Jeremy G. Thompson, Hannah M. Brown and Melanie L. Sutton-McDowall

Measuring embryo metabolism to predict embryo quality


Journal compilation © IETS 2016

Originally Published at: http://dx.doi.org/10.1071/RD15340

PERMISSIONS


Green Open Access

All journals published by CSIRO Publishing allow authors to deposit the Accepted version of their manuscript into an institutional repository or put it on a personal website, with no embargo.

The Accepted version is the author-created, peer-reviewed, accepted manuscript. The Publisher’s edited or typeset versions cannot be used. The institutional repository should be that of the institution employing the author at the time the work was conducted or PubMed Central. We ask that authors link to the published version on the CSIRO Publishing website, wherever possible.

19 September 2016

http://hdl.handle.net/2440/101131
TITLE: Measuring embryo metabolism to predict embryo quality.


ADDRESS: ARC Centre of Excellence for Nanoscale BioPhotonics Robinson Research Institute, School of Medicine, The University of Adelaide, Adelaide 5005 Australia.

Corresponding Author: Jeremy G Thompson, School of Medicine, The University of Adelaide, Adelaide 5005, Australia.

Email: jeremy.thompson@adelaide.edu.au
ABSTRACT

Measuring the metabolism of early embryos has the potential to be used as a prospective marker for post-transfer development, either alone or in conjunction with other embryo quality assessment tools. This is necessary to maximise the opportunity of couples to have a healthy child from assisted reproduction (ART) and for livestock breeders to efficiently improve the genetics of their animals. Nevertheless, although many promising candidate substrates (such as glucose uptake) and methods (such as metabolomics utilizing different spectroscopic techniques) have been promoted as viability markers, none have yet been widely used clinically or in livestock production. Here we review the major techniques that have been reported; these are divided into indirect techniques, where measurements are made from the embryo’s immediate micro-environment, or direct techniques that measure intracellular metabolic activity. Both have strengths and weaknesses, the latter ruling out some from contention for use in human ART, but not necessarily for use in livestock embryo assessment. We also introduce a new method, multi- (or hyper-) spectral analysis, which measures naturally occurring autofluorescence. Several metabolically important molecules have fluorescent properties, which we are pursuing in conjunction with improved image analysis as a viable embryo quality assessment methodology.
INTRODUCTION

Over several decades, metabolic determination of oocyte and embryo quality has been promoted as an adjunct, if not primary, method for predicting subsequent development. The ability to predict development following embryo transfer is enormously attractive to both human clinical laboratories and cattle embryo production laboratories (the two largest applications of embryo production technology). For IVF clinics, selecting the embryo with the highest implantation potential enables single embryo transfer, alleviating the health complications arising from multiple births for both mother and infants (Gardner and Sakkas 2003). In cattle embryo production, minimising recipient returns to oestrus following transfer is an economic advantage. Yet today, neither clinical ART units nor cattle veterinarians routinely perform embryo metabolic assessment prior to transfer. In contrast, other techniques (Figure 1), such as morphology grading are routinely applied (e.g. Gardner et al. 2000), albeit highly reliant on the skills and experience of the embryologist. The development of “Time lapse” systems (Meseguer et al. 2011; Herrero and Meseguer 2013) has taken morphokinetics to a greater predictive capacity, and has confirmed that the timely progression of cellular division is indicative of embryo competence. In addition, pre-implantation genetic screening of human embryos (Figure 1) has emerged from a criticised clinical technique of assessment, due to the poor predictability of ploidy status by early methods (especially fluorescence in situ hybridisation, FISH) and the high degree of ploidy errors within individual blastomeres (Vanneste et al. 2009; Harper and Sengupta 2012), to a more robust predictive method using comparative genomic hybridisation, especially when applied to blastocyst stage embryos combined with vitrified cycles (Schoolcraft and Katz-Jaffe 2013).
As highlighted in several recent reviews (Krisher and Prather 2012; Leese 2012; Lonergan and Fair 2014; Gardner and Harvey 2015; Krisher et al. 2015b), metabolic studies have been fundamental in the grounding behind embryo culture media formulations and provide valuable insights into what aspects of metabolism are associated with embryo quality, or more so, what aspects are associated with failed development or embryo stress. So where does this leave the measurement of metabolism as a prospective embryo quality assessment technology? Do current techniques have the scope to be used routinely? Has the need for determining metabolic markers of quality been overtaken? In this paper we assess the state of the field and provide a view of where the field should head.

### METHODS FOR MEASURING METABOLISM OF EMBRYOS

#### Indirect Measures of Metabolism

Indirect measures of embryo metabolism rely on a change in substrate concentration in the immediate micro-environment surrounding the embryo. Typically this is the media surrounding the embryo, often referred to as ‘spent’ culture media (Figure 2). The benefit of such techniques is that theoretically, there is no impact to the embryo, thus regarded as non-invasive.

**NAD(P)H-based assays for carbohydrates and carboxylic acids**

Inspired by the work of Oliver Lowry, Henry Leese devised fluorometric assays for measuring ATP, glucose and lactate from tissues (Leese and Bronk 1972), based on the oxidation and/or reduction of nicotinamide adenine dinucleotides (NAD(P)H) and their fluorescent properties. Indeed, these assays are used routinely in many automated substrate analysis systems today, due to their high sensitivity and
capability to measure from small volumes. These assays were based on the discovery of Oliver Lowry that the reduced forms of NAD(P)H were fluorescent molecules (emission maxima 460 nm) under UV excitation wavelengths (330-350 nm), whereas the oxidised forms of both (NAD(P)^+) were not, described in an account of his work (Lowry 1990). Lowry recognised that as these were co-factors required for dehydrogenase enzymes; by harnessing this property, he could measure the activity of these enzymes. Leese built on this concept and with John Biggers and colleagues, scaled down the assay system to measure fluorescence from nanolitre and picolitre samples, enabling the ability to measure the metabolite turnover of a single COC and embryo (Leese et al. 1984). As dehydrogenases metabolise carbohydrates (with the primary interest focussed on glucose) and carboxylic acids (pyruvate and lactate, via lactic acid dehydrogenase), substrate appearance or disappearance from the embryo culture medium is measurable over time, enabling estimates of metabolic activity; examples include: (Gardner and Leese 1988; Leese et al. 1994; Thompson et al. 1996a; Butcher et al. 1998).

**Spectrophotometric techniques**

Metabolomics is the term generally used to describe the identification and quantification of multiple metabolites in a single analysis. Measurement of a broad range of substrates and metabolites allows not only measurement of substrate turnover but also provides a better estimation of changes in metabolic pathway activity and downstream targets such as redox control and proliferation, and as such is a much more powerful discovery technique than targeted substrate analysis (Krisher, Heuberger et al., 2015). With this definition in mind, metabolomics combines two technologies; firstly the separation (gas chromatography, high performance liquid
chromatography (HPLC)) and then the detection (mass spectrometry, near infrared, nuclear magnetic resonance, Raman spectrometry) of larger numbers of metabolites within ‘spent’ culture media compared to other analytical methods. Both quantitative and/or qualitative measurements can be performed (depending on the technology used), with quantitative measures requiring standards, which may reduce the number of substrates to be measured with accuracy. Application of one spectrometry platform (near infrared spectrometry, NIR) for spent human embryo culture media analysis was initially favourable (Sakkas 2014). Nevertheless, several randomised control trials could not support initial results (Vergouw et al. 2014) and for now the application of NIR has been abandoned, until technology refinements or alternatives are developed. Indeed, metabolomics of spent culture media is still actively pursued using alternative platforms (mass spectrometry; (Krisher et al. 2015a).

Amino acid analysis within ‘spent’ medium has shown promise as a predictive tool for subsequent embryo quality. Most amino acid analyses have utilised HPLC separation following a fluorescent tagging method that enables detection following separation (Lamb and Leese 1994). Subsequent reports have identified that amino acid appearance and disappearance from ‘spent’ medium can predict sex, ploidy status, embryo development and post-implantation survival (Houghton et al. 2002; Brison et al. 2004; Picton et al. 2010; Sturmey et al. 2010).

Polarographic electrodes

Polarographic scanning electrodes quantify the concentration of a single molecular species, dependent on their sensing mechanism. For example, measurement of ions usually requires a specific ionophore (Trimarchi et al. 2000b).
Undoubtedly the widest application is for the measurement of dissolved O$_2$, especially in relation to embryo metabolism (Trimarchi et al. 2000a; Shiku et al. 2001; Lopes et al. 2007). Oxygen consumption by embryos has been proposed as an obvious candidate for determining embryo viability, as oxidative phosphorylation is critical for development (Houghton et al. 1996). Oxygen consumption should accurately reflect the rate of ATP production via oxidative phosphorylation and therefore the energy demand within an embryo. Several studies demonstrated that O$_2$ demand in mouse and bovine embryos increases with the onset of compaction and blastulation (Houghton et al. 1996; Thompson et al. 1996b). In a retrospective study of O$_2$ consumption in cattle embryos followed by embryo transfer, Lopes and colleagues (Lopes et al. 2007) found that blastocysts with the highest implantation success were in the ‘mid-range’ of consumption measurements, supporting the ‘Quiet embryo hypothesis’ (Leese 2002) (see section below). Nevertheless, day 3 human embryos may be selected on their O$_2$ consumption rate, as a retrospective analysis of implanting embryos had a higher average consumption than non-implanting embryos (Tejera et al. 2012). Polarographic O$_2$ electrodes coupled with Time lapse morphokinetics for embryo assessment was prototyped by the Danish company, “Unisense Pty Ltd”. However, they abandoned the O$_2$ sensing aspect as it became clear that replacing probes between patients would be mandatory and therefore technically challenging and commercially unviable, especially as Time-lapse microscopy alone was proving a better predictor of embryo quality than other morphometry methods. Apart from their wide use of O$_2$ measurements in a variety of applications, polarographic electrodes are also capable of measuring other gases such as NO and CO$_2$ in addition to both cations and anions.
Intracellular Measurements

Intracellular measurements by their definition must involve measuring metabolic activity within the embryo itself, and therefore cannot be regarded as non-invasive (Figure 2). The challenge is therefore to determine the extent of impact on the embryo whilst measuring metabolism. This poses potential regulatory safety issues, especially on long-term outcomes following transfer, for this technology to be clinically useful.

Non-toxic colorimetric and fluorometric dyes

Brilliant Cresyl Blue (BCB) is an supravital stain (oxazine family) which has been successfully used to segregate fully grown germinal vesicle stage oocytes from more immature oocytes, with subsequent embryo transfers proving this assay is non-toxic (Opiela and Katska-Ksiazkiewicz 2013). The assay is dependent on the activity of the X-linked enzyme, glucose-6-phosphate dehydrogenase (G6PDH), whereby fully grown oocytes exposed to BCB remain blue (low enzyme activity), whereas growing oocytes metabolise the stain and become clear. G6PDH activity reduces during development to the blastocyst stage in the mouse (Brinster 1966; De Schepper et al. 1993), with levels much lower than in the oocyte. Other than measuring activity in oocytes, there has been no attempt to measure G6PDH in embryos for viability determination. BCB staining has been assessed for determining the sex of blastocyst stage embryos (Williams 1986), and in doing so demonstrating there is little toxicity with this procedure. However, other sexing technologies (FACS –separated sperm and embryo biopsy-DNA analysis) have surpassed its relatively weak capacity for sex selection. Furthermore, such assays are certain to fall foul of national regulatory authorities, especially for human embryo application. Nevertheless, it is quite feasible
that non-toxic dyes sensitive to metabolic activity can still have application in other species, such as domesticated ruminants.

Most fluorescent probes are unusable for determining metabolic activity for viability assessment, as many will either have an inherent toxicity, or become toxic due to the chemical interaction that creates the fluorescent capacity of the probe. Thus probes such as the mitochondrial respiratory dyes JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) and the Mitotracker probes (carboryanine or rosamine-based probes) are not practical measures of viability for post-transfer work, but remain proven research tools. On the other hand, non-metabolised probes may have a role in relating to viability post-transfer. For example, glucose uptake into an embryo can be measured using 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-deoxyglucose (6-NBDG), a fluorescent glucose analogue that is not metabolised (Zander et al. 2006), and is brightly (green) fluorescent at relatively low concentrations. As yet, as far as we know, there has been no attempt to determine post-transfer viability with this particular probe.

Radiolabel isotopes

Most of the early studies on embryo metabolism were conducted utilising radio-labelled substrates, in particular glucose and pyruvate. Depending on which carbon/hydrogen atom was labelled, the production of $^{14}$CO$_2$ or $^3$H$_2$O indicated the activity of different metabolic pathways. For example, the production of $^{14}$CO$_2$ from [1-$^{14}$C] glucose measured activity through the pentose phosphate pathway (PPP) and tricarboxylic acid (TCA) cycle. Likewise, the production of $^3$H$_2$O from [5-$^3$H] glucose is indicative of glycolytic activity (Rieger and Guay 1988; Rieger and Loskutoff 1994). These measurements primarily utilised a ‘Hanging Drop’ assay,
where oocytes or embryos were incubated in ~3 μl of culture media containing the radiolabelled substrates in the lid of a centrifuge tube. The drop was then suspended by capping the lid over a reservoir containing solutions of NaOH or NaHCO₃, which acts as a metabolite “trap” (O'Fallon and Wright 1987). Following the principle of mass transfer meant that greater than 95% of the metabolised label was trapped over a 3-4 h period of time (Rieger and Guay 1988). Because of the use of radioisotopes, the technique is very sensitive, capable of measuring pathway activity in single embryos (O'Fallon and Wright 1986; Rieger and Guay 1988; Thompson et al. 1991; Rieger and Loskutoff 1994; Downs and Utecht 1999). Radioisotope-labelled substrates have never been utilised for embryo transfer and post-natal development assessment, due to the radioactivity involved, even though in reality the levels are relatively harmless, as only β-emitters are normally utilised. Furthermore, there has never been an assessment of whether these cause mitochondrial or DNA damage to the embryo. These assays still remain useful for research, so their demise as a routine method to investigate metabolism is most likely related to institutional and ethical reluctance to support radioisotope-based tools and embryo transfer.

**Autofluorescence**

Researchers utilising fluorescence microscopy will be familiar with autofluorescence within specimens. However, most will view it as a nuisance as autofluorescence is the cause of background fluorescence that may decrease the contrast in fluorescence with a specific fluoroprobe. However, there is a diversity of endogenous molecules that are fluorescent (Table 1, (Ramanujam 2000)). Significantly fluorescent molecules are NAD(P)H (as previously discussed), flavin adenine dinucleotide (FAD), collagen and porphyrins (Table 1). Because of their
fluorescent properties and roles in metabolism, NAD(P)H and FAD are widely used together, especially as the ratio can be regarded as a *de facto* measure of the intracellular redox state. The majority of NAD(P)H is represented by NADH, and just as significantly, FAD fluorescence is associated with mitochondrial activity, as the vast majority of FAD/FADH$_2$ is localised there (Heikal 2010). A drawback is that the excitation and emission spectrum of NADH and NADPH are very similar. Use of these fluorophores as measures of metabolic activity within embryos was pioneered by Dumollard and colleagues (Dumollard *et al.* 2007; Dumollard *et al.* 2009), who successfully measured changes in metabolism during the process of fertilisation and subsequent embryo development over periods of time, particularly investigating the influence of substrate changes in the medium on FAD and NAD(P)H fluorescence. The power of this approach was subsequently demonstrated by Banrezes and colleagues (Banrezes *et al.* 2011), whereby changing the levels of pyruvate and lactate in the pronuclear embryo medium and observing the ensuing redox alterations, they observed altered fetal growth related to the redox state at this early stage. Not only did this study demonstrate a new developmental regulatory insight that is energy sensitive at the pronuclear stage, but also that the measurement of autofluorescence has seemingly no consequences to viability and can be utilized with subsequent embryo transfer. However, one cannot rule out a biological impact of laser exposure, and will be dependent on laser energy utilised and length of exposure and frequency.

Accompanying the development of fluorescence microscopy, textural image analysis has also evolved to measure different pixel attributes, such as distribution, co-localisation and patterning, in addition to pixel intensity. This can improve the quality of information from microscopic images, whether they are fluorescent or not. Ultrasound sonography, dermatology and cancer research are fields that routinely use
advanced imaging matrices to assess variations in patterns of pixel characteristics,
described in a textural context, such as wrinkles, smoothness, uniformity and entropy
of images (Murata et al. 2001; Castellano et al. 2004; Alvarenga et al. 2007). In
comparison, image analysis within the pre-implantation research field is largely
limited to measurements of fluorescence intensity. We have begun to assess textural
analyses of early cleavage stage embryos to gain further information other than
intensity, an example of which has been applied to examining oocytes following
different cumulus-oocyte complex treatments (Sutton-McDowall et al. 2015a).

MEASURING EMBRYO METABOLISM – WHAT ARE WE MEASURING?

In situ vs. ex vivo embryo metabolism?

Pre-implantation stage embryos survive in the reproductive tract and are
dependent on a histotrophic substrate and protein supply, where some of these are
oviduct specific proteins (Killian 2004). It is widely accepted, yet not demonstrated,
that the microenvironment of substrates in the luminal fluid of the maternal tract (in
particular the oviduct) surrounding the early embryo is not constant but in a state of
flux. It is very likely the reproductive tract environment has a high degree of
sensitivity to maternal signals. Supporting this are the elegant observations by Leese
and colleagues (Dickens et al. 1993; Cox and Leese 1995) who measured rapid
changes in secretory behaviour of cultured oviduct epithelial cells when treated with
stimulatory ATP. Furthermore, the volume of oviductal fluid relative to luminal
surface area is small and cumulus-oocyte complexes (COCs) and embryos are in very
close proximity to the oviductal wall (for an excellent ex vivo visualisation of this,
view the videos found in (Kolle et al. 2009). No doubt this facilitates sperm-oocyte
collision, but changes in local luminal fluid composition are likely to occur as well.
Like cumulus cells (Aardema et al. 2013; Lolicato et al. 2015), a function of the zona pellucida surrounding the embryonic cells is possibly to buffer the oocyte and subsequent blastomeres from sudden shifts in substrate (and soluble gas) concentrations, in addition to its other protective and sperm-binding, capacitation and fertilization roles. Nevertheless, metabolic activity of embryos in situ could feasibly be more dynamic than what occurs within a drop in a petri dish. Perhaps this is why measurement of several metabolic parameters such as glucose, carboxylic acids, amino acids and oxygen uptake has such a broad range of values when assayed immediately following collection (Leese 2012). Embryos are thought of as ‘developmentally plastic’, an awkward term commonly used to describe the tolerance, or adaptation (with variable success), to different media formulations during in vitro culture. In actual fact, it appears that adaptability is an inherent feature of early embryo development (Leese 2012). Here then is the conundrum for all past and present work on embryo metabolism – we speculate on what ‘normal’ metabolism in situ really means. Our best attempts to measure this metabolism is restricted to immediate measures within an ex vivo environment following collection, where we know that within 3 h, the metabolic pattern between freshly flushed mouse embryos and cultured embryos can be markedly different (Lane and Gardner 1998). The assumption made is that this reflects the metabolic profile in situ. Until we develop such assays that allow us to track metabolism in situ we should speculate with caution on the relationship of what we are measuring in vitro and what occurs in situ. Perhaps in the future, the application of photonic fibres and nanoparticles will provide better access to embryos to measure their metabolism in situ.

Changes with stage of development
The widely accepted pattern of embryonic metabolism (measured under *in vitro* conditions, Figure 3) for most species examined, including human and cow, is that pre-compaction (early cleavage) stages of development are dependent on oxidative phosphorylation (Thomson 1967; Leese 1995; Thompson *et al.* 2000). Then as compaction and blastulation occur, glycolysis increases (Figure 3), even in the presence of O₂. This is not to say that post-compaction development does not require oxidative phosphorylation. Indeed, it is clear that within the blastocyst stage of most species examined, trophectoderm cells are reliant on oxidative phosphorylation for their higher energy demands, whereas the inner cell mass cells are highly glycolytic. Some have likened this metabolic profile to the ‘Warburg effect’ (Krisher and Prather 2012) observed in some tumour cells, where despite the availability of O₂, significant lactate production occurs relative to the uptake of glucose, rather than glucose oxidation via the TCA cycle and oxidative phosphorylation (Krisher and Prather 2012). Fatty acid metabolism contributing to oxidative phosphorylation is now recognised as a fundamental requirement in several species (Paczkowski *et al.* 2013), most likely meeting the oxidative phosphorylation requirement. Exceptions to this picture are the rat embryo, where blastocysts were produced in the presence of oxidative phosphorylation inhibitors (Brison and Leese 1991), and the rabbit embryo, where the reliance for oxidative phosphorylation from fatty acid oxidation is continuous from the 1-cell stage, most likely to enable the substantial proliferation that occurs within the embryo (Kane 1979). Although we have a picture of major changes in metabolism for several species, it is clear that the degree of substrate uptake and metabolic pathway preference throughout development is variable amongst such species, as recently summarised for mouse, cow and pig in the review by Krisher and Prather (Krisher and Prather 2012). As embryos of other species are
investigated, further departures from what is regarded as the ‘characteristic pattern’ of mammalian embryo metabolism will no doubt emerge.

In vitro composition of medium and influence of physical parameters, such as embryo density and gas composition.

The metabolism of the preimplantation stage embryo is also significantly influenced by the culture environment. This can be divided into 1) the culture media formulation, especially the energy substrate availability, supplemental protein concentration and influence of anti-apoptotic/mitogenic growth factors; 2) the influence of intrinsic factors during culture (e.g. the impact of autocrine and paracrine growth factors, or the presence (deliberate or otherwise) of somatic cells, to create a co-culture system; 3) the impact of extrinsic factors, such as gas composition, most notably the partial pressure of oxygen used for culture, but also CO₂.

Arguably, one of the least understood aspects of in vitro culture is the influence of the embryo itself on the culture environment, even if it is being deliberately measured. Often described as a “static” culture system, the culture media composition itself within the near-universally applied microdrop under mineral oil, is continuously changing. In particular, the smaller the culture drop, or the density of embryos per unit volume of media, the more change to media composition will occur over a period of time. Indeed, this is the whole basis for assays that measure the temporal change in substrate content as a proxy measure for substrate uptake. With specific reference to metabolomics, changes to substrates and metabolites reflect both the initial concentration and the substrate movement into or from the embryo, with a broad range in differences in concentration observable over time; some being undetectable (which will also depend on sensitivity of detection systems) and others
possibly at a point of significant depletion that may impact metabolic pathway
activity. Recently, Krisher and colleagues (Krisher et al. 2015a) argued that as long
as sufficient substrate levels were “available” to embryos, and the difference in
concentration of substrates was small relative to their appearance or disappearance
from the medium, then issues of significant depletion during the measurement period
would be avoided. Culture in larger volumes or as single embryos (Lane and Gardner
1992; Keefer et al. 1994) will impact both developmental potential and metabolism
and is thought to increase embryo stress, due to the waning influence of autocrine and
paracrine growth factors. As Krisher and colleagues (Krisher et al. 2015a) concluded,
“Metabolic measurements should occur in optimal volumes to best reflect metabolism
of a viable embryo, as well as to be clinically relevant”. As such, the metabolic
profile of an embryo is uniquely dependent on media formulation and volume,
causing difficulties if extrapolating from one culture system to another (Sakkas 2014).
The most influential extrinsic factor that varies significantly in measurement of
metabolism is gas composition. Systematic reviews of the literature addressing the
influence of O₂ conclude that a low O₂ atmosphere (5-7%) has a positive impact on
developmental consequences, especially post-compaction development. Yet much of
the work conducted measuring metabolism has been performed in air-based
atmospheres (Wale and Gardner 2013). Atmospheric O₂ levels are associated with
oxidative stress and altered gene expression profiles in blastocysts compared with low
O₂ embryo culture (Harvey 2007; Amin et al. 2014). In particular, low O₂ levels will
increase hypoxia inducible factor activity (HIFs), especially post-compaction
(Thompson and Kind, unpublished observations;(Harvey 2007), which then work to
adapt the metabolism of cells to enable growth under such conditions.
The Quiet embryo hypothesis

Is a higher metabolism better for embryo health? If the question is directed to ATP turnover alone, then the answer appears to be ‘yes’ (Van Blerkom 2011; Fragouli et al. 2015). But ATP turnover is derived from the sum of glycolytic and oxidative phosphorylation activity and the demand for cellular energy, and this turnover is in the order of tens of seconds in embryos (Leese 1991), revealing that a simple measure of ATP content alone at a single point in time does not measure rate of turnover. A central constituent to this important energy equation is how mitochondria behave, or put another way, their efficiency to generate ATP during in vitro culture in the face of demand, which is a major determinant of embryo health (Fragouli et al. 2015). It was Henry Leese and colleagues (Leese 2002; Leese et al. 2007; Leese et al. 2008) who noted that the most viable embryos were neither associated with the highest, nor lowest metabolic readout(s), when measuring key metabolic parameters such as glucose uptake, net amino acid uptake and O2 uptake. The ‘Quiet embryo hypothesis’ was drawn from metabolic profiles measured between in vivo derived and in vitro produced embryos or from retrospective analysis of metabolic parameters measured prior to embryo transfer. Leese concluded that embryos with a high probability of further development have an efficient metabolism, therefore an efficient utilisation of substrates, particularly within mitochondria. The juxtaposition is that embryos with very high metabolic levels do so as they are stressed, and likely to generate higher levels of reactive oxygen species (free radicals) from mitochondria, thus setting the embryo on a self-destructive course. This hypothesis is both supported and argued against in the ensuing literature. The major criticism (Gardner and Wale 2013) is that many of the founding studies analysed to develop the hypothesis utilized sub-optimal incubation conditions during the analysis.
period, particularly the use of atmospheric O₂ levels. Under such conditions, the levels of glucose uptake, particularly post-compaction, correlate with subsequent viability post-transfer in mice and human embryos, thereby demonstrating that the metabolic assessment environment is fundamental to the capacity of metabolism to be considered as an indicator of subsequent development. One common element of the arguments for and against the ‘Quiet embryo hypothesis’ is that in vitro cultured embryos are more stressed than their in vivo derived counterparts. Several stress activated signalling pathways operate within embryos, including sirtuins, AMP-dependent kinase (AMPK), Hypoxia Inducible Factors (HIFs) and Stress Activated Protein Kinases (SAPK, or JNK), and such have the capacity to rapidly modify metabolism; this is comprehensively reviewed by Puscheck and colleagues (Puscheck et al. 2015). It is feasible that with increasing and also different types of stress, metabolic relationships with competence change in non-linear patterns, thereby adding to the confusion about what is predictive of competence. Perhaps the real implication of the current debate is that our ability to accurately measure embryonic stress by metabolic measures with current capabilities remains unsatisfactory. A new hypothesis is helping to shed light on this (Brison et al. 2014), in that embryonic stress is associated with heterogeneity in metabolic profiles between individual blastomeres, with the ability for further development related to not only synchrony in division but synchrony and homogeneity of metabolic change during development. This is particularly so for pre-compaction stages, as post-compaction gap-junction formation enables cell-cell communication and therefore at least there is capacity for attaining some metabolic homogeneity (Brison et al. 2014). This attractive hypothesis is being actively researched and points to the need for more intracellular metabolic readouts that can be compared between blastomeres of each embryo, as
these may be more powerful than an ‘averaged’ readout examined within ‘spent’ medium.

A NEW APPROACH - MULTISPECTRAL ANALYSIS

Multi- (or hyper-) spectral imaging has been widely used in food quality monitoring (Huang et al. 2014). Its application to cellular biology has only been recent, as at a research level, there is a requirement for significant computing input, statistical data management and hardware. At a cellular level, spectral analysis is an alternative metabolomics approach using the spectral properties of the endogenous fluorophores within cells, with the capacity to measure differences within and between individual cells (and therefore an embryo) (Table 1). The application of multiple excitation wavelengths, whether by generation with a tuneable laser over a wide range of wavelengths, or by using multiple excitation diodes (up to 18 different excitation wavelengths), enables a broad spectral pattern to be generated, which then requires analysis. It can be used either to identify a naturally fluorescent substrate or product, such as NADH (either in a free- or protein bound-state), or provide a picture of the degree of spectral shifts associated with variation in cellular metabolism. We are currently assessing the technology for embryo quality predictive capacity during development of early embryos, with our partners (Sutton-McDowall et al. 2015b).

CONCLUSIONS

Our understanding of embryo metabolism has grown considerably over the past two decades. There is unambiguous evidence that embryo viability and embryo metabolism are closely interrelated at the experimental level. Significant new insights into the importance of metabolic sensing pathways in regulating metabolism and
viability are rapidly emerging, such as sirtuins, AMPK and HIFs, giving a clearer picture as to how flexible embryos are at adapting to different conditions. Nevertheless, differences in media composition from various laboratories and manufacturers (where mostly the formulation is not available, apart from a list of constituents) provide barriers for ‘spent’ media metabolomics to provide a predictive assessment of viability. However, some success with measuring glucose plus lactate level changes in media under low O\textsubscript{2} atmospheres and amino acid appearance/disappearance have been identified as predictive of further development. Alternative approaches that have developed with the advent of advancing microscope and imaging technology and computing power, such as spectral analysis of multiple endogenous fluorophores during the development period, holds great promise for determining intracellular metabolic activity. When this is coupled with Time-lapse morphokinetics, and possibly in conjunction with extracellular metabolomics, then current limitations should be resolved and this poses the best hope for accurately assessing embryonic developmental potential.
REFERENCES


21


Kane, M.T. (1979) Fatty acids as energy sources for culture of one-cell rabbit ova to viable morulae. *Biol. Reprod.* **20**, 323-332


**FIGURE LEGENDS**

Figure 1. Images representing the three major technique groups for assessing quality of embryos prior to transfer: Morphometry techniques; Metabolic techniques; Biopsy techniques.

Figure 2. Techniques for determining metabolic activity in embryos under *in vitro* conditions, which can feasibly be used to determine embryonic health prior to embryo transfer.
Figure 3. Major changes in metabolism (glycolysis and oxidative phosphorylation), RNA and protein synthesis in a generalised mammalian embryo. Adapted from Thompson et al. (2005) “Adaptive responses of early embryos to their microenvironment and subsequent consequences”. In “Early Life Origin of Health and Disease”, Eds. Wintour, M. and Owens, J. Landes Bioscience Publishing, Texas, USA.
Table 1. Excitation and emission maxima of endogenous fluorophores.

<table>
<thead>
<tr>
<th>Endogenous Fluorophores</th>
<th>Excitation maxima (nm)</th>
<th>Emission maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>280</td>
<td>350</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>275</td>
<td>300</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>260</td>
<td>280</td>
</tr>
<tr>
<td><strong>Structural Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>325, 360</td>
<td>400, 405</td>
</tr>
<tr>
<td>Elastin</td>
<td>290, 325</td>
<td>340, 400</td>
</tr>
<tr>
<td><strong>Enzymes and coenzymes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAD, Flavins</td>
<td>450</td>
<td>535</td>
</tr>
<tr>
<td>NADH</td>
<td>290, 351</td>
<td>440, 460</td>
</tr>
<tr>
<td>NADPH</td>
<td>336</td>
<td>464</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin A</td>
<td>327</td>
<td>510</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>335</td>
<td>480</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>390</td>
<td>480</td>
</tr>
<tr>
<td><strong>Vitamin B&lt;sub&gt;6&lt;/sub&gt; compounds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>332, 340</td>
<td>400</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>335</td>
<td>400</td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>330</td>
<td>385</td>
</tr>
<tr>
<td>Pyridoxic acid</td>
<td>315</td>
<td>425</td>
</tr>
<tr>
<td>Pyridoxal 5'-phosphate</td>
<td>330</td>
<td>400</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>275</td>
<td>305</td>
</tr>
<tr>
<td><strong>Lipids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipids</td>
<td>436</td>
<td>540, 560</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>340 – 395</td>
<td>540, 430 – 460</td>
</tr>
<tr>
<td>Ceroid</td>
<td>340 – 395</td>
<td>430 – 460, 540</td>
</tr>
<tr>
<td><strong>Porphyrs</strong></td>
<td>400 – 450</td>
<td>630, 690</td>
</tr>
</tbody>
</table>

* NADH, reduced nicotinamide dinucleotide; NAD(P)H, reduced nicotinamide dinucleotide phosphate; FAD, flavin adenine dinucleotide. Taken from Ramanujam, N. (2000) Fluorescence spectroscopy of neoplastic and non-neoplastic tissues. Neoplasia 2(1-2), 89-117.