A Low-Cost Time-Lapse Imaging System Developed for Documentation of

Human Pre-implantation Embryogenesis in Clinical IVF Settings

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Glossary

**Aneuploidy**: The presence of an abnormal number of chromosomes in a cell (Griffiths, 1970)

**Blastocoel** (blastocyst cavity): A fluid-filled cavity that forms in early development within the blastocyst. Initially the dividing cells form a solid cellular mass (morula) continued cell division and compaction lead to the formation of this space or cavity. In humans, this cavity is present during the end of the first week and into the second week of development (Hill, 1997).

**Blastocyst**: Term used to describe the hollow cellular mass that forms in early development. The blastocyst consists of cells forming an outer trophoblast layer, an inner cell mass and a fluid-filled cavity. The blastocyst inner cell mass is the source of true embryonic stem cells capable of forming all cell types within the embryo. In humans, this stage occurs in the first and second weeks after the zygote forms a solid cellular mass (morula stage) and before implantation (Hill, 1997).

**Blastocyst hatching**: Term used to describe the process of blastocyst emerging from the surrounding protective zona pellucida. In human development this process occurs at the end of the first week (day 5-7). The location of hatching from the ZP can occur at the original spermatozoa entry site for fertilization, and must complete before implantation can commence (Hill, 1997).

**Blastomere**: Term used to describe the first cells formed by mitotic cell division of the zygote (Hill, 1997).

**Early stage cleavage**: A series of rapid cell divisions following fertilization in early embryos; cleavage divides the embryo without increasing its mass (Hill, 1997).

**Embryogenesis**: The formation and development of the embryo (Stedman, 2012).

**Mitochondria assessment**: An assessment procedure for embryos produced through in vitro fertilization (IVF) that evaluates the quantity of mitochondrial DNA found in the outermost layer of cells in a five-day old embryo; Suggested to predict embryo competence (Diez-Juan, 2015).

**Morphokinetics**: The morphology and kinetic behavior of the embryo throughout development (Basile, 2014).

**Morula**: (Latin, *morula* = mulberry) An early stage in post-fertilization development when cells divide rapidly (embryonic cell cycle) producing a solid mass of cells (12-15 cells) with a "mulberry" appearance. Cell proliferation occurs still enclosed within the zona pellucida. This stage is followed by formation of a cavity in this cellular mass (blastocyst stage). In humans, morula stage of development occurs during the first week following fertilization (Hill, 1997).
Multiple birth: A pregnancy that results in the birth of more than one infant (Hill, 1997).

Pre-implantation genetic diagnosis (screening): (PGD/S) A screening procedure for embryos produced through in vitro fertilization (IVF) for genetic diseases that would generate developmental abnormalities or serious postnatal diseases. Embryonic cells are removed and screened for chromosomal disorders or genetic diseases before the embryo transfer (Hill, 1997).

Pre-implantation: Involving, or being an embryo before uterine implantation (Stedman, 2012).

Trophectoderm: Embryology term used to describe the earliest layer formed in the blastocyst that will for the trophoblast layer. These cells contribute to extraembryonic structures of the placenta (Hill, 1997).

Zona Pellucida: The transparent more or less elastic non-cellular outer layer or envelope of a mammalian ovum that is composed of glycoproteins (Hill, 1997).
Abstract:

Infertility is a disease of the reproductive system that affects many women of various ages, races, and socioeconomic factors across the world (CDC, WHO, 2017). The expensive nature of treatments for infertility, such as in vitro fertilization (IVF), has been combated with a simplified culture system (SCS) developed in the Van Blerkom lab (Van Blerkom et al., 2013). This recent advancement in clinical IVF procedures has prompted an effort to develop additional, low-cost technology able to help assess pre-implantation embryogenesis development and competence. This study examines the development and application of a low-cost, simplified, time-lapse imaging system that has shown efficacy in documenting embryo development from the pronuclear to the hatched blastocyst stage in the simplified culture system.
Introduction:

Infertility is a disease of the reproductive system in which an individual is not able to conceive or have a child naturally (ASRM, 2017). Conception is a complex process with many steps that requires healthy, functioning biological components. Some of these components include production of healthy sperm and eggs, unblocked fallopian tubes, ability of the fertilized egg (embryo) to implant to the uterus, embryo quality, and a hormonal environment sufficient for embryo development. If any of these factors are affected, infertility can result.

Infertility is a widespread and common medical condition with an estimated 12% of women impacted by infertility in the United States alone (CDC, 2017). A physician generally diagnoses infertility after one year of unsuccessful conception or pregnancy, and it is at this stage that treatments are suggested. Some of these treatments consist of lifestyle alterations, medication, surgery on reproductive organs, and assisted reproductive technology (ART) (Gunby et al., 2002).

ART has become a popular choice to combat infertility, and significant progress has been made with new medical developments. A key advancement that has become a mainstream, first line treatment for infertility is in vitro fertilization (IVF), which offers treatment to couples that would otherwise be unable to have a child biologically. This procedure consists of surgical removal of eggs from the ovaries, and then mixing of the eggs with sperm outside of the body, in vitro, where the fertilized eggs are then monitored and examined. The fertilized eggs, or embryos, are then transferred back to the woman, ideally resulting in pregnancy and birth. This technique was introduced and made popular by Nobel laureate Bob Edwards in 1978, and since then, an estimated 5 million babies
have been born using IVF. The generation of children born from this method has been
described as the IVF-baby-boom and numbers continue to climb as the popularity and
demand for IVF increases.

However, IVF treatments are expensive. The road to having a child through this
technique can be quite a costly endeavor with a current estimated base cost of $12,400 US
dollars for one IVF cycle (ASRM, 2017). This price does not include the medications and
often-suggested add-on technology and evaluations, such as pre-implantation genetic
diagnosis (PGD), mitochondrial assessments, and time-lapse imaging. These additional
techniques can provide valuable, and sometimes critical information about the status of the
procedure, but cost thousands of dollars. The average success rate after one IVF cycle is
only 33%, so additional cycles are often necessary (ASRM, 2017, CDC 2017). Though
infertility treatments are widely available in the West and other well-developed nations,
the expensive nature of IVF severely limits those who can afford these medical treatments.

Perhaps because of the expensive nature of the treatment, a stereotype of
individuals most in need of infertility treatments and who pursue IVF has developed.
Often these individuals are described as, “career-driven, wealthy, Caucasian, over-40-
year-old women who delayed becoming pregnant until after [they] had established
[themselves] independently” (Inhorn, van Balen, 2002). This notion may be true to some
extent, as the rise in IVF popularity and demand, particularly in the West, has been
attributed to some women waiting later in life to have children; however, infertility is a
global phenomenon (World Health Organization, 2017). Though these women may be
included in those seeking reproductive treatments, unfortunately, infertility does not
discriminate; it affects people of all races, gender, and socioeconomic standings.
It is a common misconception that infertility is most abundant in developed nations, where infertility treatments are accessible and mainstream; however, the populations most affected by infertility actually reside in developing nations. An estimated 186 million women, or one in four couples, experience infertility in these developing, low-resourced areas (World Health Organization, 2017). Those in need of infertility diagnosis and treatment often do not receive the necessary medical attention due to procedures being unaffordable, inaccessible, or nonexistent. Women affected by infertility in developing nations are largely impacted by societal stigmas and consequences that impact individuals and communities on a social, psychological, and economic scale (World Health Organization, 2017). For example, as a consequence of infertility, women in Third World areas are often excluded from social or communal groups, and experience social isolation, marital instability, and financial abandonment by family and the community as a consequence of infertility (Inhorn, van Balen, 2002). Though the societal consequences of infertility vary widely based on the culture of the area, women are inevitably being impacted by infertility across the world. Women affected by infertility in these developing nations, as well as around the world, are all equally entitled to accessible, affordable fertility care and family planning as a universal reproductive right.

**Background:**

Because of the unaffordable and limiting structure of standard IVF practices, many women have been overlooked and left behind in receiving the medical attention they need. However, recent developments in the Van Blerkom lab have made the concept of affordable IVF treatments a reality. The development of the simplified culture system for
clinical IVF purposes has significantly reduced the cost of treatments and does not require complex infrastructure commonly used in the West, which is largely unavailable for low-resourced areas. The system has been proven to be safe and effective with over 100 babies born using this system.

The simplified culture system has significantly reduced the base cost of IVF procedures using the method described (Van Blerkom et al., 2013). Current efforts in the lab have now been focused on developing low-cost alternatives for the additional technology commonly used in standard Western IVF practices.

Although laboratory techniques for embryo assessment have become increasingly more popular, they have also become more and complex. Some of these techniques as mentioned before, are pre-implantation genetic screening (PGS), mitochondria assessments, and time-lapse imaging, and have been shown to provide important information on early embryo development after fertilization. The latter has gained increasing popularity because of suggested increased implantation success rate and a reduced risk of multiple pregnancies (Hlinka, D., et al., 2012, Kaser DJ, Racowsky C, 2014, Meseguer, M, Rubio et al., 2013). Time-lapse imaging on pre-implantation embryogenesis is a non-invasive tool that can assess embryo morphokinetics and morphological features that could serve as potential empirical biomarkers for developmental competence (Rienzi et al., 2015, Racowsky et al., 2015, Motato et al., 2016). Morphokinetic features of early embryogenesis provide important information on the likelihood of embryo competence and implantation success (Dal Canto M. et al., 2012, Conaghan J, 2014, Kaser et al., 2014, Meseguer et al., 2011). Studies also suggest that certain morphokinetic characteristics in early embryogenesis may also be indicative of
numerous chromosomal abnormalities (Chavez et al., 2012, Campbell et al., 2013, Basile et al., 2014, Rienzi et al., 2015). Time-lapse imaging provides a continuous observation of embryogenesis, which is an important improvement given that many early stage cleavage abnormalities could likely be missed by static observation methods currently used in standard IVF clinics.

This useful technology, however, is expensive. Embryoscope, a popular time-lapse imaging tool currently used in many IVF clinics costs ~$150,000 U.S. dollars to purchase. Time-lapse imaging using the Embryoscope costs the patient around $500-$800 in addition to the overall IVF treatment cost. Because of the useful, albeit expensive, nature of the current time-lapse imaging available for clinical use, an effort to develop a low-cost time-lapse imaging system used in conjunction with the simplified culture system developed in the Van Blerkom lab has been investigated and ultimately achieved. This study describes the development and potential applications of a multifunctional time-lapse and video streaming capacity that is low-cost while still capable of producing high-quality, morphological and morphogenetic information from the pronuclear to the hatched blastocyst stage. Specifically, the time-lapse technique developed in this study proved to provide additional, valuable information on the early morphological changes, cleavage patterns, and developmental dynamics of the embryo using a low-cost, off-the-shelf approach.
Materials and Methods

The Simplified Culture System

The simplified culture system (SCS) for low-cost IVF utilizes two gas-tight vacutainers. The vacutainers were 10 mL borosilicate glass tubes stoppered with airtight rubber gauges, typically used for blood collection in routine phlebotomy. The first tube contained a ‘continuous’ culture medium that supports preimplantation embryogenesis from fertilization through the early blastocyst stage of development. For this study, Global Total medium was used for all embryo cultures. The second tube contained 10 mg of sodium bicarbonate that generates CO$_2$ when a solution of citric acid is added. The two tubes were then connected with a medical grade plastic tube with two 16-gauge syringe needles at each end. The syringe needles connected by the tube were then inserted into the rubber stoppers of each tube. Because the CO$_2$ generator in one tube becomes pressurized, under the conditions described, connection of the tubes drives CO$_2$ into the culture tube producing O$_2$ and CO$_2$ percentages similar to those used for standard incubator-based IVF (i.e., CO$_2$ in air; triple gas: N$_2$, CO$_2$, O$_2$). The development and use of this system in clinical settings is described in detail by Van Blerkom et al. (2013).

Embryos donated to research:

For the development of a low-cost time-lapse imaging system, as described in this paper, both fresh and thawed human embryos were available for research. Fresh embryos transported from an affiliated IVF clinic were available for this study under the following circumstances: (i) triploid at early (10-14 hour) pronuclear check; (ii) meiotically
immature at insemination but subsequently matured to metaphase II and fertilized normally in the SCS; and (iii) unsuitable for transfer after pre-implantation genetic screening on day 3 (e.g., trisomic or monosomic). Thawed pronuclear and early cleavage stage embryos cryopreserved by controlled rate freezing or vitrification were donated to research with written consent by patients who requested termination of cryostorage.

Embryo transfer to culture and positioning on microscope:

Fresh and thawed pronuclear and early cleavage stage embryos (2-to-4-cells) were maintained in HEPES buffered Global T for 5 minutes before being withdrawn in 25μl of the same medium into a 20-gauge, 90 mm stainless steel, luer-lock needle attached 1.0 mL gas-tight syringe (type 1750; Hamilton Instruments, Reno, NV). This was followed by 10μl of air and 10μl of medium containing up to six embryos. The needle was inserted through an ethanol-sterilized stopper of the culture tube, and embryos were deposited directly into 1 to 1.2 mL of culture medium, which had been equilibrated with the CO₂-generating culture tube for a minimum of eight hours. For each series of cultures used for streaming and time-lapse, one extra culture tube was used to confirm pH, which was consistently between 7.32-7.36.

After embryo placement, the tubes were kept in a vertical position for five to ten minutes, and then slowly tilted to a near horizontal position on an adjustable stage of an inverted microscope such that the area containing embryos was directly over the objective lens. Once embryo location was identified, the culture tube was secured in place at an angle of ~10 degrees above the microscope stage with a small piece of modeling clay placed near the culture tube stopper. In the present study, six to eight embryos could be
located as a group, and, when necessary, closer juxtaposition was obtained by moving the stage in a gentle back and forth motion using the Y-axis stage adjuster. Alternatively, centrifugation at low speed (~100-150 rpm) after embryo deposition in a standard clinical swinging bucket centrifuge for 2 minutes usually clustered embryos at the bottom of the culture tube, which then moved as a group into position for imaging once the tube was placed horizontally and the stage moved back and forth appropriately. Pre-coating with a binding agent that could aid in stabilizing embryo position (e.g., poly-l-lysine) was unnecessary; once positioned above the objective lens, the embryos usually remained fixed, allowing for the identification of each individual embryo during prolonged imaging based on their individual morphological signatures. Some minor repositioning was occasionally required during blastocyst hatching owing to embryo-generated movement during its emergence from the zona pellucida and subsequent expansion.

**Incubation for SCS and time-lapse imaging**

For this project, alternative methods to standard incubation were used. The SCS is a closed system, and can traditionally be incubated by low cost materials such as a water bath, thermal beads, thermoses, or with a standard incubator. However, for time-lapse imaging purposes, these methods were not compatible for continuous imaging on a microscope. For the purpose of time-lapse imaging, two methods were primarily used: a standard microscope incubation chamber, or, as a low-cost alternative, an air curtain generated by a portable electric (ceramic) heater. For the first method, the chamber was assembled around the microscope with an external source of heated air blowing into the chamber at a controlled temperature. The temperature was assessed with a thermocouple.
around the SCS tube containing the embryos, as well as with a standard mercury thermometer. Once the temperature was configured, it remained constant at 37°C.

The second method was developed in an effort to maintain the low-cost nature of the project, and provide an alternative to the chamber method previously described for such events that a microscope with an incubation chamber was not readily available. In this method, a heater was placed parallel to the microscope at a determined distance. The vertical position of the heater was achieved by placing it on an elevation block. The temperature of the SCS was monitored using a thermocouple probe. The tip of the wire thermocouple probe was secured to the glass culture tube using Parafilm and positioned at the imaging location of embryos in culture. Typically, the heater remained constantly on, but if long intervals between cultures occurred, the heat was turned on six to eight hours prior to use. Once these spatial and temporal parameters were established, the temperature surrounding the culture tube remained constant and could be monitored periodically with a mercury thermometer or thermocouple probe placed on the side of the culture tube. Once the embryos were positioned in the culture tube on the microscope stage, the setup for both methods remained undisturbed unless minor repositioning was required. Under the conditions, several relatively inexpensive inverted microscopes can be operational in a single darkened room with the ambient temperature remaining undisturbed because the presence of laboratory personnel is infrequent and video streaming allows viewing of embryos in real time at an external location.

Maintenance of a constant 37°C for prolonged periods at the site of embryo culture on a microscope stage is essential for the described time-lapse system to be applied reliably to the SCS in a clinical setting. Both methods provided a consistent and effective
incubation temperature that is necessary for embryo development. However, for the time-lapse and video streaming purposes explored in this study, the chamber method was primarily used because of convenience and availability.

Microscopy Imaging

Preliminary studies with different optical formats (bright-field, phase contrast, differential interference contrast) showed that imaging of embryos taken through the glass culture tube were clear and distortion-free using low power objectives and bright-field illumination. For these studies, plan-apochromatic 10X and 20X objectives with numerical apertures between 0.30 and 0.45 were effective on a variety of inverted microscopes (Leitz Diavert and DMIL; Nikon Diaphot and Eclipse TE300) and here, results are shown using the Leitz (DMIL) and Nikon (Eclipse TE300) instruments. Although long working distance objectives are preferable, placement of the culture tube directly above the objective lens where embryos were located provided a depth of field that was sufficient to resolved cellular and nuclear details.

Low-Light Video Microscopy

Preliminary studies examined the effectiveness of digital and analog video cameras from different manufactures with respect to cost versus imaging quality of human embryos using the glass culture tubes, bright-field optics, and very low illumination levels. Because embryos are colorless, monochromatic (i.e., black and white) we show examples of two analog cameras that met these criteria: (i) a Panasonic WV-BP110 CCTV and (ii) a Cohu high performance CCD (models 4910 and 4912: Cohu Inc., San Diego, CA.).
Transformation of an analog to a digital signal for computer-based video streaming and time-lapse imaging required an analog-to-digital converter (e.g., Canopus ACDV110: Grass Valley, Hillsboro, OR.) The inclusion of a 2mm-thick, 630nm long, pass filter in the optical path significantly enhanced contrast and blocked potential blue-to-UV emission from the halogen light source. The intensity of illumination was attenuated by first closing the field diaphragm to its lowest aperture setting and then reducing the level of illumination until adequate image quality of pronuclear- to-blastocyst stages were evident on a video monitor. Typically, the intensity of red illumination was just above the lowest setting on each microscope, and barely visible to the naked eye. In order to obtain an approximation of the level of light experienced by embryos during culture, the sensor of a digital luxmeter was centered on the objective lens where embryos would be located and measurements made in lux units.

**Time-lapse imaging and recording software**

There are many software programs with time-lapse functions available online designed for a variety of purposes, but have limited functionality in cell biology, including the documentation of human embryo morphokinetics. However, after screening the relative advantages and disadvantages of various softwares, the field was narrowed to four candidates that were well suited to imaging embryos (e.g., EvoCam5, Security Spy, SwiftCapture, Gawker). EvoCam 5.0 (evological.com) was selected as applicable to the SCS based on cost effectiveness and resident features and functions.

The features and functions of EvoCam that distinguished this software for selection are as follows: high-resolution in low light conditions, support of a wide variety
of digital and analog cameras, ability to save still time-lapse images to archive (saved as raw JPEGs), capability to compile and export time-lapse video files, manual control of image/recording brightness, contrast, and saturation, capacity for simultaneous real-time video streaming, and high quality, stable, time-lapse imaging of pre-implantation embryogenesis.

In addition to clear online user manuals, the EvoCam software included a suite of sophisticated functions useful for recording embryo morphokinetics, such as contemporaneous auto brightness and contrast settings, as well as programmable settings for taking images at predetermined times and specified intervals (eg. seconds, minutes, hours). When this function was combined with a simple multi-event timer that turned the microscope’s light source on or off at predetermined times, imaging can be discontinuous and set to when important morphokinetic events determined to be relevant for competence assessment are likely to occur in an IVF program. These events include pronuclear dissolution, first cleavage division, morulation, cavitation, inner cell mass delineation, and blastocyst hatching.

Time-lapse data is saved as JPEGs or as QuickTime files, which facilitates image processing for playback. In the present study, images were usually taken at 5-minute intervals for up to 165 hours (day 0.5-to-day 7). It is worth noting that relatively low-cost, analog, monochromatic video cameras were found to provide surprisingly acceptable image quality and resolution. For use with the SCS, one potentially advantageous feature of these programs is that embryos from multiple microscopes can be streamed, viewed, and recorded simultaneously, which may be an important factor for adoption by IVF
programs that would want to add the capacity for multiple patients while still remaining low cost.

**Time-lapse image post-production processing**

Unprocessed images for video streaming and time-lapse recording were usually of sufficiently high quality to detect developmentally significant nuclear and whole embryo morphokinetic activities. For playback and archival purposes, after completion of the time-lapse recording, individual images were compiled and exported for post-production editing, if necessary, to improve brightness and contrast levels, using Adobe Lightroom software (Adobe Systems, San Jose, CA.). If severe flickering occurred during playback, processed images were exported as a file for ‘deflickering’ using LRTimelapse 4 software (LRTimelapse.com). This inexpensive and user-friendly editing step produced high quality and fluid time-lapse sequences exported as ‘Quick Time files (Apple Inc.) using a standard video rate of 30 frames/second. However, with the time-lapse program noted, raw images typically produced sequences of high resolution and sufficient quality to detect competence-associated morphokinetic and morphological events and features without the need for post-recording processing. The images from time-lapse sequences shown below were all unprocessed, but benefited from improved contrast with the 630nm filter in the optical path. Numerous time-lapse sequences are easily archived on commonly available high capacity external drives.

**Video streaming and conferencing**

Video conferencing during real-time streaming or time-lapse playback provided a potentially important clinical dimension to the SCS because it allowed for multiple
observers at different locations to communicate and comment on developmental aspects relevant to the normality of embryogenesis that may be related to quality control or embryo selection for transfer, or both. Simultaneous video conferencing can be achieved through additional applications such as Skype, WebEx, or Face Time. The Skype application allows users to have video conferencing with multiple contacts and is an easily downloadable freeware platform, while Face Time is pre-installed on current Apple devices.
Results and Data:

Time-lapse incubation set up:

Figure 1A shows the chamber incubation method that was primarily used for this study. A Plexiglas chamber surrounding the Nikon Eclipse microscope had a heat source in the back right corner that blew warm air into the chamber. A standard mercury thermometer and digital thermometer on the stage next to the culture tube monitored the temperature continuously. An analog camera was inserted into the port on the left side of the base of the inverted microscope. The analog signal was transmitted to the analog-to-digital converter and displayed on the computer monitor.

Figure 1B shows the alternative incubation system. The figure shows the spatial configuration of an inverted microscope and a common, household portable electric heater that generates a 37°C ‘air curtain’. This is an example of an open incubation system for time-lapse and real-time video system described. The horizontal positioning of the heater was calculated to be most efficient and consistent at 13 inches away from the base of the microscope. The vertical distance was obtained using a stabilizing block, which positioned the heater to the same approximate height as the microscope stage. A logging digital thermometer verified the optimal horizontal and vertical positioning of the heater with the wire thermocouple probe secured on the bottom of the culture tube. This position of the thermocouple was directly above the objective lens where the embryos were located in culture. The culture tube was slightly elevated from horizontal and held in place by a small piece of modeling clay. Once the parameters of heater distance, height, and BTU output were established and the stability of the temperature was confirmed, the ambient
heat of the microscope stage and embryos in culture was maintained at a temperature normal for human pre-implantation embryogenesis. The thermal stability was best maintained in a small room that could accommodate several microscopes. An inexpensive line conditioner with voltage regulation was incorporated to provide a constant, ‘clean’ AC power supply to the microscope, camera, and computer.

**Time-lapse recording and video streaming:**

Figures 2-5 are representative images taken from time-lapse imaging using the EvoCam5 software. The figures indicate the typical resolution quality obtained and morphokinetics of critical developmental landmarks or abnormalities. The images were taken at the times indicated on the figure panels. Images of pronuclear dissolution, cleavage, and blastocyst hatching were recorded and streamed continuously using the time-lapse software (see above). Multiple observers, located in different countries, were given temporary site access to the streaming feed at www.mcdb.embryocam.edu/push.

**Pronuclear dissolution:** (Figure 2A)

Two pronuclear stage (PN) embryos shown in figure 2, panels A1-6, had been cryopreserved by slow cooling at 14 hours and 25 min after conventional IVF and exhibit normal pronuclear membrane dissolution (black arrow), however, occurring somewhat earlier for the embryo on the right. These images demonstrate the clarity with which cytoplasmic and nuclear structures are detectable through the simplified culture tube. Images also display the clearly detectable localization of nucleoli (n; white arrow, panels A1-2).
1-cell to 3-cell division: (Figure 2B)

Figure 2, B1-8 show what appears to be normal pronuclear membrane dissolution 21hrs 10 minutes after monospermic fertilization with conventional IVF (accessory sperm can be seen on the zona pellucida, SP panel B1). The first cleavage happened at an appropriate estimated time for normal development; however, the first cleavage division produced three blastomeres. This phenomenon is normally associated with dispermic fertilization (triploidy) and a tripolar spindle (Zhan et al., 2016, Hlinka, D., et al., 2012, Kalatova, Beata, et al., 2015). Subsequent cleavage divisions resulted in an apparently stage-and-time appropriate six-cell embryo with uniform blastomeres and no fragmentation. Standard embryologic static observations at the pronuclear stage and cleavage patterns of day 2 or 3 would have likely missed this abnormality in early cytokinesis. Additionally, this normal appearing embryo would have likely been classified as transferrable on day 3, but with a high probability of the embryo being chromosomally mosaic (Zhan et al., 2016, Hlinka, D., et al., 2012, Kalatova, Beata, et al., 2015).

Imaging for embryos in figure 2 was done at the same low intensity, 630 nm filter illuminations, but the embryo in figure 2B has a notably darker cytoplasm compared to the embryos in figure 2A. The differential cytoplasmic density was another interesting feature of this embryo observed using standard bright-field optics. Phenotypic variations, such as the cytoplasmic differences shown, are not atypical between early embryos and are usually patient-specific; however, the origin for this differential density is unclear.
2-cell to 5-cell cleavage: (Figure 3A1-10)

Figure 3, panels A1-10, shows another abnormal cleavage in early embryogenesis, resulting in a normal appearing embryo. For this embryo, the abnormal cleavage happened at the 2-cell stage, affecting one blastomere with potential downstream consequences of chromosomal aneuploidy/mosaicism of mitotic origin (Nigg, Erich A, 2001). The sequence in figure 3 shows an apparently normal pronuclear dissolution at 19 hours 20 minutes after conventional IVF (PN, arrow, panel A1), followed by a normal first cell division into two blastomeres at 22 hours. The 2-cell embryo shows ideal symmetry, morphology, and no signs of fragmentation. However, the nascent blastomere (indicated by an astric, panels A2-3) undergoes abnormal cleavage in the next cell division cycle, and divides into a 3-cell, which all appear to be mononucleated (n, panels A4-A5). A second cleavage for the other blastomere occurs ~2 hours later and produces a 5-cell embryo (panel A6-A7) where all blastomeres appear uniform in size and shape and at least 3 cells are mononucleated (arrows; the fifth cell is out of the plane of focus in panel A7). The embryo continues to divide first to a 6-cell embryo (panels A8-A9) then to an 8-cell embryo (panel A10). The final 8-cell embryo appears developmentally stage-and-time appropriate, and would likely be incorrectly classified as morphologically normal for transfer on day 3. This phenomenon of 2-to-5-cell division of mononucleated cells is likely to result in severe chromosomal abnormalities (aneuploidy), most likely of mitotic origin. It is apparent that the normal cell division checkpoints and regulations were defective or non-functioning in this particular division, resulting in 3 mononucleated cells (Nigg, Hartwell).
2-cell reverse cleavage: (Figure 3A1-5)

One other abnormal cleavage pattern visualized with the time-lapse system developed was a 2-cell reabsorption, or ‘reverse cleavage’ (Liu et al., 2014, Liu et al., 2014, Hlinka et al., 2012, Desai et al., 2014). Figure 3 shows the progressive formation of a 2-cell embryo after pronuclear membrane dissolution. Between 20-40 minutes after the completion of the first cleavage, an apparent blastomere fusion, or reversion to 1-cell embryo, occurs, perhaps due to incomplete cytokinesis. This phenomenon, referred to as ‘reverse cleavage’, has been described and characterized as abnormal development often resulting in “significantly compromised embryo development, culminating in poor implantation potential” (Yanhe, 2014). This abnormal phenomenon, however, would have likely been missed using static inspection techniques.

The morula, early cavitation and blastocyst stages: (Figure 3C1-5)

Figure 3, panels C1-5 shows the normal process of compaction (panels C1-3) and cavitation (panels C3-5) at the morula stage, where fluid accumulation (region indicated by an asterisk) begins the formation of what will later be the blastocyst cavity or blastocoel. Subsequent normal embryo expansion and thinning of the zona pellucida was clearly visible with the use of the time-lapse imaging system developed in this project. This imaging system also demonstrated efficacy in visualizing both normal and abnormal blastocyst hatching events. Here we describe three distinct blastocyst hatching phenomenon that have been characterized as key to embryo implantation success and competence (Motato et al., 2016, Kirkegaard et al., 2013, Desai et al., 2014, Dal Canto et al., 2012).
Failed Blastocyst Hatching: Zona breakers (Figure 4C1-14)

Figure 4, panel A1-14 depicts a time-lapse sequence of embryogenesis of a thawed, 8-cell embryo (panel 1; day 2.5 post insemination; slow-cooling) through morula (panel 3), cavitation (panel 4-5), blastocoel expansion (panels 6-9), with attempted and failed hatching (panels 9-14). This embryo exhibited an abortive effort to hatch where only a few trophectoderm cells initially emerged from a small, naturally occurring rent in the zona pellucida. Despite evident zona thinning (panels 2,5-6,10), during blastocoel expansion (panels 6 and 8, large asterisk), clear delineation of the inner cell mass (ICM), thinning of trophectoderm (TR), and several cycles of blastocoel collapse and re-expansion, no further extra-zonal expansion of the emerged cells occurred. At the final collapse (panel 10), the initial trophectoderm expressions that penetrated and extended through the zona pellucida (black arrow, panels 7-14), described and termed as ‘zona breakers’, detached from the underlying cells and retained motility for some hours before motion cessation and subsequent lysis (Sathananthan, 2003).

Blastocyst Hatching: (Figure 4A1-2, B1-8)

Figure 5A and 5B show the expansion and hatching of human blastocysts. This sequence confirms that the time-lapse imaging system developed and described here has sufficient resolution and capabilities to clearly visualize the hatching process, ICM, and cell divisions within the trophectoderm. This sequence also provides a proof of principle that time lapse imaging combined with the simplified culture system is capable of supporting, and does not hinder, embryogenesis of human pre-implantation embryos.
Panels A1-2 demonstrate the ability of the time-lapse system to detect cell division within the trophectoderm. The dark region indicated by an arrow in panel A1 is a trophectoderm cell nucleus in cytokinesis, and in panel A2, two nascent nuclei after the completion of cell division (arrow). Cells showing a darker nuclear phenotype prior to and after cytokinesis were commonly observed with time-lapse imaging in the trophectoderm of embryos in culture located directly above the objective lens.

Figure 5, panels B1-8 show the expansion and hatching progression of embryos from the same patient donated to research. The sequence begins ~4 hours after the embryo on the right-hand side had fully emerged, which was at an earlier stage for the embryo on the left (arrow). The age of these embryos when the first blastocyst had completely emerged was about 5 days and 20 hours after conventional insemination (panel B1). For the embryo on the left-hand side, blastocoel expansion continued during hatching (panels B1-8), which was completed approximately 10 hours after its sibling was free of the zona pellucida (panels B6). This sequence demonstrates the clarity of imaging that was obtained using the time-lapse system developed, as evidenced by the cellular detail obtained as the embryo progressively emerges from within the zona pellucida (ZP, white arrow). It also demonstrates significant differences in the timing of complete emergence for two sibling embryos that appeared stage-and-time appropriate when fertilized and throughout development in culture. This sequence displays significant differences in the timing of complete emergence for the two embryos; however, the continued expansion of the later hatching embryo (panel B7) resulted in two blastocysts of the same approximate size and diameter (panel B8).
Blastocyst hatching continued: (Figure 5C)

Similarly to the blastocysts described above, a second case of sibling embryos were donated to research and visualized using the time-lapse system developed. Time-lapse imagings of blastocyst hatching, panels C1-9, show an earlier hatched blastocyst on the right, with a sibling embryo on the left (arrow). Panels C1-3 show the emergence of the blastocyst from the zona pellucida ~5 hours after the sibling embryo on the right. The later hatching embryo emerged from the zona pellucida (panel C4, arrow) and continued its expansion post hatching (panels C7-11). The earlier hatching embryo on the right continued expanding (panels C1-6), and then collapsed (panels C7-8). The white asterisk in panel B8 indicates the most compact state of blastocoel collapse. After the initial collapse, the embryo on the right re-expanded, and both embryos continued to expand (panels C9-11) until final collapse (panel C12). Though both embryos continued expanding post-hatching, the later hatching embryo on the left (arrow) never achieved the same diameter as its sibling before the final collapse (panel C12).
Figure 1A

Figure 1A legend:
Figures 1A and B show the typical in-house setup for time-lapse recording of pre-implantation human embryogenesis using culture tubes as described in the text for the simplified culture system (SCS).

Figure 1A shows a conventional incubation chamber assembled around the Nikon Eclipse TE300 microscope. A monochromatic analog camera is inserted on the left side of the microscope. A 630nm filter is installed at the light pass located at the top of the inverted microscope. The heater located on the right side of the base of the microscope blows warm air (37°C) into the incubation chamber. The SCS containing embryos for imaging is positioned on the microscope stage and secured with a small piece of modeling clay. Temperature is recorded using a thermocouple probe and a standard, high accuracy mercury thermometer (not shown). The monitor displays a live feed of the recording from the analog camera and sends the signal to an analog-to-digital converter box. The digital signal is transmitted on a desktop computer and recorded using the time-lapse software described (not shown).
Figure 1B legend:
Figure 1B shows the open incubation method with ambient 37°C supplied by a curtain of hot air from a common electrical heater using a Leica DMIL inverted microscope. Temperature at the site of culture was assessed by both thermocouple/digital thermometer and standard, high accuracy mercury thermometer. The other components used in this system, including the line conditioner, analog black and white video camera, location of a 630nm filter, and analog-to-digital signal converter, computer and stabilizing blocks used to position the heater with respect to distance from the center of the microscope stage, are indicated in the figures. Modeling clay was used to both secure the culture tube and to slightly elevate it so that the bottom of tube sat directly above the objective lens. This is where embryos are deposited and remain in place during recording. The image being recorded by time-lapse and streamed online to multiple external observers is shown on the desktop monitor. The monitor below shows the analog feed of time-lapse recording mode only in B.
Figure 2

Selected sequence from a time-lapse video showing normal pronuclear juxtaposition (panel A1) after conventional insemination in the SCS glass culture tube, followed by pronuclear membrane (PN) dissolution for the embryo on the right (panel A2) and later for the embryo in the left (panel A5). These images were recorded near the bottom of the culture tube that was positioned directly above a 10X objective lens using bright-field optics and filtered 630nm, low light illumination (0.05lux). The resolution was sufficient to detect individual nucleolar precursor bodies (n) in each pronucleus. In figure B, the oocyte was naturally darker, probably due to high lipid content. Pronuclear evolution (PN, arrows, panels B1-4), membrane dissolution (panels B5, 6), and the first (panel B7) and second cleavage divisions (panel B8) are shown in this sequence. Of particular note is the first division into three cells (panel B7) followed in 16 hours by a second division that produced a normal-appearing 6-cell embryo (panel B8). Time from conventional insemination (SP, sperm) is shown at the lower right.
Figure 3 legend:
In A1-10, a time-lapse sequence, beginning at 19 hours after insemination, shows an abnormal pattern of early cleavage divisions detected by time-lapse. Panel A1 shows a normally fertilized egg near the end of the pronuclear membrane dissolution stage, which is followed by an apparently normal first cleavage division some 3 hours later (panels A2, 3). However, what time-lapse revealed was that two hours later, the nascent blastomere indicated by an asterisk in panel A3 divided again into three apparent cells (panel A4), each of which was clearly mononucleated (panel A5). At 36 hours, the second blastomere divided normally producing a 5-cell embryo (panel A6). The three cells from the prematurely dividing blastomere shown in panel A5 were still mononucleated (indicated by arrows in panel A7). The 5th cell, the product of an apparently normal cell division of the second blastomere in A5, is out of the plane of focus in panels A6 and 7. Continued cell divisions produced blastomeres of uneven size (panels A8 and 9), but at 64 hours, the embryo appears morphologically normal and by static evaluation, would likely have been considered stage appropriate for transfer (panel A10).
Figure 3B1-5 shows a ‘reverse cleavage’ phenomenon. The post pronuclear dissolution stage embryo in panel B1 undergoes an apparently normal first cell division as shown in panels 2-4. However, approximately 1 hour after the completion of cytokinesis (panel 5), an abrupt reversion to a single cell is observed representing a phenomenon termed ‘reverse’ cleavage.
The panels in figure 3C show normal cleavage divisions (panels 1,2), morulation (panel 3), and early fluid accumulation (asterisks, panels 3-5) accompanied by a progressive thinning of the zona pellucida as the embryo expands (arrows, panels 3-5). Time from conventional insemination (SP, sperm) is shown at the lower right.
Figure 4 legend:
Panels A1-14 show progressive embryogenesis from cleavage (panels 1,2), morula (panel 3), cavitation (large asterisk, panel 4) and blastocyst expansion with formation of the blastocoel cavity (large asterisk, panels 5,6,8) and delineation of the trophectoderm (TR, panel 7) and inner cell mass (ICM, panel 8). However, hatching failed despite the elaboration of trophectodermal surface specializations though to facilitate hatching (‘zona breakers’, arrow, panels 8-14) and several cycles of blastocoel collapse and re-expansion. The ‘zona breakers’ detached from the embryo during the final collapse yet retain motility within and on the surface of the zona pellucida for some hours later (panels 11-14). A progressive thinning of the zona pellucida, which is a normal feature of the expansion phase, is evident by comparing thickness from cleavage to expanded blastocyst stages (small asterisk, panels 2,4,5,6,10). Time from the start of culture at the 10-cell stage is shown at the bottom of each panel.
Figure 5A-B

Figure 5A legend:
The clarity of blastocyst expansion and hatching, as well as the ability to resolve fine morphokinetic details though the glass culture tube of the SCS using a simplified time-lapse system, is shown in panels A-to-C. The arrow in panels A1 and 2 shows that nuclear segregation during cytokinesis in the trophectoderm is clearly detectable. Panels 1-8 in figure B are from a longer sequence of embryogenesis that in this figure, begins for two sibling embryos about 4 hours (panel B1) after the embryo on the right had emerged completely from the zona pellucida. While the earlier hatched embryo continued to expand, its slower sibling on the left completed its emergence some 10 hours later (panel 7) but by 16 hours (panel 8), had expanded to reach a diameter that was similar to that of its earlier emerging sibling. Both embryos hatched in a continuous, fluid motion with no cycles of collapse or expansion of the blastocyst cavity (blastocoel).
Figure 5C

Figure 5C legend:
The clarity of blastocyst expansion and hatching, and the ability to resolve fine morphokinetic details though the glass culture tube of the SCS using a simplified time-lapse system is shown in panels A-to-C. Panels 1-12 in figure 5C show blastocyst hatching for the embryo on the left that begins ~5 hours after the earlier hatched embryo on the right. The earlier hatched embryo continues to expand post-hatching with clear visualization of the inner cell mass (panels 1-6). This embryo undergoes blastocoel collapse (panels 7-9) with the smallest contractile state indicated by a white asterisk at panel 8. Panels 9-11 show blastocyst re-expansion for the embryo on the right. Blastocyst hatching for the embryo on the left is indicated in panels 1-8, with final emergence of the zona pellucida (indicated with black arrow) in panel 8). The later hatching embryo on the left continues expansion in panels 9-11 with clear visibility of the inner cell mass in panel 9. Both embryos experience a final collapse indicated in panel 12. The later hatching embryo (left) failed to reach the final size that the earlier hatching sibling embryo achieved (right).
Discussion

Time-lapse imaging of human pre-implantation embryogenesis provides observational analysis to optimize embryo selection for transfer. The morphology and morphokinetic patterns of early embryogenesis provide substantial evidence for the normality and potential implantation success. The use of time-lapse observation on early stages of embryogenesis has been shown to improve embryo selection, leading to higher implantation rates and lower instances of multiple births (Lundin, 2001). Specifically, evaluation of specific and relevant developmental stages during embryogenesis, such as, pronuclear dissolution, early cleavage patterns, and compaction/morulation, have been shown as strong biological indicators of embryo potential (Lundin, 2001). Time-lapse imaging provides a non-invasive method to determine embryo competence and potential chromosomal abnormality based on kinetic behavior (Basile, 2014). Embryo aneuploidy is a major cause of implantation failure with IVF procedures (Campbell, 2013).

Observational analysis of morphokinetic patterns during early embryo development provides insight for embryo selection and de-selection methods. Time-lapse imaging has been shown to provide relevant data to determine high-risk aneuploidy embryos without the need for pre-implantation genetic screening (PGS) (Campbell et al., 2013). Though valuable information can be obtained using time-lapse imaging, it is expensive and not widely accessible in the clinical IVF practices. This study shows that significant abnormalities at time-and-stage specific developmental points can be seen and evaluated using the low-cost system developed.
Detection of significant embryo developmental stages

Pronuclear (PN) morphology has been supported as a prominent embryo selection parameter in standard clinical IVF observations (Azzerello et al., 2012, Payne et al., 1997). These static observations, however, often do not assess the important PN morphological changes over time, and the single observation approach may be defective in effectively assessing critical time-and stage specific PN kinetics. The timing of pronuclear breakdown (PNB) has been shown as a strong indicator of embryo competence and viability (Azzerello et al., 2012). This study examines the PNB stages of early embryogenesis, and shows that this critical developmental marker can be visualized and assessed using the low-cost system developed.

Early cleavage patterns:

Early cleavage patterns in pre-implantation embryogenesis are also key indicators of embryo viability (Zhan, Qiansheng, et al., 2016). The 1-cell to 3-cell embryo described in this study was observed using the time-lapse system developed. This phenomenon has been characterized as “tripolar mitosis,” where the cell divides using typical molecular mechanisms of mitosis, but results in three daughter cells instead of the phenotypically normal two cells (Kalatova et al., 2014). Embryos that exhibit this cell division rate have been characterized as having “impaired pregnancy-yielding potential.” These 1-cell to 3-cell embryos can continue to develop with a morphologically normal appearance, but have a high likelihood of being chromosomally defective (Kalatova et al., 2014). A normal appearing embryo that underwent abnormal division patterns could be evaluated as competent and selected for embryo transfer if this phenomenon was missed by the
standard static observation method used in clinical IVF. This makes time-lapse imaging an extremely useful tool in embryo assessment and selection.

An abnormal early cleavage pattern that occurred beyond the 1-cell stage was observed in a 2-cell to 5-cell embryo. Abnormal, unequal cell division has been associated with high probability of chromosomal abnormality of the mitotic origin (Nigg et al., 2001, Hartwell et al., 1989). The phenomenon of 2-to-5-cell division of mononucleated cells observed in this study was likely to result in an embryo with significant chromosomal abnormalities.

The ‘reverse cleavage’ phenomenon observed in this study shows one other abnormal early cleavage pattern associated with poor implantation potential (Liu et al., 2014). Research suggests that reverse cleavage (RC) patterns in early embryogenesis are associated with implantation failure and embryo incompetence (Liu et al., 2014). RC has been characterized as an event that could happen at any point, or multiple times, during the first 3 days of culture (Liu et al., 2014). RC was observed in this study during the 2-cell division, and further validates the claim that time-lapse imaging has clinical value for embryo assessment and selection techniques.

**Morulation, compaction, and blastocyst hatching**

Time-lapse imaging using the system described in this study was also effective for observation of morphokinetic patterns during morulation, compaction and spontaneous blastocyst hatching. Competent blastocysts exhibited continuous expansion and thinning of the zona pellucida that was typically progressive, with few instances of blastocoel collapse, until the embryo was fully emerged. Competent embryos seen in this study also
demonstrated normal morphokinetic patterns of continued expansion post hatching. Instances of failed hatching observed with the time-lapse system could suggest defective embryogenesis in patients experiencing a low implantation success rate using IVF. This time-lapse system was able to identify patterns of blastocyst hatching that related to spontaneous hatching competence.

Time-lapse imaging in this study also confirms the occurrence of a focal cluster of highly motile trophodermal processes that extend through the zona pellucida at the fully expanded blastocyst stage. This phenomenon was originally described by Sathananthan et al., who classified this cluster as ‘zona breakers,’ suggesting that they function in an enzymatic way to create a hole in the zona pellucida for blastocyst hatching. It remains to be determined, however, whether failed hatching could be a consequence of the absence of ‘zona breakers,’ an insufficient activity level of these cellular extensions to create a rent in the zona pellucida sufficient for embryo emergence, or a bioenergetics deficiency in the embryo that limits the fluid transporting function of the trophoderm to re-inflate the blastocoel after a number of collapse/re-expansion cycles.

Benefits and application of time-lapse imaging in clinical IVF settings

The findings of this study demonstrate that morphological and morphokinetic characteristics suggested to be embryo competence biomarkers observed with this low-cost system is of equal quality and value to the expensive time-lapse systems that are currently commercially available. This study also demonstrates that clinically relevant information on pre-implantation embryogenesis can be obtained using a low-cost platform from readily available components described here that combines sophisticated, high-
complexity technology with simplified, low-cost components. The time-lapse imaging preformed in this study was able to distinguish abnormal occurrences in early embryogenesis that have been characterized as high-risk for aneuploidy and likely resulted in implantation failure.

The benefits to documentation of abnormal and normal morphokinetics from the pronuclear to the hatched blastocyst stage by adding a sophisticated, yet low-cost, time-lapse imaging system could have wide spread clinical application. In addition to detecting embryo competence, the software used also has a live, real-time video streaming component. Access to live video streaming could provide embryologists continuous access to developmental assessments, potentially from remote viewing locations. This could be potentially advantageous to programs adopting a low-cost IVF platform, such as the use of the simplified culture system. This video streaming capacity could also enhance the training methods used at clinics and provide contemporaneous observation and communication with colleagues at remote locations for assistance in respect to embryo quality control, embryo performance, and selection criteria.

Although time-lapse imaging may not be necessary for every patient, it could be particularly beneficial to patients with certain qualifications. Specific instances that time-lapse imaging could be particularly beneficial are in cases of certain maternal age, previous implantation failure after several cycles of IVF, high proportion of aneuploidy embryos identified by PGS in multiple cycles, recurrent miscarriages with embryos designated as stage and morphologically appropriate at transfer, or for low-cost programs if only a single IVF attempt is possible and pre-implantation genetic screening is not an option.
Conclusion

Time-lapse imaging has shown clinical and experimental value. The morphokinetic information that time-lapse imaging provides has been characterized as providing substantial information on embryo competence and pre-implantation success (Dal Canto et al., 2012, Zhan et al., 2016, Athayde et al., 2014, Conaghan et al., 2016, Findikli et al., 2014, Gardner et al., 2016). Specifically, the pronuclear, early cleavage, morulation/compaction, and blastocyst hatching stages can suggest the normality of embryogenesis, as well as potential aneuploidy. These key developmental stages, and associated abnormalities, have been clearly visualized and assessed using the low-cost time-lapse system developed, demonstrating that significant abnormalities at time-and-stage specific developmental points can be seen and evaluated using the system developed. Although the extensive use of time-lapse imaging in clinical IVF settings may remain controversial, the technology and methods developed in this study provide a potential option for the use of inexpensive time-lapse technology to enhance embryo selection methods that could result in an increase in the number of successful pregnancies.
References:


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