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(54) Title: ASSESSING METABOLIC CONDITION OF SPERM

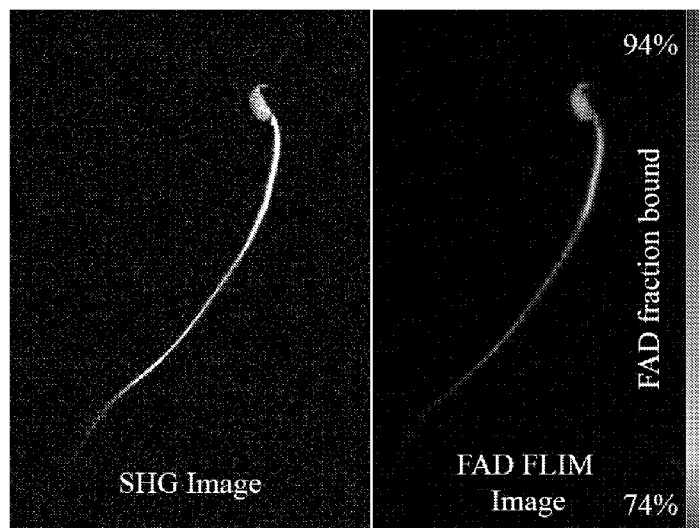


FIG. 1

(57) Abstract: Disclosed herein are methods of assessing the quality and fertility of a sperm cell by metabolic imaging, such as using fluorescence intensity, fluorescence lifetime imaging microscopy (FLIM) and/or second harmonic generation (SHG) and applications of the same for assisted reproductive technology (ART). A method of assessing the quality of a sperm cell, the method comprising subjecting a sperm cell to at least one of: a fluorescence lifetime imaging microscope (FLIM) measurement; a fluorescence intensity measurement; and second harmonic generation (SHG) measurement.

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ASSESSING METABOLIC CONDITION OF SPERM

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 62/105,647 filed January 20, 2015, the contents of which are incorporated herein by reference in their entirety.

TECHNICAL FIELD

[0002] The present invention relates to methods for assessing the metabolic condition of sperm, and can be used, e.g., in assessment of sperm in assisted reproductive technologies (ART).

BACKGROUND

[0003] 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 births 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 infants 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. alone (Bromer and Seli, 2008).

[0004] In the U.S. today, 2/3 of all ART cycles are performed using intra-cytoplasmic sperm injection (ICSI) (SART, 2012). ICSI involves choosing one sperm to inject directly into an egg to enable fertilization to take place. In normal fertilization, sperm is selected based on their ability to penetrate the egg and fertilize it. In ICSI, this natural mechanism does not take place, as the embryologist selects the sperm. As a result, embryos that are derived from ICSI have a higher rate of genetic abnormalities. However, there are limited options to determine which sperm is most viable for injection. Most embryologists look at sperm motility and morphology and choose a mobile, morphologically normal looking sperm at random to inject, with no way to choose between the sperm cells. As a result, many oocytes are injected with poor quality sperm cells, halting development of the embryo. This phenomenon is a contributing factor to the low success rates in assisted reproductive technologies today, that hover at a low 30%. Given the low ovarian reserve and poor egg

quality of many older women using assisted reproductive technologies, clinicians would not want to waste the limited number of good eggs on poor quality or non-viable sperm. Additionally, when fertilization does not occur, embryologists oftentimes do not know if the sperm or the egg caused the problem, leading to uncertainty in the path forward for a patient. Accordingly, there is an acute need in the art for methods to improve assessment of sperm prior to *in vitro* fertilization.

[0005] Also, additional methods for assisting selection of good quality sperm for freezing sperm from donors would be highly desirable.

BRIEF DESCRIPTION OF THE DRAWINGS

[0006] FIG. 1 shows a second harmonic generation (SHG) image and a fluorescence-lifetime imaging microscopy (FLIM) image of sperm.

DETAILED DESCRIPTION

[0007] The technology described herein relates to the use of fluorescence-lifetime imaging microscopy (FLIM) or second harmonic generation (SHG) to assess the quality of sperm based on morphology and/or metabolic condition of sperm. Applicants have surprisingly discovered that the metabolic condition of sperm can be used as an indicator of sperm cell capacity for fertilization. Applicants submit that by selecting sperm with better metabolism, embryologists can reduce the probability of the sperm having aneuploidy or other defects and should ultimately be able to increase success rates for *in vitro* fertilization. After the sperm is selected using the methods described herein, an embryologist can inject the selected sperm into an oocyte for *in vitro* fertilization.

[0008] The methods described herein can also be used for diagnosing sperm quality in general. In some embodiments, the methods described herein can be used for selecting sperm for ART, including intra-cytoplasmic sperm injection (ICSI). The methods may also be used to diagnose infertility based on quality, such as morphology and/or metabolic condition of sperm cell(s). In some aspects, the methods can be used to provide a general viability “score” for a sperm donor based on, e.g., average metabolic score of sperm cells in a sample. In some further aspects, the methods provide a diagnostic tool for infertility allowing a tool to assist detecting metabolic defects or abnormalities in individual sperm cells or in an aggregate sample of sperm cells. When a sperm cell is determined to be within normal parameters using any one or a combination of the methods described herein it can then be further selected for and used in ART, such as ICSI or cryopreserved. One can also perform

any of the methods as described and claimed herein with sperm that is either fresh or previously cryopreserved.

[0009] Contrary to the present methods, wherein simply the motility of the sperm provides the suggestion that the sperm cell is viable and provides best chance of fertilization, the inventors here provide that metabolic activity instead of or in combination with current methods can provide a much more accurate way of assessing, determining, and/or diagnosing fertility and selecting sperm cells for ART. In some aspects of all the embodiments, the methods described herein are performed *in vitro*, e.g., using sperm taken from, e.g., a human subject. In some aspects of all the embodiments, the methods of the present invention can be used in combination with measurements of sperm motility and sperm count to provide a more comprehensive assessment or a more accurate score of the fertility of the sperm donor.

[0010] It has generally been thought that the metabolic activity of a sperm cell is of no or limited value in assessing sperm viability or potential for fertilization. However, here, the Applicants show that the metabolic activity provides important information about fertilization capacity and can significantly enhance the potential of selecting a single sperm cell to fertilize an egg in ART or provide additional information of the general fertility of the sperm donor when applied to assessing metabolic activity of a sperm cell sample in, e.g., averaging the metabolic activity of the sperm in an aggregate sample.

[0011] Metabolic imaging can be performed using, e.g., FLIM or by measuring intensity of a fluorophore signal from the sperm cell, such as 1-photon absorption illumination or continuous wave illumination to get 1-photon absorption or a photodiode, halide lamp, halogen lamp, or mercury lamp for performing epifluorescence (1-photon), and confocal microscopy, which uses a continuous wave laser. 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, single-photon or two-photon excitation microscopy, and multiphoton tomography. In some aspects of the methods described herein, the intensity of the fluorophore signal, e.g. only the intensity and not the lifetime, can be measured using non-FLIM methods, e.g., 1-photon absorption illumination or continuous wave illumination to get 1-photon absorption or a photodiode, halide lamp, halogen lamp, or mercury lamp for performing epifluorescence (1-photon), and confocal microscopy, which uses a continuous wave laser.

[0012] The lifetime of the fluorophore signal, rather than its intensity alone, can be used to create the image in FLIM. This has the advantage of minimizing the effect of photon

scattering in thick layers of sample. In some aspects of all the embodiments as described herein and claimed, the sperm can be imaged on a surface, i.e., instead of in the layers of a sample. 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. These decay rates are sensitive to the micro-environment of the fluorophore (e.g. pH, O₂ concentration, viscosity). Thus, FLIM measurements utilize both lifetime and intensity information to detect changes in the chemical state of the mitochondria where the fluorophores are found.

[0013] Fluorescence lifetimes can be determined in the time domain by using a pulsed source.

[0014] Time-correlated single-photon counting (TCSPC) can be 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 extracted. 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.

[0015] 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 the 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.

[0016] 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, two 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 there are more than one lifetime species in the sample.

[0017] 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. Konig 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, Rae, 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 cosmetic compounds.

[0018] While FLIM has previously been described as a method of assaying cells in general (Ghukasyan et al., *J. Phys. Chem. C* 2009, 113, 11532-11540), and for oocyte or embryo quality assessment (WO2014110008), it has not previously been suggested in connection with assessing sperm quality, particularly for diagnostic purposes or for viability for ART. We have surprisingly found that metabolic activity of the sperm is indicative of its fertilization capacity and that thus metabolic imaging by, e.g., certain FLIM measurements or fluorescence intensity or SHG can be used to diagnose infertility and/or to select a preferable

or better quality sperm cell for ART to increase success of ART methods, including those methods that use single sperm injection.

[0019] The metabolic condition of sperm as an indicator for sperm health has been previously overlooked in ART. This is partly due to the fact that sperm does not undergo cell division, so it was not clear why metabolism would be an important factor for sperm viability. Sometimes metabolically dead sperm cells have been used and have even been preferred due to their immobility, which is required for intracytoplasmic sperm injection (ICSI). Contrary to the common belief, the inventors herein provide that using the assessment of metabolic activity, and selecting the cells that are metabolically healthier, can provide significant improvement for success of ART.

[0020] In addition, there are a lot fewer mitochondria in a sperm cell than in, e.g., an oocyte, and the mitochondria of the sperm are not injected to the oocyte but solely fuel the sperm through its travel. Thus, it has been believed and was previously expected that the autofluorescence level is a lot lower in a sperm cell than in an oocyte and thus not even suitable for metabolic assessment using sperm cell autofluorescence. Based on expected low autofluorescence level, sperm cells were not expected to be an optimal target for fluorescence intensity or FLIM assay or measurements as one would have been expected to have to use much more powerful exposure to image sperm than, e.g., an oocyte and thus increase the potential of the method harming the sperm cell in the process. Contrary to these expectations, Applicants have shown that fluorescence intensity, FLIM as well as SHG can be used to analyze sperm without harming it, and further that these methods provide valuable information in regard to the fertilization capacity of the sperm.

[0021] As used herein, the term “second harmonic generation (SHG)” refers to the phenomenon wherein light of a particular frequency is passed through a nonlinear material and is in part converted to light of twice the original frequency.

[0022] In one aspect of the technology described herein, the method comprises subjecting a test sperm cell or the sperm from a clinical sample from a donor to a microscope capable of measuring intensity of a fluorophore signal from the sperm cell, such as 1-photon absorption illumination or continuous wave illumination to get 1-photon absorption or a photodiode, halide lamp, halogen lamp, or mercury lamp for performing epifluorescence (1-photon), and confocal microscopy, which uses a continuous wave laser; or the intensity and the lifetime of the fluorophore from the sperm cell, such as FLIM, performing the microscopy, such as FLIM, and acquiring the image, such as a fluorescence lifetime histogram of autofluorescence of endogenous nicotinamide adenine dinucleotide (NADH) or flavin adenine

dinucleotide (FAD) for the test or clinical sperm cell from the donor. In some embodiments, the test sperm cell looks morphologically normal but the metabolic activity is abnormal, and the sperm cell can be discarded or considered non-optimal for ICSI. Sperm morphology, can be determined, e.g., using SHG. SHG can be used to identify ordered structures such as the flagella, comprised of a microtubule bundle. Other morphology, of the head, for example, can also be detected by NADH autofluorescence. In some aspects of all the embodiments of the invention a combination of FLIM and SHG is used. Sperm cell morphology includes, but is not limited to, shape, length, width of head, width of flagella, and width of the overall sperm.

[0023] In some embodiments, the sperm metabolic activity can be determined by single-photon or by a two-photon fluorescence.

[0024] In some embodiments, the sperm metabolic activity can be determined by an intensity-only method, such as such as 1-photon absorption illumination or continuous wave illumination to get 1-photon absorption or a photodiode, halide lamp, halogen lamp, or mercury lamp for performing epifluorescence (1-photon), and confocal microscopy, which uses a continuous wave laser.

[0025] In some aspects of all the embodiments of the invention, the sperm morphology can be determined by second harmonic generation (SHG).

[0026] In some aspects of all the embodiments of the invention, the measurement can comprise 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 sperm cell that has been analyzed can either be selected for injection or discarded as not optimally fit for ICSI. The sperm cells can be analyzed in any suitable cell culture medium. Suitable culture media for use with mammalian sperm must be "non-capacitating", in that they do not cause premature capacitation of the sperm cells.

[0027] NADH and FAD are known to be involved in the metabolic process of a cell. NADH serves as a coenzyme and a principal electron donor within the cell for both oxidative phosphorylation (aerobic respiration) and glycolysis (anaerobic respiration). The absorption and fluorescence spectra of NADH (the reduced form) have been well characterized at different levels of organization, e.g., 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).

[0028] The FLIM curves of NADH from 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.

[0029] 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 are typically determined by using a least-squares fit. However, a Bayesian inference approach may allow even more precise parameter estimates and reference estimates with fewer photons. This would allow even less light to be used, improving reliability while minimizing sample exposure. In some embodiments, the data can be fit using a least squares fit (LSQ). In some embodiments, the data can be fit using a Bayesian method.

[0030] In some embodiments, the 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.

[0031] In some embodiments, the 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 filtered centered around about 460 nm.

[0032] Flavin adenine dinucleotide (FAD) is a redox cofactor involved in several important reactions in cellular metabolism.

[0033] In some embodiments, the autofluorescence of FAD can be analyzed with FLIM using a wavelength of about 850-900 nm in two-photon fluorescence excitation and using an emission bandpass filtered centered around about 550 nm.

[0034] In some embodiments, the 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 filtered centered around about 550 nm.

[0035] One can combine the analysis using FLIM of NADH and FAD by simply subjecting 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. The analysis typically takes a short time, such as 30 seconds to a few minutes and can be multiplexed and automated.

[0036] In some embodiments, bound NADH and/or FAD are measured. In some embodiments, unbound NADH and/or FAD are measured. In some embodiments, overall concentration of NADH and/or FAD can be obtained by quantifying the fluorescence intensity.

[0037] In some embodiments, a plurality of FLIM images of the sperm cell can be obtained at different time points. In some embodiments, a plurality of SHG measurements of the sperm cell can be obtained at different time points.

[0038] In one aspect, described herein is a method of diagnosing male infertility, the method comprising the steps of obtaining a sperm sample from a male; subjecting a sperm cell or a plurality of sperm cells to at least one of: fluorescence lifetime imaging microscope (FLIM) and/or a diode-based measurement of fluorescence intensity to obtain a fluorescence lifetime histogram of auto-fluorescence of endogenous NADH and FAD for the sperm cell or plurality of sperm cells; averaging the fluorescence lifetime histogram of NADH auto-fluorescence and FAD auto-fluorescence over the sperm cell or plurality of sperm cells, or the cytoplasm of the sperm cell or sperm cells, or mitochondria of the sperm cells or test cells to assay measurements for fraction bound, short and long lifetimes, and average brightness for both FAD and NADH; combining the measurements for fraction bound, short and long lifetimes, and average brightness for both FAD and NADH to obtain a metabolic score; comparing the metabolic score to a reference metabolic score to obtain a metabolic score for the sperm cell or an average metabolic score of the plurality of the sperm cells; and diagnosing infertility when the metabolic score is below a reference metabolic score. In one aspect, described herein is a method for screening sperm for sperm banking the method comprising the steps of obtaining a sperm sample from a male; subjecting a sperm cell or a plurality of sperm cells to at least one of: fluorescence lifetime imaging microscope (FLIM) and/or a diode-based measurement of fluorescence intensity to obtain a fluorescence lifetime histogram of auto-fluorescence of endogenous NADH and FAD for the sperm cell or plurality of sperm cells; averaging the fluorescence lifetime histogram of NADH auto-fluorescence and FAD auto-fluorescence over the sperm cell or plurality of sperm cells, or

the cytoplasm of the sperm cell or sperm cells, or mitochondria of the sperm cells or test cells to assay measurements for fraction bound, short and long lifetimes, and average brightness for both FAD and NADH; combining the measurements for fraction bound, short and long lifetimes, and average brightness for both FAD and NADH to obtain a metabolic score; comparing the metabolic score to a reference metabolic score to obtain a metabolic score for the sperm cell or an average metabolic score of the plurality of the sperm cells; and cryopreserving the sperm sample when the metabolic activity score or the average metabolic activity score is at or above the reference profile metabolic activity score. The foregoing methods measure 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] In any one of the methods and aspects of the invention, in the step of subjecting one can expose a sperm cell to one or more forms of illumination, for example, fast pulsed lasers like ti-sapphire or standard light source, such as, 1-photon absorption. To get a standard fluorescence measurement, one can use various types of illuminators to get 1-photon absorption. For example, one can use photodiode, halide lamp, halogen lamp, or mercury lamp to do epifluorescence (1-photon) or confocal microscopy, which uses a continuous wave laser.

[0040] In SHG subjecting the sperm cell in general refers to a scattered signal obtained from the sperm cell.

[0041] Thus, in some aspects and embodiments, the invention provides a method comprising the steps of assessing the quality of the sperm cell with at least one of the following methods: fluorescence intensity, FLIM and/or SHG.

[0042] The most commonly observed autofluorescing molecules are NADPH (NADH) and flavins, such as FAD; the extracellular matrix can also contribute to autofluorescence because of the intrinsic properties of collagen and elastin (Monici M. (2005). "Cell and tissue autofluorescence research and diagnostic applications". *Biotechnol Annu. Rev.* 11: 227–56). Generally, proteins containing an increased amount of the amino acids tryptophan, tyrosine and phenylalanine also show some degree of autofluorescence (Julian M. Menter (2006). "Temperature dependence of collagen fluorescence". *Photochem. Photobiol. Sci.* 5 (4): 403–410). Thus, any of the autofluorescent molecules can be used in the methods as described herein and in any of the claims.

[0043] To assay the cells with FLIM, one can use at least three parameters, namely, short lifetime, long lifetime, and fraction bound to enzymes, that are extracted from FLIM

measurements of, e.g., NADH and FAD in sperm cells. Binding to enzymes causes the lifetime of the fluorophore to shift significantly, explaining the strong correlation in all three parameters. However, the combination of all these parameters provides the most reliable reading of the metabolic state of the cell. The metabolic score can be applied to compare between sperm and/or sperm samples to select the sperm for further use, either for, e.g., ICSI or cryopreservation, that show the best metabolic score indicating they have the best chance of survival and successful fertilization. The scores represent the metabolic health of the sperm, 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 can provide a more accurate assessment of the metabolic state of the target sperm. In some embodiments, the metabolic score can be compared to a reference score and/or value, e.g., the score and/or value for a healthy sperm and/or plurality of healthy sperm. In some embodiments, the reference score can be, e.g., an averaged score for multiple sperm and/or samples of sperm. In some embodiments, the metabolic score can be compared to the metabolic score obtained for other sperm cells and/or samples without comparison to an reference value, e.g., to select and/or identify the most metabolically active sperm cells and/or samples within a group thereof.

[0044] FLIM measurements can be performed using a system such as a tabletop microscope.

[0045] FLIM measurements can be performed using a variety of options including, but not limited to, two-photon excitation with a point detector, one-photon excitation with a point detector, or one-photon detection with an area detector (e.g., a camera). Table 1 presents a summary of non-limiting examples of options used for FLIM.

Table 1.

	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
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[0046] In some embodiments, fluorescence intensity/FLIM/SHG measurements can be used to determine the sperm mitochondria morphology, quantity of mitochondria, or mitochondrial distribution, which can be used to indicate whether the sperm cell is normal or abnormal or has better or worse capacity for fertilization.

[0047] In some embodiments, fluorescence intensity/FLIM/SHG measurements can also be used to determine the number of mitochondria present in the sperm cell, which can be used to indicate whether the sperm cell is normal or abnormal or has better or worse capacity for fertilization.

[0048] In some embodiments, FLIM can also be used to measure sperm motility. For example, sperm motility can be determined from fluorescence image sequences. In another example, sperm motility can be determined from the amount of blur in a single image. The sperm motility can also be used to indicate whether the sperm cell is normal or abnormal.

[0049] In some embodiments, fluorescence intensity/FLIM measurements can be used to measure any autofluorescent markers in a cell such as tryptophan, flavonoids, riboflavin, folic acid, retinoic acid, flavoproteins and determine whether they have normal or abnormal characteristics to provide an assessment for better or worse capacity for fertilization.

[0050] In some embodiments, sperm viability can be determined from one or more factors selected from the group consisting of morphology, metabolism, other autofluorescence, and motility.

[0051] In some embodiments, SHG can be used to determine the microscopic order of various subcellular structures (SHG signal level), including flagella, DNA, and sperm head structures and whether they are ordered normally or abnormally or to indicate fertilization potential.

[0052] In some embodiments, sperm viability/fertility can be determined from assessing the morphology and subcellular ordering.

[0053] In some embodiments, morphology can comprise mitochondrial morphology.

[0054] The quality of a sperm cell can be assessed by one or a combination of the following: motility, viability or correlates of viability such as swimming speed or genomic integrity, growth/development, fertilization rate/cleavage rate/embryo quality/blastocyst formation/pregnancy/live birth, and morphology.

[0055] In some aspects of the invention, one can also use the methods of the present invention to assess a collection of individual sperm cells to provide an average score of the metabolism of sperm in a sample. For example, one can assess the motility and sperm count in a sample, such as 0.2-1.5 ml sample, and in addition, one can provide an average metabolic activity of the sperm cells in the sample. This assessment may be performed from fresh sperm sample, prior to freezing or IVF procedures, or can be performed after thawing the sperm cell sample.

[0056] Fluorescence intensity/FLIM and/or SHG measurements on a test sperm cell can be used to form a measurement profile for the cell or multiple cells if averaging the measurements.

[0057] As used herein, the term “measurement profile” refers to a profile or matrix that comprises one or more values and/or descriptors; each value is obtained from a measurement performed on the test sperm cell. In some embodiments, the measurement profile is a fluorescent intensity and/or FLIM profile/SHG profile, which means that all the measurements done on the test sperm cell are fluorescent intensity and/or FLIM measurements/SHG measurements. A normal sperm cell or an average or range from a population of fertile/normal sperm cells or measurements from successfully fertile sperm cells can be used to provide a reference profile for comparison purposes. This would be a “normal” fertility profile. The measurement profile can be compared with the reference fertility profile to determine the viability of the test sperm cell or to assess the sperm cells in an aggregate to provide the donor a general fertility score based on the average metabolic activity of the sperm cells in any given sample. In some aspects, one can use an average of normal cell measurements as the reference value. In some aspects, one can use the distribution of values from normal sperm cells as a range of normal values for the comparisons.

[0058] The “reference value” or “reference profile” as referred to herein, is typically assessed using normal healthy cells of comparable origin, such as normal healthy sperm. The reference values and/or profiles 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. In some embodiments, a sperm cell can be determined to be healthy and/or suitable for use in sperm banking and/or ART when the FLIM profile, fluorescence intensity profile, and/or SHG profile for that cell or a cell from the same sample is determined to be a viable profile. A viable profile can be a profile that is not statistically significantly different from a reference profile obtained from a healthy cell and/or population of healthy cells, e.g. the profile obtained for sperm cell(s) that are demonstrated to be fertile and/or healthy.

[0059] The invention also provides methods for assessing success in any efforts to alter fertility, such as dietary or pharmaceutical interventions. For example, one can assess the metabolic activity of a sperm sample at a first time point before an attempt to improve the sperm quality by lifestyle choices or pharmaceutical intervention, and then assess the metabolic activity of the sperm sample at a second or a subsequent time point after the attempt to improve the sperm quality by lifestyle choices or pharmaceutical intervention. By following the metabolic activity of the sperm one can monitor the success of one or more interventions aimed at improving the sperm quality.

[0060] In some aspects, in the case of low fertility, one can select a sperm cell with the values closest to the normal sperm, which can be determined by a skilled artisan on a cell-by-cell basis when comparing the cell's metabolic viability or morphology or a combination thereof to the normal sperm values, such as ranges or averages from sperm that has shown to be fertile.

[0061] In some embodiments, a reference profile and/or reference level can be a pattern and/or level that varies over time, e.g., that varies with the age of the man, time of day of the collection of the sperm, frequency of collection of the sperm and so forth. By way of non-limiting example, the pattern and/or level of any aspect of a sperm cell described herein can vary with time. Certain patterns of variance can be indicative of a healthy sperm cell and/or an abnormal sperm cell. Such reference profiles and/or reference levels can be determined by measuring sperm cells (or a population of sperm cells) over time.

[0062] Metabolic activity, measured by a technique such as fluorescence intensity or FLIM in combination with morphological assessment with, e.g., SHG can provide a more standardized and automated way of assessing multiple sperm cells in a non-invasive manner to provide a better and faster screening of sperm cells for, e.g., ART or for diagnostic purposes or for cryopreservation of the sperm.

[0063] In some embodiments, the motility of the sperm cell is slowed for imaging purposes. For example, the sperm cell can be placed in a viscous solution. As used herein, a "viscous solution" refers to any solution with a viscosity that reduces the motility of healthy sperm by at least 50% as compared to the motility in semen, e.g., at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more. In another example, the tail of the sperm cell can be excised, e.g., by using a laser beam. The use of a laser beam for tail excision can avoid the use of chemicals for immobilization, which may hamper the metabolic assessment. Thus, in some aspects of the invention, the methods include excision of the tail of the sperm

prior to subjecting the metabolic imaging, such as FLIM. In another example, sperm can be immobilized on a surface.

[0064] The sperm cells for use in the invention are collected from a male animal (for example a human subject) by any suitable method including masturbation, prostate massage, the use of an artificial vagina (for example as part of a breeding mount used for collection from male horses, cattle or other non-human animals), vibroejaculation and electroejaculation. Under some circumstances collection may involve use of a collection condom or retrieval directly from testes by testicular sperm extraction (TESE) may be used. Collection involving ejaculation is generally favored because it will result in a sample of sperm cells suspended in semen and therefore more likely to be properly matured. The sperm cells may be collected "fresh" or may have been collected previously and frozen for a period of storage and then thawed when required for use. They may optionally be subjected to a pre-treatment step before use. Optionally the cells may have been subjected to a preceding treatment, washing or dilution step. In some embodiments, the test sperm cells are stored in a sperm bank. The sample obtained from a subject and/or patient by any of the foregoing methods can be referred to herein as a "clinical sample" or "clinical sperm sample."

[0065] The methods described herein can be used in conjunction with methods to maintain sperm ex vivo, e.g., by cryopreservation. By way of non-limiting example, a clinical sperm sample or portion thereof can be assessed according to the methods described herein after collection to determine if the sperm are normal and/or healthy enough to warrant preservation, e.g., is the sperm suitable for ART. Alternatively, a clinical sperm sample or portion thereof can be assessed according to the methods described herein after cryopreservation (e.g., after thawing the sample) to determine if the preservation and/or thawing steps have rendered the sperm unsuitable for ART.

[0066] In some embodiments, the pattern, value, and/or measurement obtained as described herein can be the average pattern, value, and/or measurement for a plurality of sperm. In some embodiments, the plurality of sperm can comprise a clinical sperm sample and/or a portion thereof. By way of non-limiting example, such average metabolic quality indicators and/or average values can be used to, e.g., identify an individual who is a suitable sperm donor (e.g. a donor whose sperm are healthy and/or suitable for ART) or to aid in the diagnosis of fertility problems experienced by a couple (e.g. by determining whether or not the male's sperm are healthy or not).

[0067] In some embodiments, the sperm cell and/or clinical sperm sample can be further subjected to existing methods of sperm analysis known in the art, e.g., determining sperm density, motility, morphology, and/or velocity.

[0068] In some embodiments of any of the aspects, multiple measurements, e.g., measurements obtained from a plurality of sperm cells are averaged. In some embodiments, the average can be obtained by obtaining a measurement and/or value for each individual sperm, e.g. FLIM curves can be determined for each of 100 sperm in a sample. The parameters of the FLIM curves and/or measurements can then be averaged to obtain an average value for the population of 100 sperm cells. Alternatively, in some embodiments, the photons from all 100 sperm cells can be binned together into a single FLIM decay curve. The resulting FLIM parameters thereby represent an average for the population of 100 sperm cells.

[0069] The various aspects of the invention primarily relate to humans. However, they may be applicable to other animals (especially other mammals) including livestock (especially horses, sheep, goats, cattle, pigs), racing animals (especially horses and camels), companion animals (including cats and dogs), wild animals (including big cats, antelopes and pandas) and research animals (including rodents such as rabbits, mice and rats).

[0070] It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such may 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.

[0071] As used herein and in the claims, the singular forms include the plural reference and vice versa unless the context clearly indicates otherwise. 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.”

[0072] All patents and other publications identified 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 present invention. 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.

[0073] Although any known methods, devices, and materials may be used in the practice or testing of the invention, the methods, devices, and materials in this regard are described herein.

Definitions

[0074] Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. Unless explicitly stated otherwise, or apparent from context, the terms and phrases below do not exclude the meaning that the term or phrase has acquired in the art to which it pertains. 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. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[0075] The term “assisted reproductive technology” refers to technology that assists in achieving pregnancy, including, but not limited to, in vitro fertilization (IVF), embryo transfer (e.g., transfer of embryos at any stage, including blastocysts), gamete intrafallopian transfer (GIFT), tubal embryo transfer (TET), intracytoplasmic sperm injection (ICSI) and intrauterine insemination (IUI).

[0076] The term “intracytoplasmic sperm injection” or “ICSI” refers to an in vitro fertilization procedure in which a single sperm is injected directly into an oocyte.

[0077] As used herein the term “comprising” or “comprises” is used in reference to compositions, methods, and respective component(s) thereof, that are useful to an embodiment, yet open to the inclusion of unspecified elements, whether useful or not.

[0078] 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 of the invention.

[0079] 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 may mean $\pm 1\%$ of the value being referred to. For example, about 100 means from 99 to 101.

[0080] 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 term “comprises” means “includes.” 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.”

[0081] Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow. Further, to the extent not already indicated, it will be understood by those of ordinary skill in the art that any one of the various embodiments herein described and illustrated can be further modified to incorporate features shown in any of the other embodiments disclosed herein.

[0082] 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.

[0083] 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.

[0084] 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.

[0085] Some embodiments of the technology described herein can be defined according to any of the following numbered paragraphs:

1. A method of assessing the quality of a sperm cell, the method comprising exposing a test sperm cell to a fluorescence lifetime imaging microscope (FLIM) and/or second harmonic generation (SHG).
2. The method of paragraph 1, further comprising acquiring a fluorescence lifetime histogram of auto-fluorescence of endogenous nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide (FAD) for the test sperm cell.
3. A method of selecting a sperm cell for assisted reproductive technologies (ART), comprising the steps of assessing the quality of the sperm cell by exposing the sperm cell to fluorescence lifetime imaging microscope (FLIM)/and/or second harmonic generation (SHG), and selecting it for ART when the sperm cell provides a viable FLIM profile/SHG profile.
4. A method of selecting a sperm for single sperm cell injection to an oocyte comprising the steps of assessing the quality of the sperm cell by exposing the sperm cell to fluorescence lifetime imaging microscope (FLIM) and/or second harmonic generation (SHG), and selecting it for ART when the sperm cell provides a viable FLIM profile and/or SHG profile.
5. A method of selecting a sperm cell for assisted reproductive technologies (ART), comprising
 - (i) assessing the quality of the sperm cell by exposing the sperm cell to fluorescence lifetime imaging microscope (FLIM) to obtain a FLIM profile and/or second harmonic generation (SHG) to obtain SHG profile;
 - (ii) comparing the FLIM/SHG profile to a reference profile; and
 - (iii) selecting the sperm cell for ART if the FLIM/SHG profile is similar to the reference profile.

6. A method of assessing the fertility potential of a sperm cell, the method comprising exposing a test sperm cell to a fluorescence lifetime imaging microscope (FLIM) and/or second harmonic generation (SHG).
7. The method of any one of the preceding paragraphs, wherein the tail of the sperm cell is excised.
8. The method of paragraph 7, wherein the tail is excised by exposing the sperm cell to a laser beam.
9. A method for determining gross morphology of a sperm cell comprising exposing the sperm cell to second harmonic generation (SHG).
10. The method of paragraph 10, wherein the gross morphology comprises shape, length, width of head and flagella and overall sperm and whether morphology is normal or abnormal.
11. A method of determining microscopic order of subcellular structures in a sperm cell comprising exposing the sperm cell to second harmonic generation (SHG), wherein the signal level of SHG provides order of the subcellular structures.
12. The, method of paragraph 11, wherein the subcellular structures are selected from flagella, DNA, and sperm head structures.

[0086] Some embodiments of the technology described herein can be defined according to any of the following numbered paragraphs:

1. A method of assessing the quality of a sperm cell, the method comprising subjecting a sperm cell to at least one of:
 - a fluorescence lifetime imaging microscope (FLIM) measurement;
 - a fluorescence intensity measurement; and
 - second harmonic generation (SHG) measurement.
2. The method of paragraph 1, further comprising acquiring a fluorescence intensity or a fluorescence lifetime histogram of auto-fluorescence of endogenous nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide (FAD) for the sperm cell.
3. A method of selecting a sperm cell for assisted reproductive technologies (ART), comprising the steps of assessing the quality of the sperm cell by subjecting the sperm cell to at least one of:
 - a fluorescence lifetime imaging microscope (FLIM) measurement;
 - a fluorescence intensity measurement; and

- a second harmonic generation (SHG) measurement, and
selecting the sperm cell for ART when the sperm cell provides a viable FLIM profile/fluorescence intensity profile/SHG profile.
4. The method of paragraph 3, wherein the ART comprises intracytoplasmic sperm injection (ICSI).
 5. A method of selecting a sperm for single sperm cell injection to an oocyte comprising the steps of assessing the quality of the sperm cell by subjecting the sperm cell to at least one of:
 - a fluorescence lifetime imaging microscope (FLIM) measurement;
 - a fluorescence intensity measurement; and
 - a second harmonic generation (SHG) measurement, andselecting the sperm cell for ART when the sperm cell provides a viable FLIM profile, viable fluorescence intensity profile, and/or a viable SHG profile.
 6. A method of selecting a sperm cell for assisted reproductive technologies (ART), comprising
 - (i) assessing the quality of the sperm cell by subjecting the sperm cell to at least one of: fluorescence lifetime imaging microscope (FLIM) to obtain a FLIM profile, a measurement of fluorescence intensity to obtain a fluorescence intensity profile, and/or second harmonic generation (SHG) to obtain SHG profile;
 - (ii) comparing the FLIM/fluorescence intensity/SHG profile to a reference profile; and
 - (iii) selecting the sperm cell for ART when the FLIM/fluorescence intensity/SHG profile is similar to the reference profile.
 7. A method of assessing the fertility potential of a sperm cell, the method comprising subjecting a sperm cell to at least one of:
 - a fluorescence lifetime imaging microscope (FLIM) measurement;
 - a fluorescence intensity measurement; and
 - a second harmonic generation (SHG) measurement.
 8. The method of any one of the preceding paragraphs, wherein the method comprises subjecting the sperm cell to a FLIM measurement.
 9. The method of any one of the preceding paragraphs, wherein the method comprises subjecting the sperm cell to SHG measurement.

10. The method of any one of the preceding paragraphs, wherein the method comprises subjecting the sperm cell to FLIM and SHG measurement.
11. A method for determining morphology of a sperm cell comprising subjecting the sperm cell to second harmonic generation (SHG), fluorescence life time imaging microscopy (FLIM) and/or fluorescence intensity imaging.
12. The method of paragraph 11, wherein the morphology comprises shape, length, width of head and flagella and overall sperm morphology.
13. The method of paragraph 11, wherein the morphology comprises mitochondrial morphology.
14. A method of determining microscopic order and non-inversion symmetry of subcellular structures in a sperm cell comprising subjecting the sperm cell to second harmonic generation (SHG), wherein the signal level of SHG provides order of the subcellular structures.
15. The, method of paragraph 14, wherein the subcellular structure is flagella or sperm head structures.
16. The method of any one of the preceding paragraphs, wherein the tail of the sperm cell has been excised.
17. The method of paragraph 16, wherein the tail has been excised by subjecting the sperm cell to a laser beam.
18. The method of any one of the preceding paragraphs, wherein the sperm is in a viscous solution.
19. The method of any of the preceding paragraphs, wherein the level of at least one autofluorescent marker other than NADH or FAD in the sperm cell is measured.
20. The method of paragraph 19, wherein the autofluorescent marker is selected from the group consisting of: tryptophan; a flavonoid; riboflavin; folic acid; retinoic acid; and a flavoprotein.
21. The method of any of the preceding paragraphs, wherein the subjecting step occurs *in vitro*.
22. The method of any of the preceding paragraphs, wherein a plurality of subjecting steps is performed at different time points.
23. The method of any of the preceding paragraphs, further comprising assaying multiple sperm cells from a clinical sperm sample, averaging one or more of the fluorescence intensity, FLIM, and/or fluorescence lifetime value measurements from the plurality

- of the sperm cells and comparing the averaged measurements to a reference value thereby providing an average metabolic score for the clinical sperm sample.
24. The method of any of the preceding paragraphs, further comprising assaying multiple sperm cells from a clinical sperm sample, averaging one or more of the morphology, intensity, and/or SHG measurements from the plurality of the sperm cells and comparing the averaged measurements to a reference value thereby providing an average metabolic score for the clinical sperm sample.
25. The method of any of the preceding paragraphs, wherein the sperm cell or another sperm cell from the same clinical sperm sample is subjected to measurement of sperm density, morphology, motility, and/or velocity.
26. The method of any of the preceding paragraphs further comprising a step of cryopreserving the sperm cell or the clinical sperm sample.
27. The method of any of the preceding paragraphs, wherein the sperm cell and/or clinical sperm sample has previously been maintained *ex vivo* or is selected to be maintained *ex vivo*.
28. A method of diagnosing male infertility, the method comprising the steps of
- a. obtaining a sperm sample from a male;
 - b. subjecting a sperm cell or a plurality of sperm cells to at least one of:
fluorescence lifetime imaging microscope (FLIM) and/or a measurement of fluorescence intensity to obtain a fluorescence lifetime histogram of auto-fluorescence of endogenous NADH and FAD for the sperm cell or plurality of sperm cells;
 - c. averaging the fluorescence lifetime histogram of NADH auto-fluorescence and FAD auto-fluorescence over the sperm cell or plurality of sperm cells, or the cytoplasm of the sperm cell or sperm cells, or mitochondria of the sperm cells or test cells to assay measurements for fraction bound, short and long lifetimes, and average brightness for both FAD and NADH
 - d. combining the measurements for fraction bound, short and long lifetimes, and average brightness for both FAD and NADH to obtain a metabolic score;
 - e. comparing the metabolic score to a reference metabolic score to obtain a metabolic score for the sperm cell or an average metabolic score of the plurality of the sperm cells; and
 - f. diagnosing infertility when the metabolic score is below a reference metabolic score.

29. A method for screening sperm for sperm banking the method comprising the steps of
- a. obtaining a sperm sample from a male;
 - b. subjecting a sperm cell or a plurality of sperm cells to at least one of:
fluorescence lifetime imaging microscope (FLIM) and/or a diode-based measurement of fluorescence intensity to obtain a fluorescence lifetime histogram of auto-fluorescence of endogenous NADH and FAD for the sperm cell or plurality of sperm cells;
 - c. averaging the fluorescence lifetime histogram of NADH auto-fluorescence and FAD auto-fluorescence over the sperm cell or plurality of sperm cells, or the cytoplasm of the sperm cell or sperm cells, or mitochondria of the sperm cells or test cells to assay measurements for fraction bound, short and long lifetimes, and average brightness for both FAD and NADH
 - d. combining the measurements for fraction bound, short and long lifetimes, and average brightness for both FAD and NADH to obtain a metabolic score;
 - e. comparing the metabolic score to a reference metabolic score to obtain a metabolic score for the sperm cell or an average metabolic score of the plurality of the sperm cells; and
 - f. cryopreserving the sperm sample when the metabolic activity score or the average metabolic activity score is at or above the reference profile metabolic activity score.
30. A method identifying a fertility enhancing agent or process, the method comprising the steps of
- a. obtaining a first sperm sample from a male at a first time point prior to administration of a test fertility enhancing agent or process;
 - b. subjecting a sperm cell or a plurality of sperm cells to at least one of:
fluorescence lifetime imaging microscope (FLIM) to obtain a FLIM profile and/or a diode-based measurement of fluorescence intensity to obtain a first fluorescence intensity profile;
 - c. obtaining a second sperm sample from the male at a second time point after administration of the test fertility enhancing agent or process;
 - d. subjecting a sperm cell or a plurality of sperm cells to at least one of:
fluorescence lifetime imaging microscope (FLIM) to obtain a FLIM profile and/or a measurement of fluorescence intensity to obtain a second fluorescence intensity profile;

- e. comparing the first and the second fluorescence intensity profiles and identifying the agent or process as fertility enhancing agent or process if the second intensity profile is higher than the first intensity profile.
31. The method of any one of the preceding paragraphs, further comprising a step of utilizing the sperm cell in assisted reproductive technology (ART).
32. The method of paragraph 31, wherein the ART comprises intra-cytoplasmic sperm injection (ICSI).

CLAIMS

What is claimed is:

1. A method of assessing the quality of a sperm cell, the method comprising subjecting a sperm cell to at least one of:
 - a fluorescence lifetime imaging microscope (FLIM) measurement;
 - a fluorescence intensity measurement; and
 - second harmonic generation (SHG) measurement.
2. The method of claim 1, further comprising acquiring a fluorescence intensity or a fluorescence lifetime histogram of auto-fluorescence of endogenous nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide (FAD) for the sperm cell.
3. A method of selecting a sperm cell for assisted reproductive technologies (ART), comprising the steps of assessing the quality of the sperm cell by subjecting the sperm cell to at least one of:
 - a fluorescence lifetime imaging microscope (FLIM) measurement;
 - a fluorescence intensity measurement; and
 - a second harmonic generation (SHG) measurement, andselecting the sperm cell for ART when the sperm cell provides a viable FLIM profile/fluorescence intensity profile/SHG profile.
4. The method of claim 3, wherein the ART comprises intracytoplasmic sperm injection (ICSI).
5. A method of selecting a sperm for single sperm cell injection to an oocyte comprising the steps of assessing the quality of the sperm cell by subjecting the sperm cell to at least one of:
 - a fluorescence lifetime imaging microscope (FLIM) measurement;
 - a fluorescence intensity measurement; and
 - a second harmonic generation (SHG) measurement, andselecting the sperm cell for ART when the sperm cell provides a viable FLIM profile, viable fluorescence intensity profile, and/or a viable SHG profile.
6. A method of selecting a sperm cell for assisted reproductive technologies (ART), comprising

- (i) assessing the quality of the sperm cell by subjecting the sperm cell to at least one of: fluorescence lifetime imaging microscope (FLIM) to obtain a FLIM profile, a measurement of fluorescence intensity to obtain a fluorescence intensity profile, and/or second harmonic generation (SHG) to obtain SHG profile;
 - (ii) comparing the FLIM/fluorescence intensity/SHG profile to a reference profile; and
 - (iii) selecting the sperm cell for ART when the FLIM/fluorescence intensity/SHG profile is similar to the reference profile.
7. A method of assessing the fertility potential of a sperm cell, the method comprising subjecting a sperm cell to at least one of:
 - a fluorescence lifetime imaging microscope (FLIM) measurement;
 - a fluorescence intensity measurement; and
 - a second harmonic generation (SHG) measurement.
8. The method of any one of the preceding claims, wherein the method comprises subjecting the sperm cell to a FLIM measurement.
9. The method of any one of the preceding claims, wherein the method comprises subjecting the sperm cell to SHG measurement.
10. The method of any one of the preceding claims, wherein the method comprises subjecting the sperm cell to FLIM and SHG measurement.
11. A method for determining morphology of a sperm cell comprising subjecting the sperm cell to second harmonic generation (SHG), fluorescence life time imaging microscopy (FLIM) and/or fluorescence intensity imaging.
12. The method of claim 11, wherein the morphology comprises shape, length, width of head and flagella and overall sperm morphology.
13. The method of claim 11, wherein the morphology comprises mitochondrial morphology.
14. A method of determining microscopic order and non-inversion symmetry of subcellular structures in a sperm cell comprising subjecting the sperm cell to second harmonic generation (SHG), wherein the signal level of SHG provides order of the subcellular structures.
15. The, method of claim 14, wherein the subcellular structure is flagella or sperm head structures.

16. The method of any one of the preceding claims, wherein the tail of the sperm cell has been excised.
17. The method of claim 16, wherein the tail has been excised by subjecting the sperm cell to a laser beam.
18. The method of any one of the preceding claims, wherein the sperm is in a viscous solution.
19. The method of any of the preceding claims, wherein the level of at least one autofluorescent marker other than NADH or FAD in the sperm cell is measured.
20. The method of claim 19, wherein the autofluorescent marker is selected from the group consisting of: tryptophan; a flavonoid; riboflavin; folic acid; retinoic acid; and a flavoprotein.
21. The method of any of the preceding claims, wherein the subjecting step occurs *in vitro*.
22. The method of any of the preceding claims, wherein a plurality of subjecting steps is performed at different time points.
23. The method of any of the preceding claims, further comprising assaying multiple sperm cells from a clinical sperm sample, averaging one or more of the fluorescence intensity, FLIM, and/or fluorescence lifetime value measurements from the plurality of the sperm cells and comparing the averaged measurements to a reference value thereby providing an average metabolic score for the clinical sperm sample.
24. The method of any of the preceding claims, further comprising assaying multiple sperm cells from a clinical sperm sample, averaging one or more of the morphology, intensity, and/or SHG measurements from the plurality of the sperm cells and comparing the averaged measurements to a reference value thereby providing an average metabolic score for the clinical sperm sample.
25. The method of any of the preceding claims, wherein the sperm cell or another sperm cell from the same clinical sperm sample is subjected to measurement of sperm density, morphology, motility, and/or velocity.
26. The method of any of the preceding claims further comprising a step of cryopreserving the sperm cell or the clinical sperm sample.
27. The method of any of the preceding claims, wherein the sperm cell and/or clinical sperm sample has previously been maintained *ex vivo* or is selected to be maintained *ex vivo*.
28. A method of diagnosing male infertility, the method comprising the steps of

- a. obtaining a sperm sample from a male;
 - b. subjecting a sperm cell or a plurality of sperm cells to at least one of:
fluorescence lifetime imaging microscope (FLIM) and/or a measurement of
fluorescence intensity to obtain a fluorescence lifetime histogram of auto-
fluorescence of endogenous NADH and FAD for the sperm cell or plurality of
sperm cells;
 - c. averaging the fluorescence lifetime histogram of NADH auto-fluorescence and
FAD auto-fluorescence over the sperm cell or plurality of sperm cells, or the
cytoplasm of the sperm cell or sperm cells, or mitochondria of the sperm cells
or test cells to assay measurements for fraction bound, short and long
lifetimes, and average brightness for both FAD and NADH
 - d. combining the measurements for fraction bound, short and long lifetimes, and
average brightness for both FAD and NADH to obtain a metabolic score;
 - e. comparing the metabolic score to a reference metabolic score to obtain a
metabolic score for the sperm cell or an average metabolic score of the
plurality of the sperm cells; and
 - f. diagnosing infertility when the metabolic score is below a reference metabolic
score.
29. A method for screening sperm for sperm banking the method comprising the steps of
- a. obtaining a sperm sample from a male;
 - b. subjecting a sperm cell or a plurality of sperm cells to at least one of:
fluorescence lifetime imaging microscope (FLIM) and/or a diode-based
measurement of fluorescence intensity to obtain a fluorescence lifetime
histogram of auto-fluorescence of endogenous NADH and FAD for the sperm
cell or plurality of sperm cells;
 - c. averaging the fluorescence lifetime histogram of NADH auto-fluorescence and
FAD auto-fluorescence over the sperm cell or plurality of sperm cells, or the
cytoplasm of the sperm cell or sperm cells, or mitochondria of the sperm cells
or test cells to assay measurements for fraction bound, short and long
lifetimes, and average brightness for both FAD and NADH
 - d. combining the measurements for fraction bound, short and long lifetimes, and
average brightness for both FAD and NADH to obtain a metabolic score;

- e. comparing the metabolic score to a reference metabolic score to obtain a metabolic score for the sperm cell or an average metabolic score of the plurality of the sperm cells; and
 - f. cryopreserving the sperm sample when the metabolic activity score or the average metabolic activity score is at or above the reference profile metabolic activity score.
30. A method identifying a fertility enhancing agent or process, the method comprising the steps of
- a. obtaining a first sperm sample from a male at a first time point prior to administration of a test fertility enhancing agent or process;
 - b. subjecting a sperm cell or a plurality of sperm cells to at least one of: fluorescence lifetime imaging microscope (FLIM) to obtain a FLIM profile and/or a diode-based measurement of fluorescence intensity to obtain a first fluorescence intensity profile;
 - c. obtaining a second sperm sample from the male at a second time point after administration of the test fertility enhancing agent or process;
 - d. subjecting a sperm cell or a plurality of sperm cells to at least one of: fluorescence lifetime imaging microscope (FLIM) to obtain a FLIM profile and/or a measurement of fluorescence intensity to obtain a second fluorescence intensity profile;
 - e. comparing the first and the second fluorescence intensity profiles and identifying the agent or process as fertility enhancing agent or process if the second intensity profile is higher than the first intensity profile.
31. The method of any one of the preceding claims, further comprising a step of utilizing the sperm cell in assisted reproductive technology (ART).
32. The method of claim 31, wherein the ART comprises intra-cytoplasmic sperm injection (ICSI).

**FIG. 1**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/014007

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 21/64 (2016.01)

CPC - G01N 21/64 (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) - C12M 1/34; C12N 5/076; C12Q 1/02; G01N 21/64 (2016.01)

CPC - C12N 5/0608, 5/061; G01N 1/30, 15/14, 21/64, 21/6408, 21/6428, 21/6458 (2016.02)

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

USPC: 422/82.08; 435/29; 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, ProQuest, PubMed.

Search terms used: sperm fluorescence lifetime imaging microscopy FLIM fluorescence intensity measurement second harmonic generation SHG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2014/0234833 A1 (INGURAN LLC) 21 August 2014 (21.08.2014) entire document	1-3, 7
Y		4-6, 8
Y	WO 2013/158658 A1 (CORNELL UNIVERSITY) 24 October 2013 (24.10.2013) entire document	4, 5
Y	WO 2014/110008 A1 (THE BRIGHAM AND WOMEN'S HOSPITAL, INC et al) 17 July 2014 (17.07.2014) entire document	6; 8
A	US 7070917 B1 (CHRISTENSEN et al) 04 July 2006 (04.07.2006) entire document	1-8, 11-15, 28-30
A	US 4,559,309 A (EVENSON et al) 17 December 1985 (17.12.1985) entire document	1-8, 11-15, 28-30
A	US 2006/0257909 A1 (HARTON et al) 16 November 2006 (16.11.2006) entire document	1-8, 11-15, 28-30

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"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

"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)

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

15 March 2016

Date of mailing of the international search report

08 APR 2016

Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/014007

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.: 9, 10, 16-27, 31, 32
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.