Morphokinetic analysis of embryo development

Failure to select a viable embryo in a given fertility treatment cycle limits the success rates of current procedures. Fertility clinics rely on a morphological assessment of embryo appearance at a few distinct time-points during development to pick a viable embryo for transfer. However, embryo development is a very dynamic process and critical stages between observations go unnoticed. This document describes the shortcomings of current IVF protocols and substantiates how improved embryo selection is possible based on a kinetic analysis of time-lapse images of embryo development. The novel diagnostic possibilities of automated time-lapse imaging are discussed and our current results are provided.

Time-lapse information on embryo development

Automated morphokinetic analysis
Introduction

Infertility is experienced by about 10% of all couples worldwide. It is a growing problem due to increasing maternal age and decreasing semen quality. Only a small percentage of infertile couples have access to infertility treatments today. Infertility treatment is an industry with a large growth potential, driven by infertile couple’s increasing acceptance of infertility treatments, decreasing tolerance of infertility, improved wealth, and increased access to reimbursement for infertility treatment costs.

The number of fertility treatments performed worldwide is currently estimated at 1.4 million per year with an annual growth rate of about 10-12%. The total turnover for the global IVF market for IVF treatments exceeds 10 billion USD annually. Despite a considerable international research effort, there are still two prominent shortcomings:

- A stagnant and unsatisfactorily low success rate. On average only every fourth or fifth initiated treatment cycle give rise to a live birth (20% to 25%)
- A large number of multiple pregnancies (twins, etc.), which pose a significant medical risk to mother and child and result in additional costs in the healthcare system.

The controlled hormonal stimulation used in IVF is designed to stimulate the ovaries to mature several eggs (oocytes) at the same time. In a typical IVF treatment cycle 8 to 12 mature eggs are retrieved from the ovaries and fertilized in vitro to generate embryos. After two to five days of in vitro culture the embryologist has to select one or more embryos (preferably a single embryo) to be transferred back to the uterus of the patient. Despite more than 30 years of research to improve the selection procedure, most transferred embryos (65-90%) cease development and degenerate shortly after transfer.

The low success rate in current IVF procedures is partly due to an inability to objectively and reliably determine which fertilized egg is most viable in each treatment cycle (among an average 8 to 12 eggs) and use this embryo for transfer. (e.g. Neuber et al. 2003; “The ability to identify the most viable embryo for transfer is of fundamental importance to assisted reproduction techniques”)

Current selection procedures are based on a morphological evaluation of the embryo at three to four chosen time points during development followed by an evaluation at the time of transfer using a standard microscope. However, it is widely recognized that the evaluation procedure needs qualitative as well as quantitative improvements.

Based on a novel proprietary approach to analyze time-lapse image series obtained as vertical stacks of images throughout the growing pre-embryo, Unisense FertiliTech

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1 The fertilized oocytes are strictly speaking pre-embryos, and only become embryos after they implant in the endometrium of the uterus. For simplicity sake, we use the term "embryo" for these cleavage stage pre-embryos in this text in accordance with practical use of the term in IVF clinics.
A/S has developed and sells an advanced incubator (the EmbryoScope™) for rapid and non-invasive viability assessment of pre-embryos used in infertility treatments. Currently (May 2011) 58 EmbryoScope™ have been installed in a total of 18 countries in Europe, USA and the Middle East. A total of 43 EmbryoScopes™ are currently used for clinical treatment cycles in Europe, and the first IVF children originating from embryos incubated in the EmbryoScope™ have already been born. Data from more than 3760 clinical IVF cycles encompassing >20,000 embryos have been uploaded to Unisense Fertility A/S for QC evaluation.

By allowing the most viable pre-embryos to be chosen for transfer, the technology will eventually increase the current success rate of infertility treatments. Proper selection of the best embryos for transfer and restricting the number of transferred embryos to the single most promising one will reduce the frequency of multiple births.

The following document will discuss the challenges and shortcomings of current IVF protocols and substantiate our expectations that improved embryo selection is possible based on a kinetic analysis of time-lapse images of embryo development. The purpose is not to discuss and disclose how to accomplish this, but merely to indicate the vast untapped diagnostic potential of a kinetic (i.e. NON-static) approach to embryo development. (For a discussion of the techniques involved see: Ramsing and Callesen 2006; Ramsing et al. 2007)

**Documented morphological selection criteria**

In classical IVF the embryos were evaluated on the day of transfer and symmetrical embryos at the most advanced stage of development were chosen for transfer. A clear correlation between embryo morphology and viability is well established, and extensively documented in the scientific literature. However it is widely recognized that even embryos with perfect morphology may fail to implant while asymmetrical embryos with poor morphology may in some cases succeed. Chromosomal defects such as frequently occurring aneuploidy (i.e. an abnormal number of chromosomes) have been shown to reduce or eliminate the viability of embryos (e.g. review by Ambartsumyan and Clark 2008). While some of these defects can lead to abnormal embryo morphology, other defects can latently be present in healthy looking embryos with perfect morphology at the time of transfer.

Sequential observation of the embryos at different times during development could reveal aberrant developmental patterns for embryos with an abnormal chromosomal content. It has been inferred that viable embryos should follow a pre-determined temporal pattern of cell divisions, and that sequential morphological observations may be used to identify embryos that follow a normal developmental pattern (e.g. Magli et al. 2007, “These observations suggest that timing is a crucial factor in determining embryonic development. In agreement with previous observations, any deviation from the expected cleavage stage has dramatic effects on implantation”)

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The sequential procedures currently applied in IVF clinics rely on three to four observations of embryo morphology to select a viable embryo for transfer. The time-points for making these observations have been chosen to give as much information as possible while avoiding odd working hours (late evening, nighttime and early morning). The observations still represent a very limited static view of a highly dynamic development process – and it is widely believed and acknowledged that important events that may indicate viability occur un-noticed between observations.

Improving embryo selection by increasing the number of times embryo morphology is assessed is counterbalanced by the inevitable stress of the observation: removing the embryo from the controlled gas and temperature environment within the incubator, temperature and pH fluctuations, handling and light stress during microscopy. This constitutes the embryologist dilemma: Observations are required to select a viable embryo – more observation gives better selection, but the embryos should preferably remain in the safe controlled environment of the incubator and observations should thus be avoided.

Early cleavage
An important developmental parameter is to use ‘early division’ to the 2-cell stage, (i.e. before 25-27 h post insemination/injection), as a quality indicator. In this approach the embryos are visually inspected 25 – 27 hours after insemination to determine if the first cell division has been completed. Several studies have documented a strong correlation between early cleavage and subsequent development potential of individual embryos e.g. (Shoukir et al. 1997; Sakkas et al., 1998; 2001; Bos-Mikich et al. 2001; Lundin et al. 2001; Petersen et al. 2001; Fenwick et al. 2002; Neuber et al. 2003; Salumets et al. 2003; Windt et al. 2004). The results of these studies and others are summarized in Table 5 in Hesters 2008:
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The graph below summarizes the pregnancy rates for early cleaving embryos (green bars) and late cleaving embryos (red bars) in these eleven publications:

All studies above found very strong and highly significant differences in viability and implantation rates between embryos with early cleavage and those that did not cleave early.

Several observers pointed out the need for more frequent observation, however, frequent visual observations with associated transfers from the incubator to an inverted microscope induce a physical stress that may impede or even stall embryo development. It is also time consuming and difficult to incorporate in the daily routine of IVF clinics. All the above studies thus rely on a single observation.

**Advantage of kinetic analysis with EmbryoScope™**

Early cleavage (EC) as outlined above has previously been assessed by a single observation. Different laboratories have been more or less stringent in maintaining a rigorous observation regimen and optimizing the regimen to account for procedural parameters that affect the timing of the first cell division such as: method of fertilization (IVF, ICSI), culture media and stimulation protocols may all affect the time at which the first cleavage occurs (Lechniak 2008; Lemmen et al. 2008; Yang et al. 2009). The clear advantage of time-lapse imaging is that the exact onset and duration of the first cell division can be automatically and precisely assessed (Ramsing and Callesen 2006). An indirect indication that cell divisions may occur too early is found in the figure above on chromosomal aneuploidy by Magli et al. 2007. The embryos that 62 hours after insemination had too high cell number (as well as those with too...
low cell numbers) were less likely to be genetically normal. It appears that among a cohort of early cleaving embryos there are some that cleave too fast, and those are likely to be genetically abnormal. Knowing the exact time of first and subsequent cell divisions may thus enable us to avoid some abnormally fast embryos. In some patients, none of the embryos have cleaved at the EC observation time-point and so assessment must be based on other parameters. The ability to observe which of the non-EC embryos cleaved first, may be important for embryo selection. With automated time-lapse imaging there is no need for laboratory personnel to be on duty at odd hours to maintain a rigorous observation regimen. Instead a complete documentation of embryo morphology for any previous time-point may be evaluated at the time of transfer. By dynamic observation of embryo development, external factors which affect division can be systematically assessed and used to correlate the existence of “windows of early cleavage” based on various factors.

Synchrony

The early cleavage stage embryo appears to be symmetric, and it is believed that the two daughter cells arising after the first division should develop in synchrony. It has been reported that synchronous appearance of nuclei after first division (Lemmen et al. 2008, Fig. 5 on this page), and synchrony of subsequent division is also significantly associated with pregnancy success and can be an indicator of embryo quality (Scott et al. 2007; Lemmen et al. 2008), and that asynchronous divisions, and embryos with odd cell numbers may have similar genetic abnormalities as arrested embryos (Hardarson et al. 2001; Magli et al. 2007). However asynchrony of cell division can only be assessed if the embryo has an odd number of cells at the exact time of observation. Unfortunately, the second and third cell division (i.e. from 2-cell to 4-cell) occur approximately 37 – 41 hours after fertilization, which in most clinics occur very early in the morning on day 2 (e.g. before 6 A.M). Very few laboratories - if any - make an attempt to evaluate the synchrony of nucleus disappearance, cytoplasmic division and nucleus reappearance.

Advantage of kinetic analysis with EmbryoScope™

Time-lapse images from the EmbryoScope™ are well suited to an accurate kinetic analysis of divisional synchrony. Images of multiple focal planes are automatically acquired and accessible at any subsequent time-point. It is thus possible to examine the detailed choreography of subsequent cell divisions at whatever time is most convenient for the lab staff. We are developing proprietary algorithms to automatically detect cell divisions based on image analysis. The algorithms for unattended detection and annotation of cytoplasmic cell division may not be able to accurately detect disappearance and re-appearance of cell nuclei, but they will facilitate operator assisted annotation.

Even cell numbers

An indirect measure of lack of divisional synchrony can be observed if embryos are in the 3-cell or the 5-cell stage at day 2 or day 3 respectively. Very convincing data
published by Scott et al. 2007, show that these embryos with uneven cell number invariably fail to implant (Scott et al. 2007 Fig. 4 reproduced below).

The duration of the 3-cell stage has been observed (Wong et al. 2010) to be linked to development to the blastocyst stage based on analysis of a dataset consisting of 100 embryos grown to day 5-6, at which time a viable embryo will normally form a blastocyst.

**Advantage of kinetic analysis with EmbryoScope™**

We have observed that time spent at an “odd” cell state can be significantly different between embryos, despite the fact that many are 4 cell embryos by the day 2 observation period. Time-lapse observation allows unattended and accurate assessment of degree of asynchrony. The figure below illustrates this.

The morphology of these two embryos were virtually identical at all normal observation time-points (17 hrs, 27 hrs, 44 hrs and 68 hrs after insemination) They were both high grade embryos. However, time-lapse images of the embryo to the left revealed synchronous divisions from the two cell stage to the four cell stage.
Substantially simultaneous cell division is indicated by the narrowly spaced bimodal peak, thus a 3 three cell stage (arrow at bimodal peak and shown as a yellow bar) of very short duration. The embryo to the right was arrested a longer time in the three cell stage (arrow at spaced peaks and shown as a wide yellow bar.) The asynchronous cell division of the right hand embryo would indicate a lower quality and the embryo to the left should be preferred for transfer.

**Blastomere size and fragmentation.**
Embryos with even sized blastomeres have higher implantation rates (Hardarson et al. 2001). A remarkable demonstration of the reduced viability of uneven sized embryos was shown by Scott et al. 2007 Fig. 3 reproduced below.

![Graph showing blastomere size and fragmentation.](image)

**Figure 3.** Day 2 cell symmetry. The percentage of even or uneven blastomeres from 1076 day 2 embryos depending on their fate: series 1.

It is likewise well established that embryos with pronounced fragmentation (i.e. small vesicles of cytoplasm without chromosomes or nucleus) have a markedly reduced viability (Neuber et al. 2003). Unfortunately, the degree of fragmentation is a notoriously difficult parameter to quantify objectively. Computing a quantitative measurement of fragmentation using image analysis is very time consuming and difficult to perform on a routine basis even at a few discrete time-points per embryo, (Hnida and Ziebe 2004). To complicate matters: Previous time-lapse studies have shown that appearance and disappearance of fragments is a dynamic process and can be very dependent upon the observation time point. Qualitative evaluation of time-lapse images have shown, fragments are often reabsorbed, and embryos which appear highly fragmented immediately after cleavage, can be very different even half an hour later (Lemmen et al. 2008; Hardarson 2002; Van Blerkom 2007).

**Advantage of kinetic analysis with EmbryoScope™**
Time-lapse allows the embryologist to observe fragmentation events as well as recovery from fragmentation. Access to a complete documentation of the embryo development will enable fragmentation estimates based on the stable residual fragmentation that is present between cell divisions, as opposed to the pronounced and highly irregular fragmentation that may occur only transiently in connection with cell division events. It is believed that the transient fragmentation that does NOT lead to permanent loss of cytoplasm as the fragments are reabsorbed is less harmful than persistent fragmentation whereby valuable biomass (i.e. resources) is lost from the cell. At present we can use this temporal information to select the most appropriate time-points to evaluate the degree of fragmentation and evenness of blastomere size (i.e. mid-points between cell division).
Multinucleation
Numerous studies have shown that multinucleation impairs embryo viability. While some embryologists have claimed that multinucleation in a single cell at the four or eight cell stage may not be detrimental as that particular blastomere may be excluded from the developing embryo, and subsequently degenerate by apoptosis. Still, the material included in that blastomere is lost, and many embryologists agree that embryos with substantial multinucleation are not viable. Recent results with embryos with a single multinucleate blastomere may indicate that even a single multinucleate cell may be detrimental (Scott et al. 2007, Figure 2 reproduced below.)

Figure 2. Day 2 state of multinucleation. The percentage of each multinucleation score for 1076 day 2 embryos depending on their fate: series 1. 1n/b, 1 nucleus per blastomere; mnv, nuclei not visible and MN, multinucleation.

Advantage of kinetic analysis with EmbryoScope™
A particular problem with routine assessment of multinucleation is that the nuclear membrane dissolves prior to cytoplasmic cell division, and only reform in the daughter cells after the division is completed. It is therefore not always possible to assess multinuclearity, when the embryo is only observed at a few defined time-points. With time-lapse microscopy it is possible to evaluate multinucleation at optimal timepoints such as midpoints between cell division events. We have observed multinucleation in some of our trials with the EmbryoScope™, and we have found cases, where multinucleate embryos were inadvertently transferred as the time-lapse images were not used for embryo selection, but only the normal observations were. An interesting and surprising finding with the EmbryoScope™ was that multinucleation at the two cell stage appears significantly less detrimental to embryo viability than multinucleation at the four cell stage or later. This is surprising as the multinucleated blastomere at the two cell stage gives rise to more progeny. However, we have frequently observed that these daughter cells of a multinucleated 2-cell embryo all appeared normal with a single nucleus.

Novel selection parameters
Besides the known and validated selection parameters outlined above, which can all be more readily and accurately quantified by time-lapse imaging, there are a number of novel and intriguing parameters that can only be assessed efficiently through time-lapse imaging. These include:
**Duration of cleavages and cell cycles**

The duration of the cytoplasmic cleavage and subsequent rearrangements of the individual blastomeres appears to be highly indicative of subsequent viability of embryos in bovine systems (Ramsing and Callesen 2006). In initial studies of bovine embryo development, the amount of cellular movements was quantified between consecutive image frames in a time-lapse image series. The resulting blastomere activity (Ramsing and Berntsen 2008) indicates the timing of cell divisions, and the duration of cellular rearrangements following a divisional event. Prolonged period of cellular rearrangements following a cell division event was a clear indicator of poor bovine embryo viability and poor developmental competence (Ramsing et al. 2007). The embryo viability was measured as the ability to develop to an expanded blastocyst. Consistently, the duration of the first cleavage (the first cytokinesis) has been shown (Wong et al. 2010) to be linked to development to the blastocyst stage in human embryos. Additionally, the second cell cycle (time between first and second cleavage) was shown to be linked to the development to the blastocyst stage and subsequent implantation. We now have an extended analysis based on time-lapse images of 247 transferred embryos with known outcome (61 implanted embryos and 186 embryos that did not implant).

**Timing of cleavages and other embryonic events**

The timing of the cytoplasmic cleavages is linked to successful implantation as shown in a retrospective analysis on time-lapse data acquired with the EmbryoScope™ on human embryos (Herrero et al. 2010). The timing of 1st, 2nd and 3rd cleavage, as well as the timing of PN formation and fading were analyzed for 159 transferred embryos with either 100% implantation (where the number of gestational sacs confirmed by ultrasound match the number of transferred embryos); or with 0% implantation (where no biochemical pregnancy was achieved). For each event the timing was measured in hours after ICSI. The table below lists data from Herrero et al. 2010, where the embryos are classified in quartiles according to the timing and grouped in two categories; the first category includes embryos with timing in the two central quartiles, and the second category includes embryos with timing in the two outer quartiles, referred to as “outside range” in the table.

<table>
<thead>
<tr>
<th>Event</th>
<th>PN formation</th>
<th>PN fading *</th>
<th>1st division *</th>
<th>2nd division *</th>
<th>3rd division *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time-range (h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.8 - 11.1 outside</td>
<td>22.3 - 25.8</td>
<td>24.4 - 28.2</td>
<td>35.3 - 40.6</td>
<td>36.0 - 41.6</td>
<td></td>
</tr>
<tr>
<td>100% implantation N (%)</td>
<td>15 (54%)</td>
<td>23 (66%)</td>
<td>23 (66%)</td>
<td>13 (72%)</td>
<td>19 (73%)</td>
</tr>
<tr>
<td>0% implantation N (%)</td>
<td>60 (49%)</td>
<td>55 (45%)</td>
<td>57 (46%)</td>
<td>43 (45%)</td>
<td>45 (45%)</td>
</tr>
</tbody>
</table>

Table presenting data from Herrero et al. 2010.
For the events of PN fading, 1st, 2nd and 3rd cleavage the abundance of 100% implanting embryos is significantly higher in the central quartiles category than in the “outside range” category. These significant differences are marked with * in the table (Chi-square test p<0.05). In conclusion, the timing of PN fading, 1st, 2nd and 3rd divisions are significantly linked to successful embryo implantation.

**Cleavage planes**

Different researchers have investigated the orientation of the initial cleavage planes relative to the pronuclear axis. They believe this could form the basis for new and powerful selection criteria. While the efficacy of such criteria is unproven, and the ability to apply these criteria automatically may be limited, it is easy to evaluate the criteria and the dataset we are currently accumulating should, be well suited to this. Abstracts presenting such studies with the EmbryoScope™ will be presented at ESHRE 2011 in Stockholm.

**Safety studies conducted with the EmbryoScope™**

In the studies listed below it is shown that the EmbryoScope™ time-lapse incubator provides incubation conditions that are at least as good as a standard laboratory incubator (More safety studies are being conducted but they are ongoing or not published yet).

A safety study was conducted at IVI Alicante Spain with the EmbryoScope™ (Cruz et al. 2011). The aim of this study was to demonstrate that incubation conditions in the EmbryoScope™ are similar to conditions in a standard laboratory incubator. A total of 478 embryos from oocyte donation cycles were included in the analysis. Fertilized embryos were randomly distributed in the EmbryoScope™ and the standard incubator (half in standard incubator and half in the EmbryoScope™). Embryo evaluation and selection were based solely on discrete time points (44h and 68h), thus additional information from time-lapse was not used for evaluation and selection of embryos. Several parameters were analyzed, e.g. embryo quality, blastocyst rate and pregnancy rates. No significant differences were found between the EmbryoScope™ and the standard incubator for all investigated parameters. In conclusion, this study demonstrates that time-lapse monitoring (image acquisition) does not impair embryo quality and implantation potential, while allowing for morphokinetic analysis of embryos.

A paired cohort retrospective analysis was performed on data from IVI Valencia Spain (Meseguer et al. 2010). To evaluate whether the EmbryoScope™ generates an environment of similar quality to a standard incubator the pregnancy rates for treatments with transfer of all embryos from either a standard incubator (N=279) or the EmbryoScope™(N=77) were compared. It is noteworthy that time-lapse information was not utilized to improve embryo selection. The biochemical pregnancy rates were 59% for the standard incubator and 64% for the EmbryoScope™, p=0.51, and the clinical pregnancy rates evaluated by observation of gestational sac(s) were 49% for the standard incubator and 55% for the EmbryoScope™, p=0.44. Both rates were slightly higher for the EmbryoScope™ than for the standard incubator cycles but the difference was not significant. Although the number of treatments in the EmbryoScope™ is limited, the data show that the EmbryoScope™ provides as good
opportunities for embryo development as the standard incubator, and we see no adverse effect of image acquisition.

**Conclusion**

Time-lapse image capture allows dynamic and flexible scoring of the developing embryo, and allows the embryologist to review and analyze the full course of embryo development at their convenience. The dramatically increased number of critical quantitative parameters that the embryologist can rely on to select a viable embryo should result in better selection of viable embryos. Our preliminary results from studies of bovine embryos (Ramsing and Callesen 2006) are merely indications of the potential that can be achieved with a fully automated and calibrated system. With regard to human embryos, successful implantation has already been shown to be linked with the exact timing of cell division events (Herrero et al. 2010).
References


