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Identification of Neutrophil Extracellular Traps in Paraffin-Embedded Feline Arterial Thrombi Using Immunofluorescence Microscopy

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Corresponding Author:	Ronald H. L. Li University of California Davis School of Veterinary Medicine Sacramento, California UNITED STATES
Corresponding Author's Institution:	University of California Davis School of Veterinary Medicine
Corresponding Author E-Mail:	rhli@ucdavis.edu
Order of Authors:	Laetitia Madeleine Marie Duler
	Nghi Nguyen
	Eric Ontiveros
	Ronald H. L. Li
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DEPARTMENT OF VETERINARY SURGICAL & RADIOLOGICAL SCIENCES
SCHOOL OF VETERINARY MEDICINE
UNIVERSITY OF CALIFORNIA
DAVIS, CALIFORNIA 95616

September 26th, 2019

Dear Dr. Stephanie Weldon,

We are excited to submit our manuscript entitled “Identification of neutrophil extracellular traps in paraffin-embedded arterial thrombi in cats using immunofluorescent microscopy
” for consideration in the Journal of Visualized Experiments (JoVE).

I confirm that this work is original. The methods described in this manuscript have not been published elsewhere nor are they currently under consideration for publication elsewhere. All authors have agreed with the submission of this work.

Hypertrophic cardiomyopathy, the most common cardiomyopathy in cats, can lead to life-threatening complications like intracardiac thrombosis and arterial thromboembolism. The pathophysiology of these thromboembolic events is complex and poorly understood. Neutrophil extracellular traps (NETs), released by neutrophils in response to systemic inflammation or pathogens, have been shown to play an integral role in intravascular thrombosis in humans, mice and dogs. However, NETs in cats with cardiogenic arterial thromboembolism have yet to be described. In this manuscript, we describe techniques to identify NETs in formaldehyde-fixed and paraffin-embedded arterial thrombi within the aortic bifurcation of cats. Identification of NETs within feline thrombi will offer new insights into the study of comparative NETosis and thrombosis.

Thank you for your consideration of this manuscript. I look forward to your review of our work and please do not hesitate to contact me if I can be of assistance during the review process.

Sincerely,

Ronald H L Li, DVM, MVetMed, PhD, DACVECC
Assistant Professor of Small Animal Emergency & Critical Care

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TITLE:

Identification of Neutrophil Extracellular Traps in Paraffin-Embedded Feline Arterial Thrombi Using Immunofluorescence Microscopy

AUTHORS AND AFFILIATIONS:

Laetitia Duler[1], Nghi Nguyen[2], Eric Ontiveros[3], Ronald H. L. Li[3]

[1]Veterinary Medical Teaching Hospital, School of Veterinary Medicine, University of California, Davis, California, USA

[2]Department of Veterinary Surgical and Radiological Sciences, School of Veterinary Medicine, University of California, Davis, California, USA

[3]Department of Veterinary Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, California, USA

Corresponding Author:

Ronald H. L. Li (rhli@ucdavis.edu)

Email Addresses of Co-authors:

Laetitia Duler (lmduler@ucdavis.edu)
Nghi Nguyen (bimnguyen@ucdavis.edu)
Eric Ontiveros (esontiveros@ucdavis.edu)

KEYWORDS:

immunofluorescence microscopy, citrullinated histone H3, neutrophil elastase, arterial thrombosis, feline hypertrophic cardiomyopathy

SUMMARY:

We describe a method to identify neutrophil extracellular traps (NETs) in formaldehyde-fixed and paraffin-embedded feline cardiogenic arterial thrombi using heat-induced antigen retrieval and a double immunolabeling protocol.

ABSTRACT:

Neutrophil extracellular traps (NETs), composed of cell-free DNA (cfDNA) and proteins like histones and neutrophil elastase (NE), are released by neutrophils in response to systemic inflammation or pathogens. Although NETs have previously been shown to augment clot formation and inhibit fibrinolysis in humans and dogs, the role of NETs in cats with cardiogenic arterial thromboembolism (CATE), a life-threatening complication secondary to hypertrophic cardiomyopathy, is unknown. A standardized method to identify and quantify NETs in cardiogenic arterial thrombi in cats will advance our understanding of their pathological role in CATE. Here, we describe a technique to identify NETs in formaldehyde-fixed and paraffin-embedded thrombi within the aortic bifurcation, extracted during necropsy. Following deparaffinization with xylene, aortic sections underwent indirect heat-induced antigen retrieval. Sections were then blocked, permeabilized, and ex vivo NETs were identified by colocalization of cell-free DNA (cfDNA), citrullinated histone H3 (citH3), and neutrophil elastase (NE) using immunofluorescence

microscopy. To optimize the immunodetection of NETs in thrombi, autofluorescence of tissue elements was limited by using an autofluorescence quenching process prior to microscopy. This technique could be a useful tool to study NETs and thrombosis in other species and offers new insights into the pathophysiology of this complex condition.

INTRODUCTION:

Cats with hypertrophic cardiomyopathy are at risk of life-threatening thromboembolic complications[1,2]. Despite the high morbidity and mortality associated with feline cardiogenic arterial thromboembolism (CATE), the underlying pathophysiology of CATE in cats is poorly understood. There are also limited diagnostic and therapeutic tools to treat and identify cats at risk of this devastating condition[3].

In addition to its role in innate immunity, neutrophils have been shown to play a role in thrombosis by releasing neutrophil extracellular traps (NETs), which are web-like networks of cell-free DNA (cfDNA) encrusted with histones and granular proteins like neutrophil elastase (NE) and myeloperoxidase. Neutrophils undergo NETs formation in response to systemic inflammation, direct encounter with pathogens, and interaction with activated platelets[4–7]. In dogs, neutrophil-derived DNA has been shown to inhibit clot lysis, while NET proteins accelerate clot formation. The ability of NETs to trap circulating cells and coagulation components is also key to their thrombogenic properties[8–11,12].

NETs are detected by colocalization of extracellular neutrophil proteins, histones, and cfDNA. Because of this, the identification and quantification of NETs in fixed tissues by immunofluorescence of deparaffinized tissues is superior to traditional hematoxylin and eosin (H&E) stain using bright field microscopy[4,5]. Several human studies using immunofluorescence microscopy identified NETs as structural components of coronary arterial thrombi, cerebral stroke thrombi, atherothrombosis, and venous thrombi[13–17]. To date, a standardized method to detect and quantify NETs in feline thrombi has not been described. Because the identification of NETs in feline cardiogenic arterial thrombi may facilitate future translational research in NETs and thrombosis, we describe techniques of NET identification and assessment in paraffin-embedded arterial thrombi in cats.

PROTOCOL:

All methods described here were performed in accordance to the guidelines of the Institutional Animal Care and Use Committee at the University of California, Davis. Necropsies and biopsies of tissues were performed with owners' consent.

1. Tissue fixation, embedding, and sectioning

1.1. Dissect out the aortic bifurcation, including the descending aorta, femoral artery, and the common iliac arteries (**Figure 1A**), shortly after humane euthanasia or death. Blunt dissect out the fascia (**Figure 1B**) before submerging it completely in 10% neutral-buffered formalin for a minimum of 24 h and no longer than 48 h.

1.2. To dehydrate the sample, first submerge in 10% neutral-buffered formalin heated to 37 °C for 1 h. Then, submerge in increasing concentrations of ethanol heated to 37 °C (70%, 95%, 100%) 2x for 1 h each. Finally, without rinsing, submerge 2x in 100% toluene heated to 37 °C for 1 h each.

1.3. Add paraffin heated to 62 °C and allow the paraffin to solidify completely overnight.

1.4. Section 2–3 µm of the paraffin-embedded tissue using a microtome and place on positively charged glass slides. Store sectioned tissues at -80 °C until further analysis.

2. Deparaffinization, rehydration, and heat-induced antigen retrieval

2.1. Use an automated system (see **Table of Materials**) to perform deparaffinization and rehydration of sections on glass slides. Place glass slides in racks and process in the following order:

2.1.1. Submerge completely in 100% xylene for 3 min. Repeat this step 2x. Do not rinse in between steps.

2.1.2. Submerge completely in decreasing concentrations of ethanol (100%, 95%, 70%) at room temperature (RT), 3x for 3 min each. Do not rinse in between steps.

2.1.3. Submerge completely in deionized water for 2 min. Repeat.

2.2. Place sections into Tris-buffered saline with 0.1 % Tween (TBST, pH = 7.6) for 2–3 min.

2.3. Fill the reservoir with deionized water heated to 100 °C. Allow the steamer chamber to equilibrate for 20 min.

NOTE: Heat-induced antigen retrieval is best performed with indirect heating generated by a steamer with a preset temperature setting, such as a food steamer.

2.4. Heat the commercially available antigen retrieval solution containing Tris and EDTA (pH = 9) to 95–97 °C on a temperature-controlled hot plate with constant stirring. Ensure that it does not boil.

NOTE: The solution should turn cloudy once it is warmed.

2.5. Pour the heated antigen retrieval solution into a slide container and place the container in the chamber of the steamer. Allow the antigen retrieval solution to equilibrate to the temperature of the steamer for 3–4 min. Ensure that the temperature of the chamber is ~95 °C.

2.6. Submerge the slides completely in the heated antigen retrieval solution and continue the application of external heating via the steamer for 20 min.

2.7. Remove the slide container from the steamer and allow the slides and the antigen retrieval solution to cool to RT. Store the diluted antigen retrieval solution at 4 °C and reuse up to 2x if needed.

2.8. Wash the slides 3x with TBST for 5 min.

3. Immunolabeling and autofluorescence quenching

NOTE: **Table 1** details the composition of the blocking buffers used in the following steps.

3.1. Incubate sections in Blocking Buffer 1 for 2 h at RT under gentle rocking (30–50 rpm). Seal with paraffin film to avoid drying.

3.2. Without washing, immediately apply 100 µL of diluted rabbit polyclonal anti-human citrullinated histone H3 (citH3) antibody (0.03 mg/mL diluted in blocking buffer 1) directly onto the slide.

3.3. Place a coverslip (24 mm x 40 mm x 0.13–0.17 mm) on each section to allow even distribution of the antibody mixture.

3.4. Incubate for 12–16 h at 4 °C with gentle rocking (30–50 rpm). Seal with parafilm film to avoid drying.

3.5. Wash 3x with TBST for 5 min.

3.6. Apply 100 µL of goat anti-rabbit antibody conjugated to Alexa Fluor 488 (diluted to a final concentration of 0.04 mg/mL or 1:50 in Blocking Buffer 1) as described in step 3.3. Incubate for 1 h at RT under gentle rocking (30–50 rpm). Protect slides from light.

3.7. Wash with TBST 3x for 5 min.

3.8. Incubate sections in Blocking Buffer 2 overnight at 4 °C under gentle rocking (30–50 rpm). Protect from light.

3.9. Wash with TBST 3x for 5 min.

3.10. Block sections in Blocking Buffer 3 as described in step 3.3 at RT for 2 h under gentle rocking (30–50 rpm).

3.11. Incubate sections with biotinylated polyclonal rabbit anti-human NE antibody (final concentration = 0.2 µg/mL in Blocking Buffer 3) at 4 °C for 12–16 h as described in steps 3.2–3.4.

3.12. Wash with TBST 3x for 5 min.

3.13. Incubate with Alexa Fluor 594 streptavidin conjugate (dilute to 1:100 or 0.02 mg/mL in Blocking Buffer 3) as described in steps 3.2–3.3 for 1 h at RT. Protect from light and seal with paraffin to prevent drying.

3.14. Wash with TBST 1x for 5 min.

3.15. Apply 100 µL of autofluorescence quenching solution mixture directly onto the sections for 1 min as instructed by the manufacturer.

3.16. Immediately wash the slides with TBST 6x for 10 min.

3.17. Cover each slide with 100 µL of 300 nM DAPI for 5 min in the dark.

3.18. Wash with TBST for 3 min. Repeat this for a total of 5x.

3.19. Apply a drop (~50 µL) of antifade mounting medium, part of the autofluorescence quenching kit, directly onto the glass slide surrounding the section. Place a coverslip (24 mm x 40 mm x 0.13–0.17 mm) gently onto the section without creating any bubbles.

3.20. Allow samples to cure overnight in the dark at 4 °C until the mounting medium has hardened for microscopic analysis with immersion lenses.

4. Neutrophil extracellular trap identification

NOTE: The following protocol utilizes an inverted epifluorescence microscope with a 1,280 x 960 digital CCD camera (see **Table of Materials**).

4.1. To locate thrombi, scan cranially to caudally along the length of the aorta, aortic bifurcation, and each femoral artery using phase contrast microscopy with a 10x objective. A thrombus is a conglomeration of tissue containing red blood cells, white blood cells, and platelets adjacent to the endothelium on phase contrast and bright field microscopy (**Figure 2A**, **Figure 2B**).

4.2. First examine sections for NETs using the DAPI channel (excitation = 357/44 nm) with 10x and 20x objectives (**Figure 2C**). Note that cfDNA appears as decondensed DNA that is not within the confines of the cytoplasm of a cell when seen on phase contrast or bright field microscopy.

4.3. Identify extracellular NE and citH3 on the green fluorescent protein channel (excitation = 470/22 nm, emission = 525/50 nm) and Texas Red channels (excitation = 585/29 nm, emission = 628/32 nm), respectively, with 10, 20, and 40x objectives.

4.4. Evaluate and analyze NETs within a thrombus using available software, such as Image J (NIH). NET formation is identified based on the colocalization of cfDNA, extracellular citH3, and NE as previously described[18]. Maintain consistent exposure time and gains of each channel throughout the acquisition of images to avoid saturation in pixel intensity.

4.5. Map each thrombus based on its proximity to the descending aorta by dividing it into three equal zones, with Zone 1 closest to the aorta, Zone 3 furthest from the aorta, and Zone 2 between Zones 1 and 3). With the operator blinded to the medical condition of each subject, take at least ten random fields in each zone. Characterize the distribution of NETs in thrombi by averaging the numbers of fields with NETs in each zone or calculating the average NET-occupying area per zone.

REPRESENTATIVE RESULTS:

Using this protocol for deparaffinization, heat-induced antigen retrieval, and double immunolabeling of paraffin-embedded thrombi, we identified NETs in feline CATE for the first time. Thrombi within the aortic bifurcation were located by fluorescence microscopy and bright field microscopy using standard H&E staining and phase contrast microscopy. On bright field microscopy, feline arterial thrombi consisted of red blood cells, leukocytes, fibrin, and platelets (**Figure 3A**). Although H&E cannot stain specific NET components, NETs frequently appeared as networks of deep purple threads of various lengths surrounding nearby erythrocytes and leukocytes (**Figure 3A**, dotted outline). A thrombus was characterized as a well demarcated structure within the vascular space on phase contrast microscopy (**Figure 2A**, **Figure 4B**). We further confirmed the presence of NETs within these areas by immunofluorescence microscopy (**Figure 3B**). Magnification of these areas revealed large aggregates of NETs, composed of cfDNA, extracellular citH3, and NE (**Figure 2C**, **Figure 3B**, white dotted outline). Using the same technique to search for thrombi and NETs in cats without CATE, we found that sheets of lyzed neutrophils could be detected occasionally in close proximity to the endothelium. Although these neutrophils displayed some morphological characteristics of NET formation, they should not be associated with any organized thrombus. We did not identify any thrombi in any of the control samples (**Figure 4A**).

Figure 5A demonstrates profound autofluorescence of clot elements like erythrocytes and collagen when imaged at the green (488 nm) wavelength, which hindered our ability to detect cfDNA and protein colocalization. We found that brief autofluorescence quenching using a commercially available kit after immunolabeling significantly increased the sensitivity of protein colocalization and NET detection, even in areas with an abundance of erythrocytes (**Figure 5B**, arrowheads).

FIGURE AND TABLE LEGENDS:

Figure 1: Representative necropsy photographs of a dissected aortic bifurcation from a cat with cardiogenic arterial thromboembolism. (**A**) The descending aorta was dissected 4–5 cm cranial (Cr) to the aortic bifurcation. (**B**) Fascia was carefully dissected out until the descending aorta (1) and femoral arteries (2,3) were clearly visible at the caudal aspect (Cd). Note the thrombus within the aortic bifurcation (*).

Figure 2: Representative phase contrast and immunofluorescence images of NETs in a thrombus found within a feline aortic bifurcation. (**A**) Phase contrast microscopy revealed a thrombus as a discrete and well demarcated structure close to the aorta. Combined phase contrast and fluorescence staining of DNA (blue) showed the presence of leukocytes and cell-free DNA within the thrombus. The boxed area consists of a large concentration of cell-free DNA. Original 10x magnification; Scale bar = 400 µm. (**B**) The boxed area in (A) was further magnified at 20x. Cell-free DNA and intracellular DNA stained with DAPI (blue), neutrophil elastase (NE), and citrullinated histone H3 (citH3) appeared green and red, respectively. (Original 20x magnification; Scale bar = 100 µm). (**C**) NETs, identified based on colocalization of cell-free DNA, extracellular NE, and citH3, were outlined (dotted line). Original 40x magnification; Scale bar = 100 µm.

Figure 3: Representative image of a feline arterial thrombus using H&E and immunofluorescence staining. (**A**) On H&E stain, large concentration of neutrophils and erythrocytes were visible. Extracellular chromatins appeared as deep purple threads of various lengths surrounded by erythrocytes and neutrophils (dotted outline, black arrow). (**B**) Neutrophil extracellular traps were easily visualized using immunofluorescence microscopy on the same thrombus (dotted outline, white arrow). NETs were identified as colocalization of cfDNA (blue), NE (green), and citH3 (red). Original 20x magnification; Scale bar = 200 µm.

Figure 4: Representative phase contrast and immunofluorescence images of aortic bifurcations. (**A**) Phase contrast and immunofluorescence images of aortic bifurcations in a cat without arterial thrombosis. Note the absence of thrombi or aggregates of neutrophils within the lumen of the aortic bifurcation from the cat without arterial thrombosis. (**B**) Phase contrast and immunofluorescence images of aortic bifurcations in a cat diagnosed with cardiogenic arterial thromboembolism. The aortic bifurcations were stained for DNA (blue), neutrophil elastase (green), and citrullinated histone H3 (red). In this case, a well demarcated thrombus bulging into the vascular wall and occupying most of the aortic lumen was noted in the cat with cardiogenic arterial thromboembolism. NETs, characterized by colocalization of cfDNA, NE, and citH3, were identified within the thrombus (dotted outline). Original 10x magnification; Scale bar = 400 µm.

Figure 5: Representative phase contrast (PC) and immunofluorescence images of arterial thrombi from two cats. The slides were stained for citH3, NE, and DNA and imaged at 488 nm (red), 595 nm (green), and 357 nm (blue) wavelengths, respectively, at 40x magnification. Cardiogenic arterial thrombi in cats had an abundance of erythrocytes (*, dotted line). (**A**) Autofluorescence from erythrocytes was most prominent across the 488 nm wavelength, diminishing the detection of colocalization signal and identification of NETs. (**B**) Quenching significantly reduced autofluorescence at the 488 nm wavelength, especially in areas with a high concentration of erythrocytes (*, dotted line). It enhanced the detection of colocalized proteins, citH3, and neutrophil elastase (arrowhead), in the presence of erythrocytes (Scale bar = 200 µm).

Supplement Figure 1: Representative immunofluorescence images of arterial thrombi from a cat. The sections were stained for DNA (blue), citH3 (red), and either myeloperoxidase (**A**) or neutrophil elastase (**B**). (**A**) Despite utilizing a feline-specific myeloperoxidase antibody (MPO, 1:5), the staining intensity of MPO remained poor. (**B**) Using a polyclonal neutrophil elastase (NE) antibody, known to cross-react with multiple species, the immunoreactivity and staining intensity were significantly higher. Note the characteristic lobulated nuclei of neutrophils surrounded by NE (arrows). Original 40x magnification; Scale bar = 100 µm.

Table 1. Composition of blocking buffers used for immunofluorescence.

DISCUSSION:

We describe a protocol to identify NETs in fixed feline cardiogenic arterial thrombi using a double immunolabeling protocol and immunofluorescence microscopy. Although only cardiogenic arterial thrombi were stained, in theory this protocol could be used for other types of thrombi and in other veterinary species. Identification of NETs within feline arterial thrombi suggests that NETs may play a role in thrombosis in cats.

Detection of NETs by immunofluorescence in fixed and paraffin-embedded tissue is superior to conventional histological stains like H&E, which often shows threads of chromatin surrounded by neutrophils[13]. Immunohistochemistry and immunofluorescence of fixed arterial thrombi allow the simultaneous detection of cfDNA and other extracellular proteins like citH3, known to be specific to NETs formation[18,19]. Because cryopreparations are suboptimal for NET detection in tissues and thrombi, we preserved our samples in 10% neutral-buffered formalin, which contains 4% formaldehyde and 10% methanol. Cell-free nucleic acids are not directly fixed by formaldehyde. Instead, they are immobilized within fixed protein structures, which are altered by partially reversible methylene bridge crosslinks induced by formaldehyde[20]. To further limit artefacts and autofluorescence caused by formic acid and ketones generated by oxidation of formaldehyde, investigators can choose to use methanol-free paraformaldehyde diluted in buffer for fixation. Because the duration of fixation affects immunoreactivity, we recommend fixation for no longer than 24 h prior to dehydration and paraffin embedding. Paraffin-embedded tissues or clots can then be stored for deparaffinization and staining.

Chemical fixation by formaldehyde and paraformaldehyde alters the tertiary structure of proteins, masking the antigens of interest and preventing the binding of antibodies to specific epitopes[21]. Antigen retrieval, a process that breaks the methylene bridge crosslinks, is essential prior to performing immunodetection in formalin-fixed tissues. Based on the authors' experience, heat-induced antigen retrieval using an alkaline retrieval solution (pH = 9 in Tris/EDTA buffer) with mild indirect heating enhances the detection of proteins and NETs while minimizing artefacts and autofluorescence. The temperature of the antigen retrieval solution should not reach the boiling point (>100 ˚C), because the denaturing of proteins can lead to nonspecific binding and background noise.

A limitation of NET identification in fixed clots is that immunofluorescence staining can be highly variable under different antigen retrieval conditions[18]. Comparable to results found by Brinkmann et al., we found that higher incubation temperatures (>55 ˚C) resulted in optimal staining of histones in the nuclei and decondensed chromatin[19]. However, we found that the staining intensity of myeloperoxidase, a granular protein found in neutrophils and NETs, was low under the proposed conditions. The poor immunoreactivity of myeloperoxidase was consistent despite the use of a feline-specific antibody (**Supplement Figure 1**). Therefore, we encourage investigators to modify the duration and conditions (e.g., pH, temperature) of the antigen retrieval process to yield a satisfactory signal based on the antigen of interest.

One of the challenges of identifying NETs in veterinary species is the lack of species-specific antibodies. To prevent the interference encountered when using primary antibodies originating from the same species, we included an additional blocking step utilizing a high concentration of rabbit immunoglobulins to saturate any remaining binding sites on the goat anti-rabbit secondary antibodies. A major disadvantage of this technique is that it is time-consuming, because it requires multiple incubation steps. Investigators should include two different controls that exclude either primary antibody in the second immunolabeling step to ensure that the secondary antibody from either immunolabelling step binds specifically to its primary antibody. The specificity of the identified NET structures can be further verified by DNase digestion or the inclusion of biological controls consisting of aortic bifurcations from cats without CATE (**Figure 5**). In addition, negative controls consisting of the same concentration of isotype control antibodies as the primary antibodies should be included to rule out nonspecific antibody interactions, nonspecific binding to Fc receptors, and cellular autofluorescence. Investigators are advised to modify this protocol based on the availability of species-specific antibodies. If no species-specific antibodies are available, we advise to first evaluate the immunoreactivity of antibodies using immunocytochemistry or evaluate the protein transcript from the species of interest for homology to the referenced transcripts.

Factors like sample fixation, inadequate deparaffinization, and the presence of specific tissue components can lead to autofluorescence in thrombi. During aldehyde fixation, amines may combine with aldehydes to form Schiff base complexes, which emit fluorescence[22]. Incomplete deparaffinization may also chemically modify the proteins in the tissue, creating autofluorescence[23]. Extracellular components in vascular samples such as collagen, elastin, and red blood cells are reported to naturally fluoresce in mammals[24,25]. Because natural or iatrogenic autofluorescence is most noticeable in the green wavelengths (excitation = 488 nm, emission = 500–550 nm), the use of far-red fluorophores may minimize some autofluorescence[26]. In the present protocol, we utilized a commercially available autofluorescence quenching kit designed to electrostatically bind to autofluorescent tissue elements. We recommend that investigators optimize the duration of autofluorescence quenching, because the manufacturer's recommendation of 5 min may diminish immunoreactivity of less abundant proteins. Alternatively, investigators can also dampen autofluorescence using Sudan Black B, 3,3'-diaminobenzidine or trypan blue[27].

Because NETs are heterogeneously distributed within a thrombus, a thorough mapping of the entire aorta and iliac arteries is recommended. Regions positive for cfDNA, citH3, and NE are then magnified. The colocalization of cfDNA, citH3, and NE has been widely used to identify NET formation and to differentiate NET formation from other forms of cell death. Unlike a recent human study that found NETs to be concentrated at the periphery of coronary thrombi, most of the NETs in feline arterial thrombi were clustered at the cranial aspect of the clot[28]. Although we used a standardized protocol to identify NETs, microscopic evaluation and quantification of NETs remains subjective. Here, we utilized a blinded and systematic method to minimize observer bias during microscopic analysis. Because the number of NETs in a sample can be influenced by the number of neutrophils, investigators can quantify NETs relative to the number of neutrophils by identifying neutrophils based on nuclear morphology, cell diameter, and expression of neutrophil-specific proteins. Another challenge of NET identification using microscopy is that NETs have ill-defined margins and they tend to merge, forming nebulous structures. This could lead to under- or overestimation of the number of NETs in any given sample. Therefore, instead of DAPI, NETs can be stained using Sytox Green for clearer identification and denotation of cell-appendant DNA from NETs formation.

We have developed a double immunolabeling protocol to identify NETs in paraffin-embedded feline arterial thrombi. Deparaffinization, rehydration, and antigen retrieval must take place before immunolabeling of citH3 and NE. This assay can be a valuable tool for the study of NET formation in cats and provide a better understanding of the pathophysiology of CATE in cats.

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DISCLOSURES:
The authors have nothing to disclose.

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23. Davis, A. S. et al. Characterizing and Diminishing Autofluorescence in Formalin-fixed Paraffin-embedded Human Respiratory Tissue. *The Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society*. **62** (6), 405–423 (2014).
24. Banerjee, B., Miedema, B. E., Chandrasekhar, H. R. Role of basement membrane collagen and elastin in the autofluorescence spectra of the colon. *Journal of Investigative Medicine: The Official Publication of the American Federation for Clinical Research*. **47** (6), 326–332 (1999).

25. Hirsch, R. E., Zukin, R. S., Nagel, R. L. Intrinsic fluorescence emission of intact oxy hemoglobins. *Biochemical and Biophysical Research Communications*. **93** (2), 432–439 (1980).
26. Billinton, N., Knight, A. W. Seeing the wood through the trees: a review of techniques for distinguishing green fluorescent protein from endogenous autofluorescence. *Analytical Biochemistry*. **291** (2), 175–197 (2001).
27. Mosiman, V. L., Patterson, B. K., Canterero, L., Goolsby, C. L. Reducing cellular autofluorescence in flow cytometry: an in-situ method. *Cytometry*. **30** (3), 151–156 (1997).
28. Ducroux, C. et al. Thrombus Neutrophil Extracellular Traps Content Impair tPA-Induced Thrombolysis in Acute Ischemic Stroke. *Stroke*. **49** (3), 754–757 (2018).

Figure 1

Click here to access/download;Figure;Fig1.psd

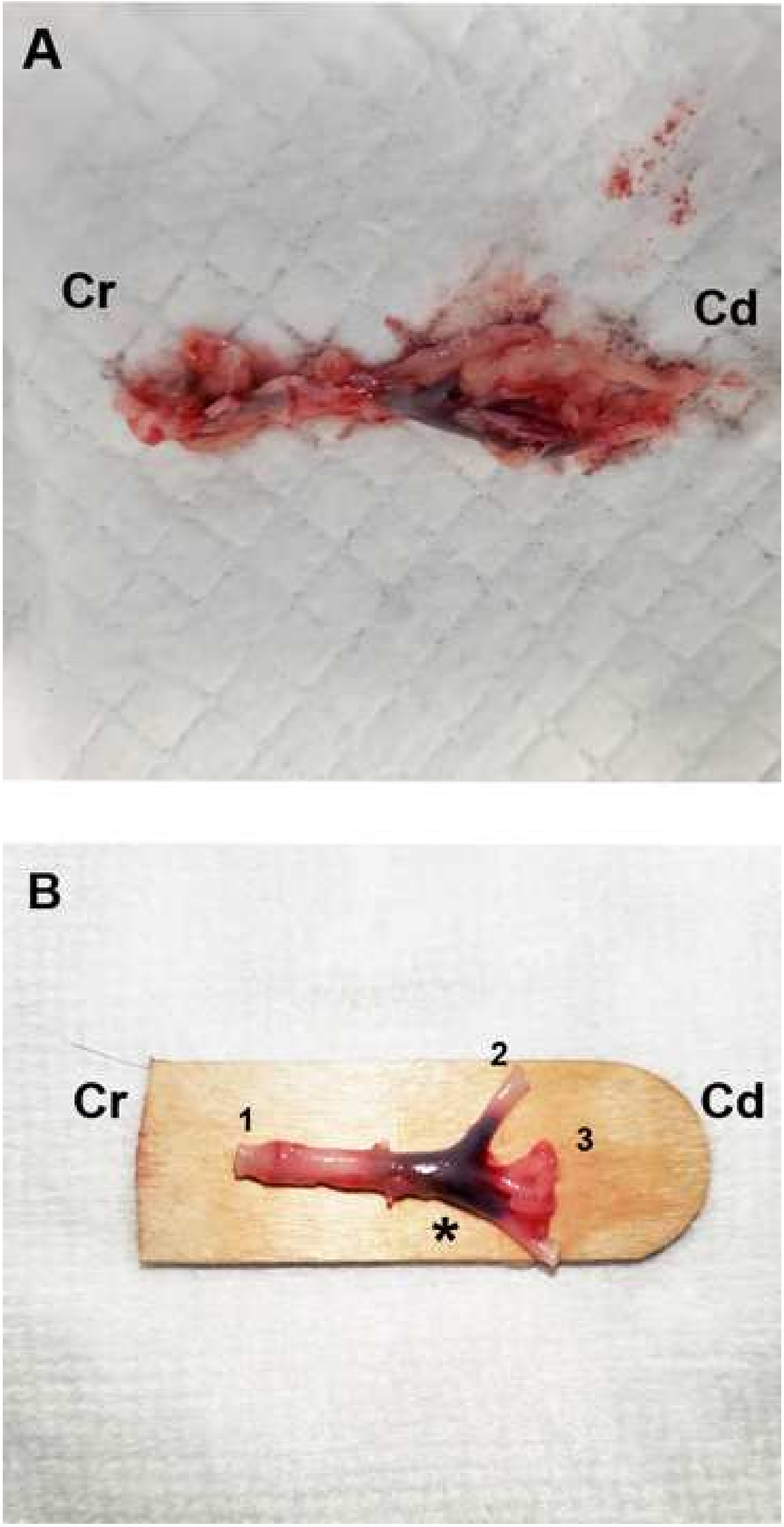


Figure 2

Click here to access/download;Figure;Fig 2.psd

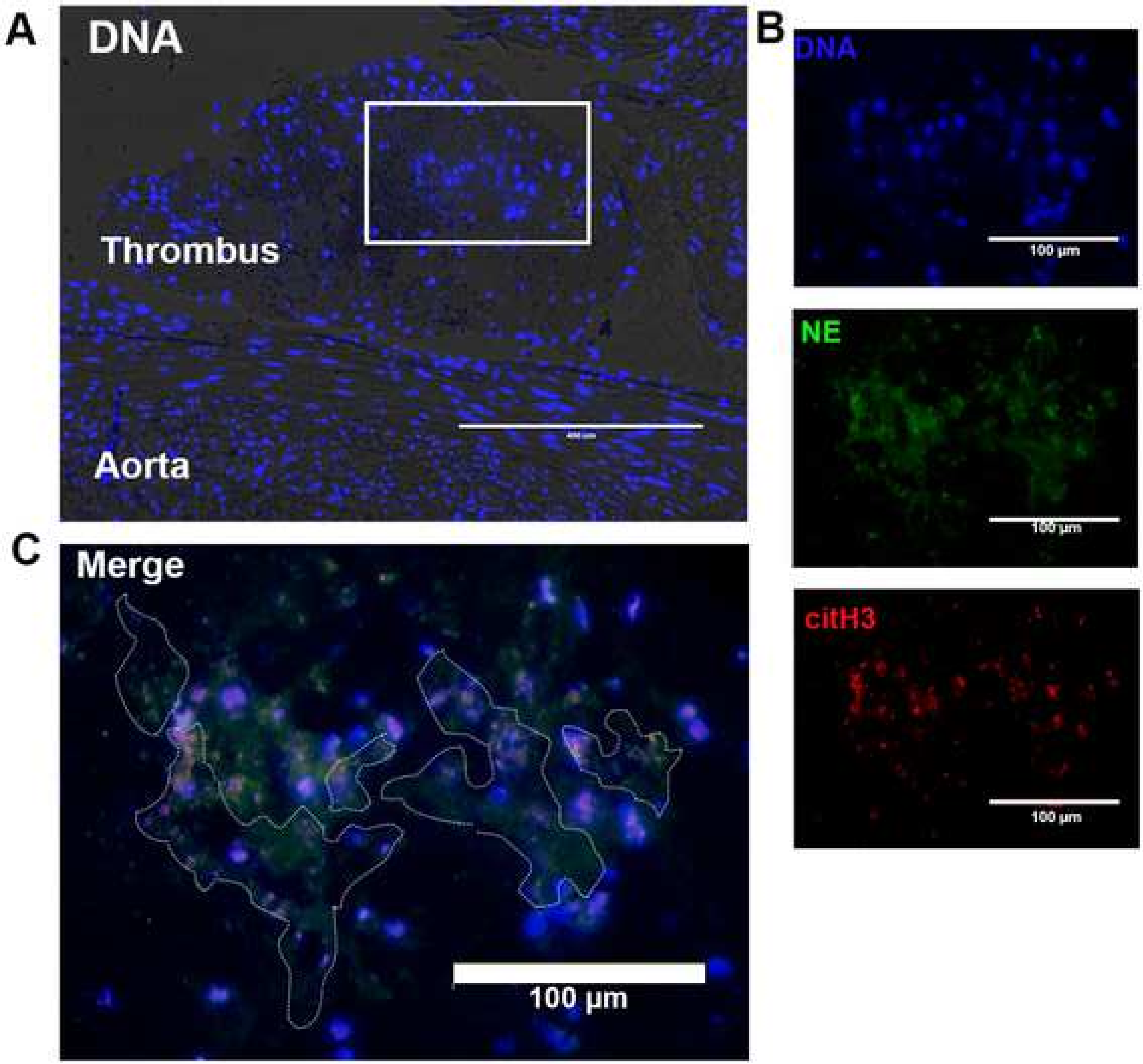
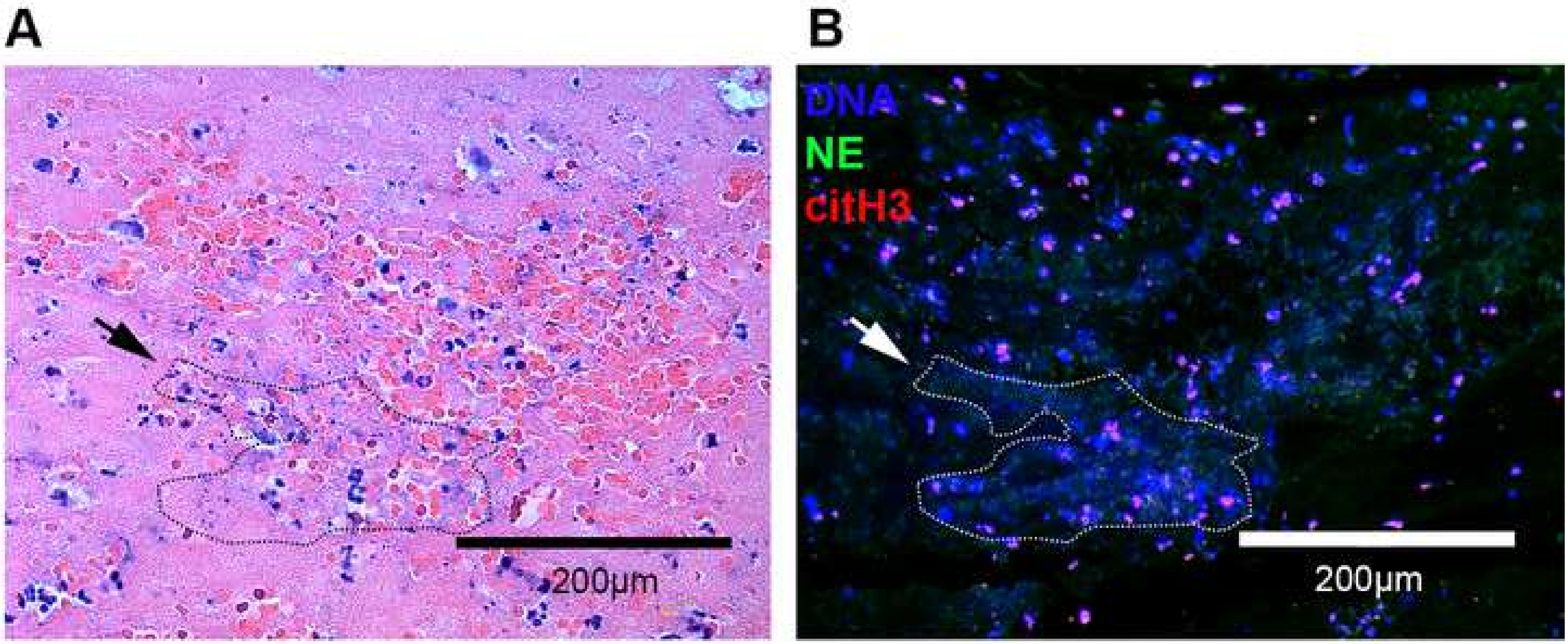


Figure 3

Click here to access/download;Figure;Fig 3.psd



Click here to access/download;Figure;Fig 4.psd

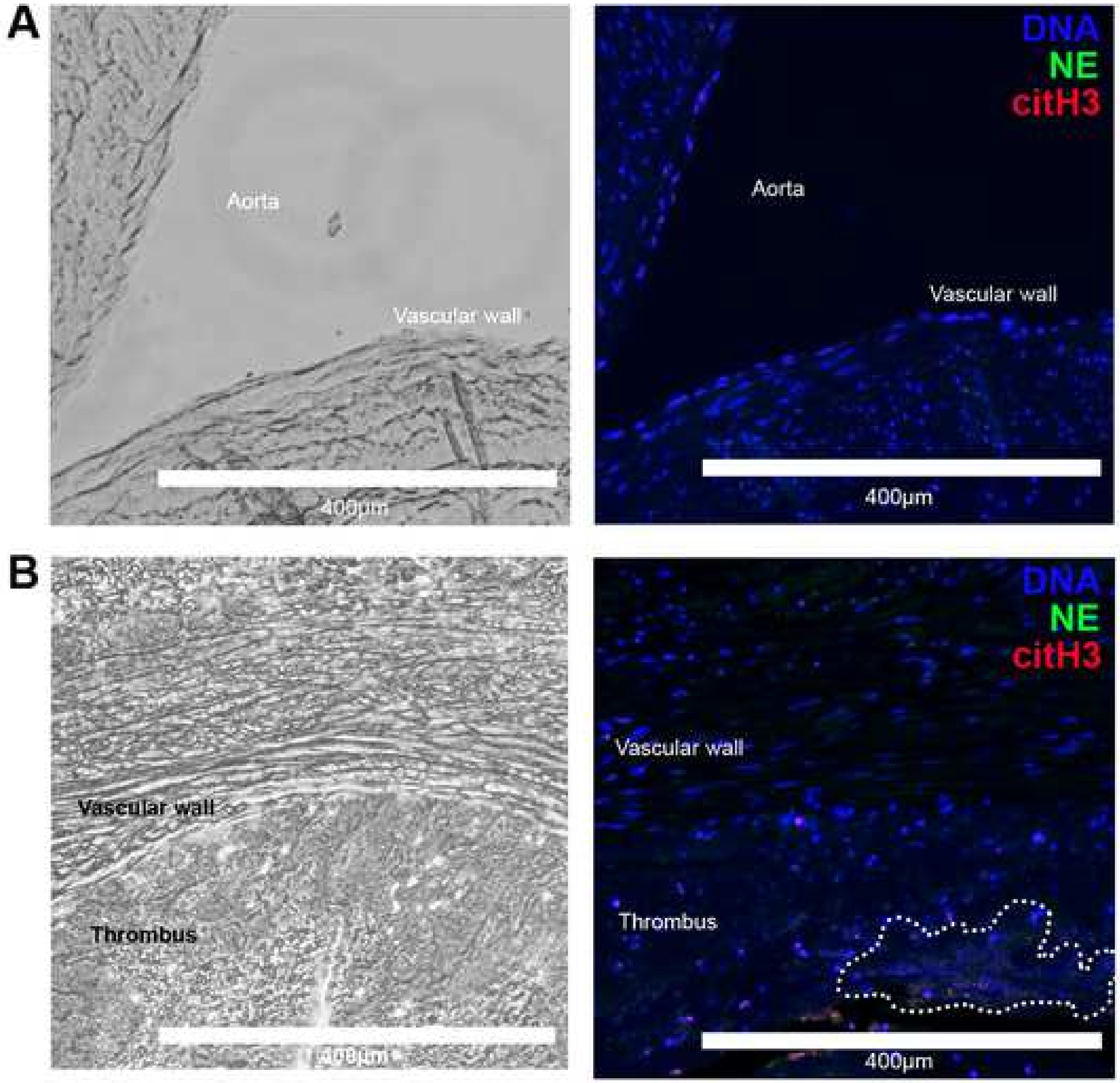
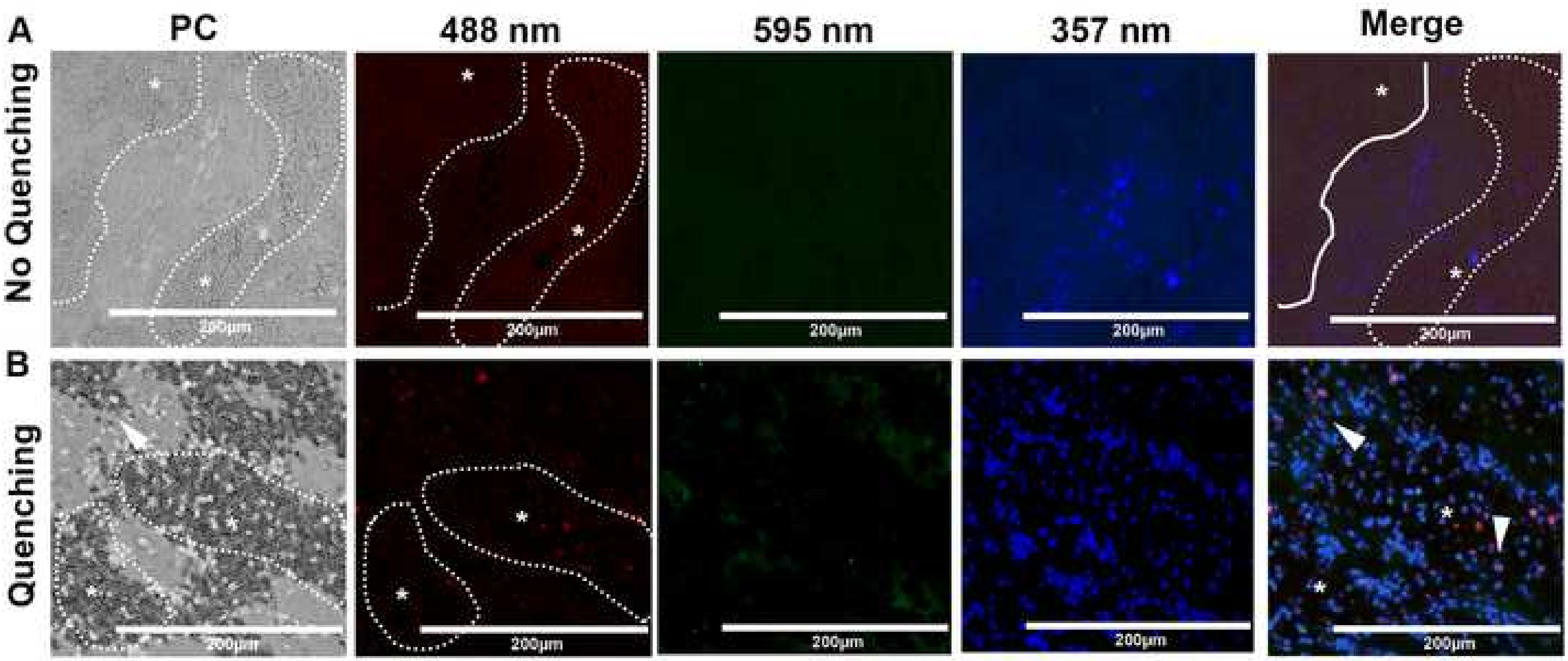


Figure 5

Click here to access/download;Figure;Fig 5.psd



Blocking Buffer	Composition
1	TBS with 0.1% Tween20, 0.1% NP40, 5% goat serum
2	TBS with 0.1% Tween-20, 10% rabbit serum, 0.1%NP-40
3	TBS with 0.1% Tween-20, 5% BSA, 0.1% NP-40

Click here to access/download;Table of Materials;Materials Table_CATE NETs.xlsx

Materials Table

Name	Company
4,6-Diamidino-2-phenylin (DAPI)	Life Technologies Corporation
Alexa Fluor 594 Streptavidin conjugate	ThermoFisher Scientific
Anti-citrullinated histone H3 antibody	Abcam
EVOS FL Cell Imaging System	ThermoFisher Scientific
EVOS Imaging System Objective 10x	ThermoFisher Scientific
EVOS Imaging System Objective 20x	ThermoFisher Scientific
EVOS Imaging System Objective 40x	ThermoFisher Scientific
Goat anti-rabbit Alexa Fluor 488 antibody	ThermoFisher Scientific
Goat serum	Jackson Immuno Research Labs
Neutrophil elastase antibody	Bioss Antibodies
NP40	Pierce
Positive charged microscope slides	Thomas Scientific
Rabbit serum	Life Technology
Target Retrieval Solution	Agilent Dako
TrueVIEW Autofluorescence Quenching Kit	Vector Laboratories

Catalog Number
D1306
Catalog # S11227
Ab5103
AMEFC4300
AMEP4681
AMEP4682
AMEP4699
Catalog # A32723
Catalog # NC9660079. Manufacturer Part # 005-000-121
Bs-6982R-Biotin
Product # 28324. Lot # EJ64292
Manufacturer No. 1354W-72
Catalog # 10510
S2367
SP-8400

Comments
NA 0.25, WD 6.9/7.45 mm
NA 0.40, WD 6.8 mm
NA 0.75, WD 0.72 mm
Rabbit polyclonal Antibody, Biotin conjugated
TRIS/EDTA, pH 9 (10x)

Click here to access/download;Rebuttal Letter;Answer to reviewers NETs identification in cats with CATE.pdf

Editorial comments

Changes to be made by the Author(s):

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.

Author response: we have thoroughly proofread the manuscript.

2. Please include email addresses of all the authors.

Author response: The email addresses of all the authors were added to the manuscript (line 20-28)

3. Please expand all abbreviation during the first-time use.

Author response: The term NETosis was ultimately removed from the manuscripts and replace by "NET formation", "NET production" or "NET release".

Author response: We ensured that all abbreviations were expanded during the 1st time use.

4. Please reword lines 136, 138-140, 141-142, 272-274, as it matches with previously published literature.

Author response:

Lines 136 (144): 3.11. Incubate sections with biotinylated polyclonal rabbit anti-human NE antibody (Final concentration: 0.2µg/ml diluted in blocking buffer 3) as described in 3.3 at 4°C for 12 to 16 hours.

Line 162 (138): Now reworded to "3.12 Wash 3 times, 5 min each, with TBST."

Line 140 (3.13): Now reworded to "3.13 Incubate with Alexa Fluor 594 streptavidin conjugate (dilute to 1:100 or 0.02mg/ml in blocking buffer 3) as described in 3.3 for 1 hour at room temperature. Protect from light and seal with paraffin to prevent drying."

*Lines 321 to 322 (Lines 273 274): Now reworded to "*Factors like sample fixation, inadequate deparaffinization and the presence of specific tissue components can lead to autofluorescence in thrombi."

5. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Author response: *Ethics statement is not required in this project because all samples were acquired at necropsy which was performed with owners' consent. We added this statement* "All methods described here were performed in accordance to the guidelines of the Institutional Animal Care and Use Committee at the University of California, Davis. Necropsy and biopsy of tissues were performed with owner consent."

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Author response: The following steps (2.1, 3.18 and 3.19) were modified using the imperative tense.

7. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

Author response: We ensured that the protocol contains only 2 to 3 action per step.

8. Please ensure that the Protocol contains only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Author response: Step 3.19 was edited to only contain actions to direct the reader.

9. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Author response: Step 1.2 (dehydration protocol using increasing concentrations of ethanol) was detailed to provide more information.

10. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution. Also, to make this a stand-alone protocol please include the dissection procedures as well starting from anesthesia process.

Author response: This was edited (see comment 5).

11. 2.1.1.-2.1.3: Please use complete sentences throughout.

Author response: These steps were edited to use complete sentences:

2.1.1 Submerge in 100% Xylene, for 3 min, 3 times.. Do not rinse in between steps.

2.1.2 Submerge in decreasing concentrations of ethanol (100%, 95%, 70%, room temperature), each for 3 min for 3 times. Do not rinse in between steps.

2.1.3 Submerge in deionized water , each for 3 min for 2 times. Do not rinse in between steps.

12. 5.1: How do you visually identify thrombi?

Author response: The following sentence was added to step 5.1: *"A thrombus is identified if a conglomeration of tissue containing red blood cells, white blood cells and platelets is present adjacent to the endothelium using phase contrast microscopy."*

13. 5.2: why only cfDNA is stained blue. Please include citation/note etc.

Author response: Cell-free DNA was differentiated from intracellular DNA based on the appearance of the chromatin. We added a note in step 5.2 to indicate the difference in appearance between cfDNA and intracellular DNA.

Lines 186 to 188: Note: cfDNA appears as decondensed DNA that are not within the confines of the cytoplasm a cell as noted on phase contrast microscopy.

14. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted the essential steps that should be included in the video.

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

All figures presented on this manuscript are original.

16. Figure 3A: Please include a scale bar.

Author response: A scale is added.

17. Figure 4: Please include a scale bar for single fluorescence image. Please make all the panels of the same size.

Author response: Figure 4 has been modified according to reviewers' suggestions. We also modified the figure as suggested. All images have scale bars and all images are the same size.

18. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the table in alphabetical order.

Author response: The table is sorted in alphabetical order and uploaded as an xls file.

Reviewers' comments

Reviewer #1:
The authors describe in the manuscript "Identification of neutrophil extracellular traps in paraffin-embedded arterial thrombi in cats using immunofluorescence microscopy" a step by step protocol how to stain NETs and investigate them with the microscope in paraffin thrombi. The method was established to reduce background signals and improve the visualization of NETs. As the analysis of tissue is nowadays more common, a well-established technique is not worse to describe.
Nevertheless, the manuscript lacks information's and in my opinion some controls that are crucial for NET quantification. Furthermore I have some detailed comments to several questions.

Major comments:
1. As the authors use different blocking buffers, it would be better to describe them as blocking buffer 1, 2 and 3. It is confusing if they describe them as new blocking buffer. Some buffers are not described exactly and therefore it cannot be reproduced. Please look carefully trough the manuscript

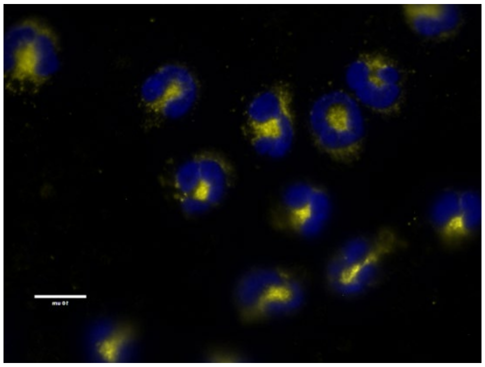
Author response: *Thank you for your suggestions. We assigned the blocking buffers numerically as suggested and listed the buffers in table format in Table 1. We added this sentence Line 137 (Step 3.1) "Table 1 details the composition of the blocking buffers used in the following steps. "*

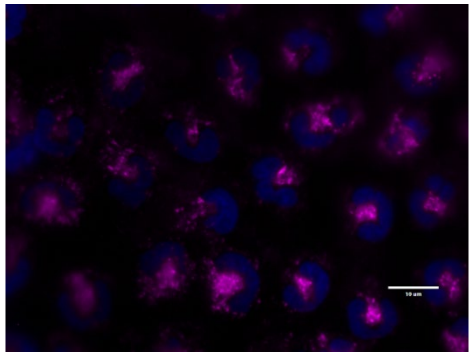
2. A description for testing the specific antibody binding is missing. Please include the preparation of an isotype control and how it is used for an adjustment of the microscope settings. Please discuss and describe if an isotype control is needed for samples coming from different animals.

Author response: *The authors agree that a detailed description of proper controls including negative controls is needed. Based on the limited space we have for the protocol, we discussed the importance of such controls in the discussion session (Lines 314 to 323). We added the following:*

Investigators should include 2 different controls that exclude either primary antibody in the second immunolabelling step to ensure that the secondary antibody from either immunolabelling step binds specifically to its primary antibody. The specificity of the identified NET structures can be further verified by DNase digestion or the inclusion of biological controls consisting of aortic bifurcations from healthy cats. In addition, negative controls consisting of the same concentration of isotype control antibodies as the primary antibodies should be included to rule out nonspecific antibody interactions, nonspecific binding to Fc receptors and cellular autofluorescence. Investigators are advised to modify this protocol based on the availability of species-specific antibodies and test the immune-specificity of antibodies using immunocytochemistry or amino acid sequence homology if no species-specific antibodies are available.

We tested the immunoreactivity of either antibodies in cats, not only via isotype control, but via testing the homology of the human nucleotide and amino acid sequences against the published/referenced feline sequences. During the process of refining the protocol, we tested feline-specific myeloperoxidase (gift from one of our colleagues) compared to a polyclonal anti-human neutrophil elastase, known to cross-reacts with multiple species. We first evaluated the Feline Neutrophil Elastase (ELANE) protein transcripts for homology to the human NE transcripts using BLAST (protein). Comparison of known feline transcripts (n=3) to known human transcripts (n=4) revealed a 99% query coverage with 75 to 77% identity and E value <4e-145. The amino acid sequence of the human histone H3: NM 001171112.1 has very high identity when compared to the feline sequence (94% positive with 0 gaps).To further confirm the specificity of the anti-human antibodies, we compare the immunoreactivity of anti-NE and anti-feline myeloperoxidase in isolated feline neutrophils using immunocytochemistry (see below). Both antibodies show localization within the cytoplasmic granules. With adequate washing and establishing optimal antibody concentrations, we found little background signals with isotype. The main issue in IF for thrombi is autofluorescence of blood cells and tissue elements – this is thoroughly discussed in the discussion section.





Neutrophil elastase Feline-specific myeloperoxidase

3. To be sure that the signals are NETs, a DNase digestion of the tissue is one option before staining. Have the authors tested this?

Author response: We did not perform a DNase digestion step but instead used aortic bifurcations in healthy cats as biological controls to determine that the structures seen in CATE are indeed NETs. However, the authors agree that this is a good way to ensure that the specificity of NET signals. We included this sentence in Discussion Section Line 318 "The specificity of the identified NET structures can be further verified by digestion using DNAase or biological controls consisting of aortic bifurcations in healthy cats."

4. Some pictures are overexposed at least in the blue channel. The authors should exchange these images with correct exposed pictures.

Author response: Thank you for pointing that out. This was likely influenced by the relatively poor quality of the uploaded figures. We have now uploaded a different format of the figures in higher quality. Please contact the editing office if you are unable to download a higher quality micrograph.

5. Line 143: Which microscope was used? Detailed information's are crucial including, camera and objective (information's found on the objective). Is a co-localization analysis possible without a confocal microscope? The authors should comment on this.

Author response: *We apologize for not including this information in the manuscript. This information is now added following Step 5. The detailed descriptions of the objective is added to the Materials Table. Based on our experiences utilizing the EVOS FL Cell imaging system, identification of NETs by colocalization technique using Image J is possible. Please refer to publications 12 and 18.*

6. Line 155: As especially the analysis by imageJ can be done with multiple ways, the authors should give one explanation for one way how they would analysis. Do the authors count positive cells, how many cells, how do they calculate NET releasing cells,

which criteria do they use for a NET positive cell? Are they analysing based on cell counting or which method do they use?

- *Author response: Thank you for your suggestions for including the methodology of how the NETs were analyzed. We first scan the entire length of the aorta and map any existing thrombi. Lines 162 to 165: "A thrombus is identified if a conglomeration of tissue containing red blood cells, white blood cells and platelets is present adjacent to the endothelium or the endocardium." We then used Image J to identify NETs based on co-colocalization of cfDNA, citH3 and NE. This method was previously published in BMC Vet Research (DOI: 10.1186/s12917-018-1523-z)*
- *Based on our experiences working in live cells and fixed tissues, it is extremely difficult to identify NETosing neutrophils in formalin-fixed tissues given the post-mortem changes, and the use of permeabilization agents. Instead, we measured the distribution of NETs within thrombi based on the anatomical location of thrombi as well as their proximity to the main descending aorta. This is now described in step 5.5. Our preliminary data suggest that the number of fields positive for NETs and the percentage of NET-occupied area were significantly higher at the proximal aspect of thrombi (closer to the descending aorta, more NETs were formed).*

7. Line 194: with the overexposed blue channel it is very difficult to identify only with DNA staining leukocytes. The authors should make this more clear e.g. arrows indicating leukocytes. Furthermore this is no possible to see in the close up pictures. The authors should try to improve the images.

Author response: We apologize for the poor quality of the images. We have uploaded images in a different file format with at least 300 dpi. Please contact the editorial office should the image quality remain inadequate for review.

8. Line 211: have the authors used for pictures in A and B the same microscope settings e.g. pin whole and smart gain,…?

We focused on the noted area (Fig 2A) at 20x magnification – hence technically the microscope setting is different as the 20x objective has a NA of 0.40 while adjusting the gains for each channel to avoid oversaturation. We included the following in step x.x
Why the authors write here pseudo-color and not in the other figures? *We have removed that as it can be confusing for readers.*
The meaning and position of arrows and arrow heads is not clear.

We clarified NETs by marking areas that we identified as NETs, instead of arrows.

Are both pictures made from one thrombus sliced after each other? If not, it could also be possible that no NETs are present? The authors should comments on this.

Author response: We assume that this comment is referring to Figure 3. These sections of the thrombus were sectioned sequentially. The authors agree that this is a very valid *point given that NETs may not be present in all sections within a thrombus. We commented on this in the Discussion Section - Line*

9. Line 259 ff: In line with this discussion the use of an isotype control is crucial and has to be included in the manuscript.

Author response: *Please see our response in Comment # 2.*

10. Line 281 ff: the standardized protocol to identify NETs is not clear described. Furthermore, subjective evaluation is becoming clearer if it is described well. Therefore this is a point that has to be included in the manuscript.

Author response: We have included a detailed description of how NETs were identified and quantified in Step 5 of the protocol.

Line 354 to 358: Although we used a standardized protocol to identify NETs, microscopic evaluation and quantification of NETs remains subjective. Herein, we utilized a blinded and systematic method to minimize observer bias during microscopic analysis. Since the number of NETs in a sample can be influenced by the amount of neutrophils, investigators can also quantify NETs relative to the number of neutrophils by identifying neutrophils based on nuclear morphology, cell diameter and expression of neutrophil-specific proteins.

11. The authors should discuss other staining's for thrombi and NETs next to thrombi in cats.

Author response:

Minor comments:
1. Line 76 small or big letters for 1A or 1b?

Author response: Thank you for picking that up. This is now corrected as the following: Figure 1A, 1B

2. Line 77 and 238 how long should the fixation be done? Minimum 24 hours or "recommend fixation for no longer than 24 hours"? Is this not depending on the size and tissue? The authors describe a minimum of formalin fixation, but is there a maximum limit as well?

Author response: The fixation time is specific to feline aortic bifurcations which are small. We added that the fixation should be "no longer than 48 hours".

3. Line 82 How long and at which temperature does the section dry? How are they stored afterwards until staining?

Author response: Line 99 we added "Store sectioned tissues at -80C until further analysis"

4. Line 87-89: is it needed to use always fresh components or how many times can these chemicals being re-used?

Author response: Thank you for your inquiry. We added this line to Line 119

"Diluted antigen retrieval solution can be stored at 4°C and reused up to 2 times."

5. Line 92: for reproducibility: what does maximum temperature mean? Give an exact value if other researches use maybe a different vegetable steamer they have to know.

Author response: Thank you for your question. The set temperature on the steamer that we use for our protocol is 100C.

6. Line 93: The authors should describe exactly the antigen retrieval solution. What are inside? Give information's as exact as possible e.g. 0.1 M EDTA final, same for TRIS. The chemical correct description is needed for reproducibility.

Author response: We use a commercially available antigen retrieval solution that unfortunately, the manufacturer does not disclose the composition of its solution. The ARS we use is included in the list of reagents with manufacturer information and catalogue number. We added " a commercially available" to Line 110.

7. Line 96 what is the temperature range in the steamer and after equilibration in the solution and is steamer again a vegetable steamer? Can point 2.3 and 2.5 run in parallel?

Author response: Thank you for your question. The steamer is a vegetable steamer. The temperature in the steamer range between 104C (against the walls) and 96C in the chamber. We would recommend performing step 2.3 (filling of the lower reservoir with water) prior step 2.5 (pouring of the heated antigen solution into a container and placement into the steamer) so the temperature equilibrates as rapidly as possible.

8. Line 103: what is the Tween concentration inside this TBST?

Author response: The Tween-20 concentration is 0.1% in this TBST. This was added in Table 1.

9. Line 105: please give order numbers for NP40 and goat serum. How long is the blocking buffer valid? Or has it to be prepared always fresh?

Author response: The NP40 and goat serum order numbers were added in the Material Table.

10. Line 106: what does constant rocking mean? Can you give a value like rpm?

Author response: We added 30 to 50 rpm. (Step 3.1)

11. Line 106 ff: is there washing step or a drying step on paper between the step 3.1 and 3.2?

Author response: There is no washing or drying in between 3.1 and 3.2. We have clarified that by adding "Without washing, immediately apply …" in step 3.2

12. Line 107 In which medium is the antibody diluted? Please refer to table with order numbers. Is the 0.03mg/ml the final concentration or the stock concentration? More detailed information's are needed.

Author response: Thank you for your suggestion. We specified in Step 3.2 that it was diluted in blocking buffer 1. In Step 3.6, diluted in blocking buffer 1 and in Step 3.13, diluted in blocking buffer 3.

13. Line 111: what does gentle rocking mean, please provide information's about rpm. What does overnight mean in hours, please give a value like 8-10 hours.

Author response: Twelve to 16 hours. We specified that in Steps 3.4.

14. Line 113 ff: describe exactly the final antibody concentration. Is the final concentration 0.04mg/ml?

Author response: Yes. We specified that in Step 3.6

15. Line 117: please describe order number of rabbit serum.

Author response: The rabbit serum order number was updated in the Material Table

16. Line 122: please describe final concentration. Is this incubation not with gentle rocking as in the other steps before?

Author response: We specified the final concentration and the specific blocking buffer in Steps 3.6 to 3.11

17. Line 126 please describe in which solution the antibody is diluted and the final concentration.

Author response: We specified the final concentration and the specific blocking buffer in Steps 3.6 to 3.11

18. Line 133: what is a sufficient amount of DAPI and how is it dissolved? Please describe more exact.

Author response: We corrected and stated that exact amount of DAPI per slide in Step 3.18. 3.18 Cover each slide with 100 µl of 300 nM DAPI for 5 min in the dark.

19. Line 149 ff: The authors should include here the usage of the isotype control and comment on possible occurring background signals of tissue in IF staining's.

Please see response to comment #2.

20. Line 196ff: is this a 10x, 20x,…. zoom magnification or objective? Please describe this only in detail in the material and methods. The information about scale bar is enough in the figure legend or as already included in each picture.

Author response: *Thank you for your suggestion. We have changed the wording to objective and included the NA of each objectives used for our analysis.*

21. Line 199: both arrows show black areas. Please put them more at specific points.

Author response: Instead of arrows, we used dotted lines to map out the area of NETs in the figure.

22. Line 208 ff: The IF picture is over saturated, therefore please provide another picture and present an isotype control. Again the arrow shows a black area. The authors should try to make clear what is the NET area.

Author response: *We apologize if that indicated NET area is not clear in the figure. Please see comment above.*

23. Line 254 ff: the authors should show examples of myeloperoxidase staining's, even if they are not working perfect. A sentence data not shown should not be used.

Author response: *These images are now shown as a supplemental figure.*

Reviewer #2:
Manuscript Summary:
This MS presents a protocol for detection of NETs in feline paraffin-embedded thrombi.

Major Concerns:
The protocol demands three overnight incubations because both primary antibodies are raised in rabbit. This is very time consuming; the anti-NE antibody is biotinylated and detected via streptavidin which may result in unspecific staining due to endogenous biotin if the method is used for NET detection in tissue samples other than thrombi. The protocol would be much quicker and possibly more specific if the authors could use an antibody crossreacting with feline NE which was not raised in rabbit, so tissue samples could be reacted with both primary antibodies simultaneously which would be detected via species-specific secondary antibodies.

Author response: *We appreciate the feedback provided by the reviewer. During the process of refining the protocol, we tested feline-specific myeloperoxidase (gift from one of our colleagues) compared to a polyclonal anti-human neutrophil elastase, known to cross-reacts with multiple species. We first evaluated the Feline Neutrophil Elastase (ELANE) protein transcripts for homology to the human NE transcripts using BLAST (protein). Comparison of known feline transcripts (n=3) to known human transcripts (n=4) revealed a 99% query coverage with 75 to 77% identity and E value <4e-145. With this information, we compared the immunoreactivity of feline-specific myeloperoxidase and NE. While the feline-specific myeloperoxidase performed well on immunocytochemistry in isolated feline neutrophils, its immunoreactivity was poor in paraffin-embedded/antigen-retrieved tissue (Lines 288 to 293). We have included this data as a supplemental figure. Although not ideal, we used a biotinylated primary antibody to avoid interferences during the second immunolabeling step since both primary antibodies originate from rabbits. We strongly advise investigators to block adequately with BSA to avoid non-specific binding and to include controls (1. Exclusion of either primary antibody in the second immunolabelling step to ensure the specificity of its primary antibody 2. Inclusion of a negative control consisting of isotype antibody of the same concentration and conditions as the primary antibody 3. Biological control using healthy cats. We have emphasized the above points by including an additional paragraph in the Discussion section.*

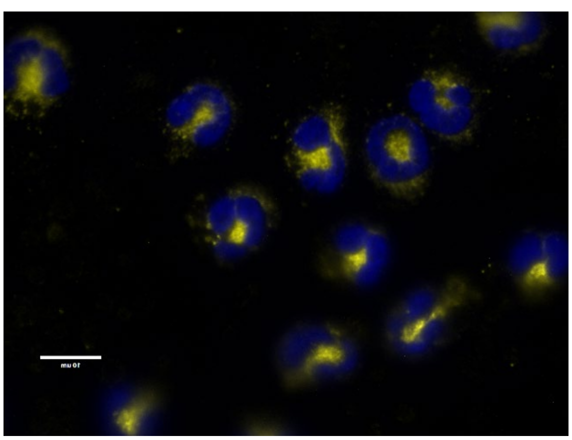
"One of the challenges of NETosis research in veterinary species is the lack of species-specific antibodies. To prevent the interference encountered when using primary antibodies originating from the same species, we included an additional blocking step utilizing a high concentration of rabbit immunoglobulins to saturate any remaining binding sites on the goat anti-rabbit secondary antibodies. A major disadvantage of this technique is that it is time consuming as it requires multiple incubation steps. Investigators should include 2 different controls that exclude either primary antibody in the second immunolabelling step to ensure that the secondary antibody from either immunolabelling step binds specifically to its primary antibody. The specificity of the identified NET structures can be further verified by DNase digestion or the inclusion of biological controls consisting of aortic bifurcations from healthy cats. In addition, negative controls consisting of the same concentration of isotype control antibodies as the primary antibodies should be included to rule out nonspecific antibody interactions, nonspecific binding to Fc receptors and cellular autofluorescence. Investigators are advised to modify this protocol based on the availability of species-specific antibodies and test the immune-specificity of antibodies using immunocytochemistry or amino acid sequence homology if no species-specific antibodies are available. "

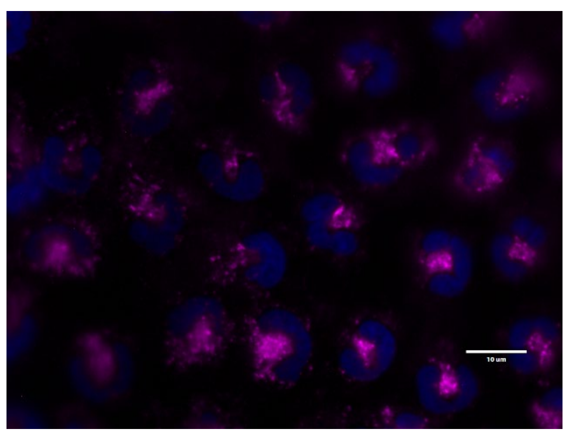
*Line 290 now reads "*The poor immunoreactivity of myeloperoxidase was consistent despite using a feline specific antibody"

We included images of tissues probed with MPO and NE to strengthen these statements as a supplemental figure.

We also evaluated the homology of the human nucleotide and amino acid sequences against the published/referenced feline sequences for Histone H3: NM 001171112.1, neutrophil elastase EF576804). The mRNA of histone H3 and NE were highly homologous (94% positive with 0 gaps, 78% positive, 2%).

To further confirm the specificity of the anti-human antibodies, we compare the immunoreactivity of anti-NE and anti-feline myeloperoxidase in isolated feline neutrophils using immunocytochemistry (see below). Both antibodies show localization within the cytoplasmic granules.





Neutrophil elastase Feline-specific myeloperoxidase

Minor Concerns:
Line 64 bright field microscopy

Author response: Thank you for your input, the sentence was modified to the following (line 71): "The presence of NETs is detected by colocalization of extracellular neutrophil proteins, histones and NE. Because of this, the identification and quantification of NETs in fixed tissues by immunofluorescence of deparaffinized tissues is superior to traditional Hematoxylin and Eosin stain using bright field microscopy".

Line 79 ethanol concentrations in reverse order, incubation times seem a bit short

Author response: That was a typing error. Please see Steps 1.2.1 to 1.2.3 for the correct protocol.

1.1 Dehydrate the sample by immersing in increasing concentrations of ethanol in the following order:

1.2.1 Submerge in 10% neutral-buffered formalin (heated to 37°C) for 1 hour.
1.2.2 Submerge in increasing concentrations of ethanol (70%, 95%, 100%, heated to 37°C) each for 1 hour for 2 times.
1.2.3 Without rinsing, submerge in 100% toluene (heated to 37°C) for 1 hour, for 2 times.

Line 81: incubation time with liquid paraffin? Does not dry but solidifies.
Author response: Step 1.3 of the protocol was edited (line 100): "1.3 Add heated paraffin (at 62°C) and allow the paraffin to solidify completely overnight."

Line 107: the antibody is diluted in which buffer?

Author response: The antibodies (line 136) were diluted in goat serum. This step and other blocking steps are clarified using Table 1 detailing the composition of different buffers used in this protocol.

Line 111: how are the specimens sealed?

Author response: The specimens were tightly sealed with paraffin film. This was added to step 3.4.

Line 146+161+164: bright field

Author response: "Light-transmission miscroscopy" was replaced by "bright field microscopy" in the manuscript.

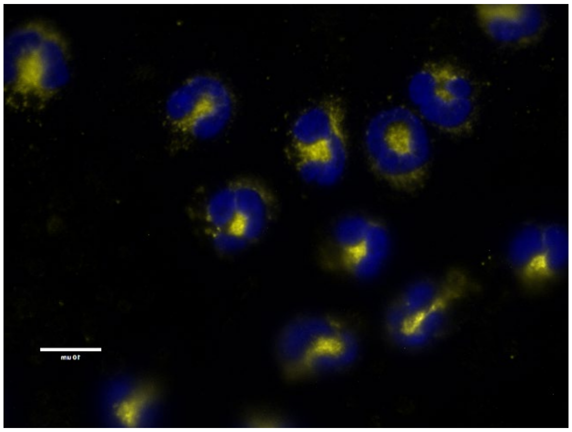
Reviewer #3:
Manuscript Summary:
Manuscript by Duler et al. is an interesting contribution into NET detection methodology in cats although the technique can be applied in any other vertebrate species (the only limiting factor being antibody availability for the species).

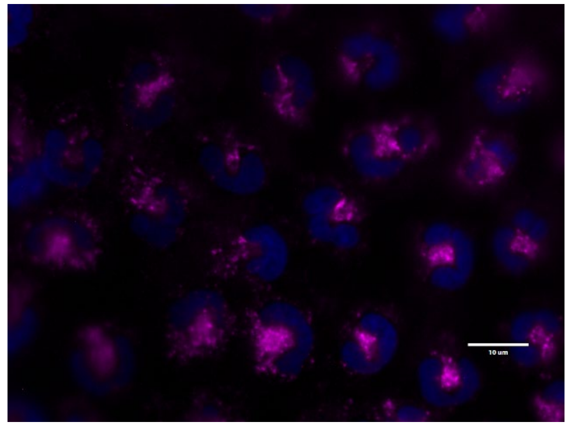
Major Concerns:
- At least in the pdf file sent out for review the images are of very poor quality (pixels) which makes their assessment very difficult while they are key for the story

Author response: *We apologize for the poor quality of the images in the PDF format. As per request by the editor, we uploaded a uploaded micrographs that are higher in quality.*

- both primary antibodies used in the protocol are against human proteins. How was their cross-specificity verified for cat epitopes?

Author response: During the process of refining the protocol, we tested feline-specific myeloperoxidase (gift from one of our colleagues) compared to a polyclonal anti-human neutrophil elastase, known to cross-reacts with multiple species. We first evaluated the Feline Neutrophil Elastase (ELANE) protein transcripts for homology to the human NE transcripts using BLAST (protein). Comparison of known feline transcripts (n=3) to known human transcripts (n=4) revealed a 99% query coverage with 75 to 77% identity and E value <4e-145. With this information, we compared the immunoreactivity of feline-specific myeloperoxidase and NE. While the feline-specific myeloperoxidase performed well on immunocytochemistry in isolated feline neutrophils, its immunoreactivity was poor in paraffin-embedded/antigen-retrieved tissue (Lines 288 to 293). We have included this data as a supplemental figure. Although not ideal, we used a biotinylated primary antibody to avoid interferences during the second immunolabeling step since both primary antibodies originate from rabbits. We strongly advise investigators to block adequately with BSA to avoid non-specific binding and to include controls (1. Exclusion of either primary antibody in the second immunolabelling step to ensure the specificity of its primary antibody 2. Inclusion of a negative control consisting of isotype antibody of the same concentration and conditions as the primary antibody 3. Biological control using healthy cats. We have emphasized the above points by including an additional paragraph in the Discussion section.





Anti-human neutrophil elastase Antibody *Anti-feline myeloperoxidase antibody*

Application of DAPI for cfDNA identification is controversial. Some papers, especially the early work on NETs, did use DAPI to stain for DNA, however, it is now recognized that the dye displays a disperse signal (cloudiness) when DNA is not condensed (as in nucleus) and strings of DNA (characteristic for NETs) cannot be seen. Moreover, it penetrates all cells (live, dead, NETting). It is strongly recommended to use one of the Sytox dyes that allow for clear denotation of cfDNA strings. With this approach one can clearly identify if NE and histones are located along the DNA threads and not within the cells (especially that the buffers contain permeabilizing detergents). If the authors still have samples, they are strongly encouraged to perform additional staining with Sytox (e.g. green) for cfDNA and for e.g. histones.

Author response: Unfortunately, we did not utilize the use of Sytox dyes as our sole DNA stain. Since the sections had to be permeabilized for detection of intracellular proteins, we do not feel that the use of a cell-impermeable nucleic acid dye provides further specificity in differentiating NETosing and necrotic cells. We have used Sytox Green in live cells and DAPI in permeabilized and paraformaldehydge-fixed cells. We have included this in our discussion as our limitation.

Lines 358 to 360: In addition, instead of DAPI, NETs can be stained using Sytox Green for clearer identification and denotation of cell-appendant DNA from NETosing neutrophils.

- no data is presented from a control tissue of unaffected cats. Some information (text) is provided at the end of "Representative results" but this must be shown on images for comparison with the CATE case

Author response: We completely agree with the reviewer's recommendation and, therefore, added an additional figure to show the the phase contrast and *immunofluorescence images of aortic bifurcations from a cat with CATE and another cat without CATE. Now Figure 4.*

- Of note, Discussion is very interesting and well written. The authors provide useful information and share their experience, e.g. on temperature adjustment for various antigen detection and provide some useful tricks. What temperature was then optimal for MPO detection (epitope retrieval) with the tested antibodies?

Despite testing for different temperatures, the immunoreactivity for MPO was low following antigen retrieval process. We have tested 2 different antibodies, including a feline specific antibody, to rule out the issue of antigen specificity.

Other important concerns:

Introduction

- "NETosis" - currently the term NETosis was reevaluated and its application is not recommended any more (except of clear cases of cell death empirically confirmed) - see Galluzzi et al. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. Cell Death Differ 2018; 25: 486- 541. Expressions such as "NET formation", "NET release", "NET production" etc. should be used instead.

Author response: We value the reviewer's opinion on this. The term NETosis was ultimately removed from the manuscripts and replaced by "NET formation", "NET production" or "NET release".

- "Neutrophils undergo NETosis in response to systemic inflammation, direct encounter with pathogens and, most importantly, interaction with activated platelets4-7." "Most importantly" for the authors of the manuscript but not in general (please remove)

Author response: This term (line 67) was removed from the manuscript.

Protocol

- p. 1, line 82, "positively charged slides" it should be explained how to obtain them (commercially available?)

Author response: These slides can be purchased commercially. We included this item in our Materials Table.

- p.2, the vegetable steamer surprisingly appears in the text out of nowhere (2.3); it should be commented on it as it is an unusual lab equipment - is it the steamer to which the authors refer to in 2.1 as a manual system? Also, examples of possible automated systems would be helpful.

Author response: Thank you for pointing that out. We added the following sentence in 2.3. "2.3 Heat-induced antigen retrieval is best performed with indirect heating generated by a steamer with a preset temperature setting Fill the reservoir with deionized water and temperature should be set at 100^{O}C. Allow the temperature of the steamer to equilibrate for 20 min.

No vegetable steamer is used step 2.1. The lab equipment mentioned in step 2.1 (deparaffinization) is an automated system using serial bathes of different concentrations of ethanol to rehydrate the samples and remove the paraffin (see step 2,1). The vegetable steamer isn't used prior to step 2.3. With this specific type of vegetable steamer, only the steaming duration can be manually adjusted. The temperature is set up to 100C and cannot be manually changed. We speficied

- Table of Materials is missing: secondary antibody for alphaH3 and streptavidin AF594

Author response: We have added the information in the Materials Table.

- 3.2, 3.4 and subsequent incubations - the slides should be placed in a humidified chamber in addition to parafilm to avoid drying

Author response: We did not have to place the slide in a humidified chamber since coverslips were used to prevent blocking buffers from evaporating during the incubations. With proper sealing using parafilm, we did not encounter any issues.

- 5.5. provide more information on how NETs can be quantified with Image J (the binary system?)

Author response: Because our protocol utilizes colocalization of cfDNA, citH3 and NE or MPO (see publications # 12, 18), we do not routinely quantify NETs using the binary system on Image J. We added a reference in Step 5.4 to provide more information. We added a step in 5 for further details in the quantification of NETs in aortic thrombi.

Figure legends/Figures
- legend to Fig. 2A, presence of cfDNA alone does not justify a claim that these are NETs

Author response: We have corrected the figure legend to "The box area consists of a large concentration of cfDNA".

- Fig. 2C the arrows do not seem to point out NETs (especially the one of the right hand side)

Author response: *We apologize if NETs were not clearly indicated in the figure. We how clarified this by encircling NETs using dotted lines on the figure. The figure legend now reads*

*"**Figure 2.** Representative phase contrast and immunofluorescence images of NETs in a thrombus found within the aortic bifurcation of a cat. (A) Phase contrast microscopy revealed a thrombus as a discrete and well demarcated structure close to the aorta. Combined phase contrast and fluorescence staining of DNA (blue) showed the presence of leukocytes and cell-free DNA within the thrombus. The boxed area consists of a large concentration of cell-free DNA. Original 10x magnification. Scale bar = 400μm. (B) The boxed area was further magnified at 20x. Cell-free DNA and intracellular DNA were stained with DAPI (blue), neutrophil elastase (NE) and citrullinated histone H3 (citH3) appeared green and red, respectively. Original 20x magnification. Scale bar = 100μm. (C) NETs ,identified based on co-localization of cell-free DNA, extracellular NE and citH3, were outlined (dotted line). Original 20x magnification. Scale bar = 100 μm.*

- Fig. 3 the arrows are located in different positions while they are supposed to point out the exact same NETs

Author response: Thank you for pointing that out. We moved the arrow in 3B so that it lines up with the black arrow in 3A. We also encircled the same area of NETs in both images. The figure legend is edited accordingly.

Representative results

- The conclusions from the fluorescence quenching experiment are not presented/described here while interpretation of these images is needed. Firstly, to verify if the quenching system worked the authors need to show images from phase contrast microscopy (how are we to identify red blood cells with fluorescence microscopy if they were not stained with any antibodies?). What is the following claim based on? "Quenching significantly reduced autofluorescence across the wavelengths and enhanced the detection of co-localized proteins within NETs and intracellular expression of citH3 (arrowhead)." The arrowheads do not seem to point out clearly to any structure.

Author response: Thank you for your suggestions. We edited figure 4 (now Figure 5) to include phase contrast images of thrombi containing an abundance of erythrocytes. To clarify, we reworded the figure legend as follow:

Representative phase contrast (PC) and immunofluorescence images of arterial thrombi from 2 cats stained for citrullinated histone H3, neutrophil elastase and cfDNA and imaged at 488 nm *(red), 595 nm (green), and 357 nm (blue) wavelengths, respectively, at 40x magnification. Cardiogenic arterial thrombi in cats have an abundance of erythrocytes (*, dotted line). (A) Autofluorescence from erythrocytes was most prominent across the 488 nm wavelength diminishing the detection of co-colocalization signal and identification of NETs. (B) Quenching significantly reduces autofluorescence at the 488 nm wavelength, especially in areas with a high concentration of erythrocytes (*, dotted line). It enhances the detection of co-localized proteins, citH3 and neutrophil elastase (arrowhead), in presence of erythrocytes. Scale bar = 200 μm.*

<u>Minor Concerns:</u>
- Introduction, p. 1, line 58 "neutrophil-deprived" or "neutrophil-derived"?

Author response: “neurophil-devrived DNA” is the correct term. This was updated in the manuscript.

- p.3, line 133, 3.18 use DAPI instead of 4',6-diamidino-2-phenylindole as the former, but not the latter, is identifiable to all

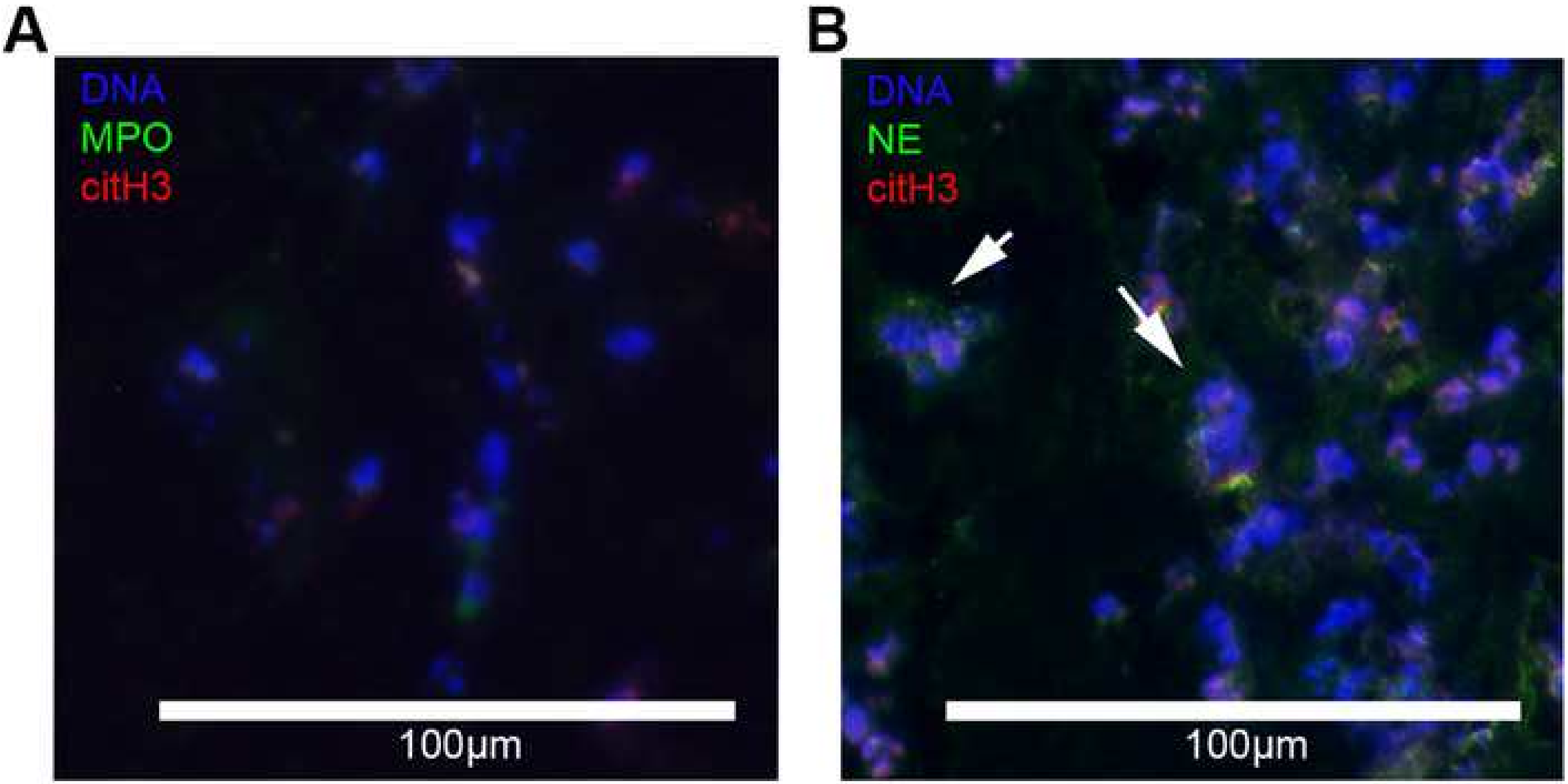
Author response: Step 3.18 was edited in the manuscript using DAPI instead of 4',6-diamidino-2-phenylindole.

- Cd is not explained in the legend to Fig. 1

Author response: Cd (caudal) is now explained in Fig 1.

Supplemental Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supple Fig1.psd



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Cambridge, MA 02140
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Author(s):	Laetitia Duler, Nghi Nguyen, Eric Ontiveros, Ronald H. L. Li

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CORRESPONDING AUTHOR

Name:	Ronald H L Li
Department:	Veterinary Surgical and Radiological Sciences
Institution:	University of California, Davis
Title:	Assistant Professor, Small Animal Emergency and Critical Care

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