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.