Chapter 13
Current and Future Preimplantation Genetic Screening (PGS) Technology: From Arrays to Next-Generation Sequencing

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Introduction

Aneuploidy is a broad term used to describe gross chromosomal imbalance in an organism. For the sake of this chapter, only aneuploidy in an embryo will be considered. Aneuploidy typically presents as either an additional chromosome (e.g., trisomy) or a missing chromosome (e.g., monosomy). Such abnormalities arise during cell division (either meiosis or mitosis) when chromosomes fail to separate equally between the two new daughter cells [1]. Aneuploidy may be present in all cells of the embryo (uniform aneuploidy) or be confined to a subpopulation of the cells (mosaicism). Aneuploidy in embryos has varied effects during reproduction, from early embryonic arrest and lack of implantation, pregnancy loss (spontaneous abortion) with trisomy 16 being the most common chromosome abnormality seen in products of conception (POC), to live born trisomic births with varying phenotypic abnormalities, the best known being Down Syndrome (trisomy 21). Aneuploidy originates during the meiotic divisions (principally in the ovary) and the early cleavage divisions (mitotic) of the preimplantation embryo. Nondisjunction, precocious separation of sister chromatids, and anaphase lag are thought to be the most common causes of aneuploidy during gamete formation and embryogenesis [1]. The impact of aneuploidy in families can be devastating, with patients being faced with the potential of pregnancy losses, stillbirths, and/or a severely affected child. In all cases, aneuploid embryos result in an unfavorable outcome for the family in

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question and are a major contributing factor to the relatively low fecundity of humans when compared with other species.

Preimplantation genetic screening (PGS) is increasingly used during in vitro fertilization (IVF) treatment and involves the cytogenetic analysis of polar bodies biopsied from oocytes, single cells (blastomeres) removed from cleavage-stage embryos, or small numbers of trophectoderm cells derived from embryos at the blastocyst stage. The intention of PGS is to reveal whether an oocyte or embryo is chromosomally normal or aneuploid, ideally allowing a single euploid embryo to be prioritized for transfer to the uterus. In theory, this strategy should lower the risk of some of the problems discussed above and improve the success rates of assisted reproductive treatment (ART). PGS is not a new concept, it was proposed alongside the earliest developments of preimplantation genetic diagnosis [2]. The ability to count chromosomes effectively in small numbers of cells from early embryos has required an evolution of diagnostic technologies, combining speed, accuracy, reproducibility, and reliability. To date, only direct analysis of chromosome copy number through embryo biopsy, and analysis of the complete chromosome complement has shown positive results in terms of improved ART outcomes [3, 4]. Indirect approaches (e.g., metabolomic and proteomic analysis of embryonic products and detailed morphokinetic analysis using time-lapse imaging technology) have yet to be convincingly associated with aneuploidy incidence across multiple laboratories.

Prior technologies aimed at counting chromosomes (e.g., fluorescence in situ hybridization (FISH) applied to polar bodies and embryonic cells) failed to show a clinical benefit in multiple randomized controlled trials (RCTs) (see meta-analysis [5], with only one RCT demonstrating an improvement in the results of ART [6]. The challenges facing FISH-based technology applied to human cleavage-stage embryos are well documented [7] and focus primarily on the safety of embryo biopsy, the importance of low diagnostic error rates, and the need to assess the copy number of all chromosomes, not just the 8–12 possible using FISH. While 24 chromosome FISH is now possible with recently released probes and protocols, technical issues related to signal interpretation and hybridization efficiency, coupled with poor clinical trial data, have signaled the end of FISH testing in eggs and embryos and its replacement with alternative methods. In particular, the advent of robust and relatively inexpensive microarray technologies, allowing rapid evaluation of all 24 chromosomes has led to arrays superseding FISH in most laboratories around the world. Despite the superior technical capabilities of array-based testing methods compared with FISH and several prospective clinical trials showing the benefit of array-based testing, the policy position of both professional and regulatory bodies on PGS has not been revised and continue to take an extremely cautious line, typically referring only to the historic and flawed FISH approach [8, 9].

Current clinical applications for PGS include first polar body, combined first and second polar body, cleavage stage, as well as trophectoderm biopsy [10]. To date, cleavage-stage biopsy has been most widely applied. Biopsy at this stage of development has long been considered to be harmless [11, 12]; however, more recent work has shown that it may reduce implantation potential especially when two cells are biopsied [13]. Concerns about the impact of cleavage-stage biopsy and the

accuracy of genetic diagnosis based upon a single cell have led to an increased clinical utilization of blastocyst analysis. The invasive nature of oocyte and embryo biopsy has led to PGS historically being used to target specific high-risk patient groups (advanced maternal age; repeated implantation failure; recurrent pregnancy loss, and severe male factor infertility). More recently, PGS has been applied to patients considered to have a good ART prognosis in an attempt to improve the success rates of IVF treatment in cycles involving single embryo transfer (SET) (a strategy used to reduce the risks of multiple gestation) [3]. Considering that PGS has a financial cost and that embryo biopsy is invasive and potentially associated with a small risk to the embryo, a robust cost–benefit analysis is essential to confirm whether or not a given patient benefits through the use of PGS [14]. This chapter explores the current methodologies employed for the purpose of PGS using microarray CGH and looks into the future to describe new technologies such as next-generation sequencing (NGS) and how this technology will shape the future of preimplantation testing.

Methods

Biopsy Strategies

A number of different embryo biopsy strategies have been used clinically for PGS. For oocyte testing, first polar body (PB1) biopsy alone and combined PB1 and PB2 strategies have both been used clinically. However, it has become clear that PB1 alone has limited applicability for PGS as up to 30 % of maternal aneuploidy will not be diagnosed if only PB1 is analyzed [15–19]. As precocious separation of sister chromatids appears to be the predominant cause of maternal meiotic aneuploidies, it is critical to biopsy PB2 as well to accurately identify all maternal aneuploidies and ensure that abnormal segregations in PB1 are not corrected in the second meiotic division. The timings of both PB1 and PB2 biopsy are also critical to the efficiency of diagnosis. This was relevant when aneuploidy screening utilizing FISH technology was popular [20] and is equally critical when using array CGH [21, 22] or newer technologies for polar body testing.

Blastocyst stage biopsy may be the optimal stage for aneuploidy screening as it partially negates the problem of mosaicism, allows analysis of meiotic aneuploidy from both the maternal and paternal complement, detects post-zygotic events (mitotic errors), and appears to have minimal impact on the developing embryo. Historically, blastocyst testing necessitated the use of embryo cryopreservation to allow enough time for testing; however, newer methodologies provide results in approximately 12 h from sample receipt permitting fresh transfer. Cryopreservation may have been viewed as a detriment to testing in the past, although it is becoming increasingly apparent that vitrification is a viable strategy to maintain or even potentially increase live birth rates following biopsy [23]. In addition, the routine use of embryo vitrification may allow clinicians and patients to overcome logistic issues

with sample transportation and diagnostic testing. It should be noted that not all embryos created during an IVF cycle successfully develop to the blastocyst stage, so not all patients will produce embryos suitable for biopsy [24]. Therefore, patient education and management of expectations are important components of PGS.

Principles of Array Comparative Genomic Hybridization

Comparative genomic hybridization (CGH) was originally designed for molecular karyotyping of tumor cells [25]. It is a method where the chromosomal count of a cytogenetically uncharacterized DNA sample can be inferred according to its ability to hybridize to target DNA sequences affixed to a solid support, such as a microscope slide, in competition with a reference DNA of known (normal) karyotype. The CGH procedure can be performed using target DNA composed of (1) metaphase chromosomes from a karyotypically normal reference male (metaphase CGH) or (2) a series of specific DNA probes derived from sites along the length of each chromosome spotted onto a glass slide [array CGH (aCGH)]. A schematic representation of the principles of CGH is shown in Fig. 13.1. Metaphase CGH is time consuming, taking 3–4 days to complete one experiment; however, it has been used clinically for PGS [26, 27]. All CGH methods require nanogram to microgram quantities of DNA for optimal performance, whereas a typical single cell contains approximately 6 pg of DNA. Consequently, whole genome amplification is required prior to the CGH procedure itself.

In the case of array CGH, the DNA spotted onto each slide can be from bacterial artificial chromosome (BAC), DNA clones (typically longer sequences) from defined chromosomal regions, or specific oligonucleotides (shorter DNA sequences). This chapter will focus mainly on the BAC clone approach as this system is the most well validated of the methods and has been used for well over 400,000 clinical preimplantation genetic samples to date. The most widely used array, 24Sure™ (Illumina Inc., San Diego, CA) contains nearly 3,000 DNA spots spaced approximately 1 Mb apart. Each clone was chosen based on minimizing copy number polymorphisms, and its location has been confirmed via reverse painting and FISH mapping.

Embryo Biopsy and Sample Handling

Following biopsy of the egg (polar body 1 and/or 2), embryo (cleavage stage), or blastocyst, the sample is washed through a number of droplets, most often phosphate buffered saline (PBS) with an additive such as polyvinyl alcohol (PVA) to reduce cell stickiness. The sample is then picked up in a small volume (<2 μL) and placed into a sterile 0.2 mL Eppendorf tube for transport to the laboratory for testing. Most embryologists perform a quick step to ensure that the cellular material and all of the fluid are collected together at the bottom of the sample tube. This can be accomplished by centrifugation or a quick flick of the tube to collect the sample

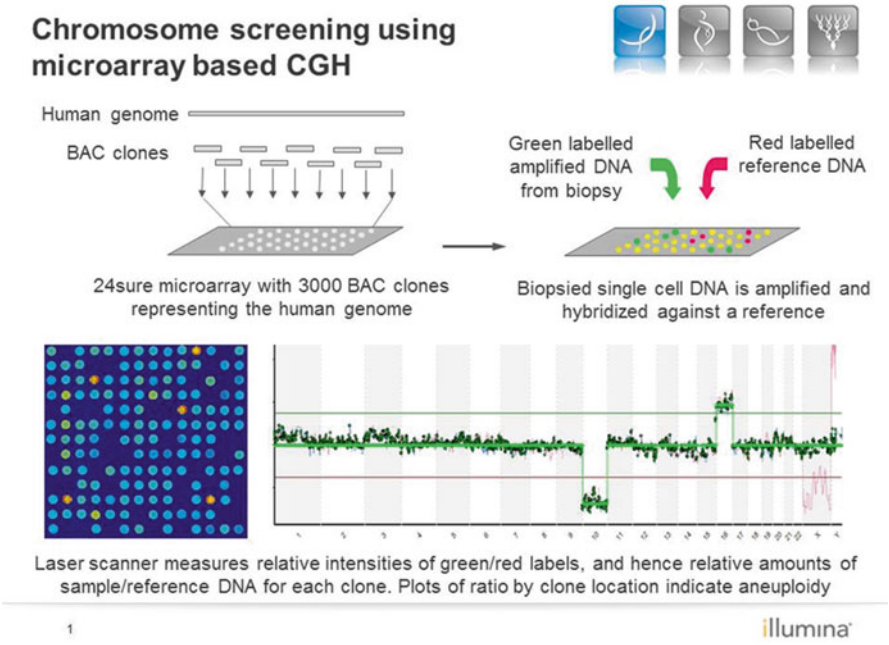

Fig. 13.1 Application of microarray technologies for PGS. Following biopsy, DNA is amplified using WGA, and then each biopsy sample is labeled with *green* fluorescent tags. *Green* biopsy sample is mixed with normal male DNA labeled with *red* fluorescent tags in equal proportions. The mixture is hybridized onto the BAC array and, following stringent washing, the slide is analyzed for the proportion of *red* and *green* fluorescence on each spot. Computer algorithms are used to assess each spot and call any gains or losses of chromosome in the test sample as compared to the normal DNA

in the bottom. Depending on the length of time the sample will have to travel, ambient temperature or wet ice (blue ice packs) can be used for shorter trips, while dry ice may be used for longer distance trips, especially in locations with warm weather at certain times of the year. While mineral oil may be used as an overlay in molecular biology experiments that require polymerase chain reaction (PCR), it should be noted that mineral oil should never be used prior to whole genome amplification (WGA) and aCGH as it inhibits the amplification process and will yield samples with no results. Of course, each laboratory will have its own standard operating procedure for sample handling ahead of transport to the testing laboratory.

Whole Genome Amplification and Labeling

A number of different WGA methods have been used historically for array CGH experiments, with the current, most often utilized method being SurePlex™ (Rubicon Genomics Inc., Ann Arbor, MI, USA and BlueGnome). This WGA kit is fragment amplification based, where self-inert degenerative primers are annealed at multiple

sites along the genome. This system was chosen because it produces optimal fragment sizes, which have been found to be reproducible between samples and are optimized for array CGH. Many of the other WGA techniques have been adapted for use in array CGH but were originally used for other purposes (e.g., single locus PCR and mutation detection). SurePlex™ is suitable because of its simple, short protocol and highly representative amplification.

Following sample receipt and accessioning in the lab, each tube is opened in a dedicated DNA amplification clean room, under a laminar flow or PCR-dedicated hood. Amplification is performed according to the manufacturer's instructions as the SurePlex™ kits have been validated for use in single cells. When using the SurePlex kit, the first step is lysis/extraction step (15 min), followed by pre-amplification steps (90 min), and finally amplification (30 min). To reduce possible contamination issues and eliminate the risk of accidental sample switches, all steps in SurePlex are performed in a single tube. The procedure is performed in a PCR machine as each step is temperature and time dependent.

After SurePlex, agarose gel electrophoresis is performed to confirm successful amplification. As the arrays can be quite expensive, it is best to ensure amplification prior to taking the sample further through the process. A smear of DNA near the top of the gel is indicative of good amplification; low molecular weight DNA or no DNA would be indicative of poor/no amplification. Following agarose gel verification of good amplification, the WGA product is labeled through nick translation with either Cy3 (green) or Cy5 (red) fluorescent tags.

Hybridization

In traditional aCGH, embryo biopsy samples labeled in one fluorescent color (e.g., green) and control reference DNA (typically a karyotypically normal male) labeled in an alternative color (e.g., red) are denatured at 74 °C to make the DNA single stranded. The single-stranded DNAs from the sample and control are then mixed together in equal proportions in hybridization buffer containing formamide and cot-1 human DNA before being adding to each 24Sure™ microarray. Microarrays are hybridized at 47 °C for at least 4 h or overnight in a humidified chamber. The length of hybridization time varies depending on the timing of biopsy, the number of samples in the lab on any given day, staffing levels, and shift patterns. During validation of the array in the lab, hybridization times as short as 3 h and as long as 16 h (overnight) were tested with no differences in diagnostic accuracy noted [28]. On the basis of these results, hybridization for at least 4 h and no longer than 16 h is deemed to be interchangeable. It should be noted, however, that shortening both labeling and hybridization may lead to suboptimal results; therefore, any protocol used clinically should be robustly validated prior to use on actual human samples.

More recent advances have led to so-called single channel aCGH. With this method, control DNA is not hybridized on each array, rather the control DNA is hybridized in each fluorescent color (Cy 3 and Cy 5), for both normal male and normal female, on separate arrays run during each experiment. Therefore, each

experimental array in single channel aCGH contains two embryo biopsy samples, one labeled in Cy3 and another labeled in Cy5. When the analysis is performed, each experimental sample is compared in silico to the male and female reference separately. Single channel aCGH allows for more samples to be run per experiment and reduces the amount of control DNA necessary in each experiment. This has also allowed the price per sample to be lower than in conventional aCGH.

Post-Hybridization Washing

Following hybridization, each microarray is washed as follows: 10 min in 2×SSC/0.05 % Tween 20 at room temperature, 10 min in 1×SSC at room temperature, 5 min in 0.1×SSC at 60 °C, and 2 min in 0.1×SSC at room temperature to remove unbound DNA.

Scanning

Each microarray slide is scanned using a dual channel fluorescent laser scanner in order to create TIFF images (e.g., ClearScan™, Illumina) showing green fluorescence at 632 nm and red fluorescence at 587 nm associated with hybridization of embryo and reference DNA samples, respectively. Raw images are loaded automatically into analytical software such as BlueFuse™ for evaluation of fluorescent signals (ratio analysis).

Scoring

Sample scoring is typically performed by a trained technologist who assesses traces for all 24 chromosomes, noting all gains and losses, as well as determining the sex of each sample. A second technologist then scores the sample blindly, with no knowledge of the initial scoring. The final result for each sample is then assigned by comparing the two scores. If discrepancies are noted between the two assessors, they are typically adjudicated by a third technologist and/or the laboratory supervisor or director. It should be noted that the current version of the BlueFuse™ software allows for automated calling of whole chromosome gains and losses; however, most laboratories do not rely on this for clinical diagnosis.

Reporting

Once results for all samples from each patient are finalized, a diagnostic report is prepared, signed off by an appropriately qualified person (on site or remotely), and shared with the referring laboratory and physician prior to embryo transfer.

Discussion

Validation

In extensive validation using single cells from known cell lines against the gold standard of karyotyping, 24Sure™ demonstrated 98 % accuracy [29]. The use of cell lines does, however, have drawbacks. During this validation, mosaicism was seen in most cell lines meaning that any one cell in the culture may or may not always have the same molecular genotype. Validation for embryo aneuploidy is perhaps even more difficult as truth data (i.e., definitive proof that the sample used as an unknown is actually the genotype that you expect it to be); this is difficult to obtain for human embryos grown in culture (due to mosaicism, for example). Human oocytes offer an interesting method for validation. One can biopsy the first and second polar body and use array CGH to analyze the chromosome complement in each sample individually. This method allows a laboratory to look at trios of data, comparing the first and second polar body to the oocyte. The expectation is to see reciprocal chromosome gains and losses from aneuploid polar body(ies) and oocyte [30].

Limitations of Array CGH

While array CGH has been shown to be highly accurate and reproducible in multiple validation studies and has been used on hundreds of thousands of embryo samples, it still has drawbacks that must be understood prior to clinical use. For example, aCGH cannot discriminate between maternal and paternal errors; it can simply elucidate chromosome gain and loss. It remains to be determined whether knowledge of the parental source of error has clinical value. Array CGH cannot distinguish between meiotic and mitotic errors of chromosome segregation; however, again the data on whether this is an important factor remains unclear. Perhaps the most clinically relevant limitation of aCGH is the fact that it cannot distinguish a euploid embryo from certain forms of triploidy (i.e., 69,XXX chromosomes) or tetraploidy (i.e., 92 chromosomes). Purely triploid and tetraploid embryos often implant, leading to pregnancy loss prior to delivery.

Competing Technologies

While array CGH has become the gold standard and most widely used method for counting chromosomes clinically, there are a number of competing platforms that could challenge this position. As with all competing technologies, there are advantages and disadvantages to each [31, 32]. Comprehensive chromosomal screening

using multiplex quantitative PCR (qPCR) [33] has been proposed as a faster and less expensive means of detecting aneuploidy. However, the qPCR systems optimized for embryo analysis have not been fully commercialized, restricting availability. Furthermore, existing qPCR systems are only applicable to trophectoderm samples and cannot be used for the analysis of polar bodies or single blastomeres. Chromosome counting can also be performed through the use of single nucleotide polymorphism (SNP) arrays. Using a combination of loss of heterozygosity, quantitative SNP calling, and analysis of patterns of SNP inheritance from parents to embryos, it is possible to detect chromosomal gains and losses [34–37]. SNP-based arrays do offer the ability to detect the parent of origin in aneuploidy cases and have been validated to reliably detect inheritance of specific genotypes allowing for nearly universal detection of many single gene defects [34]. However, SNP arrays also have a much longer protocol (24 h+ in most cases), are typically more expensive than alternative methods, and typically require parental DNA ahead of testing adding to the cost and time needed for this type of array.

Noninvasive Indirect Methods of Determining Aneuploidy

It is appealing to consider noninvasive approaches to embryo selection. Weak correlations exist between the presence of embryonic aneuploidy and morphological aspects of embryo development following retrospective analysis [38, 39]. These findings have stimulated the field of morphokinetic analysis during IVF, with an attempt to identify aneuploidy in a real-time clinical setting. Analysis of time-lapse imaging during embryo growth demonstrates that certain morphologic features and/or developmental timings of the embryo may have some relationship to aneuploidy [40, 41]. This data, if confirmed in larger data sets with appropriate subgroup analysis stratified by maternal age and in multiple clinics, may provide some useful information to place embryos in the order of priority for transfer. However, it does not appear that morphokinetics will have the capacity to provide the same level of specificity and accuracy yielded by aneuploidy testing using array CGH. Another promising morphokinetic approach is to assess dynamic fragmentation patterns within early embryos but again, this currently does not identify specific aneuploidies and only provides a relative risk of abnormality for each embryo [42]. Currently, no morphokinetic parameter or set of parameters has been shown to be able to discriminate between euploid and simple aneuploid (e.g., trisomy 21) embryos. In addition to morphokinetics, measurement of specific metabolites or combinations of biologically relevant molecules in culture medium has been suggested as a method to predict the viability of an embryo. However, none of these methods have been proven to be able to differentiate between general chromosomal aneuploidy and specific aneuploidy in prospective controlled studies.

Next-Generation Sequencing for Chromosome Counting

Next-generation sequencing (NGS) may supercede all other methodologies as it promises several advantages over all other techniques [43]. The term *next-generation sequencing* (NGS) describes several distinct methods that share in common an ability to provide huge quantities of DNA sequence data from the samples analyzed, rapidly, and at relatively low cost. There are two ways in which NGS can be employed for the detection of aneuploidy screening. The first involves biopsy of cells from embryos followed by whole genome amplification, after which the DNA is broken into small fragments and then subjected to NGS. The sequence of each fragment is compared to the sequence of the human genome, allowing its chromosome of origin to be determined. The relative proportion of fragments attributable to each chromosome is indicative of its copy number—e.g., an increase in the proportion of DNA fragments derived from an individual chromosome (relative to a chromosomally normal sample) is evidence of a trisomy. The second way that NGS can be used for aneuploidy detection involves the use of multiplex PCR (rather than WGA) to simultaneously amplify multiple specific loci on each chromosome. After amplification, the mixture of DNA fragments is analyzed using NGS, and the number of sequences attributable to each chromosome is calculated. A deviation from the expected number of DNA fragments for a particular chromosome is indicative of aneuploidy.

NGS promises several advantages compared with other technologies for screening aneuploidy in embryos, but perhaps the most important is its potential to reduce costs. Although each NGS experiment remains relatively expensive, costs per sample can be lowered significantly by simultaneously sequencing large numbers of embryos, thus sharing expenses across multiple samples. This strategy also has the effect of reducing the proportion of the genome analyzed from each embryo, which may mitigate ethical concerns related to NGS, as it prevents reliable analysis of individual genes. However, at this time, the cost to analyze a single sample from an embryo by NGS is comparable to the cost of analyzing the same sample by current methods like aCGH. This is likely to change over time as sequencing costs continue to drop as the technology improves.

Following extensive validation, NGS has been used to screen embryos in clinical cycles leading to the birth of healthy children in the United States [30] and China [43]. In addition to the clinical utility, a recent paper details the validation of NGS-based PGS in one laboratory as compared to the current standard of care aCGH (24sure) in one laboratory [44]. In the future, the extraordinary power of NGS may be used to evaluate additional aspects of embryo biology, relevant to viability assessment. Furthermore, as NGS provides DNA sequence information, it also has the potential to be used for the targeted detection of specific mutations responsible for inherited disorders at the same time as screening for aneuploidy.

With innovations such as NGS, we are entering a new and exciting era in preimplantation genetics which is well positioned to enable greater use of the single embryo transfer strategy for IVF patients. The next few years will see less expensive tests and analyses that provide a more detailed insight into embryo viability than

those currently available. Superior embryo viability screening and lower costs, resulting in increased patient access, will likely contribute to a significant improvement in the success rates of IVF. A number of randomized clinical trials are already underway to confirm whether or not this prediction is correct.

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