TR&D2: Scalable 3D microbioreactors for large-scale cell production

Goal: Current cell manufacturing infrastructure mainly depends on the use of conventional stirred tank bioreactors (STRs) for centralized cell production. This process of cell production is inherently associated with low cell densities, slow growth rates, and high cell damage or death during the production process. To satisfy the demand for producing a large number of cells, bioreactors are typically expanded in volume. However, simply increasing volume significantly increases cost and in turn brings scalability challenges due to inefficient production scales. Instead of merely scaling up bioreactor volume or quantity, we prioritize the development of a miniaturized cell culture system. A microbioreactor will be designed to mimic the three-dimensional stress-free tissue-like microenvironment found in vivo, overcoming the inefficiencies of traditional large-volume bioreactors. In a laboratory setting, we have already demonstrated groundbreaking efficiency, achieving 250 times increase in cell density compared to STRs, 3,000-fold cell expansion per passage, and reducing production costs by 90%. This significant leap in efficiency highlights the scalability and affordability of our microbioreactor, making it a potential game-changer for cell manufacturing. We will advance this microbioreactor design suitable for large-scale cell production and test the capability of new microbioreactors in producing hiPSC-derived beta cells.

Published and Preliminary Data:

Limitations of Current Cell Culture Technologies. Currently, the industry is trying to scale up cell production using 3D bioreactors such as stirred tank bioreactors (**STRs**). However, these bioreactors have un-addressable problems, including excessive cell aggregation, substantial shear stress-induced cell death, unacceptable batch-to-batch and cell line-to-cell line variations, and difficult scaling up. Consequently, STRs have low cell yield – for instance, human pluripotent stem cells (hPSCs) typically expand<10 fold/passage to yield ~2x10^6 cells/mL. The cell mass occupies ~0.4% of the culture volume. The largest scale demonstrated is ~1x10^{10} hPSCs/batch. In recent studies to make hPSCs-derived cardiomyocytes (hPSC-CMs) in small STRs (100 mL), the yield varied from 40 million to 125 million cells, and the product purity varied from 28% to 88% in 6 batches.

Hydrogel Tube Microbioreactors. The root cause is that STRs provide cells with a highly stressed microenvironment. We hypothesize that i) a cell-friendly microenvironment can substantially improve cell culture efficiency, and ii) such a microenvironment can be created with hydrogel microtubes (Fig 1a). First, hydrogel tubes can protect cells from negative shear stress. Second, the hydrogel has abundant nanopores that allow nutrients and growth factors in the medium to pass through the tube walls. Third, the tube diameter can be controlled under the diffusion limit of human tissue (~400 μm), ensuring efficient mass transport throughout the culture. Finally, tubes provide free spaces for cells to interact and grow, which is critical to achieving high viability, growth rate, and yield.

To test our hypothesis, we developed technologies to fabricate hydrogel microtubes using alginates (AlgTubes) (Fig 1). We demonstrated that hPSCs could be cultured in AlgTubes with high viability (>95%), achieving up to 3,000-fold expansion per passage and a yield of 5x10^8 cells/mL - 250 times higher than standard STRs. All cells retained pluripotency and normal karyotypes after 10 passages in AlgTubes.

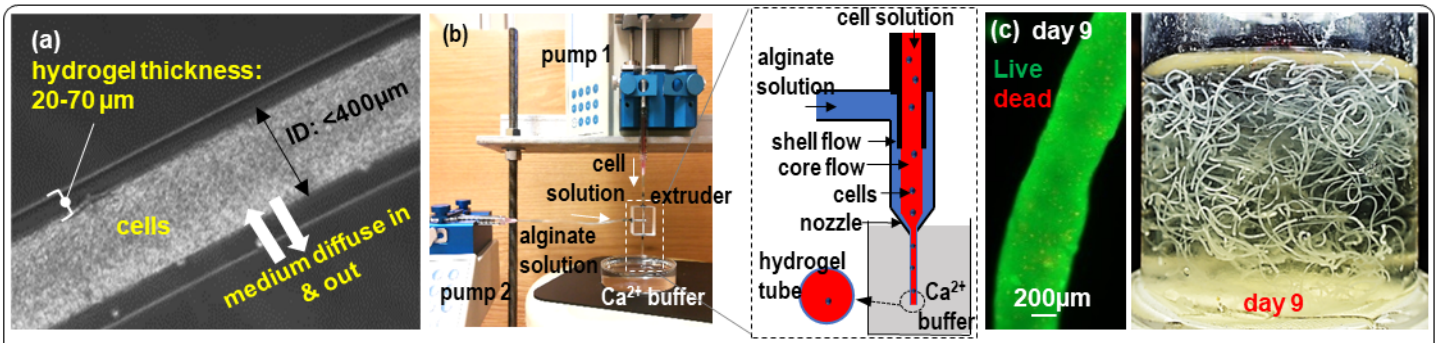

Fig 1. **(a)** Design principles. Inner diameter is ≤400μm and hydrogel wall thickness is between 20 to 70μm. Nutrients can freely diffuse through the hydrogel shell. **(b)** Process AlgTubes. A cell solution and an alginate solution is pumped into the central channel and side channel of a custom-made micro-extruder, respectively, to form coaxial core-shell flows that are extruded into a $CaCl_2$ buffer. The shell alginate flow is instantly crosslinked by Ca^{2+} to generate a hydrogel tube. Subsequently, the $CaCl_2$ buffer is replaced by a cell culture medium and cells are grown in the tubes. **(c)** Single cells expand quickly to fill the tube with minimal cell death. 1x10^9 cells (white cell mass) can be produced with merely 2 mL hydrogel tubes contained in a small bioreactor.

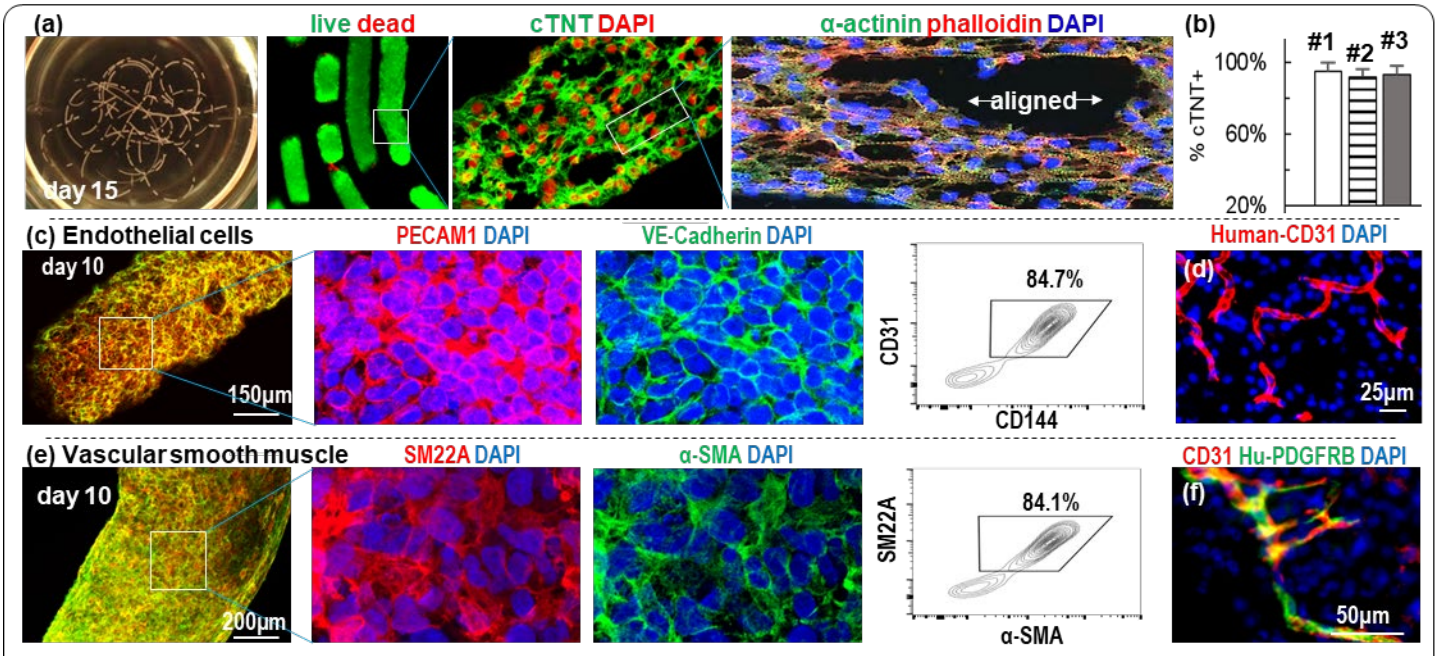

Fig 2. Human iPSCs were differentiated into cTnT+ CMs **(a, b)**, PECAM1+/VE-Cadherin+ ECs **(c, d)** and SM22A+/α-SMA+ VSMCs **(e, f)** in AlgTubes with high purity and viability. The differentiation was consistent between 3 iPSC lines (b). ECs and VSMCs formed functional blood vessels in mice (d, f).

Moreover, hPSCs could be successfully differentiated into functional cell types (Fig 2), such as cardiomyocytes, endothelial cells, vascular smooth muscle cells, and neural stem cells in AlgTubes. The resulting cells exhibited gene expression and phenotype comparable to those produced using current methods. These findings support our hypothesis and underscore the transformative potential of hydrogel tube microreactors.

Given the diverse phenotypes of mammalian cells, it is crucial to have multiple material options for fabricating hydrogel tube microbioreactors. We also developed hydrogel tubes made with collagen proteins. No existing technologies can quickly process collagen proteins into stable microtubes. We therefore developed a novel instrument including a micro-extruder and a cooling box to address the problem (**Fig 3**). ColTubes are formed by combining rapid pH and temperature rise (**Fig 3e**). We also designed micro-extruders that can process multiple ColTubes in parallel for large-scale cell production (**Fig 3f**). We found that ColTubes could be

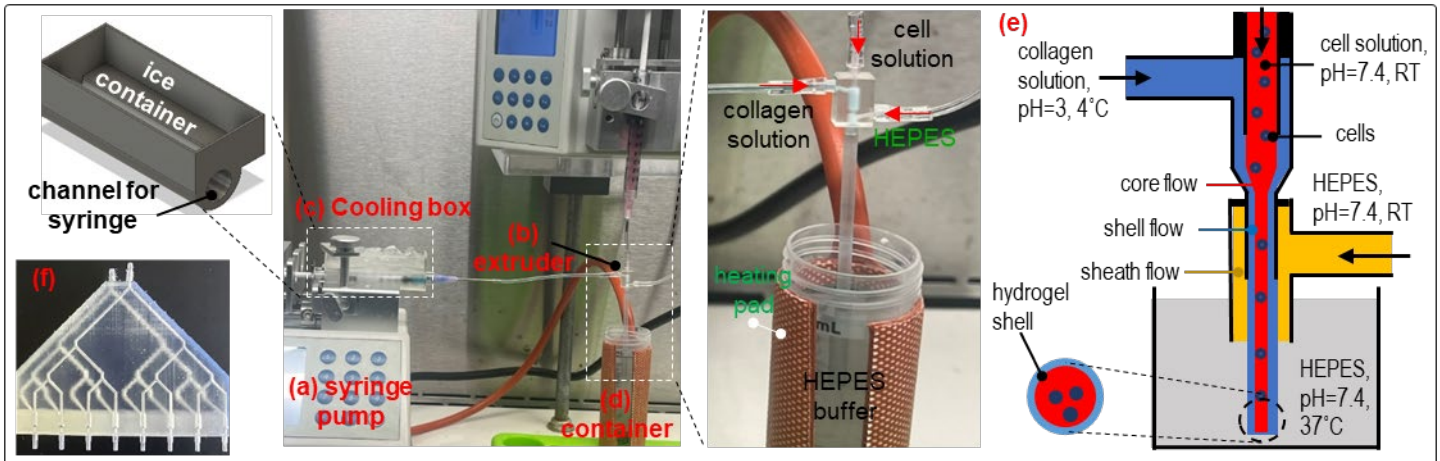

Fig 3. The ColTube processing setup consists of 3 syringe pumps with syringes **(a)**, a micro-extruder **(b)**, a cooling box **(c)**, and a conical tube or container (with a heating pad) containing HEPES buffer **(d)**. The 3 syringes contain a cell solution at room temperature (RT, pH = 7.4), an ice-cold collagen solution (pH = 3.0), and a HEPES buffer (RT, pH = 7.4), respectively. The cooling box has a channel for holding syringe 1. Ice is loaded into the box to maintain the collagen solution in syringe 1 at a temperature below 4°C. The 3 solutions are pumped into the micro-extruder to form coaxial core-shell-sheath flows that are extruded into the heated HEPES buffer. The collagen solution is neutralized by the HEPES buffer in both the sheath flow and the conical tube. Additionally, the collagen solution is rapidly heated by the HEPES in the conical tube. The shell collagen flow rapidly forms a hydrogel tube as a result of the simultaneous pH neutralization and temperature increase **(e)**. The extruder can be multiplexed for making multiple ColTubes in parallel **(f)**.

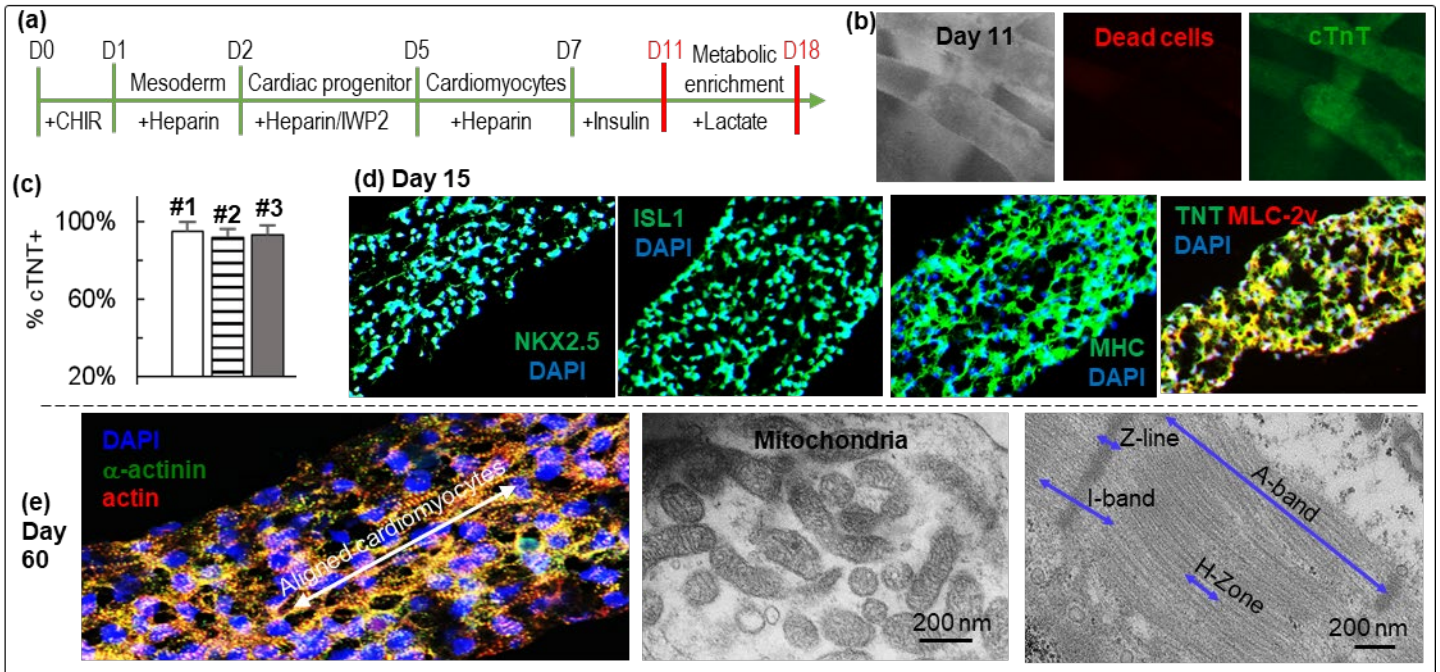

Fig 4. (a) hPSC-CM differentiation protocol. **(b)** Most day 11 cells were live and cTnT+. **(c)** >98% purity was achieved for 3 hPSC lines. **(d)** hPSC-CMs expressed common CM markers. **(e)** Ultrastructure of hPSC-CMs after 60 days in ColTubes. Cells were aligned, had abundant mitochondria, A-band, H-zone, Z-line and I-bands.

fabricated using collagen proteins derived from various species and tissues, such as collagen isolated from rat tails, bovine skin, and human placenta. This extrusion system is the first and only scalable technology that can rapidly process ColTubes without compromising cell viability.

These collagen hydrogel tubes exhibit high cell culture efficiency comparable to alginate hydrogel microtubes, while offering enhanced robustness and adhesion (Fig 4). We believe collagen and alginate hydrogel tube microbioreactors, together, will enable scalable, cost-effective, and efficient cell production for a broad range of applications.

Substantial Improvement, New Capabilities, and Transformative Potential of Hydrogel Tube Microbioreactors.

Preliminary data shows that our microbioreactors can remove all the problems associated with STRs and offer substantial improvements, as summarized in Table 1. ColTubes would enable the following new capabilities:

Enable industrial-scale cell production: The high cell density and expansion fold/passage have significant impacts on large-scale cell production. For instance, to produce 1012 hPSCs from 1x107 seeds, our modeling shows it requires 1,365 liters total culture volume, 51 days, and 9 passaging operations using current STRs (Table 1b)55. A production that is technically and economically challenging. The same production can be done with 4 liters of ColTubes, 20 days, and 1 passaging55. The production cost is estimated to be 10% of the cost of using STRs55. It is achieved by cutting ~90% of facility footprint needs, ~50% of medium consumption, ~50% of production time, and ~80% of labor needs. It also cuts the logistic cost by allowing localized production due to its compact size and automation.

Accelerate cell therapy development: Developing a cell therapy is expensive and time-consuming, partly due to the effort required for bioprocessing

Table 1: AlgTubes vs current Stirred Tank Bioreactor (STRs)

(a)	STR	AlgTubes
Volumetric yield	~$2x10^6$ cells/mL	$5x10^8$ cells/mL
Expansion/passage	<10-fold	3000-fold
Batch variation	large	minor
Scalability	10s liter or 10^{10}cells	No up limitation
Long-term culture	Hard to culture cells >1 month	Up to 9 months tested
(b) bioprocess to make $1x10^{12}$ cells from $1.0x10^7$ seeds		
# of passaging	9	1
Production time	40 days	20 days
Total culture volume	1365 liters	2 liters
Production cost	high	10% of STRs

*The numbers are based on hPSCs.

development. For example, developing hPSC-CMs typically starts with small-scale 2D culturing (10^9 cells) for rodent studies, followed by scaling up with STRs for large animal tests and further for clinical trials. Each scaling step requires extensive bioprocess optimization and cell characterization to ensure consistent product safety and efficacy, adding significant time and cost. ColTubes are scalable and can be used across all development stages, providing consistent cells and enabling seamless translation from preclinical studies to clinical applications.

Broaden laboratory research: ColTubes allow research labs to conduct studies requiring large quantities of cells, such as 3D printing a heart or screening 1 million compounds. These studies demand ~10^{10} cells, which are challenging to produce using STRs. These quantities can be generated with just 20 mL ColTubes in a flask.