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Good practice recommendations for the use of time-lapse technology[†]

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STUDY QUESTION: What recommendations can be provided on the approach to and use of time-lapse technology (TLT) in an IVF laboratory?

SUMMARY ANSWER: The present ESHRE document provides 11 recommendations on how to introduce TLT in the IVF laboratory.

WHAT IS KNOWN ALREADY: Studies have been published on the use of TLT in clinical embryology. However, a systematic assessment of how to approach and introduce this technology is currently missing.

STUDY DESIGN, SIZE, DURATION: A working group of members of the Steering Committee of the ESHRE Special Interest Group in Embryology and selected ESHRE members was formed in order to write recommendations on the practical aspects of TLT for the IVF laboratory.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The working group included 11 members of different nationalities with internationally recognized experience in clinical embryology and basic science embryology, in addition to TLT. This document is developed according to the manual for development of ESHRE recommendations for good practice. Where possible, the statements are supported by studies retrieved from a PUBMED literature search on 'time-lapse' and ART.

MAIN RESULTS AND THE ROLE OF CHANCE: A clear clinical benefit of the use of TLT, i.e. an increase in IVF success rates, remains to be proven. Meanwhile, TLT systems are being introduced in IVF laboratories. The working group listed 11 recommendations on what to do before introducing TLT in the lab. These statements include an assessment of the pros and cons of acquiring a TLT system, selection of relevant morphokinetic parameters, selection of an appropriate TLT system with technical and customer support, development of an internal checklist and education of staff. All these aspects are explained further here, based on the current literature and expert opinion.

LIMITATIONS, REASONS FOR CAUTION: Owing to the limited evidence available, recommendations are mostly based on clinical and technical expertise. The paper provides technical advice, but leaves any decision on whether or not to use TLT to the individual centres.

WIDER IMPLICATIONS OF THE FINDINGS: This document is expected to have a significant impact on future developments of clinical embryology, considering the increasing role and impact of TLT.

STUDY FUNDING/COMPETING INTEREST(S): The meetings of the working group were funded by ESHRE. S.A. declares participation in the Nordic Embryology Academic Team with meetings sponsored by Gedeon Richter. T.E. declares to have organized workshops for Esco and receiving consulting fees from Ferring and Gynemed and speakers' fees from Esco and honorarium from Merck and MSD. T.F. received consulting fees from Vitrolife and Laboratoires Genévrier, speakers' fees from Merck Serono, Gedeon Richter, MSD and Ferring and research grants from Gedeon Richter and MSD. M.M. received sponsorship from Merck. M.M.E. received speakers' fees from Merck, Ferring and MSD.

WHAT DOES THIS MEAN FOR PATIENTS?

In most fertility treatments (IVF and ICSI), eggs retrieved from the woman are fertilized with sperm from the man in the laboratory. These fertilized eggs are then cultured in the laboratory for a few days before being transferred as embryos to the woman's womb or frozen. During this process, embryologists regularly check the development of the embryos under the microscope to make sure they are developing well and to be able to pick the best embryo to be transferred. To have good-quality embryos and a good chance of pregnancy, it is important that the embryos are cultured in a stable environment, which means using an incubator with a fixed temperature and oxygen level. Alterations of such conditions should be kept to a minimum, but inevitably embryologists will need to take the embryos out of the incubator at least once a day to monitor their development.

Time-lapse technology (TLT) systems are used in some laboratories to facilitate embryo monitoring. In a TLT incubator, images of embryo development are recorded at regular intervals of 5–15 min. This allows the embryologists to assess embryo development thoroughly in a dynamic fashion without removing them from the incubator. Some TLT systems use specific computer programs that assist in the assessment of embryos based on changes in shape/structure occurring over time and help in the ranking of embryos depending on their developmental ability.

The TLT has been introduced in several IVF labs, and studies have reported on the clinical outcomes (pregnancy rates) after its use. Overall, the studies do not allow us to conclude that better pregnancy rates can be achieved after using a TLT system compared with standard incubation and assessment. However, using a TLT system may have other benefits, for example a better and more flexible management of laboratory workload. Drawbacks of the time-lapse technology include the need for specific training on using this type of equipment. By describing the benefits, drawbacks and impact of a TLT system, this paper provides recommendations for good practice, which will help embryologists to make decisions about the choice of a specific device and help them to use it appropriately.

Patients are sometimes offered use of the time-lapse technology (with or without extra costs). The information on the benefits, drawbacks and impact of a time-lapse system in this paper will help patients in discussing the topic at the IVF clinic and deciding on how to proceed.

Introduction

An optimal incubation environment and accurate embryo selection are two defining factors for the successful outcome of IVF treatment. During *in vitro* culture, embryos are typically assessed by morphological grading in order to predict embryo developmental competence and implantation potential. The features evaluated may include the morphology of pronuclei (PN) and nucleoli, stage-specific number and size of blastomeres, fragmentation, multinucleation, blastocyst expansion and inner cell mass (ICM) and trophectoderm (TE) appearance (Cummins *et al.*, 1986; Scott, 2003; Ahlstrom *et al.*, 2011; Fulka *et al.*, 2015; De los Santos *et al.*, 2016; Otsuki *et al.*, 2017). Traditional morphological evaluation is performed at static time points and thus provides a 'snap-shot' of embryo development. Furthermore, it usually requires physical removal of the embryos from the incubator, exposing them to fluctuations in temperature, pH and oxygen levels. Crucially, this approach has limited ability to predict embryo developmental competence and ongoing pregnancy, with high intra- and inter-observer variability (Rijnders and Jansen, 1998; Guerif *et al.*,

2007). 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 (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). Although this was undoubtedly a step in the right direction, the limitations of static morphology evaluation were not overcome.

Although time-lapse technology (TLT) was introduced in ART many years ago (Payne *et al.*, 1997), it was not until 2010 that TLT shifted from a mere observation of human embryos while in culture, to a selection and prediction tool. Wong *et al.* (2010) described an algorithm able to predict blastocyst formation by day 2 of embryo culture, based on cell division timings (Wong *et al.*, 2010). The year of 2011 marked the official introduction of TLT in the embryology laboratory, when embryo implantation was shown to be associated with specific cell division timing parameters, introducing the term 'morphokinetics' (Meseguer *et al.*, 2011). The introduction of TLT has enabled both an increase in the number of observations and the dynamic assessment

of developing embryos. In parallel, TLT offers an uninterrupted culture environment, minimizing embryo handling and the need to expose embryos to conditions outside of the incubator (Meseguer et al., 2012).

A TLT system typically comprises a stand-alone incubator with one or more integrated inverted microscopes coupled to a digital camera. Alternatively, and less commonly, an optical system can be placed inside a conventional incubator. In both cases, digital images are collected at regular intervals and at different focal planes throughout embryo development and subsequently processed into videos. With this information, 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, Rubio et al., 2014).

This 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 needs. This paper is not intended as a manual on the use of TLT, nor does it provide a systematic description of clinical evidence. A meta-analysis of randomized controlled trials (RCT) assessing clinical outcomes after TLT was recently published (Armstrong et al., 2019).

Materials and Methods

ESHRE good practice recommendations are developed based on the *Manual for development of recommendations for good practice* (Vermeulen et al., 2018), which can be consulted at the ESHRE website (www.eshre.eu/guidelines). The manual describes a nine-step procedure for writing recommendations documents by the working group supported by the ESHRE methodological expert.

The current paper is the result of a 2-day consensus meeting and three online meetings of the working group. In preparation of the consensus meeting, information was collected by means of published surveys, manufacturer information and narrative reviews. In addition, relevant published data were collected from a literature search. We searched PUBMED from insertion to 23 January 2019 combining search terms (including MESH terms and synonyms) for ART/IVF and time-lapse. Papers not in English or not focused on TLT for ART were excluded. All other references were assessed, and relevant papers selected. Each working group member prepared a draft of a pre-allocated section, after which these were discussed until consensus within the group was reached. After the meeting, all 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. Fourteen people participated in the stakeholder review and submitted comments (Supplementary Data). 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 (www.eshre.eu/guidelines).

Recommendations

A list of recommendations for clinics before getting started with TLT is provided below.

List of recommendations for clinics before getting started with time-lapse technology in human IVF

- Clearly identify the reasons to introduce a TLT system

- Assess pros and cons of acquiring a TLT system, both financially and operatively

- Identify whether morphokineticparameters will be used in selection/deselection /ranking of embryos

- Identify (from scientific literature) the morphokineticparameters of interest and assess how to monitor and use them

- Find the suitable system based on considerations of culture conditions/systems and other costs, including hardware maintenance and software upgrades

- Evaluate technical/customer support available, including accessibility, the level of embryologist support and the expertise the manufacturer will provide to your team

- Seek appropriate installation and training from the manufacturer/distributor

- Develop an internal checklist, based on a user requirement specification for the system, identifying and matching what the clinic/laboratory wants in a system e.g. type of gas, humidity, footprint, capacity, type of dish, software, cost, supply chain and manufacturer support.

- Once introduced in the lab, find the appropriate system settings

- Identify and train one or more embryologists (depending on the size of the laboratory) who will develop the role of "TLT referent"; the designated person(s) will be responsible for the annotation of morphokineticvariables (to avoid initially inter-operator variations with other members of staff) and for the implementation of quality control programs

- Educate clinic staff on the current evidence behind TLT in order to counsel patients alongside offering the technology.

TLT: time-lapse technology.

Why clinics can use TLT (significance of TLT) for embryo assessment

The identification of the embryo with the best prognosis remains an unmet need in IVF. This section will evaluate whether morphokinetic embryo assessment by TLT may assist in achieving this goal.

Embryo assessment based on fertilisation 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., TLT enabled Coticchio and colleagues to draw an in-depth map of events occurring during fertilisation, which may be putative indicators of embryo quality (Payne et al., 1997; Coticchio et al., 2018). 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 cytoplasmic halo

appearance ➔ disappearance; halo appearance ➔ PN fading; PN fading ➔ first cleavage (t2); and (iv) male PN appearance ➔ male PN fading (tPNf) (Coticchio et al., 2018). Further studies assessing these markers as predictors of embryo quality on day 5 and clinical outcome are required, but TLT is the only existing technology that enables assessing embryos based on such criteria. Although time of polar body emission (tPB2) and PN morphology did not predict live birth, tPNf was associated with live birth, i.e. the tPNf of zygotes resulting in live birth was significantly longer than the tPNf of the no live birth group (Azzarello et al., 2012). Furthermore, it was reported that erratic PN 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 (Table I), mostly undetectable with static embryo assessment, have been described and correlated with embryo quality, chromosomal status and implantation potential. Future studies are still needed to standardize, sub-categorize and more clearly define irregularly cleaved embryos (Lagalla et al., 2017).

Wong and colleagues have shown that blastocyst development can be predicted with high sensitivity (94%) and specificity (93%) based on parameters identified by tracking an embryo up to the four-cell stage, namely, the time interval between the end of the first mitosis and the initiation of the second (duration of two-cell stage) and the time interval between the second and third mitoses (duration of the three-cell stage) (Wong et al., 2010).

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

Five-cell cleavage timing and intervals during two cleavages (t5 and s2, cc2) (Table II) were shown to be the most predictive parameters for embryo viability and implantation (Wong et al., 2010; Meseguer et al., 2011). Recently, an association between irregular division (Liu et al., 2014a; Desai et al., 2018b), start time of blastulation (tSB), expansion (tEB), the interval tEB-tSB and both ploidy and aneuploidy status with odds of live birth was reported (Desai et al., 2018b, Fishel et al., 2018).

Since the routine introduction of TLT, there have been numerous attempts to assess the clinical and biological significance of the parameters described in Table II. A non-exhaustive summary of these studies is available in Table III. However, it is difficult to compare the outcomes of these different studies since the methodologies used are not consistent.

TLT and ploidy status
Embryo ploidy status is probably the most critical factor impacting an embryo's implantation potential. PGT for aneuploidy (PGT-A) has greatly improved over the last few years and allows the accurate evaluation of embryo chromosomal status. However, PGT-A is not permitted in some countries, and there remains some debate regarding its cost-effectiveness and/or clinical relevance (Dahdouh et al., 2015; Sermon et al., 2016; Griffin and Ogur, 2018; Neal et al., 2018; Penzias et al., 2018; Rosenwaks et al., 2018; Lee et al., 2019; Somigliana et al., 2019). 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 (Chavez et al., 2012; Campbell et al., 2013). 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 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). However, the combination of PGT-A with morphokinetic analysis may help in selecting the embryo with the highest implantation potential (Rocafort et al., 2018).

Training/teaching

TLT provides an excellent tool for teaching embryology and standardizing assessment. Since embryos can be examined without removal from the incubator to assess their morphology and dynamic events, the time factor is no longer an issue and detailed assessment is also feasible *a posteriori*. Visual examples of standard morphology assessment and examples of normal and unusual, probably abnormal, cleavage patterns can easily be stored and used as learning material.

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

Quality control

Intra- and inter-observer variability impacts on static morphological embryo scoring and evaluation of morphokinetic criteria (Sundvall et al., 2013). Several factors can affect precision and reproducibility of morphokinetic annotation by TLT; examples include the selection of an appropriate focal plane for the observation of spatially restricted events, consensus on when to annotate events that are occurring gradually (e.g. pronuclear formation or compaction) and mere definition of the parameters of interest. 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 due to assessment of morphokinetic behaviours not amenable to precise quantitative measurement 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 fading, nuclear appearance and disappearance at the two-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

Table I Atypical human embryo cleavage features observed with time-lapse technology versus classic embryo morphology assessment once per day.

Name of feature	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)
Differently sized pronuclei	Difference in pronuclear areas immediately before pronuclear membrane fading	Exclusively	(Otsuki et al., 2017)
Pronuclei reappearance	Pronuclei fading and reappearance	Exclusively	(Coticchio et al., 2018)
Aberrant behaviour of female pronucleus	Extrusion of the third PB 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 three 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 fertilisation	Better	(Barrie et al., 2017)
Direct cleavage	Cleavage of zygote to three cells (trichotomous mitosis) or one blastomere to three cells in the first ($t3$-$t2 = 0$) or second cell division cycle (two cells to five or six cells), but this should be distinguished from rapid cleavage ($t3$-$t2 < 5$ h)	Exclusively	(Athayde Wirka et al., 2014, Barrie et al., 2017, Fan et al., 2016, Lagalla et al., 2017, Meseguer et al., 2011, Rubio et al., 2012, Zhan et al., 2016)
Reverse cleavage	Fusion of two cells into one blastomere	Exclusively	(Barrie et al., 2017, Desai et al., 2014, Goodman et al., 2016, Liu et al., 2014b)
Blastomere movement	Prolonged blastomere movement induced by delay in pronuclear fading and first cell division	Exclusively	(Ezoe et al., 2019)
Multinucleation	Blastomere with >1 nucleus	Better	(Balakier et al., 2016, Desai et al., 2014, Ergin et al., 2014, Goodman et al., 2016, Hashimoto et al., 2016)
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)
Cell exclusion	Exclusion of one or more blastomeres from the formation of compact morula or blastocyst	Better	(Coticchio et al., 2019, Lagalla et al., 2017)
Blastocyst collapse	Complete or almost complete disappearance of blastocoel and consequent blastocyst shrinkage	Better	(Bodri et al., 2016b, Kovacic et al., 2018, Marcos et al., 2015)

TLT: time-lapse technology

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 II). Good intra- and inter-observer agreement was also reported in more recent studies (Adolfsson and Andershed, 2018; Storr et al., 2018).

Table II 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 fading; 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 blastocoel 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 and time intervals	Psyn	Syngamy, time from PN fading to the first cytokinesis
	Not mentioned	Time between nuclear envelope breakdown and subsequent division to two cells
	s2	Time between division to three cells and subsequent division to four cells
	s3	Time between division to five cells and subsequent division to eight cells
	ECC1	Duration of the first cell cycle (t2-tPB2)
	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 blastocoel 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. Table adapted from consensus paper (Ciray *et al.*, 2014). Time zero (t0) may change from one study to another (mid-time for ICSI, standard IVF insemination, tPB2 or TPNf). These inconsistencies have to be taken into account when comparing data from different studies (Kaser and Racowsky, 2014).
*Parameters with the highest concordance between operators.

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, number of focal planes (Z resolution) or in the quality of images collected. These differences may have implications for events occurring rapidly (e.g. pronuclear fading) or for morphological characteristics requiring precise description (e.g. arrangement of nuclear precursor bodies). Nevertheless, a 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 PB and pronuclear formation, and another spanning the eight-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 in order 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. 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

Table III 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 fading	Embryo quality on day 3	(Coticchio et al., 2018)
Time interval PN fading ➜ first cleavage (t2)	Embryo quality on day 3	(Coticchio et al., 2018)
Time interval male PN appearance ➜ male PN fading	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)
	Implantation	(Aguilar et al., 2014, Chamayou et al., 2013, Kirkegaard et al., 2013c, 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., 2016a, Wu et al., 2016)
	Blastocyst formation	(Mizobe et al., 2018)
	Top-quality blastocyst formation	(Mizobe et al., 2016a)
	Embryo quality on day 3	(Coticchio et al., 2018)
t3	Implantation	(Meseguer et al., 2011)
t4	Implantation	(Carrasco et al., 2017, Freour et al., 2013, Meseguer et al., 2011, Mizobe et al., 2016a, Wu et al., 2016)
	Top-quality blastocyst formation	(Mizobe et al., 2016a)
t5	Implantation	(Meseguer et al., 2011)
t6	Top-quality blastocyst formation	(Storr et al., 2015)
t7	Implantation	(Carrasco et al., 2017)
	Top-quality blastocyst formation	(Storr et al., 2015)
t8	Implantation	(Dal Canto et al., 2012)
	Top-quality blastocyst formation	(Storr et al., 2015)
tn	Implantation	(Chamayou et al., 2013, Kirkegaard et al., 2013c)

Continued

Table III Continued.

Markers	Prediction/outcome	Reference
Mean duration of two-cell stage	Implantation	(Meseguer et al., 2011) (Rubio et al., 2012)
	Expanded blastocyst formation	(Dal Canto et al., 2012)
	Blastocyst development	(Conaghan et al., 2013, Cruz et al., 2012, Wong et al., 2010)
Mean duration of three-cell stage	Implantation	(Meseguer et al., 2011)
	Blastocyst development	(Conaghan et al., 2013, Cruz et al., 2012, Wong et al., 2010)
	Expanded blastocyst formation	(Dal Canto et al., 2012)
tM	Top-quality blastocyst formation	(Storr et al., 2015)
	Blastocyst formation and implantation	(Chamayou et al., 2013, Kirkegaard et al., 2013c, Motato et al., 2016)
	No difference in implantation	(Chamayou et al., 2013, Kirkegaard et al., 2013c)
tSC	Implantation	(Chamayou et al., 2013, Kirkegaard et al., 2013c)
tSB	Top-quality blastocyst formation	(Fishel et al., 2018, Storr et al., 2015)
	Implantation	(Goodman et al., 2016, Mizobe et al., 2017)
tB	Top-quality blastocyst formation	(Storr et al., 2015)
	Implantation	(Chamayou et al., 2013, Kirkegaard et al., 2013c)
tEB	Blastocyst formation and implantation	(Motato et al., 2016)
	Implantation	(Chamayou et al., 2013, Kirkegaard et al., 2013c)
s3	Blastocyst formation	(Cetinkaya et al., 2015)
	Top-quality blastocyst formation	(Storr et al., 2015)
	Blastocyst formation and implantation	(Motato et al., 2016)
	Implantation	(Carrasco et al., 2017, 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)
	Live birth	(Kovacic et al., 2018)

implement appropriate programmes of quality control and assurance (De los Santos *et al.*, 2016).

On a different level, TLT has significant relevance for other laboratory activities. For example, differences in embryo morphokinetics, as 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). TLT also offers the opportunity to sharpen the sensitivity of mouse embryo assays. Wolff *et al.* (2013) reported that a morphokinetic algorithm was able to detect alterations in mouse embryo development caused by media contaminants and lots of toxic mineral oil, while the same culture conditions did not affect blastocyst rate (Wolff *et al.*, 2013). Deviant morphokinetic patterns can therefore represent an early warning of altered culture conditions.

Implications of TLT

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 on 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, and the embryo will metabolize them according to its demand—so called 'single-step media' (Summers *et al.*, 1995). 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. Randomisation of mature oocytes was carried out and followed by 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 PN (tPNf) and cleavage up to five-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 a 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 a 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 single-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 of single-step or sequential media on implantation and pregnancy rates. The uninterrupted culture, which avoids the need for media replenishment and 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). Exposure of embryos to atmospheric oxygen tension is associated with a higher production of reactive oxygen species (ROS) (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). There is evidence that embryo culture in 5% rather than ambient oxygen leads to improved pregnancy and life-birth (Meintjes *et al.*, 2009; Kovacic *et al.*, 2010; Bontekoe *et al.*, 2012). Such benefits of lower O_2 levels will almost certainly apply to TLT incubators (and as such is recommended by the supplier).

To address this, Wale and Gardner (2010) cultured murine embryos in low (5%) or high (20%) O_2 concentrations for the first 2 days, followed by culture in the same or reciprocal O_2 concentrations for a further 2 days. They reported irreversible and detrimental effects of atmospheric oxygen on mouse embryo development 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 O_2 at any stage had significantly fewer cells compared with the 5% O_2 counterparts. In human, Kirkegaard *et al.* (2013a) found 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.*, 2013a). Since the delayed development after culture in ambient O_2 was seen in the precompaction 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, while the embryos of many other mammals require culture in groups. For instance, contrary to grouped embryos, mouse embryos cultured individually are more sensitive to the stress caused by atmospheric O_2 (Kelley and Gardner, 2016). It is speculated that grouping such embryos may lower local O_2 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 (O'Neill, 2008; Ebner *et al.*, 2010).

Kelley and Gardner (2016) were the first to use the time-lapse technique to measure the influence of embryo density on cleavage

behaviour. Although detectable from t2 (20% O_2) and t3 (5% O_2), the significant delay in individual culture culminated at the eight-cell stage (5% O_2, 1.29 h) or blastocyst stage (20% O_2, 4.76 h) (Kelley and Gardner, 2016). In a follow-up study, 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 ICM (Kelley and Gardner, 2017).

It is important to stress that with current TLT systems the ideal group culture is not possible due to the design of the commercially available culture dishes. There are two types of dishes, which have either 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 multiple microwell type better supports embryo development compared with single culture in individual drops (Chung et al., 2015). A similar effect can be achieved by simply increasing the volume of individual droplets so that they have contact with each other, but this may not be in compliance with some of the manufacturer's recommendations for dish preparation. Importantly, with the current dimensions of the dishes, and particularly the distance between microwells, any potential paracrine action of embryotrophic factors is very unlikely (Gopichandran and Leese, 2006; Ebner et al., 2010).

In a mouse model, 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 smaller increase of osmolality (e.g. 12 mOsm/kg) as compared to 10- and 20-µl drops. Using dishes specifically designed for time-lapse imaging, Kelley and Gardner (2017) reported that in volumes of 2 and 20 µl, only a 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).

Although the optimal osmolality for human embryo culture is still unclear, for physiological reasons most of the culture media today are specified to fall in a range of 270–290 mOsm (Sunde et al., 2016) or even lower (Baltz, 2012). However, it would be expected that in vitro culture of embryos will be performed within their range of osmotic tolerance (Wale and Gardner, 2016). In terms of hyperosmolality, embryo osmotic tolerance is up to 320 mOsm or even higher (e.g. 350 mOsm) considering the fact that current culture media contain strong osmolytes such as the amino acid glycine (Baltz and Tartia, 2010).

A humid atmosphere, as shown in non-TLT incubators (Fawzy et al., 2017), could counteract potential adverse effects since it may reduce the effect of fluctuations in osmolality (Yumoto et al., 2019). This stabilisation effect was strongly related to the drop volume and the mineral oil layer used (Yumoto et al., 2019). Furthermore, any theoretical drawback strongly depends on the starting osmolality of the culture medium, the length of in vitro culture (oocytes are most sensitive to hypo- or hyperosmolality) and whether the medium is changed on day 3 or not. It should be stressed, however, that the gradual changes in osmolality reported above never reached critical values (Swain et al., 2012; Kelley and Gardner, 2017; Yumoto et al., 2019). Using mineral oil which has been preequilibrated in a humid incubator overnight would further reduce osmotic stress (Yumoto et al., 2019).

To summarize, the current method of culturing embryos for 5–6 days in medium-sized drops of single-step or sequential media covered with mineral oil does not appear to affect osmolality and, as a consequence, development of the embryos. It is, however, strongly recommended to work with reduced oxygen.

Management of staff time, work-flow, staff training

A key strategic decision associated with investing in TLT is deciding how to implement the technology. No matter which approach is chosen, a TLT system will have a significant impact on the logistics of the laboratory. TLT eliminates the necessity of assessing embryos at fixed time points (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011), instead providing the flexibility of reviewing the developmental history at any appropriate time, possibly even remotely from the laboratory. This flexibility can improve efficiency as it allows better planning and timing of specific tasks (i.e. fertilisation check, embryo biopsy) and use of equipment (such as inverted microscopes). Importantly, instead of basing clinical decisions on single and static assessments, more information is available with TLT for ranking and selecting embryos. When initially implemented, staff members usually want to spend a lot of time looking at the videos generated by TLT. They will learn a lot about early embryonic development, and 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 ensure the availability of sufficient resources during the introduction and training of staff and to manage any effect on laboratory productivity. However, once accustomed to the technology, staff members will become more efficient at making annotations.

Since TLT does not require physical removal of the embryos from the incubator, staff members can perform a more thorough assessment. The possibility to 'scroll back and forward' allows users to review the continuum of development, which 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 their deselection or ranking strategy more confidently and incisively. Laboratories may opt to only annotate in detail the morphologically good- and fair-quality embryos on the day of transfer and to give a simple morphological score for any remaining poor-quality 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 with reference to their patient population. In doing so, clinics would be wise to consider a range of factors including, but not limited to, the number of units available, patient characteristics, their medical histories, the number of embryos available, day of transfer and enrolment in a PGT programme.

Staff training

Laboratories need to have appropriate standard operating procedures for tissue culture and, where used, assessment of embryos using TLT. In addition, an appropriate training programme for staff members

should be implemented, as part of a quality control programme. The training programme must clearly contain information on how to operate the TLT system and include an evaluation of the relevant time-lapse parameters. Importantly, some time-lapse parameters appear to be more difficult to assess with high consistency (Sundvall et al., 2013). Therefore, any TLT training programme should be complemented with training in static morphological assessment.

It is important to inform medical and nursing staff of the new routines concerning assessment and culture. TLT can not only be used to increase understanding of embryo development but also as an important aid in making embryo assessments more descriptive, hence facilitating exchange of information amongst operators with different roles forming the IVF team.

How to introduce TLT

Different TLT systems

Currently, there are several commercially available TLT Systems. The choice of the system can be based on practical considerations, such as the laboratory workload, dimensions and the budget, or on the specifications of the individual systems. The key features of systems currently commercially available are summarized in Table IV.

As outlined in above, 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. This may represent an important consideration when choosing a TLT system.

In addition, factors influencing a decision might include the nature of the computer software used for visualisation and analysis, and the options for annotation, which may be manual, guided or automated. 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 clinics to independently validate their own approach for embryo selection (see below).

Safety

Installation

Introducing a TLT incubator in the laboratory should start with the installation performed in accordance with manufacturer's instructions and should be accompanied by operational and performance quali-fication. As with any incubation system, TLT requires a connection to an external monitoring/alarm system, which must be tested prior to clinical use (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 suggesting a 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) reported a significant reduction in the rate of first cleavage in hamster zygotes when exposed to <500 nm [blue] light for 30 min (Umaoka et al., 1992). These data were confirmed in a more sensitive hamster model, also showing that 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 related to the spectral composition of the light, but also to the intensity and exposure time (Oh et al., 2007).

Exposure frequency and duration. In a TLT 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 of 80-ms exposure times does not significantly affect the fertili-sation 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, if any, of light exposure on embryos from exposure during time-lapse observations. Intuitively, it is expected that in TLT incubators, embryos are more exposed to light. However, scalar irradiance and therefore light exposure in TLT systems are 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 TLT incubators consisting of individual chambers, light exposure is reduced even further. Thus, the use of TLT can standardize variations in light exposure between patients.

Culture environment stability. Compared to conventional embryo assessment, stability of the key environmental parameters may be maintained with TLT (temperature: 0.09–0.2°C; CO_2: 0.1–0.4%; O_2: 0.3–0.5%). Short recovery times for these different parameters 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, some studies report that culture in integrated TLT systems may improve embryo development compared to standard incubators (Alhelou et al., 2018; Barberet et al., 2018; Cimadomo et al., 2018; Sciorio et al., 2018; Mascarenhas et al., 2019), while other studies do not confirm this superiority (Cruz et al., 2011; Kirkegaard et al., 2012a; Park et al., 2015; Insua et al., 2017).

Morphokinetic algorithms for embryo selection

Several teams have worked on developing algorithms aimed at stan-dardizing and refining embryo quality evaluation and embryo selection. An algorithm predicting development to the blastocyst stage was first described in 2010, and later validated and adapted (Wong et al., 2010; Meseguer et al., 2011; Conaghan et al., 2013; Rubio et al., 2014; Basile et al., 2015). Although concerns were raised on the reproducibility of the algorithms and cleavage anomalies in previous attempts (Freour et al., 2015; Kirkegaard et al., 2015; Neyer et al., 2015; Barrie et al., 2017), a tendency towards better clinical outcomes was concluded (Petersen et al., 2016; Pribenszky et al., 2017).

Table IV 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	Integrated	Integrated
External dimensions (W × D × H mm)		530 × 860 × 381	220 × 80 × 110	550 × 600 × 500	600 × 560 × 440	785 × 596 × 380; 960 × 700 × 325	625 × 500 × 300	625 × 500 × 300
Specific culture dish		Single culture	Group culture	Single culture (shared medium)	Single culture	Single or group culture (shared medium)	Group culture (shared medium)	Group culture (shared medium)
Specifications	Number of focal planes	11 (max.)	3 to 11	11	Up to 17—typically 7	3 to 7	Up to 11	Up to 11
	Time between acquisitions	15 min (adjustable between 15 and 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	5 min
	Camera (megapixels)	1.3	5	2.2 (3 px/μm)	1.3 (3 px/μm)	1.25	5	5
	Type of microscopy	Oblique illumination	Brightfield (Hoffman modulation)	Brightfield (Hoffman modulation)	Brightfield (Hoffman Modulation)	brightfield	brightfield	brightfield/darkfield
	Embryo illumination for image acquisition	Red LED	Amber LED (590 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	0.008 s	0.2 to 0.005 s	<0.02 s; <32 s/day/embryo	<0.032 s; <31 s/day/embryo (7 focal planes)	0.064 s	<0.005 s; ≈164 s/day/embryo	<0.005 s; <0.009 s ≈203 s/day/embryo
Software	Morphokinetic annotation	Yes, manual	Yes, manual, guided/semi-automated	Yes, manual, guided/semi-automated	Yes, manual, guided/semi-automated	yes, manual and automated	yes, manual, semi-automated and automated	yes, manual, semi-automated and automated
	Predictive algorithm	/	Yes, or defined by user	Yes, or defined by user	Yes, or defined by user	defined by user	defined by user	yes

Continued

Table IV Continued.

		System A	System B	System C	System D	System E	System F	System G
Costs	General	Culture dish	Culture dish + software	Culture dish + software	Culture dish + software	culture dish + software	culture dish + software	culture dish + software
	Gas consumption		N/A	N_2: max 5 L/h, typical 2–3 L/h; CO_2: max 2 L/hr, typical 0.5 L/hr	N_2: <10 L/h Typical 3 L/h CO_2: <1 L/h	N_2: 3–5 L/hr CO_2: 1-2 L/hr	N_2 & CO_2: 3.6 L/hr	N_2 & CO_2: 3.6 L/hr
	Type of gas	Built-in gas mixer	N/A	Integrated gas mixer	Integrated gas mixer	built in gas mixer; premixed not required	premixed	premixed
	Recovery time (min)	Temperature: 10–12 Gas: 5–6	N/A	CO_2 <5; O_2 <3	CO_2 and temperature <5; O_2 <15 (with a 30-s door opening, typical door opening is 4 s)	temperature <1; gas <3	temperature <1; CO_2 <3; humidity 4 h (for full recovery)	temperature <1; CO_2 <3; humidity 4 h (for full recovery)
Dry or humid culture system		Dry	N/A	Dry	Dry	dry	dry or humid, independently on each chamber	dry or humid, independently on each chamber
pH monitoring		Performed by placing a petri dish with a media sample and oil cover inside the embryo chamber	N/A	Specific pH validation dish	Performed by placing a petri dish with a media sample and oil cover inside the embryo chamber	built in pH measuring system	performed by placing a petri dish with a media sample and oil cover inside the embryo chamber	performed by placing a petri dish with a media sample and oil cover inside the embryo 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	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	individual sensors for temperature, humidity and CO_2. individualized heating elements; shared gas supplying all the chambers individually

Continued

Table IV Continued.

	System A	System B	System C	System D	System E	System F	System G
Impact of compartment failure	If one fails in terms of temperature, the rest still works	-	Failure of entire unit (if only the computer systems fails, the incubator is not affected)	Failure of entire unit (if only the computer systems fails, the incubator is not affected)	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.	the damaged compartment can be deactivated.
Impact of camera failure	Incubator works as a regular benchtop	N/A	Incubator works as a regular benchtop	Incubator works as a regular benchtop	incubator works as a regular benchtop	incubator works as a regular benchtop; the remaining non affected cameras work without problem	incubator works as a regular benchtop; the remaining non affected cameras work without problem
Electronic record systems integration	Manually	Possible to integrate	Possible to integrate	Possible to integrate	under development	possible to integrate	possible to integrate
Other	Remote access to the images	Remote access to the images	Culture dishes automatically registered using a barcode labelling; remote access to the images	Remote access to the images	remote access to the images	remote access to the images, dry contact alarm surveillance	remote access to the images, dry contact alarm surveillance

Information was gathered from manufacturers' brochures and through contact with local distributors, from November 2018 until June 2019.

Table V 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.*, 2018a, 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.*, 2016b, 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)
	Fertilisation technique	(Bodri *et al.*, 2015, Cruz *et al.*, 2013, Inoue *et al.*, 2019, Joergensen *et al.*, 2014, Kim *et al.*, 2017, Kirkegaard *et al.*, 2013b, Kirkegaard *et al.*, 2013c, Kirkegaard *et al.*, 2016, Liu *et al.*, 2015)
	Biopsy	(Bar-El *et al.*, 2016, Kalma *et al.*, 2018, Kirkegaard *et al.*, 2012b)
	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.*, 2016a, Bronet *et al.*, 2015, Huang *et al.*, 2019, Serdarogullari *et al.*, 2014, Zeyad *et al.*, 2018)

If possible, each laboratory introducing TLT should perform a proper validation, based on appropriate sample size or post-hoc power analysis, certifying the value of each variable introduced and the corrections for putative confounders that could influence the algorithms (Table V) (Carrasco *et al.*, 2017).

Evidence of a clinical benefit of TLT

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

The latest Cochrane review (nine RCTs, 2955 women) (Armstrong *et al.*, 2019) reported insufficient evidence for differences in live birth rate (odds ratio (OR0 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) for TLT combined with embryo selection software versus conventional incubation and assessment. Likewise, a putative benefit of TLT was not demonstrated by meta-analyses (Polanski *et al.*, 2014; Armstrong *et al.*, 2015a; Racowsky *et al.*, 2015; Chen *et al.*, 2017; Armstrong *et al.*, 2018). Conversely, one meta-analysis, using a different methodological approach, has suggested a beneficial effect of TLT compared to conventional incubation and assessment, respectively, reporting a significantly higher ongoing pregnancy rate (51.0 versus 39.9%; OR 1.54, 95% CI 1.21–1.97), a significantly lower early pregnancy loss (15.3 versus 21.3%; OR 0.66, 95% CI 0.47–0.94) and a significantly increased live birth rate (44.2 versus 31.3%; OR 1.67, 95% CI 1.13–2.46) (Pribenszky *et al.*, 2017).

Cumulative live birth rates were assessed in a recent retrospective study of 1882 cycles comparing time-lapse and conventional incubation/assessment (Mascarenhas *et al.*, 2019): the study showed similar cumulative live birth rates for time-lapse and conventional incubation/assessment (51.7 versus 51.2%; OR 1.02, 95% CI: 0.85–1.22), although fresh embryo transfer live birth rates were higher for TLT cycles (36.8 versus 33.9%, adjusted OR 1.28, 95% CI: 1.05–1.57).

The main reason for the controversy over TLT efficacy is the fact that it entails two distinct components, i.e. an undisturbed incubation environment and embryo selection through imaging software. In this respect, these two components have not been effectively distinguished in the majority of studies, possibly masking the weight of the effect of better culture conditions or improved embryo selection on the reported outcomes (Armstrong *et al.*, 2015b). Additional confounders that may explain the heterogeneity amongst studies include different days of assessment, different endpoints, the wide array of morphokinetic timings assessed, inter- and intra-operator variation in annotating and the various other confounding factors listed in Table V.

Importantly, no safety issues have been reported following embryo culture in TLT incubators, and obstetric and perinatal outcomes, such as duration of gestation, congenital malformations and birth weight, are comparable (Costa-Borges *et al.*, 2016; Insua *et al.*, 2017; Kovacs *et al.*, 2019) or better (Mascarenhas *et al.*, 2019) compared to standard incubation.

Despite the current lack of evidence from RCTs for a clinical benefit of TLT, it is reasonable to assume that, compared with static observations, continuous embryo monitoring in an undisturbed environment will offer more information into embryo development and is expected to enhance the identification of good-prognosis embryos for clinical

use. In order to firmly establish a putative beneficial effect of TLT, more well-designed and sufficiently powered RCTs reporting on live births and perinatal outcomes are necessary.

Current state of TLT

Although in-house systems have existed since the late 1990s, TLT became commercially available for human IVF in 2009. The large volume of published articles, communications in congresses and active communication on the internet and in conventional media from IVF centres using TLT suggest a vigorous implementation rate of this technology in IVF laboratories throughout the world. Surprisingly, almost no data are currently available on the global use of TLT. Scotland represents a somewhat unique area, since the Scottish government funding has enabled all four publicly funded (UK National Health Service) assisted conception units within the country to invest in TLT (Thomas Freour, personal communication). Besides this specific case, only two surveys could be found reporting on TLT implementation rate and use. The first study by Dolinko et al. reported the results of an online survey of 294 IVF laboratory directors in the USA on TLT use (Dolinko et al., 2017). In total, 162 (55%) responded, with 35 laboratories (17%) reporting that they run at least one TLT system. The presence and 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 survey of 210 laboratory directors in all 105 IVF laboratories in France (Boueilh et al., 2018). Amongst the 78 respondents (response rate 37%), 30 (39%) reported using TLT clinically. Amongst non-users, 11 (23%) reported plans to invest in TLT within the next 2 years. Unlike the situation in the USA, TLT implementation was not associated with the number of IVF cycles performed in France. Although these two 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 a conclusion of the overall use of TLT worldwide. Altogether, these elements highlight the relevance of the present guidelines in order to help patients and clinics benefit as much as possible from TLT. A more global picture of the TLT market would be interesting in order to evaluate its current use and trends in IVF practice and to find opportunities for cost-effectiveness and medical studies.

Current and future research perspectives

In comparison with the rapid technical development of TLT together with other technologies for basic research in cell biology, the TLT in clinical embryology remains in its infancy 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 for exploring 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 multiparameter analyses.

Artificial intelligence (AI), or machine learning, describes a non-biased approach to multiparameter 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. Khosravi and colleagues used AI and TLT and, by analysing more than 10 000 embryos, developed a model that was able to predict blastocyst quality with an AUC of >0.98 (Khosravi et al., 2019). Using a similar approach, 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 blastocysts that went on to give a foetal 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 characterized 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 in 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 (Table III). Moreover, with a 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 forward, 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 during oocyte maturation (Duncan et al., 2012; Holubcova et al., 2015; Patel et al., 2015; Zielinska et al.,

2015; Capalbo et al., 2017). Furthermore, exciting developments in fluorescence live-cell imaging of human embryos (Hashimoto et al., 2016), fluorescent light sheet in toto imaging of developing mouse embryos (Strnad et al., 2016) and a combination of the fluorescent time-lapse with comparative genomic hybridisation (Chavez et al., 2012) or single-cell sequencing (Daughtry et al., 2019) of individual blastomeres may help in discovering mechanisms of aneuploidy during cleavages of primate and human embryos.

There is growing interest in using advanced label-free imaging techniques to gain a molecular-level understanding of cellular function (Kasprowicz et al., 2017). Such approaches can yield additional information on the physiology of the cell, including details of metabolic processes, since many metabolites and enzymes exhibit autofluorescent 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 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 flavin adenine dinucleotide (Sutton-McDowall et al., 2017). Similarly, Sanchez et al. (2018) used fluorescent lifetime imaging (to detect mitochondrial dysfunction (Sanchez et al., 2018).

The search for objective robust biomarkers of embryo viability continues, although to date, and despite significant research effort, no single reliable biomarker with a sufficiently high predictability of live birth 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.

Should TLT data be shared with patients

The introduction of TLT in assisted reproduction has raised many questions and concerns, mostly related to its clinical relevance in IVF and its impact on reproductive outcome. However, the sustained implementation of this technology has raised many additional logistic questions associated 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, but is there any need to link the information provided with product used to obtain such information? Since many TLT 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 when day 3 transfer is performed and four for blastocyst (day 5–6) transfers. Additionally, accurate time-stamping of images is crucial; for example, fertilisation (18 h), day 2 (44 h) and day 3 (68 h), with the intention to accurately describe the development of the embryo up to blastocyst stage (116 h), as described in the Istanbul Consensus (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology,

2011). Important technical difficulties appear when patients present high numbers of oocytes to be fertilized, more than those that can be placed in a single slide; this may necessitate the need for several TLT slides per patient, each being considered as a 'new' or 'different' patient for the TLT software. This could result in reports being not immediately compatible and cause 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 embryos that are vitrified. The amount of information that may 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 complex information means it may be very difficult for patients to fully understand it at first mention, or that it 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 clinicians should 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, which may not improve the cumulative outcome by itself, but may impact time to pregnancy (Kovacs and Lieman, 2019). The additional financial expenses should be taken into consideration and also the most suitable indication, which is still unknown. However, it is wise to explain that TLT still lacks a convincing evidence base to prove any clinical efficacy, although it may provide otherwise unknown information on embryo quality and development. In addition, TLT may help to counsel couples in decisions making regarding further treatment, donor egg use, adoption, etc.

There are few publications that provide scientific evidence of possible benefits of sharing TLT data with the patients. Blomqvist et al. explored patient-oriented aspects of new technologies by a prospective, observational questionnaire with a sample size of more than 200 patients. Interestingly, the majority of them found that viewing the videos and obtaining a copy of it relevant, but only if the treatment resulted in a viable pregnancy (Blomqvist et al., 2017). Reinforcing the importance of the videos, Bui et al. reported the relevance of remote access to images of developing embryos during an IVF cycle. In their study of over 100 patients, the majority surveyed indicated that viewing their embryo images during the cycle enhanced their experience of IVF treatment (Bui et al. 2018).

Currently, the working group considers that '*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 in the future will improve its power as an adjunctive test to select embryos with the highest implantation potential/deselect embryos with lowest implantation potential*'.

Conclusion

TLT has been introduced into human IVF as a routine procedure only in the last decade, much later than in other fields of biosciences, and yet it has led to major changes in the way that 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, but thus far, studies to effectively assess the efficacy of TLT for embryo selection have lacked sufficient rigour to demonstrate unequivocally any substantial improvement in pregnancy rate/live birth 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 a strategic vision for this technology. This manuscript has attempted to collate recommendations to assist with 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 monitoring 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, but 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 its potential for research. Making patients aware of the benefits and limits of TLT is not simple, but every effort should be made to inform them in a meaningful and unbiased fashion. The promise that TLT may evolve into a full-blown embryo selection modality, once combined with AI and non-invasive analytical approaches, is 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 imperative for embryologists and IVF laboratories.

Supplementary data

Supplementary data are available at *Human Reproduction Open* online.

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