Metabolic profiling of *in vitro* fertilization embryos using hyperspectral imaging (HYSPLANT)

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ABSTRACT

The prevalence of infertility is a growing health concern in Europe. *In vitro* fertilization is the most common treatment which involves fertilization, culture of the embryos and subsequent transfer to the mother. However, there is a lack of reliable technologies to select the most competent embryos for implantation, which compromises treatment success and forces patients to undergo repeated cycles of embryo transfer. In this report, we describe a new hyperspectral imaging framework to profile the metabolism of mammalian embryos and reveal their health status. We outline our main findings and the roadmap to bring the technology to the market.

Keywords: hyperspectral imaging; embryos; in vitro fertilization; reproduction.

1. INTRODUCTION

One out of six couples suffers from subfertility problems [1]. While assisted reproduction techniques (ART), including *in vitro* fertilization (IVF), have helped to overcome many subfertility problems, in a typical IVF cycle, only 30% of the cultured embryos transferred to the mother manage to implant and give rise to successful pregnancy [2]. A common procedure to artificially increase IVF success rate is to transfer multiple embryos, but this strategy has the undesirable side effect of multiple pregnancies, which are associated with significant health complications. Mothers have higher hospitalization rates and caesarean delivery, and the babies may be premature, may have low birth weight and up to 40 times higher risk of early infant death [3]. Therefore, the main challenge for IVF clinics is to transfer single embryos, for which they need to be able to select those that have the highest chance of implantation.

Metabolic balance and evolution are crucial for embryo development and implantation [4]. Numerous metabolites are naturally auto-fluorescent (AF), which may allow to measure metabolic status using optical microscopy methods [5]. However, AF signals are difficult to capture in a convenient and reliable way. In order to enable implantation prediction, the IVF field needs a direct and non-invasive method to determine the metabolic profile and correlate it with the implantation success rate. Hyper-spectral (HS) imaging is an optical technique that provides full spectral information at every pixel of an image, potentially encoding rich information about the metabolic activity [6]. However, it has been challenging to apply it to IVF because of several factors, including complex data analysis, biologically ambiguous data interpretation, and risk of photo-damaging the embryos.

In the ATTRACT phase 1 project, we developed a HS imaging and image analysis approach that overcomes these limitations. It has the advantage of unmixing large numbers of AF metabolites (5+) in low signal-to-noise ratios (SNR) conditions. Our method has minimal photo-toxicity and provides insight into embryos' viability and implantation potential. We used this technique to obtain comprehensive metabolic profiles of mammalian embryos in a direct and minimally invasive way. Also, we were able to score the implantation success rate in a high-throughput manner, thanks to a proprietary 3D *ex vivo* implantation matrix (3D ExVIM) developed during previous research in our lab (US patent 10213282). Our results are a proof-of-concept of our technology (TRL 2-3) and establish the basis for developing a device for selecting competent embryos in IVF clinics.

2. STATE OF THE ART

Nowadays, IVF clinical procedures rely heavily on the experience of an embryologist to identify viable embryos. Time-lapse incubators and pre-implantation genetic screening [5] use morphological and genetic criteria, respectively, to assist the embryologist with

embryo quality grading *in vitro*. However, these techniques do not provide insight into embryos' implantation probability, and thus lack diagnostic potential (Tab.1).

Metabolic activity is a core measure of embryo physiology [4] and health. It is traditionally assessed by methods that are hard to translate into clinical tools [5], since they are slow, technologically demanding, and/or invasive (i.e. NMR or Raman Spectroscopy). Interestingly, in the two main energy-generation pathways of the embryo (oxidative phosphorylation and glycolysis), there are key naturally auto-fluorescent (AF) metabolites, such as NADH and FAD. AF is the basis for several fluorescence-based methods for measuring metabolic activity, mainly Fluorescence Lifetime Imaging Microscopy (FLIM) [7], and Hyperspectral (HS) microscopy [6] (Tab.1).

FLIM allowed the detection of mitochondrial dysfunction in mouse embryos [8]. However, most metabolites are undistinguishable for FLIM, because they have very similar fluorescence life-times and their spectra are overlapping. This limitation precludes comprehensive metabolic profiling of embryos using FLIM, and the transfer of this technology to the market.

Tab. 1. IVF diagnostic technologies: benchmarks.

Name of technology	Pros	Cons
PGS – preimplantation genetic screening	Available in IVF clinics. Detects genetic abnormalities.	No predictive value for implantation. Only applicable for specific genetic markers.
Time-Lapse	Available in IVF clinics. Detects morphologic abnormalities.	No predictive value for implantation. Lack of statistical significance according to retrospective studies.
FLIM	Good discrimination between free and bound NADH.	Limited number of metabolites can be analysed. Slow. Requires expensive detectors and electronics. Cannot provide morphological insight.
HS + multi-variate analysis	Good discrimination of 5+ metabolites. Fast. Provides physiological and morphological information.	Requires precise calibration. Data interpretation requires training.

A promising alternative for measuring AF metabolites is HS imaging. The full AF spectrum can be obtained at each image pixel, thus encoding rich metabolic data that could be useful for embryo selection. Unmixing the overlapping spectra remains a challenge, which we have solved here by using multivariate statistics capable of simultaneously differentiating at least 5 fluorescent labels *in vivo*. Our solution includes powerful de-noising algorithm that permits operations at very low SNR. These SNRs are ~50x lower than those of FLIM, and ~20x lower than alternative HS unmixing methods such as linear unmixing and principal component analysis (PCA).

3. BREAKTHROUGH CHARACTER OF THE PROJECT

In this project, we have accomplished a breakthrough in the IVF field. We have made several significant advances, and will continue to do so as follows:

(i) We have introduced a direct, quantitative, and non-invasive way to measure embryo metabolics via HS imaging and subsequent multivariate statistical analysis (Tab.1). The quantification is enabled by developing an HS analysis approach and applying it into the AF domain.

(ii) Current optical methods are limited to 1-2 metabolic spectral signatures, and often rely on indirect information, such as image sharpness [6]. The results of our HS approach has an unparalleled advantage in unmixing a large number of closely overlapping AF spectra (currently 5, theoretically up to 32) with low SNR. The strategy provides direct access to bio-relevant information such as the concentration and distribution of various metabolites. This asset will allow us to generate new knowledge and understanding of metabolic activity as a crucial factor for embryo development and implantation.

(iii) We are now able to correlate embryos' HS metabolic profiles with their implantation success rate in a high-throughput manner, by using 3D ExVIM. This method allowed us to go beyond current knowledge and study metabolites in so far unexplored conditions (after hatching) during implantation, thus obtaining a complete picture of the entire implantation process. 3D ExVIM must be understood as a tool to accelerate laboratory research towards clinical studies.

(iv) Combining the unique set of developments outlined above, in a Phase 2 of the project we will create the first prediction tool for embryo implantation, based on quantitative, direct and non-invasive measurements of the embryos' metabolic signature.

(v) In a phase 2 we will also empower our HYSPLANT analysis using complementary deep learning algorithms. Such algorithms can achieve outstanding classification capabilities when trained with thousands of images. Our metabolic libraries can lay the foundation of a deep learning training data set for embryo classification.

Altogether, our HS protocol provides a quantitative and minimally invasive technology to assess embryos' implantation probability in a robust and reliable way. In this report, our scientific results are accompanied by a business strategy showing not only that our technology is disruptive, but that the path towards the market is reliable and necessary.

4. PROJECT RESULTS

1) To lay the foundations of the methodology, we have performed and verified calibration of the HS imaging technique with respect to:

a) imaging conditions (Fig.1). We identified optimal imaging conditions (laser wavelength and power, pixel dwell time, number of pixels, etc.) that provide the following advantages: (i) simultaneous excitation of 5+ metabolites with a single wavelength scan, minimizing photo-damage while maximizing the information obtained; (ii) use of a laser irradiation dose that is high enough to obtain biologically meaningful spectral signatures of the different metabolites, while also being low enough to avoid photo-damage. To verify the safety of the imaging methodology, we performed a measurement test in 200+ embryos, and did not observe statistically significant difference in subsequent development between illuminated embryos and non-illuminated controls.

b) Pure metabolic solutions. We calibrated the method with pure metabolic solutions of the 5 metabolites of interest. This allowed us to verify that the spectral unmixing of complex AF metabolic signatures inside the embryo is working properly.

2) We showed that we can use the developed methodology to obtain HS metabolic fingerprints of mouse embryos (Fig.1). In an embryology context, we can detect and distinguish three times the number of metabolites (6) compared to the current state of the art (FLIM). Therefore, we can obtain a more comprehensive picture of mammalian embryos' metabolism. These data will allow us to more clearly identify and distinguish viable embryos from non-viable ones.

3) HS data are multi-dimensional and therefore unintuitive to analyse and interpret. We successfully adopted and tailored a particular HS data analysis procedure and software that allows straightforward visualization of the HS data in 2D space, ultimately facilitating data interpretation (Fig.1). Additionally, a de-noising algorithm permits operation at very low signal-to-noise ratio (SNR): ~50x lower compared to FLIM and ~20x lower compared to alternative HS linear unmixing methods. This method, combined with our data acquisition procedure from 1), allows imaging at a lower laser dose and a much faster speed. Indeed, typical FLIM imaging of 1 metabolite in an embryo takes about 3-5 minutes, whereas HS imaging to differentiate 5+ metabolites with similar settings takes under a minute. Thus, we have significantly improved the imaging efficiency compared to the state of the art.

4) As implantation occurs inside the mother, it is generally difficult to observe the process in a non-invasive way. Moreover, it is usually necessary to sacrifice large numbers of animals in implantation studies. Finally, with the current standard methods it is not possible to keep track of individual transferred embryos between metabolic characterization and implantation. Our group uses 3D ExVIM, a high-throughput procedure for assessing embryo implantation potential. This set-up allows optical access, keeping embryos individualized and therefore connecting each metabolic profile to single implanting or non-implanting embryos. This approach puts us in the unique position to perform high-throughput optical studies of the implantation process and to correlate implantation success of individual embryos to their respective metabolic profile.

5) Based on the above results, we created a standard operating procedure (SOP) for obtaining HS metabolic fingerprints of embryos, including HS imaging, data analysis, and evaluation of implantation in the 3D ExVIM (Fig.1).

6) Using the above results, we have started to build a library of metabolic profiles of "healthy" and "non-healthy" embryos (Fig.1). This core resource is necessary to achieve successful embryo selection. This library includes the metabolic profiles of more than 100 healthy embryos and as many unhealthy ones. The characterization of "unhealthy" embryos is fundamental, since it allows us not only to contrast healthy and unhealthy embryos, but also to better understand the stress that embryos are inevitably subjected to in an *in vitro* environment. The health status of the embryos is validated in a high-throughput manner using the 3D ExVIM.

7) We identified numerous biologically important characteristics related to various healthy and unhealthy features, determined through HS imaging. They will be used as predictive factors in the future implantation prediction software (Fig.1). An example is represented by the concentration and localization of various metabolites in different embryo structures (mitochondria, cytoplasm, inner cell mass, trophectoderm), at distinct developmental stages.

5. FUTURE PROJECT VISION

5.1. Technology Scaling

In this section, we present the main steps required in ATTRACT Phase 2 to scale up the Technology Readiness Level (TRL) we achieved in ATTRACT Phase 1.

TRL3. Design the Proof-of-Concept (PoC) instrument (M2, BRH, Plate manufacturer). Establish the preliminary design of system and sample chambers. Negotiate OEM manufacturing contract. Build, test and calibrate the system.

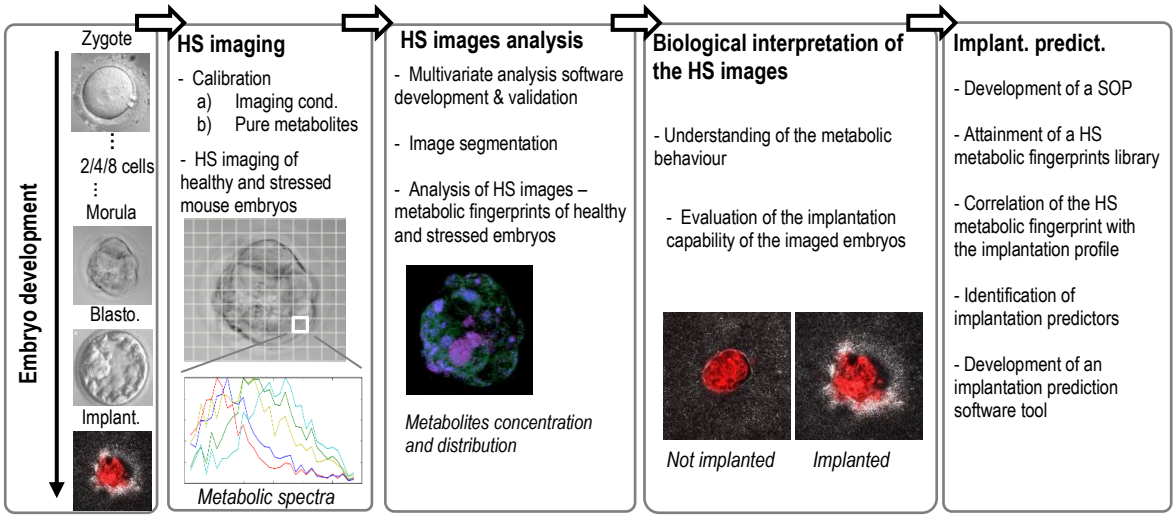

Fig. 1 HYSPLANT workflow and results

TRL4. Develop and validate the PoC instrument (BRH, Dexeus). Test and validate the PoC instrument to ensure that it meets the scientific and technical requirements. Assess the instrument's validity in obtaining the required outcome (embryo implantation prediction), defined by the validation protocol.

TRL4. Set up a design strategy for the imaging prototype (BRH, M2, MOS, Dexeus).
Use the market research for understanding customers' (IVF clinics) requirements in terms of functionality, workable space, desired usability, level of user competency, etc., and ensure that the final product will meet the market demand. Identify IVF clinics (Dexeus and collaborators) to perform the work and recruit a number of tests sites covering the spread of the market.

TRL 4-5. Develop the imaging prototype (M2, MOS-BCN). Understand the requirements of the defined imaging device prototype, and design the system, based on the established working envelope. Test the assembled system and run standard calibrations as final steps.

TRL5-6. Validate the prototype in a first relevant environment (Research lab BRH)
Run validation trials to ensure that the prototype meets market needs (BRH). Feedback to M2 and MOS-BCN to evaluate different aspects of the system and determine its effectiveness in delivering the required outcomes.

TRL7. Prototype validation in a second relevant environment (IVF Clinics)
Run validation and feedback trials required from final users (embryologists and doctors) to get the ultimate proof of the system feasibility (Dexeus).

5.2. Project Synergies and Outreach

To address the challenges of Phase 2, new partners will be added to the consortium.
The microscope manufacturing company M-Squared (M2, Scotland) is the ideal addition to our consortium, since its expertise combines the optics and manufacturing capability necessary at initial (prototyping) and advanced (scale up) TRL stages.
MOS Design, a Barcelona-based design company, will also join the team to fill the gap between the high-tech, design (carried out by **M2 and BRH**) and the user-friendliness required by the end-users (embryologists).
We will contact a plate manufacturer, e.g. **the company Ibidi**, a world expert in manufacturing microscope imaging chambers based in Germany. It will contribute to the project by engineering culture plates tailored to our device.
IBEC and M2 communication departments will develop a marketing plan for the dissemination and public disclosure of the results of ATTRACT Phase 2. We will use social networks, short videos, our own web, the ATTRACT web, and press communication.

5.3. Technology application and demonstration cases

We will implement two technology demonstration cases in ATTRACT Phase 2, to bring concrete benefit to the areas of scientific research and societal challenges.

At TRL5, BRH will use the prototype system to measure the implantation capacity and define the use protocol of embryos donated to the consortium project by Dexeus patients.

At TRL7, we will use the defined protocol to study embryos from patients in Dexeus IVF clinic. Additional prototypes will be delivered to collaborating clinics to run additional tests.

These demonstrations are expected to solve health and demographic challenges. Health issue: 1 out of 7 seven couples suffer from infertility of different degrees. However, the clinical efficacy of IVF is below 30%, in terms of pregnancy success per embryo transferred. Demographic issue: European society is undergoing progressive ageing and declining birth rate. By increasing IVF efficiency, the success of our project has the potential to improve health and block or even reverse the demographic trend.

5.4. Technology commercialization

The BRH is an Open Innovation Laboratory initiative that promotes market-oriented research activities. Structural funding at the BRH is already supported by the VC Scranton Enterprises BV. After completion of ATTRACT phase 1, we presented our TRL roadmap to Scranton, who agreed to lead a second round of investment when the company and the technology require it, in order to accelerate the process of reaching the market.

Envisioned risks

A market risk is the fact that any technology, including disruptive ones like ours, may not necessarily be adopted easily by the market. To address this challenge, Jorge Fuentes (Technology commercialization business expert) and Mònica Valls (MSc. in Biology & Business) are leading the project's business strategy and market analysis. They are studying several aspects of the market (lobbies, distribution channels, etc.) and are planning a company roadmap accordingly. Moreover, to mitigate the corresponding financial risks, again we have the support of Scranton BV. For manufacturing and distribution, we are planning a strategic alliance with M2.

5.5. Liaison with Student Teams and Socio-Economic Study

It has been a very fruitful experience to collaborate with MSc. Students from TeSI program (Technology for Social Innovation from ESADE, UPC and IED) in ATTRACT Phase 1. It helped us redefine our product to better suit users' needs and reach a greater market. Samuel Ojosnegros had fluid communication with the students, obtaining outstanding results from over 150 surveys and 56 interviews with IVF clinics worldwide. Therefore, we are truly motivated and committed to continue our collaboration with MSc. students and to contribute to the expert-driven socio-economic study in ATTRACT Phase 2 with interviews, technology impact references or any other form envisaged.

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