Organisms-on-a-chip

Manuela Teresa Raimondi[1,*]

[1]Polytechnic University of Milano, Department of Chemistry, Materials and Chemical Engineering "Giulio Natta", 20133 piazza L. da Vinci, Milano, Italy

Abstract. The progression of fibrosis is frequently related to a failed healing process, and it may affect many tissues and organs causing severe consequences including post-infarct heart insufficiency, post-injury limb paralysis, cirrhosis, nephropathy, retinopathy, failure of implanted devices and even resistance to chemotherapy in solid tumours. Experimental models, both in vitro and in vivo, are widely used for studies of basic pathophysiology, and for pre-clinical testing of pharmacological therapies counteracting fibrosis. However, conventional models are not able to realistically reproduce the revascularization aspect of the inflammatory reaction that leads to the generation of fibrotic tissue. In this talk, I will present the most advanced frontier in this sector, represented by the new concept of an "organism-on-a-chip". This is a hybrid model, in which an organ-on-a-chip is implanted sub-cute in a living organism, such as a mouse or an embryonated avian egg, thus eliciting a foreign-body reaction with the formation of a fibrotic microenvironment. In an organism-on-a-chip, the fibrotic reaction can be guided using microscopic scaffolds incorporated in the implanted chip. The fibrotic microenvironment can then be imaged longitudinally, in high resolution, in single and multiphoton optical microscopy, with the added advantage of significantly reducing animal sacrifice. The optical measurements performed in the platform address parameters of the extra-cellular matrix (stiffness, collagen density, collagen orientation, coefficient of diffusion) and vascularity (number of blood vessels, length, blood velocity and vessel permeability).

1 Models of fibrotic progression: state-of-the-art

Great research efforts are currently devoted to the development of new modelling tools for understanding fibrotic progression in many diseases and in performing preclinical tests on antifibrotic drugs. Fibrosis can adversely affect wound healing of the heart and spinal cord after an acute damage, entire organs including the liver and the kidney, encapsulation of an implanted biomaterial, and can cause drug resistance in a solid tumour. Realism of the study models in these fields is directly proportional to the potential of clinical translation of the results. Even in animal models of fibrotic progression developed from human cells, anti-fibrotic agents have a low effectiveness resulting from their poor penetration into fibrotic masses and suboptimal pharmacokinetics.

1.1 Humanised organoids and assays *in vitro* for fibrosis research

To overcome these limitations, new "humanized" cell models are being developed *in vitro*. Cell spheroids and organoids from human cells are scarcely reproducible and cannot include interactions with the tumour extra-cellular matrix. Microfluidic-based platforms can instead recreate on micro tissues *in vitro* complex functional aspects of the fibrotic micro environment. For example, a breast tumour-on-chip may reproduce differential fibroblast activation modulating extra-cellular matrix diffusivity causing treatment resistance. The sector of organ-on-chips has evolved at a level that these platforms are now starting to be integrated into the drug development pipeline, in partial replacement of animal studies. However, even the most advanced organ-on-chip platform lacks a functional microvascular network. At the state of the art, microvascular networks generated *in vitro* aren't stable enough in time, in terms of alignment, hierarchy and permeability, to maintain physiological flows in the vessels' lumens. This limits the realism of these platforms in reproducing fibrotic progression, because it is precisely signalling between endothelial cells, fibroblasts and macrophages that gives rise to the real organ stiffening behaviour.

1.2 Models of fibrotic progression with intravital imaging *in vivo*

Alternatively, an evolving metastasis, or the fibrotic encapsulation of a polymeric scaffold, can be imaged *in vivo* with two-photon intravital microscopy, through a permanent imaging window surgically implanted in a rodent. For example, observation of a human breast cancer xenograft, around 4 mm in diameter, through a mammary imaging window allowed to monitor tissue revascularisation and oxygenation at various time points of tumour progression. However, in this model optical

*Corresponding author: manuela.raimondi@polimi.it

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distortions limited the accuracy of parameter quantifications on intravital two-photon images, due to significant changes in the tissue refractive index with light penetration depth. Most importantly, after 11 days of observation, cancer cells were necrotic in the tumour core and revascularisation occurred only on the tumour periphery thus recapitulating the very initial phase of inflammation that gives rise to progressive fibrotic stiffening of a tumour. As a matter of fact, tumour stiffening takes several months to develop in adult animals, while longitudinal intravital imaging studies can hardly exceed one month. Long-term imaging acquisitions on the same animal must in fact be progressively shortened, due to increasing suffering of the animal by repeated and prolonged (>1h) inhalation anaesthesia.

2 Models of fibrotic progression in embryonated avian eggs

The development of tissue is significantly faster in embryonic organisms. Also, embryos can be long-term two-photon imaged intravitally without compromising their development. The embryonic organism most widely used in pharmacological research is the embryonated chicken egg, not considered a living animal by national legislations worldwide. A chick embryo that has not reached the 14th embryonic development day does not experience pain and can be used for experimentation without any ethical restrictions or prior protocol approval, nor does it require an animal facility. The avian embryo is widely used in studies with human cells because it's immune-tolerant, i.e. its adaptive immune response based on T-cells and antibodies is immature and does not reject xenogeneic material like human cancer cells. Instead, the avian innate immune response based on macrophages is active throughout embryonic development, making an avian embryo model potentially highly predictive, although not entirely human, in replicating an innate response like fibrosis. This experimental model allows to measure *in vivo* the effect of therapeutic agents injected in the embryonic circulation, on pharmacokinetics, toxicity, pro/anti-angiogenicity, tissue repair and wound healing induced on the embryo and/or, most interestingly, on a breathing structure in contact with the egg shell, called chorioallantoic membrane (CAM). Transparent eggshells may improve optical accessibility of the CAM to intravital microscopy.

2.1. Measurement of a foreign-body fibrotic reaction in an organism-on-a-chip

We have recently developed and patented an innovative platform, consisting of a micro-patterned imaging window implantable *in vivo* [1]. To validate this window in an *in vivo* environment, we implanted the window in chicken embryos extracted from the egg shell and maintained in culture *ex ovo* (Fig. 1).

Inside these micro scaffolds, we measured by label-free two-photon microscopy the infiltration of cells and micro vessels, and the collagen content at 4 days after implantation. Then, we euthanized and fixed the embryos with a paraformaldehyde solution. We dissected embryos to perform immunofluorescence treatments focused on immune response labelling to confirm all the data collected by two-photon imaging. We performed single-photon multi-stack acquisitions of the immune-labelled infiltrated scaffolds comparing linear and nonlinear microscopy in terms of cellular density, blood vessels infiltration and collagen-I content. Confocal and two-photon observations at implantation sites demonstrated massive cell infiltration inside the micro scaffolds, with cell density triplicated in 24 hours, and a capillary density almost six-fold greater in the micro scaffolds than in surrounding control tissue. Second harmonic generation signal showed a highly oriented layer of collagen-I inside the micro scaffolds [2].

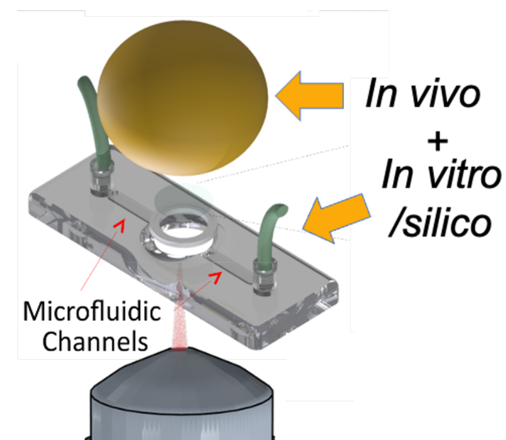

Fig. 1. The emerging concept of an organism-on-a-chip. A microfluidic organ-on-a-chip is implanted in a living organism and imaged intravitally in high resolution, to obtain robust optical quantifications of parameters related to the foreign body reaction (such as cellularity, vascularisation, extra-cellular matrix stiffness).

Our preliminary data show that implantation of micro scaffolds does elicit a foreign-body fibrotic reaction, dependent on the scaffold micro architecture and well quantifiable in real time *in vivo*. In conclusion, thanks to the robust optical measures allowed in vivo, the new organism-on-a-chip platform has the potential to shorten the approval process for drugs, using a combined efficacy/safety approach that lowers the risks.

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