

## Towards a full karyotype screening of interphase cells: ‘FISH and chip’ technology

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### Abstract

Numerical chromosome aberrations are incompatible with normal human development. Our laboratories develop hybridization-based screening tools that generate a maximum of cytogenetic information for each polar body or blastomere analyzed. The methods are developed considering that the abnormality might require preparation of case-specific probes and that only one or two cells will be available for diagnosis, most of which might be in the interphase stage. Furthermore, assay efficiencies have to be high, since there is typically not enough time to repeat an experiment or reconfirm a result prior to fertilization or embryo transfer. Structural alterations are delineated with breakpoint-spanning probes. When screening for numerical abnormalities, we apply a Spectral Imaging-based approach to simultaneously score as many as ten different chromosome types in individual interphase cells. Finally, DNA micro-arrays are under development to score all of the human chromosomes in a single experiment and to increase the resolution with which micro-deletions can be delineated. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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### 1. Introduction

Carriers of balanced translocations have an elevated risk of producing aneuploid germ cells due to disturbed homologue pairing. The resulting partial or total aneuploidies lead to spontaneous abortions, stillbirth or severe deficiencies and disease. Assisted reproductive technology now offers couples at risk several diagnostic approaches to reduce the risk of carrying an affected fetus. If the woman carries the abnormality, first polar bodies can be analyzed immediately after oocyte harvest. Following in vitro fertilization, pre-implantation

genetic analysis (PGD) can be performed on individual blastomeres biopsied from 3-day-old embryos. Since most of the embryonic cells will be found in interphase stage, the diagnostic approach will have to work reliably with either the less condensed chromatin in interphase cell nuclei or the highly condensed DNA in polar bodies (PB's).

Our collaborating laboratories have long been involved in the development of nucleic acid hybridization-based procedures for the rapid detection of structural and numerical chromosome abnormalities. Here, we report the present state of hybridization-based technologies for interphase cell analysis in PGD. Since only one or two cells are available for analysis, our approaches are geared towards obtaining a maximum of cytogenetic information per experiment.

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## 2. Material and methods

For more than a decade, our laboratories have been involved in the development of technologies for analysis of interphase and metaphase cells. In collaboration with scientists at the University of California, San Francisco, and the St Barnabas Medical Center, Livingston, researchers at the E.O. Lawrence Berkeley National Laboratory study the chromosomal composition of blastomeres with regard to numerical as well as structural aberrations. The technical aspects of our probe preparation and multicolor detection protocols have been published previously (Weier et al., 1994; Jossart et al., 1996; Cassel et al., 1997).

A major goal of our technical developments is to maximize the number of chromosomal targets that can be scored simultaneously. Briefly, probes specific for repeated DNA on chromosomes 15, X, and Y purchased from Vysis (Downers Grove, IL) were labeled with either a green or red fluorochrome (Spectrum Green or Spectrum Orange, respectively). The probes specific for chromosome 9, 13, 14, 16, 18, 21, and 22 were prepared in house and labeled by random priming (BioPrime Kit, GIBCO/LTI, 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) (Weier et al., 1995), or Cy3-dUTP (Amersham, Arlington Heights, IN). Bound biotinylated probes were detected with avidin-Cy5, and bound digoxigenin-labeled probes were detected with Cy5.5-conjugated antibodies against digoxin (Sigma, St. Louis, MO). Between 0.5 and 3  $\mu$ l of each probe along with 1  $\mu$ l human COT1™ DNA (1 mg/ml, GIBCO/LTI) and 1  $\mu$ l salmon sperm DNA (20 mg/ml, 3'–5', Boulder, CO) were precipitated with 1  $\mu$ l glycogen (Roche Molecular Biochemicals, 1 mg/ml) and 1/10 volume of 3 M sodium acetate in 2 volumes of 2-propanol, air dried and resuspended in 3  $\mu$ l water, before 7  $\mu$ l of hybridization master mix [78.6% formamide (FA, GIBCO/LTI), 14.3% dextran sulfate in  $2.9 \times$  SSC, pH 7.0 (1  $\times$  SSC is 150 mM NaCl, 15 mM Na citrate)] were added. This gave a total hybridization mixture of 10  $\mu$ l.

All blastomeres used in the probe developments were obtained from embryos donated by patients enrolled in the IVF Programs of The University of California, San Francisco, or The Institute for Reproductive Medicine and Science of Saint Barnabas Medical Center. In accordance with guidelines set by the internal review boards of these Medical Centers, written consent was obtained from the patients in each case. Embryo biopsies and blastomere fixations were carried out as described (Munné et al., 1994, 1996). As indicated below, embryos used for some studies had arrested development or were morphologically abnormal.

## 3. Results and discussion

Traditional filter based microscope systems limited fluorescence in situ hybridization (FISH) experiments to the simultaneous use of typically no more than three to five differently labeled probes for interphase analysis (Munné and Weier, 1996). This is sufficient to detect structural alterations in interphase and metaphase cells or score a few chromosomes in interphase cells (Munné et al., 1994; Munné and Weier, 1996; Munné et al., 1996). We prepared case-specific breakpoint-spanning probe contigs to identify intrachromosomal rearrangements such as inversions or deletions (Cassel et al., 1997). The same approach can be used to demonstrate interchromosomal rearrangements such as reciprocal translocations (Munné et al., 1998a; Fung et al., 1999; Weier et al., 1999). The case-specific probes allow one to discriminate between a normal karyotype, aneuploid cells, and a balanced karyotype carrying the derivative chromosomes. A less time consuming and, thus, less expensive approach using DNA probes that bind distal to the respective breakpoints can only be used to count the number of chromosome copies and thus cannot discriminate between the normal and the balanced karyotypes (Munné et al., 1998b).

### 3.1. Detection of structural chromosome aberrations

Our scheme for the detection of structural alteration is based on the preparation and hybridization of two differently labeled DNA probes that bind on both sides of the respective chromosome breakpoints (Cassel et al., 1997). One probe will be detected in the green fluorescence wavelength interval, while the second probe is made such that it fluoresces red. Normal homologues lack the rearrangement and the probes produce large hybridization domains that appear either red or green in the fluorescence microscope. At the same time, we counterstain the DNA with 4,6-diamino-2-phenylindole (DAPI) which fluoresces blue under ultraviolet light excitation. If the cell contains a derivative chromosome, the hybridization result will show a red/green associated or partially overlapping signal indicative of the translocation event. Thus, we detect structural alteration and score homologues at the same time.

Our work is greatly facilitated by access to resources created in the course of the International Human Genome Sequencing Project, such as large insert genomic DNA libraries (bacterial or yeast artificial chromosomes (BACs or YACs, respectively), high resolution physical maps or collections of cytogenetically mapped DNA probes (Chen et al., 1996; Kim et al., 1996; Korenberg et al., 1999). This enables us to prepare case-specific probe sets suitable for interphase cell analyses of most patient cells within a few weeks.

Once optimized, these probe sets allow to rapidly determine the exact number of normal chromosomes and derivative chromosomes in somatic cells from translocation carriers as well as their germ cells or offspring. So far, however, these procedures failed to produce the desired increase in pregnancy rates in cases where one spouse carried a balanced reciprocal translocation. Our concern is that the impaired homologue pairing in the carriers leads to gain or loss of other chromosomes which remains undetected in assays scoring only the translocation chromosomes.

The recent introduction of Spectral Imaging (SI) now allows one to interrogate a much larger number of targets, thus producing a more comprehensive picture of the chromosomal composition of the cells. SI allows an investigator to discriminate an increased number of fluorescent probes by exciting fluorescent molecules over a broad spectral range and by recording the fluorescence emission spectral using an interferometer.

### 3.2. Detection of numerical chromosome abnormalities using Spectral Imaging (SI)

Chromosome abnormalities occur with astonishing frequency in humans, being present in an estimated 10–30% of all fertilized eggs. Over 25% of all the miscarriages are monosomic or trisomics, making aneuploidy the leading known cause of pregnancy loss. Ideally, one likes to detect aneuploidy involving any of the 24 human chromosomes for preimplantation genetic and prenatal diagnosis. Thus, an analytical method to enumerate as many chromosomes as possible in few interphase cells is highly desirable. Using a set comprised of seven chromosome-specific probes (chromosome 10, 14, 16, 18, 22, X and Y) hybridized to lymphocyte interphase nuclei, we demonstrated that Spectral Imaging system provides a significant improvement over conventional filter-base microscope systems for enumeration of multiple chromosomes in interphase nuclei (Fung et al., 1998b).

Using mostly yeast or bacterial artificial chromosome probes for cytogenetic analyses of blastomeres and detection of structural alterations, we are building panels of probes to simultaneously score 10 or more different chromosomes. Further increases in the number of probes is complicated due to occasional overlap of chromosome domains or local variation in hybridization efficiency. We developed a 10-chromosomes probe set (chromosomes 9, 13, 14, 15, 16, 18, 21, 22, X and Y) for the purpose of labeling DNA targets most frequently associated with aneuploidy and spontaneous abortions and tested its application in PGD (blastomeres from abnormal human preimplantation embryos) and prenatal diagnosis (uncultured amniocytes obtained by amniocentesis). Results demonstrated in-

creasing levels of background fluorescence on different cells after hybridization in the order: (uncultured amniocytes) >> (blastomeres) > (interphase cells from lymphocytes). All blastomeres fixed for this study ( $N = 25$ ) spread very well. Fourteen nuclei (56%) showed interpretable hybridization results, and most of them were karyotyped as abnormal, since all those cells were from 1 and 3 PN human embryos, and had arrested development or were morphologically abnormal. The signals from 11 nuclei (44%) were faint. This may be related to the quality of the embryos, since all of them were developing abnormally. The fixation of uncultured amniocytes on slides for Spectral Imaging analysis turned out to be somewhat difficult. Most nuclei were not flattened out, presenting a problem due to the limited focal depth. Overlapping signal domains were a problem in uncultured amniocytes, where only about 20% of all cells showed interpretable spreads. In summary, Spectral Imaging has demonstrated advantages for evaluating numerical chromosomal abnormalities in single interphase cells. Its utility for chromosome scoring, however, remains limited due to chromosome domain overlap.

### 3.3. DNA micro-arrays (chips)

The DNA micro-arrays represent an exciting new technology with applications ranging from gene expression profiling to determination of gene copy number changes in tumors. We are presently investigating this approach in which the DNA probes are immobilized on glass slides as a strategy complementing FISH studies. The DNA from the embryonic cells is labeled in one color (e.g. red), while an equal amount of a reference DNA probe is labeled in a different color (e.g. green). These differentially labeled DNA are combined, denatured and hybridized to a DNA micro-array or 'chip' in a quantitative manner. Results are obtained after reading the micro-arrays with specially designed fluorescence scanners as red/green or red/infrared fluorescence ratios. After normalization, every increase or decrease from the average ratio indicates an abnormal number of copies of the hybridization target.

In practice, the DNA contained in a single cell is not sufficient to generate measurable signals. The commonly used protocols therefore include a DNA in vitro amplification step using a random primer or oligonucleotide with arbitrary sequence prior to labelling. The DNA to be immobilized can be obtained by standard isolation protocols or by in vitro DNA amplification. We use a DNA spotter based on the design published by Brown's group at Stanford University (Schna et al., 1996). This allows us to spot small amounts of DNA on poly-L-lysine coated glass slides with a 100–200 micron pitch. A 288-spot DNA micro-array like the test array depicted schematically in Fig. 1 then measures no more

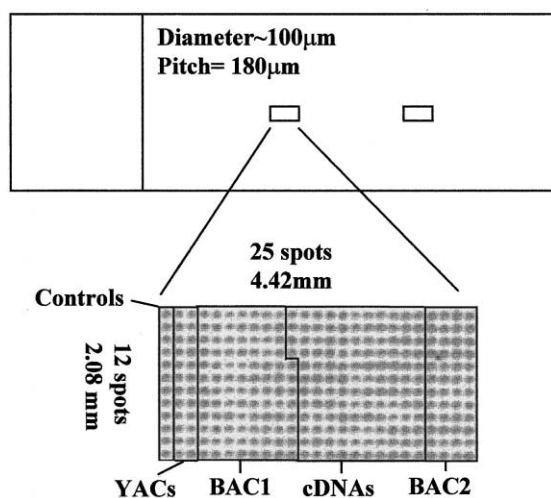


Fig. 1. Prototype DNA micro-array for technology development. The chip contains from left to right: control DNA spots (six samples), PCR amplified YAC DNA (12 clones), BAC array 1 (46 clones for copy number determination in breast cancer research), cDNA (62 tyrosine kinase cDNA for expression profiling), and BAC array 2 (24 clones for chromosome enumeration in PGD). All samples are spotted in duplicate. The total number of DNA spots of 300 arrayed with a 180  $\mu\text{m}$  pitch leads to array dimensions of  $2.08 \times 4.42 \text{ mm}^2$ . Each slide contains two identical micro-arrays.

than a few square millimeters. This small size of micro-arrays is an advantage over larger arrays prepared on nylon filters, because it requires less amount of labeled DNA sample in the hybridization reaction.

We are presently preparing DNA test chips to develop the methods and study parameters such as probe preparation, hybridization conditions and chip reader performance. Our long term objective is the development of reliable procedures to detect structural as well as numerical abnormalities using a combination of 'FISH and chip' technology. Chips to be used in those studies will carry several hybridization targets per chromosome arm, thus allowing a more detailed gene dosage determination or delineation of full or partial aneusomies than the FISH-based assays.

#### 4. Uncited reference

Fung et al., 1998a

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