# Microfluidic technology: new opportunities to develop physiologically relevant *in vitro* models

## Integrated microfluidic platform for the *in vitro* pre-implantation culture of individual mammalian embryos and their *in situ* characterization

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***Abstract*—Here, we report the development of an integrated sensing platform for the field of assisted reproductive technologies (ART), and more specifically for the pre-implantation culture of mammalian embryos and their *in situ* characterization through evaluation of their metabolic activity. The entire platform consists of a nanoliter-culture chamber, with an integrated oxygen sensor to monitor the respiratory activity of individual embryos, as a marker for their viability and developmental competence. We first discuss the key-advantages of microfluidic technology to realize such an integrated sensing platform. We next present the culture device, and its validation on mouse embryos. This first stage validation reveals that microfluidics supports the full-term development of mouse embryos down to the single embryo level with birth rates comparable to group culture in conventional formats. In a second step, the device is upgraded for the culture of human embryos, and tested on donated frozen-thawed embryos. Finally, we describe an oxygen sensor consisting of an ultra-microelectrode array (UMEA) to be integrated in the culture device. Using this UME-based sensor, we also propose a novel measurement approach at short timescales, which allows reducing drastically the amount of oxygen consumed through the electrochemical measurements. Current work focuses on the integration of the sensor in the culture platform and its validation on biological materials. The integrated platform is currently tested on spheroids, which are used as surrogates of mammalian embryos, before it is applied on mouse embryos.**

***Keywords—microfluidics, integrated sensing platform, assisted reproductive technologies, mammalian embryos, ultra-microelectrode arrays, oxygen sensing.***

## I. INTRODUCTION

Microfluidics can be defined as a technology allowing the accurate manipulation of small volumes, in the low nanoliter range, using structures having micrometric dimensions [1]. Microfluidics, which is also know as lab-on-a-chip technology (LOC), has reached a mature state and has become very popular in the field of life sciences due to the numerous advantages it offers. Specifically, microfluidic devices enable faster, more sensitive and reproducible analysis using lower amounts of reagents. Furthermore, microfluidics lends itself well to the realization of complex, highly parallelized and integrated platforms.

Originally, microfluidic developments have been driven by the field of bioanalysis. However, applications of LOC have recently diversified and extended to cellular investigations, for which microfluidics presents additional advantages [2, 3]: exquisite control, both spatially and temporally, on a cell microenvironment and the possibility to create *in vivo*–like conditions thanks to the high level of confinement and the laminar character of the flow; dynamic culture conditions; and a unique capability to integrate smart capabilities such as sensors to monitor *in situ*, in real-time and in a non-invasive manner a cell microenvironment and/or a cell behavior. Microfluidic technology is currently utilized for a great variety of applications, ranging from single cell analysis and experimentation [4] to 3D cell culture and investigation [5], and the development of physiologically relevant *in vitro* models, known as organ-on-chip platforms [6].

Here, we focus on one particular field of applications that can greatly benefit from the use of microfluidic technology, for all the aforementioned reasons, which is the field of assisted reproductive technologies (ART) [7, 8]. ART, which include all methods to achieve pregnancy by using artificial means, are increasingly used worldwide, with a total of more than 5 million babies conceived with these techniques. For ART, microfluidics can specifically provide alternative approaches for the various *in vitro* steps of the entire treatment, and thereby remedy currently encountered issues.

In this paper, we particularly focus on two separate steps of the *in vitro* ART protocol, which are namely the pre-implantation culture of the embryos, and their characterization with the purpose of monitoring their growth and identifying embryos with the highest developmental competence for transfer. Specifically, we first report a microfluidic platform developed for the culture of mammalian embryos, and its successful application for the culture of individual mouse embryos. Next, we present the early validation of this microfluidic platform for the culture of donated frozen-thawed human embryos. For the *in situ* characterization of the embryos, we introduce an electrochemical oxygen sensor and a novel sensing principle aiming at minimizing the amount of oxygen consumed during the measurements. Finally, we present the integration of this sensor in a culture microfluidic platform and its early validation and tumor spheroids, used here as surrogates for mammalian embryos.

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## II. MICROFLUIDIC DEVICE FOR THE PRE-IMPLANTATION CULTURE OF MOUSE EMBRYOS

As a first step, a platform is developed for the culture of mammalian embryos, to evaluate if microfluidics can support the pre-implantation growth of individual embryos, down to the single embryo level, leading as well to full-term development. This culture device includes a nanoliter culture chamber, in which embryos are trapped, and from which they can easily be retrieved, for their transfer or further experimentation (Fig. 1) [9]. The microfluidic device is fabricated from PDMS (polydimethylsiloxane) by soft-lithography techniques and it is bonded to a glass substrate.

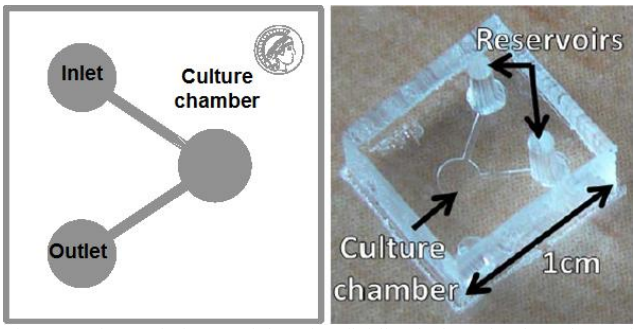


Fig. 1. Design and picture of the microfluidic platform for the culture of individual mouse embryos. The platform includes a culture chamber having a nanoliter volume, and which is equipped with structures at its inlet and outlet to prevent the embryo(s) from escaping during the culture. Interestingly, the structures at the inlet still allow retrieving the embryos for further experimentation or their transfer.

As for any new technology developed for ART, this culture device is first tested on an animal model, which is the mouse here. Naturally fertilized embryos are collected from mice, and these zygotes are distributed in groups of 20 or 5 embryos, or isolated as individual embryos. These embryos are subsequently cultured in microfluidic devices with a culture chamber of 30 or 270 nL, or, as a control, in 5-µL droplets of medium, which are covered with mineral oil to alleviate evaporation issues. Two end-points are considered to evaluate the embryo developmental potential: on one hand, their pre-implantation growth after 4-5 days of *in vitro* culture, which is examined as a blastocyst rate, the blastocyst being the stage the embryos should have reached at that time of their development; and, on the other hand, their full-term growth, or birth rate. For this second end-point, part of the embryos are recovered from the culture platforms after 3.5 days of *in vitro* culture, and transferred into pseudo-pregnant mice to allow them to complete their development.

All microfluidic culture conditions tested here (1, 5, and 20 embryos, and chamber volumes of 30 and 270 nL) give blastocyst rates higher than 90%, while conventional droplet culture for the same embryo group sizes yields significantly lower developmental rates (30-75%), the exact value depending on the embryo group size (Fig. 2) [9]. Regarding single embryo culture, the microfluidic approach proves to be highly promising with a *ca*. 95% blastocyst rate, compared to a low 30% for the droplet format (Fig. 2) [9].

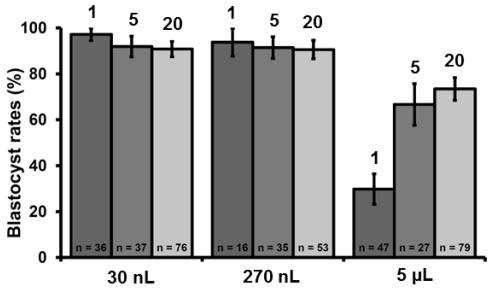


Fig. 2. Pre-implantation development or blastocyst rate for mouse embryos cultured as groups of 5 or 20, or individually, in microfluidic chambers having a volume of 30 or 270 nL or in 5-µL droplets (control).

As a next stage, the embryo full-term development is considered to ensure the used *in vitro* culture conditions do not harm the embryos. Overall birth rates are similar for both culture platforms, microfluidic devices and conventional droplets, and they amount to *ca*. 30% (Fig. 3). However, when examining single embryo culture, significantly superior performance is found in the microfluidic setting (>30% *vs*. 20% for the droplet format), and the smaller the volume for the culture, the higher the birth rate (Fig. 3) [9]. These results collectively suggest that the use of confined culture conditions benefits to embryo growth, down to the single embryo level, and this not only at the pre-implantation stage but also in terms of full-term development. This advantage given by confinement can, for instance, be explained by the fact that embryos secrete growth factors promoting their development [10], and that they can create a niche with a high concentration in these growth factors in a nanoliter chamber. In contrast, in a larger volume, these growth factors are highly diluted, which therefore results in impaired or delayed embryo development, as observed here in droplet culture.

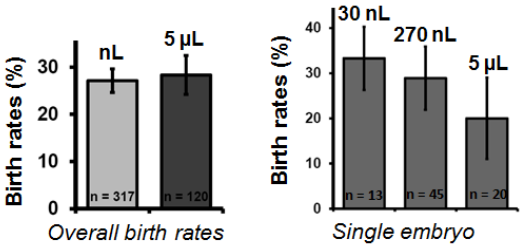


Fig. 3. Full-term development of mouse embryos cultured in microfluidic devices with culture chambers of 30 or 270 nL, or in droplets (control). *Left*: Overall birth rates in droplets *vs*. microfluidics, and *Right*: birth rates obtained for single embryo culture in the three culture volumes tested here – 30 & 270 nL (microfluidics), and 5 µL (droplets).

## III. MICROFLUIDIC SYSTEM FOR THE *IN VITRO* CULTURE OF HUMAN EMBRYOS

This single embryo culture approach and microfluidic platform is subsequently tested on human embryos. The design of the microfluidic platform is upgraded to include a larger culture chamber (*ca*. 640 nL) to accommodate human embryos, which are roughly twice larger in size [11], the device being still produced from PDMS and glass using the same fabrication process. Donated human embryos are utilized in this second

stage validation (CCMO authorization number NL38300.000.11), which have been kept in liquid nitrogen. These embryos, which are already at day 4 of their development, are first thawed before they are cultured for up to 72 h in the microfluidic devices and, as a control, in 25-µL droplets of medium covered with mineral oil. In total, 120 embryos are included in the study and randomly distributed between the microfluidic systems and the conventional droplets. Embryos are graded at different time points, after 24, 28, 48 and 72 h, in terms of blastocyst rate and stage to evaluate their developmental progression.

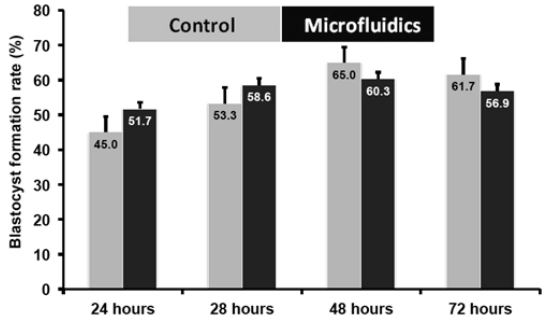


Fig. 4. Developmental rate of single human embryo cultured in PDMS-glass microdevices and conventional droplets, as controls. The developmental rate is evaluated as the blastocyst formation rate, at different time points (24, 28, 48 and 72 h) after introduction of the embryos in the respective culture platforms [11].

This second stage validation confirms the ability of PDMS microfluidic systems to support the growth of single embryos. However, similar growth rates are found for both culture platforms, and no clear advantage of the microfluidic system is seen (Fig. 4) [11]. This difference in outcome, compared to the mouse study, can be explained by the fact that at day 4, human embryos have already undergone essential steps in their development; for instance, genome activation already takes place before the 8-cell stage (day 3 of development).

## IV. Oxygen Sensor for In Situ Monitoring of the Embryo Growth and Developmental Competence

As a next step towards the development of an integrated platform for single embryo culture and characterization, an oxygen sensor is developed. Oxygen, which is an overall marker for the embryo metabolism, has been acknowledged as an indicator for embryo viability and developmental competence [12]. We have developed an electrochemical sensor consisting of an array of ultra-microelectrodes (UME's) acting as a working electrode. The first prototype for this sensor includes square arrays of 16, 25 or 36 UME's, which have each a diameter of 2 microns and an inter-electrode distance of 20 microns (Fig. 5) [13]. All electrodes (UME array, counter electrode and reference electrode) are fabricated from Pt on glass, and the UME's are patterned in an oxide-nitride-oxide insulating layer.

First, the reducing potential for dissolved oxygen is determined, and a reducing potential of -0.2 V is found in phosphate buffer. To minimize the oxygen consumption during the electrochemical measurements, an innovative sensing protocol is developed [13]. Specifically, a short (< 5 ms) pulse at -0.2 V is applied and the reducing current continuously measured. At this short time-scale, the diffusion profile around the UME's remains in the linear regime, so that the sensor behavior obeys the Cottrell equation. Subsequently, the dissolved oxygen concentration can be derived from:

$$I\sqrt{t} = mnFA_{UME}\sqrt{\frac{D}{\pi}}C$$

where $m$ is the number of UMEs in the sensors, $n$ the number of electrons involved in the reaction, $F$ the Faraday constant, $A_{UME}$ the surface area of each UME, $D$ the diffusion constant of oxygen, and $C$ the dissolved oxygen concentration.

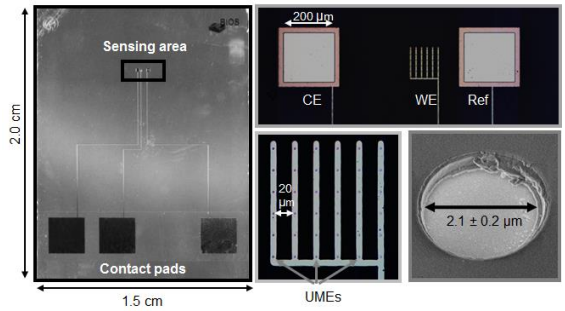


Fig. 5. Design of the electrochemical sensor consisting of an array of ultra-microelectrodes. The sensor includes a working electrode (UMEA), a counter and a reference electrodes, all fabricated from Pt on a glass substrate. Each sensor includes a square array of UMEs with here 36 UMEs patterned in an ONO insulating layer. Each UME is ca. 2 microns in diameter, and the UME's are spaced apart by 20 microns [13].

Using this novel sensing principle, the proposed UME-based sensor is calibrated by varying the concentration in dissolved oxygen through nitrogen bubbling, while monitoring the sensor response as well as the actual dissolved oxygen concentration using a commercial sensor from Unisense. An excellent linear correlation is found between the dissolved oxygen concentration and the sensor response for a 36-UME sensor, with a sensitivity of 0.49 $nA.s^{-0.5}.L.mg^{-1}$ (Fig. 6). Finally, the amount of oxygen consumed through the measurements is evaluated; for a 5-ms pulse, *ca*. 63 fmol of oxygen are consumed, which is significantly lower than when using standard microelectrodes [13].

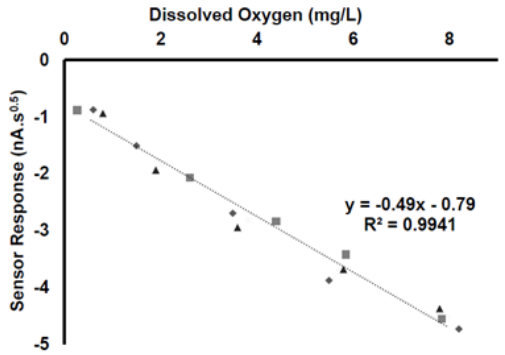


Fig. 6. Calibration of the UMEA sensor in phosphate buffer using the short measurement time protocol. The dissolved oxygen concentration is varied and measured concomitantly with an external sensor and the UMEA-based integrated sensor. Since the UMEA is operated at short measurement times, its response is expressed as $nA.s^{-0.5}$. [13]

Next, the UMEA sensor is integrated in a microfluidic device for combined embryo culture and characterization. To that end, the design of the microfluidic device is altered to include a trapping site for the capture of a single embryo in the close vicinity of the oxygen sensor (Fig. 7). So far, this integrated sensing platform has solely been tested on tumor spheroids, which are utilized as surrogates for embryos for a first stage validation of the integrated sensing platform. After introduction of a spheroid in the device, its oxygen consumption is monitored over time using the integrated sensor and the novel short measurement time approach. Typically, after 2 h monitoring, a decrease of ca. 1 mg/L in dissolved oxygen is measured in the device compared to atmospheric conditions. As a next step, the sensing device is going to be tested on mouse embryos.

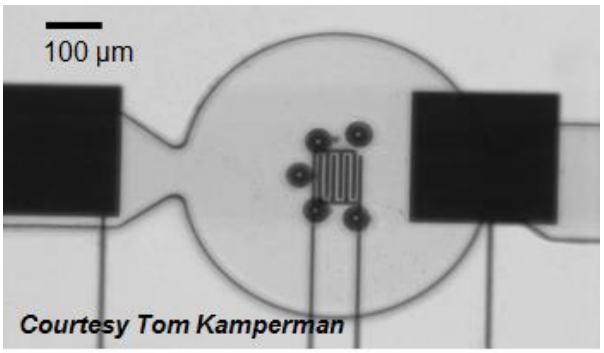


Fig. 7. Picture of the integrated sensing device, which consists of a microfluidic layer with trapping structures for the capture of a single embryo, and a glass substrate which includes the UMEA-based sensor. The sensor is located exactly in the trapping structure.

## V. CONCLUSION

The ultimate goal of this project is to develop an integrated platform for combined single embryo culture and characterization based on non-invasive and metabolic parameters. Towards this goal, in a first step, we have realized and successfully validated on mouse embryos a microfluidic platform for single embryo culture. After its adaptation, the same platform has been tested on donated human embryos. In parallel, we have developed an electrochemical sensor consisting of an array of ultra-microelectrodes to monitor *in situ* in the culture chamber the embryo respiratory rate, as an overall marker of its metabolism and viability. We have notably proposed a novel sensing principle to be utilized in combination with this sensor, to minimize the amount of oxygen consumed by the electrochemical measurements. Current work focuses on the integration of the proposed sensor in the culture microfluidic platform, the validation of the entire platform on spheroids which are used as surrogates for embryos, before the integrated sensing platform can safely be evaluated on mouse embryos.

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