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EMBRYO BIOLOGY

Could time-lapse embryo imaging reduce the need for biopsy and PGS?

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Abstract
Purpose To review relevant studies examining the relationship between embryo morpho-kinetics and aneuploidy.
Methods Search of Pubmed and Medline using relevant keywords pertaining to morphology, morphokinetics and embryonic aneuploidy, as well as examination of various reference lists and conference proceedings.
Results An abundance of publications, both preliminary and peer-reviewed, have emerged regarding the usefulness of time-lapse imaging in tracking embryo development and improving embryo selection. Recently, these publications have explored ability to not only predict blastocyst formation and implantation, but also the ability to detect embryonic chromosomal aneuploidy. Of the two peer-reviewed retrospective studies on morpho-kinetics and embryonic aneuploidy, one demonstrates that early cleavage timings can indicate chromosomal complement, while the other demonstrates that key events following the maternal-zygotic transition can be markers of aneuploidy. A recent paper also demonstrates improved outcomes following IVF using a selection algorithm to identify embryos at "low risk" of chromosomal abnormalities. However, the predictive nature of these events and timings is far from ideal. Additionally, results may be dependent upon the day of biopsy and method utilized for chromosomal assessment.
Conclusion With continued effort, the combination of multiple morphologic endpoint assessments and developmental timings and refinement of modeling systems may improve the predictive ability to determine embryonic aneuploidy. This may help select a subset of embryos that are less likely to carry chromosomal abnormalities and improve assisted reproductive outcomes. However, embryo biopsy, followed by preimplantation genetic screening/comprehensive chromosomal screening still remains the most reliable method to assess chromosomal complement of preimplantation embryos.

Keywords Time-lapse · Morphokinetics · Aneuploidy · Embryo · Blastocyst

Capsule Time-lapse imaging of embryos may provide insight into chromosomal status.

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Introduction

While real-time video or time-lapse imaging of embryo development is not a new concept [11, 33, 38, 46, 47, 51, 54, 61, 76, 77], only recently have commercially available devices been developed that permit wide-spread clinical implementation. This has resulted in an abundance of publications utilizing time-lapse imaging of human preimplantation embryos [5, 15–18, 35, 41–43, 47, 62, 82].

Imaging systems suitable for clinical use that permit a more-or-less continuous monitoring of preimplantation embryo development within the incubator environment offer unique opportunities to non-invasively visualize time points and aspects of embryo morphokinetics not previously feasible in the traditional IVF lab, with the intent of finding more accurate predictors of embryo quality. This emerging approach carries the advantage of avoiding unnecessary environmental stressors, such as pH or temperature fluctuations, associated with removal of cells from the incubator environment for the routine static observations at discrete time points. In addition, unique incubators used by at least one commercially available imaging system, as well as unique culture dishes with microwells or other approaches to permit individual embryo tracking, may further improve the embryo microenvironment and subsequent embryo development [66, 69]. Though concerns about the detrimental impact of light exposure from extended microscopic imaging of oocytes and

embryos may exist, especially in rodent species [9, 19, 36, 58, 59, 70, 71, 75], these embryo-specific imaging systems have all attempted to validate safety, and reduction in light intensity through use of low intensity source or darkfield or LED lighting sources, and/or brief exposure times of only a few seconds, seem to be enough to alleviate concerns or possible detrimental effects with human cells.

As mentioned, the true strength of time-lapse embryo imaging is the ability to observe developmental events normally missed by current static embryo visualization approaches, as well as to determine the timing of specific morphological occurrences and permit comparison between embryos. These events may provide key additional information to permit improved embryo selection, with the desire of improving clinical outcomes while promoting single embryo transfer. For example, recent time-lapse imaging publications focus on early mitotic events in cleavage stage embryos and timing between the first three cell divisions with the ability to predict blastocyst formation [17, 35, 82]. Other studies relate early embryo mitotic division timings to implantation success [53]. As will be discussed, more recent studies have attempted to utilize real-time embryo imaging to permit insight into the chromosomal status of individual embryos.

Aneuploidy occurs in a surprisingly high percentage of human preimplantation embryos, with estimates that 50–80 % may have chromosomal abnormalities [28, 78]. This high aneuploidy rate has obvious ramifications on limiting success of assisted reproduction. While preimplantation genetic screening (PGS), now also often referred to as comprehensive chromosomal screening (CCS), of all 24 chromosomes using new SNP or CGH arrays, qPCR or nextGen approaches are more informative and accurate compared to prior FISH techniques [28, 73], and these technologies may help improve embryo selection and assisted reproductive outcomes [26, 27, 64], embryo biopsy remains an invasive procedure that could compromise embryo quality. Additionally, the increased cost of biopsy and PGS/CCS may be prohibitive. Thus, non-invasive morphologic assessment methods to determine embryonic aneuploidy could be extremely beneficial as a means of improving embryo selection.

The idea of using embryo morphology to gain insight into embryo chromosomal compliment is not new, but has had limited success [80]. Various studies have utilized static assessment at distinct time points, often during cleavage stages, to try to show some link to embryo aneuploidy; though correlations are weak and not overly reliable [20, 21, 34, 50, 55, 57]. It was also suggested that a sequential embryo scoring system, utilizing information from days 1 to 3 of development, including endpoints like multi-nucleation, symmetry and number of blastomeres may help select euploid embryos [25]. However, again, accuracy of this approach is limited. Emerging morphokinetic analysis of embryo development offers the ability to not only compile numerous morphological endpoints, but their specific timings as well, which may perhaps be incorporated into a predictive algorithm to improve ability to non-invasively determine chromosomal status.

This review explores the potential for a non-invasive means of determining embryonic aneuploidy through examination of embryo morphology and timing of distinct morphologic events. This is accomplished through the description of major morphologic events occurring during preimplantation embryo development and examination of the existing literature on static morphologic assessment and relation to embryonic chromosomal complement. Recent kinetic data obtained using time-lapse imaging, much of which is preliminary, of these major morphologic events is then reviewed, focusing on studies that attempt to correlate timing of these events to embryonic aneuploidy.

Methods

Pubmed and Medline databases were searched using relevant keywords pertaining to embryo morphology, morphokinetics, and embryonic aneuploidy. Relevant papers were examined and included reference lists searched to locate additional studies. Published conference proceedings were also examined and preliminary studies related to embryo morphokinetics and aneuploidy analyzed. No time limits or other restrictive criteria were utilized. The last date of search was April, 2013.

Data analysis focused on correlation between embryo morphokinetics and aneuploidy, examining each major preimplantation embryo morphological event and relevant timings, as well as the analytical methods used to examine chromosomal status.

Results

Pronucleii

One of the first morphologic events evident and assessed during IVF is appearance of and assessment of pronucleii. Identification of the correct number of pronucleii is useful in identifying those subsequent embryos that may become aneuploid [23]. It is well-known that 3PN embryos can continue development to the blastocyst stage, but these are likely to be aneuploid. Similarly, 1PN embryos can also continue development and may be aneuploid, though they could also be the result of simply missing observation of a transient 2nd pronucleii. While this fertilization assessment can be done using a single static time point assessment ~16–18 h following insemination, use of time-lapse embryo imaging can help ensure improved accuracy of pronucleii

assessment, permitting visualization of transient pronucleii that may have formed or disassembled slightly earlier or later than "normal" and thus help ensure selection of euploid embryos for transfer. However, more subtle aspects of pronuclear morphology may be indicative of chromosomal complement. At 16 h post-insemination following ICSI or IVF, embryos were rolled to permit adequate visualization and static assessment of pronuclear positioning (5 patterns) was assessed. Subsequent day 3 biopsy with FISH analysis indicated that euploid embryos were only present in two pattern groups; those with juxtaposed pronucleii either centrally or peripherally located (32 % and 41 % respectively [31]. Using the same analytic approach, location of the polar bodies in respect to the longitudinal axis of pronucleii was also correlated to aneuploidy. Of the 3 groupings, those in line with or at 90° angles had the greatest number of euploid embryos (36 % and 33 %, respectively); significantly greater rates than the other group [31]. Static assessment of nuclear precursor bodies (NPBs) at 16–17 within pronucleii also seems to be related to embryonic aneuploidy. At least seven studies, with grading systems ranging from 3 to 6 grouping methods, have found some level of correlation between NPB organization (number, size, synchrony, polarity) and chromosomal complement following day 3 embryo biopsy with FISH analysis [7, 22, 30–32, 40]. It should also be noted that incorporation of three PN morphologic assessments (polar body alignment, PN positioning, NPB scoring) into a single scoring systems with 8 categories indicated that 4 of these categories yielded significantly higher rates of euploid embryos compared to the remaining 4 groups [31].

It can be appreciated that, if assessment of subtle location or other morphologic indicators is predictive of aneuploidy, that consistent timing of these assessments is crucial for adequate comparisons. Time-lapse imaging offers a mean's to ensure these timing windows are not missed. This may be especially true in regard to PN assessments. At least one preliminary study indicates that timing of pronuclear appearance, dissolution, or size, had no correlation to embryo ploidy when analyzing 24 chromosomes using qPCR following trophectoderm biopsy of resulting blastocysts [68] (Table 1). Similarly, a recent publication also found no correlation between time of PN fading and aneuploidy with TE biopsy and 24 chromosome microarray [13, 14] (Table 1). Whether specific timing of pronuclear abuttal, or even more detailed analysis of NPB patterns/dynamics can help improve prediction of chromosomal status of resulting embryos remains unknown. Importantly, the ability to "roll" or orient embryos to accurately visualize PN and/or NPB positioning/alignment, factors which static assessment studies indicate may be indicative of aneuploidy, is likely not feasible, or at least not as easily performed when using current time-lapse imaging systems that promote uninterrupted culture of cells. Also important to note, use of ICSI or IVF would appear to important variables to consider with regard to PN timing windows, as the exact time of sperm penetration and signs of fertilization will likely differ between these methods.

Upon closer examination of the above mentioned studies, it becomes apparent that the studies that find correlation between PN/NPB scoring systems use day 3 biopsy with FISH analysis. No predictive ability of such PN scoring systems is apparent with blastocyst biopsy and 24 chromosome analysis. This finding may be an important factor in interpretation of older static observation data in relation to newer studies using time-lapse imaging.

Multi-nucleation

Similar to pronucleii, static observation of blastomere multinucleation on Day 2 has been associated with embryo aneuploidy as confirmed by day 3/4 biopsy and FISH analysis [4, 44, 67]. Using 9 probe FISH analysis, aneuploid embryos were present 85 % of the time when multinucleation was apparent within blastomeres on day 2, while only 78 % aneuploidy was apparent with mono-nucleation [4]. Similarly, 5 probe FISH indicated day 2 multinucleation yielded 76.5 % aneuploidy, compared to only 50.9 % of mono-nucleated control embryos [44]. The same correlation to aneuploidy appears to be true for multinucleation on day 3 [44, 67]. Day of multinucleation appearance, number of nuclei per cell and number of multinucleated cells per embryo did not appear to increase aneuploidy rates [44]. Importantly, these multinucleated embryos may continue development and still form good morphology blastocysts. Thus, identifying this multinucleation appears critical to isolate those embryos that are most likely to be euploid for selection and transfer.

At least one study using time-lapse imaging has examined occurrence of multinucleation and relationship to aneuploidy. No significant difference was apparent in rates of aneuploid versus euploid embryos with 2-cell embryo multinucleation as assessed by TE biopsy and 24 chromosome SNP array analysis [13, 14] (Table 1). Apparently in agreement with this finding, another preliminary study using time-lapse imaging found that multinucleation of 2-cell embryos had no significant impact on subsequent implantation rates compared to mono-nucleated embryos (47.9 % vs. 52.1 %, respectively) [6] (Table 1).

The ability to detect multinucleation over several time points, ensuring that this transient occurrence is not missed, may be extremely beneficial in selection of euploid embryos. However, while static embryo assessment using day 3 biopsy and FISH indicates the importance of this morphologic assessment, no correlative data exists from time-lapse studies using trophectoderm biopsy. Additional studies are likely required to validate this finding. It should also be mentioned that not all time-lapse embryo imaging devices may be able to detect multinucleation. While identification of multinucleation may

Table 1 Summary of time-lapse studies evaluating various morphokinetic events and timings and correlation to embryonic aneuploidy

Timing endpoint assessment	Biopsy type	Diagnosis approach	Imaging system	Aneuploidy correlation	Reference
Prolonged duration of 1st cytokineses, time between 1st and 2nd mitosis	Day 2	aCGH	Eeva™	Yes	[15]
1st division, 2nd division, appearance of 4th blastomere, 3rd division	Trophectoderm	aCGH	Embryoscope®	No	[65]
PN appearance, PN disappearance, cleaving timing, compaction, cavitation	Trophectoderm	qPCR	Embryoscope®	No	[68]
1st cytokinesis, 2nd mitotic division, synchronicity of 3rd/4th cells	Polar body & Trophectoderm	aCGH	Eeva™	Yes	[29]
Cleavage times until the 8cell stage, 2–3cell division, 3–4cell division,	Day 3	FISH	Embryoscope®	No	[12]
Shorter 5 to 8– cell division (3rd synchrongy division)	Day 3	FISH	Embryoscope®	Yes	[12]
"Optimal" timings of 2nd synchrony, time to 5-cell, 2nd cell cycle	Day 3	FISH or aCGH	Embryoscope®	No	[10]
Duration 1st and 2nd mitosis synchrony 2nd & 3rd cell cycle, blastulation duration, 2cell multinucleation	Trophectoderm	aCGH or SNP array	Embryoscope®	No	[13]
Delayed compaction, cavitation, full blastocyst formation	Trophectoderm	aCGH or SNP array	Embryoscope®	Yes	[14]
Early compaction	Trophectoderm	aCGH	Embryoscope®	Yes	[52]
Syngamy, cavitation	Trophectoderm	aCGH	Embryoscope®	No	[52]
First sign of fragmentation	Trophectoderm	aCGH	Embryoscope®	No	[56]

be feasible using brightfield imaging devices, this can only occur if the blastomeres are in the correct focal plane, and existing darkfield devices are limited and may not be able to visualize these structures.

Timing of mitotic divisions

The area receiving the most recent widespread attention in regard to finding a means of predicting embryo ploidy has focused on the timings of early mitotic divisions. As mentioned, information gained from time-lapse imaging during the first 3 mitotic divisions have clearly been shown to be related to blastocyst formation, pregnancy and perhaps implantation [17, 35, 53, 81, 82], which may infer selection of euploid embryos.

Specifically, duration of the first mitotic division with in 14.3±6.0 min, time between first and second mitosis (2-cell to 3-cell transition) within 11.1±2.2 h and time between the second and third mitosis (3-cell to 4-cell transition) within 1.1±1.6 h gave the ability to predict blastocyst formation from frozen/thawed zygotes with 94 % and 93 % sensitivity and specificity, respectively [82]. A similar study using another imaging device demonstrated that shortened timing between the second and third mitosis related to improved blastocyst morphologic grading [35]. This was also confirmed by Cruz et al. [17], who, in a retrospective cohort study, also showed that timings of early cleavage divisions correlated to blastocyst formation and quality score [17]. As will be discussed, there may be a relationship between blastocyst morphology and ploidy, so timing of early mitotic division may also lend insight into embryonic aneuploidy. Similarly, time-lapse imaging revealed that division to the 5-cell stage (48.8–56.6 h), time between the first and second mitosis (11.9 h), time between the second and third mitosis (<0.76 h), as well as timing to reach the 2-cell, 3-cell and 4-cell stages correlated with implantation [53]. Again, this may suggest selection of euploid embryos. However, blastocysts can be aneuploid at relatively high rates, and, despite implantation, aneuploidy may still be present.

Numerous preliminary publications have attempted to correlated timings of early cleavage cell divisions with embryo chromosomal status with limited success. Using the Embryoscope®, timings of the first mitotic division, division from 2 to 3 cells, appearance of the 4th blastomere and the third mitotic division were recorded and resulting 76 blastocysts biopsied and analyzed using 24 chromosome aCGH [65] (Table 1). No significant differences were apparent between aneuploid and euploid embryos using differences in early cleavage timings. Another similar preliminary study using the Embryoscope® also found no correlation between early cleavage timing and blastocyst aneuploidy following blastocyst biopsy and qPCR for all chromosomes [68] (Table 1). Other small preliminary studies report similar non-correlation findings of various early cleavage timings and aneuploidy following blastocyst biopsy and various chromosomal analysis approaches [52, 65] (Table 1). These preliminary data agree with a recent peer-reviewed publication that examined time of the 2nd cell division (2 to 3 cells), time of the third cell cycle

(3 to 5 cells) as well as time and synchrony of the 2nd and 3rd cell divisions (2 to 4 cells, or, 4 to 8 cells, respectively) [13, 14]. In this study, using trophectoderm biopsy and 24 chromosome screening, though later morphologic timings and event appeared to predict chromosomal status of embryos, early cleavage timings had no correlation with aneuploidy (Table 1).

Interestingly, at least two preliminary reports and one peer-reviewed study indicate a possible correlation between early cleavage timings and embryo aneuploidy. Utilizing day 3 biopsy and 9 probe FISH, 122 embryos analyzed indicated that while no difference were apparent in cleavage time to the 8-cell stage or duration or cleavage from 2 to 3-cell or 3 to 4-cell, euploid embryos had a significant shorter time to cleavage from the 5-cell to the 8-cell stage compared to aneuploid embryos [12] (Table 1). Similarly, utilizing a darkfield imaging approach and automated cell tracking system, a correlation between timing of early mitotic events and embryo ploidy was reported [15, 29] (Tables 1 and 2). In a preliminary study, 18 embryos were analyzed using polar body biopsy and some using trophectoderm biopsy. Using aCGH for chromosomal analysis, 62.5 % of embryos (5/8) that exhibited "normal" timings of duration of first cytokinesis, time of 2nd mitosis and synchrony of the 3rd and 4th cell appearance, were euploid, while only 40 % (4/10) with "abnormal" timings were euploid [29]. In agreement, peer reviewed data from the same group analyzed development of 75 human zygotes that were monitored for 2-days with image collection at 5 min intervals. Cleavage stage embryos were then disassembled and ploidy determined for each blastomere via 24 chromosome aCGH. It was determined that 75 % of embryos were aneuploid. Authors then attempted to determine if normally diagnosed embryos displayed any unique mitotic timing characteristic compared to the aneuploid counterparts. It was determined that aneuploid embryos had a larger standard deviation of timings related to the interval of the first cytokinesis, as well as time for cleavage from the 2-cell to 3-cell and 3-cell to the 4-cell stage. It was also determined that the degree of aneuploidy impacted cell cycle timings. As a result, only ~30 % of aneuploid embryos fell within the time windows of euploid embryos (14.4±4.2 min, 11.8±0.71 h, 0.96±0.84 h, respectively) (Table 2). These timings were determined to give a sensitivity and specificity of 100 % and 66 % to predict embryonic euploidy.

Importantly, though difference in imaging devices and slight variances in cleavage timing windows due to variable starting points or embryo sources may help explain differential outcomes between the above mentioned studies in regard to ability to predict aneuploidy with mitotic timings, again, an explanation may lie in the stage of the embryos biopsied and analyzed. As with PN and multinucleation analyses, correlations were only found in the study where cleavage embryos were biopsied, while no correlations were found in studies with trophectoderm biopsy.

Day 2/3 morphology

Static assessment of day 2 and day 3 fragmentation and/or blastomere size/symmetry are common indicators used for embryo selection for transfer. Numerous studies exist showing correlation between various day 2/day 3 morphologic parameters, such has cell number or degree of fragmentation [8, 25, 55, 83]. However, the majority of these studies utilized day 3 biopsy and FISH. More recent preliminary studies have examined the correlation between morphology and aneuploidy using 24 chromosome screening. One study using static assessment and 46 chromosome aCGH examined 452 day 3 biopsied embryos and correlated ploidy with day 3 morphology [49]. Those embryos with less than 6 cells at time of biopsy had higher rates of aneuploidy than those with >6 cells (59.3 % vs. 74.35). Additionally, embryos with >15 % fragmentation were more aneuploid that those with <15 % (66.1 % vs. 23.6 %). Another preliminary study examining 1915 embryos using day 3 biopsy and aCGH found that embryos with >9cells on day 3 were more likely to be aneuploid [45]. However, not all studies indicate an association between cleavage morphology and aneuploidy. One preliminary study using day 3 biopsy and 24 aCGH found no correlation to aneuploidy with day 2 or 3 fragmentation when using <10 % or 11–25 % cutoffs [79].

Complicating the association of cleavage stage morphology to aneuploidy, it is well-known that embryo development is a dynamic process, and cell number and degree of fragmentation can change rapidly as fragments are extruded or absorbed during development. Thus, if a time-lapse imaging system were able to refine a time window for these

Table 2 Morphokinetic data using the Eeva™ time-lapse imaging system that demonstrates early cleavage stage event timings frozen/thawed human embryos may correlate to aneuploidy

Chromosomal status	n	Duration 1st cytokinesis (min)	Interval between 1st & 2nd mitosis (min)	Interval between 2nd & 3rd mitosis (min)
Normal	8	14.2±4.2	11.8±0.71	0.96±0.84
Meiotic error	9	117.2±166.5	4.0±5.2	2.0±4.3
Mitotic error	21	36.0±66.9	6.4±6.6	2.0±3.9
High mosaic	13	52.7±89.8	3.5±6.2	2.2±4.1
Low mosaic	12	17.9±16.8	9.5±5.6	1.8±3.8

Table adapted from [15]

observations and then track and incorporate these assessments into a standardized algorithm, it may be more useful in determining embryonic aneuploidy than a subjective single time point assessment. In fact, a recent study using the time-lapse imaging EEVA system and day 3 biopsy to correlate early cleavage timings with embryo ploidy indicated that embryo fragmentation on day 2 of development may help improve ability to detect aneuploidy embryos when used with these mitotic timing intervals [15]. Furthermore, a large number of aneuploid and triploid, but not euploid embryos, showed fragmentation, though fragmentation alone offered very little predictive ability. However, a preliminary study using the Embryoscope® for time-lapse embryo analysis with blastocyst biopsy was unable to show any correlation between the timing of first fragmentation, appearance or degree of fragmentation to the chromosomal status of human blastocysts [56] (Table 1). A similar study also using the Embryoscope® with blastocyst biopsy and qPCR of all chromosomes also showed no correlation between aneuploidy and blastomere symmetry or fragmentation [68] (Table 1).

Again, similar to possible limitations previously mentioned, while conflicting data in the correlation of day 2/3 morphology/fragmentation may lie in the inherent variability of the visual assessment or in the thresholds used, differing days of biopsy (day 3 vs. blastocyst) may explain differential findings. Additional future studies using time-lapse analysis may attempt to determine if prior described patterns of fragmentation [2, 3] correlate to embryonic aneuploidy, and newly developed software that can non-subjectively track embryo fragmentation and possibly reabsorption of fragments during time-lapse imaging may further refine the objectiveness and usefulness of this approach [15].

Compaction and blastocyst formation

In addition to the early mitotic events in embryo development that may provide morphologic indicators of embryo ploidy, later morphologic event may provide further insight. Blastocyst culture is used as an additional positive selection tool in many IVF cases. It is known that blastocysts of higher morphological grades results in higher pregnancy and implantation rates. Use of established morphological grading standards that are known to help improve pregnancy rates may also provide insight into chromosomal status. Indeed, 500 human blastocysts were biopsied and ploidy determined using CGH arrays [1]. The chromosomal status was then correlated with blastocyst morphologic grade as determined by the Gardner grading scale. It was shown that 50 % of hatching and hatched blastocysts (grade 5/6) were euploid compared to only 37.5 % of grade 1/2 blastocysts. The grade of both the ICM and TE were also correlated with aneuploidy, with lower grades being more aneuploid. Various preliminary reports also indicate that blastocyst development and quality appear to be positively related to euploid status [24, 37, 39, 48, 63, 79]. These findings may help partially explain why examination of trophectoderm biopsy and attempts to correlate to early morphology or morphokinetic timings do not tend to prove successful. Analysis of blastocysts may be skewing the data by only observing a select pool of the embryos, which tend to be more euploid. A significant portion of aneuploid embryos may be arresting prior to blastocyst formation. Also important to note, not all studies indicate a correlation between blastocyst morphology and aneuploidy and a significant portion of "poor" morphology blastocysts can still be euploid. One preliminary study indicates that blastocyst morphology had no relation to aneuploidy, and that CCS resulted in 22 % of single embryo transfer cases having an alternate embryo transferred other than the highest graded embryo [26, 27]. As a result, these patients had a 77 % clinical pregnancy rate.

Interestingly, a recent time-lapse publication using trophectoderm biopsy and CGH and SNP arrays suggests that, using insemination as the starting point, the time to compaction (comp) as well as time to full blastocyst formation (blast) were significantly slower in aneuploid versus euploid embryos (comp: 85.1 h vs. 79.7 h; blast: 110.9 h vs. 105.9 h, respectively). Similarly, onset of cavitation was significantly slower in single and multiple aneuploid embryos (103.4 and 101.9 h) compared to euploid (95.1) [13, 14] (Table 3). Using two of these variables, cavitation and blastocyst formation, a classification model was developed with three risk categories, with an area under the ROC curve of 0.72.

It should be noted that his delay in compaction with aneuploidy contradicts at least one preliminary study, where compaction was actually accelerated in aneuploid embryos compared to euploid [52] (Table 1). No differences were apparent when looking at rates of blastocyst expansion or hatching between groups. Additionally conflicting data exist regarding the ability of blastocyst formation timing to predict aneuploidy. A preliminary report analyzing blastocysts from 53 patients were grouped into those that formed expanded blastocyst at <110 h and those that formed >110 h [68] (Table 1). No differences in rates of aneuploidy were apparent between groups. Another study using trophectoderm biopsy also indicated time to cavitation did not differ between aneuploid or euploid blastocysts [52] (Table 1).

Importantly, observing an adequate number of embryos, and determining the right time window is essential in determining if timing of morphologic events offers predictive ability with respect to aneuploidy. Additionally, reduction in subjectivity is important, as detection of subtle differences may be instrumental in determining aneuploidy. For example, in determining rates of expanded blastocyst formation as a specific time-point cutoff, more specific classification of "expanded" may be useful, utilizing blastocoels diameter or volume measurements to help in classification. Furthermore,

Table 3 A. Timings from post maternal-zygotic events obtained from fresh human embryos using the Embryoscope™ that that may permit prediction of aneuploidy. B. Incorporation of collected timings and formulation of a relative risk assessment with potential predictive ability (tables adapted from [13, 14])

Chromosomal Status	n	Compaction (hpi)	Cavitation (hpi)	Blastocyst formation (hpi)	
Euploid	35	79.7 (56.3–107.6)	95.1 (85.2–113.9)	105.9 (86.8–122.3)	
Single aneuploid	30	80.7	103.4 (79.8–121.5)	109.2	
Multiple aneuploid	28	85.1 (64.9–113.0)	101.9 (86.8–129.4)	110.9 (90.1–137.0)	
Risk class	n	Cavitation Time (hpi)	Blastocyst Formation (hpi)	Incidence	Probability
Low	36	<122.9	<96.2	0.36	0.37
Medium	49	<122.9	≥96.2	0.69	0.69
High	12	≥122.9		1.00	0.97

hpi hours post-insemination

number of expansion/collapsing events may also prove insightful if use together with other apparently predictive morphologic criteria. Coupling this time of morphokinetic data with traditional blastocyst grading may further improve any predictive power.

Conclusion

With emerging sequencing technology and other approaches to further reduce costs to patients, PGS/CCS may become more affordable and easier to offer, however, the invasive nature of biopsy still persists. It has been shown using time-lapse imaging in mouse embryos, that removal of one blastomere from a 4-cell embryo can delay blastocyst formation and increase the number of expansion/collapse events during blastocyst development [72, 74]. Similar effects are also apparent following biopsy on day 3 in human embryos [41–43]. While trophectoderm biopsy is less damaging than biopsy on day 3, avoidance of biopsy is likely best to maintain embryo quality. Thus, a non-invasive means to assess embryonic aneuploidy, such as morphokinetic timings, would be a powerful tool.

Though a non-invasive means to assess embryonic aneuploidy is appealing, conflicting data exist on the ability to use morphologic or morphokinetic embryo grading criteria to accurately predict chromosomal complement. This may stem, in part, from the day of biopsy used in various studies, as the vast majority of studies that indicate a correlation between morphology and timing of morphologic events are evident when using day 3 biopsy and only a few studies indicate a correlation when utilizing trophectoderm biopsy. With known issues of cleavage stage mosaicism and recent data indicating improved accuracy and reliability of results using blastocyst biopsy, the reliability of those studies using day 3 biopsy should be examined closely.

While the advantage of determining embryonic aneuploidy as early as possible is obvious, and use of early cleavage timings alone would be a convenient and simplified approach, advocating a complete move to day 3 transfer may be a bit premature. Rather, the ability to utilize and combine multiple morphologic endpoint assessments throughout embryo development may further improve aneuploidy predictive ability [25]. Combining the endpoint timings from the two recent peer-reviewed retrospective publications on morphokinetics and correlation to aneuploidy, using information from both pre- and post-MZT events, may improve predictive ability. However, this remains to be proven. This approach would presumably help account for any possible chromosomal "self-correction" and permit consideration of chromosomal contributions of the male gamete.

Importantly, having the right time-lapse imaging system to integrate ability to view all possible or relevant morphologic parameters during fertilization and cleavage development, while also then being able to incorporate traditional blastocyst morphology scoring, would undoubtedly be useful in refining this selection process; though confirmation of this cumulative approach and efficacy/accuracy remains to be demonstrated. Certainly, automated tracking and algorithms would be extremely useful to be able to integrate multiple endpoint assessments while also reducing subjectivity, though this may not be an easy task.

It should be mentioned, that while these visual observation and morphokinetics may help select euploid embryos, the accuracy of this approach at the moment is far from ideal. Sequencing approaches still remain the most reliable method of assessing embryo chromosomal complement. Thus, PGS/CCS will still undoubtedly have clinical applications. However, with the right combination of key morphologic events and timings, non-invasive visual observations may be accurate enough to identify "at risk" embryos and aid in selection of those more likely to be chromosomally normal. This may be a useful tool in perhaps avoiding biopsy and PGS/CCS in select younger patients, who are less susceptible to rates of aneuploidy, or at least reducing the number of embryos to be biopsied and analyzed for all patients. Supporting this approach, a recent submission used an aneuploidy risk model based on morphokinetic timings and demonstrated a significant improvement in fetal heart beats and

live-birth in the "low risk" group compared to "medium risk" [13, 14]. Ideally, the strengths of both approaches would be utilized, with the chromosomal status of this subset of "select" embryos identified from time-lapse being validated using PGS/CCS, with genetic material at the blastocyst stage, either from trophectoderm biopsy, or perhaps with a less invasive approach to obtain genetic material, such blastocoel fluid sampling [60].

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