Video Article

A Net Mold-based Method of Scaffold-free Three-Dimensional Cardiac Tissue Creation

Yang Bai[1,2], Enoch Yeung[2], Cecillia Lui[2], Chin Siang Ong[2], Isaree Pitaktong[2], Chenyu Huang[2], Takahiro Inoue[2], Hiroshi Matsushita[2], Chunye Ma[2], Narutoshi Hibino[2]

[1]Department of Cardiac Surgery, The First Hospital of Jilin University

[2]Division of Cardiac Surgery, Johns Hopkins Hospital

Correspondence to: Narutoshi Hibino at nhibino1@jhmi.edu

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Abstract

This protocol describes a novel and easy net mold-based method to create three-dimensional (3-D) cardiac tissues without additional scaffold material. Human-induced pluripotent stem-cell-derived cardiomyocytes (iPSC-CMs), human cardiac fibroblasts (HCFs), and human umbilical vein endothelial cells (HUVECs) are isolated and used to generate a cell suspension with 70% iPSC-CMs, 15% HCFs, and 15% HUVECs. They are co-cultured in an ultra-low attachment "hanging drop" system, which contains micropores for condensing hundreds of spheroids at one time. The cells aggregate and spontaneously form beating spheroids after 3 days of co-culture. The spheroids are harvested, seeded into a novel mold cavity, and cultured on a shaker in the incubator. The spheroids become a mature functional tissue approximately 7 days after seeding. The resultant multilayered tissues consist of fused spheroids with satisfactory structural integrity and synchronous beating behavior. This new method has promising potential as a reproducible and cost-effective method to create engineered tissues for the treatment of heart failure in the future.

Video Link

The video component of this article can be found at https://www.jove.com/video/58252/

Introduction

The goal of current cardiac tissue engineering is to develop a therapy to replace or repair the structure and function of injured myocardial tissue[1]. Methods to create 3-D cardiac tissue models exhibiting the important contractile and electrophysiological properties of native cardiac tissue have been rapidly expanding[2,3]. A variety of strategies have been explored and used in studies[4,5]. These methods range from the use of specific synthetic and natural bioactive hydrogels, such as gelatin, collagen, fibrin, and peptides[6], to bio-ink deposition technologies[2] and bioprinting technologies[7].

It has been shown that scaffold-free methods can produce comparable tissues as biomaterial-based methods, without the drawbacks of incorporating foreign scaffolding material[8]. Oren Caspi *et al.* demonstrated that the incorporation of various types of cells enables the generation of highly vascularized human engineered cardiac tissue[9]. Chin *et al.* developed a 3-D printing method for cardiac patch creation from spheroids. Resulting patches are composed of cardiomyocytes, fibroblasts, and endothelial cells in a 70:15:15 ratio[10]. Spheroids have been shown to be effective "building blocks" of scaffold-free cardiac tissue creation, as they are resistant against hypoxia and possess sufficient mechanical integrity for implantation[11,12]. Previous studies have demonstrated several fabrication methods for spheroid creation, including the use of the hanging drop method, spinner flasks[13], microfluidic systems[14], and non-adherent culture surfaces uncoated or coated with agarose micro-molds[15]. In this protocol, we use the hanging drop device, which contains micropores for condensing hundreds of spheroids at one time.

This study presents a novel and efficient scaffold-free method for cardiac tissue creation, which includes manually seeding the spheroids into a square mold cavity and incubating the tissue on a shaker for maturation. Under usual static culture conditions, oxygen diffusion is limited to the outer aspects of the tissue construct, resulting in central necrosis. However, with the net mold, all the spheroids seeded into the mold are immersed in media with a constant fluidic motion, allowing for the increased diffusion of nutrients and oxygen. Additionally, this mold-based method allows for the simultaneous creation of different-sized tissue patches with minimal manual effort and the resultant tissue can be easily removed from the mold. This novel method allows for the efficient and reproducible creation of scaffold-free, multilayered cardiac patches.

Protocol

1. Preparation of Cardiomyocytes

1. Coat 6-well plates with basement membrane matrix and culture human-induced pluripotent stem cells (hiPSCs) as previously described[17].

2. Differentiate hiPSCs into hiPSC-CMs using previously described methods[18].
3. At 16 - 18 d post-differentiation, suspend the cardiomyocytes by rinsing each well with 2 mL of 1x phosphate-buffered saline (PBS) without calcium or magnesium, followed by incubation with 1 mL/well of trypsin or cell dissociation reagent (see **Table of Materials**) for 5 min at room temperature.
4. Neutralize the trypsin or cell dissociation reagent (see **Table of Materials**) using an equal volume of Roswell Park Memorial Institute (RPMI) cell media supplemented with B-27 (RPMI/B-27 cell media). Pipette up and down to loosen any adhered cells.
5. Collect the suspended cardiomyocytes with a 10-mL serological pipette and transfer them to a 50-mL conical tube.
6. Centrifuge the cell suspension at 250 x *g* for 5 min at room temperature to obtain a cell pellet.
7. Resuspend the pellet in 10 mL of RPMI/B-27 cell media.
8. Combine 20 µL of cell suspension with an equal amount of 0.4% Trypan blue solution and mix gently.
9. Use a manual hemocytometer to count and obtain the concentration and cell viability of the cell suspension.

2. Preparation of Fibroblasts

1. Initiate a culture of a human cardiac fibroblast (HCF) (adult ventricular type) cell line as described previously[16].
2. Suspend the HCFs by incubating them with an appropriate amount of trypsin or cell dissociation reagent (see **Table of Materials**) for 5 min at room temperature[16]. For a T175 flask, 10 mL of trypsin or cell dissociation reagent was used.
3. Neutralize the trypsin or cell dissociation reagent (see **Table of Materials**) using an equal volume of medium, then transfer the sample to a 50-mL conical tube and centrifuge the cell suspension at 250 x *g* for 5 min at room temperature to obtain a pellet.
4. Resuspend the pellet in 10 mL of fibroblast growth medium (see **Table of Materials**).
5. Combine 20 µL of the HCF cell suspension with an equal amount of 0.4% trypan blue solution and mix gently.
6. Use an automated cell counter or manual hemocytometer to count and obtain the concentration and cell viability of the new cell suspension.

3. Preparation of Endothelial Cells

1. Initiate a culture of a human umbilical vein endothelial cell (HUVEC) line as described previously[17]. Suspend the HUVECs by incubating them with an appropriate amount of trypsin or cell dissociation reagent (see **Table of Materials**) for 3 min at room temperature[17]. Neutralize the trypsin or cell dissociation reagent (see **Table of Materials**) using an equal volume of medium, then transfer to a 50-mL conical tube and centrifuge the cell suspension at 250 x *g* for 5 min at room temperature to obtain a pellet.
   NOTE: For a T175 flask, 10 mL of trypsin or cell dissociation reagent was used.
2. Resuspend the pellet in 10 mL of endothelial cell growth medium (see **Table of Materials**).
3. Combine 20 µL of the HUVEC suspension and stain it with an equal amount of 0.4% Trypan blue solution and mix gently.
4. Use an automated cell counter or a manual hemocytometer to count and obtain the concentration and cell viability of the cell suspension.

4. Creation of Hanging Drop Spheroids

1. Place the hanging drop device which contains 850 micropores (each with a diameter of 350 µm) into sterile 6-well plates.
2. Isolate the three types of cells as described above: hiPSC-CMs, HCFs, and HUVECs. Combine them in a ratio of 70% iPSC-CMs, 15% HCFs, and 15% HUVECs in a 50-mL conical tube with RPMI/B-27 cell media at a concentration of 2,475,000 cells per mL.
3. Dispense 4 mL of the cell suspension (2,475,000 cells per mL) to each well of an ultra-low attachment hanging drop system (with a micropore diameter of 350 µm) seated in a 6-well plate.
4. Spheroids will spontaneously form within 12 h of dispensing the cell suspension into the hanging drop device. Continue to culture for a total of 72 h (3 d) at 37 °C, 5% CO_2, and 95% humidity for the maturation of the spheroids.
5. After 72 h of culture, harvest the spheroids through the pores at the bottom of the hanging drop system by placing the device into a dish with medium and swirling it gently to release the spheroids from the hanging drop device.

5. 3-D Patch Creation Using the Novel Net Mold

1. The base, bottom square plate, bottom net, and 10 layered side nets are assembled to create the novel mold with the aid of a pair of sterile forceps. First, stack and align the base, bottom square plate, and bottom net using the corner posts. Then, stack and layer the 10 side nets in alternating directions to create a net of fine stainless-steel prongs.
2. Flush the filling base with PBS or medium to reduce the surface tension and, then, transfer the assembled mold onto the filling base.
3. Wet the net mold cavity with PBS or medium in the same method. Slowly fill the spheroids into the presupposed cavity of the desired size (2 x 2 x 1 mm, 4 x 4 x 1 mm, or 6 x 6 x 1 mm).
   Note: Make sure that 120% of the anticipated volume of spheroids is fed into the cavity so that the cell aggregates are in tight connection and stay static.
4. Assemble the top net and top square plate onto the corner posts and secure the stoppers and holding tubes to prevent a washout of the spheroids.
5. Transfer the filled net mold system to a 6-well plate with 6 mL of RPMI/B-27 cell media or into a sterile container with enough medium to cover the entire assembled mold.
6. Incubate the 6-well plate with the filled mold system on a swinging shaker for 7 - 10 days at 3,000 - 4,000 x *g*.
   NOTE: The duration of the incubation is decided by the size of the cardiac patches. With larger patches, the incubation time will need to be increased for cardiac patch maturation.

6. Removal of the Patch from the Novel Net Mold

1. Remove the mold system from the media and place it on the sterilized handling mat in a sterile 10-cm dish.

2. Remove the holding tube, stoppers, the top square plate, and the top net. Carefully slide out the layered side nets one by one with a pair of sterile forceps. After decannulation, an intact cardiac patch is obtained atop the bottom net.
3. Pick up the bottom net with the patch using sterile forceps and transfer the bottom net into a 35-mm dish with 5 mL of RPMI/B27 media. Gently loosen the patch from the bottom net by slowly swirling the net in the dish, or with a sterile cell scraper. After detachment from the bottom net, the cardiac tissue can be cultured with RPMI/B27 media.
4. Continue to incubate the free-floating 3-D cardiac patch in the 35-mm dish. Functional synchronous beating can be observed as early as 24 h after the removal from the mold.
5. After removing the patch from the net mold, observe that its shape is maintained with integrity and the intensity of synchronous beating increases with time.
   NOTE: The 3-D net mold-based cardiac patches exhibited electrical integration of component cardiospheres after decannulation. We observed a beating frequency ranging from 60 to 80 beats per minute that persisted for more than 60 days.

Representative Results

In our experiments, we utilized a cell suspension of 70% iPSC-CMs, 15% HCFs, and 15% HUVECs in RPMI/B-27 cell media at a concentration of 2,475,000 cells per mL. After creating the cell suspension, we dispensed 4 mL of the cell suspension to each well of an ultra-low attachment hanging drop system, as described in step 4.3 of the protocol. The use of the hanging drop system resulted in the spontaneous formation of hundreds of beating spheroids after 3 days of culture at 37 °C, 5% CO_2, and 95% humidity. The spheroids were easily observed to be beating under light microscopy at 4X and 10X magnification with an average diameter of 350 µm after 72 h of culture (**Figure 1**).

At the end of step 6.5 of the protocol, after harvesting the spheroids, the mold was easily seeded with simple pipetting methods. Culturing of the mold allowed for a fusion of the spheroids; subsequent removal of the mold resulted in an intact cardiac patch with mechanical integrity. After removal from the mold and 24 h of culture, functional and synchronous beating of the cardiac tissue was observed, confirming the mechanical integration of the spheroids (**Figure 2**, left). We also observed that the multiple layers of spheroids were beating spontaneously in a coordinated fashion on light microscopy (**Figure 2**, right).

An immunohistochemical analysis of the net mold-based 3-D cardiac patch was also performed with the cardiac marker troponin T. Confocal imaging showed that the well-aligned cardiomyocytes all exhibited troponin T expression (**Figure 3**).

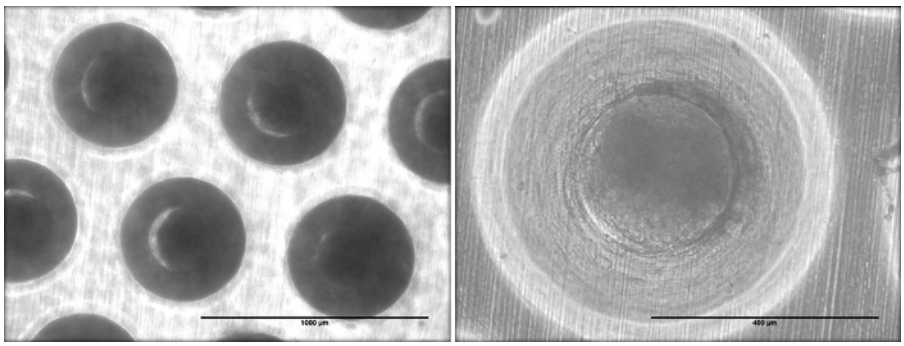

Figure 1: Creation of hanging drop spheroids. Human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), human cardiac fibroblasts (HCFs), and human umbilical vein endothelial cells (HUVECs) were isolated and used to generate a cell suspension with 70% iPSC-CMs, 15% HCFs, and 15% HUVECs, at a concentration of 2,475,000 cells per mL. After 72 h, the spontaneously formed spheroids in each micropore were easily observed to be beating under light microscopy. The scale bar in the left panel = 1,000 µm; the scale bar in the right panel = 400 µm. Please click here to view a larger version of this figure.

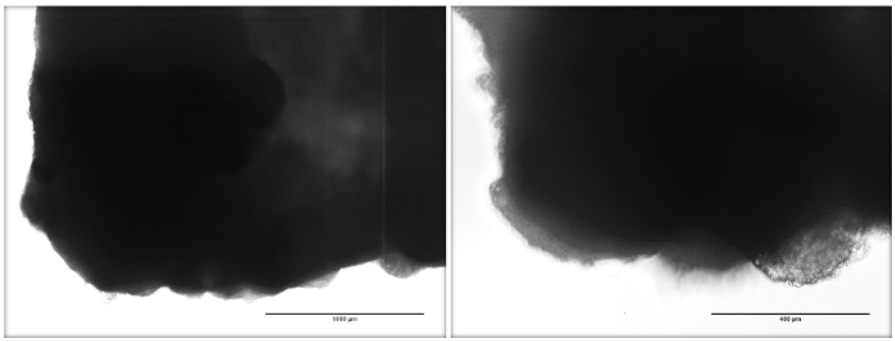

Figure 2: Creation of a multilayered cardiac tissue displaying integration of the component spheroids. After detachment from the net mold, the 3-D cardiac patch maintained its shape and demonstrated synchronous beating, indicating the mechanical integration of the spheroids, as shown in the left panel (where the scale bar = 1,000 µm). The stacked multilayers of spheroids were inspected with light microscopy, as shown in the right panel (where the scale bar = 400 µm), and observed to be beating spontaneously. Please click here to view a larger version of this figure.

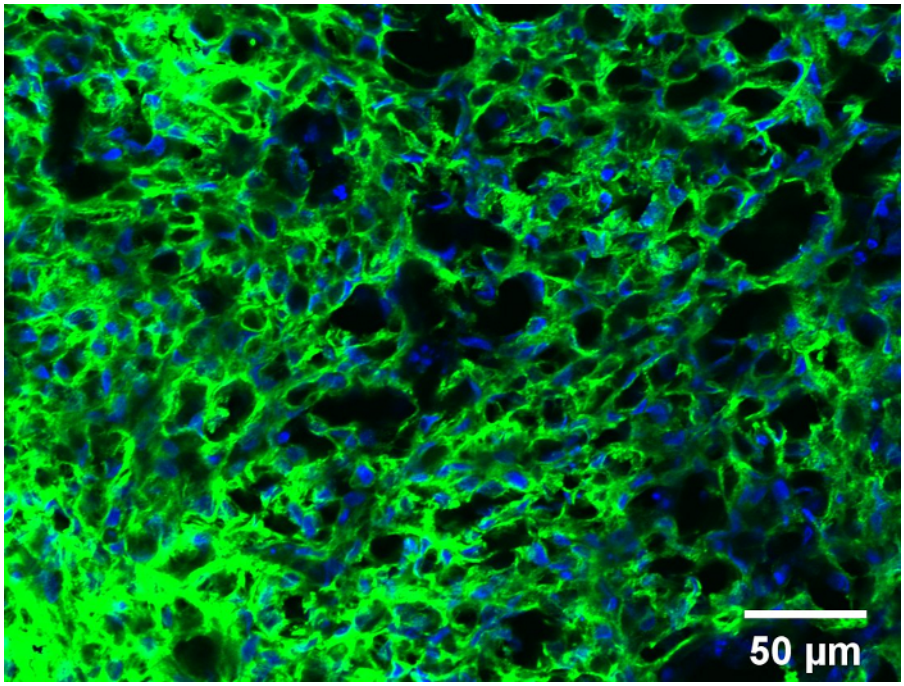

Figure 3: Immunofluorescence evaluation of the cardiac tissue. On immunofluorescence analysis, the net mold-based 3-D cardiac tissue was found to consist of well-aligned cardiomyocytes with troponin T expression. The scale bar = 50 µm. Please click here to view a larger version of this figure.

Discussion

The significance of this method lies in its reproducibility and the effectiveness of the resultant multilayered cardiac tissue. In the field of cardiac tissue engineering, one of the current goals is to identify a method to construct beating, multilayered, and functional 3-D cardiac patches. We report an efficient and reproducible method of creating multilayered cardiac tissues by direct manual seeding of spheroids composed of cardiomyocytes, endothelial cells, and fibroblasts into a novel net mold. The net mold used in this method has a variety of different sizes for the creation of tissues ranging from 2 x 2 x 1 mm to 6 x 6 x 1 mm. The techniques we have described can also be modified for different mold shapes and systems depending on the desired tissue geometry. The described cell concentrations and ratios were optimized previously for hiPSC-CMs, and modifications to a different cell type will require additional evaluation.

The *in vitro* creation of 3-D cardiac tissues using bioprinter technology has been explored with the hope of developing therapeutic and diagnostic models. However, current bioprinting methods are cumbersome and require significant time and the use of ancillary support structures such as needles or scaffold materials. The presence of these ancillary support structures decreases the diffusion of nutrients and oxygen[18]. Therefore, central necrosis is a major concern in current 3-D cardiac constructs, due to the lack of capillaries to supply oxygen and nutrients[19]. Sakaguchi *et al.* applied the method of sandwiching oriented cell sheets and using vascular bed bioreactors to develop an endothelial network[20]. In the net mold system presented here, the mold cavity can allow sufficient diffusion of nutrients and oxygen without the requirement of any supporting structures or scaffolds. Furthermore, the net mold-based method is efficient and does not require specialized skills for both the seeding of the spheroids into the mold and the maintenance of the subsequent patch. As such, it is a quick and inexpensive manner to acquire synchronously beating and functional cardiac patches.

To optimize the spheroid diameter and the number of spheroids used in each hanging drop device, we carried out troubleshooting and modifications regarding the cell types and quantities. For this net mold-based method, the spheroid diameter and the number of spheroids used were of paramount importance to create a functional cardiac tissue of adequate size. We tried different cell concentrations to optimize the best spheroid size for the cardiac tissue creation. Three types of cells were used to create cardiac spheroids by the hanging drop device, which included 70% iPSC-CMs, 15% HCFs, and 15% HUVECs. Under light microscopy, the spontaneously formed spheroids in each micropore were easily observed to be beating after 72 h. We observed that the spheroids' diameter increased with the cell concentration of the hanging drop device. We tried different concentrations of spheroids, from 100 spheroids to 300 spheroids in each device, with 4 mL of RPMI/B27 medium. With numerous trials, we found that the concentration of 2,475,000 cells per mL yielded an optimal diameter of the resultant spheroids. With the optimized diameter of spheroids, we experienced effortless collection and seeding of the spheroids in the stacked net mold, which were important steps to the net mold-based method for multilayer cardiac tissue creation.

The most critical step of the protocol is the optimization of the spheroid size. It is worth mentioning that the spheroids used in this method are smaller than the size of spheroid bodies used in other bioprinting techniques[21]. Larger spheroids have been found to have central necrosis due to insufficient diffusion of nutrition and oxygen[22]. With spheroids with a smaller diameter, adequate diffusion of nutrients and oxygen to the center of each spheroid is more easily accomplished, increasing the cellular viability in all areas of the resultant patch. In the protocol presented here, a spheroid diameter of 350 µm allows for improved cell viability and more contact surface area between the spheroids, which we believe contributes to the successful creation of the cardiac tissue using this net mold. Therefore, this is a critical step in the described methodology. To adapt the protocol to other cell types, additional spheroid size optimization experiments are required.

The limitations of this approach include the inability to precisely control the stacking of the spheroids. This results in areas of non-uniformity of the resulting tissue patches and heterogenous thickness. Moreover, although the actual hands-on time for the seeding of the spheroids is minimal, the creation of large multilayered patches requires an extended culture time to allow for the fusion of the spheroids.

As for future applications, this novel net mold-based method is an efficient and reproducible method of creating 3-D, scaffold-free cardiac tissues with minimal manual effort. The resulting multilayered tissues consist of fused spheroids with satisfactory structural integrity and synchronous beating behavior. This net mold-based method presents a cost-effective and scalable alternative to current bio-fabrication methods of engineered tissues and has the potential to create clinically applicable cardiac tissues for the treatment of heart failure with the goal of replacing ischemic or dysfunctional cardiomyocytes[23]. Additionally, this method can be utilized to create cardiac tissue for patient-specific screenings of potential novel drug therapies[24].

Disclosures

The authors have nothing to disclose.

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