Quantifying Neutrophil Extracellular Trap Release in a Combined Infection-Inflammation NET-Array Device

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① Prime the NET-array device with fibronectin

1X wash with assay buffer →

② Add isolated primary human neutrophils to the device and let settle in the wells

Isolated neutrophils from blood sample

1X wash with assay buffer →

③ Add bacterial cell suspension (MOI 10) with or without the inflammatory mediators

Bacterial suspension + Cytokine solution

Time-lapse imaging →

④ Seal the device with press-to-seal cover; add assay buffer on top

⑤ Immunocytochemistry on-chip to study heterogeneity of proteins on NETs

⑥ Automated image analysis

Time-lapse NETs assay

Live neutrophils
PAO1
Dead cell DNA

100 μm

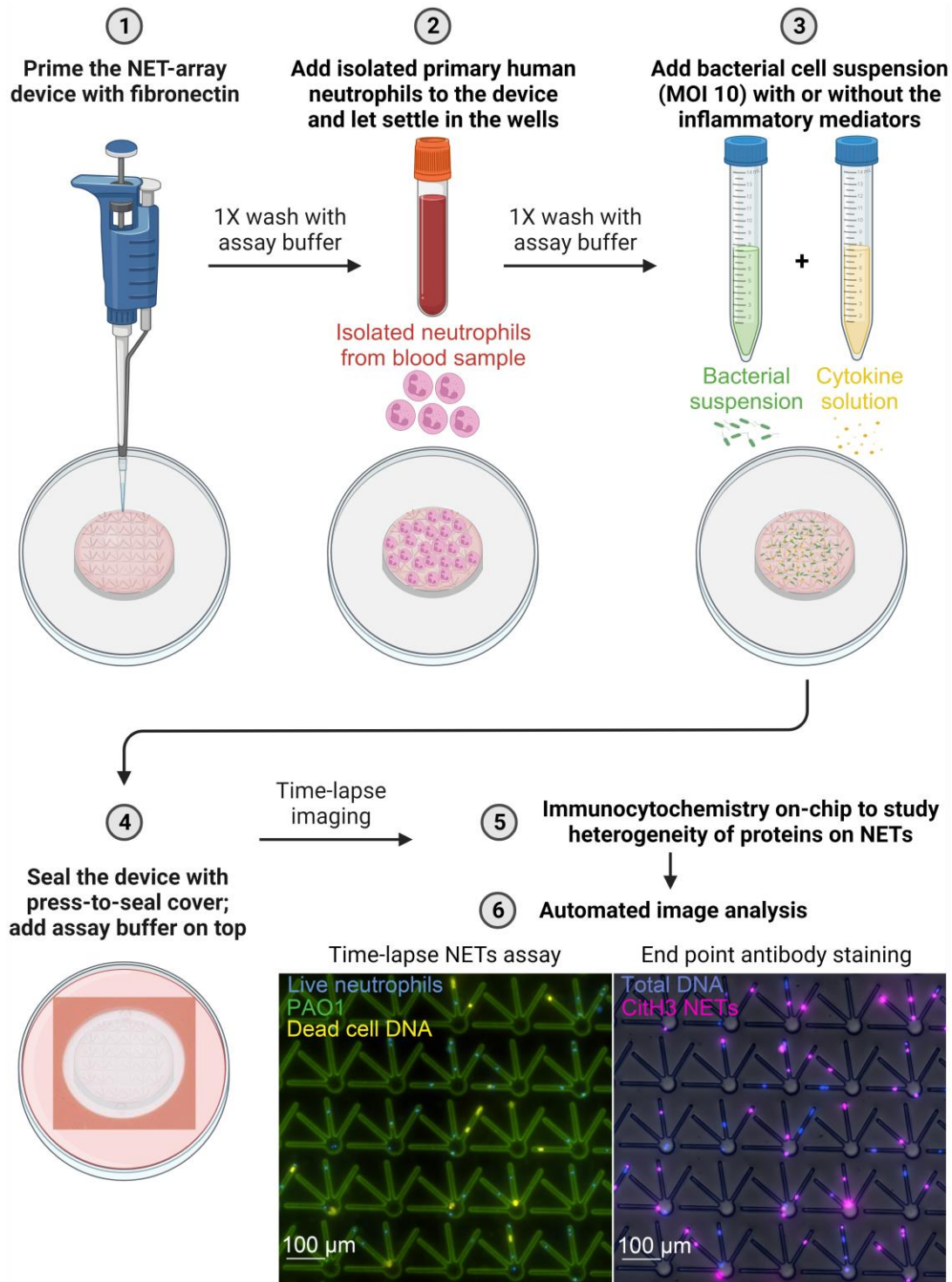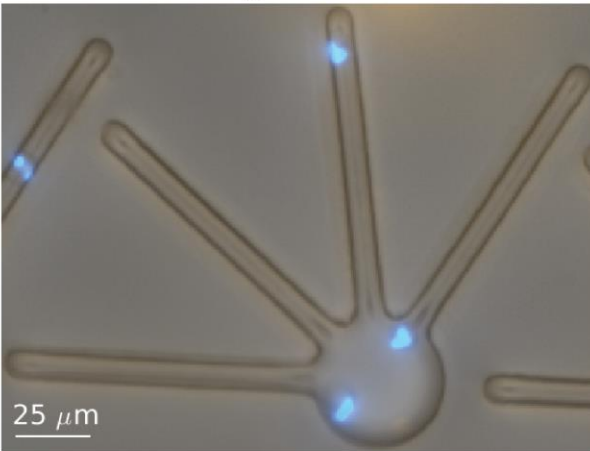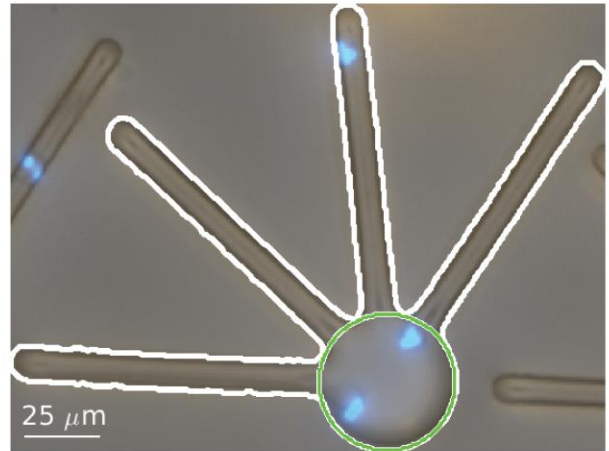End point antibody staining

Total DNA
CitH3 NETs

100 μm

Figure S1. Detailed schematic of the experimental workflow developed to quantify NET release dynamics in the combined infection-inflammation NET-array device.

A. Reference image

B. Well detection

C. Cell detection and analysis

1 hr. — 32 μm² ; 36 μm² ; 34 μm²

2 hr. — 214 μm²

3 hr.

4 hr.

5 hr. — 188 μm²

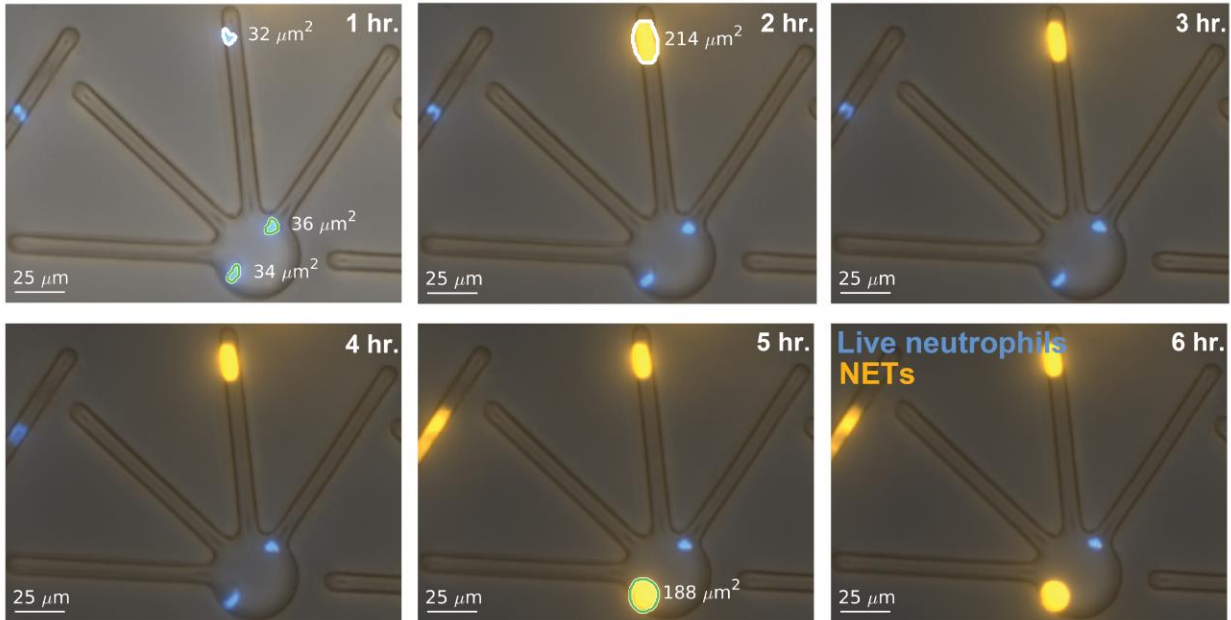6 hr. — Live neutrophils / NETs

25 μm

Figure S2. Computer-vision based image processing to automate the quantification of individual NETs released in different locations of the NET-array device. Blue: live neutrophil DNA (Hoechst 33342 stained); Orange: NETs (sytox orange stained). **(A)** Brightfield reference image of a single well overlayed with live cells at t = 1 hr. **(B)** Well detection followed by identification of chamber (boundary marked in green) and loop (boundary marked in white) **(C)** Cell detection of live (blue) and dead (orange) cells across all the time points. Area (in μm²) of dead cells is computed at their first detection time point.
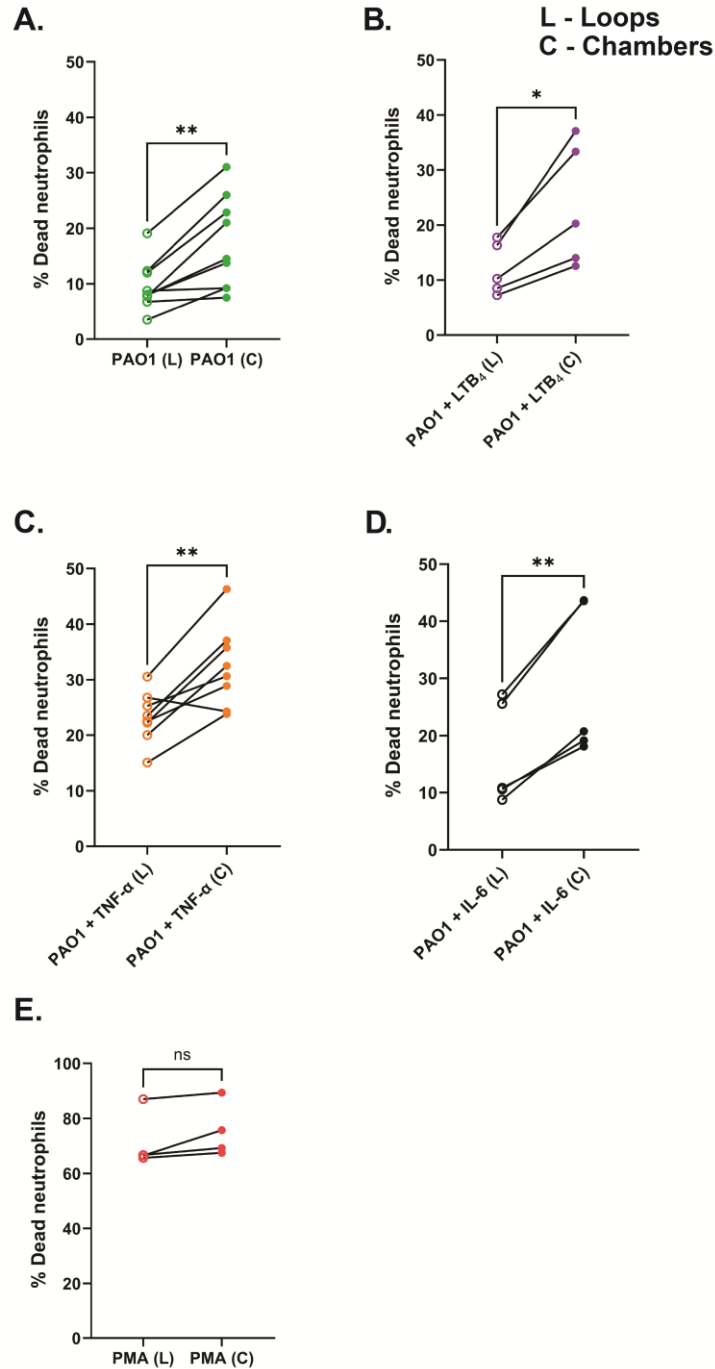
Figure S3. Comparative analysis showing lower percentage of dead neutrophils in the loops (L) relative to the chambers (C) of the device, when challenged with *P. aeruginosa* (PAO1) bacteria with or without the inflammatory mediators. (A) Neutrophils + PAO1 (no inflammatory mediator) **(B)** Neutrophils + PAO1 + 20 nM LTB$_4$ **(C)** Neutrophils + PAO1 + 20 ng/mL TNF-α and **(D)** Neutrophils + PAO1 + 50 ng/mL IL-6 and **(E)** Positive control (20 nM PMA). Data are represented as mean $\pm$ SEM and is collected from 5 to 9 separate healthy volunteers, except for positive control where n = 4 donors. ns – not significant, * $p < 0.05$, ** $p < 0.01$; two-tailed paired student t test used.

Video

Video S1. Video demonstrating the spatiotemporal dynamics of NETs released to _P. aeruginosa_ (PAO1) bacteria in 5 × 5 wells of the NET-array device over a period of 6 hrs., under conditions listed. Blue: live neutrophil DNA (Hoechst 33342 stained); Orange: Dead cell DNA (sytox orange stained); Green: PAO1-GFP. **(A)** Negative control (neutrophils alone) **(B)** Positive control (20 nM PMA) **(C)** Neutrophils + PAO1 (no inflammatory mediator) **(D)** Neutrophils + PAO1 + 20 nM LTB_4 **(E)** Neutrophils + PAO1 + 20 ng/mL TNF-α and **(F)** Neutrophils + PAO1 + 50 ng/mL IL-6.