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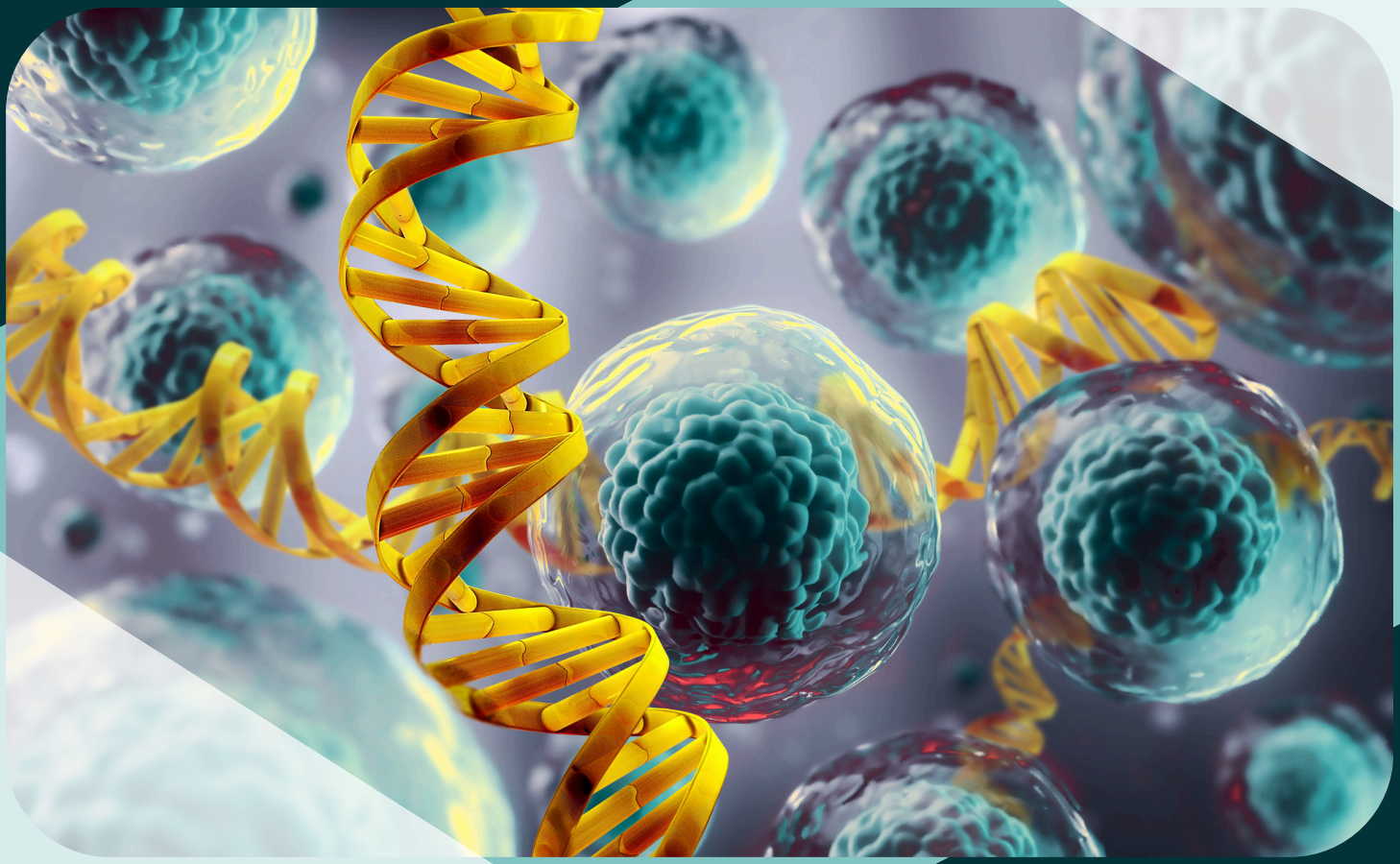

Beyond basic biology: elevating stem cell potential with advanced cell models

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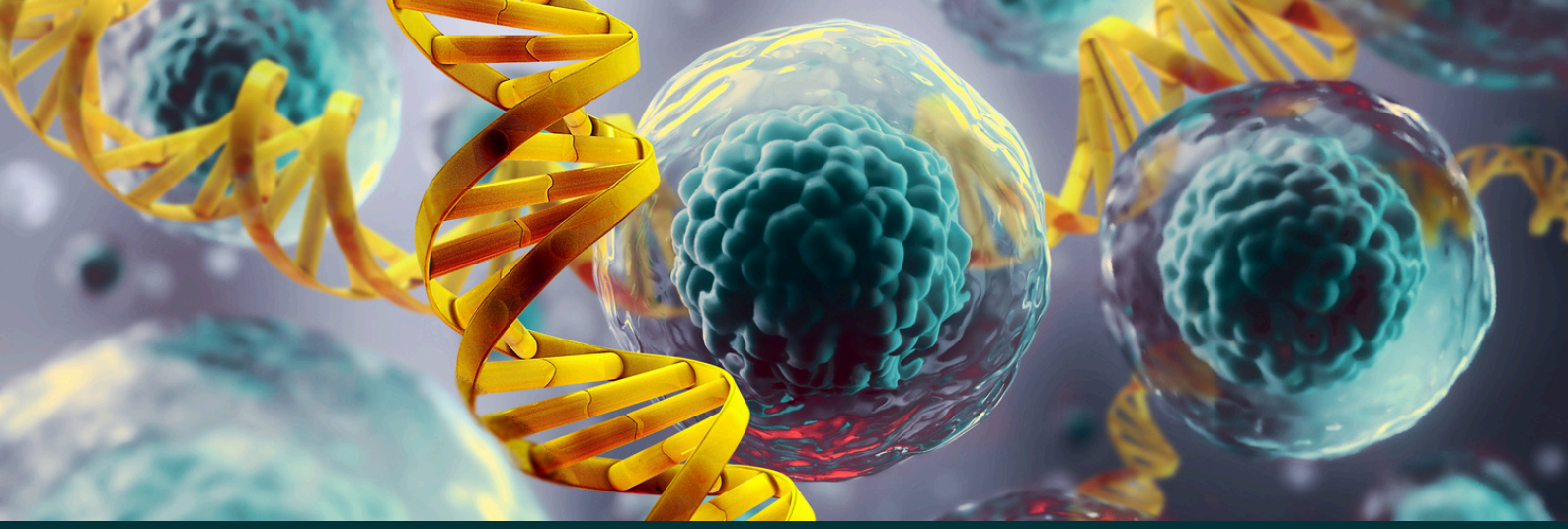

Beyond basic biology: elevating stem cell potential with advanced cell models

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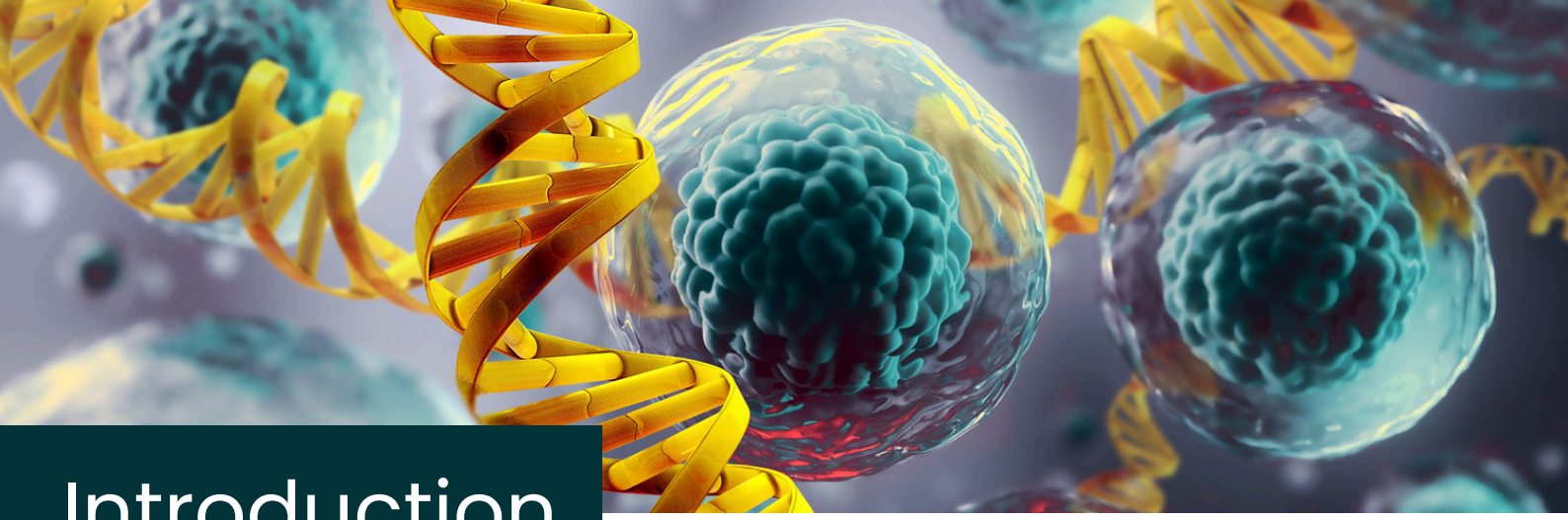

Introduction

Stem cells hold immense potential in research and therapeutic development. One area where they are being utilized is in the development of advanced cell models, such as organoids. Stem cell-derived advanced cell models closely resemble human physiology and therefore can be used to study complex biological processes and disease mechanisms. In drug development, these models provide a reliable platform for screening potential therapeutics and assessing toxicity, reducing reliance on animal testing and improving prediction of human responses. Furthermore, patient-specific stem cells facilitate personalized medicine approaches, allowing for the development of tailored treatments based on individual genetic profiles.

However, the cultivation and maintenance of stem cells present significant challenges. These cells are notoriously high maintenance and expensive to work with, requiring precise culture conditions and constant monitoring to ensure they retain their unique ability to differentiate into multiple cell types. The pluripotency, viability and homogeneity of stem cells are critical factors that must be preserved throughout research and therapeutic processes.

In this eBook, we look at how advanced cell models are elevating stem cell potential. We also explore solutions that are available for the culturing, maintenance and characterization of stem cells, to ensure models are of the highest quality.

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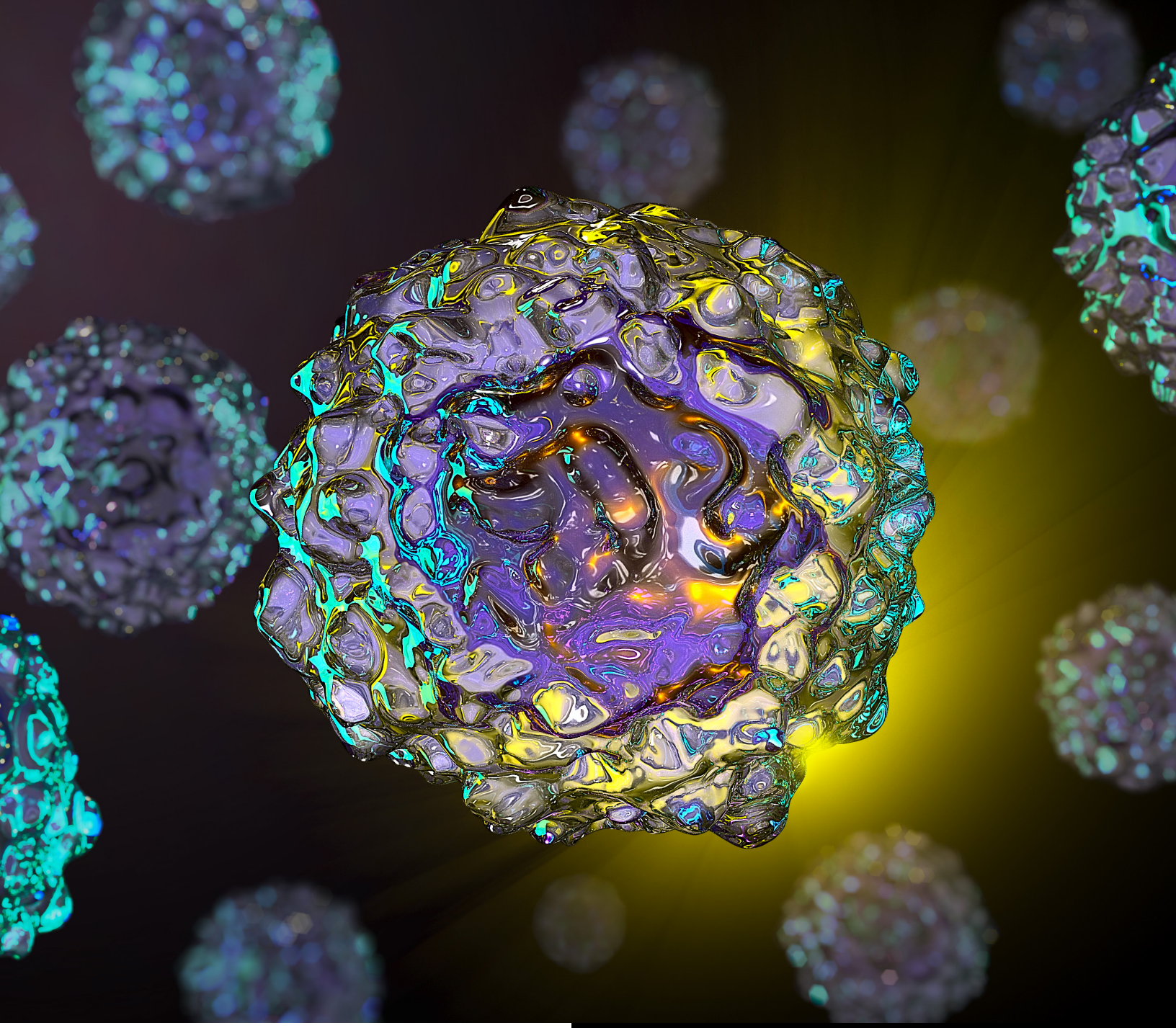

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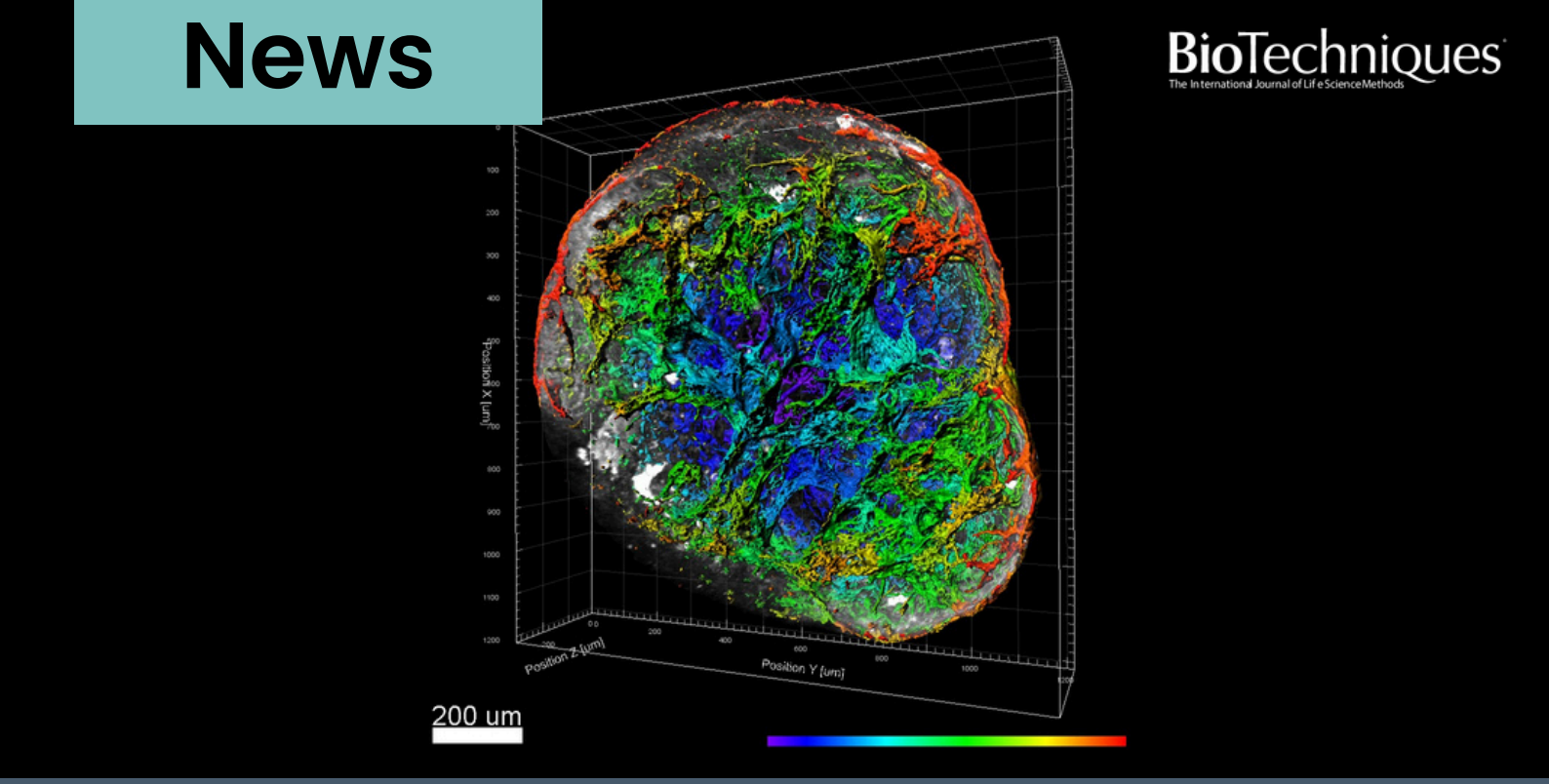

A vascular organoid developed by Takanori Takebe *et al.* Credit: Cincinnati Children's

Exposing the circulatory impacts of COVID-19 with vascular organoids

Researchers have developed human-derived vascular organoids that can be infected with SARS-CoV-2 to investigate its impact on blood circulation, revealing a potential target for therapeutic development.

A recent research collaboration between Cincinnati Children's Hospital Medical Center (OH, USA) and Tokyo Medical and Dental University (Japan), led by Takanori Takebe (of both organizations), has successfully developed vascular organoids to investigate the circulatory effects of COVID-19. Using these models, the team has been able to identify key mechanisms involved in the disruption to blood caused by SARS-CoV-2 and piece together a potential therapeutic solution.

When COVID-19 first ripped through the globe, one of the key factors identified in its deadly pathogenesis was the impact it had on the circulation of blood in acutely ill patients; both deep vein and microvascular thromboses were observed, alongside pulmonary embolisms. Unfortunately, the mechanisms behind these symptoms were not understood and traditional rodent model studies were of no help, as the rodent response to infection with SARS-CoV-2 was too disparate from our own.

In order to address this gap in our understanding and to facilitate the development of more effective therapeutics for COVID-19, the research team set out to design a more appropriate model to investigate the mechanisms responsible for the circulatory effects of the disease. To do this, they successfully developed infection-competent human induced PSC (iPSC)-derived vascular organoids, dubbed iVOs.

The team then conducted a longitudinal proteome analysis of critically ill patients' serum alongside an analysis of single-cell transcriptomics data derived from the bronchoalveolar lavage fluid of three healthy

controls, three moderate and six severe COVID-19 patients.

From this investigation the team found that a deviancy in the amplification cycle regulated by complement factor B and D (CFD) played a key role in COVID-19's impact on the circulatory system. So, to combat the source of these symptoms, the team established a long-acting, pH-sensitive monoclonal antibody therapeutic that targets CFD. This monoclonal antibody was used to treat both the newly established vascular organoids and a cohort of macaque monkey models, both infected with COVID-19.

"After conducting many proteomic, genetic and other studies, we determined that the severe vascular damage and thrombosis associated with COVID-19 can be mitigated by a long-acting monoclonal antibody that targets the complement amplification cycle regulated by factor D (CFD)," Takebe revealed.

While these results were observed in both the organoid and macaque models, they were only mild. Next, the team want to investigate the development of a clinical-grade monoclonal antibody to target CFD with the hope that it will one day be used to successfully treat COVID-19.

Written by Tristan Free, Senior Editor, *BioTechniques*

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1. Kawakami E, Saiki N, Yoneyama Y *et al.* Complement factor D targeting protects endotheliopathy in organoid and monkey models of COVID-19. *Cell Stem Cell* 30(10), P1315–1330 (2023).

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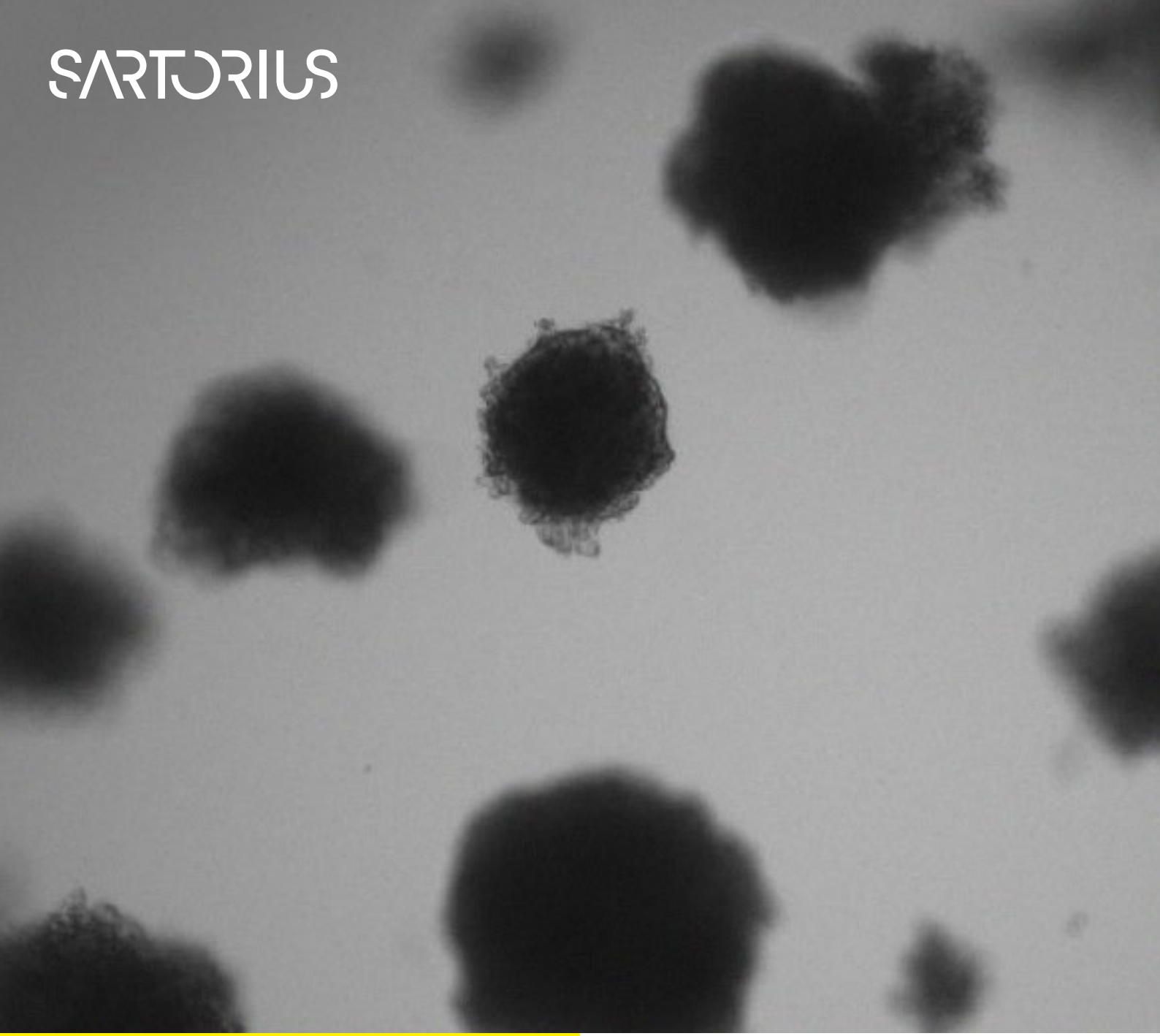


Cell Selection and Retrieval

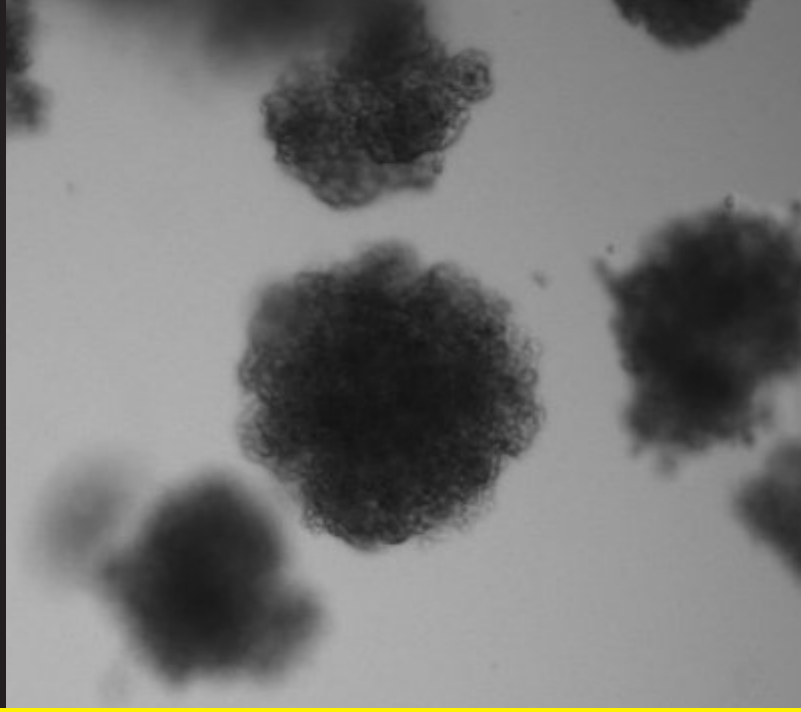
Organoids and Spheroids

Automated Workflows for the High-Throughput Selection and Picking of Complex 3D Structures

- Automated scanning, detection and gating of complex 3D structures based on a range of morphological parameters
- Organoid sizes from 80 µm to 3.5 mm
- Successful embedding of spheroids and organoids in 100% Matrigel into plates with or without cell culture membranes
- Low (1 µL) media injection volumes
- No aspiration of neighbouring clones

Organoid and Spheroid Research

Organoids are self-organizing, 3-dimensional systems which retain many physiological characteristics of the native tissue from which they are derived. Accordingly, these miniaturized models have significant advantages over the use of traditional immortalized cell lines in providing accurate information on human disease modelling and can be used in the fields of drug screening, rare disease research, personalized medicine, and many others.



Key Advantages of the CellCelector in Organoid Research

- Automated scanning, detection and gating of complex 3D structures based on morphological and fluorescence parameters
- Gentle picking of a wide range of organoid sizes, ranging from 80 µm to 3.5 mm
- No changes in 3D structure or morphology following picking and transfer
- Organoid transfer with exceptionally low (1 µL) injection volumes of surrounding media into either 100% hydrogel, liquid media or any other medium
- Successful embedding of spheroids and organoids in 100% Matrigel into plates with or without cell culture membranes
- Full documentation of transferred organoids – from source vessel to destination plate

Organoid Research: CellCelector Advantages

The CellCelector Flex has a number of inherent hardware features which are crucial for generating successful results within within various organoid applications:

Cooled Destination Plates

The use of the optional cooled deck tray can maintain hydrogel temperature at ~0 °C, thus preventing any polymerization before the organoid structure is deposited (Fig. 1). Increasing the temperature of deck tray up to 40 °C subsequently facilitates optimal polymerization.

Further information on the cooled deck tray can be found in the "**CellCelector Sample Deposition**" technical flyer.

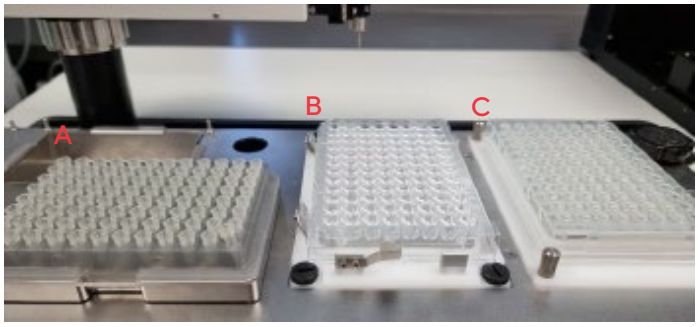


Figure 1: Cooled destination deck tray. (A) Rack containing 500 µm plastic PrecisionTips; (B) 96 well destination tray for organoid transfer; and (C) Cooled PCR plate containing 30 µl Matrigel in each well at approximately 0°C.

Automatic Morphology Measurements and Gating

Automatically identify desirable organoids based on a range of morphological parameters, including area, diameter, sphericity, and the presence of neighbouring organoids (Fig. 2).

Further information on object measurements and gating can be found in the "**CellCelector Image and Image Analysis**" technical flyer.

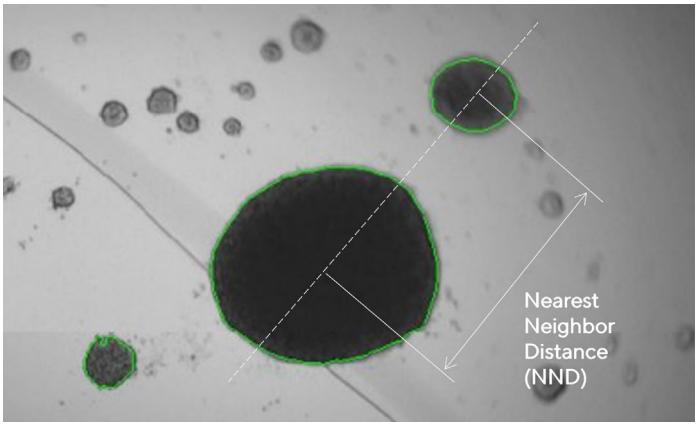


Figure 2: Nearest Neighbour Distance between a large heart organoid and its satellite organoid

Automated Picking Correction for Organoids in Suspension

Non-adherent organoids may move between scanning and picking. By using the automated correction pick-up functionality, organoids which might have moved can be easily picked within a pre-defined detection area (Fig. 3).

Further information on the automatic picking correction functionality can be found in the "**CellCelector Picking and Transfer**" technical flyer.

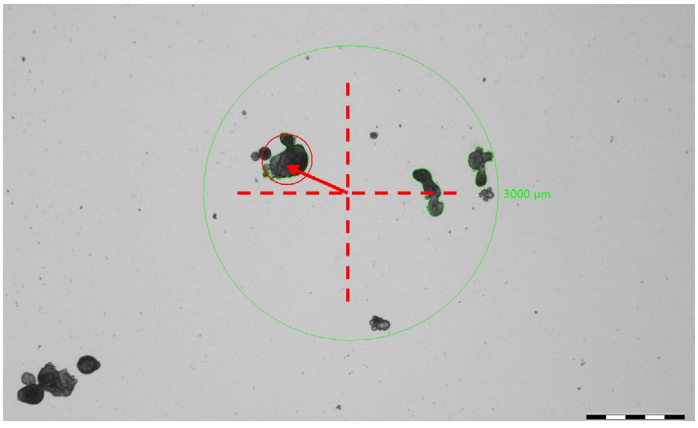


Figure 3: Automated picking correction for lung organoids in suspension

Picking From and Deposition Into 100% Hydrogel

Picking From Different Hydrogels

Organoids can be easily picked from a variety of hydrogels or liquid media without disturbing surrounding structures. In this example, organoids were efficiently picked from Matrigel® despite high organoid density across different planes (Fig. 4).

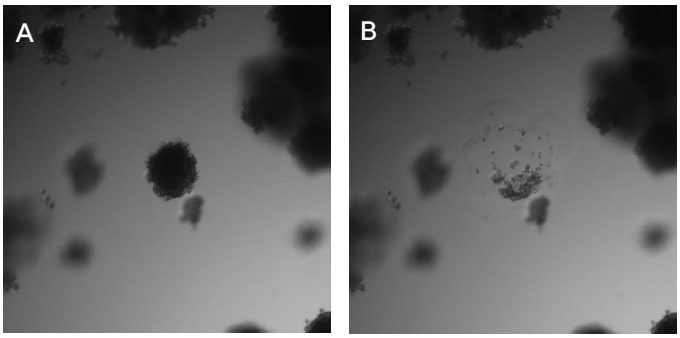


Figure 4: Accurate organoid selection and transfer from areas of high organoid density, (A) before and (B) after selection and transfer

Bubble-Free Deposition Into Hydrogel

Controlling aspiration speed, volume and destination temperature parameters allows 100% bubble-free organoid deposition into small volumes of hydrogel (<10 µL) or liquid media. Different approaches can be taken to achieve this. Destination plates can be kept at a continuously low temperature by the cooled deck tray allowing small hydrogel volumes to be aspirated and deposited without any polymerization, before the organoid is deposited directly into the hydrogel (Fig. 5). Conversely, both the hydrogel and the organoid can be aspirated in a single movement, before bubble-free deposition into the destination plate of choice (Fig. 6).

A key feature of both approaches are the low media volumes (<1 µL) aspirated with the organoid before deposition, therefore ensuring the organoids are surrounded by the optimal environment required for further growth and development.

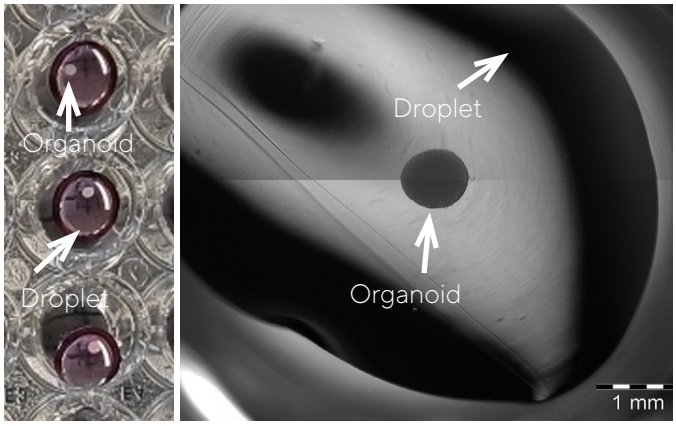


Figure 5: Photograph and scanning of the destination plate to verify deposits and the absence of air bubbles

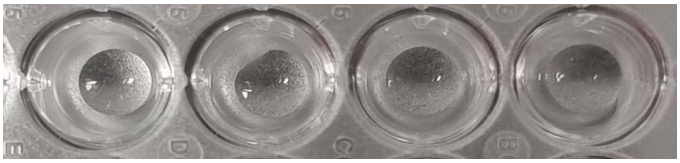


Figure 6: Photograph of bubble-free 10 µL and 20 µL Geltrex® droplets in U-bottom 96 well plates 90 mins after initial deposition

Morphology Preservation Following Transfer

Comparison of organoid images before (from the source plates) and after (from the destination plate) transfer shows that the organoids retain their morphology and structure due to the very gentle transfer. Additional downstream analysis confirmed internal structure preservation (Fig. 7).

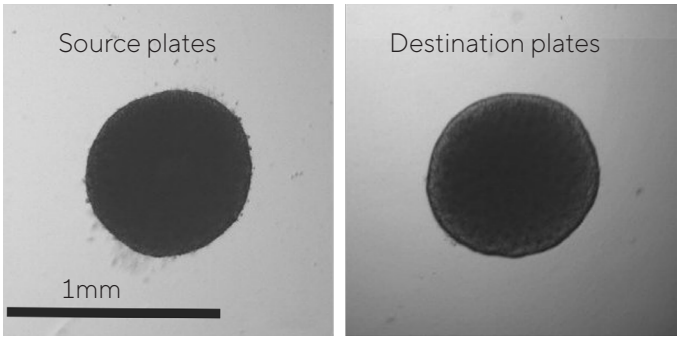


Figure 7: 700 µm heart organoids maintained their structure following gentle transfer

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REVIEW ARTICLE

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Synthetic scaffolds for 3D cell cultures and organoids: applications in regenerative medicine

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ABSTRACT

Three-dimensional (3D) cell cultures offer an unparalleled platform to recreate spatial arrangements of cells *in vitro*. 3D cell culture systems have gained increasing interest due to their evident advantages in providing more physiologically relevant information and more predictive data compared to their two-dimensional (2D) counterpart. Design and well-established fabrication of organoids (a particular type of 3D cell culture system) are nowadays considered a pivotal achievement for their ability to replicate *in vitro* cytoarchitecture and the functionalities of an organ. In this condition, pluripotent stem cells self-organize into 3D structures mimicking the *in vivo* microenvironments, architectures and multi-lineage differentiation. Scaffolds are key supporting elements in these 3D constructs, and Matrigel is the most commonly used matrix despite its relevant translation limitations including animal-derived sources, non-defined composition, batch-to-batch variability and poorly tailorable properties. Alternatively, 3D synthetic scaffolds, including self-assembling peptides, show promising biocompatibility and biomimetic properties, and can be tailored on specific target tissue/cells. In this review, we discuss the recent advances on 3D cell culture systems and organoids, promising tools for tissue engineering and regenerative medicine applications. For this purpose, we will describe the current state-of-the-art on 3D cell culture systems and organoids based on currently available synthetic-based biomaterials (including tailored self-assembling peptides) either tested in *in vivo* animal models or developed *in vitro* with potential application in the field of tissue engineering, with the aim to inspire researchers to take on such promising platforms for clinical applications in the near future.

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Introduction

Cell cultures are a widely used as an *in vitro* tool in which cells, under proper and controlled conditions, can live and grow. Their main goals are: (1) improving the understanding of cellular biology and living tissues self-organization; (2) providing precious insights for developmental biology; (3) shining a light on the mechanisms of tissue diseases; (4) the development of novel tissue engineering and/or drug-based therapies. 2D cell cultures have been used to study different cellular types *in vitro*, assessing cell proliferation, differentiation, survival, 2D migration, trans-differentiation [1–3]. However, due to their simplified setup, 2D cultures cannot thoroughly simulate the heterogeneous microenvironments and complex processes observed *in vivo,* such as cell signaling, biochemistry and 3D geometry: yielding results poorly predictive of an *in vivo* milieu [4–7].

One of the most exciting recent advancements in life science has been the introduction of 3D cell cultures, where cells grow, proliferate and differentiate into 3D microenvironments that are made of natural or a synthetic extracellular matrix (ECM)-like scaffolds [8]. Compared to 2D counterparts, the 3D cultures are biophysically and biochemically more similar to *in vivo* tissues/organs, yielding to a controlled biomimicry of an *in vivo* milieu, recapitulating cell-cell and cell-matrix interactions [9,10]. In recent years, the term "organoid" has been used to defined a particular 3D cell culturing methodology that incorporates some key features of an organ. They feature heterogenous and organ-specific cell types exhibiting spatial organization similar to the

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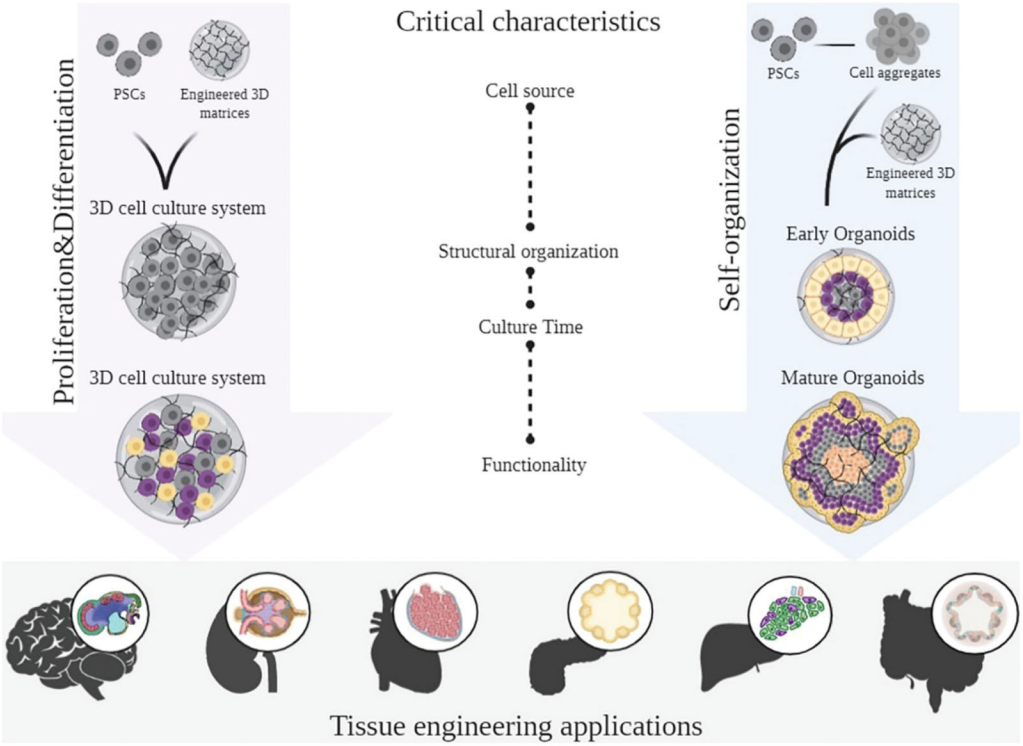

Figure 1. Strategies to create 3D cell culture systems and organoids. The formation of 3D cell culture systems (left) depends on integration of PSCs into an engineered 3D matrix.

in vivo and are capable of reproducing some functions of the selected organ [11]. However, despite their exciting potential in biomedical applications, existing organoid cultures have significant drawbacks. They typically require animal-derived ECMs substrates, such as Matrigel or similar products, characterized by a poorly defined composition and batch-to-batch variability, raising concern on results reproducibility, pathogen transfer and translational potential. That is why increasing efforts have been dedicated to develop bioinspired and fully-synthetic materials that could replace naturally-derived matrices, potentially generating protocols that are more reproducible and translatable into clinical applications [12]. Nonetheless, it is important to note that some 3D constructs made of synthetic biomaterials are perfused with serum-containing media, or other animal extracts, required for cell differentiation and/or organoid formation in poorly biomimetic substrates [13].

In this Review, we provide a detailed overview of the recent relevant advances in the field of "synthetic" 3D cell culture systems and organoids as advanced tools for regenerative medicine and tissue engineering, "keeping an eye" on their clinical applications. From now on, we will consider "3D cell culture systems" and "organoids" as similar but distinct concepts. On the other hand, the term "3D constructs" will stand for both models.

Differences and similarities between 3D cell culture systems and organoids

It is important to define 3D cell culture systems and organoids, highlighting their differences and similarities (Figure 1). Firstly, 3D constructs are made of cells embedded into biomaterial-made scaffolds. Biomaterials provide structural and mechanical support for cell cultures. They are used as substrates mimicking ECM and, in some cases, they can be tailored to coax different cell behaviors [8,14]. On the other hand, mostly used cells are induced-pluripotent stem cells (iPSCs), pluripotent stem cells (PSCs), adult stem cells (AdSCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs) or adult primary tissue-derived cells. The selection of appropriate media, growth factors and morphogens are fundamental to preserve functional characteristics of cells and to obtain the desired 3D constructs. Technologies such as cell encapsulation [15], 3D printing [16], air-liquid interface [17,18] and microfluidic organ-on-a-chip [19] have promoted the development specific 3D constructs applicable in various biomedical fields (see the following paragraphs).

Both 3D cell culture systems and organoids provide artificial microenvironments in which cells grow, differentiate and can interact with each other (and with the encapsulating biomaterial) in all three-dimensions, mimicking what happens in the *in vivo* milieu.

The definition of organoid is still evolving, but it can be summarized in four points: (i) two or more interacting cell types; (ii) 3D multi-cellular structure; (iii) self-organization of multiple distinct cell phenotypes into specific supra-cellular structures found in organs; and (iv) functional properties resembling the corresponding native tissue. Indeed, an organoid is a subtype of 3D cell culture system that mimics its corresponding tissue in terms of morphology, structure and, in particular, functionality (Figure 1) [11]. Self-organization is the key feature of organoids because it stands for multicellular structures coming from stem/progenitor cells differentiation/proliferation/migration and exhibiting remarkable similarities to the *in vivo* organ cyto-architecture. While in organoids, organ-specific cell types self-organize into local structures mimicking those of the corresponding tissue/organ, in 3D cell culture systems. A uniformly random cell organization prevails, making them more approximative replicas of the target organ. Also, 3D culture systems and organoids can be maintained in culture for long timeframes (up to 1 year) [20] and they can be expanded after dissociation and subsequent re-plating, enabling the generation of clonal cultures [21,22].

Different research groups reported the development of 3D constructs that model (albeit incompletely) amongst other organs: brain [17,23], intestine [24,25], kidney [26] and liver [27]. Their main application fields range from tissue engineering [18] and drug discovery [28] to stem cell biology [29] and disease modeling [30] (Figure 2). 3D cell culture systems and organoid achievements will be presented in the following paragraphs considering the just mentioned differences and similarities.

Random organization, brief culture times, intensive cell proliferation and differentiation are key characteristics of 3D cell culture systems. Conversely, cell aggregates in clusters, highly organized structures, extended culture times and functional properties resembling biological tissue portray organoid formation. Researchers have applied these protocols to produce *in vitro* sections of several organs (or even whole organs), such as: brain, kidney, heart, pancreas, liver and intestine.

Engineered 3D matrices for cell constructs formation

Scaffolds are the key-supporting elements of 3D constructs and are also used as standalone components in tissue engineering to repair and restore damaged

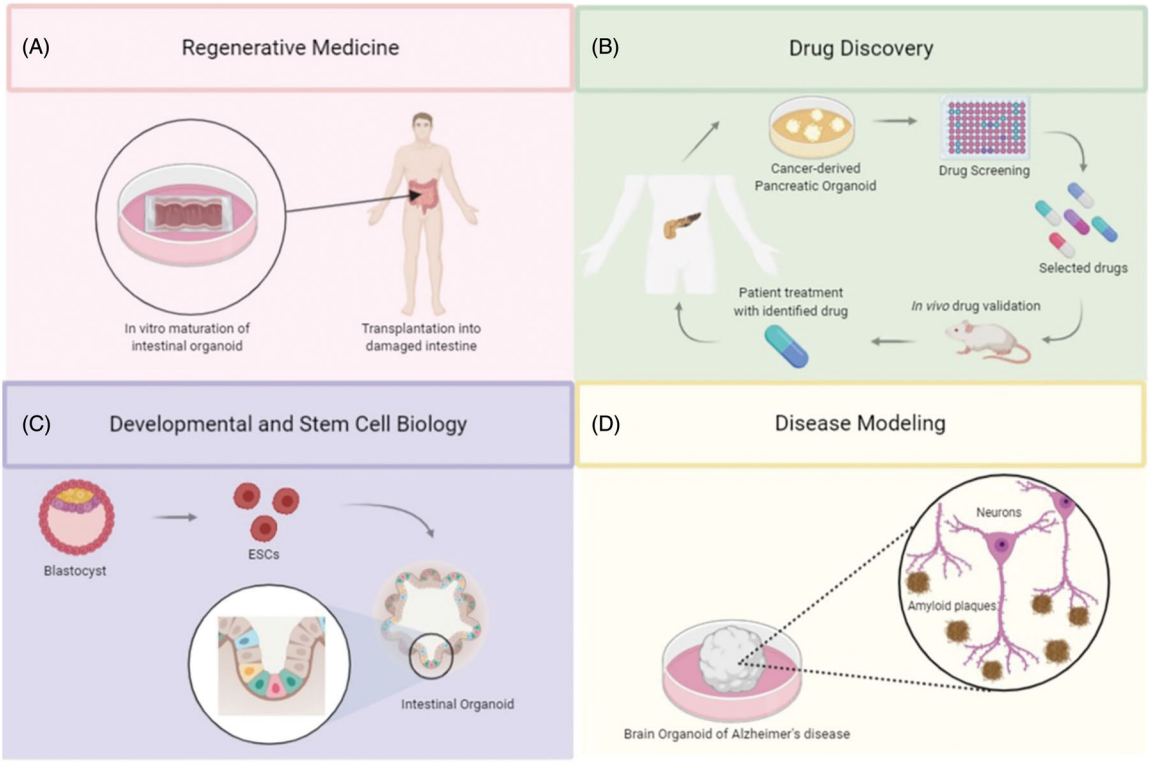

Figure 2. Potential applications of engineered 3D constructs. (A) organoids derived from healthy donor cells could be used as a bioengineered tissue for regenerative medicine purposes. (B) Organoids can be used to identify specific drugs: patient-specific organoids may help to identify the best customized drug for each patient. (C) In the field of developmental and stem cell biology, organoids can be used to better understand the principles of development, homeostasis and regeneration. (D) Lastly, organoids represent a useful tool for the study of disease modeling because they aim to mimic *in vitro* the complexity of a diseased organ. Hence, they are used to study *in vitro* particular pathologies, difficult to reproduce in *in vivo* experiments.

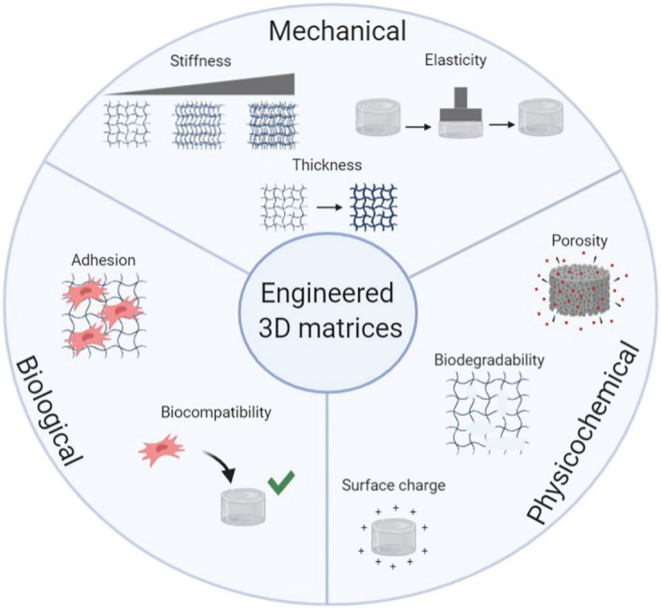

Figure 3. Mechanical, physicochemical and biological parameters of biomimetic scaffolds to be tuned in accordance with specific *in vitro* and/or *in vivo* applications.

tissues. Scaffolds are made of 3D porous, fibrous or permeable biomaterials intended to confer mechanical support to cultured cells and mimic native ECM *via* biological moieties. [8]. Besides mechanical support, hydrogels feature other critical cues regulating cell homeostasis and migration: this is the case for surface chemistry, porosity, degradation rate, micro-/nanotopography (Figure 3). 3D scaffolds are generally highly porous with interconnected pore networks facilitating nutrient/oxygen diffusion and waste removal. Highly porous biomaterials enable effective release of bioactive molecules such as: cytokines [31], inhibitors [32], drugs [33] and antibiotics [34]. Indeed, porosity and micro-/nanotopography should be tailored and optimized for each 3D cell culture system. As an example, proliferation and differentiation of MSCs cultured in 3D matrices are affected by material porosity and fiber diameter: the higher porosity and larger the fiber diameters, the higher the cell proliferation rate. On the contrary, thinner fibers resulted in lower cell attachment and caused MSCs with spherical morphologies [35].

Moreover, increasing the net positive charge on tissue engineering scaffolds results in improved proliferation and the spreading of cells because of their negatively charged membrane proteins [36]. This is the case for myoblast cells showing elongated morphologies when seeded onto substrates with positive net surface charges [37].

Because of the above, scaffold design is of paramount importance and must be customized toward the target tissue of the desired application to provide the best possible impact.

The natural ECM is a highly hydrated, organized, heterogeneous, bioactive and dynamic structure that regulates cell function. The main components of native ECM are fibrous proteins of collagen and elastin, fibronectin, laminin, and glycosaminoglycans [38]. ECM composition varies considerably from tissue to tissue and changes during disease and aging [39,40]. Moreover, the mechanical properties of biological tissues differ greatly, and elastic modulus range from the 11 Pa of intestinal mucus to the 20 GPa of the cortical bone [41]. Changing the elastic moduli of the cellular environment can lead to differences in cellular responses, in term of: adhesion, morphology and differentiation [42]. For example, it has been demonstrated that scaffolds featuring the *in vivo* brain mechanical properties, enhanced neuronal differentiation of neural stem cells while it was not the case for stiffer hydrogels [43,44]. On the other hand, stiff hydrogels were necessary to obtain appropriate osteo-inductive cell differentiation [45]. Hence, deep characterization and understanding of ECM is essential to design 3D constructs "replicating" distinct tissues and, as such, effective in regenerative medicine applications.

Scaffolds can be made from natural sources or from synthetic polymers, or, as a third option, they can be synthetic but engineered to mimic the biological activities of ECM (ECM-like scaffolds) *via* biological active motifs (such as IKVAV and YIGSR laminin-derived sequences, RGD-based sequences, DGRGDSVAYG osteogenic cell adhesion motif, and so on [46]) found in the ECM. Lastly, natural and synthetic biomaterials can be

mixed together to obtain scaffolds with a hybrid composition [8]. A significant advantage of synthetic ECM-like scaffolds is the possibility to better control mechanical properties, biomimetics or the permeability of the matrix, in order to modulate the cell fate [47].

Hydrogels are the preferred choice to develop 3D constructs *in vitro* [48–50]. In general, hydrogels are made of 3D networks with hydrophilic polymer chains entrapping large amounts of water. Also, hydrogel matrices can better mimic viscoelastic and topographical properties of ECM and, by means of moieties decorating their nanostructures, may also favor cell–cell or cell-matrix interactions [51].

Naturally-derived hydrogels are still widely used by the scientific community to create 3D constructs, despite their serious limitations. They can be used as a gold standard in *in vitro* studies due to their availability, ease of use and versatility for culturing different types of cells. Also, they have natural adhesive properties and sustain many physiological cell functions. Theirs assets are superior cell viability, high proliferation and satisfactory cell differentiation, yielding networks of the cell phenotypes typically observed *in vivo* [52]. Matrigel and similar products, like Geltrex or Cultrex, are the most commonly used *in vitro* matrices derived from extracts of Engelbreth-Holm-Swarm mouse tumors. They contain gelatinous mixtures of ECM components, including: laminin, collagen type IV, entactin, and heparan sulfate proteoglycans, as well as some growth factors, such as TGF-β and FGF [53]. Naturally-derived materials also includes: collagen [54], agarose [55], alginate [56] and silk fibroin [57]. They are widely used *in vitro* for the production of organoids in the fields of: developmental and stem cell biology [58], disease modeling [59] and drug discovery [60]. Nonetheless, their applicability in advanced biomedical technologies for translational medicine is hampered by: batch-to-batch composition variability, limited chemical tunability, risk of pathogen-transfer and the presence of xenogeneic components [12,61]. Indeed, numerous studies have identified different amounts of: growth factors [53,62], the presence of xenogenic contaminants [63,64] and unexpected variations in mechanical properties [65,66] between batches and within a single batch. As an example, it was demonstrated that the elastic modulus could be doubled in two analyzed batches (400 Pa and 840 Pa) [65]. Others reported heterogeneous mechanical properties within the same Matrigel scaffold, revealing local areas with higher elastic moduli [66].

Therefore, synthetic polymeric scaffolds are considered by the scientific community to be more advantageous over natural-derived hydrogels because of their reproducible mechanical, chemical and biological properties, that can be tuned by changing their composition and molecular weight [67] or by adding crosslinker molecules [68,69] and multifunctional moieties [70].

Advanced biomaterials with translational potential

In general, biomaterials should feature some goal characteristics: nontoxic, low-immunogenic, chemically inert and elicit a negligible inflammatory response. In particular, clinically relevant biomaterials are characterized by a well-defined composition, low batch-variability and the absence of animal derivatives, such as serum, ECM extract and so forth.

Thanks to the advances in nanomedicine and nanotechnology, a myriad of synthetic hydrogels is now being developed to obtain scaffolds with defined biological, biochemical, and biophysical features, aiming to provide effective alternatives to Matrigel and a number of new products potentially translatable into clinics. The most common synthetic scaffolds and their application are listed in Table 1. Among them, the most widely used synthetic polymers are: poly(ethylene glycol) (PEG) [27,71,85], polylactide-co-glycolide (PLG) [18], poly-L-lactic acid (PLLA), polycaprolactone (PCL) (or a combination of them) [91] and also self-assembling peptides [75].

Self-assembling peptides: biomimetic scaffolds

Distinct from synthetic polymeric hydrogels, self-assembling peptide (SAP) hydrogels are advanced nanostructured hydrogels integrating biofunctional, mechanical and morphological cues in order to reliably mimic the ECM.

In general, the mostly used and advanced SAPs in tissue engineering comprise, in their self-assembling sequence, alternated charged hydrophilic and hydrophobic amino acid residues [96] or hydrophilic heads linked to hydrophobic tails [97]. SAPs can spontaneously self-assemble upon exposure to an external stimulus to form nano-fibrous structures that, at the nanoscale level, form stable β-sheet structures and self-assemble into twisted nanofibers and/or flat sheets (Figure 4(A,B)) [98].

SAPs are versatile matrices that can be customized to fabricate *in vitro* microtissues or 3D cell cultures (Table 1). Multiple bioactive motifs have been successfully added to self-assembling backbones without hampering the SAP self-assembling propensity. This approach provides controllable and simultaneous

Table 1. 3D constructs made of synthetic (or semi-synthetic) hydrogels, key characteristics and tissue engineering applications.

Organ	Scaffold composition	Technology used	Cell source	Description
	PEG	Cell encapsulation for 3D cell culture system	Human iPSC- and ECS-derived NPC Co-culture of NPC, endothelial cells, mural cells, and microglia precursors	Long term *in vitro* neural differentiation (up to 49 days) with upregulation of neuronal and glial mRNA and presence of glutamate neurotransmitter [71] *In vitro* development of highly organized 3D cell culture system characterized by neuronal and glial cells, organized vascular network and microglia with ramified morphology [72]
	RADA16 bi-functionalized with -RGD and -IKVAV	Cell encapsulation for 3D cell culture system	NPCs/NSCs	Nervous regeneration in sciatic nerve defect, intracerebral hemorrhage and spinal cord transection [73]
	RADA16 functionalized with BDNF-derived peptide	Cell encapsulation for 3D cell culture system	hUC-MSCs and active astrocytes	Application in TBI for cortical coloboma: reduced injured brain cavities and reduced reactive gliosis surrounding the implants [74]
	Multifunctionalized SAP (HYDROSAP)	Cell encapsulation for 3D cell culture system	Human NSCs	Pre-differentiated human NSCs *in vitro* showed high percentage of neuronal markers, better NSCs engraftment and improved behavioral recovery when implanted in spinal cord animal model [75]. Long-term *in vitro* 3D cell culture system showed expression of neurotransmitters and presence of myelinic proteins in serum-free conditions [76]
	Semi-synthetic GelMA	Bioprinting for 3D cell culture system	sNPCs and OPCs	sNPCs differentiate into neurons projecting axons throughout the scaffold channels, and OPCs differentiate into oligodendrocytes that myelinate axons creating an effective relay across the spinal cord injury site [77]
		Cell encapsulation for 3D cell culture system	iPSC-derived NSCs	Enhanced axonal regeneration, improved functional recovery, and inhibited inflammation in spinal cord injuries [78]
	PDMS	Bioprinting for 3D cell culture system	iPSCs	Design of 3D engineered system for the generation of custom-made kidney tubules [79,80]
	PEGDMA	Bioprinting for 3D cell culture system	BMSCs	3D printed PEGDA hydrogel patch with microchannels seeded with BMSCs to treat myocardial infarction [81]
	RADA16 functionalized with QHREDGS	Cell encapsulation for 3D cell culture system	MSCs	Improvement of cardiac function, reduction of infarct size, collagen content and cell apoptosis in myocardial infarction animal model [82]
	Hydroxybutyl chitosan HBC	LbL and Bioprinting for organoid	human iPSCs-derived cardiomyocytes, NHFC, HMVEC	Fabrication of native organ-like 3D cardiac tissue with one-direction cell alignment and vascular network formation[83]
	Semi-synthetic GelMA	Bioprinting for 3D cell culture system	Cardiomyocytes	Fabrication of 3D culture system of aligned cardiomyocytes resembling myofibril alignment of native myocardium [84]
	PEG functionalized with RGD	Cell encapsulation for 3D cell culture system	PI isolated from rats	Controlled delivery of VEGF enhanced vascularization within extrahepatic islet transplantation site, improving islet *via*bility and function *in vivo* [85]
		Microfluidics for organoid	PI isolated from mice	*In vivo* tracking of PEG-encapsulated islets in murine model of autoimmune diabetes demonstrated long-term protection [86]
	Dextran hydrogel functionalized with RGD	Cell encapsulation for organoid	Patient derived-human PI	Long-term expansion of HO in a chemically defined hydrogel and serum-free medium showed presence of pancreas ductal cells with similar characteristic of native tissue [87]
	Patented bioscaffold based on polysaccharide-polyamine copolymers	Cell encapsulation for organoid	PI isolated from mice	Bioscaffolds seeded with islets were assembled into a pancreas-like organoid: neo-vascularization around the implant site, and reduced hyperglycemia were detected in recipients [88]
	PEG	Cell encapsulation for organoid Microfluidics for organoid	iPSCs-derived hepatic progenitors human hepatocytes and fibroblasts	Fabrication of liver organoids resembling human liver in terms of morphology, gene expression and protein secretion [27] Organ-on-a-chip of liver featuring hepatocytes with morphology and activity (secretion of albumin) similar to those of *in vivo* cells [89]

(*continued*)

Table 1. Continued.

Organ	Scaffold composition	Technology used	Cell source	Description
	PEG functionalized with RGD	Cell encapsulation for organoid	Rat cholangiocytes	Fabrication of fully-synthetic scaffold for development of bile duct model: biophysical and biochemical parameters of scaffold affected cyst morphogenesis [90]
	PLLA and PCL	Cell encapsulation for 3D cell culture system	iPSCs-derived hepatocytes-like cells	Scaffold supported the iPSCs differentiation into hepatocytes-like cells *in vitro* [91]
	PGA	Cell encapsulation for organoid	Human cholangiocytes	Functional secretory capacity and formation of tubular structures similar to native tissue when transplanted in mice [92]
	PEG-4MAL	Cell encapsulation for organoid	Human PSCs	Intestinal organoids with performances similar to Matrigel when delivered to injured mouse colon [93] Hydrogel seeded improved colonic wound repair and transplanted human intestinal organoids engraftment [94]
	PEG functionalized with RGD	Cell encapsulation for organoid	Intestinal stem cells	Well-defined hydrogel for intestinal organoid cultures but supplemented with animal derived-laminin-111 [13]
	PEVA and PGS	Cell encapsulation for organoid	Intestinal stem cells	Long-term evaluation of artificial intestine in piglets with short bowel syndrome [95].

exposure of different functional motifs from self-assembled nanofibers, and boosted cell adhesion and proliferation [46]. Another important feature is the incorporation of short glycine spacers (2–4 Gly), between the self-assembling backbone and the functional motif, providing distancing and enough degrees of freedom to the functional motifs themselves, thus warranting satisfactory exposure of functional motifs to cell receptor/membrane binding (Figure 4(C,D)) [99].

SAPs may act as scaffolds for a wide range of biomedical applications ranging from: neural regeneration, angiogenesis and cardiac tissue regeneration to homeostasis and bone repair, but also for dental pulp and cartilage regeneration [100]. Furthermore, SAPs have been tested in clinical trials while many are being studied, thus showing their promising safety and therapeutic potential. For example, monomeric SAP P11-4, applied either in combination with fluoride varnish or polymeric SAP matrix, was a superior treatment for early caries compared to fluoride varnish alone in the treatment of non-cavitated occlusal caries in humans[101]. PuraStat peptide, based on RADA16, is currently used in clinics as an hemostat to control acute gastrointestinal blending, providing an effective control of different types of gastrointestinal hemorrhage when conventional hemostatic methods fail [102].

During *in vitro* studies, SAPs are used as physical supports for different cell types to generate 3D constructs. SAPs can embed pancreatic islets (PI) for their *in vitro* culturing and subsequent transplantation by protecting their viability and function over time. As represented in Figure 4(E), SAPs, contrarily to standard *in vitro* free-floating cultures, can help PI to maintain their primary islet structure throughout several weeks *in vitro* thanks to their soft biomechanics, nanostructured milieu and a protein-like composition similar to native pancreatic ECM [103]. Also, a wide range of publications demonstrated that SAPs can be used as serum-free reproducible supports fostering neural stem cells (NSCs) spreading and entanglement by conferring adhesive cues to enhance their proliferation, differentiation and maturation [1,43,97]. RADA16-BMHP1 [104], FAQ-LDLK12 [1] and multi-functionalized HYDROSAP [75] can support neuronal differentiation (βIII-Tubulin cells) of NSCs similarly to a natural derived matrix (Cultrex) [76]. Neuronal and oligodendroglial differentiation of NSCs cultured on synthetic SAPs were similar to Matrigel and Cultrex, with the advantage of being xenogeneic contaminant-free (Figure 4(F)). HYDROSAP was recently used [76] to design a 3D cell culture system achieving cell proliferation, neural differentiation, entanglement and neuronal maturation in long term cultures of densely seeded human NSCs. In Figure 4, a SAP-based 3D cell culture system (Figure 4(G)) is depicted, that is made of uniformly distributed human NSCs after one week's culture (Figure 4(H)), subsequently self-organized into structures resembling a neural organoid after 8 weeks *in vitro* (Figure 4(I)).

Building 3D scaffolds

Encapsulating cells in a hydrogel structure offers numerous features for tissue engineering applications, including protecting encapsulated cells from immune system and mediating interactions with the host environment [105,106]. Conventional protocols on cell encapsulation for the fabrication of artificial nanostructures with biological activities comprise cells mixing in

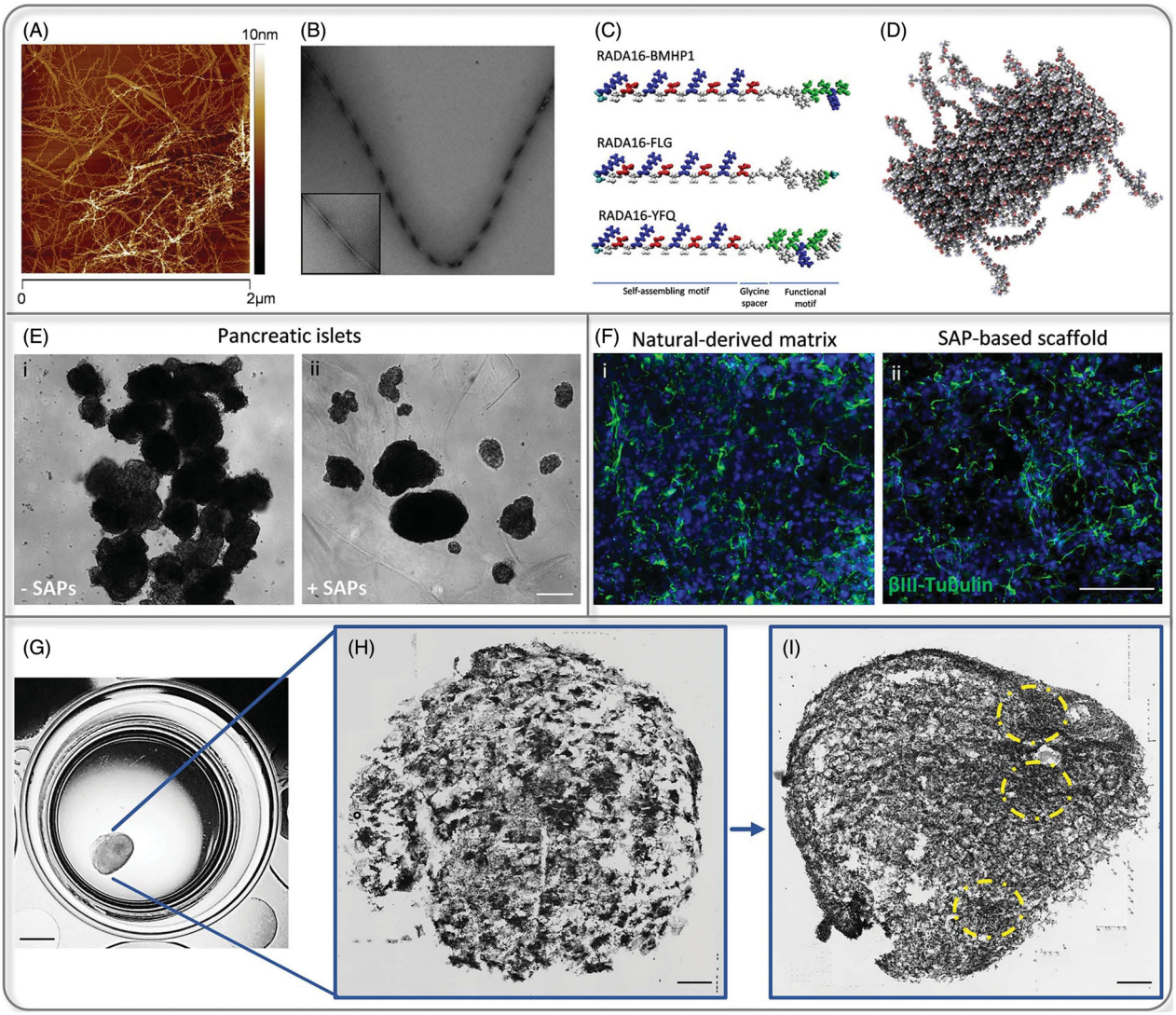

Figure 4. SAPs properties and applications. (A) AFM image of SAPs shows disposition of nanofibers into twisted protofibrils and flat sheets, while (B) TEM observation confirms a regular twisted protofibril structure. (C) Molecular representation of RADA16 self-assembling backbone linked to PFSSTKT (BMHP1), FLGFPT and YFQRYLI functional motifs interspaced with a di-Glycine spacer. Once exposed to physiological pH solutions these SAPs self-assemble into cross-beta structures (D): functional motifs hang from the double-layered RADA16 β-structure and are available for cell-receptors binding to trigger cell-specific responses. (Ei) SAPs prevent islets aggregation and maintain unaltered PI native structure in *in vitro* culture compared to free-floating PI (Eii). Functionalized SAPs (Fi) are an excellent alternative to gold standard natural-derived matrices (Fii): for example, neurons stained with βIII-tubulin marker (in green) show extended process and entangled neuronal network in both conditions. A 3D cellularized patch composed of differentiating human NSCs and functionalized SAP was obtained *in vitro* (G) and maintained in culture for 1 week (H) and for 8 weeks (I) in order to study self-organization and dense neural network formation. The different morphology represented in Haematoxylin-Eosin staining shows aggregates of clustered cells (H) giving rise to a proliferated, widespread and differentiated progeny (I), featuring also self-organized cellular proto-structures (dashed circles). Scale bar 100 μm for E and F; 5 mm for G; 500 μm for H and I.

a liquid solution followed by hydrogel gelation (Figure 5(A)) [107].

However, several artificial biologically active structures lack heterogenicity, and, as such, they fail to recapitulate the complexity of native tissues. Recently, novel strategies including microfluidics (Figure 5(B)), air-liquid interface (Figure 5(C)), layer-by-layer (Figure 5(D)) and 3D bioprinting cultures (Figure 5(E)), were adopted to produce advanced platforms of cell encapsulation systems. In the following paragraphs, we focus our discussion on the most recent advances in each of the mentioned approaches.

Hydrogel scaffolds for cell encapsulation

As mentioned before, conventional protocols on cell encapsulation include the fabrication of 3D constructs

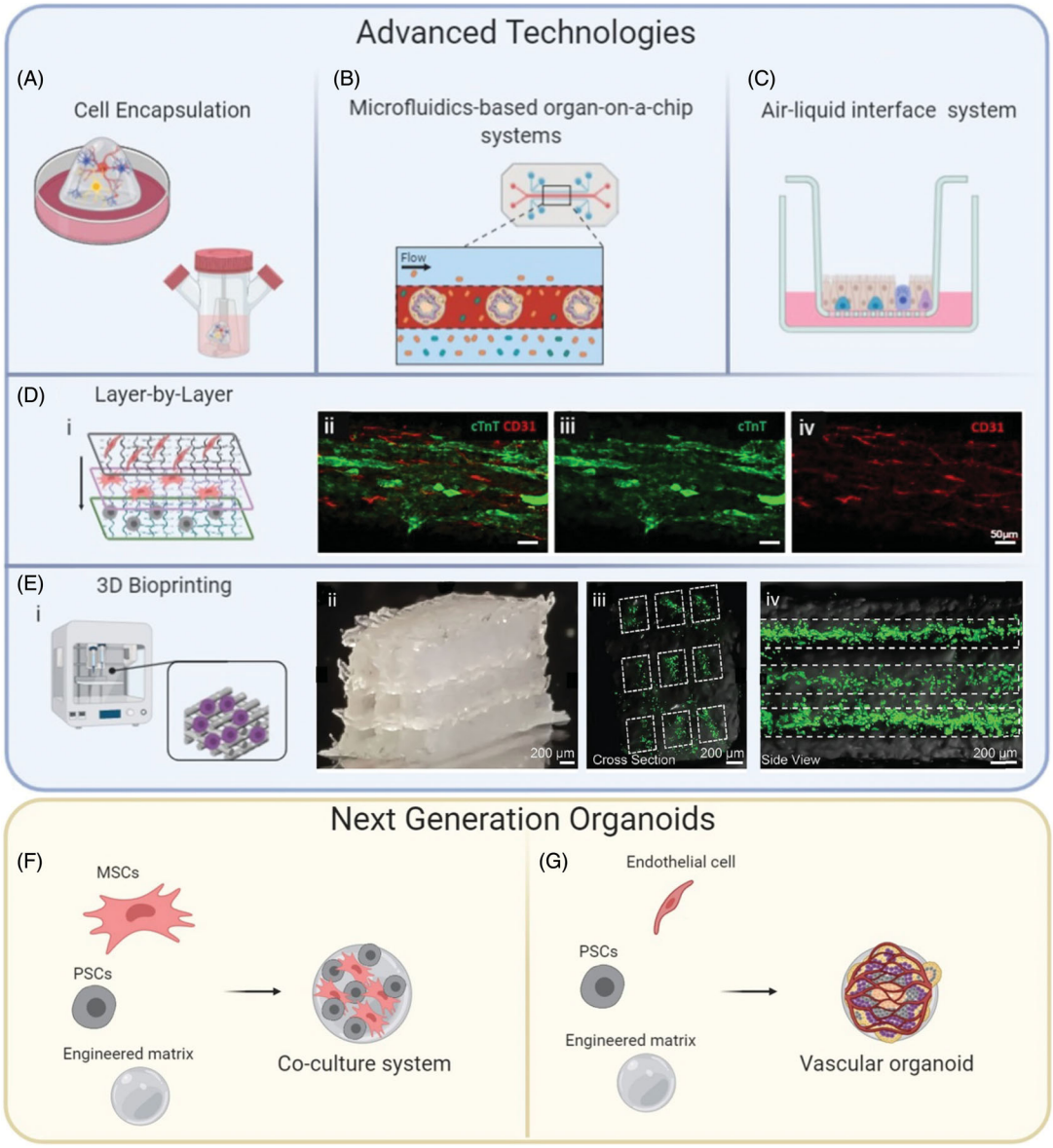

Figure 5. Strategies for creating 3D cell constructs. (A) Bioreactors are used to increase nutrient exchange in hydrogels scaffold with encapsulated cells; (B) Organoids can be integrated into *ad hoc* organ-on-chips (allowing better controlled nutrient and gas exchange) to recapitulate 3D tissue architectures/functions and also fostering connection among multiple pre-formed "organs"; (C) ALI systems are suited for organotypic cultures and organoids grown on permeable filter enabling enhanced oxygen transport; (Di) in LbL technique a multilayer 3D structure is produced: each layer can be made of different cell types and/or biomaterials to better recapitulate the complexity of native tissues; (Dii-iv) fabrication of orientation-controlled 3D cardiac tissue with vascular network (Div) and presence of cardiac troponin T (Diii) by using LbL technique; (Ei) 3D bioprinted structures allows precise cell-positioning and can be produced with a considerable degree of versatility and customization; (Eii) construction of neuro-compatible 3D bioprinted spinal cord-like culture system; (Eiii) cross-sectional, and (Eiv) longitudinal side views of live cells (in green) after 3 days from printing. Other promising steps forward for organoid technology are represented by the co-culturing of different cell types for the formation and the spatial self-organization of diverse tissue-specific interactions within a single organoid (F): in particular, endothelial cells can be used to create vascular networks *in vitro* to provide organoids with better nutrients exchange (G). (D,ii), (D,iii) and (D,iv) were licensed under the terms of Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/legalcode) from Ref. [83]. (E,ii), (E,iii) and (E,iv) are reproduced from Ref. [77] with permission (Copyright Wiley-VCH).

for a wide range of applications [105]. For 3D neuronal cultures, a multi-functionalized SAPs hydrogel was customized to encapsulate NSCs and promote cell adhesion, neuronal differentiation and neurite outgrowth [76]. The use of a spinning bioreactor tackles one of the major challenges in 3D constructs manufacturing, that is the lack of vascularization, i.e. nutrients exchange (Figure 5(A)). By means of dynamic culture conditions, cells constantly receive an increased amount of nutrients and oxygen [59]. Thanks to spinning bioreactors, cerebral organoids were maintained in culture up to 10 months, obtaining regions reminiscent of the cerebral cortex, choroid plexus, retina and meninges. Cruz-Acuña and colleagues proposed a cell-encapsulating hydrogel based on fully synthetic four-armed, maleimide-terminated PEG-4MAL macromer. They encapsulated human PSC-derived 3D spheroids, generating a robust and highly reproducible protocol for *in vitro* human organoid (HO) studies, with performances similar to those of Matrigel [93]. Thanks to its wide range of applications, their system could be adapted to a variety of different tissues, animal models and delivery sites. For example, localized in the *in vivo* delivery of PEG-4MAL hydrogel-encapsulated HOs to injured mouse colon, resulted in an enhanced engraftment of HOs and accelerated colonic wound repair [94].

Microfluidics-based organ-on-a-chip systems

Microfluidics organ-on-a-chip-based cell culture devices are valuable tools that enable co-culturing of 3D constructs in a spatially controlled manner (e.g. controlled perfusion flow and gradient control of cytokines) to better simulate tissue- and organ physiology (Figure 5(B)). Different studies on engineered 3D constructs-on-a-chip were proposed. Among them, Schepers and colleagues [89] developed a liver chip: primary human hepatocytes (or iPSC-derived hepatocyte-like cells) and fibroblasts were encapsulated in a small PEG microtissue. The resulting 3D culture system was placed into the microfluidic device, obtaining: (1) enhanced transport of oxygen and nutrients; (2) stable secretion of albumin for more than 28 days; (3) maintained inducible cytochrome P450 enzyme activity over time. Also, iPSC-derived hepatocyte-like cells featured morphology and activity resembling hepatocytes and biliary cells [89]. Such a combination of organ-on-a-chip and iPSCs could be generalized to other microfluidic tissue models.

In a recent study, microfluidic encapsulation was applied to obtain a 3D pancreas culture system. By using microfluidic PEG-based encapsulation, authors reduced microgel size down to eight-fold compared to traditional techniques, simplifying transplantation within highly vascularized tissue, enhancing safety and the translatability of their approach [86].

Air-liquid interface (ALI) systems

ALI technique was primarily used to mimic respiratory tract epithelia *in vitro* [108]. It was then applied to *in vitro* skin models, becoming a routine approach in clinics for wound healing [109]. Generally, in ALI, the top layer of the cultured cells is exposed to air while the basal surface is in contact with the culture medium. 3D constructs produced with ALI are cultured in a gel and are directly exposed to air instead of being submerged in a culture media (Figure 5(C)). To the best of the author's knowledge, there are no publications in the literature regarding synthetic hydrogel-based 3D constructs produced *via* ALI.

Nevertheless, because of the promising results of ALI, organoids are made of natural-derived hydrogels. It is worth spending a few words on cerebral [17], kidney [110] and lung [18,111] ALI systems. Among them, cerebral organoids cultured with the ALI technique, generated extensive axonal outgrowths, growth-cones formation, presence of various cortical neuronal identities and thick axon tracts with different morphologies, including long projections within (and away from) the organoid. Such built-*in vitro* tracts were capable of eliciting coordinated muscle contractions in co-cultured mouse spinal cord–muscle explants. This approach could also be a useful tool to study axon guidance, tract formation, and connectivity in human systems [17].

In the case of lung organoids, PLG and PCL hydrogel scaffolds were used to encapsulate human PSC-derived lung organoids (made of Matrigel) to enhance transplant efficiency, leading to tube-like structures that resemble both the structure and cellular diversity of adult airways. The different properties of the scaffold (degradation and pore size) could also be tuned to influence the final properties of explant to be achieved upon transplantation into immunocompromised mice: e.g. the number and size of airways structures or the size of the overall explant [18].

Layer-by-layer (LbL)

LbL is a process based on the sequential addition of a series of layers, creating a multilayer 3D structure (Figure 5(Di)) [50]. This technology was often used in co-cultures (specifically for vascularized 3D constructs), with each layer made of different cell types and/or different biomaterials, with the aim of creating a highly defined and organized construct. A recent study reported the fabrication of a native organ-like 3D cardiac tissue with an oriented structure and vascular

network (Figure 5(Di–iv)) [83]. Further discussion of this protocol will be conducted in next section (see "Co-culture systems and vascular organoids").

3D Bioprinting

Bioprinting has emerged as a promising process for creating 3D structures by using a bio-ink, where individual cells or spheroids are dispersed into hydrogels in a LbL fashion [112]. The main advantage of bioprinting is the ability to produce 3D constructs with high precision over shape, size, and positioning of cells, thus allowing a specified personalized treatment (Figure 5(Ei)). For this purpose, Joung et al. [77] designed a 3D bioprinted spinal cord-like culture system (Figure 5(Eii–iv)) in which iPSC-derived spinal neuronal progenitor cells (sNPCs) and oligodendrocyte progenitor cells (OPCs) were precisely positioned within neurocompatible Gelatin methacryloyl (GelMA) scaffold. sNPCs differentiation into neurons showed physiological spontaneous calcium flux, whereas OPCs differentiated into oligodendrocytes capable of myelinating axons. In the future, this approach could help to rebuilding axonal connections into the tissue damaged area by creating an effective relay across the injury site.

Scaffolds from SAPs are of particular interest for bioprinting due to their synthetic essence and biomimetic properties. In a recent work [113], two different SAP tetramers (specifically, CH-01 and CH-02) were tested in a 3D cell culture system of mouse myoblasts to assess the effectiveness of such peptide-based bio-inks in long-term cell cultures. The authors demonstrated preserved cell viability, enhanced growth and alignment. Authors demonstrated that SAPs could be promising bio-inks for 3D bioprinting and tissue engineering applications.

3D Cell culture systems and organoid platforms for tissue engineering applications

3D Organotypic brain/spinal cultures

Brain is the most powerful and complicated organ of the human body in terms of its structure and function. The limited understanding of the human brain is reflected by the lack of effective treatments for various neurological disorders and in nervous regenerative medicine in general. *In vitro* design and development of 3D neural tissues *in vitro*, aiming to replicate the brain structure, development and function, remains a great challenge [114].

In a recent work, a tailored PEG diacrylate-cross-linked porous scaffold was applied to support the expansion and long-term differentiation of both human iPSC- and ESC-derived neural precursor cells (hPSC-NPCs) in 3D neural cell cultures [71]. These authors demonstrated a terminal *in vitro* neural differentiation (at 49 DIV) with upregulation of neuronal and glial mRNA, as well as a spontaneous calcium flux concomitant with the presence of the glutamate neurotransmitter [71]. Long term *in vitro* differentiation was also adopted to create a SAP-based 3D cell culture system with densely seeded human NSCs for the treatment of spinal cord injury (SCI) [75]. The success of this work was to direct *in vitro* stem cell differentiation into defined phenotypes prior to transplantation. Thanks to a multi-functionalized SAP hydrogel, human NSCs thoroughly: proliferated, differentiated and maturated *in vitro* for 6 weeks, revealing randomly organized electrically active neuronal networks expressing GABAergic, glutamatergic and cholinergic neurons, as well as mature oligodendrocytes expressing the myelin basic protein marker. Moreover, implantation in a subacute SCI rodent model revealed that pre-differentiated human NSCs (1) yielded to an increased presence of transplanted stem cell progeny, (2) enhanced expression of markers for phosphorylated neurofilaments, GABAergic and glutamatergic neurons, (3) decreased astrogliosis, (4) reduced the immune response and (5) improved behavioral recovery, compared to standard NSC-derived progenitors' progeny. Despite no supracellular complex structure nervous components were detected in 3D constructs. This approach did not involve the use of animal derivatives (serum, ECM extracts or other xenogeneic components) and, as such, still holds a high translation potential into clinics. Lastly, the same authors validated the protocol demonstrating its reproducibility with other different human stem cell lines [76].

Another therapeutic strategy for SCI repair was proposed by Wang's group [78]. iPSC-derived NSCs were photo-encapsulated into the semi-synthetic GelMA hydrogel, whose mechanical properties had been previously tuned to favor cell survival and differentiation. Their 3D cell culture system was used to fill the lesion site of the injured spinal cord and its neuroregenerative potential was evaluated up to 6 weeks *in vivo*. Results showed that exogenous cellular transplantation enhanced axonal regeneration, caused extensive axonal outgrowth, improved functional recovery and inhibited inflammation, with concomitant nervous regeneration at the injury/graft site.

In the realm of SAP hydrogels, Wu's group used RADA16 functionalized with RGD cell adhesion peptide and IKVAV neurite outgrowth peptide [73]. A 3D construct of NPCs/NSCs was used with three nerve injury

models: sciatic nerve defect, intracerebral hemorrhage and spinal cord transection. The bi-functionalized RADA16 supported NPCs/NSCs differentiation toward neural and glial phenotypes without adding extra soluble growth factors, providing a more permissive environment for nerve regeneration than RADA 16-I hydrogel alone [73].

Endodermal organoids

Artificial 3D liver cultures gained great importance for the treatment of end-stage liver failure, aiming to become a valid alternative to conventional liver transplantation therapy. Hepatocytes, hepatic stellate cells, Kepffer cells, liver sinusoidal endothelial cells are the most important cell types in the liver that can be reprogrammed from various stem cells, using a specific differentiation-induction method [115].

ECM-cholangiocytes interactions are essential for the formation of the biliary tree. When embedded in natural ECM-like Matrigel, cholangiocytes are able to self-organize into polarized monolayers enclosing a central lumen, termed a cyst. Authors designed a fully synthetic hydrogel with defined mechanical properties [90], cholangiocytes were integrated into artificial liver culture systems made with PEG crosslinked with different peptides sensitive to metalloproteinase degradation and functionalized with the RGD motif. Results revealed that the overall elastic modulus of the construct correlates with the presence of RGD in a dose-dependent manner, and, consequently, on the formation of large cysts, that in some cases were similar to those obtained with Matrigel. Authors have highlighted the translational potential of their approach, making use of pure synthetic scaffolds to develop their bile duct models [90]. Similarly, Rashid's group [27] created bioengineered liver organoids using an inverted colloid crystal PEG-scaffold and iPSC-derived hepatic progenitors. They obtained a more physiologically relevant liver phenotype compared to Matrigel 3D models. Additional improved results were obtained by functionalizing hydrogels with selected ECM proteins. After implantation into the livers of immune-deficient mice, morphology, gene expression, protein secretion, and drug metabolism resulted similar to adult tissue, hence suggesting the achievement of a bioengineered platform suited for a range of mechanistic and clinical organoid-related applications for the near future.

Even if not fully synthetic, a noteworthy example is also provided by Tysoe *et al.*, describing a protocol for the fabrication of a functional bioengineered biliary tissue [92]. Cholangiocytes organoids were produced from Matrigel and subsequently embedded in polyglycolic acid (PGA) scaffolds for 4 weeks. They decided the use of a PGA matrix because of its biodegradability, flexibility and lack of inflammatory response *in vivo*. Also, it was easily processed into tailored architectures. The resulting organoids expressed biliary markers similar to the primary tissue. This displayed functional secretory capacity, responded to hormonal signals and, after being transplanted under the kidney capsule of immunocompromised mice, they formed tubular structures capable of long-term survival and expressing standard biliary markers. Also, cells expanded and populated the PGA scaffolds, generating a confluent construct expressing cholangiocytes markers and exhibiting biliary functions *in vitro*.

In another study, PLLA/PCL hybrid fibers successfully supported the differentiation of human IPSCs toward hepatocytes-like cells, capable of expressing biochemical and molecular liver markers [91]. PLLA/PCL hybrid fibers are degradable, cost-effective, flexible, and biocompatible polymers with nanostructures similar to ECM and ideal for the differentiation of cells from soft tissues [116]

Therapeutic approaches for type 1 diabetes (T1D) include: pancreas transplantation, exogenous insulin administration and immunosuppressive therapies. However, these clinical applications are limited due to insufficient tissue compatible donors, side effects of exogenous insulin administration and increased infections due to chronic immunosuppression treatment [117]. An alternative approach is to use insulin-producing PI embedded in 3D engineered scaffolds for their implantation into diabetic recipients [118]. In a recent study, a patented bioscaffold, based on polysaccharide-polyamine copolymers, was employed to correct hyperglycemia in streptozotocin-induced and autoimmune-driven non-obese diabetic mouse models [88]. Results *in vitro* demonstrated that PI seeded into the biomaterial remained viable for 4 weeks *in vitro* and small molecules could diffuse passively in- and out- of the bioscaffold. After 2 weeks of culturing *in vitro*, intraperitoneal implantation of scaffolds seeded with donor-derived syngeneic islets reduced hyperglycemia levels, supported long term cell viability and improved metabolic hormone balances in recipient diabetic mice. The implanted scaffold assembled into a pancreas-like organoid substructure that re-organized the ECM compartment and recruited endothelial progenitors for neovascularization to prevent hypoxia. This strategy recapitulates physiological islet microenvironments and supports long-term insulin production as a reaction to elevated blood glucose levels. These features highlight

its feasibility for effective insulin restoration and opens the door to additional applications in case of other types of organ failure by using this new class of biomaterials [88].

Huch's group developed a tunable biomimetic hydrogel based on dextran polymers modified with a RGD motif-decorated hyaluronic acid [87]. They found that this modified polymer supported organoid formation and maintained the epithelial morphology of the organoids: with hyaluronic acid, acting as a crosslinker. This preserved the undifferentiated state of human embryonic stem cells, whereas cleavable dextran allowed the expansion of the human pancreatic organoids. They reported a long-term expansion of human pancreatic organoid from both fresh and cryopreserved pancreas tissue from human donors, in a chemically defined, serum-free medium. *In vitro* genomic stability, cell differentiation and *in vivo* safety also demonstrated: their work may potentially pave the way for a novel treatment of diabetes. It also boosted further studies of epithelial biology and pancreatic disease modeling. In further studies, the incorporation of "adhesive" peptides into PEG hydrogels modulated islet viability and function, with RGD-functionalized PEG gels featuring the highest insulin responsiveness [86]. Also, controlled vascular endothelial growth factor (VEGF) delivery from PEG hydrogels enhanced functional vascularization within islet transplant sites, supporting islet viability and function [85].

As mentioned in the section on "hydrogel scaffolds for cell encapsulation," PEG hydrogels were used for the development of *in vitro* human intestinal organoids (HIOs), [93,94]. Subsequently, the same authors also used RGD-decorated PEG hydrogels to obtain the formation of HIOs in a similar manner to those formed in Matrigel (positive control). However, to achieve such a milestone result (in terms of cell differentiation, morphogenesis and organoid formation) it was necessary to supplement the PEG-based hydrogels with animal-derived Laminin-111 [13]. This was doable because this protocol was designed for the growth of mouse intestinal stem cell colonies and organoids, but it may require additional optimization and customization to be translated to humans.

In short bowel syndromes, a novel preclinical approach in neonatal piglets was established using intestinal stem cells cultured into nonabsorbable poly(-ethylene-vinyl acetate) (PEVA) or bioabsorbable poly(-glycerol sebacate) (PGS) bioscaffolds [95]. While permanent PEVA had excellent handling properties and supported cellular proliferation *in vitro*, it failed to integrate into the surrounding tissue *in vivo*. Because of these findings, authors opted for degradable PGS, obtaining a clinically relevant model of 3D artificial crypt-villus architectures with remarkable similarity to native intestines.

Mesodermal organoids

In the human body, cardiac tissue has a very complex structure: as such, the effects of cardiomyocytes alignment on excitation-contraction coupling, shortening and force development have to be considered as a relevant parameter in the design of 3D synthetic patches. In regenerative medicine, cardiac patches are now considered as a promising therapeutic technology for the treatment of diseased heart tissue [119]. Ideally, cardiac patches should be designed to provide mechanical support and, at the same time, favor damaged tissue regeneration. Numerous natural and synthetic polymeric patches (such as Collagen, Gelatin, PEG, PLLA, PCL) have been tested in heart infarction animal models [120,121]. Currently, patch bioprinting is the most used approach in this field because it allows to fabricate patches with heart-like structures [122].

On the other hand, MSCs transplantation has been employed in multiple clinical trials because they effectively reduced infarct size and restored heart function after myocardial infarction [123].

Others incorporated MSCs into different biomaterials to create 3D cell culture systems to facilitate cell survival, proliferation and differentiation [124,125]. Recently, RADA16 functionalized with QHREDGS functional motif was seeded with bone marrow mesenchymal stem cells (BMSCs) and used as a 3D system to efficiently improve angiogenesis and overall heart function in an acute myocardial infarction animal model, where it reduced scar size and cell apoptosis [82]. Schook's group bioprinted a 3D culture system using polyethylene glycol dimethacrylate (PEGDMA) hydrogel patch and BMSCs. Cell-loaded micro-channelled patches with desired diameter were fabricated *via* a 3D bioprinter with the aim of draining cell-secreted growth factors to the host tissue. Authors detected the secretion of pro-angiogenic factors (i.e. interleukin-8 IL-8 and vascular endothelial growth factor VEGF), pigment epithelium-derived (PEDF) anti-angiogenic factor, and a series of cytokines including tissue inhibitor of metalloproteinase-1 (TIMP-1), and antiapoptotic cytokines [81]. The 3D printed patches prevented abnormal fibrosis resulting from acute ischemic injury [81].

In another study, cellular shape and orientation in 3D culture systems were tuned to obtain a 3D cardiac tissue with structural and functional cues similar to

native tissue and cells were encapsulated in 3D GelMA scaffolds *via* Microscale Continuous Optical Printing. Encapsulated neonatal mouse ventricular cardiomyocytes preferentially aligned along the engineered microarchitecture and displayed morphology and myofibril alignment of native myocardium. Authors proposed that this physiologically relevant 3D culture system mimicking the microarchitecture and function of ventricular myocardium as a promising *in vitro* model for studies of cardiac diseases [84].

Kidneys are responsible for essential body functions, including the filtering of waste products and minerals from blood, maintenance of fluids and acid-base balance, and erythropoietin production. Nephrons are kidney's functional units, and their permanent loss of functionality leads to chronic kidney disease. To date, there is no tissue engineering technology suited to model human kidney diseases or for drug screenings. Hence, a bioengineered kidney would be extremely needed. Recent work lays a solid basis for the engineering of anatomically relevant human kidney tissue *in vitro*: using polydimethylsiloxane (PDMS) synthetic scaffolds and a kidney-derived cell line Xinaris'group developed a system to rapidly obtain custom-made 3D tubules with typical renal epithelial properties [79,80].

Co-culture systems and vascular organoids

Co-culturing of different cell types allows for the formation and the spatial self-organization of diverse cellular structures within a single 3D construct (Figure 5(F)). In particular, co-culture systems were used to promote vascularization by integrating endothelial cells (or their progenitors) during organoid formation (Figure 5(G)) [126].

Tsukamoto *et al.* reported a new method for the fabrication of 3D cardiac tissue with heart specific structure, cell orientation and vascular network by using LbL, cell accumulation and 3D printing techniques [83]. They co-cultured human IPSC-derived cardiomyocytes, normal human cardiac fibroblasts (NHCF) and human cardiac microvascular endothelial cells (HMVEC) in hydroxybutyl chitosan (HBC). Their tailored 3D cardiac tissue with contractile properties (making it similar in function to living cardiac tissue), oriented structures and a vascular network may be a useful tool for cardiac regenerative medicine and pharmaceutical applications in the near future (Figure 5(Di–iv)). For these reasons, this assessment result indicates that it is possible to fabricate cardiac tissue with a function similar to a living body using the techniques outlined in this study

Co-culture systems were also adopted to produce a multi-component neural 3D construct, combining human ESC-derived neural progenitor cells, endothelial cells, mural cells and microglia precursor cells, all cultured on PEG hydrogel. Cell differentiation and self-organization produced a 3D neuronal/glial network, an organized vascular tree, and a microglia population with ramified morphologies [72]. Differently from ordinary Matrigel-based organoids[72], their neural model mimicking a "developing brain" were produced with a fully synthetic biomaterial formed with thiol-ene chemistry. Besides, several recent studies have demonstrated that synthetic hydrogels formed using thiol-ene chemistry are promising for *in vitro* modeling approaches of neural tissue, including neurotoxicity screening, potency testing, directed differentiation, and neural cell cultures [127,128].

Also, a double cell transplantation system was adopted to treat traumatic brain injury (TBI) and authors co-cultured human umbilical cord mesenchymal stem cells (hUC-MSCs) with active astrocytes in RADA16 functionalized with a functional peptide derived from BDNF. After implantation, authors detected reduced injured brain cavities and reduced reactive gliosis surrounding the implants. Moreover, the chosen functionalization fostered the proliferation and neuronal differentiation of hUC-MSCs *in vivo*, because BDNF has been shown to promote neurotrophy, cell proliferation, neuronal differentiation and neurite outgrowth [129,130]. Such an approach may constitute a novel therapeutic strategy for cortical coloboma caused by TBI, with realistic potential application in clinics [74].

Future perspectives

The introduction of 3D cell culture systems can be considered as an important move forward for the overall life-science field. However, just recently, researchers are tackling the issue of translating such an advanced technology to the clinics. This will likely bring organoids from "benchtop to bed" but, at the same time, it will compel more selective choices such as using artificial biomimetic substrates, well-defined cell lines. This is likely, a dedicated regulatory framework evaluating the risks of medium/long-term *in vitro* manipulations of the "bioprostheses" before implantation into patients.

While various functional hydrogels have been developed for the regeneration of different tissue and organs, synthetic hydrogels still exhibited some limitations like modest mechanical properties (fragility, poor resilience) and randomly oriented structures at the nanoscale. This is also the case of self-assembling

peptides, where these issues are currently being addressed by respectively chemical cross-linking [68], co-assembling of multiple species featuring different flexibility at the molecular scale and physical [131] magnetically driven assembling [132]. Also, the complexity of different biological signals found in the ECM recently began to be addressed with multiple scaffold functionalizations [75]. Nonetheless it has not to be forgotten that more complex scaffolds may have a hard time in being evaluated and approved by regulatory agencies for human use because of the contemporaneous presence of multiple biological cues with potentially more unexpected side-effects.

On the other hand, it is not hard to foresee the pros of *in vitro* "matured" 3D constructs vs standard cell therapy with controlled differentiation favoring appropriate cell engraftment. Pre-organized cell networks are capable of ready mimicking of organ functionalities, chances of assembling multiple different organoids to elevate the complexity (and probability of success) of the implants. As such, the field is deemed to bring the next generation therapies in regenerative medicine in the near future.

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Application Note

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Solutions for the Culturing, Maintaining and Characterization of Induced Pluripotent Stem Cells

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Introduction

From drug discovery to organoid modeling of disease, stem cells are increasingly being used in research as a vital tool for scientific investigation. The current trend away from animal models and the push to more relevant systems for simulating the human body require flexible and specific tools to achieve this goal. Induced Pluripotent Stem Cells (iPSCs) are produced from normal tissue, through the forced expression of key transcription factors[1], providing a limitless supply of these precious cells for research and development. Due to the very specialized nature of these cells, their maintenance and culture is more intensive than most cell lines. For this reason, it is important that solutions for the culture and maintenance of these cell types are readily and widely available. Characterization of stem cells can be difficult and unreliable, depending on the methodology used, which is why it is important to develop robust techniques for monitoring stem cells throughout culture and experimental testing. If conditions are not optimal during the maintenance of iPSCs, their pluripotency can be lost.

Reproducibility is highly prized in research and automated solutions can provide high levels of consistency in method and data generation. The CellCelector Flex is an automated platform for targeted cell identification and picking that is not only highly accurate, but also very gentle on cells, providing an ideal solution when working with delicate iPSCs. The Incucyte® Live-Cell Analysis platform automates the imaging processes of iPSC workflows, allowing cells to be monitored over time to analyze changes in morphology and colony formation from within the incubator. This limits the disturbance to precious iPSC culture plates, but also enables real-time tracking of cell growth and health metrics.

Find out more: www.sartorius.com/ipscs

Further characterization of iPSCs can be performed on the iQue® Advanced Flow Cytometer, investigating changes in expression of pluripotency markers integral to maintaining stemness, providing an overview of the status of iPSCs.

Many traditional methods for culturing, monitoring and characterizing iPSCs can:

1. Be inconsistent and unreliable, resulting in seeded populations with high levels of heterogeneity, cell death and differentiation
2. Require regular disturbance of culture plates to monitor growth and confluency, with no integrated options for analysis
3. Demand large volumes of precious sample for analysis, resulting in less material for downstream applications
4. Necessitate the use of a variety of techniques to measure multiple characteristics

This application note discusses the novel solutions provided by Sartorius platforms for the culture, maintenance, and characterization of iPSCs, during research and development.

Methods

The following methods outline a flexible, in-depth workflow for growth and characterization of iPSCs using multiple Sartorius platforms.

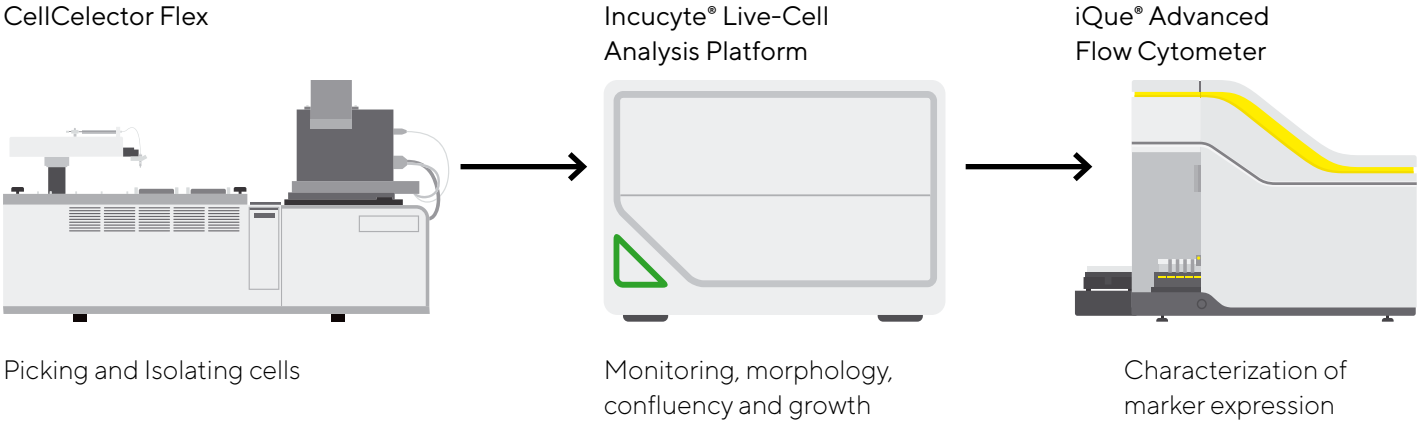


Figure 1. Schematic showcasing the use of Sartorius platforms in iPSC culture.
Using the three Sartorius instruments, CellCelector Flex, Incucyte® and iQue®, iPSCs can be picked and seeded, pluripotency tested, and growth and confluency monitored.

Cell Culture and Maintenance

Picking and seeding iPSCs

Individual cells and colonies were picked using the CellCelector Flex with the Adherent Colony Picking Module and seeded into tissue culture plates for further expansion and downstream processing. Images were taken prior to and post picking to monitor and record the effects of colony manipulation using the CellCelector Flex. Propidium Iodide (PI) staining was undertaken on iPSC colonies after seeding by adding PI at a concentration of 500 nM and incubating for 3 minutes, rinsing twice with PBS and resuspending in growth medium (mTESR Plus) for imaging.

Thawing and Culturing iPSCs

Cells (ATCC-DYS0100 cells derived from human foreskin fibroblasts) were thawed and plated onto Vitronectin XF™ (1:25 dilution in CellAdhere™ Dilution Buffer) precoated 6-well plates at a seeding density of $1x10^6$ cells/well in 1 mL growth medium (mTESR™ Plus) supplemented with Y-27632 (ROCK inhibitor, 10 µM) and incubated at 37°C. iPSCs were monitored using the Incucyte® system to assess confluency, colony formation, and general cell morphology and health. The confluence of colonies was analyzed using the integrated Incucyte® AI confluence software algorithm. Passages were performed every 3-4 days at approximately 60-70% confluence using Gentle Cell Dissociation Reagent and replated at $1x10^5$ cells/well. Medium changes were performed daily during the week, while double volume medium changes were performed on Friday to account for no medium changes over the weekend. For the non-optimized iPSC culture, cells were grown as above except using RPMI 1640 medium supplemented with 10% FBS, L-glutamine 2 mM, Penicillin/Streptomycin 100 µg/mL.

Characterization and Monitoring of Pluripotency

Pluripotency Characterization: iQue®

iPSCs were dissociated to single cells during passage and at specified timepoints using Gentle Cell Dissociation Reagent. Single cell suspensions were stained with cell surface marker antibodies (in PBS + 2% FBS) for one non-pluripotent marker, SSEA-1, and two pluripotency markers, SSEA-4 and TRA-1-60, in addition to the iQue® Membrane Integrity (B/Red) Dye, for viability analysis. Cells were seeded at $2x10^4$ cells/well in a V-bottom 96-well plate and stained with the cocktail of antibodies described (RT in the dark for 30 minutes). To wash plates, PBS + 2% FBS (100 µl) was added, prior to centrifugation (300 x g, 5 minutes), then aspirated. Plates were shaken (3000 rpm, 60 seconds) and the samples resuspended in PBS + 2% FBS (20 µL), prior to being analyzed on the iQue®. Analysis of data was performed using the iQue Forecyt® software after compensation had been optimized for each of the antibodies.

Monitoring Pluripotency and Cell Health: Incucyte®

During the experiments, iPSCs were monitored for changes in morphology and confluency using the Incucyte® Live-Cell Analysis platform. Cultured iPSCs lines were monitored by high definition (HD) phase contrast at 4-hour intervals using a repeating scan schedule at 10X. Nuclear to cytoplasmic ratios were calculated by staining iPSC nuclei using the Incucyte® Nuclight Rapid Red Dye (1:1000) and measuring the cytoplasmic area (confluence mask) and the nuclear area (fluorescence mask) using basic masking to quantify pluripotency/normal iPSC morphology.

Intracellular and Surface Marker Studies

iPSC and control THP-1 cells were seeded at $2x10^4$ cells/well in a V-bottom 96-well plate and fixed, permeabilized and stained according to the protocol found in the following tech note: Intracellular Staining Assay for iQue® Platform. Pluripotency markers, SSEA-4, TRA-1-60, Oct 3/4 and Sox-2 were analyzed, while SSEA-1 expression was used as a marker for non-pluripotency. Analysis was performed on the iQue Forecyt® software after compensation had been optimized for each of the antibodies.

Results

Developing workflows for the culture and characterization of stem cells such as iPSCs is vital in producing consistent, reproducible and robust data. Using the Sartorius platforms showcased here (Figure 1), we can highlight the benefits of the approaches described for culturing iPSCs that are healthy and pluripotent while monitoring and characterizing these stem cells for key markers of health and stemness.

Picking iPSCs Using the CellCelector Flex Is Fast, Gentle and Reliable

It is important when working with any cell system, but notably stem cells such as iPSCs, to maintain good cell health. The data here highlights the delicate, gentle picking and seeding capability of the CellCelector Flex. When stained with Propidium Iodide (PI), a stain that indicates cell death, manual manipulation of iPSCs produces an

increased number of PI positive cells when compared to the CellCelector Flex, indicative of fewer healthy cells (Figure 2A). The CellCelector Flex colony also has less debris and more tightly defined borders (Figure 2B). The flexibility and power of the CellCelector Flex is exemplified by its capabilities, it is able to pick single iPSCs or whole iPSC colonies from a tissue culture plate. This provides the opportunity to select ideal colonies from cultures on a standard plate for further propagation. Additionally, portions of colonies can be selected for further culture. This is useful if a portion of the colony spontaneously differentiates. Differentiated sections can be removed or pluripotent sections can be picked for passaging or analysis (Figure 2C-F).

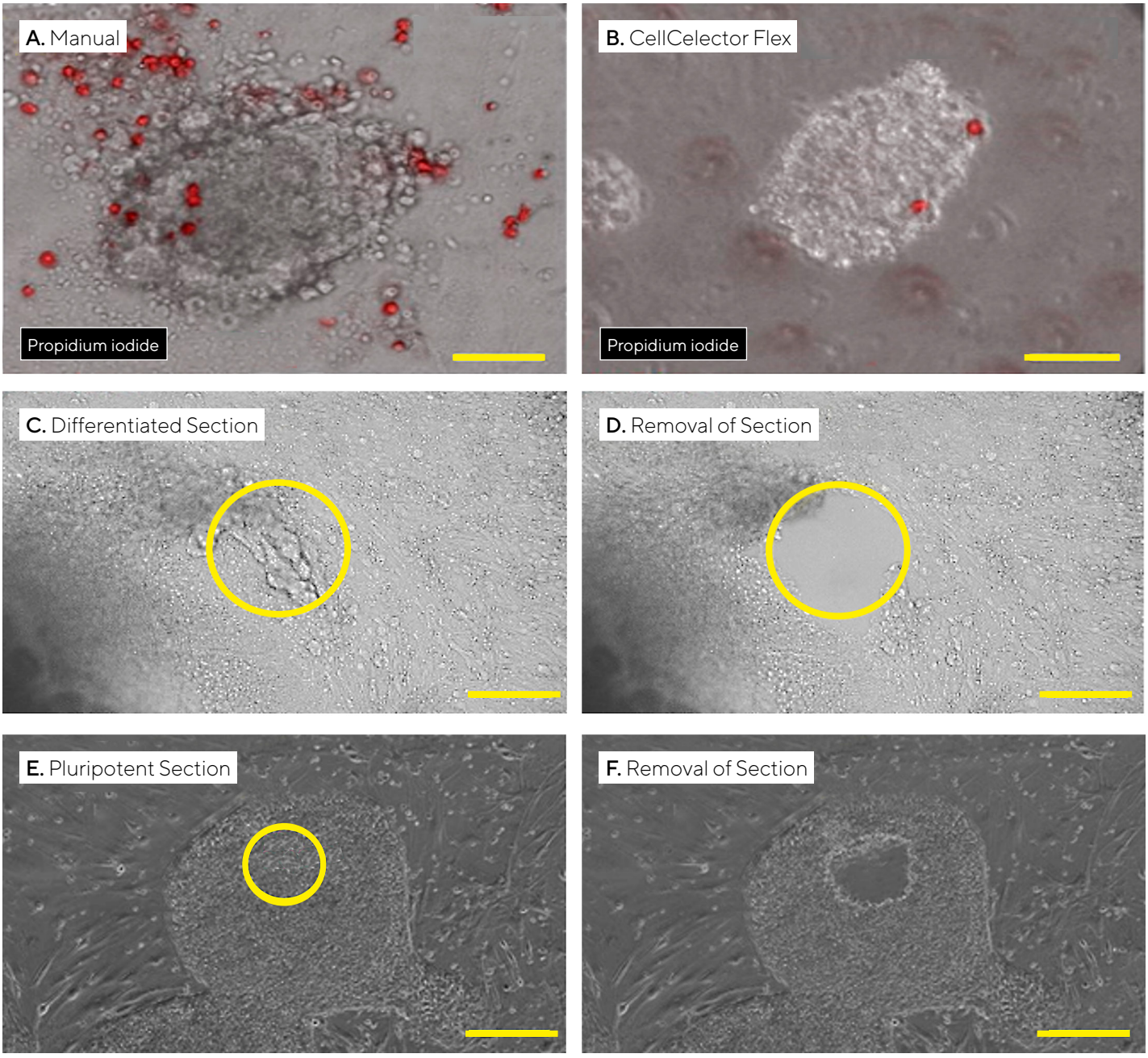


Figure 2. Picking iPSCs using the CellCelector Flex is accurate, fast, gentle and reliable.
Micrographs taken using the CellCelector platform highlighting iPSC colonies selected by the system. (A) Manually and (B) CellCelector picked and seeded iPSC colony stained with propidium iodide (PI) to identify cell death. (C) Micrograph depicting an area of differentiation in a stem cell colony prior to picking with the CellCelector. (D) The same area of the culture plate shown in (C) after removal. (E) Micrograph of a large iPSC colony grown on a feeder layer, prior to picking a section of pluripotent cells. The bottom right of the colony has indications of spontaneous differentiation. (F) The colony in (E) after picking using the CellCelector Flex, the area of pluripotent cells targeted by the machine has been collected for further culture. Scale bar equals 500 µm.

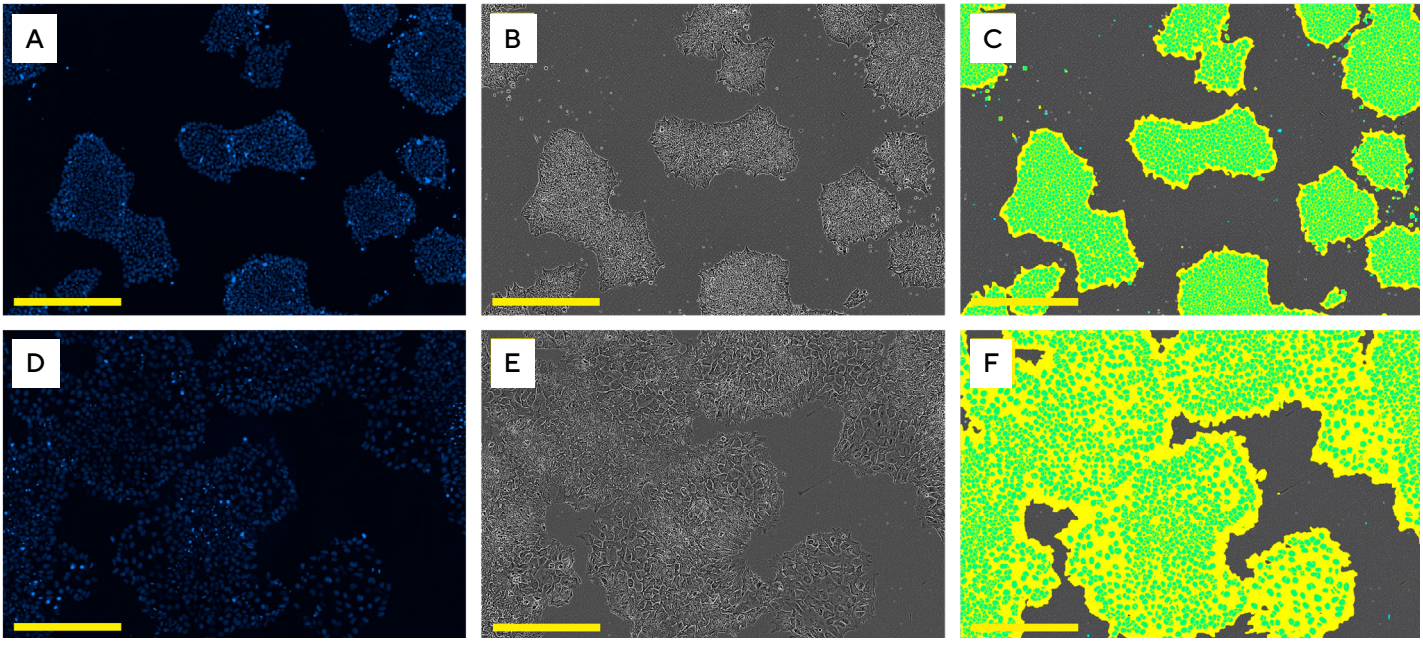
Monitoring Morphology and Pluripotent Potential During iPSC Culture

The CellCelector Flex can be used within the same workflow as another Sartorius platform, the Incucyte® Live-Cell Analysis platform. This system provides tools for monitoring cells during culture within the incubator, so changes in morphology can be recorded and analyzed without requiring removal of culture plates. In the following case, losses in morphological indicators of pluripotency can be observed, recorded, and subsequent analysis can be performed to quantify these changes.

Incucyte® images of iPSCs after 2 days in culture, show a marked difference in morphology between the optimized and non-optimized culture conditions. iPSCs grown in optimized conditions form tightly packed colonies with clearly defined edges, that 'glow' under phase images (Figure 3B), by contrast, non-optimized iPSCs are much more spread out and no longer form tightly packed colonies, they are beginning to resemble fibroblast cells (Figure 3E). Nuclear staining using Incucyte® Nuclight Rapid Red Dye also highlights the separation of the cells when grown in non-optimized conditions (Figure 3D), nuclei are much more spread out and lose the tight distribution found in optimized conditions (Figure 3A). Quantification of these morphological differences was performed using the Incucyte® Adherent Cell-by-Cell scan at 10X magnification and nuclear and cytoplasm area measurements were made using the Basic Analyzer and AI Confluence analysis (micrographs in Figure 3C, F) using the following equation to provide a nuclear/cytoplasm ratio, a standard measurement used when studying iPSCs.

$$\frac{\text{total nuclei area}}{\text{total cytoplasmic area}} = \text{nuclear/cytoplasm ratio}$$

The graph in Figure 3G illustrates the reduction in this ratio in the non-optimized conditions, from 0.6 to 0.4. The more iPSC like, and thus pluripotent, a cell is, the higher the nuclear/cytoplasm ratio.



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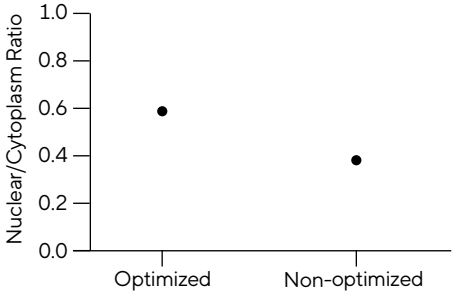

Figure 3. Monitoring morphology and pluripotent potential during iPSC culture. Incucyte® images of iPSCs grown under optimized (mTESR Plus) and non-optimized (RPMI) conditions. (A, D) Fluorescent images of iPSCs stained with Nuclight Rapid Red Dye comparing nuclear density between conditions. (B, E) Phase contrast images of the same iPSCs showing morphological differences between the two variables. (C, F) Analysis masking on the Incucyte® depicting confluency and nuclear masking that can be used to determine the nuclear/cytoplasm ratio illustrated in (G). Scale bar equals 400 µm.

Changes in iPSC Marker Expression Analyzed with the iQue® Flow Cytometry Platform

To investigate further the losses in pluripotency in iPSCs when cultured in non-optimal conditions, surface marker expression of specific pluripotency markers can be analyzed with the iQue® Flow Cytometry Platform, requiring as little as 10 µL per sample.

iPSCs grown in non-optimized conditions show rapid loss of pluripotency marker expression compared to optimized conditions (Figure 4). This indicates a loss in pluripotency correlating with the data collected on the Incucyte® platform (Figure 3). After 2 days in culture (Figure 4A), analysis of non-optimized conditions shows a decrease in expression of pluripotency markers SSEA-4 (97.3 ± 0.8%), TRA-1-60 (89.8 ± 0.9%), and the pluripotent population (34.6 ± 0.3%), with a further decrease after 4 days of treatment (SSEA-4 63.4 ± 2.9%, TRA-1-60 58.9 ± 2.9%, pluripotent population 19.3 ± 3.0%) when compared with optimized conditions (Figure 4B). In contrast, for the optimized iPSCs, no marked differences in expression profile over the time course of these studies was observed (95 ± 0.4% for pluripotent markers and less than 1.8 ± 0.5% for SSEA-1). (Figure 4A, B). In addition, the increase in non-pluripotent marker SSEA-1 expression (57.5 ± 0.7%) is clear as early as 2 days post treatment (Figure 4A) and remains high throughout culture.

In Figure 4C, (dot plots taken directly from iQue Forecyt® software) there is a clear shift in SSEA-1 expression between the optimized (1.63% SSEA-1 positive) and non-optimized conditions (57.5 % SSEA-1 positive) (upper two dot plots). The lower plots further illustrate the shift away from pluripotent marker expression in the non-optimized conditions, where the optimized iPSCs present a compact population in the upper right quadrant of the plot (SSEA-4+, TRA-1-60+) while the non-optimized iPSCs present a much more spread population shifting into the TRA-1-60 negative portion of the plot.

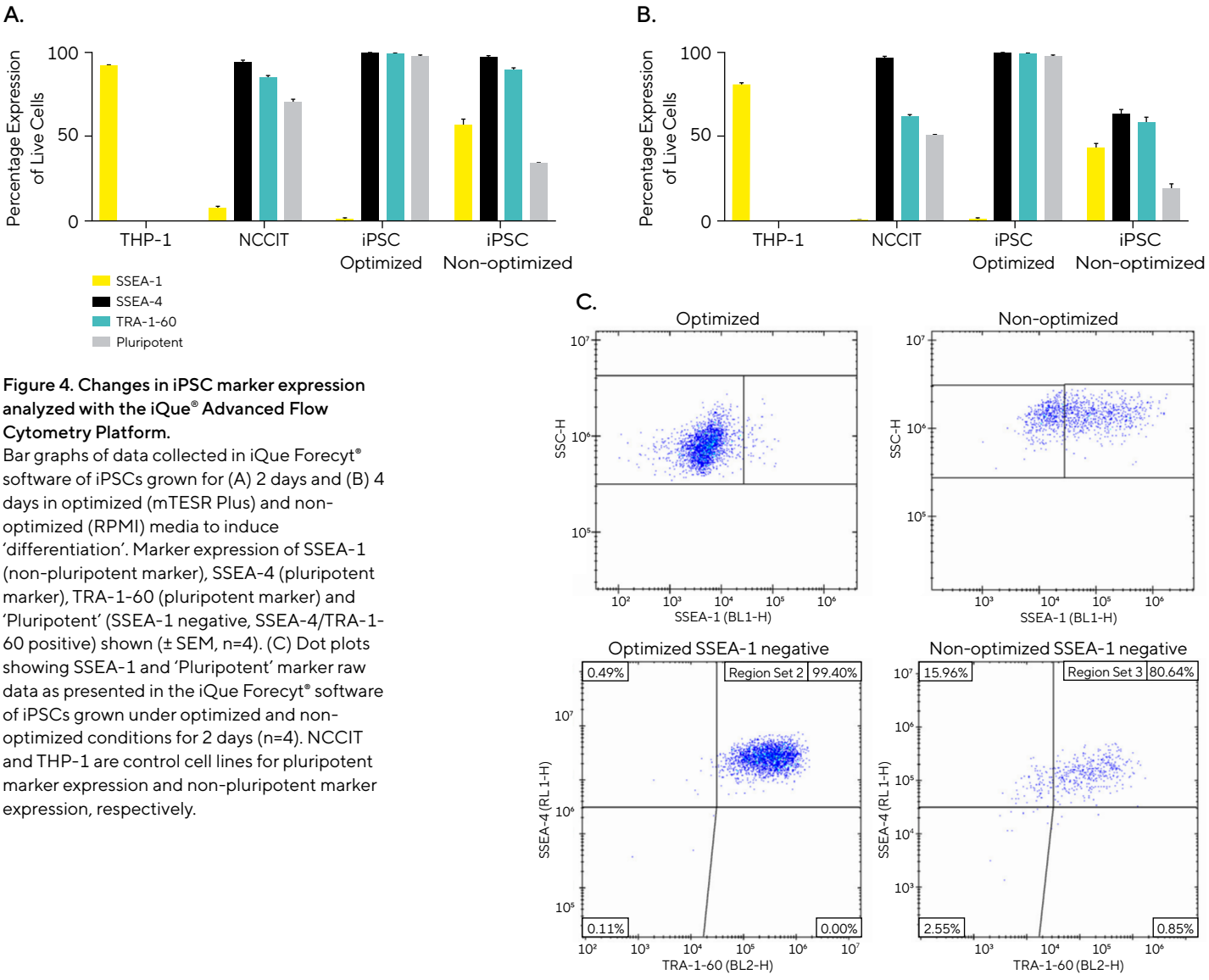

Figure 4. Changes in iPSC marker expression analyzed with the iQue® Advanced Flow Cytometry Platform.
Bar graphs of data collected in iQue Forecyt® software of iPSCs grown for (A) 2 days and (B) 4 days in optimized (mTESR Plus) and non-optimized (RPMI) media to induce 'differentiation'. Marker expression of SSEA-1 (non-pluripotent marker), SSEA-4 (pluripotent marker), TRA-1-60 (pluripotent marker) and 'Pluripotent' (SSEA-1 negative, SSEA-4/TRA-1-60 positive) shown (± SEM, n=4). (C) Dot plots showing SSEA-1 and 'Pluripotent' marker raw data as presented in the iQue Forecyt® software of iPSCs grown under optimized and non-optimized conditions for 2 days (n=4). NCCIT and THP-1 are control cell lines for pluripotent marker expression and non-pluripotent marker expression, respectively.

Surface and Intracellular Marker Staining Provides Solutions for High-Throughput Cellular Characterization

Using the iQue® Flow Cytometry Platform to monitor intracellular markers in addition to surface markers further characterizes the pluripotency of cells.

Using THP-1 cells as a non-pluripotent control, iPSCs were fixed, permeabilized and stained for the surface markers SSEA-1, SSEA-4 and TRA-1-60, in addition to the intracellular markers Oct 3/4 and Sox 2 (Figure 5). Dot plot data taken directly from iQue Forecyt® software, clearly show the expression of pluripotency markers SSEA-4, TRA-1-60, Oct 3/4 and Sox 2 in iPSC cells (black) and the non-pluripotent marker, SSEA-1, only expressed in the THP-1 control cell line (yellow) (Figure 5A). The heatmap in Figure 5B illustrates this expression pattern in a plate view configuration, where black is high expression and yellow is low expression, exemplifying the flexibility of data presentation in the iQue Forecyt® software. Analysis of this data as a bar graph in Figure 5C further highlights the contrasting expression profiles of the two cell types. The ability to characterize a range of marker expression in cell lines, including iPSCs, via a flexible multiplexed workflow, exemplifies the power and utility of Sartorius platforms such as the iQue® Advanced Flow Cytometer.

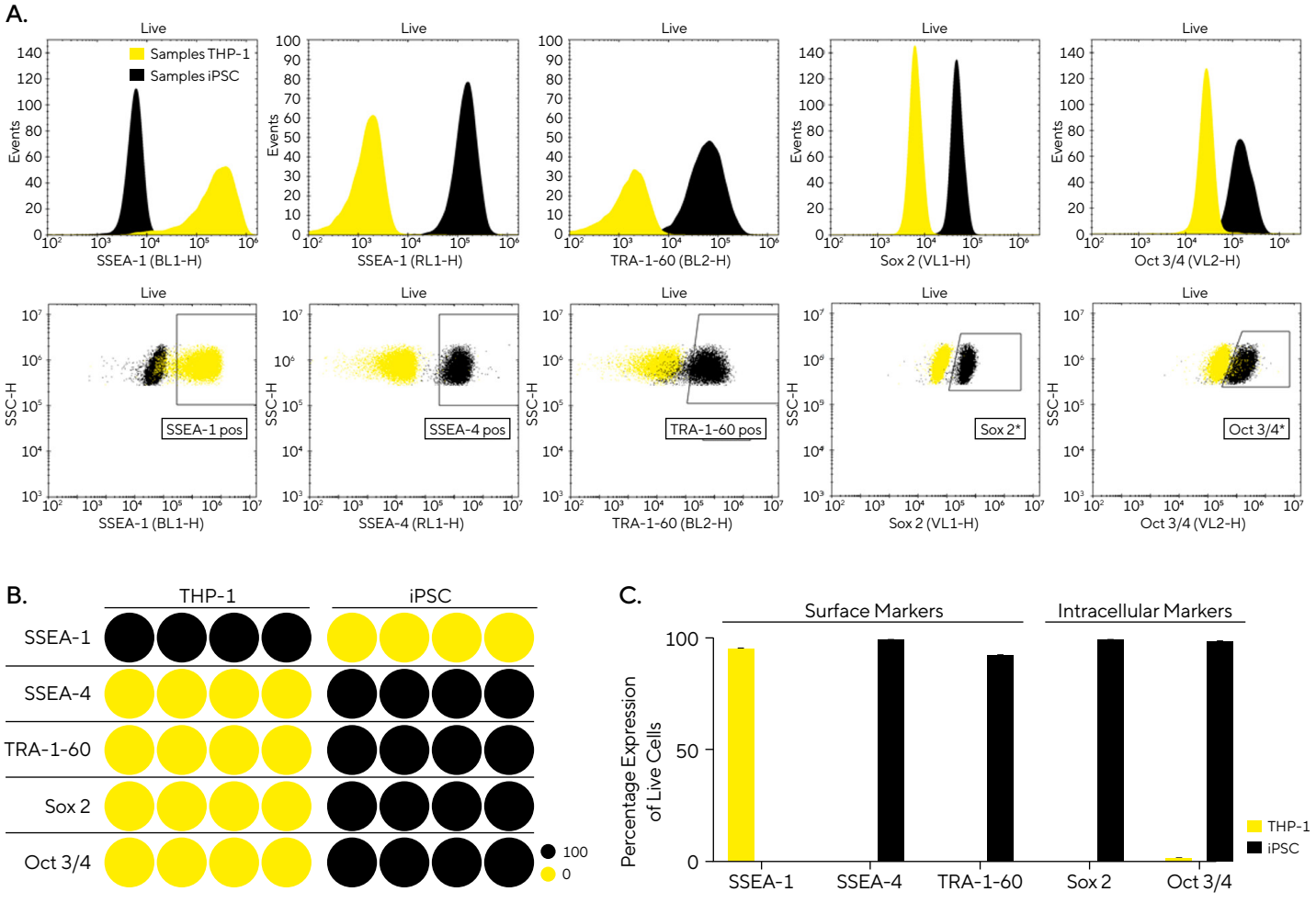

Figure 5. Surface and intracellular marker staining provides solutions for high throughput cellular characterization.
SSEA-1 was used as a marker of normal, non-pluripotent cells, while SSEA-4, TRA-1-60, Sox 2, and Oct 3/4 were all used to characterize pluripotent cells. (A) Histograms and dot plots created in the Forecyt software system for iQue®, showing the expression of various surface and intracellular markers in iPSC and control cells (n=4). (B) Heatmap from iQue Forecyt® illustrating the expression of the same markers, representing the plate map and expression profile per well. (C) Bar graph showing marker expression data in 3rd party software (± SEM, n=4).

Conclusions

iPSCs are increasingly used in many areas of research, requiring specific conditions for optimal growth, to maintain pluripotency, viability, and propagation potential. These requirements are often expensive and methods for monitoring iPSC status can be complex and time intensive, requiring multiple complicated techniques and solutions.

Using various Sartorius platforms throughout an iPSC culture workflow, we have shown how we can successfully pick and seed iPSCs, monitor their morphological status and characterize their pluripotency using the CellCelector Flex, the Incucyte® Live-Cell Analysis platform and the iQue® Advanced Flow Cytometer. The key advantages of using this combined workflow over conventional methods are:

1. Consistency and reliability, the CellCelector Flex can reproducibly pick specific iPSC colonies for further testing or culture, maintaining high levels of cell health
2. The ability to monitor delicate iPSC line culture morphology and growth characteristics without removing plates from the incubator
3. Minimal sample volumes required to characterize precious cell types, with minimal attrition for downstream requirements
4. Multiplexing experiments providing flexibility for the characterization of multiple metrics, such as surface and intracellular marker expression, using the same platform

The data presented here showcases the advantages of using a streamlined workflow combining multiple Sartorius systems for the culture, monitoring and characterization of iPSCs for several applications from drug development, disease modeling and clinical therapy research.

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REVIEW

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The Potential of Pancreatic Organoids for Diabetes Research and Therapy

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ABSTRACT

The success of clinical transplantation of pancreas or isolated pancreatic islets supports the concept of cell-based cure for diabetes. One limitation is the shortage of cadaver human pancreata. The demand–supply gap could potentially be bridged by harnessing the self-renewal capacity of stem cells. Pluripotent stem cells and adult pancreatic stem cells have been explored as possible cell sources. Recently, a system for long-term culture of proposed adult pancreatic stem cells in a form of organoids was developed. Generated organoids partially mimic the architecture and cell-type composition of pancreatic tissue. Here, we review the attempts over the past decade, to utilize the organoid cell culture principles in order to identify, expand, and differentiate the adult pancreatic stem cells from different compartments of mouse and human pancreata. The development of the culture conditions, effects of specific growth factors and small molecules is discussed. The potential utility of the adult pancreatic stem cells is considered in the context of other cell sources.

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Organoids; adult stem cells; progenitor cells; pancreas; beta cell; stem cell therapy; diabetes; CD133; LGR5; ALDH; PROCR; transplantation; beta cell regeneration

1. Introduction

One great breakthrough in the field of stem cell (SC) research is the recent development of the organoid cell culture system. It can be loosely described as an "organ in a dish,"[1] as it simulates organ-like growth *in vitro*.[2] Thus, the organoids gradually make solid organs accessible for stem cell research, which until recently was possible for hematopoietic tissue only.

The cell source in the organoid culture can either be pluripotent stem cells or primary epithelial stem/progenitor cells with the potential to differentiate into organ-specific cell types.[1,3,4] According to a generally accepted definition, organoids have the intrinsic capacity to expand cells and spontaneously grow into self-organized three-dimensional (3D) structures, which at least partially mirror tissue architecture, cell-type composition, and functionality of a given organ.[2,5–8] As elegantly demonstrated in the intestinal organoids, the stem cells differentiate into organ-specific lineages under specific culture conditions, which activate or inhibit specific signaling pathways.[9,10] The extracellular factors directing the fate of stem/progenitor cells were originally conceptualized for hematopoiesis as the "stem cell niche."[11]

The term organoid was first used in 1946 to describe the tissue of dermoid cystic "teratoma."[12] Sometimes the term organoid is applied incorrectly to spheroids[2] or islet-like cell clusters, which also form 3D cellular structures, but the cells are not attached to the extracellular matrix and the culture media is different from the media used for the organoid culture.[13]

Organoids provide a useful tool for disease modeling,[4,14] drug testing,[15,16] or cancer research.[17] They also represent an opportunity to study tissue–pathogen interaction *in vitro*.[18,19] Organoids can help in studying stem cell niches[20] and organ development.[3] In this context, pancreatic organoids have been employed to investigate pancreatic ductal adenocarcinoma,[21,22] pancreas development,[23–25] or cystic fibrosis,[26] and to screen drugs targeting pancreatic diseases.[27,28] No information has yet been published on pancreatic organoids derived from diabetic patients in order to investigate the disease pathogenesis, or to screen candidate drugs for diabetes.[29] However, pancreatic

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organoids have been researched as the potential cell source for therapy of diabetes.

Diabetes is a chronic metabolic disease characterized by lost control over blood glucose. Type 1 diabetes is caused by an absolute deficiency of insulin. In type 2 diabetes, relative insulin deficiency results from an increased insulin resistance. At present, the type 1 diabetic patients are mostly managed by the administration of synthetic insulin.[30,31] Nevertheless, a small fraction of patients are already cured from diabetes by transplantation of beta cells within cadaver pancreas (more than 48,000 patients)[32] or isolated pancreatic islets (more than 1900 allograft and autograft recipients).[33] Availability of the cell-based cure of diabetes is circumscribed by the limited amount cadaver pancreata. The demand–supply gap could potentially be bridged by beta cells derived from self-renewing stem cells. The three possible sources of SCs explored over the past two decades include the embryonic stem cells (ESC), induced pluripotent stem cells (iPSC), and adult pancreatic stem cells.

The first insulin-secreting cells derived from genetically modified ESCs were reported to normalize glycemia in streptozotocin-induced diabetic mice in 2000.[34] In the following years, the therapeutic potential of ESCs and iPSC has been extensively tested, and high efficacy multi-stage differentiation approaches were developed.[35,36] In 2014, Rezania and Pagliuca independently described protocols for *in vitro* differentiation of human pluripotent stem cells through pancreatic progenitors into beta cells that reversed diabetes when transplanted into mice.[37,38] However, the pluripotent stem cells carry the potential for mutagenesis, chromosomal aberrations, and carcinogenesis,[39] mainly due to the unphysiological number of cell divisions needed for meaningful therapeutic application.[40,41] The safety can be significantly improved by cell encapsulation or by molecules that degrade the remaining undifferentiated cells.[42] While the encapsulation also avoids the requirement of immunosuppression, making it safer for patients, fibrosis of the capsule hinders proper vascularization (https://viacyte.com/press-releases/two-year-data-from-viacytes-step-one-clinical-trial-presented-at-ada-2018/ STEP ONE clinical trial). On the other hand, an open system capsule allows a better degree of vascularization at the cost of immunosuppression. Bioengineered pluripotent stem cell therapy and their bottlenecks were reviewed elsewhere.[43]

Several biotechnology companies have already driven their successful research into pre-clinical and clinical trials.[44] Viacyte developed pancreatic progenitors, which differentiate from embryonic stem cells with almost 100% efficiency into islet cells, which are implanted into diabetic patients in closed or open capsule systems (Clinical trials NCT04678557, NCT03163511 https://www.clinicaltrials.gov/ct2/show/NCT04678557?term=viacyte&draw=1&rank=1 and https://www.clinicaltrials.gov/ct2/show/NCT03163511?term=viacyte&draw=1&rank=2).[45] Vertex recently registered a clinical trial to test SC-based islet cell therapy VX-880 in patients with hypoglycemia unawareness syndrome (NCT04786262 https://clinicaltrials.gov/ct2/show/NCT04786262?term=VX-880&draw=2&rank=1). Another clinical-stage company Kadimastem, which previously tested clinical grade ESC derived astrocytes (NCT03482050 https://clinicaltrials.gov/ct2/show/NCT03482050), has announced effectiveness of their microencapsulated islet-like clusters (IsletRx https://www.kadimastem.com/post/kadimastem-announces-successful-preclinical-results-of-its-cell-therapy-treatment-for-insulin-depend) in immunocompetent diabetic mice.

While pluripotent SCs appear to be a promising source of cells to cure diabetes, serious complications can still occur in clinical trials. It is therefore reasonable to consider alternative sources of insulin-producing cells, such as the adult pancreatic stem cells, which only recently became amenable to research thanks to the advancement of the organoid cell culture, as reviewed below.

2. Principles of pancreatic organoid culture

2.1 Basic principles of organoid culture

Organoid culture requires three key components: the extracellular matrix substitute, culture media, and source cells. The extracellular matrix substitutes provide specific attachment sites for cell adhesion molecules and three-dimensional support for the constituent cells. The media provide specific

soluble factors to modulate signaling pathways or chromatin state. The cell source, from which pancreatic organoids are originated, can either be unselected, such as crude ductal fragments,[22,46–50] or highly selected single cells carrying putative stem cell markers.[46,47,51–53] The capacity of organoids to expand over numerous passages proves the presence of stem/progenitor cells in the original preparation, as well as the ability of the culture conditions to sustain them.[46,47,54,55] By the same token, a failure to expand suggests either the absence of stem/progenitor cells or an inadequate media/matrix composition. When organoid cultures are initiated from unselected raw islet-depleted pancreatic tissue comprising multiple cell types, the organoids gradually prevail, while the other cell types diminish.[52] The size of pancreatic organoids ranges between 50 and 2000 µm, dependent on the culture duration, as shown in Figure 1 top (we are grateful to Folia Biologica for the permission to reproduce data recently generated in our lab),[52] and media composition. Once an organoid is established, some of the constituent cells can proliferate or differentiate, in response to the appropriate stimuli. Organoid cells are grown within a drop or a ring of Matrigel surrounded with medium, which is changed every 1–3 days. The passaging of organoids (generally every 7–10 days) consists of mechanical or enzymatic digestion of the basement membrane, releasing whole organoids that are further dissociated into small cell clusters or individual cells, that are subsequently replated in new Matrigel drop and culture medium. The three basic components of the organoid culture are discussed in the following chapters.

2.2 Three-dimensional extracellular matrices

Extracellular matrix in 3D organoid culture system permits the growth and expansion of cells in both horizontal and vertical planes, thus distinguishing it from the standard 2D culture system. The

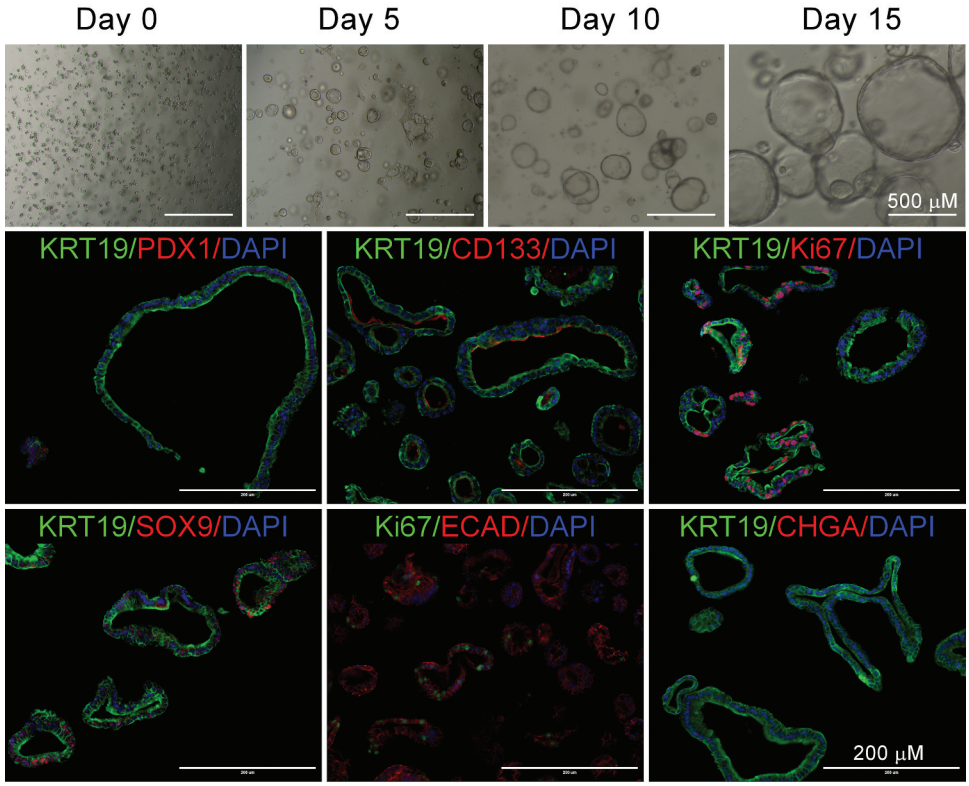

Figure 1. Example of organoid culture established from adult human pancreatic CD133^{+} cells. Top: Phase contrast microscopy visualizing the expansion of organoids over two weeks. Scale bar: 500 µm. Bottom: Immunohistochemistry visualizing the constituent cells at Day 15, indicating the proliferation activities (Ki67) and cell types (duct, KRT19; epithelial, ECAD; endocrine, CHGA; pancreatic progenitors PDX1, SOX9), including the original CD133^{+} cells. Scale bar: 200 µm.

mammalian cells grown on a flat surface as opposed to the three-dimensional space are subjected to different mechano-chemical cues, causing differences in cytoskeleton rearrangement, gene expression, cell shape, and function. In two-dimensional culture, no gradients of nutrients and signal molecules are possible. Also, the cells are forced to apical-basal polarity, reducing lateral cell-to-cell adhesions, which are critical for development and function of cells,[56] including beta cells.[57] Sensitivity to such mechano-chemical cues was demonstrated for stem cells and pancreatic progenitors.[58,59] In 3D culture systems, the individual cells utilize a range of surface adhesion molecules to reaggregate among themselves and to interact with fibrillar proteins of the matrix; consequently, chemical gradients are generated.[60–62] 3D organoid cultures can further be enhanced by co-culturing with additional cell types, e.g. endothelial or mesenchymal cells, to provide signals, which at present cannot be delivered by defined chemical components.[1,63]

The most commonly used artificial extracellular matrix substitute is Matrigel/BME (Basement membrane extract), which generates 3D scaffold by rapid spontaneous gelification at 37°C. This assortment of gelatinous proteins of the extracellular matrix derived from Engelbreth-Holm-Swarm tumor cell line was shown to support the stem cells' self-renewal potential and preserve their undifferentiated state.[64] The major Matrigel components comprise ~60% laminin, ~30% collagen IV, ~8% nidogen/entactin, and Heparan sulfate.[65,66] A number of poorly defined growth factors naturally bound to the matrix were reduced in some commercial variants (e.g. BME2) to improve chemical definition and standardization. Matrigel has two major limitations: the xenogeneic cancer cell line origin, hindering its medical use; and the batch to batch variation, hampering research reproducibility.[67,68]

Synthetic matrices are devoid of growth factors, which are substituted in defined media, thus advancing the organoid field toward Good manufacturing practice (GMP). Defined binding sites for integrins and for other cell adhesion molecules are attached to branched polymers interconnected by a crosslinker, thus forming a hydrogel network. Three such compounds were tested in pancreatic organoid culture. First, the poly(ethylene glycol) (PEG)-based hydrogel was covalently functionalized with laminin-1, and substrates of the FXIIIa enzyme, enabling the crosslinking by Thrombin-activated factor XIIIa. Similar to Matrigel, this hydrogel also supported the morphology, cluster formation, and progenitor maintenance of pancreatic embryonic organoids, but was less potent.[54] Second, another PEG-based hydrogel was functionalized with Integrin receptor binding motif containing sequence Arg-Gly-Asp (RGD) while using maleimide for crosslinking. It supported the growth of intestinal organoids derived from human embryonic/induced pluripotent stem cells,[69] but the morphology of human pancreatic organoids was altered.[70] Third, a dextran polymer was functionalized with RGD and crosslinked with hyaluronic acid. It supported pancreatic organoid morphology and simplified the passaging by digestion with dextranase. However, the expansion was slow and limited to only 5–6 passages in 100 days.[48] Additionally, apoly-isocyanopeptide-based hydrogel functionalized with human recombinant laminin-111 supported organoids derived from adult liver,[71] making it promising for developmentally related pancreatic organoids. Novel variants of synthetic hydrogels were recently reviewed elsewhere.[72]

2.3 Basic medium for expanding pancreatic organoids

Culture media for pancreatic organoids were developed from the medium originally established by Sato for intestinal organoids.[73] Similarly, Sato's medium was adopted for derivation of organoids from normal or tumor tissue of other digestive organs, including colon,[74] stomach,[75] and liver.[76] Sato's medium comprises Advanced DMEM/F12 medium and three key growth factors, EGF, Noggin, and R-Spondin-1, hence ENR medium.[73] The rationale for selecting these growth factors is following. EGF was shown to potentiate proliferation, while suppressing differentiation of pancreatic endocrine embryonic progenitors during development and *in vitro*.[77] Noggin, a member of the transforming growth factor superfamily, inhibits the bone morphogenetic protein signaling pathway,

which is fundamental for solid organ development,[78] including pancreas.[79] R-Spondin-1 is an agonist of LGR5 receptor[80] of the Wnt-β-catenin signaling pathway (Wnt),[81] which, in turn, is essential for development and for self-renewal of several types of adult stem cells.[82,83]

The Basic Medium for pancreatic organoids is the ENR Medium enriched by additional factors, such as FGF10, Nicotinamide, N-acetylcysteine, and B27 (contains 21 ingredients, mostly antioxidants, and enzymes, such as catalase and superoxide dismutase). FGF10, a natural product of mesenchymal cells, supports the proliferative capacity of PDX1$^+$ (Pancreatic and duodenal homeobox 1) pancreatic progenitors,[84] and helps to integrate the growth and the differentiation during pancreatic development.[85] Nicotinamide is an inhibitor of sirtuin (a regulator of epigenetic silencing), and poly(ADP-ribose) polymerase (PARP), which regulate protein deacetylation and DNA repair.[86,87] Nicotinamide was shown to promote survival and differentiation of human pluripotent stem cells after individualization.[87] Within this review, we call it "Basic Medium."

Basic Medium was variously supplemented with Wnt pathway ligand, WNT3A (WENR medium);[74] hormone Gastrin; Rho-associated protein kinase (ROCK) inhibitor, Y-27632; inhibitors of TGFβ pathway, A83-01 or SB431542; Prostaglandin E2; Hepatocyte growth factor; an inhibitor of histone deacetylases, Trichostatin A; an inhibitor of glycogen synthase kinase 3β, CHIR99021; and an activator of adenylyl cyclase, Forskolin, Tables 1, Table 2. Gastrin is produced by G-cells of the developing[88] and neonatal pancreas,[89] where it has documented proliferative activity. Inhibitor of the ROCK signaling pathway diminished the dissociation-induced apoptosis in embryonic stem cells *in vitro*.[90] The contribution of the individual soluble factors to the expansion capacity of pancreatic organoids was systematically evaluated over 6 months (20 passages) in Basic Medium supplemented with WNT3A, Gastrin, A83-01, and Prostaglandin E2.[22] Both human and mouse organoids failed to expand beyond the passages 3–5, when one of the following factors was omitted: EGF, Noggin, R-Spondin-1, WNT3A, Prostaglandin E2, or Nicotinamide. The omission of A83-01 or Gastrin allowed for at least ten passages.[22]The protein factors

Table 1. Effect on organoid proliferation and differentiation by selected proteins and small molecules.

Factor*	When the factor is absent from media	Reference no.
EGF (133 kDa, 1207 AA) promotes cell proliferation	• In adult organoids, human: reduced size and expansion (2–5 passages)	46, 22
	• In fetal organoids, mouse/human: reduced proliferation, improved differentiation	55
Noggin (58 kDa, 232 AA) inhibits Bone morphogenic protein pathway	• In adult organoids, human: reduced size and expansion (4 passages)	22
	• In adult organoids, mouse: reduced expansion (2 months)	46
	• In embryonic organoids, mouse: increased cystic morphology	54
R-Spondin-1 (~28kDa, 263 AA) activates Lgr5 in Wnt/β-catenin pathway	• In adult organoids, human: reduced size and expansion of (3 passages)	22
	• In adult organoids, mouse: reduced expansion (2–5 passages)	46
WNT3A (39 kDa, 352 AA) activates Wnt/β-catenin signaling pathway	• In adult organoids, human: reduced expansion (approx. 3 passages)	22
FGF10 (23 kDa, 208 AA), mesenchymal factor	• In mouse adult organoids: reduced expansion (2–5 passages)	46
	• In fetal organoids, mouse/human: slower expansion	55
	• In mouse embryonic organoids: reduced acinar diff. and *Pdx1* expression	54
	• In mouse embryonic organoids: no effect after 4 days	54
FGF1 (17 kDa, 155 AA), mesenchymal factor	• In embryonic organoids, mouse: improved endocrine differentiation	54
	• In embryonic organoids, mouse: diminished *Pdx1* expression	
VEGF (27 kDa, 232 AA), vascular endothelial growth factor	• In adult organoids, human: reduced engraftment efficiency (<3 months)	48
Nicotinamide (MW 122.12) a vitamin B3 form	• In adult organoids, human: reduced expansion (4 passages)	22
	• In adult organoids, mouse: reduced expansion (<2 months)	46
Y-27632 (MW 247.34) inhibits ROCK (Rho-associated protein kinase)	• In fetal organoids, mouse/human: cell proliferation dramatically decreased	55
	• In embryonic organoids, mouse: reduced org. formation, none *Pdx1*$^+$	54
A83-01 (MW 421.52) inhibits TGFβ pathway	• In adult organoids, human: reduced expansion (from 20 to 10 passages)	22
Prostaglandin E2 (MW 352.47)	• In adult organoids, human: reduced expansion (5 passages)	22

*) UniProtKB/Swiss-Prot: https://genecards.weizmann.ac.il; MW, molecular weight.

Table 2. Expansion of pancreatic organoids; cell origin, media composition, and results.

Cell selection	Basic Medium modifications*	Cultivation time, No. of passages	Doubling time [hrs]	Reference no.
Mouse, hand-picked duct fragments	Gastrin	10 months	~60	46
Mouse, *Lgr5*$^{+}$	Gastrin, ROCK inhibitor	>4 months	-	
Mouse, *Ptf1A*$^{+}$	Gastrin	3–4 passages	-	
Mouse, EpCAM^{+} TSQ^{+}	Gastrin, ROCK inhibitor	1 month, no proliferation	-	
Mouse, hand-picked single ducts	Gastrin	-	-	49
Human, islet-depleted fragments	Gastrin, A83-01; w/o Nicotinamide	10 passages	67	47
Human, ALDH^{high} (from organoids)	Gastrin, A83-01, ROCK inhibitor; w/o Nicotinamide	-	-	
Human, hand-picked duct fragments	WNT3A, Gastrin, A83-01, Prostaglandin E2	20 passages, 6 months	-	22
Human, CD133^{+}	w/o: B27, N-acetylcysteine	>3 months	-	53
Human, CD133^{+}	Prostaglandin E2, HGF, Trichostatin A, CHIR99021, SB431542	>5 months	72	52
Human, islet-depleted fragments	Gastrin, A83-01, Prostaglandin E2, Forskolin	~5 passages, 70 days	73	50
Mouse, Procr^{+} cells from islets	B27, ITS, EGF, FGF2, heparin, endothelial cells; w/o:Basic Medium	20 passages, 6 months	-	161

*Basic Medium: Advanced DMEM/F12, B27, N-acetylcysteine, EGF, Noggin, R-Spondin-1, Nicotinamide, FGF10

can be derived either from specifically designed cell lines, that express these factors and secrete them into the medium,[91] or these factors can be added into the medium in the form of pure recombinant proteins, which are amenable for GMP.

2.4 Media for pancreatic organoid differentiation

Unlike the intestinal organoids, no universal medium has yet been developed for differentiation of pancreatic organoids toward beta cells. Several approaches were tested. The simplest one is the exclusion or reduction of growth factors that stimulate cell proliferation, such as EGF or R-Spondin-1.[46,47,52,53,55] Nicotinamide, known to induce differentiation and maturation of fetal pancreatic endocrine cells in non-organoid culture,[92] is commonly used in organoid culture.[47,52,53,93] The genes required for differentiation of organoid stem cells are presumably inaccessible for the developmental transcription factors. The accessibility of such genes can be improved by chromatin state modulation, using small molecules inhibiting nuclear epigenetic modifiers, such as DNA methyltransferases.[52] More direct approach employed in some pancreatic organoid studies is transdifferentiation *in vitro* by introducing the key transcription factors by viral[49,53] or non-viral[52] means. Detailed information about differentiation approaches are described in the chapter Organoids derived from Adult Pancreas.

3. Lessons from Embryogenesis and adult organ regeneration

Organoids derived either from embryonic or adult pancreatic tissue could potentially serve as a model system to accelerate the study of some aspects of pancreas and beta cell development. This is particularly important for research of human pancreas, given the limited accessibility. The designing of organoid culture systems draws from the knowledge obtained from studies of the development and regeneration of pancreas and other epithelial organs.

Pancreas develops from dorsal and ventral buds of the foregut endoderm in close contact with endothelium.[94–97] The early cellular development as well as the late maintenance of the differentiated state are orchestrated by a hierarchical cascade of stage-specific combinations of transcription factors.[98–100] The development of pancreas is divided into three major stages: a primary transition (E9.5–12.5), a secondary transition (E12.5-birth), and the early postnatal period until weaning.[101] During the primary transition period, the pancreatic progenitors proliferate

Table 3. Differentiation of expanded organoids from adult murine and human pancreata.

Cell source	*In vitro* differentiation	*In vivo* differentiation	Maturation by histology	C-peptide secretion	Reference no.
Mouse, $EpCAM^+$ TSQ^- CAG^+	-	Re-aggregated with embryonic cells, transplanted under kidney capsule	2.5% $insulin^+$ (all mono-hormonal)	*In mice*: detectable, response not tested	46
Mouse, hand-picked single ducts	Lentiviral delivery: *Pdx1, MafA, Ngn3-WT*	-	7% $insulin^+$	-	49
	Lentiviral delivery: *Pdx1, MafA, Ngn3-phosphomutant*	-	28% $insulin^+$ (33% coexpressed somatostatin)	-	
	Plus medium modification: ROCK inhibitor; IWP-2, w/o R-Spondin-1, EGF for the last 2 days	-	61% $insulin^+$ (41% coexpressed somatostatin)	-	
	Plus last 2 days the three genes turned off	-	61% $insulin^+$ (87% mono-hormonal)	-	
Human, islet-depleted fragments	Nicotinamide containing medium	Transplanted under kidney capsule in normoglycemic or hyperglycemic mice	1.5% $insulin^+$ (mono-hormonal)	*In mice*: detectable, not glucose-responsive	47
Human, $CD133^+$	Adenoviral delivery: *PDX1, MAFA, NGN3, PAX6*; Medium: Retinoic acid; w/o R-spondin	Transplanted under kidney capsule	7–11% $insulin^+$ (all mono-hormonal)	*In vitro*: not glucose-responsive; *In mice*: detectable*	53
Human, $CD133^+$	mRNA delivery: NGN3; Medium: RepSox, PP2, ISX-9, GSK126, 5-aza-2′-deoxycytidin, Forskolin	-	5% $insulin^+$ (some coexpressed somatostatin)	*In vitro*: not glucose-responsive	52
Mouse, $Procr^+$ cells from islets	B27, ITS, EGF, FGF2, heparin, endothelial cells	Organoid transplanted under the kidney capsule were already differentiated	*in vitro*: 30% mono-hormonal $insulin^+$cells, 70% bi-hormonal $insulin^+$ cells	*In vitro*: glucose-responsive, *In mice*: glucose-responsive	161

*After 3 g/kg of glucose, after o/n starving, there was a trend to incresed C-peptide levels, but statistical significance is not reported.

within thickening endoderm, which comes into a close proximity of endothelial cells of dorsal aortae.[102] The endothelial cells initiate evagination of endoderm,[94] which leads to tubulogenesis and branching. During this period, bipotent pancreatic progenitors form the stalk/trunk domains, which give rise to ductal and endocrine cells, while multipotent progenitors present in the tip domains generate endocrine, ductal, and acinar cells.[103] Most of the endocrine cell differentiation occurs within the secondary transition period, hand in hand with the extensive exocrine differentiation. Within the secondary transition period, extensive exocrine and most of the endocrine cell differentiation occurs. Multipotent progenitors of the tip domains lose their multipotency and differentiate into acinar cells.[99] In the original model of islet formation, the differentiating $Ngn3^+$ endocrine progenitors, derived from bipotent trunk progenitors, undergo epithelial-mesenchymal transition allowing for cell delamination from the ducts, migration toward blood vessels and aggregation within newly formed islets.[101] However, recent model omits both the epithelial-mesenchymal transition and cell migration. In the course of islet formation, the cell contacts between the precursors are maintained, bi-layered nascent ”peninsulas” are formed, where alpha cells first develop in the outer layer (E13.5), while beta cells gradually appear beneath them, attached to the epithelial cord producing differentiating cells (E14.5). This spatiotemporal collinearity gradually leads to the core-mantel architecture typical for mature mouse islets.[104] Functionally, mouse beta cells mature only after birth, coincident with weaning.[105] In the unique human islet architecture, all endocrine cells are attached to blood vessels, heterologous alpha–beta cell contacts are favored, and homologous beta–beta cell contacts are permitted.[106] In the formation of human islets, a coalescence of few small ”peninsulas” was proposed.[104]

Since the 1880s, adult pancreatic cells were considered undividing, but the capacity of pancreas to regenerate remained tenable, due to the early observations of mitotic figures in pancreatic cells, after partial pancreatectomy.[107] In humans, beta cells noticeably increase their numbers only in the first few years of life and during pregnancy.[108,109] In 1993, Bonner-Weir proposed two pathways of pancreas regeneration after 90% pancreatectomy in mice, when observing a) the replication of preexisting endocrine and exocrine cells, and b) the

proliferation and differentiation of ductal cells, forming new lobules, which contained new beta cells, reminiscent of embryonic development.[110] Two decades later, the regeneration of adult beta cells remains unsettled. Some genetic cell tracing studies support the original hypothesis[110] of beta cell neogenesis from adult ductal progenitors after injury inflicted by duct ligation[111,112] or genetic cell targeting.[113] Other fate mapping studies failed to find evidence for beta cell neogenesis from adult pancreatic progenitors.[111,114–119] Additional genetic cell tracing studies combined with pancreatic duct injury in rodents convincingly demonstrated the replication of beta cells as the dominant regenerative mechanism during adult life[120,121] even after partial pancreatectomy[120] or targeted beta cell depletion.[122] Significance of these complex experiments has been thoroughly reviewed elsewhere.[107,123–131] Finally, transdifferentiation of alpha to beta cells was identified as a source of beta cell regeneration after near-total beta cell ablation in adult mice.[132] After puberty, this capacity of alpha cells was not altered by age until senescence.[133] In juvenile age, dedifferentiation of delta cells was observed, accompanied by subsequent proliferation and differentiation into beta cells after almost complete beta cell ablation.[133]

An unexpected heterogeneity of regeneration strategies was discovered among epithelial organs, ranging from the presence of multiple types of adult stem cells within adult tissues to the natural plasticity of differentiated cells, which de-differentiate into stem cells and in turn are capable of tissue regeneration.[134] The existence of facultative adult progenitors was proposed in some tissues, including liver and pancreas. A picture is emerging, where the $Lgr5^+$ crypt collumnar cells residing at the bottom of the intestinal crypt serve as the intestinal stem cells under normal conditions, while under other conditions, such as radiation injury, the radiation-resistant +4 cells replenish not only the differentiated intestinal cell population, but also lost $Lgr5^+$ crypt collumnar stem cells.[135] LGR5 was also used as a positive selection marker to generate adult pancreatic organoids.[46]

4. Organoids derived from embryonic and fetal pancreatic progenitors

Unlike in adult pancreas, the existence of pancreatic progenitors in the embryonic and fetal tissues is undoubted. The following three studies[54,55,93] represent the first attempts to apply the principles of organoid culture in order to replicate the main features of pancreas development *in vitro*.

Greggio[54] optimized culture conditions and achieved expansion and partial differentiation of pancreatic organoids. The organoids originated from single cells or small groups of multipotent pancreatic progenitors isolated from embryonic (E10.5) mice. The mice were genetically modified (e.g. *Ngn3-EYFP, Pdx1-nGFP, Sox2-Cre x R26R-lacZ, Sox2-Cre x R26R-YFP, Neurog3 knockout*) in order to visualize the lineage tracing with fluorescent or histochemistry labels, in time lapse experiments. The efficiency of organoid structure formation was found to depend on the number of $Pdx1^+$ cells per cluster, giving rise to the organoid (100% efficiency was achieved when at least 12 $Pdx1^+$ cells were present per cluster),[54] suggesting the community effect.[136] Within seven days, the largest organoids formed lobulated tubes with duct cells marked with mucin. The periphery of terminal buds was crowned with $PTF1A^+/SOX9^+/PDX1^+$ cells, giving rise to acinar cells (15–20%). In the organoid core bipotent progenitor cells ($HNF1B^+$) differentiated into polarized ductal cells with rare endocrine differentiation. The frequency of mono-hormonal C-peptide$^+$ cells increased up to ~0.7% by omission of FGF1, which however was necessary in the first four days for the proliferation of $Pdx1^+$ progenitor cells. Similarly, ROCK inhibitor was indispensable for the survival of progenitors expressing *Pdx1*. When the organoids were dissociated and transplanted into E13.5 pancreatic explants (a transplantation assay), the endocrine differentiation increased within 10 days up to 4%, indicating an incompleteness of the artificial niche provided by the *in vitro* culture conditions.[54]

Sugiyama[93] described an alternative protocol for partial reconstitution of pancreas development, using spherical organoids. Transgenic mice (e.g. *Sox9-eGFP/Ngn3-tdTomato, MIP-GFP*) were used

for cell selection and tracking. FACS-sorted fetal (E11.5) $NGN3^{-}/SOX9^{+}$ multipotent progenitors (expressing also *Pdx1, Hnf6, Tcf2* and *Hes1*, but not endocrine markers) were seeded at clonal density and cultured in Matrigel mixed with mesenchymal cells in PrEBM medium supplemented with FGF10, IGF1, Retinoic acid, insulin, and Transferrin. While the organoids expanded for three passages only, the progenitors here differentiated into multilayered spheres, with the inner layer of $mucin^{+}$ duct-like cells, and the periphery with $SOX9^{-}$ cells. A subset of $SOX9^{-}$ cells expressed the endocrine markers, e.g. Ngn3, insulin, C-peptide, and glucagon. The endocrine cells on the outer surface were separated from the inner lining of $mucin^{+}$ cells by another cell layer. The differentiation into glucose-responsive insulin-secreting cells was enhanced by a combination of Nicotinamide, physiological level of oxygen, reduced concentration of FGF10, and in the presence of mesenchymal cells. Uniquely, twofold increase in glucose-stimulated (3 vs. 20 mM) C-peptide secretion was observed *in vitro*.[93]

Bonfanti[55] took advantage of transgenic mice (*Pdx1-eGFP x Ins1-mRFP*) to prospectively select and monitor fetal (E12 or E13) pancreatic progenitors in order to study the differentiation dynamics in organoids derived from a single cell.[55] Unlike the previous group, the authors demonstrated a high efficiency of organoid formation from individual progenitor cells, however with only partial endocrine differentiation.

In the same paper, Bonfanti investigated the impact of EGF on human fetal pancreatic organoids with the conclusion that EGF potentiates the organoid expansion while suppressing the differentiation toward endocrine fate.[55] Human progenitors were isolated after abortion 8–11 weeks post conception; mesenchymal cells were removed and the remaining epithelial tissue was digested, dissociated, mixed with Matrigel and cultured in Basic Medium supplemented with Gastrin and ROCK inhibitor. In the presence of EGF, the organoids expanded for at least 5 months, without changes in their cystic morphology. The organoid architecture contained ductal structures with cell polarization, as detected by *MUC1* expression. In the absence of EGF, the organoids grew smaller, some assumed dense rather than cystic morphology, and the possible passage number dropped to ten. Significantly, a spontaneous differentiation toward endocrine fate (e.g. chromogranin A, insulin, glucagon, somatostatin) and acinar fate (e.g. *PTF1A*) was observed. The authors also observed a decreased proliferation rate after excluding ROCK inhibitor, FGF10, or R-Spondin-1. Their findings demonstrated a similar response between human and mouse fetal progenitors to the same environmental cues.[55]

5. Organoids derived from adult pancreas

Enzymatic digestion of adult pancreas releases endocrine islets, and non-endocrine fragments comprising the acinar, centro-acinar, and ductal components, which somewhat differ in the tissue density.[137] Enrichment of the starting material with the prospective progenitor cells was achieved using techniques, such as tissue fragment separation on density gradient, hand-picking of duct fragments, and single cell sorting using putative markers of the proposed pancreatic stem/progenitor cells, as summarized in Table 2. The mouse and human organoid studies discussed below explored the utility of cell surface markers LGR5, CD133, and PROCR, as well as intracellularly expressed cell markers ALDH and PTF1A. Beta cell differentiation was induced by media composition and/or manipulating key transcription factors, as summarized in Table 3.

Azzarelli[49] transdifferentiated mouse ductal organoids *in vitro* using lentiviral-mediated overexpression of transcription factors *Pdx1, MafA*, and *Neurogenin3*. The organoid culture was initiated from hand-picked ducts. Neurogenin3 was administered in two forms: the wild type and a more stable phospho-mutant. The best percentage of transdifferentiation into insulin positive cells (28% versus 7% in the wild type) was observed in organoid cells treated with the stable mutant of Neurogenin3. Significantly, the differentiation further increased up to 61% of insulin positive cells, when Wnt pathway was inhibited (addition of IWP-2, omission of R-Spondin-1) and EGF was removed for the last two days of culture. Moreover, when viral expression of all the transcription factors was turned off, the proportion of monohormonal insulin-producing cells increased. In

spite of this success, the measurement of glucose responsiveness was inconclusive due to the small number of organoid cells.[49]

A potential selection marker for adult pancreatic progenitors is LGR5, a plasma membrane receptor for R-Spondin-1, involved in the signaling of canonical Wnt pathway.[138] Its deletion is neonatally lethal.[139] It was identified in stem cells of the small intestine, colon,[83] and in long-lived cycling stem cells in hair follicle.[140] Lgr5$^+$ cells in the intestinal crypt were shown to generate all epithelial lineages over more than a year of follow up *in vivo* study.[141] Additionally, liver injury induced the expression of Lgr5 on proposed facultative liver progenitors, as documented by their capacity to expand *in vitro* in a Wnt-pathway-dependent organoid culture.[76] Huch[46] stimulated the emergence prospective facultative progenitors *in vivo* by applying the partial duct ligation model in genetically modified adult mice (e.g. *ECad-CFP, CAG-EGFP, Lgr5-LacZ*). After pancreatic duct ligation, a population of *Lgr5*$^+$ stem/progenitor cells appeared in the ducts. Huch[46] concluded that adult *Lgr5*$^+$ cells are bipotential progenitors capable of endocrine and ductal differentiation. In this study, hand-picked ductal fragments were embedded in Matrigel and grown in Basic Medium in Matrigel and grown in Basic Medium supplemented with Gastrin. Ductal fragments formed budding cyst-like organoids composed of duct-like progenitors (expressing *Sox9, Pdx1, Muc1, Krt19*). The complete medium allowed the organoids expansion for more than ten months, while the omission of EGF, Noggin, R-Spondin-1, FGF10, or Nicotinamide grossly reduced the number of possible passages. The capacity of FACS-sorted single *Lgr5*$^+$ cells to form organoids was demonstrated with a colony-forming efficiency of 16%, that was in agreement with organoid studies of other digestive organs.[75] The potential of the *Lgr5*$^+$ pancreatic progenitors to differentiate into ductal and endocrine lineages was demonstrated *in vivo* using organoids derived from single epithelial non-endocrine cells (EpCAM$^+$ TSQ$^-$). After 6 weeks of expansion, the organoids were dissociated, re-aggregated, and mixed with late embryonic pancreatic cells (E13.5). Additional stem cell niche signals were provided by transplanting this cell mixture under the kidney capsule of immunodeficient mice. Mainly, differentiated ductal (KRT19$^+$) were observed. Histology also revealed 5% of endocrine cells (synaptophysin$^+$), half of which were mono-hormonal insulin$^+$ and C-peptide$^+$. However, C-peptide in the plasma was not reported.[46]

Adult Ptf1a$^+$ acinar cells *in vivo* were shown to regain aspects of embryonic multipotentiality under injury, and subsequent conversion into mature beta cells was revealed.[142] PTF1A (Pancreas Transcription Factor 1a) is selectively expressed in pancreas, retina, spinal cord, brain, and enteric nervous system. It is indispensable in controlling the expansion of multipotent progenitor cells as well as the specification and maintenance of the acinar cells.[143] Huch[46] took advantage of the organoid culture in order to substantiate the hypothesis of the acinar cells as the adult pancreatic progenitors. The authors used PTF1A marker to select single cells for the organoid formation, but their expansion ceased four passages later.

Loomans[47] identified in adult human pancreatic organoids an abundant cell population characterized by a high aldehyde dehydrogenase (ALDH) activity, and proposed it as a new marker for adult pancreatic progenitors. ALDH/RALDH (Aldehyde dehydrogenase) participates in All-trans Retinoic acid synthesis, which regulates gene expression by activating specific nuclear receptors during development in various tissues, including pancreas.[144,145] In the organoid study by Loomans et al., the transcription profile of ALDH$^+$ cells (*CPA1, PDX1, MYC*, and *PTF1A*) corresponded with multipotent embryonic progenitors described by Zhou.[103] ALDH^{high} cells constituted a quarter of the cells in the primary organoids, which were derived from fragments of islet-depleted pancreatic tissue and cultured in Matrigel and Basic Medium supplemented with Gastrin and TGFβ inhibitor (A83-01), with Nicotinamide omitted. ALDH^{high} cells were predominantly localized at the tips of the budding organoids, which expressed mucin-1 at the luminal side and were maintained for at least 10 passages. ALDH^{high} cells selected from the primary organoids formed secondary cyst-like colonies, which in turn also expanded, suggesting stemness of the original cells. No endocrine differentiation of the secondary colonies was reported. A small cell fraction (0.5%) in the primary organoids differentiated *in vitro* into

insulin positive cells, when Nicotinamide containing differentiation medium was used.[146] No glucagon positivity was reported. One month after the primary organoids were transplanted under the kidney capsule of either normoglycemic or hyperglycemic immunodeficient mice, 1.5% of organoids cells were insulin positive. Human C-peptide was detectable in mouse plasma, but was not glucose-responsive, indicating failed maturation *in vivo*. Glucagon was detected in mono-hormonal cells only.[47] While ALDH$^+$ cells were scarcely detectable in normal adult pancreas,[147] they become abundant in regenerating conditions in human (patients with pancreatitis or T1D) as well as in mice (early postnatal, pregnancy),[148] corroborating their facultative progenitor status. In this context, Rovira,[147] by means of the centro-acinar /terminal ductal cells expressing ALDH1, published the first attempt to utilize the principles of mouse organoid culture in order to identify the putative adult pancreatic progenitor cell. Sorted ALDH positive cells were used to generate clonal pancreatospheres. The spheres contained cells co-expressing ALDH and SOX9, suggesting self-renewal capacity, which however was supported by only miniscule expansion (three passages). Interestingly, spontaneous C-peptide secretion occurred, but glucose responsiveness was tested at 0 vs. 11 mM glucose levels (the standard glucose testing levels are 3 vs. 20 mM), nor was it statistically evaluated.[147]

Another potentially useful selection marker for adult pancreatic progenitors is CD133 (AC133, Prominin-1), a plasma membrane protein with a large extracellular loop. It was identified in undifferentiated embryonic stem cells,[149] and organ-committed stem cells,[150] such as hematopoietic[151] and neural[152] stem cells. Histology of pancreas demonstrated the presence of CD133$^+$ cells within adult ducts.[153,154] Several groups utilized it for positive selection of the prospective bipotent or multipotent pancreatic progenitors, employing antibody-based FACS[53,155–157] or MACS[52,158] cell sorting.

Lee[53] transdifferentiated human CD133$^+$ cells derived pancreatic organoids into insulin-secreting cells, using ectopic overexpression of four principal islet transcription factors (PDX1, MAFA, Neurogenin3, and PAX6). The CD133$^+$ cells, selected by FACS from islet-depleted pancreatic tissue, were positive for the ductal marker cytokeratin-19, and negative for acinar and endocrine markers. Single-layer organoids were expanded for up to 3 months in Basic Medium with the omission of B27 supplement. The transdifferentiation protocol involved adenoviral vectors expressing the transcription factors inspired by Zhou.[159] After a few days of R-Spondin-1 withdrawal and supplementation with Retinoic acid, the organoids were cultured for subsequent two weeks in a differentiation medium, composition of which was less important than the timing. The differentiated spheres comprised 7–11% insulin positive cells, which secreted C-peptide into the medium, irrespective of the glucose level in the range 2–11 mM. A trend toward glucose-responsiveness (2.4×) was observed only when glucose was increased from starvation level (0.1 mM). However, C-peptide response to non-glucose secretagogues (KCl, sulphonylurea) was observed. After transplantation of transdifferentiated spheres under the kidney capsule of non-diabetic immunodeficient mice, human C-peptide was detectable in plasma for two weeks and a trend toward glucose responsiveness was observed (statistics for these functional observations was not provided).[53]

Koblas[52] achieved reprogramming of human CD133$^+$ organoid cells into insulin-producing cells by the means of non-viral non-integrative introduction of Neurogenin3 in a combination with small molecules altering signaling pathways and epigenetic state. CD133$^+$ cells were immunomagnetically separated from islet-depleted pancreatic tissue and cultivated in Matrigel with Basic Medium replenished with HGF, Prostaglandin E2, Trichostatin A, CHIR99021, and ALK5 inhibitor (SB431542). Single-layer organoids were expanded for at least 5 months. Neurogenin3 was introduced in the form of synthetic mRNA. Differentiation medium contained RepSox, PP2, ISX-9, GSK126, 5-aza-2′-deoxycytidin, and Forskolin. After the differentiation, the organoids comprised ~40% chromogranin A positive cells, including 5% insulin positive cells.

C-peptide was detected in the medium at low levels ($1/10^3$ of that produced by the same amount of islets), which failed to respond to glucose challenge, possibly due to insufficient expression of MAFA and PAX6. However, the secretion of C-peptide was KCl-responsive. A double-hormonal subpopulation co-expressing insulin and somatostatin was observed, further corroborating the immature character of insulin-producing cells. Glucagon positive cells were not observed.[52] The non-integrative approach taken in this study avoids virus-induced inflammatory response and oncogenic transformation.[160]

The above discussed studies achieved various degrees of expansion of pancreatic organoids derived from different subpopulations of pancreatic duct cells. The maximum length of the growth of human organoids reached six months (20 passages), while ten months for mice. Cyst-like oval shape organoids generally comprised a single layer of predominantly undifferentiated cells oriented around a central lumen (rather than elongated ducts),[22,46–53] as exemplified in Figure 1. The culture conditions and the results of the presented studies are summarized in Table 1–3.

Wang et al.[161] uniquely searched for the adult endocrine progenitors within the pancreatic islets. The authors identified a novel population of Procr$^+$ progenitors within adult mouse pancreatic islets, using single-cell RNA sequencing (scRNA-seq), and demonstrated their capacity to ameliorate hyperglycemia in diabetic mice. PROCR (EPCR, CD201) is an endothelial receptor for protein C, which was previously identified on hematopoietic stem cells,[162] cultured cord blood cells,[163] blood vascular endothelial stem cells,[164] and breast cancer cells.[165] More than 7000 individual cells isolated from islet-enriched preparation were analyzed by scRNA-seq and mapped to clusters representing ten known cell types (alpha, beta, delta, PP, duct, acinar, endothelial, immune, mesenchymal, and stellate cells), and an additional cluster of previously unknown cells was identified. Unique signature genes in this cluster included Wnt pathway agonist gene *R-spondin-1* and Wnt target gene *Procr,* suggesting stem cell character. These Procr$^+$ islet progenitors had the capability to form expanding organoids, to differentiate into four endocrine cell types including insulin-producing beta-like cells, which ameliorated diabetes after transplantation. In Procr-mGFP-2A-LacZ mouse model, Procr$^+$ cells were identified from all pancreas exclusively in the islets, each containing only few such cells. The lineage tracing in adult Procr-CreERT2; RosaConfetti mice several months after tamoxifen pulse revealed clones comprising approximately seven cells. In 70% of the clones, all endocrine cell types were present, while 30% of the clones contained beta cells only. One cell in each clonal population was hormone negative, which was the proposed progenitor cell. When FACS-isolated Procr$^+$ cells were plated at a clonal density in serum-free medium supplemented with B27, ITS, EGF, FGF2, and heparin, one out of 15 cells formed a colony (Procr$^-$ cells failed to form colonies). However, Procr$^+$ cells could not be maintained for more than 7 days. Co-culture with endothelial cells was revealed as the key factor allowing long-term culture (more than 20 passages) of Procr$^+$ derived organoids. At 15th passage, the organoids contained all hormone$^+$ cell types, and secreted insulin and C-peptide (approximately ten-fold and fivefold, respectively) into the medium in the glucose-regulated manner. When 1000 organoids were transplanted under the kidney capsule of diabetic mice, glucose level was ameliorated below 10 mM, which was similar to the control group of diabetic mice transplanted with 300 natural islets. One month after transplantation, intraperitoneal glucose tolerance test (IPGTT) demonstrated the improvement of glucose tolerance to a similar degree as 300 islets transplanted to a control group. Graftectomy after 125 days led to an abrupt blood glucose increase to 25 mM. The clustering of scRNA-seq data from explanted graft revealed alpha, beta, delta, and PP cells matching well with the primary islet cell clusters.[161]

6. Potential clinical application of adult pancreatic organoids

The first and the only reported attempt to develop a large-scale GMP-level manufacturing procedure for the expansion of organoids for clinical purposes comes from Dossena et al.[50] The enzymatic digestion was replaced by a mechanical one, thus avoiding manual duct picking. The organoids were expanded using BME2 matrix and Basic Medium supplemented with Gastrin, Prostaglandin E2, Forskolin, and A83-01. The cystic morphology

and the expression profile (positivity for *SOX9, PDX1*, and *MUC*) typical for adult ductal organoids were observed. The authors were able to expand the islet-depleted pancreatic tissue, derived from a single cadaver donor, up to 250×10^6 pancreatic organoids with the prospect of future differentiation into insulin-secreting cells.[50]

The total number of organoid cells reached approximately 250×10^9 cells.[50] So far, the best published human differentiation rate achieved *in vitro* by clinically amenable means reached insulin positivity in 5% of organoid cells.[52] If these two approaches were combined, one could hope for 13×10^9 of cells, which however would not yet be functional (steady secretion of $1/10^3$ of the amount of insulin secreted by isolated islets).[52] Drawing from the experience with clinical islet transplantation, the minimum number of beta cells to achieve insulin independence is estimated to be 10^9 of functional beta cells per recipient, given the estimated 1140 beta cells per islet equivalent.[166] Some authors demonstrated up to 60% differentiation rate into insulin positive cells, in the case of mouse organoids.[49] While the means were unsuitable for clinical practice, these results suggest that a highly efficient though partial transdifferentiation is possible.

The safety of pancreatic organoids derived from adult stem cells for clinical application has not yet been properly evaluated. Only a few pilot studies performed transplantation of expanded or differentiated organoids into the mice without detection of any malignant transformations after maximal observation time for 3 months.[48,53]

7. Perspective

The existence of adult pancreatic progenitors or facultative progenitors has been contested for decades. Historically, three major assays have been devised to prove the existence of a stem cell in a given tissue: lineage tracing *in vivo*, clonogenic growth *in vitro*, and cellular transplantation.[124] Here, we reviewed the attempts to resolve the issue by the means of pancreatic organoid culture, which was applied to all four histological compartments of pancreas, comprising the acinar, and centro-acinar /terminal ductal, ductal, and islet cells.

The acinar and centro-acinar/terminal ductal cells failed to expand in long-term organoid culture, suggesting the absence of the stem cells. Although the ductal cells expanded in the long term and differentiated into insulin-producing cells, suggesting the presence of stem/progenitor cells, the differentiated cells remained unresponsive to glucose. Unlike the insulin-secreting cells derived from pluripotent stem cells,[167] the insulin positive cells derived from the organoid culture failed to mature *in situ* after transplantation under the kidney capsule. These findings need to be interpreted with caution, because under the current culture conditions even the embryonic/fetal stem-cells-derived organoids failed to completely differentiate unless co-cultured with mesenchymal cells. One explanation for failed differentiation can be the epigenetic state of the organoid cells preventing sufficient induction of key transcription factors involved in the differentiation process. The beta cell apparatus for metabolically regulated insulin release is quite complex,[168] and beta cells naturally mature only after weaning from fat-based to carbohydrate-based food.[105] This suggests that current adult non-islet derived organoid cultures might have failed to provide the adequate developmental and maturation cues.

Only $Procr^+$ cells isolated from adult mouse islets constituted organoid culture capable of the long-term expansion as well as the differentiation into glucose-responsive insulin-producing cells, which were uniquely able to ameliorate blood glucose in diabetic mice after transplantation.[161] Repeated supplementation of fresh endothelial cells to the organoid co-culture was necessary. The essential role of endothelial cells not only for embryogenesis, but also for proper beta cell function in adulthood was previously described,[106,169,170] with the dependence of beta cell on signaling cues from basal membrane, where beta cell cannot synthesize.

Clinical application of adult pancreatic progenitors for stem cell therapy of diabetes is challenged not only by doubtful existence of the adult pancreatic bipotential progenitors, but also by the inaccessibility of the proper pancreatic niche. The unparalleled success of clinical transplantation of hematopoietic stem cells was facilitated by the accessibility of the appropriate niche to the natural adult stem cells. Experimentally,

hyperglycemia was indeed ameliorated in mice by transdifferentiation of pancreatic acinar cells *in situ*, taking advantage of the natural pancreatic niche.[159] While current evidence supports other sources of beta cell regeneration,[131] the existence of adult bipotential pancreatic progenitors has not yet been abandoned.[129,130] The co-expression of *Pdx1, Sox9, Nkx6.1*, and *Hnf6 (Onecut-1)* is characteristic for the bipotent endocrine/duct progenitors during embryogenesis, and the same transcription factors are also detected within the pancreatic organoids.[46,47,52]

Although a large-scale expansion of human adult pancreatic organoids was recently developed, partially at GMP level for putative clinical application, more research would be necessary to develop truly efficient and safe approach. Another drawback of the adult pancreas as the source for cell-based therapy seems to be rather complicated access to this potential cell source, the inherent variability among different donors, and technical difficulties associated with the up-scaling of organoid culture.

Nevertheless, pancreatic organoid culture represents a potentially helpful tool bringing otherwise inaccessible and complex organ in a dish. Hypothetically, a pancreatic biopsy could be used for this purpose, similar to the intestinal organoids from cystic fibrosis patients.[16] Diabetes-associated genetic variants could also be introduced into organoids via CRISPR/Cas9 approach. At present, however, the identification of prospective pancreatic progenitor appears to be the most attractive goal for the use of pancreatic organoid system.

At the moment, adult pancreatic organoids are far from becoming a cell source for clinical application, but they remain an invaluable research tool for the investigation of beta cell development and regeneration, which makes more refinement effort worthwhile. It is intriguing whether the recently discovered Procr^{+} islet progenitor cells in the adult mice exist also in human islets, constituting a novel source of potentially curative beta cell replacement therapy or *in situ* regeneration. The lessons learned from the differentiation approach so successful in Procr^{+} islet progenitors might be applicable for the putative extra-islet pancreatic progenitors, thus moving the ten years old field forward.

Abbreviations

ALDH	Aldehyde dehydrogenase
ALK5	Activin receptor-like kinase 5
BME	Basement membrane extract
CD133	Prominin 1
ECAD	E-cadherin
ECM	Extracellular matrix
EGF	Epidermal growth factor
ENR	EGF+Noggin+R-Spondin-1
EpCAM	Epithelial cell adhesion molecule
ESCs	Embryonic stem cells
EZH2	Enhancer of zeste homolog 2
FACS	Flourescent activated cell sorting
FGF1	Fibroblast growth factor 1
FGF10	Fibroblast growth factor 10
GMP	Good manufacturing practices
HGF	Hepatocyte growth factor
HNF1B	Hepatocyte nuclear factor-1 beta
HNF6	Hepatocyte nuclear factor 6, Onecut-1
CHGA	Chromogranin A
CHIR99021	Inhibitor of glycogen synthase kinase 3ß
IGF-1	Insulin-like growth factor 1
IPGTT	Intraperitoneal glucose tolerance test
iPSC	Induced pluripotent stem cells
ISX-9	Izoxazole 9
ITS	Insulin-Transferrin-Selenium
IWP-2	Inhibitor of WNT Production-2
Ki67	Proliferation marker
KRT19	Cytokeratine 19
LGR5	Leucine-rich-repeat-containing G-protein-coupled receptor 5
MACS	Magnetic-activated cell sorting
MAFA	C-maf musculoaponeurotic fibrosarcoma oncogene homolog A
MUC1	Mucin 1
NGN3	Neurogenin3
NKX6.1	Nirenberg and Kim homeobox 6.1
PAX6	Paired box gene 6
PDX1	Pancreatic and duodenal homeobox 1
PEG	Poly(ethylene glycol)
PP2	4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d] pyrimidine
PrEBM	Prostate epithelial cell growth basal medium
Procr	Protein C receptor
PTF1A	Pancreas associated transcription factor 1A
RepSox	Inhibitor of TGFβ type I activin like kinase receptor (ALK5)
RFD	Tripeptide Arg-Gly-Asp
R-Spondin-1	Roof plate-specific Spondin-1
SC	Stem cell
scRNA-seq	Single-cell RNA sequencing
SOX9	SRY-Box Transcription Factor 9
TGFβ	Transforming growth factor β superfamily
TSQ	Fluorescent chelator for Zn^{2+} ionts (6-methoxy-8-p-toluenesulfonamidoquilone)
VEGF	Vascular endothelial growth factor (A)
Wnt	Wingless-INT-β-catenin signaling pathway
WNT3A	Wnt family member 3A
Y-27632	ROCK inhibitor

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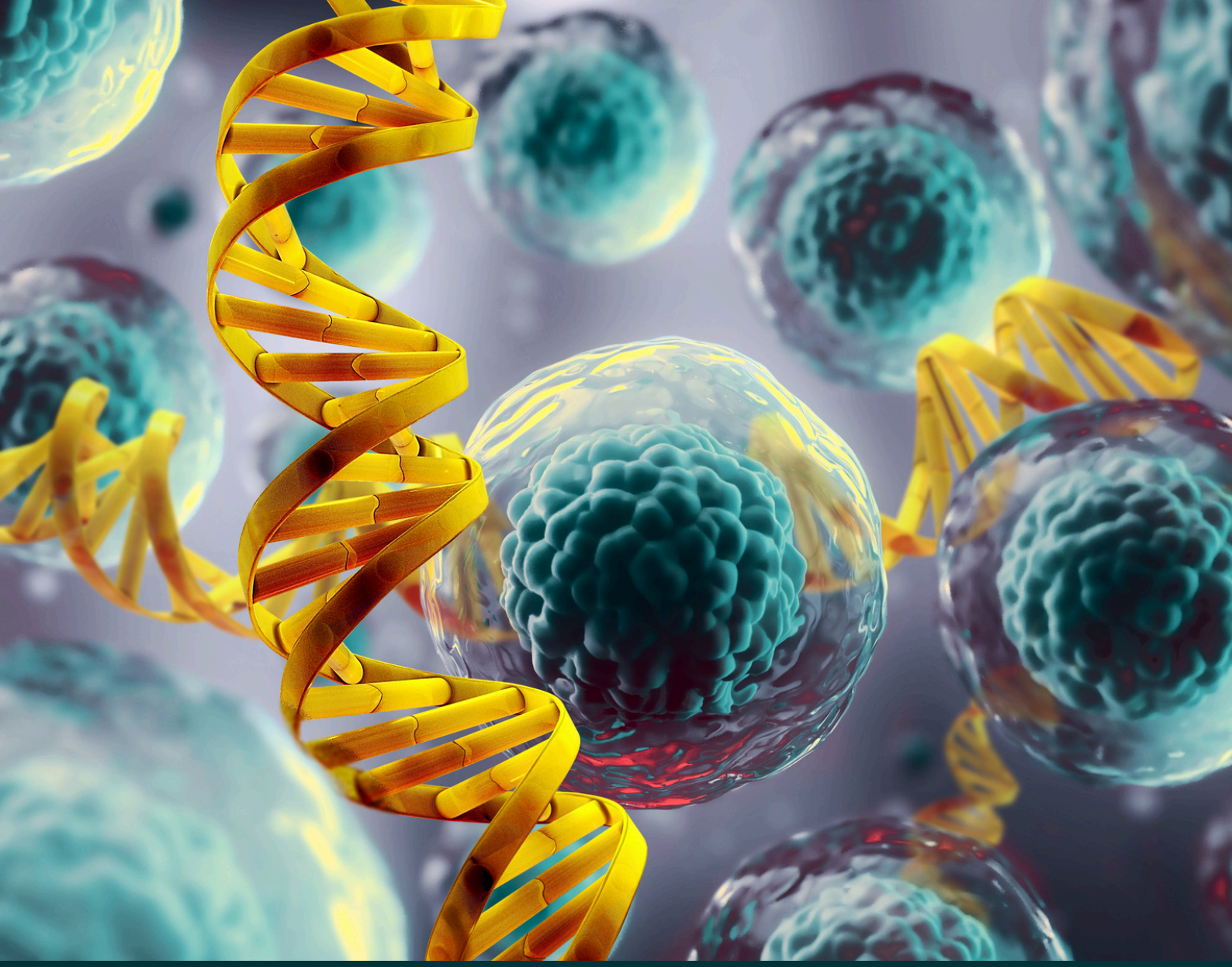

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