Published in final edited form as:

Methods Mol Biol. 2014 ; 1181: 69–81. doi:10.1007/978-1-4939-1047-2_7.

Native Cardiac Extracellular Matrix Hydrogels for Cultivation of Human Stem Cell-Derived Cardiomyocytes

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Summary

Biomaterial scaffolds made of native and synthetic materials are designed to serve as a structural and informational template for cell attachment and tissue formation. The use of native extracellular matrix (ECM) is of special interest for the culture of cardiac stem and progenitor cells due to the presence of intrinsic regulatory factors regulating cardiac function. We describe here how to obtain native ECM hydrogels from porcine hearts for the culture of human embryonic, induced pluripotent, and somatic stem cells for cardiac tissue engineering and regenerative medicine applications.

Keywords

Native; Heart; Cardiac; Extracellular matrix; ECM; Hydrogel; *In vitro*; Stem cells

1. Introduction

In our body, cells reside in the extracellular matrix providing tissue-specific molecular, structural and mechanical signals that regulate cell behavior. In culture, biomaterial scaffolds provide the functions of the native ECM during the formation and subsequent culture of cells and engineered tissue constructs. Hydrogels are the most frequently used scaffolds for the study of living cells and the delivery of cells to repair sites. Ideally, a hydrogel should mimic the native tissue matrix in order to provide a “natural” environment for the cells. In addition, it is of interest to have a range of hydrogel properties, as the environments encountered by cells can change with time, location within a tissue region, and the state of health or disease.

We have developed a method for generating hydrogels from a number of tissues, including the heart. For research purposes, we routinely derive hydrogels from animal heart tissues (such as porcine or murine) and human heart tissues that we obtain through IRB protocols (e.g., explanted human hearts, cores taken during the implantation of the pump in LVAD patients). Using this method, we can also obtain hydrogels consisting of tissue matrix from a

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specific region (such as the left or right ventricle) or tissue matrix associated with disease (such as the infarct scar). The resulting hydrogels have tunable properties and can be blended with other components, such as collagen. The hydrogels are easy to use, as cells are simply mixed with hydrogel solution and culture medium, and polymerize after being incubated briefly in a culture well or being delivered into the heart muscle itself.

We investigated the effects of cardiac ECM on differentiation and maturation of cardiomyocytes derived from human embryonic stem cells (hESCs). To this end, we prepared a series of hydrogels by blending decellularized ECM with collagen type I at varying ratios. We found that hydrogels with high ECM content increased the expression of cardiac marker troponin T, promoted the maturation of cellular ultrastructure, and improved the contractile function of cardiac cells. The ability of native cardiac ECM hydrogel to induce cardiac differentiation of hESCs without the addition of soluble factors makes it an attractive biomaterial for basic studies of cardiac development and potentially for cell delivery into the heart. We describe here the preparation of hydrogels derived from native heart ECM and the use of these hydrogels for the culture of human cardiac stem and progenitor cells.

2. Materials

Prepare all decellularization solutions using deionized water. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing hazardous materials (*see* Note 1). Most solutions can be sterile-filtered to improve sterility.

2.1. Decellularization Reagents

1. Hypertonic phosphate buffered saline (PBS): 2X solution in water.
2. Trypsin (Life Technologies, Grand Island, NY, USA): 0.02% solution, 1.5mM EDTA, in water.
3. Tween-20[®] (Sigma-Aldrich, St. Louis, MO, USA): 3% solution, 1.5mM EDTA, in water.
4. Sodium deoxycholate (Sigma-Aldrich, St. Louis, MO, USA): 102mM solution in water (*see* Note 2).
5. Peracetic acid (Sigma-Aldrich, St. Louis, MO, USA): 0.1% solution, 4% ethanol, in water (*see* Note 3).
6. PBS: 1X solution, sterile.
7. Deionized water: sterile.
8. Penicillin/Streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

¹Sodium deoxycholate is a lung irritant and should be handled under a chemical fume hood. Peracetic acid is corrosive to the skin and eyes and hazardous in case of inhalation. Peracetic acid waste should be disposed according to Environmental Health & Safety guidelines.

²Warming the solution to 37°C aids solubility. Return solution to room temperature before use.

³Peracetic acid solution should be prepared fresh on the day of use.

2.2. Decellularization Components

1. Tweezers.
2. 50mL centrifuge tubes (Falcon, BD Biosciences, San Jose, CA, USA).
3. 50mL centrifuge tube drain caps (*see* Note 4).
4. Orbital shaker (Bellco, Vineland, NJ, USA).
5. Four-way tube racks (Fisher Scientific, Dallas, TX, USA).
6. Sterile filters.

2.3. Hydrogel Reagents

1. Cardiac extracellular matrix powder, lyophilized (Prepared in section 3.2).
2. Pepsin from porcine gastric mucosa, lyophilized, 3,200–4,500 units/mg protein (Sigma-Aldrich, St. Louis, MO, USA).
3. Hydrochloric acid: 0.01 N HCl in water, pH 1.8.
4. Sodium hydroxide: 0.1 N NaOH in water, sterile.
5. 1X PBS: sterile.
6. 10X PBS: sterile.
7. Storage solution: 1% Penicillin/Streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in 1X PBS, sterile.

2.4. Hydrogel Components

1. Tweezers.
2. Scalpel or razor blade.
3. Cutting board.
4. Paper towels.
5. Liquid nitrogen.
6. Pestle and mortar.
7. Kimwipes.
8. Rubber band.
9. FreezeZone Lyophilizer (Labonco, Kansas City, MO, USA).
10. Magnetic stir bar.
11. Magnetic stir plate.
12. Parafilm.

⁴Drain caps can be made by drilling a concentric pattern of 1 mm diameter holes through 50 mL tube caps. Alternatively, tubes may be uncapped and solutions poured out carefully to avoid loss of tissue.

13. Microcentrifuge tubes.

2.5. Cell Culture Reagents

1. StemPro34 Kit (Invitrogen, 10639-011) (*see* Note 10).
2. DMEM/F12 (Cellgro)
3. Penicillin/streptomycin (Sigma)
4. PBS (with Ca^{2+} and Mg^{2+}) (Cellgro)
5. PBS (without Ca^{2+} and Mg^{2+}) (Cellgro)
6. Glutamine (Invitrogen) (*see* Note 11).
7. Knockout serum replacement (Gibco, 10828-028)
8. Fetal calf serum
9. DNase (Millipore, 260913)
10. Transferrin (Roche, 10652202) Store transferrin into 2ml aliquots at 4°C.
11. Ascorbic acid (Sigma, A4544) (*see* Note 12).
12. Monothioglycerol (Sigma, M6145) (*see* Note 13).
13. Bovine serum albumin: 30% (Sigma).
14. Cytokines

All cytokines are stored either in the original supplied form or aliquots after reconstitution at -20 to -70°C . They are stable as the supplied form for 12 months, and 3 months after reconstitution unless otherwise indicated. If stored at 2 to 8 °C under sterile condition, it must be discarded after 1 month of use.

- i. BMP4 (R&D Systems, 314-BP). Reconstitute at 10 $\mu\text{g}/\text{ml}$ in sterile 4mM HCl containing 0.1% BSA.
- ii. VEGF (R&D Systems, 293-VE). Reconstitute at 5 $\mu\text{g}/\text{ml}$ in sterile PBS containing 0.1% BSA.
- iii. DKK (R&D Systems, 5439-DK/CF). Reconstitute at 50 $\mu\text{g}/\text{ml}$ in sterile PBS containing 0.1% BSA.
- iv. bFGF (Invitrogen, 13256-029). Reconstitute at 10 $\mu\text{g}/\text{ml}$ in sterile PBS containing 0.1% BSA. Stable for up to 6 months at -20°C after reconstitution.

¹⁰StemPro 34 contains a basal liquid medium and frozen supplement. If combined, it should be consumed within 2 weeks. Otherwise, store the medium in 50 mL aliquots and the supplements in 1.3 mL aliquots. Combine right before using.

¹¹Warm 200X L-glutamine in 37°C water bath until it is dissolved completely. Store in 5 mL aliquots at -20°C . Once thawed, use within three weeks.

¹²Prepare a stock solution of 5 mg/mL ascorbic acid in cold distilled water. Reconstitute on ice and vortex periodically until completely dissolved. Filter, aliquot, and store at -20°C . Once opened, discard the excess.

¹³Store monothioglycerol in 1 mL aliquots at 4°C. It is strongly recommended to aliquot monothioglycerol to minimize the amount of oxidation due to repeated opening of the stock bottle.

- v. Activin A (R&D Systems, 338-AC/CF). Reconstitute at 10 µg/ml in sterile PBS containing 0.1% BSA.
15. Trypsin-EDTA (Sigma, T4799). Final concentration is 0.25%. (*See Note 14*).
 16. Collagenase type I (Sigma, C0130). Reconstitute collagenase type I at 2 mg/mL in sterile PBS (with Ca²⁺ and Mg²⁺) containing 20% fetal calf serum. Filter and store as 12 mL aliquots at −20°C. (*See Note 15*).

2.6. Cell Culture Components

1. Base Medium
 - a. StemPro34 Kit
 - b. Glutamine (1%)
 - c. Transferrin (150 µg/mL)
 - d. Ascorbic acid (50 ng/mL)
 - e. Monothioglycerol (0.4 µM)
2. Aggregation Medium
 - a. Base Medium
 - b. BMP4 (0.5 ng/mL)
3. Stage I medium
 - a. Base Medium
 - b. BMP4 (10 ng/mL)
 - c. bFGF (5 ng/mL)
 - d. Activin A (3 ng/mL)
4. Stage II medium
 - a. Base Medium
 - b. VEGF (10 ng/mL)
 - c. DKK1 (150 ng/mL)
5. Stage III medium
 - a. Base Medium
 - b. VEGF (10 ng/mL)
 - c. bFGF (5 ng/mL)
6. Stop Medium

¹⁴Weigh appropriate amount of Trypsin-EDTA and add to warm PBS (without Ca²⁺ and Mg²⁺). Allow to dissolve for 15min. Once dissolved, filter and aliquot. Store at −20°C.

¹⁵Freeze-thaw cycles will damage the enzyme solution. Recommended maximum is 1 freeze-thaw cycle.

- a. DMEM/F12
 - b. Penicillin/Streptomycin (0.5%)
 - c. Glutamine (0.5%)
 - d. Knockout serum replacement (2.5%)
 - e. Fetal calf serum (50%)
 - f. DNase (30 µg/mL) Reconstitute with cold distilled water at 1 mg/mL. Filter and store in 1 mL aliquots at –20°C. Use once and discard the rest.
7. Ultra low attachment 6-well culture plate (Corning Costar, 3471)
 8. 24-well plate (BD Falcon)
 9. Hypoxic incubator/hypoxia chamber

2.7. Fluorescence Activated Cell Sorting (FACS) Analysis and Immunofluorescence Staining Components

1. Iscove's Modified Dulbecco's Medium (IMDM) (Cellgro, 15-016-CV)
2. Collagen solution for coating the chamber slides: Prepare 3 mg/mL stock solution of collagen in 0.1% acetic acid. Dilute the stock solution 1:20 in distilled water. Apply this solution to the surface of the slides ensuring even coating. Air dry at room temperature (under UV light for sterilization).
3. Triton X-100
4. Goat serum
5. 4% Paraformaldehyde: Prepare 8% stock solution by mixing 8 g paraformaldehyde in 100 mL distilled water. Heat to 60°C while stirring. Slowly add 1–3 drops 1M NaOH until solution is clear. Filter and store at 4°C. Prior to use, mix stock solution with equal volume of PBS.
6. Mounting medium (VectaShield)
7. Primary antibodies
 - i. Cardiac troponin T (clone 13–11, LabVision Neomarkers MS-295)
 - ii. KDR (R&D Systems, FABSP3P)
 - iii. C-kit (R&D Systems, FAB332A)
8. Microscope slides
9. Coverslips
10. Tweezer
11. 12×75mm tubes with cell strainer cap (BD Falcon). Wet the tubes first with a small amount of buffer.
12. Syringes

13. 20 gauge needles

3. Methods

Use deionized water for all steps unless indicated otherwise. Following decellularization, all steps should be performed using sterile technique and instruments. To improve sterility, the last steps should be performed inside a laminar flow hood when possible.

3.1. Decellularization of Heart Tissue

1. Procure porcine hearts from pigs (65–70 kg) immediately following euthanasia. Process immediately or place in saline (4°C) for no more than 24 h.
2. Thoroughly clean the heart tissue by removing excess connective tissue, blood, and debris, and rinsing with water at room temperature.
3. Blot the excess water, wrap in non-stick wax paper, and freeze at –80°C for at least 24 h. Maintain the shape of the heart in order to facilitate the preparation of thin myocardial tissue slices.
4. Slice frozen myocardium (left and right ventricles) into thin (< 1 mm) slices using a meat slicer. Blot the excess water and record the weight of each slice.
5. Place each slice within a 50 mL tube, add 40 mL of water, and load each tube into a four-way tube rack mounted on an orbital shaker (*see* Note 5). Wash for 5 min.
6. Drain water and replace with 40 mL of 2X PBS. Wash for 15 min.
7. Calculate the appropriate volume of decellularization solution for each slice according to a ratio of 15 g tissue mass to 100 mL of solution. Drain 2X PBS and replace with amount of 0.02% trypsin solution calculated for each slice based on mass of tissue. Wash all slices with trypsin for 2 h.
8. Drain the trypsin from each tube, replace with water, and wash for 5 min. Do an additional wash with 2X PBS for 15 min (40 mL each).
9. Drain 2X PBS and replace with amount of 3% Tween-20[®] solution calculated for each slice based on the mass of the tissue (15 g tissue mass to 100 mL of solution). Wash for 2 h.
10. Drain Tween-20[®], replace with water, and all slices for 5 min. Do an additional wash with 2X PBS for 15 min.
11. Drain 2X PBS and replace with amount of 102 mM sodium deoxycholate solution calculated for each slice based on mass of tissue. Wash all slices with sodium deoxycholate for 2 h.
12. Drain sodium deoxycholate, replace with water, and wash for 5 min. Do an additional wash with 2X PBS for 15 min.

⁵The shaker should be maintained at approximately 200 rpm throughout decellularization.

13. Drain 2X PBS and replace with amount of 0.1% peracetic acid solution calculated for each slice based on mass of tissue. Wash and sterilize all slices for 1 h.
14. Drain peracetic acid, replace with *sterile* 1X PBS, and wash for 15 min.
15. Replace 1X PBS with *sterile* water and wash for 5 min. Repeat wash.
16. Drain water, replace with *sterile* 1X PBS, and wash for 15 min.
17. Store tissues in *sterile* 1X PBS with 1% penicillin/streptomycin.
18. Decellularization can be confirmed by DNA quantification and histological analysis. To quantify DNA content following decellularization, use a Quant-iT PicoGreen Assay Kit (Invitrogen) according to the manufacturer's instructions. Briefly, digest ~10 mg samples in 100 μ L Proteinase K solution for 24 hrs at 60°C. (Prepare proteinase K solution by mixing 2.2 mg proteinase K in 2 mL TE buffer, provided with PicoGreen Assay Kit) For histological analysis (such as hematoxylin and eosin staining, fix samples in Accustain (Sigma) for 20 min at room temperature, then prepare samples for paraffin embedding, sectioning, and staining.

3.2. Preparation of Native ECM Hydrogels

1. Use a scalpel to cut decellularized heart tissue slices into smaller sections. Blot sections with a Kimwipe to remove excess water.
2. Snap-freeze sections by immersing the tissues in a small amount of liquid nitrogen (~5 mL) in a mortar. Pulverize the frozen tissue sections into a fine powder using a mortar and a pestle (*see* Note 6).
3. Collect the frozen ECM powder in a tube, cover the uncapped tube with a Kimwipe, secure the Kimwipe with a rubber band, and load into a lyophilizer (*see* Note 7). Lyophilize frozen ECM powder overnight.
4. After lyophilization, mix the ECM powder with pepsin in a ratio of 10:1 w/w per 100 mL 0.01 N HCl. Otherwise, for long-term storage at room temperature, wrap the cap of the tube containing the lyophilized cardiac ECM powder with parafilm.
5. Digest the solution for 48 hrs at room temperature under constant stirring using a magnetic stir bar and plate until the solution becomes viscous with no visibly undigested granules.
6. Aliquot the 10 mg/mL digested ECM solution and freeze aliquots at -80°C to terminate pepsin digestion (*see* Note 8).

3.3. Reconstitution of Native ECM Hydrogels

1. Thaw frozen aliquot of 10 mg/mL digested ECM solution at room temperature.

⁶When snap-freezing, flatten tissue sections out as much as possible to aid in the pulverization process. Sections frozen in a globular form are significantly more difficult to pulverize.

⁷A Kimwipe is used to cover the uncapped tube during lyophilization so that during retrieval electrostatic interactions do not result in the loss of any ECM powder.

⁸A bicinchoninic acid assay (BCA assay) may be performed to determine total solubilized protein concentration.

2. Place thawed aliquot of 10 mg/mL digested ECM solution and sterile hydrogel reagents (0.1 M NaOH, 10X PBS, 1X PBS) on ice and allow to cool to 4°C.
3. Generate 1 mL of 6 mg/mL cardiac ECM hydrogel by adding 60 µL 0.1 N NaOH, 67 µL 10X PBS, 275 µL 1X PBS, and 600 µL cardiac ECM digest in a micro-centrifuge tube and mix thoroughly with pipette tip (*see* Note 9). Keep cold at all times.
4. Add desired volume of mixture to cell culture plate and incubate at 37°C for 45 min.
5. Recover cardiac ECM hydrogel for the *in vitro* culture of cardiac stem cells or other desired applications.

3.4 Encapsulation of Human Embryonic Bodies in Native ECM Hydrogels

1. Human embryonic stem cells (hESCs) are maintained in 6-well ultra low attachment plates under hypoxic condition for 4 days in aggregation medium (day 0) and then in Stage I medium (day 1 to 4) to form human embryonic bodies (hEBs, aggregates of hESCs) and induce mesoderm differentiation (Yang 2008 and Duan 2011). The seeding density of hESCs is 0.5–1 million cells/well of a 6-well plate. Use 2 mL media for every 0.5–1 million cells.
2. At day 4, hESCs have formed hEBs. (*see* Note 16).
3. Thaw native ECM hydrogel overnight at 4°C the night before the encapsulation.
4. Coat 24-well plates with 50 µl hydrogel solution/well and incubate at 37°C for at least 30 min to allow for gelation before encapsulating the hEBs.
5. Remove hEBs with a 5 mL pipette and pool 3 wells of a 6-well plate per 15 mL conical tube.
6. Allow hEBs to settle for 20 min in hypoxic incubator/chamber to separate hEBs from single cells and cell debris.
7. Remove supernatants carefully and leave hEBs undisturbed at the bottom of the tube.
8. Wash hEBs with 10 mL IMDM medium supplemented with antibiotics (penicillin/streptomycin) to wash out the residual cytokines (*see* Note 17).
9. Centrifuge the hEBs at 800 rpm for 5 min and remove supernatant.

⁹Different concentrations of ECM hydrogels (ranging from 2 – 6 mg/mL) can be prepared by varying the volume of ECM digest. For concentrations below 6 mg/mL, the volume of 1X PBS should be increased to obtain the final desired volume. Alternatively, cell culture media or a cell suspension may be used in place of the 1X PBS reagent for encapsulation of cells within the cardiac ECM hydrogel.

¹⁶Gentle closing and opening of the incubator door is critical during this period to prevent disturbing the aggregation process.

¹⁷This step is critical because the cytokines BMP4 and Activin A, if not completely washed out, will affect the next stage induction even at very low concentrations. The washing must be gentle enough not to disturb the aggregates, as they are relatively easy to break apart. Aggregation is key for successful differentiation. Harvest for FACS analysis at day 4–7 to ensure mesoderm induction and cardiovascular progenitor specification in the hEBs (Section 3.5) (Yang 2008).

10. Resuspend hEBs in culture medium either with or without growth factors at a concentration of 0.5 million cells/mL. See Section 3.5 for counting method of hEBs.
11. Add 50 µl of hEB suspension to 1 mL of ECM hydrogel solution (*see* Note 18).
12. After gentle mixing, transfer 250 µl of each hEB/hydrogel mixture to a coated well of a 24-well plate and incubate at 37°C for 30–60 min to gel.
13. Add 2 mL of fresh Stage II medium or base medium to each well and maintain the culture in hypoxia chamber (*see* Note 19).
14. At day 8, remove culture plates from hypoxia chamber and change culture medium to Stage III medium or base medium (*see* Note 20).
15. Place culture plates in hypoxia chamber for additional 4 days.
16. Repeat step 12, and incubate cells in Stage III medium or base medium at ambient oxygen levels for the rest of the induction procedure.
17. Stain for cardiac markers at day 14–16 (See section 3.6 for staining of cardiac troponin T).

3.5 Disassociation of Human Embryonic Bodies for Cell Counting or FACS Analysis

1. Pool 1–3 wells of hEBs in a 15 mL conical tube for 15–20 min to separate hEBs from cell debris.
2. Aspirate medium and add 2 mL Trypsin-EDTA.
3. Incubate at 37°C for 5 min and then stop the reaction with 1 mL Stop Medium.
4. Prepare a single cell suspension by mechanically passing hEBs 4–6 times through a 20 gauge needle
5. Add 4 mL IMDM supplemented with 10% fetal calf serum
6. Centrifuge at 1000 rpm for 5 min.
7. Remove supernatant and resuspend cells in IMDM supplemented with 5% fetal calf serum (500µl/well)
8. Take 100 µl of the cell suspension for cell counting using trypan blue, Use the rest of the cells for FACS analysis.
9. For FACS analysis, filter the suspension through round-bottom tubes with a cell strainer cap to remove remaining cell aggregates and ensure a uniform single cell suspension.

¹⁸Cut off the tip of a 200 µL pipette tip for easier transportation of hEBs.

¹⁹Gently add medium along the sidewall of the culture well and try not to disturb the newly formed gel. Here we provide two types of culture medium that can be used: stage induction medium (with growth factors) and base medium alone (without growth factors). Note that stage induction medium is optimized for maximizing the percentage of cardiac progenitor cells without the addition of native hydrogel. We find that hydrogel/collagen without the addition of growth factors achieved significantly better outcome than collagen hydrogels with the supplement of growth factors (Duan 2011).

²⁰Avoid using vacuum for medium aspiration because of the fragility of the hEB/hydrogel mixture. Gently remove medium with a 1000 µL pipette.

10. Stain for desired antibodies according to the manufacture's instructions and perform FACS analysis. We use KDR and c-kit to monitor the induction of cardiac mesoderm.

3.6 Cardiac Troponin T Staining for hEBs Encapsulated in Native ECM Hydrogel

1. Gently remove culture medium from one well of the 6-well ultra low attachment plate.
2. Add 1 mL collagenase type I (containing 10 µg/ml DNase) and incubate at 37°C for 1 hr.
3. Aspirate hEBs with a 5 mL pipette into a 15 mL conical tube and add 9 mL of IMDM.
4. Wash hEBs by gently pipetting up and down 3 times.
5. Centrifuge at 800 rpm for 5 min.
6. Repeat steps 2–6 from Section 3.5.
7. Remove supernatant and resuspend cells in Stage III medium.
8. Seed cells on microscope slides coated with type I collagen and culture for 2 days.
9. Fix cells in 4% paraformaldehyde for 15 min at room temperature.
10. Wash fixed cells 3 times in PBS (without Ca^{2+} and Mg^{2+}).
11. Quench cells with 0.5M NH_4Cl in PBS containing 0.1% bovine serum albumin for 15 min.
12. Permeabilize cells using PBS containing 5% fetal calf serum and 0.1% Triton X-100 for 10 min at room temperature.
13. Wash cells in PBS for 5 min. Repeat three times.
14. Block cells for 15 min in blocking buffer (10% goat serum in PBS containing 0.1% BSA)
15. Stain cells with primary antibody (cardiac troponin T) overnight at 4°C in blocking buffer (*see* Note 21).
16. Wash cells with PBS containing 0.1% BSA and 2.5% NaCl for 15 min.
17. Stain cells with secondary antibodies according to the manufacture's instructions for 2 hrs at room temperature. Cover cells to protect from light.
18. Stain cell nuclei with Topro3 for 5 min according to the manufacture's instructions. (*see* Note 22).
19. Wash twice with PBS containing 2.5% NaCl and 0.1% BSA, 5 min each.
20. Wash with PBS alone for 5 min.

²¹Ensure that slides are stored in a moist environment to prevent desiccation of staining solution overnight.

²²If fluorescence microscope has ultraviolet excitation, use mounting medium containing DAPI to label cell nuclei.

21. Mount slides with mounting medium. Coverslip and seal with nail polish.
22. Image with fluorescence microscope.

Acknowledgments

This work was supported by the NIH (grants HL076485 and EB002520), NYSTEM (grants C026721A and CO28119), and starter funding from Columbia University Technology Ventures Office.

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