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Multicolour detection of every chromosome as a means of detecting mosaicism and nuclear organisation in human embryonic nuclei

Turner KJ,¹* Fowler KE,²* Fonseka GL¹, Griffin DK¹ and Ioannou D³

1. School of Biosciences, University of Kent, Canterbury, Kent, UK
2. School of Human and Life Sciences, Canterbury Christ Church University, Canterbury, UK
3. Department of Human and Molecular Genetics, Herbert Wertheim College of Medicine, Florida International University, Miami, FL, USA.

*Turner KJ and Fowler KE are joint first authors
Griffin DK is the corresponding author
Introduction

Fluorescence *in situ* Hybridisation (FISH) revolutionised the study of cytogenetics in the late 1980s, enabling basic scientists and clinicians to visualise specific chromosome regions within the nucleus. It provided, for the first time, a direct link between the microscope and DNA sequence. The technique uses fluorescently labelled short stretches of DNA (probes) that have a high level of sequence complementarity to specific sections of a chromosome. Following denaturation of chromosomal (target DNA) and probe, hybridisation is allowed to occur under specific conditions (e.g. temperature, concentration of formamide) to allow high affinity between target and probe DNA. By the early 1990s FISH was adopted by fertility centres worldwide as means of sex determination in preimplantation embryos from couples at risk of transmitting X-linked disorders [1, 2]. Shortly after, FISH found additional roles in the identification of unbalanced translocations and in chromosome copy number screening (e.g. embryo, sperm aneuploidy). Since then, the rapid increase in the use of *in vitro* fertilisation (IVF) and preimplantation genetic diagnosis (PGD) globally has not only enabled huge advancements in reproductive medicine, but has also provided a unique opportunity to study the cytogenetics of human embryos at the earliest stages of development. With the ultimate goal of developing diagnostic tests and improving patient care, those embryos produced by IVF cycles that are not deemed for transfer represent a valuable source of sample material under appropriate ethical justification. Nowadays, FISH has been replaced with newer technologies for the purposes of PGD using single cells; first by array CGH, then by single nucleotide polymorphism arrays (SNP) or quantitative real-time polymerase chain reaction (qRT-PCR) and most recently by next generation sequencing (NGS) [3]. Concurrently with the application of these newer technologies, the platform of evaluating the chromosomal status of preimplantation embryos has shifted from blastomere to trophoderm biopsy (blastocyst stage). Despite the advancement in technologies, the cell-by-cell analysis of blastomeres or available blastocysts within an embryo with the new techniques is still prohibitively expensive. As a result, FISH remains very much an invaluable resource for the study of...
cytogenetics in preimplantation embryos in terms of evaluating the level of mosaicism and at a more research level the nuclear organisation of chromosomes at this early stage of development. The purpose of this review is to give an overview of the history of FISH in PGD/PGS, cover the reasons why it fell out of favour and indicate how it may, with recent adaptations, be used as a tool for research and “follow up” of clinical cases.

A brief history of FISH and its use for PGS

The assessment of chromosome copy number in preimplantation embryos is the essence of preimplantation genetic screening (PGS), a commonly elected procedure in couples where advanced maternal age (AMA), recurrent miscarriage, recurrent implantation failure (RIF) or male factor infertility is implicated. It is widely believed that aneuploidy (presence of an extra or missing chromosome) is present in approximately 0.6% of live newborn infants, 6% of stillbirths, and 60% of spontaneous abortions [9, 10]. Moreover, numerical chromosome abnormalities are present in 60-70% of embryos generated by IVF (at the blastomere stage) [11-14], whereas it can reach levels of >50% in the blastocyst stage [15]. Although the majority of chromosome copy number abnormalities are lethal, aneuploidies involving a few specific chromosomes survive to term. On this basis, following a rise in the use of FISH for sex determination in the early 1990s and the availability of multicolour probes, the use of FISH was expanded to the detection of aneuploidy in order to selectively implant embryos more likely to be fully euploid.

The rationale behind the use of PGS in infertile couples requiring assisted reproductive technology (ART), is to increase pregnancy rates, since morphology alone does not suffice to distinguish a euploid from an aneuploid embryo. Therefore, by transferring euploid embryos, the chances of a viable pregnancy should be higher. The logic of this hypothesis is generally accepted in the field of reproductive medicine and can have particular application in women of AMA, couples with RIF, repeated miscarriage or severe male factor infertility [20, 21]. Initially from the 1990s to 2010, FISH
was used to perform diagnosis on the chromosomal complement of polar bodies and blastomeres [22, 23]. A total of eight chromosomes, six autosomal chromosomes (13, 15, 16, 18, 21 and 22) and the sex chromosomes were more commonly tested in IVF clinics through PGS as these were known to be involved in aneuploidies detected in spontaneous abortions and in trisomic live births [11, 24]. Despite the initial reports for an increase in implantation rates, reduction in trisomic offspring and spontaneous abortions [25, 26], criticism emerged since these reports were non-randomised, had poor experimental design, inadequate control groups and lack of report on live births [26].

From 2004 to 2010, eleven randomised control trials (RCTs) showed that PGS with FISH did not increase delivery rates, some studies showed the contrary and sparked a huge debate in the field. The reasons [26-29] for the reduced efficiency of PGS-FISH are beyond the scope of this review however they extended from technique-inherent limitations to biological (e.g. high levels of mosaicism in cleavage stage embryos, biopsy stage). This opened up different methodological approaches for the analysis of all chromosomes using genome wide platforms (e.g. aCGH, SNP-array, NGS), prompted multi-centre RCTS [30, 31] and parallel with improvements in culturing [32] and cryopreservation of embryos (e.g., vitrification) [33, 34] the diagnostic platform was shifted from day 3 to day 5 (blastocysts), making at the same time FISH an outdated technology for the complete chromosomal complement analysis in a PGS setting.

24 chromosome FISH on single cells

During the time since FISH was first popularised, the technique has evolved considerably to see the development of directly labelled, multicolour, commercially available probes with shorter hybridisation times and greater hybridisation efficiencies, which has enabled the ability to study up to 12 chromosomes within the same nucleus at once [4]. Better still, whole chromosome paints for all 24 chromosomes soon became commercially available by mixing fluorochromes to produce secondary colours. However, the difficulty with taking this approach is that overlapping signals in the
interphase nucleus are not easily distinguishable from one another. To circumvent this problem therefore, we developed a new ‘multilayer’ approach to 24-chromosome FISH, enabling comprehensive analysis of copy number for each chromosome in the karyotype. Based on a previously published protocol termed ‘re-FISH’, six spectrally distinct probes were used, in four consecutive rounds of FISH to visualise all 24 chromosomes [5]. The setup of 6 fluorochromes and 4 rounds of hybridization was selected to maximise the outcome of chromosome copy number, while reducing the rounds of re-probing of nuclei and thus increase the chances of signal efficiency. In addition the probes for the chromosomes that constitute each round of hybridisation were categorised based on availability of centromeric sequences for that particular chromosome or not.

The first three rounds of hybridisation (that can be inter-changeable) use probes against centromeric sequences; round one for chromosomes 1, 3, 4, 6, 7 and 8, round two for chromosomes 9, 10, 11, 12, 17 and 20, round three for chromosomes 2, 15, 16, 18, X, Y whereas the fourth round uses unique sequence targets for 6 chromosomes that do not have unique centromeric probes, chromosomes 5, 13, 14, 19, 21 and 22. The protocol can be completed within 24 hours, since the hybridisation times for the centromeric layers is 15-30 minutes and overnight for the unique sequence layer, fitting in a clinical setting and tailored for different applications (e.g. embryo versus sperm aneuploidy). The fast hybridisation times for the centromeric layers are possible due to the highly repetitive sequences (α-satellite) used to generate the respective probes.

A bespoke capturing system is necessary in order to be able to image all fluorochromes in separate channels plus the DAPI counterstain in a different channel. In-house, we used a modified version of Digital Scientific’s SmartCapture, and this novel approach that has been previously validated in different cell types [5], offers a powerful research tool in the identification of chromosome copy number that can be applied to different cell types. It also allows for the simultaneous assessment of nuclear organisation; that is, the so-called “nuclear address” of chromosomes (or sub-chromosomal
regions and/or loci) within the nucleus. For this feature a custom-script for Image J (freely-
downloadable software) is required and more details have been previously published here [6, 7].

The main advantage of this approach is the ability to assess the levels of mosaicism in individual cell
populations, particularly early human preimplantation development. While cell-by-cell analysis is
certainly technically feasible (and potentially more accurate) using array CGH or NGS, the costs
involved are prohibitively expensive. Practical applications include the “follow up” validation of PGS
cases and assessing the levels of mosaicism in cleavage stage, morula or blastocyst embryos. In the
latter case, blastulation represents the first visible stage of differentiation of the human embryo and
study of mosaicism at this stage is attracting great interest in the scientific literature at the moment
[8].

The methodology

Material used for our studies have been mostly “follow up” aneuploid PGS cases, the collaborating
clinics were the London Bridge Fertility Centre and the Lister Fertility Clinic. The protocol involves six
Kreatech fluorochromes, namely PlatinumBright™: 405 (blue), 415 (light blue/aqua), 495 (green),
547 (light red/orange), 590 (dark red), 647 (far red) plus the DAPI counterstain in a four-stage
probing and re-probing strategy. All probes for this protocol were synthesized by Kreatech
Diagnostics using the Universal Linkage Labeling System (KBI-40060):

http://www.kreatech.com/rest/products/repeat-freetm-poseidontm-fish-dna-
probes/preimplantation-genetic-screening/multistar-24-fish.html, including six unique sequence
targets for chromosomes 5, 13, 14, 19, 21 and 22 and the remaining 18 centromeric probes. These
are shown in figures 1 and 2. The highly repetitive nature of the remaining unique centromeric
targets meant that hybridisation times could be reduced to 15-30 minutes, however the unique
sequence probes required overnight hybridisation. The choice of fluorochromes for each individual
probe relied on combining the strongest signals with the least strong fluorochromes and vice versa.
For instance chromosome 18 (one of the brightest and most reliable probes) was labelled with the blue (the least bright) fluorochrome. Table 1 illustrates the final probe-fluorochrome combinations. Human IVF embryo nuclei are fixed to slides by standard protocols; slides are washed in PBS for 2 minutes and dehydrated and dried using an ethanol series. Pepsin treatment removes excess protein (1 mg/ml pepsin in 0.01 M HCl, 20 min at 37 °C), then the slides are rinsed in distilled water and PBS, followed by a paraformaldehyde (1% in PBS) fix at 4 °C for 10 min, followed by another PBS and distilled water wash and an ethanol dehydration and drying step. The four probe combinations are dissolved in hybridization mix (Kreatech standard protocols). It is important to pre-denature the probes at 73 °C for 10 min before application on the slide, then co-denaturation of probe and chromosomes proceeds at 75°C for 90 seconds in a “Thermobrite-StatSpin” before hybridization at 37°C. The hybridization period for the first three (alpha, beta, gamma) rounds of hybridization (centromeric probes) is for 30 min, whereas for the final round (omega), it is overnight. Post-hybridization washes are for 1 min 30 s in 0.7× SSC, 0.3%Tween 20 at 72 °C followed by a 2 min in 2×SSC at room temperature. Slides are mounted in Vectashield containing 0.1 ng/μl of DAPI (Vector labs) before microscopy and image analysis. After analysis and image capture, slides are washed in 2×SSC at room temperature to remove the coverslip and then washed for 30 seconds in distilled water (72°C) to remove the bound probe. An ethanol series precedes air-drying before continuation to the next round of hybridization. The protocol is the same for the second, third and final rounds with the following exceptions: The overnight hybridization time for the final round (previously mentioned), pepsin and paraformaldehyde treatment are only required for the first round; the post-hybridization wash time is reduced with every round from 90 s (first round of hybridization) to 50–60 s (second round) to 30 s (third and final rounds). Microscopy analysis, at least in our hands, is performed on an Olympus BX-61 epifluorescence microscope equipped with a cooled CCD camera (by Digital Scientific—Hamamatsu Orca-ER C4742-80) and using the appropriate filters. To enable analysis of the fluorochromes for image acquisition two communicating filter wheels (Digital Scientific UK) with the appropriate filters were used. The recommended filters by the probe
Chromosome mosaicism in human preimplantation development

Early studies that assessed chromosome copy number in IVF preimplantation embryos discovered that a large proportion of human embryos are mosaic. The incidence and mechanisms of aneuploidy and mosaicism are extensively reviewed elsewhere [16] and therefore this review will not cover this topic in detail. Briefly however, the term mosaicism can be defined as the presence of two or more cell populations with different chromosome constitutions in a single embryo. Mosaicism can be “general” (proportions of aneuploid and euploid embryos are roughly equal in each lineage) or “confined” (where one karyotype predominates in each germ layer e.g. the trophectoderm). Several different mechanisms can lead to mosaicism including: anaphase lag, endoreplication and nondisjunction [17]. Anaphase lag manifests as the impediment of movement during anaphase of one homologous chromosome (meiosis) or one chromatid (mitosis) resulting in failure of connection to cellular spindle apparatus, or slow movement towards the pole of the cell and thus the ‘lagging’ chromosome is not integrated in the nucleus. Endoreplication describes a variation of the cell cycle that involves replication of the entire genome in the absence of cell division, leading to a polyploid cell; interestingly, evidence suggests that many cells in a diploid organism are polyploid [18]. Nondisjunction is the failure of homologous chromosomes to separate either in meiosis I, meiosis II (sister chromatid separation) or during mitosis. The existence of both monosomy and trisomy for the same chromosome in an embryo is indicative of nondisjunction as the predominant mechanism for embryo mosaicism. The literature suggests that anaphase lag is the predominant mechanism by which mosaicism occurs in preimplantation embryos [16]. Furthermore, mosaicism can be caused by any one of numerous factors albeit paternal, maternal or exogenous such as culture media or
possibly controlled ovarian hyperstimulation during in vitro fertilization (IVF) [16]. Also noteworthy, is that embryo mosaicism can be classified into a number of categories, ranging from normal (all blastomeres being normal diploid), minor mosaic (more than 50% of nuclei are normal), major mosaic (more than 50% of nuclei are abnormal) and chaotic mosaicism (random segregation of chromosomes) [19]. It is thought that chaotic mosaicism arises due to chromosome loss and gains through no specific mechanism, characterised by nuclei depicting randomly different chromosome complements. A final, and perhaps most important, consideration is whether the embryos was euploid or aneuploid from the outset. Mosaics that were originally aneuploid tend to have the majority of cells with the same abnormality. Those that were euploid from the outset however tend to acquire abnormalities that may or may not have subsequent clinical relevance. The issue of mosaicism is still one that is vexing practitioners of PGS and, although FISH (even 24 chromosome FISH) is no longer used for diagnostic purposes, it may still find a use for establishing the level of mosaicism in cleavage stage, morula and blastocyst embryos.

Results to date

A preliminary study assessing mosaicism in whole embryos from day 3 blastomeres that were not transferred, thus were surplus material to IVF was performed by Ioannou et al. using the above 24-FISH assay [38]. The type of mosaicism in that cohort of embryos [data shown in table 2] supported previous findings of diploid/aneuploidy being the predominant pattern [12, 17]. Munne et al.[39] suggest that this form of mosaicism originates in the first few cleavage divisions and persists due to failure of cell cycle checkpoint control during cleavage stage [40]. Another study by Fonseka et al., (unpublished) indicated that mosaic embryos demonstrated more of chaotic mosaicism pattern; this was in contrast to the study by Munné and colleagues who reported that aneuploid mosaicism was the most common type of mosaicism seen in preimplantation embryos [39]. Results have also demonstrated that morphologically poor embryos had higher rates of polyploidy and diploid mosaicism. These types of studies are now performed on cells from the blastocyst stage (since this is
now the preferred biopsy stage, used by the novel genome wide platforms) and allow the evaluation of the level of mosaicism [8] and types of aneuploidy. Our initial results, albeit on embryos we knew to be aneuploid, indicated patterns of mosaicism more complex that previously appreciated.

In a second round of experiments, we extended the study further on a larger number of embryos, some for the same patient. In these set of experiments we looked at a larger number of embryos, some from the same patient. Figure 3 shows example images on each of the embryos and table 3 summarises the results.

Taken together, our results suggest that 24 chromosome FISH has great potential in unravelling the mysteries of chromosome mosaicism, one of the most hotly debated topics currently in preimplantation genetics. The ability to assay every chromosome on a cell by cell basis is particularly attractive. Our results suggest that embryo aneuploidy is not highly significantly correlated to maternal age, probably due, in part, to the large preponderance of post-zygotic (mitotic) errors. Of these, chromosome loss is the most common (presumably due to anaphase lag), followed by chromosome gain (endoreplication) whereas 3:1 mitotic non-disjunction of chromosomes appears to be rare in human preimplantation development.

**Nuclear Organisation**

Another feature, with a more research oriented scope that the 24-FISH platform can provide is the simultaneous assessment of the nuclear organization in preimplantation embryos. The term nuclear organisation or “nuclear architecture” describes the spatial and temporal topology of chromosomes or sub-chromosomal compartments (e.g. genes) within the nucleus that forms a fully functional nuclear landscape. With the popularisation of FISH in the early 1990s allowing visualisation of chromosomes in the interphase nucleus came a flurry of studies that sought to address chromosome position *in situ*. These led to the realisation of the now widely accepted concept that, within the nucleus, chromosomes are not randomly distributed but are organised into discrete regions known
as chromosome territories (CTs) [41-45]. Between these chromosome territories, inter-chromatin compartments containing macromolecular complexes are positioned. These are required for DNA replication, transcription, gene splicing and DNA repair and as such, the location of a chromosome within the nuclear volume is directly related to its accessibility to nuclear machinery [41]. The strict order of chromosome territories is believed to play a vital role in the regulation of gene expression, DNA replication, damage, and repair, controlling all cellular functions and development [41, 46-53]. Evidence to support the hypothesis for a link between position and function is provided from studies of cellular differentiation processes. Examples include the repositioning of the immunoglobulin gene cluster, the Mash1 locus during neural induction [54] [55] the HoxB1 gene in mouse embryos [56], the repositioning of adipogenesis genes during porcine mesenchymal stem cell adipogenesis [57] and sex chromosome movement during porcine spermatogenesis [58] just to name a few. In addition evidence supports that perturbation of nuclear organisation is correlated with certain diseases like laminopathies [59, 60] and certain cancers (promyelotic leukaemia, breast) [61]. Because of observations in different cell types and organisms [62, 63] proximity patterns of chromosomes, were identified leading to the proposal of two models (gene density and chromosome size) for the radial arrangement of CTs.

The gene density model for nuclear organization postulates that gene rich chromosomes occupy more central regions of the nuclear volume whereas gene poor chromosomes are localized toward the periphery [64-70]. This model originated from observations in proliferating lymphoblasts and fibroblasts and can be seen in primates, old world monkeys, rodents, birds (excluding chicken) and cattle. The chromosome size model of nuclear organization originated from observations in flat ellipsoid fibroblasts, quiescent, and senescent cells. In this scenario smaller chromosomes are positioned towards the nuclear interior and larger chromosomes toward the outermost regions of the nuclear membrane [71-73]. Furthermore, the chromocentric model (seen in human sperm) where chromosomes are positioned with their centromeres toward the interior of the nucleus
(forming chromocentres) and their telomeres extending toward the nuclear periphery forming dimers and tetramers [74-77].

Other models proposed later, included the chromosome territory interchromatin compartment (CT-IC) model, which described the existence of two domains in the nuclei called chromosome territories (CT) and interchromatin compartments (IC) [78] the lattice model, which suggested that fibres from different chromosomes were able to intermingle to a certain extent at the edges of CTs [79] and finally, the interchromatin network (ICN) model, which explained the long range intermingling of distal chromosome regions belonging to the same chromosome, or between regions of different chromosomes via the ‘looping out’ of chromatin within and between chromosome territories respectively [48].

Although there are many studies that have addressed nuclear organisation in a range of cell types from a wide spectrum of species, few studies have investigated nuclear organisation in the human embryo, and only one study has assessed the positioning of all 24 chromosomes [38]. Moreover, evidence presented thus far is not clear-cut. In studies that have assessed the nuclear positions of a subset of chromosomes (13, 16, 18, 21, 22, X and Y) in cleavage stage embryos (day 3-4), Mackenzie et al. [80] found central positioning of chromosomes 13, 18, 21 and X and peripheral positioning of chromosomes 16, 22 and Y, whereas both studies from Diblik [81] and Finch [82] found a random distribution of these chromosomes (with the exception of chromosome 18, that showed a central localisation [81]). The reason behind the discrepancies observed could be due to number of factors, both technical (e.g. method of fixation and method of position analysis), or biological (e.g. the quality of the embryos used, which in any study akin to these, were likely deemed unsuitable for transfer due to developmental, morphological or genetic abnormalities). Nonetheless, despite these differences, there is clear evidence to suggest that nuclear organisation of totipotent cells originating from the cleavage stage preimplantation human embryo differs significantly to that of
committed cell lines [38, 82], suggesting a functional role for chromosome positioning during development and differentiation. Furthermore, there is evidence to suggest that although chromosome positioning remains unperturbed in embryos with poor morphology compared to those of higher morphological grade, nuclear organisation is significantly altered in embryos with chromosome copy number abnormalities [80-82]. The biological mechanism behind this phenomenon remains as yet rather elusive, as shown by the fact that the nature and extent of re-organisation in aneuploid blastomeres is not consistent among the literature. While MacKenzie et al. [80] report that an extra copy of a specific chromosome results in re-distribution from central to peripheral regions of the nucleus, the study from Finch et al. [82] reports that euploid blastomeres adopt a relaxed state of nuclear organisation in which chromosomes were positioned randomly and that aneuploidy was associated with central positioning of chromosomes [80, 82]. The study from the Diblik group on the other hand, identified that of the chromosomes assessed, only chromosome 18 showed differential positioning in blastomeres possessing an extra copy, and that this difference was characterised by a shift from a random to a peripheral location [81]. It is noteworthy however, that Finch et al. [82] highlight the difficulty in extrapolating conclusions regarding the shift of specific chromosomes in relation to an extra copy (of the same chromosome), given the small subset of chromosomes assayed and any additional chromosomal abnormalities in chromosomes that were not investigated.

Assessing nuclear organization of human embryos by 24 chromosome FISH

With the introduction of 24-colour FISH, the aforementioned shortfall could be addressed and previous observations could be expanded upon with the inclusion of the topology for each chromosome in the karyotype [38]. In order to measure nuclear organization we extrapolated 3D data from 2D preparations thus: For each probe, the question was asked whether a non-random pattern of distribution of the FISH signal could be identified in each embryo. If so, we asked which part of the nucleus was preferentially occupied with reference to five “shells,” each representing
equal portions of the nucleus (from interior to periphery). We employed an ImageJ “macro” that divided each image of a nucleus into separate RGB planes (red and green for two of the six signals, blue for the DAPI counterstain) and then converted the blue image to a binary mask from which 5 concentric regions of interest (shells) of equal area were created. The proportion of signal in each channel contained within each shell was measured relative to the total signal for that channel within the area covered by the binary mask. The output of these results was pasted to an Excel spreadsheet for statistical analysis. To compensate for the fact that we were deriving 3D information from a flattened 2D object, the proportion of signal within each shell was normalised against the DAPI density measured within that shell as a function of the amount of DNA measured. The results are represented as a histogram and a $\chi^2$ “goodness of fit” test was performed to test whether the nuclear position of the signal was non-randomly distributed to a specific shell ($p<0.05$) or “not discernible from a random pattern” (NDRP).

As shown in table 4, by and large, our results showed that, human embryos at the morula or blastocyst stage (day 4 or 5 respectively) appear to adopt a chromocentric pattern of nuclear organisation, with almost all centromeric signals residing in the inner-most regions of the nuclear volume (with the exception of chromosome 5 predominantly identified at the nuclear periphery and chromosome 19, which showed a random distribution) [38]. This was an interesting finding that was consistent with results from studies in embryos from mice [83]. However the chromocentric arrangement seen in mice embryos appears to be consistent throughout development [83], whereas evidence for this in human embryos is partial from cleavage stage data, where 3 out of the 8 chromosomes investigated had a peripheral distribution [80]. Since nuclear organisation is subjected to alteration during the process of differentiation in other cell types [58, 63, 84-93], it is possible that earlier findings from a small number of chromosomes assessed in cleavage stage embryos indicate a more fluid nature of nuclear organisation in totipotent blastomeres. At the blastocyst stage however, which is the earliest differentiation event, a more ordered organisation with spatial and
temporal cues important for embryo development appears. Supporting this evidence is the fact that committed cells (e.g., lymphocytes) adopt a different pattern of organization compared to embryos (assessed on day 3 or 5 post fertilisation), as shown in table 4 [38]. In the future it would be interesting to compare the organisation by following an embryo (surplus to IVF) from blastomere to blastocyst stage, but more importantly apply this technique into a larger number of cells from blastocyst stage and stratify any organisation data based on the indication for IVF (e.g. AMA, RIF).

In terms of the organisation of preimplantation embryos and aneuploidy status, our results have not revealed a difference between the individual cells (from the whole embryo) that were classified as “normal” for the needs of the study compared to the aneuploid ones. A partial explanation for this could be either due to the probes used, that targeted a predominantly heterochromatic proportion of the chromosome, small in size and therefore difficult to observe a potential noticeable difference using it as a single reference point, or more importantly the fact that the single cells assessed from the whole embryo, originated from unsuitable for transfer blastocysts that were probably already compromised in terms of their developmental potential.

The use of different probes (e.g. whole chromosome territories or a combination of reference points on the chromosome) and if applicable better quality blastocysts could help to address the issues regarding ploidy and genome organisation when the whole karyotype is investigated with 24 colour FISH.

Furthermore, a better appreciation about the organisation of preimplantation embryos will be possible by moving from 2D to 3D and the development of a more automated protocol that will allow to render the captured images into 3D models. Software like that is currently available. Currently, all studies that have assessed nuclear organisation in the blastomeres of human embryos have utilised 2D analysis techniques using centromere specific probes. The use of whole
chromosome paints, combined with 3D analysis will provide a more complete map about the
topology of chromosomes and how this might be related to the development of the human
preimplantation embryo.

**Conclusion**

In conclusion, it seems that, while FISH is mostly “dead and buried” for the mainstream use in PGS, it
still has a place for the assessment of mosaicism and for the study of nuclear organization. The
development of a 24 chromosome protocol extends the power of this analysis and we would like to
hope that it will still find an application as a result.
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Turner KJ,1* Fowler KE,2* Fonseka GL1, Griffin DK1 and Ioannou D3

1. School of Biosciences, University of Kent, Canterbury, Kent, UK
2. School of Human and Life Sciences, Canterbury Christ Church University, Canterbury, UK
3. Department of Human and Molecular Genetics, Herbert Wertheim College of Medicine, Florida International University, Miami, FL, USA.

*Turner KJ and Fowler KE are joint first authors

Griffin DK is the corresponding author
Introduction

Fluorescence *in situ* Hybridisation (FISH) revolutionised the study of cytogenetics in the late 1980s, enabling basic scientists and clinicians to visualise specific chromosome regions within the nucleus. It provided, for the first time, a direct link between the microscope and DNA sequence. The technique uses fluorescently labelled short stretches of DNA (probes) that have a high level of sequence complementarity to specific sections of a chromosome. Following denaturation of chromosomal (target DNA) and probe, hybridisation is allowed to occur under specific conditions (e.g. temperature, concentration of formamide) to allow high affinity between target and probe DNA. By the early 1990s FISH was adopted by fertility centres worldwide as means of sex determination in preimplantation embryos from couples at risk of transmitting X-linked disorders [1, 2]. Shortly after, FISH found additional roles in the identification of unbalanced translocations and in chromosome copy number screening (e.g. embryo, sperm aneuploidy). Since then, the rapid increase in the use of *in vitro* fertilisation (IVF) and preimplantation genetic diagnosis (PGD) globally has not only enabled huge advancements in reproductive medicine, but has also provided a unique opportunity to study the cytogenetics of human embryos at the earliest stages of development. With the ultimate goal of developing diagnostic tests and improving patient care, those embryos produced by IVF cycles that are not deemed for transfer represent a valuable source of sample material under appropriate ethical justification. Nowadays, FISH has been replaced with newer technologies for the purposes of PGD using single cells; first by array CGH, then by single nucleotide polymorphism arrays (SNP) or quantitative real-time polymerase chain reaction (qRT-PCR) and most recently by next generation sequencing (NGS) [3]. Concurrently with the application of these newer technologies, the platform of evaluating the chromosomal status of preimplantation embryos has shifted from blastomere to trophectoderm biopsy (blastocyst stage). Despite the advancement in technologies, the cell-by-cell analysis of blastomeres or available blastocysts within an embryo with the new techniques is still prohibitively expensive. As a result, FISH remains very much an invaluable resource for the study of
cytogenetics in preimplantation embryos in terms of evaluating the level of mosaicism and at a more research level the nuclear organisation of chromosomes at this early stage of development. The purpose of this review is to give an overview of the history of FISH in PGD/PGS, cover the reasons why it fell out of favour and indicate how it may, with recent adaptations, be used as a tool for research and “follow up” of clinical cases.

**A brief history of FISH and its use for PGS**

The assessment of chromosome copy number in preimplantation embryos is the essence of preimplantation genetic screening (PGS), a commonly elected procedure in couples where advanced maternal age (AMA), recurrent miscarriage, recurrent implantation failure (RIF) or male factor infertility is implicated. It is widely believed that aneuploidy (presence of an extra or missing chromosome) is present in approximately 0.6% of live newborn infants, 6% of stillbirths, and 60% of spontaneous abortions [9, 10]. Moreover, numerical chromosome abnormalities are present in 60-70% of embryos generated by IVF (at the blastomere stage) [11-14], whereas it can reach levels of >50% in the blastocyst stage [15]. Although the majority of chromosome copy number abnormalities are lethal, aneuploidies involving a few specific chromosomes survive to term. On this basis, following a rise in the use of FISH for sex determination in the early 1990s and the availability of multicolour probes, the use of FISH was expanded to the detection of aneuploidy in order to selectively implant embryos more likely to be fully euploid.

The rationale behind the use of PGS in infertile couples requiring assisted reproductive technology (ART), is to increase pregnancy rates, since morphology alone does not suffice to distinguish a euploid from an aneuploid embryo. Therefore, by transferring euploid embryos, the chances of a viable pregnancy should be higher. The logic of this hypothesis is generally accepted in the field of reproductive medicine and can have particular application in women of AMA, couples with RIF, repeated miscarriage or severe male factor infertility [20, 21]. Initially from the 1990s to 2010, FISH
was used to perform diagnosis on the chromosomal complement of polar bodies and blastomeres [22, 23]. A total of eight chromosomes, six autosomal chromosomes (13, 15, 16, 18, 21 and 22) and the sex chromosomes were more commonly tested in IVF clinics through PGS as these were known to be involved in aneuploidies detected in spontaneous abortions and in trisomic live births [11, 24]. Despite the initial reports for an increase in implantation rates, reduction in trisomic offspring and spontaneous abortions [25, 26], criticism emerged since these reports were non-randomised, had poor experimental design, inadequate control groups and lack of report on live births [26].

From 2004 to 2010, eleven randomised control trials (RCTs) showed that PGS with FISH did not increase delivery rates, some studies showed the contrary and sparked a huge debate in the field. The reasons [26-29] for the reduced efficiency of PGS-FISH are beyond the scope of this review however they extended from technique-inherent limitations to biological (e.g. high levels of mosaicism in cleavage stage embryos, biopsy stage). This opened up different methodological approaches for the analysis of all chromosomes using genome wide platforms (e.g. aCGH, SNP-array, NGS), prompted multi-centre RCTS [30, 31] and parallel with improvements in culturing [32] and cryopreservation of embryos (e.g., vitrification) [33, 34] the diagnostic platform was shifted from day 3 to day 5 (blastocysts), making at the same time FISH an outdated technology for the complete chromosomal complement analysis in a PGS setting.

24 chromosome FISH on single cells

During the time since FISH was first popularised, the technique has evolved considerably to see the development of directly labelled, multicolour, commercially available probes with shorter hybridisation times and greater hybridisation efficiencies, which has enabled the ability to study up to 12 chromosomes within the same nucleus at once [4]. Better still, whole chromosome paints for all 24 chromosomes soon became commercially available by mixing fluorochromes to produce secondary colours. However, the difficulty with taking this approach is that overlapping signals in the
interphase nucleus are not easily distinguishable from one another. To circumvent this problem therefore, we developed a new ‘multilayer’ approach to 24-chromosome FISH, enabling comprehensive analysis of copy number for each chromosome in the karyotype. Based on a previously published protocol termed ‘re-FISH’, six spectrally distinct probes were used, in four consecutive rounds of FISH to visualise all 24 chromosomes [5]. The setup of 6 fluorochromes and 4 rounds of hybridization was selected to maximise the outcome of chromosome copy number, while reducing the rounds of re-probing of nuclei and thus increase the chances of signal efficiency. In addition the probes for the chromosomes that constitute each round of hybridisation were categorised based on availability of centromeric sequences for that particular chromosome or not.

The first three rounds of hybridisation (that can be inter-changeable) use probes against centromeric sequences; round one for chromosomes 1, 3, 4, 6, 7 and 8, round two for chromosomes 9, 10, 11, 12, 17 and 20, round three for chromosomes 2, 15, 16, 18, X, Y whereas the fourth round uses unique sequence targets for 6 chromosomes that do not have unique centromeric probes, chromosomes 5, 13, 14, 19, 21 and 22. The protocol can be completed within 24 hours, since the hybridisation times for the centromeric layers is 15-30 minutes and overnight for the unique sequence layer, fitting in a clinical setting and tailored for different applications (e.g. embryo versus sperm aneuploidy). The fast hybridisation times for the centromeric layers are possible due to the highly repetitive sequences (α-satellite) used to generate the respective probes.

A bespoke capturing system is necessary in order to be able to image all fluorochromes in separate channels plus the DAPI counterstain in a different channel. In-house, we used a modified version of Digital Scientific’s SmartCapture, and this novel approach that has been previously validated in different cell types [5], offers a powerful research tool in the identification of chromosome copy number that can be applied to different cell types. It also allows for the simultaneous assessment of nuclear organisation; that is, the so-called “nuclear address” of chromosomes (or sub-chromosomal
regions and/or loci) within the nucleus. For this feature a custom-script for Image J (freely-downloadable software) is required and more details have been previously published here [6, 7]. The main advantage of this approach is the ability to assess the levels of mosaicism in individual cell populations, particularly early human preimplantation development. While cell-by-cell analysis is certainly technically feasible (and potentially more accurate) using array CGH or NGS, the costs involved are prohibitively expensive. Practical applications include the “follow up” validation of PGS cases and assessing the levels of mosaicism in cleavage stage, morula or blastocyst embryos. In the latter case, blastulation represents the first visible stage of differentiation of the human embryo and study of mosaicism at this stage is attracting great interest in the scientific literature at the moment [8].

The methodology

Material used for our studies have been mostly “follow up” aneuploid PGS cases, the collaborating clinics were the London Bridge Fertility Centre and the Lister Fertility Clinic. The protocol involves six Kreatech fluorochromes, namely PlatinumBright™: 405 (blue), 415 (light blue/aqua), 495 (green), 547 (light red/orange), 590 (dark red), 647 (far red) plus the DAPI counterstain in a four-stage probing and re-probing strategy. All probes for this protocol were synthesized by Kreatech Diagnostics using the Universal Linkage Labeling System (KBI-40060): http://www.kreatech.com/rest/products/repeat-freetm-poseidontm-fish-dna-probes/preimplantation-genetic-screening/multistar-24-fish.html, including six unique sequence targets for chromosomes 5, 13, 14, 19, 21 and 22 and the remaining 18 centromeric probes. These are shown in figures 1 and 2. The highly repetitive nature of the remaining unique centromeric targets meant that hybridisation times could be reduced to 15-30 minutes, however the unique sequence probes required overnight hybridisation. The choice of fluorochromes for each individual probe relied on combining the strongest signals with the least strong fluorochromes and vice versa.
For instance chromosome 18 (one of the brightest and most reliable probes) was labelled with the blue (the least bright) fluorochrome. Table 1 illustrates the final probe-fluorochrome combinations.

Human IVF embryo nuclei are fixed to slides by standard protocols; slides are washed in PBS for 2 minutes and dehydrated and dried using an ethanol series. Pepsin treatment removes excess protein (1 mg/ml pepsin in 0.01 M HCl, 20 min at 37 °C), then the slides are rinsed in distilled water and PBS, followed by a paraformaldehyde (1% in PBS) fix at 4 °C for 10 min, followed by another PBS and distilled water wash and an ethanol dehydration and drying step. The four probe combinations are dissolved in hybridization mix (Kreatech standard protocols). It is important to pre-denature the probes at 73 °C for 10 min before application on the slide, then co-denaturation of probe and chromosomes proceeds at 75°C for 90 seconds in a “Thermobrite-StatSpin” before hybridization at 37°C. The hybridization period for the first three (alpha, beta, gamma) rounds of hybridization (centromeric probes) is for 30 min, whereas for the final round (omega), it is overnight. Post-hybridization washes are for 1 min 30 s in 0.7× SSC, 0.3%Tween 20 at 72 °C followed by a 2 min in 2×SSC at room temperature. Slides are mounted in Vectashield containing 0.1 ng/μl of DAPI (Vector labs) before microscopy and image analysis. After analysis and image capture, slides are washed in 2×SSC at room temperature to remove the coverslip and then washed for 30 seconds in distilled water (72°C) to remove the bound probe. An ethanol series precedes air-drying before continuation to the next round of hybridization. The protocol is the same for the second, third and final rounds with the following exceptions: The overnight hybridization time for the final round (previously mentioned), pepsin and paraformaldehyde treatment are only required for the first round; the post-hybridization wash time is reduced with every round from 90 s (first round of hybridization) to 50–60 s (second round) to 30 s (third and final rounds). Microscopy analysis, at least in our hands, is performed on an Olympus BX-61 epifluorescence microscope equipped with a cooled CCD camera (by Digital Scientific—Hamamatsu Orca-ER C4742-80) and using the appropriate filters. To enable analysis of the fluorochromes for image acquisition two communicating filter wheels (Digital Scientific UK) with the appropriate filters were used. The recommended filters by the probe
Chromosome mosaicism in human preimplantation development

Early studies that assessed chromosome copy number in IVF preimplantation embryos discovered that a large proportion of human embryos are mosaic. The incidence and mechanisms of aneuploidy and mosaicism are extensively reviewed elsewhere [16] and therefore this review will not cover this topic in detail. Briefly however, the term mosaicism can be defined as the presence of two or more cell populations with different chromosome constitutions in a single embryo. Mosaicism can be “general” (proportions of aneuploid and euploid embryos are roughly equal in each lineage) or “confined” (where one karyotype predominates in each germ layer e.g. the trophectoderm). Several different mechanisms can lead to mosaicism including: anaphase lag, endoreplication and nondisjunction [17]. Anaphase lag manifests as the impediment of movement during anaphase of one homologous chromosome (meiosis) or one chromatid (mitosis) resulting in failure of connection to cellular spindle apparatus, or slow movement towards the pole of the cell and thus the ‘lagging’ chromosome is not integrated in the nucleus. Endoreplication describes a variation of the cell cycle that involves replication of the entire genome in the absence of cell division, leading to a polyploid cell; interestingly, evidence suggests that many cells in a diploid organism are polyploid [18]. Nondisjunction is the failure of homologous chromosomes to separate either in meiosis I, meiosis II (sister chromatid separation) or during mitosis. The existence of both monosomy and trisomy for the same chromosome in an embryo is indicative of nondisjunction as the predominant mechanism for embryo mosaicism. The literature suggests that anaphase lag is the predominant mechanism by which mosaicism occurs in preimplantation embryos [16]. Furthermore, mosaicism can be caused by any one of numerous factors albeit paternal, maternal or exogenous such as culture media or
possibly controlled ovarian hyperstimulation during in vitro fertilization (IVF) [16]. Also noteworthy, is that embryo mosaicism can be classified into a number of categories, ranging from normal (all blastomeres being normal diploid), minor mosaic (more than 50% of nuclei are normal), major mosaic (more than 50% of nuclei are abnormal) and chaotic mosaicism (random segregation of chromosomes) [19]. It is thought that chaotic mosaicism arises due to chromosome loss and gains through no specific mechanism, characterised by nuclei depicting randomly different chromosome complements. A final, and perhaps most important, consideration is whether the embryos was euploid or aneuploid from the outset. Mosaics that were originally aneuploid tend to have the majority of cells with the same abnormality. Those that were euploid from the outset however tend to acquire abnormalities that may or may not have subsequent clinical relevance. The issue of mosaicism is still one that is vexing practitioners of PGS and, although FISH (even 24 chromosome FISH) is no longer used for diagnostic purposes, it may still find a use for establishing the level of mosaicism in cleavage stage, morula and blastocyst embryos.

**Results to date**

A preliminary study assessing mosaicism in whole embryos from day 3 blastomeres that were not transferred, thus were surplus material to IVF was performed by Ioannou et al. using the above 24-FISH assay [38]. The type of mosaicism in that cohort of embryos (data shown in table 2) supported previous findings of diploid/aneuploidy being the predominant pattern [12, 17]. Munne et al.[39] suggest that this form of mosaicism originates in the first few cleavage divisions and persists due to failure of cell cycle checkpoint control during cleavage stage [40]. Another study by Fonseka et al., (unpublished) indicated that mosaic embryos demonstrated more of chaotic mosaicism pattern; this was in contrast to the study by Munné and colleagues who reported that aneuploid mosaicism was the most common type of mosaicism seen in preimplantation embryos [39]. Results have also demonstrated that morphologically poor embryos had higher rates of polyploidy and diploid mosaicism. These types of studies are now performed on cells from the blastocyst stage (since this is
now the preferred biopsy stage, used by the novel genome wide platforms) and allow the evaluation of the level of mosaicism [8] and types of aneuploidy. Our initial results, albeit on embryos we knew to be aneuploid, indicated patterns of mosaicism more complex that previously appreciated.

In a second round of experiments, we extended the study further on a larger number of embryos, some for the same patient. In these set of experiments we looked at a larger number of embryos, some from the same patient. Figure 3 shows example images on each of the embryos and table 3 summarises the results.

Taken together, our results suggest that 24 chromosome FISH has great potential in unravelling the mysteries of chromosome mosaicism, one of the most hotly debated topics currently in preimplantation genetics. The ability to assay every chromosome on a cell by cell basis is particularly attractive. Our results suggest that embryo aneuploidy is not highly significantly correlated to maternal age, probably due, in part, to the large preponderance of post-zygotic (mitotic) errors. Of these, chromosome loss is the most common (presumably due to anaphase lag), followed by chromosome gain (endoreplication) whereas 3:1 mitotic non-disjunction of chromosomes appears to be rare in human preimplantation development.

**Nuclear Organisation**

Another feature, with a more research oriented scope that the 24-FISH platform can provide is the simultaneous assessment of the nuclear organization in preimplantation embryos. The term nuclear organisation or “nuclear architecture” describes the spatial and temporal topology of chromosomes or sub-chromosomal compartments (e.g. genes) within the nucleus that forms a fully functional nuclear landscape. With the popularisation of FISH in the early 1990s allowing visualisation of chromosomes in the interphase nucleus came a flurry of studies that sought to address chromosome position *in situ*. These led to the realisation of the now widely accepted concept that, within the nucleus, chromosomes are not randomly distributed but are organised into discrete regions known
as chromosome territories (CTs) [41-45]. Between these chromosome territories, inter-chromatin compartments containing macromolecular complexes are positioned. These are required for DNA replication, transcription, gene splicing and DNA repair and as such, the location of a chromosome within the nuclear volume is directly related to its accessibility to nuclear machinery [41]. The strict order of chromosome territories is believed to play a vital role in the regulation of gene expression, DNA replication, damage, and repair, controlling all cellular functions and development [41, 46-53]. Evidence to support the hypothesis for a link between position and function is provided from studies of cellular differentiation processes. Examples include the repositioning of the immunoglobulin gene cluster, the Mash1 locus during neural induction [54] [55] the HoxB1 gene in mouse embryos [56], the repositioning of adipogenesis genes during porcine mesenchymal stem cell adipogenesis [57] and sex chromosome movement during porcine spermatogenesis [58] just to name a few. In addition evidence supports that perturbation of nuclear organisation is correlated with certain diseases like laminopathies [59, 60] and certain cancers (promyelotic leukaemia, breast) [61]. Because of observations in different cell types and organisms [62, 63] proximity patterns of chromosomes, were identified leading to the proposal of two models (gene density and chromosome size) for the radial arrangement of CTs.

The gene density model for nuclear organization postulates that gene rich chromosomes occupy more central regions of the nuclear volume whereas gene poor chromosomes are localized toward the periphery [64-70]. This model originated from observations in proliferating lymphoblasts and fibroblasts and can be seen in primates, old world monkeys, rodents, birds (excluding chicken) and cattle. The chromosome size model of nuclear organization originated from observations in flat ellipsoid fibroblasts, quiescent, and senescent cells. In this scenario smaller chromosomes are positioned towards the nuclear interior and larger chromosomes toward the outermost regions of the nuclear membrane [71-73]. Furthermore, the chromocentric model (seen in human sperm) where chromosomes are positioned with their centromeres toward the interior of the nucleus
(forming chromocentres) and their telomeres extending toward the nuclear periphery forming
dimers and tetramers [74-77].

Other models proposed later, included the chromosome territory interchromatin compartment (CT-IC) model, which described the existence of two domains in the nuclei called chromosome territories (CT) and interchromatin compartments (IC) [78] the lattice model, which suggested that fibres from different chromosomes were able to intermingle to a certain extent at the edges of CTs [79] and finally, the interchromatin network (ICN) model, which explained the long range intermingling of distal chromosome regions belonging to the same chromosome, or between regions of different chromosomes via the ‘looping out’ of chromatin within and between chromosome territories respectively [48].

Although there are many studies that have addressed nuclear organisation in a range of cell types from a wide spectrum of species, few studies have investigated nuclear organisation in the human embryo, and only one study has assessed the positioning of all 24 chromosomes [38]. Moreover, evidence presented thus far is not clear-cut. In studies that have assessed the nuclear positions of a subset of chromosomes (13, 16, 18, 21, 22, X and Y) in cleavage stage embryos (day 3-4), Mackenzie et al. [80] found central positioning of chromosomes 13, 18, 21 and X and peripheral positioning of chromosomes 16, 22 and Y, whereas both studies from Diblik [81] and Finch [82] found a random distribution of these chromosomes (with the exception of chromosome 18, that showed a central localisation [81]). The reason behind the discrepancies observed could be due to number of factors, both technical (e.g. method of fixation and method of position analysis), or biological (e.g. the quality of the embryos used, which in any study akin to these, were likely deemed unsuitable for transfer due to developmental, morphological or genetic abnormalities). Nonetheless, despite these differences, there is clear evidence to suggest that nuclear organisation of totipotent cells originating from the cleavage stage preimplantation human embryo differs significantly to that of
committed cell lines [38, 82], suggesting a functional role for chromosome positioning during development and differentiation. Furthermore, there is evidence to suggest that although chromosome positioning remains unperturbed in embryos with poor morphology compared to those of higher morphological grade, nuclear organisation is significantly altered in embryos with chromosome copy number abnormalities [80-82]. The biological mechanism behind this phenomenon remains as yet rather elusive, as shown by the fact that the nature and extent of reorganisation in aneuploid blastomeres is not consistent among the literature. While MacKenzie et al. [80] report that an extra copy of a specific chromosome results in re-distribution from central to peripheral regions of the nucleus, the study from Finch et al. [82] reports that euploid blastomeres adopt a relaxed state of nuclear organisation in which chromosomes were positioned randomly and that aneuploidy was associated with central positioning of chromosomes [80, 82]. The study from the Diblik group on the other hand, identified that of the chromosomes assessed, only chromosome 18 showed differential positioning in blastomeres possessing an extra copy, and that this difference was characterised by a shift from a random to a peripheral location [81]. It is noteworthy however, that Finch et al. [82] highlight the difficulty in extrapolating conclusions regarding the shift of specific chromosomes in relation to an extra copy (of the same chromosome), given the small subset of chromosomes assayed and any additional chromosomal abnormalities in chromosomes that were not investigated.

Assessing nuclear organization of human embryos by 24 chromosome FISH

With the introduction of 24-colour FISH, the aforementioned shortfall could be addressed and previous observations could be expanded upon with the inclusion of the topology for each chromosome in the karyotype [38]. In order to measure nuclear organization we extrapolated 3D data from 2D preparations thus: For each probe, the question was asked whether a non-random pattern of distribution of the FISH signal could be identified in each embryo. If so, we asked which part of the nucleus was preferentially occupied with reference to five “shells,” each representing
equal portions of the nucleus (from interior to periphery). We employed an ImageJ “macro” that divided each image of a nucleus into separate RGB planes (red and green for two of the six signals, blue for the DAPI counterstain) and then converted the blue image to a binary mask from which 5 concentric regions of interest (shells) of equal area were created. The proportion of signal in each channel contained within each shell was measured relative to the total signal for that channel within the area covered by the binary mask. The output of these results was pasted to an Excel spreadsheet for statistical analysis. To compensate for the fact that we were deriving 3D information from a flattened 2D object, the proportion of signal within each shell was normalised against the DAPI density measured within that shell as a function of the amount of DNA measured. The results are represented as a histogram and a $\chi^2$ “goodness of fit” test was performed to test whether the nuclear position of the signal was non-randomly distributed to a specific shell (p<0.05) or “not discernible from a random pattern” (NDRP).

As shown in table 4, by and large, our results showed that, human embryos at the morula or blastocyst stage (day 4 or 5 respectively) appear to adopt a chromocentric pattern of nuclear organisation, with almost all centromeric signals residing in the inner-most regions of the nuclear volume (with the exception of chromosome 5 predominantly identified at the nuclear periphery and chromosome 19, which showed a random distribution) [38]. This was an interesting finding that was consistent with results from studies in embryos from mice [83]. However the chromocentric arrangement seen in mice embryos appears to be consistent throughout development [83], whereas evidence for this in human embryos is partial from cleavage stage data, where 3 out of the 8 chromosomes investigated had a peripheral distribution [80]. Since nuclear organisation is subjected to alteration during the process of differentiation in other cell types [58, 63, 84-93], it is possible that earlier findings from a small number of chromosomes assessed in cleavage stage embryos indicate a more fluid nature of nuclear organisation in totipotent blastomeres. At the blastocyst stage however, which is the earliest differentiation event, a more ordered organisation with spatial and
temporal cues important for embryo development appears. Supporting this evidence is the fact that committed cells (e.g., lymphocytes) adopt a different pattern of organization compared to embryos (assessed on day 3 or 5 post fertilization), as shown in table 4 [38]. In the future it would be interesting to compare the organisation by following an embryo (surplus to IVF) from blastomere to blastocyst stage, but more importantly apply this technique into a larger number of cells from blastocyst stage and stratify any organisation data based on the indication for IVF (e.g. AMA, RIF).

In terms of the organisation of preimplantation embryos and aneuploidy status, our results have not revealed a difference between the individual cells (from the whole embryo) that were classified as “normal” for the needs of the study compared to the aneuploid ones. A partial explanation for this could be either due to the probes used, that targeted a predominantly heterochromatic proportion of the chromosome, small in size and therefore difficult to observe a potential noticeable difference using it as a single reference point, or more importantly the fact that the single cells assessed from the whole embryo, originated from unsuitable for transfer blastocysts that were probably already compromised in terms of their developmental potential.

The use of different probes (e.g. whole chromosome territories or a combination of reference points on the chromosome) and if applicable better quality blastocysts could help to address the issues regarding ploidy and genome organisation when the whole karyotype is investigated with 24 colour FISH.

Furthermore, a better appreciation about the organisation of preimplantation embryos will be possible by moving from 2D to 3D and the development of a more automated protocol that will allow to render the captured images into 3D models. Software like that is currently available. Currently, all studies that have assessed nuclear organisation in the blastomeres of human embryos have utilised 2D analysis techniques using centromere specific probes. The use of whole
chromosome paints, combined with 3D analysis will provide a more complete map about the topology of chromosomes and how this might be related to the development of the human preimplantation embryo.

**Conclusion**

In conclusion, it seems that, while FISH is mostly “dead and buried” for the mainstream use in PGS, it still has a place for the assessment of mosaicism and for the study of nuclear organization. The development of a 24 chromosome protocol extends the power of this analysis and we would like to hope that it will still find an application as a result.
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