Journal of Visualized Experiments

Production of extracellular matrix fibers via sacrificial hollow fiber membrane cell culture

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58791R1
Full Title:	Production of extracellular matrix fibers via sacrificial hollow fiber membrane cell culture
Keywords:	scaffold; Tissue engineering; Extracellular Matrix; Regenerative medicine; membrane
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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)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Department of Orthopedics, University of Arkansas for Medical Sciences, Little Rock, Arkansas

UNIVERSITY OF ARKANSAS

College of Engineering
Department of Biomedical Engineering

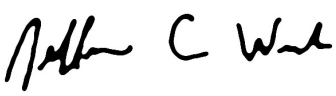
July 13, 2018

Dear Editor,

Our group explores strategies toward the repair of tissues through the engineering of novel implantable biomaterials. To this end, we have developed a class of extracellular matrix (ECM) implants isolated by the solvent degradation of cultured polymeric foams and hollow fiber membranes.

In this manuscript we describe specific protocols for the fabrication, cell culture, and degradation of our sacrificial scaffolds as well as processing of the ECM constructs for implantation. The hollow fiber membranes described in this manuscript allow diffusion of cell culture nutrients but trap the high molecular weight ECM secreted by cells, after which the cell culture scaffold can be dissolved using a solvent to yield ECM constructs which can be decellularized for implantation. We believe that these protocols will be of significant interest to the JoVE Bioengineering audience. Dr. Nandita Singh has assisted us through the submission of this invited manuscript.

Sincerely,



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TITLE:

Production of Extracellular Matrix Fibers via Sacrificial Hollow Fiber Membrane Cell Culture

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KEYWORDS:

Scaffold, tissue engineering, regenerative medicine, fibers, membrane, extracellular matrix

SUMMARY:

The goal of this protocol is production of whole extracellular matrix fibers targeted for wound repair which are suitable for preclinical evaluation as part of a regenerative scaffold implant. These fibers are produced by the culture of fibroblasts on hollow fiber membranes and extracted by dissolution of the membranes.

LONG ABSTRACT:

Engineered scaffolds derived from extracellular matrix (ECM) have driven significant interest in medicine for their potential in expediting wound closure and healing. Extraction of extracellular matrix from fibrogenic cell cultures *in vitro* has potential for generation of ECM from human- and potentially patient-specific cell lines, minimizing the presence of xenogeneic epitopes which has hindered the clinical success of some existing ECM products. A significant challenge in *in vitro* production of ECM suitable for implantation is that ECM production by cell culture is typically of relatively low yield. In this work, protocols are described for the production of ECM by cells cultured within sacrificial hollow fiber membrane scaffolds. Hollow fiber membranes are cultured with fibroblast cell lines in a conventional cell medium and dissolved after cell culture to yield continuous threads of ECM. The resulting ECM fibers produced by this method can be decellularized and lyophilized, rendering it suitable for storage and implantation.

INTRODUCTION:

Implantable surgical scaffolds are a fixture of wound repair, with over one million synthetic polymer meshes implanted worldwide each year for abdominal wall repair alone[1]. However, following implantation the synthetic materials polymers traditionally used in the fabrication of these scaffolds tend to provoke a foreign body response, resulting in inflammation deleterious to the function of the implant and scarring of tissue[2]. Further, as the predominant synthetic mesh materials (*i.e.,* polypropylene) are not appreciably remodeled by the body, they are generally applicable to tissues where scarring can be tolerated, limiting their clinical usefulness toward the treatment of tissues with higher-order function such as muscle. While there are many surgical mesh products which have been applied with clinical success, recent manufacturer recalls of synthetic surgical meshes and complications from interspecies tissue implants highlight the importance of maximizing implant biocompatibility, prompting the FDA to tighten regulations on surgical mesh manufacturers[3,4]. Implantation of scaffolds derived from patients' own tissues reduces this immune response, but can result in significant donor-site morbidity[5]. Extracellular matrix (ECM) scaffolds produced *in vitro* are a possible alternative, as decellularized ECM scaffolds exhibit excellent biocompatibility, particularly in the case of autologous ECM implants[6].

Because of the limited availability of patient tissue to harvest for autologous implantation and the risk of impeding function at the donor site, the ability to produce ECM scaffolds *in vitro* from the culture of human cell lines or, if possible, a patient's own cells is an attractive alternative. The primary challenges in the manufacture of substantial amounts of ECM *in vitro* is the sequestration of these difficult-to-capture molecules. In previous work, we have demonstrated that ECM can be produced by culturing ECM-secreting fibroblasts in sacrificial polymeric foams that are dissolved after the culture period to yield ECM which can be decellularized for implantation[7-10]. As ECM produced in foams tend to adopt the internal architecture of the foams, hollow fiber membranes (HFMs) were explored as a sacrificial scaffold for production of threads of ECM. Described herein are methods tasked for lab scale manufacturing of cell culture quality hollow fiber membranes and the extraction of bulk extracellular matrix fibers from the same following a period of fibroblast culture. This static culture approach is readily adoptable by laboratories containing standard mammalian cell culture equipment. ECM produced by this approach could be applied toward a variety of clinical applications.

PROTOCOL:

1. Production of Extracellular Matrix Using Sacrificial Hollow Fiber Membranes

CAUTION: N-methyl-2-pyrrolidone is an irritating solvent and reproductive toxicant. Exposure to NMP may cause irritation to the skin, eyes, nose, and throat. Solvent-resistant personal protective equipment should be used when handling NMP. Use of NMP should be performed within a fume hood.

1.1. Preparation of polysulfone polymer solution for hollow fiber membranes

1.1.1. Weigh 70 g of polysulfone pellets (molecular weight of 35 kD) in a weigh boat using an analytical balance.

1.1.2. Transfer 314 mL (323.3 g) of N-methyl-2-pyrrolidone (NMP) to a clean borosilicate flask.

1.1.3. Place a stir bar in the flask with NMP and place flask onto stirrer. Set the stirrer to a moderate rate of rotation.

1.1.4. Use a dry funnel to slowly insert the prepared 70 g of polysulfone into the flask with NMP.

1.1.5. Allow the polymer "dope" solution to stir for three days at room temperature, or until homogenized.

1.2. Setting concentricity and cleaning of the hollow fiber membrane spinneret

Note: Spinnerets are commercially available; correct spinneret dimensions are critical to successful membrane fabrication and are listed in the **Table of Materials**. Handle the spinneret very gently, as the spinneret needle is particularly fragile.

1.2.1. Use a screwdriver or drill with an appropriate screwdriver bit to carefully disassemble the spinneret.

1.2.2. Gently flow acetone through the inlet and outlet of the spinneret, collecting any acetone and residual polymer in a glass beaker for disposal.

1.2.3. Secure the upper body of the spinneret with the needle facing upward in a table vise.

1.2.4. Place the outlet (bottom) body of the spinneret onto the upper body.

1.2.5. Move the outlet body of the spinneret laterally while holding the upper body fixed on the vise until the spinneret needle is directly in the center of the outlet body.

1.2.6. Carefully and slowly reinsert the screws connecting the two bodies of the spinneret while ensuring that the needle visible at the spinneret outlet remains centered.

1.3. Fabrication of polysulfone hollow fiber membranes

1.3.1. Prepare a "bore solution" containing 45 mL of N-methyl-2-pyrrolidone and 255 mL of deionized water, forming a 15% mixture of NMP with deionized water.

1.3.2. Mount a concentric hollow fiber membrane spinneret 8 cm above the surface of a room-temperature tap water bath.

1.3.3. Connect the polymer inlet of the spinneret and the bore inlet of the spinneret to two

separate steel pressure vessels having an inner volume of at least 350 mL. Both vessels must have check valves at their outlets.

1.3.4. Connect each of the pressure vessels to the regulators of separate N_2 gas cylinders

1.3.5. Use a funnel to insert the prepared dope solution (~315 mL) into the pressure vessel connected to the polymer inlet of the spinneret. Ensure that the top of the vessel is tightly sealed.

1.3.6. Use a funnel to insert the prepared bore solution (300 mL) into the pressure vessel connected to the bore inlet of the spinneret. Ensure that the top of the vessel is tightly sealed.

1.3.7. Open the check valve for the bore vessel first, then the polymer vessel second. If the spinneret is sufficiently clean, the bore solution will begin to stream out of the spinneret outlet.

1.3.8. Pressurize both vessels to 1 PSI. Visually confirm that both polymer and bore solutions are exiting the spinneret, with a continuous white filament precipitating as the combined streams contact the water bath.

1.3.9. Use long forceps to guide the nascent fiber under the rollers in the center of the bath, and then wind the nascent fiber around a rotating wheel connected to a motor.

1.3.10. Set the take-up wheel and motor to rotate at a rate of approximately two meters per minute.

1.3.11. Make minor adjustments to the regulators or take-up wheel speed as needed until a steady-state flow is achieved. The nascent fiber should form a 90° angle with the surface of the water bath.

1.3.12. Close the N_2 cylinder regulators and pressure vessel check valves and disengage the take-up wheel motor after all polymer and bore solution has been extruded from the vessels.

1.3.13. Remove the prepared hollow fiber membranes and place them into a deionized water bath for three days, exchanging deionized water once per day to remove residual solvent.

1.3.14. Sterilize the hollow fiber membranes by autoclaving them at 121 °C for 30 minutes or by immersing them for one day in 70% ethanol.

1.4. Cell seeding of hollow fiber membranes

Note: All cell culture procedures should be performed within a biosafety cabinet.

1.4.1. Treat the hollow fiber membranes with a 20 µg/mL solution of bovine plasma fibronectin in PBS (pH = 7.4) to promote cell attachment.

1.4.1.1. Weigh 1 mg of powdered bovine plasma fibronectin in a weigh boat and dissolve it in 1 mL of sterile PBS (pH = 7.4) in a sterile 1.5 mL microcentrifuge tube.

1.4.1.2. Incubate the 1 mg/mL bovine plasma fibronectin solution in the incubator for 30 minutes. Add the solution to 49 mL of sterile PBS in a sterile 50 mL conical centrifuge tube.

1.4.1.3. Incubate the fibers in the prepared 50 mL of bovine plasma fibronectin in an incubator at 37 °C with 5% CO_2 for 1 hour. Recollect the fibronectin solution for later use and store in a sterile 50 mL conical centrifuge tube at 4 °C.

1.4.2. Use sterile microscissors to cut the fibers to desired length (<6 cm).

1.4.3. Seed fibroblast cells directly into the lumina of the hollow fiber membranes at a seeding density of 100,000 cells per fiber using a 21-gauge needle with 1 mL syringe.

Note: A practicable target for preparing the cell-suspension for loading in the syringe is a suspension density of 100,000 cells for every 30 microliters of medium.

1.4.4. Place six seeded fibers into a 6 cm diameter Petri dish. Allow the seeded fibers to incubate at 37 °C with 5% CO_2 for five minutes.

1.4.5. Remove the seeded fibers from incubation and culture them for up to 3 weeks in DMEM/F-12 containing final concentrations of 50 µg/mL L-ascorbic acid and 150 µg/mL L-ascorbic acid 2-phosphate (freshly prepared and sterile filtered), 5 ng/mL TGF-β1 (from sterile filtered aliquots stored at -20 °C), and 1% penicillin-streptomycin in a 6 cm Petri dish within a 5% CO_2 incubator at 37 °C. Exchange cell medium every two days.

1.5. Extraction of extracellular matrix from cultured hollow fiber membranes

1.5.1. Place cultured fibers into individual scintillation vial using forceps.

1.5.2. Place up to 5 mL of NMP into each vial using a glass pipette.

1.5.3. Immerse the hollow fibers in NMP, performing a total of three exchanges of NMP. Slowly aspirate the old NMP using a 1 mL pipette to prevent tearing of extracted ECM.

Note: When adding fresh NMP, it is helpful to tilt the vial and allow the NMP run down the sides, otherwise the remaining ECM scaffold is subjected to shear which may cause tearing.

1.5.4. Remove NMP and rinsed the resulting ECM thread 3 times in deionized water.

1.5.5. Cut a 1/32-inch thick sheet of silicone rubber to a length of 3 inches, width of 1 inch and cut an 8 mm by 4 mm rectangular mold in the center of the length of rubber.

1.5.6. Place the prepared piece of rubber onto a standard 3-inch by 1-inch microscopy slide and autoclave at 121 °C for 30 minutes.

1.5.7. Lay each ECM fiber side by side in the 8 mm by 4 mm silicone mold until there is no visible open space.

1.5.8. Place the mold into a 50 mL conical centrifuge tube and freeze at -80°C until completely frozen.

1.5.9. Lyophilize frozen ECM mesh overnight or until completely dry. Store the mesh at 4°C until ready for decellularization.

2. Decellularization of Extracellular Matrix

2.1. Prepare a solution of 1% (w/w%) sodium dodecyl sulfate (SDS) in deionized water.

2.2. Incubate extracted extracellular matrix in the mold in 1% SDS for 24 hours at room temperature on a rocker with gentle agitation.

2.3. Rinse extracted extracellular matrix three times in sterile phosphate-buffered saline (PBS) with gentle agitation, using 3 mL of PBS per rinse.

Note: Rinses should be performed gently to minimize tearing of prepared scaffolds.

2.4. Prepare 1 L of DNAse/RNAse digestion buffer.

Note: DNAse/RNAse digestion buffer may be stored for several months at 4°C.

2.4.1. Weigh 1.54 g of Tris HCl, 308 mg of $MgCl_2$, and 56 mg of $CaCl_2$ in separate weigh boats.

2.4.2. Combine Tris HCl, $MgCl_2$, and $CaCl_2$ with 1 L of deionized water in a large flask with a stir bar and stir until all components are dissolved.

2.4.3. Sterile filter the digestion buffer with a 0.22 µm filter into a sterile 1 L borosilicate bottle and store at 4°C.

2.5. Prepare 5 mL of DNAse/RNAse digestion solution.

Note: Digestion solution should be used within 24 hours of preparation.

2.5.1. Weigh 0.125 mg (50 kU) of DNAse I in a weigh boat and add to 5 mL of digestion buffer.

2.5.2. Add 75 µL of RNAse A stock solution to the digestion buffer.

2.6. Add 32 µL of the digestion solution to each prepared mold and incubate at 4°C for 6 hours.

2.7. Aspirate the digestion solution and rinse three times in sterile PBS.

2.8. Aspirate the PBS and incubate scaffolds overnight in 10% penicillin-streptomycin in PBS at 4°C.

2.9. Aspirate the penicillin-streptomycin and rinse scaffolds three times in sterile PBS.

2.10. Place the mold into a 50 mL conical centrifuge tube and freeze at -80°C.

2.11. Lyophilize the decellularized ECM mesh overnight or until completely dry.

2.12. Transfer the lyophilized scaffolds within a biosafety hood to a sterile container at 4°C pending use.

REPRESENTATIVE RESULTS:

Successful production of extracellular matrix from sacrificial scaffolds is contingent on appropriate scaffold fabrication, cell culture, and solvent rinse procedures. Fabrication of the hollow fiber membranes is performed using a dry-jet wet-spinning system assembled from commercially available components (**Figure 1**) which uses extrusion of polymer solution through the annulus of a commercially available steel spinneret (inner diameter = 0.8 mm, outer diameter = 1.6 mm) to generate a nascent tube of polymer solution which precipitates into a hollow fiber membrane upon contact with a water bath.

An example process for ECM extraction from HFMs is illustrated in **Figure 2**. **Figure 3A** shows a transverse cross-section of polysulfone HFMs fabricated under this protocol, exhibiting outer and inner layers of finger-like pores characteristic of an asymmetric membrane. In this protocol, cells are seeded specifically in the inner lumen of the membrane and cultured in 6 cm diameter Petri dishes (**Figure 3B**), with cells tending to proliferate on all surfaces of the membrane. Cultured membranes can then be subjected to batch NMP and deionized water rinsing in standard glass scintillation vials (**Figure 3C**), producing translucent threads of ECM (**Figure 3D**). ECM-producing cells remain viable inside HFMs throughout the 3-week period of culture (**Figure 4**).

HFMs cultured for three weeks with primary rat skeletal muscle fibroblasts (RSMF) were dissolved via three exchanges of N-methyl-2-pyrrolidone, after which they were rinsed three times in deionized water. The extracted matrix, normally being translucent in appearance when hydrated (**Figure 5A**), will tend to cloud upon hydration if not subjected to appropriate solvent rinsing due to the presence of residual polymer. It should also be noted that the ECM remaining after dissolution of the membrane is somewhat fragile, requiring care in handling with fine forceps. ECM fibers assembled into meshes and then lyophilized exhibit an off-white color and fibrous appearance with a gross longitudinal alignment (**Figure 5B**).

FIGURE LEGENDS:

Figure 1: Illustrated process flow for casting hollow fiber membranes. Hollow fiber membranes are manufactured using the ubiquitous non-solvent induced phase separation method (NIPS) using a system prepared from commercially available components listed in the table of specific materials. Polysulfone in NMP (17.8 w/w%) and NMP bore solutions (15 w/w% NMP in water) are extruded from separate stainless steel vessels by pressurized N_2 into a spinneret, generating a nascent hollow fiber membrane at the spinneret outlet which fully precipitates upon contact with the tap water precipitation bath. Nascent hollow fiber is manually guided under and over guides and allowed to collect on a rotating motorized take-up wheel at a rate of 2.3 meters per minute.

Figure 2: Illustrated production of ECM from cultured hollow fiber membranes. Hollow fiber membranes are seeded with fibroblasts and cultured for three weeks, followed by exchange of NMP 3x and exchange of deionized water 3x.

Figure 3: Culture and extraction of ECM. Asymmetric mesoporous hollow fiber membranes **(A)** were cultured for three weeks with RSMF cells in DMEM/F-12 with supplemented ascorbic acid and TGF-β **(B)**. Cultured hollow fibers (**C, D** top) were dissolved via 3 exchanges in n-methyl-2-pyrrolidone then rinsed three times in deionized water, resulting in continuous threads of ECM (**D**, bottom). High magnification scanning electron microscopy micrograph of the ECM fiber surface **(E)**.

Figure 4: Cell viability on hollow fiber membranes. Representative hollow fiber membranes cultured with RSMF cells for three weeks were longitudinally sectioned using a fine razor to reveal the luminal surface of the HFMs and subjected to live-dead staining with Calcein AM and EthD-1. Viability staining revealed a confluent layer of viable cells with negligible EthD-1 fluorescence.

Figure 5: Assembly of ECM implant. Individual extracellular matrix threads (n = 30) derived from culture of RSMF cells were placed lengthwise into a silicone mold **(A)** and decellularized by 1% SDS followed by treatment with DNAse I, RNAse A, and penicillin-streptomycin. Decellularized ECM was then lyophilized, yielding an off-white mesh with a fibrous appearance and longitudinal architecture **(B)**.

DISCUSSION

The processes described enable the production of bulk ECM biomaterials *in vitro* using hollow fiber membranes cast by a dry-jet wet spinning system allowing for inexpensive bulk production of membranes as well as standard cell culture equipment. While the membranes fabricated in this protocol are intended for use in cell culture, the system described can also be adapted for the production of membranes for separation purposes, with pore size distribution and hollow fiber dimensions tunable by varying spinneret dimensions, polymer used, dope and bore flow rate, take-up speed, and environmental conditions.[13] Though the protocol detailed employs hollow fiber membranes, in principle any dissolvable cell culture scaffold with

appropriate transport properties such as open cell foams could be used, as demonstrated in previous work[7-9]. This general approach appears useful for the production of ECM scaffolds by sacrificial scaffolds which can more faithfully mimic the internal architecture of tissues. Implants produced by this protocol in particular exhibit a gross alignment (**Figure 5B**), which may be of particular benefit toward the reconstruction of highly aligned tissues such as tendons, ligaments and skeletal muscle.

Regular exchange of medium and, in particular, supplementation of medium with ascorbic acid and TGF- β is crucial to production of ECM, as ascorbic acid is an essential enzyme in collagen biosynthesis, and TGF- β induces the synthesis of several ECM proteins[10]. Additionally, thorough rinsing of ECM with NMP must be performed, otherwise residual polymer will remain in the extracted ECM, appearing as a white film during water rinses. Care must be taken to not overly agitate ECM during membrane dissolution, as it is relatively fragile. Collected ECM scaffolds intended for implantation must be subjected to a decellularization step as described to remove xenogeneic epitopes to minimize a potential host foreign body response.

The significance of this technique lies in its production of a biocomplex scaffold of whole extracellular matrix which can be remodeled by the body's own wound-healing processes. By using this approach to produce ECM from cells specific to a target species, it may be possible to minimize the foreign body response which hinders the clinical effectiveness of this class of biomaterials; by using cells specific to an individual, the foreign body response may be lessened further. This approach also allows for the production of ECM targeted toward particular tissues, with recent reports suggesting that tissue-specific ECM may be particularly effective in certain applications[12]. As this protocol allows for production of ECM across various cell lines, implants combining ECM from several cell types (*e.g.* muscle, nervous, endothelial) could be used to tailor implants to more faithfully approximate the structure and chemical complexity of target tissues. While the ECM meshes presented here were originally intended as scaffolds for wound repair, they may also have use as platforms for investigations into cell-ECM interactions, durotaxis, and biosensing. In particular, this approach lends the investigator the ability to produce ECM from specific cell types of interest which may allow for new insights into the biological significance of tissue-specific ECM structure, composition and function.

Potential improvements to the ECM production techniques presented here could include scale-up via the use of dynamic and pre-conditioning bioreactors as well as exploration of alternative sacrificial scaffold materials and architectures. In particular, transition to a solventless scaffold removal process would improve the safety of the extraction process; sacrificial scaffolds composed of materials which are degradable by enzymes, such as regenerated cellulose, may allow for solventless ECM extraction[14]. While the tensile strength of these materials is below those of synthetic materials, there exist several avenues for improvement of these scaffolds, including exploration of various culture conditions, media formulations, and sacrificial scaffold geometries. Recent genetic engineering advances could be leveraged to produce pro-fibrotic cell lines, which in combination with platforms for ECM capture could enable the production of scaffolds satisfying clinical demands. Further, existing dynamic culture systems such as continuous flow-loop hollow fiber membrane bioreactors facilitate high rates of nutrient

exchange conducive to greater and more rapid ECM production, and may be of particular interest in leveraging this technique for production of larger quantities of ECM for biological research and clinical use.

ACKNOWLEDGMENTS:

Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under award number R15AR064481, the National Science Foundation (CMMI-1404716), as well as the Arkansas Biosciences Institute.

DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

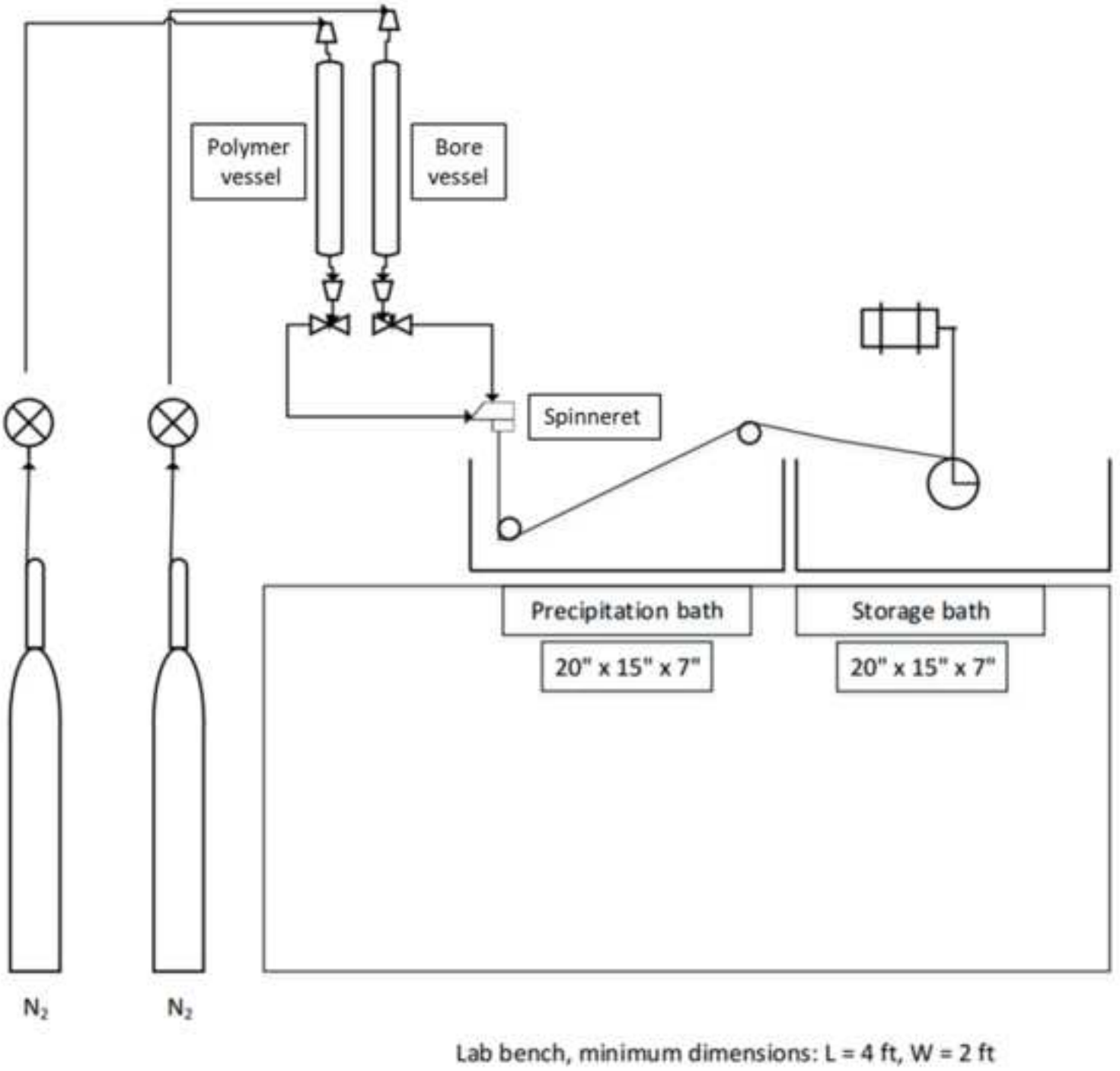

Figure 2

Figure 3

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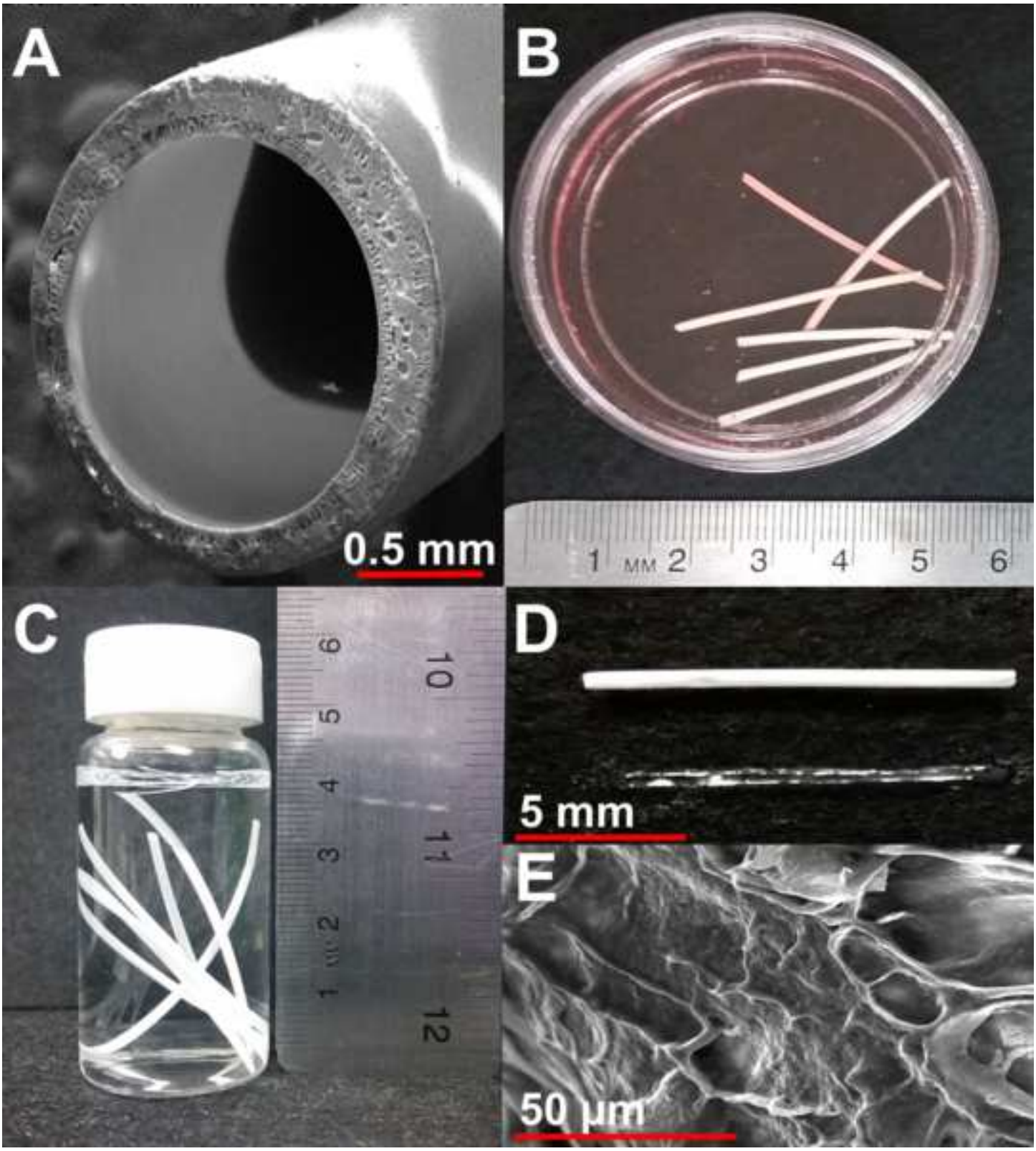

Figure 4

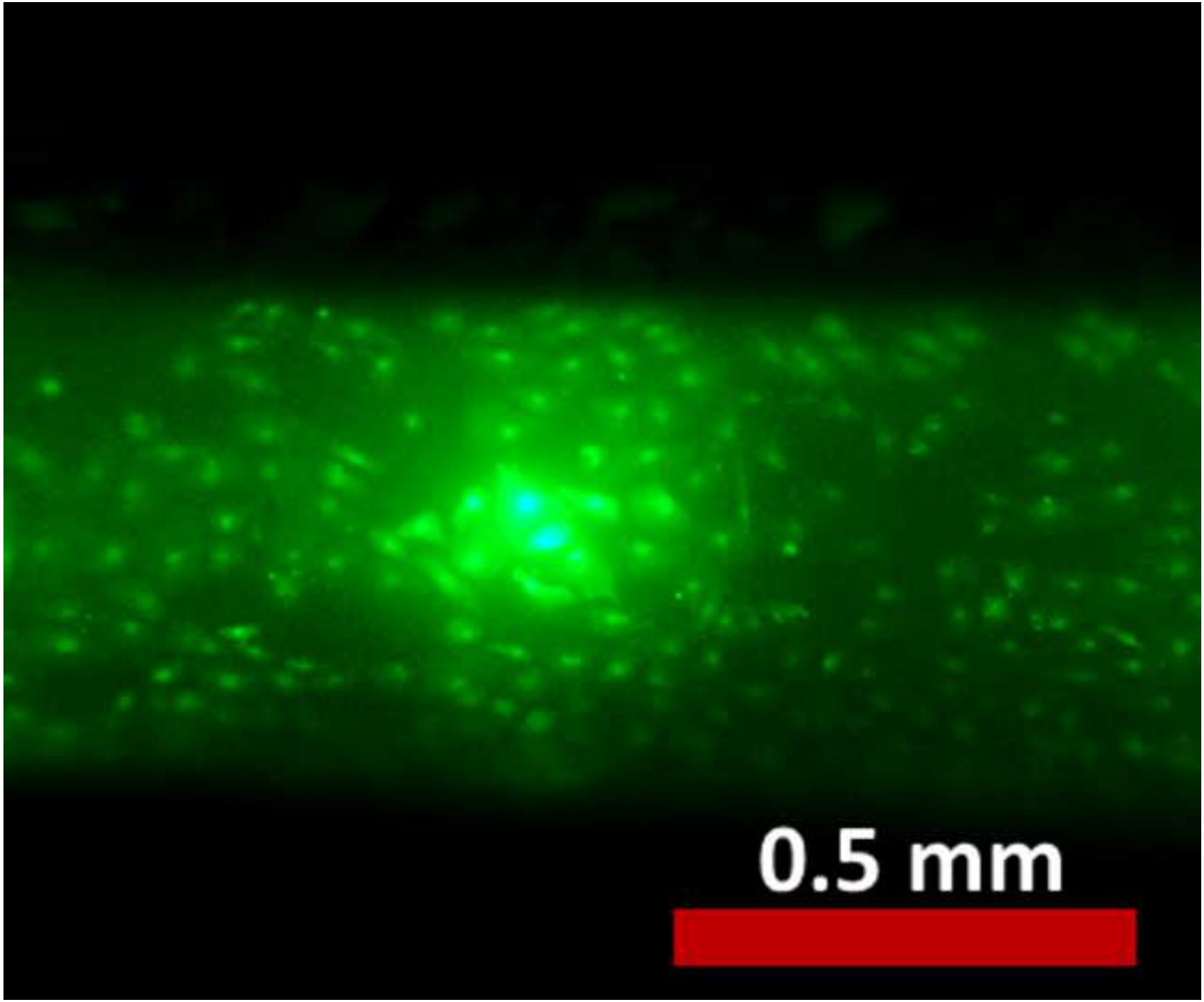

Figure 5

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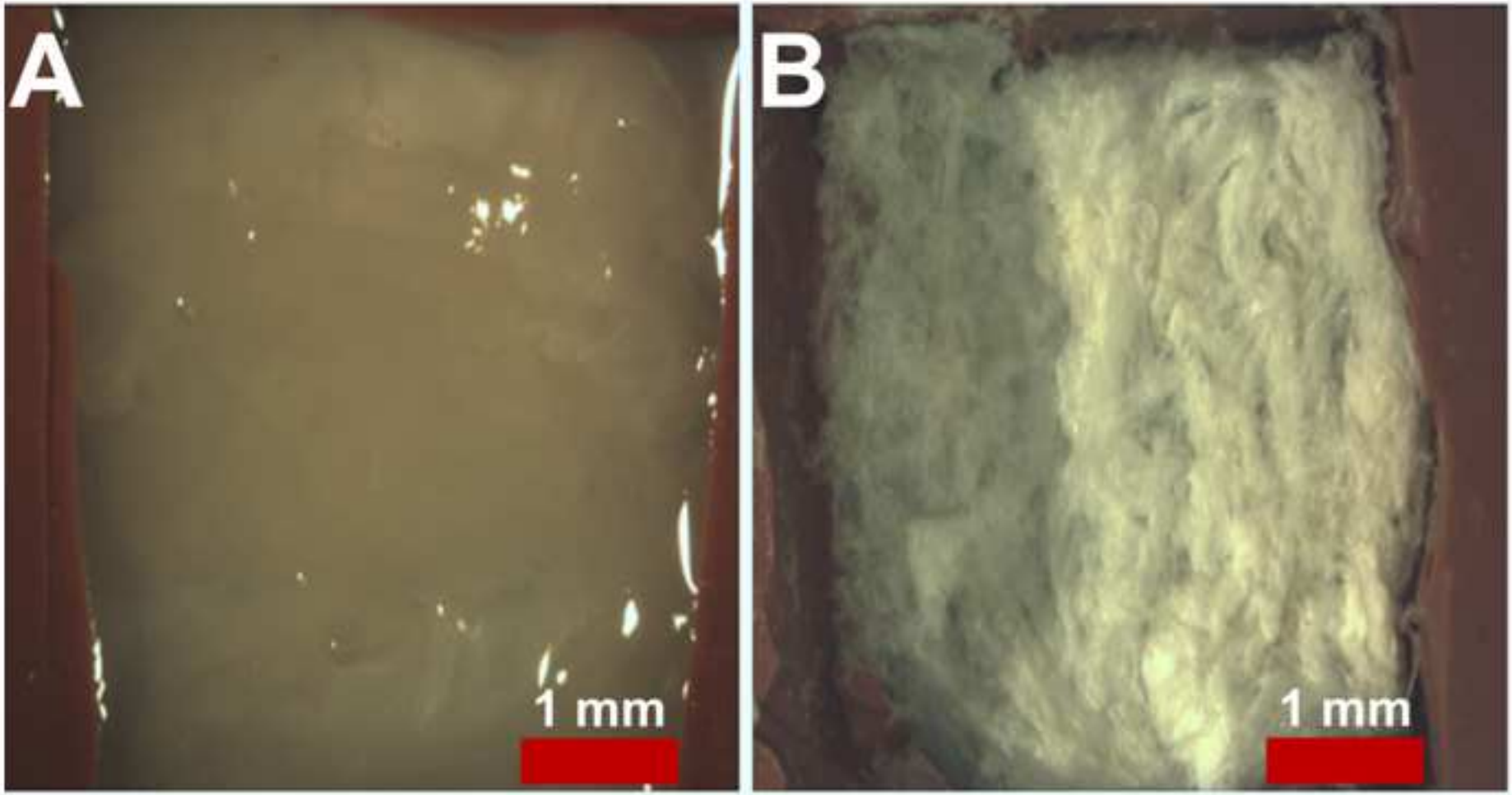

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1/32 inch thick silicone rubber	Grainger	B01LXJULOM	
20 mL Scintillation Vials, Borosilicate glass, Disposable - VWR	VWR	66022-004	With attached white urea cap and cork foil liner
3 inch by 1 inch microscopy slides	VWR	75799-268	
4C refrigerator	Thermo Fisher Scientific	FRGG2304D	Any commercial 4C refrigerator will suffice.
50 mL tubes	VWR	21008-178	
6-well cell culture plates	VWR	10062-892	Alternative brands may be used
Acetone	VWR	E646	Alternative brands may be used
Bore vessel	McMaster-Carr	89785K867	6 ft 316 steel tubing
Bovine Plasma Fibronectin	Thermo Fisher Scientific	33010018	Comes as 1 mg of lyophilized protein
$CaCl_2$	VWR/Amresco	97062-590	
Cell Culture Incubator w/ CO_2			Any appropriate CO2-supplied mammalian cell incubator will suffice.
Disposable Serological Pipets, Glass - Kimble Chase	VWR	14673-208	Alternative brands may be used
DMEM/F-12, HEPES	Thermo Fisher Scientific	11330032	Warm in water bath at 37°C for 30 minutes prior to use

DNase I	Sigma-Aldrich	DN25-10MG	
Dope vessel	McMaster-Carr	89785K867	6 ft 316 steel tubing
Ethanol	VWR	BDH1160	Dilute to 70% for sterilization
Fetal Bovine Serum, qualified, US origin - Gibco	Thermo Fisher Scientific	26140079	Mix with growth media at 10% concentration (50mL in 500mL media)
Four 1/4-inch to 1" reducing unions	Swagelok	SS-1610-6-4	One reducing union for each inlet and outlet of each vessel
Freeze-dryer/lyophilizer	Labconco	117 (A65312906)	Any lyophilizer will suffice.
Hexagonal Antistatic Polystyrene Weighing Dishes - VWR	VWR	89106-752	Any weigh boat will suffice
Hollow fiber membrane immersion bath			34L polypropylene tubs may be used or large bath containers can be fabricated from welded steel sheets
Hollow Fiber Membrane Spinneret	AEI	http://www.aei-spinnerets.com/specifications.html	Made to order. Inner diameter = 0.8 mm, outer diameter = 1.6 mm
Hot plate/stirrer	VWR	97042-634	
Human TGF-β1	PeproTech	100-21	
L-Ascorbic acid	Sigma-Aldrich	A4544-25G	
L-Ascorbic acid 2-phosphate	Sigma-Aldrich	A8960-5G	

L-glutamine (200 mM) - Gibco	Thermo Fisher Scientific	25030081	Mix with growth media at 1% concentration (5mL in 500mL media)
MgCl2	VWR/Alfa Aesar	AA12315-A1	
Minus 80 Freezer	Thermo Fisher Scientific	UXF40086A	Any commercial -80C freezer will suffice.
N2 gas cylinders (two)			
NIH/3T3 cells	ATCC	CRL-1658	Alternative fibrogenic cell lines may be used.
N-methyl-2-pyrrolidone	VWR	BDH1141	Alternative brands may be used
Penicillin/Streptomycin Solution - Gibco	Thermo Fisher Scientific	15140122	Mix with growth media at 0.1% concentration (0.5 mL in 500mL media)
Polysulfone	Sigma-Aldrich	428302	Any polysulfone with an average Mw of 35,000 daltons may be used
Portable Pipet-Aid Pipetting Device - Drummond	VWR	53498-103	Alternative brands may be used
PTFE tubing (1/4-inch inner diameter)	McMaster-Carr	52315K24	Alternative brands may be used.
Rat skeletal muscle fibroblasts			Independently isolated from rat skeletal muscle. Alternative fibrogenic cell lines may be used.
RNase A	Sigma-Aldrich	R4642	
Silicone sheet	McMaster-Carr	1460N28	

Take-up motor	Greartisan	B071GTTSV3	200 RPM DC Motor
Tris HCl	VWR/Amresco	97063-756	
Two needle valves	Swagelok	SS-1RS4	

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Article Title: Production of extracellular matrix fibers via sacrificial hollow fiber membrane cell culture

Signature: Jeff Wolchok Digitally signed by Jeff Wolchok DN: cn=Jeff Wolchok, o=University of Arkansas, ou=Biomedical Engineering, email=jwolchok@uark.edu, c=US Date: 2018.07.13 17:22:04 -05'00'

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The authors would like to thank the reviewers for their valuable time and effort in evaluating our manuscript. The following constitute our responses (**shown in bold**) to the reviewers' comments. Revisions within the manuscript are highlighted as well.

Referee 1

Major Concerns:

The advantage of this method is not fully addressed. Is high yield of ECM by this method compared to the other methods? Dose the ECM produced by this method function in vivo? The short abstract (summary) should be rewritten more precisely what they did, and the discussion part should be more related to the results we present.

> **The summary has been edited for clarity while staying within the 50-word maximum.**
>
> **Additional detail has been added to the representative results and discussion sections, particularly regarding the use of the hollow fiber casting system, as well as specific references to figure panels not previously specifically referenced in the text.**

Minor Concerns:

1. The all abbreviations should be clear indicated (for example: Line 317: DI, line 323: SEM; Line 325 HFM?)

> **"DI" at line 317 has been clarified to "deionized water"**
>
> **"SEM" at line 323 has been clarified to "scanning electron microscopy"**
>
> **"Title of figure 3 has been clarified to "cell viability on hollow fiber membranes"**

2. Line 121: how did they calculate "17.8 w/w%", should be "22.3% w/w"?

> **The mass of Polysulfone to be used has been corrected to 70 g in section 1.1 of "Production of extracellular matrix using sacrificial hollow fiber membranes"**
>
> **The volume of NMP to be used for the polymer solution has been corrected to 314 mL (323.3g, density NMP = 1.03 g/mL) in section 1.2 of "Production of extracellular matrix using sacrificial hollow fiber membranes"**
>
> **w/w% = (weight solute/weight solution)*100**
>
> **w/w% = (70g/(70+323.3g))*100 = 17.8 w/w%**

3. Line 204: what is container used for solution?

“a sterile 1.5 mL microcentrifuge tube” has been specified in section 4.1 of “Production of extracellular matrix using sacrificial hollow fiber membranes” for preparing the 1 mg/mL stock solution of bovine plasma fibronectin in PBS.

“a sterile 50 mL conical centrifuge tube” has been specified in section 4.1 for preparing the diluted solution of bovine plasma fibronectin in PBS (50 mL)

pH of sterile PBS to be used has also been specified in section 4.1

4. Line 245: "3." Should be "2."?

The section header number for “Decellularization of extracellular matrix” has been corrected from “3.” to “2.”

5. Line 257: this is not a step, should label as a note

The step previously labeled as 1.3.2 has been relabeled as a note.

6. Line 271-274: How to prepare Dnase 50KU? KU is KiloUnit?

Units for preparation of DNAse I and RNAse A in section 2.1.5 of “Decellularization of extracellular matrix” have been clarified. U (enzyme unit, kU => kilounits) refers to the amount of an enzyme necessary to catalyze the reaction of 1 micromole of substrate per minute.

7. in Figure 3, a fibroblast cell marker should be stained and present.

The authors recognize that evidence of fibroblast phenotype could be helpful and point to the original research publication where several lines of fibroblasts were used.

Roberts, K., Schluns, J., Walker, A., Jones, J. D., Quinn, K. P., Hestekin, J., & Wolchok, J. C.. Cell derived extracellular matrix fibers synthesized using sacrificial hollow fiber membranes. *Biomedical Materials*, *13*(1), (2017)

Referee 2:

Minor Concerns:

lines 198- 260 are highlighted and I was not sure of the purpose.

Sections were highlighted in yellow which the authors judged to be of interest for portrayal in the JoVE methods video as directed in the JoVE Instructions for Authors.

Reviewer #3:

Major Concerns:
none

Minor Concerns:
(1) In addition to its therapeutic significance, this high-yield ECM production method can be of interest to researchers working in biomechanics, cellular migration (e.g. durotaxis) or biosensing given the recent findings on ECM-bound nanovesicles, the exosome-like cargo-carrying biomarker particles which are now identified to be an integral and functional key component of the ECM. Given the strong medical/clinical significance it is reasonable that the authors put the weight for the motivation of the method to these areas, but it would broaden the perspective of the reader if a few sentences are included pointing to the research fields for which the method can be useful for, other than biomedicine.

The authors recognize the potential broader utility of this process and have added relevant commentary in the discussion section.

(2) It is not clear if all required elements of the HFM Spinneret from AEI are commercially available. In the earlier research articles by the authors (Biomedical Materials 2018), the Spinneret from the same vendor, seem to contain custom-built parts. This part of the protocol is not marked to be filmed. To be able to assure the efficient reproduction of the method by researchers who are not familiar with hollow fiber membrane fabrication, it would be good if the authors add to the associated parts of the protocol a note (section 2 or 3), to guide the reader on if the Spinneret is operated exactly in the form it was received from the vendor, if/what modifications have been applied or if such fibers could alternatively be purchased directly from the companies.

The authors have added language in the discussion clarifying that all components necessary for the hollow fiber membrane system are commercially available. The spinnerets themselves are available can be ordered from a AEI Inc., manufactured to desired dimensions listed in the Table of Specific Materials and language to this effect has been added in a note within section 1.2. A hyperlink to the manufacturer's website with schematics of the spinneret itself has also been provided in the Table of Specific Materials.

Figure 2 is not referred to in the main text of the article except for panel A (line 296). Figure 4A is not referred to in the main text of the article, just panel B (line 310).

All panels are now referenced in the text

Editorial comments:
Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The authors have proofread the manuscript for spelling and grammar.

2. Please add an author list and then provide the full postal address of each affiliation

The author list has been provided and postal codes have been added to each affiliation address.

3. Please provide an email address for each author.

Email addresses for each author have been provided.

4. Please spell out each abbreviation the first time it is used.

The first instance of ECM in the long abstract has been defined.

The first instance of SEM in the body has been defined.

The title of figure 3 was rephrased to eliminate need for abbreviation.

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

Section numbering has been modified to conform to the JoVE Instructions for Authors

6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

Several steps have been corrected to use the imperative tense. Mislabeled notes have been appropriately labeled.

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Additional detailed steps have been added throughout the protocol sections and extra clarifying details added to several preexisting steps. Additional details referring specifically to construction of the mesh scaffold displayed in figure 5 has been added under sections 1.5 and 2.1 of the protocol. An authoritative reference on hollow fiber membrane characterization has also been added.

Some examples:

A schematic of the spinneret as Figure 1 would greatly aid in the protocol.

A process flow diagram of the hollow fiber membrane casting system has been included as figure 1. A hyperlink to the manufacturer website with detailed schematics and descriptions of the spinneret itself have been added to the list of materials.

4.1: Please specify the incubation temperature.

Incubation temperature has been provided.

4.4: How large is the petri dish?

Diameter of the petri dish has been provided (6 cm).

4.5: Please specify the culture conditions.

Additional culture condition information has been provided.

8. Figure 1: Please use the lowercase x to denote the number of times the step should be repeated.

Figure 1 caption has been amended to use the lowercase x convention.

9. Please also describe other panels of Figures 2 and 4 in the Representative Results.

All figure panels are now referenced in the text.

10. References: Please do not abbreviate journal titles.

Journal title abbreviations have been amended except where abbreviations are used in the official title of the journal (e.g., “The AAPS Journal).