*The authors thank the editors and reviewers for their thoughtful comments. We have carefully considered the contents of the review and have taken steps to improve the manuscript based on these insights. Included below are detailed responses to each comment, in addition to the location of the changes. All changes have been underlined in the revised copy of the manuscript.*

**Editorial comments:**

Changes to be made by the Author(s) regarding the written manuscript:

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

*We have reviewed the manuscript and made any necessary corrections.*

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

<https://www.future-science.com/authorguide/archivesharearticle>

*Above is a link detailing the re-print permissions for authors that are attempting to utilize figures previously published in Biotechniques, the source of some of the figures used in this manuscript. The permissions indicate that “use [of] all or part an article and abstract in personal compilations or other scholarly publications of their own work” is permitted “provided that authors give appropriate acknowledgment to the journal and publisher, and cite the full bibliographic reference for the article”*

3. Please provide an email address for each author.

*These have now been included on the title page.*

4. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

*The authors thank the editor for their suggestion. We have rewritten the short abstract to more clearly emphasize the protocol and its applications. Underlined revisions can be found on page 1 of the manuscript.*

5. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

*We have amended the introduction to clearly state the goal of this method. Underlined revisions can be found on page 2 of the manuscript.*

6. Please spell out each abbreviation the first time it is used.

*All abbreviations have been defined at their first use.*

7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

*All personal pronouns have been removed from the protocol.*

8. 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.

*The protocol has been amended to reflect the changes described above.*

9. Please add more details to your 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 you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:

2.5: Please specify the size of the syringe needles used in this step.

*The size of the needle has been added to the protocol step on page 3.*

2.6: Which needle is removed, the one connected to the inert gas tank?

*The authors have specified which needle to remove on page 3.*

5.2.2: Are the incubation conditions the same as in 5.2.1?

*Details on the humidified chamber used in 5.2.2 were added on page 5.*

5.2.3: Please mention how to collect the samples and how to concentrate using a centrifugal filter. What are the centrifugation parameters?

*Details on the centrifugation parameters have been added to step 5.2.3 on page 5.*

5.4: What type of plate is used here?

*This step in the protocol is now 6.2. The plate referenced here is the peptide zymography gel.*

5.8: Please give an example of the filters and fluorophore used in this step. Please combine 5.8 and 5.8.1.

*These two steps in the protocol have been combined into step 7.1. An example of the filters used in this step has been added on page 6.*

10. Please include single-line spaces between all paragraphs, headings, steps, etc.

*The authors have confirmed that all spaces are single line spaces.*

11. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

*Please see response to #13.*

12. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

*Please see response to #13.*

13. Please include all relevant details that are required to perform the step in the highlighting. 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 highlighted.

*The authors have highlighted all the steps in the protocol that they wish to be included in the filming process.*

14. Figure 1: Please change “Hour/Hours” to “h” and include a space between numbers and their temperature units (4 °C, 37 °C).

*The units have been adjusted accordingly to the editor’s suggestions.*

15. Figure 2: Please label panels A and B. What is the unit for the numbers in the left panel? Please change “n=3±SD” to “n=3, mean ± SD” or remove it from the figure since this has been defined in the figure legend.

*The labels for panels A and B have been added to figure 2. The units for the molecular weight labels have also been added. “n=3±SD” has been removed from the figure.*

16. Figure 3: Please provide the unit for the numbers in the left two panels.

*The units for the molecular weight labels have been added.*

17. Table 1: Please make the number 2 in H2O a subscript.

*The number 2 has been made a subscript throughout Table 1.*

18. Discussion: Please describe any limitations of the technique.

*The authors thank the editors for their suggestion. We have added to the discussion section in order to describe some of the limitations of the technique on page 8 of the manuscript.*

**Reviewers' comments:**

Reviewer #1:  
  
Manuscript Summary:  
The authors developed a fluorescent peptide zymography method to detect a wider range of proteases in complex biological samples. Although the application of zymography has been widely reported for sensing of various proteases, the present method with several tunable peptides demonstrated enhanced sensitivity and expanded the library of detectable targets. Furthermore, in terms of proteases detection, the work is technically important.  
  
Major Concerns:  
1. In the introduction part, the author said "Furthermore, the high tunability of our method allows for the simultaneous detection of numerous proteases, a result that was previously unattainable by traditional, native substrate zymography". Then how to design the specific substrate peptide which covalently couple to the polyacrylamide polymer network in multiplex detection?

*We recognize this was not clear in the original manuscript and have clarified how we develop peptide substrates in the introduction on page 2 based on commercially available MMP substrates.*

2. In protocol 5.5, "until the molecular weight standard indicates that the proteases of interest are within the peptide resolving gel layer", how to judge whether the proteases of interests are within the peptide resolving gel layer?

*Please see our reply to question 3 below.*

3. How to ensure that numerous proteases of interest are all within the peptide resolving gel layer when multiple proteases with relatively large molecular weight differences are detected simultaneously?

*Given known protease molecular weights (35-100 kDa for most MMPs), the molecular weight standards can be used to estimate when the proteases of interest are within the peptide resolving gel layer – which has a visible orange color. While we describe a two-layer resolving gel approach in this manuscript to save on reagent use, the entire resolving gel could be made with the fluorogenic peptide substrates or the size of the peptide resolving gel layer can be adjusted to accommodate all proteases of interest. We have clarified these points in the protocol on page 5, step 6.2.*

Minor Concerns:  
1. In protocol 5.7, "Incubate gels at 37 °C under gentle agitation for 24 hours", how to control the area of the enzyme reaction? How to reduce the dispersion of enzyme bands in 24 hours?

*The authors thank the reviewer for their question. All of the steps following electrophoresis of the peptide zymography gels are similar to those in traditional gelatin zymography. As such, the gel should be fully submersed within the developing buffer solution during the overnight incubation. Any proteases that become activated under the development conditions and are capable of cleaving the incorporated substrate can then be detected by fluorescent imaging. The authors have clarified this on page 5.*

2. In figure 3A and 3C, there are two bands of MMP-9. In figure 3A, B, C, there are more than two bands of HT 1080 and MDA-MB-231, yet there is only one band in D, E and F. Please explain the reason for this result.

*Figure 3A, B, C correspond to gels made with the LACW peptide and Figure D, E, and F correspond to gels made with the QGIW peptide. Because of the difference in peptide sequence, each peptide will have a different sensitivity to each protease, as demonstrated in Figure 2. In this figure it is possible that the QGIW is either not cleaved by both forms of MMP-9 or not detected due to the lower sensitivity of the QGIW peptide to MMP-9 (Figure 2). We have added text clarifying this issue.*

Reviewer #2:  
  
Manuscript Summary:  
Zymography is a useful technique that is inexpensive and does not require antibodies as the authors mention. Technique described here of using crosslinked fluorogenic peptides could be a helpful addition to the field.  
  
Major Concerns:  
None

Minor Concerns:  
Authors state that zymography has suffered due to a limited number of degradable substrates available for proteases/enzymes to work on, but it might be a bigger limitation for renaturing and refolding proteins into their native/active conformation after the electrophoresis and partial denaturation by SDS.

*The authors thank the reviewer for their recommendations. We recognize this limitation in zymographic techniques in general and have now acknowledged it in the discussion on page 8.*

Reviewer #3:  
  
Manuscript Summary:  
This manuscript describes detail methodology of results previously published in BioTechniques (2018). Except for the abstracts (see below), manuscript is well-written and all essential information (materials, equipment, and methods) are included. Below are concerns (mostly minor) that authors should take into consideration when revising.  
  
Minor Concerns:  
  
1. I have some concerns about the title of the manuscript. Clearly, the method detects the protease activity not the proteases themselves. I suggest that title be changed to "Detection of protease activity…"

*The authors believe that due to the limitations on zymography for renaturing and refolding proteins as well as separating MMP-inhibitor complexes, “protease activity” can, in some sense, be misleading. However, the authors also recognize that we do not directly measure protease expression and therefore, we have amended the title of the manuscript to reflect that.*

2. Both the short and long versions of the abstracts are poorly written. Both of them need to be re-written.  
  
Abstract: The main texts are easy to read, but for some reason (written by different person?), the abstracts are written in cluttered sentences and even wrong grammar. For example, the last sentence "Fluorescent peptide zymography with several tunable peptides demonstrated enhanced the sensitivity and expanded the library of detectable proteases by zymographic techniques" needs to be corrected. Also, "tunable peptide" is not very clearly defined. What is "highly transient nature of physical entrapment?" Why not just say that the small molecules diffuse out of the gel easily? Instead of "This technique was applied to the detection of proteolysis by purified enzymes and conditioned media… by electrophoresis" authors can just say "This new type of gel was used to run purified enzymes and conditioned media, and the protease activity was detected by direct fluorescence of the protein band without staining." Authors should strive to write clear sentences.

"Copolymerization" typically represents two monomers making a co-polymer, and it suggests that the multiple components are covalently incorporated into the polymer chain. Therefore, readers will likely mis-interpret "copolymerization of short, fluorescent peptide" as peptide (which has polymerizable moiety) covalently conjugated to polymer during the polymerization process. Therefore, I suggest replacing "copolymerization of peptide…" with "incorporation of peptide during the polymerization (gelation) process."

Short abstract: First sentence should be how fluorescent peptide zymography was produced.

*The authors thank the reviewer for their suggestions. The long and short abstracts have been re-written to provide a clearer summary of the method and key findings. Furthermore, copolymerization has been replaced with “incorporation” in the long abstract and throughout the manuscript. These revisions can be found underlined on page 1.*  
3. Azido-PEG3-maleimide is not a cross-linker. Traditionally, the term "crosslink" is used for describing connection between polymer chains. The utility of azido-PEG3-maleimide is conjugating peptide to the polymer backbone. Therefore, grafting molecule or linker molecule is more appropriate.

*The authors thank the reviewer for this observation. We have replaced any references to azido-PEG3-maleimide as a “crosslinker” to a “linker molecule” throughout the manuscript.*   
  
4. Vial 2 is introduced abruptly without introducing vial 1. Make sure to describe both solutions (or both vials). Also, it would be helpful to clearly list what is in the two vials.

*A description of the contents of both vials has been introduced on page 4.*  
5. It is unclear how azido group adds to the polymer chain during the polymerization reaction. Authors mentioned this in the original BioTechnique paper. I wonder if authors still believe in their original conjecture that azido group adds to the amino side chain. I think it is much more likely that a radical reaction to the azido group results in incorporation of the peptide directly on the polymer backbone (not side chain), since azido can partake in radical reactions. Amino group, on the other hand, is known to be inert toward clink chemistry (hence click reaction is used a lot in bioconjugation in the presence of amino groups). Authors can take this opportunity to re-think and perhaps provide more likely scenario for covalent link between peptide and polymer backbone (which is the main idea behind this work).

*We agree that it remains unclear within the literature and the field how the azido group reacts with the acrylamide/poly-acrylamide. However, data from our previous work (Deshmukh et al. Biotechniques 2018), indicates stable incorporation of the fluorogenic peptide only upon inclusion of the linker molecule. Upon further thought and review of the literature, the mechanism may be what the reviewer suggests or possibly a mechanism in which*

*1) hydrolysis of the amide occurs followed by 2) the Schmidt reaction. Amide groups within the polyacrylamide structure can be hydrolyzed to carboxylic acids under the basic conditions of the polyacrylamide solution. This is followed by a reaction between the azide functional group in the linker and the carboxylic acid. However, because both these mechanisms remain speculative without literature support specifically about the azido/acrylamide reaction, we have taken out all mentions of the chemical mechanism beyond that the fluorogenic peptide is stably incorporated.*

6. For the polymerization reaction of the peptide resolving gel, I wonder if there is any effect on whether peptide was added first and allowed to react to the linker molecule before polymerization or it was added directly to the polymerization reaction. This is because the thiol group of cysteine is known to be a good chain transfer agent and that it can reduce the MW of the polymer.

*This is an intriguing possibility. We have generated zymogram gels in which 1) we pre-incubate the peptide with the linker and then add this to the acrylamide solution prior to polymerization or 2) the linker and peptide are added directly to the acrylamide solution prior to polymerization. We have observed no differences in how far the samples or ladder migrate during electrophoresis with either approach – which would be one indication of changes in the polyacrylamide network. We did, however, observe increased sensitivity to a concentration gradient of MMP-9 with approach 1 (see below). However, since we have carefully optimized approach 2, found this protocol to empirically work with many different kinds of samples, and either approach does not significantly change the experimental results, we would prefer to describe the original approach (#2) throughout the protocol and results. We are including the results here for appraisal by the reviewers and editors, but we believe these results may be distracting to the reader if included in the protocol and not provide additional benefit. If advised by the editor, we can include this data in the manuscript.*

T:\Labs\Leight\Shared Lab\2-Publications\JOVE- Peptide Zymography\Figures\MMP-14 Peptide Pre-Incubation\MMP-14 pre incubation_MMP9 Concentration Dependence1.tifT:\Labs\Leight\Shared Lab\2-Publications\JOVE- Peptide Zymography\Figures\MMP-14 Peptide Pre-Incubation\MMP-14_MMP9 Concentration Dependence1.tif

Approach 2: Direct addition of LACW Peptide and azido-PEG3-maleimide linker to the polyacrylamide solution

Approach 1: Pre-Incubation of LACW Peptide w/ azido-PEG3-maleimide linker prior to addition to the polyacrylamide solution

|  |  |
| --- | --- |
| **Approach** | **EC50** |
| Approach 1- Pre-incubation | 18.02 |
| Approach 2- Direct addition | 28.06 |

T:\Labs\Leight\Shared Lab\2-Publications\JOVE- Peptide Zymography\Figures\MMP-14 Peptide Pre-Incubation\MMP-9 Titration.tif7. There is no information about the sample buffer and running buffer. If it is conventional buffers, it needs to be explicitly said so in the manuscript.

*Details about the contents of the sample and running buffers have been added to the protocol on page 5.*  
8. In the representative results section, the peptides should be written from N terminus to C terminus (Cystein on the left side).

*The authors have reviewed the orientation of the peptide and would like to note that the cysteine is in fact in the C-terminal position. Therefore, the orientation of the notation is correct.*  
9. Line 291: "in to" should be "into"

*These changes have been made as per the reviewers request*