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EMBRYO BIOLOGY

# Non-invasive imaging of mouse embryo metabolism in response to induced hypoxia

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## Abstract

**Purpose** This study used noninvasive, fluorescence lifetime imaging microscopy (FLIM)-based imaging of NADH and FAD to characterize the metabolic response of mouse embryos to short-term oxygen deprivation. We investigated the response to hypoxia at various preimplantation stages.

**Methods** Mouse oocytes and embryos were exposed to transient hypoxia by dropping the oxygen concentration in media from 5–0% over the course of ~1.5 h, then 5% $O_2$ was restored. During this time, FLIM-based metabolic imaging measurements of oocyte/embryo cohorts were taken every 3 minutes. Experiments were performed in triplicate for oocytes and embryos at the 1- to 8-cell, morula, and blastocyst stages. Maximum hypoxia response for each of eight measured quantitative FLIM parameters was taken from the time points immediately before oxygen restoration.

**Results** Metabolic profiles showed significant changes in response to hypoxia for all stages of embryo development. The response of the eight measured FLIM parameters to hypoxia was highly stage-dependent. Of the eight FLIM parameters measured, NADH and FAD intensity showed the most dramatic metabolic responses in early developmental stages. At later stages, however, other parameters, such as NADH fraction engaged and FAD lifetimes, showed greater changes. Metabolic parameter values generally returned to baseline with the restoration of 5% oxygen.

**Conclusions** Quantitative FLIM-based metabolic imaging was highly sensitive to metabolic changes induced by hypoxia. Metabolic response profiles to oxygen deprivation were distinct at different stages, reflecting differences in metabolic plasticity as preimplantation embryos develop.

**Keywords** Embryo metabolism · Hypoxia · Noninvasive assessment · Metabolic imaging · FLIM · Mitochondria

## Introduction

In the 1960s, it was established that metabolites such as glucose, pyruvate, and lactate are essential for mammalian embryo development in vitro [1–4]. Further studies showed that there is an intricate relationship between specific metabolites and embryo metabolism over the course of an embryo's preimplantation development [5–8]. For example, early in development (pre-compaction), embryos rely heavily on pyruvate as their preferred metabolic substrate, but by the blastocyst stage, they shift towards a glucose-dependent metabolism [6]. As the site of ATP production in the electron transport chain (ETC), mitochondria play a pivotal role in embryonic development [9–11]. Oxygen is a key mitochondrial regulator, and the presence or absence of oxygen preferentially shifts metabolic functions. Oxygen consumption of the preimplantation mammalian embryo also changes over time; just before implantation, a blastocyst has higher oxygen consumption than its cleavage-stage embryo counterpart

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[12, 13]. Furthermore, oxygen level is a critical element in embryo culture. It is well established that embryos cultured in atmospheric oxygen levels (approximately 21%) have poorer embryo development than those cultured in levels more closely mimicking in vivo oxygen tension (5%) [14, 15]. The importance of oxygen levels has also been highlighted in numerous studies examining gene expression and epigenetic effects when comparing mammalian embryos cultured in low versus high oxygen environments [8, 9, 16, 17]. The ability of mammalian embryos to cope with oxygen fluctuations is, however, poorly understood, as real-time embryo metabolism and response has been previously difficult to measure.

As early as 1980, an association was shown between glucose uptake of bovine embryos and viability [18]. A number of studies have subsequently used ultramicrofluorimetric techniques to investigate embryo metabolism [6]. Leese, Gardner, and colleagues showed that glucose uptake is strongly related to the viability of mouse and human embryos and, in particular, can differentiate between embryos of similar morphology [19–22]. Routine assessment of the metabolic integrity of embryos as a selection tool has, however, remained difficult due to the complexities of the previous techniques.

The two mainstays of embryo selection are standard morphologic assessment and preimplantation genetic testing (PGT) using trophectoderm biopsy. Each of these modalities has advantages and drawbacks, but neither provides information about metabolic function. Metabolic imaging of NADH and FAD using fluorescence lifetime imaging microscopy (FLIM) can provide real-time, noninvasive, quantitative characterizations of cellular metabolic state [23–26]. For example, Sanchez et al. successfully used FLIM to evaluate quantitative metabolic differences between old versus young oocytes and mutant versus wildtype oocytes for a metabolic mutation, Clpp [27]. FLIM has also been shown to be capable of detecting metabolic shifts over development and shifts due to chemical perturbations using mouse embryos [28].

These studies suggest that metabolic imaging has the potential to be an objective, noninvasive tool for assessing viability of oocytes and embryos. As a research tool, metabolic imaging provides quantitative measures that may allow for a more precise characterization of embryo metabolism and a means of elucidating how metabolism changes in early embryo development. The aim of this study was to use FLIM to measure metabolic responses of mouse oocytes and embryos to transient hypoxia exposure at various preimplantation developmental stages.

## Materials and methods

### Imaging system and FLIM measurements

FLIM measurements were performed on a Nikon TE300 microscope with a Nikon 20X objective (0.75 NA) with a galvanometer scanner and a TCSPC module (SPC-150, Becker and Hickl). Two-photon excitation was supplied via a Ti:sapphire pulsed laser (M-Squared Lasers) with an 80-MHz repetition rate and ~ 150 fs pulse width. 750 nm illumination (30 mW) and a 447/60 nm bandpass filter were used for imaging NADH fluorescence. 845 nm illumination (50 mW) and a 550/88 nm bandpass filter were used for imaging FAD fluorescence. The spectrum of NADPH is almost identical to that of NADH [29], so our measurements contain contribution from both these molecules. We still refer to this as the "NADH" signal for simplicity. Illumination intensities were calibrated by measuring power output through the objective. Fluorescence was detected in the epi direction with a hybrid detector (HPM-100–40, Becker and Hickl). For several acquisitions, second harmonic generation (SHG) spindle imaging was detected simultaneously with FAD imaging by a single-photon counting detector (PMC-150, Becker-Hickl), placed in the forward-scattered direction, with combined 650 short-pass and 440/20 nm bandpass filters. Scans were acquired for 60 s in the mid-plane of the oocytes/embryos. Acquisitions were performed using custom LabVIEW software.

### Oocyte/embryo handling

Cryopreserved oocytes and 1-cell stage mouse embryos (EmbryoTech, Haverhill MA), from crosses between B6C3F1 females and B6D2F1 males, were thawed and transferred to pre-equilibrated dishes. Research was conducted under a protocol approved by Harvard University's Institutional Animal Care and Use Committee (IACUC), which has a Letter of Assurance (File No. A3593-01) from the National Institutes of Health Office of Laboratory Animal Welfare. Embryos were imaged in Vitrolife Primo Vision nine-well dishes in KSOM media (MR-121-D, Millipore-Sigma) in an on-stage incubator (Ibidi, 10918), at 37C, 5% $CO_2$, and 5% $O_2$ (when not undergoing experimental hypoxia) (Fig. 1a). Prior to imaging, embryos were stored in a table-top commercial incubator (Panasonic MCO5MPA) and either equilibrated (for 1-cell experiments) or cultured out to the appropriate stage then equilibrated prior to imaging.

### Oxygen deprivation experiments

Oocytes or embryos at the following stages were evaluated: oocyte; 1-cell, 2-cell, and 3- to 8-cell; morula and blastocyst. For all experiments, embryos were equilibrated at 5% oxygen on the microscope under a 1050 μL overlay of mineral oil (Vitrolife Ovoil$^{TM}$). The volume of the oil overlay was calibrated with prior experiments using an oxygen-sensitive ruthenium dye (Sigma 544981) to measure the $O_2$ exchange rate within the media droplet (data not shown).

Oocytes/embryos were imaged every 3 min. at 5% oxygen for 10 min. to obtain baseline data, and then the gas was

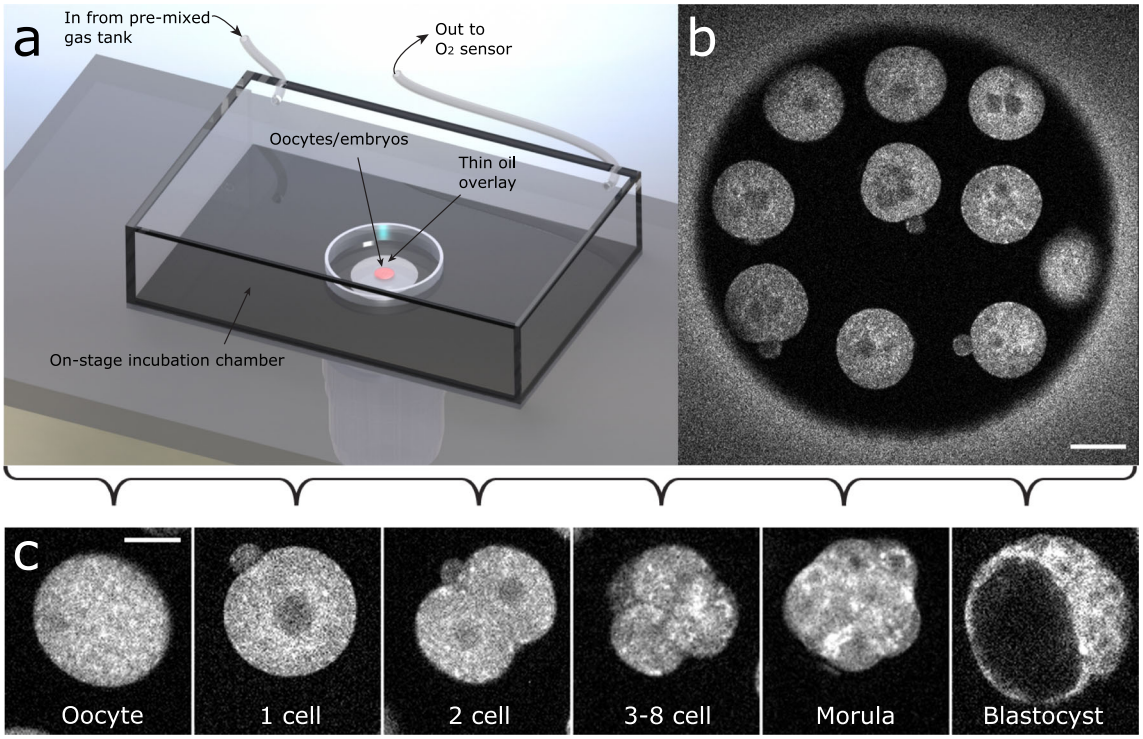


**Fig. 1** (**a**) A schematic of the on-stage, enclosed incubation system, which allows for exchange of pre-mixed gases with 0 or 5% O2. Oocytes and embryos were imaged continuously in a microwell dish with an oil overlay of fixed thickness to produce consistent O2 exchange rates with the media. (**b**) An NADH fluorescence intensity image of embryos at the 1-cell stage generated from a FLIM measurement. 50 μm scale bar. (**c**) Representative images of the six stages of development, for which oxygen deprivation experiments were performed. 50 μm scale bar

quickly displaced with 0% oxygen, 5% $CO_2$ gas, inducing an exponential exchange of oxygen tension in the media. After imaging for 90 min under 0% oxygen, 5% oxygen gas was then restored, and measurements were continued for 30 more min. A UV Flux 25% Oxygen Sensor (CO2meter.com) was used to confirm the oxygen concentration of gas coming out of the on-stage incubation chamber's output. In control experiments, samples were imaged for the same total amount of time (130 min.) but maintained at 5% oxygen throughout. Oxygen drop experiments were performed in triplicate. Following oxygen deprivation experiments, a subset of early stage 1-cell ($n$ = 8), 2-cell ($n$ = 11), and 4-cell ($n$ = 11) embryos were subsequently cultured out to the blastocyst stage as a quality control check. All 30/30 embryos developed to blastocyst.

## Data analyses

We used open source, supervised machine learning-driven segmentation software ilastik [30], to separate intracellular regions from background. The algorithm was trained on representative embryo NADH and FAD intensity images, wherein the user manually draws over the regions to specify their classification (e.g., mitochondrial, cytoplasmic, background) as described previously[27, 31]. For each embryo, photon arrival times from the intracellular region were binned into an arrival time histogram, and the histogram was fit to a bi-exponential decay model to account for engaged and non-engaged populations of fluorophore: $P(t) = A((1 - F)\exp(-t/\tau_1) + F\exp(-t/\tau_2)) + B$. Here, the two exponentials represent population of fluorophore (NADH or FAD) that is engaged or unengaged with enzyme, which have distinct fluorescence decay lifetimes. This fit yields the following quantitative parameters: intensity (sum of all photons registered in histogram), fraction of NADH or FAD engaged (F), short lifetime ($\tau_1$), and long lifetime ($\tau_2$) for both NADH and FAD images, as described previously [31]. Intensities are represented in custom units, calculated as total fluorescence photons detected within the intracellular region divided by the number of intracellular pixels, divided by the number of integrated scans of the field of view.

Triplicate data runs were first averaged separately and inspected to verify that triplicates for each group exhibited the same qualitative behaviors. Then, samples from all triplicates were combined and averaged together to obtain the final results. For individual embryo measurements, error bars were calculated from the error of the measurements, while for cohort averages, standard errors were calculated from inter-embryo parameter variation.

Statistical comparisons were performed for each FLIM parameter by comparing the initial mean value (under normal oxygen) to the mean value observed just before O2 restoration (maximum hypoxic). Significant changes were determined using paired *t* tests.

## Results

We cultured oocytes and embryos in an onstage-incubated chamber setup to facilitate oxygen exchange (Fig. 1a). Approximately ten oocytes or embryos were placed in a single well for measurements (Fig. 1b). The intensity images obtained from FLIM allow the morphology of oocytes and embryos to be clearly visualized, making the stage of the embryos straightforward to identify (Fig. 1c). The brighter areas in these images are regions with a higher density of mitochondria[31], as NADH and FAD are both highly enriched in mitochondria.

We first studied the impact of hypoxia on FLIM parameters in 1-cell embryos. Upon dropping oxygen, the 1-cell embryos *individually* exhibited large increases in NADH intensity, but these intensities reverted back to their original value upon restoration of 5% $O_2$ (Fig. 2a). In contrast, embryos cultured on stage in constant 5% $O_2$ exhibit no large change in intensity over a similar time period, demonstrating that the observed changes are indeed caused by the oxygen drop (Fig. 2b). To better characterize this response, we quantified changes in FLIM parameters for each embryo. This revealed that dropping oxygen leads to an increase in some parameters, such as NADH intensity and FAD fraction engaged, and a decrease in other parameters, such as FAD intensity and NAD fraction engaged (Fig, 2c, left). FLIM parameters remained constant in control embryos held at constant oxygen levels (Fig. 2C, right). Thus, hypoxia induces large changes in FLIM parameters that can be readily measured in individual 1-cell embryos. In 1-cell embryos we observed significant embryo-to-embryo variation in FLIM parameters, but all embryos exhibited similar shifts in parameters upon decreasing oxygen levels (Fig. 3a, blue and orange curves). Averaging the individual embryo response curves together allowed these trends to be more easily seen (Fig. 3a, black).

We next repeated the oxygen drop experiments for oocytes ($n$ = 26), 2-cell embryos ($n$ = 22), 3- to 8-cell embryos ($n$ = 37), morula stage embryos ($n$ = 20), and blastocyst stage embryos ($n$ = 15) (sample sizes represent individuals from all triplicates combined for each group). It has previously been shown that FLIM parameters change greatly over the course of embryo development [28, 31]. We therefore quantified the response to hypoxia at different stages by measuring the relative change in FLIM parameters induced by oxygen deprivation, which varied greatly with stage (Fig. 3b). When oxygen was restored after 90 min. of hypoxia, nearly all FLIM parameters showed apparent restoration towards their pre-hypoxic values. This argues that the metabolic changes due to short-term hypoxia may be largely reversible. Interestingly, we also observed that spindles disappeared in response to hypoxia, but reappeared once oxygen was restored and the cells proceed to divide (see *supplemental video*). This further suggests that the response to short-term hypoxia is reversible.

To more clearly visualize how the response to hypoxia varies across stages, we quantified the maximum response values (i.e., the values just before $O_2$ restoration) for each FLIM parameter at each embryo stage (Fig. 4). We observed a trend for many FLIM parameters that oocytes and earlier stage embryos show greater responses to hypoxia than later stage embryos. For example, under hypoxic conditions, NADH intensity of 1-cell embryos increased by 29.0 ± 3.6%, [$p = 3.8 \times 10^{-14}$], while at the blastocyst stage it changed by just −1.3 ± 7.9%, [$p = 0.95$]. However, some FLIM parameters deviate from this trend. Blastocyst NADH fraction engaged changed by −5.4 ± 2.8% [$p = 0.006$], and blastocyst FAD fraction engaged changed by 0.6 ± 0.4% [$p = 0.03$]. Those changes are almost as great as those observed at the 1-cell stage, where NADH fraction changed by −6.5 ± 1.2% [$p = 9.0 \times 10^{-11}$ ], and FAD fraction changed by 0.7 ± 0.4% [$p = 0.004$]. While the response to hypoxia varied between two stages, each stage showed highly significant changes in at least several FLIM parameters, indicating that oxygen plays an important role throughout development. NADH and FAD intensity shifts varied inversely to each other in all cases, with NADH increasing and FAD decreasing at low oxygen levels, except at the 3–8 cell and morula stages.

## Discussion

FLIM-based metabolic imaging is able to detect and quantify metabolic changes in response to oxygen deprivation. The metabolic response to hypoxia varies depending on the stage of preimplantation development at the time of oxygen deprivation. This phenomenon mirrors the known shift in metabolic function before and after the cleavage stage. The fact that almost all parameters returned to their baseline values after 5% $O_2$ suggests that these measurements reflect acute physiological changes, rather than more complex adaptations due to activation of signaling pathways. Finally, SHG imaging in supplemental video shows a spindle disappearing during hypoxic conditions, but then reappearing when 5% oxygen was restored, and the zygote continued to divide. These observations argue that brief periods of hypoxia cause short-term metabolic stress, but did not cause significant long-term damage or apoptosis until at least the blastocyst stage.

As a first-order observation, NADH and FAD intensity show similar response patterns, with major changes in early stages and smaller changes later in development. These

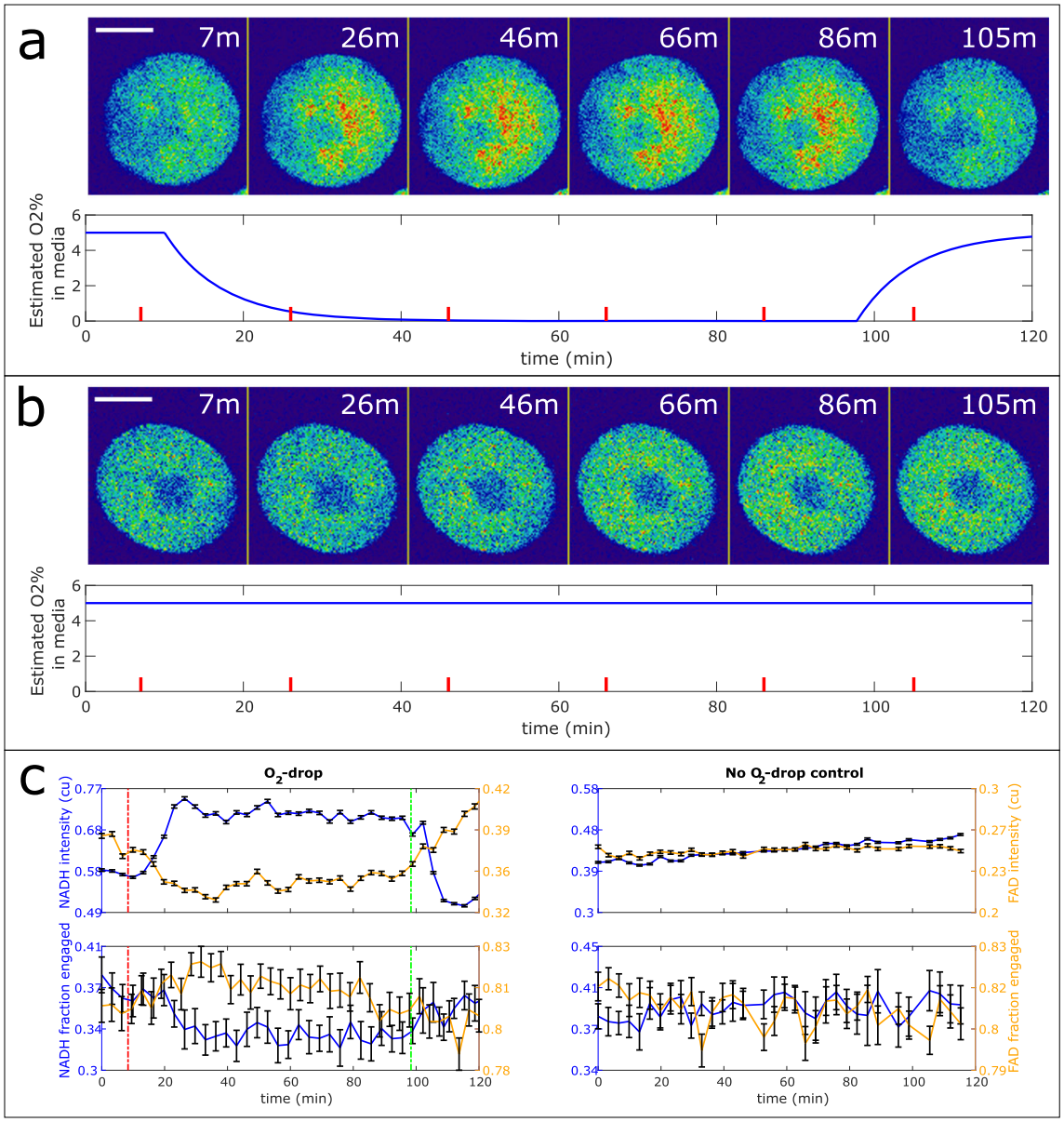


**Fig. 2** (**a**) FLIM NADH imaging of a 1-cell embryo under experimental hypoxic conditions. Estimated oxygen concentration percentage in media shown below the corresponding embryo images. NADH intensity (pseudocolored) increases during hypoxia, and recovers when 5% oxygen is restored. 50 μm scale bar. (**b**) 1-cell control embryo with no oxygen deprivation undergoes no significant change in brightness. 50 μm scale bar. (**c**) Corresponding quantitative metabolic parameters for individual embryos displayed in A (left plots) and B (right plots). Four (4) metabolic parameters displayed: NADH intensity and fraction engaged (blue) and FAD intensity and fraction engaged (orange). The oxygen deprivation samples show significant changes in all four (4) parameters in response to hypoxia. Here, the vertical dashed red line indicates the time when the oxygen drop was initiated, and the later green line indicates the oxygen restoration time. Control samples (at 5% oxygen throughout) show no significant metabolic change. Consistent data ranges displayed for comparison with standard error bars

molecules play central roles in mitochondria, where $NAD^+$ and FAD are reduced by the Krebs cycle to form NADH and $FADH_2$, which are in turn oxidized to provide electrons to the ETC. The redox shift we see in response to hypoxia at early stages, in which NADH intensity increases and FAD intensity decreases, is consistent with the expected changes resulting from the ETC shutting down in hypoxic conditions. Interestingly, these redox shifts are largely absent with blastocysts, even though blastocyst consumes significantly more oxygen than earlier stage embryos [32, 33]. NADH is also a key component in many metabolic reactions in the cytoplasm, including glycolysis. It is well-established that there is a shift in metabolism during pre-implantation development, in which later stage embryos preferentially utilize glucose over pyruvate and increasingly perform glycolysis [6, 7, 34]. Other metabolic processes may also be related to an increase in NADH, including the pentose phosphate pathway. It is possible that the FLIM signal from later

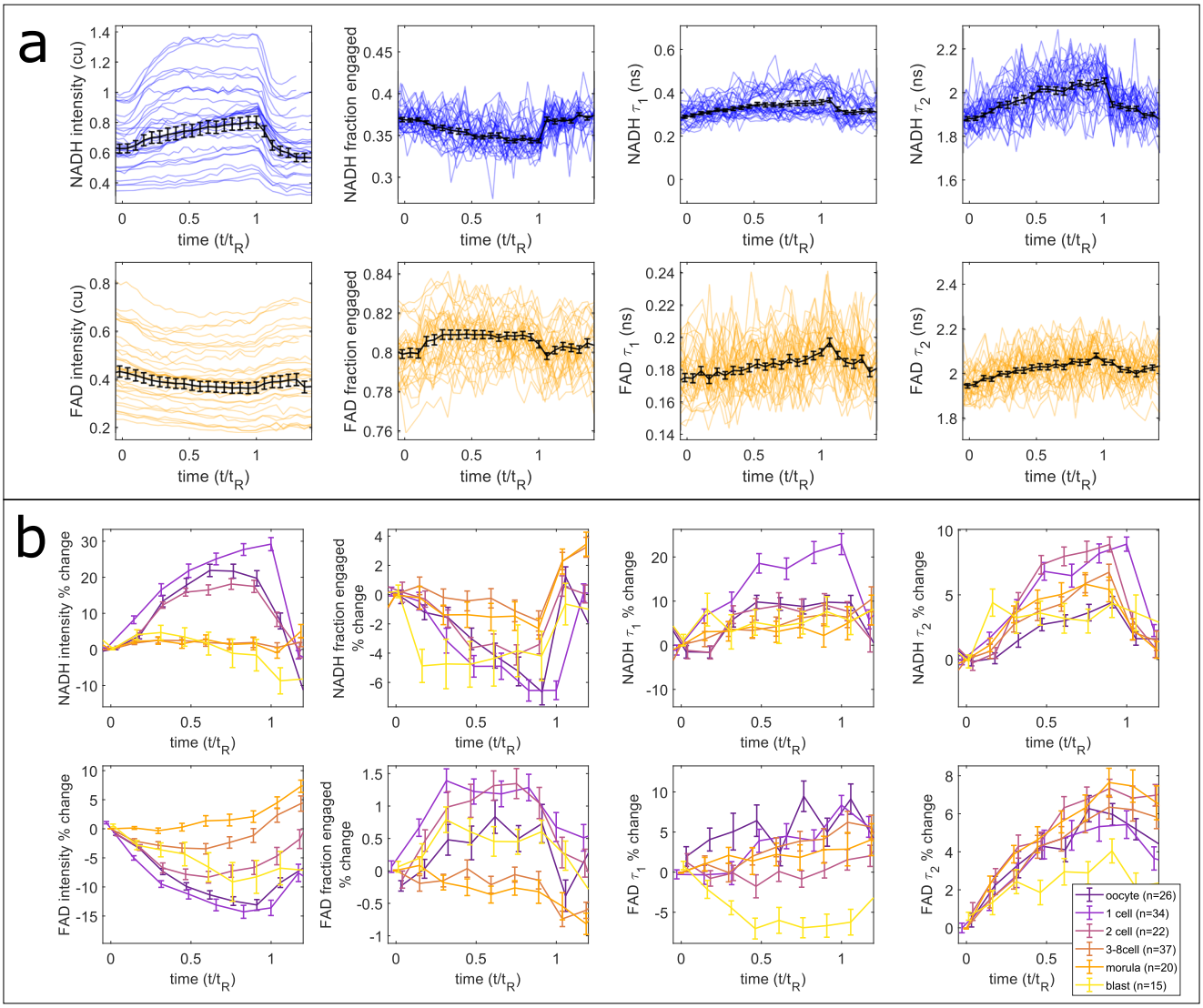


**Fig. 3** (**a**) Changes in absolute values of metabolic parameters in response to oxygen deprivation for embryos at the 1-cell stage. Time courses for individual embryos for all eight parameters shown using light traces. Each trace represents an individual embryo. Average metabolic parameter values are shown in black with standard error bars displayed. Time is represented in scaled units where oxygen drop begins at $t = 0$ and is restored at $t = 1$. (**b**) Average changes in metabolic response to oxygen deprivation of all embryos, arranged by stage of development. Standard error bars are displayed. Time is represented in scaled units where oxygen drop begins at $t = 0$ and is restored at $t = 1$

stage embryos is increasingly dominated by redox reactions in the cytoplasm instead of mitochondria, which would explain their relative insensitivity to hypoxia. Future work could test that possibility by performing higher magnification imaging to separately measure FLIM signals from mitochondria and cytoplasm and by studying how FLIM parameters respond when inhibiting different pathways at different stages.

The in vivo conditions during preimplantation development may provide insight into these shifts. These measured metabolic changes may reflect physiologic changes in the low oxygen environment just prior to implantation. At the cleavage stage, a mammalian embryo travels through the oviduct or fallopian tube, which has an estimated oxygen concentration of 5–7% [35]. By blastocyst stage, the embryo has entered the uterus, which is an even lower oxygen tension environment (approximately 2–3%) [35]. It has been postulated that blastocysts exhibit a Warburg effect, similar to cancer cells, i.e., they undergo anaerobic glycolysis even in the presence of oxygen [36, 37]. This adaptation may allow the blastocyst to withstand near-anoxic conditions at the time of implantation [38]. For example, at the blastocyst stage, there are documented shifts towards using the pentose phosphate pathway and gluconeogenesis [39–42].

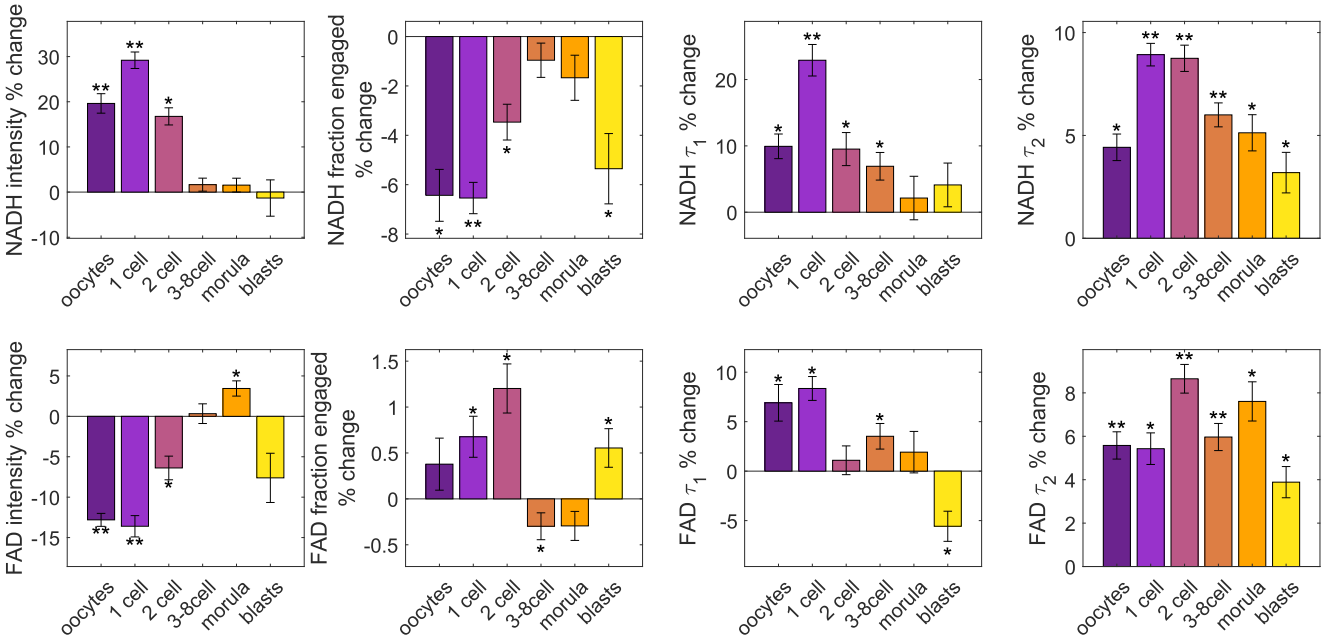


**Fig. 4** Bar plots show the maximum response values of all eight FLIM parameters for the following developmental preimplantation stages: unfertilized oocytes, 1 cell embryos, 2 cell embryos, 3-8 cell embryos, compaction or morula stage embryos and blastocysts. Bar plots projecting upwards from zero showed positive relative change and those projecting downwards showed negative change. Standard error bars are displayed, with asterisks indicating the following p-values: (*:$p < 0.05$) and (**:$p <$ 10-8)

In addition to the biologic insights provided by FLIM parameters in this study, these results further demonstrate potential for FLIM to be used as an embryo selection tool in a clinical setting. These data show that FLIM is able to precisely quantify differences in metabolism between individual embryos. Since the inception of human IVF, there has been a need to preferentially select the most viable embryo to transfer. Currently, the available options for embryo selection are using morphology, which is noninvasive but subjective, or using embryo biopsy with PGT (preimplantation genetic testing), which is objective but invasive [43]. Metabolic characteristics have already been shown to be useful in selecting viable embryos in several species, including humans [18–22]. The use of metabolic imaging is promising, as it would be objective yet non-invasive [31, 43]. Together with previous studies demonstrating the relative safety of the FLIM assay's illumination on mouse embryos [27, 31, 43], sperm DNA, and oxidative stress, clinical trials are now needed to directly evaluate the techniques clinical potential.

There are limitations to our study. Firstly, the reason for differences in FLIM results between embryos at similar stages needs to be addressed. Whether this is signal drift or true metabolic differences is crucial to this technology being used as a future clinical tool. Secondly, a mouse model may not be entirely generalizable to human gametes with associated clinical implications. Future studies on human embryos will be important to determine the metabolic response unique to human oocytes and embryos and to assess this technologies potential use as an embryo selection tool.

Our results have highlighted the intricate nature of metabolic response to hypoxic challenges at different stages of embryo development. The multiparametric FLIM data show that early cleavage and post-genome activation embryos exhibit different responses to the important environmental stress of hypoxia. Applications such as FLIM and other non-invasive platforms will allow us to better understand how eggs and embryos cope with metabolic stressors in relation to their stage of development. This technology could play a role in improving in vitro embryo culture conditions. Furthermore, FLIM may be a useful tool in embryo selection by noninvasively imaging human embryos in a clinical setting in the future.

**Availability of data and material** Available upon request

**Authors' contributions** EAS primarily contributed to study conception and design and data acquisition, analysis, and interpretation; drafted and critically revised the manuscript; and approved the final version for publication. TS contributed to study conception and design and data acquisition, analysis, and interpretation; drafted and critically revised the manuscript; and approved the final version for publication. DS contributed to study conception and design and data interpretation; critically revised the manuscript; and approved the final version for publication. MV contributed to design and data interpretation, and approved the final version for publication. DJN contributed to study conception, design and data interpretation, critically revised the manuscript, and approved the final version for publication.

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## Compliance with ethical standards

**Conflicts of interest/Competing interests** TS and DJN co-hold patent US20150346100A1 pending for metabolic imaging methods for assessment of oocytes and embryos and patent US20170039415A1 issued for nonlinear imaging systems and methods for assisted reproductive technologies.

**Code availability** Available upon request

## References

1. Wales RG, Brinster RL. The uptake of hexoses by pre-implantation mouse embryos in vitro. J Reprod Fertil. 1968;15:415–22.
2. Brinster RL. Studies on the development of mouse embryos in vitro. II. The effect of energy source. J Exp Zool. 1965;158:59–68.
3. Brinster RL. Studies on the development of mouse embryos in vitro. IV. Interaction of energy sources. J Reprod Fertil. 1965;10:227–40.
4. Biggers JD, Whittingham DG, Donahue RP. The pattern of energy metabolism in the mouse oöcyte and zygote. Proc Natl Acad Sci U S A. 1967;58:560–7.
5. Hewitson LC, Leese HJ. Energy metabolism of the trophectoderm and inner cell mass of the mouse blastocyst. J Exp Zool [Internet]. 1993;267:337–43 Available from: http://www.ncbi.nlm.nih.gov/pubmed/8228868.
6. Leese HJ, Barton AM. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. J Reprod Fertil. 1984;72:9–13.
7. Gardner DK, Leese HJ. Non-invasive measurement of nutrient uptake by single cultured pre-implantation mouse embryos. Hum Reprod. Oxford University Press. 1986;1:25–7.
8. Chason RJ, Csokmay J, Segars JH, DeCherney AH, Armant DR. Environmental and epigenetic effects upon preimplantation embryo metabolism and development. Trends Endocrinol Metab. 2011;412–420. https://doi.org/10.1016/j.tem.2011.05.005.
9. Takahashi M. Oxidative stress and redox regulation on in vitro development of mammalian embryos. J Reprod Dev. 2012:1–9. https://doi.org/10.1262/jrd.11-138n.
10. Van Blerkom J. Mitochondria as regulatory forces in oocytes, preimplantation embryos and stem cells. Reprod Biomed Online [Internet]. 2008;16:553–69 Available from: http://www.ncbi.nlm.nih.gov/pubmed/18413065.
11. Harvey AJ. Mitochondria in early development: Linking the microenvironment, metabolism and the epigenome. Reproduction BioScientifica Ltd. 2019:R159–79. https://doi.org/10.1530/REP-18-0431.
12. Dumollard R, Carroll J, Duchen MR, Campbell K, Swann K. Mitochondrial function and redox state in mammalian embryos. Semin Cell Dev Biol Elsevier Ltd. 2009:346–53. https://doi.org/10.1016/j.semcdb.2008.12.013.
13. Magnusson C, Einarsson B, Nilsson BO. Oxygen consumption by the mouse blastocyst at activation for implantation. Acta Physiol Scand. 1986;127:215–21.
14. Wale PL, Gardner DK. Oxygen Regulates Amino Acid Turnover and Carbohydrate Uptake During the Preimplantation Period of Mouse Embryo Development1. Biol Reprod. Oxford University Press (OUP). 2012;87. https://doi.org/10.1095/biolreprod.112.100552.
15. Swain JE. Controversies in ART: can the IVF laboratory influence preimplantation embryo aneuploidy? Reprod BioMed Online. Elsevier Ltd. 2019:599–607. https://doi.org/10.1016/j.rbmo.2019.06.009.
16. Duranthon V, Watson AJ, Lonergan P. Preimplantation embryo programming: Transcription epigenetics, and culture environment. Reproduction. 2008;135:141–50.
17. Zeng F, Baldwin DA, Schultz RM. Transcript profiling during preimplantation mouse development. Dev Biol. 2004. https://doi.org/10.1016/j.ydbio.2004.05.018.
18. Renard JP, Philippon A, Menezo Y. In-vitro uptake of glucose by bovine blastocysts. J Reprod Fertil. 1980;58:161–4.
19. Leese HJ. Non-invasive methods for assessing embryos. Hum Reprod. Oxford University Press. 1987;2:435–8.
20. Gardner DK, Leese HJ. Assessment of embryo viability prior to transfer by the non-invasive measurement of glucose uptake. J Exp Zool. 1987;242:103–5.
21. Lane M, Gardner DK. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. Hum Reprod [Internet]. 1996;11:1975–8 Available from: http://www.ncbi.nlm.nih.gov/pubmed/8921074.
22. Gardner DK, Wale PL, Collins R, Lane M. Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. Hum Reprod. Oxford University Press. 2011;26:1981–6.
23. Papkovsky DB, Dmitriev RI. Imaging of oxygen and hypoxia in cell and tissue samples. Cell Mol Life Sci [Internet]. 2018;75:2963–80 Available from: http://www.ncbi.nlm.nih.gov/pubmed/29761206.
24. Hink MA, Bisselin T, Visser AJWG. Imaging protein-protein interactions in living cells. Plant Mol Biol [Internet]. 2002;50:871–83 Available from: http://www.ncbi.nlm.nih.gov/pubmed/12516859.
25. Wang Y, Shyy JY, Chien S. Bioengineering in Cell and Tissue Research: Fluorescence Live-Cell Imaging: Principles and Applications in Mechanobiology. Proteins [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2008;10:1–38. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18647110
26. Van Munster EB, Gadella TWJ. Fluorescence Lifetime Imaging Microscopy (FLIM). Adv Biochem Eng Biotechnol. 2005:143–75. https://doi.org/10.1007/b102213.
27. Sanchez T, Wang T, Pedro MV, Zhang M, Esencan E, Sakkas D, et al. Metabolic imaging with the use of fluorescence lifetime imaging microscopy (FLIM) accurately detects mitochondrial dysfunction in mouse oocytes. Fertil Steril. 2018;110:1387–97.
28. Ma N, de Mochel NR, Pham PD, Yoo TY, Cho KWY, Digman MA. Label-free assessment of pre-implantation embryo quality by the Fluorescence Lifetime Imaging Microscopy (FLIM)-phasor approach. Sci Rep. Nature Publishing Group. 2019;9:1–13.
29. Ghukasyan V, Heikal A. Natural biomarkers for cellular metabolism: biology, techniques, and applications. 2014.
30. Berg S, Kutra D, Kroeger T, Straehle CN, Kausler BX, Haubold C, et al. ilastik: interactive machine learning for (bio) image analysis. Nat Methods. Nat Res Forum. 2019;16:1226–32.
31. Sanchez T, Venturas M, Aghvami SA, Yang X, Fraden S, Sakkas D, et al. Combined non-invasive metabolic and spindle imaging as potential tools for embryo and oocyte assessment. Hum Reprod [Internet]. 2019;34:2349–61 Available from: https://academic.oup.com/humrep/article/34/12/2349/5643744.
32. Houghton FD, Thompson JG, Kennedy CJ, Leese HJ. Oxygen consumption and energy metabolism of the early mouse embryo.

Mol Reprod Dev [Internet]. 1996 [cited 2020 Apr 9];44:476–85. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8844690
33. Mills RM, Brinster RL. Oxygen consumption of preimplantation mouse embryos. Exp Cell Res. Academic Press. 1967;47:337–44.
34. Leese HJ. History of oocyte and embryo metabolism. Reprod Fertil Dev. CSIRO. 2015:567–71. https://doi.org/10.1071/RD14278.
35. Ng KYB, Mingels R, Morgan H, Macklon N, Cheong Y. In vivo oxygen, temperature and pH dynamics in the female reproductive tract and their importance in human conception: A systematic review. Hum Reprod Update. Oxford University Press. 2018;24:15–34.
36. Redel BK, Brown AN, Spate LD, Whitworth KM, Green JA, Prather RS. Glycolysis in preimplantation development is partially controlled by the Warburg Effect. Mol Reprod Dev. 2012;79:262–71.
37. Krisher RL, Prather RS. A role for the Warburg effect in preimplantation embryo development: Metabolic modification to support rapid cell proliferation. Mol Reprod Dev. 2012;79:311–20.
38. Bagheri D, Kazemi P, Sarmadi F, Shamsara M, Hashemi E, Daliri Joupari M, et al. Low oxygen tension promotes invasive ability and embryo implantation rate. Reprod Biol. Elsevier Sp. z o.o. 2018;18:295–300.
39. O'Fallon JV, Wright RW. Quantitative determination of the pentose phosphate pathway in preimplantation mouse embryos1. Biol Reprod. Oxford University Press (OUP). 1986:34, 58–64. https://doi.org/10.1095/biolreprod34.1.58.
40. O'Fallon JV, Wright RW. Calculation of the pentose phosphate and Embden-Myerhoff pathways from a single incubation with [U-14C]- and [5-3H]glucose. Anal Biochem. 1987;162:33–8.
41. Gardner HG, Kaye PL. Characterization of glucose transport in preimplantation mouse embryos. Reprod Fertil Dev. 1995;7:41–50.
42. Comizzoli P, Urner F, Sakkas D, Renard JP. Up-regulation of glucose metabolism during male pronucleus formation determines the early onset of the S phase in bovine zygotes1. Biol Reprod. Oxford University Press (OUP). 2003;68:1934–40.
43. Sanchez T, Seidler EA, Gardner DK, Needleman D, Sakkas D. Will non-invasive methods surpass invasive for assessing gametes and embryos? Fertil Steril. Elsevier Inc. 2017:730–7. https://doi.org/10.1016/j.fertnstert.2017.10.004.
44. Dumollard J, Carroll MR, Duchen K, Campbell K, Swann K. Mitochondrial function and redox state in mammalian embryos. Semin Cell Dev Biol 2009;20(3):346–53.