Micro structured tools for cell modeling in the fourth dimension

Raimondi, Manuela, Barzaghini, Bianca, Bocconi, Alberto, Conci, Claudio, Martinelli, Chiara, et al.

Manuela T. Raimondi, Bianca Barzaghini, Alberto Bocconi, Claudio Conci, Chiara Martinelli, Alessandra Nardini, Carolina Testa, Stephana Carelli, Giulio Cerullo, Giberto Chirico, Riccardo Gottardi, Roberto Osellame, Andrea Remuzzi, Matteo Laganà, Emanuela Jacchetti, "Micro structured tools for cell modeling in the fourth dimension," Proc. SPIE 11786, Optical Methods for Inspection, Characterization, and Imaging of Biomaterials V, 117861T (20 June 2021); doi: 10.1117/12.2593332

SPIE.

Event: SPIE Optical Metrology, 2021, Online Only

Micro structured tools for cell modeling in the fourth dimension

Manuela T. Raimondi*[a,h,i,j], Bianca Barzaghini[a], Alberto Bocconi[a], Claudio Conci[a], Chiara Martinelli[a], Alessandra Nardini[a], Carolina Testa[a,b], Stephana Carelli[e,f], Giulio Cerullo[c], Giberto Chirico[g], Riccardo Gottardi[h,i,j], Roberto Osellame[c,d], Andrea Remuzzi[k], Matteo Laganà[l] and Emanuela Jacchetti[a]

[a]Dept. of Chemistry, Materials and Chemical Engineering "G. Natta", [b]Dept of Electronics, information and Bioengineering, [c]Dept. of Physics and [d]Istituto di Fotonica e Nanotecnologie IFN-CNR, Polytechnic University of Milan, 32 Piazza L. da Vinci, 20133 Milano, Italy; [e]Department of Biomedical and Clinical Sciences "L. Sacco" and [f]Pediatric Clinical Research Centre Fondazione "Romeo ed Enrica Invernizzi", University of Milan, Via Grassi 74, 20157 Milan, Italy; [g]Dept. of Physics "G. Occhialini", Bicocca University of Milan, Room 4044, Building U01, 1 Piazza della Scienza, 20126 Milano, Italy; [h]Dept. of Pediatrics, Perelman School of Medicine and [i]Dept. of Bioengineering, School of Engineering and Applied Sciences, University of Pennsylvania, [j]Children's Hospital of Philadelphia, Room 1016G, Abramson Research Center, 3615 Civic Center Blvd, Philadelphia, PA USA 19104; [k]Dept. of Management, Information and Production Engineering, University of Bergamo, via Salvecchio 19, 24129 Bergamo, Italy; [l]Gemma Prototipi Studio, Longone al Sedrino (CO), Italy

ABSTRACT

We describe several bioengineered cell models developed by our group. We develop tools for cell culture allowing fluorescence diagnostics on the cellularised constructs cultured within, both in 3D and prolonged culture times extending to several weeks. These cell models proved able to recapitulate *in vitro* several slowly developing biological processes such as the regeneration of a cartilaginous tissue by cartilage cells, the formation of a bone metastasis by breast cancer cells, the instruction of adaptive immune cells as occurs in a lymphnode, and the neuroprotective effect on pathological neurons of mesenchymal stem cell secretome. We also scaled down these tools in the aim to better control stem cell function in our models, by applying two-photon laser polymerization to fabricate micro scaffolds for stem cell expansion. We were able to condition mesenchymal stem cells, neural precursor cells and embryonic stem cells towards maintenance of a greater stemness and multipotency/pluripotency, compared to conventional flat culture. This result opens an avenue towards a safer use of these cells for stem cells therapies. Finally, we describe our new revolutionary concept of implanting the cell model in a living organism to regenerate a vascularized network anastomosed to the host, allowing for studies involving interactions with the host immune system.

Keywords: bioengineering, cell modeling, mechanobiology, optical imaging, microfluidics, metastasis, neurodegeneration, stem cell niche

*manuela.raimondi@polimi.it; phone (USA) 001 215 398 8536, (Italy) 0039 338 5756238; nichoid.polimi.it

1. CELL MODELLING IN SPACE AND TIME

Our group aims at engineering cell models able to recapitulate slowly developing biological processes. Years ago, we started with modelling tissue regeneration *in vitro* [1]. The engineering of a tissue *in vitro* is a very slow process that requires several weeks of culture before an extra-cellular matrix can develop and fill the scaffold pores and remodel to acquire aspects of functionality. We could analyze these three-dimensional (3D) constructs only at the end of several weeks of culture and we were desperately seeking a solution to monitor tissue development non-destructively during long-term culture.

Optical Methods for Inspection, Characterization, and Imaging of Biomaterials V, edited by Pietro Ferraro, Simonetta Grilli, Monika Ritsch-Marte, Christoph K. Hitzenberger, Proc. of SPIE Vol. 11786, 117861T · © 2021 SPIE · CCC code: 0277-786X/21/$21 · doi: 10.1117/12.2593332

1.1 The Miniaturized Optically Accessible Bioreactor (MOAB)

To advance the field, we incorporated a perfusion bioreactor on a microscopy slide [2]. The platform consists of a high-tech microscopy slide with three culture chambers built on it, allowing to inspect cellularised constructs non-destructively in fluorescence at various time points of perfused culture. For example, collagen secreted by cartilage cells can be visualized on live cells under perfused culture using an intravital fluorescent label (Figure 1).

Optical accessibility is guaranteed in the platform both in standard and in confocal microscopy, both in straight and inverted set-up. The main advantages of this approach are: 1) easiness of opening/closing of each chamber, possible on a repetitive basis; 2) modularity of the culture chambers in terms of geometry, dimensions, independency; 3) modularity of the operative conditions in terms of chamber connection, chamber number, independency of the cultured cell model in each chamber; 3) low-cost mass production by injection molding of plastics, allowing for a single-use of the bioreactors, despite the high performance.

To monitor the local environment of cultured cells in tissue-engineered constructs, we used computational modelling to predict mass transport of nutrients and oxygen in the system, in the aim to control tissue development in function of these parameters over long culture times, up to several weeks. For example, we modeled tissue development in function of mass transport coupled to the fluid dynamic environment acting on the cultured cells [3,4]. Using a system for oxygen monitoring, we were also able to monitor oxygen concentration inside the culture chambers at the individual cell scale [5]. In this way, optical accessibility at a high resolution and control over culture parameters over long culture times was finally guaranteed in cellularised constructs engineered *in vitro*, both in space and time (the fourth dimension). Below we describe some main applications of this approach that we used to model slowly developing biological processes *in vitro*.

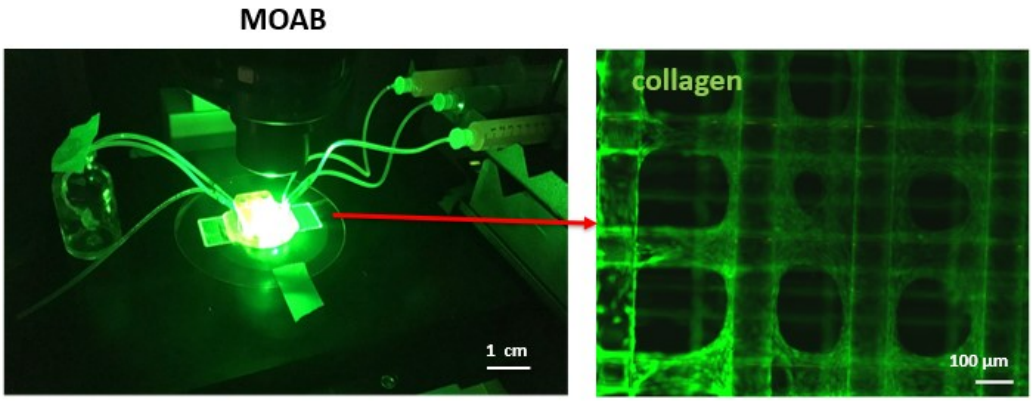


Figure 1. The MOAB cell culture platform mounted on an upright fluorescence microscope. (Left) Set-up of an experiment of engineering of cartilaginous tissue. (right) Real-time monitoring of cell secretion of collagen by cartilage cells. The MOAB is and the tissue under development is stained with 5-(4,6-dichlorotriazinyl) amino fluorescein (5-DTAF), the product of the reaction of amino fluorescein with cyanuric chloride, a nonspecific amine-reactive fluorescent dye for visualizing collagen fibers in live tissue, both for in vivo healing experiments and for in situ mechanical studies.

1.2 The bone perivascular niche-on-a-chip to study metastatic colonization and to test anticancer agents

We had the opportunity to test the MOAB platform in the field of cancer research in various applications. Initially, we were interested cancer cell visualization and selective cell capture. We used direct photo-patterning of hyaluronic acid baits onto a fouling release surface, for selective visualization and capture of CD44-positive cancer cells [6]. More recently, we had the opportunity to model a slowly developing process related to cancer: the formation of a bone metastasis by breast cancer cells. Metastasis to distant organs is the leading cause of death in many cancers (e.g. breast, prostate). Disseminated breast cancer tumor cells leave the blood stream through the microvasculature and colonize

distant organs like the bone marrow. Endothelial cells and bone marrow stem cells mediate tumor cell proliferation in the perivascular tumor niche [7].

The MOAB allowed us to add the effect of interstitial flow to the existing *in vitro* models. We found that interstitial fluid flow up-regulates endothelial sprouting. Endothelial cells self-assembled in a loose vascular bed in static culture, and in a more dense vascular bed when perfused, likely by increased convective transport of angiogenetic signals [8]. We were able to visualize both the green-fluorescent cancer cells and the red-fluorescent endothelial cells in fluorescence microscopy under bioreactor culture. In this model of metastatic niche, we measured a significant increase in cancer cell survival without flow, compared to what measured with flow, specifically when cancer cells where put in co-culture with stromal cells and endothelial cells.

Our most important finding was that cancer cells proliferated less under interstitial flow but presented a significantly greater resistance to traditional chemotherapeutic drugs like Sunitinib, likely because this drug targets proliferative cells. Sunitinib is in fact a chemotherapeutic agent targeting receptor tyrosine kinases (including VEGFR, PDGFRβ, c-KIT) in proliferative cancer cells, failing to inhibit breast cancer cells exposed to interstitial flow. The MOAB can thus be successfully used for testing chemotherapy drugs against bone metastases.

1.3 The lymphnode-on-a-chip to study adaptive immunity and to test cancer immunotherapies and vaccines

Another slowly developing class of interactions are typical of our adaptive immune system. For example, dendritic cells flow in a lymphnode, adhere to reticular cells and express receptors of T-cells to make them join and instructed them. This process of lymphnode instruction can be modelled in the MOAB by seeding yellow-fluorescent stromal cells on a scaffold, then by introducing dendritic cells suspended in the perfused culture medium. Dendritic cells can be visualized while adhering to the fibroblast reticular cells, and while migrating on the scaffold. Furthermore, their activation can be visualized by inserting a fluorescent protein gene on the promoter of the gene encoding for the T-cell receptor ICAM-1. When cyan-fluorescent antigen-specific T-cells were subsequently introduced with flow, we monitored that they tended to adhere to the dendritic cells while crawling on the scaffold [9]. This lymphnode instruction model can be used to test cancer immunotherapies and vaccines, that are both therapeutic approaches with slowly developing effects.

1.4 The perineural niche-on-a-chip to study neurodegeneration and to test neuroprotective therapies

Another very slowly developing process is chronic neurodegeneration. The MOAB can be used to model in vitro the 3D cell microenvironment in long term chronic neurodegeneration, to test the effect of frontier therapies [10]. For example, it's an effective tool to model the neuroprotective effect of mesenchymal stem cell secretome on neuronal-like cells subjected to oxidative stress [11]. To model this process, we inserted human SHSY-5Y neuron-like cells with an oxidative damage resembling the one induced by Alzheimer's disease, in adhesion on a polystyrene scaffold. We cultured these neurons for several days under conditioning with neuroprotective factors secreted by bone marrow cells. We could measure a significantly higher viability of the conditioned neurons, suggesting a neuroprotective action exerted by the secretome of bone marrow cells [12].

More complex mechanisms of neurodegeneration can be modelled by connecting multiple devices representing separate compartments of a functional "axis" like the microbiota-gut-brain axis that connects microbiota, gut, immune system, and brain [13]. In this way, we can recapitulate *in vitro* very complex mechanisms that involve very far *apparata* of the entire human organism, for example the effect of microbiome-secreted neurotoxins on the development of Alzheimer's disease [14] and relevant therapeutics.

2. SCALING DOWN TO THE INDIVIDUAL CELL SCALE

Our original curiosity-driven question was: what are the parameters influencing cell response that end up in the regeneration and remodeling of a tissue? The possibility to observe live cells in space and time (4D) led us to the formulation of a hypothesis to answer this question. My hypothesis was that when the cell modifies its morphology, for example while crawling on the scaffold in the MOAB, the nucleus senses such deformation through a direct mechanical link between the cell cytoskeleton and the nuclear pore complexes [15]. When the cell spreads, the nuclear envelope is

strained and this in turn activates the nuclear pore complexes that become more permeable to protein trafficking [16]. This mechanism increases the permeability of the nuclear envelope to the nuclear import of the signals called "transcription factors" involved in key master switches that dictate cell function such as the transformation of a stem cell into a differentiated cell.

We managed to predict theoretically the diffusive flux of a transcription factor with increasing cell deformation [17]. Initially, we formulated several multiphysics computational models in which we were able to couple the deformation of individual cells to the diffusive nuclear import flow of molecules, by assuming a strain-dependent diffusion coefficient at the nuclear membrane [18]. However, to experimentally validate this hypothesis we had to control the morphology of the cell nuclei in space and time, at the scale of individual cells.

Many lessons learned *in situ* from studies on stem cell niches dictated the design specifications for the cell culture substrate. Firstly, we needed to isolate the mechanical contribution of cell deformation from the contribution of biochemical signals in a well-defined mechanical environment, structurally and chemically stable in space and time. Therefore, we excluded the use of exogenous biochemical signals, biodegradable 3D scaffolds and hydrogels [19]. Also, stem cells self-organize spontaneously in space and time by using autocrine and paracrine signaling [20], thus we also needed to maintain cells in 3D contact between each other to allow for physiologic signaling to take place. Thus, we excluded the use of single-cell manipulation techniques like single-cell adhesive substrates and wells, and optical tweezers. Finally, we needed to monitor nuclear entry of signals that regulate gene expression, so-called transcription factors, in space and time.

2.1 The NICHOID culture substrate

We managed to satisfy all the design specifications detailed above by fabricating micro scaffolds with a technique called two-photon laser polymerization. This is a micro stereolithography technique in which polymerization is induced in hybrid organic-inorganic photopolymers based on Silicon and Zirconium (for example, SZ2080). A femtosecond-pulsed laser beam induces two-photon adsorption in the resin, which polymerizes in a volume very close to the focus. The sample can be moved in space while keeping the laser on focus and this allows to literally "write" the microstructure in the resin with an astonishing resolution down to 100nm. The advantages with this technique over others are that the geometries micro-fabricated are totally arbitrary [21,22]. Also, several photo resins available for two-photon polymerization are inert, hydrophilic thus highly biocompatible [23].

Thanks to this microfabrication technique, we invented the NICHOID micro scaffold (Figure 2), a microscopic lattice able to maintain cell adhesion in 3D in a very isotropic configuration, allowing full cell-to-cell contact and physiologic paracrine signaling. The acronym was conceived to sound like "organoid" but with an emphasis on recapitulating the anatomical stem cell niches present in the tissue. Initially, it took great efforts to reduce the autofluorescence of the material to allow for the visualization of fluorescently labelled cells. We changed the photo initiator component in the photopolymer and finally solved this problem [24].

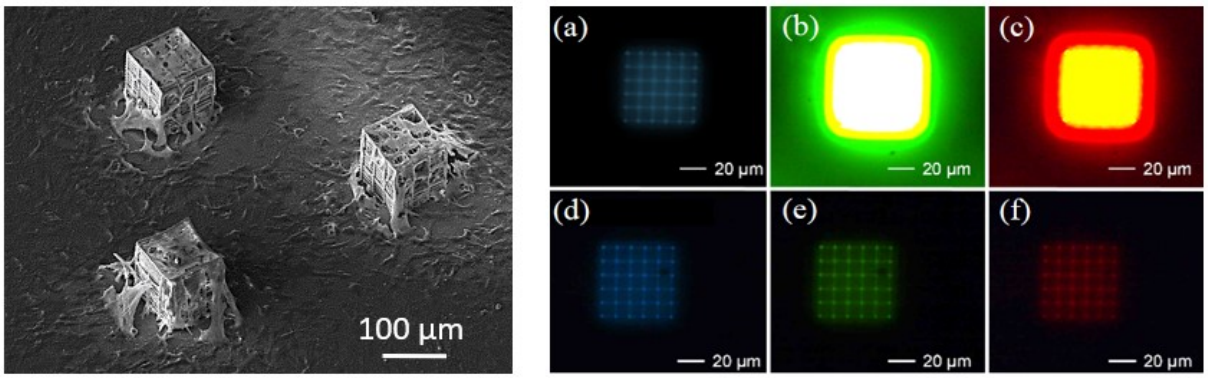


Figure 2. (Left) The NICHOID micro scaffolds seeded with adhering mesenchymal stem cells and imaged in scanning electron microscopy at 3 weeks of expansion. Mesenchymal stem cell density was 50% greater in the NICHOID (0.18 million cells/cm^2)

compared to flat (0.12 million cells/cm^2). (Right) Autofluorescence in the micro-scaffolds formed with Irg (bottom row) photo initiator is optimized compared to Bis (top row) in channels (a,d) blue (DAPI), (b,e) green (FITC) and (c,f) red (TRITC). Adapted from [24].

2.2 The mesenchymal stem cell niche-on-a-chip

The NICHOID was conceived as a cell adhesion substrate able to mimic *in vitro* the physical and geometric constraints present in the native stem cell niche in order to control stem cell shape, thus its function, during *ex vivo* culture. We found that mesenchymal stem cells migrated in the NICHOID micro scaffolds and tended to colonize. The substrate induced a 50% higher cell density than flat controls; cells that migrated in the micro scaffolds tended to adhere and home inside the scaffold lattice [25]. Most importantly, human mesenchymal stem cells maintained a significantly greater stemness and multipotency, as compared to flat-cultured controls, when adhering to the micro scaffolds [26].

Indeed, with fluorescence recovery after photobleaching (FRAP) measurements we confirmed our hypothesis that cell spreading was a primary factor determining the permeability of the nucleus to MyoD, a transcription factor involved in mesenchymal stem cell differentiation [2728]. We found this transcription factor significantly more localized in the nucleus of cells cultured on flat with respect to cells cultured in the micro scaffolds. Most importantly, we were able to visualize the fluorescence recovery of GFP-MyoD, a parameter proportional to the nuclear flow, on the entire nuclei in space and time in the two conditions, NICHOID and flat. We discovered that in the NICHOID, the import flow of this transcription factor is slower and is maintained very homogeneous in time, because the particles flow inside the nucleus from all the directions in space. On flat-cultured controls, instead, not only the import flow is faster, but it occurs preferentially from the peripheral areas of the nucleus. We concluded that in flat conditions, the layer of cytoplasm in contact with the cell nuclear top and bottom surfaces is very thin, likely providing for limited transcription factor availability. Thus, the import flows of transcription factors through the nuclear membrane occur primarily from a lateral ring-shaped surface. We called this the “effective surface” in transport.

With regard to the intracellular forces developed by mesenchymal stem cells adhesion we found that, compared to flat, cells adhered to the internal lattice of the NICHOID with reduced expression of structural proteins that form the focal adhesions, including beta actin and vinculin, as shown by our co-localization studies. Also, the number and dimension of the focal adhesions were significantly lower that on flat-cultured cells. This 3D adhesion configuration induced a significantly more spherical nuclear shape in NICHOID-cultured cells, compared to the discoidal shape of flat-cultured cells. Thus, the micro scaffold was able to release the tension from the cell nuclei and this induced a very significant effect on cell gene expression [29].

Specifically, the NICHOID promoted spontaneous inhibition of MSC differentiation [30] and up-regulation of stemness genes, e.g. the leukemia inhibitory factor (LIF), and cytokines such as interleukin 6 (IL6) and colony-stimulating factor 3 (CSF3) [31] that promote function and mobilization of hematopoietic stem cells [32]. This result is coherent with the current knowledge on the architecture of the native hematopoietic stem cell niche, in which mesenchymal stromal cells show a typical stellate morphology in 3D, with few thin extended extrusions that contact the mature hematopoietic stem cells, likely regulating primary aspects of their function [33].

Mesenchymal stem cells are increasingly used in stem cell therapy. They are found primarily in bone marrow and adipose tissue in the adult, can differentiate in many cell types including bone, cartilage, fat [34]. There are currently 300 major clinical trials underway worldwide for conditions such as ischemic cardiomyopathy, spinal cord injury, lung cancer, type 1 diabetes, and stroke. Due to the very limited survival rate of mesenchymal stem cells after transplantation, many recent approaches focus on the delivery of their “secretome” containing the signals secreted by cells. These signals are already proven to control inflammation, promote angiogenesis, and to contrast fibrosis while promoting healing processes for several tissues like cartilage and neural tissue.

A critical issue in this regard is the tendency of these cells to lose stemness and multipotency, thus function, when expanded *in vitro* in order to obtain clinically significant cell dosages. Considering the multipotency reprogramming effect of the NICHOID and its potential in therapeutic applications, we scaled up the fabrication of the micro scaffolds in the aim to extend their use to expand mesenchymal stem cells and other typologies of stem cells, as well. We used multi-foci parallelization with a spatial light modulator (SLM) that basically splits the incoming single laser beam into multiple

laser beams that are all focused on one sample at the same time. After ten years of optimisation, we are now able to cover a surface of one squared cm with ten thousand micro-lattices in only two hours of continuos laser writing [35,36].

2.3 The embryonic stem cell niche-on-a-chip

Pluripotent cells obtained by genetic reprogramming from adult cells have a potential to repair tissues in many clinical settings such as retinal damage, spinal cord injury and cardiac injury. The translation of these pluripotent cells into the clinics is far away due to the risk of teratoma formation, a major obstacle to pluripotent stem cell-based therapeutics. Pluripotent cells are currently mainly exploited as models for drug discovery and toxicity screening, but they tend to spontaneously differentiate when expanded [37]. However, given our impressive results on mesenchymal stem cells, we tested the ability of the NICHOID to maintain cell stemness and pluripotency during expansion also in pluripotent cells [38,39]. We cultured mouse embryonic stem cells in the NICHOID without chemical conditioning. Cells simultaneously maintained the Octamer4 pluripotency gene switched on and several differentiation genes switched off [26]. Others have found similar effects thereon [40]. The NICHOID can thus be regarded as a new method for at least partially reprogramming pluripotency in cells in view of their use as a frontier research tool in biology.

2.4 The neural progenitor cell niche-on-a-chip

We tested the micro scaffolds on a cell model with a higher potential than embryonic stem cells to be translated to the clinics: neural progenitors [41]. We expanded erythropoietin-secreting neural progenitor cells (NPCs) on the NICHOID and, consistently with our previous results on mesenchymal stem cells and on embryonic stem cells, we demonstrated a significant upregulation of pluripotency genes and downregulation of differentiation genes after expansion, compared to conventional culture [42]. Furthermore, NPCs detached from the NICHOID retained a memory of pluripotency induction. Most importantly, we tested the safety and efficacy of these cells *in vivo* in an experimental model of Parkinson's disease. We measured greater functional recovery indexes indicative of greater therapeutic power of cells grown inside the NICHOID. The presence in the animal brain of Tyrosine Hydroxylase, a key enzyme of dopamine synthesis, confirmed the behavioral data measured on animals [43].

3. CONNECTING THE CELL MODEL TO A VASCULAR NETWORK

No matter how complex is the mechanism under investigation and how complex is the platform that we conceive to model such a mechanism, an important limitation of most cell models is that the interstitial flow of culture medium is not anastomosed to the capillaries engineered within the construct. Microvascular networks regenerated *in vitro* can effectively interact with the other cell populations present in the cell model, but they are usually not perfused. This limits the possibility to model crucial biological mechanisms involving the intravasation and extravasation of cells, for example cancer cells, red blood cells or circulating cells of the immune system [44], and their interactions with the other cells forming the organoid in culture [45].

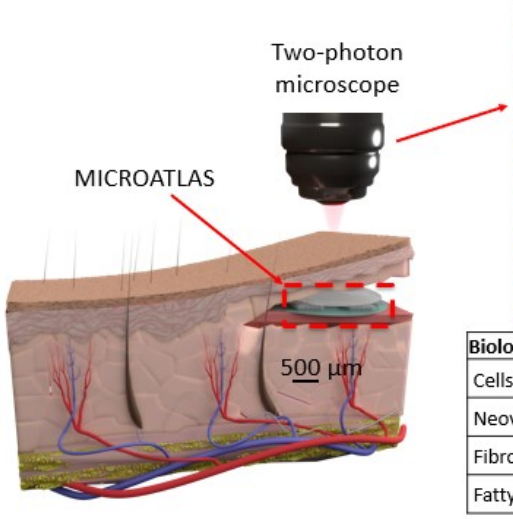
To anastomose micro vessels to a perfused system mimicking the human circulation, there are two possible approaches. One is to engineer the micro vessels *in vitro* and anastomose them to the circuit that perfuses the culture medium [21,46]. Our innovative approach, instead, is to implant the cell model in a living organism, for example a mouse. The main advantage of this approach is that the immune system of the animal can interact in a realistic way with the cell model. Other advantages are that the cell model can be miniaturized and thus the platform can be minimally invasive and potentially optically accessible in multiphoton microscopy. In this case, a fluorescent ultra-precise scaffold can be exploited not only to guide vascular regeneration, but also for correction of optical aberrations during imaging.

3.1 The Microscopy Atlas (MICROATLAS)

The cell model can be implanted in a living organism [47]. Subsequently, it can be invaded by endothelial cells of the host and be revascularized while interacting with the host immune system. We recently invented this concept of the

MICROATLAS, a miniaturized imaging window that can be used for the intravital quantification of the host response to implantation of a heterologous tissue or a biomaterial, or to administration of a therapeutic agent. It's basically a miniaturized subcutaneous window chamber for time-lapse analyses (Figure 3). The device incorporates a micro scaffold for in vivo tissue regeneration, which also acts as a reference geometry, allowing to 3D reposition the field of view during repeated intravital observations.

Thanks to 3D correction of optical aberration, we were able to visualize and quantify, label-free, cell invasion, neovascularization and collagen synthesis in the micro-scaffold [48]. We obtained the first validation *in vivo* of our platform by implanting the MICROATLAS in the chorioallantoic membrane of an embryonated chicken egg cultured *ex ovo*. The circulatory system of the developing avian embryo has several vascular shunts found only during the embryonic stages. These vascular shunts allow systemic venous return entering the right atria to bypass the nonventilated lungs and they are called "chorioallantoic vessels".



Biological structure	Label-free emission
Cells	Autofluorescence
Neovascularisation	Autofluorescence
Fibrosis (collagen)	Second harmonic
Fatty Infiltrate	Third harmonic

Figure 3. The MICROATLAS cell culture platform implanted sub-cute and imaged *in vivo* in two-photon microscopy.

To date, with label-free two-photon microscopy we were able to visualize cells, microvascular networks and collagen in the micro scaffolds. We visualized effectively the micro vascular network regenerated in the scaffolds in green autofluorescence, and the collagen secreted by cells in blue autofluorescence, by label-free two-photon emission. We found a significantly greater density of blood vessels and collagen fibers in the scaffold, compared to the unimplanted areas of the chorioallantoic membrane surrounding the device. Further quantifications are in progress.

Acknowledgements.

European Research Council (ERC projects NICHOID, G.A. 646990, NICHOIDS, G.A. 754467, and MOAB, G.A. 825159); European Commission (FET-OPEN project IN2SIGHT, G.A. 964481); European Space Agency (ESA project NICHOID-ET, G.A. 4000133244/20/NL/GLC); National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs projects MOAB, G.A. NC/C01903/1 and NC/C019201/1); Italian Ministry of University and Research (MIUR-FARE project BEYOND, G.A. R16ZNN2R9K); Fondazione Social Venture Giordano Dell'Amore, Cariplo Factory, Politecnico di Milano, Fondazione Bassetti and Fondazione Triulza (S2P project 2021).

REFERENCES

[1] Raimondi, M.T., "Engineered Tissue as a Model to Study Cell and Tissue Function from a Biophysical Perspective," Curr. Drug Discov. Technol. **3**(4), 245–268 (2007).

[2] Laganà, M. and Raimondi, M. T., "A miniaturized, optically accessible bioreactor for systematic 3D tissue engineering research," Biomed. Microdevices **14**(1), 225–234 (2012).

[3] Raimondi, M. T., Causin, P., Laganà, M., Zunino, P. and Sacco, R., "Multiphysics computational modeling in cartilage tissue engineering," [Studies in Mechanobiology, Tissue Engineering and Biomaterials], Springer, 267–285 (2013).

[4] Nava, M. M., Raimondi, M. T. and Pietrabissa, R., "A multiphysics 3D model of tissue growth under interstitial perfusion in a tissue-engineering bioreactor," Biomech. Model. Mechanobiol. **12**(6), 1169–1179 (2013).

[5] Raimondi, M. T., Giordano, C. and Pietrabissa, R., "Oxygen measurement in interstitially perfused cellularized constructs cultured in a miniaturized bioreactor," J. Appl. Biomater. Funct. Mater. **13**(4), e313–e319 (2015).

[6] Credi, C., De Marco, C., Molena, E., Nava, M. M., Raimondi, M. T., Levi, M. and Turri, S., "Direct photo-patterning of hyaluronic acid baits onto a fouling-release perfluoropolyether surface for selective cancer cell capture and immobilization," Mater. Sci. Eng. C **62**, 414–422 (2016).

[7] Ghajar, C. M., Peinado, H., Mori, H., Matei, I. R., Evason, K. J., Brazier, H., Almeida, D., Koller, A., Hajjar, K. A., Stainier, D. Y. R., Chen, E. I., Lyden, D. and Bissell, M. J., "The perivascular niche regulates breast tumour dormancy," Nat. Cell Biol. **15**(7), 807–817 (2013).

[8] Marturano-Kruik, A., Nava, M. M., Yeager, K., Chramiec, A., Hao, L., Robinson, S., Guo, E., Raimondi, M. T. and Vunjak-Novakovic, G., "Human bone perivascular niche-on-a-chip for studying metastatic colonization," Proc. Natl. Acad. Sci. U. S. A. **115**(6), 1256–1261 (2018).

[9] Raimondi, M. T., Donnaloja, F., Barzaghini, B., Bocconi, A., Conci, C., Parodi, V., Jacchetti, E. and Carelli, S., "Bioengineering tools to speed up the discovery and preclinical testing of vaccines for SARS-CoV-2 and therapeutic agents for COVID-19," Theranostics **10**(16), 7034–7052 (2020).

[10] Izzo, L., Tunesi, M., Boeri, L., Laganà, M., Giordano, C. and Raimondi, M. T., "Influence of the static magnetic field on cell response in a miniaturized optically accessible bioreactor for 3D cell culture," Biomed. Microdevices **21**(1) (2019).

[11] Chierchia, A., Chirico, N., Boeri, L., Raimondi, I., Riva, G. A., Raimondi, M. T., Tunesi, M., Giordano, C., Forloni, G. and Albani, D., "Secretome released from hydrogel-embedded adipose mesenchymal stem cells protects against the Parkinson's disease related toxin 6-hydroxydopamine," Eur. J. Pharm. Biopharm. **121**, 113–120 (2017).

[12] Tunesi, M., Fusco, F., Fiordaliso, F., Corbelli, A., Biella, G. and Raimondi, M. T., "Optimization of a 3D dynamic culturing system for in vitro modeling of Frontotemporal Neurodegeneration-relevant pathologic features," Front. Aging Neurosci. **8**(JUN) (2016).

[13] Trapecar, M., Wogram, E., Svoboda, D., Communal, C., Omer, A., Lungjangwa, T., Sphabmixay, P., Velazquez, J., Schneider, K., Wright, C. W., Mildrum, S., Hendricks, A., Levine, S., Muffat, J., Jasmine Lee, M., Lauffenburger, D. A., Trumper, D., Jaenisch, R. and Griffith, L. G., "Human physiomimetic model integrating microphysiological systems of the gut, liver, and brain for studies of neurodegenerative diseases" (2021).

[14] Raimondi, M. T., Albani, D. and Giordano, C., "An Organ-On-A-Chip Engineered Platform to Study the Microbiota–Gut–Brain Axis in Neurodegeneration," Trends Mol. Med. **25**(9), 737–740 (2019).

[15] Garcia, A., Rodriguez Matas, J. F. and Raimondi, M. T., "Modeling of the mechano-chemical behaviour of the nuclear pore complex: Current research and perspectives," Integr. Biol. (United Kingdom) **8**(10), 1011–1021 (2016).

[16] Lin, D. H., Stuwe, T., Schilbach, S., Rundlet, E. J., Perriches, T., Mobbs, G., Fan, Y., Thierbach, K., Huber, F. M., Collins, L. N., Davenport, A. M., Jeon, Y. E. and Hoelz, A., "Architecture of the symmetric core of the nuclear pore," Science (80-.). **352**(6283), 56–64 (2016).

[17] Nava, M. M., Raimondi, M. T. and Pietrabissa, R., "Bio-chemo-mechanical models for nuclear deformation in adherent eukaryotic cells," Biomech. Model. Mechanobiol. **13**(5), 929–943 (2014).

[18] Nava, M. M., Fedele, R. and Raimondi, M. T., "A strain-dependent diffusivity model to study the nuclear import of mechanobiological transcription factors," Proc. Annu. Int. Conf. IEEE Eng. Med. Biol. Soc. EMBS **2015-Novem**, 1857–1860, Institute of Electrical and Electronics Engineers Inc. (2015).

[19] Raimondi, M. T., Balconi, G., Boschetti, F., Di Metri, A., Azmi Mohammed, S. A., Quaglini, V., Araneo, L.,

Galvéz, B. G., Lupi, M., Latini, R. and Remuzzi, A., "An opto-structural method to estimate the stress-strain field induced by cell contraction on substrates of controlled stiffness in vitro," J. Appl. Biomater. Funct. Mater. **11**(3), 143–150 (2013).

[20] Rompolas, P., Mesa, K. R. and Greco, V., "Spatial organization within a niche as a determinant of stem-cell fate," Nature **502**(7472), 513–518 (2013).

[21] Rayner, S. G., Howard, C. C., Mandrycky, C. J., Stamenkovic, S., Himmelfarb, J., Shih, A. Y. and Zheng, Y., "Multiphoton-Guided Creation of Complex Organ-Specific Microvasculature," Adv. Healthc. Mater. **2100031**, 1–6 (2021).

[22] Song, J., Michas, C., Chen, C. S., White, A. E. and Grinstaff, M. W., "From Simple to Architecturally Complex Hydrogel Scaffolds for Cell and Tissue Engineering Applications: Opportunities Presented by Two-Photon Polymerization," Adv. Healthc. Mater. **9**(1), 1–13 (2020).

[23] Raimondi, M. T., Eaton, S. M., Nava, M. M., Laganà, M., Cerullo, G. and Osellame, R., "Two-photon laser polymerization: From fundamentals to biomedical application in tissue engineering and regenerative medicine," J. Appl. Biomater. Funct. Mater. **10**(1), 56–66 (2012).

[24] Raimondi, M. T., Nava, M. M., Eaton, S. M., Bernasconi, A., Vishnubhatla, K. C., Cerullo, G. and Osellame, R., "Optimization of femtosecond laser polymerized structural niches to control mesenchymal stromal cell fate in culture," Micromachines **5**(2), 341–358 (2014).

[25] Raimondi, M. T., Eaton, S. M., Laganà, M., Aprile, V., Nava, M. M., Cerullo, G. and Osellame, R., "Three-dimensional structural niches engineered via two-photon laser polymerization promote stem cell homing," Acta Biomater. **9**(1), 4579–4584 (2013).

[26] Nava, M. M., Piuma, A., Figliuzzi, M., Cattaneo, I., Bonandrini, B., Zandrini, T., Cerullo, G., Osellame, R., Remuzzi, A. and Raimondi, M. T., "Two-photon polymerized 'nichoid' substrates maintain function of pluripotent stem cells when expanded under feeder-free conditions," Stem Cell Res. Ther. **7**(1) (2016).

[27] Boeri, L., Jacchetti, E., Soncini, M., Negro, A., Albani, D. and Raimondi, M. T., "Advantages and limitations of a supernegative GFP in facilitating MyoD intracellular tracking," Methods Appl. Fluoresc. **8**(2) (2020).

[28] Jacchetti, E., Nasehi, R., Boeri, L., Parodi, V., Negro, A., Albani, D., Osellame, R., Cerullo, G., Matas, J. F. R. and Raimondi, M. T., "The nuclear import of the transcription factor MyoD is reduced in mesenchymal stem cells grown in a 3D micro-engineered niche," Sci. Rep. **11**(1), 3021 (2021).

[29] Kirby, T. J. and Lammerding, J., "Emerging views of the nucleus as a cellular mechanosensor," Nat. Cell Biol. **20**(4), 373–381 (2018).

[30] Parodi, V., Jacchetti, E., Bresci, A., Talone, B., Valensise, C. M., Osellame, R., Cerullo, G., Polli, D. and Raimondi, M. T., "Characterization of mesenchymal stem cell differentiation within miniaturized 3d scaffolds through advanced microscopy techniques," Int. J. Mol. Sci. **21**(22), 1–21 (2020).

[31] Remuzzi, A., Bonandrini, B., Tironi, M., Longaretti, L., Figliuzzi, M., Conti, S., Zandrini, T., Osellame, R., Cerullo, G. and Raimondi, M. T., "Effect of the 3D Artificial Nichoid on the Morphology and Mechanobiological Response of Mesenchymal Stem Cells Cultured In Vitro," Cells **9**(8) (2020).

[32] Rahman, N., Brauer, P. M., Ho, L., Usenko, T., Tewary, M., Zúñiga-Pflücker, J. C. and Zandstra, P. W., "Engineering the haemogenic niche mitigates endogenous inhibitory signals and controls pluripotent stem cell-derived blood emergence," Nat. Commun. **8**(May) (2017).

[33] Flores-Figueroa, E. and Gratzinger, D., "Beyond the niche: Myelodysplastic syndrome topobiology in the laboratory and in the clinic," Int. J. Mol. Sci. **17**(4) (2016).

[34] Carelli, S., Messaggio, F., Canazza, A., Hebda, D. M., Caremoli, F., Latorre, E., Grimoldi, M. G., Colli, M., Bulfamante, G., Tremolada, C., Di Giulio, A. M. and Gorio, A., "Characteristics and properties of mesenchymal stem cells derived from microfragmented adipose tissue," Cell Transplant. **24**(7), 1233–1252 (2015).

[35] Ricci, D., Nava, M. M., Zandrini, T., Cerullo, G., Raimondi, M. T. and Osellame, R., "Scaling-up techniques for the nanofabrication of cell culture substrates via two-photon polymerization for industrial-scale expansion of stem cells," Materials (Basel). **10**(1) (2017).

[36] Zandrini, T., Shan, O., Parodi, V., Cerullo, G., Raimondi, M. T. and Osellame, R., "Multi-foci laser microfabrication of 3D polymeric scaffolds for stem cell expansion in regenerative medicine," Sci. Rep. **9**(1) (2019).

[37] Park, H. J., Yang, K., Kim, M. J., Jang, J., Lee, M., Kim, D. W., Lee, H. and Cho, S. W., "Bio-inspired oligovitronectin-grafted surface for enhanced self-renewal and long-term maintenance of human pluripotent stem cells under feeder-free conditions," Biomaterials **50**(1), 127–139 (2015).

[38] Joddar, B. and Ito, Y., "Artificial niche substrates for embryonic and induced pluripotent stem cell cultures," J.

Biotechnol. **168**(2), 218–228 (2013).
[39] Hazeltine, L. B., Selekman, J. A. and Palecek, S. P., "Engineering the human pluripotent stem cell microenvironment to direct cell fate," Biotechnol. Adv. **31**(7), 1002–1019 (2013).
[40] Bakhtina, N. A., Müller, M., Wischnewski, H., Arora, R. and Ciaudo, C., "3D Synthetic Microstructures Fabricated by Two-Photon Polymerization Promote Homogeneous Expression of NANOG and ESRRB in Mouse Embryonic Stem Cells," Adv. Mater. Interfaces, 2001964 (2021).
[41] Rey, F., Barzaghini, B., Nardini, A., Bordoni, M., Zuccotti, G. V., Cereda, C., Raimondi, M. T. and Carelli, S., "Advances in Tissue Engineering and Innovative Fabrication Techniques for 3-D-Structures: Translational Applications in Neurodegenerative Diseases," Cells **9**(7) (2020).
[42] Rey, F., Pandini, C., Barzaghini, B., Messa, L., Giallongo, T., Pansarasa, O., Gagliardi, S., Brilli, M., Zuccotti, G. V., Cereda, C., Raimondi, M. T. and Carelli, S., "Dissecting the effect of a 3D microscaffold on the transcriptome of neural stem cells with computational approaches: A focus on mechanotransduction," Int. J. Mol. Sci. **21**(18), 1–37 (2020).
[43] Carelli, S., Giallongo, T., Rey, F., Barzaghini, B., Zandrini, T., Pulcinelli, A., Nardomarino, R., Cerullo, G., Osellame, R., Cereda, C., Zuccotti, G. V. and Raimondi, M. T., "Neural precursors cells expanded in a 3D micro-engineered niche present enhanced therapeutic efficacy in vivo," Nanotheranostics **5**(1), 8–26 (2021).
[44] Graney, P. L., Ben-Shaul, S., Landau, S., Bajpai, A., Singh, B., Eager, J., Cohen, A., Levenberg, S. and Spiller, K. L., "Macrophages of diverse phenotypes drive vascularization of engineered tissues" (2020).
[45] Ingber, D. E., "Is it Time for Reviewer 3 to Request Human Organ Chip Experiments Instead of Animal Validation Studies?," Adv. Sci. **7**(22), 2002030 (2020).
[46] Wright, M. E., Yu, J. K., Jain, D., Maeda, A., Yeh, S. C. A., DaCosta, R. S., Lin, C. P. and Santerre, J. P., "Engineering functional microvessels in synthetic polyurethane random-pore scaffolds by harnessing perfusion flow," Biomaterials **256** (2020).
[47] Dondossola, E., Holzapfel, B. M., Alexander, S., Filippini, S., Hutmacher, D. W. and Friedl, P., "Examination of the foreign body response to biomaterials by nonlinear intravital microscopy," Nat. Biomed. Eng. **1**(1), 1–10 (2017).
[48] Conci, C., Jacchetti, E., Zandrini, T., Sironi, L., Collini, M., Chirico, G., Cerullo, G., Osellame, R. and Raimondi, M. T., "Quantification of the foreign body reaction by means of a miniaturized imaging window for intravital nonlinear microscopy," Biomed. Sci. Eng. **3** (2020).