human reproduction

### Noninvasive metabolic profiling of cumulus cells, oocytes, and embryos via fluorescence lifetime imaging microscopy: a mini-review

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**ABSTRACT:** A major challenge in ART is to select high-quality oocytes and embryos. The metabolism of oocytes and embryos has long been linked to their viability, suggesting the potential utility of metabolic measurements to aid in selection. Here, we review recent work on noninvasive metabolic imaging of cumulus cells, oocytes, and embryos. We focus our discussion on fluorescence lifetime imaging microscopy (FLIM) of the autofluorescent coenzymes NAD(P)H and flavine adenine dinucleotide (FAD+), which play central roles in many metabolic pathways. FLIM measurements provide quantitative information on NAD(P)H and FAD+ concentrations and engagement with enzymes, leading to a robust means of characterizing the metabolic state of cells. We argue that FLIM is a promising approach to aid in oocyte and embryo selection.

Key words: metabolism / noninvasive / assessments / fluorescence lifetime imaging microscopy / embryo selection

 $^{\dagger}\text{The}$  authors consider that the first two authors should be regarded as joint First Authors.

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Noninvasive measures of metabolism of cumulus cells, oocytes, and preimplantation embryos using fluorescence lifetime imaging microscopy. ICM: inner cell mass; TE: trophectoderm; FAD+: flavine adenine dinucleotide.

### Introduction

A major goal in ART is to select the single embryo with the highest developmental potential from within a patient's embryo cohort (Gardner and Sakkas, 2003; Kirkegaard *et al.*, 2015; Kovacs and Lieman, 2019; Zaninovic and Rosenwaks, 2020). Selecting a single high-quality embryo to transfer reduces the number of potential embryos implanted and, in turn, decreases the number of embryo transfers a patient must undergo (Gardner and Sakkas, 2003), alleviating the risks associated with multiple pregnancies (Dudenhausen and Maier, 2010).

Currently, the most common method used to assess oocyte and embryo quality is to evaluate their morphology at discrete time points (Schoolcraft et al., 1999; Tesarik and Greco, 1999; Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). More detailed information can be obtained by timelapse microscopy (Goodman et al., 2016), which can be analyzed more objectively with machine learning algorithms (Fernandez et al., 2020; Leahy et al., 2020; Zaninovic and Rosenwaks, 2020). However, it is still unclear if timelapse microscopy is beneficial for embryo selection (Ahlström et al., 2022) and approaches based on morphology or morphokinetics fail to provide information on embryo physiological or genomic state (Wong et al., 2014). Preimplantation genetic testing for aneuploidies (PGT-A) has increasingly been used to evaluate the ploidy of blastocysts (Forman et al., 2013; Munné, 2018). However, PGT-A is invasive and its use remains controversial (Cornelisse et al., 2020; Simopoulou et al., 2021). Thus, improved methods for embryo selection would be beneficial for improving ART success.

### The importance of metabolism for oocyte and embryo development

#### Cumulus-oocyte complex

Granulosa and cumulus cells (CCs) are specialized somatic cells that enclose the oocytes (Zhou et al., 2016). At early stages of oogenesis, there is a crosstalk between granulosa and CCs and the surrounded oocytes (Sutton et al., 2003; Richani et al., 2021) that helps support



**Figure 1. Metabolic cooperation and variability in oocyte maturation and embryo development.** (**A**) Depiction of metabolic cooperativity between the oocyte and the enclosing cumulus cells. (**B**) Variations in metabolism are observed through human oocyte growth and maturation and (**C**) throughout preimplantation embryo development. (**D**) Metabolic variations are also observed between embryos from the same patient and between patients. (**E**) Endogenous coenzymes, such as NAD(P)H and flavine adenine dinucleotide (FAD+), are natural biomarkers of metabolism involved in pathways including glycolysis, fermentation, and mitochondrial respiration. Their autofluorescent properties enable noninvasive imaging of the metabolic state of cells. Created using BioRender.com.

oocyte growth (Downs et al., 2002; Huang and Wells, 2010; Richani et al., 2021), maturation (Dumesic et al., 2015), and enable CCs differentiation (Sutton et al., 2003; Gilchrist et al., 2008) (Fig. 1A). Throughout this article we will focus on CCs, as they are coupled with the oocyte via gap junctions and paracrine signals (Anderson and Albertini, 1976). CCs mitochondrial activity, using measures of mitochondrial DNA copy number, immunofluorescent probes, or measures of membrane potential via flow cytometry, have been linked with oocyte maturation (Anderson et al., 2018; Lan et al., 2020) and the acquisition of developmental competence (Eppig, 1991; Albertini et al., 2001; Lu et al., 2022). Therefore, measurements of CCs metabolic state might provide a means to assess oocyte quality (Ogino et al., 2016; Desquiret-Dumas et al., 2017; Fontana et al., 2020).

Oocyte metabolism, in particular mitochondria, plays key roles in supplying energy and metabolic precursors to support oocyte maturation and further embryo development (Hoshino, 2018; Fabozzi *et al.*, 2021) (Fig. 1B). Oocyte mitochondrial dysfunction has been linked with decreased quality and implantation potential (Wilding *et al.*, 2001; Eichenlaub-Ritter *et al.*, 2011; Zhao and Li, 2012) and is known to impact spindle assembly and chromosome segregation during nuclear maturation, however, the mechanisms by which they do so remain unclear (Eppig, 1996; Tatone *et al.*, 2011). Hence, the assessment of oocyte metabolism may help elucidate these mechanisms and provide a measure of their quality (Tan *et al.*, 2022a).

#### **Preimplantation embryos**

As the oocyte matures and is fertilized, further intricate metabolic shifts occur. The preimplantation embryo subsequently undergoes metabolic changes through development (Lane and Gardner, 2000; Chason *et al.*, 2011; Gardner and Harvey, 2015; Harvey, 2019) that are necessary to produce developmentally competent embryos (Gardner *et al.*, 2001; Leese, 2012) (Fig. IC) and are also involved in cell fate specification (Chi *et al.*, 2020; Zhu and Zernicka-Goetz, 2020). These dynamic variations in metabolic pathways are interwoven with the viability of the embryo (Van Blerkom *et al.*, 1995; Harvey, 2019; Gardner, 2015), which led to the development of the 'Quiet embryo hypothesis', suggesting that embryos that have a less active metabolism have higher developmental potential (Leese *et al.*, 2007; Leese, 2012; Santos Monteiro *et al.*, 2021). However, the validity of this hypothesis is still uncertain (Gardner *et al.*, 2011; Tejera *et al.*, 2012).

Additionally, metabolic state varies across embryos between different patients and within the cohort of embryos from the same patient (Venturas et al., 2021, 2022) (Fig. 1D). It is unclear what determines these variations, but oocyte- or embryo-specific characteristics, such as stage, ploidy, or time since fertilization, appear to influence their metabolic profiles (Gardner and Sakkas, 2003; Rosenwaks, 2017; Sanchez et al., 2017; Shah et al., 2020; Santos Monteiro et al., 2021; Venturas et al., 2022). To this end, measures of metabolism could aid in selecting the embryo with the highest implantation potential from within a patient's cohort.

#### Assessments of metabolism

Measures of metabolism can be performed via the addition of fluorescent dyes to label structures like mitochondria (Gorshinova *et al.*, 2017; Al-Zubaidi *et al.*, 2019), or measures of mitochondrial DNA copy number (Fragouli *et al.*, 2015; Kumar *et al.*, 2021). However, these techniques are invasive and their utility in clinical IVF remains uncertain (Ogino *et al.*, 2016; Desquiret-Dumas *et al.*, 2017; Kumar *et al.*, 2021).

Gene expression in CCs is a potentially noninvasive surrogate marker of oocyte metabolism and quality, but so far has not been predictive of clinical outcome (Hamel *et al.*, 2010; Fragouli *et al.*, 2014; Green *et al.*, 2018; Racowsky and Needleman, 2018). More direct, noninvasive measures of metabolism quantify the uptake and secretion of metabolites in the media surrounding the embryos or oocytes (Conaghan *et al.*, 1993; Urbanski *et al.*, 2008), or via measures of embryo oxygen consumption levels (Lopes *et al.*, 2010; Kurosawa *et al.*, 2016). These methods require highly specialized skills and equipment, such as near-infrared or mass spectrometry, high-performance liquid chromatography, or microarrays. Despite the reported associations of levels of metabolites (Vergouw *et al.*, 2008), proteins (Katz-Jaffe *et al.*, 2006), and amino acids (Brison *et al.*, 2004) with embryonic developmental potential, these methods have not yet been clinically useful (Vergouw *et al.*, 2008; Hardarson *et al.*, 2012).

Some intracellular molecules with integral roles in cellular physiology can be specifically probed with optical microscopy (Heikal, 2010; Cheng and Xie, 2012). Hence, several groups have focused on developing methods to optically measure intracellular metabolic function using techniques such as Raman spectroscopy, confocal imaging, and hyperspectral (McLennan et al., 2020; Tan et al., 2022a) or fluorescence lifetime imaging microscopy (FLIM) (Sanchez et al., 2019; Shah et al., 2020; Venturas et al., 2022; Tan et al., 2022b), with the aim to develop them for clinical use. These methods are noninvasive, avoiding the potential interference with biological functions associated with exogeneous dyes. A broad array of autofluorescent molecules involved in metabolic functions can be probed with hyperspectral microscopy (Sutton-McDowall et al., 2017; Santos Monteiro et al., 2021; Tan et al., 2022a,b). Hyperspectral microscopy is showing some promise for its potential clinical application (Sutton-McDowall et al., 2017; Tan et al., 2022a) and has recently been used to measure the association between embryo metabolic state and ploidy (Santos Monteiro et al., 2021).

NADPH, NADH, and flavine adenine dinucleotide (FAD+) are some of the most abundant, autofluorescent metabolites. These molecules have received particular attention because of their strong association with mitochondrial function (Heikal, 2010). These molecules are endogenous electron carriers involved in many metabolic pathways, including glycolysis, fermentation, and mitochondrial respiration (Chance and Williams, 1955) (Fig. 1E). Hence, these coenzymes have a diagnostic potential as noninvasive biomarkers of the cellular metabolic state and mitochondrial anomalies (Klaidman *et al.*, 1995; McLennan *et al.*, 2020; Tan *et al.*, 2022b). Measurements of their fluorescence intensities are correlated with their concentrations (Heikal, 2010) and have been used to assess mitochondrial function (Klaidman et al., 1995; Dumollard et al., 2009; Santos Monteiro et al., 2021).

# Fluorescence lifetime imaging microscopy

Besides fluorescence intensity, the advanced microscopic technique of FLIM enables additional measurements of the molecule's fluorescence lifetime (Heikal, 2010; Becker, 2012): the time it takes for a fluorescent molecule to return to ground state after excitation (Jablonski, 1933) (Fig. 2A). The fluorescence lifetime of a molecule is independent of its concentration, but depends on its molecular conformation, which can be altered by its environment (Suhling et al., 2004; Ghukasyan and Heikal, 2014). Both NAD(P)H and FAD+ have short and long lifetime components, depending on whether or how these molecules are engaged with an enzyme (Lakowicz, 2006). Changes in metabolic states can be measured by the change of their fluorescence lifetimes (Skala et al., 2007). Taken together, FLIM provides a quantitative characterization of cellular metabolic states in terms of eight metabolic parameters, including intensities, fluorescence lifetimes, and enzyme engagement of NAD(P)H and FAD+ (Becker, 2012; Ma et al., 2019; Sanchez et al., 2019) (Fig. 2B).

FLIM, like all light microscopy techniques, requires exposing the sample to illumination, raising the concern of potential damage. Indeed, it is well known that excessive light exposure in conventional microscopy (Masters and So, 2008) or laser pulses during biopsy (Bradley et al., 2017) can harm biological material. However, using low levels of illumination can eliminate such adverse effects (Nakahara et al., 2010; Scott et al., 2013) and it has been shown that FLIM illumination exposure during single or timelapse FLIM imaging does not disrupt the viability of mouse embryos or increase the levels of reactive oxygen species (Sanchez et al., 2018; Seidler et al., 2020). Despite these findings, safety in mouse embryos does not necessarily generalize to human embryos. Additionally, FLIM timelapse illumination does not appear to produce changes in FLIM parameters during human blastocyst expansion (Venturas et al., 2022), or impact maturation rates of human oocytes when compared to control (Pietroforte et al., 2022). However, in order to use this technique in a clinical setting, additional studies are needed to demonstrate its safety in human oocytes or preimplantation embryos. Laser intensity, time of exposure, and frequency of imaging should all be carefully studied.

## Potential applications of FLIM in ART

FLIM has previously been applied in other fields, and its utilization in clinical ART is showing great promise (Sanchez *et al.*, 2018; Ma *et al.*, 2019; Venturas *et al.*, 2021, 2022; Yang *et al.*, 2021) (Fig. 2C).

## Noninvasive FLIM assessments of cumulus cell metabolic state

Recent studies have evaluated the potential application of noninvasive measurements of CCs metabolism as a surrogate for oocyte quality



**Figure 2.** Noninvasive assessments of intracellular metabolic state from fluorescence lifetime imaging of NAD(P)H and FAD+. (A) Fluorescence lifetime imaging (FLIM) of cumulus cells, oocytes, and embryos. FLIM can be used to noninvasively measure metabolic state of cumulus cells, oocytes, and embryos. (B) Fluorescence decay curves from FLIM. Fitting the fluorescence decay curves to a double exponential function provides information on the fluorescence intensity, lifetime, and enzyme engagement of NAD(P)H and flavine adenine dinucleotide (FAD+), with a total of eight metabolic parameters. (C) Evaluation of cellular morphological features and patient clinical characteristics could potentially be used in synergy with quantitative measurement of the metabolic state of cumulus cells, oocytes, or embryos in order to help select the best embryo from within a patient's cohort. Ns: nanoseconds; au: arbitrary units. Created using BioRender.com.

(Richani et al., 2021; Venturas et al., 2021; Tan et al., 2022b). Measuring the metabolic state of CCs using noninvasive methods offers several advantages. First, CCs are frequently removed or trimmed and discarded when performing ART treatments, therefore measuring their metabolic state is completely noninvasive. Additionally, the greater variance observed between oocytes from the same patient than between patients (Venturas et al., 2021) implies that quantitative measures of CCs metabolism could be used to assess oocyte quality from within a patient's cohort. These metabolic changes might be associated with oocyte-specific characteristics, such as their maturity. In this regard, metabolic profiles of CCs characterized by FLIM have been associated with oocyte maturation status (Anderson et al., 2018; Venturas et al., 2021). CCs showed distinct FLIM parameters depending on whether they enclosed immature oocytes or mature metaphase II (MII) oocytes (Venturas et al., 2021). Additionally, patient-specific factors, like maternal age, and hormone levels have been shown to also influence CCs and oocyte metabolic state (Venturas et al., 2021; Lu et al., 2022). These observations suggest that patient clinical characteristics should be factored in when developing prediction algorithms for oocyte viability. However, because CCs undergo a process of expansion and detach from the oocyte during maturation (Nikoloff, 2021), measurements of their metabolism are a less direct assessment of oocyte physiology. Whether CCs metabolic profile is associated with oocyte viability has yet to be determined.

### Noninvasive FLIM assessments of oocyte metabolic state

Oocyte metabolic state has long been linked with their physiological state and quality (Dumollard et al., 2007; Sanchez et al., 2018; Scott et al., 2018; Richani et al., 2021). However, robust and guantitative techniques to measure oocyte metabolic state noninvasively have yet to be established. Recent studies using FLIM to measure oocyte metabolism in mice demonstrated that FLIM can be used to identify oocytes with metabolic impairments (Sanchez et al., 2018) and showed the impact of age on oocytes metabolic state (Sanchez et al., 2018). Maternal age, among other factors, negatively impacts oocyte quality, in particular because of increased rates of aneuploidy (Eichenlaub-Ritter et al., 2011; Cimadomo et al., 2018). However, the precise relation between oocyte metabolism and correct chromosome segregation has not been established. Therefore, noninvasive measurements of metabolism could provide a means to study this relation (Sanchez et al., 2017, 2018; Scott et al., 2018; Yang et al., 2021). Preliminary work has found distinct FLIM parameters in oocytes that mature and those that do not (Pietroforte et al., 2022). FLIM could

help improve the understanding of the interconnection between nuclear and cytoplasmic maturation of the oocytes or perhaps help predict which eggs will mature (Tan *et al.*, 2022a). Additionally, media and conditions of culture can also affect metabolic pathways and nutrient utilization pathways (Gardner *et al.*, 2011; Kleijkers *et al.*, 2015). FLIM measurements of the intracellular metabolic state provide a means to study the metabolic relation of the oocyte with its environment, how this is related to the acquisition of developmental competence (Tan *et al.*, 2022a) and potentially aid in improving culture conditions (Bertoldo *et al.*, 2020; Pollard *et al.*, 2021). Measuring the metabolic profile of single oocytes from a patient's cohort could help in selecting the oocyte with the highest implantation potential.

### Noninvasive FLIM assessments of embryo metabolic state

It has long been known that embryo metabolism, in particular glucose uptake, is associated with embryo viability (Renard et al., 1980; Van Blerkom et al., 1995; Gardner et al., 2001; Leese et al., 2007; Gardner, 2015); however, it is technically challenging to noninvasively measure embryo metabolism with the sensitivity and robustness that would be required for ART clinical applications. FLIM of NAD(P)H and FAD+ is promising in this regard, as recent work shows that it is capable of measuring intricate metabolic shifts throughout preimplantation development in mouse (Ma et al., 2019; Sanchez et al., 2019) and human blastocysts throughout blastocyst expansion and hatching (Shah et al., 2022; Venturas et al., 2022). It was found that embryo metabolic profiles not only change throughout development but also vary between blastocysts from the same patient and between patients. These profiles are associated with the day of development but are not associated with embryo morphological grades (Venturas et al., 2022), which can suggest that both of these assessments may provide synergistic information, aiding separately in embryo selection (Tejera et al., 2012). Additionally, blastocyst metabolism was also associated with the ploidy status in human embryos (Shah et al., 2022). Whether assessment of blastocyst metabolism via FLIM is associated with embryo implantation should be further explored.

### Mechanistic studies and interpretation of FLIM measurements

One of the challenges of using FLIM measurements to help select oocytes and embryos based on their metabolic state is to understand what physiological information is encoded by FLIM measurements and to what extent they are predictive of their developmental competence. The majority of FLIM studies so far are correlative, demonstrating the sensitivity of FLIM parameters to metabolic perturbations or changes in cell physiology (Sanchez et al., 2018, 2019; Ma et al., 2019; Venturas et al., 2021, 2022; Shah et al., 2022), but how to relate FLIM measurements to activities of specific metabolic pathways remains largely unknown. Knowing the relation between FLIM parameters and specific metabolic activities will better inform the interpretation of the correlations between FLIM measurements and patient characteristics that have been observed in clinical data (Venturas et al., 2021). The

goal is to quantitatively interpret FLIM measurements of NAD(P)H and FAD+ in terms of activities of specific metabolic pathways, such as respiration, glycolysis, and fermentation in oocytes, CCs, and embryos, and to understand mechanistically how metabolic defects impact developmental competence in these systems. This would provide more relevant information on how to use these noninvasive FLIM measurements to select oocytes and embryos in ART.

Biophysical models provide useful tools to interpret FLIM measurements by mapping FLIM parameters into biologically meaningful quantities. Biophysical models have enabled mechanistic interpretations of FLIM of NADH in MII mouse oocytes. MII oocytes remain in a guasimetabolic steady state with constant FLIM parameters for many hours, making quantitative metabolic perturbations easier to interpret, hence providing an ideal system to relate FLIM parameters to specific metabolic activities. A coarse-grained biophysical model of NADH redox reactions has enabled the prediction of metabolic fluxes, i.e. the turnover rate of metabolites, within single oocytes from FLIM measurements of NADH (Yang et al., 2021). Specifically, this model enables the prediction of mitochondrial oxygen consumption rate (OCR) for single oocytes from noninvasive FLIM imaging of NADH. Previously, it has been proposed that OCR correlates with oocyte viability (Scott et al., 2008; Tejera et al., 2011), but it is unclear what cellular processes control OCR. Prediction of OCR from FLIM measurements has demonstrated that the OCR of oocytes is insensitive to perturbations in cellular energy demand and nutrient supply, despite significant sensitivity of NADH FLIM parameters (intensity, fluorescence lifetimes, enzyme engagement) to these perturbations. In contrast, an oocyte's OCR is sensitive to direct mitochondrial perturbations. These results show that OCRs of oocytes are determined by intrinsic properties of mitochondria, rather than by cellular energy demand or nutrient supply (Yang et al., 2021). This apparent OCR homeostasis also implies the existence of an unknown mechanism of metabolic regulation that maintains the global metabolic flux at the expense of redistribution of specific metabolic fluxes. Combining NADH redox modeling with detailed biophysical models of mitochondrial metabolism will help identify the rewiring of metabolic fluxes in the oocytes, providing biological insights into how metabolic perturbations impact oocyte viability.

Recent work has revealed subcellular metabolic heterogeneity, including spatial variations in the mitochondrial membrane near the meiotic spindle in mouse oocytes (Al-Zubaidi et al., 2019). Such subcellular metabolic heterogeneity may be associated with oocyte viability. For example, abnormal distributions of mitochondria correlate with a decrease in oocyte developmental competence (Yu et al., 2010; Liu et al., 2016), highlighting the potential utility of probing subcellular metabolic heterogeneity to predict oocyte viability. In addition to single-cell averaged OCR, NADH redox modeling also enables prediction of OCR at different locations within the same cell by taking advantage of the subcellular resolution of FLIM measurements. A subcellular OCR gradient exists within a single oocyte, where mitochondria closer to the oocyte periphery display a higher OCR than those at the center of the oocyte (Yang et al., 2021) (Fig. 3A). This metabolic gradient is caused by enhanced proton leak in peripherally located mitochondria, suggesting the existence of distinct subpopulations of mitochondria within a single oocyte. However, it is unclear how these metabolic variations arise and how they impact the viability and developmental competence of the oocyte. Do mitochondria of different intrinsic activities move into different locations of the oocyte during maturation or are mitochondria



**Figure 3. FLIM of NAD(P)H reveals spatiotemporal metabolic variations in mouse oocytes and developing embryos. (A)** Fluorescence lifetime imaging (FLIM) of NADH reveals a spatial gradient of the average NADH fluorescence lifetime in mouse metaphase II (MII) oocytes (left). An NADH redox model (middle) is used to interpret FLIM measurements and predicted a subcellular gradient of flux through the mitochondrial electron transport chain (ETC), or equivalently oxygen consumption rate (right). (**B**) FLIM of NAD(P)H reveals spatiotemporal metabolic variations in terms of the average NAD(P)H fluorescence lifetime during mouse preimplantation embryo development. Inner cell mass (ICM) and trophectoderm (TE) display different metabolic states.

responding to heterogeneous local signals? Since cumulus–oocyte crosstalk is crucial in oocyte maturation, it is natural to ask how CCs impact these metabolic heterogeneities and regulate oocyte viability. Previous work has shown that oocytes matured with or without CCs *in vitro* display different developmental competence (Zhang *et al.*, 2012). Combining biophysical modeling of metabolic crosstalk and highresolution imaging of cumulus–oocyte complexes by FLIM during oocyte maturation provides a method to address these questions.

Developing embryos display complex spatiotemporal metabolic dynamics. Understanding these variations may help guide embryo selection based on metabolic profiles. Recent work has highlighted the importance of spatiotemporal control of mitochondrial metabolism in oogenesis (Rodríguez-Nuevo et al., 2022) and early embryo development (Nagaraj et al., 2017). As discussed above, FLIM metabolic profiles of human blastocysts are associated with their developmental stage, but not with their morphological assessment (Venturas et al., 2022). Understanding these correlations will require relating FLIM measurements to metabolic activities of the embryo. Earlier work has shown that embryo metabolism transitions from a respiration dominant mode to a hybrid mode of respiration and fermentation at the blastocyst stage, which provides a starting point to interpret variations in FLIM parameters. Mouse embryos provide a model system to study metabolic variations. FLIM of NAD(P)H and FAD+ has revealed intricate spatiotemporal dynamics throughout mouse preimplantation embryo development (Fig. 3B) (Sanchez et al., 2019). Notably, a striking metabolic heterogeneity between the inner cell mass and trophectoderm has been observed in both mouse (Fig. 3B) and human blastocysts (Venturas et al., 2022), suggesting a potential connection between metabolic variation and cell fate specification (Kumar et al., 2018; Chi et al., 2020). Single-cell and spatial transcriptomics have helped elucidate cell lineage specification in early embryos (Peng et al., 2020; Meistermann et al., 2021). Combining FLIM with transcriptomics and metabolic perturbations should help elucidate the causes and consequences of these metabolic variations, their role in cell fate specification (Peng et al., 2020; Zhu and Zernicka-Goetz, 2020), and guide embryo selection in ART. It is well known that different cells within an embryo can exhibit different ploidies (Popovic et al., 2019; Capalbo et al., 2021). Since FLIM can provide information on the metabolic state of individual cells within an embryo, it would be interesting to use this technique to determine if this genetic mosaicism leads to metabolic heterogeneity.

### Conclusion

It remains an open challenge to select oocytes and embryos with the highest developmental competence in ART. Extensive studies (Gardner et al., 2011; Thompson et al., 2016) have revealed associations between metabolic state and embryo developmental competence. Since FLIM of NAD(P)H and FAD+ can be used to

quantitatively characterize the metabolic states of CCs, oocytes, and embryos in a label-free and noninvasive manner (Ma et al., 2019; Sanchez et al., 2019; Venturas et al., 2022), it is a promising tool for selecting oocytes and embryos. Furthermore, recent studies on CCs, oocytes, and embryos have demonstrated that FLIM can sensitively detect metabolic variations not only across samples between different patients but also within samples from the same patient (Venturas et al., 2021, 2022). In addition, metabolic variations in oocytes and embryos have been associated with oocyte maturity, ploidy status (Shah et al., 2022), and embryo developmental stages but not with embryo morphology (Venturas et al., 2022). These results suggest that metabolic characterizations can be combined with patient clinical characterization and morphological evaluations to provide a synergistic approach for the selection of oocytes and embryos. Initial work in mouse indicates minimal photodamage from FLIM measurements (Sanchez et al., 2018), but further safety studies on human oocytes and embryos will be necessary. Biophysical models aid the interpretation of FLIM measurements and will provide a mechanistic basis for oocyte and embryo selection (Yang et al., 2021). Establishing the potential predictive power that FLIM can have will ultimately require future studies determining the extent of association between FLIM measurements and ART outcome.

### Data availability

No new data were generated or analysed in support of this research.

### **Authors' roles**

M.V. and X.Y. contributed to the conceptualization of the idea of the review and drafted the manuscript. D.S. and D.N. critical revision of the article.

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### **Conflict of interest**

D.N. is an inventor on patent US20170039415A1.

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