

# Journal of Visualized Experiments

## Immunofluorescence Labelling of Human and Murine Neutrophil Extracellular Traps in Paraffin-Embedded Tissue --Manuscript Draft--

<b>Article Type:</b>	Invited Methods Article - JoVE Produced Video
<b>Manuscript Number:</b>	JoVE60115R1
<b>Full Title:</b>	Immunofluorescence Labelling of Human and Murine Neutrophil Extracellular Traps in Paraffin-Embedded Tissue
<b>Keywords:</b>	Neutrophil Granulocytes; Polymorphonuclear Leukocytes; Neutrophil Extracellular Traps (NETs); paraffin-embedded tissue; antigen retrieval; antibody labelling; immunofluorescence
<b>Corresponding Author:</b>	Volker Brinkmann, Dr. rer. nat. Max-Planck-Institut für Infektionsbiologie Berlin, Berlin GERMANY
<b>Corresponding Author's Institution:</b>	Max-Planck-Institut für Infektionsbiologie
<b>Corresponding Author E-Mail:</b>	brinkmann@mpiib-berlin.mpg.de
<b>Order of Authors:</b>	Volker Brinkmann, Dr. rer. nat. Ulrike Abu Abed
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US\$4,200)
Please indicate the <b>city, state/province, and country</b> where this article will be <b>filmed</b> . Please do not use abbreviations.	Berlin, Germany

**TITLE:**

Immunofluorescence Labelling of Human and Murine Neutrophil Extracellular Traps in Paraffin-Embedded Tissue

**AUTHORS AND AFFILIATIONS:**

Ulrike Abu Abed<sup>1,2</sup>, Volker Brinkmann<sup>1</sup>

<sup>1</sup>Microscopy Core Facility, Max Planck Institute for Infection Biology, Berlin, Germany

<sup>2</sup>Cellular Microbiology, Max Planck Institute for Infection Biology, Berlin, Germany

Email address of co-author:

Ulrike Abu Abed (abed@mpiib-berlin.mpg.de)

Corresponding author:

Volker Brinkmann (brinkmann@mpiib-berlin.mpg.de)

**KEYWORDS:**

neutrophil granulocytes, polymorphonuclear leukocytes, neutrophil extracellular traps (NETs), paraffin-embedded tissue, antigen retrieval, antibody labelling

**SUMMARY:**

Neutrophil extracellular traps (NETs) are three-dimensional structures generated by stimulated neutrophil granulocytes. It has become clear in recent years that NETs are involved in a wide variety of diseases. Detection of NETs in tissue may have diagnostic relevance, so standardized protocols for labelling NET components are required.

**ABSTRACT:**

Neutrophil granulocytes, also called polymorphonuclear leukocytes (PMN) due to their lobulated nucleus, are the most abundant type of leukocytes. They mature in the bone marrow and are released into the peripheral blood, where they circulate for about 6–8 h; however, in tissue, they can survive for days. By diapedesis through the endothelium, they leave the blood stream, enter tissues, and migrate towards the site of an infection following chemotactic gradients. This protocol is an illustration of how they combat invading microorganisms by phagocytosis, degranulation, and generation of neutrophil extracellular traps (NETs). NETs are the result of a process called NETosis, which leads to the release of nuclear, granular, and cytoplasmic components either from living (vital NETosis) or dying (suicidal NETosis) neutrophils. In vitro, NETs form cloud-like structures, which occupy a space several times larger than that of the cells from which they descended. The backbone of NETs is chromatin, to which a selection of proteins and peptides originating from granules and cytoplasm are bound. Thereby, a high local concentration of toxic compounds is maintained so that NETs can capture and inactivate a variety of pathogens including bacteria, fungi, viruses, and parasites, while diffusion of the highly active NET components leading to damage in neighboring tissue is limited. Nevertheless, in recent years it has become apparent that NETs, if generated in abundance or cleared insufficiently, do have pathological potential ranging from autoimmune diseases to cancer. Thus, detection of NETs in

tissue samples may have diagnostic significance, and the detection of NETs in diseased tissue can influence the treatment of patients. Since paraffin-embedded tissue samples are the standard specimen used for pathological analysis, it was sought to establish a protocol for fluorescent staining of NET components in paraffin-embedded tissue using commercially available antibodies.

## INTRODUCTION:

Neutrophil extracellular traps (NETs) have a complex three-dimensional structure. High resolution scanning-electron microscopic analysis revealed that they consist of smooth fibers with a diameter of 15–20 nm (chromatin) studded with globular domains of >25 nm which presumably consist of an assortment of about 30 granular and cytoplasmic proteins and peptides<sup>1,2</sup>. When generated in vitro from isolated neutrophils, NETs can be identified by staining of two or more of their components by immunofluorescence (e.g., histones and neutrophil elastase [NE]). In unstimulated polymorphonuclear leukocytes (PMN), histones are located exclusively in the nucleus, while NE is contained in granules. During NETosis, NE enters the nucleus, where it processes histones<sup>3,4</sup>. NETs are characterized by colocalization of components, which are clearly separated in unstimulated neutrophils. Since currently no antibodies exist that exclusively detect NET specific epitopes (i.e., do not react with naïve neutrophils), detecting colocalization of neutrophil proteins in NETs is the only way to identify NETs.

In tissue, NETs can hardly be detected by conventional histological stains (e.g., by staining with hematoxylin/eosin [H&E], which depicts NETs as a diffuse pale bluish tinge). Only when highly abundant, NETs can clearly be distinguished with H&E staining, such as in thrombi<sup>5</sup>. Since NETs are diffuse per se, tissue structure has to be preserved optimally, so cryo-preparations that are prone to induce freezing artifacts are suboptimal for analysis of NETs in tissue. Instead, formaldehyde fixation and paraffin embedding has been shown to preserve NET structure in tissue well<sup>2</sup>. (Immuno-)histological inspection of sections of paraffin-embedded tissue is the standard method for pathological analysis. As tissue in paraffin blocks is conserved even at room temperature (RT), tissue specimens from daily diagnostic work can be compared with specimens that have been prepared years ago, with questions and techniques that have recently arisen. NETs in tissue have not been detected until recently, and detailed analysis of NET formation and removal during the course of diseases may lead to new insights into pathogenesis. The use of paraffin-embedded tissue has the invaluable advantage to allow the analysis of archival material and to conduct retrospective studies<sup>6</sup>. Undeniably, these benefits come at a cost. Before embedding into paraffin, tissue has to be formaldehyde-fixed, dehydrated and heated above 50 °C. These procedures induce tissue autofluorescence as well as epitope masking.

To limit fixation artifacts, fixation time should be kept to a minimum, so the size of tissue samples should not exceed an area of 20 mm x 30 mm with ~3 mm thickness. Samples of this size are completely fixed after overnight incubation at RT in formaldehyde, ideally followed by direct dehydration and paraffin embedding. Alternatively, fixed samples can be stored for 1 or 2 days in buffer. For immunofluorescence studies, the fixative should be freshly prepared from paraformaldehyde dissolved in a suitable buffer (e.g., phosphate-buffered saline [PBS] or Tris-buffered saline [TBS]). During fixative preparation, the temperature must be kept below 60 °C to

prevent formation of formic acid. Formalin, which is a standard fixative for pathology, should not be used since it contains methanol, other aldehydes, ketones, and formic acid. These impurities lead to increased epitope masking and significant tissue autofluorescence.

For successful immunolabelling, epitope masking has to be reverted in a process called antigen retrieval. Tissue sections are heated in a suitable buffer, which is believed to break methylene bridges, so epitopes become accessible to the antibodies<sup>7</sup>. For detection of NETs, heat-induced epitope retrieval (HIER) is preferred to other methods such as the use of proteolytic enzymes. Routinely, immunolabelling of paraffin sections is carried out with a single antibody followed by a peroxidase-labelled secondary antibody, which is detected with a precipitating substrate. Compared to immunofluorescence, enzyme-based detection methods have a lower spatial resolution due to diffusion of the substrate precipitate, and generally, simultaneous detection of more than one antigen is limited<sup>6</sup>.

As currently no antibodies exist that exclusively bind to NETs, this protocol is used to label two proteins, histone 2B, which is nuclear, and neutrophil elastase, which is localized in granules. In unstimulated neutrophils, these proteins are separated, but they are colocalized in neutrophils undergoing NETosis and in NETs. The simultaneous detection of two antigens can be achieved with two primary antibodies raised in different hosts and two species-specific secondary antibodies labelled with different fluorochromes. This report is a standardization of our previously published protocol<sup>8</sup> and uses a combination of two commercially available antibodies that reliably stain NET components in paraffin-embedded tissue, both fresh and archival, of human and murine origin.

## **PROTOCOL:**

Tissue protocols were approved by Landesamt für Gesundheit und Soziales, Berlin, Germany (G0121/16). Experiments were conducted in accordance with the European directive 2010/63/EU on Care, Welfare and Treatment of Animals.

### **1. Fixation, dehydration, paraffin embedding, sectioning, mounting of sections**

1.1. Prepare 2% formaldehyde solution by dissolving paraformaldehyde in TBS (pH 7.4). Avoid heating above 60 °C. Cool to RT. Formaldehyde solution can be stored at -20 °C.

1.2. Place fresh tissue (here: mouse lung) into a glass Petri dish with TBS. With a scalpel, dissect into pieces not exceeding 20 mm x 30 mm x 3 mm in size. Immerse specimen into 2% formaldehyde solution in TBS.

NOTE: The volume of fixative should be at least 20x that of the tissue.

1.3. Fix at RT for 8–20 h. Transfer specimens to TBS. Place into cassettes.

1.4. Dehydrate using a series of ethanol (70%, 80%, 90%, 96%, 100%, 100%), with each step lasting 1 h.

133  
134 1.5. Clear specimens 2x in 100% xylene (dimethylbenzene), each step 1 h.

135  
136 1.6. Soak in paraffin 2x at 60 °C (melting temperature 54–56 °C), each step 1 h.

137  
138 1.7. Mount specimens using embedding molds, use the cassette bottom as a cover.

139  
140 1.8. Let paraffin solidify and remove embedding molds.

141  
142 1.9. Prepare 3 µm tissue sections, and let them float on 37 °C water bath.

143  
144 1.10. Float sections on water surface onto adhesive glass slides (**Table of Materials**).

145  
146 1.11. Incubate sections on glass slides overnight at 40 °C to bond the tissue to the glass.

147  
148 **2. Rehydration, heat-induced epitope retrieval, staining, mounting, microscopic analysis**

149  
150 2.1. Place sections on glass slides in racks and submerge them into the media used for  
151 dehydration and clearing, in reverse order, with each step for 5 min: 2x 100% xylene, ethanol  
152 series (2x 100%, 96%, 90%, 80%, 70%).

153  
154 2.2. Heat a water bath with a temperature-controlled hot plate to 70 °C. Place a jar filled with  
155 heat-induced epitope retrieval (HIER)-buffer (pH 9; **Table of Materials**), containing 10% glycerol  
156 as a temperature buffer, into a water bath. When the HIER buffer has reached 70 °C, place the  
157 rack with slides into buffer jar.

158  
159 2.3. Incubate slides for 120 min at 70 °C.

160  
161 2.4. Remove the jar from the water bath and let it cool to RT. Rinse sections 3x with deionized  
162 water and once with TBS (pH 7.4).

163  
164 2.5. Carefully remove liquid from slides between sections with rolled filter paper or cotton swab,  
165 leaving sections hydrated.

166  
167 2.6. Create barrier around sections with a hydrophobic barrier pen.

168  
169 2.7. Incubate sections in blocking buffer (1% bovine serum albumin [BSA], 2% normal donkey  
170 serum, 5% cold water fish gelatin and detergents [**Table of Materials**] in TBS) at RT for 30 min to  
171 prevent unspecific binding.

172  
173 2.8. Dilute primary antibodies at a concentration of 1 µg/mL in blocking buffer.

174  
175 NOTE: For human and mouse NETs, a combination of rabbit antibody against neutrophil elastase  
176 and chicken antibody against Histone 2B can be used (**Table of Materials**).

2.9. Place slides into a moist chamber. Remove blocking buffer and add the diluted primary antibodies without washing to the sections using sufficient volume to prevent drying. Seal moist chamber with paraffin film and incubate overnight at RT.

2.10. Wash sections 3x with TBS for 5 min each.

2.11. Prepare working solution of secondary antibodies in blocking buffer. For detection of primary antibodies, use secondary antibodies raised in donkey and pre-absorbed against serum proteins from multiple species (**Table of Materials**). Cover tissue sections with working solution of secondary antibodies. Transfer slides to moist chamber, seal with paraffin film. Incubate for 1 h at RT.

NOTE: Donkey anti rabbit conjugated to Cy2 and donkey anti chicken conjugated to Cy3 can be combined with a DNA counterstain.

2.12. Wash sections 3x for 5 min each with TBS, then once for 5 min with deionized water.

2.13. Cover sections with mounting medium (**Table of Materials**) and apply cover glass avoiding bubble formation.

2.14. Let mounting medium solidify, then analyze immunofluorescence using a wide-field microscope with appropriate band pass filters or a confocal microscope.

#### **REPRESENTATIVE RESULTS:**

Using this protocol, NET components can successfully be detected in paraffin-embedded tissue both of human and murine origin. In unstimulated neutrophils, H2B is located exclusively in the nucleus and neutrophil elastase in granules; consequently, their fluorescence signals do not overlap. In contrast, during NETosis and after NET formation, NE, H2B, and DNA partly colocalize. If imaged as green, red, and blue signals, areas of colocalization are depicted as whitish overlay (**Figure 1G**, **Figure 2D**, and **Figure 3D**). This overlay can also be quantified using image analysis software. Pixels from overlapping signals that are positive for green, red, and blue have been used to create a purple overlay depicting NET areas in **Figure 1G'**, **Figure 2D'**, and **Figure 3D'**. Some of the cells in **Figure 1** and **Figure 2** have lobulated nuclei but are negative for NE. It is believed that these cells are eosinophil granulocytes.

If the tissue sections have a thickness of 2–3  $\mu\text{m}$ , they can be analyzed by wide-field microscopy using 10x or 20x objectives. An example is presented in **Figure 1**, which depicts a section of human appendicitis tissue stained for NE (green), H2B (red) and Hoechst 33342 (blue). Panels A, C, E, and G are from an area of the section containing NETs, while panels B, D, F, and H are from a different area of the same section, which contains numerous neutrophils (**Figure 1B**, NE) but no NETs. Areas with massive NET formation can easily be found even at low magnifications, since all three NET components colocalize, often in stringy extracellular structures, which in the overlay of the three channels appear as whitish extracellular fibers (**Figure 1G**), purple overlay in **Figure**

1G'.

The staining patterns of both tissue areas are clearly different, with NE contained in granules (**Figure 1B**) and DNA in nuclei (**Figure 1F**). Interestingly, the staining for H2B is rather weak in neutrophil-rich areas (**Figure 1D**) compared to NET-containing tissue (**Figure 1C**). This could be due to the size of the antibody (IgY has 180 kD compared to 150 kD for IgG), which may prevent binding to compact chromatin in intact nuclei, while access to H2B is facilitated if the chromatin is decondensed as is the case in NETs (**Figure 1C**).

For higher resolution, confocal microscopes or widefield microscopes with deconvolution have to be used to minimize out-of-focus blur. **Figure 2** depicts a NET-rich area from the same human appendicitis specimen. It is a maximum projection of a confocal stack. NE (green, **Figure 2A**) is found in granules but is also abundant extracellularly, where it colocalizes with H2B (red, **Figure 2B**) and DNA (blue, **Figure 2C**). The extracellular colocalization results in a whitish color combination (**Figure 2D**). These pixels positive for green, red, and blue have been used to create a purple overlay presenting NETs in **Figure 2D'**.

**Figure 3** is a detail of a central section from a mouse lung infected with *Mycobacterium tuberculosis* (*M. tb*). Antigen retrieval and staining conditions are the same as for **Figure 1** and **Figure 2**. Again, colocalization of all three NET components is clearly visible as whitish areas between neutrophils, which has been used to create a purple layer indicating NETs in **Figure 3D'**.

The specificity of the staining is demonstrated in **Supplementary Figure 1**, which depicts negligible staining with control antibodies, and **Supplementary Figure 1A,B** depicts rabbit non-immune serum (green) and chicken anti-GFP IgY (red). **Figure 1C,D** shows the staining with secondary antibodies alone, the combination was the same as was used in **Figure 1**, **Figure 2**, and **Figure 3**. **Supplementary Figure 1A,C** shows a section of human appendicitis similar to **Figure 1** and **Figure 2**. **Supplementary Figure 1B,D** is mouse lung tissue similar to **Figure 3**. DNA is stained with Hoechst 33342. Scale bar represents 25  $\mu$ m.

#### FIGURE LEGENDS:

**Figure 1: Wide-field fluorescence microscopy of a paraffin section of a human appendicitis sample.** Panels A, C, E, and G depict a tissue area with NETs, while panels B, D, F, and H show a different area of the same section that is rich in neutrophils but without NET formation. Staining is against NE (**A,B**; green), H2B (**C,D**; red), and DNA (**E,F**; blue). **Figure 1G,H** represents the overlay of all three channels. Pixels with an intensity between 80 and 256 in all colors represent areas of overlapping staining for NE, H2B, and DNA and are considered to be derived from NETs or neutrophils undergoing NETosis. These pixels have been pseudo-colored as purple in panel **G'**, where they form a large area; and in panel **H'**, where only small spots are found. Images were taken with a wide-field microscope using a 20x objective, and scale bar represents 25  $\mu$ m.

**Figure 2: Confocal fluorescence microscopy of NET components in a human appendicitis sample.** The same tissue section used in **Figure 1** was used. (**A**) Staining against NE, (**B**) Depiction

of H2B, and (C) Hoechst 33342 staining of DNA. (D) The overlay of all three channels. Colocalization of all three signals is pseudo-colored purple in panel D'. For this, pixels with an intensity >80 in all three colors were detected using Volocity 6.3. The purple area depicts NETs or neutrophils undergoing NETosis. The images were taken with a confocal microscopy as Z-stacks and presented as maximum projection. Scale bar represents 25 µm.

**Figure 3: Confocal fluorescence microscopy of NET components in a mouse lung infected with *M. tb*.** It is a detail from the central part of a section of a complete lung with massive neutrophil infiltration. (A) NE staining, (B) H2B staining, and (C) DNA staining. (D) The overlay of all three channels. The purple overlay in panel D' indicates pixels with intensity values of >80 in all three colors indicating neutrophils undergoing NETosis as well as NETs. The images were taken as a Z-stacks with a confocal microscope and presented as maximum projection. Scale bar represents 25 µm.

**Supplementary Figure 1: Staining control with unrelated primary antibodies.** Human (A,C) and murine tissue (B,D) as was used for Figure 1 and Figure 2, and Figure 3, respectively, were stained with unrelated primary antibodies (A,B) or without primary antibodies (C,D). As control primary antibodies in panels A and B, serum from a non-immunized rabbit and a chicken IgY against GFP were applied. Secondary antibodies were the same as shown in all other staining and also used in panels C and D. As expected, in all conditions, negligible background staining was detected, illustrating the specificity of the antibodies used for Figure 1, Figure 2, and Figure 3. The images were taken with a confocal microscope, DNA stained with Hoechst 33342, and scale bar represents 25 µm.

## DISCUSSION:

With growing awareness of the role of NETs during pathogenesis<sup>9</sup>, their detection in tissue from patients or experimental animals is gaining increasing importance. Paraffin-embedded tissue samples have a number of advantages compared to other tissue preparations (e.g., sections of cryopreserved specimens). The tissue preservation in paraffin-embedded samples is clearly superior, and once embedded, the samples are preserved for decades, enabling retrograde studies. For detection of NETs, which are filigree structures, good sample conservation is a precondition ruling out the use of cryopreserved material, which is prone to tissue damage due to ice crystal formation that can give rise to artifacts morphologically resembling NETs.

For optimal preservation, tissue from experimental animals should be fixed shortly after death, ideally by perfusion, to avoid autolysis. As fixative, freshly prepared or freshly thawed solutions of paraformaldehyde in a suitable buffer like TBS or PBS are optimal. This is chemically defined in contrast to formalin preparations used for standard histology, and induces less tissue autofluorescence. In contrast, human tissue often is not fixed directly after excision, and as fixative, normally a 10% dilution of formalin is utilized which contains 10% to 15% of methanol as a stabilizer to prevent polymerization, as well as formic acid, other aldehydes, and ketones. Often, the tissue is stored in this fixative for extended amounts of time before embedding. The result can be autolysis (depending on the time between excision and fixation), and excessive formation of methylene bridges due to the overfixation. Formaldehyde fixation induces changes

in the tertiary structure of proteins by formation of intra- and inter-molecular methylene bridges<sup>10</sup>. Non-proteinaceous cell components like nucleic acids, carbohydrates and lipids are not fixed directly, but immobilized in the three-dimensional protein network. For successful labelling, methylene bonds have to be broken to expose the epitopes in the process of antigen retrieval. This is accomplished by heating of tissue sections mounted onto microscope slides in a suitable heat-induced antigen retrieval buffer (HIER buffer)<sup>11</sup>. Our previous study analyzed the influence of pH and temperature of HIER buffers on the detection of NET components in paraffinized tissue, and it was found that successful tissue pretreatment for one component often is suboptimal for a second component<sup>8</sup>.

In the meantime, it has been found that heating the tissue to 70 °C in HIER buffer at a pH of 9 is a good compromise for many NET components and will retain good tissue preservation, which is often compromised at higher temperatures. It is proposed to use the retrieval time indicated as a starting point, but especially with archival specimens of unknown fixation parameters, the staining intensity can be unsatisfactory. In this case, prolongation of or higher temperature during the retrieval procedure can improve staining efficiency. This can also influence the histone 2B staining of the IgY antibody used in our protocol. As shown in **Figure 1**, decondensed chromatin can be found in neutrophils undergoing NETosis as well as in NETs stains much stronger with this antibody compared to chromatin in resting neutrophils. This is probably due to restricted access of the 180 kDa IgY molecule to compact nuclei and may differ after increased epitope recovery. This may intensify binding efficiency even in areas of condensed chromatin, which will result in stronger fluorescence in normal nuclei of neutrophils and other cells. The difference in staining efficiency between neutrophils undergoing NETosis and non-stimulated neutrophils could be less pronounced than depicted in **Figure 1**. Areas of NET formation still would be identified by the co-localization of NE, H2B, and DNA.

The fixation procedure can also induce significant autofluorescence of the tissue, mainly in the bluish/greenish part of the spectrum. It is important to avoid most of this autofluorescence by using adequate narrow bandpass fluorescence filter sets (wide-field) or detector settings (confocal) that match the emission maximum of the fluorochrome used with blue excitation, e.g., Cy2, Alexafluor 488. In extreme cases of autofluorescence, the bluish/greenish part of the spectrum should be avoided. Instead, fluorescence signals can be detected easier in the far red part of the spectrum (e.g., secondary antibodies coupled to Cy 5 or Alexafluor 635), but this requires filterless black/white cameras or confocal microscopes. Since the human eye is rather insensitive beyond 600 nm, far red fluorescence signals can hardly be detected using oculars. In any case, it is important to use negative controls (e.g., non-immune sera/isotype controls instead of primary antibodies or omitting primary antibodies) to determine suitable exposure times or detector settings.

Tissue sections of 1–2 µm can be analyzed with wide-field microscopes using 10x or 20x objectives. These lenses provide a large focal depth, so (nearly) the entire tissue section will be in focus. This can be used to quickly scan tissue sections for areas of NET formation if they are sufficiently large (as in **Figure 1**). The colocalization of the green (NE), red (H2B), and blue (DNA) channels results in a white staining indicating areas of NET formation (**Figure 1G**). For higher

magnifications, confocal or wide-field microscopes with deconvolution are necessary. **Figure 2** shows details of the human appendicitis specimen also used for **Figure 1**. In **Figure 2A**, NE localizes to small dots (granules), but a large proportion is extracellular, often forming stripes which overlap with H2B (**Figure 2B**) and DNA (**Figure 2C**). This colocalization results in a whitish overlay indicating NETs (**Figure 2D**). A very similar pattern can be found in the lung section of a mouse infected with *M. tuberculosis* (**Figure 3**).

The specimens presented here are characterized by areas with a high degree of NET formation which can be identified even at low magnification. Depending on the tissue density and respective stimulus, NET formation can be much less pronounced, down to NET formation of small groups of neutrophils (for an example in myocarditis, see a previous publication<sup>12</sup>). It is believed that this protocol will help foster more research on NETs in tissue and hopefully aid in unravelling unrecognized roles of NETs in the formation or prevention of diseases.

#### ACKNOWLEDGMENTS:

The funding source of this work is the Max Planck Society. We thank Arturo Zychlinsky for critically reading the manuscript and Philippe Saikali for the mouse tissue.

#### DISCLOSURES:

The authors have nothing to disclose.

#### REFERENCES:

1. Brinkmann, V. et al. Neutrophil extracellular traps kill bacteria. *Science*. **303** (5663), 1532-1535, doi:10.1126/science.1092385, (2004).
2. Urban, C. F. et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against *Candida albicans*. *PLOS Pathogens*. **5** (10), e1000639, doi:10.1371/journal.ppat.1000639, (2009).
3. Fuchs, T. A. et al. Novel cell death program leads to neutrophil extracellular traps. *Journal of Cell Biology*. **176** (2), 231-241, doi:10.1083/jcb.200606027, (2007).
4. Papayannopoulos, V., Metzler, K. D., Hakkim, A., Zychlinsky, A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *Journal of Cell Biology*. **191** (3), 677-691, doi:10.1083/jcb.201006052, (2010).
5. de Boer, O. J., Li, X., Goebel, H., van der Wal, A. C. Nuclear smears observed in H&E-stained thrombus sections are neutrophil extracellular traps. *Journal of Clinical Pathology*. **69** (2), 181-182, doi:10.1136/jclinpath-2015-203019, (2016).
6. Robertson, D., Savage, K., Reis-Filho, J. S., Isacke, C. M. Multiple immunofluorescence labelling of formalin-fixed paraffin-embedded (FFPE) tissue. *BioMed Central Cell Biology*. **9**, 13, doi:10.1186/1471-2121-9-13, (2008).
7. Rait, V. K., Xu, L., O'Leary, T. J., Mason, J. T. Modeling formalin fixation and antigen retrieval with bovine pancreatic RNase A II. Interrelationship of cross-linking, immunoreactivity, and heat treatment. *Laboratory Investigation*. **84** (3), 300-306, doi:10.1038/labinvest.3700041, (2004).
8. Brinkmann, V., Abu Abed, U., Goosmann, C., Zychlinsky, A. Immunodetection of NETs in Paraffin-Embedded Tissue. *Frontiers in Immunology*. **7**, 513, doi:10.3389/fimmu.2016.00513, (2016).

9. Papayannopoulos, V. Neutrophil extracellular traps in immunity and disease. *Nature Reviews Immunology*. **18** (2), 134-147, doi:10.1038/nri.2017.105, (2018).
10. Cattoretti, G. et al. Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. *Journal of Pathology*. **171** (2), 83-98, doi:10.1002/path.1711710205, (1993).
11. Yamashita, S., Okada, Y. Mechanisms of heat-induced antigen retrieval: analyses in vitro employing SDS-PAGE and immunohistochemistry. *Journal of Histochemistry and Cytochemistry*. **53** (1), 13-21, doi:10.1177/002215540505300103, (2005).
12. Weckbach, L. T. et al. Midkine drives cardiac inflammation by promoting neutrophil trafficking and NETosis in myocarditis. *Journal of Experimental Medicine*. **216** (2), 350-368, doi:10.1084/jem.20181102, (2019).

Figure 1

[Click here to access/download;Figure;Fig. 1.tif](#)

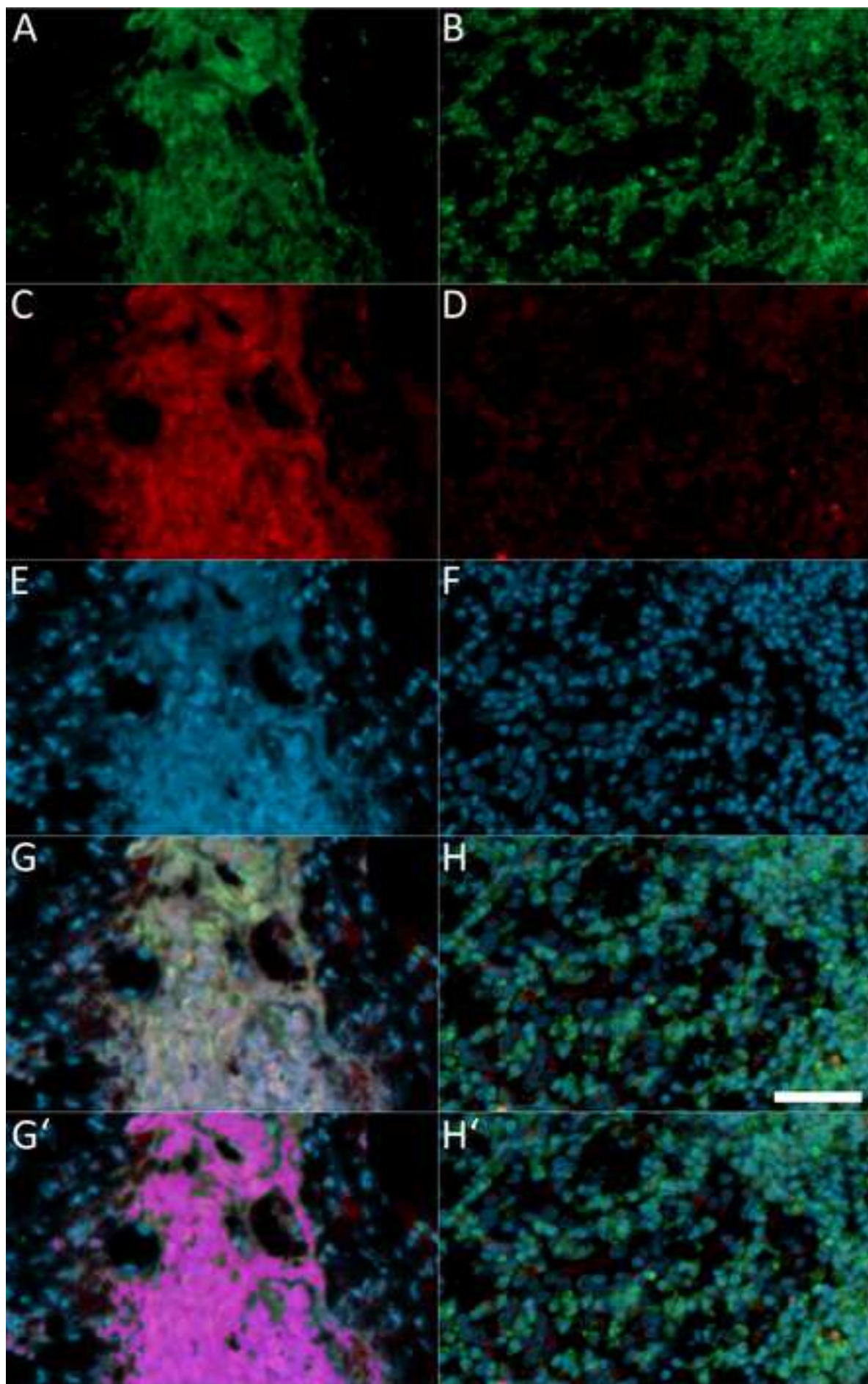


Figure 2

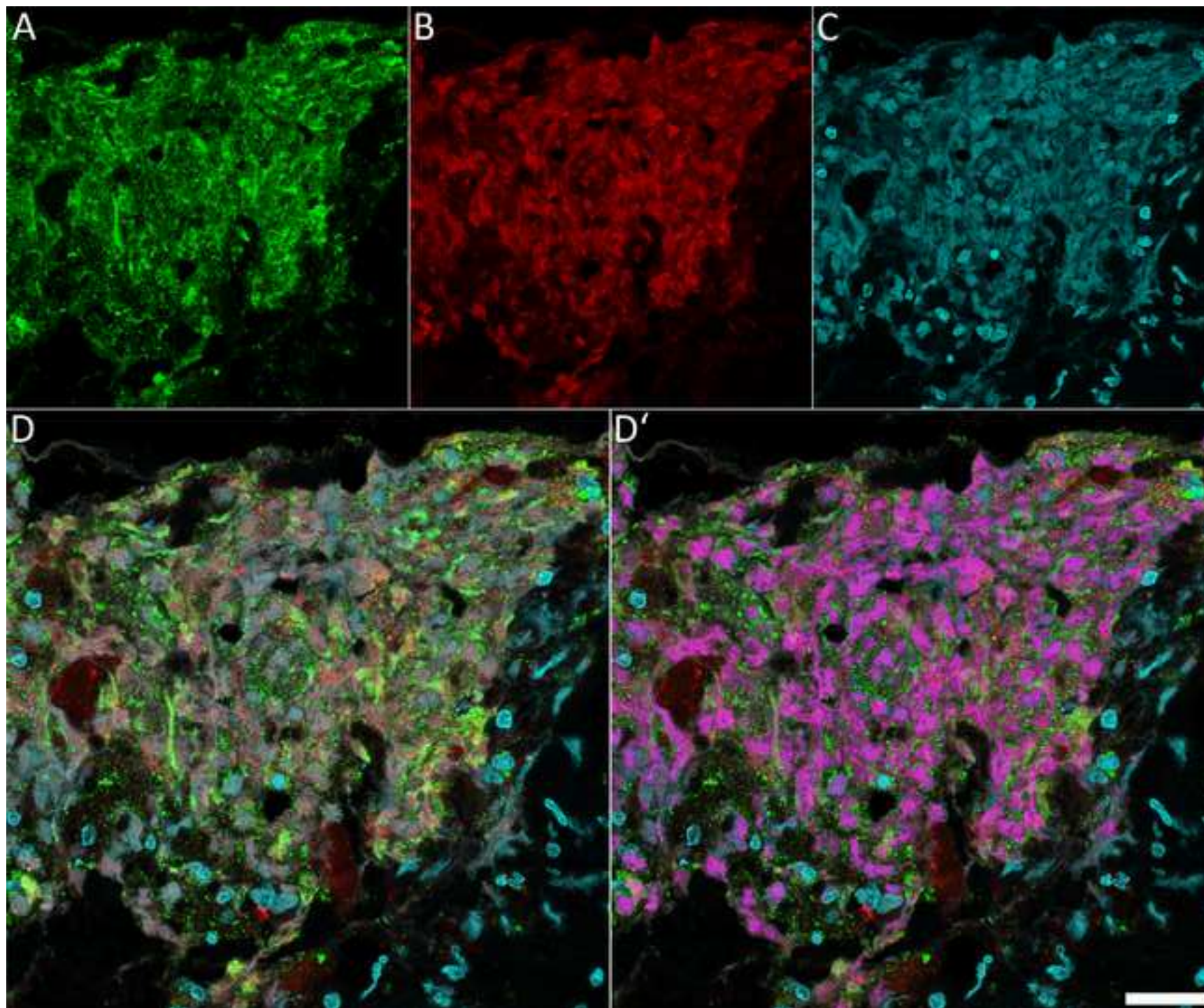
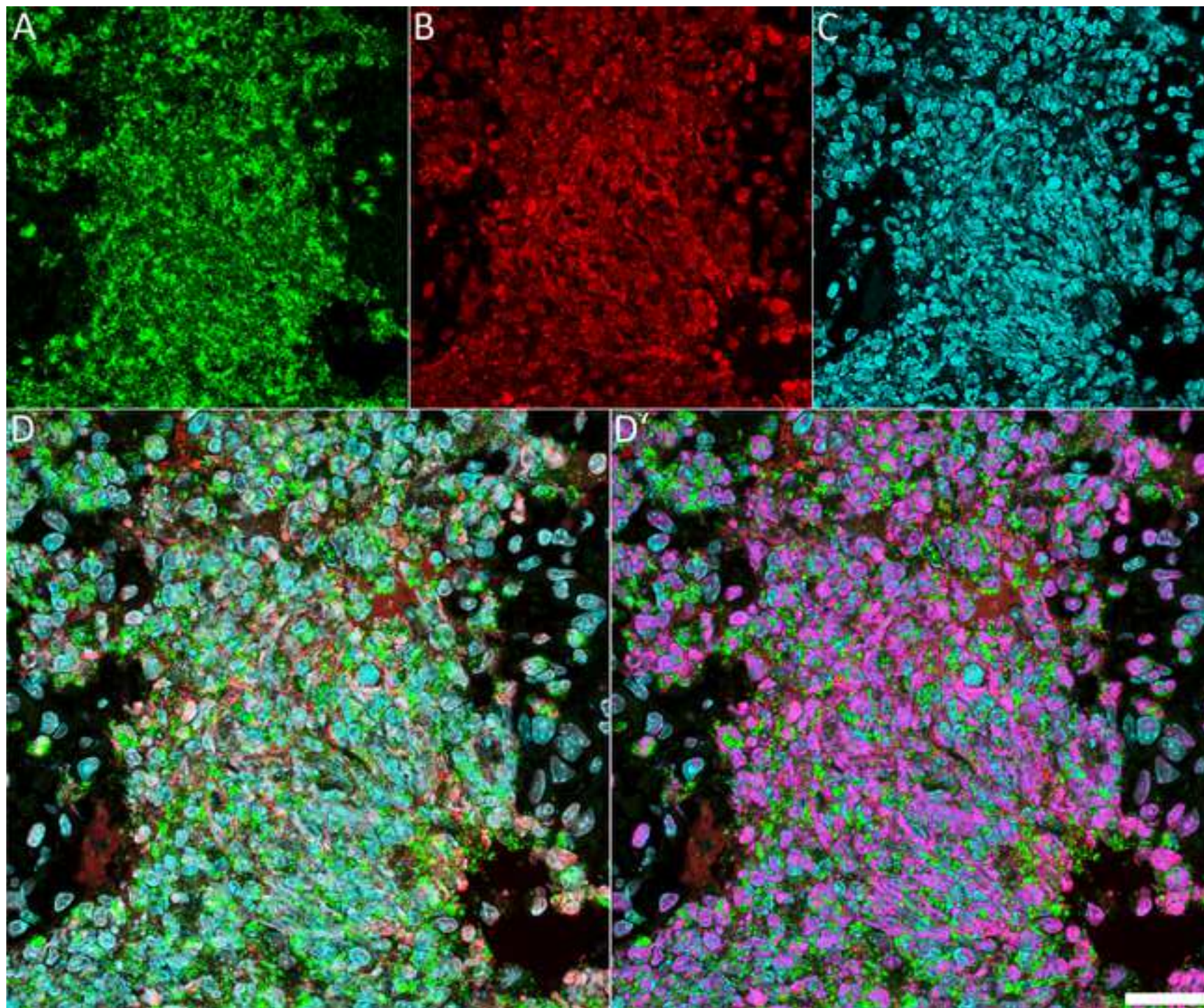
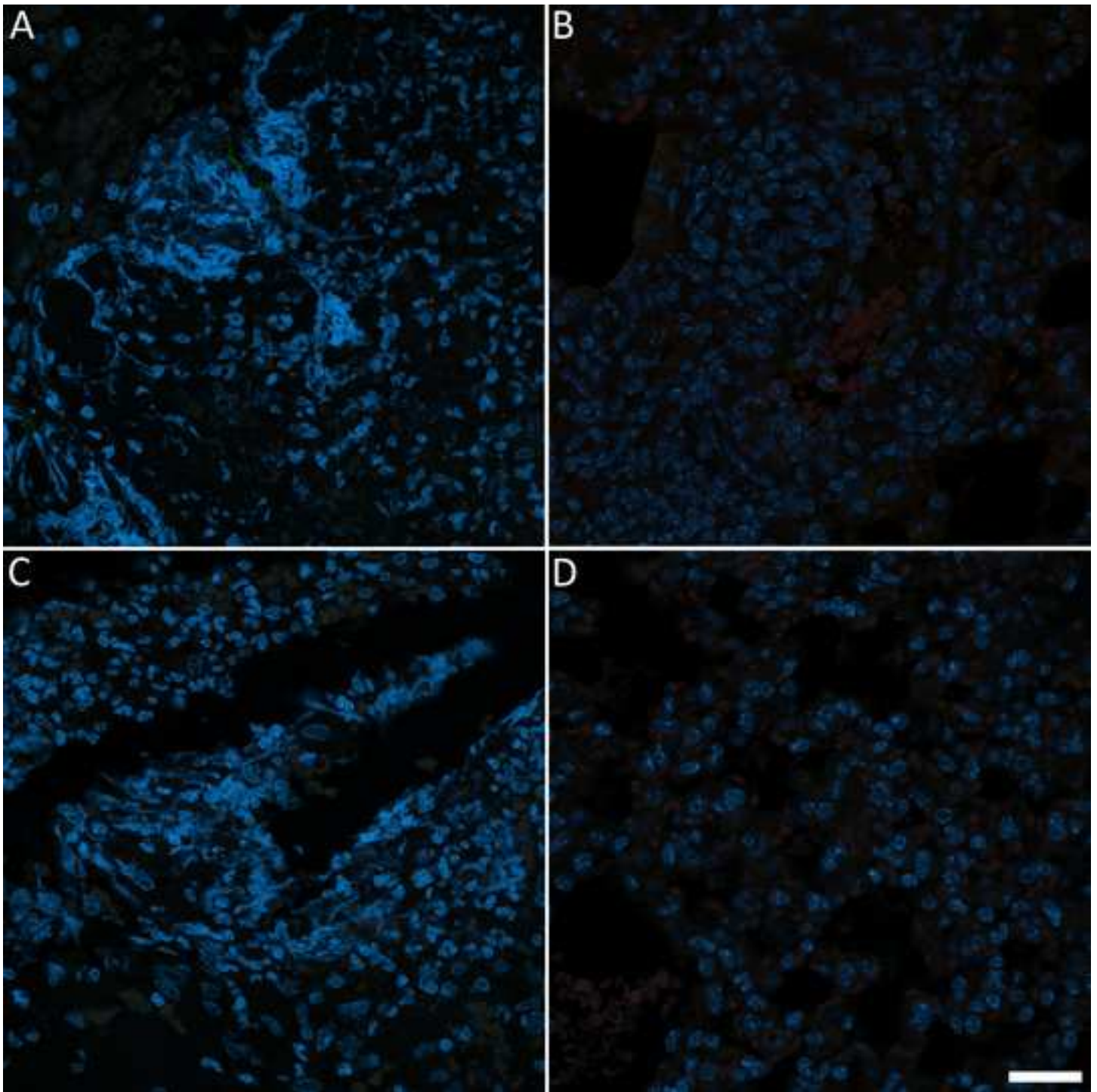


Figure 3





<b>Name of Material/ Equipment</b>	<b>Company</b>	<b>Catalog Number</b>
bovine serum albumine	Sigma	A7906
chicken anti Histone 2B antibody	Abcam	ab 134211
cold water fish gelatine	Sigma-Aldrich	G7765
donkey anti chicken antibody, Cy3 conjugated, for multiple labelling	Jackson Immuno Research	703-165-155
donkey anti rabbit antibody, Cy2 conjugated, for multiple labelling	Jackson Immuno Research	711-225-152
embedding cassettes	Roth	K116.1
embedding molds	Sakura	4162
embedding station	Microm	AP280
ethanol	Roth	9065.4
filter paper, rolled	OCB	
forceps	Dumont	7a
glass cylinder with 20 cm diameter	VWR	216-0075
glycerol	Roth	3783.1
HIER buffer pH 9	Scytex	TES999
Hoechst 33342	Sigma	14533
incubator (dry, up to 40 °C)	Memmert	MMIPP30
moist chamber (plastic box with tight lid)	Emsa	508542
Mowiol 40-88	Aldrich	32.459-0
normal donkey serum	Merck	S30
PAP-pen ImmEdge	Vector Lab	H-4000
paraffin microtome	Microm	355S
paraformaldehyde	Aldrich	16005
petri dishes	Schubert /Weiss	7020051
rabbit anti ELANE antibody	Atlas	HPA068836
racks, jars for microscope slides	Roth	H554.1 H552.1
scalpel	Braun	Fig.22
Superfrost Plus glass slides	Thermo Scientific	J1800AMNZ
temperature-controlled hot plate	NeoLab	D6010
tissue processor for paraffin embedding	Leica	TP1020
Tris buffered saline TBS	VWR	788
 Triton X100	 Roth	 3051.4
 Tween 20	 Fluka	 93773
water bath 37 °C	GFL	1052
widefield or confocal microscope	Leica	DMR, SP8
xylene (dimethylbenzene)	Roth	9713.3

## Comments/Description

alternatively Cy5 conjugate, 711-175-152

water bath included



1 Alewife Center #200  
Cambridge, MA 02140  
tel. 617.945.9051  
[www.jove.com](http://www.jove.com)

## ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Immunofluorescence labelling of human and murine Neutrophil Extracellular Traps in Paraffin-embedded tissue
Author(s):	Ulrike Abu-Abed, Volker Brinkmann

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.

## 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

## 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 be 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:	Dr. Volker Brinkmann	
Department:	Core Facility Microscopy	
Institution:	Max Planck Institute for Infection Biology	
Title:		
Signature:		Date: April 4th, 2019

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

Max-Planck-Institut für Infektionsbiologie  
Dr. Volker Brinkmann  
Wissenschaftlicher Zentralbereich Mikroskopie

Max-Planck-Institut für Infektionsbiologie  
Schumannstraße 21/22 Campus Charité Mitte D-10117 Berlin

Xiaoyan Cao, Ph.D.  
Review Editor

Berlin, den 16.05.2019

Dear Dr. Cao,

We now submit a revised version of our manuscript. We have changed the MS according to your requests and following the suggestions of the reviewers and think that now the MS is clearly improved compared to the initial version. Especially, we have removed commercial language from the protocol text and have included a new paragraph "Table of Materials". An ethic statement on the use of histological samples is now included.

We thank the reviewers for their thoughtful comments that have led to numerous changes throughout the manuscript. Following the request of reviewer 1 we have prepared an additional figure, supplementary Figure 1, to reveal staining specificity.

Comments to reviewer 1:

We thank the reviewer for his/her valuation of the usefulness of our protocol. As requested, we have now included images of tissue stained with unrelated primary antibodies (serum from naïve rabbits and chicken anti-GFP IgY) as well as sections stained only with secondary antibodies. Since these only show low tissue autofluorescence, we did not submit these images, but include them now as Supplementary Figure 1.

In 2016, we have published a similar protocol to stain NET components in paraffin-embedded tissue containing various antibody combinations as well as different ways of antigen retrieval. We have now standardized this protocol and condensed it to just one pair of antibodies and one way of antigen retrieval which

Max-Planck-Institut  
für Infektionsbiologie



in our hands stains effectively NETs in both human and murine tissue, even if overfixed. As requested, we have added the citation of our first protocol. We thank the reviewer for pointing out the oversimplified statement on the survival time of neutrophils and have changed the text accordingly.

We have restructured the discussion and discussed other retrieval conditions from our first NET staining protocol to the discussion.

We thank the reviewer for pointing out our error concerning the size bars of Figs. 2 and 3. We have now corrected it to 25  $\mu\text{m}$ .

We have noted that during submission of the manuscript, the original TIFF images were converted and compressed to PDF files with low resolution. The original files can be downloaded and have at 300 dpi sizes of at least 13.8 cm x 17.6 cm. As requested by reviewer 2, we have restructured the figures to accommodate a new subfigure that depicts NET areas as purple overlay. We hope that this adds to easier identify areas with colocalization of NE, H2B and DNA.

Reviewer 2:

We thank the reviewer for his/her encouraging remarks. We have incorporated all requested text changes. We have restructured the discussion and agree with the reviewer that this eliminates some redundancies in the text. We have omitted the final sentences.

To indicate NETs in the figures, we have added a second overlay panel with all pixels brighter than grey value 80 in green/red and blue highlighted in purple to better visualize NETs.

We hope that in its present form this protocol is now suitable for publication with JoVE.

Kind regards,



Dr. Volker Brinkmann