



# Non-invasive evaluation of embryo quality for the selection of transferable embryos in human *in vitro* fertilization-embryo transfer

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The ultimate goal of human assisted reproductive technology is to achieve a healthy pregnancy and birth, ideally from the selection and transfer of a single competent embryo. Recently, techniques for efficiently evaluating the state and quality of preimplantation embryos using time-lapse imaging systems have been applied. Artificial intelligence programs based on deep learning technology and big data analysis of time-lapse monitoring system during *in vitro* culture of preimplantation embryos have also been rapidly developed. In addition, several molecular markers of the secretome have been successfully analyzed in spent embryo culture media, which could easily be obtained during *in vitro* embryo culture. It is also possible to analyze small amounts of cell-free nucleic acids, mitochondrial nucleic acids, miRNA, and long non-coding RNA derived from embryos using real-time polymerase chain reaction (PCR) or digital PCR, as well as next-generation sequencing. Various efforts are being made to use non-invasive evaluation of embryo quality (NiEEQ) to select the embryo with the best developmental competence. However, each NiEEQ method has some limitations that should be evaluated case by case. Therefore, an integrated analysis strategy fusing several NiEEQ methods should be urgently developed and confirmed by proper clinical trials.

**Keywords:** Assisted reproductive technology; Cell-free nucleic acids; Culture media; Embryo; MicroRNA; Non-invasive; Secretome; Time-lapse imaging

## Introduction

Over the past half-century, strategies have been developed to evaluate and select competent preimplantation embryos for uterine transfer in human *in vitro* fertilization-embryo transfer (IVF-ET) pro-

grams. In the early days, morphological characteristics including fragmentation and other features of embryos observed by an optical microscope were mainly used to evaluate the quality and developmental potential of embryos [1]. However, simple daily microscopic observations by clinical embryologists had limitations in accurately predicting the developmental capacity of embryos.

Recently, techniques for efficiently evaluating the state and quality of embryos using time-lapse monitoring systems (TLMSs) and various molecular genetic approaches have been introduced. In particular, TLMS could select viable embryos without concerns regarding observer-variability and disturbances of culture conditions [2]. Various studies have searched for optimal morphokinetic parameters during TLMS, which could enhance the probability of blastocyst formation, aneuploidy, and finally implantation. Analyses of implantation-related morphokinetic parameters during TLMS have facilitated the development of several clinical algorithms as promising tools for the evaluation and prediction of embryos destined to become the

Received: August 8, 2022 · Revised: November 9, 2022 ·

Accepted: November 10, 2022

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\*This project was financially supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (NRF-2018R1D-1A1B07046419 to Jaewang Lee and NRF-2020R1F-1A1071918 to Jin Hyun Jun).

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most competent blastocysts [3].

Artificial intelligence (AI) programs based on deep learning technology and big data analysis of TLMS have been developed and applied as a method for the non-invasive evaluation of embryo quality (NiEEQ). The clinical effectiveness of NiEEQ for human IVF-ET programs has just begun to be reported, and there are several algorithms to predict the implantation potential of day-3 or day-5 embryos. Although the application of NiEEQ alone may not be perfect for selecting the best embryos, more advanced information about the physiological and genetic state of embryos could provide insights into all aspects of the embryos' intrinsic characteristics [4].

Furthermore, advanced and sensitive molecular genetic approaches have been successfully applied to spent embryo culture media (SECM), which can be easily obtained during *in vitro* embryo culture. It is possible to analyze small amounts of cell-free DNA (cfDNA), mitochondrial DNA, microRNA, and long non-coding RNA secreted from embryos using real-time polymerase chain reaction (PCR) or digital PCR, as well as next-generation sequencing (NGS) [5-7]. In addition, studies have evaluated the developmental ability of embryos by analyzing substrates and metabolites produced during *in vitro* culture, which will be discussed further below [8-11]. Many recent studies have evaluated the correlation between the results of SECM analysis and the embryos' developmental competence. However, the results obtained from those methods can be affected by various external sources of contamination and have the disadvantages of needing relatively expensive equipment, having high costs, and requiring special expertise.

This review provides an overview of the current status of NiEEQ, including TLMS and advanced molecular biological methods in SECM analysis. We also describe the need to develop a method for integrated analysis to overcome the several limitations of each NiEEQ system that has been used in recent years.

## TLMS for the selection of the best embryos for transfer

In human IVF-ET programs, embryo cleavage is observed daily by microscopy during *in vitro* culture, and the quality of embryos is determined by the number of blastomeres, cell symmetry, percentage of fragmentation, and other parameters on the day of transfer. The quality of blastocysts is also assessed according to the blastocysts' expansion state and the appearance of the inner cell mass (ICM) and trophoctoderm cells (TE) [12]. Transferable embryos are traditionally selected through a time-point observation of morphological features by trained clinical embryologists with expertise in embryo evaluation [13]. However, there are some limits in accurately predicting the developmental capacity of embryos by microscopic observations. In-

ter- and intra-observer variations can occur in embryo grading, even when it is performed by expert clinical embryologists [14].

For this reason, the TLMS was developed and applied in human IVF-ET programs [15]. A time-lapse system allows the complete observation of developing embryos in the IVF laboratory within stable culture conditions [16]. Initially, valuable knowledge was obtained through the TLMS during *in vitro* culture of pre-implantation embryos in animal models, such as mice and cows, and the TLMS provided precise information on developmental dynamics by making it possible to recognize important morphological changes of the embryo state [17,18]. The advantages of this system include a reduced need for handling and human risk, uninterrupted culture conditions, the ability to detect abnormal events that would otherwise not be noticed, and reduced inter- and intra-observer variability [16,19].

Through TLMS, various morphokinetic markers of developing embryos to predict blastocyst formation have been proposed, as shown in Table 1 [20-35]. In addition to blastocyst formation, morphokinetic markers associated with embryo implantation have been identified, as shown in Table 2 [22,24,25,34,36-50]. Several algorithms using a combination of morphokinetic variables have been introduced and successfully applied in human IVF-ET to select embryos with higher developmental capacity and implantation potential.

The known implantation data (KID) score is an interesting algorithm to improve embryo selection and predict implantation and live birth. The KID score algorithm attempts to rank embryo quality and optimize embryo selection prior to transfer based on conventional morphology grading [51]. Several reports have demonstrated the efficacy of the KID score algorithm and other similar programs using AI. The KID scores of day-5 blastocysts were found to be inversely proportional to maternal age, but directly proportional to blastocyst morphological grade [52]. This finding indicates that the KID score model works well to select blastocysts with higher implantation potential in patients with advanced maternal age.

More recently, the iDAScore algorithm has been developed; this is a deep learning-based annotation scoring system to predict the viability of embryos and the likelihood of implantation and pregnancy. Automatic embryonic ranking systems with AI have demonstrated higher performance with respect to successful implantation and pregnancy prediction than conventional morphological grading systems for the selection of transferable embryos [53]. The area under the curve (AUC) for the iDAScore was comparable to or higher than those of the KID score and Gardner criteria for young and older groups. In particular, for younger women, the AUC of iDAScore was 0.72, which was greater than those of the other two models.

For the KID score, strongly predictive morphokinetic variables were identified (time to 2 cells, duration of the second cell cycle below or above a threshold) with regard to implantation and live birth

**Table 1.** Various morphokinetic markers to predict blastocyst formation in time-lapse monitoring systems

| Study                         | Study design                             | No. of evaluated embryos | Origin of embryos                  | Time-lapse monitoring system                       | Identified predictive marker                    |
|-------------------------------|--|--------------------------|------------------------------------|--|---|
| Wong et al. (2010) [20]       | Retrospective study                      | 100                      | Supernumerary frozen 2PN           | Modified Olympus IX-70/71; CKX-40/41 BioStation CT | First cytokinesis, P2 and P3                    |
| Hashimoto et al. (2012) [21]  | Experimental study                       | 80                       | Donated human embryos for research | BioStation CT                                      | Durations of second and third mitotic divisions |
| Hlinka et al. (2012) [22]     | Retrospective study                      | 180                      | Clinical IVF routine               | Primo vision                                       | c2, c3, and c4; i2, i3, and i4                  |
| Cruz et al. (2012) [23]       | Retrospective cohort study               | 834                      | Oocyte donation cycles             | EmbryoScope  | t4, s2, DC3 cells, and tM; UN2 cells            |
| Chamayou et al. (2013) [24]   | Retrospective study                      | 224                      | Fresh oocyte ICSI cycles           | EmbryoScope  | t1, t2, t4, t7, t8, tC–tF, and s3               |
| Kirkegaard et al. (2013) [25] | Prospective cohort study                 | 571                      | Fresh oocyte ICSI cycles           | EmbryoScope  | First cytokinesis, t3, and DC3 cells            |
| Conaghan et al. (2013) [26]   | Prospective multicenter study            | 233                      | Fresh oocyte ICSI cycles           | Eeva   | P2 and P3                                       |
| Kirkegaard et al. (2014) [27] | Prospective multicenter study            | 1,519                    | Fresh oocyte ICSI cycles           | EmbryoScope  | P2 and P3                                       |
| Cetinkaya et al. (2015) [28]  | Retrospective observational cohort study | 3,354                    | Clinical IVF routine               | EmbryoScope  | CS2   |
| Yang et al. (2015) [29]       | Prospective observational study          | 345                      | Metaphase I donated for research   | Primo vision                                       | Cleavage patterns                               |
| Milewski et al. (2015) [30]   | Prospective observational study          | 432                      | Fresh oocyte ICSI cycles           | EmbryoScope  | t2, t5, cc2, and SC                             |
| Storr et al. (2015) [31]      | Prospective cohort study                 | 380                      | Fresh oocyte ICSI cycles           | EmbryoScope  | s3, t8, and tEB                                 |
| Motato et al. (2016) [32]     | Retrospective study                      | 7,483                    | Clinical IVF routine               | EmbryoScope  | tM and t8–t5                                    |
| Coticchio et al. (2018) [33]  | Retrospective observational study        | 500                      | Fresh oocyte ICSI cycles           | EmbryoScope  | PN appearance during fertilization              |
| Zaninovic et al. (2019) [34]  | Retrospective multicenter study          | 27,316                   | Fresh oocyte ICSI cycles           | EmbryoScope  | t3, t3–t2, t5, t3–tPNF and t5–tPNF              |
| Desai et al. (2019) [35]      | Retrospective observational study        | 716                      | Fresh oocyte ICSI cycles           | EmbryoScope  | Cleavage patterns                               |

PN, pronuclei; CT, computed tomography; P2, time of division from 2 to 3 cells; P3, time of division from 3 to 4 cells; IVF, *in vitro* fertilization; c2, time between 3 and 4 cells; c3, time between 5 and 8 cells; c4, time between 9 and 16 cells; i, interphase; t4, time of cleavage to 4 cells; s2, the second synchronization parameter (t4–t3); DC, direct cleavage; tM, time from insemination to compaction into the morula stage; UN, uneven blastomere size; ICSI, intracytoplasmic sperm injection; t1, time of cleavage to 1 cell; t2, time of cleavage to 2 cells; t7, time of cleavage to 7 cells; t8, time of cleavage to 8 cells; tC–tF, time of pronuclei appearance to disappearance; s3, the third synchronization parameter (t8–t5); t3, time of cleavage to 3 cells; CS2, cleavage synchronicity from 2 cell; t5, time of cleavage to 5 cells; cc2, the second round of cleavage (t3–t2); SC,  $s_{t2} \times \text{odds ratios}_{t2} + s_{t5} \times \text{odds ratios}_{t5} + s_{cc2} \times \text{odds ratios}_{cc2}$ ; tEB, time from insemination to expanded blastocyst; tPNf, time from insemination to pronuclei fading.

following IVF in a retrospective study comprising 2,827 embryos [54]. For the AI-based automated iDAScore, two parameters were identified (blastocyst grading and direct cleavage) using retrospective data from 18 IVF clinics consisting of 115,832 embryos, of which 14,644 embryos were assessed using the KID score [55].

Another AI-based model, termed Life Whisperer (LW), was developed by assessing the images of 8,886 embryos from 11 IVF clinics and provided time-saving and higher accuracy for successful pregnancy [56]. The LW model significantly improves the predictive accuracy of embryologists for viable and non-viable embryos. The weighted overall accuracy was 64.3% for embryo viability, with an improvement of 24.7% over embryologists' accuracy. This model showed a sensitivity of 70.1% and specificity of 60.5% for viable em-

bryos, while still showing a bias toward high sensitivity.

The DynScore, constructed in 2021, is a model calculated with the Gaussian distributions of the "a" coefficients (defined as the estimated number of maximum cells at 72 hours equivalent to the asymptote of the logistic curve). Logistic regression was performed using morphokinetic parameters from the first 3 days of 1,186 embryos, and the model output was highly predictive of blastocyst formation, with an AUC above 0.9 [57]. Although this model used a machine learning system with reinforcement capacity to predict the fate of embryos, it was only useful for specific types of patients, and it was not able to predict pregnancy.

Deep learning models have achieved good prediction results for successful pregnancy and fetal heartbeat following selected blasto-

**Table 2.** Various morphokinetic markers to predict implantation potential in time-lapse monitoring systems

| Study                         | Study design  | No. of evaluated embryos  | Origin of embryos                               | Time-lapse monitoring system                                | Identified predictive marker                                |
|-------------------------------|---|---------------------------|---|---|---|
| Lemmen et al. (2008) [36]     | Retrospective study   | 19                        | IVF/ICSI cycles                                 | Nikon Diaphot 300 microscope with camera in a closed system | Nuclei appearance in the first blastomere                   |
| Meseguer et al. (2011) [37]   | Retrospective study   | 247                       | ICSI cycles                                     | EmbryoScope   | t5, s2, cc2, UN 2 cell, MN 4 cell, and DC 1–3 cells         |
| Azzarello et al. (2012) [38]  | Prospective study   | 159                       | ICSI cycles                                     | EmbryoScope   | PN breakdown  |
| Hlinka et al. (2012) [22]     | Retrospective study   | 114                       | ICSI cycles                                     | Primo Vision  | c2, c3 and c4; i2, i3, and i4                               |
| Rubio et al. (2012) [39]      | Retrospective multi-center study                            | 5,225 (1,659 transferred) | IVF cycles from donated and autologous oocytes  | EmbryoScope   | DC 2–3 cells  |
| Freour et al. (2013) [40]     | Retrospective analysis and prospectively collected database | 191                       | ICSI cycles                                     | EmbryoScope   | t4 and s3   |
| Chamayou et al. (2013) [24]   | Retrospective study   | 178                       | ICSI cycles                                     | EmbryoScope   | cc3   |
| Kirkegaard et al. (2013) [25] | Prospective cohort study                                    | 84                        | ICSI cycles                                     | EmbryoScope   | None  |
| Rubio et al. (2014) [41]      | Prospective randomized control trial                        | 2,638                     | ICSI cycles from donated oocytes                | EmbryoScope   | t5, s2, cc2, UN 2 cell, MN 4 cell, and DC 1–3 cells         |
| Aguilar et al. (2014) [42]    | Retrospective cohort study                                  | 1,448                     | ICSI cycles from donated oocytes                | EmbryoScope   | Time to 2PB, PF, and length of S-phase                      |
| Basile et al. (2015) [43]     | Retrospective multi-center study                            | 1,122                     | ICSI cycles from donated and autologous oocytes | EmbryoScope   | cc2, t3, t5, UN 2 cell, MN 4 cell, and DC 1–3 cells         |
| Vermilyea et al. (2014) [44]  | Retrospective multi-centric study                           | 331                       | IVF/ICSI cycles                                 | Eeva  | P2 and P3   |
| Freour et al. (2015) [45]     | Retrospective study   | 528                       | ICSI cycles                                     | EmbryoScope   | t5, s2, cc2, UN 2 cell, MN 4 cell, and DC 1–3 cells         |
| Dominguez et al. (2015) [46]  | Retrospective cohort study                                  | 28                        | ICSI cycles from donated oocytes                | EmbryoScope   | cc2   |
| Adamson et al. (2016) [47]    | Prospective concurrent cohort study                         |                           | ICSI and IVF cycles from autologous oocytes     | Eeva  | P2 and P3   |
| Goodman et al. (2016) [48]    | Prospective randomized control trial                        | 2,092                     | ICSI and IVF cycles from autologous oocytes     | EmbryoScope   | cc2, s2, t5, s3, tSB, MN, and irregular division            |
| Coello et al. (2017) [49]     | Retrospective observational cohort study                    | 429                       | ICSI cycles                                     | EmbryoScope   | Vitrified/warmed blastocyst morphology and collapse pattern |
| Zaninovic et al. (2019) [34]  | Retrospective multi-center study                            | 816                       | Fresh oocyte ICSI cycles                        | EmbryoScope   | t3–t2   |
| Barberet et al. (2019) [50]   | Retrospective cohort study                                  | 232                       | ICSI cycles                                     | EmbryoScope   | PN appearance and MN 2 cells                                |

IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; t5, time of cleavage to 5 cells; s2, the second synchronization parameter (t4–t3); cc2, the second round of cleavage (t3–t2); UN, uneven blastomere size; MN, multinucleated; DC, direct cleavage; PN, pronuclei; c2, time between 3 and 4 cells; c3, time between 5 and 8 cells; c4, time between 9 and 16 cells; i, interphase; t4, time of cleavage to 4 cells; s3, the third synchronization parameter (t8–t5); cc3, the third round of cleavage (t8–t4); PB, polar body; PF, time of pronuclei fading; t3, time of cleavage to 3 cells; P2, time of division from 2 to 3 cells; P3, time of division from 3 to 4 cells; tSB, time of starting blastulation.

cyst transfer [58,59], and a few studies have reported the prediction of embryo euploidy [60–62]. The correlation between euploidy and embryo morphokinetics has been widely studied, as shown in Table 3 [60–74]. Using a known data set of single static embryo images, the Embryo Ranking Intelligent Classification Algorithm was developed

to rank embryos based on ploidy and implantation potential [75]. The euploid prediction algorithm, with comprehensive consideration of morphokinetic parameters, patient age, and ploidy state determined by preimplantation genetic testing (PGT) improved the predictive efficiency and accuracy (the AUC of 0.80) [76]. To improve the

**Table 3.** Various morphokinetic markers to predict embryo euploidy in time-lapse monitoring systems

| Study                             | Study design   | No. of evaluated embryos | Time-lapse monitoring system | Biopsy day after fertilization | Analysis methods of PGD | Identified predictive marker  |
|-----------------------------------|--|--------------------------|------------------------------|--------------------------------|-------------------------|---|
| Chavez et al. (2012) [60]         | Prospective observational study                        | 75                       | Custom-built microscope      | Day 3                          | aCGH                    | P1, P2, P3, and fragmentation   |
| Campbell et al. (2013) [61]       | Retrospective cohort study                             | 98                       | EmbryoScope                  | Day 5                          | aCGH/SNP array          | tSB and tB  |
| Basile et al. (2014) [62]         | Retrospective cohort study                             | 504                      | EmbryoScope                  | Day 3                          | aCGH                    | t5–t2 and cc3   |
| Rienzi et al. (2015) [63]         | Longitudinal cohort study                              | 455                      | EmbryoScope                  | Day 5                          | CCS                     | None  |
| Chawla et al. (2015) [64]         | Retrospective cohort study                             | 460                      | EmbryoScope                  | Day 3                          | aCGH                    | tPNf, t2, t5, cc2, cc3, and t5–t2   |
| Vera-Rodriguez et al. (2015) [65] | Prospective observational study                        | 85                       | Eeva                         | Day 3                          | aCGH                    | Time between PN disappearance and the start of 1st cytokinesis; 3 to 4 cell |
| Mumusoglu et al. (2017) [66]      | Retrospective cohort study                             | 415                      | EmbryoScope                  | Day 5                          | aCGH                    | t9, tM, tSB, tB, and tEB,   |
| Desai et al. (2018) [67]          | Retrospective analysis of prospectively collected data | 767                      | EmbryoScope                  | Day 5                          | aCGH/NGS                | Dysmorphisms, tSB, tEB, and tEB-tSB   |
| Rocafort et al. (2018) [68]       | Retrospective study                                    | 1,482                    | Eeva                         | Day 5                          | NGS                     | P2 and P3   |
| Huang et al. (2019) [69]          | Retrospective observational study                      | 188                      | EmbryoScope                  | Day 5                          | aCGH                    | Starting time to blastocyst expansion                                       |
| Yap et al. (2019) [70]            | Retrospective study                                    | 807 Patients             | EmbryoScope                  | Day 5                          | NGS                     | KIDscore D5 algorithm (score 6.0–9.9)                                       |
| Gazzo et al. (2020) [71]          | Retrospective study                                    | 492                      | EmbryoScope                  | Day 5                          | NGS                     | KIDscore D5 algorithm   |
| Ozbek et al. (2021) [72]          | Retrospective analysis of prospectively collected data |                          | EmbryoScope                  | Day 5                          | NGS                     | Cleavage patterns   |
| Urich et al. (2022) [73]          | Prospective observational study                        | 212                      | EmbryoScope                  | Day 5                          | NGS                     | t2 and t3   |
| De Gheselle et al. (2022) [74]    | Retrospective cohort study                             | 539                      | EmbryoScope                  | Day 5                          | NGS                     | tPB2, tPNa, and t7  |

PGD, preimplantation genetic diagnosis; aCGH, array-based comparative genomic hybridization; P1, time of division from 1 to 2 cells; P2, time of division from 2 to 3 cells; P3, time of division from 3 to 4 cells; SNP, single-nucleotide polymorphism; tSB, time of stating blastulation; tB, time from insemination to formation of a full blastocyst; t5, time of cleavage to 5 cells; t2, time of cleavage to 2 cells; CCS, comprehensive chromosome screening; tPNf, time from insemination to pronuclei fading; cc2, the second round of cleavage (t3–t2); cc3, the third round of cleavage (t8–t4); PN, pronuclei; t9, time of cleavage to 9 cells; tM, time from insemination to compaction into the morula stage; tEB, time from insemination to expanded blastocyst; NGS, next-generation sequencing; tPB2, time of 2nd polar body extrusion; tPNa, time from insemination to pronuclei appearance; t7, time of cleavage to 7 cells.

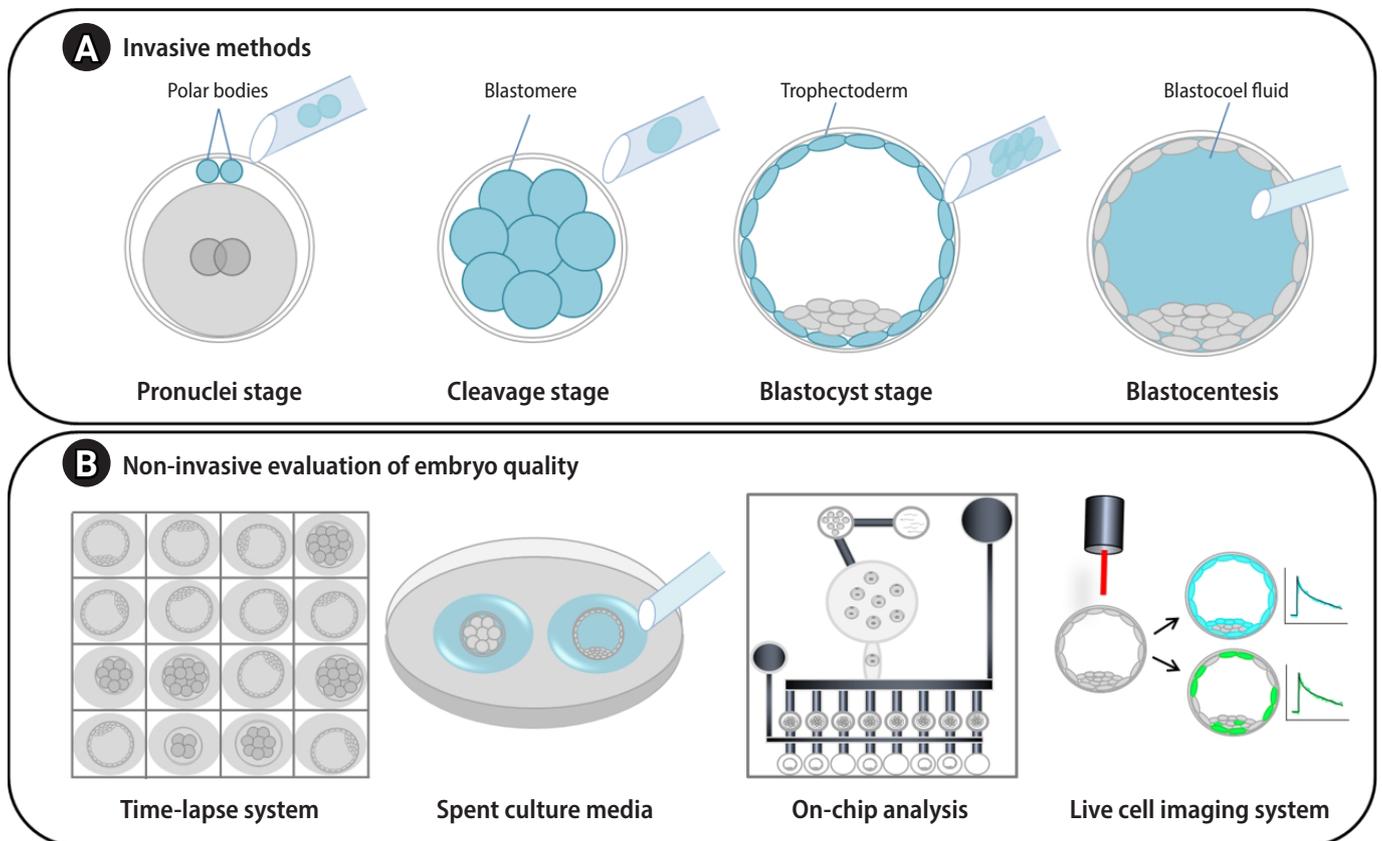
benefits of the AI model in terms of prediction of clinical pregnancy, validation for AI applications with a large-scale dataset is needed. The morphological features of embryos are not absolute, and do not fully represent the potential of embryos for successful implantation.

Despite their widespread application, embryo morphological assessments with TLMS have limited predictive power, especially for genetic variations and metabolic competence. Several studies have clearly demonstrated that embryo morphology and time to blastocyst formation are linked to embryo metabolism. Many approaches rely on intracellular measurements or quantification of metabolites in the spent media to detect the metabolic activity of the whole em-

bryo. Those methods are either invasive or require highly specialized skills. Non-invasive techniques to measure embryonic development and metabolic state may assist in improving embryo selection in clinical laboratories.

### Development and advancement of molecular biological methods for the analysis of SECM

Traditionally, invasive biopsy of pre-implantation embryos is performed for PGT to identify inherited or *de novo* euploidy or aneuploidy (Figure 1A). However, aneuploid cells are preferentially elimi-



**Figure 1.** Invasive and non-invasive methods for evaluation of embryo quality. (A) For invasive methods, genetic materials can be obtained by polar body biopsy at the pronuclei stage, blastomere biopsy for cleavage-stage embryos, and trophoblast biopsy or blastocoel fluid aspiration for blastocyst-stage embryos. (B) For non-invasive methods, image analysis of embryos using time-lapse monitoring systems; spent culture media analysis with quantitative real-time polymerase chain reaction (PCR), digital PCR, and on-chip-analyses; and live cell image analyses using advanced microscopic systems have been applied.

nated from mosaic embryos via processes of apoptosis or expulsion of cells during compaction. This is a cause of misdiagnosis or poor pregnancy outcomes. It was found that autophagy-mediated apoptosis eliminated aneuploid cells in a mouse model of chromosome mosaicism [77].

Many researchers have used non-invasive methods to determine the metabolic and genetic state of embryos concerning their viability and pregnancy outcomes for IVF patients (Figure 1B). During *in vitro* culture of human embryos, a variety of macromolecules, including proteins, nucleic acids, genetic material, and extracellular vesicles are present in SECM. Of many molecules, the level of GDF9 in human SECM was linked to embryo quality and viability [78]. Interestingly, various miRNA populations have been detected in the SECM, and these miRNAs may influence genes impacting early embryo development [79]. Profiling the secretome in SECM provides potential diagnostic biomarkers for embryo quality and ploidy [80-82]. Interestingly, a comparative analysis of the metabolomic profiles of SECM on day 5 found two different clusters of metabolite composition between euploid and aneuploid embryos with good morphology. Fur-

thermore, untargeted metabolomics of SECM by high-performance liquid chromatography–mass spectrometry identified potential biomarkers of embryos with good morphology that would undergo unsuccessful implantation [83]. In a preliminary report, three artificial neural networks that combined morphological variables and proteins using blastocyst image analysis and proteomic profiles of SECM were able to predict live birth, with an AUC of 1.0 in receiver operating characteristic curve analysis [84]. The researchers suggested that their model may provide an efficacious tool to select the embryo most likely to lead to a live birth in a euploid cohort. It may be applied to reduce the number of transferred embryos per patient to prevent complicated multiple pregnancies.

The reported levels of ploidy agreement between non-invasive SECM samples and biopsied embryonic cells vary widely [85]. A study found various cfDNAs in SECM from 57 embryos of seven IVF patients, and their genetic testing by array-based comparative genomic hybridization was consistent with TE biopsy [86]. Furthermore, single-cell bisulfite sequencing of SECM identified cfDNAs derived from human blastocysts, cumulus cells, and polar bodies, and de-

tected cellular origin and chromosome aneuploidy. The DNA methylation-based approach decreases the risk of contamination by maternal components, which interfere with a genetic diagnosis [87].

The greatest advantage of non-invasive genetic testing is cost-effectiveness due to the lack of fees for embryo biopsy, and it is useful as first-line PGT [88,89]. The efficiency of this method has been restricted by technical complications associated with DNA contamination and low sensitivity, resulting in clinical misdiagnoses [90]. In many cases, a small sample size reduces the reliability of the results of non-invasive PGT. Larger-scale and well-designed studies testing embryo-derived and extra-embryonic genetic material are warranted to shed light on the mechanisms and potential dynamics of embryo mosaicism.

Another issue to be considered for non-invasive genetic testing is SECM preparation. Group culture is not suitable, and it is necessary to place only one embryo in each small drop of culture medium. This aspect strongly affects culture conditions by evaporation and leads to excessive use of culture dishes. Modification of the culture platform on which gametes, embryos and media flow are handled may offer benefits including rapid fluid manipulation and feasibility of usage. The microfluidic method utilizes fluid movement along microchannels in a micro- or nano-environment during cell culture, while the embryos remain largely undisturbed [91]. Microfluidics platforms facilitate the easy manipulation or removal of gametes/embryos dealing with small volumes and the examination of metabolomic activity and profiles, offering a feasible non-invasive predictor of embryo quality [91,92]. Some lab-on-a-chip devices have met with a certain degree of success in adherent cell systems [93,94]. The technical development of integrative automation for more complex procedures within the same platform remains a work in progress. Embryo culture and subsequent analysis on the same platform offer the ability to reduce cell handling and the potential introduction of laboratory errors.

Electron carriers, such as nicotinamide adenine dinucleotide and flavin adenine dinucleotide, have recently been used to characterize variations in the metabolic state obtained using fluorescence lifetime imaging microscopy (FLIM) [95]. This measuring system allows the observation of distinct metabolic states between ICM and TE, and makes it possible to detect variations in individual blastocysts from the same patient and between patients. However, the association between FLIM data and embryo ploidy has not yet been fully elucidated.

## Clinical outcomes of TLMS and SECM analysis in human IVF-ET programs

TLMS for pre-implantation embryos in human IVF-ET programs

provides more embryo information as a non-invasive tool. However, it has been debated whether using a TLMS could improve the clinical outcomes compared with conventional evaluation systems. These TLMSs have been applied to clinical practice since the early 2000s, and many clinical trials have been reported. This review discusses the overall trend and future directions through a review of meta-analyses of clinical trials.

The first meta-analysis on the efficiency of TLMS was reported in 2014 [96]. The authors suggested that TLMS does not significantly offer the likelihood of achieving clinical and ongoing pregnancy in blastocyst transfer. They concluded that more research is needed to improve the quality of the available evidence and to investigate the usefulness of TLMS interventions for the selection of transferable embryos.

Thereafter, several meta-analyses were published until 2019, and all suggested that it is difficult to confirm a significant difference between TLMS and conventional methods [97-100]. In a Cochrane review published in 2019, the authors concluded that there was insufficient good-quality evidence of differences in live birth, ongoing pregnancy, miscarriage and stillbirth, and clinical pregnancy rates between TLMS and conventional methods.

However, a recent meta-analysis reported that TLMS interventions were effective [101]. Two randomized controlled clinical trials demonstrated the efficacy of TLMS in various conditions in the last 6 years [102,103]. Many retrospective studies using TLMS have reported statistically significantly higher rates of pregnancy success compared to traditional methods [54,104-107]. In addition, the KID score and iDAScore, using AI algorithms based on deep learning, have been developed and their applications are expanding in human IVF-ET programs [3,51,53,108,109]. The fully automated iDAScore model reduces manual evaluation and eliminates bias due to inter- and within-observer variability [55].

However, a couple of studies have reported that the evidence for significant advantages of TLMS remains unclear [110-112]. Elective single cleavage-stage embryo transfer with TLMS did not have any advantages over conventional observation in young women with good ovarian reserve [111]. That study also suggested that single blastocyst transfer with TLMS does not increase the likelihood of ongoing pregnancy compared to conventional observation; in particular, the use of a TLMS to choose blastocysts for fresh single embryo transfer on day 5 did not improve ongoing pregnancy rate compared to morphology alone [112].

One of the important challenges in the field of PGT of preimplantation embryos is the use of non-invasive procedures [113-115], with the aim of improving PGT cost-efficiency and safety. The collection of SECM is not a difficult procedure, and it can be safely performed on all cultured embryos. It does not require special expertise in embryo

manipulation, unlike invasive biopsies of embryos and blastocysts. Since this method avoids all detrimental effects of suboptimal micro-manipulations and the potential risks caused by invasive procedures, it does not affect the embryo development and reproductive potential. Moreover, SECM can be collected at any pre-implantation developmental stage; even cleaved embryos with fewer than six cells on day 3 and early blastocysts can be tested, unlike invasive PGT, which is based on embryo biopsy or blastocentesis. Hence, PGT by SECM might be particularly suitable for cultured growing embryos with low implantation potential that cannot be tested by invasive PGT. In fact, SECM-PGT is relatively fast, taking less than 12 hours from SECM collection to genetic analysis [116-118], and the results may be available before embryo transfer or cryopreservation. If there is a positive diagnosis, another SECM sample should be collected after 24 hours of incubation for confirmation. Many published reports have suggested that SECM is a potential alternative source of embryonic DNA, indicating that SECM-PGT is a promising procedure for the genetic testing of all developing embryos [119,120]. However, before implementing SECM-PGT in clinical practice, it is necessary to improve its reliability [121]. The standardization of SECM-PGT and establishment of guidelines are also essential to enable reliable comparisons of results and to verify the consistency of results among IVF-ET centers.

Debate continues regarding the reliability of alternative sources of genetic materials for embryo evaluation, although cfDNAs from SECM have been successfully detected and amplified. Discrepancies have been found regarding the concordance of the embryonic genetic state obtained from SECM and other DNA sources, including polar bodies, embryos, and TE biopsies, and whole embryos. There have been many discussions and suggestions on standardization and validation methods in several review papers on this issue [90,113,122-125]. With the latest advanced methods, such as NGS and mass spectrometry, which have recently emerged as superior analysis methods, it has become possible to verify the source of the genetic sample used for analysis and assess the probability of accurately estimating the genetic state of the embryo [126]. However, mosaicism, multinucleation, blastomere fragmentation, and contamination of SECM are still difficult to overcome. In particular, it is not easy to accurately distinguish genetic material of maternal or paternal origin and from the embryo.

## Conclusion

The ultimate goal of human IVF-ET programs is to achieve a healthy pregnancy and birth, ideally from the selection and transfer of the single best, most competent embryo. The effectiveness of NiEEQ in clinical applications of human IVF-ET programs has been pur-

sued intensively. However, each non-invasive method, such as TLMS and SECM analysis, has limitations that must be handled case by case. Since the evaluation of the embryo state using TLMS is based on morphological criteria, it is impossible to confirm variation at the actual genome and gene expression level. It is also difficult to reflect differences according to the culture conditions of each laboratory and the characteristics of each individual. In SECM analysis, alterations of nucleic acids and metabolites may appear depending on the presence or absence of cumulus cells or sperm that can be cultured if they attach to the fertilized oocytes. In order to overcome these limitations of NiEEQ, it would be ideal to develop integrated analysis methods through the fusion of complementary methods.

Rapidly developing, deep learning and AI algorithms with big data analysis can play a crucial role in improving and assisting many methods of both TLMS and SECM analysis. Several studies are being conducted to support the application of various techniques by developing automated annotation programs for the morphological dynamics of TLMS and genetic analysis of SECM. This advanced computational approach is expected to provide fast, robust, and reliable results while reducing bias in the interpretation of data and selection of the best embryo.

In the near future, it is expected that new integrated NiEEQ methods will emerge that can combine the advantages and compensate for the disadvantages of these two methods. We need to develop an integrated NiEEQ for the best embryo selection to achieve a healthy pregnancy and birth.

## Conflict of interest

Jin Hyun Jun is an editorial board member of the journal but was not involved in the peer reviewer selection, evaluation, or decision process of this article.

No other potential conflicts of interest relevant to this article were reported.

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Conceptualization: JHJ. Data curation: JK, JL. Formal analysis: JHJ, JK. Funding acquisition: JL, JHJ. Methodology: JK, JL. Visualization: JHJ, JK. Writing—original draft: JK. Writing—review & editing: JHJ, JL.

## References

- Montag M, Liebenthron J, Koster M. Which morphological scoring system is relevant in human embryo development? *Placenta* 2011;32 Suppl 3:S252–6.
- Lundin K, Park H. Time-lapse technology for embryo culture and selection. *Ups J Med Sci* 2020;125:77–84.
- 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.
- 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.
- Lei CX, Sun XX. Character of cell-free genomic DNA in embryo culture medium and the prospect of its clinical application in preimplantation genetic testing. *Reprod Dev Med* 2022;6:51–6.
- Stigliani S, Anserini P, Venturini PL, Scaruffi P. Mitochondrial DNA content in embryo culture medium is significantly associated with human embryo fragmentation. *Hum Reprod* 2013;28:2652–60.
- Zhang Q, Ji H, Shi J, Wang L, Ding L, Jiang Y, et al. Digital PCR detection of mtDNA/gDNA ratio in embryo culture medium for prediction of embryo development potential. *Pharmgenomics Pers Med* 2021;14:521–31.
- Heo YS, Cabrera LM, Bormann CL, Smith GD, Takayama S. Real time culture and analysis of embryo metabolism using a microfluidic device with deformation based actuation. *Lab Chip* 2012;12:2240–6.
- Lee YS, Thouas GA, Gardner DK. Developmental kinetics of cleavage stage mouse embryos are related to their subsequent carbohydrate and amino acid utilization at the blastocyst stage. *Hum Reprod* 2015;30:543–52.
- Finger BJ, Harvey AJ, Green MP, Gardner DK. Combined parental obesity negatively impacts preimplantation mouse embryo development, kinetics, morphology and metabolism. *Hum Reprod* 2015;30:2084–96.
- Lee YS, Gardner DK. Early cleaving embryos result in blastocysts with increased aspartate and glucose consumption, which exhibit different metabolic gene expression that persists in placental and fetal tissues. *J Assist Reprod Genet* 2021;38:3099–111.
- Nasiri N, Eftekhari-Yazdi P. An overview of the available methods for morphological scoring of pre-implantation embryos in in vitro fertilization. *Cell J* 2015;16:392–405.
- Minasi MG, Greco P, Varricchio MT, Barillari P, Greco E. The clinical use of time-lapse in human-assisted reproduction. *Ther Adv Reprod Health* 2020;14:2633494120976921.
- Baxter Bendus AE, Mayer JF, Shipley SK, Catherino WH. Interobserver and intraobserver variation in day 3 embryo grading. *Fertil Steril* 2006;86:1608–15.
- Paternot G, Debrock S, D'Hooghe T, Spiessens C. Computer-assisted embryo selection: a benefit in the evaluation of embryo quality? *Reprod Biomed Online* 2011;23:347–54.
- Mio Y, Maeda K. Time-lapse cinematography of dynamic changes occurring during in vitro development of human embryos. *Am J Obstet Gynecol* 2008;199:660.
- Kim J, Kim SH, Jun JH. Prediction of blastocyst development and implantation potential in utero based on the third cleavage and compaction times in mouse pre-implantation embryos. *J Reprod Dev* 2017;63:117–25.
- Sugimura S, Akai T, Somfai T, Hirayama M, Aikawa Y, Ohtake M, et al. Time-lapse cinematography-compatible polystyrene-based microwell culture system: a novel tool for tracking the development of individual bovine embryos. *Biol Reprod* 2010;83:970–8.
- Basile N, Caiazzo M, Meseguer M. What does morphokinetics add to embryo selection and in-vitro fertilization outcomes? *Curr Opin Obstet Gynecol* 2015;27:193–200.
- Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM, et al. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotechnol* 2010;28:1115–21.
- Hashimoto S, Kato N, Saeki K, Morimoto Y. Selection of high-potential embryos by culture in poly(dimethylsiloxane) microwells and time-lapse imaging. *Fertil Steril* 2012;97:332–7.
- Hlinka D, Kalatova B, Uhrinova I, Dolinska S, Rutarova J, Rezacova J, et al. Time-lapse cleavage rating predicts human embryo viability. *Physiol Res* 2012;61:513–25.
- Cruz M, Garrido N, Herrero J, Perez-Cano I, Munoz M, Meseguer M. Timing of cell division in human cleavage-stage embryos is linked with blastocyst formation and quality. *Reprod Biomed Online* 2012;25:371–81.
- Chamayou S, Patrizio P, Storaci G, Tomaselli V, Alecci C, Ragolia C, et al. The use of morphokinetic parameters to select all embryos with full capacity to implant. *J Assist Reprod Genet* 2013;30:703–10.
- Kirkegaard K, Kesmodel US, Hindkjer JJ, Ingerslev HJ. Time-lapse parameters as predictors of blastocyst development and pregnancy outcome in embryos from good prognosis patients: a prospective cohort study. *Hum Reprod* 2013;28:2643–51.
- Conaghan J, Chen AA, Willman SP, Ivani K, Chenette PE, Boostanfar R, et al. Improving embryo selection using a computer-automated time-lapse image analysis test plus day 3 morphology: results from a prospective multicenter trial. *Fertil Steril* 2013;100:

- 412–9.
27. Kirkegaard K, Campbell A, Agerholm I, Bentin-Ley U, Gabrielsen A, Kirk J, et al. Limitations of a time-lapse blastocyst prediction model: a large multicentre outcome analysis. *Reprod Biomed Online* 2014;29:156–8.
  28. Cetinkaya M, Pirkevi C, Yelke H, Colakoglu YK, Atayurt Z, Kahraman S. Relative kinetic expressions defining cleavage synchronicity are better predictors of blastocyst formation and quality than absolute time points. *J Assist Reprod Genet* 2015;32:27–35.
  29. Yang ST, Shi JX, Gong F, Zhang SP, Lu CF, Tan K, et al. Cleavage pattern predicts developmental potential of day 3 human embryos produced by IVF. *Reprod Biomed Online* 2015;30:625–34.
  30. Milewski R, Kuc P, Kuczynska A, Stankiewicz B, Lukaszuk K, Kuczynski W. A predictive model for blastocyst formation based on morphokinetic parameters in time-lapse monitoring of embryo development. *J Assist Reprod Genet* 2015;32:571–9.
  31. Storr A, Venetis CA, Cooke S, Susetio D, Kilani S, Ledger W. Morphokinetic parameters using time-lapse technology and day 5 embryo quality: a prospective cohort study. *J Assist Reprod Genet* 2015;32:1151–60.
  32. Motato Y, de los Santos MJ, Escriba MJ, Ruiz BA, Remohi J, Meseguer M. Morphokinetic analysis and embryonic prediction for blastocyst formation through an integrated time-lapse system. *Fertil Steril* 2016;105:376–84.
  33. Coticchio G, Mignini Renzini M, Novara PV, Lain M, De Ponti E, Turchi D, et al. Focused time-lapse analysis reveals novel aspects of human fertilization and suggests new parameters of embryo viability. *Hum Reprod* 2018;33:23–31.
  34. Zaninovic N, Nohales M, Zhan Q, de Los Santos ZM, Sierra J, Ros-enwaks Z, et al. A comparison of morphokinetic markers predicting blastocyst formation and implantation potential from two large clinical data sets. *J Assist Reprod Genet* 2019;36:637–46.
  35. Desai N, Gill P. Blastomere cleavage plane orientation and the tetrahedral formation are associated with increased probability of a good-quality blastocyst for cryopreservation or transfer: a time-lapse study. *Fertil Steril* 2019;111:1159–68.
  36. Lemmen JG, Agerholm I, Ziebe S. Kinetic markers of human embryo quality using time-lapse recordings of IVF/ICSI-fertilized oocytes. *Reprod Biomed Online* 2008;17:385–91.
  37. Meseguer M, Herrero J, Tejera A, Hilligsoe KM, Ramsing NB, Remohi J. The use of morphokinetics as a predictor of embryo implantation. *Hum Reprod* 2011;26:2658–71.
  38. Azzarello A, Hoest T, Mikkelsen AL. The impact of pronuclei morphology and dynamicity on live birth outcome after time-lapse culture. *Hum Reprod* 2012;27:2649–57.
  39. Rubio I, Kuhlmann R, Agerholm I, Kirk J, Herrero J, Escriba MJ, et al. Limited implantation success of direct-cleaved human zygotes: a time-lapse study. *Fertil Steril* 2012;98:1458–63.
  40. Freour T, Dessolle L, Lammers J, Lattes S, Barriere P. Comparison of embryo morphokinetics after in vitro fertilization-intracytoplasmic sperm injection in smoking and nonsmoking women. *Fertil Steril* 2013;99:1944–50.
  41. Rubio I, Galan A, Larreategui Z, Ayerdi F, Bellver J, Herrero J, et al. Clinical validation of embryo culture and selection by morphokinetic analysis: a randomized, controlled trial of the EmbryoScope. *Fertil Steril* 2014;102:1287–94.
  42. Aguilar J, Motato Y, Escriba MJ, Ojeda M, Munoz E, Meseguer M. The human first cell cycle: impact on implantation. *Reprod Biomed Online* 2014;28:475–84.
  43. Basile N, Vime P, Florensa M, Aparicio Ruiz B, Garcia Velasco JA, Remohi J, et al. The use of morphokinetics as a predictor of implantation: a multicentric study to define and validate an algorithm for embryo selection. *Hum Reprod* 2015;30:276–83.
  44. VerMilyea MD, Tan L, Anthony JT, Conaghan J, Ivani K, Gvakharia M, et al. Computer-automated time-lapse analysis results correlate with embryo implantation and clinical pregnancy: a blinded, multi-centre study. *Reprod Biomed Online* 2014;29:729–36.
  45. Freour T, Le Fleuter N, Lammers J, Spingart C, Reignier A, Barriere P. External validation of a time-lapse prediction model. *Fertil Steril* 2015;103:917–22.
  46. Dominguez F, Meseguer M, Aparicio-Ruiz B, Piqueras P, Quinonero A, Simon C. New strategy for diagnosing embryo implantation potential by combining proteomics and time-lapse technologies. *Fertil Steril* 2015;104:908–14.
  47. Adamson GD, Abusief ME, Palao L, Witmer J, Palao LM, Gvakharia M. Improved implantation rates of day 3 embryo transfers with the use of an automated time-lapse-enabled test to aid in embryo selection. *Fertil Steril* 2016;105:369–75.
  48. 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.
  49. Coello A, Meseguer M, Galan A, Alegre L, Remohi J, Cobo A. Analysis of the morphological dynamics of blastocysts after vitrification/warming: defining new predictive variables of implantation. *Fertil Steril* 2017;108:659–66.
  50. Barberet J, Bruno C, Valot E, Antunes-Nunes C, Jonval L, Chammas J, et al. Can novel early non-invasive biomarkers of embryo quality be identified with time-lapse imaging to predict live birth? *Hum Reprod* 2019;34:1439–49.
  51. Boucrot L, Tramon L, Saulnier P, Ferre-L'Hotellier V, Bouet PE, May-Panloup P. Change in the strategy of embryo selection with time-lapse system implementation-impact on clinical pregnancy rates. *J Clin Med* 2021;10:4111.

52. Goto S, Kadowaki T, Tanaka S, Hashimoto H, Koikeguchi S, Shiotani M. Prediction of pregnancy rate by blastocyst morphological score and age, based on 1,488 single frozen-thawed blastocyst transfer cycles. *Fertil Steril* 2011;95:948–52.
53. Ueno S, Berntsen J, Ito M, Uchiyama K, Okimura T, Yabuuchi A, et al. Pregnancy prediction performance of an annotation-free embryo scoring system on the basis of deep learning after single vitrified-warmed blastocyst transfer: a single-center large cohort retrospective study. *Fertil Steril* 2021;116:1172–80.
54. Sayed S, Reigstad MM, Petersen BM, Schwennicke A, Wegner Hausken J, Storeng R. Time-lapse imaging derived morphokinetic variables reveal association with implantation and live birth following in vitro fertilization: a retrospective study using data from transferred human embryos. *PLoS One* 2020;15:e0242377.
55. Berntsen J, Rimestad J, Lassen JT, Tran D, Kragh MF. Robust and generalizable embryo selection based on artificial intelligence and time-lapse image sequences. *PLoS One* 2022;17:e0262661.
56. VerMilyea M, Hall JM, Diakiw SM, Johnston A, Nguyen T, Perugini D, et al. Development of an artificial intelligence-based assessment model for prediction of embryo viability using static images captured by optical light microscopy during IVF. *Hum Reprod* 2020;35:770–84.
57. Giscard d'Estaing S, Labrune E, Forcellini M, Edel C, Salle B, Lornage J, et al. A machine learning system with reinforcement capacity for predicting the fate of an ART embryo. *Syst Biol Reprod Med* 2021;67:64–78.
58. Tran D, Cooke S, Illingworth PJ, Gardner DK. Deep learning as a predictive tool for fetal heart pregnancy following time-lapse incubation and blastocyst transfer. *Hum Reprod* 2019;34:1011–8.
59. Khosravi P, Kazemi E, Zhan Q, Malmsten JE, Toschi M, Zisimopoulos P, et al. Deep learning enables robust assessment and selection of human blastocysts after in vitro fertilization. *NPJ Digit Med* 2019;2:21.
60. Chavez SL, Loewke KE, Han J, Moussavi F, Colls P, Munne S, et al. Dynamic blastomere behaviour reflects human embryo ploidy by the four-cell stage. *Nat Commun* 2012;3:1251.
61. Campbell A, Fishel S, Bowman N, Duffy S, Sedler M, Thornton S. Retrospective analysis of outcomes after IVF using an aneuploidy risk model derived from time-lapse imaging without PGS. *Reprod Biomed Online* 2013;27:140–6.
62. Basile N, Nogales Mdel C, Bronet F, Florensa M, Riqueiros M, Rodrigo L, et al. Increasing the probability of selecting chromosomally normal embryos by time-lapse morphokinetics analysis. *Fertil Steril* 2014;101:699–704.
63. Rienzi L, Capalbo A, Stoppa M, Romano S, Maggiulli R, Albricci L, et al. No evidence of association between blastocyst aneuploidy and morphokinetic assessment in a selected population of poor-prognosis patients: a longitudinal cohort study. *Reprod Biomed Online* 2015;30:57–66.
64. Chawla M, Fakih M, Shunnar A, Bayram A, Hellani A, Perumal V, et al. Morphokinetic analysis of cleavage stage embryos and its relationship to aneuploidy in a retrospective time-lapse imaging study. *J Assist Reprod Genet* 2015;32:69–75.
65. Vera-Rodriguez M, Chavez SL, Rubio C, Reijo Pera RA, Simon C. Prediction model for aneuploidy in early human embryo development revealed by single-cell analysis. *Nat Commun* 2015;6:7601.
66. Mumusoglu S, Yarali I, Bozdog G, Ozdemir P, Polat M, Sokmensuer LK, et al. Time-lapse morphokinetic assessment has low to moderate ability to predict euploidy when patient- and ovarian stimulation-related factors are taken into account with the use of clustered data analysis. *Fertil Steril* 2017;107:413–21.
67. Desai N, Goldberg JM, Austin C, Falcone T. Are cleavage anomalies, multinucleation, or specific cell cycle kinetics observed with time-lapse imaging predictive of embryo developmental capacity or ploidy? *Fertil Steril* 2018;109:665–74.
68. Rocafort E, Enciso M, Leza A, Sarasa J, Aizpurua J. Euploid embryos selected by an automated time-lapse system have superior SET outcomes than selected solely by conventional morphology assessment. *J Assist Reprod Genet* 2018;35:1573–83.
69. Huang TT, Huang DH, Ahn HJ, Arnett C, Huang CT. Early blastocyst expansion in euploid and aneuploid human embryos: evidence for a non-invasive and quantitative marker for embryo selection. *Reprod Biomed Online* 2019;39:27–39.
70. Yap WY, Lee CS, Lim YX, Lim MW. Relationship between euploidy rates and D5 KIDScore of blastocysts derived from embryo-scope. *Reprod Biomed Online* 2019;39:e39.
71. Gazzo E, Pena F, Valdez F, Chung A, Bonomini C, Ascenzo M, et al. The Kidscore D5 algorithm as an additional tool to morphological assessment and PGT-A in embryo selection: a time-lapse study. *JBRA Assist Reprod* 2020;24:55–60.
72. Ozbek IY, Mumusoglu S, Polat M, Bozdog G, Sokmensuer LK, Yarali H. Comparison of single euploid blastocyst transfer cycle outcome derived from embryos with normal or abnormal cleavage patterns. *Reprod Biomed Online* 2021;42:892–900.
73. Urich M, Ugur MR, Li F, Shamma FN, Hammoud A, Cottrell HN, et al. Comparison of two culture media on morphokinetics and ploidy status of sibling embryos. *Zygote* 2022;30:410–5.
74. De Gheselle S, Jacques C, Chambost J, Blank C, Declerck K, De Croo I, et al. Machine learning for prediction of euploidy in human embryos: in search of the best-performing model and predictive features. *Fertil Steril* 2022;117:738–46.
75. Chavez-Badiola A, Flores-Saiffe-Farias A, Mendizabal-Ruiz G, Drakeley AJ, Cohen J. Embryo Ranking Intelligent Classification

- Algorithm (ERICA): artificial intelligence clinical assistant predicting embryo ploidy and implantation. *Reprod Biomed Online* 2020;41:585–93.
76. Huang B, Tan W, Li Z, Jin L. An artificial intelligence model (euploid prediction algorithm) can predict embryo ploidy status based on time-lapse data. *Reprod Biol Endocrinol* 2021;19:185.
77. Singla S, Iwamoto-Stohl LK, Zhu M, Zernicka-Goetz M. Autophagy-mediated apoptosis eliminates aneuploid cells in a mouse model of chromosome mosaicism. *Nat Commun* 2020;11:2958.
78. Li J, Li C, Liu X, Yang J, Zhang Q, Han W, et al. GDF9 concentration in embryo culture medium is linked to human embryo quality and viability. *J Assist Reprod Genet* 2022;39:117–25.
79. Rio PD, Madan P. Does miRNA expression in the spent media change during early embryo development? *Front Vet Sci* 2021;8:658968.
80. Botros L, Sakkas D, Seli E. Metabolomics and its application for non-invasive embryo assessment in IVF. *Mol Hum Reprod* 2008;14:679–90.
81. Ferrick L, Lee YS, Gardner DK. Metabolic activity of human blastocysts correlates with their morphokinetics, morphological grade, KIDScore and artificial intelligence ranking. *Hum Reprod* 2020;35:2004–16.
82. Huang J, Yao Y, Jia J, Zhu X, Ma J, Wang J, et al. Chromosome screening of human preimplantation embryos by using spent culture medium: sample collection and chromosomal ploidy analysis. *J Vis Exp* 2021;(175):e62619.
83. Eldarov C, Gamisonia A, Chagovets V, Ibragimova L, Yarigina S, Smolnikova V, et al. LC-MS analysis revealed the significantly different metabolic profiles in spent culture media of human embryos with distinct morphology, karyotype and implantation outcomes. *Int J Mol Sci* 2022;23:2706.
84. Bori L, Dominguez F, Fernandez EI, Del Gallego R, Alegre L, Hickman C, et al. An artificial intelligence model based on the proteomic profile of euploid embryos and blastocyst morphology: a preliminary study. *Reprod Biomed Online* 2021;42:340–50.
85. Hanson BM, Tao X, Hong KH, Comito CE, Pangasnan R, Seli E, et al. Noninvasive preimplantation genetic testing for aneuploidy exhibits high rates of deoxyribonucleic acid amplification failure and poor correlation with results obtained using trophectoderm biopsy. *Fertil Steril* 2021;115:1461–70.
86. Shamoni 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.
87. Chen Y, Gao Y, Jia J, Chang L, Liu P, Qiao J, et al. DNA methylome reveals cellular origin of cell-free DNA in spent medium of human preimplantation embryos. *J Clin Invest* 2021;131:e146051.
88. Leaver M, Wells D. Non-invasive preimplantation genetic testing (niPGT): the next revolution in reproductive genetics? *Hum Reprod Update* 2020;26:16–42.
89. D'Alessandro A, Federica G, Palini S, Bulletti C, Zolla L. A mass spectrometry-based targeted metabolomics strategy of human blastocoele fluid: a promising tool in fertility research. *Mol Biosyst* 2012;8:953–8.
90. Brouillet S, Martinez G, Coutton C, Hamamah S. Is cell-free DNA in spent embryo culture medium an alternative to embryo biopsy for preimplantation genetic testing? A systematic review. *Reprod Biomed Online* 2020;40:779–96.
91. Heo YS, Cabrera LM, Bormann CL, Shah CT, Takayama S, Smith GD. Dynamic microfunnel culture enhances mouse embryo development and pregnancy rates. *Hum Reprod* 2010;25:613–22.
92. Mancini V, McKeegan PJ, Schrimpe-Rutledge AC, Codreanu SG, Sherrod SD, McLean JA, et al. Probing morphological, genetic and metabolomic changes of in vitro embryo development in a microfluidic device. *Biotechnol Prog* 2021;37:e3194.
93. Herrmann M, Roy E, Veres T, Tabrizian M. Microfluidic ELISA on non-passivated PDMS chip using magnetic bead transfer inside dual networks of channels. *Lab Chip* 2007;7:1546–52.
94. Eteshola E, Balberg M. Microfluidic ELISA: on-chip fluorescence imaging. *Biomed Microdevices* 2004;6:7–9.
95. Venturas M, Shah JS, Yang X, Sanchez TH, Conway W, Sakkas D, et al. Metabolic state of human blastocysts measured by fluorescence lifetime imaging microscopy. *Hum Reprod* 2022;37:411–27.
96. Polanski LT, Coelho Neto MA, Natri CO, Navarro PA, Ferriani RA, Raine-Fenning N, et al. Time-lapse embryo imaging for improving reproductive outcomes: systematic review and meta-analysis. *Ultrasound Obstet Gynecol* 2014;44:394–401.
97. Racowsky C, Kovacs P, Martins WP. A critical appraisal of time-lapse imaging for embryo selection: where are we and where do we need to go? *J Assist Reprod Genet* 2015;32:1025–30.
98. Pribenszky C, Nilseid AM, Montag M. Time-lapse culture with morphokinetic embryo selection improves pregnancy and live birth chances and reduces early pregnancy loss: a meta-analysis. *Reprod Biomed Online* 2017;35:511–20.
99. Chen M, Wei S, Hu J, Yuan J, Liu F. Does time-lapse imaging have favorable results for embryo incubation and selection compared with conventional methods in clinical in vitro fertilization? A meta-analysis and systematic review of randomized controlled trials. *PLoS One* 2017;12:e0178720.
100. Armstrong S, Bhide P, Jordan V, Pacey A, Marjoribanks J, Farquhar C. Time-lapse systems for embryo incubation and assessment in assisted reproduction. *Cochrane Database Syst Rev* 2019;5:CD011320.

101. Liu Y, Qi F, Matson P, Morbeck DE, Mol BW, Zhao S, et al. Between-laboratory reproducibility of time-lapse embryo selection using qualitative and quantitative parameters: a systematic review and meta-analysis. *J Assist Reprod Genet* 2020;37:1295–302.
102. Wang S, Ding L, Zhao X, Zhang N, Hu Y, Sun H. Embryo selection for single embryo transfer on day 3 based on combination of cleavage patterns and timing parameters in in vitro fertilization patients. *J Reprod Med* 2016;61:254–62.
103. Kovacs P, Matyas S, Forgacs V, Sajgo A, Molnar L, Pribenszky C. Non-invasive embryo evaluation and selection using time-lapse monitoring: results of a randomized controlled study. *Eur J Obstet Gynecol Reprod Biol* 2019;233:58–63.
104. Aparicio-Ruiz B, Basile N, Perez Albala S, Bronet F, Remohi J, Meseguer M. Automatic time-lapse instrument is superior to single-point morphology observation for selecting viable embryos: retrospective study in oocyte donation. *Fertil Steril* 2016;106:1379–85.
105. Fishel S, Campbell A, Foad F, Davies L, Best L, Davis N, et al. Evolution of embryo selection for IVF from subjective morphology assessment to objective time-lapse algorithms improves chance of live birth. *Reprod Biomed Online* 2020;40:61–70.
106. Hur YS, Ryu EK, Hyun CS, Yang SH, Yoon SH, Lim KS, et al. Retrospective study of single vitrified-warmed blastocyst transfer cycles according to the presence of morphokinetic variables. *Clin Exp Reprod Med* 2018;45:52–5.
107. Chera-Aree P, Thanaboonyawat I, Thokha B, Laokirkkiat P. Comparison of pregnancy outcomes using a time-lapse monitoring system for embryo incubation versus a conventional incubator in in vitro fertilization: an age-stratification analysis. *Clin Exp Reprod Med* 2021;48:174–83.
108. Reigner A, Girard JM, Lammers J, Chtourou S, Lefebvre T, Barriere P, et al. Performance of day 5 KIDScore morphokinetic prediction models of implantation and live birth after single blastocyst transfer. *J Assist Reprod Genet* 2019;36:2279–85.
109. Kato K, Ueno S, Berntsen J, Ito M, Shimazaki K, Uchiyama K, et al. Comparing prediction of ongoing pregnancy and live birth outcomes in patients with advanced and younger maternal age patients using KIDScore day 5: a large-cohort retrospective study with single vitrified-warmed blastocyst transfer. *Reprod Biol Endocrinol* 2021;19:98.
110. Magdi Y, Samy A, Abbas AM, Ibrahim MA, Edris Y, El-Gohary A, et al. Effect of embryo selection based morphokinetics on IVF/ICSI outcomes: evidence from a systematic review and meta-analysis of randomized controlled trials. *Arch Gynecol Obstet* 2019;300:1479–90.
111. Meng Q, Xu Y, Zheng A, Li H, Ding J, Xu Y, et al. Noninvasive embryo evaluation and selection by time-lapse monitoring vs. conventional morphologic assessment in women undergoing in vitro fertilization/intracytoplasmic sperm injection: a single-center randomized controlled study. *Fertil Steril* 2022;117:1203–12.
112. Ahlstrom A, Lundin K, Lind AK, Gunnarsson K, Westlander G, Park H, et al. A double-blind randomized controlled trial investigating a time-lapse algorithm for selecting day 5 blastocysts for transfer. *Hum Reprod* 2022;37:708–17.
113. Farra C, Choucair F, Awwad J. Non-invasive pre-implantation genetic testing of human embryos: an emerging concept. *Hum Reprod* 2018;33:2162–7.
114. Munne S. Status of preimplantation genetic testing and embryo selection. *Reprod Biomed Online* 2018;37:393–6.
115. Kuliev A, Rechitsky S. Preimplantation genetic testing: current challenges and future prospects. *Expert Rev Mol Diagn* 2017;17:1071–88.
116. Wu H, Ding C, Shen X, Wang J, Li R, Cai B, et al. Medium-based noninvasive preimplantation genetic diagnosis for human  $\alpha$ -thalassemias-SEA. *Medicine (Baltimore)* 2015;94:e669.
117. 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.
118. Li P, Song Z, Yao Y, Huang T, Mao R, Huang J, et al. Preimplantation genetic screening with spent culture medium/blastocoel fluid for in vitro fertilization. *Sci Rep* 2018;8:9275.
119. Ou Z, Deng Y, Liang Y, Chen Z, Sun L. Improved non-invasive pre-implantation genetic testing for beta-thalassemia using spent embryo culture medium containing blastocoelic fluid. *Front Endocrinol (Lausanne)* 2022;12:793821.
120. Shitara A, Takahashi K, Goto M, Takahashi H, Iwasawa T, Onodera Y, et al. Cell-free DNA in spent culture medium effectively reflects the chromosomal status of embryos following culturing beyond implantation compared to trophoctoderm biopsy. *PLoS One* 2021;16:e0246438.
121. Yin B, Zhang H, Xie J, Wei Y, Zhang C, Meng L. Validation of preimplantation genetic tests for aneuploidy (PGT-A) with DNA from spent culture media (SCM): concordance assessment and implication. *Reprod Biol Endocrinol* 2021;19:41.
122. Vera-Rodriguez M, Diez-Juan A, Jimenez-Almazan J, Martinez S, Navarro R, Peinado V, et al. Origin and composition of cell-free DNA in spent medium from human embryo culture during pre-implantation development. *Hum Reprod* 2018;33:745–56.
123. Li J, Liu Y, Qian Y, Zhang D. Noninvasive preimplantation genetic testing in assisted reproductive technology: current state and future perspectives. *J Genet Genomics* 2020;47:723–6.
124. Rogers A, Menezes M, Kane SC, Zander-Fox D, Hardy T. Preim-

- plantation genetic testing for monogenic conditions: is cell-free DNA testing the next step? *Mol Diagn Ther* 2021;25:683–90.
125. Sialakouma A, Karakasiliotis I, Ntala V, Nikolettos N, Asimakopoulos B. Embryonic cell-free DNA in spent culture medium: a non-invasive tool for aneuploidy screening of the corresponding embryos. *In Vivo* 2021;35:3449–57.
126. Pais RJ, Sharara F, Zmuidinaite R, Butler S, Keshavarz S, Iles R. Bioinformatic identification of euploid and aneuploid embryo secretome signatures in IVF culture media based on MALDI-ToF mass spectrometry. *J Assist Reprod Genet* 2020;37:2189–98.