Editors and Reviewers Comments Responses

*The authors would like to thank the editors and reviewers for their insightful comments which greatly improved the manuscript. The authors carefully considered editorial and reviewers’ comments and revised the manuscript accordingly. Changes made to the main manuscript are underlined. Moreover, responses to reviewers and their corresponding location in the revised manuscript can be found below.*

**Editorial comments:**  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

*The manuscript has been read thoroughly and all lingual issues were corrected.*

2. 1.1, 2.1, 3.1, 3.2: Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.  
*Steps 1.1, 2.1, 3.1 and 3.2 were revised and rewritten in the imperative tense (Protocol section, steps 1.1, 2.1, 3.1 and 3.2).*

3. 2.1.1: What volume of FBS is used?  
*We typically process 100 mL of FBS at one time, although the protocol could be scaled up or down, depending on user needs. This has been clarified in the manuscript (Protocol section, step 2.1.1).*

4. 3.2.1: Please describe how this is done and specify the concentration of trypsin and the reaction conditions.  
*Step 3.2.1 was further clarified and manuscript was revised accordingly (Protocol section, step 3.2.1).*

5. Figure 2: Please change “hr” to “h” for the time unit.  
*Figure was revised, and hr changed to h throughout the manuscript.*

6. Table 2: Please define SD.  
*SD is the standard deviation. Table 2 was revised accordingly.*

7. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.  
*Authors would like to thank the editors for their comments to improve this manuscript. As a supplement to notes mentioned in the protocol that elaborate on critical steps and considerations the assay user should be aware of, the discussion section was revised to expand on possible sources of variability and ways to address them (Discussion section).*

**Reviewers' comments:**  
  
Reviewer #1:  
  
This manuscript describes the development of an assay to measure two cellular functions simultaneously in a 3D hydrogel culture system without further sample treatments, such as matrix digestion. The assay is an adaptation of a previously published method. Here, sample size is reduced, saving time and volume of reagents used. The system seems to give reproducible results for this A375 cell system. This interesting assay contributes to the development of high throughput analyses of 3D cell culture systems.  
  
The introduction gives a concise background of the topic and establishes the significance of the work. The methodology described here is adequate and seems to be simple, appropriate for general use in cellular studies, and bioengineering. The experiments are well carried out and the results are clear. Overall, manuscript data appear to be correct and thorough.  
  
There are just a couple of questions:  
(1) Since gels are polymerized within the 96 well plate, I understand the hydrogel is stuck to the bottom of the well. Then, is it important that some parts of the gel are not equally exposed to media? Especially for longer term experiments? And is this assay still reproducible at later time points?

*PEG is a very hydrophilic material, with the final hydrogel composed of greater than 95% water, enabling rapid diffusion with the surrounding media and no loss of cell viability near the bottom of the well. The timing of the assay is dependent on cell type and cell density, at later time points all of the fluorogenic MMP peptide will be cleaved and the signal will plateau. Therefore we recommend using the cell density optimization experiment described here to determine the appropriate cell density to be within the linear range of the assay at the 24 hour time point. We have more clearly addressed this in the text (Discussion section, 2nd paragraph).*

(2) Minor comments: line 111, LAP initiator is also commercially available (since this is pointed out for Irgacure) and line 196, please describe lamp.  
*The manuscript was revised and LAP was noted as commercially available (Protocol section, step 1.3). The lamp description was added to the manuscript (Protocol section, step 3.2.6), model number (95-0006-02) is listed in the materials list table.*

Reviewer #2:  
  
Manuscript Summary:  
The work describes the protocol for detecting MMP activity in cell-laden hydrogels formed by thiol-ene photopolymerization.  
  
Major Concerns:  
No major concern.  
  
Minor Concerns:  
Line 61: "poly (ethylene glycol)' should be revised as 'poly(ethylene glycol)'.

*Manuscript was revised accordingly.*

Line 64: Please specify which MMPs were the target of analysis.

*The MMP-degradable peptide sequence used here for the fluorogenic sensor was previously developed for specificity to MMP-11 and MMP-14 (Mucha et .al 1998). This has been clarified in the manuscript (Results section, 1st paragraph).*

Line 71: 'phenomenon' should be replaced with 'phenomena'.  
*Corrected.*

Line 72: 'necessitate 3D in vitro systems for further study' should be rewritten as a separate sentence.  
*Manuscript was revised and recommended changes have been made (Introduction section, 2nd paragraph).*

Line 85: 'described' should be replaced with 'required'.  
*Corrected (Introduction section, 3rd paragraph).*

Line 101: Please specify the type of fluorophore used.  
*The fluorogenic peptide was labeled with fluorescein (fluorophore) and dabcyl (quencher). The manuscript was revised to include this information (Protocol section, step 1.1, Results section, 1st Paragraph, and Figure 1).*

Line 117: Since the hydrogels were also crosslinked by MMP sensitive peptide, please comment on whether there is competition between cleavage of MMP-sensitive linker vs. cleavage of MMP-sensitive fluorescent probe.  
*There may be competition between the cleavage of the MMP-degradable crosslinker and fluorescent MMP-degradable sensor. However, in a separate manuscript currently under revision, we have found experimentally that varying the amount of crosslinker did not affect cleavage of the fluorescent sensor across a range of collagenase concentrations.*

Line 196: Please specify the type of UV light source and wavelength.  
*The lamp specifications (UVL-56 Handheld UV Lamp, UVP, Upland, CA) were added to the manuscript (Protocol section, step 3.2.6). The UV light source produces UV-A light at a long UV wavelength (365nm).*

Lines 279-281: Please rewrite these two sentences. It's not clear what the authors mean by 'limited in measuring cellular functions'/  
Using 3D culturing systems to measure cellular function has been challenging due to the difficulty of cellular retrieval and limited cell number, limiting subsequent biological assays in the type of assay and/or the number of possible conditions. This is now more clearly described in the discussion *(Discussion section, 1st paragraph).*

Line 297: Please discuss why the readings have high variation at lower cell density.  
Figure 1B: A collagenase activity 'standard curve' should be established (not just 0, 10, 1000 ug/mL).

*At lower seeding densities, cells produce weak signal that overlaps with the background noise. This overlap creates variability within triplicates and between experiments on different days.*

*A standard curve was performed for the MMP sensor using a range of collagenase concentrations (0 to 2000 µg/mL) (Figure 3). From this curve, the working range was calculated as, three standard deviations above the minimum (background) and below the maximum detected signals. Sample signals that fall within the working range can be considered statistically significantly different from background noise with 99.7% confidence. Hydrogels incubated with collagenase enzyme at 0, 10 and 1000 µg/mL are used as positive controls in the cell density experiment (Figure 4) to indicate where the signals produced by cells fall within the working range of the assay. Manuscript was revised and necessary changes were made to reflect the discussed issue (Discussion section, 2nd paragraph).*