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Human oocytes and embryos viewed by time-lapse videography, and the development of an embryo deselection model

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Parts of Chapters 2, 3, 4, 5 and Appendices 3-10 are not included in this version of the thesis.

EDITH COWAN UNIVERSITY

School of Medical and Health Sciences

Human oocytes and embryos viewed by time-lapse videography,

and the development of an embryo deselection model

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by

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ABSTRACT

Despite its wide application today, in vitro fertilization (IVF) treatment continues to have relatively low efficacy, largely due to inaccuracy in selecting the best quality embryo(s) from the cohort for transfer. Novel methodologies for improved selection are being developed, and time-lapse observation of human embryos is gaining increasing popularity due to the more detailed morphokinetic information obtained plus uninterrupted culture conditions. The morphokinetic information enables the use of quantitative timings in developmental milestones of embryos and qualitative measures of abnormal biological events, to assist embryo selection/deselection. This project aimed to identify current limitations in the use of such measures and to develop recommendations for improvement in clinical application.

In the current study, most data were collected retrospectively from infertile couples seeking IVF treatment at a fertility clinic, with consent to use time-lapse incubation (Embryoscope) for embryo culture. Comparisons of time-lapse measures were made between embryos with confirmed implantation and non-implantation outcomes following uterine transfers. Thereafter, an embryo deselection model was proposed based on the retrospective findings, followed by prospective validation.

It was found in the current study that the reference starting time point (t0) in certain existing time-lapse systems was inaccurate due to (i) the early biological variations between sibling oocytes, (ii) technical limitations in current equipment and protocols, and (iii) different insemination methods used (Papers 1&2). The above variations may be minimized by using pronuclear fading (PNF, a biological time point) as t0 rather than insemination (a procedural time point) (Paper 2). An example of such application was the comparison of embryo development between patients with high and low serum progesterone levels on the trigger-day (Paper 3). Furthermore, the growth rate of embryos reported in the literature is subject to multiple clinical or laboratory factors, and this was in agreement with the present study where a published time-lapse algorithm emphasizing quantitative timing parameters was shown to lose its

discriminatory power in implantation prediction when applied in two different laboratories (Paper 4). Interestingly, the qualitative measures seemed to have better inter-laboratory transferability due to the embryo growth patterns appearing independent of clinical and technical factors (Paper 4). Two novel qualitative measures were reported in the present study, namely reverse cleavage and less than 6 intercellular contact points at the end of the 4-cell stage, showing negative correlations with embryo implantation outcomes (Papers 5&6). A qualitative embryo deselection model was therefore proposed, including several qualitative measures with implantation rates being potentially increased from 22.4% to 33.6% (Paper 6). Finally, an embryo deselection model combining both qualitative and quantitative measures was reported with the use of PNF as t0, showing significant prediction of implantation outcomes in embryos regardless of insemination method (Paper 7).

In conclusion, this thesis demonstrates the usefulness of time-lapse embryo selection during IVF treatment in one specific laboratory. However, any new time-lapse parameter or model for embryo selection requires external validation by properly designed large-scale studies. Future clinical research and the development of integrated engineering and computer technology may further improve the efficacy of time-lapse selection of human embryos.

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LIST OF PUBLICATIONS

CHAPTER 2

Liu Y, Chapple V, Roberts P, Ali J, Matson P. Time-lapse videography of human oocytes following intracytoplasmic sperm injection: events up to the first cleavage division. Reproductive Biology. 2014;14(4):249-56. (Paper 1)

Liu Y, Chapple V, Feenan K, Roberts P, Matson P. Time-lapse videography of human embryos: Using pronuclear fading rather than insemination in IVF and ICSI cycles removes inconsistencies in time to reach early cleavage milestones. Reproductive Biology. 2015;15(2):122-5. (Paper 2)

CHAPTER 3

Liu Y, Copeland C, Chapple V, Roberts P, Feenan K, Matson P. The relationship between embryo quality assessed using routine embryology or time–lapse videography and serum progesterone concentration on the day of ovulatory trigger in in vitro fertilization cycles. Asian Pacific Journal of Reproduction. 2015;4(2):140-6. (Paper 3)

Liu Y, Copeland C, Stevens A, Feenan K, Chapple V, Myssonski K, Roberts P, Matson P. Assessment of human embryos by time-lapse videography: a comparison of quantitative and qualitative measures between two independent laboratories. Reproductive Biology. 2015;15(4):210-6. (Paper 4)

CHAPTER 4

Liu Y, Chapple V, Roberts P, Matson P. Prevalence, consequence, and significance of reverse cleavage by human embryos viewed with the use of the Embryoscope time-lapse video system. Fertility and Sterility. 2014;102(5):1295-300 (Paper 5)

Liu Y, Chapple V, Feenan K, Roberts P, Matson P. Clinical significance of intercellular contact at the four-cell stage of human embryos, and the use of abnormal cleavage patterns to identify embryos with low implantation potential: a time-lapse study. Fertility and Sterility. 2015;103(6):1485-91 (Paper 6)

CHAPTER 5

Liu Y, Chapple V, Feenan K, Roberts P, Matson P. Time-lapse deselection model for human day 3 in vitro fertilization embryos: the combination of qualitative and quantitative measures of embryo growth. Fertility and Sterility. In Press. (Paper 7)

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LIST OF ABBREVIATIONS

ANOVA   Analysis of variance

AUC   Area under the curve

CFC   Canberra Fertility Centre

CI   Confidence interval

CV   Coefficient of variation

DC   Direct cleavage

EP   Elevated serum progesterone

eSET   Elective single embryo transfer

FN   Fertility North

GnRH   Gonadotrophin-releasing hormone

ICC   intra-class correlation coefficient

ICCP   Intercellular contact point

ICSI   Intracytoplasmic sperm injection

IVF   In vitro fertilization

KID   Known implantation data

LSD   Least significant difference

MII   Metaphase II

MN   Multinucleation

OCC   Oocyte-cumulus-complex

OHSS   Ovarian hyperstimulation syndrome

OR   Odds ratio

pb2   Second polar body

PNF   Pronuclear fading

PQE   Poor quality embryo

RC   Reverse cleavage

rFSH	Recombinant follicle-stimulating hormone
ROC	Receiver operating characteristic
SD	Standard deviation
SEM	Standard error of mean
SPSS	Statistical package for the social sciences
U-IVF	Universal IVF medium

CHAPTER 1: General introduction

1.1 Human in vitro fertilization (IVF) and embryo selection

On average, 9% of the global population are suffering from infertility (1). Following the birth of the first baby conceived through IVF in 1978 (2), IVF together with its emerging derivative technologies have become one of the most effective options for infertility management. IVF treatment usually involves the creation of multiple embryos per cycle, however, the potential to implant after uterine transfer for each individual embryo in the same cohort is not necessarily equal (3). Therefore, it is crucial to maximize the capability of identifying the very best quality embryo(s) from the cohort for transfer, so that the treatment efficacy is maintained at the highest posible level (4). The most widely used non-invasive embryo assessment in IVF laboratories is the morphology based evaluation via static observations (3), which may be performed on either cleavage or blastocyst stage embryos (5). Morphology assessment at the cleavage stage takes into consideration cell count, degree of fragmentation, and symmetry of blastomeres at specific checkpoints, and/or pronuclear analysis (5); whilst blastocyst evaluation involves the combination of developmental stage judged by the volume of cavity, and the patterns of how the cells organize in the inner cell mass and trophectoderm (5, 6). These approaches are considered relatively safe and cost effective to apply, by simply involving static observations only. However, selections based on the limited observations, mostly once or twice daily with various timings of observations, may result in subjective gradings, or even inaccurate embryo selections as a result (7). Aiming at improved embryo selection, additional complementary methods have been explored over the last few decades, which will be briefly reviewed in this section.

The preimplantation genetic testings of human embryos, which involves the removal of one or more cells from the embryo for chromosomal study or certain single gene disorder detection, have been discussed and practiced since a couple of decades ago (8, 9). More recently, comprehensive chromosome screening has been reported to be effective in the improvement of embryo selection by analyzing the chromosome/DNA

quality of the embryos (10). The sampling procedures may involve different biopsy approaches for the required analysis, which is generally considered invasive (11). The sample collected could be either a blastomere from a cleavage stage embryo, or a few trophectoderm cells from a blastocyst (11). The associated risks on the subsequent embryonic development and well-being of offspring following embryo biopsy have not yet been fully understood, although the removal of excessive blastomeres was considered to be one of the contributors to adverse subsequent outcomes (12). A recent time-lapse study revealed a delay after blastomere biopsy of 8-cell stage human embryos to reach the compaction stage, while the growth rate towards subsequent milestones after the initial delay was unaffected (13). Furthermore, since the first report of a live birth after the use of a combination of polar body biopsy and chromosomal analysis via fluorescent in-situ hybridization in 1995 (14), rapid progress has been achieved over the last 20 years as indicated by the improved accuracy of testing results (4). It is however still debatable as to (i) whether or not the improvement has been properly validated (15), (ii) which patient groups are more/less likely to benefit from this technique (16), and (iii) its cost-effectiveness when applied in routine clinical practice (17).

Apart from the intactness of an embryo at the chromosome/DNA level, its viability may also be reflected by certain physiological activities (4). The dynamic profilings of such biomarkers including certain types of proteins (18) or metabolites (19) were reported to be associated with blastocyst development after the analysis of spent culture media, while being independent of morphological gradings (4). A few biomarkers, such as the consumption of glucose (20) or oxygen (21) at specific developmental stages of the embryo, were succesfully linked with subsequent implantation outcomes. Additionally, amino acid profiling was proposed to have good potential for the improvement of non-invasive embryo selection (22, 23), however, the effect of oxygen level used in the culture environment should be taken into consideration before drawing any firm conclusions (24, 25). Interestingly, some of the investigated markers such as glucose uptake and amino acid utilization were also found to vary between embryos with different genders (26). However, a recent randomized controlled trial studying metabolomic profiling of spent culture media of human day 3 embryos reported no significant differences observed in live birth results after embryo

selection using near-infrared spectroscopy in addition to morphology compared to using morphology alone (27). Further research is warranted on how to incorporate such factors into the embryo selection process.

Time-lapse technology has only been introduced into human IVF in recent years (28, 29), but is showing great potential in improving treatment outcomes (30), most likely due to the optimized embryo culture conditions via closed incubation and/or improved embryo selection based on morphokinetic information collected via continuous observations (31). The traditional approach for morphological assessment of human embryos is carried out at a low frequency (mostly 24 hour intervals or even longer) which is still being widely used today (5), while the emergence of time-lapse imaging in human IVF has no doubt offered us the opportunity to look more closely at embryo development in a non-invasive manner. Due to the large amount of information obtained, a good number of novel morphokinetic markers generated after continuous monitoring of human early embryo development have been reported to be associated with the subsequent implantation outcomes (4, 32, 33). Although it's wise to take precautions when introducing time-lapse into clinical routine, as suggested in a few review articles (33-36), great potential does exist in this field for further improved embryo selection due to the range of unexplored aspects regarding early human embryo development. The following sections will review the current application of time-lapse technology in human IVF, then specify some limitations of existing commercially available time-lapse systems, upon which a series of study aims in the thesis are introduced.

1.2 Application of time-lapse technology in human IVF

Time-lapse observations of animal embryos were reported as early as the 1950's (37), but the same had not been applied to human embryos until the late 1990's (38). This was about two decades after the successful birth of the first IVF baby (2), likely due to technical limitations in the incubator manufacturing and automated high resolution imaging technology. Early time-lapse monitoring of human embryos was carried out with in-house equipment mainly for experimental purposes, with embryos/oocytes

being observed one at a time (38, 39). Starting from the early 2010's, time-lapse monitoring of human embryos for treatment purposes has been emerging in the literature, with the use of commercially available time-lapse equipment or devices (28, 29). So far, there have been a few options of these available on the commercial market, either in the form of a separate device (e.g., the Eeva or Primo Vision system) that can be placed in the chamber of a traditional incubator, or an integrated incubator and time-lapse camera (e.g., the Embryoscope). Two recent randomized clinical trials comparing the conventional and time-lapse (Embryoscope) incubators did not find significant differences with regard to embryo development and pregnancy/implantation rates (40, 41). Despite the encouraging results regarding time-lapse selection of human embryos reported by a couple of recent large-scale retrospective studies and randomized trials (30, 31), others have expressed caution about applying this technology routinely before a full validation has been completed (33-36). In this section, the current application of quantitative and qualitative measures generated by the time-lapse technology is reviewed in terms of (i) timing parameters that were identified at different embryonic developmental stages, namely up to first cleavage division post fertilization, cleavage stage, and morula or blastocyst stage; and (ii) abnormal biological events.

1.2.1 Early biological events up to the first cleavage division

Early experimental studies on donated human oocytes showed a large variation in timings for early biological events following sperm injection, including second polar body extrusion, appearance of male or female pronuclei, and pronuclear abuttal (38). The same study (38) also reported that the quality of subsequent day 3 embryos was significantly correlated with the timings for second polar body extrusion and pronuclear abuttal, or the synchrony between appearance of male and female pronuclei. Later in 2008, Mio et al. (39) reported a detailed time-lapse video, demonstrating a series of biological evens following sperm entry via conventional IVF. In the same year, Lemmen et al. (42) identified the differences in early stage milestones between embryos fertilized via IVF and intracytoplasmic sperm injection (ICSI), and reported that early pronuclear fading or first cleavage division was significantly associated with larger cell numbers in the subsequent day 2 embryos. More recently,

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using modern time-lapse equipment, Azzarello et al. (43) found that embryos resulting in live birth had a later timing for pronuclear fading than those without live birth, while the pronuclear morphology scores and timing for second polar body extrusion were not associated with live birth results. However, Aguilar et al. (44) successfully correlated implantation results and early events occurring within the first cell cycle, including timings for second polar body extrusion and pronuclear fading after sperm injection, and the S-phase (time between pronuclear appearance and disappearance). Although time-lapse technology offers a great opportunity for clinical embryologists to reveal more details regarding early biological events in human embryo development, conflicting results are still present. Considering the young stage this area of science is at, further exploration is surely warranted.

1.2.2 Morphokinetics at cleavage stage

Over the last 15 years, significant improvement has been achieved in embryo selection via the introduction of extended culture and embryo transfer at blastocyst stage (5, 45). Although blastocyst culture is being used in a high proportion of clinics globally due to the benefits of significantly optimized live birth rates per embryo transfer (46), concerns still exist with regard to the potential adverse obstetric outcomes and increased risk of epigenetic disorders or long-term impact on the health of offspring following blastocyst transfers rather than transfers at cleavage stage (47-51), despite conflicting results from other groups (52-56). The inconsistent conclusions may be attributed to the different interpretations of observed differences, as pointed out by Gardner (57) recently, depending on whether or not to consider the contribution of oxygen concentration used in the culture system. Nevertheless, this topic is still under debate (58) until further evidence becomes available based on more up-to-date large-scale studies. Furthermore, patients with a low number of embryos available in the treatment cycle may have a high risk of zero blastocyst formation, and subsequent cancelled transfer as a result, in which case extended culture is often not preferred (59, 60). Consequently there has been a demand for improved selection at an earlier stage without the need for extended culture of embryos (59). Following the emergence of time-lapse technology used in human IVF laboratories, a number of studies have focused on the use of morphokinetic parameters at cleavage stage for prediction of

blastulation (61-63), or implantation results (28, 64). A range of predictive cleavage stage timing parameters have been reported, such as t3 (time from insemination to 3-cell stage), t4 (time from insemination to 4-cell stage), t5 (time from insemination to 5-cell stage), t8 (time from insemination to 8-cell stage), cc2 (duration of 2-cell stage), s2 (duration of 3-cell stage) and s3 (time interval between 5- and 8-cell stages) and other relative timing expressions (33).

One of the most influential day 3 embryo selection algorithms so far was reported by Meseguer et al. (28) in 2011, combining t5, cc2 and s2 into a hierarchical algorithm after applying a few excluding and discarding criteria. Assessed against a number of criteria in this algorithm, day 3 embryos were then classified into 10 grades (A+, A-, B+, B-, C+, C-, D+, D-, E, F with descending quality) of which the reported implantation rates ranged from 66% to 8% in the first 9 grades (28). This algorithm was recently modified by the same group and tested on a larger-scale at a multicenter level, by (i) using t3 as the primary selection criterion instead of t5 which has been downgraded to a tertiary criterion in the modified version, (ii) removal of s2 from the system, and (iii) modification of optimal ranges for t5 and cc2 (65). However the predictive power of the algorithm did not appear as impressive as in the original report in 2011.

Whilst the majority of publications regarding time-lapse selection models for human embryos were performed using the Embryoscope, there was another model created through the use of the Eeva system (29, 64). Although in this system dark-field imaging facilitates the capability of auto-detecting initial cell divisions (29), the nuclear status of blastomeres could be difficult to evaluate due to limited visibility (66), which may lead to inaccurate judgment, e.g., differentiation between blastomere and large non-nucleated fragmentation. In this model, two morphokinetic parameters P2 (cc2, duration of 2-cell stage) and P3 (s2, duration of 3-cell stage) were used to structure Eeva grades, and may be used in the conjunction with conventional day 3 morphology assessment to predict blastulation (64, 67).

In spite of the fact that a good number of early cleavage timing parameters are evidently associated with embryo viability, it is still not conclusive regarding how to organize those parameters for improved discriminatory power with both satisfactory sensitivity and specificity (35). Furthermore, as a few recent studies have shown inconsistent prediction of implantation outcomes after applying the published models on embryos created in different laboratories (68-70), further investigations are required to uncover the reasons behind those findings.

1.2.3 Morphokinetics at morula and blastocyst stages

While the vast majority of time-lapse studies have focused on early stage markers during embryo development (33), there have also been a few reports exploring later stage markers of embryo viability during morula and blastocyst formation (63, 71-74). The time for embryos to reach morula stage has been reported to be associated with the formation and quality of subsequent blastocysts (63, 74), and implantation results (72). Although two later stage timing parameters, timings to reach start of blastulation and full blast stage, have been proposed to be used for prediction of ploidy status of embryos (71) and implantation outcomes (72), conflicting results were reported by another independent laboratory who failed to reproduce the proposed classification model based on a selected population of poor –prognosis patients (73).This highlights the need to consider patient-related confounders, such as female age, when interpreting correlations between timing parameters of observed embryos and implantation outcomes, as stated by Kirkegaard et al. (75), who also reported that timing parameters at blastocyst stage were more affected by patient-related factors than those at cleavage stage.

1.2.4 Abnormal biological events

Apart from the quantitative parameters (i.e., continuous timing data) as mentioned above, another benefit of having embryos continuously monitored is the capability of detecting abnormal biological events during embryo development (76). Due to the dynamic nature of embryo development, it would be very difficult to identify such events via static observations in the conventional embryo incubation system. For

example, multinucleation (MN) in human IVF embryos has been studied for more than a decade (77). Despite its value for clinical application being widely accepted, the accuracy and feasibility of assessment are limited due to the various cell cycle stages at time of observation. According to a recent time-lapse study (78), only 27.6% of embryos with MN were identified within the timing window proposed by the consensus meeting (5) for embryo observation.

A few abnormal cleavage patterns of early stage human embryos were reported recently (62, 79, 80). Direct cleavage (DC) 2-3 cells was referred to as an embryo undergoing a tri-polar cell division, resulting in 3 daughter cells instead of 2 as seen in the typical cell division (62). The occurrence of such event has been witnessed at both the first and second cleavage cycles of early embryo development, with the prevalence at 8.3-13.7% (79, 80) and 9.2% (79) respectively. It can be expected that such types of cell division would very likely result in uneven distribution of the duplicated chromosomes into 3 daughter cells, which requires future aneuploidy studies to confirm. However, currently available publications do show reduced subsequent blastulation (62, 79) and implantation rates (79, 80) of affected embryos. More recently, the presence of collapse at blastocyst stage was also shown to be adversely correlated with implantation results, so potentially can be used as another late stage predictive marker for implantation (81). The above indicates that qualitative measures, with the outcome being either positive or negative, may be used as deselection parameters as an additional advantage of time-lapse culture of human embryos.

1.3 Limitations in existing time-lapse systems in human IVF

Although extensive time-lapse research has been carried out during the last 5 years since the introduction of commercially available time-lapse equipment into clinical practice (28, 29), this field of science is still at a young age. A number of implantation predictive parameters have been reported by either individual laboratories or even multicenter studies, but there is still a lack of wide range consensus regarding the usefulness of time-lapse monitoring on embryo selection (33-36). In spite of the recent publication of the first guidelines aiming to standardize parameters that could be

generated by the time-lapse equipment/device (76), there is still room for improvement on the current timing systems. Encouragingly a few embryo selection models have been published so far (28, 64, 71), however, the reproducibility has been raised as a potential barrier to their wider application (68-70). The current project focuses on the following 2 major limitations of current systems, and explores their potential causes followed by the proposal of corresponding solutions.

1.3.1 Limited accuracy and certainty of current timing systems

The vast majority of current time-lapse studies were based on ICSI embryos only (33), likely due to the ease of defining a start time point (t0), sperm injection, to time subsequent biological events. In contrast, IVF embryos mostly have uncertain sperm entry time because of the reduced visualization due to the presence of cumulus cells around the oocytes at time of fertilization. Although the published guidelines have proposed using the time of mixing sperm and oocyte-cumulus-complex in conventional IVF as a t0 (76), the actual sperm entry time for each individual oocyte is mostly unknown, being later than the recorded time point. It is widely acknowledged that ICSI technique should not be overused due to the potential risk posed to subsequent offspring considering its invasive nature (82), therefore time-lapse technology should not be constrained by ICSI cases only.

Even for the ICSI cases, where the time point of sperm injection for each individual oocyte can be recorded, it may not be easy to do so in certain types of time-lapse systems. For example, the Embryoscope incubator may contain up to 6 Embryoslides, each of which carries up to 12 embryos. However, the current timing system only allows a single t0 to be defined for the whole Embryoslide, instead of a different t0 for each individual embryo. Since the whole ICSI procedure may take considerable time for the completion of the entire cohort of oocytes, only the middle time of the whole procedure can be used as t0 as suggested by the published guidelines (76). Furthermore, nuclear mature oocytes (metaphase II) produced in the same cohort may not have the same degree of cytoplasmic maturity, and this may be partially indicated by the fact that sibling metaphase II (MII) oocytes extrude their second polar bodies

at a wide range of times after sperm injection (38, 44). So these should lead to the exploration of an alternative t0 to increase the accuracy and certainty in the current timing systems, with the potential to remove the reported inconsistencies in the timings of early stage development between ICSI and IVF embryos (83, 84).

1.3.2 Limited reproducibility of published algorithms

In recent years, a number of time-lapse prediction models have been published, including a day 3 model to predict implantation potential (28) and a day 5 model to predict ploidy status (71). However, despite the impressive results shown in the original reports, there have been other studies by independent laboratories showing reduced discriminatory power or reproducibility after employing the reported models (68-70, 73). The reason behind this is unclear but may involve multiple patient- and laboratory-related factors, and the time in culture, as blastocyst time-lapse parameters are more affected by patient-related factors than cleavage parameters (75). Therefore, the current project intends to explore potential explanations for such inconsistent findings, upon which solutions could be developed to improve outcomes.

1.4 Thesis aims

In accordance with the limitations in current time-lapse systems as discussed above, the current thesis is structured as a series of related publications, measuring and defining various morphokinetic parameters (see appendix 1), with each individual publication serving a single aim. Aims in different chapters/sections are shown below:

Aim 1: To explore variations in timings of early biological events in human MII oocytes following sperm injection, and their relationship with subsequent cleavage divisions (**Section 2.2**).

Aim 2: To propose the use of pronuclear fading as an alternative biological reference starting time point for timing subsequent cleavage divisions (**Section 2.3**).

Aim 3: To compare traditional embryology parameters and embryo morphokinetics by using pronuclear fading as a reference starting time point according to the trigger-day serum progesterone level (**Section 2.3**).

Aim 4: To investigate variations in embryo growth rates between two independent IVF laboratories for potential explanation of the poor inter-laboratory reproducibility after applying a published algorithm (**Section 3.3**).

Aim 5: To report two novel qualitative time-lapse parameters, namely reverse cleavage and less than 6 intercellular contact points at the end of the 4-cell stage, followed by the proposal of a time-lapse qualitative deselection model (**Chapter 4**).

Aim 6: Based on the findings in previous chapters, to develop a universal time-lapse deselection model for day 3 human embryos regardless of insemination method, by combining both qualitative and quantitative measures (**Chapter 5**).

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CHAPTER 2: Variations in early biological milestone timings in human oocytes following fertilization

2.1 Prelude

Conventional static observations on human embryos are used as routine protocol in IVF programs today but only provide limited dynamic information regarding the early embryonic development of fertilized oocytes. This chapter takes the opportunity offered by modern time-lapse technology to investigate the timings of early biological milestones of oocytes following fertilization. Two papers were included in this chapter with citation information shown below. Paper 1 focused on the variations over time for human MII oocytes to reach early events up to the first cleavage division post ICSI, including second polar body extrusion, pronuclear fading and first mitotic division. Based on the findings in Paper 1, Paper 2 explored the weakness of the current timing system (Embryoscope) used in the conventional IVF and ICSI cycles, which may be inaccurate when timing biological events by using "insemination" as the start time point (t0). Additionally, this paper proposed that pronuclear fading should be used as t0 for avoidance of such uncertainty in developmental timings at early stage post IVF or ICSI insemination. Concomitantly, there's a potential to build a timing system to describe embryo development that is compatible to both insemination methods.

Liu Y, Chapple V, Roberts P, Ali J, Matson P. Time-lapse videography of human oocytes following intracytoplasmic sperm injection: events up to the first cleavage division. Reproductive Biology. 2014;14(4):249-56. Epub 2014/12/03. (Paper 1)

Liu Y, Chapple V, Feenan K, Roberts P, Matson P. Time-lapse videography of human embryos: Using pronuclear fading rather than insemination in IVF and ICSI cycles removes inconsistencies in time to reach early cleavage milestones. Reproductive Biology. 2015;15(2):122-5. Epub 2015/06/09. (Paper 2)

Liu, Y., Chapple, V., Roberts, P., Matson, P. (2014). Prevalence, consequence, and significance of reverse cleavage by human embryos viewed with the use of the Embryoscope time-lapse video system. *Fertility and Sterility, 102*(5), 1295-3000. doi:10.1016/j.fertnstert.2014.07.1235. The abstract of the article is available at http://ro.ecu.edu.au/ecuworkspost2013/173/

Liu, Y., Chapple, V., Feenan, K., Roberts, P., & Matson, P. (2015). Clinical significance of intercellular contact at the four-cell stage of human embryos, and the use of abnormal cleavage patterns to identify embryos with low implantation potential: A time-lapse study. *Fertility and Sterility, 103*(6), 1485-1491. doi: 10.1016/j.fertnstert.2015.03.017.
The abstract of the article is available at http://ro.ecu.edu.au/ecuworkspost2013/948/

CHAPTER 3: Factors affecting embryo morphokinetics

3.1 Prelude

After the introduction of time-lapse technology into human IVF programs, the observation of dynamic behaviours of embryos in culture became possible. It has been reported that embryo morphokinetics could be affected by different female patient populations, type of ovarian stimulation protocols and hormone levels, sperm DNA integrity, and embryo culture systems. In this chapter, Paper 3 investigated the impact of the trigger-day serum progesterone on embryo quality assessed via both routine embryology and time-lapse videography. In addition, Paper 4 compared embryo morphokinetic features expressed by both quantitative and qualitative measures between two independent laboratories, which had various patient profiles and embryo culture conditions (Paper 4). Also, in this paper, both the implanting and non-implanting embryos were analysed separately.

Liu Y, Copeland C, Chapple V, Roberts P, Feenan K, Matson P. The relationship between embryo quality assessed using routine embryology or time–lapse videography and serum progesterone concentration on the day of ovulatory trigger in in vitro fertilization cycles. Asian Pacific Journal of Reproduction. 2015;4(2):140-6. (Paper 3)

Liu Y, Copeland C, Stevens A, Feenan K, Chapple V, Myssonski K, Roberts P, Matson P. Assessment of human embryos by time-lapse videography: a comparison of quantitative and qualitative measures between two independent laboratories. Reproductive Biology. 2015;15(4):210-6. (Paper 4)

Pages 54-90 are not included in this version of the thesis due to copyright restrictions. These pages include the following 2 articles:

Liu, Y., Copeland, C., Chapple, V., Roberts, P., Feenan, K., & Matson, P. (2015). The relationship between embryo quality assessed using routine embryology or time–lapse videography and serum progesterone concentration on the day of ovulatory trigger in in vitro fertilization cycles. *Asian Pacific Journal of Reproduction, 4*(2), 40-46. doi: 10.1016/S2305-0500(15)30011-7.
The abstract of the article is available at http://ro.ecu.edu.au/ecuworkspost2013/1584

Liu, Y., Copeland, C., Stevens, A., Feenan, K., Chapple, V., Myssonski, K., Roberts, P., & Matson, P. (2015). Assessment of human embryos by time-lapse videography: A comparison of quantitative and qualitative measures between two independent laboratories. *Reproductive Biology, 15*(4):210-216. doi: 10.1016/j.repbio.2015.09.001.
The abstract of the article is available at *http://ro.ecu.edu.au/ecuworkspost2013/1583*

CHAPTER 4: Qualitative deselection measures of human embryos observed *via* time-lapse videography

4.1 Prelude

Embryo selection is normally performed through the identification of the embryo(s) with highest implantation potential, but could also be achieved by excluding those with the lowest implantation potential. The chance of picking the right embryo(s) from the cohort could be significantly increased by reducing the number of candidate embryos *via* deselection. This chapter focused on embryo deselection using qualitative measures generated via time-lapse monitoring. Such parameters (mostly abnormal cleavage patterns of embryos), are very difficult to detect *via* conventional static observations (usually once daily). Paper 5 investigated the prevalence of reverse cleavage in observed embryos and the impact on subsequent embryonic development. Furthermore, embryos affected by reverse cleavage had very low implantation rates following transfer. Similarly, Paper 6 reported another parameter that could be potentially used for embryo deselection, namely the degree of intercellular contact at the end of the 4-cell stage. Also, it was proposed in this paper that a qualitative algorithm incorporating a number of time-lapse deselection parameters may aid the improvement of implantation rates of embryos after deselection.

Liu Y, Chapple V, Roberts P, Matson P. Prevalence, consequence, and significance of reverse cleavage by human embryos viewed with the use of the Embryoscope time-lapse video system. Fertility and Sterility. 2014;102(5):1295-300 (Paper 5)

Liu Y, Chapple V, Feenan K, Roberts P, Matson P. Clinical significance of intercellular contact at the four-cell stage of human embryos, and the use of abnormal cleavage patterns to identify embryos with low implantation potential: a time-lapse study. Fertility and Sterility. 2015;103(6):1485-91 (Paper 6)

Liu, Y., Chapple, V., Roberts, P., Matson, P. (2014). Prevalence, consequence, and significance of reverse cleavage by human embryos viewed with the use of the Embryoscope time-lapse video system. *Fertility and Sterility, 102*(5), 1295-1300. doi:10.1016/j.fertnstert.2014.07.1235.
The abstract of the article is available at http://ro.ecu.edu.au/ecuworkspost2013/173/

Liu, Y., Chapple, V., Feenan, K., Roberts, P., & Matson, P. (2015). Clinical significance of intercellular contact at the four-cell stage of human embryos, and the use of abnormal cleavage patterns to identify embryos with low implantation potential: a time-lapse study. *Fertility and Sterility, 103*(6), 1485-1491. doi:10.1016/j.fertnstert.2015.03.017
The abstract of the article is available at http://ro.ecu.edu.au/ecuworkspost2013/948/

CHAPTER 5: Clinical application – an embryo deselection model by use of time-lapse videography

5.1 Prelude

As illustrated in Chapter 2, considerable uncertainty exists in the timings of early developmental milestones following fertilization of human oocytes, while the use of PNF as a biological reference starting point for timing subsequent developmental stages may minimize such uncertainty in both IVF and ICSI cases. Furthermore, Chapter 4 reported two time-lapse qualitative parameters for embryo deselection, and a deselection model incorporating a number of such parameters was also proposed. Whilst in Chapter 3, inter-laboratory comparisons showed better reproducibility of such qualitative parameters compared with the quantitative ones. Therefore, in this chapter, Paper 7 focused on the clinical application based on the above findings and proposed an embryo deselection model combining both the qualitative and quantitative parameters. In this model, embryos may be graded under the same criteria regardless of the insemination method used, by using PNF as the reference starting time point. The qualitative component of this model may also be used separately for better inter-laboratory transferability as discussed in Chapter 4.

Liu Y, Chapple V, Feenan K, Roberts P, Matson P. Time-lapse deselection model for human day 3 in vitro fertilization embryos: the combination of qualitative and quantitative measures of embryo growth. Fertility and Sterility. In Press. (Paper 7)

Pages 135-155 are not included in this version of the thesis due to copyright restrictions. These pages include the following article:

Liu, Y., Chapple, V., Feenan, K., Roberts, P., & Matson, P. (2016). Time-lapse deselection model for human day 3 in vitro fertilization embryos: the combination of qualitative and quantitative measures of embryo growth. *Fertility and Sterility*, *105*(3), 656-662. doi: 10.1016/j.fertnstert.2015.11.003
The abstract of the article is available at http://ro.ecu.edu.au/ecuworkspost2013/1547/

CHAPTER 6: General discussion and conclusions

6.1 Alternative biological starting point for timing parameters (Chapter 2)

At the beginning of this project (section 2.2) we investigated the times of early developmental events in human oocytes following sperm injection by using the Embryoscope, one of the commercially available time-lapse incubators. Morphometric and morphokinetic analysis was performed assisted by the Embryoviewer software. The timings of three major biological events were analyzed, namely second polar body extrusion, pronuclear fading and first mitotic cleavage division. We found a large variation in the timings of second polar body extrusion post ICSI amongst nuclear mature (MII) oocytes, which is in agreement with previous publications (1, 2). This biological milestone seems important since the morphometric analysis in this section showed a shrinkage of the oocytes post injection but a cessation of reduction in size occurred at second polar body extrusion regardless of its timing. Furthermore, the timings to reach subsequent early stage milestones seemed to vary less after the extrusion of the second polar body. The above phenomenon may be a reflection of the diverse maturity status in the cytoplasm of the MII stage oocytes from the same cohort at time of sperm injection. This indicates that the variations in cytoplasmic maturity of the MII oocytes may confound the timings of subsequent early developmental milestones if using sperm injection, a procedural time point, as the reference starting time point t0; while the use of a biological t0 such as second polar body extrusion or any early events beyond, may minimize the variations caused by such an artificial factor, i.e., sperm entry timing determined by ICSI protocol in the laboratory.

In routine practice where only static observations are involved, the timing variations in the development of early human embryos, fertilized via either conventional IVF or ICSI, are difficult to identify. Unified time points for observations at different stages of embryo culture, regardless of insemination method, have been outlined in the recent consensus (3). Following the application of time-lapse technology in human IVF laboratories, it has become feasible to reveal timing differences between IVF and ICSI

embryos. Pronuclear fading is one of the earliest biological events that can be easily observed in both IVF and ICSI embryos. A number of recent time-lapse studies reported that ICSI embryos spent significantly less time reaching various early cleavage stage milestones (4-7) in comparison to their IVF counterparts when using insemination as t0, while by using pronuclear fading as t0 the early timing differences between IVF and ICSI embryos could be removed (4). In section 2.3, similar results were reported in timings up to the 5-cell stage in both our overall embryo population and the implanting embryos. However, these studies, including the present study, were retrospective and based on overall embryo populations, therefore patient-related clustering and confounding effects in such design may increase the risk of misinterpreting the observed results due to the influence of patient factors (8). Considering the above mentioned facts, future properly designed large-scale prospective studies are suggested to confirm these preliminary findings. Moreover, the method of statistical analysis should be carefully selected to minimize the risk of misinterpretation of observed results (8).

The majority of currently published time-lapse studies were based on ICSI embryos probably due to the difficulties in defining the sperm entry time point in conventional IVF cases. However an ideal timing system, in particular a system proposed for embryo selection, would be able to accommodate all embryos created via either insemination method. Indeed conventional IVF is a crucial component in the world of ART. Evidence available at present implies pronuclear fading could be considered in the future timing systems for embryo selection, due to its potential benefits of improved certainty and compatibility.

6.2 Factors affecting embryo morphokinetics (Chapter 3)

In section 3.2, the potential effect of trigger-day serum progesterone level during IVF treatment was evaluated on the subsequent oocyte viability. In order to reflect the oocyte quality, embryology performance (conventional IVF cycles only) was examined via both conventional morphology and morphokinetic assessment. ICSI cases were not included to avoid confounding effects from paternal factors. In the

morphokinetic analysis, pronuclear fading was used as t0 to time subsequent early embryo development so that the variations caused by uncertain sperm entry timing could be minimized. Although no significant impact of high progesterone level per follicle was found on the subsequent embryo development; the retrospective design, restricted range of progesterone concentrations, and absence of multivariate analysis in the present study may limit the conclusions drawn. Future prospective randomized studies should attempt to minimize the patient-related confounding variables (8) as a number of reports have linked a range of factors to altered embryo morphokinetics. These factors include embryo culture conditions such as oxygen level (9) or culture media (10), ovarian stimulating protocols (11, 12), and patient populations (8, 13-15). Meanwhile studies with different findings have been reported (16, 17), but it would not be surprising to see varying morphokinetics of embryos created and cultured in different clinics given the diversity in patient populations, culture conditions and ovarian stimulating protocols.

To verify the above assumption regarding between-laboratory variation, section 3.3 compared the embryo morphokinetics of both implanting and non-implanting embryos using KID data between two independent Australian IVF clinics. Furthermore, to avoid potential confounding factors from inter-operator variations with regard to embryo annotation, this study only involved one embryologist who had retrospectively annotated all included embryos from both clinics. Interestingly, between the two clinics, both implanting and non-implanting embryos had significantly different morphokinetics at early developmental stages. The variations seemed reasonable when looking at the differences in laboratory protocols and patient profiles between two clinics, as presented in Tables 12 & 13. This leads to a potential explanation of the recently reported low reproducibility or transferability of some published embryo classification models, which are mainly based on timing parameters of embryo development (18-21). An ideal embryo selection model or algorithm, similar to other diagnostic pathology tests (22), should have transferable reference ranges against which to compare the growth of an embryo for selection purposes. While considering different embryo development profiles in different laboratories as presented in Section 3.3, one may expect different prediction power when using a model with reference ranges externally created. These models may not lose their discriminatory power when

tested within the same clinic or group, but it would be wise for an external user to take precautions when directly applying the published models.

6.3 Qualitative deselection of embryos via time-lapse monitoring (Chapter 4)

Chapter 4 concentrated on the qualitative measures that can be potentially used for embryo deselection. Similar parameters have been reported previously, such as direct cleavage where a blastomere undergoes multi-polar cell division resulting in more than 2 daughter cells with uneven distribution of chromosomes (23, 24). Reverse cleavage and less than 6 ICCPs at the 4-cell stage are two abnormal cleavage patterns which have been reported individually in two separate papers (Sections 4.2 & 4.3), and they are only detectable via continuous monitoring. These events potentially impact the chromosomal integrity or intercellular communication in early human embryos, and as a result, those with such observed abnormalities are expected to have reduced viability. Better embryo selection could be expected by combining both qualitative and quantitative parameters. However, precautions must be taken when new time-lapse users apply embryo selection models including quantitative parameters due to the variations that exist between clinics as discussed in the previous section. While the qualitative parameters only involve two outcomes of measurement (either positive or negative and being independent of embryo growth rates), better inter-laboratory reproducibility could be achieved when used separately. This assumption was validated in Section 3.3, where embryo morphokinetics (quantitative parameters) varied between two independent laboratories (Table 14) while the occurrence of abnormal biological events (qualitative parameters) appeared more similar between the same two laboratories (Table 16). Additionally, a qualitative embryo deselection model was proposed at the end of section 4.3, by including a number of abnormal biological events observed via time-lapse monitoring, and it showed significantly improved implantation rates by simply deselecting embryos displaying such abnormal biological events.

Since several time-lapse systems are currently available commercially, the transferability of the above mentioned qualitative parameters between different time-lapse systems may also require further validation. One of the potential issues might be the difficulty in identifying certain biological events in specific time-lapse systems. For example, the identification of direct cleavage involves the confirmation of an extra blastomere(s) by the visualization of a nucleus (nuclei), such that it can be distinguished from large non-nucleated fragmentation. Also, the detection of reverse cleavage involves the confirmation of nuclear disappearance before cell fusion or failed cytokinesis followed by its reappearance. However time-lapse systems using dark-field imaging, such as the Eeva system, may have difficulties confirming the above, therefore potentially leading to different conclusions from other systems such as the Embryoscope.

6.4 Embryo deselection model combining both quantitative and qualitative measures (Chapter 5)

Based on the findings in Chapters 2 to 4, Chapter 5 proposed a deselection model for human day 3 embryos, including both qualitative and quantitative parameters. Compared to the day 3 algorithm published by Meseguer et al. (25) in 2011, this model may lead to the following benefits: (i) it is applicable to both IVF and ICSI embryos by using PNF as t0, (ii) the qualitative deselection module may be used separately for new time-lapse users when collecting KID data to determine their own reference ranges for timing parameters, and (iii) it offers an easier decision making process for new time-lapse users, i.e., selecting those with a shorter t5_PNF after the exclusion of extreme cases (direct cleavage from 1-3 or 2-5 cells). The transferability issue is still not completely resolved in the current model, and it is clearly stated that individual laboratories must determine their own reference ranges for timing parameters based on KID data. However, this model provides a methodology that may be adopted by new time-lapse users to calculate their own reference ranges for the timing parameters. Additionally, one can still benefit by using the qualitative deselection model alone.

This model was constructed using retrospective data, followed by prospective validation using a relatively small sample size. Although preliminary tests showed similar predictive power in two different culture media suites within the same laboratory, future large-scale prospective validation is warranted, ideally involving a different laboratory by creating their own reference ranges after applying the same methodology. Retrospective studies are at frontline of methods to identify potential biomarkers for human embryo selection, as a means of initially identifying various parameters for further investigation. However, confounding factors in such retrospective analyses are difficult to control, potentially leading to misinterpretation of observed effects. So it is important for retrospective findings to be validated by subsequent prospective studies with a controlled design, as demonstrated in recent publications (26, 27). Whilst the randomized controlled trials are considered to be one of the preferred tools to test the effectiveness of a study objective because of the better controlled confounding factors via randomization, such study design is not without limitations such as extended duration, high running costs, difficulty in participant recruitment, and the inability to change protocol in response to subsequent findings.

6.5 Limitations and perspectives

Although promising, the application of time-lapse technology in clinical IVF practice is still in its infancy with room for improvement and refinement. One of the constraints is that embryos in time-lapse culture are not able to be rolled for better viewing angles, whilst rolling embryos under the microscope during conventional observations is quite often used by the embryologists to gain better three dimensional information, especially when assessing the inner cell mass at the blastocyst stage. In Section 4.3, the evaluation of intercellular contact of the 4-cell stage human embryos was occasionally affected by a suboptimal viewing angle and not being able to roll the embryos. Future time-lapse systems with an extra dimension of imaging on the embryos would potentially address this issue by the addition of an extra camera, i.e., a total of two cameras scanning the embryos in both horizontal and vertical planes.

As defined by current policy at the clinic where this study was performed, embryos included in the present study were all cultured and transferred 3 days post oocyte collection. Consequently, the effect of reported parameters such as reverse cleavage and less than 6 ICCPs at the end of the 4-cell stage on the subsequent blastulation were not investigated. Although day 3 morphology score and implantation were used as the two major end points for assessing their impact, it would no doubt provide further insights by including blastulation analysis since blastocyst culture is currently widely used globally as a routine practice. The blastulation rates of embryos classified into different grades (A+ to F) as described in Section 5.2 would lead to further understanding of the relationship between early embryo morphokinetics and subsequent blastulation or blastocyst quality. The implantation rate of A+ embryos was 52.9% as shown in Figure 9, which appears comparable with the implantation rate of blastocysts. It is not clear based on currently available literature whether the morphokinetics of early embryo development is only predictive of blastulation instead of implantation, or time-lapse parameters are implantation predictive independent of blastulation. Subsequent future research may focus on the blastulation of embryos with different grades in the proposed model. If morphokinetic features of early embryo development were predictive of blastulation only, the options may rely on the individual strategy at different clinics to either use blastocyst culture as a tool of selection with lower running costs, or transfer the embryos at an earlier developmental stage after time-lapse analysis with minimized potential external physical/biochemical stress caused by in vitro culture. However, if the early morphokinetic features were independent of blastulation, the parameters could then be used in combination with blastocyst grading for improved selection. The recent promotion of single stage culture media combined with extended culture of embryos in a time-lapse incubator may well facilitate the development of such a protocol (28).

Another limitation of the present studies was the relatively small sample sizes, and their retrospective nature. As pointed out in a recent study by Kirkegaard et al. (8), retrospective studies may carry a high risk of patient-based confounding factors, therefore the observed differences in embryo development may be misinterpreted when treating embryos as independent observations. Further prospective large-scale randomized controlled studies are certainly necessary to validate the reported findings,

so that both known and unknown patient-related confounders could be equally distributed via the randomization of patients (8). Furthermore, the preliminary report of reverse cleavage in the present study only analyzed the implantation outcomes of 22 affected embryos. Although the extremely low implantation rate reached statistical significance in comparison to the unaffected counterparts, the small sample size did not allow the evaluation of different types of reverse cleavage nor the developmental stage related impact on the subsequent implantation results. Indeed, less impact on the embryo viability could be expected when such abnormal events occurred at later developmental stages. In future studies, at the preliminary phase, it would be useful to explore blastulation rates for embryos with type I or II reverse cleavage occurring at different developmental stages. At the second phase, those that have successfully reached blastocyst stage and been cryopreserved may be used for implantation assessment in subsequent frozen transfer cycles. However, since they (grade Es in the reported deselection model) are expected to be given low priority for transfer, it may take a considerable amount of time to gather a good sample size for statistical analysis. Correlating with future findings, further detailed grading systems may be developed/upgraded.

Evidence regarding the inter- and intra-operator reproducibility in time-lapse parameters on human embryos is currently limited. In a recent report, the reproducibility of time-lapse timing parameters appeared to be at satisfactory to good levels, while qualitative parameters (only multinucleation and evenness at the 2-cell stage were included in this study) seem to be less reproducible (29). It has been demonstrated previously in conventional embryo morphology grading systems that training sessions provided to the participating embryologists may be an effective way to improve inter- and intra-operator consistencies (30, 31), however the same has not yet been performed on the time-lapse assessment systems. Furthermore, the reproducibility of the identification of abnormal cleavage patterns, such as direct cleavage, reverse cleavage and <6ICCPs at the end of the 4-cell stage as reported in the present study, has also not been evaluated. All embryo annotations in the present study were performed by the same embryologist in order to avoid inter-operator variations, so future studies should investigate such inter-operator issues by involving multiple operators, preferably with the addition of training sessions to validate their

effect on the improvement of consistency. At the laboratory level, however, the reproducibility of qualitative parameters, which are measured as either positive or negative and independent of embryo growth rates, are considered to have better inter-laboratory reproducibility in comparison to the quantitative parameters as shown in Section 3.3. This again will need further evaluations by involving more laboratories with multiple levels of comparisons.

A number of published studies comparing morphokinetic timing parameters between embryo groups did not consider the underlying confounding impact of abnormal cleavage patterns (4, 6). For example, embryos displaying direct cleavage have a faster cleavage rate, therefore the group of embryos which have a larger proportion of such embryos would demonstrate a faster growth rate than the group with a smaller proportion of such embryos. Consequently, when analysing implantation prediction by certain timing parameters, a group of embryos with faster growth rates cannot be said to have better viability than their slower counterparts if the directly cleaved embryos have not been excluded from analysis. Comparisons of quantitative parameters in the present study have excluded direct cleavage at 1- or/and 2-cell stages and, as a result, data analysis is expected to be more representative. One example is the distribution of certain timing parameters of embryos with implantation/non-implantation outcomes in Section 5.2. The t5_PNF shifted from a bell shaped curve when including the direct cleaved embryos, to a linear shape after excluding such embryos (Figure 10). This change of shape enables an easier way for new time-lapse users when selecting embryos based on t5_PNF, by picking the one with the shortest t5_PNF without concerns about the unknown optimal ranges for t5_PNF in their specific laboratory.

Additional novel qualitative parameters could be revealed in the future research. Dynamic features that could not possibly be observed in the conventional protocol, such as blastomere rolling, cytoplasmic wave and cytoplasmic string at the blastocyst stage have not yet been fully investigated. However the accurate description or quantification of such parameters may require further development of corresponding computer assisted software. Currently available software like the Embryoviewer have shown such potential in the morphometric analysis as illustrated in Section 2.2, where

the size of oocyte was represented by the computer calculated surface area around the oolemma (Figure 1). However the accuracy of current protocol still needs further improvement since the oocyte is not always a perfect sphere. As a result, the oocyte size may be (i) misrepresented by the measurement on a single image captured in one focal plane of the oocyte, which could actually be an oval shaped oocyte at a 3-D level; or (ii) underrepresented/overrepresented due to the irregularity of oolemma outline. Further, current measurement functions in the Embryoviewer software still require manually drawn lines or circles, therefore the accuracy and ease of use could potentially be improved by introducing more advanced image analysis software.

The emergence of novel quantitative and qualitative time-lapse parameters may lead to improved laboratory performance monitoring. In the conventional laboratory management, proportion parameters such as day 2/3 good quality embryo rates or blastulation rates have been used as key performance indicators. Apart from these parameters, now we have the option of using continuous timing parameters. This approach could result in increased sensitivity due to the statistical advantage of continuous data compared to proportion data. Abnormal cleavage events revealed via time-lapse monitoring could also supplement the effectiveness of the use of continuous timing parameters. Furthermore, continuous data may also be very useful when assessing different products/procedures by comparing timing parameters of randomized sibling embryos in the time-lapse culture.

Although the majority of time-lapse human embryo studies have been performed with the use of the Embryoscope, there are other commercially available options such as the Primo Vision or Eeva systems. One parameter or model developed and validated in one time-lapse system, does not necessarily ensure its usefulness in another time-lapse system. Therefore, future studies may involve not only the multicenter prospective validation of the proposed parameters and selection models, but also careful testing with the use of alternative time-lapse systems. In addition, current advantages and disadvantages between existing systems may encourage the acceleration of technical evolution in modern manufacturing, with each individual time-lapse system being upgraded more frequently as a result.

6.6 Conclusions of thesis

The main conclusions are summarized as the following:

- Human oocytes reduce in size after sperm injection, but shrinkage is terminated at second polar body extrusion.

- Second polar body extrusion occurs at various times after sperm injection.

- Pronuclear fading may be used as an alternative biological reference starting time point for timing subsequent cleavage divisions.

- Inconsistent timings of early cleavage divisions relative to insemination are present between IVF and ICSI embryos, but the inconsistencies may be removed by using pronuclear fading as a reference start point.

- Serum progesterone level per follicle on the trigger injection day does not affect subsequent embryo morphokinetics.

- A review of literature showed a range of factors affecting embryo morphokinetics, including patient or culture condition related variables.

- Embryo growth rates differ between different laboratories, in both implanting and non-implanting embryos.

- Embryo selection algorithms emphasizing quantitative timing parameters may lose their discriminatory power when introduced into a different clinic due to various factors affecting embryo growth.

- Time-lapse parameters for qualitative deselection are effective in identifying embryos with low implantation potential, with better inter-laboratory reproducibility than quantitative time-lapse parameters.

- Embryos displaying certain abnormal cleavage patterns such as direct cleavage should be excluded when comparing timing parameters to avoid a confounding impact.

- The embryo deselection model proposed in the current study is effective to discriminate embryos with different implantation potential, regardless of insemination method.

- Both quantitative and qualitative time-lapse parameters are predictive of embryo implantation results, but quantitative parameters may require laboratory specific reference ranges.

- This thesis has identified a number of potential time-lapse approaches for embryo deselection. These findings showed clinical usefulness in a preliminary prospective study, but these findings need to be confirmed by larger prospective randomized controlled trials.

6.7 References

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APPENDICES

Appendix 1: List of description of time-lapse parameters of human embryos

Time-lapse parameters	Descriptions
Tpb2	Time (hour) from insemination to 2nd polar body extrusion
Tpnf	Time (hour) from insemination to pronuclear fading
T2 or T2_insem	Time (hour) from insemination to 2-cell stage
T3 or T3_insem	Time (hour) from insemination to 3-cell stage
T4 or T4_insem	Time (hour) from insemination to 4-cell stage
T5 or T5_insem	Time (hour) from insemination to 5-cell stage
T8 or T8_insem	Time (hour) from insemination to 8-cell stage
T2_pnf	Time (hour) from pronuclear fading to 2-cell stage
T3_pnf	Time (hour) from pronuclear fading to 3-cell stage
T4_pnf	Time (hour) from pronuclear fading to 4-cell stage
T5_pnf	Time (hour) from pronuclear fading to 5-cell stage
T8_pnf	Time (hour) from pronuclear fading to 8-cell stage
CC2 (T3-T2)	Time (hour) from 2-cell to 3-cell stage
S2 (T4-T3)	Time (hour) from 3-cell to 4-cell stage
T5_T4	Time (hour) from 4-cell to 5-cell stage
S3 (T8-T5)	Time (hour) from 5-cell to 8-cell stage
MN	Multinucleation
DC	Direct cleavage where one cell divided into 3 or more daughter cells
RC	Reverse cleavage where a) two daughter cells fuse after separation, or b) cell fails to separate after karyokinesis
<6ICCP	Less than 6 intercellular contact points at the end of the 4-cell stage

Appendix 2: Use of the Embryoscope- Standard Operating Procedure

FertilityNorth
Fertility & Endometriosis Treatment Centre

SOP

Use of the Embryoscope™

Purpose:

To describe how to use the Embryoscope for the uninterrupted culture of embryos and their continuous monitoring, aiming to improve embryo selection based on morphokinetic information acquired.

Associated Documents:

LB-S-482	Laboratory Emergency Procedure
LB-S-520	Culture Media Requirements and Set Up
LB-S-536	Laboratory Opening and Closing Procedure and Quality Control
LB-S-602	Control and Storage of Laboratory Supplies and Disposables
LB-S-611	Embryo Transfer
LB-S-615	Vitrification
LB-S-617	Embryo Culture, Assessment and Selection
LB-S-618	Blastocyst Culture, Assessment and Selection
LB-S-624	Laboratory Environment
LB-S-693	Fertilisation Check and 24 Hour Check
LB-S-1021	Use of Geotech G100
LB-S-1025	Use of Thermometer 4610
LB-S-1046	Maintenance of Laboratory Equipment
LB-F-140	Embryology Laboratory Weekly Quality Control
LB-F-176	Embryology ICSI Worksheet
LB-F-177	Embryology IVF Worksheet
	EmbryoScope & EmbryoViewer User Manual_2013 ver 4.3
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Definitions:

DC	Direct Cleavage
ET	Embryo Transfer
FN	Fertility North

Use of the
Embryoscope™

ICCP	Intercellular Contact Points
ICSI	Intracytoplasmic Sperm Injection
IVF	In Vitro Fertilisation
JHC	Joondalup Health Campus
KID	Known Implantation Data
MN	Multi Nucleation
PN	Pronuclear
QC	Quality Check
RC	Reverse Cleavage
SOP	Standard Operating Procedure.
TVOA	Transvaginal Ovum Aspiration
UPS	Uninterruptible Power Supply

Responsibilities:

Role or Department	Responsible for:
Embryologists	• Ensuring ES dishes are correctly prepared to minimise osmolality increases in the micro droplets of media. • Ensuring oocytes / embryos are correctly loaded into the microwells of ES dishes, to ensure the capture of images are not obstructed. • Ensuring that the focus is checked for each oocyte / embryo. • Ensuring all embryos are annotated for morphokinetic time parameters and abnormal cleavage patterns as outlined in this SOP. • Ensuring embryo selection is performed in accordance with the algorithm developed in house as described in this SOP.
Laboratory Manager	• Ensuring that Embryology staff are adequately trained and supported to allow this procedure to take place in accordance with the documented protocol.

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Embryoscope™

1   Introduction

1.1 Blastocyst culture is widely used to minimise multiple pregnancies without compromising the pregnancy rate per embryo transfer (ET). However embryo utilisation rates are often reduced, potentially resulting in cancelled cycles with no blastocyst(s) available for ET on day 5 or 6. Furthermore, concerns have been raised over the prolonged *in vitro* culture of embryos in a number of publications that have reported subsequent epigenetic side effects. Therefore an optimised embryo selection method, without extended culture, is warranted.

1.2 The Embryoscope™ is an advanced piece of equipment which is used for viewing the dynamic development of human embryos in IVF clinics. The continuous monitoring of embryos following fertilisation can enable a more accurate grading of multiple embryos from the same cohort, improving the chance that the best embryo(s) with highest implantation potential can be selected for ET.

1.3 Research at FN has shown Abnormal Cleavage Events such as Reverse Cleavage and <6 Intra Cellular Contact Points (ICCPs) at the end of the 4 cell stage negatively impacts on embryo implantation (Liu et al., 2015b; Liu et al., 2014).

1.4 An improved grading algorithm based on Embryoscope™ data has been retrospectively constructed using FN's Known Implantation Data (KID), which has showed improved predictive accuracy after 3 days culture (Liu et al., 2015c). The algorithm may be used for both IVF and ICSI embryos by using pronuclear (PN) fading as the reference starting time point rather than insemination (Liu et al., 2015a).

1.5 FN now uses this algorithm prospectively for the grading and selection of embryos cultured in the Embryoscope™.

2   Preparation

2.1 Entering a New Patient / Procedure No.

2.1.1   For new patients, information is registered in the EmbryoViewer® system at least one day before TVOA.

2.1.2   Click on the [View All Patients] button on the home screen of EmbryoViewer® system. Then click on the [New] (new) button to create new patient.

2.1.3   Add in Patient name, DOB and File no. as shown below. Other fields may be left blank. Click on the [Save] button after all items have completed.

Use of the
Embryoscope™

2.1.4 To create a new treatment Procedure no., click on the [New] button and add the Procedure No. (e.g. 35142).

2.1.5 Subsequent Procedure No.s may be added for future treatment cycles following 2.1.4.

2.2 EmbryoSlide® Preparation

2.2.1 Refer to *LB-S-520 Culture Media Requirements and Set Up* for the preparation of EmbryoSlides® (ES dishes).

2.2.2 ES dishes are pre-equilibrated in the Embryoscope, if the Embryoscope is full, dishes are pre-equilibrated in the Galaxy Incubator (top shelf).

2.2.3 The EmbryoSlides® are depicted below.

The EmbryoSlide® culture dish

3 Placing Embryos into the EmbryoScope™

3.1 Loading Embryo(s) into the EmbryoSlide®

3.1.1 Fertilised eggs are placed into EmbryoSlide® after fertilisation check on day 1 post TVOA (*see LB-S-693 Fertilisation Check and 24 Hour Check*).

3.1.2 Eggs are carefully placed in the centre of the microwells ensuring no bubbles are created and all existing bubbles removed.

3.2 Placing the EmbryoSlide® into the EmbryoScope™

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Use of the
Embryoscope™

3.2.1 The EmbryoScope™ incubator door is unlocked by pressing the 'Add Slide' button on the home screen of EmbryoScope™. The lid light turns from red to green indicating it is ready to open, whilst a dialog box is shown on the screen (see below).

3.2.2 Open the lid of the door and carefully insert the EmbryoSlide® into the slide holder using the handling fin, the handling fin will be facing outwards. Press "OK" on the screen after the door is closed.

3.2.3 Sometimes briefly opening the door may cause a visual alarm, turning one of the buttons below yellow (Temperature, CO_2, O_2), if so press the alarming button and press the 'Reset Alarm' to acknowledge.

3.3 Linking the EmbryoSlide® to the Patient ID

3.3.1 After a new EmbryoSlide® has been added and the door closed, a dialog box will pop up requesting 'Patient ID' (see below).

3.3.2 Type in the 'Patient ID' and locate the patient. Select a 'Treatment ID' No. (Procedure No. as added in 2.1.4) **OR** create a new Treatment ID by typing in the Procedure No. in the appropriate box (see below). Press the 'Done' button when complete.
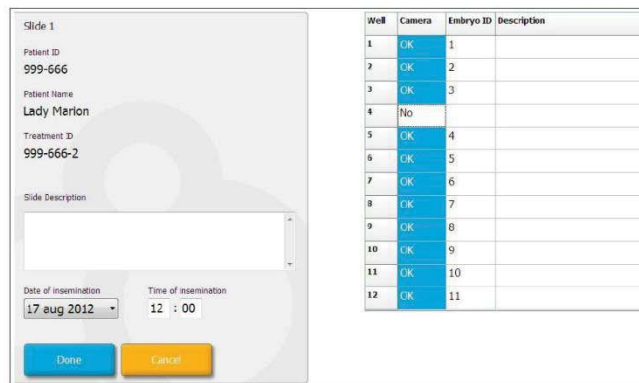
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3.4 EmbryoSlide® Details

3.4.1 When the EmbryoSlide® has been successfully linked with the Patient and Procedure No., a dialog box will appear, add the Insemination date and time. In the table on the right, the 'Camera' column indicates whether or not time-lapse images are required for each individual well (indicated as 'OK' or 'NO'). Press on the cell to switch between 'OK' and 'NO'. Check all information is correctly entered before pressing the 'Done' button.

3.4.2 A dialog box will ask if an additional slides are required for same treatment. Click 'YES' if required and repeat the above steps, otherwise click on 'NO' to proceed.

3.5 Auto Focusing

3.5.1 The EmbryoScope™ will automatically scan a number of focal planes for each microwell and will choose the most informative plane as the main focus. (For a full EmbryoSlide® this may take a few minutes to complete.)

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3.5.2 The automatically chosen focus plane however must be checked as on occasion the image may not be optimum and manual adjustment is required.

3.5.3 On the Embryoscope touch screen, click on the Patient's slide, click on the first oocyte / embryo and click the 'Live' tab.

3.5.3.1 Locate the most suitable focal plane by clicking the "up" and "down" arrows on the right of the image until the oolemma is in sharp focus.

3.5.3.2 The actual microwell position can be adjusted by clicking the arrows within the image box (up, down, left, right), or by holding the image and dragging across the screen.

3.5.3.3 The adjusted settings must be saved by pressing the 'New Focus' button. (The brightness of the image can be adjusted by modifying the exposure time after selecting 'Adjust Camera', however this is not often required).

3.5.3.4 Press 'Next Well' to navigate through the remaining microwells.

3.5.4 Once all the microwells have been checked, the screen must be returned to the Home screen; click on the 'Video' tab and press 'Home'.

4   Navigating the Touch Screen of the EmbryoScopeTM

4.1 Home Screen

4.1.1 Each EmbryoSlide® has an overview tab that can be enlarged by either tapping on the touch screen of EmbryoScope™ (see picture below) or by double clicking on the tab on EmbryoViewer® (computer software).

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4.1.2 Underneath the slide tabs on the touchscreen are 4 function buttons, "Add Slide", "Pause All", "Check…" and "End".

4.1.2.1 New slides can be inserted after tapping "Add Slide" button (the button is deactivated when all the 6 slide positions are occupied).

4.1.2.2 The "Pause All" button enables the pause and access of all running EmbryoSlides®, for example, when a change media is required for all EmbryoSlides® together (however this action would be rare).

4.1.2.3 The "Check…" opens the menu for checking of gas concentrations and temperature.

4.1.2.4 The "End" button opens the close program menu (for the entire system, not individual slides).

4.1.3 The lower panel shows the running conditions. When an alarm is triggered, the colour of the corresponding button turns red. A prolonged alarm condition will trigger an audio alarm (when an alarm is recovering it turns from red to yellow). Alarms can be acknowledged by tapping on the coloured button and pressing the "Reset Alarm" button (see 3.2.3).

4.1.4 The "Viewer" button opens the EmbryoViewer® connection menu.

4.1.5 The "Settings" button opens the general settings menu for functions like image acquisition frequency.
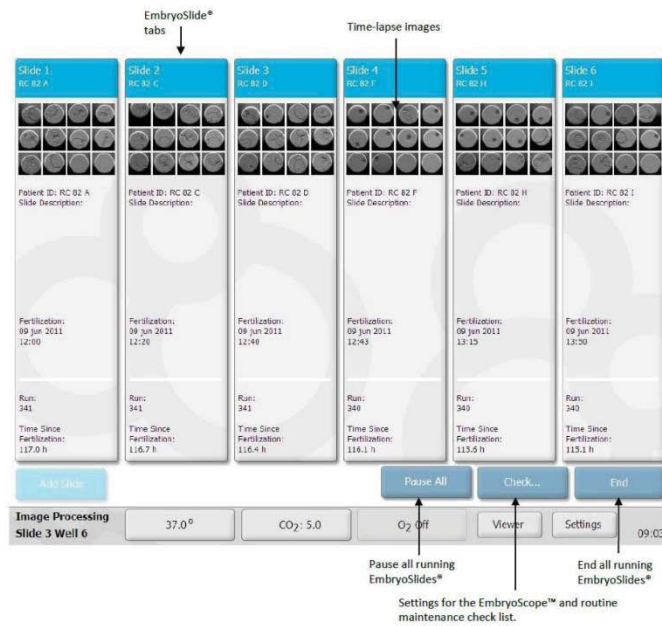
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4.2 Viewing Embryos

4.2.1   Embryo are viewed and annotated using the EmbryoViewer® (computer software), see below.
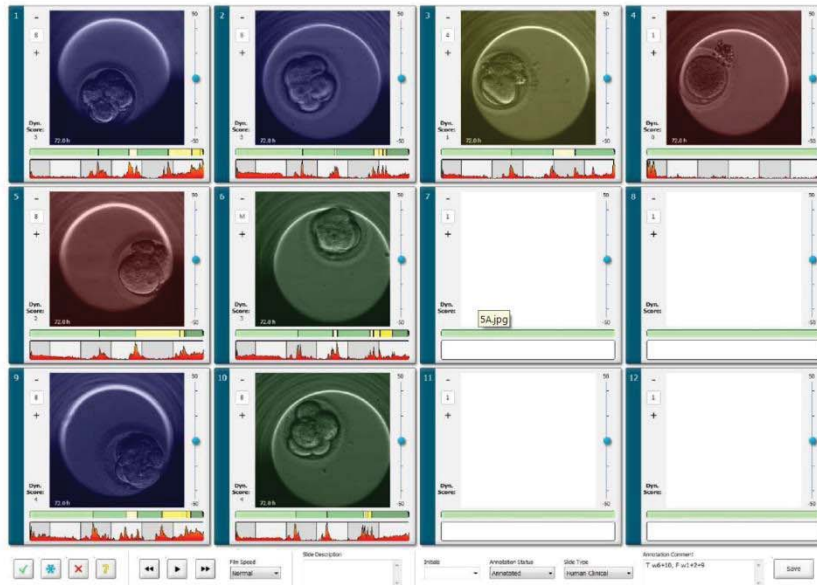
4.2.2   In the EmbryoViewer®, the left panel displays a group of function buttons

4.2.2.1   Dark blue such as [View All Patients] indicates the option is available but not selected / active;

4.2.2.2   Light blue such as [Compare & Select] indicates the option is selected / active;

4.2.2.3   Grey [Annotate] means the action is disabled.

4.2.3   The 'View Running' button opens the home screen showing an overview of the EmbryoSlides® in culture.

4.2.4   The 'View all patients' shows a list of all patient records and the 'Patient Details' button shows the record of the selected patient.

4.2.5   The 'Database' and 'Settings' sections are not frequently used, refer to the manufacturer's user manual if more information is required.

4.2.6   Each time-lapse video can be played by using [◄◄] [►] [►►] buttons or using the scroll wheel. Film speed can be adjusted by clicking on the drop box and choosing "fast", "normal" or "slow".

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4.2.7 An embryo can be indicated for transfer, freezing, discard or that a later decision is required using [✓] [✱] [✗] [?] buttons respectively, click the appropriate button and click over the embryo to highlight; the embryo will turn green, blue, red and yellow respectively.
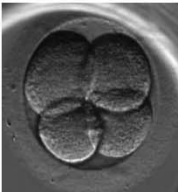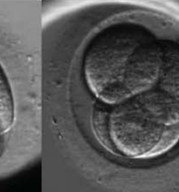
4.3 Performing Annotations

4.3.1 Annotations can be done on up to 3 embryos at the same time by selecting 1-3 embryos at the [View Slide] page then clicking on [Annotate] as shown below. Otherwise click each embryo one at a time. Scroll the wheel clockwise to play the video forward and anti-clockwise to play backwards.

4.3.2 The annotation of each embryo must follow the order below (see Annotation Table below). If the embryo stops developing / arrests at any stage, go to straight to 4.4.

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4.2.7 An embryo can be indicated for transfer, freezing, discard or that a later decision is required using ☑ ✱ ✖ ❓ buttons respectively, click the appropriate button and click over the embryo to highlight; the embryo will turn green, blue, red and yellow respectively.

4.3 Performing Annotations

4.3.1 Annotations can be done on up to 3 embryos at the same time by selecting 1-3 embryos at the [View Slide] page then clicking on [Annotate] as shown below. Otherwise click each embryo one at a time. Scroll the wheel clockwise to play the video forward and anti-clockwise to play backwards.

4.3.2 The annotation of each embryo must follow the order below (see Annotation Table below). If the embryo stops developing / arrests at any stage, go to straight to 4.4.

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Order	Annotation	Action	Notes
1.	PN Status	Record the PN status of the embryo at the earliest possible time point.	
2.	PN Fade	Record the PN fade at the first frame that the embryo shows complete fading of both PN [PN faded]	**Both PN must be completely invisible.**
3.	2 Cell Stage	Record the time of the beginning of the 2-cell stage, i.e. the first frame where 2 daughter blastomeres completely separate [- 2 +]	**For Type 1 RC cases (see No. 17), do not record cell division until the cells have completely separated, showing no more merging.**
4.	2 Cell Stage (Evenness)	Record the evenness of the 2 daughter blastomeres.	**If the evenness / fragmentation status changes over time, add another record at the corresponding time point to reflect the new status.**
5.	2 Cell Stage (Fragmentation)	Record the degree of fragmentation.	
6.	2 Cell Stage (MN)	Record the number of blastomeres with MN **OR** NA if the status cannot be confirmed.	**Cell divisions can generate large non-nucleated fragments, so it is very important to differentiate large fragments from blastomeres. This can be determined by confirming the presence of a nucleus / nuclei in the newly generated blastomere. If the cell is actually a fragment, change the fragmentation status without increasing the cell count. If large fragments are misreported as cells, the abnormal cleavage event of DC (see No. 18) may be misreported.**
7.	2 Cell Stage (Nuclear disappearance)	Record the time points of nuclear disappearance (NDAP) for each of the 2 blastomeres (NDAP1/2 for the first blastomere and NDAP 2/2 for the second).	
8.	3 Cell Stage	Record the time of the 3-cell stage as per No. 3	
9.	4 Cell Stage	Record the time of the 4-cell stage as per No. 3	
10.	4 Cell Stage (MN)	Record the number of blastomeres with MN **OR** NA if the status cannot be confirmed	
11.	4 Cell Stage (No. ICCPs) *An Abnormal Cleavage Event*	Record the Intercellular Contact Point (ICCP) status <u>at the end</u> of the 4-cell stage. If the 4 blastomeres <u>have 6 ICCPs</u> then select 'no' under the '1 Plate' option If the 4 blastomeres have <6 ICCPs i.e. 4 or 5 ICCPs <u>at the end</u> of the 4 cell stage select 'yes" under the '1 Plate' option [1 plate] 4 ICCP 5 ICCP 6 ICCP	

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12.	5 Cell Stage	Record the time of 5-cell stage as per No. 3	**Record at the first frame where the two (three in the DC cases) resulting daughter cells are completely separate.**
13.	6 Cell Stage	Record the time of 6-cell stage as per No. 3	
14.	7 Cell Stage	Record the time of 7-cell stage as per No. 3	
15.	8 Cell Stage	Record the time of 8-cell stage as per No. 3	
16.	9+ Cell Stage	Record the time of 9-cell stage as per No. 3	**The ES cannot record cell numbers greater than 9, therefore for embryos with >9 cells, document the actual cell number on the worksheet only.**
17.	Reverse Cleavage *An Abnormal Cleavage Event*	RC includes Type 1 and Type 2, which can be documented by selecting either '1' or '2' in the drop box of the 'Reverse cleavage' option. Reverse cleave ▢ ▾ Type 1 RC = when 2 daughter cells **completely** separate (*at least one frame showing complete separation*), after the disappearance of nucleus / nuclei, followed by the re-joining of the 2 resulting cells, resulting in a reduction in cell count. Type 2 RC = when blastomeres fail to split after the disappearance of nucleus / nuclei, followed by the re-appearance of nucleus / nuclei. In some cases, the movement on the cell membrane and cytoplasm may be very slight or very hard to detect. Thus it is very important **to track the nuclear disappearance and re-appearance** during the cell cycle.	**RC may be documented at any time point it occurs.** **The temporary increase in cell count before the RC should not be recorded**, only document that type 1 RC has occurred. (This type of RC accounts for around 10% of RC embryos).
18.	Direct Cleavage *An Abnormal Cleavage Event*	There are two descriptions of DC; a) DC is the division of 1 cell into 3 or more daughter cells in any cell cycle. This is documented in annotations as a comment in the comment box. b) DC is a reduced cell stage time from 2->3 cells and 4->5 cells, categorised as <5 hours. The EmbryoViewer will calculate this (see 4.4.5).	**DC may be documented at any time point it occurs *OR* the Embryoscope will calculate as a short cell stage at the 2 cell and 4 cell stages (<5 hours duration).**

4.4 Embryo Grading and Selection

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4.4.1 To obtain the final Embryo Grades the following algorithm is used (see 4.4.7). A series of questions in the form of a flow chart must be answered to give the embryo a final ES Grade.

NOTE: A Conventional morphology grade is also documented for each embryo as per *LB-S-617 Embryo Culture, Assessment and Selection*. Both the ES Grade and the Conventional grade are documented on *LB-F-176 Embryology ICSI Worksheet* or *LB-F-177 Embryology IVF Worksheet*.

4.4.2 Starting at the bottom of the algorithm, embryos with poor conventional morphology, by 64 hours post insemination are given a Grade F. Categorised as;

- ≥8 cells with >50% fragmentation
- 6-7 cells with 20-50% fragmentation
- 5 cells with >10% fragmentation

4.4.3 Embryos displaying any of the 3 Abnormal Cleavage Events, at any given time, are given a Grade E. Categorised by;

- <6 ICCP at the end of the 4-cell stage
- RC - Type 1 or Type 2
- DC – Either 1 cell direct to 3+ cells, or the duration of 2-cell stage or 4-cell stage is less than 5 hours

4.4.4 To distinguish between Grades D – A+, the user needs to know the following parameters. Go to [Compare & Select] and select the '**06052015**' model.

The EmbryoViewer will display the following information;

	Pri. T5PNF	Sec. S2	cell68	CC2	t4t5
Description	Length of time taken to reach 5 cells post PN fade	Length of time taken to divide from 2 to 4 cells	Cell number at 68 hours post insemination	Length of the 2-cell stage (required to classify DC)	Length of the 4-cell stage (required to classify DC)

NOTE: Ignore the 'Current Score' column in the table, as this is the Embryo Grade given by Fertilitech's Algorithm. Input the Fertility North Grade manually to each embryo as described below.

4.4.5 The final Grade must be typed in manually to the EmbryoViewer. Click each embryo and add the Grade to the 'Dynamic score' box, and click 'Save'.

4.4.6 The embryo(s) with highest Grade will be selected for transfer. If there are embryos of equal grade, other parameters such as conventional morphology grading, MN at the 2 cell / 4 cell stage and S3 value (time taken for the completion of the 5->8cell stage) may be taken into consideration. The latter of which can be found in the **06052015** model.
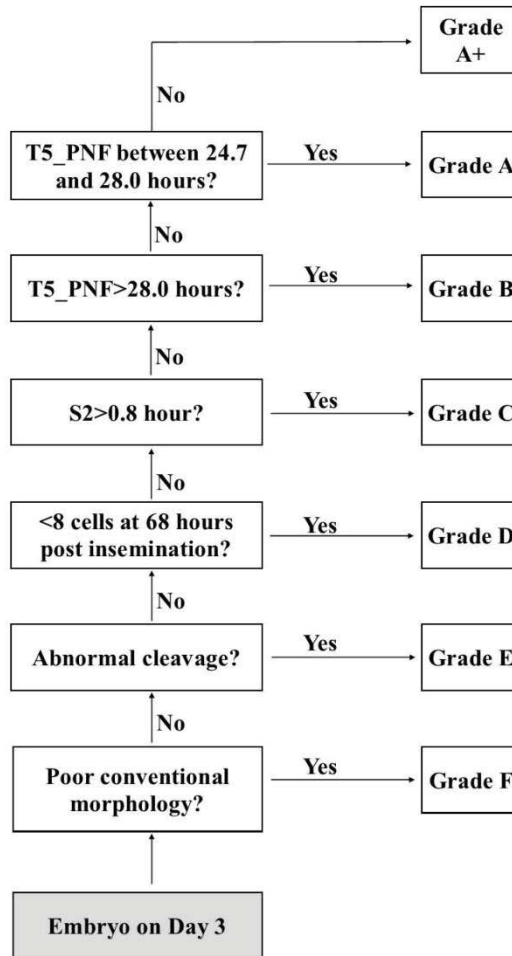
4.4.7 Embryos given a Grade F are discarded, Grades A+ - E may be vitrified as per *LB-S-615 Vitrification*.

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4.4.8 Embryos that are borderline for freezing, may be removed from the EmbryoScope and transferred to the conventional incubators for Blastocyst culture (refer to *LB-S-618 Blastocyst Culture, Assessment and Selection*).
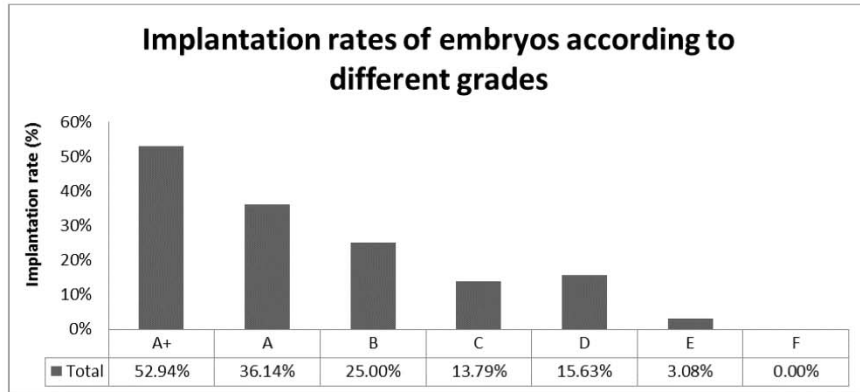
4.4.9 Grading Algorithm

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4.4.10 The implantation rates for embryos with grades A+, A, B, C, D, E and F are shown below, which are based on a retrospective data analysis from 270 transferred embryos at FN, with known implantation data.

Implantation rates of embryos according to different grades

	A+	A	B	C	D	E	F
■ Total	52.94%	36.14%	25.00%	13.79%	15.63%	3.08%	0.00%

5   Ending Slides

5.1 When the fate of every embryo has been determined, press the "End" button and unlock the incubator door. The EmbryoSlide® then can be removed from EmbryoScope™ and embryo(s) for ET / Vitrification can be transferred into the appropriate dishes (*refer to LB-S-611 Embryo Transfer and LB-S-615 Vitrification*).

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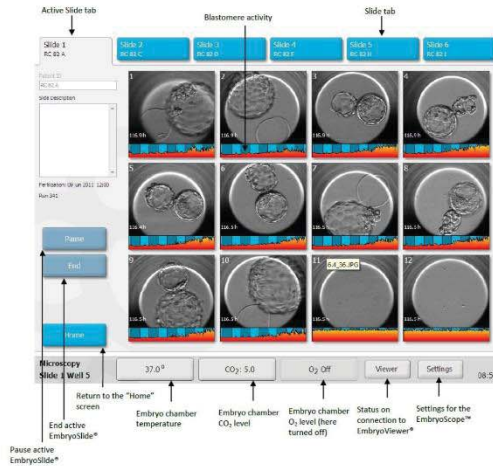
6   Quality Control

6.1 Daily Monitoring

6.1.1   Every morning at Laboratory Opening QC check, the EmbryoScope™ is checked for the Temperature, CO_2 and O_2 concentration, readings of which are displayed at the bottom of the home screen. All readings are recorded on the *LB-F-140 Embryology Laboratory Weekly Quality Control* (*see LB-S-536 Laboratory Opening and Closing Procedure and Quality Control*).

6.1.2   A detailed history of each parameter can be viewed by tapping on the relevant button. For example, temperature history will be shown as follows and any unexpected change(s) can be easily identified.

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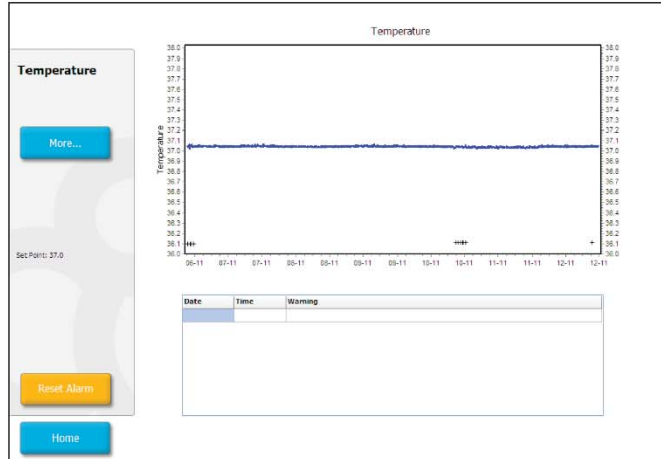
6.2 Weekly QC Checks

6.2.1 Weekly measurements are taken using calibrated machines (*see LB- S-536 Laboratory Opening and Closing Procedure and Quality Control*).

6.2.2 To measure gas / temperature the incubator must be placed in 'Incubator Check' mode. Tap for the Check... button, located at the bottom right of the home screen. To select the appropriate mode, the other modes most be passed through – press the 'Next' button to action.

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6.3 Gas Measurements

6.3.1 A gas sample port can be found by removing the service lid on top of the Embryoscope™.

6.3.2 Connect the Geotech G100 CO_2 / O_2 analyser to the Embryoscope™ as shown below (refer to *LB-S-1021 Use of Geotech G100* for details on use).

6.3.3 Wait for the readings to settle and document on *LB-F-140 Embryology Laboratory Weekly Quality Control*, see *LB-F-140 Embryology Laboratory Weekly Quality Control* for reference ranges.

Gas sample port
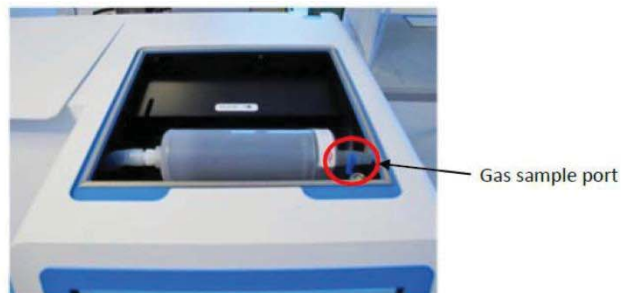
6.3.4 If the readings are within range proceed to 6.4 by tapping the [Next] button. If the reading(s) are out of range, re-calibrate following 6.5.

6.4 Temperature Measurements;

6.4.1 Refer to *LB-S-1025 Use of Thermometer 4610* for directions on the Digital Thermometer use (and Probe 4611).

6.4.2 When ready for the temperature check, open the door of the Embryoscope™ incubator. Remove the transparent cover over the slide holder, and fully insert the probe into the temperature port shown as below.

Insert the YSI probe

Slide holder

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6.4.3 Close the door on the **THICKER** part of the cable and leave the whole **THINNER** part in the chamber.

6.4.4 Wait 10 minutes for the reading to settle, and document on *LB-F-140 Embryology Laboratory Weekly Quality Control, see LB-F-140 Embryology Laboratory Weekly Quality Control* for the reference range.

NOTE: According to the user manual, the temperature reading represents the core temperature of the slide holder; however the slide holder is 0.2°C higher than the temperature in the bottom of the microwells (where the embryos are located). Therefore the Embryoscope temperature range is 37.2°±0.5°C rather than 37.0°±0.5°C to accommodate this difference.

6.4.5 If the reading is within range, return to the Home screen by pressing 'Next' and 'Done'. If the reading is out of range go to 6.6.

6.5 Re-calibration of Gas Concentration (CO$_2$ or O$_2$)

6.5.1 In the set point menu, navigate to the CO$_2$ or O$_2$ menu item by pressing the "left" or "right" arrow, unitl the CO$_2$ or O$_2$ concentration is displayed `CO2` or `O2`.

6.5.2 Press and hold the "down" arrow for three seconds, until the display stops flashing `------` to enter the calibration menu. When the change of menu is successful, the display will show `CO.c` or `O2.c`.

6.5.3 Press and hold the `SP` button, the display will alternate between `CO.c` or `O2.c` and `CAL`. This indicates the Embryoscope™ is ready for calibration.

6.5.4 While still holding the `SP` button, press either the "up" or "down" button, to increase or decrease the corresponding gas concentration for calibration. The concentraion is adjusted by 0.1% each time the "up" or "down" button is pressed.

6.5.5 Release the `SP` button to save the calibration. Press and hold "up" arrow until the display stops flashing `------` to return to the set point menu.

6.5.6 Leave for 1-2 hours and repeat 6.3 to re-check the measurement.

6.6 Re-calibration of Temperature

6.6.1 In the set point menu, navigate to the temperature menu item `37.0` by pressing the "left" or "right" arrow.

6.6.2 Press and hold the "down" arrow for three seconds, until the display stops flashing `------` to enter the calibration menu. When the change of menu is successful, the display will show `t.c 37.0`.

6.6.3 Press and hold the `SP` button, the display will alternate between `t.c 37.0` and `CAL 37.0`. This

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indicates the Embryoscope™ is ready for calibration.

6.6.4 While still holding the (SP) button, press either the "up" or "down" button ⟨↔⟩, to increase or decrease the temperature for calibration. The temperature is adjusted by 0.05 °C each time the "up" or "down" button is pressed.

6.6.5 Release the (SP) button to save the calibration. Press and hold "up" arrow ⟨↔⟩ until the display stops flashing ▬▬▬▬▬ to return to the set point menu.

6.6.6 Leave for 1-2 hours and repeat 6.4 to re-check the measurement.

7   Maintenance

7.1 Cleaning

7.1.1   Weekly Cleaning Check

7.1.1.1   A weekly cleaning check is performed at the end of the gas & temperature checks as shown below, when the Incubator is empty and the incubator is in 'Cleaning check' mode.
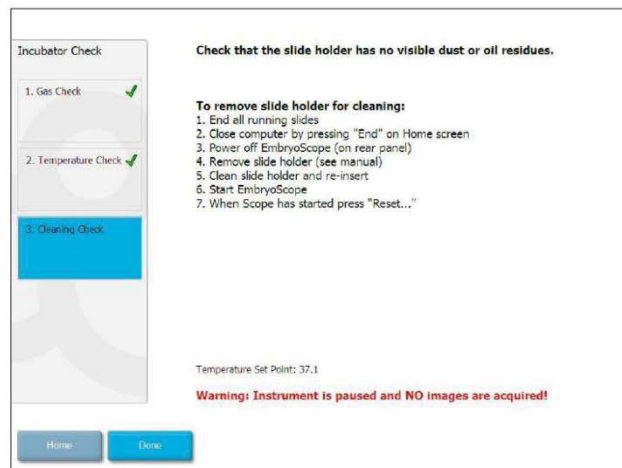
7.1.1.2   Open the incubator door and visually inspect the EmbryoSlide® holder and the whole chamber. If there are any liquid residue or particles present, perform the cleaning procedure as per 7.1.2.1.

7.1.2   Quarterly Cleaning

7.1.2.1   The Embryoscope™ is cleaned once every three months unless required at the weekly check. Before opening the door, ensure no embryos are in the incubator. Whilst in 'Cleaning Check' mode (see 7.1.1.1), open

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the incubator door and remove the plastic protector. Spray Oosafe® directly onto a Kimwipe and wipe all accessible surfaces inside the incubator. Follow this with Sterile water and a Kimwipe.

7.2 Gas Supply

7.2.1 The Embryoscope™ at FN is equipped with both CO_2 and N_2 supplies via a Gas Manifold system; the embryos are cultured under 6% CO_2, 5% O_2 and 89% N_2. See *LB-S-602 Control and Storage of Laboratory Supplies and Disposables* for further information regarding the gas supply.

7.3 Gas Alarm

7.3.1 When the pressure of one or more gas lines is lower than the set point, an audible alarm is sent from the manifold to the Gas alarm panel next to the Galaxy Incubator (refer to *LB-S-624 Laboratory Environment* for further details on the Manifold system including how to change cylinders).

7.4 Gas Ordering

7.4.1 BOC supplies the gas to FN, see *LB-S-602 Control and Storage of Laboratory Supplies and Disposables* for further details.

7.5 Power Supply

7.5.1 The Embryoscope™ is supplied with backup power from the JHC Emergency grid. In addition it is connected to an UPS battery back-up which can supply power for 8 hours (see *LB-S-482 Laboratory Emergency Procedure* for further details).

7.6 Servicing

7.6.1 Servicing is provided by UNISENSE FERTILITECH twice a year (refer to *LB-S-1046 Maintenance of Laboratory Equipment* for further details). In between scheduled services, remote monitoring takes place by UNISENSE. Individual technical support is available by contacting Palle at pbl@fertilitech.com.

7.6.2 An alternative email contact (response within 2 working days) is available at support@fertilitech.com

7.6.3 Emergency contact (24/7 support): +45 7023 0500

8 Alarms

8.1 Incubator Alarms - An audible alarm is triggered by any of the following;

8.1.1 Temperature out of the range 37.2±0.5°C

8.1.2 CO_2 concentration out the range 6.0±0.5%

8.1.3 O_2 concentration is out the range of 5.0±1.0%.

8.1.4 CO_2 or N_2 inlet pressure is lower than 0.2 bar.

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8.1.5 The alarm will also show on the Embryoscope™ touch screen. Red indicates a parameter is alarming, yellow indicates a past alarm that has been acknowledged. Refer to 3.2.3 for how to reset an alarm.

8.2 Computer Alarms - The failure of the integrated computer can trigger an audible alarm. In this instance incubator is unaffected, but the time-lapse imaging would be disrupted. Conditions that may result in computer alarms include;

8.2.1 Loss of power

8.2.2 Computer system failure

8.2.3 Disconnection between incubator and computer

8.2.4 The Embryoscope™ software is not functioning or turned off

8.2.5 A dialog on the screen has not been acknowledged

8.2.6 More than 1 hour pause of EmbryoSlides®

8.2.7 Embryoscope™ software has been in check mode for more than 30 minutes

8.2.8 Incubator door is kept open for more than 20 seconds

8.2.9 Temperature within parts of the Embryoscope™ >55°C

8.2.10 Internal computer temperature >50°C

NOTE: The computer alarm cannot be reset, the condition causing the alarm must be resolved.

8.3 External Monitoring

8.3.1 External Alarm System - The Embryoscope™ is connected to the eviSENSE 24 hour external monitoring system, see *LB-S-561 Labguard (Evisense)* for details.

9 Emergency Removal of EmbryoSlides®

9.1 In the case of an absolute emergency situation such as a power failure, the EmbryoSlide(s)® can be removed following the steps below.

9.2 Ensure the main switch on the back of the Embryoscope™ is switched off, even in the event of a power failure.
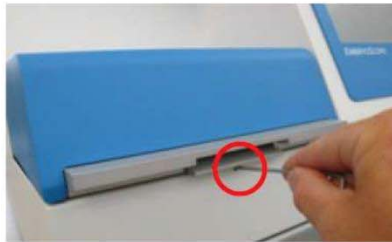
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9.3 The Emergency Instructions together with a 2.5mm Allen key are stored underneath the service lid on the top of the Incubator.

9.4 The incubator door can be unlocked by inserting and pressing the 2.5 mm Allen key into the hole as shown below, and pressing down the red door lock.

9.5 Open the door and remove the transparent cover as shown below.

9.6 Use the 2.5 Allen Key to pull the EmbryoSlides® holder towards the loading area so that enough space is available to access and remove the EmbryoSlides®.

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9.7 When emergency conditions have been resolved, replace the transparent cover and close the incubator door. The Embryoscope™can now be switched on.

9.8 Embryos should not be returned for culture in the Embryoscope™ for at least 3 hours, to allow the temperature and gas culture conditions to fully recover.

9.9 Temperature and gas checks must be performed (as per 6.3 & 6.4) before embryos are replaced into the Embryoscope™.

END OF DOCUMENT

Appendices 3-10 are not available in this version of the thesis.