Video Article

Epithelial Cell Repopulation and Preparation of Rodent Extracellular Matrix Scaffolds for Renal Tissue Development

Joseph S. Uzarski[1,2], Jimmy Su[1,2,3,4], Yan Xie[1,2], Zheng J. Zhang[1,2], Heather H. Ward[5], Angela Wandinger-Ness[6], William M. Miller[7,8], Jason A. Wertheim[1,2,3,4,8,9]

[1]Comprehensive Transplant Center, Feinberg School of Medicine, Northwestern University

[2]Department of Surgery, Feinberg School of Medicine, Northwestern University

[3]Department of Biomedical Engineering, Northwestern University

[4]Simpson Querrey Institute for BioNanotechnology in Medicine, Northwestern University

[5]Department of Internal Medicine, University of New Mexico HSC

[6]Department of Pathology, University of New Mexico HSC

[7]Department of Chemical and Biological Engineering, Northwestern University

[8]Chemistry of Life Processes Institute, Northwestern University

[9]Department of Surgery, Jesse Brown VA Medical Center

Correspondence to: Jason A. Wertheim at jason.wertheim@northwestern.edu

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Abstract

This protocol details the generation of acellular, yet biofunctional, renal extracellular matrix (ECM) scaffolds that are useful as small-scale model substrates for organ-scale tissue development. Sprague Dawley rat kidneys are cannulated by inserting a catheter into the renal artery and perfused with a series of low-concentration detergents (Triton X-100 and sodium dodecyl sulfate (SDS)) over 26 hr to derive intact, whole-kidney scaffolds with intact perfusable vasculature, glomeruli, and renal tubules. Following decellularization, the renal scaffold is placed inside a custom-designed perfusion bioreactor vessel, and the catheterized renal artery is connected to a perfusion circuit consisting of: a peristaltic pump; tubing; and optional probes for pH, dissolved oxygen, and pressure. After sterilizing the scaffold with peracetic acid and ethanol, and balancing the pH (7.4), the kidney scaffold is prepared for seeding via perfusion of culture medium within a large-capacity incubator maintained at 37 °C and 5% CO_2. Forty million renal cortical tubular epithelial (RCTE) cells are injected through the renal artery, and rapidly perfused through the scaffold under high flow (25 ml/min) and pressure (~230 mmHg) for 15 min before reducing the flow to a physiological rate (4 ml/min). RCTE cells primarily populate the tubular ECM niche within the renal cortex, proliferate, and form tubular epithelial structures over seven days of perfusion culture. A 44 µM resazurin solution in culture medium is perfused through the kidney for 1 hr during medium exchanges to provide a fluorometric, redox-based metabolic assessment of cell viability and proliferation during tubulogenesis. The kidney perfusion bioreactor permits non-invasive sampling of medium for biochemical assessment, and multiple inlet ports allow alternative retrograde seeding through the renal vein or ureter. These protocols can be used to recellularize kidney scaffolds with a variety of cell types, including vascular endothelial, tubular epithelial, and stromal fibroblasts, for rapid evaluation within this system.

Video Link

The video component of this article can be found at https://www.jove.com/video/53271/

Introduction

As the number of patients suffering from end-stage renal failure continues to increase, there is a severe and growing shortage in the number of donor kidneys available for transplantation. The inability to meet the demand of a continually rising number of candidates wait-listed for kidney transplantation has prompted research in kidney organ engineering with the ultimate goal of developing customized, implantable kidney grafts on demand[1,2]. Building functioning kidney tissue from a patient's own cells would eliminate the need for lifelong immunosuppression, decrease the amount of time patients spend on dialysis waiting for a kidney transplant, and extend life-saving transplantation to more patients with chronic kidney disease.

The first step toward bioengineering a kidney tissue using patient-derived cells is to develop a scaffold that serves as a supportive substrate for renal parenchyma (*e.g.* tubular epithelial), stroma fibroblast, and vascular cell growth. Biomaterial scaffolds derived from natural organ extracellular matrices (ECMs) have several characteristics that make them desirable for use in tissue engineering, including their natural biological composition; appropriate macro- and microstructure to endow physiological function; and cellular biocompatibility, promoting cell adhesion, migration, and constructive tissue remodeling[3]. A promising method to produce scaffolds for renal tissue regeneration is through

decellularization of allogeneic or xenogeneic kidneys that preserve much of the complex natural protein composition of the kidney ECM[4], retain the inherent architectural intricacy of the organ, and overcome the difficulty associated with bottom-up engineering of thick cellularized tissues by providing a vascular supply to developing cells after scaffold recellularization[5].

Perfusion decellularization is a process in which detergents, enzymes, or other cell-disrupting solutions are uniformly delivered through the vascular network of the organ[6]. This strategy has been established as an efficient process to derive acellular organ-based ECM scaffolds as three-dimensional (3D), biological templates for whole-organ engineering[6-8], as evidenced by the development of acellular renal templates from discarded human kidneys[9] and xenogeneic kidneys obtained from large-animal (*e.g.* pig[10], goat[11]) and rodent sources[12]. In particular, the use of small animal models such as rodents requires fewer cells and culture media, which is especially helpful for organ recellularization studies in which cell numbers are usually limited, as is the case with stem cell-derived tissues. The goal of the described decellularization protocol is to produce an acellular renal ECM that can be used as a 3D scaffolding system for regeneration of kidney structures, including nephron tubules that are repopulated in the present example with human renal cortical tubular epithelial (RCTE) cells. We previously described our rigorous evaluation of an optimal, detergent-based rat kidney decellularization protocol[7], which is more rapid (approximately one day) than other methods previously reported (Ross *et al*.- 5 days[12], Song *et al*.- 4.5 days[13]), and exposes the organ to a considerably lower concentration (0.1%) of the denaturant sodium dodecyl sulfate (SDS) during decellularization than prior reports[12-15].

A limited number of studies have described the use of rodent kidneys for decellularization and subsequent use as a 3D scaffold for cellular repopulation (reviewed elsewhere[1])[12-16]. In this protocol, we provide a detailed description of our previously established, optimal decellularization strategy for producing acellular kidney scaffolds from Sprague Dawley rat kidneys[7]. Using custom-designed perfusion bioreactors capable of dual seeding and maintenance perfusion culture[17], we recellularize the acellular kidney scaffolds with human RCTE cells, which consistently repopulate the tubular component in these decellularized matrices, proliferate, and survive in perfusion culture for over a week. We further demonstrate our use of the resazurin perfusion assay – an inexpensive, non-cytotoxic, and non-invasive metabolic assessment previously used for cytotoxicity studies[17]– to provide an indication of cell viability and proliferation within the recellularized kidneys over time[7].

Protocol

ETHICS STATEMENT:All procedures involving animals were performed according to guidelines approved by the Institutional Animal Care and Use Committee of Northwestern University.

1. Kidney Decellularization

1. Prepare decellularization solutions. Prepare the following volumes of reagents for each kidney to be decellularized, plus one additional volume (*e.g.*, for 4 kidneys, prepare 5,000 ml Triton X-100 for step 1.1.3):
    1. Prepare 500 ml reverse osmosis water (ROH_2O). Note: Alternatively, deionized water may be used in steps where ROH_2O is indicated.
    2. Prepare 1,000 ml Triton X-100, 1% (v/v) in ROH_2O. Slowly add 10 ml Triton X-100 to 990 ml water in a large beaker under rapid stirring on a stir plate. Allow the reagent to dissolve completely before use (at least 10 min).
    3. Prepare 1,000 ml Triton X-100, 1% (v/v) in ROH_2O (separate volume).
    4. Prepare 1,000 ml sodium dodecyl sulfate (SDS), 0.1% (v/v) in ROH_2O. Slowly add 5 ml SDS (20% stock solution) to 995 ml water in a large beaker under fast stirring on a stir plate. Allow the reagent to mix uniformly before use (at least 10 min).
    5. Prepare 500 ml ROH_2O (separate volume).

2. Prepare kidneys for decellularization.
    1. Recover a kidney from a male Sprague Dawley rat (250-300 g) as previously described[7].
        1. Anesthetize the rat by intraperitoneal injection of pentobarbital (50 mg/kg body weight). Frequently examine the depth of anesthesia (every 10-15 min) by monitoring respiratory function, heart rate, and toe pinch response during surgery. If the animal has an elevated respiration rate or positive pedal reflex, administer a supplemental dose (with 1/3 to 1/4 of the initial dose) of pentobarbital.
        2. Perform a longitudinal midline abdominal incision to expose the kidneys, abdominal aorta, and the inferior vena cava.
        3. Inject 2,000 USP heparin Units/kg body weight into the penile vein.
        4. Mobilize both kidneys by gentle dissection. Carefully separate the kidney from the perirenal fat, while keeping the renal capsule surrounding the kidney intact. Perfuse the kidneys with cold saline (10 ml) through infra-renal abdominal aorta.
    2. Insert a 24 gauge catheter into the renal artery, tightly ligate the catheter to the artery, and (using a syringe) gently perfuse the kidney with 10 ml cold phosphate-buffered saline (PBS) to completely clear the organ of blood.
    3. Immerse kidney in 25 ml PBS within a Petri dish and place in a -20 °C freezer to gradually freeze the organ (thereby inducing cell lysis) for storage until decellularization.
    Note: if desired, the ureter and/or renal vein may also be cannulated for retrograde seeding, though not described in this protocol.
    4. Completely thaw the frozen kidney (equilibriate at room temperature (RT)), and gently perfuse with 10 ml of PBS to check the ligated renal artery for leaks. If leaks or significant resistance are observed, re-catheterize the renal artery.

3. Prepare equipment for decellularization. Assemble the decellularization perfusion system as depicted in **Figure 1B**.
    1. Connect one 8” length of peristaltic pump tubing (**Figure 1D, f**) to two sufficient lengths (>36” recommended) of 1/16” inner diameter (ID) silicone rubber tubing (**Figure 1D, h**). Use two male Luer lock to 1/8” barbed adapters joined by one female Luer x female Luer adapter to join segments of tubing (**Figure 1D, j**). Insert an additional male Luer lock to 1/8” barbed adapter (**Figure 1D, c**) into the downstream end of the perfusion line for attachment to the renal artery catheter.
    2. Connect each peristaltic pump tubing segment to the 4-roller pump head using a large pump cartridge.
    3. After placing the upstream end of silicone rubber tubing in the first solution reservoir (ROH_2O), hold the “Prime” button to fully prime each perfusion circuit with solution. CRITICAL: Verify that no bubbles remain entrapped in the tubing, and that the upstream end (left

open to draw fluid) of the silicone rubber tubing is secured below the liquid-air interface of the reagent reservoir, as depicted in **Figure 1B**.

4. Perform the decellularization protocol at RT[7].
    1. Connect the renal artery catheter of each thawed kidney to the end of the perfusion circuit tubing downstream from the pump, ensuring that no air bubbles are entrapped in the catheter. Allow the kidney to be suspended along the inner wall of an empty 5 L beaker (perfusate collection reservoir, see **Figure 1B**) so that the renal artery is not kinked or coiled.
    2. Adjust the pump drive to 5 ml/min, and press the “Start” button. Confirm that each kidney is perfusing by observing solutions drip from the bottom of the organ.
    3. Perfuse each kidney with the following reagents as described in **Figure 1A**
        1. Perfuse with 500 ml ROH_2O at 5 ml/min for 1 hr, 40 min.
        2. Perfuse with 1,000 ml 1% Triton X-100 at 5 ml/min for 3 hr, 20 min.
        3. Perfuse with 1,000 ml 1% Triton X-100 at 1 ml/min for 16 hr, 40 min.
        4. Perfuse with 1,000 ml 0.1% SDS at 5 ml/min for 3 hr, 20 min.
        5. Perfuse with 500 ml ROH_2O at 5 ml/min for 1 hr, 40 min.
        Note: Each decellularized kidney may be stored in PBS (without additives) in a 50 ml conical tube at 4 °C for a maximum of two weeks before use.

2. Perfusion Bioreactor Assembly, Kidney Sterilization, and Preparation for Recellularization

1. Prepare bioreactor vessels.
    1. Wash the glass bioreactor reservoirs (body component) and bioreactor heads with warm, dilute dish detergent solutions (*e.g.* 1% dish detergent solution in tap water) and rinse thoroughly with tap water and then ROH_2O. Allow components to dry completely.
    2. Apply a small volume (~5 ml) of siliconizing reagent to the bottom of each reservoir. Ensure that reagent wets the entire bottom surface for several seconds, then drain excess fluid.
    3. Allow the reservoirs to dry in a fume hood at RT O/N. Alternatively, reservoirs may be dried in an oven at 100 °C for 30 min to expedite drying and improve durability of the coating, which is intended to prevent attachment of cells to the bottom of the glass bioreactor reservoir.

2. Prepare perfusion circuit components for sterilization (**Figure 1D**).
    1. Cut the following lengths of tubing (for each bioreactor):
        1. Cut two 25” lengths of 1/16” ID silicone rubber tubing (**Figure 1D, h**).
        2. Cut one 8” length of peristaltic pump tubing (**Figure 1D, f**).
        3. Cut one 4.25” length of 1/16” ID silicone rubber tubing.
        4. Cut two 1” lengths of thick-walled 1/16” ID silicone rubber tubing (**Figure 1D, g**).
        5. Cut one 2” length of 1/4' ID x 0.5' outer diameter (OD) silicone rubber tubing (**Figure 1D, e**).
    2. Prepare the following Luer adapters (for each bioreactor):
        1. Prepare 10 male Luer lock to 1/8' barbed adapters (**Figure 1D, c**)
        2. Prepare 2 male Luer plugs (**Figure 1D, a**)
        3. Prepare 2 female Luer caps (**Figure 1D, b**)
        4. Prepare 5 female Luer x female Luer adapters (x5; **Figure 1D, d**)
    3. Wash all tubing and Luer adapters with warm, dilute dish detergent solutions, rinse thoroughly in tap water and then ROH_2O, and allow them to dry.

3. Assemble perfusion circuits.
    1. Slip 1” length of thick-walled 1/16' ID silicone rubber tubing over inlet Luer acceptor attached to the head of the bioreactor. Place male Luer lock to 1/8” ID barb adapter into open end of tubing. Repeat for the other inlet Luer acceptor, and connect a female Luer cap to seal off port (intended for ureteral or venous seeding techniques not described in this protocol).
    2. Connect each 25” segment of 1/16” ID silicone rubber tubing to the 8” segment of peristaltic pump tubing using male Luer lock to 1/8' ID barb and female Luer x female Luer adapters (**Figure 1D, j**).
    3. Connect one open end of silicone rubber tubing to the media perfusate outlet on the outside of the bioreactor head. Connect the 4.25” length of 1/16” ID tubing to the opposite (inner) side of the media perfusate outlet on the inside of the bioreactor head.
    4. Connect a male Luer lock to 1/8' ID barbed adapter to the remaining open end of the perfusion circuit tubing. Connect a female Luer x female Luer adapter to the male Luer lock. Connect the female Luer x female Luer adapter to the open male Luer lock leading into the inlet Luer acceptor on the bioreactor. This will serve as the media perfusate inlet leading directly into the catheterized renal artery.
    5. Ensure that no ports on the bioreactor lid are left open or exposed. Tightly close the vacuum valve on bioreactor body.
    6. Fit the bioreactor head over the reservoir body, with an O-ring sandwiched in between the identical grooves lining each component. Place a metallic clamp over the interface to hold the two components together.
    7. Fit the vent port on the bioreactor head with a 0.2 micron vent filter, connecting the two components using a 2” length of 1/4' ID x 0.5' OD silicone rubber tubing (**Figure 1D, e**). Open the vent valve.
    8. Slightly loosen the red screw cap on the bioreactor head.
    9. Place the remaining Luer adapters and 6” serrated specimen forceps in an autoclavable pouch and seal.

4. Autoclave the assembled bioreactors and male Luer plugs.
5. Prepare the following reagents for scaffold sterilization and medium perfusion before seeding (volumes are listed per decellularized kidney):

1. Within a fume hood, prepare a 50 ml 0.1% peracetic acid, 4% ethanol solution by adding 2.56 ml peracetic acid solution (39% stock concentration) and 40 ml 200 proof ethanol to ~957 ml ROH_2O in a 1 L bottle. Mix thoroughly by repeatedly inverting bottle, and place in biological safety cabinet.
2. Prepare a 150 ml 1x PBS solution, pre-sterilized by autoclaving.
3. Prepare 50 ml DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep).

6. Collect the remaining items for perfusion bioreactor assembly. Place all items in a biological safety cabinet. CRITICAL: Ensure that the biological safety cabinet is fitted with working electrical sockets to power the digital pump drive. Alternatively, use an extension cord to connect the pump drive to external electrical sockets outside the cabinet.
    1. Collect decellularized kidneys.
    2. Collect autoclaved bioreactors with attached perfusion circuits.
    3. Collect autoclaved pouch containing Luer adapters and forceps.
    4. Collect digital pump drive with attached 4-roller head, and large pump cartridges (1 per perfusion circuit).

7. Complete the perfusion circuit as described in **Figure 1C** under sterile conditions within a biological safety cabinet.
    1. Connect a 3-way stopcock (**Figure 1D, i**) between the male Luer lock to 1/8' ID barb adapter near the media perfusate inlet and the female Luer x female Luer adapter. Leave all three ports on the stopcock open.
    2. Remove the red screw cap on the bioreactor head and pipette 50 ml of the 0.1% peracetic acid, 4% ethanol solution into the medium reservoir through the opening.
    3. Connect the peristaltic pump tubing segment to the pump head using a large pump cartridge. Adjust the flow rate to 5 ml/min, press “Start”, and allow the circuit to prime.
        1. When the liquid fills the perfusion line and reaches the remaining open port of the three-way stopcock, plug the port using a male Luer plug **(Figure 1D, a)**.
        2. Allow the circuit to fully prime until no air is observed in the perfusion circuit tubing or on the inside of the inlet Luer acceptor. If necessary, increase the flow rate temporarily to expel bubbles from the perfusion line. When fully primed, stop the pump drive.
    4. Carefully plug the female Luer end of the renal artery catheter into the male inlet Luer acceptor on the inner surface of the bioreactor head using the sterilized 6” forceps. Make sure the connection is tight and that no air is left in the catheter. Allow the kidney to gently hang from renal artery catheter, so that the renal artery does not twist or kink.
    5. Tighten the metallic clamp holding together the bioreactor head and body so that the bioreactor reservoir is tightly sealed. Close the red screw cap on the bioreactor head.

8. Sterilize kidneys at RT by perfusion with 0.1% peracetic, 4% ethanol solution at 5 ml/min for 1 hr, then three sequential 1-hr perfusions at RT with 50 ml PBS at 5 ml/min.
    1. Changing solutions:
        1. After 1 hr, stop the pump and open red screw cap. Using a sterile (autoclaved) Pasteur pipette, carefully aspirate all of the solution from the bioreactor reservoir. Leave the perfusion circuit fully primed.
        2. Pipette 50 ml of fresh solution into the bioreactor reservoir, close the screw cap, and start the pump.

9. After the final PBS rinse, aspirate PBS from the reservoir and pipette in 50 ml DMEM/F12 medium supplemented with 10% FBS and 1% Pen-Strep. Close the screw cap, and transfer the bioreactor with the attached perfusion circuit to a large-capacity incubator maintained at 37 °C and 5% CO_2.
10. If necessary, transfer the pump drive to the incubator. Connect bioreactor perfusion circuit to the pump, and perfuse the kidneys at 4 ml/min for at least 1 hr prior to seeding.

3. Kidney Recellularization with Renal Cortical Tubular Epithelial Cells

1. Warm sufficient volumes of DMEM/F12 (supplemented with 10% FBS and 1% Pen-Strep) and cell dissociating enzyme for cell lifting to 37 °C. Suggested volumes are listed below:
    1. Prepare 10 ml cell dissociating enzyme and 10 ml DMEM/F12 per 175 cm^2 culture flask for lifting.
    2. Prepare 5-10 ml DMEM/F12 per kidney to be seeded.

2. Collect a sufficient number of culture flasks for the desired seeding concentration (4 x 10^7 immortalized human RCTE cells[18] per kidney results in maximal engraftment of RCTE cells using this seeding strategy).
3. Lift cells from the flasks using cell dissociating enzyme. Aspirate medium from flask, then pipette 10 ml cell dissociating enzyme into flask. Place flask in 37 °C incubator to expedite dissociation.
4. Check flask on a phase contrast microscope every 2 min until full dissociation of cells from flask surface is observed. Note: Incubation time will vary depending on the level of confluence of the cells, but RCTE cells will require approximately 15 min to fully dissociate.
5. Take a sample of dissociated cell suspension for counting prior to pelleting. Dilute the cell suspension with an equal volume of pre-warmed DMEM/F12 medium, and centrifuge at 232 x g relative centrifugal force (RCF) for 5 min.
6. Count the cells during centrifugation. Dilute the obtained sample with an equal volume of Trypan Blue, and pipette 10 µl into each end of a hemacytometer for counting. Calculate necessary pellet dilution volume to obtain the desired seeding concentration (2 x 10^7 cells/ml).
7. After centrifugation, dilute the pellet with an appropriate volume of culture medium to obtain a final concentration of 2 x 10^7 cells/ml. Draw up 2 ml of the seeding suspension into a sterile 5 ml syringe.
8. Transfer the perfusion bioreactor to a biological safety cabinet. Turn the stopcock valve to close flow to the seeding port. Remove the male Luer slip plug from the stopcock, and connect the syringe loaded with the seeding suspension.
9. Quickly transfer the perfusion circuit back to incubator, and use the large pump cartridge to secure the peristaltic pump tubing segment to the pump head.

10. Cell seeding: Close the stopcock valve port pointing toward pump. Slowly inject the cell suspension into kidney, ensuring that the entire suspension is transferred from the syringe into the stopcock and perfusion line. Turn the stopcock valve to close flow from the syringe, and start the pump at 25 ml/min for 15 min.
11. After 15 min, lower the pump flow rate to 4 ml/min. Exchange medium (100 ml volume for subsequent changes) the following day and thereafter every two days.

4. Evaluation of Cell Viability and Proliferation using Resazurin Perfusion Assay

1. Prepare resazurin reagent. Dissolve 110.5 mg resazurin sodium salt in 100 ml of PBS under stirring, and dilute 1:10 by adding 5 ml of the resulting solution to 45 ml fresh PBS to create a 440 µM resazurin stock solution. Filter-sterilize (using a 0.2 µm syringe filter), and store the stock solution in a light-protected 50 ml conical tube at 4 °C.
2. Prepare resazurin-supplemented media controls. Prepare a 10% solution of resazurin reagent in culture medium (*e.g.* 5 ml resazurin stock solution + 45 ml culture medium) to create resazurin working solution. Autoclave a 10 ml volume of the resazurin working solution in a light-protected container. Note: This will completely reduce the resazurin compound to resorufin, and will serve as a positive control for calculating percent resazurin reduction.
3. Aliquot 1 ml each of resazurin working solution (oxidized), autoclaved resazurin working solution (reduced), and culture medium alone (blank) into separate 1.5 ml collection tubes. Place the open tubes in the same incubator as the perfusion bioreactors.
4. Transfer the kidney perfusion circuit from the incubator to a biological safety cabinet. Remove the screw cap from the bioreactor head, and aspirate culture medium from the bioreactor reservoir using a Pasteur pipette.
5. Pipette 10 ml of resazurin working solution into reservoir, close screw cap, and transfer the kidney perfusion circuit back to the incubator.
6. Start the pump at 4 ml/min and allow reagent to perfuse through the kidneys for exactly 1 hr.
7. After 1 hr, stop the pump, and transfer the kidney perfusion circuit to the biological safety cabinet.
8. Collect the conditioned (partially reduced) resazurin solution, and add 100 ml culture medium to the bioreactor reservoir. Transfer perfusion circuit to incubator, and resume flow (4 ml/min).
9. Pipette 100 µl (x 3 replicates) conditioned resazurin solution, resazurin working solution, autoclaved working solution, and culture media blank into a black (opaque) 96-well assay plate. Read fluorescence intensity (excitation: 540/35; emission: 590/20) using a spectrophotometer.
10. Calculate percent reduction as a ratio of fluorescence intensities normalized by the fluorescence intensity (FI) generated by the oxidized resazurin medium (ORM, or resazurin working solution not exposed to cells) or the reduced resazurin medium (RRM, or autoclaved working solution): % reduction = 100 x {[FI(conditioned resazurin solution) - FI(ORM)] / [FI(RRM) - FI(ORM)]}. To normalize results, multiply % reduction by circulating volume (10 ml) and divide by incubation time (1 hr).

Representative Results

Kidneys sequentially perfused with water and dilute detergent solutions (1% Triton X-100, 0.1% SDS) according to a previously established, optimal decellularization protocol (see **Figure 1A, B**)[7], become progressively more transparent over a 26 hr period, as shown in **Figure 2A**. The resulting acellular kidney scaffold is devoid of cells and retains a cohesive renal ECM supported by an intact renal capsule, which is undamaged following the perfusion protocol. By the final detergent perfusion (SDS), the kidney's vascular network, and in particular the interlobar vessels, are prominently displayed in the decellularized scaffold, owing to the greater thickness of these blood vessels relative to the comparatively thin basement membrane of the nephron tubules (see **Figure 2A, B**). The entire organ is cleared of native cells, leaving behind the intact basement membrane network of glomeruli, tubules, and collecting ducts, and the ECM of decellularized blood vessels, including internal and external elastic laminae of cortical interlobular arteries (see **Figure 2B, C**). In addition to the larger vessels, the microvascular basement membranes within glomeruli also retain structural integrity (see **Figure 2B, C**).

Decellularized kidneys are stored in PBS at 4 °C to limit the natural hydrolytic deterioration of the renal ECM, and should be used within 2 weeks of decellularization. We have previously described in detail the design of a custom perfusion-based kidney bioreactor that is used for both seeding decellularized rodent kidney scaffolds, and long-term culture of the recellularized organs under flow[17]. For recellularization, a perfusion circuit composed of small-diameter silicone rubber and fatigue-resistant PharMed pump tubing is used to route culture medium from the bioreactor reservoir, to a peristaltic pump, and back to the inlet Luer acceptor to which the catheterized renal artery is connected at the inner face of the bioreactor head (see **Figure 1C**). After sterilizing the decellularized kidney by perfusion with a peracetic acid/ethanol mixture, the perfusion system is prepped for seeding by circulating standard culture medium (containing 10% FBS to improve cell-ECM adhesion) through the scaffold within the incubator.

The decellularized kidneys may now serve as acellular ECM templates for recellularization, which we have previously performed using induced pluripotent stem cell-derived endothelial cells for revascularization[7]. Here, we demonstrate the utility of this scaffold and perfusion bioreactor system for tubulogenesis using human-derived renal cortical tubular epithelial (RCTE) cells. RCTE cells are seeded into kidney scaffolds through the renal artery under a high-pressure perfusion protocol that has been described previously[7,17].RCTE cells infused in this manner home primarily to the cortical regions of the kidney, where they preferentially repopulate the periglomerular tubules (see **Figure 3A**). Few cells embed within glomeruli at day 1 post-seeding, and by day 7, glomeruli are virtually devoid of cells. During perfusion culture, medium is changed every 2 days, at which point the resazurin perfusion assay is concurrently performed to provide comparative assessments of cell viability over time (see **Figure 3B**). As supported by the resazurin reduction results, RCTE cells proliferate within the 3D ECM, forming tightly organized, patent tubular structures by day 7. While the majority of these cells occupy the cortical regions of the renal ECM, after 7 days of antegrade perfusion culture many RCTE-lined tubules are present in the outer medullary and papillary tubules and collecting ducts (see **Figure 4**). After repopulation with RCTE cells and one week of perfusion culture, the transparency observed following decellularization is lost, and the recellularized kidney appears opaque and closer in appearance to its native state (see **Figure 4**).

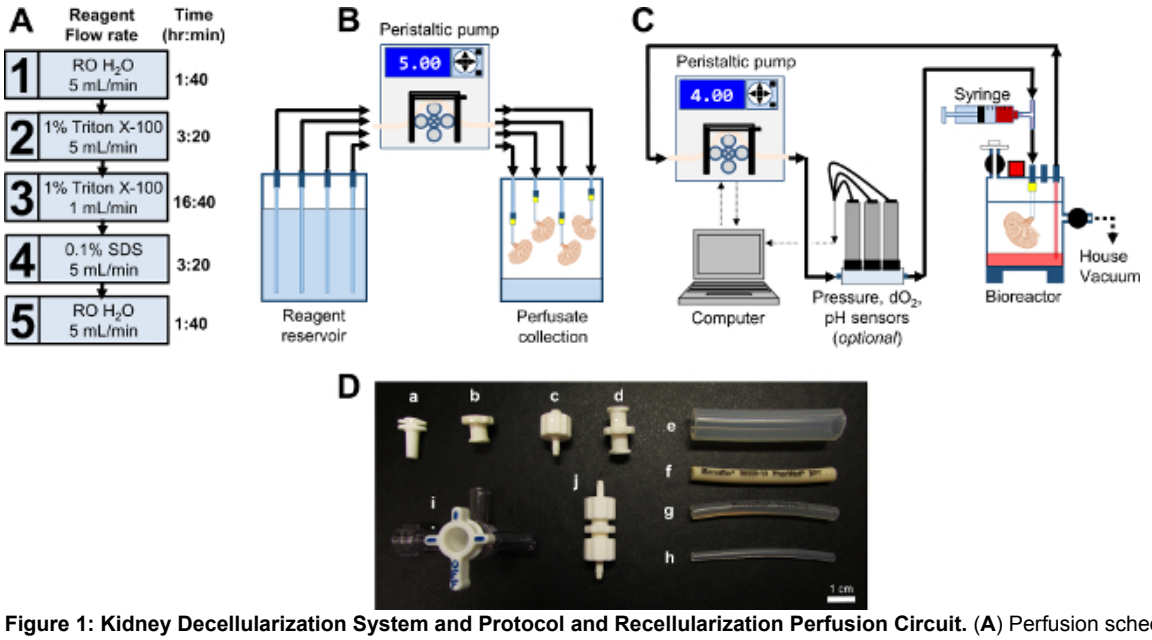

Figure 1: Kidney Decellularization System and Protocol and Recellularization Perfusion Circuit. (**A**) Perfusion schedule of reagents for decellularization of rodent kidneys. The reagents used for decellularization, volumetric flow rate, and duration of each step are shown. (**B**) Perfusion decellularization set-up. Solutions are pumped unidirectionally from a reagent reservoir to a perfusate collection container through individual flow lines for each kidney undergoing decellularization. Up to four kidneys may be decellularized per peristaltic pump. (**C**) Perfusion circuit for seeding and culture of recellularized kidney scaffolds. Cells are loaded through a syringe connected directly upstream of the Luer inlet acceptor. Optional in-line sensors for monitoring pressure, dissolved oxygen (dO_2), and pH may be placed upstream of bioreactor. The digital peristaltic pump may be controlled by computer, and negative pressure (partial vacuum) may be applied to aid ureteral seeding. (**D**) Components used to create perfusion lines for decellularization (**B**) and recellularization (**C**). (a) male Luer plug, (b) female Luer cap, (c) male Luer lock to 1/8" barbed adapter, (d) female Luer to female Luer adapter, (e) ¼" ID silicone tubing, (f) peristaltic pump tubing, g: thick-walled 1/16" ID silicone rubber tubing, (h) 1/16" ID silicone rubber tubing, (i) three-way stopcock, (j) coupler used to connect segments of tubing by combining two 1/8" barb to male Luer adapters (c) using a female Luer to female Luer coupler (d). Please click here to view a larger version of this figure.

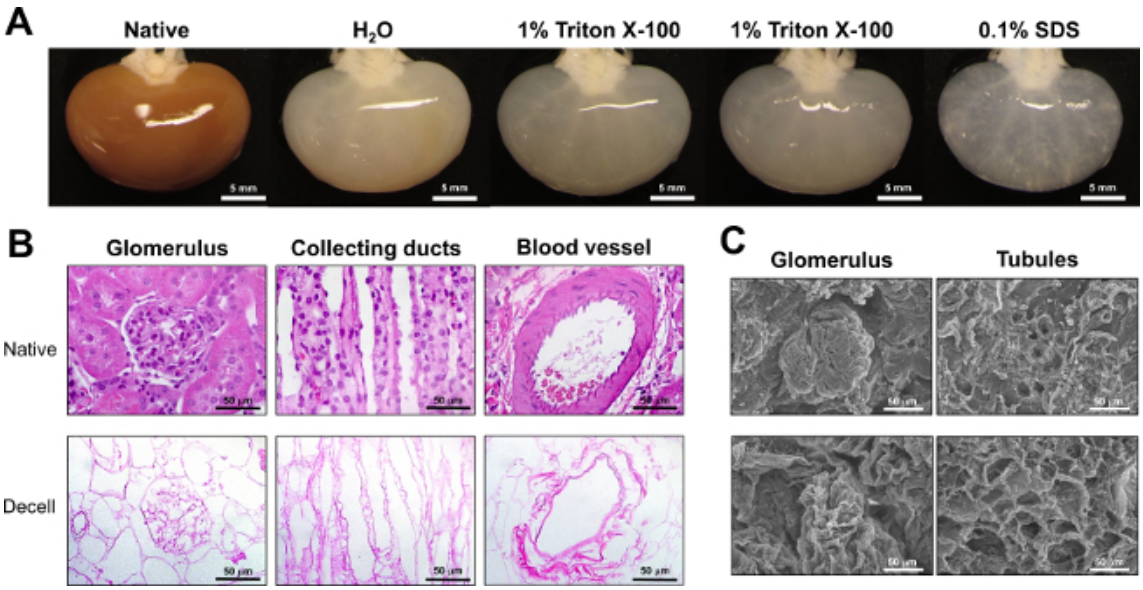

Figure 2: Representative Results of Kidney Decellularization. Representative gross and microscopic images are shown of kidneys before and following decellularization. (**A**) Time lapse series of a kidney undergoing decellularization. Images are shown immediately following perfusion with each reagent specified. Following perfusion of 0.1% SDS (sodium dodecyl sulfate), preserved vascular network is visible. (**B**) Representative images of native or decellularized kidneys sectioned and stained with hematoxylin and eosin (H&E). The ECM architecture of microstructural features, including glomeruli, collecting ducts, and blood vessels, is well-preserved following decellularization. (**C**) Scanning electron micrographs comparing glomeruli and tubules in native (top row) and decellularized (bottom row) kidneys. Following cell removal, open tubules are prevalent throughout the kidney. Please click here to view a larger version of this figure.

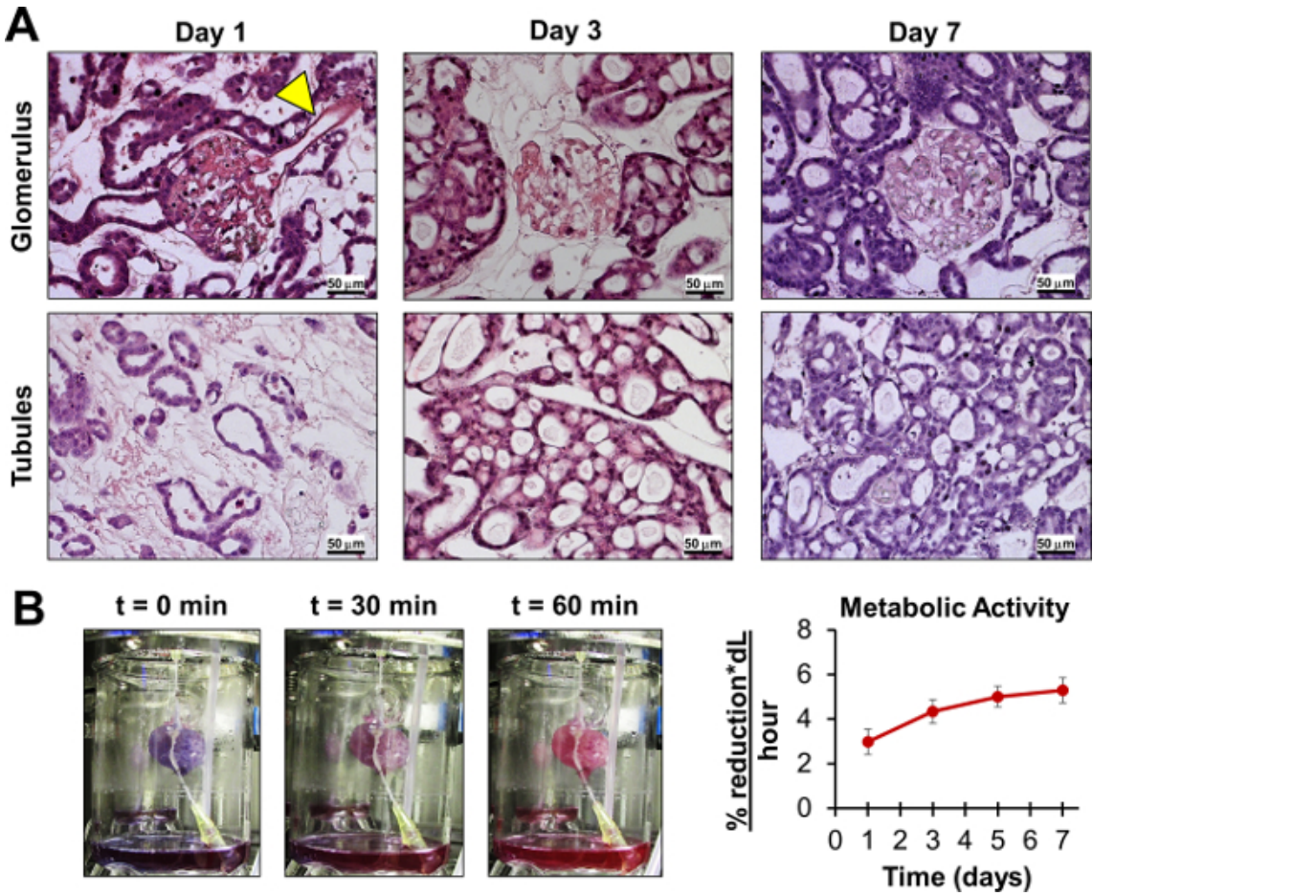

Figure 3: Representative Results of Human Renal Cortical Tubular Epithelial Recellularization of Decellularized Rat Kidneys. Kidneys are recellularized through a high-pressure antegrade arterial perfusion technique that results in RCTE cell adhesion primarily to the renal tubules of the cortex. (**A**) Representative high-magnification images (20X) of hematoxylin and eosin-stained histological sections of recellularized scaffolds 1, 3, or 7 days after seeding. Top row shows that the cells occupy the periglomerular tubular space despite being injected through the arterial vasculature, indicating their preference for the former renal ECM niche. Yellow arrowhead points to an afferent arteriole with a lumen that is devoid of cells. Lower row shows cortical tubules where RCTE cells proliferate, with increasing cell density over one week, and form tightly packed tubular epithelial structures. (**B**) A small (10 ml) volume of resazurin-supplemented culture medium is recirculated through kidneys at the time of media changes. During 60 min of perfusion, cells reduce the oxidized resazurin compound (blue) to resorufin (red), which produces a highly fluorescent signal in proportion to the number of cells within the kidney. Consistent with the observed increase in cell density seen within the histological images, percent resazurin reduction increases over culture time with cell growth, providing a non-invasive indication of both cell viability and proliferation during maintenance culture. Results are presented as mean ± standard deviation (n = 4 recellularized kidneys). Please click here to view a larger version of this figure.

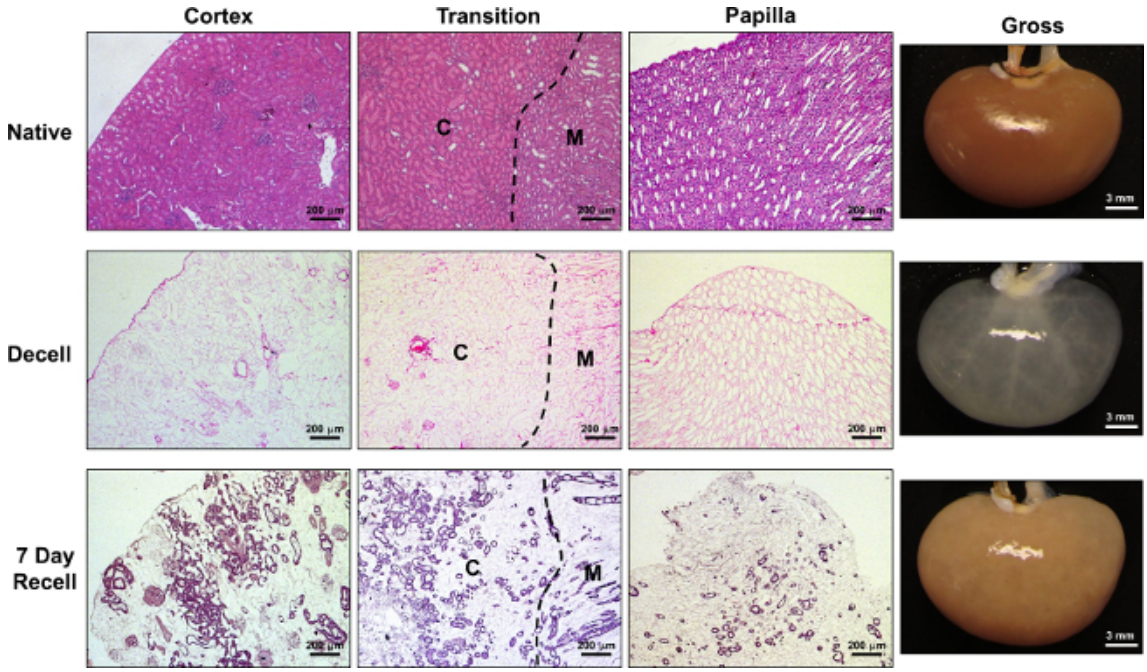

Figure 4: Representative Results: Comparison of Native, Decellularized, and Recellularized Kidneys. Low magnification histological images show the renal cortex, transition zone between the cortex and outer medulla, and medullary papilla regions in native kidneys (top row), decellularized (Decell; middle row), and 7-day RCTE-recellularized (7 Day Recell; bottom row) scaffolds. The dashed line indicates the approximate border between the renal cortex (C) and outer medulla (M). Gross images show a kidney in its native state after procurement, following decellularization, and 7 days following seeding of RCTE cells through the renal artery. Please click here to view a larger version of this figure.

Discussion

The described decellularization protocol consistently produces a completely acellular kidney ECM that serves as a 3D template for culture of human renal cortical tubular epithelial cells (both proximal and distal tubule-derived), in addition to vascular endothelial cells[7,17]. The cannulated renal vasculature serves as the key feature for uniform delivery of reagents and cells throughout the scaffold within a bioreactor set-up, thus enabling the perfusion decellularization, cell seeding, long-term perfusion culture, and the resazurin perfusion protocols. As such, proper cannulation of the renal artery prior to organ perfusion is critical, and special care must be taken to ensure that the renal artery is not obstructed or damaged, and that the catheter is secured. The Sprague Dawley rats from which the kidneys are recovered must be systemically heparinized to avoid clotting within the vasculature during organ procurement, as intravascular clots cannot be removed, and may inhibit complete decellularization of the kidney.

The perfusion bioreactor used for seeding and perfusion culture of recellularized kidneys is designed to allow multiple seeding methods *in situ*[17]. In addition to the arterial injection technique described here, cells may be injected retrograde through the catheterized ureter or renal vein. Furthermore, the bioreactor body is fitted with a valve that permits application of negative pressure (partial vacuum; see **Figure 1C**), for ureteral seeding. Regardless of the seeding strategy utilized, perfusion of culture medium antegrade through the renal artery is critical for adequate nutrient (*e.g.* oxygen, glucose) delivery to seeded cells.

The described recellularization protocol demonstrates our use of decellularized renal matrices to serve as templates specifically for epithelial tubulogenesis. However, we have previously shown that the renal matrix also supports re-endothelialization of the retained vascular network and may be lined with human induced pluripotent stem cell-derived endothelial cells, which is an important observation for eventual long-term transplantation of recellularized kidneys in animal models by preventing thrombotic occlusion of the renal vasculature[7]. A current obstacle to the eventual scale-up of kidney recellularization protocols to large-animal kidney scaffolds is the substantially greater number of cells required to repopulate human-sized kidney scaffolds. Efficient seeding strategies, such as the high-pressure arterial injection technique described above, are critically needed to maximize the number of cells that engraft within the decellularized ECM. Given the heterogenous cellular composition of the native kidney, multiple seeding methods may ultimately be required to effectively repopulate the various extracellular renal niches with diverse cell types that collectively perform the numerous functions of the kidney, including filtration, reabsorption, concentration of urine, and hormone synthesis.

Finally, the resazurin perfusion assay demonstrated in this article provides a non-invasive, non-toxic assessment of cell viability and proliferation during long-term culture[7,17,18]. The assay provides instantaneous feedback on the metabolic state of cells growing within kidney scaffolds, and when regularly performed in between periodic medium exchanges, can be used to characterize cell proliferation over time. The resazurin reagent is inexpensive, the assay requires little time to perform (1 hr), and it is a more conservative analytical method to characterize cell proliferation or metabolic trends compared to histological evaluation, which requires terminal sacrifice of the recellularized scaffold. The resazurin perfusion assay can also be adapted for evaluation of cell populations during growth within other recellularized organs or tissues.

Disclosures

The authors have nothing to disclose.

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