Extended Abstract

Development of a non-invasive method for the *in vitro* monitoring of 3D cell cultures

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Mesenchymal stromal cells (MSC) show great promise in regenerative medicine due to their remarkable properties. MSC are isolated from tissues and expanded *in vitro* to achieve relevant quantities for cell-based therapies and can be further seeded to three-dimensional (3D) platforms and differentiated for tissue engineering purposes. Current methodologies to examine 3D-cultures are based on endpoint analysis, which require cell samples to be sacrificed and enhances the risk of contamination due to regular handling. **Methods:** In this work, a perfusion bioreactor system was used to dynamically cultivate MSC in 3D platforms. An apparatus was coupled to the reactor to non-invasively acquire medium samples from the centre of the platforms, thus enabling the monitoring of the cultivation by biological parameter analysis and preventing the usage of endpoint methodologies. Prior to the cultivation inside the reactor, the cytotoxicity of the reactor material was assessed, and the cell seeding to collagen membranes and alginate hydrogels was optimized. Additionally, the diffusional capacity and the MSC differentiation in these scaffolds were characterized. **Results:** Cells remained viable for up to 7 days when cultivated under perfusion inside the reactor, as demonstrated via calcein-AM/PI staining. Glucose/lactate monitoring within the cell-constructs was possible due to sampling acquired with the apparatus, which enabled to predict the cell behaviour during the cultivation in a non-invasive manner. **Conclusion:** These findings support the potential of using this novel concept of coupling the non-invasive apparatus to the perfusion reactor, towards the creation of an optimized 3D-culture monitoring system.

Keywords: MSC; regenerative medicine; dynamic cultivation; perfusion bioreactor; 3D cultivation; 3D cultivation monitoring

1. Introduction

Mesenchymal stromal cells (MSC) are adult multipotent stem cells capable of self-renewal and able to differentiate into specialized cell types. They are promising in cell-based therapies and tissue engineering due to their role in the regulation of immune [1] and inflammatory responses [2] and tissue regeneration mechanisms [3].

Human MSC (hMSC) were first discovered within the bone marrow [4], but other sources have been identified since then, including adipose tissue [5]. Despite the source heterogeneity of hMSC, a minimal set of criteria must be met to characterize these cells; MSC must be plastic-adherent, capable of trilineage differentiation (adipogenic, chondrogenic, osteogenic) and express specific surface markers ($CD105^+$, $CD73^+$, $CD90^+$, $CD45^-$, $CD14^-$, $CD19^-$ and $HLADR^-$) [6]. Standardized protocols for MSC isolation, culture, expansion and differentiation, as well as tight quality controls are determinant for an effective and reproducible MSC based therapy.

Two-dimensional (2D) culture systems have been the protocol of choice to study adherent cultures due to their simplicity and easy handling. However, this type of monolayer method is limited, since cells do not exist in an isolated 2D microenvironment *in vivo*. In turn, 3D cell cultures provide physiologically relevant cues that better mimic the *in vivo* microenvironment; 3D allows cells to exhibit a morphology and migration mode similar to that found *in vivo*, which determines their biological activity [7] and enhance the degree of cell interaction with their immediate surroundings, greatly affecting their cellular function [8, 9]. 3D platforms for the cultivation of MSC may have two formats: scaffold-free, such as aggregates, or scaffold-based, including porous microcarriers, scaffolds and hydrogels.

Bioreactors that accommodate dynamic culture conditions play an important role in growing cells *in vitro* that more closely resemble the physiological environment, since 3D platforms can be integrated in those systems, be subjected to mechanical cues, and the transport of mass and energy are improved [10]. Dynamic bioreactors suitable for MSC culture can be mechanically or hydraulically driven. In the former, such as the stirred tank and wave reactors, cell constructs (*e.g.* aggregates, microcarriers, gel beads) are grown in suspension; these reactors are more appealing for large expansion of MSC [11]. In turn, hydraulically driven reactors, which are operated with perfusion, operate smaller scale cultivations due to their limited scalability [10, 11]. However, these are better suited for tissue engineered applications due to their capacity to mimic the *in vivo* microcirculation and, compared to the mechanically driven, the mass transfer (resultant from the continuous exchange of media) is enhanced [12].

Regarding the monitoring of a cell culture, two main approaches are possible: either perform endpoint analysis, in which cells are sacrificed (*e.g*. lysed or fixed), or perform real-time monitoring without sacrificing cell samples, allowing to non-invasively conclude about the dynamics within a cell population over culture time; this is of most interest in cell-based and tissue engineering applications. Given the importance and rising interest of 3D cell culture, in combination with the lack of an affordable, robust, effective and simple-to-use techniques to analyse it, a

considerable effort has recently been given in developing non-invasive real-time assays to monitor 3D cultivations. Nonetheless, the retrieval of samples from 3D cultures and reactors, or the integration of monitoring tools within these systems, are challenging.

The current work focuses on the establishment of an *in vitro* monitoring system for the non-invasive analysis of 3D cultivation. The system comprises a mini-bioreactor, that supports 3D cultivation and operates under perfusion to better mimic physiological conditions. Additionally, an apparatus is coupled to the reactors for a non-invasive medium sampling during cultivation. Such sampling enables for the continuous control of the cell viability via biological parameters analysis, while preventing the need for the currently used endpoint methodologies to examine 3D cultures.

2. Methods

Standard cell culture

Human adipose-derived MSC (adMSC) were retrieved from vials stored in the cell bank of Research Group Kasper. Each vial (1 ml) was suspended in 10 ml of basal medium and centrifuged at $500 \times g$ for 5 min . The supernatant was discarded and the pellet resuspended in 0.5 ml of expansion medium. The cells were counted using a haematocytometer and the suspension was transferred to a T-flask, where cells were cultivated in expansion medium until reaching 80-90% of confluence, point in which cells were passaged. For that, the flask was rinsed with PBS and then incubated (37 ºC, 5% CO_2, 21% O_2) with Accutase® (Sigma Aldrich). After 15 min, expansion media was added to the flask and the suspension was transferred to a falcon tube and centrifuged (5702, Eppendorf). The supernatant was discarded, the pellet resuspended in medium, and the cells were counted. Finally, the cell suspension was cultivated in new T-flasks or used in experiments. Expansion medium was changed every 2-3 days during cultivation.

Cytotoxicity of the reactor material

MSC in passage were cultivated (4,000 cells/cm^2) in wells of a well-plate with expansion medium for 2 days, followed by a cultivation period of 4 days in the presence of cylinder-shaped pieces (1 mm thick, 5 mm of diameter) of different materials: parylene-coated High Temp resin 1× or 4× autoclave-sterilized (HT-P1× and HT-P4×, respectively), non-parylene-coated HT resin autoclave-sterilized (HT) or γ-irradiation-sterilized (HT-γ), Clear resin 1× UV light-sterilized (C-UV1×), and UV-cured Clear resin (C(+)UVc); all resins are from Formlabs. At the end of the cultivation, the cell viability was assessed via calcein-AM/PI staining (documented by fluorescence microscope, Leica), MTT (documented by bright field microscope, Leica; absorbance measured on a plate-reader, Infinite® M1000 Pro, Tecan) TOX8 (fluorescence measured on a plate-reader) and glucose and lactate concentrations (YSI 2700 SELECT, YSI Incorporated).

3D cell culture in collagen membranes

MatriStypt® or MatriDerm® were cut to the desired size, placed in plastic culture plates and UV light (250 nm) sterilized for 30 min, each side. MSC were seeded to dry or medium soaked matrices at 5,000-20,000 cells/cm^2. The cells were seeded statically or dynamically, and in both settings cell suspension was added on top of the membranes, with the latter being followed by a centrifugation step at 500 rpm for 5 min (Heraeus™ Megafuge™ 16, Thermo Scientific). After 1h of incubation to allow cell adhesion, expansion medium was added to the membranes, which were finally incubated for 3 days before being used in further experiments.

In the cell culture optimization experiments, MatriStypt® seeded with different methodologies were cultivated in a standard manner for up to 11 days; calcein-AM/PI staining was performed regularly.

MSC used in the reactor cultivations and 3D-differentiation experiments were seeded statically to hydrated membranes.

3D cell culture in alginate hydrogel

Sodium alginate (powder) was UV light sterilized for 30 min before preparing a 1.2% (w/v) alginate solution. This was accomplished by dissolving sodium alginate in expansion medium supplemented with 1% (v/v) gentamycin. MSC in passage were added to the alginate solution at 100,000-300,000 cells/ml; the gels were casted by adding the alginate-cell suspension to well-plates and topping that suspension with 100 mM $CaCl_2$ in a volume ratio of 1:2. Finally, the plates were incubated for 8h to allow the gel cross-linkage before being used in further experiments.

In the cell culture optimization experiment, gels seeded with different densities were cultivated in a standard manner for 5 days; calcein-AM/PI staining was performed regularly.

Gels used in the reactor cultivations and 3D-differentiation experiments were seeded at 100,000 and 300,000 cells/ml, respectively.

Standard cultivation of 3D platforms

Standard cultivation of seeded 3D platforms was performed in the optimization of cell culture experiments and in the assemblage of controls for the cultivation inside the perfusion bioreactor. It comprised on cultivating seeded alginate gels or membranes in culture plates, such as well-plates or petri dishes, filled with expansion medium up to the respective working volume. Medium was changed every 2-3 days during the cultivation period.

Transport dynamics in 3D platforms

A pair of membranes (MatriStypt® or MatriDerm®) or an alginate gel, in the absence of cells, were transferred to separate reactors; the reactors were coupled to the apparatus for medium sampling from within the 3D platforms. The reactor circuits were operated under perfusion (constant rate, 10 rpm) with expansion medium. After 2h, the medium was changed for PBS (20 ml) to remove glucose/lactate from the platforms and perfusion was performed for 48h.

Finally, PBS was changed for expansion medium (15 ml) to replace the biomolecules in the platforms. Medium samples from within the platforms were acquired occasionally with the apparatus to monitor the glucose/lactate concentration within the platforms.

3D cell culture inside the reactor

The medium container of the perfusion circuit was filled with expansion medium (15 ml) supplemented with 1% (v/v) gentamycin. For static cultivation, the inlet and outlet of the reactor were closed with caps, and the chamber was filled with the same medium (3 ml). Seeded pairs of membranes or alginate gels were inserted in separate reactors; depending on the experiment setting, the apparatus for non-invasive medium sampling was coupled to the reactors, to allow medium sampling from the centre of the cell constructs. The reactors were operated under perfusion (constant rate; 4, 5 or 10 rpm) and/or static conditions for up to 7 days, without the cultivation medium being changed. The cultivation was assessed via calcein-AM/PI on cell samples, or via glucose/lactate measurement on medium samples.

adMSC trilineage differentiation in 2D culture

The surface of well bottoms were coated with 2 µg/cm^2 of fibronectin and left to dry at room temperature for 1h. The wells were rinsed with PBS and then seeded with cells (4,000-7,000 cells/cm^2) and medium. Upon reaching 100% confluence, the medium was changed to the respective differentiation solution (Table 1).

adMSC trilineage differentiation in 3D culture

Medium soaked MatriDerm® (round-shaped, 0.28 cm^2) were statically seeded at 20,000 cells/cm^2; 100 µl of cell-alginate suspension (300,000 cells/ml) were casted in wells of 96-well plates, as described previously. The plates were incubated overnight. On the day after, the medium was changed to the respective differentiation solution (Table 1).

In both settings (2D and 3D), cells were cultivated for 21 days under hypoxia (5% O_2) or normoxia (21% O_2), with the medium being changed every 2-3 days. On day 21, cells were fixated with ethanol (osteogenesis and chondrogenesis) or paraformaldehyde (adipogenesis) according to the respective staining. Chondrogenesis was assessed via Alcian Blue, adipogenesis via Oil Red O, and osteogenesis via Alizarin Res S, von Kossa and the pairing of Calcein and DAPI. All stainings were documented with the bright field microscope, except Calcein and DAPI, which were documented by fluorescence microscopy.

Table 1 Differentiation media and respective composition.

Media	Composition
Adipogenic medium (ADM)	1% (v/v) gentamycin in NH AdipoDiff Media
Chondrogenic medium I (CDM I)	1% (v/v) gentamycin in NH ChondroDiff Media
Chondrogenic medium II (CDM II)	1% (v/v) ITS+, 50 µg/ml L-ascorbate-2-phosphate, 100 nM dexamethasone, 40 µg/ml L-proline, 10 ng/ml TGF-β, 1% (v/v) gentamycin in DMEM-high glucose
Osteogenic medium I (ODM I)	1% (v/v) gentamycin in NH OsteoDiff Media
Osteogenic medium II (ODM II)	5 mM β-glycerophosphate, 50 µg/ml L-ascorbate-2-phosphate, 100 nM dexamethasone in expansion medium

3. Results and discussion

An overview of the developed work is provided in (Figure 1); it highlights the major investigated topics (each represented by a block), and how they are interrelated.

Cytotoxicity evaluation of the reactor material

Prior to performing the cell cultivation inside the bioreactors, the cytotoxicity of their manufacturing material was assessed to conclude which is the most appropriate.

Concerning the optical assays (Figure 2A), the production of formazan (MTT) by the HT-P and the C(+)UVc samples was of the same extent and similar to the control, suggesting that cells remained with their metabolic capacity unaltered; there was a considerably less production of the purple crystal by the HT-γ samples, and no significant conversion of MTT was detect in the HT or C-UV1× samples. In the calcein-AM/PI, cells cultivated in the presence of HT-P or C(+)UVc resembled the control in the viability and morphology, and there was a considerably higher number of viable cells for these conditions than in the HT-γ samples; no viable cells were detected on the samples cultivated in the presence of HT or C-UV1×.

The results obtained in the quantitative assays are consistent with the optical assays. The cell count (Figure 2B) and metabolic activity – MTT (Figure 2C) and TOX8

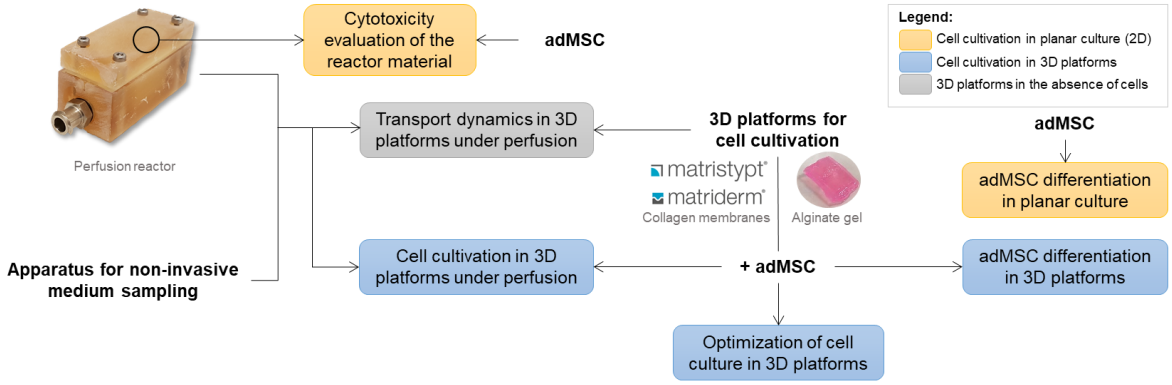

Figure 1 Block diagram of the developed work. Each block represents a major investigated topic.

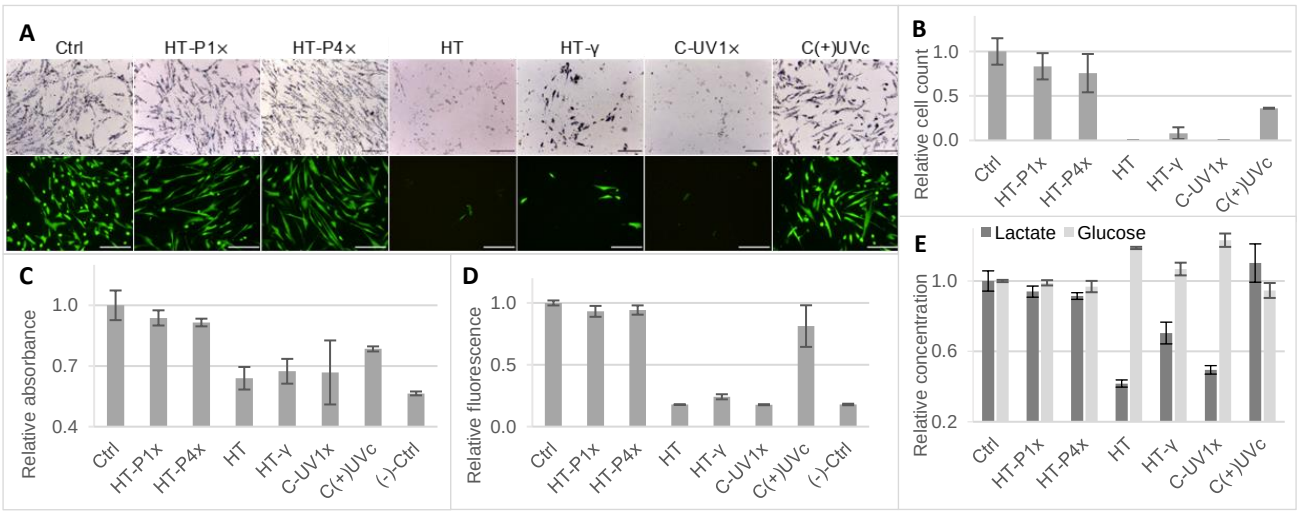

Figure 2 Optical assays (A) MTT (top row) and calcein-AM/PI staining (bottom row); quantitative assays (B) cell count, (C) MTT, (D) TOX8 and (E) lactate and glucose concentrations on cells cultivated for 4 days in the absence of pieces of material in fresh medium (Ctrl) or in the presence of pieces of HT-P1x, HT-P4x, HT, HT-γ, C-UV1x and C(+)UVc. Calcein-AM/PI, MTT and TOX8 assays were performed to conclude about cell viability: calcein-AM stains live cells in green; PI stains dead cells in red; live cells metabolize the yellow-coloured MTT into the purple-coloured formazan crystals, and the blue-coloured resazurin into its red-coloured reduced form (TOX8). Scale bar represents 250 µm. Data represents mean ± SD from n = 3 replicates for each condition; data presented is relative to the control; expansion medium was used as negative control, (-)-Ctrl, in the MTT and TOX8 assays.

(Figure 2D) – were highest for the control, followed closely by the samples cultivated in the presence of HT-P. Compared to these conditions, there was a lower number of viable cells for the C(+)UVc condition, and consequently a lower metabolism rate in the MTT and TOX8 assays. The cell viability was negligible for the HT and C-UV1× conditions, which translated into a low metabolic activity that resembled the negative control.

Regarding the glucose/lactate metabolism (Figure 2E), the highest consumption of the sugar and production of the metabolite were detected in the HT-P and C(+)UVc samples, which were similar to the control. The degree of glucose metabolism by the cells cultured in the presence of HT-γ was less pronounced compared to the previous three conditions. The lowest glucose conversion into lactate was observed in the samples cultivated in HT or C-UV1×.

In general, these findings indicate that the non-parylene coated High Temp resin (HT) and the untreated Clear resin (C-UV1×.) have a negative effect on cells, as they became metabolically inactive and presumably death, suggesting that these materials are cytotoxic.

It is worth noting the positive effect of the parylene-coating and the UV light hardening treatment on the cytocompatibility of the High Temp and Clear resins, respectively. The results illustrate that cells remained viable in the presence of HT-P or C(+)UVc, which was not verified when the High Temp resin was not coated with parylene or the UV light hardening treatment was not applied to the Clear resin after the 3D-printing.

To conclude, the results suggest that the HT-P resin is the best suited for cell cultivation, since for all the different conditions studied, it was the best material to enable the cells to maintain their viability, morphology and metabolic activity. Although the UV-cured Clear resin has shown promise of being cytocompatible, the results obtained for the HT-P resin were globally preferable. Accordingly, the bioreactors employed to cultivate cells under perfusion were chosen to be manufactured with this material.

Optimization of cell culture in MatriStypt®

The cell seeding methodology determines the cultivation efficacy in 3D platforms. Hence, this study had the goal of: i) conclude which initial cell density is best suited for this platform; ii) compare cell seeding on dried *vs.* medium-soaked (hydrated) membranes; and iii) compare dynamic (centrifugation) *vs.* static seeding.

Squares of Matristypt® were pre-incubated, or not, in expansion medium and then statically or dynamically seeded at a given cell concentration: 5,000; 10,000; 15,000 or 20,000 cells/cm^2. The membranes were cultivated for up to 11 days and documented via calcein-AM/PI staining.

First, none of the employed seeding methods had a negative impact in the viability or in the morphology of the cells, as they presented a fibroblast-like shape and no red-coloured cells were observed upon calcein-AM/PI staining.

Regarding the cell density, 5,000 cells/cm^2 is not adequate due to a considerable area without cells that translated in a low fluorescent signal; the results obtained for the 10,000 and 15,000 cells/cm^2 were comparable and had an appropriate signal intensity. Confluence was visible in samples seeded at 20,000 cells/cm^2 on dry membranes, thus densities up to 20,000 cells/cm^{2}are preferable due to a more intense fluorescent signal, while preventing confluence.

Concerning the seeding on dried or hydrated membranes, the medium present in the latter allowed for an even cell distribution along the surface. In the dried matrix, the cells remained concentrated on the spot they were

seeded, and a negative cell density gradient was created between that spot and the borders of the membrane. As the seeding to dried matrices did not allow to evenly distribute the cells throughout the matrix surface, and the extent of cell attachment appeared comparable between both conditions, the cell seeding to medium-soaked matrices is advantageous.

Regarding the static and dynamic seeding, there was no apparent difference between these conditions. The parameters of the dynamic seeding were adapted from the work developed by Zhang *et al.* [13], in which 1 mm-thick scaffolds obtained from cancellous bone were centrifuged thrice at 500 rpm for 2 min and then turned over for centrifuging as one cycle. Although the thickness is similar to that of MatriStypt®, the stiffness of the cancellous bone and the collagen matrix is distinct, hence the seeding parameters that fits the cancellous bone scaffold might not adjust to the collagen membrane. Further studies tackling different centrifugation settings, using robust methods to analyse the scaffold centre, should be performed to assess the effect of dynamic cell seeding to MatriStypt®.

Finally, when cells are seeded on MatriStypt®, they cross through the membrane and settle on the bottom (no cells observable on the top of the matrices at days 3-4). However, on days 10-11, cells were visible on the top surface, indicating that they migrated inside MatriStypt® in the upward direction. Moreover, a higher number of cells were present on the top of the hydrated matrices compared to the dried matrices, suggesting that soaking MatriStypt® in medium prior to cell seeding might aid the cells to migrate (Results available in [14]).

Optimization of cell culture in alginate hydrogel

Gels were casted at 100,000 or 200,000 cell/ml, cultivated for up 5 days and documented via calcein-AM/PI staining. The methodology employed to entrap cells within the alginate polymer allowed cells to maintain their viability throughout the cultivation, since all cells were stained green and the number of viable cells appeared to remain constant, independently of the initial cell concentration. Therefore, casting gels by mixing cell suspension in alginate 1.2% (w/v) followed by the addition of 100 mM $CaCl_2$ in a ratio 1:2, and subsequent incubation for 8h, is an adequate method to cultivate cells within the alginate polymer.

No significant difference was noticed between the studied cell densities; however, higher densities are preferable once it allows for a higher fluorescent signal. (Results available in [14]).

Experimental setup of the perfusion circuit

The perfusion chamber and respective lid are 3D-printed with the HT-P resin. The chamber has a cubical shape with trimmed vertical edges and an inner volume of 3 ml. As depicted in Figure 3, a scaffold holder fixes the scaffold between the lid and the chamber so that medium flows parallel to its surface; the holder bottom has an open cross structure that supports the scaffold, while allowing medium to perfuse the scaffold. In addition, the reactor design allows for the insertion of the apparatus – to gather medium samples – in between the scaffold and its platform holder.

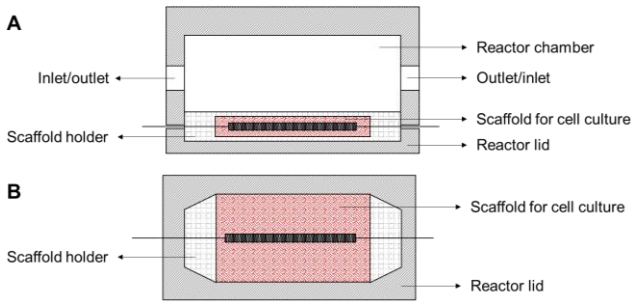

Figure 3 (A) Side and (B) bottom views of the perfusion chamber body (figures not in scale).

The circuit of a single perfusion bioreactor system is illustrated in Figure 4. The main circuit includes a medium container, a reactor chamber, a multi-channel peristaltic pump (that allows for several perfusion circuits to operate in parallel) and the tubing for continuous circulation of medium, to provide oxygen and nutrients during cultivation; the gas exchanges occur through a 0.2 µm sterile filter. The sampling circuit consists on the apparatus that retrieves samples from within the cell 3D platforms in a non-invasive manner; the samples are further analysed by biological parameter analysis, such as glucose/lactate measurement.

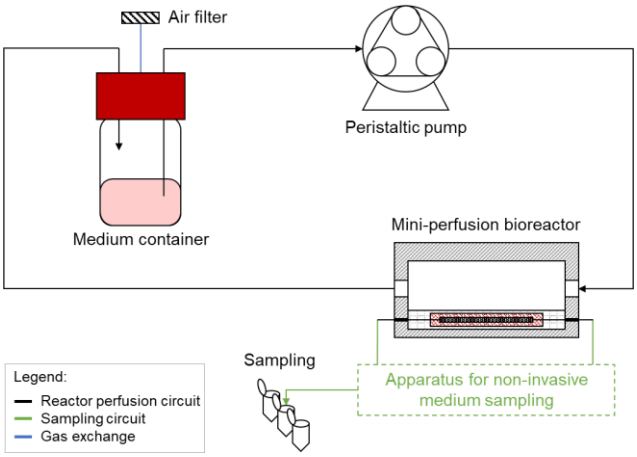

Figure 4 Circuit of a single perfusion bioreactor system. Each perfusion system comprises a medium container, a micro-perfusion reactor and a multi-channel peristaltic pump; the sampling system is composed by the apparatus to acquire samples from within the scaffold.

Transport dynamics in 3D platforms under perfusion

Prior to the cell cultivation, it is relevant to understand the transport of glucose/lactate inside the addressed 3D platforms - alginate hydrogel and collagen membranes. By assessing the transport of these molecules inside the platforms under perfusion and in the absence of cells, this

study aims to: i) compare the diffusional capacity between the different platforms; ii) evaluate the platforms capacity to supply glucose and retrieve lactate; and iii) aid in the discrimination of the glucose/lactate profile due to cell

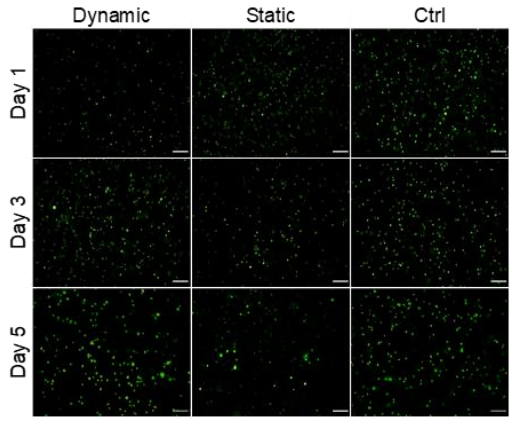

Figure 6 Calcein-AM/PI of alginate gels on days 0, 1, 3 and 5 of cell cultivation under perfusion (10 rpm) or static conditions or in well-plates (Ctrl). Calcein-AM stains live cells in green; PI stains dead cells in red. Scale bar represents 250 µm.

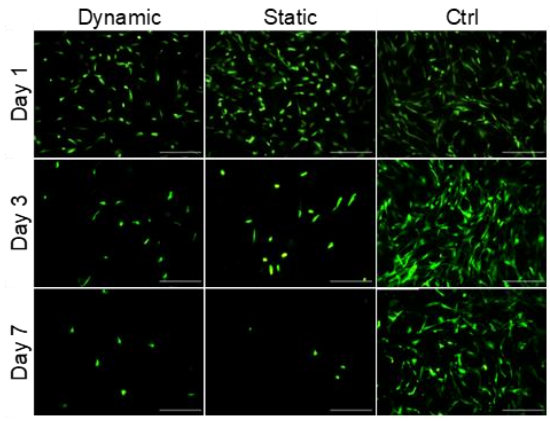

Figure 5 Calcein-AM/PI of the bottom of Matristypt® on days 1, 3 and 7 of cell cultivation under perfusion (10 rpm) or static conditions or in well-plates (Ctrl). Calcein-AM stains live cells in green; PI stains dead cells in red. Scale bar represents 250 µm

metabolism or diffusional transport when cultivating cells in the forthcoming experiments.

When the platforms were perfused with PBS, the retrieval of glucose and lactate was more efficient in the membranes compared to the alginate gel, and faster in MatriDerm® than in MatriStypt®. After changing PBS for medium, the reposition of the molecules was achieved with better ease in the membranes, moreover, concentrations equal to those in the medium being perfused were achieved within the membranes. The reposition rate of the molecules in alginate was lower than the membranes, and after 100 h of perfusion with medium, the molecules concentration within the gel were not able to meet those in the perfusion medium. These findings suggest that the collagen membranes have better diffusional capacities then the gel. (Results available in [14]).

Cell cultivation in 3D platforms under perfusion

First, an endpoint analysis via calcein-AM/PI was performed in alginate gel and MatriStypt® to allow an optical monitoring of the cell cultivation under perfusion. This has the advantage of allowing a relatively easy qualitative comparison between the different studied conditions, but presents a series of drawbacks: it obliges the frequent opening of the bioreactor, enhancing the risk of contamination; it is time and reagent consuming; and samples of gel/membrane have to be manipulated and sacrificed.

Alginate gels were casted at 100,000 cells/ml and cultivated for 5 days under perfusion (constant rate, 10 rpm) or static conditions. Gels were also standard cultivated in well-plates, to serve as controls; calcein-AM/PI staining was performed occasionally (Figure 5).

With regard to the cultivation inside the bioreactors, an increase of viable cells was observed in the gel cultivated under perfusion, while a decrease was detected in the gel of the static bioreactor. Under dynamic conditions, cells have access to a reposition of glucose and oxygen and retrieval of lactate within the gel, while in static conditions, cells might get deprived from the necessary nutrients, and a toxic amount of lactate might get accumulated within the gel, thus resulting in a loss of viability. The cell viability in the gel under perfusion was comparable to the standard cultivation platform (control), and advantageous compared to the gel cultivated in the reactor under static conditions. These findings indicate that coupling an alginate gel to the mini-perfusion bioreactor shows promise in MSC cultivation.

MSC were seeded (15,000 cells/cm^2) on MatriStypt® and cultivated for 7 days under perfusion (constant rate, 10 rpm) or static conditions. Membranes were also cultivated in a standard manner in well-plates, to serve as controls; calcein-AM/PI was performed occasionally (Figure 6).

The degree of cell viability throughout the 7 days of cultivation was comparable between the dynamic and static conditions, suggesting that the medium renewal did not offer an advantage to the perfused cells, nor inflicted cell damage. After one day of cultivation, the density of viable cells on the reactor membranes were similar to the controls, however from day 3 onwards there was a significant loss of viability in both conditions, accompanied by a loss of the fibroblast-like morphology characteristic of MSC.

The loss of viability might have resulted from the cultivating setting, membrane handling and manipulation when acquiring the samples, or even due to cell detachment from the membranes. The last hypothesis is unlikely, since the membranes were inserted in the reactors with their bottom facing the lid, preventing them from being released to the chamber. Relatively to the cultivation setting, the coupling of MatriStypt® with the perfusion reactor is not as promising as the alginate; cells cultivated in gels are likely more protected due to being encapsulated within a polymer chain, thus less susceptible to external hazards, such as medium flow or constant handling when performing cell sampling.

In order to circumvent the hurdles of performing calcein-AM/PI, or another endpoint analysis, a second approach was performed. This is based on taking samples of medium contained within the platforms In a non-invasive manner with the aid of the apparatus. The glucose and lactate concentrations in the samples were measured to infer about the cell metabolism, thus enabling to predict the cell

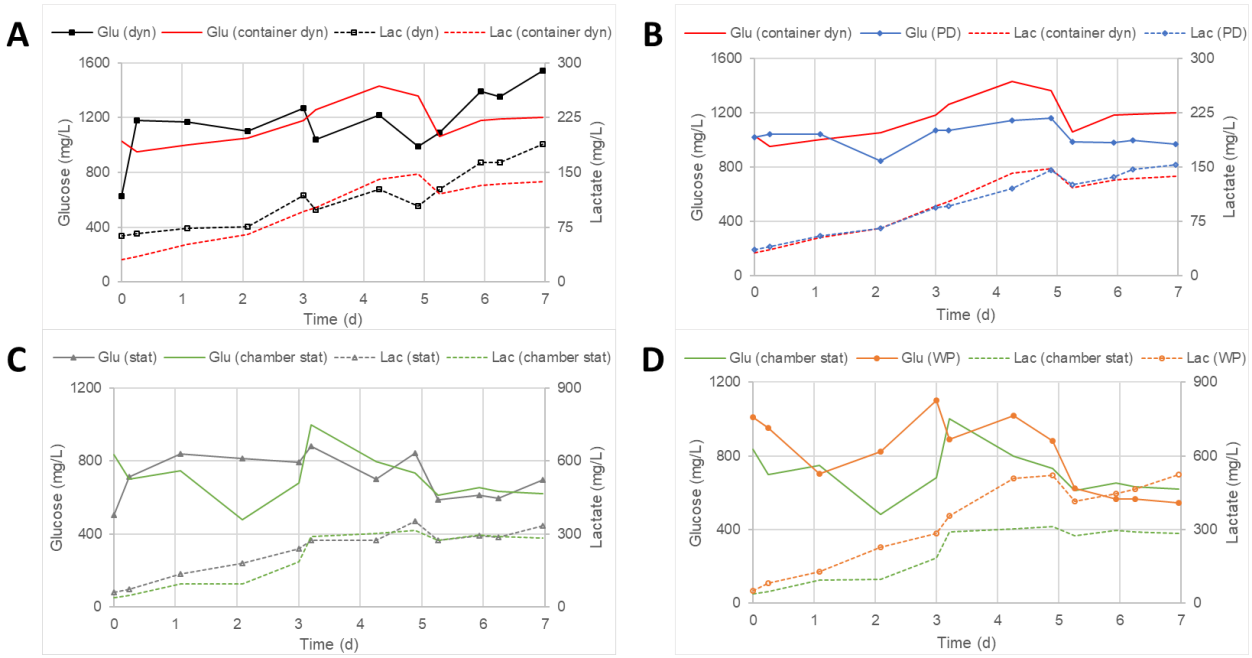

Figure 7 Glucose (Glu) and lactate (Lac) profiles throughout 7 days of cultivation in samples acquired with the apparatus from the between of MatriDerm® pairs cultivated (A) under perfusion (constant rate, 4 rpm) or (C) under static conditions. Samples of culture medium were acquired manually from: (A and B) the medium container of the perfusion circuit; (B) a petri dish with a pair of seeded membranes; (C and D) the reactor chamber of the static cultivation; and (D) a well-plate with a pair of seeded membranes.

viability. Compared to the first approach, this greatly decreases the risk of contamination, it is considerably less time and reagent consuming and most importantly, non-invasive. This approach was performed in all of the previously addressed 3D platforms: alginate gel, MatriStypt® and MatriDerm® (results only shown for the latter).

MSC were seeded (16,000 cells/cm^2) on MatriDerm® and cultivated for 7 days under perfusion (constant rate, 4 rpm) or static conditions. Two pair of membranes were also cultivated in a standard manner in a well-plate or a petri dish, to serve as controls. Medium samples were taken occasionally in a non-invasive manner with the apparatus (reactors), or manually (chamber of the static reactor, container of the perfusion circuit and controls) to monitor glucose/lactate (Figure 7); calcein-AM/PI was performed on the last day (Figure 8).

Attention was paid to cultivating the same number of cells in all conditions and elaborating appropriate controls to the cell constructs being cultivated in the reactors. A pair of membranes was cultivated in a petri dish containing 15 ml of medium, the same volume flowing in the perfusion circuit; a second pair of membranes was cultivated in a well-plate containing 3 ml of medium, the same volume inside the static reactor chamber. The variations of glucose/lactate in the container of the perfusion circuit and in the chamber of the static reactor were monitored and compared to those inside the petri dish and well-plate, respectively, which was possible since medium samples from these platforms were acquired using the same methodology (manual sampling with a micropipette).

Concerning the dynamic cultivation (Figure 7A), the concentrations of glucose and lactate between the MatriDerm® pair were similar to those of the medium container throughout the 7 days of the experiment, indicating that the flow rate of 4 rpm was such that allowed for the reposition of glucose and prevented the accumulation of lactate in the cell construct. Up to day 5, both glucose and lactate profiles indicate that cells were metabolically active since the former varied with a negative tendency, whereas the later varied positively. The notorious increase of these molecules from day 5 onwards is possibly due to the acquisition of samples by the apparatus, as there is no plausible explanation for that occurrence and the concentrations of these molecules in the medium container remained with the same tendency as before.

The increase of lactate in the medium container closely resembled the increase of this molecule in the respective control (Figure 7B); the resemblance of the glucose variation was not as evident. While in the control there was a slight tendency for a decrease in glucose, in the medium container of the perfusion circuit the opposite was observed, which might be explained by a stronger degree of evaporation in the container than in the control. The consumption of different C-sources, such as glutamine, by the perfused cells; or a higher metabolic activity in the consumption of glucose by the cells in the control, compared to the cells cultivated dynamically, might as well have contributed to that occurrence. These findings indicate that the cultivation of seeded MatriDerm® under perfusion at a constant flow of 4 rpm inside the mini-perfusion reactor allows for a sufficient supply of glucose and retrieval of lactate that surpasses the metabolic rate of the cells;

furthermore, cells remain as metabolically active as in a standard cultivation setting, and presumably with a similar degree of cell viability.

Regarding the static cultivation inside the reactor (Figure 7C), the initial increase of glucose inside the cell construct suggest that the fresh medium was able to cross through the membranes and supply glucose (up to 800 g/L), but naturally in less extent compared to the dynamic cultivation (1000 g/L). From day 1 onwards, the glucose diminished at a constant rate, both in between the pair of membranes and in the reactor's chamber, suggesting that glucose was being consumed by the cells. As expected, due to the lack of fluid motion, the accumulation of lactate within the cell construct could not be prevented in a static setting, with values of 300 mg/L being reached by day 3, about three times more compared to the same period in the dynamic setting, thereby indicating an advantage of cultivating cells under perfusion inside the micro-perfusion reactor. The increase of lactate up to day 3 indicates that cells were metabolically active, thus viable. However, from day 3 onwards, the variation of lactate stagnated, indicating that the cells became metabolically inactive and presumably death. The fact that the concentration of glucose remained constant from day 5 onwards supports the addressed hypothesis of cell death.

As observed in the control of the static reactor (Figure 7D) a more intense decrease of glucose (up to 600 mg/L) and increase of lactate (up to 500 mg/L) compared to the static cultivation in the reactor did not prevent cells from being metabolically active and viable. Hence, the morbidity observed inside the static reactor is likely related to the depletion of oxygen inside the reactor, since a lower air volume was available in that static platform compared to the well-plate.

These findings suggest that, when cultivating seeded MatriDerm® inside the reactors, it is preferable to perfuse the cells rather than cultivating them under static conditions.

As depicted in (Figure 8), a comparable degree of viability was visible between both controls, as well as the maintenance of the fibroblastic-like morphology characteristic to MSC after the 7-day period of cultivation. Compared to the controls, a lower viability and a loss of the fibroblastic morphology were detected in the pair of membranes cultivated inside both reactors.

Viable cells were visible in both membrane pairs cultivated inside the reactors, but while there were intense green-coloured cells in the pair cultivated under perfusion, in the pair cultivated under static conditions the cells were stained with a dim colour. This suggests a higher degree of viability in the perfused membranes, probably due to the constant supply of nutrients and oxygen and retrieval of lactate.

In contrast, a higher density of cells was detected in the membranes cultivated under static conditions inside the reactor, indicating that the flow of the dynamic cultivation might have caused some cells to detach. Furthermore, the presence of bright cells on the surface of the perfused membranes interfered with the detection of cells further within the fibrous mesh, due to contrast properties of the software responsible for documenting the cell samples.

MSC cultivated in 3D scaffolds were shown to present two distinct forms of adhesion, either flattened, in which cells firmly adhere to a single section of the scaffold, resembling planar culture; or bridged, in which cells attach to multiple scaffold components, thereby exhibiting a 3D conformation [15, 16]. When under perfusion, flattened cells are subject to shear stress at their exposed surface similar to those cultivated in planar culture, whereas bridged cells sense the flow perpendicularly to their cytoskeleton and therefore, are more sensitive and susceptible to the effects of fluid-induced shear forces [15, 16].

The fact that the calcein-AM/PI staining showed a loss in the cell number (Figure 8), while the glucose/lactate monitoring suggested that cells were viable inside the dynamic reactor (Figure 7), might be explained by the detachment of cells that were more susceptible to the flow, such as bridging cells/or cells closest to the surface. Moreover, the loss of the fibroblastic-like morphology of the cells on the membrane surfaces under perfusion contributes to this hypothesis, that only flattened cells did remain attached to the surface even though the harsh conditions. These results are evidence that several cell characteristics dependent on mechanical forces and stimuli, such as adhesion and proliferation, are more variable and complex to interpret in 3D scaffolds under perfusion than those in conventional 2D platforms.

With regard to the effect of the position of the membranes in the reactors, there was no apparent difference in the cell viability between the membranes facing the lid or the chamber within the dynamic reactor. As with the static reactor, a higher cell density was detected on the membrane in the chamber side, which is to be expected since the closer to the lid, the lesser the availability of oxygen and nutrients due to diffusional limitations within the membranes.

Interestingly, MatriDerm® showed the capacity for the cells to migrate across its collagen-elastin mesh, since green-coloured cells were visible in both surfaces in all cultivation conditions (results not shown).

Globally, these results show promise in cultivating MSC in MatriDerm® inside the mini-perfusion reactor coupled to an apparatus for a non-invasive medium sampling. MatriDerm® shows a decent diffusional capacity

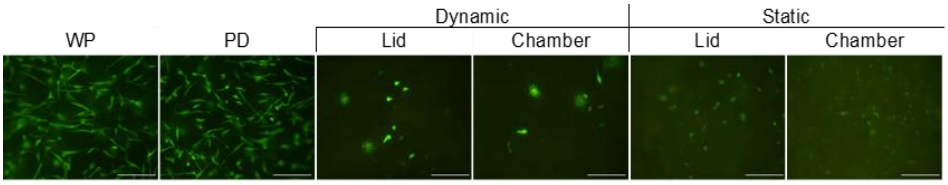

Figure 8 Calcein-AM/PI of the top of MatriDerm® on day 7 of cultivation under perfusion (4 rpm) conditions or statically in a well-plate (WP) or in a petri dish (PD). Calcein-AM stains live cells in green; PI stains dead cells in red. Scale bar represents 250 µm.

and enables cells to migrate within its fibers, even when under perfusion. The monitoring of the medium inside the container of the perfusion circuit, in addition to the monitoring of the medium within the cell constructs, aids in the elucidation of how the samples acquired with the apparatus allow to predict the cell viability. Further studies using optimized experiment settings should be performed to better understand the relation between the profile of glucose/lactate within the cell constructs and the cell viability. In addition, it would be interesting to exploit different conditions, such as different flow rates, to evaluate its impact on the cell detachment, in order to improve the dynamic cultivation inside the mini-perfusion reactor.

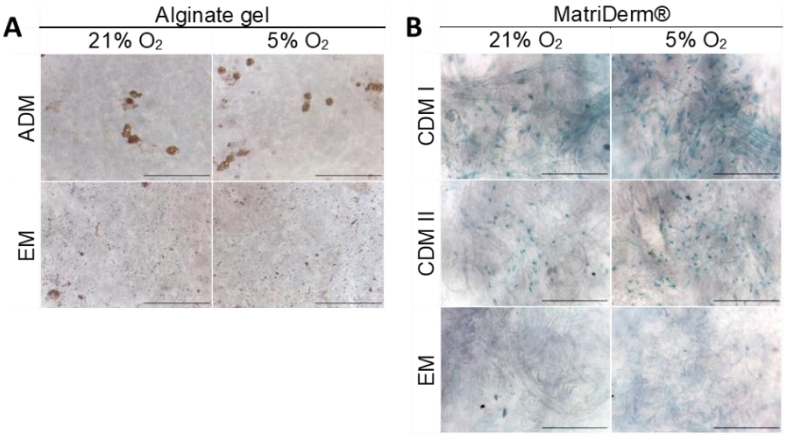

Figure 9 (A) Oil Red O at day 21 on adMSC cultivated in alginate gel in adipogenic differentiation medium (ADM) or expansion medium (EM). Oil Red O stains lipid vacuoles in red. (B) Alcian Blue at day 21 on adMSC cultivated in MatriDerm® in manufactured or handmade chondrogenic differentiation medium (CDM I and II, respectively), or EM. Alcian Blue stains glycosaminoglycans in blue. Cells were cultivated under 21% O_2 or 5% O_2 conditions. Scale bar represents 250 µm.

Adipose-derived MSC differentiation

One of the aims of coupling the mini-perfusion bioreactor to the apparatus is not exclusively to non-invasively monitor 3D cultivation of cells, but also monitor tissue cultivation.

The trilineage differentiation of adMSC was performed in planar culture, to infer if they satisfy one of the minimal criteria that characterize MSC, and in 3D platforms, with the aim of evaluating the capacity of adMSC to differentiate in those platforms and generate a functional tissue to be used in future studies inside the novel monitoring device, and ultimately, create an *in vitro* model. The impact of the oxygen concentration and media formulations on the adMSC differentiation potential were assessed.

In the 2D differentiation (results available in [14]), chondrogenesis was not detected, either due to low cell densities that resulted in a negligible production of ECM (Alcian Blue stains glycosaminoglycans characteristic of the cartilage ECM), inappropriate Alcian Blue staining methodology, or due to a partial trilineage differentiation capacity of this adMSC cell line.

Lipid droplets were detected in cells cultivated in the adipogenic medium, demonstrating that adMSC are capable to differentiate into the adipogenic lineage. There was no apparent dependence of adipogenesis on the oxygen supply, which stands in agreement with a body of literature that reported no relevant effect of hypoxia on the adipogenic potential of MSC [17, 18, 19].

The osteogenic differentiation was favoured under hypoxia in the manufactured osteogenic medium (ODM I), whereas only normoxic conditions did allow the differentiation in the handmade osteogenic medium (ODM II). These findings demonstrate that, in addition to the impact of the oxygen tension, adMSC differentiation is dependent on several other cultivation conditions, with a critical factor being the composition of the differentiation culture medium.

In general, the staining protocols to detect the trilineage differentiation of MSC in planar culture proved not to be suitable for the addressed 3D platforms. Nevertheless, golden lipid droplets were visible in alginate gel cultivated in adipogenic medium upon Oil O Red staining (Figure 9A), confirming adipogenesis. In turn, chondrogenesis and osteogenesis were observed in MatriDerm®.

Alcian Blue staining (Figure 9B) was detected and distinguishable between both chondrogenic media and the control. Regarding the media, the amount of glycosaminoglycan was more pronounced in the samples cultivated with the manufactured chondrogenic medium (CDM I). As for the oxygen supply, hypoxia favoured the chondrogenic potential of adMSC, independently of the chondrogenic medium used, since in both CDM there was a more intense blue colorization in samples cultivated under 5% O_2 compared to those cultivated in 21% O_2. This result is in accordance to what is globally accepted by the scientific community, that chondrogenesis is supported by hypoxia [20, 21, 22].

Although not as evident as the aforementioned stainings, the Calcein/DAPI staining on MatriDerm® enabled the detection of calcium deposits, does indicating osteogenesis (results not shown).

In order to facilitate the detection of MSC differentiation in 3D platforms, more adequate experiment settings are necessary. For instance, an optimization of the fixation, staining and rinsing protocols for each platform; perform different methodologies to assess the differentiation, such as the examination of the expression of specific markers to each lineage; or the usage of 3D platforms that permit a clear optical evaluation and do not interfere with the methodology of analysis.

These results show the feasibility of differentiating MSC in the considered 3D platforms and to possibly generate, in the future, a functional tissue construct to be cultivated inside the novel monitoring device or to serve as an *in vitro* model.

4. Conclusion and future prospects

Endpoint analysis of a cell culture, such as the widely employed calcein-AM/PI staining, requires the frequent handling of cell samples, which enhances the risk of contamination, influences the cultivation conditions and sacrifices cells; furthermore, it is time, reagent and labour consuming.

The present work confirms the feasibility of cultivating 3D cell constructs inside a mini-perfusion bioreactor connected to an apparatus to acquire medium samples from within the scaffolds. The measurement of glucose and lactate in those samples enables a non-invasive monitoring of the cultivation of cell constructs under perfusion, demonstrating a great advantage compared to endpoint analysis.

Additional experimentation should be conducted to optimize this non-invasive 3D monitoring system, notably: exploit tissue cultivation, evaluate different biological parameters in the samples - such as oxygen tension, pH and specific expression markers - implement devices enabling the real-time measurement of those parameters, and tackle different experiment settings - such as flow rate and oxygen supply. This would significantly contribute for the improvement and establishment of an advanced and standardized non-invasive monitoring device for 3D cell and tissue cultivations.

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