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Enrichment of extracellular matrix proteins from tissues and digestion into peptides for mass spectrometry analysis.

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Abstract:	<p>The extracellular matrix (ECM) is a complex meshwork of cross-linked proteins that provides biophysical and biochemical cues that are major regulators of cell proliferation, survival, migration, etc. The ECM plays important roles in development and in diverse pathologies including cardio-vascular and musculo-skeletal diseases, fibrosis, and cancer. Thus, characterizing the composition of ECMs of normal and diseased tissues could lead to the identification of novel prognostic and diagnostic biomarkers and potential novel therapeutic targets. However, the very nature of ECM proteins (large in size, cross-linked and covalently bound, heavily glycosylated) has rendered biochemical analyses of ECMs challenging. To overcome this challenge, we developed a method to enrich ECMs from fresh or frozen tissues and tumors that takes advantage of the insolubility of ECM proteins. We describe here in detail the decellularization procedure that consists of sequential incubations in buffers of different pH and salt and detergent concentrations and that results in 1) the extraction of intracellular (cytosolic, nuclear, membrane and cytoskeletal) proteins and 2) the enrichment of ECM proteins. We then describe how to deglycosylate and digest ECM-enriched protein preparations into peptides for subsequent analysis by mass spectrometry.</p>
Author Comments:	<p>Boston, March 16, 2015</p> <p>Dear Editors,</p> <p>We are pleased to submit a revised version of our manuscript that includes all the changes requested by the reviewers. Edits of the manuscript appear in blue in the revised version of the manuscript. In addition, the sections of the protocol that we</p>

would like to have included in the video are highlighted in yellow.

Please find in a separate document our point-by-point response to the editorial and peer review comments.

We hope you will find our revisions satisfactory and our article suitable for production and publication in the Journal of Visualized Experiments.

With best regards,

Alexandra Naba, on behalf of all the co-authors.

January 10, 2015

Dear Editors,

We are pleased to submit a revised version of our manuscript that includes all the changes requested. We include in a separate file the list of all the changes made to address each of the comments raised.

We hope you will find our revisions satisfactory and our article suitable for production and publication in the Journal of Visualized Experiments.

With best regards,

Alexandra Naba, on behalf of all the co-authors.

Dear Editors,

In response to the invitation of Dr. Nandita Singh, JoVE Senior Science Editor, we wish to submit our manuscript entitled "Enrichment of extracellular matrix proteins from tissues and digestion into peptides for mass spectrometry analysis" for consideration for publication in the Journal of Visualized Experiments.

As you are no doubt aware, the extracellular matrix (ECM) is a vital and dynamic component of the tissue microenvironment contributing to homeostasis and diseases such as fibrosis, vascular diseases and cancers. Due to the very nature of extracellular matrix proteins (large size, extensive post-translational modifications, insolubility), the ECM has been difficult to analyze biochemically and, consequently, until recently we did not have a detailed understanding of its composition and changes therein.

We have previously developed a proteomics-based approach to characterize the composition of in vivo ECMs (or "matrisome"). We present in detail in this manuscript and video 1) how to decellularize tissues and enrich for ECM proteins and 2) how to deglycosylate and digest ECM-rich protein preparations into peptides for subsequent analysis by mass spectrometry. The unique multimedia format offered by JoVE will allow us to demonstrate how to handle ECM-rich protein preparations and highlight the visual cues that can help researchers monitor the quality of their experiments.

Thank you for inviting us to submit our manuscript, we hope you will find it suitable for publication in Journal of Visualized Experiments.

Alexandra Naba, on behalf of all the co-authors.

Additional Information:	
Question	Response
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Boston, March 16, 2015

Dear Editors,

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TITLE:

Enrichment of extracellular matrix proteins from tissues and digestion into peptides for mass spectrometry analysis.

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KEYWORDS:

Extracellular Matrix, Matrisome, Proteins, Decellularization, Sequential solubilization, Deglycosylation, Tryptic digestion, Proteomics, Mass Spectrometry

SHORT ABSTRACT:

This protocol describes a procedure for enriching ECM proteins from tissues or tumors and deglycosylating and digesting the ECM-enriched preparations into peptides to analyze their protein composition by mass spectrometry.

LONG ABSTRACT:

The extracellular matrix (ECM) is a complex meshwork of cross-linked proteins that provides biophysical and biochemical cues that are major regulators of cell proliferation, survival, migration, etc. The ECM plays important roles in development and in diverse pathologies including cardio-vascular and musculo-skeletal diseases, fibrosis, and cancer. Thus, characterizing the composition of ECMs of normal and diseased tissues could lead to the

identification of novel prognostic and diagnostic biomarkers and potential novel therapeutic targets. However, the very nature of ECM proteins (large in size, cross-linked and covalently bound, heavily glycosylated) has rendered biochemical analyses of ECMs challenging. To overcome this challenge, we developed a method to enrich ECMs from fresh or frozen tissues and tumors that takes advantage of the insolubility of ECM proteins. We describe here in detail the decellularization procedure that consists of sequential incubations in buffers of different pH and salt and detergent concentrations and that results in 1) the extraction of intracellular (cytosolic, nuclear, membrane and cytoskeletal) proteins and 2) the enrichment of ECM proteins. We then describe how to deglycosylate and digest ECM-enriched protein preparations into peptides for subsequent analysis by mass spectrometry.

INTRODUCTION:

The extracellular matrix (ECM) is a complex meshwork of cross-linked and glycosylated proteins that provides architectural support and anchorage for cells and defines, in part, tissues' biomechanical properties^{1,2}. ECM proteins also signal to the cells either directly through their receptors (e.g integrins, syndecans, etc.) or by modulating growth factor signaling³. The ECM thus provides biophysical and biochemical cues that are major regulators of cellular processes such as proliferation, survival, polarization, differentiation, migration, etc.

The ECM plays key roles in physiology, development and aging⁴. Moreover, several pathologies, such as cardio-vascular diseases, fibrosis, musculo-skeletal diseases, cancers, are caused by, or result in, ECM alterations. Furthermore, the ECM contributes to the maintenance of stem cell niches and identifying key ECM molecules that support stemness will have direct application in tissue engineering and regenerative medicine⁵. However, despite its importance, the ECM has remained, until recently, underexplored⁶.

In silico analysis has revealed that the matrisome, defined as the ensemble of ECM and ECM-associated proteins, comprises the products of several hundred genes in both the human and mouse genomes^{1,7,8}. However, the insolubility of ECM proteins has hindered the systematic characterization of the composition of *in vivo* extracellular matrices of normal and pathological specimens. We recently demonstrated that this insolubility could be turned to advantage and be used to enrich ECM proteins⁸⁻¹⁰. We and others further demonstrated that mass spectrometry was a method of choice to characterize the composition of ECMs^{8-10, 13}.

We describe here a decellularization procedure that consists of sequential incubations in buffers of different pH and salt and detergent concentrations. This procedure results in the extraction (or depletion) of cytosolic, nuclear, membrane and cytoskeletal proteins and the enrichment of ECM proteins. We then describe how to digest ECM-enriched protein preparations into peptides for subsequent analysis by mass spectrometry.

Using the procedures detailed and illustrated here, we have successfully enriched and characterized by mass spectrometry the extracellular matrices from ten different tissues and tumor types: normal murine lung⁸, human and murine colon^{8,9}, human liver⁹, human colorectal tumors and derived liver metastases⁹, melanoma xenografts⁸, mammary tumor xenografts¹⁰, murine pancreatic islets and murine insulinomas (Naba et al., unpublished). Comparison of the different matrisomes revealed tissue- and tumor-specific ECM signatures that could further be used as potential diagnostic or prognostic biomarkers.

We believe that this procedure can be applied to other specimens with no or relatively minor modifications.

PROTOCOL:

NOTE: The procedure can be conducted on fresh or flash frozen tissues. We recommend perfusing highly vascularized tissues with PBS at the time of dissection to eliminate red blood cells and plasma proteins. We do not recommend conducting the procedure on fixed tissues as fixation (i.e chemical crosslinking) interferes with decellularization and can also significantly compromise subsequent mass spectrometry analysis. For the entire procedure, we recommend the use of low-retention tubes and pipette tips to maximize protein and peptide recovery.

1. Section 1: Decellularization of tissues or tumors

NOTE: Before starting, prepare the reagents and add protease inhibitors (provided with the Compartment Protein Extraction kit) to the desired volume of each buffer. All the buffers and samples should be kept on ice for the duration of the experiment except the Buffer CS that needs to be kept at room temperature to prevent SDS precipitation.

NOTE: The protocol uses a series of incubation in buffers of different pH and containing different amount of salts and detergents to sequentially extract intracellular proteins and enrich for insoluble ECM proteins (Figure 1, Table 1; see also Discussion for alternatives to the use of the commercial kit detailed here). The volumes of reagents given below are for 100mg of tissues or tumors (see Table 1) and need to be adjusted appropriately.

1.1) Homogenize 100mg of tissue in 500 μ L of Buffer C containing protease inhibitors using a tissue homogenizer until the tissue is completely disrupted and a homogenous suspension is obtained.

NOTE: Add deoxyribonuclease I (final concentration: 200 μ g/mL, reconstituted according to the manufacturer's instructions) and ribonuclease A (final concentration: 20 μ g/mL, reconstituted according to the manufacturer's instructions) to Buffer N.

1.2) Sequential extraction of intracellular soluble proteins

1.2.1) Extraction of cytosolic proteins

Incubate the homogenate on a tube rotator for 20 min at 4 °C. Save a small aliquot (10 μ L to 20 μ L) of the homogenate for subsequent western blot analysis (see Expected Results section and Figure 2).

1.2.2) Centrifuge the homogenate at 16,000 x g for 20 min at 4 °C. Collect the supernatant in a clean tube, this will constitute the cytosolic (C) fraction of the western blot analysis. Flash freeze this fraction and store at -80 °C.

1.2.3) To wash, resuspend the pellet in 400 μ L of Buffer W containing protease inhibitors and incubate the sample on a tube rotator for 20 min at 4 °C. Centrifuge the homogenate at 16,000 x g for 20 min at 4 °C. Discard the supernatant.

1.2.4) To extract, nuclear proteins, resuspend the pellet in 150μL of Buffer N containing protease inhibitors, deoxyribonuclease I and ribonuclease A and incubate the sample on a tube rotator for 30 min at 4 °C.

1.2.5) Centrifuge the sample at 16,000 x g for 30 min at 4 °C and collect the supernatant in a clean tube. Repeat this step once: after centrifuging the sample for the second time, add the supernatant to the previous supernatant: this will constitute the nuclear (N) fraction of the western blot analysis. Flash freeze this fraction and store at -80 °C. Then, perform washings as per step 1.2.3.

1.2.6) To extract membrane proteins, resuspend the pellet in 100μL of Buffer M containing protease inhibitors and incubate the sample on a tube rotator for 30 min at 4 °C. Centrifuge the sample at 16,000 x g for 30 min at 4 °C.

1.2.7) Collect the supernatant in a clean tube: this will constitute the membrane (M) fraction of the western blot analysis. Flash freeze this fraction and store at -80 °C.

1.2.8) To extract cytoskeletal proteins, resuspend the pellet in 200μL of Buffer CS containing protease inhibitors and incubate the sample on a tube rotator for 30 min at room temperature. Note that the pellet will not fully dissolve. We suggest disrupting the pellet by pipetting up and down until observing disruption of the pellet.

1.2.9) Centrifuge the sample at 16,000 x g for 30 min at room temperature. Collect the supernatant in a clean tube. Note at this point a further marked decrease in the size of the pellet (see video).

1.2.10) Resuspend the pellet in 150μL of Buffer C containing protease inhibitors and incubate the sample on a tube rotator for 20 min at 4 °C. Centrifuge the sample at 16,000 x g for 20 min at 4 °C.

1.2.11) Collect the supernatant and add it to the previous supernatant: this will constitute the cytoskeletal (CS) fraction of the western blot analysis. Flash freeze this fraction and store at -80 °C.

1.2.12) Perform additional washes. Resuspend the pellet in 500μL of PBS containing protease inhibitors and incubate the sample on a tube rotator for 5 min at 4 °C. Centrifuge the sample at 16,000 x g for 5 min at 4 °C. Discard the supernatant. Repeat this step three times.

NOTE: All traces of detergents need to be removed by extensive washes prior to digestion of the proteins into peptides (see Section 3). At this point, the ECM-enriched pellet can be flash-frozen and kept at -80 °C. Note that the size of the pellet will depend on the amount of insoluble (ECM) proteins in the starting material and the efficiency of the decellularization.

2. Section 2: Monitoring the quality of the decellularization/ECM enrichment by SDS-PAGE and western blot.

2.1) Mix 10-20uL aliquot of total tissue extract and 50uL aliquots of intermediate fractions with Laemmli Buffer containing 100mM DTT. Resuspend the ECM-enriched, insoluble fraction in 3X Laemmli Buffer containing 100mM DTT.

Note: the elevated concentrations of DTT and SDS assist in solubilization of ECM proteins that are relatively insoluble.

2.2) Separate the proteins by SDS-PAGE and transferred onto nitrocellulose membranes.

2.3) Perform immune-blots using antibodies to monitor proteins representative of each of the cytosolic, nuclear, membrane, cytoskeletal and ECM fractions (see Representative Results section, Table 2 and Figure 2).

3. Section 3: In-solution digestion of proteins to peptides for mass spectrometry analysis

NOTE: The pellet obtained after the decellularization procedure and removal of SDS is highly enriched in insoluble ECM proteins. For further analysis by mass spectrometry these proteins need to be digested into peptides. Note that, as a consequence of ECM protein insolubility, it is not possible at this step to measure the protein concentration of the sample. We thus provide volumes of reagents to digest the ECM-enriched samples into peptides based on the size (mm) or dry weight of the ECM-enriched pellet (Table 3). The solutions of ammonium bicarbonate, urea, DTT, iodoacetamide and trifluoroacetic acid all need to be freshly prepared.

3.1) Resuspend the ECM-enriched sample by adding the appropriate volume of 8M urea to the ECM-enriched pellet and add DTT at a final concentration of 10mM (see Table 3). Incubate with continuous agitation at 1,400 rpm for 2 hours at 37 °C.

NOTE: at this point the ECM proteins will not be fully dissolved and the visibly large protein particles should not be discarded by centrifugation or filtration. The suspension will clear upon deglycosylation and digestion (see video).

3.2) Alkylation

3.2.1) Prepare the iodoacetamide solution in HPLC-grade water. Cool the sample to room temperature and add the iodoacetamide to a final concentration of 25mM. For complete alkylation, the DTT:iodoacetamide ratio should be between 1:2.5 and 1:3.

3.2.2) Incubate in the dark for 30 min at room temperature.

3.3) Deglycosylation:

Note: deglycosylation is needed to remove carbohydrate side chains that interfere with identification of peptides modified by N-linked glycosylation.

3.3.1) Dilute to 2M urea with 100 mM ammonium bicarbonate pH 8.0 and add the appropriate amount of PNGaseF (see Table 3). Incubate with continuous agitation at 1,400 rpm for 2 hours at 37 °C.

3.4) Digestion

3.4.1) Add Lys-C and incubate with continuous agitation at 1,400 rpm for 2 hours at 37 °C. Add the trypsin and incubate with continuous agitation at 1,400rpm overnight at 37 °C.

NOTE: the ECM-rich suspension that began cloudy upon initial reconstitution in 8M urea appears clear after overnight digestion (see video).

3.4.3) Add a second aliquot of trypsin to the sample and incubate with continuous agitation at 1,400 rpm for an additional 2 hours at 37 °C.

3.5) Acidification

3.5.1) Upon completion of the digestion, inactivate the trypsin by acidifying the sample with freshly prepared 50% trifluoro-acetic acid (TFA). The sample should reach pH <2. We suggest adding 1 – 1.5μL of 50% TFA at a time and using 1μL of the peptide solution to measure the pH of the solution using pH paper (see video).

3.5.2) Centrifuge the acidified sample at 16,000 x g for 5 min at room temperature. Collect the supernatant in a clean low-retention tube. At this point, the peptide solution can be stored at -20 °C.

3.6) Desalting

Note: that this last step is usually conducted at a mass spectrometry facility according to their own preferred methods.

3.6.1) Prior to proteomics analysis, desalt the samples and peptides eluted with freshly prepared 60% acetonitrile, 0.1% trifluoroacetic acid, followed by concentration in a vacuum concentrator. Resuspend the peptides in freshly prepared 3% acetonitrile, 0.1% trifluoroacetic acid⁸.

NOTE: that after desalting, the concentration of the peptide solution can be measured by spectrophotometry (see Expected Results section).

3.6.2) Now analyze the sample by mass spectrometry, again according to the optimal procedures of the mass spectrometry facility.

NOTE: We encourage researchers interested in characterizing the composition of ECMs using mass spectrometry to refer to our other publications⁸⁻¹⁰ and website <http://matrisomeproject.mit.edu>¹¹ that provide further detailed explanation, including LC-MS/MS parameters, mass spectrometry data search for protein identification and data analysis using the *in silico* matrisome annotation tool we developed⁸.

REPRESENTATIVE RESULTS:

Quality control of the decellularization procedure:

The efficiency of the decellularization can be monitored by analyzing the protein content of each fraction by western blot. Table 2 lists proteins of diagnostic value to assess the quality of the decellularization procedure. Figure 2A shows the efficient extraction in the intermediate

fractions of cytosolic (GAPDH), nuclear (histones), membrane ($\beta 1$ integrin) and cytoskeletal (actin) proteins, whereas no ECM proteins (collagen I) is detected in these fractions (Figure 2). In turn, the final pellet is enriched for ECM proteins and largely depleted of intracellular proteins (Figure 2A). Figure 2B presents satisfactory intracellular protein depletion (no histone is detected in the ECM-rich fraction), although, actin can still be detected in the ECM-rich fraction and depletion of monomeric collagen I - apparent molecular weight ~ 110 kDa, presumptively corresponding to unassembled collagen I - can be observed in the CS fraction.

We also routinely monitor additional ECM proteins such as fibronectin and laminin, although, in some tissues these components can be partially solubilized in earlier fractions⁸⁻¹⁰. For example, fibronectin also occurs as soluble plasma fibronectin that is not incorporated into the ECM. Perfusion of the tissue prior to extraction reduces plasma fibronectin concentration but does not always eliminate it. In some tissues, laminins are found loosely associated with the cell surface or the ECM and extract in intermediate fractions. If this occurs it can be addressed by altering extraction conditions (see Discussion).

Note that the enrichment of ECM proteins and concomitant depletion of intracellular components is based on the relative solubility of proteins in the different buffers. This differs among different tissues – in some cases the histones and actin are more readily extracted than in others. Also note that, although histones are expected to be extracted in the N fraction (Figure 2B), we often observe a more complete depletion of histones in the M or CS fraction (Figure 2A and B).

Indicative peptide concentration expected.

The concentration of the peptide solution obtained after digestion, acidification and desalting can be measured by spectrophotometry either by measuring the absorbance of the peptide solution using the 280 nm wavelength corresponding to tryptophan, tyrosines, or using the 205 nm wavelength corresponding to absorbance of the peptide bonds.

We measured the concentration of the peptide solutions obtained from the decellularization of three murine lungs samples (82mg, 100mg and 100mg respectively) prepared in parallel and obtained from each: 424ng/ μ L, 450 ng/ μ L and 580ng/ μ L of peptides respectively.

Identification of ECM peptides by mass spectrometry.

Mass spectrometric analysis of the composition of ECM-enriched protein samples, prepared as described here, showed that $>70\%$ of the signal intensity corresponds to ECM and ECM-associated proteins⁸⁻¹⁰.

FIGURE LEGENDS:

Table 1: Volume of reagents from Compartmental Extraction kit to decellularize 100mg of tissue or tumor.

This table lists the composition and the volume of each buffer used to conduct the decellularization of 100mg of tissue or tumor.

Table 2: Diagnostic proteins to monitor the quality of the decellularization procedure.

This table lists examples of proteins that are characteristics of each subcellular compartment (cytosol, nucleus, plasma membrane, cytoskeleton and ECM) that can be used to monitor the quality of the decellularization procedure and the efficiency of the ECM-enrichment.

Table 3: Volume of reagents to digest ECM-enriched samples into peptides.

This table lists the reagents used to resuspend ECM-enriched protein samples and reduce, alkylate, deglycosylate and digest proteins samples into peptides prior to mass spectrometry analysis.

Figure 1: Scheme of the experimental procedure.

Schematic workflow of the protocol to decellularize tissues (Section 1), control the quality of the decellularization and evaluate the ECM enrichment (Section 2) and digest ECM-enriched protein samples into peptides prior to mass spectrometry analysis (Section 3).

Figure 2: Quality control of the decellularization procedure by western blot.

Western blots were performed on murine lung (A) and human mammary carcinoma xenograft (B) samples using the following antibodies: rabbit anti-actin (clone 14-1) and rabbit anti- β 1 integrin antibodies produced in our laboratory; rabbit anti-collagen I, mouse anti-GAPDH and rabbit anti-pan-histones antibodies were from Millipore. Following primary antibody incubation, the membranes were washed and incubated in the presence of HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibody from Jackson ImmunoResearch Laboratory. Finally, the membranes were washed and incubated in Western Lightning™ Chemiluminescence Reagent (PerkinElmer LAS).

* indicates minimal residual histone contamination of the ECM-rich fraction.

** indicates partial depletion of monomeric (presumptively unassembled) collagen I in the CS fraction.

*** indicates residual actin contamination of the ECM-rich fraction.

The smear detected with the anti-collagen antibody represents different levels of post-translational modifications (e.g. cross-linking, glycosylation)

DISCUSSION:

Although we employed this exact procedure to enrich the ECM from ten tissues and tumor types⁸⁻¹⁰, modifications of the protocol should be considered in the following instances:

1) Detection of ECM proteins in the intermediate fractions.

Extracellular matrices from different tissues or tumor types may differ in their extractability/insolubility, as discussed above for fibronectin and laminins. For example, it is thought that the ECM of fibrotic tissues or remodelling tissues turns over very dynamically and thus one might observe a higher proportion of ECM proteins in those tissues to be more readily extractable¹². Depending on the fraction in which ECM proteins are detected, we suggest reducing the incubation time of the step causing the extraction of ECM proteins or omitting that step.

2) Detection of a significant proportion of intracellular components in the ECM-enriched pellet. In some tissues or tumor types the ratio cells:ECM is particularly high (e.g. liver, spleen, non-desmoplastic tumors). In that case, a significant contamination of the ECM-enriched fraction by

intracellular proteins (in particular cytoskeletal proteins and/or histones) can be observed. To deplete intracellular proteins efficiently, we suggest repeating twice the incubation in Buffer M and/or Buffer CS (both containing detergents, this usually depletes abundant intracellular proteins). Another alternative would be to use alternative buffers with higher detergent concentrations, with the caveat that this may lead to the depletion of relatively more soluble ECM proteins as well (see next paragraph).

3) Alternative to using a commercial kit.

We were unable, for proprietary reasons, to obtain the precise composition of the buffers from the supplier of the Compartmental Protein Extraction kit. However, we have included in Table 1 notes based on our own experience using home-made buffers with defined detergent (NP-40, sodium deoxycholate and SDS) concentrations to conduct similar extractions. A recent study also highlighted the importance of the pH of decellularization buffers to retain ECM proteins¹³.

The method presented here relies on the fact that ECM proteins are intrinsically more insoluble than most intracellular proteins. However, the decellularization method described here certainly extracts soluble components present within the ECM such as some growth factors or ECM-remodeling enzymes. Although ECM-associated proteins tightly bound to ECM proteins were detected by proteomics in samples prepared as described, this method may be too stringent to fully profile the composition of matrisome-associated proteins.

The advantage of the method presented here over other methods is that it can be tailored to the nature of the ECM of interest: intermediate steps can be omitted or repeated to prevent the loss of ECM proteins or increase the depletion of contaminating intracellular proteins respectively. This method also only uses minimal amounts of detergents that are rinsed off to prevent their interference with subsequent peptide preparation and mass spectrometry. Finally, the method described here to digest ECM-rich protein preparations into peptides has also the advantage of not requiring proteins to be soluble and can be conducted on “crude” ECM-enriched fractions.

Alternative decellularization methods utilizing chaotropes (such as guanidine hydrochloride) to study the composition of ECMs by mass spectrometry have been reported in the literature (reviewed in ¹⁴) and have been used in combination with mass spectrometry to characterize the ECM composition of cartilage^{15,16}, heart¹⁷, mammary gland¹⁸ and vascular¹⁹ and glomerular²⁰ basement membranes.

Decellularization methods employing trypsin to digest out cells should not be used if ECMs are enriched for subsequent proteomics analyses, as trypsinization will result in partial ECM digestion and loss of ECM proteins and peptides. Similarly, if collagenase digestion were to be used to aid tissue disruption, it would need to be monitored carefully as it causes ECM digestion and loss of ECM proteins and peptides.

In-solution vs. in-gel digestion? ECM proteins are cross-linked and highly insoluble and, even when resuspended in 3X Laemmli buffer (containing 6% SDS) and 100mM DTT, separate poorly on SDS gels. Thus in-gel digestion is not a preferred method.

It is worth noting that, while not discussed here, the composition of each of the intermediate fractions collected during the decellularization could also be analyzed by mass spectrometry. This may be particularly valuable when studying very small samples (i.e. human biopsies) or when information is desired on other cellular fractions.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1: Scheme of the experimental procedure.

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Section 1: Decellularization / ECM Enrichment

Section 3: In-solution digestion of proteins to peptides for MS analysis

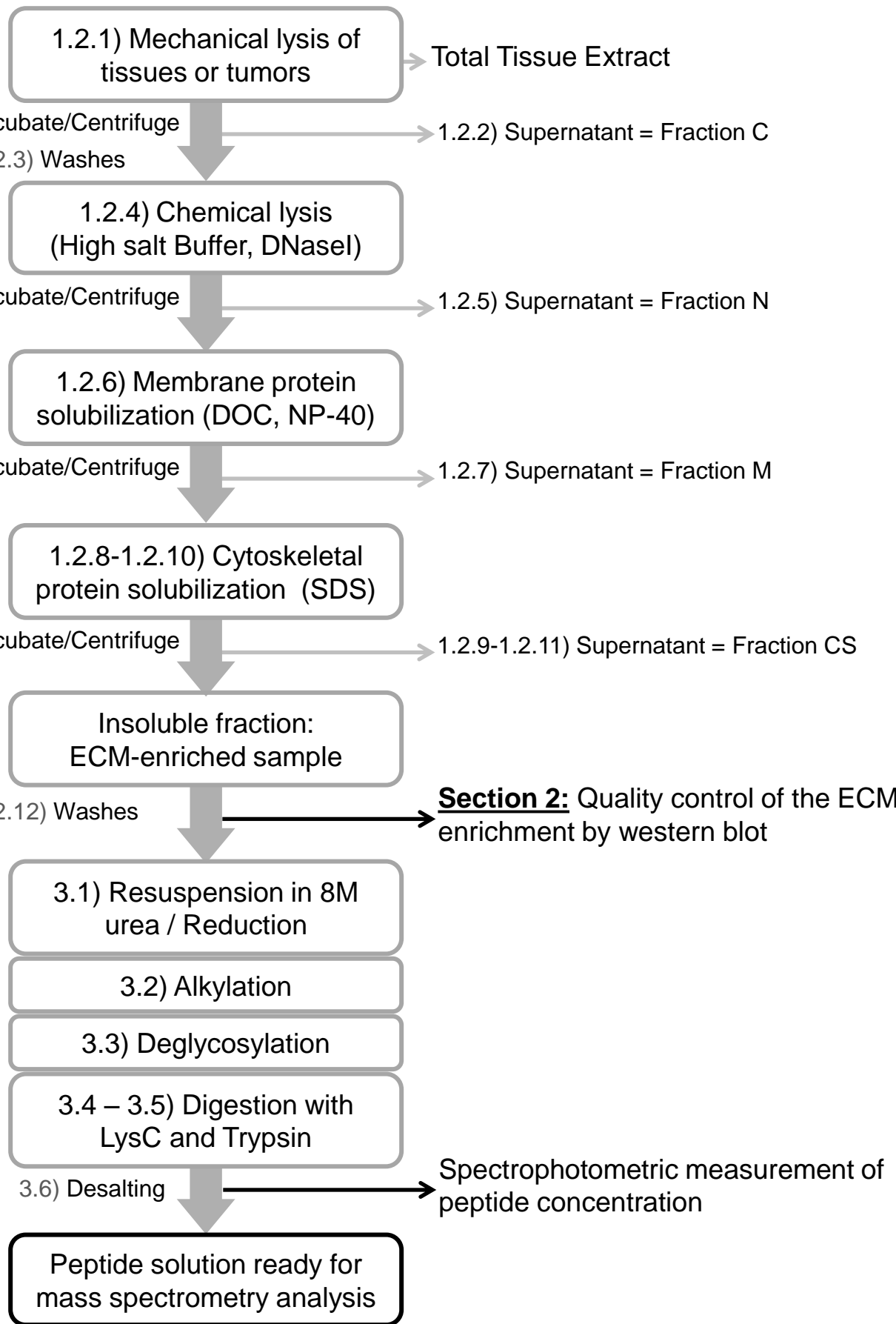
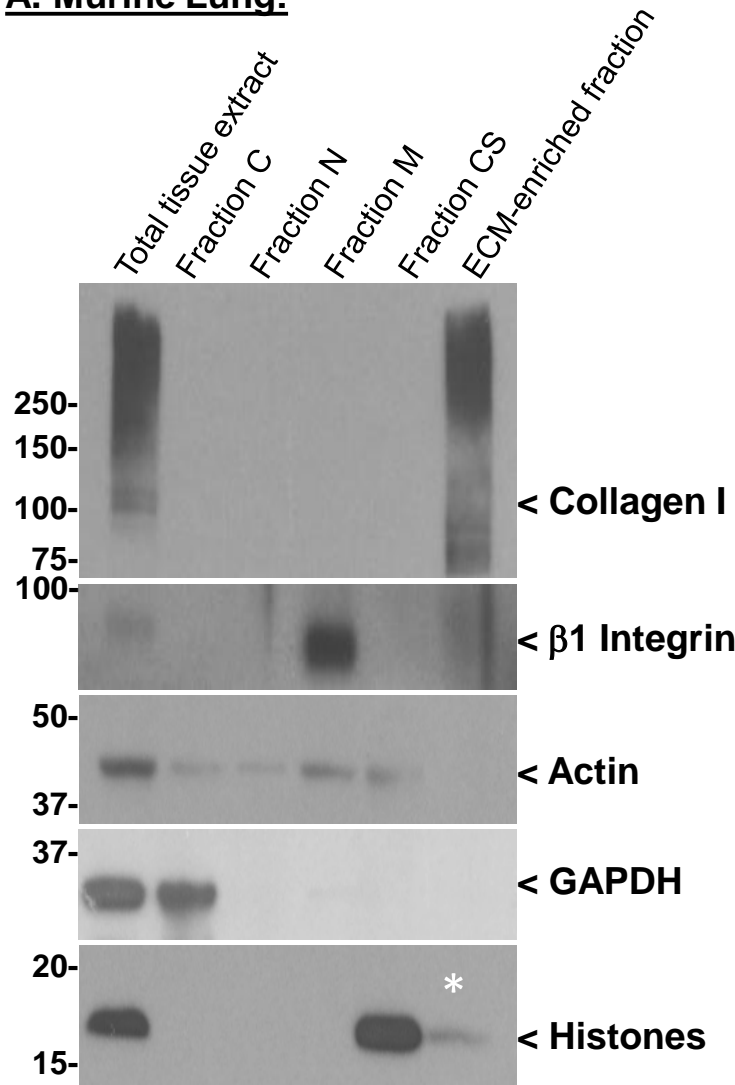


Figure 2: Quality control of the decellularization procedure by western blot.

A. Murine Lung:



B. Mammary carcinoma xenograft:

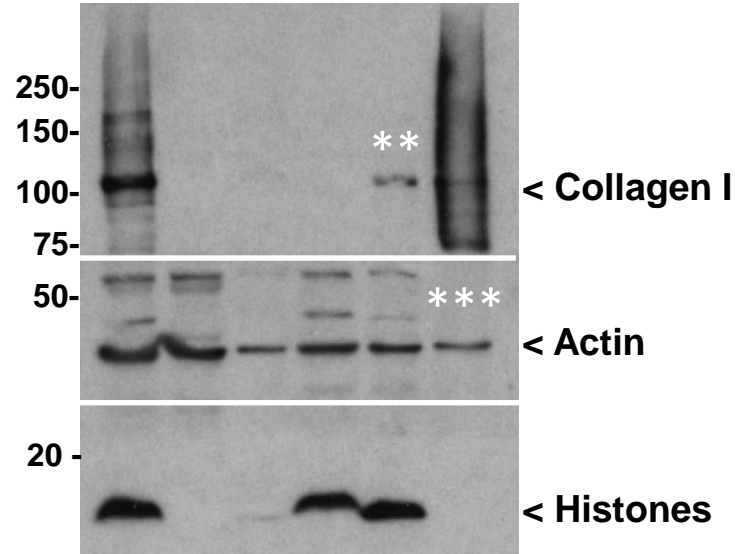


Table 1

[Click here to download Table: Table1_Naba_Final.xlsx](#)

Table 1: Volume of reagents from Compartmental Extraction kit to decellularize 100mg (wet weight) of tissue or tumor.

A cocktail of protease inhibitors is provided as a 50X solution and needs to be added to each buffer².

Reagents from Compartment Protein Extraction Kit	Volume for 100mg of tissue	Composition (based on Millipore datasheet cat#2145 ¹)
Buffer C	500µL	HEPES (pH7.9 ³), MgCl ₂ , KCl, EDT ⁴ , Sucrose, Glycerol, Sodium Orthovanadate ⁵
Buffer W	400µL	HEPES (pH7.9), MgCl ₂ , KCl, EDTA, Sucrose, Glycerol, Sodium Orthovanadate
Buffer N	150µL x2	HEPES (pH7.9), MgCl ₂ , NaCl, EDTA, Glycerol, Sodium Orthovanadate
Buffer W	400µL	HEPES (pH7.9), MgCl ₂ , KCl, EDTA, Sucrose, Glycerol, Sodium Orthovanadate
Buffer M	100µL	HEPES (pH7.9), MgCl ₂ , KCl, EDTA, Sucrose, Glycerol, Sodium deoxycholate (DOC) ⁶ , NP-40 ⁶ , Sodium Orthovanadate
Buffer CS	200µL	PIPES (pH6.8), MgCl ₂ , NaCl, EDTA, Sucrose, Sodium Dodecyl Sulfate (SDS) ⁷ , Sodium OrthoVanadate
Buffer C	150µL	HEPES (pH7.9), MgCl ₂ , KCl, EDTA, Sucrose, Glycerol, Sodium Orthovanadate
1X PBS	500µL/wash	-

Notes:

¹ For proprietary reasons, we were unable to obtain the precise composition of the buffers from the supplier of the kit, but we include here some notes based on our own experience using home-made buffers to conduct similar extractions.

² Protease inhibitor: it is advisable to include a variety of inhibitors against cysteine, serine and threonine peptidases, serine esterases, divalent cation-dependent metalloproteinases etc. Many commercially available protease inhibitor cocktails exist.

³ pH above 7.0 is an effective inhibitor of lysosomal proteases.

⁴ EDTA (usually used at 2mM) is an effective inhibitor of divalent cation-dependent proteases.

⁵ Sodium orthovanadate is a phosphatase inhibitor. A typical effective concentration would be 0.5-5 mM.

⁶ NP40 at 0.1-0.5 % is sufficient to solubilize most membrane lipids. The combination of NP40 and DOC - often used at equal concentrations (e.g., 0.5 % of each) is often used as a more stringent extraction that still leaves many protein-protein interactions intact.

⁷ SDS is a more stringent ionic detergent (CMC 0.1%). It can also be used in combination with the other two detergents SDS/NP40/DOC 0.1/0.5/0.5% as an intermediate stringency buffer.

Table 2: Diagnostic proteins to monitor the quality of the decellularization procedure:

Intracellular Compartment	Diagnostic Proteins
Cytosolic proteins	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
Nuclear proteins	Histones, Lamins, Nucleoporin
Membrane proteins	Integrins, Transferrin Receptor
Cytoskeletal proteins	Actin, Tubulin, Vimentin
Basement membrane ECM proteins	Collagen IV, Nidogens, Laminins
Interstitial ECM proteins (interstitial)	Collagen I, Collagen III, Collagen VI, Fibronectin

For recommendation on antibodies, see our publications⁸⁻¹⁰

Table 3: Volume of reagents to digest ECM-enriched samples into peptides.

Reagents	Preparation	Final Concentration / Amount for 1mm-thick pellet (~5-10mg dry weight)	Volume for 1mm-thick pellet (~5-10mg dry weight)
Ammonium bicarbonate (NH ₄ HCO ₃)	100mM solution in HPLC-grade water	-	-
Urea	8M solution in 100mM ammonium bicarbonate	8M	50µL
Dithiotreitol	Reconstitute in HPLC-grade water at 500mM	10mM	1µL
Iodoacetamide	Reconstitute in HPLC-grade water at 500mM	25mM	2.5µL
Peptide -N-Glycosidase F (PNGaseF)	Commercial solution at 500U/µL	1000U	2µL
Endoproteinase LysC, mass spectrometry-grade	Reconstitute in HPLC-grade water at 0.5 µg/µL	1µg	2µL
Trypsin, mass spectrometry-grade (<i>round 1</i>)	Commercial solution at 0.5 µg/µl	3µg	6µL
Trypsin, mass spectrometry-grade (<i>round 2</i>)	Commercial solution at 0.5 µg/µl	1.5µg	3µL
Trifluoro-acetic acid (TFA)	50% solution in HPLC-grade water	-	2 – 5µL
Acetonitrile (<i>elution</i>)	60% solution with 0.1% TFA in HPLC-grade water	-	500 µL
Acetonitrile (<i>reconstitution</i>)	3% solution with 0.1% TFA in HPLC-grade water	-	100 µL

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Section 1			
Tissue homogenizer: Bullet blender + beads	Next Advance	BB24-AU	http://www.nextadvance.com/api/index.cfm/products.info/c/421/Bullet-Blender
Compartmental Extraction kit	Millipore	2145	http://www.emdmillipore.com/US/en/product/Compartment-Protein-Extraction-Kit-MM-NE-2145
Deoxyribonuclease I	Sigma-Aldrich	DN25	http://www.sigmaaldrich.com/catalog/product/sigma/dn25?lang=en&region=US
Ribonuclease A	Qiagen	19101	http://www.qiagen.com/products/catalog/sample-technologies/dna-sample-technologies/plasmid-dna/rnase-a/
Section 3			
HPLC-grade water	Sigma-Aldrich	34877	http://www.sigmaaldrich.com/catalog/product/sial/34877?lang=en&region=US
Urea	Sigma-Aldrich	U4883	http://www.sigmaaldrich.com/catalog/product/sigma/u4883?lang=en&region=US
Dithiotreitol (DTT)	Thermo Scientific	20291	http://www.piercenet.com/product/dithiothreitol-dtt
Ammonium bicarbonate (NH ₄ HCO ₃)	Sigma-Aldrich	9830	http://www.sigmaaldrich.com/catalog/product/fluka/09830?lang=en&region=US
Iodoacetamide	Sigma-Aldrich	A3221	http://www.sigmaaldrich.com/catalog/product/sigma/a3221?lang=en&region=US
Peptide -N-Glycosidase F (PNGaseF)	New England Biolabs	P0704S	https://www.neb.com/products/p0704-pngase-f
Endoproteinase LysC, mass spectrometry-grade	Wako	125-05061	http://www.wako-chem.co.jp/english/labchem/product/life/Lys-C/index.htm
Trypsin, mass spectrometry-grade	Promega	V5111	https://www.promega.com/products/mass-spectrometry/proteases-and-surfactants/sequencing-grade-modified-
Trifluoro-acetic Acid	Sigma-Aldrich	T6508	http://www.sigmaaldrich.com/catalog/product/sial/t6508?lang=en&region=US
Acetonitrile, mass spectrometry-grade	Thermo Scientific	51101	http://www.piercenet.com/product/acetonitrile-acn-lc-ms-grade
Desalting columns: Oasis HLB 1 cc, 10 mg Sorbent <i>per</i> Cartridge	Waters	186000383	http://www.waters.com/waters/partDetail.htm?partNumber=186000383



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Author(s):

Alexandra NABA; Karl R. Clauser; Richard O. Hynes

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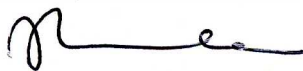
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Response to the editorial comments:

- Authors should put the state in the address.

[We have now corrected this \(see Title page\).](#)

- Manuscript should be edited to typographical errors throughout, particularly punctuation.

[We have checked throughout for such errors and corrected them, when found.](#)

- Second sentence of short abstract should read “for analysis of their composition...”

[We have now corrected this.](#)

- Continuity: 1.2.3-1.2.7: It is not clear why such a large section of the extraction protocol is skipped for filming. It is well within the length limit.

[We can certainly include at least some of the steps \(1.2.2, the first repeat of 1.2.3 and 1.2.5\) in the video if you think there is time. We have now highlighted these steps in yellow in the text \(see page 5\).](#)

- Additional detail is required:

-1.1.1, 1.2.3, 1.2.4, 1.2.6, 1.2.8, 1.2.12: Which protease inhibitors? How much?

[As indicated page 4, the cocktail of protease inhibitors used is provided as a 50X solution with the kit from Millipore. We have now added alternative options in Table 1.](#)

-3.4: The note indicates overnight digestion, but 3.4 only includes 4 hr worth of digestion.

[This was a mistake. We have now corrected this in the manuscript \(see page 7\).](#)

-Line 340: Reviewed in where?

[We have now added the reference in the text.](#)

- Unnecessary branding should be removed:

-1.1.1: Bullet, Polytron (homogenizer); 3.1.1, 3.3, 3.4: Thermomixer; 3.6: Speed-Vac

[We have now removed unnecessary branding from the text.](#)

- Results: If possible, please show that ECM peptides were subsequently identified by mass spec.

[We have extensively reported in previous publications \(cited in the manuscript\) the efficiency of the methods described here at generating ECM peptides. We have now added a comment to the Representative Result section of the manuscript.](#)

- Discussion: What are the future directions and critical steps?

[We have now added comments to the Discussion \(see page 12\).](#)

- References: Journal titles are not abbreviated.

[We have now corrected this and have added, when available the DOIs.](#)

Response to the reviewers' comments

All three reviewers raised questions about the fact that the decellularization protocol described here uses a commercial kit. Despite our repeated attempts, we were unable, for proprietary reasons, to obtain the precise composition of the buffers from the supplier of the kit. However, we have now included in Table 1 notes based on our own experience using home-made buffers to conduct similar extractions. We have also extended the paragraph on “modifications of the protocol” in the Discussion section (see page 11).

Reviewer #1:

Manuscript Summary:

The manuscript by Naba et al describes a methodology for the extraction and enrichment of extracellular matrix (ECM) prior to analysis by mass spectrometry (MS). Overall the paper is well written and presented, although there are a number of points which require either clarification or rationale. This manuscript would be a valuable contribution to the current literature.

Major Concerns:

Abstract: This could be improved by a brief explanation of why we need to study ECM in addition to why this approach is beneficial.

We have now added two sentences to the abstract.

The abstract also mentions the use of different pH buffers, yet I could not see this explained further in the main text.

We have now commented this in Table 1 and in the Discussion.

Introduction: Again, the purpose of these studies was missing. The detailed investigation of disease tissue has potential to identify novel disease biomarkers and this is only briefly mentioned in line 89-90. This could be expanded.

As acknowledge by reviewer #3, we have addressed the purpose of characterizing the composition of ECMs in the introduction.

The comment (L97) regarding the use of fixed tissue is not referenced and it should be more open. Whilst fixation may reduce yield, it does not prohibit identifications and there are a number of studies reporting the MS analysis of formalin fixed tissue following laser dissection. It may well be possible, with further method optimisation, to improve yield from fixed tissue.

Although proteins from formaldehyde-fixed tissues can certainly be analyzed by mass spectrometry, the crosslinking induced by formaldehyde would prevent efficient decellularization. That is why we do not recommend using formaldehyde-fixed tissues for the protocol described in this manuscript. We have now rephrased the sentence in the manuscript for clarification (see page 4).

Protocol:

1. The authors use a commercially available kit but do not comment on the purpose of the different buffers. These all appear to be very similar from Table 1. Could (non-commercial) alternatives be used?

[See our comment above.](#)

2. The article would be improved if rationale for each step could be clearly stated.

[We have added lead-in sentences to address this point.](#)

3. Figure 1 could be improved with direct reference to the steps described in the text (e.g. there is no mention of the nuclear fraction).

[We have now added to Figure 1 references to specific sections of the protocol.](#)

4. L179 indicates the use of 3x Laemmli buffer for the ECM action extraction, presumably to improve solubility. This could be emphasised and explained.

[Extracellular matrix proteins are well known to be highly insoluble and yes, the use of 3X Laemmli buffer, together with a high concentration of reducing agent \(100mM DTT\) contributes to solubilizing ECM proteins prior to SDS-PAGE. We have added a sentence to note this \(see page 6\).](#)

5. Section 3: It should be clearer that the digestion of proteins is an 'in-solution' method rather than an 'in-gel' method. What are the advantages of the 'in solution' technique?

[We have now changed the title of section 3 and added a short paragraph in the Discussion to make this point clearer \(see page 12\). ECM proteins are cross-linked and highly insoluble and, even when resuspended in 3X Laemmli buffer \(containing 6% SDS\) and 100mM DTT, separate poorly on SDS gels. Thus in-gel digestion is not a preferred method.](#)

6. Section 3.3 describes deglycosylation of the sample. It would be useful to know (in the representative results) how this compares to preparations without this step. Does it improve yield as suggested?

[We have added a sentence to explain the purpose of deglycosylation \(see page 7\).](#)

7. The note (L220) could appear closer to Section 3.1 for clarity.

[We have now added a note to Section 3.1.](#)

8. In section 3.5 I am unclear why the digestion is stopped. Do you not want to see complete digestion? Explanation for this step would help.

[This was a poor choice of words. We actually do let the digestion go until completion. We rephrased the sentence to indicate that the acidification inactivates the trypsin \(see page 8\).](#)

9. L230 measurement of the pH with pH paper could result in substantial loss of the precious and small volume sample, depending on the size of the paper. This could be clarified.

We have now added a note indicating the extremely small volume (1-2µl) used to perform the pH test (see page 8) and this will be demonstrated in the video. Note that this amounts to, at most, a few percent of the total sample.

10. In note (L244) it is not clear which steps should be performed in the MS facility- all of section 3?

What are the specific requirements (low contamination, fresh reagents...)?

We apologize for any misunderstanding. In our mind, and that is why we are providing this detailed protocol and video, steps 3.1 to 3.5 can be conducted in any labs. To this end, we have indicated in the introductory note to section 3 that all reagents need to be freshly prepared and listed in Table 2 mass-spectrometry-grade reagents to be used.

The desalting step, however, is often performed at a mass spectrometry facility according to their own preferred methods. We have now rephrased that sentence.

Results and discussion are well written.

Thank you.

A comment about the use of enzymes (e.g. collagenase) to aid tissue disruption would be helpful- what are the advantages and disadvantages?

Collagenase digestion will disrupt the ECM, which we want to avoid. If it were to be used that would need to be monitored carefully (for example by western blot). We now address this point in the Discussion (see pages 11-12).

It would also be helpful to know what are the measures of a successful experiment? Is this % enrichment of ECM proteins? Finally it would be useful to add some detail about the downstream analysis of MS data, although could be a paper in itself.

We have described extensively in previous publications 1) the parameters used to conduct LC-MS/MS and 2) the downstream analysis of MS data and believe, as the reviewer suggests, that discussing this is beyond the scope of this manuscript. However, we have now added a sentence and references to our publications at the end of Section 3 (see page 8).

Minor Concerns:

I would avoid using the term etc and the use of a full stop after min or oC mid-sentence.

We have now corrected this in the manuscript.

Reviewer #2:

Major Concerns: N/A

Minor Concerns:

The manuscript of Drs. Naba et al proposes a mass spectrometry-usable sample preparation for the analysis of extracellular matrix from different tissues. The second version of the manuscript is greatly improved; the changes make the procedure much more easy to follow.

The protocol and the selection of buffers seem to be developed by the authors until one understands that the authors use an extraction kit from Millipore. That needs to be stated upfront. Now, it only becomes clear in the text when one tries to understand the composition of the buffers. Buffer C and N differ only by the inclusion of NaCl for KCl. Why the difference? Why does the exchange of NaCl for KCl make buffer C make more efficient in extracting nuclear components. Why a different pH for buffer CS? It would be helpful for the reader to understand the selection of buffers and reagent to adapt the procedure to possibly other tissues.

[See our comment above.](#)

In testing the various fractions I was missing Western blots for non-collagenous ECM proteins, such as fibronectin and laminin. They may behave quite differently in the extraction procedure than collagen I. [This is a good point. Collagen I, as well as collagen IV and VI are reproducibly insoluble throughout the extractions. In other words, it would be worrisome if they were extracted in the intermediate fractions. As suggested by the reviewer, other matrix components can be more readily “extractable”, this is true for example for the soluble form \(plasma\) fibronectin, this can also be true for laminins which, in some tissues can be retained at the surface of the cells and are not as insoluble as collagen I or III. We now address this issue further in the Representative Results and Discussion sections \(see page 9 and page 11\).](#)

Reviewer #3:

Manuscript Summary and Overall Comments:

The authors of this article describe a detailed protocol for the sequential extraction of proteins from homogenized tissues, resulting in the enrichment of highly insoluble extracellular matrix (ECM) proteins. They then go on to describe a method whereby the enriched insoluble ECM proteins are deglycosylated and enzymatically digested, allowing for their improved characterization by mass spectrometry (MS).

Overall the manuscript is well written, concise and informative. In the introduction, the authors provide clear background regarding the important role played by ECM proteins in several pathologies, as well as the difficulties in comprehensive biochemical characterization of ECM proteins associated with their relative insolubility. The steps laid out in the protocol, as well as the tables listing the various reagents necessary to carry out the methods, appear to be of sufficient detail. In addition, the authors include important comments in their discussion regarding troubleshooting of their methods when preparing tissues with more/less soluble ECM proteins or contaminating insoluble cellular proteins.

[Thank you.](#)

As many standard techniques used for protein preparation/analysis (via MS or otherwise) tend to omit a large proportion of insoluble proteins, such as those present in the ECM, publication of methods such as those presented here are an important step in achieving more comprehensive characterization of the proteins present in both healthy and diseased tissues. I would therefore recommend that this article be

accepted for publication in JOVE with only minor revisions. I have noted additional comments below that the authors should address prior to publication.

Major Comments:

1) While the authors have described an effective method for the enrichment and MS-analysis of ECM proteins, this appears to come at the expense of MS-analysis of many cellular proteins, which become solubilized in detergent-based solutions. Although this is likely acceptable for studies targeting ECM proteins alone, it might preclude more comprehensive MS-analysis of precious tissue samples (i.e. human biopsies). Can the authors comment on any alternative methods (e.g. types of decellularization or sample cleanup) which might allow for analysis of both fractionated cellular and ECM proteins? [As noted by the reviewer, the purpose of this manuscript and video is to highlight a method to characterize the composition of extracellular matrices using proteomics. However, it is worth noting that the composition of each of the “intermediate fractions” generated during the decellularization could be analyzed by mass spectrometry. We have now clarified this in the discussion section \(see page 11\).](#)

2) The authors recommend tissue homogenization prior to the sequential extraction of cellular proteins, which might physically disrupt many ECM protein-protein interactions, resulting in more easily solubilized ECM proteins. Is this step necessary for efficient extraction of cellular proteins? Have the authors attempted a similar protocol using minced tissues instead (perhaps skipping ahead to the more harsh decellularizing detergents), and if so does it result in differences in the proteins detected in the downstream insoluble ECM pellet, or as visualized across the various protein fractions in QC western blots?

[We have not tried to perform the experiment without disrupting the tissues.](#)

3) The bulk of the methods used in the sequential extraction of proteins from tissue homogenates relate to a commercially available kit sold by Millipore. Can the authors provide the precise makeup (i.e. concentrations) of the various extraction reagents used in the kit? And if not, could the authors comment on, or provide references to more specific decellularization techniques so that implementation of their downstream enzymatic methods does not rely on the continued sale of a commercially available product?

[See our comment above.](#)

Minor Comments:

1) The second sentence in the short abstract (lines 39-40) requires editing, as it appears to be missing some words.

[We have rephrased the short abstract.](#)

2) Line 109 is missing the word "to."

[Thank you, we have now corrected this.](#)

3) The authors may want to provide more detail regarding the desalting technique used prior to proteomic analysis (e.g. activating, rinsing, eluting from column; or reference to the product manual).

As indicated in the manuscript, the desalting step is normally conducted at the mass spectrometry facility according to standard procedure and each facility's preferences. We have now added a reference to a previous publication in which we have described in detail the desalting step including materials and reagents used (see page 8). However, other MS facilities will use their own preferred methods, which we have also now indicated in the text (see page 8).

4) In line 344 "results" should be changed to "result."

Thank you, we have now corrected this in the manuscript.