We wish to thank the reviewers and editor for their careful critique of our manuscript. We are encouraged by the positive remarks, and found the criticism very helpful in strengthening the impact of our work and the clarity of our manuscript. We have revised the manuscript to address the reviewers’ comments. The changes are highlighted in the revised manuscript. The point-by-point answers to the reviewer comments are provided below:

**Changes recommended by the JoVE Scientific Review Editor:**  
  
• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

This has been addressed.  
  
• **Protocol Language:** Please ensure that ALL text in the protocol section is written in the imperative tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.  
  
Example NOT in imperative tense: “PEGDA is weighed out and then dissolved in phosphate buffered saline”.  
  
Example in imperative tense: “Weight out PEGDA and dissolve it in phosphate buffered saline.”

This has been addressed.  
  
• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more details to the following protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure that all additional details in the protocol section are written in the imperative tense, as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.).  
1) Line 146: Please provide column MWCO, duration for separation etc.

We have added this information. 5KDa MWCO and a reference.

2) Line 148: Please add a reference for Ellman’s assay.

I have added this reference.

3) Line 155: How much solution?

Typically, 800~1000 ul, this has been added to the text.

4) Line 166: Where is the punch hole made?

“Punch out multiple hydrogels from the gel rectangle to serve as replicates and control samples.”

5) Line 175: How is the photomask prepared?

I added a step (5.1) to include how to design the photomask and prepare it for use.

6) Line 178: Is the line light the same as in previous steps?

Yes, I have made this clear in the text.

7) Line 188: What is the growth medium used? What volume to you recommend?

Medium is EGM-2 without growth factors at volume of 250ul for a 48 well plate. This has been added to step 6.1

8) Section 6: There are no steps describing cell seeding on the hydrogel, please add them and include cell concentration per hydrogel.

I have added step 7.5 to include this.  
  
• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE’s 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. There must also be a one-line space between each protocol step.

This has been corrected.  
  
• **Protocol Highlight:** 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, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight 2.75 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps that match the title and abstract. Please see JoVE’s 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.  
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.  
2) Some of your shorter protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.  
3) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.  
4) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length (2.75 pages or less). Please see JoVE’s instructions for authors for more information.  
5) Notes cannot be filmed and should be excluded from highlighting.

I have highlighted sections that is content for filming. Sections: 1, 3, 5, 6, 7  
  
• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

I have added and edited discussion to address each of these topics listed above.

• **Figures:**  
1) Please provide each figure as an individual PDF, TIFF, JPEG or PNG files.

This has been done.

2) Please remove the text “Fig.#....” from all figures.

This has been fixed.  
  
• **Tables:** Please upload each table as an individual Excel file, instead of embedding in the manuscript doc.

This has been addressed.  
  
• **Figure/Table Legends:** Please place legends directly below the Representative Results text. This has been moved.

• **References:** Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi:DOI (YEAR).]  
1) Please abbreviate all journal titles.  
2) Please include volume, issue numbers, and DOIs for all references.

This has been completed. Unsure the format to cite manufacture’s online protocols (see reference numbers 15-18).

• **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an in INDIVIDUAL xls/xlsx file. Please include items such as reagents, protein, columns, filters, cells, cell culture media, etc.  
This has been provided in the excel spreadsheet.

• Please define all abbreviations at first use (e.g. LAP).

Yes, this has been corrected.

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

Yes, this has been corrected  
  
• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

All figures are original.

**Comments from Peer-Reviewers:**   
  
**Reviewer #1:**  
*Manuscript Summary:*  
This manuscript is well organized and clearly presented. All the steps are described and easy to follow.

**Reviewer #2:**  
*Manuscript Summary:*  
The paper described a photo pattern technique that allowed proteins to be patterned on hydrogel surface.  
  
*Major Concerns:*  
None  
  
*Minor Concerns:*  
1) the full name of PEGDA and LAP should be provided when they are first mentioned in the paper (line 90).

This has been corrected.

2) Line 172, "small volume of protein solution", it's not obvious what protein solution this is. I presume it is 2-iminothiolane-treated solution. It would be much clearer if the authors can specify it.

Yes, this was unclear. I have corrected it to say “pipette a small volume of thiolated protein solution”.

3) There are a few minor grammar mistakes throughout the text. e.g. line 218, "volume is should just", line 225 "UV exposure time for should total ....", line 231, "unable create immobilized...", etc.

I have corrected these grammar mistakes along with others I found during edits for resubmission.   
  
*Additional Comments to Authors:*  
N/A  
  
**Reviewer #3:**  
*Manuscript Summary:*  
This manuscript describes a protocol to create a layer of patterned protein on biocompatible PEG hydrogel. The PEG gel incorporated fibronectin to support cell proliferation, and the stiffness of the gel is tunable. The patterned protein can potentially modulate the cell migration/differentiation. This work gives a general method to control the cell behavior via applying spatial biochemical cues.  
  
*Major Concerns:*  
Lack of experimental details  
We added more details in the revised manuscript.

*Minor Concerns:*  
N/A photochemistry   
  
*Additional Comments to Authors:*  
1. Line 68, the description of "multiple photopolymerization steps" is not accurate, it's photopolymerization followed by click chemistry.

Yes, I agree with this correction of word choice. I corrected the sentence to read “To create bioactive hydrogels, a photopolymerization hydrogel forming step is followed by a thiol-ene bioconjugation reaction.

2. The authors should give more significance in protocol, such as:  
a. Line 104, what's LAP's function

I added a sentence to state LAP is the photoinitiator to initiate the polymerization reaction of PEGDA.

b. Line 98, why having fibronectin in precursor solution

I added a sentence to state fibronectin is added to allow cells to attach to the PEG hydrogel.

c. Line 127, what's the sources of sulfhydryl and why it need protection

I changed the diction and added a sentence. EDTA is important as it protects from thiols forming disulfide bonds. Thiol groups must remain in their reduced form to allow for the reaction to occur.

d. Line 171, protein patterning, what's the objective to introduce another protein (i.e., growth factor) besides fibronectin.

I added the following sentence: “Add the thiolated protein to the surface of the hydrogel for surface patterning. The objective of introducing this protein is to examine how cell respond to its embolized spatial signal.”

3. The authors should give more experimental details  
a. Line 146, details about purify protein using desalting column

I added the following sentence: “Add the thiolated protein solution, prepared in step 2, to the surface of the hydrogel for surface patterning. The objective of introducing this protein is to examine how cell respond to its spatial signal.”

b. Line 141 and 142, the ratio range 2-20 and 1-8 is kind of too broad, what's the ratio in this work?

The 2-20 ratio is a reference from the manufacturer’s protocol. I have removed that statement and added a reference to the manufacturer’s protocol. I have made it more clear what was used in this work. In this work, 8:1 molar ratio was used.

c. Line 203, seeding density

Yes, I have added the seeding density of 75,000 cells /cm2.

**d. The protocol of getting results of Figure 2 and Figure 3**

I have added an additional section for rheology testing of PEG hydrogels (Step 4) and evaluation of bioactivity (Step 8).

4. Line 225 and 226, the content in brackets should be "Figure 2B" and "Figure 2C", respectively.

Yes, this has been corrected.