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Spectral Imaging in Preconception/Preimplantation Genetic Diagnosis of Aneuploidy: Multicolor, Multichromosome Screening of Single Cells

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Purpose: *Our purpose was to evaluate the utility of spectral imaging for multicolor, multichromosome enumeration in human interphase cell nuclei.*

Methods: *Chromosome-specific probes labeled with different fluorochromes or nonfluorescent haptens were obtained commercially or prepared in-house. Metaphase spreads, interphase lymphocytes, or blastomeres cells were hybridized with either 7 or 11 distinctly different probes. Following 46 hr of hybridization, slides were washed and detected using either a filter-based quantitative image processing system (QUIPS) developed in-house or a commercial spectral imaging system.*

Results: *The filter-based fluorescence microscope system is preferred for simultaneous detection of up to seven chromosome targets because of its high sensitivity and speed. However, this approach may not be applicable to interphase cells when 11 or more targets need to be discriminated. Interferometer-based spectral imaging with a spectral resolution of approximately 10 nm allows labeling of chromosome-specific DNA probes with fluorochromes having greatly overlapping emission spectra. This leads to increases in the number of fluorochromes or fluorochrome combinations available to score unambiguously chromosomes in interphase nuclei.*

Conclusions: *Spectral imaging provides a significant improvement over conventional filter-based microscope systems for enumeration of multiple chromosomes in interphase nuclei, although further technical development is necessary in its application to embryonic blastomeres. When applied to preconception/preimplantation genetic diagnosis, presently available probes for spectral imaging are expected to detect abnormalities responsible for 70–80% of spontaneous abortions caused by chromosomal trisomies.*

KEY WORDS: aneuploidy; diagnosis; interphase cells; preconception; preimplantation genetic diagnosis; fluorescence in situ hybridization; spectral imaging.

INTRODUCTION

Numerical aberrations involving parts of or entire chromosomes have detrimental effects on mammalian embryonic, fetal, and postnatal development. While all autosomal monosomies lead to periimplantation loss and most trisomies lead to early fetal loss, some numerical imbalances may come to term and give rise to karyotypically and phenotypically abnormal offspring (1,2). Extrapolating results from normal conceptuses, where first-trimester spontaneous abortions due to trisomies alone reach an estimated 27% of all spontaneous abortions (2–4), suggests that failure of implantation and early fetal loss in in vitro fertilization (IVF) programs are likely to be caused by similar mechanisms requiring precise control of timed gene expression and thus affected by any change in gene dosage. Conversely, preconception and preimplantation genetic diagnosis (PGD) will facilitate the selection of chromosomally normal oocytes for fertilization or diploid embryos for transfer. Thus, PGD is expected to increase IVF success rates, thereby resulting in higher implantation rates per embryo transferred and lower rates of spontaneous abortion.

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Because aneuploidy of any of the 24 human chromosomes will have catastrophic consequences, the desired PGD test will score all chromosomes for their correct number and alert the investigator, if the number of any of the autosomes or gonosomes found in a cell deviates from the normal value of either one or two, dependent on target chromosome and cell type. Our research and development therefore focus on assays that allow rapid scoring of all human chromosomes in few individual cells.

This imposes very stringent conditions on the assay performance. Unlike typical situations found in tumor cell research, when many thousands of cells are available for analysis and failure in the analysis of some or most of the cells may be tolerable, our assay has to provide reliable information based on the analysis of only one or two cells, i.e., polar bodies or blastomeres. We also strive to analyze or score all chromosomes at the same time, which represents a major advancement beyond state-of-the-art analytical PGD techniques. With a background of experience in the biopsy, fixation, and fluorescence in situ hybridization (FISH) analysis of single blastomere cells using five or fewer chromosome-specific probes (5), it is now possible to investigate the technical capabilities of advanced hybridization and detection systems.

Spectral karyotyping (SKY) has recently been developed for genomewide screening of metaphase chromosome spreads for translocations involving nonhomologous chromosomes (6). In that implementation, the technique is based on hybridization of 24 chromosome-specific DNA probe libraries that can be resolved by image spectrofluorometry. We adapted the SKY technique to enumerate a large number of chromosome-specific probe hybridization domains in interphase cell nuclei. We focused our effort on scoring of human chromosomes 3, 4, 7, 13, 14, 16, 18, 21, 22, X, and Y, because trisomies involving these chromosomes account for approximately 70% of trisomies present in early embryos, as inferred from spontaneous abortions or trisomic conceptions that reach term (2,3). Among them, trisomy 16 alone accounts for almost 29% of all trisomy-based spontaneous abortions (2). Probes were obtained from commercial sources or prepared in-house and labeled with fluorochromes or nonfluorescent haptens, such as biotin and digoxigenin (7,8), requiring immunocytochemical detection. In this study, we report our experience in probe labeling and detection and compare the utility of SKY with the quantitative image processing system (QUIPS) based on computer-controlled selection of fluorescence excitation and emission filters (9).

MATERIALS AND METHODS

Cells

Metaphase spreads were prepared from phytohaemagglutinin-stimulated short-term cultures of normal male lymphocytes following standard procedures (10). Following hypotonic treatment with 75 m *M* KCl and fixation with 3:1 methanol:acetic acid, the cells were dropped on ethanol-cleaned slides inside a CDS-5 Cytogenetic Drying Chamber (Thermotron Industries, Holland, MI) at 25°C and 47.5% humidity. Slides were stored for at least 2 weeks in ambient air at room temperature, then placed in sealed plastic bags under nitrogen at −20°C until use.

Blastomere Preparation

Blastomeres from embryos donated for research were biopsied following the procedure described by Munné *et al.* (11). All our procedures followed protocols approved by the UCSF Committee on Human Research Internal Review Board. Individual blastomeres were fixed on microscope slides in methanol:acetic acid and their position was marked as described by Munné *et al.* (11). Blastomere cell samples were used within a week following fixation.

Probes

Ultraviolet excitation and the blue fluorescence emission range were used for observation of fluorescence from 4′,6-diamino-2-phenyl indole (DAPI; Calbiochem, La Jolla, CA), which was applied to counterstain DNA. Chromosome-specific DNA probes were labeled with dyes that fluoresce in the green, orange/red, or infrared wavelength interval, respectively (Table I). Tables IIA and B list DNA probes used for 7 and 11 chromosome-labeling reactions, respectively. The probe labeling scheme and detection of biotin- or digoxigenin-labeled probes is included in Table II. As much as possible, we used commercially

Table I. Spectral Characteristics of Fluorochromes Used

	Spectrum Green	FITC	Cy3	Spectrum Orange	Rhodamine	Cy5
Absorbance, max. (nm)	485	490	552	545	554	646
Fluorescence, max. (nm)	538	520	568	590	573	663

Table II. Fluorescence Labeling Scheme Using 7 (A) and 11 (B) DNA Probes

A

Chromosome	Spectrum Green	FITC	Spectrum Orange	Digoxigenin (rhodamine)	Biotin (Cy5)
10	33%	—	67%	—	—
14	—	80%	—	20%	—
16	—	—	—	50%	50%
18	100%	—	—	—	—
22	—	—	—	—	100%
X	—	—	100%	—	—
Y	67%	—	33%	—	—

B

Chromosome	Spectrum Green	FITC	Cy3	Spectrum Orange	Digoxigenin (rhodamine)	Biotin (Cy5)
3	—	100%	—	—	—	—
4	—	—	—	—	—	100%
7	100%	—	—	—	—	—
13	—	—	—	100%	—	—
14	—	25%	—	—	75%	—
16	—	33%	—	—	33%	33%
18	—	—	100%	—	—	—
21	—	—	—	—	25%	75%
22	—	—	—	—	100%	—
X	50%	—	—	50%	—	—
Y	75%	—	—	—	—	25%

available probes. The probes specific for repeated DNA on human chromosomes 7, 10, 13, 18, X, and Y were labeled with either a red fluorochrome (Spectrum Orange; Vysis, Downers Grove, IL) or a green fluorochrome (Spectrum Green; Vysis). A Cy3 fluorescent probe (Amersham, Arlington Heights, IL) was used to stain specifically the centromeric region of chromosome 18. The probe specific for repeated DNA of chromosome 16 was prepared from clone pHUR195 (12) following standard procedures of plasmid DNA isolation and random priming (13). The locus-specific DNA probes for chromosomes 3, 4, 14, 21, and 22, respectively, were obtained from PFGE-purified DNA of yeast artificial chromosome (YAC) clones as described (14,15). Following excision of the YAC-containing bands from the PFGE gels and β-agarase digestion of the gel slices (14), the pure YAC DNA was polymerase chain reaction–amplified using mixed-base primers (DOP-PCR) (13,16). The DNA for chromosomes 3, 4, 14, 16, 21, and 22 was labeled by random priming incorporating biotin-14-dCTP (part of the BioPrime kit; GIBCO/LTI, Gaithersburg, MD), digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN), fluorescein-12-dUTP (Boehringer Mannheim), or Cy5-11-dCTP (Amersham).

In Situ Hybridization

Approximately 20–40 ng of each probe along with 4 μl of COT1 DNA (1 mg/ml; GIBCO/LTI) and 4 μl of salmon sperm DNA (20 mg/ml, 3′–5′; Boulder, CO) was precipitated with 1 μl of glycogen (Boehringer Mannheim; 1 mg/ml) and 2-propanol and resuspended in 3 μl of water, before 7 μl of hybridization master mix [78.6% formamide (FA; GIBCO/LTI), 14.3% dextran sulfate in 2.9× SSC, pH 7.0 (2× SSC is 300 m*M* NaCl, 30 m*M* Na citrate)] was added. The hybridization mixture was denatured at 76°C for 5–7 min, then allowed to preanneal at either 20°C for 20 min or 37°C for 60 min. The slides were denatured for 3–3.5 min at 76°C in 70% FA/2× SSC, pH 7.0; dehydrated in 70, 80, and 100% ethanol for 2 min each step; and allowed to air-dry. Finally, the hybridization was allowed to proceed for approximately 46 hr in a moisture chamber at 37°C.

After hybridization, the slides were washed three times in 50% FA 2× SSC for 10 min, followed by two washes in 2× SSC for 10 min and one wash in 0.4× SSC for 5 min at 43°C. Bound biotinylated probes were detected by incubation of slides with avidin-Cy5 (Vector, Burlingame, CA) and digoxigenin-labeled probes were detected with anti-digoxigenin/rhodamine

[Boehringer Mannheim; 2 mg/ml in PNM (13)]. Subsequently, slides were washed three times in 2× SSC at 20°C for 10 min each. Finally, the slides were mounted in 8 μl of DAPI [0.5 μg/ml in antifade solution (0.1% p-phenylenediamine dihydrochloride; Sigma, St. Louis, MO), 0.1 × PBS (GIBCO/LTI), 45 m*M* $NaHCO_3$, 82% glycerol, pH 8.0] (11).

Quantitative Image Processing System (QUIPS)

Fluorescence microscopy was performed on a Zeiss Axioskop microscope equipped with a computer-controlled filter wheel (Ludl Electronic, Hawthorne, NY) to select individual excitation wavelength intervals and a quadruple-color filter set for fluorescence emission centered around 460 nm (DAPI, blue), 520 nm (FITC, green), 573 nm (rhodamine, red) and 663 nm (Cy5, infrared) (ChromaTechnology, Brattleboro, VT)(8,9). Images were collected using a cooled CCD camera (Photometrics, Tucson, AZ) connected to a Sun Sparc station (SUN Inc., Mountain View, CA).

Spectral Imaging

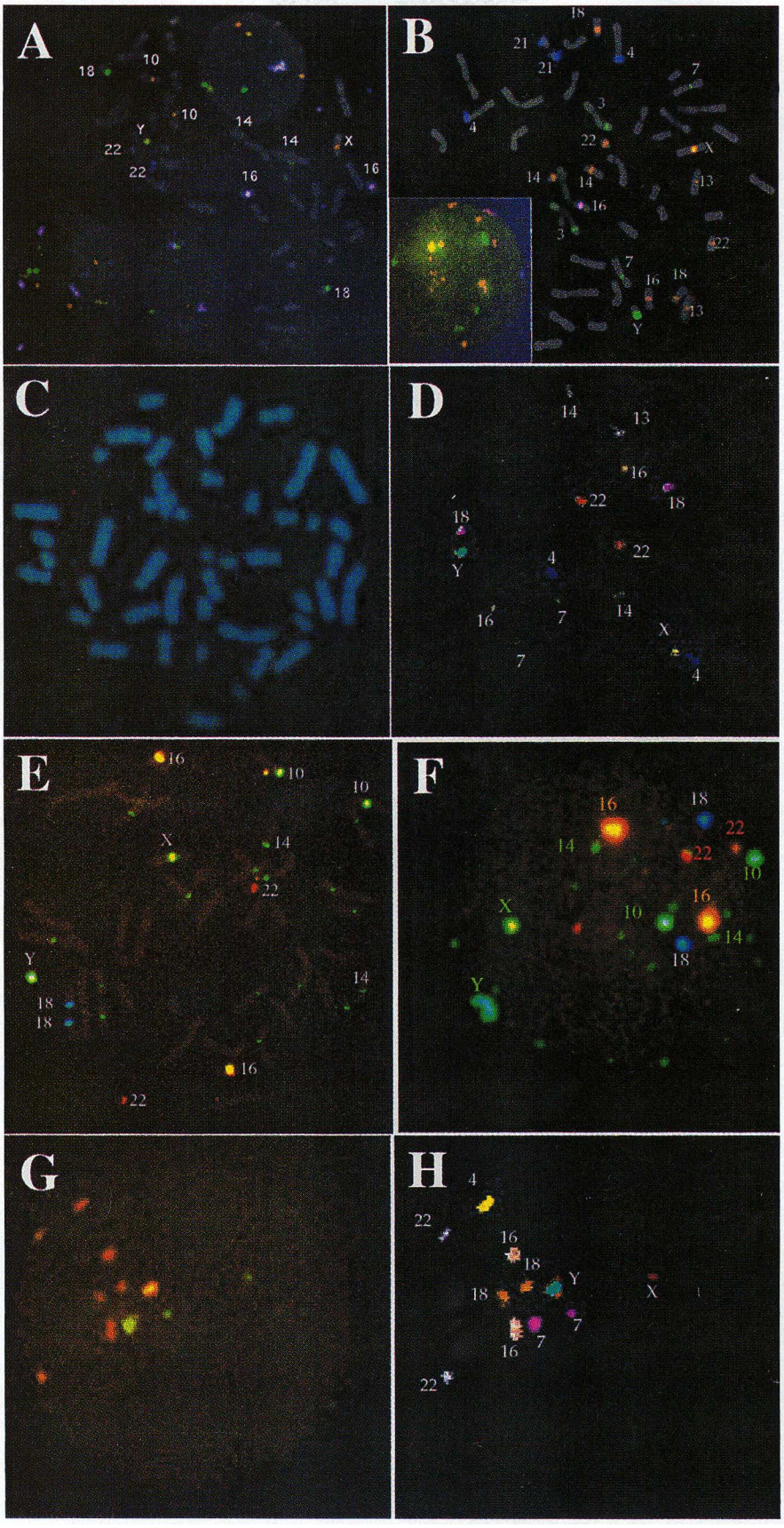
Spectral images were acquired and analyzed with the SD200 spectral bioimage system (Applied Spectral Imaging, Ltd., Migdal Haemek, Israel). The SD200 imaging system attached to an inverted microscope (Axiovert, Zeiss, Thornwood, NY) by means of a C-mount consisted of an optical head coupled to a multiline CCD camera (Hamamatsu, Bridgewater, NJ) to capture images at discrete interferometric steps, which were stored as an image stack in a Pentium 486/200-MHz computer. Next, each interferogram was Fourier-transformed, resulting in a fluorescence spectrum for each pixel of the image (6). The multiple-band pass filter set was custom-designed (Chroma Technology, Brattleboro, VT) to provide broad emission bands (giving a fractional spectral reading from ~450 to ~850 nm)(6). In a typical experiment, the spectral image was generated by acquiring 80–120 interferometric frames per object. After Fourier transformation, the measured spectrum at each pixel was divided into three spectral ranges (roughly 450–500, 500–650, and 650–850 nm, respectively), and the three ranges were assigned to different base colors (blue, green, red). This allowed the emission spectra to be converted to RGB display colors for visualization on a color monitor. Using the images of one or two metaphase spreads, individual chromosomes and their associated spectra were identified. A reference spectrum library was constructed based on spectra from known chromosomes and a classification color was assigned to each reference spectrum. Applying this spectrum-based classification library to the image of interphase nuclei, the individual emission spectra and, thus, specifically labeled chromosomes in the image could be identified and highlighted in classification colors. The determination of the number of copies of each chromosome in interphase cells was based on scoring the number of separate domains that matched the respective reference spectrum.

RESULTS

Lymphocyte Studies

The filter-based QUIPS system discriminated all seven chromosome-specific probes from set 1 (Table IIA). In this set, five chromosome-specific targets were labeled with distinct fluorochromes or color combinations, while chromosome 10- and Y-specific targets were hybridized with a combination of Spectrum Green- and Spectrum Orange-labeled probes at a 1:2 and a 2:1 ratio, respectively. With the exception of the Cy5-labeled probes specific for chromosomes 16 and 22, respectively, all hybridization domains could be identified by eye. Images were recorded as monochrome frames in the order DAPI–FITC–rhodamine–Cy5. With exposure times ranging from 0.2 to 2.0 sec, photobleaching was not found to impose significant problems. For display of the hybridization results, we found it useful to display the DAPI image in grayscale format and to overlay the FITC (display color green), rhodamine (display color red) and Cy5 (display color blue) images as shown in Figs. 1A and B. Approxi-

Fig. 1. *(opposite)* Hybridization results observed with the filter-based QUIPS or the spectral imaging system. The QUIPS system performed well when analyzing up to 7 chromosome-specific signals (A) but was limited to its four registration wavelength intervals (blue, green, red, infrared) and three display colors (red, green, blue) when 11 chromosomes were hybridized with probes with partly overlapping emission spectra (B). The spectral imaging system produced a similar display image, allowing the discrimination of seven probes in metaphase (C) as a well as interphase cells (D), but full spectra were available for detailed analysis. When analyzing the hybridization pattern of the 11-chromosome probe set, the DAPI metaphase image (E) was essential to define reference spectra of hybridization signals (F). This guided the analysis of interface cells. Similarly, tinted signals seen in display colors (G) could be resolved using prerecorded fluorescence spectra (H).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mately 70% of interphase nuclei showed the expected number of signals (Fig. 1A).

The interpretation of hybridization signals from the 11-chromosome set (Table IIB) was facilitated on metaphase spreads, where chromosome size and centromere location provided additional information for chromosome identification. In interphase cells, however, targets labeled with probes fluorescing in about the same wavelength interval were indistinguishable with the filter-based QUIPS system. In our set 2 (Table IIB), for example, the green probes for chromosome 3 (FITC) and chromosome 7 (Spectrum Green) as well as the red probes for chromosomes 13, 18, and 22 (labeled with Spectrum Orange, Cy3, and rhodamine, respectively) could not be distinguished by eye (Fig. 1B).

The spectral imaging system, in comparison, provided an entire spectrum for each picture element ("pixel") in the image. The system was calibrated by hybridization of the probe sets to metaphase spreads prepared from lymphocytes from a normal male donor (Figs. 1C and D). Hybridization of chromosome-specific probes was recorded together with the DAPI image (Fig. 1C). The fluorescence spectra of individual hybridization domains could then unambiguously be assigned to particular chromosomes and stored in the computer as a library of reference spectra. The procedure allowed us to record and identify all chromosome-specific targets using the seven-chromosome set (Figs. 1E and F) in metaphase and interphase cells, respectively. Cross-hybridization signals revealing a different fluorescence spectrum and, thus, a different probe composition could easily be identified as nonspecific signals. The main problem during the spectral imaging analysis, however, remained the loss of weak signals due to a thresholding step in the image analysis routine. In typical experiments, we were unable reliably to detect signals from our weaker chromosomes 3-, 13-, and 21-specific probes (Fig. 1D). With this exception, approximately 70% of interphase nuclei showed the expected number of signals (Fig. 1D).

When signals were above the threshold, the spectral imaging system could identify a chromosome-specific fluorescence spectrum even in the background of high levels of autofluorescence. As demonstrated in the example in Figs. 1G and H, signals from chromosomes 4-, 16-, 18-, and 22-specific targets appeared similar (red) when seen by eye or recorded using the filter-based system (Fig. 1G). However, background substraction and comparison with stored library spectra allowed unambiguous target identification using the spectral imaging system (Fig. 1H).

Blastomere Studies

Our studies on blastomeres were confined to the spectral imaging approach. Hybridization of the 7- and 11-probe sets on individual blastomeres produced results showing high levels of autofluorescent background in our material. While individual hybridization domains were distinguishable, the classification of signal spectra was complicated. When we applied a reference spectrum library recorded from metaphase spreads, the spectral imaging system misclassified signals and produced images with even higher levels of nonspecific background. The results suggested that the reference libraries recorded from metaphase spreads with a low level of autofluorescent background cannot be applied to blastomere preparations with much higher autofluorescence. Thus, further development of this approach using oocytes or blastomeres will require a refinement of fixation conditions to reduce the levels of autofluorescence background.

DISCUSSION

The spectral imaging system compares favorably to the filter-based QUIPS fluorescence microscope generating a wealth of information that allows the identification of many, if not all, human chromosomes with distinctly labeled probes. That all 24 human chromosome types can be unambiguously labeled and identified using chromosome painting probes has previously been demonstrated on metaphase spreads (6). For two reasons, the situation in interphase cell nuclei is much more complicated. First, interphase chromosomes occupy partially overlapping domains that are very difficult to resolve with whole-chromosome painting probes (17,18). Second, interphase cell nuclei, even those that were fixed and dropped on glass slides, possess a three-dimensional structure with several layers of DNA on top of one another (19,20). In the present labeling and hybridization scheme, most chromosome-specific probes are comprised of several probes, each labeled separately with a different reporter molecule. These individual probes are then combined at predefined ratios to produce the chromosome-specific spectrum. Spatial overlap constitutes a severe problem for the spectral imaging system, because as currently used FISH is not quantitative. Thus the fluorescence intensity is not a good measure for the number of chromosomes labeled, and spatially overlapping probes would produce the same spectrum as probes labeled with multiple reporters.

If any system seems appropriate to solve the overlap problem, it will be a spectral imaging rather than a discrete fluorescence wavelength interval, i.e., filter-based system. The spectral imaging system used in our study offers an ~10 nm resolution and allowed us to resolve separated but close fluorescence spectra of dyes such as Vysis' Spectrum Green and Boehringer Mannheim's FITC. Extrapolating these results, the spectral imaging system should be able to resolve at least 40 dyes in the 450 nm to 850 nm interval, if such dyes were available. It will be up to the commercial suppliers to develop and provide to the scientific community the necessary fluorochromes for this novel application. Given many recent developments, this task might be accomplished in the very near-future.

A major concern relates to the time needed for image acquisition and analysis using the spectral imaging system. Most filter-based systems take four to six images with individual exposure times below 2 sec. While exposure and readout of any spectral imaging are determined by the attached camera, interface, and computer system, in general, image capture requires significantly longer times, resulting in significant photobleaching. This is due to the high number of interferometric frames to be recorded in the spectral imaging system. In fact, we found that our specimens needed to be exposed for at least 2 min, which led to significant bleaching, and that it was often impossible to acquire a second image from the same specimen.

The first results using blastomere samples did not lead to informative images due to high levels of autofluorescent background. This background was observed irrespective of the probe combinations and was considered a fixation artifact. The results of Munné *et al.* (19,21) have demonstrated convincingly that blastomeres can be biopsied, fixed, and analyzed by FISH with minimal background fluorescence. Careful review of our protocol and alignment with the procedures described by Munné *et al.* is expected to solve this problem. The blastomere results also suggested that libraries of reference spectra recorded from the metaphase spreads may not be useful for classification of signals in the background of high autofluorescence levels.

In summary, spectral imaging has demonstrated its value for multitarget chromosome enumeration in interphase cell nuclei. Although image capture is very time-consuming in its present state, the development of additional fluorochromes and chromosome-specific probes will facilitate the enumeration of all chromosomes in single cells. This will enable a more comprehensive analysis of polar bodies and blastomeres, complementing existing PGD techniques.

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