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(54) Title: AUTOMATIC SURVEILLANCE OF IN VITRO INCUBATING EMBRYOS

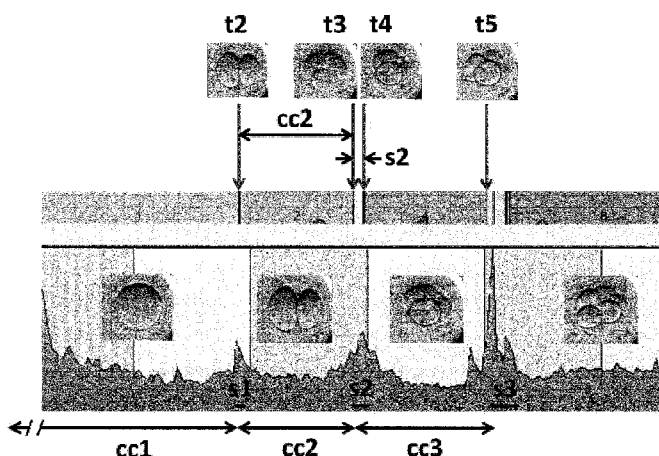


Fig. 1

(57) Abstract: A computer implemented method for automatically detecting variations and/or abnormalities in the developmental conditions of in vitro incubating embryos, the method comprising the steps of: a) obtaining a first dataset comprising morphokinetic parameters relating to the development of a first group of embryos, b) obtaining a second dataset comprising morphokinetic parameters relating to the development of a second group of embryos, c) modifying the first and second datasets by extracting morphokinetic parameter outliers from the first dataset and or the second datasets, d) calculating the difference between specific morphokinetic parameters from the modified first dataset and the corresponding morphokinetic parameters from the modified second dataset, and monitoring said morphokinetic difference thereby detecting variations in the developmental conditions of the first and second group of embryos.

Automatic surveillance of in vitro incubating embryos

The present invention relates to a system and a method for automatically surveying the developmental conditions of in vitro incubating embryos. The present invention may be applied within quality control of embryo handling in relation to IVF to ensure that the quality of transferred embryos is maintained. Thus, the present invention may be a tool for fertility clinics to survey and maintain a high implantation potential of in vitro incubating embryos.

Background of invention

Infertility affects more than 80 million people worldwide. It is estimated that 10% of all couples experience primary or secondary infertility. In vitro fertilization (IVF) is an elective medical treatment that may provide a couple who has been otherwise unable to conceive a chance to establish a pregnancy. It is a process in which eggs (oocytes) are taken from a woman's ovaries and then fertilized with sperm in the laboratory. The embryos created in this process are then placed into the uterus for potential implantation.

Quality Control (QC) is an important issue in IVF clinics to monitor the quality of the treatment and the different processes in the clinics that has an effect on the developmental conditions of the embryo. Quality control can be conducted by monitoring the development of running averages in outcome variables like

- Biochemical pregnancy rates (number of HCG positive per transferred embryos)
- Fetal heart beat rates (number of fetal heart beats per transferred embryos)
- Abortion rate (number of abortions per implanted embryos)
- KID ratio (the number of known implantations (defined either as fetal heart beat or HCG positive) per known outcome)

The value of this kind of monitoring is undisputable but the monitoring is slow to respond due to a limited number of transferred embryos and an even lower amount of known implantation embryos in a specific clinic. A further problem is the time-lag due to the delay when waiting for the results of HCG test, scanning outcome and abortion information. The sensitivity is limited due to the discrete binomial outcome of the

process (pregnant/not pregnant, aborted/not aborted, implanted/not implanted), which means that a large population is needed in order to evaluate if a significant change has occurred. This induces a further delay in the quality control monitoring.

5 **Summary of invention**

10 If the quality control shall pick up on e.g. failure to comply to best practice (e.g. errors in the handling of the embryos), toxicity of lab utensils or consumables (e.g. toxic oil or media) or environmental changes (contamination of laboratory air) then the response
15 needs to be accurate, fast and sensitive. Accurate so that correcting measures are only set in place if something is really wrong, fast so that modifications can be implemented as quickly as possible and sensitive so that even small changes in the performance can be detected and corrected. Therefore, a sensitive and fast responding tool for monitoring the clinic procedures is needed.

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One embodiment of the invention therefore relates to a computer implemented method for automatically detecting variations and/or abnormalities in the developmental conditions of in vitro incubating embryos, the method comprising the steps of:

- 20 a) obtaining a first dataset comprising morphokinetic parameters relating to the development of a first group of embryos,
b) obtaining a second dataset comprising morphokinetic parameters relating to the development of a second group of embryos,
c) modifying the first and second datasets by extracting morphokinetic parameter outliers from said datasets,
25 d) calculating the difference between specific morphokinetic parameters from the modified first dataset and the corresponding morphokinetic parameters from the modified second dataset,
e) monitoring said morphokinetic difference thereby detecting variations in the developmental conditions of the first and second group of embryos.

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It is known that the morphokinetic variables are important indicators for embryo implantation success rates. In the present invention morphokinetic parameters are utilized as quality control indicators and thereby form an alternative to monitoring the pregnancy rates or related variables which are delayed by definition. The big
35 advantage of using morphokinetic parameters is that the number of embryos in a clinic

is around one magnitude higher than the number of embryos transferred and the variables are available as soon as the incubation is ended (2-5 days after fertilization). Furthermore, the number of available morphokinetic parameters is around one magnitude higher than the number of embryos, because each embryo "produces" a plurality of morphokinetic parameters during the different developmental stages. So by monitoring the morphokinetic parameters instead of the pregnancy rates both speed and accuracy may be increased. Since e.g. the timings of cleavages are continuous variables instead of discrete the sensitivity of the statistical tests that can be used to test if two groups are significantly different are greatly increased.

The present invention is most naturally applied to human embryos, but may also be applied within monitoring of any mammal embryos.

Definitions and embryo quality parameters

Cleavage time is defined as the first observed timepoint when the newly formed blastomeres are completely separated by confluent cell membranes, the cleavage time is therefore the time of completion of a blastomere cleavage. In the present context the times are expressed as hours post ICSI microinjection or post time for mixing of semen and oocyte in IVF, i.e. the time of insemination. This is the time of the deliberate introduction of sperm into the ovum. However, herein the term fertilization is also used to describe this timepoint. Thereby the cleavage times are as follows:

- t2: Time of cleavage to 2 blastomere embryo
- t3: Time of cleavage to 3 blastomere embryo
- t4: Time of cleavage to 4 blastomere embryo
- t5: Time of cleavage to 5 blastomere embryo
- t6: Time of cleavage to 6 blastomere embryo
- t7: Time of cleavage to 7 blastomere embryo
- t8: Time of cleavage to 8 blastomere embryo

Duration of cell cycles is defined as follows:

- cc1 = t2: First cell cycle.
- cc2 = t3-t2: Second cell cycle, duration of period as 2 blastomere embryo.

- $cc2b = t4 - t2$: Second cell cycle for both blastomeres, duration of period as 2 and 3 blastomere embryo.
- $cc3 = t5 - t3$: Third cell cycle, duration of period as 3 and 4 blastomere embryo.
- $cc2_3 = t5 - t2$: Second and third cell cycle, duration of period as 2, 3 and 4 blastomere embryo.
- $cc4 = t9 - t5$: Fourth cell cycle, duration of period as 5, 6, 7 and 8 blastomere embryo.

Short and long cell cycles

In an embryo with a direct cleavage, the time between one of the cell divisions appear to be inadequate for DNA replication of the whole genome. For the first three cell cycles:

- Cell cycle 1, $cc1$, which is not a standard cell cycle as it combines the process of fertilization, pronuclei formation and disappearance, etc. - until the advent of the first cell division at which time-point the maternal and paternal alleles have been combined in the embryonic genome. The combined genome has presumably been replicated in advance of the cell division from zygote to 2-cell. $cc1 = t2$.
- Cell cycle 2, $cc2$, the time from completion of the first cell division resulting in two cells to the next cell division resulting in three cells. i.e. $cc2 = t3 - t2$.
- Cell cycle 3, $cc3$, is the time required for the third round of DNA replication. When a four cell embryo divides to five cells it should have five full copies of the genome and the two newest cells in the five-cell embryo should have successfully completed their third round of DNA replication. As the fastest cell to divide is most likely the one that initially appeared after the first cleavage of the two cell embryo to three cells, the best estimate for the fastest cell cycle is: $cc3 = t5 - t3$, the time of cleavage to five cells minus the time of cleavage to three cells.

Short cell cycle embryos, SCC

In short cell cycle embryos any of the following conditions are found:

- $cc1 = t2 < 15$ hr, or
- $cc2 = t3 - t2 < 5$ hr, or

- $cc3 = t5 - t3 < 5 \text{ hr}$

Long cell cycle embryos, LCC

A similar concept, which might be equally useful is "Long cell cycle embryos". These embryos are embryos that develop very slowly with unusual long durations for their cell cycles. The long cell cycle embryos have a similar poor prognosis and are significantly less likely to implant than more normal embryos (as is shown below). However, the class of "long cell cycle embryos" is less clearly defined than the short cell cycles as the distribution at the high end is continuous with a gradually decreasing implantation potential. It is therefore less clear how to define the boundaries for this class. The boundaries for the long cell cycle embryo class has been chosen such that the LCC class correlate with a markedly reduced implantation potential similar to the one found for short cell cycle embryos. A long cell cycle class is thus defined with a comparable size to the short cell cycle class of embryos:

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Long cell cycle embryo, LCC, imply

- $cc1 = t2 > 32 \text{ hr}$, or
- $cc2 = t3 - t2 > 20 \text{ hr}$, or
- $cc3 = t5 - t3 > 25 \text{ hr}$

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Medium cell cycle embryos, MCC

All embryos that fall between the groups, i.e. embryos where:

- $cc1$ is from 15 to 32 hrs, and
- $cc2$ from 5 to 20 hrs and
- $cc3$ from 5 to 25 hrs.

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are classified as medium cell cycle embryo. These are the "normal" embryos with a normal cleavage pattern. The MCC embryos typically constitute about 60% of all embryos, which can be used to compare embryo development from clinic to clinic. E.g. the first group of embryos may be selected among MCC embryos.

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Synchronicities are defined as follows:

- $s2 = t4 - t3$: Synchrony in division from 2 blastomere embryo to 4 blastomere embryo.
- $s3 = t8 - t5$: Synchrony in division from 4 blastomere embryo to 8 blastomere embryo.

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- s3a = t6-t5; s3b = t7-t6; s3c = t8-t7: Duration of the individual cell divisions involved in the development from 4 blastomere embryo to 8 blastomere embryo.

5 Cleavage period: The period of time from the first observation of indentations in the cell membrane (indicating onset of cytoplasmic cleavage) to the cytoplasmic cell cleavage is complete so that the blastomeres are completely separated by confluent cell membranes. Also termed as duration of cytokinesis.

10 Fertilization and cleavage are the primary morphological events of an embryo, at least until the 8 blastomere stage. Cleavage time, cell cycle, synchrony of division and cleavage period are examples of morphological embryo parameters that can be defined from these primary morphological events and each of these morphological embryo parameters are defined as the duration of a time period between two morphological events, e.g. measured in hours.

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A normalized morphological embryo parameter is defined as the ratio of two morphological embryo parameters, e.g. cc2 divided by cc3 (cc2/cc3), or cc2/cc2_3 or cc3/t5 or s2/cc2.

20 The duration of a plurality of cell cycles (e.g. CC1, CC2, CC3 and CC4) can be combined to form a common normalized parameter:

$$CC_{norm} = \sqrt{\sum_{all\ i} \left(\frac{CCi - CCi_{median}}{CCi_{median}} \right)^2}$$

where CCi e.g. is selected from CC1 to CC4. In one embodiment of the invention a high value of CC_{norm} indicates a poor embryo quality as one or more of the variables CCi is far from the median, i.e. it is not the absolute values of CCi that are used, but the mutual relation of the variables. The median may be calculated based on the whole population or parts of the population (e.g. embryos with known and positive implantation). Another equivalent variable using the logarithmic value instead (lCC_{norm}) may also be useful in assessing embryo quality.

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$$lCC_{norm} = \sqrt{\sum_{all\ i} w_i \log \left(\frac{CCi - CCi_{median}}{CCi_{median}} \right)^2}$$

Likewise the synchronicity S_i of the cell divisions (e.g. S2, S3 and S4) may be combined to form a common normalized parameter:

$$S_{norm} = \sqrt{\sum_{all\ i} \left(\frac{S_i}{S_{i_{median}}} \right)^2}$$

- In one embodiment of the invention a high value of S_{norm} indicates a poor embryo quality as one or more of the synchronicities is long compared to the. Another equivalent variable using the logarithmic value instead (IS_{norm}) may also be useful in assessing embryo quality.

$$IS_{norm} = \sqrt{\sum_{all\ i} w_i \log \left(\frac{S_i}{S_{i_{median}}} \right)^2}$$

- The variables CC_{norm} and S_{norm} may be calculated based on the first, second, third or fourth cell cycle, depending on the duration of the incubation.

- In some examples a morphokinetic parameter for embryos which is used in accordance with certain embodiments of the invention may be established by combining a plurality of morphokinetic characteristics relating to the development of the respective embryos. For example, in some implementations the morphokinetic parameter may be derived by obtaining values for a plurality of morphokinetic characteristics relating to the development of an embryo during an observation period, for example characteristics such as cell cleavage times ($t_2, t_3, t_4 \dots$) and / or differences between subsequent cell cleavage times / divisions ($t_3-t_2, t_4-t_3, t_5-t_4 \dots$) and / or cycle durations ($cc1, cc2, cc3 \dots$ etc). A value for a continuous variable may then be determined by combining differences between the obtained values for the plurality of characteristics and corresponding reference values in a pre-defined manner. The reference values may, for example, be determined from values for the plurality of characteristics obtained for one or more reference embryos of known development potential (e.g. KID positive embryos). A morphokinetic parameter for an embryo which is to be used in accordance with certain embodiments of the invention may thus be established from the continuous variable. In this respect the morphokinetic parameter may correspond with the development potential for an embryo determined in accordance with the principles set

out in co-pending patent application PCT/EP2013/063240 filed 25 June 2013, the entire contents of which are incorporated herein by reference.

5 In accordance with some example implementations the step of combining differences between the obtained values for the plurality of morphokinetic characteristics and the corresponding reference values may take account of weighting values associated with each of the reference values. The weighting values may, for example, be statistically determined from values for the plurality of characteristics obtained for a plurality of reference embryos of known development potential. For example, the weighting values 10 may be determined from a variance of the relevant values obtained for the plurality of reference embryos.

Some example implementations of the present invention may further comprise obtaining values for a further plurality of characteristics relating to the development of the embryo during the observation period; determining a value for a further continuous 15 variable by combining differences between the obtained values and corresponding reference values for the further plurality of characteristics in a further pre-defined manner; and establishing the development potential for the embryo based also on the determined value for the further continuous variable.

20 In some examples a morphokinetic characteristic for embryos may be determined by combining / aggregating differences for various characteristics seen for each embryo and corresponding reference values (e.g. determined for a KID positive population of embryos) to generate what might be referred to as a generalized irregularity variable (GIV), which in some cases may be defined as:

$$GIV = \frac{1}{n} \sqrt{\sum_{\substack{\text{all } i \text{ in} \\ \text{sequence}}} \frac{(cci - cci_m)^2}{cci_v}}$$

Where cci is a series of cell cycle durations observed for an embryo, cci_m is the corresponding series of average cell cycle durations seen in a reference group of embryos (e.g. a positive KID population from patients under the age 35), and cci_v are the corresponding variance values associated with the reference population. The 30 parameter n is the number of cell cycle durations comprising the series cci. The differences (cci - cci_m) are normalized by the variance values cci_v as part of the

combining. This means that differences for particular cell cycles (values of i) which exhibit relatively high variance in the sample population contribute less to the value of GIV than differences for cell cycle which exhibit relatively low variance in the sample population. The differences ($cci - cci_m$) are squared in the combining, and this means the contribution to GIV is the same for a given difference, regardless of whether it is positive or negative (i.e. whether cci is longer or shorter than cci_m). The series cci may comprises durations for various different cell cycles in different implementations as discussed further below.

Generally speaking GIV as defined above is low when the study embryo exhibits a regular cleavage pattern and is high when the embryo exhibits an irregular cleavage pattern.

In some examples a morphokinetic characteristic for embryos may be referred to as a generalized time variable (GTV) defined as:

$$GTV = \frac{1}{k} \sum_{\substack{\text{all } j \text{ in} \\ \text{sequence}}} \frac{\Delta t_j - \Delta t_{j_m}}{\Delta t_{j_v}}$$

Where Δt_j is a series of differences in times for subsequent cell divisions observed for an embryo, Δt_{j_m} is the corresponding series of average values seen in a reference group of embryos (e.g. the positive KID population from patients under the age 35), and Δt_{j_v} are the corresponding variance values associated with the reference population. The parameter k is the number of values comprising the series Δt_j . The differences ($\Delta t_j - \Delta t_{j_m}$) are normalized by the variance values Δt_{j_v} as part of the combining. This means that differences for particular cell divisions (values of j) which exhibit relatively high variance in the sample population contribute less to the value of GTV than for those which exhibit relatively low variance in the sample population. In this example the contribution to GTV for each time difference depends on whether the difference in times for a particular pair of subsequent cell divisions is faster or slower than the average seen in the positive KID population (i.e. whether the difference is positive or negative).

The pending application PCT/DK2012/050236 filed at 29.06.2012 and entitled "Adaptive embryo selection criteria optimized through iterative customization and

collaboration" from the same applicant relates to the issue of adapting embryo quality criteria across populations of embryos cultures under different incubation conditions, e.g. in different clinics. This application is hereby incorporated by reference in its entirety. However, quality parameters like CC_{norm} , ICC_{norm} , S_{norm} and IS_{norm} may help to ensure that quality models will be directly applicable across different populations of embryos cultured under different incubation conditions, because they are based on variables that are insensitive to differences in running conditions. Another example of that is quality parameters based on relative time periods (e.g. $CC2/CC3$), variables divided with a central estimate of that variable (e.g. mean or median, e.g. $cc2/cc2_median$) or using target intervals where the center is scaled according to a central estimate and the boundaries are scaled according to a variance estimate (e.g. variance, standard deviation, percentiles).

The following discrete (binary) variables can be used

- MN2: Multi nucleation observed at the 2 blastomere stage; can take the values "True" or False".
- MN2val: the number of multinuclear cells at the 2 cell stage (0,1,2).
- MN4: Multi nucleation observed at the 4 blastomere stage; can take the values "True" or False".
- MN4val: the number of multinuclear cells at the 4 cell stage (0,1,2,3,4).
- EV2: Evenness of the blastomeres in the 2 blastomere embryo; can take the values "True" (i.e. even) or "False" (i.e. uneven).

Blastocyst related parameters

A blastocyst quality criterion is an example of an embryo quality criterion. The following blastocyst related parameters may be used:

Initial compaction (IC) describes the first time a compaction between 2 or more blastomeres is observed. Compaction is a process wherein an intensification of the contacts between the blastomeres with tight junction and desmosomes result in reduction of the intercellular space and a blurring of the cell contours (see fig. 3). Compaction can be seen at the 6-8 cell stage but rarely do the embryos cleave to 16 cell or more before compaction occurs.

Compaction/Morula (M) is defined as the first time where no plasma-membrane between any blastomeres are visible. When compaction is complete no plasma-membranes between any of the blastomeres forming the compaction are visible and the embryo can be defined as a morula. The stage is characterized by a process with an intensification of the contacts between the blastomeres with tight junction and desmosomes resulting in reduction of the intercellular space and a blurring of the cell contours. Compaction/Morula can sometimes be seen at the 6-8 cell stage during the 3rd division (synchrony) period (S3) but most often compaction/Morula is seen after S3 close to or right in the beginning of the fourth synchrony period (S4). Rarely do the embryos cleave to 16 cell or more before compaction occurs.

Initial differentiation of trophectoderm (IDT) is defined as the first time during the compaction stage where distinct trophectoderm cells are recognized. It describes the onset of differentiation of the trophectoderm cells. The blastomeres gradually become flattened and elongate creating a barrier between the outside environment and the inner cell part of the morula.

Onset of cavitation/early blastocyst/blastocyst (BI) is defined as the first time a fluid-filled cavity, the blastocoel, can be observed. It describes the initiation of the transition period between the compaction and the blastocyst stage of the embryo. Embryos often remain in this transition stage for a period of time before entering the actual blastocyst stage. Onset of cavitation usually appears immediately after differentiation of the trophectoderm cells. The outer layer of the morula with contact to the outside environment begins to actively pump salt and water into the intercellular space, as a result of which a cavity (the blastocoel) begins to form.

Initial differentiation of inner cell mass (IDCIM) defined as the first time after OC (onset of cavitation) the inner cell mass can be recognized. IDCIM describes the initiation of inner cell mass development. An eccentrically placed cluster of cell connected of gap junction where the boundaries between the cells seem not well defined.

Onset of expansion of the blastocyst (EB) is defined as the first time the embryo has filled out the perivitelline space and starts moving/expanding Zona Pelucidae. EB describes the initiation of the embryos expansion. As the blastocyst expands the zona

pellucida becomes visibly thinner.

Hatching blastocyst (HB) is defined as the first time a trophectoderm cell has escaped / penetrated the zona pellucida.

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Fully hatched blastocyst (FH) is defined as when hatching is completed with shedding zona pellucida.

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Number of Contractions (NC (X)) describes the number of contractions (X) the embryo undergoes after the onset of cavitation. In many embryos the contractions can be quite large and lead to a large reduction of the embryonic volume. A contraction is defined as a reduction in the cross sectional surface area of the embryo of more than 15%.

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Degree of vacuolization (VC (X); $X=\{0, 1, 2, 3\}$) describes the extent of vacuolization after the embryo has undergone compaction. The degree of the vacuoles is rated by a 0-3 scale (0 = no vacuolization; 1 = small degree of vacuolization where small vacuoles appear after compaction but the embryonic development does not appear to be affected; 2 = moderate degree of vacuolization where large vacuoles appear after compaction and embryonic development is affected to some extent; 3 = severe vacuolization where very large vacuoles appear after compaction and embryonic development is severely affected. In this incidence the vacuoles can be mistaken for the blastocyst cavitation.

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Compaction – de-compaction-compaction (CDC) describes a phenomenon where an onset compaction of the embryo had started but becomes disrupted of cleavage. The cell boundaries of the blastomeres become visible again but after a while the embryo returns into a compacted composition.

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Partial Compaction (PC) describes an uneven compaction where one or more of the blastomeres are not included in the compaction.

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In humans embryonic gene activation (EGA) typically occurs on day 3, around the 8-cell stage. Before EGA embryos are observed to translate only maternally inherited mRNA, i.e. that mRNA which is present in the oocyte when it is fertilized. The mRNA is localized in different parts of the oocyte, so that as the oocyte/zygote divides it is

segregated into different blastomeres. This segregation is thought to underlie much of the differentiation of cells that occurs before EGA. After EGA the embryo begins to transcribe its own DNA, cells become motile and cell division becomes asynchronous. Since the cells are now transcribing their own DNA, this stage is where differential expression of paternal genes is first observed. The transition around EGA is also referred to as midblastula or midblastula transition.

Rearrangement of cellular position = Cellular movement (see below)

Cellular movement: Movement of the center of the cell and the outer cell membrane. Internal movement of organelles within the cell is NOT cellular movement. The outer cell membrane is a dynamic structure, so the cell boundary will continually change position slightly. However, these slight fluctuations are not considered cellular movement. Cellular movement is when the center of gravity for the cell and its position with respect to other cells change as well as when cells divide. Cellular movement can be quantified by calculating the difference between two consecutive digital images of the moving cell. An example of such quantification is described in detail in the pending PCT application entitled "Determination of a change in a cell population", filed Oct 16 2006. However, other methods to determine movement of the cellular center of gravity, and/or position of the cytoplasm membrane may be envisioned e.g. by using FertiMorph software (ImageHouse Medical, Copenhagen, Denmark) to semi-automatically outline the boundary of each blastomere in consecutive optical transects through an embryo.

Other parameters

Organelle movement: Movement of internal organelles and organelle membranes within the embryo which may be visible by microscopy. Organelle movement is not Cellular movement in the context of this application.

Movement: spatial rearrangement of objects. Movements are characterized and/or quantified and/or described by many different parameters including but restricted to: extent of movement, area and/or volume involved in movement, rotation, translation vectors, orientation of movement, speed of movement, resizing, inflation/deflation etc. Different measurements of cellular or organelle movement may thus be used for different purposes some of these reflect the extent or magnitude of movement, some

the spatial distribution of moving objects, some the trajectories or volumes being afflicted by the movement.

Embryo quality is a measure of the ability of said embryo to successfully implant and develop in the uterus after transfer. Embryos of high quality have a higher probability of successfully implant and develop in the uterus after transfer than low quality embryos. However, even a high quality embryo is not a guarantee for implantation as the actual transfer and the woman's receptivity highly influences the final result.

Viability and quality are used interchangeably in this document. Embryo quality (or viability) measurement is a parameter intended to reflect the quality (or viability) of an embryo such that embryos with high values of the quality parameter have a high probability of being of high quality (or viability), and low probability of being low quality (or viability). Whereas embryos with an associated low value for the quality (or viability) parameter only have a low probability of having a high quality (or viability) and a high probability of being low quality (or viability)

Description of Drawings

Fig. 1. Nomenclature for the cleavage pattern showing cleavage times (t_2 - t_5), duration of cell cycles (cc_1 - cc_3), and synchronies (s_1 - s_3) in relation to images obtained.

Fig. 2: The development of the embryo until the blastocyst stage. The number refers to the number of blastomeres in each stage. The letters a to e refer to the following kinetic parameters. a: Compaction/Morula (M), b: Initial differentiation of trophectoderm (IDT), c: Onset of cavitation/early blastocyst/blastocyst (BI), d: Onset of expansion of the blastocyst (EB), e: Hatching Blastocyst (HB). Initial Compaction (IC) can be observed between t_5 and compaction/morula (a), if present usually IC precedes compaction/morula by minutes to a few hours. Partial compaction (PC) can be observed between stages a and c if present. Vacuolization" (VC(X)) and contractions (NC(X)) can be observed between stages a and d+ if present.

Fig. 3 shows the variation of morphokinetic parameters (in this case t_2 , t_3 and t_5) as a function of the culture medium in a fertility clinic. The total period runs from February 2011 to June 2011. Of the three media used (A, B, C) media A provided the worst

embryo development (latest cell division timing and t2, t3 and t5 are all higher for media A). Media A also provided worse implantation rates and pregnancy rates. Media B and Media C both provided normal embryo development and high implantation and pregnancy rates. Applying the present invention to surveillance of morphokinetic parameters of embryos developing in different media can reveal these problems online as they progress.

Fig. 4a. A series of images showing where the time of t2 (time of cleavage where a 2 blastomere embryo is created, i.e. the time of resolution of the cell division) is seen to happen at 22.9 hours.

Fig. 4b. A series of images showing direct cleavage to a 3 blastomere embryo. Cleavage from 1 to 3 cells happens in one frame, thus t3 = t2 and CC2 is thereby less than 5 hours, i.e. an example of an SCC embryo.

Fig. 5. Mouse embryo development with varying temperature of the incubation medium (see example 1).

Fig. 6. Duration between various cell divisions for mouse embryos for varying temperatures of the incubation medium (see example 1).

Figs. 7a-c. Direct cleavage has been analysed in a large data material containing 4,181 IVF treatments from eight IVF clinics which involve a total of 32,382 retrieved oocytes that were inseminated and placed in EmbryoScope incubators. 18,024 annotated embryos have been evaluated of which 5491 were transferred. As shown in fig. 7a short cell cycles were found in 4709 of the 18024 annotated embryos (26.1%) and long cell cycles in 2464 of 18024 embryos (13.7%). The remaining 10851 embryos (60.2%) were MCC. The abundance of both SCC and LCC was reduced among the transferred embryos: SCC 14.1% (776 of 5491) and 9.2% (503/5491) as shown in fig. 7b. The remaining 76.7% was "normally" dividing MCC embryos. The KID ratio is shown in fig. 7c. The two aberrant classes SCC and LCC display a significantly reduced KID ratio. The relative implantation ratio was reduced to $8.3\%/17.7\% = 47\%$ of the MCC rate for SCC embryos and $7.2\%/17.7\% = 41\%$ of the MCC rate for LCC embryos. In one embodiment of the invention SCC and/or LCC embryos are therefore defined as embryos with morphokinetic parameter outliers. Further, variation in the relative

amount of SCC and/or LCC embryos may be an indicator of change in developmental conditions and by monitoring this variation early warnings may be provided.

5 Figs. 8 and 9 show the variation in the average of the morphokinetic parameters t2, t3, t4, t5, t8, s2, cc3 and t8-t5 for embryos cultures in different lots of oil. The differences are clearly seen and especially oil lot "L1" is significantly different, possibly due to a small amount of a toxic substance that leads to markedly lower embryo quality. The embryos cultured in L1 are seen to develop more slowly and with a longer cell cycle (cc3). For the cleavage times the difference is more pronounced for the latest
10 morphokinetic parameters t5 and t8. Thus, monitoring the morphokinetic parameters is a sensitive and fast responding tool to survey the quality of incubating embryos.

Figs. 10-14. Experiments were conducted with controlled amounts of TX-100 (Triton X-100) in culture media for embryos from hybrid mice. The leftmost columns are the
15 control group with no TX-100 and the five other groups have increasing amounts of TX-100, up to 0.002%. Fig. 11 shows the blastocyst rate at 96 hours, i.e. the rate of embryos having reached the blastocyst stage at 96 hours. Only group 5 with most TX-100 is significantly different. Fig. 12 shows the variation of t2, t3, t4, t5 and t8 across the groups and a trend towards a slower development with increasing amounts of TX-
20 100 is seen with the most pronounce differences at the later cleavage times. Fig. 13 shows the variation of s2, cc3 and t8-t5 across the groups with no clear trends other than most groups have higher values than the control group. Fig. 14 shows the variation for the timings of different blastocyst stages and a clear trend towards a slower development with increasing amounts of TX-100 is seen.

25

Detailed description of the invention

A way to identify a viable embryo in a cohort of embryos from an IVF treatment would
30 be to compare the recorded temporal pattern of cell division, represented by the morphokinetic parameters, to the recorded temporal patterns of cell division from embryos in past treatment cycles. A viable embryo would be characterized by having morphokinetic parameters that match the recorded morphokinetic parameters from embryos that implanted and resulted in a live birth in the past. In selecting the embryo
35 for transfer that display morphokinetic parameters resembling those of positive

embryos (i.e. embryos from ongoing or successfully completed pregnancies) and differ where possible from the majority of negative embryos (i.e. those embryos that failed to implant or gave rise to clinical abortions) it would be possible to improve the likelihood of obtaining a pregnancy and to achieve the desired outcome of the fertility treatment.

5 The present invention turns this known principle upside down: By monitoring the morphokinetic parameters undesirable differences or trends may be detected much sooner and this may give rise to early warnings pointing to e.g. unintended differences in embryo handling. On the other hand morphokinetic parameter surveillance may also be used to alleviate fears after multiple implantation failures, because morphokinetic
10 parameter analysis can show that embryo development is indeed normal.

Several factors have been shown to effect embryo development, e.g. the timing of cell divisions. The factors that have been shown to influence embryo development, and consequently the derived morphokinetic parameters, include: Temperature, media
15 composition, pH, CO₂ and oxygen, growth factors, cultivation vessel etc. Other factors such as patient age, etiology, BMI, stimulation protocol (agonist/antagonist, type of hormone rFSH/hMG), embryo handling (pipettes, fertilization method, assisted hatching, removal of blastomeres, polar bodies or trophectoderm cells by biopsy) and even experience and competence of the person handling the embryos, have been
20 proposed by various scientists to influence embryo development and in particular the timing of cellular events such as cell cleavage.

Thus, one embodiment of the invention applies within quality control in a fertility clinic by comparing average cleavage patterns of embryos in recent treatment cycles with
25 cleavage patterns from past cycles. Temporal changes in general morphokinetic parameters for good quality embryos may indicate an unintended change in protocol, such as bad lot of media, problems with incubators, pipette tips, etc.

For continuous monitoring the steps of the claimed method can be repeated and the
30 second dataset can thus be continuously updated with the most recent embryo data, such as the most recent embryo data selected from a specific time period or selected from a predefined number of the most recent embryos, such as the most recent embryos from a predefined time period, such as number of hours, days, weeks or months. Said predefined number of embryos or predefined time period may
35 advantageously be determined as a user input. The developmental conditions can

thereby be continuously surveyed.

5 The method is computer implemented and thus the functionality of issuing a warning when the morphokinetic difference is above a predefined level may advantageously be implemented. The type of the warning may be dependent of the severity of the detected difference, e.g. green light for normal, yellow light for possible problems and red light for a serious quality issue.

10 The first group of embryos will typically be a reference group or control group and the second group of embryos may be the group of embryos that are monitored and compared to the control group. The detected morphokinetic difference between the groups of embryos can be determined by standard statistical methods known in the art, e.g. the predefined level for issuing a warning may be determined as a predefined level above the standard deviation of one or more of the morphokinetic parameters from the first dataset.

15 Morphokinetic parameter outliers may be defined as relative outliers, i.e. the outermost 3% of the population. However, it may be an advantage to define morphokinetic parameter outliers as absolute outliers, i.e. a morphokinetic parameter is defined as an outlier if it outside a predefined absolute number. This is the case for SCC embryos and LCC embryos, as defined herein, that are examples of absolute outliers. The lower limit of 5 hours for the SCC embryos comes from the fact that 5 hours is not enough time for DNA replication of the whole genome.

25 The morphokinetic parameter outliers may be excluded from the first datasets and/or from the second dataset. Monitoring the variation in the morphokinetic parameters may be a good indicator for quality issues as many of the morphokinetic parameters are quality parameters for the embryo quality. However, it may be an advantage to monitor the number or frequency or distribution of morphokinetic outliers, in particular in the second dataset, as any variation in the outliers may be an indicator of quality issues.

30 Only the specific morphokinetic parameters that are outliers may be excluded from the dataset. However, it may be an advantage to exclude all data from specific embryos that have one or more morphokinetic outlier in their development.

35

It may be that the second group of embryos is a subset of the first group of embryos such that the second dataset is a subset of the first dataset.

The method according to any of the preceding claims, wherein the morphokinetic parameters are selected from the group of:

- 5 the time of cleavage to a n blastomere embryo, tn , where $n = \{1, \dots, 8\}$,
duration of cell cycles $cc1$, $cc2$, $cc2b$, $cc3$, $cc2_3$ and $cc4$,
synchronicities $s2$, $s3$, $s3a$, $s3b$ and $s3c$,
number of blastomeres at specific predefined timepoints,
10 timing, extent or duration of cellular and/or organelle movement,
any combination of these morphokinetic parameters.

The following may be selected as a morphokinetic parameter:

$$CC_{norm} = \frac{1}{n} \sqrt{\sum_{all i} \left(\frac{CCi - CCi_m}{CCi_v} \right)^2}$$

- 15 wherein $i = \{1, 2, 3, 4\}$, cc_i_m is the mean value or median value of cc_i and cc_i_v is the variance of cc_i .

- In one embodiment of the invention the first and/or second group of embryos are embryos that has been fertilized, preserved and/or incubated under a specific set of conditions. The first group of embryos may have been fertilized under a different set of conditions than the second group of embryos. These conditions may be selected from the group of: type of fertilization treatment, preservation such as cryopreservation, incubating temperature, type of media, specific incubator, specific treatment such as hormonal treatment, hormonal treatment, aspiration, male factor, specific incubator, incubation temperature, type of media, type of oil, PGD Treatment, transfer,
20 cryopreservation, thawing, time outside incubator, number of fertilization treatments.

- In one embodiment of the invention the second group of embryos are embryos that originate from predefined embryo donors. The predefined embryo donors may be selected from the group of: individuals younger or older than a predefined age, embryo donors in a specific treatment or stimulation protocol, embryo donors in a specific fertilization treatment, embryo donors with a specific diagnosis such as hereditary chromosomal diseases, Hiv, Hep, PCO's, individuals with current or past exposure to radiation or hazardous chemical substances, individuals with current or past drug use,
30

individuals with a BMI higher or lower than a predefined level, smokers, non-smokers, individuals with normal or abnormal menstrual cycle.

5 The second group of embryos may be selected as embryos from specific patient groups (e.g. younger patients), patients with specific diagnosis (e.g. endometriosis), specific fertilization treatments (e.g. ICSI), embryos that has received special pre-treatment (e.g. cryopreserved).

10 A specific problem arises if the clinic occasionally has patients with very deviating cleavage patterns. Then the number of these patients may strongly influence the average of the timings. In this case monitoring of the above mentioned variables on selected groups of embryos may be a solution.

15 The lengths of the cell cycles cci ($cc1$, $cc2$, $cc3$ and $cc4$) are all important variables in determining the quality of the embryo. Combinations of the morphokinetic parameters may be used to construct a new morphokinetic parameter that is resilient to the rarely occurring extremes in timing of the specific cleavage events

$$CC_{norm} = \frac{1}{n} \sqrt{\sum_{all\ i} \left(\frac{CCi - CCi_m}{CCi_v} \right)^2}$$

Where the CCi_m is the median (average) value of cci and cci_v is the variance of cci . These can e.g. be calculated on the group of KID positive embryos, from all transferred embryos or from all embryos. If a maximum limit x is set for CC_{norm} (e.g. 3) it means
20 that if the CCi 's from the second group of embryos on average fall more than x standard deviations (of the KID positive, transferred or all) from the average value of the first group of embryos (e.g. KID positive, transferred or all) that data will be excluded.

25

To monitor that best laboratory practice is being observed it will be possible to isolate embryos that have undergone a specific treatment to monitor the variables for these embryos to see if there is a change over time, i.e. the second group of embryos is selected among embryos with this specific treatment. Also groups of embryos that have
30 been handled by a specific person of the laboratory or clinic staff can be selected as the second group of embryos to evaluate if changes has occurred over time or if there is a general difference between staff performance.

In a further embodiment of the invention the first group of embryos comprise preimplantation data from implanted embryos that have resulted in ongoing pregnancies, live born babies, fetal heart beat (FHB), and/or gestational sacs. I.e. the first group is selected to reflect high quality embryos with proven track record.

5

In yet another embodiment of an embryo dataset (e.g. a first or second embryo dataset) comprise morphokinetic parameters for

- 1) all embryos in a group of monitored embryos, or
- 2) a functionally defined subgroup from the group of embryos.

10

I.e. all embryos in group of monitored embryos (i.e. all embryos ever monitored in a certain clinic) can be selected as the frame of reference for the statistical calculations.

Or just a subgroup is selected where this subgroup is functionally defined. Examples of functionally defined subgroups:

- all fertilized embryos in the group,
- 15 - embryos that have divided to at least a predefined number of cells at a predefined number of hours after insemination, such as divided to at least 7 cells 68 hours after insemination,
- embryos that have less than a predefined percentage of fragmentation at a predefined hours after insemination, e.g. less than 20% fragmentation 68 hours
- 20 after insemination,
- embryos that are not multinucleated at a certain cell stage, e.g. at the four cell stage,
- embryos that have been classified as "Good quality embryos" (GQE) by a qualified embryologist,
- 25 - embryos that have been chosen for freeze or transfer,
- embryos that have been chosen for transfer, and/or
- embryos that have implanted.
- Embryos selected by excluding poorly developing embryos, e.g. by excluding Scc and/or Lcc embryos or by employing other exclusion criteria as e.g. described in
- 30 pending applications PCT/DK2012/05018 or EP 12174432.0, the latter filed at 29.06.2012 and entitled "Embryo quality assessment based on blastocyst development".

In a further embodiment of the invention the morphokinetic parameters are selected from the group of:

35

- the timing and/or duration cell-division periods and inter-division periods,
- the timing and/or duration of: cleavage times, cleavage periods and cell cycle times;
- the timing and/or duration of divisional stages and quiet stages,
- 5 – synchrony of cell divisions;
- timing, extent or duration of cellular and/or organelle movement,
- timing, extent or duration of quality criteria, such as quality criteria as described in PCT/DK2012/05018
- Blastocyst quality criteria as described in EP 12174432.0

10

In a further embodiment of the invention the morphokinetic parameters are selected from the group of:

- the timing and/or duration cell-division periods and inter-division periods, determined for the first, second, third, fourth, fifth and/or sixth cell division;
- 15 – the timing and/or duration of: cleavage times, cleavage periods and cell cycle times determined for the first, second, third, fourth, fifth and/or sixth cell division;
- the timing and/or duration of divisional stages and quiet stages determined for the first, second, third, fourth, fifth and/or sixth cell division;
- synchrony of the second and third cell division;
- 20 – timing, extent or duration of cellular and/or organelle movement determined for the first, second, third, fourth, fifth and/or sixth cell division;
- timing, extent or duration of cellular and/or organelle movement determined in between the first, second, third, fourth, fifth and/or sixth cell division;

25

In a further embodiment of the invention said second group of embryos dataset is substantially smaller than the first group of embryos, such as 2 times smaller, such as 5 times smaller, such as 10 times smaller, such as 50 times smaller, such as 100 times smaller, such as 200 times smaller, such as 500 times smaller, such as 1000 times smaller.

30

In a further embodiment of the invention the embryos are cultured and/or monitored in an incubator. Preferably the embryos are monitored through image acquisition, e.g. by means of time-lapse microscopy equipment, such as image acquisition at least once per hour, preferably image acquisition at least once per half hour such as image acquisition at least once per twenty minutes, such as image acquisition at least once

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per fifteen minutes, such as image acquisition at least once per ten minutes, such as image acquisition at least once per five minutes, such as image acquisition at least once per two minutes, such as image acquisition at least once per minute.

- 5 The method according to the invention may be computer implemented or at least partly computer implemented thereby providing an efficient customizable tool for fertility clinics. I.e. the method according to the invention may be implemented in automated incubator systems for culturing and monitoring embryos, such as human embryos. By implementing the present invention in such automated incubator systems, the selection
- 10 processes and the quality control of e.g. culture media and other culturing conditions, may be more or less automated, i.e. fully manual with the software assisting the users with proposed decisions, semi-automatic or fully automatic with the incubator system making all the decisions, including early warnings, based on data analysis.
- 15 In a further aspect the invention relates to a system having means for carrying out the methods described above. Said system may be any suitable system, such as a computer comprising computer code portions constituting means for executing the methods as described above.
- 20 The system may further comprise means for acquiring images of the embryo at different time intervals, such as the system described in WO 2007/042044.

In a yet further aspect the invention relates to a data carrier comprising computer code portions constituting means for executing the methods as described above.

25

Late stage monitoring

- The search for prognostic factors that predict embryo development and the outcome of in vitro fertilization (IVF) treatment have attracted considerable research attention as it
- 30 is anticipated that knowledge of such factors may improve future IVF treatments. One promising predictive factor is the precise timing of key events in early embryo development. Studies that involve imaging have been limited to measurements of early development, such as pronuclear formation and fusion, and time to first cleavage (Nagy, Z.P. 1994, Fenwick, J. 2002, Lundin, K. 2001, Lemmen, J.G. 2008). An
- 35 important finding of the time-lapse analysis is a correlation between the early cleavage

pattern to the 4-cell stage and subsequent development to the blastocyst stage. Morphokinetic analysis on the development of bovine embryos have also been published, where timing, duration and intervals between cell cleavages in early embryo development successfully predicted subsequent development to the expanded
5 blastocyst stage (Ramsing 2006, Ramsing 2007).

The present inventors have performed a large clinical study involving many human embryos and monitoring the development, not only until formation of a blastocyst, but further until sign of implantation of the embryo. In this study important differences in the
10 temporal patterns of development between the embryos that implanted (i.e. embryos that were transferred and subsequently led to successful implantation) and those that did not (i.e. embryos that were transferred but did not lead to successful implantation) were observed. By using implantation as the endpoint, not only embryo competence for blastocyst formation, but also subsequent highly essential processes such as hatching
15 and successful implantation in the uterus is assessed.

It has been found that there exists an optimal time range for parameters characterizing the embryonic cell divisions. The observations support the hypothesis that the viability of embryos is associated with a highly regulated sequence of cellular events that begin
20 at the time of fertilization. In this clinical study on exclusively good quality embryos, it has been confirmed that an embryo's capability to implant is correlated with numerous different cellular events. The complexity, structure and parameters in the models must be adaptable to different clinical situations like incubation temperature, transfer times, culture media and other.

25 Timing of early events in embryonic development correlates with development into a blastocyst, and the development into a blastocyst is a necessity for a successful implantation and thus the formation of a blastocyst is a quality parameter in itself. However it has been found that the development into a blastocyst does not necessarily
30 correlate with successful implantation of the embryo.

Supporters of early implantation at day 2 have argued that the extended culture of embryos to the blastocyst stage at around day 5 give rise to potential risks because the culture period is significantly prolonged which may disrupt embryo integrity. However,
35 an extended culture period to the blastocyst stage has several advantages. Cultured

human embryos have an average blastocyst formation rate of only approx. 30-50%, and by extending the culture period a large part of the low quality embryos have automatically been excluded, by not forming the blastocyst. Furthermore, after EGA at around the 5-8 blastomere stage the embryos own DNA controls the development. By
5 evaluating the embryos at the blastocyst stage high quality embryos can be identified with a higher degree of certainty.

Thus, the data allows the detection of blastocyst related developmental criteria for implantation potential. The results in particular indicate that timing of late events, such
10 as the onset of cavitation, are a consistently good indicator of implantation potential, and that the discrimination between implanting and non-implanting embryos is improved when using blastocyst quality criteria, e.g. tBI as opposed to the earlier events (t2, t3 and t4). Data indicate that incubating the embryos to the blastocyst stage can give additional important information that will improve the ability to select a viable
15 embryo with high implantation potential. Furthermore, monitoring the embryos at the later stages up to the blastocyst stages may give better indication of quality issues and as shown in figs. 10-14 the implications of e.g. a toxic oil are more pronounced at the later stages. It may therefore be an advantage to include blastocyst quality criteria in the herein selected morphokinetic parameters, i.e. selected for quality surveillance.

20 The following list a number of embryo quality (selection) criteria and blastocyst quality criteria that may be applied singly or combined in groups to assess embryo quality and consequently assess quality issues when monitoring and automatically surveying developmental conditions.

25 *Multiple variables*

Multiple variables may be used when choosing selection criteria. When using multiple variables it can be an advantage that the variables are selected progressively such that initially one or more of the variables that can be determined early with a high accuracy
30 are chosen, e.g. t2, t3, t4 or t5. Later other variables that can be more difficult to determine and is associated with a higher uncertainty can be used.

Normalized or relative parameters

It is unlikely that selection criteria derived from morphokinetic parameters would be
35 universally applicable as the developmental conditions may vary from clinic to clinic. It

may therefore be an advantage to define normalized morphokinetic parameters, e.g. normalized morphological parameters based on two, three, four, five or more parameters selected from the group of t2, t3, t4, t5, t6, t7 and t8. By normalizing the parameters the time of fertilization may be "removed" from the embryo quality assessment. Further, a normalized morphological embryo parameter may better describe the uniformity and/or regularity of the developmental rate of a specific embryo independent of the environmental conditions, because instead of comparing to "globally" determined absolute time intervals that may depend on the local environmental conditions, the use of normalized parameters ensure that specific ratios of time intervals can be compared to "globally" determined normalized parameters, thereby providing additional information of the embryo development. Further, the normalized morphokinetic parameters may provide additional regarding quality issues in the embryo development.

Thus, a blastocyst quality criterion may be to determine 1) the duration of a first time period from fertilization until translation of maternally inherited mRNA in the blastomeres is completed and 2) the duration of a second time period from initiation of transcription of the blastomeres own DNA to said blastocyst stage are determined, and wherein a blastocyst quality criterion is the ratio of said first and second time periods.

Thus, a blastocyst quality criterion may be to determine 1) the duration of a first time period from fertilization to a 5 blastomere embryo and 2) the duration of a second time period from the 5 blastomere embryo to said blastocyst stage are determined, and wherein a blastocyst quality criterion is the ratio of said first and second time periods.

The time from fertilization to the blastocyst stage is thereby divided into two time periods and the ratio between these time periods is a blastocyst quality criterion. The reason for dividing at the 5 blastomere stage is that this is approx. the time of embryonic gene activation. Thus, determine 1) the duration of a first time period from fertilization until translation of maternally inherited mRNA in the blastomeres is completed and 2) the duration of a second time period from initiation of transcription of the blastomeres own DNA to said blastocyst stage are determined, and wherein a blastocyst quality criterion is the ratio of said first and second time periods.

A corresponding blastocyst quality criterion can be provided by determining 1) the duration of a first time period from fertilization to blastocyst, and 2) the duration of a second time period from initiation of transcription of the blastomeres own DNA to said blastocyst stage and taking the ratio of these time periods. This ratio provides
 5 information on how much of the total time period from fertilization to blastocyst the embryo's own DNA is in control.

These ratios can be seen as measures of the relative development speed in a certain period relative to the overall development speed until that stage. Any variation in these
 10 ratios may indicate that the embryos are accelerating or decelerating in their development and this may be an indicator of quality issues in the developmental conditions.

The abovementioned blastocyst stage may be selected from the group of: initial
 15 compaction (IC), compaction/morula (M), initial differentiation of trophectoderm cells (IDT), early blastocyst (ERB), onset of cavitation/blastocyst (BI), expansion of blastocyst (EB), first contraction (CPS(1)), second contraction (CPS(2)), third contraction (CPS(3)), fourth contraction (CPS(4)), fifth contraction (CPS(5)), sixth contraction (CPS(6)), seventh contraction (CPS(7)), hatching (HB), and fully hatched
 20 (FH). Thus, t_{IC} is the time from fertilization to initial compaction, t_M is the time from fertilization to compaction/morula, etc.

Examples of embryo quality criteria

A blastocyst quality criterion is determination of $t_{B_i} - t_i$ wherein t_{B_i} is selected from the
 25 group of $\{t_M, t_{BI}, t_{EB}\}$ and t_i is selected from the group of $\{t_5, t_6, t_7 \text{ and } t_8\}$.

A blastocyst quality criterion is determination of $(t_{B_i} - t_i) / t_i$ wherein t_{B_i} is selected from the group of $\{t_M, t_{BI}, t_{EB}\}$ and t_i is selected from the group of $\{t_5, t_6, t_7 \text{ and } t_8\}$.

30 A blastocyst quality criterion is determination of $(t_{B_i} - t_i) / t_{B_i}$ wherein t_{B_i} is selected from the group of $\{t_M, t_{BI}, t_{EB}\}$ and t_i is selected from the group of $\{t_5, t_6, t_7 \text{ and } t_8\}$.

A blastocyst quality criterion is determination of the time from fertilization to a blastocyst stage.

35

A blastocyst quality criterion is determination of one or more of the following morphological blastocyst parameters:

tIC = time from fertilization to initial compaction,

tM = time from fertilization to compaction/morula,

5 tIDT = time from fertilization to initial differentiation of trophectoderm cells,

tERB = time from fertilization to early blastocyst,

tBI = time from fertilization to onset of cavitation,

tEB = time from fertilization to expansion of blastocyst,

tCPS(1) = time from fertilization to first contraction,

10 tCPS(2) = time from fertilization to second contraction,

tCPS(3) = time from fertilization to third contraction,

tHB = time from fertilization to hatching, and

tFH = time from fertilization to fully hatched.

15 A blastocyst quality criterion is determination of one or more of the following morphological blastocyst parameters:

tBI – tM,

tEB – tB,

tHE – tEB,

20 tEB - tM

tBI – tCPS(1),

tBI – tCPS(2),

tEB – tCPS(1),

tEB – tCPS(2),

25 tCPS(2) - tCPS(1),

tCPS(3) - tCPS(2).

A blastocyst quality criterion is determination of the absolute or relative 2D and/or 3D expansion of the blastocyst.

30

A blastocyst quality criterion is determination of the diameter and/or the volume of the embryo at the onset of expansion.

A blastocyst quality criterion is determination of the maximum diameter and/or the maximum volume of the blastocyst before hatching.

The normalized morphological embryo parameter may be selected from the group of

- 5 $cc2/cc2_3 = (t3-t2)/(t5-t2),$
 $cc3/cc2_3 = (t5-t3)/(t5-t2),$
 $cc3/t5 = 1 - t3/t5,$
 $s2/cc2 = (t4-t3)/(t3-t2),$
 $s3/cc3 = (t8-t5)/(t5-t3),$ and
 10 $cc2/cc3 = (t3-t2)/(t5-t3).$

A quality criterion is determination of the extent of irregularity of the timing of cell divisions when the embryo develops from 4 to 8 blastomeres.

- 15 A quality criterion is determination of the maximum cleavage time for each blastomere when the embryo develops from 4 to 8 blastomeres.

- 20 A quality criterion is determination of the ratio between the maximum cleavage time for each blastomere when the embryo develops from 4 to 8 blastomeres and the duration of the total time period from 4 to 8 blastomeres; $\max(s3a,s3b,s3c)/s3.$

Combination with measurements of movement

- 25 The quality criteria discussed above may also be combined with determinations of movement of the embryo, such as i) determining the extent and/or spatial distribution of cellular or organelle movement during the cell cleavage period; and/or ii) determining the extent and/or spatial distribution of cellular or organelle movement during the inter-cleavage period thereby obtaining an embryo quality measure.

- 30 Volumes within the zona pellucida that are devoid of movement (or similarly areas in a projected 2D image of the embryo that remain stationary) are an indication of "dead" zones within the embryo. The more and larger these immotile "dead" zones the lower the probability of successful embryo development. Large areas within a time-lapse series of embryo images without any type of movement (i.e. neither cellular nor organelle movement) indicates low viability. Organelle movement should generally be
 35 detectable in the entire embryo even when only comparing two or a few consecutive

frames. Cellular movement may be more localized especially in the later phases of embryo development.

5 The cell positions are usually relatively stationary between cell cleavages (i.e. little cellular movement), except for a short time interval around each cell cleavage, where the cleavage of one cell into two leads to brief but considerable rearrangement of the dividing cells as well as the surrounding cells (i.e. pronounced cellular movement). The lesser movement between cleavages is preferred. Thus, any unexpected variation in cellular or organelle movement may be an indicator of quality issues in relation to the developmental conditions.

In relation to movement during cleavage and inter-cleavage periods we also refer to PCT application WO 2007/144001.

15 Further examples of morphokinetic parameters

As noted above, morphokinetic parameters to be used in accordance with certain embodiments of the invention may be established by combining a plurality of morphokinetic characteristics relating to the development of embryos. For example, in some implementations a morphokinetic parameter may be derived by obtaining values for a plurality of morphokinetic characteristics relating to the development of an embryo during an observation period, for example characteristics relating to any of cell cleavage times, time differences between pairs of cell divisions, and cell cycle durations. A value for a continuous variable may be determined by combining differences between the obtained values and corresponding reference values. The reference values may, for example, be determined from values for the plurality of characteristics obtained for one or more reference embryos of known development potential (e.g. KID positive embryos). A morphokinetic parameter may then be based on the determined value for the continuous variable.

30 Thus a method for determining a morphokinetic parameter for an embryo (referred to here as a study embryo) by generating a value for a continuous variable from a plurality of characteristics relating to the development of the embryo in accordance with an implementation of an embodiment of the invention may comprise various steps as follows:

In a first step S1 a plurality of characteristics relating to the development of the study embryo during an observation period are obtained. These characteristics may fundamentally be based on cleavage times determined using conventional time-lapse embryonic imaging. One or more characteristics may be based on the timing of pronuclei fading / disappearance (tPNfading (or tPNf)).

In one example the characteristics may comprise a series of cell cycle durations cci for a sequence of cell cycles. For example, the plurality of characteristics may comprise a series of values: cc2a (= t3 - t2); cc2b (= t4 - t2); cc3a (= t5 - t3), cc3b (= t6 - t3), cc3c (= t7 - t4), cc3d (= t8 - t4). That is to say, for this example the sequence comprises durations for all cell cycles from cc2a to cc3d (i.e. all cell cycle durations up to an eight-blastomere embryo except for cc1). If there are particular cell cycle durations that are not measured for a given embryo (e.g. because the timing of a relevant cleavage event cannot be properly determined because of inadequate measurement or because the cleavage event has not occurred by the end of the incubation time (tEnd)), the missing cell cycle(s) may be left out of the sequence.

In a second step S2 average and variance values seen in a population of one or more reference embryos of known development potential (e.g. KID positive embryos) for characteristics corresponding to those obtained for the study embryo in step S1 are obtained. These may, for example, be read from a memory or other storage of an apparatus executing the method. The average and variance values may be obtained through retrospective analysis of images of embryos that proceeded to successful implantation. The embryos for which the average and variance values are obtained for a given study embryo may be referred to as reference embryos. The reference embryos may in some cases comprise embryos that have been expected at the same clinic as the study embryo, for example to help take account of inter-clinic variations associated with different incubation conditions. That is to say, step S2 may also comprise selecting an appropriate grouping of reference embryos for which to obtain the average and variance values based on characteristics of the study embryo. The average and variance values may be determined in accordance with conventional statistical analysis techniques, for example potentially involving the discarding of outlier data, and so forth. It will be appreciated the term "average" is used broadly herein to refer to a typical / representative / indicative value for a parameter seen in a sample

population. In this regard, the average may, for example, correspond to a mean, mode or median value of the relevant characteristic for the reference population (positive KID population).

- 5 In a third step S3 a difference between the value of each characteristic seen for the study embryo and the corresponding average characteristic associated with the population of known development potential (e.g. KID positive) embryos is determined.

- 10 In a fourth step S4 a morphokinetic parameter for the study embryo (corresponding to a continuous variable) is determined by combining / aggregating the differences determined for each characteristic in a way which is weighted by the respective variance values. Thus in one specific example, a morphokinetic parameter (GIV) is defined as:

$$GIV = \frac{1}{n} \sqrt{\sum_{\substack{\text{all } i \text{ in} \\ \text{sequence}}} \frac{(cci - cci_m)^2}{cci_v}}$$

15

- Where cci is the series of cell cycle durations observed for the study embryo, cci_m is the corresponding series of average cell cycle durations seen in a reference group of embryos (e.g. the positive KID population from patients under the age 35), and cci_v are the corresponding variance values associated with the reference population. The parameter n is the number of cell cycle durations comprising the series cci. The differences $(cci - cci_m)$ are normalized by the variance values cci_v as part of the combining. This means that differences for particular cell cycles (values of i) which exhibit relatively high variance in the sample population contribute less to the value of GIV than differences for cell cycle which exhibit relatively low variance in the sample population. The differences $(cci - cci_m)$ are squared in the combining, and this means the contribution to GIV is the same for a given difference, regardless of whether it is positive or negative (i.e. whether cci is longer or shorter than cci_m).
- 20
- 25

- Generally speaking GIV is low when the study embryo exhibits a regular cleavage pattern and is high when the embryo exhibits an irregular cleavage pattern.
- 30

The particular morphokinetic parameter based on the particular sequence of cell cycle durations $cci = cc2a; cc2b; cc3a; cc3b; cc3c$ and $cc3d$ in this example may be referred to herein as a first generalized irregularity variable GIV1.

- 5 Thus, steps S1 to S4 discussed above represents a process for establishing a morphokinetic parameter for an embryo in accordance with an embodiment of the invention. It will be appreciated that similar methods may be used to establish a morphokinetic parameter for an embryo using different characteristics relating to the development of the study embryo and / or by combining the characteristics in a
10 different way to generate a different morphokinetic parameter.

For example, while the first generalized irregularity variable GIV1 as described above is based on durations of cell cycles $cc2a, cc2b, cc3a, cc3b, cc3c$ and $cc3d$ (or at least the durations of the ones which are measured / not missing), other generalized irregularity
15 variables may be based on durations of other cell cycles. For example, the following variations may be defined to provide different morphokinetic parameters for use in accordance with embodiments of the invention:

GIV2 (second generalized irregularity variable): similar to GIV1, but also including $cc1$,
20 i.e. GIV2 may be calculated in a similar manner to GIV1, but based on $cci = cc1, cc2a, cc2b, cc3a, cc3b, cc3c$ and $cc3d$.

GIV3 (third generalized irregularity variable): only including the second-generation cell cycles ($cc2a$ and $cc2b$), i.e. GIV3 may be calculated in a similar manner to GIV1, but
25 based on $cci = cc2a$ and $cc2b$.

GIV4 (fourth generalized irregularity variable): only including the shortest cell cycle for the second- and third generations ($cc2a$ and $cc3a$), i.e. GIV4 may be calculated in a similar manner to GIV1, but based on $cci = cc2a$ and $cc3a$.
30

Whereas the above example generalized irregularity variables are based on cell cycle durations defined with respect to cell cleavage times, it will be appreciated that other generalized irregularity variables may be based on other timings (and / or durations between timings) which are associated with other embryonic developmental events.
35 For example, a generalized irregularity variable used as a morphokinetic parameter in

accordance with some embodiments of the invention may be established using timings defined relative to the time of pronuclei fading (tPNf). One example, which may be referred to as GIV5, can be defined as follows:

- 5 GIV5 (fifth generalized irregularity variable): comprising (t3 - tPNf) and cc3a.

In each case, if any of the characteristics comprising the generalized irregularity variable are missing for an embryo (e.g. because they were not properly measured or had not occurred before the end of incubation time), the corresponding characteristic
10 may be left out of the calculation of the continuous variable (morphokinetic parameter), with the value of n being correspondingly reduced.

Other morphokinetic parameters may be determined from characteristics relating to the development of the study embryo other than cell cycle durations.

15

For example, a variation of the method discussed above with reference to steps S1 to S4 may comprise the following steps:

In a first step T1 the characteristics relating to the development of the study embryo
20 may instead comprise a series of time differences Δt_j between subsequent cell divisions (or morphological stages). For example, the plurality of characteristics may comprise a series of values: $\Delta t_1 (= t_2)$; $\Delta t_2 (= t_3 - t_2)$; $\Delta t_3 (= t_4 - t_3)$; $\Delta t_4 (= t_5 - t_4)$, $\Delta t_5 (= t_6 - t_5)$, $\Delta t_6 (= t_7 - t_6)$, $\Delta t_7 (= t_8 - t_7)$ - i.e. the time differences between subsequent cell divisions up to the eight blastomeres stage. That is to say, for this example the
25 sequence comprises all times between subsequent cell divisions from a single cell to an eight-blastomere embryo, or at least all of these times that are deemed to be properly measured (i.e. not missing).

In a second step T2 average and variance values seen in a population of one or more
30 reference embryos of known development potential (e.g. KID positive embryos) for the characteristics obtained for the study embryo in step T1 are obtained.

In a third step T3 a difference between the value of each characteristic seen for the study embryo and the corresponding average characteristic associated with the
35 population of known development potential (e.g. KID positive) embryos is determined.

In a fourth step T4 a morphokinetic parameter for the study embryo (corresponding to a continuous variable) is determined by combining / aggregating the differences determined for each characteristic in a way which is weighted by the respective variance values. Thus in one specific example, a morphokinetic parameter (GTV) is defined as:

$$GTV = \frac{1}{k} \sum_{\substack{\text{all } j \text{ in} \\ \text{sequence}}} \frac{\Delta t_j - \Delta t_{j_m}}{\Delta t_{j_v}}$$

Where Δt_j is the series of differences in times for subsequent cell divisions observed for the study embryo, Δt_{j_m} is the corresponding series of average values seen in a reference group of embryos (e.g. the positive KID population from patients under the age 35), and Δt_{j_v} are the corresponding variance values associated with the reference population. The parameter k is the number of values comprising the series Δt_j . The differences $(\Delta t_j - \Delta t_{j_m})$ are normalized by the variance values Δt_{j_v} as part of the combining. This means that differences for particular cell divisions (values of j) which exhibit relatively high variance in the sample population contribute less to the value of GTV than for those which exhibit relatively low variance in the sample population. In this example the contribution to GTV for each time difference depends on whether the difference in times for a particular pair of subsequent cell divisions is faster or slower than the average seen in the positive KID population (i.e. whether the difference is positive or negative).

This morphokinetic parameter GTV may be referred to herein as a generalized time variable, GTV. Generally speaking GTV is low when the study embryo exhibits a relatively fast development and is high when the embryo exhibits a relatively slow development.

The particular morphokinetic parameter based on the particular sequence $\Delta t_1 (= t_2)$; $\Delta t_2 (= t_3 - t_2)$; $\Delta t_3 (= t_4 - t_3)$; $\Delta t_4 (= t_5 - t_4)$, $\Delta t_5 (= t_6 - t_5)$, $\Delta t_6 (= t_7 - t_6)$, $\Delta t_7 (= t_8 - t_7)$, as in this example, may be referred to herein as a third generalized time variable GIV3.

It will again be appreciated that similar methods may be used to establish a morphokinetic parameter for an embryo using different characteristics relating to the development of the study embryo.

- 5 For example, while the third generalized time variable GTV3 as described above is based on all times between subsequent cell divisions from a single cell to an eight-blastomere embryo, other generalized irregularity variables may be based on other sequences of time differences. For example, the following variations may be defined to provide different morphokinetic parameters for use in accordance with embodiments of
10 the invention:

GTV1 (first generalized time variable): similar to GTV3 but using only the time difference of the last two cell divisions observed up to the 8 blastomere state. I.e. $\Delta t_i = (t_8 - t_7)$, if t_7 and t_8 are annotated; or $\Delta t_i = (t_7 - t_6)$ if t_8 is missing and t_6 and t_7 are
15 annotated; or $\Delta t_i = (t_6 - t_5)$ if t_8 and t_7 are missing and t_5 and t_6 are annotated; or $\Delta t_i = (t_5 - t_4)$ if t_8 , t_7 and t_6 are missing and t_4 and t_5 are annotated; or $\Delta t_i = (t_4 - t_3)$ if t_5 to t_8 are missing and t_3 and t_4 are annotated; or $\Delta t_i = (t_3 - t_2)$ if t_4 to t_8 are missing and t_2 and t_3 are annotated; $\Delta t_i = t_2$ if t_3 to t_8 are missing and t_2 is annotated. In each case the parameter k is 1.

20 GTV2 (second generalized time variable): similar to GTV1 but with the later division time (cleavage time) in a pair replaced with t_{End} (the end of the incubation time) where timings are missing. I.e. $\Delta t_i = (t_8 - t_7)$, if t_7 and t_8 are annotated; or $\Delta t_i = (t_{\text{End}} - t_7)$ if t_8 is missing and t_7 is annotated; or $\Delta t_i = (t_{\text{End}} - t_6)$ if t_7 and t_8 are missing and t_6 is
25 annotated; or $\Delta t_i = (t_{\text{End}} - t_5)$ if t_6 to t_8 are missing and t_5 is annotated; or $\Delta t_i = (t_{\text{End}} - t_4)$ if t_5 to t_8 are missing and t_4 is annotated; or $\Delta t_i = (t_{\text{End}} - t_3)$ if t_4 to t_8 are missing and t_3 is annotated; or $\Delta t_i = (t_{\text{End}} - t_2)$ if t_3 to t_8 are missing and t_2 is annotated. Mean and variance values for the reference population may be calculated based on Δt_i without substitution. For GTV2 the parameter k is always 1.

30 GTV3 (third generalized time variable): as discussed above, this morphokinetic parameter uses timings between all consecutive divisions that are not missing from the data to the 8 blastomere stage. i.e. $\Delta t_i = ((t_8 - t_7), (t_7 - t_6), (t_6 - t_5), (t_5 - t_4), (t_4 - t_3), (t_3 - t_2), t_2)$. If a Δt_i from this sequence is missing for a particular embryo, it is omitted from the

calculation. The parameter k is the number of Δt_i used in the calculation for a particular embryo.

5 GTV4 (fourth generalized time variable): similar to GTV3 but using all consecutive divisions with substitution of the last division time with incubation end time if missing. $\Delta t_i = ((t_8 - t_7), (t_7 - t_6), (t_6 - t_5), (t_5 - t_4), (t_4 - t_3), (t_3 - t_2), t_2)$. If a t_i is missing it is substituted with the t_{End} . Mean and variance values for the reference population may be calculated based on Δt_i without substitution. K is the number of Δt_i used in the calculation for that particular embryo.

10

GTV5 (fifth generalized time variable): similar to GTV3 but using timings for the full cell cycles. I.e. $\Delta t_i = ((t_8 - t_4), (t_4 - t_2), t_2)$. If a Δt_i is missing it is omitted. k is the number of Δt_i used in the calculation for a particular embryo.

15

GTV6 (sixth generalized time variable): similar to GTV5 but using all full cell cycles with substitution of the last division time with t_{End} if missing. $\Delta t_i = ((t_8 - t_4), (t_4 - t_2), t_2)$. If a t_i is missing it is substituted with t_{End} . Mean and variance is calculated based in the Δt_i without substitution. k is the number of Δt_i used in the calculation for that particular embryo.

20

GTV7 (seventh generalized time variable): similar to GTV2 but using only the period from insemination to the last annotated timing. I.e. $\Delta t_i = t_8$ if t_8 is annotated, $\Delta t_i = t_7$ if t_8 is missing and t_7 is annotated, $\Delta t_i = t_6$ if t_7 and t_8 are missing and t_6 is annotated, and so on. For GTV7 the parameter k is always 1.

25

GTV8 (eighth generalized time variable): similar to GTV3 but using t_8 if it is annotated and otherwise using t_{End} if t_8 is missing. For GTV8 the parameter k is always 1.

30 GTV9 (ninth generalized time variable): similar to GTV2 but using stages up to the blastocysts stage for evaluation on day 5 post insemination. $\Delta t_i = (t_B - t_{SB})$, if both t_{SB} and t_B are annotated; or $\Delta t_i = (t_{End} - t_{SB})$, if t_B is missing and t_{SB} is annotated; or $\Delta t_i = (t_{End} - t_M)$, if t_{SB} and t_B is missing and t_M is annotated; or $\Delta t_i = (t_M - t_8)$, if t_M to t_B is missing and t_8 is annotate; or $\Delta t_i = (t_{End} - t_7)$, if t_8 to t_B is missing and t_7 is annotated; or $\Delta t_i = (t_{End} - t_6)$ if t_7 to t_B are missing and t_6 is annotated; or $\Delta t_i = (t_{End} - t_5)$ if t_6 to t_B are missing and t_5 is annotated; or $\Delta t_i = (t_{End} - t_4)$ if t_5 to t_B are missing and t_4 is annotated;

35

or $\Delta t_i = (t_{\text{End}} - t_3)$ if t_4 to t_B are missing and t_3 is annotated; or $\Delta t_i = (t_{\text{End}} - t_2)$ if t_3 to t_B are missing and t_2 is annotated. Mean and variance may be calculated based on the Δt_i without substitution. For GTV9 the parameter k is always 1.

- 5 GTV10 (tenth generalized time variable): similar to GTV4 but using all stages up to the blastocyst stage. For evaluation on day 5 post insemination. $\Delta t_i = ((t_B - t_{SB}), (t_{SB} - t_M), (t_M - t_8), (t_8 - t_7), (t_7 - t_6), (t_6 - t_5), (t_5 - t_4), (t_4 - t_3), (t_3 - t_2), t_2)$. If a timing is missing it is substituted with the t_{End} . Mean and variance is calculated based on the Δt_i without substitution. For GTV10 the parameter k is the number of Δt_i used in the calculation for that
- 10 particular embryo.

Embryo

- 15 In some cases the term "embryo" is used to describe a fertilized oocyte after implantation in the uterus until 8 weeks after fertilization at which stage it becomes a foetus. According to this definition the fertilized oocyte is often called a pre-embryo until implantation occurs. However, throughout this patent application we will use a broader definition of the term embryo, which includes the pre-embryo phase. It thus encompasses all developmental stages from the fertilization of the oocyte through
- 20 morula, blastocyst stages hatching and implantation.

- An embryo is approximately spherical and is composed of one or more cells (blastomeres) surrounded by a gelatine-like shell, the acellular matrix known as the zona pellucida. The zona pellucida performs a variety of functions until the embryo
- 25 hatches, and is a good landmark for embryo evaluation. The zona pellucida is spherical and translucent, and should be clearly distinguishable from cellular debris.

- An embryo is formed when an oocyte is fertilized by fusion or injection of a sperm cell (spermatozoa). The term is traditionally used also after hatching (i.e. rupture of zona
- 30 pellucida) and the ensuing implantation. For humans the fertilized oocyte is traditionally called an embryo for the first 8 weeks. After that (i.e. after eight weeks and when all major organs have been formed) it is called a foetus. However the distinction between embryo and foetus is not generally well defined.

During embryonic development, blastomere numbers increase geometrically (1-2-4-8-16- etc.). Synchronous cell cleavage is generally maintained to the 16-cell stage in embryos. After that, cell cleavage becomes asynchronous and finally individual cells possess their own cell cycle. Human embryos produced during infertility treatment are usually transferred to the recipient before 16-blastomere stage. In some cases human embryos are also cultivated to the blastocyst stage before transfer. This is preferably done when many good quality embryos are available or prolonged incubation is necessary to await the result of a pre-implantation genetic diagnosis (PGD).

Accordingly, the term embryo is used in the following to denote each of the stages fertilized oocyte, zygote, 2-cell, 4-cell, 8-cell, 16-cell, morula, blastocyst, expanded blastocyst and hatched blastocyst, as well as all stages in between (e.g. 3-cell or 5-cell)

15

Examples

Example 1

Development for three different groups of mouse embryos incubated in three different temperatures of the incubation medium were investigated under similar conditions, i.e. only the temperature differed between the three different groups. The temperature of the incubation media was assessed by measuring the temperature of the slideholder using a YSI precision thermometer.

The three different temperatures were 36.5°C (33 embryos), 37.5°C (63 embryos) and 38.5°C (35 embryos), respectively. Nearly all mouse embryos reached the blastocyst stage as seen in the below table.

Temperature of slide holder (°C)	N	Blastocyst rate (%)
36.5	33	100
37.5	63	98
38.5	35	100

The table below shows the measured average timing for different cell divisions, the morula and blastocyst stage.

Temp. (°C)	2 cells (t2)	3 cells (t3)	4 cells (t4)	5 cells (t5)	6 cells (t6)	7 cells (t7)	8 cells (t8)	9+ cells (t9)	Morula	Blastocyst
36.5	4.61	26.36	27.57	35.91	36.30	37.10	37.54	44.54	49.45	67.46
37.5	3.75	23.43	24.25	32.12	32.54	33.19	33.63	40.72	45.85	59.27
38.5	3.06	21.96	22.50	30.62	30.97	31.43	31.87	39.06	44.29	55.03

These data have been plotted in three graphs shown in fig. 5. The difference between various cell divisions is shown in fig. 6. The data and the graphs show that increasing the temperature of the medium clearly speeds up the development.

5

In order to assess the difference in development a relative rate coefficient k can be defined. If k is set to 1 at base temperature (T_b) the following relationship can be assumed:

$$k(T) = 1 + \alpha(T - T_b)$$

10

where T is the temperature in °C and α is the temperature dependency coefficient.

The expected time t for a given temperature T , relative to $t(T_b)$, is inversely proportional to $k(T)$:

$$t(T) = t(T_b)/k(T)$$

15

The above linear simplification offers the advantage of only requiring the estimation of a single parameter. Conversely, it is probably only valid within a narrow temperature range. However, in the case of human embryo incubation, the expected maximum temperature span would be somewhat below $\pm 1^\circ\text{C}$, such that the practical influence of non-linearity can be considered negligible.

20

Optimising $k(T)$ and $t(T)$ by utilisation of the above listed mouse embryo data, using the time of division to 5 cells (t_5), α is estimated to 0.080 ± 0.015 (95 % CI).

25

The Q_{10} value is calculated as:

$$Q_{10} = \left(\frac{R_2}{R_1} \right)^{10/(T_2 - T_1)}$$

where R is the rate and T is the temperature.

30

Utilising the above parameter, the mouse embryo data, and the $\pm 1^\circ\text{C}$ span in the experiment, the above equation yields a Q_{10} of 2.22, which is inside the normally

expected range of 2 - 3 in biological systems (Reyes et al., 2008, Mammalian peripheral circadian oscillators are temperature compensated. J. Biol. Rhythms 23: 95-98).

- 5 The same calculations have been performed for a set of data from 1397 human embryos extracted from different clinics. The incubation conditions for these human embryos are therefore not as similar as the above mentioned mouse embryos. However, the clinics belong to the same chain of IVF clinics using the same instrumentation. All embryos have been transferred with homogenised procedures,
10 besides temperature. Utilising t_5 here again, and optimising according to $k(T)$ and $t(T)$, the estimate for α becomes 0.058 ± 0.028 (95 % CI).

- In contrast to the mouse embryos these human embryos have been incubated under slightly different conditions. The extracted human embryo data are therefore not
15 comparable to the same degree as the mouse embryo data. However, again the data from the human embryos indicate that a higher temperature of the medium speeds up the development. By constantly monitoring the morphokinetic parameters this increase in development speed may be detected and provide an early warning of a problem. This problem may be related to an undesirable increase in temperature, which may be
20 corrected before any embryos are actually transferred.

Claims

1. A computer implemented method for automatically detecting variations and/or abnormalities in the developmental conditions of in vitro incubating embryos, the method comprising the steps of:
 - a) obtaining a first dataset comprising morphokinetic parameters relating to the development of a first group of embryos,
 - b) obtaining a second dataset comprising morphokinetic parameters relating to the development of a second group of embryos,
 - c) modifying the first and second datasets by extracting morphokinetic parameter outliers from the first dataset and or the second datasets,
 - d) calculating the difference between specific morphokinetic parameters from the modified first dataset and the corresponding morphokinetic parameters from the modified second dataset, and
 - e) monitoring said morphokinetic difference thereby detecting variations in the developmental conditions of the first and second group of embryos.
2. The method according to claim 1, wherein step c) is optional.
3. The method according to claim 1, wherein step c) is non-optional.
4. The method according to any of the preceding claims, wherein the second dataset comprises the most recent embryo data.
5. The method according to any of the preceding claims, wherein steps a) – d) are repeated and the second dataset is continuously updated with the most recent embryo data.
6. The method according to any of the preceding claims, wherein the second dataset comprises the most recent embryo data selected from a specific time period or selected from a predefined number of the most recent embryos.
7. The method according to any of the preceding claims, further comprising the step of issuing a warning when the morphokinetic difference is above a predefined level.

8. The method according to claim 7, wherein said predefined level is determined as a predefined level above the standard deviation of one or more of the morphokinetic parameters from the first dataset.

5 9. The method according to any of the preceding claims, wherein steps a) – d) are repeated thereby continuously surveying the developmental conditions.

10 10. The method according to any of the preceding claims, wherein at least one of the morphokinetic parameter outliers are defined as relative outliers.

11 11. The method according to any of the preceding claims, wherein at least one of the morphokinetic parameter outliers are defined as absolute outliers.

15 12. The method according to any of the preceding claims, further comprising the step of monitoring the number of extracted outliers

13. The method according to any of the preceding claims, wherein the morphokinetic parameter outliers are excluded from the datasets.

20 14. The method according to any of the preceding claims, wherein all morphokinetic parameters from embryos with one or more morphokinetic parameter outliers are excluded from the datasets.

25 15. The method according to any of the preceding claims, wherein the second group of embryos is a subset of the first group of embryos such that the second dataset is a subset of the first dataset.

30 16. The method according to any of the preceding claims, wherein the second group of embryos is selected as most recent embryos, such as a predefined number as the most recent embryos, such as the most recent embryos from a predefined time period, such as number of hours, days, weeks or months.

35 17. The method according to claim 16, wherein said predefined number of embryos or predefined time period is determined as a user input.

18. The method according to any of the preceding claims, wherein the morphokinetic parameters are selected from the group of:

- the time of cleavage to a n blastomere embryo, tn , where $n = \{1, \dots, 8\}$,
- duration of cell cycles $cc1$, $cc2$, $cc2b$, $cc3$, $cc2_3$ and $cc4$,
- synchronicities $s2$, $s3$, $s3a$, $s3b$ and $s3c$,
- number of blastomeres at specific predefined timepoints,
- timing, extent or duration of cellular and/or organelle movement,
- any combination of these morphokinetic parameters.

19. The method according to any of the preceding claims, further comprising the step of calculating running mean values or running median values of the morphokinetic parameters.

20. The method according to any of the preceding claims, further comprising the step of calculating mean (average) values, median values, variance values and/or standard deviation values of the morphokinetic.

21. The method according to any of the preceding claims, wherein the following is a morphokinetic parameter:

$$CC_{norm} = \frac{1}{n} \sqrt{\sum_{all\ i} \left(\frac{CCi - CCi_m}{CCi_v} \right)^2}$$

wherein $i = \{1, 2, 3, 4\}$, $cc i_m$ is the mean value or median value of $cc i$ and $cc i_v$ is the variance of $cc i$.

22. The method according to any of the preceding claims, wherein at least one morphokinetic parameter for an embryo is established by obtaining values for a plurality of characteristics relating to the development of the embryo during an observation period; and determining a value for the at least one morphokinetic parameter by combining differences between the obtained values and corresponding reference values for the plurality of characteristics in a pre-defined manner.

23. The method of claim 22, wherein the reference values are determined from values for the plurality of characteristics obtained for at least one reference embryo.

24. The method of claim 22 or 23, wherein the step of combining differences between the obtained values and the reference values takes account of weighting values associated with each of the reference values.

5

25. The method of claim 24, wherein the weighting values are statistically determined from values for the plurality of characteristics obtained for a plurality of reference embryos of known development potential.

10

26. The method of claim 25, wherein the weighting values are determined from a variance of the values obtained for the plurality of reference embryos.

15

27. The method of any of claims 22 to 26, wherein the morphokinetic parameter represents a measure of regularity in the morphological developments of the embryo.

28. The method of any of claims 22 to 27, wherein the plurality of characteristics comprise a plurality of cell cycle durations for the embryo, cci .

20

29. The method of any of claims 22 to 28, wherein the plurality of characteristics comprise a plurality of differences in time between subsequent cell divisions for the embryo, Δt_j .

25

30. The method according to any of the preceding claims, wherein at least one of the morphokinetic parameters is based on a generalised irregularity variable, GIV, determined according to:

$$GIV = \frac{1}{n} \sqrt{\sum_{\substack{\text{all } i \text{ in} \\ \text{sequence}}} \frac{(cci - cci_m)^2}{cci_v}}$$

30

where cci is a series of cell cycle durations for an embryo, cci_m is a corresponding series of average cell cycle durations seen in a reference population of embryos, cci_v is a corresponding series of variance values for the respective cell cycle durations seen in the reference population, and n is the number of cell cycle durations comprising the series of cell cycle durations.

31. The method according to any of the preceding claims, wherein at least one of the morphokinetic parameters is based on a generalised time variable, GTV, determined according to:

$$GTV = \frac{1}{k} \sum_{\substack{\text{all } j \text{ in} \\ \text{sequence}}} \frac{\Delta t_j - \Delta t_{j_m}}{\Delta t_{j_v}}$$

where Δt_j is a series of differences in times for subsequent cell divisions for an embryo, Δt_{j_m} is a corresponding series of average values seen in a reference population of embryos, Δt_{j_v} is a corresponding series of variance values for the respective differences in times for subsequent cell divisions seen in the reference population, and k is the number of differences in times comprising the series.

32. The method according to any of the preceding claims, wherein morphokinetic outliers are selected from the group of: directly cleaving 1→3 cell embryos with cc2<5 hours, directly cleaving 2→5 cell embryos, short cell cycle embryos, long cell cycle embryos, and embryos experiencing multinuclearity.

33. The method according to any of the preceding claims, wherein short cycle embryos (SCC) and/or long cycle embryos (LCC) are defined as embryos with morphokinetic parameter outliers.

34. The method according to any of preceding claims, wherein said first and/or second group of embryos are embryos that has been fertilized, preserved and/or incubated under a specific set of conditions.

35. The method according to claim 34, wherein the first group of embryos have been fertilized under a different set of conditions than the second group of embryos.

36. The method according to any of claims 34 to 35, wherein said conditions are selected from the group of: type of fertilization treatment, preservation such as cryopreservation, incubating temperature, type of media, specific incubator, specific treatment such as hormonal treatment, hormonal treatment, aspiration, male factor, specific incubator, incubation temperature, type of media, type of oil, PGD Treatment, transfer, cryopreservation, thawing, time outside incubator, number of

fertilization treatments.

37. The method according to any of the preceding claims, wherein said second group of embryos are embryos that has been handled by a specific person.

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38. The method according to any of the preceding claims, wherein said second group of embryos are embryos that originates from predefined embryo donors.

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39. The method according to claim 38, wherein said predefined embryo donors are selected from the group of: individuals younger or older than a predefined age, embryo donors in a specific treatment or stimulation protocol, embryo donors in a specific fertilization treatment, embryo donors with a specific diagnosis such as hereditary chromosomal diseases, Hiv, Hep, PCO's, individuals with current or past exposure to radiation or hazardous chemical substances, individuals with current or past drug use, individuals with a BMI higher or lower than a predefined level, smokers, non-smokers, individuals with normal or abnormal menstrual cycle.

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40. The method according to any of the preceding claims, wherein said first group of embryos are selected from all implanted embryos, implanted embryos where the outcome is known, implanted embryos that have resulted in ongoing pregnancies, live born babies, fetal heart beat (FHB), and/or gestational sacs.

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41. The method according to any of the preceding claims, wherein said second group of embryos are selected from the group of:

- a) all fertilized embryos,
- b) embryos that have been chosen for freeze or transfer,
- c) embryos that have been chosen for transfer,
- d) embryos that have been classified as "Good quality embryos" (GQE) by a qualified embryologist,
- e) embryos that have implanted.

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42. The method according to any of the preceding claims, wherein the embryos are monitored through image acquisition, such as image acquisition at

least once per hour, such as image acquisition at least once per half hour, such as image acquisition at least once per twenty minutes, such as image acquisition at least once per fifteen minutes, such as image acquisition at least once per ten minutes, such as image acquisition at least once per five minutes, such as image acquisition at least once per two minutes, such as image acquisition at least once per minute.

43. The method according to any of the preceding claims, wherein the embryos are monitored by means of time-lapse microscopy equipment.

44. The method according to any of the preceding claims, wherein the embryos have been fertilized in vitro.

45. The method according to any of the preceding claims, wherein the embryos are cultured and/or monitored in an incubator.

46. The method according to any of the preceding claims, wherein the embryos are human embryos.

47. A system for determining embryo quality comprising means for carrying out the steps of any of claims 1 to 46.

48. A computer comprising computer code portions constituting means for executing the methods according to claim 1 to 46.

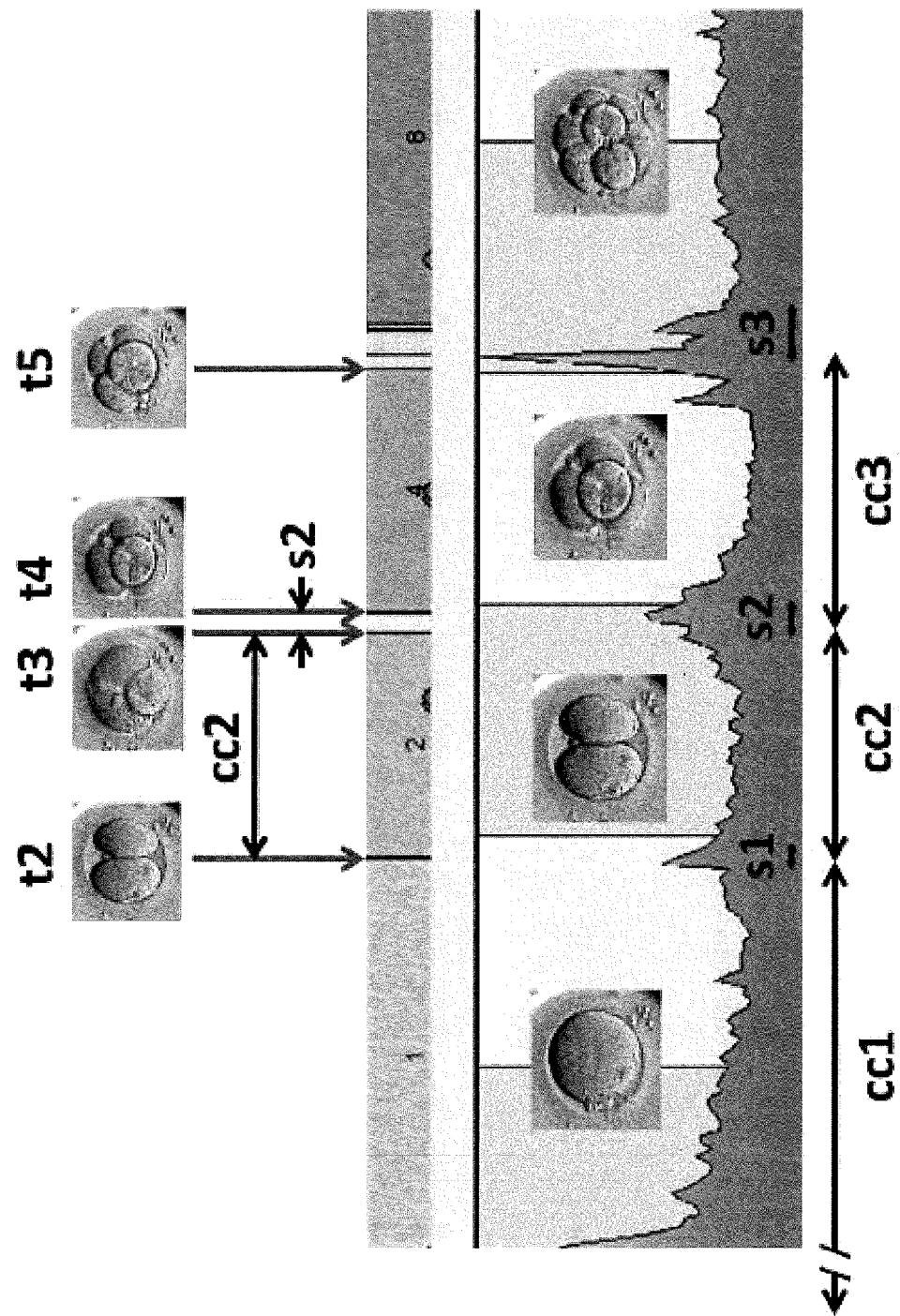
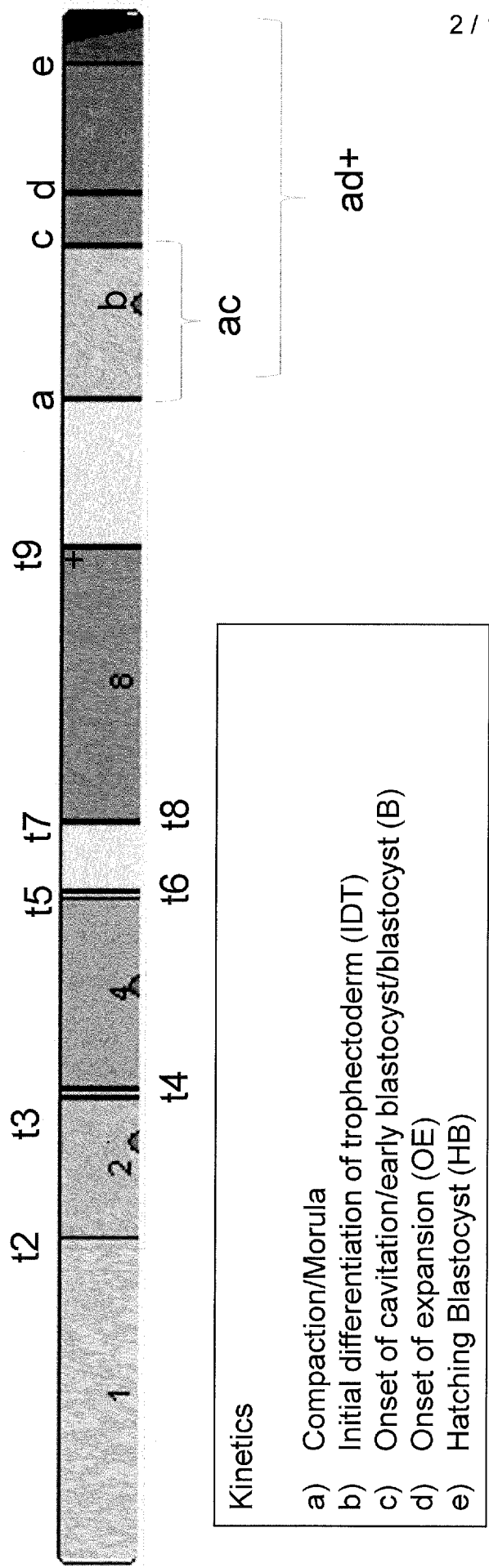


Fig. 1



Morphology

t5-a) Interval where Initial Compaction (IC) can be observed if present usually IC precedes "a" by minutes to a few hours.

a-c) Interval where "Partial compaction" (PC) can be observed if present.

a-d+) interval where "Degree of vacuolization" (VC(X)) and number of contractions (NC(X)) can be observed if present.

Fig. 2

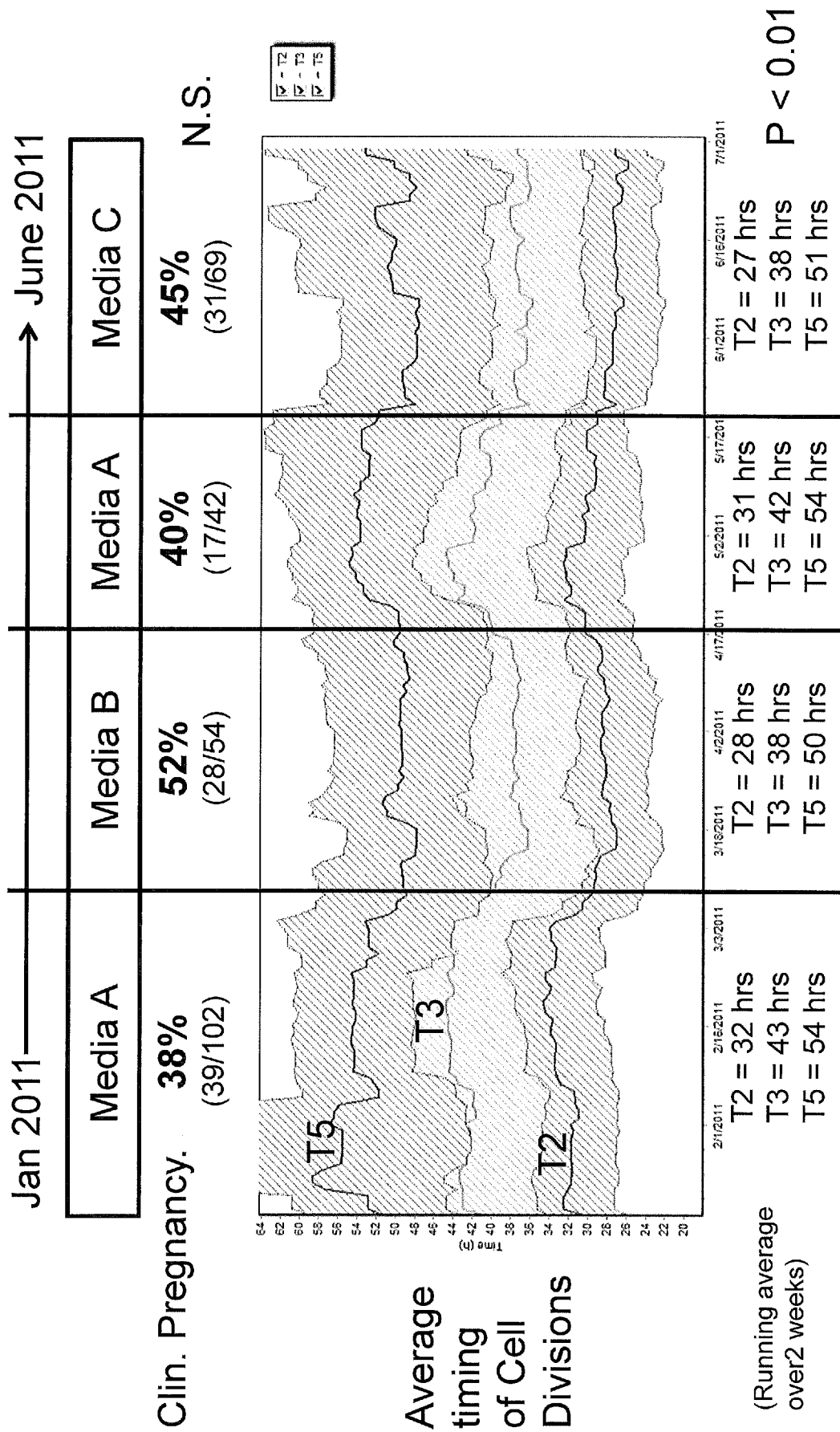


Fig. 3

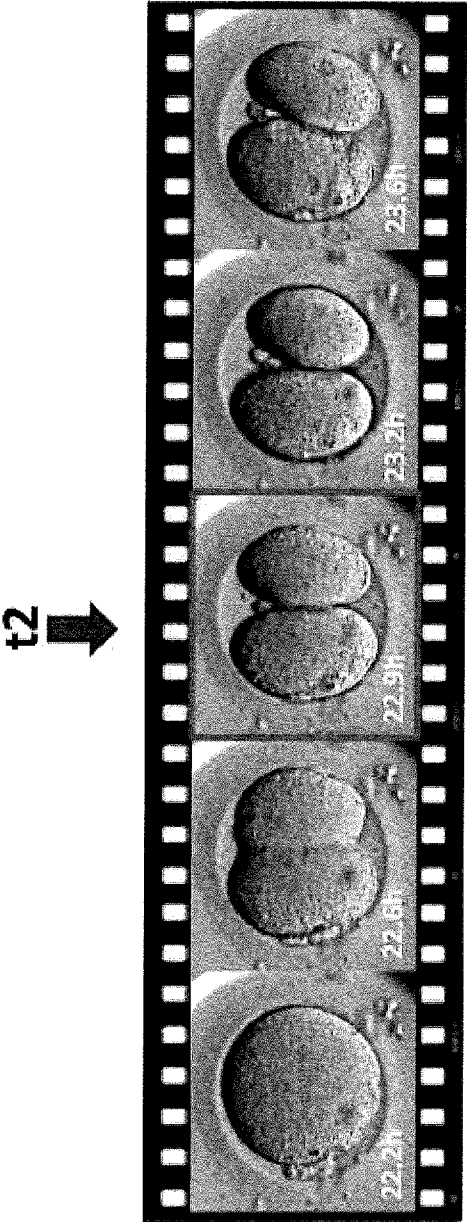


Fig. 4a

$t_3=t_2$ 

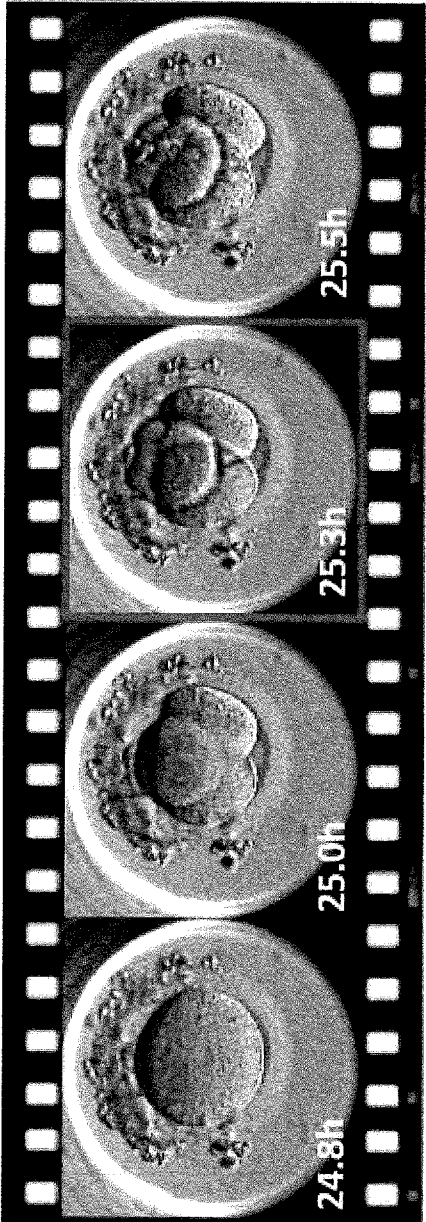


Fig. 4b

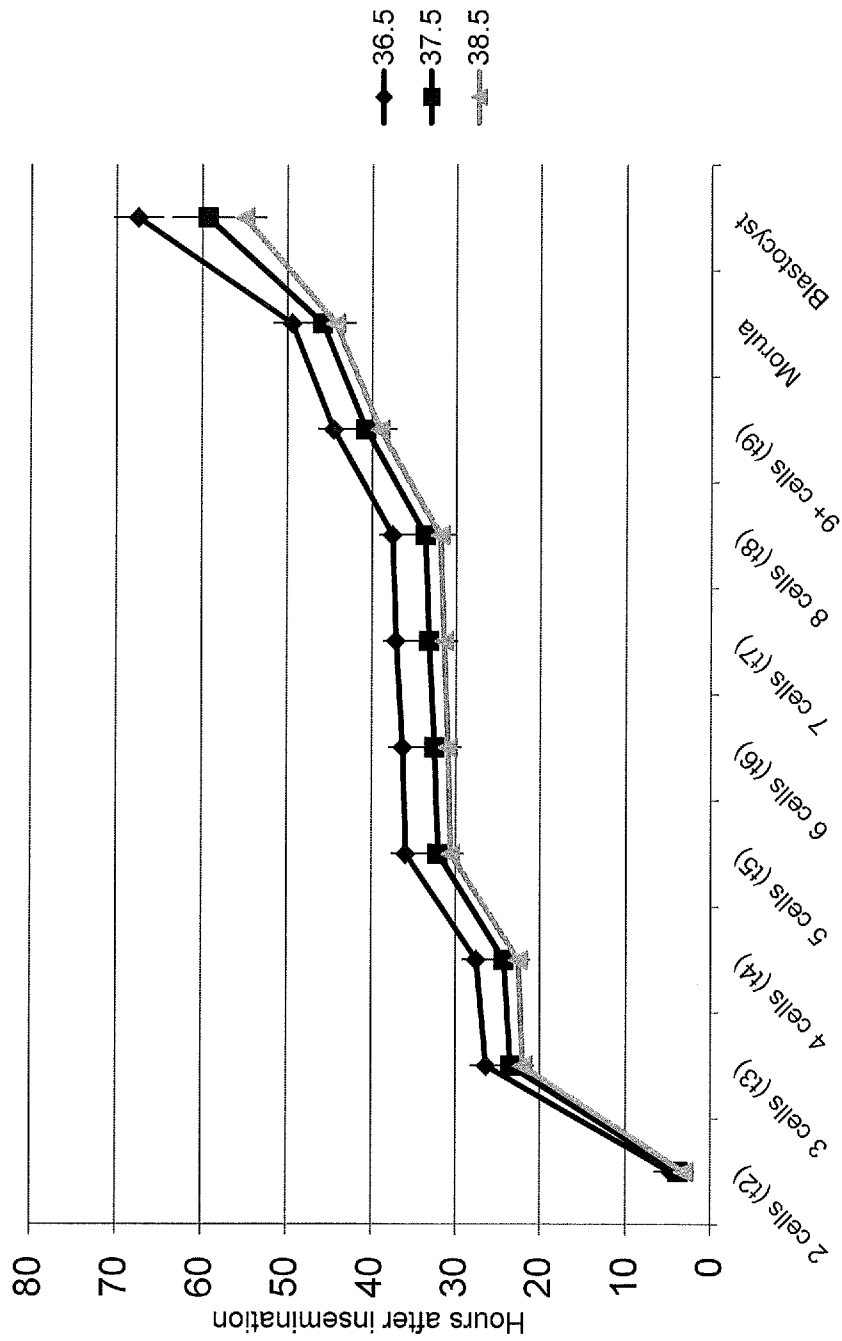


Fig. 5

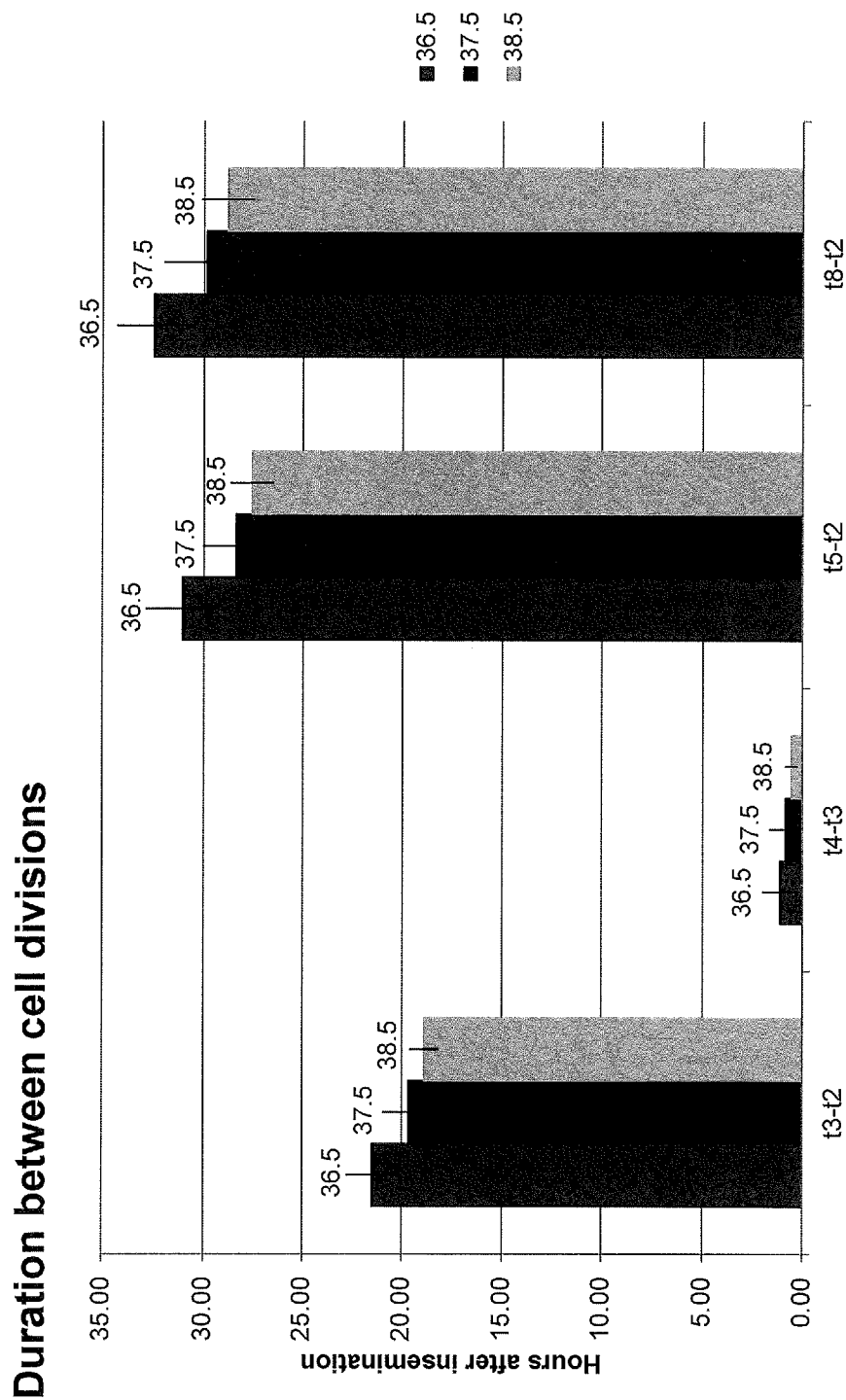


Fig. 6

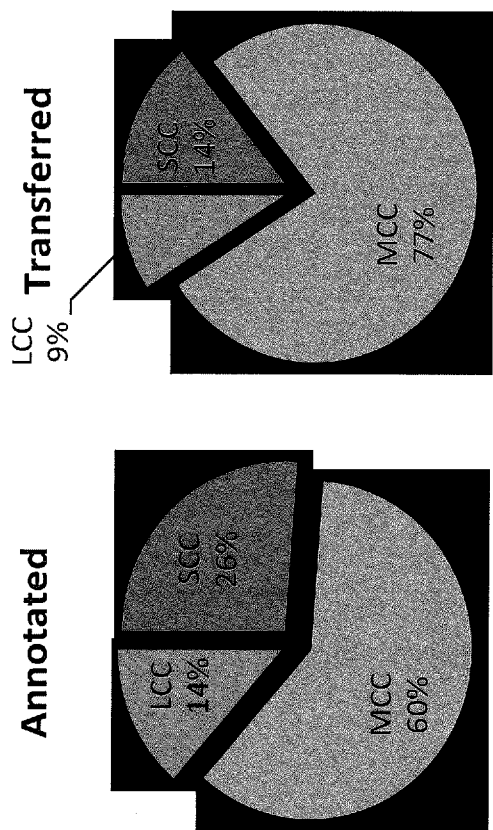


Fig. 7a

Fig. 7b

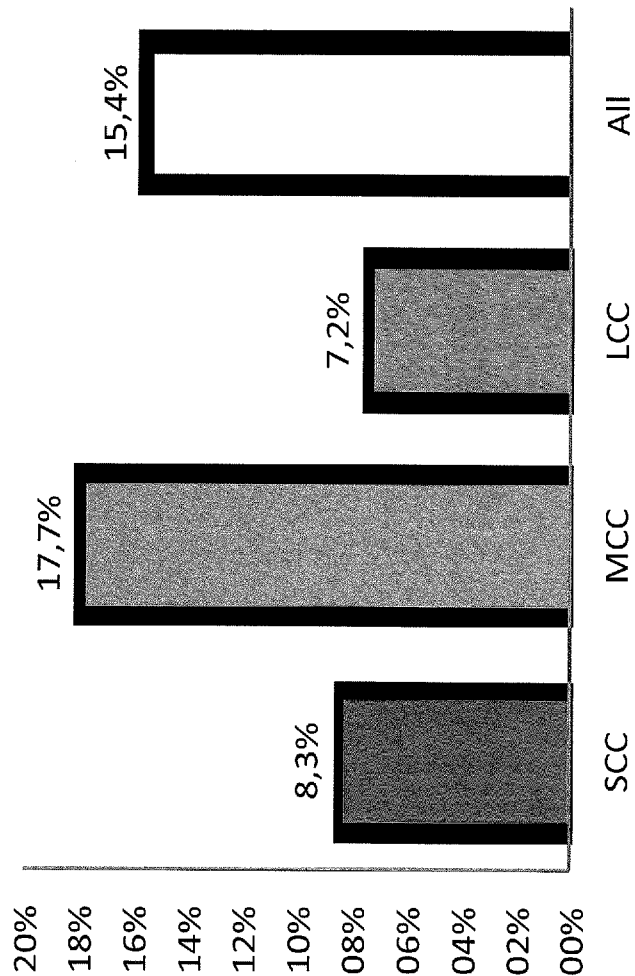


Fig. 7c

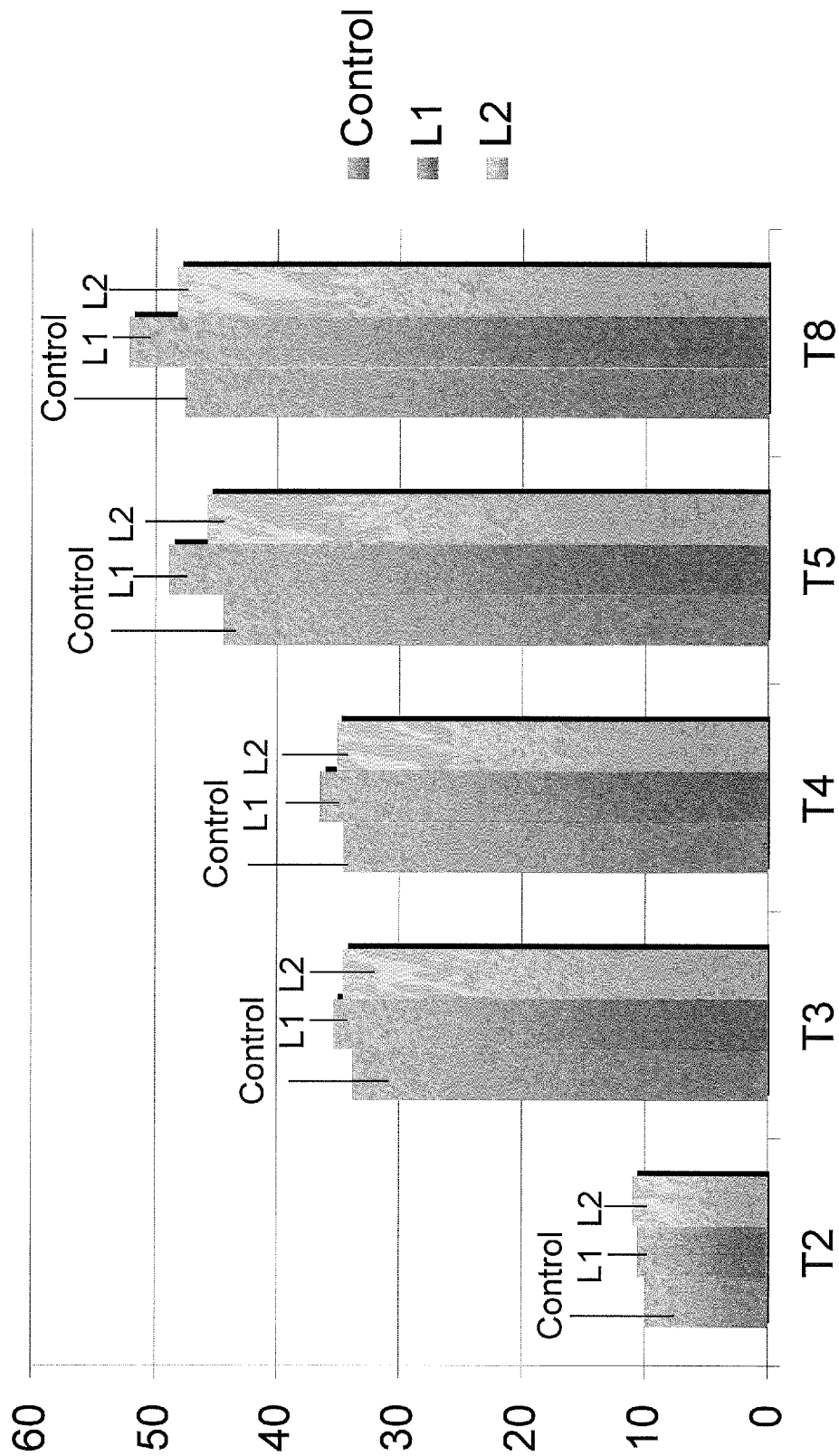


Fig. 8

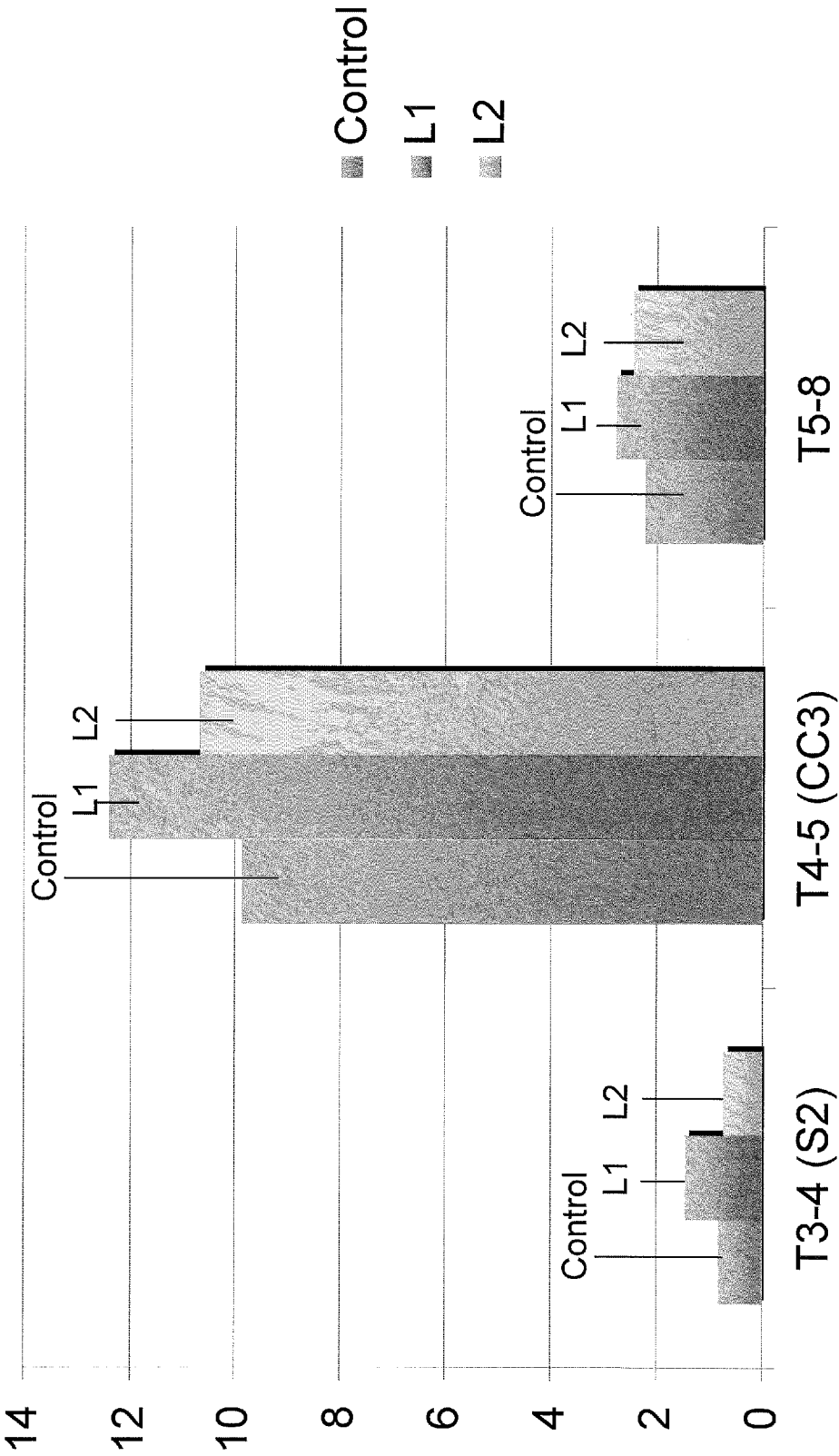


Fig. 9

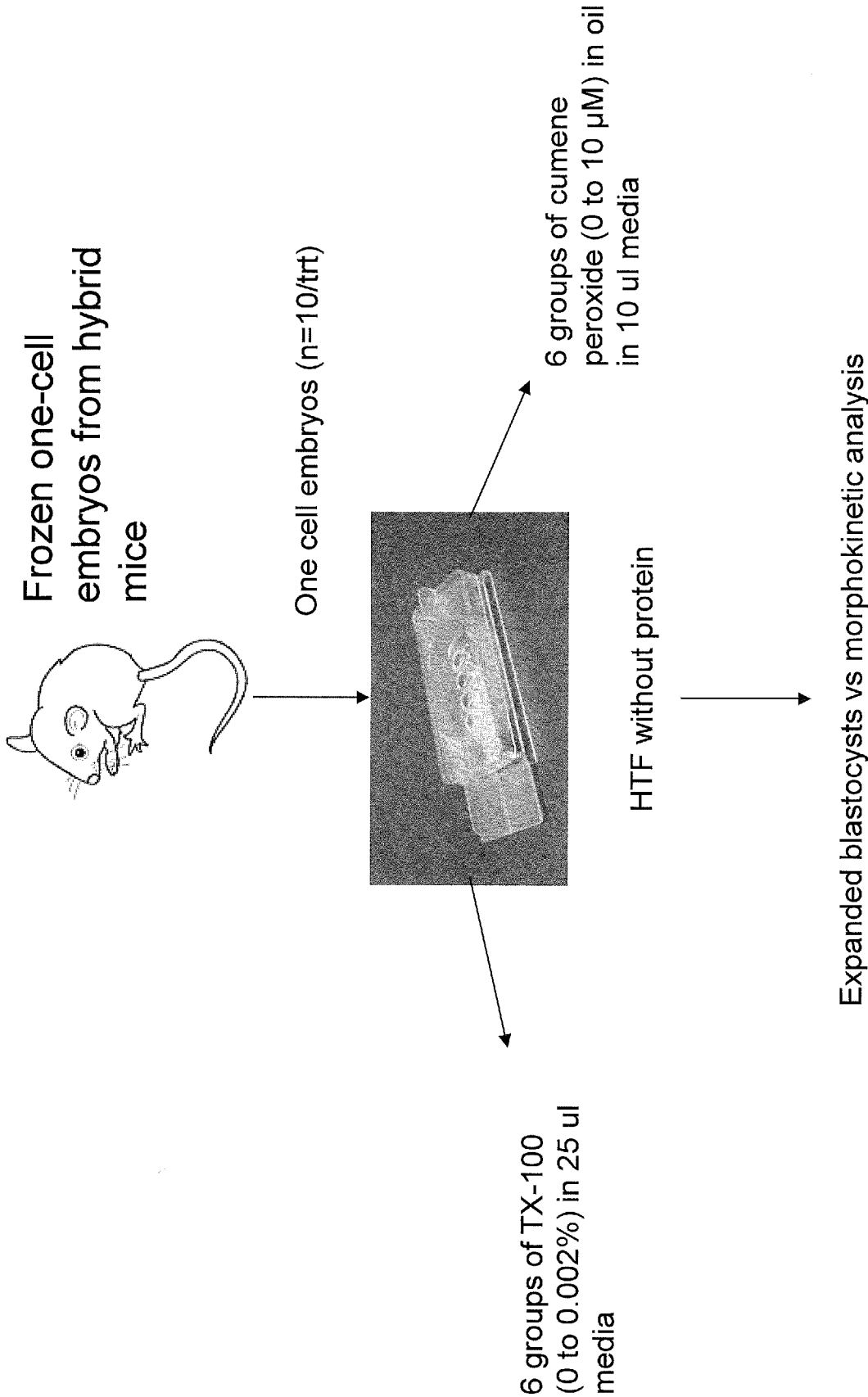


Fig. 10

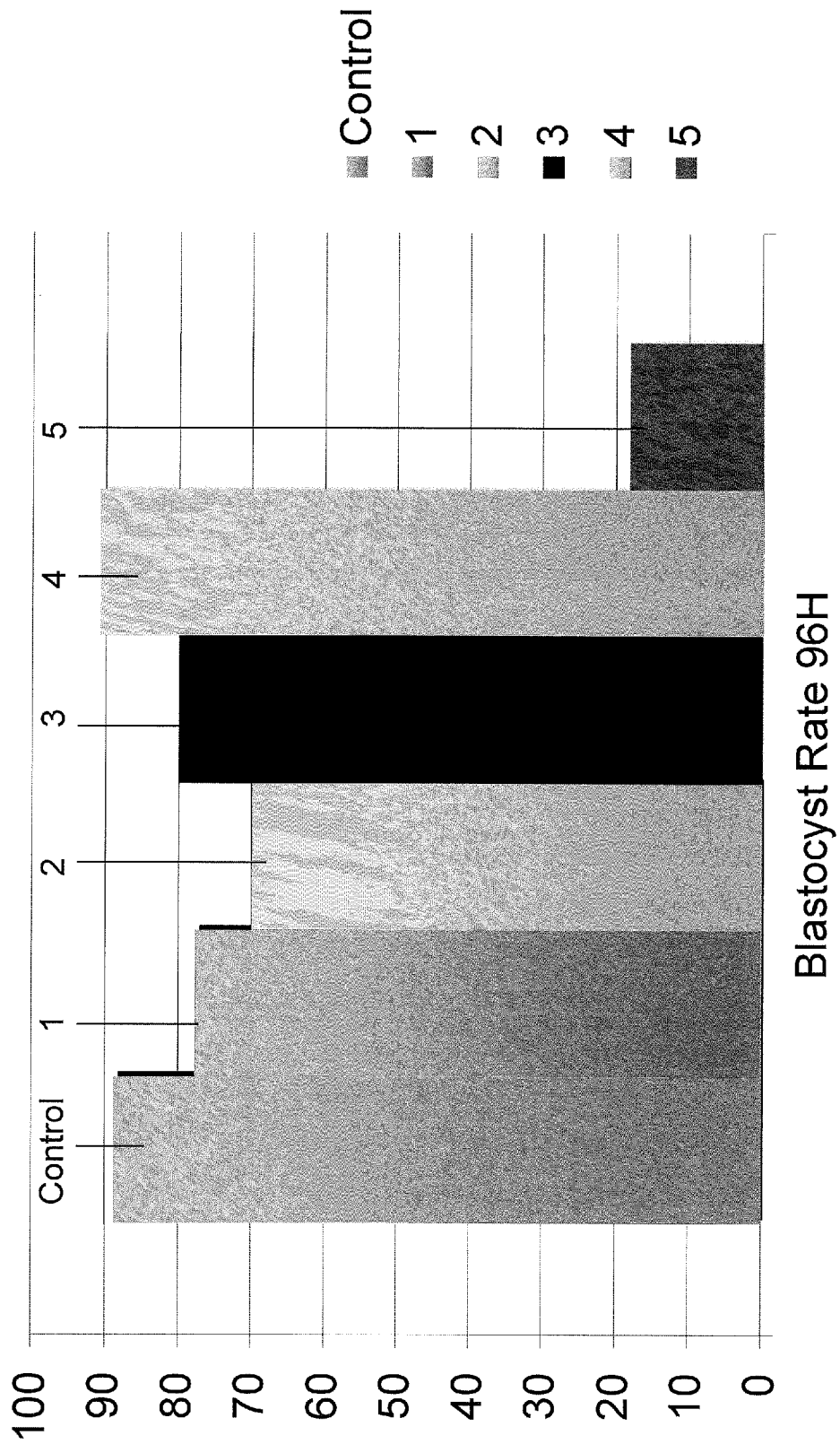


Fig. 11

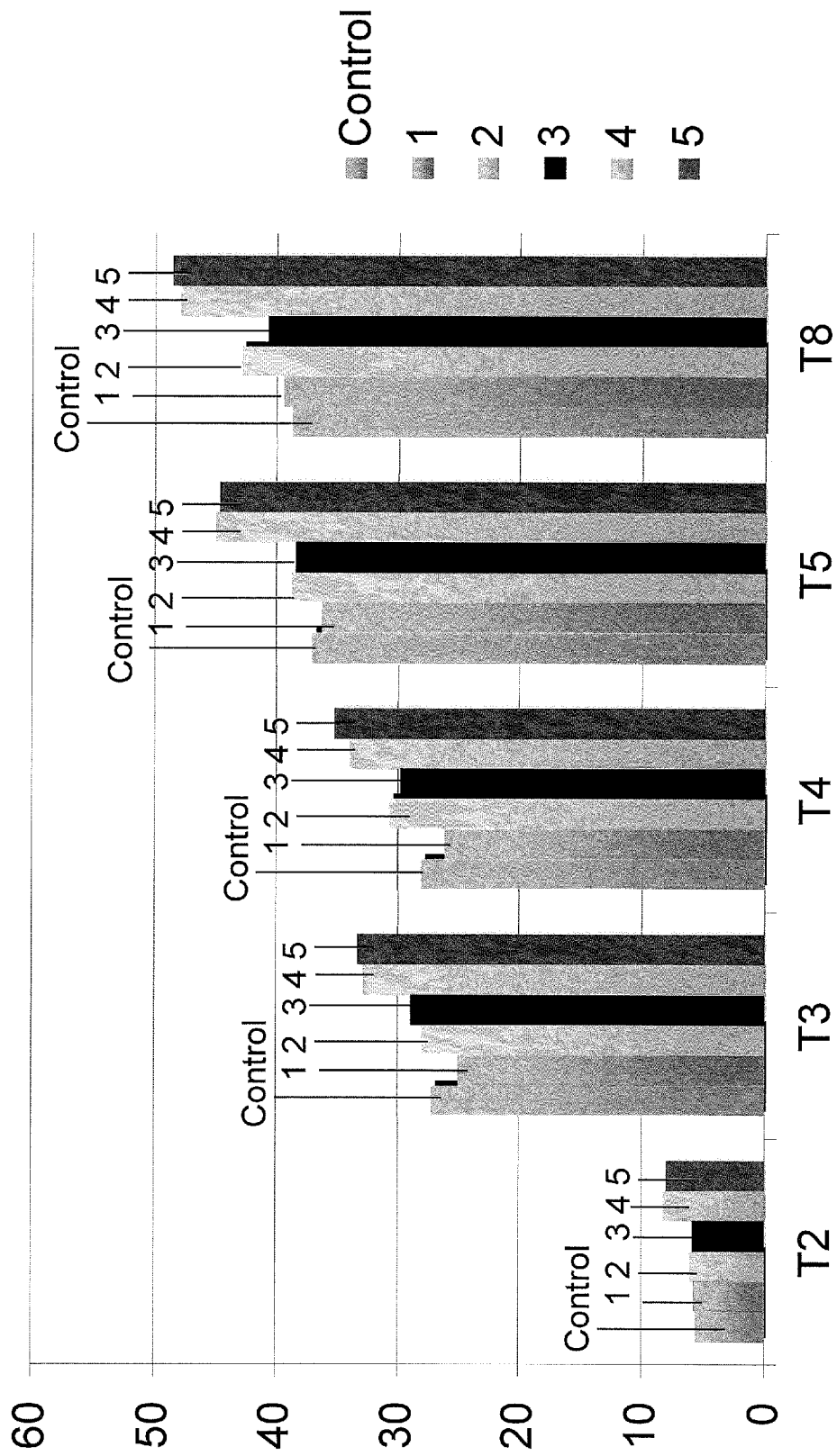


Fig. 12

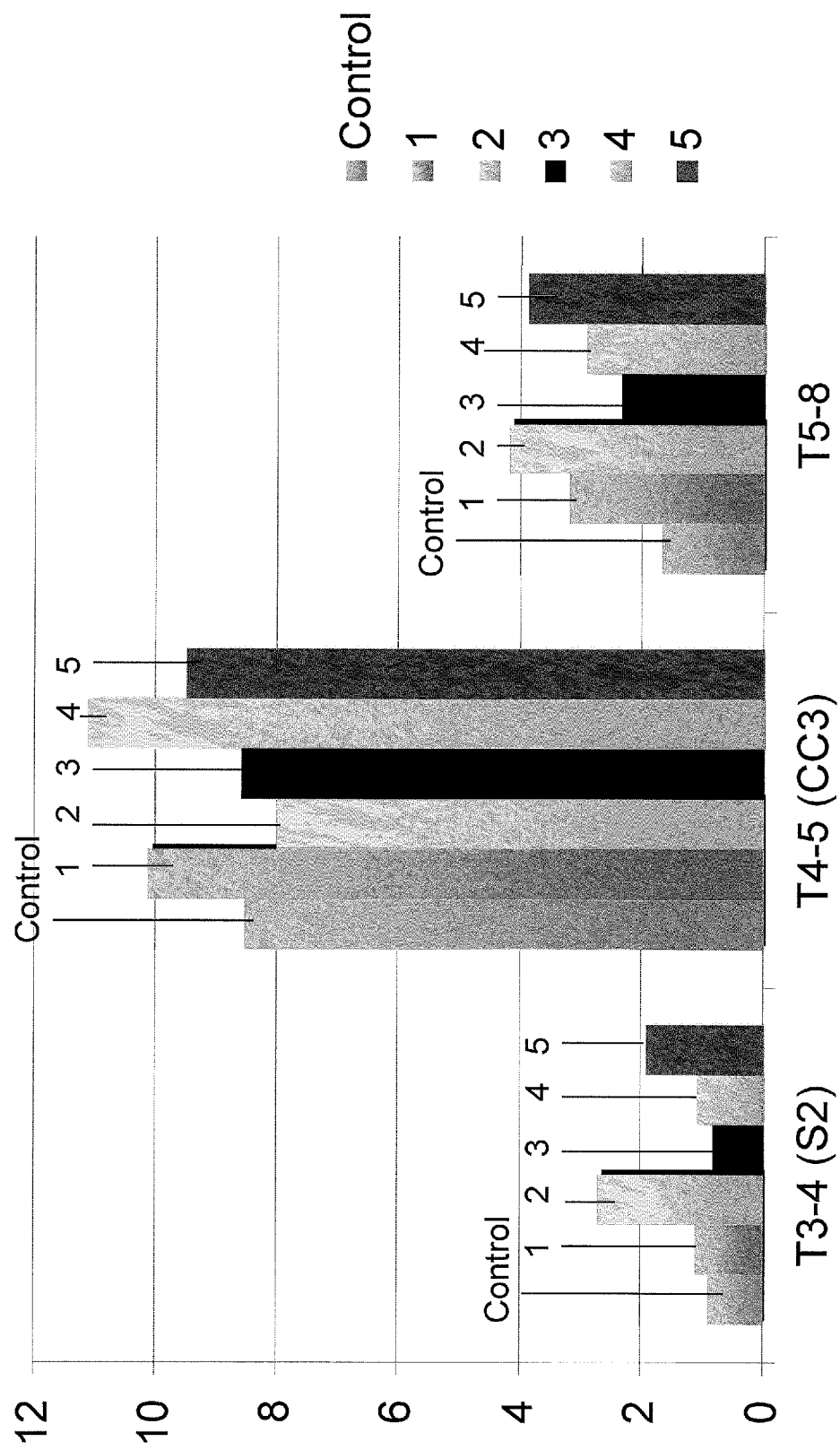


Fig. 13

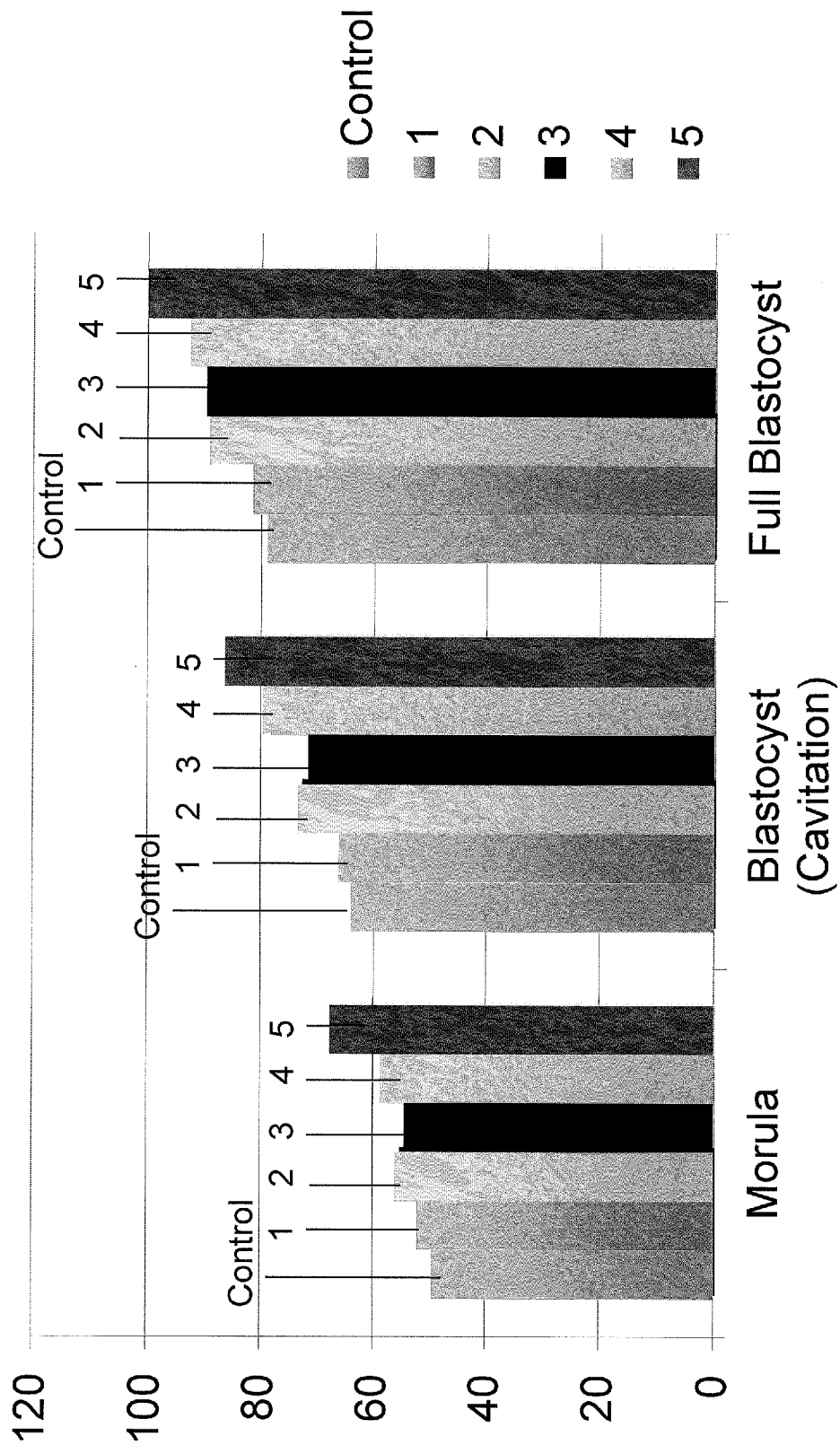


Fig. 14

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/067888

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/073 G06K9/00 C12N5/02
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N G06K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, CHEM ABS Data, EMBASE, INSPEC, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HERRERO J ET AL: "Linking successful implantation with the exact timing of cell division events obtained by time-lapse system in the embryoscope", FERTILITY AND STERILITY, ELSEVIER SCIENCE INC, NEW YORK, NY, USA, vol. 94, no. 4, 1 September 2010 (2010-09-01), page S149, XP027250457, ISSN: 0015-0282 [retrieved on 2010-08-30] the whole document ----- -/-	1-48



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

19 September 2013

Date of mailing of the international search report

26/09/2013

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Bayer, Martin

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/067888

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	MARIABEATRICE DAL CANTO ET AL: "Cleavage kinetics analysis of human embryos predicts development to blastocyst and implantation", REPRODUCTIVE BIOMEDICINE ONLINE, vol. 25, no. 5, 2 August 2012 (2012-08-02), pages 474-480, XP055079707, ISSN: 1472-6483, DOI: 10.1016/j.rbmo.2012.07.016 the whole document -----	1-48
X	CONNIE C WONG ET AL: "Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage", NATURE BIOTECHNOLOGY, vol. 28, no. 10, 1 October 2010 (2010-10-01), pages 1115-1121, XP055079709, ISSN: 1087-0156, DOI: 10.1038/nbt.1686 the whole document -----	1-48

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Information on patent family members

International application No

PCT/EP2013/067888

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