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REVIEW

New approaches to embryo selection

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Markus Montag obtained his PhD at the German Cancer Research Centre and worked as a post-doc at NUH, Singapore with Professor SC Ng, the 'father' of the first subzonal insemination baby. Returning from Singapore, he worked as laboratory director in a private IVF unit before he became Director of the Reproductive Biology Laboratory at the University Clinics of Bonn in 1995. In 2011 he took over the position of Director of the Reproductive Biology Laboratory at the University Clinics of Heidelberg. He is a co-founder of the fertility preservation network FertiProtekt and started one of the largest cryobanks for ovarian tissue. He is actively involved in counselling IVF centres all over the world and in education of young people in the field. His main research areas include the use of lasers in assisted reproduction, cryobanking of ovarian tissue, oocyte activation, polarization microscopy, polar body biopsy and time-lapse imaging.

Abstract Embryo selection has been an important topic since the introduction of assisted reproduction, with embryo morphology being the most obvious criterion. Although morphology serves as indicator for overall IVF laboratory quality, its statistical assessment limits the possibility to identify the most implantation-competent embryos. In order to reach a direct picture of the developing embryo, invasive procedures such as preimplantation genetic screening or transcriptome and proteome analysis of biopsied embryonic tissue were initially prioritized and are still under investigation. More recently, focus has shifted towards noninvasive techniques that maintain the integrity of the embryo. Metabolomic profiling of culture medium from growing embryos attracted much research. Although successful in a pilot study, that approach failed in a randomized controlled trial. Other metabolomics studies are on their way but not yet available for routine clinical use. The most promising strategy at present is the combined evaluation of morphology and developmental kinetics using time-lapse imaging. This has brought new insights into certain characteristics that enable deselection of embryos at an early stage of development and to identify others with high potential for successful implantation. However, there is still considerable room for improvement. Further strategies will most likely involve the combination of several different approaches.

KEYWORDS: embryo selection, morphokinetics, morphology, noninvasive, time-lapse imaging

Introduction

Identification of the embryo with the highest potential to implant and establish an ongoing pregnancy is a primary aim in human assisted reproduction. This task is undertaken every day by embryologists worldwide during the treatment of couples that wish to conceive by IVF. The optimal scenario is the transfer of a single embryo which gives rise to a singleton pregnancy. However, the mean implantation rate of an individual embryo after transfer for women below the age of 35 in the USA was 36.8% in 2010 (Cohen et al., 2012), meaning that even in this patient group with a good prognosis, the majority of embryos transferred will not succeed. This success rate is far below the expectations of couples seeking treatment. Consequently, transferring more than one embryo to enhance the chances of pregnancy is routine in most IVF clinics. This has the consequence of producing a high rate of twins born after assisted reproductive treatment, with all the attendant risks that this entails. Hence embryo selection has been a 'hot topic' of investigation almost since the beginning of the era of IVF. Significant progress has been made in recent years and this article gives an overview of current embryo selection strategies and their

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clinical usefulness particularly to enhance successful single-embryo transfer.

Strategies for embryo selection

Embryo selection is based on methods that can give a direct or indirect clue regarding the potential of a given embryo to implant. These methodologies are based on either invasive or noninvasive procedures that are applied at various stages of development from the oocyte to cleavage-stage embryos and up to the blastocyst stage (**Table 1**).

Invasive embryo selection

Invasive procedures do require the removal of a compartment of the oocyte or the embryo for further analysis. The most commonly applied invasive test is preimplantation genetic screening (PGS) of the chromosomal constitution of polar bodies, blastomeres or trophectoderm cells (Goossens et al., 2012; Harper et al., 2012). However, there is still an ongoing debate if the patient may benefit from PGS and which methodology should be applied (Geraedts et al., 2011), although results from a recently published randomized pilot study revealed a benefit for single-embryo transfer after trophectoderm biopsy and array comparative genomic hybridization (Yang et al., 2012). Other genetic investigations focus on the presence of chromosome instability and especially copy number variations (Vanneste et al., 2012). Although these data provide new insight into fundamental biology, the clinical value in assisted reproduction is not yet clear. As embryo selection based on genetic analysis is a major topic in its own right, it will not be discussed within this article.

Other invasive methods include the investigation of the transcriptome of polar bodies (Reich et al., 2011), oocytes and blastocysts (Kakourou et al., 2013) and reveal large numbers of transcripts, many with unknown function. Although one has to acknowledge that these studies will help to better understand the orchestration of embryo development, their overall impact is in basic research and they are currently of limited use in patient treatment.

The most critical aspect of invasive studies is the potential impact of biopsy and manipulation on subsequent embryo development. The removal of a single blastomere does negatively influence further embryo development and blastocyst formation as shown by a recent time-lapse study (Kirkegaard et al., 2012a). In view of this, the positive effect gained by analysis of a prognostic marker may be counterbalanced by the negative effect of biopsy and blastomere removal. Moving towards the trophectoderm biopsy may overcome that issue provided that diagnosis is accomplished within a reasonable time to allow for a fresh transfer preferably on day 5. However, apart from this, the investigation of the transcriptome or proteome of trophectoderm cells may simply not give conclusive information regarding the inner cell mass that is in fact the origin of the fetus.

Table 1 Overview of embryo selection strategies.

Method	*Nature*	*Applicability*	*Benefit regarding pregnancy rates*	*Reference*
PGS				
Polar bodies	Invasive	Selected patients only	Not yet proven	Geraedts et al. (2011)
Blastomeres	Invasive	Selected patients only	No, biopsy impacts embryo development	Geraedts et al. (2011)
Trophectoderm	Invasive	Selected patients only	Selected patients only	Yang et al. (2012)
Transcriptomics				
Blastomeres	Invasive	Experimental	Not yet proven	Kakourou et al. (2013)
Granulosa cells	Noninvasive	Experimental	Not yet proven	Hamel et al. (2008)
Cumulus cells	Noninvasive	Experimental	Not yet proven	Assou et al. (2010)
Embryo morphology	Noninvasive	Standard procedure in embryology	Proven, but with limitations	Montag et al. (2011) ESHRE (2012)
Blastocyst culture	Noninvasive	Established in most laboratories	Proven, but with limitations	Cruz et al. (2012) Glujovsky et al. (2012)
Metabolomics	Noninvasive	Experimental	No, according to one RCT	Hardarson et al. (2012)
Oxygen measurement	Noninvasive	Experimental	Not yet proven	Tejera et al. (2012)
Time-lapse monitoring	Noninvasive	Clinically applied	Highly promising; but requires confirmation	Meseguer et al. (2012)

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Noninvasive embryo selection

In view of the shortcomings of invasive embryo selection, the use of noninvasive selection methods seems a better strategy towards identification of potential embryos without the risk of possible impacts due to the investigation itself.

Morphology: appearance can deceive us

The most obvious parameter that can easily be assessed from the oocyte up to the blastocyst stage is morphology. Consequently, morphology was the first parameter assessed in detail in the early days of IVF and numerous studies have linked oocyte and embryo morphology to success rates. However, the moderate increase in pregnancy rates over the last two decades (Cohen et al., 2012) is most likely due to better laboratory practice and not to the use of morphology-based selection. Embryologists from ALPHA and European Society of Human Reproduction and Embryology (ESHRE) have recently elaborated some of the pitfalls of morphology to grade oocytes and embryos at a consensus meeting at Istanbul (ALPHA and ESHRE, 2011a,b). According to that consensus paper, the only morphological parameter relevant at the oocyte stage is the presence of smooth endoplasmic reticulum (ALPHA and ESHRE, 2011a,b; Otsuki et al., 2004). Smooth endoplasmic reticulum has been shown to result in poor pregnancy outcome expressed by high rates of biochemical pregnancy losses and early miscarriage (Otsuki et al., 2004).

For morphology-based selection at cleavage stages, a major obstacle that has been identified is variation in the practice in the embryology laboratory. Morphological assessment is not always done at constant time points and scoring embryo quality is highly prone to subjectivity. Established embryo scoring systems are relatively crude, being based on the number of blastomeres and embryo quality (Steer et al., 1992). But variations in the timing of assessment on day 2 may reveal embryos still at the 2-cell stage early on day 2 or already at the 4-cell stage if assessed later. This subjectivity in judgment of embryo quality can result in highly variable embryo scores. The difficulty of the morphological approach becomes even more apparent while looking at examples of the variety in morphological appearance of oocytes, zygotes and embryos that were recently published in an atlas of human embryos (ESHRE, 2012). Furthermore morphological appearance changes rapidly at times of cleavage, which may be relevant for day-2 and day-3 evaluations (Montag et al., 2011) and can result in a classification that is not objectively correct. In addition, morphologically variable embryos may nonetheless go on to implant successfully.

Culture selection: survival of the fittest!

The selection of viable embryos by extended culture up to the blastocyst stage seems to be another useful approach, since many embryos undergo developmental arrest at early stages. Blastocyst culture has been widely used since the turn of the century following the discussion on the composition of culture media and their use in a sequential or single step approach (Gardner and Lane, 1998; Summers et al., 2000). However, while it seems obvious on how to score early cleavage-stage embryos, blastocysts look much more diverse and several different scoring systems have been proposed (Dokras et al., 1993; Gardner et al., 2000; Kovacic et al., 2004).

Extended culture to day 5 has been subjected to meta-analysis, and the evolution of blastocyst transfer is well covered by the Cochran database from 2005 to 2012. The 2005 evaluation found a significant benefit of blastocyst culture and transfer regarding pregnancy and live birth rates (Blake et al., 2005). In 2007 this statement was only valid for good prognosis patients (Blake et al., 2007), and the most recent report (Cruz et al., 2012; Glujovsky et al., 2012) found a slight benefit for blastocyst culture but looking at cumulative pregnancy rates, day-2/3 transfers were far more efficient. It may well be that such an evaluation overlooks the total number of children born, as one would assume that transfer of two blastocysts on day 5 may result in higher twin rates compared with the transfer of two embryos on day 2/3. Therefore a new discussion has started on the potential of day-5 culture allowing for single-embryo transfer in combination with vitrification of surplus blastocysts. However, this approach has not been investigated in large cohorts nor has the effect on cumulative live birth rates been ascertained.

Consequently the use of blastocyst culture as a standard treatment option for all patients is still under discussion, although combining extended culture with other diagnostic tests in order to achieve better results or to enable selection of a single embryo for transfer have been proposed (Seli et al., 2010).

Analysis of follicle vascularity and follicular fluids

Predicting embryo quality on the base of follicular characteristics attracted much interest. Three-dimensional ultrasonography and power Doppler angiography were used to assess follicular vascularity but the results were too heterogeneous to establish a robust model applicable in clinical practice (Mercé et al., 2006). Likewise the composition of the follicular fluid regarding various substances has been investigated in relation to pregnancy outcome (Andersen, 1993; Lédée et al., 2011; Shaikly et al., 2008). Although some biochemical markers are still under investigation, none of them is used routinely in the clinic.

Gene expression of cumulus cells: an indirect approach

Studies on gene expression profiling in mural granulosa and cumulus cells are among the technologies that are highly promising (Hamel et al., 2008). The transcriptome of these cells can serve as a biomarker for oocyte competence (Ouandaogo et al., 2011) and does indirectly allow predicting the viability and quality of the resulting embryo (Assou et al., 2010; van Montfoort et al., 2008). Analysis of transcriptional networks has already identified key regulator genes (Assidi et al., 2011). These approaches enable a search for the best prognostic strategy to use for embryo selection — either by focusing on selected target genes or by looking at the whole cumulus cell transcriptome. However, at present, the time has not yet come to apply this technology in a standard IVF programmes. Barriers to imple-

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mentation include the lack of a ready-to-use devices giving consistent results with suggested marker genes is still lacking and because the costs are still prohibitive.

Metabolomics: hope and reality

The development of sequential culture media for blastocyst culture focused awareness on the composition of culture media and the requirements of cleavage-stage embryos. It was then only a short step to determine what embryos utilize from and release into the medium during their growth to see if there is a correlation with implantation (Gardner et al., 2001).

These investigations were focused on known candidates such as pyruvate, glucose or amino acids (Gardner et al., 2012; Leese, 2012) that were analysed in spent culture medium from embryos cultured individually. It was found that pyruvate as well as glucose consumption correlate with developmental competence of mouse, bovine and human embryos (Gardner et al., 2001; Lane and Gardner, 1996; Renard et al., 1980). Similar results were reported using changes in amino acid profiles in culture media from embryos (Leese, 2012). However, both tests also have other potential uses. For example, glucose consumption of embryos after compaction does allow prediction of the sex of an embryo and even live birth (Gardner et al., 2011) and amino acid profiling has been used for identification of aneuploidy embryos (Picton et al., 2010). Although these test procedures are of proven prognostic value, their applicability in the clinical routine is at present not warranted due to the technical complexity of the diagnosis and the need for adequate equipment with the capacity for high sample throughput (Sturmey et al., 2010).

The technologies presented so far were based on known target molecules; another approach investigated the overall change of the presence of functional groups in culture media. Functional groups are a characteristic of most molecules with metabolic activity. Therefore, such an approach identifies a variety of changes at the same time instead of investigating defined target molecules. The use of techniques such as near infrared spectroscopy, derived from organic chemistry, is based on comparing the absorption spectra of spent media from cultured embryos versus control media and resulted in the calculation of an algorithm giving a so-called viability score (Botros et al., 2008; Nagy et al., 2008). Initial pilot studies showed a significant correlation between the viability score and the implantation rate of human embryos (Ahlstrom et al., 2011). However, a randomized follow-up study was terminated prematurely after an interim analysis showed no significant difference between the control group and the study group (Hardarson et al., 2012). It is widely believed that the study suffered from a methodological bias due to differences in the set up of the pilot study and the follow-up study. Given this, the underlying principles and concepts are probably still valid; however, there is no commercial device that can yet be used clinically in the USA, although they are under development.

Oxygen respiration measurements

Oxygen is a biomarker that has been investigated in human oocytes and blastocysts as long ago as 1986 using micro-spectrophotometry (Magnusson et al., 1986). It took another 15–20 years until direct measurement of the oxygen concentration in the culture well was possible. Initial studies analysing the respiration pattern of bovine oocytes and embryos (Lopes et al., 2007) prepared the way for comparable investigations on human oocytes and embryos (Scott et al., 2008). Results of these studies revealed the potential of respiration measurement to detect oocyte competence as well as to differentiate in later developmental stages between embryos having the capacity to grow to blastocysts (Tejera et al., 2011, 2012).

However, although the results presented from all experimental studies so far were promising, the technology never entered clinical routine due to the risk of cross-contamination that was inevitably associated with the use of a single oxygen electrode for all individually cultured embryos.

Interestingly, the first device that was initially developed for oxygen respiration measurements of oocytes and embryos already enabled time-lapse imaging (Scott and Ramsing, 2007) and was later modified into a routine stand-alone time-lapse imaging system.

Time-lapse imaging: the breakthrough?

Scoring embryos by morphology has been adapted to the daily routine in the IVF laboratory and is driven by two considerations: (i) to reduce the disturbance of embryo culture as much as possible, which results in assessing embryos only once per day while some laboratories even skip embryo assessment on day 2 or day 4; and (ii) to check embryo development at a time slot that fits to the workload of the laboratory. This approach is not conducive to reproducible analysis and developmental time-points for all embryos from different patients.

Embryo development is driven by the kinetics of the cell cycle and by fundamental molecular and cellular mechanisms. It is likely that static observation is incapable of giving a true reflection of the dynamic process of embryonic growth and some important events may be missed or overlooked. The solution to this is time-lapse imaging of embryo development by remote viewing into the culture dish while the embryo remains undisturbed in the incubator. Several experimental and laboratory-specific time-lapse approaches have been developed (Adachi et al., 2005; Lemmen et al., 2008; Mio and Maeda, 2008). However, using this methodology as a general tool requires robust imaging technology in combination with specifically adjusted incubation conditions.

This became possible with the recent introduction of commercial time-lapse systems (Pribenszky et al., 2010; Scott and Ramsing, 2007). Culture by incubation in a closed time-lapse imaging system does not harm embryos compared with the traditional standard incubation approach and results in similar overall embryo development (Kirkegaard et al., 2012b). However, as time-lapse imaging allows following changes of morphology over time it has enabled the exploration of completely new embryo grading strategies. Coupled with sophisticated image analysis algorithms, specific aspects of embryo development have been found to relate to the ability of an embryo to implant. This has led to the possibility to deselect embryos with a low or close to zero chance of developing to the blastocyst stage or to

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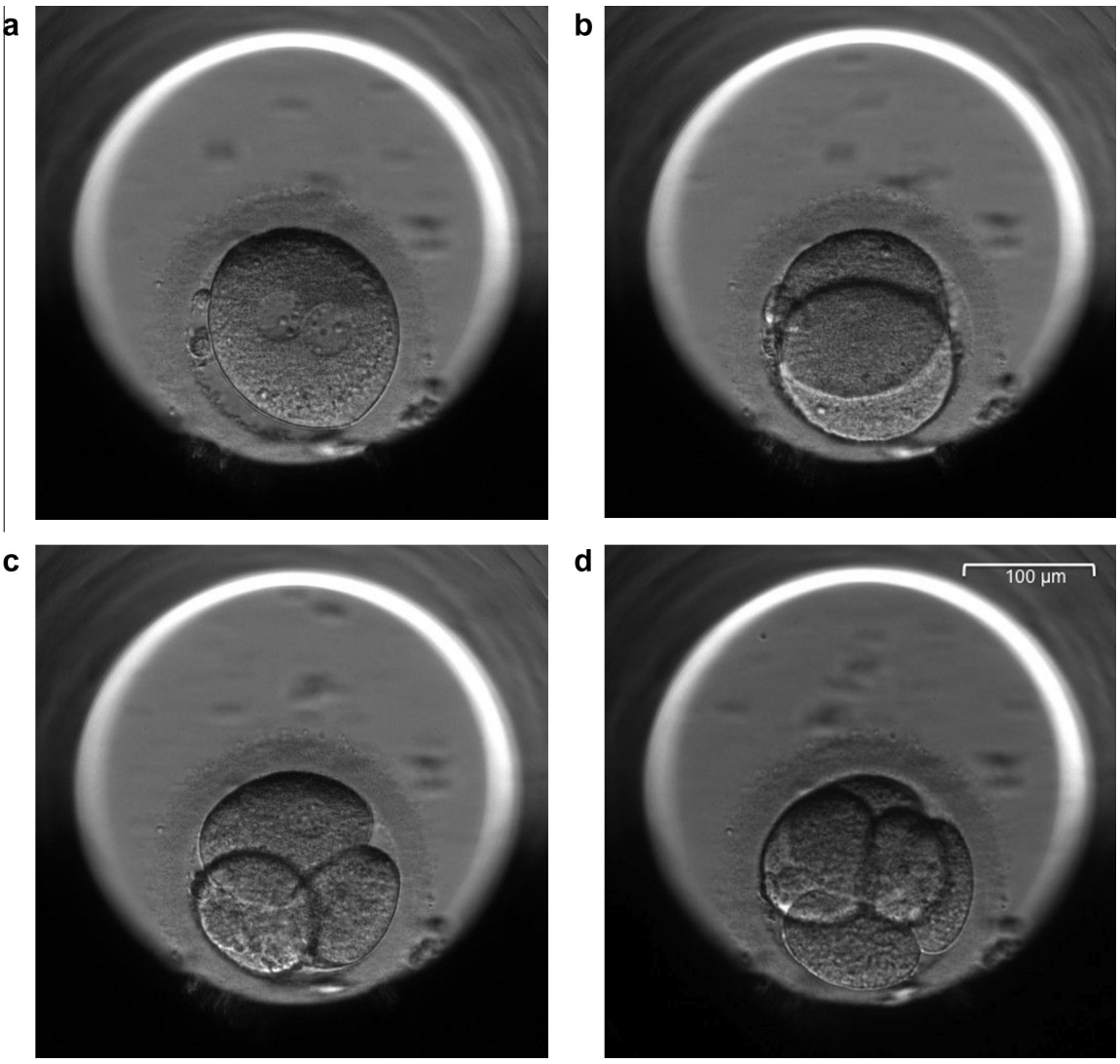

Figure 1 Time-lapsed based deselection of embryos: direct cleavage. An embryo is shown at the 2PN stage at 23.1 h post intracytoplasmic sperm injection (ICSI) (A). This cell showed direct cleavage as it divided after completion of the first cleavage at 27.1 h post ICSI (B) and within less than 5 h from 2 to 3 cells (31.5 h; C). Interestingly the upper blastomere present at the 3-cell stage cleaved directly into 3 cells giving a 5-cell embryo at 38.5 h after ICSI (D). Based on current literature and irrespective of further development, this embryo has a chance to implant that is far below 2% (Rubio et al., 2012). The individual pictures were taken from a time-lapse imaging sequence recorded in a time-lapse monitoring and incubation system (EmbryoScope, Unisense FertiliTech, Aarhus, Denmark).

implant. Predictive parameters include direct cleavage from the 1- to 3-cell stage or the 2- to 5-cell stage within less than 5 h (Rubio et al., 2012) (**Figure 1**). Furthermore, the synchrony of cleavage cycles seems to be predictive for blastocyst development (Cruz et al., 2012; Wong et al., 2010) (**Figure 2**). These and other findings have opened the possibility for the development of selection algorithms that may allow the identification of embryos with a high implantation potential (Meseguer, Herrero et al., 2011).

A recent multicentre study has shown that applying time-lapse imaging selection strategies enabled a relative increase of the pregnancy rate of 20% (Meseguer et al., 2012). Summarizing these data, it seems as if morphokinetics by time-lapse imaging is at present the only biomarker that holds a true potential to be of clinical value for selecting embryos.

Most of the clinical results on time-lapse imaging were derived by the use of one integrated system and the encouraging data reported so far need to be confirmed by other studies. However, while assessing the benefit of a specific technology in embryology, the fact that a number of independent publications have reached similar conclusions is a good indicator for the prospects of this approach. In view of this, time-lapse imaging has definitely entered the field of embryology and is probably the most potent and influential technology for objective embryo selection. It also offers the potential for studying dynamic aspects of the process of embryo attachment itself using in-vitro models, as discussed by Grewal et al. (2008) and Weimar et al. (2013).

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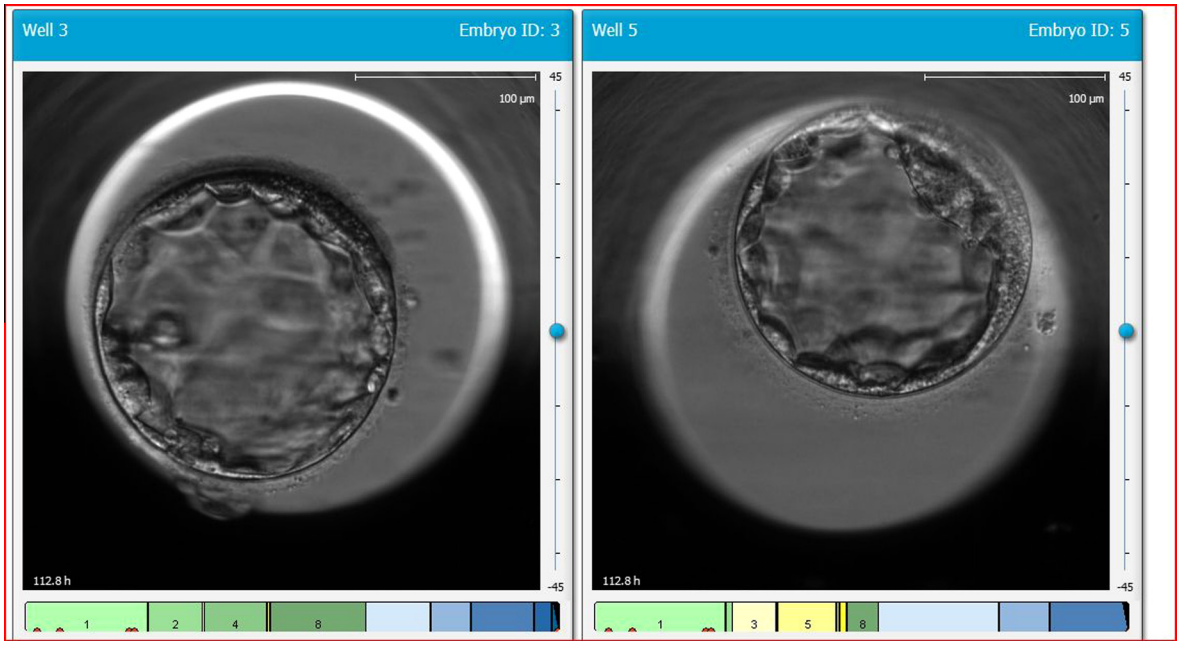

Figure 2 Detection of cleavage cycle asynchrony by time-lapse imaging. Two blastocysts are shown at 112.8 h post ICSI (A, B). Embryo development was followed closely by time-lapse imaging. Bars below the images depict the timeline for embryo development and numbers (1, 2, 4 or 8) indicate the embryonic cell stage. Stages with an extended duration due to DNA replication (1, 2, 4 or 8 cells) are shown in shades of green and transient stages (3, 5, 6 or 7 cells) are shown in yellow. Stages of compaction and blastulation are indicated in blue. Red dots denote from left to right: extrusion of polar body 2, pronuclear appearance and pronuclear fading. Time-lapse monitoring of the embryo on the left revealed synchronous division cycles from the 2- to 4-cell stage and 4- to 8-cell stage. In contrast, the embryo to the right immediately divided from the 2- to the 3-cell stage and spent a long time in the 3- to 5-cell stage prior reaching the 8-cell stage that was of short duration and followed by a longer time spent prior compaction. The embryo on the left had a better history of even cell numbers and synchronous cleavage cycles. The asynchronous cell division of the right hand embryo could indicate a lower quality and the embryo to the left should probably be preferred for transfer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Conclusions

Selection and deselection of embryos with the best prospect of implantation remains as topical as ever in today's busy IVF laboratories and is a vital part of the effort to raise success rates. Several invasive approaches have been developed over the last three decades but have so far not fulfilled the expectations when translated into normal clinical practice.

Among the noninvasive approaches, the 'tried and tested' morphology evaluation has lost credibility as a means to grade embryo potential because the outer appearance at a single time point does not necessarily reflect the embryo's developmental potential. Other noninvasive methodologies are on the way from research into routine practice. Some such as metabolomics are still awaiting implementation into clinical routine and a major reason may be that commercial solutions are still undergoing development and validation. However, others such as time-lapse imaging, are already in the implementation process and are providing new insights into the traditional approach of morphometric assessment. Morphokinetic embryo analysis, which is the change of embryo morphology over time, is by far the most important noninvasive embryo selection tool today. It already can be applied to identify embryos likely to develop to the blastocyst stage and it may also have great potential when deciding which embryo to transfer in elective single-embryo transfer. If combined with developing technologies to assess physiological embryo properties such as metabolomics, these may constitute the backbone of every embryology laboratory's workflow in the future.

Finally, although embryo development in the laboratory is considered to be the most influential aspect in IVF success, one should never forget that implantation is a multistep process. Beside the embryo and the embryologist, the physician doing the transfer and the endometrial receptivity of the patient are additional key players in determining the success of implantation, and the role of both should not be neglected. These aspects of the process are discussed in the accompanying articles included in this symposium by Fatemi and Popovic-Todrovic (2013) and Edgell et al. (2013).

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