of the extra chromosome during embryonic cell mitosis, while the progenitor cells of the placenta remain trisomic. The trisomic cells can be confined to trophoblast layers, chorionic villus stroma, or both. In many instances, the level of mosaicism detected by CVS does not

reflect the level in the term placenta as a whole, which can vary considerably (Henderson et al., 1996).

To date, many genetic studies have examined floating villi and the cells they contain, including the trophoblast populations. Very little is known about the genotype of human CTBs that arise from anchoring villi and subsequently invade the uterine wall. Interestingly, in mice the analogous population of invasive trophoblasts undergoes endoreduplication (MacAuley et al., 1998; Nakayama et al., 1998). A few reports suggest the possibility that invasive human CTBs have an elevated ploidy level (hypertetraploid and hyperoctaploid) (Wakuda and Yoshida, 1992; Zybina et al., 2002). To identify the most common chromosomal changes in invasive CTBs, **a full karyotype of these cells is desirable**. ~~**We wanted to fully karyotype these cells.**~~ With almost all cells available for analysis in interphase, we needed to develop a reliable protocol to enumerate all 24 human chromosomes.

We devised a rapid enumeration procedure based on the hybridization of chromosome-specific probes and Spectral Imaging (SIm) analyses to ~~**fully**~~ karyotype single interphase cells. SIm is a powerful technique in which standard emission filters in a fluorescence microscope are replaced with an interferometer to record high-resolution spectra from fluorescently stained specimens. This bio-imaging system combines the techniques of fluorescence optical microscopy, charged coupled device imaging, and Fourier spectroscopy, **as well as** sophisticated software for digital image analysis. The power of this technology has been demonstrated by specific **whole-chromosome** staining of all 24 human chromosomes in metaphase spreads, termed “Spectral Karyotyping (SKY)”(Garini et al., 1996; Schröck et al., 1996). SKY has been applied in cancer studies (Schröck et al., 1996; Zitzelsberger et al., 1999, 2001), prenatal diagnosis (Ning et al., 1999), and human oocytes and polar body analysis (Márquez et al., 1998). Our group pioneered the development of protocols for simultaneous enumeration of 10 chromosomes in interphase nuclei using SIm (Fung et al., 1998, 2000), and applied it to the aneuploidy screening for human preimplantation embryos.

Our goal is to fully karyotype single interphase cells using FISH and SIm. We are in the process of developing three sets of probes and scoring eight chromosome types per hybridization. Existing SIm instrumentation can record fluorescence spectra with about 10nm resolution. This allows us to label probes uniquely with commercially available fluorochromes. Following image acquisition, the probes are removed and a different set of probes are hybridized to score a second group of chromosomes. By repeating the cycle of probe removal, hybridization and image acquisition once more with a third set of probes, we will have uniquely marked and recorded all 24 human chromosome types. In the present study, we demonstrate the use of SIm to score eight chromosomes per hybridization experiment in blastomeres and the feasibility of five repeated hybridizations on CTBs, both of which will form the basis for our proposed full karyotyping technique.

MATERIALS AND METHODS

Cells

Control metaphase spreads were made from phytohemagglutinin-stimulated short-term cultures of normal male lymphocytes according to the procedure described by Harper and Saunders (Harper and Saunders, 1981). Fixed lymphocytes were dropped on ethanol-cleaned slides in a CDS-5 Cytogenetic Drying Chamber (Thermatron Industries, Inc., Holland, MI) at 25°C and 45%-50% relative humidity.

Blastomeres were obtained from discarded embryos. All procedures followed protocols approved by the UCSF Committee on Human Research regarding use of embryos for research. Prior written consent was obtained from all donors. Individual blastomeres were incubated in a hypotonic solution of 1% Na-citrate, 6 mg/ml bovine serum albumin in water for 5 min before being placed on microscope slides and fixed with a solution of methanol / acetic acid (3/1 or 1/1, vol./vol.) (Tarkowski, 1966).

CTBs were isolated from human placentas. Portions of the placenta and basal plate (fetal-maternal interface) were collected immediately after elective pregnancy terminations. Written informed consent was obtained from all patients and full Institutional Review Board approval was obtained. Tissue sections of the fetal-maternal interface were fixed, embedded in OCT **(optimum cutting temperature, formulation of water-soluble sucrose and resins, Sakura Tissue-Tek OCT Compound)** and frozen in liquid nitrogen (Damsky et al., 1992). CTBs were isolated from the remaining placental tissues according to our published methods (Librach et al., 1991). The major steps included removal of the syncytium and release of CTBs by sequential enzymatic digestions (collagenase followed by trypsin digestion). The resulting cells were enriched using Percoll gradient centrifugation. Isolated cells were counted and adjusted to 10^6 cells/ml in serum-free medium (SFM). ~~**The purity of cell preparations was assessed after staining of a small fraction of cells with the anti-cytokeratin antibodies 7D3 (Damsky et al., 1992). Only preparations with at least 90% of placental cells were used in the**~~

~~**experiments**.~~ Ten-microliter aliquots of CTBs, 10 µl of fetal bovine serum and 200 µl of SFM were combined, spun onto Cytospin® microscope slides (Thermo Shandon, Pittsburg, PA) and stored at –20°C.

FISH and repeated hybridizations

Tissue sections (5 µm) cut from the **frozen** placental blocks were placed on silanized slides, and then fixed in Carnoy's fixative (acetic acid:methanol, 1:3) for 5 min. The slides were washed twice with 2x SSC (0.3 M NaCl, 0.03 M Na_3 citrate·$2H_2O$, pH 7.0; 5 min each), treated with 100 µg/ml pepsin **(Amresco, Solon, OH)** in 0.01 N HCl for 20 min at 37°C, washed with 2x SSC (twice for 5 min each) and subsequently again dried on a heat block for 5 min at 45°C. After incubation in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 min, the slides were washed twice with 2x SSC (5 min each time) and re-dried on a heat block for 5 min at 45°C. The **denaturation and** hybridization followed published protocols using Vysis probes for X, Y and 16 (Vysis, Inc.)

Fixed, isolated cells on slides were pretreated with 50 µg/ml pepsin ~~**(Amresco, Solon, OH)**~~ in 0.01 N HCl for 5-13 min at 37°C before immersion in PBS for 5 min. Next, the slides were incubated in 1% PFA in PBS for 5 min, then in PBS for 5 min. After a sequential change of ethanol (70%, 80%, 100%; 2 min per step), the slides were air dried. Cells fixed on slides were denatured for 5 min at 76°C in 70% formamide (FA) (Invitrogen, Gaithersburg, MD)/2× SSC and then dehydrated in 70%, 80%, and 100% ethanol (2 min per step) before air-drying. Meanwhile, the hybridization mixture was denatured for 5 min at 76°C, and pre-annealed for 30-90 min at 37°C. The denatured hybridization mixture was then applied to each slide, ~~**and cover slips were added and sealed with rubber cement.**~~ **and the** hybridization proceeded for 40 h at 37°C. After hybridization, all slides were washed three times (10 min each time, 43°C) in 50% FA/2× SSC, then twice in 2× SSC (10 min each time, 43°C). **The cells were stained with**

~~**slides were mounted with 8 µl of**~~ 4′,6-diamidino-2-phenylindole (DAPI, 0.5 µg/ml, Calbiochem, La Jolla, CA) dissolved in anti-fade medium (Fung et al., 2001a,b)**.**

Table 1 lists the fluorochrome labeling scheme for eight chromosome-specific DNA probes. Probes specific for repeated DNA on chromosomes 15, X, and Y were purchased from Vysis Inc. ~~**(now a wholly owned subsidiary of Abbott Molecular)**~~ and labeled with either a green or red fluorochrome (Spectrum Green or Spectrum Orange, respectively). **Locus-specific DNA probes for chromosome 13 (YAC 900g6), chromosome 18 (YAC 945b6), chromosome 21 (YAC 141g6), and chromosome 22 (YAC 849e9) were obtained from yeast artificial chromosome (YAC) (Genethon/CEPH library) (Weissenbach et al. 1992) purchased from Research Genetics. The probe specific for satellite II DNA of chromosome 16 was prepared from clone pHUR195 (Moyzis et al. 1987). Each DNA was labeled by random priming** ~~**The DNAs from clones specific for chromosomes 13, 16, 18, 21, and 22 were labeled by random priming**~~ (BioPrime kit, Invitrogen, Gaithersburg, MD) incorporating biotin-14-dCTP (part of the BioPrime kit), digoxigenin-11-dUTP (Roche Molecular Biochemicals, Indianapolis, IN), fluorescein-12-dUTP (Roche Molecular Biochemicals) (Fung et al., 2001a,b), or Cy3-dUTP (Amersham, Arlington Heights, IN). **All DNA probes were checked by using short term cultures of lymphocytes from a healthy male donor. Between 0.5 and 3 µl of each probe, plus 4 µl human COT1™** DNA (1 mg/ml, Invitrogen) and 1 µl salmon sperm DNA (20 mg/ml, 3'-5', Boulder, CO) were precipitated with 1 µl glycogen (Roche Molecular Biochemicals, 1 mg/ml) and 1/10 volume of 3M sodium acetate in 2 volumes of 2-propanol**,** air-dried and resuspended in 3 µl water, before 7 µl of hybridization master mix (78.6% FA, 14.3% dextran sulfate in 2.9x SSC, pH 7.0) was added. This gave a total hybridization mixture of 10 µl.

We also performed repeated hybridizations using five different sets of chromosome-specific probes to score 12 chromosomes (3, 6, 8, 9, 10, 11, 12, 16, 17, 18, X, and Y, Table 3). Different labeled centromere enumerator probes (CEP) (all CEP probes were from Vysis, Inc.) were combined in hybridization probe sets. After hybridizing the first probe set (CEP X

SpectrumGreen™, CEP Y SpectrumAqua, CEP 16 SpectrumRed) ~~**and acquiring images from six different areas of the slide**~~, we removed the probes by twice incubating the slide in **0.1**× SSC (5 min each time; 43°C), followed by denaturation for 2 min at 76°C and dehydration in a graded ethanol series (70%, 80%, 100%). Next, we denatured a mixture of CEP 8 SpectrumOrange and CEP 12 SpectrumGreen probes (set 2) and proceeded with an overnight hybridization. The process of probe stripping, denaturation and re-hybridization was performed three more times. Subsequent probe sets used were CEP 18 SpectrumOrange and CEP 17 SpectrumGreen (set 3), CEP 10 SpectrumOrange and WCP 9 SpectrumGreen (set 4), and a combination of a Cy5-labeled probe for chromosome 3 **(cat.#A75003, Biological Detection Systems, Inc., Pittsburg, PA)** ~~**Oncor, Gaithersburg, MD)**~~ with CEP 6 SpectrumOrange and CEP 11 SpectrumGreen (set 5).

Signal detection and data analysis

Signals were visualized by using fluorescence microscopes equipped with filters for DAPI, FITC, rhodamine, and Spectrum Aqua excitation and detection from various manufacturers. Spectral images were acquired using an SD200 SpectraCube™ spectral imaging system. ~~**with a Xenon light source (Applied Spectral Imaging, Ltd., Migdal Haemek, Israel). The SD200 imaging system attached to a Nikon E600 microscope consisted of an optical head (Sagnac interferometer) coupled to a multi-line CCD camera (Hamamatsu, Bridgewater, NJ) to capture images at discrete interferometric steps.**~~ The multiple band pass filter set used for fluorochrome excitation was custom-designed (SKY-1, Chroma Technology, Brattleboro, VT) to provide broad emission bands (giving a fractional spectral reading from ~ 450 nm to ~ 850 nm). ~~**DAPI images were recorded using a DAPI-specific optical filter set**~~. The spectral information was displayed by assigning red, green and blue colors (RGB color image) to three areas of interest in the spectrum. Based on the measured spectrum for each signal domain, a spectral classification algorithm compared the

measured spectra with reference spectra allowing the assignment of a pseudocolor to all points in the image that had the same spectrum. ~~**This algorithm forms the basis for chromosome identification by spectral karyotyping.**~~ Thus, a classification color image and a karyotype table were obtained. The probe set shown in Table 1 was hybridized to metaphase spreads with known chromosome identities to build the reference spectra library. This library was essential for karyotyping metaphase spreads and interphase nuclei.

The scoring criteria used for both tissue and isolated cells eliminate any nuclei that are overlapping or ruptured. When analyzing cells in tissue sections, only the individual nuclei showing at least one signal per required chromosome (X, Y, 16 for male samples or X, 16 for female samples) were scored. Analysis of tissue section from different parts of the placenta, at least 40 cells were scored in each part. Hybridization signals were counted according to the criteria published by Hopman *et al.* (Hopman et al., 1986): pairs that were spaced less than the diameter of a signal domain were counted as one chromosome, and pairs that were farther apart than the diameter of a signal domain were counted as two chromosomes.

RESULTS AND DISCUSSION

~~**Our goal is to fully karyotype single interphase cells using FISH and SIm. We are in the process of developing three sets of probes and scoring eight chromosome types per hybridization. Existing SIm instrumentation can record fluorescence spectra with about 10nm resolution. This allows us to label probes uniquely with commercially available fluorochromes. Following image acquisition, the probes are removed and a different set of probes are hybridized to score a second group of chromosomes. By repeating the cycle of probe removal, hybridization and image acquisition once more with a third set of probes, we will have uniquely marked and recorded all 24 human chromosome types. In the present study, we demonstrate the use of SIm to score eight chromosomes per hybridization experiment in blastomeres and the feasibility of five repeated hybridizations on CTBs, both of which will form the basis for our proposed full karyotyping technique.**~~

Multi-color FISH, Spectral Imaging analysis for interphase cells

Present FISH technology using commercially available probes (i.e., probes from Vysis, Inc.) limits ploidy analysis of **interphase cells** to a maximum of 5 chromosomes per hybridization ~~**experiment based on filter-based microscopes**~~. Using SIm, we now can routinely score eight to ten chromosomes in single interphase cells ~~**nuclei**~~ per hybridization (Fung et al., 1998, 2000, 2001b). In the present study, six fluorochromes (**FITC, Spectrum** Green, Spectrum Orange, Cy3, Cy5, and Cy5.5) were used to label eight DNA probes (Table 1). The emission maxima of **FITC, Spectrum Green**, Spectrum Orange (or Cy3), Cy5, and Cy5.5 are **525 nm, 530 nm**, 592 nm, 678 nm, and 702 nm, respectively. The emission spectra of Cy3 probes prepared in house and the commercially available Spectrum Orange labeled DNA probes were found to be identical, therefore these two fluorochromes were indistinguishable. Fig.1A shows the RGB pseudo-color image of a normal metaphase spread hybridized with the

probe set shown in Table 1. The image was acquired using a SIm system and **a SKY-1 filter**. Fig.1B is the corresponding inverted DAPI image **acquired using a SIm system and a DAPI filter**. The signals in the RGB color image were selected by manually drawing contour lines (red) around each signal domain. To make the signals in Figs. 1A and 1D **more** clearly visible, the contour lines are not shown. As shown in Figs. 1A-1B, a total of 14 signals were counted. After the spectrum of each signal was compared to the reference spectra library, the classification color image (Fig. 1C) and a karyotype table (data not shown) were constructed. The size and shape of the signals in the classification color image (Fig. 1C) were the same as the size and shape of each contour (red, Fig. 1B). Figs.1D-1F shows results of our SIm analysis of one blastomere. In the karyotype table (Fig. 1F), chromosomal signals were grouped such that signals from the RGB color image (Fig. 1D) were aligned with corresponding images from the classification color image (Fig. 1E). The normal karyotype (Fig. 1F) showed two copies each of chromosome 13, chromosome 15, chromosome 16, chromosome 18, chromosome 21, chromosome 22, and one copy each of the X and Y chromosomes, as expected for a diploid interphase nucleus.

Chromosome enumeration in tissue sections from the fetal-maternal interface

We have found that a subset of freshly isolated CTBs from uncomplicated pregnancies have numerical chromosomal abnormalities (Weier et al., 2005). When hybridized with three chromosome enumerator DNA probes (CEP X, CEP Y, and CEP 16), we observed that many CTBs displayed a continuum of CEP X signals that ranged from closely spaced pairs to widely separated signals. In general, the copy number of chromosome X was greater than the copy number of chromosome 16. We studied a total of 14 placental specimens (8 male and 6 female specimens) from uncomplicated pregnancies, as shown in Table 2. Hybridization signals were scored in the nuclei of cells from the basal plate (BP), syncytiotrophoblasts (ST) and the mesenchyme cells in the villous core of floating villi (VC). **At least 40 cells were scored in**

each part. **Progenitor CTBs and densely packed CTBs in columns were not scored due to overlap of nuclei**. ~~**The results shown in Table 2 report the fraction of normal diploid cells, i.e., male cells showing (X,Y,16,16) and female cells showing (X,X,16,16) in percent of the total number of cells scored.**~~ In our analyses of 5 µm sections, we scored cells as 'normal' when they showed a diploid chromosome complement [(X,Y,16,16) or (X,X,16,16)]. The fraction of 'hyperdiploid cells' is comprised of cells showing gains of chromosomes. Cells lacking one signal were scored as 'hypodiploid'. The group of 'multi' is comprised of cells showing gains or loss of more than one chromosome. The fact that incomplete nuclei were included in the analyses is expected to overestimate the fraction of hypodiploid cells, and underestimate the fraction of diploid and hyperdiploid cells. We have found that the fraction of aneuploid cells increases as a function of gestational age, and aneuploid cells exist in all three compartments studied. The results also show that the cells in the basal plate had a higher fraction of hyperdiploid cells, suggesting that CTBs accumulate aneuploidies as they differentiate along the invasive pathway. Finally, analysis of tissue sections showed that the spatial distribution of the aneuploid trophoblasts appeared to be random, suggesting that cells acquire aneusomies sporadically as opposed to clonal expansion of an aneuploid CTB subset.

The rates of hyperdiploid cells in tissue sections in the present study were ~15% (first trimester), ~25% (second trimester), and ~34% (at term), when we combined the numbers of both" "hyper" and "multi" categories in Table 2. Isolated cells from the same placentas were analyzed, and previously published: the mean percentage of hyperdiploid cells among isolated CTBs was 16.9 % for first trimester samples, and 32.5% for second trimester and term samples (Weier et al., 2005). On average, more than 20% of non-overlapping CTBs were indeed hyperdiploid, although all cytospin preparations evidently contain a fraction of clumped cells. Thus, there were no significant differences between results obtained with isolated cells and the corresponding tissue sections. Therefore, the measured of ploidy levels of cells in present study were not due to overlapping nuclei.

Repeated hybridization on isolated CTBs

Comparative genomic hybridization (CGH) has been proposed as a rapid approach to chromosome enumeration in fetal and placental tissues (Yu et al., 1997; Levy et al., 2000; Lestou et al., 2000; Lomax, et al., 2000; Barrett et al., 2001; Tabet et al., 2001). However, the spatial distribution of the aneuploid CTBs in tissue appeared to be random. Tissue heterogeneity makes the CGH approach unlikely to provide meaningful information about the frequency and type of aneusomy in individual CTBs. Therefore, we performed repeated hybridizations to score 12 chromosomes (3, 6, 8, 9, 10, 11,12, 16, 17, 18, X, and Y) in individual CTBs **(Fig. 2, Fig. 3, Table 3).** We hybridized the isolated CTBs with the first set of probes **(CEP X, CEP Y, and CEP 16; Fig. 2B, Fig. 3B)**. ~~**The number of signal domains in Fig. 2 is listed in Table 3. The sample was found to be a female**~~. We then removed the probes and rehybridized the cells with **the second set of probes (CEP 12 and CEP 8; Figs. 2C and 3C)** ~~**a probe mixture of CEP 8-Spectrum Orange and CEP 12-Spectrum Green probes (set 2, Fig.2).**~~ The process of probe stripping, denaturation and re-hybridization was repeated three more times **with the third set (CEP 17 and CEP 18; Figs. 2D and 3D), the fourth set (WCP 9 and CEP 10; Figs. 2E and 3E), and the fifth set (CEP 11, CEP 6, and CEP 3; Figs. 2F and 3F)**. ~~**The succeeding probe sets were comprised of CEP18-Spectrum Orange and CEP 17-Spectrum Green (set 3, Fig. 2D), CEP 10-Spectrum Orange and chromosome 9-specific Spectrum Green-labeled probe (this turned out to be a whole chromosome painting probe)(set 4, Fig. 2E), and a combination of a Cy5-labeled probe for chromosome 3 with CEP 6-Spectrum Orange and CEP 11-Spectrum Green (set 5, Fig. 2F).**~~ The five repeated hybridizations allowed us to score 12 different chromosomes in each of these cells. Upon close examination of the images, it appeared **that a few cells retained a small amount of CEP 17-Spectrum Green after the third probe removal step**. ~~**This indicates a need to further optimize the procedure and include quality control steps such as visual inspection prior to re-hybridization.**~~ In the

experiment described here, **however**, it did not lead to misinterpretations, since the green probe in set 4 ('CEP-9') turned out to be a WCP probe. **We found the WCP 9 probe was very easy to score in this study, since the hybridization domains showed up with excellent contrast and were nicely separated, as shown in Fig. 2E. Our lab had demonstrated the use of high complexity WCP probes for chromosome enumeration in interphase cells (Kuo et al., 1991). Thus, with WCP 9-scoring this easy, we did not feel a need to repeat the hybridizations using a CEP 9 probe.**

The single hyperdiploid CTB in Fig. 2 contained one copy of chromosome 16, two copies of chromosomes 3, 8, 9, 10, 12, and 18, three copies of chromosome 11, four copies of chromosome X and 17, as well as five copies of chromosome 6. Fig. 3 shows the FISH images of a group of CTBs, and the number of hybridization domains of seven single cells is listed in Table 3. Some of the signals were not shown clearly in Fig.3, due to their location in different focal planes and their uneven intensity. Scoring signals visually on the microscope is quite different from judging FISH signals from lower resolution digital images, because of the limited depth-of-focus of high magnification microscope lenses. Whenever we noted a problem with FISH signals in different planes [in the Z-direction], we took images in two or more planes to document the results. If not scored and recorded immediately, results were evaluated taking all images from the 'Z-stack' into account. Further analysis of **69** cells from the same CTB preparation, **as shown in Figs. 2 and 3,** revealed an aneuploidy rate of 97.3%. The average copy number (mean ± standard deviation) of each chromosome was as follows: chromosome 3, **2.50** ± 0.89; chromosome 6, 2.66 ± 0.87; chromosome 8, 2.24 ± 0.83; chromosome 9, **2.03 ± 0.69**; chromosome 10, **2.54 ± 0.94**; chromosome 11, **2.62 ± 0.90**; chromosome 12, 2.43 ± 1.07; chromosome 16, **2.15 ± 0.76**; chromosome 17, **1.93 ± 0.72**; chromosome 18, **2.29 ± 0.93**; and chromosome X, **2.72 ± 1.09**. The molecular cytogenetic analysis of CTBs suggests that aneu-ploidy is an important cellular **development during** normal placentation, possibly limiting the

proliferative and invasive potential of CTBs.

The results obtained with this case of female CTBs demonstrated that: 1) this cell type can be hybridized repeatedly; 2) dehydration is important to keep the cell flat after probe stripping; 3) **more than 90% of the scored CTBs were involved in aneuploidy** ~~**All seven non-overlapping cells in this field of view were aneuploid (Table 3)**~~; 4) all of the 10 scored autosomes and the X chromosomes are involved in aneuploidy, although they show different rates of aneusomy; 5) the number of copies of the autosomes per cell ranged from 1 to 5; none of the chromosome types were found to be entirely absent; 6) some of the extra chromosomes showed up in paired arrangements, most notable the chromosomes 6, 11, 12 and X.

The challenges of FISH and SIm

The detection and enumeration of chromosome-specific signal domains in interphase cells is often complicated by reduced **probe penetration** into the interphase nuclei, overlapping or overly spread signals, and high levels of nuclear autofluorescence. Therefore, challenges are the definition of suitable, minimally overlapping hybridization targets, optimization of cell pretreatment, hybridization and removal conditions as well as the interactive digital image processing to increase the rate of analysis. The repeated probe stripping and re-hybridization is actually far less challenging than generally expected. Similar schemes with only 2-3 probes have been used before by us in PGD and by others in prenatal analysis (Zhen, 1998). **CGH might be an alternative technique for karyotyping single cells (Wells et al., 2000, 2002) for PGD, but technical protocols are not yet sufficiently robust for routine clinical applications. Another shortfall of CGH for single cell analysis is the fact that it does not allow one to perform control experiments on the same cell, since the DNA needs to be in vitro amplified using the polymerase chain reaction (Erlich, 1999), thus destroying the nuclear morphology. FISH assays, on the other hand, can be repeated at least 10 times on the same cell (Walch et al., 2001) allowing repeated tests in situations with ambiguous**

results. ~~Using single probes in as many as ten repeated hybridizations, a recent report described studies of tumor tissue sections with negligible loss of DNA (Walch et al., 2001).~~

While developing probe sets for SIm analysis of interphase cells, we had to optimize several hybridization parameters such as target DNA preparation, probe labeling, and fluorochrome selection. The ideal probe set should be comprised of bright, single-copy locus-specific probes rather than DNA repeat probes to avoid cross-hybridization and domain clustering. In general, single copy probes like those prepared from bacterial artificial chromosome (BAC) or YAC clones require blocking of interspersed repeats (LINEs, SINEs), which is commonly achieved by addition of unlabelled COT1 DNA. Probes that target DNA repeats, on the other hand, can often be prepared highly specific and need minimal blocking or no blocking during hybridization. Thus, single copy and DNA repeat probes are best used separately. We also notice that ratio-labeling schemes did not work with the same efficiency. Often, one fluorochrome yielded stronger signals than the other fluorochrome when both were bound on the same DNA. For example, the intensity of probes detected with Cy5.5 was usually much stronger than the intensity of Cy3 labeled probes. This effect was likely a combination of different quantum efficiencies of dyes, probe labeling index, energy transfer and detection sensitivity. In filter-based microscope systems, signals from weaker probes are typically enhanced through longer exposure times. The SD-200 Spectral Imaging system, however, uses the same exposure time for each interferogram in an exposure series. To adjust the ratio of Cy3 fluorescence to Cy5 or Cy5.5 fluorescence, the Cy3 probes were thus used at a higher concentration than their Cy5/5.5 counterparts. Carefully choosing a DNA target and the appropriate fluorochrome-labeled nucleotide triphosphates remain important issues for SIm analysis. **Nevertheless, Spectral Imaging analysis is powerful by comparing the spectrum of signals with the reference spectrum library, and eliminating nonspecific signals.** Ideally, we would like to label each

type of DNA probe in a set with a unique reporter molecule, i.e., performing nine-color, eight-target SIm analysis.

In conclusion, the technical developments presented in this work demonstrate the feasibility to score all chromosomes per cell using a combination of SIm and repeated hybridizations. It will be interesting to see if we can confirm previously published observations that the chromosomal abnormalities in CTBs are comprised mostly of chromosome gains. Knowledge about the distribution of aneusomies across the chromosome types will allow us to estimate the consequences of specific chromosome gains or losses for cell survival. Although not shown in this paper, we have found that the invasive, aneuploid CTBs show a very low level of bromodeoxyuridine incorporation, suggesting that they had exited the mitotic cell cycle. This explains the difficulties of culturing CTBs in vitro and a failure to prepare metaphase spreads (Weier et al., 2005).

Interestingly, the unproportionally high rate of chromosomal gains compared to losses might also explain why amniotic fluid sampling and interphase FISH analysis often fail to detect the expected number of chromosomes in uncultured samples (Bryndorf et al., 1997). The hypothesis that many chromosomal gains in invasive second trimester CTBs are compatible with cell survival, but not proliferation, while most losses appear to accelerate cell death and/or apoptosis, **remains challenging**. Since invasive CTBs acquire an endothelial phenotype during invasion and remodeling of maternal uterine blood vessels (Zhou et al., 1997), our observation of a high fraction of aneuploid CTBs in uncomplicated pregnancies also cautions the interpretation of interphase FISH results using fetal cells isolated from maternal blood.

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Table 1

Fluorochrome labeling scheme for chromosome-specific DNA probes [a]

Chromosome	FITC	Spectrum Green	Spectrum Orange	Cy3	Biotin (Cy5)	Digoxogenin (Cy5.5)
13	+					+
15			+			
16				+		+
18				+	+	
21						+
22					+	
X		+	+			
Y		+				

[a] Probes labeled with biotin or digoxigenin were detected with avidin-Cy5 and Cy5.5 conjugated antibodies against **digoxin**, respectively.

Table 2

Fractions of normal, hyper- and hypodiploid cells in placental cell compartments based on scoring of chromosomes 16, X, and Y in 5 μm thick tissue sections

Placental samples	Number	Gestational age weeks	Area [a]	Norm [b] %	Hyper %	Hypo %	Multi %
First trimester	6	8.5	ST	66.7	4.0	19.0	10.3
			VC	70.3	6.3	14.0	9.4
Second trimester	5	19.9	BP	50.4	22.4	15.4	11.8
			ST	59.4	11.6	14.6	14.4
			VC	68.6	3.2	17.6	10.6
Term	3	33.3	BP	36.7	22.0	18.7	22.6
			ST	52.3	17.3	12.7	17.7
			VC	58.0	8.3	20.0	13.7

[a] BP: cytotrophoblasts in the basal plate; ST: syncytiotrophoblasts; VC: mesenchyme cells in the villous core of floating villi. In the deciduas detached from the placenta in first trimester placental samples, the BP subpopulation was excluded. **Only the individual nuclei showing at least one signal of each chromosome (X, Y, 16 for male samples or X, 16 for female samples) were scored. Analysis of tissue section from different parts of the placenta, at least 40 cells were scored in each part**. **Progenitor CTBs and densely packed CTBs in columns were not scored due to overlap of nuclei**.

[b] Norm: cells with diploid chromosome complement; Hypo: hypodiploid cells; Hyper: hyperdiploid cells; Multi: cells with aneuploidy of multiple chromosomes.

Table 3

Number of hybridization domains in cytotrophoblasts [a]

	Green	Red	Green	Red	Green	Red	Green	Red	Green	Red	Cy5
Cell #	Chr.X [b]	Chr.16	Chr.12	Chr.8	Chr.17	Chr.18	Chr.9	Chr.10	Chr.11	Chr.6	Chr.3
Cell 1	**2**	**2**	**2**	**2**	**3**	**2**	**2**	**2**	**2**	**2**	**2**
Cell 2	**4**	**2**	**4**	**2**	**1**	**2**	**2**	**2**	**2**	**3**	**2**
Cell 3	**3**	**2**	**2**	**2**	**1**	**2**	**2**	**2**	**2**	**2**	**3**
Cell 4	**1**	**2**	**2**	**2**	**1**	**2**	**2**	**2**	**3**	**3**	**5**
Cell 5	**2**	**3**	**2**	**2**	**2**	**2**	**2**	**1**	**2**	**2**	**2**
Cell 6	**1**	**2**	**2**	**2**	**2**	**2**	**1**	**1**	**2**	**2**	**3**
Cell 7	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**

[a] Results are from the seven non-overlapping cells shown in **Fig. 3A-F**.

[b] All the cells were female; no Y signals (CEP Y SpectrumAqua) were detected.

FIGURE LEGENDS

Figure 1. Fluorescence *in situ* hybridization results analyzed with the Spectral Imaging system on different human cell types. (A) The red, green and blue (RGB) colors of a metaphase spread from normal male lymphocytes **was acquired using a SIm system and a SKY-1 filter**, showing a total of 14 signals. The **representative chromosomes of each signals were labeled.** (B) The inverted DAPI image of the metaphase spread with contour lines **(red)** indicating the position of the chromosome-specific signals. **This was acquired using a SIm system and a DAPI filter.** (C) The classification color image corresponding to A **obtained by comparing the spectra for each signals with reference spectra.** (D) The RGB color image of a human blastomere. (E) The classification color image corresponding to D. (F) The karyotype table showing a total of 14 signals (chr.13 x2, chr.15 x2, chr.16 x2, chr.18 x2, chr.21 x2, chr.22 x2, chr.X, and chr.Y) in the blastomere. Please note that the contour lines in A and D are not shown.

Figure 2: Fluorescence *in situ* hybridization analysis of aneuploid cytotrophoblast cells isolated from the fetal-maternal interface. (A) DAPI image of a single cell. (B) Hybridization of probes for chromosomes X (green), 16 (red) and Y (blue) showed a single hybridization domain for the chromosome 16-specific probe, and paired hybridization signals for the chromosome X-specific probe (arrowheads). No chromosome Y-specific signals was detected. (C) Probes for chromosome 12 (green) and 8 (red) showed 2 signals each in the nucleus of this cell. (D) We detected two chromosome 18-specific signals in this cell (red), while the chromosome 17-specific probe showed four hybridization domains (green). Please note that one of the green signals is in a different focal plane. (E) Hybridization signals for chromosome 9- (WCP 9, green) and chromosome 10 (red) – specific probes suggested two copies of each of

these chromosomes. Please note the excellent delineation of the chromosome 9 – specific DNA by the green WCP 9 probe. (F) The hybridization of a mixture containing probes for chromosomes 11 (green), 6 (red) and 3 (infrared) revealed three green, five red and two infrared signals in this nucleus. The chromosome 3-specific signals are shown in light blue superimposed on the DAPI image (grayscale) and are indicated by blue arrows. The green arrows point to two chromosome 11-specific signals in a rather large distance from one another.

Figure 3. Fluorescence *in situ* hybridization analysis of cytotrophoblast aneuploidy. **The DNA probes were identical to those mentioned in Fig.2.** ~~**(see Table 3 for details).**~~ Repeated hybridizations to cytotrophoblasts revealed chromosome-specific aneusomy patterns. (A) The nuclei were visualized by DAPI staining. ~~**The white arrows indicate the cell number.**~~ (B-F) FISH results of five repeated hybridizations of the chromosomes **indicated in the lower left.** ~~**The green and red arrows indicate the paired signals**~~. **The cell identifiers are indicated in A, and the number of hybridization domains in images B-F are listed in Table 3. The signals were scored using multi pass filters with single wavelength excitation. Some of the signals were not shown clearly in B-F, due to their location in different focal planes and their uneven intensity.**