

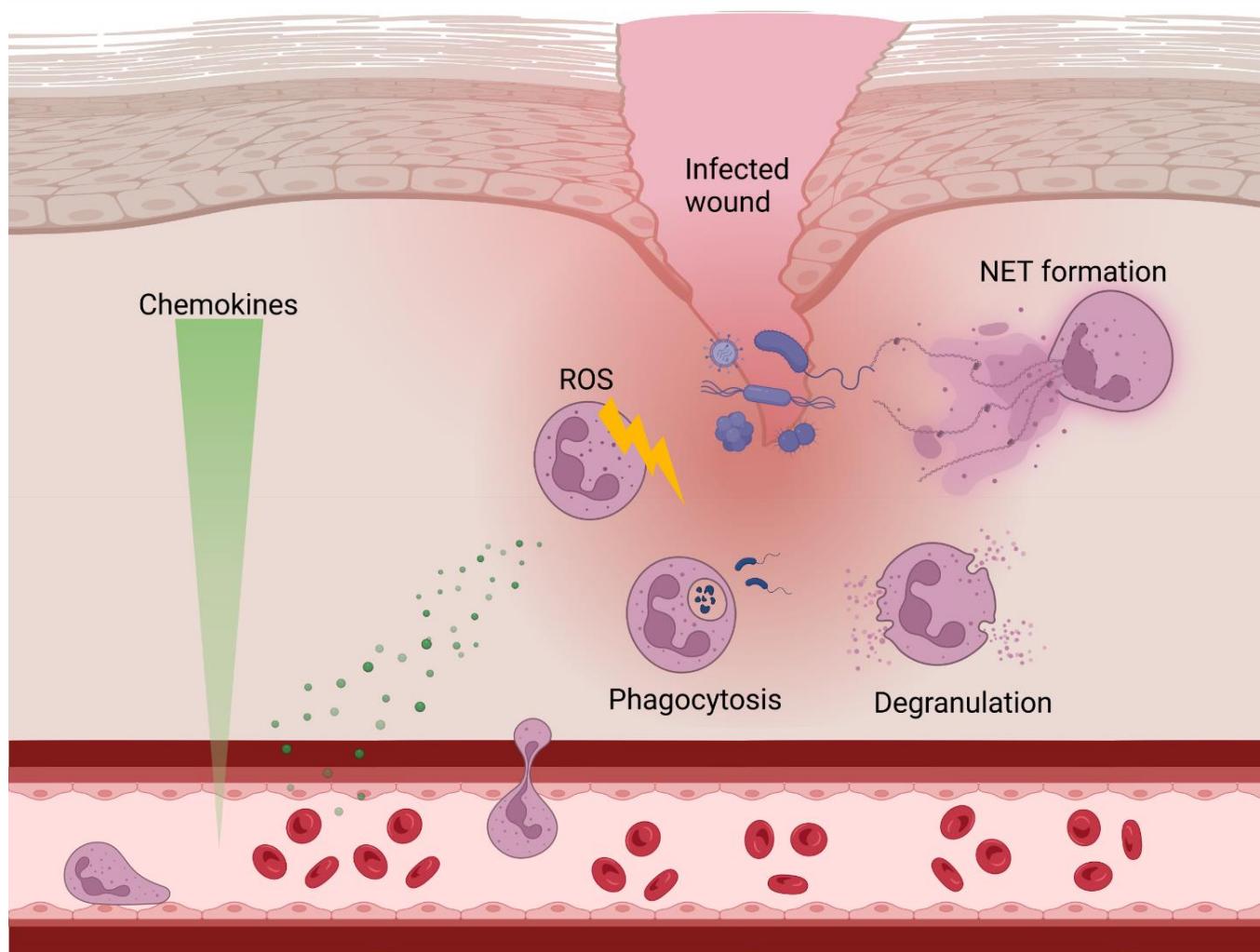


Comparison of NET quantification methods based on immunofluorescence microscopy: hand-counting, semi – automated and automated evaluations

Department Meeting
Institute for Biochemistry
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Timo Henneck

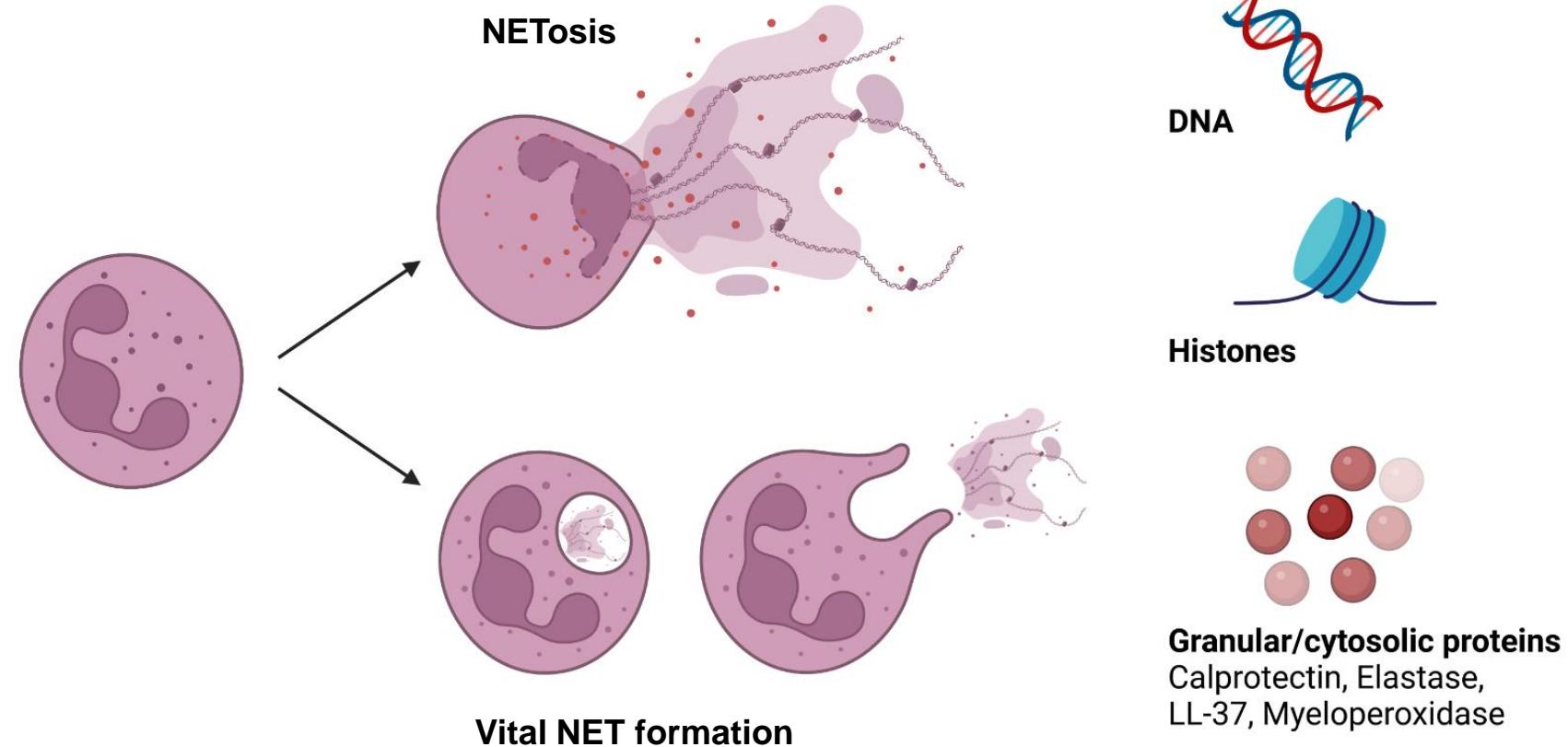
Neutrophils – the first responders of innate immunity

- Most abundant innate immune cell
- Transmigration to infection site
- Different modes of action

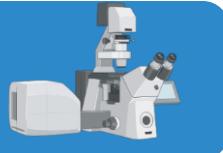


Neutrophil extracellular trap (NET) formation

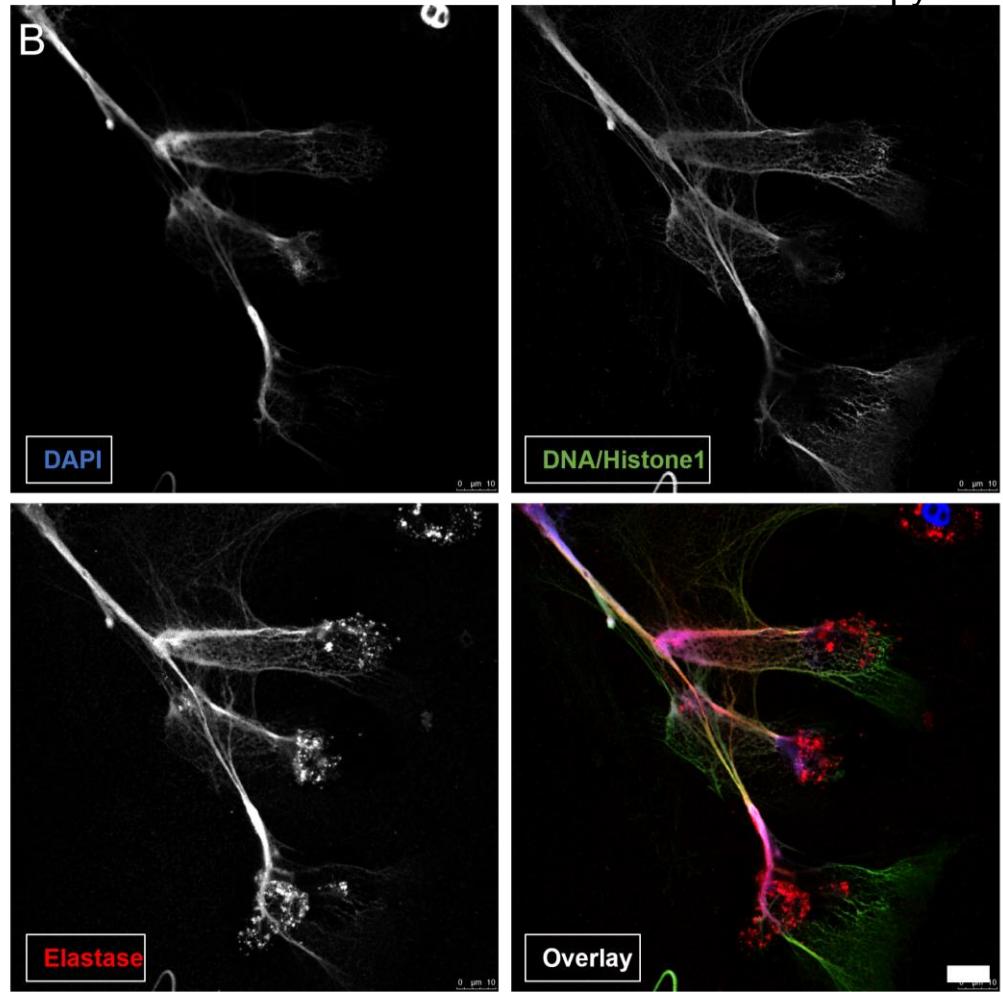
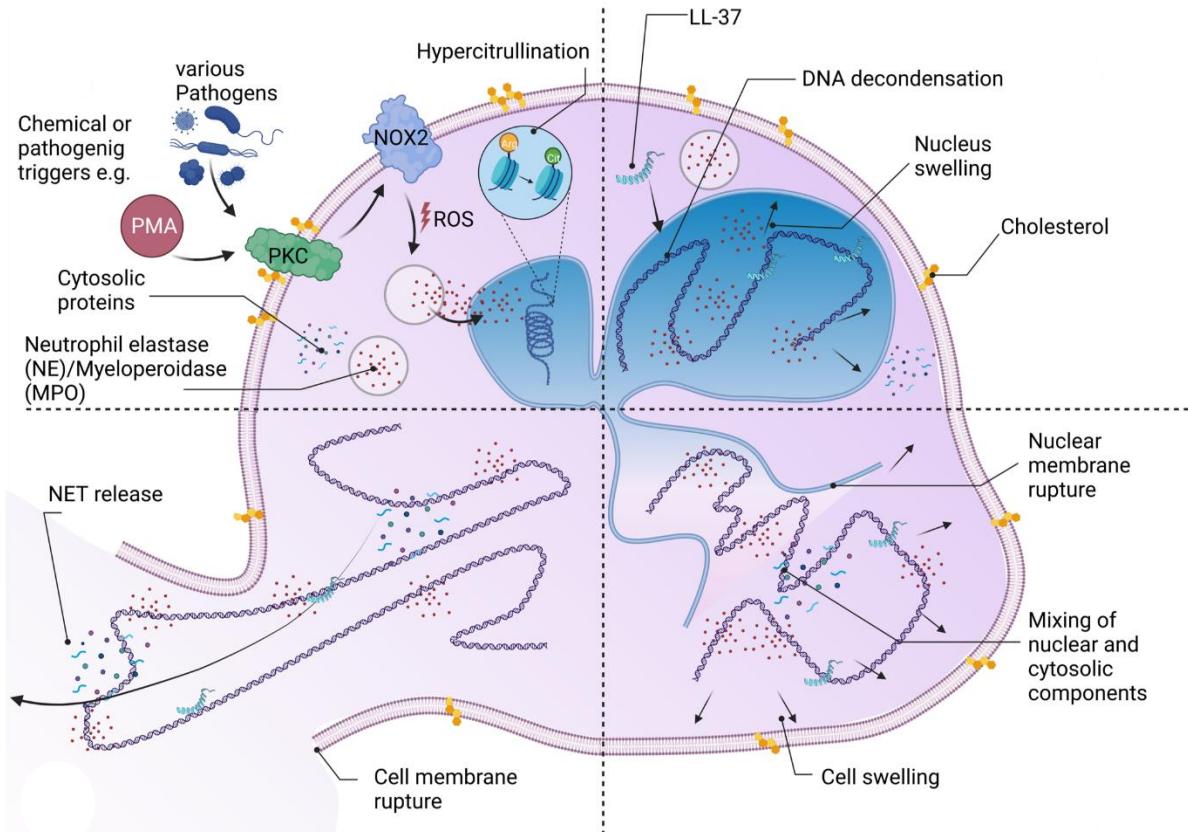
- Antimicrobial action
- Different stimuli can trigger NET release
- NETosis
- Vital/vesicular NET formation
- NET structure: DNA backbone, histones, proteins



Classical NETosis in detail – IFM as tool



Confocal microscopy



- Induced by various stimuli (pathogens, chemicals)
- Multiple markers used in IFM to target NET structures

PMA: Phorbol 12-myristate 13-acetate
PKC: Protein kinase C
NOX2: NADPH oxidase
ROS: reactive oxygen species
IFM: Immunofluorescence microscopy

Aims

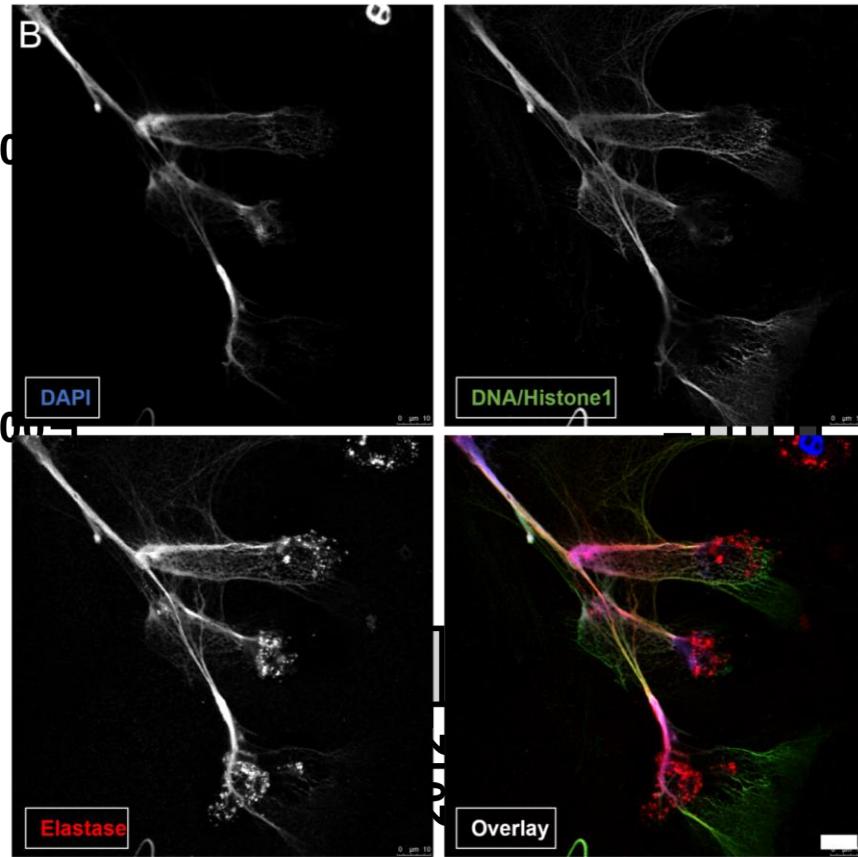


Determine status quo of NET quantification based on literature

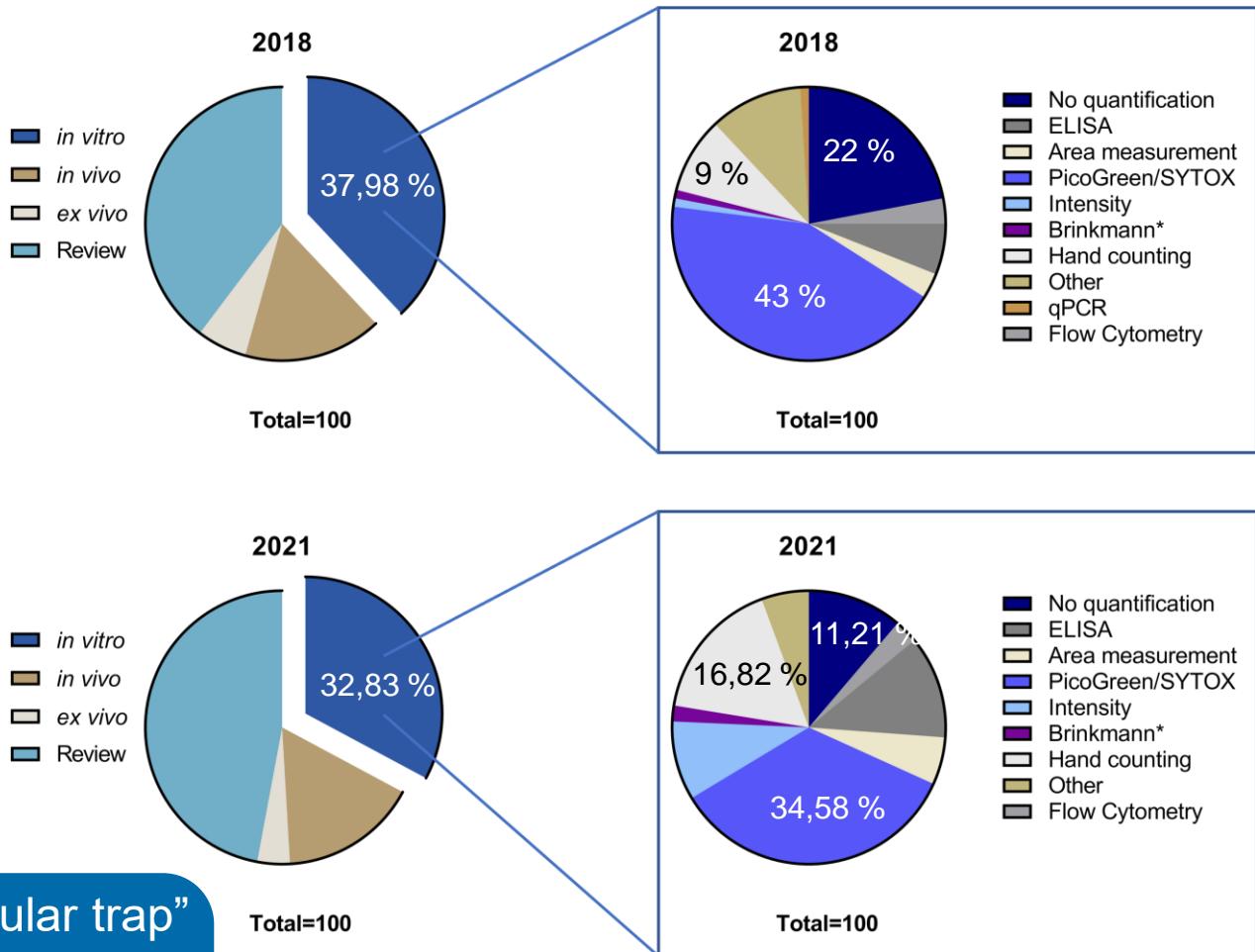
Evaluation of a program based semi automated and fully automated quantification tool for analysis of immune fluorescence images of NET formation

Why is it relevant? Increasing interest in NET research

Publications/year



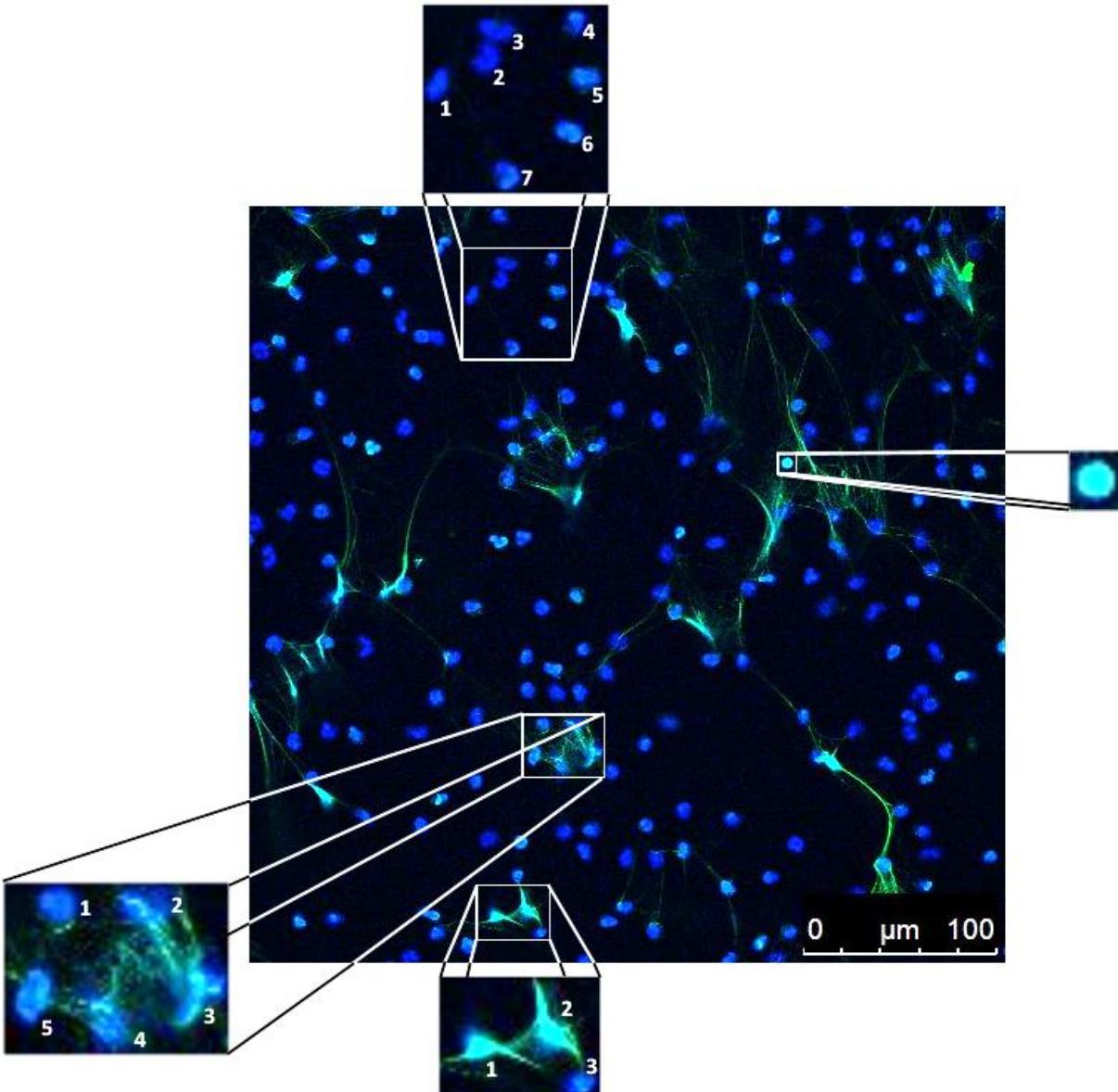
- Literature screening - Pubmed: “neutrophil extracellular trap”
- Identification and quantification of NETs
- Visualization crucial in NET research
- DNA intercalating dye not sufficient



Hand counting – what do you want to count

Define criteria:

- What is a NET
 - Off-shoot
 - Enlarged nucleus and (green) staining
 - + decondensed (puffy)
 - + blurry rim
 - All cells touching an off-shoot
- What is not a NET
 - Small blue or (green) dots
 - Lobulated structures



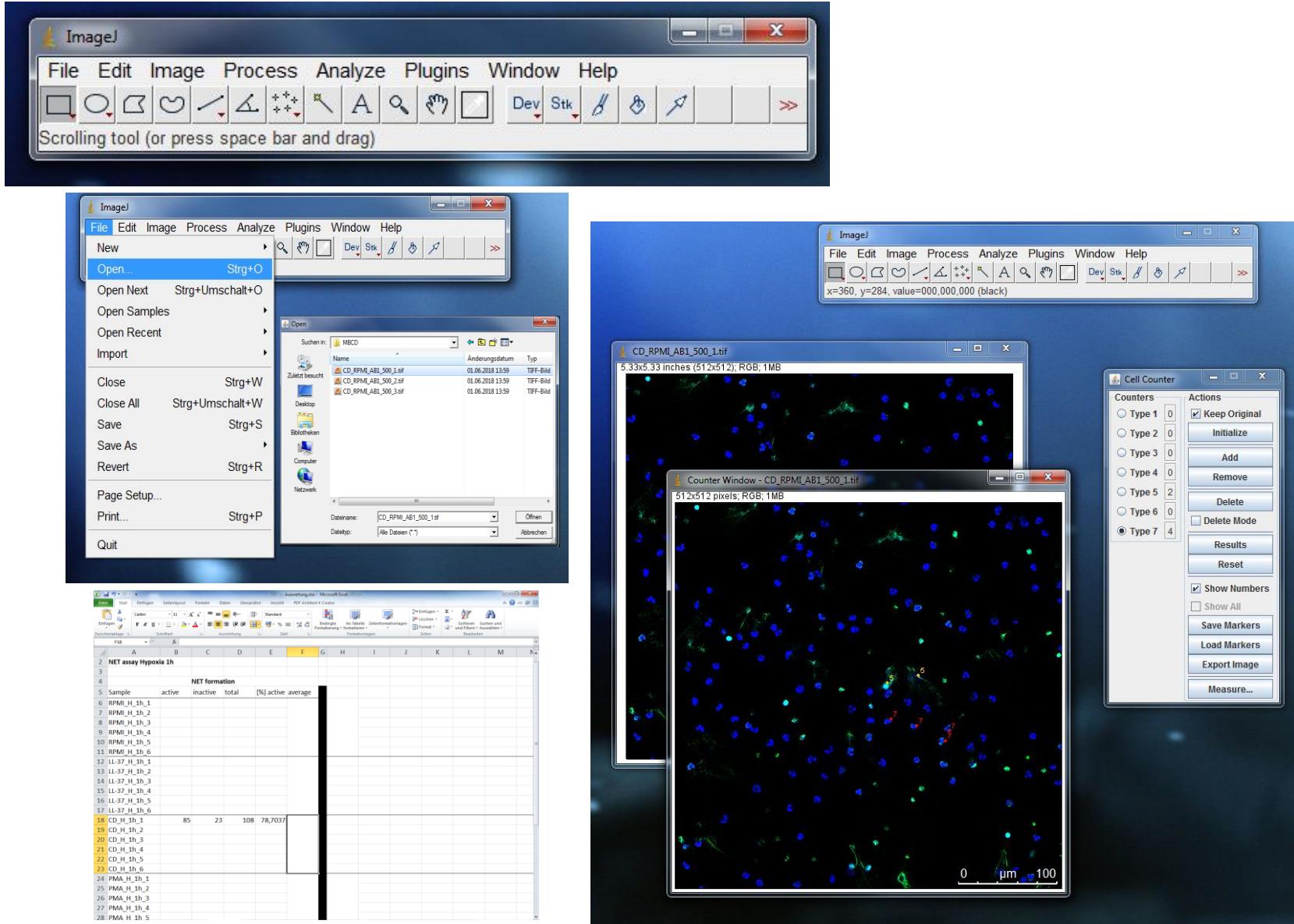
Hand counting – the tool to say goodbye to your weekend



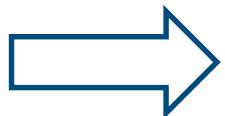
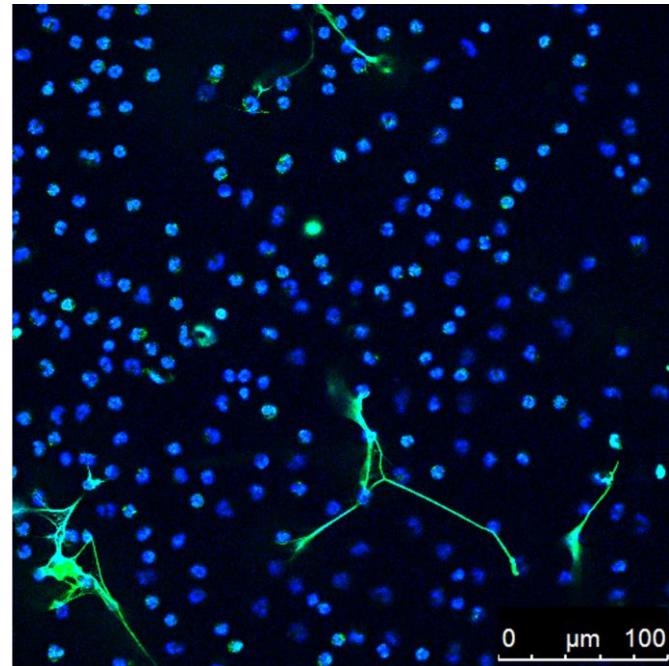
ImageJ - Cell counter plugin

- Mark cell as NET positive or negative on every image
 - ~ 150 cells per Image
 - Easily > 200 images per experiment
 - 2-5 min per image
- Training intensive
- Time intensive
- Bias prone
- Reproducibility/comparability

Need for a reliable alternative

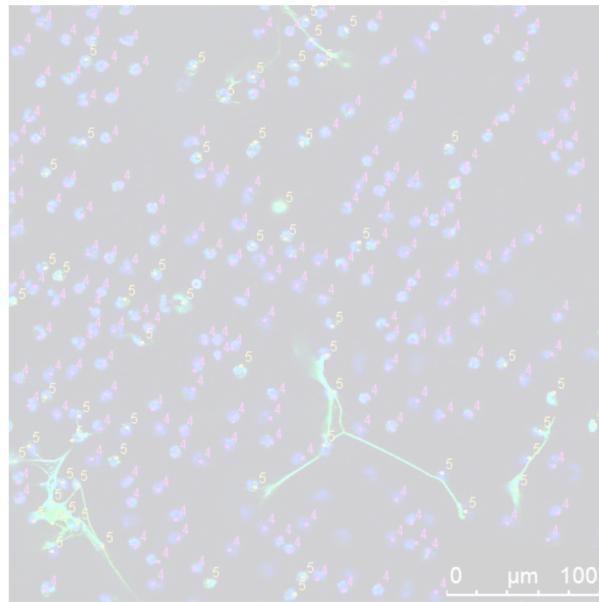


Semi automated quantification in ImageJ (Brinkmann)



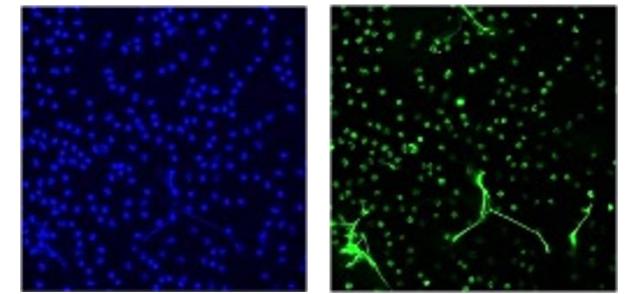
Hand counting

Marking cells individually
with cell counter

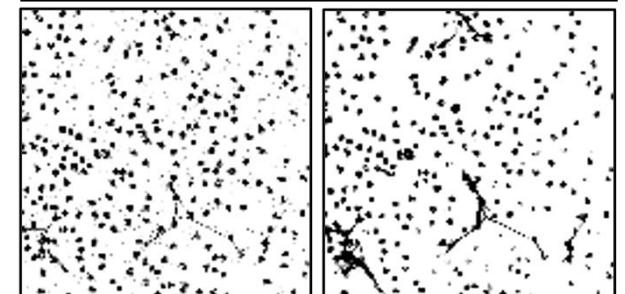


- Freely available software
- Relatively easy to apply
- Applied in the field to some extend
- Fast

Semi automated



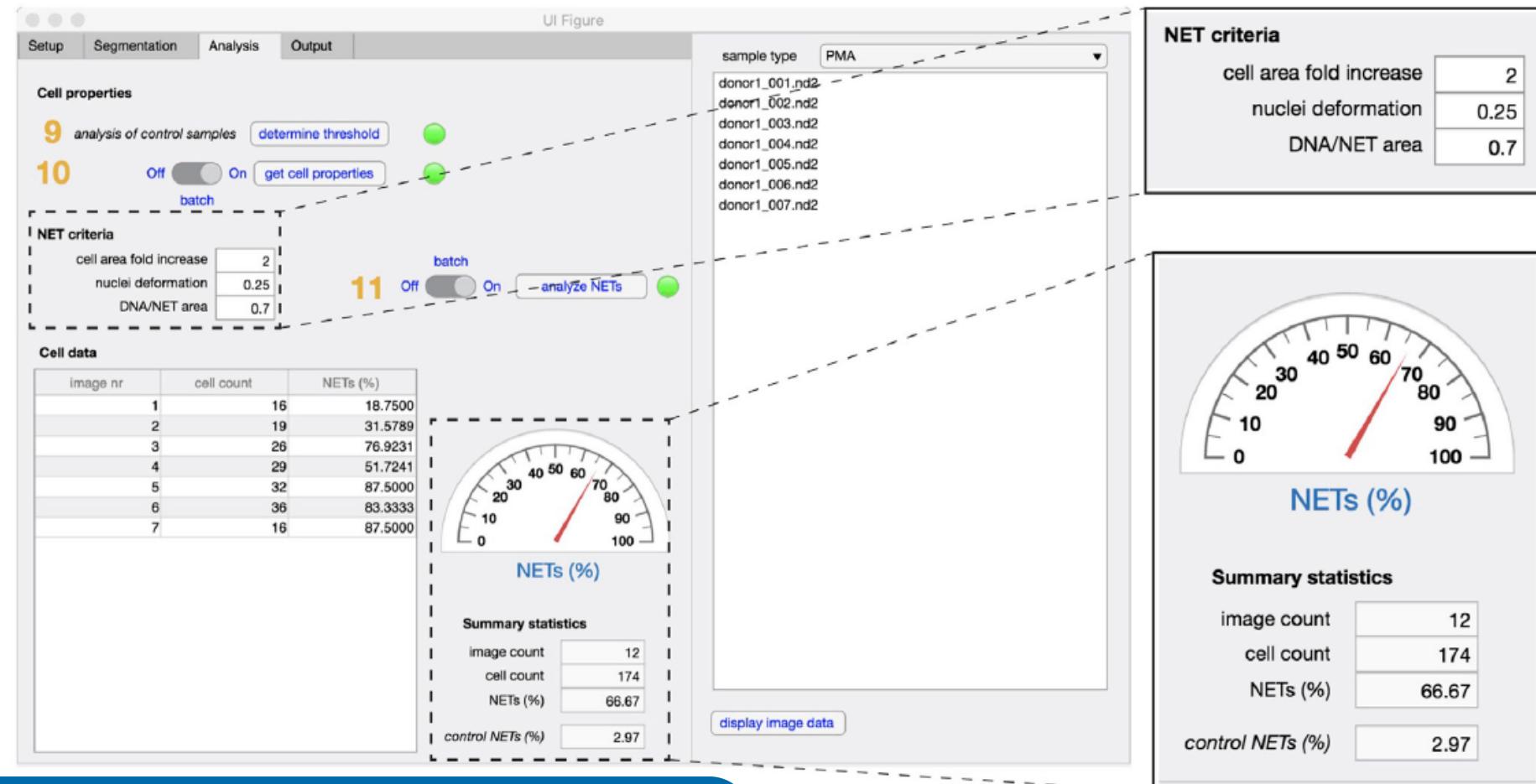
ImageJ:
8Bit → threshold determination →
min/max size → analysis



Count of events

$$\frac{\text{count DNA/Histone}}{\text{count DAPI}} * 100 = \text{NETosis [\%]}$$

NETQUANT – fully automated



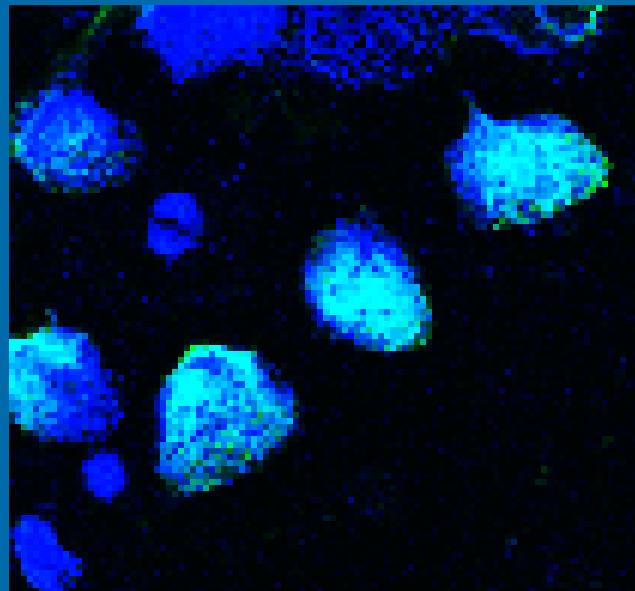
- Automated quantification after initial setup
- Multiple formats usable (e.g. tif, nd2)
- Requires MATLAB license
- Fast (if processing works error free)

Comparison of the 3 tools with 2 image sets

Image set 1

Own images

Staining: DAPI (blue) DNA/Histone (green)



Hand

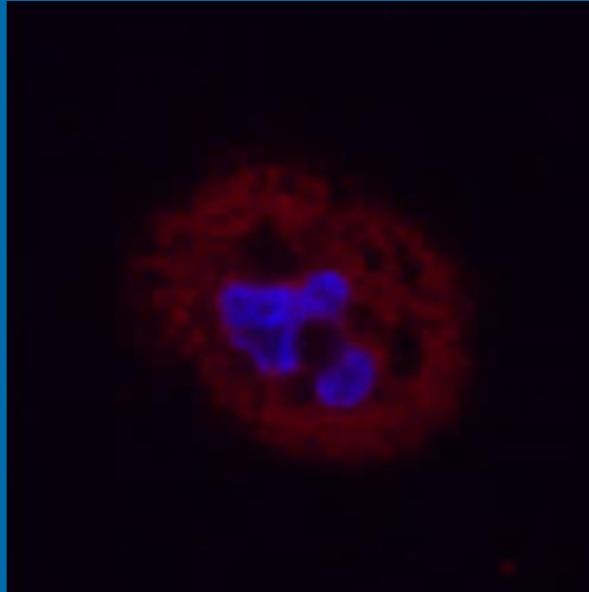
Semi-automated

NETQUANT

Image set 2

Provided by NETQUANT for program setup

Staining: DAPI (blue), Neutrophil Elastase (red)

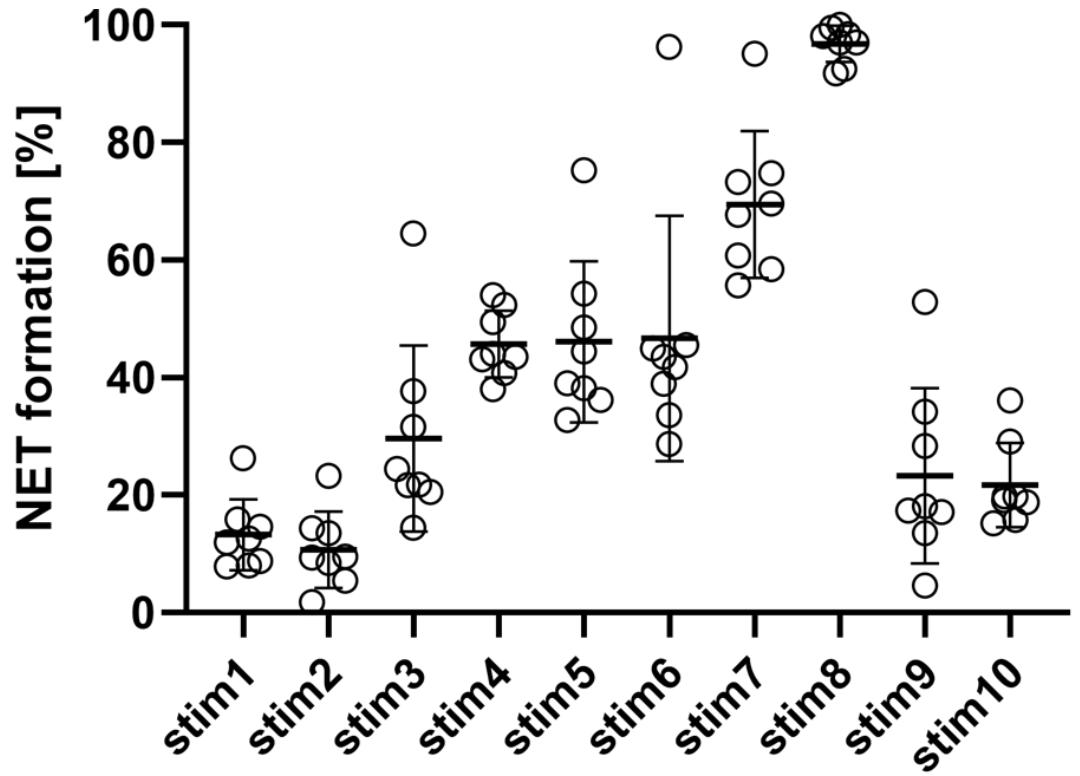
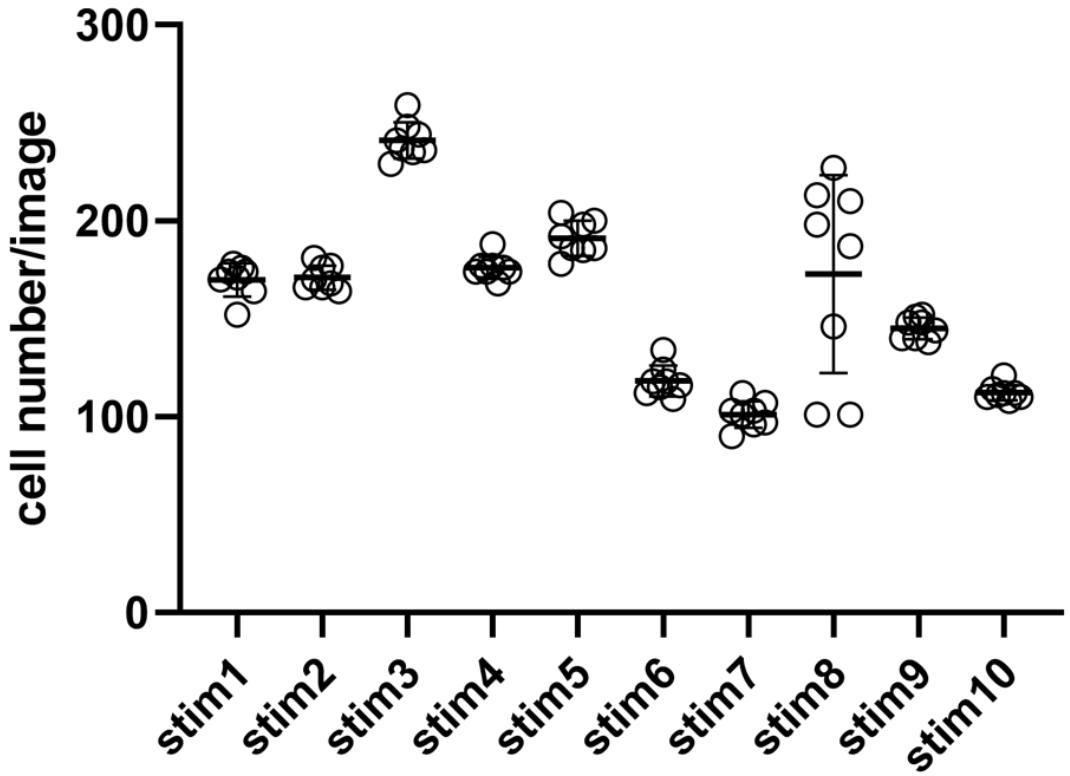
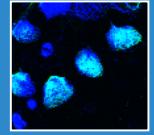


Hand

Semi-automated

NETQUANT

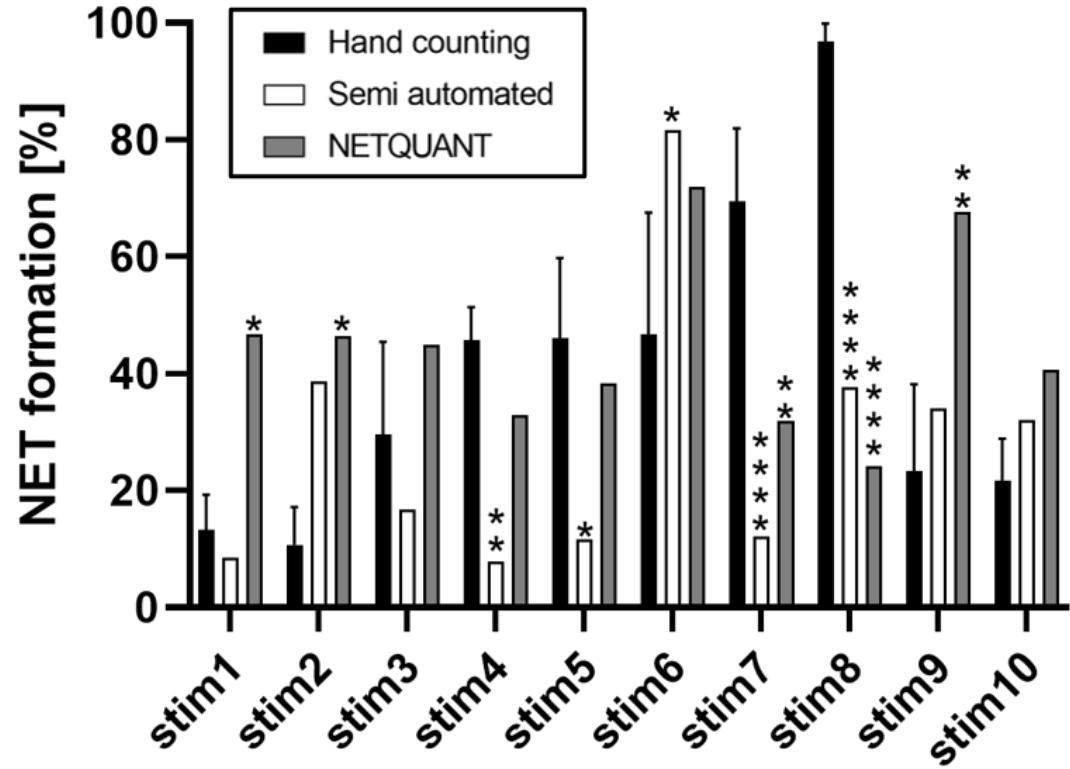
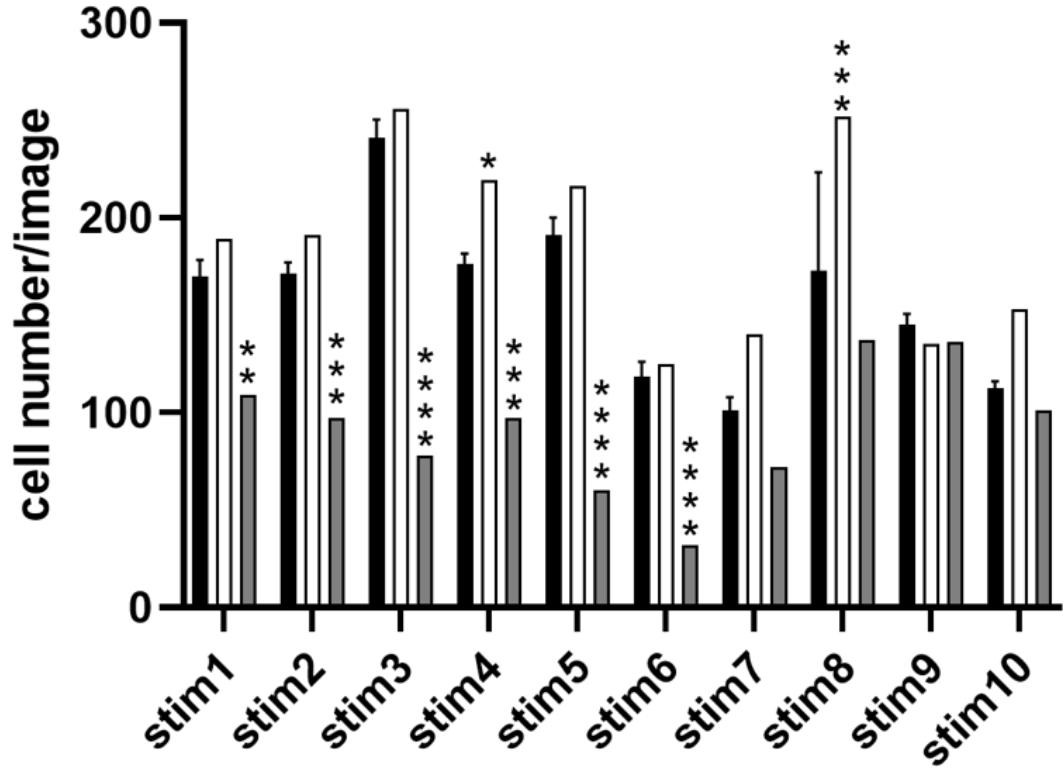
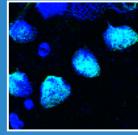
Different people – different opinions I



Multiple people quantified image set 1

- Cell number determination can already show deviations
- Effect enhanced for NET formation – criteria interpretation

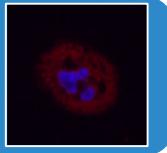
Programs see numbers and NETs differently I



Program values compared to hand counting

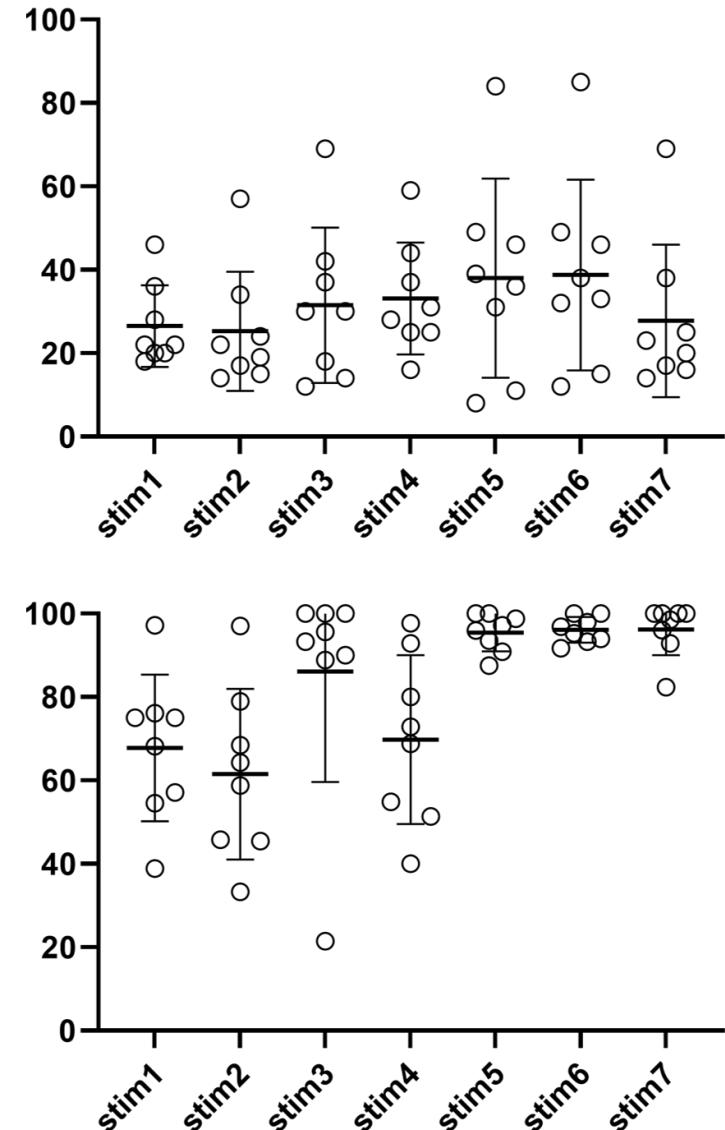
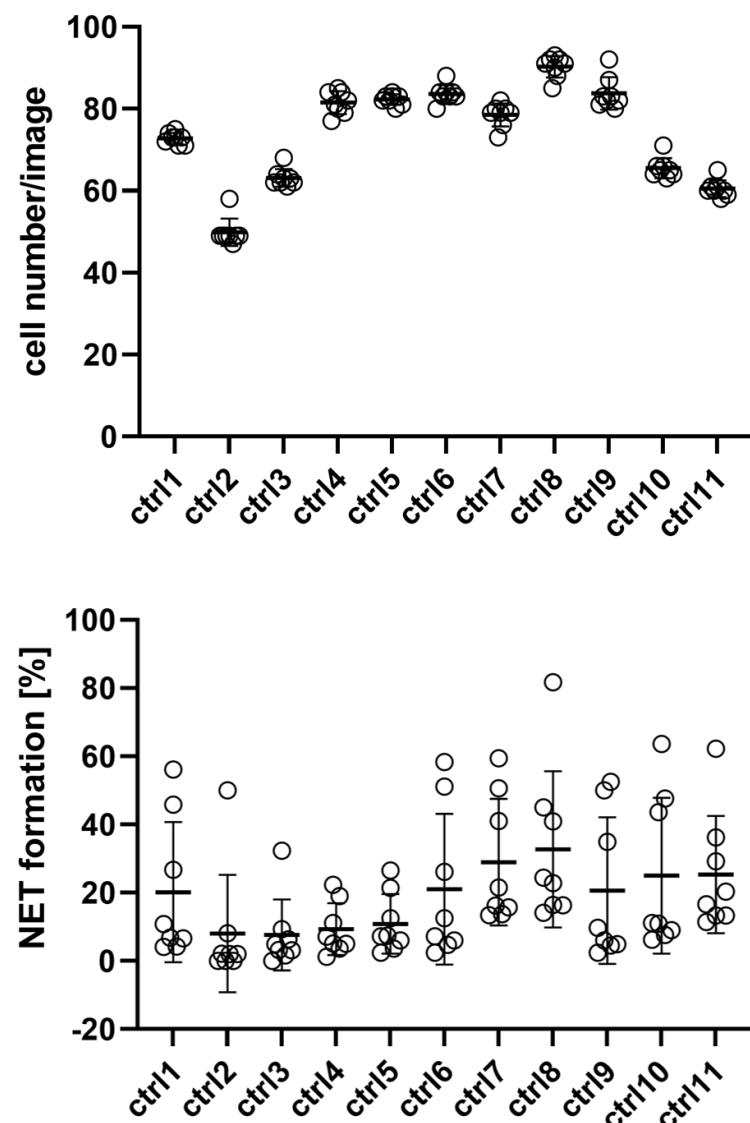
- Cell number: NETQUANT often lower, Semi automated close to Hand counting
- NET formation: NETQUANT and Semi automated mostly far from Hand counting

Different people – different opinions II

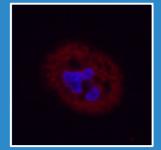


Multiple people quantified image set 2

- Similar for cell number in unstimulated group, diverse in stimulated group
- Heterogenous results for NET formation in both groups
- Different staining technique than our standard – no training

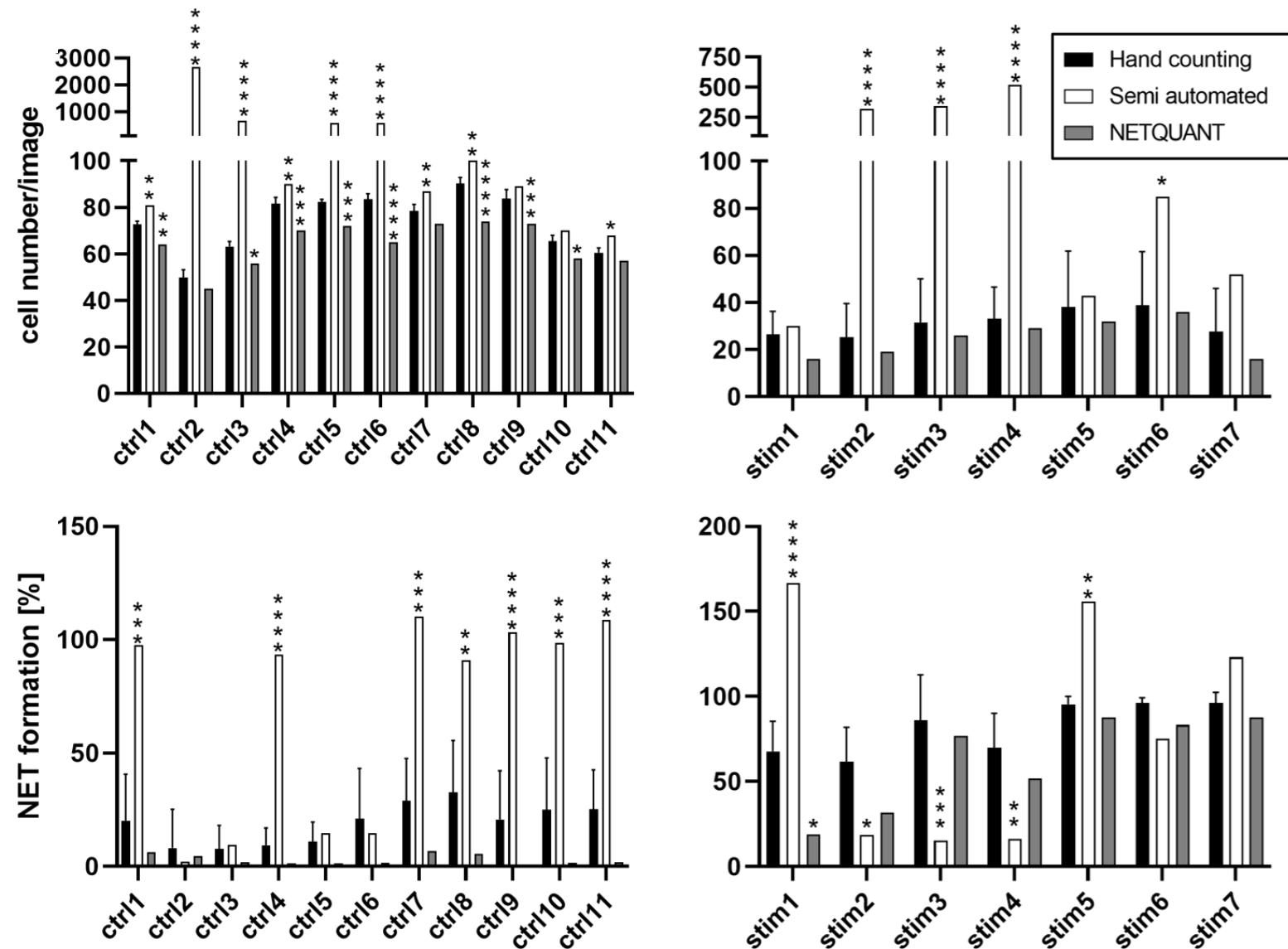


Programs see numbers and NETs differently II



Program values compared to hand counting

- Cell number: High overestimation by Semi automated
- NET formation: Semi automated far above other methods



Where do discrepancies come from?

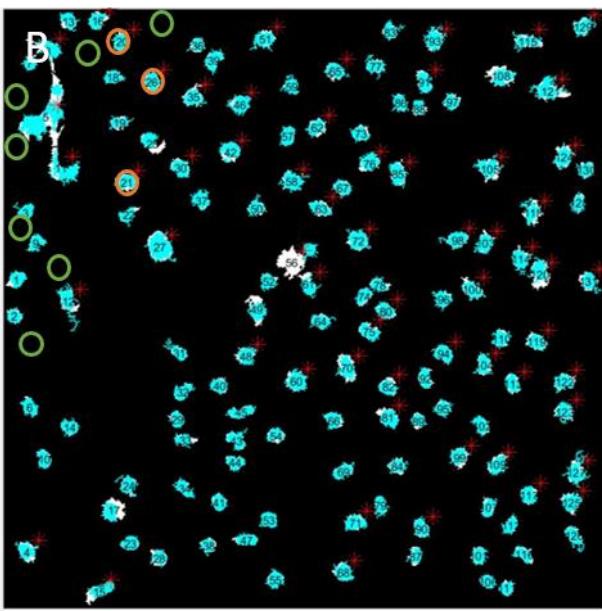
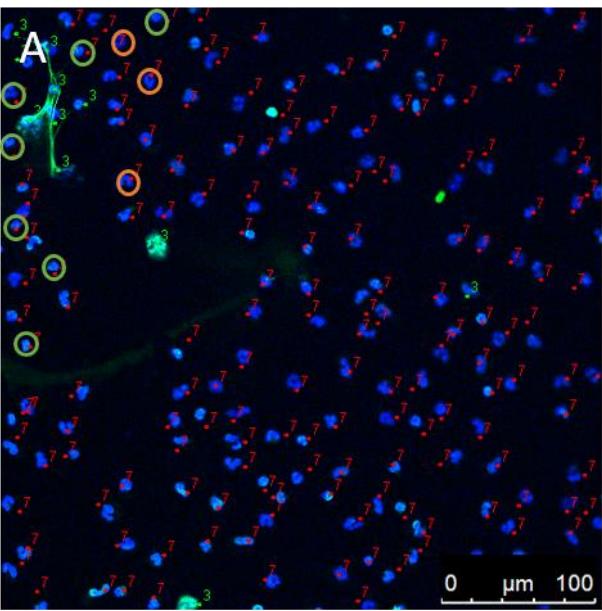
NETQUANT

- Loss of cells
- Negative cells seen positive

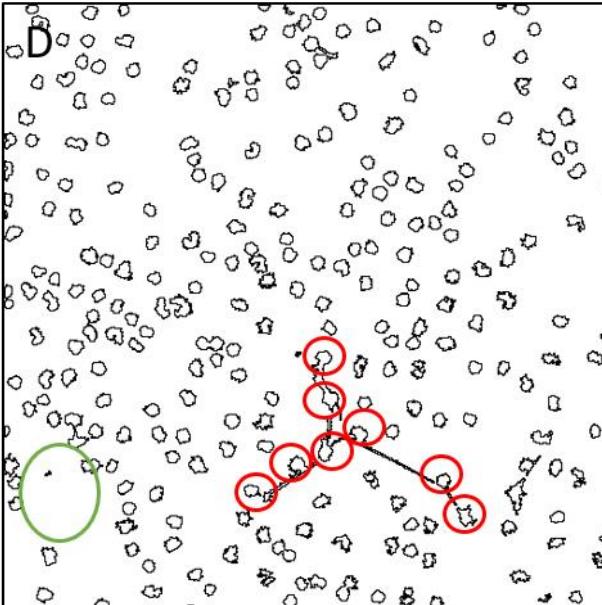
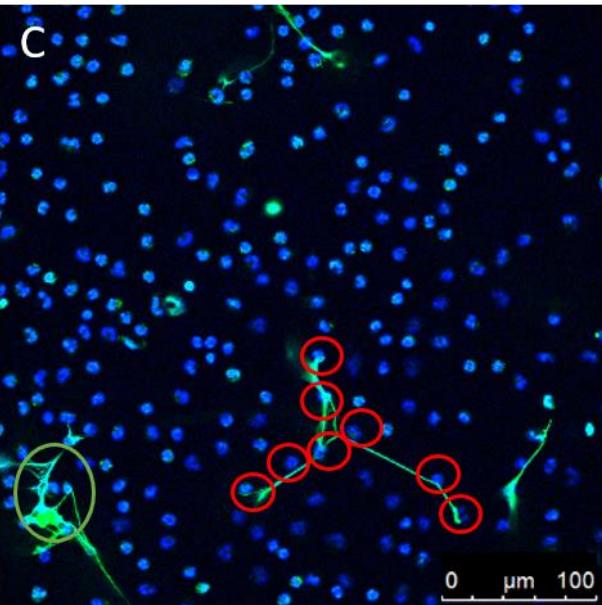
Semi automated

- Loss of cells or NET structures
- Fusion events

NETQUANT

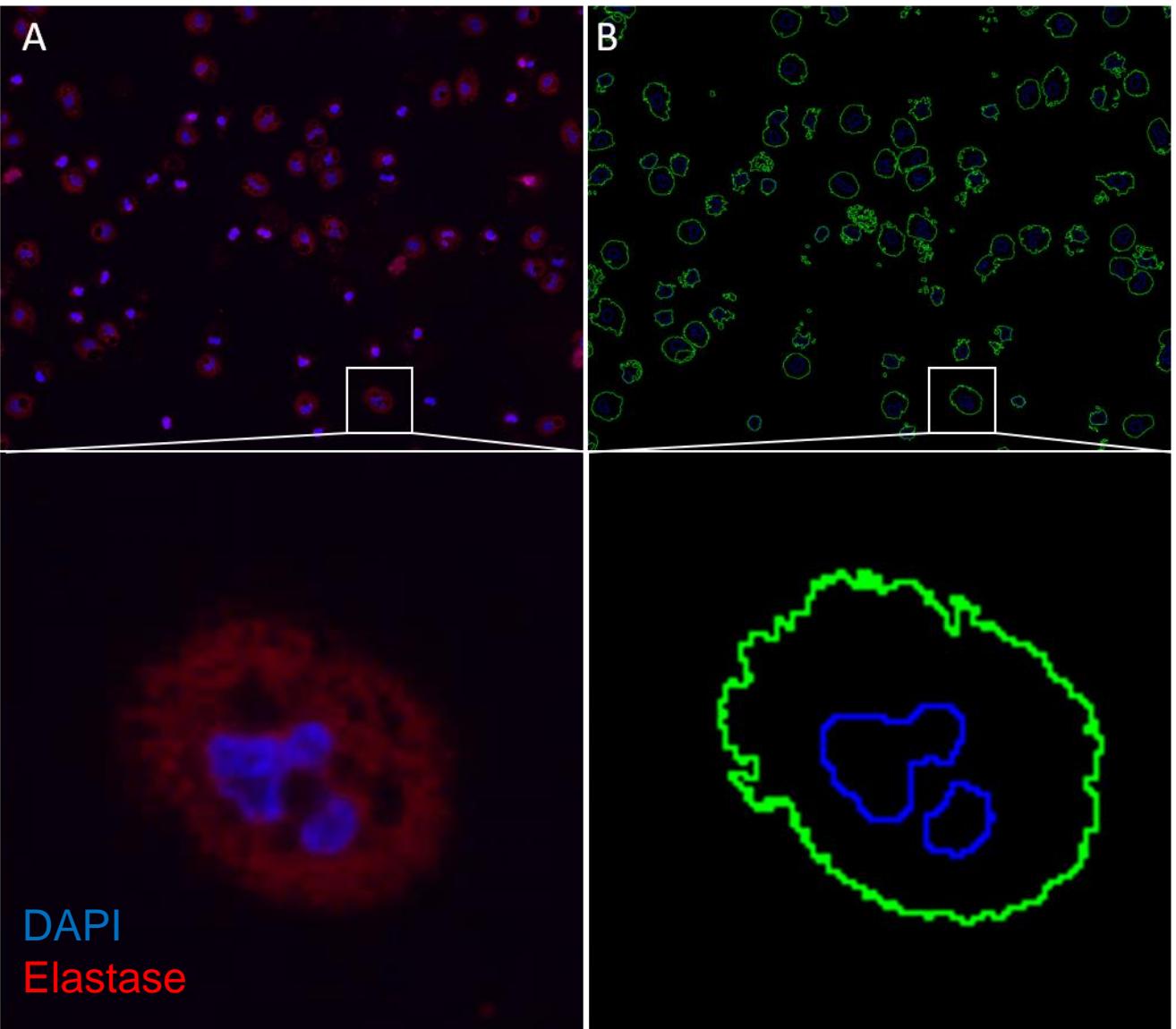


Semi automated

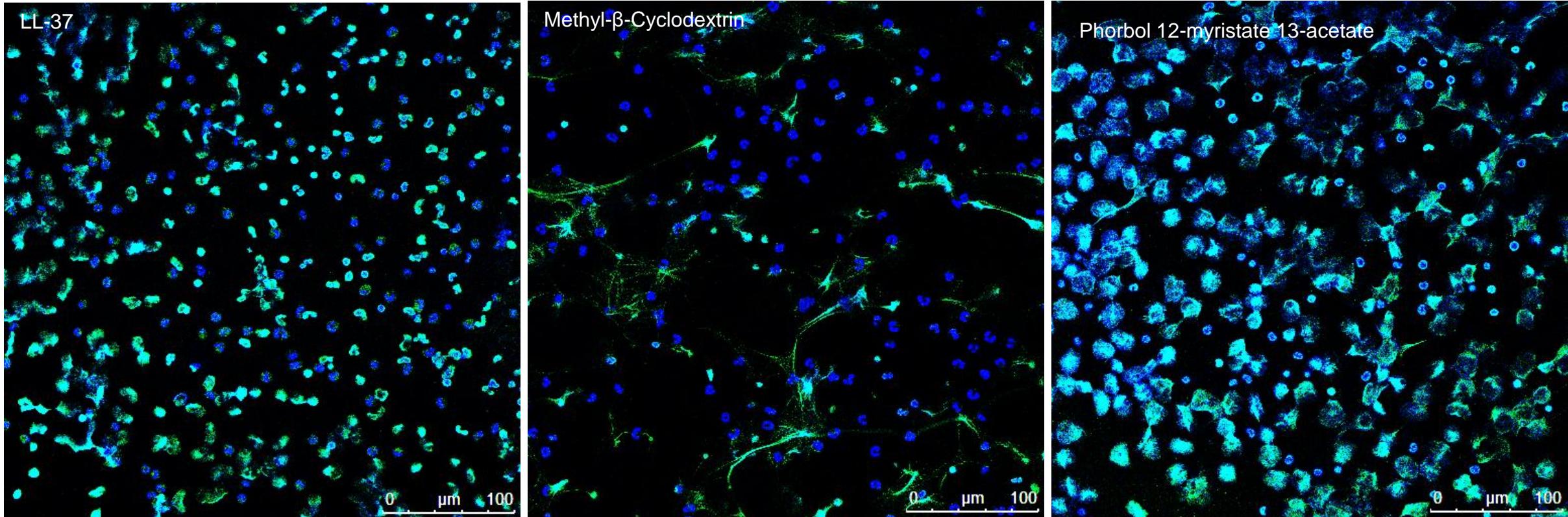


Elastase as single NET marker

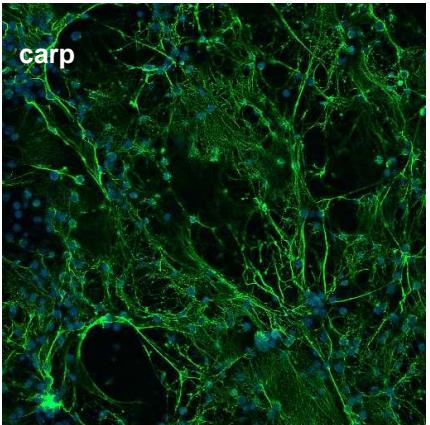
- Semi automated quantification
- Separated channels for DAPI and NET marker
 - Outlines drawn by ImageJ
 - Events counted in each channel
 - Elastase also present in inactive cells, creating a false positive event in NET channel
- NET channel / DNA channel



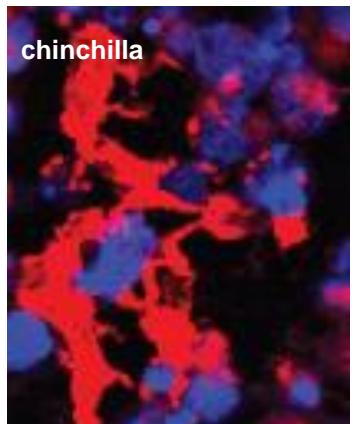
NET heterogeneity due to stimulus



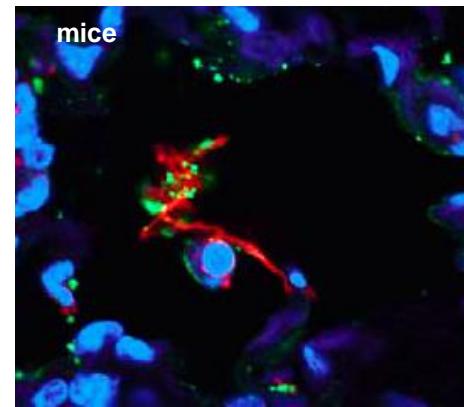
NET heterogeneity due to species



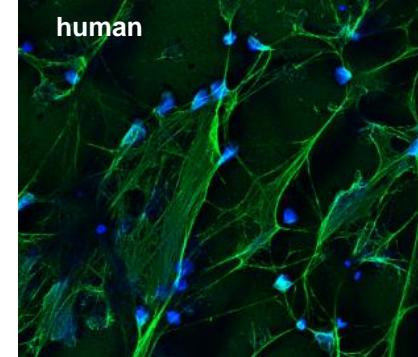
Brogden et al., *Dev. Comp. Immunol.*, 2014;
Brogden et al., *Fish and Shellfish Immunol.* 2012



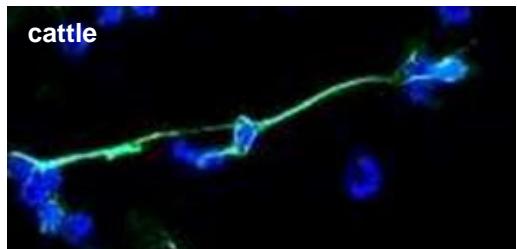
Short et al., *Infect. Immun.*, 2013



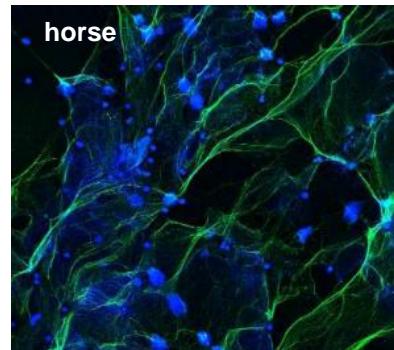
Berends et al., *J. Innate Immun.*, 2010



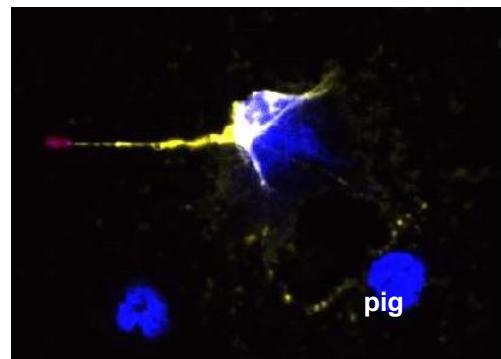
Neumann et al., *J. Innate Immun.*, 2014;
Branitzki-Heinemann et al., *Biochem. J.* 2012



Jerjomiceva et al., *J. Innate Immun.*, 2014



Schumski et al., *in preparation*



de Buhr et al., *Microbiology*, 2013

Discussion: What to choose out of imperfect options?

- Hand counting not perfect
- Awareness for obvious down sides (bias, time) – good description in material and methods needed
- Still broadly used for IFM image quantification
- Flexible
- Programs were not able to reach the standard of hand counting – no program widely accepted in the field
- Strongly fixed on certain staining conditions
 - Possible application for heavily standardized staining and quantification protocol
- Heterogenous morphology too challenging (staining, stimulus, species, population, disease)
- Bias: users set thresholds, cut off values → improvement with less bias compared to hand counting?
- Programs need to develop further
- Deep learning
 - For now: too complicated for users, too expensive
- Single read out not sufficient anymore:
- More markers for IFM (here only one marker + DNA dye)
- Combination with additional techniques (ELISA, Scanning EM, Transmission EM)

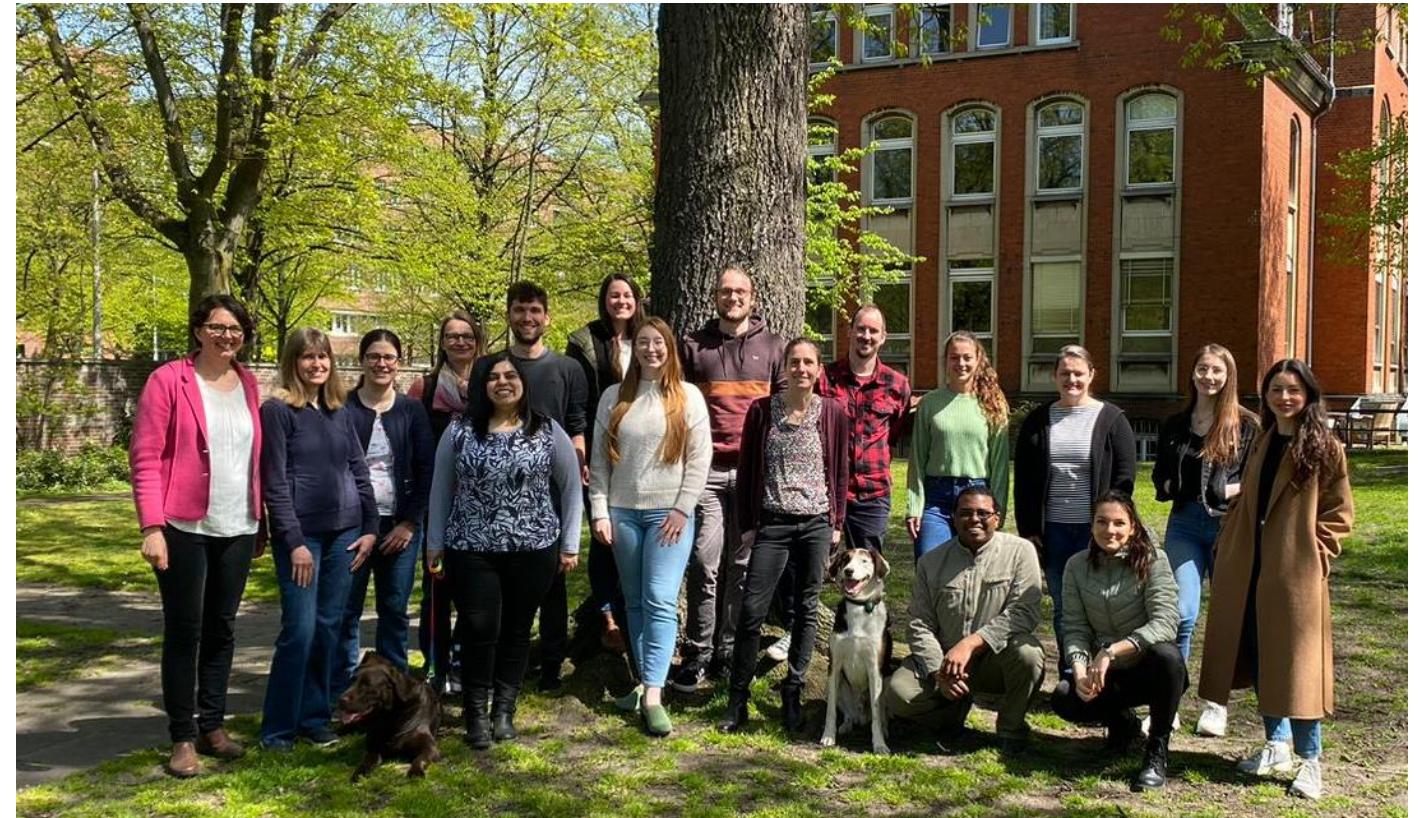
Conclusion: Hand counting remains the method of choice

But: Has to be well described and supported by additional methods

Thank you for your attention! Questions?

Thanks to all Coauthors:

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Nicole de Buhr
Katja Branitzki-Heinemann
Maren von Köckritz-Blickwede



AG Infection Biochemistry
Institute for Biochemistry

Recently accepted



Figures created with Biorender



Image set 1, control group

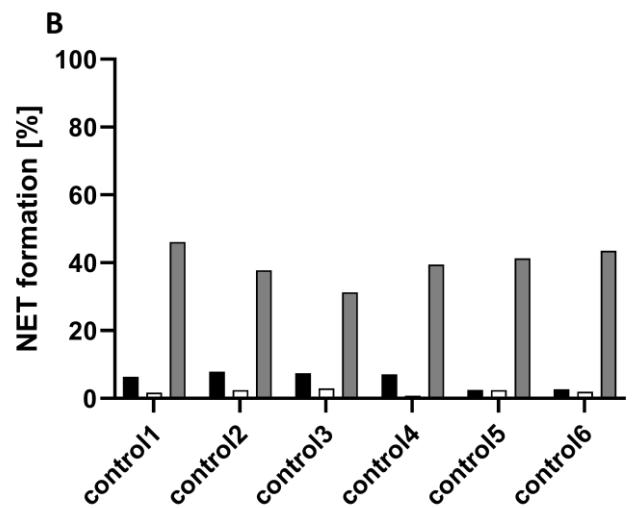
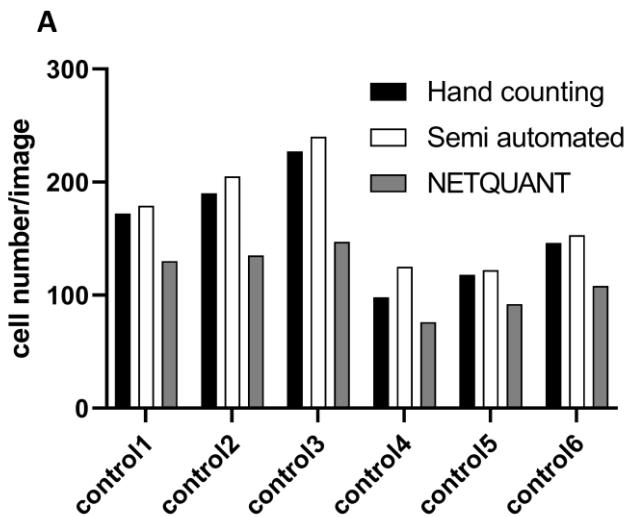
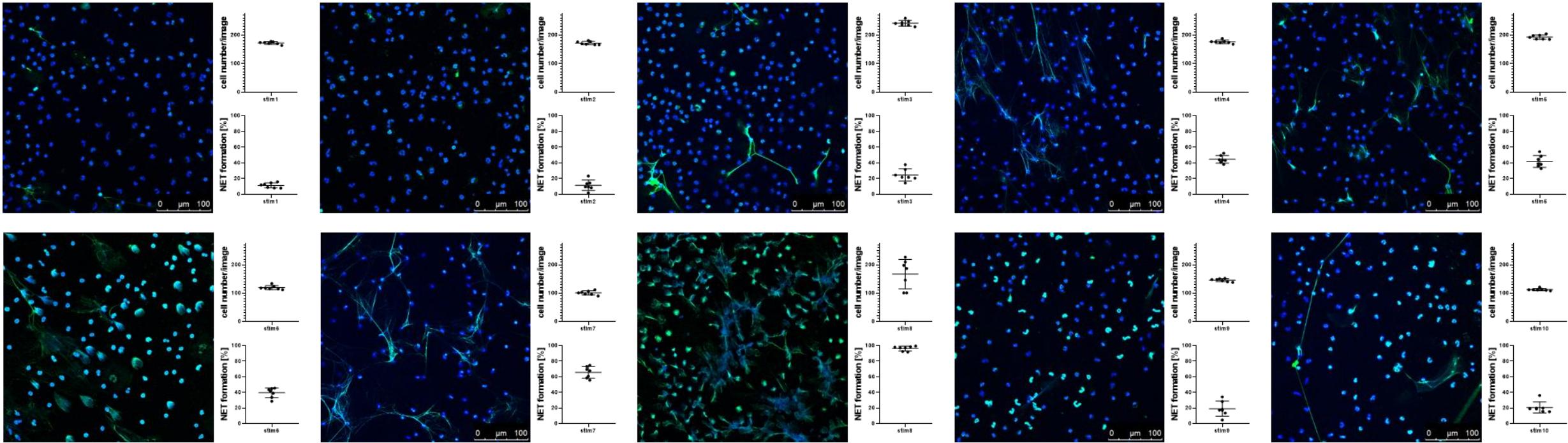


Image set 1



NETs in Scanning electron microscopy

