Will noninvasive methods surpass invasive for assessing gametes and embryos?

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The need to identify the most viable embryo following in vitro fertilization (IVF) was established early in the history of human IVF. The stalwart of identifying the best embryos has been morphology. Other techniques have however seen wide acceptance, including the use of preimplantation genetic screening, even though concerns exist over the invasive nature of the technique. Alternatively, noninvasive assessment technologies have tried to determine an embryo's viability through measurements of factors in the media or by imaging of the embryo. We present data showing that the metabolic blueprint of an embryo is linked to viability, and argue that analysis of metabolic function, using either spent medium or by novel microscopies, could provide the basis for selecting the embryo with the highest viability. This review therefore asks, "Will noninvasive methods surpass invasive for assessing gametes and embryos?" We examine the current state of research on noninvasive technologies, including novel optical methods, and conclude noninvasive embryo viability assessment will assist in embryo selection for transfer. (Fertil Steril® 2017;108:730–7. ©2017 by American Society for Reproductive Medicine.)

Key Words: Embryo assessment, IVF, embryo metabolism, noninvasive, microscopy

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MODERN IVF HAS TRANSCENDED CLASSIC MORPHOLOGICAL EMBRYO ASSESSMENT

The need to identify the most viable embryo following in vitro fertilization (IVF) was established early in the history of human IVF, when the only available information about the embryo could be obtained from cleavage rates and morphology patterns. Indeed, early studies, including those by Bob Edwards, stated that "cleavage rates on a certain day and overall embryo morphology were valuable in choosing which embryo to transfer" (1). Forty years later, the development of an accurate quantitative method to assess embryo viability, leading to improved pregnancy rates, while allowing the transfer of a single embryo, remains one of the main challenges for IVF clinics (2).

The significant health risks associated with multiple pregnancies has been the motivation to transfer as few embryos as possible, without compromising the chance of achieving pregnancy. To this end, IVF centers have used grading systems based upon semi-quantitative descriptors of the morphology of the pronucleate oocyte, cleavage-stage embryo, morula or blastocyst (3). Pronucleate oocyte grading systems evaluated pronuclear size and position, nucleoli number and distribution, and cytoplasmic appearance (4–6). Cleavage-stage embryo-grading systems include criteria such as the uniformity of blastomeres, percentage of fragmentation, rate of cleavage, and blastomere multinucleation (6–9). However, the ability of morphology alone to accurately identify viable pre-compacted embryos has been limited. For example, even embryos with irregular morphological and morphokinetic parameters at early stages of development can lead to normal live births (10). When assessing cleavage stage embryos, one is primarily considering the quality of the oocyte, as limited embryonic genes have been activated (11, 12). With the introduction of improved culture systems capable of supporting the human embryo throughout the preimplantation period, blastocyst grading systems were developed to account for the degree of expansion, zona thinning, and the quality of the trophectoderm and inner cell mass (13, 14), thereby assessing true embryonic

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traits post embryonic genome activation. This approach has led to lowering the number of embryos transferred, thereby leading to a reduction in the incidence of twins and all but eliminating high order multiple gestations (15–17).

The ultimate aim of any embryo selection system is to identify the cleavage stage embryo, or blastocyst, that is most likely to implant and develop into a healthy baby. Although used extensively for over three decades, classic morphologic assessment does not provide any insight into the physiology of the embryo, and although extremely useful in embryo deselection, when faced with embryos of the same morphology such assessment cannot identify the embryo with the highest viability.

In this review, we focus on why noninvasive assessment can still provide an ultimate means of embryo assessment. We ask the question, "Will noninvasive methods surpass invasive for assessing gametes and embryos?" While we acknowledge that it is possibly too early in the development of some of the technologies to provide strong clinical evidence, it is important that the assisted reproductive technology community is aware of the continued pursuit of reliable noninvasive embryo selection techniques.

QUANTITATIVE ASSESSMENT OF EMBRYO VIABILITY THROUGH INVASIVE MEANS

Invasive assessment has been driven by the hypothesis, "… if a euploid embryo can be identified, its ability to lead to a normal live birth will be greatly enhanced." The first reported use of an invasive method in humans was for the treatment of sex linked diseases (18, 19). This initial procedure was based on single blastomere analysis using fluorescence in situ hybridization (FISH), but was eventually modified by adding in further chromosome labels so that aneuploid embryos could be deselected. Although the validity of selecting a euploid embryo has never been challenged, the quest for technologies to accurately measure chromosome content without compromising the embryo has faced numerous hurdles. The greatest such barrier came from the technical challenges to perform FISH on a single biopsied cell. A series of trials in 2006-2008 led many to question its validity (20–22). In recent years, the advent of trophectoderm biopsy and preimplantation genetic screening (PGS), powered by dramatic advances in molecular based screening technologies including the development of next generation sequencing platforms, have provided significant improvement in embryo selection by comprehensive chromosome screening (23–26). However, embryo mosaicism has emerged as a new challenge for PGS methods (27, 28). Concerns also persist over possible damage from the biopsy (29). Additionally, not all euploid embryos are developmentally competent; thus, screening for aneuploidy does not necessarily guarantee a viable embryo.

THE HOLY GRAIL OF NONINVASIVE EMBRYO ASSESSMENT?

Noninvasive assessment has been driven by two hypotheses: morphological determinants exist during the preimplantation stages that can be related to an embryo's viability (or certainly lack thereof); and quantitatively measuring factors in the media or noninvasively in the embryo, in particular metabolic signatures, can provide the basis for selecting the embryo with the highest viability.

The importance of metabolic activity for embryo development and viability has been established over several decades (30). Early studies revealed a clear correlation between adenosine triphosphate (ATP) levels and embryo viability in mouse (31), and subsequent works identified the pivotal role of glucose metabolism in oocyte and embryo viability (32). Significantly, viability has been linked to the consumption of oxygen, an essential component of oxidative metabolism (33). As metabolic function is known to be essential for viability, any assay for measuring the metabolic state will logically provide valuable information for assessing the health of embryos.

Several noninvasive methods aiming to determine embryo viability have been put forward as candidates, including proteomics (34), birefringence imaging (35), measurement of respiration rate (36), amino acid turnover (37–39), soluble human leukocyte antigen-G (40), and pyruvate uptake (41). More recently, a rejuvenation of morphological assessment has been attempted using time lapse imaging also by quantifying morphokinetic features of development. Although such an approach looks promising with regards to the ability to deselect embryos for transfer (42, 43), one is still only making morphological assessments of the embryo, and not actually quantifying any physiological parameters. Unfortunately, it has so far failed to provide significant improvements in viability prediction (44).

The most consistently repeatable parameter linked to embryo viability across several mammalian species has been embryonic glucose uptake and its utilization (30, 45, 46). As early as 1970, Menke and McLaren (47) revealed that mouse blastocysts, developed in basic culture conditions, lost their ability to oxidise glucose. This initial observation was followed by several studies that elucidated changes in embryo metabolism associated with loss of developmental capacity in vitro (48). In 1980, Renard et al. (49), observed that day-10 cattle blastocysts which had a glucose uptake greater than 5 μg/hour developed better, both in culture and in vivo after transfer, than those blastocysts with a glucose uptake below this value. In 1987, using the relatively new technique of noninvasive microfluorescence, Gardner and Leese (46) measured glucose uptake by individual day-4 mouse blastocysts prior to transfer to recipient females. Blastocysts that went to term had a significantly higher glucose uptake in culture than those embryos that failed to develop after transfer. This work was built upon by Lane and Gardner (50), who went on to reveal that not only was the rate of glucose consumption linked to viability after transfer, but so was the metabolic fate of said glucose. Blastocysts that exhibited excessive lactate production, i.e. not showing a normal physiological profile, were shown to have very limited viability (50).

Analysis of the relationship between human embryo nutrition and subsequent development in vitro (38, 46) was subsequently undertaken by Gardner et al., (46) who determined that glucose consumption on day 4 by human embryos was twice as high in those embryos that went on to form blastocysts. Gardner and colleagues (51) then went on

to confirm a positive relationship between glucose uptake and human embryo viability on both day 4 and day 5 of development. Regrettably, the complex nature of the microfluorometric method, including dedicated equipment and technical staff, has greatly impeded its entrance into mainstream use for clinical IVF. However, a potential means to accurately analyze small volumes of culture media is through the use of microfluidics. A proof-of-principle study was performed by Urbanski et al. (52) who developed a chip using soft-lithography and polydimethylsiloxane, capable of analyzing sub-microliter volumes of media. Such devices represent a means of accurately quantifying nutrient and metabolite levels (53).

BARRIERS TO NONINVASIVE ASSESSMENT

Noninvasive assessment of preimplantation embryos has therefore remained largely limited to the use of morphology. As with the technical advances seen in molecular-based PGS technologies, the main hurdle to noninvasive screening is not in the validity of the hypothesis, but in the validity of the technology. A prime example is the implementation of metabolomics to assess embryo viability. Metabolomics is the study of the unique chemical fingerprints that specific cellular processes leave behind, in this case the embryo. Metabolomics was evaluated in human IVF for noninvasive profiling of embryo culture media. Analyses were performed on media samples from embryos that led to pregnancy or no pregnancy (54–56). The technologies studied included proton nuclear magnetic resonance, Raman spectroscopy, and near-infrared spectroscopy (54–58). All three technologies revealed a correlation with embryo "viability," as assessed in vitro, and with pregnancy outcome. Following a series of initial promising studies, a prototype instrument, which generated a Viability Score to grade each embryo, was introduced for clinical use. The studies were however terminated due to the assessment scores being highly susceptible to instrument variability. Many of the commercial instruments showed poor repeatability within instrument, and poor reproducibility between instruments. For example, individual algorithms created on master instruments were vulnerable to interference when loaded onto other instruments. Indeed, larger studies using single-embryo transfer had been conducted to assess the precision and reproducibility of the viability score, and to evaluate the efficacy of the method in identifying the single best embryo of a patient's cohort. Two of those single-embryo transfer studies failed to show a difference in implantation and clinical pregnancy rates using metabolomic profiling of spent embryo culture media (59, 60). Data examining outcomes after multiple embryo transfers were however more promising (61), indicating that score variability was definitely more detrimental in a single embryo transfer scenario.

The technological challenges to develop a noninvasive system cannot be underestimated; however, just as PGS has progressed from FISH to NGS, the field of noninvasive assessment is also evolving. It is envisaged that further studies will be forthcoming on human embryo metabolism and its relationship to IVF outcome.

NOVEL OPTICAL METHODS FOR ASSESSING GAMETES AND EMBRYOS

It could be argued that exposure of embryos to any outside artificial conditions, including illumination necessary for any kind of imaging, is "invasive." Although technically not completely noninvasive, in the context of this review we consider imaging to be noninvasive (providing wavelengths out of the ultraviolet and blue end of the spectrum are avoided). This nomenclature does, however, take into account studies that have shown some impact of light on embryo viability (62) and that the proviso always exists that safety must be determined prior to the introduction of such "noninvasive" techniques into routine care.

Noninvasive embryo assessment can now be performed by a number of novel imaging platforms. The main prerequisite of such imaging approaches is the information being measured should be obtained without the use of labels, probes, or prolonged exposure times, to assess the embryo or oocyte.

Raman spectroscopy (56, 63), Fourier transform infrared spectroscopy (64), fluorescence-lifetime imaging microscopy (FLIM) (65) and hyperspectral analysis (66) can all be used to provide additional spectroscopic data on embryos, and they thus have the potential to produce superior clinical tools for embryo assessment. However, a number of hurdles still exist. In particular, expense and ease of use must be improved. Finally, credible data showing that the methodologies are safe and do indeed predict embryo viability must also follow. Mitochondria, which play key roles in cellular respiration, biosynthesis pathways, Ca^{2+} regulation, apoptosis, and the production of reactive oxygen species, are critical for successful embryo development (67–69). Embryo viability has been found to correlate with proxies for mitochondria physiological state, such as ATP levels, O_2 consumption, glucose oxidation and membrane potential (31,70–73). Artificially disrupting mitochondria in oocytes and early embryos results in loss of viability, defects in maturation and fertilization, and errors in cell division (74–79). These results support the hypothesis that measurements of mitochondria function will provide the basis to select high quality embryos for transfer.

Cells have long been known to contain molecules or factors that emit autofluorescent signals. Numerous endogenous fluorophores exist in cells including amino acids, structural proteins, enzymes and coenzymes, vitamins, and lipids (80). This intrinsic property of cells can therefore be captured using different types of spectral analysis. The main advantage of this type of approach is that it avoids the use of any labels of probes. Nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) are autofluorescent coenzymes involved in numerous biochemical reactions within the cell, the states of which are directly connected to cellular respiration. Hence they make ideal candidates for biomarkers of mitochondrial function. There is a long history of using NADH and FAD fluorescence to noninvasively study mitochondria in cells, tissues, and intact organisms (68, 69, 76, 81, 82). One of the most effective approaches to assess NADH and FAD in vivo, is metabolic imaging by FLIM,

performed with time correlated single photon counting (27). This established technique creates a histogram of times that the fluorophores remain in their excited state. The fluorescent lifetimes of NADH and FAD change depending on whether they are bound to proteins or soluble, with the precise values depending on the local environment. The total amount of fluorescence produced is related to the amount of NADH and FAD present. Thus, a properly calibrated metabolic imaging system can be used to measure the concentration of NADH, the concentration of FAD, the fraction of each bound to protein, and it can provide information on the local environment of both the soluble and protein bound pools. The technique yields quantitative parameters, which provide a relatively direct measure of the state of mitochondria in individual embryos (Fig. 1).

The effectiveness of FLIM-based metabolic imaging to sensitively measure metabolic states has been established in many studies, including the differentiation of various cancer cell types (83) and the observation of distinct metabolic profiles for stem cells at different stages of differentiation (84). Metabolic imaging of oocytes and embryos has great potential in IVF because it provides an objective measurement of the physiological state of their mitochondria, and is rapid, noninvasive, and quantitative (Fig. 2).

While such an approach as FLIM is promising, it is important to note that a causative relationship between abnormal mitochondrial functions and clinically relevant embryo loss has not been established. Furthermore, it has not been demonstrated that mitochondria function is correlated with viability in a cohort of embryos from an individual patient (which is required for a useful selection tool). Future systematic studies using this approach could resolve these issues, and establish the utility of metabolic imaging, both as a selection tool in IVF and as a research tool for the field of fertility.

Other types of microscopic analysis are also being examined in the realm of embryology. Hyperspectral analysis (66) investigates overall autofluorescent signatures and their cellular distribution. Similar to FLIM, this technique has also been shown in other cell systems to provide insights into cellular processes (84–86), including embryos (87). Recently, this type of analysis was shown for the first time to noninvasively extract biologically relevant and quantitative information from cells and tissues. It was able to identify and document autofluorescence by multispectral imaging whereby a spectrum is taken at each pixel in the image, generating about a million such spectra from cellular areas with varying molecular composition (66). Individual cells are segmented out and their images processed to generate multiple, mathematically defined cellular features. The endogenous cell fluorophores examined included NADH, nicotinamide adenine dinucleotide phosphate (NADPH), FAD and flavin mononucleotide, N-retinylidene-N-retinylethanolamine, cytochrome C, and proteins including abundant species like collagen and elastin.

Optical coherence microscopy (OCM) is a further promising technique. This intriguing approach is based on optical coherence tomography (OCT) (88), which can image structural information of a sample by measuring the interference between back-scattered light from the sample with a reference signal. The main benefits of this technique are superior sample penetration and axial resolution; however, OCT is typically limited in its transverse (XY plane) imaging resolution. OCM overcomes this transverse resolution limitation by combining OCT with confocal microscopy, providing a noninvasive means of performing high-resolution, 3-dimensional imaging of subcellular components.

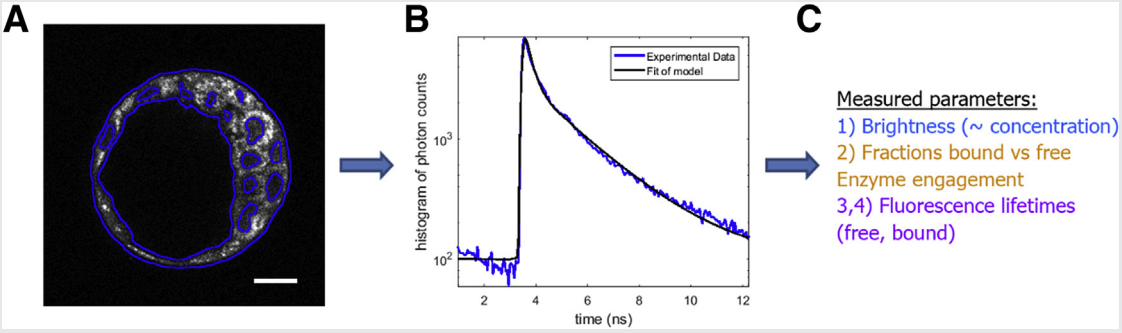
This intriguing approach was recently shown to provide detailed information on early embryonic development, without requiring sample pre-processing or labeling (89). The technique images the internal motion of cytoplasm using custom scanning and signal processing protocols, and it provides a type of high-resolution intracellular time-lapse imaging. Karnowski et al. (89) used mouse and pig oocytes and embryos, and visualized them through fertilization and the first embryonic division, as well as at selected stages of oogenesis and preimplantation development. While OCM appears to yield more structural information than bright field imaging, its utility is still based upon the same fundamental premise as current techniques: that morphological and morphokinetic properties are highly indicative of embryo viability. As morphology appears not to be totally predictive of embryo viability, it remains to be seen whether OCM will contribute as much selective power as techniques that directly sample the biochemistry of embryos. However this type of combination of novel microscopy and embryology is another excellent example that may represent a new chapter in imaging-based preimplantation embryo diagnostics.

NOVEL METHODS FOR ASSESSING SPERM

A number of novel new technologies are also being developed which may serve the purpose of allowing better sperm selection prior to classical IVF or intracytoplasmic sperm injection (90–93). These include the use of Raman Spectroscopy to noninvasively distinguish the DNA packaging and protamine content between normal and abnormal cells (94). In a number of studies it has been shown that the relative protein content per cell and DNA packaging efficiencies are distributed over a relatively wide range for sperm cells with both normal and abnormal shape. These findings indicate that single cell Raman spectroscopy could be a valuable tool in assessing the quality of sperm cells. This technology in combination with microfluidic sperm cell sorting (95, 96) will hold great promise for sperm selection techniques that may translate to improved IVF outcomes by selecting the more competent paternal genome component.

Although these sperm assessment techniques are intriguing, the biggest challenge is identifying evidence that selecting sperm in such a way will be of major significance on the outcome of IVF. It has been previously discussed (93) in relation to sperm selection that our greatest challenge to creating predictive sperm assessment models is the multifactorial nature of fertility. We are not only dealing with the many attributes of the spermatozoon, but also a significant impact from the egg and uterus.

FIGURE 1



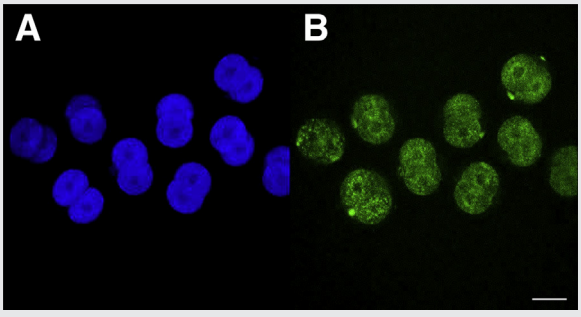
Metabolic imaging on individual embryos. (**A**) FLIM measurements of nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide (FAD) produce fluorescence images, such as this NADH image of a mouse blastocyst. Because NADH and FAD are concentrated in the mitochondria, these images provide reflect mitochondrial distribution. Images can be segmented with processing techniques to identify the embryo and separate it from the background. (**B**) Photon arrival time counts that occurred within the embryo region can be pooled together into a single fluorescence-lifetime imaging microscopy histogram, representing the average state of the embryo. This histogram can be fit to a theoretical model of two exponentially decaying species–one for bound molecule and one for free. (**C**) From these fits, each measurement produces 4 quantitative parameters that are sensitive to changes in mitochondrial state. NADH and FAD measurements can also be taken in tandem for a total of up to 8 parameters. Bar = 20 μm.

Sanchez. Noninvasive embryo assessment revisited. Fertil Steril 2017.

NON-OPTICAL METHODS FOR ASSESSING GAMETES AND EMBRYOS

Other areas are developing that could allow non-microscopic methods to be used to measure factors noninvasively. In addition to the use of microscopy, there have also been advances in technologies to investigate cell free DNA and RNA in the culture media surrounding embryos (97–99). One area of particular interest is the recent publication reporting the assessment of cell free DNA in embryo culture media being used to assess the ploidy status of embryos. Xu et al. (98) performed NGS on the spent culture media used to culture human blastocysts (n = 42) and obtained the ploidy information of all chromosomes. They validated these results by comparing each with their corresponding whole donated embryo and obtained a high correlation for identification of chromosomal abnormalities (sensitivity, 0.882, and specificity, 0.840). They subsequently reported chromosome screening on IVF embryos from seven couples with balanced translocation, azoospermia, or recurrent pregnancy loss and reported six successful clinical pregnancies. Although promising, concerns exist as to the validity of measuring cell free DNA in embryo culture media (100).

FIGURE 2



Example of digitalized metabolic imaging on individual 2-cell mouse embryos. (**A**) Fluorescence-lifetime imaging microscopy measurements of nicotinamide adenine dinucleotide and (**B**) flavin adenine dinucleotide produce fluorescence images that can subsequently be quantified by specific algorithms incorporating information related to the auto- fluorescent signal of each embryo (Fig. 1). Bar = 50 μm.

Sanchez. Noninvasive embryo assessment revisited. Fertil Steril 2017.

In addition to technologies that are completely novel to IVF, recent improvements of older format assays, such as immunoassays, also shows some promise. One example is a novel ultrasensitive digital immunoassay platform (101–103) developed almost 10 years ago. In a recent study (104) we used this platform to assess media for interleukin-6 (IL-6). Media samples were analyzed for IL-6 (pg/ml) expression and then matched to a coded database to determine if implantation of the transferred embryo had occurred. We found that from 71 blastocysts which failed to implant and from 60 embryos that implanted the resultant IL-6 values (mean±standard deviation) in the media were significantly different. These digitalized immunoassays could be the basis for a clinically accessible platform allowing IVF laboratories to identify previously undetectable markers in the embryo culture media and quantify them more accurately and reproducibly.

WILL NONINVASIVE METHODS EQUAL OR SURPASS INVASIVE METHODS?

The success of comprehensive chromosome screening has created the gold standard for aneuploidy assessment techniques. When a euploid embryo is identified we now expect a live birth rate approaching the 60% range (23–26). This number does pose an interesting dilemma however, what is

happening to the other 40% of euploid embryos? We can attribute some failures to uterine asynchrony (105, 106), and some to paternal influences or male factor (93, 107). Although many other female factors could contribute to transfer outcome (108), our focus has been on the ability to select the embryo with the highest probability of initiating and maintain a successful and healthy pregnancy.

The ability to quantify metabolism more accurately using a noninvasive platform may serve to identify those euploid embryos that are more likely to lead to a live birth. The question remains what, if any, correlation exists between euploid status and metabolic competence? If euploid embryos also show a higher metabolic competence, then the ability to use noninvasive metabolic assessment of embryos will be equivalent to the current invasive techniques. If the novel optical technologies being developed can both identify aneuploidy and metabolic competence, as suggested by the OCM and FLIM technologies, then the future may involve noninvasive platforms.

Currently, hurdles remain to achieving the long-held goal of quantitating embryo viability in IVF. The safety, accuracy and utility of these technologies is far from established. However, research into the novel noninvasive techniques as described herein, and their implementation into IVF, holds promise for the development of superior, noninvasive embryo viability assessment tools, which are likely to play increasingly important roles in coming years.

REFERENCES

1. Edwards R, Fishel S, Cohen J. Factors influencing the success of in vitro fertilization for alleviating human infertility. J In Vitro Fert Embryo Transf 1984;1:3–23.
2. Gardner DK, Meseguer M, Rubio C, Treff NR. Diagnosis of human preimplantation embryo viability. Hum Reprod Update 2015;21:727–47.
3. Gardner DK, Balaban B. Assessment of human embryo development using morphological criteria in an era of time-lapse, algorithms and 'OMICS': is looking good still important? Mol Hum Reprod 2016;22:704–18.
4. Payne JF, Raburn DJ, Couchman GM, Price TM, Jamison MG, Walmer DK. Relationship between pre-embryo pronuclear morphology (zygote score) and standard day 2 or 3 embryo morphology with regard to assisted reproductive technique outcomes. Fertil Steril 2005;84:900–9.
5. Montag M, Van der Ven H. German Pronuclear Morphology Study Group. Evaluation of pronuclear morphology as the only selection criterion for further embryo culture and transfer: results of a prospective multicentre study. Hum Reprod 2001;16:2384–9.
6. Weitzman VN, Schnee-Riesz J, Benadiva C, Nulsen J, Siano L, Maier D. Predictive value of embryo grading for embryos with known outcomes. Fertil Steril 2010;93:658–62.
7. Ciray HN, Karagenc L, Ulug U, Bener F, Bahceci M. Early cleavage morphology affects the quality and implantation potential of day 3 embryos. Fertil Steril 2006;85:358–65.
8. Hesters L, Prisant N, Fanchin R, Mendez Lozano DH, Feyereisen E, Frydman R, et al. Impact of early cleaved zygote morphology on embryo development and in vitro fertilization-embryo transfer outcome: a prospective study. Fertil Steril 2008;89:1677–84.
9. Pelinck MJ, Hoek A, Simons AH, Heineman MJ, van Echten-Arends J, Arts EG. Embryo quality and impact of specific embryo characteristics on ongoing implantation in unselected embryos derived from modified natural cycle in vitro fertilization. Fertil Steril 2010;94:527–34.
10. Stecher A, Vanderzwalmen P, Zintz M, Wirleitner B, Schuff M, Spitzer D, et al. Transfer of blastocysts with deviant morphological and morphokinetic parameters at early stages of in-vitro development: a case series. Reprod Biomed Online 2014;28:424–35.
11. Schultz RM. The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. Hum Reprod Update 2002;8:323–31.
12. Braude P, Bolton V, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature 1988;332:459–61.
13. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. Fertil Steril 2000;73:1155–8.
14. Balaban B, Yakin K, Urman B. Randomized comparison of two different blastocyst grading systems. Fertil Steril 2006;85:559–63.
15. Bromer JG, Ata B, Seli M, Lockwood CJ, Seli E. Preterm deliveries that result from multiple pregnancies associated with assisted reproductive technologies in the USA: a cost analysis. Curr Opin Obstet Gynecol 2011;23:168–73.
16. Wolner-Hanssen P, Rydhstroem H. Cost-effectiveness analysis of in-vitro fertilization: estimated costs per successful pregnancy after transfer of one or two embryos. Hum Reprod 1998;13:88–94.
17. Storgaard M, Loft A, Bergh C, Wennerholm UB, Soderstrom-Anttila V, Romundstad LB, et al. Obstetric and neonatal complications in pregnancies conceived after oocyte donation: a systematic review and meta-analysis. BJOG 2017;124:561–72.
18. Handyside AH, Pattinson JK, Penketh RJ, Delhanty JD, Winston RM, Tuddenham EG. Biopsy of human preimplantation embryos and sexing by DNA amplification. Lancet 1989;1:347–9.
19. Handyside AH, Kontogianni EH, Hardy K, Winston RM. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. Nature 1990;344:768–70.
20. Twisk M, Mastenbroek S, van Wely M, Heineman MJ, van der Veen F, Repping S. Preimplantation genetic screening for abnormal number of chromosomes (aneuploidies) in in vitro fertilisation or intracytoplasmic sperm injection. Cochrane Database Syst Rev 2006:CD005291.
21. Mastenbroek S, Twisk M, van Echten-Arends J, Sikkema-Raddatz B, Korevaar JC, Verhoeve HR, et al. In vitro fertilization with preimplantation genetic screening. N Engl J Med 2007;357:9–17.
22. Hardarson T, Hanson C, Lundin K, Hillensjo T, Nilsson L, Stevic J, et al. Preimplantation genetic screening in women of advanced maternal age caused a decrease in clinical pregnancy rate: a randomized controlled trial. Hum Reprod 2008;23:2806–12.
23. Chen M, Wei S, Hu J, Quan S. Can Comprehensive Chromosome Screening Technology Improve IVF/ICSI Outcomes? A Meta-Analysis. PLoS One 2015;10:e0140779.
24. Dahdouh EM, Balayla J, Garcia-Velasco JA. Comprehensive chromosome screening improves embryo selection: a meta-analysis. Fertil Steril 2015;104:1503–12.
25. Dahdouh EM, Balayla J, Garcia-Velasco JA. Impact of blastocyst biopsy and comprehensive chromosome screening technology on preimplantation genetic screening: a systematic review of randomized controlled trials. Reprod Biomed Online 2015;30:281–9.
26. Scott RT Jr, Upham KM, Forman EJ, Hong KH, Scott KL, Taylor D, et al. Blastocyst biopsy with comprehensive chromosome screening and fresh embryo transfer significantly increases in vitro fertilization implantation and delivery rates: a randomized controlled trial. Fertil Steril 2013;100:697–703.
27. Capalbo A, Ubaldi FM, Rienzi L, Scott R, Treff N. Detecting mosaicism in trophectoderm biopsies: current challenges and future possibilities. Hum Reprod 2017;32:492–8.
28. Franasiak JM, Scott RT Jr. Embryonic aneuploidy: overcoming molecular genetics challenges improves outcomes and changes practice patterns. Trends Mol Med 2014;20:499–508.
29. Scott RT Jr, Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: a randomized and paired clinical trial. Fertil Steril 2013;100:624–30.
30. Bowman P, McLaren A. Viability and growth of mouse embryos after in vitro culture and fusion. J Embryol Exp Morphol 1970;23:693–704.

31. Quinn P, Wales RG. The relationships between the ATP content of preimplantation mouse embryos and their development in vitro during culture. J Reprod Fertil 1973;35:301–9.
32. Sutton-McDowall ML, Gilchrist RB, Thompson JG. The pivotal role of glucose metabolism in determining oocyte developmental competence. Reproduction 2010;139:685–95.
33. Overstrom EW, Duby RT, Dobrinsky J, Roche JF, Boland MP. Viability and oxidative metabolism of the bovine blastocyst. Therioheology 1992;37:269.
34. Katz-Jaffe MG, Gardner DK. Symposium: innovative techniques in human embryo viability assessment. Can proteomics help to shape the future of human assisted conception? Reprod Biomed Online 2008;17:497–501.
35. Montag M, Koster M, van der Ven K, van der Ven H. Gamete competence assessment by polarizing optics in assisted reproduction. Hum Reprod Update 2011;17:654–66.
36. Scott L, Berntsen J, Davies D, Gundersen J, Hill J, Ramsing N. Symposium: innovative techniques in human embryo viability assessment. Human oocyte respiration-rate measurement–potential to improve oocyte and embryo selection? Reprod Biomed Online 2008;17:461–9.
37. Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, Humpherson PG, et al. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. Hum Reprod 2004;19:2319–24.
38. Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, Rutherford AJ, et al. Non-invasive amino acid turnover predicts human embryo developmental capacity. Hum Reprod 2002;17:999–1005.
39. Sturmey RG, Bermejo-Alvarez P, Gutierrez-Adan A, Rizos D, Leese HJ, Lonergan P. Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. Mol Reprod Dev 2010;77:285–96.
40. Warner CM, Lampton PW, Newmark JA, Cohen J. Symposium: innovative techniques in human embryo viability assessment. Soluble human leukocyte antigen-G and pregnancy success. Reprod Biomed Online 2008;17:470–85.
41. Conaghan J, Hardy K, Handyside A, Winston R, Leese HJ. Selection criteria for human embryo transfer: a comparison of pyruvate uptake and morphology. J Assist Reprod Genet 1993;10:21–30.
42. Liu Y, Copeland C, Stevens A, Feenan K, Chapple V, Myssonski K, et al. Assessment of human embryos by time-lapse videography: A comparison of quantitative and qualitative measures between two independent laboratories. Reprod Biol 2015;15:210–6.
43. Petersen BM, Boel M, Montag M, Gardner DK. Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on Day 3. Hum Reprod 2016;31:2231–44.
44. Goodman LR, Goldberg J, Falcone T, Austin C, Desai N. Does the addition of time-lapse morphokinetics in the selection of embryos for transfer improve pregnancy rates? A randomized controlled trial. Fertil Steril 2016;105:275–85.
45. Gardner DK, Leese HJ. Assessment of embryo viability prior to transfer by the noninvasive measurement of glucose uptake. J Exp Zool 1987;242:103–5.
46. Gardner DK, Lane M, Stevens J, Schoolcraft WB. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. Fertil Steril 2001;76:1175–80.
47. Menke TM, McLaren A. Mouse blastocysts grown in vivo and in vitro: carbon dioxide production and trophoblast outgrowth. J Reprod Fertil 1970;23:117–27.
48. Gardner DK. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. Theriogenology 1998;49:83–102.
49. Renard JP, Philippon A, Menezo Y. In-vitro uptake of glucose by bovine blastocysts. J Reprod Fertil 1980;58:161–4.
50. Lane M, Gardner DK. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. Hum Reprod 1996;11:1975–8.
51. Gardner DK, Wale PL, Collins R, Lane M. Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. Hum Reprod 2011;26:1981–6.
52. Urbanski JP, Johnson MT, Craig DD, Potter DL, Gardner DK, Thorsen T. Noninvasive metabolic profiling using microfluidics for analysis of single preimplantation embryos. Anal Chem 2008;80:6500–7.
53. Thouas G, Potter DL, Gardner DK. Microfluidic devices for the analysis of gametes and embryo physiology. In: . In: Gardner DK, Seli E, Wells D, Sakkas D, editors. Human gametes and preimplantation embryos: assessment and diagnosis. New York: Springer; 2013:281–99.
54. Scott R, Seli E, Miller K, Sakkas D, Scott K, Burns DH. Noninvasive metabolomic profiling of human embryo culture media using Raman spectroscopy predicts embryonic reproductive potential: a prospective blinded pilot study. Fertil Steril 2008;90:77–83.
55. Seli E, Botros L, Sakkas D, Burns DH. Noninvasive metabolomic profiling of embryo culture media using proton nuclear magnetic resonance correlates with reproductive potential of embryos in women undergoing in vitro fertilization. Fertil Steril 2008;90:2183–9.
56. Seli E, Sakkas D, Scott R, Kwok SC, Rosendahl SM, Burns DH. Noninvasive metabolomic profiling of embryo culture media using Raman and near-infrared spectroscopy correlates with reproductive potential of embryos in women undergoing in vitro fertilization. Fertil Steril 2007;88:1350–7.
57. Seli E, Vergouw CG, Morita H, Botros L, Roos P, Lambalk CB, et al. Noninvasive metabolomic profiling as an adjunct to morphology for noninvasive embryo assessment in women undergoing single embryo transfer. Fertil Steril 2010;94:535–42.
58. Vergouw CG, Botros LL, Roos P, Lens JW, Schats R, Hompes PG, et al. Metabolomic profiling by near-infrared spectroscopy as a tool to assess embryo viability: a novel, non-invasive method for embryo selection. Hum Reprod 2008;23:1499–504.
59. Vergouw CG, Kieslinger DC, Kostelijk EH, Botros LL, Schats R, Hompes PG, et al. Day 3 embryo selection by metabolomic profiling of culture medium with near-infrared spectroscopy as an adjunct to morphology: a randomized controlled trial. Hum Reprod 2012;27:2304–11.
60. Hardarson T, Ahlstrom A, Rogberg L, Botros L, Hillensjo T, Westlander G, et al. Non-invasive metabolomic profiling of Day 2 and 5 embryo culture media: A prospective randomized trial. Hum Reprod 2011;27:89–96.
61. Sfontouris I, Lainas G, Sakkas D, Iliadis G, Zorzovilis I, Petsas G, et al. Assessment of embryo selection using non-invasive metabolomic analysis as an adjunct to morphology indicates improvement in implantation with fetal cardiac activity rates. Hum Reprod 2011;26:i86.
62. Ottosen LD, Hindkjaer J, Lindenberg S, Ingerslev HJ. Murine pre-embryo oxygen consumption and developmental competence. J Assist Reprod Genet 2007;24:359–65.
63. Downes A, Mouras R, Bagnaninchi P, Elfick A. Raman spectroscopy and CARS microscopy of stem cells and their derivatives. J Raman Spectrosc 2011;42:1864–70.
64. Ami D, Mereghetti P, Natalello A, Doglia SM, Zanoni M, Redi CA, et al. FTIR spectral signatures of mouse antral oocytes: molecular markers of oocyte maturation and developmental competence. Biochim Biophys Acta 2011;1813:1220–9.
65. Stringari C, Cinquin A, Cinquin O, Digman MA, Donovan PJ, Gratton E. Phasor approach to fluorescence lifetime microscopy distinguishes different metabolic states of germ cells in a live tissue. Proc Natl Acad Sci U S A 2011;108:13582–7.
66. Gosnell ME, Anwer AG, Mahbub SB, Menon PS, Inglis DW, Adhikary PP, et al. Quantitative non-invasive cell characterisation and discrimination based on multispectral autofluorescence features. Sci Rep 2016;6:23453.
67. Chappel S. The role of mitochondria from mature oocyte to viable blastocyst. Obstet Gynecol Int 2013;2013:183024.
68. Van Blerkom J. Mitochondrial function in the human oocyte and embryo and their role in developmental competence. Mitochondrion 2011;11:797–813.
69. Dumollard R, Duchen M, Carroll J. The role of mitochondrial function in the oocyte and embryo. Curr Top Dev Biol 2007;77:21–49.
70. Van Blerkom J, Davis PW, Lee J. ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. Hum Reprod 1995;10:415–24.
71. Acton BM, Jurisicova A, Jurisica I, Casper RF. Alterations in mitochondrial membrane potential during preimplantation stages of mouse and human embryo development. Mol Hum Reprod 2004;10:23–32.

72. Komatsu K, Iwase A, Mawatari M, Wang J, Yamashita M, Kikkawa F. Mitochondrial membrane potential in 2-cell stage embryos correlates with the success of preimplantation development. Reproduction 2014;147:627–38.
73. Lane M, Gardner DK. Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. Hum Reprod 1998;13:991–7.
74. Reynier P, May-Panloup P, Chretien MF, Morgan CJ, Jean M, Savagner F, et al. Mitochondrial DNA content affects the fertilizability of human oocytes. Mol Hum Reprod 2001;7:425–9.
75. Zeng HT, Ren Z, Yeung WS, Shu YM, Xu YW, Zhuang GL, et al. Low mitochondrial DNA and ATP contents contribute to the absence of birefringent spindle imaged with PolScope in in vitro matured human oocytes. Hum Reprod 2007;22:1681–6.
76. Zander-Fox DL, Fullston T, McPherson NO, Sandeman L, Kang WX, Good SB, et al. Reduction of mitochondrial function by FCCP during mouse cleavage stage embryo culture reduces birth weight and impairs the metabolic health of offspring. Biol Reprod 2015;92:124.
77. Mayevsky A, Chance B. Oxidation-reduction states of NADH in vivo: from animals to clinical use. Mitochondrion 2007;7:330–9.
78. Lane M, Gardner DK. Mitochondrial malate-aspartate shuttle regulates mouse embryo nutrient consumption. J Biol Chem 2005;280:18361–7.
79. Mitchell M, Cashman KS, Gardner DK, Thompson JG, Lane M. Disruption of mitochondrial malate-aspartate shuttle activity in mouse blastocysts impairs viability and fetal growth. Biol Reprod 2009;80:295–301.
80. Ramanujam N. Fluorescence spectroscopy of neoplastic and non-neoplastic tissues. Neoplasia 2000;2:89–117.
81. Dumollard R, Ward Z, Carroll J, Duchen MR. Regulation of redox metabolism in the mouse oocyte and embryo. Development 2007;134:455–65.
82. Dumollard R, Carroll J, Duchen MR, Campbell K, Swann K. Mitochondrial function and redox state in mammalian embryos. Semin Cell Dev Biol 2009;20:346–53.
83. Walsh AJ, Cook RS, Manning HC, Hicks DJ, Lafontant A, Arteaga CL, et al. Optical metabolic imaging identifies glycolytic levels, subtypes, and early-treatment response in breast cancer. Cancer Res 2013;73:6164–74.
84. Quinn KP, Sridharan GV, Hayden RS, Kaplan DL, Lee K, Georgakoudi I. Quantitative metabolic imaging using endogenous fluorescence to detect stem cell differentiation. Sci Rep 2013;3:3432.
85. Reyes JM, Fermanian S, Yang F, Zhou SY, Herretes S, Murphy DB, et al. Metabolic changes in mesenchymal stem cells in osteogenic medium measured by autofluorescence spectroscopy. Stem Cells 2006;24:1213–7.
86. Renkoski TE, Banerjee B, Graves LR, Rial NS, Reid SA, Tsikitis VL, et al. Ratio images and ultraviolet C excitation in autofluorescence imaging of neoplasms of the human colon. J Biomed Opt 2013;18:16005.
87. Sutton-McDowall M, Gosnell ME, Anwer AG, White M, Purdey M, Abell AD, et al. Hyperspectral microscopy can detect metabolic heterogeneity within bovine post-compaction embryos incubated under two oxygen concentrations (7% versus 20%). Hum Reprod 2017;32:2016–25.
88. Podoleanu AG. Optical coherence tomography. J Microsc 2012;247:209–19.
89. Karnowski K, Ajduk A, Wieloch B, Tamborski S, Krawiec K, Wojtkowski M, et al. Optical coherence microscopy as a novel, non-invasive method for the 4D live imaging of early mammalian embryos. Sci Rep 2017;7:4165.
90. Berkovitz A, Eltes F, Lederman H, Peer S, Ellenbogen A, Feldberg B, et al. How to improve IVF-ICSI outcome by sperm selection. Reprod Biomed Online 2006;12:634–8.
91. Said TM, Land JA. Effects of advanced selection methods on sperm quality and ART outcome: a systematic review. Hum Reprod Update 2011;17:719–33.
92. Carrell DT. Contributions of spermatozoa to embryogenesis: assays to evaluate their genetic and epigenetic fitness. Reprod Biomed Online 2008;16:474–84.
93. Sakkas D, Ramalingam M, Garrido N, Barratt CL. Sperm selection in natural conception: what can we learn from Mother Nature to improve assisted reproduction outcomes? Hum Reprod Update 2015;21:711–26.
94. Huser T, Orme CA, Hollars CW, Corzett MH, Balhorn R. Raman spectroscopy of DNA packaging in individual human sperm cells distinguishes normal from abnormal cells. J Biophotonics 2009;2:322–32.
95. de Wagenaar B, Dekker S, de Boer HL, Bomer JG, Olthuis W, van den Berg A, et al. Towards microfluidic sperm refinement: impedance-based analysis and sorting of sperm cells. Lab Chip 2016;16:1514–22.
96. de Wagenaar B, Berendsen JT, Bomer JG, Olthuis W, van den Berg A, Segerink LI. Microfluidic single sperm entrapment and analysis. Lab Chip 2015;15:1294–301.
97. Shamonki MI, Jin H, Haimowitz Z, Liu L. Proof of concept: preimplantation genetic screening without embryo biopsy through analysis of cell-free DNA in spent embryo culture media. Fertil Steril 2016;106:1312–8.
98. Xu J, Fang R, Chen L, Chen D, Xiao JP, Yang W, et al. Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for in vitro fertilization. Proc Natl Acad Sci U S A 2016;113:11907–12.
99. Capalbo A, Ubaldi FM, Cimadomo D, Noli L, Khalaf Y, Farcomeni A, et al. MicroRNAs in spent blastocyst culture medium are derived from trophectoderm cells and can be explored for human embryo reproductive competence assessment. Fertil Steril 2016;105:225–35.
100. Hammond ER, McGillivray BC, Wicker SM, Peek JC, Shelling AN, Stone P, et al. Characterizing nuclear and mitochondrial DNA in spent embryo culture media: genetic contamination identified. Fertil Steril 2017;107:220–8.
101. Pleil JD, Angrish MM, Madden MC. Immunochemistry for high-throughput screening of human exhaled breath condensate (EBC) media: implementation of automated Quanterix SIMOA instrumentation. J Breath Res 2015;9:047108.
102. Walt DR. Optical methods for single molecule detection and analysis. Anal Chem 2013;85:1258–63.
103. Rissin DM, Kan CW, Campbell TG, Howes SC, Fournier DR, Song L, et al. Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations. Nat Biotechnol 2010;28:595–9.
104. Seidler E, Gemani D, Ocalii O, Sakkas D. Utilization of a novel ultrasensitive digital immunoassay platform to measure interleukin-6 in blastocyst culture media. Fertil Steril 2017;107:e16.
105. Ruiz-Alonso M, Blesa D, Diaz-Gimeno P, Gomez E, Fernandez-Sanchez M, Carranza F, et al. The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure. Fertil Steril 2013;100:818–24.
106. Nejat EJ, Ruiz-Alonso M, Simon C, Meier UT. Timing the window of implantation by nucleolar channel system prevalence matches the accuracy of the endometrial receptivity array. Fertil Steril 2014;102:1477–81.
107. Jenkins TG, Carrell DT. The paternal epigenome and embryogenesis: poising mechanisms for development. Asian J Androl 2011;13:76–80.
108. Pinborg A, Wennerholm UB, Romundstad LB, Loft A, Aittomaki K, Soderstrom-Anttila V, et al. Why do singletons conceived after assisted reproduction technology have adverse perinatal outcome? Systematic review and meta-analysis. Hum Reprod Update 2013;19:87–104.