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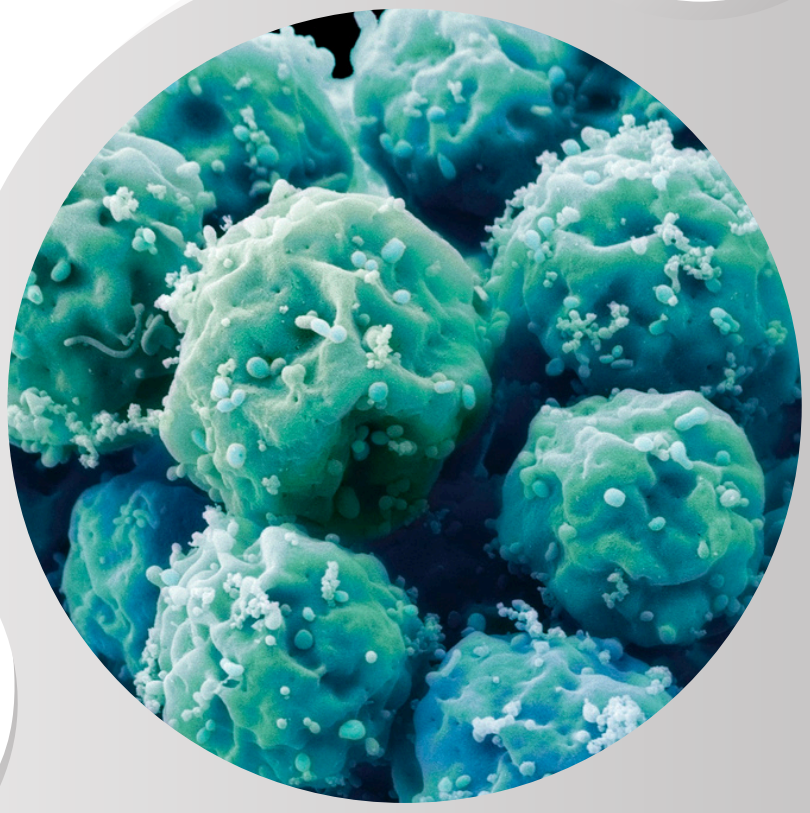
Scaling up 3D cell cultures with advanced microencapsulation technology

Discover how microencapsulation can enable reproducible and scalable suspension culturing of 3D cell aggregates such as spheroids and organoids in bioreactor systems.



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Scaling up 3D cell cultures with advanced microencapsulation technology

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Highlights

- 3D cell aggregates provide biomimetic culture conditions;
- Microencapsulation enables reproducible and scalable suspension culturing of 3D cell aggregates such as spheroids and organoids in bioreactor systems;
- IN-AIR MICROFLUIDICS™ technology offers a mild, reproducible, and scalable process for the encapsulation and suspension culture of 3D cell aggregates.

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Abstract

Cell therapies offer significant potential for treating severe diseases, including CAR-T cells for cancer, insulin-producing cells for diabetes, and various (stem) cells for tissue restoration. Despite their potential, numerous challenges hinder widespread adoption. Scaling cell cultures is difficult due to issues like uncontrolled cell merging and exposure to hazardous shear forces in bioreactors. Moreover, cell therapies often face problems such as immune responses and rapid cell clearance post-injection.

Novel 3D cell culture techniques offer solutions by more closely mimicking the native cellular environment, enhancing cell viability, and improving scalability. These techniques, including the use of spheroids and organoids, are pivotal for the effective development and application of cell therapies. Various methods like hydrogel domes, microwells, and bioreactor suspension cultures are used for forming 3D cell aggregates, each with distinct advantages and limitations in terms of scalability, reproducibility, and handling.

Recent advancements in microencapsulation technologies, such as IN-AIR MICROFLUIDICS™, offer promising solutions by combining scalability with high resolution and biocompatibility. This method facilitates the production of uniform microcapsules, enhancing the efficiency and consistency of 3D cell cultures and thereby improving clinical and industrial translation of cell therapies.

The promise and challenge of cell therapies

ATMPs

Advanced therapy medicinal products (ATMPs) are medicines for human use that are based on cells, tissues, or genes. These so-called cell and gene therapies offer groundbreaking new opportunities for the treatment of disease and injury.[1]

Myriad cell-based therapies are currently trying to find their way to the market, such as CAR-T cells to treat cancer, insulin-producing cells against diabetes, and (stem) cells for restoring skin, musculoskeletal, cardiac, intestinal, and nervous systems.[2]

Overall, ATMPs hold the promise of revolutionizing the treatment landscape for a variety of serious and life-threatening conditions by providing highly targeted, effective, and personalized therapies. However, multiple grand challenges still need to be solved to unlock the full potential of cell therapies on a global scale.

Scaling cell culture is challenging

As the key ingredient for many ATMPs, cells and tissues need to be produced in copious amounts. Suspension or bioreactor cultures (in a stirred tank) in principle offer a promising route for large-scale cell expansion, but typically are associated with poor reproducibility due to uncontrolled merging of cells and exposure to hazardous shear forces.[3] Furthermore, live cells typically need to be stored at ultra-low temperatures (<-80 °C), which requires a complex ultra-cold chain that causes a significant logistic burden.[4] Finally, several challenges still hamper the successful application of the cell-based product, such as shear stress-induced cell damage during injection, rapid clearance of cells from the site of injection, and unwanted immune responses against (allogeneic) cells post-injection.[5]

Novel 3D cell culture techniques are expected to significantly contribute to overcoming the challenges associated with the global implementation of cell therapies by enhancing biological relevance, scalability, and efficacy.

3D cell culturing

3D cell cultures more closely resemble the native habitat of cells in our body as compared to conventional 2D cultures on tissue culture plastic.[6] Furthermore, cell aggregation has been shown to support long-term maintenance of cell viability, proliferation, function, and phenotype during in vitro culturing and injectable therapy models.[7-10] Consequently, 3D cell aggregate culturing contributes to improved efficacy, safety, reproducibility, and scalability of cell therapies and plays an important role in the development and manufacturing of advanced therapy medicinal products (ATMPs).

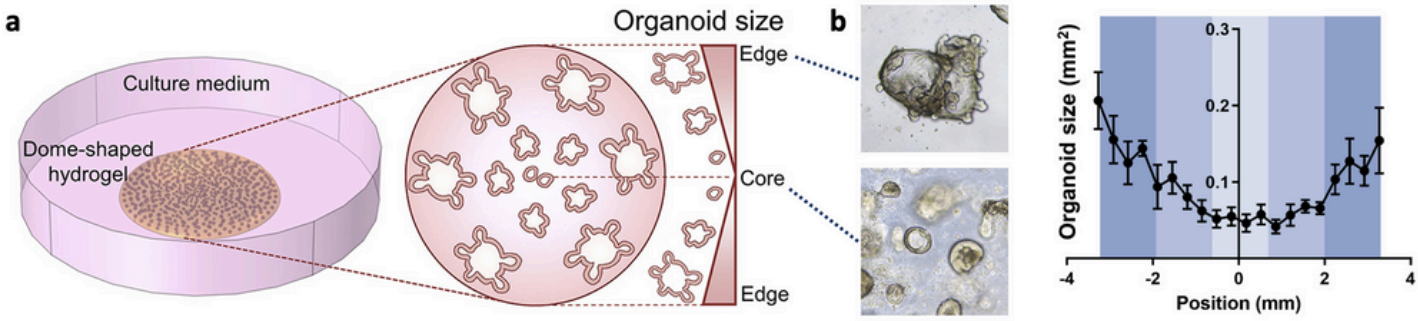
The field mainly distinguishes two types of 3D cell aggregates, namely spheroids and organoids. Spheroids are spherical cellular clusters that can be cultured as free-floating aggregates. They are often regarded as having limited complexity and are primarily used as simple yet effective models for tumor organization. Organoids represent 3D multi-cell units with a level of complexity that emulates the structure and function of an organ. Both spheroids and organoids are being extensively used for research, development, and therapeutic applications, including ATMPs.

Pros and cons of different 3D aggregate culture methods

Formation of 3D cell aggregates requires spatial confinement of cells and sufficient time for the cells to cluster. Various technologies have been developed to support cell aggregate formation, as previously reviewed,[11,12] and which we briefly cover here.

Conventional 3D culture approaches rely on the encapsulation of cells into hydrogels. For instance, **hydrogel** domes randomly seeded with multiple cells or cell clusters can be used, as shown in **Figure 1**.

Figure 1. Hydrogel dome-based organoid culture.[13]



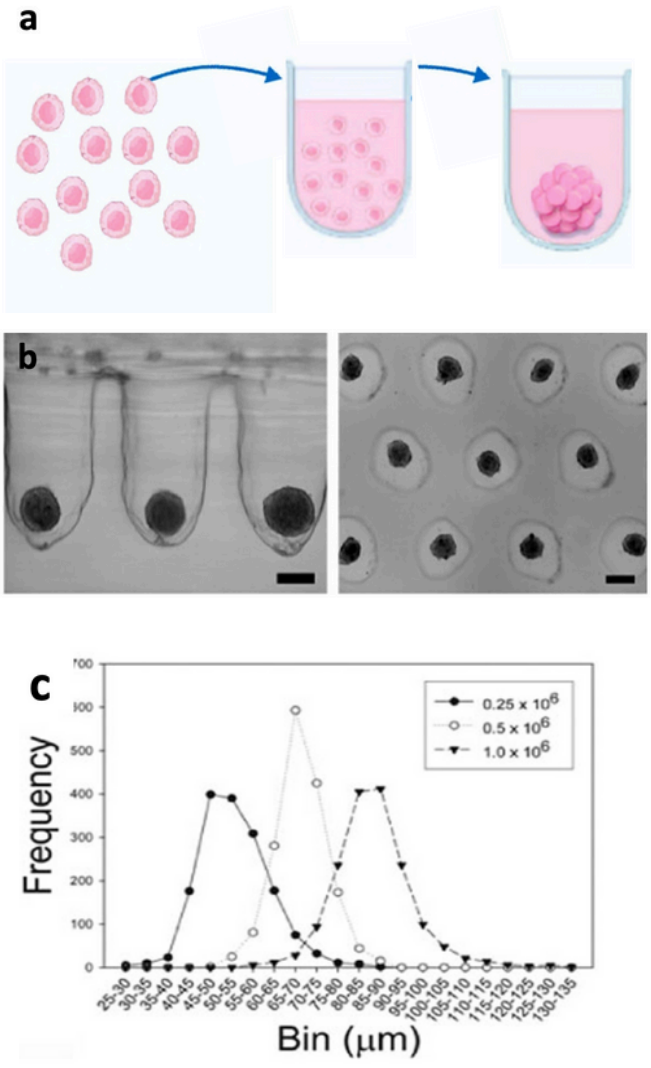
(a) Schematic representation of organoids cultured in hydrogel domes.

(b) The resulting organoids are characterized by a large size variation, dependent on organoid positioning within the hydrogel dome. Organoids in the center of the hydrogel dome are typically smaller compared to ones found at the edge.

Cell-material interactions can be tailored to emulate the native cellular microenvironment and support organoid formation. Besides the common use of chemically undefined materials (e.g., Matrigel®), cell-laden bulk hydrogels typically also lack reproducibility due to the presence of different aggregate sizes and diffusional gradients, as well as challenging optical readouts.[13] Furthermore, hydrogel domes are difficult to scale due to the labor-intensive handling and limitations in tissue culture plastic surface area on which the constructs are typically cultured.

Microwells are associated with easy handling, readout, and consistency but lack scalability. In practice, cells are deposited in non-adherent microwell-plates and allowed to self-assemble (by gravitational force and cell-cell interaction) into aggregates, of which the size and shape are controlled by adjusting the seeding density and microwell dimensions (**Figure 2**).[14] Although this process can produce highly homogenous 3D aggregates and is compatible with industrialized automated imaging systems, the 2D well-plate format limits throughput and requires either intensive manual labor or complex automated handling systems (i.e., robotization) for industrialization.

Figure 2. Microwell-based aggregate culture.[12,14]



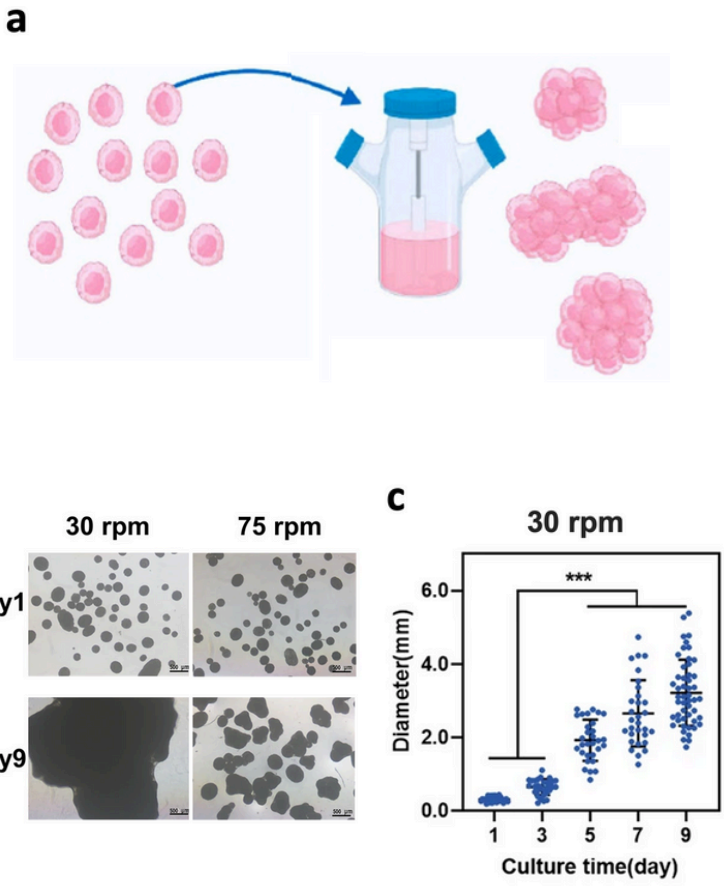
(a) Schematic representation of cells cultured in non-adherent microwells.

(b) Cells are enabled to self-assemble into 3D aggregates due to their adherent nature and the non-adherent properties of the microwells.

(c) Highly controlled aggregate sizes can be obtained due to the provided confinement. Scale bars indicate 200 µm.

Suspension cultures in bioreactors (or spinner flasks) offer the most scalable 3D culture route, but are associated with random merging of cell aggregates as well as external shear forces acting on cells, which causes inconsistent and suboptimal outcomes such as necrotic cores, cell shedding, and uncontrolled stemness or differentiation (**Figure 3**).[15] Moreover, standard dynamic bioreactor cultures require meticulous handling to maintain viability and prevent overgrowth.

Figure 3. Bioreactor-based suspension culture.[12,15]



(a) Schematic of suspension culture[14] of adherent cells in a bioreactor. Culturing adherent cells inside a bioreactor leads to the formation of cellular aggregates.

(b,c) Bioreactor-based aggregate formation leads to a wide variation in aggregate sizes, due to random merging and external shear forces. Stirring speed is one of the main parameters that can be used to control aggregate size, but due to the variation in shear forces also induces functional differences in aggregates.

(d) As an example, Zhang et al. showed significant variations in Nanog expression, a stemness marker, in mesenchymal stem cell aggregates when different stirring speeds were used.

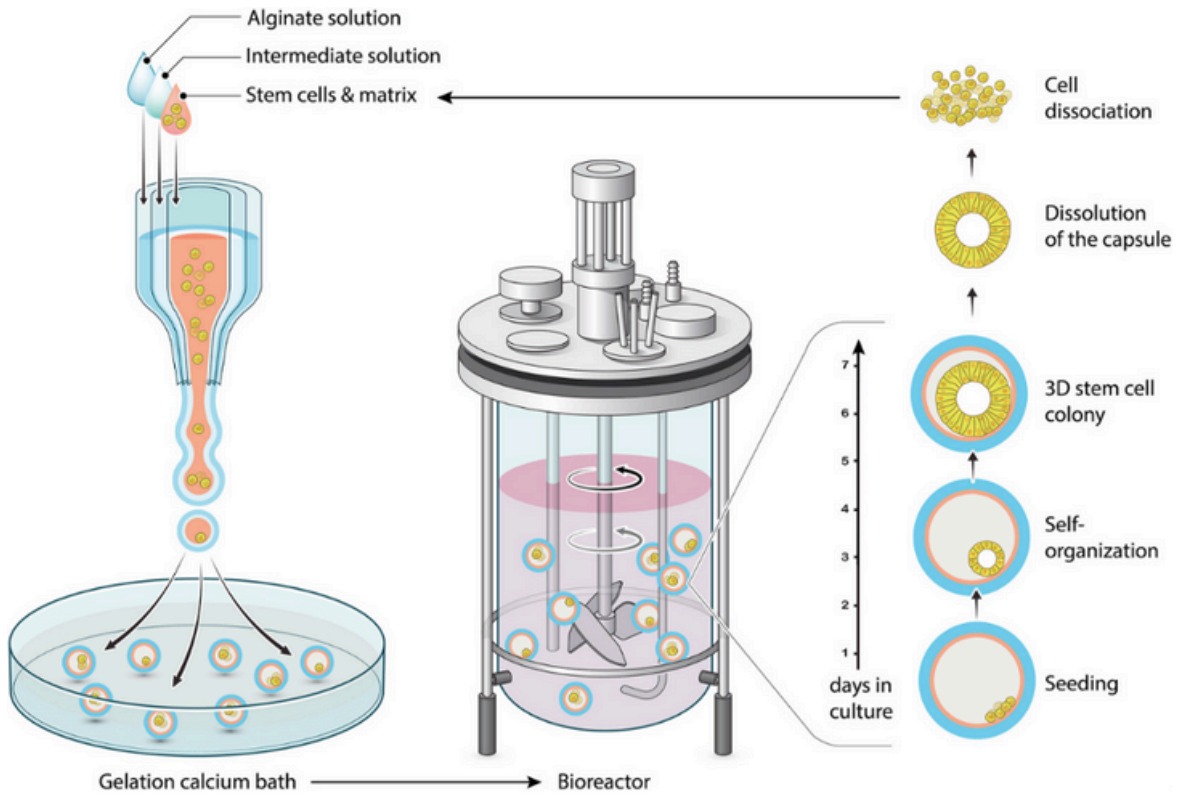
Microencapsulation unlocks scalable 3D cell culture

The ideal solution to produce and maintain 3D cell aggregates at clinically or industrially relevant scales combines the reproducibility of microwells, biocompatibility of hydrogels, and scalability of a bioreactor-based culturing approach. Microencapsulation (or compartmentalization) of cells into capsules that are compatible with conventional bioreactor culture offers a potential solution to this end.[13]

To facilitate cell culture, microgels or microcapsules are made of a **semipermeable polymer membrane** that supports the exchange[16] of nutrients and waste products while providing a shielded and confined microenvironment. This approach enables cell compartmentalization, aggregation, proliferation, differentiation, and even maturation into functional microtissues, such as organoids, while being cultured in suspension.

To facilitate harvesting and multiple consecutive 3D cell cultivation cycles, a temporary or **sacrificial capsule** can be used. For example, capsules comprising a Matrigel® core and calcium-crosslinked alginate shell have been reported for the repetitive encapsulation, 3D expansion, and release of stem cells [17] (**Figure 4**).

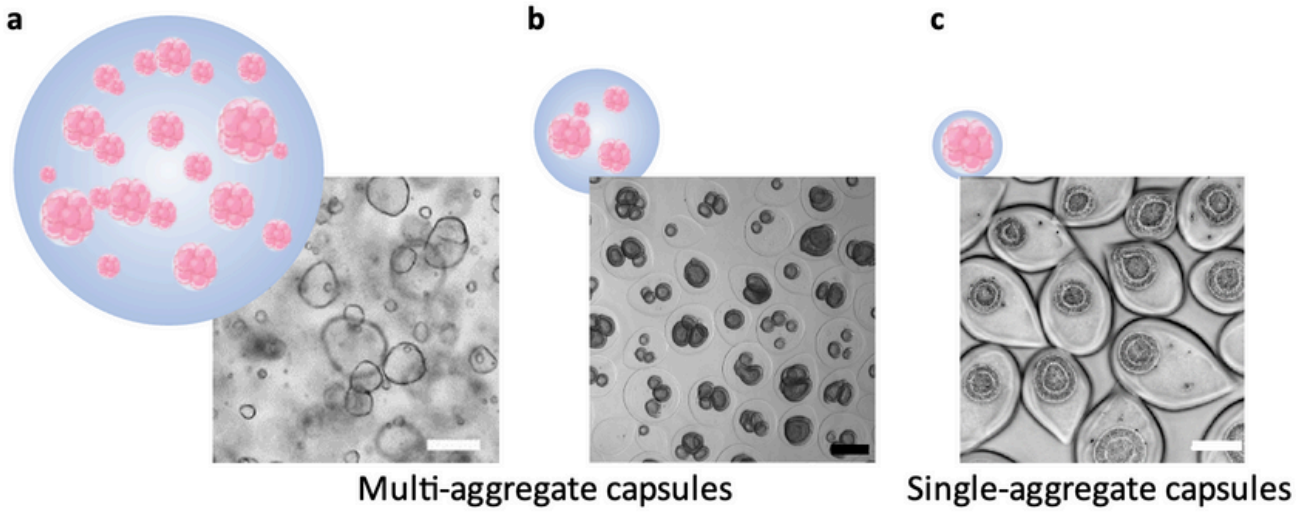
Figure 4. Microcapsule-based suspension culture.[17]



Schematic showing the formation of cell-laden microcapsules containing an intermediate layer of Matrigel® using a multi-axial microfluidic nozzle. After cell encapsulation within a core-shell microcapsule, cells can be cultured inside a bioreactor and form uniform aggregates in a fully shear-force protected manner. When sacrificial materials are chosen, aggregates can be harvested from the capsules at the end of the culture.

One of the primary benefits of microcapsules is the ability to control the **spatial resolution** of 3D cell culture constructs, which is of utmost importance for consistency and reproducibility. Gradients of nutrients, waste products, and cytokines (e.g., growth factors) over the construct should be minimized as they cause heterogeneity in cell viability and function throughout the 3D culture (**Figure 5**).

Figure 5. Multi-aggregate and single-aggregate capsules.[17,18]



(a) Multiple cell aggregates inside a macrocapsule (> mm). Photograph shows a zoomed image of the larger hydrogel dome.

(b) Multiple cell aggregates inside microcapsules (0.5-1 mm).

(c) Single aggregates inside microcapsules (< 500 µm).

(White scale bars indicate 200 µm, black scale bars indicate 500 µm)

Ultimately, microcapsules should be completely uniform in shape and size, and downsized to the single-aggregate level, to ensure maximum control and predictability over the 3D aggregate microenvironment and fate. Such precise, biocompatible, and scalable production of single-cell or single-organoid capsules requires a sophisticated microencapsulation process, but would also combine the advantages of both dynamic bioreactor culture and static microwell cultures.

Table 1. Qualitative comparison of 3D cell aggregate culturing methods.

Data partially sourced from:[11]

Parameter	Dynamic culture in bioreactors[11]	Static culture in microwells[11]	Dynamic encapsulated culture in bioreactors
Shear stress	Medium – high	Low	Low
Mass transfer	High	Low	Medium - high
Aggregate diameter control	Medium	High	High
Aggregate homogeneity	Low	High	High
Aggregate formation time	Low – medium	Low – medium	Low
Aggregate yield	High	Medium	High
Equipment costs	Medium – high	Medium – high	High
Culture medium expenditure per aggregate	High	Low	Medium – high
Cell manipulation	Low	Medium – high	Medium
Process reproducibility	Medium	High	High
Up-scaling potential	High	Medium	High
Ease of final aggregate harvesting	High	Low – medium	Medium

Microencapsulation processes

The encapsulation of cells into microcapsules typically comprises two essential steps. First, a liquid solution containing cells is dispersed into individual droplets. Secondly, the droplets are solidified through crosslinking or precipitation. Various methods can be used to form a dispersion containing the so-called cell-laden precursor droplets, including patterning, molding on or within solid substrates, emulsification in an immiscible liquid, and atomization in a gas.[12]

Biocompatibility

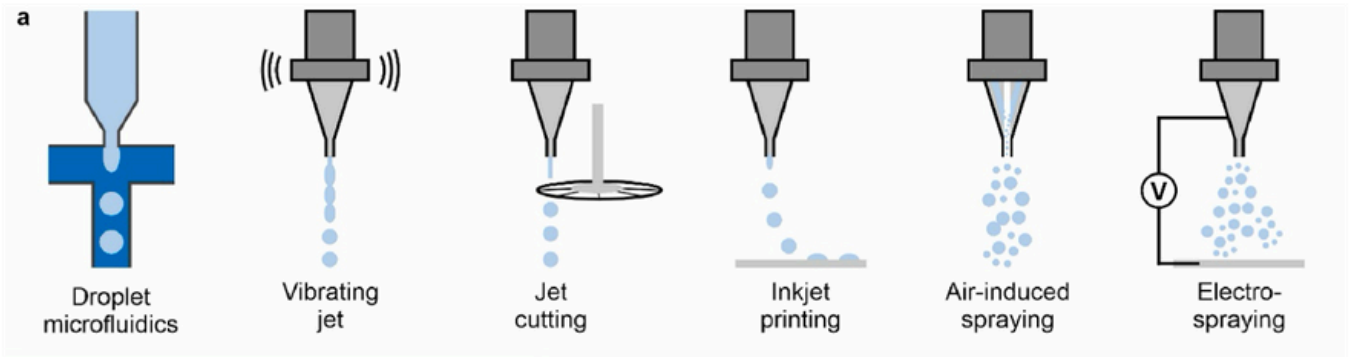
From a **chemical biocompatibility** perspective, relatively mild process solutions are preferred since these are in direct contact with the cells and have the inherent risk of eventually being transferred with the ATMP into the patient. Although highly flexible and accurate (i.e., high resolution), conventional droplet microfluidics relies on a non-solidifying co-flow to drive droplet pinch-off and prevent system clogging. The co-flow liquid typically comprises an immiscible oil/surfactant phase that poses a risk to the cells as well as the patient, for example, due to hydrocarbon oil's alleged effects on adjuvanticity and autoimmunity.[19] Any emulsion-based cell encapsulation process would thus require extensive purification as well as stringent quality control before therapeutic application. Notably, fluorocarbon (i.e., PFAS, also known as 'forever chemicals') oils are also not uncommon for these applications,[20] which may pose an unknown long-term risk for the cells, operators, end users, and environment.

Effectively dispersing liquids into (cell-laden) precursor droplets in a gas phase requires sufficient energy to overcome liquid surface tension. If too much energy is added or not well-focused on the droplet pinch-off, this may result in lethal shear forces on the cells and thus affect **mechanical biocompatibility**. For example, jet cutting enables effective dispersion even of high-viscosity liquids by smashing a wire through a continuous jet. However, this process also causes severe deformation of the droplets and potential disruption of the cells, as well as some product loss due to liquid sticking to the rotating cutting wire.

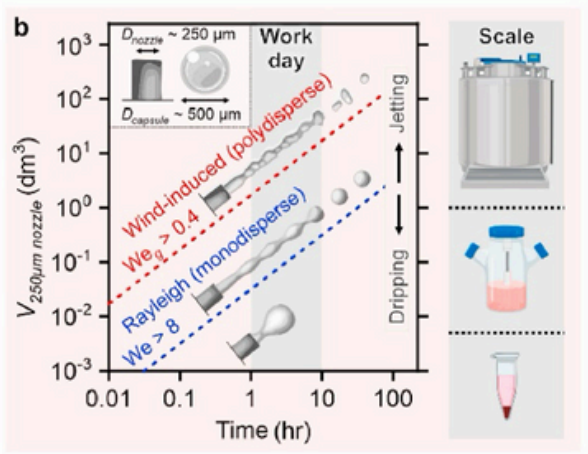
A dripping or drop-on-demand process, as used in inkjet-based droplet generation, exerts less shear stress on cells during droplet pinch-off but is limited in terms of throughput, flexibility of the ink composition (due to viscosity limitations), and droplet size (**Figure 6**). Notably, traditional printing onto a solid substrate may also affect cell survival due to droplet deformations upon impact on the substrate.[21] Continuous printing strategies such as vibrating jet offer a relatively fast continuous droplet production route, although this method is less efficient when high-resolution spatiotemporal deposition (e.g., single-cell printing) is required.

Other common droplet generation methods are based on introducing a gas co-flow (e.g., air-induced dripping or spraying) or an electric field (e.g., electro-spraying). Although effective and scalable, these methods typically result in relatively polydisperse products. Employing electric potential to pull droplets from a so-called Taylor cone also creates the risk of reduced cell viability and even abrupt electrical discharge (i.e., sparks).[22,23]

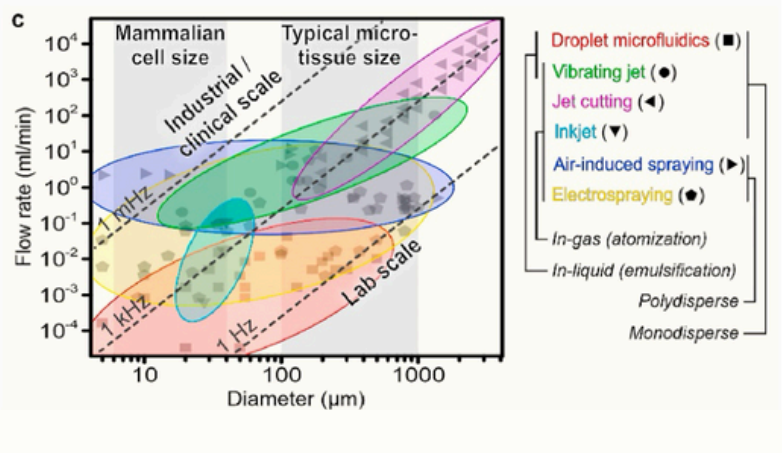
Figure 6. Comparison of microfluidic droplet generation methods.[12]



. **(a)** Schematic representation of various continuous droplet generation methods.



(b) Production volume per time unit for different flow regimes based on Weber number. Reactor sizes are indicated on the right side to illustrate the amount of droplets required to fill reactors.



(c) Per-nozzle flowrates of a variety of microfluidic droplet generation methods.

Resolution and throughput

Droplet production and stabilization processes play an essential role in final product quality and batch-to-batch variation. Particularly uniformity in size, shape, and composition of the microcapsule has an effect on the viability, function, and fate of encapsulated cells (**Figure 5**). Important factors to this end include (i) the minimum droplet size, which dictates the microcapsule resolution; (ii) the variation in droplet size, which largely prescribes the microcapsule size distribution; and (iii) the droplet solidification process, which plays an important role in the stability and shape of the microcapsule.

Droplet solidification is in principle uncoupled from the dispersion process and can be based on, among others, freezing, coacervation, evaporation, or crosslinking induced by, for example, radiation, temperature, ions, radicals, and/or enzymes. The introduction or initiation of the solidification mechanism can have a significant effect on the capsule quality or cell survival. For example, photo-induced radical crosslinking may cause cytotoxicity,[24] whereas ionic crosslinking by impacting droplets into a crosslinker bath can cause significant capsule deformation and capsule merging.[17,25]

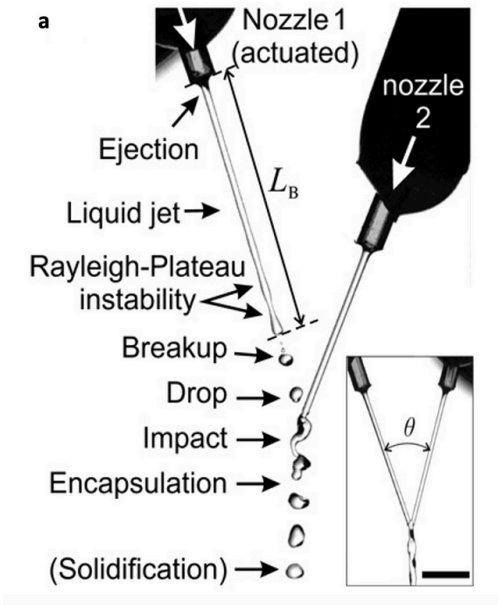
From a **scalability** perspective, a continuous process is preferred. Among the wide variety of continuous microencapsulation processes available (**Figure 6**), continuous jet-based approaches offer the highest throughput, while droplet microfluidics provides the highest level of flexibility via in-line control over processed liquids and cells, but typically at the cost of throughput and production scale.[12]

Overall, conventional microencapsulation methods seem to have a trade-off in biocompatibility, resolution, and scalability. We hypothesize that a combination of multiple continuous process technologies is necessary to enable rapid and scalable production of microtissues with high resolution, biocompatibility, and uniformity.

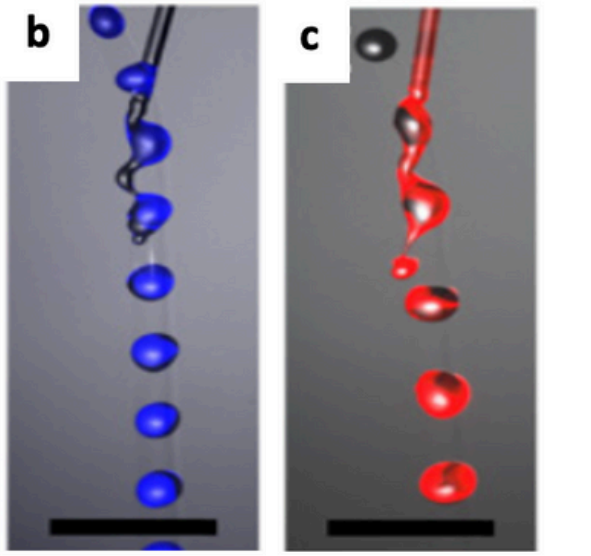
IN-AIR MICROFLUIDICS™

IN-AIR MICROFLUIDICS™ is a microencapsulation technology that combines multiple liquid jets in mid-air (**Figure 7**).[25] The technology offers the biocompatibility, uniformity, and throughput of vibrating jet technology, combined with the in-line control and flexibility (e.g., in microparticle shape and composition) of a microfluidic process.

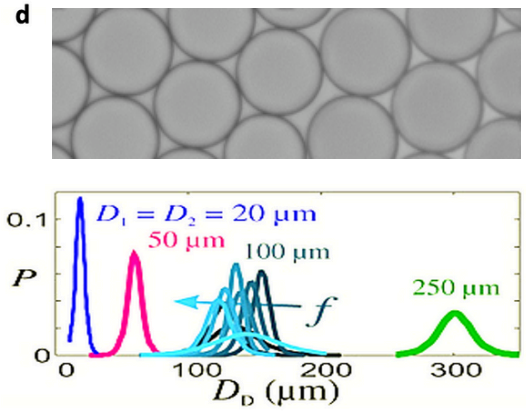
Figure 7. IN-AIR MICROFLUIDICS™ enables high-throughput monodisperse microdroplet production.[25]



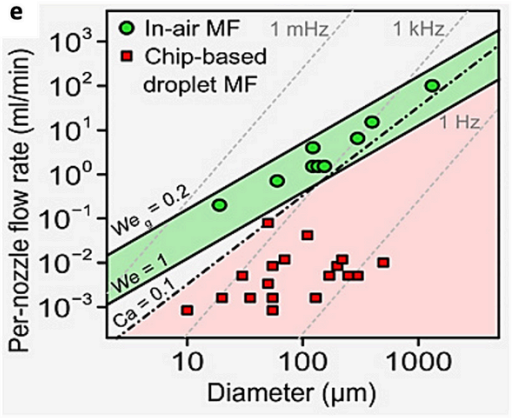
(a) Photograph of the basic operating mode of in-air microfluidics where a monodisperse droplet train is impacted on an uninterrupted liquid jet.



(b-c) Zoomed photographs show the droplet train highlighted in blue and the uninterrupted jet highlighted in red.



(d) Monodisperse microparticles of a wide range of sizes can be produced with narrow size distributions.



(e) Unparalleled per-nozzle flow rates as compared to conventional chip-based droplet microfluidics. Scalebars indicate 1 mm.

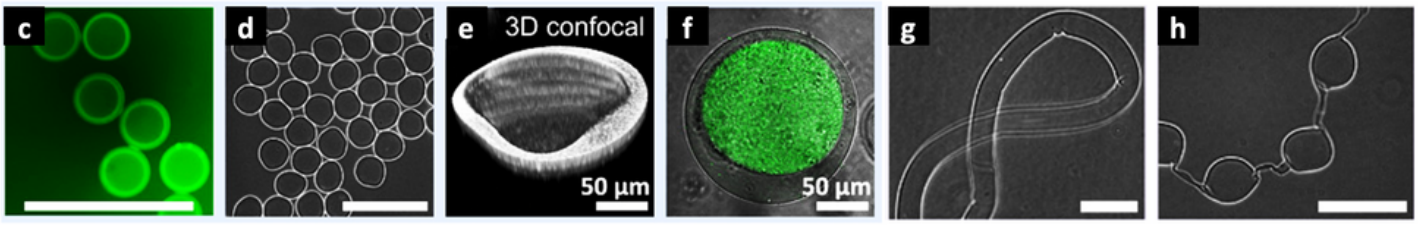
Scalable, reproducible, and flexible

As a liquid jet-based technology, IN-AIR MICROFLUIDICS™ is up to 1000x faster (i.e., per nozzle) than conventional chip-based microfluidics, readily enabling kg-scale production of monodisperse microcapsules (**Figure 7**). Furthermore, nozzle parallelization empowers production capacities of multiple tons per year, indeed facilitating industrial and clinical translation (**Figure 8**). IN-AIR MICROFLUIDICS™ enables the production of a wide variety of micromaterial designs, including matrix-type particles, core-shell capsules, and fibers with excellent control over size, shape, and composition (**Figure 7**).

Figure 8. IN-AIR MICROFLUIDICS™ allows for controlled and scalable production of a wide variety of microparticle designs.[25]

(a) Industrial-scale IN-AIR-MICROFLUIDICS™ machine to produce multiple tons of microparticles per year.

(b) Lab-scale IN-AIR-MICROFLUIDICS™ machine for cleanroom-grade production up to kilograms per day.

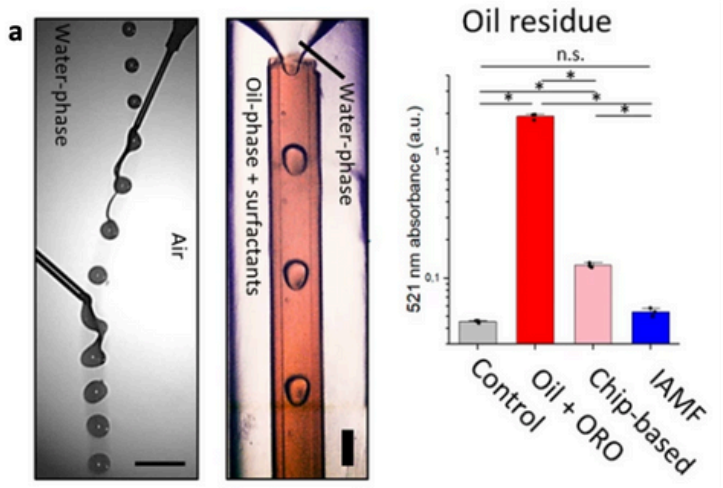


(c-h) varying microparticle compositions that can be readily produced with the IN-AIR-MICROFLUIDICS™ technology. Scale bars indicate 200 µm, unless stated otherwise.

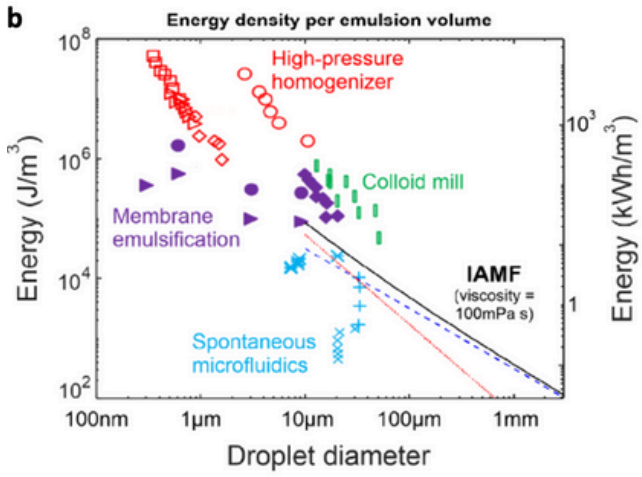
Clean and gentle

In contrast to chip-based microfluidics, the IN-AIR MICROFLUIDICS™ process can produce capsules without using oils and emulsifiers, making it inherently clean and biocompatible (**Figure 9**). Furthermore, on-the-fly solidification of droplets into shape-stable capsules prevents the merging of capsules and their contents upon collection without the necessity for a high-voltage electrical field, which increases safety for the encapsulated live products and operator. Moreover, as opposed to conventional dripping or jetting in a bath, the impact angle of jets can be easily tuned to allow for gentle liquid impact, which prevents capsule deformation and ensures high sphericity and well-centered cores. The gentle nature of the IN-AIR MICROFLUIDICS™ process further roots in the low energy density used for droplet pinch-off. Consequently, for the dispersion process, almost no excessive energy is used that can translate into lethal heat and or shear stresses (**Figure 9**).

Figure 9. IN-AIR MICROFLUIDICS™ enables clean and gentle microencapsulation.[26,27]



(a) As opposed to chip-based microfluidics, IN-AIR-MICROFLUIDICS™ does not require oils or emulsifiers for capsule formation. Scale bars indicate 200 µm.



(b) Energy density per emulsion volume of IN-AIR MICROFLUIDICS™ compared to other droplet generation methods.

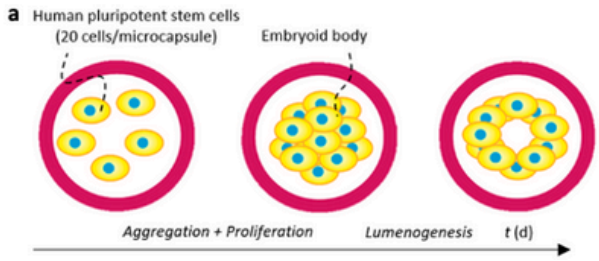
Showcase:

Scalable, reproducible, and xeno-free 3D pluripotent stem cell culture

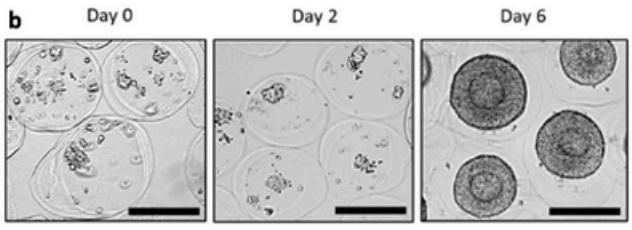
Existing organoid production methods often face limitations in throughput (i.e., gram scale), size control (i.e., polydisperse), and composition (e.g., xenogeneic Matrigel®),[28] which hinder their translation to clinical and industrial applications.

Van Loo et al. recently demonstrated high-throughput production (i.e., thousands per second)[26] and long-term culture of live 3D embryoid bodies enabled by IN-AIR MICROFLUIDICS™. Specifically, triple-jet in-air microfluidics enabled the generation of monodisperse and xenogeneic-free (i.e., without Matrigel®) semi-permeable alginate-based hydrogel microcapsules, serving as microbioreactors for the controlled proliferation, aggregation, and organization of pluripotent stem cell aggregates (**Figure 10**). The process resulted in nearly 100% efficient formation of individually encapsulated spherical 3D embryoid bodies that developed a single lumen surrounded with radially oriented cells positive for multiple primed pluripotency markers. The fate of microencapsulated 3D cell cultures thereby showed remarkable resemblance to natural early embryonic development.[29]

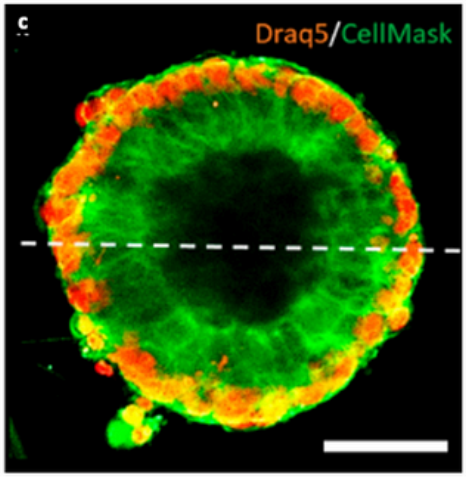
Figure 10. Microencapsulated pluripotent stem cells undergo autonomous aggregation and cavitation.[29]



(a) Schematic representation of pluripotent stem cell encapsulation, aggregation and lumenogenesis.

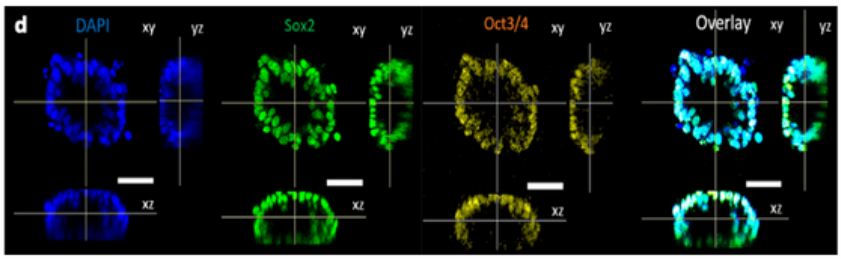


(b) Microscopic photographs of encapsulated pluripotent stem cells during each step of the process.

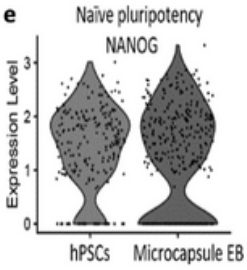


(c) Fluorescent microscopic photograph of an embryoid body with stained nuclei (Draq5) and cell membranes (CellMask). Cells have self-organized into a radially oriented structure.

Figure 10. Microencapsulated pluripotent stem cells undergo autonomous aggregation and cavitation.[29]



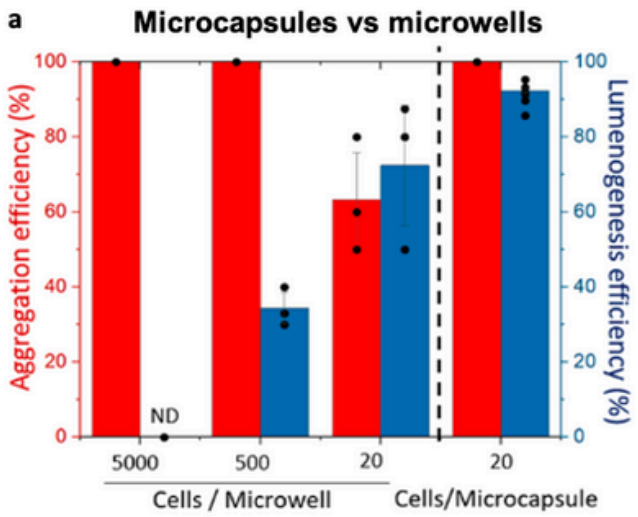
(d) Confocal slices of an embryoid body with fluorescently labelled nuclei (DAPI) and pluripotency markers (Sox2 and Oct3/4).



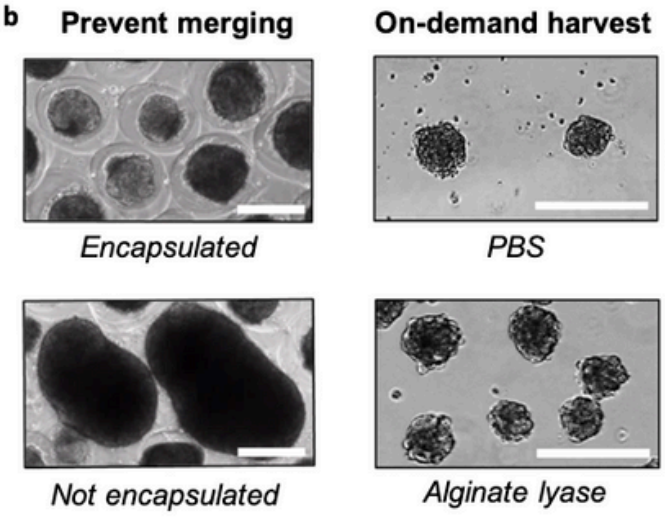
(e) Quantification of the expression levels of the naïve pluripotency marker Nanog, which in microencapsulated embryoid bodies was near identical to microwell cultured human pluripotent stem cells aggregates. Black scale bars indicate 100 µm, white scale bars indicate 50 µm.

Importantly, capsules prevented overgrowth and merging of cellular aggregates during suspension culture, while enabling their on-demand retrieval through cytocompatible capsule dissolution using phosphate-buffered saline or alginate lyase (**Figure 11**). Moreover, a direct comparison with a microwell-plate culture format revealed that the microcapsules enabled more efficient embryonic cell aggregation with smaller cell numbers, as well as more efficient (single) lumen formation, plausibly by offering a more spatially confined niche.

Figure 11. Microencapsulation of cells allows for efficient microtissue formation, culture, and retrieval.[26]



(a) Aggregate formation in microcapsules is more efficient than in microwells, requiring a smaller number of embryonal stem cells and facilitating a more efficient lumen formation.



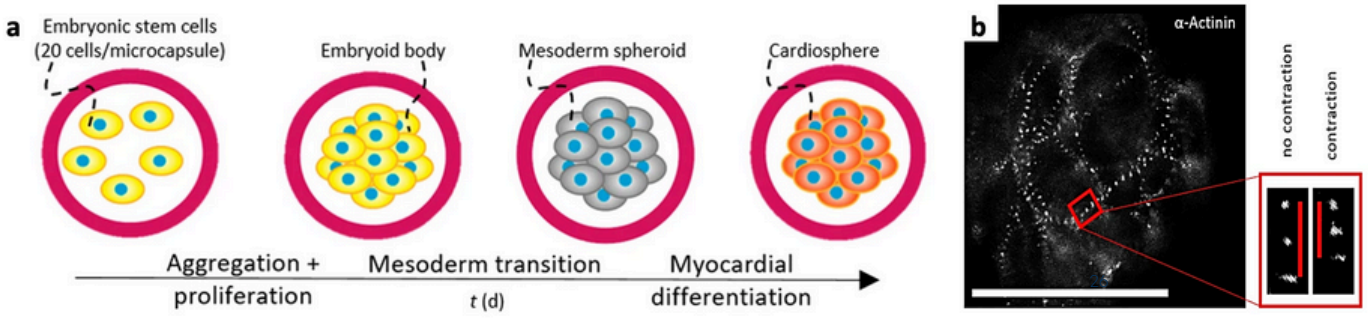
(b) Microencapsulation in alginate capsules enables the formation of cellular aggregates without the risk of merging. Aggregates can be easily harvested using PBS or alginate lyase. Scale bars indicate 200 µm.

Showcase:

Functional spheroid production

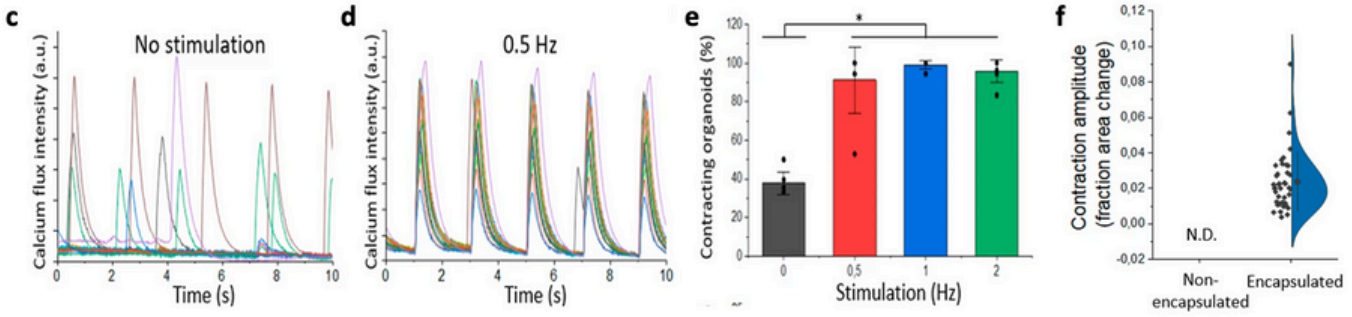
Microencapsulated embryoid bodies (see showcase above) could be readily differentiated into functional (i.e., beating) cardiac spheroids (**Figure 12**). The cardiospheres comprised well-organized contractive sarcomeres and were highly responsive to external electric stimulation. Microencapsulated cardiospheres based on pluripotent stem cells expressing a fluorescent α-Actinin fusion protein readily enabled the real-time visualization of cells' sarcomere Z-lines and thereby contraction quantification. Interestingly, such contraction quantification was not feasible using non-encapsulated cardiospheres that were grown atop tissue culture plastic, plausibly due to their partial adherence to each other and the culture substrate and thereby contraction limitation.

Figure 12. Encapsulated embryonic stem cells can differentiate into functional cardiospheres.[26]



(a) Schematic representation of the process from encapsulating embryonal stem cells to myocardial differentiation to form cardiospheres.

(b) α-Actinin staining in cardiospheres (including contracted and non-contracted state displayed in the inset) after 18 days of culture.



(c,d) Calcium flux intensity measurements of spontaneous (c) and electrically stimulated (d) contracting cardiospheres.

(e) Relative amount of contracting cardiospheres with or without electrical stimulation.

(f) Contraction amplitude quantified as fraction area change in non-encapsulated and encapsulated cardiospheres. Scale bar indicates 100 µm.

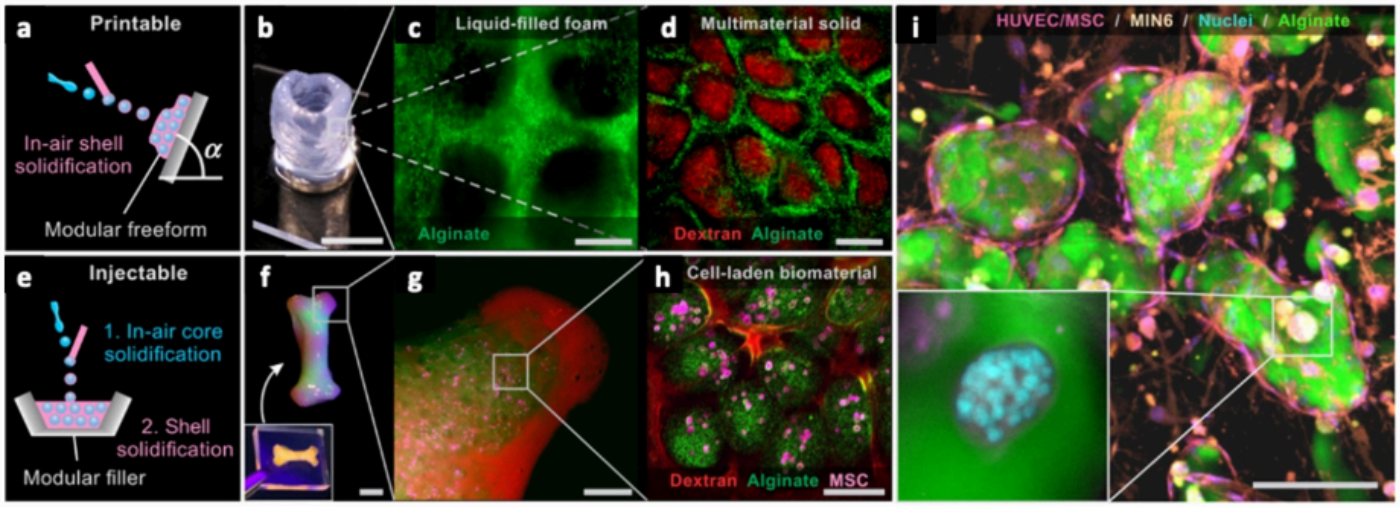
Showcase:

Direct 3D printing of pancreas-like tissue

Creating large living constructs with intricate complexity that resembles the structure and function of our native tissues is among the key goals of tissue engineering. However, manufacturing methods often lack the resolution, biocompatibility, or the throughput to achieve that goal.

Visser et al demonstrated that IN-AIR MICROFLUIDICS™ can be used for the direct 3D printing of modular freeform constructs with an internal multi-material cellular structure (**Figure 13**).[25] Alternatively, the same technology could be used for injection molding of such modular constructs containing mesenchymal stem cell (MSC)-laden microniches of alginate within a continuous dextran hydrogel network. Finally, a multi-scale modular material was printed that contained insulin-producing MIN6 cell aggregates embedded within a fibrin gel laden with a co-culture of human umbilical vein endothelial cells (HUVECs) and MSCs forming an interstitial angiogenic network. The engineered tissue construct thereby partially mimicked the basic structure and function of native pancreatic tissue.

Figure 13. Direct 3D printing using IN-AIR MICROFLUIDICS™ [25]



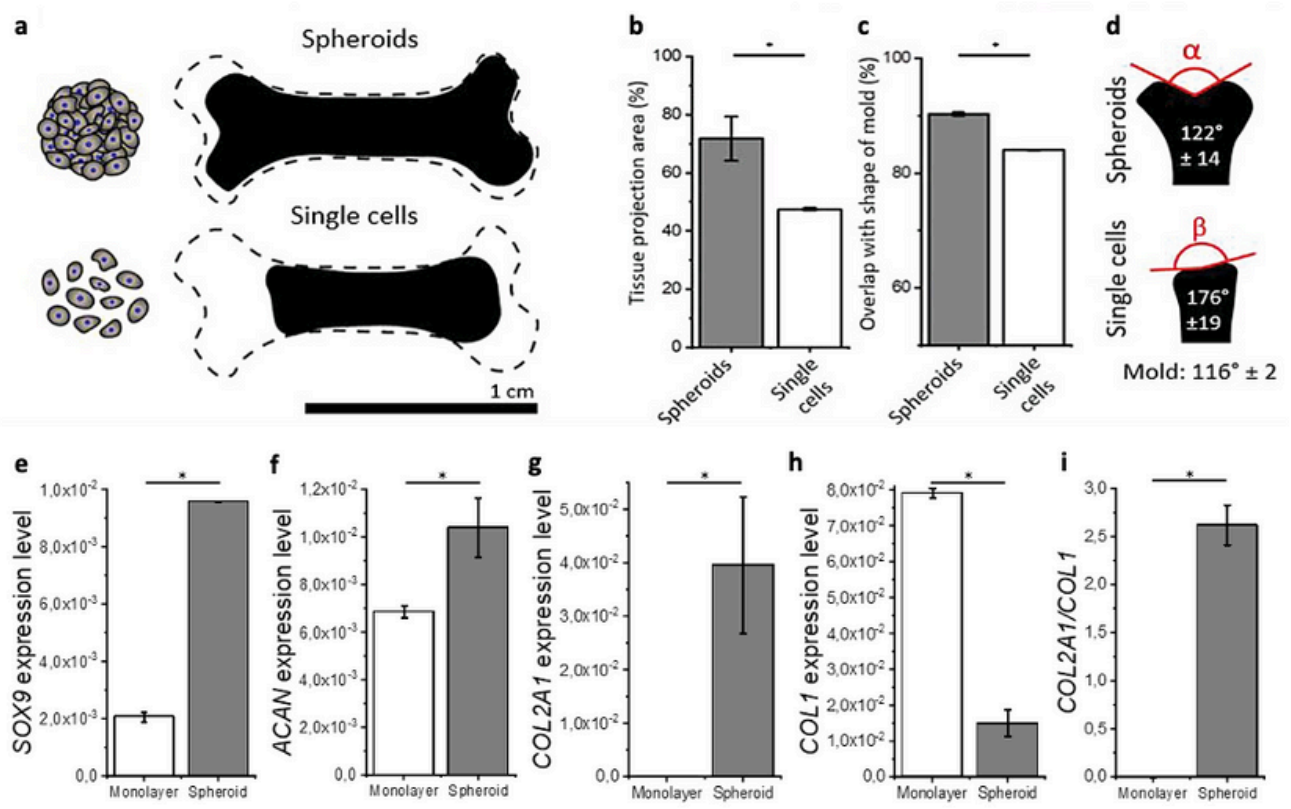
(a) IN-AIR MICROFLUIDICS™ can be utilized to directly deposit compartmentalized hydrogels onto a surface and form **(b,c)** liquid-filled foams or **(d)** multi-material solids. **(e)** Multi-scale modular biomaterials can also be deposited into a mold to create **(f,g)** shaped hydrogels, which, if used in conjunction with cell-encapsulation, can form a **(h)** cell-laden material with intricate cellular complexity. **(i)** Modular tissue-engineered construct consisting of alginate microgels generated with IN-AIR MICROFLUIDICS™ containing MIN6 cells positioned within a fibrin-based hydrogel matrix laden with MSCs and HUVECs forming a cellular network. Scale bars indicate 1 cm (b,f), 5 mm (g), and 100 µm (c,d,h,i).

Showcase:

Single-step biofabrication of cartilage-mimicking tissue

In line with the previous showcase, van Loo et al utilized IN-AIR MICROFLUIDICS™ for the direct generation of an engineered tissue construct comprising chondrogenic cell spheroids.[30] Specifically, the in-air process was utilized to encapsulate human primary chondrocytes into a compartmentalized hydrogel with an open (cellular) structure. Subsequent to 3D cell aggregation, cartilaginous spheroids could be harvested from the compartmentalized hydrogels using alginate lyase, readily enabling the construction of shape-stable, centimeter-sized biomaterial-free living tissues through spheroid injection molding (**Figure 14**). The study furthermore revealed that spheroids cultured within these compartmentalized hydrogels surpassed 2D monolayer cell cultures, particularly in terms of chondrogenic behavior (i.e., higher SOX9, ACAN, COL2A1, and lower COL1).

Figure 14. Engineering shape-stable chondrogenic tissue constructs.[30]



(a) Spheroids harvested from compartmentalized hydrogels show better shape stability when injected into a mold as compared to single cells. **(b-d)** Quantification of the shape stability of spheroids harvested from compartmentalized hydrogels compared to single cells after injection into a mold. **(e-i)** Expression levels of chondrogenic marker genes SOX9, ACAN, and COL2A1, and of the fibrocartilage marker COL1, as well as the ratio between COL2A1 and COL1 levels as an indication of cartilage phenotype.

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