Journal of Visualized Experiments

The MUB40 Peptide can be Used to Detect Neutrophil Mediated Inflammation Events

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58367R3
Full Title:	The MUB40 Peptide can be Used to Detect Neutrophil Mediated Inflammation Events
Keywords:	MUB40; Peptide; Retro-Inverso; Neutrophil; inflammation; biomarker; Lactoferrin; in vivo
Corresponding Author:	Mark Charles Anderson Institut Pasteur Paris, FRANCE
Corresponding Author's Institution:	Institut Pasteur
Corresponding Author E-Mail:	mark.anderson@pasteur.fr
Order of Authors:	Mark Charles Anderson
	Louise Injarabian
	Benoit S. Marteyn
	Regis Tournebize
	Antonin Andre
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	28 rue du Dr Roux, Paris, France, 75015

TITLE:

The MUB_{40} Peptide for Use in Detecting Neutrophil-Mediated Inflammation Events

AUTHORS & AFFILIATIONS:

Mark C. Anderson[1,2], Louise Injarabian[1,2,3], Antonin Andre[1,2,3], Regis Tournebize[1,2,4], Benoit S. Marteyn[1,2]

[1]Institut Pasteur, Unité de Pathogénie Microbienne Moléculaire, Paris, France

[2]Institut Pasteur, INSERM Unité 1202, Paris, France

[3]Université Bordeaux Segalen, Institut de Biochimie et Génétique Cellulaires, Bordeaux, France

[4]Institut Pasteur, Unit of Technology and Service Photonic BioImaging, Paris, France

Corresponding Author:

Benoit S. Marteyn

benoit.marteyn@pasteur.fr

KEYWORDS:

MUB_{40}, peptide, retro-inverso, neutrophil, inflammation, biomarker, lactoferrin, *in vivo*

SUMMARY:

Here, we present a protocol to detect the presence of neutrophils in fixed/permeabilized histology sections and assess the activation state of live purified neutrophils. In particular, the MUB_{40} peptide binds lactoferrin present in neutrophil-specific and tertiary granules. Exposure of the granule contents through either permeabilization or neutrophil activation allows for the marking of neutrophils.

ABSTRACT:

Here, we provide a protocol involving the use of MUB_{40}, a synthesized peptide with the ability to bind glycosylated lactoferrin stored at high concentrations in specific and tertiary granules of neutrophils. This protocol details how MUB_{40} conjugated directly to a fluorophore can be used to stain neutrophils in fixed/permeabilized tissues as well as how this can be used in live-cell imaging to assay for neutrophil activation and de-granulation. Neutrophil detection methods are limited to species-specific monoclonal antibodies, which are not always suitable for certain applications. MUB_{40} does not penetrate the cell membrane and is thus excluded from lactoferrin stored in non-activated/non-permeabilized neutrophils. MUB_{40} has the added benefit of recognizing lactoferrin from a broad host range, making it especially useful for comparing results in studies involving multiple research models, reducing the number of duplicate reagents, and simplifying protocols through single-step staining.

INTRODUCTION:

Neutrophils are one of the primary arms of the innate immune system and are routinely recruited to the sites of inflammation around the body. The study of neutrophils has been largely impaired by their short lifespan *in vitro* (less than 8 h) and by limited detection tools under basal conditions or after activation. Here, we present a well-tested protocol for the broad and specific detection of mammalian neutrophils in fixed/permeabilized samples. We also provide a detailed protocol

for staining live neutrophils with MUB_{40}. Using the live neutrophil staining protocol, the timing and location of neutrophil activation can be pinpointed. This protocol is ideal for researchers who wish to study neutrophil activation or granule release. Beyond their antimicrobial functions, neutrophils are now appreciated as immunomodulatory cells involved in a wide range of diseases and immune responses (innate and adaptive)[1,2]. Neutrophils are present in most inflammatory tissues, at high numbers in infected tissues, in inflammatory tumors[3], during IBS and Crohn's flare-ups[4], and in areas of non-infectious inflammation such as the synovia of Rheumatoid Arthritis (RA) patients[5]. Neutrophils contain four classes of pre-formed granules named azurophil (α), specific ($\beta1$), tertiary ($\beta2$) granules, and secretory vesicles (γ)[6]. Upon migration to an inflammatory site, recruited neutrophils become activated and sequentially secrete granule contents (composed of adhesion, antimicrobial compounds and immunomodulatory molecules), which promotes further recruitment and contributes to infection resolution (but also to host tissue damage)[7]. While neutrophils are considered a critical aspect of innate immunity, to date there are few detection reagents available to study them, and even fewer that can be used to assess the activation state of living neutrophils.

Current methods of detecting neutrophils rely on monoclonal antibodies generated against cell-surface exposed antigens, such as Ly-6G[2] or proteins specifically stored in neutrophil granules under basal conditions (*e.g.,* myeloperoxidase, lactoferrin). Advantages of monoclonal antibodies include their strong binding, sensitivity, and versatility under various assay conditions. However, there are several downsides to using monoclonal antibodies and anti-Ly-6G in particular. These downsides include the specificity, as Ly-6G is present on a majority of myeloid cells in bone marrow and on all granulocytes including eosinophils; thus, deciphering neutrophils from eosinophils with this marker requires more complexes approaches[8]. Another frequent tradeoff with monoclonal antibodies is their often-limited host specificity range, making comparison studies with more than one animal species difficult. A third drawback of antibody detection methods, especially when using live cells or *in vivo,* is their potential to disrupt cell function or lead to cell activation. For example, the administration of anti-Ly-6G to mice is commonly applied to neutrophil depletion and transient neutropenia[9]. It has additionally been demonstrated that antibody injection may stimulate neutrophil antitumor function[10]. Finally, antibody detection of neutrophils does not reveal the activation state of the cell.

We have identified a 40-amino acid peptide called MUB_{40}, which can be used in a number of assays to label neutrophils under *in vitro* or *in vivo* conditions as well as assay the activation status of live neutrophils[11]. MUB_{40} is derived from MUB_{70}[12], a domain of the *Lactobacillus reuteri* surface mucus-binding protein originally described for its ability to bind mucus[13,14]. MUB_{40} interacts with glycosylated lactoferrin that is present at high concentrations in neutrophil-specific and tertiary granules. MUB_{40} can be exposed to these granules through standard permeabilization steps present in histopathology or fluorescence-activated cell sorting (FACS) protocols for robust staining of fixed neutrophils. When live neutrophils are kept in an inactivated state, the MUB_{40} peptide is excluded from the granule contents, and cells do not stain positive with the marker. Upon activation, MUB_{40} can bind to exposed lactoferrin and lead to robust staining with the peptide. Thus, MUB_{40} can be used to determine the activation state of purified neutrophils, making it an attractive marker to follow the infectious process. The ability to bind to live activated

neutrophils also allows MUB_{40} to be used as a tool to detect areas of neutrophil inflammation in live animal models (*e.g.,* a mouse arthritis model[15]). The protocols in this manuscript detail how fluorescently labeled MUB_{40} can be used to reveal neutrophils in histology tissues and how to assay the activation of live purified neutrophils *in vitro*.

PROTOCOL:

All methods described here have been reviewed and approved by the French organizations CoRC (Comité de Recherche Clinique), CPP (Comité de Protection des Personnes), and CNIL (Commission Nationale Informatique et Liberté).

1. Staining Neutrophils in Histopathology Tissue with Fluorescently Labelled MUB_{40}

1.1. To obtain the tissue for histopathology, fix tissue/biopsy sections in a suitable volume of 4% paraformaldehyde (PFA) for 30 min up to 4 h depending on the thickness of the sample. Place the samples in a 4 °C refrigerator during fixation.

Note: Alternative fixation methods/timings can be substituted based on the user's unique needs.

1.1.1. Remove the PFA, add 16% sucrose solution with enough volume to completely cover the sample, and place the sample at 4 °C for 4 h up to overnight.

1.1.2. Remove 16% sucrose solution and add 30% sucrose solution at a large enough volume to completely cover the sample, and place it at 4 °C for 4 h up to overnight.

1.1.3. Remove the 30% sucrose solution and blot off excess solution from the tissue with a paper towel. Place the tissue section in optimal cutting temperature (OCT) media and snap-freeze in 2-methylbutane cooled in a dry ice-ethanol bath (-72 °C). Once frozen solid (after ~2 min), remove blocks from the 2-methylbutane and place them on dry ice until all blocks are finished and ready for long-term storage. Store the frozen blocks at -80 °C for long-term storage.

1.2. The night before tissue blocks are to be cut, remove them from the -80 °C freezer and place them at -20 °C overnight to equilibrate.

1.2.1. Using a cryostat, cut OCT-embedded tissue into 10- to 30 μm-thick slices and adsorb to high-quality glass slides.

Note: Thicker or thinner slices can be used depending on the experimental requirements.

1.2.2. With a wax pen or other hydrophobic method, draw a box around the tissue slice on the glass slide and let dry.

1.2.3. Prepare the staining solution by performing a 1:1000 dilution of MUB_{40}-Cy5 (or other fluorescent MUB_{40} version, 1 mg/mL stock solution) in 0.1% saponin-PBS solution. The optimal final MUB_{40} concentration should be 1 μg/mL.

Note: Lower final concentrations may be used (0.1-0.01 μg/mL) but may lead to lower signal intensity.

CAUTION: Saponin powder can cause breathing irritation. Make sure to weigh saponin in a cabinet or protected area. Alternative permeabilization methods can be used such as Triton. Add additional markers at manufacturer-recommended concentrations (*e.g.*, 4',6-diamidino-2-phenylindole (DAPI), phalloidin, fluorescent antibodies).

1.2.4. Gently dip the glass slide into a solution of 0.1% saponin-PBS three times.

1.2.5. Carefully blot away excess liquid around the tissue section and add enough staining solution to completely cover the tissue section. Place the slide into a humidity chamber to avoid evaporation and incubate it for 1 h at room temperature. Keep it protected from light.

1.2.6. Gently dip the slide into 0.1% saponin-PBS solution 3x. Then, gently dip the slide into PBS solution 3x. Finally, gently dip the slide into distilled H_2O 3x. Carefully dry excess liquid from the slide.

1.2.7. Add a small amount (~20 μL) of mounting medium (20 mM Tris pH 8.0, 0.5% N-propyl gallate, 90% glycerol) next to the tissue slice and gently lay down a glass coverslip on top of the glass slide. Gently and evenly press the coverslip onto the tissue section and, using a paper towel, carefully remove any mounting media that extrudes around the edges of the coverslip.

1.2.8. Place the mounted slide in a dark cabinet for 24 h to allow the mounting media to solidify. Alternatively, place the mounted slide at 37 °C for 1 h.

1.3. Image the tissue slices on a fluorescent microscope according to the desired acquisition method.

2. Visualization of Neutrophil Activation with Retro-Inverso-MUB_{40}-Cy5 Peptide

2.1. Obtain human neutrophils using the following protocol. Prepare the following solutions and place them in an anoxic cabinet overnight. Exposure to oxygen will begin to activate neutrophils and further induce cell death[16,17].

Note: Neutrophils may be alternatively purified "on the bench" for MUB_{40}-labeling experiments; however, be aware that oxygen exposure will begin to affect neutrophil activation.

2.1.1. Prepare the following solution:
Solution 1 [sodium chloride (NaCl) 0.9%]: add 4.5 g of NaCl to 500 mL of H_2O. Filter the solution.
Solution 2 (dextran 6%): add 6 g of dextran in NaCl 0.9% solution to 100 mL.

Solution 3 (washing buffer): dissolve ethylenediaminetetraacetic acid (EDTA) in PBS pH 7.2 to a final EDTA concentration of 2 mM. Immediately prior to use, dissolve bovine serum albumin (BSA) in the PBS/EDTA solution such that the final BSA concentration is 10% w/v.

2.1.2. Centrifuge blood collection tubes at 650 x g for 20 min without breaks.

2.1.3. Without disrupting separated blood, transfer the blood collection tubes to an anoxic cabinet and collect plasma fractions (top) in a 50 mL conical tube.

2.1.4. Remove the plasma-containing tube from the anoxic cabinet and centrifuge at 2900 x g for 20 min with breaks to pellet the platelets.

2.1.5. Without disrupting the pelleted platelets, gently transfer the plasma tube back into the anoxic cabinet and pipet the platelet poor plasma into a fresh 50 mL tube (hereafter called "plasma").

2.2. Separation of red blood cells from leukocytes

2.2.1. Using the tubes containing the red blood cells (RBCs) from step 2.1.3., combine the RBCs into a conical 50 mL tube (5 blood collection tube contents per 50 mL tube).

2.2.2. Add NaCl 0.9% until the total volume reaches 44 mL. Add 6 mL of dextran 6% (last).

2.2.3. Gently mix the blood/dextran mixture by inverting the tube 10-20 times. Let the tube sediment for at least 30 min.

2.2.4. While sedimentation is occurring, prepare a Percoll gradient by adding 4.2 mL of Percoll solution to 5.8 mL of plasma in a 15 mL conical tube, and mix well by inverting the tube.

2.2.5. Collect the top fraction of the dextran while trying to avoid pipetting red blood cells.

2.2.6. Tighten the screw cap, remove the dextran tube from the anoxic chamber, and centrifuge the tube at 300 x g for 10 min with breaks.

2.2.7. Carefully place the tube back into the anoxic chamber without disturbing the pelleted cells, and remove the liquid via pipetting.

2.2.8. Gently re-suspend the cell pellet in 1 mL of plasma and very slowly add to the top of the Percoll solution. Be careful not to induce mixing of the layers when pipetting the cells on top.

2.2.9. Carefully remove the Percoll solution tube from the anoxic chamber (avoiding mixing), and centrifuge at 800 x g for 20 min without breaks. Peripheral blood mononuclear cells (PBMCs) will remain on the Percoll solution surface, and neutrophils will pellet with the RBCs.

2.3. Removal of contaminating RBCs

2.3.1. Prepare 14 mL of washing buffer solution by adding 700 µL PBS + 10% BSA to 14 mL of washing buffer.

2.3.2. Place the Percoll solution tube back into the anoxic chamber. Remove the Percoll and PBMCs via pipetting and re-suspend the pelleted cells in 1 mL of washing buffer solution. The pellet to be re-suspended will have a red color due to contaminating RBCs.

2.3.3. Add 200 µL of anti-CD235a (glycophorin) magnetic beads to the re-suspended cells, gently mix, and incubate for a minimum of 15 min. This step loads the contaminating RBCs with magnetic beads so that they can be removed.

2.3.4. Wash the separation column with 2 mL of washing buffer 3x.

2.3.5. While holding a 15 mL conical tube under the separation column, slowly pipette the neutrophil/RBC mixture to the top of the column. Collect the cells in the 15 mL conical tube as they flow through. The flow-through should be cloudy and lose its red color due to the RBCs remaining bound to the column.

2.3.6. Add 2 mL of washing buffer to the top of the column and collect in the same 15 mL conical tube. By the end of the 2 mL wash, the flow-through should be clear, signifying that all the neutrophils have been collected.

2.3.7. Gently mix the tube to ensure even distribution of neutrophils and collect 10 µL for cell counting. Note the final volume for cell counting purposes. Enumerate the total number of cells with any standard cell counting method.

2.3.8. Remove the neutrophil tube from the anoxic chamber, and centrifuge the neutrophils at 300 x g for 20 min with breaks.

2.3.9. Place sedimented neutrophils back into the anoxic chamber and remove the washing buffer via pipetting. Re-suspend the neutrophil pellet in plasma with a maximum cell density of 10^7 PMN/mL.

2.4. Enumeration of purified neutrophils and visualization using MUB_{40}

2.4.1. Add 1 µL of RI-MUB_{40}-Cy5 (1mg/mL) to 1 mL of Rosewell Park Memorial Institute (RPMI) 1640 medium without phenol red. Mix by pipetting well.

2.4.2. For the purposes of this protocol, the result has been outlined as if a potent neutrophil activating reagent, N-formylmethionyl-leucyl-phenylalanine (FMLP), is added. Add 1 µm of FMLP to the RPMI/RI-MUB_{40}-Cy5 tube and mix via pipetting up and down.

NOTE: Each experimental procedure will be different from this point forward, depending on the questions being addressed.

2.4.3. Transfer the 1 mL RPMI/RI-MUB_{40}-Cy5/FMLP mixture to a glass bottom microscopy dish. To this dish, add a volume of purified cells corresponding to 10^6 total neutrophils. Mix by gently pipetting.

2.4.4. Place the dish into an inverted fluorescent microscope and start image acquisition. For example, it is recommended to acquire baseline images prior to the addition of a neutrophil-activating reagent such as FMLP or bacteria. In this example, start the acquisition of RPMI/RI-MUB_{40}-Cy5/neutrophils, and at a later timepoint, pipette the neutrophil-activating reagent into the glass dish and continue image acquisition.

Note: Modifications to these steps can be made based on scientific needs.

2.4.5. Process acquired images/time-lapse movies as pertains to the specific microscope used.

REPRESENTATIVE RESULTS:

Results of MUB_{40}-stained tissues from histopathology slides typically reveal individual cells scattered throughout the tissue. MUB_{40} stains lactoferrin, which is present in neutrophil granules and compartmentalized. Thus, typically seen are punctate staining or several large separated areas of signal coming from individual neutrophils (**Figure 1**). It is helpful to add a second cell marker such as DAPI to help co-localize the MUB_{40} signal with the stained cell. The total number of detected cells depends upon the number of neutrophils present in the field of view and can vary dramatically depending upon the source and strength of the immune response. Shown here are representative images from purified fixed human neutrophils (**Figure 1A**) and from a tissue biopsy of an ulcerative colitis patient (**Figure 1B**). Also shown are images taken from a relatively low level of neutrophils in the lungs of mice infected with *Klebsiella pneumoniae* (**Figure 1C**) and a high level of neutrophils in the colon of a guinea pig infected with *Shigella sonnei* (**Figure 1D**).

Results using live, purified neutrophils typically show little-to-no cell staining at early time points, and gradually increase in RI-MUB_{40} staining over time as more neutrophils become activated. Shown here is a time course series of images from an experiment using purified human neutrophils infected with fluorescent *S. sonnei* (**Figure 2**). Depending on the strength of the activating signal, neutrophils may begin staining with MUB_{40} rapidly, so it is recommended to start acquiring images prior to the addition of activators. When cells become activated and RI-MUB_{40} positive, they should exhibit a similar staining profile to that of fixed and permeabilized cells, which give a punctate staining. The optimal concentration of MUB_{40} for both live and fixed cell staining is 1 μg/mL (**Figure 3**). Lower concentrations (0.1-0.01 μg/mL) can be used but result in lower signal intensity. It is also recommended to use a DIC image or other live-cell stain to differentiate which cells are displaying an RI-MUB_{40} signal.

FIGURE AND TABLE LEGENDS:

Figure 1: MUB_{40}-stained neutrophils of human, mouse, and guinea pig origin. (A) Representative MUB_{40} staining (green) of neutrophils purified from healthy human donors. Cell nuclei are stained with DAPI (blue). (B) Staining of human neutrophils in tissue from an ulcerative colitis biopsy. Neutrophils are revealed with MUB_{40} (green) and cell nuclei are stained with DAPI (blue). (C) Histopathology from the lung of a mouse infected with *Klebsiella pneumoniae*. Mouse neutrophils are revealed in the tissue using MUB_{40} (green) and cell nuclei are stained with DAPI (blue). (D) Histopathology from the colon of a guinea pig infected with *Shigella sonnei*. Neutrophils responding to the infection are revealed in the tissue with MUB_{40} (green). *S. sonnei* bacteria (red) express the fluorescent dsRED protein. Cell nuclei are stained with DAPI (blue).

Figure 2: Live neutrophils stain with MUB_{40} only when activated. Selected images from a time lapse series involving purified human neutrophils infected with *S. sonnei* in the presence of RI-MUB_{40}. In the first set of images (top) a neutrophil encounters a *S. sonnei* bacterium (red) shown with a green arrow. Over the time course, the bacterium is internalized by the neutrophil and digested. As the neutrophil becomes activated from the bacteria, it stains progressively stronger with RI-MUB_{40}. Images on the left are fluorescent channels overlaid with DIC images. Images on the right are fluorescent channels only. Images were taken at 5-min intervals.

Figure 3: Effect of different MUB_{40} concentrations on neutrophil staining. Representative staining of fixed/permeabilized neutrophils with various concentrations of MUB_{40}-Cy5. Purified human neutrophils were fixed and stained with the indicated concentrations of MUB_{40}-Cy5. Cell nuclei are stained with DAPI (blue).

DISCUSSION:

Here, two assays are described in which the MUB_{40} peptide is used to study neutrophil inflammation and activation. We show how MUB_{40} can reveal neutrophils present in histopathology sections or show their activation state using live purified neutrophils. The critical steps for using MUB_{40} as a staining reagent are the same as with any other fluorescent detection method. Care must be taken to ensure compatibility of fluorescent signals and that adequate washing steps have been taken to remove background staining. The most important considerations to achieve good staining results are the quality of the microscope, glass slides/cover slips, and the tissue sections. When viewing live purified neutrophils, the most important steps are in the purification process itself. Neutrophils are sensitive and can be activated by a number of different signals. In order to ensure that the experiment produces meaningful results, care must be taken to keep the purified neutrophils inactive until they are ready to be used. This can be achieved by performing the neutrophil purification protocol in an anoxic chamber to limit neutrophil exposure to oxygen.

Both a limitation and advantage of MUB_{40}, depending upon the experimental question being addressed, is that MUB_{40} only stains activated neutrophils unless they are somehow permeabilized. This can be a huge advantage if the experiment calls for following inflammation in a live animal or purified cell, but it may be a disadvantage if the experiment requires detection of all live neutrophils regardless of activation state. A second potential limitation to this protocol is the fact that lactoferrin is present in various bodily secretions such as tears, milk, and mucus.

For histopathology staining in which these secretions are maintained (*e.g.,* colonic biopsies in which the mucus layer is intact), MUB_{40} will also stain the mucus layer along with any present neutrophils. In practice, this has yet to affect our analysis, but it should be noted as a potential signal source.

Currently, MUB_{40} is the only neutrophil biomarker that can differentiate between activated and non-activated live neutrophils. Also, MUB_{40}, unlike antibody detection methods, does not appear to alter the function or survival of neutrophils *in vitro* or *in vivo*. For example, addition of specific antibodies against live neutrophils can be an activating signal affecting neutrophil function or survival in the host. This makes MUB_{40} a very useful reagent for research involving neutrophil activation or granule release *in vitro* or *in vivo*. Additionally, MUB_{40} has a broad host specificity range and can be directly conjugated to a number of different fluorophores, allowing for use in single-step staining assays across multiple hosts. Thus, MUB_{40} staining is comparable between mouse, human, and other mammalian targets, and it simplifies and reduces the number of reagents needed to detect neutrophils.

While this protocol focuses specifically on histopathology and live cell imaging using MUB_{40}, we have also successfully used the peptide in FACS sorting and live animal *in vivo* imaging. We anticipate that MUB_{40} will be amenable to many different assays involving the detection of neutrophils or visualization of inflammatory events in the body, and that future studies and protocols will be developed to further leverage the spectrum of MUB_{40} uses, particularly using non-invasive imaging technics.

ACKNOWLEDGMENTS:

This work was supported by the Fondation Laurette Fugain (LF-2015-15) (B.S.M.) and ANR JCJC grants (ANR-17-CE15-0012) (B.S.M.).

DISCLOSURES:

B.S.M. is listed as an inventor on MUB_{40} patents:

MUB_{40}: European Patent EP11290403.2, 09/09/2011.

RI-MUB_{40}: European Patent EP no. 17306746.3, 11/12/17.

REFERENCES:

1. Nathan, C. Neutrophils and immunity: challenges and opportunities. *Nature Reviews Immunology.* **6** (3), 173–182 (2006).
2. Nicolás-Ávila, J. Á., Adrover, J. M., Hidalgo, A. Neutrophils in Homeostasis, Immunity, and Cancer. *Immunity.* **46** (1), 15–28 (2017).
3. Coffelt, S. B., Wellenstein, M. D., de Visser, K. E. Neutrophils in cancer: neutral no more. *Nature Reviews Cancer.* **16** (7), 431–446 (2016).
4. Wright, H. L., Moots, R. J., Bucknall, R. C., Edwards, S. W. Neutrophil function in inflammation and inflammatory diseases. *Rheumatology.* **49** (9), 1618–1631 (2010).
5. Glennon-Alty, L., Hackett, A. P., Chapman, E. A., Wright, H. L. Neutrophils and redox stress in the pathogenesis of autoimmune disease. *Free Radical Biology & Medicine*. (2018).

6. Borregaard, N., Cowland, J. B. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood.* **89** (10), 3503–3521 (1997).
7. Borregaard, N. Neutrophils, from marrow to microbes. *Immunity.* **33** (5), 657–670 (2010).
8. Rose, S., Misharin, A., Perlman, H. A novel Ly6C/Ly6G-based strategy to analyze the mouse splenic myeloid compartment. *Cytometry Part A: The Journal of the International Society for Analytical Cytology.* **81** (4), 343–350 (2012).
9. Shi, C., Hohl, T. M., Leiner, I., Equinda, M. J., Fan, X., Pamer, E. G. Ly6G+ neutrophils are dispensable for defense against systemic Listeria monocytogenes infection. *Journal of Immunology.* **187** (10), 5293–5298 (2011).
10. Albanesi, M., Mancardi, D. A., *et al.* Neutrophils mediate antibody-induced antitumor effects in mice. *Blood.* **122** (18), 3160–3164 (2013).
11. Anderson, M. C., Chaze, T., *et al.* MUB40 Binds to Lactoferrin and Stands as a Specific Neutrophil Marker. *Cell Chemical biology.* **25** (4), 483–493.e9 (2018).
12. Coïc, Y.-M., Baleux, F., *et al.* Design of a specific colonic mucus marker using a human commensal bacterium cell surface domain. *Journal of Biological Chemistry.* **287** (19), 15916–15922 (2012).
13. Roos, S., Jonsson, H. A high-molecular-mass cell-surface protein from Lactobacillus reuteri 1063 adheres to mucus components. *Microbiology.* **148** (Pt 2), 433–442 (2002).
14. Boekhorst, J., Helmer, Q., Kleerebezem, M., Siezen, R. J. Comparative analysis of proteins with a mucus-binding domain found exclusively in lactic acid bacteria. *Microbiology.* **152** (Pt 1), 273–280 (2006).
15. Mancardi, D. A., Jönsson, F., *et al.* Cutting Edge: The murine high-affinity IgG receptor FcγRIV is sufficient for autoantibody-induced arthritis. *Journal of Immunology.* **186** (4), 1899–1903 (2011).
16. Walmsley, S. R., Print, C., *et al.* Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. *Journal of Experimental Medicine.* **201** (1), 105–115 (2005).
17. Monceaux, V., Chiche-Lapierre, C., *et al.* Anoxia and glucose supplementation preserve neutrophil viability and function. *Blood.* **128** (7), 993–1002 (2016).

Figure 1

Click here to download Figure Figure 1.tif

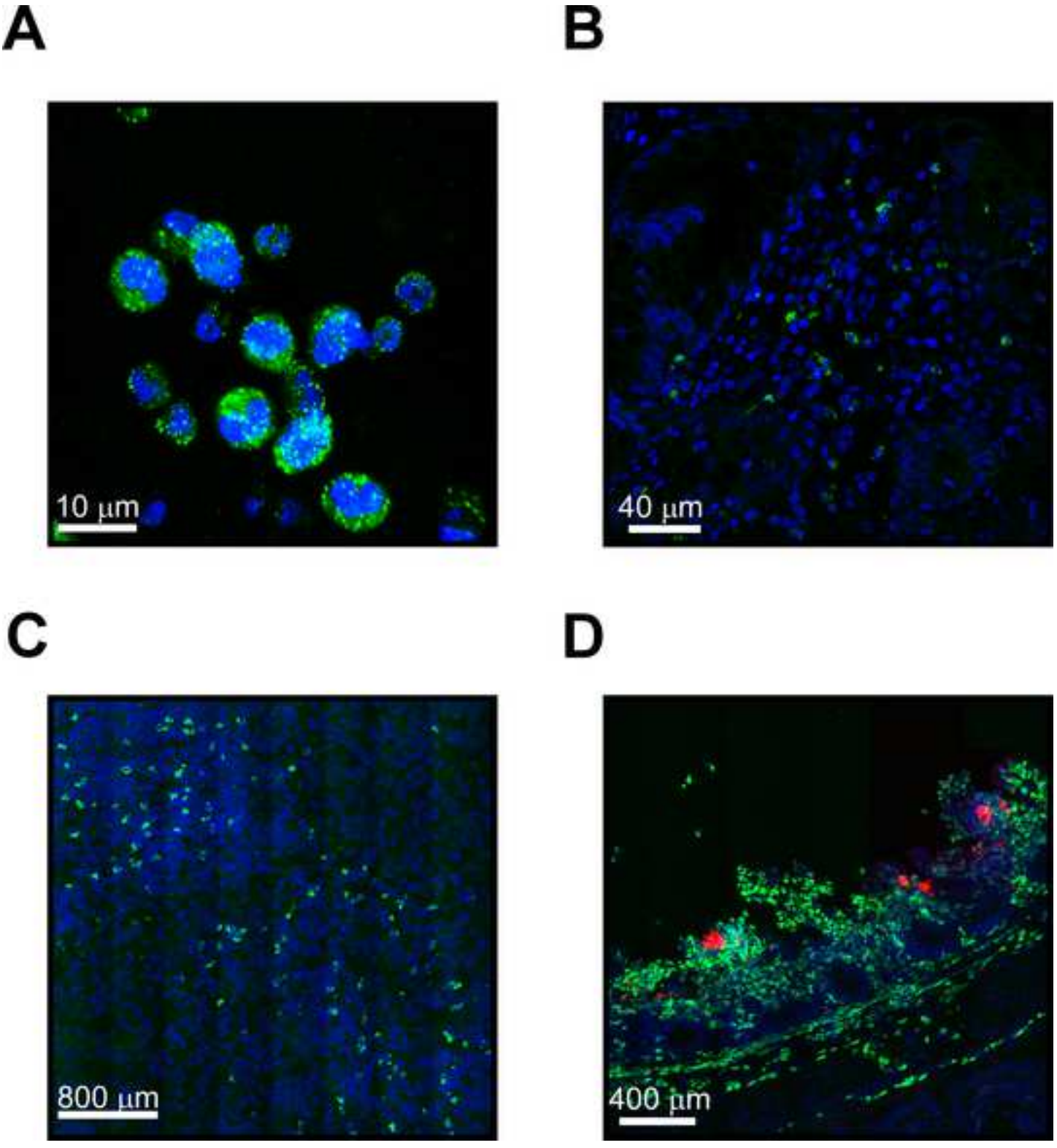


Figure 2

Click here to download Figure Figure 2.tif

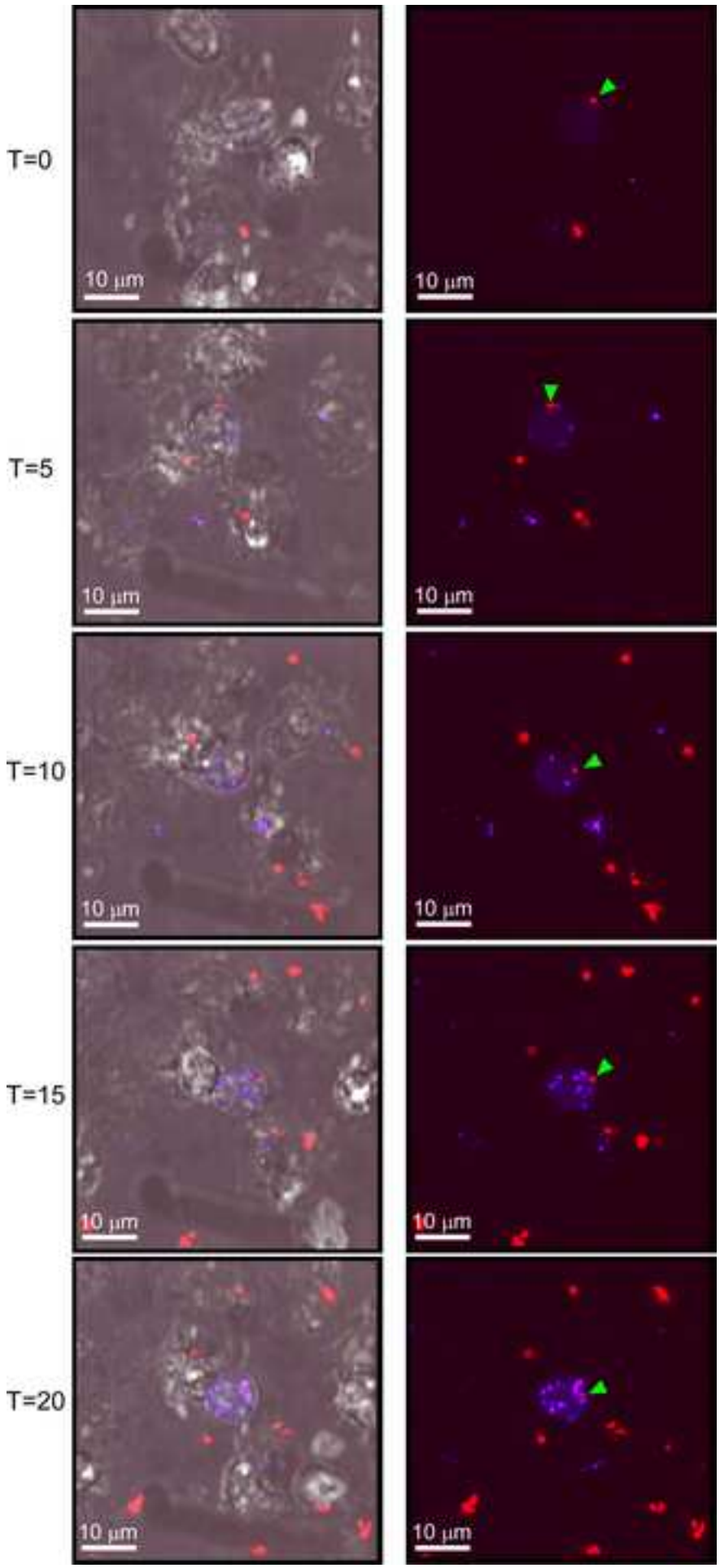
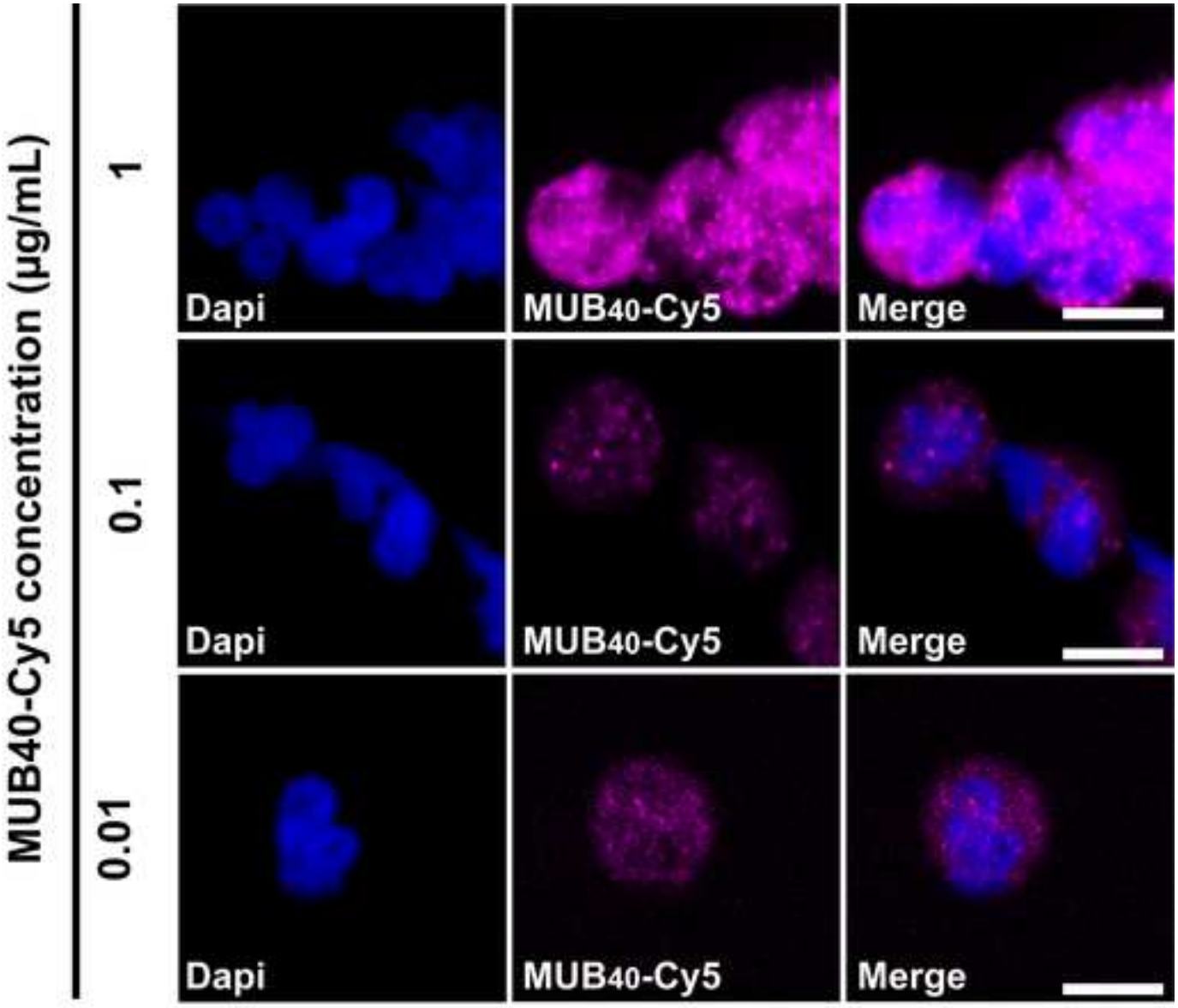


Figure 3

Click here to download Figure Figure 3.tif



Materials Table

Click here to download Table of Materials JoVE_Materials.xlsx

Name of Material/ Equipment	Company
MUB40-Cy5	Benoit Marteyn
RI-MUB40-Cy5	Benoit Marteyn
Parformaldehyde	Sigma-Aldrich
Sucrose	Sigma-Aldrich
Optimal Cutting Temperature Compound (OCT	Sakura Finetek USA
2-Methylbutane	Sigma-Aldrich
Ethanol	Sigma-Aldrich
Leica Cryostat	Leica Biosystems
Glass microscopy slides	Fisher Scientific
Cover slips	Thor Labs
Dako pen	Sigma-Aldrich
Saponin	Sigma-Aldrich
DAPI	Sigma-Aldrich
Alexa fluor 488 Phalloidin	Fisher Scientific
Prolong Gold antifade Mountant	Fisher Scientific
Fluorescent microscope	Various
Anoxic Cabinet	Various
Sodium Chloride NaCL	Sigma-Aldrich
EDTA	Sigma-Aldrich
MACS BSA buffer	Miltenyi Biotec
Percoll	Sigma-Aldrich
CD235a glycophorin magnetic microbeads	Miltenyi Biotec

LS columns	Miltenyi Biotec
RPMI-1640 without Phenol Red	Sigma-Aldrich

Catalog Number	Comments/Description
benoit.marteyn@pasteur.fr	Fluorescent MUB40 peptide (available wi
benoit.marteyn@pasteur.fr	Retro-inverso fluorescent MUB40 peptid
16005	Fixative for histology
S7903	Solution used to remove excess PFA
4583	Used to freeze tissue before cryostat sec
M32631	Used to freeze tissue before cryostat sec
1.00974	Used for dry ice bath to freeze tissue
CM1520	Used to section tissues
12-518-101	
CG15CH	Hi precision coverslip
Z377821	Used to prevent liquid loss during tissue s
47036	Used to permeabilize cells for IF staining
10236276001	Stains DNA
A12379	Binds actin
P36930	Prolongs IF signal and resistance to photo
Various	Used to image IF slides
Various	Used for the purification of live inactivate
S7653	
E9884	Washing buffer component
130-091-376	Washing buffer component
P4937	Gradient for neutrophil purification
130-050-501	Used to remove contaminating RBCs

130-042-401	Used in the removal of RBCs from neutro
R8755	Used for neutrophil assays

ith other conjugated fluorophores)

e. Synthesized with D-amino acids and resistant to proteases (available with other conjugated fluorophores)

tioning

tioning

staining

obleaching

ed neutrophils

›phils

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	The TUEB40 peptide can be used to detect neutrophil mediated inflammation events
Author(s):	Anderson MA, Injarabian L, Andrè A, Tournebize R, Marteyn BS

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☒ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	MARTEYN Benoit S
Department:	Pathogenicity Molecular Niches
Institution:	Institut Pasteur
Title:	principal investigator
Signature:	
Date:	July, 15th 2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: The FluB40 peptide can be used to detect neutrophil Mediated

Author(s): Anderson MA, Injarabian L, Marteyn BS Inflammat events

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove
JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

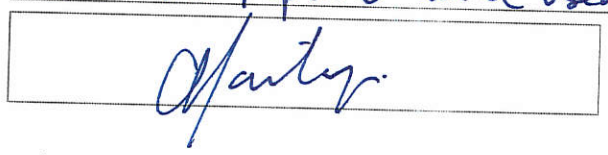
CORRESPONDING AUTHOR:

Name: Benoit S Nauleyn

Department: Cell Biology and Infection

Institution: Institut Pasteur / Inserm 1202

Article Title: The THB40 peptide can be used to detect neutrophil mediated inflammatio events

Signature: 

Date: April 26th 2018

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

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 MUB_{40} staining to work correctly, we believe that they will help the reader be able to perform MUB_{40} 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 MUB_{40}.

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 MUB_{40} 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.

MUB_{40} binds to a glycosylation moiety present on lactoferrin. MUB_{40} 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 MUB_{40} can cross lipid bilayers of live intact cells. Thus we believe that only permeabilization/activation allows access of MUB_{40} 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.