NET formation extended

PMA Pathway: PMA -> PKC -> NADPH oxidase -> ROS -> chromatin decondensation -> NETs release

Ionomycin Pathway: Ionomycin -> calcium level increase -> PAD4 -> histone citrullination -> NETs release

Raptinal Pathway: Raptinal -> Apoptosis induction -> Mitochondrial Outer Membrane Permeabilization (MOMP) -> caspase activation (caspase 9/3) -> gasdermin E cleavage -> GSDME-N -> membrane pores -> lytic NET release

Nigericin Pathway: Nigericin -> Potassium ions efflux with Hydrogen ions influx -> NLRP3 inflammasome -> caspase-1 directed gasdermin D cleavage -> GSDMD-N -> pore formation -> DNA release

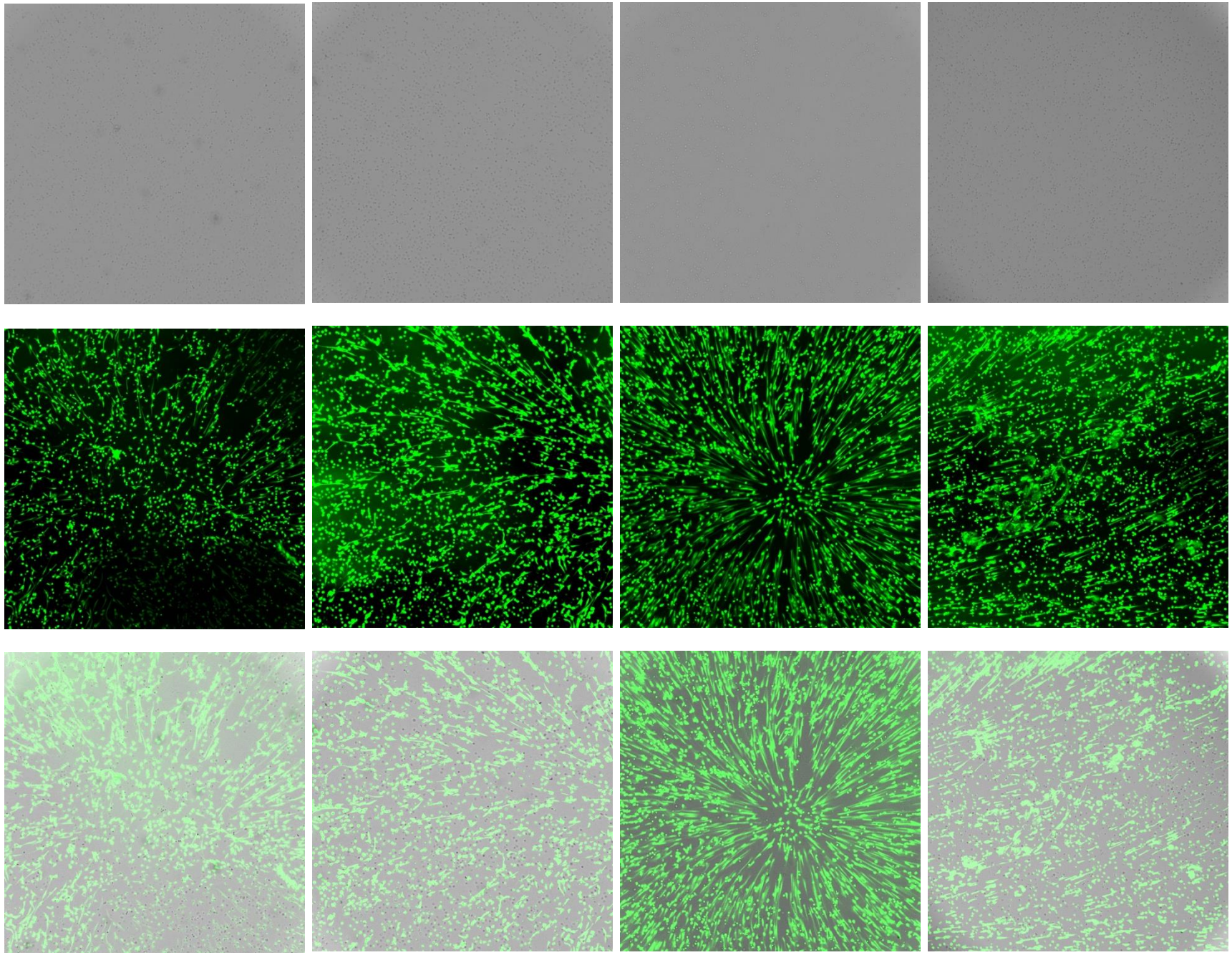
The two pathways above are not directly associated with NETosis but do promote NET structure.

Val-boropro Pathway: pyroptopsis -> DPP8/9 inhibition -> CARD8 (human) or NLRP1 (mouse) -> Caspase-1 (human), Caspase-11 (mouse) -> lytic cell death (slightly slower)

Hypothesis: Investigating different compounds with apoptosis and pyroptopsis on NET formation.

Methodology:
Extract the neutrophils from bone marrow
Add the compounds to the cell
Use the device to observe the NETs

10x Microscope



BF

GFP

Merged

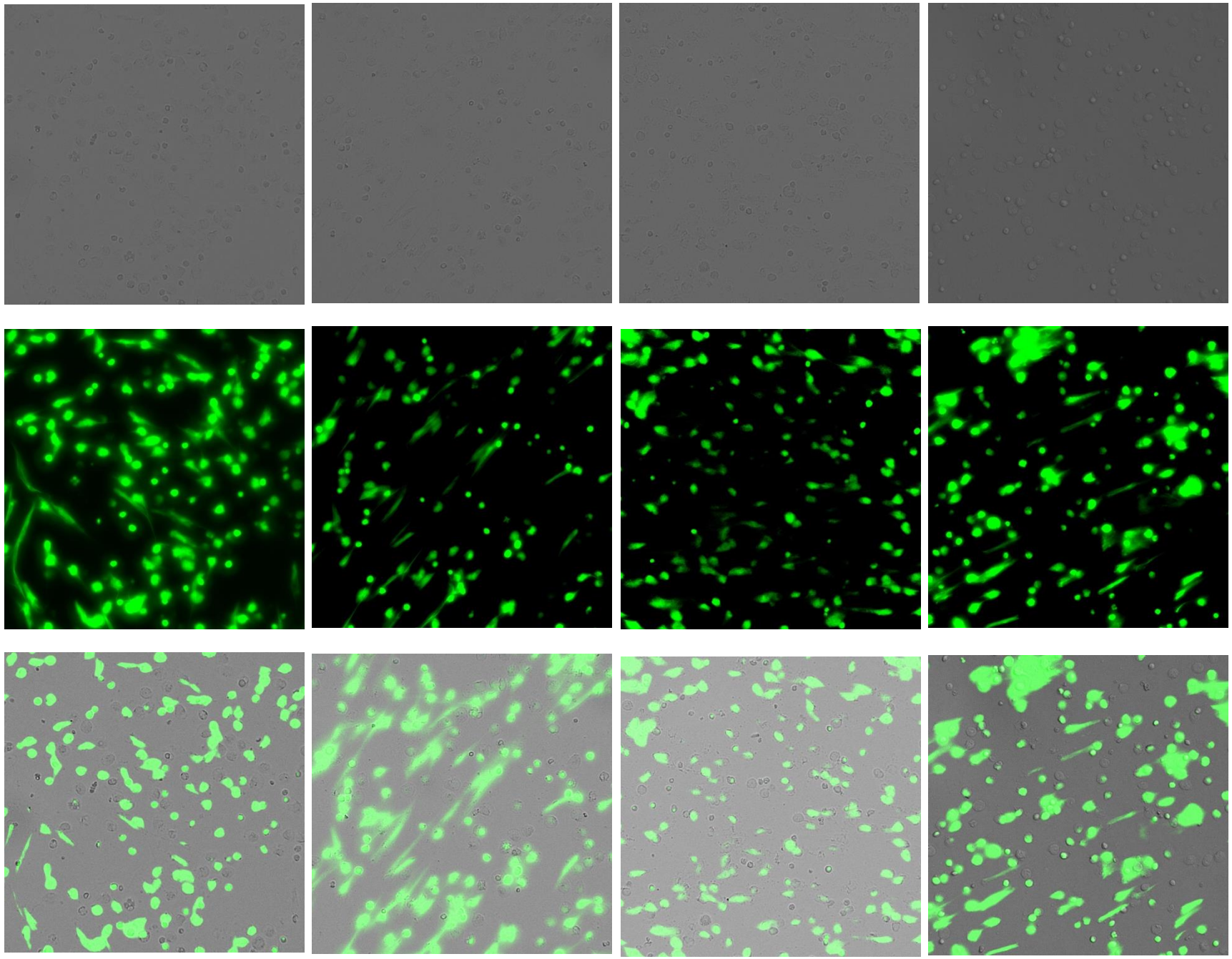
PMA

PMA

Ionomycin

Ionomycin

40x Microscope



BF

GFP

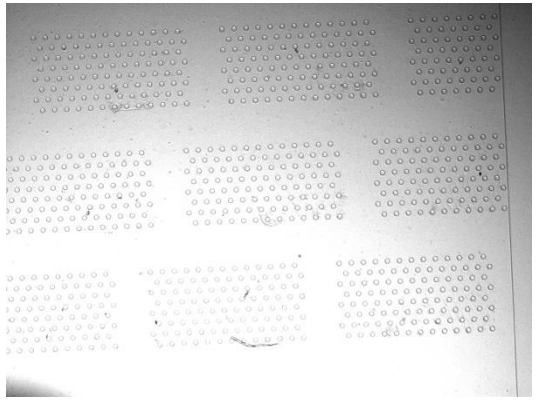
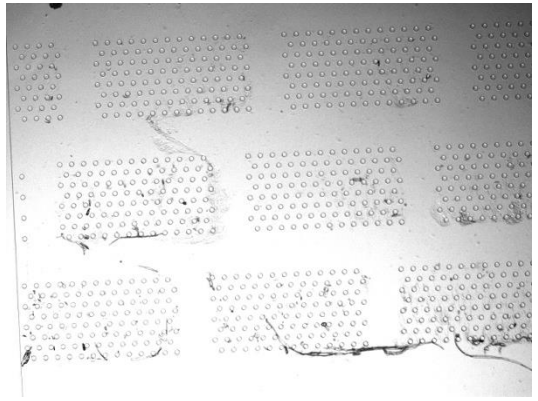
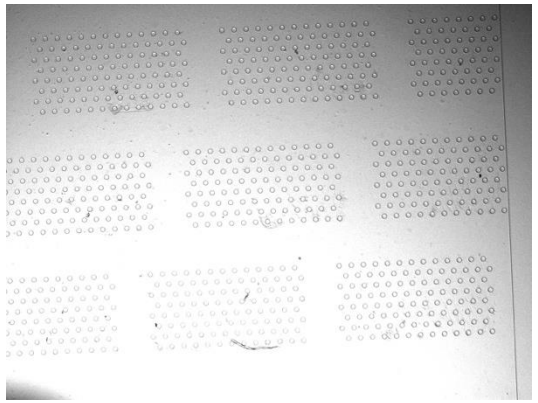
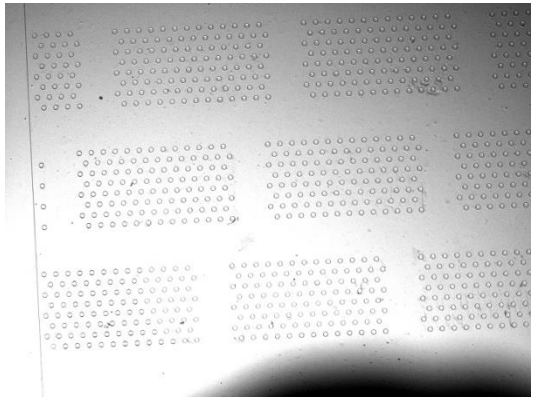
Merged

PMA

PMA

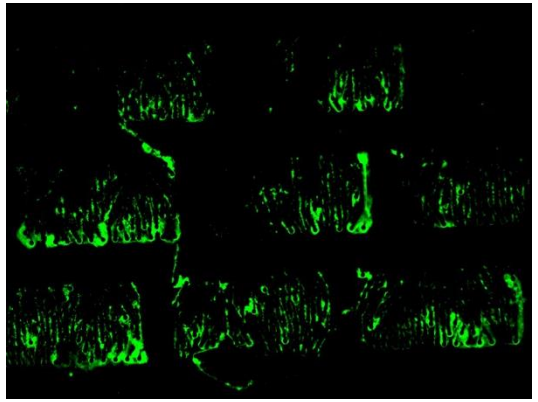
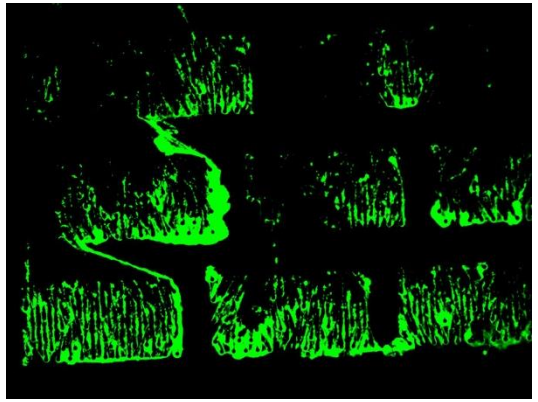
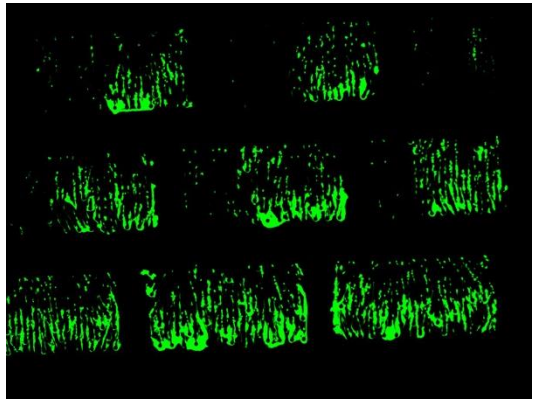
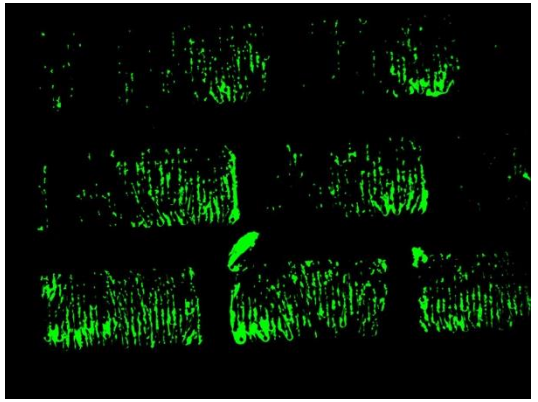
Ionomycin

Ionomycin

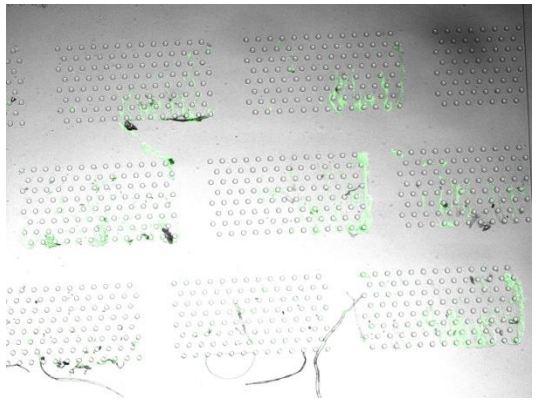
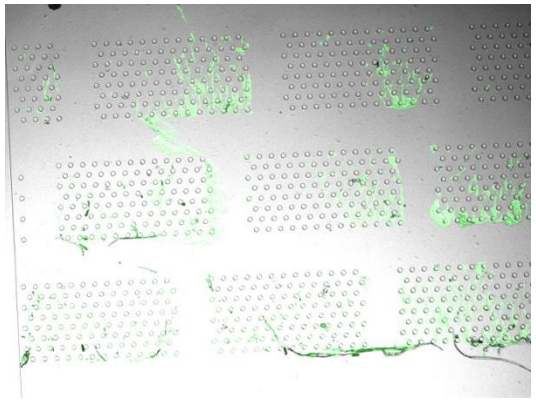
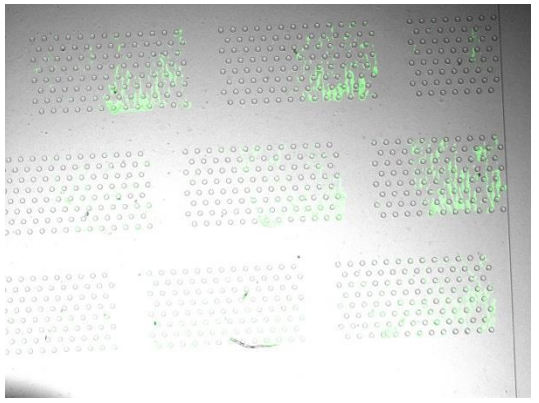
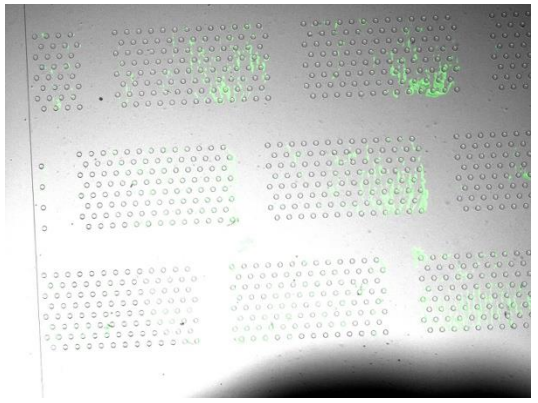





10x Device

BF

GFP

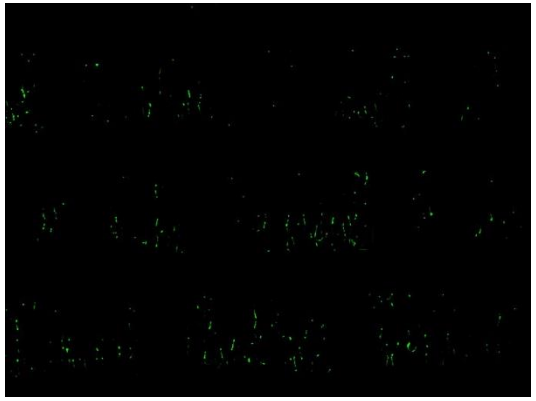
Merged

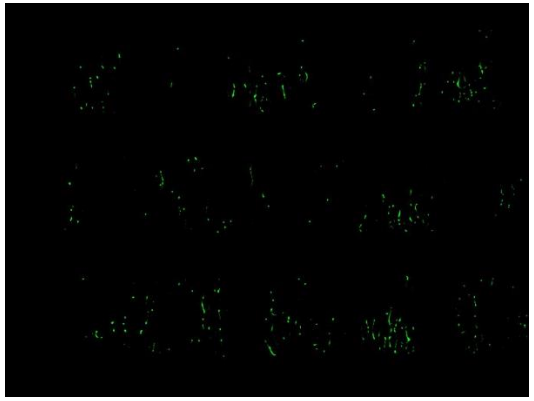
PMA

PMA

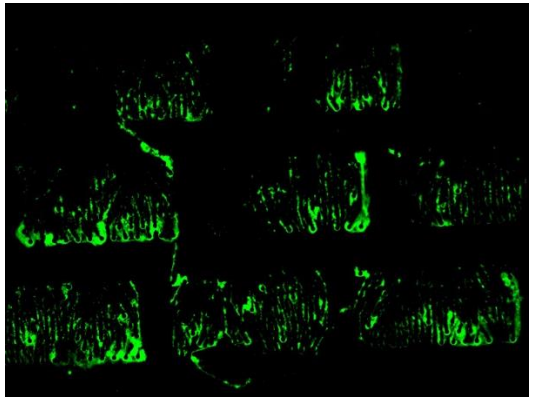
Ionomycin

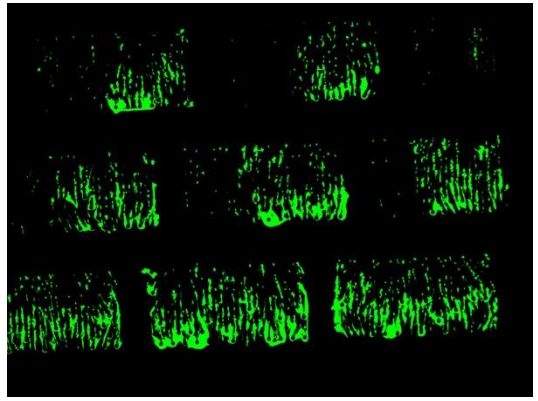
Ionomycin

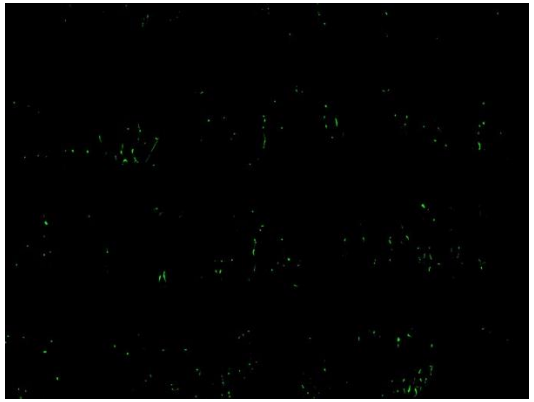




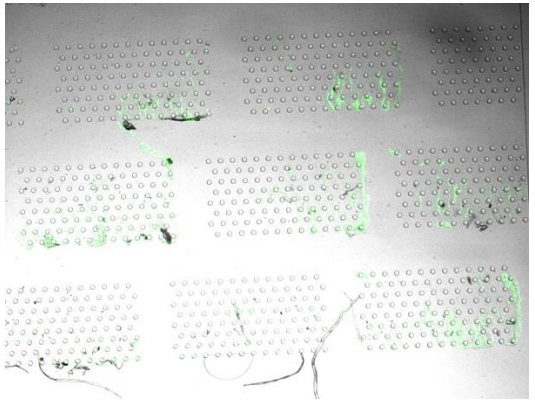
GFP

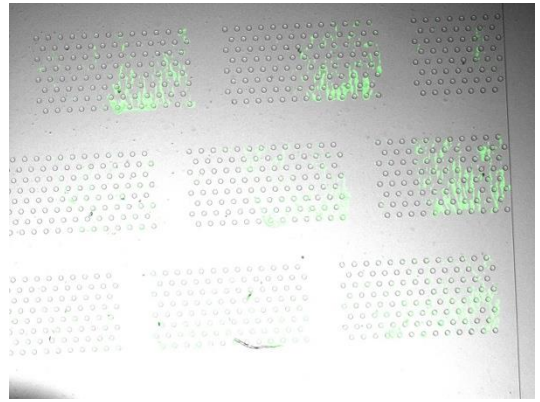






GFP





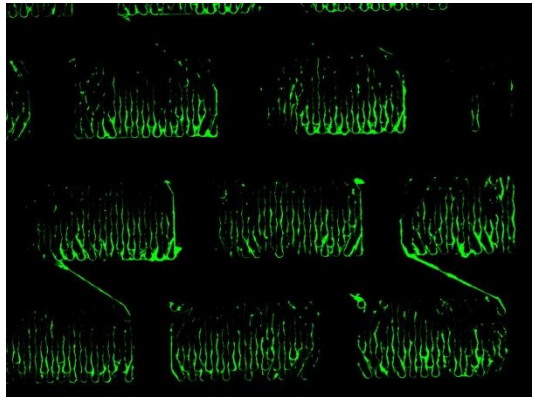
Blank

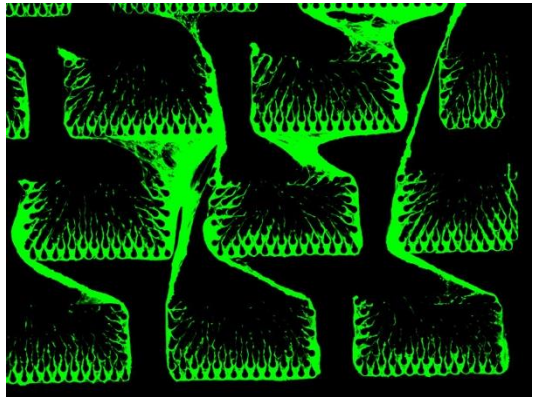
Blank

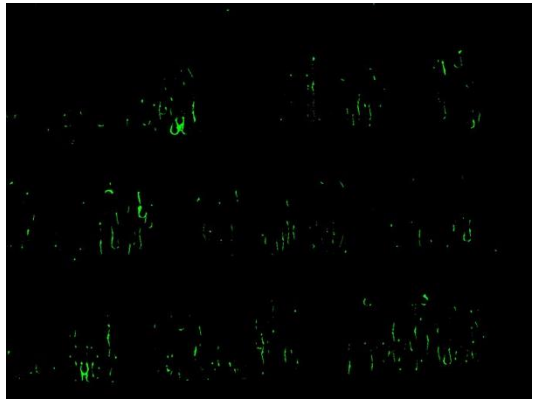
PMA

Ionomycin

10x Device

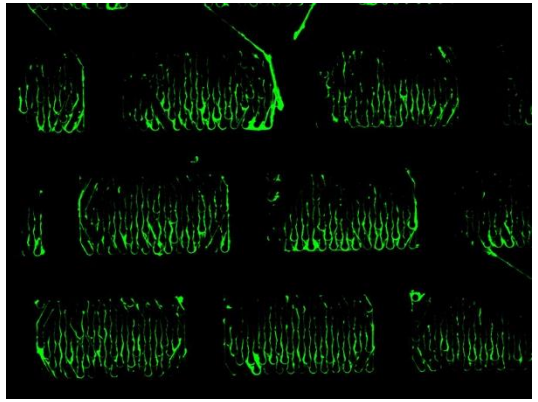


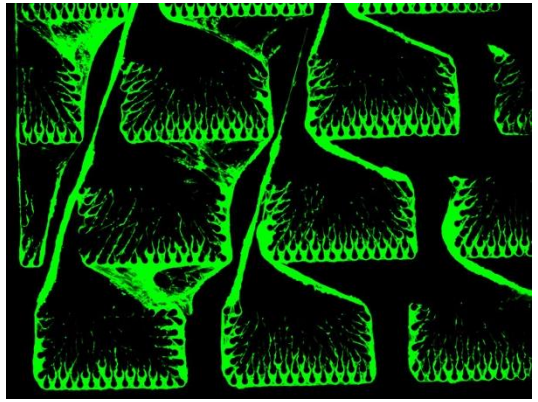


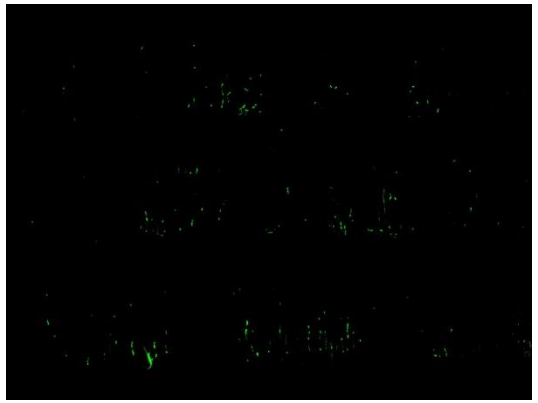


10x Device

GFP 1







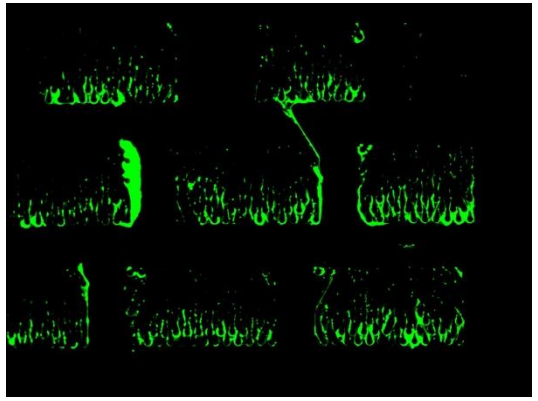
GFP 2

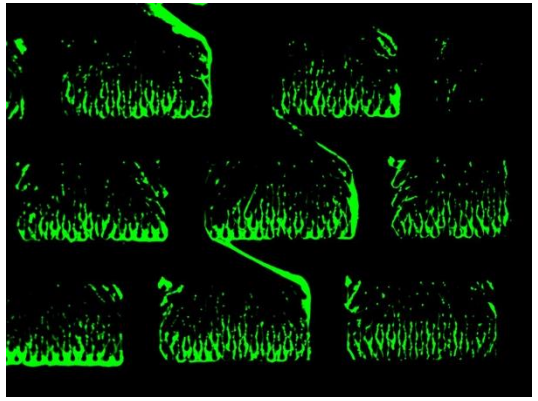
PMA

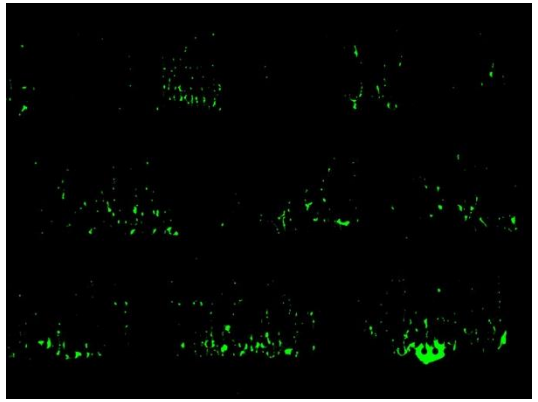
Ionomycin

Blank

2025-06-10

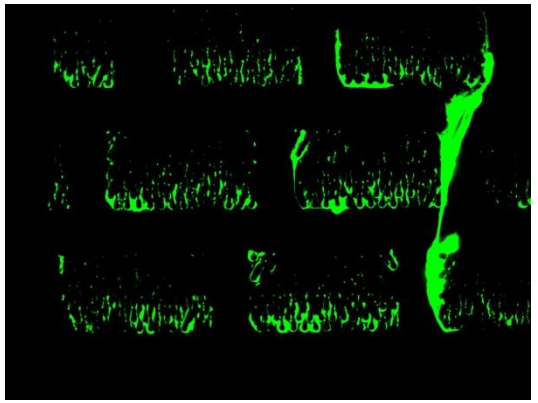


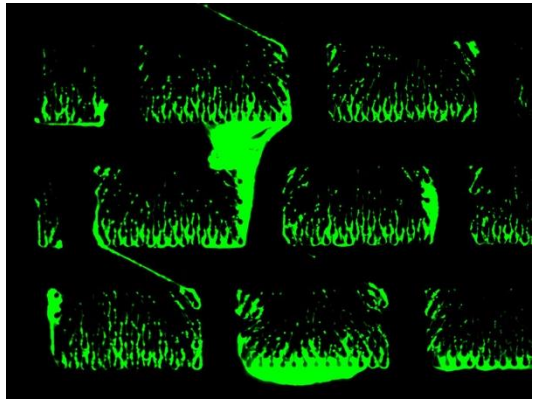


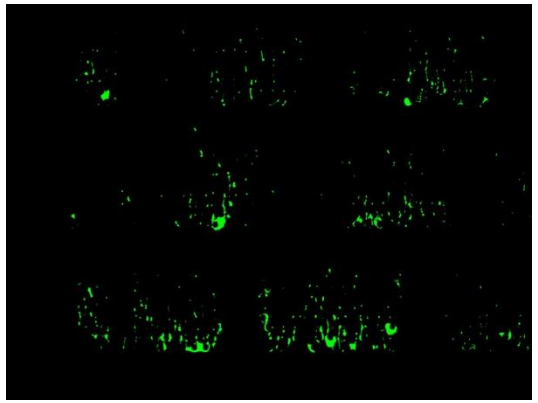


10x Device

GFP 1







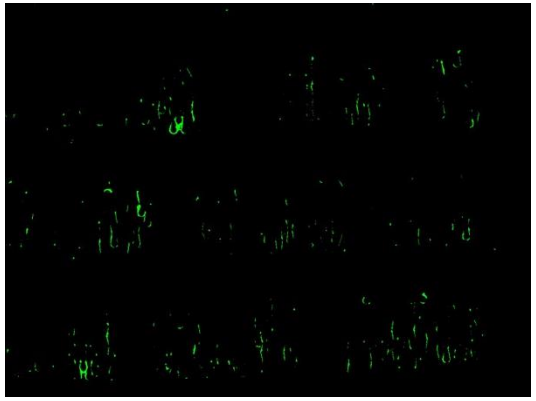
GFP 2

PMA

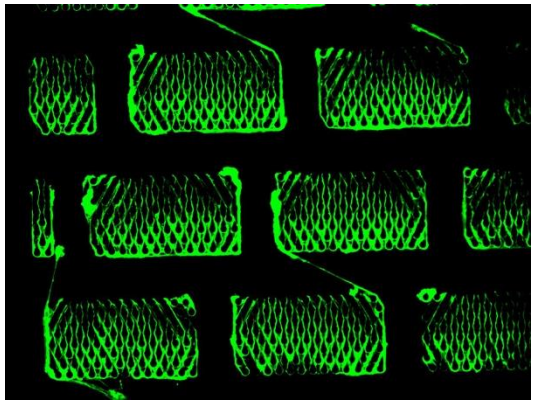
Ionomycin

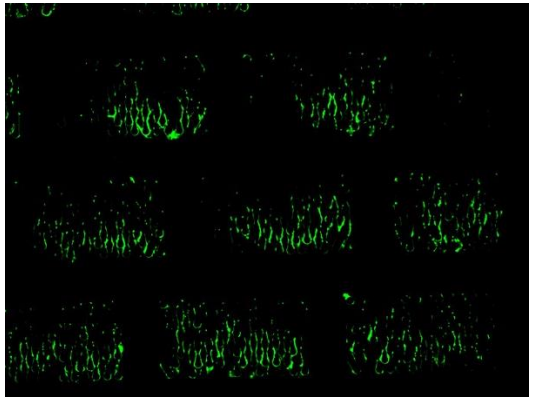
Blank

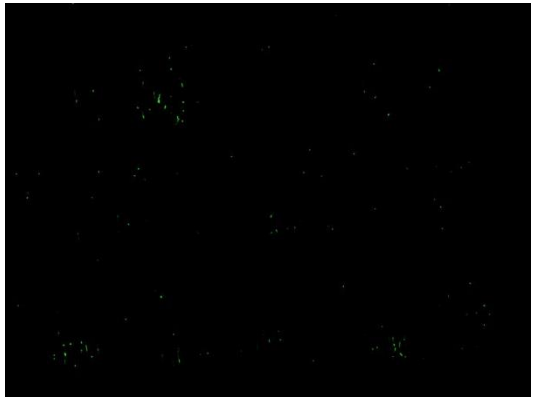
2025-06-12

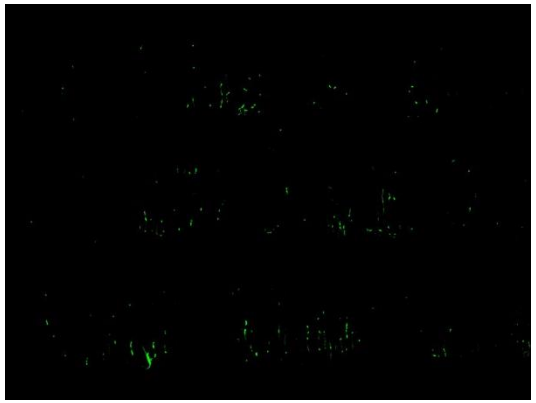


GFP

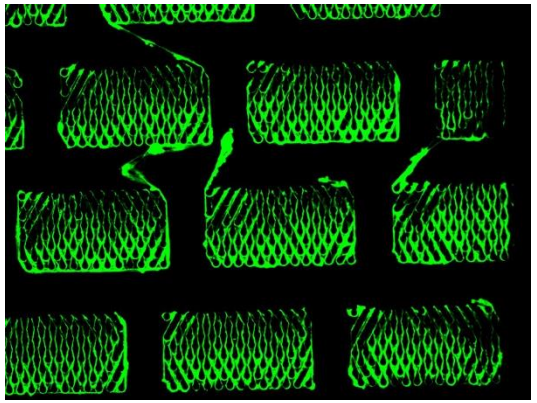


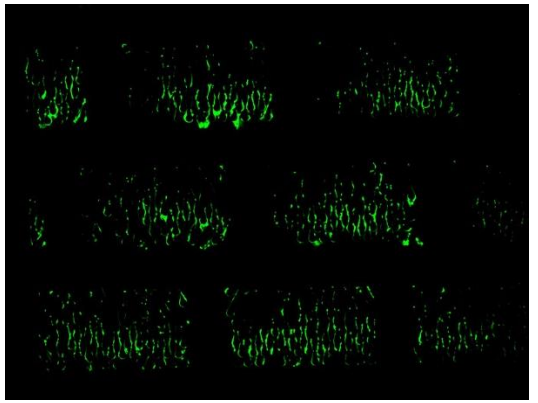


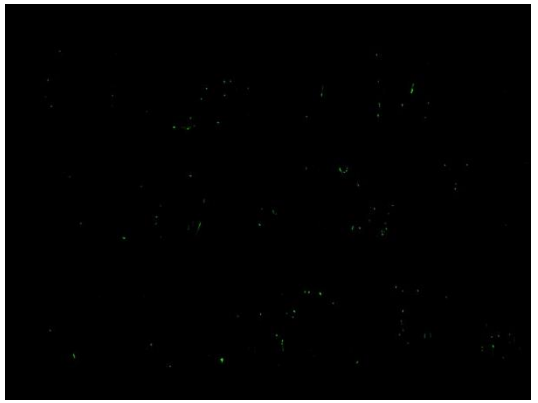




GFP







Blank

Raptinal

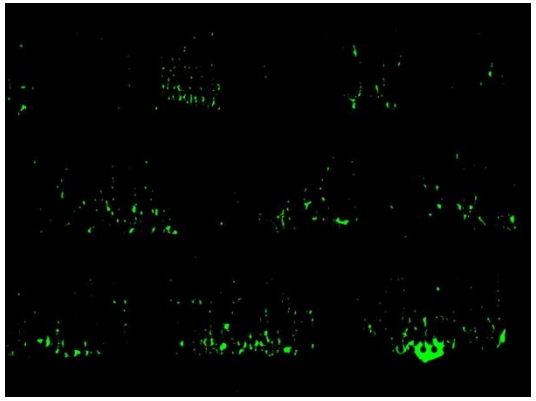
Nigericin

Val-boropro

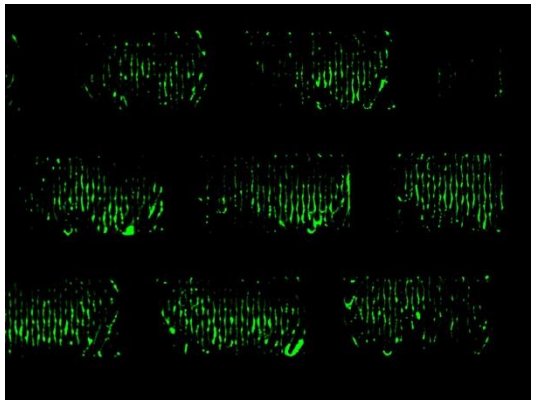
Conc.: 5 micromoles

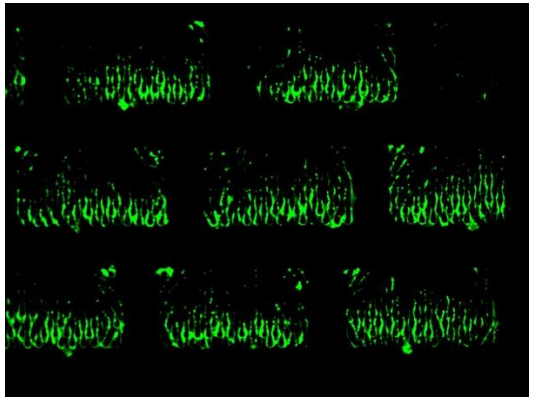
2025-06-10

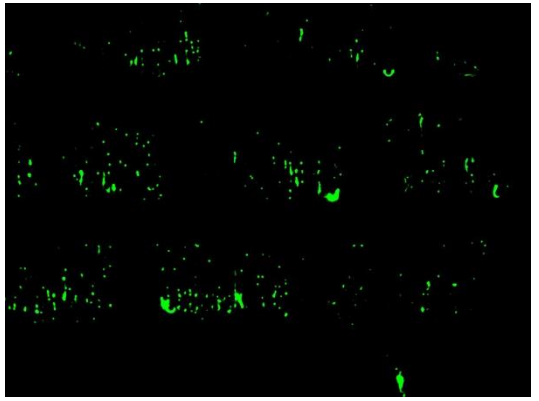
10x Device

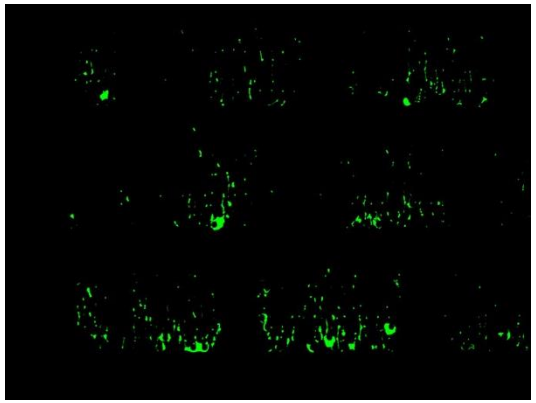


GFP

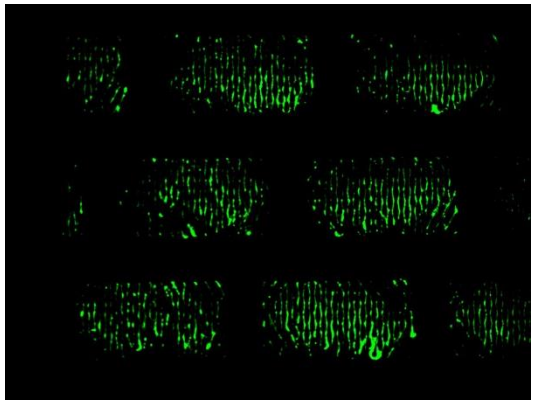


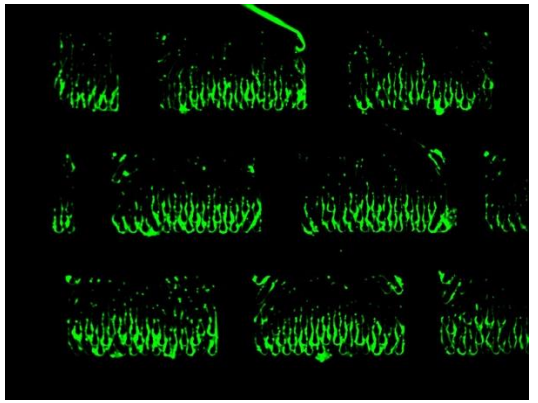


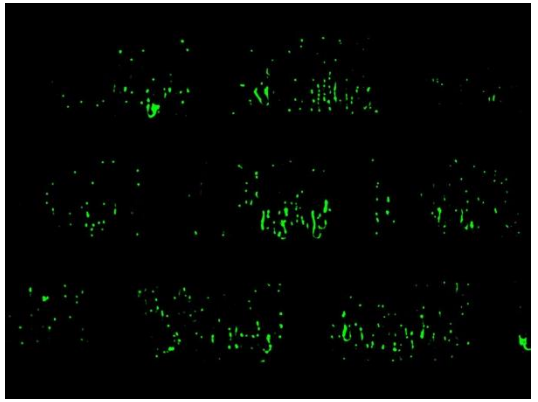




GFP







Blank

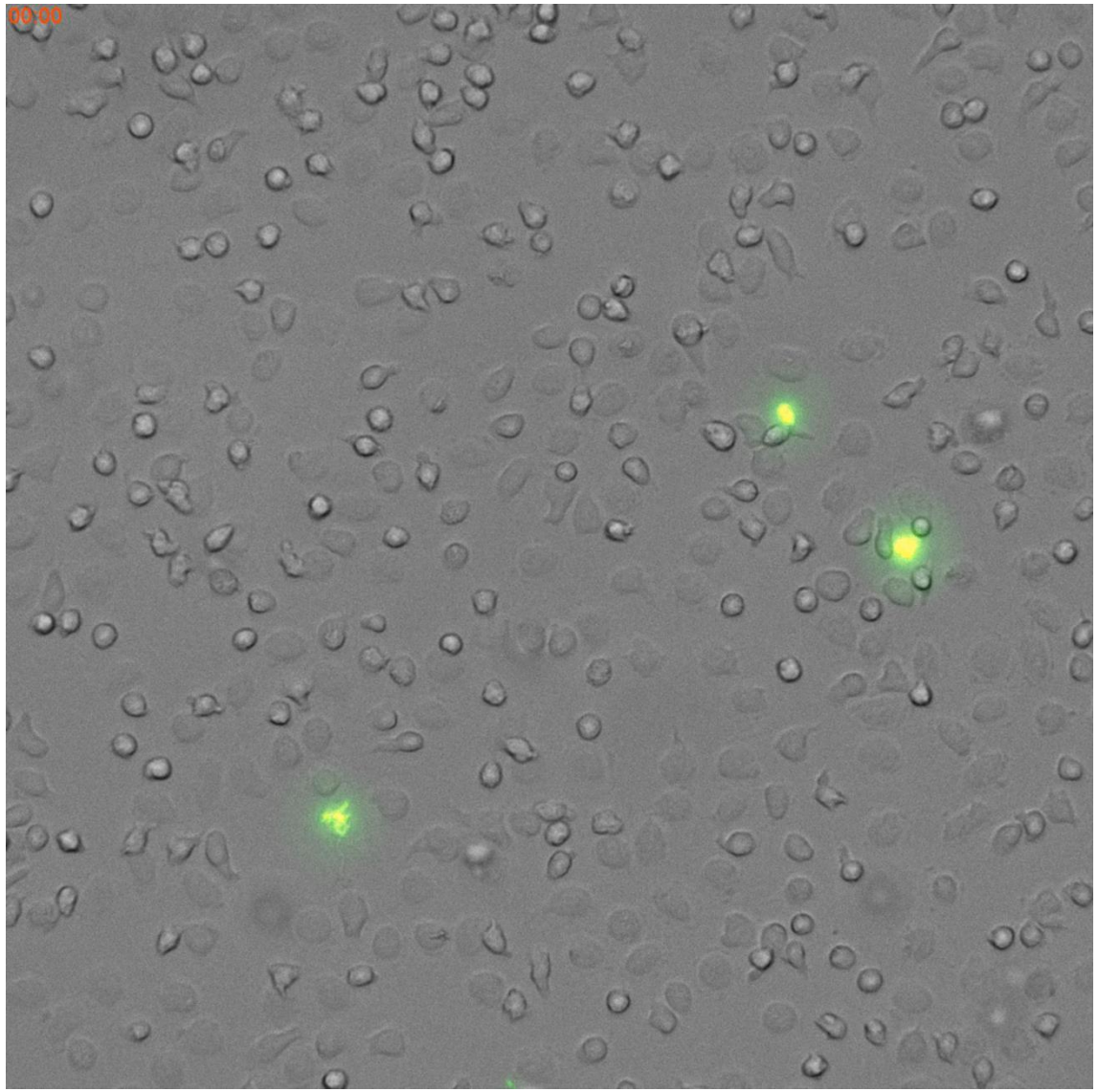
Raptinal

Nigericin 2x conc

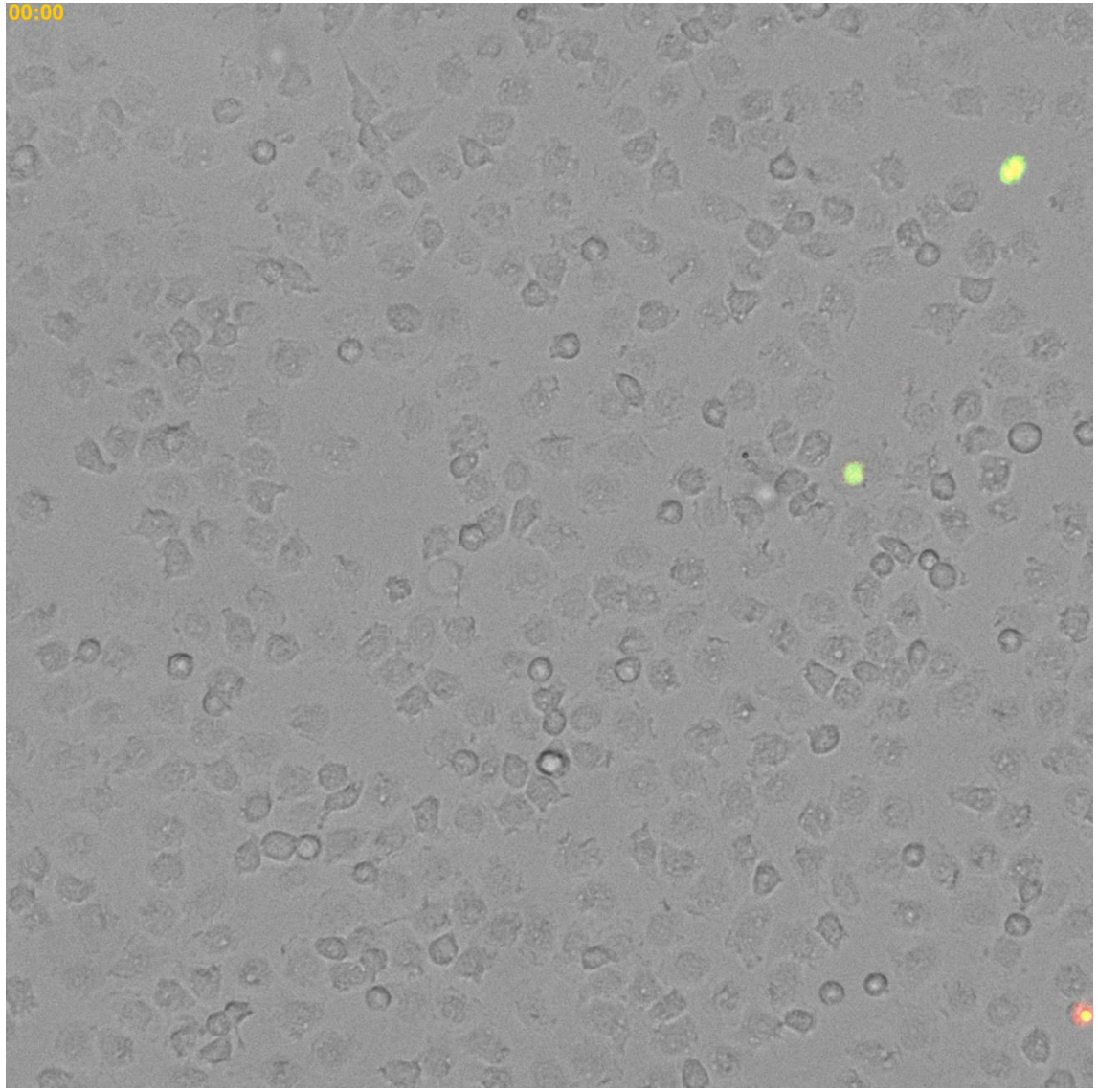
Val-boropro 10x conc

2025-06-12

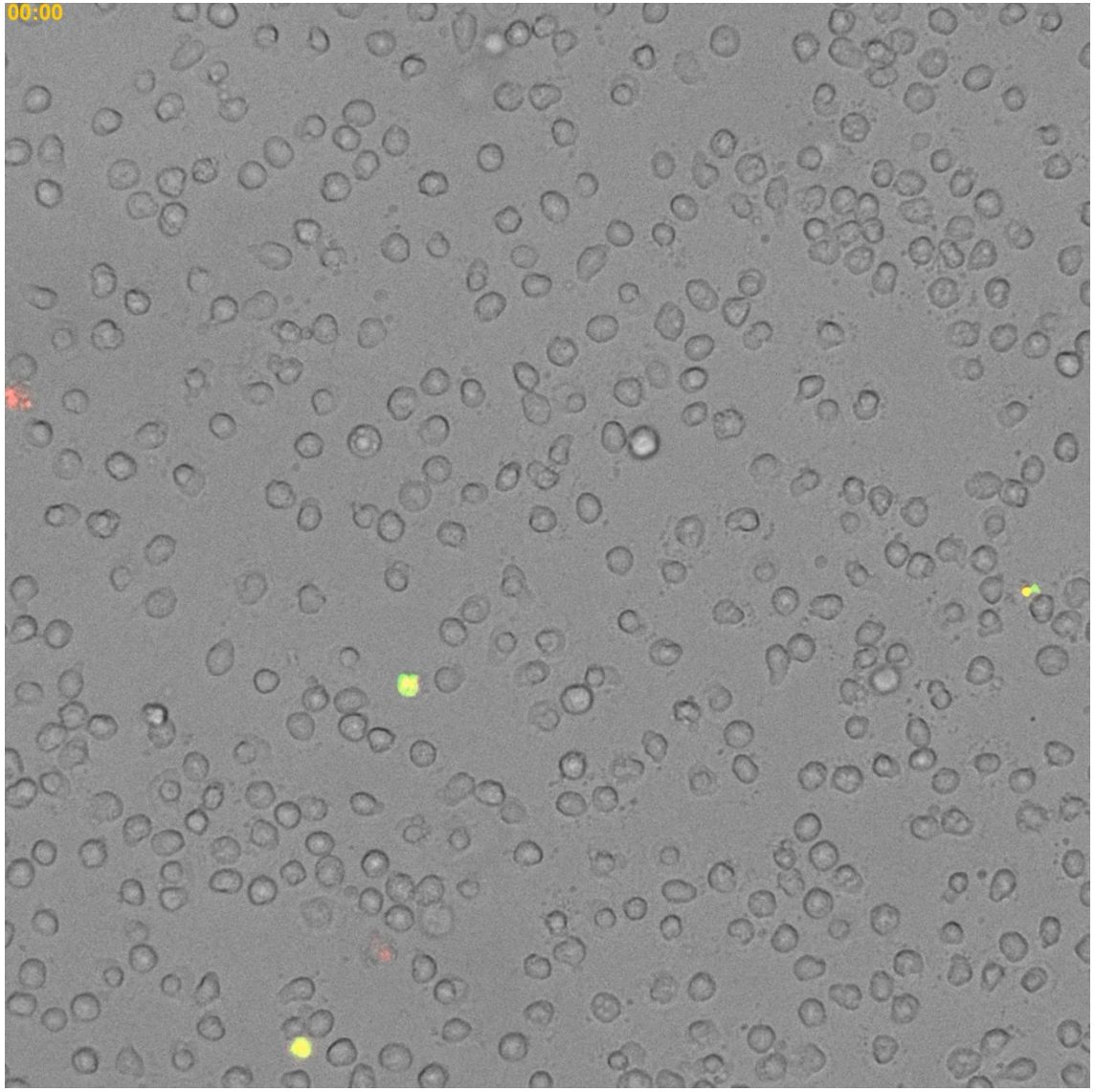
10x Device



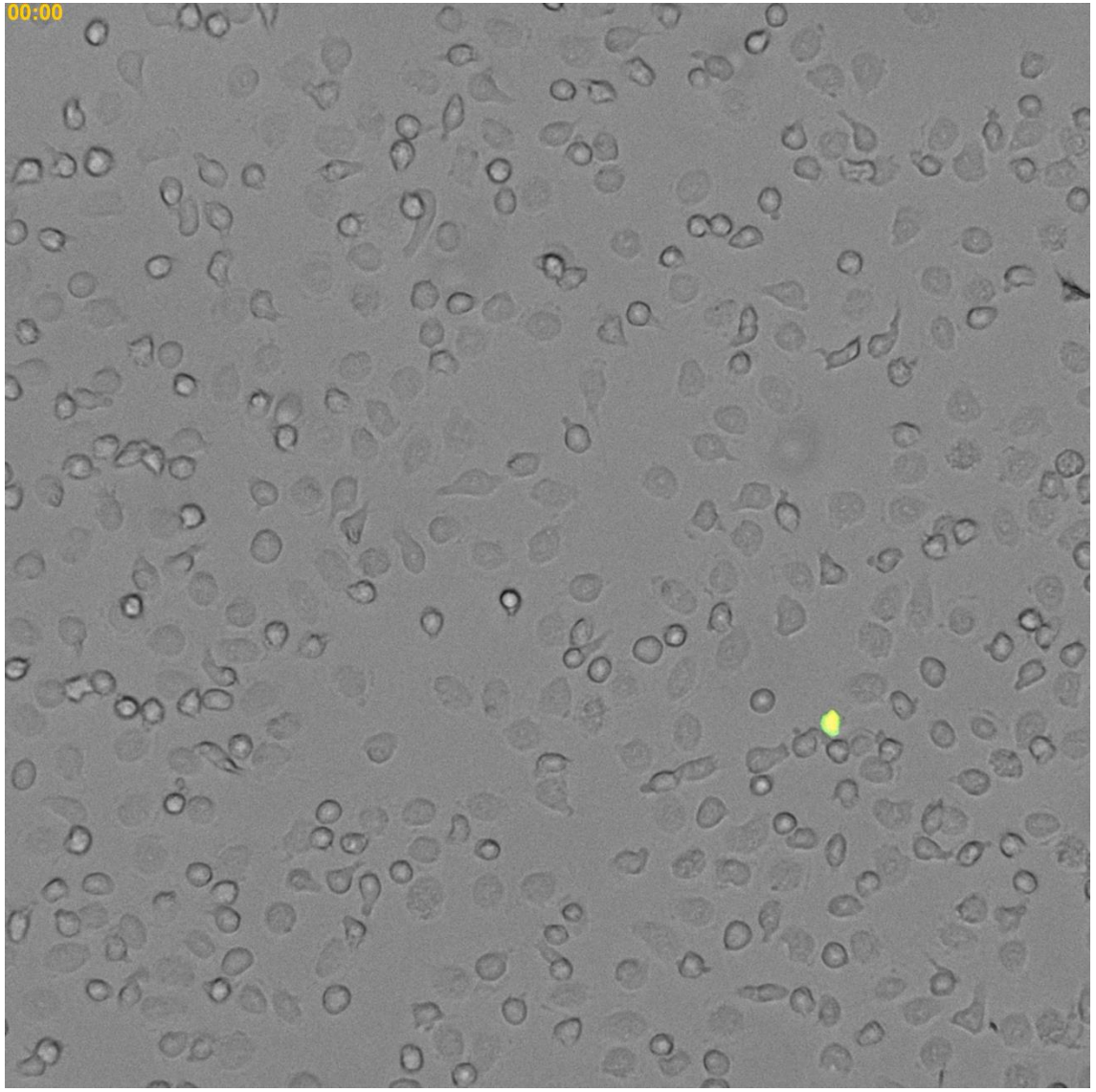
Blank



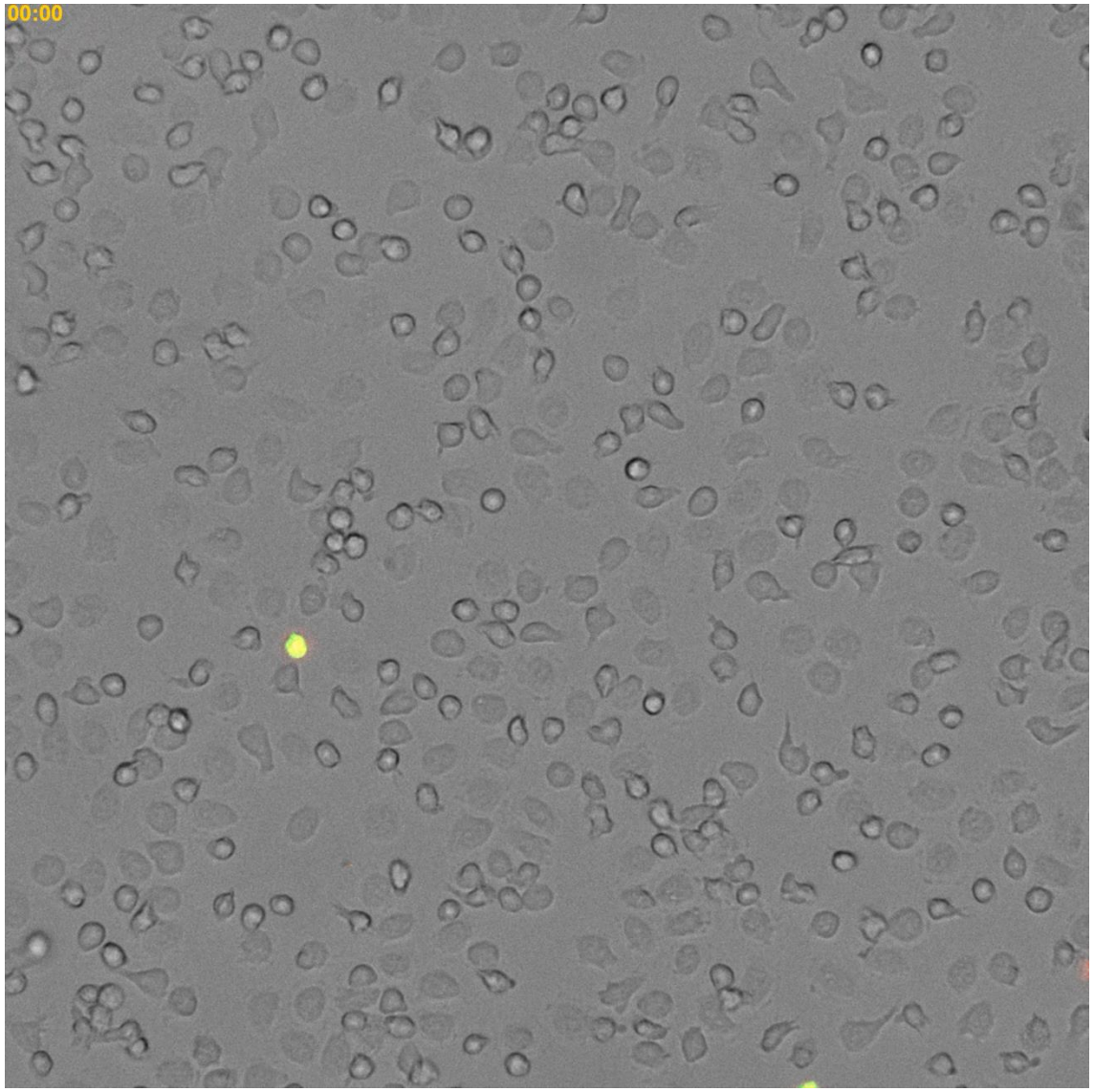
PMA



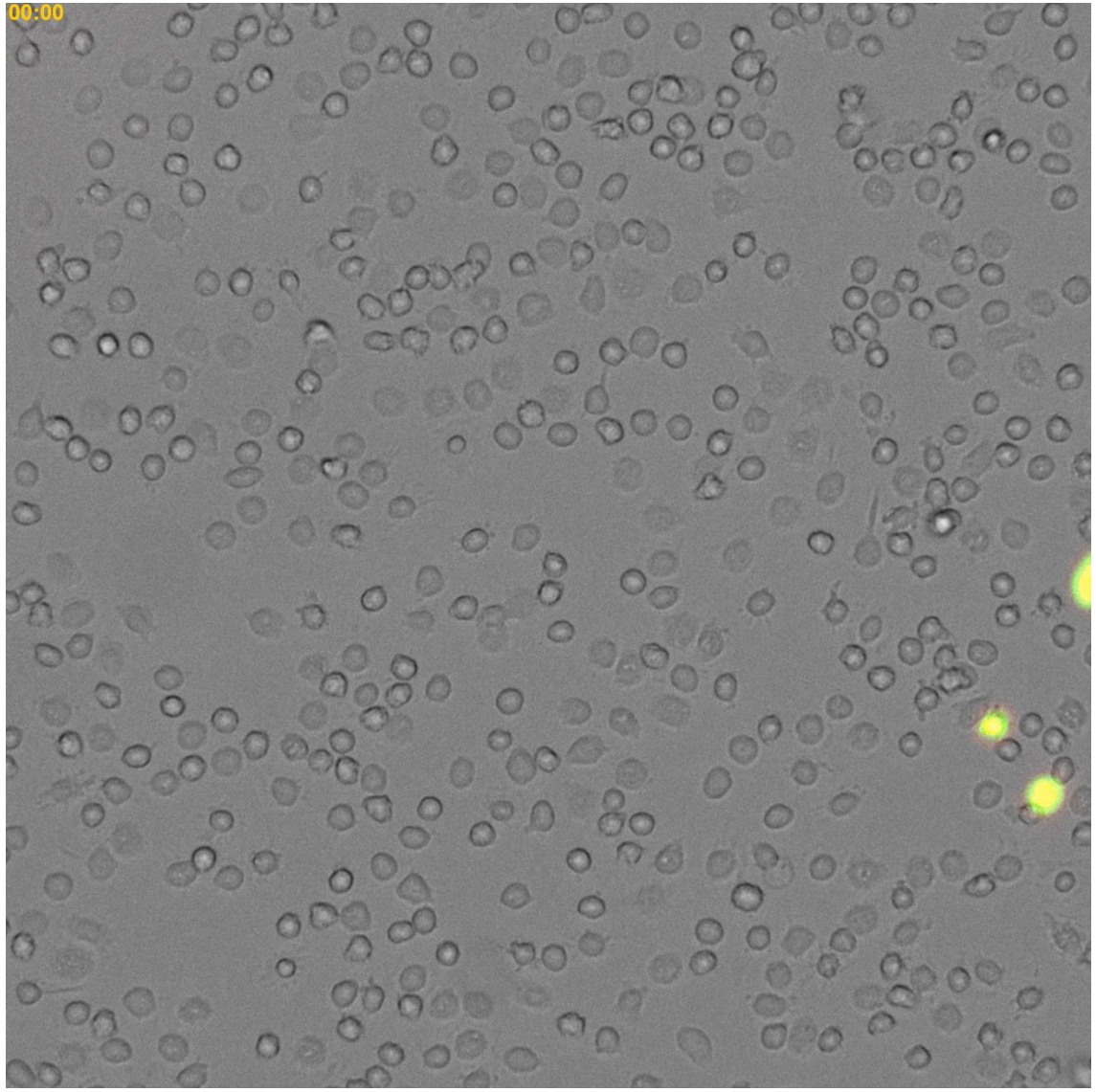
Ionomycin



Raptinal



Val-boropro



Nigericin

Death rate at 4 hours:

Blank: 30%

PMA: 93%

Ionomycin: 95%

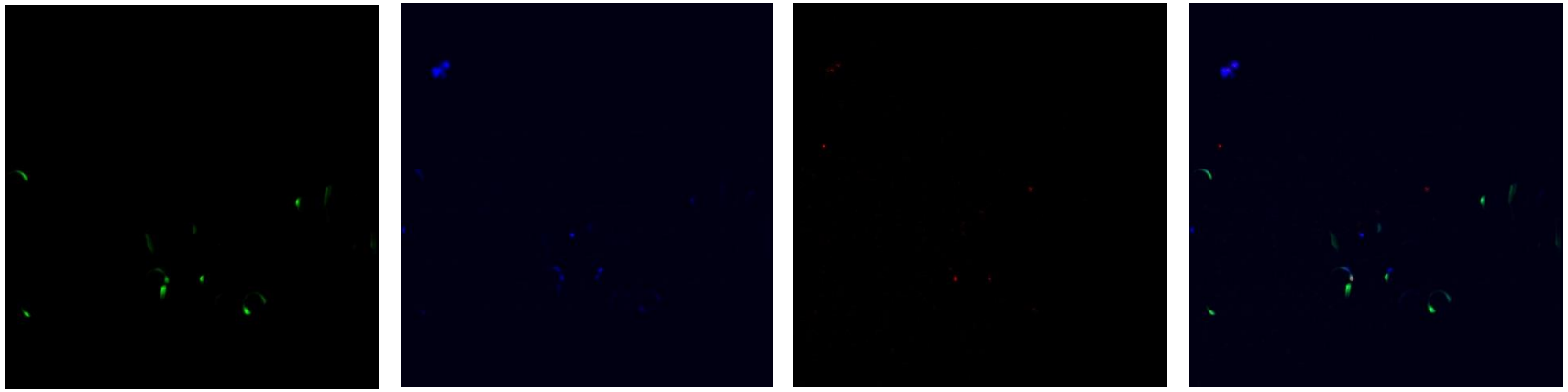
Raptinal: 70%

Val-boropro: 25%

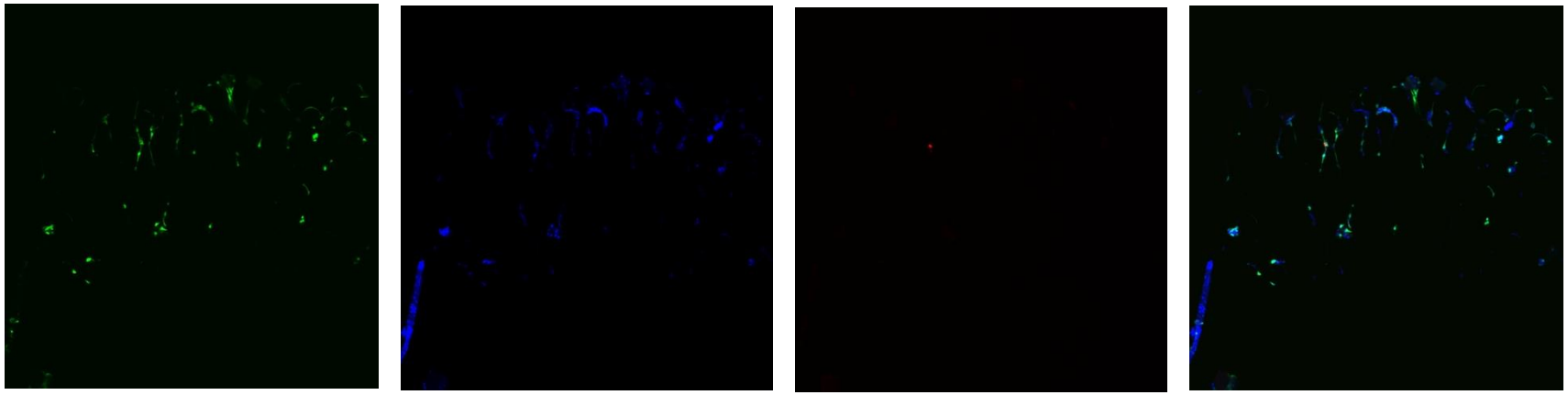
Nigericin: 85%

Device

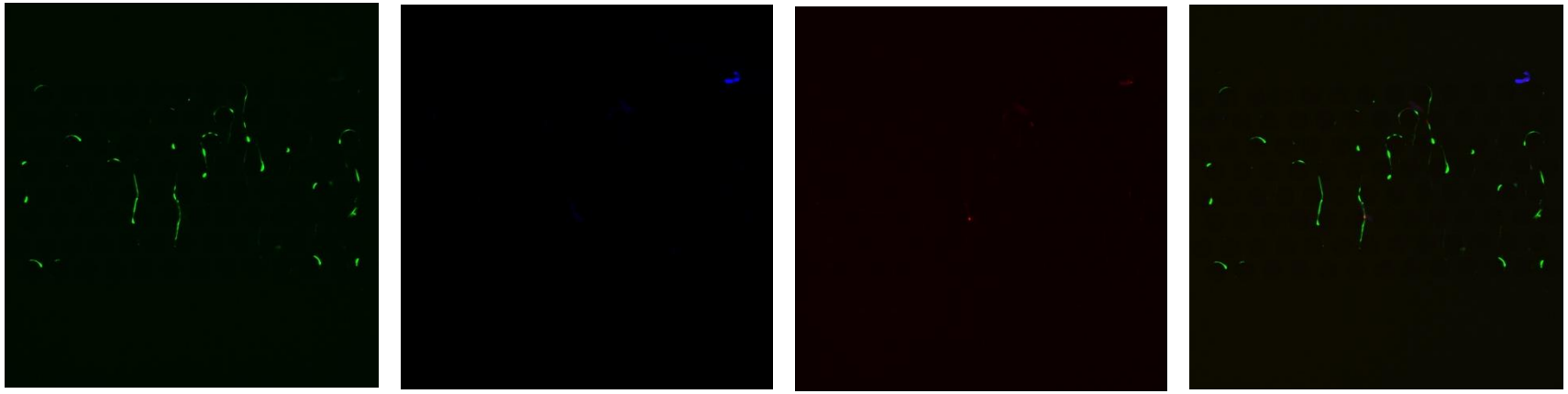
2025-06-13



Blank 10x



Nigericin 10x

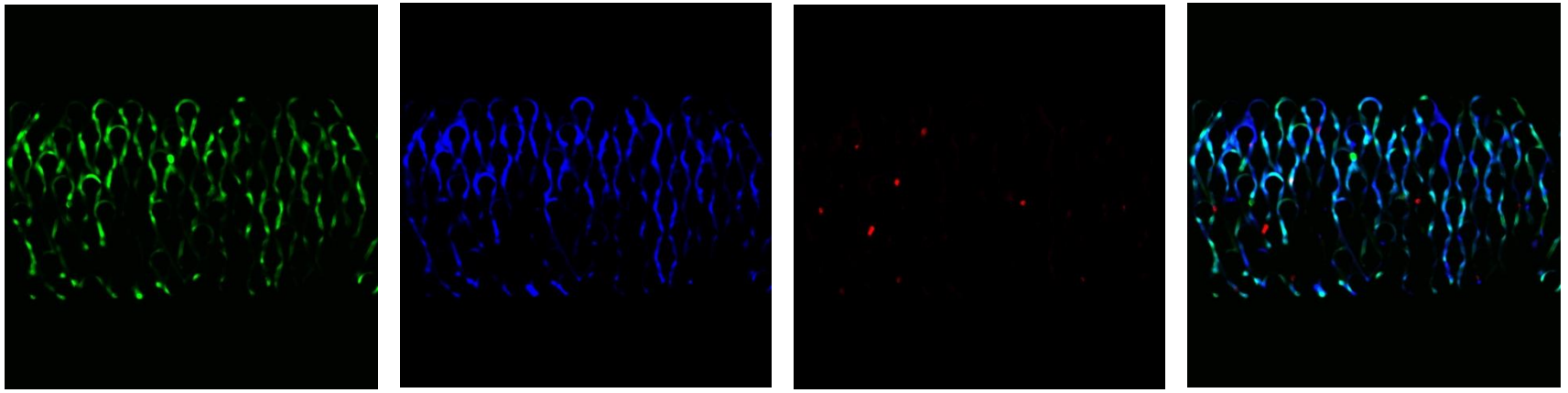


Val-boropro 10x

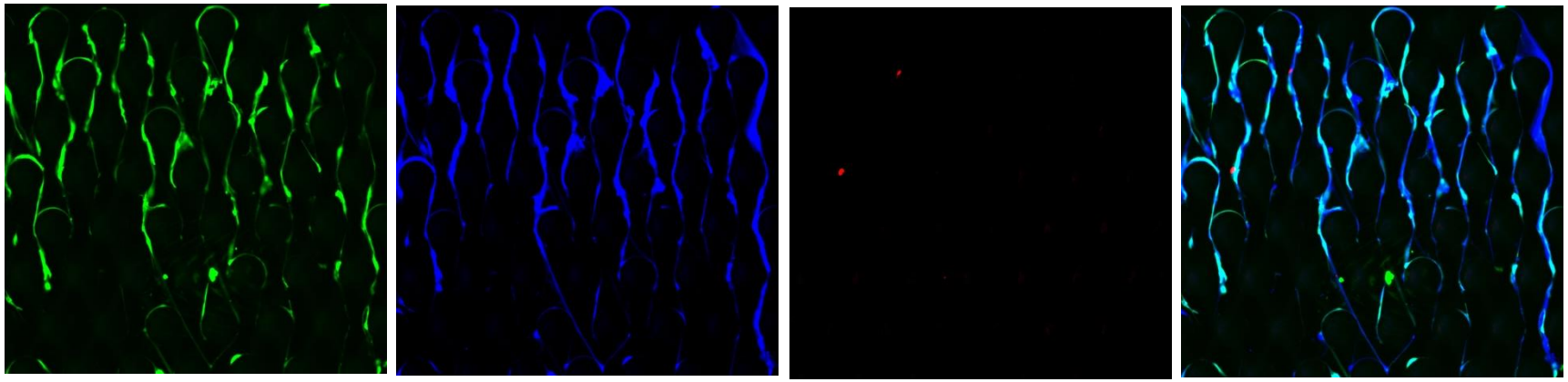
G B R Composite

Device

2025-06-13



PMA 10x

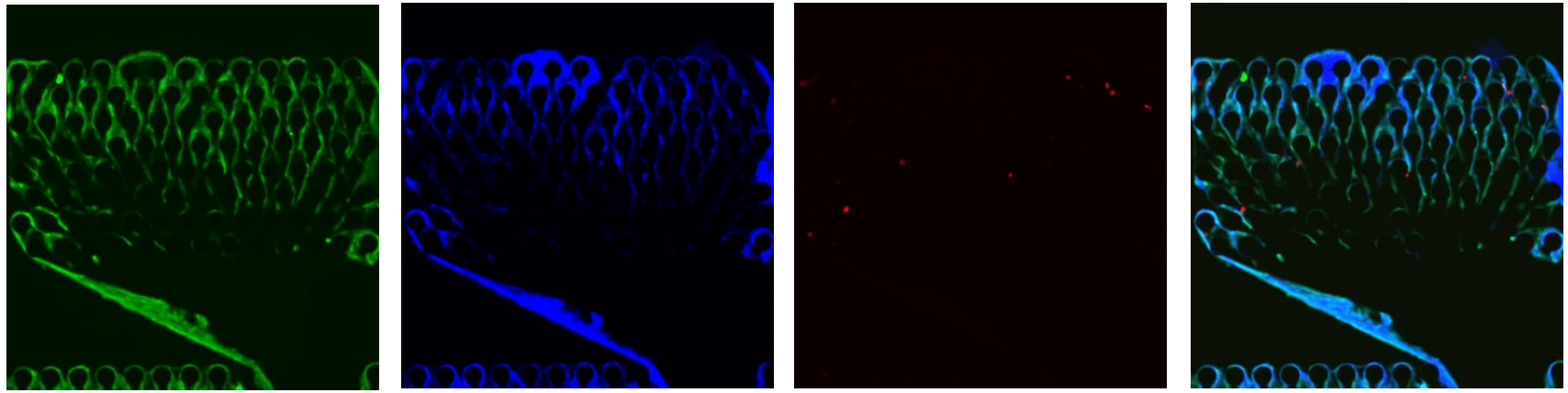


PMA 20x

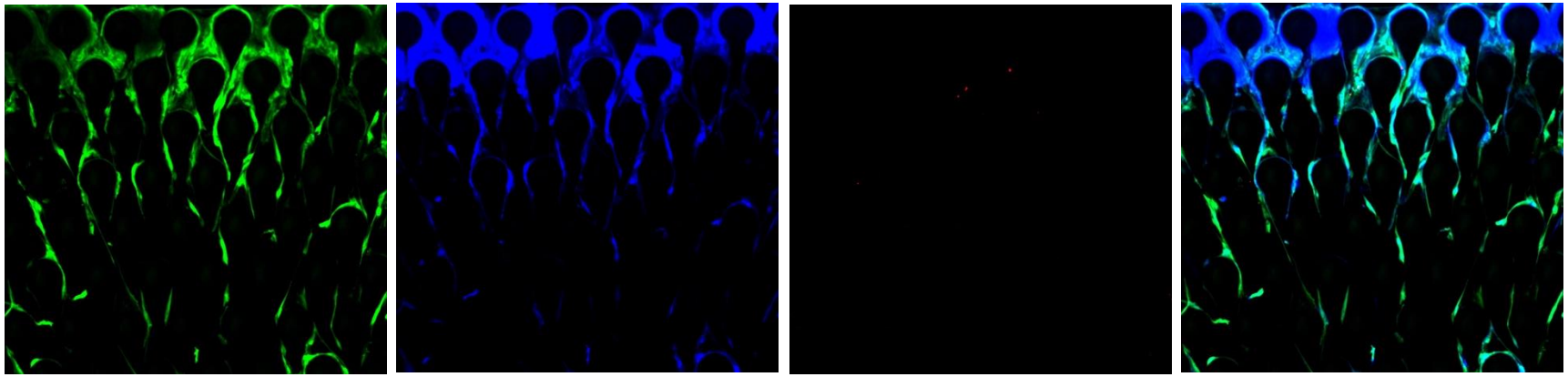
G B R Composite

Device

2025-06-13



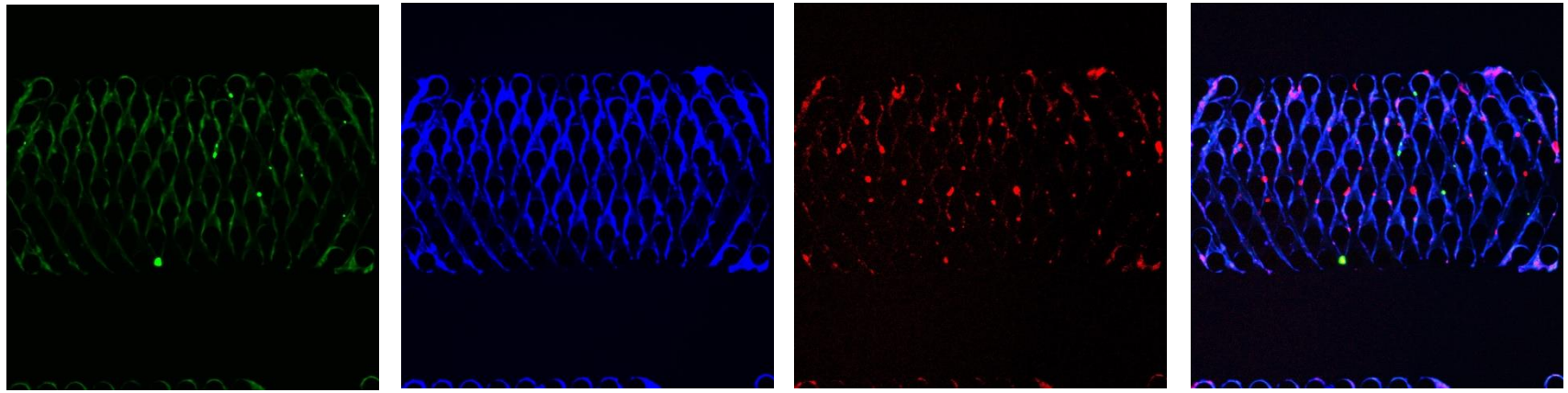
Ionomycin 10x



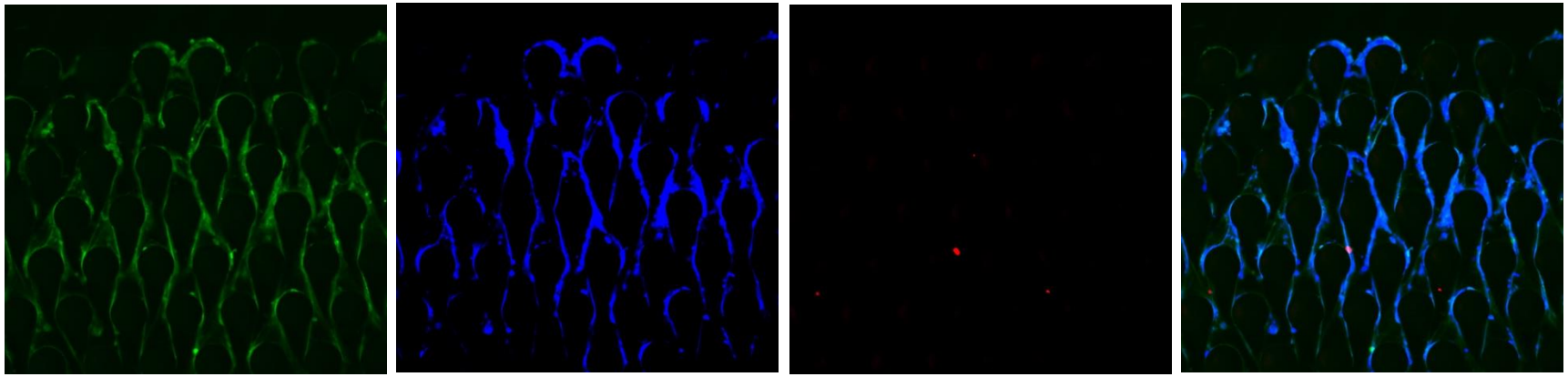
Ionomycin 20x

Device

2025-06-13



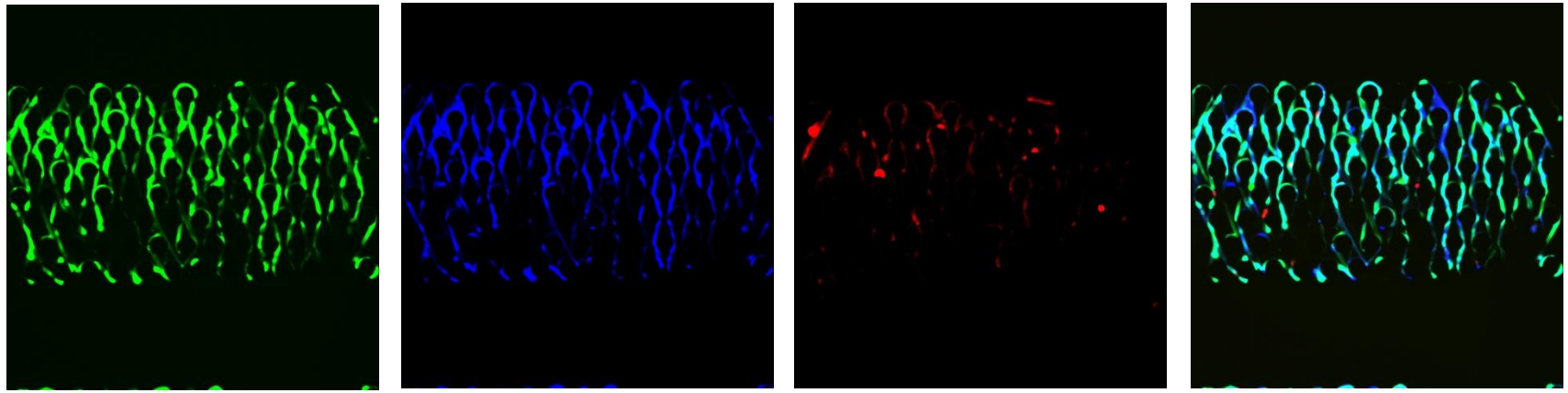
Raptinal 10x



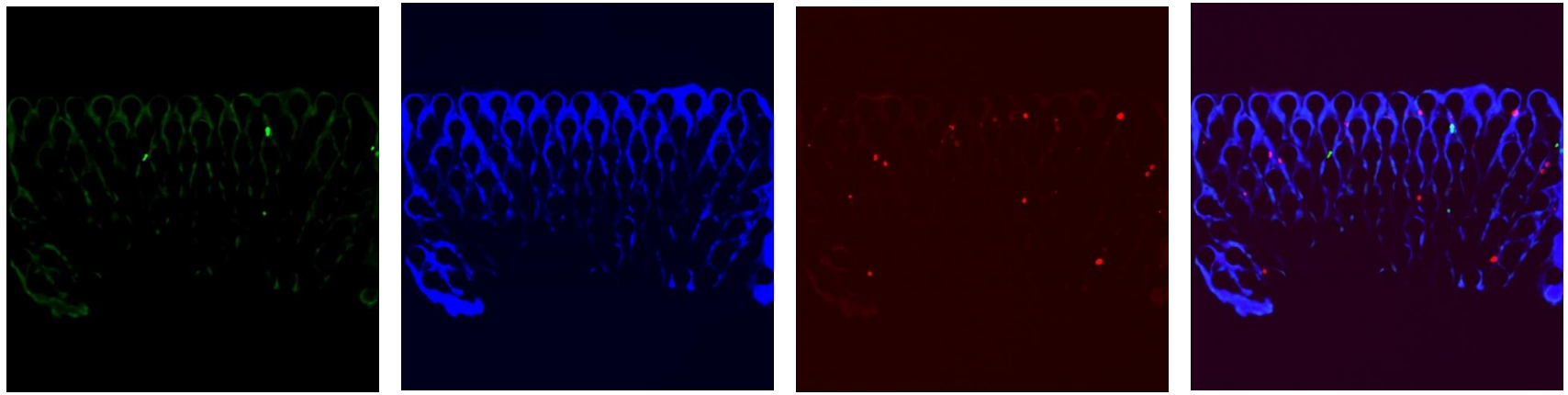
Raptinal 20x

Device

2025-06-13



PMA 10x



Ionomycin 10x

NET formation pathway summarized

1. Neutrophil activation with ROS production (or PAD4 activation)

2. Histone modification and chromatin unwinding (PAD4 + MPO)

3. Nuclear rupture and NET release (cell death, not vesicle secretion)

4. NET deployment

Chromatin (in nature)

Chromatin: Complex of DNA and protein in eukaryotic cells, double helix, DNA wrapped around the histones

Chromatin decondensation: From compact chromatin fibers to a more relaxed form

Observed both in mitosis and meiosis

Occasions:

Mitosis (G1 entry, telophase)

Embryogenesis (zygote genome activation)

Cellular Reprogramming

NET formation

Driving Processes:
Histone Acetylation, Histone Phosphorylation, Histone Citrullination, Non-Histone Factors, DNA Demethylation

Chromatin decondensation in neutrophils

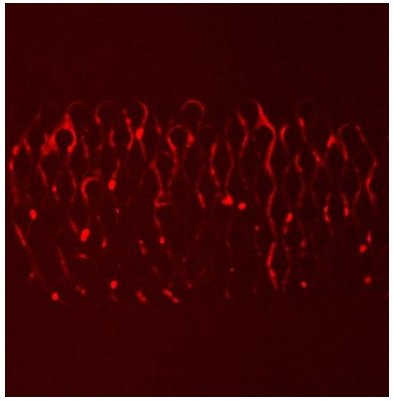
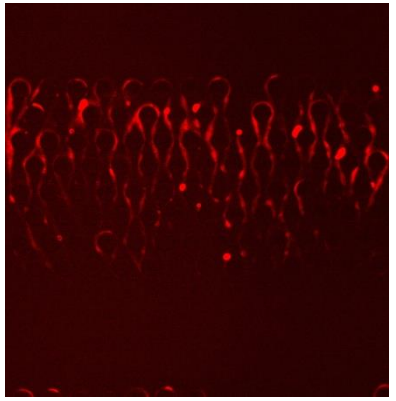
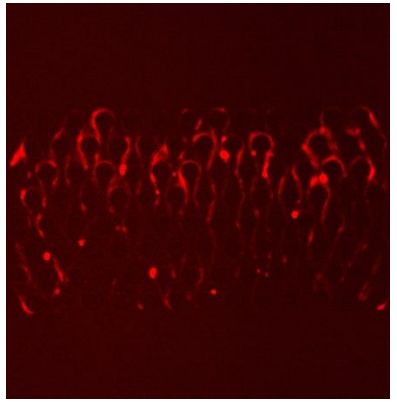
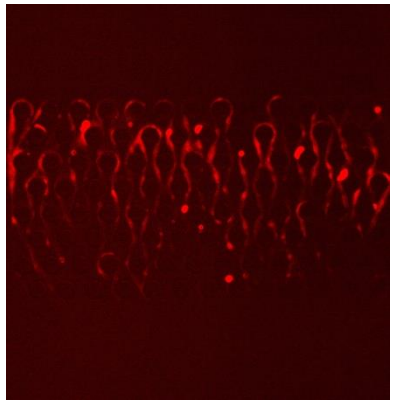
PAD4: nuclear enzyme, activated by calcium
Arginine residues to citrulline (histone citrullination)
Weakening histone and DNA binding, loosen up the chromatin

Neutrophil Elastase: serine protease, stored in granules
When neutrophil is activated, NE translocated to the nucleus and cleaves histones
Works complementally with PAD4
Mice deficient of NE is shown to have less chromatin decondensed (knock out potential?)

MPO: Also, a granule enzyme, associates with NE
Generates oxidants from hydrogen peroxide
NETs are decorated with MPOs

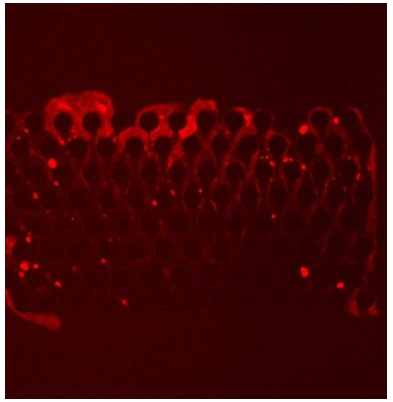
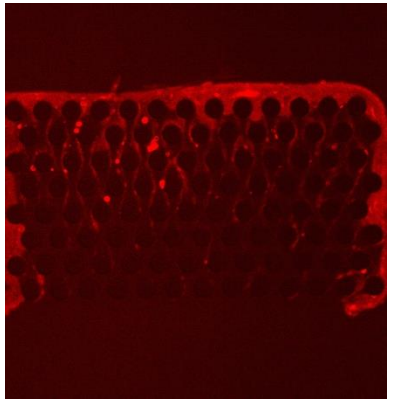
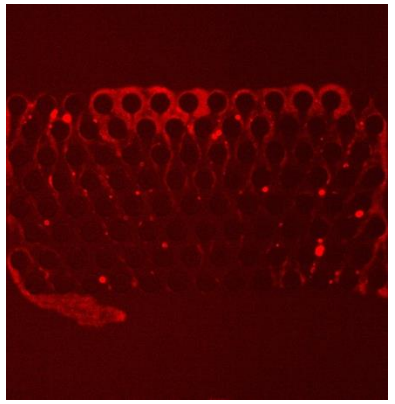
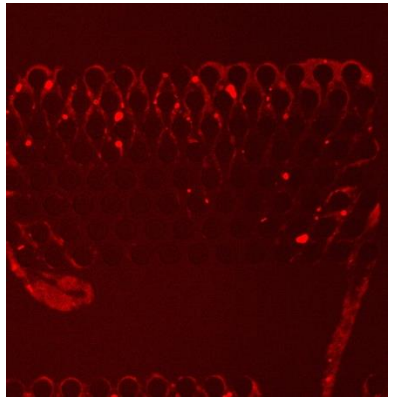
NADPH Oxidase and ROS: ROS -> PAD4

Raptinal: Lacks MPO but has PAD4, so is citrullinated (in tumor cells)
In tumor cells, NET-like structures are called NEPs

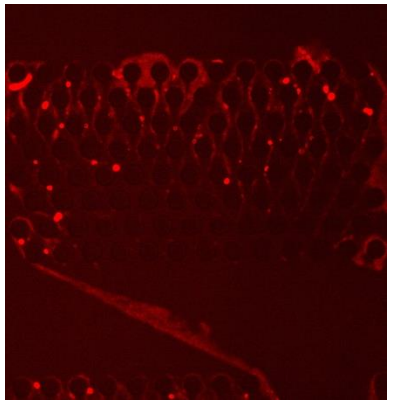
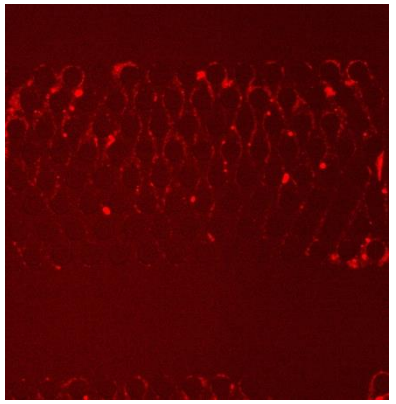
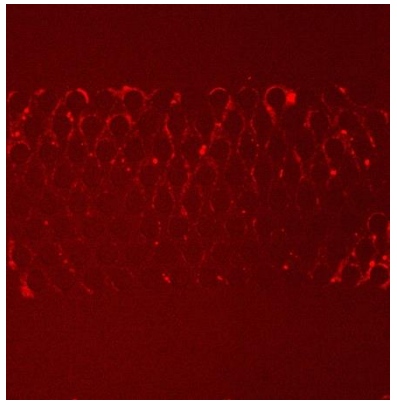
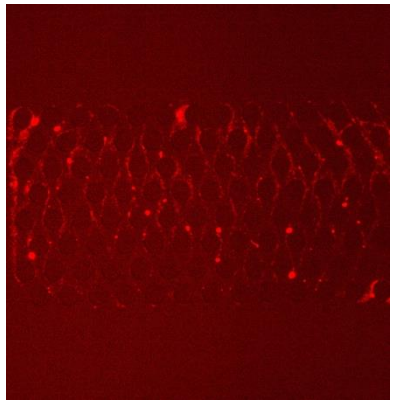





10x Device

PMA

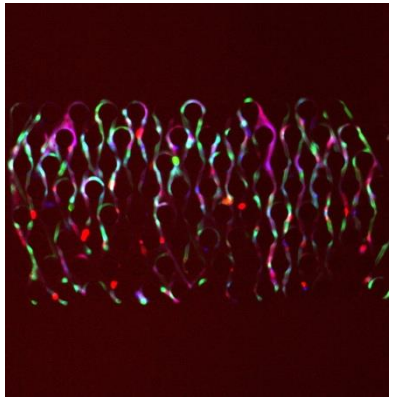
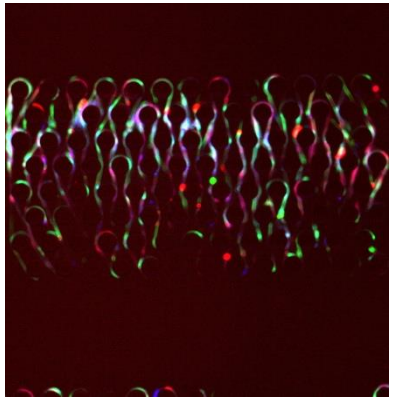
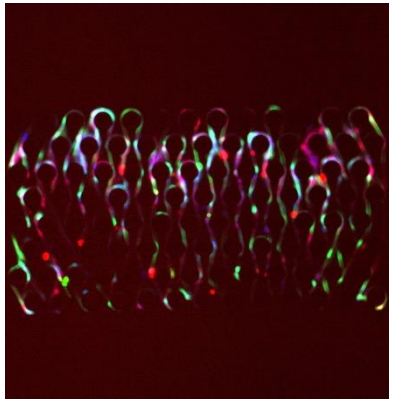
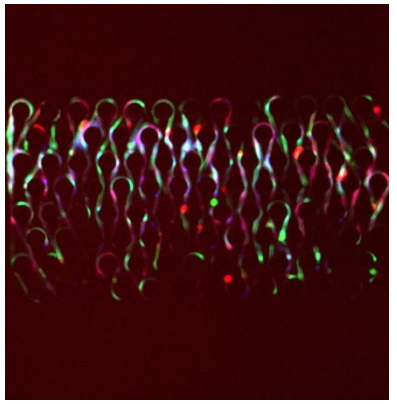





Ionomycin

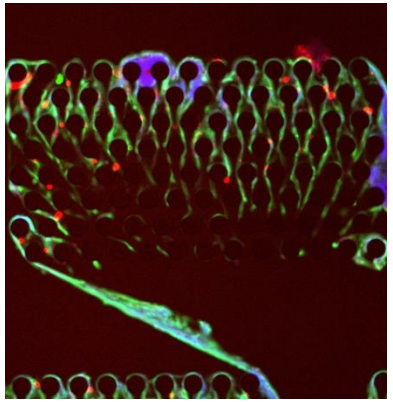
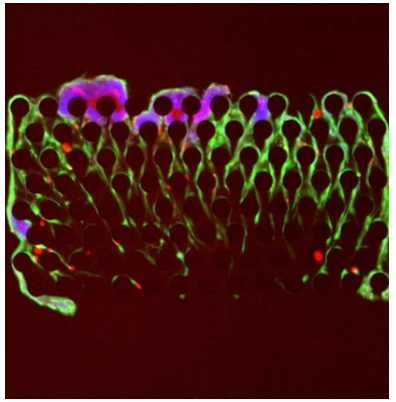
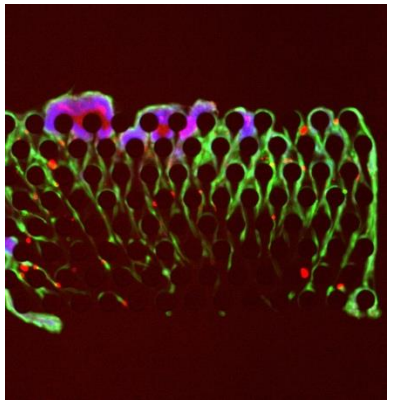
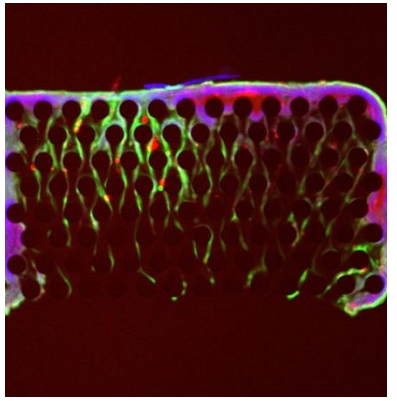
Raptinal

2025-06-13

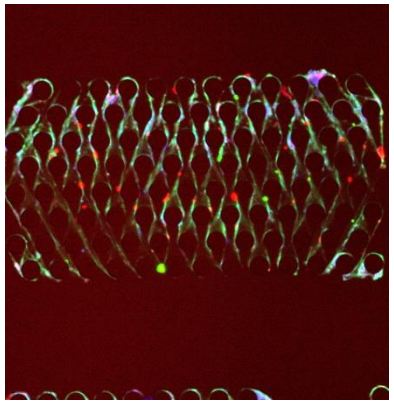
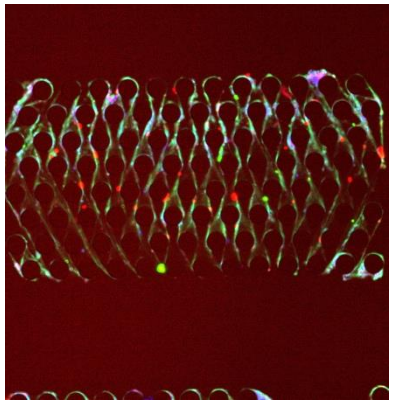
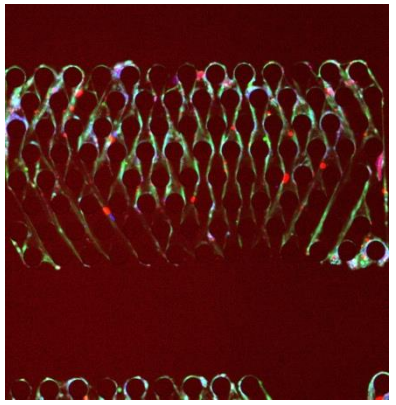
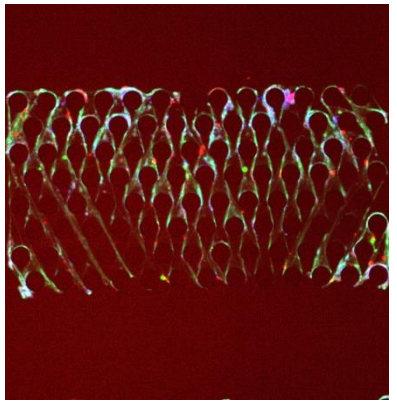





10x Device

PMA

Ionomycin

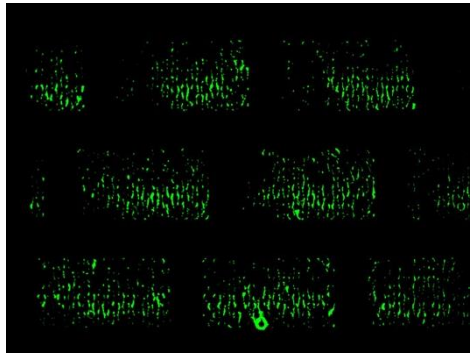





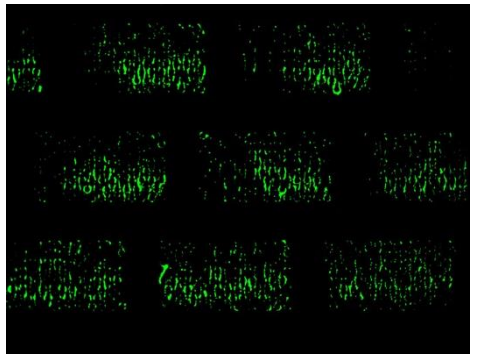
Raptinal

2025-06-13

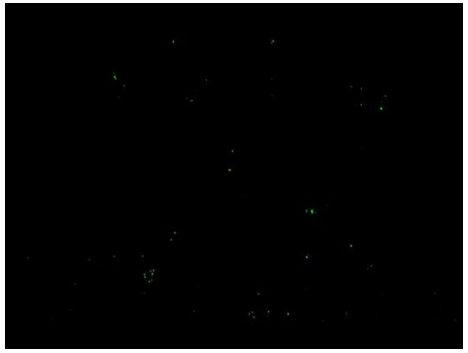
10x Device

Blank

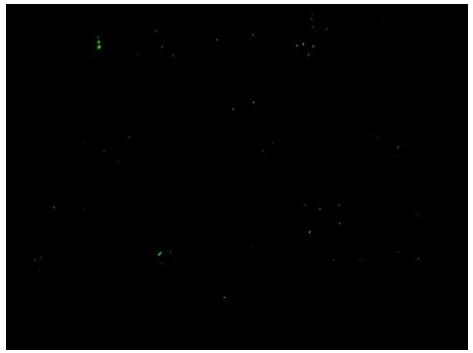


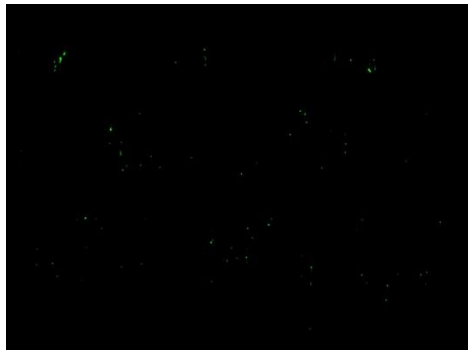


PMA 5 micromoles

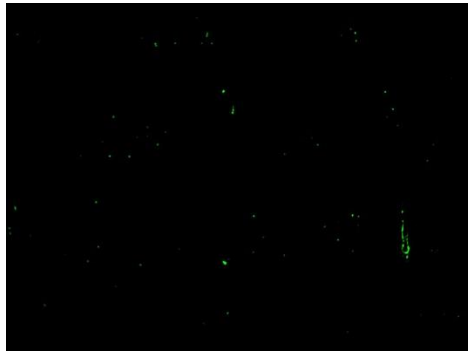


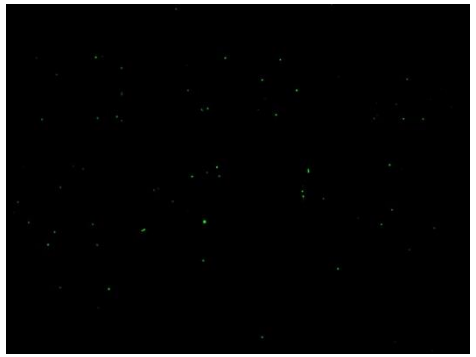
Blank





PMA 500 nanomoles



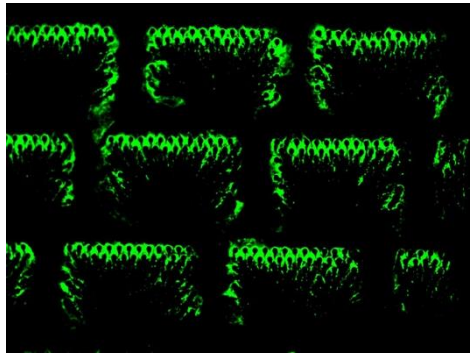


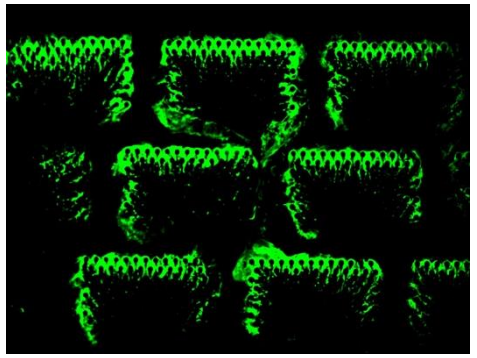
PMA 50 nanomoles

2025-06-17

10x Device

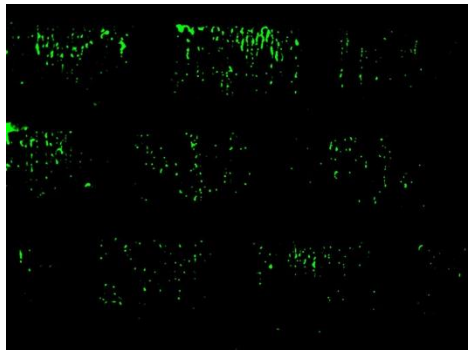
Blank

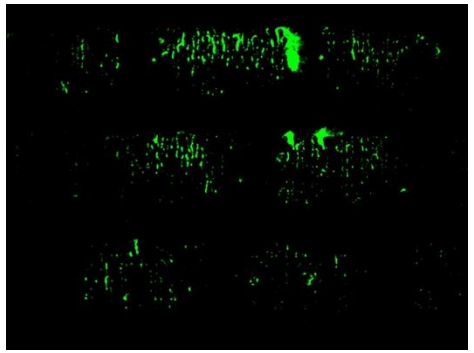




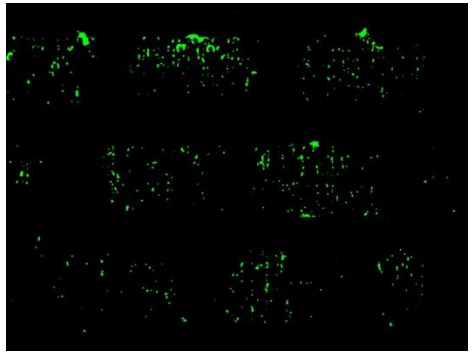
PMA 5 micromoles

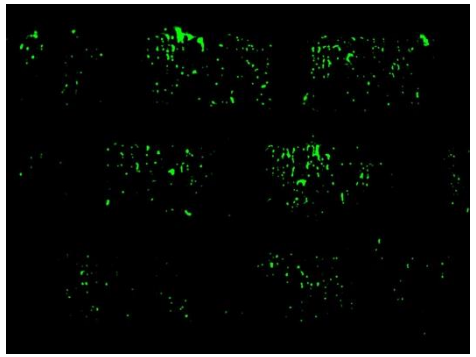
Blank





PMA 500 nanomoles



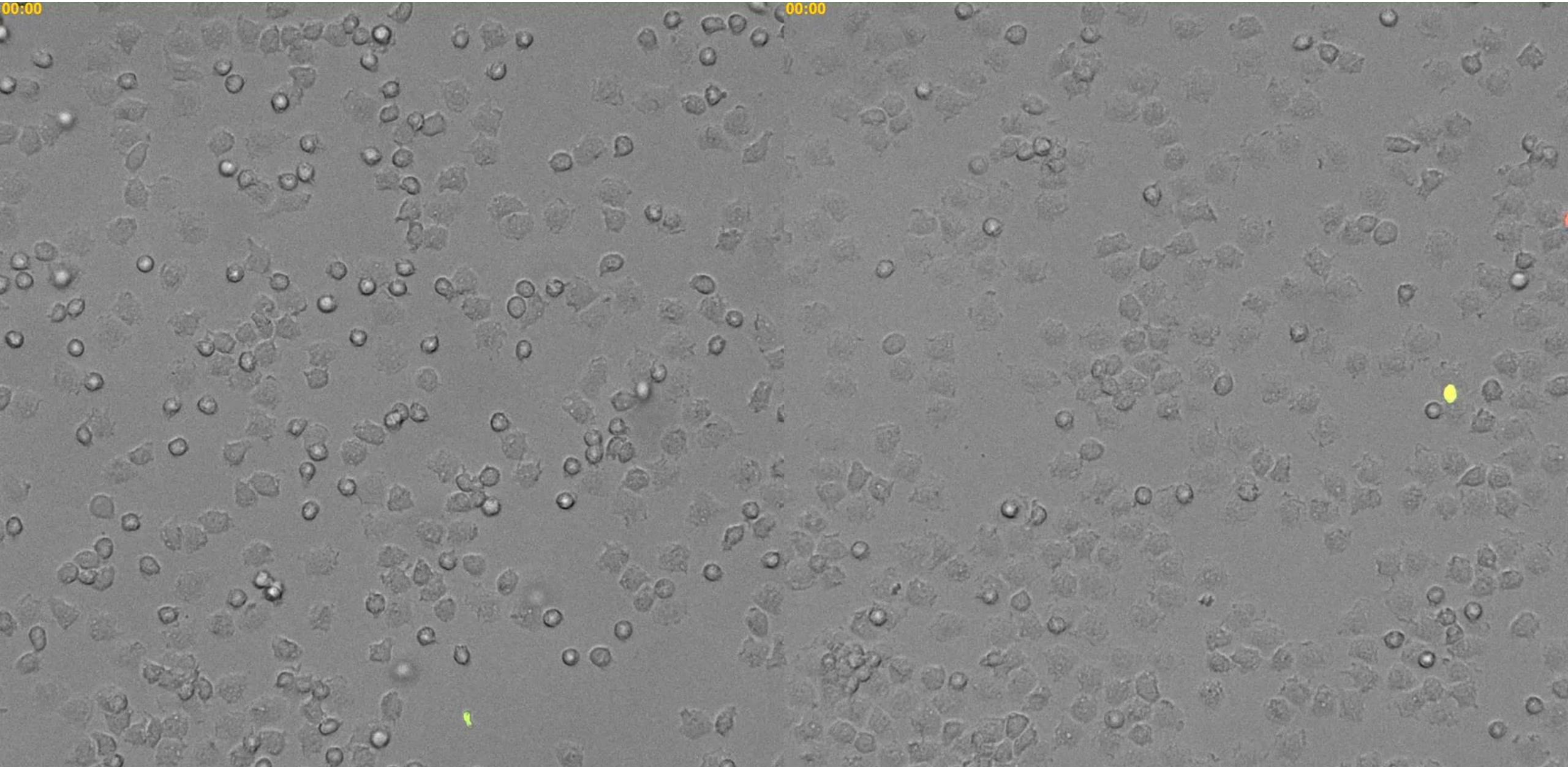


PMA 50 nanomoles

2025-06-24

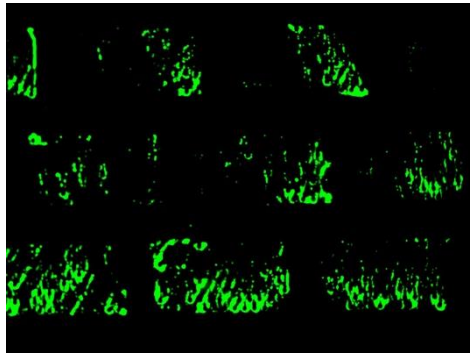
2025-06-17

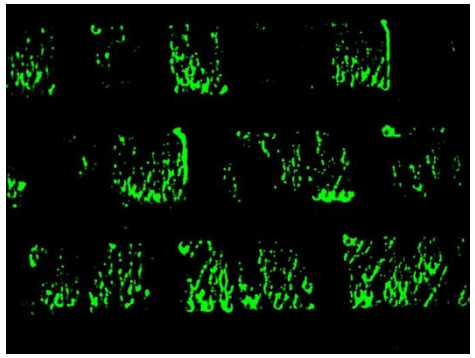
PMA 500 nanomoles / 5 micromoles



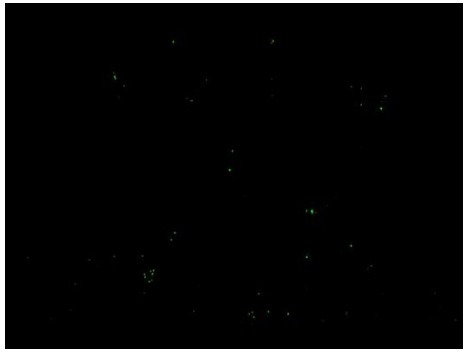
10x Device

Blank

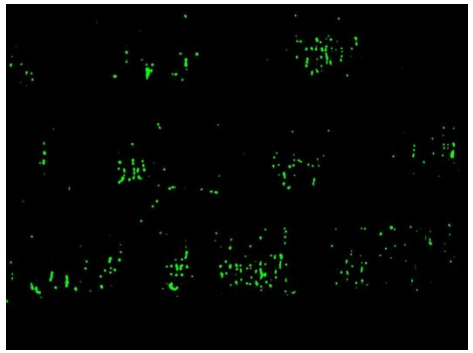


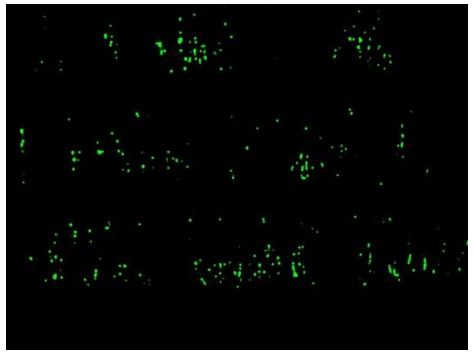


Ionomycin 5 micromoles

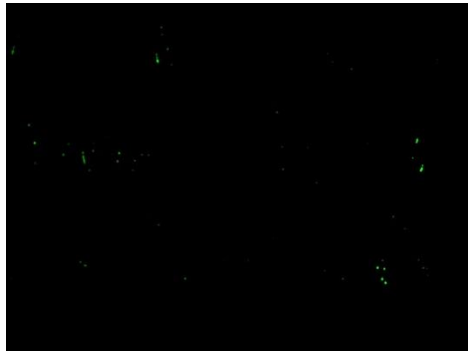


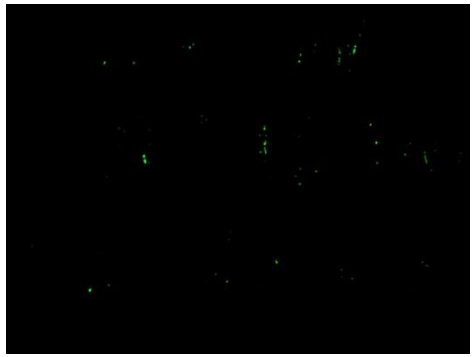
Blank





Ionomycin 500 nanomoles



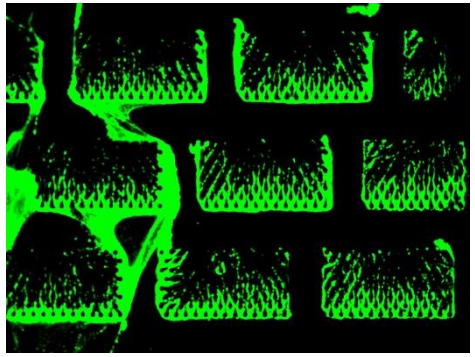


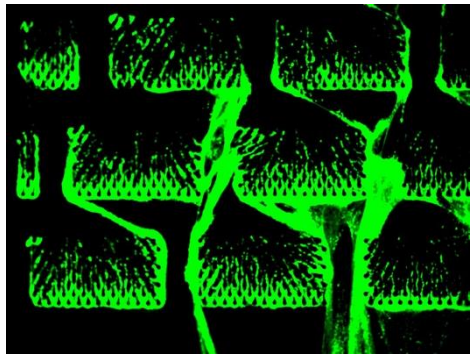
Ionomycin 50 nanomoles

2025-06-17

10x Device

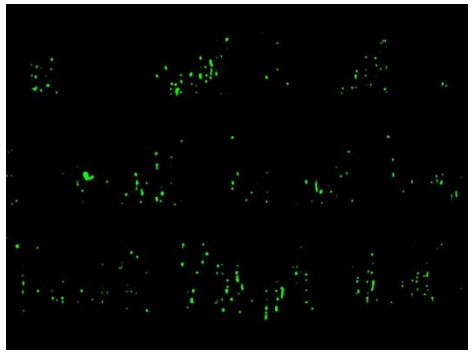
Blank

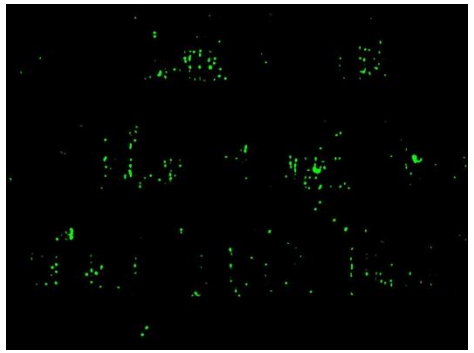




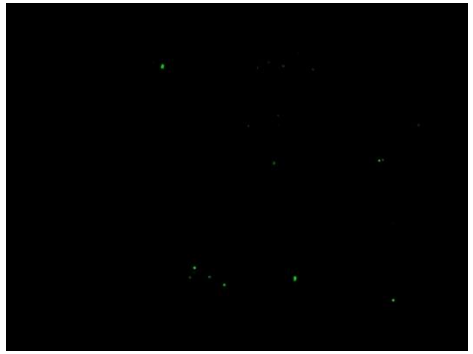
Ionomycin 5 micromoles

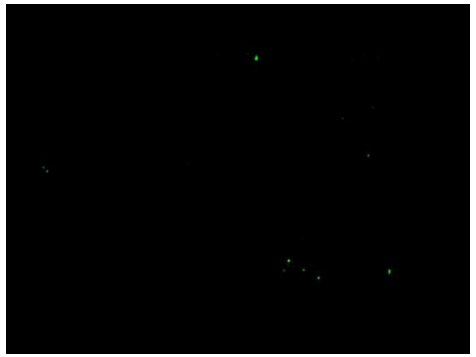
Blank





Ionomycin 500 nanomoles



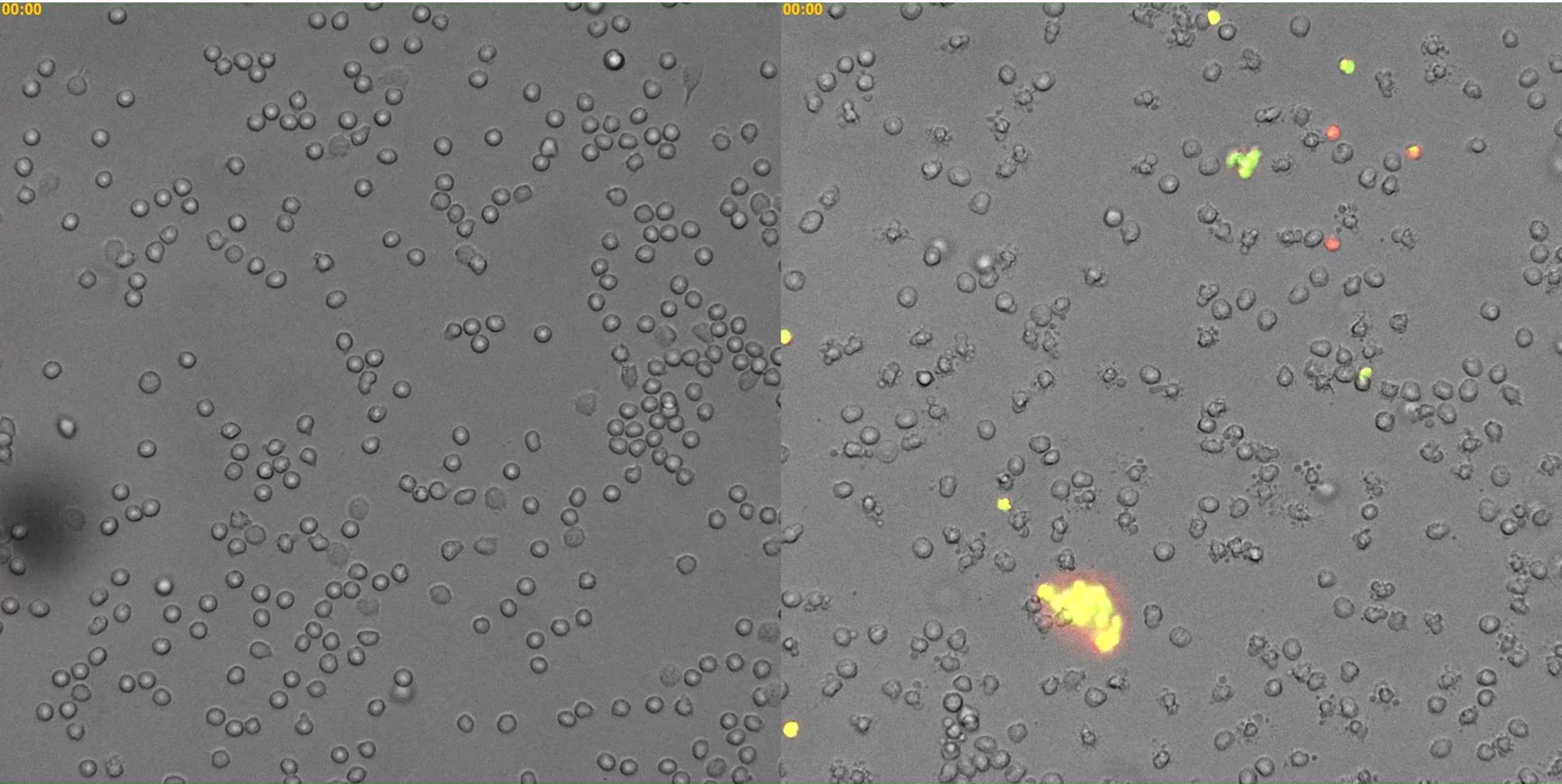


Ionomycin 50 nanomoles

2025-06-24

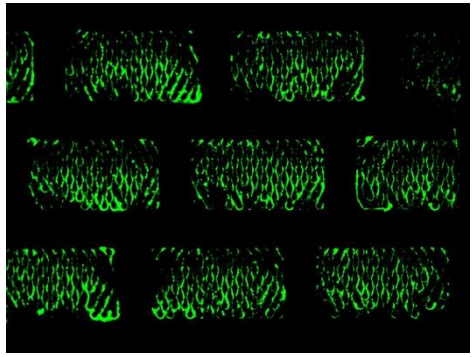
2025-06-17

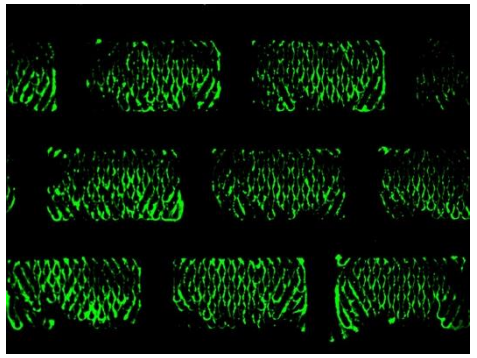
Ionomycin 500 nanomoles / 5 micromoles



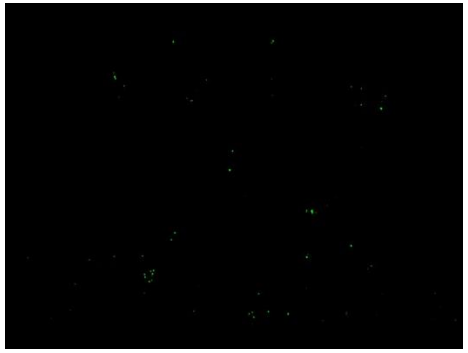
10x Device

Blank

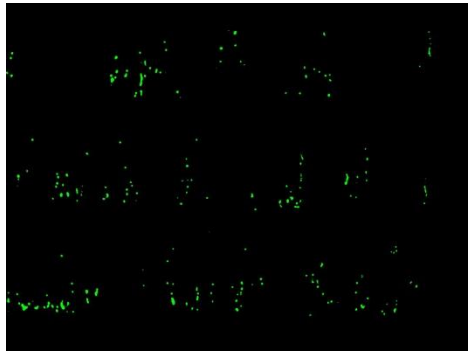


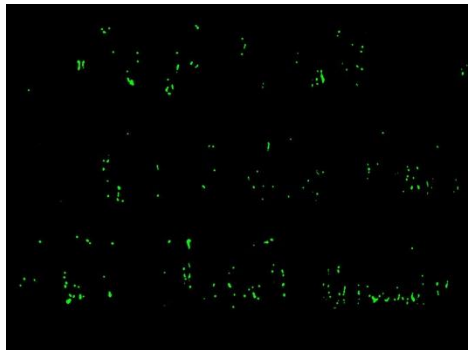


Raptinal 5 micromoles

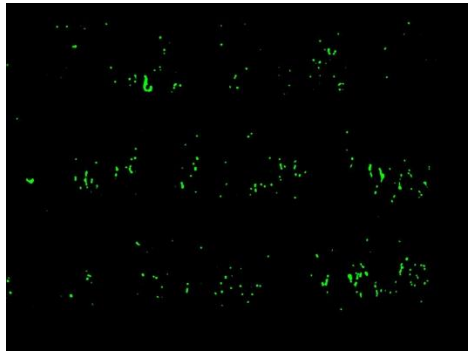


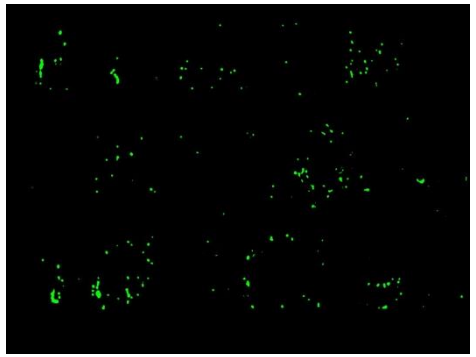
Blank





Raptinal 500 nanomoles



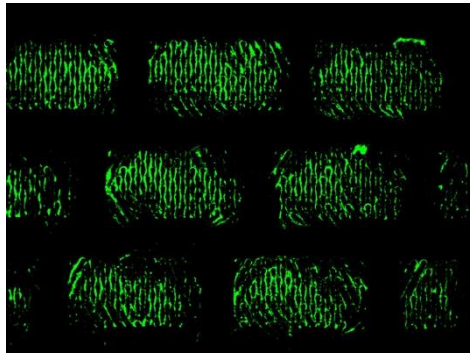


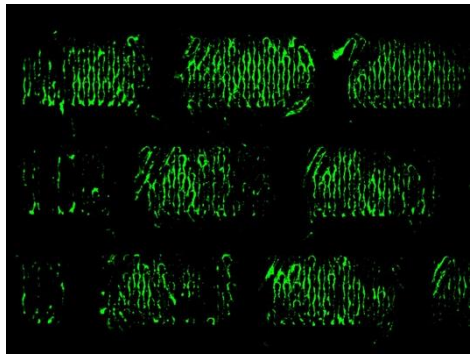
Raptinal 50 nanomoles

2025-06-17

10x Device

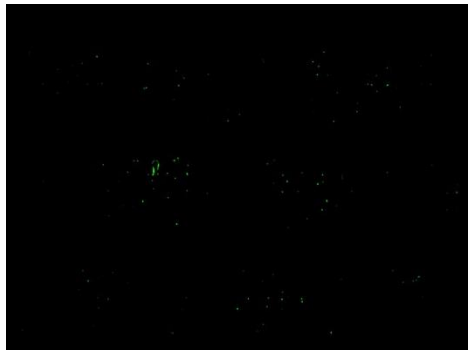
Blank

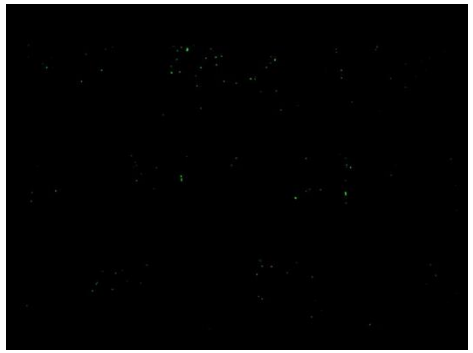




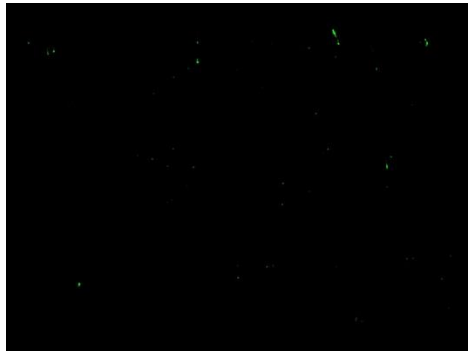
Raptinal 10 micromoles

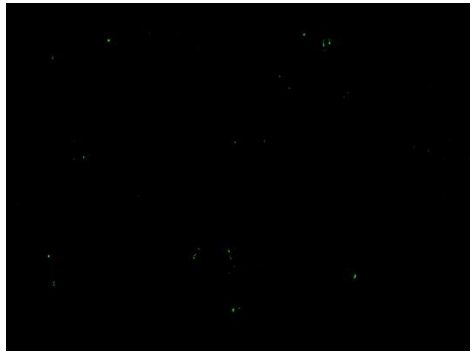
Blank





Raptinal 1 micromoles



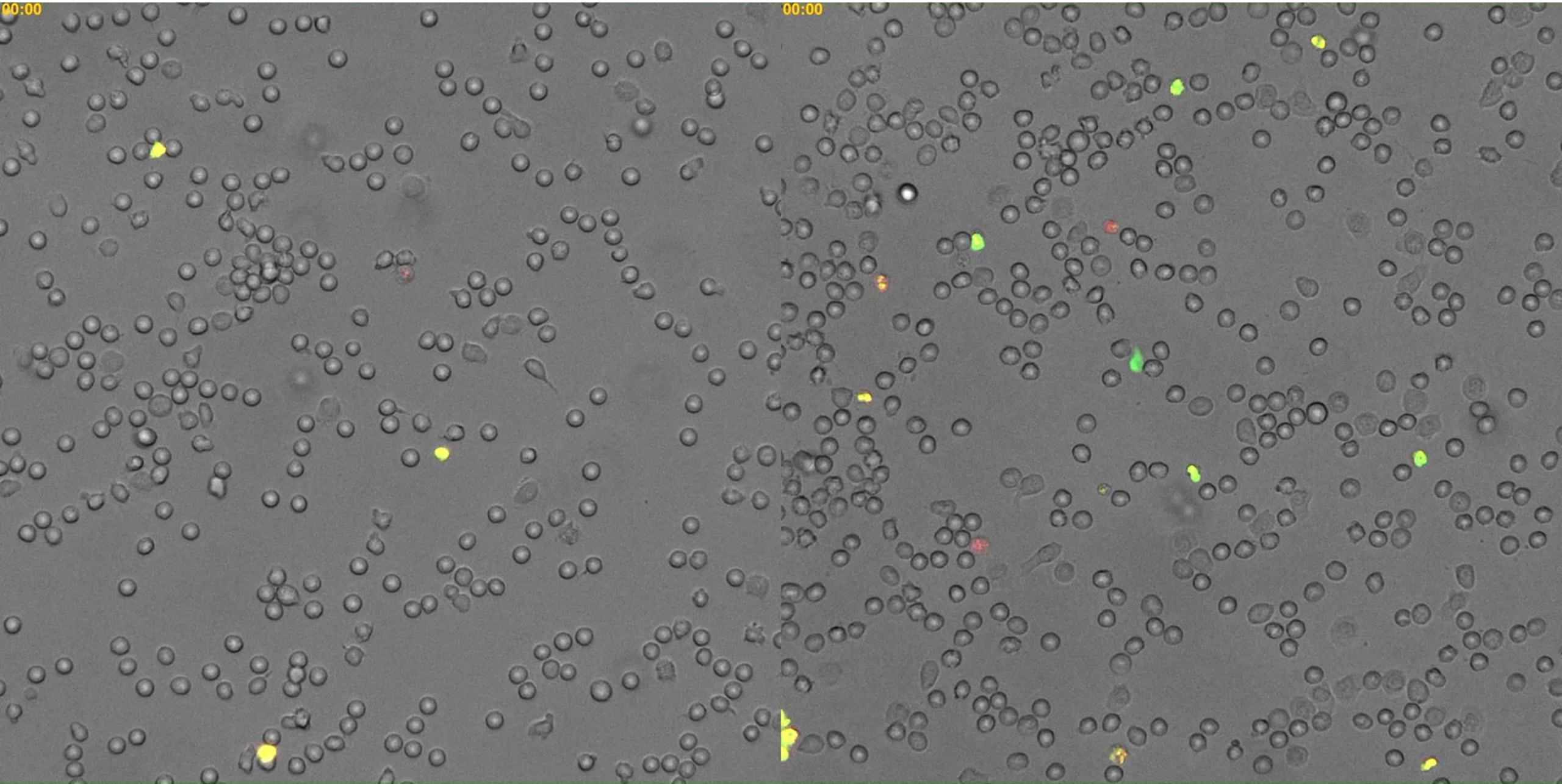


Raptinal 100 nanomoles

2025-06-24

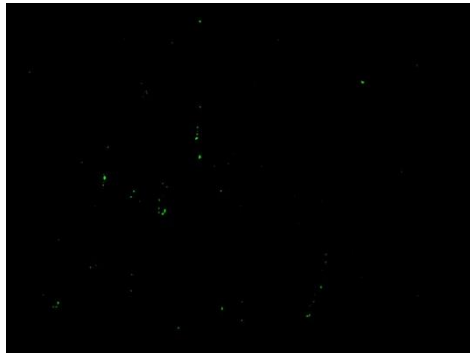
2025-06-17

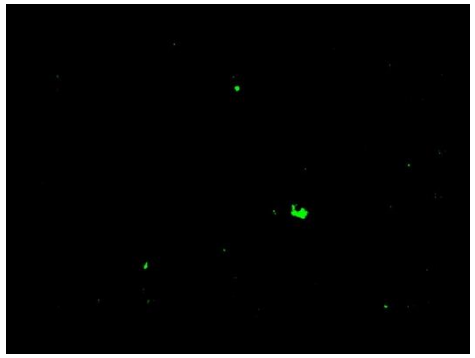
Raptinal 500 nanomoles / 5 micromoles



10x Device

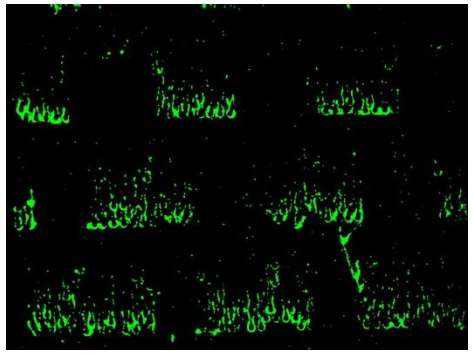
Blank

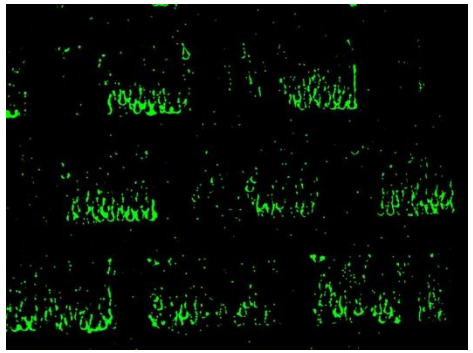




PMA PAD4-Knockout

Blank



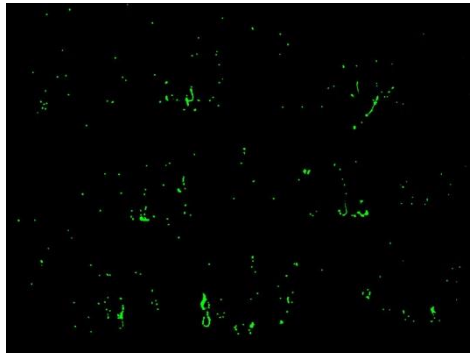


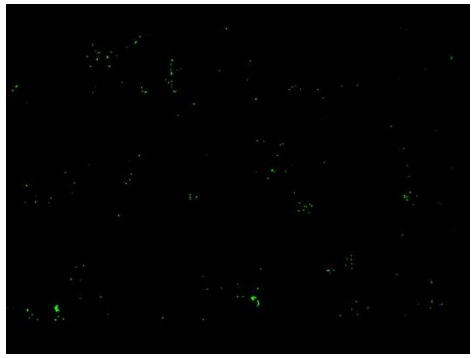
PMA Wildtype

2025-06-27

10x Device

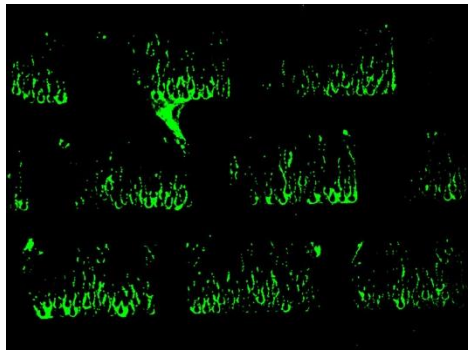
Blank

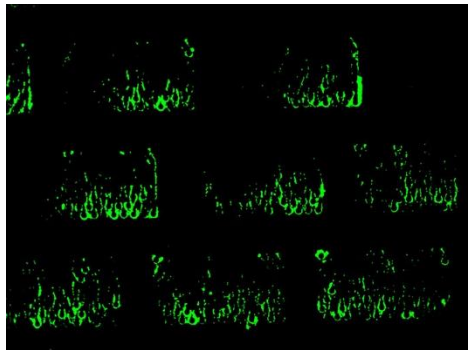




Ionomycin PAD4-Knockout

Blank





Ionomycin Wildtype

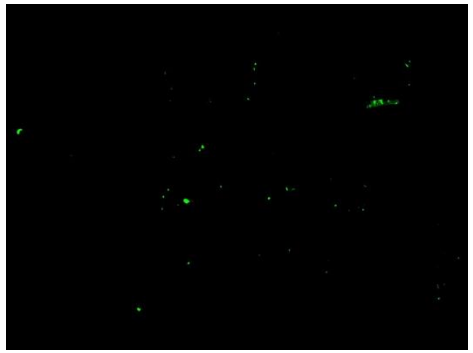
2025-06-27

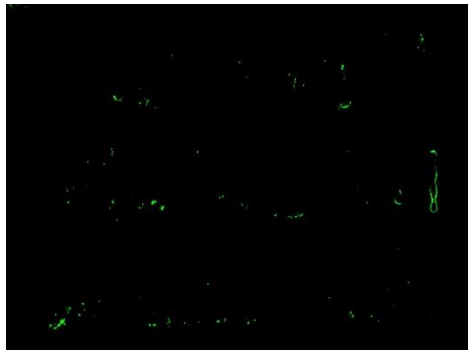
10x Device

Blank

Raptinal PAD4-Knockout

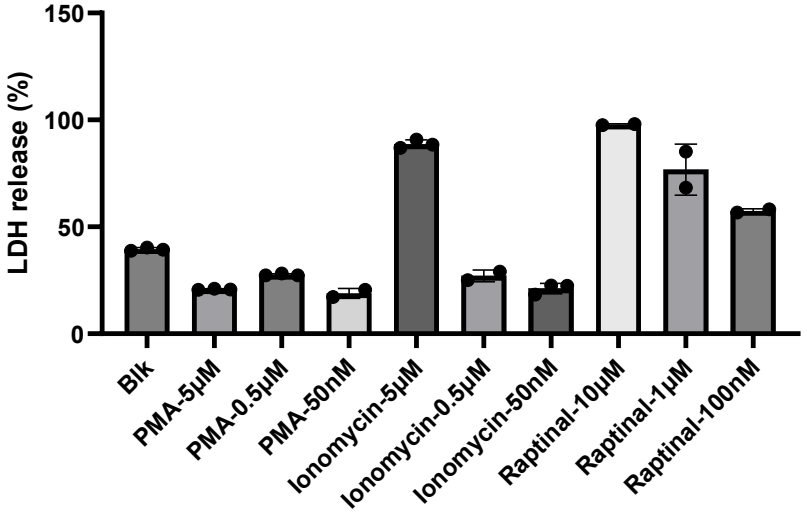
Blank





Raptinal Wildtype

2025-06-27



ASC-critine neutrophil , 4h, LDH release (lysis death)
Normal death rates at 4h (low conc., high conc.):
PMA: 55%, 98%
Ionomycin: 100%, 100% (Both are all dead at 3:40)
Raptinal: 5%, 95%

Ionomycin: At 4h all dead, but LDH release vastly different
Raptinal: At 4h massive difference in cell death rate, but LDH release very similar (gradient among different conc.)
PMA: Needed to be repeated, but apparently there is no lysis death

Further plans: Repeating Knockout, bacteria induction, etc.

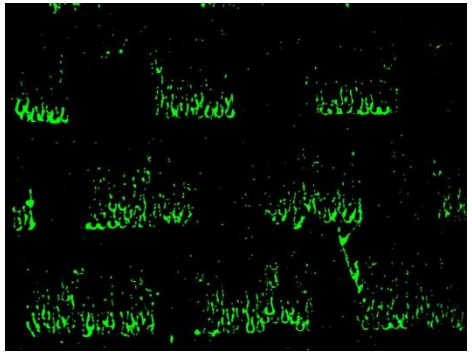
Citations

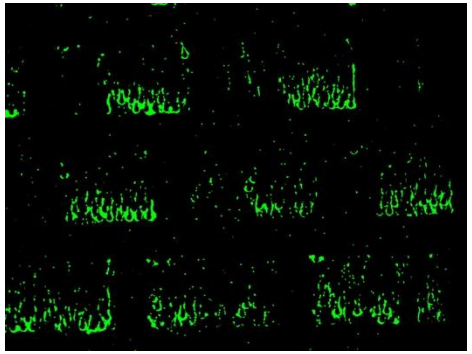
Mice NE Deficiency: (Kolaczkowska, E., Jenne, C. N., Surewaard, B. G. J., Thanabalasuriar, A., Lee, W.-Y., Sanz, M.-J., Mowen, K., Opdenakker, G., & Kubes, P. (2015, March 26). Molecular mechanisms of net formation and degradation revealed by intravital imaging in the liver vasculature. Nature News. https://www.nature.com/articles/ncomms7673#:~:text=The%20enzymes%2C%20peptidyl%20arginine%20deiminase,DNase%20has%20been%20shown)

MPO: Usher, A. K., & Stockley, R. A. (2013, November 13). *The link between chronic periodontitis and COPD: A common role for the neutrophil? - BMC medicine*. BioMed Central. https://bmcmedicine.biomedcentral.com/articles/10.1186/1741-7015-11-241#:~:text=ROS%20have%20been%20implicated%20in,release%20is%20shown%20in%20Figure%C2%A04

Raptinal Apotopsis: Park, W.-Y., Gray, J. M., Holewinski, R. J., Andresson, T., So, J. Y., Carmona-Rivera, C., Hollander, M. C., Yang, H. H., Lee, M., Kaplan, M. J., Cappell, S. D., & Yang, L. (2023, March 27). *Apoptosis-induced nuclear expulsion in tumor cells drives S100A4-mediated metastatic outgrowth through the rage pathway*. Nature News. https://www.nature.com/articles/s43018-023-00524-z

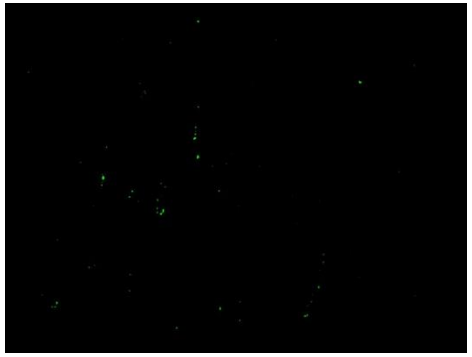
Blank

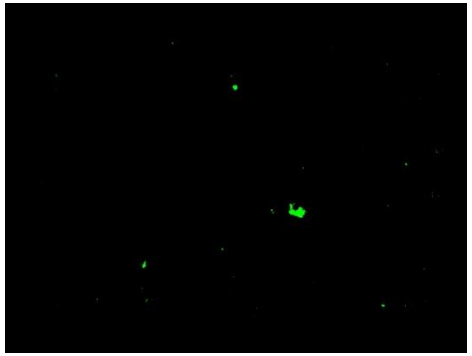




PMA Wildtype

Blank

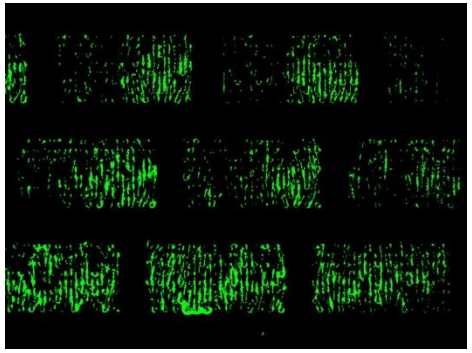


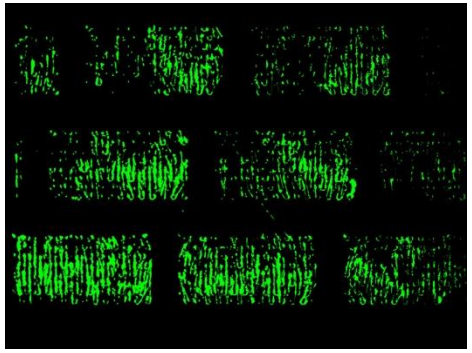


PMA PAD4-Knockout

2025-06-27

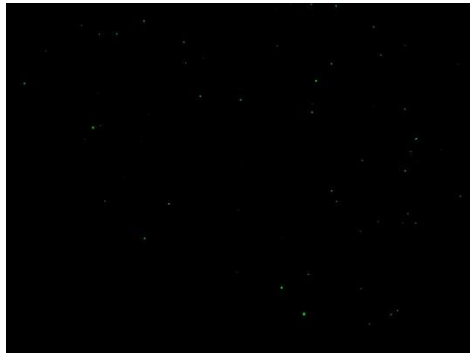
Blank





PMA Wildtype

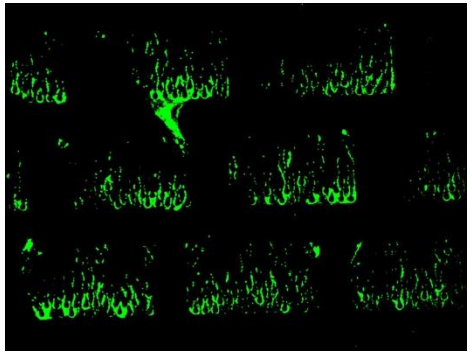
Blank

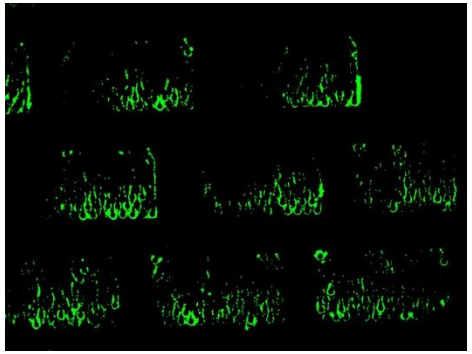


PMA PAD4-Knockout

2025-07-01

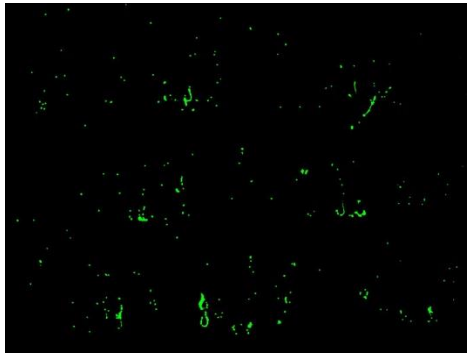
Blank

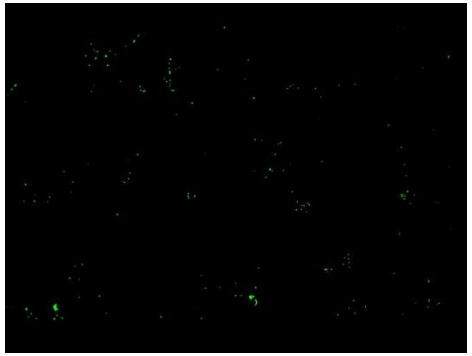




Ionomycin Wildtype

Blank

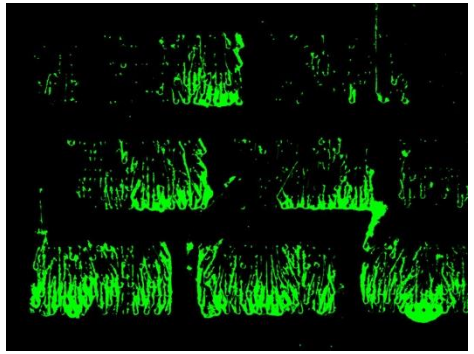


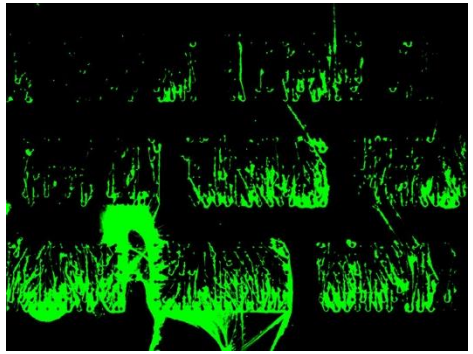


Ionomycin PAD4-Knockout

2025-06-27

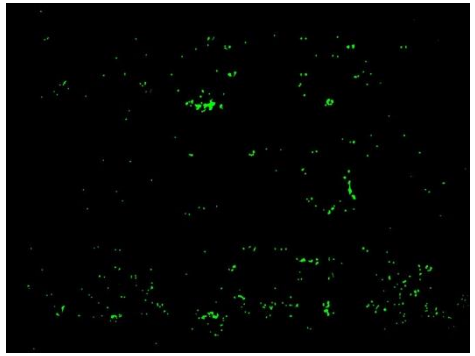
Blank

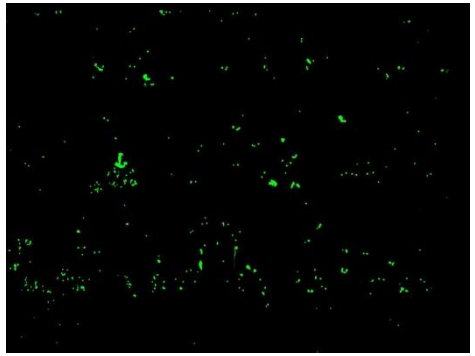




Ionomycin Wildtype

Blank

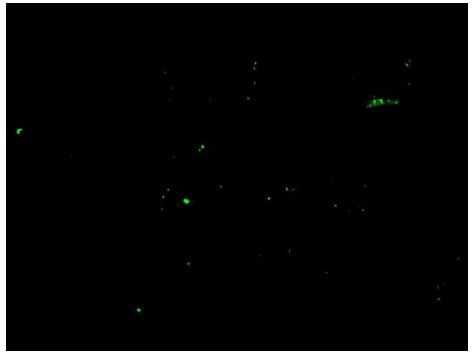


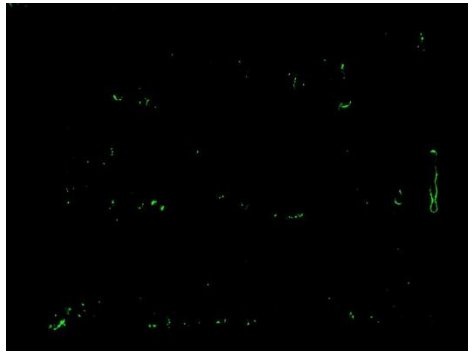


Ionomycin PAD4-Knockout

2025-07-01

Blank





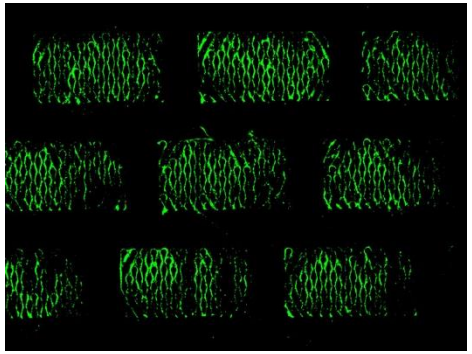
Raptinal Wildtype

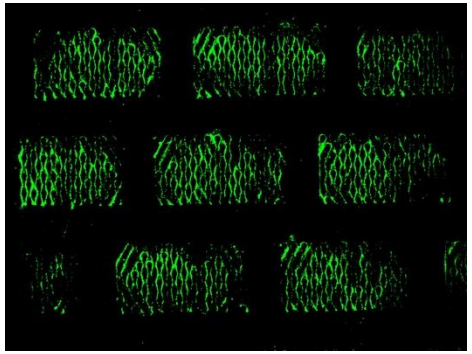
Blank

Raptinal PAD4-Knockout

2025-06-27

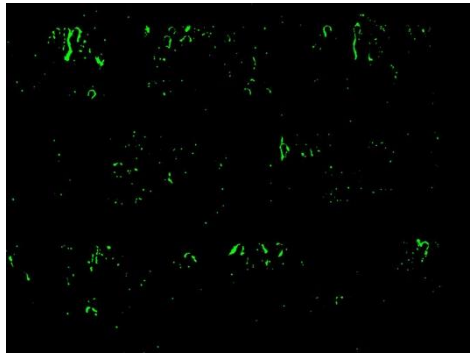
Blank

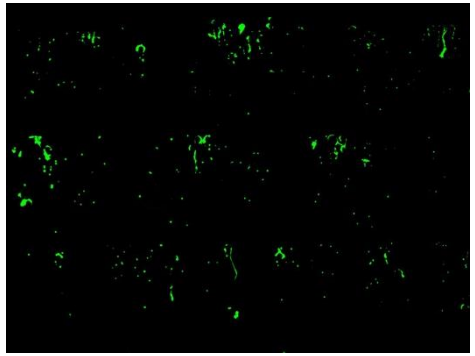




Raptinal Wildtype

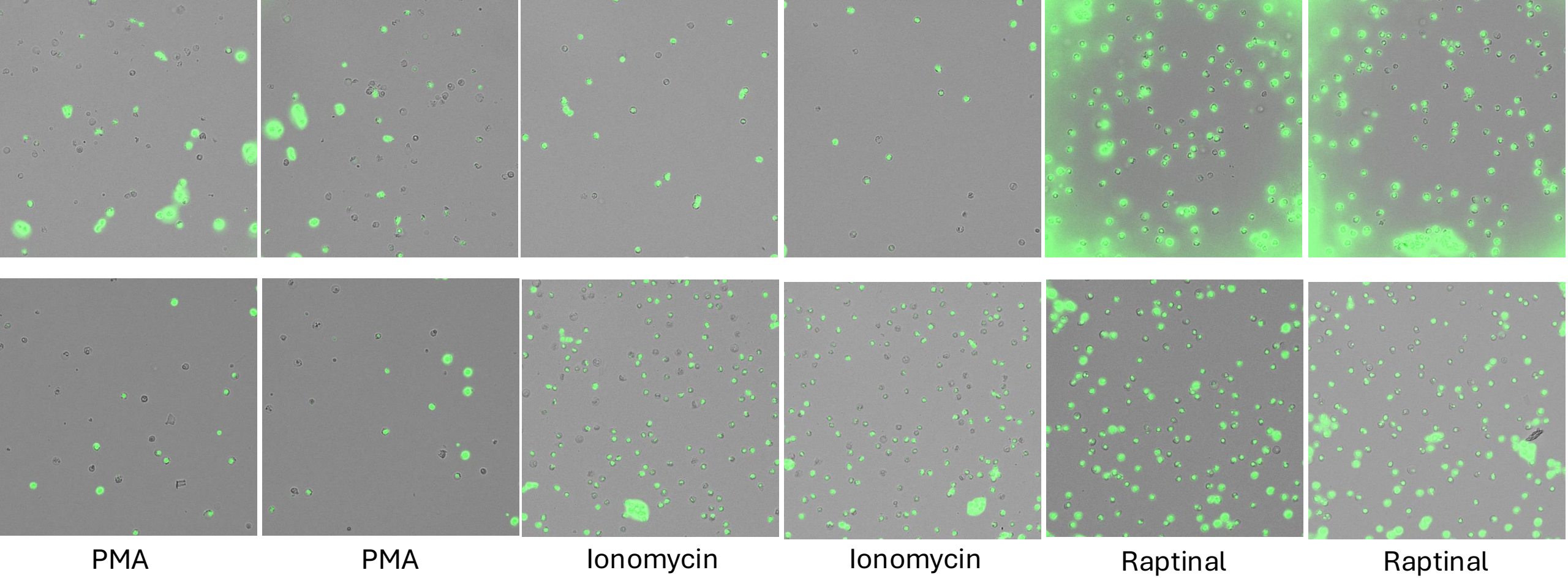
Blank



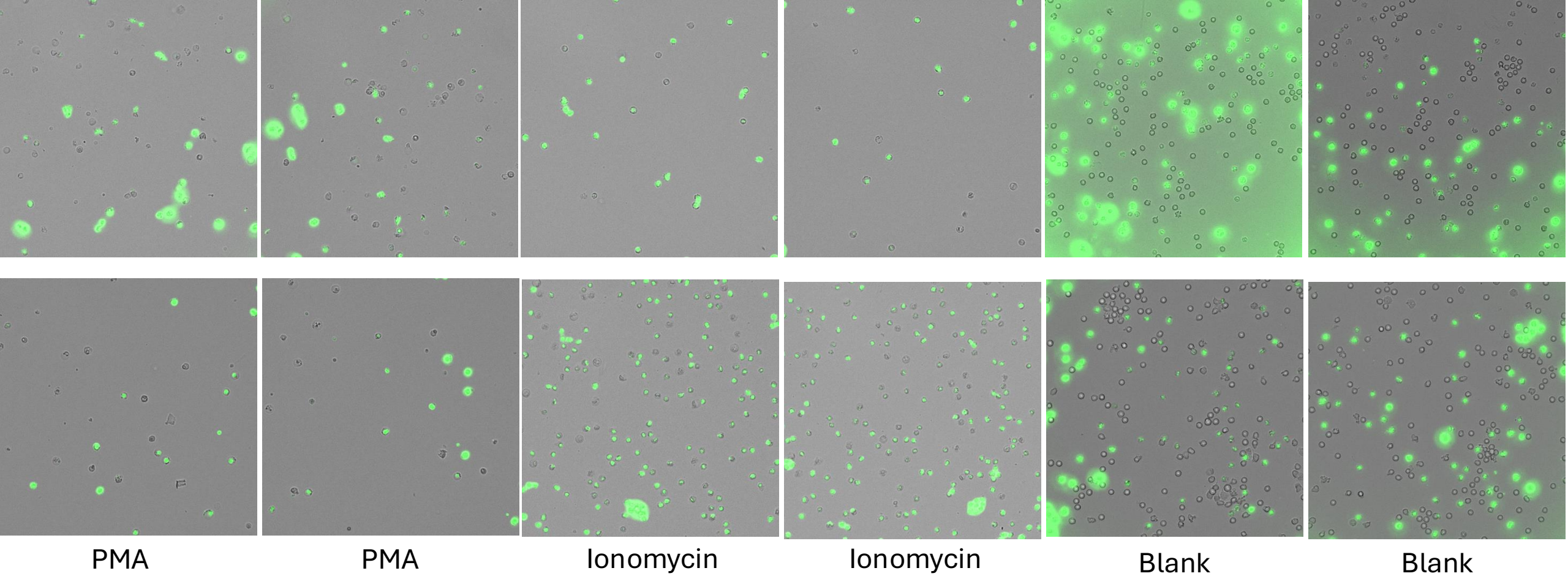


Raptinal PAD4-Knockout

2025-07-01

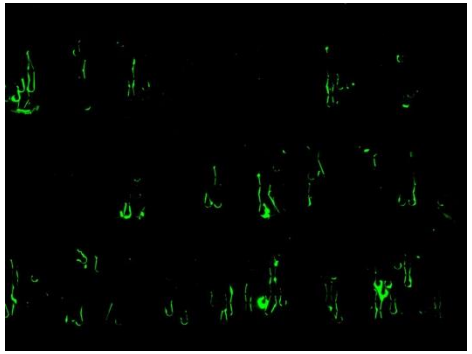


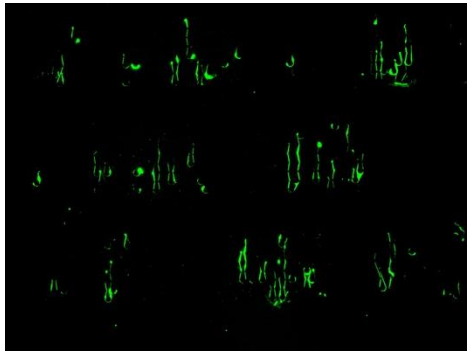
2025-07-01, WT above, KO below



2025-07-01, WT above, KO below

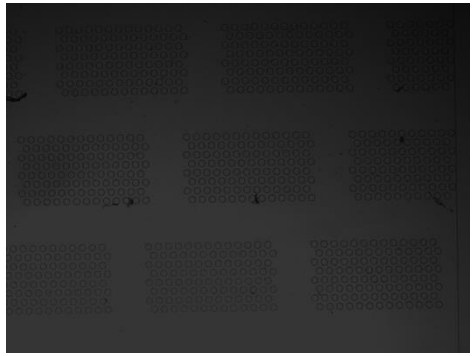
Blank

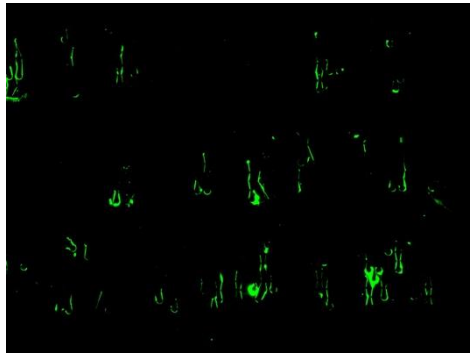




Spontaneous Death Pico

Blank

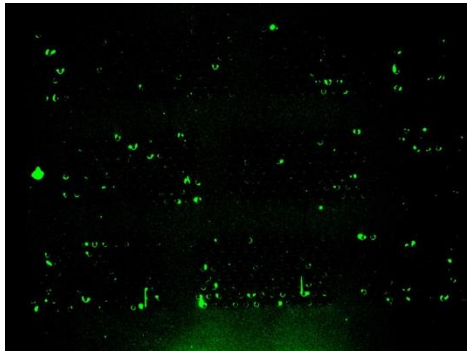


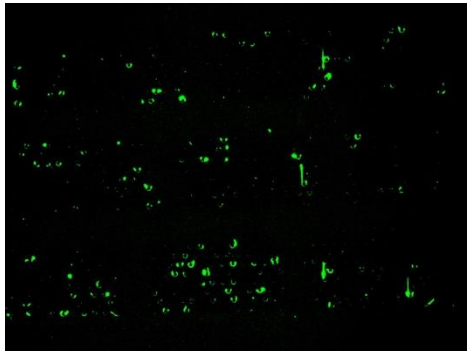


Spontaneous Death Pico

2025-07-03

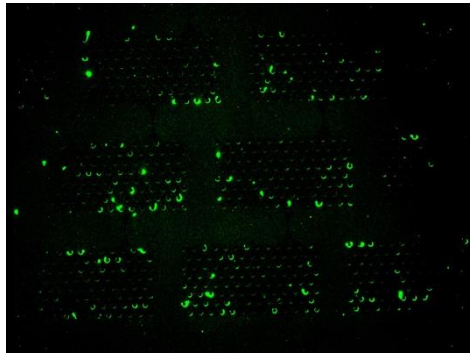
Blank

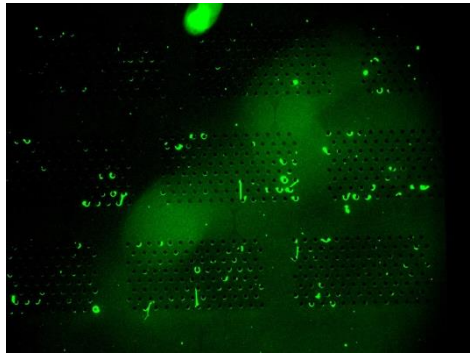




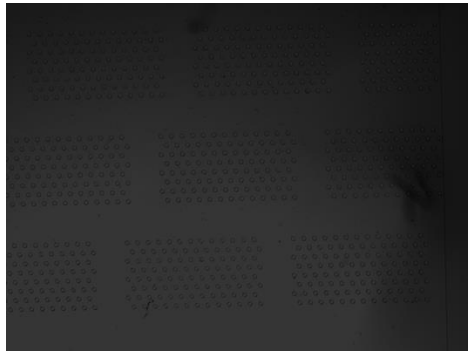
Spontaneous Death Plate
2x cells

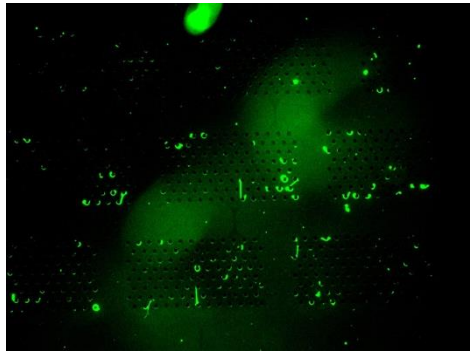
Blank





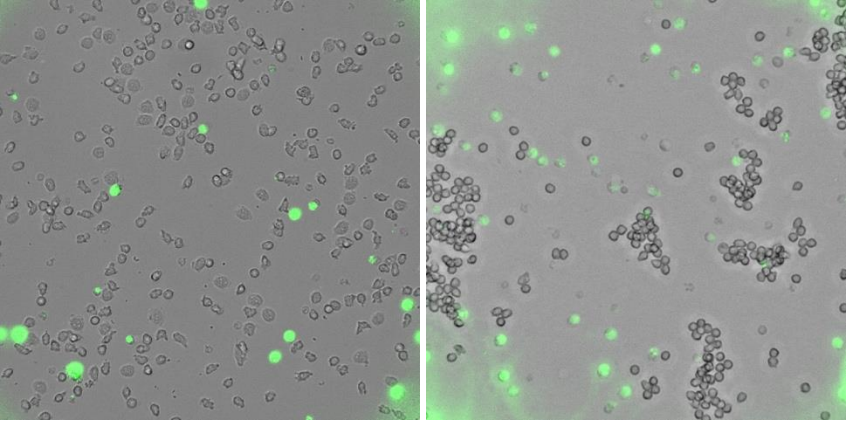
Spontaneous Death Plate
2x cells

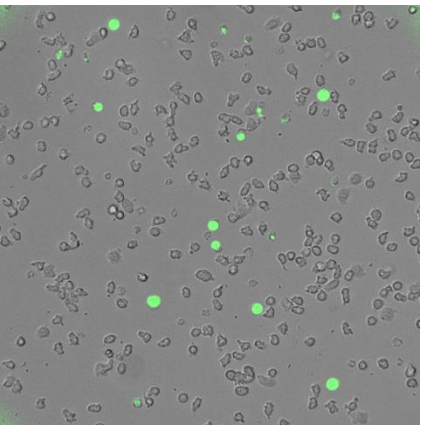


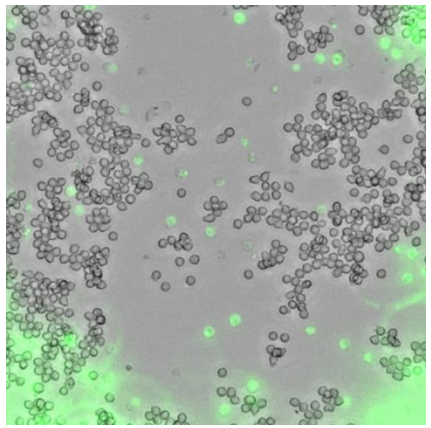


Spontaneous Death Plate
2x cells

2025-07-03







B2

C3

2025-07-03, Spontaneous Death

NET formation pathway summarized

- 1. Neutrophil activation with ROS production (or PAD4 activation)
- 2. Histone modification and chromatin unwinding (PAD4 + MPO)
- 3. Nuclear rupture and NET release (cell death, not vesicle secretion)
- 4. NET deployment
- **Pathway is different in mice and humans**
- **Right now, our experiments only in mice neutrophils**

Mouse neutrophil, in vitro, PMA PAD4 dependency

- Literature: “PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps” PMC , pmc.ncbi.nlm.nih.gov/articles/PMC2931169/. Accessed 7 July 2025.

- Eghbalzadeh, Kaveh. “Compromised Anti-inflammatory Action of Neutrophil Extracellular Traps in PAD4-Deficient Mice Contributes to Aggravated Acute Inflammation After Myocardial Infarction” Compromised Anti-inflammatory Action of Neutrophil, 1 Oct. 2019 www.frontiersin.org/journals/immunology/articles/10.3389/fimmu.2019.02313/full. Accessed 7 July 2025.

Neutrophils trap and kill bacteria by forming highly decondensed chromatin structures, termed neutrophil extracellular traps (NETs). We previously reported that histone hypercitrullination catalyzed by peptidylarginine deiminase 4 (PAD4) correlates with chromatin decondensation during NET formation. However, the role of PAD4 in NET-mediated bacterial trapping and killing has not been tested. Here, we use PAD4 knockout mice to show that PAD4 is essential for NET-mediated antibacterial function. Unlike $PAD4^{+/+}$ neutrophils, $PAD4^{-/-}$ neutrophils cannot form NETs after stimulation with chemokines or incubation with bacteria, and are deficient in bacterial killing by NETs. In a mouse infectious

MI. Noteworthy, $PAD4^{-/-}$ neutrophils were unable to release NETs upon *ex vivo* stimulation with ionomycin or PMA, but produced significantly higher amounts of reactive oxygen species (ROS). Increased levels of circulating cell-free DNA, mitochondrial DNA and cardiac troponin were found in $PAD4^{-/-}$ mice in the acute phase of MI when compared to WT mice. Reduced cardiac expression of *IL-6, IL-10,* and M2 marker genes, as well as increased *TNF*-α expression, suggested a pro-inflammatory state. $PAD4^{-/-}$ mice displayed significantly

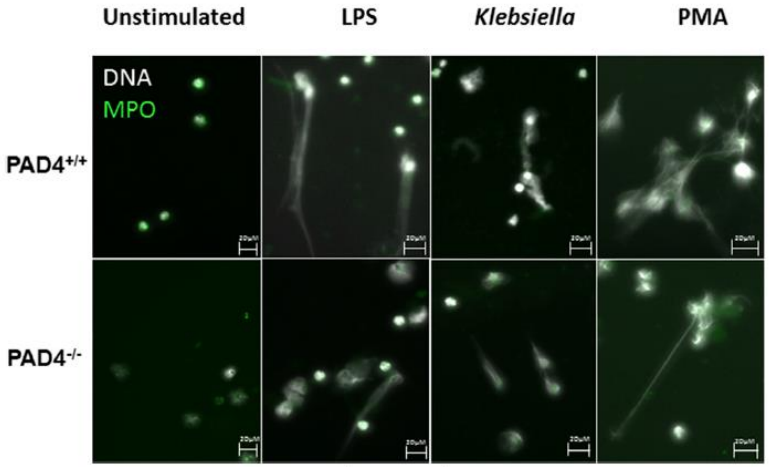


FIGURE 5. Ex vivo NET formation does not rely on PAD4. PAD4$^{+/+}$ and PAD4$^{-/-}$ neutrophils were stimulated with medium control, LPS, PMA, or UV-radiated *Klebsiella* for 3 h. NETs were visualized with immunofluorescence of MPO (in green) or DNA (DAPI staining in white).

During murine pneumosepsis, CitH3 levels were increased in the lungs of PAD4$^{+/+}$ but not of PAD4$^{-/-}$ mice. Combined light and electron microscopy showed NET-like structures surrounding *Klebsiella* in areas of CitH3 staining in the lung; however, these were also seen in PAD4$^{-/-}$ mice with absent CitH3 lung staining. Moreover, cell-free DNA and nucleosome levels were mostly similar in both groups. Moreover, *Klebsiella* and LPS could still induce NETosis in PAD4$^{-/-}$ neutrophils. Both

Mouse neutrophil, in vitro, PMA PAD4 independency (?)

Pneuomonia-induced

- Literature: van, "Cornelis. “Role of Peptidylarginine Deiminase 4 in Neutrophil Extracellular Trap Formation and Host Defense during Klebsiella pneumoniae- Induced Pneumonia-Derived Sepsis” PubMed, 9 July 2018, pubmed.ncbi.nlm.nih.gov/29987161/. Accessed 10 July 2025.

- Induced pneumonia-derived sepsis
- Not relevant

Human neutrophil, in vitro, PMA PAD4 independency

PKC is required for PMA, *C. albicans* and GBS induced NETosis. Only PMA and nigericin require calcium to make NETs

To test whether ROS were required for NETosis, we pre-incubated neutrophils with pyrocatechol before stimulation. As expected, ROS was absolutely required for PMA-induced NETosis (Figure 3D). Conversely,

to induce NETosis.

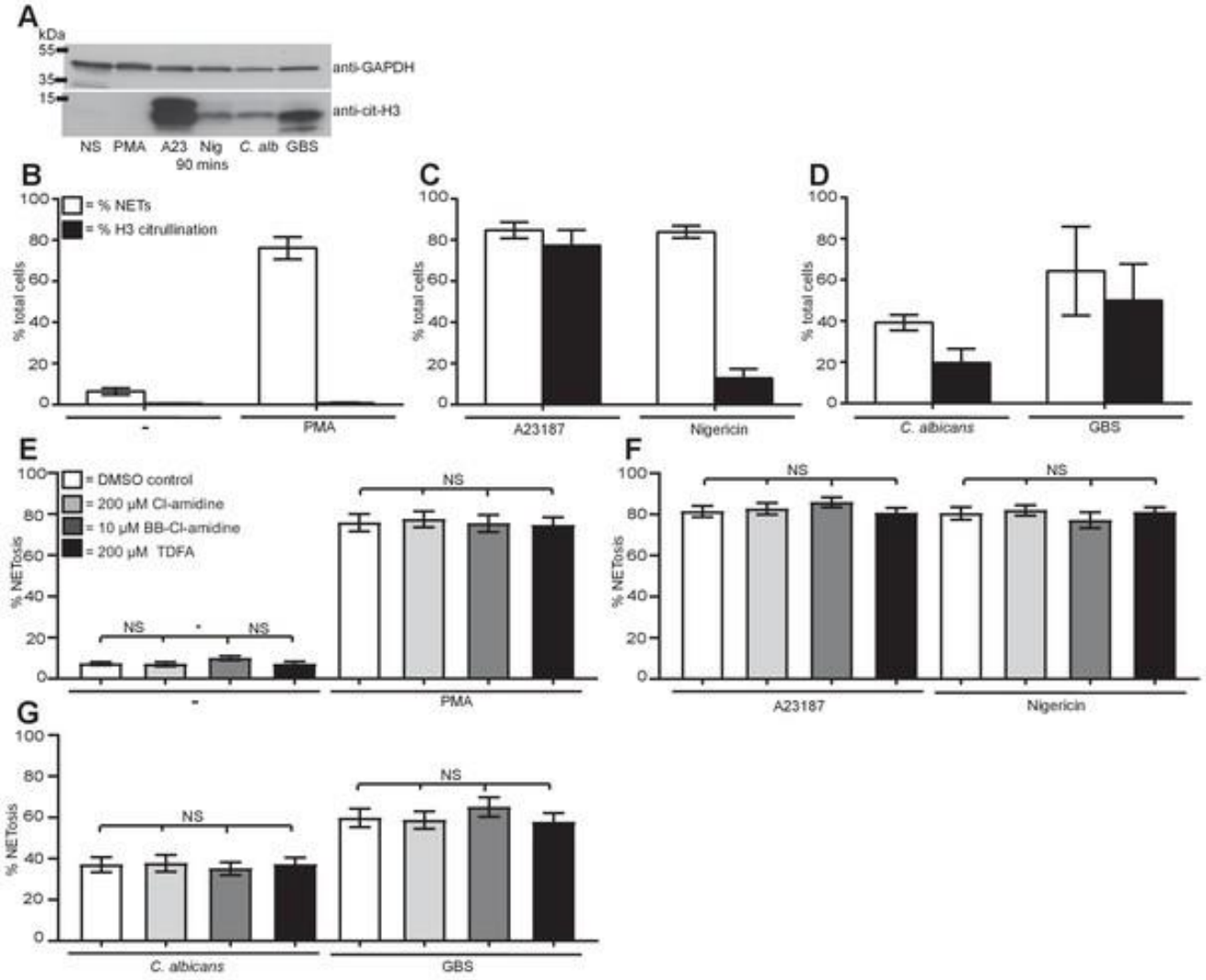
Conversely, activation with the ionophores A23187 and nigericin does not require PKC, ROS, MPO or NE or transcriptional activation and calcium only has a limited role. Histone H3 is citrullinated upon activation by these stimuli; however, pre-treatment with PAD inhibitors does not affect the ability of the neutrophils to make NETs. This suggests that citrullination (of histone H3 at least) is a consequence of NETosis, and that PAD4 is not required for NET formation. Ionophore and PMA-induced NETosis appears to be distinct, at least in the few components of the signal transduction cascade already described.

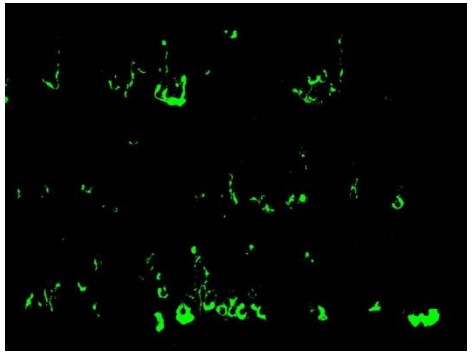
We next explored if citrullination was required for NET formation. Neutrophils were pre-treated with three inhibitors of PAD proteins: Cl-amidine and BB-Cl-amidine, both of which inhibit PAD2 and PAD4, and Thr-Asp-F-amidine (TDFA), a potent specific PAD4 inhibitor. Treating neutrophils with Cl-amidine, BB-Cl-amidine or TDFA did not induce NETosis spontaneously (Figure 6E). PMA induced NETosis was not affected by these inhibitors, consistent with the data obtained with GSK199, another PAD4-selective inhibitor (Figure 6E) (Lewis et al., 2015). Importantly, in response to A23187 or nigericin stimulation, NETosis remained intact after incubation with the three inhibitors (Figure 6F).

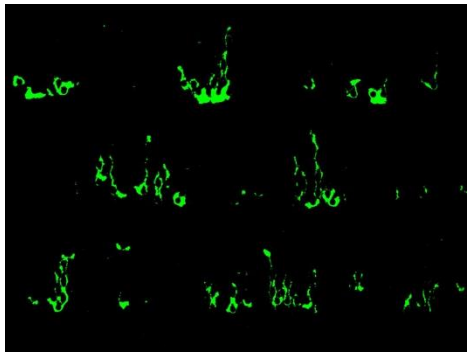
- Literature: Kenny, Elaine F. “Diverse stimuli engage different neutrophil extracellular trap pathways” ELife, 2 June 2017, elifesciences.org/articles/24437. Accessed 7 July 2025.

Human neutrophil, in vitro, PMA PAD4 independency

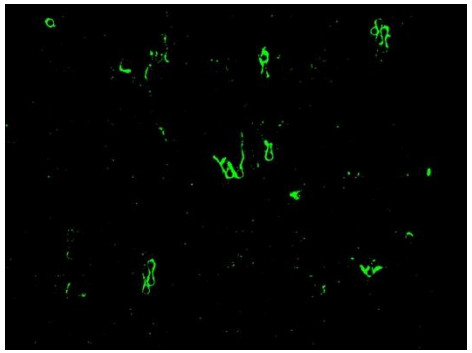
- Literature: Kenny, Elaine F. “Diverse stimuli engage different neutrophil extracellular trap pathways” ELife, 2 June 2017, elifesciences.org/articles/24437. Accessed 7 July 2025.
- (Figure 6E is PAD4 knockout)

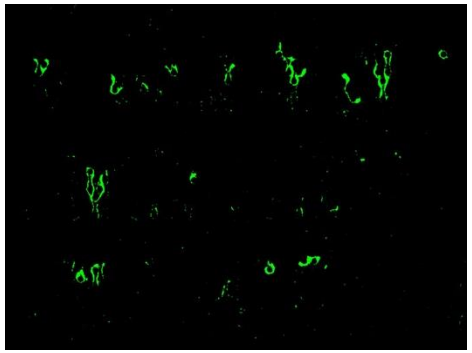






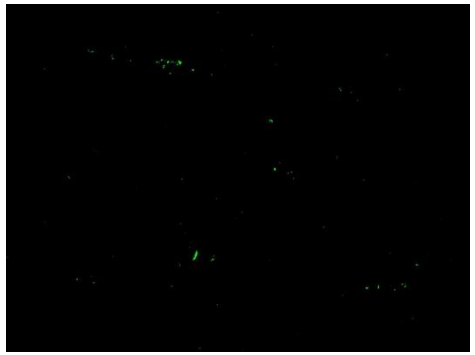
Spontaneous Death
- Percoll, 24h





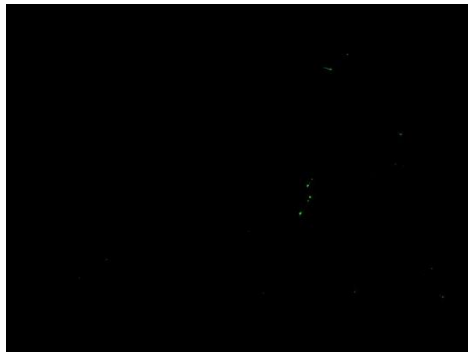
Spontaneous Death
- Percoll, 48h

2025-07-09,07-10, Spontaneous Death

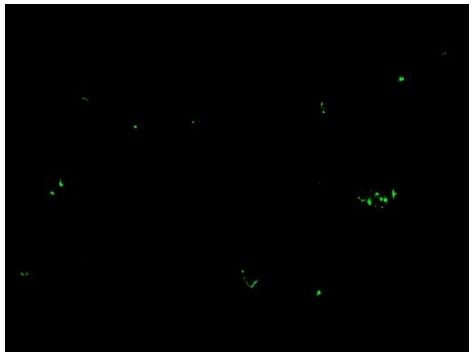
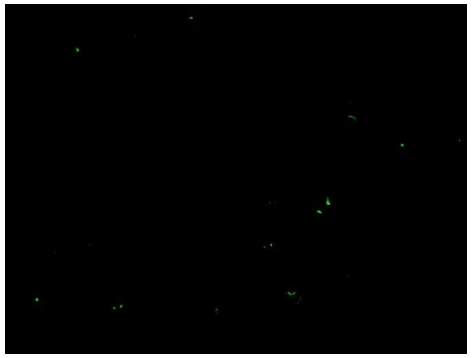


Spontaneous Death
- Percoll – Histopaque
- 2x cells, 24h

Spontaneous Death
- Percoll – Histopaque, 48h




Spontaneous Death
- Percoll – Negative Selection, 24h

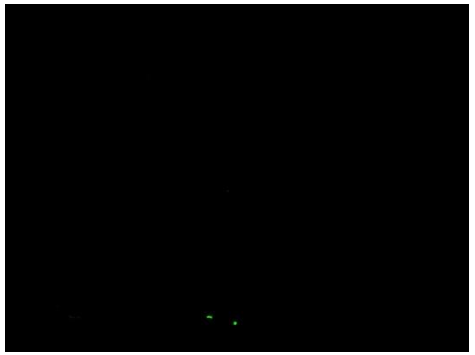



Spontaneous Death
- Percoll – Negative Selection
- 2x cells, 24h


Spontaneous Death
- Percoll – Negative Selection
- 48h

2025-07-09,07-10, Spontaneous Death

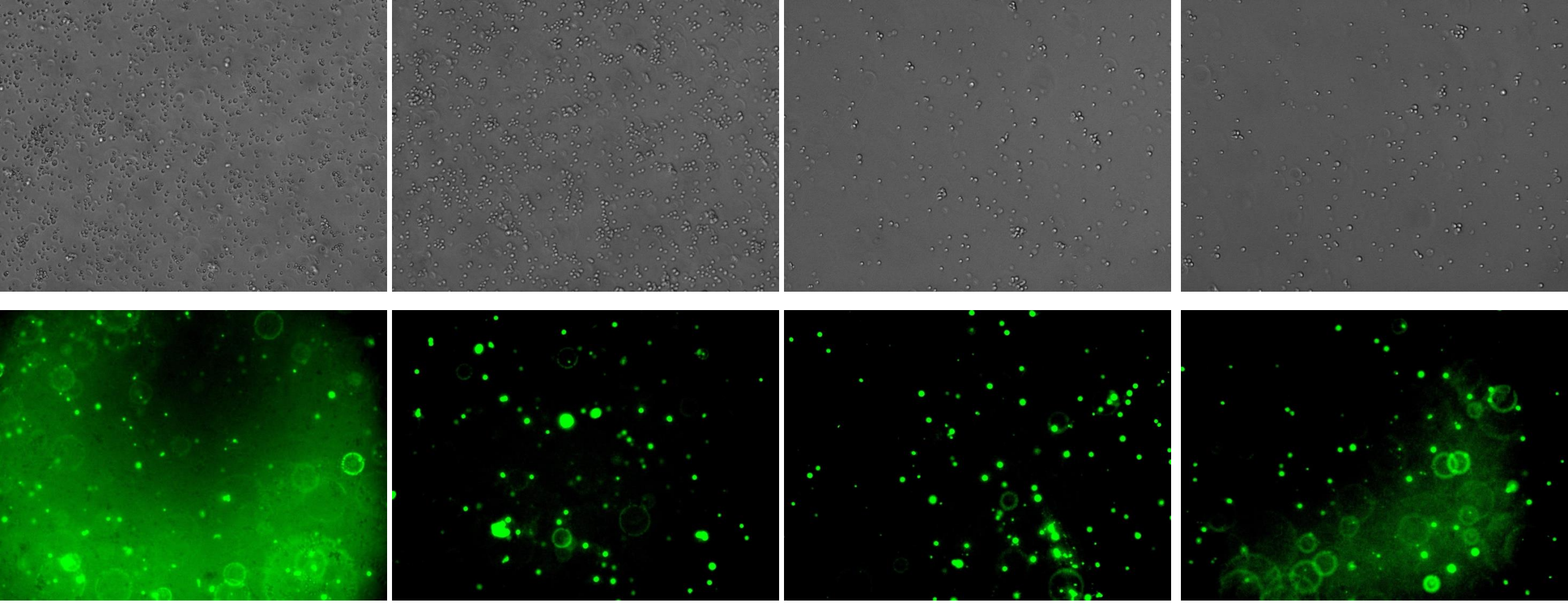

Spontaneous Death
- Percoll – Histopaque
- Negative Selection, 24h

Spontaneous Death
- Percoll – Histopaque
- Negative Selection
- 2x cells, 24h

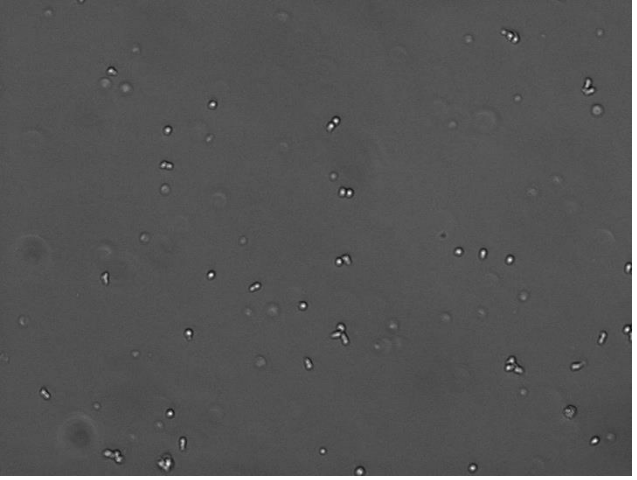

Spontaneous Death
- Percoll – Histopaque
- Negative Selection
- 2x cells, 48h

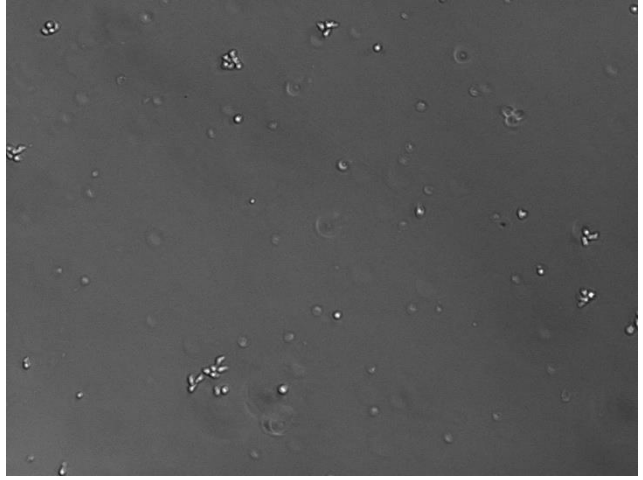
2025-07-09,07-10, Spontaneous Death

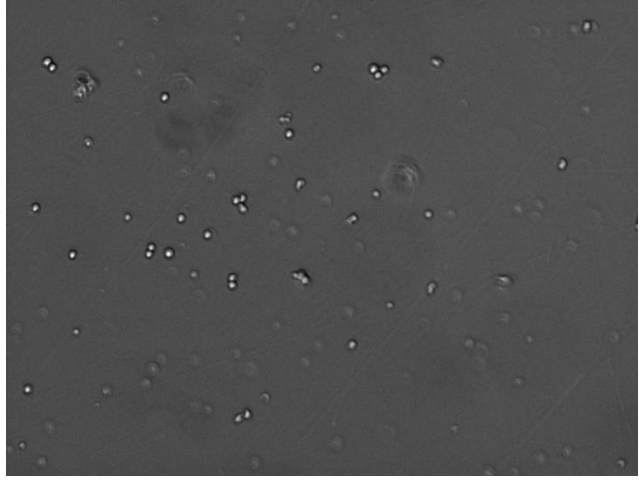


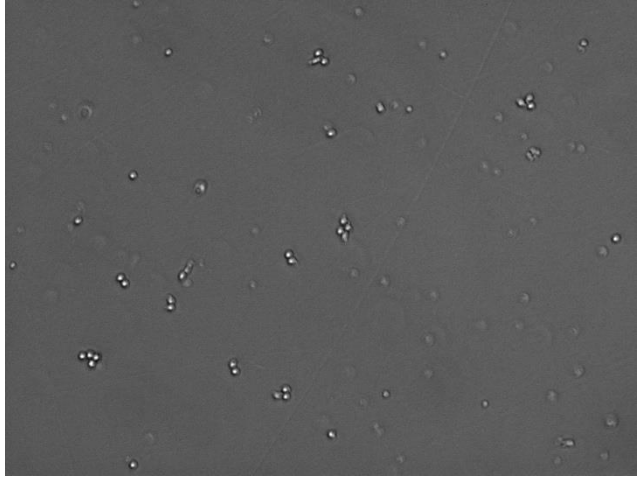
Percoll | Percoll | Percoll - Histopaque | Percoll - Histopaque

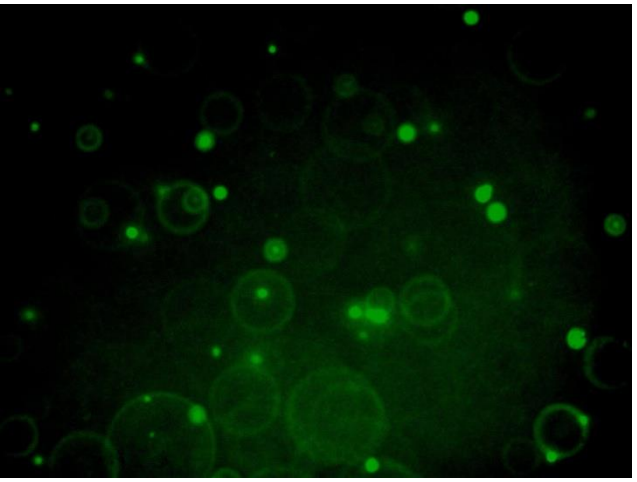
2025-07-09, Spontaneous Death, 24h

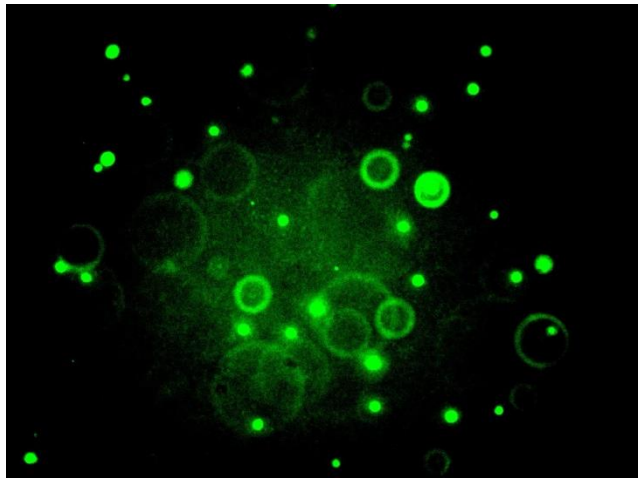


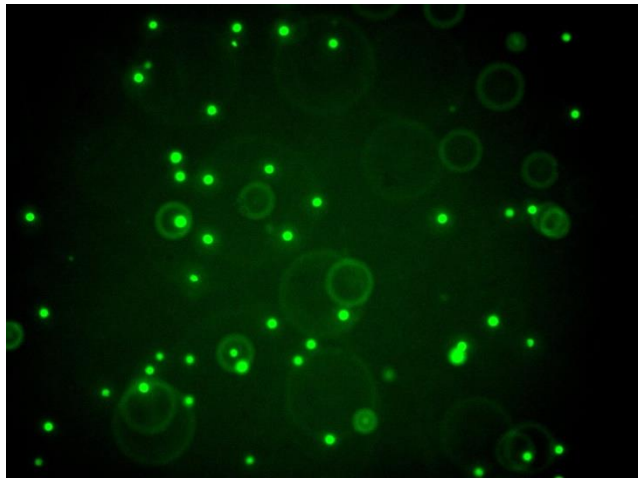


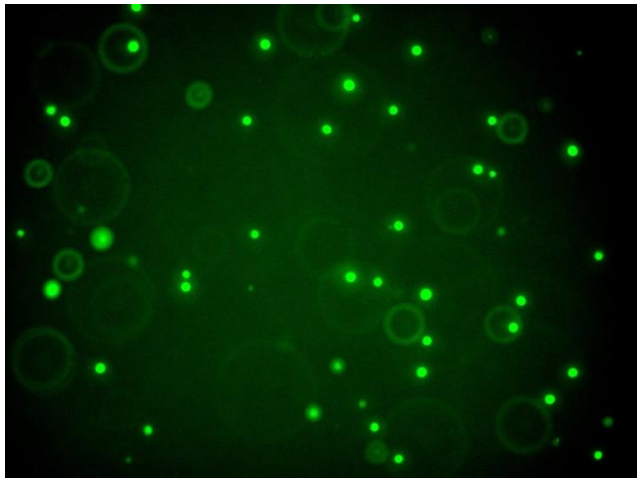












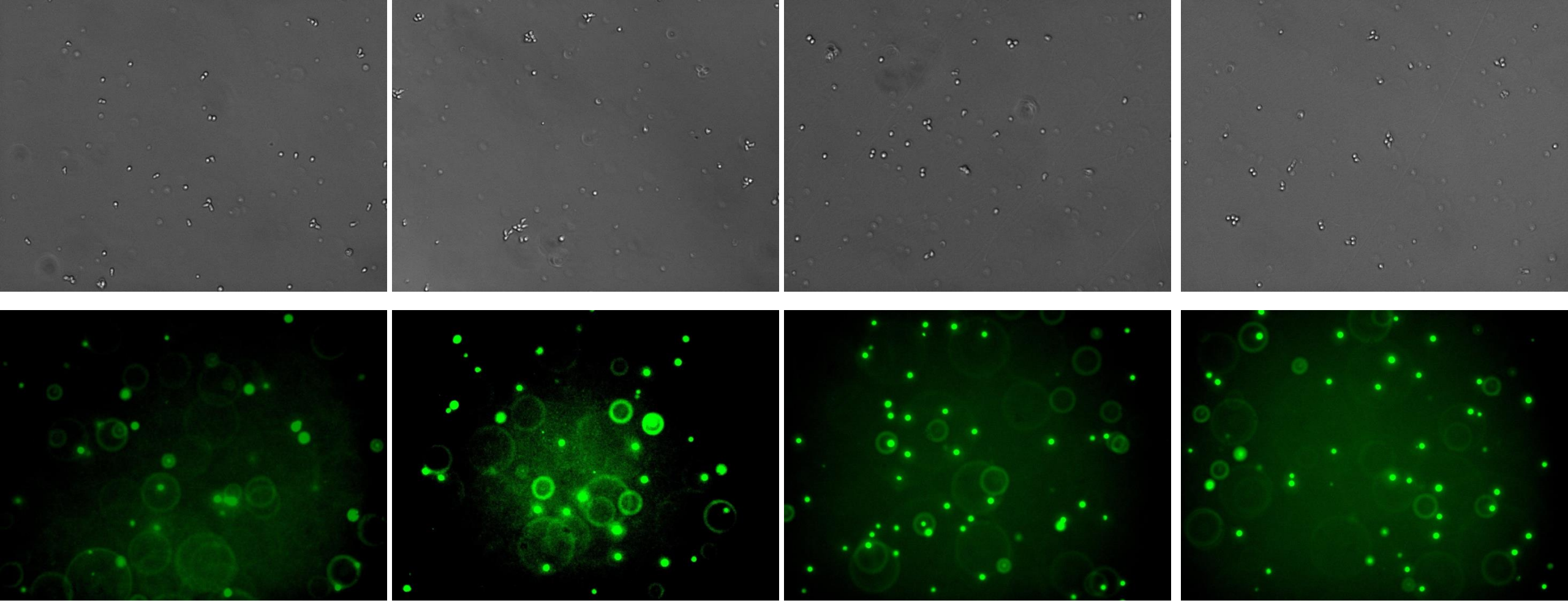
Percoll – Negative Selection

Percoll – Negative Selection

Percoll – Histopaque - Negative Selection

Percoll – Histopaque - Negative Selection

2025-07-09, Spontaneous Death, 24h



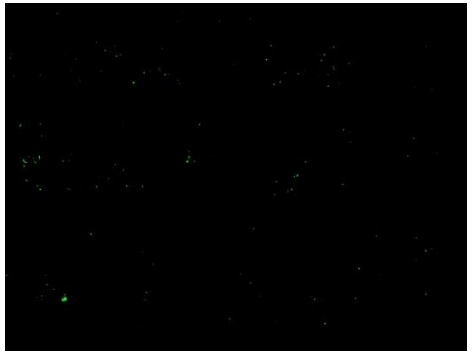
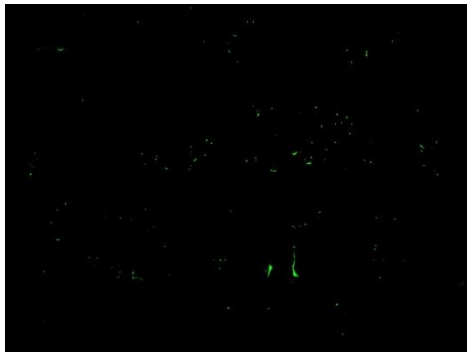
10

Percoll – Negative Selection

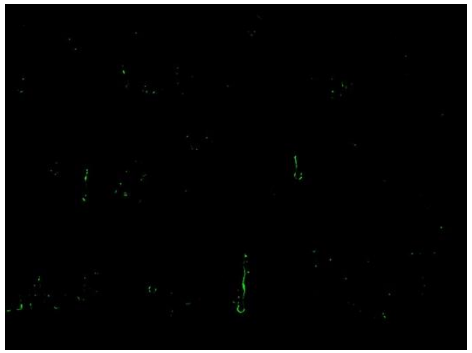
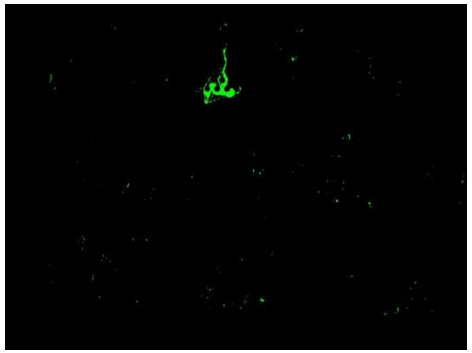
Percoll – Histopaque - Negative Selection

Percoll – Histopaque - Negative Selection

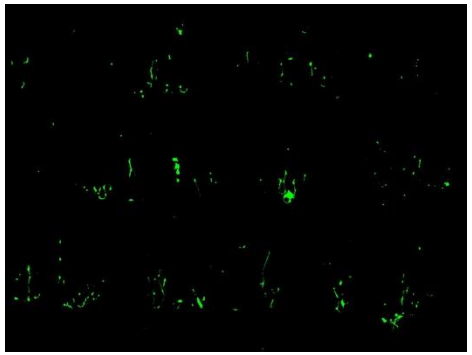
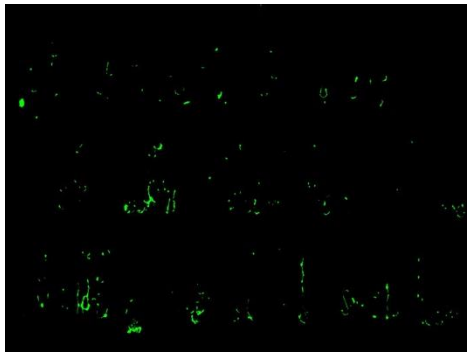
2025-07-09, Spontaneous Death, 24h

S. Aureus
ATCC 10390

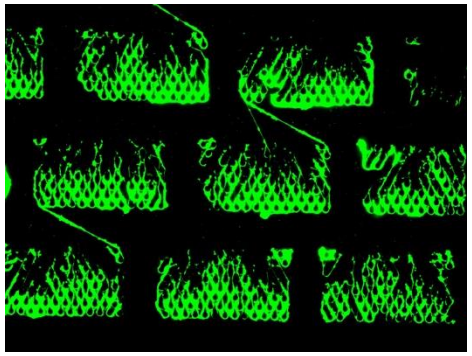



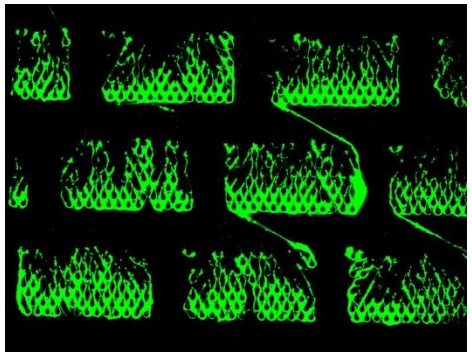
S. Aureus
ATTC BAA-1717

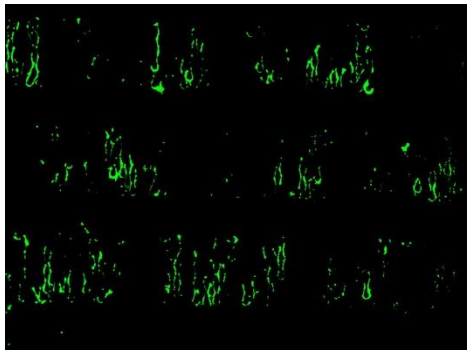
K12
ATCC MG1655 700926

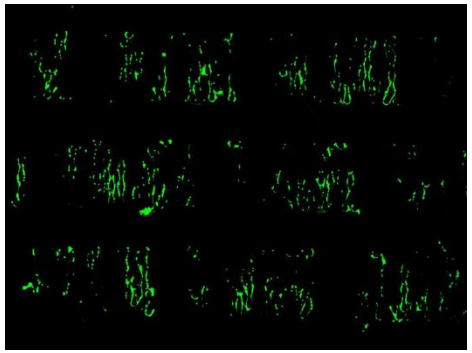
2025-07-10, Bacteria induced NETs



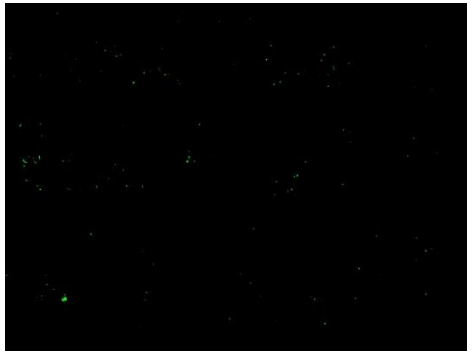
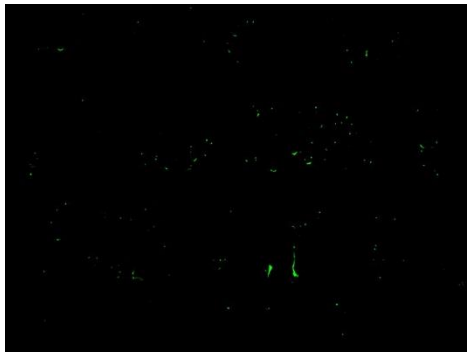


Bacillus cereus
ATCC 14579

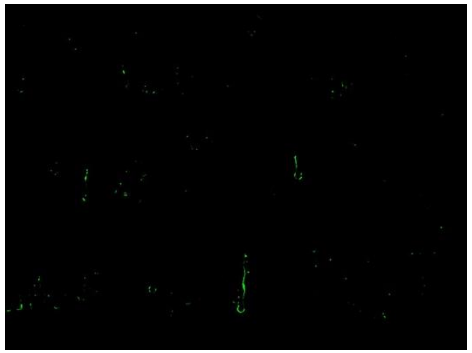
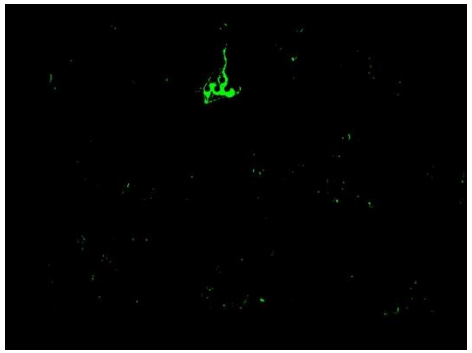




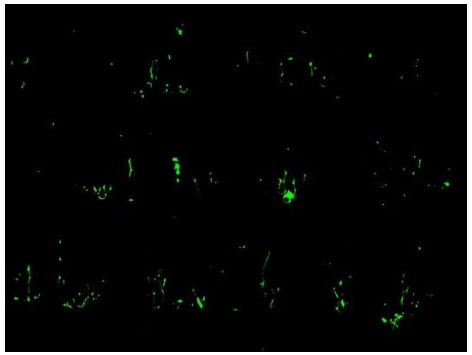
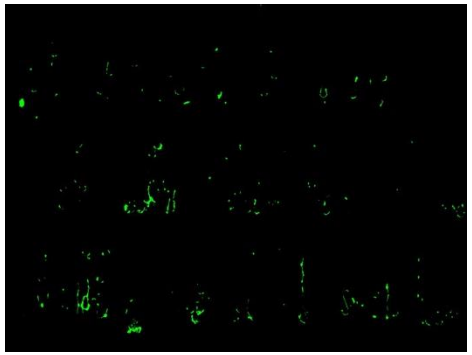
A7
ATCC 700928

S. Aureus
ATCC 10390

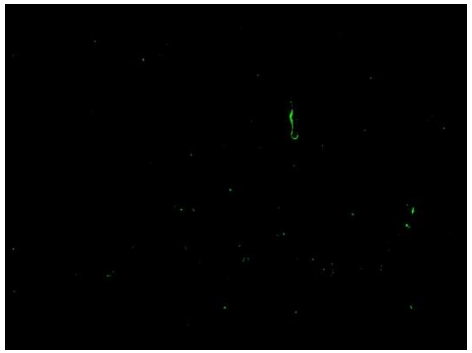
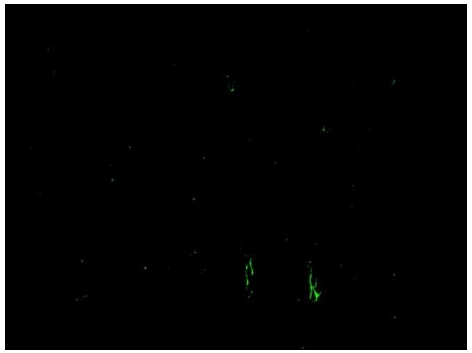
S. Aureus
ATTC BAA-1717

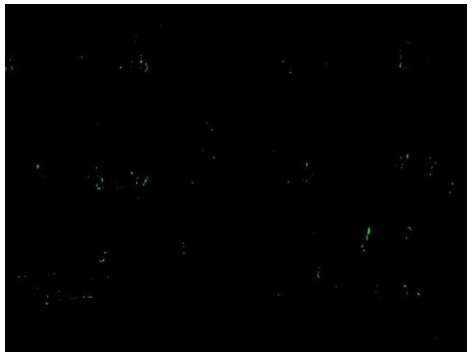
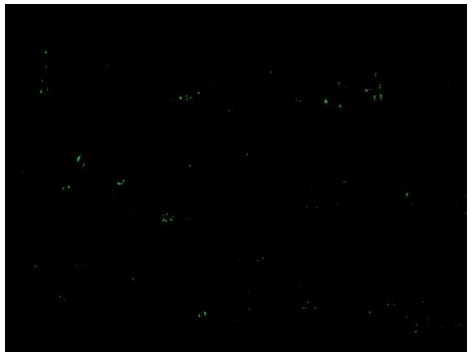
K12
ATCC MG1655 700926

2025-07-10, Bacteria induced NETs, 4h, 1% FBS


S. Aureus
ATCC 10390

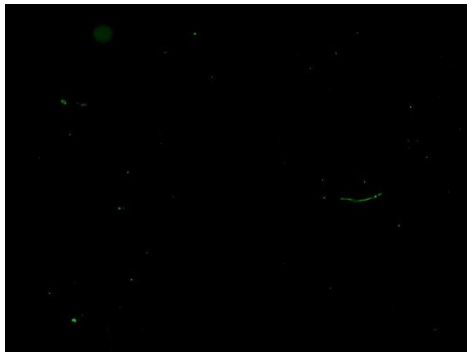



S. Aureus
ATTC BAA-1717

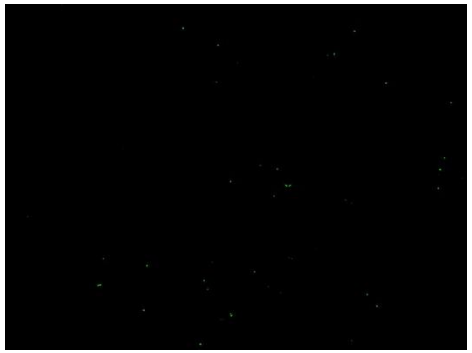



K12
ATCC MG1655 700926

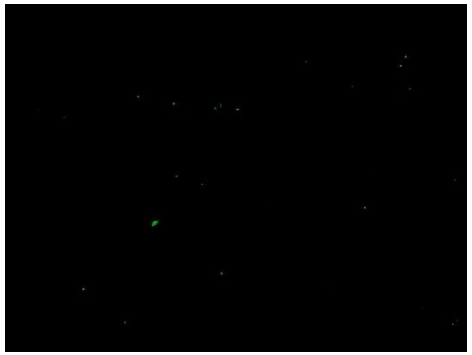
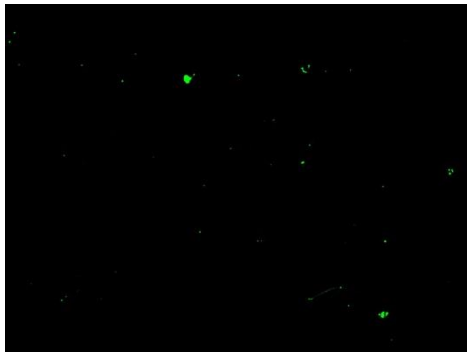
2025-07-15, Bacteria induced NETs, 4h, 1% FBS

S. Aureus
ATCC 10390

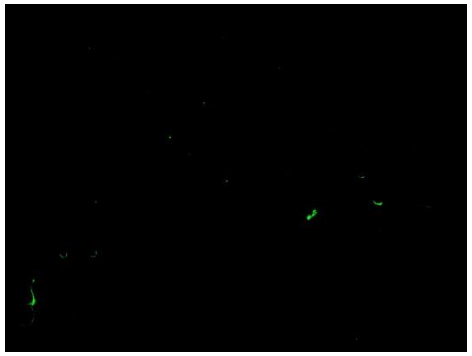



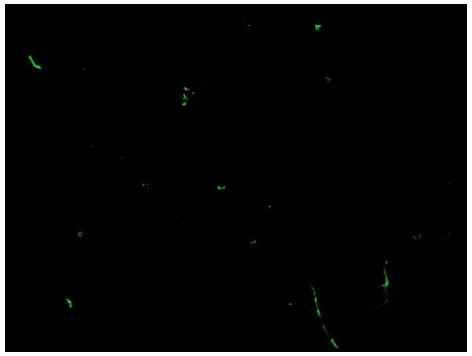
S. Aureus
ATTC BAA-1717

K12
ATCC MG1655 700926

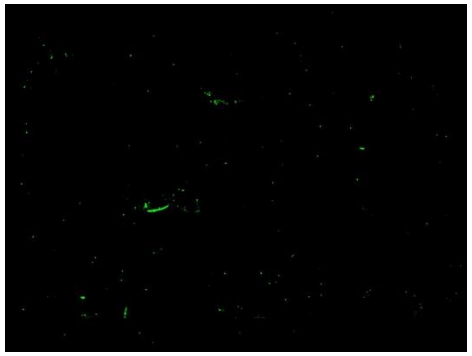
2025-07-15, Bacteria induced NETs, 8h, 1% FBS

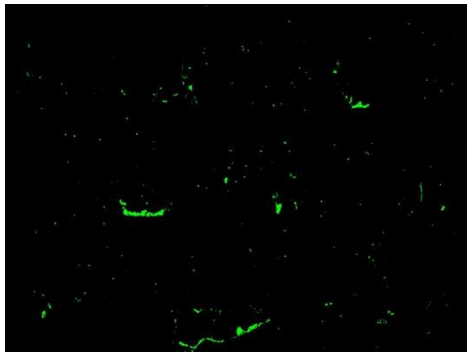




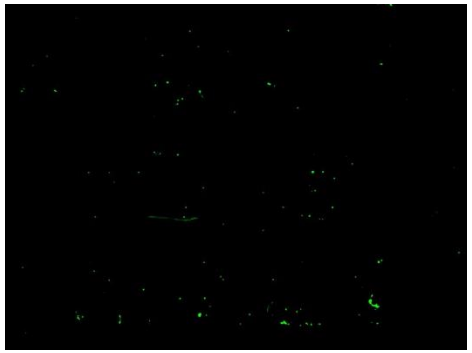
S. Aureus
ATTC BAA-1717

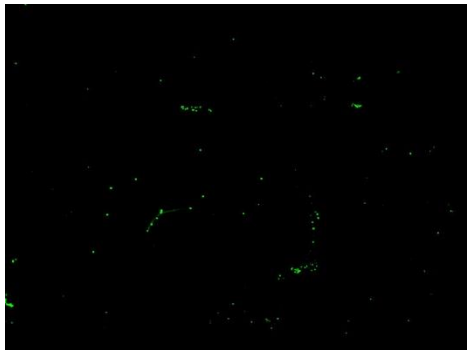
2025-07-15/16, Bacteria induced NETs, 24h, 1% FBS





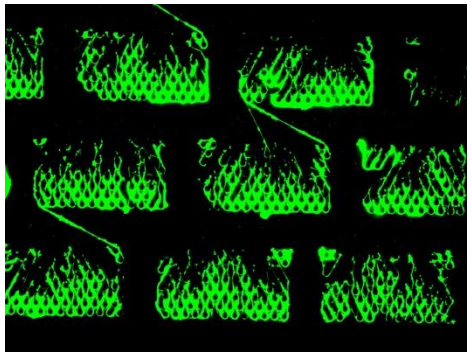
S. Aureus
ATCC 10390

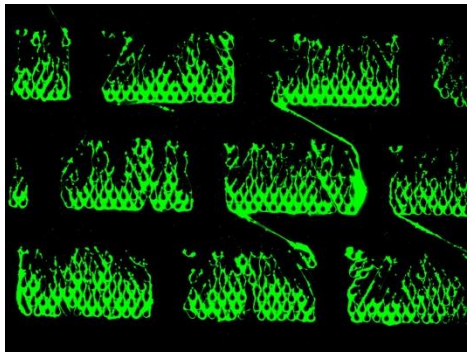




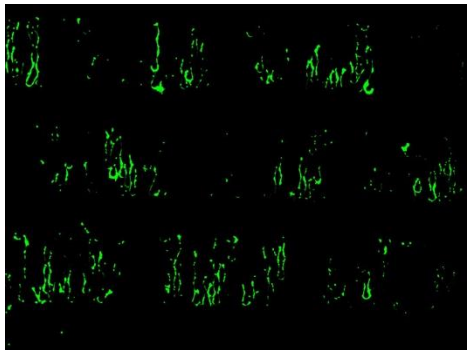
S. Aureus
ATTC BAA-1717

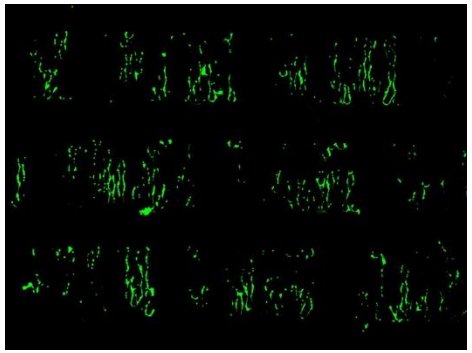
2025-07-17, Bacteria induced NETs, 4h, 1% FBS





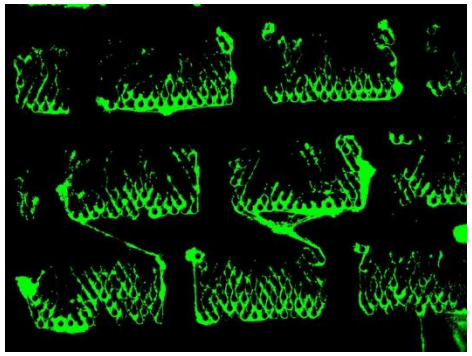
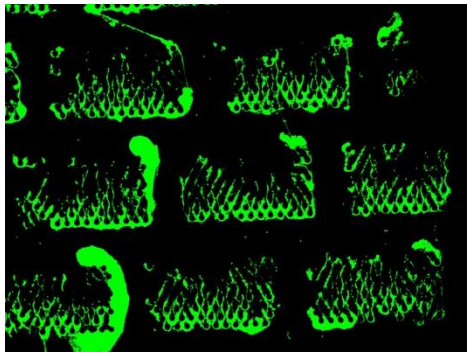
Bacillus cereus
ATCC 14579



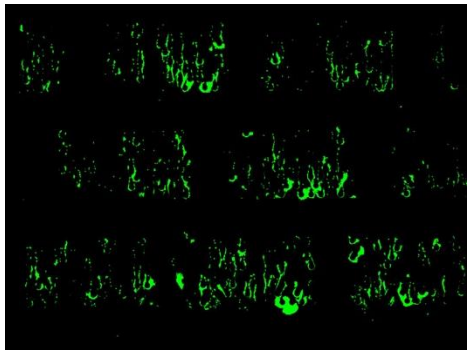
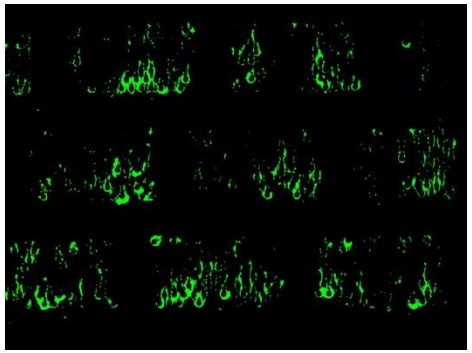


A7
ATCC 700928

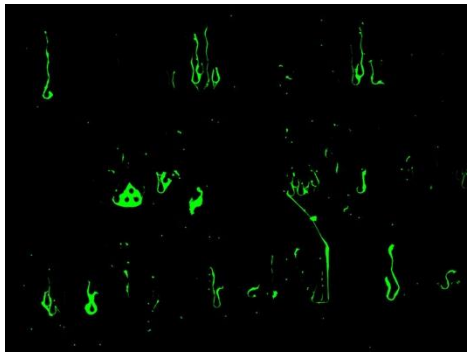
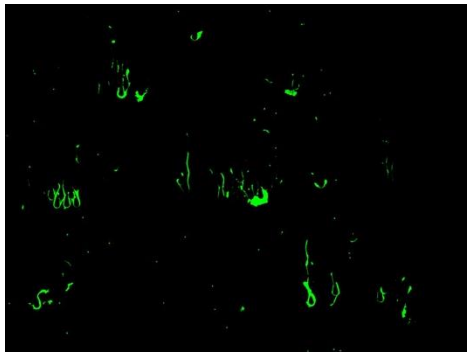
2025-07-10, Bacteria induced NETs, 4h, 1% FBS

Bacillus cereus
ATCC 14579

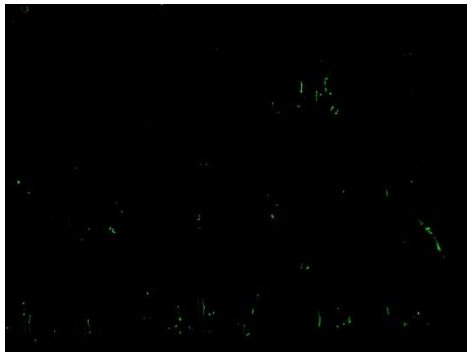



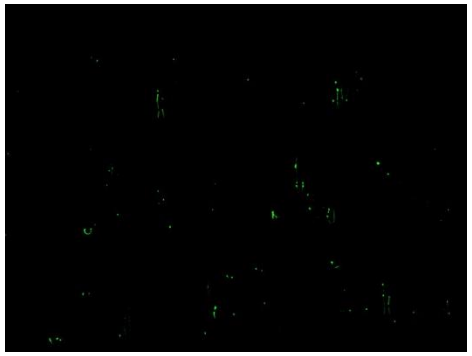
A7
ATCC 700928

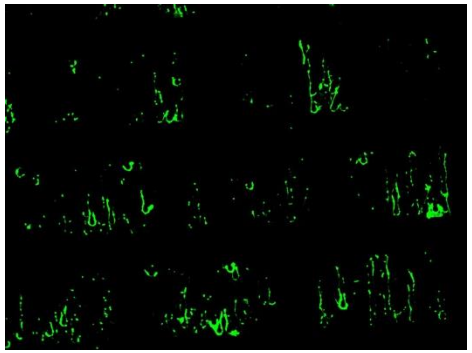
Triton

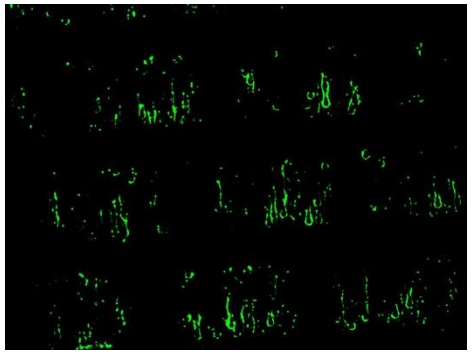
2025-07-15, Bacteria induced NETs, 4h, 1% FBS





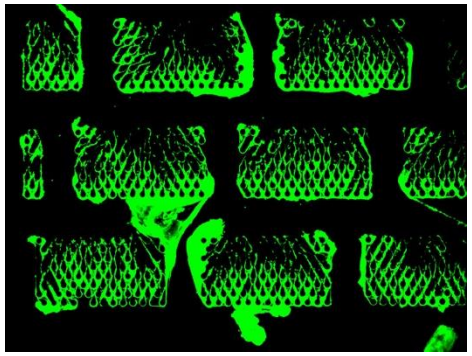
Blank

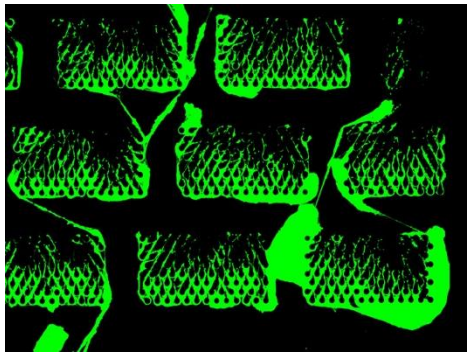




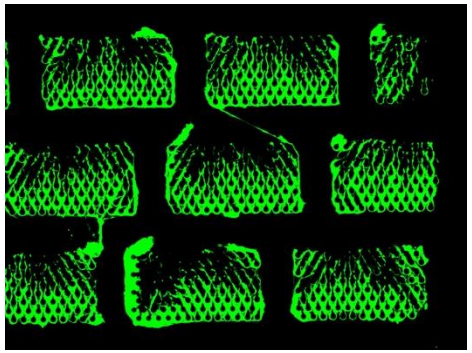
A7
ATCC 700928

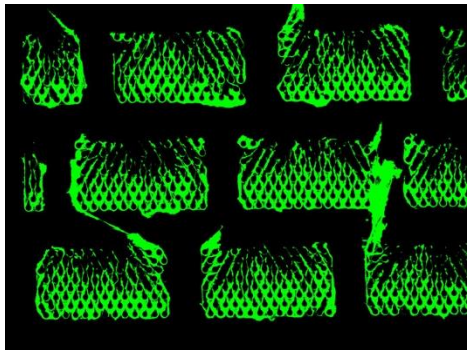
2025-07-15, Bacteria induced NETs, 8h, 1% FBS





Bacillus cereus
ATCC 14579



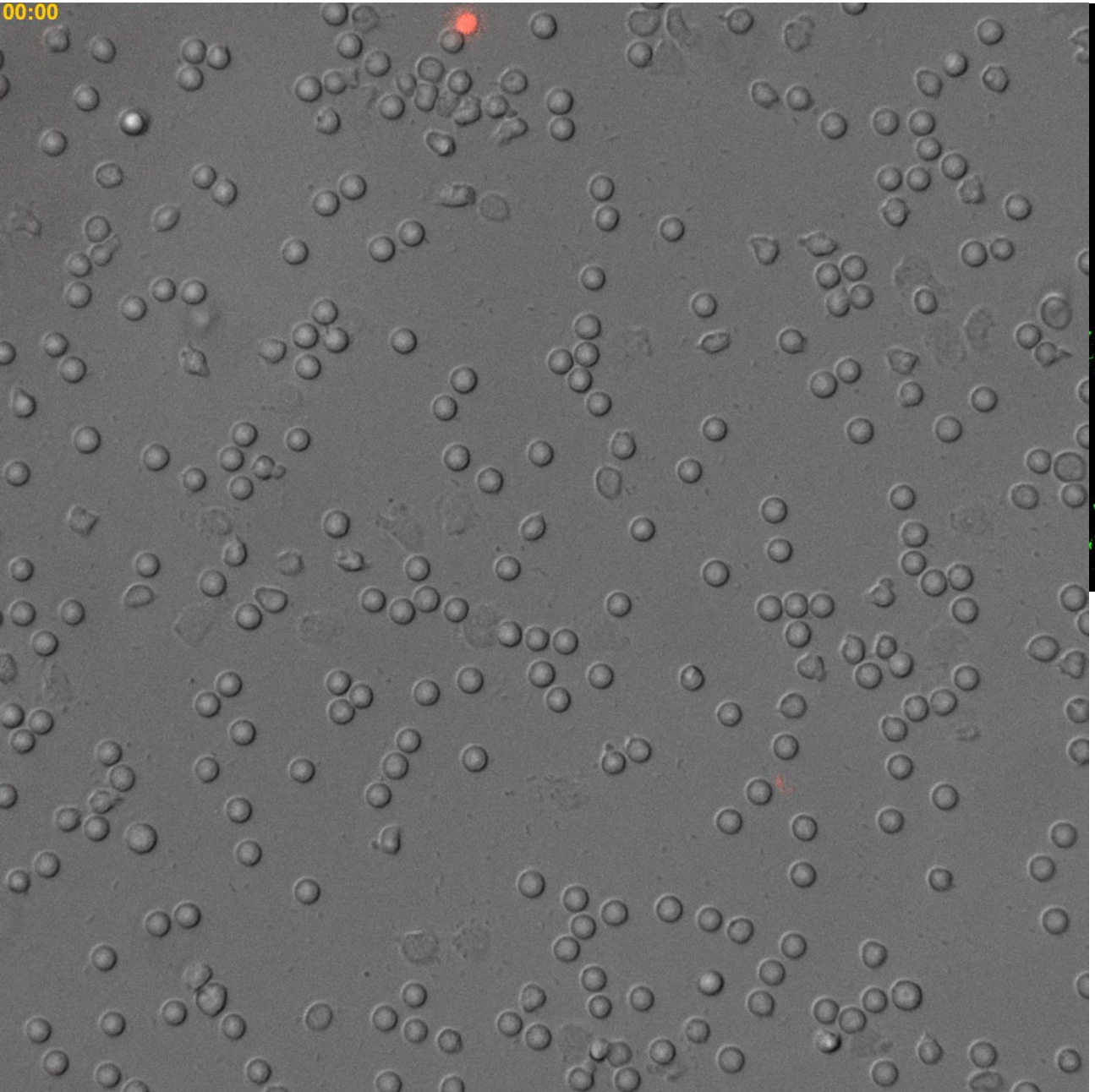


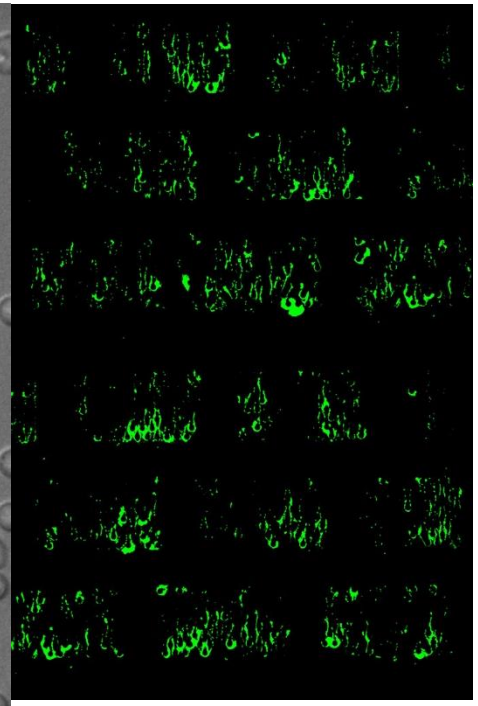
Ionomycin

2025-07-17, Bacteria induced NETs**, 4h, 10% FBS**

2025-07-15

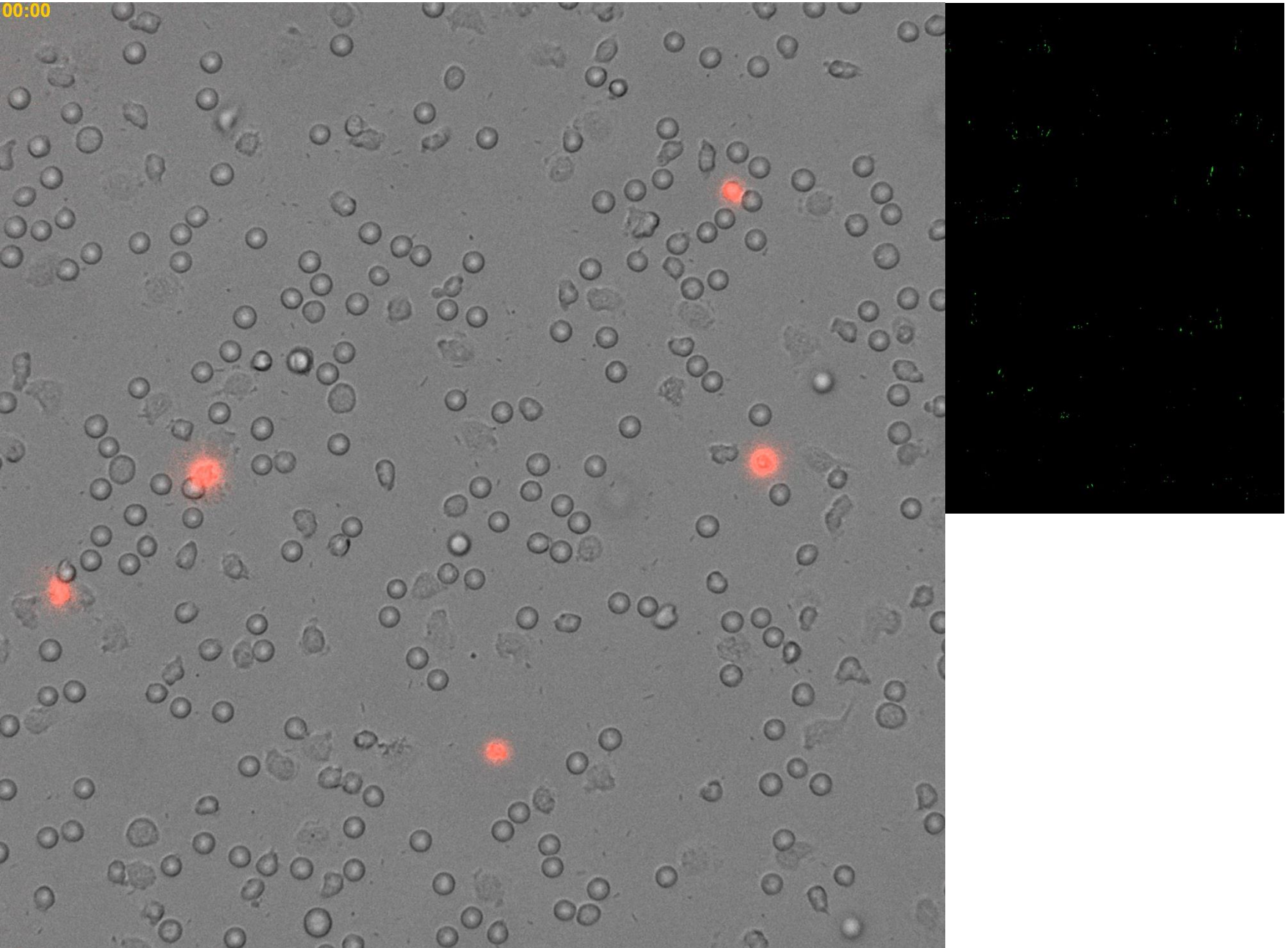
A7





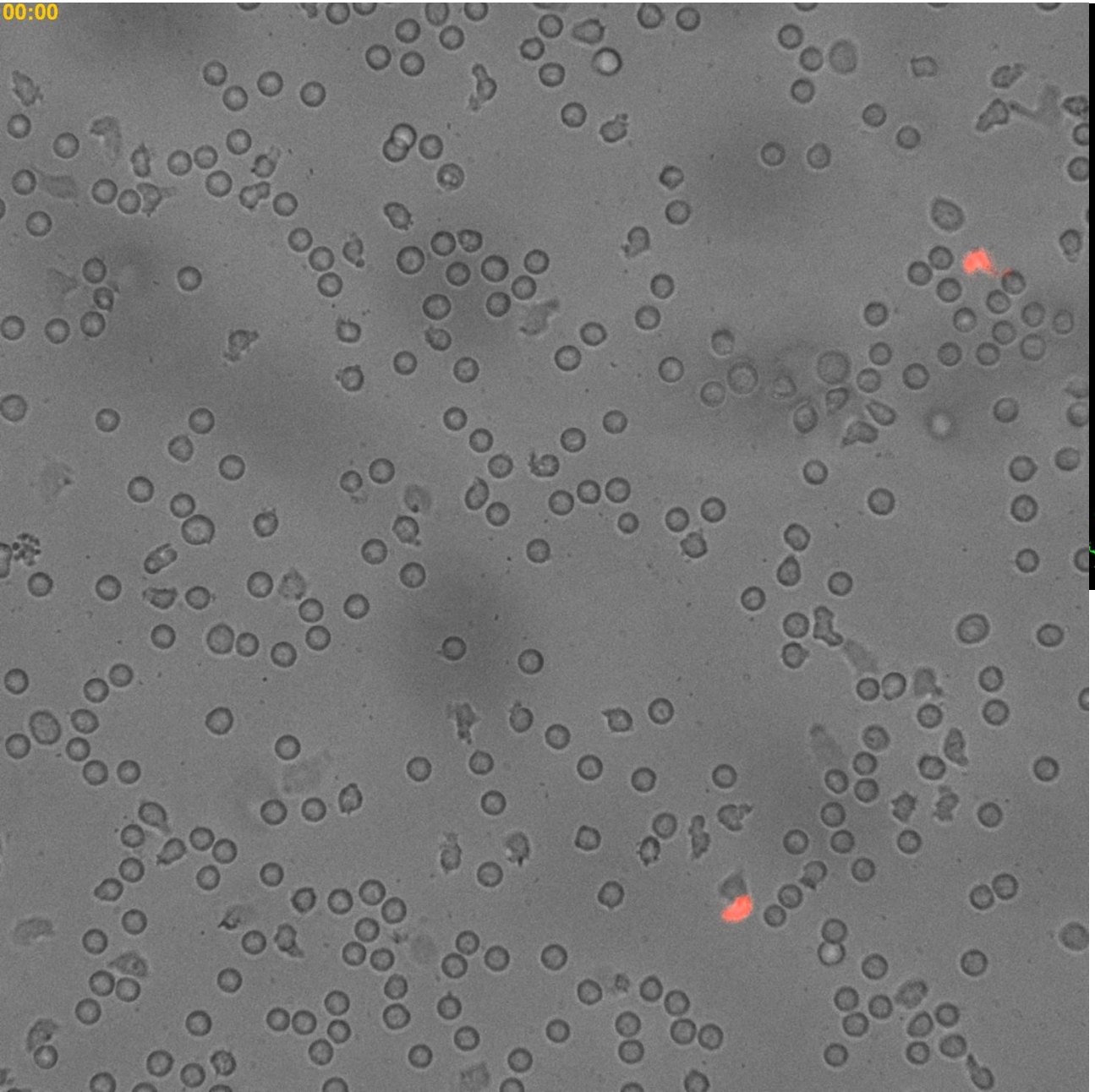
2025-07-15

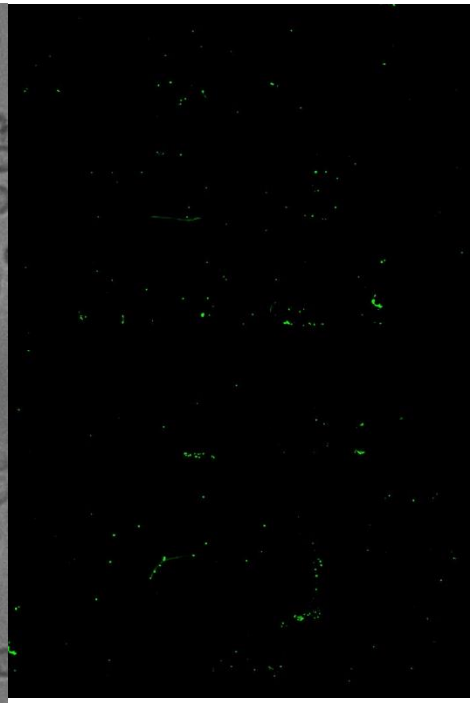
K12



2025-07-15

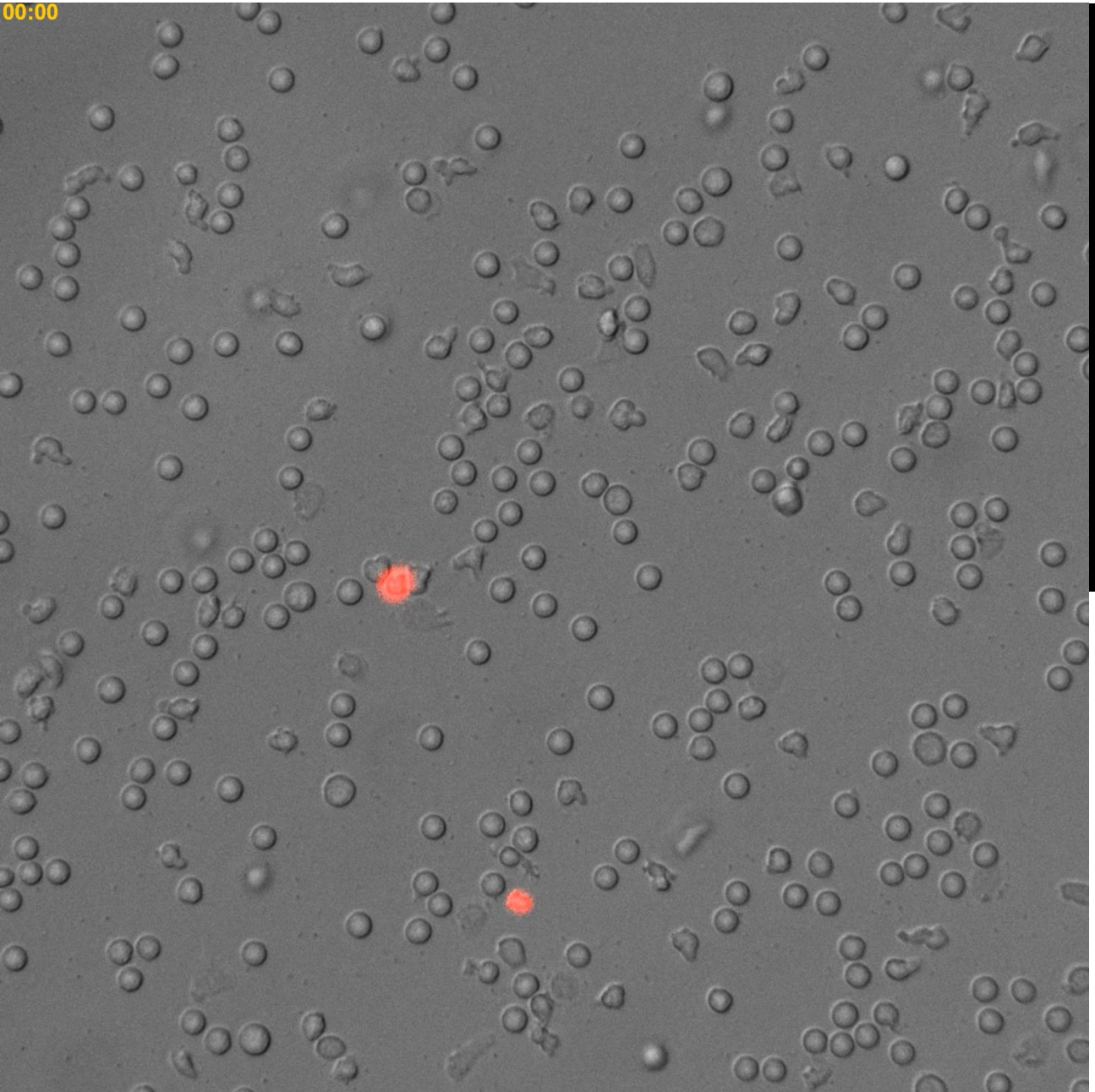
Sa

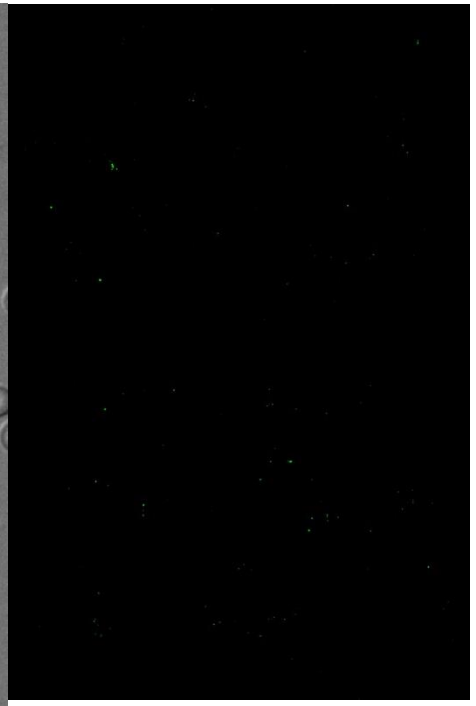




2025-07-15

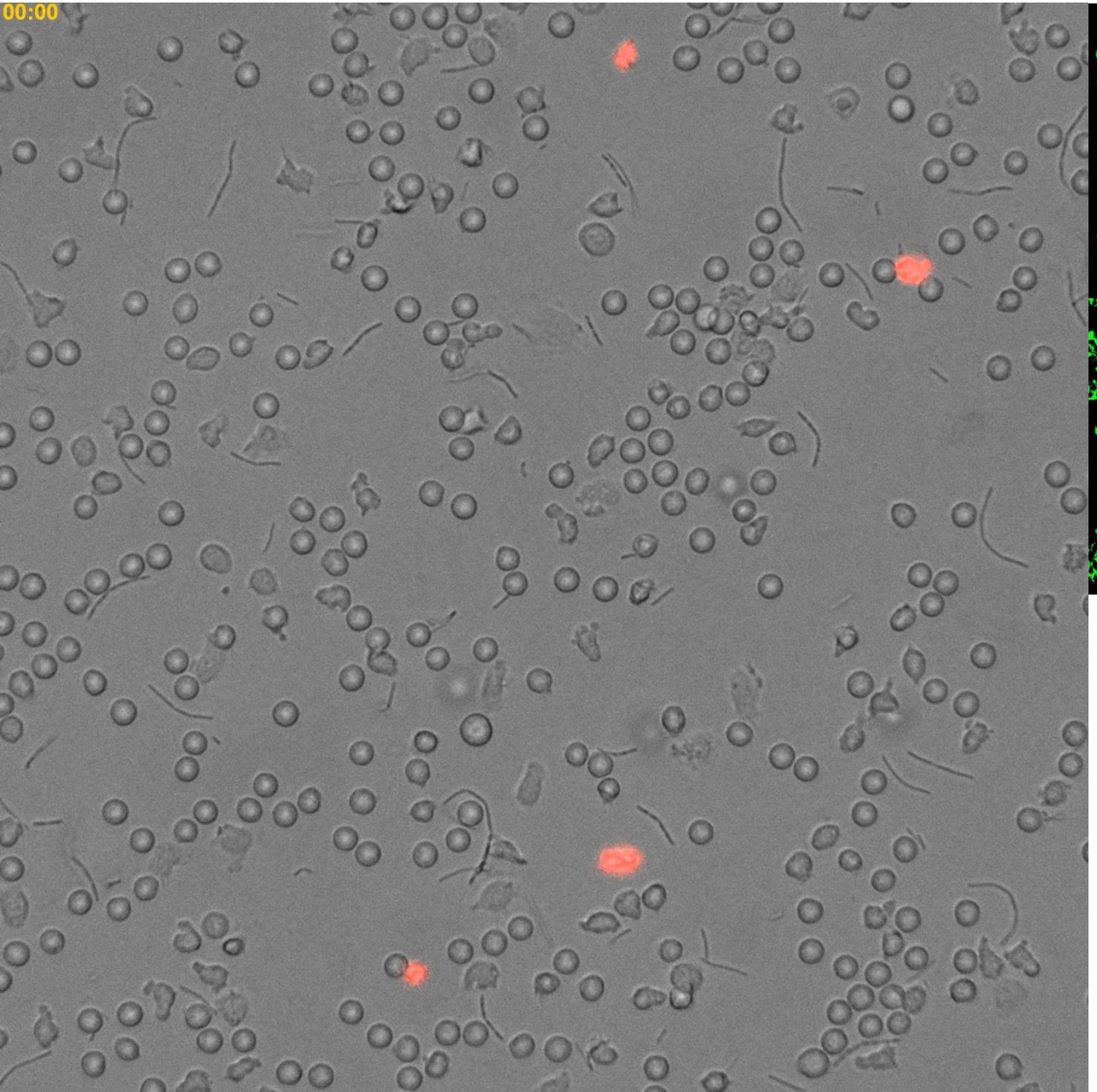
10390

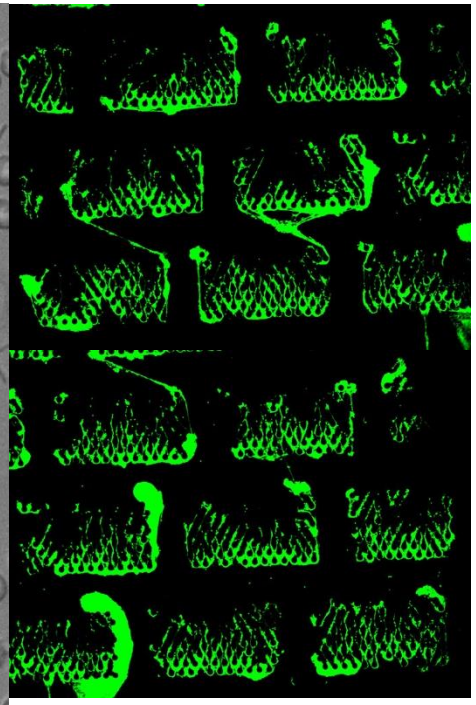




2025-07-15

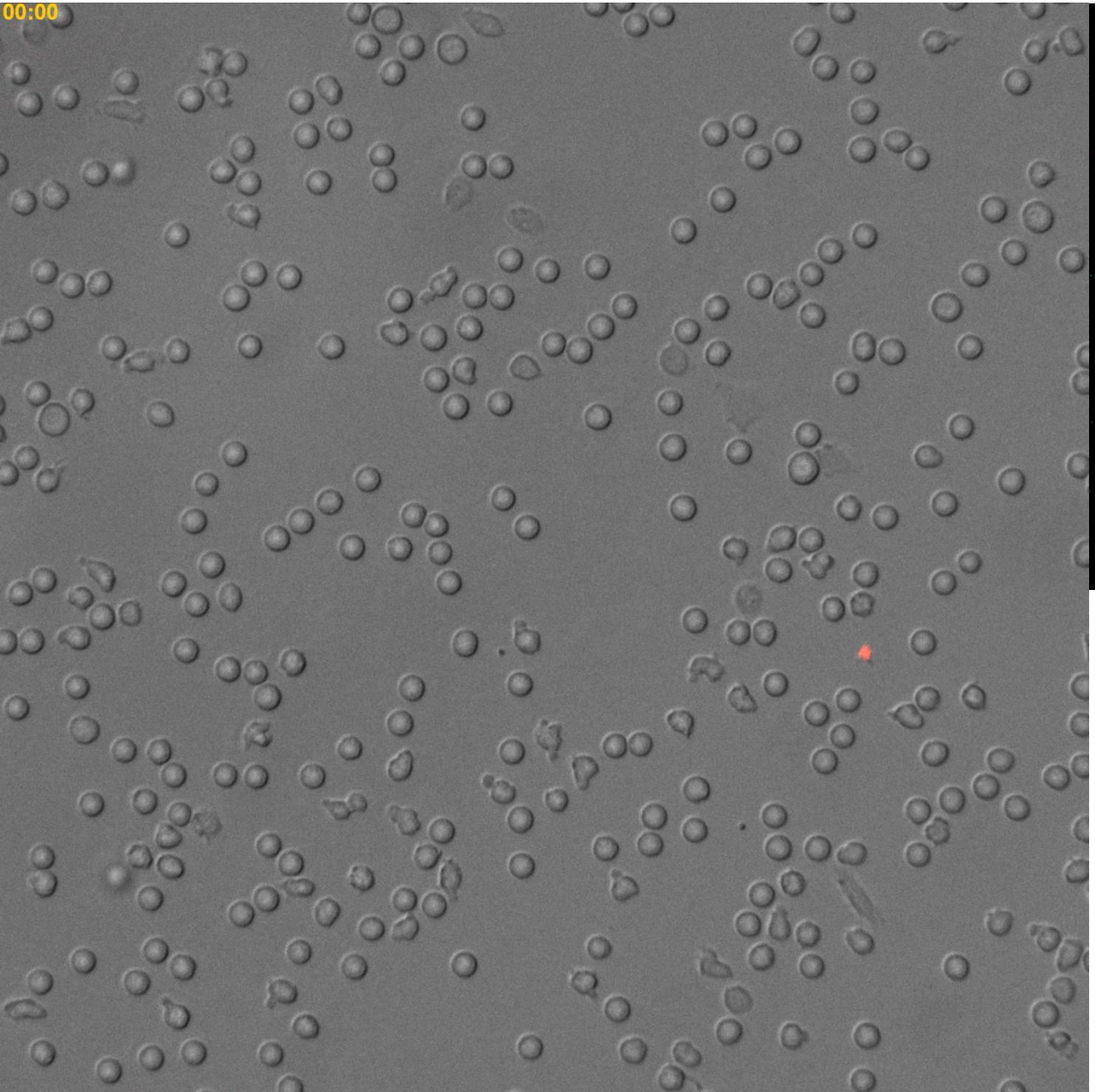
Bacillus

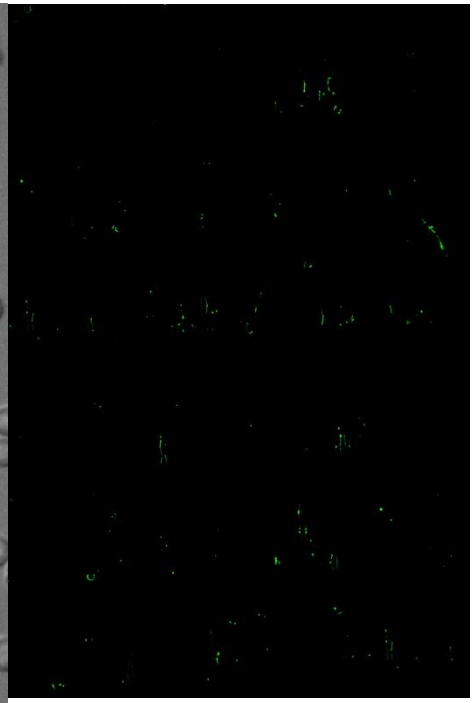




2025-07-15

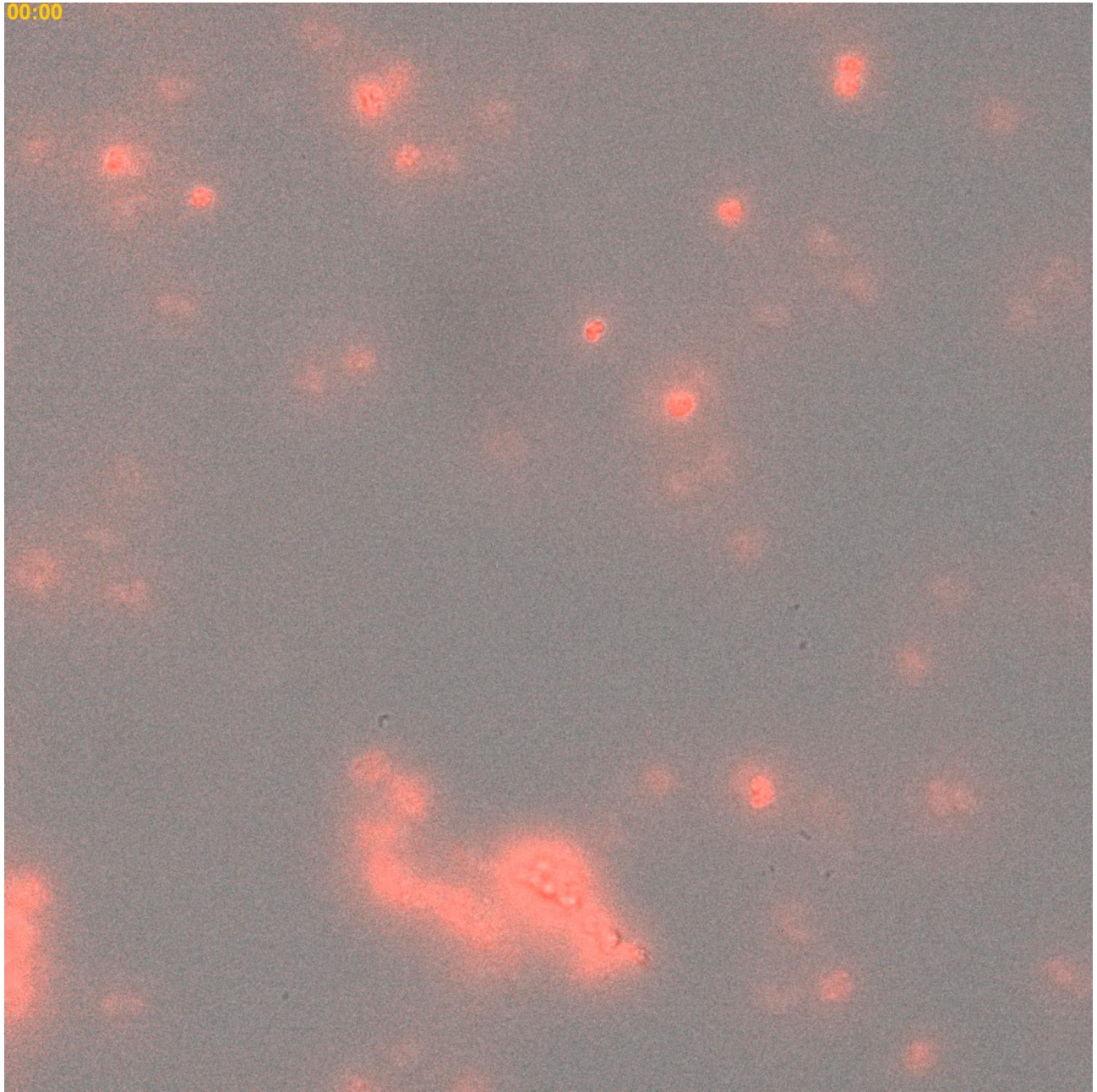
Blank (8h NET)





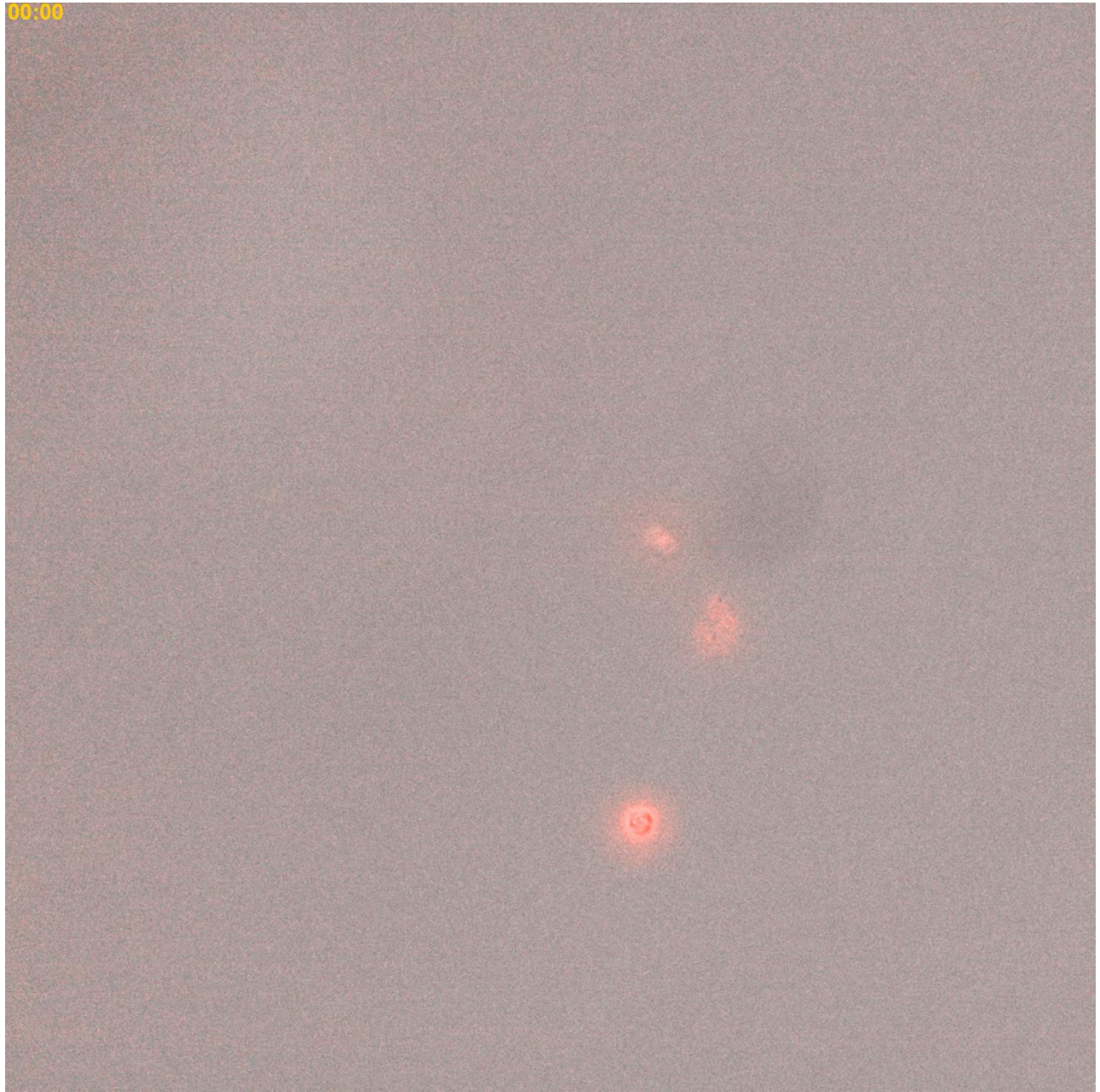
2025-07-15

Triton



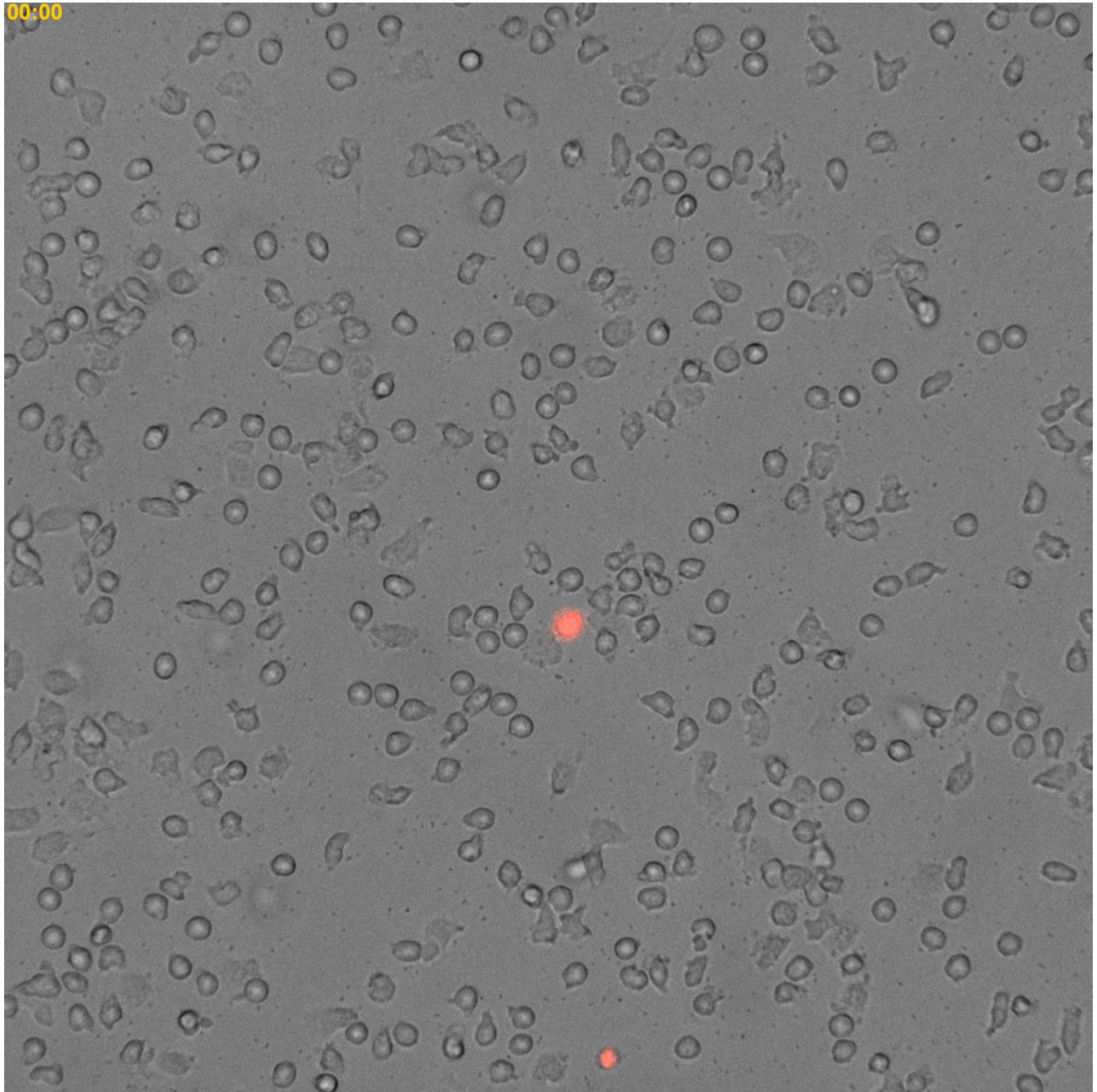
2025-07-15

Lysis Buffer



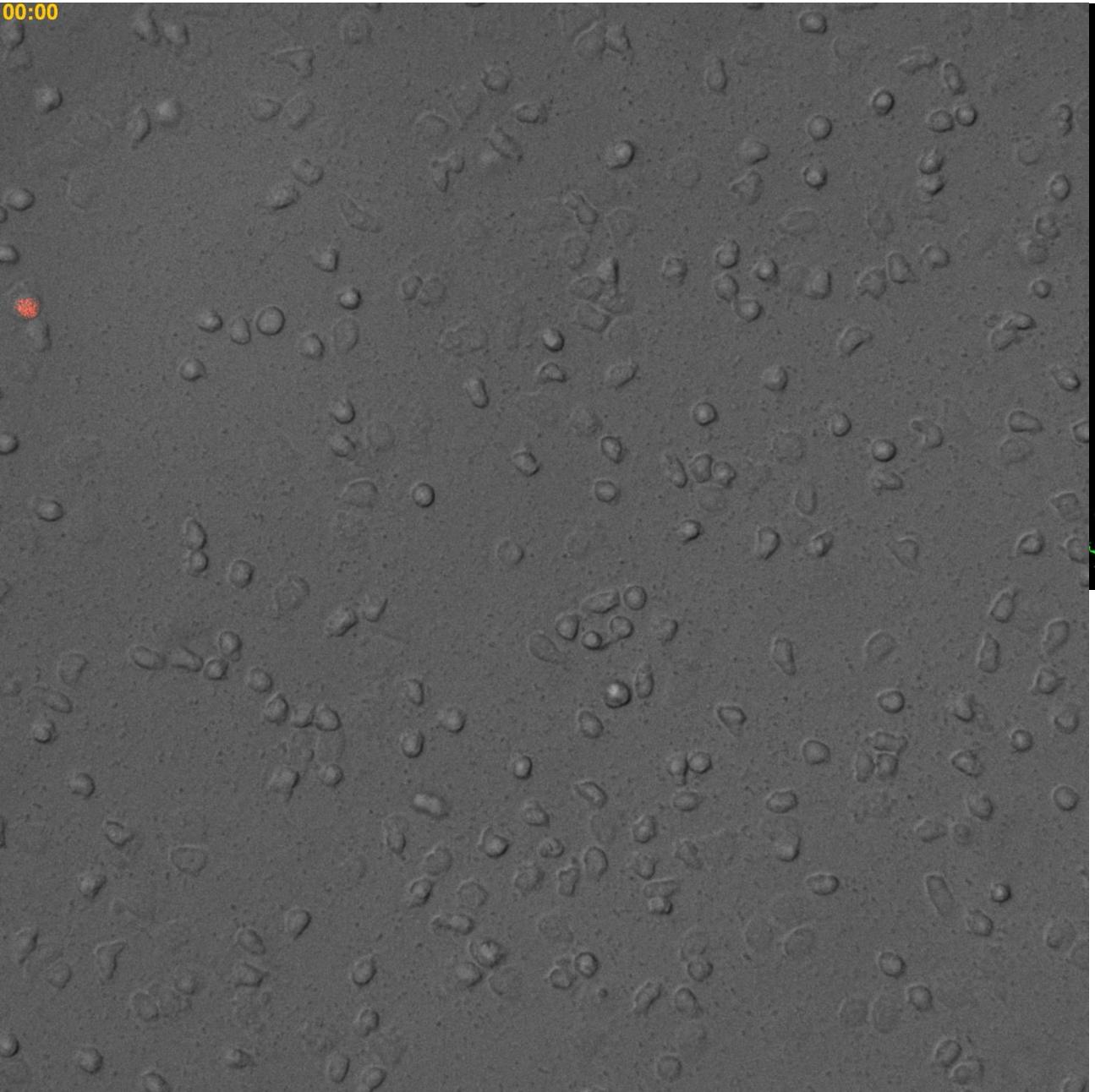
2025-07-17

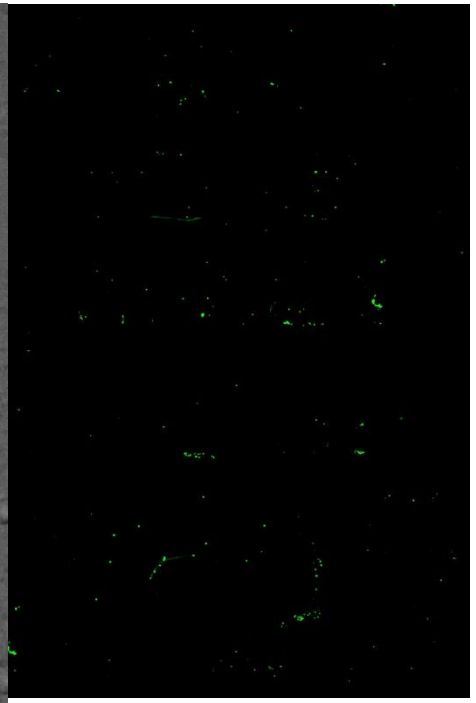
Sa MOI=50



2025-07-17

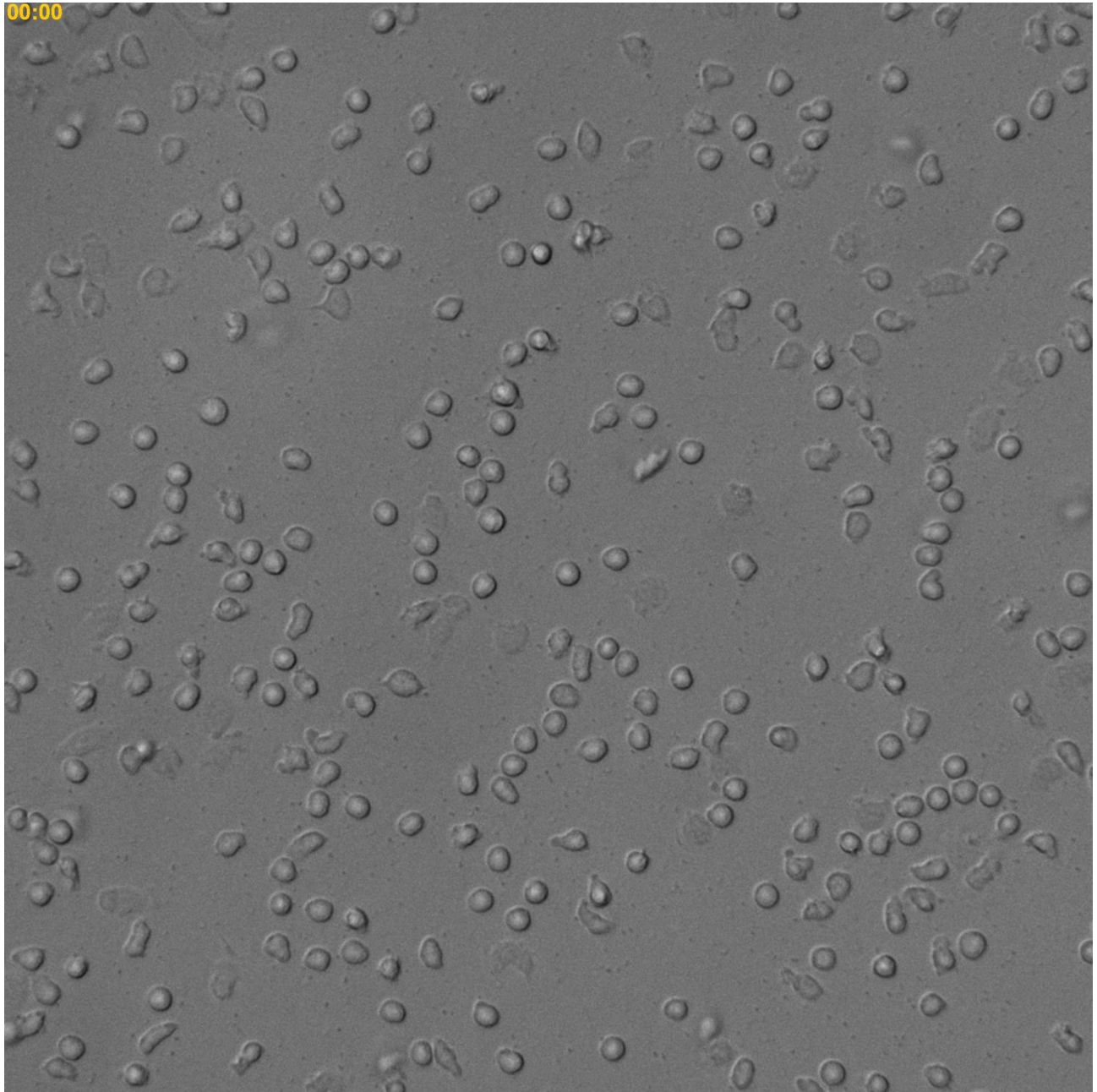
Sa MOI=200





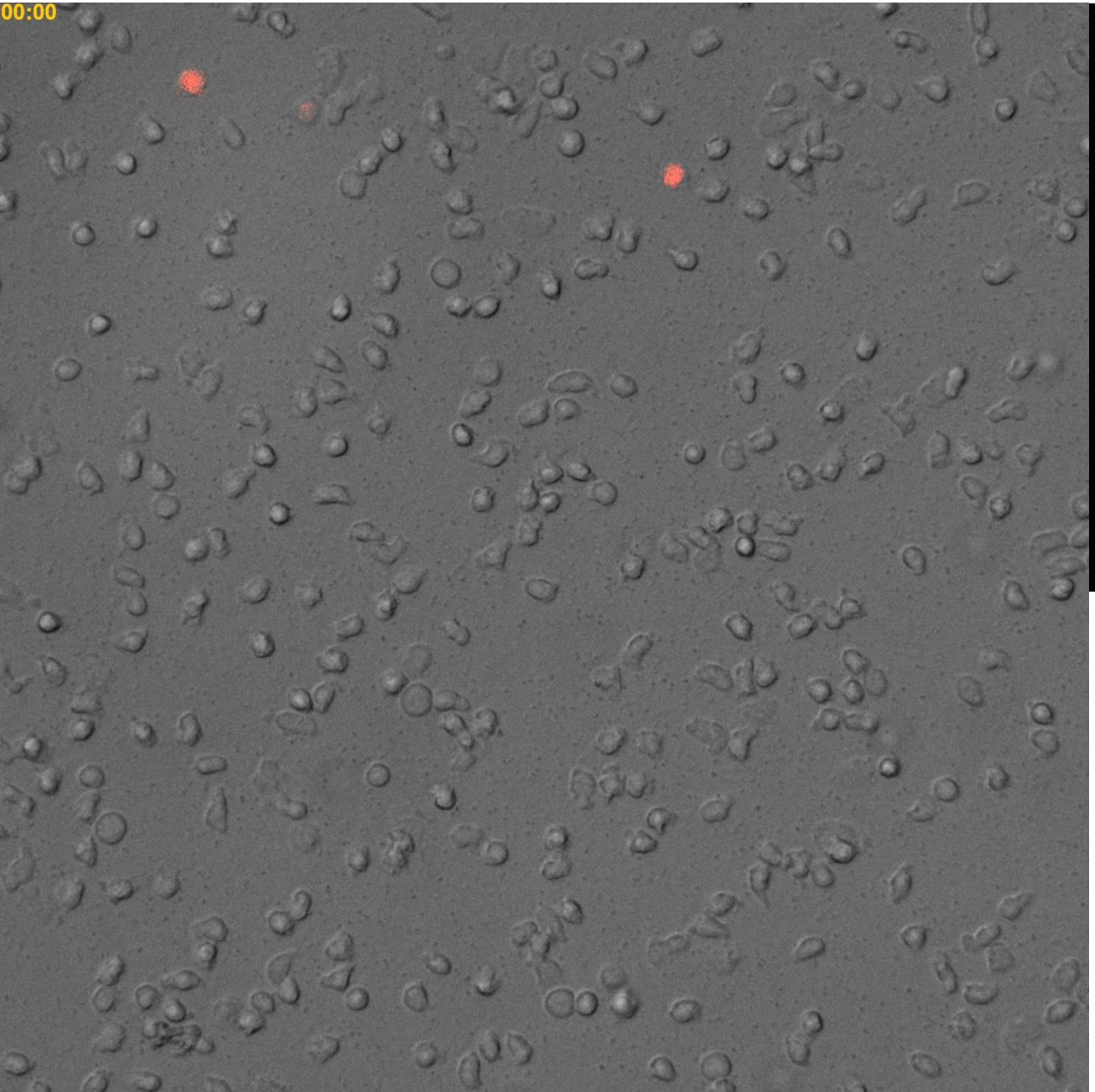
2025-07-17

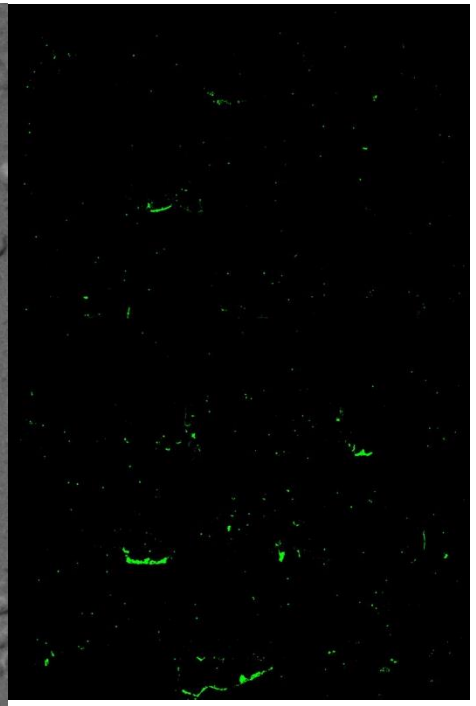
10390 MOI=35



2025-07-17

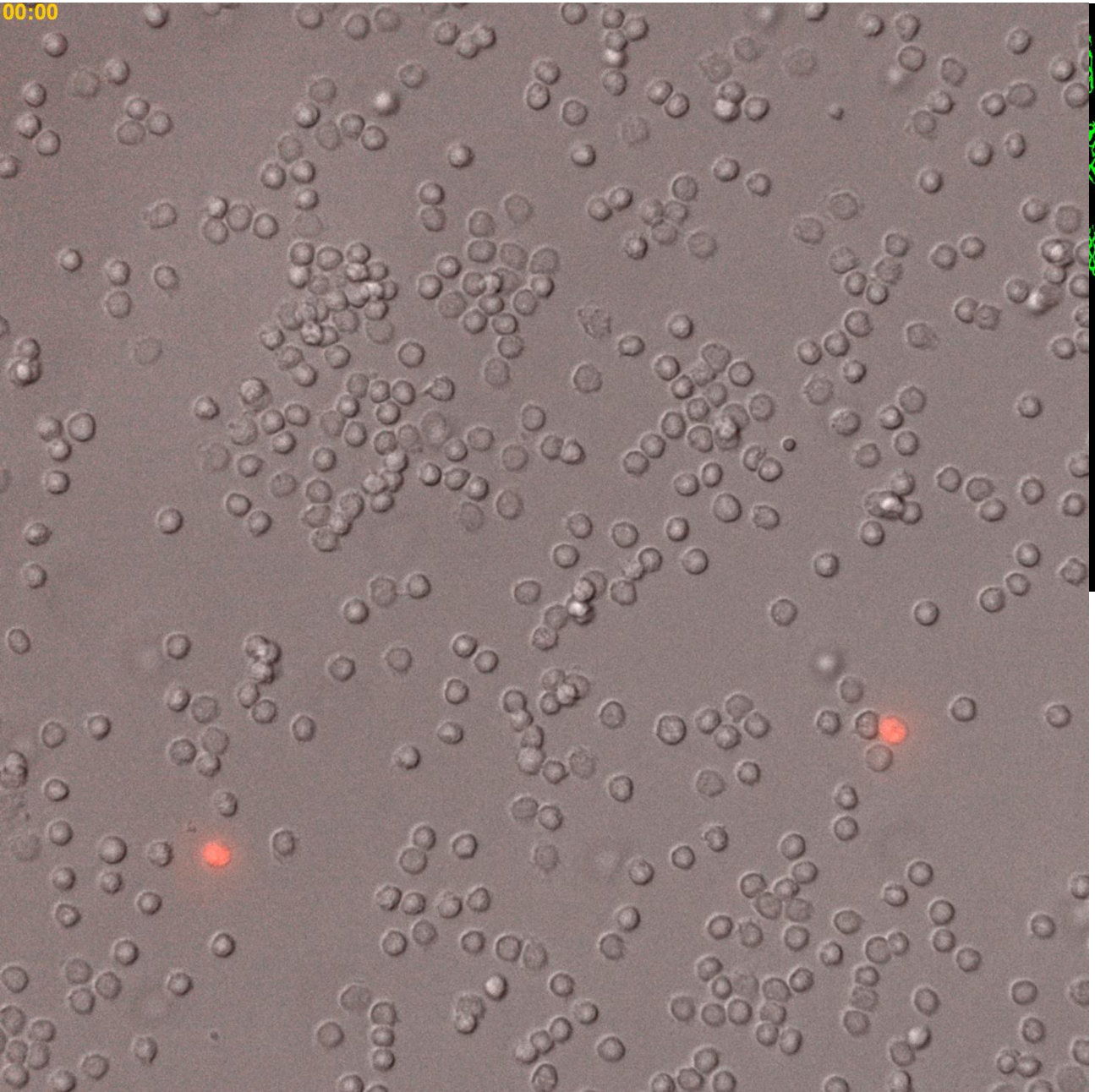
10390 MOI=140

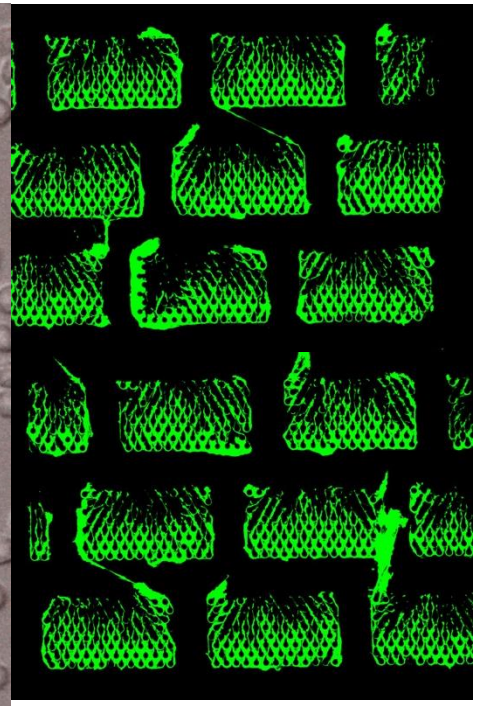




2025-07-17

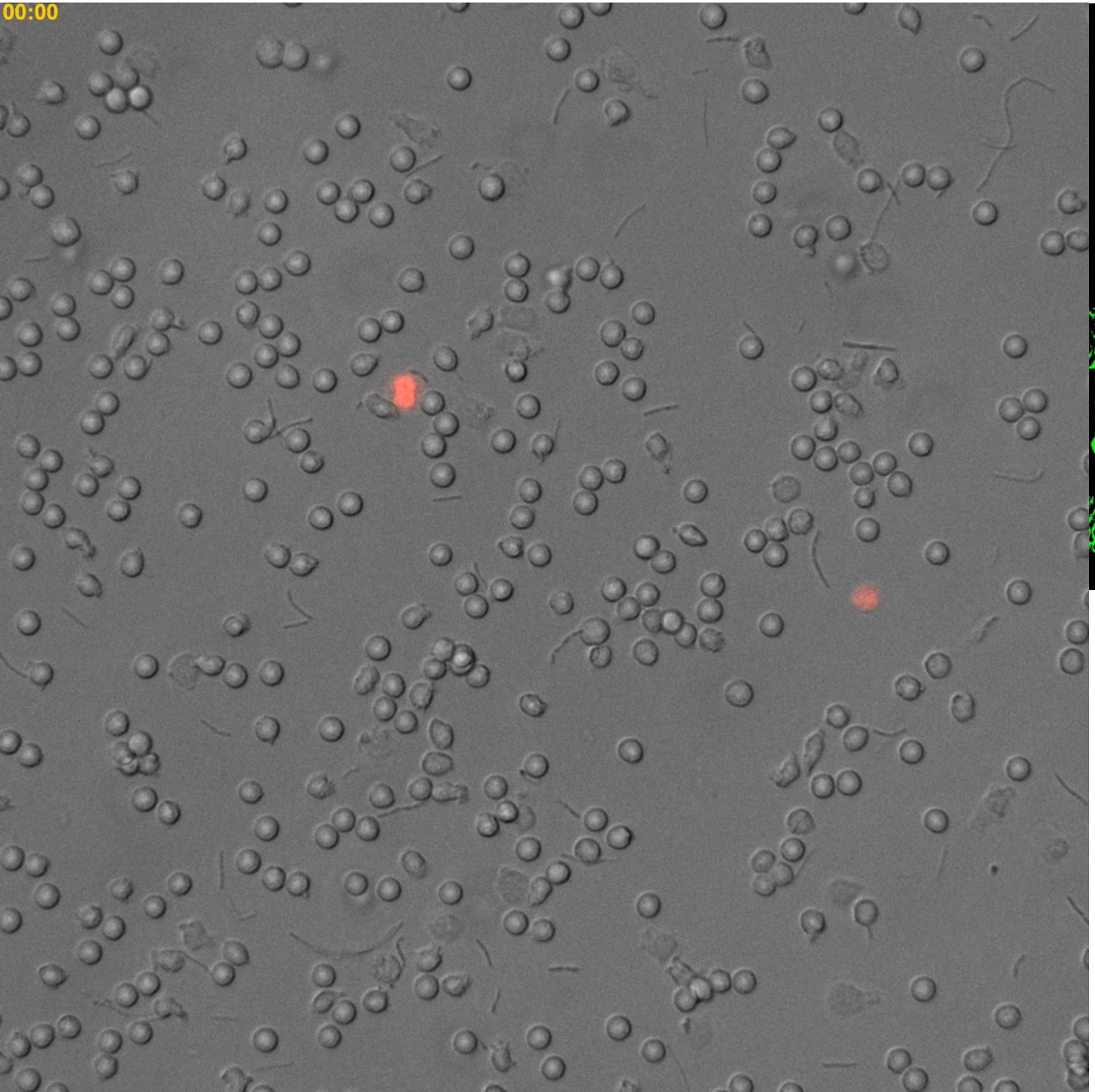
Ionomycin

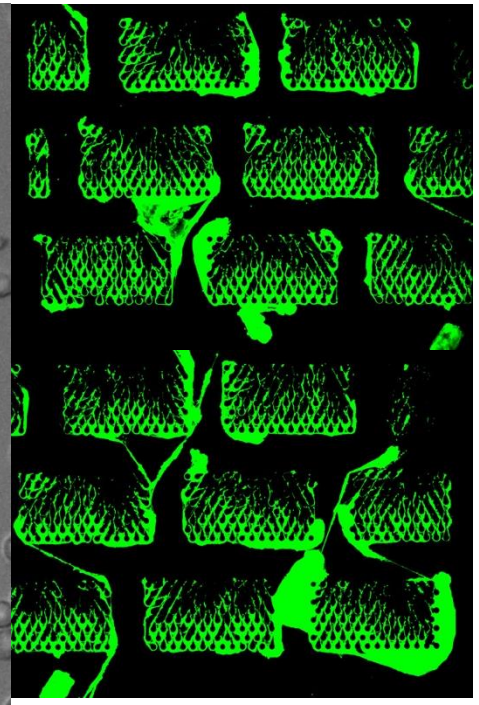




2025-07-17

Bacillus MOI=5





Protocol Change

After we get our NETs from ionomycin:

We add 400 μL of bacteria at a concentration of 5×10^{8}/mL through the device.

Then we wash it with 200 μL of PBS, repeated 8 times.

Add 50 μL of DNase through the device.

Incubate the device at 37°C for 30 minutes.

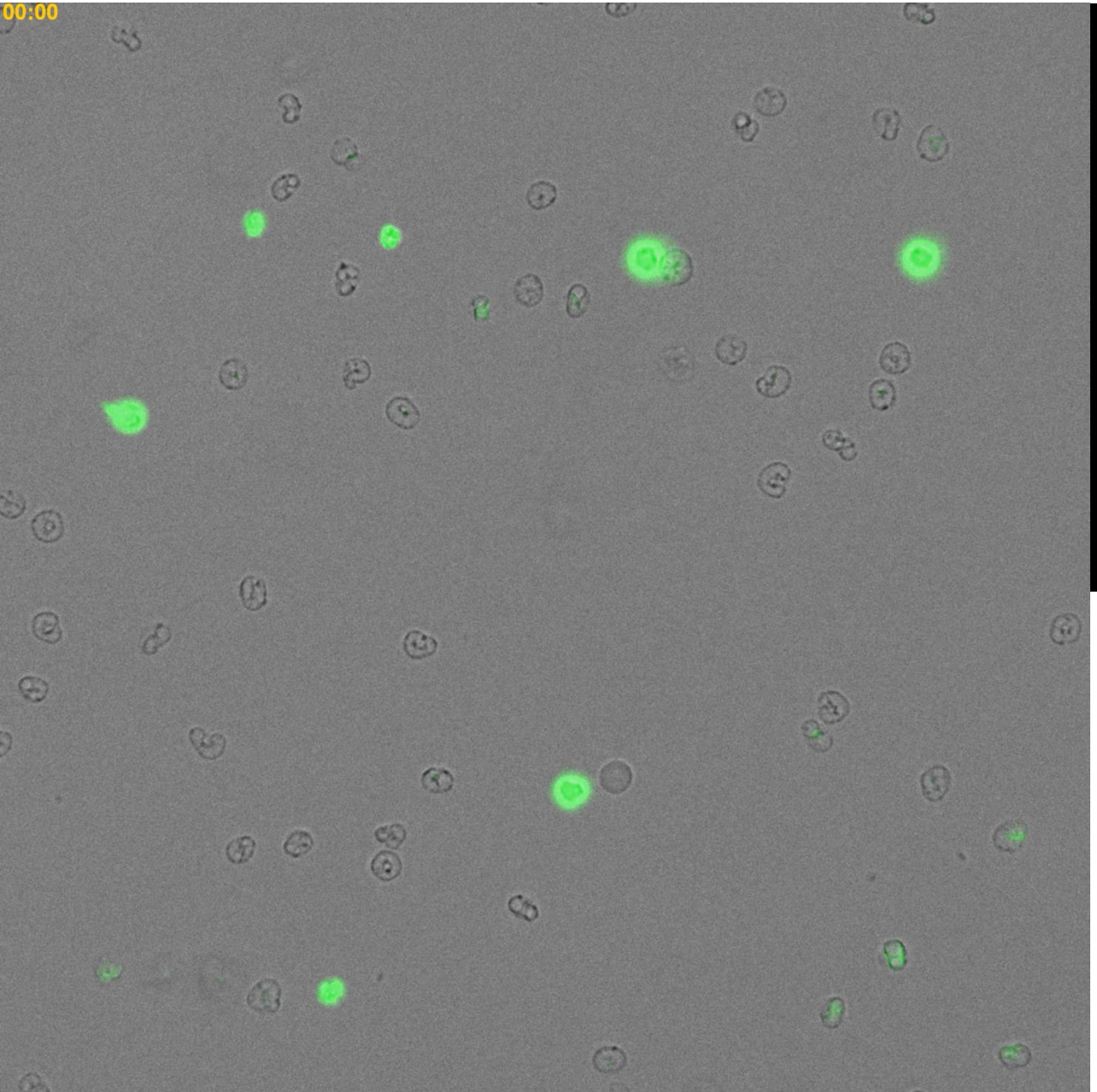
Place the device under the microscope.

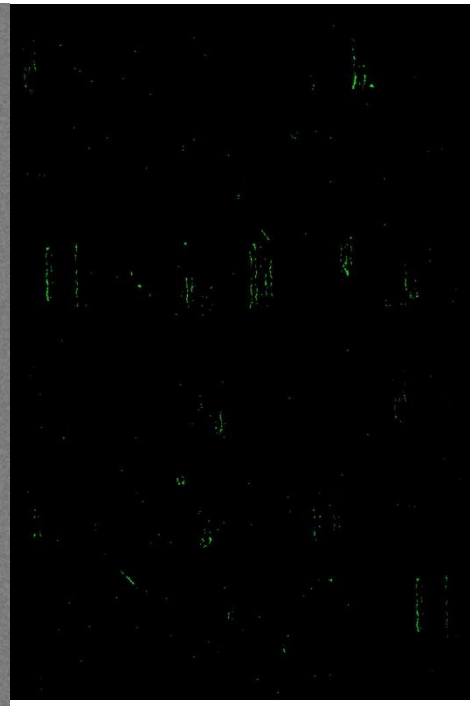
Use 1 mL of PBS to collect the bacteria from the device.

Dilute the collected bacteria and spread on an agar plate for counting.

2025-07-22

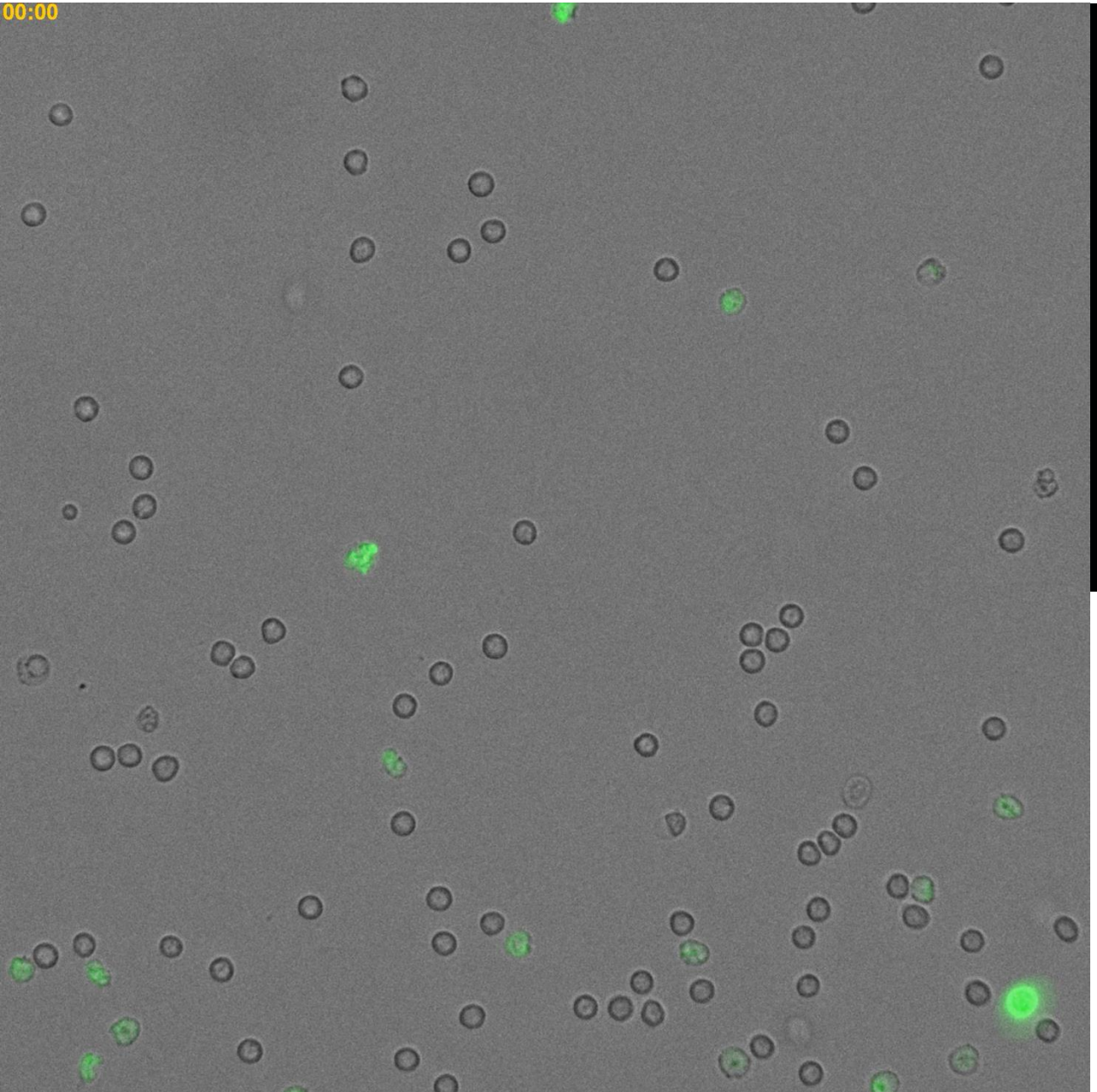
Triton

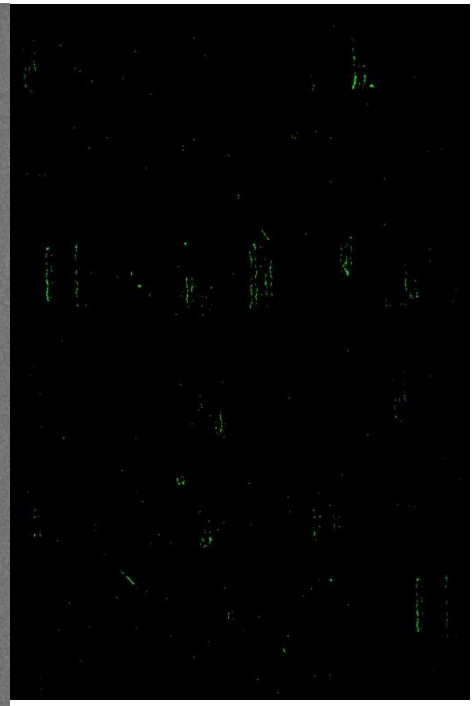


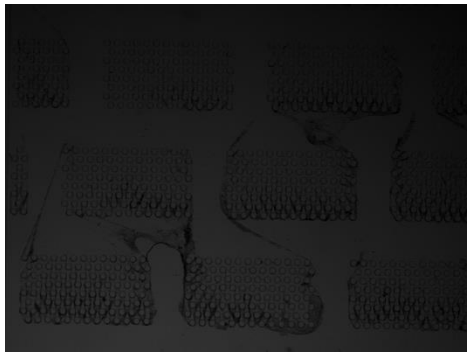


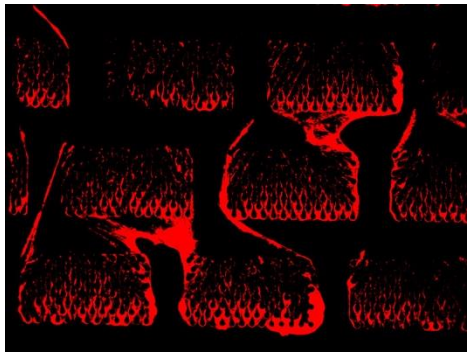
2025-07-22

Triton

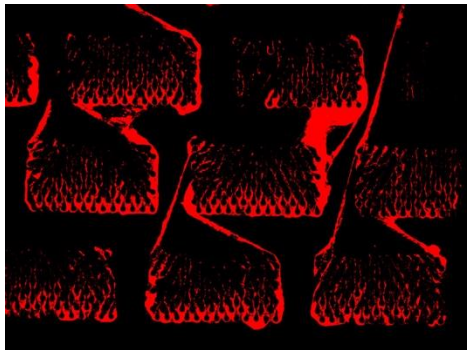








Ionomycin View 1

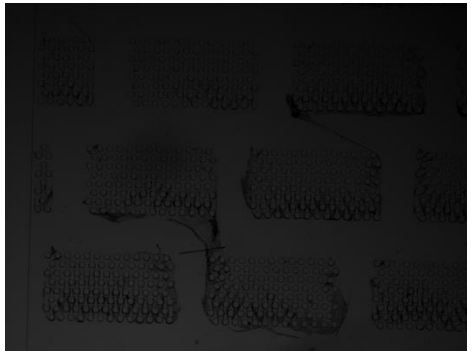
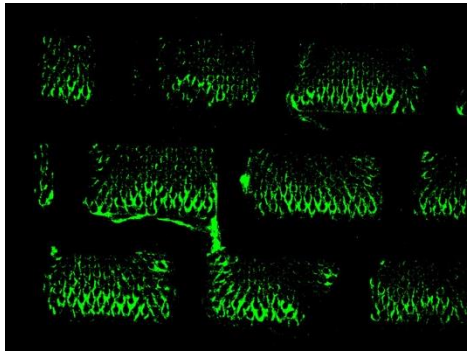
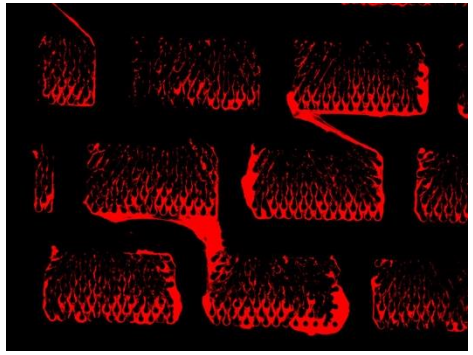


Ionomycin View 2

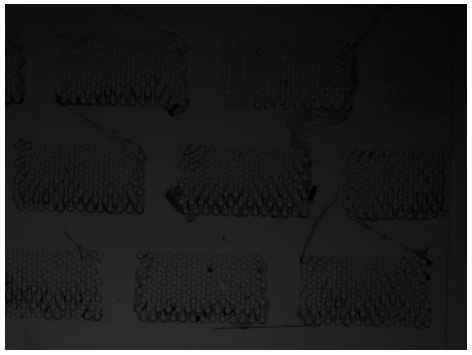
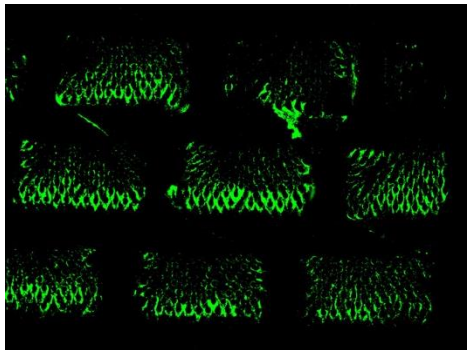
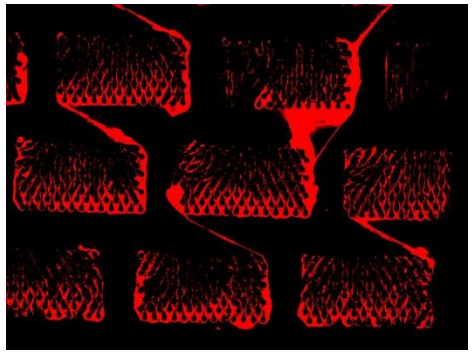
BF

Cy5

2025-07-22, Bacteria induced NETs, 4h, 1% FBS

Ionomycin View 1
After adding bacteria
and washing

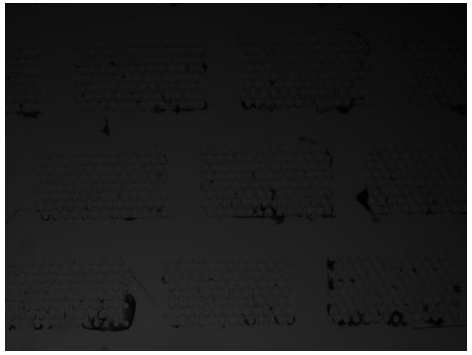
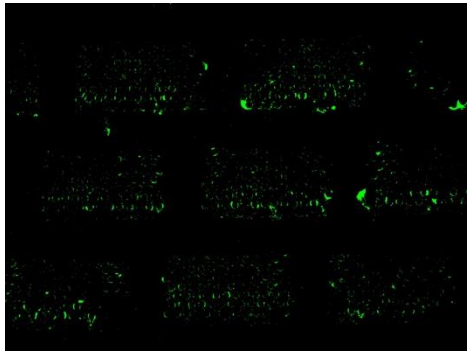
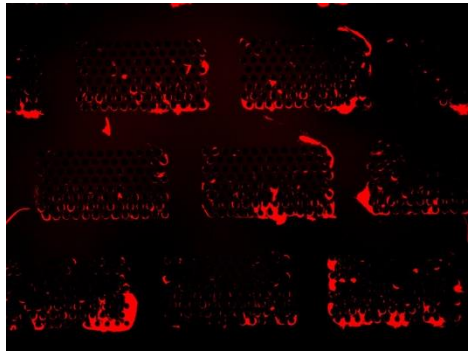




Ionomycin View 2
After adding bacteria
And washing

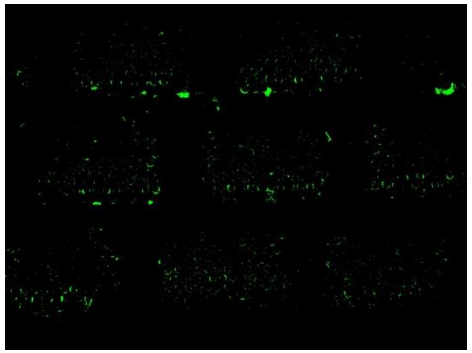
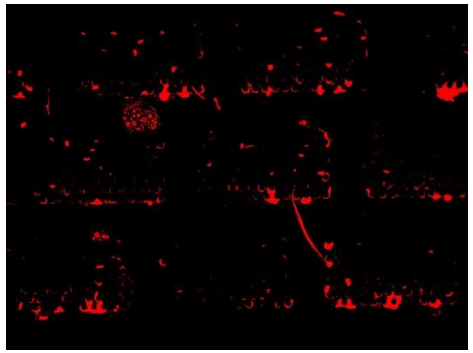
BF GFP Cy5

GFP is bacteria (they are green in nature), and Cy5 is the NET.
The red staining is weaker than the green staining,
but same luminance settings have been used (from before)

2025-07-22, Bacteria induced NETs, 4h, 1% FBS

Ionomycin
Before flushing

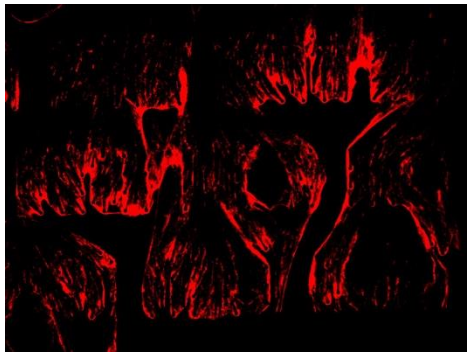
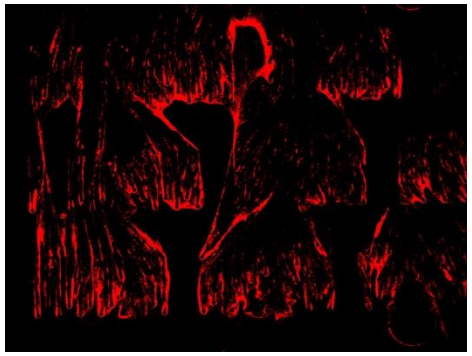
Ionomycin View 2
After flushing

BF

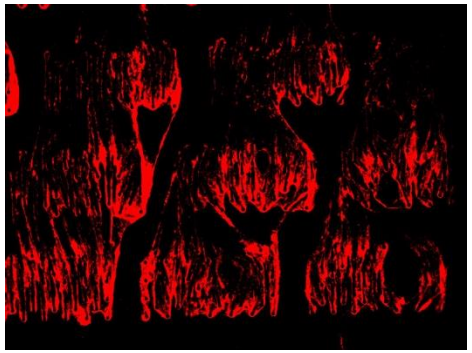
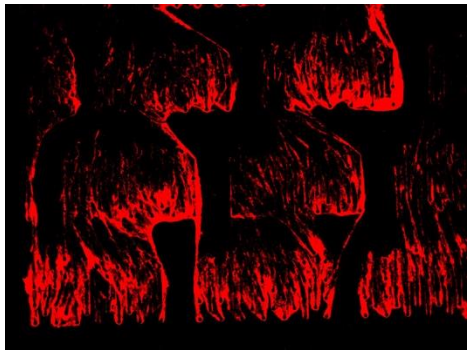
GFP

Cy5

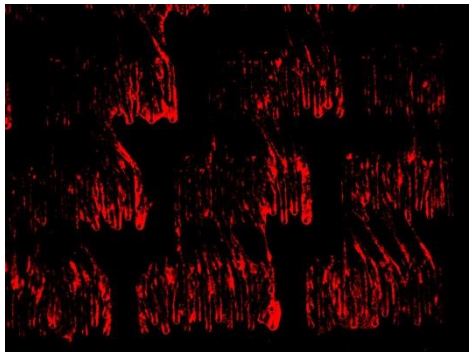
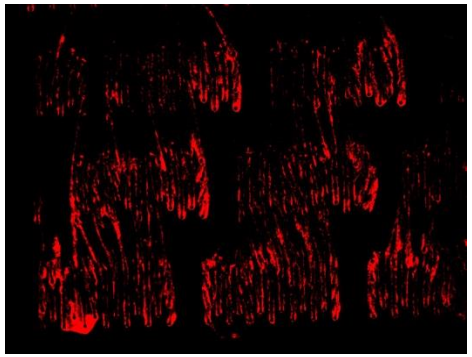
2025-07-22, Bacteria induced NETs**, 4**h, 1% FBS

Ionomycin

Ionomycin

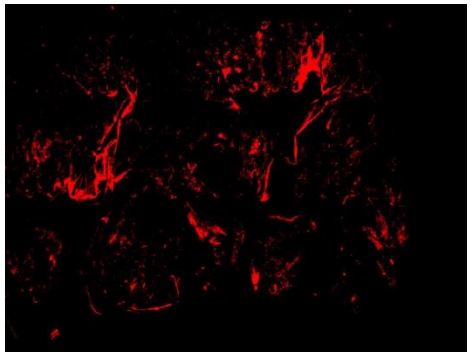
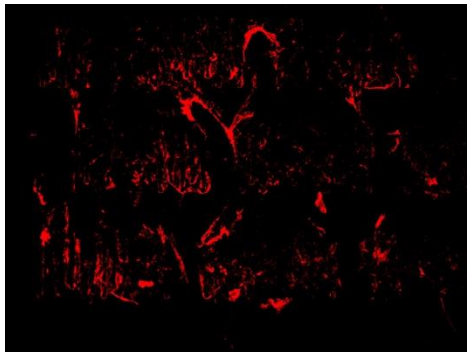



Ionomycin

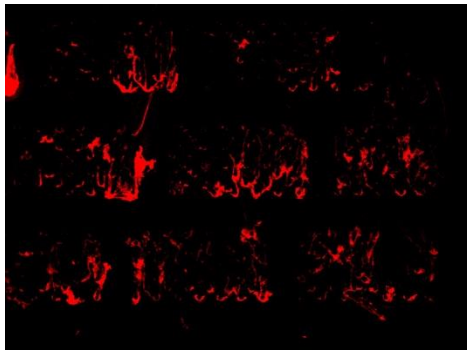
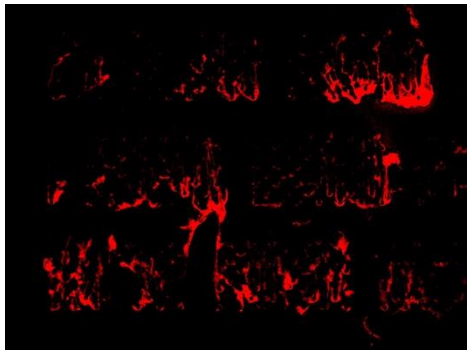
Cy5

Cy5

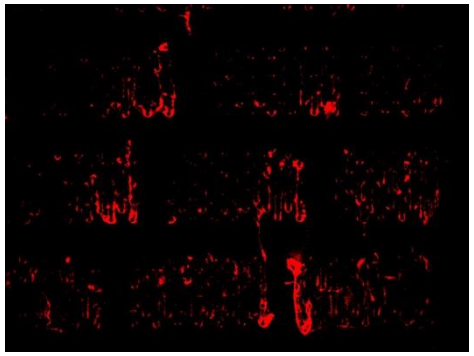
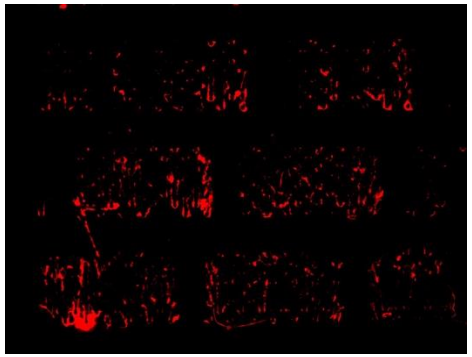
2025-07-25, Bacteria induced NETs**, 3**h, 1% FBS, **400** microliters of cells

Ionomycin
After adding enzyme 2

Ionomycin
After adding enzyme 3

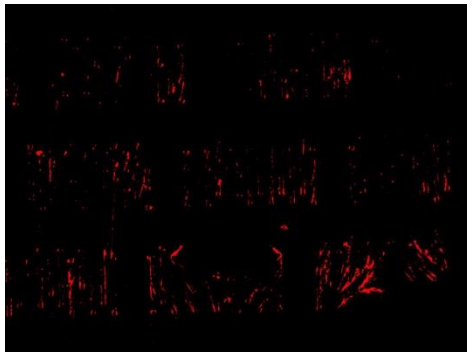



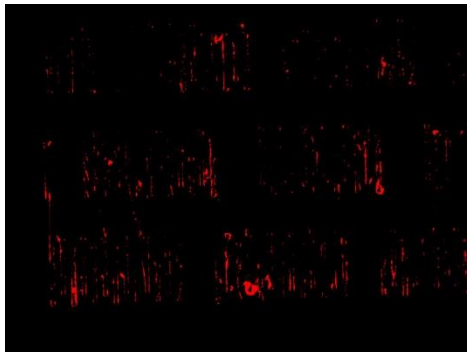
Ionomycin
After adding enzyme 4

Cy5

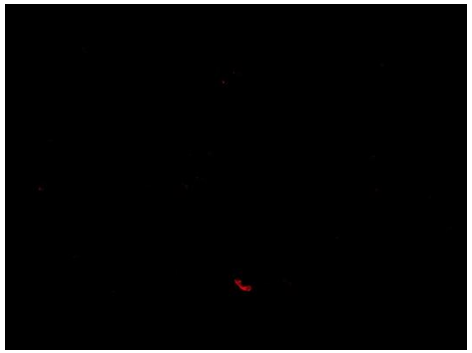
Cy5

2025-07-25, Bacteria induced NETs**, 3**h, 1% FBS , **400** microliters of cells



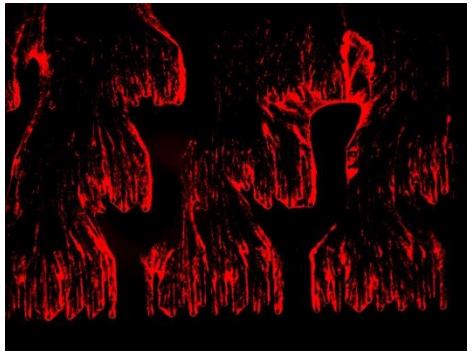
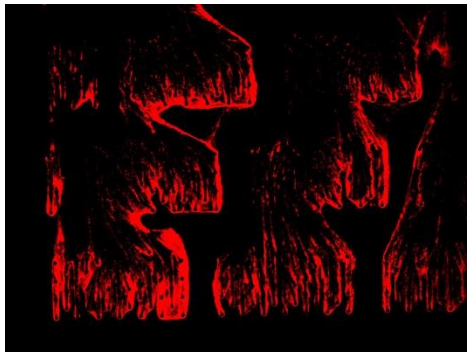
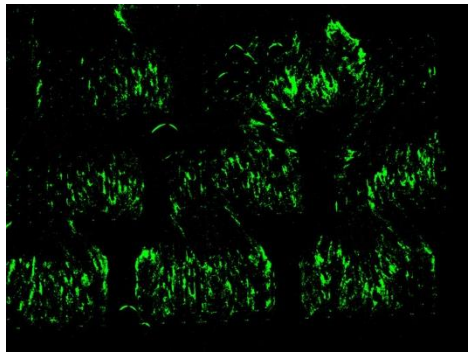


Ionomycin
Before adding DNase

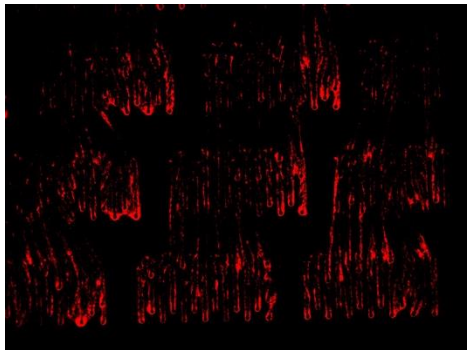
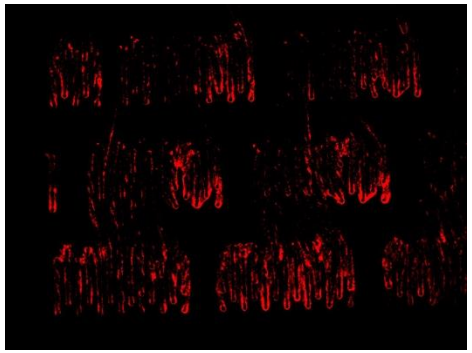
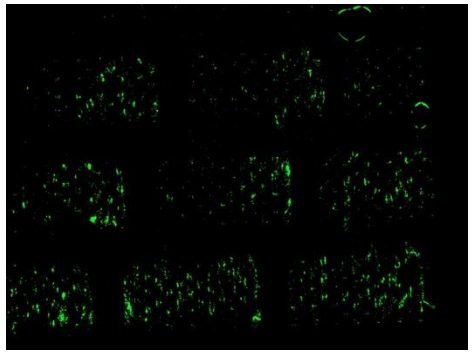


Ionomycin
Before adding DNase

2025-07-25, Bacteria induced NETs**, 3**h, 1% FBS , **200** microliters of cells (? Must repeat)

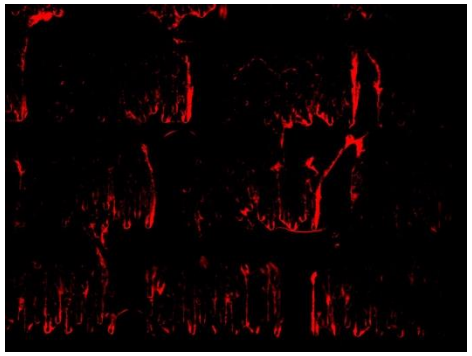
Ionomycin View 1

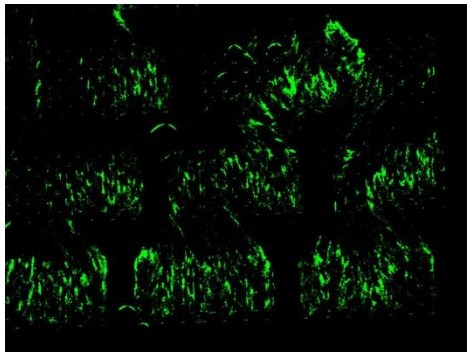




Ionomycin View 2

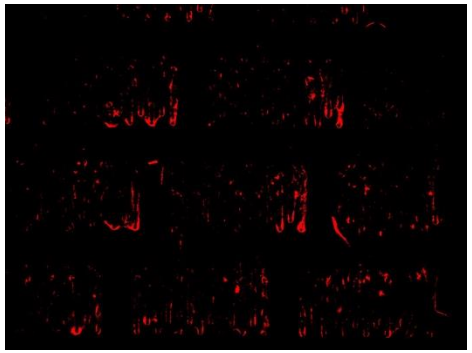
Cy5 Cy5 Bacteria

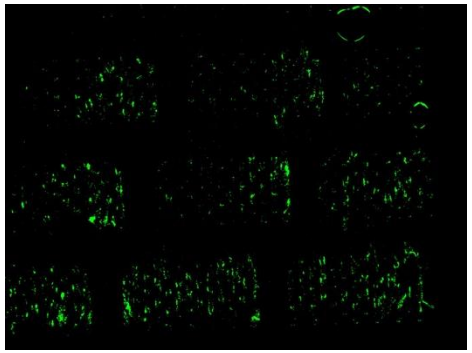
2025-07-25, Bacteria induced NETs**, 4**h, 1% FBS, before incubation, 1% FBS





Ionomycin View 1



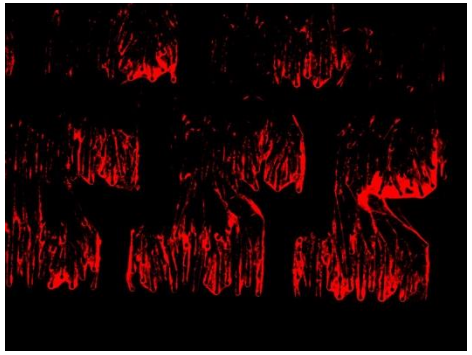


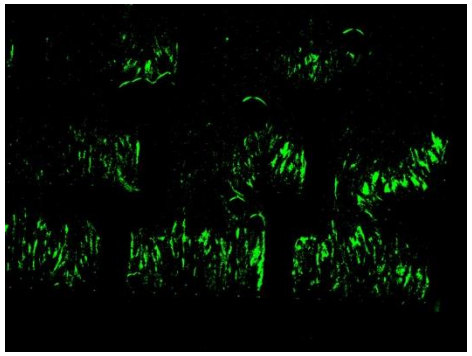
Ionomycin View 2

Cy5

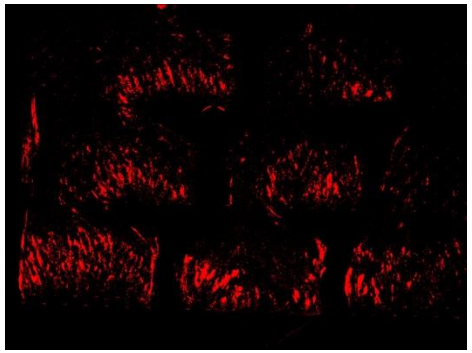
Bacteria

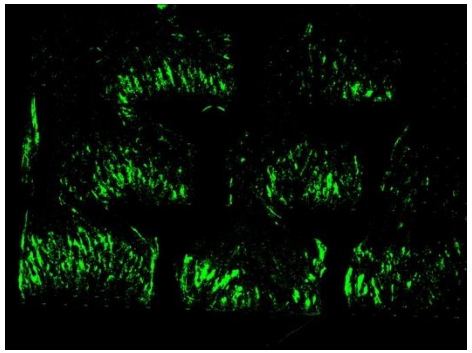
2025-07-25, Bacteria induced NETs**, 4**h, 1% FBS, after incubation, 1% FBS





Ionomycin View 1



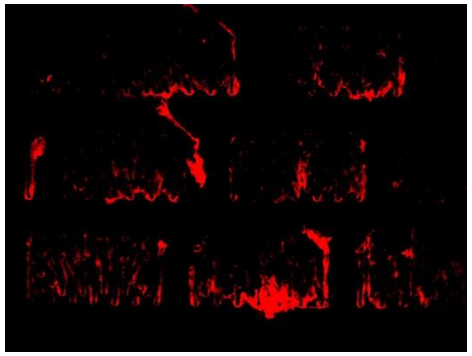


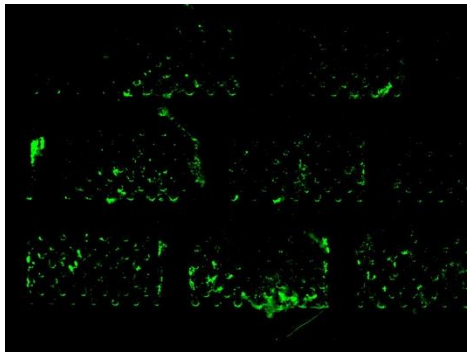
Ionomycin View 2

Cy5

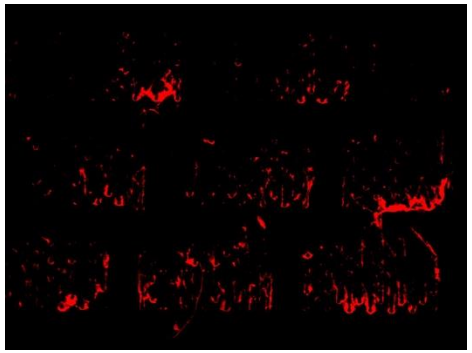
Bacteria

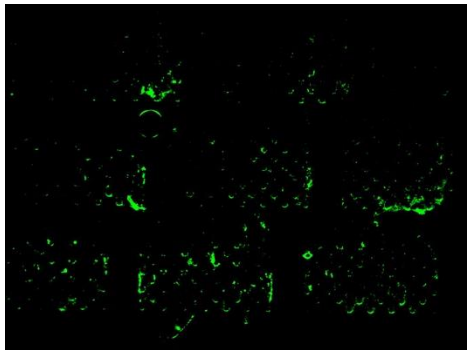
2025-07-25, Bacteria induced NETs**, 5**h, 1% FBS, before incubation, 1% FBS





Ionomycin View 1





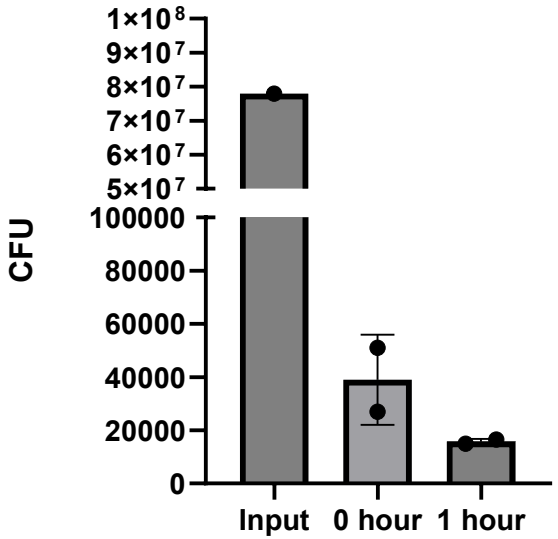
Ionomycin View 2

Cy5

Bacteria

2025-07-25, Bacteria induced NETs**, 5**h, 1% FBS, after incubation, 1% FBS

20250727 Bacteria trapping and killing



Project Summary

2025

Timeline

All systems in vitro mouse neutrophils:
Pre-06-03: Training
06-03 NETs in plate wells (PMA, Iono)
06-05 NETs in devices (PMA, Iono)
Conclusion: Both produce NETs in wells and devices, Ionomycin more

06-10 NETs in devices repeat (PMA, Iono, Raptinal, Nigericin, Val-boropro)
06-12 NETs in devices repeat (PMA, Iono, Raptinal, Nigericin, Val-boropro)
Cell death videos (PMA, Iono, Raptinal, Nigericin, Val-boropro)
Conclusion:
PMA, Ionomycin and Raptinal all consistently produces NETs
Nigericin has some NETs. Val-boropro has too low of cell death rate.

Timeline

06-13 Cell death videos (PMA, Iono, Raptinal, Nigericin, Val-boropro)
Images of different staining (PMA, Iono, Raptinal, Nigericin, Val-boropro)

06-17 NETs of concentration gradient (PMA, Iono, Raptinal)
Cell death videos of concentration gradient (PMA, Iono, Raptinal)
Conclusion: NETs decrease with the concentration decrease

06-24 LDH release (PMA, Iono, Raptinal)
06-27 NETs of PAD4 Knockout and WT (PMA, Iono, Raptinal)
07-01 NETs of PAD4 Knockout and WT (PMA, Iono, Raptinal)
Cell death video (PMA, Iono, Raptinal)
Conclusion: In WT NETs, in PAD4 KO no NETs (or less NETs)
Ionomycin kills all in both KO and WT, but only in WT NETs are observed
Raptinal has a big difference in death rate of KO and WT, and a huge difference in NET formation, but LDH remains the same
PMA has a big difference in death rate of KO and WT, but no lysis death at all

Timeline

07-03 NETs of Spontaneous Death
07-09 NETs of Spontaneous Death Repeat
NETs of different protocols - Percoll, Histopaque, Negative Selection
Conclusion: Only Percoll has a little NETs (possible due to RBCs)
Percoll + Histopaque, + Negative Selection, + Histopaque + Negative Selection, all no NETs

07-10 NETs of bacteria induction (S. Aureus 10390, S. Aureus BAA, K12, Bacillus Cereus, A7)
07-15 NETs of bacteria induction Repeat (S. Aureus 10390, S. Aureus BAA, K12, Bacillus Cereus, A7, Triton)
Cell death videos (S. Aureus 10390, S. Aureus BAA, K12, Bacillus Cereus, A7, Triton, Lysis buffer)
07-17 NETs of bacteria induction Repeat (S. Aureus 10390, S. Aureus BAA, K12, Bacillus Cereus, A7)
Cell death videos (S. Aureus 10390 (two different MOI), S. Aureus BAA (two different MOI), Bacillus Cereus, Ionomycin)
Conclusion: Bacillus Cereus can induce lot of NETs (close to Ionomycin)
A7 has NETs (bit less than Raptinal)
S. Aureus 10390, S. Aureus BAA, K12 have no NETs in our system

NETs of 1% FBS and 10% FBS (Bacillus Cereus, Ionomycin)
Conclusion: FBS does not affect NET formation

Timeline

07-22 NETs of Triton
Conclusion: Despite total death in 5 minutes, no NETs

07-22 NETs for bacteria trapping and killing (Ionomycin, 3h, 4h, 5h)
07-29 NETs for bacteria trapping and killing repeat (Ionomycin, 3h, 4h, 5h)
07-31 NETs for bacteria trapping and killing repeat (Ionomycin, 4h, 5h)

ImageJ Macros

20250620_organzize_images.bat:
Takes all current directory tif images
Automatically categorizes them into the subfolders of cell video
/* A folder named Template must be provided in the same directory with the correct structure
This can be accomplished by either generating a Template folder by using create_template_folders, or by copying the Template folder from the disk. */

20250620_composite_macro_workflow.jim:
Takes three subfolders, "BF", "PI", "Speck", *number of* images
Processes them into a video automatically

20250620_convert_to_gfp.jim:
Takes all current directory tif images
Converts them to GFP and store them in the subdirectory GFP under the current directory
Same exposure time / gain (no automatic adjustment for individual images, quantification purposes)
(This can be freely edited in the raw script)

20250703_convert_gfp_bf_manual.ijm:
Takes a folder, where there are subfolders containing a bright field image and a GFP image
Loads the GFP image and allows you to set the brightness manually
Outputs the composite image

Protocol Change

After we get our NETs from ionomycin:

Add **300 µL of bacteria** at a concentration of **5×10^8/mL** through the device.

Wash it with **200 µL of PBS**, **repeated 8 times.**

Add **50 µL of DNase** through the device. **Don't collect the liquid sucked out (at this moment nothing is digested yet)**

Incubate the device at 37°C for 10 minutes.

Add **50 µL of DNase** through the device. **Collect the liquid sucked out.**

Incubate the device at 37°C for 10 minutes.

Place the device under the microscope.

Use 1 mL of PBS to collect the bacteria from the device.

Dilute the collected bacteria and spread on an agar plate for counting.

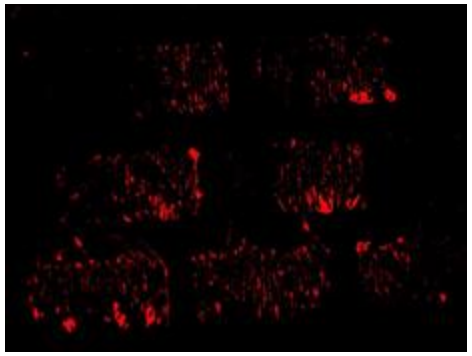
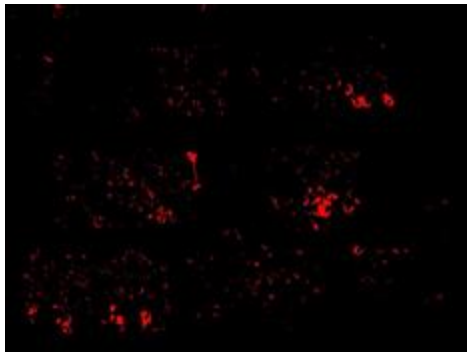
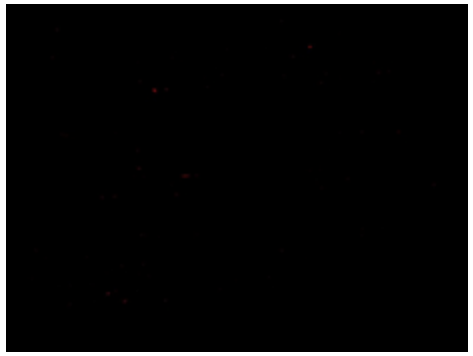
Protocol Change

We include **two sets of devices** in our batch:

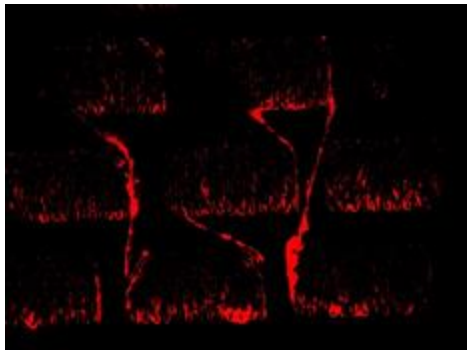
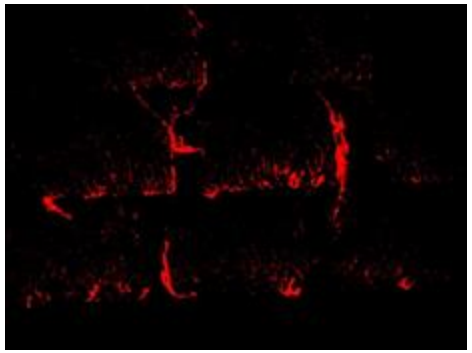
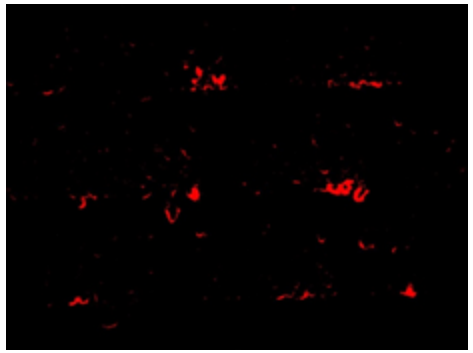
We culture the cells for 4 hours after adding Ionomycin, then:

1. We **directly add** the cells, bacteria and DNase (and culture) two times and see it under the microscope.

1. We add the cells and bacteria, then we **incubate the device for an hour,** take it out and add the DNase.

Ionomycin
High conc.
DNase

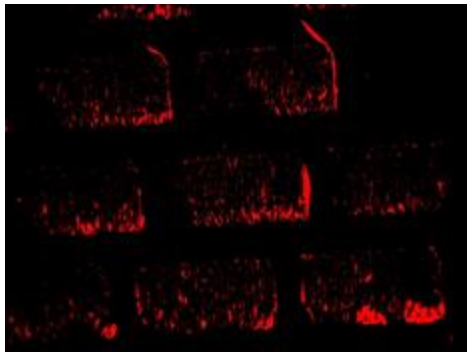
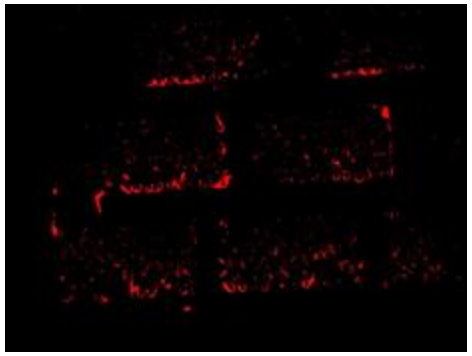
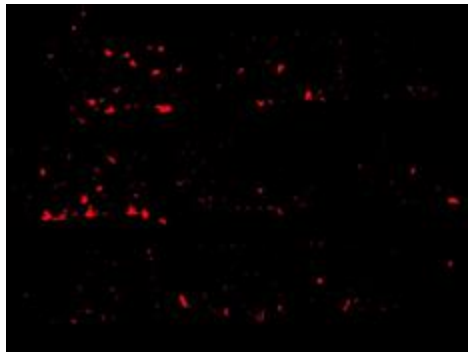
Ionomycin
Low conc.
DNase

0
min

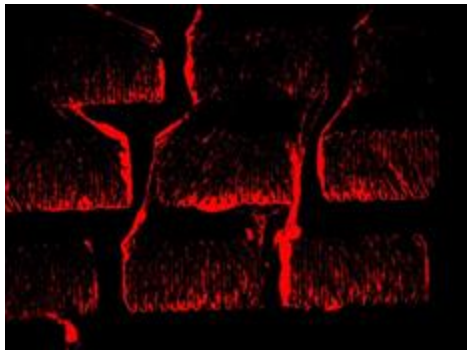
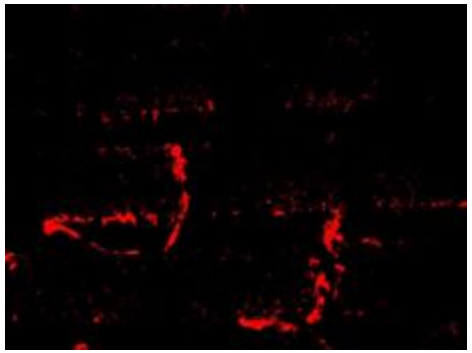
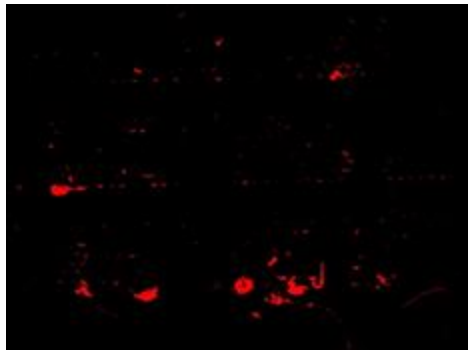
10 min
1*DNase

20 min
2*DNase

2025-07-29, NET killing and trapping**, 3h**, 1% FBS**, 300**

Ionomycin
High conc.
DNase

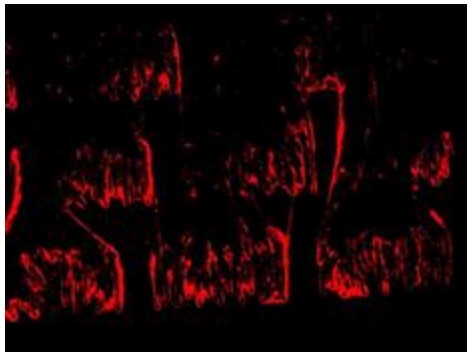
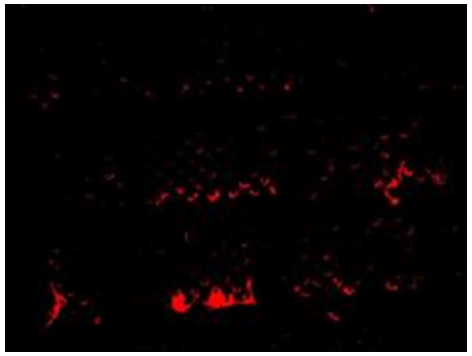
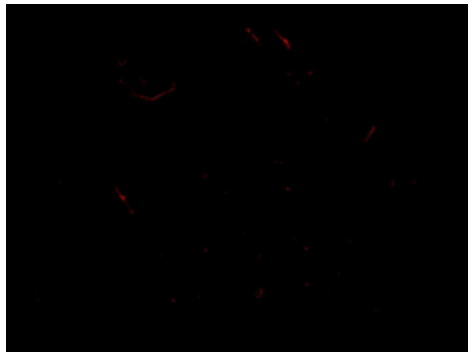
Ionomycin
Low conc.
DNase

0
min

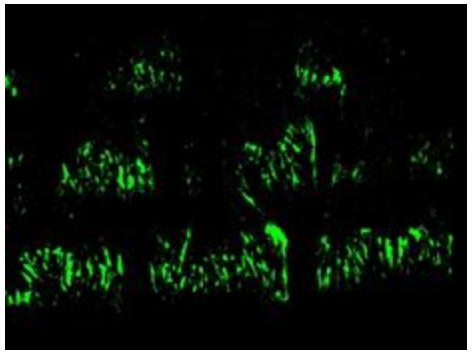
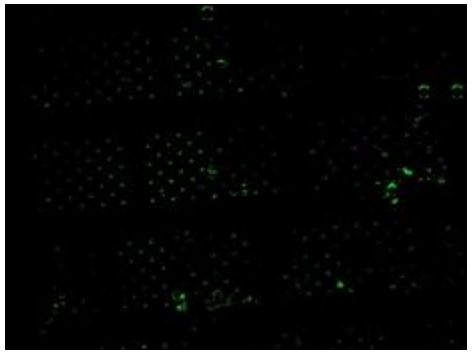
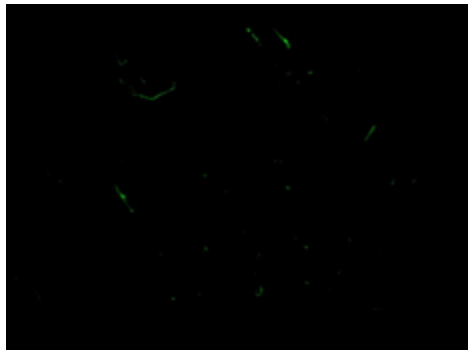
10 min
1*DNase

20 min
2*DNase

2025-07-29, NET killing and trapping**, 3h**, 1% FBS**, 400**

NETs

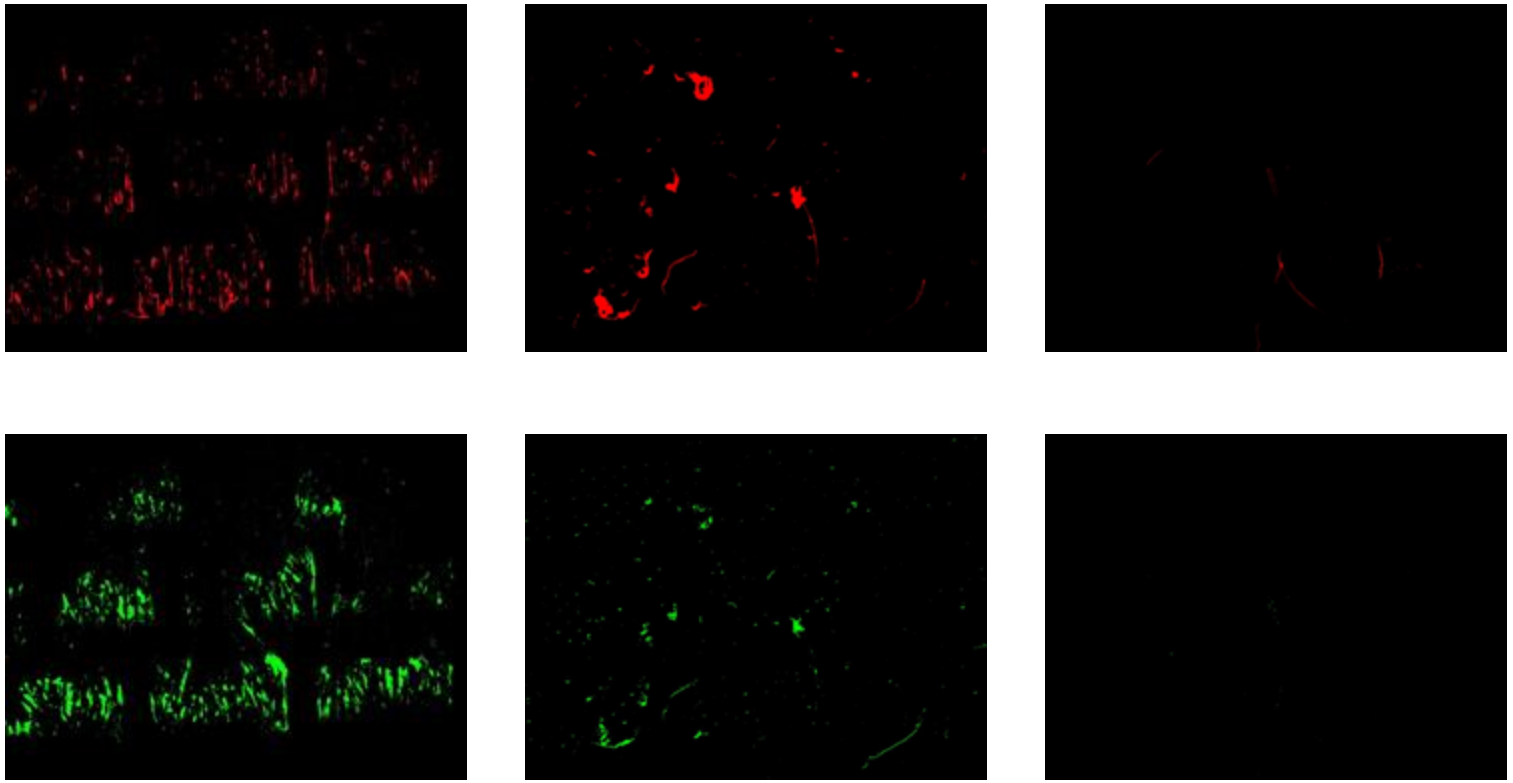
Bacteria

0
min

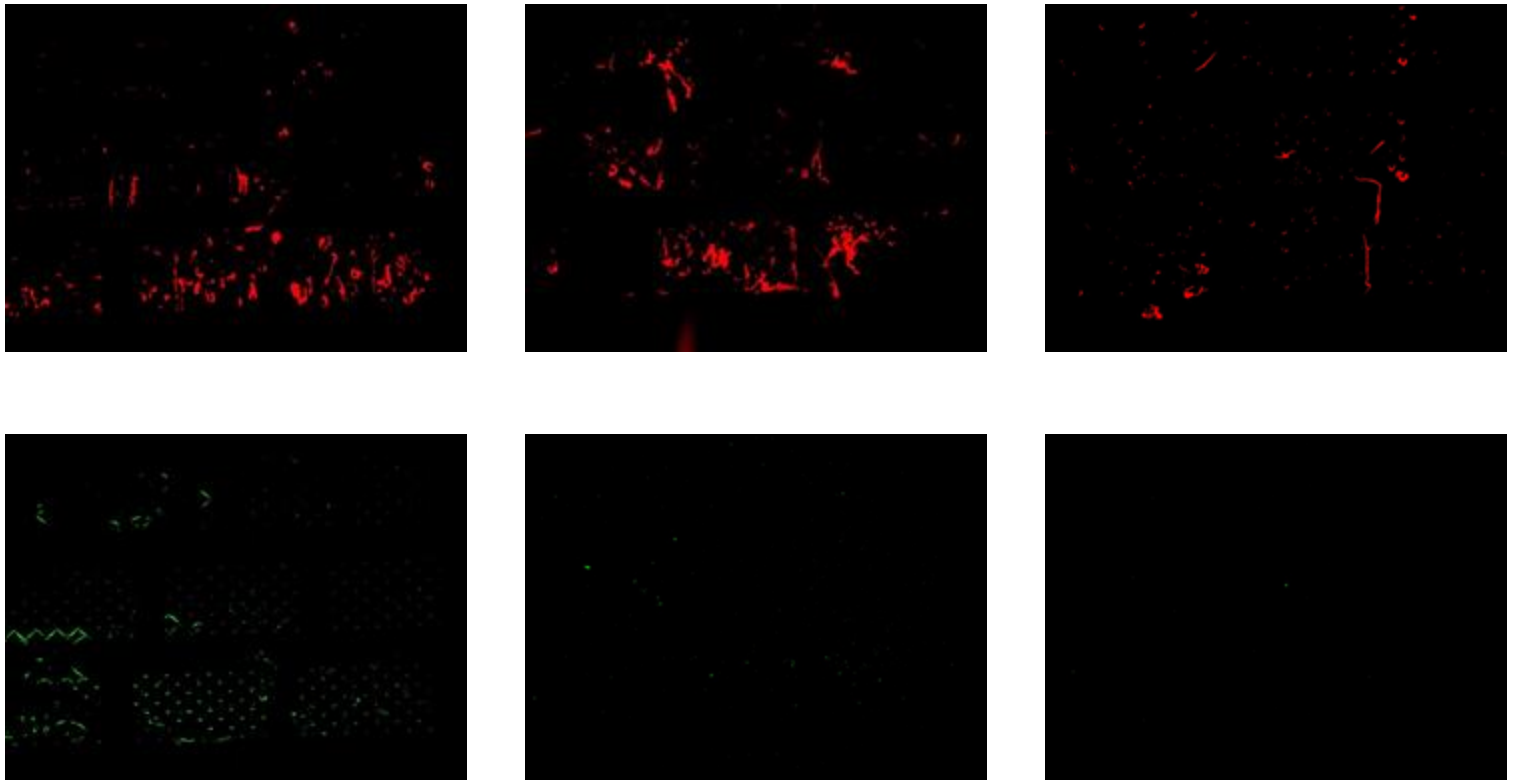
Group 1
Add enzymes
immediately

Group 2
Incubate 1 hour
more

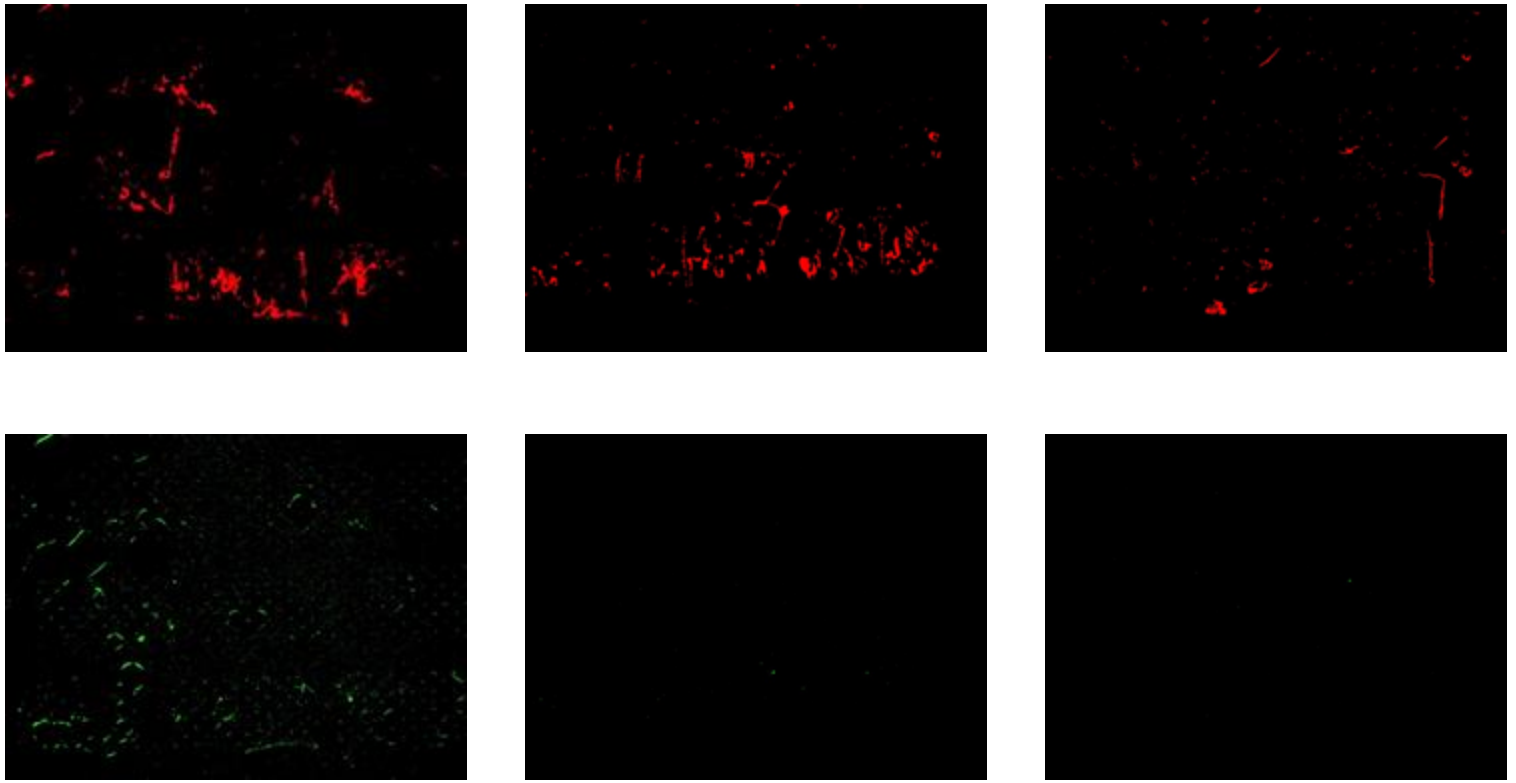
2025-07-29, NET killing and trapping**, 4h**, 1% FBS**, 400**



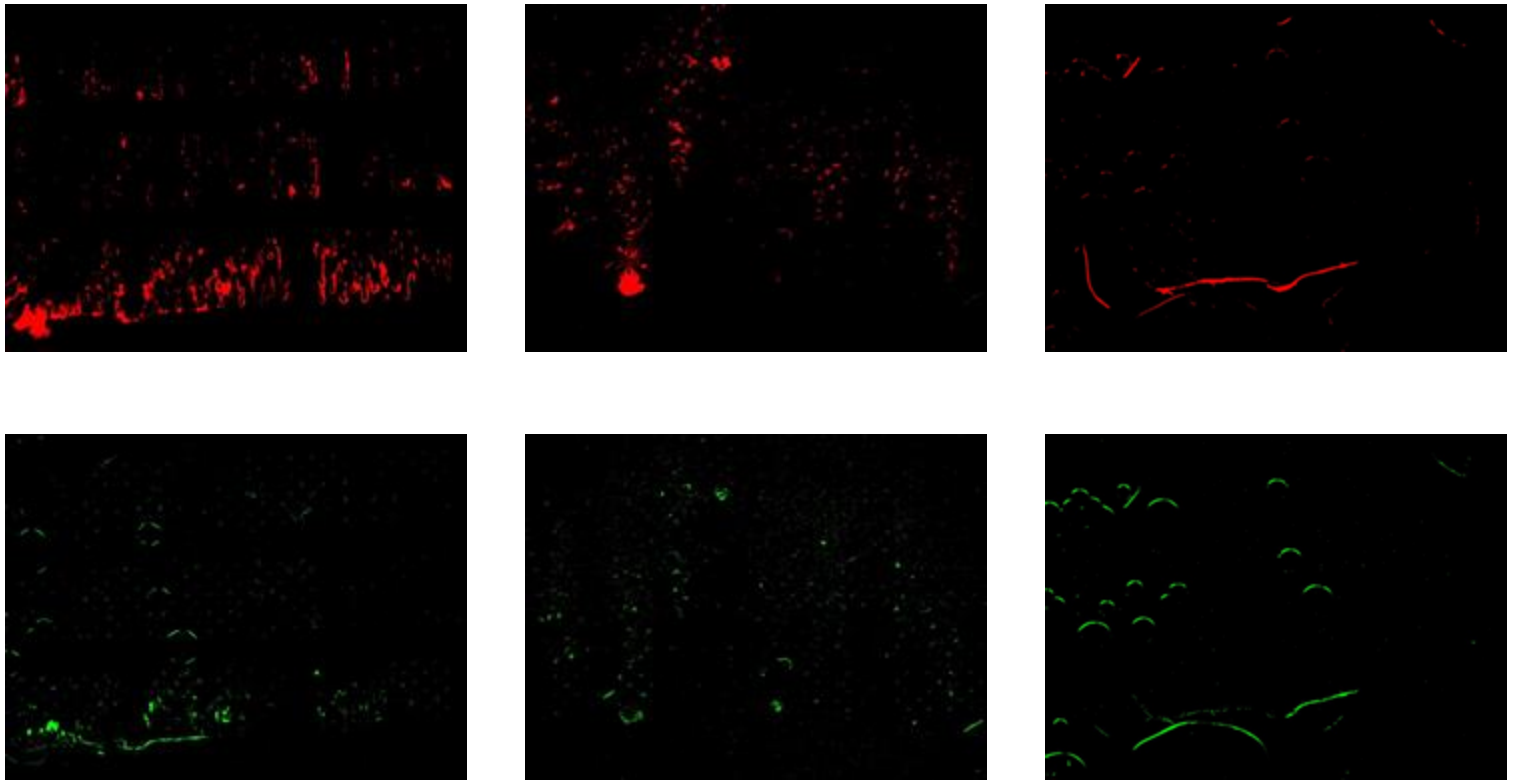
2025-07-29, NET killing and trapping**, 4h**, 1% FBS**, 400**



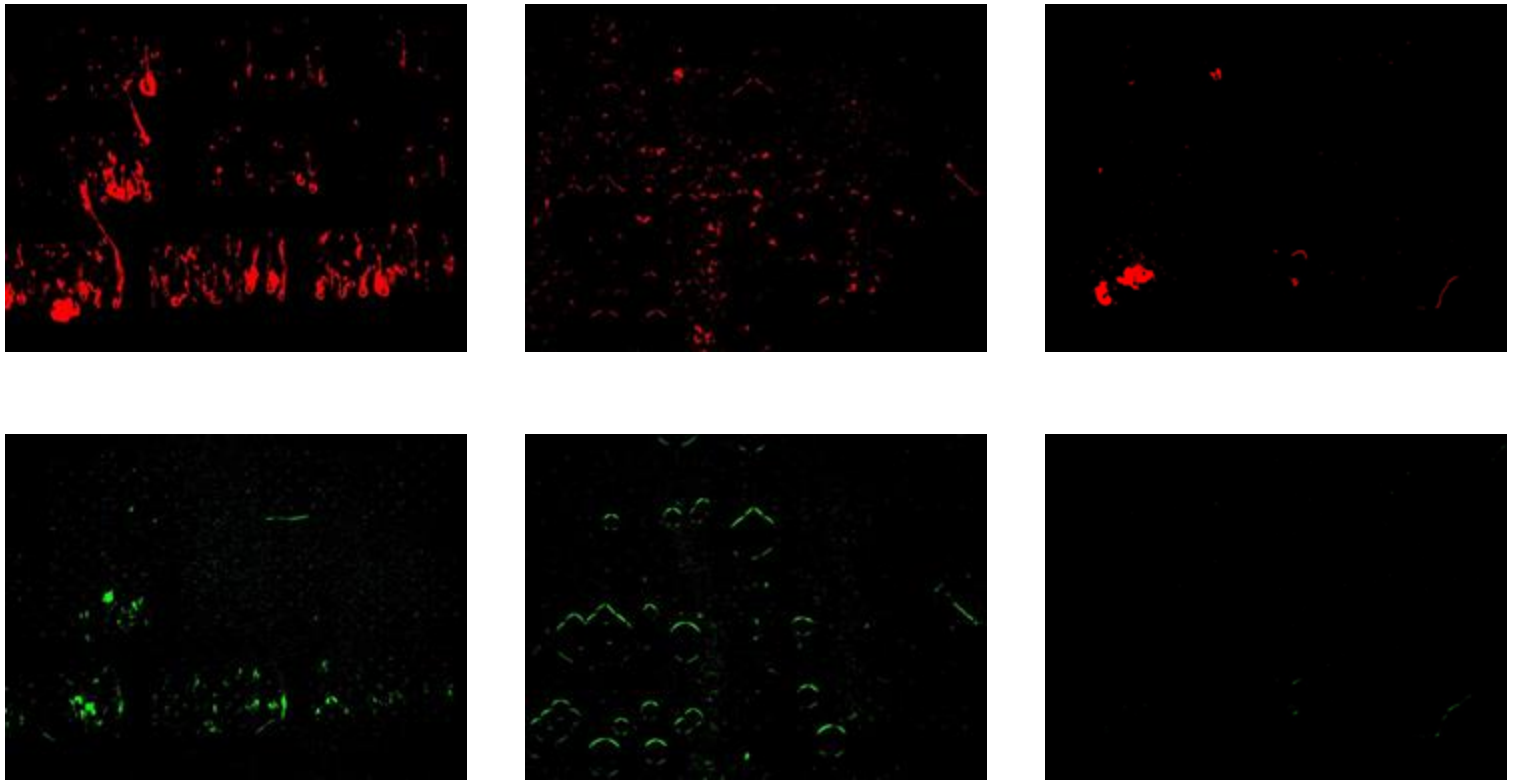
2025-07-31, NET killing and trapping**, 4h**, 1% FBS**, 400**



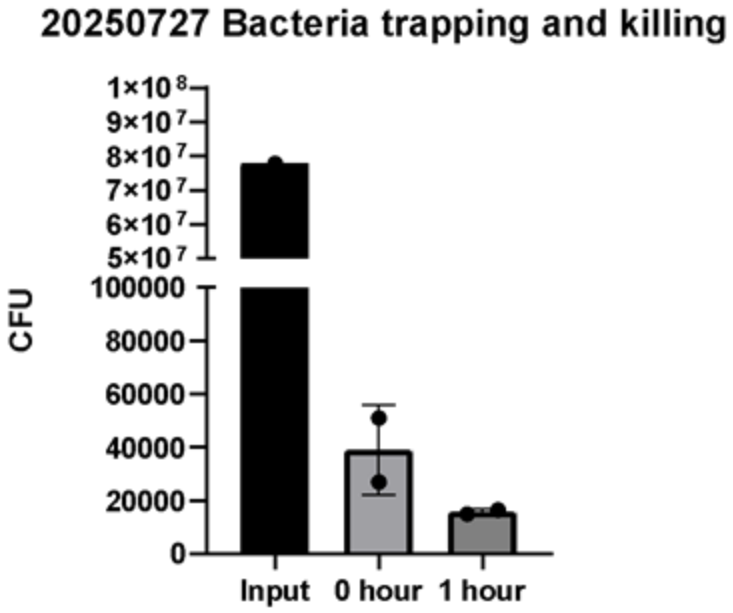
2025-07-31, NET killing and trapping**, 4h**, 1% FBS**, 400**



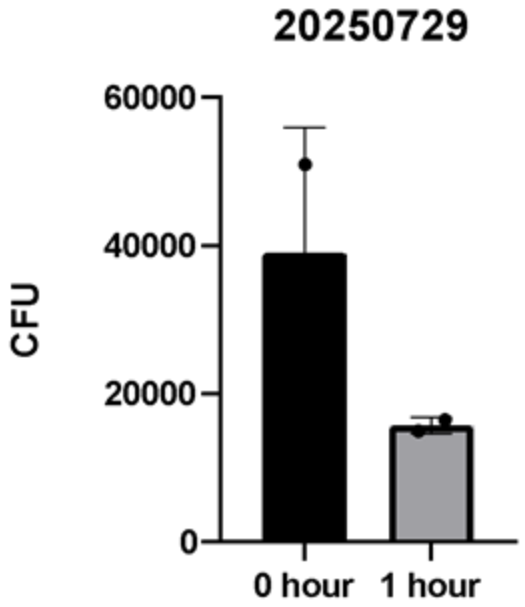
2025-07-31, NET killing and trapping**, 4h and 1h of incubation with enzymes**, 1% FBS**, 400**



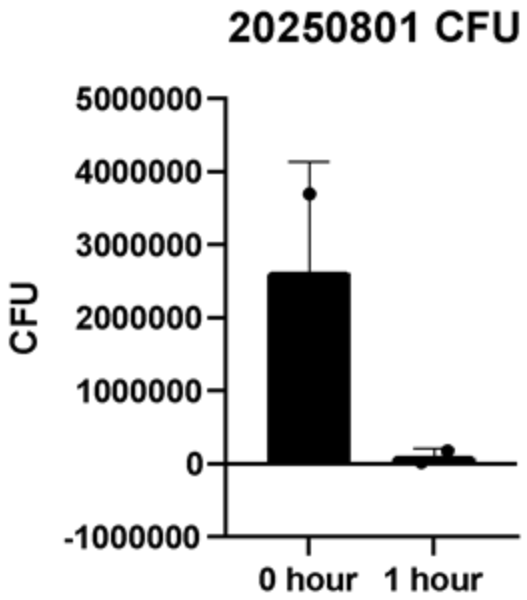
2025-07-31, NET killing and trapping**, 4h and 1h of incubation with enzymes**, 1% FBS**, 400**



Once digestion
(100ul/1.5ml)



Twice digestion
(100ul+100ul/1.5ml)



Twice digestion
(100ul+100ul/1.5ml)