Title

Tissue engineering: construction of a multicellular 3D scaffold for the delivery of layered cell sheets.

Authors

Turner, William S
Sandhu, Nabjot
McCloskey, Kara E

Publication Date

2014-10-01

DOI

10.3791/51044

Peer reviewed

Journal of Visualized Experiments

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

--Manuscript Draft--

Manuscript Number:	
Article Type:	Invited Methods Article - JoVE Produced Video
Section/Category:	JoVE Bioengineering
Keywords:	Cell Delivery; Matrices; Tissue Engineering; Cardiac Patch; Cell Sheet Engineering.
Corresponding Author:	William Turner UNITED STATES
First Author:	William Turner
Order of Authors:	William Turner
	William S Turner
	Nabjot Sandhu
	Kara E McCloskey
Abstract:	Some tissues, such as the adult human hearts, are unable to adequately regenerate after damage. Strategies in tissue engineering (TE) propose innovations to assist the body in recovery and repair. For example, TE approaches may be able to attenuate heart remodeling after myocardial infarction. As with any functional tissue, successful regeneration involves the proper delivery of specific cell types (cardiomyocytes, endothelial-cells, and most likely smooth-muscle-cells, as well as cardiac-fibroblasts) and environmental cues favoring integration and survival. Engineered cardiac-tissues address elements of soluble signals, cell-to-cell interactions or the use of extracellular matrix materials as delivery vehicles, where other techniques of cell injection ignore these tenants. To date, a patch design combining these ingredients has yet to be examined due to various levels of difficulty in manipulation of the engineered materials. We have developed a method of patch construction that combines cell-encapsulation, cell-sheet-engineering, as well as, the use of natural fibrous matrices for the purpose of delivering cells for tissue regeneration. We demonstrate that cells with pre-established cell-to-cell interaction, in a sheet can be integrated into this construction. Our Patch architecture provides a nutrition rich protective environment permissive of allowing cellular retention. This patch design can be applied to other organs other than heart with minimal design and material changes, and is meant to be a quickly built, off-the-shelf product for regenerative therapies. This protocol has five detailed steps. First we use the temperature-sensitive Poly(N-isopropylacrylamide) pNIPAAM to coat our tissue culture dishes. Then we culture our tissue specific cells on the surface of those plates to form cell-sheets with strong lateral adhesions. Thirdly, we create a base for the patch by combining porous matrix with neo-permissive hydrogels and endothelial cells. Finally, we lift the cell-sheets from the pNIPAAM coated dishes and transfer the sheet to the base element, making the complete construct.

UNIVERSITY OF CALIFORNIA

BERKELEY • DAVIS • IRVINE • LOS ANGELES • MERCED • RIVERSIDE • SAN DIEGO • SAN FRANCISCO

SANTA BARBARA • SANTA CRUZ

School of Engineering
5200 North Lake Rd
Merced, CA 95343

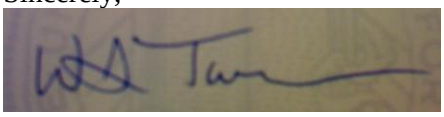
April 2, 2013

Dear Editor,

Please find an attached manuscript by W.S. Turner et al, titled "Tissue Engineering: Construction of a Multicellular 3D Scaffold for the Delivery of Layered Cell Sheets and Matrix," which we feel would be a superb candidate for publication in the Journal of Visualized Experiments. This original submission relates our technique for construction of a tissue engineering patch that can be applied to solid organs of the body. The patch is unique in its ability to offer a shelter for protecting cells in a nutrient rich environment as well as organizing multiple cell types into unique patterns.

Thank you for your consideration. We look forward to hearing your impressions.

Sincerely,



William S. Turner, Ph.D.

Suggested 5 peer reviewers with their institutional affiliation and email address.

Kent Leach
University of California, Davis
jkleach@ucdavis.edu

Michelle Khine
University of California, Irvine
mkhine@uci.edu

Bob Dennis
University of North Carolina, Chapel Hill
bob@unc.edu

Buddy Ratner
University of Washington
ratner@uweb.engr.washington.edu

Glenn Prestwich
University of Utah
gprestwich@pharm.utah.edu

Jan Nolta
University of California, Davis
jan.nolta@ucdmc.ucdavis.edu

TITLE:

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

Authors:
William S. Turner, Nabjot Sandhu, Kara E. McCloskey

Authors: institution(s)/affiliation(s) for each author:
William S. Turner
School of Engineering
University of California, Merced
5200 N Lake Rd,
Merced, CA 95343
wturner5@ucmerced.edu

Nabjot Sandhu
School of Engineering
University of California, Merced
nsandhu3@ucmerced.edu

Kara E. McCloskey
School of Engineering
University of California, Merced
kmccloskey@ucmerced.edu

Corresponding author:
William S. Turner

Keywords:
Cell Delivery, Matrices, Tissue Engineering, Cardiac Patch, Cell Sheet Engineering

Short Abstract:

The assembly of multiple materials and cell types into a composite "patch" is necessary to recapitulate highly organized structures, such as heart and muscle. Injection of cells and/or single materials alone has shown variable success in other organ systems and limited success in cardiac regeneration.[1-7] Currently, stem cell-derived cells are delivered to damaged tissue using a variety of delivery methods including: direct cell injection, coronary artery perfusion (for the case of the heart), and multiple material carriers like hydrogels, synthetic polymers, and natural matrices. Our method of patch construction is aimed at enhancing cell packaging by using a combination of cell sheet and materials-based components for the delivery of both tissue specific functional cells and endothelial cells. This design combines strengths of both materials and multiple cell types. The base fibrous matrix, provides the physical strength to the construct which is also filled with a neo-vessel permissive hydrogel with endothelial cells for establishing vascularization and integration of the cell sheets.

Long Abstract:

Some tissues, such as the adult human hearts, are unable to adequately regenerate after damage.[8,9] Strategies in tissue engineering (TE) 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.[10] As with any functional tissue, successful regeneration of cardiac tissue involves the proper delivery of specific cell types (cardiomyocytes, endothelial cells, and most likely smooth muscle cells, as well as cardiac fibroblasts) and environmental cues favoring integration and survival. Engineered cardiac tissues address elements of soluble signals, cell-to-cell interactions or the use of extracellular matrix materials as delivery vehicles, where other techniques of cell injection ignore these tenants.[9,11,12] To date, a patch design combining these ingredients have yet to be examined due to various levels of difficulty in manipulation of the engineered materials. Here, we have developed a method of patch construction that combines cell encapsulation, cell sheet engineering, as well as, the use of natural fibrous matrices for the purpose of delivering cells with the specific purpose of tissue regeneration. Furthermore, we demonstrate that cells with pre-established cell-to-cell interaction, in a sheet can be integrated into this construction. Patch architecture is aimed at providing a

nutrition rich protective environment permissive of allowing cellular retention. This patch design can be applied to other organs other than heart with minimal design and material changes, and is meant to be a quickly built, off-the-shelf product for regenerative therapies. This protocol has five detailed steps. First we use the temperature sensitive Poly(*N*-isopropylacrylamide) pNIPAAM to coat our tissue culture dishes. Then we culture our tissue specific cells on the surface of the coated plates to form sheets of cells with strong lateral adhesions. Thirdly, we create a base for the patch by combining porous matrix with neo-permissive hydrogels and endothelial cells. Finally, we lift the cell sheets from the pNIPAAM coated dishes and transfer the sheet to the base element, making the complete construct.

Protocol Text:

The overall production process for our layered cellular patch is outlined by the flowchart in **Figure 1**. Here we use pNIPAAM-treated surfaces to culture muscle cells. These cells are incubated at 37°C for a minimum of 24 hours at confluence to establish lateral connections between adjacent cells. To release the cell sheet, the plate is subjected to temperatures below 32°C. The cell sheet is then moved to the base matrix containing a neovascular-permissive hydrogel with vascular endothelial cells embedded into a stronger fibrous matrix.

1.) Creation of pNIPAAM-coated plates

1.01) Weigh out 2.6 grams of pNIPAAM (Sigma-Aldrich)

1.02) Make a 60% toluene / 40% hexane (Sigma-Aldrich) solution

1.03) Dissolve the 2.6 grams of pNIPAAM in the 60%tolune/40%hexane solution.

1.04) Heat the mixture to 60°C.

1.05) Cut filter paper into a circle and place paper in the Buchner funnel.

1.06) Weigh a glass beaker.

1.07) Filter the solution through Buchner Funnel into the glass beaker (hexane will melt plastics)

1.08) Vacuum the beaker and contents overnight.

Note: Until it is reacted with isopropyl it will oxidize so make sure it does not come into contact with oxygen.

1.09) Weigh the beaker to establish the weight of the pNIPAAM.

1.10) Add the same amount of isopropyl alcohol in weight as the pNIPAAM creating a 50/50 solution.

1.11) Place the solution on surface to coat, allowing it to sit for 5 minutes under UV light.

1.12) Wash the plate with warm PBS 2x before use.

2.) Creation of Cell Sheets

Note 1: 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 we have done here. Pre-coated thermo-sensitive plates are also offered by a number of vendors.

Note 2: This protocol is for culture using a 35mm dish.

2.01) Isolate the cell population. (This method is dependent on the individual derivation procedures and the type of muscle cells used.) Rat aortic smooth muscle cells (RAOSMC) are used in this example. These are primary smooth muscle cells isolated from the abdominal aorta of a rat and used below passage # 50 (Lonza).

2.02) Wash the cells with 2ml of warm PBS.

2.03) Add 3ml of trypsin for 5 minutes.

2.04) Inhibit trypsin by addition of culture media, or phosphate buffer solution (PBS) containing 10% Fetal Bovine Serum (FBS).

2.05) Count the cells.

2.06) Spin the cells at 1000rpm for 5min.

2.07) Aspirate the supernatant and resuspend the cells in SmGM2 plus bullet kit (Lonza) culture medium.

2.08) Place the media containing the cells on a 35ml thermo-sensitive plate – pNIPAAM coated plate at a concentration that will achieve 100% confluence. For RAOSMCs we have determined that number to be 100,000 cells/cm^2. However, due to loss of cells during the passing, we use 120% of our final value.

2.09) Place into an incubator at 37C overnight.

3.) Preparation of Foundational Matrix

3.01) Various 3D fibrous matrices can be used to layer strong fibrous matrix between the delicate cell sheets. Some examples include: gelfoam (Pfizer), bioglass, natural acellularized materials[13] or nanospun materials[14,15] The porcine urinary bladder matrix (UBM) used in these studies was generously provided from our collaborator, Dr Badylak.[16]

3.02) Prior to use, matrix characteristics should be determined including the lack of cellular content if decellularized matrix is used,[14,15] cell specific viability, and void space.[17]

3.03) Sterilize the fibrous matrix with 40 Gray (40 J/kg), over a 10 min period.[18]

3.04) Cut the matrix into a desired size and shape. Here, we use a hole-punch to cut a 4mm diameter circle.

4.) Seeding Endothelial Cells into a Neovascular Permissive Hydrogel

4.01) Endothelial cells can be obtained from primary variety of sources, including differentiation from stem or progenitor cells. Here, we use HuVECs (Invitrogen).

4.02) Any permissive hydrogel can be used as long as the cross-linking time is short enough to allow the cells stay viable. Here, we use HyStem (produced by Glycosan, a subsidiary of Biotime; Alameda, CA)

4.03) Preparation of the hydrogel is done in accordance with the company protocol.

4.04) Collect endothelial cells and disperse into a single cell solution using 1x trypsin (Accutase or Cell Dissociation Buffer could also be used for single cell dispersion)

4.05) Deactivate the trypsin enzyme by using an equal amount of soybean trypsin inhibitor or 10% FBS in PBS, collecting the solution/cells into a 15ml falcon tube.

4.06) Count the cells, and calculate the volume needed for the patch dimensions (previously quantified). For a 4mm patch we will use 2 million endothelial cells.

4.07) Extract 2 million cells, and place into a new 15ml falcon tube.

4.08) Spin at (1000rpg) for 5 minutes.

4.09) Aspirate the supernatant, leaving the cells as a pellet in a falcon tube

4.10) Add 4/5 of the total volume desired from the 1:1 mixture of HyStem:Gelatin-S.

4.11) Resuspend the endothelial cells in the mixture

4.12) Place resuspended cells onto the base fibrous matrix cut in Step 2.

4.13) Add 1/5 of the total volume desired from the Extralink (crosslinker).

4.14) Incubate for 1 hour at 37C.

5. Isolation of Cell Sheets

5.01) Remove the 35mm pNIPAAM -treated plates from the incubator and place in a cell culture hood at room temperature.

5.02) Quickly remove the media from the cells, and add in 2ml of 6% gelatin that has been heated to 37°C.

5.03) While the gelatin is still warm, place the metal lattice into the gelatin, submerging it below the surface of the gelatin (Movie 1).

5.04) Place the plate onto ice for 5 to 7 minutes, allowing the gelatin to harden.

5.05) After 7 minutes, use a spatula to carefully ring the plate, and then use forceps to lift the metal lattice from the plate (the 6% gelatin, and the cell sheet should lift with the lattice)

5.06) Move the cell sheet to the dish with the based matrix with hydrogel, and carefully set the lattice on top of the construct. (The apical side of the cell sheet will still be in the top position)

5.07) Add 2ml of warm media (37C).

5.08) Incubate overnight allowing the sheet of cells to adhere to the hydrogel surface, and remove the lattice after 24 hours

Representative Figures and 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 (UpCell) can also be used for creating the cell sheets. Special topological surfaces are used to specifically pattern (i.e. align) the cells[19].

The base fibrous matrix can be generated from decellularizing native tissue matrix or electronspun. Here, we show the fibrous material sheet cut to a 4mm 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 that we used here has been previously characterized and published.[17]

The hydrogels containing endothelial cells are cross-linked after application of the HyStem liquid components to the fibrous matrix. Fluorescence/transmission microscopy shows living cells stained with Calcien 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, (UpCell plates; Nunc). RAOSMC are plated on the pNIPAAM treated surface for at least 16 hours at 37C. 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 hours, the plate of cells must be moved to room temperature for the a drop below 32°C, and using ice for 5-8 minutes 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 RAOSMC 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 pNIPAAM or purchased UpCell plates was difficult and often resulted in tearing of the sheets **(Figure 3F)**. Therefore, we needed to come up with a solution for transferring the sheets. 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** top down view). Figure 4A represents an early patch design composite picture combining the mitrotracker red dyed RAOSMC **(Figure 4B)**, calcien 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 5E** is the composite of the mitotraker red (**Figure 4F**), the Calcein AM green HuVECs (**Figure 4G**) and the transmitted light (**Figure 4H**). The bottom up view (base matrix, HA/HuVEC), and cell sheet images of each component is show in **Figures 5I-L**. The composite images is **Figure 5I**, with the individual parts represented in **Figure 5J-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**), our matrix has a special quality of fluorescing at the same wavelength as Dapi. Therefore we can use the ultraviolet excitation of the matrix to clearly separate it 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.

Tables and Figures:

Figure 1: Flow Chart. PDMS plates are treated with pNIPAAM monomer solution. Then cell-sheets are created by seeding cells onto the surface of the coated plate. The plate is kept at an incubation temperature of 37°C allowing the cells to reach confluence and establish lateral connections to neighboring cells. Upon the drop in temperature, the pNIPAAM changes its conformation and become hydrophobic, thus allowing the cell sheet to detach (yellow disk). Concurrently endothelial cells are captured in a neovascular permissive hydrogel (Glycosan HyStem-C from BioTime, Inc) by crosslinking the mixture after placing it upon the surface of the fibrous matrix (white disk). The cell sheet is then transferred on top the combination (Matrix + Hydrogel) completing the construction of the patch.

Figure 2: A. The fibrous matrix is punched into a round shape 4mm in diameter to create small patches. The endothelial cells are then embedded in the fibrous matrix using a hydrogel. **B.** HuVECs stained with mitotracker green (Calcein AM) suspended in HA hydrogels on the surface of the base matrix (10x).

Figure 3: Cell Lifting - Creation of Cell Sheets: A. RAOSMC (1million) were cultured on Upcell-treated 35mm-dishes for 16 hours. The cells are adherent to the surface at 37C. (10x) **B.** RAOSMC were lifted by placing the plate on ice for 5-7 minutes. **C.** Edge of the lifted cell sheet created on UpCell surface (10x). **D.** RAOSMC grown on 60/40 Hexan-Toluen/NIPAAM coated 35mm standard tissue culture plates. (4x) **E.** When cooled, the cell sheets contracted and folded as they detached from the surface of the NIPAAM plate. (4x) **F.** Due to the fragility of the single-cell sheet, the cell sheet often gets damage during lifting or other manipulations. (4x) **G.** We, therefore, introduced a metal screen support to aid the transfer of the cell sheets. Here, the RAOSMC - stained Neutral Red (Invitrogen) – were grown on a pNIPAAM coated plate and then lifted onto 6% gelatin with a porous metal screen support **H.** and transferred to another plate without damage. (4x) **I.** The larger RAOSMC cell sheet layer (pink color) was then placed on top of the fibrous patch/hydrogel (arrow). The metal screen can then be easily removed, washed, autoclaved, and reused.

Figure 4: Final 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), RAOSMCs in two sheets stained with **B.** Mitotracker Red, with **C.** HuVECs stained with green, and **D.** in transmitted light. **E.** Composite image of a cell-sheet combined with the matrix/HA-hydrogel containing Calceing AM stained HuVECs (green) **F.** Red fluorescent RAOSMCs in a sheet. G. Green fluorescent HuVECs suspended in a hydrogel. **H.** Transmission image of (E). **I.** Composite image looking from the bottom of the patch upwards through the base matrix (not stained), HA containing HuVECs (green), and cell sheet of RAOSMCs (red), respectively. **J**. Red fluorescent RAOSMCs sheet, as seen through the matrix **K.** Green fluorescent HuVECs. **L**. Transmission image of the patch construct. **M**. Composite picture of cell-sheets (red) over the base matrix (autofluorescent-blue). Cell sheets were maintained without tearing in making patches by implementing the use of the gelling/metal lattice construction technique. **N**. Cells in the sheet cover the base matrix, increased fluorescence at the edges is due to bunching of the cells. **O**. Transmission image of the construct. **P.** Natural matrix is autofluorescent in the Dapi (blue wavelength).

Discussion: The method presented in this manuscript is a fairly low cost means of creating a cell sheet for the purpose of tissue engineering applications. Creating pNIPAAM coated plates within a university laboratory can be standardized with this method. The most significantly challenging component of this protocol centers on the manipulation of the cell sheet. The cell sheet is rather fragile and will tear if manipulated with forceps. Moreover, when the cell sheet is not held in place, it tends to contract. The current construction allows for the use of multiple cell types and some organization. The use of cell constructs (sheets) rather than single cells, may increase the likelihood of survival and integration for tissue engineered regenerative medicine applications. It is now possible to work with implanting tissues rather than just the cells that make them up. Furthermore, this will allow scientist to study the nature of tissue structure in its importance for cell survival and function after implantation.

Acknowledgments: This work was funded by a New Faculty Award II from the California Institute of Regenerative Medicine (CIRM; RN2-00921-1), NIH-funded National Research Award (F32-HL104924), and CIRM Training Grant (TG21163). Materials were provided by: Glycosan Biosystems Inc / BioTime and Dr. Stephen Badylak (University of Pittsburgh)

Disclosures: I have nothing to disclose.

Table of Specific Reagents and Equipment:

Name of the reagent	Company	Catalogue number	Comments (optional)
Calcein-AM	Invitrogen	C3099	Cell tracker / live dye
Lysotracker Red	Invitrogen	L7528	Cell tracker
Neutral Red	Sigma	N7005	Visible Cell dye

Supplementary Movie:

The process by which we can transfer 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.

References:

1 Etzion, S. *et al.* Influence of embryonic cardiomyocyte transplantation on the progression of heart failure in a rat model of extensive myocardial infarction. *J Mol Cell Cardiol* **33**, 1321-1330, doi:10.1006/jmcc.2000.1391 S0022-2828(00)91391-9 [pii] (2001).

2 Koh, G. Y., Soonpaa, M. H., Klug, M. G. & Field, L. J. Strategies for myocardial repair. *J Interv Cardiol* **8**, 387-393 (1995).

3 Li, R. K. *et al.* Construction of a bioengineered cardiac graft. *J Thorac Cardiovasc Surg* **119**, 368-375, doi:S0022522300946403 [pii] (2000).

4 Muller-Ehmsen, J. *et al.* Rebuilding a damaged heart: long-term survival of transplanted neonatal rat cardiomyocytes after myocardial infarction and effect on cardiac function. *Circulation* **105**, 1720-1726 (2002).

5 Reinecke, H., Zhang, M., Bartosek, T. & Murry, C. E. Survival, integration, and differentiation of cardiomyocyte grafts: a study in normal and injured rat hearts. *Circulation* **100**, 193-202 (1999).

6 Roell, W. *et al.* Cellular cardiomyoplasty improves survival after myocardial injury. *Circulation* **105**, 2435-2441 (2002).

7 Soonpaa, M. H. *et al.* Potential approaches for myocardial regeneration. *Ann N Y Acad Sci* **752**, 446-454 (1995).

8 Jakob, P. & Landmesser, U. Current Status of Cell-Based Therapy for Heart Failure. *Curr Heart Fail Rep*, doi:10.1007/s11897-013-0134-z (2013).

9 Chen, Q.-Z., Harding, S. E., Ali, N. N., Lyon, A. R. & Boccaccini, A. R. Biomaterials in cardiac tissue engineering: Ten years of research survey. *Materials Science and Engineering: R: Reports* **59**, 1-37 (2008).

10 Tongers, J., Losordo, D. W. & Landmesser, U. Stem and progenitor cell-based therapy in ischaemic heart disease: promise, uncertainties, and challenges. *Eur Heart J* **32**, 1197-1206, doi:10.1093/eurheartj/ehr018 ehr018 [pii] (2011).

11 Masuda, S., Shimizu, T., Yamato, M. & Okano, T. Cell sheet engineering for heart tissue repair. *Adv Drug Deliv Rev* **60**, 277-285, doi:S0169-409X(07)00249-9 [pii] 10.1016/j.addr.2007.08.031 (2008).

12 Laflamme, M. A. *et al.* Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol* **25**, 1015-1024, doi:nbt1327 [pii] 10.1038/nbt1327 (2007).

13 Song, J. J. & Ott, H. C. Organ engineering based on decellularized matrix scaffolds. *Trends Mol Med* **17**, 424-432, doi:10.1016/j.molmed.2011.03.005 S1471-4914(11)00063-3 [pii] (2011).

14 Badylak, S. F. *et al.* The use of extracellular matrix as an inductive scaffold for the partial replacement of functional myocardium. *Cell Transplant* **15 Suppl 1**, S29-40 (2006).
15 Wang, Y. *et al.* Lineage restriction of human hepatic stem cells to mature fates is made efficient by tissue-specific biomatrix scaffolds. *Hepatology* **53**, 293-305, doi:10.1002/hep.24012 (2011).
16 Gilbert, T. W. *et al.* Collagen fiber alignment and biaxial mechanical behavior of porcine urinary bladder derived extracellular matrix. *Biomaterials* **29**, 4775-4782, doi:10.1016/j.biomaterials.2008.08.022 S0142-9612(08)00622-4 [pii] (2008).
17 Turner, W. S. *et al.* Cardiac tissue development for delivery of embryonic stem cell-derived endothelial and cardiac cells in natural matrices. *J Biomed Mater Res B Appl Biomater* **100**, 2060-2072, doi:10.1002/jbm.b.32770 (2012).
18 Turner, W. S. *et al.* Human hepatoblast phenotype maintained by hyaluronan hydrogels. *J Biomed Mater Res B Appl Biomater* **82**, 156-168, doi:10.1002/jbm.b.30717 (2007).
19 Luna, J. I. *et al.* Multiscale biomimetic topography for the alignment of neonatal and embryonic stem cell-derived heart cells. *Tissue Eng Part C Methods* **17**, 579-588, doi:10.1089/ten.TEC.2010.0410 (2011).

Figure 1
Click here to download high resolution image

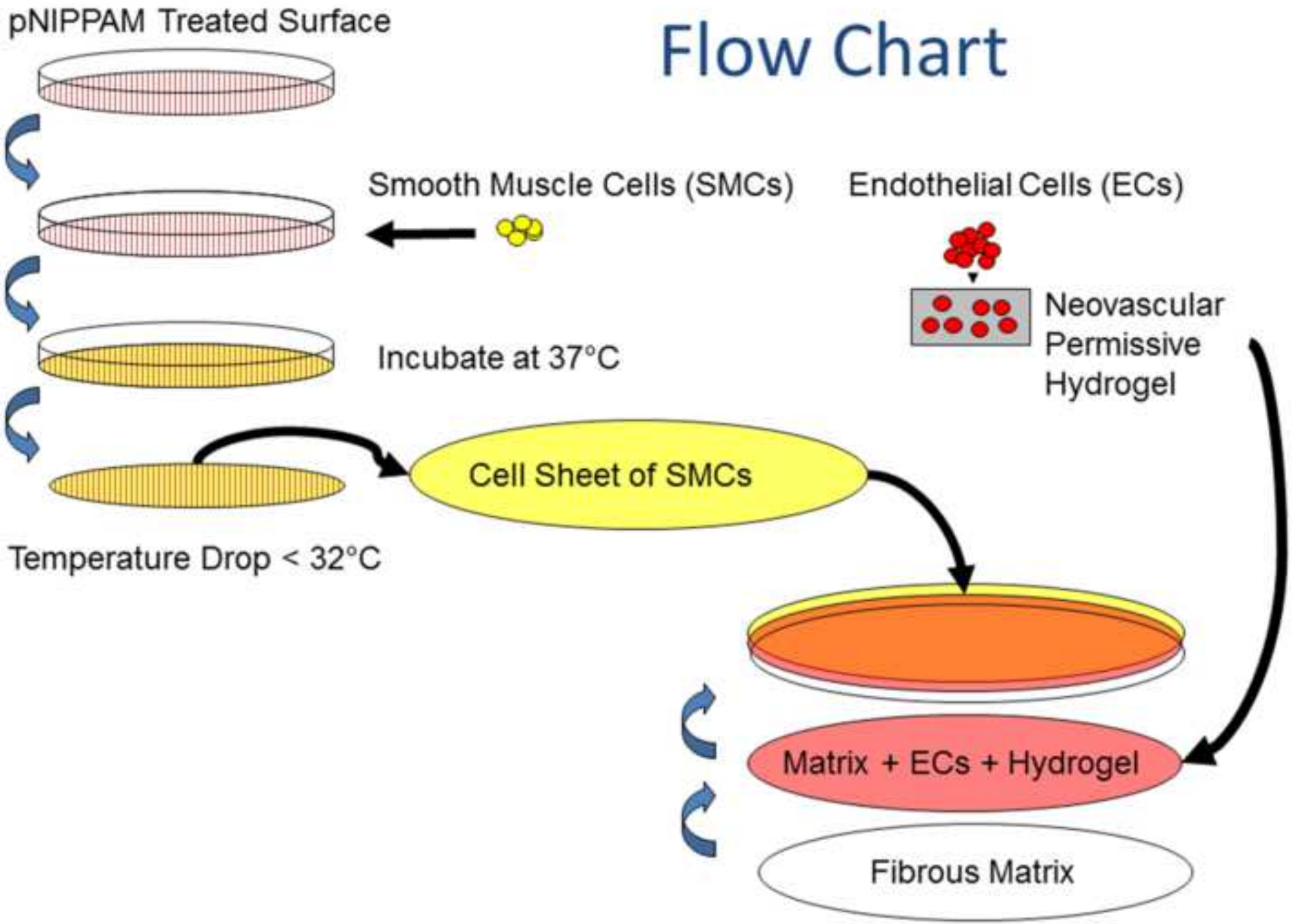


Figure 2
Click here to download high resolution image

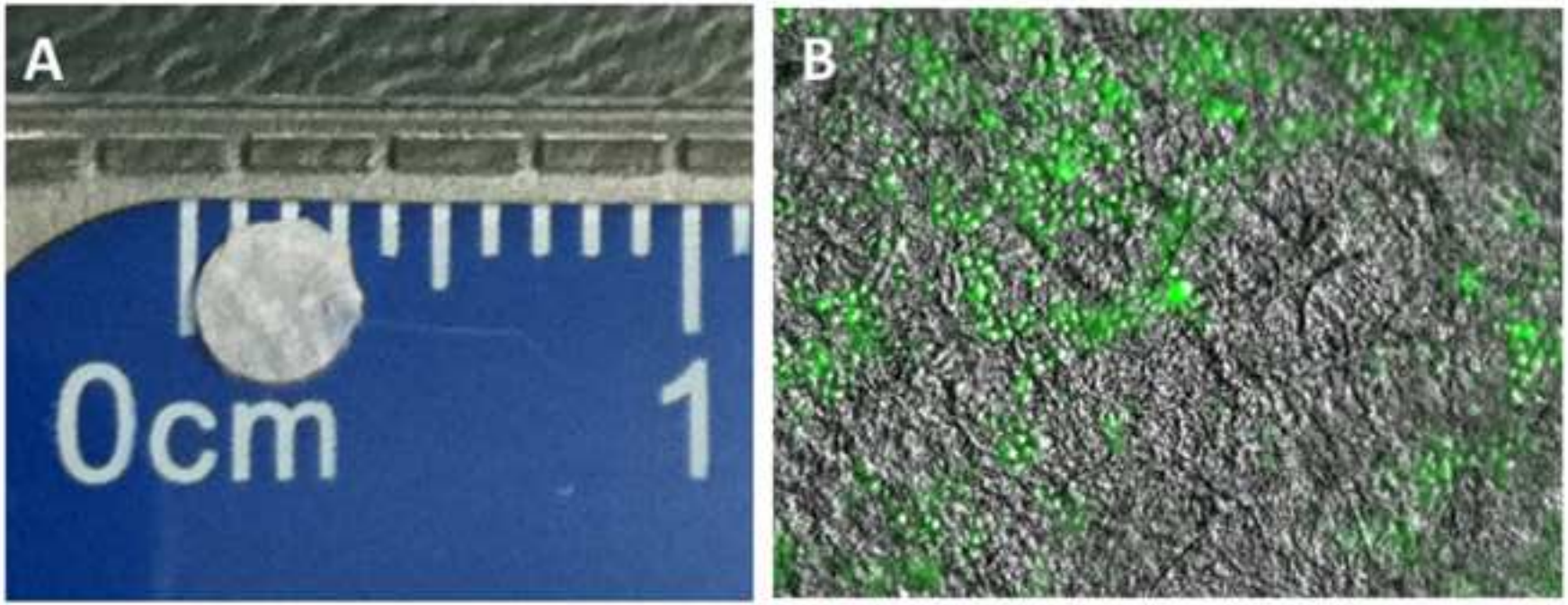


Figure 3
Click here to download high resolution image

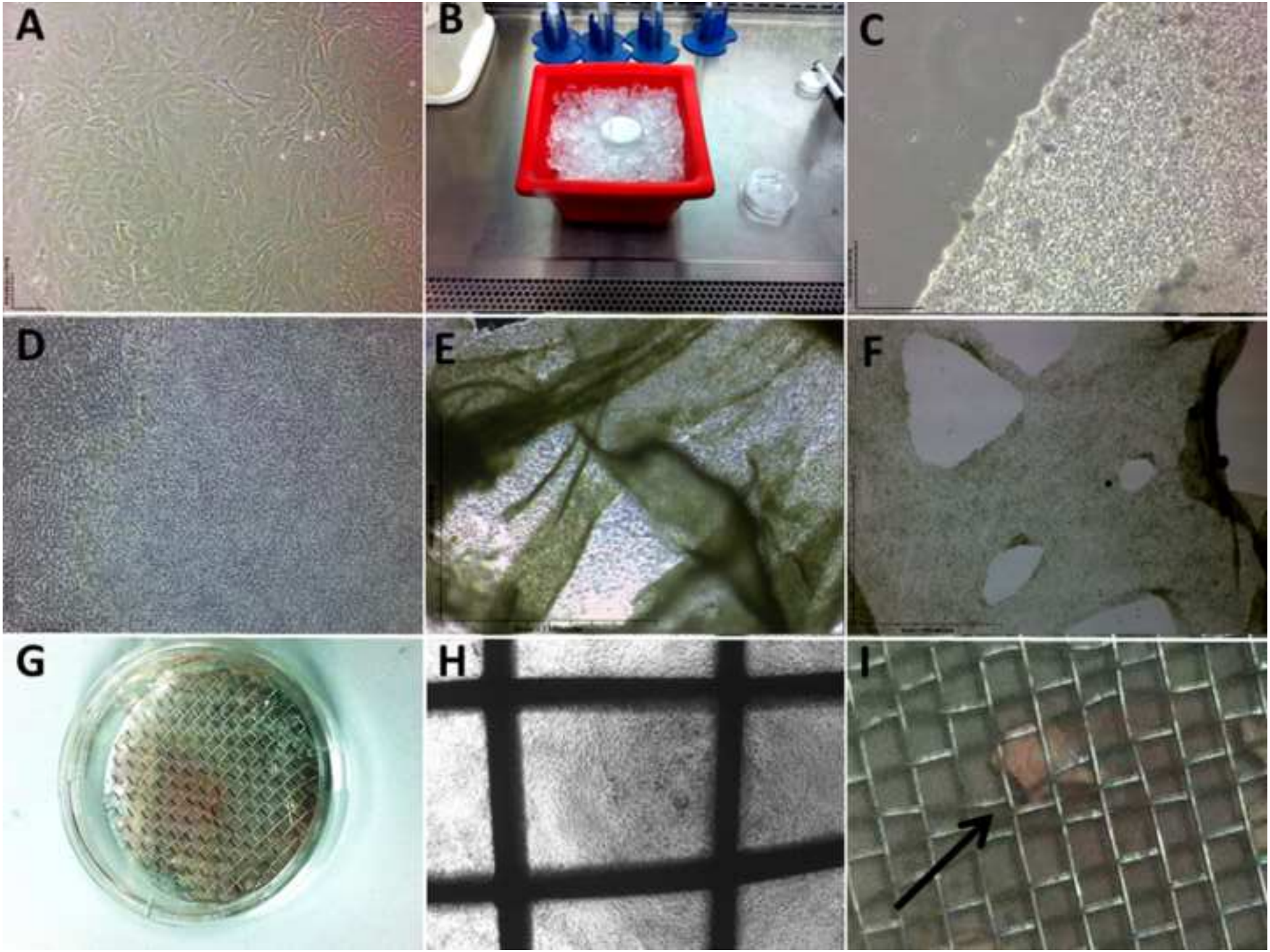


Figure 4
Click here to download high resolution image

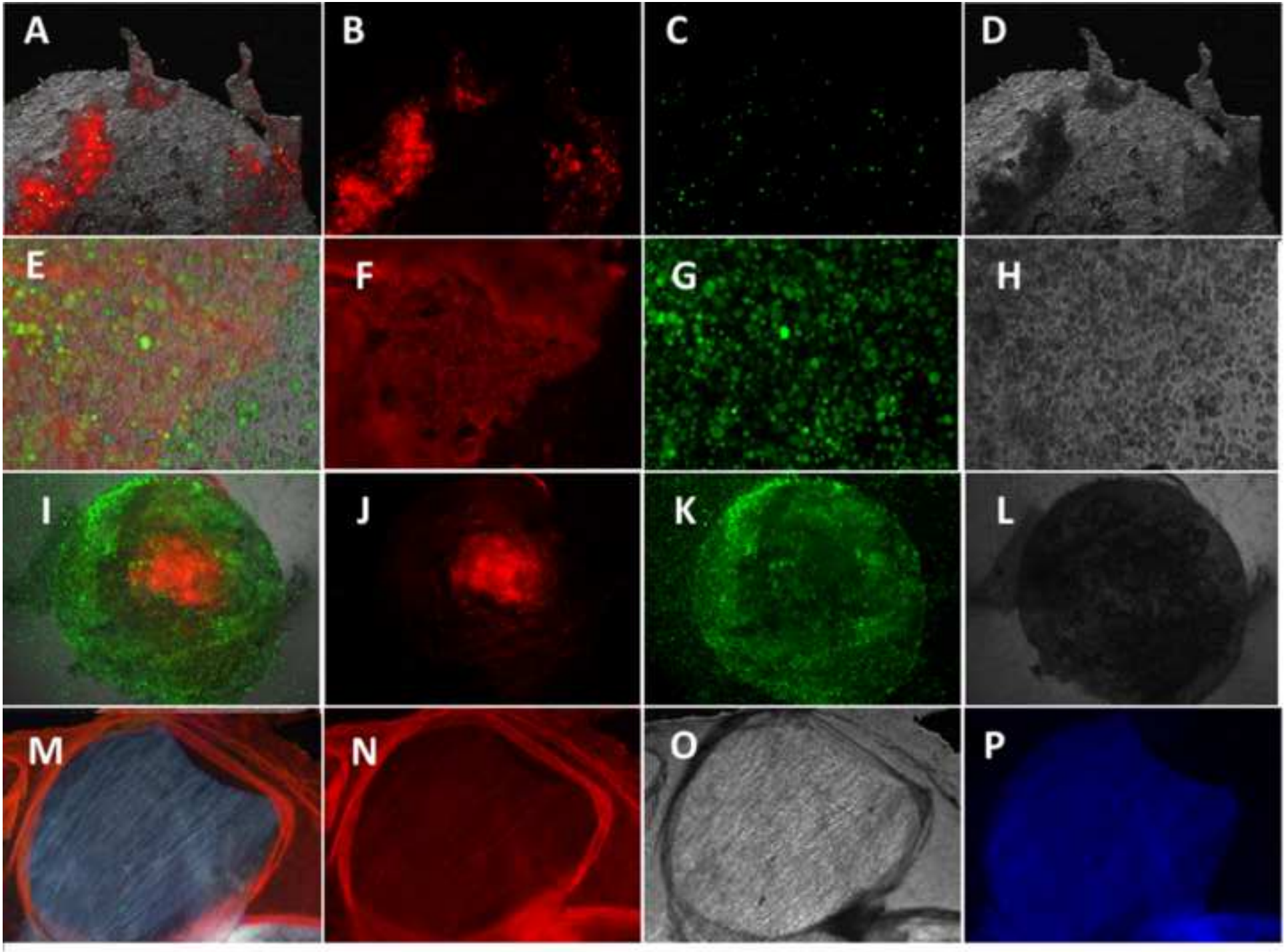


*Table of Reagents/ Materials Used

Click here to download Table of Reagents/ Materials Used: Table of Specific Reagents Final.docx

Table of Specific Reagents:

Name of the reagent	Company	Catalogue number	Comments (optional)
Calcein-AM	Invitrogen	C3099	Cell tracker / live dye
Lysotracker Red	Invitrogen	L7528	Cell tracker
Neutral Red	Sigma	N7005	Visible Cell dye

17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Author(s): William S. Turner, Nabjot Sandhu, Kara E. McCloskey

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☐ Standard Access ☐ Open Access

Item 2 (check one box):

☐ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the

Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including,

without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

AUTHOR:

Name: William S. Turner

Department: School of Engineering

Institution: University of California, Merced

Article Title:

Signature: William S. Turner Digitally signed by William S. Turner DN: cn=William S. Turner, o=University of California - Merced, ou=School of Engineering, email=wturner5@ucmerced.edu, c=US Date: 2013.04.04 14:27:46 -07'00' Date: 04/04/13

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy as a PDF to the *JoVE* submission site upon manuscript submission (preferred);
2) Fax the document to +1.866.381.2236; or
3) Mail the document to *JoVE* / Attn: *JoVE* Editorial / 17 Sellers St / Cambridge, MA 02139

For questions, please email editorial@jove.com or call +1.617.945.9051.

MS # (internal use):