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Video Article

Tissue Engineering: Construction of a Multicellular 3D Scaffold for the Delivery of Layered Cell Sheets

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Abstract

Many tissues, such as the adult human hearts, are unable to adequately regenerate after damage.[2,3] Strategies in tissue engineering propose innovations to assist the body in recovery and repair. For example, TE approaches may be able to attenuate heart remodeling after myocardial infarction (MI) and possibly increase total heart function to a near normal pre-MI level.[4] As with any functional tissue, successful regeneration of cardiac tissue involves the proper delivery of multiple cell types with environmental cues favoring integration and survival of the implanted cell/tissue graft. Engineered tissues should address multiple parameters including: soluble signals, cell-to-cell interactions, and matrix materials evaluated as delivery vehicles, their effects on cell survival, material strength, and facilitation of cell-to-tissue organization. Studies employing the direct injection of graft cells only ignore these essential elements.[2,5,6] A tissue design combining these ingredients has yet to be developed. Here, we present an example of integrated designs using layering of patterned cell sheets with two distinct types of biological-derived materials containing the target organ cell type and endothelial cells for enhancing new vessels formation in the “tissue”. Although these studies focus on the generation of heart-like tissue, this tissue design can be applied to many organs other than heart with minimal design and material changes, and is meant to be an off-the-shelf product for regenerative therapies. The protocol contains five detailed steps. A temperature sensitive Poly(*N*-isopropylacrylamide) (pNIPAAM) is used to coat tissue culture dishes. Then, tissue specific cells are cultured on the surface of the coated plates/micropattern surfaces to form cell sheets with strong lateral adhesions. Thirdly, a base matrix is created for the tissue by combining porous matrix with neovascular permissive hydrogels and endothelial cells. Finally, the cell sheets are lifted from the pNIPAAM coated dishes and transferred to the base element, making the complete construct.

Video Link

The video component of this article can be found at http://www.jove.com/video/51044/

Introduction

Injection of cells and/or single materials alone has shown variable success in other organ systems and limited success in cardiac regeneration.[5,7-12] Currently, stem cell-derived cells are delivered to damaged tissue using a variety of delivery methods including: direct cell injection into tissue and perfusion into the blood supply.[13-17] Others have implanted cells alone, materials alone and/or in combination with material carriers to help regenerate damaged organs.[18-21] This design combines multiple strategies that provide material strength, patterning in multiple materials and multiple cell types.

Specifically, the base acellularized fibrous matrix provides the foundational physical strength to the construct, making it suitable for suturing in into the patient, if necessary. The void spaces in the base matrix are filled with endothelial cells in a neovascular permissive hydrogel[22] for rapidly establishing vascularization of the implanted construct. This composite is then integrated with pre-patterned cell sheets that allow enhanced cell-to-cell communication, more closely mimic the native tissue.[1,23-25] The overall production process for the layered cellular patch is outlined by the flowchart in **Figure 1**.

Protocol

1. Creation of pNIPAAM-coated Plates

1. Dissolve the 2.6 g of pNIPAAM in 2 ml of a 60% toluene/40% hexane solution.
2. Heat the mixture to 60 °C for 10 min stirred, until the pNIPAAM is dissolved.
3. Cut filter paper into a 60 mm diameter circle and place paper in the Buchner funnel.
4. Filter the solution through Buchner Funnel into the pre-weighed glass beaker (do not use plastics, as hexane will melt plastics).
5. Place the beaker and contents into a bell vacuum (24 psi) O/N (16 hr). Note: Until the residue is reacted with isopropyl it will oxidize so make sure it does not come into contact with oxygen.
6. Weigh the beaker to establish the weight of the pNIPAAM.

7. Add isopropyl alcohol to the pNIPAAM, creating a 50/50 w/w solution.
8. Place 2 ml of the solution on the surface of the tissue culture plate, and coat for 5 min under UV light.
9. Wash the plate with 2 ml of warm PBS twice before using for cell culture.

2. Creation of Cell Sheets

Note: Cell sheets of primary cells for the target organ can be created using a number of different methods, or by coating tissue culture surfaces with thermo-responsive polymer as described here. Pre-coated thermo-sensitive plates are also offered by a number of vendors.

Note: This protocol is for culture using a 35 mm dish. Briefly, cells are first incubated at 37 °C for a minimum of 24 hr at confluence to establish lateral connections between adjacent cells. To release cell sheets, plates are subjected to temperatures below 32 °C. The cell sheet is then transferred to the strong base fibrous matrix containing a neovascular permissive hydrogel with vascular endothelial cells.

1. Isolate the cell population. Note: This method is dependent on the individual derivation procedures and the type of cells. Rat aortic smooth muscle cells (RASMC) are used in this example. These are primary smooth muscle cells isolated from the abdominal aorta of a rat.
2. Wash the cells with 2 ml of warm PBS.
3. Add 3 ml of trypsin (or other cleaving/disassociating solution) to the cells for 5 min.
4. Inhibit the trypsin by the addition of 3 ml of the culture media, or phosphate buffer solution (PBS) containing 10% Fetal Bovine Serum (FBS).
5. Collect the cells in a conical tube and count an aliquot.
6. Spin the cells at 1,000 rpm (228 x g) for 5 min.
7. Aspirate the supernatant and resuspend the cells in their growth media (SmGM2 plus bullet kit culture medium is used for RASMC).
8. Place the media containing the cells on a 35 mm thermo-sensitive plate – pNIPAAM coated plate at a concentration that will achieve 100% confluence. Note: For RASMCs that number was determined to be 100,000 cells/cm^2. However, due to loss of cells during the passing, 120% of that value is used.
9. Place into an incubator at 37 °C O/N. Note: It is important to maintain the cells at 37 °C to maintain the cell adhesion to the plate.

3. Preparation of Foundational Matrix

Note: Various 3D fibrous matrices can be used to layer strong fibrous matrix between the delicate cell sheets. Some examples include: gelfoam, bioglass, natural acellularized materials[26] or nanospun materials[27,28] The porcine urinary bladder matrix (UBM) used in these studies was generously provided from our collaborator, Dr Badylak.[29]

1. Prior to use, determine matrix characteristics including the lack of cellular content if decellularized matrix is used,[27,28] cell specific viability, and void space.[22]
2. Cut the pre-sterilized matrix into a desired size and shape. Note: Here, a hole-punch is used to cut a 4 mm diameter circle.

4. Seeding Endothelial Cells into a Neovascular Permissive Hydrogel

Note: Endothelial cells can be obtained from a variety of sources, including differentiation from stem or progenitor cells. Here, HuVECs are used.

1. Use any permissive hydrogel (fibrin, collagen gels) as long as the cross-linking time is short enough to allow the cells stay viable. Note: Here, a Hyaluronan (HA) based gel cross-linked with a disulfide bridge is used.
2. Prepare the HA hydrogel in accordance with the company protocol.
3. Collect the endothelial cells and disperse into a single cell solution using 1x trypsin. Note: Accutase or Cell Dissociation Buffer could also be used for single cell dispersion.
4. Deactivate the trypsin enzyme by using an equal amount of soybean trypsin inhibitor (if it is important that cells do not come into contact with serum) or 10% FBS in PBS, collecting the solution/cells into a 15 ml conical tube.
5. Count the cells, and calculate the volume needed for the patch dimensions (previously quantified). Note: For a 4 mm patch, here 2 million endothelial cells are used.
6. Extract 2 million cells, and place into a new 15 ml conical tube.
7. Spin at (228 x g) for 5 min.
8. Aspirate the supernatant, leaving the cells as a pellet in a conical tube.
9. Mix the HA and Gelatin liquid materials in a 1:1 ration. Then add 80% of the total volume into the conical tube containing the pellet.
10. Resuspend the endothelial cells in the 1:1 HA /Gelatin mixture
11. Place the suspended cells in the HA /Gelatin mixture into the base fibrous matrix from Step 2.
12. Add 1/5 (20 percent) of the total volume desired of the cross-linker
13. Incubate for 1 hr at 37 °C.

5. Isolation of Cell Sheets

1. Remove the 35 mm pNIPAAM-treated plates containing the cells from the incubator and place in a cell culture hood at RT.
2. Quickly aspirate the media from the cells, and add 2 ml of 6% normal gelatin that has been heated to 37 °C.
3. While the gelatin is still warm, place the metal lattice into the gelatin, submerging it below the surface of the normal gelatin (Movie 1).
4. Place the entire plate onto ice for 5 to 7 min, allowing the gelatin to harden.
5. After 7 min, use a spatula to carefully separate the gelatin edges from the side of the plate, and then use forceps to lift the metal lattice from the plate Note: The 6% gelatin, and the cell sheet should lift with the lattice.
6. Move the cell sheet to the dish and place on top of the base fibrous matrix-hydrogel combination, carefully setting the lattice on top of the construct. Note: The apical side of the cell sheet will still be in the top position.

7. Add 2 ml of warm media (37 °C).
8. Incubate O/N allowing the sheet of cells to adhere to the hydrogel surface.
9. Remove the metal lattice after the solution warms (approximately 1 hr), or the next day.

Representative Results

The flow diagram (**Figure 1**) shows the overall method of making the multilayered patch. Cell sheets are detached from the pNIPAAM treated plate by dropping the temperature below 32 °C. Then the cell sheet is placed on top of the cross-linked hydrogel containing the endothelial cells seeded into the underlying fibrous matrix (**Figure 1**). The pretreated thermo-sensitive plates can also be used for creating the cell sheets. Special topological surfaces are used to specifically pattern (*i.e.,* align) the cells[30].

The base fibrous matrix can be generated from decellularizing native tissue matrix or electrospun. Here, the fibrous material sheet was cut to a 4 mm diameter for the patch base (**Figure 2A**). The characterization of this material is important for determining the amount of hydrogel that can be used to fill the void spaces. The matrix used here has been previously characterized and published.[22]

The hydrogels containing endothelial cells are cross-linked after application of the HA hydrogel liquid components to the fibrous matrix. Fluorescence/transmission microscopy shows living cells stained with Calcein AM (**Figure 2B**) that have been captured in the cross-linked hydrogel.

The process of creating the cell sheet is imaged (**Figure 3**), including comparison between our own pNIPAAM-coated plates and pre-coated plates purchased from a vendor. RASMC are plated on the pNIPAAM treated surface for at least 16 hr at 37 °C. This minimum time allows the cells to establish their lateral boarder adhesions with neighboring cells (**Figure 3A**). Note: The cells must be at confluence to establish these lateral boarders. After culturing for at least 16 hr, the plate of cells must be moved to RT for the a drop below 32 °C, and using ice for 5-8 min speeds up the cooling process (**Figure 3B**) . The temperature drop changes the conformational contact angle of the material coating allowing the cell sheet to lift off of the plate. **Figure 3C** shows the cell sheet lifting from the plate.

Plates coated in the laboratory worked well, after some optimization, for creating and moving the cell sheets. **Figure 4D** shows a confluent monolayer of RASMC prior to transfer. When the cells were allowed to lift, the sheets tended to fold and stick to itself (**Figure 3E**)**.** In fact, manipulating the cell sheets on in-house created pNIPAAM or those purchased was difficult and often resulted in tearing of the sheets (**Figure 3F**). Therefore, a solution for transferring the sheets was developed. Once the cells are removed from the incubator and start to cool, 6% gelatin was used to cover the cells with an additional metal lattice embedded within the gel (**Figure 3G**). As the plate cooled, the cells lifted, and the gelatin hardens. Using forceps, the gelatin- lattice and cell sheet can be removed together from the culture plate; all at the same time (**Figure 3H**). Then these three components are placed on top of the based construct (**Figure 3I**). Here, the cell sheet (pink) is much larger than the underlying base matrix (thick white matrix under the pink cell sheet). The cell sheet can easily be trimmed to size.

The final cell patch (**Figure 4**) is created by layering the cell sheet onto the preformed complex of the patch base and permissive hydrogel. From bottom to top, the patch consists of a fibrous matrix seeded with hyaluronan hydrogel containing the endothelial cells, and then the cell sheet is layered on top of this matrix. Early attempts to manipulate the cell sheets without the use of the gelatin/lattice apparatus resulted in very small cell sheets that often folded and were torn (**Figure 4A-D** viewed from above). **Figure 4A** represents an early patch design composite picture combining the mitotracker red dyed RASMC (**Figure 4B**), calcein AM green fluorescent HuVECs (**Figure 4C**), and the transmitted light image (**Figure 4D**). Closer 10x images are supplied for the cell sheet and HA/Hydrogel without matrix (**Figure 4E-H**). **Figure 4E** is the composite of the mitotraker red (**Figure 4F**), the Calcein AM green HuVECs (**Figure 4G**) and the transmitted light (**Figure 4H**). The view from below (base matrix, HA/HuVEC), and cell sheet images of each component is show in **Figures 4I-L**. The composite images is **Figure 4I**, with the individual parts represented in **Figure 4J-L**, **Figure 4J** shows the cell sheet (red), the endothelial cells (green) are in **Figure 4K**, and the transmission image (**Figure 4L**) The composite image (**Figure 4M**) shows the patch with a uniform cell-sheet covering the entire area of the matrix without any folding or tearing. **Figures 4M-P** are also from the bottom looking up into the matrix. **Figure 4N** is the cell sheet, containing Neutral Red. Again, thick red strips appear around the matrix because the sheet folds in these areas directly adjacent to the edge of the matrix. Transmission light imaging (**Figure 4O**) shows the morphology of the cells, and the structure of the matrix. Finally (**Figure 4P**), the matrix has a special quality of fluorescing at the same wavelength as Dapi. Therefore, the ultraviolet excitation of the matrix is used to clearly view it separate from the cell sheet (bright red). The edges of the patch can be trimmed to remove any edges of the sheet that are hanging over the edges of the matrix.

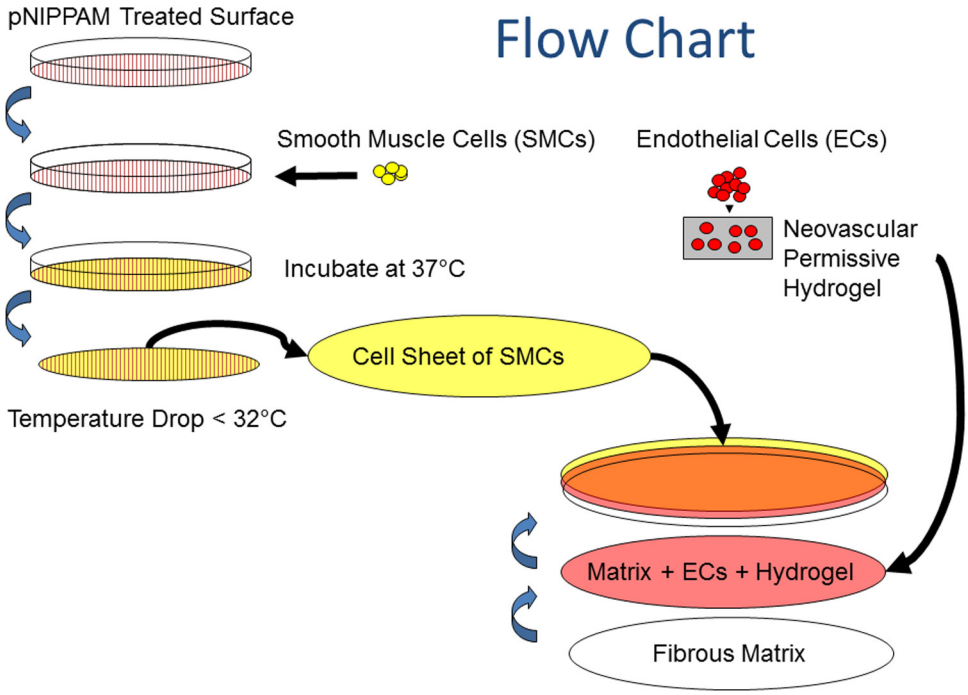

Figure 1. Flow Chart of Tissue Assembly. Cell-sheets are created by seeding cells onto the thermoresponsive pNIPAAM-coated plates, and allowing enough time for cells to reach confluence and establish lateral connections to neighboring cells. The cell sheet is released by reducing the temperature (yellow disk). Concurrently, endothelial cells are embedded into a neovascular permissive HA hydrogel, injected into voids space of the stronger base fibrous matrix, and chemically crosslinked (white disk). The cell sheet (yellow disk) is then layered with the endothelial matrix combination (white), as needed. Please click here to view a larger version of this figure.

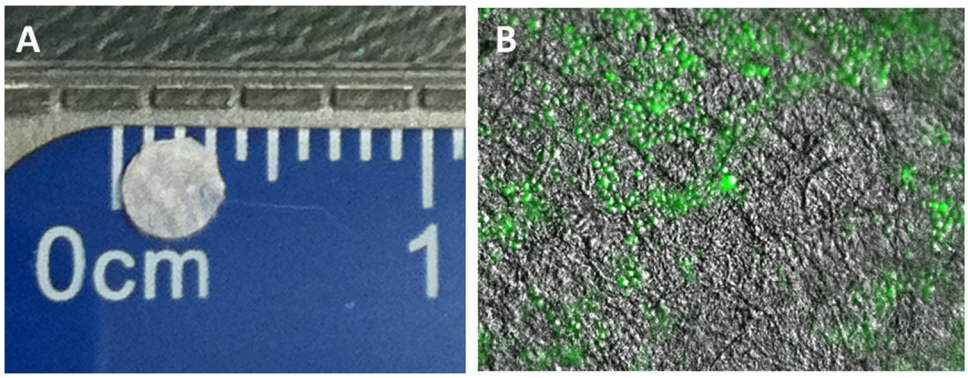

Figure 2. Images of Base Matrix and Endothelial Encapsulation. (A) The stronger base fibrous matrix is punched into a round shape. Here a 4 mm in diameter is used based on the *in vivo* animal model. **(B)** HuVECs stained with mitotracker green (Calcein AM) suspended in HA hydrogels on the surface of the base matrix (10X). Please click here to view a larger version of this figure.

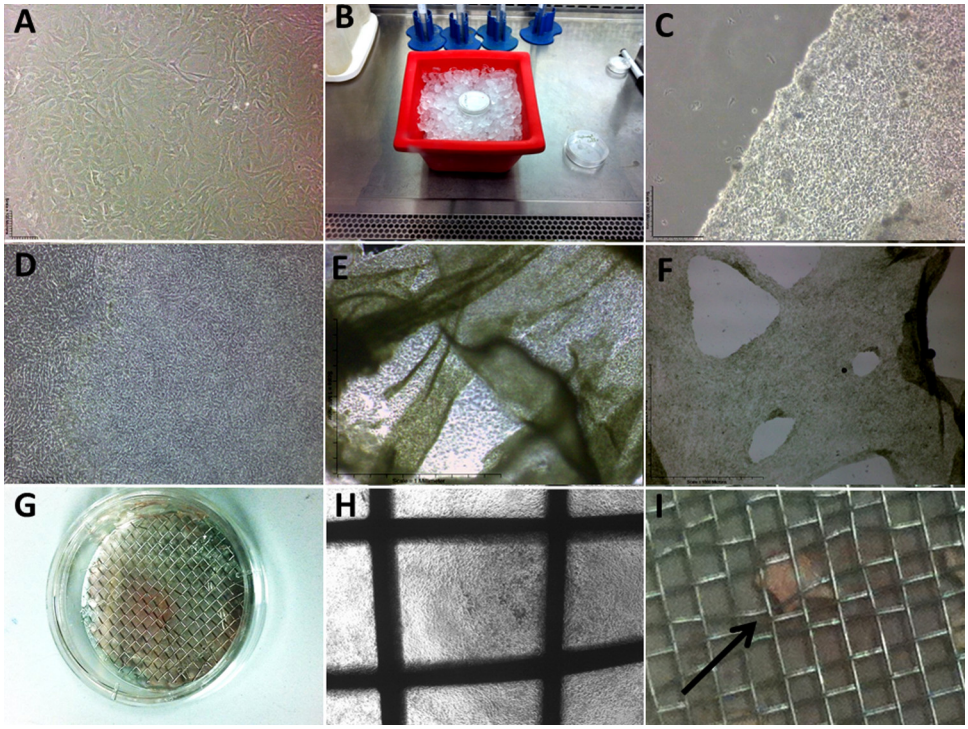

Figure 3. Creation of Cell Sheets. (A) RASMC cultured on pNIPAAM-treated 35 mm-dishes for 16 hr (10X). **(B)** RASMC lifted after placing the plate on ice for 5-7 min. **(C)** Image of the edge of a cell sheet releasing from the commercially-purchased thermoresponsive cell culture dish (10X). **(D-F)** Images depict similar results for the generation of cell sheets from in-house created pNIPAAM-coated dishes. **(D)** Confluent RASMC grown on 35 mm standard tissue culture plates (4X). **(E)** After cooling, the cell sheets contracted and folded as they detached (4X). **(F)** Due to the fragility of the single-cell sheet, the cell sheets are often damaged during lifting or other manipulations (4X). **(G)** In order to mitigate perforations in the cell sheet, a metal screen was used as a physical support to aid the transfer of the cell sheets. RASMC were stained with Neutral Red, covered with 6% gelatin and a porous metal screen support, and allowed to harden. **(H)** With the screen support, cell sheets are transferred with minimal damage (4X). **(I)** The larger RASMC cell sheet layer (pink color) was then placed on top of the stronger base fibrous patch-hydrogel combination (arrow). Please click here to view a larger version of this figure.

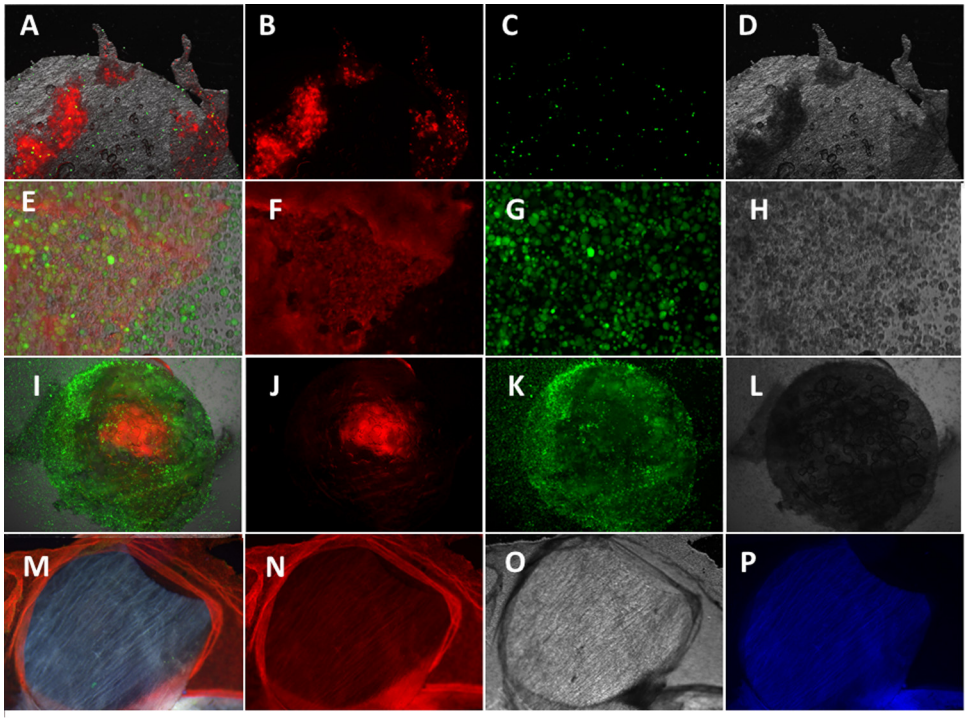

Figure 4. Images of Layered Cellular Patch. Cells were cultured on a pNIPPAM coated surface and then moved as a sheet to the surface of the fibrous matrix. Early trials that were not transferred with the gelatin/metal lattice resulted in small tattered patches (**A-D**). (**A)** Composite image of (**B-D**), **(B)** RASMCs in two sheets stained with Mitotracker Red, and **(C)** HuVECs stained with green, and **(D)** in transmitted light. **(E)** Composite image of a cell-sheet combined with the stronger base fibrous matrix-hydrogel combination containing Calceing AM stained HuVECs (green), and **(F)** Mitotracker Red RASMCs. **(G)** Green fluorescent HuVECs suspended in a hydrogel. **(H)** Transmission image of the stronger base fibrous matrix-hydrogel combination. **(I)** Composite image looking from the bottom of the patch upwards through the stronger base fibrous matrix (not stained), HA containing HuVECs (green), and cell sheet of RASMCs (red), respectively. **(J)** Red fluorescent RASMCs sheet, as seen through the base fibrous matrix-hydrogel combination. **(K)** Green fluorescent HuVECs. **(L)** Transmission image of the patch construct. **(M)** Composite picture of cell-sheets (red) over the base fibrous matrix-hydrogel combination (autofluorescent-blue). **(N)** Cells in the sheet cover the base fibrous matrix-hydrogel combination show increased fluorescence at the edges is due to bunching of the cells. **(O)** Transmission image of the construct. **(P)** Natural fibrous base matrix is autofluorescent in the DAPI (blue wavelength). Please click here to view a larger version of this figure.

Movie 1. The process of transferring the sheets of cells is highlighted in the supplemental movie submitted with the document. The movie shows, in step-wise process, the removal of the cells from the incubator; replacement of the media with 6% gelatin, the insertion of the metal screen, chilling of the cells on ice, transfer of the cells from the pNIPAAM coated dish to another dish, an image of the cells during transfer, and finally the removal of the screen from the sheet.

Discussion

The critical steps in the protocol include: coating the plate surfaces with the thermoresponsive polymer and manipulating the cell sheets after cooling the plates. Because different cells exhibit different physical properties, like adhesivity, the lifting time should be optimized for each different cell type. The second, and most significantly challenging component of this protocol, centers on the manipulation of the cell sheet, a critical aspect of methods for tissue assembly. The single cell layer in the cell sheet is quite fragile and can tear easily if manipulated with forceps. Moreover, when the cell sheets are not held in place, they tend to contract and can fold easily. The proposed cell sheet transfer using a supportive screen aids the manipulation of the fragile cell sheet.

The design methodology presented in this manuscript provides a fairly low cost approach to creating a cell sheet for a variety of tissue engineering applications. Moreover, creating pNIPAAM coated plates within a university laboratory can be standardized with this method and modifications to the protocol can be made to accommodate multiple cell types, and surfaces. The use of cell sheets, rather than unorganized single cells, aids tissue assembly, and may also increase the likelihood of survival and integration of implanted tissues. However, the *in vivo* delivery method (not mentioned in this protocol) of these constructs will need to be optimized for each tissue type. Future applications include explorations and optimized methodologies for generating tissues for specific organ systems.

Disclosures

We have nothing to disclose.

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