Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58314R2
Full Title:	Fabricating a Kidney Cortex Extracellular Matrix-Derived Hydrogel
Keywords:	bioengineering; Tissue Engineering; extracellular matrix; hydrogel; kidney; endothelial cells
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
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TITLE:

Fabricating a Kidney Cortex Extracellular Matrix-Derived Hydrogel

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KEYWORDS:

Bioengineering, tissue engineering, extracellular matrix, hydrogel, kidney, endothelial cells

SUMMARY:

Here we present a protocol to fabricate a kidney cortex extracellular matrix-derived hydrogel to retain the native kidney extracellular matrix (ECM) structural and biochemical composition. The fabrication process and its applications are described. Finally, a perspective on using this hydrogel to support kidney-specific cellular and tissue regeneration and bioengineering is discussed.

ABSTRACT:

Extracellular matrix (ECM) provides important biophysical and biochemical cues to maintain tissue homeostasis. Current synthetic hydrogels offer robust mechanical support for *in vitro* cell culture but lack the necessary protein and ligand composition to elicit physiological behavior from cells. This manuscript describes a fabrication method for a kidney cortex ECM-derived hydrogel with proper mechanical robustness and supportive biochemical composition. The hydrogel is fabricated by mechanically homogenizing and solubilizing decellularized human kidney cortex ECM. The matrix preserves native kidney cortex ECM protein ratios while also enabling gelation to physiological mechanical stiffnesses. The hydrogel serves as a substrate upon which kidney cortex-derived cells can be maintained under physiological conditions. Furthermore, the hydrogel composition can be manipulated to model a diseased environment which enables the future study of kidney diseases.

INTRODUCTION:

Extracellular matrix (ECM) provides important biophysical and biochemical cues to maintain tissue homeostasis. The complex molecular composition regulates both structural and functional

properties of tissue. Structural proteins provide cells with spatial awareness and allow for adhesion and migration[1]. Bound ligands interact with cell surface receptors to control cell behavior[2]. Kidney ECM contains a plethora of molecules whose composition and structure varies depending on anatomical location, developmental stage, and disease state[3,4]. Recapitulating the complexity of ECM is a key aspect in studying kidney-derived cells *in vitro*.

Previous attempts at replicating ECM microenvironments have focused on decellularizing whole tissue to create scaffolds capable of recellularization. Decellularization has been performed with chemical detergents such as sodium dodecyl sulfate (SDS) or non-ionic detergents, and it utilizes either whole organ perfusion or immersion and agitation methods[5-13]. The scaffolds presented here preserve the structural and biochemical cues found in native tissue ECM; furthermore, recellularization with donor-specific cells has clinical relevance in reconstructive surgery[14-19]. However, these scaffolds lack structural flexibility and are therefore incompatible with many current devices used for *in vitro* studies. To overcome this limitation, many groups have further processed decellularized ECM into hydrogels[20-24]. These hydrogels are compatible with injection molding and bioink and circumvent micrometer scale spatial constraints that decellularized scaffolds place on cells. Furthermore, molecular composition and ratios found in native ECM are preserved[3,25]. Here we demonstrate a method to fabricate a hydrogel derived from kidney cortex ECM (kECM).

The purpose of this protocol is to produce a hydrogel that replicates the microenvironment of the kidney cortical region. Kidney cortex tissue is decellularized in a 1% SDS solution under constant agitation to remove cellular matter. SDS is commonly used to decellularize tissue because of its ability to quickly remove immunological cellular material[6,7,9,26]. The kECM is then subject to mechanical homogenization and lyophilization[5,6,9,11,26]. Solubilization in a strong acid with pepsin results in a final hydrogel stock solution[20,27]. Native kECM proteins that are important for structural support and signal transduction are preserved[3,25]. The hydrogel can also be gelled to within one order of magnitude of native human kidney cortex[28-30]. This matrix provides a physiological environment that has been used to maintain the quiescence of kidney-specific cells compared to hydrogels from other matrix proteins. Furthermore, matrix composition can be manipulated, for example, through the addition of collagen-I, to model disease environments for the study of renal fibrosis and other kidney diseases[31,32].

PROTOCOL:

Human kidneys were isolated by LifeCenter Northwest following ethical guidelines set by the Association of Organ Procurement Organizations. This protocol follows animal care and cell culture guidelines outlined by the University of Washington.

1. Preparation of Human Kidney Tissue

1.1. Preparation of decellularization solution

1.1.1. Sterilize a 5000 mL beaker and a 70 x 10 mm stir bar.

1.1.2. Mix 1:1000 (weight:volume) sodium dodecyl sulfate (SDS) in autoclaved deionized water in the beaker. Leave the solution on a stir plate at approximately 200 rpm for 24 h or until the SDS is completely dissolved.

Note: Typically, 2500 mL of 1% SDS solution is sufficient to decellularize a single human kidney.

1.1.3. Transfer the solution to a 500 mL sterile vacuum filter and filter it into sterilized sealable containers.

1.2. Processing of kidney tissue

1.2.1. Wash and autoclave a pair of forceps, two hemostat clamps, a pair of general service grade scissors, two scalpel blade handles, a 1000 mL beaker covered with aluminum foil, and a 36 x 9 mm stir bar.

1.2.2. Line a tissue culture hood with underpad. Place the beaker, a sterile tissue culture dish (150 x 25 mm), and the whole kidney organ into the hood. Fill the beaker with 500 mL of 1% SDS solution.

Note: Human kidneys were received on ice from LifeCenter NorthWest.

1.2.3. Place the kidney in the sterile tissue culture dish (**Figure 1A**). Remove all perirenal fat by lightly shaving around the renal capsule with a scalpel (**Figure 1B**).

1.2.4. Make a shallow 8-10 cm incision with the scalpel, just deep enough to break open the renal capsule without damaging the underlying cortex tissue, across the superior end of the kidney. Remove the renal capsule by peeling it away from the cortex tissue with two hemostat clamps (**Figure 1C**).

1.2.5. Bisect the kidney along the coronal plane by using the scalpel along the lateral side of the kidney (**Figure 1D**). Isolate cortex tissue from both halves by carving out the medullar region with the scalpel (**Figure 1E**) and dice the cortex tissue into 0.5 cm^3 pieces (**Figure 1F**). Remove any large visible vessels.

1.3. Isolation of extracellular matrix

1.3.1. In a tissue culture hood, fill a 1000 mL beaker with 500 mL of 1% SDS solution. Place the diced cortex tissue and stir bar into the beaker containing SDS solution. Cover the beaker with autoclaved aluminum foil and place it on a stir plate at approximately 400 rpm outside of the tissue culture hood.

1.3.2. After the cortex tissue has been on the stir plate for 24 h, bring the beaker into a tissue culture hood and add a 40 µm sterile cell strainer made with nylon mesh. Fill a separate 1000 mL beaker with 200 mL of bleach and place it in the tissue culture hood.

1.3.2. Pipette out the SDS solution through the cell strainer into the beaker containing bleach. Pipette out all SDS solution until only decellularized tissue and the cell strainer remain in the beaker.

Note: The cell strainer should prevent any tissue from being removed during solution aspiration.

1.3.3. Leave the cell strainer in the beaker and fill with 500 mL of fresh SDS solution. Cover the beaker with the same aluminum foil and place onto a stir plate at the same speed as before.

1.3.4. Repeat steps 1.3.1-1.3.3 every 24 hours with fresh SDS solution for a total of five days.

1.3.5. Rinse decellularized tissue with autoclaved DI water every 24 h for 3 days total, following the technique outlined in steps 1.3.1-1.3.3.

1.3.6. Rinse decellularized tissue with cell culture grade water every 24 h for 2 days total, following the technique outlined in steps 1.3.1-1.3.3.

1.3.7. Repeat steps 1.3.1-1.3.2. Transfer the decellularized tissue (referred to as kECM from this point on) into a 30 mL self-standing conical tube and fill it with cell culture grade water until all the tissue is submerged.

2. Fabrication of Hydrogel Stock Solution

2.1. Mechanical processing of decellularized tissue

2.1.1. In a tissue culture hood, mechanically homogenize the kECM within the conical tube with a tissue homogenizer for 2 min.

Note: Homogenized kECM should resemble an opaque solution with no visible pieces of ECM.

2.1.2. Submerge the conical tube containing the kECM in liquid nitrogen until boiling surrounding the tube no longer persists. Store the kECM at -4 ˚C overnight.

2.2. Lyophilization of frozen decellularized tissue

2.2.1. Slightly loosen the conical tube cap to allow for gas exchange and place the tube into a lyophilization machine. Lyophilize the kECM for three days or until it resembles a fine white powder. Store at -4 ˚C.

2.3. Chemical digestion and solubilization of gel

2.3.1. Autoclave a 20 mL scintillation vial and cap, a 15.9 x 7.9 mm stir bar, and one pair of fine-tip forceps.

2.3.2. Weigh the lyophilized kECM and calculate the volume of HCl and mass of pepsin needed to solubilize the kECM to a 3% (30 mg/mL) solution using the following equations, where m_{pepsin} is the mass of pepsin, m_{tissue} is the mass of lyophilized tissue, and V_{HCl} is the volume of 0.01 N HCl:

$$m_{pepsin}(mg) = \frac{m_{tissue}(mg)}{10}$$

$$V_{HCl}(mL) = \frac{m_{tissue}(mg)}{30(\frac{mg}{mL})}$$

2.3.3. In a tissue culture hood, add porcine gastric pepsin, 0.01 N HCl, and the stir bar to the scintillation vial, and leave it on a stir plate at approximately 500 rpm until all the pepsin has dissolved. Transfer the lyophilized kECM to the scintillation vial and leave the solution on a stir plate at approximately 500 rpm for three days.

3. Hydrogel Gelation

3.1 Kidney ECM hydrogel preparation

3.1.1. Gel the hydrogel by mixing the kECM hydrogel stock solution with 1 N NaOH, 10x Media Supplement (M199), and cell culture media. Keep all the solutions on ice.

Note: Final gel concentrations of 7.5 mg/mL were used for cell culture. 1 mL of kECM gel was sufficient for cell culture experiments presented.

3.1.1.2. Determine the volume of workable kECM gel produced and volume of stock kECM hydrogel needed by using the following equation, where V_{final} is the volume of gel created, $V_{stock\ kECM}$ is the volume of stock kECM hydrogel needed, $C_{stock\ kECM}$ is the concentration of the stock kECM hydrogel, and C_{final} is the concentration of the final gel:

$$V_{stock\ kECM} = \frac{V_{final} * C_{final}}{C_{stock\ kECM}}$$

3.1.1.3. Determine the volume of neutralizing reagents needed by using the following equations, where V_{NaOH} is the volume of 1 N NaOH, V_{10X} is the volume of M199 10X media supplement, and V_{1X} is the volume of cell culture media:

$$V_{NaOH} = 0.022 * V_{stock\ kECM}$$

$$V_{10X} = 0.1 * V_{stock\ kECM}$$

$$V_{1X} = V_{final} - V_{stock\ kECM} - V_{NaOH} - V_{10X}$$

3.1.1.4. In a tissue culture hood, pipette the neutralizing reagents (NaOH, M199, and cell culture media) into a sterile 30 mL self-standing conical tube. Mix the neutralizing reagent solution with a microspatula.

3.1.1.5. Use a sterile 1 mL syringe to transfer the appropriate volume of stock kECM hydrogel to the neutralizing reagent solution. Use a microspatula to gently mix the solution until a homogeneous in color hydrogel solution is obtained.

Note: Avoid introducing air bubbles by stirring slowly and gently.

3.1.1.6. To incorporate cells into the kECM hydrogel, subtract 10 µL of cell culture media (V_{1X}) from the neutralizing solution volume calculations in step 3.1.1.3.

3.1.1.6.1. Suspend cells into 10 µL of cell culture media. Determine the number of cells to be suspended by using the following equation, where $\#_{cells}$ implies the number of cells to suspend and V_{final} is the volume of gel created:

$$\#_{cells} = 300{,}000 \left(\frac{cells}{mL}\right) * V_{final}(mL)$$

Note: 300,000 cells/mL is the concentration of cells used in the kECM gel.

3.1.1.6.2. Pipette the 10 µL of cell suspended solution into the final kECM gel after the kECM stock solution has been mixed with neutralizing reagent solution. Stir the solution with a microspatula until the cells are evenly distributed.

3.1.2. Use a 1 mL syringe to fill a desired cell culture device with the kECM hydrogel.

3.1.3. Allow the gel to set at 37 ˚C for 1 h before transferring or plating cells.

REPRESENTATIVE RESULTS:

The kECM hydrogel provides a matrix for kidney cell culture with similar chemical composition as the native kidney microenvironment. To fabricate the hydrogel, kidney cortex tissue is mechanically isolated from a whole kidney organ and diced (**Figure 1**). Decellularization with a chemical detergent (**Figures 2A.1-A.3**) followed by rinses with water to remove detergent particles (**Figures 2A.4-A.6**) yields isolated kidney cortex ECM. Histological evaluation confirms typical basal laminar proteins such as collagen-IV and laminin and structural proteins such as collagen-I are preserved, with vitronectin as the only noted exception **(Figure 2B)**. Furthermore, protein composition, including preservation of isoforms, in the ECM remains consistent with observed values in native kidney ECM (**Figure 3**). The ECM (**Figure 4A**) is mechanically homogenized and lyophilized (**Figure 4B**) then solubilized to produce the final kECM hydrogel (**Figure 4C**). The kECM hydrogel appeared opaque with small amounts of visible tissue and was not as viscous as traditional collagen-I hydrogel. Rheological measurement of gelled kECM at a

concentration of 15 mg/mL revealed a complex modulus (dynamic elastic modulus) of around 800 Pa over the linear range of strain values, significantly greater than that of 7.5 mg/mL collagen-I ($p = 1.602E-14$). Human kidney peritubular microvascular endothelial cells (HKMECs) cultured on collagen-I, kECM, and a 1:1 mixture gel showed differences in phenotype, specifically in CD31 expression around cell surfaces and junctions (**Figure 5**). HKMECs cultured on collagen-I displayed uniform CD31 expression while HKMECs cultured on the two gels containing kECM displayed reduced CD31 expression in uneven distributions. Matrix type did not appear to affect the high PV1 and low VWF expression in the HKMECs.

FIGURE AND TABLE LEGENDS:

Figure 1: Isolation of kidney cortex tissue. Mechanical processing of a whole kidney organ to isolate the cortex tissue as a base material for the kECM hydrogel. Mechanical isolation begins with (A) the removal of the perirenal adipose tissue. Large pieces of adipose tissue can be torn away from the kidney with hemostat clamps. Remaining pieces of adipose tissue can be removed by running a scalpel against the renal capsule at an angle. (B) The renal capsule is best removed by making a shallow incision along the superior end of the kidney and (C) peeling the renal capsule away from the underlying tissue with hemostat clamps. (D) Bisecting the kidney along the coronal axis allows for the visualization of the cortex and medulla regions. (E) Isolation of the cortex tissue is best done by carving out pieces of the medulla tissue with a scalpel. The color of the cortical region is noticeably darker than that of the medullar region and can be used differentiate the two anatomically distinct tissues. Final processing of the cortex tissue involves (F) dicing the tissue into 0.5 cm^3 pieces to aid in subsequent decellularization.

Figure 2: Decellularization of cortex tissue. Visual and histological characterization of decellularized tissue. (A.1) Submerging diced cortex tissue in 1% SDS solution causes lysing of cells and removal of cellular material. After (A.2) 1 h and (A.3) 24 h, the tissue begins to lose color, indicating cellular matter is being removed. By (A.4) 120 h the tissue is blanched and only the ECM remains. Rinses with water at (A.5) 24 h and (A.6) 120 h show no visible changes to the tissue. (B) Immunofluorescence staining of untreated and decellularized cortex tissue reveals near complete removal of cellular matter and preservation of major structural proteins (collagen-IV = Col-IV; laminin = LAM; fibronectin = FN; heparin sulfate proteoglycans = HSPG; and vitronectin = VN). Scale = 100 μm.

Figure 3: Mass spectroscopy of decellularized tissue. Analysis of decellularized cortex tissue by mass spectrometry to determine ECM protein composition. All ratios are measured as mass percent. (A) Structural and other proteins associated with the basal lamina were present with collagen-IV and -I being the most highly represented. (B.1) Collagen-IV A1 and A2 chains, ubiquitous in all basement membranes, were conserved. Collagen-IV A3 and A5 chains, present only in basement membranes of the glomerulus, were also detected. (B.2) Common isoforms of laminins and (B.3) collagen-I were also detected. This figure was reproduced with permission[12].

Figure 4: Fabrication of kECM hydrogel. Mechanical and chemical processing of decellularized cortex tissue yields a workable kECM hydrogel with sufficient mechanical properties following gelation with neutralizing reagents. (A) Decellularized cortex tissue is mechanically homogenized

with a tissue homogenizer until no visible pieces of ECM remain. (B) A coarse powder was yielded after 3 days under lyophilization. (C) Solubilization in HCl and chemical digestion with pepsin in a scintillation vial resulted in a workable kECM hydrogel. The hydrogel was opaque and of a low viscosity. (D) Physical characterization of the kECM hydrogel following gelation with neutralizing reagents. Rheological experiments were performed with a 30 mm diameter parallel plate system. The sample edges were protected with mineral oil and the loading platform was set at 37 ˚C. The kECM hydrogel was allowed to gel for 1 h prior to testing. Viscoelastic properties of the gel were measured with a strain sweep between 0.01 to 20%. Three samples of both kECM and collagen-I were tested (n = 3).

Figure 5: Cell growth characterization. Characterization of morphological differences in HKMECs grown on different matrices. Hydrogels were mixed with neutralizing reagents and set at 37 ˚C for 45 min in open-faced polydimethylsiloxane molds. HKMECs were seeded on the surface of the gels and kept in culture for 72 h before being fixed and stained. Immunofluorescent images of HKMECs cultured in (A and D) 7.5 mg/mL collagen-I gel; (B and E) 7.5 mg/mL kECM gel; and (C and F) 7.5 mg/mL 1:1 mixture gel. (A–C): red = CD31; green = VWF; blue = nuclei; scale bar = 50 µm. (D–F): red = F-actin; green = PV1; blue = nuclei; scale bar = 50 µm. This figure was reproduced with permission[12].

DISCUSSION:

Matrices provide important mechanical and chemical cues that govern cell behavior. Synthetic hydrogels are able to support complex 3-dimensional patterning but fail to provide the diverse extracellular cues found in physiological matrix microenvironments. Hydrogels derived from native ECM are ideal materials for both *in vivo* and *in vitro* studies. Previous studies have used decellularized ECM hydrogels to coat synthetic biomaterials to prevent host immunological responses[33,34], to differentiate stem cells[35-38], as a substrate for 2D and soft lithography cell culture[39-42], and in preparation of bioinks for 3-dimensional printing[43-46]. ECM hydrogels provide cells with proper signaling to initiate adhesion and control further proliferation and differentiation[2,47].

The ratios and composition of major components of the ECM, such as collagens, laminins, elastin, fibronectin, and glycosaminoglycans, are highly dependent on the anatomical location and functionality of the tissue[48-50]. It is well established that using nonspecific or nonnative ECM-derived hydrogels will elicit improper responses from cells[21,51-54]. In the kidney, ECM composition varies widely between anatomical locations[1,2]. It is, therefore, important to differentiate between regions such as the cortex, medulla, or papilla before fabricating hydrogels for experimental use.

The kECM matrix fabricated here preserves native kidney cortex ECM protein ratios following decellularization while also enabling gelation to a physiologically relevant mechanical stiffness[3,25,28-30]. Serum albumin, a blood plasma protein, was detected in trace amounts within the decellularized cortex tissue, possibly due to binding to heparin that escaped decellularization and rinsing[55,56]. Although pepsin, a non-native chemical to kidney cortex ECM, is present in the stock kECM solution, it only accounts for 2.5% (w/v) of the final gelled product. Furthermore,

pepsin becomes deactivated with the addition of the neutralizing reagent solution[57].

The kECM hydrogel serves as a substrate upon which kidney cortex-specific cells can be maintained under physiological conditions. We demonstrated that this matrix can support HKMEC growth on a planar surface. These HKMECs maintained a quiescent physiological state as determined through functional assays, phenotypic expression, and genetic expression[58]. In contrast, these cells became activated when collagen-I, a matrix component correlated with kidney fibrosis, was added to the kECM hydrogel at a 1:1 ratio[59]. By comparison, when human umbilical vein endothelial cells were cultured on collagen-I, they were quiescent, and when mixed with kECM they became activated[12]. Accordingly, collagen-I is known to be an integral component of the basement membrane composition within the umbilical cord and is decreased under pathological states such as preeclampsia[38,60]. These results highlight the importance of providing cells with tissue-specific cues to maintain quiescence and also how manipulation of the ECM milieu can affect homeostasis.

Whereas all steps in the outlined procedure are important, several steps are critical in ensuring the viability of the fabricated hydrogel. The degree of homogenization of the decellularized cortex tissue will vary based on the technique or equipment used. It is important to find a technique that will best homogenize the tissue. During solubilization, the pH should be kept at 3-4 to ensure the pepsin is active. When mixing the hydrogel with neutralizing reagents, the gel must be mixed thoroughly and without the introduction of air bubbles. If a uniform mixture is difficult to obtain, check the pH of the gel solution to ensure it is neutral.

The representative results presented in this method demonstrate how a physiologically relevant matrix for the *in vitro* study of kidney cells can be achieved. Decellularized kidney cortex ECM provides an ideal base material to support kidney-derived cell growth as ECM protein ratios are conserved in the final hydrogel product[3,25]. The gelled product can also achieve physiologically relevant mechanical properties[28-30]. The kECM hydrogel allows for proper cell-matrix interactions and has been shown to elicit greater physiological behavior from kidney cells than collagen-I when cultured on a planar surface. Furthermore, the hydrogel can be seeded with kidney-specific cells prior to gelation to model a more physiologically relevant 3-dimensional environment. The composition of the kECM hydrogel can also be easily manipulated. For example, mixing the hydrogel with varying amounts of collagen-I prior to gelation could produce matrices that mimic progressive states of renal fibrosis[31,32]. The tunability of this ECM-derived hydrogel to mimic known diseased ECM compositions warrants further investigation and will enable the future study of kidney diseases.

ACKNOWLEDGMENTS:

The authors would like to acknowledge the Lynn and Mike Garvey Imaging Laboratory at the Institute for Stem Cell and Regenerative Medicine and LifeCenter NorthWest. They would also like to acknowledge the financial support of National Institutes of Health grants, UH2/UH3 TR000504 (to J.H.) and DP2DK102258 (to Y.Z.), NIH T32 training grant DK0007467 (to R.J.N.), and an unrestricted gift from the Northwest Kidney Centers to the Kidney Research Institute.

DISCLOSURES:

The authors have nothing to disclose.

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Isolation of kidney cortex tissue

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Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Preparation of Kidney Tissue			
5000 mL Beaker	Sigma-Aldrich	Z740589	
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich	436143	
Sterile H_2O			Autoclaved DI H_2O
Stir Bar (70 x 10 mm)	Fisher Science	14-512-128	
500 mL Vacuum Filter	VWR	97066-202	
Stir Plate	Sigma-Aldrich	CLS6795420D	
1000 mL Beaker	Sigma-Aldrich	CLS10031L	
Forceps	Sigma-Aldrich	F4642	Any similar forceps may be used
Scissor-Handle Hemostat Clamp	Sigma-Aldrich	Z168866	
Dissecting Scissors	Sigma-Aldrich	Z265977	
Scalpel Handle, No. 4	VWR	25859-000	Any similar scalpel handle may be used
Scalpel Blade, No. 20	VWR	25860-020	Any similar scalpel blade may be used
Stir Bar (38.1 x 9.5 mm)	Fisher Science	14-513-52	
Absorbent Underpad	VWR	82020-845	
Petri Dish (150 x 25 mm)	Corning	430597	
Autoclavable Biohazard Bag	VWR	14220-026	
Sterile Cell Strainer (40 um)	Fisher Science	22-363-547	
Cell Culture Grade Water	HyClone	SH30529.03	
30 mL Freestanding Tube	VWR	89012-778	
Fabrication of ECM Gel			
Tissue Homogenizer Machine	Polytron	PCU-20110	
Freeze Dryer	Labconco	7670520	
20 mL Glass Scintillation Vials and Cap	Sigma-Aldrich	V7130	
Stir Bar (15.9 x 8 mm)	Fisher Science	14-513-62	
Pepsin from Porcine Gastric Mucosa	Sigma-Aldrich	P7012	

0.01 N HCl	Sigma-Aldrich	320331	Dilute to 0.01 N HCl with cell culture water
Kidney ECM Gelation			
1 N NaOH (Sterile)	Sigma-Aldrich	415413	Dilute to 1 N in cell culture grade water
Medium 199	Sigma-Aldrich	M4530	
15 mL Conical Tube	ThermoFisher	339651	
Cell Culture Media	ThermoFisher	11330.032	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12)
Fetal Bovine Serum (FBS)	Gibco	10082147	
Antibiotic-Antimycotic 100X	Life Technologies	15240-062	
Insulin, Transferrin, Selenium, Sodium Pyruvate Solution (ITS-A) 100X	Life Technologies	51300-044	
1 mL Syringe	Sigma-Aldrich	Z192325	
Microspatula	Sigma-Aldrich	Z193208	

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Author(s): Hiraki, Harrison; Nagao, Ryan; Zheng, Ying; Himmelfarb, Johnathan

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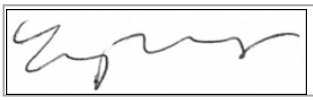
Signature:	 Date: 4/7/2018

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Vineeta Bajaj
Review Editor
Journal of Visualized Experiments

July 17, 2018

Subject: Revision and Resubmission of manuscript JoVE58314

Dear Dr. Bajaj,

Thank you for your email dated July 10, 2018, containing editor comments and the opportunity to revise our paper on 'Fabricating a Kidney Cortex Extracellular Matrix-Derived Hydrogel'. We appreciate your helpful comments in the protocol section. Editor comments are included below, immediately followed by responses indicating how we addressed each comment. Changes to the manuscript are shown in red.

The manuscript has been thoroughly proofread and spelling and grammatical corrections have been made. Correct reference for use of previously published figures have been added to figure captions 2, 3, and 5 and explicit reprint permission has been obtained. The revisions prompted by the editor's comments are minor and require no further explanation beyond what appears in our responses below.

We hope the manuscript will suit the *Journal of Visualized Experiments,* but are happy to consider further revisions. We thank you for your continued interest in our research.

Sincerely,

Ying Zheng
Associate Professor
Department of Bioengineering
University of Washington

Editor Comments and Author Responses, and Manuscript Changes

1. *Line 26, Please expand during first time use.*

Response: Thank you for pointing out a lack of proper definition here. ECM has been initially defined on line 26 and subsequent repetitive defining of ECM was removed on line 35.

2. *Line 54, This is commercial. Please use generic term instead. Maybe non-ionic detergent? The commercial term can be sufficiently referenced in the table of materials.*

Response: We appreciate you catching this mistake. On line 55, we have used non-ionic detergent and included the catalogue number for the Triton-X100 used in the materials table.

3. *Line 108, Please include a note stating where is the kidney obtained from? Is it fresh or frozen and other necessary details.*

Response: Thank you for addressing that a clarification is needed at this point in the protocol. We have included a note on line 109 stating where the kidney was obtained from and its condition.

4. *Line 116, How? using what?*

Response: Thank you for your comment to improve the clarity of the protocol. In lines 119-120 we state that a scalpel should be used and describe how the kidney should be bisected.

5. *Line 117, Using what?*

Response: Thank you again for your insight into how to improve the clarity of the protocol. In lines 120-121 we have stated that a scalpel was used to perform this step.

6. *Line 124, Aluminum foil?*

Response: We appreciate your comment. We have changed the use of tinfoil to aluminum foil on lines 102, 128, and 142.

7. *Line 128, Also transwell is a commercial term. Please use generic term and refer to the commercial term in the table of materials.*

Response: We appreciate you pointing out the use of a commercial term throughout the protocol section. We have changed transwell to nylon mesh cell strainer on lines 132, 135, 136, 139, and 141.

8. *Lines 131-132, How much? Are you pipetting out or in?*

Response: Thank you for your clarifying comment. On line 135 we added that the SDS solution should be pipetted out of the beaker and that all of the SDS solution in the beaker should be removed.

9. *Lines 132-133, Please make this a note or convert to imperative tense.*

Response: We appreciate your insight. We have made the explanation of the cell strainer a note on line 139.

10. *Lines 141-145, To be done after 5 days wash with SDS? ,So the washes goes for 10 days?*

Response: Thank you for pointing out a potentially confusing part of the protocol. We have reworded steps 1.3.5 and 1.3.6 on lines 146-150 to better explain the work done.

11. *Lines 155-156, This is done in the conical tube?*

Response: We appreciate you bringing up this part of the protocol that is not well defined. We have reworded step 2.1.1 on lines 160-161 to explain that the homogenization of the ECM should take place within the conical tube.

12. *Line 158, Converted to a note since not in imperative tense. Also, removed the highlight since notes cannot be filmed.*

Response: We appreciate your edit that made the protocol section more consistent with the use of notes!

13. *Lines 174-176, Calculation steps cannot be filmed, so highlights removed.*

Response: Thank you for your comment. We have removed highlights on all steps within the protocol that are only calculations.

14. *Lines 193-194, What volume and concentration was used?*

Response: We appreciate your comment and agree that the information should be included in the protocol. We have added to the note on lines 199-200 explaining what volume and concentration of kECM gel was needed to perform the experiments outlined in the results section.

15. *Lines 198-200, Please use imperative tense.*

Response: Thank you for pointing out this flaw in our protocol section. We have made corrections to the wording within the protocol section to remove any text that is not in imperative tense.

16. *Lines 218-223, Please make substeps as we cannot have paragraphs of text in the protocol section.*

Response: We appreciate your comment to improve the protocol section. We have split step 3.1.2 into multiple substeps and a note in lines 219-227.

17. *Lines 225-227, How many cells and how much culture media?*

Response: We appreciate your comment and agree that this is necessary information to add. We have better outlined how much cell culture media and cells are needed to incorporate in the gel in step 3.1.1.6 and substeps in lines 229-242.

Gmail

Harrison Hiraki <hirakih@gmail.com>

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