

Review Article

Tips and Tricks in Fluorescence In-situ Hybridization (FISH)-based Preimplantation Genetic Diagnosis /Screening (PGD/PGS)

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ABSTRACT

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As numerical and structural defects in chromosomes are an inevitable consequence of IVF, Pre-implantation genetic diagnosis and screening (PGD/PGS) methods are used for detecting abnormalities in embryos before implantation to the uterus to increase the successful rate of IVF. Pre-implantation genetic diagnosis and screening approaches can be achieved by different techniques such as NGS, CGH and FISH. Among these approaches, FISH-based PGD/PGS is challenging in that it requires experience and skill to increase its facility and validity. Therefore, based on literature review and our experiences obtained from genetic laboratory of Yazd Reproductive Sciences Institute (Yazd, Iran), we were determined to discuss these challenges. After reviewing the available protocols and articles, we compared results of different methods for performing pre- and post-examination FISH process. Required samples in each section were obtained from embryo in cleavage or blastocyst stage. According to our team's experience, we recommend the cleavage stage biopsy and our modified fixation method. Also, we do not recommend more than two round hybridization on the same cell. Many studies have shown that FISH-based PGD is an efficient method for decreasing IVF failure in infertile patients. This paper introduces the best biopsy and fixation method and, includes some useful tips and tricks on type and number of probe, removing the cytoplasm, denaturation and hybridization, data evaluation and scoring criteria.

Introduction

Pre-implantation genetic diagnosis (PGD), pre-implantation genetic screening (PGS) and aneuploidy screening (PGD-AS) are a couple of methods introduced to screen embryos for common chromosomal abnormalities in order to improve the efficiency of in vitro fertilization (IVF). All these approaches can be achieved by different techniques including next generation sequencing (NGS), comparative genomic hybridization (CGH) and fluorescence in-situ hybridization (FISH) and consequently have caused conflicting results. FISH-based PGD is the technique of choice for analysis of the chromosomal complement of biopsied cells. FISH can be used for social sexing or for detecting X-linked genetic diseases, inherited chromosome rearrangements and aneuploidy screening. The most frequently used indications for PGS include female infertility with advanced maternal age (AMA; define as ≥ 35 years), husband and wife with normal karyotypes but recurrent pregnancy loss (RPL; at least three previous miscarriages) or with repeated implantation failure (RIF; three or more failed embryo transfers) and severe male factor infertility. Further indications have progressively been suggested including a previous affected child, low quality embryo, previous radiotherapy and single embryo transfer (SET) [1-3].

The use of FISH-based PGD as a molecular cytogenetic approach would pose some challenges in both practicalities and signal interpretation. First of all, the desirable cells achieved by biopsy from embryo need to be

fixed within a pre-defined area on the slide to enable its localization following FISH. This procedure would be really demanding as due care and skill is required for confirming that the cytoplasm has been removed, and that the nucleus is intact and detectable. Also, it is necessary to have an accurate scoring and visible interpretation rule to avoid the risk of mistake. However, in expert hands, the FISH is a strong technique for PGD-AS in clinical practices. This paper could be helpful for technicians and researchers who work in the field of fluorescent-based cytogenetic and focuses on the technical aspects and the limitations of FISH. Required blastomeres for studying efficiency of different fixation and biopsy methods were obtained from arrested embryo in cleavage stage while other comparisons are based on collected data from FISH-based PGS on patient's samples. Generally, our paper contains some useful and simple tips on accurate selection of type and number of probes, especially with the aim of PGS, best biopsy method, slide preparation, blastomere fixation, removing the cytoplasm, denaturation and hybridization, data evaluation and scoring criteria.

Recommendations for FISH probes

The type and number of FISH probes that are used depend on the indications mentioned above. In sexing, regardless of X and Y specific probes, the application of one autosomal probe is highly recommended [4]. The autosomal probe is used to distinguish between trisomy X and triploidy as well as tetrasomy X and

tetraploidy [1-3]. A probe set containing alpha-satellite X, Y, and one autosomal chromosome with low polymorphism rate is optional [3]. If multiple rounds of FISH are being used, the X and Y specific probes should be applied in the first round of hybridization. The hybridization efficiency of commercial probes, range from 95% to 99%. Some hybridization failures would happen if DNA is not completely denatured at the target sequence of probe. Moreover, some probes have cross-hybridization with sequences on the other chromosomes. These cases should be documented carefully and be considered during signal recording and analysis [5, 6]. For investigating chromosome rearrangement, the elective probe set should detect all possible products of the rearrangements. If ideal probe set is not available, you can use existing probe mix, provided that they just could not detect unbalanced products to be non-viable or to have a very low frequency [7, 8].

In FISH-based PGS, the number of chromosomes that can be investigated simultaneously is restricted by the number of applied filters in currently fluorescent microscopes, the number of available fluorochromes (only 5 different fluorescent dyes are available in commercial kits) as well as the number of biopsied cells (usually a single cell). Also, a maximum of 5 chromosomes can be analyzed simultaneously in one cell, because only five fluorochromes on the optical spectrum have necessary separation for efficient detection. Therefore, for analyzing more than 5 chromosomes, two or more rounds of washing and hybridization must be performed. Unfortunately, multiple hybridization rounds performed on the same

cell may increase the cell missing and FISH errors. Thus, the PGD technician usually performs a maximum of three rounds of hybridization with up to 15 probes [9]. However, our experience suggests that after second hybridization rounds, increasing background signal and noise, overlapping new signals with each other or with remaining effect of previous signals result in decreasing efficiency of analysis (Figure 1). Also, the cell missing during the third round is significant (Table 1). Considering the limitations mentioned and high rates of cell missing and FISH error in third hybridization round, for performing PGS in cleavage stage embryo, we recommend two cells biopsy as well as two rounds of hybridization together with a perfect fixation method. By reports from a retrospective study, there is no difference in successful IVF rate between non-biopsied embryos and embryos from which two cells are taken [10]. As a result, biopsy of two cells and two rounds of hybridization allows screening 16 -20 chromosomes in one embryo.

Aneuploidies in chromosomes 22, 16, 21, and 15 are common in cleavage-stage. On the other hand, the abnormalities in the chromosomes X, Y, 13, 18 and 21 are able to reach the term. Therefore, the minimum number of recommended chromosomes for analyzing PGS includes X, Y, 13, 15, 16, 18, 21, 22 [11-13]. Also, with a slight modification of the standard eight-FISH probe panel (adding chromosomes 8, 14, 17, and 20), it would be possible to analyze effectively up to 12 probes and thus eliminate the need for multiple hybridization rounds. Based on the literature,

although even investigation of five chromosomes (X, Y, 13, 18, 21) in embryos causes a significant reduction in spontaneous abortion cases, further examination on other factors

expanding the number of analyzed chromosomes brings about an increase in the IVF success rate as well.

Table 1. Cell missing and signal efficiency in repeated hybridization round on same nucleus in FISH based PGS

No. of hybridization round	No. of nuclear loss in each round	No. of non-analyzable nucleus *
First	3 out of 60 (5%)	4 out of 60 (6%)
Second	5 out of 60 (13%)	11 out of 60 (18%)
Third	11 out of 60 (18%)	18 out of 60 (30%)

*due to partial or complete nuclear loss, background signal and overlapping signals

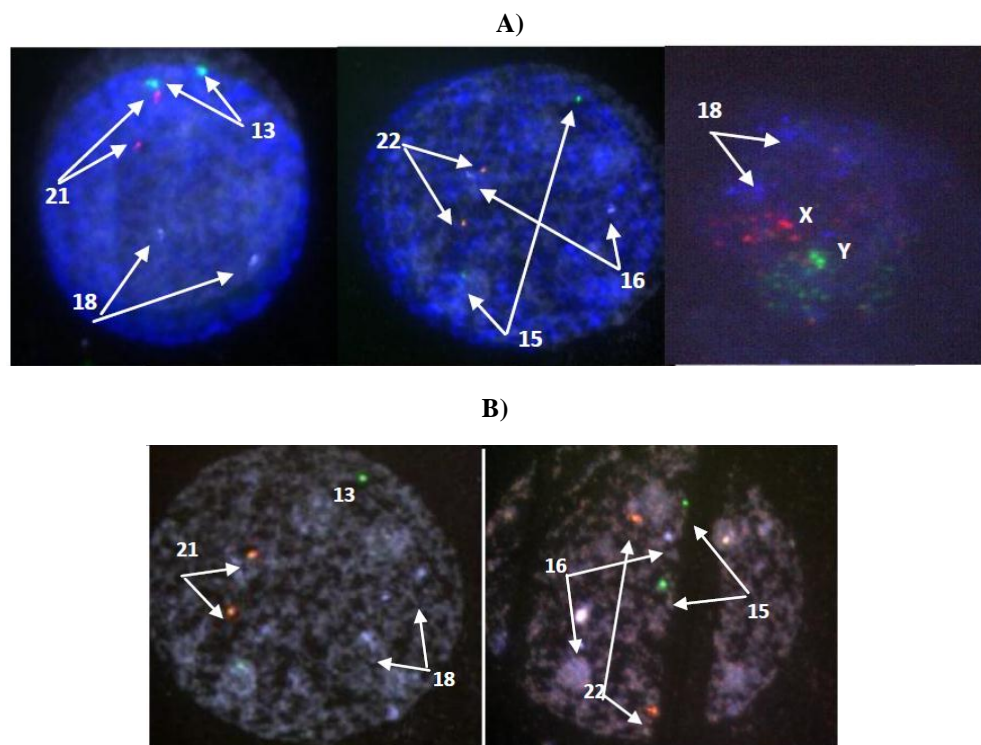


Fig. 1. A) The images from left to right show the results from three FISH rounds on a blastomer. After each round of wash/hybridization, lower core density, more overlap or splitted signals and generally non-informative results was observed. B) In images from second and third round of hybridization, the over manipulation of fixed blastomer lead to complete or partial removal of nucleus; thus, some results would be lost. Consequently with regard to observations from these images which showed significant cell loss during different rounds of FISH, more than two rounds of hybridization is not recommended.

Type of biopsy methods in FISH-based PGD

Biopsied cells for FISH-based PGD can be achieved by different methods: taking away the first and the second polar body from the unfertilized oocyte or the zygote; removal of one or two blastomeres at the day-3 cleavage stage, or removal of numerous cells at the

blastocyst stage. Recently, some protocols have applied polar body biopsy together with day-3 single-cell analysis. Each method has advantages and limitations with respect to its impact on viability of remaining embryo and the accuracy of information obtained from biopsied cells. Here, by comparing our findings with the

published studies pertaining to advantages and disadvantages of each biopsy technique for FISH-based PGS, we have summarized a whole series of results in the table 2.

Polar body biopsy and FISH

Using polar body (Pb) biopsy gives direct information about PbI and PbII, which allows us only to investigate maternal genetic content [14]. The first and the second Pb can be removed individually or at the same time [15]. However, from the screening point of view, the Pb biopsy is a worthwhile alternative, because numerical chromosomal disorders mostly arise in the meiosis of the oocyte. Moreover, the Pb biopsy is a practical option when there are some legal or ethical restrictions for working on embryo [16]. As only maternally inherited genome abnormalities could be inspected by Pb, using this method has encountered a serious problem. Furthermore, fragmentation of the chromatin in the biopsied Pbs often leads to the technical problems during FISH analysis and most probably in PCR analysis [17].

Cleavage stage biopsy and FISH

Cleavage-embryo biopsy of single blastomere allows the analysis of male and female genetic contribution to the final embryo by means of FISH analysis at the single-cell level [18]. One

or two blastomeres are usually removed from the embryo in the morning of Day 3, at about 68–72h after microinjection [19]. The totipotency of embryonic blastomeres inspires the confidence that removal of few cells (1 or 2) from blastocyst does not affect the ability of remaining cells to differentiate in all the cell lineages required for a correct development [20].

Number of blastomeres for FISH-based PGD

One of the principal issues concerning cleavage stage biopsy is the number of cells to aspirate. The analysis of one or two blastomeres for PGD using FISH has been discussed [21]. The number of cells needed for PGD depends on a balance between two aspects: an exact, correct diagnosis of the embryo and sustaining its implantation potential [22]. In 2010, Harper et al. [23] estimated that approximately 90% of IVF clinics perform embryo biopsy and PGD on day 3 of the embryos’ development when the embryo is typically composed of 6-8 cells. Despite the fact that some laboratories recommend biopsy with a single blastomere [21, 24] and some others suggest two [10, 25, 26], there is currently no general consensus among clinics on how many blastomeres are needed for obtaining a confirmative PGD results.

Table 2. Comparison of the PGD results from three biopsy methods and after two rounds of FISH.

Biopsy stage	No. results	Max No. of analyzed chromosomes	Max No. of analyzed cells	Best signal quality without overlapping	Complete cell loss
FISH on one blastomere at cleavage stage (N=45)	2/40 (5%)	8	1	++	5/45 (11%)
FISH on two blastomeres at cleavage stage (N =46)	3/85 (3.5%)	16	2	+++	7/92 (7.5%)
FISH on 5-20 cells on blastocyst (N =25)	3/25 (12%)	8	10	+	0/25 (0%)

N= number of analysed embryo

Since the ultimate aim of PGD is the birth of a healthy child, the diagnostic potential of FISH technique for evaluating chromosomal aneuploidies must be efficient, accurate and reliable. High efficiency rates have been reported in the literature following first round of probing, ranging between 95.2% [27] and 97% [28, 29]. As to re-probing a single blastomere for the second round, an efficiency rate of 95% is reported [30]. However, it is still a matter of controversy whether one or two blastomeres should be analyzed for PGD. Analyzing two cells per embryo may increase the accuracy of analysis and the number of chromosomes for screening, but may also have a detrimental effect on the developmental and implantation capacity of the embryo [31, 32]. On the contrary, if only a single blastomere is analyzed, there is a more likelihood that the results may not represent the chromosomal content of the remaining embryo due to misdiagnosis [10, 20, 33]. Furthermore, the correct interpretation of fluorescent signals on a single cell is not always evident thus two-cell analysis certainly provides a more reliable results especially taking mosaic cases into account. As high level of mosaicism had been previously reported in the cleavage stage of embryos, the analysis of two or even more blastomeres was introduced. Nonetheless, it has been shown that embryos with low-moderate chromosome mosaicism on day 3 often undergo self-correction during their development to the blastocyst stage [22]. Recent studies have highlighted that the removal of 2 cells from an 8-cell embryo can

result in impaired implantation potential [24, 34]. Therefore, the European Society of Human Reproduction and Embryology (ESHRE) PGD consortium recommended the biopsy of just one cell in PGS cycles: the aim of PGS is to improve embryo implantation and the removal of more than one cell would not be beneficial for the embryo [35].

In order to choose the right blastomere to aspirate, the presence of a clearly visible nucleus should be considered because multinucleation or anucleation is frequently observed in the cleavage stage of embryos [36]. However, some additional aspects can significantly influence the outcome of the biopsy, including size, orientation, shape and volume of the blastomere. The ESHRE [37] PGD consortium guidelines (2010) suggest that while the biopsy of more than one blastomere can have detrimental effect on clinical outcomes, the removal of two blastomeres may be required in some cases in order to improve the diagnostic accuracy of the test. To help maintain both embryo viability and diagnostic accuracy, ESHRE recommends that two blastomeres only be taken from embryos that consist of six or more blastomeres on day 3 [38]. Brodie et al. [39] only considered embryos ≥ 7 blastomeres in size suitable for two blastomeres biopsy. This is in contrast with the findings reported by Goossens et al. [24] and De Vos et al. [21], who biopsied one or two blastomeres from embryos ≥ 6 blastomeres in size. In general, any decision on this issue is based on the

difference in biopsy criteria being used by different laboratories.

Blastocyst biopsy and FISH

The blastocyst-stage biopsy consists of removing 5 to 10 trophoctoderm cells on day 5 or 6 of the embryo development [40]. Retrieval of 5 to 10 trophoctoderm cells from a 100- or 150-cell blastocyst corresponds with the lower fraction of cells lost in embryo (3.3% to 10%) whereas removing one or two blastomeres from a 6- to 8-cell embryo reduces the cell content by 12.5% to 33% [21]. Blastocyst biopsy also provides more starting blastomere than day-3 biopsy, which would theoretically lead to improving the sensitivity and specificity of PGD and is associated with lower rates of mosaicism [41]. This technique is cost-effective because fewer embryos are tested, and it has been associated with increased chance of live birth in the last decade [42]. However, embryologists working in PGD-PGS units should gain experience with blastocyst embryo culture and vitrification if frozen embryo transfer is to be performed. It has been recently shown that trophoctoderm biopsy has no impact on blastocyst reproductive potential whereas biopsy in the cleavage-stage results in 39% reduction in implantation rate.

For optimizing the biopsy procedure, our team compared different methods in three groups: I) the one-cell cleavage stage biopsy (from 45 embryos in 7-8 cells stage); II) the 2-cell cleavage stage biopsy (from 46 embryos in 7-8 cells stage); and III) 5 to 10 cells- blastocyst biopsy (from 25 embryos). Generally, the blastocyst biopsy provided more reliable results due to analyzing more

cells, particularly in mosaic cases in which the cells might partially or completely be lost during fixation. However, according to our experience, 2-cells cleavage stage is recommended for FISH-based PGD/PGS. Firstly, on the condition that a cell is lost, another cell remains for analysis. Secondly, the good quality and big size of blastomere nucleus in this stage leads to higher accuracy and informative signals as well as investigating more chromosome number (in PGS) in second round of hybridization. However, in blastocyst biopsy, small size of nucleus will result in weak signals in the first round of FISH and overlapping signals in the second round. Moreover, following blastocyst biopsy, time limitation for transferring the embryo would be the main drawback.

Slide preparation

It is recommended that before slide preparation, they are labeled with the case number and then make a circle (approximately 3-5 mm diameter) on the slide using a diamond pen. Here, there are two points that should be mentioned: location on a slide and type of slides. Regarding the first one, we suggest that instead of using beneath the slide, the circle should be formed on top of it. In this way, the cell location will be much easier under the fluorescent microscope. On the other hand, care should be taken about generation of splinter from rubbing the diamond pen. It is recommend to get rid of the splinter and dipping the slides into a jar filled with fixative solutions for a few seconds. This solution could be a combination of methanol/acetic acid or methanol/HCL. Then, the slides are dried and used for fixing the

biopsied blastomeres. The other point is about what kind of slide would be better for fixing biopsied cells an amine-coated (optional) or a conventional glass slide? Our experience suggests that from the standpoint of fixing the biopsied cells, there is no difference between the types of slides. Even applying the amine-coated slides, especially those coated manually, results in difficulties during image analysis. It is due to the production of a mask by poly-L-lysine along the slide that increases the background noise and FISH error. After selecting a good slide according to fixation method, labeling, and scoring circle, the slide is washed in fixative, and followed by one of the blastomeres fixation techniques. It should be noted that any dirt or debris on the slide can be mistaken as the real signals or the covered part of the nucleus can result in not detecting a signal.

Type of fixation methods in FISH-based PGD

One of the most important factors affecting the single cell FISH-based PGD results is the fixation efficiency. The traditional fixation technique based on air-dry method was later modified in different ways by others, for instance applying fixative solution methanol/ acetic acid (Carnoy). Generally, there are two main methods for fixation of blastomere nuclei on microscope slides. One is the traditional fixation method based on methanol and acetic acid [43], and the second the Tween: HCl method. [44]. There are a few fixation methods, each with their advantages and disadvantages. Some studies have compared the available methods based on the number of

blastomeres loss, analyzable nucleus and FISH errors. Therefore, considering other studies, our team modified the method based on carnoy, comparing it with the other three methods. The blastomeres were biopsied from arrested embryos at 4 to 8 cells stage and were randomly assigned to four groups: one undergoing fixation by our modified method and blastomers from three other groups fixed by protocol described by Velilla et al. [45]. One of the most important problems in methanol/acetic acid method is that the blastomere may be lost during adding of hypotonic solution and mixing fixative with hypotonic solution containing cell [46].

For elimination of the above problem, we modified methanol/acetic acid method and fixed the blastomeres as follow: A drop of hypotonic solution was placed within the circle, then the aspirated blastomeres were transferred into hypotonic solution (for 1-5 min.). This procedure makes a boundary around the drop thus avoiding losing the cell. Addition of hypotonic solution was gently continued until blastomere lysed completely and the cytoplasm dispersed before drying buffer. The best sign to ensure removing of the cytoplasm is observation of budding on the membrane. It is really essential, because without removing cytoplasm here, probe could not penetrate the nucleus in the hybridization step. The nucleus should remain on the slide, then it must be exposed to gentle heat for a moment. This prevents moving the the blastomere, and the cell nuclei are fixed using several drops of the fixative. Then, the slide is air-dried at room temperature and is proceeded

with hybridization. None of the fixation methods are certainly better than the other and each laboratory should utilize methods that have the most experience and that the best informative results are obtained under

their own working conditions [47]. Our observations suggest that modified fixation method obtains maximum informative results and minimum cell loss. Our comparative results are summarized in table 3.

Table 3. Rates of cell loss and informative nucleus depending on different fixation methods and studies

Method	No. of fixed	Cell loss(%)	Lack of nuclei	Informative* nucleus (%)
Present study				
1 (our modified method)	55	2 (3.6)	5	48 (90.5)
2	60	5 (8.3)	7	49 (89)
3	55	6 (10.9)	10	38 (77)
4	48	5 (10.4)	8	36 (83.7)
Study 1 E Velilla et al.				
1	110	4 (3.6)	15	89 (84)
2	106	3 (2.8)	22	71 (68.9)
3	114	3 (2.6)	10	92 (82.9)
Study 2 Dozortsev et al.				
1	16	2 (12.5)	ND	13 (81)
2	16	1 (6.25)	ND	14 (87)
3	18	0 (0.0)	ND	18 (100)
Study 3 Xu et al.				
1	121	26 (21.5)	ND	76 (62.8)
2	131	8 (6.1)	ND	60 (45.8)

* lack of informative signals result from the following: complete or partial lack of nucleus because of analyzing a no nucleus blastomere or removing during biopsy or fixation, reducing nuclear areas during fixation, no probing due to lack of proper cytoplasm removing and/or due to signal overlap or splitting
 ND = not determined

In situ hybridization of blastomere nuclei

Fixing the blastomere according to the mentioned method reduces the rate of losing the cell significantly. However, several steps in pretreatment process increase the possibility of loss of the cell. Therefore, it is recommended to reduce the steps as much as possible. For example, if you are sure that the cytoplasm is removed completely when exposed to the hypotonic solution, it is not necessary for pretreatment with salting buffers or pepsin solutions. On the other hand, it is highly

recommended to minimize the time of exposure to different pretreatment solutions. Follow these steps for hybridizing probe on blastomere: Pretreat the slides in 2 x saline sodium citrate (SSC), pH 7.0 at 37°C for 10 min. (optional), post-fix in 1% buffered formaldehyde in 1X phosphate buffer solution (PBS)/20 mM MgCl2 at -4°C for 5 min., wash the slide for 5 min. in 1X PBS at room temperature (optional), incubate the slides 2-10 min. (depending on cytoplasm remaining around blastomere) in 0.005% Pepsin solution in 0.01 M HCl at 37°C,

wash the slides for 5 min. in 1X PBS at room temperature, dehydrate slides in 70%, 85% and 100% ethanol for 1 min. each, then in methanol for 5 min (optional), and air dry the slides, apply 2 µl of probe mixture, and cover with a 9 x 9 mm cover slip, then seal with Rubber Cement, co-denature the sample and probe on a hot plate at 75-78°C for 5-10 min., incubate at 37°C in a humidified chamber (2-20 h depending on type of probe (Fast/overnight). Humidity and temperature should be controlled and be made stable, because it has a significant role in different steps of FISH procedure. Humidity is necessary for efficient hybridization even though overhumidity results in splitting of the signals. Carefully, remove the rubber cement, then slide off cover slips. Immerse slides in (0.4X SSC/ 0.3% tween 20) for 2 min. at 72°C ($\pm 1^\circ\text{C}$), then in (2X SSC/ 0.1% tween 20) for 2 min. at room temperature. Rinse the slides twice in sterile distilled water (optional), and air dry at room temperature. Apply 4 µl counterstain and apply glass cover slip, seal the edges of it with clear nail varnish, then proceed with microscopy. The stringency of PH, temperature and times of pre- and post-hybridization are important. If the conditions of this step are too severe, the signals will be weak. If the conditions is not severe enough, it may result in non-specific hybridization on the other sequences and chromosomes that could be incorrectly interpreted as signals.

Execution of more than one round of hybridization on each nucleus is the routine step in PGS to increase the number of chromosomes analyzed. This approach is effective, but it requires a lot of skill and care to achieve

analyzable signals in later rounds. In order to get rid of the remaining signal from previous round, it is recommended to wash the slides in 4X SSC or 1X PBS for 10 min. at room temperature and then expose the nucleus to bright light for a few hours. It needs to be carefully considered that when analyzing subsequent rounds of FISH, the position of signals on the nucleus at different rounds should be compared to determine that they are new signals or have remained from prior rounds of FISH. It is well documented that multiple denaturation and hybridization during second and third round of FISH, causes nucleus degeneration and reduced efficiency of the results [48].

Fluorescence microscopy and causes of misdiagnosis

For optimal visualization, use a regularly calibrated microscope equipped with a 100 W mercury lamp and a x63 or x100 fluorescent objective. Score signals by single band-pass filters for each fluorochrome in the test. Each nucleus should be scored by two analysts. A general guideline is necessary for scoring a single signal. However, the judgment based on experience needs to be exercised to interpret the signals of varying size, intensity, and separation. We used Applied Spectral Imaging (ASI) software to capture an image of the nucleus for confirmation of the visual diagnosis and for image archiving as part of the laboratory quality assurance plan. There are a number of possible causes of misdiagnosis. Although, some diagnostic errors are related to the sample or slide mislabeling and mis-identification, many of these errors are specific

to the technology or methodology used. These are like any laboratory technique. FISH-based PGD has a number of limitations which can lead to incorrect interpretation of the results and a potentially IVF failure. Dubious and error in diagnosis ranges from 1-20 % of the embryos undiagnosed [49, 50].

Errors in FISH based PGD could be divided in two groups: first, errors caused by technical complications concerning FISH technique, and second those caused by mosaicism. Different PGD laboratories reported different error rates of 4% [11, 51, 52] to 50% [52-54]. According to the Preimplantation Genetic Diagnosis International Society (PGDIS) guidelines [55, 56], error rates under 10% is acceptable. Centers with greater error rates should not offer PGD unless on an experimental basis until their results are improved. Effective signal diagnosis depends on the quality of biopsy, fixation and hybridization stages. Even with performing these procedures in the best way, signals may overlap or split. Occasionally, the target sequence of a specific probe on homologous chromosomes is overlaid during fixation. When subjected to FISH, it leads to overlapping two signals and is interpreted as one signal. Such signals will appear larger than normal signals. In this condition, reprobng of the nucleus with a new probe in the second round of FISH which is located elsewhere on the understudied chromosome can resolve the problem. On the other hand, target sequence of a specific probe can split and result in difficulties in signal interpretation [57]. A criterion that has been used in the past for scoring dubious signals include assessing size and distance between the

signals. At the best condition, the existence of two average signal diameters between two signals is the minimum distance to confirm that two signals are separate [58]. Recently, Hardarson et al. [59] reported that the existence of one domain (signal-width) distance between two signals is enough to score them separate. Another way to decide on dubious signals is no result rescue in which the new probe is used in the second round of hybridization that binds to another target of the same chromosome. Rehybridizing will help to distinguish between a split signal and two real separate signals [11, 60, 61]. Another technical difficulty relates to dirt or debris which can cover some signals or be considered as a signal by mistake. Any spot similar to a signal that is detectable through all microscopic filters is very possibly to be dirty. Ordinarily, the fixed nucleus is somewhat flat and all of the signals can be imagined in a single focal plane. Occasionally, the signals are not seen together because they are in dissimilar depths within the nucleus. Therefore, for confirming that all signals are imagined it maybe necessity to capture more than one image by each filter. All limitations and weaknesses mentioned above for FISH-based PGS are also raised about the diagnosis of the chromosomal rearrangements by FISH. However, it is essential to apply a combination of probes to analyze chromosome imbalance in embryos from translocation carriers. Applying combination of probes allows detecting all possible rearrangements [5].

Error rates due to mosaicism

The existence of chromosomal mosaicism is reported in half of the early human embryos,

but its rate varies extensively in the literature. This variation can be related to the patient population, types of hormonal stimulation, calculation standards, the overall quality, developmental stage and condition of the embryo. Large studies on the cleavage-stage embryos indicate that mosaicism rates between 25% to 30% [29, 32, 52, 62-65]. Rate of false-positive or false-negative due to mosaicism in the experienced laboratories has been assessed to be around 4.3% and 1.3%, respectively [52]. This means that the chromosomal pattern of blastomere biopsied for PGD by FISH may not reflect the karyotype of remaining embryo and could result in IVF failure or abnormal live birth. This cannot be considered as a misdiagnosis but as a biological source of error or inherent limitation of FISH-based diagnosis. Some clinic centers for IVF have suggested that biopsy of two blastomeres from each embryo would be more verifiable to distinguish mosaicism. Although mosaicism due to false positive errors can affect the result of analysis, there has been no evidence concerning that as a major issue in PGD [66]. Total FISH error rates are estimated as low as 7% by different researchers [11, 51] 2% of which is due to technical difficulties and the rest is related to mosaicism. Hence, the effect of mosaicism on PGD errors is just part of the <10% complete error rate accounted by experienced laboratories in IVF centers.

Conclusions

Over the years, FISH-based technique has not lost its place among the new technologies. The advent of new approaches like CGH and NGS

makes the FISH-based PGD technique a widely conventional method for analyzing the chromosomal aneuploidies and translocations. However, the cost-effectiveness and reliability of FISH-based techniques makes it as one of the feasible approaches in many clinics which do not have access to the new technologies such as CGH and NGS. Therefore, our clinical experience in FISH-based methods has persuaded us to publish some tips and tricks about pre- and post-examination of FISH process that might favorably affect the results.

First of all, the best type and number of probes are selected according to the number of applied filters in fluorescent microscope, the number of available fluorochromes, and the number of biopsied cells. To analyze more chromosomes, two rounds of washing and hybridization could be performed on the same cell but not more. Unfortunately, after second hybridization rounds, rising cell missing, background signal, noise and overlapping signals lead to decreasing efficiency and validity of FISH results.

Therefore, biopsy of two cells and two rounds of hybridization are recommended for screening the mosaicism and more chromosomes in one embryo. Moreover, our observations suggest the biopsy on cleavage stage embryo because of the big size and quality of nucleus which is necessary for FISH- based analyzing.

Regarding blastocyst biopsy, small size of nucleus would be an indicative issue, so that it could result in weak signals in first round and overlapping signals in second round of FISH. On the other hand, time limitation for embryo transferring after blastocyst biopsy is a vital

drawback for performing FISH in this stage. Also, our observations showed that, compared with other methods, our modified fixation method would obtain maximum informative results and minimum cell lost. Although FISH-based PGD must be performed according to strict protocols, there are always some difficulties that may arise from procedure and lead to misinterpretation of the signals and adverse outcomes. However, these protocols vary between laboratories and should be optimized according to the laboratory working

conditions. We believe that by using the tips and tricks suggested in this article, FISH-based PGD will become an affordable and efficient tool for selecting euploid embryos and increasing success in IVF.

Conflict of Interest

The authors declare that they have no competing interests.

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