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

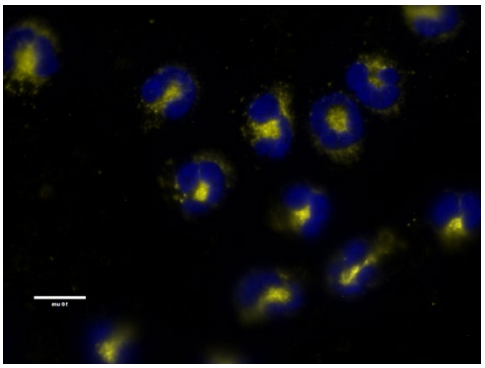
Author response: *Thank you for your suggestions. We assigned the blocking buffers numerically as suggested and listed the buffers in table format in Table 1. We added this sentence Line 137 (Step 3.1) "Table 1 details the composition of the blocking buffers used in the following steps. "*

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.

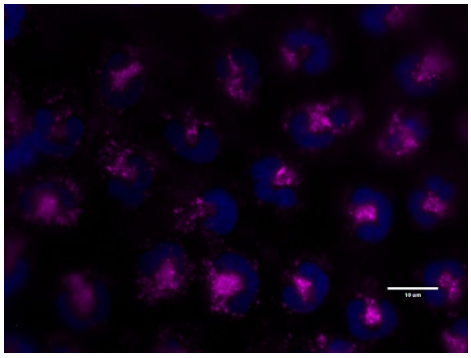
Author response: *The authors agree that a detailed description of proper controls including negative controls is needed. Based on the limited space we have for the protocol, we discussed the importance of such controls in the discussion session (Lines 314 to 323). We added the following:*

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.*

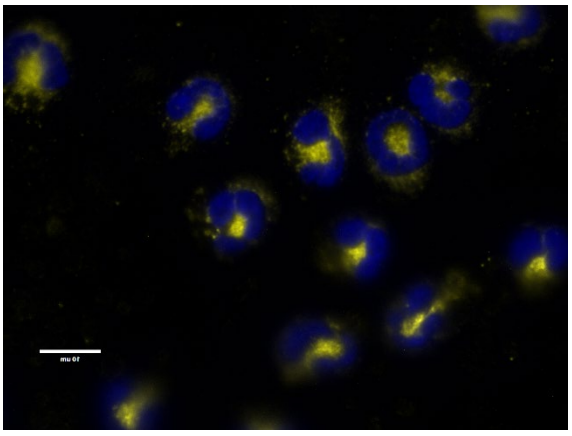
"One of the challenges of NETosis research in veterinary species is the lack of species-specific antibodies. To prevent the interference encountered when using primary antibodies originating from the same species, we included an additional blocking step utilizing a high concentration of rabbit immunoglobulins 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. "

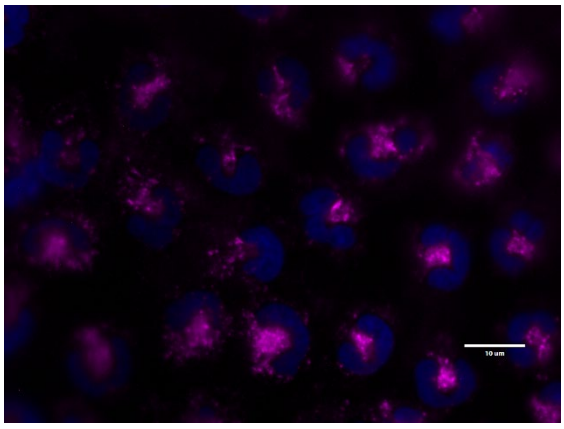
*Line 290 now reads "*The poor immunoreactivity of myeloperoxidase was consistent despite using a feline specific antibody"

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^{O}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.

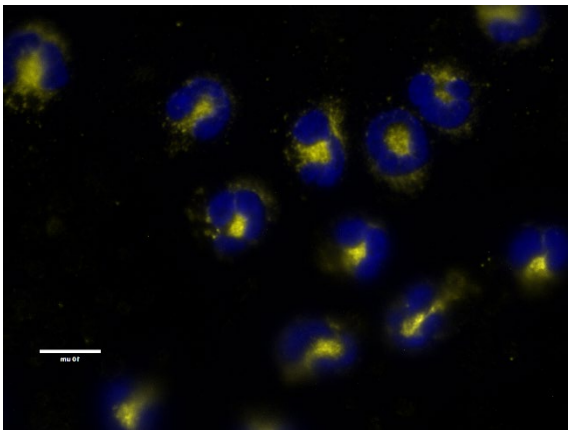
Reviewer #3:
Manuscript Summary:
Manuscript by Duler et al. is an interesting contribution into NET detection methodology in cats although the technique can be applied in any other vertebrate species (the only limiting factor being antibody availability for the species).

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

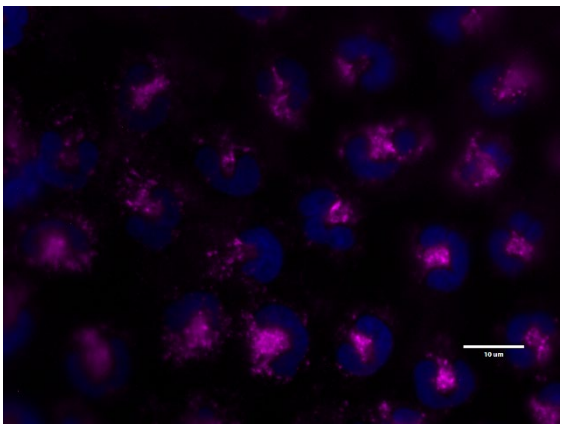
Author response: *We apologize for the poor quality of the images in the PDF format. As per request by the editor, we uploaded a uploaded micrographs that are higher in quality.*

- 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.*

Minor Concerns:

- 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.