# Morphological and Metabolic Assessment of Oocytes and Embryos

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## Introduction

The non-invasive assessment of preimplantation embryos has been largely limited to the use of morphology and has become the primary tool of the embryologist for selecting which embryo(s) to replace. Since the early years of in vitro fertilization (IVF) it was noted that embryos cleaving faster and those of better morphological appearance were more likely to lead to a pregnancy. Indeed, Edwards and colleagues noted only a few years after the birth of Louise Brown "that cleavage rates on a certain day and overall embryo morphology were valuable in choosing which embryo to transfer" [1]. In 1986, one of the initial large studies ($N = 1{,}539$ embryos) examining the usefulness of embryo morphology was published by Cummins et al. [2] and reported that embryo quality scores were valuable in predicting success. Indeed Cummins et al. [2] calculated an embryo development rating based on the ratio between the time at which embryos were observed at a particular stage after insemination and the time at which they would be expected to reach that stage of a hypothetical "ideal" growth rate with a cell cycle length of 11.9 h. Using this scoring system, "normally" growing embryos scored 100, however the scoring system was evidently never assessed prospectively. The following year a study by Puissant et al. [3] reported the grading of embryos based on the amount of anucleate fragments expelled during early cleavage and on developmental speed. They found that embryos endowed with a high score were more often associated with pregnancy and in particular with the occurrence of multiple pregnancy. Interestingly, they already proposed that in the event of a high score: "It might be warranted to replace only two embryos when these conditions are fulfilled." Here already, in the 1980s, the simple but important concept was introduced that identifying a better embryo will allow us to transfer fewer embryos.

In addition to the classical parameters of cell number and fragmentation, numerous other characteristics have now been examined including: pronuclear

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morphology, early cleavage to the 2-cell stage, top quality embryos on successive days and various forms of sequential assessment of embryos (see reviews by [4–6]). One could therefore make a case that morphological assessment systems have evolved over the past decade but in effect very little has changed in the way most IVF laboratories examine embryos routinely. A close examination of the original Cummins et al. [2] paper shows that we have not really progressed in our routine assessment of morphology. One significant change however has been the ability to culture and assess blastocyst stage embryos routinely and this has helped dramatically to improve the ability to select embryos on the basis of morphology [7]. The main question is however: "How far can morphological assessment of the cleavage stage embryo go in the identification of viable embryos?"

In this review the history and progress of both morphological and metabolic assessment will be examined. The review will conclude with an evaluation of where these technologies will take us in the future.

## The Changing Practice of IVF Will Challenge Classic Morphological Assessment

The drive to reduce the risk of multiple pregnancies as a consequence of IVF means that clinics around the world must transfer fewer embryos to each patient than in the past without compromising the chance of achieving a pregnancy. In order to accomplish this goal, IVF centers long used grading systems that contain semi-quantitative descriptors of the morphology of the early zygote, embryo or blastocyst (Fig. 1). Zygote grading systems evaluate pronuclear size and position, nucleoli number and distribution, and cytoplasmic appearance [8–10]. Several different criteria including the uniformity of blastomeres, percentage of fragmentation, rate of cleavage, and blastomere multinucleation are used to grade early stage cleaved embryos [10–13]. Later stage blastocyst grading systems evaluate expansion, zona thinning, and quality of the trophectoderm and inner cell mass [14, 15]. Some systems look at each stage separately and some have combined different stages and incorporate them into a "graduated" or "cumulative" embryo score [16–19].

The ultimate aim of any grading system has been to identify the zygote, embryo or blastocyst that is most likely to implant and become a healthy baby. The morphologic assessment is taken into account when deciding how many embryos to replace with the ultimate goal being a single embryo transfer (SET). Given the well-known morbidity associated with multiple pregnancy [20–22], many programs are shifting toward elective single embryo transfer (eSET). Although several countries have enacted legislation to allow the transfer of only one or two embryos [23, 24], the USA and many other countries currently have no laws to limit the number of embryos transferred but do have recommended limits issued by professional societies to encourage eSET. The American Society of Reproductive Medicine suggests that eSET is appropriate for women under age 35 with a good prognosis and a "top quality embryo" available. However, despite the recommendation the national rates

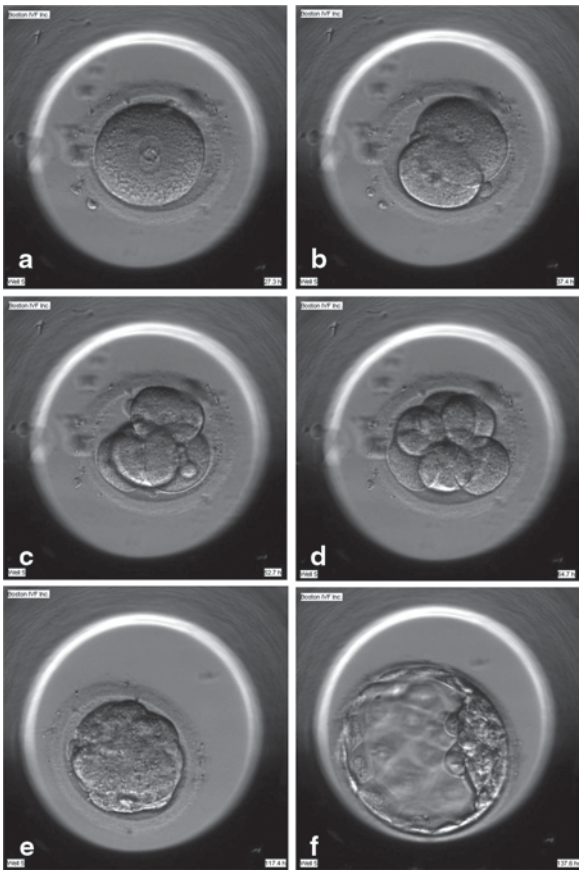


**Fig. 1** An embryo development sequence taken from a real time morphology system. The real time morphology system allows continuous monitoring of the embryo without the need of removing it from the incubator. The time after insemination is annotated on the bottom right hand corner. This series depicts an embryo that progressed to the Expanded Blastocyst stage from **a** a two pronuclear embryo, **b** a two-cell embryo, **c** a four-cell embryo with some minor fragmentation, **d** a seven-cell embryo, **e** a compacted embryo and **f** an expanding blastocyst

of eSET in the USA remain below 7%. In a positive sign the eSET rates are increasing in the younger age groups and are up to 11.2% in the less than 35 group [25]. The question remains for all IVF physicians and embryologists: What criteria do we use to help us pick the best embryo for transfer?

## *Cleavage Stage Assessment*

The usefulness of morphology has been shown numerous times. Recently, a large grading study using the Society for Assisted Reproductive Technology (SART) database found that day 3 morphology was indeed useful when correlating to live birth [26]. Relationships were identified between live birth, maternal age, and morphology of transferred day 3 embryos as defined by cell number, fragmentation, and blastomere symmetry. Logistic multiple regressions and receiver operating characteristic curve analyses were applied to determine specificity and sensitivity for correctly classifying embryos as either failures or successes. Live birth rate

was positively associated with increasing cell number up to eight cells (<6 cells: 2.9%; 6 cells: 9.6%; 7 cells: 15.5%; 8 cells: 24.3%; and >8 cells: 16.2%), but was negatively associated with maternal age, increasing fragmentation, and asymmetry scores. An area under the receiver operating curve (AUC) of 0.753 (95% confidence interval 0.740–0.766) was derived, with a sensitivity of 45.0%, a specificity of 83.2%, and 76.4% of embryos being correctly classified with a cutoff probability of 0.3.

Interestingly, when similar models were applied to some sequential scoring systems they appear to not have helped as much as expected. Models built using Day 1, 2 or 3 scores independently on the re-sampled data sets showed that Day 1 evaluations provided the poorest predictive value (median AUC = 0.683 versus 0.729 and 0.725, for Day 2 and 3). Combining information from Day 1, 2 and 3 marginally improved discrimination (median AUC = 0.737). Using the final Day 3 model fitted on the whole dataset, the median AUC was 0.732 (95% CI, 0.700–0.764), and 68.6% of embryos would be correctly classified with a cutoff probability equal to 0.3. The authors concluded that Day 2 or Day 3 evaluations alone are sufficient for morphological selection of cleavage stage embryos. The derived regression coefficients can be used prospectively in an algorithm to rank embryos for selection. It could be argued however that when static morphological systems are challenged with SET they will struggle to be as predictive. The usefulness of some sequential systems has been shown by Belgian groups which developed characteristics that constituted a "top quality" embryo [19–22] and showed improvement when transferring one embryo only.

The most impressive static morphology based selection results have been reported using the blastocyst scoring systems. A number of these have been developed but the most widely used system is that referred to as the Gardner Blastocyst Alphanumeric Scoring System.

## *The Blastocyst*

It could be argued that the best static morphological selection tool available to us has been right under our noses all along [27–29]. For an embryo to form a blastocyst in culture it has already been challenged by the in vitro environment and is also a complete expression of the embryos ability to develop distinct tissue types and proceed through embryonic genome activation, reflecting both maternal and paternal genome expression. Selection of embryos up to the 8-cell stage is not always reflective of these challenges. In contrast, many laboratories argue that it is difficult to culture embryos to the blastocyst stage and also many patients fail to have blastocysts for transfer. This however is not the experience of all laboratories as demonstrated by Marek et al. [30] whereby the cancellation rates for transfer after retrieval for day 3 compared with day 5 transfer were 2.9 vs 6.7%, respectively. Another program also found that there was no difference in the percent of patients not having

an embryo transfer on day-5 (2.8%) compared to day-3 (1.3%) [31]. Both studies, more than 10 years ago, concluded that using extended embryo culture in a nonselective manner for couples undergoing IVF was feasible.

A propensity of studies has shown that blastocyst transfer is more successful than transfer of cleavage stage embryos. The most recent Cochrane Data base analysis [32, 33] has shown that there was evidence of a significant difference in implantation rate and live birth rate per couple favoring blastocyst culture. The most recent report showed that in 1510 women the Live Birth Rate was 31% for Day 2–3 and 38.8% for Day 5–6. Although this report did not show a difference in cumulative pregnancy rates, it would be expected that more up to date data will also lead to improvements in cryopreservation of blastocysts as more vitrification data is published [34]. This data indicates that vitrified blastocysts are virtually equal to fresh blastocysts in their viability [34].

In order to select the best blastocyst for transfer, in humans, three morphological parameters have routinely been used, i.e. degree of blastocoele expansion and appearance of both the trophectoderm (TE) and the inner cell mass (ICM) (Fig. 1). Although it has been shown that blastocysts with highest scores for all three parameters achieve highest implantation rates, their independent ability to predict pregnancy outcome has recently come under scrutiny. Ahlstrom et al. [35] performed a retrospective analysis of 1117 fresh day 5 single blastocyst transfers and examined the live birth outcome related to each morphological parameter. Whereas all three morphological characteristics had a significant effect on live birth however, once adjusted for known significant confounders, it was shown that TE was the only statistically significant independent predictor of live birth outcome. They concluded that a strong TE layer is essential at this stage of embryo development, allowing successful hatching and implantation. The final barrier to performing routine blastocyst culture was the ability to cryopreserve them successfully. This has now been put to rest with the success of vitrification where success rates are being reported equivalent to fresh transfers [34]. The added benefit may also be that transferring on frozen cycles, as compared to fresh stimulated cycles, may convey further benefits to the developing fetus such as improved weight at live birth [36].

## Real Time Morphology

Since the late 1980s numerous groups examined the possibility of time lapse video imaging of embryos. Indeed Cohen and colleagues published a number of studies on the prognostic value of morphologic characteristics of cryopreserved embryos [37, 38], while Payne et al. [39] used video imaging to examine polar body extrusion and pronuclear formation. Hardarson et al. [40] also used video imaging to observe the internalization of cellular fragments in a human embryo. More recently, Lemmen et al. [41] used time lapse recordings to examine kinetic markers of human embryo quality in particular when cleaving from the 1 to 2 cell stage. A number

of commercial time lapse systems are now on the market or being developed for the market, including the Embryoscope, Auxogyn and Primovision. One system (The Embryoscope$^{TM}$) is a combined incubator and time-lapse system and has had numerous publications indicating an equivalent or elevated clinical pregnancy rate, which was attributed to a combination of stable culture conditions and the use of morphokinetic parameters for embryo selection [42]. The time lapse system produces high quality videos with the capability of annotating each individual embryo (Fig. 1). This time lapse system looks extremely promising and some algorithms have already been developed that claim to improve pregnancy rates [43]. Interestingly the algorithms rely more on de-selecting embryos that cleave abnormally than proactively selecting the best embryo. A second system has also been developed which aims to assist in the early prediction of which embryo will form a blastocyst [44]. These authors have published mouse data indicating progression to the blastocyst stage can be predicted with > 93 % sensitivity and specificity by measuring three dynamic, noninvasive imaging parameters by day 2 after fertilization, before embryonic genome activation. They have now also showed predictability with Human euploid embryos using similar strategies [45]. None of the time lapse systems have however undergone a rigorous clinical trial as yet to show whether they provide an overall benefit for improving single embryo transfer pregnancy rates. This data is eagerly anticipated. The real time imaging systems could however provide other benefits including the ability to monitor the embryos without removing them from the incubator. This more stable and consistent culture will limit changes in temperature and pH that the embryo experiences when being manipulated and examined outside the incubator.

## Embryo Metabolism as a Means of Assessing Viability

### *Glucose*

In 1980, Renard et al. [46], observed that Day 10 cattle blastocysts which had an elevated glucose uptake developed better, both in culture and in vivo after transfer than those blastocysts with a lower glucose uptake. Numerous studies have since validated this original observation in different species. In 1987, using non-invasive microfluorescence, Gardner and Leese [47] measured glucose uptake by individual Day-4 mouse blastocysts prior to transfer to recipient females. Those embryos that went to term had a significantly higher glucose uptake in culture than those embryos that failed to develop after transfer. Similar studies have validated this technology and shown that the glycolytic rate of mouse blastocysts could also be used to select embryos for transfer prospectively [48]. Interestingly this study only examined morphologically identical mouse blastocysts with equivalent diameters and rated them according to metabolic criteria, as either “viable” or “non viable” prior to transfer. Those selected as viable had a fetal development of 80 % while embryos

that exhibited an abnormal metabolic profile (compared to in vivo developed controls), developed at a rate of only 6%. Clearly, this data provides unequivocal evidence that glucose metabolism is linked to embryo viability.

Recently, Gardner et al. [49] determined that glucose consumption on Day 4 by human embryos was twice as high in those embryos that went on to form blastocysts. They also found that blastocyst quality affected glucose uptake. Poor quality blastocysts consumed significantly less glucose than top scoring embryos. In studies on amino acid turnover by human embryos, Houghton et al., [50] determined that alanine release into the surrounding medium on Day 2 and Day 3 was highest in those embryos that did not form blastocysts. Brison et al. [51] have reported that changes in concentration of amino acids in the spent medium of human zygotes cultured for 24 h in an embryo culture medium containing a mixture of amino acids using High Performance Liquid Chromotography. They found that asparagine, glycine and leucine were all significantly associated with clinical pregnancy and live birth. Unfortunately we are still waiting for an easy to use methodology to assess these parameters. The studies performed on nutrient uptake and the subsequent viability of the human embryo have all used techniques that are still difficult to use routinely. The problem of adapting more difficult laboratory techniques to measure metabolism has led to the question of how else can the metabolic profile of an embryo be investigated?

Another approach that has been examined is one that performs a more systematic analysis of the inventory of metabolites that are present in the media an embryo is cultured in. One drawback of using this approach is that one needs to create an algorithm that relates to embryo function, whereas the other approach relies more on a candidate metabolite assessment.

## *Metabolomics*

The complete array of small-molecule metabolites that are found within a biological system constitutes the metabolome and reflects the functional phenotype [52]. Metabolomics, is the systematic study of this dynamic inventory of metabolites, as small molecular biomarkers representing the functional phenotype in a biological system. Using various forms of spectral and analytical approaches, metabolomics attempts to determine metabolites associated with physiologic and pathologic states [53]. As has been observed with the examination of individual metabolites such as glucose, investigation of the metabolome of embryos, as detected in the culture media they grow in, using targeted spectroscopic analysis and bioinformatics has also shown differences in viability of embryos. In an initial proof of principle study Seli et al. [54] established that these differences are detectable in the culture media using both Raman and Near Infrared (NIR) spectroscopy. Briefly, a statistical formula was used to assign a relative "embryo viability score"—equating to embryo reproductive potential—and it was found that this score correlated to positive or negative implantation outcomes. Interestingly when human embryos of similar morphology

**Table 1** Studies examining the clinical utility of the Near Infra Red (NIR) spectrometry system indicated that although some ability was evident in improving pregnancy results it was not consistent enough. The Hardarson study examined both Day 2 and 5 single embryo transfers, the Vergouw study examined Day 3 single embryo transfers while the final two studies combined different days of transfer

| Type of NIR instrument | Study type | Outcome | Morphology | Morphology plus viametrics (NIR) |
|---|---|---|---|---|
| Prototype Hardarson et al. [61] | Single embryo transfer | Live birth rate | Day 2: 22/83 (26.5%) | Day 2: 27/87 (31.0%) |
| | – | – | Day 5: 36/80 (45.0%) | Day 5: 30/77 (39.0%) |
| Prototype Vergouw et al. [60] | Single embryo transfer | Live birth rate | Day 3: 68/163 (41.7%) | Day 3: 61/146 (41.8%) |
| Commercial Economou et al. (*Unpublished*) | Double embryo transfer | Clinical pregnancy rate | 8/28 (29%) | 16/28 (57%) |
| Commercial Sfontouris et al. (62) | Multiple embryo transfer | Clinical pregnancy implantation rate | 41/86 (47.7%) 66/257 (25.7%) | 21/39 (53.9%) 35/102 (34.3%) |

were examined using the same NIR spectral profile their viability scores varied remarkably in relation to morphology indicating that the metabolome of embryos that look similar differ significantly [55, 56].

Although numerous preliminary studies [55–59] showed a benefit of this technique they were largely based on retrospective studies and performed in a single research laboratory as distinct from a real clinical setting. Recently, a number of clinical studies have been reported using either a prototype or commercial version of the Molecular Biometrics Inc. NIR system showing inconsistent results (Table 1). The largest of these studies were performed as Randomized Clinical Trials after SET [60, 61]. All studies compared standard Morphological techniques for embryo selection versus using the NIR system to rank embryos within a cohort that had good morphology and were being selected for either transfer or cryopreservation. In the Gothenburg study [61] both day 2 and day 5 SETs were included. Although not significant, the results indicated a possible benefit of embryo selection through addition of NIR on day 2 transfer. However it failed to show any benefit for selection of day 5 SET. Interestingly, the benefits of selecting a single good quality blastocyst on day 5 have also been found to be beneficial in many other studies.

One of the underlying problems encountered in the NIR system was that the threshold of signal distinguishing between a viable and non-viable embryo was susceptible to signal noise. As a consequence this method, that had been established and cross-validated on a larger scale, proved problematic because of the technical platform itself. This was not dissimilar to the situation faced by aneuploidy screening of embryos, whereby using FISH has proved to be inadequate [63] while it now appears that modern comprehensive screening techniques are providing more consistent results [64].

It is beyond question that markers do exist in the spent embryo culture media indicative of viability. The major benefits of a non invasive type of technology is the fact that the technology can be used on spent media and the time taken to assess

the samples is very short, making it possible to perform the analysis just prior to ET. So far NIR spectroscopy, when tested in stringent clinical trials, does not appear to consistently improve the chance of selecting a single embryo for a viable pregnancy and these types of technology appear to need further development before being used as an objective marker of embryo viability.

### *Oxygen and Reactive Oxygen Species*

Other techniques have also been reported to measure metabolic parameters in culture media; however, they have yet to be diligently tested in a clinical IVF setting. These include the self-referencing electrophysiological technique, which is a non-invasive measurement of the physiology of individual cells and monitors the movement of ions and molecules between the cell and the surrounding media [65, 66]. An alternative approach measures oxygen consumption of developing embryos using a microsensor system. Interestingly, although this technology has been shown to correlate with bovine blastocyst development, it was less successful in predicting mouse embryo development [67, 68]. A more recent study has shown some benefits by examining oxygen consumption from individual embryos close to the time of transfer and showing that the oxygen consumption pattern was associated with successful implantation [69].

Some emphasis is now being placed on the relationship between reactive oxygen species (ROS) levels in culture media and the outcome of IVF cycles. This idea was first introduced by Nasr-Esfahani and Johnson in 1990 as an explanation of abnormal development of mouse embryos in vitro. In the human, a study by Bedaiwy et al. [70] has shown that increasing levels of ROS generation in Day 3 in vitro embryo culture media may have a detrimental effect on in vitro embryo growth parameters, as well as clinical pregnancy rates in IVF and ICSI cycles.

## Conclusion

Analysis of embryo morphology and the development of suitable grading systems have greatly assisted in the selection of human embryos for transfer. We are however fast approaching a revolution in the way we assess embryos prior to transfer. It is highly likely that all IVF laboratories will contain some type of real time imaging system in the future which will allow both assessment of morphology and the ability to retain embryos in a constant temperature and pH without moving them for assessment. As a significant adjunct to morphology we will be using either non-invasive and/or invasive methods more routinely to help in selecting which single embryo to transfer and cryopreserve. The non-invasive analysis of embryo physiology and function using metabolic parameters will definitely be one tool that will allow us to better quantify embryo viability. The addition of such technologies will be of immense value in helping both clinicians and embryologists to more confidently select the most viable single embryo within a cohort, helping us reach the goal of all our patients to achieve pregnancy.

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