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A Rapid, Scalable Method for Isolation, Functional Studies and Analysis of Cell-Derived Extracellular Matrix

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Abstract:	The extracellular matrix (ECM) is recognized as a diverse, dynamic and complex environment that is involved in multiple cell-physiological and pathological processes. However, isolation of ECM, from tissues or cell culture, is complicated by the insoluble and cross-linked nature of the assembled ECM proteins and by the potential contamination of ECM extracts with cell surface and intracellular proteins. Here, we describe a method for use with cultured cells which is rapid, and reliably removes cells to isolate a cell-derived ECM for downstream experimentation. Through use of this method, the isolated ECM and its components can be visualized by immunofluorescence microscopy in situ. The dynamics of specific ECM proteins can be tracked by tracing the deposition of a tagged protein by fluorescence microscopy, both before and after the removal of cells. Alternatively, the isolated ECM can be extracted for biochemical analysis such as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. At larger scale, full proteomics analysis of the isolated ECM by mass spectrometry can be made. By conducting ECM isolation under sterile conditions, sterile ECM layers can be obtained for functional or phenotypic studies with any cell of interest. The method can be applied to any adherent cell type, is relatively easy to perform, and can be linked to a wide repertoire of experimental designs.
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Question	Response
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

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Dear Mr Upponi,

Thank you for your letter of 25 July and the reviewers' comments. We are pleased to return here our revised manuscript and full set of responses.

Yours sincerely,

Josephine Adams

TITLE:
A Rapid, Scalable Method for the Isolation, Functional Study, and Analysis of Cell-Derived Extracellular Matrix

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SHORT ABSTRACT:
The extracellular matrix plays a major role in defining the microenvironment of cells and in modulating cell behavior and phenotype. We describe a rapid method for the isolation of cell-derived extracellular matrix, which can be adapted to different scales for microscopic, biochemical, proteomic, or functional studies.

LONG ABSTRACT:
The extracellular matrix (ECM) is recognized as a diverse, dynamic, and complex environment that is involved in multiple cell-physiological and pathological processes. However, the isolation of ECM, from tissues or cell culture, is complicated by the insoluble and cross-linked nature of the assembled ECM and by the potential contamination of ECM extracts with cell surface and intracellular proteins. Here, we describe a method for use with cultured cells that is rapid and reliably removes cells to isolate a cell-derived ECM for downstream experimentation.

Through use of this method, the isolated ECM and its components can be visualized by *in situ* immunofluorescence microscopy. The dynamics of specific ECM proteins can be tracked by tracing the deposition of a tagged protein using fluorescence microscopy, both before and after the removal of cells. Alternatively, the isolated ECM can be extracted for biochemical analysis, such as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. At larger scales, a full proteomics analysis of the isolated ECM by mass spectrometry can be conducted. By conducting ECM isolation under sterile conditions, sterile ECM layers can be obtained for functional or phenotypic studies with any cell of interest. The method can be applied to any adherent cell type, is relatively easy to perform, and can be linked to a wide repertoire of experimental designs.

INTRODUCTION:
Over the last few decades, the extracellular matrix (ECM) has become recognized as a diverse, dynamic, and complex environment that is involved in multiple cell-

physiological and pathological processes. At the tissue level, the ECM influences cell signaling, motility, differentiation, angiogenesis, stem cell biology, tumorigenesis, fibrosis, etc[1,2]. The study of ECM organization and ECM-dependent processes thus has wide implications on cell biology and tissue physiology. To reach a mechanistic understanding of ECM composition, organization, and functional properties, methods for the accurate isolation of ECM are required. Whereas the earliest identification of ECM proteins relied upon isolation from tissues[3], the preparation of ECM from cultured cells has now become more prevalent.

The isolation and analysis of cell-derived ECM is complicated for two main reasons. Firstly, the presence of cells and their abundant intracellular proteins can make it difficult to isolate the ECM as a discrete extracellular structure. Indeed, some ECM proteins have roles inside the cell as well as in the ECM[4]; therefore, the efficient removal of intracellular proteins from cell-derived ECM is vital if the study of proteins within the ECM is not to be confused with their roles inside the cell. Secondly, cell-derived ECM is composed of many large, oligomeric proteins, which are often covalently cross-linked upon ECM assembly and are therefore insoluble in standard detergents. These properties can complicate extraction and the further analysis of ECM. To address these issues, a method is required that enables the efficient separation of ECM proteins from cellular components.

Several methods have been described in the literature for the isolation of ECM from either cell culture or tissue extracts. Many of these methods are aimed at the extraction of the abundant ECM protein, collagen, from tissues and include the use of neutral salts[3], acidic conditions[5,6], or pepsin[7]. Isolation of total ECM from tissue extracts often involves decellularization of the tissue prior to the isolation of the ECM. For example, a sequential extraction method has been described that isolates ECM from human cardiac tissue[8]. First, loosely-bound ECM proteins were extracted with 0.5 M NaCl before sodium dodecyl sulphate (SDS) was used to remove the cells. Finally, the remaining ECM proteins were extracted using 4 M guanidine[8]. ECM can also be solubilized from decellularized samples using 8 M urea[9]. Other techniques use detergents, such as deoxycholic acid[10], to extract both cells and ECM before separating insoluble ECM proteins from the soluble cell lysate by centrifugation.

The method for isolation and analysis of cell-derived ECM described in this report provides a reproducible method for removing cell material, leaving cell-derived ECM that can be analyzed by *in situ* immunofluorescence or extracted for further biochemical analysis. This method can be adapted for any adherent cell type and can be scaled up for downstream procedures, such as immunoblotting or mass spectrometry, or for utilization of the isolated ECM in functional studies. The method can also be used in conjunction with confocal microscopy of live cells to track ECM deposition of a tagged protein of interest in real time. This is achieved through the use of a gridded, glass-bottomed dish. Overall, the approach provides an accurate isolation of cell-derived ECM and also the scope to identify and monitor the deposition and dynamics of individual ECM proteins.

PROTOCOL:

1. Removal of Cells with Ammonium Hydroxide Solution

1.1) Prepare adherent cells by plating at the appropriate density.

NOTE: The cells can be any adherent cell type that produces sufficient ECM for analysis. Here, we describe the use of COS-7 cells, an African green monkey kidney fibroblast-like cell line that contains SV-40 viral DNA sequences; RCS, a rat chondrosarcoma cell line; or normal human dermal fibroblast (HDF) strains from juvenile foreskin. HDF are used from passage 1 to passage 8 only.

1.1.1) Plate the cells on coverslips for the imaging of live cells and ECM, for fluorescence microscopy studies of fixed ECM, or for preparation of cell-derived ECM for small-scale functional assays. Plate the cells on cell culture dishes for SDS-PAGE analysis, immunoblot, or proteomics studies.
NOTE: The cell culture conditions (number of cells to plate and culture medium) will depend on the cell type. The cell number to plate will need to be established empirically for the particular cell line or cell strain.

1.1.2 Allow a suitable time for the cells to deposit ECM, typically >16 h. NOTE: The time period will depend on the cell type and will need to be determined empirically. If conducting ectopic expression, allow appropriate time for the expression of the transfected protein of interest and for the deposition of ECM.

1.2) In an extractor hood, prepare 20 mM ammonium hydroxide in a suitable vessel by diluting the stock solution 1/14 with de-ionized H_2O.

1.2.1) Remove the cells from the incubator and gently wash the cells to remove culture medium by adding phosphate-buffered saline (PBS) without Ca^{2+}/Mg^{2+}; gentle pour it against the walls of the dish. Rock the dish twice and remove the liquid with a plastic transfer pipette. Repeat twice more.

1.3) In an extractor hood, tilt each dish and remove the PBS from step 1.2 with a plastic transfer pipette. Add 3 mL of ammonium hydroxide per 100-mm dish and incubate them at room temperature for 5 min. During the 5-min incubation period, gently agitate the dish every minute to ensure the lysis of all the cells. Steps 1.4-1.7 will also be carried out in the extractor hood.
NOTE: Alternative cell removal reagents include 2 M or 8 M urea, which are incubated with the cells for 10 min.

1.4) Add copious amounts of de-ionized H_2O to each dish, at least 20 mL per 100-mm dish, with rocking. Dispose of the ammonium hydroxide-solubilized material—which is composed of ammonium hydroxide, lysed cells, and de-ionized H_2O—by aspiration with a transfer pipette. Transfer this waste solution into a container for liquid waste.

1.5) Wash the insoluble ECM layer in copious de-ionized H_2O four more times to ensure the complete removal of all the ammonium hydroxide-solubilized material.

1.6) For examination of the ECM by immunofluorescence, fix the ECM by adding 2% paraformaldehyde (PFA; 3 mL per 100-mm dish) at room temperature for 10 min.
Caution: PFA is very hazardous in case of skin or eye contact and severe overexposure can produce lung damage, choking, unconsciousness, or death. It should only be handled in an extractor hood, and personal protective equipment should be worn.

1.7) Wash each fixed sample twice with PBS, as in step 1.2, and dispose of the PFA solution into a suitable liquid waste container. If necessary, store the ECM samples in PBS at 4 °C before preparing them for fluorescence microscopy.

2. Tracking Deposition of an ECM Protein by Confocal Microscopy Imaging of Live Cells

2.1) Count the cells under a hemocytometer. For COS-7 cells, plate 250,000 cells onto a 60-mm cell culture dish for transfection.
NOTE: For live cell imaging, the cells must be transfected with a vector encoding the ECM protein of interest, fused in frame with a genetically-encoded fluorescent tag. This is to enable the identification of the ECM protein of interest during live-cell imaging.

2.2) After a suitable time for protein expression (*e.g.,* 16 h) remove the cells from the dish with trypsin-EDTA solution, count them in a hemocytometer, and plate ~150,000 cells onto a 35-mm gridded, glass-bottomed dish for imaging. Allow 2 h for the cells to reattach and to begin ECM deposition (the time period will depend on the cell type and must be established empirically).

NOTE: Use a cell medium that does not contain phenol red, as this can give a red tinge that affects image quality.

2.3) During the above 2-h period, set up a confocal microscope with a 37 °C incubator chamber and a CO_2 supply. Allow the temperature in the chamber and in the dish to stabilize to 37 °C before imaging; this can take up to 1 h.

2.4) Incubate the cells plated onto the 35-mm gridded dish with a fluorescent cell marker for 30 min. Then, wash the cells twice in phenol red-free culture medium prior to imaging.
NOTE: A cytoplasmic fluorescent marker can be used to visualize cell volumes, or a membrane-incorporating marker can be used to visualize cell edges.

2.5) Using the 20X objective and phase contrast on a confocal microscope, identify an appropriate grid square that contains a number (>3) of adherent, healthy cells. Confirm the expression of the target protein of choice in the cells within the selected grid square using the 63X or 100X objectives and the appropriate wavelengths under fluorescence microscopy.

2.6) Set up fluorescence and phase-contrast time-lapse imaging using a z-stack software. Set the “Begin” stack to be just below the ECM layer and the “End” stack to be just above the cell body. Set the z-step size to 0.3 µm and the z-volume to between a 5- and 10-µm depth in total, depending on the cell type. This will give between 15-30 z-sections per time point.

2.7) Capture fluorescence (for red fluorescent protein (RFP), excitation = 555 nm and emission = 584 nm) and phase-contrast images periodically over a set time period. For example, use the time-lapse software to set the time interval at 2 min and the duration to 2 h. Select the “start” button to begin capture of z-section images over the defined time period.

2.8) At the end of the time period, remove the cells from dish, as described in steps 1.1 – 1.5, to confirm the localization of the fluorescently-tagged protein of interest in the ECM.

2.9) Use phase-contrast imaging and the 20X objective to relocate the relevant grid square.

2.10) Switch to the 63X objective and identify the ECM layer under fluorescence microscopy. Compare this image to the pre-extraction image

3. Sterile Preparation of Cell-Derived ECM for Cell Functional Assays

3.1) Grow one population of cells (the "matrix producer" cells) on coverslips and another population (the "test" cells) in standard culture in a 100-mm cell culture dish for at least 24 h.

NOTE: These can be any two different adherent cell types, and the experimental design can include transfection of one or both cell populations to express a fluorescently-tagged ECM protein.

3.2) In a laminar flow hood, as used for cell culture, prepare sterile solutions of PBS without Ca^{2+}/Mg^{2+}, 20 mM ammonium hydroxide (see step 1.3), and de-ionized H_2O by passing each through a sterile 0.2-µm filter into an appropriate sterile container.

3.3) Maintaining sterile technique, gently wash the "matrix producer" cells on coverslips three times with sterile PBS (see step 1.2).

3.4) Remove the PBS by aspiration with a sterile pipette and dispose it in a suitable liquid waste container. Add 20 mM sterile ammonium hydroxide (3 mL/100-mm dish) and incubate them for 5 min with rocking at intervals.

3.5) Add copious amounts of sterile de-ionized H_2O to the "matrix producer" dish, at least 20 mL per 100-mm dish, with rocking, and pour away the cell lysate into a suitable waste container.

3.6) Repeat step 3.5 four more times to ensure the complete removal of the ammonium hydroxide-solubilized material.

NOTE: The ECM deposited by the "matrix producer" cells on the coverslips then provides a sterile ECM for functional assays with any test cells of interest.

3.7) Incubate the test cells from the cell stock dish with 1.5 mL of trypsin-EDTA solution per 100-mm dish (0.05% w/v trypsin and 0.02% w/v EDTA in Hank's balanced salt solution) until the test cells are detached from the dish. Add cell medium, centrifuge the test cells at 400 x g for 5 min, and remove the supernatant liquid.

3.8) Resuspend the test cells in fresh, sterile medium and count them in a hemocytometer. Plate an appropriate number of these test cells onto the sterile, isolated ECM on coverslips. Culture them in the appropriate cell medium for 2-3 h, or for the time period required for the experimental design.

3.9) To examine the organization of the actin cytoskeleton, fix the test cells in 2% PFA and stain them with fluorescein isothiocyanate (FITC)-phalloidin (excitation = 490 nm and emission = 525 nm) to visualize F-actin and with 4',6-diamidino-2-phenylindole (DAPI; excitation = 358 nm and emission = 461 nm) to visualize the nuclei[11].
NOTE: Compare the morphology and actin organization in the test cells plated on heterologous cell-derived ECM with test cells plated on their own ECM.

4. Isolation of ECM for SDS-PAGE, Immunoblot Analysis, or Proteomics
4.1) Grow adherent cells of interest on 100-mm cell culture dishes (5 to 10 dishes for proteomics or 1-2 dishes for immunoblotting) for 24-96 h, as appropriate for the cell type, to allow secretion and deposition of ECM.

4.2) Remove the cells as described in steps 1.1-1.5.

4.3) Tilt each plate at an angle to drain residual H_2O to the bottom of the dish, and carefully remove any remaining H_2O with a p200 pipet until the plate is completely dry.

4.4) In parallel, heat SDS-PAGE sample buffer containing 100 mM dithiothreitol (DTT) to 95 °C for 2 min using a heat block, and then add it to the plate. 200 µL of sample buffer per 100-mm plate is appropriate for samples to be examined by immunoblot.

4.4.1) To analyze ECM by mass spectrometry, achieve a more concentrated sample by collecting the SDS-PAGE sample buffer from the dish (as described in steps 4.5 and 4.6, below), re-heating it to 95 °C, and using the same aliquot across 2-3 additional plates.

4.5) Use a cell scraper to thoroughly scrape the ECM off the dish, ensuring that all areas of the dish have been scraped.

4.6) With the cell scraper, gather the sample to one side of the dish and remove it with a 200-µL pipette tip into a tube.

4.7) Transfer any residual SDS-PAGE sample buffer extract from the cell scraper tip into the tube to minimize the loss of ECM material. For a concentrated sample, re-heat the same SDS-PAGE sample buffer aliquot to 95 °C and re-use it across 2-3 additional plates.

4.8) Resolve the ECM proteins by SDS-PAGE[12], using either 7% or 4-7% gradient polyacrylamide gels.
NOTE: It is convenient to use pre-stained protein markers.

4.9) To increase the resolution of the high-molecular-weight ECM proteins, extend the gel running time such that the 37 kDa molecular weight marker runs close to the bottom of the gel and molecular weight markers <37 kDa have run off the gel.

4.10) For proteomic analysis of ECM proteins, stain the gel with a protein stain and capture an image. Cut bands of interest from the gel, digest them with trypsin, and

analyze them by matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry[13].

4.11) Alternatively, for a more comprehensive analysis of the ECM extract and to analyze the entire sample, slice the gel lane into sections, digest them with trypsin, and perform liquid chromatography-mass spectrometry (LC-MS)[14].

4.12) Alternatively, for immunoblotting[15], transfer proteins from the SDS-PAGE gel onto polyvinylidene fluoride (PVDF) membrane at 15 V for at least 1 h 10 min (semi-dry method) to ensure the transfer of the high-molecular-weight ECM proteins. Visualize the total protein transferred to the membrane with a Ponceau S stain.

4.13) Following step 4.11, use antibodies to establish the presence and/or make a semi-quantitative analysis of individual ECM proteins[15].

4.13.1) Block the membrane with 2% (w/v) milk in TBS-Tween 20 (blocking buffer) overnight at 4 °C. Dilute specific antibodies to ECM proteins in blocking buffer and incubate with the membrane for 1.5 h at room temperature (antibody to fibronectin diluted 1/600 and antibody to thrombospondin-1 diluted 1/150; Supplementary Table 1).

4.13.2) Test the quality of the ECM isolation by re-probing the same blot with antibodies to abundant intracellular proteins, such as actin or tubulin (anti-actin diluted 1/10,000 and anti-tubulin diluted 1/5,000).

REPRESENTATIVE RESULTS:

The protocol described in steps 1.1-1.5 acts to remove cells and leave behind the cell-derived ECM. The protocol was carried out on COS-7 cells grown for 96 h on glass coverslips, and the cell-derived ECM was analyzed by fluorescence microscopy. The ammonium hydroxide treatment removed the cells effectively, as established by the loss of the cell nuclei and actin cytoskeleton, according to DAPI and phalloidin staining, respectively (**Figure 1**). The extraction of cells with 2 M urea was not as effective as ammonium hydroxide; traces of DAPI and phalloidin staining were detected after treatment. 8 M urea had a similar effect to ammonium hydroxide (**Figure 1**). Next, cell-derived ECM was visualized before and after the ammonium hydroxide treatment (steps 2.1-2.11). COS-7 cells were transfected with a monomeric red fluorescent protein (mRFP)-tagged thrombospondin-1 C-terminal trimer, (mRFPovTSP1C)[11] and grown on a 35-mm dish with a gridded glass base.

Two hours post-plating, a suitable grid square that contained multiple healthy cells expressing mRFPovTSP1C was visualized under a confocal microscope. A reference phase contrast image was taken of this area, and then fluorescence time-lapse imaging was carried out every 1 min for 2 h (**Figure 2A**). Cells were then removed using ammonium hydroxide, and the same grid square on the dish was relocated under phase contrast and then re-analyzed by fluorescence microscopy. The cells were no longer present in the grid square, but mRFPovTSP1C remained within the ECM, detected by fluorescence microscopy as characteristic puncta (**Figure 2A**). Any mRFPovTSP1C deposited in the ECM prior to cell removal was identified by comparing the fluorescence pattern pre- and post-removal of cells. The

fluorescent puncta identified in both images (**Figure 2A**, example arrowed) are inferred to be associated with the ECM.

Ammonium hydroxide treatment was then used to isolate native ECM from cultured, adherent cells. Human dermal fibroblasts (HDF) were grown on glass coverslips for 96 h. Cells were removed using ammonium hydroxide, and the ECM was probed with an anti-collagen I antibody, which demonstrated a characteristic fibrillar and meshwork staining pattern[16] (**Figure 2B**). Fibronectin patterning was examined around fixed, non-permeabilized rat chondrosarcoma cells (RCS) and within the ECM after the removal of the RCS cells (**Figure 2C**). The ammonium hydroxide isolation of ECM was also carried out under sterile conditions so that "test" cells could be re-plated onto the ECM for phenotypic or functional studies. For example, "test" COS-7 cells were plated for 2 h onto sterile ECM produced by RCS cells, and then they were fixed, permeabilized, and analyzed for F-actin organization by FITC-phalloidin staining, in comparison to COS-7 cells producing their own ECM (steps 3.1-3.9) (**Figure 3**).

At a larger scale, the method was used to isolate ECM for biochemical procedures. For example, RCS cells were grown on two 100-mm cell culture dishes for 7 days, and the procedures in steps 4.1-4.7 were followed. Proteins in the cell-derived ECM were collected by scraping them into hot SDS-PAGE sample buffer and were separated by SDS-PAGE on a 7% polyacrylamide gel under reducing conditions. The gel was stained for protein, and the four major bands were each isolated and analyzed by mass spectrometry (**Figure 4A**). A number of ECM proteins, including fibronectin, thrombospondin 1 (TSP1), thrombospondin 5/cartilage oligomeric matrix protein (TSP5 / COMP), and matrilin-1 were identified (**Figure 4B**).

Alternatively, smaller-scale preparations of ECM can be evaluated by immunoblots. For example, cell-derived ECM from COS-7 cells ectopically expressing mRFPovTSP1C was separated by SDS-PAGE, transferred to a PVDF membrane, and probed for RFP with an anti-RFP antibody (**Figure 4C**). This technique is sensitive enough to detect differences in deposition between a native trimer of TSP1 (mRFPovTSP1C) and an engineered pentamer of the TSP1 C-terminal region (mRFP–TSP-5-1C)[17] (**Figure 4C**). To investigate the endogenous ECM of HDF, the presence of specific proteins in cell lysate or the isolated ECM was examined by immunoblotting. The characteristic ECM proteins fibronectin and thrombospondin-1 were detected in the ECM, whereas the abundant intracellular proteins α-tubulin and β-actin were absent from the isolated ECM (**Figure 4D**).

FIGURE LEGENDS:

Figure 1. Comparison of Methods of Cell Removal.

COS-7 cells were grown at high density for 96 h on coverslips, fixed in 2% PFA, and permeabilized in 0.5% Triton X100, or were treated as indicated for cell removal. Coverslips were then stained with FITC-phalloidin to visualize F-actin and DAPI to visualize nuclei. Scale bar = 25 μm. This figure is modified from an original publication in the Journal of Cell Science[11].

Figure 2. Identification of ECM Proteins in Isolated ECM.

(**A**) Phase-contrast and fluorescence images of COS-7 cells expressing mRFPovTSP1C and grown on a 35-mm dish with a glass-bottomed, gridded base for

2 h. Images are shown before and after the removal of the cells by ammonium hydroxide. The edge of the grid letter is indicated in the phase-contrast images with a dotted line. White arrows indicate examples of fluorescent puncta that were present before and after cell removal. (**B**) Detection of collagen I in HDF ECM by indirect immunofluorescence. HDF were grown for 96 h and removed with ammonium hydroxide, and the ECM was stained for human fibrillar collagen I. The ECM was also stained with FITC-conjugated anti-rabbit secondary antibody alone. (**C**) Localization of fibronectin around RCS cells or within isolated RCS ECM, as detected by indirect immunofluorescence. RCS cells were grown for 48 h, fixed in 2% PFA, and stained for fibronectin and with DAPI. In parallel dishes, cells were removed with 20 mM ammonium hydroxide and the ECM was stained for fibronectin and with DAPI. Cells were also stained with FITC-conjugated anti-mouse IgG antibody alone.

Figure 3. Use of Sterile ECM for Functional Studies.
RCS “matrix producer” cells were grown for 48 h on glass coverslips and then treated with 20 mM ammonium hydroxide under sterile conditions to remove the cells. COS-7 “test” cells were plated on coverslips with or without isolated RCS ECM for 2 h, fixed in 2% PFA, permeabilized in 0.5% Triton X100, and stained with FITC-phalloidin to visualize F-actin and DAPI to visualize nuclei. COS-7 cells plated on RCS ECM spread more extensively and formed large microfilament bundles (example arrowed) and edge ruffles.

Figure 4. Identification of Proteins from Isolated ECM by SDS-PAGE, Mass Spectrometry, or Immunoblotting.
(**A**) RCS cells were grown for 7 days and removed with 20 mM ammonium hydroxide; the ECM was scraped up into hot SDS-PAGE sample buffer containing 100 mM DTT. The ECM preparation was separated by SDS-PAGE on a 7% polyacrylamide gel under reducing conditions. Four significant bands (arrows 1-4) were identified by protein staining. (**B**) Proteins from RCS ECM bands 1-4 were identified by MALDI mass spectrometry. (**C**) COS-7 cells expressing either mRFPovTSP1C (trimer) or mRFP–TSP-5-1C (pentamer) were cultured for 48 h and removed with 20 mM ammonium hydroxide, and the was ECM isolated into hot SDS-PAGE sample buffer containing 100 mM DTT. The ECM preparation was separated by SDS-PAGE on a 7% polyacrylamide gel under reducing conditions, transferred to a PVDF membrane, and probed with an anti-RFP antibody. This panel is modified from an original publication in Bioscience Reports[17]. (**D**) HDF were grown for 96 h and then treated either with 2% deoxycholic acid in PBS (cell lysate) or with 20 mM ammonium hydroxide to remove cells. The ECM was scraped into hot SDS-PAGE sample buffer. The two fractions were separated by SDS-PAGE on a 10% polyacrylamide gel under reducing conditions, transferred to a PVDF membrane, and probed with antibodies, as indicated. In each panel, the positions of molecular weight markers are indicated in kDa.

DISCUSSION:
Critical steps within the protocol
The method has some critical steps that must be followed to ensure the successful isolation and analysis of ECM. For example, ammonium hydroxide is used to remove the cells and to isolate ECM for analysis. Although ammonium hydroxide is stable in aqueous solutions in the dark and can be stored at room temperature, bottles must

be tightly re-sealed between each use. Because the gas may evaporate from the solution, thus reducing the concentration, we strongly recommend starting a fresh bottle every 6-8 weeks. In addition, the working solution should be freshly made for each experiment. It is also essential that the cells are fully removed with copious washes in de-ionized water. If these steps are not performed adequately, cellular material may remain on the dish and contaminate downstream analyses. If cells have been grown for 10 days or longer, additional water washes may be needed. It is important to note that the isolated ECM layer is typically very thin in comparison to the cells[11], so care is needed when identifying the ECM during imaging. For effective extraction of the isolated ECM into SDS-PAGE sample buffer, it is essential that the sample buffer contains a reducing agent. For the most effective removal of the ECM, boiling-hot sample buffer must be used. For experiments in which ECM samples will be resolved by SDS-PAGE electrophoresis for protein staining or proteomics, it is critical to achieve a concentrated sample by scraping several plates-worth of ECM into the same aliquot of sample buffer.

Modifications and troubleshooting

The production and secretion of ECM proteins can vary significantly between cell types, so time periods for secretion and deposition of ECM should be determined *de novo* for each cell type. It is also important to be aware that ECM composition will change dynamically in relation to cell density and time in culture[18]. This factor should be taken into account when designing experiments. Clearly, the selection of a cell type for this method is important, particularly when extracting ECM for analysis by mass spectrometry, which may require a substantial amount of total ECM protein.

Limitations of the technique

Cells that secrete very low levels of ECM proteins will be more challenging for use with this method. Non-adherent cells, 3D cell cultures, or cell invasion assays are unsuitable at present for ECM isolation by this method. The method is most suitable for use with standard “2-dimensional” cell cultures. Because the ammonium hydroxide extraction removes the cells completely, ECM associated with the upper sides of cells and secreted, but non-cross-linked, ECM proteins are also removed, leaving on the dish a thin layer of assembled ECM from below and around the bases of the cells[11,17]. It is complex to quantify how much ECM is released by the ammonium hydroxide extraction, because the released material also includes ECM proteins that are intracellular either prior to secretion or after uptake from the cell surface.

Significance of the technique with respect to existing/alternative methods

The ability to conduct a wide range of experiments with isolated ECM is important in the context of cell biology and tissue physiology, and it also has implications for the fields of tissue regeneration and tissue engineering. The method has advantages over gels formed from purified collagens or other purified ECM proteins in that the cells assemble complex ECM structures at their native size, with native cross-linking mechanisms and with individual ECM proteins present in ratios appropriate to the cell type of origin. For example, the proteomic study of RCS ECM demonstrated abundant matrilins and COMP/TSP5, as expected for a cartilaginous ECM[19,20] (**Figure 4**). In general, the analysis of cell-derived ECM is hampered by its nature as an extensive, multi-protein network that results in covalent crosslinking and the insolubility of many ECM proteins, and also by the potential contamination of ECM

extracts with intracellular proteins. A multi-step procedure designed to isolate the ECM fraction from tissues for proteomic analysis yielded 8% of ECM proteins in the total set of proteins identified. However, peptides from ECM proteins were 73% of the total peptides identified. This method for isolation and analysis of cell-derived ECM rapidly and reliably removes cellular material whilst retaining ECM proteins. This method can be used for a range of cell types and downstream applications and facilitates the analysis of ECM biology and cell-ECM interactions in cell culture. Our protocols detail how the method can be applied at different scales to address a wide range of experimental questions with regard to ECM organization, dynamics, or composition, and to the functional effects of isolated ECM on cells.

Future applications or directions after mastering this technique
Looking to the future, the method could be adapted for use with 3D cell cultures; this would expand its physiological relevance. Decellularized native tissue has great potential as a scaffold for the regeneration of lost or damaged tissue and as an alternative to transplantation, such as after heart failure[22]. It has the potential to overcome the problems of donor availability and immunorejection. Future applications of the method described in this report could investigate its use with 3D cell cultures or tissue decellularization, which would further increase the scope of this approach.

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DISCLOSURES:
The authors declare that they have no competing financial interests.

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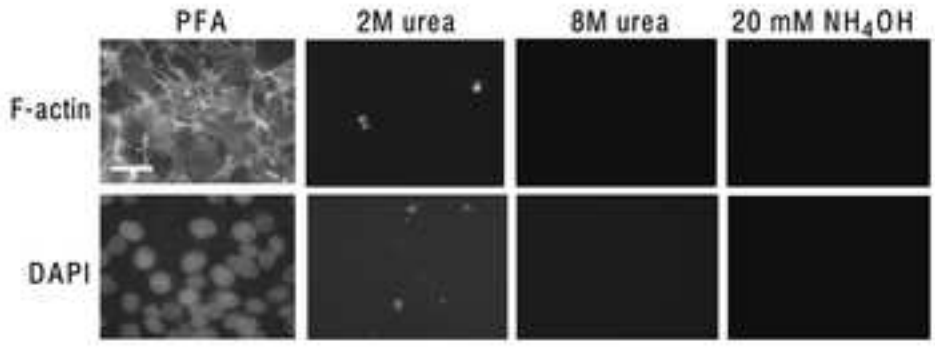

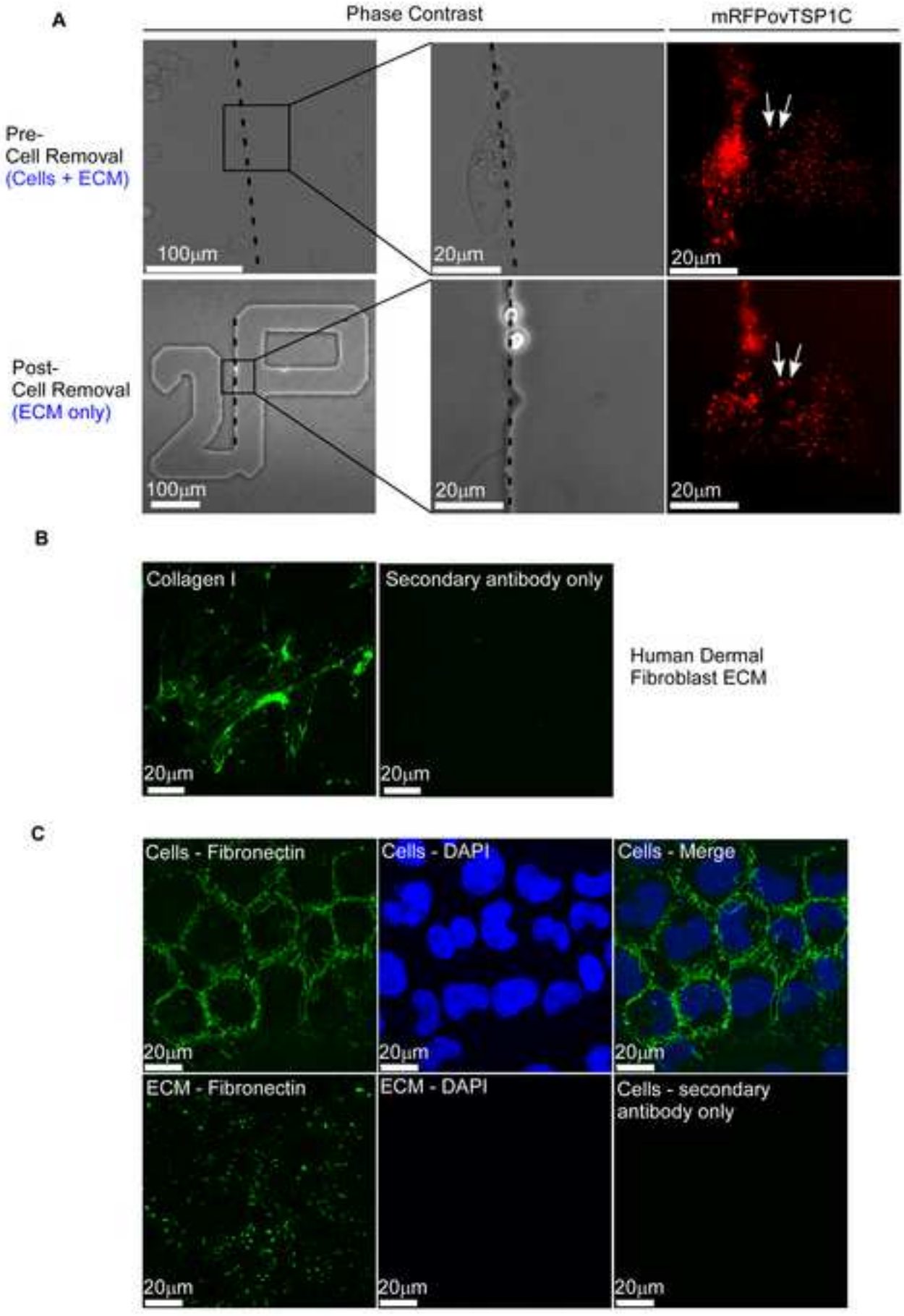

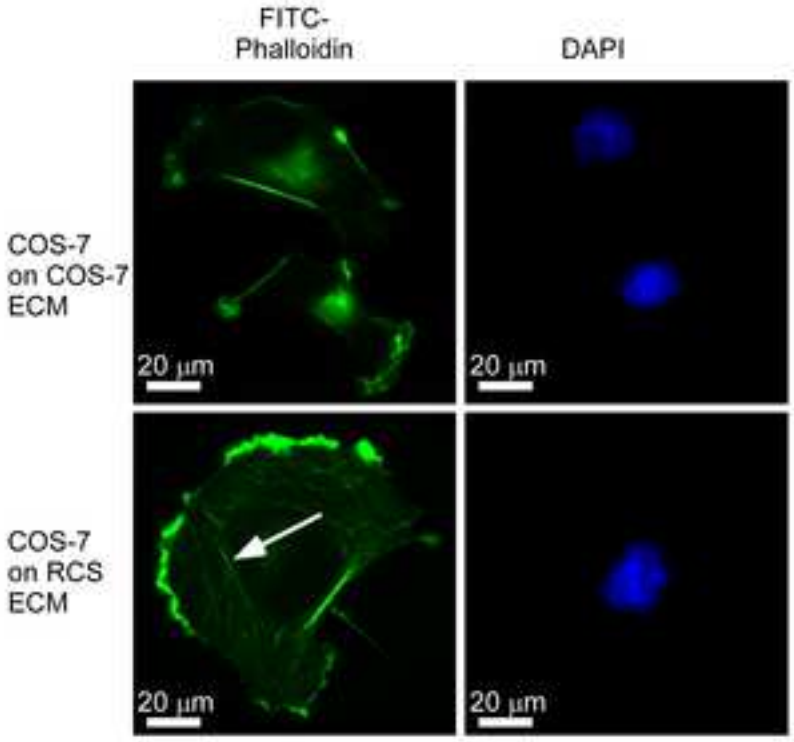

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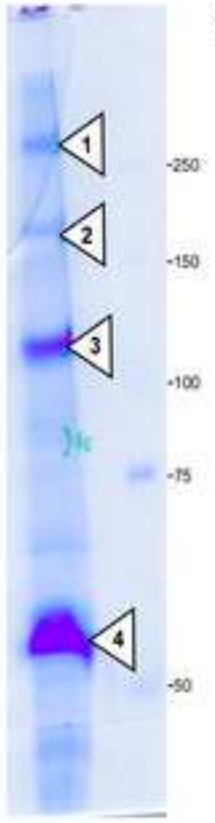
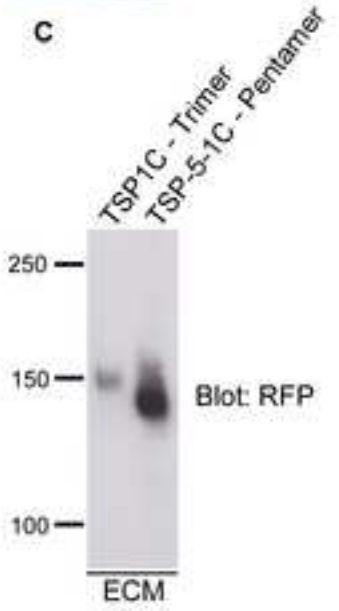


B

Band	Protein Identification	LTQ	
		MASCOT score	No. Peptides /(Coverage)
1	Fibronectin	5081	76 (44%)
	Matrilin-1	1200	18 (50%)
	Matrilin-3	335	6 (17%)
	Thrombospondin-1	234	5 (4%)
	Thrombospondin-5/COMP	124	2 (3%)
	Chondroitin sulphate proteoglycan 4	122	3 (1%)
2	Thrombospondin-1	3321	50 (43%)
	Matrilin-1	1176	17 (43%)
	Fibronectin	657	10 (5%)
	Matrilin-3	457	7 (17%)
	Thrombospondin-5/COMP	167	3 (5%)
3	Thrombospondin-5/COMP	2140	29 (51%)
	Matrilin-1	954	14 (35%)
	Collagen α1(IX)	809	12 (16%)
	Matrilin-2	716	11 (13%)
	Matrilin-3	491	7 (15%)
	Thrombospondin-1	250	6 (4%)
	Proline arginine-rich leucine-rich repeat protein	113	2 (8%)
4	Matrilin-1	2540	35 (66%)
	Matrilin-3	1672	22 (50%)

C



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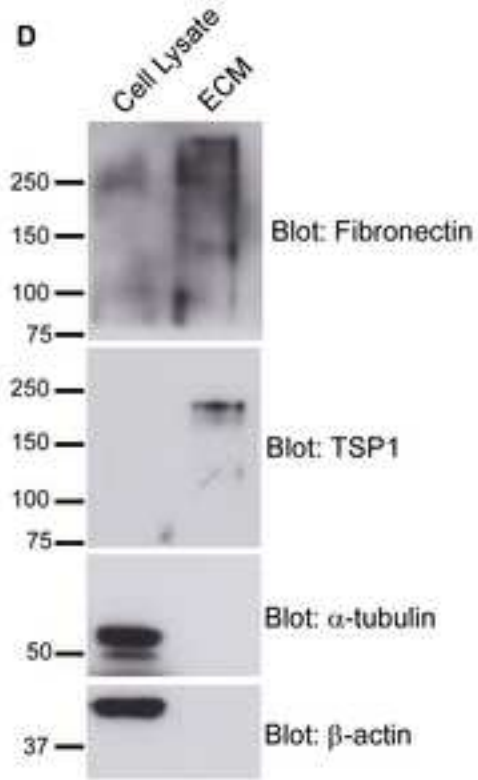

Excel Spreadsheet- Table of Materials/Equipment

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JoVE_Materials_Table.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Antibody to COL1A1	Novus	NB600-408	Rabbit polyclonal. Reacts with other fibrillar collagens as well as collagen I. IF: 1/200
Antibody to fibronectin	Sigma	F3648	Rabbit polyclonal. WB: 1/600 for 1.5 hr. IF: 1/200
Antibody to thrombospondin 1	ThermoFisher	MA5-13398	Mouse monoclonal clone A6.1. WB: 1/150 for 1.5 hr
Antibody to RFP	Abcam	ab62341	Rabbit polyclonal. WB: 1/2000 for 2 hr
Antibody to β-actin	Sigma	A1978	Mouse monoclonal clone AC-15. WB: 1/10000 for 1.5 hr
Antibody to α-tubulin	Sigma	T9026	Mouse monoclonal clone DM1A. WB: 1/5000 for 1.5 hr
Cell Tracker Green	ThermoFisher	C2925	Fluorescent cell marker. Use at 1 μM for 30 min
Fluorescein isothiocyanate (FITC)-Phalloidin	Sigma	P-5282	Stock = 50mg/ml in DMSO.1/50 for 45 min
FITC-conjugated goat anti-mouse IgG	Sigma	F8771	IF: 1/50 for 1 hr
FITC-conjugated sheep anti-rabbit IgG	SIgma	F7512	IF: 1/50 for 1 hr
Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG	LI-COR	926-80010	WB: 1/50000 for 1 hr
HRP-conjugated goat anti-rabbit IgG	LI-COR	926-80011	WB: 1/100000 for 1 hr
Vectorshield mounting medium with DAPI	Vector	H-1200	Store at 4 C in the dark
Dulbecco's Modified Eagle's Medium	Sigma	D6429	Warm before use
Fibroblast growth medium	PromoCell	C-23010	Warm before use, supplement with 50 μg / mL ascorbic acid
Phenol red-free DMEM	ThermoFisher	21063-029	Warm before use
Trypsin-EDTA solution	Sigma	T3924	Warm before use
Gridded glass-bottomed dish	MatTek	P35G-2-14-C-GRID	
NH_4OH, 28% - 30% solution	Sigma	221228	Dilute to 20 mM in de-ionised water. Once opened store in the dark, tightly capped.
Paraformaldehyde, 16 % solution	Alfa Aesar	43368	Dilute to 2 % (v/v) in PBS
Deoxycholic acid	Sigma	D2510	Use at 2 % (v/v) final concentration
Triton X-100	Sigma	T8787	Dilute to 0.5 % (v/v) final concentration

GelCode Blue stain reagent	ThermoFisher	24590	Protein stain for SDS-PAGE gels
Precision Plus protein standards	Biorad	161-0374	Protein standards for SDS-PAGE gels
Trans-Blot SD Semi Dry transfer cell	Biorad	1703940	Efficient transfer of high molecular weight proteins
PVDF transfer membrane	Millipore	ISEQ00010	Immobilon-PSQ
Ponceau S stain	Sigma	P7170	Reversible protein stain for PVDF membrane
WesternSure Enhanced chemiluminescence (ECL) substrate	LI-COR	926-80200	
High performance chemiluminescence film	GE Healthcare	28906837	
Sterile filtration unit, MILLEX-GV	Millipore	ML481051	
SP8 AOBS confocal laser scanning microscope	Leica		Environmental chamber needed for temperature and CO_2 control (Life Imaging Services)

Key: IF, Immunofluorescence;
WB, Western Blot

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•Formatting:
-Please include spaces between numbers and units.
Response: Done.

-1.1.2 would be better placed as a note, or re-worded. E.g. "Place coverslips in X location for X time to allow deposit of ECM"
Response: The informational comments in Section 1 are all now formatted as notes. The protocol points have been reworded where necessary.

-References - Please add DOIs where possible, and abbreviate Journal titles.
Response: Done. Journal titles are formatted as in the Endnote template for JoVE. Please let us know if any further changes are needed.

•Grammar:
-Title – "functional studies" should be "functional study"
Response: Thank you, but we have not adopted this proposal as we wish the title to convey that the ECM can be used for many different functional studies.
-Line 265: …"ensuring all areas of the dish have been scrapped"
Response: We do not agree with this proposal. The correct word is 'scraped'.
-Line 131: "Lyzed cells" – Lysed.
Response: The text is written in USA English as requested. Therefore we believe the spelling should be 'lyzed'.

•Visualization: The highlighted protocol is relatively short. We recommend highlighting most of section 3.
Response: Thank you. We have highlighted 3.2-3.9.

•Additional detail is required: 1.1.1 – What medium is used? What is the seeding density? What are the incubation conditions?
Response: The medium, seeding density and incubation conditions will depend on the cell type being used and will need to be determined empirically. We have now included a note in the text (after 1.1.1) to explain this.

•Results: Please label what the numerical ladder values at the side of the SDS-PAGE represent in 4A. This also applies to 4C and 4D.
Response: We had stated in the figure 4 legend that '…all molecular mass markers are given in kDa'.
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Response: Thank you, we have changed the wording in figures 1 and 4 to indicate that they are modified with permission. Both of the journals of the original publications allow re-use of figure panels by authors.

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Reviewers' comments.
We thank the reviewers for their positive appraisal of this manuscript.
Reviewer #1:
Manuscript Summary:
This manuscript describes methods for the production of cell derived extracellular matrices and the use of these matrices in a number of different applications. This is a very timely paper and likely to be of high interest in the field. The text is well written and organized. In places the flow of the paper might be improved and it will be important to make clear the exact qualities/nature of the matrix this protocol generates compared to other published methods:
1. The NOTE on page 1, line 147 might be better placed at the end of section 1.3 instead. It is not clear why step 1.6 should not be followed with these methods of cell removal?
Response: Thank you for pointing this out. We have moved the note into section 1.3.

2. I found section 2 a little hard to follow:
a. An introduction about what might be transfected, or if not about using cells stably expressing labelled ECM components might help to set the scene
b. section 2.2 presumably refers only to transfected cells - as it reads it is not clear if non-transfected cells should be plated and then after 16 hours replated.
c. I can understand why you would want to follow labelled ECM components over time using this method but the value of section 2.8-2.10 - re-examining the matrix after cell removal is not clear - what might be the purpose of this part of the experiment?
Responses:
a. Thank you, we have added some explanatory phrases to section 2 (in 2.1 and 2.4) to guide a reader.
b. Because the live-imaging method relies on expression of a fluorescently-tagged ECM protein, we have removed the reference to untransfected cells.
c. The purpose of these steps is to identify which elements of the fluorescent signal are definitely within the ECM. This has been clarified in the text.

3. Section 3.1, and throughout section 3, might benefit from distinguishing the two cell populations to be used as 'matrix producers' and 'test cells' or similar, to make it clearer that one cell type will be used to make a matrix and the other to assess the effect of the matrix on cell behavior.
Response: Thank you for this comment. The terminology has been added.

4. The efficiency of cell removal using this method is shown nicely in figure 1, but

what is the efficiency of matrix retention - how much of the original matrix remains in situ and how much is lost in the wash fractions?
Response: Because we allow at least 16 h for ECM deposition, cells are not detached by the PBS washes. Soluble proteins within the conditioned media are washed away by the PBS washes. Secreted but non-crosslinked ECM proteins are removed during the NH4OH extraction, along with ECM that is associated to the upper surfaces of the cells (hence the reduced signal in immunofluorescence, as shown for fibronectin in Fig. 2). It is difficult to quantify how much "original matrix" remains, because, without the ECM isolation step, the signal obtained will include ECM proteins that are intracellular: either prior to secretion or after uptake from the cell surface. We have added this point on p10.

5. Figure 2c confirms effective removal of cells but here much of the fibronectin also seems to have disappeared with the cells, moreover the organization of the amount that is left looks quite different to that present before cell removal. Is the matrix architecture significantly altered by cell removal using this technique? Is the matrix that is left at all 3 dimensional, or it is a planar substrate? Does this method act by stripping off the top layer of matrix together with the cells, leaving behind only any matrix that is attached firmly enough to the plate/glass, or can it be optimized to dissociate the cells leaving intact a multidimensional substrate (e.g. Mao and Schwarzbauer 2005)? If not, is there any way to mitigate the effect this change in matrix architecture might have on further functional studies using these matrices?
Response: Thank you for the questions. We have expanded the discussion of limitations to the method on p10.

6. For global proteomic analyses e.g. by LC-MS, how does the efficiency of this method compare to that of ECM enrichment techniques reported to extract ECM from tissues (but also applicable to cell studies)(e.g. by Naba et al, 2011)?
Response: Naba et al. 2011 reported that 8% of the unique proteins identified were "core matrisome" proteins. This is now stated on p10.
For the reviewer's information, we have begun to conduct proteomic analysis of the ECM of HDF isolated by our method. 10% of the proteins identified correspond to "ECM" according to GO terms and manual curation. Because these data are part of an ongoing, unpublished study, we have not included this information in the manuscript text.
Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
This is a very clearly written methods paper. I think we could generate cell-derived ECM in our own lab based on the protocol presented, as well as published work from the Adams lab.

Major Concerns:
I have no major concerns other than those addressed by the authors all ready---that a method for extracting ECM from 3D cultures would also be helpful.

Minor Concerns:
Could the authors address transmembrane proteins like integrins? For example, is it known whether the low divalent cation conditions of the PBS washes are sufficient to undo integrin-ECM interactions? Or are the extracellular portions of these transmembrane proteins part of the "matrix fraction?" Similarly, what about newly secreted matrix proteins that aren't yet cross-linked into the matrix or soluble growth factors and cytokines? Are these removed with the NH4OH washes? The answers to these questions would affect data interpretation in functional analyses of cell-derived matrix.
Response: Because we allow at least 16 h for ECM deposition, cells are not detached by the PBS washes. Soluble proteins within the conditioned media are washed away by the PBS washes. Secreted but non-crosslinked ECM proteins are removed during the NH4OH extraction, along with ECM that is associated to the upper surfaces of the cells (hence the reduced signal in immunofluorescence, as shown for fibronectin in Fig. 2). Whether integrin fragments can be retained within the ECM is an interesting point. In an unpublished ECM proteome from dermal fibroblasts we have detected peptides from integrins α_5, α_v, β_1 and β_5. Because these are unpublished data we have not included them in the manuscript text.

Additional Comments to Authors:
N/A

Manuscript:

Responses to JOVE editorial comments are given in red:

1. Please adjust the numbering of your protocol section to follow JoVE instructions for authors, 1. should be followed by 1.1) and then 1.1.1) if necessary and all steps should be lined up at the left margin with no indentations.

The numbering has been adjusted in the protocol section on pages 5-8 as instructed and all the steps are lined up at the LH margin with no indentation.

2. Please add a one line space between each step and sub-steps of your protocol section.

A one line space has been added between steps and sub-steps on pages 5-8 of the protocol.

3. Please specify the cell line used in the protocol where applicable.

We have specified which cell lines were used in step 1.1 on page 5 and step 2.1 on page 6. We have also emphasised that the protocol can be used for any adherent cells which produce sufficient ECM for analysis in step 1.1 on page 5 and with the statement “cells of interest” in steps 3.1 and 4.1 on page 7.

4. In step 2 how are the cells washed? Please provide details.

The details of the washing step have been added in step 1.2 on page 5.

5. Step 3 is a run-on sentence. Please split the sentence and provide details as to how the solution is prepared, how the PBS is removed, etc.

The sentence has been split in step 1.3 on page 5. Details have been provided as to how the solutions are prepared in the extractor hood and removed with a plastic transfer pipette.

6. In step 4-5 what is the hydroxide-solubilized material is the author referring to?

An explanation of the hydroxide-solubilized material has been included in step 1.4 on page 5.

7. Please define all the abbreviations upon its first occurrence. PBS, EMC, etc.

All abbreviations are now defined upon first usage.

8. In step 6 please provide a caution statement for paraformaldehyde.

A caution statement for paraformaldehyde is now included in step 1.6.

9. In step 7 how are the washing carried out?

A description of the washing step is provided in step 1.2 on page 5. The reader is directed to this step from step 1.7.

10. Is step 8 a NOTE?

Step 8 on page 5 has been changed to a note as it is not an essential part of the protocol but is referred to in the results section.

11. Please re-write steps of your protocol section in imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.). Please try to avoid usage of phrases such as “should be”, “could be”, “would be” and write in the active/imperative style.

The tense has been changed where appropriate to the imperative.

12. For instance see step 1 in section 2.

Please see response to point number 11.

13. Prior to step 1, at what point are the cells counted and how?

Steps 2.1 and 2.2 on page 6 have been re-written to provide an explanation of the method for cell counting.

14. Phenol red-free media is not mentioned previously in section 1 and seems discontinuous. Please provide the details.

A description of phenol red and the effect it has on imaging has been provided in step 2.2 on page 6.

15. In step 4 is the temperature stabilized to 37 degree C? If so, please specify.

In step 2.3 on page 6 it is now specified that the temperature is stabilized to 37 degree C in the chamber.

16. In step 5 how many cells?

A minimum number of cells is now specified in step 2.5 on page 6.

17. In step 6 please specify the wavelengths used?

The wavelengths for maximum excitation and emission of RFP are now included in step 2.7 on page 6.

18. If z-stack imaging step is to be filmed then please provide details as to how this is carried out. Optionally, please provide a reference.

More detail has been provided for the z-stack imaging process in step 2.6 on page 6.

19. In step 8, how is this carried out?

More detail is provided on how the time-lapse microscopy images are captured In step 2.7 on page 6.

20. In section 3, step 1, please specify the cell used.

We now note in step 3.1 on page 7 that any adherent cell types can be used and that the two cell populations can be different cells.

21. In step 2 how are these prepared? Any precautions to be used?

More detail is provided in step 3.2 on page 7 on the constitution of the PBS. The reader is now referred to step 1.3 on page 5 for more information on preparations and precautions.

22. In step 3 how are the cells washed?

In step 3.3 on page 7 the reader is now referred to step 1.2 on page 5 for more details on the washing step.

23. In step 4 how is the PBS removed?

Details are now provided in step 3.4 on page 7 on how to remove the PBS.

24. In step 5 please replace copious with the actual amount.

In step 3.5 on page 7 copious has been explained by "at least 20 ml per 100 mm dish".

25. In step 6, please specify the step number.

In step 3.6 on page 7, the step number is referred to is step 3.5.

26. In step 7, what is the concentration of trypsin – EDTA used? how much is used? How long is it incubated in? Please provide details of the harvesting step.

The concentration of the trypsin-EDTA is now included as % weight by volume and details of the amount, timing of incubation and the harvesting step are included in step 3.7 on page 7.

27. What happens post-centrifugation between step 7-8?

In step 3.8 on page 7 details of the resuspension and counting of cells post-centrifugation is now included.

28. In step 8 how are cells counted?

The use of a hemocytometer to count the cells is stated in step 3.8 on page 7.

29. Please provide a reference for step 9 and provide the different wavelengths used.

A reference is now provided for step 3.9 on page 7 along with the emission and excitation maxima for FITC and DAPI.

30. Again in section 4, specify the cells.

In step 4.1 on page 7 it is stated that any adherent cells of interest may be used.

31. In step 4 how is this heated?

In step 4.4 on page 8 it is specified that the SDS-PAGE sample buffer is heated to 95 °C using a heat-block.

32. Please provide a citation for step 8, 9 10,11, 12.

Citations are now provided for steps 4.8, 4.9, 4.10, 4.11, and 4.12 on page 8.

33. In step 12 please provide the antibody details, and dilutions. Please do not use brand names or commercial names.

Antibody details and dilutions are now included in step 4.12 on page 8.

34. Please make a note that cited step should not be in the highlighting for visualization.

No highlighting has been included for any of the cited steps.

35. After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10 page limit for the protocol text, but there is a 2.75 pages limit for filmable content. If your protocol is longer than 3 pages, please highlight (in yellow) 2.75 pages (or less) of text to identify which portions of the protocol are most important to include in the video; i.e. which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVEs instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

Regarding the protocol steps important to be included in the video, we have highlighted the steps we consider would be crucial to show. These cover about 1.5 pages of text. There are additional steps that could be included, but we would need input from JoVE on how accessible these are for videoing. These

steps include section 3.2-3.6, which are carried out in a laminar flow hood, also 2.5 and 2.8-2.9, to demonstrate how the ECM is identified on the same square of a gridded dish before and after cell imaging and removal of cells. We have not highlighted these sections, as we would need more information on how the video will be designed.

36. If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.

The appropriate phrases have been included in the legends for figure 1 on page 13 and figure 4C on page 14 as they have both been published previously by our laboratory. Both Journals allow authors to reproduce figures from their papers without specific reprint permission.

37. In the figures, please specify a scale bar and define the scale units in the figure legends.

Scale bars and units are specified in figures 1, 2 and 3.

38. Please define all error bars (SD, SEM) in the legends of their respective figures.

There are no error bars in any of the figures.

39. Please make sure that the “Discussion” is written under the following sections.
a. Critical steps within the protocol.
b. Modifications and troubleshooting.
c. Limitations of the technique.
d. Significance of the technique with respect to existing/alternative methods.
e. Future applications or directions after mastering this technique.

The discussion on page 11-12 has been rearranged to reflect the sections listed above and the headings have been included.

40. IMP: Please copyedit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

The entire manuscript has been copyedited by native English speakers and the text adjusted to American-English.

41. NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.

Please see all line-by-line responses above.