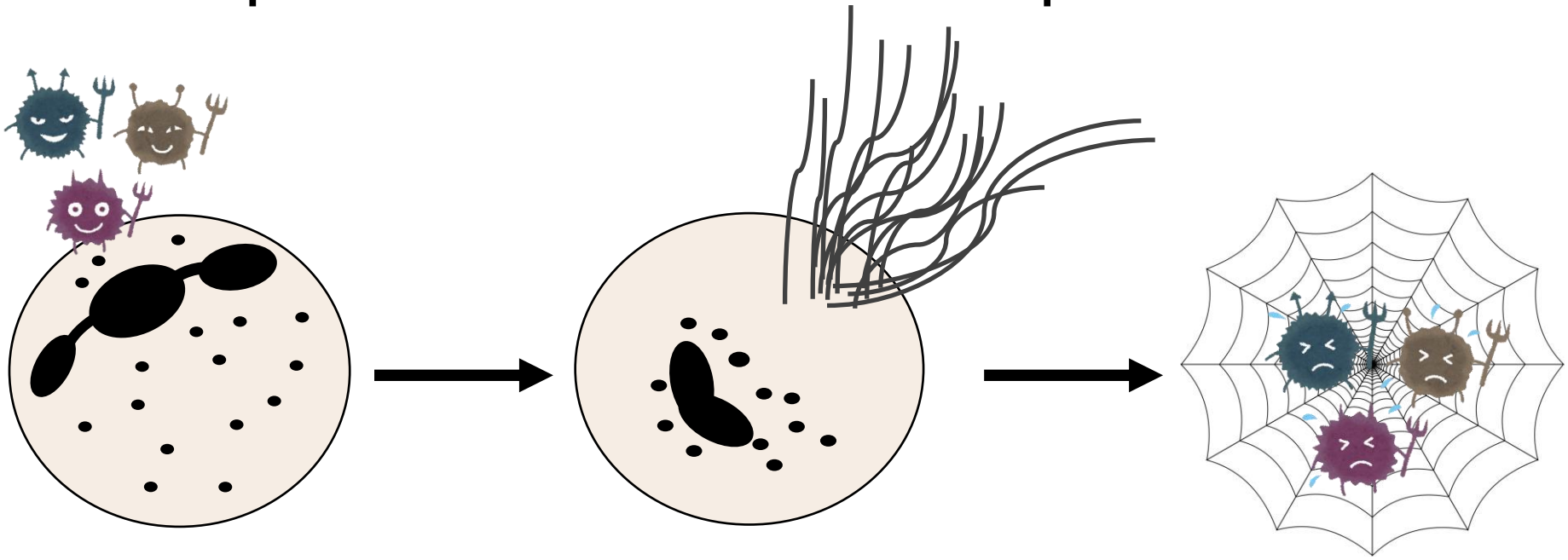
Visualization of neutrophil extracellular traps (NETs) in histopathology and cytology specimens

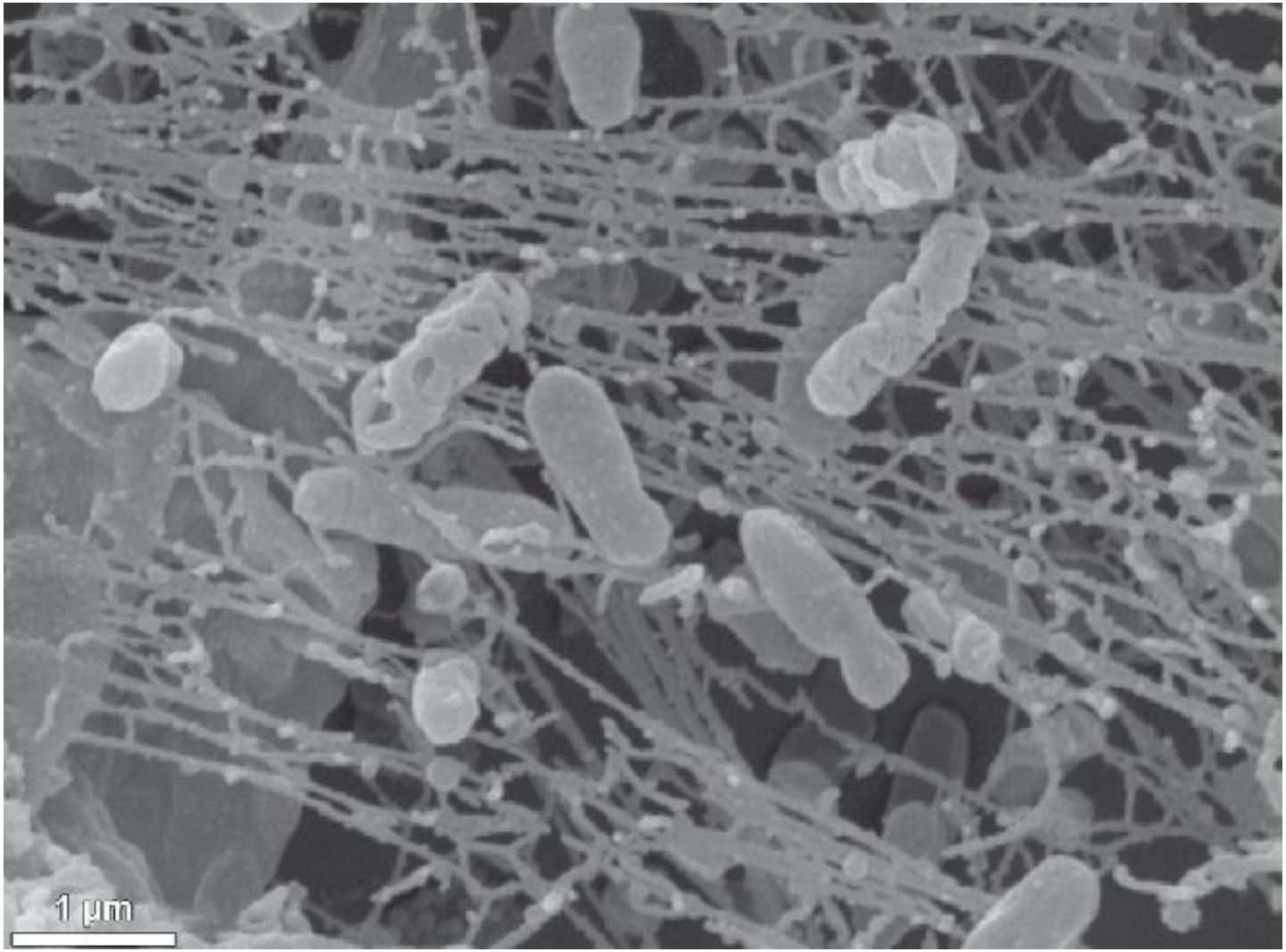
Neutrophil extracellular traps (NETs) trap to kill microorganisms by forming spiderweb-like structures. NETs are composed of degraded chromatin and granules of neutrophil origin. NETs can be visualized in formalin-fixed, paraffin-embedded sections and in cytology specimens by immunostaining for lactoferrin, citrullinated histone H3 and myeloperoxidase. NETs can be distinguished from fibrinogen gamma chain-positive fibrin fibrils. NETs are suspected in HE- and Pap-stained specimens, particularly when the deposited fibrils appear thin and basophilic.

Presented by Prof. Kazuya Shiogama at the Department of Morphology and Cell Function, Fujita Health University, Toyoake, Aichi, Japan, at the 13th Japan-China Joint Seminar on Histochemistry and Cytochemistry held in Kobe, Japan in October, 2019.

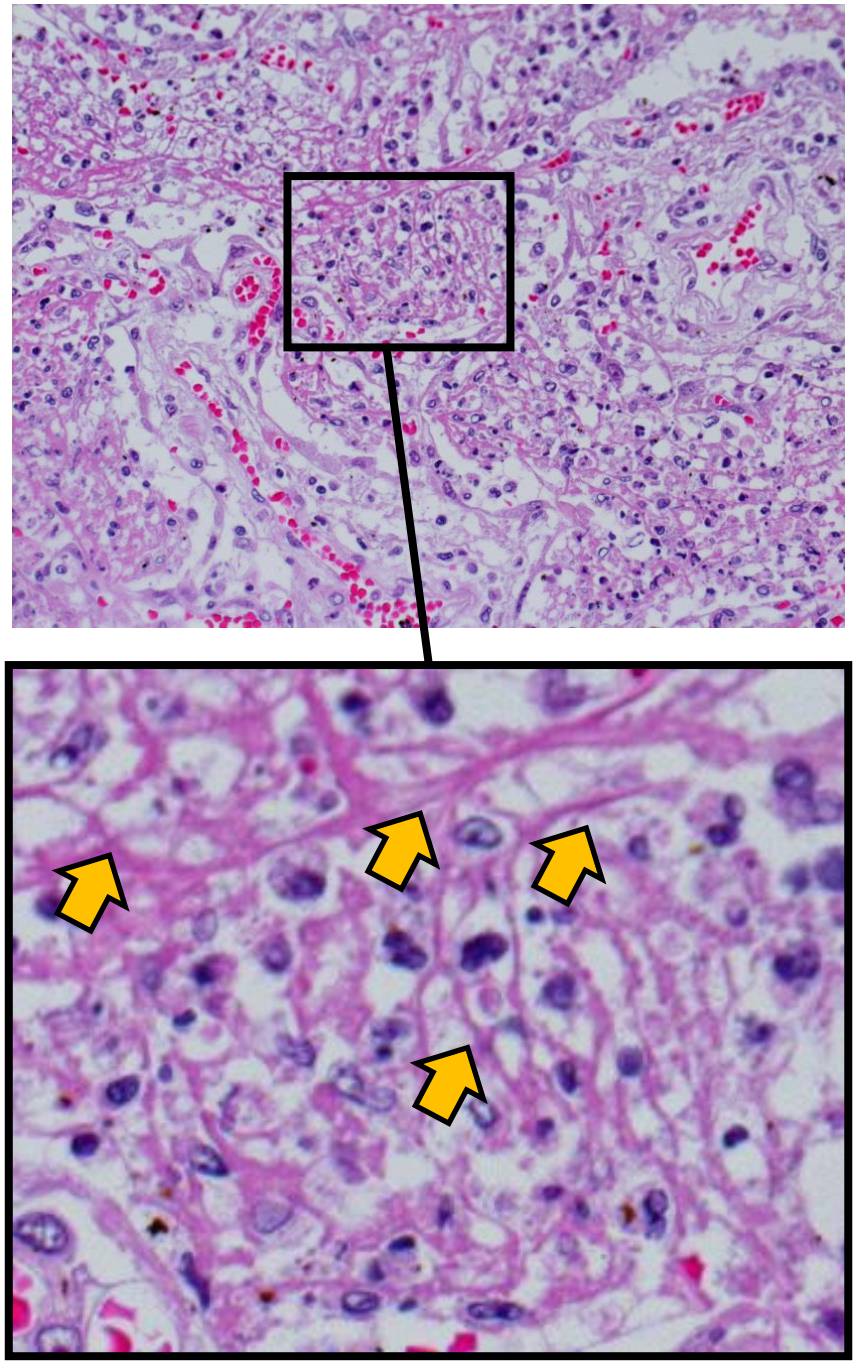
Neutrophil Extracellular Traps : NETs



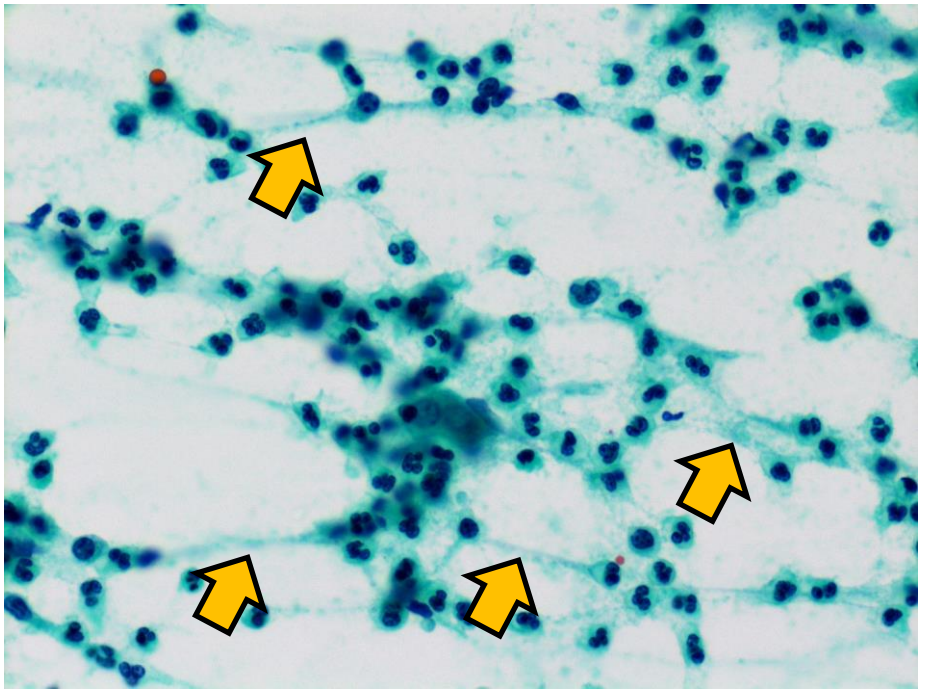
In 2004, Brinkmann and colleagues reported that stimulated neutrophils can produce spiderweb-like extracellular fibrils called neutrophil extracellular traps (NETs) that trap to kill microorganisms. NETs are composed of degraded chromatin and granules of neutrophil origin.



SEM of in-vitro induced NETs entrapping Pseudomonas bacteria:
Cited from Figure 1 by Obermayer A, et al. Emerging evidence supports the hypothesis that neutrophil extracellular traps are a major factor in genesis and progression of chronic obstructive pulmonary disease. J Immunol Sci 2018; 2(5): 31-37. doi: 10.29245/2578-3009/5.1161



Both fibrinand NETs are included in the fibrillar materials in a lung abscess lesion.



Detection of NETs in formalin-fixed, paraffin-embedded sections of fibrinopurulent inflammation

Ref.-1. Shiogama K, et al. Visualization of neutrophil extracellular traps and fibrin meshwork in human fibrinopurulent inflammatory lesions: I. Light microscopic study. *Acta Histochem Cytochem* 2016; 49 (4): 109-116. doi: 10.1267/ahc.l6015

Ref.-2. Onouchi T, et al. Visualization of neutrophil extracellular traps and fibrin meshwork in human fibrinopurulent inflammatory lesions: II. Ultrastructural study. *Acta Histochem Cytochem* 2016; 49 (4): 117-123. doi:10.1267/ahc.l6016

Ref.-3. Onouchi T, et al. Visualizationof neutrophil extracellular traps and fibrin meshwork in human fibrinopurulent inflammatory lesions: III. Correlative light and electron microscopic study. *Acta Histochem Cytochem* 2016; 49 (5): 141-147. doi: 10.1267/ahc.l6028

Aims:

Shiogama K et al. *Acta Histochem Cytochem* 49 (4): 109-116, 2016.
Onouchi T et al. *Acta Histochem Cytochem* 49 (4): 117-123, 2016.
Onouchi T et al. *Acta Histochem Cytochem* 49 (5): 141-147, 2016.

We immunohistochemically investigated how NETs are involved in the process of fibrinous inflammation using formalin-fixed, paraffin-embedded (FFPE) and cytology specimens.

Detection of NETs using FFPE sections

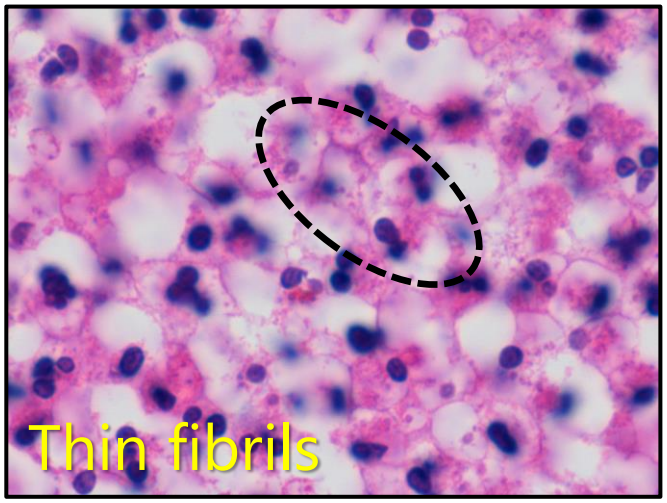
Samples:

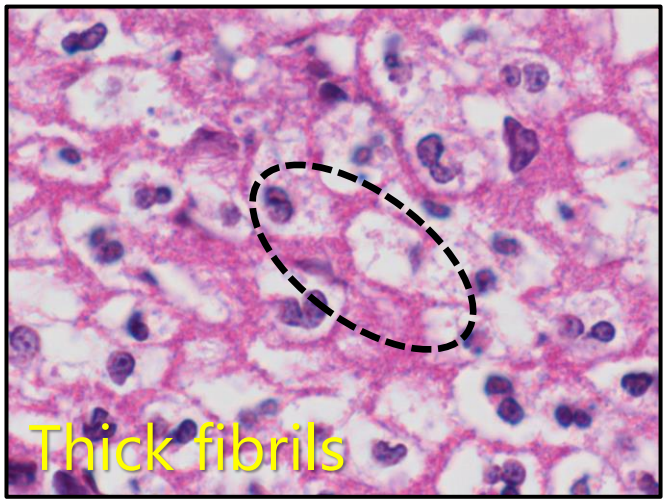
A total of 25 tissue specimens with fibrinopurulent inflammation

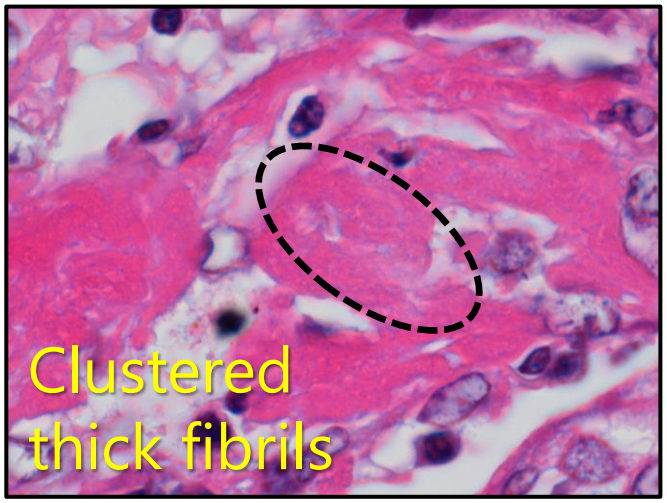
Immunohistochemical markers

Antibodies	Target
Citrullinated Histone H3 (Cit-H3)	NETs fibrils
Lactoferrin (LF)	
Myeloperoxidase (MPO)	
Fibrinogen gamma chain (FGG)	Fibrin fibrils

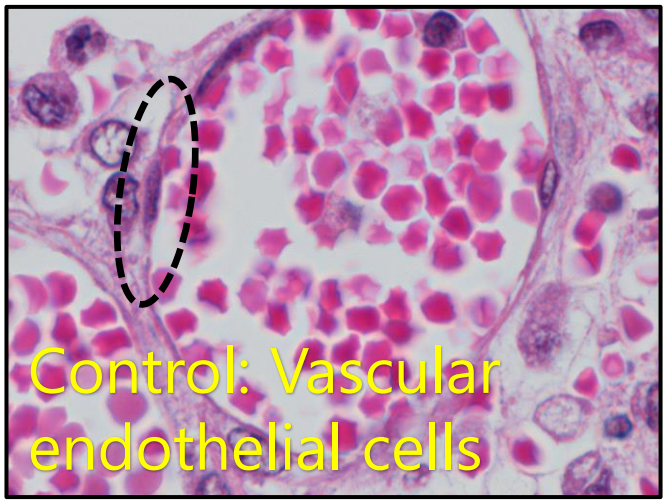
Hypercitrullination of Histone H3 plays an important role in chromatin decondensation.





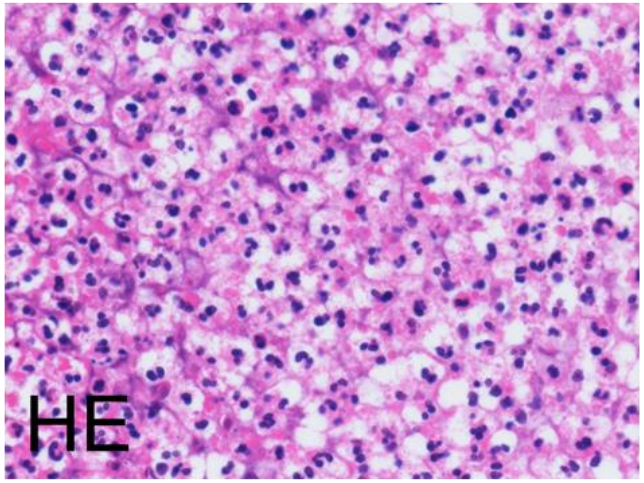


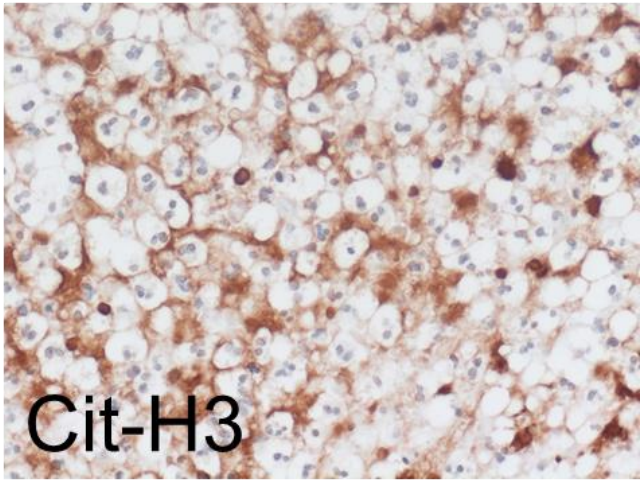
categorization of the fibrils into three types

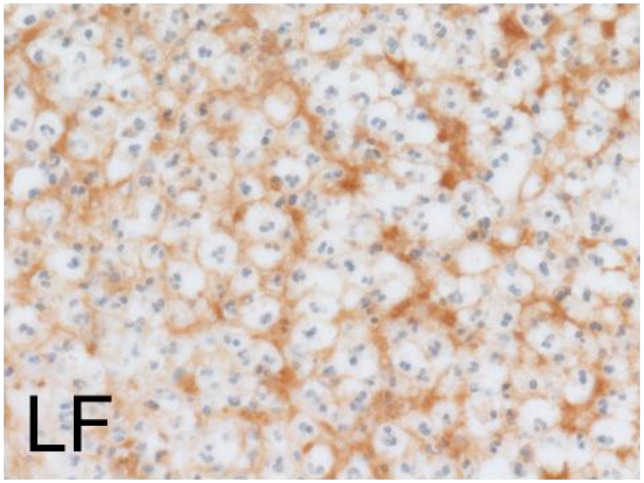


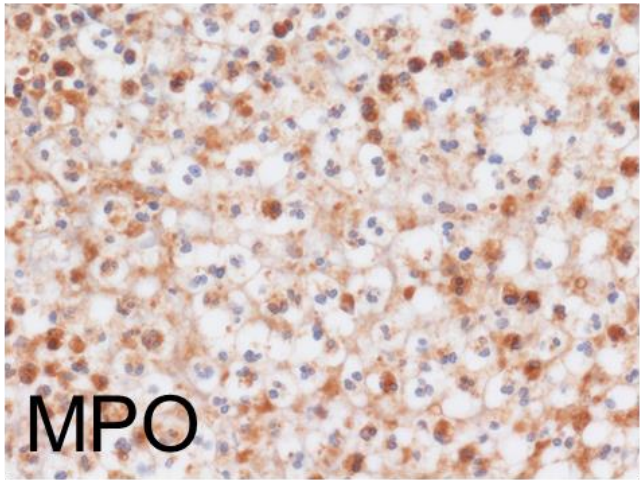
Thin fibrils

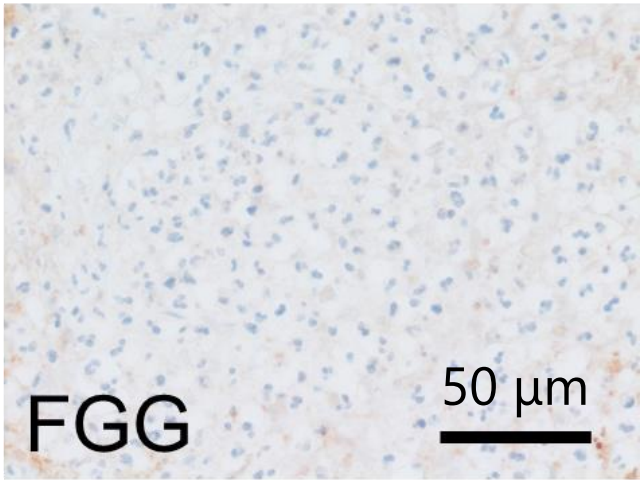
Case 1:
Appendicitis









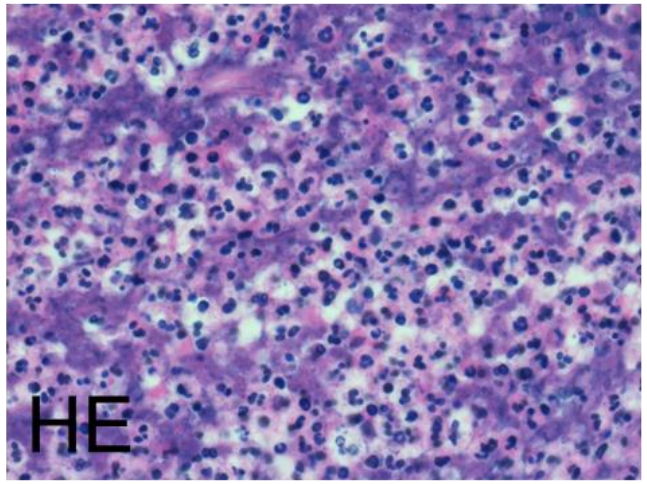


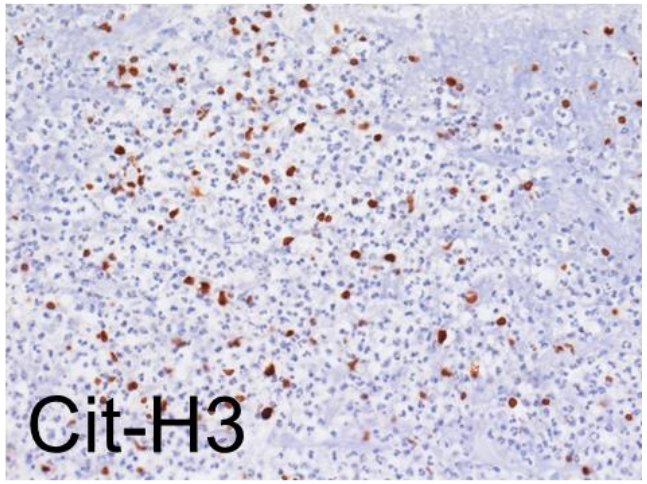
Thin eosinophilic fibrils belonged to NETs.

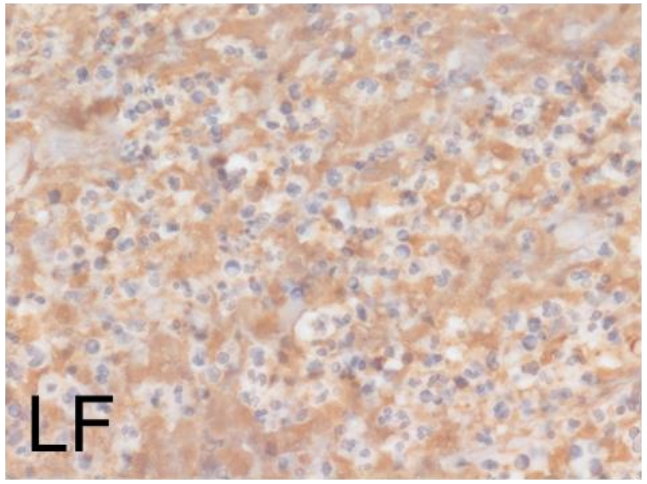
[Cit-H3 (+) /LF (+) /MPO (+) /FGG (−)]

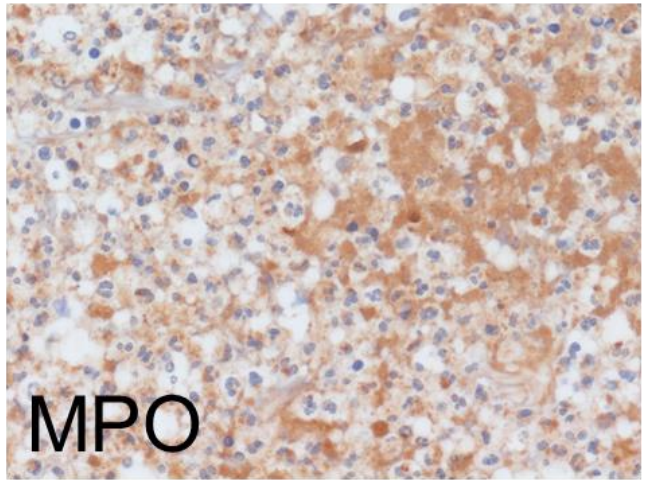
Thin fibrils

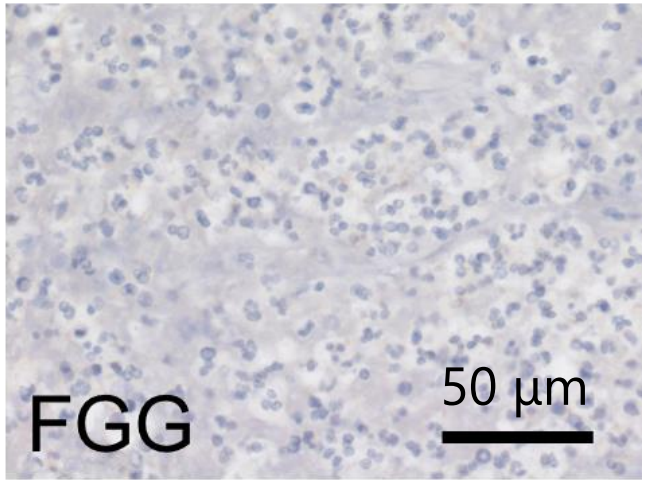
Case 2:
Cholecystitis









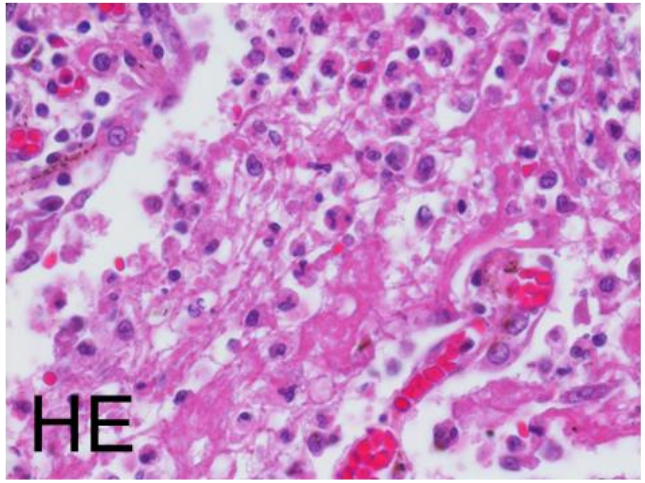


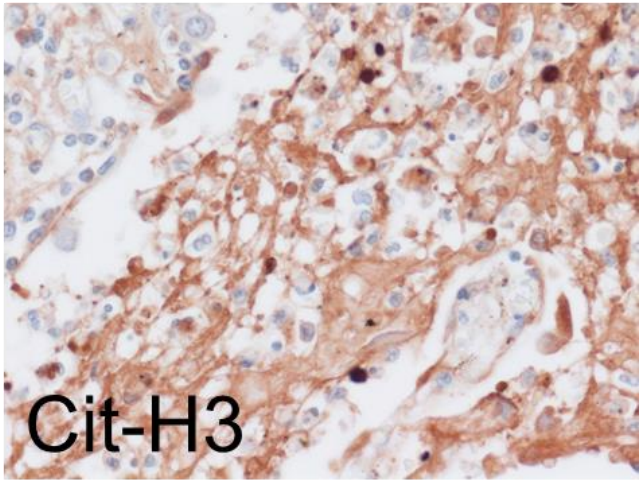
Cit-H3 showed negative immunoreactivity in chromatin-rich basophilic thin NETs fibrils.

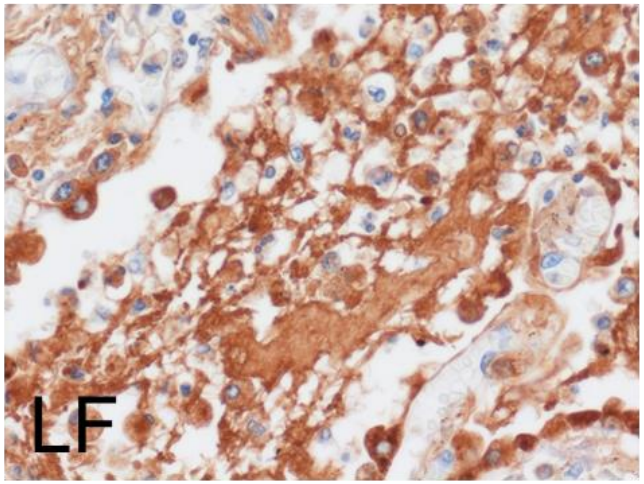
[Cit-H3 (−) /LF (+) /MPO (+) /FGG (−)]

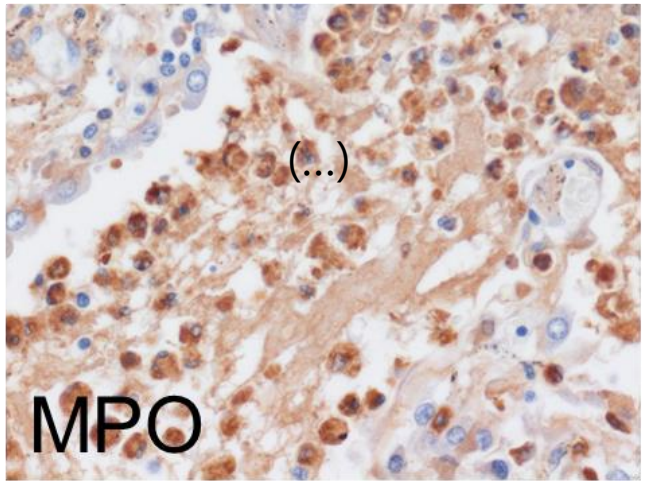
Thick fibrils

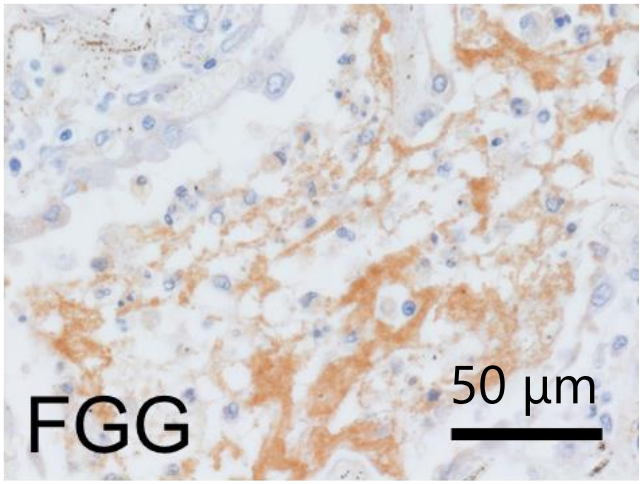
Case 3:
Legionnair's disease









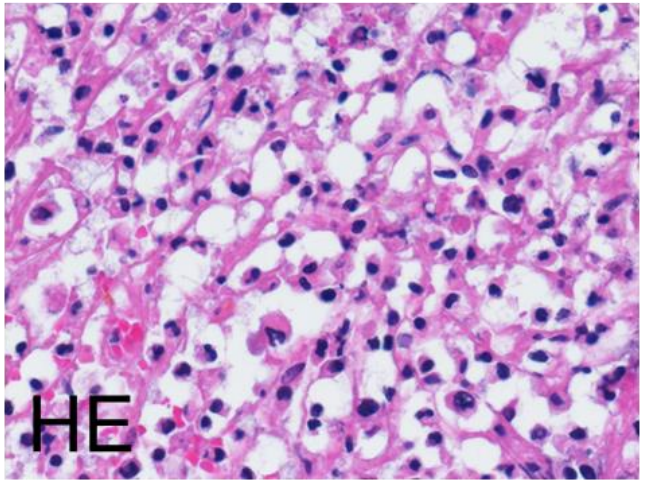


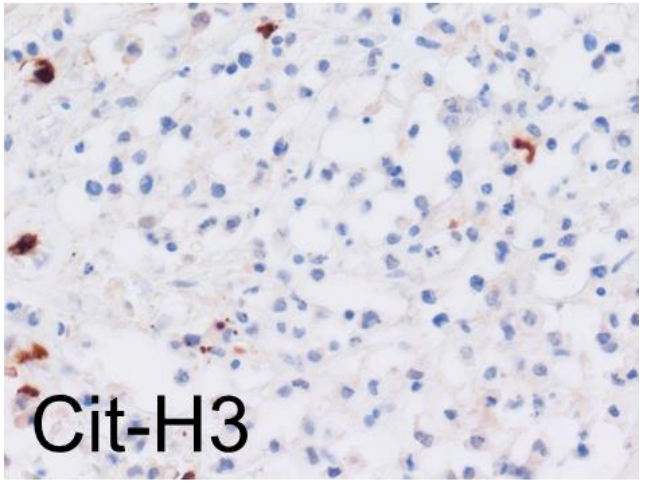
Thick fibrils are composed of both NETs and fibrin.

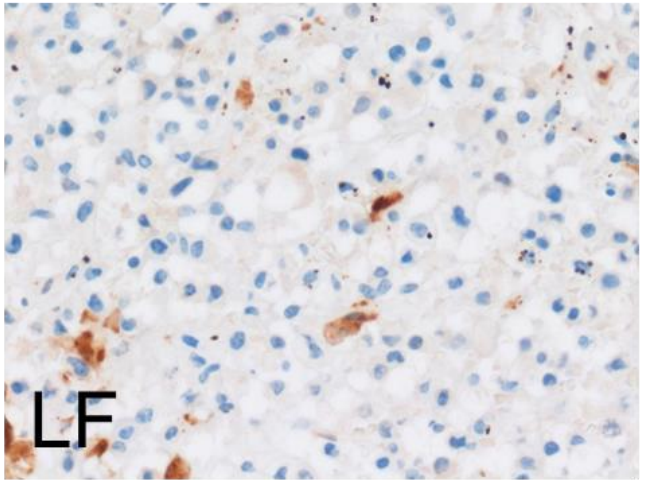
[Cit-H3 (+) /LF (+) /MPO (+) /FGG (+)]

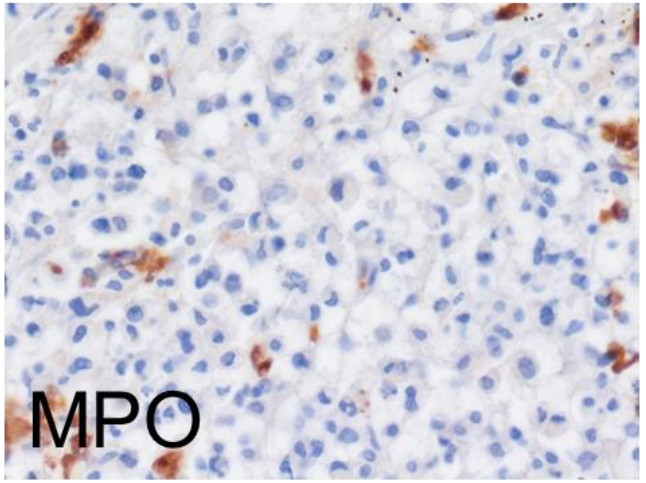
Thick fibrils

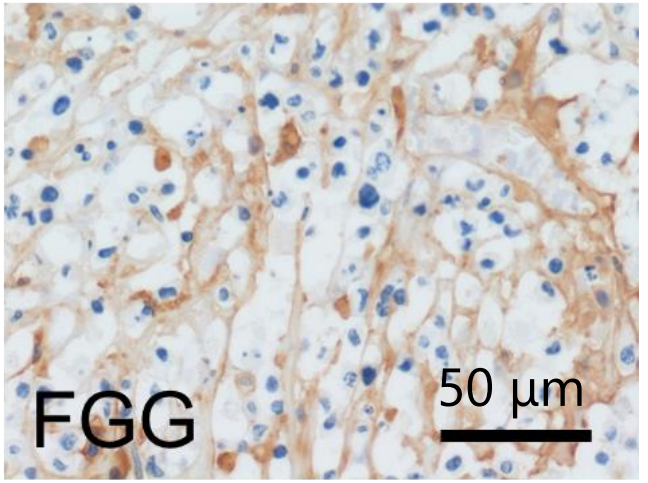
Case 4:
Abscess of liver









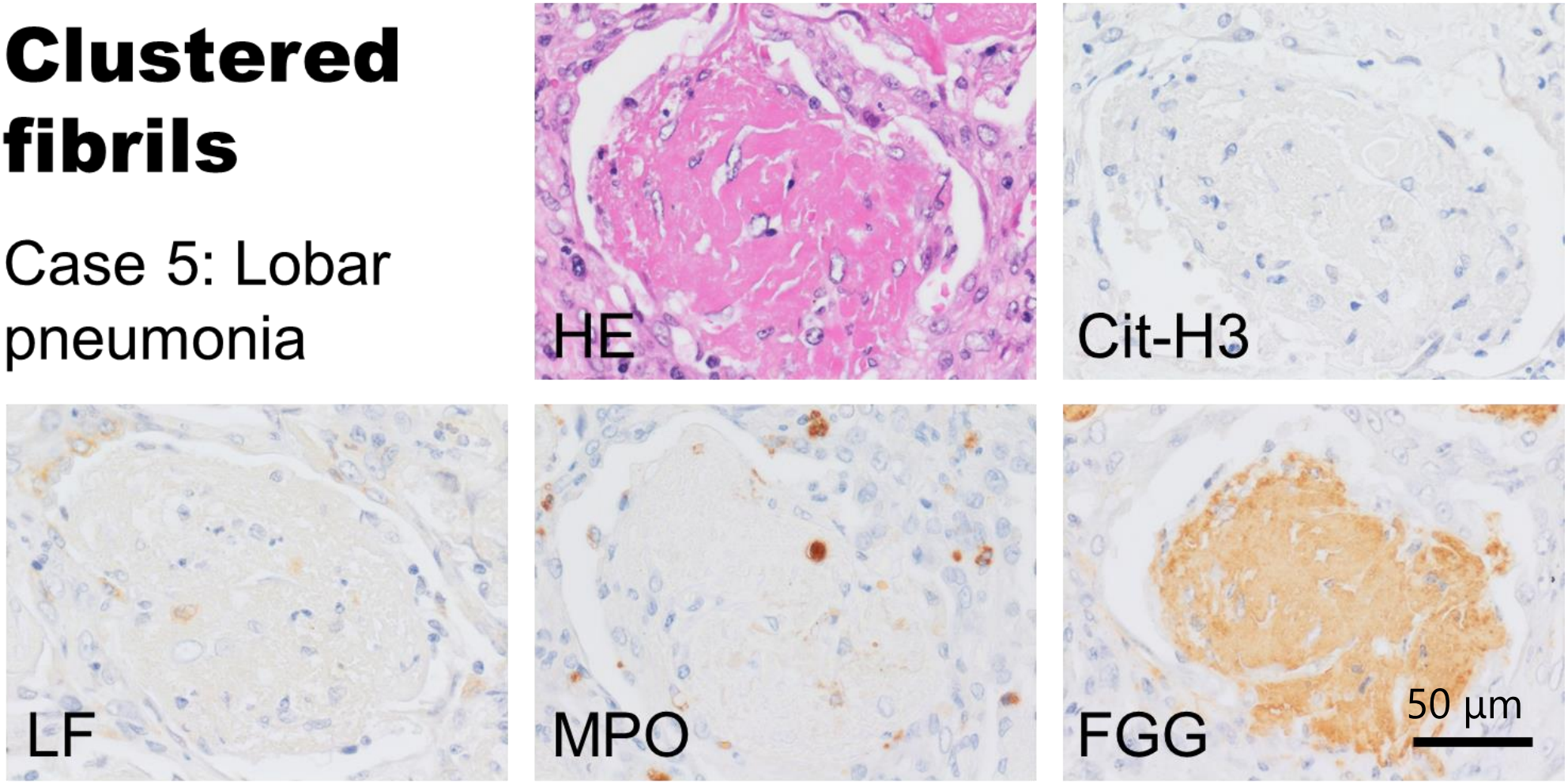


Thick fibrils are composed of fibrin alone.

[Cit-H3 (−) /LF (−) /MPO (−) /FGG (+)]

Clustered fibrils

Case 5: Lobar pneumonia



Clustered thick fibrils are solely composed of fibrin.

[Cit-H3 (−) /LF (−) /MPO (−) /FGG (+)]

Comparison with fibril thickness and NETs markers

	Cit-H3	LF	MPO
Thin fibrils (n=18)	14 (78%)	18 (100%)	17 (94%)
Thick fibrils (n=23)	7 (30%)	18 (78%)	11 (48%)
Clustered fibrils (n=9)	0 (0%)	0 (0%)	0 (0%)

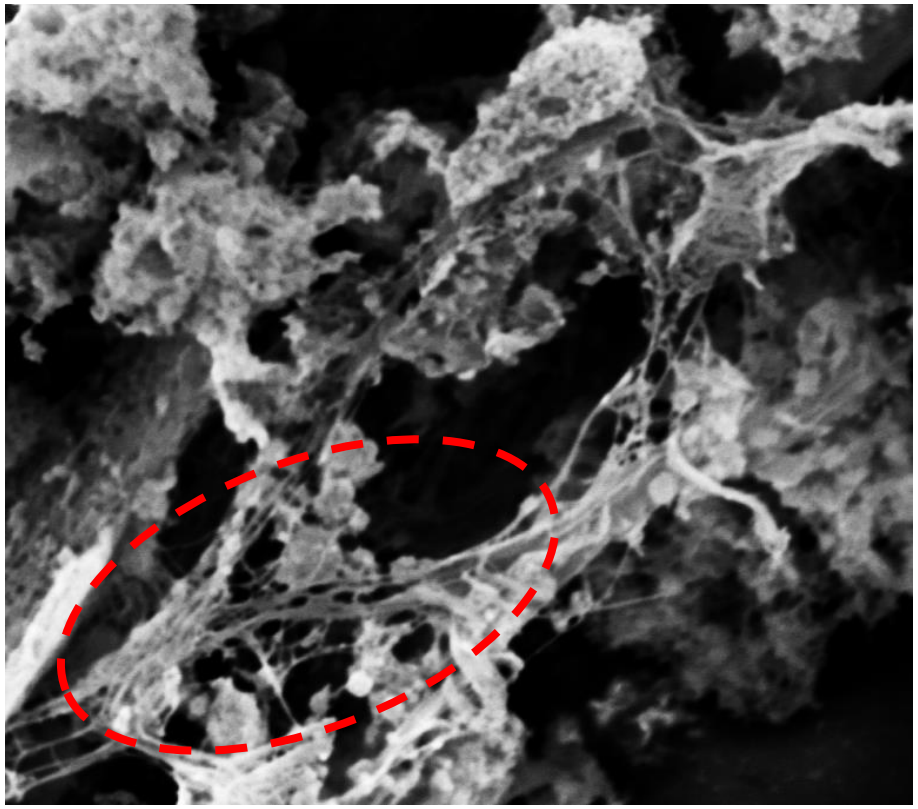
Lactoferrin is the best NETs marker regardless of fibril size.

Relationship between fibril thickness and LF/FGG immunoreactivities

	NETs & fibrin LF＋/FGG＋	NETs LF＋/FGG－	Fibrin LF－/FGG＋
Thin fibrils (n=18)	7 (39%)	14 (78%)	0 (0%)
Thick fibrils (n=23)	18 (78%)	0 (0%)	17 (74%)
Clustered fibrils (n=9)	0 (0%)	0 (0%)	9 (100%)

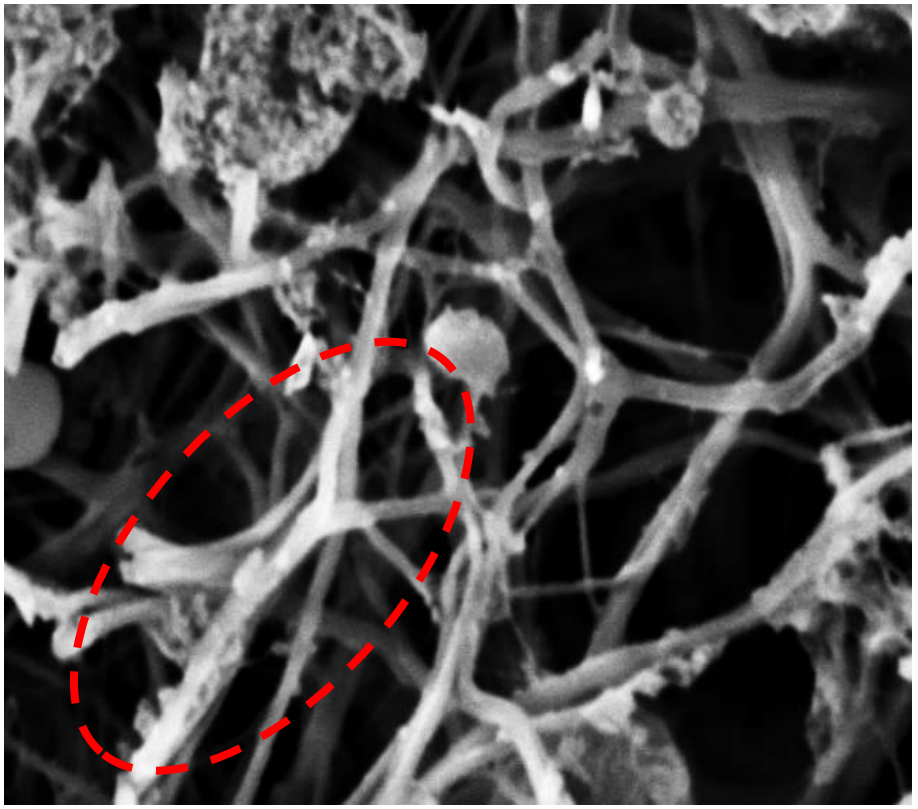
Scanning Electron Microscope

NETs



98±13 nm

Fibrin

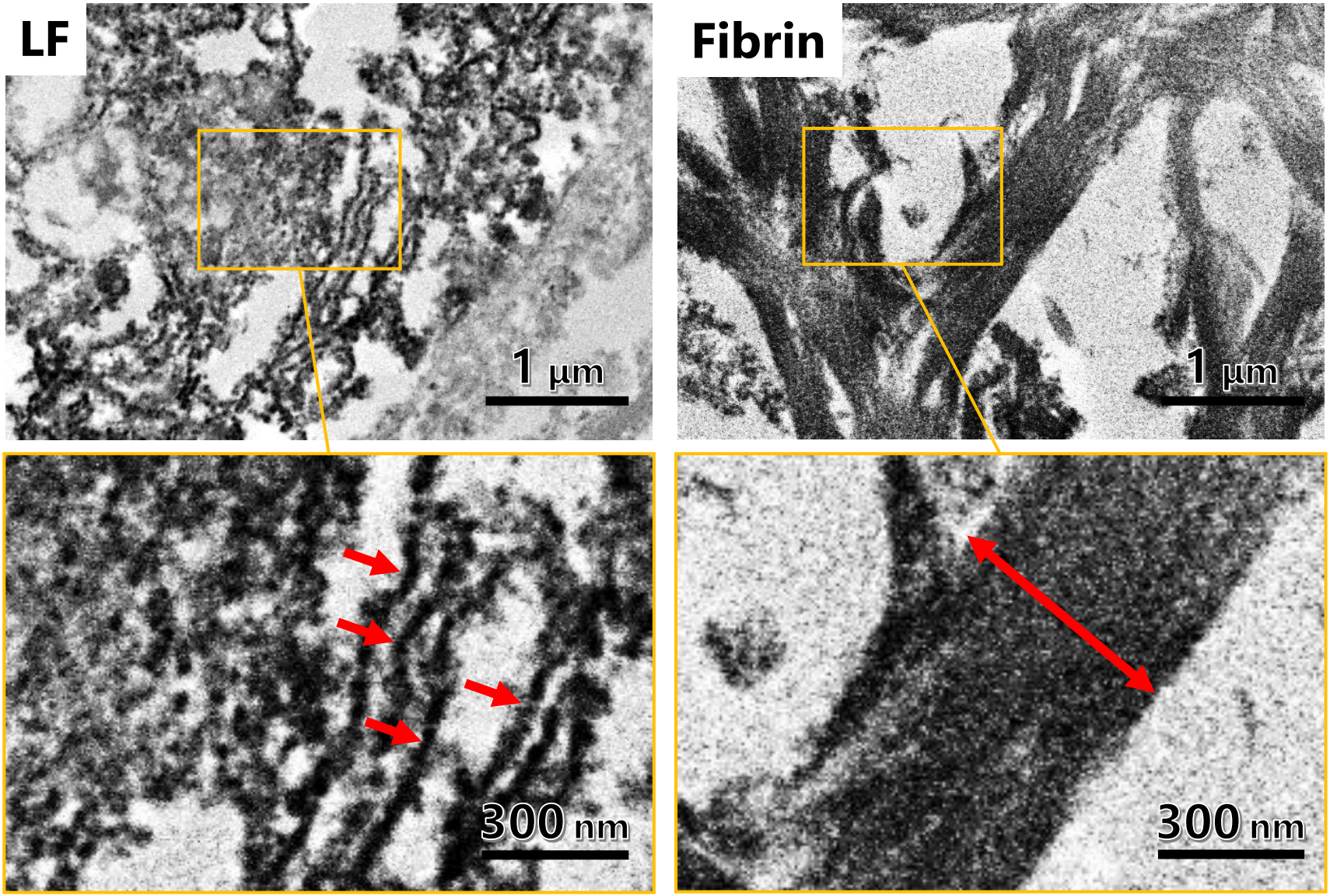


466±98 nm

p<0.01

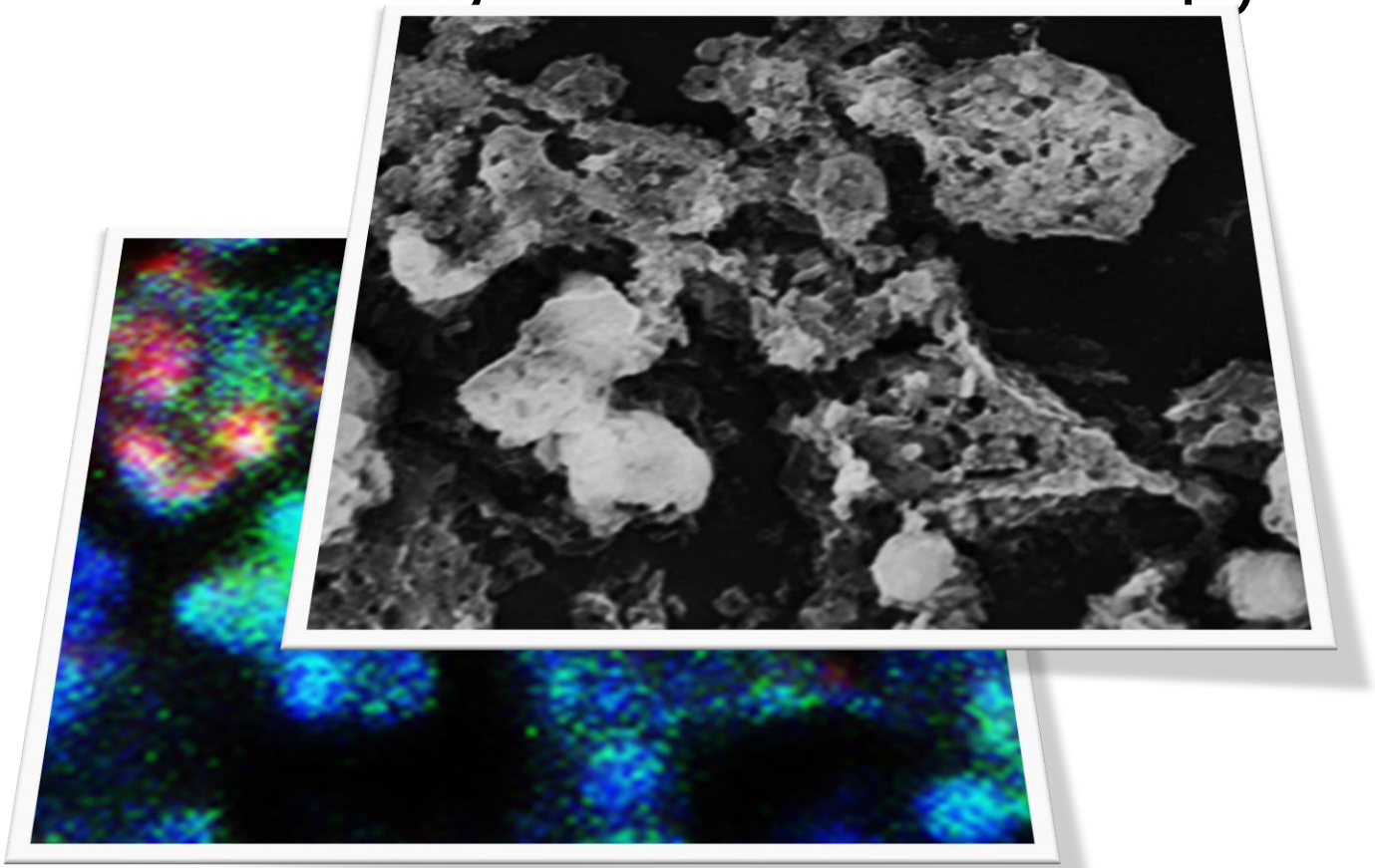
Immunoelectron microscopy

(Pre-embedding immunoelectron microscopy)

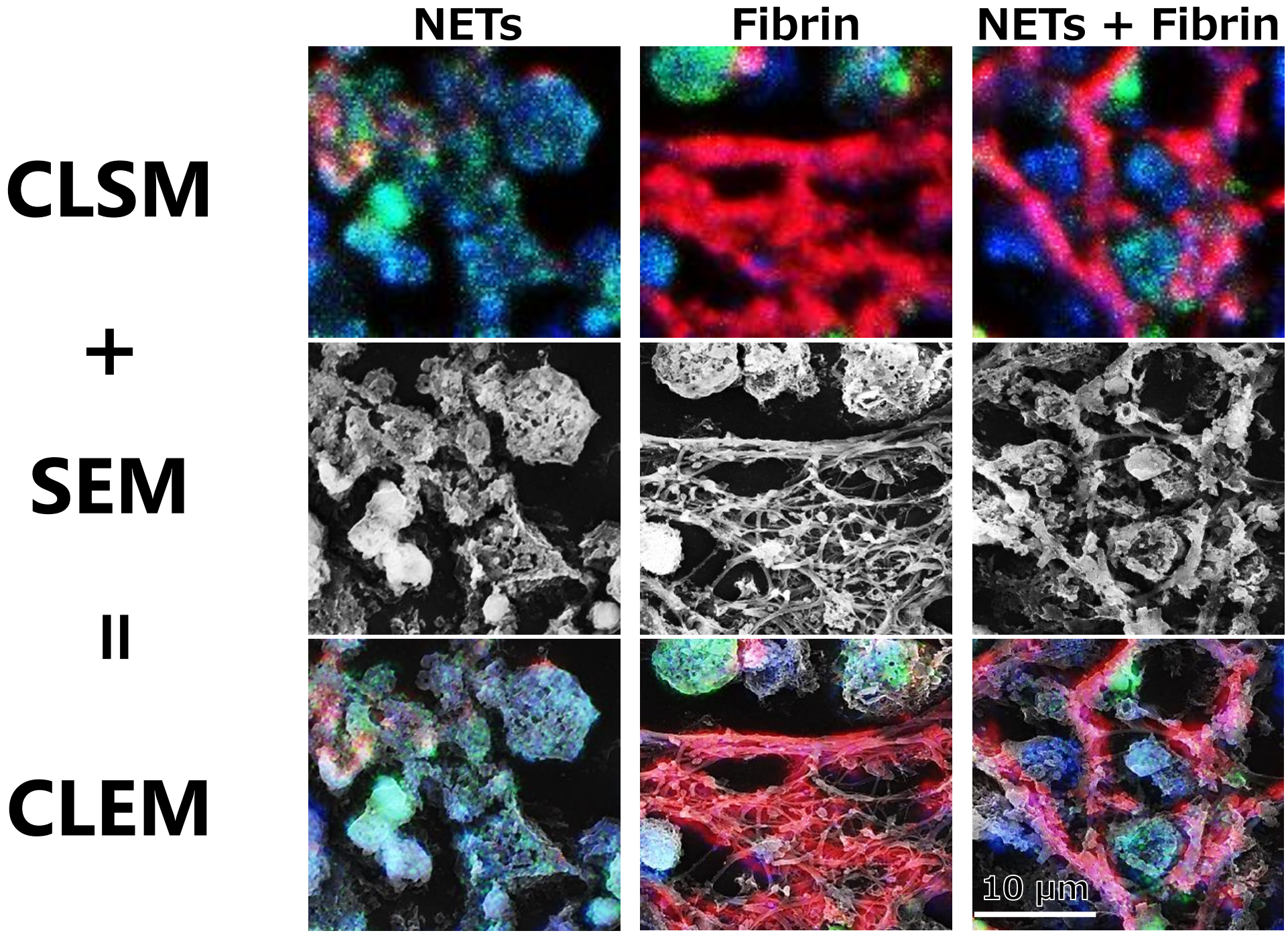


Correlative Light and Electron Microscopy: CLEM

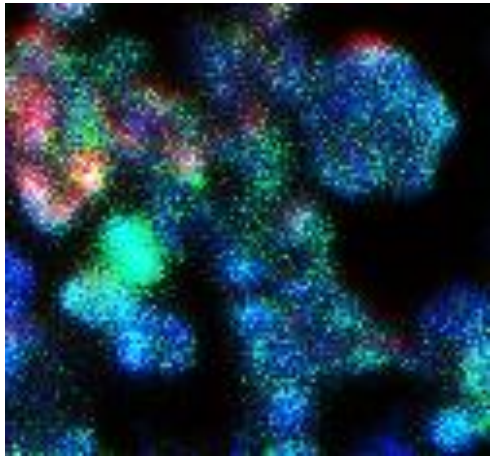
Scanning Electron Microscopy: SEM



Confocal Laser Scanning Microscope: CLSM


NETs
Fibrin
NETs + Fibrin
CLSM
+
SEM
=
CLEM
10 µm

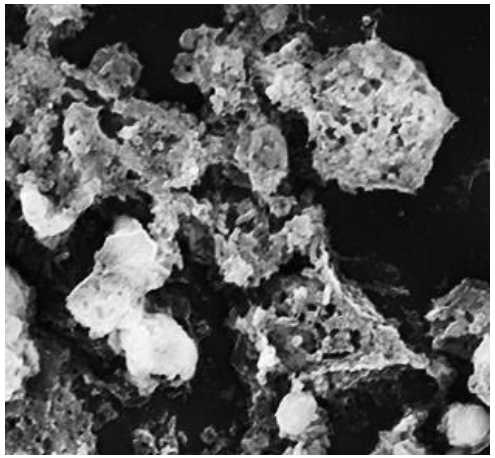
CLEM for NETs detection

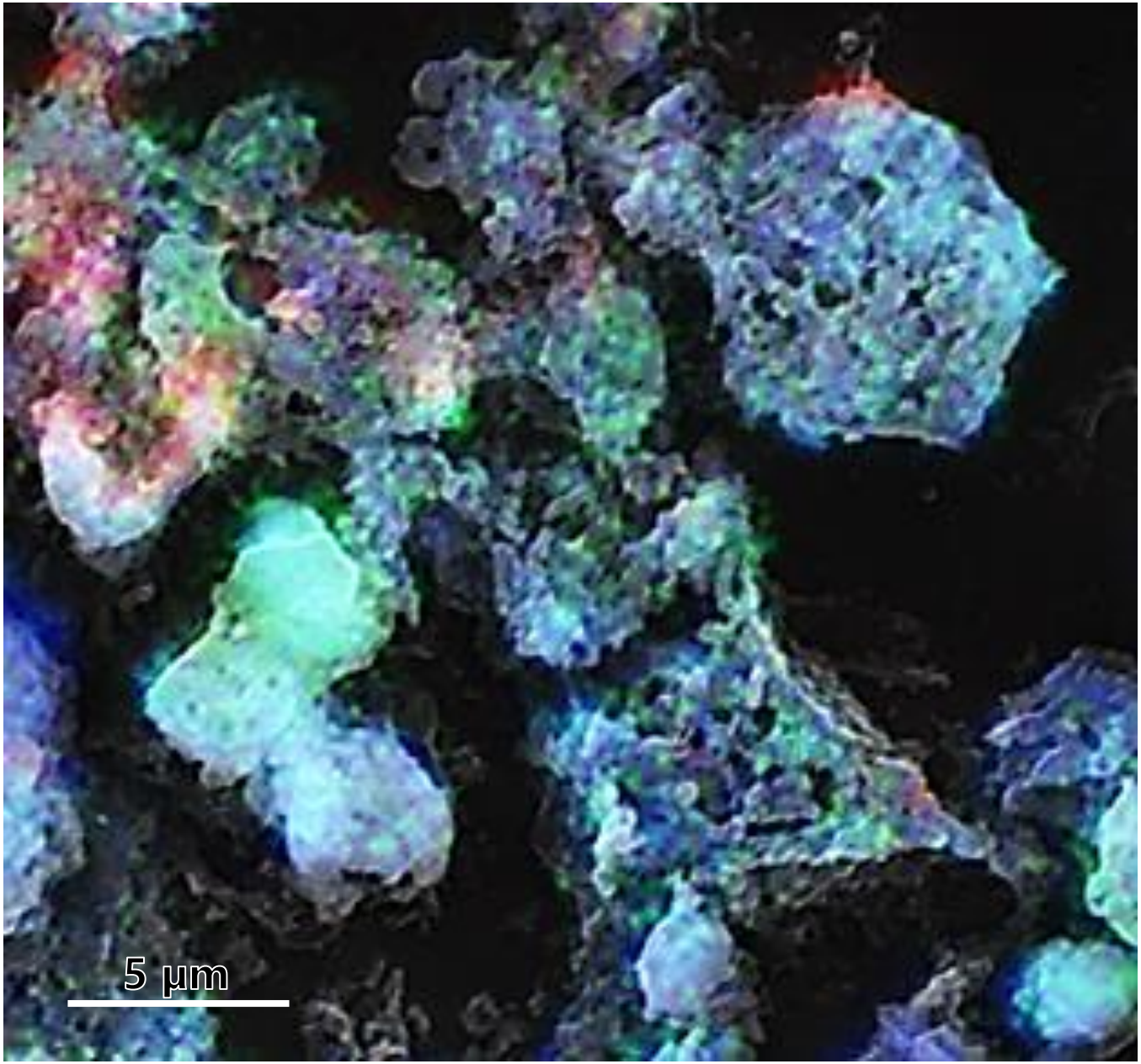


LF (green)

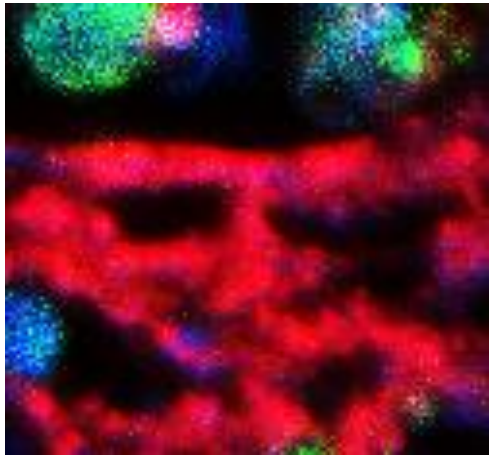
DAPI (DNA: blue)

+



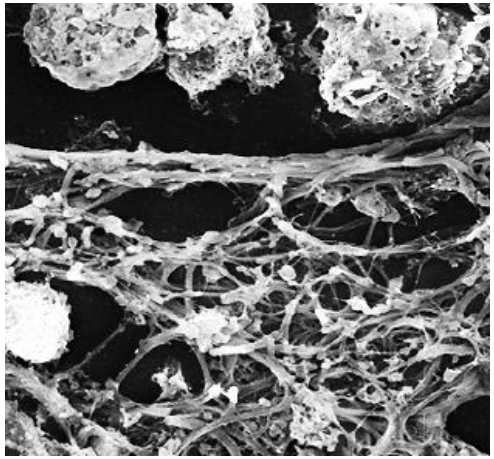


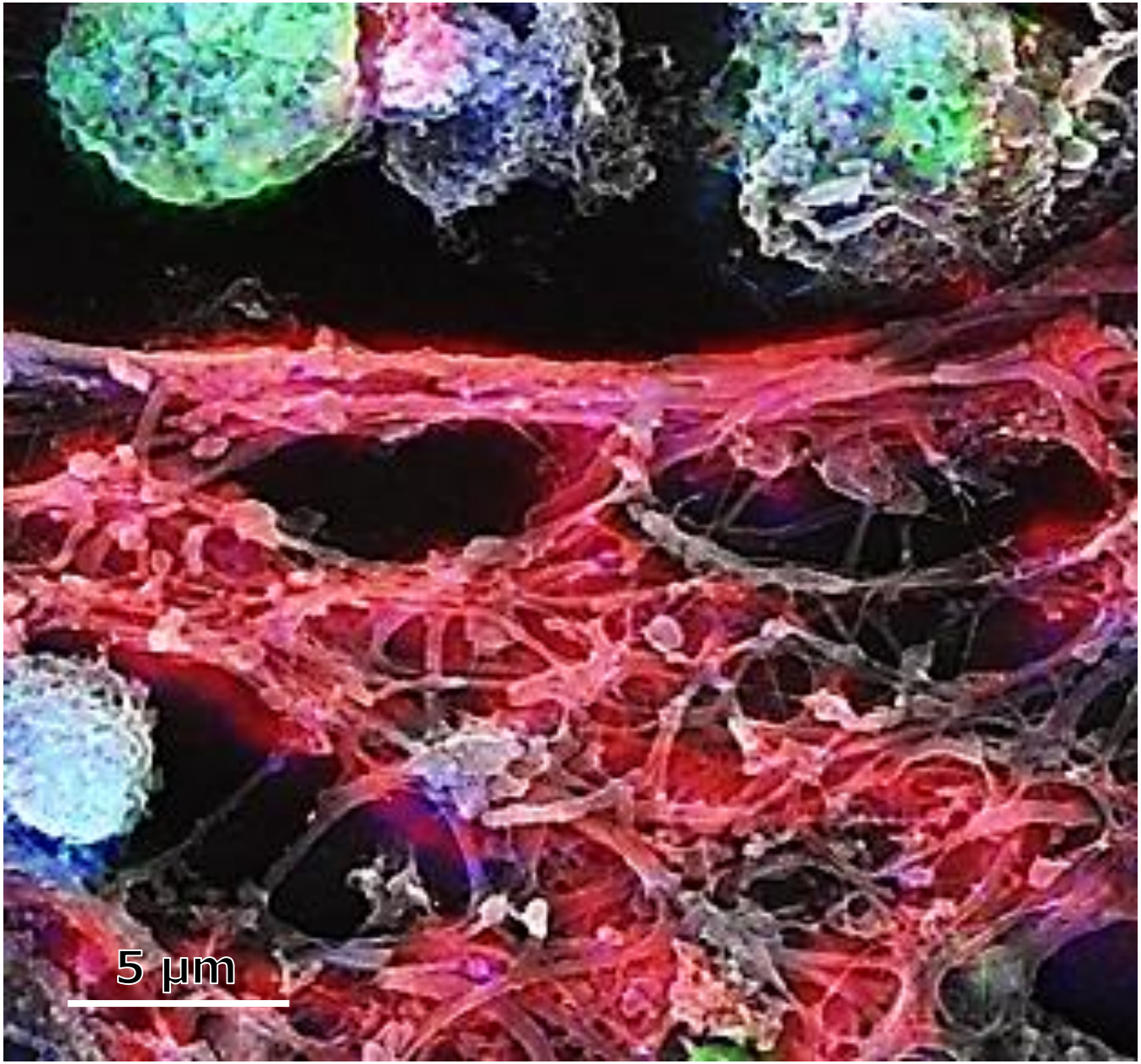
CLEM for fibrin detection



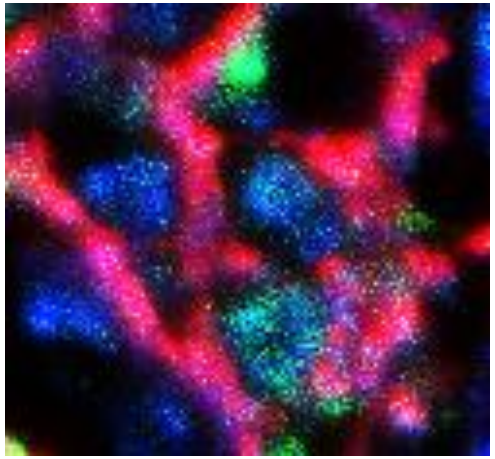
FGG (red)

+



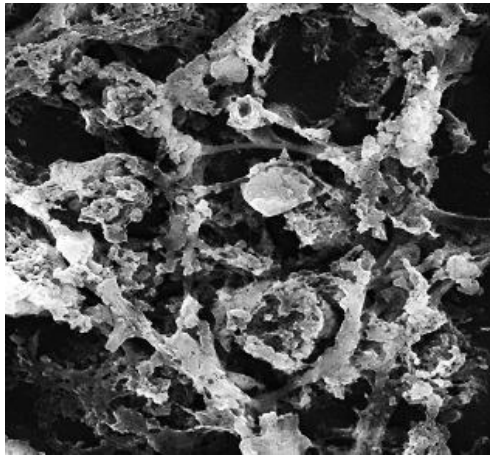


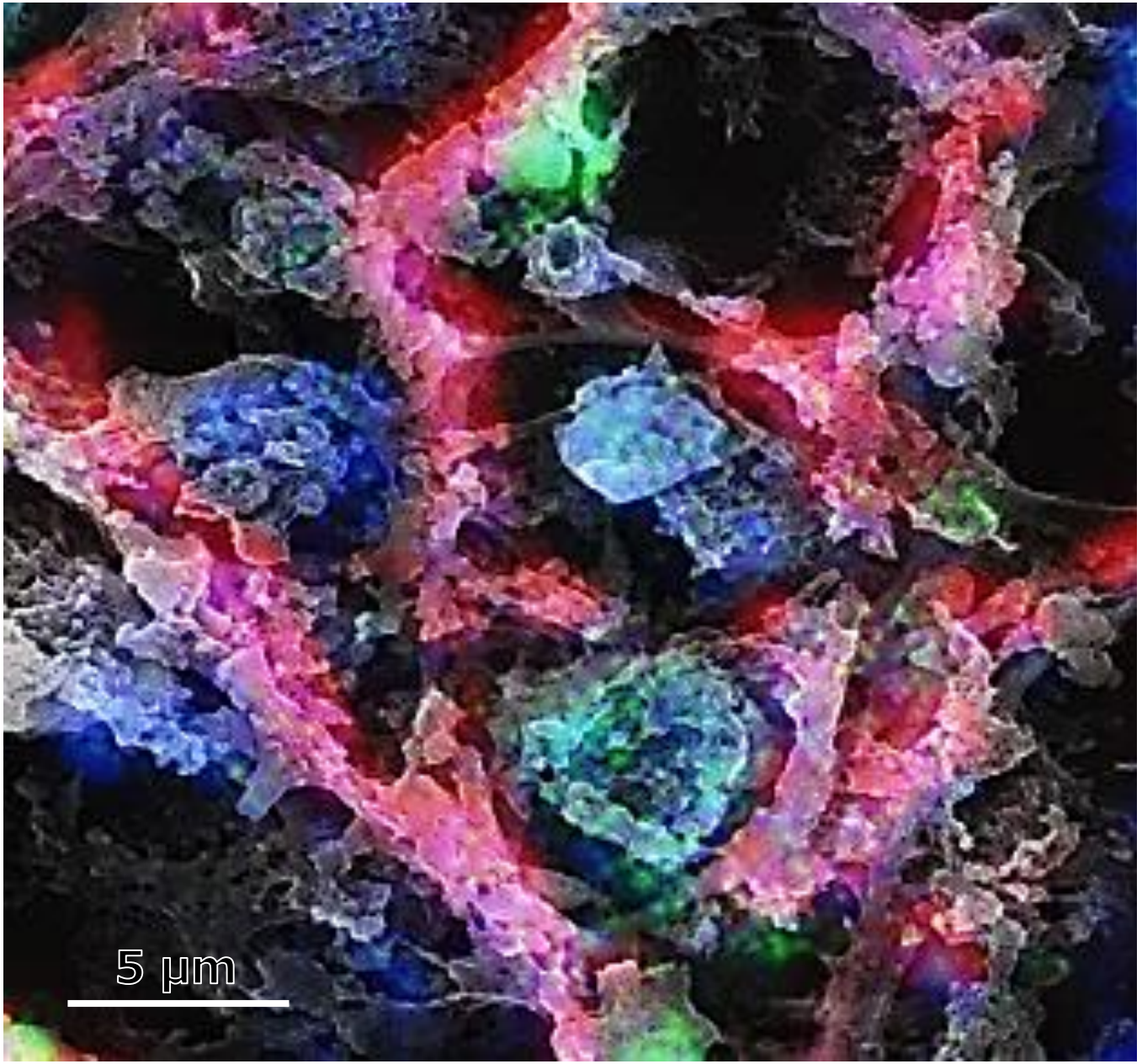
CLEM for NETs and fibrin detection



LF (green)
DAPI (DAPI)
FGG (red)

+



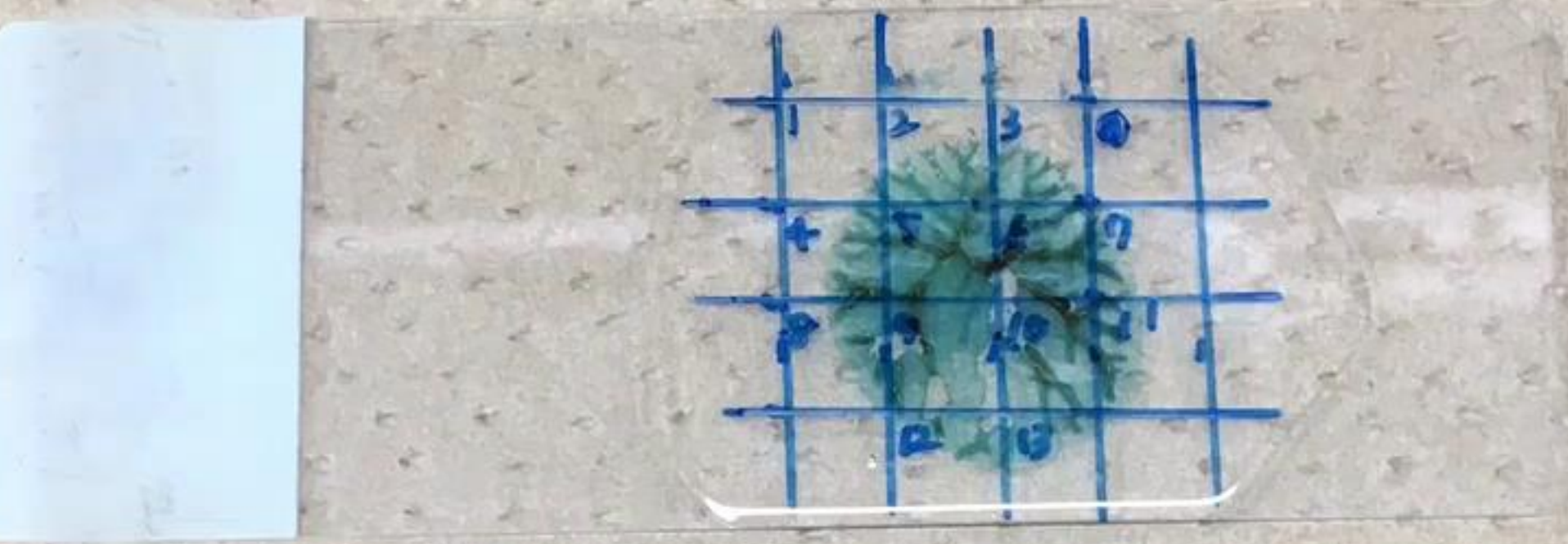


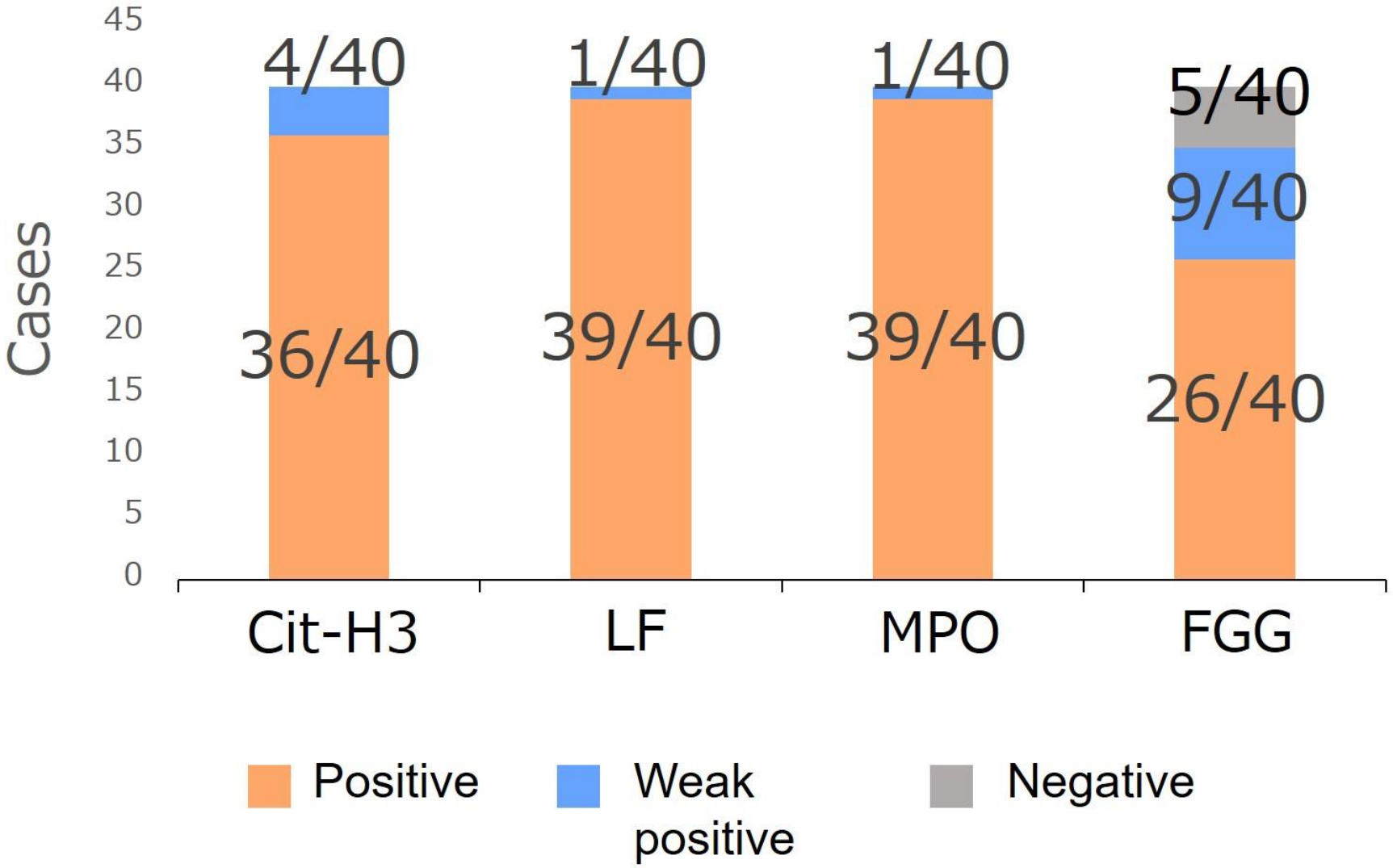
Detection of NETs using cytology specimens

Samples:

a total of 40 fine-needle aspiration (FNA) smears from abscess of the breast

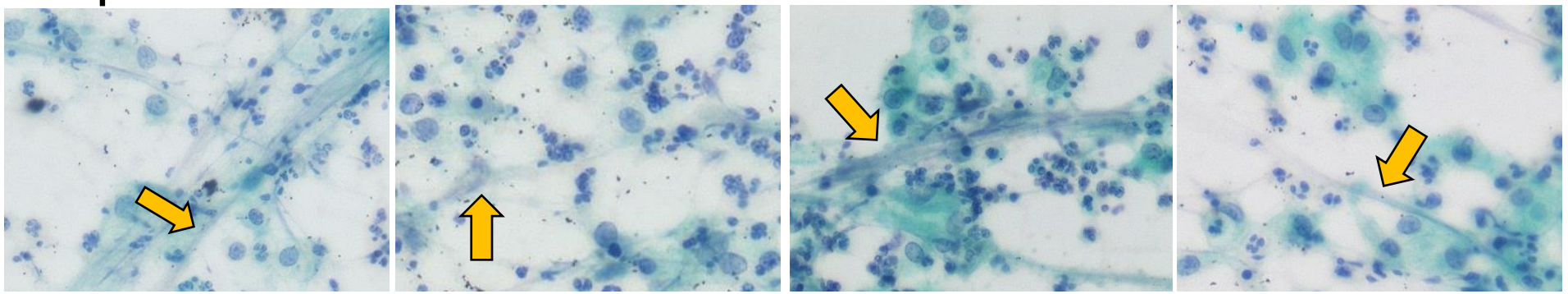
ell tranfer technique with cutting into piece



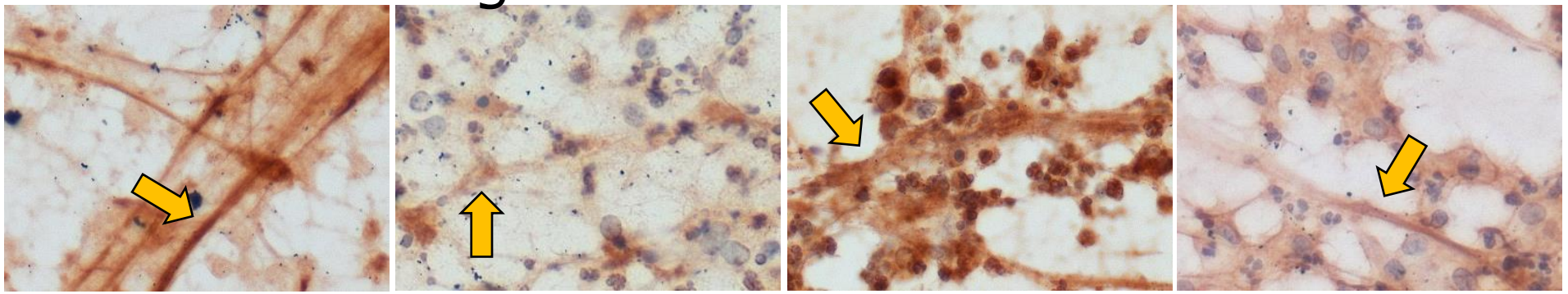


NETs markers were positive in all cytology specimens.
Fibrin was negative in some specimens.

Pap stain



Immunostaining

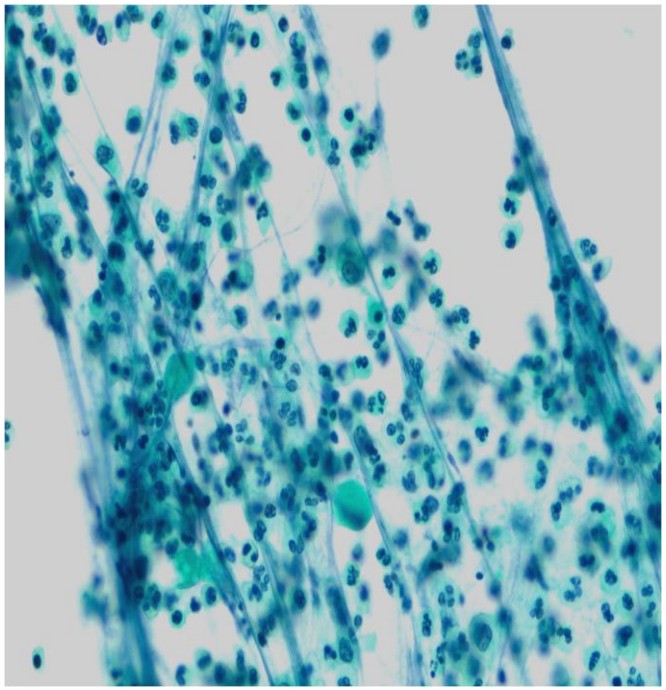


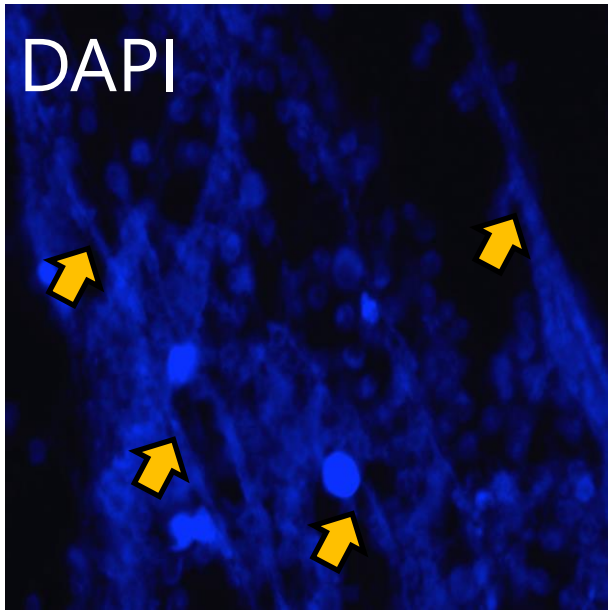
Cit-H3 LF MPO Fibrin

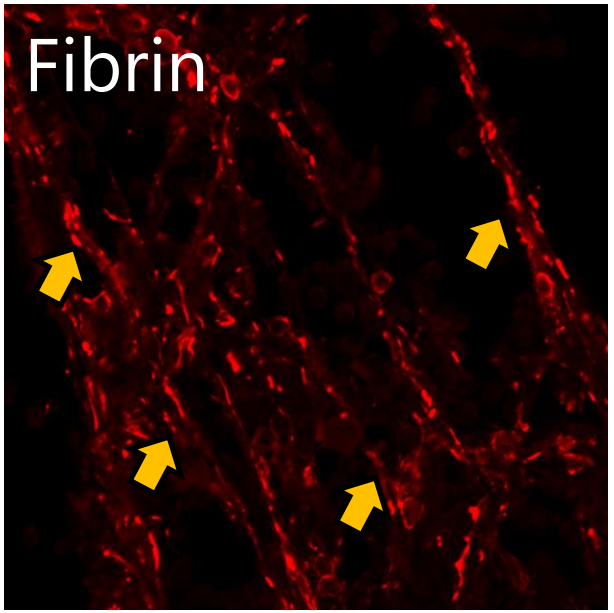
Re-immunostaining was performed after pap staining.
Mixed expression of NETs and fibrin was seen.

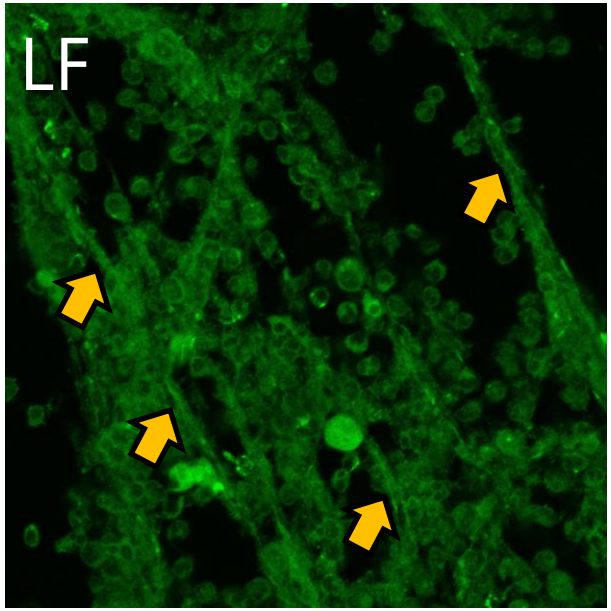
Co-localization of NETs and fibrin

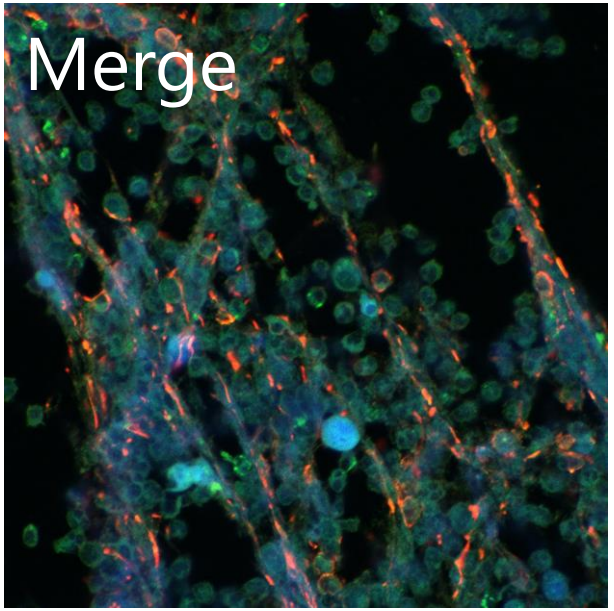
Observation by CLSM

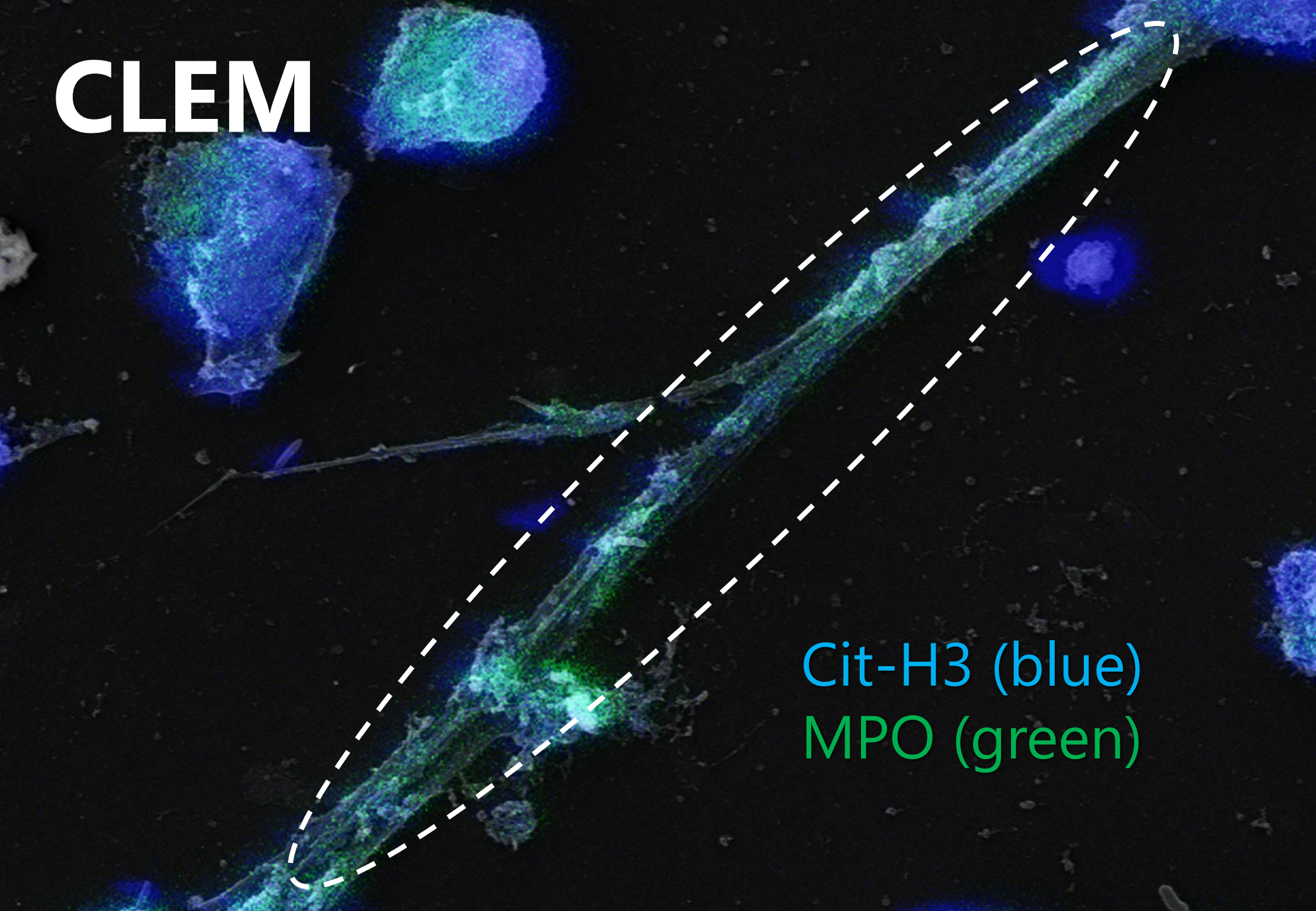




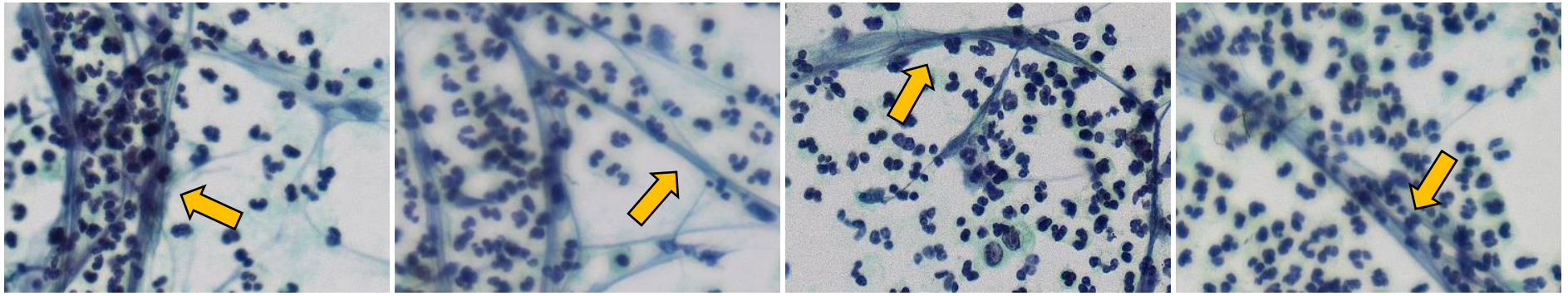




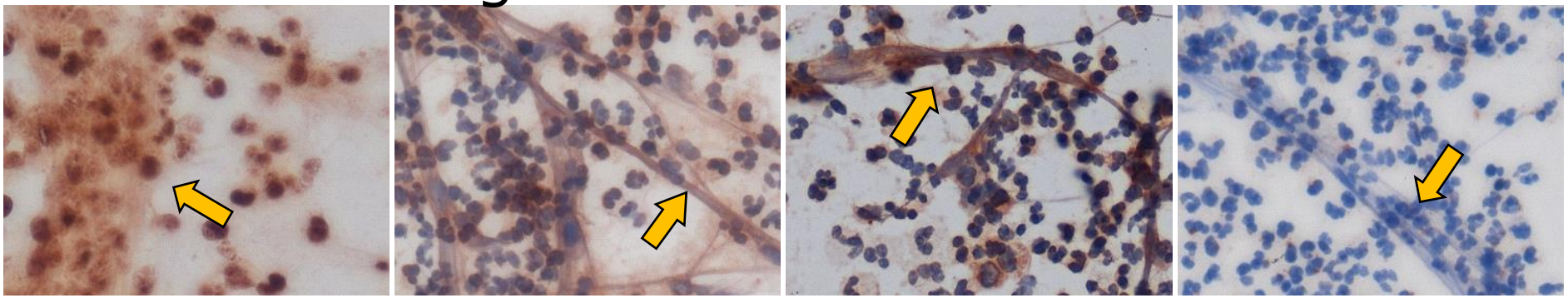



CLEM
Cit-H3 (blue)
MPO (green)

Pap. stain

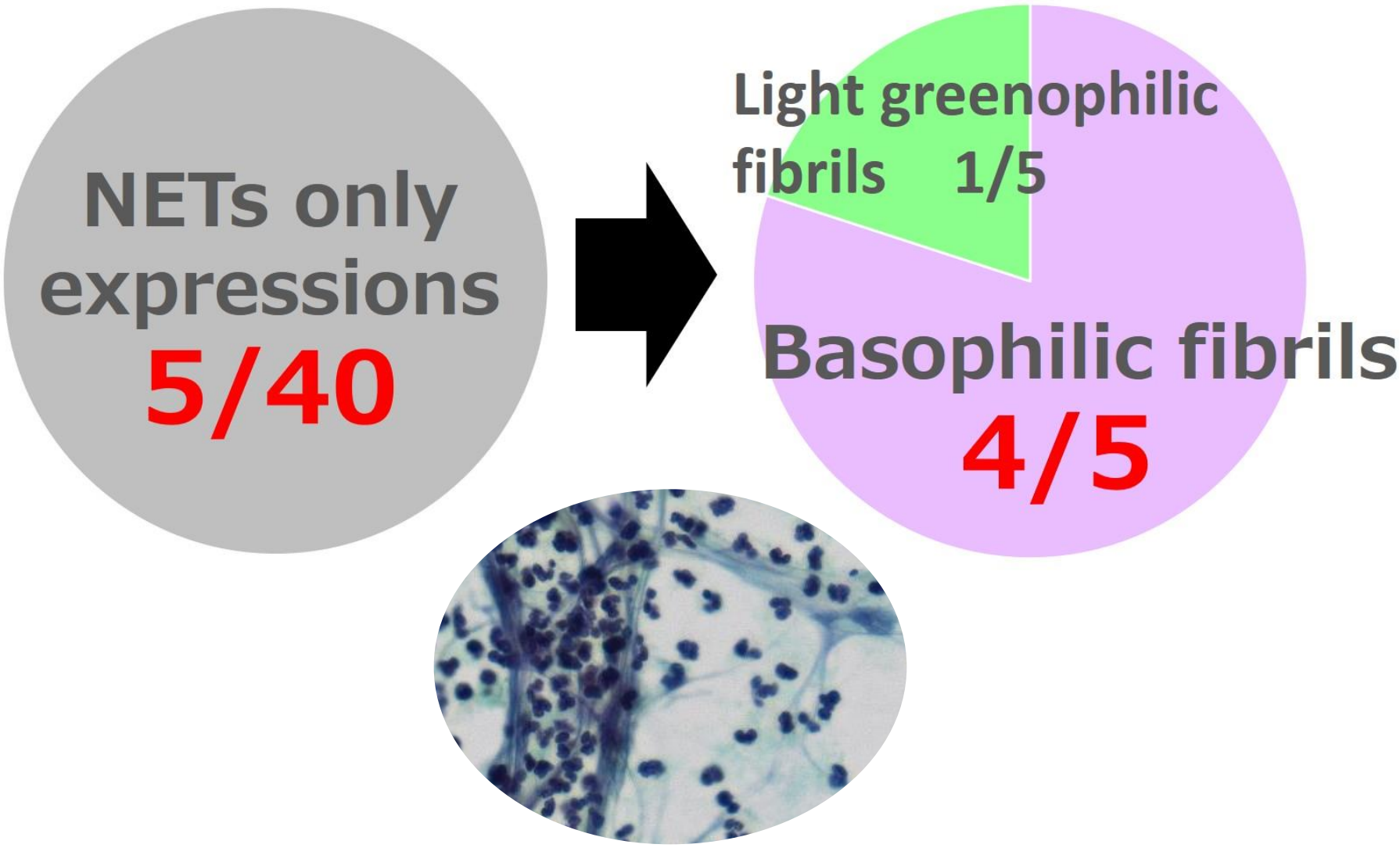


Immonostaining

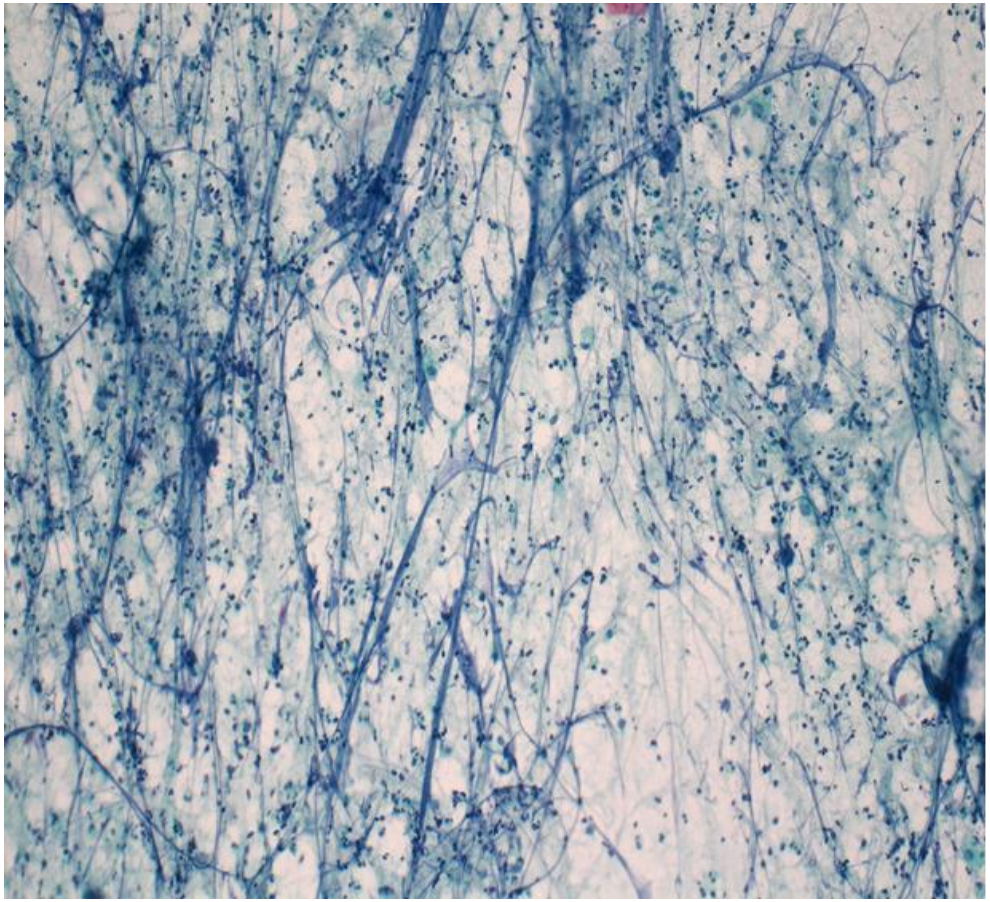


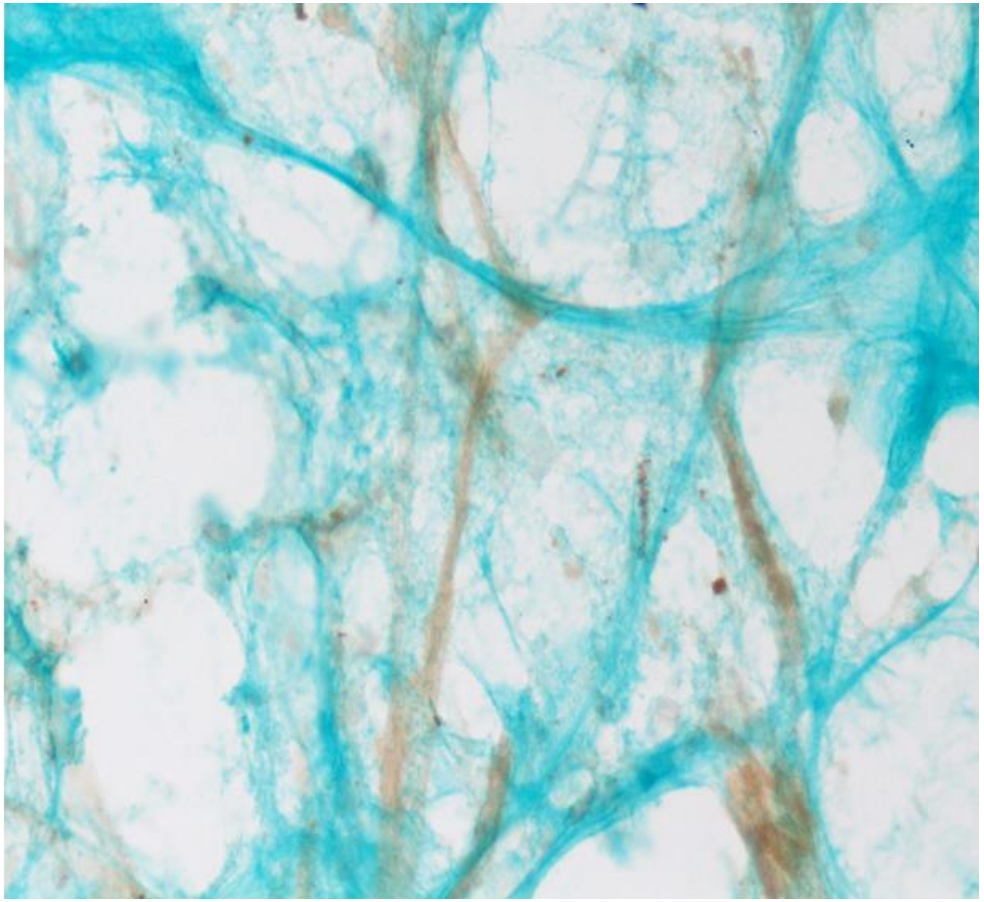
Cit-H3 LF MPO Fibrin

Re-immunostaining was done after pap staining.
NETs without fibrin immunoreactivity are seen.



Chromatin DNA-rich basophilic fibrils are likely to be composed of NETs.





NETs: Cit-H3 (brown)
Mucin: Alcian blue (blue)

NETs can be distinguished from mucin.

Conclusions

We established a reliable immunohistochemical technique for identifying NETs and fibrin in FFPE sections of exudative inflammatory lesions.

NETs are suspected in HE- and Pap-stained specimens, particularly when the deposited fibrils appear thin and basophilic.