Response to Editor and Reviewer comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

**We have re-read through the manuscript and made corrections to spelling and grammar.**

2. Figures: Please change the unit “µM” to “µm”.

**Figures have been corrected.**

3. Figure 2: It would be helpful to label the time when the images were taken.

**Time points were added.**

4. Please provide an email address for each author.

**Email addresses have been added.**

5. 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 …”

**We have rephrased the short abstract.**

6. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

**The long abstract has been rephrased.**

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

**The introduction has been rephrased with a clear statement of the overall method goal.**

8. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

**Spaces have been added between all numbers and units.**

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

**We have revised the protocol to remove personal pronouns.**

10. 1.2.1: Please change “μM” (concentration unit) to “μm” (length unit).

**Changed.**

11. Representative Results: Please refer to all of the figures.

**All figures are now referenced in representative results.**

12. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:  
a) Critical steps within the protocol  
b) Any modifications and troubleshooting of the technique  
c) Any limitations of the technique  
d) The significance with respect to existing methods  
e) Any future applications of the technique

**We have revised the Discussion section to address these points more clearly.**

13. References: Please do not abbreviate journal titles.

**Journal titles are no longer abbreviated.**

Manuscript Summary:  
This manuscript outlines a novel and interesting approach to the labelling of neutrophils in vitro and in vivo. TH manuscript outlines each step in the procedure in a fixed tissue assay and in a live cell activation assay.  
  
Major Concerns:  
  
The paper spends too much time discussing protocols such as tissue fixation and freezing which are not particular to this protocol. The steps up to 1.2.3. feel unnecessary unless there is a specific reason that the steps must be carried out like this for the MUB40 to work correctly. This is the same for the neutrophil isolation procedure, (steps 2.1 - 2.3.8) while interesting, that is not the focus of this method.

**While we agree that covering steps 1.1-1.2.3. are not essential for MUB40 staining to work correctly, we believe that they will help the reader be able to perform MUB40 staining as we have described in all of our papers. This will help the reader be able to replicate our staining procedure exactly, if they so choose. We have added several statements explaining that initial fixation and tissue freezing procedures are open to modification.**

**Steps 2.1-2.3.8. are crucial to obtain live neutrophils that are in an inactive state. Exposure to oxygen during purification will shorten the duration of time that the neutrophils can be used before they become activated and stain with MUB40.**  
  
More time could be spent discussing different concentrations of the probes which could be used for different purposes and most importantly the specific detection parameters which would be optimal for the detection of MUB40. While this is of course specific to each individual microscope, giving an idea of the excitation/emission wavelengths would be beneficial.

**We have added a figure showing the affect of different concentrations of the probes on neutrophil staining. This is now Figure 3 and is referenced in the protocol section and representative results sections.**  
  
The authors mention the probe can be used for in vivo detection, this would be an extremely interesting example to present as this methodology would be far more challenging, what are the concentrations to be used in vivo, what is the percentage of labelling?

**We are still working to fine tune the *in vivo* imaging protocols and it will require a much greater amount of resources/time to complete.**  
  
For both the fixed tissue and the activation assay it would be helpful to add an additional marker to identify the non-activating neutrophils for comparison.

**For fixed/permeabilized tissue, all neutrophils are stained due to the permeabilization step. For the live neutrophil staining, the addition of a "second" antibody based marker is itself an "activating" signal which defeats the purpose of the MUB40 stain.**   
  
Minor Concerns:  
  
In the reviewer copy of the PDF, the figure legends were missing.  
  
1.1.1/1.1.2 Are both sucrose steps essential?

**For optimal results, both sucrose steps are essential.**  
  
1.1.3 Is there a more specific way to describe the freezing, what temperature for the ethanol?

**We have added the temperature of the dry-ice ethanol bath as -72 C.**  
  
1.2.3. Is sapoinin essential or can other detergents such as Trition be used?

**Other permeabilization techniques can be used. This has been referenced in the protocol.**  
  
2.2.1. Are the 'blood cells ' in this line referring to the plasma above? This is a bit unclear

**We have reworded step 2.2.1. to better explain that the tubes in question are from step 2.1.2. and are in reference to the sedimented red blood cells.**

Reviewer #2:  
  
Manuscript Summary:  
The manuscript written by Anderson et al. and submitted to JOVE, describes a novel peptide that labels extracellular lactoferrin and marks the sites of neutrophil activation with a fluorescent signal. This work presents an innovative method that has the potential to detect neutrophil activation in vitro and in vivo and to help to better understand neutrophil functions. Please find my major and minor comments below:  
  
Major Concerns:  
- the authors describe the use of the MUB40 peptide on PFA-fixed tissue samples. According to the protocol, MUB40 has to be added after fixation of the sample by repeated exposure to higher concentrations of sucrose and after its slicing in OCT medium. Lactoferrin will not be enzymatically functional in these samples at the time of peptide addition, so how will be there fluorescent signal generated on fixed tissues? My understanding was that MUB40 has to be enzymatically cleaved by lactoferrin to become fluorescent.

**MUB40 binds to a glycosylation moiety present on lactoferrin. MUB40 does not require any enzymatic cleavage to work. The sucrose wash steps are used to remove excess PFA and to add "volume" to the fixed cells which helps to maintain the integrity of the tissue.**

- fixation could also lead to permeabilization of cells in a fixed tissue specimen. Chances are, the MUB40 peptide can penetrate into cells in fixed samples and stain lactoferrin in non-activated neutrophils, as well. How sure is it in vivo that the peptide only marks extracellular lactoferrin informative of activated neutrophils?

**It is possible that fixation steps lead to permeabilization of cells. In our protocols we always ensure that all cells are permabilized by using a gentle saponin treatment after fixation. We have not tested whether PFA fixation alone is sufficient to permeabilize all cells.**

**We have not seen any evidence that MUB40 can cross lipid bilayers of live intact cells. Thus we believe that only permeabilization/activation allows access of MUB40 to its lactoferrin target.**

- figure 2 images are of bad quality and should be replaced with images with higher resolution

**We have recompiled the image at 600 dpi.**  
  
Minor Concerns:  
- last word in line 42, correct: "azurophil"

**Corrected.**