The use of human pluripotent stem cells (hPSCs) provide great potential to produce large quantities of cardiomyocytes *in vitro* which can potentially be used for high-throughput pharmaceutical drug testing and myocardial repair. For either application, highly reproducible 3D tissues, that can mimic the native myocardium, are desirable to better mimic the heart's natural physiology. Initially, stem cell differentiation protocols used a 3D embryoid body (EB) aggregation approach, resembling developmental steps primarily in their structural nature. Due to several obstacles, i.e. insufficient cardiomyocyte production and reproducibility, state-of-the-art cardiac differentiation protocols transitioned into 2D sheets and a fully defined media including small molecules. With the ability to successfully produce stem cell derived cardiomyocytes, improvements in total cardiomyocyte number per area, cardiomyocyte maturation, and fully functional 3D cardiac tissues with minimum cell handling steps by overcoming 2D cardiomyocyte dissociation, is desired. Thus, the objective of this study was to create 3D hydrogels to culture and directly differentiate human induced pluripotent stem cells (hiPSCs) into mature, aligned, and synchronously contracting engineered cardiac tissues.

IMR90-1 hiPSCs were cultured and expanded on hESC qualified Matrigel in mTeSR-1 media. HiPSCs were dissociated into clumps or single cells, counted, centrifuged, and resuspended in PEG-fibrinogen precursor solution at 60×10^6 cells/mL, which consisted of PEG-fibrinogen polymer precursor, triethanolamine (TEOA), N-vinylpyrrolidone (NVP), and the photoinitiator Eosin Y. The cell-PEG-fibrinogen mixture was transferred to a polydimethylsiloxane (PDMS) mold on acrylated glass and photocrosslinked using a visible light source to form 3D microislands. Microislands were cultured in mTeSR-1 media for three days followed by initiation of differentiation (day 0). Stem cell viability 24 hours post-encapsulation was assessed using LIVE/DEAD assay, which showed that clump and single hiPSCs remained viable in their new, 3D microenvironment. Starting on day 0, cardiac differentiation timeline follows previously published differentiation protocols (Lian, X. et al, 2012). Temporal changes throughout cardiac differentiation were assessed by phase contrast microscopy, immunohistochemistry, RT-qPCR, transmission electron microscopy (TEM), and electrophysiology. First isolated areas of contractions within microislands were visible on day 7 of differentiation. Spontaneously contracting areas and force of contraction increased over time, resulting in fully and synchronously contracting 3D cardiac microislands which remained their function for over three months. Proliferating cell nuclear antigen (PCNA) immunostaining of microislands on day 10 of differentiation showed that proliferating cells within microislands were primarily located on tissue edges. Cardiac localization, alignment, and maturation were assessed by staining engineered cardiac microislands with α-sarcomeric actinin (aSA, cardiac marker) and connexin 43 (Cx43, gap junction protein) on day 10, day 20, day 30, and three months. Sarcomeres were visible at all four time points but developed a more defined and highly aligned structure within microislands over time and showed mature gap junctions at three months. Pluripotency gene Oct4 and cardiac genes myosin heavy chain 6 and 7 (MYH6, MYH7) expression of hiPSCs, day 0, day 10, and day 20 microislands were compared to time-matched 2D cardiac sheets. RT-qPCR revealed a significant decrease in Oct4 gene expression in day 0 3D microislands and 2D sheets compared to hiPSCs. The MYH7/MYH6 ratio, which was used to determine cardiac maturation, showed an increase in 2D sheets and 3D microislands from day 10 to day 20. Although ratios increased in both culture systems, 3D cultured cells matured at a slower rate

compared to 2D cardiomyocytes initially, but showed features of mature cardiomyocytes after three months using TEM. Lastly, contracting microislands were dissociated to obtain electrophysiological characteristics. Recording of calcium transients in human cardiomyocytes were obtained using an IonOptix Myocyte Calcium and Contractility Recording System. Samples were loaded with Fura-2AM dye and perfused with Tyrode's solution. Cardiomyocytes were paced from 0.5-1.5 Hz and stimulated at 30 V. Both 2D and 3D cultured cardiomyocytes showed 1:1 correspondence to electrical stimuli of up to 1.5 Hz.

In this study we showed that clump and single hiPSCs can be cultured and directly differentiated into contracting 3D cardiac tissues using the biomaterial PEG-fibrinogen. In this new, 3D microenvironment hiPSCs remained viable, proliferated, and formed highly reproducible synchronously contracting cardiac tissues. Although cardiac differentiation in 3D progresses slower initially compared to 2D cultured cardiomyocytes, 3D cultured cells responded to pacing frequencies up to 1.5 Hz, showed fully aligned and well defined sarcomeres, and remained contractile functions over several months that led to fully mature cardiomyocytes *in vitro*, which mimics features of native human heart development. This one-step encapsulation procedure of hiPSCs into PEG-fibrinogen produces highly reproducible, physiologically relevant, aligned, and mature 3D engineered cardiac tissues *in vitro* for possible uses in drug-testing or myocardial injections.