DOI: 2016.075

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Keywords: meiomapping, karyomapping, polar body, oocyte, oocyte activation, meiosis, meiotic recombination, crossovers, aneuploidy, egg, chromosome, chromosome segregation, SNP, single nucleotide polymorphism, calcium ionophore, haplotyping, embryo, Whole genome amplification, WGA, SNP genotyping, Comparative Genomic Hybridizations, CGH, macro, excel

Editorial summary: This protocol describes how to perform polar body biopsy, artificial activation of human oocytes and SNP genotyping to generate genome-wide maps of female meiotic recombination and chromosome segregation outcomes.

Generating meiomaps of genome-wide recombination and chromosome segregation in human oocytes

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Abstract

We have developed a protocol for generating genome-wide maps (meiomaps) of recombination and chromosome segregation for the three products of human female meiosis: the first and second polar bodies (PB1 and PB2) and the corresponding oocyte. PB1 is biopsied and the oocyte is artificially activated by exposure to calcium ionophore, after which PB2 is biopsied and collected with the corresponding oocyte. The whole genome of the polar bodies and oocytes are amplified by multiple displacement amplification and together with maternal genomic DNA are genotyped for approximately 300,000 SNPs genome-wide by microarray. Informative maternal heterozygous SNPs are phased using a haploid PB2 or oocyte as a reference. A simple algorithm is then used to identify the maternal haplotypes for each chromosome, in all of the products of meiosis for each oocyte. This allows mapping of crossovers and analysis of chromosome segregation patterns. The protocol takes a minimum of 3-5 days and requires a clinical embryologist with micromanipulation experience and a molecular biologist with basic bioinformatic skills. It has several advantages over previous methods; importantly, the use of artificial oocyte activation avoids the creation of embryos for research purposes. Also, compared with next generation sequencing, targeted SNP genotyping is cost effective and simplifies the bioinformatic analysis, since only one haploid reference sample is required to establish phase for maternal haplotyping. Finally, meiomapping is more informative than copy number analysis alone for analysis of chromosome segregation patterns and using this protocol, we have provided new insights that may lead to improvements in assisted reproduction for the treatment of infertility.

Introduction

Chromosome segregation errors in human female meiosis are the principal cause of embryo aneuploidy leading to pregnancy loss, abnormal pregnancies and live births affected by chromosomal disorders[1]. The causes of these errors are not completely understood however, they increase exponentially with maternal age over 35 years and are associated with reduced and/or altered patterns of genetic recombination[2]. The evidence for this has been based largely on population studies and analysis of genetic markers in trisomic pregnancies and live births[2]. Furthermore, analysis of markers close to the centromere in common trisomies, for example, trisomy 21, which causes Down syndrome, has demonstrated the presence of both maternal homologues in most cases. Classically, therefore, most trisomies were thought to arise by non-disjunction of the bivalent chromosomes in the first meiotic division (meiosis I) of oogenesis.

The use of *in vitro* fertilisation (IVF) for the treatment of infertility opened up the possibility of studying these processes directly in human gametes and early preimplantation embryos. Early pioneering studies by Angell and colleagues, in which human oocytes at various stages were karyotyped, indicated that rather than whole chromosome non-disjunction, premature separation of sister chromatids (PSSC) at meiosis I was the major mechanism causing segregation errors[3]. However, karyotyping oocytes and embryo cells is technically challenging and identification of chromosomes by banding patterns is generally not possible. More recently, the development of methods for whole genome amplification from single or small numbers of cells biopsied from preimplantation embryos and array based comparative genomic hybridisation (array CGH) has allowed chromosome copy number analysis for all 23 pairs of chromosomes. Copy number analysis by array CGH of all three products of female meiosis, the first and second polar bodies (PB1 and PB2) and the corresponding fertilised oocyte, demonstrated a high incidence of aneuploidy in the oocytes of women of advanced maternal age[4]. It also confirmed that the predominant mechanism causing segregation errors in meiosis I for all affected chromosomes is PSSC with only a small minority showing whole chromosome non-disjunction.

Whole genome amplification at the single cell level also allows the use of other genomics methods including single nucleotide polymorphism (SNP) microarrays and next generation sequencing (NGS). Genome-wide SNP genotyping of both parents and a close relative of known disease status, allows informative parental SNPs to be phased and compared to the genotype of embryo biopsy samples as a universal linkage-based test (karyomapping) for preimplantation genetic diagnosis (PGD) of single gene defects[5,6]. Since karyomapping maps the parental origin of each chromosome present in the embryo cell(s), meiotic trisomies, monosomies and deletions can be detected by the presence or absence of one or both parental chromosome haplotypes. Furthermore, the transitions between parental haplotypes distal from the centromeres provided the first high resolution data for meiotic recombination at the single cell level[5].

To identify recombination events at the chromatid level and analyse chromosome segregation errors in human oocytes, requires maternal haplotype analysis of all three products of meiosis i.e. PB1, PB2 and the oocyte (collectively referred to as 'oocyte-PB trios') (Fig 1). Here we describe a protocol for: the artificial activation of donated human oocytes, which causes the resumption and completion of meiosis II and avoids the need to create fertilised embryos for research purposes; the biopsy of PB1 and PB2 before and after activation and the isolation of the activated oocyte (Fig 2); whole genome amplification and genome-wide SNP genotyping; and maternal haplotype analysis using a simple algorithm based on Mendelian segregation of maternal SNP alleles (meiomapping), similar in principle to karyomapping, to generate genome-wide maps of recombination in oocyte-PB trios. Using this protocol, we recently demonstrated selection for maternal recombination rates, described a novel reverse segregation pattern of chromosome segregation and presented the first evidence for meiotic drive[7].

Development of the protocol

The meiomap protocol was developed as a tool to study recombination and chromosome segregation in human female meiosis. A previous method used donated oocytes, which were fertilised by intracytoplasmic sperm microinjection (ICSI) using donated sperm, following biopsy of PB1[8]. After PB2 biopsy, the male and female pronucleus were removed by micromanipulation and the whole genome

of the polar bodies and female pronucleus (FPN) were amplified by Multiple Annealing and Looping Based Amplification Cycles (MALBAC). Maternal haplotyping was then achieved by NGS and bioinformatic analysis of multiple triads.

To avoid the ethical issues involved in creating fertilised embryos for research purposes and the additional manipulation required to remove the pronuclei, we based our protocol on an established method for artificial activation of oocytes arrested in metaphase of the second meiotic division (MII) with calcium ionophore (A23187)[9-14]. The activated MII oocytes resume meiosis and extrude the PB2. Furthermore, artificial activation has been used clinically in cases of ICSI failure and live births reported[15-19]. Sources of human oocytes may include cryopreserved oocytes no longer required for therapeutic use or theoretically, immature oocytes, at germinal vesicle or metaphase of the first meiotic division (MI), matured *in vitro* to MII. These are widely available, since a proportion of oocytes at these stages are collected in routine IVF cycles. Irrespective of the source of oocytes, informed patient or donor consent is mandatory.

Our choice of method for whole genome amplification was based on the availability of commercial PCR-based libraries and multiple displacement amplification (MDA) methods are used routinely for chromosome copy number analysis by array CGH or NGS, and for karyomapping by SNP genotyping. However, for SNP genotyping the call rates are higher and the resulting resolution of the meiomap analysis is also higher with MDA. Finally, targeted sequencing of a defined set of SNPs using a microarray is cost effective and simplifies the bioinformatic analysis since the same loci are analysed in the maternal DNA and oocyte-PB trios.

To map the positions of crossovers in the oocyte-PB trios, an algorithm is used to phase heterozygous maternal SNPs distributed across each chromosome (including the non-pseudoautosomal region of the X chromosome and excluding the Y chromosome) using a haploid PB2 or activated oocyte as a reference. This results in two notional maternal haplotypes at consecutive loci across each chromosome: Maternal haplotype 1 and 2 colour coded in yellow and green, respectively (Table 1). The genotypes at all of these informative loci are then examined in each of the other trio samples and compared to the reference. If the sample genotype is homozygous

and identical to the reference it is Maternal haplotype 1 (yellow). If homozygous for the other SNP allele then it is Maternal haplotype 2 (green). Finally, if heterozygous it is colour coded in blue or if neither haplotype is detected for that SNP this is depicted in grey (Table 1 & Figure 3). Occasionally, heterozygous SNPs call in homozygous (haploid) regions and we refer to these as 'allele drop in' (ADI) events that generate low-level background 'noise' in the data (e.g. Table 1: SNP 13 in PB2). Please note that because the SNP genotyping software assumes that all samples are diploid, it calls haploid SNP loci in PB2 or the activated oocyte as either 'AA' or 'BB'. We therefore refer here to these calls as 'homozygous' to distinguish these loci from genuinely heterozygous calls in, for example, PB1 where two chromatid regions from both maternal homologues are present.

Theoretically, all maternal heterozygous SNPs should be informative. In practice, however, selecting heterozygous maternal SNPs, which are, as an additional requirement, genotyped as homozygous in the chosen reference, significantly improves the consistency of haplotype calling. (Please note that because of the principle of independent segregation, each chromosome in the reference has a random grandparental origin).

When the colour coded SNPs are displayed in columns as a consecutive series for each chromosome, the transitions which occur as a result of recombination (or 'crossovers') are evident (Figure 3). For PB1, each recombination is marked by a transition from a heterozygous (blue) region to a homozygous region for one of the two maternal haplotypes (yellow or green) or *vice versa* (Figure 4). For PB2 and the activated oocyte, the transitions occur between the two homozygous maternal haplotypes (yellow to green or green to yellow; Figure 4). At this stage, the reference sample, which for phasing purposes is assumed to have no recombination, displays only one maternal haplotype *i.e.* all SNPs coded yellow. However, in most cases the reference chromosome will in fact be recombinant. This has the effect of creating an apparent recombination in all of the other haploid samples at that position (common crossover). This is because each SNP along a chromosome is compared to the reference and where they match they are assigned the same haplotype as the reference (yellow) and where they differ they are assigned the opposing haplotype (green). A recombination in the reference therefore causes a 'switch' in haplotype at

that position across all other haploid samples from that donor (Figure 3 & 4). Each sample, therefore, has a combination of true crossovers and common crossovers. Secondary analysis is then required to identify the common crossovers, restore them to the reference sample and remove them from all of the others (Figure 4).

Applications of the method

This protocol is an important advance to human oocyte research within an ethical framework that facilitates a new era of investigation into various aspects of female meiosis. It does not require the creation or destruction of fertilized human embryos and the protocol was paramount to uncovering several novel findings[7]. These include a previously unknown meiotic segregation pattern termed 'reverse segregation'; in which both homologs undergo a meiosis II-like division at meiosis I and the two non-sister chromatids segregate at meiosis II[7]. This work has also shed new light on the meiotic origin of both monosomies and trisomies. It has led to direct evidence showing that lower, genome-wide recombination rates are associated with increased missegregation of chromosomes in human female meiosis, suggesting that higher recombination rates are selected for in the female germline[7]. The studies also revealed direct evidence of meiotic drive, in which non-recombinant chromatids are preferentially excluded from the oocyte[7]. This protocol is central for further oocyte-PB trio analysis and is being used by the Human MeioMap Project to explore human female meiosis on a broader population scale (www.sussex.ac.uk/lifesci/hoffmannlab/research/meiomap).

Currently the protocol is optimised for oocytes arrested at metaphase II (MII). However, it could also theoretically be applied to surplus immature, germinal vesicle (GV) or metaphase I (MI) stage, oocytes from human IVF cycles that are otherwise unused and can be matured *in vitro* to the metaphase stage of the second meiotic division. This would require an additional *in vitro* maturation step of up to 48 hours culture before starting the protocol[14]. Nevertheless, these oocytes represent a potentially large resource that, with patients' informed consent, could enable easy and ethical access to material for the study of human female meiosis. Application of this method will also provide a validation tool to assess various *in vitro* maturation protocols.

Genome-wide mapping of female meioses can also be achieved by analysing the SNP genotype of PB1, PB2 and fertilised embryos (embryo-PB trios). For embryo-PB trios, however, the presence of the paternal genome in the embryo samples has to be taken into account. One option is to genotype both parents and perform karyomap analysis[5]. Unlike meiomap analysis in which all heterozygous maternal SNP loci are potentially informative, karyomapping identifies two sets of informative SNP loci for the paternal and maternal chromosomes, in which one parent is heterozygous and the other homozygous. Heterozygous informative SNPs are then phased, typically using a child or, with embryo-PB trios, a sibling embryo, as reference and compared to the genotype of each sample. This has the advantage that crossovers between the paternal chromosomes can be identified in the same embryos. However, the resolution is lower because two distinct sets of informative SNPs are used to define the parental haplotypes and the number of these SNP loci is significantly reduced. Alternatively, meiomap analysis as described here can be used with a PB2 or an embryo as reference, with the additional requirement that both the reference and sample SNP genotypes are homozygous. Compared with oocyte-PB trios, this again reduces the number of informative SNP loci and the resolution, but is still effective. For purposes of brevity, we have referred to oocyte-PB trios only throughout the protocol since in all other aspects, the protocol is the same.

In theory, meiomapping or karyomapping of polar bodies alone could be used for PGD to identify maternal meiotic errors resulting in aneuploidy in the corresponding fertilised embryo, to diagnose the inheritance of maternal structural chromosome defects or for linkage-based detection of single gene defects. However, neither of these protocols have been validated for clinical use and establishing which maternal haplotype is associated with a genetic defect is still required. This would require some knowledge of familial genotypes, such as the genotype of an affected child or relative.

Comparison to other methods: Advantages and Limitations

Until the introduction of genome-wide sequence analysis[8] and meiomapping, studies of the relationship between recombination and meiotic chromosome segregation in humans were confined to population-based studies[20-23]. Most of these analyses are limited to trisomies that are compatible with *in utero* development and cannot

ascertain the meiotic origins of monosmies, the vast majority of which do not give rise to clinically recognised pregnancies. Furthermore, by only examining one product of meiosis; the oocyte (without the information from polar bodies), chromosome missegregation patterns and total recombination rates can only be estimated.

One major advantage of meiomapping is its power to detect global recombination rates in human oocytes. Previously, the only method of detecting recombination across all 23 homologs was through cytological analysis of Mlh1 (that marks crossovers) in fetal oocytes[24,25]. However, this method cannot determine crossovers that may have arisen through Mlh1-independent pathways (e.g. Mus81-dependent crossovers, which account for <10% crossovers [26]). Meiomapping provides an additional advance over these cytological assessments in that it provides information about which of the four chromatids in a bivalent recombined at any given crossover.

The second major advantage of meiomapping is its capacity to reveal the chromosome segregation patterns that result in aneuploidy. Previously this could only be inferred from pericentromeric SNPs in trisomic conceptions[20,27], which are potentially subject to interpretation errors. More recently, however, chromosome copy number analysis of polar bodies by array CGH has enabled new insight into the prevalence of segregation errors in preimplantation embryos following IVF[4,28,29]. Array CGH differs from meiomapping in that it can report chromosome copy number losses or gains in the polar bodies, which can in turn reveal any losses/gains in the fertilised oocyte, but cannot distinguish maternal haplotypes and therefore whether homologs or sister chromatids missegregated.

To obtain similar results to our protocol, Hou *et. al* used intracytoplasmic sperm injection (ICSI) to induce resumption of the second meiotic division in mature (MII arrested) human oocytes[8]. The female pronucleus was then removed from the resulting fertilised embryos thus effectively destroying them. Our oocyte activation protocol is less technically involved in that the whole oocyte is tubed rather than having to use micromanipulation to remove the pronuclei and does not result in the creation or destruction of human embryos for research purposes. Furthermore, our protocol results in an activation rate equal to or higher than performing ICSI in a

clinical IVF setting[30]. Hou *et. al* used a sequencing-based approach[8], however, we validated our protocol using genome-wide SNP arrays. This protocol is theoretically platform-independent, relying only on the detection of genome-wide polymorphisms. Therefore, with minor adaptation, the protocol will evolve with advances in single cell technology, including next generation sequencing, which has the potential to increase resolution. Although our method could be adapted to a sequencing-based approach, the efficacy and resolution depends on the read depth and level of SNP overlap across all samples (including maternal and reference genotypes). At present, the depth of sequencing required for our approach is not cost-effective.

An advantage of our simple Mendelian approach compared to other advanced bioinformatic algorithms, which identify haplotypes by analysing multiple reference samples[8], is that a single haploid reference (PB2 or oocyte) is sufficient to phase maternal heterozygous SNPs and detect the majority of recombination events. However, the single reference needs to be from a second oocyte-PB trio in order to identify all crossover events in the two trios (Table 2). Otherwise, reciprocal crossovers between sister chromatids in the bivalent segregating to PB2 and oocyte, at precisely the same locus, will not be visible. For optimal detection of all possible crossovers three or more oocyte-PB trios are required (Table 2). The identification of common crossovers in the second, and at least one other trio, then allows assignment of these to the reference sample. Furthermore, availability of multiple reference samples can be helpful to resolve rare ambiguous crossovers (for example at the extreme ends of chromosomes). It also becomes necessary to use an alternative reference if there are one or more aneuploidy or structural chromosome abnormalities in the original reference (see Troubleshooting Table 3).

The need to have three or more oocyte trios to detect all of the recombination events and chromosome segregation errors is an inherent limitation (Table 2). At present the protocol does not allow for the separation of the two sister chromatids in the PB1. Reciprocal crossovers in PB1 cannot be detected as they exist in heterozygous regions of PB1[7]. Additionally, not all recombination events are visible when chromosome segregation errors have taken place. For example, when an extra chromatid segregates to the PB1 this will obscure the recombination events on these

three chromatids so that the only detectable recombination event for that bivalent will be on the single chromatid in the PB2 or oocyte.

Meiomapping relies upon efficient single cell whole genome amplification (WGA) and accurate SNP genotyping. Although the protocol for collection and processing of the single cell samples is well established for clinical diagnostic use, amplification failure does occasionally occur (<5%) and is highly operator dependent.. Nevertheless, if two out of three elements in an oocyte-PB trio are successfully amplified and genotyped, all of the recombinations can be identified (owing to the reciprocal nature of crossovers). In our initial study, 34 out of 40 (85%) oocytes activated and the corresponding trios were amplified but many samples were lost in shipment between labs[7]. Of the remaining samples, all 13 complete oocyte-trios were analysed successfully. Also, irrespective of the method used, WGA results in a certain level of amplification errors[31]. Random failure to amplify one of the parental SNP alleles, or allele dropout (ADO), for example, is relatively common at the single cell level and here results in erroneous homozygous calls in heterozygous regions (Table 1). More rarely, allele drop in (ADI) can also occur and result either in erroneous heterozygous or homozygous genotype calls. Finally, variation of amplification across the genome at the sequence level results in a significantly increased proportion of SNPs, which cannot be genotyped, ‘no calls’, and lower overall call rates. These types of errors are particularly evident in heterozygous regions, which are typically present in PB1. Indeed, with the beadarray and associated software used here, heterozygous calls are much more likely to result in no calls. Overall call rates exceeding about 60% are usually satisfactory for meiomapping purposes. However, at the lower end of this range, heterozygous regions, whilst easily identifiable, have poorly defined boundaries. The location of the crossover is therefore best identified from either of the haploid elements (PB2 or oocyte). Fortunately, with these ADI is rare and no call rates are also much lower so that the transition from one continuous series of SNP alleles for one maternal haplotype, in most cases, switches to the next haplotype across adjacent informative SNPs. This is reflected in the lower resolution of recombination events in PB1 compared with PB2 or oocyte.

Further limitations include the distribution of SNP loci genotyped on the array. The coverage of the array in this protocol exceeds 90% of the genome. However, there are large gaps on the q arms of chromosomes 1 and 9 and recombination in these regions requires careful interpretation (See Troubleshooting Table 3). Also, the density of SNP markers varies across the genome and consequently so does the resolution of the position of recombination events.

Finally, any studies of human oocytes (or embryos) are limited by their restricted availability from clinics involved in fertility treatment and legal regulations. They must be ethically sourced with patients' informed consent preferably by taken by someone not directly involved in the research.

Experimental Design

Level of expertise: Access to human oocytes and embryos for research is only possible by collaborating with an IVF clinic with suitably qualified personnel and the expertise to stimulate the ovaries appropriately and collect the oocytes etc. The protocol for meiomapping itself requires a clinical embryologist with micromanipulation experience to perform polar body biopsy, activate the oocytes and isolate the activated oocytes, and a molecular biologist with basic bioinformatic skills. With suitable training, the clinical embryologist can collect the samples and perform the whole genome amplification. The amplified DNA can either be sent to a service lab for SNP genotyping, or this can be performed in-house if SNP arrays are available. Basic bioinformatic skills and knowledge of programming, for example, using Visual Basic for Applications (VBA) in Microsoft Excel, is necessary to develop the tools to analyse and display the SNP data (see example in Supplementary Data).

Genomic DNA: Genomic DNA from the oocyte donors is obtained by standard methods using commercially available kits. Sources of DNA include blood samples, buccal cells (kits can be sent in the post to the donors) or cumulus cells removed from oocytes at the time of egg collection.

Meiomapping: Informative maternal heterozygous SNP loci are identified and phased by reference to the genotype of a haploid PB2 or oocyte. This establishes two maternal haplotypes across each chromosome. The genotypes of the polar bodies and activated oocytes are then compared to the reference haplotype and crossovers identified by transitions between heterozygous and homozygous regions (typically in PB1) or between opposing haplotypes in PB1, PB2 or activated oocytes. Finally, common crossovers present in all samples are identified and reassigned to the reference sample. This reveals the pattern of unique crossover events in the oocyte-PB trios, which allows the chromosome segregation patterns to be interpreted.

Recombination events can be seen by comparing the samples to a haploid (PB2 or oocyte) reference. The reference needs to be from an independent oocyte-PB trio from the same donor and ideally with a high call rate (>60%) and low heterozygous call rate (~1-2%). The requirement for an independent reference sample necessitates at least two oocyte-PB trios per donor. It is desirable to have multiple samples from the same donor, which can help overcome problems associated with aneuploidy in the reference sample or common crossovers very close to other crossovers (see Troubleshooting Table 3 & Table 2).

Bioinformatics techniques and analysis: The SNP calls for each chromosome and each sample are imported into a Microsoft Excel spreadsheet for primary analysis (Supplementary Data). Each SNP is classified as ‘AB’ (both haplotypes), ‘AA’, or ‘BB’ (each grandparental haplotype). Regions of heterozygosity (both haplotypes present) are defined by the presence of blocks of AB calls. Homozygous regions are compared SNP-by-SNP to a reference sample; where the SNPs match those of the reference they are assigned ‘haplotype 1’ (yellow), where they differ they are ‘haplotype 2’ (green). This results in a pattern of haplotype switching or breakpoints, which represent crossovers. Any crossovers present on the reference chromatid will manifest as ‘common crossovers’ across all samples (Figure 3 & 4; red boxed region). These are removed and replaced to the reference as part of the secondary analysis (see step 48). Chromosome segregation is assessed at the end following crossover mapping as part of the secondary analysis. Non-canonical segregation patterns manifest as reciprocal losses or gains of chromosomes or

chromatids or in the case of reverse segregation as heterozygous SNPs surrounding the centromere in PB1.

Materials

CRITICAL: The reagents and equipment specified below for the culture and micromanipulation of human oocytes and embryos are for illustration purposes only. Individual IVF clinics will vary in their practices, which are optimised for clinical use.

REAGENTS:

- Mature (metaphase II arrested) Oocytes

CAUTION: Any experiments involving human oocytes must conform to relevant Institutional and National regulations. In the UK they must be obtained through an HFEA-licensed clinic in accordance with the HFE Act and with approval of the local research ethics service. Fully informed patient consent must be obtained in all cases.

- Oocyte culture medium (Quinn's Advantage cleavage medium; Sage, USA, ART-1026)
- Oocyte handling medium (Sage HEPES buffered medium, ART-1023)
- Human serum albumin (Sage HSA, ART-3001)
- Mineral oil (Sage culture oil, ART-4008)
- Hyaluronidase solution (SAGE, ART-4007-A)
- Phosphate Buffered Saline (PBS) (Dulbecco's Phosphate Buffered Saline, Sigma-Aldrich, USA, D8537)
- Calcium ionophore (Sigma-Aldrich, USA, A23187)
- Dimethyl Sulphoxide (DMSO) (Sigma-Aldrich, USA, D2650)
- WGA Kit (Sure MDA; Illumina, USA, PR-40-405102-00)
- Genomic DNA from oocyte donors obtained by using any appropriate commercially available kit (see Experimental Design for more details)

EQUIPMENT:

CRITICAL: All equipment should be situated in a dedicated tissue culture facility suitable for the culture of human gametes and embryos.

- Vertical Class 2 flow cabinet work station (IVF Tech, Stenløse DK)
- Stereo microscope (Leica Microsystems M80, Germany)
- 37°C heated work surface (IVF Tech, Stenløse DK).

- Incubators for embryo media equilibration and embryo activation culture (Sanyo MCO-5M, Sanyo Electric Co. Ltd, Japan- MINC Benchtop incubator, COOK Medical, Bloomington USA)
- Inverted microscope (Nikon Eclipse TE2000-U, Nikon Japan) equipped with two micromanipulators (NT-88-V3,Narishige, Japan) fitted with syringes attached to the holding and micro-pipettes.
- Inverted microscope (Nikon IX-70 microscope; Nikon Ltd, Japan) equipped with Hoffman Modulation contrast (Nikon Ltd, Japan), 37°C heating stage (Tokai Hit Thermo Plate, Tokai Hit CO.Ltd, Japan) and a laser objective (Saturn laser 5; Research Instruments, Falmouth UK)
- Holding micro-pipette (Holding pipette; TPC, Australia)
- Aspiration micro pipette for biopsy (zona drilling pipette; TPC, Australia)
- Stripper hand piece and plastic denuding pipette tips with a range of diameters (120µm,130µm and 170µm lumen; Flexipet denuding pipette,COOK Medical, Ireland)
- Culture dishes appropriate for culture system, either with or without time lapse incubation (EmbryoSlide; Unisense Fertilitech, Denmark/ Vitrolife Göteborg- or Falcon Primaria dish 60x15mm; Corning Incorporated, USA)
- Biopsy dishes (Nunc IVF ICSI Dish; Thermo Scientific, USA)
- Karyomapping Beadchip (HumanKaryomap-12 v1.0 BeadChip, Illumina, San Diego CA, USA)
- 0.2ml PCR tubes (any supplier)
- Thermocycler machine
- Computer with Visual Basic enabled Microsoft Excel (Microsoft Office; Microsoft, USA)
- **OPTIONAL:** Time lapse incubator (EmbryoScope; Unisense Fertilitech, Denmark/ Vitrolife Göteborg) for embryo cell culture and tracking.

REAGENT SET-UP:

Calcium ionophore stock solution. Dilute 20.94mg of calcium ionophore in 10ml of DMSO to make 4mM stock solution. Store at -20°C for up to 3 months

Working activation culture medium. Add 100ul of calcium ionophore stock solution to 3.9ml of Sage cleavage medium for a final 100uM working solution. Add 10% (v/v) HSA. Store at 3-5°C for up to a week.

Oocyte culture medium. Add 10% HSA to Sage cleavage medium (without calcium ionophore). Store at 3-5°C for up to a week.

Oocyte handling medium. Add 10% HSA to Sage HEPES buffered media. Store at 3-5°C for up to a week.

EQUIPMENT SET-UP:

Oocyte holding dishes: Make two 25µl drops of oocyte culture medium (one for washing, one for culture) per oocyte within culture dishes (Falcon Primaria) and overlay with mineral oil until the drops are entirely covered. Dishes should be allowed to equilibrate in incubator set at 37°C in 6% CO2 and 5% O2 for a minimum of 3 hours prior to use and within 24 hours of preparation.

Activation dishes: Make two 25µl drops of working activation culture medium (one for washing, one for culture) per oocyte within culture dishes (Falcon Primaria) and overlay with mineral oil until the drops are entirely covered. Dishes should be allowed to equilibrate in incubator set at 37°C in 6% CO2 and 5% O2 for a minimum of 3 hours prior to use and within 24 hours of preparation.

Post-activation dishes: This can be done using either EmbryoSlides, if culturing in an Embryoscope, or ordinary culture dishes if standard culture is used.If standard Culture is used make two 25µl drops of oocyte culture medium (one for washing, one for culture) per oocyte within culture dishes (Falcon Primaria) and overlay with mineral oil until the drops are entirely covered. If embryoscope culture is used fill all 12 numbered wells of the EmbryoSlide with 25µl of oocyte culture medium ensuring the micro-well at the bottom is free of bubbles. Completely fill the wash wells (with no micro-well) with the same medium and overlay with mineral oil until the wells are entirely covered.

Both culture dishes and EmbryoSlides should be allowed to equilibrate in an incubator set at 37°C in 6% CO2 and 5% O2 for a minimum of 3 hours prior to use and within 24 hours of preparation.

Biopsy dishes: Make two 10µl drops of oocyte handling medium per oocyte (one wash drop, one working drop) within biopsy dishes and overly with mineral oil. Allow

to equilibrate on heated stage (37°C) for a minimum of 20 minutes prior to use and use within one hour.

PROCEDURE:

CRITICAL: All oocyte culture is performed in a humidified incubator set at 37°C in 6% CO2 and 5% O2.To enable tracking of the oocytes and their corresponding PBs throughout the protocol, individual samples must remain separated. Thus, all culture drops, wells and tubes should be uniquely numbered and recorded to allow traceability throughout the experiment.

Oocyte preparation TIMING 5-10 mins

CRITICAL: For best results the protocol should begin 38-40 hours post LH surge (natural cycle) or administration of HCG trigger (stimulated cycle). If using vitrified oocytes, the protocol should begin 1-2 hours post oocyte warming (protocol for warming will depend on procedure used in clinic. We used the Kitazato CryoTop method[32]).

1. Obtain oocytes collected by surgical means ~35 hours post LH surge (peak of circulating Luteinising Hormone) or administration of Human Chorionic Gonadotrophin (HCG) injection. Remove all cumulus cell and the cells of the corona radiata from the collected oocyte [28]. Move all mature MII oocytes to an oocyte holding dish containing oocyte culture medium (see reagent set up) ready for biopsy and activation.

   CRITICAL STEP: It is imperative to ensure complete removal of surrounding cumulus cells before proceeding. Incomplete removal of all maternal somatic cells surrounding the oocyte could result in maternal contamination and will be apparent in the downstream SNP analysis. We recommend removal of cumulus cells by pipetting the oocytes up and down with a 130µm pipette in hyaluronidase solution. At this point oocytes may be vitrified for batching purposes prior to starting the protocol[33].

First polar body (PB1) biopsy. TIMING 15-30 mins

CRITICAL The activation and biopsy part of the protocol (steps 2 to 34) could feasibly be performed by a single operator. However, it is recommended that 2

operators work in tandem at time points where certain activities can be performed in parallel (e.g. biopsy and tubing) to avoid delays in processing, preserving the sample's integrity, especially when performing the protocol on more than six oocytes at the time. The biopsy should be performed as soon as possible following polar body extrusion. See troubleshooting table 3 for advice on polar body fragmentation.

2. At a stereo microscope work station, move the prepared oocytes using a 170um stripper from the culture dish in Step 1 to a biopsy dish (see reagent set-up) with individually numbered 10 µl microdrops of HEPES medium + 10% HSA under Sage oil. Use the one available wash drop per oocyte to ensure that each oocyte is free from any cellular debris by gently pipetting the oocyte up and down several times before allocating them to their biopsy working drop. The number of oocytes per dish is dependent on the skill of the operator. CRITICAL STEP: We recommend that no more than 15 minutes should pass between the oocytes entering the dish and being removed after biopsy (from 1-6 oocytes per dish)
3. Place the biopsy dish containing the oocytes on the inverted microscope (with micromanipulators and laser attached) to give a clear view of the PB and secure the oocyte by suction with the holding pipette ensuring that the oocyte is just above the bottom of the dish (Figure 2A).
4. Rotate the oocyte using the aspiration pipet until the PB is clearly in focus at 12 to 1 o'clock. If done correctly, the zona pellucida should also be clearly in focus (Figure 2A). CRITICAL STEP: It is essential to maintain a gentle yet constantly full seal between the holding pipette and the oocyte as even a small break in the seal could result in a poor outcome.
5. Once the oocyte is correctly positioned for biopsy make a small aperture with a series of laser pulses in the zona pellucida, no larger than the diameter of the aspiration pipette, adjacent to the PB on the side of the aspiration pipette. Work inwards from the outer surface of the zona. CRITICAL STEP: Attention must be given so as not to fire a laser pulse at the cytoplasm of the oocyte or PB.
6. Insert the aspiration pipette through the opening in the zona and position the lumen of the pipet adjacent to the PB.

7. Use the aspirator to create a small amount of suction and slowly withdraw to remove the PB. If the PB is still attached to the oocyte, break the intercellular bond using a single laser pulse, on the lowest intensity setting, at the cell junction to release the bond. CRITICAL STEP: Ideally the polar body should not fully enter the biopsy pipette. The suction created should simply allow the polar body to be drawn through the breach in the zona. Complete aspiration of the polar body may result in lysis, which may not affect amplification but makes tubing unreliable. If PB1 is fragmented during biopsy, ensure all fragments are removed for tubing.

Oocyte activation. TIMING 45-50mins

8. Move the biopsy dish containing the biopsied oocytes to a stereo microscope work station immediately after completing biopsy of all oocytes contained within the dish. CRITICAL STEP: Care must be taken during this step onwards so as not to exert too much pressure on the biopsied oocytes when pipetting. Slow pipetting with gentle aspiration and expulsion is recommended using 170um stripper pipettes.
9. Identify both the PB and the oocyte under the stereo microscope. Use a 170um stripper pipette to transfer individual oocytes, one at a time, to individually numbered drops in an activation culture dish, leaving the biopsied PB1 behind in the biopsy dish for immediate tubing.
10. Use the available wash drops to gently wash each oocyte before allocating them to their activation culture drop.

CRITICAL STEP: care must be taken not to disturb the biopsied polar body when removing the oocytes from the biopsy dish. Failure to do so may result in the polar body being lost

11. Begin a 40 minute timer from the time the last oocyte is transferred to the activation culture dish and put the dish in the incubator until the 40 minute timer ends.

CRITICAL STEP: At this point, further PB1 biopsy can be performed following Steps 2-11). If so a second operator should perform the tubing procedure below in parallel.

PB1 Tubing. TIMING 15-30mins

CRITICAL: *Prepare liquid nitrogen bath in advance for PB storage*

12. Add 1ul of PBS to the appropriate number of 0.2 ml, RNase and DNase free thin walled, flat cap PCR tubes for the number of polar bodies and label each tube appropriately.
13. To avoid evaporation and contamination, close the cap on the tubes gently (loose cap). CRITICAL STEP: Caps should be closed but easily releasable for depositing the samples. The 1uL of PBS should remain at the bottom of the tubes. If small drops form on the side of the tube, give the tubes a quick spin on the micro centrifuge prior to depositing the samples.
14. Focus on the polar body from Step 7 with the stereo microscope using the mirror to create contrast for best visibility.
15. Set the stripper pipette to 1uL and using a 120um pipette tip prime as much as possible with PBS. Depress the plunger all the way, aspirating PBS into the pipette until the plunger is entirely released. CRITICAL STEP: For best results change to a new stripper tip for each sample.
16. Enter the drop with the pipette tip whilst gently expelling a small amount of PBS to ensure no oil blocks the tip. No more than 0.5uL should need to be expelled. CRITICAL STEP: If any oil droplets are visible in the pipette tip, try to clear it by vigorous aspiration in clean PBS or replace it and restart from Step 15.
17. Place the pipette tip over and adjacent to the PB and release the plunger entirely (PB should enter the pipette and remain there). CRITICAL STEP: Ensure that the plunger and the pipette are not moved abruptly at this stage as the sample could be lost.
18. Open a pre-primed and labelled PCR tube from Step 12 and hold the tube between thumb and index finger so as to have a clear view through the tube from top to bottom.
19. Insert the pipette containing the sample into the tube so that the tip enters the PBS. CRITICAL STEP: Avoid touching the sides of the tube or the bottom if possible.
20. Expel the contents of the pipette into the tube stopping when 1uL mark is reached. This should be when there is a point of more resistance when depressing the plunger.
21. Keep the plunger depressed to this point and re-enter the sample drop to expel the remaining PBS whilst observing any objects exiting the pipette.

Pipette up and down once or twice with the media in the drop to ensure the PB is not stuck to the pipette wall. If the PB can no longer be seen in the pipette tip, tightly cap the tube. Move on to the remaining samples repeating Steps 12-21.

22. Once all samples have been tubed, briefly centrifuge them to ensure all the samples and media are in the bottom of the tubes.
23. Submerge the bottom of every tube (not the entire tube) in a shallow liquid nitrogen bath to snap freeze and store tubes in racks at -20^{0}C.

PAUSE POINT. Samples can be stored at -20^{0}C for several days prior to WGA. For best results WGA should be performed within 3 days.

Post activation culture. TIMING 16-18hrs

CRITICAL Ideally, post activation culture should be performed within a time-lapse incubator (set to 37°C, 6% CO_2 and 5% O_2). This enables easy assessment of PB2 extrusion and pronuclear formation without having to repeatedly remove oocytes from the incubator. It also allows monitoring of embryo development, if using embryos instead of activated oocytes. For our protocol we specifically use the EmbryoScope system that has its own proprietary dishes (EmbryoSlides), however, we also provide below options for proceeding without the use of a time-lapse incubator.

24. After 40 minutes of activation in culture (using samples from Step 9), remove the activation culture dish containing the oocytes from the incubator and move it to the stereo microscope work station.
25. Follow option A for post-activation culture using the Embryoscope time-lapse incubator, or option B to proceed if a time-lapse incubator is not available:

A. Post-activation culture using Embryoscope time-lapse incubator

i) Move each oocyte to a separate well of an EmbryoSlide with a 170um pipette tip. Wash using gentle pipetting in the provided wash wells prior to depositing in the final post-activation culture micro well (see reagent set-up). CRITICAL STEP: Ensure each oocyte is positioned in the centre of the micro-well and that all bubbles are removed for best visibility on the time lapse system.

ii) Move the EmbryoSlide to the EmbryoScope incubator unit and insert, following the prompts. CRITICAL STEP: It is important that the slide is seated in its position securely using substantial downward force.

B. Post-activation culture if time lapse incubator is not available

i) Move the oocytes to a post-activation dish containing individually labelled 30ul drops of post activation culture medium using a 170um stripper. Wash each oocyte thoroughly by gentle pipetting in a wash drop within the dish prior to depositing in the final culture drop.

ii) Place the dish in a standard incubator (set at 37°C in 6% CO2 and 5% O2) until assessment of PB2 extrusion 16-18 hours after the oocytes are moved to post activation culture. “?TROUBLESHOOTING”

Assessing activation. TIMING 5-10mins

26. Follow option A to assess activation using the Embryoscope time-lapse incubator, or option B to proceed if a time-lapse incubator is not available:

A. Assessing activation using Embryoscope time-lapse incubator

i) After ~ 16 hours of post-activation culture, use the EmbryoScope time lapse viewer to scroll through the images of each oocyte whilst the EmryoScope slide containing the oocytes remains in the incubator unit. Scrolling at different focal depths may be necessary to properly identify PB2 extrusion. Pronuclei may also become visible by this time however normal activation is not dependent on it.

B. Assessing activation if time lapse incubator is not available

i) After ~ 16 hours of post-activation culture, move the dish containing the oocytes to the inverted microscope work station. Gradually focus through the oocytes to identify the presence of PB2. Again, pronuclei may also be visible by this time however normal activation is not dependent on it. If PB2 is not visible, check again in two hours.

PB2 Biopsy. TIMING 15-30mins

27. Repeat Steps 2-7 to biopsy PB2 observed in step 25

CRITICAL STEP: Ensure extra care is taken when pipetting and that suction with the holding pipette (Steps 3-4) avoids the existing aperture in the zona pellucida (Figure 2B).

CRITICAL STEP**:** If PB2 biopsy is to be performed on more than one dish of activated oocytes (recommended maximum 6 oocytes per biopsy dish) a second operator should perform the tubing procedure below in parallel.

PB2 Tubing. TIMING 15-30mins

28. Repeat steps 12-23 whist the oocyte remains in the drop with the PB2.
    CRITICAL STEP: It is important that the oocyte and PB2 are well separated in the drop and that the oocyte is avoided when pipettes enter the drop (steps 16, 17 & 21) (Figure 2). If working in tandem with another operator, move biopsy dish to inverted microscope for zona removal procedure (step 29-32).

    PAUSE POINT If working alone, it is possible to leave the biopsy dish, now containing only the oocytes (devoid of their PB1 and PB2), on the heated stage of the stereo microscope for up to two hours

Zona removal. TIMING 10-20mins

29. Place the dish containing the oocytes on the inverted microscope (with micromanipulators and laser attached) to give a clear view of the zona pellucida and secure the oocyte by suction with the holding pipette ensuring that the oocyte is just above the bottom of the dish. Be careful to avoid the two existing apertures in the zona.
30. Once in position, make a large aperture in the zona pellucida using a series of laser pulses. The aperture should be between a quarter to a half of the zona around. Ablate the zona using laser pulses approximately the same diameter of the zona thickness, working in a circular fashion (figure 2C).
31. Insert the aspiration pipette under the zona and gently expel medium to displace the oocyte so that it is free from the zona. Ensure that the pipette contains sufficient media and be careful not to blow any bubbles. Gentle manual manipulation with the aspiration pipette to move the oocyte may also be necessary to help the displacement process.

32. Whilst still attached to the zona move the holding pipette to the edge of the drop and deposit the zona there leaving the oocyte in the centre of the drop.

Oocyte tubing. TIMING 15-30mins

33. Repeat steps 12-23 to complete the tubing of the oocyte. CRITICAL STEP: avoid the empty zona pellucida when aspirating the oocyte (steps 16-17).

Whole genome amplification (WGA) and SNP genotyping. TIMING: 1-2 days

CRITICAL: for further details refer to Natesan *et. al* 2014[6].

34. Use a 10ul Gilson pipette to bring the three PCR tubes containing each of the trio samples (from steps 20,28 & 33) to an end volume of 4 µl with PBS.
35. Amplify the DNA by Multiple displacement amplification (MDA) using a kit according to the manufacturer's instructions (SureMDA, Illumina).
36. Process 400 ng of donor genomic DNA and 8 µl of each of the WGA products from the trio samples (PB1, PB2 and oocyte) on SNP genotyping beadchips (Human CytoSNP-12 or HumanKaryomap-12 v1.0 BeadChip, Illumina, San Diego CA, USA) for ~300K SNPs, following the instructions provided. CRITICAL STEP: This step in the protocol can be outsourced to a third party if necessary. If so, the samples can be shipped in racks on ice to the commissioned laboratory. Do not ship on dry ice. Ensure care is taken with the parcel and shipping time does not exceed 3 hours.
"?TROUBLESHOOTING"

Meiomapping. TIMING: 1-2 days or as long as required.

CRITICAL: Steps 41 [Query to author: Do you mean step 44 here?] onwards are carried out using a series of Visual Basic for Applications (VBA) macros in Microsoft Excel[34]. An example data set can be found in Supplementary Data (contact authors for further information regarding macros).

37. Import data generated from SNP genotyping into Microsoft Excel and sort according to (1) chromosome and (2) base pair (bp) position. Save file and archive following step 39.

38. Import the SNP genotype data for each of the 23 chromosomes into a second Excel spreadsheet for Meiomapping and arrange in columns side-by-side for each chromosome and each sample. CRITICAL STEP: Note that the original data and processed data are stored in separate Excel workbooks to keep the size of the files manageable.

39. Calculate the call rates and heterozygous call rates for all samples (please see ‘Comparison to other methods’ section for description of call rates).

40. Select a reference sample (either a PB2 or activated oocyte)

CRITICAL STEP**:** Note that to maximise the number of informative SNP loci a reference sample with a high call rate and low heterozygous call rate (ADI) should be selected. The call rates should exceed 80% although anything above 60% is acceptable and the heterozygosity rate should be in the region of 1-2%.

41. Identify all Informative maternal heterozygous SNP loci genotyped as homozygous in the reference sample (AA or BB), and defined as Maternal haplotype 1 (yellow) for each chromosome. See Table 1.

42. Compare sample genotypes at informative maternal SNP loci for each chromosome to the reference genotype and identify as (1) homozygous for the same allele – Maternal haplotype 1 (yellow), (2) homozygous for the other allele – Maternal haplotype 2 (green), (3) heterozygous – combination of Maternal haplotypes 1 and 2 (blue), or (4) no call (grey). See Figure 3 and Table 1. See troubleshooting table 3 for explanation of anomalies caused by aneuploidy in the reference sample.

43. Display the results of the previous step as a consecutive series of informative SNPs in vertical columns, colour coded with their bp position, for each chromosome. Arrange the vertical columns side-by-side for each sample and each chromosome (Figure 3). “?TROUBLESHOOTING”

44. Use a macro that detects transitions in maternal haplotypes (based on the colour of each cell) or manually annotate the positions of any crossovers indicated by the transitions described above For manual annotation, copy and

paste the informative SNPs flanking the recombination into the adjacent right-hand column, including the colour coding and bp position (Figure 3, enlarged region). See troubleshooting table 3 for advice on very closely spaced crossovers and for tagging transitions between heterozygous and homozygous haploblocks. CRITICAL STEP: Each recombination should always be present in two out of three of the products of meiosis at the same position. “?TROUBLESHOOTING”

45. Import the flanking SNP positions for each recombination event into a separate worksheet.

46. Remove common crossovers from each non-reference sample and add to the reference sample (Figure 4). CRITICAL STEP**:** It is essential to identify and remove common crossovers from all other samples and represent them only in the reference chromatid. Note that reciprocal recombination between sister chromatids in PB2 and the activated oocyte, one of which is the reference, will not be visible in the non-reference component either and needs to be added back to this component as well as the reference (Figure 4; red dotted boxed region). “?TROUBLESHOOTING”

47. Calculate the position of all crossovers as an average of the base pair position of the two SNPs flanking a crossover. For example, the average of two SNPs flanking the crossover shown in the enlarged box in Figure 3 is 39,416,076 (green SNP position: 39,354,517, yellow SNP position: 39,477,635) and so the crossover is inferred to have occurred at position 39,416,076.

48. Correct the haplotype colours across each true recombination following the removal of the common crossovers.

49. Display the pattern of crossovers in each component of each trio according to its position to generate the MeioMap (Figure 4). See troubleshooting table 3 about crossovers near to centromeres on chromosomes 1 and 9. “?TROUBLESHOOTING”

50. Use further macros within Excel or manual counting to perform secondary analysis of the numbers and positions of the crossovers Detect various chromosome segregation errors by inferred atypical patterns of homozygous and heterozygous regions in the three components in the MeioMap. Please refer to Ottolini et al (2015) for comprehensive description of the various patterns[7].

TIMING

Day One:
- Preparing oocytes: 5 – 10 minutes (step 1)
- PB1 biopsy: 15-30 minutes (steps 2-7)
- Oocyte activation: 45-50 minutes (steps (8-11)
- PB1 tubing: 15-30 minutes (steps 12-23)
- Post activation culture: 16 – 18 hours (overnight; steps 24-25)

Day Two:
- Assessing activation: 5 – 10 minutes (step 26)
- PB2 biopsy: 15-30 minutes (step 27)
- PB2 tubing: 15-30 minutes (step 28)
- Zona removal: 10-20 minutes (step 29-32)
- Oocyte tubing: 15-30 minutes (step 33)
- Whole Genome Amplification (WGA) and shipment of samples: ~ 4 hours (step 34)
- **Time taken to receive the SNP genotyping results will depend on the turnaround time of the service lab.**

Day Three onwards:
- Meiomapping: The time taken will depend on the number of samples as the rate limiting step is the manual annotation of the crossover points, but as a guide allow 1-2 full working days. This stage of the protocol is all computer-based and so is less time-sensitive (steps 35-48)

TROUBLESHOOTING

See Table 3 for troubleshooting guidance.

ANTICIPATED RESULTS

After mapping all of the crossovers genome-wide across all samples the data can be represented as a complete MeioMap (Figure 5A) with three columns for each chromosome representing the PB1, PB2 and activated oocyte. Note that for any given crossover, two out of the three elements are involved (i.e. each crossover is reciprocal). Additionally, every haploblock segregates in the predicted Mendelian ratios (2 green and 2 yellow chromatids at any given position). In the example shown, all chromosomes followed normal meiotic segregation pattern.

Non-canonical chromosome segregation patterns can also be detected in the meiomaps. Examples include reverse segregation where both homologs segregate their sister chromatids at meiosis I[7], precocious separation of sister chromatids and meiosis II non-disjunction (Figure 5B). Note that these non-canonical segregation events do not always result in a loss/gain in the oocyte.

Structural chromosome abnormalities can also be detected in MeioMaps. Frequently these are characterised by duplications on one arm of a metacentric chromosome. For example, a region of heterozygosity may exist on the q arm of PB1 and on the p arm of PB2 or oocyte (as shown in the example in Figure 5C). Structural chromosome abnormalities can also manifest as missing segments of a chromosome (Figure 5C; bottom)[Query to author: Editor has changed text, please check it is OK].

The total number of crossovers genome-wide together with the number of chromosome segregation errors can be counted for each oocyte-PB trio and used for subsequent data analysis.

CFI

The authors declare that they have no competing financial interests.

Author contributions statement

CO, AC, DC, LR and FU were responsible for donor consenting, oocyte collection and oocyte activation. LR, FU, AH oversaw ethical and legal regulation in Italy and the UK. AC, CO and DC carried out amplification, SNP array and array CGH experiments. AH, LJN and CO analysed the SNP data. AH and LJN generated the figures. AH, LJN and CO wrote the manuscript. AH, ERH and LJN edited the manuscript. All authors proof-read and accepted the manuscript.

		Haplotype				MeioMap					
						PB1 Haplotype		PB2 Haplotype		oocyte Haplotype	
SNP no.	MAT	1 REF	PB1	PB2	oocyte	1	2	1	2	1	2
1	AA	Not informative									
2	BB	Not informative									
3	AB	AA	AB	AA	BB	A	B	A			B
4	AB	BB	BB	NC	AA	B					A
5	AB	AA	AB	AA	NC	A	B	A			
6	AB	AA	AB	AA	BB	A	B	A			B
7	AB	AA	NC	BB	AA				B	A	
8	AB	BB	AB	NC	BB	B	A			B	
9	AB	AA	BB	BB	NC		B		B		
10	AB	BB	AB	NC	BB	B	A			B	
11	AB	BB	BB	AA	AA	B			A		A
12	AB	AA	AA	BB	NC	A			B		
13	AB	BB	BB	AB	AA	B		B	A		A
14	AB	BB	BB	AA	AA	B			A		A
15	AB	AA	AA	BB	BB		B	A		A	
16	AB	AA	AA	NC	BB		B			A	
17	AB	BB	BB	AA	BB		A	B			A
18	AB	BB	BB	AA	AA		A	B		B	

Table 1. Table showing representation of phasing of maternal SNPs in oocyte-PB trios and illustrating some of the technical limitations that accompany SNP genotyping. Only heterozygous maternal SNPs are informative for phasing. The reference sample is from an independent oocyte-trio from the same donor. Where the sample SNP matches the reference (light pink) it is assigned maternal haplotype 1 and is colored yellow in the MeioMap. Where the sample SNP differs from the reference, it is assigned maternal haplotype 2 and colored green. Where both SNPs are present (AB) the region is heterozygous and is colored blue in the MeioMap. When neither SNP calls, the SNP is colored grey. Crossovers between homozygous regions of PB2 and oocyte occurs at SNP 6 as detected by a switch from yellow to green SNPs (and *vice versa*). A crossover between heterozygous and homozygous

regions in PB1 and oocyte, respectively occurs at SNP 10. A ‘common crossover’ (see Development of Protocol & Figure 4) occurs at SNP 14. Allele Drop Out (ADO) refers to when one SNP of a particular haplotype fails to call. If this occurs within a block of homozygosity, this will manifest as a ‘no call’ where neither haplotype is present for that particular SNP. In contrast, ADO in an otherwise heterozygous block will manifest as only one haplotype being present for that particular SNP (e.g. one green cell surrounded by a block of blue cells either side). Allele Drop In (ADI) refers to when the opposing haplotype calls for one particular SNP in an otherwise homozygous region (e.g. one blue cell surrounded by a block of green or yellow cells either side). Examples of allele drop out (ADO) and allele drop in (ADI) are as follows: ADO in homozygous region (grey cell, SNP 4 PB2) leads to loss of resolution; ADO in heterozygous region (SNP 4 or 9 in PB1) leads to loss of heterozygosity and creates ‘noise’ in the data; Double ADO (SNP 7 in PB1) results in no call and a reduced resolution; ADI without ADO (SNP 13 in PB2) results in erroneous heterozygous calls and ‘noise’ in the data; ADI with accompanying ADO at the same position (SNP 17 in oocyte) leads to erroneous call of opposing haplotype and ‘noise’ in the data.

Table 2. The relationship between the number of oocyte-PB trios per donor and the detection and assignment of crossovers by meiomap analysis

No. of oocyte-PB trios	Crossovers detected and assigned
1	All crossovers between homologous chromosomes detected but cannot be assigned to the specific chromatid in PB2 or oocyte. Meiomapping cannot detect crossovers occurring within heterozygous regions of the chromosome remaining in PB1.
2	All crossovers between homologous chromosomes detected and assigned to PB2 or oocyte in non-reference trio.

	Crossovers between sister chromatids in PB2 and oocyte are detected but cannot be assigned to a specific trio. Repeating the meiomap analysis with the reference in the second trio allows assignment in both trios
≥3	All crossovers between homologous chromosomes detected and assigned to PB2 or oocyte in all trios. Repeating the meiomap analysis with a second reference in a different trio allows complete assignment in the original ref trio and the other trios Crossovers between sister chromatids detected in all trios.

Table 3: Troubleshooting table

Step No.	Problem	Possible Reason	Possible Solution
2	Fragmentation of the polar bodies.	Biopsy taking place too long after polar body extrusion.	If fragmentation occurs, try to extract and tube all fragments and make a note of the sample that can be referred back to if any non-mendelian losses or gains are observed. Performing the biopsy immediately after polar body extrusion should minimise risk of fragmentation.
25	Inefficient oocyte activation.	Calcium ionophore not fully solubilised in stock solution.	Ensure calcium ionophore is fully solubilised in DMSO when preparing stock solution.
36	Poor DNA amplification.	DNA may have partially degraded.	Keep samples on ice and tubes capped whenever possible to minimise exposure to nucleases. You can confirm the DNA amplification step by running the samples on a 1% agarose gel.
43	Erroneous SNP calling across all samples for one particular chromosome.	Aneuploidy (chromatid loss or gain) in the reference sample.	Aneuploidy in the reference requires that you use an alternative reference for that chromosome only. This should restore the normal haplotype patterning to the samples.
44	Very closely positioned crossovers that give rise to very short haplotype blocks (1).	These could be genuinely closely spaced crossovers (see below). Alternatively, they may be a crossover very close to a common crossover. In the rare event that a crossover occurs at the exact same SNP position as a common crossover, this will render the common crossover invisible in this sample.	If this is caused by proximity to a common crossover, this should be apparent from presence of the common crossover across all samples. If a crossover occurs at the exact same position to a common crossover in the reference this will be detectable by the common crossover ‘missing’ from one sample. In this scenario, it

			becomes advantageous to have multiple samples from the same donor in order to expose the sample with the 'missing' common crossover. In either case, the true pattern of crossovers can be confirmed by using an alternative reference for that chromosome.
44	Very closely positioned crossovers that give rise to very short haplotype blocks (2).	Although crossovers are generally widely spaced (owing to crossover interference), sometimes two crossovers can occur in close proximity.	It is therefore worth setting the minimum number of consecutive SNPs that are required to define a haploblock. This is also applicable to crossovers that occur very close to telomeres. If allele drop in (ADI) rates are particularly high you may want to increase the threshold of consecutive SNPs.
44	Tagging SNPs flanking a transition from heterozygous to homozygous region.	Regions of heterozygosity indicate the presence of two non-sister chromatids, typically in PB1, tend to have variable levels of allele drop out and 'no calls' (Table 1). This may make it hard to judge where to 'tag' the last SNP of the heterozygous block.	The nearest available heterozygous (blue) SNP to the crossover should therefore be tagged along with the first available SNP of the next haplotype (yellow or green).The reciprocal transition in PB2 or the activated oocyte can be used to align the precise position of the recombination event.
49	Crossovers occurring across the centromere of chromosome 1 or 9.	Large regions of repetitive sequences surround the centromeres of both chromosomes 1 and 9 and are therefore absent from the SNP array.	Since crossovers cannot be mapped directly in these regions be aware of haplotype switches either side of the centromere, which would indicate a crossover somewhere in this 'blind' region which can be arbitrarily assigned to either the p or q arm.

Figure 1. Schematic showing an adult oocyte completing the first and second meiotic divisions. In the adult ovary, primary oocytes contain 23 pairs of homologous chromosomes held together by crossovers in the 'bivalent' configuration. Oocytes complete meiosis I *in vivo* and extrude one set of homologs into the first polar body (PB1), the mature oocyte then arrests at metaphase II. Completion of meiosis II normally only occurs upon fertilisation, but we utilise a calcium ionophore to trigger meiosis II without fertilisation by sperm. At meiosis II one pair of sister chromatids are extruded to the second polar body (PB2) resulting in a haploid oocyte.

Figure 2. Images showing oocyte activation and sequential biopsy of first and second polar bodies. (A) The first polar body (PB1) is biopsied and tubed ('polar body 1 biopsy'; steps 11-22). (B) The mature oocyte is then activated using a calcium ionophore and the second polar body (PB2) is then biopsied ('polar body 2 biopsy'; steps 27-30). Note the appearance of a pronucleus in the activated oocyte (black arrow). The position of the laser is shown by a red cross within a green circle. (C) Finally, the activated oocyte is isolated from the zona pellucida, ready for tubing (steps 31-32). Bars: 15 µm. Permission to perform this experiment was obtained from the Ethical Review board at GENERA clinic, Italy.

Figure 3. Screenshot of SNP patterns in a MeioMap showing a region of a single chromosome across an oocyte-polar body trio. Each cell represents the position of a single SNP and opposing maternal SNPs are shown as yellow or green cells. When both sets of maternal SNPs are present (i.e. in heterozygous regions), the cells are coloured blue. Grey cells denote SNPs that have failed to call (ADO). Transitions between haplotypes mark points of crossovers. Crossovers are 'tagged' (step 44) by copying the two SNPs flanking a crossover into the right-hand adjacent column. The red dashed box illustrates where a crossover has occurred in the reference resulting in a 'common crossover' at the same position across all homozygous samples. The boxed area is enlarged on the right to show the SNP positions in each cell. In this example the SNPs flanking the crossover are 123 kb apart. Screenshot is from data imported into a Microsoft Excel worksheet. [MPS: It is not necessary to be able to read the specific numbers on the right of this figure.]

Figure 4. Informative SNPs are phased using a reference chromatid to reveal positions of haplotype switches (crossovers). (A) A haploid cell containing a single chromatid (either PB2 or Oocyte) with a good call rate is chosen as the reference chromatid. Oocyte-PB trios from the same individual are compared to the reference chromatid and crossovers are mapped at positions where the haplotypes switch from

the ‘assumed ancestral phasing’ in the reference. When a crossover occurs on the reference chromatid, this manifests as a ‘common crossover’ that appears across all homozygous samples at the same position (dotted red boxes). (B) These common crossovers are identified, removed and replaced to the reference chromatid using a macro. This process is repeated with a second reference to confirm crossover positions and the original ancestral phase of the two maternal haplotypes (C).

Figure 5. Anticipated results of Meiomapping. (A) An example of a complete Meiomap of an oocyte-PB trio. The three columns correspond to polar body 1 (PB1), polar body 2 (PB2) and oocyte. Green and yellow maternal haplotypes are shown and where both are present (in PB1) this is represented in blue. Regions where SNPs are not available on the array are shown in white (centromeres of chromosomes 1 and 9) or gray (rDNA). Black boxes indicate the centromeres. (B) Three example meiomaps (of a single chromosome) following non-canonical chromosome segregation patterns. This includes reverse segregation where both homologs segregate their sister chromatids at meiosis I, characterised by a PB1 with heterozygous SNPs (blue) around the centromere[7]. In the example of precocious separation of sister chromatids, an extra chromatid segregated into the PB1 with a corresponding loss in PB2. Meiosis II non-disjunction is characterised by a normal PB1 (two chromatids homozygous at the centromere) but with a gain and corresponding loss in the PB2/oocyte. Schematic representations of the inferred patterns of chromosome segregation shown to the right. (C) Two examples of structural chromosome rearrangements. Top example; an extra segment from the p-arm of chromosome 8 segregated to the oocyte. Bottom example; a large segment of the q-arm of chromosome 22 is missing in the oocyte. Schematic representations of the inferred patterns of chromosome segregation shown to the right.

Supplementary Data

An example Excel spreadsheet containing the raw data and assimilated MeioMap for chromosome 1 with three oocyte-PB trios.

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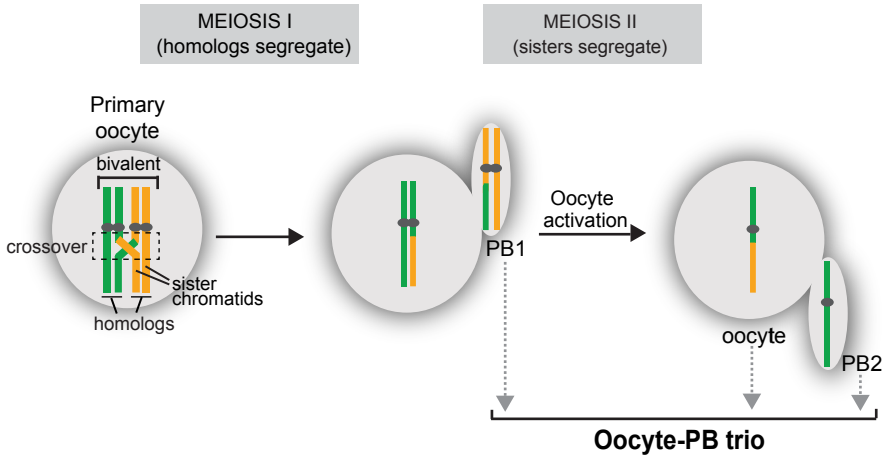
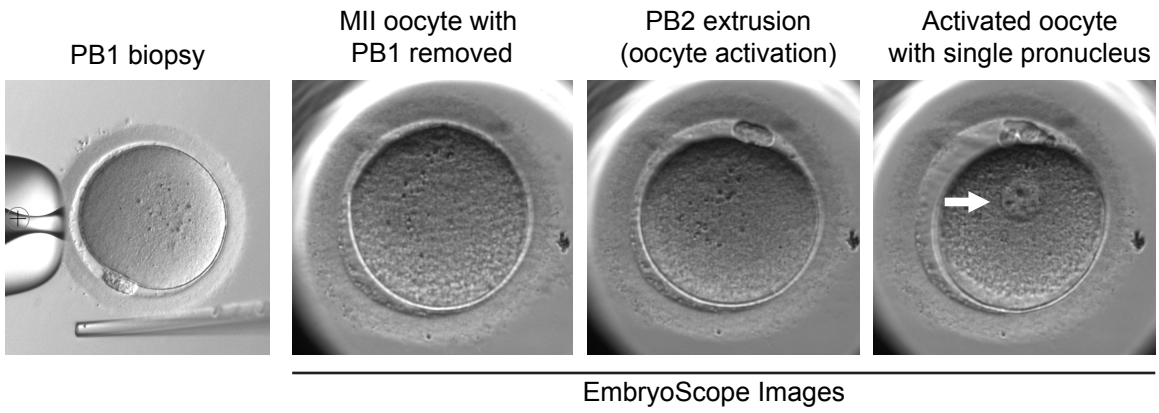
Fig.1


MEIOSIS I
(homologs segregate)
MEIOSIS II
(sisters segregate)
Primary
oocyte
bivalent
crossover
sister
chromatids
homologs
PB1
Oocyte
activation
oocyte
PB2
Oocyte-PB trio


PB1 biopsy
MII oocyte with PB1 removed
PB2 extrusion (oocyte activation)
Activated oocyte with single pronucleus
EmbryoScope Images

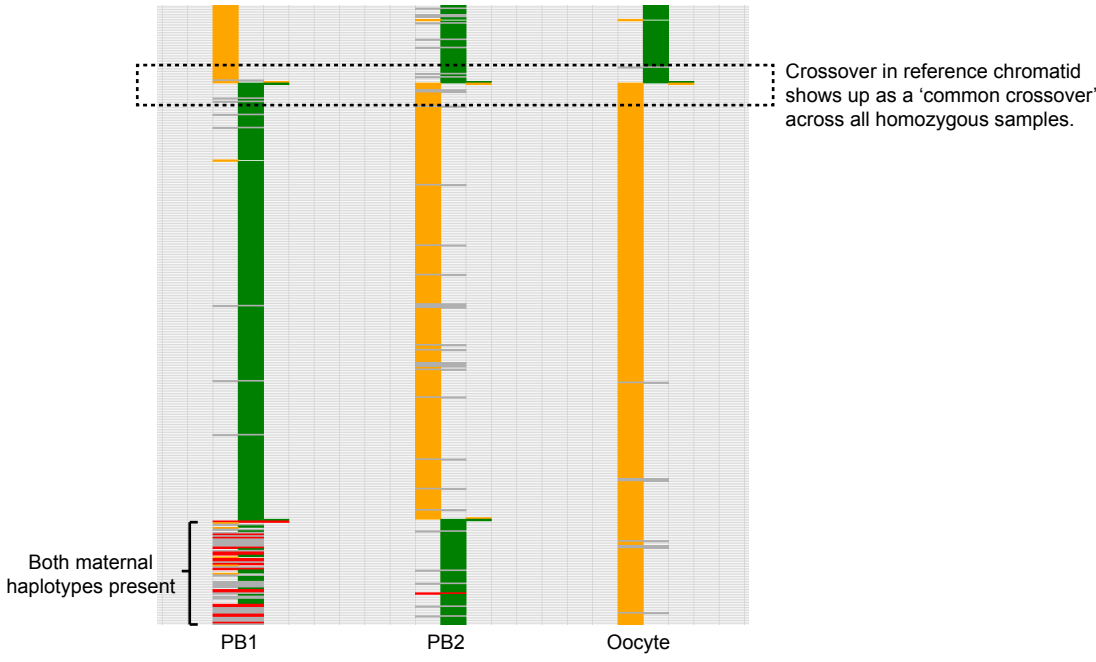
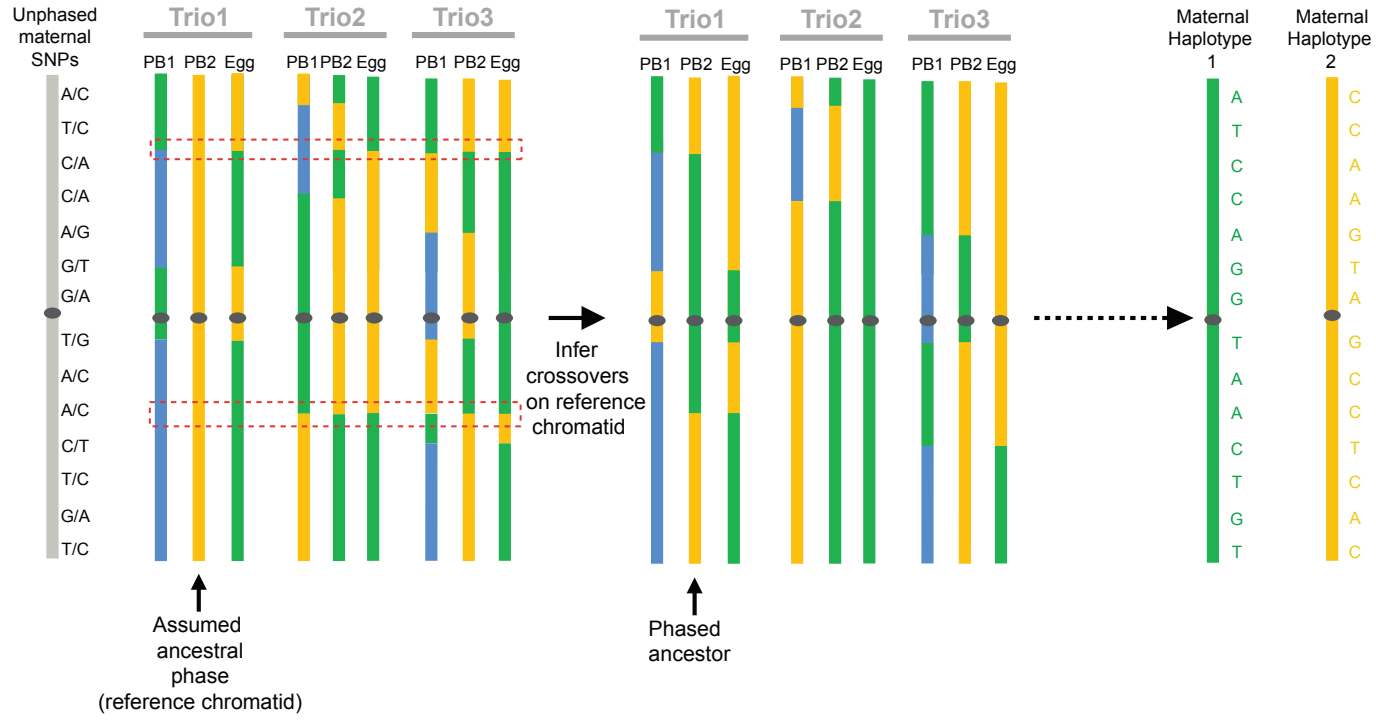

Crossover in reference chromatid shows up as a 'common crossover' across all homozygous samples.
Both maternal haplotypes present
PB1
PB2
Oocyte

Fig. 4


Unphased maternal SNPs
A/C
T/C
C/A
C/A
A/G
G/T
G/A
T/G
A/C
A/C
C/T
T/C
G/A
T/C
Trio1
Trio2
Trio3
PB1 PB2 Egg
PB1 PB2 Egg
PB1 PB2 Egg
Assumed ancestral phase (reference chromatid)
Infer crossovers on reference chromatid
Trio1
Trio2
Trio3
PB1 PB2 Egg
PB1 PB2 Egg
PB1 PB2 Egg
Phased ancestor
Maternal Haplotype 1
A T C C A G G T A A C T G T
Maternal Haplotype 2
C C A A G T A G C C T C A C