(54) Title: FLIM FOR DIFFERENTIATING OLD AND YOUNG OOCYTES

Fig. 5

(57) Abstract: The invention provides novel non-invasive in vitro meth-
ods for assessing the metabolic condition of oocytes and/or embryos with
fluorescence lifetime imaging microscope, that can be used, for example,
in assessment of oocytes and embryos in assisted reproductive technolo-
gies. The method allows differentiating between older and younger oo-
cytes.
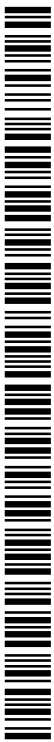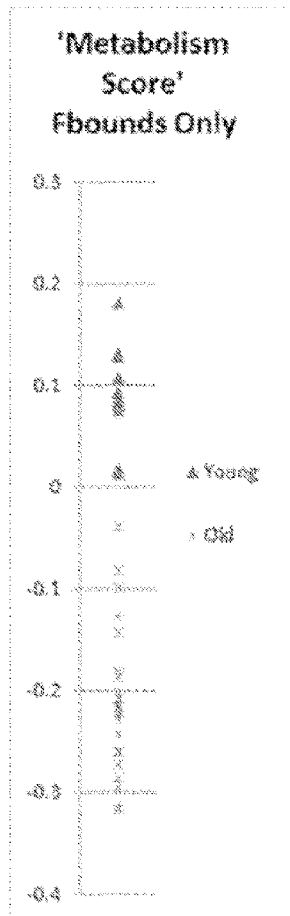
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT,

LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

FLIM FOR DIFFERENTIATING OLD AND YOUNG OOCYTES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 62/135,488 filed March 19, 2015, the contents of which are incorporated herein by reference in their entirety.

GOVERNMENT SUPPORT

[0002] This invention was made with government support under 0959721 awarded by The National Science Foundation. The government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] The invention relates to methods for assessing the metabolic condition of oocytes, and can be used, e.g., in assessment of oocytes in assisted reproductive technologies. Specifically, the invention relates to differentiating cells, such as oocytes that are more viable from the ones that are less viable, such as differentiating between older and younger oocytes. The methods can be also be used to select the most viable embryos for implantation or cryopreservation.

BACKGROUND

[0004] Assisted Reproductive Technology (ART) has revolutionized the treatments of human infertility in the past 30 years and has become ubiquitous. ART currently accounts for over 1% of birth in the U.S. (SART, 2005). However, success rates for ART are low, only 10% - 35% of cycles result in successful birth, leading to high costs and use of multiple embryos, which in turn gives rise to high rates of multiple gestations. Multiple gestations greatly increase mortality rates and suffering for both infant and mothers, and produce substantial financial costs. It has been estimated that complications from multiple pregnancy from ART account for approximately one billion dollars of health care cost annually in the U.S. (Bromer and Seli, 2008).

[0005] One of the major reasons for the low success rate of ART is the absence of reliable methods for selecting the highest quality embryo(s) for transfer. The lack of methods for assessing embryo quality has led to substantial efforts to develop improved assays of embryo viability. The current most reliable method for predicting embryo quality is to examine embryo morphology prior to transfer using standard transmitted light microscopy systems, with some clinics exploring the utility of time-lapsing imaging using specialized microscopes such as the EmbryoScope by Unisense or EPIC by Auxogyn. However, selection criteria

generally remain subjective and only result in the ~35% success rate quoted above. Newly proposed non-microscopy based methods, using genomic, transcriptomic or proteomic based assays (Uyar, et al, 2012), require a biopsy of the embryo and are thus invasive and significantly reduce rates of embryo survival (Scott et al., 2006). A metabolomic approach which initially showed promise was to assay the metabolic state of the embryo by measuring changes in metabolites in the embryo culture media (Botros, et al. 2008). However, a prospective randomized trial has recently failed to show that utilization of such metabolomic assessment improves selection over morphologic evaluation alone (Vergouw et al, 2012).

[0006] Moreover, oocytes from aging women are often not optimal quality for IVF. Having a method to rank the oocytes based on their viability prior to IVF to allow IVF only on the most viable oocytes to be used in the procedure would be an important advance.

[0007] Minimally invasive, simple and reliable methods for assessing embryo or oocyte viability for assisted reproductive technologies would provide a significant advance for improving the safety of the mother and the fetus, and would also reduce the costs of the assisted reproductive technologies by reducing the number of times one has to try implantation to get pregnant and also by reducing multiple gestations.

SUMMARY OF THE INVENTION

[0008] We provide an integrated, automated system for rapidly determining oocyte and embryo quality. The quality of an oocyte and/or embryo can refer to the health, metabolic activity, suitability for successful embryo (and/or fetal) development, and/or likelihood of successful embryo (and/or fetal) development. The described methods are minimally invasive and allow ranking or scoring of the oocytes or embryos based on their metabolic activity, where one or more of the most metabolically active oocytes or embryos can be used in methods of in vitro fertilization. We have discovered that a direct analysis of the metabolic state of these cells without additional manipulations of the cells or the cell culture media can be performed reliably and rapidly, and in most cases minimally invasively using fluorescence lifetime imaging microscopy (FLIM) of a combination of NADH and/or FAD in an in vitro assay. Specifically, here we provide an approach to combine FLIM data from both FAD and NADH. We show that combining fraction bound, short and long lifetimes, and average brightness for both FAD and NADH provides a "metabolic score" for an oocyte or embryo that can be used in selecting the most viable oocytes and/or embryos for IVF applications.

[0009] Previously, methods of using FLIM to assess metabolic state of oocytes and embryos were described in a PCT Application Publication No. WO 2014/110008 incorporated herein

2

by reference in its entirety. We provide further improved methods for assessing oocytes and embryos either using oocytes or complete embryos or by using granulosa or cumulus cells associated with the oocytes or one or more cells biopsied from the embryo by analyzing their metabolic state using autofluorescence from nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) with FLIM and combining the measurements of fraction bound, short and long lifetimes, and average brightness to provide a "metabolic score" for each of the analyzed cells. Cells with objectively measured adequate metabolic state, or as indicated by fluorescent lifetime of protein fraction of bound and/or free NADH or protein fraction bound and/or free FAD using both short and long lifetimes, and average brightness an oocyte or embryo can "rate" or "score" the oocytes/embryos that are subjected to the analysis and allow selection of the oocyte or oocytes or embryo or embryos that have the best chance of viability in IVF. Thus, the oocytes or embryos with the most optimal metabolic score can then be selected for in vitro fertilization or implantation, and/or the oocytes/embryos with lowest metabolic activity can be discarded from in vitro fertilization or implantation.

[0010] Previously, we showed that unperturbed oocytes exhibit a range of values of alpha and beta (Figure 1, circles). As seen in Figure 1, oocyte quality depends on oocyte metabolic state. Oocyte metabolic state can be rapidly, non-invasively, and quantitatively measured by Fluorescence Lifetime Imaging Microscopy (FLIM) of FAD or NADH. Two parameters (alpha and beta) were extracted from FLIM measurements of NADH (or FAD) in oocytes. In the example, we used mouse oocytes but similar calculations are expected to work for human oocytes, as the mammalian cells, such as mouse and human cells are relatively similar, particularly relating to their metabolic state, at this stage. Each point corresponds to data from a single oocyte. Perturbing oocytes by specific metabolic inhibitors (black crosses), or non-specific damage (triangles) causes both parameters to increase. Unperturbed oocytes (circles) exhibit a range of values of alpha and beta. These parameters are indicative of oocyte quality.

[0011] Here, we add to the parameters by assessing the combination of fraction bound, short and long lifetimes, and average brightness to provide an even more accurate assessment of the metabolic state. Using this system, we can, for example, differentiate between younger and older oocytes (see Figures 2-4).

[0012] One can perform the FLIM assessment by comparing the NADH/FAD autofluorescence lifetime, including fraction bound, short and long lifetimes, and average

brightness to a reference distribution which provides a convenient way of selecting viable and non-viable oocytes/embryos.

[0013] One can also take all available oocytes collected from a woman and subject them to the analysis and select the oocytes with most optimal or healthiest metabolic score that then have the best potential for IVF, without having any external reference as to the score. Similarly, or in addition, one can take all fertilized eggs from an IVF procedure and subject them to the FLIM assessment as set forth above, and select the embryo or embryos for implantation based on their ranking in the metabolic rate – starting the implantation with the embryos with the most optimal metabolic score compared to the others in the analysis. The FLIM process can also be used in previously cryopreserved oocytes and embryos to select the most viable ones from those.

[0014] The lifetime distribution of NADH/FAD can be approximated as a sum of two exponentials. The parameter α is the ratio of the amplitude of the two exponentials, the parameter, β, is the lifetime of the longer exponential. Increase in both alpha and beta of NADH was found to be indicative of damage in the embryo/oocyte. Thus, typically, cells with increased alpha and beta values compared to a reference value are discarded from ART methods as they would be considered damaged and alpha and beta less than a reference value would be selected for ART methods as their metabolic activity is indicative of healthy activity.

[0015] The FLIM measurements according to the methods of the invention can be performed with extremely low levels of illumination, which is far less than is currently used in *in vitro* fertilization clinics to determine the morphology of oocytes and embryos. Therefore, the FLIM measurements performed according to the methods of the present invention will not perturb oocytes and embryos and are thus as non-invasive as possible.

[0016] Accordingly, we provide a method for assessing the quality of an oocyte or an embryo, the method comprising (a) exposing an embryo or a test cell or a plurality of test cells selected from an oocyte or an oocyte-associated cumulus cell or a cell from an embryo to a fluorescence lifetime imaging microscope (FLIM) to acquire a fluorescence lifetime histogram of auto-fluorescence of endogenous NADH and FAD for the embryo or the test cell; (b) averaging the fluorescence lifetime histogram of NADH auto-fluorescence and FAD auto-fluorescence over the entire embryo, test cell or test cells, or the cytoplasm of the test cell or cells, or mitochondria of the test cell or cells to assay measurements for fraction bound, short and long lifetimes, and average brightness for both FAD and NADH (c)

combining the measurements for fraction bound, short and long lifetimes, and average brightness for both FAD and NADH to obtain a metabolic score; (d) comparing the metabolic score between the plurality of test cells to order them based on the metabolic score to determine the cells with the most optimal metabolic activity; and (e) selecting the embryo or oocyte or embryos or oocytes with the most optimal metabolic score for in vitro fertilization or for implantation or cryopreservation.

[0017] The cells can be fresh or previously cryopreserved.

[0018] One or more best scoring cells or embryos, i.e., the cells with most optimal metabolic state can be selected for further processes, such as IVF, implantation or cryopreservation.

[0019] In some aspects of all the embodiments of the invention, the method comprises a step of establishing the statistical significance by fitting lifetime histograms to a sum of two exponentials and the parameters of the measured cells are deemed to fall within or not fall within the range of parameters found in the healthy cells.

[0020] In some aspects of all the embodiments of the invention, the parameters to be compared between the measurements and the healthy cells are alpha, defined as the ratio of the amplitude of the two exponentials, and beta, defined as the lifetime of the longer exponential.

[0021] In some aspects of all the embodiments of the invention, the maximum value of alpha from the healthy cells is a specific value within the range 1.0 - 4.0 and the corresponding maximum value of beta of healthy cells is in the range 2000 ps - 3000 ps.

[0022] In some aspects of all the embodiments of the invention, the FLIM is performed using a wavelength of about 740 nm in two-photon fluorescence excitation and using an emission bandpass filtered centered around about 460 nm.

[0023] In some aspects of all the embodiments of the invention, the FLIM is performed using a wavelength of about 340 nm in one-photon fluorescence excitation and using an emission bandpass filtered centered around about 460 nm.

[0024] In some aspects of all the embodiments of the invention, the FLIM is performed in the frequency domain instead of the time domain.

[0025] In some aspects of all the embodiments of the invention, the FLIM is performed using a wavelength of about 900 nm in two-photon fluorescence excitation and using an emission bandpass filtered centered around about 550 nm.

[0026] In some aspects of all the embodiments of the invention, the FLIM is performed using a wavelength of about 450 nm in one-photon fluorescence excitation and using an emission bandpass filtered centered around about 550 nm.

[0027] In all aspects of the invention, the methods are performed in vitro. The noninvasive nature of the methods allow them to be performed without harming the embryos or oocytes.

[0028] These and other aspects of the invention, as well as various advantages and utilities, will be more apparent with reference to the detailed description of the preferred embodiments and to the accompanying drawings.

BRIEF DESCRIPTION OF DRAWINGS

[0029] Figure 1 shows data from FLIM imaging of NADH in mouse oocytes. Non-linear microscopy was used to assess the quality of live oocytes and embryos. FLIM curves from individual oocytes were fit to the sum of two exponentials. The parameter α, is the ratio of the amplitude of the two exponentials, the parameter, β, is the lifetime of the longer exponential in picoseconds. Each point corresponds to data from a single oocyte. Unperturbed oocytes exhibit a range of values of α and β. Perturbing oocytes by specific metabolic inhibitors (circles) or non-specific damage (triangles) causes both parameters to increase.

[0030] Figure 2 shows fraction of FAD and NADH molecules bound to enzymes in young and old mouse oocytes.

[0031] Figure 3 shows long lifetime values for NADH and FAD (measured in nanoseconds) in young and old mouse oocytes.

[0032] Figure 4 shows fraction of FAD and NADH molecules bound to enzymes in young and old mouse oocytes.

[0033] Figure 5 shows an example of a "metabolic score" measurement. As can be seen, young eggs can be easily distinguished from the old eggs.

[0034] Figure 6 depicts a graph of two-photon measurements.

[0035] Figure 7 depicts a graph of one-photo measurements.

DETAILED DESCRIPTION OF THE INVENTION

[0036] We have discovered that the metabolic state of an oocyte or an embryo can be directly assessed using FLIM imaging of the autofluorescence of the cellular metabolites inside the oocyte or the embryo or using one or more cumulus cells that surround the oocyte or a cell biopsied from the embryo. Oocytes and embryos with acceptable or best available metabolic status can then be selected for in vitro fertilization or implantation and oocytes and embryos having abnormal metabolic status can be discarded from further assisted reproductive methods. The methods provide a way to analyze embryos/oocytes with a process that is

much less invasive than any method we are aware of. The embryos/oocytes can be fresh or previously cryopreserved. With the scoring method presented herein, one can also select the most viable embryos/oocytes for cryopreservation.

[0037] The methods for assessing embryo quality are useful for predicting which embryos have the greatest potential for implantation in order to: (i) increase pregnancy rates with assisted reproductive technologies; (ii) decrease multiple pregnancy rates by justifying transfer of the single "best" embryo; and (iii) appropriately select suitable embryos for cryopreservation and (iv) increase the efficiency of offspring from transgenic intervention. The methods for assessing embryo quality are also useful for assessing the impact or effect of current invasive procedures on the oocyte and embryo, including: (i) intracytoplasmic sperm injection; and (ii) blastomere biopsy for pre-implantation cytogenetic diagnosis.

[0038] In the current methods we apply FLIM to detection of metabolic state of embryos and/or oocytes by direct analysis of the embryos or oocytes, or by analysis of cells from embryos or analysis of cumulus or granulosa cells surrounding the oocytes. The currently described method measures FLIM parameters that include fraction bound, short and long lifetimes, and average brightness for both FAD and NADH; and combines these parameters into a comprehensive "metabolic score."

[0039] For example, using our assay, we have demonstrated that oocytes originating from young and old mice have clearly differentiated metabolic activity as measured by Fluorescence Lifetime Imaging Microscopy (FLIM). In our examples, we showed that oocyte metabolic state can be rapidly, non-invasively, and quantitatively measured by a combination of FLIM of NADH and FAD.

[0040] To assay the cells, one can use at least three parameters, namely, short lifetime, long lifetime, and fraction bound to enzymes, that are extracted from FLIM measurements of NADH and FAD in oocytes, embryos or oocyte-associated cumulus cells.

[0041] Binding to enzymes causes the lifetime of the fluorophore to shift significantly, explaining the strong correlation in all three parameters (see, e.g., Figures 2-4). However, we have discovered that the combination of all these parameters provides the most reliable reading of the metabolic state of the cell and allows, e.g., distinguishing between the metabolic activity in young and old oocytes. The metabolic score can be applied to compare between oocytes and/or embryos to select the oocytes/embryos for further use, either for IVF or implantation or cryopreservation, that show the best metabolic score indicating they have the best chance of survival.

[0042] Similar calculations are expected to work for human oocytes, as mammalian cells are relatively similar, particularly relating to their metabolic state, at this stage. In Figures 2-4, one can see a clear separation in the parameter values between young and old oocytes. Given that it is clearly established that oocyte quality declines with age, these data indicate that differences in parameter values are associated to differences in oocyte quality.

[0043] As used herein, "oocyte" refers to a female germ cell. The oocyte analyzed according to the methods of the invention is obtained prior to fertilization, and the analysis is performed in vitro.

[0044] "Pre-implantation embryo" or "embryo" is used herein to refer typically to an in vitro fertilized oocyte with two pronuclei (up to and including a blastocyst) but which has not implanted in the lining of the female reproductive tract. In general, in one embodiment, the pre-implantation embryo contains between about 2 and about 8 cells (i.e., the embryo is assessed between about 18 and about 70 hours post-fertilization), although these ranges may vary among species. Typically, the quality assessment for a human or a mouse embryo is performed on an embryo containing between 2 and 8 cells.

[0045] "Cell from an embryo" refers to a single cell biopsied from the embryo. Embryo biopsy is a procedure that involves removing one or more cells from the embryo before it is transferred to the mother's uterus. It is typically performed for testing the embryo for specific genetic disorders. The methods of the present invention can be performed to the cells prior to performing a genetic analysis.

[0046] Given the intimate physical and metabolic contact between the cumulus granulosa cells and the oocyte, FLIM-acquired data of these heterologous cells has been shown to correlate with oocyte health. Such an approach circumvents any potential harm of illumination to the oocytes, and also circumvents any need for clinical trials to prove safety. The terms "cumulus cell" and "granulosa cell" as used herein refer to cumulus cells that are specialized granulosa cells surrounding and nourishing the oocyte. These cells surround the fully-grown oocyte to form a cumulus-oocyte complex ("COC"). The terms cumulus oophorus cells, cumulus granulosa cells, cumulus oophorous granulosa cells, granulosa-cumulus cells are used to make a distinction between these cells and the other functionally different subpopulation of granulosa cells at the wall of the Graafian follicle. Cumulus cells provide key products for the acquisition of developmental competence and differ from granulosa cells in their hormonal responses and growth factors they produce. The absence of cumulus cells or insufficient numbers of cumulus cells impairs embryo production. Denuded oocytes in culture cannot undergo normal fertilization with standard insemination. Cumulus

cells are required for the successful maturation of oocytes. These cells synthesize an abundant muco-elastic extracellular matrix, which promotes oocyte extrusion from the follicle, a 20-40 fold increase in the volume of the cumulus mass, and probably also functions as a selective barrier for sperm (Salustri, 2000). For cellular and molecular events during oocyte maturation and the formation of the extracellular matrix of the cumulus-oocyte complex see also: Russel and Salustry (2006) and Kimura et al (2007). Cumulus cells show high expression of many enzymes of the glycolytic pathway and also neutral amino acid transporters. Their expression is promoted by paracrine factors secreted by oocytes (Eppig et al, 2005; Sugiura et al, 2005), which themselves are unable to take up L-alanine and poorly metabolize glucose for energy production and thus depend on cumulus cells for their provision (Biggers et al, 1967; Colonna and Mangia, 1983; Donahue and Stern, 1968; Eppig et al, 2005; Haghighat and Van Winkle, 1990; Leese and Barton, 1984, 1985). As the cumulus cells provide the growth environment for the oocyte, their metabolic state can be used as a proxy of the metabolic state of the oocyte they support. Accordingly, in one aspect of all the embodiments of the invention the method uses one or more cumulus cells to select an oocyte for in vitro fertilization or for excluding an oocyte from in vitro fertilization. The cumulus cells are obtained from around an oocyte prior to insemination or ICSI, and the analysis is performed in vitro.

[0047] The cells are typically placed in a well suitable for imaging and comprising cell culture medium at 37°C.

[0048] The "reference value" as referred to herein, is typically assessed using normal healthy cells of comparable origin, such as normal healthy oocytes, normal healthy embryos, cumulus cells around a normal healthy oocyte or cells from a normal healthy embryo. The reference values are typically a range from averaged experiments, and are typically pre-determined although assays including a healthy reference cell of similar origin are also provided.

[0049] The methods of the invention use a device that is typically a self-contained, microscope setting, such as a box, such as a table-top microscope box that is typically used at in vitro fertilization clinics to select oocytes for fertilization and embryos for transfer.

[0050] The methods are based on use of fluorescence lifetime imaging microscopy (FLIM) of metabolic state of the oocytes or embryos. The interior of the device comprises, or consists essentially of the microscope and all the peripherals, as well as an environmental chamber enclosing the microscope stage. A small slot in the microscope exterior allows custom, multi-well plates containing granulosa cells, oocytes or embryos or cells biopsied from an embryo to be inserted onto the microscope stage. A screen monitor, such as a touch-screen

9

monitor, for example located on the device's exterior, contains controls and displays acquired data.

[0051] The device can be used on oocytes and embryos as well as cumulus cells and cells isolated from an embryo that can be acquired by any procedure and placed in any media. Thus the methods are easily compatible with the current practices in *in vitro* fertilization clinics and other settings where assessment of embryos or oocytes is performed.

[0052] Living cells possess an intricately regulated system of energy-producing and energy-utilizing chemical reactions. Metabolic reactions that are involved in energy generation break down macromolecules such as carbohydrate, lipid, or protein. Most of the energy-generating metabolic pathways of the cell eventually result in the production of acetyl coenzyme A (acetyl CoA). For example, carbohydrates are metabolized to pyruvate, which is oxidized to acetyl CoA by the pyruvate dehydrogenase system.

[0053] Acetyl CoA is completely oxidized in a cyclic series of oxidative reactions alternately referred to as the tricarboxylic acid (TCA) cycle, the Krebs cycle or the citric acid cycle. Although certain of the TCA cycle enzymes are also found in the cytosol, where the enzymes function in other metabolic pathways, all of the TCA cycle enzymes are located in the mitochondria. The oxidation of acetyl CoA in one complete TCA cycle results in the production of two CO_2 molecules, one high energy phosphate bond (such as that present in GTP) and four reducing equivalents, i.e., three NADH and one $FADH_2$ from three NAD+ and one FAD, respectively.

[0054] Measurement of NADH and FAD has been previously used in cell cultures and analyses of precancerous epithelia (Skala et al., PNAS 104(49): 19494-19499, 2007). Skala et al. showed that a decrease in protein-bound NADH, and an increase in protein bound FAD fluorescence lifetimes were associated with different states of epithelial cancer. It has also been shown that the fluorescent lifetime of free and protein-bound NADH decrease with hypoxia (Bird et al. Cancer Res. 65: 8766-8773, 2005; Schneckenburger et al., J. Fluorescence 14: 649-654, 2004) and decrease in pre-cancers of hamster cheek pouch model of oral cancer in vivo (Skala et al. J. Biomed. Opt. 12:024014, 2007).

[0055] It has been suggested that measuring pyruvate levels in oocyte/embryo culture media as a means of assessing metabolic activity is inadequate for a number of reasons. For example, the contribution of pyruvate to the media from cumulus cells surrounding the oocyte cannot be predicted. In addition, the metabolic profile of the oocyte/embryo is complicated by the changing nutrient (e.g., substrate) requirements of the developing embryo from immature oocyte to blastocyst. In the oocyte and early stage embryo, the vast majority

of pyruvate that is taken up by the cell is channeled via acetyl CoA into the mitochondrial TCA cycle and oxidative phosphorylation (Wales, R. et al, (1970) Aust. J. Biol. Sci. 23, 877-887). Pyruvate is required to support the first and second cleavage divisions of the embryo in culture (Biggers, J. et al, (1967) Proc. Natl. Acad. Sci. U.S.A. 58(2), 560-567), whereas glucose is unable to support development until the four-cell stage. Glucose is the predominant nutrient required by the blastocyst (Brinster, R. et al, (1966) Exp. Cell Res. 42, 303-315). Cells in culture exhibit different nutrient requirements over time. For example, the oxidative metabolism of cultured cells declines over time; such cells become increasingly dependent on anaerobic glycolysis with concomitant lactate production (Morgan, M. et al, (1981) Biosci. Rep. 1, 669-686). Thus, for example, mouse blastocysts which have been cultured in vitro produce almost twice as much lactate as blastocysts which are freshly collected (Gardner, D. et al, (1990) J. Reprod. Fertil. 88, 361-368). Therefore, the simple measurement of pyruvate (nutrient) in culture media is not necessarily a reliable or accurate measure of the mitochondrial or metabolic status of the oocyte/embryo.

[0056] The oocyte/embryo contains a steady-state concentration of NADH that is present in the cell(s) as a result of metabolic reactions taking place in the mitochondria (e.g., tricarboxylic acid cycle and electron transport) and in the cytosol (e.g., glycolysis). Although one might expect to find a correlation between metabolic activity and oocyte/embryo viability, past efforts failed to establish any such direct correlation. For example, a study by Conaghan et al, J. Assist. Reprod. Genet 10(1): 21-30, 1993, reported that pyruvate uptake by human embryos was not predictive of those that successfully implanted.

[0057] The findings in U.S. Patent No. 5,541,081 suggested that to obtain information about the metabolic state of an embryo/oocytes using NADH, one must first reduce the endogenous NADH concentration of the oocyte/embryo by placing it in a control medium and obtaining at least one control NADH fluorescence measurement. After the control measurement, the oocyte/embryo was then subjected to a different medium with a nutrient for a time period and the change in the NADH concentration was observed. This analysis is not only time consuming but also subjects the embryo/oocyte to an additional and unnecessary stress when it is moved from special medium to another.

[0058] Contrary to the prior reported methods for embryo assessment, the method of the present invention allows direct analysis of NADH and/or FAD inside an embryo/oocyte/cumulus cell/cell from embryo without subjecting the embryo/oocyte/cumulus cell/cell from embryo to changes in its culture medium. Due to the specificity of FLIM, we have shown that we can observe the fluorescent lifetime of protein

bound and free NADH and/or FAD which we have found is indicative of the metabolic activity of the embryo/oocyte and predictive of, e.g., implantation success.

[0059] Fluorescence-lifetime imaging microscopy (FLIM) is an imaging technique for producing an image based on the differences in the exponential decay rate of the fluorescence from a fluorescent sample. FLIM can be used as an imaging technique in confocal microscopy, two-photon excitation microscopy, and multiphoton tomography.

[0060] The lifetime of the fluorophore signal, rather than its intensity, is used to create the image in FLIM. This has the advantage of minimizing the effect of photon scattering in thick layers of sample.

[0061] A fluorophore which is excited by a photon will drop to the ground state with a certain probability based on the decay rates through a number of different (radiative and/or nonradiative) decay pathways. To observe fluorescence, one of these pathways must be by spontaneous emission of a photon. This can be utilized for making non-intensity based measurements in chemical sensing.

[0062] Fluorescence lifetimes can be determined in the time domain by using a pulsed source.

[0063] Time-correlated single-photon counting (TCSPC) is usually employed. More specifically, TCSPC records times at which individual photons are detected by something like a photo-multiplier tube (PMT) or an avalanche photo diode (APD) after a single pulse. The recordings are repeated for additional pulses, and after enough recorded events one is able to build a histogram of the number of events across all of these recorded time points. This histogram can then be fit to a function that contains parameters of interest, and thus the parameters can be accordingly be extracted. 16~64 multichannel PMT systems have been commercially available, whereas the recently demonstrated CMOS single-photon avalanche diode (SPAD)-TCSPC FLIM systems can offer additional low-cost options.

[0064] Pulse excitation is still used in the gating method. Before the pulse reaches the sample, some of the light is reflected by a dichroic mirror and gets detected by a photodiode that activates a delay generator controlling a gated optical intensifier (GOI) that sits in front of your CCD detector. The GOI only allows for detection for the fraction of time when it is open after the delay. Thus, with an adjustable delay generator, one is able to collect fluorescence emission after multiple delay times encompassing the time range of the fluorescence decay of the sample.

[0065] Alternatively, fluorescence lifetimes can be determined in the frequency domain by a phase-modulated method. The intensity of a continuous wave source is modulated at high

frequency, by an acousto-optic modulator for example, which will modulate the fluorescence. Since the excited state has a lifetime, the fluorescence will be delayed with respect to the excitation signal, and the lifetime can be determined from the phase shift. Also, y-components to the excitation and fluorescence sine waves will be modulated, and lifetime can be determined from the modulation ratio of these y-components. Hence, 2 values for the lifetime can be determined from the phase-modulation method. Consequently, if the lifetimes that are extracted from the y-component and the phase do not match, it means that you have more than one lifetime species in your sample.

[0066] FLIM has primarily been used in biology as a method to detect photosensitizers in cells and tumors as well as FRET in instances where ratiometric imaging is difficult. The technique was developed in the late 1980s and early 1990s (Bugiel et al. 1989. König 1989) before being more widely applied in the late 1990s (Oida T, Sako Y, Kusumi A (March 1993). "Fluorescence lifetime imaging microscopy (flimscopy). Methodology development and application to studies of endosome fusion in single cells". Biophys. J. 64 (3): 676–85). In cell culture, it has been used to study EGF receptor signaling (Wouters FS, Bastiaens PI (October 1999). "Fluorescence lifetime imaging of receptor tyrosine kinase activity in cells". Curr. Biol. 9 (19): 1127–30) and ErbB1 receptor trafficking (Verveer PJ, Wouters FS, Reynolds AR, Bastiaens PI (November 2000). "Quantitative imaging of lateral ErbB1 receptor signal propagation in the plasma membrane". Science 290 (5496): 1567–70). FLIM imaging is particularly useful in neurons, where light scattering by brain tissue is problematic for ratiometric imaging (Yasuda R (October 2006). "Imaging spatiotemporal dynamics of neuronal signaling using fluorescence resonance energy transfer and fluorescence lifetime imaging microscopy". Curr. Opin. Neurobiol. 16 (5): 551–61). In neurons, FLIM imaging using pulsed illumination has been used to study Ras (Harvey CD, Yasuda R, Zhong H, Svoboda K (July 2008). "The spread of ras activity triggered by activation of a single dendritic spine". Science 321 (5885): 136–40), CaMKII, Rac, and Ran (The design of Forester (fluorescence) resonance energy transfer (FRET)-based molecular sensors for Ran GTPase, in press P. Kalab, J. Soderholm, Methods (2010) family proteins). FLIM has also been used in clinical multiphoton tomography to detect intradermal cancer cells as well as pharmaceutical and cosmetical compounds.

[0067] The cells in the present method can be analyzed in any suitable cell culture medium used for embryo/oocyte/cumulus cell/cell from embryo. Thus, the present methods avoid subjecting the cells to any extraordinary medium changes. Moreover, because there is no

need to change the metabolic state of the cell, like e.g., in the '081 patent, no additional time is needed for the analysis, making the analysis fast and convenient.

[0068] The measurement consists of acquiring a single FLIM image per cell, which can be obtained rapidly and non-invasively or minimally invasively. Typically, it takes about 1-5 minutes, sometimes 30 seconds to 2 minutes to load a sample containing the cells to be studied and only seconds to acquire the data after which the oocyte/embryo that has been analyzed can either be selected for further fertilization or implantation or discarded as not optimally fit for these procedures.

[0069] The FLIM measurement is taken directly inside the embryo using the autoflourescence of nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide (FAD) both molecules involved in cellular metabolism.

[0070] In the methods of the present invention, the acquired data are typically subsequently averaged over the entire cell, because subcellular information is unnecessary, producing one FLIM curve per cell.

[0071] While we have used mouse embryos and oocytes in our examples provided herein, the assay will not need to be altered when analyzing human cells. The metabolic state of human and mouse embryos and oocytes are comparable during these stages of development and thus the results obtained with mouse oocytes/embryos can be directly applied to human cells as well.

[0072] In essence, the method of the invention comprises exposing a test cell to a fluorescence lifetime imaging microscope (FLIM) to acquire a fluorescence lifetime histogram of auto-fluorescence of endogenous NADH and/or FAD for the test cell. The test cell can be an oocyte or an oocyte-associated cumulus cell or an embryo or a cell from the embryo.

[0073] Before the exposure, the test cell can be in or be placed in any normal cell culture medium used in maintaining the embryos/oocytes at the clinic. Culture media for embryo development should meet the metabolic needs of pre-implantation embryos by addressing amino acid and energy requirements based on the specific developmental stage of the embryo.

[0074] Various culture media are and have been used in ART methods and any of them can be used in the methods of the invention. The following provides some examples of culture media. Culture media, like Earle, Ham's F10, Tyrode's T6 and Whitten's WM1 were based on different salts and were constructed to support the development of somatic cells and cell lines in culture. These culture media, known as physiological salt solutions were used by

14

Robert Edwards for his first successful In Vitro Fertilization (IVF). These media were formulated for use with or without serum supplementation, depending on the cell type being cultured. The Ham's Nutrient Mixtures were originally developed to support growth of several clones of Chinese hamster ovary (CHO) cells, as well as clones of HeLa and mouse L-cells.

[0075] Menezo et al., 1984, suggested adding serum albumin as a source for amino acids. The serum protein ensures that oocytes and embryos do not adhere to the glass surface of the pipette used to manipulate them. The medium entitled B2, is still in use today. In 1985 Quinn et al. published in the journal Fertility and Sterility a formula entitled Human Tubal Fluid (HTF), which mimics the in vivo environment to which the embryo is exposed. The formulation of HTF was based on the known chemical composition of the fluids in human fallopian tubes as known at that time. This medium is based on a simple balanced salt solution without amino acids; however, the concentration of potassium was adjusted to that measured in the human female reproductive tract. This medium was found to be better compared with earlier media developed.

[0076] The supplementation of the HTF medium with either whole serum or with serum albumin became a gold standard for the production of culture medium for human embryos transferred on day 2 or day 3 of culture, for example 1-4 days in culture, 2-3 days in culture, 2-4 days in culture.

[0077] It is typically considered that culturing embryos involves addressing specific needs depending on the developmental stage of the embryo. Energy source requirements evolve from a pyruvate-lactate preference while the embryos, up to the 8-cell stage, are under maternal genetic control, to a glucose based metabolism after activation of the embryonic genome that supports their development from 8-cells to blastocysts. This observation lead to the development of the first commercial media. The culture media developed was based on HTF: both media were free of inorganic phosphate, glucose and amino acids. Pool and his colleagues formulated HTF which was free of glucose and phosphate. Cleaving embryos use pyrovate and lactate as energy sources and non-essential amino acids (NEAA) for protein metabolism. From the 8-cell stage the major energy source is glucose and for protein metabolism the embryos use essential amino acids (EAA). These findings lead to development of composition of two culture media G1 and G2 that are to be used in sequence. G1 supports the in-vitro development of the fertilized oocyte, the zygote, to the 8-cell stage, and G2 from 8-cells to blastocyst. Several modifications to these media also exist and are

15

well known to one skilled in the art. Sequential media are now being used successfully in IVF treatment all over the world.

[0078] Any of the above-discussed media may be used when imaging the test cells according to the methods of the invention.

[0079] The typical composition of the embryo culture medium includes: culture media containing a phosphate buffer or Hepes organic buffer are used for procedures that involve handling of gametes outside of the incubator, flushing of follicles and micromanipulation. The pH and osmolality for most culture media utilize a bicarbonate/CO_2 buffer system to keep pH in the range of 7.2-7.4. The osmolarity of the culture medium should be in the range of 275-290 mosmol/kg. Similar conditions should optimally be maintained while imaging the cells according to the methods of the invention.

[0080] In addition, the human oocyte is temperature-sensitive and a humidified incubator with a temperature setting of 37.0-37.5°C should be used for oocyte fertilization and embryo culture. Similar temperature should optimally be maintained while imaging the cells according to the methods of the invention.

[0081] Embryos should be cultured under paraffin oil, which prevents evaporation of the medium preserving a constant osmolarity. The oil also minimizes fluctuations of pH and temperature when embryos are taken out of the incubator for microscopic assessment. Paraffin oil can be toxic to gametes and embryos; therefore, batches of oil must be screened and tested on mouse embryos before use in culture of human embryos. The oil does not need to be removed to perform the FLIM analysis of the invention.

[0082] The medium is also composed of 99% water. Purity of the water is important, and is typically achieved by ultrafiltration.

[0083] Albumin or synthetic serum are typically added in concentrations of 5 to 20% (w/v or v/v, respectively). The commercial media typically includes synthetic serum in which the composition is well known.

[0084] Commercial IVF media typically comprises, for example, one or more of the following components: synthetic serum, recombinant albumin, salt solution in MTF, NaCl, KCl, KH_2PO_4, $CaCl_22H_2O$, $MgSO_47H2O$, $NaHCO_3$, and carbohydrates.

[0085] Carbohydrates are present in the female reproductive tract. Their concentrations vary throughout the length of the oviduct and in the uterus, and are also dependent on the time of the cycle.

[0086] Together with the amino acids the carbohydrates are the main energy source for the embryo. Culture media that support the development of zygotes up to 8-cells contain

pyruvate and lactate. Some commercial media are glucose free, while others add a very low concentration of glucose to supply the needs of the sperm during conventional insemination.

[0087] Media that support the development of 8-cell embryos up to the blastocyst stage contain pyruvate and lactate in low concentrations and a higher concentration of glucose.

[0088] Amino acids supplement of the culture medium with amino acids is necessary for embryo development. Media that support the development of zygotes up to 8-cells are often further supplemented with non-essential amino acids. Proline, serine, alanine, aspargine, aspartate, glycine, glutamate. Media that support the development of 8-cell embryos up to the blastocyst stage are typically supplemented with essential amino acids: Cystine, histadine, isolucine, leucine, lysine, methionine, valine, argentine, glutamine, phenylalanine, therionine, tryptophane.

[0089] The majority of ART laboratories use culture media containing antibiotics to minimize the risks of microbial growth. The most commonly used antibiotics being Penicillin (β-lactam Gram-positive bacteria disturbs cell wall integrity) and Streptomycin (Aminoglycoside Gram-negative bacteria disturbs protein synthesis). The anti-bacterial effect of penicillin is attributed to its ability to inhibit the synthesis of peptidoglycan, unique glycoproteins of the bacterial cell wall. Streptomycin and gentamycin belong to the aminoglycoside group of antibiotics which exert their antibacterial effect by inhibiting bacterial protein synthesis. The use of genthamicine is still controversial and it is not being used by every laboratory.

[0090] Often, the culture medium also comprises EDTA which is used as a chelator in medium that supports the embryo from the zygote stage to 8-cells and prevents abnormal glycolysis.

[0091] As noted before, the methods of the invention are not dependent on the type of the medium. The cells should remain in the medium and conditions they are cultured to avoid additional stress to them during the FLIM analysis.

[0092] The method further comprises averaging the fluorescence lifetime histogram of NADH auto-fluorescence or FAD auto-fluorescence or both over the entire test cell, or the cytoplasm of the test cell, or mitochondria of the test cell.

[0093] To be able to select the healthy cells for further assisted reproductive methods, the method also comprises comparing the averaged fluorescence lifetime histogram from the test cell to an averaged fluorescence lifetime histogram reference value to determine if the measured averaged fluorescence lifetime histogram from the test cell differs statistically from that of the reference value. The comparing is typically made using a non-human machine

17

typically using a computer executable software which includes a comparison between a reference value and the value from each individual cell FLIM analyses.

[0094] Similarly to NADH, also autofluorescence of FAD can be analyzed using FLIM in the methods of the present invention.

[0095] The FLIM curves of NADH crom cells exhibit a double exponential decay with a long lifetime (about 2.5 nanoseconds (ns)) corresponding to protein bound NADH and a short lifetime (about 0.4 ns) corresponding to free NADH. Thus, the FLIM curve is a double exponential with the relative fraction of the long and short lifetimes reflecting the relative fraction of protein bound and free NADH. This provides a direct readout of the metabolic state of the cell (Lacowciz et al., 1992). The long lifetime might vary from 1-3 (nanoseconds) ns and the short life time might vary from 0.2 – 0.7 ns.

[0096] The precise value of these lifetimes in the free and bound states depends on a variety of cellular factors, such as pH (Ogikubo et al., 2011). The relative fraction of the long and short lifetimes, and the precise value of these lifetimes is typically determined by using a least-squares fit. However, Bayesian inferences approaches, may allow even more precise parameter estimates and reference estimates with fewer number of photons, allowing even less light to be used to obtain further reliability and further minimizing sample exposure.

[0097] The absorption and fluorescence spectra of NADH (the reduced form) have been well characterized at different levels of organization, i.e., in solution, mitochondria and cell suspensions, tissue slices, and organs in vitro and in vivo. NADH has an optical absorption band at about 300 to 380 nm and a fluorescence emission band at 420 to 480 nm. The spectra are considered the same, although there are small differences in the shape and maxima of the spectra for different environments and measurement conditions. However, there is a universal agreement that the intensity of the fluorescence band, independent of the organization level of the environment, is proportional to the concentration of mitochondrial NADH (the reduced form), particularly when measured in vivo from a tissue (see, e.g., review by Avraham Mayevsky and Gennady G. Rogatsky, Am J Physiol Cell Physiol February 2007 vol. 292 no. 2 C615-C640).

[0098] Accordingly, in some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of about 740 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around about 460 nm.

[0099] In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of from about 720 nm to about 760

nm in two-photon fluorescence excitation and using an emission bandpass filter centered around from about 400 nm to about 485 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of from about 720 nm to about 760 nm in two-photon fluorescence excitation and using an emission bandpass filter of about 50 nm bandwidth centered around from about 400 nm to about 485 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of from about 720 nm to about 760 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around about 460 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of from about 720 nm to about 760 nm in two-photon fluorescence excitation and using an emission bandpass filter of about 50 nm bandwidth centered around about 460 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of about 740 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around about 460 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of about 740 nm in two-photon fluorescence excitation and using an emission bandpass filter of about 50 nm bandwidth centered around about 460 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of about 750 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around about 460 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of about 750 nm in two-photon fluorescence excitation and using an emission bandpass filter of about 50 nm bandwidth centered around about 460 nm.

[00100]   In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of from 720 nm to 760 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around from 400 nm to 485 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of from 720 nm to 760 nm in two-photon fluorescence excitation and using an emission bandpass filter of about 50 nm bandwidth centered around from 400 nm to 485 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of from 720 nm to 760 nm in two-

photon fluorescence excitation and using an emission bandpass filter centered around 460 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of from 720 nm to 760 nm in two-photon fluorescence excitation and using an emission bandpass filter of 50 nm bandwidth centered around 460 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of 740 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around 460 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of 740 nm in two-photon fluorescence excitation and using an emission bandpass filter of 50 nm bandwidth centered around 460 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of 750 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around 460 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can be analyzed with FLIM using a wavelength of 750 nm in two-photon fluorescence excitation and using an emission bandpass filter of 50 nm bandwidth centered around 460 nm.

[00101]   In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can also be analyzed with FLIM using a wavelength of from about 300 nm to about 380 nm in one-photon fluorescence excitation and using an emission bandpass filtered centered around about 550 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can also be analyzed with FLIM using a wavelength of from about 300 nm to about 380 nm in one-photon fluorescence excitation and using an emission bandpass filter of about 80 nm bandwidth centered around about 550 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can also be analyzed with FLIM using a wavelength of about 340 nm in one-photon fluorescence excitation and using an emission bandpass filter centered around about 460 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can also be analyzed with FLIM using a wavelength of about 340 nm in one-photon fluorescence excitation and using an emission bandpass filter of about 80 nm bandwidth centered around about 460 nm.

[00102]   In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can also be analyzed with FLIM using a wavelength of from 300 nm to 380 nm in one-photon fluorescence excitation and using an emission bandpass filtered

centered around 550 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can also be analyzed with FLIM using a wavelength of from 300 nm to 380 nm in one-photon fluorescence excitation and using an emission bandpass filter of 80 nm bandwidth centered around 550 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can also be analyzed with FLIM using a wavelength of 340 nm in one-photon fluorescence excitation and using an emission bandpass filter centered around 460 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of NADH can also be analyzed with FLIM using a wavelength of 340 nm in one-photon fluorescence excitation and using an emission bandpass filter of 80 nm bandwidth centered around 460 nm.

[00103]   In some aspects of all the embodiments of the invention, the direct autofluorescence of FAD can be analyzed with FLIM using a wavelength of from about 810 nm to about 950 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around from about 550 nm to about 600 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of FAD can be analyzed with FLIM using a wavelength of from about 810 nm to about 950 nm in two-photon fluorescence excitation and using an emission bandpass filter of about 80 nm bandwidth centered around from about 550 nm to about 600 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of FAD can be analyzed with FLIM using a wavelength of from about 810 nm to about 950 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around about 550 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of FAD can be analyzed with FLIM using a wavelength of from about 810 nm to about 950 nm in two-photon fluorescence excitation and using an emission bandpass filter of about 80 nm bandwidth centered around about 550 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of FAD can be analyzed with FLIM using a wavelength of about 845 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around about 550 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of FAD can be analyzed with FLIM using a wavelength of about 845 nm in two-photon fluorescence excitation and using an emission bandpass filter of about 80 nm bandwidth centered around about 550 nm. In some aspects of all the embodiments of the invention, the direct autofluorescence of FAD can be analyzed with FLIM using a wavelength of about 900 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around about 550 nm. In some aspects of all the embodiments of the

invention, the direct autofluorescence of FAD can be analyzed with FLIM using a
wavelength of about 900 nm in two-photon fluorescence excitation and using an emission
bandpass filter of about 80 nm bandwidth centered around about 550 nm.

[00104]   In some aspects of all the embodiments of the invention, the direct
autofluorescence of FAD can be analyzed with FLIM using a wavelength of from 810 nm to
950 nm in two-photon fluorescence excitation and using an emission bandpass filter centered
around from 550 nm to 600 nm. In some aspects of all the embodiments of the invention, the
direct autofluorescence of FAD can be analyzed with FLIM using a wavelength of from 810
nm to 950 nm in two-photon fluorescence excitation and using an emission bandpass filter of
80 nm bandwidth centered around from 550 nm to 600 nm. In some aspects of all the
embodiments of the invention, the direct autofluorescence of FAD can be analyzed with
FLIM using a wavelength of from 810 nm to 950 nm in two-photon fluorescence excitation
and using an emission bandpass filter centered around 550 nm. In some aspects of all the
embodiments of the invention, the direct autofluorescence of FAD can be analyzed with
FLIM using a wavelength of from 810 nm to 950 nm in two-photon fluorescence excitation
and using an emission bandpass filter of 80 nm bandwidth centered around 550 nm. In some
aspects of all the embodiments of the invention, the direct autofluorescence of FAD can be
analyzed with FLIM using a wavelength of 845 nm in two-photon fluorescence excitation
and using an emission bandpass filter centered around 550 nm. In some aspects of all the
embodiments of the invention, the direct autofluorescence of FAD can be analyzed with
FLIM using a wavelength of 845 nm in two-photon fluorescence excitation and using an
emission bandpass filter of 80 nm bandwidth centered around 550 nm. In some aspects of all
the embodiments of the invention, the direct autofluorescence of FAD can be analyzed with
FLIM using a wavelength of 900 nm in two-photon fluorescence excitation and using an
emission bandpass filter centered around 550 nm. In some aspects of all the embodiments of
the invention, the direct autofluorescence of FAD can be analyzed with FLIM using a
wavelength of 900 nm in two-photon fluorescence excitation and using an emission bandpass
filter of 80 nm bandwidth centered around 550 nm.

[00105]   In some aspects of all of the embodiments of the invention, the direct
autofluorescence of FAD can be analyzed with FLIM using a wavelength of from about 400
nm to about 450 nm in one-photon fluorescence excitation and using an emission bandpass
filter centered around about 550 nm. In some aspects of all of the embodiments of the
invention, the direct autofluorescence of FAD can be analyzed with FLIM using a
wavelength of from about 400 nm to about 450 nm in one-photon fluorescence excitation and

using an emission bandpass filter of about 80 nm bandwidth centered around about 550 nm. In some aspects of all of the embodiments of the invention, the direct autofluorescence of FAD can be analyzed with FLIM using a wavelength of about 450 nm in one-photon fluorescence excitation and using an emission bandpass filter of about 80 nm bandwidth centered around about 550 nm. In some aspects of all of the embodiments of the invention, the direct autofluorescence of FAD can be analyzed with FLIM using a wavelength of about 450 nm in one-photon fluorescence excitation and using an emission bandpass filter centered around about 550 nm.

[00106]   In some aspects of all of the embodiments of the invention, the direct autofluorescence of FAD can be analyzed with FLIM using a wavelength of from 400 nm to 450 nm in one-photon fluorescence excitation and using an emission bandpass filter centered around 550 nm. In some aspects of all of the embodiments of the invention, the direct autofluorescence of FAD can be analyzed with FLIM using a wavelength of from 400 nm to 450 nm in one-photon fluorescence excitation and using an emission bandpass filter of 80 nm bandwidth centered around 550 nm. In some aspects of all of the embodiments of the invention, the direct autofluorescence of FAD can be analyzed with FLIM using a wavelength of 450 nm in one-photon fluorescence excitation and using an emission bandpass filter of 80 nm bandwidth centered around 550 nm. In some aspects of all of the embodiments of the invention, the direct autofluorescence of FAD can be analyzed with FLIM using a wavelength of 450 nm in one-photon fluorescence excitation and using an emission bandpass filter centered around 550 nm.

[00107]   The embryo analyzed according to the methods of the invention is a pre-implantation embryo, and the analysis is performed in vitro. Similarly, a cell obtained from an embryo is obtained from a pre-implantation embryo and the cell biopsy is performed in vitro.

[00108]   One can combine the analysis using FLIM of NADH and FAD by simply exposing the cells sequentially to wavelength suitable for NADH and then to FAD or wavelength suitable for imaging the fluorescence lifetime of FAD and then NADH. Accordingly, in some aspects of all the embodiments of the invention the method comprises a sequential analysis of FAD and NADH.

[00109]   The analysis typically only takes a short time, such as 30 seconds to 5 minutes and can be multiplexed and automated.

[00110]   Thus, we provide a method for assessing the quality of an oocyte or an embryo, the method comprising (a) exposing, in a medium, granulosa cell(s), oocyte, the

23

embryo or cell(s) from the embryo to a fluorescence lifetime imaging microscopy (FLIM) to acquire a fluorescence lifetime histogram of auto-fluorescence of NADH in the granulosa cells, oocyte, the embryo or cell(s) from the embryo; (b) averaging the fluorescence lifetime histogram of auto-fluorescence of NADH over the entire granulosa cell, oocyte, the embryo or cell(s) from the embryo; (c) fitting the averaged fluorescence lifetime histogram of auto-fluorescence of NADH to a sum of two exponentials; and (d) selecting for in vitro fertilization or implantation the oocyte or embryo when one detects a cell, whether granulosa cells, oocyte, the embryo or cell(s) from the embryo, in which the alpha is less than an alpha reference value and the beta is less than a beta reference value or discarding the oocyte or embryo from in vitro fertilization when one detects a cell, whether granulosa cells, oocyte, the embryo or cell(s) from the embryo, in which the alpha is equal or more than the alpha reference value and if the beta is equal or more than the beta reference value. The cell can be an embryo, oocyte, or a cell extracted from an embryo or a cumulus cell surrounding an oocyte. In some aspects, the method further comprises a step of in vitro fertilizing the oocyte when one detects a cell, either oocyte or one or more cumulus cell from around the oocyte where the alpha is less than an alpha reference value and the beta is less than a beta reference value, or implanting the embryo when one detects a cell from the embryo or an embryo where the alpha is less than an alpha reference value and the beta is less than a beta reference value.

[00111]   In some aspects of all the embodiments of the invention, the alpha reference value is between 1 and 4, such as 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, or 2-3, or 2-4, or 1-2, or 1-3. In some aspects of all the embodiments of the invention, the alpha reference value is 1.7.

[00112]   In some aspects of all the embodiments of the invention, the beta reference value is 2000 ps - 3000 ps, or 2000-2500, or 2500-3000, or 2000-2250, or 2000-2750. In some aspects of all the embodiments the beta reference value is 2250 ps (picoseconds). Figure 1 provides an example of suitable alpha and beta values for evaluation of oocytes.

[00113]   A typical analysis of the metabolic health of an oocyte includes calculating comprehensive "metabolic scores" for oocytes or embryos. The scores represent the metabolic health of the oocyte or embryo, and therefore the general viability. One way to calculate the score comprises or consists of deriving from all the parameters obtained in one FLIM measurement, namely, (1) fraction bound, (2) short and long lifetimes, and (3) average

brightness for both FAD and NADH. Combination of the parameters may provide a more accurate assessment of the metabolic state of the target oocyte or embryo.

[00114]   An example of the calculation of the scores is provided in Figure 5, in which the positive score is reflective of better health compared to the negative scores.

[00115]   While our preliminary analyses have been performed using mouse oocytes as a model, the metabolic state of oocytes and embryos in both humans and mice is practically identical. Therefore, the reference values obtained from the metabolic state FLIM analysis in a mouse cumulus cell, oocyte and/or embryo, can be directly applied to human oocyte and embryo analysis. The reference values can also be obtained from human cumulus cell, oocyte and/or embryo.

[00116]   In some aspects of all the embodiments of the invention, the FLIM of NADH is performed using a wavelength of about 740 nm in two-photon excitation and using an emission bandpass filter centered around about 460 nm.

[00117]   In some aspects of all the embodiments of the invention, one obtains FLIM images at different times instead of only acquiring a single image. This kind of time-lapse data would be richer, and thus can provide more information about the cells. However, it will require cells to be in the device for longer periods of time and they would be exposed to more light, thereby slightly increasing the invasiveness of the method.

[00118]   The described method is typically performed using a system that contains nearly all of the components of a traditional microscope. Thus the method can be combined with analysis of morphological time-lapse data as well.

[00119]   There are three possible options for performing the FLIM measurements, all of which can be used in the methods of the present invention: two-photon excitation with a point detector, one-photon excitation with a point detector, or one-photon detection with an area detector (i.e. a camera). The table below indicates the advantages of each of the options. We used the two-photon method in our examples.

	Two-Photon Point Detector	One-Photon Point Detector	One-Photon Camera
Sensitivity	Highest	Medium	Lowest
Cost	Highest	Medium	Lowest
Robustness	Lowest Robustness	Medium Robustness	Most Robust
Complexity	Simple Optical Setup with Stage Scanning Imaging	Complex Optical Setup with Stage Scanning Imaging	Simple Optical Setup

Safety Considerations	Long Wavelength (reduces possible damage)	Shorter Wavelength	Shorter Wavelength

[00120] In some embodiments, the methods described herein can relate to obtaining, e.g., an average metabolic score for a subject and/or a sample obtained from the subject. In some embodiment, a plurality of test cells from a clinical sample can be assayed according the methods described herein such that an individual metabolic score is obtained for each of the test cells and the individual metabolic scores averaged to obtain an average metabolic score for the plurality of test cells. In some embodiments, the, e.g., standard deviation, of the average metabolic score can be obtained and/or reported. Such average metabolic scores can permit a determination of whether the average egg obtained from a subject will be suitable for, e.g., IVF and thus whether the subject should continue with IVF or how aggressive an approach to IVF should be considered for the individual subject. In some embodiments, the average metabolic score can be compared to reference values, e.g., metabolic scores for subjects who have previously attempted IVF and a probability of future success thereby provided.

[00121] In some embodiments of any of the aspects, the FLIM data is analyzed by fitting the lifetime histograms to a model. In some embodiments of any of the aspects, the FLIM data is analyzed by phasor analysis.

[00122] For convenience, the meaning of some terms and phrases used in the specification, examples, and appended claims, are provided below. Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. The definitions are provided to aid in describing particular embodiments, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. If there is an apparent discrepancy between the usage of a term in the art and its definition provided herein, the definition provided within the specification shall prevail.

[00123] For convenience, certain terms employed herein, in the specification, examples and appended claims are collected here.

[00124] The terms "decrease", "reduced", "reduction", or "inhibit" are all used herein to mean a decrease by a statistically significant amount. In some embodiments, "reduce,"

"reduction" or "decrease" or "inhibit" typically means a decrease by at least 10% as compared to a reference level (e.g. the absence of a given treatment) and can include, for example, a decrease by at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99% , or more. As used herein, "reduction" or "inhibition" does not encompass a complete inhibition or reduction as compared to a reference level. "Complete inhibition" is a 100% inhibition as compared to a reference level. A decrease can be preferably down to a level accepted as within the range of normal for an individual without a given disorder.

[00125]   The terms "increased", "increase", "enhance", or "activate" are all used herein to mean an increase by a statically significant amount. In some embodiments, the terms "increased", "increase", "enhance", or "activate" can mean an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level. In the context of a marker or symptom, a "increase" is a statistically significant increase in such level.

[00126]   As used herein, a "subject" means a human or animal. Usually the animal is a vertebrate such as a primate, rodent, domestic animal or game animal. Primates include chimpanzees, cynomologous monkeys, spider monkeys, and macaques, e.g., Rhesus. Rodents include mice, rats, woodchucks, ferrets, rabbits and hamsters. Domestic and game animals include cows, horses, pigs, deer, bison, buffalo, feline species, e.g., domestic cat, canine species, e.g., dog, fox, wolf, avian species, e.g., chicken, emu, ostrich, and fish, e.g., trout, catfish and salmon. In some embodiments, the subject is a mammal, e.g., a primate, e.g., a human. The terms, "individual," "patient" and "subject" are used interchangeably herein.

[00127]   Preferably, the subject is a mammal. The mammal can be a human, non-human primate, mouse, rat, dog, cat, horse, or cow, but is not limited to these examples.

Mammals other than humans can be advantageously used as subjects that represent animal models of infertility. A subject can be male or female.

[00128]   A subject can be one who has been previously diagnosed with or identified as suffering from or having a condition in need of treatment (e.g. fertility related conditions) or one or more complications related to such a condition, and optionally, have already undergone treatment for the condition or the one or more complications related to the condition. Alternatively, a subject can also be one who has not been previously diagnosed as having the condition or one or more complications related to the condition. For example, a subject can be one who exhibits one or more risk factors for the condition or one or more complications related to the condition or a subject who does not exhibit risk factors.

[00129]   A "subject in need" of treatment for a particular condition can be a subject having that condition, diagnosed as having that condition, or at risk of developing that condition.

[00130]   As used herein, the terms "treat," "treatment," "treating," or "amelioration" refer to therapeutic treatments, wherein the object is to reverse, alleviate, ameliorate, inhibit, slow down or stop the progression or severity of a condition associated with a disease or disorder. The term "treating" includes reducing or alleviating at least one adverse effect or symptom of a condition, disease or disorder associated with a condition. Treatment is generally "effective" if one or more symptoms or clinical markers are reduced. Alternatively, treatment is "effective" if the progression of a disease is reduced or halted. That is, "treatment" includes not just the improvement of symptoms or markers, but also a cessation of, or at least slowing of, progress or worsening of symptoms compared to what would be expected in the absence of treatment. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s), diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, remission (whether partial or total), and/or decreased mortality, whether detectable or undetectable. The term "treatment" of a disease also includes providing relief from the symptoms or side-effects of the disease (including palliative treatment).

[00131]   The term "test cell" as used herein denotes a cell taken or isolated from a biological organism, e.g., a oocyte from a subject. The test sample can be freshly collected or a previously collected sample.

[00132]   In some embodiments, the methods, assays, and systems described herein can further comprise a step of obtaining a test cell from a subject. In some embodiments, the subject can be a human subject.

[00133]   The term "statistically significant" or "significantly" refers to statistical significance and generally means a two standard deviation (2SD) or greater difference.

[00134]   Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used in connection with percentages can mean ±1%.

[00135]   As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the method or composition, yet open to the inclusion of unspecified elements, whether essential or not.

[00136]   The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[00137]   As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment.

[00138]   The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The abbreviation, "e.g." is derived from the Latin exempli gratia, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g." is synonymous with the term "for example."

[00139]   Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art to which this disclosure belongs. It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such can vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims. Definitions of common terms in immunology and molecular biology can be found in The Merck Manual of Diagnosis and Therapy, 19th Edition, published by Merck Sharp & Dohme Corp., 2011 (ISBN 978-0-

911910-19-3); Robert S. Porter *et al.* (eds.), The Encyclopedia of Molecular Cell Biology and Molecular Medicine, published by Blackwell Science Ltd., 1999-2012 (ISBN 9783527600908); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8); Immunology by Werner Luttmann, published by Elsevier, 2006; Janeway's Immunobiology, Kenneth Murphy, Allan Mowat, Casey Weaver (eds.), Taylor & Francis Limited, 2014 (ISBN 0815345305, 9780815345305); Lewin's Genes XI, published by Jones & Bartlett Publishers, 2014 (ISBN-1449659055); Michael Richard Green and Joseph Sambrook, Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (2012) (ISBN 1936113414); Davis *et al.*, Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (2012) (ISBN 044460149X); Laboratory Methods in Enzymology: DNA, Jon Lorsch (ed.) Elsevier, 2013 (ISBN 0124199542); Current Protocols in Molecular Biology (CPMB), Frederick M. Ausubel (ed.), John Wiley and Sons, 2014 (ISBN 047150338X, 9780471503385), Current Protocols in Protein Science (CPPS), John E. Coligan (ed.), John Wiley and Sons, Inc., 2005; and Current Protocols in Immunology (CPI) (John E. Coligan, ADA M Kruisbeek, David H Margulies, Ethan M Shevach, Warren Strobe, (eds.) John Wiley and Sons, Inc., 2003 (ISBN 0471142735, 9780471142737), the contents of which are all incorporated by reference herein in their entireties.

[00140]   Other terms are defined herein within the description of the various aspects of the invention.

[00141]   All patents and other publications; including literature references, issued patents, published patent applications, and co-pending patent applications; cited throughout this application are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the technology described herein. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

[00142]   The description of embodiments of the disclosure is not intended to be exhaustive or to limit the disclosure to the precise form disclosed. While specific

embodiments of, and examples for, the disclosure are described herein for illustrative purposes, various equivalent modifications are possible within the scope of the disclosure, as those skilled in the relevant art will recognize. For example, while method steps or functions are presented in a given order, alternative embodiments may perform functions in a different order, or functions may be performed substantially concurrently. The teachings of the disclosure provided herein can be applied to other procedures or methods as appropriate. The various embodiments described herein can be combined to provide further embodiments. Aspects of the disclosure can be modified, if necessary, to employ the compositions, functions and concepts of the above references and application to provide yet further embodiments of the disclosure. These and other changes can be made to the disclosure in light of the detailed description. All such modifications are intended to be included within the scope of the appended claims.

[00143]   Specific elements of any of the foregoing embodiments can be combined or substituted for elements in other embodiments. Furthermore, while advantages associated with certain embodiments of the disclosure have been described in the context of these embodiments, other embodiments may also exhibit such advantages, and not all embodiments need necessarily exhibit such advantages to fall within the scope of the disclosure.

[00144]   The technology described herein is further illustrated by the following examples which in no way should be construed as being further limiting.

[00145]   Some embodiments of the technology described herein can be defined according to any of the following numbered paragraphs:

1.   A method for assessing the quality of an oocyte or an embryo, the method comprising

(a)   exposing an embryo or a test cell or a plurality of test cells selected from an oocyte or an oocyte-associated cumulus cell or a cell from an embryo to a fluorescence lifetime imaging microscope (FLIM) to acquire a fluorescence lifetime histogram of auto-fluorescence of endogenous NADH and FAD for the embryo or the test cell;

(b)   averaging the fluorescence lifetime histogram of NADH auto-fluorescence and FAD auto-fluorescence over the entire embryo, test cell or test cells, or the cytoplasm of the test cell or cells, or mitochondria of the test cell or cells to assay measurements for fraction bound, short and long lifetimes, and average brightness for both FAD and NADH

(c)   combining the measurements for fraction bound, short and long lifetimes, and average brightness for both FAD and NADH to obtain a metabolic score;

(d)   comparing the metabolic score between the plurality of test cells to order them based on the metabolic score to determine the cells with the most optimal metabolic activity; and

(e)   selecting the embryo or oocyte or embryos or oocytes with the most optimal metabolic score for in vitro fertilization or for implantation or cryopreservation.

2.   The method of paragraph 1, wherein the FLIM is performed in the time domain.

3.   The method of paragraph 1, wherein the FLIM is performed in the frequency domain.

4.   The method of any one of paragraphs 1-3, wherein the FLIM is performed using a wavelength about 720 about 760 nm in two-photon fluorescence excitation and using an emission bandpass filter about 400 nm to about 485 nm.

5.   The method of any one of paragraphs 1-4, wherein the FLIM is performed using a wavelength of from about 720 to about 760 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around about 460 nm.

6.   The method any one of paragraphs 1-5, wherein the FLIM is performed using a wavelength of from about 720 to about 760 nm in two-photon fluorescence excitation and using an emission bandpass filter of about 50 nm bandwidth centered around about 460 nm.

7.   The method of any one of paragraphs 1-5, wherein the FLIM is performed using a wavelength of about 740 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around about 460 nm.

8.   The method of any one of paragraphs 1-5, wherein the FLIM is performed using a wavelength of about 740 nm in two-photon fluorescence excitation and using an emission bandpass filter of about 50 nm bandwidth centered around about 460 nm.

9.   The method of any one of paragraphs 1-5, wherein the FLIM is performed using a wavelength of about 750 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around about 460 nm.

10.   The method of any one of paragraphs 1-5, wherein the FLIM is performed using a wavelength of about 750 nm in two-photon fluorescence excitation and using an emission bandpass filter of about 50 nm bandwidth centered around about 460 nm.

11.   The method of any one of paragraphs 4-10, wherein the FLIM data of the autofluoresence of NADH is analyzed.

12.   The method of any one of paragraphs 1-11, wherein the FLIM is performed using a wavelength about 810 nm about 950 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around from about 550 to about 600 nm.

13.   The method of any one of paragraphs 1-12, wherein the FLIM is performed using a wavelength of from about 810 nm to about 950 nm in two-photon fluorescence excitation and using an emission bandpass filter of about 80 nm bandwidth centered around from about 550 to about 600 nm.

14.   The method of any one of paragraphs 1-13, wherein the FLIM is performed using a wavelength of from about 810 nm to about 950 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around about 550 nm.

15.   The method of any one of paragraphs 1-13, wherein the FLIM is performed using a wavelength of from about 810 nm to about 950 nm in two-photon fluorescence excitation and using an emission bandpass filter of about 80 nm bandwidth centered around about 550 nm.

16.   The method of any one of paragraphs 1-13, wherein the FLIM is performed using a wavelength of about 900 nm in two-photon fluorescence excitation and using an emission bandpass filter centered around about 550 nm.

17.   The method of any one of paragraphs 1-13, wherein the FLIM is performed using a wavelength of about 900 nm in two-photon fluorescence excitation and using an emission bandpass filter of about 80 nm bandwidth centered around about 550 nm.

18.   The method of any one of paragraphs 1-13, wherein the FLIM is performed using a wavelength of about 845 nm in two-photon fluorescence excitation and using an emission bandpass filtered centered around about 550 nm.

19.   The method of any one of paragraphs 1-13, wherein the FLIM is performed using a wavelength of about 845 nm in two-photon fluorescence excitation and using an emission bandpass filter of about 80 nm bandwidth centered around about 550 nm.

20.   The method of any one of paragraphs 12-19, wherein the FLIM data of the autofluoresence of FAD is analyzed.

21.   The method of any one of paragraphs 1-3, wherein the FLIM is performed using a wavelength of from about 300 nm to about 380 nm in one-photon fluorescence excitation and using an emission bandpass filter centered around about 550 nm.

22.   The method of any one of paragraphs 1-3 and 21, wherein the FLIM is performed using a wavelength of from about 300 nm to about 380 nm in one-photon fluorescence excitation and using an emission bandpass filter of about 80 nm bandwidth centered around about 550 nm.

23.   The method of any one of paragraphs 1-3, and 21-22, wherein the FLIM is performed using a wavelength of about 340 nm in one-photon fluorescence excitation and using an emission bandpass filter centered around about 460 nm.

24.   The method of any one of paragraphs 1-3 and 21-23, wherein the FLIM is performed using a wavelength of about 340 nm in one-photon fluorescence excitation and using an emission bandpass filter of about 80 nm bandwidth centered around about 460 nm.

25.   The method of any one of paragraphs 21-24, wherein the FLIM of the autofluoresence of NADH is analyzed.

26.   The method of any one of paragraphs 1-3 and 21-25, wherein the FLIM is performed using a wavelength of from about 400 nm to about 500 nm in one-photon fluorescence excitation and using an emission bandpass filter centered around about 550 nm.

27.   The method of any one of paragraphs 1-3 and 21-26, wherein the FLIM is performed using a wavelength of from about 400 nm to about 500 nm in one-photon fluorescence excitation and using an emission bandpass filter of about 80 nm bandwidth centered around about 550 nm.

28.   The method of any one of paragraphs 1-3 and 26-27, wherein the FLIM is performed using a wavelength of about 450 nm in one-photon fluorescence excitation and using an emission bandpass filter centered around about 550 nm.

29.   The method of any one of paragraphs 1-3 and 26-27, wherein the FLIM is performed using a wavelength of about 450 nm in one-photon fluorescence excitation and using an emission bandpass filter of about 80 nm bandwidth centered around about 550 nm.

30.   The method of any one of paragraphs 26-29, wherein the FLIM of the autofluoresence of FAD is analyzed.

31.   The method of any one of paragraphs 1-30, further comprising assaying a plurality of test cells from a clinical sample such that an individual metabolic score is obtained for each of the test cells and averaging the individual metabolic scores to obtain an average metabolic score for the plurality of test cells.

32.   The method of paragraph 31, where the average metabolic score is a patient-level score.

33.   The method of any of paragraphs 1-32, wherein the FLIM data is analyzed by fitting the lifetime histograms to a model.

34.   The method of any of paragraphs 1-32, wherein the FLIM data is analyzed by phasor analysis.

[00146]   Some embodiments of the technology described herein can be defined according to any of the following numbered paragraphs:

1.   A method for assessing the quality of an oocyte or an embryo, the method comprising

(a)   exposing an embryo or a test cell or a plurality of test cells selected from an oocyte or an oocyte-associated cumulus cell or a cell from an embryo to a fluorescence lifetime imaging microscope (FLIM) to acquire a fluorescence lifetime histogram of auto-fluorescence of endogenous NADH and FAD for the embryo or the test cell;

34

(b)   averaging the fluorescence lifetime histogram of NADH auto-fluorescence and FAD auto-fluorescence over the entire embryo, test cell or test cells, or the cytoplasm of the test cell or cells, or mitochondria of the test cell or cells to assay measurements for fraction bound, short and long lifetimes, and average brightness for both FAD and NADH

(c)   combining the measurements for fraction bound, short and long lifetimes, and average brightness for both FAD and NADH to obtain a metabolic score;

(d)   comparing the metabolic score between the plurality of test cells to order them based on the metabolic score to determine the cells with the most optimal metabolic activity; and

(e)   selecting the embryo or oocyte or embryos or oocytes with the most optimal metabolic score for in vitro fertilization or for implantation or cryopreservation.

2.   The method of paragraph 1, wherein the FLIM is performed in the time domain.

3.   The method of paragraph 1, wherein the FLIM is performed in the frequency domain.

4.   The method of any one of paragraphs 1-3, wherein the FLIM is performed using a wavelength between about 720 and about 760 nm in two-photon fluorescence excitation and using an emission bandpass filter of any wavelength range between about 400 nm to about 485 nm.

5.   The method of paragraph 4, wherein the FLIM data of the autofluoresence of NADH is analyzed.

6.   The method of any one of paragraphs 1-11, wherein the FLIM is performed using a wavelength between about 810 nm and about 950 nm in two-photon fluorescence excitation and using an emission bandpass filter of any wavelength range between about 550 and about 600 nm.

7.   The method of paragraph 6, wherein the FLIM data of the autofluoresence of FAD is analyzed.

8.   The method of any one of paragraphs 1-7, wherein the FLIM is performed using a wavelength between about 300 nm and about 380 nm in one-photon fluorescence excitation and using an emission bandpass filter of any wavelength range centered around about 550 nm.

9.   The method of paragraph 8, wherein the FLIM of the autofluoresence of NADH is analyzed.

10.   The method of any one of paragraphs 1-9 and 22-26, wherein the FLIM is performed using a wavelength between about 400 nm and about 500 nm in one-photon fluorescence

excitation and using an emission bandpass filter of any wavelength range centered around about 550 nm.

11.   The method of paragraph 10, wherein the FLIM of the autofluoresence of FAD is analyzed.

12.   The method of any one of paragraphs 1-11, further comprising assaying a plurality of test cells from a clinical sample such that an individual metabolic score is obtained for each of the test cells and averaging the individual metabolic scores to obtain an average metabolic score for the plurality of test cells.

13.   The method of paragraph 12, where the average metabolic score is a patient-level score.

14.   The method of any of paragraphs 1-13, wherein the FLIM data is analyzed by fitting the lifetime histograms to a model.

15.   The method of any of paragraphs 1-13, wherein the FLIM data is analyzed by phasor analysis.

EXAMPLES

Example 1

[00147]   We showed that oocytes originating from young and old mice have clearly differentiated metabolic activity.

[00148]   Oocyte metabolic state can be rapidly, non-invasively, and quantitatively measured by Fluorescence Lifetime Imaging Microscopy (FLIM) of NADH and FAD. Three parameters (short lifetime, long lifetime, and fraction bound to enzymes) are extracted from FLIM measurements of NADH and FAD in oocytes. Binding to enzymes causes the lifetime of the fluorophore to shift significantly, explaining the strong correlation in all three parameters. In the example, we used morphologically normal mouse oocytes.

[00149]   Similar calculations are expected to work for human oocytes, as mammalian cells are relatively similar, particularly relating to their metabolic state, at this stage.

[00150]   In Figures 2-4, each point corresponds to data from a single oocyte. 20 oocytes originating from old mice and 12 oocytes originating from young mice were tested. Gray dots represent oocytes originating from old mice and red dots represent oocytes originating from young mice.

[00151]   The Figures demonstrate a clear separation in the parameter values between these two groups. Given that it is clearly established that oocyte quality declines with age, this indicates that differences in parameter values are associated to differences in oocyte quality.

Example 2

[00152]   We obtained preliminary data on FLIM of NADH in mouse oocytes. The preliminary data was acquired on a FLIM system. The microscope consists of a ti-sapphire femtosecond laser (Spectra-Physics), an inverted microscope base (Nikon), a scan head (Becker & Hickl), a hybrid PMT detector (Hamamatsu), and electronics for time correlated single photon counting (Becker & Hickl). This microscope was assembled to acquire the preliminary data.

[00153]   Oocytes were placed in a medium on the microscope stage and imaged. A single image of each oocyte was analyzed by averaging the FLIM data over the entire oocyte. The acquired fluorescence lifetime histogram from NADH, averaged over the entire oocyte, was fit to a sum of two exponentials. The parameter alpha, is the ratio of the amplitude of the two exponentials, the parameter, beta, is the lifetime of the longer exponential (in picoseconds).

[00154]   Unperturbed oocytes exhibit a range of values of alpha and beta (Figure 1, circles). As seen in Figure 1, oocyte quality depends on oocyte metabolic state. Oocyte metabolic state can be rapidly, non-invasively, and quantitatively measured by Fluorescence Lifetime Imaging Microscopy (FLIM) of FAD or NADH. Two parameters (alpha and beta) are extracted from FLIM measurements of NADH (or FAD) in oocytes. In the example, we used mouse oocytes but similar calculations are expected to work for human oocytes, as the mammalian cells, such as mouse and human cells are relatively similar, particularly relating to their metabolic state, at this stage. Each point corresponds to data from a single oocyte. Perturbing oocytes by specific metabolic inhibitors (black crosses), or non-specific damage (triangles) causes both parameters to increase. Unperturbed oocytes (circles) exhibit a range of values of alpha and beta. These parameters are indicative of oocyte quality.

[00155]   Alpha and beta both increase when oocytes are perturbed by either applying a metabolic inhibitor (Figure 1, black circles) or greatly increasing laser power to intentionally damage the oocytes (Figure 1, triangles). Such damage does not occur at the low laser powers used for imaging.

[00156]   Our results show that low values of alpha and beta are indicative of oocyte and embryo health. All perturbed oocytes are found in the upper quadrant with alpha greater than 1.7 and beta greater than 2250. This shows that these values of alpha and beta can be used as cutoffs with oocytes above these values rejected as likely being unhealthy, and oocytes below these values deemed healthy and selected for use.

[00157]   The metabolism of human and mouse oocytes are very similar, and thus, cutoff values of alpha and beta established for mice can be used for human analyses as well.

[00158]   The values of alpha and beta are obtained quantitatively, rapidly, and objectively. Thus the described approach is highly useful in in vitro fertilization clinics.

REFERENCES

[00159]   The references cited herein and throughout the specification are herein incorporated by reference in their entirety.

[00160]   Botros, L., Sakkas, D. and Seli, E. Metabolomics and its application for non-invasive embryo assessment in IVF. Molecular Human Reproduction Vol.14, No.12 pp. 679–690, 2008.

[00161]   Lakowciz, J.R., Szmacinski, H., Nowaczyk, K., and Johnson, M.L. Fluorescence lifetime imaging of free and protein-bound NADH. PNAS. 1992. 89. 1271-1275

[00162] Ogikubo, S., et al,. Intracellular pH Sensing Using Autofluorescence Lifetime Microscopy. Journal of Physical Chemistry B. 2011. 115, 10385-10390.

[00163] Scott RT, Kerry F, Su J, Tao X, Scott K, Treff N. Comprehensive chromosome screening is highly predictive of the reproductive potential of human embryos: a prospective, blinded, non-selection study. Fertil Steril 2012; 97:870-5.

[00164] Vergouw CG, Kieslinger DC, Kostelijk EH, Botros LL, Schats R, Hompes PG, Sakkas D, Lambalk CB. Day 3 embryo selection by metabolomic profiling of culture medium with near-infrared spectroscopy as an adjunct to morphology: a randomized controlled trial. Hum Reprod. 2012; 27(8):2304-11.

[00165] Seli, E. and Brommer, J.G. Assessment of embryo viability in assisted reproductive technology: shortcomings of current approaches and the emerging role of metabolomics. Current Opinion in Obstetrics and Gynecology. 2008, 20:234–241

[00166] Society for Assisted Reproductive Technology (SART). Assisted reproductive technology success rates. National summary and fertility clinic reports. Atlanta, GA: Centers for Disease Control and Prevention; 2005

[00167] Stringaria, C., Cinquinb, A., Cinquinb, O., Digmana, M.A., Donovan, P.J., and Grattona, E. Phasor approach to fluorescence lifetime microscopy distinguishes different metabolic states of germ cells in alive tissue. PNAS. 2011. 108: 33. 13582–13587

[00168] Uyar A, Seli E. Embryo assessment strategies and their validation for clinical use: a critical analysis of methodology. Curr Opin Obstet Gynecol. 2012 Jun;24(3):141-50

[00169] Zipfel WR, et al. (2003) Live tissue intrinsic emission microscopy using multiphotonexcited native fluorescence and second harmonic generation. Proc Natl Acad Sci USA 100:7075–7080.Botros, L., Sakkas, D. and Seli, E. Metabolomics and its application for non-invasive embryo assessment in IVF. Molecular Human Reproduction Vol.14, No.12 pp. 679–690, 2008

[00170] Lakowciz, J.R., Szmacinski, H., Nowaczyk, K., and Johnson, M.L. Fluorescence lifetime imaging of free and protein-bound NADH. PNAS. 1992. 89. 1271-1275

[00171] Ogikubo, S., et al,. Intracellular pH Sensing Using Autofluorescence Lifetime Microscopy. Journal of Physical Chemistry B. 2011. 115, 10385-10390.

[00172] Scott RT, Kerry F, Su J, Tao X, Scott K, Treff N. Comprehensive chromosome screening is highly predictive of the reproductive potential of human embryos: a prospective, blinded, non-selection study. Fertil Steril 2012; 97:870-5.

[00173]   Vergouw CG, Kieslinger DC, Kostelijk EH, Botros LL, Schats R, Hompes PG, Sakkas D, Lambalk CB. Day 3 embryo selection by metabolomic profiling of culture medium with near-infrared spectroscopy as an adjunct to morphology: a randomized controlled trial. Hum Reprod. 2012; 27(8):2304-11.

[00174]   Seli, E. and Brommer, J.G. Assessment of embryo viability in assisted reproductive technology: shortcomings of current approaches and the emerging role of metabolomics. Current Opinion in Obstetrics and Gynecology. 2008, 20:234–241

[00175]   Society for Assisted Reproductive Technology (SART). Assisted reproductive technology success rates. National summary and fertility clinic reports. Atlanta, GA: Centers for Disease Control and Prevention; 2005

[00176]   Stringaria, C., Cinquinb, A., Cinquinb, O., Digmana, M.A., Donovan, P.J., and Grattona, E. Phasor approach to fluorescence lifetime microscopy distinguishes different metabolic states of germ cells in alive tissue. PNAS. 2011. 108: 33. 13582–13587

[00177]   Uyar A, Seli E. Embryo assessment strategies and their validation for clinical use: a critical analysis of methodology. Curr Opin Obstet Gynecol. 2012 Jun;24(3):141-50

[00178]   Zipfel WR, et al. (2003) Live tissue intrinsic emission microscopy using multiphotonexcited native fluorescence and second harmonic generation. Proc Natl Acad Sci USA 100:7075–7080.

Example 3

[00179]   Figure 6 depicts a graph of absorption(2 photon)-emission spectra graph for NADH and FAD. Lines indicate the laser illumination wavelengths for NADH (orange) and FAD (blue). The emission filters which isolate the fluorescence are shown on the left.

[00180]   As indicated by the data, ranges suitable for use in the methods described herein can include:

- NADH excitation - 720-760nm. Above 760 nm, NADH excitation decreases
- NADH emission - 400-485nm. Below 400 nm, the signal captured is not appreciably higher and above 485 nm, FAD fluorescence is captured and contaminates the signal
- FAD excitation - 810-950nm. Below 810 nm, NADH excitation occurs and contaminates the signal. Above 950 nm, the FAD excitation decreases.
- FAD emission - 500-600nm. NADH fluorescence is not a particular concern because it is not excited at 845 nm, thus permitting a broad spectrum for FAD emission. The range of 500-600 captures nearly the entire area under the curve.

[00181]   For 1 photon excitation, as depicted by Figure 7, the following ranges are suitable for use in the methods described herein.

- NADH excitation - 300-380nm
- FAD excitation - 400-500nm, this range avoids exciting NADH with wavelengths less than 400 nm

CLAIMS

We claim:

1.   A method for assessing the quality of an oocyte or an embryo, the method comprising

(a)   exposing an embryo or a test cell or a plurality of test cells selected from an oocyte or an oocyte-associated cumulus cell or a cell from an embryo to a fluorescence lifetime imaging microscope (FLIM) to acquire a fluorescence lifetime histogram of auto-fluorescence of endogenous NADH and/or FAD for the embryo or the test cell;

(b)   averaging the fluorescence lifetime histogram of NADH auto-fluorescence and/or FAD auto-fluorescence over the entire embryo, test cell or test cells, or the cytoplasm of the test cell or cells, or mitochondria of the test cell or cells to assay measurements for fraction bound, short and long lifetimes, and average brightness for both FAD and/or NADH

(c)   combining the measurements for fraction bound, short and long lifetimes, and average brightness for FAD and/or NADH to obtain a metabolic score;

(d)   comparing the metabolic score between the plurality of test cells to order them based on the metabolic score to determine the cells with the most optimal metabolic activity; and

(e)   selecting the embryo or oocyte or embryos or oocytes with the most optimal metabolic score for in vitro fertilization or for implantation or cryopreservation.

2.   The method of claim 1, wherein the FLIM is performed in the time domain.

3.   The method of claim 1, wherein the FLIM is performed in the frequency domain.

4.   The method of any one of claims 1-3, wherein the FLIM is performed using a wavelength between about 720 and about 760 nm to excite NADH with two-photon fluorescence excitation and using an emission bandpass filter of any wavelength range between about 400 nm to about 485 nm to isolate fluorescence from NADH.

5.   The method of claim 4, wherein the FLIM data of the autofluoresence of NADH is analyzed.

6.   The method of any one of claims 1-11, wherein the FLIM is performed using a wavelength between about 810 nm and about 950 nm to excite FAD with two-photon fluorescence excitation and using an emission bandpass filter of any wavelength range between about 550 and about 600 nm to isolate fluorescence from FAD.

7.   The method of claim 6, wherein the FLIM data of the autofluoresence of FAD is analyzed.

8.   The method of any one of claims 1-7, wherein the FLIM is performed using a wavelength between about 300 nm and about 380 nm to excite NADH with one-photon fluorescence excitation and using an emission bandpass filter of any wavelength range centered around about 550 nm to isolate fluorescence from NADH.

9.   The method of claim 8, wherein the FLIM of the autofluoresence of NADH is analyzed.

10.   The method of any one of claims 1-9 and 22-26, wherein the FLIM is performed using a wavelength between about 400 nm and about 500 nm to excite FAD with one-photon fluorescence excitation and using an emission bandpass filter of any wavelength range centered around about 550 nm to isolate fluorescence from FAD.

11.   The method of claim 10, wherein the FLIM of the autofluoresence of FAD is analyzed.

12.   The method of any one of claims 1-11, further comprising assaying a plurality of test cells from a clinical sample such that an individual metabolic score is obtained for each of the test cells and averaging the individual metabolic scores to obtain an average metabolic score for the plurality of test cells.

13.   The method of claim 12, where the average metabolic score is a patient-level score.

14.   The method of any of claims 1-13, wherein the FLIM data is analyzed by fitting the lifetime histograms to a model.

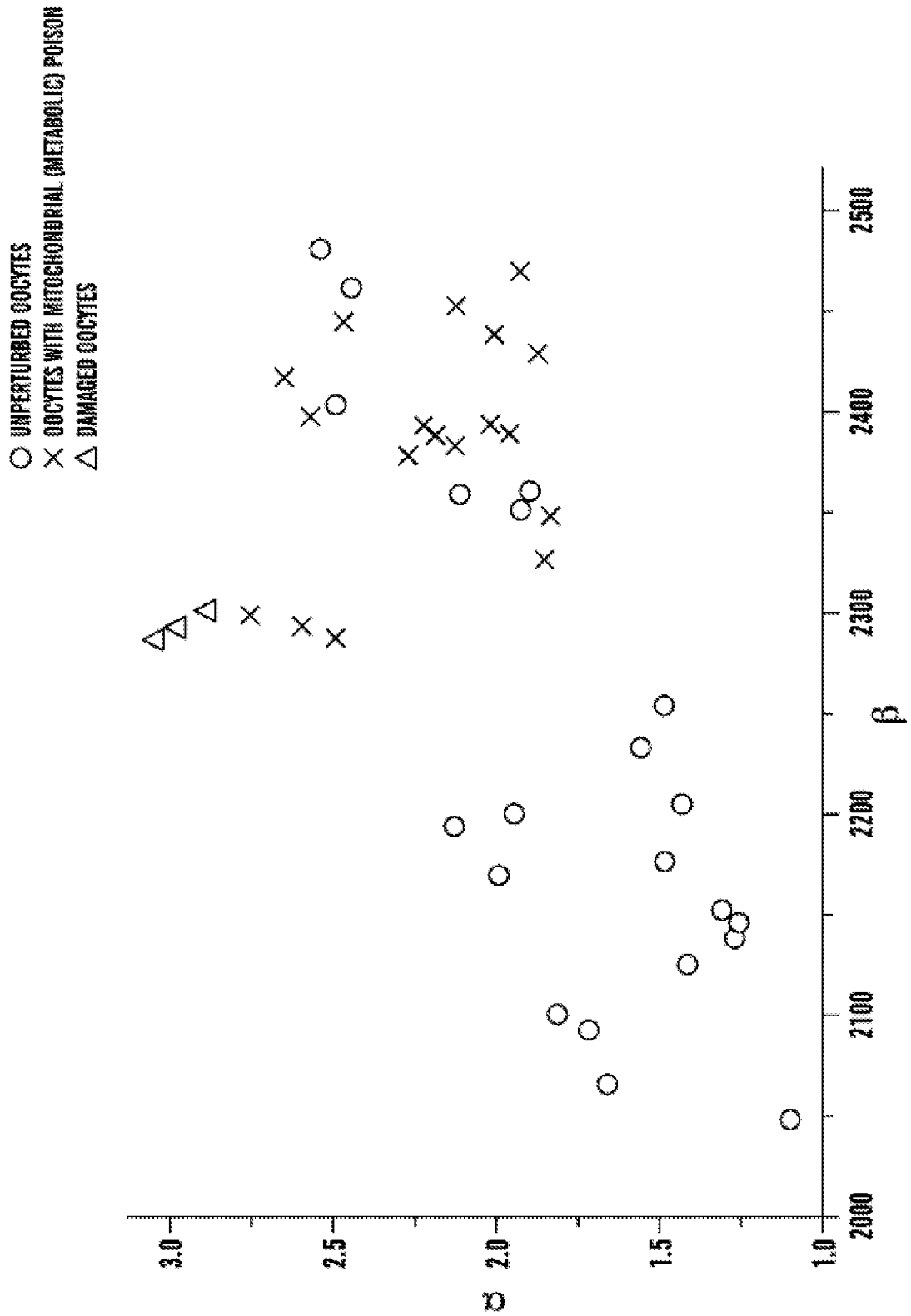15.   The method of any of claims 1-13, wherein the FLIM data is analyzed by phasor analysis.
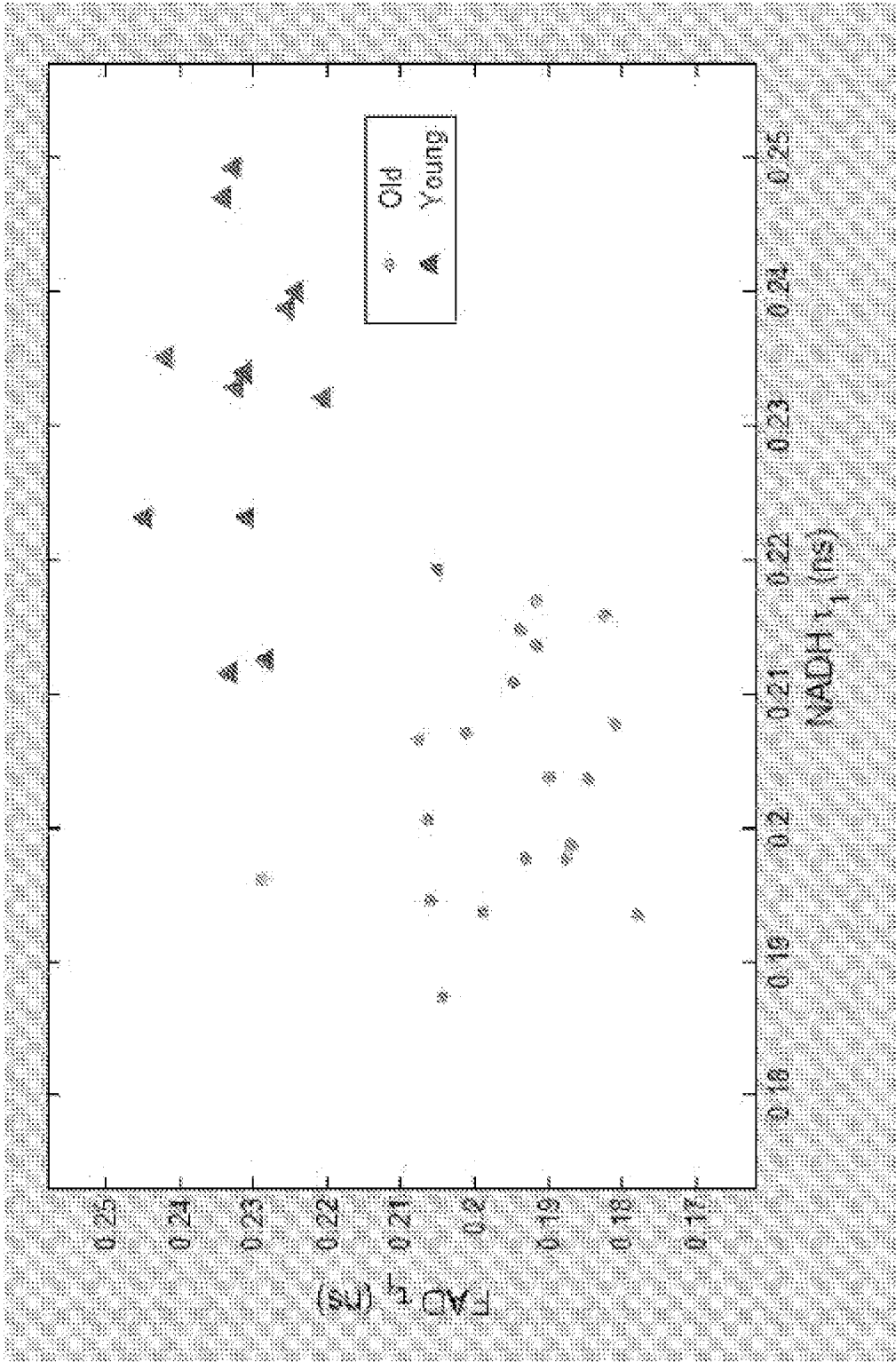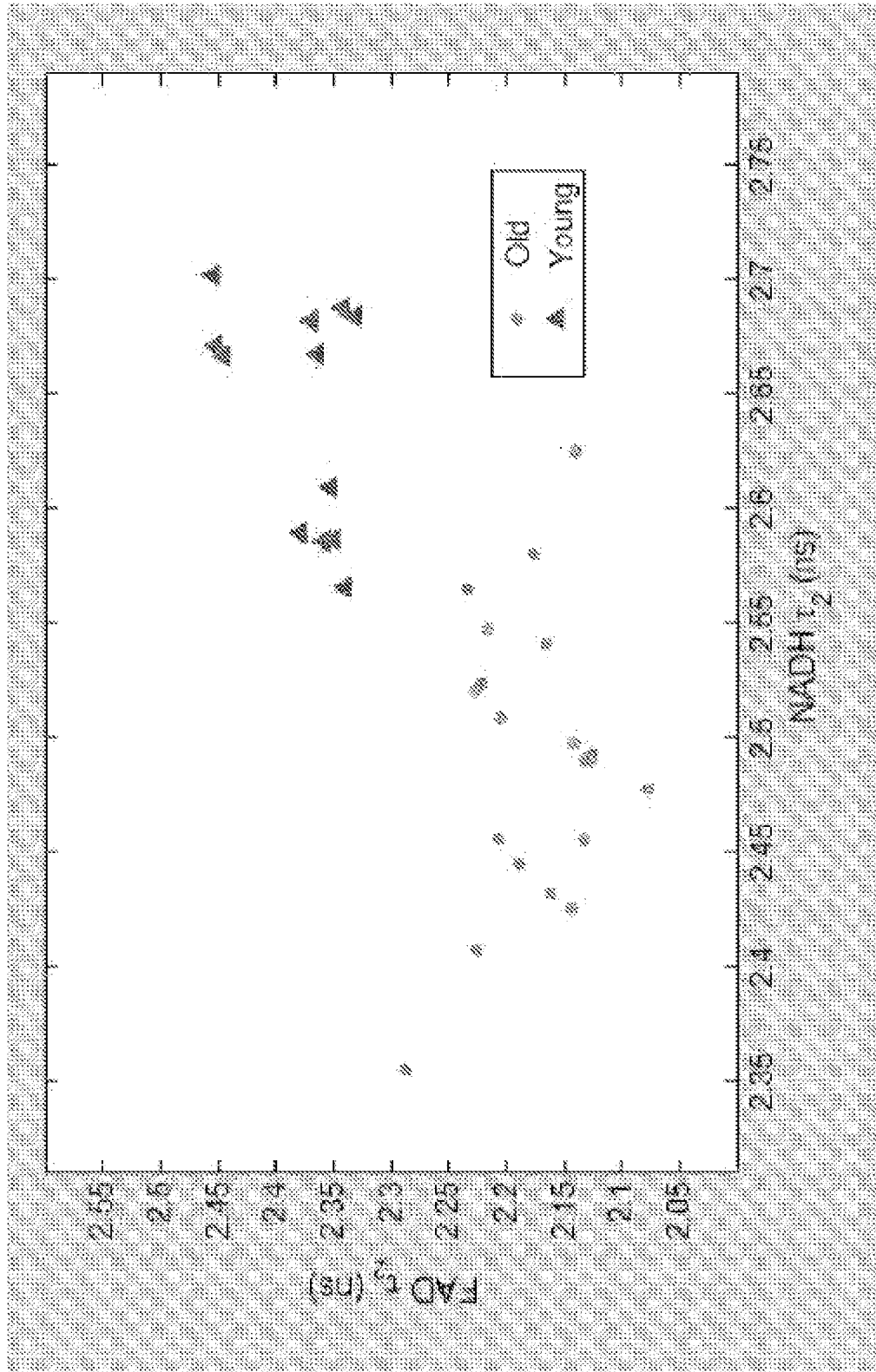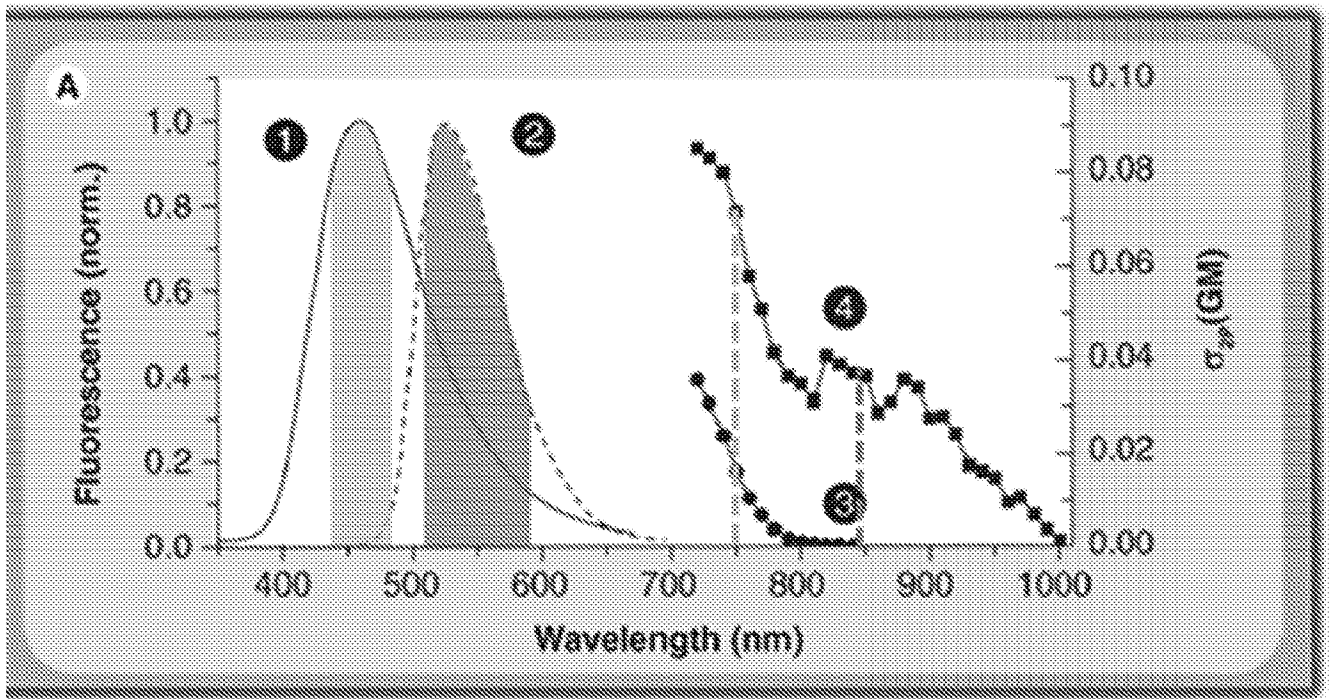
FIG. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6
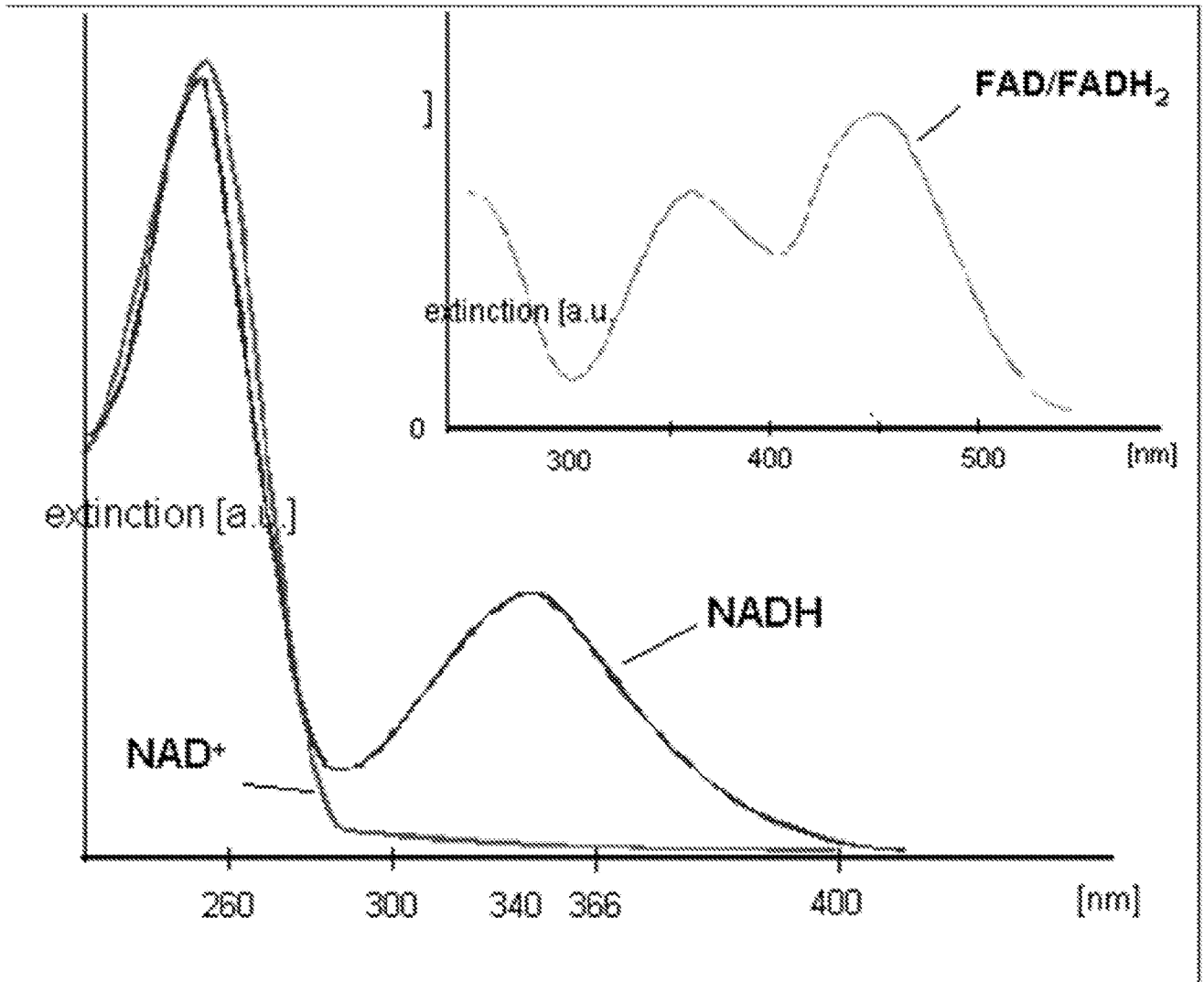
Fig. 7

INTERNATIONAL SEARCH REPORT	International application No.
	PCT/US2016/020245

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 5/073, C12N 5/075; C12Q 1/00; G01N 21/64 (2016.01)

CPC - C12M 1/3476; C12N 5/0603, C12N 5/0609; G01N 21/6408, G01N 21/6458 (2016.02)

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)

IPC(8) - C12N 5/073, C12N 5/075; C12Q 1/00; G01N 21/64 (2016.01)

CPC - C12M 1/3476; C12N 5/0603, C12N 5/0609; G01N 21/6408, G01N 21/6458 (2016.02)

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

USPC - 435/25; 436/172 (keyword delimited)

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

PatBase, Google Patents, Google Scholar, PubMed.

Search terms used: embryo oocyte FLIM fluorescence lifetime imaging autofluorescence lifetime brightness metabolic score

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2014/110008 A1 (THE BRIGHAM AND WOMEN'S HOSPITAL, INC) 17 July 2014 (17.07.2014) entire document	1-5
A	US 5,541,081 A (HARDY et al) 30 July 1996 (30.07.1996) entire document	1-5
A	WO 2013/006948 A1 (CASPER et al) 17 January 2013 (17.01.2013) entire document	1-5
A	US 8,497,063 B2 (SCHENK et al) 30 July 2013 (30.07.2013) entire document	1-5
A	US 2012/0276578 A1 (STRINGARI CHIARA et al) 01 November 2012 (01.11.2012) entire document	1-5

☐ Further documents are listed in the continuation of Box C.	☐ See patent family annex.

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier application or patent but published on or after the international filing date	"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
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"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
"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	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 April 2016	**17 MAY 2016**

Name and mailing address of the ISA/	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450	Blaine R. Copenheaver
Facsimile No. 571-273-8300	PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

Box No. I   Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

Form PCT/ISA/210 (continuation of first sheet (1)) (January 2015)

Box No. II	Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-15
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III	Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.