Title: Hyperspectral microscopy can detect metabolic heterogeneity within bovine post-compaction embryos incubated under two oxygen concentrations (7% versus 20%)

Running Title: Metabolic heterogeneity in embryos

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Abstract

Study Question:

Can we separate embryos cultured under either 7% or 20% oxygen atmospheres by measuring their metabolic heterogeneity?

Summary Answer:

Metabolic heterogeneity and changes in metabolic profiles in morula exposed to two different oxygen concentrations were not detectable using traditional fluorophore and two-channel autofluorescence but were detectable using hyperspectral microscopy.

What is Known Already:

Increased genetic and morphological blastomere heterogeneity is associated with compromised developmental competence of embryos and currently forms the basis for embryo scoring within the clinic. However, there remains uncertainty over the accuracy of current techniques, such as PGS and time-lapse microscopy, to predict subsequent pregnancy establishment.

Study Design, Size, Duration:

The impact of two oxygen concentrations (7% = optimal and 20% = stressed) during post-fertilisation embryo culture was assessed. Cattle embryos were exposed to the different oxygen concentrations for 8 days (D8; embryo developmental competence) or 5 days (D5; metabolism measurements). Between 3-4 experimental replicates were performed, with 40-50 embryos per replicate

used for the developmental competency experiment, 10-20 embryos per replicate for the fluorophore and two-channel autofluorescence experiments and a total of 21-22 embryos used for the hyperspectral microscopy study.

Participants/Materials, Setting, Methods:

In-vitro produced (IVP) cattle embryos were utilised for this study. Post fertilisation, embryos were exposed to 7% or 20% oxygen. To determine impact of oxygen concentrations on embryo viability, blastocyst development was assessed on D8. On D5, metabolic heterogeneity was assessed in morula (on-time) embryos using fluorophores probes (active mitochondria, hydrogen peroxide and reduced glutathione), two-channel autofluorescence (FAD and NAD(P)H) and 18-channel hyperspectral microscopy.

Main Results and the Role of Chance:

Exposure to 20% oxygen following fertilisation significantly reduced total blastocyst, expanded and hatched blastocyst rates by 1.4-, 1.9- and 2.8-fold respectively compared to 7% oxygen ($P < 0.05$), demonstrating that atmospheric oxygen was a viable model for studying mild metabolic stress. The metabolic profiles of D5 embryos was determined and although metabolic heterogeneity was evident within the cleavage stage (i.e. arrested) embryos exposed to fluorophores, there were no detectable difference in fluorescence intensity and pattern localisation in morula exposed to the two different oxygen concentrations ($P > 0.05$). While there were no significant differences in two-channel autofluorescent profiles of morula exposed to 7% and 20% oxygen (main effect, $P > 0.05$), morula that subsequently progressed to the blastocyst stage had

significantly higher levels of FAD and NAD(P)H fluorescence compared to arrested morula ($P < 0.05$), with no change in the redox ratio. Hyperspectral autofluorescence imaging (in 18-spectral channels) of the D5 morula revealed highly significant differences in four features of the metabolic profiles of morula exposed to the two different oxygen concentrations ($P < 0.001$). These four features were weighted and their linear combination revealed clear discrimination between the two treatment groups.

Limitations, Reasons for Caution:

Metabolic profiles were assessed at a single time point (morula), and as such further investigation is required to determine if differences in hyperspectral signatures can be detected in pre-compaction embryos and oocytes, using both cattle and subsequently human models. Furthermore, embryo transfers should be performed to determine the relationship between metabolic profiles and pregnancy success.

Wider Implications of the Findings:

Advanced autofluorescence imaging techniques, such as hyperspectral microscopy, may provide clinics with additional tools to improve the assessment of embryos prior to transfer.

Study Funding/Competing Interest(s):

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Key Words:

Embryo, Pre-implantation, Metabolism, Heterogeneity, Oxygen, Embryo Culture, Autofluorescence, Hyperspectral Microscopy.

INTRODUCTION

Pre-implantation embryos respond to their surrounding environment and both in-vivo and in-vitro environments can influence developmental outcomes, such as implantation and ongoing pregnancy (reviewed by (Fleming, et al., 2015) Early development prior to embryonic genomic activation and compaction (4-8 cell stage in humans and the 8-cell stage in ruminants) is particularly sensitive to environmental perturbations, partly because there is limited communication between blastomeres within the pre-compaction embryo. Communication between individual blastomeres begins with the initiation of connexion formation (the building blocks of gap junctions), coinciding with polarisation and compaction (Bloor, et al., 2002, Eckert, et al., 2004, Houghton, 2005).

Cellular morphological heterogeneity forms the basis of morphological embryo grading in the clinic, with healthier embryos having no obvious morphological heterogeneity (Gardner, et al., 2007). An increase in the appearance of blastomere heterogeneity is associated with poor quality embryos (Brison, et al., 2014).

Another form of blastomere heterogeneity within human embryos is their karyotype. The extent of chromosomal mosaicism within pre-compaction (Day 3) human embryos is such that a single biopsied cell may not reflect accurately the chromatin profile of the remaining blastomeres within an embryo (Voullaire, et al., 2000, Wells and Delhanty, 2000). More accurate techniques of assessing mosaicism within trophectoderm cells recovered by biopsy of blastocyst stage embryos has revealed a greater incidence than first thought, and increased

mosaicism appears to be associated with a reduction in pregnancy rates (Munne and Wells, 2017), although this association remains controversial (Gleicher, et al., 2014).

There is a surge of interest from human IVF clinics to apply time-lapse microscopy to predict embryo developmental outcomes. Time-lapse measures morphokinetic parameters such as the timing of syngamy, periods between cytokinesis, and oolema ruffling and abnormal cytokinetic events resulting in uneven blastomere sizes (reviewed by (Wong, et al., 2013), as these parameters that may correlate with embryo developmental outcomes (VerMilyea, et al., 2014). Nevertheless, morphokinetic analysis over the first three days of development that predict subsequent blastocyst development does not predict implantation (Chamayou, et al., 2013).

A recent hypothesis (Brison, et al., 2014) connects increasing cellular stress with increased blastomere metabolic heterogeneity. Measuring embryo metabolism is viewed by many researchers as the cellular parameter most likely to reflect embryo developmental outcomes (Thompson, et al., 2016). However, most of the current methodologies measuring embryo metabolism cannot measure individual blastomere metabolic heterogeneity. Non-invasive measurements of substrate turnover in spent culture media using microfluorometric analyses or spectrometer-based metabolomics (Krisher, et al., 2015) represent the metabolic profile of the whole embryo, but not the metabolic variation between blastomeres. Furthermore, such metabolic measurements are usually performed at a single time point of development. Hence, alternative non-invasive technologies are

needed to measure individual blastomere metabolism, preferably in real-time and throughout development. This should provide the clearest relationship between blastomere metabolic variation and assessment of embryo stress, which is likely to reflect developmental outcome.

Metabolic differences between oocytes and embryos can be determined temporally and in real time using autofluorescence. Published examples include examinations of metabolism during fertilization of mouse oocytes (Dumollard, et al., 2004) or of the impact of recombinant oocyte secreted-factors on metabolism of cattle cumulus-oocyte complexes (Sutton-McDowall, et al., 2012, Sutton-McDowall, et al., 2015). Most studies involve two-channel excitation wavelengths, specifically targeting the two metabolic cofactors, NAD(P)H and FAD. Recently, we explored a broader spectral approach, hyperspectral imaging of embryo autofluorescence, which involved up to 18 spectral channels. Autofluorescence can be used to provide insights into metabolism, for example through separately identified bound- and free-NADH and NADPH (e.g. Fluorescence Lifetime Imaging Microscopy, Cinco et al 2016). Furthermore, our laboratory has explored the utility of Grey Level Co-occurrence Matrices (GLCM) applied to embryo autofluorescence, to increase the capacity for discrimination between different treatments applied to embryos during development (Tan, et al., 2016). It has been recently demonstrated that hyperspectral microscopy of cell autofluorescence and features analysis with GLCM was able to distinguish biomolecular and metabolic differences in cultured somatic cells and embryos (Gosnell, et al., 2016a, Gosnell, et al., 2016b).

The oxygen concentration within the reproductive tract during development is lower than in the atmosphere (Fischer and Bavister, 1993). Many studies across several species demonstrate that low oxygen levels (typically 5-7%) for culture of pre-implantation embryos improve developmental outcomes compared to atmospheric levels (Thompson, et al., 1990, Thompson, 2000, Bontekoe, et al., 2012, Guo, et al., 2014, Gardner, 2016). Relative to low oxygen levels, exposure to atmospheric oxygen concentrations increases reactive oxygen species levels and of oxidative damage, and both gene and protein expression profiles within embryos reflect a greater degree of cellular stress (Wale and Gardner 2016). Hence, exposure to a low oxygen concentration relative to atmosphere during development was employed in this study as a model for inducing cellular stress. We routinely utilize 7% O_2 for ruminant embryo culture, based on our previous publication (Thompson, et al., 1990).

The aim of this study was to assess the metabolic heterogeneity of pre-implantation cattle embryos cultured under 7% and 20% O_2 levels, with a view to determine whether autofluorescence has potential for clinical assessment of embryo development outcomes. Firstly, embryos were imaged using a combination of fluorescence stains to assess blastomere heterogeneity between the two treatments. This was followed by examination with two non-invasive autofluorescence techniques by confocal microscopy examining two excitation channels, and its extension, hyperspectral imaging, using bespoke 18 spectral channels with varying excitation and emission bandwidths. Their data were, respectively, analysed using GLCM and through combined features of colour and morphology.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals and reagents were purchased from Sigma Aldrich.

Oocyte and Embryo Culture

Cattle ovaries were transported from a local abattoir in warm saline (30-35 °C) and follicular contents were aspirated from 3-8 mm follicles using an 18-gauge needle and a 10 ml syringe. The aspirate was allowed to settle and intact cumulus-oocyte complexes (COCs) with greater than four cell layers of compact cumulus and ungranulated ooplasms were selected in undiluted follicular fluid and washed twice in IVM medium. IVM medium was VitroMat (IVF Vet Solutions, Adelaide Australia) + 4 mg/ml fatty acid free (FAF) BSA (MP Biomedicals, Solon OH USA) + 0.1 IU/ml FSH (Puregon; Organon, Oss Netherland). Groups of 40-50 COCs were transferred into 500 µl of pre-equilibrated IVM medium, overlaid with paraffin oil and cultured for 23 h at 38.5°C, 6% CO_2 in humidified air.

At the completion of IVM, COCs were washed once in wash medium (VitroWash; IVF Vet Solutions, + 4 mg/ml FAF BSA) and transferred into 500 µl of IVF medium (VitroFert, IVF Vet Solutions; + 4 mg/ml FAF BSA + 10 IU/ml heparin + 25 µM penicillamine + 12.5 µM hypotaurine + 1.25 µM epinephrine), overlaid with paraffin oil. Two straws of bull sperm of proven fertility were thawed and prepared using a discontinuous Percoll gradient (45%:90%; GE Healthcare) and

sperm were added to IVF wells at a final concentration of 1 x 10^6 sperm/ml. COCs and sperm were co-cultured at 38.5°C, 6% CO_2 in humidified air.

After 23 h of COC and sperm co-culture (day 1; D1), presumptive zygotes were mechanically denuded of cumulus cells by repeat pipetting in wash medium, washed once in cleavage medium (VitroCleave, IVF Vet Solutions; + 4 mg/ml FAF BSA) and five embryos were transferred into 20 µl drops of pre-equilibrated cleavage medium, overlaid with paraffin oil. Presumptive zygotes were cultured in 7% or 20% O_2, with 6% CO_2 in nitrogen balance at 38.5 °C.

On day 5 (D5), embryos were washed once in blastocyst medium (VitroBlast, IVF Vet Solutions; + 4 mg/ml FAF BSA) and groups of five embryos were transferred into 20 µl drops of pre-equilibrated blastocyst medium, overlaid with paraffin oil. Embryos were cultured until day 8 (D8) in either 7% or 20 %O_2, with 6% CO_2 in nitrogen balance at 38.5 °C.

Developmental Competence of Embryos Following Culture at 7% or 20% O_2

Embryo developmental stage was assessed on D8. In order to determine the influence of different oxygen concentrations post-fertilisation on embryo quality, four experimental replicates were performed with 40-50 COCs per treatment within replicates.

Anti-Oxidant, Reactive Oxygen Species and Mitochondrial Activity in Day 5 Cleavage Stage Embryos and Morula

On D5, embryos were classified as cleavage stage (delayed, pre-compaction) or morula (on-time, post-compaction) and live embryos were co-stained with peroxyfluor-1 (PF1), monochlorobimane (MCB) and Mitotracker Red CMXROS (MTR, Invitrogen; Carlsbad CA, USA), as previously described (Sutton-McDowall, et al., 2015). Briefly, PF1 is an aryl boronate probe that fluoresces on reaction with hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS) produced by mitochondria; MCB fluoresces when bound to low weight thiol compounds, with the highest affinity for reduced glutathione (GSH; representative of 99% of fluorescence intensity; Keelan, et al., 2001), an anti-oxidant, and MTR is a rosamine based fluorophore that accumulates within active mitochondria (membrane potential dependent).

Embryos were washed and stained in wash media, beginning with a brief wash and then each incubated at 38.5°C in semi-darkness with 20 μM PF1 for 1 h, briefly washed and moved into 12.5 μM MCB for 30 m, briefly washed and then incubated with 200 nM MTR for 15 m. Embryos were then washed once and transferred into 2 μl drops of wash medium, and overlaid with paraffin oil, in glass bottomed confocal dishes (Cell E&G, Houston TX, USA). Fluorescence was visualised using a Fluoview FV10i (Olympus; Tokyo Japan) confocal microscopy (MCB: excitation = 358 nm and peak emission = 461 nm; PF1: excitation = 496 nm and peak emission = 519 nm; MTR: excitation = 578 nm and peak emission = 598 nm) at 90x magnification, at a single z plane. Laser settings, magnification and image settings remained constant across replicates. Individual experimental runs were

normalised to fluorescence beads (InSpeck, Invitrogen) to account for any variations in the instrument between runs.

Image processing and analysis of laser scanning confocal microscopy images was performed using Image J software (NIH). Prior to analysis, images were converted from Olympus image files (oif) to 8-bit grey scale tiff images and positive staining areas/region of interest (ROI) were selected using the threshold selection function within ImageJ. The mean intensity and texture of the ROI was determined using batch macros for intensity and grey-level co-occurrence matrix (GLCM), both available online (http://rsb.info.nih.gov/ij/plugins/index.html) and as per Sutton-McDowell et al. (Sutton-McDowall, et al., 2015).

Three texture feature analyses were conducted within the GLCM analysis. "Orderliness" (predictability) of fluorescence is measured by angular secondary moment (ASM), with increasing ASM values indicating more predictable patterns and decreased heterogeneity. "Contrast" measures the variability in fluorescence intensity as a broad measure of fluorescence homogeneity, with increasing values indicating increased heterogeneity. "Correlation" relates to the linear dependence of grey levels on neighbouring pixels (Haralick, et al., 1973), predictive of organelle patterning.

Three experimental replicates were performed with 10-20 embryos per treatment and developmental stage within each replicate.

Redox State of Morula as a Predictor of Developmental Competence

The autofluorescence (AF; NAD(P)H and FAD) of D5 morula embryos was measured using laser scanning confocal microscopy to determine if there was a relationship between redox state and embryo developmental potential. On D5, morula-stage embryos cultured in 7% and 20% O_2 were washed once in wash medium and transferred into individual 2 µl of wash medium in confocal dishes, overlaid with mineral oil. Embryos (+AF) were imaged for NAD(P)H (excitation = 405 nm and emission = 420-520 nm) and FAD (excitation = 473 nm and emission = 490-590 nm) using a Fluoview FV10i confocal microscope at 90x magnification and at a single z plane. Embryos were washed once in blastocyst medium and then transferred into 2 µl drops of blastocyst media and cultured individually. Control embryos (without AF) were washed once in blastocyst medium and then transferred into 2 µl drops of blastocyst medium. All embryos were cultured for a further three days (D8) in either 7% or 20 %O_2, 6% CO_2 in nitrogen balance at 38.5 °C to assess whether the AF results on D5 (morula stage) correlated with development to the blastocyst stage on D8. Small groups of embryos were imaged per replicate (5-10 per dish per replicate, 4 replicates). Embryo handling in atmosphere was limited to less than 10 mins during the image capture period to minimise stress.

Hyperspectral Analyses of Morula

Day 5 embryos were air transported (3 h travel) to perform hyperspectral analysis on live morula-stage embryos (7% O_2 = 21 embryos and 20% O_2 = 22 embryos), as per previous publications (Gosnell, et al., 2016a, Gosnell, et al., 2016b). Briefly,

spectral autofluorescence was measured using an Olympus IX71 epifluorescent microscope (Olympus), fitted with a multi LED light source (Prizmatix Ltd; Givat-Shmuel, Israel). Hyperspectral microscopy utilises 18 spectral channels: 12 separate excitation wavelength ranges (generated by 12 different light emitting diodes) and 4 emission wavelength bands, covering excitation wavelengths of 334-495 nm and emission wavelengths of 447-700 nm (see Table 1 for channel details). Images were captured using an iXon 885 camera (Andor; Belfast, UK). The power densities and exposures (~1 s) were comparable to standard fluorescence microscopy (Gosnell, et al., 2016b). At these exposures, no detectable photobleaching was observed.

Embryos were transferred into glass bottomed confocal dishes containing cleavage medium and hyperspectral images were taken at five different z planes in 10 μm increments, one image at the equatorial plane of focus of the embryo (widest diameter), two images above and two images below. For each spectral channel a composite, multi-focus image reconstruction was applied prior to analysis (Gosnell, et al., 2016a, Gosnell, et al., 2016b).

Hyperspectral Image Analysis

Image analysis was performed as per Gosnell et al. (Gosnell, et al., 2016a, Gosnell, et al., 2016b). Images containing cells were segmented to define a perimeter around the cells' fluorescent cytoplasm. The boundaries of the fusing cells of morula were manually segmented, based on the differential interference contrast (DIC) images taken simultaneously with the hyperspectral images, creating regions of interest (ROI) representing seemingly continuous areas (either single

blastomeres or very compacted blastomeres). The median value of the non-zero pixels was subtracted from all non-zero pixels in order to greatly reduce the sharp edge around the perimeter of the ROIs, whilst retaining most of the obvious texture. Pixels within each ROI were used to calculate the features in each of these cell regions.

Custom-designed software was used to determine multiple mathematically defined cellular features, such as cell spectra, channel values and morphological patterns (for the definition of features see Gosnell, et al., 2016b). A total of 33,000 feature algorithms were used in the analysis. Following feature selection process and their ranking (carried out as in Gosnell, et al., 2016a), four such features were identified to have strong correlation with embryo classes examined here (culture at 7% or 20% O_2) and, taken together, they could demonstrate significant differences between embryos cultured in the two O_2 concentrations. Descriptions of the four features A-D are provided in the Supplementary Material.

Colour Spectral Plots

Autofluorescence data obtained in 18 channel hyperspectral imaging were projected onto a three-dimensional space using Principal Component Analysis with the top three PCA variables capturing most of the intensity variance. The three new variables were then used to make false colour images (red/green/blue), which are representative of spectral information in autofluoresencence. These false colour images are provided to highlight differences of cellular biochemistry within the embryos.

Statistical Analysis

Differences between treatments were analysed using a general linear model using version 22 (IBM) SPSS software, with oxygen concentrations built in as the main effect. Embryo development data was arcsine transformed prior to analyses. Data is presented as means ± SEM and P-values less than 0.05 were considered significant.

To analyse hyperspectral autofluorescence feature data, a discrimination plot was created by performing linear discrimination analysis (LDA) of the four hyperspectral features (selected out of 33,000 hyperspectral features). The linear combinations of the best four features obtained by LDA most sensitively captured the differences between 7% and 20% O_2 cultured embryos.

RESULTS

Developmental Competence of Embryos Following Culture in 7% or 20% O_2

From Day 1, putative zygotes were cultured in 7% or 20% O_2 and on-time embryo development was determined on Day 8. There were no differences in cleavage rates (Table 2). However, blastocyst development was significantly reduced, as expected, following culture in 20% O_2 (Table 2, $P < 0.05$), particularly in advanced stages of blastocyst development, with hatched blastocyst rates reduced 2.8-fold in the presence of atmospheric oxygen levels ($P < 0.05$). Hence, the model for embryo stress using low and high oxygen concentration was validated.

Metabolic Heterogeneity in Day 5 Cleavage Stage Embryos and Morula

The effects of 7% versus 20% oxygen concentration on reactive oxygen species (ROS) production, anti-oxidants and mitochondrial activity within Day 5 embryos was determined. In addition to measuring the mean fluorescence intensity, texture analysis (grey level co-occurrence matrix; GLCM, examining three features) was performed to predict the uniformity and frequency of the patterns of fluorescence throughout the embryos.

Differences in colour from the merged fluorophore staining between morula and arrested cleavage stage embryos was evident (Figure 1). The variability in the merged fluorescence images between blastomeres within the cleavage stage embryos demonstrated metabolic heterogeneity among blastomeres of arrested cleavage stage embryos, whereas such visually clear heterogeneity was not evident at the morula stage.

Only images of morula were used for subsequent analyses. The fluorescence intensity of MCB (GSH) was significantly higher in embryos cultured in 20% O_2 compared to 7% O_2, (Table 3; 7% = 160.5 ± 3.8 vs. 20% = 173.4 ± 2.7 intensity values; $P = 0.007$) and the 'predictability' (ASM) of fluorescence intensity patterns was also higher in morula cultured in 20% O_2 (Table 3; ASM, a measure of orderliness), with no significant differences in contrast and correlation measurements of MCB fluorescence. The correlation (predictability of fluorescence intensity at the pixel level) of PF1 fluorescence was also higher in 20% O_2 exposed embryos, compared to the 7% treatment group (Table 3; $P < 0.001$).

Redox State of Morula as a Predictor of Metabolic Heterogeneity and Developmental Outcome Following Autofluorescence Measurement

To determine if conventional autofluorescence measurements by confocal microscopy of morula (Day 5) was predictive of heterogeneity and embryo developmental outcomes, FAD and NAD(P)H fluorescence were measured in morula cultured in 7% or 20% O_2. Following measurement, morula were cultured individually and the development was then assessed on Day 8 (Figure 2A). An additional cohort of morula was removed from group culture into individual drops to serve as a "no confocal microscopy" controls. Neither oxygen concentration affected the number of morula that reached the blastocyst stage on Day 8 during culture and fluorescence measurement (Table 4; main effects, $P > 0.05$), suggesting that the positive impact of reduced oxygen was primarily during the pre-compaction period of culture.

In contrast to the analysis with fluoroprobes, there were no significant autofluorescence differences in intensity (and therefore redox ratio) or texture between oxygen concentrations measured in Day 5 morula (data not shown, main effect, $P > 0.05$). However, both FAD and NAD(P)H intensity were significantly higher in Day 5 morula that developed to the blastocyst stage on Day 8, compared to that in morula that arrested (Figure 2B and C; $P < 0.05$). There was no significant differences in the redox ratio (Figure 2D, FAD:NAD(P)H), demonstrating that increases in both FAD and NAD(P)H metabolism was predictive of further development, suggesting that increased metabolic activity is associated with advanced development in this model.

Hyperspectral Analyses of Morula

Colour spectral plots (composite two-dimensional images) were created to highlight differences in cellular biochemistry, registered in all 18-hyperspectral channels and z-stacks of five planes in which images were captured (Figure 3). False Principle Component Analysis-derived colours were applied to the hyperspectral image stacks as indicated in the Methods, with multiple hues within embryos indicating enhanced variation in metabolic signatures. Representative images are presented in Figure 3, with exposure to 20% O_2 resulting in morulae with more hues (represented by Figure 3D-F) compared to morulae cultured in 7% O_2 where the hues were more uniform (represented by Figure 3A-C).

Furthermore, a suite of 33000 textural features was applied to the two-dimensional z-stack reconstructions of embryo images. Of these, four features revealed differences that were significantly different between morula exposed to the different oxygen concentrations ($P < 0.001$; Figure 4). Morula cultured in the presence of 7% O_2 had significantly higher mean $\log_{10}$ intensity of the brightest 10% pixels in channel 5 (Excitation: 365 nm, Emission: 587 nm) compared to morula exposed to 20% O_2 ($P < 0.001$; Figure 4A). Likewise, the standard deviation of pixel intensity in channel 15 (Excitation: 495 nm, Emission: 587 nm) was significantly higher in the 7% O_2 group compared to 20% O_2 (Figure 4D). The other two features were significantly higher in the 20% O_2 treatment group. Specifically, the "blobbiness" feature (indicative of increased disorderliness of signal intensity) of channel 18 (Excitation: 495 nm, Emission: 635 nm) and the mean intensity ratio of channel 11:channel 13 (Excitation: 425 nm, Emission: 587

nm and Excitation: 455 nm, Emission: 587 nm) were both significantly higher in the 20% O_2 treatment (Figures 4B & C respectively; $P < 0.001$).

The values of the four features for each ROI in each embryo were then linearly transformed using Linear Discrimination Analysis (LDA) with respect to the two groups (7% O_2 and 20% O_2). A clear discrimination between the 7% and 20% O_2 treatment groups was apparent, with significant differences in the mean values of clusters (Figure 5; $P < 0.001$).

Discussion

We employed a well-characterised model of cell stress (7% O_2 vs 20% O_2) in a bovine embryo model to evaluate techniques that may quantify responses to cellular stress. The ruminant embryo is exquisitely sensitive to oxygen levels during culture (Tervit, et al., 1972, Thompson, et al., 1990). Our results clearly support the observations of others that low oxygen culture reduces cell stress and enhances pre-implantation development during in-vitro culture and therefore should be the preferred atmosphere for human embryo culture (Katz-Jaffe, et al., 2005, Rinaudo, et al., 2006, Gardner and Wale, 2013, Gardner, 2016).

We also employed fluorescent probes to target specific aspects of cellular metabolism to observe whether embryo culture under either 7% or 20% oxygen atmospheres induced different levels of metabolic heterogeneity in either arrested (pre-compaction, cleavage stage) or embryos that had developed 'on-time' (post-compaction, morula). It was visually evident that metabolic

heterogeneity is present in the arrested embryos (Figure 1). In extreme cases, blastomeres within the same embryo that appear morphologically similar (shape, size, density) under phase contrast microscopy had obvious variations in the intensity of fluorescence. As expected, using the same fluorophore probes at the morula stage revealed fewer differences in intensity and patterns in fluorescence between the two culture conditions (as determined by texture analysis). We would expect less heterogeneity at this stage of development, due to the establishment of cell-cell communication. Nevertheless, GLCM analysis revealed that MCB fluorescence was more consistent (predictable) when embryos were cultured under 20% O_2 compared to 7% O_2. MCB fluorescence is specific for reduced thiols, of which glutathione (GSH) is the most abundant. While increased developmental competence in oocytes is correlated with increased levels of reduced glutathione (de Matos and Furnus, 2000), it might also be associated with a response to increased stress (Zeng, et al., 2014).

Autofluorescence of somatic cells has previously been shown to measure differences in redox state, as the strongest fluorescence signals are derived from the autofluorescence of NAD(P)H (Excitation: 405 nm and Emission: 420-520 nm) and FAD (Excitation: 473 nm and Emmision: 490-590 nm) (Skala and Ramanujam, 2010). Two-channel autofluorescence measurement has been used for distinguishing differences in redox activity in oocytes and embryos as a result of fertilisation and cleavage (Dumollard, et al., 2007a, Dumollard, et al., 2007b), and the impact of different culture environments for oocyte in-vitro maturation and early development (Zeng, et al., 2014, Sutton-McDowall, et al., 2015, Sutton-McDowall, et al., 2016). Two-channel autofluorescence measurements were

applied at the morula stage to embryos cultured under 7% and 20% O_2 concentrations but neither autofluorescence channel was able to discriminate a difference between the two culture conditions. However, higher levels of autofluorescence were associated with the ability of morula to develop to the blastocyst stage, which may have a clinical application as an embryo diagnostic tool.

Four image analysis features were able to discriminate differences in hyperspectral images of embryos cultured under either 7% or 20% oxygen. Unlike the two-channel autofluorescence measurements, the more detailed 18 channel hyperspectral microscopy and more sophisticated textural analysis was sensitive enough to detect differences between morula exposed the two different culture conditions. Hyperspectral autofluorescence analysis has been shown to successfully separate differences between somatic cells in monolayer cultures, demonstrating that cellular heterogeneity exists in large populations of cells (Gosnell, et al., 2016a, Gosnell, et al., 2016b). Further studies are needed to determine if differences can be seen in earlier stages of development, such as pre-compaction, zygotes or oocytes, which could incorporate additional features beyond the four that were used in the current study. In addition, further studies are required in animals, then feasibly in IVF clinics, to evaluate the utility of hyperspectral analysis by examining its predictive capacity for post-transfer pregnancy establishment, which was logistically impossible to conduct in the current study. Nevertheless, the data presented here describes an important new tool to discriminate two populations of embryos that are difficult to separate on morphology and less detailed metabolic measures alone. This approach to

548 discriminate between embryos may be an adjunct tool to combine with other
549 diagnostic methods to provide accurate embryo viability assessment.
550

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557

558 Authors' Roles

559 M.S.M. and J.T. conceived the idea for the study and experimental design; M.S.M.
560 and M.W. conducted the embryology, fluorophore and two-channel
561 autofluorescence experiments; M.P. and A.D.A. synthesised and developed the PF1
562 fluorophore; M.G., A.A. and E.G. conducted the hyperspectral microscopy and
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574 Conflict of Interest

575 The authors declare no conflict of interest.

576

577 References

578 Bloor DJ, Metcalfe AD, Rutherford A, Brison DR, Kimber SJ. Expression of cell
579 adhesion molecules during human preimplantation embryo development. *Mol*
580 *Hum Reprod* 2002;8: 237-245.

581

582 Bontekoe S, Mantikou E, van Wely M, Seshadri S, Repping S, Mastenbroek S. Low
583 oxygen concentrations for embryo culture in assisted reproductive technologies.
584 *The Cochrane database of systematic reviews* 2012;7: CD008950.

585

586 Brison DR, Sturmey RG, Leese HJ. Metabolic heterogeneity during
587 preimplantation development: the missing link? *Hum Reprod Update* 2014;20:
588 632-640.

589

590 Chamayou S, Patrizio P, Storaci G, Tomaselli V, Alecci C, Ragolia C, Crescenzo C,
591 Guglielmino A. The use of morphokinetic parameters to select all embryos with
592 full capacity to implant. *J Assist Reprod Genet* 2013;30: 703-710.

593

de Matos DG, Furnus CC. The importance of having high glutathione (GSH) level after bovine in vitro maturation on embryo development effect of beta-mercaptoethanol, cysteine and cystine. *Theriogenology* 2000;53: 761-771.

Dumollard R, Duchen M, Carroll J. The role of mitochondrial function in the oocyte and embryo. *Current topics in developmental biology* 2007a;77: 21-49.

Dumollard R, Marangos P, Fitzharris G, Swann K, Duchen M, Carroll J. Sperm-triggered [Ca2+] oscillations and Ca2+ homeostasis in the mouse egg have an absolute requirement for mitochondrial ATP production. *Development* 2004;131: 3057-3067.

Dumollard R, Ward Z, Carroll J, Duchen MR. Regulation of redox metabolism in the mouse oocyte and embryo. *Development* 2007b;134: 455-465.

Eckert JJ, McCallum A, Mears A, Rumsby MG, Cameron IT, Fleming TP. PKC signalling regulates tight junction membrane assembly in the pre-implantation mouse embryo. *Reproduction* 2004;127: 653-667.

Fischer B, Bavister BD. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *J Reprod Fertil* 1993;99: 673-679.

Fleming TP, Velazquez MA, Eckert JJ. Embryos, DOHaD and David Barker. *J Dev Orig Health Dis* 2015;6: 377-383.

619 Gardner DK. The impact of physiological oxygen during culture, and vitrification
620 for cryopreservation, on the outcome of extended culture in human IVF. *Reprod*
621 *Biomed Online* 2016;32: 137-141.
622
623 Gardner DK, Stevens J, Sheldon CB, Schoolcraft WB. Analysis of blastocyst
624 morphology. In Elder K and Cohen J (eds) *Human preimplantation embryo*
625 *selection*. 2007. Informa Healthcare, London, pp. 79-87.
626
627 Gardner DK, Wale PL. Analysis of metabolism to select viable human embryos for
628 transfer. *Fertil Steril* 2013;99: 1062-1072.
629
630 Gleicher N, Kushnir VA, Barad DH. Preimplantation genetic screening (PGS) still
631 in search of a clinical application: a systematic review. *Reproductive biology and*
632 *endocrinology : RB&E* 2014;12: 22.
633
634 Gosnell ME, Anwer AG, Cassano JC, Sue CM, Goldys EM. Functional hyperspectral
635 imaging captures subtle details of cell metabolism in olfactory neurosphere cells,
636 disease-specific models of neurodegenerative disorders. *Bba-Mol Cell Res*
637 2016a;1863: 56-63.
638
639 Gosnell ME, Anwer AG, Mahbub SB, Menon Perinchery S, Inglis DW, Adhikary PP,
640 Jazayeri JA, Cahill MA, Saad S, Pollock CA *et al.* Quantitative non-invasive cell
641 characterisation and discrimination based on multispectral autofluorescence
642 features. *Sci Rep* 2016b;6: 23453.
643

644 Guo N, Li Y, Ai J, Gu L, Chen W, Liu Q. Two different concentrations of oxygen for
645 culturing precompaction stage embryos on human embryo development
646 competence: a prospective randomized sibling-oocyte study. *Int J Clin Exp Pathol*
647 2014;7: 6191-6198.
648
649 Haralick RM, Shanmuga K, Dinstein I. Textural Features for Image Classification.
650 *Ieee T Syst Man Cyb* 1973;Smc3: 610-621.
651
652 Houghton FD. Role of gap junctions during early embryo development.
653 *Reproduction* 2005;129: 129-135.
654
655 Katz-Jaffe MG, Linck DW, Schoolcraft WB, Gardner DK. A proteomic analysis of
656 mammalian preimplantation embryonic development. *Reproduction* 2005;130:
657 899-905.
658
659 Keelan J, Allen NJ, Antcliffe D, Pal S, Duchen MR. Quantitative imaging of
660 glutathione in hippocampal neurons and glia in culture using
661 monochlorobimane. *J Neurosci Res* 2001;66: 873-884.
662
663 Krisher RL, Heuberger AL, Paczkowski M, Stevens J, Pospisil C, Prather RS,
664 Sturmey RG, Herrick JR, Schoolcraft WB. Applying metabolomic analyses to the
665 practice of embryology: physiology, development and assisted reproductive
666 technology. *Reprod Fertil Dev* 2015.
667

668 Munne S, Wells D. Detection of mosaicism at blastocyst stage with the use of
669 high-resolution next-generation sequencing. *Fertil Steril* 2017;107: 1085-1091.
670
671 Rinaudo PF, Giritharan G, Talbi S, Dobson AT, Schultz RM. Effects of oxygen
672 tension on gene expression in preimplantation mouse embryos. *Fertil Steril*
673 2006;86: 1252-1265, 1265 e1251-1236.
674
675 Skala M, Ramanujam N. Multiphoton redox ratio imaging for metabolic
676 monitoring in vivo. *Methods Mol Biol* 2010;594: 155-162.
677
678 Sutton-McDowall ML, Mottershead DG, Gardner DK, Gilchrist RB, Thompson JG.
679 Metabolic differences in bovine cumulus-oocyte complexes matured in vitro in
680 the presence or absence of follicle-stimulating hormone and bone
681 morphogenetic protein 15. *Biol Reprod* 2012;87: 87, 81-88.
682
683 Sutton-McDowall ML, Purdey M, Brown HM, Abell AD, Mottershead DG, Cetica
684 PD, Dalvit GC, Goldys EM, Gilchrist RB, Gardner DK *et al.* Redox and anti-oxidant
685 state within cattle oocytes following in vitro maturation with bone
686 morphogenetic protein 15 and follicle stimulating hormone. *Mol Reprod Dev*
687 2015;82: 281-294.
688
689 Sutton-McDowall ML, Wu LL, Purdey M, Abell AD, Goldys EM, MacMillan KL,
690 Thompson JG, Robker RL. Nonesterified Fatty Acid-Induced Endoplasmic
691 Reticulum Stress in Cattle Cumulus Oocyte Complexes Alters Cell Metabolism
692 and Developmental Competence. *Biol Reprod* 2016;94: 23.

693

694 Tan TC, Ritter LJ, Whitty A, Fernandez RC, Moran LJ, Robertson SA, Thompson JG,
695 Brown HM. Gray level Co-occurrence Matrices (GLCM) to assess microstructural
696 and textural changes in pre-implantation embryos. *Mol Reprod Dev* 2016;83:
697 701-713.

698

699 Tervit HR, Whittingham DG, Rowson LE. Successful culture in vitro of sheep and
700 cattle ova. *J Reprod Fertil* 1972;30: 493-497.

701

702 Thompson JG. In vitro culture and embryo metabolism of cattle and sheep
703 embryos - a decade of achievement. *Anim Reprod Sci* 2000;60-61: 263-275.

704

705 Thompson JG, Brown HM, Sutton-McDowall ML. Measuring embryo metabolism
706 to predict embryo quality. *Reprod Fertil Dev* 2016;28: 41-50.

707

708 Thompson JG, Simpson AC, Pugh PA, Donnelly PE, Tervit HR. Effect of oxygen
709 concentration on in-vitro development of preimplantation sheep and cattle
710 embryos. *J Reprod Fertil* 1990;89: 573-578.

711

712 VerMilyea MD, Tan L, Anthony JT, Conaghan J, Ivani K, Gvakharia M, Boostanfar
713 R, Baker VL, Suraj V, Chen AA *et al.* Computer-automated time-lapse analysis
714 results correlate with embryo implantation and clinical pregnancy: a blinded,
715 multi-centre study. *Reprod Biomed Online* 2014;29: 729-736.

716

717 Voullaire L, Slater H, Williamson R, Wilton L. Chromosome analysis of
718 blastomeres from human embryos by using comparative genomic hybridization.
719 *Hum Genet* 2000;106: 210-217.
720
721 Wells D, Delhanty JD. Comprehensive chromosomal analysis of human
722 preimplantation embryos using whole genome amplification and single cell
723 comparative genomic hybridization. *Mol Hum Reprod* 2000;6: 1055-1062.
724
725 Wong C, Chen AA, Behr B, Shen S. Time-lapse microscopy and image analysis in
726 basic and clinical embryo development research. *Reprod Biomed Online* 2013;26:
727 120-129.
728
729 Zeng HT, Richani D, Sutton-McDowall ML, Ren Z, Smitz JE, Stokes Y, Gilchrist RB,
730 Thompson JG. Prematuration with cyclic adenosine monophosphate modulators
731 alters cumulus cell and oocyte metabolism and enhances developmental
732 competence of in vitro-matured mouse oocytes. *Biol Reprod* 2014;91: 47.
733
734

735 Figure Legends

736 **Figure 1**. Representative images of Day 5 arrested (cleaved) and on-time (morula)
737 embryos cultured in 7% and 20% O_2 and labeled with fluorescence probes. MCB
738 = monochlorobimane (reduced glutathione); PF1 = perfluoxy 1 (hydrogen
739 peroxide) and MTR = Mitotracker Red CMXRos (active mitochondria). The scale
740 bar represents 50 μm.

741

742 **Figure 2**. Autofluorescence (AF) measured on a laser scanning confocal
743 microscope in two channels (representative of FAD and NAD(P)H) of morula (M)
744 on day 5, in relation to embryo developmental stage on day 8. A) Experimental
745 design; B) FAD; C) NAD(P)H and D) redox ratio (FAD:NAD(P)H). Dot plots indicate
746 individual embryos, with bars representing mean units ± SEM. UnBl = unexpanded
747 blastocyst; ExBl = expanded blastocyst and HBl = hatched blastocyst. Asterisks
748 indicate significant differences to the morula group ($P < 0.05$).

749

750 **Figure 3**. Representative images of composite two-dimensional reconstructions
751 of the 90 images (5 z-planes and 18 spectral channels) of each morula. False
752 colours produced from the top three components in Principal Component Analysis
753 (PCA) indicate variability in metabolic signatures, with an increased number of
754 hues in a single embryo relating to increased heterogeneity. A-C = 7% O_2 and D-E
755 = 20% O_2.

756

757 **Figure 4.** Hyperspectral signatures of day 5 morula exposed to 7% or 20% O_2.
758 Feature values are A) the $\log_{10}$ of the mean pixel intensity of the brightest 10%
759 pixels in channel 5 (Ex: 365nm, Em: 587nm; $P < 0.000026$); B) blobbiness feature
760 (measuring how well pronounced are various irregular oval shapes) in channel 18
761 (Ex: 495 nm, Em: 635 nm; $P < 0.00003$); C) mean cellular pixel intensity ratio of
762 channel 11:channel 13 (Ex: 415nm, Em: 587 nm vs. Ex: 455 nm, Em: 587nm; P
763 <1e-06) and D) standard deviation of pixel intensity of channel 15 (Ex: 495 nm,
764 Em: 587 nm; $P < 0.000084$). Dot plots represent individual regions of interests and
765 the bars with shading represent mean $\pm$ SEM.

Figure 5: A plot obtained using Linear Discriminant Analysis (LDA) of the four hyperspectral signature features A-D that varied significantly between morula cultured in 7% and 20% O_2. The LDA helps to optimally separate the two classes of embryos. Crosses = 7% O_2; circles = 20% O_2 treatment. Crosshairs = means of the clusters. These means are significantly different ($P < 0.001$).

Supplementary Material

Hyperspectral algorithms

Our selection of the most informative cellular features arrived at the following four best features (**Features 1-4**).

Feature 1 refers to the log of the mean pixel intensity of the brightest 10% of pixels in channel 5 (ex: 365 nm, em: 587 nm).

Feature 2 calculations relate to the blobbiness of images (channel 8; ex: 495 nm, em: 635 nm), a measure of the pixel intensity blobs values disorder. The blob image intensity image was first filtered using a continuous wavelet transform filter (Mexican hat wavelet). The multidimensional mexihat wavelet function is equivalent to the laplacian of a multidimensional Gaussian:

$$\square(\square,\square,\square) = -\frac{1}{\square\square^4}\left(1-\frac{\square^2+\square^2}{2\square^2}\right)\square^{-(\square^2+\square^2)/2\square^2}$$

Where x and y are the matrix coordinates and σ is a scaling value. Small values make the wavelet filter sensitive to fine spatial detail.

To calculate the wavelet filter output for a given input image, the image matrix of intensity values was convoluted with the 2D wavelet function defined at a particular scale value. The smallest scale starting at one provides a filter with the highest frequency sensitivity producing high output values in regions where there are very small blobs. This convolution was performed by transforming the wavelet function matrix and image using the 2D fast Fourier transform algorithm, multiplying the matrices element wise, then transforming the answer back into the spatial domain (Antoine, et al., 2004). For the feature of interest in this work (Feature 2), a scale value of 2 was used, and then the wavelets filtering were performed. The wavelet-filtered images contained a high contrast pattern of many small blobs.

Furthermore**,** the local entropy was determined, based on the calculation of pair-wise entropy values of a centre pixel with its local neighbouring pixel intensities. The size of the neighbourhood chosen here were nine pixels from the centre pixel. Thus 360 neighbouring pixel intensity values were used to calculate entropy at each pixel site. Each of the □ neighbour pixel intensity values were binned into one of 16 intensity bins, equally spanning the 0-255 ranges of grey intensity levels. From this, we construct an estimated probability distribution □(□□) for the neighbouring pixel intensity values occurring within a bin □□.

$$\text{Local entropy} = -\sum_{□} □(□_{□})□□□_{2}□(□_{□})$$

Maximum entropy or uncertainty occurs when distribution is normal or random, therefore any local structure were likely to yield a lower entropy value. Local entropy is defined for a specific pixel. To calculate the local entropy feature for a cell, local entropies were added for each pixel in that cell.

Feature 3 is the mean intensity ratio of channels 11 and 13. The mean pixel intensity value of signal in channel 11 (ex: 425 nm, em: 587 nm) was divided by the mean pixel intensity value of signal in channel 13 (ex: 455 nm, em: 587 nm).

Feature 4 is the standard deviation of channel 15-pixel intensity. The pixel intensity dispersion, as measured by the standard deviation, was calculated in channel 15.