**JoVE manuscript 55051R1, Responses to Reviewers’ comments**

**Editorial comments:**

•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’.

•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.

*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.   
                                             
•JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

*Response*: Done.  
                                                
•IMPORTANT: Please copy-edit 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.

*Response:* Thank you, we have checked again for typographical errors and the corrections are all indicated in the text.  
                                           
•NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.

*Response:* All responses are included in this document.

**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 α5, αv, β1 and β5. Because these are unpublished data we have not included them in the manuscript text.  
  
*Additional Comments to Authors:*  
N/A