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Research Article

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High Throughput Production and Characterization of Primary Rat Hepatocyte Spheroids

Birincil Sıçan Hepatosit Sferoidlerinin Yüksek Verimle Üretimi ve Karakterizasyonu

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ABSTRACT

Developing in vitro models to explore cell biology and physiology holds significant importance in biotechnology, cancer research, drug discovery, toxicity testing, and the emerging fields of tissue engineering and regenerative medicine. The conventional two-dimensional (2D) approaches of mammalian cell culture (2D) have limitations in replicating all of the mechanical and biochemical signals in vivo. Culturing cells as spheroids provides a three-dimensional environment that more accurately mimics physiological conditions compared to 2D culture. In this study, a method for high-throughput spheroid formation using primary rat hepatocytes (PRHs). Using a high throughput platform, the effect of varying concentrations of cell culture media supplements was investigated on spheroid formation. Additionally, different cell seeding densities were assessed and characterized for 7 days. The average diameter and circularity of PRH spheroids remained stable on days 2, 4, and 7, regardless of the initial seeding density. Moreover, PRH spheroids demonstrated high viability (> 90 %) for up to 7 days across all seeding cell densities. These results demonstrated that this technique enables straightforward, large-scale, consistent, and repeatable spheroid manufacturing and presents an alternative approach for future applications.

Key Words

Primary rat hepatocytes, spheroids, 3D cell culture, self-assembly.

ÖZ

Hücre biyolojisi ve fizyolojisini keşfetmek için in vitro modeller geliştirmek, biyoteknoloji, kanser araştırmaları, ilaç keşfi, toksisite testleri, doku mühendisliği ve rejeneratif tıp alanlarındaki araştırmalar için büyük önem taşımaktadır. Memeli hücre kültürüne yönelik geleneksel iki boyutlu (2B) yaklaşımlar, in vivo ortamlarda mevcut olan mekanik ve biyokimyasal sinyallerin taklit edilmesinde sınırlamalara sahiptir. Hücrelerin 3 boyutlu (3B, küresel) kültürlenmesi, 2B kültüre kıyasla fizyolojik olarak canlılardaki dokulara daha benzer olan modellerin ortaya çıkarılması sağlamaktadır. Bu çalışmada, birincil sıçan hepatositler (BSH) kullanılarak yüksek verimli 3B kültür (sferoid, küremsi yapılar) oluşumu için bir yöntem geliştirilmiştir. Kullanılan yüksek verimli platform ile değişen konsantrasyonlarda hücre besi ortamı takviyelerinin BSH sferoidlerinin oluşumu üzerindeki etkisi araştırılmıştır. Ek olarak, 7 gün boyunca farklı hücre ekim yoğunluklarının BSH sferoidlerinin fiziksel özelliklerine etkisi incelenmiştir. BSH sferoidlerinin ortalama çapının ve daireselliğinin, başlangıç ekim hücre yoğunluğundan bağımsız olarak 2, 4 ve 7. günlerde sabit kaldığı gözlenmiştir. Ayrıca BSH sferoidleri, kullanılan üç farklı hücre ekim yoğunluğunda da canlılığını (>%90) 7 gün boyunca korumuştur. Sonuçlar, bu tekniğin basit, büyük ölçekli, tutarlı ve tekrarlanabilir BSH sferoidlerinin üretimine olanak sağladığını ve gelecekteki uygulamalar için alternatif bir yaklaşım sunduğunu göstermektedir.

Anahtar Kelimeler

Birincil sıçan hepatositler, sferoid, 3 boyutlu (3B) kültür, öz toplanma.

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INTRODUCTION

Spheroids, three-dimensional cellular aggregates, have become significant attention as invaluable models for studying cell biology and physiology [1, 2]. Traditional two-dimensional (2D) cell culture methods, while informative, fall short in replicating the intricate cellular interactions and microenvironments found in vivo [3]. The shift toward spheroid culture arises from a recognition that these structures better mimic physiological conditions, providing a more accurate representation of normal cellular function. The unique advantages of spheroids have presented their ability to bridge the gap between conventional 2D cultures and in vivo systems. The increased cell-to-cell interactions including adhesion and junctional connections within spheroids contribute to a microenvironment closely resembling natural tissues and organs [2]. Moreover, 3D culturing is preferred, as certain cells have demonstrated a tendency to lose their physiological phenotypes and functions when cultured in two-dimensional platforms; however, these phenotypes can be reinstated by culturing cells in 3D conditions that emulate physiological settings [3]. This feature is particularly relevant in the fields of biotechnology, cancer research, drug discovery, toxicity testing, and the emerging fields of tissue engineering and regenerative medicine.

Various strategies have been developed to generate spheroids efficiently. One widely used technique is the hanging drop, known for its simplicity and cost-effectiveness, where spheroids form in Petri dishes or conventional well plates through the combined effects of gravity and surface tension. This method has proven successful for various cell types [4, 5]. Mechanical methods for spheroid generation involve cultivating cells in suspension within bioreactors [6, 7]. This dynamic system facilitates controlled cell collision through gentle convective forces generated by an impeller, promoting aggregate formation [6, 7]. Additionally, biological compounds like the arginylglycylaspartic acid peptide, which carries the signal sequence mediating cell binding, can induce aggregate formation [8]. Matrigel®, a gelatinous protein mixture derived from mouse tumor cells, is another example that mimics the natural 3D tissue architecture by replicating the ECM [9-11]. Polymer-based techniques are also employed, such as the direct addition of methylcellulose to the culture medium, elevating its viscosity to induce cell aggregation [12]. Another approach involves coating multiwell plates with non-adherent polymer hydrogels to hinder cell adhesion to surfaces and promote cell aggregation [13]. Commercially available ultra-low attachment (ULA) well plates, provide a straightforward method for obtaining spheroids at a cost between traditional hanging drop techniques and more advanced methods [2]. Recently, advanced technological methods have emerged worldwide, with two notable approaches. The first involves the creation of microfluidic systems, particularly beneficial in scenarios demanding system miniaturization, providing precise control over fluid elements and shear stress to facilitate spheroid formation [14-16]. The second approach revolves around additive manufacturing, commonly referred to as 3D printing, offering meticulous control over the intended geometry and diameter [17, 18]. The choice of method often depends on the specific requirements of the experiment or application, with an overarching goal of producing spheroids that closely mimic the in vivo environment.

Despite notable advancements in spheroid production techniques, translating these methods into high-throughput platforms presents a considerable challenge. The reproducible, robust miniaturization of 3D culture models is still a significant hurdle for researchers. In this study, a method for efficiently generating spheroids at high throughput using primary rat hepatocytes (PRHs) and Corning® Elplasia® Round Bottom Plates was established. First, the influence of varying concentrations of fetal bovine serum (FBS, 5%, 10%) supplements in cell culture media was evaluated on spheroid formation. Notably, it was observed that FBS at concentrations of 5% and 10% in Williams E media was crucial, promoted improved spheroid formation, and ensured the structural integrity of PRH spheroids. Additionally, the effect of different cell seeding densities was assessed. The properties (diameter, circularity) of PRH spheroids were characterized for different culture days. Across all seeding densities (100, 150, 200 cells/microwell), circularity consistently exceeded 0.95, confirming the successful formation of spheroids. The average diameter and circularity of PRH spheroids remained consistent on days 2, 4, and 7, regardless of the initial seeding density. Noteworthy PRH spheroids showed high viability (>90%) for up to 7 days across all seeding cell densities. This method allowed for simple, scalable, and reproducible spheroid production, presenting an alternative approach in emerging fields such as tissue engineering, regenerative medicine, cancer research, drug discovery, and toxicity testing.

MATERIALS and METHODS

Materials

William's E Medium, (no phenol red), primary hepatocyte maintenance supplement (CM4000), penicillin-streptomycin (10000 U/mL), and fetal bovine serum (FBS) were purchased from GibcoTM by Thermo Fisher Scientific (Waltham, MA, USA). 24 well Elplasia plates were supplied from Corning (NY, USA). GenClone 25-508 Dulbecco's phosphate-buffered saline (DPBS) was purchased from Genesee Scientific (Research Triangle Park, NC, USA). Live cell imaging solution (1X), ethidium homodimer-1 (EthD-1), calcein AM and Hoechst 33342, trihydrochloride, and trihydrate were purchased from Invitrogen by Thermo Fisher Scientific (Carlsbad, CA, USA).

Primary rat hepatocyte (PRH) isolation

Freshly isolated primary rat hepatocytes (PRHs) were obtained from adult female Lewis rats (10-12 weeks old, 180-200g) sourced from Charles River Laboratories, USA. The isolation protocol #2011N000111, approved by the Institutional Animal Care and Use Committee (IACUC) at Massachusetts General Hospital (MGH), was carried out by the Cell Resource Core (CRC). Approximately 300-400 million primary hepatocytes, exhibiting 85-95% viability as determined by hemocytometer after trypan blue staining, were promptly seeded in collagen-coated 12-well plates following the isolation process.

Primary rat hepatocyte (PRH) culture for 3D spheroid formation

Corning Elplasia plates feature microcavities within each well, incorporating an ultra-low attachment surface to facilitate the uniform generation of multiple spheroids. As a cell culture media, Williams E medium is mixed with a primary hepatocyte maintenance supplement, penicillin-streptomycin (0.5% v/v), and fetal bovine serum (5 and 10 v/v%). Before seeding PRHs, the wells were wetted with the 1 mL cell culture medium and centrifuged (500 g, 1 min) to eliminate any trapped air from the microcavities. Well-plates were checked if there were any microcavities with trapped air since suspended cells may not properly settle into these structures. 55400 cells/mL (100 cells/microcavity), 83100 cells/mL (150 cells/microcavity), and 110800 cells/mL (200 cells/microcavity) were added to wells and gently rocked to distribute the cells. PRHs were incubated for 48 hours at 37 °C with 5% CO2 without any media change. After PRH spheroids formation (48 hours), half media exchanges by adding droplets of culture media were performed as gently as possible to reduce the chances of disturbing the spheroids (Figure 1).

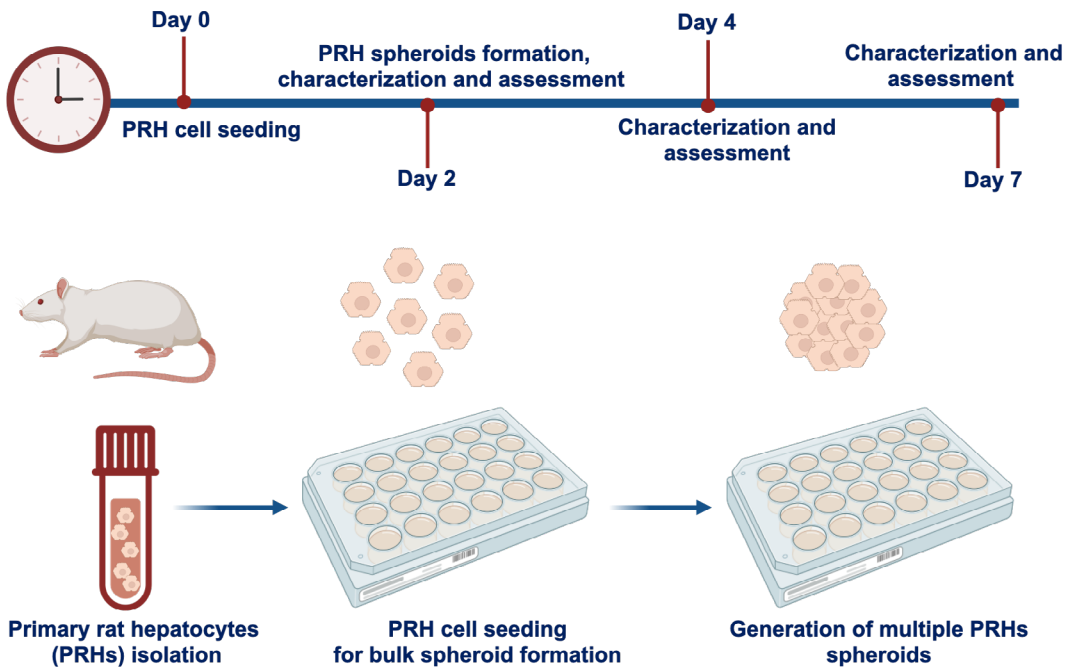

Figure 1. Schematic profile depicting the generation of multiple primary rat hepatocyte (PRH) spheroids. PRHs were seeded at various cell densities in Elplasia plates and incubated for 48 hours at 37°C with 5% CO2, resulting in the formation of multiple spheroids. Characterization and assessment of the spheroids were conducted on days 2, 4, and 7.

Cell viability assessment

Cell viability was assessed using a standard live/dead assay kit. The fluorescent dye calcein AM was utilized to evaluate the cell viability, while ethidium homodimer was employed to appraise cell membrane integrity via fluorescence imaging. The staining solution was prepared by combining 10 mL of live cell imaging solution (1X), 5 µL of calcein AM reagent, 2 µL of Hoechst 33342, and 20 µL of ethidium homodimer-1. Subsequently, cell media were removed from the wells, and the wells were rinsed with 1 mL of Live cell imaging solution (1X). Next, 1 mL of the staining solution was added to the wells, and the plate was incubated at 37°C with 5% CO2 for 40 minutes. Following incubation, the staining solution was replaced with 1 mL of the cell imaging solution, and viability was assessed using the EVOS M5000 microscope (ThermoFisher Scientific, AMF5000, Waltham, MA). Viable cells were identified by the exclusion or minor presence of ethidium homodimer (red) and the retention of calcein (green), while cells stained with ethidium homodimer (red) were categorized as non-viable. The intensity of spheroid fluorescence images was quantified using ImageJ, by adjusting the threshold and subsequently calculating the mean gray value.

Statistical analysis

Quantitative data were expressed as the mean ± standard error of the mean (SEM) and were derived from three distinct wells (n=3). The t-test was applied to check the statistical significance. p-values below 0.05 were considered statistically significant.

RESULTS and DISCUSSION

The impact of fetal bovine serum (FBS) was initially investigated on the migration and formation of primary rat hepatocyte (PRH) spheroids. PRHs were cultured in William E medium with varying concentrations of FBS

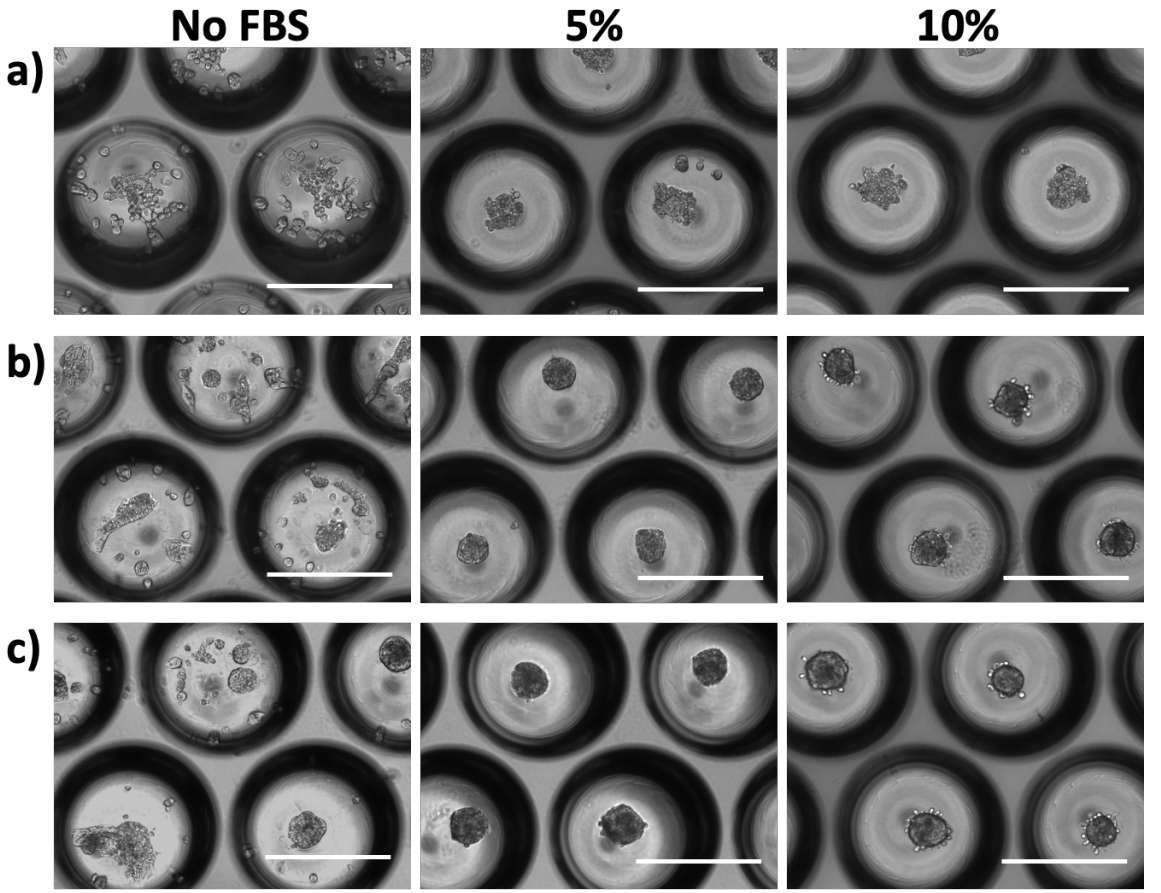

Figure 2. Effect of fetal bovine serum (FBS) concentration on the generation of PRH spheroids. PRHs were cultured in Williams E media with varying FBS concentrations. Spheroid formation was assessed on days a) 1, b) 2, and c) 4. Cell seeding density: 110800 cells/mL (200 cells/microwell). Image scale bar: 400 µm. A minimum of 50 spheroids in three different wells were evaluated in each experiment (n=50, N=3).

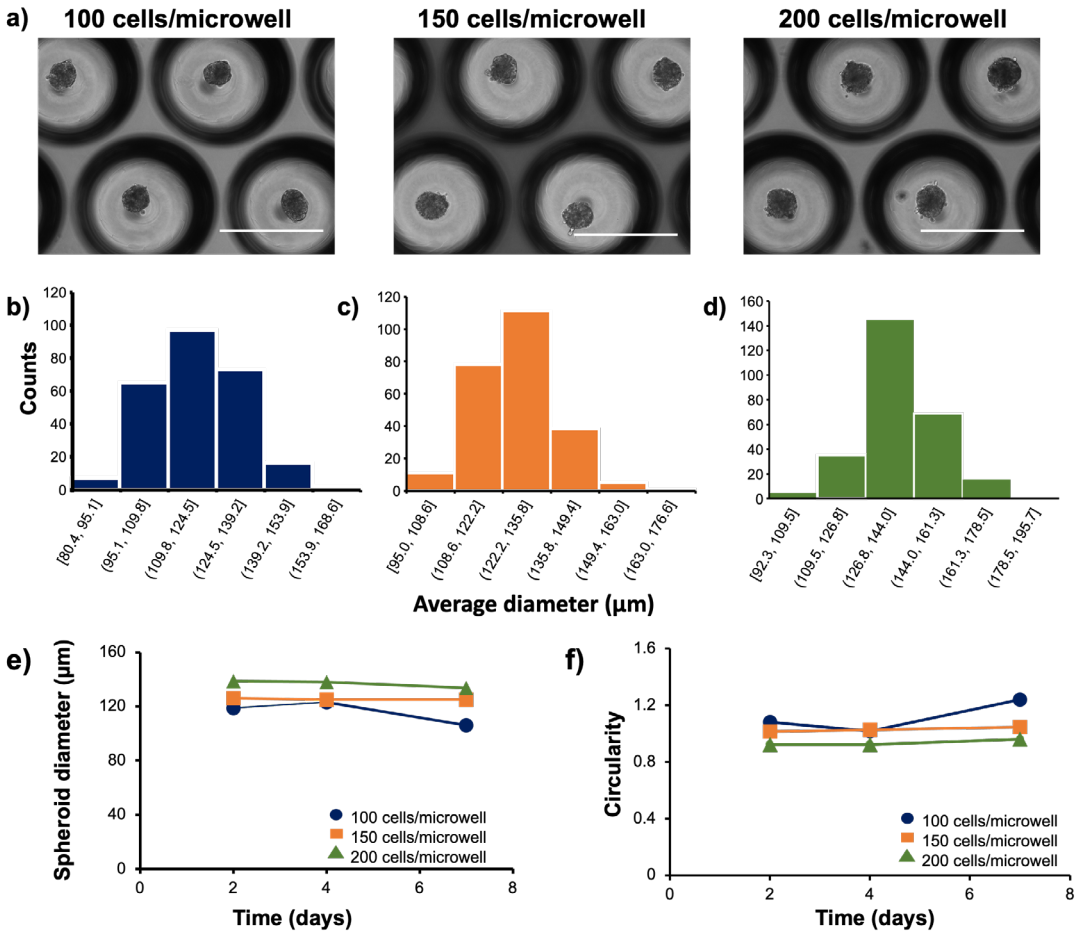

Figure 3. Characterization of PRH spheroids on different days. a) Phase contrast images of PRH spheroids with varying seeding densities on day 2, average PRH spheroids diameter with seeding densities of b) 100 cells/microwell, c) 150 cells/microwell, d) 200 cells/microwell on day 2, e) average PRH spheroid diameter on days 2, 4, and 7 with varying seeding densities, f) circularity of PRH spheroids on days 2, 4, and 7. FBS concentration: 5 v/v%. Image scale bar: 400 µm. To calculate the average spheroid diameter and circularity, a minimum of 50 spheroids in three different wells were evaluated in each experiment (n=50, N=3). Standard error bars are in between (0.005-0.015) and are smaller than the size of the marker.

over 4 days. Notably, the FBS-free medium showed limited spheroid formation capacity on days 1, 2, and 4. However, the addition of 5% and 10% FBS significantly improved the formation of PRH spheroids (Figure 2). Under both 5% and 10% FBS concentrations, PRH spheroids formed within 2 days, with no significant difference observed between the two concentrations (Figure 2). FBS, a widely used supplement in cell and tissue culture media, plays a crucial role in promoting proliferation and maintaining cellular activities [19, 20, 21]. Furthermore, it provides essential compounds, including hormones, vitamins, and binding factors [22]. Thus, these results demonstrated that FBS facilitated PRH spheroid formation, and contributed to the establishment of 3D models with greater ease. 5% FBS concentration was selected for further studies described below.

Subsequently, different cell seeding densities (100, 150, and 200 cells/microwell) were examined and various properties of the spheroids were analyzed, including average diameter on day 2, spheroid size, and circularity up to 7 days. Phase contrast images of PRH spheroids with varying seeding densities revealed that spheroids formed on day 2 regardless of the initial seeding density (Figure 3a). The average diameter of PRH spheroids ranged from 120- 140 µm and increased with higher initial seeding densities (Figures 3b, c, d). The control of spheroid size has been identified as a limitation in rocked and spinner flask cultures in previous reports [23]. This high-throughput production technique successfully addressed this limitation, producing size-controlled spheroids depending on the application. Moreover,

a spheroids size of 125-175 µm is ideal due to ease of oxygenation and better resembling the thickness of hepatic plates (usually 2-3 cells thick at most) [24-27]. Consequently, these three different seeding densities were appropriate for further cell analyses tailored to diverse research purposes. Then, the spheroid diameter was assessed on days 2, 4, and 7 (Figure 3e). PRH spheroids sustained their shape, integrity, and diameter for up to 7 days. Circularity plays a crucial role for spheroids, which provides to maintain a linear gradient of oxygen tension, mirroring the natural microarchitecture of the liver. The circularity of PRH spheroids was analyzed over a 7-day culture period in the microwells. These results indicated that seeding densities of 100, 150, and 200 cells per microwell were favorable for preserving the circularity (>0.92) of PRH spheroids for 7 days (Figure 3f).

Further, a live/dead assay on PRH spheroids was conducted for three different seeding densities (cultured with an initial density of 100, 150, and 200 cells per microwell) (Figure 4). On 2, 4, and 7 days of culture, the results revealed a high viability of PRH spheroids for all seeding densities (Figure 4). The fluorescence image intensities of PRH spheroids were also quantified using ImageJ and the intensity values were normalized based on day 2 (Figure 5). Quantification of intensity values also confirmed that PRH spheroids demonstrated high viability, surpassing 90%, throughout the 7 days of culture for three different seeding densities (100, 150, 200 cells/microwell) (Figure 5).

CONCLUSION

In this study, a method for the high-throughput production of spheroids was established using primary rat hepatocytes (PRHs). The impact of varying concentrations of cell culture media supplements was investigated on spheroid formation. Notably, FBS at concentrations 5% and 10 facilitated PRH spheroid formation and contributed to the establishment of 3D models with greater ease. The 5% FBS concentration was selected for further experiments. The effect of various cell seeding densities and the properties of PRH spheroids were also evaluated. Across all seeding densities (100, 150, 200 cells/microwell), circularity was higher than 0.95, confirming the satisfactory spheroid formation. The average diameter and circularity of PRH spheroids remained stable on days 2, 4, and 7, irrespective of the initial seeding density. Remarkably, these spheroids demonstrated high viability (> 90 %) for up to 7 days across all seeding cell densities. Thus, this technique facilitates straightforward, large-scale, consistent, and repeatable spheroid manufacturing. It offers an alternative approach in emerging fields such as tissue engineering, regenerative medicine, cancer research, drug discovery, and toxicity testing.

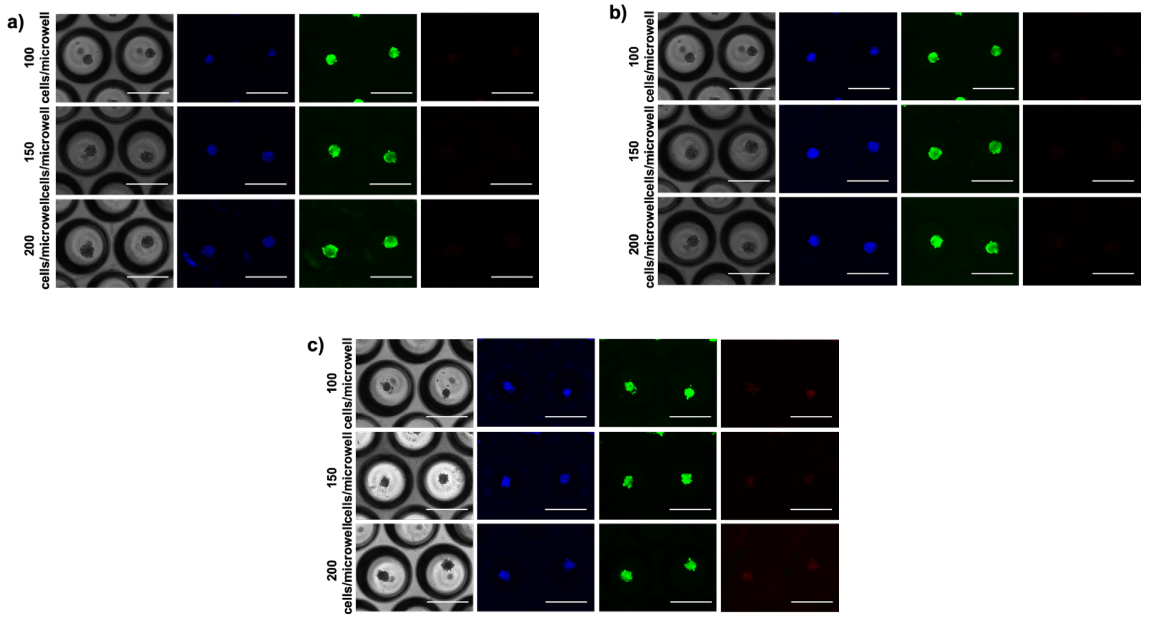

Figure 4. PRH spheroid staining on days a) 2, b) 4, and c) 7. According to staining images, PRH spheroids exhibited high viability for up to 7 days for all seeding densities. PRH spheroids were stained by calcein AM (live), Hoechst 33342 (nuclei), and ethidium homodimer (dead). FBS concentration: 5 v/v%. Image scale bar: 400 µm. Three different wells were evaluated in each experiment (N=3).

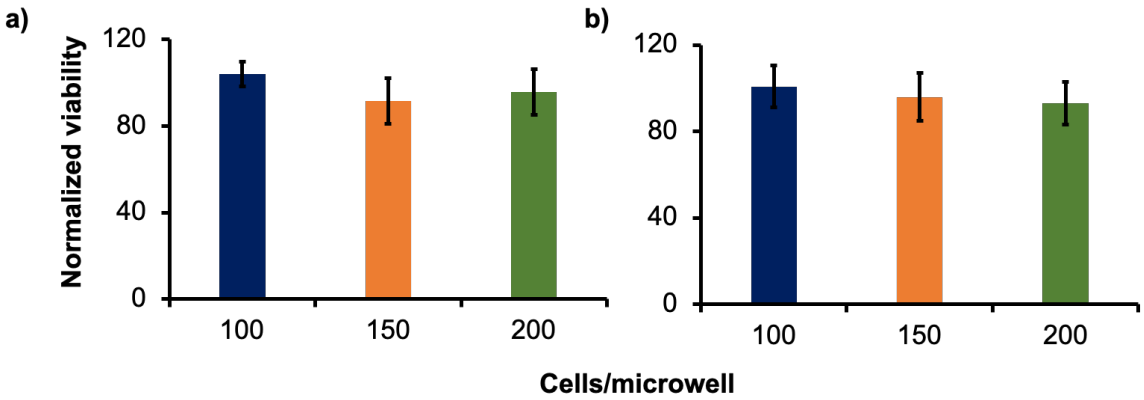

Figure 5. Normalized PRH spheroids viability on days a) 4 and c)7. Fluorescence image intensities of PRH spheroids were quantified using ImageJ and the intensity values are normalized based on day 2. Quantification of intensity values verified that PRH spheroids exhibited high viability, surpassing 90%, throughout the 7-day period across three distinct seeding densities. FBS concentration: 5 v/v%. Three different wells were evaluated in each experiment (N=3). Error bars represent the standard error of the mean.

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