Title: Non-invasive, label-free optical analysis to detect aneuploidy within the inner cell mass of the preimplantation embryo

Running title: Label-free optical analysis for embryo aneuploidy

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28 **Abstract**

29 **Study question:** Can label-free, non-invasive optical imaging by hyperspectral microscopy
30 discern between euploid and aneuploid cells within the inner cell mass of the mouse
31 preimplantation embryo?

32 **Summary answer:** Hyperspectral microscopy shows a variance in metabolic activity which
33 enables discrimination between euploid and aneuploid cells.

34 **What is known already:** Euploid/aneuploid mosaicism affects up to 17.3% of human
35 blastocyst embryos with trophectoderm biopsy or spent media currently utilised to diagnose
36 aneuploidy and mosaicism in clinical in vitro fertilisation. Based on their design, these
37 approaches will fail to diagnose the presence or proportion of aneuploid cells within the fetal
38 lineage (inner cell mass (ICM)) of some blastocyst embryos.

39 **Study design, size, duration:** The impact of aneuploidy on cellular metabolism of primary
40 human fibroblast cells and mouse embryos was assessed by a fluorescence microscope
41 adapted for imaging with multiple spectral channels (hyperspectral imaging). Primary human
42 fibroblast cells with known ploidy were subjected to hyperspectral imaging to record native
43 cell fluorescence (euploid n= 467; aneuploid n= 969). For mouse embryos, 50-70 individual
44 euploid and aneuploid blastomeres (8-cell stage embryo) and chimeric blastocysts (40-50 per
45 group: euploid; aneuploid; or 1:1 and 1:3 ratio of euploid:aneuploid) were utilised for
46 hyperspectral imaging.

47 **Participants/materials, setting, methods:** Two models were employed: (i) Primary human
48 fibroblasts with known karyotype and (ii) a mouse model of embryo aneuploidy where mouse
49 embryos were treated with reversine, a reversible spindle assembly checkpoint inhibitor,
50 during the 4- to 8-cell division. Individual blastomeres were dissociated from reversine
51 treated (aneuploid) and control (euploid) 8-cell embryos and either imaged directly or used to
52 generate chimeric blastocysts with differing ratios of euploid:aneuploid cells. Individual
53 blastomeres and embryos were subjected to hyperspectral imaging. Changes in cellular
54 metabolism were determined by quantification of metabolic cofactors (inferred from their

autofluorescence signature): reduced nicotinamide adenine dinucleotide (NAD(P)H), flavins with the subsequent calculation of the optical redox ratio (ORR: Flavins/[NAD(P)H + Flavins]). Mathematical algorithms were applied to extract features from the autofluorescence signals of each cell/blastomere/inner cell mass to discriminate between euploid and aneuploid.

Main results and the role of chance: An increase in the relative abundance of NAD(P)H with a decrease in flavins led to a significant reduction in the ORR for aneuploid cells in both primary human fibroblasts and individual mouse blastomeres ($P < 0.05$). Mathematical algorithms were able to achieve good separation between (i) euploid and aneuploid primary human fibroblast cells, (ii) euploid and aneuploid mouse blastomeres cells and (iii) euploid and aneuploid chimeric blastocysts and (iv) 1:1 and 1:3 chimeric blastocysts. The accuracy of these separations was supported by receiver operating characteristic curves with areas under the curve of 0.85, 0.99, 0.87 and 0.88, respectively. We believe that the role of chance is low as multiple cellular models (human somatic cells and mouse embryos) demonstrated a consistent shift in cellular metabolism in response to aneuploidy as well as the robust capacity of mathematical features to separate euploid and aneuploid cells in a statistically significant manner.

Limitations, reasons for caution: There would be added value in determining the degree of embryo mosaicism by sequencing the inner cell mass (ICM) of individual blastocysts to correlate with metabolic profile and level of discrimination achieved using the mathematical features approach.

Wider implications of the findings: Hyperspectral imaging was able to discriminate between euploid and aneuploid human fibroblasts and mouse embryos. This may lead to the development of an accurate and non-invasive optical approach to assess mosaicism within the ICM of human embryos in the absence of fluorescent tags.

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84 **Key words:** hyperspectral microscopy/ pre-implantation/ mosaicism/ aneuploidy/

85 autofluorescence/ NAD(P)H/ Flavins/ embryo assessment/ non-invasive/ cellular metabolism/

86 optical microscopy

1. Introduction

During the early days of life, errors occur during mitotic cell division resulting in the emergence of aneuploidy: a deviation from the expected 46 chromosomes. Mitotic aneuploidy during preimplantation embryo development can lead to mosaic blastocyst embryos, containing both euploid cells (expected number of chromosomes) and aneuploid cells. The incidence of mosaicism in the blastocyst is as high as 30-40% (Fragouli, *et al.*, 2011, Huang, *et al.*, 2019, Northrop, *et al.*, 2010), with euploid/aneuploid mosaicism reported to vary from 2.0 to 17.3% (Capalbo, *et al.*, 2014, Fragouli, *et al.*, 2011, Huang, *et al.*, 2019, Johnson, *et al.*, 2010, Northrop, *et al.*, 2010). Animal studies show that while a lower proportion of aneuploid cells in the embryo is tolerated, a higher proportion of such cells leads to an increased incidence of pregnancy loss (Bolton, *et al.*, 2016). Thus, the determination of aneuploid cell proportions would likely aid in stratification of embryo quality and improve clinical in vitro fertilisation (IVF) success. To this end, genetic testing of preimplantation human embryos is widely employed albeit amongst considerable debate over the accuracy and safety of these clinical tools.

Preimplantation genetic testing for aneuploidy (PGT-A) is the most widely used method to detect aneuploidies in clinical IVF. This involves an invasive biopsy of trophoctoderm cells followed by sequencing. However due to the random nature of mosaicism, this biopsy will not provide a measure of the proportion of aneuploid cells in the ICM (fetal cells) or the remainder of the TE (placental cells) in some embryos (Gleicher, *et al.*, 2017). This is because aneuploid cells can be restricted to the TE, while the ICM is chromosomally normal (and vice versa), resulting in false positives (or negatives)(Vera-Rodriguez, *et al.*, 2017). This can lead to 1) erroneous disposal of false-positive embryos that would have otherwise resulted in a healthy offspring or 2) transfer of false-negative embryos, which may result in implantation failure, pregnancy loss or foetal abnormality. Further, transfer of biopsied embryos leads to a 3-fold increased risk of preeclampsia: a pregnancy complication with

elevated risk of morbidity and mortality for mother and fetus and a life-time increased risk of cardiovascular disease (Mastenbroek, *et al.*, 2011).

More recently, an alternative method for preimplantation genetic testing has emerged using cell-free DNA (cfDNA) released by embryos into the culture media (Rubio, *et al.*, 2019, Vera-Rodriguez, *et al.*, 2018). This approach, termed non-invasive preimplantation genetic testing for aneuploidy (niPGT-A), has shown high concordance with TE biopsy (Feichtinger, *et al.*, 2017, Huang, *et al.*, 2019). However, other publications report highly variable results which is thought to arise from differences in culture method, level of embryo mosaicism (Xu, *et al.*, 2016), and potential contamination of maternal DNA in the spent medium (Vera-Rodriguez, *et al.*, 2018). Importantly, this technique assumes that cfDNA originates equally from the ICM and TE (Kuznyetsov, *et al.*, 2020), which has not yet been proven. Therefore, it is probable that this approach will fail in some embryos to detect aneuploidy within the ICM (false negative) or mis-diagnose genetically normal ICM (false positive) (Gleicher, *et al.*, 2019). Currently, it appears that accurate detection of mosaicism within the divergent lineages of the blastocyst embryo can only be achieved through destructive dissection of the embryo (Gleicher, *et al.*, 2017, Taylor, *et al.*, 2016).

Aneuploidy results in altered cell metabolism in primary human fibroblasts and cancer cells (Newman, *et al.*, 2019, Sheltzer, 2013, Warburg, 1956), yeast (Sheltzer, *et al.*, 2011, Torres, *et al.*, 2007), and human embryos (Fragouli, *et al.*, 2015, Picton, *et al.*, 2010). As metabolites and co-enzymes of metabolic pathways are naturally fluorescent, including nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADPH) and flavins, there is potential to exploit this fluorescence as a non-invasive indicator of aneuploidy. Importantly, these endogenous fluorophores can be directly distinguished by their excitation and emission profiles without the need for exogenous fluorescence labels (Campbell, *et al.*, 2019). Both NAD(P)H and flavins are increasingly used to reflect cellular metabolism in oocytes and early embryos (Aparicio, *et al.*, 2013, Dumollard, 2004, Sugimura, *et al.*, 2014, Sutton-McDowall, *et al.*, 2016, Thompson, *et al.*, 2016).

Hyperspectral microscopy is a label-free approach used to non-invasively identify native endogenous fluorophores within a cell (Mahbub, *et al.*, 2017). This technique excites endogenous fluorophores with a wide range of excitation and emission wavelengths as opposed to using single (or dual) excitation wavelength(s). Notably, hyperspectral imaging has demonstrated the capability to discriminate between different types of cancer cells (Campbell, *et al.*, 2019, Gosnell, *et al.*, 2016b), bovine embryos with good and poor developmental potential (Santos Monteiro, *et al.*, 2020, Sutton-McDowall, *et al.*, 2017), as well as quantifying the metabolic content of oocytes from young and aged mice (Bertoldo, *et al.*, 2020).

In the present study, we determine whether hyperspectral imaging is able to discriminate between euploid and aneuploid primary human fibroblasts and mouse embryos. Aneuploid mouse embryos were generated using reversine, a spindle assembly inhibitor, and used to create mosaic blastocysts with different ratios of euploid/aneuploid cells (Bolton, *et al.*, 2016). As current clinical tools are limited in their capacity to detect aneuploidy within the foetal cell lineage of the blastocyst, the aim of this study was to determine whether hyperspectral imaging coupled with mathematical feature extractions was able to discriminate between the ICM of embryos with varying proportions of aneuploid cells. Further, we assessed the safety of this approach using post-transfer viability.

2. Materials and methods

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

2.1 Animals and ethics

Female (21-23 days) and male (6-8 weeks old) CBA x C57BL/6 first filial (F1) generation (CBAF1) mice were obtained from Laboratory Animal Services (LAS; University of Adelaide, SA, Australia) and maintained on a 12 h light:12 h dark cycle with rodent chow

and water provided *ad libitum*. All experiments were approved by the University of Adelaide's Animal Ethics Committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2 Media for gamete and embryo handling and culture

All gamete and embryo culture took place in media under paraffin oil at 37 °C in a humidified atmosphere of 5 % O₂, 6 % CO₂ with the balance as N₂. All media were pre-equilibrated at least 4 h prior to use. All handling procedures were carried out at 37 °C. Mouse reproductive tissues were collected in Research Wash Medium (ART Lab Solutions, SA, Australia) supplemented with 4 mg/ml of low fatty acid bovine albumin (BSA; MpBio). Research Fertilisation Medium and Cleave Medium (ART Lab Solutions, SA, Australia) were supplemented with 4 mg/ml low fatty acid BSA and used for *in vitro* fertilisation (IVF) and embryo culture, respectively. Embryo vitrification and warming were carried out in Handling Medium (HM; alpha Minimal Essential Medium; αMEM; Gibco by Life Technologies, CA, USA) containing: 10 mM HEPES-buffered αMEM medium supplemented with 6 mM NaHCO₃, 50 mg/L gentamicin sulfate, 5.56 mM glucose and 2 mM glutamax. Before use, HM was supplemented with 5 mg/ml low fatty acid BSA.

2.3 Isolation of COCs and *in vitro* fertilisation

Female mice were administered intraperitoneally (i.p.) with 5 IU equine chorionic gonadotrophin (eCG; Braeside, VIC, Australia), followed by 5 IU (I.P.) human chorionic gonadotrophin (hCG; Kilsyth, VIC, Australia) 48 h later. Mice were culled by cervical dislocation 14 h post-hCG administration and oviducts removed. Ovulated COCs were harvested by gently puncturing the ampulla using a 29-gauge needle. Male mice with proven fertility were culled by cervical dislocation one hour prior to IVF. Spermatozoa were released from the vas deferens and the caudal region of the epididymis by blunt dissection in Research

Fertilisation Medium and allowed to capacitate for 1 h at 37 °C, 5 % O₂, 6 % CO₂ with the balance as N₂. Mature COCs were then co-cultured with 10 µl of capacitated spermatozoa for 4 h at 37 °C in an atmosphere of 5 % O₂, 6 % CO₂ with the balance as N₂. Resulting presumptive zygotes were transferred into Research Cleave Medium (in groups of 10; 2 µl per embryo) and cultured at 37 °C in an atmosphere of 5 % O₂, 6 % CO₂ with the balance as N₂. Fertilisation rate was scored 24 h later, with embryos allowed to develop to the 4-cell or blastocyst stage.

2.4 Euploid/ Aneuploid/ Chimeric Embryo Generation

Generation of chimeric embryos was performed as described in (Bolton, *et al.*, 2016), using reversine, a biomolecule inhibitor of monopolar spindle 1-like 1 kinase, which is crucial for the normal functioning of the spindle assembly checkpoint (SAC) (Santaguida, *et al.*, 2010). Briefly, during the 4- to 8-cell division, embryos were cultured in the absence (euploid) or presence of 0.5 µM reversine (aneuploid) diluted in Research Cleave Medium. A group of non-treated 8-cell embryos were set aside to culture directly to the blastocyst stage to investigate whether the presence of the zona pellucida altered the autofluorescence profile of embryos. The remaining 8-cell embryos were incubated in 0.05 % pronase diluted in MOPS to remove the zona pellucida. Individual blastomeres were then separated in calcium and magnesium free medium (Research Wash Medium without calcium and magnesium) using a STRIPPER Micropipette Handle (CooperSurgical, Trumbull, Connecticut) fitted with a 30-35 µm biopsy pipette (TPC micropipettes; CooperSurgical, Trumbull, Connecticut). Following separation, individual blastomeres were washed thoroughly in Research Wash Medium and either subjected to imaging using the hyperspectral microscope or reaggregated in 2% phytohaemagglutinin M (ThermoFisher Scientific, Waltham, Massachusetts) in Research Cleave Medium to generate chimeric blastocysts: euploid; aneuploid; 1:1 or 1:3 ratio of euploid:aneuploid. Agglutinated chimeric 8-cell embryos were then cultured overnight at 37 °C, 5 % O₂, 6 % CO₂ with the balance as N₂. Chimeric embryos were only included if there was aggregation of all 8 blastomeres and development to the morula stage. This is to ensure

218 imaging was only performed on blastocysts that resulted from the agglutination of all 8 cells,
219 and thus reflective of their allocated ratio of euploid:aneuploid cells.

220 For hyperspectral imaging of individual blastomeres, 8-cell embryos were vitrified and
221 shipped from the University of Adelaide (UoA) to the University of New South Wales (UNSW)
222 in a dry shipper to maintain cryogenic temperature. For the imaging of individual blastomeres,
223 8-cell embryos were warmed and individual blastomeres separated using the biopsy
224 micropipette as described above. For hyperspectral imaging of chimeric embryos, chimeric
225 morulas were created at UoA as described above, subjected to vitrification, and shipped in a
226 dry shipper to UNSW. Chimeric morulas were then subjected to embryo warming and allowed
227 to develop to the blastocysts stage prior to hyperspectral imaging.

228

229 *2.5 Embryo Vitrification and Warming*

230 We utilised the CryoLogic vitrification method (CVM) for the vitrification of 8-cell
231 and morula embryos. A NUNC four-well dish (ThermoFisher Scientific, Waltham,
232 Massachusetts) was set up with 600 µl of HM, equilibration solution (ES; HM supplemented
233 with 10 % each ethylene glycol (EG) and DMSO), and vitrification solution (VS; HM
234 supplemented with 16 % each for EG and DMSO and 0.5 M sucrose). After pre-warming to
235 37 °C, 8-cell or morula embryos were washed twice in HM, followed by transfer into ES for 3
236 min. Embryos were then transferred into a VS drop for 30-45 s before loaded onto a Fibreplug
237 (Cryologic,Pty. Ltd, Victoria) and immediately vitrified using the CVM system, followed by
238 storage in a straw in a liquid nitrogen tank.

239 For embryo warming, HM supplemented with decreasing concentrations of sucrose
240 (0.3M, 0.25M and 0.15M) were prewarmed to 37 °C. Storage straws containing 8-cell or
241 morula embryos were kept immersed in liquid nitrogen prior to use. The Fibreplug was
242 removed from the straw and quickly submerged in HM containing 0.3M sucrose for 30 s,
243 followed by transfer to decreasing concentrations of sucrose. Prior to imaging, 8-cell embryos

were recovered in Research Cleave Medium for 2 h then dissociated into individual blastomeres as described above. Chimeric embryos at the morula stage were transferred to Research Cleave Medium and cultured overnight at 37 °C, 5 % O₂, 6 % CO₂ with the balance as N₂ until they reached the blastocysts stage. Survival of the warmed embryos was assessed morphologically, based on the presence of inner cell mass and blastocoel cavity. Blastocysts were then subjected to hyperspectral imaging.

2.6 Tissue Culture of Primary Human Fibroblasts

Primary human fibroblasts (Coriell Institute for Medical Research, USA) with known karyotypes (46, XY (GMO0970); 46, XX (GMO3525); triploid (GM01672); trisomy: 13 (GM02948), 18 (GM01359), 21 (AG06922), XXX (GM04626), and XXY (GM03184)) were cultured in MEM-E (Minimum Essential Medium Eagle with Earle's salts, 2.2 g/L sodium bicarbonate and, supplemented with 2 mM L-glutamine (Gibco), 1% MEM Non-Essential Amino Acids Solution (Gibco, ThermoFisher Scientific, Waltham, Massachusetts), 100 U/ml penicillin-streptomycin, and 10% fetal calf serum (FCS; Gibco). Cell cultures were maintained in 5 % CO₂ in air at 37°C. Using a 35 mm glass bottom dish (Ibidi, Martinsried, Planegg, Germany), 1 x 10⁵ cells were plated and cultured overnight. Prior to imaging, cells were washed twice with PBS without calcium and magnesium. Cells were imaged in Hank's balanced salt solution (Gibco, ThermoFisher Scientific, Waltham, Massachusetts).

2.7 Hyperspectral and differential interference contrast (DIC) Imaging

In this work, the hyperspectral microscopy system was built by adapting a standard fluorescence microscope (Olympus iX83™) fitted with a 60x silicon U12™ series objective to capture and characterised fluorescence of native endogenous fluorophores found within cells (Gosnell, *et al.*, 2016a, Gosnell, *et al.*, 2016b). A hyperspectral excitation lamp (Quantative™, Australia) comprising multiple low-powered light-emitting diodes (LEDs) with selected bands of excitation wavelengths produced by the LEDs (centred at 334 nm, 365 nm, 385 nm, 395 nm,

405 nm, 415 nm, 425 nm, 435 nm, 455 nm, 475 nm, and 495 nm, each about 10 nm wide) were used to excite cellular autofluorescence. The subsequent emission, through the use of three epifluorescence filter cubes with the emission bands centred at 447 nm (60 nm wide), 587 nm (35 nm wide), and 700 nm long pass, allowed us to record the autofluorescence signal of cell samples in a number of defined narrowbands (± 5 nm) to generate specific and defined spectral channels, covering a broad excitation (345 nm-505 nm) and emission (414 nm-675 nm) wavelength ranges (see Table I for details of spectral channels). In total, 67 specific spectral channels were available to measure single photon-excited emission, or autofluorescence signals of the biological sample. Images were captured by an electron multiplying CCD (Nuvu™ 1024, Canada) operated below -80 °C to minimise sensor-induced noise in the image data. The sensor is made out of 1024×1024 (13 μm × 13 μm) pixels. We used image acquisition times of up to 5 seconds per channel, with multiple averaging (typically 3-5 times) to optimise image quality and minimise any potential photodamage to the cells in each channel. Each data set was supplemented with a brightfield image of the sample which was used as a broad reference.

In this study, we also utilised an additional hyperspectral microscopy system (UoA) to investigate light-induced damage (photodamage) on embryo development. This hyperspectral system was built with a similar concept by adapting to a standard epifluorescence microscope (Nikon Eclipse TiE) and fitted with a multi-LED light source (Prizmatix Ltd, Givat-Shmuel, Israel). These low power LEDs provide 40 spectral channels: 21 excitation wavelength ranges and three emission wavelength filters, covering excitation wavelengths from 340 to 664 nm and emission wavelengths from 440 – 715 nm (see Table II for details of spectral channels). Images were captured by a digital camera C1140, OCRA Flash 4.0 (Hamamatsu) using all 40 spectral channels.

For imaging, blastomeres, morula embryos and chimeric blastocysts were transferred onto a glass bottomed confocal dish (Ibidi, Martinsried, Planegg, Germany) containing prewarmed Research Wash Medium overlaid with paraffin oil. Hyperspectral images were

taken by adjusting the input light beam to specifically focus on the equatorial plane of blastomeres and morula embryos (i.e. widest diameter) and the ICM of individual blastocysts.

2.8 Hyperspectral Data Analysis

The analysis of hyperspectral image data in this study included image preparation, classification and unmixing of individual fluorophores. Firstly, image preparation was carried out to remove image artefacts, such as background fluorescence, Poisson's noise, dead or saturated pixels, and illumination curvature across the field of view, as described in detail in previous work (Habibalahi, *et al.*, 2019, Mahbub, *et al.*, 2017, Rehman, *et al.*, 2017). At the beginning of each experiment, two calibration images were captured using the hyperspectral system: a "background" reference image of a culture dish with medium only, and another with calibration fluid only. The "background" reference image was included and subtracted from all images with cells to remove any potential, unavoidable background signals generated from the glass-bottomed dish or potential fluorescence contamination of microscope optics, which may form an additional fluorescence source to all spectral images. The microscope system was calibrated with a mixture of 30 μ M NADH and 18 μ M riboflavins whose spectrum spans across all our spectral channels. The excitation and emission spectra of this calibration fluid was measured using the fluorimeter (FluoroMax Plus-c; Horiba, Japan) and imaged on the hyperspectral microscope across all spectral channels. The calibration images were then used to correct the same spectrum measured with the hyperspectral system to be able to assign the unmixed fluorophores as well as to flatten the uneven illumination of fluorescence images generated by using our custom-made GUI software. Next, samples were manually segmented by using the DIC image taken concurrently with the hyperspectral images, creating a region of interest (ROI) in preparation for the unmixing process. For fibroblasts and individual blastomeres the ROI was drawn around the cell membrane that was clearly visible on the DIC image. For blastocysts, the ROI was manually drawn around the ICM by an experienced embryologist, avoiding the apical cells where TE might contaminate the signal.

Secondly, following image segmentation with generated region of interest (ROI) for each single channel image of each sample, the autofluorescence data were then subjected to feature analysis. The mathematical algorithms used here employed various transformations to capture various aspects of cell spectra and patterns in the cell images such as average intensity per cell of autofluorescent signals in each channel, the ratios of such average channel intensities for each pair of channels (see Table III), various types of spatial, frequency, spectral, geometric, morphological and statistical information (Gosnell, *et al.*, 2016b); altogether approximately 33,000 cellular features were identified and used in the analysis. Next, depending on the selection of cell/embryo groups under consideration, indicative features which passed an ANOVA test ($P < 0.005$) were ranked according to their significance for classification into the specific groups, correlated features were removed, and the highest ranked uncorrelated features were selected as described previously (Gosnell, *et al.*, 2016a), yielding a different feature set for each different group comparison. A total of 10 features (see Table III for the list of features) with the highest ranking were selected for the classification of human cells (refer Figure 2) and 7 features were used for mouse individual blastomeres (refer Figure 4 and 5; D, and E) and blastocysts (refer Figure 6; D and E). These selected feature sets generated optimal separation between the groups under consideration. Further, these selected features for human cells, mouse blastomeres and blastocysts were projected onto a two-dimensional (2D) space created by linear discrimination analysis (LDA) (Jombart, *et al.*, 2010). This procedure maximises the distance between the two distinct groups of cells (euploid and aneuploid) across all cell types while minimizing the variance within each group (Jombart, *et al.*, 2010). The 2D space is spanned by two canonical variables that are linear combination of selected features described above (10 features for human cells; 7 features for mouse blastomeres and blastocysts) (Naganathan, *et al.*, 2008). Following this, a receiver operating characteristics (ROC) curve was generated and the area under the curve (AUC) was calculated to evaluate the accuracy of the separation between groups across cell types (Hand, *et al.*, 2001).

Lastly, unmixing is the process where the spectral characteristic of the cells related to identified molecular fluorophores is extracted from the total autofluorescence signal. This is verified by comparison with known characteristics of the fluorophores at typical cellular concentrations and their relative abundance level (percentage of total fluorophore content) is calculated on a pixel by pixel basis. In this study, a linear mixing model (LMM) was adapted to perform the unmixing (Gosnell, *et al.*, 2016a, Mahbub, *et al.*, 2017). The model assumes that the fluorescent signal of each pixel is a linear combination of the endmember component spectra with their linear coefficients (weights) corresponding to the concentration of the molecules responsible for these component spectra. An unsupervised unmixing algorithm, Robust Dependent Component Analysis (RoDECA) (Mahbub, *et al.*, 2017) was then employed to detect the native fluorophores and calculate their relative abundance in the hyperspectral dataset across cell types. The accuracy of RoDECA in separating and quantifying the spectral signals of specific fluorophores from the complex noise in the cellular environment was established previously (Mahbub, *et al.*, 2019, Mahbub, *et al.*, 2017). Amongst the identified fluorophores across all cell types, metabolic co-factors - NAD(P)H and flavins were able to be identified (data not shown) and therefore were used for subsequent analysis for metabolic activity. The optical redox ratio was calculated and defined as the intensity of flavins divided by the sum of intensity of NAD(P)H and flavins which reflects the activity of the mitochondrial electron transport chain, and therefore of cellular metabolism (Kolenc, *et al.*, 2019, Yong, *et al.*, 2019)

2.9 Assessment of photodamage following hyperspectral imaging

Zona-enclosed embryos were generated by IVF and cultured as described above. Embryos at the morula stage of development were exposed to light in a hyperspectral microscope under identical conditions as during imaging at UoA (Table II). Non-imaged embryos were included and subjected to identical handling procedures as the imaged embryos

(i.e. time out of the incubator). Both groups of embryos were cultured for a further 24 h when blastocyst development was scored, and embryos were assessed for DNA damage.

Imaged and non-imaged embryos were stained for γ H2AX to quantify for DNA damage as previously described (Brown, *et al.*, 2018). Briefly, blastocysts were fixed in 4% paraformaldehyde (PFA) diluted in PBS for 30 min at room temperature. Following fixation, embryos were washed with PBV (PBS containing 0.3 mg/ml of polyvinyl alcohol) and permeabilized for 30 mins in 0.25 % v/v Triton-X100 in PBS. Embryos were then blocked with 10 % goat serum (Jackson Immuno, Philadelphia, Pennsylvania) diluted in PBV for 1 h. After blocking, embryos were incubated overnight at room temperature in the dark with anti- γ H2AX primary antibody (Cell Signalling Technology, Danvers, MA) at 1:200 dilution with 10% goat serum in PBV. A negative control was included where embryos were incubated in the absence of the primary antibody. On the following day, embryos were washed 3 times in PBV before incubation for 2 h at room temperature in the dark with anti-rabbit Alexa Fluor 594-conjugated secondary antibody (Life Technologies, Carlsbad, CA) at 1:500 dilution with 10 % goat serum in PBV. Embryos were then counterstained with 3 mM of 4',6-diamidino-2-phenylindole (DAPI). Lastly, embryos were washed 3 times in PBV and transferred onto a glass microscope slide with DAKO mounting medium (Dako Inc., Carpinteria, California) and enclosed with a coverslip using a spacer (ThermoFisher Scientific, Waltham, Massachusetts). Embryos were imaged on an Olympus FV3000 Confocal Laser Scanning microscope (Olympus, Tokyo, Japan). Embryos were excited at laser 405 nm (Emission Detection Wavelength: 430 nm to 470 nm) for DAPI and excited at laser 594 nm (Emission Detection Wavelength: 610 nm to 710 nm) for γ H2AX. Image acquisition occurred at 60x magnification with immersion oil. A z-stack was captured for each embryo with maximum projection created and fluorescence intensity measured using Image J software (National Institute of Health) (Sutton-McDowall, *et al.*, 2015, Tan, *et al.*, 2016). Instrument settings were kept constant across all experimental replicates.

Embryo transfer experiments were conducted following hyperspectral imaging on the microscope at UNSW (Table I). Blastocyst embryos were transferred into the uterine horn of pseudo-pregnant Swiss female mice 2.5 days post-coitum with vasectomised males. On the day of transfer, blastocysts were warmed as described above. Embryo warming was performed 2 hours prior to embryo transfer to allow sufficient time for recuperation from potential heat-shock induced following warming. Post-warming survival of embryo was about 80-85 % for imaged and non-imaged groups. Embryo transfer was performed under anaesthesia with isoflurane. Eight morphologically normal, expanded blastocysts were transferred to each uterine horn (16 embryos transferred per mouse). Imaged and non-imaged embryos were transferred to different recipients. Number of pups from each female recipient was recorded on delivery and monitored for downstream offspring health. At post-natal day 21, offspring were weaned, weighed and assessed for gross facial abnormalities.

2.10 Statistical Analysis

All hyperspectral data analyses were carried out using Matlab (R2017b) software. All statistical analyses were carried out on GraphPad Prism Version 8 for Windows (GraphPad Holdings LLC, CA, USA). Data were subjected to normality testing using D'Agostino-Pearson Omnibus normality test prior to analysis. Data were either analysed by unpaired student t-test or Mann-Whitney test (due to data not following normal distribution), as indicated in the figure legends. Data are presented as mean $\pm$ standard error of mean (SEM). Statistical significance was set at P-value < 0.05 .

3. Results

Autofluorescence: imaging, analysis and cell discrimination of euploid and aneuploid primary human fibroblasts

We utilised primary human fibroblast cells with known karyotypes (euploid; triploid and trisomies: 13, 18, 21, XXX, and XXY) to determine whether hyperspectral microscopy can discriminate between euploid and aneuploid cells based on their autofluorescence spectra. Following hyperspectral imaging, linear unmixing was applied to determine the relative abundance of the metabolic co-factors NAD(P)H and flavins. Further, features were applied to the autofluorescence images to discriminate between euploid and aneuploid cells.

The abundance of NAD(P)H was found to be significantly higher in aneuploid compared to euploid fibroblast cells (Figure 1A, $P < 0.05$). In contrast, the abundance of flavins was significantly lower in aneuploid cells in comparison to euploid (Figure 1B, $P < 0.01$), which was reflected in a significantly lower optical redox ratio (Figure 1C, $P < 0.0001$). To further explore these differences, heat maps were generated to visualise the localisation and abundance of NAD(P)H and flavins within individual cells. As observed with the quantified data, the heat map shows a higher intensity of NAD(P)H in aneuploid cells compared to euploid (Figure 1D). A similar correlation was also observed for flavins (Figure 1E), with a higher abundance seen in euploid fibroblasts compared to aneuploid. Interestingly, there was heterogeneity in the location and abundance of fluorophores between cell type (aneuploid vs euploid) and within cells (Figure 1D and E).

Next, we extracted features from the hyperspectral data and conducted analysis which demonstrated that hyperspectral features can effectively discriminate between euploid and aneuploid human fibroblasts. Features identified are described in Table III. The cellular vectors of the selected and most discriminative features were plotted in a 2D discriminative space generated by LDA and span by two canonical variables which demonstrated clear clustering of euploid and aneuploid fibroblast cells (Figure 2A). To determine the accuracy of this separation, a ROC curve was generated and displayed a high accuracy in discriminating euploid and aneuploid fibroblast cells, with an AUC value of 0.85 (Figure 2B).

453 *Autofluorescence: imaging, analysis and cell discrimination of euploid and aneuploid primary*
454 *mouse blastomeres*

455 We next determined whether individual aneuploid blastomeres from mouse embryos
456 showed a similar shift in the abundance of endogenous fluorophores and whether features
457 analysis could separate euploid and aneuploid blastomeres. Reversine was used to induce
458 aneuploidy during the 4- to 8-cell division of the embryo at which point individual blastomeres
459 were dissociated and imaged. Compared to euploid blastomeres, the abundance of NAD(P)H
460 in aneuploid blastomeres was significantly higher (Figure 3A, $P < 0.01$), while the abundance
461 of flavins was significantly lower (Figure 3B, $P < 0.0001$). These changes were reflected in a
462 significantly lower optical redox ratio in aneuploid blastomeres (Figure 3C; $P < 0.0001$) when
463 compared to euploid blastomeres. Heat maps generated for the cellular distribution of
464 NAD(P)H and flavins confirmed our findings in overall abundance for these metabolic co-
465 factors between euploid and aneuploid individual blastomeres (Figure 3D and E).

466 The extraction of features from the hyperspectral data showed that the chosen
467 algorithms could effectively discriminate between euploid and aneuploid mouse blastomeres.
468 Features identified are described in Table III. As for human primary fibroblasts, cellular vectors
469 of selected and discriminative features were plotted in a 2D space generated by LDA and span
470 by two canonical variables showing a clear clustering of euploid and aneuploid blastomeres
471 (Figure 4A). To determine the accuracy of this separation, a ROC curve was generated and
472 displayed very high accuracy with an AUC value of 0.99 (Figure 4B).

473

474 *Autofluorescence: imaging, analysis and cell discrimination of mouse blastocysts with*
475 *differing ratios of euploid:aneuploid cells*

476 We next sought to determine whether the inner cell mass (ICM) of blastocyst embryos
477 with differing proportions of aneuploid cells displayed differences in native fluorophores. We
478 employed a model of euploid:aneuploid mosaicism (Bolton, *et al.*, 2016), where reversine was

used to induce aneuploidy during the 4- to 8-cell division after which, individual blastomeres were dissociated and reaggregated with untreated (euploid) blastomeres to form the desired proportion of euploid:aneuploid (euploid; aneuploid; 1:1 or 1:3, respectively). Chimeric blastocyst embryos were imaged where the plane of interest, the ICM, was focused on. Unmixing of endogenous fluorophores showed no significant differences in the relative abundance of NAD(P)H (Figure 5A), flavins (Figure 5B) or the optical redox ratio (Figure 5C) between euploid and aneuploid chimeric blastocysts. Similarly, 1:1 and 1:3 chimeric blastocysts demonstrated no significant difference in NAD(P)H abundance level (Figure 6A). In contrast, the abundance of flavins was significantly lower in 1:3 chimeric blastocysts compared to 1:1 (Figure 6B, $P < 0.001$), which was reflected in a significantly lower optical redox ratio (Figure 6C, $P < 0.001$). The cellular vectors of the selected and most discriminative features (refer Table III for list of features) were plotted which demonstrated clear clustering, albeit with some degree of overlapping of euploid and aneuploid (Figure 5D) as well as 1:1 and 1:3 (Figure 6D). To determine the accuracy of this separation, a ROC curves were generated and displayed high accuracy in discriminating euploid and aneuploid chimeric blastocysts (Figure 5E; AUC value of 0.87) and 1:1 vs 1:3 (Figure 6E; AUC value of 0.88).

Due to the process of generating chimeric blastocysts, imaging and analysis occurred in the absence of a zona pellucida. We set out to confirm that the presence of the zona pellucida yielded similar results for the metabolic fluorophores, NAD(P)H and flavins. Zona pellucida enclosed blastocysts were imaged and compared to chimeric, zona free, blastocysts. Fluorophore unmixing showed no significant difference between zona pellucida-enclosed and zona pellucida-free embryos (data not shown).

Safety of hyperspectral imaging

To assess whether photodamage had occurred in response to imaging, untreated morula stage mouse embryos were subjected to either hyperspectral imaging (*imaged*) or not imaged

(*non-imaged*). Imaging of morula embryos did not affect their ability to develop to the blastocyst stage (Figure 7A) nor the level of DNA damage within the subsequent blastocyst embryo (Figure 7B) compared to non-imaged embryos.

Next, we examined whether hyperspectral imaging impacted live birth rate or weight at weaning following transfer of imaged and non-imaged blastocysts to pseudopregnant mice. Imaged embryos resulted in the birth of live pups (Figure 7C). There was no significant difference in livebirth rate between non-imaged and imaged blastocysts (non-imaged: 47 full-term pups from 72 transferred embryos (65.3%); vs. imaged: 49 full-term pups from 80 transferred embryos (61.3%), data not shown). No gross facial deformities were noted across treatment groups. Finally, there was no significant difference in weight at weaning between pups derived from imaged and non-imaged embryos (Figure 7D).

4. Discussion

Clinical assessment of embryo aneuploidy remains a highly debatable topic due to the limitations and safety concerns of current approaches. Thus, there remains a need for an accurate, non-invasive clinical test for the detection and assessment of embryo aneuploidy. The present study showed that the metabolic activity of primary human fibroblasts, mouse blastomeres and the ICM of chimeric embryos was significantly altered due to the presence of aneuploidy. Further, we show that feature analysis of hyperspectral images was able to discriminate between euploid and aneuploid cells and embryos. Finally, we present data demonstrating that hyperspectral imaging poses no harm to embryo and offspring health using the assessments described herein.

Hyperspectral microscopy coupled with feature analysis demonstrated successful capture and identification of endogenous fluorophore abundance across different cell types with aneuploidy. In all cell types, aneuploidy was associated with an altered abundance of metabolic cofactors, which resulted in a lower optical redox ratio, and thus metabolic activity.

It is important to note the NAD(P)H fluorescence may be attributed to either NADH, cytosolic NADPH, or both, due to their near identical spectral properties (Galeotti, *et al.*, 1970, Rehman, *et al.*, 2017). Therefore, the results presented here could be ascribed to an increase in NADPH, which plays a key role in regulating oxidative stress, or NADH, which is essential for ATP synthesis via oxidative phosphorylation (reviewed in Ying, 2008). Elevated NAD(P)H may indicate increased oxidative stress due to an increase in oxidative phosphorylation, which generates reactive oxygen species (ROS) as a by-product. Supporting this, aneuploid mouse embryonic fibroblasts have been shown to contain higher levels of ROS than euploid cells (Li, *et al.*, 2010). Alternatively, the shift could also be due to a preferential shift to glycolysis, leading to increased NAD(P)H and decreased flavin abundance observed with aneuploidy. Indeed, cancer cells which have a high rate of aneuploidy are known for their dependence on glycolysis (Zhang, *et al.*, 2013) to satisfy the significant biosynthetic demands of uncontrolled proliferation, also known as the Warburg effect (Liberti, *et al.*, 2016). It is also interesting to note that in mouse embryos, a shift in metabolic pathway from oxidative phosphorylation to glycolysis occurs naturally during development to adapt to changes in micronutrient availability in the reproductive tract and energy demands of the embryo (Thompson, *et al.*, 2016). Therefore, it is tempting to postulate that aneuploidy may accelerate this process of change in metabolic pathway for mouse blastomeres and potentially chimeric blastocysts, leading to the observed altered metabolic profile. Collectively, our results align with the current understanding of how aneuploid cells display altered cellular metabolism compared to euploid cells (Williams, *et al.*, 2008, Zhu, *et al.*, 2018) and confirms the capability of label-free hyperspectral microscopy to discriminate cells based on cellular autofluorescence (Campbell, *et al.*, 2019, Gosnell, *et al.*, 2016b, Mahbub, *et al.*, 2019).

Furthermore, our results show the spatial localisation and abundance of endogenous fluorophores linked to metabolism. This provides further information on how fluorophores behave differently in aneuploid cells and that heterogeneity exists within and between cells. The different spatial profile of fluorophores in aneuploid cells observed in the current study

may be associated with the altered localisation of chromosomes known to occur in aneuploid blastomeres (McKenzie, *et al.*, 2004). Overall, the results on spatial localisation of endogenous fluorophores demonstrates that aneuploid cells display an altered metabolic profile compared to euploid cells. To our knowledge, this is the first report of altered cell metabolism in mouse embryos in response to aneuploidy.

The safety of hyperspectral imaging was also assessed in the current study. This is of critical importance as existing literature shows that light exposure can be damaging to the embryo, negatively impacting preimplantation development and implantation, as well leading to increased levels of DNA damage within the embryo (Bognar, *et al.*, 2019, Oh, *et al.*, 2007, Takahashi, *et al.*, 1999). Our data showed that hyperspectral imaging did not affect blastocyst formation, level of DNA damage, or subsequent postnatal outcomes when compared to embryos that were not imaged. Collectively, these results demonstrate that hyperspectral imaging of the preimplantation embryo does not impact developmental competence, pregnancy outcome, and offspring health in a mouse model.

It is important to note that the present study utilised a mouse model to elucidate the impact of aneuploidy on metabolism and thus further interpretation or direct correlation to human embryos using these findings warrants caution. Furthermore, aneuploidy induced by reversine is random (Bolton, *et al.*, 2016), and may not reflect the types of aneuploidy observed clinically. However, we do not expect an effect of aneuploidy type on cell autofluorescence as variability in chromosome content yields a consistent transcriptional response in human cell lines compared to euploidy (Durrbaum, *et al.*, 2014). Accurate determination of the proportion of aneuploid cells within the ICM of individual blastocysts to correlate with altered metabolism and level of discrimination between euploid and aneuploid embryos is of interest. Finally, the predictive power of hyperspectral microscopy to detect differing proportions of aneuploid cells within the ICM vs TE and associate with subsequent developmental outcomes should be the basis of future study.

5. Conclusions

Hyperspectral imaging combined with feature analysis can accurately detect changes in metabolism associated with aneuploidy and discriminate between euploid and aneuploid human fibroblasts, mouse blastomeres, and blastocysts. No differences in developmental competence, DNA damage, and postnatal outcomes were observed in embryos subjected to hyperspectral imaging. Together, this work demonstrates that hyperspectral imaging of autofluorescence represents a new non-invasive approach to detect metabolic variance in embryos with aneuploidy. In the future, hyperspectral imaging of autofluorescence may lead to a more accurate and non-invasive assessment of the ploidy status of the divergent cell lineages of the blastocyst embryo.

Author contributions:

KRD conceived the idea for the study. TCYT, SBM, CAC, JMC, AH, SM, EG, and KRD were involved in experimental design and data analysis. TCYT, SBM, CAC, JMC and AH conducted experiments and performed data analysis. TCYT and KRD wrote the first draft of most of the manuscript, with specific sections written by SBM, AH, CAC, DJXC and EG. TCYT, SBM, AB and CAC generated the figures. All authors edited and approved the manuscript.

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614

615 **Conflict of interest statement**

616 The authors declare no conflict of interest.

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812

813 **Table I** Specification of spectral channels with their respective excitation, emission, dichroic
814 mirror wavelengths and laser powers for hyperspectral system in UNSW, Sydney, Australia.

Spectral Channel	Wavelength (nm)			Power (μ W)	Exposure time (s)
	Excitation	Emission	Dichroic mirror		
1	345	414	389	2.80	0.2
2	345	451	389	2.90	0.2
3	345	575	552	2.70	1.5
4	345	594	552	2.90	2.0
5	345	675	552	2.00	2.0
6	490	575	552	3.98	0.8
7	505	575	552	8.40	0.1
8	490	594	552	4.11	0.1
9	505	594	552	8.63	0.1
10	490	675	552	3.29	0.9
11	505	675	552	6.90	2.0
12	358	414	389	2.76	2.0
13	371	414	389	3.10	2.0
14	377	414	389	2.10	2.0
15	381	414	389	0.87	2.0
16	358	451	389	2.54	2.0
17	371	451	389	3.14	2.0
18	377	451	389	2.17	2.0
19	381	451	389	0.90	2.0
20	358	575	552	3.02	2.0
21	371	575	552	4.60	2.0
22	377	575	552	8.52	2.0
23	381	575	552	10.19	2.0
24	391	575	552	6.92	2.0
25	397	575	552	8.45	0.01
26	400	575	552	9.20	2.0
27	403	575	552	4.90	2.0
28	406	575	552	9.73	2.0
29	412	575	552	13.51	2.0
30	418	575	552	14.80	2.0
31	430	575	552	10.21	2.0
32	437	575	552	14.31	2.0
33	457	575	552	8.33	2.0
34	469	575	552	11.89	2.0
35	476	575	552	12.86	2.0
36	358	594	552	3.13	2.0
37	371	594	552	4.84	2.0
38	377	594	552	8.95	2.0
39	381	594	552	10.73	2.0
40	391	594	552	7.26	2.0

Spectral Channel	Wavelength (nm)			Power (μ W)	Exposure time (s)
	Excitation	Emission	Dichroic mirror		
41	397	594	552	8.81	2.0
42	400	594	552	9.55	2.0
43	403	594	552	5.30	2.0
44	406	594	552	10.15	0.01
45	412	594	552	14.25	2.0
46	418	594	552	15.49	2.0
47	430	594	552	10.74	2.0
48	437	594	552	14.88	2.0
49	457	594	552	8.62	2.0
50	469	594	552	12.34	2.0
51	476	594	552	13.35	2.0
52	358	675	552	2.47	2.0
53	371	675	552	3.89	2.0
54	377	675	552	7.15	2.0
55	381	675	552	8.62	2.0
56	391	675	552	5.82	2.0
57	397	675	552	7.19	2.0
58	400	675	552	7.60	2.0
59	403	675	552	4.13	2.0
60	406	675	552	8.06	2.0
61	412	675	552	11.44	2.0
62	418	675	552	12.41	2.0
63	430	675	552	8.49	0.01
64	437	675	552	11.96	2.0
65	457	675	552	6.85	2.0
66	469	675	552	10.00	2.0
67	476	675	552	10.82	2.0

816 **Table II** Specification of spectral channels with their respective excitation, emission, dichroic
817 mirror wavelengths and laser powers for hyperspectral system in UoA, SA, Australia.

Spectral Channel	Wavelength (nm)			Power (μ W)	Exposure time (s)
	Excitation	Emission	Dichroic mirror		
1	367	475	442	3.21	5.0
2	372	475	442	3.60	5.0
3	378	475	442	4.35	4.0
4	384	475	442	4.38	4.0
5	388	475	442	4.30	4.0
6	394	475	442	6.44	4.0
7	401	475	442	9.91	4.0
8	409	475	442	10.30	4.0
9	418	475	442	12.84	4.0
10	340	593	561	3.27	4.0
11	367	593	561	3.26	4.0
12	372	593	561	3.68	4.0
13	378	593	561	4.39	4.0
14	384	593	561	4.41	4.0
15	388	593	561	4.31	4.0
16	394	593	561	6.50	4.0
17	401	593	561	10.06	4.0
18	409	593	561	10.79	4.0
19	418	593	561	15.64	4.0
20	430	593	561	21.90	4.0
21	443	593	561	27.80	4.0
22	455	593	561	26.50	4.0
23	465	593	561	26.70	4.0
24	475	593	561	31.40	4.0
25	495	593	561	18.36	4.0
26	501	593	561	16.75	4.0
27	401	593	561	6.40	4.0
28	409	593	561	8.08	10.0
29	418	593	561	14.06	4.0
30	430	715	695	22.81	4.0
31	443	715	695	30.00	4.0
32	455	715	695	28.60	4.0
33	465	715	695	29.00	4.0
34	475	715	695	34.00	4.0
35	495	715	695	20.81	4.0
36	501	715	695	19.82	4.0
37	597	715	695	18.67	4.0
38	639	715	695	37.70	4.0
39	664	715	695	49.00	4.0
40	White	593	561	3.21	0.01

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Table III Number of features identified and used for analysis. Distinct features were selected according to experimental objectives and cell types listed as follows: 10 features were used to separate between euploid and aneuploid human fibroblast cells in Figure 2; 7 features were used to distinguish between euploid and aneuploid mouse blastomeres in Figure 4; euploid and aneuploid blastocysts in Figure 5D and 5E; and 1:1 and 1:3 chimeric blastocysts in Figure 6D and 6E.

Figures in this manuscript	Features	Description of methodology used for analysis
Figure 2	1	Mean value of channel 26 divided by mean value of channel 58
	2	Mean value of channel 4 times by mean value of channel 10
	3	Contrast value of channel 63
	4	Mean value of channel 67 divided by mean value of channel 42
	5	Mean value of channel 50 divided by mean value of channel 9
	6	Mean value of channel 14 divided by mean value of channel 64
	7	Kurtosis value of second principal component image
	8	Energy value of channel 63
	9	Mean value of second principal component image divided by mean value of channel 48
	10	Mean value of channel 19 divided by mean value of channel 52
Figure 4	1	Mean value of channel 55 divided by mean value of top 40 percent pixel values channel 37
	2	Mean value of first principal component image divided by mean value of top 40 percent pixel values of channel 46
	3	Mean value of first principal component image divided by mean value of channel 56
	4	Mean value of first principal component image divided by mean value of top 40 percent pixel values of channel 58
	5	Mean value of channel 67
	6	Mean value of first principal component image divided by mean value of channel 18
	7	Mean value of first principal component image divided by mean value of channel 32
Figure 5D, 5E	1	Mean value of channel 47 divided by mean value of top 40 percent pixel values of channel 24
	2	Mean value channel of 47 divided by mean value of channel 5
	3	Variance value of channel 56
	4	Skewness value of channel 15
	5	Mean value of second principal component image divided by mean value of channel 18
	6	Skewness value of channel 38
	7	Kurtosis value of channel 28

Figure 6D, 6E	1	Mean value of channel 28
	2	Mean value of channel 41 divided by mean value of top 40% channel 39
	3	Mean value channel 32 times by mean value of top 40% channel 40
	4	Skewness value of channel 60
	5	Mean value of second principal component image divided by mean value of third principal component image
	6	Mean value of channel 8 divided by mean value of channel 36
	7	Mean value of channel 8 divided by mean value of top 40 percent pixel values channel 9

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Figure Legends

Figure 1 Metabolic activity is altered in aneuploid human primary fibroblasts. Primary human fibroblasts with known karyotypes (euploid [male and female] or aneuploid [triploid and trisomies: 13, 18, 21, XXX, and XXY]) were imaged using hyperspectral microscopy. Intracellular autofluorescence signals were subjected to unmixing for quantification of the relative abundance of NAD(P)H (A) and Flavins (B). The optical redox ratio (Flavins/[NAD(P)H + Flavins]) was also calculated (C). Heat maps for euploid and aneuploid fibroblast cells were generated and show the localisation and abundance of fluorophores NAD(P)H and Flavins with representative cells shown in D and E, respectively. Scale bar = 20 μ m. Data were analysed using Mann-Whitney test and presented as mean $\pm$ SEM. Four to six independent experimental replicates were performed; $n = 467$ for euploid cells; $n = 969$ for aneuploid cells. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.001$.

Figure 2 Mathematical algorithms separate euploid from aneuploid in primary human fibroblast cells. The autofluorescence data for each human fibroblast cell was subjected to feature analysis using a suite of mathematical algorithms as described in *materials and methods*. A cluster separation graph was generated using each cell (A, *blue*: male and female euploid cells; *red*: Triploid and Trisomies 13, 18, 21, XXX, XXY). Canonical values represent combinations of multiple features. (B) A receiver operating characteristic (ROC) curve was generated to determine the accuracy of separation between euploid and aneuploid cells (area under the curve (AUC) = 0.85). $n = 364$ for euploid cells; $n = 400$ for aneuploid cells.

Figure 3: Aneuploidy results in an altered metabolism in mouse blastomeres. Aneuploidy was induced during the 4- to 8-cell division in the presence of reversine (0.5 μ M). Eight-cell embryos were dissociated into individual blastomeres and imaged. Aneuploid blastomeres (reversine treated) were compared to euploid mouse blastomeres (control). Intracellular

autofluorescence signals were subjected to unmixing for quantification of the relative abundance of NAD(P)H (A) and Flavins (B). The optical redox ratio (Flavins/ [NAD(P)H + Flavins]) was also calculated (C). Heat maps for euploid and aneuploid individual mouse blastomeres were generated and depict localisation and abundance of fluorophores NAD(P)H and Flavins with representative blastomeres shown in D and E, respectively. Scale bar = 20 μ m. Data were analysed using Mann-Whitney test and presented as mean $\pm$ SEM. Five independent experimental replicates were performed; $n = 39$ for euploid; $n = 44$ for aneuploid blastomeres. * $P < 0.05$, **** $P < 0.001$.

Figure 4: Mathematical algorithms separate euploid and aneuploid mouse blastomeres.

The autofluorescence data for each mouse blastomere was subjected to feature analysis using mathematical algorithms as described in *materials and methods*. A cluster separation graph was generated using each cell (A, *blue*: euploid; *red*: aneuploid). Canonical values represent combinations of multiple features. (B) A receiver operating characteristic (ROC) curve was generated to determine the accuracy of separation between euploid and aneuploid cells mouse blastomeres (area under the curve (AUC) = 0.99). Five independent experimental replicates were performed; $n = 61$ euploid; $n = 71$ aneuploid blastomeres.

Figure 5: Euploid and aneuploid chimeric blastocysts show no difference in metabolic fluorophores but separation achieved using mathematical algorithms.

Chimeric euploid and aneuploid blastocysts were generated as described in *materials and methods*. Chimeric blastocysts were subjected to hyperspectral imaging with field of view adjusted to focus on the inner cell mass (ICM). Intracellular autofluorescence signals were subjected to unmixing for quantification of the relative abundance of NAD(P)H (A) and Flavins (B). The optical redox ratio (Flavins/ [NAD(P)H + Flavins]) was also calculated (C). Autofluorescence spectra for each ICM was subjected to feature analysis using mathematical algorithms as described in *materials*

and methods. A cluster separation graph was generated for euploid (*blue*) and aneuploid (*red*) chimeric blastocysts (D). Canonical values represent combinations of multiple features. (E) A receiver operating characteristic (ROC) curve was also generated to show separation between euploid and aneuploid ICM (area under the curve (AUC) = 0.87). Data in A-C are presented as mean $\pm$ SEM and were analysed by either unpaired Student's t-test (A) or Mann-Whitney test (B and C). Eight independent experimental replicates were performed; $n = 34$ euploid; $n = 39$ aneuploid chimeric blastocysts

Figure 6: A higher ratio of aneuploid cells within the inner cell mass (ICM) of chimeric blastocysts leads to altered metabolism. Chimeric 1:1 and 1:3 (euploid:aneuploid) blastocysts were generated as described in *materials and methods* and subjected to hyperspectral imaging with field of view adjusted to focus on the ICM. Intracellular autofluorescence signals were unmixed to quantify of the relative abundance of NAD(P)H (A) and Flavins (B). The optical redox ratio (Flavins/ [NAD(P)H + Flavins])) was also calculated (C). A cluster separation graph was generated for chimeric blastocysts to show separation between 1:1 (euploid:aneuploid, *blue*) and 1:3 (euploid:aneuploid, *red*) blastocysts (D). Canonical values represent combinations of multiple features. (E) A receiver operating characteristic (ROC) curve was generated to show separation between 1:1 and 1:3 blastocysts (area under the curve (AUC) = 0.83). Data in A-C are presented as mean $\pm$ SEM and were analysed by either unpaired Student's t-test (A) or Mann-Whitney test (B and C). Eight independent experimental replicates were performed; $n = 30$ 1:1 chimeric blastocysts; $n = 37$ 1:3 chimeric blastocysts. ** $P < 0.01$.

Figure 7: Hyperspectral imaging does not affect completion of preimplantation embryo development, the level of DNA damage within the embryo or postnatal outcomes following embryo transfer. Embryos at the morula stage were imaged using the hyperspectral

microscope and compared with non-imaged embryos for their capacity to reach the blastocyst stage of development (A) and the level of DNA damage within the blastocyst (B). Data were expressed as a percentage of γ H2AX /DAPI (B). In a separate experiment, chimeric euploid blastocysts were generated and either imaged or not imaged using hyperspectral microscopy. Blastocysts were transferred to pseudo-pregnant females with imaged blastocysts resulting in the birth of live pups (C). Weights of pups at weaning (day 21) were assessed for each group (D). Data were analysed by Mann-Whitney test and presented as mean $\pm$ SEM, $n = 50$ embryos per group (A) and $n = 11$ embryos per group (B) representative of 5 experimental replicates. For D, $n = 72$ non-imaged and 88 imaged blastocysts transferred into 5 and 6 pseudopregnant recipients, respectively.

Figure 1

932 **Figure 2**

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Figure 5

Figure 6

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