BIOLOGY AND MICROFLUIDICS

A discussion in November 2012 between researchers at Industrial Research Limited, University of Canterbury and University of Auckland, and Reg Harris, about the use of microfluidics to facilitate the growth of human tissue

Key Challenges

Reg Harris: One of the biggest challenges is to grow complex tissue with a steady blood supply to provide a continuous flow of \( O_2 \) and nutrients to deep layers [beyond 200 µm?] and to provide a route for the withdrawal of \( CO_2 \) and other waste products.

Industrial Research Limited: We can fabricate surface layers made of a silicone material [PDMS] that is known to have relatively high gas permeability. These could feature (1) capillaries for fluid flow and (2) patterned textures for guided flow.

We have experience with a variety of fluid transport mechanisms in complex, confined systems. We could contribute to innovations regarding use of different transport mechanisms, and characterisation/engineering of substrates [e.g. surface modification, engineering of charge properties].

The picture illustrates how two parallel flows introduced, at the top left, into a single microfluidic channel show little tendency to mix.

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1 For insight go to Chemical Society Reviews, 2010, Issue 3, pages 889 to 1220 http://pubs.rsc.org/en/journals/journalissues/cs#issueid=cs039003?type=current&issnprint=0306-0012 and the editorial article by van den Berg, Craighead and Yang: “Perhaps the most important driving force for microfluidics is the ability to control minute amounts of liquid in a very precise way” and “The excellent fit between typical microchannel sizes [10-100µm] and the size of a human cell [typically 10µm] is probably the reason for the great interest from biologists”. The ‘minute amounts’ referred to are measured in nL, pL and fL.

2 Dr Mike Arnold, Principal Research Scientist and Team Leader, Nano and Micro Fluidics; Dr Geoff Willmott, Senior Scientist Nano and Micro Fluidics; Andrea Bubendorfer, Research Scientist Nano and Micro Fluidics; Dr Jeremy Wu, Research Scientist Advanced Materials.

3 Dr Volker Nock, Lecturer, Department of Electrical and Computer Engineering

4 Dr Cather Simpson, Department of Chemistry and Physics; Director The Photon Factory.
The following notes are applicable to more than one question:

- We have a broad collaborative network in relevant physical sciences via organisations such as the MacDiarmid Institute for Advanced Materials and Nanotechnology [www.macdiarmid.ac.nz] and ANZ Nano and Microfluidics [www.anzmicrofluidics.org]

- We particularly note the work of MacDiarmid Institute colleagues Dr John Evans [University of Otago, Christchurch], Dr Maan Alkaisi [University of Canterbury] and their teams regarding reconstruction of cellular textures in vitro using polymer nanoimprinting, and also that of Dr Volker Nock [also University of Canterbury] on cell culture with PDMS devices with measurement of oxygen concentrations.

- The NMF team at IRL Wellington is just down the hall from our Applied Maths team, which is capable of high-performance computer modelling to support our [or others’] work. Their specific experience is in microfluidic transport rather than cellular science. There is a further capability in fluidic [not just microfluidic] modelling being developed at IRL Parnell.

University of Canterbury: Similar to IRL, we have extensive capabilities in the design fabrication of polymer microfluidic devices. In regards to supply of O₂ and nutrients to cells cultured in vitro, we can contribute experience in species transport modelling and the design and fabrication of silicone-based microfluidic devices with integrated gas exchangers. In particular, we specialise in the control of O₂ gradients based on device geometry, as well as in the in situ measurement of dissolved O₂ in culture media based on fluorescent oxygen sensors developed in-house.

University of Auckland: At Auckland, these activities are housed in the Microfabrication Facility in the School of Chemical Sciences and in the Photon Factory in the Faculty of Science. We also have a strong BioMems group. The active research groups are run by Prof David Williams [Chemistry], Dr Bryon Wright [Chemistry], Dr Donald Wlodkowic [Chemistry, Biological Sciences], and Dr Cather Simpson [Chemistry, Physics].

In our various laboratories and centres, we have sophisticated capabilities in the design and fabrication of polymer microfluidic devices in environments that range from clean room to PC1 standards. We also have laser micromachining at our disposal, for more exotic polymer microfluidics, and for constructing devices in quartz, glass, or other ‘hard’ materials. These latter capabilities can be particularly useful for combining microfluidics and optics.

RH: Another big challenge is to develop in vitro physiological systems for studying fundamental biological phenomena. Vascular cell biology, in particular, is an area of research with great biomedical relevance, where the dynamic 3D micro-environment found in vivo [and characterised, for example, by shear stresses, growth factor gradients, co-cultures and cellular migrations] needs to be mimicked to be understood properly.

Again, how might your organisation be able to assist in meeting this challenge?

IRL: We have experience and capability in:
• Materials with matched properties for fabrication
• Flow geometries optimised to match internal flow pressures
• Textured surfaces to facilitate cellular migrations
• Chemically modified surfaces
• Micro-actuation.

We can construct and study model experimental systems where the environmental factors mentioned can be specifically isolated, measured and/or controlled. We are capable of associated theoretical development relating to physical phenomena.

Our expertise and experience [theory and practical] with tunable nanopores could be applicable to studies of mechanical stresses on biological materials, cellular membranes, and particularly to transmembrane transport via ion channels.

In this picture a number of different microchannel geometries are shown: these have been trialled to force the mixing of parallel streams of fluids.

UoC: As mentioned above, we would be able to contribute our experience with contact-free fluorescence sensors to characterize the dynamic effects in the 3D microenvironment. Techniques established for O₂ visualisation are currently being extended to 3D imaging and can be extended further to characterise other small molecule factors relevant to the cellular microenvironment. We have previously demonstrated gradient-based control and detection of O₂ in microfluidic devices which can be easily adapted to other solutes.

UoA: We have projects in which we use our lasers and 3D control to manufacture tailored structures for such studies. For example, we recently began a collaboration to examine the behaviour of the arterial branches from the mouse aorta, and another project is underway to model the architecture and flow through human sinus cavities. These models can be manufactured out of a wide array of materials – essentially micro-scale, 3D sculpting.

Connective Tissue

RH: The major types of connective tissue within which we might expect microfluidics to operate are connective tissue proper [loose (including ‘standard’ and adipose) and dense forms], supporting connective tissue [cartilage, a nonvascularised tissue (including hyaline, elastic and fibro-) and bone] and fluid connective tissue [blood and lymph]. The functions of each are different; consider the following examples of ‘solid’ connective tissues:

• ‘Standard’ loose connective tissue cushions organs; provides support but permits independent movement and provides, via phagocytes, defence against pathogens. [under the dermis of skin, digestive tract, respiratory and urinary tracts; between muscles, around blood vessels, nerves and around joints]
• Adipose tissue provides padding and cushions shocks; insulates [reduces heat loss] and stores energy reserves. [deep to the skin, especially at sides, buttocks, breasts; padding around eyes and kidneys]
- **Dense connective tissue** provides firm attachment; conducts pull of muscles; reduces friction between muscles; stabilises relative positions of bones and helps prevent overexpansion of organs [such as the urinary bladder]. [between skeletal muscles and skeleton (tendons); between bones (ligaments); covering skeletal muscles; capsules of internal organs]
- **Hyaline cartilage** provides stiff but somewhat flexible support and reduces friction between bony surfaces. [between tips of ribs and bones of sternum; covering bone surfaces at synovial joints; supporting larynx (voice box), trachea and bronchi; forming part of nasal septum]
- **Elastic cartilage** provides support but tolerates distortion without damage and returns to original shape. [auricle of external ear; epiglottis; acoustic canal; cuneiform cartilages of larynx]
- **Fibrocartilage** resists compression; prevents bone-to-bone contact and limits relative movement. [pads within knee joint; between pubic bones of pelvis; intervertebral discs separating vertebrae]
- **Bone** is divided into five main types with functions that are mechanical [protection, structural, movement, sound transduction]; synthetic [blood production]; and metabolic [mineral storage, growth factor storage, fat storage, acid-base balance, detoxification, endocrinial control].

**IRL:** As regeneration is the main aim, we could look at nano- or micro-patterning a surface so that collagen or other extra-cellular polymers adhere readily, perhaps even selectively, to it.

Some degree of orientation could possibly be achieved this way. Scaffolds for cells could be made by laser-drilling of polymer films, or by etching of blocks of composites. These are microfabrication tasks, the microfluidics could be used in addition as described above.

The picture shows an array of 20-micron sized wells etched into glass following laser-writing. Further etching can give a filter structure.

If, on the other hand, the aim is to mimic these tissues or create prostheses, then the three types of cartilage could be replaced by various custom-modified elastomers, with appropriate tailoring to give reasonable ranges of hardness.

As an example, PDMS is biologically compatible, non-toxic, stable and insoluble in aqueous environments. It is also widely amenable to the uptake of fillers which change its mechanical properties, and is easily formed into textures/patterns, e.g. by micromoulding. There is also a significant scope in modification based on the ability to vary molecular weight, terminal groups, degree of branching and cross-linking, etc, and a range of polymerisation techniques are available. PDMS also combines well with a number of different polymers to form multiphase systems with diverse properties [see for example: http://www.cosmeticbreastsurgeon.co.uk/images/Basilone%20product%20information.pdf].

Adding to this, we have the capability to study the mechanical and transport properties relevant to such model tissues.

**RH:** Irrespective of the answer[s] to the foregoing question and assuming IRL were able to choose the tissue type[s] to start with.....
IRL: We would offer the same answer, to match our capability and [limited] experience also. However, we would be prepared to consider whatever is most useful to the frontline health researcher [who will know better in respect of clinical applications]. Our role can be to take on the challenge of providing research in whichever direction is most useful.

UoC: Currently we have most experience with the formation of muscle tissue based on experiments related to the effects of micro- and nano-scale surface patterns on cells. At this stage microfluidics play only a minor role in tissue formation. To a lower degree with have experience in the formation of cartilage tissue based on a collaboration with Dr Tim Woodfield [University of Otago Christchurch Department of Orthopaedic Surgery and Centre for Bioengineering]. Similar as to the previous, microfluidics have not yet been used specifically for these cultures.

UoA: We address this broad issue along several directions. We have just secured major funding to explore the laser machining of a wide variety of materials, including bio-mimetic materials and biological tissues. While we are not primarily interested in recreating tissue complexity or substrates, we have technical capabilities in this space that could enable research by others.

Our second major area of focus here is understanding and controlling precise, microscopic surgery with femtosecond laser pulses, in particular. The advent of the femtosecond laser led to the development of many biomedical and biomedical engineering tools that exploit the very high pulse energies, and ultrashort pulse duration associated with these pulses. Under the right conditions, single mitochondria can be ablated from within a cell, for example, to explore the impact on cellular function. On a larger scale, the laser can be used to target specific anatomical systems or subsystems within an organism.

Tackling Complexity

RH: The application of microfluidics to expedite the growth of a reasonably homogenous tissue construct [say skin or bone] poses one level [the ‘easiest’?] of scientific and technical difficulty. But its application in respect of more complex entities such as a liver, with its outer fascia layer; columns and sinuses; blood vessels including veins, arteries and their smaller subdivisions; bile ducts and nerves poses more daunting challenges, particularly in any quest to grow a complete new organ ‘from scratch’.

IRL: First of all, we would need a greater understanding [we are not anatomy aces] of the structure and cellular constituents of such tissues and their likely interactions in order to see how such an assembly could be possible. Then we work out how to handle the individual entities separately, characterise and optimise.

Create a scaffold to set-up a multiple entity system, seed appropriate layers, slowly increasing the three-dimensionality of the scaffold/system.
We have previously discussed a method of preparing a seed layer to capture individual cells in specifically located areas, *via* selectively patterned patches to bond cells. This could be expanded on by directing cells where best to be positioned and grow, with carefully constrained growth. We would prefer a methodical approach – maybe the key point is recognising that it’s quite possible we can create the scaffolds on small enough scales to reach the complexity required.

**Technology Partnering**

**RH:** No one technology can be expected to provide ‘all the answers’ to facilitating the growth of human tissue. Recognising this, and cognisant of the difficulties of building clinic-ready tissue-engineered vascular grafts, a team of researchers has developed PLLA/PCL scaffold [that releases heparin, an anticoagulant] by a combination of electrospinning [ES] and fused deposition modelling [FDM]. Scaffolds are produced by ES in tubular shape and then FDM is used to armour the tube with a single coil of PCL on the outer layer to improve mechanical properties. Scaffolds are then seeded with hMSCs [human mesenchymal stem cells] and assayed in terms of morphology, mechanical tensile strength, cell viability and differentiation. This particular scaffold design allows the generation of both a drug delivery system amenable to surmount thrombogenic issues and a microenvironment able to induce endothelial differentiation [genesis of the inner layer of cells in blood vessels]. At the same time, the PCL external coiling improves mechanical resistance of the microfibrous scaffold. By the combination of ES and FDM, and exploiting the biological effects of heparin, an *ad hoc* differentiating device for hMSCs seeding and subsequent induction of differentiation into vascular endothelium, is developed.

What technology partnering involving microfluidics promise the best frameworks for facilitating the growth of vascularised human tissue? What are the reasons?

**IRL:** A whole heap of these. The work by Dr Volker Nock and others at *University of Canterbury/Otago Medical School/MacDiarmid Institute for Advanced Materials and Nanotechnology* is certainly of relevance here.

Currently there is little activity in cell or tissue culture at *IRL*, yet this will be necessary complement to our work as an on-going test of the suitability of whatever structures we fabricate. Such a facility and expert staff should include also characterisation ability, such as access to a confocal microscope and perhaps rapid genotyping.

Laser fabrication, such as at the Photon Factory in the *University of Auckland* [Dr Cather Simpson, Department of Physics] could be of use for some structures.

Whilst PDMS might be of use for permanent structures, for some tissue types only a temporary support would be required. Therefore some expertise in methods of fabricating structures in biodegradeable materials would likely be useful.

We should reiterate that our present strengths are in (i) microfabrication (ii) physical characterization *e.g.* transport, mechanics: theoretical and practical.
Three-Dimensionality

**RH:** It might reasonably be assumed that systems for culturing cells, thence tissue, will have more biological or clinical relevance if the cells exhibit 3D phenotypes similar to those *in vivo*. This leads to consideration of the extent to which microfluidic platforms and their capability can be three-dimensionalised.

In recent years a common approach for vascularised microfluidic systems has been to stack and assemble 2D vascularised polymer films [usually made by micromolding techniques] into large 3D devices suitable for transplantation. This appears to have been reasonably successful but entails a cumbersome, difficult-to-upscale, process requiring multiple fabrication and masking steps.

It is not always clear from the literature whether stack-and-assemble platforms apply to the 3D microfluidic research being reported; the examples below fall into this category but should provide an appreciation of the range of work carried out:

- a microfluidic channel-based system that allows cells to be perfusion-cultured in 3D, with carcinoma cell lines, hepatocytes and primary progenitor cells maintaining their 3D cyto-architecture and cell specific functions.
- the culturing of two mammalian cell lines and primary mammalian cells [bone marrow mesenchymal stem cells] in a gel-free 3D microfluidic cell culture system, with the cells displaying 3D cellular morphology, cellular functions and differentiation capability, thus affirming the versatility of the system as a 3D cell perfusion culture platform for anchorage-dependent mammalian cells.
- a microfluidic perfusion plate that enables high quality cell culture in a 3D matrix. The array in focus contains 32 independent micro-chambers designed to be user filled with an extracellular matrix [ECM], and the cells can be overlaid or embedded in the gel for long term perfusion culture.
- a microfluidic assay of endothelial cell sprouting and migration into an interpenetrating polymer semi-network hyaluronic acid-collagen hydrogel which acts as ECM providing an enhanced *in vivo*-mimicking 3D microenvironment to cells [with the microfluidic chip giving a well-controlled gradient of growth factor to the latter].
- through harnessing electrostatic discharge phenomena the embedment of branched 3D microvascular fluidic networks inside plastic substrates. This process, said to be nearly instantaneous, reproducibly generates highly branched tree-like microchannel architectures similar to those of naturally occurring vasculature [see picture below]. This method is claimed by its developers to be applicable to a variety of polymers, and to potentially enable production of organ-sized tissue scaffolds containing embedded vasculature.

In what ways might your organisation be able to contribute to the three-dimensionalisation of microfluidic systems for facilitating the growth of human tissue?

IRL: This question requires some speculation into the kind of innovations that we could be involved with viz 3D scaffold patterning? Cell-specific patterning? The following relate:

- Possibly a 3D printer able to print in a biodegradable medium [some type of gel?] that would act as the scaffold and growth limiter may be useful for printing spaces within which cells/tissues can grow. Note that, currently, 3D printers have coarser spatial resolution than microfabrication, so we’re necessarily talking about some innovative combination of effects.

- Controlled basic characterisation of scaffolds made elsewhere.

- What we can already do is to fabricate 2D array of hexagonal close-packed pores. This is done by controlled anodisation of aluminium. The template is essential anodic aluminium oxide. The pores have a very narrow size distribution, and we can tailor the diameters from 20nm to 300nm. The third dimension would be the thickness of the template i.e. the length of the pore channels. This can be done up to 0.5mm, allowing a huge aspect ratio if that is required. What we do typically is to design templates based on end-users’ requirements. If this is the length scale you are interested in, and provided the template material [amorphous aluminium oxide] is compatible, then there may be opportunities.

- In addition, we are also looking at titanium-based cellular structures as well as 3D printing of ceramic materials. The anodisation technology can be applied to titanium, making nanoporous TiO$_2$ surfaces on titanium substrates.

UoC: Devices similar to the one shown above have been fabricated at the High Voltage Laboratory at UoC, mainly in wood and plexiglas, but this could be extended to more relevant materials. While most microfluidic devices designed locally to this day have been investigating 2D monolayers of cells, a degree of three-dimensionality could be easily attained by integration of hydrogels into the devices. In particular, the work by Dr Kenny Chitcholtan [Otago Medical School] regarding the growth of 3D tumors using hydrogels and non-adhesive surfaces and their response to drugs compared to monolayers should be noted. While currently well-based, there are plans to move some of these experiments into microfluidic platforms in the future.

Reaching the ‘Real world’

RH: The following questions are undoubtedly linked.....

First, what would the main challenges be in modelling biological processes occurring in lab-scale microfluidic systems for application at-scale?

IRL: In the case of cellular systems, more knowledge of the cellular requirements [nutritional, co-factor, surface sensitivity, population-sensing, etc]: In short, advanced cell biology. Without this the unpredictable
nature of the system and inability to take into account all the assumptions needed would defeat the project.

However, in the case of an immobilised enzyme device, the knowledge required is already available in many cases.

**Second, what would the main challenges be in scaling up of microfluidic systems from the lab bench to production manufacturing or clinical application?**

**IRL:** This is a difficult question to answer because we don’t know what we are addressing! A number of cases can be distinguished even at this very early stage:

- **Device yield:** lab devices can be acceptable if the yield is just 1%, yet they are then unlikely to be an economic proposition for industry.
- **If the device uses cells added during manufacture, a reliable source that isn’t going to get infected is required. Storage and shelf-life are also big issues here.**
- **If cells are to be grown in the device *in vivo*, or for any implantable device, then a mild procedure for achieving sterility could be an issue.**
- **Production quantities of devices will likely require larger throughput than existing microfabrication facilities in New Zealand are designed for. Therefore a change of technology may be required e.g. from small-scale moulding or embossing to tape embossing. Tooling costs can be significant at this stage.**
- **Stand-alone nature:** a lab device can have many associated instruments, power supplies etc. Not so an implantable one.
- **Finally conventional scale-up challenges i.e. cost [time, materials] required for scale-up.**

Outside all this are the IP, safety and regulatory issues that must have already been clear before we started [!].