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ORIGINAL ARTICLE ***Embryology***

Proposed guidelines on the nomenclature and annotation of dynamic human embryo monitoring by a time-lapse user group

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STUDY QUESTION: Can the approach to, and terminology for, time-lapse monitoring of preimplantation embryo development be uniformly defined in order to improve the utilization and impact of this novel technology?

SUMMARY ANSWER: The adoption of the proposed guidelines for defining annotation practice and universal nomenclature would help unify time-lapse monitoring practice, allow validation of published embryo selection algorithms and facilitate progress in this field.

WHAT IS KNOWN ALREADY: An increasing quantity of publications and communications relating to time-lapse imaging of *in vitro* embryo development have demonstrated the added clinical value of morphokinetic data for embryo selection. Several articles have identified similar embryo selection or de-selection variables but have termed them differently. An evidence-based consensus document exists for static embryo grading and selection but, to date, no such reference document is available for time-lapse methodology or dynamic embryo grading and selection.

STUDY DESIGN, SIZE AND DURATION: A series of meetings were held between September 2011 and May 2014 involving time-lapse users from seven different European centres. The group reached consensus on commonly identified and novel time-lapse variables.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Definitions, calculated variables and additional annotations for the dynamic monitoring of human preimplantation development were all documented.

MAIN RESULTS AND THE ROLE OF CHANCE: Guidelines are proposed for a standard methodology and terminology for the of use time-lapse monitoring of preimplantation embryo development.

LIMITATIONS, REASONS FOR CAUTION: The time-lapse variables considered by this group may not be exhaustive. This is a relatively new clinical technology and it is likely that new variables will be introduced in time, requiring revised guidelines. A different group of users from those participating in this process may have yielded subtly different terms or definitions for some of the morphokinetic variables discussed. Due to the technical processes involved in time-lapse monitoring, and acquisition of images at varied intervals through limited focal planes, this technology does not currently allow continuous monitoring such that the entire process of preimplantation embryo development may be visualized.

WIDER IMPLICATIONS: This is the first time that a group of experienced time-lapse users has systematically evaluated current evidence and theoretical aspects of morphokinetic monitoring to propose guidelines for a standard methodology and terminology of its use and study, and its clinical application in IVF. The adoption of a more uniform approach to the terminology and definitions of morphokinetic variables within this developing field of clinical embryology would allow practitioners to benefit from improved interpretation of data and the sharing of best practice and experience, which could impact positively and more swiftly on patient treatment outcome.

† The authors consider that the first two authors should be regarded as joint first authors.

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Key words: embryo assessment / embryo development / morphology / developmental kinetics / time-lapse monitoring

Introduction

Preimplantation embryo development is a dynamic event. Assessment of embryo viability, on the other hand, is commonly based on observations of morphology at pre-defined intervals (ALPHA Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011a,b). The introduction of time-lapse technology enables almost continuous monitoring of embryo development through frequent multiple image acquisitions without potentially compromising viability due to the interruption of culture conditions (Wright *et al.*, 1990; Oh *et al.*, 2007). This technology generates comprehensive information regarding morphology and kinetics of embryo development and facilitates observation of dynamic, and often transient, events occurring between static observation periods. Together, these have been defined as 'morphokinetic' variables (Meseguer *et al.*, 2011).

The potential impact of several confounding factors on morphokinetic variables, such as age (Leibenthron *et al.*, 2012; Hampl and Stěpán, 2013), ploidy (Chavez *et al.*, 2012; Campbell *et al.*, 2013a,b), ovarian reserve (Fréour *et al.*, 2012), infertility indication (Wissing *et al.*, 2012), ovarian response to stimulation (Muñoz *et al.*, 2013), gas composition during *in vitro* culture (Meseguer *et al.*, 2012), culture media (Ciray *et al.*, 2012; Basile *et al.*, 2013), embryo biopsy (Terada *et al.*, 2009; Kirkegaard *et al.*, 2012; Kroener *et al.*, 2012), fertilization method (Lemmen *et al.*, 2008; Cruz *et al.*, 2013), cryopreservation of sperm (Garcia *et al.*, 2012), female body mass index (Bellver *et al.*, 2013) and female smoking habits (Fréour *et al.*, 2013), have been assessed. Strong correlations between embryo kinetics and embryo viability have been demonstrated in various studies (Pribenszky *et al.*, 2010; Wong *et al.*, 2010; Cruz *et al.*, 2011; Meseguer *et al.*, 2011; Azzarello *et al.*, 2012; Campbell *et al.*, 2013b; Chamayou *et al.*, 2013; Herrero *et al.*, 2013; Aguilar *et al.*, 2014). These studies have been challenged by a recent publication, reporting live births following the transfer of blastocysts with 'deviant' morphokinetic profiles (Stecher *et al.*, 2014), which has highlighted the need for IVF clinics to proceed with caution when applying embryo exclusion or selection criteria based on time-lapse literature, mainly due to the fact that, to date, all the studies relating to embryo selection parameters have been retrospective, and randomized control trials are required to establish and confirm morphokinetics parameters which enhance selection of the embryo with greatest implantation potential.

In order to allow the comparison of these morphokinetic variables of embryos within a cohort, between patients and most importantly according to their outcome, such as implantation and live birth, measurements associated with embryo development can be recorded or annotated (automatically or by the embryologist) to allow retrospective analysis. Correct annotation is key in order to exploit reliable information from time-lapse systems and 'almost perfect agreement within and between observers has been reported in a clinic utilizing this technology (Sundvall *et al.*, 2013). Such data can be utilized to build models, or algorithms, which aid prospective embryo selection by distinguishing between the morphokinetic parameters of embryos with known outcomes. For this, standardization of the nomenclature and time of annotations is a prerequisite.

Several clinical time-lapse devices with automatic image capture and software are currently available for IVF practice. The systems vary in several ways including device design (modular within a standard incubator or integrated incubation-camera system), type of microscopy and image acquisition utilized (bright or dark field, single or multiple focal planes and frequency of image collection), culture method (single or group embryo culture) and annotation (automatic, semi-automatic or manual) and in the style of embryo selection software tools available (fixed universal algorithm or customisable software) (Campbell, 2014). Whilst this document aims to provide generic guidelines, some of the parameters, such as those associated with pronuclear morphology and dynamics, described within this document may not be clearly observable with a dark field time-lapse system. Additionally, the ability to record or annotate each parameter defined may be restricted by the device or software available.

The aim of this document is to propose guidelines on the nomenclature of morphokinetic parameters and how and when they should be annotated uniformly. The morphological characteristics of static observations established by ALPHA and ESHRE consortiums were used as reference criteria (ALPHA Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011a,b) and time-lapse publications and abstracts were used as a resource. These parameters trace the development of embryos produced from fresh, or cryopreserved then warmed/thawed oocytes, and follow their development until *in vitro* culture is completed (up to the blastocyst stage). It is recognized that users with specialist interests may choose to annotate in greater detail during particular periods of embryo development and that, for practical purposes, the number of parameters annotated routinely may need to be streamlined by practitioners.

Definitions for dynamic monitoring of human preimplantation embryo development

In the following definitions, '*t*' represents time and references to 'frames' refer to images generated by time-lapse photography during *in vitro* culture (Fig. 1, Supplementary data, Video S1 and Table I). Appearance and fading, of a described variable, are represented by 'a' or 'f' respectively and cell, or episode, number are represented by 'n'.

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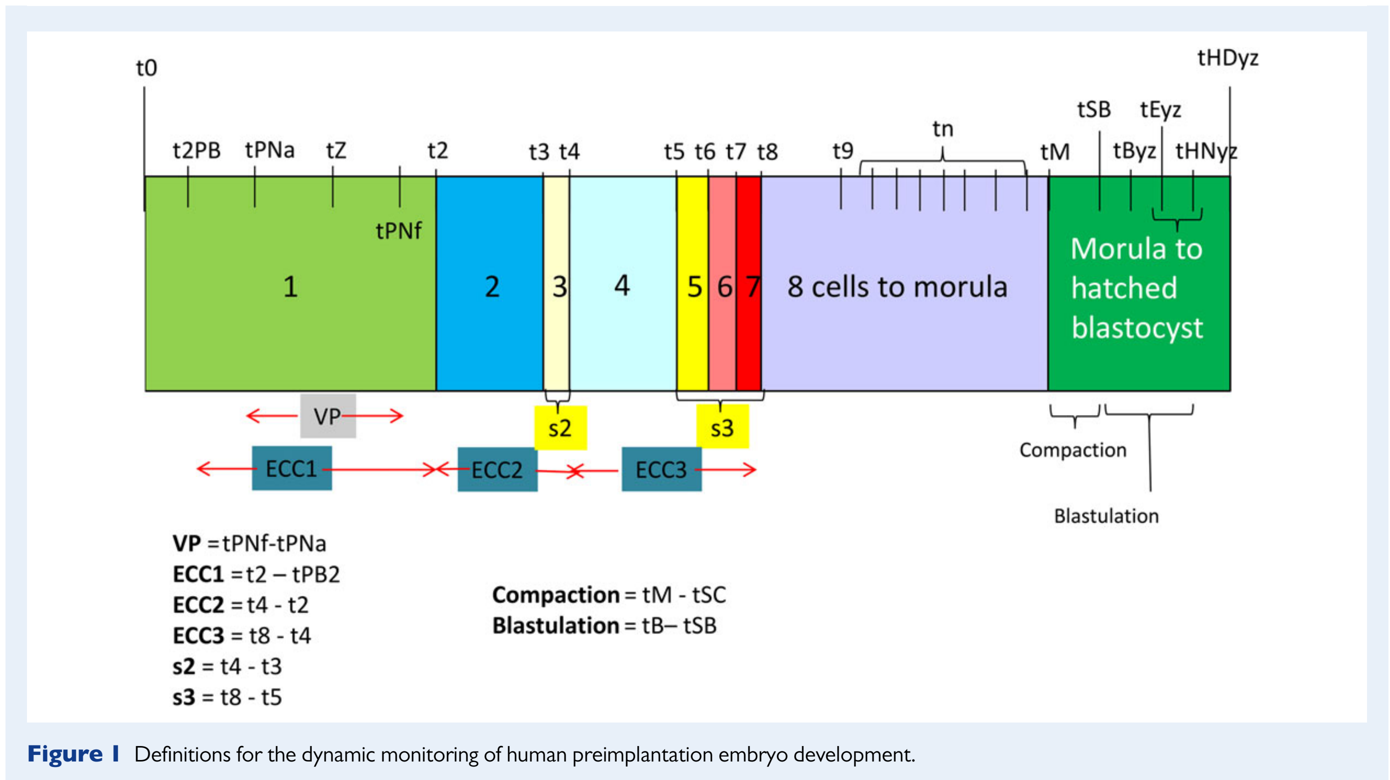

Figure 1 Definitions for the dynamic monitoring of human preimplantation embryo development.

Table I Summary of Morphokinetic variables and proposed definitions.

Timings	
Time	**Definitions of expected events**
t0	Time of IVF or mid-time of micro/injection (ICSI/IMSI)
tPB2	The second polar body is completely detached from the oolemma
tPN	Fertilization status is confirmed
tPNa	Appearance of individual pronuclei; tPN1a, tPN2a; tPN3a..
tPNf	Time of pronuclei disappearance ; tPN1f; tPN2f. . .
tZ	Time of PN scoring
t2 to t9	Two to nine discrete cells
tSC	First evidence of compaction
tMf/p	End of compaction process (last frame before cavity formation) 'f' corresponds to fully compacted; 'p' corresponds to partial compaction
tSB	Initiation of blastulation.
tByz	Full blastocyst (last frame before zona starts to thin) 'y' corresponds to morphology of inner cell mass; 'z' corresponds to morphology of trophectoderm cells
tEyz	Initiation of expansion; first frame of zona thinning
tHNyz	Herniation; end of expansion phase and initiation of hatching process
tHDyz	Fully hatched blastocyst

Each timing defines the first time lapse frame in which the expected phenomenon is observed or detected.

It is recommended that, during manual assessment, time-lapse images are rewound in order to ensure that the correct time point has been selected for the annotation of each variable. In addition, at the outset, users are advised to decide whether non-recording of a variable will indicate its absence or that all variables within the clinic's standard operating procedure will be recorded as absent or present.

t0: The time at which insemination occurs in conventional IVF. For ICSI/IMSI, where the time-lapse monitoring system and practice allows, the time of the sperm injection may be recorded, per oocyte but otherwise, it is the mid-time point from when injection begins and ends for that patient's cohort of oocytes. This time point is used as a start time for the variables below which are measured in hours post insemination/injection, unless otherwise stated.

tPB2: The time at which the second polar body (PB2) is extruded. This is annotated at the first frame in which PB2 appears completely detached from the oolemma. The extrusion of the second polar body can be obscured depending on the position of the oocyte in the well or by cumulus cells in routine IVF insemination.

tPN: The time at which fertilization status is confirmed. It is recommended to annotate fertilization immediately before fading of pronuclei (tPNf) hence coinciding to tZ (time of pronuclear scoring), since no further observational dynamic changes are expected to occur. Appearance of individual pronuclei may be further annotated as tPNna ('n' for individual pronuclei in the order of appearance: 'a'): e.g. tPN1a, tPN2a, tPN3a the initial time at which the first, second, third, etc. pronuclei become visible.

tPNf: The time when both (or the last) PN disappear. This annotation is made at the first frame whereby the embryo is still at the 1-cell stage but pronuclei can no longer be visualized. Pronuclear fading may be further recorded according to individual pronuclei, tPN1f, tPN2f, etc. to

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denote the time at which the first, second or additional pronuclei fade (i.e. similar to annotation of their appearances).

tZ: The time of time-lapse PN assessment. PN are dynamic structures; they move and their morphology can change between tPNa and tPNf (Azzarello *et al.*, 2012). It has recently been reported that the movement of the pronuclei within the cytoplasm and fading of nuclear membranes may be indicative of subsequent blastocyst development potential and hence a novel parameter providing an early indication of the embryo's developmental potential (Wirka *et al.*, 2013). Changes in pronuclear appearance and position may coincide with movement of the nucleolar precursor bodies (NPBs) inside pronuclei, allowing differential PN scoring to be deduced. The time-lapse user group recommends annotation of PN scoring, if required, at the last frame before the pronuclei disappear (i.e. tPNf) because the alteration in pronuclear morphology has been completed.

t2: The time of the first cell cleavage, or mitosis. t2 is the first frame at which the two blastomeres are completely separated by individual cell membranes.

t3: The first observation of three discrete cells. The three cells stage marks initiation of the second round of cleavage.

tn: The first time these numbers of discrete cells are observed (until compaction of blastomeres prevents visualization of individual cells).

tSC: The first frame in which evidence of compaction is present; the initial frame that any (two) cells start to compact is observed. The precise timing of initiation of compaction may be difficult to observe due to the increased number of cells and the type of compaction (partial or complete; as described below).

tMf/p: This marks the end of the compaction process; when observable compaction is complete. The morula may be fully or partially compacted, where f is full and p is partial; the morula has excluded material. The degree and time of compaction has been reported to be associated with blastocyst formation and quality (Ivec *et al.*, 2011).

Dynamic developmental stages of blastocyst formation cannot easily be scored using existing static grading schemes (Gardner and Schoolcraft, 1999), for example the time when the blastocoel constitutes less than half the volume of the embryo (early blastocyst) may not be differentiated with certainty from when it is greater than or equal to half of the volume of the embryo (blastocyst). Therefore the time-lapse user group recommends employment of a novel scoring system for depicting the developmental stage of blastocysts, while it is recommended that the morphology of the inner cell mass (*y*) and trophectoderm (*z*) are graded in agreement with the static parameters within the time frame described for dynamic developmental stages, at fixed time points. This group acknowledges that blastomere biopsy may alter the dynamics of embryo development and blastocyst expansion thereby confounding morphokinetic comparisons with non-biopsied embryos (Kirkegaard *et al.*, 2012). However, facilitative laser breaching of the zona pellucida, at the early cleavage stage, to facilitate herniation of trophectoderm for biopsy, has been reported not to impact downstream development to the full blastocyst stage, compared with unbreached controls (Campbell *et al.*, 2013a).

tSB = initiation/start of blastulation. The first frame when initiation of a cavity formation is observed.

tByz = full blastocyst. The last frame before the zona pellucida starts to thin.

tEyz = initiation of expansion. The first frame when the zona pellucida starts to thin.

tHNyz = herniation. The first frame where extrusion of cell(s) from the zona is observed. This marks the end of the expansion phase and the initiation of the hatching process.

tHDyz = hatched blastocyst. The first frame where the embryo is detached from the zona as a whole.

Calculated variables of dynamic monitoring of human preimplantation embryo development

Calculated variables refer to durations of either events to occur or particular morphologies to become visible and they comprise the period in between initiation and termination or appearance and disappearance, respectively (Table II).

Duration of events related to dynamics of early preimplantation period

PN duration (VP: visible pronuclei)

VP is the time period in which the pronuclei are visible. It is calculated as VP = tPNf − tPNa. If pronuclei are annotated individually, the duration for each can be calculated (e.g. tPN1f − tPN1a).

Duration of cell cycles

The cell cycle is an orderly sequence of events in which a cell duplicates its contents and then divides into two (Fig. 2). The duration of the cytoplasmic cleavage and subsequent rearrangements of the individual blastomeres appears to be highly indicative of subsequent viability of embryos (Ramsing and Callesen, 2006). Prolonged cell cycles can be due to DNA repair or cellular rearrangement prior to cleavage (Ramos and de Boer, 2011).

Cell cycle duration is calculated using time-lapse annotation either according to a single cell division or as a round of mitosis whereby the number of blastomeres doubles. For the first cell cycle, as development begins with the single cell, these are the same. However, the second cell cycle begins with two cells, both of which should subsequently divide, forming two daughter cells each. There are therefore two individual blastomere cell cycles but a single embryo cell cycle, which results in the doubling from two to four cells.

Figure 2 provides a schematic to represent the blastomere cell cycles (cc) and the rounds of divisions herein defined as embryo cell cycles (ECC), resulting in the doubling from two to four, and from four to eight, cells. The cell cycle for blastomere 'a' is calculated as t3 − t2 and documented as cc2a, and for blastomere b as t4 − t2, and documented as cc2b. The cell cycle whereby the embryo reaches four cells from two cells (ECC2) is also calculated (t4 − t2). So, the time that the last cleaving blastomere takes to cleave (from t2 to t4) equates to the duration of the ECC; all individual blastomeres cleave within this time frame. The same applies for the third cell cycle. The duration of the embryo's third cycle (ECC3) is the time it takes the embryo to develop from four to eight cells, and includes four blastomere/cell cycles; a, b, c and d. cc3a is t5 − t4; cc3b is t6 − t4; cc3c is t7 − t4 and cc3d is t8 − t4. ECC3 is t8 − t4 (Fig. 3).

An alternative annotation termed 'synchronization', as defined below, is recommended as a simpler alternative:

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Table II Summary of calculated variables of dynamic monitoring of human preimplantation embryo development.

Annotations	Timings: Calculated duration of events		Dynamic event	
VP	tPNf-tPNa		PN Duration	
ECC1	t2 − tPB2		Duration of first cell cycle	
ECC2	t4 − t2	cc2a = t3 − t2 cc2b = t4 − t2	Duration of second embryo cell cycle	Duration of single blastomere cell cycle
ECC3	t8 − t4	cc3a = t5 − t4 cc3b = t6 − t4 cc3c = t7 − t4 cc3d = t8 − t4	Duration of third embryo cell cycle	Duration of single blastomere cell cycle
s2	t4 − t3		Synchronization of cell divisions	
s3	t8 − t5		Synchronization of cleavage pattern	
dcom	tMf-tSC (full compaction) tMp-tSC (partial compaction)		Duration of compaction	
dB	tB-tSB		Duration of blastulation	
dexp	tHN-tE		Duration of blastocyst expansion	
dcol	tBCend(n)-tBCi(n)		Duration of blastocyst collapse; 'n' is number of episodes of collapse and re-expansion	
dre-exp	tre-exp end(n)-tre-expi(n)		Duration of re-expansion	
dHN	tHN-tHD		Duration of herniation	

It comprises calculation of events related to dynamics of early and late preimplantation period.

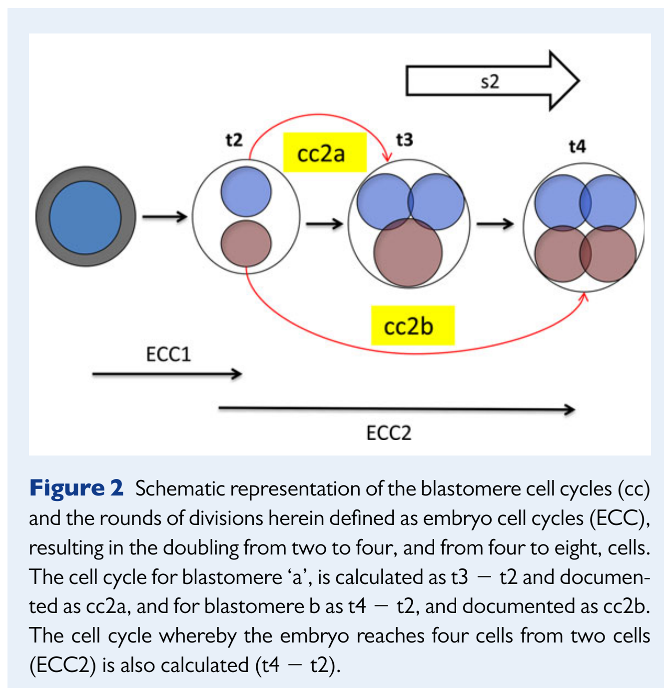

Figure 2 Schematic representation of the blastomere cell cycles (cc) and the rounds of divisions herein defined as embryo cell cycles (ECC), resulting in the doubling from two to four, and from four to eight, cells. The cell cycle for blastomere 'a', is calculated as t3 − t2 and documented as cc2a, and for blastomere b as t4 − t2, and documented as cc2b. The cell cycle whereby the embryo reaches four cells from two cells (ECC2) is also calculated (t4 − t2).

Synchronization

Early embryo development follows a geometric sequence cleavage pattern {1 cell, 2 cells, 4 cells, 8 cells….} as mentioned above, and therefore synchronization can be measured as the time sister cells take to divide into two new cells, reaching the next step in the geometric sequence.

s2 = The synchronicity of the two blastomere divisions within the second cell cycle, calculated as t4 − t3.

s3 = The synchronicity of the four blastomere divisions within the third cell cycle, calculated as t8 − t5.

Duration of cytokinesis (dck)

The duration of each cytokinesis may also be calculated (related to speed of the event and image capture capacity) from the first frame where a cleavage furrow is observed and the time point when cytokinesis is completed.

Duration of events related to dynamics of late preimplantation period

Duration of compaction (dcom)

This is the time period from initiation to cessation of compaction. For full compaction (dcom) = tMf-tSC. For partial compaction (dcom) = tMp-tSC.

Duration of blastulation (dB)

This is the time period from initiation of blastulation to full blastocyst formation (tB-tSB).

Duration of blastocyst expansion (dexp)

This is the time period from initiation of expansion to herniation (tHN-tE).

Duration of blastocyst collapse/re-expansion (dcol/dre-exp)

Regular cycles of expansion and collapse are physiological during blastocyst development (Hardarson et al., 2012). For collapse, these are

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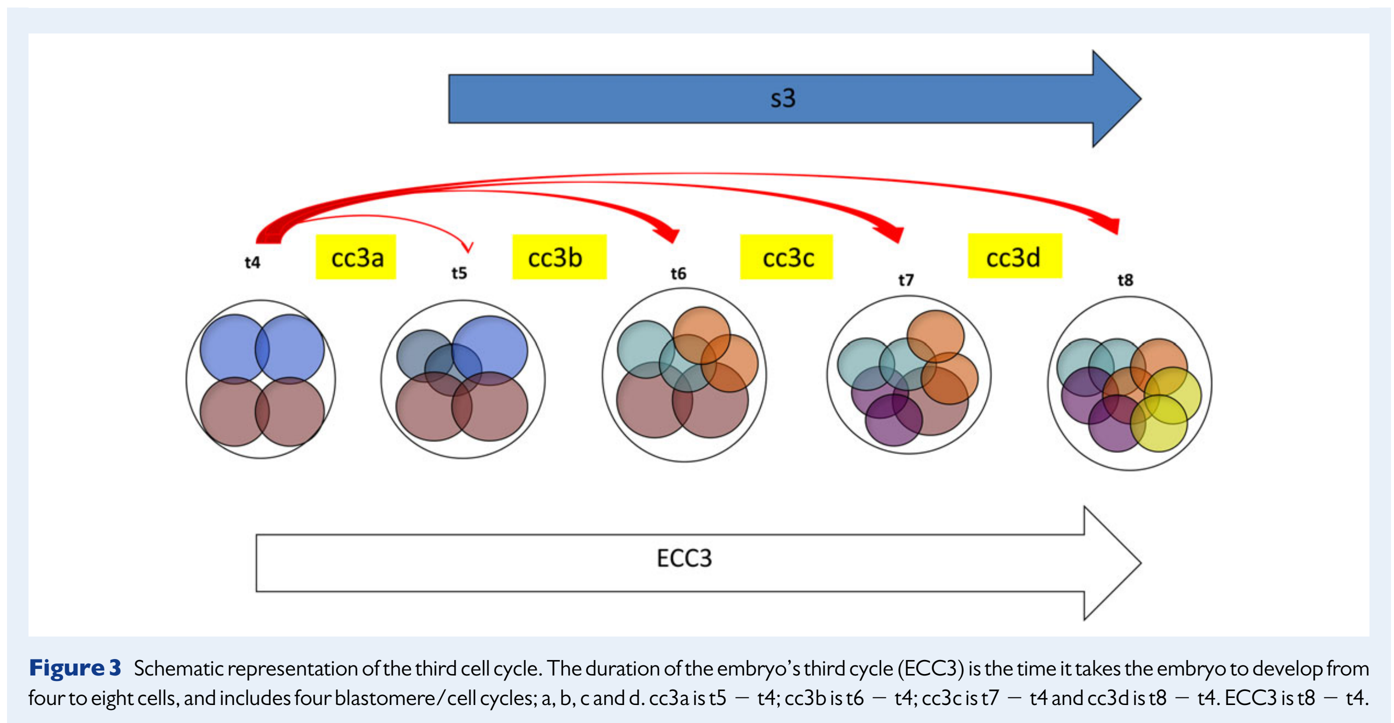

Figure 3 Schematic representation of the third cell cycle. The duration of the embryo's third cycle (ECC3) is the time it takes the embryo to develop from four to eight cells, and includes four blastomere/cell cycles; a, b, c and d. cc3a is t5 − t4; cc3b is t6 − t4; cc3c is t7 − t4 and cc3d is t8 − t4. ECC3 is t8 − t4.

defined as tBCi(n) and tBCend(n), for initiation and completion of the episode, respectively, where 'n' corresponds to the subsequent episodes (for example, tBCi1 = first expansion of blastocyst cavity which follows tBCend1 = first collapse of the blastocoel). The duration of each phase of expansion-contraction cycle can be calculated; the initiation of collapse is annotated according to the first frame in which the blastocoel volume visibly decreased when compared with the volume (diameter) on the previous frame. The final frame prior to the initiation of re-expansion (tBCend) marks the end of the collapse episode. The period in between is the 'duration of collapse (dcol)' and is tBCend(n) − tBCi(n). The initiation of re-expansion is annotated according to the first frame in which the blastocoel volume visibly increased compared with the volume (diameter) on the previous frame. The duration of re-expansion (dre-exp) is tre-expend(n) − tre-expi(n).

Duration of herniation (dHN)

This is the time period from initiation of herniation to hatching (tHN-tHD).

Additional annotations

The proposed annotation for initiation or cessation of the appearance of these events is described below. From these, the duration, or visible period for the event may be calculated as follows: d(event) = t(event)(end) − t(event)(i) (Table III).

Smooth endoplasmic reticulum clusters

Aggregation of smooth endoplasmic reticulum (SER) in oocytes has been associated with suboptimal outcome in some patients (Ebner *et al.*, 2008) (Supplementary data, Video S2). Presence of SER should be annotated as the time points for the appearance, tSER(i) and disappearance, tSER(end).

Fragmentation

To annotate stage-specific fragmentation, *x*%ftn should be used, where *x* is the percentage of fragmentation and tn is the last cell division that was completed. The time-lapse user group suggests the annotation of embryo fragmentation associated with blastomere number, at the final frame prior to the subsequent round of cleavage, because, as development proceeds, embryonic fragments may remain as separate units, or alternatively, may be reabsorbed by the same blastomere from which they were produced or fuse with a neighbouring blastomere (Hardarson *et al.*, 2001; Chavez *et al.*, 2012). For example: '10%ft2' means presence of 10% fragmentation during the final frame 't' at the 2-cell stage.

Annotation of nuclear morphology

nMONO (mononucleated) where 'n' represents the number of blastomeres in which a unique nucleus is seen.

nBI (binucleated) number of blastomeres in which two nuclei per cell are visible.

nMULTI (multinucleated) number of blastomeres in which more than two nuclei are visible. This definition includes micronuclei.

Any cytoplasmic structure without any visible nucleus during the entire cell cycle should be considered as fragments regardless of their sizes.

The time of appearance ('a') and fading ('f') of nuclei can be annotated among individual blastomeres enabling calculation of duration of nuclear visibility. For example, 't4MONO1(a)' depicts the time when the single (mono) nucleus becomes visible in the 'first blastomere' (chosen arbitrarily) of a 4-cell embryo, while t4mono3(f) depicts the fading time of the single nucleus in the third blastomere of the 4-cell embryo.

Blastomere (a)symmetry

Asymmetry of blastomeres is physiological in certain periods of mitotic cycles (e.g. those excluding 2, 4 and 8-cell stages) and such patterns contribute positively to overall embryo selection parameters (Sela *et al.*,

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Table III Summary of additional dynamics annotations.

Additional annotations	
Time	**Definitions of special annotations**
devent	Duration of event = t(end)-t(i)
tSER(i)	Appearance of SER
tSER(end)	Disappearance of SER
x%ftn	Stage-specific fragmentation '*x*' is percentage of fragmentation 'tn' is last cell division that was completed
nMONO	'n' is number of mononucleated blastomeres
nBI	'n' is number of binucleated blastomeres
nMULTI	'n' is number of multinucleated blastomeres
Even/Uneven cells	Symmetry of blastomere sizes
tTM	Trichotomous mitosis
tFu	Time of cell fusion
tPA	Time of planar blastomere arrangement
tRoll	Time of embryo rolling without divisions
tRoll (i)	Initiation of embryo rolling
tRoll(end)	End of embryo rolling
tCW	Timing of cytoplasmic waves
tCS	Timing of cytoplasmic strings
tV S/M	Presence of vacuoles 'S' represents single vacuole; 'M' represents multiple vacuoles
tG	Appearance of granularity of the cytoplasm

Each timing defines the first time lapse frame in which the expected phenomenon is observed or detected.

2012). Therefore it is recommended that blastomere (a)symmetry is annotated at the end of 2-, 4- and 8-cell stages, during which symmetry of blastomeres is considered optimal morphology. The degree of deviation (i.e. uneven/severe uneven) from normal should be considered as a compromise from embryo viability potentially accompanied with aneuploidy (Hardarson *et al.*, 2001). Cells are described uneven (at the 2-, 4-, 8-cell stages) if blastomeres are more than one third different from sibling cells in size (Puissant *et al.*, 1987). The time-lapse user group encourages annotating alterations in the evenness of blastomeres at these cell stages at the beginning to the end of a particular blastomere division, which may have an impact on subsequent embryo viability.

Irregular cleavage events

Rapid cleavage

Rapid cleavage is a novel dynamic event, which was first described and defined by Rubio *et al.* (2011) as a 'direct cleavage from two to three cells occurring in <5 h'. They have been reported to occur in around 14% of all embryos and they were demonstrated to be one of the most conclusive embryo de-selection parameters, since they compromise implantation capacity (Rubio *et al.*, 2011, 2012).

The time-lapse user group consider that this phenomenon described by Rubio and colleagues is more accurately defined as 'rapid cleavage'. Rapid cleavage describes cleavage of a single cell to two daughter cells occurring faster than the 'normal' duration, which is yet to be defined specific to each cell cycle. This phenomenon may be related to errors in cell cycle mechanisms, which result in this premature cytokinesis. Such irregular cleavage patterns can occur at any cell stage but are most readily identified during early cleavage embryo stage development. As the optimal or normal duration of early cleavage events has not been defined, rapid cleavage cannot yet be annotated as present or not, unless an arbitrary value is introduced. Rather, the durations associated with cleavages can be calculated from the annotation of the embryo reaching sequential cell stages and if and when the normal cell cycle duration is defined, precocious or rapid cleavage can then be identified against normal limits and based on clinical evidence.

Trichotomous mitosis

The first demonstration of, what this proposal is referring to as, trichotomous mitosis was performed and reported by Kola *et al.* (1987) who, employing time-lapse cinematography, described it as an event occurring at the first cleavage of tripronucleated oocytes in order to prevent them developing into triploid embryos (Supplementary data, Video S3). Kola's group considered the spindle forming at the first cleavage in such zygotes, to be tripolar. A year earlier, Angell *et al.* (1986) introduced the concept of direct cleavage and a rapid second division, although they were unable to distinguish between the two irregular phenomena as time-lapse imaging was not employed.

This irregular cleavage event, an aberrant cleavage from a single cell directly to three daughter cells (irrespective of the number of pronuclei), defined as trichotomous mitosis is observable using time-lapse imaging. As in rapid cleavage, this type of irregular division may also occur at any stage of development. Trichotomous mitoses and rapid cleavages can be distinguished from each other as different developmental patterns, possibly associated with differing biological events. Trichotomous mitosis may be associated with errors in spindle apparatus (e.g. tripolar). Trichotomous mitosis, as defined above, can be calculated, for example if $t3 - t2 = 0$, when the embryo did not, at least observably within the acquired images, exist at the 2-cell stage as it cleaved directly to three cells or in a general formula if $t(n) - t(n-1) = 0$. This may be annotated on observation as tTM.

Cell fusion (independent of compaction)

This is defined as a reduction in the number of cells of an embryo during its development due to the merging, or fusion, of cells giving the appearance of a reversed cleavage event (Supplementary data, Video S4). This phenomenon must be distinguished from fragment internalization or re-absorption through identification of a nucleus within the cells involved, prior to occurrence of this event. It is also distinguishable from the merging of cells during compaction preceding morula formation. In an observational study of 1698 zygotes, this phenomenon was observed in 10% of all embryos (Hickman *et al.*, 2012). Although this study demonstrated that cell fusion did not impair embryo development to the blastocyst stage, and was not associated with embryo ploidy, further research is needed to determine the clinical significance of embryos exhibiting such cell fusion, and the mechanisms that cause embryos to undergo this process. When observed, this should be annotated as tFu.

Planar arrangement. Cleavage planes of a 4-cell-stage embryo are considered as normal when they are perpendicularly orientated and blastomeres are tetrahedrally arranged. Some embryos may display

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parallel-orientated cleavage axes and are non-tetrahedral or planar possibly due to the disruption of the mitotic spindle (Ebner *et al.*, 2012).

The reported incidence of planar embryos ranges between 3% (Ebner *et al.*, 2012) and 21% (Cauffman *et al.*, 2014). While some associated them with poor implantation capacity (Ebner *et al.*, 2012, Paternot *et al.*, 2014), others reported similar clinical outcomes to tetrahedral embryos (Cauffman *et al.*, 2014). When observed, this may be annotated on observation, tPA.

Embryo rolling. Time-lapse monitoring allows the visualization of embryo rolling; the blastomeres move on themselves without dividing. Prolonged periods of cellular rearrangements following a cell division event can be indicators of poor embryo viability and poor developmental competence as well as poor implantation potential (Ramsing *et al.*, 2007; Cruz *et al.*, 2012). When observed, its beginning and termination should be annotated as tRoll(i) and tRoll(end), respectively.

Cytoplasmic waves. Mouse studies have shown a correlation between rhythmic cytoplasmic movements in oocytes and subsequent development to the blastocyst stage (Ajduk *et al.*, 2011). These movements are caused by contractions of the actomyosin cytoskeleton triggered by calcium oscillations induced by fertilization. Similar waves have been identified in human oocytes; however, they were not correlated with embryo development (Swann *et al.*, 2012). When observed, the waves may be annotated tCW(i) and tCW(end), for their beginning and termination, respectively.

Cytoplasmic strings. Cytoplasmic strings can often be observed in the blastocyst traversing the blastocoel (Supplementary data, Video S5). They are commonly present in early blastocysts and may withdraw as the blastocyst expands. Their persistence in the expanded blastocyst has been associated with poor embryo quality, poor media conditions or a breakdown in polarization (Scott, 2000; Hardarson *et al.*, 2012). More recently, and using time-lapse data from transferred blastocysts, the presence of cytoplasmic strings was observed not to compromise viability (unpublished data).

Time-lapse annotation of the presence or absence of cytoplasmic strings is recommended in order to retrospectively assess their potential value in assessment of embryo viability. When observed, the proposed recommendation for annotation is tCS.

Zona pellucida. The time-lapse user group accepts possible effects on patient or cycle specific basis and proposes annotating exceptional observations regarding the morphology of the zona pellucida. As the appearance of the zona pellucida remains the same throughout the pre-expansion stages, its annotation does not require to be related to time.

Vacuoles. The time-lapse user group proposes the annotation of the presence of vacuoles and the time and duration of appearance. When observed, the proposed guideline for annotation is tV S/M (i) and tV (end), where S refers to a single vacuole; M to multiple vacuoles. As used for other annotations, 'I' and 'end' should be used to annotate the first appearance of vacuoles (i) and when they are no longer visible (end). Measurement of their dimensions may be possible with time-lapse device software tools.

Granularity. Cytoplasmic granularity is poorly defined and its impact on embryo development and viability is not clearly understood. It may be persistent or transitional and should be annotated at the time it is observed and, if applicable, ends. When observed, the proposed guideline for annotation is tG (i) and tG (end).

Discussion

In recent years, the number of studies relating to dynamic development of the preimplantation human embryo development under *in vitro* conditions has increased rapidly and this trend is expected to continue. This paper proposes definitions and annotations for events occurring and observed during dynamic development of the preimplantation human embryo development. During the preparation of this paper, a review paper, incorporating a proposal to standardize nomenclature for time-lapse embryo imaging has been published (Kaser and Racowsky, 2014). While some nomenclature is common, these dedicated guidelines describe and define the dynamic events of preimplantation embryo development more comprehensively, with supporting information and resources. Furthermore, with regard to the important objective of defining clear guidelines in order to maximize its utilization, the current proposal lacks some definitions used by Kaser and Racowsky (e.g. 'formation of expanding blastocyst') since they have been considered too subjective and finite, by this group, for dynamic monitoring as is discussed within. However, others have been highlighted within this document, omitted by Kaser and Racowsky, which have been reported to have clinical significance (e.g. irregular cleavage events).

The major challenge in proposing guidelines for time-lapse technology in IVF arises from the nature and variability of observable events; that they are dynamic. Dynamic events are more difficult to define compared with static observations since the additional dimension of time comes into play. A specific dynamic event may be regarded to occur at its initiation, i.e. the first frame of observation, its end, i.e. that it has been completed, or anytime in between while the event progresses, for example the time when the defined morphology according to static assessments is observed. Examples of such confusions appear as the literature, surrounding morphokinetics of human embryo development, accumulates: pronuclear fading (PNf) as described by this time-lapse user group has been previously defined as the last frame (recorded as tC) where both pronuclei were observed (Chamayou *et al.*, 2013), with a difference of one consecutive frame and as pronuclear disappearance (Lemmen *et al.*, 2008) or pronuclear breakdown (Azzarello *et al.*, 2012). The importance of consensus is enhanced in situations where reference point(s) are established. As an example, time of insemination during ICSI has been defined as 'midway through ICSI' (Campbell *et al.*, 2013a), where other previous studies refer to the time where the procedure has been completed (generally referred as 't0' as in Chamayou *et al.*, 2013). It is of utmost importance that future studies refer to a consented terminology in order to minimize the negative impact of dynamics of these events, and instead provide a benefit gained through this novel technology.

During dynamic monitoring of preimplantation embryo development, some events are easier to observe and define than others. Cleavage-stage cell cycles are such examples since the beginning or termination of dividing blastomeres can clearly be observed through distinct cell membranes unless there is extensive fragmentation. Due to the relative objectivity, ease of study and annotation, early morphokinetic events have been most studied and consequently they feature more frequently, than later events, in algorithms assessing developmental competence

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and/or implantation prediction. For example, time between division from two to three cells and from three to four cells have been found predictive in two different algorithms; those described by Wong et al. (2010) and Meseguer et al. (2011) and referred to as P2 and P3 by the former or cc2 and s2 by the latter groups, respectively. In addition to these parameters, Wong et al. (2010) suggested P1, which referred to the duration of first cytokinesis and Meseguer et al. (2011), t5 as being the duration for the embryo to reach the 5-cell stage, as an adjunct. In the currently proposed terminology, descriptions suggested in earlier publications have been used as much as possible; however, some have been modified with the intention of simplification and/or clarification. For example, terminology regarding definition of cell cycles has been changed in this proposal; the second cell cycle, as the time the embryo takes to cleavage from two to three cells, is now collectively called CC2, additionally, the time the embryo requires to cleave from three to five cells is called the CC3 (Wong et al., 2010; Meseguer et al., 2011; Herrero et al., 2013; Basile et al., 2014). CC3 was demonstrated as a key parameter associated with greater implantation rates compared with other downstream parameters (Herrero et al., 2013). In this document, the term ECC2 has been proposed, which spans the period where the embryo develops from two to four cells and is divided to CC2a and CC2b, as divisions from two to three and to four cells, respectively. Likewise, the third embryo cell cycle refers (ECC3) to cleavage from four to eight cells and now proposed to be subdivided to CC3a-d in accordance to consecutive blastomere divisions. Because cell cycle terminology and subsequent calculations may be confusing to future investigators, use of terminology regarding synchronization is encouraged. Nevertheless, findings and calculations previously reported, which show a relationship between the duration of blastomeres cell cycles are not invalidated by this proposed terminology.

It is expected that uptake of proposed guidelines for time-lapse terminology will enable utilization of events occurring at later stages of embryo development. Without consensus in terminology and annotation, morula and blastocysts stages of development may be more difficult to track and evaluate, as events are more complex, transitional, overlapping and optically restricted due to the increased number of cellular and a-cellular structures. There are few studies assessing morphokinetics of later development stages yet diversity among terminology and annotations are nevertheless evident. Time of full blastocyst formation has been annotated as tB and described as 'blastocoel filling the embryo with <10% increase in diameter' and time of expanded blastocyst as 'blastocyst increased in diameter by >30% in diameter concomitant to initiation of zona thinning' (Campbell et al., 2013a,b); however, tB has also been defined as the frame in which 'a crescent-shaped area began to emerge from the morula' in the study by Chamayou et al. (2013) and the successive frame was the time of expanded blastocyst (tEB), consistent with the 'increase of the overall volume of the embryo and expansion of the blastocoel cavity'. Many of these discrepancies may refer to concepts that are easily understandable. However, the rapid increase in the number of publications concerning time-lapse technology may trigger an endless list of definitions based on the researchers point of view unless a consensus between time-lapse users is not reached.

Through introduction of this novel technology, many events, which had remained obscure with static observation, have come to light. Some examples of such events are abnormal cleavage patterns like trichotomous and reverse cleavages, and rolling of the embryo. Terminology regarding description of these novel events may cause more confusion. For example 'irregular divisions' have been collectively referred to as those from one to three and/or two to five cells in <5 h (Campbell et al., 2013a,b); the former has also been defined as abrupt division (Meseguer et al., 2011), direct (Rubio et al., 2012), tri-polar cleavage (Wong et al., 2010) or very recently as abnormal cleavage (Wirka et al., 2014). A round of mitosis, in the early human preimplantation embryo has been documented to occur 'normally' 10 to 12 h (Cummins et al., 1986). It is yet to be established how much viability may be compromised in those embryos cleaving between 5 and 10 h.

It should be noted that the time-lapse user group does not propose it being necessary to annotate all the parameters listed in this proposal. It is also likely that once the technology becomes available to a wider population of professionals, events that may have escaped current attention will also be described. An update of this current paper will then become a necessity. As stated earlier, the ultimate goal for successful integration of morphokinetic parameters into clinical practise should include: (i) their statistical and biological significance and reproducibility, (ii) their validation through prospective clinical studies proving safety, efficacy and practical utility and (iii) development of reliable technology to measure and quantify such markers (Wong et al., 2013).

Within this rapidly progressing and promising area of reproductive medicine, practitioners now have an additional and increasingly reliable tool for improving embryo selection. The more time-lapse images are interrogated and annotated, and data output is standardized, the more we may understand whether an optimal morphokinetic profile exists. With this, will come the possibility to develop new, and fine tune existing, morphokinetic embryo selection criteria. Time-lapse monitoring is a tool which provides precise information on embryo development, but also has the potential to train, educate and most importantly enhance clinical outcome.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

H.N.C. played a role in the original conception of the manuscript, and in its design and drafting and gave final approval. A.C. was involved in critical and grammatical revision and drafting of the manuscript and gave final approval. I.E.A. took part in the organizing the meetings, drafted the manuscript, and acquired pictures and videos and gave approval. J.A. was involved in writing the manuscript and critical revision and gave final approval. S.C. took part in the original conception and design of the manuscript and writing of the article and gave final approval. M.E. played a role in drafting and critical revising of article and gave final approval. S.S. played a role in writing the manuscript and critical revision and gave final approval.

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Conflict of interest

H.N.C. is a scientific consultant for ESCO, supplier of Miri TL. I.E.A. is a minor shareholder in Unisense Fertilitech, supplier of the EmbryoScope. Full disclosures of all participants are presented herein. The remaining authors have no conflict of interest.

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