CSIRO PUBLISHING

SPECIAL ISSUE | REVIEW
https://doi.org/10.1071/RD22217

Reproduction, Fertility and Development

Non-invasive assessment of oocyte developmental competence

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Published: 18 October 2022

Cite this:
Tan TCY and Dunning KR (2023) *Reproduction, Fertility and Development*, **35**(1–2), 39–50.
doi:10.1071/RD22217

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ABSTRACT

Oocyte quality is a key factor influencing IVF success. The oocyte and surrounding cumulus cells, known collectively as the cumulus oocyte complex (COC), communicate bi-directionally and regulate each other's metabolic function to support oocyte growth and maturation. Many studies have attempted to associate metabolic markers with oocyte quality, including metabolites in follicular fluid or 'spent medium' following maturation, gene expression of cumulus cells and measuring oxygen consumption in medium surrounding COCs. However, these methods fail to provide spatial metabolic information on the separate oocyte and cumulus cell compartments. Optical imaging of the autofluorescent cofactors – reduced nicotinamide adenine dinucleotide (phosphate) [NAD(P)H] and flavin adenine dinucleotide (FAD) – has been put forward as an approach to generate spatially resolved measurements of metabolism within individual cells of the COC. The optical redox ratio (FAD/[NAD(P)H + FAD]), calculated from these cofactors, can act as an indicator of overall metabolic activity in the oocyte and cumulus cell compartments. Confocal microscopy, fluorescence lifetime imaging microscopy (FLIM) and hyperspectral microscopy may be used for this purpose. This review provides an overview of current optical imaging techniques that capture the inner biochemistry within cells of the COC and discusses the potential for such imaging to assess oocyte developmental competence.

Keywords: autofluorescence, cellular metabolism, FAD, NAD(P)H, non-invasive, oocyte assessment, optical imaging, optical redox ratio.

Introduction

In recent years, the social trend of delayed childbearing (pregnancy occurring in women 35 years and older) is one of the growing reasons for subfertility (Johnson *et al.* 2012; Adashi and Gutman 2018). Advanced maternal age, along with increased incidence of lifestyle factors such as obesity and diabetes, have contributed to the statistic that one in six couples have trouble conceiving (Chambers *et al.* 2017). Many studies have associated maternal age with oocyte developmental competence (Cimadomo *et al.* 2018; Krisher 2018; Lee *et al.* 2021; Ntostis *et al.* 2022). Oocyte developmental competence is defined as the ability of an oocyte to successfully resume meiosis, undergo fertilisation and preimplantation embryo development, implant and result in the birth of a healthy offspring (Dunning *et al.* 2010). Importantly, oocyte quality is a key limiting factor in determining the success rate of an IVF cycle (Gilchrist *et al.* 2008).

The predominant method used to assess oocyte quality is morphological inspection prior to fertilisation (Lasienë *et al.* 2009). Several reviews discuss, and cover in detail, the morphological predictors of oocyte quality, which include assessments of cumulus expansion, zona pellucida, perivitelline space, polar body cytoplasm, and meiotic spindle analysis using polarisation microscopy (Ebner *et al.* 2003; Wang and Sun 2007; Lasienë *et al.* 2009). However, oocyte selection based on morphological criteria are highly subjective and dependent on the skill of the embryologist in predicting competency (Wang and Sun 2007; Richani *et al.* 2021).

There are many studies focused on understanding the metabolism of the cumulus oocyte complex (COC) (O'Brien *et al.* 1996; Ferguson and Leese 2006; Dunning *et al.* 2010; Sutton-McDowall *et al.* 2010; Hemmings *et al.* 2012) and how various metabolic pathways impact

oocyte quality (for review, see Richani *et al.* 2021). However, current methods used to measure metabolism are potentially limited in their ability to predict oocyte developmental potential, as they mostly focus on the follicular fluid or 'spent medium' following oocyte maturation, which are largely reflective of the somatic follicular cells and not the oocyte (Richani *et al.* 2021). In this review, we will discuss how optical approaches can provide spatial metabolic information on the separate oocyte and cumulus cell compartments, as well as the potential for such imaging to assess oocyte developmental competence.

Metabolic co-dependence of the oocyte and cumulus cells

Substrates for mammalian oocyte metabolism primarily come from the surrounding environment (*in vivo* or *in vitro* culture) which include glucose, pyruvate, lactate and glutamine. Oocytes regulate their metabolism during development, with mitochondria being a vital organelle for this.

Mitochondria

Mitochondria play a multifaceted role in supporting the development of the mammalian oocyte and embryo. They are membrane-bound organelles present in almost all eukaryotic cells. The mitochondria in mammalian oocytes are different from the typical structure found in somatic cells in that they are more spherical and contain fewer, irregular cristae. Therefore, the mitochondria found in oocytes are less efficient at producing ATP compared to somatic cell mitochondria (Bentov *et al.* 2011). As such, mature oocytes contain the largest number of mitochondria (~100 000) (May-Panloup *et al.* 2005), far more than sperm (~50–75) (Hirata *et al.* 2002) or somatic cells (~80–2000) (Cole 2016) to meet their energy requirements. Importantly, the number of mitochondria within an oocyte is one of the important factors for oocyte and subsequent embryo quality. This is because they are maternally inherited organelles, and the early stages of embryo development are entirely dependent on the starting pool of mitochondria from the oocyte until the blastocyst-stage embryo (van der Reest *et al.* 2021). Mitochondrial dysfunction has been associated with fertilisation failure (Santos *et al.* 2006), abnormal embryo development (Ge *et al.* 2012), implantation failure (Wai *et al.* 2010) and increased incidence of aneuploidy (Fragouli *et al.* 2015).

The primary function of mitochondria is to generate ATP through oxidative phosphorylation, which is supported by the tricarboxylic acid (TCA) cycle. The TCA cycle generates electron carriers: reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$), mainly from catabolism of glucose and fatty acids (May-Panloup *et al.* 2021). Oxidative phosphorylation involves five large multi-enzymatic complexes embedded within the cristae of the inner mitochondrial membrane. The electron transport chain (ETC) functions by the transfer of electrons using dedicated electron carriers along the mitochondrial membrane, while pumping protons into the intermembrane space, giving rise to the mitochondrial membrane potential (van der Reest *et al.* 2021). The proton gradient generated by the transfer of electrons across the ETC is ultimately used to power the synthesis of ATP from ADP by the ATP synthase (May-Panloup *et al.* 2021). The ratio of reduced to oxidised electron donors (NADH/NAD+ and $FADH_2$/FAD) in the mitochondrial matrix space forms an effective measurement of overall metabolic activity – the redox ratio (Dumollard *et al.* 2009; Skala and Ramanujam 2010).

Metabolism in mammalian oocytes: a requirement for oocyte developmental competence

During maturation, oocytes exhibit a high energy demand required for a series of nuclear and cytoplasmic changes. The mammalian oocyte and surrounding cumulus cells communicate bi-directionally (Eppig 2001). These two cell types are connected by gap junctions (Anderson and Albertini 1976), forming the cumulus oocyte complex (COC). Substrate utilisation, especially glucose and fatty acids, provide the oocyte with sufficient ATP to attain developmental competence (Sutton-McDowall *et al.* 2010; Dunning *et al.* 2011).

Glucose is one of the key substrates for the COC to generate ATP. The oocyte itself has a low capacity for glucose metabolism due to the lack of phosphofructokinase activity (Dumesic *et al.* 2015). The highly glycolytic cumulus cells therefore play an important role in metabolising glucose to pyruvate, which is then supplied via gap junctions to the oocyte for ATP production. (Biggers *et al.* 1967; Yeo *et al.* 2009; Sutton-McDowall *et al.* 2010; Dumesic *et al.* 2015). Pyruvate is converted into acetyl coenzyme A (Acetyl-CoA) in the oocyte mitochondria, which enters the TCA cycle and electron transport chain (ETC) to produce ATP. Concurrently, the oocyte ensures its own supply of pyruvate by up-regulating glycolytic genes in cumulus cells via secretion of growth differentiation factor-9 (Sugiura *et al.* 2007; Gilchrist *et al.* 2008). A small portion of glucose is metabolised by the pentose phosphate pathway in cumulus cells (Sutton-McDowall *et al.* 2010), which involves the conversion of glucose-6-phosphate dehydrogenase (generated from glucose) to ribose 5 phosphate, producing NADPH. The molecule NADPH is important for the biosynthesis of fatty acids and nucleotides, and for maintaining the balance between reduced and oxidised glutathione, which acts as an antioxidant against reactive oxygen species (ROS) (Gutnisky *et al.* 2014).

In recent years, the importance of lipid metabolism, specifically fatty acid- or β-oxidation, for oocyte developmental potential has been shown (Ferguson and Leese 2006; Sturmey *et al.* 2006; Dunning *et al.* 2010). Activated fatty acids (fatty acyl-CoA) are transported into mitochondria by the carnitine palmitoyl transferase 1. Fatty acyl-CoA is then metabolised via β-oxidation in the mitochondria matrix and enters the TCA cycle to generate NADH and $FADH_2$ (Dunning *et al.* 2010). During ovulation, the maturing COC increases the use of energy from lipid metabolism, as metabolism of fatty acids has the capacity to produce 106 ATP, which is 3.5-fold more than glucose (Valsangkar and Downs 2013; Dunning *et al.* 2014). As the metabolism of fatty acids and glucose are crucial for oocyte development, it is important to maintain balanced energy homeostasis within oocytes and cumulus cells, which further supports subsequent embryo development (Dumollard *et al.* 2007; Blacker and Duchen 2016).

Assessment of oocyte developmental competence

To date, much of the work has focused on finding methods to select the most viable embryo for transfer, while research into developing assessments for oocyte developmental potential has received far less attention. Importantly, the quality of the oocyte cannot be improved by altering laboratory practices, as it is determined prior to oocyte pick-up from the ovary. Therefore, oocyte quality is the first checkpoint for a successful pregnancy, and is important not only for fertilisation, but also for subsequent development (Gilchrist *et al.* 2008).

Clinically, all morphologically normal oocytes are fertilised and selection is mainly focused on the embryo (Richani *et al.* 2021). As developmental competency of an embryo is highly dependent on the oocyte it is derived from, selection of an oocyte with good developmental potential is likely to improve IVF success (Richani *et al.* 2021). This then raises a challenge; do we have a clinically reliable and accurate diagnostic to select good quality oocytes?

Potential biomarkers in follicular fluid, culture medium and cumulus cells

There has been significant interest in finding potential biomarkers of oocyte developmental competence in follicular fluid, 'spent' culture medium and cumulus cells. Profiling of follicular fluid is a promising approach as the material is collected directly from the environment in which the oocyte developed and matured. Some of the potential biomarkers linked with oocyte quality include cytokines and growth factors such as granulocyte-colony stimulation factors, interleukins and growth differentiation factor-9 (Mendoza *et al.* 2002). Similarly, the presence and abundance of fatty acids in follicle fluid has also been shown to associate with oocyte quality (Jungheim *et al.* 2011; Feng *et al.* 2022). Additionally, proteomic (Ambekar *et al.* 2013; Shen *et al.* 2017) and metabolomic (Wu *et al.* 2007; O'Gorman *et al.* 2013) analysis of follicular fluid showed correlations between metabolites and oocyte developmental competence.

Analysis of IVM 'spent medium' has been proposed as a potential predictor for oocyte competence, particularly fertilisation success. In a mouse model, developmentally competent COCs that were subsequently fertilised demonstrated an increase in glucose consumption and lactate secretion, compared to those that did not fertilise (Preis *et al.* 2005). In a separate study, the turnover of amino acids were significantly different between bovine oocytes that failed to fertilise and those that subsequently developed to the blastocyst-stage (Hemmings *et al.* 2012). Specifically, oocytes that failed to fertilise utilised significantly more glutamine and produced significantly higher amounts of alanine compared to those that subsequently developed to the blastocyst-stage (Hemmings *et al.* 2012).

Cumulus cell gene expression, involved in cell signalling, homeostasis and metabolism was also proposed as a non-invasive viability marker (McKenzie *et al.* 2004; Fragouli *et al.* 2012). Similarly, in human cumulus cells, genes involved in metabolism and extracellular matrix formation were expressed at a significantly higher level when derived from an oocyte that resulted in a live birth (Gebhardt *et al.* 2011). Taking a slightly different approach, multiple studies have shown that telomere length in cumulus cells was associated with oocyte quality. (Cheng *et al.* 2013; Ozturk *et al.* 2014).

Despite the generation of a substantial body of research to develop potential predictors for oocyte developmental competence, these methods have not been routinely implemented. Perhaps this is due to the limitations of these approaches, which include (1) confounding factors caused by varying clinical practice; (2) small sample size; and (3) measurements of the entire COC that cannot detect heterogeneity between the oocyte and cumulus cell compartments. Therefore, development of a reliable, accurate and non-invasive tool to assess oocyte quality would be highly desirable.

New tools to non-invasively measure oocyte metabolism

Metabolic co-factors: NAD(P)H and FAD

In the 1950s, Britton Chance first discovered and began to characterise the naturally fluorescent metabolic coenzymes: NADH and FAD, and associated these molecules with cellular metabolism (Chance 1952; Chance *et al.* 1962*a*). His research started in isolated cells (Chance *et al.* 1964; Scholz *et al.* 1969), and subsequently explored the potential of these

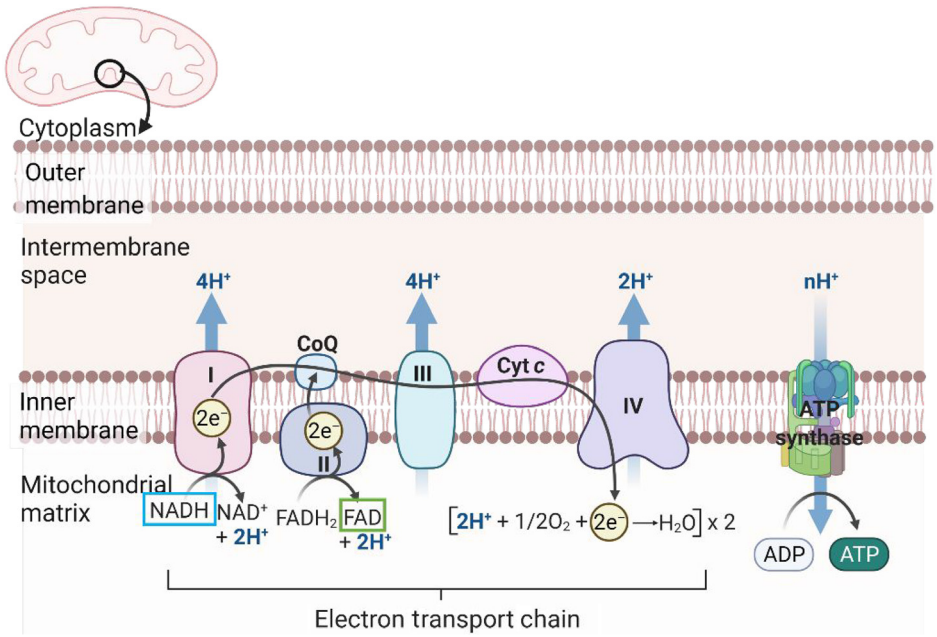

Fig. 1. Non-invasive assessment of cellular metabolism via optical imaging of autofluorescent co-factors used in the electron transport chain. The electron transport chain (ETC) consists of five protein complexes embedded within the inner mitochondria membrane. The electron carriers (NADH and $FADH_2$) produced from TCA cycle are used to fuel the ETC by transporting electrons along the complexes and pump proton ions (H+) into the intermembrane space. Complex I (NADH dehydrogenase) accepts electrons from NADH, and passes them to co-factor ubiquinone Q (CoQ). At the same time, four hydrogen ions will be pumped into the intermembrane space. Complex II (succinate dehydrogenase) accepts electrons from $FADH_2$ and passes them to CoQ. Ubiquinone Q passes these electrons to complex III (cytochrome *c* dehydrogenase) which also pumps four hydrogen ions from the matrix into the intermembrane space. Two electrons are the passed to complex IV, a cytochrome *c* reductase, which will bind to oxygen and hydrogen ions to form a water molecule (H_2O). The hydrogen ions pumped from the matrix to the intermembrane space form a proton gradient which powers ATP synthase to pump hydrogen ions back to the matrix and form ATP from ADP. The activity of ETC can be measured by optical imaging of the autofluorescent cofactors NAD(P)H (blue box) and FAD (green box) and provides a spatially resolved indicator of metabolism via the optical redox ratio (FAD/[NAD(P)H + FAD]). Figure generated using BioRender.

molecules as reliable indicators of cell metabolism in other tissues such as the liver (Ji *et al.* 1979; Quistorff and Chance 1986), bladder and brain (Chance *et al.* 1962*b*). In mitochondria, NADH and FAD are directly involved in the production of ATP through oxidative phosphorylation (Fig. 1). These metabolic co-factors exist either in the oxidised (NAD^+, $NADP^+$, FAD) or reduced (NADH, NADPH and $FADH_2$) state. Among these, only NADH, NADPH and FAD are autofluorescent. Therefore, quantifying the fluorescence intensity of these molecules has been used to identify the metabolic changes in living cells or tissues. The autofluorescence of these coenzymes (both bound and free) can be detected using specific excitation and emission bands (Table 1) (Georgakoudi and Quinn 2012; Kolenc and Quinn 2019). It is important to note that NADH and NADPH have near identical spectral properties, generally excited at 335–350 nm and emitted at 440–460 nm and are therefore collectively referred as NAD(P)H. On the other hand, FAD is excited at 450 nm and reaches peak emission at approximately 520 nm. To understand the connection between redox state and metabolism, it is important to understand the roles of NADH and FAD in oxidative phosphorylation.

Roles of NADH and FAD in oxidative phosphorylation

During cellular metabolism, substrates such as glucose and fatty acids are broken down to produce ATP. During glycolysis, a glucose molecule is catabolised to pyruvate, producing ATP and reducing NAD^+ to NADH. Following glycolysis, pyruvate is shuttled into the mitochondria and broken down to acetyl-CoA by pyruvate dehydrogenase. Acetyl-CoA will then enter the TCA cycle, which begins

Table 1. Excitation and emission of endogenous fluorophores.

Metabolic coenzymes	Peak single-photon excitation wavelength (nm)	Peak two-photon excitation wavelength (nm)	Peak single-photon emission wavelength (nm)	Peak two-photon emission wavelength (nm)
NADH (bound)	335–350	690–730	440–450	440–450
NADH (free)	335–350	690–730	450–470	450–460
NADPH	335–350	690–730	450–470	450–460
FAD (bound)	365, 455	700–730	515–525	515–525
FAD (free)	375, 450	700–730	515–525	515–525

Data sourced from (Georgakoudi and Quinn 2012; Kolenc and Quinn 2019).

to break down carbon-based molecules to CO_2, producing electron carriers: NADH and $FADH_2$. During oxidative phosphorylation, TCA cycle generated NADH and $FADH_2$ revert back to their oxidised form in the ETC–NAD+ and FAD by the enzyme complexes I (NADH dehydrogenase) and II (succinate dehydrogenase), respectively (Fig. 1). Electrons are passed to coenzyme Q and complex III (cytochrome *c* reductase). The ultimate fate of these electrons is the conversion of oxygen to water at complex IV (cytochrome *c* oxidase). Simultaneously, proton ions are pumped into the intermembrane space of the mitochondria by complex I, III and IV. This creates a proton gradient, which is used to power ATP synthase to produce ATP (Osellame *et al.* 2012).

The ratio of reduced to oxidised electron carriers indicates the metabolic state of cells that primarily rely on oxidative phosphorylation. As mentioned earlier, only NAD(P)H and FAD are naturally fluorescent molecules, thus, the ratio of FAD/(FAD + NAD(P)H), can be utilised to measure the cell redox state (referred to as optical redox ratio; ORR) (Chance *et al.* 1979). During oxidative phosphorylation, the autofluorescence of NADH decreases when oxidised to non-fluorescent NAD+ (at complex I), while the autofluorescence of FAD increases due to oxidation of $FADH_2$ (at complex II), resulting in a higher ORR, indicative of increased ATP production (Georgakoudi and Quinn 2012). On the other hand, the ORR will decrease in the event of hypoxia or the need to increase glycolysis despite the presence of oxygen (Warburg effect), as commonly observed in cancer cells or cells undergoing high proliferation in response to disease or injury (Liberti and Locasale 2016). It is important to acknowledge that not all NADH fluorescence is associated with the bound state in oxidative phosphorylation, but can also be contributed from bound- and free-NADPH and free-NADH found in the cytoplasm (Blacker and Duchen 2016). The same is true for FAD; although the majority of FAD is found within the mitochondria and associated with oxidative phosphorylation, a small fraction of FAD fluorescence can be either attributed to electron transfer flavoproteins during the fatty acid oxidation or reduced by dithionite which is not related to cellular metabolism (Kunz and Kunz 1985).

Autofluorescence: label-free optical imaging to measure cellular metabolism

Autofluorescence within cells is commonly perceived as an interfering factor due to the impact on contrast when using a fluorescently labelled probe (Staikopoulos *et al.* 2016). However, a wide range of endogenous fluorophores that are integral to cellular function such as lipids, vitamins and amino acids have been identified as naturally fluorescent (Andersson *et al.* 1998; Croce and Bottiroli 2014). With advancements in microscopies and the ability to capture high-resolution images, autofluorescence recorded by optical imaging is able to measure the inner biochemistry of cells. Thus, this presents an opportunity for a non-invasive measurement that can directly measure the cellular metabolism of an individual oocyte, and potentially determine their developmental potential. As NADH and FAD are directly associated with mitochondrial activity (Blacker and Duchen 2016), both have been utilised to monitor the metabolic changes of oocytes and embryos in the complete absence of exogenous tags (Dumollard *et al.* 2004, 2007; Sutton-McDowall *et al.* 2012, 2015; Nohales-Córcoles *et al.* 2016). Importantly, we and others have shown that optical imaging has the capacity to capture the dynamic and heterogenic aspects of metabolic differences that exist between cells of the COC and the embryo (Dumollard *et al.* 2004, 2007; Denisenko *et al.* 2005; Brison *et al.* 2014; Sutton-McDowall *et al.* 2015; Tan *et al.* 2022*a*). To achieve this, various optical imaging approaches have been proposed, including standard fluorescence microscopy, Raman spectroscopy, laser scanning confocal microscopy and hyperspectral microscopy. In our recent study, we found that the ORR was an accurate reflection of metabolism in the oocyte compartment when compared with oxygen consumption measured in the whole COC (Fig. 2; Tan *et al.* 2022*a*). These findings demonstrate that label-free optical imaging of metabolic cofactors is a sensitive assay for measuring metabolic changes and has the potential to assess oocyte developmental competence.

Although optical imaging is deemed as non-invasive for living cells or tissues, this cannot be assumed to be the case for oocytes and embryos. Existing literature shows that light exposure can be detrimental to embryo development and lead to increased levels of DNA damage

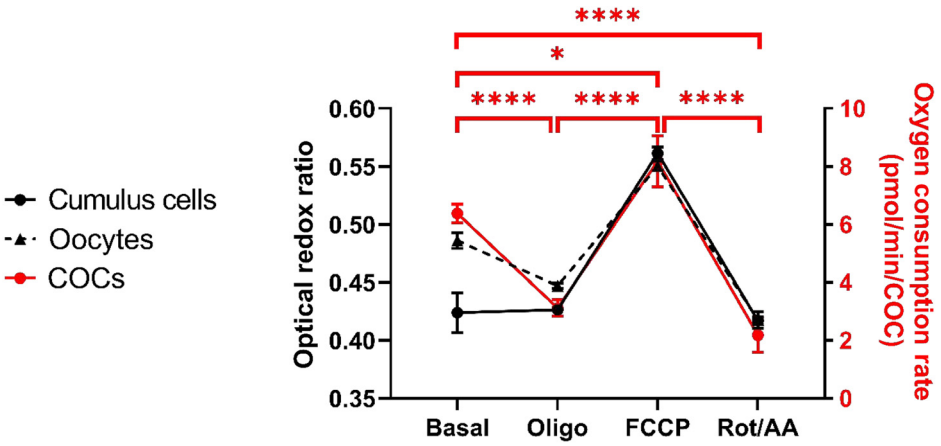

Fig. 2. Optical imaging of autofluorescent metabolic cofactors in cumulus oocyte complexes reflects changes in oxygen consumption rate. Metabolism was measured in immature cumulus oocyte complexes (COCs) in response to metabolic inhibitors of the electron transport chain: oligomycin (oligo: 2.0 μM), carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP: 1 μM) and Rotenone/antimycin A (Rot/AA: 2.5 μM). The changes in metabolism in response to these inhibitors were measured by either laser scanning confocal microscopy (intracellular NAD(P)H and FAD) or extracellular flux analysis (oxygen consumption rate). The optical redox ratio for the oocyte and cumulus cell compartments were compared with the oxygen consumption rate for whole COCs. Data are presented as mean ± s.e.m.; optical redox ratio: $n = 12$ COCs per drug treatment, four independent experimental replicates; oxygen consumption rate: $n = 16$ wells (20 COCs/well; oxygen consumption rate was normalised using the number of COCs per well and presented as pmol/min/COC), four independent experimental replicates. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Figure adapted from Tan *et al.* (2022*a*).

(Takenaka *et al.* 2007; Campugan *et al.* 2022). Therefore, the safety of any imaging modality must be assessed in detail before implementing to human IVF.

Standard fluorescence microscopy

Early autofluorescence work by Chance was not focused on providing a non-invasive assessment tool, therefore the general localisation of NAD(P)H fluorescence intensity within living cells was initially measured using a fluorimeter (Chance and Thorell 1959). This was followed by the development of the Chance Redox Scanner which allowed the capture of images (Quistorff *et al.* 1985). Two-photon excited fluorescence (TPEF) microscopy has emerged as another imaging tool to assess metabolic activity and cellular responses. In TPEF microscopy, molecules are excited through the simultaneous absorptions of two near-infrared photons with half the energy and double the wavelength (Quinn *et al.* 2013). This allows for imaging at higher penetration depths and reduces potential photodamage in the out-of-focus regions (Georgakoudi and Quinn 2012). However, longer wavelengths are not ideal for high resolution three-dimensional (3D) imaging (Swoger *et al.* 2014).

Laser scanning confocal microscopy

Confocal microscopy has become a standard imaging modality for biological applications when requiring 3D images. Laser scanning confocal microscopy operates by focal point illumination with a high-power laser. The 'confocal pinhole' is an aperture placed in front of the detector to reject any out-of-focus light and allow images to be taken at different depths (Blacker and Duchen 2016). This allows for image acquisition of high-resolution subcellular structures and provides detailed localisation of molecules of interest. This imaging approach was utilised widely to investigate a range of developmental processes in different cell types such as epithelial and endothelial cells in the cornea (Masters *et al.* 1993), lung tissue (Erokhina *et al.* 2019), plant cells (Roshchina 2012) and embryonic cells (Heppert *et al.* 2016). Traditionally, most of the studies that employ confocal microscopy use an exogenous tag or molecular probe to monitor cellular processes and biochemistry within the cells (Johnson 2010), such as DAPI for nucleus, Mitotracker for mitochondria and Mitosox for superoxide and antibodies. There is increasing interest in using confocal microscopy to capture metabolic cofactors, NAD(P)H and FAD, in oocytes and embryos (Dumollard *et al.* 2004, 2007; Sutton-McDowall *et al.* 2016, 2017; Santos Monteiro *et al.* 2021). Despite the promising results from these studies, a pitfall of this approach is the use of lasers which makes it difficult for clinical implementation due to the high probability of photodamage (Campugan *et al.* 2022).

Fluorescence lifetime microscopy (FLIM)

Fluorescence lifetime microscopy (FLIM) can capture autofluorescence and is able to provide discrimination between NADH and NADPH (Blacker *et al.* 2014). It may offer a more sensitive approach to detect metabolic cofactors by measuring the time a fluorophore remains in an excited state before being emitted, allowing the discrimination between bound- and free-NADH, and separating the NADH fluorescence from NADPH (Drozdowicz-Tomsia *et al.* 2014; Sanchez *et al.* 2019). Indeed, in reproductive medicine research, FLIM has proven successful in assessing the metabolic processes of oocytes (Sanchez *et al.* 2018, 2019) and provides spatial distribution of metabolic cofactors in the cytoplasm and mitochondrial regions. However, FLIM involves the use of complex laser and detection electronics for image acquisition, making it challenging for clinical translation.

Raman microscopy

Raman spectroscopy is a label-free imaging method based on inelastic scattering of monochromatic light, allowing direct evaluation of organelle structures and molecular biochemical processes inside living cells (Windom and Hahn 2013). With the well-developed knowledge that metabolism is linked to oocyte quality, Raman spectroscopy was proposed as a non-invasive method to investigate the correlation between metabolomic profile and embryo quality, as well as an indicator of developmental potential and pregnancy outcome (Seli *et al.* 2007; Scott *et al.* 2008; Perevedentseva *et al.* 2019). Raman spectroscopy was also employed to study lipid metabolism by quantifying lipid content of mouse oocytes (Bradley *et al.* 2016). However, a drawback of this approach is the use of lasers (Yakubovskaya *et al.* 2019), which potentially have downstream impacts on embryo development depending on the laser wavelength and dose of laser radiation. A single study by Perevedentseva *et al.* (2019) demonstrated that although the development rate from 2-cell to blastocyst-stage was not impacted in mouse embryos, the cell number of blastocysts was significantly reduced in the Raman-imaged group compared to control. This suggests that Raman spectroscopy may not be suitable for implementation in an IVF setting.

Hyperspectral imaging

A promising imaging modality with capacity to capture cellular autofluorescence in the oocyte and embryo is hyperspectral microscopy. This form of imaging employs multiple light-emitting diodes of varying excitation and emission wavelengths to record autofluorescent signatures. This broad-spectrum approach (excitation from 340 nm to 750 nm) allows for the detection of a range of endogenous fluorophores, not limited to NAD(P)H and FAD, thereby providing insight into the metabolism and content of a cell (Gosnell *et al.* 2016*a*). Hyperspectral imaging can detect subtle differences in metabolic signatures between disease and healthy states: pancreatic cancer cells (Gosnell *et al.* 2016*a*); olfactory cells (Gosnell *et al.* 2016*b*); and articular cartilage tissue (Mahbub *et al.* 2019). In the field of reproductive biology, hyperspectral microscopy demonstrated the capacity to discern between good and poor quality bovine embryos (Sutton-McDowall *et al.* 2017; Santos Monteiro *et al.* 2021) and oocytes denuded of cumulus cells from young and aged mice (Bertoldo *et al.* 2020). Additionally, mathematical algorithms applied to autofluorescence signals obtained from hyperspectral imaging were able to discriminate between different cell populations (Gosnell *et al.* 2016*a*; Sutton-McDowall *et al.* 2017; Habibalahi *et al.* 2019; Mahbub *et al.* 2021; Tan *et al.* 2022*b*). Our recent work successfully demonstrated the ability of hyperspectral imaging to separately measure metabolism in the oocyte and cumulus cell compartments of the intact COC, when compared with conventional laser scanning confocal microscopy (Fig. 3; Tan *et al.* 2022*a*). With the ability to capture metabolic heterogeneity between cells within an embryo and between compartments of a COC (cumulus cells vs the oocyte), hyperspectral microscopy may be a potential non-invasive approach to detect metabolic changes associated with oocyte and embryo quality.

Implications of non-invasive oocyte assessment for livestock breeding

Apart from human IVF, non-invasive assessment of oocyte quality could be implemented in the livestock industry. *In vitro* produced (IVP) embryos are widely used in the livestock industry to improve genetic gain, especially for cattle (Pontes *et al.* 2009). However, the efficiency of IVP is still far from optimal, with the pregnancy rate remaining below 50% (Ealy *et al.* 2019). To date, the industry continues to rely on the subjective analysis of oocyte and embryo quality by an embryologist based on morphological classification (Wrenzycki 2021). A plethora of basic research on new technologies to assess oocyte and embryo health exist in the clinical space. However, there are relatively few occurrences of this in livestock species. This is perhaps due to methods such as time-lapse imaging and metabolomic profiling being logistically challenging and expensive (McLennan *et al.* 2020). Therefore, there exists an opportunity to provide the research field with imaging modalities that are cost-effective and easy to use. This may lead to an amplification of research in this area which if successful, will result in the development of an accurate diagnostic tool for oocyte developmental competence, delivering better outcomes in breeding efficiencies and positively impacting the livestock industry.

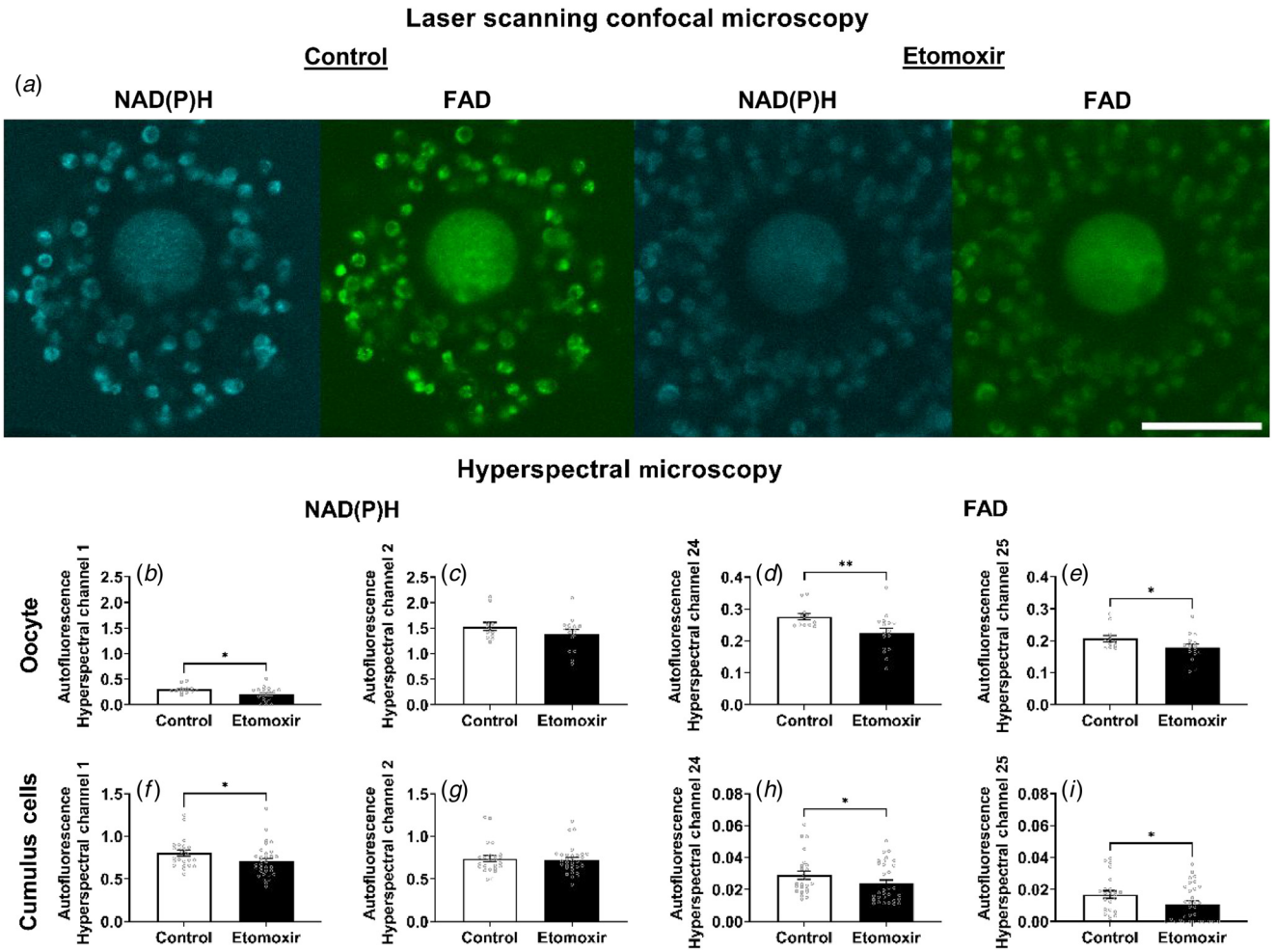

Fig. 3. Hyperspectral microscopy detects metabolic changes associated with oocyte quality. Cumulus oocyte complexes (COCs) were matured *in vitro* in the absence or presence of etomoxir. Etomoxir is a known inhibitor of fatty acid metabolism (β-oxidation), leading to poor quality oocyte. The metabolic response to etomoxir was measured by laser scanning confocal microscopy (*a*) or hyperspectral microscopy (*b*–*i*) for intracellular NAD(P)H and FAD. Representative images of confocal microscopy for NAD(P)H and FAD autofluorescence are shown in (*a*). Hyperspectral microscopy was employed due to its low energy dose, making it more clinical compatible. Autofluorescence intensity was quantified for the oocyte (*b*–e) and cumulus cells (*f*–*i*) in hyperspectral channels that match the spectral properties of NAD(P)H: channel 1 (*b* and *f*) and channel 2 (*c* and *g*); and FAD: channel 24 (*d* and *h*) and channel 25 (e and *i*). Data were analysed by a two-tailed unpaired Student's *t*-test (*b*–*d*, *i*) or Mann–Whitney test (e–*h*). Data presented as mean ± s.e.m., three independent experimental replicates; $n = 12$ for control COCs, $n = 16$ for etomoxir-treated COCs. $^{*}P < 0.05$, $^{**}P < 0.01$. Figures adapted from Tan *et al.* (2022*a*).

Future directions

Existing selection methods for oocyte developmental competence are not predictive for pregnancy success. Therefore, there is a need to develop a viability assessment tool that is accurate, non-invasive, and importantly, predicts pregnancy success and live birth. Considering the importance of metabolism in determining oocyte developmental potential and the limitations of current methods in failing to detect metabolic heterogeneity between cells, label-free optical imaging of endogenous fluorophores may be a promising tool for measuring oocyte developmental competence. These advanced technologies combine non-invasive, spatially resolved, real time metabolic measurements. These metabolic measurements and their correlation with subsequent developmental outcomes will provide us with further understanding on how the dynamic metabolic changes in the COC impacts developmental competence. Importantly, metabolic imaging of COCs, following either oocyte pick up (OPU) or *in vitro* maturation (IVM), may facilitate a ranking of oocytes based on developmental competence leading to improved live birth rates. Although not the focus of this review, such imaging of resultant embryos may provide additional information to that acquired at the oocyte stage. This burgeoning field

of research will undoubtedly benefit both clinical and agricultural IVF.

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Data availability. Data sharing is not applicable as no new data were generated or analysed during this study.

Conflicts of interest. The authors declare no conflicts of interest.

Declaration of funding. K.R.D. is supported by a Mid-Career Fellowship from the Hospital Research Foundation (C-MCF-58-2019). K.R.D and T.C.Y.T. acknowledge support from the National Health and Medical Research Council (APP2003786).

Author contributions. T.C.Y.T. wrote the first draft of the manuscript. K.R.D. conceived the review content, provided critical feedback and edited the manuscript. All authors edited and approved the manuscript.

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