

Preparation of Decellularized Biological Scaffolds for 3D Cell Culture

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Abstract

The biggest challenge of designing and implementing an *in vitro* study is developing a microenvironment that most closely represents the interactions observed *in vivo*. Decellularization of tissues and organs has been shown to be an effective method for the removal of potentially immunogenic constituents while preserving essential growth factors and extracellular matrix (ECM) proteins necessary for proper cell function. Enzymatic digestion of decellularized tissues allows these tissue-specific components to be reconstituted into bioactive hydrogels through a physical crosslinking of collagen. In the following protocol, we describe unique decellularization methods for both dermis and urinary bladder matrix (UBM) derived from porcine tissues. We then provide details for hydrogel formation and subsequent three-dimensional (3D) culture of two cell types: NIH 3T3 fibroblasts and C2C12 myoblasts.

Key words Hydrogel, Extracellular matrix, Scaffolds, Cell culture, Decellularization

1 Introduction

It has been long understood that the extracellular matrix (ECM) not only provides structural support but also regulates cell growth [1, 2], survival, maturation, differentiation [3, 4], and development [5] of resident cells [6]. While many components of the ECM are conserved across several tissue types, each tissue is believed to possess a unique composition [7, 8]. Recently, scaffolds have been derived from ECM sourced from a variety of tissues including skin, fat, pericardium, heart, skeletal muscle, and liver for both *in vivo* and *in vitro* experiments [6, 9–12]. These scaffolds have been observed to assist with constructive remodeling or the formation of site-appropriate tissue when used as a biomaterial *in vivo* [13] and in some cases these ECM scaffolds have tissue-specific effects on cellular behavior [10].

Despite this unique interplay between the ECM and resident cells, *in vitro* studies often assess cell behavior on coatings consisting of either a single purified protein or directly on polystyrene

tissue culture dishes [14]. These methods do not accurately mimic the complexity of the extracellular microenvironment and may significantly alter outcomes observed in vitro, making it difficult for studies to translate appropriately in vivo [6].

The use of ECM matrices during in vitro experiments is becoming increasingly common as these matrices are believed to better mimic the native cellular environments [6, 14, 15]. Here we describe the methods involved in decellularizing porcine dermis and UBM as well as the preparation of ECM hydrogels for subsequent three-dimensional (3D) culture of two cell types: NIH 3T3 fibroblasts and C2C12 myoblasts. Both porcine dermis and UBM are decellularized through mechanical delamination, followed by chemical and enzymatic washes. The resultant ECM scaffolds are solubilized using a hydrochloric acid and pepsin solution then reconstituted as hydrogels and seeded with fibroblasts or myoblasts. While the methods described below are designed for specific tissues and cell types, the basic principles can be applied for the decellularization of multiple tissue types and the subsequent formation of 3D cell culture models.

2 Materials

2.1 Reagents for Decellularization of Porcine Dermis and Urinary Bladder

1. Tissues: Porcine full thickness skin and urinary bladder acquired from a local abattoir.
2. Distilled H₂O (diH₂O).
3. Peracetic Acid (PAA) solution: 0.1% PAA and 4% ethanol. Mix diH₂O, 100% ethanol, and 15% PAA in the ratios shown in Table 1 based on tissue weight being decellularized.
4. 0.25% trypsin solution: Add 20 mL of 2.5% trypsin stock solution (10× solution in Hank's balanced salt solution) to 180 mL of diH₂O.
5. 3% H₂O₂: Add 20 mL of 30% H₂O₂ to 180 mL of diH₂O.
6. 70% ethanol.

Table 1
Volumes to prepare PAA solution based upon tissue weight

Tissue weight (g)	Volume diH ₂ O (mL)	Volume ethanol (200 proof, mL)	Total liquid volume (mL)	Volume of PAA (15%, mL)
10	192	8	200	1.33
20	384	16	400	2.67
30	576	24	600	4.00

7. Triton X-100 solution: 1% Triton X-100 in 0.26% EDTA and 0.69% Tris. Weigh out 56 mg of EDTA and 138 mg of Tris, combine in a graduated cylinder and then fill with diH₂O up to 200 mL. Transfer 198 mL of this EDTA and Tris solution to a flask and add 2 mL of Triton X-100 to this flask while a magnetic stir bar mixes the solution.
8. Phosphate-buffered saline (PBS, 1×): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄ in diH₂O.

2.2 Reagents and Equipment for Enzymatic Digestion, and Hydrogel Formation

1. Porcine pepsin.
2. 0.1 N HCl.
3. 0.1 N NaOH.
4. 10× PBS.
5. 1× PBS.
6. Lyophilizer.
7. Mill with a size 40 mesh screen.
8. Non-humidified incubator.

2.3 Reagents for 3D Cell Culture

1. Cell lines: NIH 3T3 fibroblasts (CRL-1658, ATCC) and C2C12 myoblasts (CRL-1772, ATCC).
2. Cell culture medium: 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin in Dulbecco's Modified Eagle Media (DMEM). In a cell culture hood (under sterile conditions), add 50 mL of FBS and 5 mL of 100× penicillin/streptomycin to 445 mL of DMEM and then sterile filter into an autoclaved bottle. Store at 4 °C.
3. Trypsin–EDTA solution: 2.5% (w/v) trypsin and 0.53 mM EDTA solution in diH₂O.
4. Sterilized stainless steel ring with an inner diameter 1.38 cm (*see Note 1*).

3 Methods

3.1 Decellularization of Porcine Dermis

1. Collect full thickness sections of skin from market weight, adult pigs. In particular, skin sections were collected from the dorsolateral flank.
2. Cut the skin into 35 cm × 50 cm rectangular sections and use a dermatome to separate the subcutaneous fat and connective tissue layers from the dermal layer. Alternatively, pre-delaminated skin may be sourced directly from your local abattoir (*see Note 2*).
3. The now isolated dermal layer can be stored until decellularization in a –80 °C freezer and thawed to room temperature before use.

Table 2
Approximate wash volumes used based on amount of tissue being decellularized

Weight (g)	5	10	15	20	25	30	35	40	45	50	55	60
Volume (mL)	100	200	300	400	500	600	700	800	900	1000	1100	1200

4. Place the dermis into the 0.25% trypsin solution and agitate within an Erlenmeyer flask using an orbital shaker at 300 rpm for 6 h. Approximate wash volumes based upon the amount of tissue being decellularized are shown in Table 2.
5. After 6 h, drain the trypsin solution and wash the dermal tissue three times in diH₂O for 15 min each while agitating at 300 rpm.
6. After the third diH₂O wash, agitate the tissue with a 70% ethanol solution for 10 h at 300 rpm.
7. Remove the 70% ethanol and replace with a 3% H₂O₂ solution and agitate for 15 min at 300 rpm.
8. After the completion of the H₂O₂ solution wash, immerse the dermis twice in diH₂O for 15 min each at 300 rpm.
9. Wash the dermis with Triton X-100 solution and shake for 6 h at 300 rpm. Drain and refill with fresh solution then agitate for an additional 16 h.
10. Follow with an additional three washes with diH₂O for 15 min each at 300 rpm.
11. Place the dermis in PAA solution and shake at 300 rpm for 2 h.
12. Wash the dermis in 1× PBS twice for 15 min at 300 rpm followed by two final washes in diH₂O again for 15 min at 300 rpm.
13. Store the decellularized dermis in the freezer at −80 °C.

3.2 Decellularization of Porcine Urinary Bladder

1. Collect urinary bladders from market weight, adult pigs.
2. Cut the apex and neck of the bladder off. Then cut along the connective tissue middle line forming a rectangular sheet that can be laid flat with the lumen side facing down.
3. Using a hard plastic scraper, stretch the tissue to approximately twice the previous area.
4. Then using a pair of scissors, lightly placed on the surface of the tissue, make partial thickness cuts down the centerline of the bladder.
5. Use forceps to carefully peel back the muscle and mucosal layers then discard. This should leave only the basement membrane and tunica propria of the urinary bladder remaining (*see Note 3*).

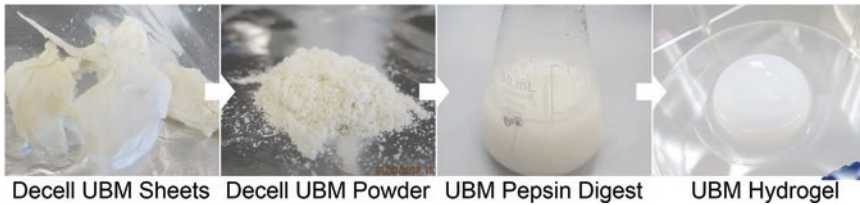


Fig. 1 Urinary bladder matrix (UBM) decellularization and hydrogel formation. Processing of tissues occurs in four general stages: (1) decellularization of tissues to remove genetic material while preserving ECM proteins, (2) lyophilization and milling of ECM into fine powder, (3) digestion of tissues using pepsin and HCl, and (4) hydrogel formation by the balancing of pH and salt concentrations and physical cross-linking of collagen at 37 °C

6. Rinse the remaining tissue in diH₂O to remove any extraneous materials.
7. Place the tissue in an Erlenmeyer flask containing PAA solution for 2 h on an orbital shaker at 300 rpm. The volume of this solution is relative to the amount of tissue being decellularized (Table 2).
8. Rinse the UBM twice with 1× PBS for 15 min each at 300 rpm.
9. Perform two final washes for 15 min each in diH₂O at 300 rpm.
10. Store the UBM in the freezer at −80 °C.

3.3 Lyophilization, Enzymatic Digestion, and Hydrogel Formation

This section describes the process of turning the solid ECM scaffold into a thermally responsive ECM hydrogel (Fig. 1).

1. Remove the frozen dermis and/or UBM from the −80 °C freezer and immediately place in the lyophilizer (*see Note 4*).
2. Ensure that the tissues are completely free of water by cutting through the thickest section (*see Note 5*).
3. After lyophilization is confirmed, grind the decellularized and lyophilized tissues into fine particles using a mill with a size 40 mesh screen (*see Note 6*).
4. Prepare 10 mL of stock solution of ECM digest at a concentration of 10 mg/mL by combining 100 mg of lyophilized and ground ECM with 10 mg porcine pepsin (final concentration 1 mg/mL pepsin) and 10 mL of 0.1 N HCl in a 25 mL Erlenmeyer flask. Adjust the necessary amount of pepsin and HCl according to the yield of ECM. Thoroughly mix the components using a magnetic stir bar for 48–72 h at room temperature until fully digested. Keep a piece of Parafilm on top of the flask to prevent evaporation (*see Notes 7 and 8*).
5. After the tissue has been fully digested, aliquot 1 mL of ECM into ten 1.5 mL microcentrifuge tubes and freeze at −80 °C until use (*see Note 9*).

Table 3

Calculated volumes of the necessary components for four concentrations for ECM hydrogel formation. All hydrogels are prepared using a starting concentration of 10 mg/mL and make a final gel volume of 0.5 mL

Starting concentration (mg/mL)	10	10	10	10
Final concentration (mg/mL)	2	4	6	8
Final volume (mL)	0.5	0.5	0.5	0.5
Volume ECM digest (μ L)	100	200	300	400
Volume 0.1 N NaOH (μ L)	10	20	30	40
Volume 10 \times PBS (μ L)	11.11	22.22	33.33	44.44
Volume 1 \times PBS (μ L)	378.89	257.78	136.67	15.56

3.4 Three-Dimensional Cell Culture of NIH 3T3 Fibroblasts and C2C12 Myoblasts In Vitro

This section describes a cell culture technique for preparation of single-celled suspension of cells and two techniques of 3D cell culture using the ECM hydrogel. All procedures should be performed using stringent cell culture techniques and all items should be sterilized prior to working with cells to minimize the potential for contamination.

1. Thaw the ECM digest on ice or at RT.
2. To prepare ECM hydrogels (*see Notes 10–13*), neutralize the pH of the ECM digest by adding 0.1 N NaOH (one-tenth digest volume), adjust the salt concentration by adding 10 \times PBS (one-ninth digest volume) and dilute the sample to the desired final ECM concentration using 1 \times PBS (*see Table 3*).

While preparing ECM hydrogels, keep ECM digest and buffers on ice until immediate use. Mix the components in a new 1.5 mL microcentrifuge tube by gently pipetting up and down until the ECM digest goes into solution. Avoid formation of bubbles.

3. Place a sterilized stainless steel ring into a 6-well plate and pipette 0.5 mL of newly prepared hydrogel solution into it while avoiding bubble formation.
4. Carefully place the plate in a non-humidified incubator at 37 °C for 1 h. Remove all bubbles prior to moving the plate into the incubator (*see Notes 14 and 15*).
5. Remove the hydrogel from the incubator and ensure that the gel has solidified (Fig. 2).

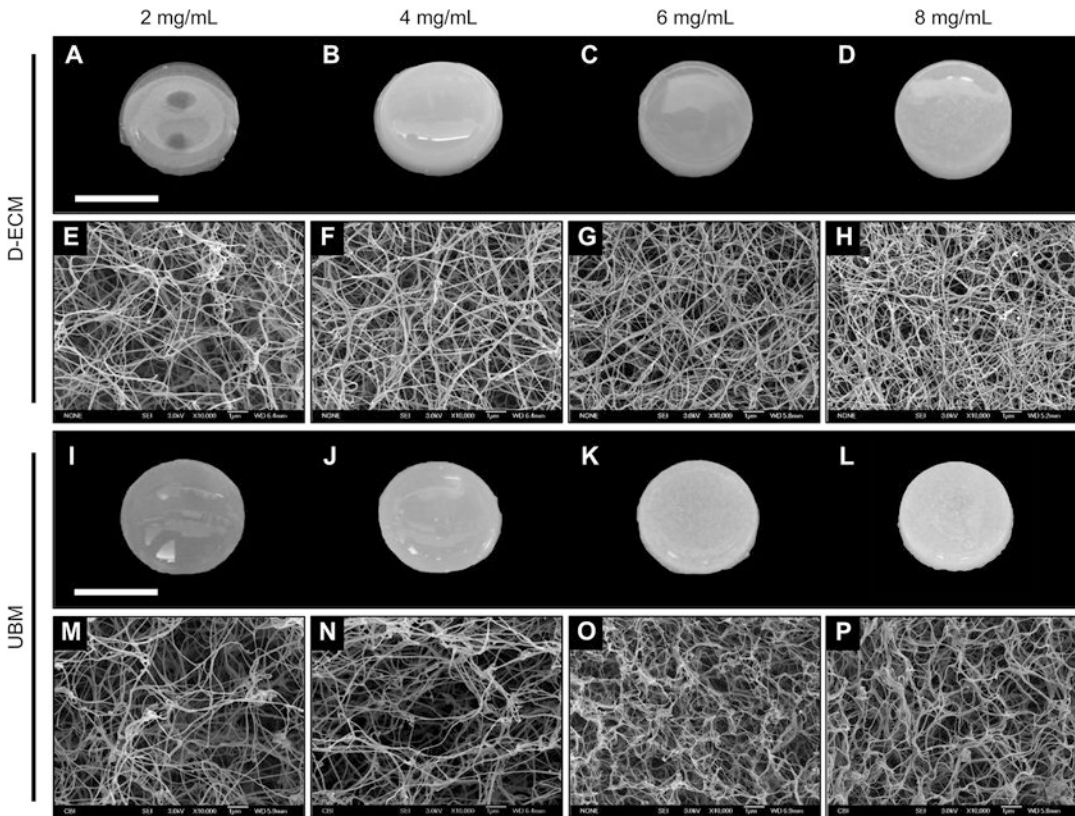


Fig. 2 Porcine dermis (D-ECM) (A–D) and UBM (I–L) hydrogels prepared at various concentrations: 2, 4, 6, 8 mg/mL. Hydrogels appear more opaque as ECM digest concentrations increase. Scanning electron microscopy provides detailed images of the ECM ultrastructure. (E–H and M–P) The fiber networks are significantly more advanced and complex at higher concentrations leading to increasing mechanical stiffness of the hydrogel. Reproduced from Wolf et al., 2012 [15] with permission from Elsevier B.V.

3.4.1 Preparation of Single-Celled Suspension and Sub-Culture of NIH 3T3 Fibroblasts and C2C12 Myoblast

1. To split cells (*see Note 16*), remove cell culture flask from the incubator and aspirate the medium without disturbing the cells.
2. Add 2–3 mL of warmed trypsin–EDTA solution to the flask and place in the incubator for 5–15 min.
3. After cells have detached, add 6–8 mL of fresh cell culture medium to the flask.
4. Transfer the cell suspension into a centrifuge tube and take an aliquot for counting e.g., using hemocytometer.
5. For subculturing, pipet the cell suspension at a seeding density of 2.25×10^5 to 3.75×10^5 cells/75 cm² for NIH 3T3 fibroblasts and 1.5×10^5 to 1×10^6 cells/75 cm² for C2C12 myoblasts into new flasks with appropriate amount of fresh cell culture medium and place in the cell culture incubator at 37 °C.

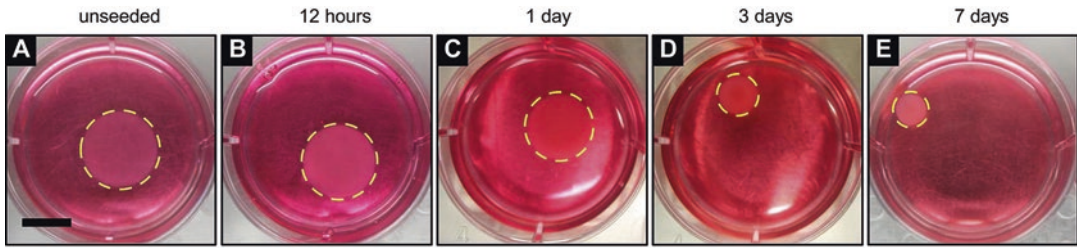


Fig. 3 In vitro cell culture of NIH 3T3 fibroblasts inside of UBM hydrogels at 6 mg/mL. Time lapse of the cell-hydrogel construct immersed in medium for 7 days shows a decrease in size due to cell contractility and migration. (A) unseeded gel, (B) 12 h, (C) 1 day, (D) 3 days, and (E) 7 days. Reproduced from Wolf et al., 2012 [15] with permission from Elsevier B.V.

3.4.2 3D Surface Culture

1. Under sterile conditions, prepare 500 μL of hydrogel at two concentrations: 6 and 8 mg/mL (*see Notes 17 and 18*).
2. Select a cell line for seeding onto the hydrogel: NIH 3T3 fibroblasts or C2C12 myoblasts (*see Note 19*).
3. Add 1 mL of cell suspension to the surface of the hydrogels for a final seeding density of 5.0×10^5 cells/cm².
4. Allow the cells about 16 h for adequate cell attachment to the hydrogel surface.
5. Once the cells have attached, remove the metal ring surrounding the hydrogel and add enough fresh medium to cover the hydrogel (Fig. 3) (*see Note 20*).
6. Replace the medium every 3 days to replenish essential nutrients for cell viability (Fig. 4) (*see Notes 21 and 22*).

3.4.3 3D Within-Gel Culture

1. Prepare single-celled suspension of cells (*see Subheading 3.4.1*).
2. Count the cells (e.g., using a hemocytometer) and adjust the cell suspension to a final concentration of 1.0×10^6 cells/mL of hydrogel. The cells for the “within-gel” culture replace the volume of the 1 \times PBS component with cell-containing medium. For example: In a 8 mg/mL hydrogel there should be 5.0×10^5 cells resuspended in 15.56 μL of cell media.
3. Pipet the pre-gel solution thoroughly to ensure uniform distribution of cells, then pipet the total mixture into the stainless steel ring.
4. Place the plate containing the cells and pre-gel mixture in a dry heat incubator at 37 $^\circ\text{C}$ for 45 min.
5. Once the gel has formed, remove the metal ring surrounding the hydrogel and add enough fresh medium to cover the hydrogel.
6. Replace the medium every 3 days to replenish essential nutrients for cell viability (*see Notes 21 and 22*).

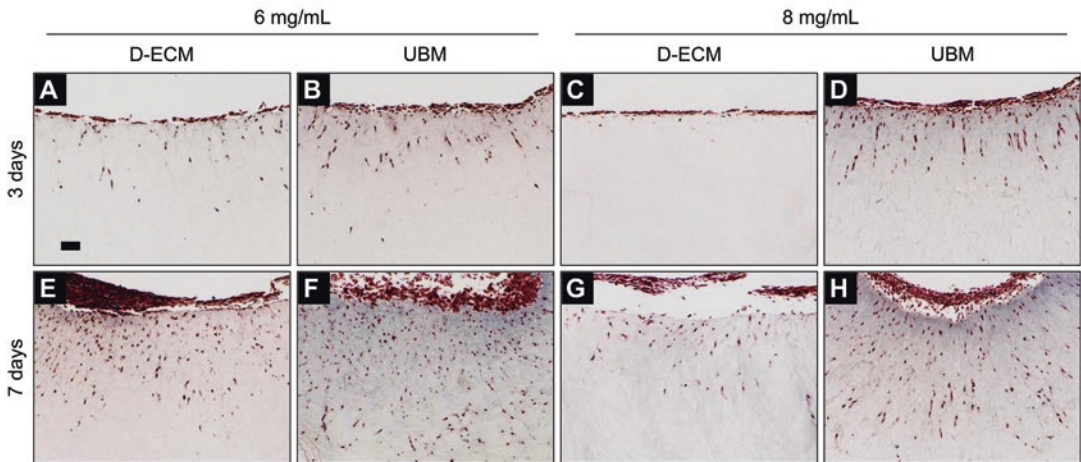


Fig. 4 NIH 3T3 fibroblasts seeded on top of both dermis and UBM hydrogels at two concentrations: 6 mg/mL and 8 mg/mL. Masson's Trichrome stained cross sections shows cell infiltration into ECM hydrogels after 3 (a–d) and 7 (e–h) days. Reproduced from Wolf et al., 2012 [15] with permission from Elsevier B.V.

4 Notes

1. Seeding rings are austenitic stainless steel with a 2.0 cm outer diameter and 1.38 cm inner diameter. Each ring has a height of 1.3 cm.
2. Isolating the dermal layer from full thickness skin without a dermatome, while not impossible, is very difficult. However, some abattoirs may sell the already isolated dermal tissue.
3. Effectiveness of the UBM decellularization protocol can be diminished if too many or too few tissue layers are removed during mechanical delamination. The basement membrane and tunica propria are approximately 100–150 μm in thickness. The tissue should appear almost but not completely transparent after delamination.
4. To freeze and lyophilize the UBM, spread the thin scaffold out on nonstick aluminum foil to increase surface area and hasten the drying process.
5. A non-lyophilized sample will make it difficult to collect the ECM from the mill. The moisture will prevent the tissue from freely flowing through the filter and into the collection chamber.
6. Using a 40 μm mesh screen with the mill provides small enough ECM particles to be easily digested. A larger mesh may lead to a longer incubation period for complete digestion of the ECM.

7. Pepsin can be difficult to weigh in small quantities due to its hygroscopic properties. It is very important to measure the desired mass of pepsin as quickly and efficiently as possible. A portion of the pepsin could be lost as it has a propensity to stick to the weigh boat during measurement. This could result in poor digestion and inaccurate final concentrations of ECM digest. A technique that has been used to fix this problem is to directly add the pepsin to the HCl on the balance. Once the desired volume of HCl is determined, pipette the acid into a weigh boat. Place the HCl and weigh boat onto the scale and “zero” the balance. Carefully add the desired amount of pepsin to the solution and add to the ECM for digestion.
8. Digesting the tissues in pepsin and HCl longer than 72 h has been shown to negatively impact both gelation and bioactivity of the hydrogels. Digestion must be monitored carefully to ensure that essential components of the ECM are conserved.
9. ECM digest should be kept to a minimal number of freeze–thaw cycles. Numerous freeze–thaw cycles are detrimental to the bioactivity of the hydrogels.
10. While preparing ECM hydrogels keep digest and buffers on ice until immediate use.
11. When creating the final concentrations for hydrogel formation, combine all buffers before adding the digest. For the “within-gel” culture, take care to add the 0.1 N NaOH and 10× PBS to the ECM digest prior to adding cells to ensure the cells are not damaged by either a basic or acidic environment. Be sure to mix the solution well by pipetting the mixture up and down.
12. The ECM digest and pre-gel solution is quite viscous and as much as 10% may be lost using normal air displacement pipettes. If available, a positive displacement pipette can reduce this volume loss and increase the precision of concentration measurements.
13. Basic mechanical characteristics of the gel can be determined using a parallel plate dynamic rheometer. The most basic characterization would be an amplitude sweep, while adding time and strain sweeps would be more encompassing.
14. Using a non-humidified chamber will assist with hydrogel gelation time. A humidified incubator adds moisture that limits the ability of the natural components in the gel to form. It is thus strongly recommended to use dry heat to prepare the hydrogels.
15. When mixing hydrogel components it can be very difficult to prevent bubble formation. However, there are ways to prevent bubbles and also to remove them. The best technique to prevent bubble formation is to slowly pipette the solution, while

keeping the pipette tip in the liquid. Once there is air in the tip bubbles will develop and can be difficult to remove. The other technique that has been successful is applying air pressure with a high volume micropipette. After the hydrogel solution has been prepared and pipetted into the metal ring, bubbles can be removed by directing large volumes of air directly toward the bubbles. The increased surface tension created by the air pressure should eliminate any residual bubbles. To create a uniform and consistent hydrogel architecture it is important to remove any and all bubbles from the gel mixture prior to gelation in the incubator.

16. Suggested volumes are based on cultures in a 75 cm² flask. The cells should be split at least twice per week at 80% confluency or less. It is important to not let the cells become confluent because this may result in contact inhibition. Media should be changed every 2–3 days. Cells may be frozen down for storage in complete growth medium supplemented with 5% (v/v) DMSO and stored in liquid nitrogen.
17. ECM derived from different tissues can be used in similar 3D cell culture experiments. However, ECM derived hydrogels from various tissue types including small intestinal submucosa, peripheral nerve, cardiac, and ovarian tissue can have varying properties. Therefore, the protocol described above may need to be adapted to work with ECM hydrogels derived from different tissues, particularly those with lower relative collagen I composition.
18. Selection of these concentrations was due to the differing mechanical and structural properties observed between these concentrations.
19. C2C12 myoblasts may be cultured both in growth and fusion medium. C2C12 myoblasts may be differentiated into myotubes using fusion medium containing DMEM with 2% horse serum, 100 U/mL penicillin and 100 µg/mL streptomycin for an additional 2 days after the 7 days of culture in growth medium. In a previous study, C2C12 myoblasts were used solely at a hydrogel concentration of 6 mg/mL.
20. For cell culture applications, it is essential that the hydrogel remains hydrated with medium to prevent the gel from drying out. A dehydrated hydrogel will result in altered mechanical properties leading to a detrimental cell response.
21. Cell viability of 3T3 fibroblasts and C2C12 myoblasts atop the ECM hydrogels can be analyzed using a Live/Dead assay kit.
22. Visualizing the cells within and atop the gels can be achieved by fixing the hydrogels in 10% neutral buffered formalin, splitting in half to expose a cross-section and embedding in paraffin. The paraffin block is then sectioned and stained with Masson's Trichrome (Fig. 5).

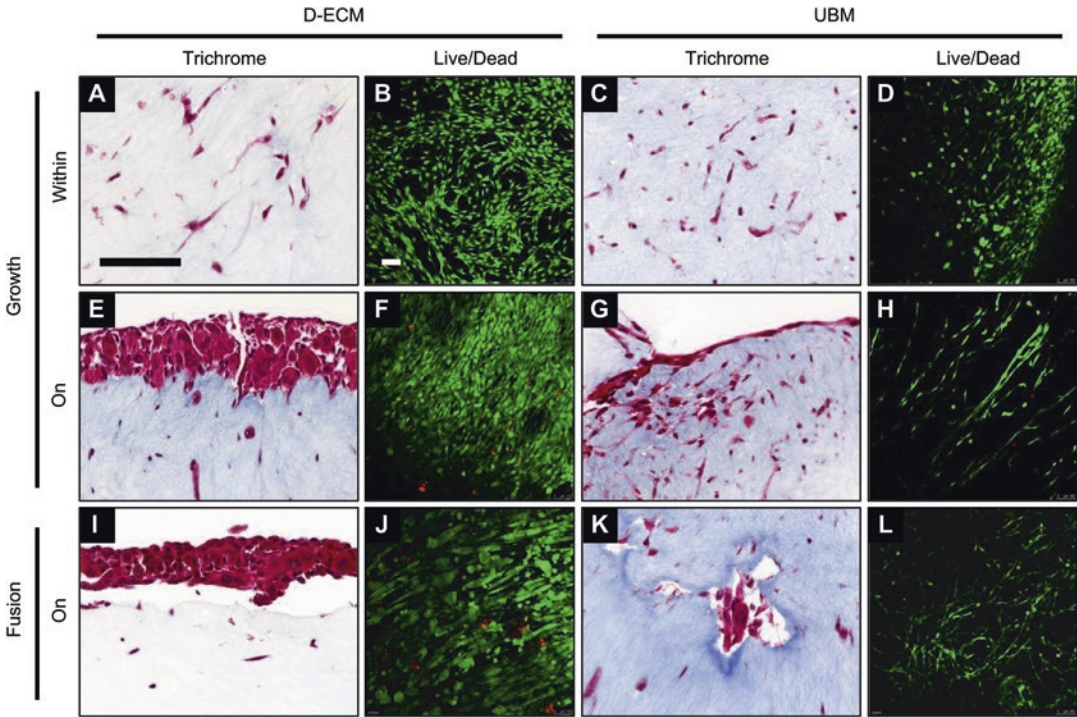


Fig. 5 C2C12 myoblasts cultured on the surface and within D-ECM (*left panel*) and UBM (*right panel*) hydrogels in both growth (**A–D** - *within gel*, **E–H** - *on top of gel*) and fusion (**I–L** - *on top of gel*) conditions (see **Note 19**). Cells were visualized and analyzed using histology and immunofluorescence staining methods. Masson's Trichrome stain was used on cross sections of the hydrogel while a Live/Dead staining of the hydrogel surface was used to visualize viable cells (*green*) and dead cell nuclei (*red*) (see **Notes 21–22**). Reproduced from Wolf et al., 2012 [15] with permission from Elsevier B.V.

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