# Chapter 9

# Shrink-Induced Biomimetic Wrinkled Substrates for Functional Cardiac Cell Alignment and Culture

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## Abstract

The anisotropic alignment of cardiomyocytes in native myocardium tissue is a functional feature that is absent in traditional in vitro cardiac cell culture. Microenvironmental factors cue structural organization of the myocardium, which promotes the mechanical contractile properties and electrophysiological patterns seen in mature cardiomyocytes. Current nano- and microfabrication techniques, such as photolithography, generate simplified cell culture topographies that are not truly representative of the multifaceted and multi-scale fibrils of the cardiac extracellular matrix. In addition, such technologies are costly and require a clean room for fabrication. This chapter offers an easy, fast, robust, and inexpensive fabrication of biomimetic multi-scale wrinkled surfaces through the process of plasma treating and shrinking prestressed thermoplastic. Additionally, this chapter includes techniques for culturing stem cells and their cardiac derivatives on these substrates. Importantly, this wrinkled cell culture platform is compatible with both fluorescence and bright-field imaging; real-time physiological monitoring of CM action potential propagation and contraction properties can elucidate cardiotoxicity drug effects.

**Key words** Stem cells, Cardiomyocytes, Cell mechanics, Alignment, Shrink film, Biomaterials, Biomimetic topography

## 1 Introduction

Heart disease, the number one cause of death in developed countries [1], is particularly difficult to treat due to the post-mitotic nature of adult cardiomyocytes (CMs). CMs have limited regenerative potential; therefore, damaged cells are not replaced nor repaired. In recent years, human pluripotent stem cells (hPSCs) have become an attractive cell source for basic and translational cardiac studies. These cells are capable of self-renewal [2–4] and differentiation into CMs [5–7], which provides an unlimited supply of cardiac cells.

Such cardiac cells from hPSCs have promising applications in high-throughput drug screening [8–10]. Current preclinical drug testing methods fail to effectively recapitulate human cardiac

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responses, thus contributing to cardiac toxicity. In fact, cardiac toxicity is the leading cause for drug removal from the market [11]. Since the discovery of hPSC-derived CMs (hPSC-CMs) over a decade ago [12], optimization of cardiac cell culture has been the objective of thousands of research reports in pursuit of a functional in vitro cardiac model.

Despite their benefits, hPSC-CMs cultured in vitro commonly display embryonic-like phenotypes, which currently prevents their adoption both for transplantation and even as an acceptable in vitro model [13]. Their dysfunctional features, including immature structural development, electrical propagation, and contractile properties [14–17], differ significantly compared to mature cardiac cells. Exposure to both biological and mechanical factors drives cardiac maturation in vivo. In the native human heart, layers of anisotropic extracellular matrix (ECM) fibrils, spanning from nano- to microscale, naturally align cardiac cells [18]. Such organization facilitates the proper development of physiological, mechanical, and electrical functions of CMs [19].

Common in vitro cell culture devices (e.g., petri dishes) fail to recreate the native microenvironment of the heart. Yet studies have shown that tuning various components of the in vitro cellular environment can induce alignment of sarcomere structures, improved action potential propagations, and greater contractile forces in CMs [20, 21]. Several micropatterning and microfabrication techniques have been implemented to provide CMs with necessary mechanical cues [22]. However, there are significant trade-offs between the cost and effectiveness of each technique. Microcontact printing is a micropatterning technique, in which proteins are directly printed onto soft substrates, aligning CMs that are cultured directly on these proteins [23–25]. While this process is relatively cheap and suitable for large culture surface areas, the proteins lack the mechanical structure of the native microenvironment. Microfabrication techniques, such as photolithography, are traditionally used to generate surface topographies composed of uniform nano- to micro-sized grooves or ridges (Fig. 1a) [18, 26–28]. These features are typically arranged in repetitive patterns that are limited by inability to capture the complex, varying structures of the ECM. Replicating the finer features (at the nm scale) of the ECM usually requires more advanced fabrication techniques, such as electro-beam lithography and nanoprint technology [29]. Using advanced technologies to produce biomimetic features over substantial areas is a timely process and requires expensive equipment not accessible to most cell culture laboratories.

Shrink film technologies provide both an inexpensive and effective method for achieving multi-scale, biomimetic wrinkles that have similar size and structure to those of native collagen bands (Fig. 1b) [19]. Shrink film is composed of polyethylene (PE), a prestressed thermoplastic that shrinks by 90 % by area when heated in a conventional oven [30]. Prior to shrinking, the surface

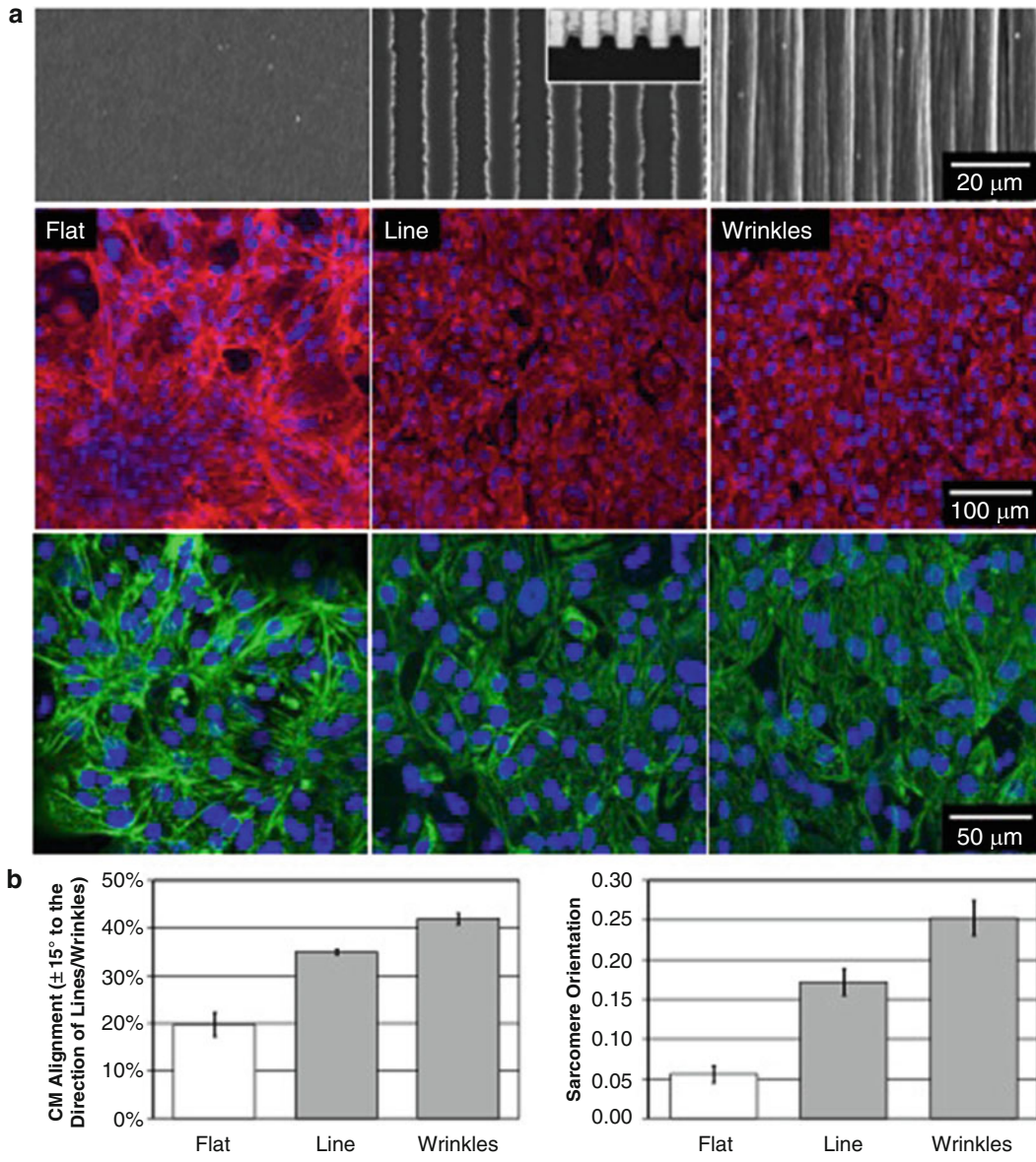


**Fig. 1** Alignment of hESC-CMs. (**a**) Comparison of (*left*) flat, (*middle*) lined, and (*right*) wrinkled substrate topographies. (*Top panel*) Scanning electron micrographs (SEM) of topographies at ×1,000 magnification. Inset is the tilted view of the cross section of lines. Inset scale bar is 20 μm. (*Middle panel*) Fluorescent images taken at ×20 magnification of f-actin. (*Lower panel*) Fluorescent images taken at ×40 magnification of α-actinin, showing hESC-CM alignment. (**b**) Bar graphs quantifying (*left*) f-actin alignment by percent aligned and (*right*) sarcomere alignment using orientation organization parameter. This figure was reproduced with permission from Biomaterials [33]

of the PE film is oxidized by a plasma machine. The formation of wrinkles depends on the stiffness mismatch between the oxidized surface layer and the shrink film. During the shrinking process the oxidized layer buckles on itself, causing multi-scale wrinkles to self-assemble on the surface [31]. These self-similar wrinkles are aligned

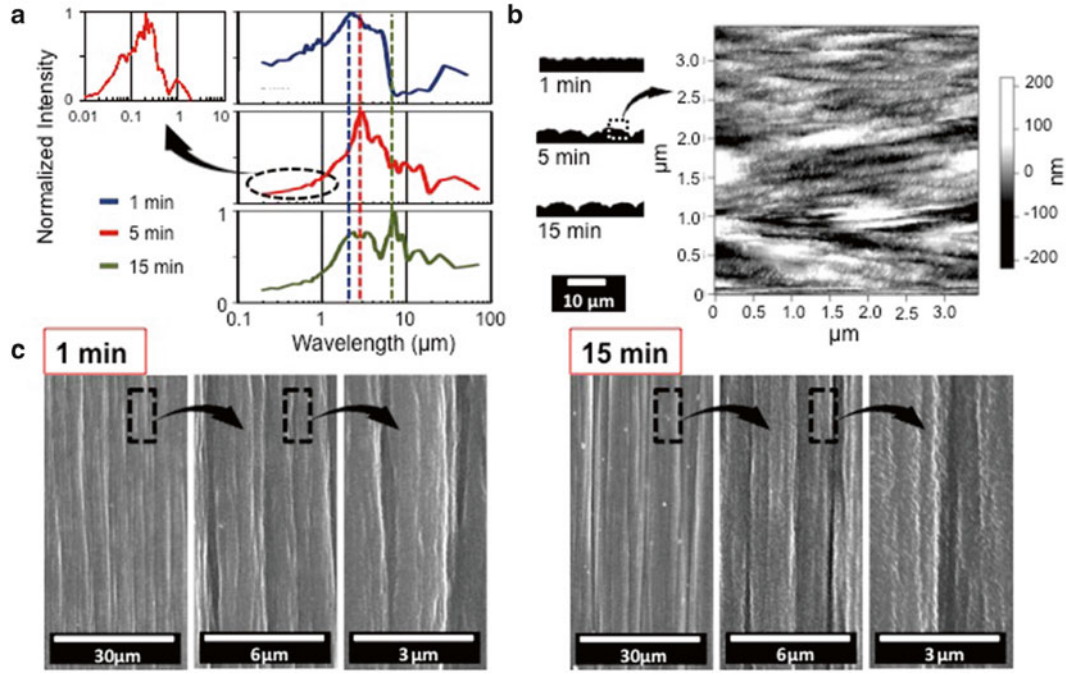


**Fig. 2** Characterization of wrinkles. (**a**) Fast Fourier transform (FFT) was performed on SEM images to determine the characteristic wavelengths of wrinkles of three different plasma conditions: 1, 5, and 15 min. Inset shows smaller wrinkles at ×20,000 magnification. (**b**) (*Left*) Bright-field image at ×150 magnification shows cross section of wrinkles at the three plasma time conditions. (*Right*) AFM of matrigel-coated wrinkles of 5-min plasma time condition. (**c**) SEM images with progressive zoom to illustrate self-similar wrinkles and wrinkle bundling of (*left*) 1-min plasma time condition and (*right*) 15-min plasma time condition

and layered in such a way that they resemble the bundled structure of the ECM. The average predominant wavelengths of the smaller wrinkles (minor wavelengths) range from 60 to 380 nm, while average wavelengths of larger wrinkles (major wavelengths) range from 1 to 7 μm (Fig. 2). This scale is consistent with natural ECM, in which individual collagen fibers measure about 300 nm and bundles of fibrils reach micrometers in size [32]. These feature sizes are easily tunable with longer oxidation periods yielding greater wrinkle bundling [30].

These biomimetic substrates have served as a platform for functional monitoring of hPSC-CMs [33]. Capturing time-varying mechanical and electrical properties of contracting CMs is crucial for in vitro characterization and detection of changes in CM behavior. Wrinkled substrates are compatible with bright-field and fluorescence-based microscopy, which provides a noninvasive option for real-time physiological monitoring and cardiotoxicity assays of hPSC-CMs. Functional anisotropic alignment of human embryonic stem cell-derived ventricular CMs (hESC-VCMs) using the multi-scale wrinkles represents a more accurate model for efficacious drug discovery and development as well as arrhythmogenicity

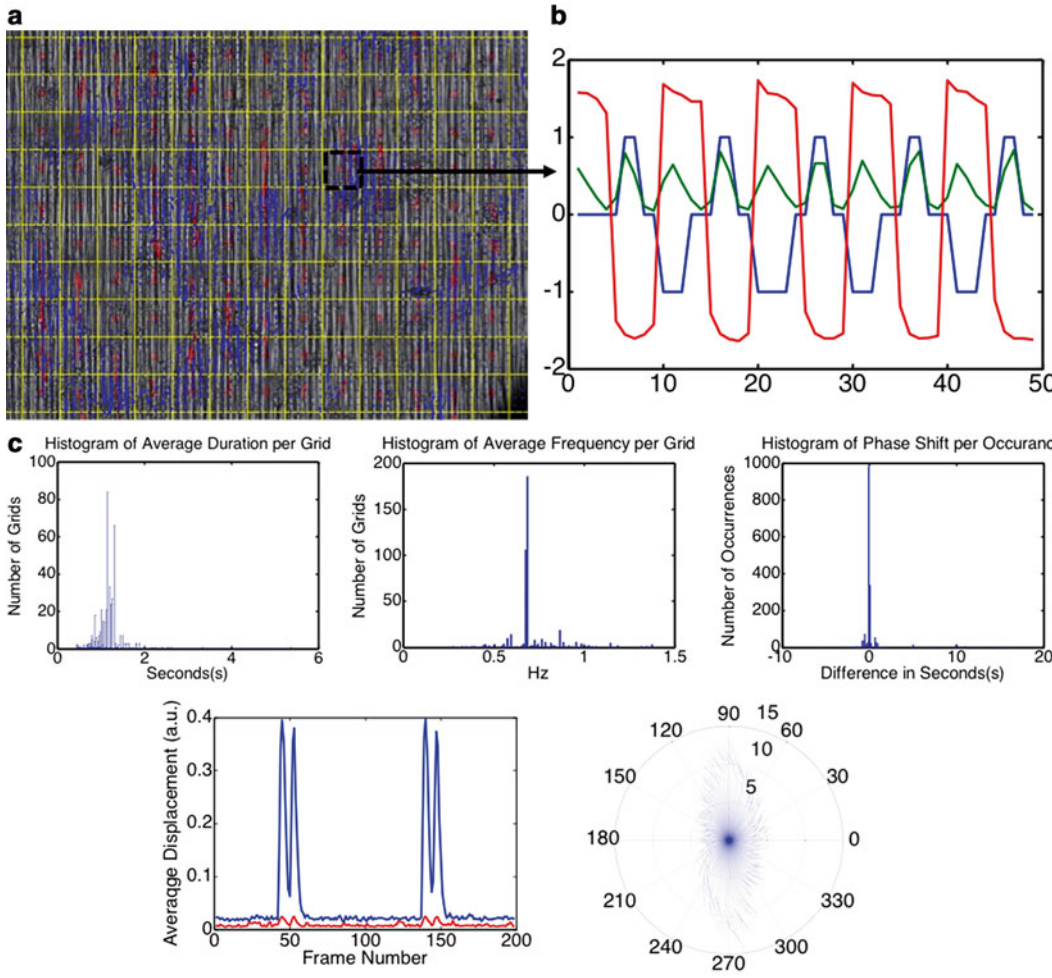


**Fig. 3** Optical flow hPSC-CM contraction analysis. (**a**) Time-lapse images are gridded (*yellow boxes*), and motion vectors (*blue lines*) are generated using the optical flow code. Average motion vectors are calculated for each grid (*red arrows*). (**b**) Contraction analysis plot. The *green line* represents displacement, and the *red line* is direction of motion vector in radians. The *blue line* is the categorical plot, with values of 1 indicating contractions and −1 indicating relaxations. (**c**) Types of analysis that can be generated by the optical flow code include histograms of contractile duration, frequency, and synchronicity (labeled as phase shift). Contractile orientation can be represented in two graphs: *x*- and *y*-motion vector trances (*bottom left*, *blue* is *y*, and *red* is *x*) and a compass plot (*bottom right*). This figure was reproduced with permission from Biomaterials [33]

screening when combined with action potential measurements [34]. When combined with time-lapse bright-field imaging and optical flow vector analysis, these biomimetic substrates provide an integrated platform capable of determining cardiotoxicity drug effects by simply characterizing CM contraction properties [33]. Optical flow analysis generates a series of motion vectors, which can be analyzed to give information regarding CM contraction duration, frequency, synchronicity, and orientation (Fig. 3).

This chapter provides the easy, inexpensive method for fabricating biomimetic plastic substrates, which takes less than 10 min [30]. The PE-wrinkled surface can be either directly cultured on or used as a master mold for transferring wrinkled features on to any other cell culture plastic via hot embossing (also detailed in this chapter). In addition, techniques for culturing stem cell-derived CMs on these surfaces are provided.

## 2 Materials

### 2.1 Shrink Film

1. Prestressed PE shrink film (Cryovac® D-film, LD935, Sealed Air Corporation) is used to create biomimetic-wrinkled surfaces.
2. Polydimethylsiloxane (PDMS) (Sylgard 184) can be used to make a stamp to transfer wrinkled features onto other cell culture plastics.
3. These methods describe hot embossing features onto a tissue culture-compatible polystyrene sheet (Grafix Clear Shrink Film), but any tissue culture plastic substrate can be substituted.
4. Equipment needed includes a plasma machine (Plasma Prep II, SPI Supplies) for oxygen plasma treatment of surfaces and a conventional oven for shrinking prestressed plastics.

### 2.2 Cell Culture

This protocol is specific to the culture and alignment of hESC-CMs. Wrinkled shrink substrates have also been proven to successfully align mouse embryonic fibroblasts (MEFs), aortic smooth muscle cells (AoSMCs), and human embryonic stem cells (hESCs) [30]. Many different protocols for the culture and differentiation of hESC-CMs exist [2, 5, 6, 12, 35–38] and are likely compatible with wrinkled shrink substrates.

#### 2.2.1 Cell Culture Media and Factors

1. Maintain hESC cells with mTeSR1 medium.
2. For differentiation procedure, use RPMI-B27 with and without insulin.
3. Supplements include 100 ng/mL activin A (R&D Systems) and 10 ng/mL BMP4 [39].

#### 2.2.2 hESC-CM Seeding

1. Use 0.25 % trypsin–EDTA to lift cells from plate.
2. Prepare plating media consisting of Dulbecco's modified Eagle medium (D-MEM) high glucose supplemented with 10 % fetal bovine serum (FBS), 1× nonessential amino acids, and 2 mM Glutamax.
3. Maintain hESC-CMs in RPMI-B27 culture media with insulin.

## 3 Methods

### 3.1 Fabrication of Biomimetic Wrinkles

#### 3.1.1 Wrinkled PE Master Mold

1. Treat a PE film with oxygen plasma for 5 min (Fig. 4I) (*see* **Note 1**).
2. On top of a glass plate, place a piece of paper that is slightly bigger than the size of the treated PE film (*see* **Note 2**).
3. Place the PE film on top of the paper-covered glass slide, and secure the shorter ends with binder clips.
4. Place the construct (PE film, paper, and glass plate) in an oven that is preheated to 150 °C for 3 min. This process will shrink the film and generate the wrinkled surface features on the PE (Figs. 1 and 4.II) (*see* **Note 3**).
5. The resulting PE-wrinkled substrate can be used directly for cell culture (skip to Subheading 3.1.4) or used as a master mold for transferring features via hot embossing (continue to Subheading 3.1.2).

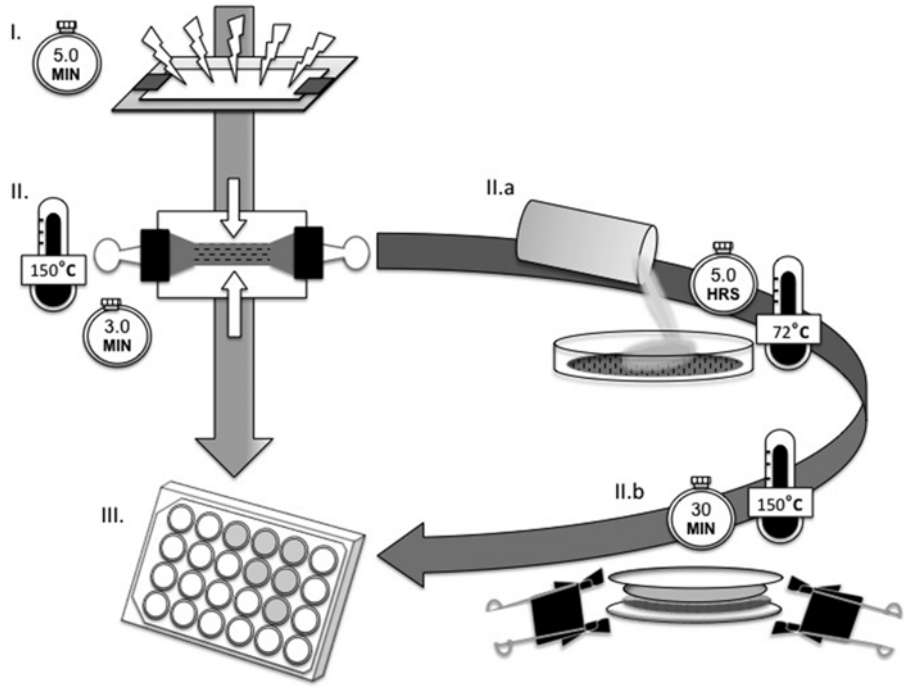


**Fig. 4** Fabricating wrinkled cell culture substrates. (I) Treat PE with oxygen plasma for 5 min. (II) Bind both ends of PE and shrink uniaxially by placing in an oven at 150 °C for 3 min [30]. (II.a) Pour PDMS on top of featured side of PE in a petri dish. (II.b) Hot emboss PS by placing on featured side of PDMS, sandwich together with glass slides and binder clips, and heat at 150 °C for 30 min. (III) Use either the original wrinkled PE substrate or the hot embossed PS substrate for cell culture

*3.1.2 PDMS-Wrinkled Stamp*

1. Inspect wrinkles under a microscope for micro-tears (*see* **Note 3**).
2. Cut out the parallel portion of wrinkled PE, and use double-sided tape to adhere it to a petri dish, featured side up.
3. Mix PDMS monomer and curing agent at a 1:10 ratio.
4. Spin PDMS mixture in a centrifuge at 2,060 × *g* for 5 min to degas.
5. Pour the degassed PDMS mixture over the wrinkled PE in the petri dish (Fig. 4.II.a). The volume of the mixture is dictated by the desired thickness of the stamp.
6. Place the petri dish under a vacuum for 3 h. Then allow it to sit at room temperature for an additional 5 h.
7. Transfer the petri dish to an oven at 72 °C for at least 5 h.
8. Remove from oven, and cut out the PDMS stamp.

*3.1.3 Hot Embossing*

1. Place a sheet of PS (or any other tissue culture plastic) atop the featured side of the PDMS stamp (*see* **Note 4**).
2. Sandwich the PS and PDMS stamp with two pieces of glass and secure together with binder clips (Fig. 4.II.b).
3. Preheat a conventional oven to 150 °C (*see* **Note 5**).
4. Heat the construct in the oven for 30 min. Features on the PDMS stamp will be hot embossed onto the PS sheet (*see* **Note 6**).
5. Remove construct from the oven, and allow it to cool to room temperature.

*3.1.4 Substrate Mounting and Sterilization*

1. Inspect wrinkles under a microscope for micro-tears.
2. Cut wrinkled substrate to desired size/shape (15 mm diameter circles are described here).
3. Pipette 500 μL of PDMS onto a 15 mm glass cover slip. Place the wrinkled substrate directly on top of PDMS to adhere it to the 15 mm glass cover slip (*see* **Note 7**).
4. Sterilize the wrinkled substrate by submerging in 70 % ethanol and treating with UV light for at least 30 min.
5. Submerge wrinkled substrate again in double-distilled water and treat with UV light for a minimum of 30 min.

### *3.2 hESC Expansion*

1. Feeder-free hPSC culture has been described elsewhere [39].
2. Begin hESC cell culture in standard tissue culture plates coated with 1:200 Matrigel®.
3. Feed cells daily with mTeSR1 medium.
4. Passage when cells reach 80–90 % confluency; during passage, plate cells in a 1:10 ratio (*see* **Note 8**).

### 3.3 Cardiac Differentiation

*Day 2*: Passage a confluent well of hPSC into three wells of a tissue culture well plate (*see* **Note 9**) [5].

*Day 1*: Exchange media with mTeSR1. Cells should be very confluent.

*Day 0*: Exchange media using RPMI-B27 (without insulin), and add a supplement of 100 ng/mL activin A for the first 24 h.

*Day 1*: Exchange media using fresh RPMI-B27 (without insulin), and add a supplement of BMP4 (10 ng/mL). Wait for 4 days.

*Day 5*: Exchange media with RPMI-B27 (with insulin). Do not add any cytokines. Following this, exchange media every 3 days using RPMI-B27 with insulin.

*Days 8, 11, 14, etc.*: Exchange media. Spontaneous contractions usually start occurring.

*Day 20*: Start the experiment (*see* **Note 10**).

### 3.4 Cell Seeding onto Wrinkled Substrates

1. Oxidize wrinkled cell culture substrates by UV/ozone for 8 min.
2. Coat surface-activated substrates with 50 μg/mL fibronectin and incubate overnight at 2–8 °C.
3. Lift cardiac cells by incubating them in 0.25 % trypsin–EDTA for 5 min.
4. Resuspend cells in "plating medium" (DMEM with 10 % FBS and 2 mM Glutamax).
5. Load cells onto fibronectin-coated wrinkled substrate at a seeding density of $5 \times 10^5$ cells/mL.
6. The following day, replace culture media using RPMI-B27 (with insulin).
7. Change media every 3–4 days.

## 4 Notes

1. This plasma procedure is specifically developed for Plasma Prep II. Optimization is required when using different plasma machines. Settings are as follows: Turn vacuum pump on, and wait for 10 min for vacuum to warm up. Turn oxygen gas valve open and bring to 7 PSI. With the sample in the chamber, turn on machine vacuum and pump down to 200 mTorr. Begin plasma treatment at a power level of 60–70 mA. Wait for 5 min (plasma chamber should glow a light blue color). Oxygen will be introduced into the chamber continuously over the 5 min. Stop plasma treatment, de-vacuum the plasma machine, and remove the sample from the chamber.

2. The paper acts as a non-sticking surface so that the film can retract freely during shrinkage. It is fine to substitute any other non-stick surface for paper.
3. When film is completely shrunk, it should resemble the shape of a bowtie, with the center being relatively narrow and parallel (Fig. 4.II). Depending on the quality and handling of the PE film, as well as plasma treatment condition, micro-tears sometimes do occur after shrinking. Micro-tears can be identified as discoloration within the parallel portion of the wrinkles. If tears do occur, the piece is deemed unusable.
4. Depending on the desired thickness of final wrinkled substrates, thicker or multiple PS sheets may be used.
5. If using a different type of thermal plastics, make sure that the oven temperature is higher than the glass transition temperature.
6. Depending on the quality and placement of PS sheets, bubbles can form and get trapped during hot embossing. If this occurs, multiple layers of PS sheets should be exchanged for a single sheet of thick, high-quality PS.
7. In general, be gentle with the cells. Aspirate and pipette media slowly. Do not place media in warm water bath; instead, slowly bring to room temperature by placing in a dark drawer.
8. The PDMS acts as glue that fastens the wrinkled substrate to the glass. Be careful not to get PDMS on top of the plastic surface or wrinkles will be compromised. It is okay to use double-sided tape instead of PDMS as long as the tape does not affect cell culture.
9. Make sure that cells are confluent in the three new wells. Many cells will die during differentiation.
10. On days 0, 1, 2, 5, and 8, do not use an aspirator when exchanging media. Use a pipette tip and exchange medium SLOWLY. Cells are very loosely attached during differentiation. It is usually best to wait before starting cardiac experiments until after day 20; however, cardiac experiments may technically begin any time after day 8.

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