Beyond the Cell

From mammalian tissue engineering to 3D plant cell culture

Recent advances applying mammalian tissue engineering to in vitro plant cell culture have successfully cultured single plant cells in a 3D microstructure, leading to the discovery of plant cell behaviours that were previously not envisaged. Animal and plant cells share a number of properties that rely on a hierarchical microenvironment for creating complex tissues. Both mammalian tissue engineering and 3D plant culture employ tailored scaffolds that alter a cell's behaviour from the initial culture used for seeding. For humans, these techniques are revolutionizing healthcare strategies, particularly in regenerative medicine and cancer studies. For plants, we predict applications both in fundamental research to study morphogenesis and for synthetic biology in the agri-biotech sector.

Mammalian tissue engineering and regenerative medicine

Tissue engineering is a suite of techniques that induces mammalian cells to develop into 3D tissues. The concept was based on the recognition that traditional 2D monolayer cell cultures cannot recapitulate the complex in vivo physiology in animal tissues. Animal cells in vivo are embedded in a hierarchical 3D structure made of neighbouring cells and the extracellular matrix (ECM). Three-dimensional in vitro cell cultures that reflect this in vivo environment can reproduce important physiological functions that are not possible for 2D cultures. For instance, it has been shown that 3D cultures of breast epithelial cells can form acini and produce milk in the presence of laminin, whereas cultures grown on flat surfaces (2D) do not.

To induce tissue-like growth in mammalian cells in vitro, a wealth of micro-engineering techniques have been developed over the last three decades. Today, 3D mammalian cell culture has become a major healthcare strategy, bringing together cross-disciplinary professionals including surgeons, biologists, materials scientists and engineers. By making use of the diverse expertise and cutting-edge technologies such as nanoscience and additive manufacturing, 3D cultures have been used for clinical delivery of implantable tissues to replace or fortify damaged body parts such as trachea, skin and blood vessels.

Mammalian tissue engineering can be broadly categorized into two platforms: scaffold-free and scaffold-embedded cultures. Scaffold-free cultures are spheroids – spheres of multicellular aggregates. Spheroids are the oldest 3D model, first documented in 1944 by Johannes Holtfreter. They are often grown to ~250 µm in diameter, though they can range between 50 µm and 5 mm. The composition consists of a hypoxic, necrotic core, a middle layer of quiescent cells and proliferating cells at the periphery. This stratified growth of spheroids leads them to resemble the avascular regions of tumours, making spheroids highly attractive for high-throughput anti-cancer drug screening. Traditional methods to generate spheroids were slow (weeks), irregular in size and hard to physically manipulate for large-scale drug screening. Recent advances including the InSphero hanging-drop technique and n3D Biosciences’ magnetic levitation method have enabled better reproducibility and faster culture time (days), leading to their wider use in drug screening and alternative non-animal-based drug/cosmetics testing.

Meanwhile, to generate an implantable tissue/organ with a defined shape and size, a scaffold-embedded culture is required. A scaffold is an engineered structure that mimics the ECM, a fibrous network that supports cell–cell and cell–environment interactions and maintains proliferation and differentiation. The architecture of the scaffold guides the eventual shape of the new tissue and organ. A diverse range of techniques have been developed to produce 3D scaffolds to mimic the topology of natural ECM; these include animal-derived gels and polymeric fibrous structures (dry scaffolds or hydrogels).

The most established hydrogels in 3D cell culture are reconstituted, de-cellularized ECM from animals, including mouse-derived MatrigelTM, first documented in 1972 by Hynda Kleinman. Animal-derived hydrogels
function well as in vitro scaffolds but suffer from poor mechanical strength and low porosity, which hinder cell migration and tissue development. A deeper problem is that animal-based scaffolds contain background ECM proteins and each batch comes from a different individual, thus giving rise to natural lot-to-lot variations that are hard to quantify or reproduce. Furthermore, transplanting animal-sourced materials can potentially cause an immune response in patients. The USA Food and Drug Administration hence discourage the use of animal-derived components in therapeutics. Consequently, researchers have been developing non-animal-based, alternative scaffolds that can provide quantifiable contents – giving rise to new polymeric fibrous scaffolds.

The design and synthesis of polymeric scaffolds have been refined over the last two decades. Fibre fabrication methods include thermally induced phase separation, freeze drying, solvent casting, electrospinning, melt spinning, solution blowing, additive manufacturing and 3D bioprinting, to name but a few\(^5\). A suitable scaffold should possess interconnected micrometre-sized pores that permit cell migration, a rough nanotopology that encourages cell–scaffold interaction and tuneable mechanical strength to suit the tissue type. The structure can be in the form of microfibres, nanofibres or synthetic hydrogels made of a hydrated polymer network.

While hydrogels and nanofibres have good cell attachment efficiency, they allow limited cell migration due to poor structural and mechanical definition. Microfibres can give a scaffold a broader range of mechanical strength than nanofibres or hydrogels; hence, scaffolds often feature a mixture of microfibres, nanofibres and/or hydrogels. In addition, fibre diameter can play an important role in the initiation of cell attachment and cell–scaffold interactions; different cell types can have different preference for the diameter range, for example, fibroblast cells attach better on nanofibres, while oligodendrocytes prefer microfibres of 2–4 μm\(^4\).

Today, research in mammalian tissue engineering is a multidisciplinary and multinational consortium, bringing together cutting-edge developments across disciplines, leading to unique and powerful discoveries and research translations from the laboratory to patients.

**From mammalian to plant tissue engineering**

A similar cross-disciplinary effort between biologists and materials scientists has led to the recent development of 3D plant tissue engineering. Plant cell culture was developed as a tool to understand fundamental plant physiology. Over the last 100 years, it has become an important area of study with both fundamental research and commercial significance\(^6\). Cultures are commonly prepared as a liquid suspension or a mass of undifferentiated tissue termed callus, which forms from tissue explants such as roots and leaves. By carefully manipulating the amounts of phytohormones and nutrients in the medium, a tissue explant can give rise to a whole new individual. This form of regeneration is commonly used for some plant species as part of stable genetic transformation protocols. Manipulation of the medium in some liquid cultures is used to study the development of cell types that are difficult to access in the whole organism, a key example being the semi-synchronous in vitro formation of the water-conducting tracheary elements\(^6\).

Plant cell culture lacks an effective tool to investigate cellular responses to structural cues that are normally found only in a tissue environment. Animal and plant cells share a number of properties that rely on a hierarchical microenvironment for creating complex tissues, though unlike animal cells, plant cells form a primary cell wall. It is the formation and modification of the wall in the turgid cell that drives growth and cellular morphogenesis (shape). It can be thought of as a type of ECM since the sum of the cell walls both makes and shapes a tissue. While it is hard to resolve individual cellular behaviour in callus and explants, suspension cultures are without the cues that normally come from being in a tissue environment. Since the cell grows according to the surrounding structure and chemistry, traditional plant cell cultures cannot reflect the complex response observed in any tissue, such as the response to external temperature, imposition of mechanical forces and phytohormone gradients. Recent plant studies employing microfluidics have been able to isolate single plant cells such as pollen with precise control over the chemical and positional cues\(^7\). However, microfluidic channels have few comparable features with the fibrous tissue ECM. Cells developing in the capillaries still lack the same 3D spatio-temporal cues afforded in a tissue structure.

Creating a 3D microenvironment that allows the plant cells to grow in a tissue-like way could lead to a wealth of physiological information previously inaccessible to plant biologists. Culture methods for animal and plant tissues already share technological innovations including aseptic culture, co-culture, bioreactors and complimentary tools such as gene transformation. It makes sense that scaffolds used for mammalian tissue engineering could be applied to 3D plant cell culture. Recently, we demonstrated this concept and described a new method for 3D plant culture\(^8\). This method uses a rapid and straightforward protocol that can be readily adapted to a wide range of plant cell types and scaffold designs. Cells from liquid cultures of the laboratory model plant Arabidopsis thaliana were cultured in non-woven scaffolds.
containing defined proportions of microfibres and nanofibres. Within 48 hours of incubation, cells attached and interacted with the fibres, which in turn strongly influenced the morphogenesis of the cells. It was found that the same features of the scaffolds required for human tissue engineering are also needed for proper integration and growth within the scaffold, including micron-sized pores and a particular nanotopography.

**Isolating single plant cell behaviour unobserved in planta**

Cells in the 3D scaffold were found to adapt their shape to fit the surrounding space and take on growth and morphological characteristics with greater complexity than observed even in native plant tissues. The spherical/oblong cells in culture developed various complex geometries and orientations of very long and twisted cells after incorporation into 3D scaffolds (Figure 1). Some cells had constrictions along their length indicating that the cell was “squeezing” through the gaps between fibres and neighbouring cells in order to elongate further and explore new “space” (Figure 1a). Other cells would wrap themselves around microfibre supports, sometimes several times (Figure 1b, c). Cells could become very elongated, stretching across the gap between fibres and resuming isotropic growth at their termini (Figure 1d). Where cells met any obstruction, whether it be a concentration of fibres or other cells, they were able to grow past each other while changing their shape.

**Future perspectives**

Three-dimensional plant tissue engineering brings a new suite of techniques for the growth and study of plant cells *in vitro*. We predict applications both as research tools to study morphogenesis and for synthetic biology in the agri-biotech sector. The technique allows researchers to give single cells a quantifiable artificial extracellular environment. Moreover, the differentiation of plant cells inside a specially tailored scaffold structure offers the potential to provide new tools to study developmental processes that have been previously inaccessible. For example, a carefully constructed scaffold might enable researchers to recreate and observe the series of oriented divisions and development of the vascular cambium. Combining transgenics with an engineered scaffold may produce new biomaterials that are carefully pre-moulded at the nanoscale.

Scaffolds may be used as vehicles for signalling components. One application we are currently working on is the generation of artificial phytohormone gradients, particularly auxin, to see how the cells respond and transduce the signal. Potentially, any active agent, such as ligands for receptors or diffusible molecules, can be encapsulated in the core of the fibres that make up the scaffold, or inside micro/nanoparticles that are then adsorbed onto the fibre surface. The micro/nanoparticle shell may be made of a biocompatible polymer such as poly(N-isopropylacrylamide), which shrinks according to changes in physiologically relevant temperature (e.g. a change from 20°C to 32°C). This swelling and shrinking of the encapsulating shell enables a level of control of the release profile of the encapsulated agent. Coupled with standard live imaging techniques, this technology will enable the real-time monitoring of subcellular responses to the agent, for example, exploring changes in growth and polarization of proteins such as PINs (membrane auxin efflux transporters) during auxin release from an adjoining fibre.

Another application for 3D scaffolds is to explore, in detail, how a cell responds to its physical environment, whether it be another cell or part of the scaffold. As a cell grows and collides with an obstacle, it is expected that the response will include changes in the cytoskeleton and cell wall to further alter the growth properties. Mechanical signals are generated by internal...
factors in a tissue, and are not purely dependent on the external environment. For example, stretching or shear may occur when a neighbouring cell grows. The exact molecular and cellular responses are not well elucidated and it is still unclear what factors are responsible for sensing and transducing the mechanical loading between the individual cells. One established method measures strain rates using whole tissues such as recording the distance from root tip (µm) and strain rate (%.h⁻¹) against time (min). However, individual cell behaviour is masked in whole tissues that behave with group dynamics. Meanwhile, in mammalian tissue engineering, collagen gel-scaffolds have been widely used to study mechanical forces generated by single cells and cell–matrix interactions in a simplified tissue model with quantifiable mechanical properties. For plant cells, hydrogel-based scaffolds could also be used to study the mechanical forces of plant cells in a similar way. The scaffold can be cut to size and placed in well-plates to embed cells during culture. Once cells have developed, the scaffold can be examined directly under a microscope. The compression of the scaffold can be measured before and after a period of growth and the average strain rate per cell can be calculated. Moreover, pressure can be applied on two ends of the gel with a tailored shape, allowing live observation of the transfer of forces from the scaffold to the cells at the nearest point of pressure and subsequently how their microtubule orientations change and influence neighbouring cells.

To scale-up applications at an industrial level, 3D plant tissue engineering techniques need to improve the labour-intensive and time-consuming scaffold fabrication and culture initiation procedures. Nonetheless, the 3D tissue constructs are quicker to achieve (10–14 days) than whole plant tissues (14–21 days growing from seeds for Arabidopsis). Similar scale-up challenges are currently limiting mammalian tissue engineering to niche products. Here, it is worth noting that the larger plant cell size (50–500 µm) compared with mammalian cells (1–10 µm) helps to lower the resolution barrier to advanced scalable technologies including additive manufacturing (3D printing). For example, methods such as melt electrospinning writing (MEW) can precisely define the shape and size of the in vitro environment with submicron fibre diameter. But the technology is limited to fabricating pore sizes ≥ 50 µm. These structures need to be modified to better capture small mammalian cells but are perfectly suited for the size of plant cells. Together with some promising new scaffold modifications that enable accelerated plant cell growth, the future of plant tissue engineering at an industrial scale is looking good.

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References