Recommendations for good practice for the use of Time-Lapse technology

Authors

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Introduction

Accurate embryo selection and optimal incubation environment are two defining factors for the successful outcome of in vitro fertilisation (IVF) treatment. During in vitro culture, embryos are typically assessed by morphological grading, in order to predict embryo developmental competence and implantation potential. The morphological features evaluated may include pronuclear alignment, stage-specific number and size of blastomeres, fragmentation, multinucleation, blastocyst expansion, inner-cell mass and trophectoderm appearance (Ahlstrom, et al., 2011, Cummins, et al., 1986, De los Santos, et al., 2016, Scott, 2003). Traditional morphological evaluation is typically performed at static time-points which provides a "snap-shot" of embryo development. However, these approaches usually require the physical removal of the embryos from the incubator, exposing them to fluctuations in temperature, pH and oxygen levels. Crucially, it has limited ability to predict embryo developmental competence and ongoing pregnancy, with high intra/inter-observer variability (Guerif, et al., 2007, Rijnders and Jansen, 1998). In an attempt to standardize morphological evaluation across different laboratories, a consensus on the timings and characteristics of morphology assessment of human embryos was published by ESHRE and Alpha Scientists in Reproductive Medicine (2011). Although this was undoubtedly a step in the right direction, the limitations of static morphology evaluation were not overcome.

Therefore, Time-lapse technology (TLT) has been introduced in clinical IVF. This has the effect of increasing the number of observations and enabling the assessment of developing embryos in a dynamic fashion. In parallel, TLT offers an uninterrupted culture environment, thus minimizing the need to remove embryos from the incubator (Meseguer, et al., 2012).

A TLT system typically comprises of a stand-alone incubator with an integrated inverted microscope coupled to a digital camera. Alternatively, an optical system can be placed inside a conventional incubator, although this approach is less commonly used. In either case, digital images are collected at regular intervals and at different focal planes throughout development, and subsequently processed into videos. Thus, TLT enables embryologists to record preimplantation embryo development in a dynamic, real-time manner and permits the interpretation of morphokinetic events more precisely. Data from these observations can be annotated and analysed using integrated TLT software, facilitating the development of more complex embryo selection/deselection algorithms (Ciray, et al., 2014).

The paper will describe different types of TLT systems, discuss the potential benefits and uses of TLT, and evaluate the impact on laboratory workflow, in order to inform IVF clinics as they

choose a system appropriate for their own circumstances. This paper is not intended as a manual on the use of TLT, nor does it provide a systematic description of clinical evidence. The most recent review of clinical evidence was published by Armstrong et al. (Armstrong, et al., 2019)

Materials and methods

European Society of Human Reproduction and Embryology (ESHRE) recommendations for good practice are developed based on the Manual for development of recommendations for good practice (N. Vermeulen, N. Le Clef, A. D'Angelo , Z. Veleva, K. Tilleman, Manual for development of recommendations for good practice, version 2018), which can be consulted at the ESHRE website (www.eshre.eu/guidelines). The manual describes a 12-step procedure for writing recommendations documents by the guideline development group, supported by the ESHRE methodological expert.

The current paper is the result of a 2-day consensus meeting of expert professionals. In preparation of the meeting, information was collected by means of published surveys, manufacturers information, and narrative reviews. In addition, relevant published data were collected from a literature search. Experts in the field each prepared a draft of a pre-allocated section, after which these were discussed until consensus within the group was reached. After the meeting, ESHRE members were invited to submit comments during stakeholder review of the draft; it was published on the ESHRE website between 21 June and 2 August 2019. Each comment was documented in a review report, and appropriate changes were made in the manuscript. A review report is published on the ESHRE website.

Recommendations

BOX: Before getting started with TLT:

- Clearly identify the reasons to introduce a TLT system
- Assess financially and operatively pros and cons to acquire a TLT system
- Identify whether morphokinetic parameters will be used in selection/deselection of embryos
- Identify from scientific literature the morphokinetic parameters of interest and assess how to monitor and use them
- Find the suitable system based on considerations on culture conditions/systems and costs
- Once introduced in the lab, find the appropriate system settings
- Identify and train one embryologist who will develop the role of “TLT referent”; the designated person will be responsible for the annotation of morphokinetic variables (to avoid initially inter-operator variations with other members of staff) and for the implementation of quality control programs

1. Why clinics can use TLT (Significance of TLT).

1.1 Embryo assessment

The identification of an embryo that is most likely to develop into a healthy child remains an unmet need in IVF. This section will evaluate whether morphokinetic embryo assessment by TLT may assist to achieve this goal.

Embryo assessment based on fertilization markers

Markers of embryo quality at early stages of development are of particular value to clinics where extended embryo culture is not feasible. Following pioneering research by Payne et al. (Payne, et al., 1997), TLT enabled Coticchio and colleagues (Coticchio, et al., 2018) to draw an in-depth map of events occurring during fertilization, which may be putative indicators of embryo quality. Twenty-eight parameters that were previously unknown or were poorly documented were described. The time-intervals between four morphokinetic events were shown to predict embryo quality on day 3. These were (1) cytoplasmic halo appearance → disappearance, (2) halo appearance → PN breakdown, (3) PN breakdown → first cleavage (t2) and (4) male PN appearance → male PN breakdown (tPNf) (Coticchio, et al., 2018). Further studies assessing these markers as predictors of embryo quality on day 5 and clinical outcome are required. However, TLT is the only existing technology that enables assessing embryos based on such criteria. Although tPB2 and PN morphology did not predict live birth, tPNf was associated with live birth; tPNf of zygotes resulting in live birth was significantly longer than the tPNf of the no live birth group (Azzarello, et al., 2012). Besides, it was reported that erratic PNs movement within the cytoplasm and delayed fading of nuclear envelopes are indicative of compromised embryo developmental potential (Athayde Wirka, et al., 2014).

Embryo assessment and cleavage features

Discrete cleavage anomalies, mostly undetectable with static embryo assessment, have been described and correlated with embryo quality, chromosomal status and implantation potential (Table 1) (Basile, et al., 2014, Desai, et al., 2018, Meseguer, et al., 2011). Wong and colleagues have shown that blastocyst development can be predicted with high sensitivity based on parameters identified by tracking embryo up to 4 cell-stage, namely, the duration of the first cytokinesis, the time interval between the end of the first mitosis and the initiation of the second and the time interval between the second and third mitoses (Wong, et al., 2010).

Guidelines were proposed on the nomenclature and annotation of the events observed during embryo development followed with a TL system (Ciray, et al., 2014). The variable and the description of the events are summarized in Table 2.

Two to five cell cleavage timing and intervals during two cleavages (t5 and s2, cc2) were shown to be most predictive parameters for embryo viability and implantation (Meseguer, et al., 2011). Recently, an association between irregular division (Desai, et al., 2018, Liu, et al., 2014), start time of blastulation (tSB), expansion (tEB), the interval tEB-tSB and aneuploidy was reported (Desai, et al., 2018). Furthermore, it was demonstrated that presence of at least two abnormal cleavage features was a bad prognosis for embryo chromosomal status (Desai, et al., 2018).

Since the development of TLT there have been numerous attempts to assess the clinical and biological significance of the parameters described in Table 2. A non-exhaustive summary of these studies is available in Table 3.

123 **Table 1:** Atypical embryo cleavage phenotypes observed with TLT or classic once per day embryo morphology
124 assessment.

Name of phenotype	Explanation	Observed exclusively or better by TLT	References
Abnormal syngamy	Erratic PN movement in the cytoplasm	Exclusively	(Athayde Wirka, et al., 2014, Coticchio, et al., 2018)
Asynchronous appearance of two pronuclei	Disappearance of one and appearance of another pronucleus	Exclusively	(Coticchio, et al., 2018)
Pronuclei reappearance	Pronuclei breakdown and reappearance	Exclusively	(Coticchio, et al., 2018)
Aberrant behaviour of female pronucleus	Extrusion of the 3rd polar body instead of female pronucleus formation	Exclusively	(Mio, et al., 2014)
Fragmentation of pronuclei	Formation of micronuclei	Better	(Mio, et al., 2014)
Fusion of pronuclei	A pronucleus formed by the fusion of two preexisting pronuclei	Exclusively	(Mio, et al., 2014)
Unipolar cleavage furrow	Appearance of cleavage furrow on one site of the zygote	Exclusively	(Hojnik, et al., 2016, Wong, et al., 2010)
Tripolar cleavage furrow	Appearance of 3 cleavage furrows on the zygote	Exclusively	(Wong, et al., 2010)
Pseudofurrows	Zygote presenting oolemma ruffling before cytokinesis	Exclusively	(Athayde Wirka, et al., 2014, Wong, et al., 2010)
Absent cleavage	Arrest in zygote stage despite normal fertilization	Better	(Barrie, et al., 2017)
Direct cleavage	Cleavage of zygote or one blastomere to three cells in the first or second cell division cycle	Exclusively	(Athayde Wirka, et al., 2014, Barrie, et al., 2017, Fan, et al., 2016, Meseguer, et al., 2011, Rubio, et al., 2012)
Reverse cleavage	Fusion of two cells into one blastomere	Exclusively	(Athayde Wirka, et al., 2014, Barrie, et al., 2017, Liu, et al., 2014)
Multinucleation	Blastomere with >1 nucleus	Better	(Desai, et al., 2014)
Internalization of cellular fragments	Fragments reabsorbed into one mother blastomere	Exclusively	(Hardarson, et al., 2002, Mio, et al., 2014)
Irregular chaotic division	Disordered cleavage behaviour with uneven cleavages and fragmentation	Better	(Athayde Wirka, et al., 2014, Barrie, et al., 2017)
Early compaction	Formation of tight junctions between blastomeres in Day-3 or even Day-2 embryos	Better	(Iwata, et al., 2014, Le Cruguel, et al., 2013)
Blastocyst collapse	Complete or almost complete disappearance of blastocoel and consequent blastocyst shrinkage	Better	(Bodri, et al., 2016, Kovacic, et al., 2018, Marcos, et al., 2015)

125

Table 2: Nomenclature of morphokinetics parameters.

	Terminology	Description of the event
	tPB2	The second polar body is completely detached from the oolemma
	tPNa	Appearance of individual pronuclei; tPN1a, tPN2a; tPN3a..
	tPNf *	Time frame of pronuclei disappearance; tPN1f; tPN2f...
	tZ	Time of PN scoring (last time frame before tPNf)
	tn *	First time frame at which an embryo reaches n number of blastomeres (e.g. t2, t3, t4)
	tTM	Trichotomous mitosis at different stages
	tSC	First evidence of compaction
	tM	Time of completion of compaction process (in case some blastomeres are excluded, it might be difficult to assess the real time frame)
	tSB	Initiation of blastulation (first frame in which the blastocyst is visible)
	tB	Full blastocyst (last frame before zona starts to thin)
	tE or tEB	Initiation of expansion; first frame of zona thinning (also called TEyB 'y' corresponds to morphology of inner cell mass; 'z' corresponds to morphology of trophectoderm cells)
	tHN	Herniation; end of expansion phase and initiation of hatching process (also called tHNyz)
	tHD or tHB *	Fully hatched blastocyst (also called tHDyz)
Dynamic events	Not mentioned	Time between nuclear envelop breakdown and subsequent division to 2 cells
	s2	Time between division to 3 cells and subsequent division to 4 cells
	s3	Time between division to 5 cells and subsequent division to 8 cells
	ECC1	Duration of the first cell cycle
	cc2	Blastomere cell cycle: Duration of the second cell cycle (a=t3-t2, b=t4-t2)
	cc3	Blastomere cell cycle: Duration of the third cell cycle (a=t5-t4, b=t6-t4, c=t7-t4, d=t8-t4)
	ECC2	Embryo cell cycle: t4-t2
	ECC3	Embryo cell cycle: t8-t4
	Blastocyst contraction	A decrease in blastocoel volume
Cryopreserved /warmed blastocyst	tRE	Time of the start of re-expansion (First frame in which the blastocoele reforms or increases in size)
	tCRE	Time of completion of re-expansion (First frame the blastocyst occupies the whole perivitelline space)

General comment: depending on the configuration of the TLT system, some events may not be seen. Adapted table from consensus paper (2011).

* parameters with the highest concordance between operators.

131 **Table 3:** Parameters with biological/clinical significance

Markers	Prediction/ Outcome	Reference
Time interval cytoplasmic halo appearance → disappearance	Embryo quality on day 3	(Coticchio, et al., 2018)
Time interval halo appearance → PN breakdown	Embryo quality on day 3	(Coticchio, et al., 2018)
Time interval PN breakdown → first cleavage (t2)	Embryo quality on day 3	(Coticchio, et al., 2018)
Time interval male PN appearance → male PN breakdown	Embryo quality on day 3	(Coticchio, et al., 2018)
PNs movement and fading	Blastocyst formation	(Athayde Wirka, et al., 2014)
Appearance of nuclei after first cleavage	Pregnancy success	(Lemmen, et al., 2008)
Duration of the first cytokinesis	Blastocyst formation	(Wong, et al., 2010)
Time interval between the end of the first mitosis and the initiation of the second	Blastocyst formation	(Wong, et al., 2010)
Time interval between the second and third mitoses	Blastocyst formation	(Wong, et al., 2010)
tPNf	Live birth	(Azzarello, et al., 2012)
	No difference in implantation	(Chamayou, et al., 2013, Kirkegaard, et al., 2013)
	Implantation	(Aguilar, et al., 2014, Wu, et al., 2016)
tPB2	Implantation	(Aguilar, et al., 2014)
Length of s-phase	Implantation	(Aguilar, et al., 2014)
t2	Implantation	(Meseguer, et al., 2011, Mizobe, et al., 2016, Wu, et al., 2016)
	Blastocyst formation	(Mizobe, et al., 2018)
	Top quality blastocyst formation	(Mizobe, et al., 2016)
	Embryo quality on day 3	(Coticchio, et al., 2018)
t3	Implantation	(Meseguer, et al., 2011)
t4	Implantation	(Freour, et al., 2013, Meseguer, et al., 2011, Mizobe, et al., 2016, Wu, et al., 2016)
	Top quality blastocyst formation	(Mizobe, et al., 2016)
t5	Implantation	(Meseguer, et al., 2011)
t6	Top quality blastocyst formation	(Storr, et al., 2015)
t7	Top quality blastocyst formation	(Storr, et al., 2015)
t8	Implantation	(Dal Canto, et al., 2012)
	Top quality blastocyst formation	(Storr, et al., 2015)
tn	No difference in implantation	(Chamayou, et al., 2013, Kirkegaard, et al., 2013)
Mean duration of 2-cell stage	Implantation	(Meseguer, et al., 2011)
	Implantation	(Rubio, et al., 2012)
	Expanded blastocyts formation	(Dal Canto, et al., 2012)
	Blastocyst development	(Cruz, et al., 2012)
Mean duration of 3-cell stage	Implantation	(Meseguer, et al., 2011)
	Blastocyst development	(Cruz, et al., 2012)
	Expanded blastocyts formation	(Dal Canto, et al., 2012)

tM	Top quality blastocyst formation	(Storr, et al., 2015)
	Blastocyst formation and implantation	(Motato, et al., 2016)
	No difference in implantation	(Chamayou, et al., 2013, Kirkegaard, et al., 2013)
tSC	Implantation	(Mizobe, et al., 2017)
	No difference in implantation	(Chamayou, et al., 2013, Kirkegaard, et al., 2013)
tSB	Top quality blastocyst formation	(Storr, et al., 2015)
	Implantation	(Goodman, et al., 2016, Mizobe, et al., 2017)
tB	Top quality blastocyst formation	(Storr, et al., 2015)
	No difference in implantation	(Chamayou, et al., 2013, Kirkegaard, et al., 2013)
tEB	Blastocyst formation and implantation	(Motato, et al., 2016)
	No difference in implantation	(Chamayou, et al., 2013, Kirkegaard, et al., 2013)
s3	Blastocyst formation	(Cetinkaya, et al., 2015)
	Top quality blastocyst formation	(Storr, et al., 2015)
	Blastocyst formation and implantation	(Motato, et al., 2016)
	No difference in implantation	(Chamayou, et al., 2013)
cc3	Implantation	(Chamayou, et al., 2013)
Blastocyst contraction	Implantation rate	(Marcos, et al., 2015, Vinals Gonzalez, et al., 2018)
tRE, tCRE	Pregnancy	(Ebner, et al., 2017)
Post thawing blastocyst re-expansion speed (tCRE-tRE)	Pregnancy and pregnancy loss	(Ebner, et al., 2017)
	No correlation with live birth	(Kovacic, et al., 2018)

TLT and ploidy status

Embryo ploidy status is probably the most critical factor impacting an embryo's implantation potential. Screening of embryo ploidy status, also known as Preimplantation Genetic Testing for Aneuploidy (PGT-A), has largely improved over the last years and allows accurate evaluation of embryo chromosomal status. However, PGT-A is not permitted in some countries, and there remains doubt regarding its cost-effectiveness and clinical relevance (Griffin and Ogur, 2018). As TLT provides extensive information on embryo development in vitro, it is postulated that morphokinetic parameters could be associated with embryo ploidy, thus providing a cheaper, faster and less invasive method for the evaluation of embryo ploidy status than PGT-A (Campbell, et al., 2013, Chavez, et al., 2012). A comprehensive review of the literature on the predictive value of morphokinetic parameters for embryo ploidy status was reported recently (Reignier, et al., 2018). A total of 13 studies were included, which had a significant heterogeneity in terms of design, inclusion criteria, embryo biopsy, statistical approach, and outcome measures. While most studies found significant differences in morphokinetic parameters between euploid and aneuploid embryos, none provided evidence sufficient to recommend the clinical use of TLT for embryo ploidy assessment. The same conclusion was reached in another contemporary review where the association between morphokinetics and aneuploidy was discussed (Zaninovic, et al., 2017).

1.2 Training/teaching

TLT provides an excellent tool for teaching embryo assessments. Since embryos can be examined without removal from the incubator to record their morphokinetic changes, the time factor is no longer an issue and detailed assessment is feasible also “a posteriori”. Visual examples of different cleavage patterns can easily be stored and used as learning material.

Officially recognised training programmes to direct staff to the use of TLT devices and morphokinetic annotation remain to be developed. Such programmes should include thorough understanding of technical and theoretical principles governing equipment operation, acquisition of manual skills to set up and maintain embryo culture conditions required by the device and attainment of competences relevant to morphokinetic annotation and treatment data input.

1.3 Quality control

Intra- and inter-observer variability impacts the traditional static morphological embryo scoring and evaluation of morphokinetic criteria. Several factors can affect precision and reproducibility of morphokinetic annotation. Examples specific to TLT include the selection of an appropriate focal plane for the observation of spatially restricted events, consensus on when to annotate events occurring gradually (e.g. pronuclear formation or compaction), and mere definition of the parameters of interest. However, initial experiences aimed at assessing intra- and inter-operator variability in annotation of morphokinetic parameters were reassuring (Sundvall, et al., 2013). Overall, inter-observer annotation, subject to possible biases, was found to have an almost perfect agreement, although the degree of conformity was not the same for the diverse parameters. For example, the measurements with the highest degree of agreement were those relevant to pronuclear breakdown, nuclear appearance and disappearance at the 2-cell stage and achievement of full blastocyst hatching. On the other hand, parameters that were less consistently annotated included pronuclear appearance, multinucleation, blastomere evenness and number of collapses during blastocyst expansion. In general, intra-observer annotations (typically subject to random errors) were characterized by an even higher, although not statistically significant, coefficient of consistency. Interestingly, in this class, the degree of agreement of each parameter reflected the same trend reported for the inter-observer comparisons (as indicated by * in table 2). Good intra- and inter-observed agreement was also reported in more recent studies (Adolfsson and Andershed, 2018, Storr, et al., 2017).

Clearly, technical differences between different TLT devices may also limit annotation consistency. For example, TLT devices may differ in time intervals between two consecutive image acquisitions or in the quality of images collected. These differences may have implications for events occurring rapidly (e.g. pronuclear breakdown) or for morphological characteristics requiring precise description (e.g. arrangement of nuclear precursor bodies), respectively. Nevertheless, comparison of two different TLT devices showed that inter-laboratory variability clusters mostly at two specific developmental intervals, one delimited by extrusion of the second polar body and pronuclear formation, and another spanning between the 8-cell and the morula stages (Martinez-Granados, et al., 2017). Overall, inter-laboratory agreement between different TLT devices was high, although it was similar or

lower compared with conventional morphological observation, depending on the equipment used (Martinez-Granados, et al., 2017). Taken together, these experiences are important to assess the reliability of the TLT approach, but they cannot be considered conclusive and call for more extensive analyses.

At present, automated annotation has not solved the question of fidelity of morphokinetic analysis. Indeed, automation requires human supervision to correct possible, but recurrent, annotation inaccuracies that may affect the performance of prediction models for embryo selection. Therefore, similar to other activities, each laboratory should implement appropriate programs of quality control and assurance (De los Santos, et al., 2016).

On a different ground, TLT has significant relevance for other laboratory activities. For example, differences in embryo morphokinetics, revealed by TLT, may be valuable endpoints against which to compare consumables, cryopreservation protocols and devices introduced in the IVF laboratory (Ferrick, et al., 2019).

2. Implications of TLT

2.1 Impact on embryo culture conditions

Culture medium

Embryos in vitro are exposed to numerous physical and chemical stressors (Wale and Gardner, 2016) which creates an environment that can impact the developing embryo. Amongst these external factors, the culture medium used is a crucial one. Improvements in culture conditions have come primarily from modifications in media formulations that have been developed according to two doctrines. On the one hand, there is the attempt to satisfy the perceived changing requirements of the human embryo in a manner that is analogous to the environmental changes it would encounter as it would move in vivo from the oviduct to the uterus (Barnes, et al., 1995). The approach to address this concept is to fine-tune media composition in order to fulfil the needs of the embryo – so called “sequential media”. On the other hand, it has been hypothesized that it is of benefit to supply all nutrients (Summers, et al., 1995) upon which the embryo will metabolize them according to its demand – so called “single-step media”. Results from studies in conventional incubators remain inconclusive as to whether one culture system is superior to the other (Sepulveda, et al., 2009, Sfontouris, et al., 2016, Werner, et al., 2016).

There arises a question of whether the increased resolution of TLT might identify more subtle differences, e.g. in morphokinetic behaviour, between sequential and single-step media?
Ciray et al. were the first to compare the two approaches to embryo culture using TLT. Randomization of mature oocytes was done following ICSI. On day 3 of culture, those embryos in sequential culture had their medium replaced whereas the single step group had their culture medium replenished with a fresh infusion of the same medium (Ciray, et al., 2012). The authors found that in single-step medium, fading of pronuclei (tPNf) and cleavage up to 5-cell stage (t2 → t5) took place significantly earlier compared to counterparts grown in sequential medium. In implanted embryos, t2 and t4 were significantly shorter with single-step medium (Ciray, et al., 2012). Recently, these data were, at least in part, confirmed by

Kazdar et al., who reported an accelerated first mitotic cell cycle (tPNf → t2) with single-step medium (Kazdar, et al., 2017).
In contrast, others have been unable to identify morphokinetic differences between embryos grown in sequential or single step culture (Basile, et al., 2013, Schiewe, et al., 2018). However, it is possible that any developmental delay at earlier times may be compensated at later stages. Indeed, in a recent multicentre trial, culture in a one-step medium designed specifically for TLT resulted in a longer t7 and t8, but by blastulation (tSB) the differences were no longer present (Hardarson, et al., 2015).

Crucially, no study has yet demonstrated any effect on implantation and pregnancy rates. The uninterrupted culture which avoids the need of media replenishment, thus, minimizing culture disruption, and stress to the embryos may be preferred for practical reasons. However, renewing media on day 3 does neither influence morphokinetics nor implantation and live-birth (Costa-Borges, et al., 2016).
Thus, data to date have been unable to demonstrate conclusive superiority of either single-step nor sequential media in terms of clinical outcomes when used in conjunction with TLT incubators.

Oxygen tension

It is now widely accepted that the oxygen tension of the mammalian female reproductive tract is between 2% and 8% (Fischer and Bavister, 1993), yet despite this, such conditions have only been replicated in vitro in the past ten years. Exposure of embryos to atmospheric oxygen tension is associated with a higher production of reactive oxygen species (Yang, et al., 1998) and may also alter gene expression (Rinaudo, et al., 2006), DNA methylation (Li, et al., 2016) and embryo metabolism (Wale and Gardner, 2012). Persuasive evidence that embryo culture in 5% rather than ambient oxygen leads to improved pregnancy and life-birth (Kovacic, et al., 2010, Meintjes, et al., 2009). Such benefits of lower oxygen levels will almost certainly apply to time-lapse incubators.
To address this, Wale and Gardner (2010) cultured murine embryos in low (5%) or high (20%) oxygen concentrations for the first two days, followed by culture in the same or reciprocal oxygen concentrations for a further 48 h. This study identified irreversible detrimental effects of atmospheric oxygen on mouse embryo development is evident from the first mitosis (Wale and Gardner, 2010). More importantly, the delay in the timing of cleavages was found to be cumulative since it became more pronounced as embryo development progressed. In addition, blastocysts that were exposed to atmospheric oxygen at any stage had significantly fewer cells compared with the 5% O_2 counterparts.

In human, Kirkegaard et al. (2013) demonstrated that timing of the third cleavage cycle (t5-t8) was faster for embryos cultured in 5% compared with embryos cultured in 20% O_2. However, no differences were observed in timing of the early and full blastocyst stages (Kirkegaard, et al., 2013). Since the delayed development after culture in ambient oxygen was seen in the pre-compaction embryo only it seems that in human, the negative influence of high oxygen may be stage-specific.

Embryo density

Human embryos are capable of in vitro development whether cultured in groups or individually. By contrast, the embryos of many other mammals require culture in groups. Interestingly, mouse embryos cultured individually are more sensitive to the stress caused by atmospheric oxygen (Kelley et al., 2016). It is speculated that grouping such embryos may lower local oxygen concentrations, and as a consequence reduce ROS (Wale and Gardner, 2010). In addition, embryotrophic factors may play a role in the better performance of group culture (Ebner, et al., 2010, O'Neill, 2008).

Kelley et al. (2016) were the first to use time-lapse technique to measure the influence of embryo density on cleavage behaviour. Although present from t2 (20% oxygen) and t3 (5% oxygen), respectively, the significant delay in individual culture culminated at 8-cell stage (5%, 1.29h) or blastocyst stage (20%, 4.76h) (Kelley and Gardner, 2016). In a follow-up time-lapse study (Kelley and Gardner, 2017) it was shown that embryos that had individual culture for the entire duration of culture, or any portion thereof, had fewer cells at blastocyst stage compared with those cultured in groups. This was especially notable in the ECM.

It is important to stress that with current time-lapse systems in principle ideal group culture is not possible due to the design of the culture dishes. Embryo culture in time-lapse incubators has to be carried out using commercially available dishes. There are two types of dishes which may either present multiple microwells under one drop of media, or single wells which require separate drops of media (both under mineral oil). There is evidence that the former type better supports embryo development compared with single culture in individual drops (Chung, et al., 2015). However, a similar effect can be achieved with simply increasing the volume of individual droplets so that they have contact to each other.

Importantly, with the current dimensions of the dishes, particularly the distance between microwells, any potential paracrine action of embryotrophic factors is very unlikely (Ebner, et al., 2010, Gopichandran and Leese, 2006).

Swain and co-workers (2012) emphasized the importance of drop size in maintaining osmolality of culture media (Swain, et al., 2012). They found that using a larger volume of medium (40 µl) resulted in a significantly lower increase of osmolality (e.g., 12 mOsm/kg) as compared to 10 µl and 20 µl drops. Using dishes specially designed for time-lapse imaging Kelley et al. (2017) reported that in volumes of 2 µl and 20 µl only minor increase of 4-5 mOsm/kg in osmolality was observed which had no effect on further growth (Kelley and Gardner, 2017). This negligible shift could be caused by absorption of water by the mineral oil overlay or due to the manipulation during sampling and measuring (Heo, et al., 2007).

To summarize, the current method of culturing embryos for five to six days in medium sized drops of single-step or sequential media covered with mineral oil does not affect osmolality, and as a consequence, development of the embryos. It is, however, strongly recommended to work with reduced oxygen.

2.2 Management of staff time, work-flow, staff training

A key strategic decision associated with investing in a TLT is deciding how to implement the technology. No matter which approach is chosen; a time-lapse system will have a remarkable impact on the logistics of the laboratory.

TLT eliminates the necessity of assessing embryos at fixed time points (2011), instead providing the flexibility of reviewing developmental history at any appropriate time, and even in a location remote from the laboratory. Importantly, instead of basing clinical decisions on sole, static assessments, more information is available for choosing which embryo/embryos to transfer/cryopreserve.

When initially implemented, staff will want to spend a lot of time at looking at film sequences generated by TLT. Staff will learn much about early embryonic development. Many questions will emerge surrounding the significance, sequence, relative timing, duration and relative importance of morphokinetic heterogeneity. It may be wise to proactively develop strategies to manage any effect on laboratory productivity. However, once accustomed to the technology staff will become more efficient at making annotations.

Since TLT does not require physical removal of the embryos from the incubator, staff can potentially undertake a more thorough assessment. The possibility to 'scroll back and forward' allows users to review the continuum of development; this should make the assessments more reliable. Moreover, the availability of the videos makes it easier to ask colleagues for a second opinion. Thus, when choosing embryos for transfer/cryopreservation, laboratories with TLT will be able to implement more incisively a deselection or ranking strategy. Laboratories may opt to annotate in detail only morphologically good quality embryos on the day of transfer and give a simple morphological score for any remaining embryos.

Policy

In most cases, laboratories are not exclusively equipped with TLT systems. When implementing a TLT approach, it is essential that clinics perform a detailed analysis to develop a tailored policy for its use. In doing so, clinics would be wise to consider a range of factors including but not limited to number of units available, patient characteristics, medical history, number of embryos available, day of transfer and enrolment in a PGT program.

Staff training

Laboratories need to have appropriate standard operating procedures (SOPs) for culture and, where used, assessment of embryos using TLT. In addition, an appropriate training program for staff, as part of a quality control program should be implemented. The training program must clearly contain information on how to operate the TLT system. Also, evaluation of the time-lapse parameters that have been identified as relevant should be included. Importantly, some time-lapse parameters appear to be more difficult to assess with high consistency than others (Sundvall, et al., 2013). Thus, any TLT training program should complement training in static morphological assessment.

3. How to introduce TLT

3.1 Different (types of) TLT systems

Currently, there are several commercially available TLT Systems. The initial choice of the system may, on one hand, be related to practical decisions such as the laboratory workload, dimensions and the budget, and on the other hand to the specifications of the individual

systems. The key features of systems currently commercially available are summarized in Table 4.

As outlined in section 2, all TLT systems currently available require a specific culture dish, supplied by the manufacturer. Most of the culture dishes are designed for single embryo culture for image analysis and traceability purposes. However, some of the culture dishes permit the sharing of culture media between compartments, in theory allowing exchange of soluble components, and are described by manufacturers as group culture (more about embryo culture in section 2). This may represent an important consideration when choosing a TLT system.

In addition, factors suggesting a decision might include the nature of the software used for visualization and analysis and the options for annotation, which may be manual, guided or automated. These features could influence the choice of TLT system, as a guided annotation may minimize the time spent on annotations. Furthermore, some companies offer predictive algorithms (Conaghan, et al., 2013, Petersen, et al., 2016) to be used on their equipment, which may incur additional costs. Nevertheless, it is important for each clinic to independently validate their own approach for embryo selection.

Table 4: Different commercially available Time-Lapse Systems

		System A	System B	System C	System D	System E	System F	System G
Incubator		integrated	placed in conventional incubators	integrated		integrated	integrated	
External dimensions (W x D x H mm)		530 x 860 x 381	220 x 80 x 110	550 x 600 x 500	600 x 560 x 440	785 x 596 x 380; 960 x 700 x 325	625 x 500 x 300	
Specific culture dish		single culture	group culture	single culture (shared medium)	single culture	single or group culture (shared medium)	single or group culture (shared medium)	
Specifications	**Number of focal planes**	11 (max.)	3 to 11	11	up to 17 – typical 7	3 to 7	up to 11	
	Time between acquisitions	15 min (adjustable between 15 to 60 min)	5 to 60 min	10 min	10 min for 7 focal planes, 2 min for a single focal plane	5 min	5 min	
	Camera (MP)	1.3	5	2.2 (3px/µm)	1.3 (3px/µm)	1.25	5	
	Type of microscopy	oblique illumination	brightfield (Hoffman Modulation)			brightfield	brightfield	brightfield/ darkfield
	Embryo illumination for image acquisition	red LED	green LED (550 nm)	red LED (630 nm)	red LED (635 nm)	red LED (635 nm)	orange LED (591 nm)	red LED (630 nm)
	Time of light exposure	0.008s	0.2 to 0.005s	<0.02s; <32s/day/embryo	<0.032s; <31s/day/embryo (7 focal planes)	0.064s	<0.005s; ≈164s/day/embryo	<0.005s; <0.009s ≈203s/day/embryo
Software	**Morphokinetic annotation**	yes, manual	yes, manual, guided/semi-automated			yes, manual and automated	yes, manual, semi-automated and automated	
	Predictive algorithm	/	Yes, or defined by user			defined by user	defined by user	Yes
Costs	**General**	culture dish	culture dish + software	culture dish + labels + software	culture dish + software	culture dish + software	culture dish +software	
	Gas consumption		N/A	N_2: Max 5 L/hr, typical 2-3L/hr CO_2: Max 2L/hr, typical 0.5 L/hr	N_2: <10 L/hr Typical 3L/hr CO_2: <1L/hr	N_2: 3-5 L/hr CO_2: 1-2L/hr	N_2 & CO_2: 3.6 L/hr	
	Recovery time (minutes)	temperature: 10-12 Gas: 5-6	N/A	CO_2 < 5; O_2 <3	CO_2 and temperature <5;	temperature <1; gas <3	temperature <1; CO_2<3; humidity 4 hours (for full recovery)	

				O_2<15 (with 30s door opening, typical door opening is 4s)		
Dry or humid culture system	dry	N/A	dry		dry	dry or humid, independently on each chamber
Capacity	12 embryos/dish; 9 dishes/incubator	16 or 9 embryos/dish; 1 dish/inverted microscope	16 embryos/dish; 15 dishes/incubator	12 embryos/dish; 6 dishes/incubator	14 embryos/dish; 6 or 12 dishes/incubator	16 embryos/dish; 6 dishes/incubator
Compartment individualization	individual sensor for temperature and heating elements; mixed gas provided into each compartment through separated gas line	N/A	shared chamber	shared chamber	individual temperature control sensor; one gas mixer supplying all the chambers individually	individual sensors for temperature, humidity and CO_2. individualized heating elements; shared gas supplying all the chambers individually
Impact of compartment failure	if one fails in terms of temperature, the rest still works	-	failure of entire unit	failure of entire unit	temperature failure – does not affect the remaining chambers; gas leak - the gas flow is adjusted in the remaining chambers	the damaged compartment can be deactivated.
Other	possible remote control	remote control	culture dishes automatically registered using a barcode labelling; remote control	remote control	remote control	remote control, dry contact alarm surveillance

Information was gathered from manufacturers brochures and through contact with local distributors.

3.2 Safety

Installation

Introducing time-lapse incubator in the laboratory should begin with installation performed in accordance with manufacturer's instructions and should be accompanied by operational and performance qualification. As with any incubation system, TLT requires a connection to an external monitoring/alarm system, that must be tested prior to clinical use, in accordance with the ESHRE revised guidelines for good practice in IVF laboratories (De los Santos, et al., 2016). Some TLT systems allow remote follow-up of system performance. In case of emergency, troubleshooting protocols should be in place and system redundancy is required, to allow, if necessary, for culture dishes to be removed and transferred to other available incubators.

Incubator

Light source

There is evidence that suggests a general negative effect of light exposure on embryo development. Light emitted at 400–500 nm (blue light) appears to be more harmful than longer wavelengths (green, orange, red light) of visible light, resulting in oxidative stress (Ottosen, et al., 2007). Umaoka et al. (1992) demonstrated that when hamster zygotes were exposed to <500 nm [blue] light for 30 minutes, there was a significant reduction in the rate of first cleavage (Umaoka, et al., 1992). These data were confirmed by Oh et al. in a more sensitive hamster model. Moreover, light emitted at 400–500 nm resulted in a decrease in blastocyst formation and reduced blastocyst quality with increasing ICM & TE cell apoptosis. However, the detrimental effects of visible light are not only directly related to the spectral composition of the light, but also to intensity and exposure time (Oh, et al., 2007).

Exposure frequency and duration

In a time-lapse incubator, an embryo may be subjected to light exposure up to 1500 times. However, even in older systems, exposing embryos approximately 300 times to white light during 80 msec exposure times, does not significantly affect the fertilization rate of ICSI, the cleavage rate, or the morphological grade of embryos compared to conventional embryo scoring (Nakahara et al., 2010). This suggests that there is little effect of light exposure on embryos from exposure during time-lapse observations. Intuitively, it is expected that in time-lapse incubators, embryos are more exposed to light. However, scalar irradiance and therefore light exposure in TLT systems is lower than with conventional morphology assessment (Li, et al., 2014, Wale and Gardner, 2016). Furthermore, over a 5 to 7-day observation period in a TLT system, the total energy dose of the total light exposure time was much lower as compared to light exposure with conventional morphology assessment (Li, et al., 2014). In time-lapse incubators consisting of individual chambers, light exposure is even further reduced. Thus, the use of TLT can standardize variations in light exposure between patients.

Culture environment

Compared to conventional embryo assessment, stability of key environmental parameters may be maintained with TLT (temperature: 0,09 °C- 0,2 °C; CO2: 0,1 % - 0.4 °C; O2: 0,3 % - 0,5 %). Short recovery times are achieved in integrated TLT systems, which are comparable to conventional bench-top incubators. Therefore, TLT provides a safe environment for embryo observation for research and clinical use. Indeed, there are some studies that report that culture in integrated TLT systems may improve embryo development compared to standard incubators (Alhelou, et al., 2018, Barberet, et

413 al., 2018, Cimadomo, et al., 2018, Mascarenhas, et al., 2019, Sciorio, et al., 2018), while other studies
414 do not confirm this superiority (Cruz, et al., 2011, Insua, et al., 2017, Kirkegaard, et al., 2012, Park, et
415 al., 2015).

416 3.3 Morphokinetic algorithms for embryo selection

417 Several teams have worked on developing algorithms aimed at standardizing and refining embryo
418 quality evaluation and embryo selection. The first attempt was conducted by Meseguer and
419 colleagues in 2011 followed by a validation, adaptation and improvements (Basile, et al., 2015,
420 Meseguer, et al., 2011, Rubio, et al., 2014). A tendency towards better clinical outcomes was
421 concluded when an algorithm was used (Pribenszky, et al., 2017), although concerns were raised on
422 the reproducibility of the results (Barrie, et al., 2017, Freour, et al., 2015, Kirkegaard, et al., 2015,
423 Neyer, et al., 2015).

424 Each and every lab introducing TLT should do their proper validation, as algorithms could be
425 influenced by several confounding factors (see Table 5).
426

Table 5: Possible confounding factors with the use of TLT algorithms.

	Parameters to consider	References
Patient-related factors	Age	(Akarsu, et al., 2017, Akhter and Shahab, 2017, Gryshchenko, et al., 2014, Kirkegaard, et al., 2016, Siristatidis, et al., 2015)
	Type of infertility	(Freis, et al., 2018, Sundvall, et al., 2015, Wissing, et al., 2014)
	Weight/BMI/Obesity	(Bellver, et al., 2013, Kirkegaard, et al., 2016, Leary, et al., 2015)
	Ovarian stimulation protocol	(Gryshchenko, et al., 2014, Gurbuz, et al., 2016, Kirkegaard, et al., 2016, Munoz, et al., 2013, Wdowiak and Bojar, 2015)
	Type of responder/ovarian reserve	(Akarsu, et al., 2017, Bhide, et al., 2017, Hojnik, et al., 2016, Rienzi, et al., 2015)
	Smoking	(Freour, et al., 2013, Salvarci, et al., 2017, Siristatidis, et al., 2015)
Gamete, embryo or laboratory-related factors	Sperm factor	(Desai, et al., 2018, Knez, et al., 2013, Lammers, et al., 2015, Mangoli, et al., 2018, Neyer, et al., 2015, Wdowiak, et al., 2015)
	Oocyte morphology	(Mizobe, et al., 2016, Otsuki, et al., 2018, Van Blerkom, 1990)
	IVM	(Dal Canto, et al., 2016, Escrich, et al., 2012, Roesner, et al., 2017, Walls, et al., 2015, Wilken-Jensen, et al., 2014)
	Fertilization technique	(Bodri, et al., 2015, Cruz, et al., 2013, Joergensen, et al., 2014, Kim, et al., 2017, Kirkegaard, et al., 2013, Kirkegaard, et al., 2013, Kirkegaard, et al., 2016, Liu, et al., 2015)
	Biopsy	(Bar-El, et al., 2016, Kalma, et al., 2018, Kirkegaard, et al., 2012)
	Cryopreservation	(Chamayou, et al., 2015, Cobo, et al., 2017, Coello, et al., 2017, De Munck, et al., 2015, Eastick, et al., 2017, Ebner, et al., 2017, Kovacic, et al., 2018, Maezawa, et al., 2014)
	Sex of the embryo	(Bodri, et al., 2016, Bronet, et al., 2015, Huang, et al., 2019, Serdarogullari, et al., 2014, Zeyad, et al., 2018)

4. Evidence of a clinical benefit of TLT

Like any new intervention, TLT should be implemented in routine clinical practice only after stringent testing that demonstrates a proven benefit for patients (Brison, et al., 2013, Harper, et al., 2017). However, a clear increase of IVF success rates with the use of TLT remains to be firmly proven.

The latest Cochrane review (9 RCTs, 2955 women) (Armstrong, et al., 2019) reported insufficient evidence of differences in live birth rate (OR 1.12, 95% CI 0.92-1.36), miscarriage rate (OR 0.63, 95% CI 0.45-0.89) or clinical pregnancy rate (OR 0.95, 95% CI 0.78-1.16) with TLT utilising embryo selection software versus conventional incubation and assessment. Likewise, a putative benefit of TLT was not

436 demonstrated by preceding meta-analyses (Armstrong, et al., 2015, Armstrong, et al., 2018, Chen, et
437 al., 2017, Polanski, et al., 2014, Racowsky, et al., 2015).

438 Conversely, one meta-analysis has suggested a beneficial effect of TLT compared to conventional
439 embryo assessment/incubation, reporting a significantly higher ongoing pregnancy rate (51.0% vs.
440 39.9%; OR 1.54, 95% CI 1.21-1.97), a significantly lower early pregnancy loss (15.3% vs. 21.3%; OR
441 0.66, 95% CI 0.47-0.94) and a significantly increased live birth rate (44.2% vs. 31.3%; OR 1.67, 95% CI
442 1.13-2.46) (Pribenszky, et al., 2017).

443 A significant cause for the controversy over TLT efficacy is the fact that it entails two distinct
444 components, i.e. undisturbed incubation environment and embryo selection through imaging
445 software. In this respect, these two components have not been effectively distinguished in the
446 majority of studies, possibly masking the weight of the effect of better culture conditions or improved
447 embryo selection on the reported outcomes (Armstrong, et al., 2015).

448 Cumulative live birth rates were assessed in a recent retrospective study of 1882 cycles comparing
449 time-lapse and conventional incubation conditions (Mascarenhas, et al., 2018). The study showed
450 similar cumulative live birth rate between time-lapse incubation and standard incubation (51.7% vs.
451 51.2%; OR 1.02, 95% CI: 0.85-1.22). Importantly, no safety issues have been reported following
452 embryo culture in time-lapse incubators, and obstetric and perinatal outcomes, such as duration of
453 gestation, congenital malformations and birth weight, are comparable to conventional incubators
454 (Costa-Borges, et al., 2016, Insua, et al., 2017, Kovacs, et al., 2019).

455 Despite the current lack of evidence from RCTs for a clinical benefit of TLT, it is reasonable to assume
456 that, compared with static observations, continuous embryo monitoring in an undisturbed
457 environment will offer more information into embryo development, and is expected to enhance the
458 identification of good-prognosis embryos for clinical use. Of note, a number of retrospective studies
459 have shown a positive association between TLT use and clinical outcome (Kirkegaard, et al., 2015).
460 Therefore, it is necessary that more RCTs with adequate design and sufficient power be conducted,
461 reporting on live births and perinatal outcomes, in order to firmly establish a putative beneficial effect
462 of TLT.

463 5. Current state of TLT

464 Although in-house systems have existed since the late 90's, TLT became commercially available for
465 human IVF in 2010. The large volume of published articles, communications in congresses, as well as
466 active communication of IVF centres using TLT on the internet and in conventional media suggest a
467 vigorous implementation rate of this technology in IVF laboratories throughout the world.
468 Surprisingly, almost no data is currently available that describes global use of TLT. Scotland represents
469 a somewhat unique area, since the Scottish government funding has enabled all 4 publicly funded (UK
470 National Health Service) assisted conception units within the country to invest in TLT (Thomas Freour,
471 personal communication). Besides this specific case, only 2 surveys could be found in the international
472 literature up to now reporting TLT implementation rate and use. The first study was reported by
473 Dolinko et al., who conducted an online survey on TLT use by 294 IVF lab directors in the USA (Dolinko,
474 et al., 2017). In total, 162 (55%) responded, with 35 laboratories (17%) reporting that they run at least
475 one TLT system. The presence/availability of TLT was positively associated with the number of IVF

cycles performed in the centre. Following this first report, a French team conducted a very similar study with an online survey to 210 lab directors in all 105 IVF laboratories in France (Boueilh, et al., 2018). Among the 78 respondents (response rate 37%), 30 (39%) reported using TLT clinically. Among non-users, 11 (23%) reported plans to invest in TLT within the 2 next years. Unlike the situation in the United States, TLT implementation was not associated with the number of IVF cycles performed in France. Although these 2 studies provide an interesting insight into TLT implementation in two different countries in terms of IVF regulation or funding policy, it is not prudent to draw conclusion of the overall use of TLT worldwide. A more global picture of the TLT market would be interesting in order to evaluate its current use and trends in IVF practice and find opportunities for cost-effectiveness and medical studies.

6. Current and future research perspectives

TLT remains in its infancy in clinical embryology and as such, there is significant scope to refine and improve the method. However, beyond this, the type of data generated, coupled with the relative ease of use and non-invasive nature of TLT means that there are exciting prospects explore fundamental developmental biology in significant detail.

Embryo selection parameters based on visual indicators of presumed quality have largely been a subjective application of a decision tree (Simopoulou, et al., 2018). The inclusion of multiple visual parameters has led to improvements in outcomes, and the widespread application of the so-called "Gardner criteria" is a good illustrative example (Gardner and Schoolcraft, 1999). This indicates the prospective value of assessing multiple parameters, and data generated by TLT will offer the opportunity for profound evolution of such multi-parameter analyses.

Artificial Intelligence (AI) or machine learning describes a non-biased approach to multi-parameter analysis. In the context of TLT, attempts are underway to use higher-powered computer-processing power to analyse large data sets of images to identify combinations of parameters that might link to embryo viability. There is little doubt that the future of AI and TLT will incorporate some degree of machine learning, to facilitate complex analysis of large data sets which will likely reveal currently unidentified combinations of visual markers. Indeed, Tran and colleagues have recently reported the development of a deep learning model to annotate automatically morphokinetic videos. The authors retrospectively analysed more than 10.000 videos from multiple centres and were able to show that their model was able to reproducibly identify images from embryos that went on to give a fetal heartbeat, with an AUC of >0.90 (Tran, et al., 2019).

An important issue that deserves discussion is whether biological justification is required for acceptance of computer-generated algorithms to select embryos based on machine-learned combinations of parameters. The very strength of adopting an objective approach of using AI to interrogate digital images free of human bias is that such a system will 'look beyond' traditional parameters of morphology and may identify unique combinations of markers that relate to embryo viability. However, in doing so, it is possible that such combinations may be unfamiliar. Furthermore, as AI systems are not able to ascribe meaning to parameters, it is possible that markers may relate to non-classical features, such as image grey scale or image texture depth (e.g. (Molder, et al., 2015)). Before adoption of such approaches, there is a requirement for robust clinical validation prior to evaluating its acceptance by the relevant stakeholders.

TLT enables research possibilities in fundamental developmental processes. For example, the immediate period after fertilisation is characterised by a number of molecular processes, each of them with its own specific dynamics. With TLT, it has been possible to observe a number of processes in their entirety including: cytoplasmic movements in oocytes during meiosis resumption (Bui, et al., 2017) and embryos (Milewski, et al., 2018), fertilisation events (Coticchio, et al., 2018), the beginning of first mitosis (Wong, et al., 2010) and the dynamics of blastocyst formation (Marcos, et al., 2015). The observation of such crucial developmental events in real time has revealed a number of new parameters that have been introduced into embryology (reviewed by (Chen, et al., 2013)). Moreover, with more detailed understanding of developmental kinetics, we may be able to ascribe key landmarks to other aspects of embryo physiology, such as embryo chromosomal status (Pennetta, et al., 2018) and response to cryopreservation (Taborin and Kovacic, 2019).

Looking forwards, it is difficult to imagine that there will not be significant improvements to the technology of TLT to drive further knowledge and understanding of early development. Such developments are likely to come from more refined image collection methods and the integration with other technologies. Development of fluorescence and confocal time-lapse imaging enables the observation of morphokinetics of organelles and chromosomes (Capalbo, et al., 2017, Duncan, et al., 2012, Patel, et al., 2015, Zielinska, et al., 2015).

There is growing interest in using advanced label-free imaging techniques to gain molecular-level understanding of cellular function (Kasprowicz, et al., 2017). Such approaches can yield additional information of the physiology of the cell, including detail on metabolic processes, since many metabolites and enzymes exhibit autofluorecent properties (Gosnell, et al., 2016). Measuring metabolic and biochemical function has long been a pursuit of those interested in the identification of biomarkers of viability. Bradley et al. (2016) have used an image-based approach called CARS (Coherent Anti-stokes Raman Scattering) to identify reliably the composition, ratio and real time change in lipid profiles of single preimplantation embryos (Bradley, et al., 2016). Sutton-McDowall et al. (2017) were able to demonstrate differences in metabolic profiles of embryos grown in hyperoxic (20%) or normoxic (7%) conditions using hyperspectral imaging to measure ratios of NAD(P)H and FAD (Sutton-McDowall, et al., 2017). Similarly, Sanchez et al (2018) used Fluorescent Lifetime Imaging (FLIM) to detect mitochondrial dysfunction (Sanchez, et al., 2018).

To date, despite significant research effort, no single reliable biomarker of embryo quality has yet been identified. This may reflect the complexity of preimplantation development. Consequently, the search for biomarkers must no longer occur in isolation; the combination of TLT with other markers of embryo physiology is a natural evolution of both fields.

7. Should TLT data be shared with patients

The introduction of TLT in assisted reproduction has raised many questions and concerns, mostly related with its clinical relevance in IVF and its impact on reproductive outcome. However, the sustained implementation of this technology into routinely applied instruments has raised many additional logistic questions related with daily practice. Some of the most pressing concerns are how we engage with the final stakeholders; our patients.

For example, we may consider whether we should declare the brand of the TLT in the reports provided to our patients. Is there any need to link the information provided with product used to obtain such information? Since many time-lapse incubators are technically similar and able to provide comparable results, it may be prudent to avoid speaking in terms of 'brands'.

TLT practitioners may also wish to consider the number of images used in describing the embryo(s) selected for transfer. Ideally, we may choose three images for the embryos whether Day 3 transfer is performed and four for Blastocysts (D5-6) transfers. Additionally, accurate timestamping of images is crucial; for example, fertilization (18h), D2 (44 h), D3 (68 h), with the intention to describe accurately the development of the embryo up to blastocysts (116h) as described by Alpha Consensus meeting (2011). Important technical difficulties appear when patients present high numbers of oocytes to be fertilized. Such a situation may necessitate the need for several TLT slides per patient, which may be considered as 'new' or 'different' patient for the TLT software. Is then possible that those reports will not be immediately compatible and may contribute a source of confusion for doctors and even patients.

In the report, together with the images of the embryos transferred (at different times) thought needs to be given as to whether to include images (at least one per embryo) of those to be vitrified, or those to be discarded. In such images, the time reference (after ICSI) or the stage of embryo development may be included as headings. The presentation of the embryos, to distinguish those embryos to be transferred from those frozen or discarded could be potentially useful. We may also consider including multiple images at different time frames of those embryos that are vitrified. In any case, the pictures should be well separated and numerated. The number of pages that should be used for a report could be debated, too many pages may create confusion and too little may result in a deficient information.

Inclusion of single static representative images does not address how to share data on morphokinetics or morphology with patients. We may add information about the timings of key landmarks in embryo development, as well as the incidence of abnormal or irregular cleavages, blastocyst collapses or multinucleation as potential parameters that may affect negatively the implantation potential. Together with this, there remains the option to share the classification of the embryos after using any of the algorithms described in the scientific literature methods of embryo development. However, the inclusion of such information may be too complex to our patients, which means very difficult to be understood or that will need extra time with the patient at consultation to explain those values.

The obligation of the medical professionals should be to inform patients objectively about the development phase of the implementation of new technology in clinical practice. Thus, the question remains what should clinicians tell their patients? We need to explain that we do not have perfect tools to identify the best embryo, but we may change the order in which the embryos are transferred based on these technologies that not improve the cumulative outcome by itself (Kovacs and Lieman, 2019), but may impact time to pregnancy. The additional financial expenses should be taken into consideration and also the proper indication which is still unknown. However, it is wise to explain that TLT still lacks a convincing evidence base to prove any clinical efficacy.

A short explanation about what TLT means to the patients, may be useful including a statement like this example; *"TLT are next generation incubators that allow a detailed real-time embryo evaluation. The continuous embryo monitoring facilitates a complete follow up and a detailed analysis of embryo*

development. With TLT it is possible to perform a study of the kinetics of embryo development and the relationship between timings of cleavages and embryo viability. This information may therefore help to identify good embryos and recognize those with numerous atypical embryo developmental patterns. However, it should be noted that in these development stages there is an extraordinary plasticity in embryo morphology and developmental dynamics and that embryos also have their own self-correction mechanisms. With more research morphokinetics may, in the future, provide a valid adjunct to select embryos with the highest implantation potential".

8. Summary/Conclusions

TLT has been routinely introduced in human IVF only in the last decade, much later than in other fields of biosciences, and yet it has marked major changes in the way embryos are observed and handled. When TLT was first adopted, expectations were high. It was hoped that dynamic observation of development would offer a more precise, non-invasive modality to assess embryo viability, with obvious implications for the efficiency of ART treatment. Many studies, although mainly retrospective, have attempted to answer the question of whether TLT brings a clinical benefit, without reaching a consensus. The hopes are not lost, however, thus far, studies to effectively assess the efficacy of TLT for embryo selection have lacked sufficient rigour to demonstrate unequivocally any substantial improvement in outcomes. Regardless of a possible direct impact on clinical outcome, TLT does confer several advantages that justify its use. Its introduction in the workflow of the IVF laboratory, however, has a multiplicity of implications requiring technical and managerial expertise, as well as strategic vision of this technology. This manuscript has attempted to collate recommendations to assist the choice, introduction, management and harnessing of the TLT in the IVF laboratory.

Based on current technology, TLT probably offers the safest and most stable embryo culture environment. Continued embryo observation has allowed us to identify previously unknown or undetectable aspects of development, some of which, such as direct cleavage of the fertilized egg into three blastomeres, have significant clinical impact. There is now awareness that chromosomal aberrations may affect embryo morphokinetics, however, not to an extent to suggest that TLT can replace PGT-A in the identification of euploid embryos. TLT devices, however, are relatively demanding pieces of equipment. Therefore, a suitable technical choice requires elements of knowledge, relevant to embryo culture conditions, consistency of use between operators and laboratories, data management, cost-benefit balance, and potential for research. To make aware patients of the benefits and limits of TLT is not simple, but every effort should be made to inform in a meaningful and unbiased fashion. The promises that TLT can evolve into a full-blown embryo selection modality, once combined with AI and non-invasive analytical approaches, are compelling. While the prediction of future achievements of TLT is a difficult exercise, there is little doubt that this technology is here to stay. Mastering its use is therefore becoming an imperative for embryologists and IVF laboratories.

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