

Physical Effect of Cold Atmospheric Plasma on Cancer Cells

Dr. Dayun Yan

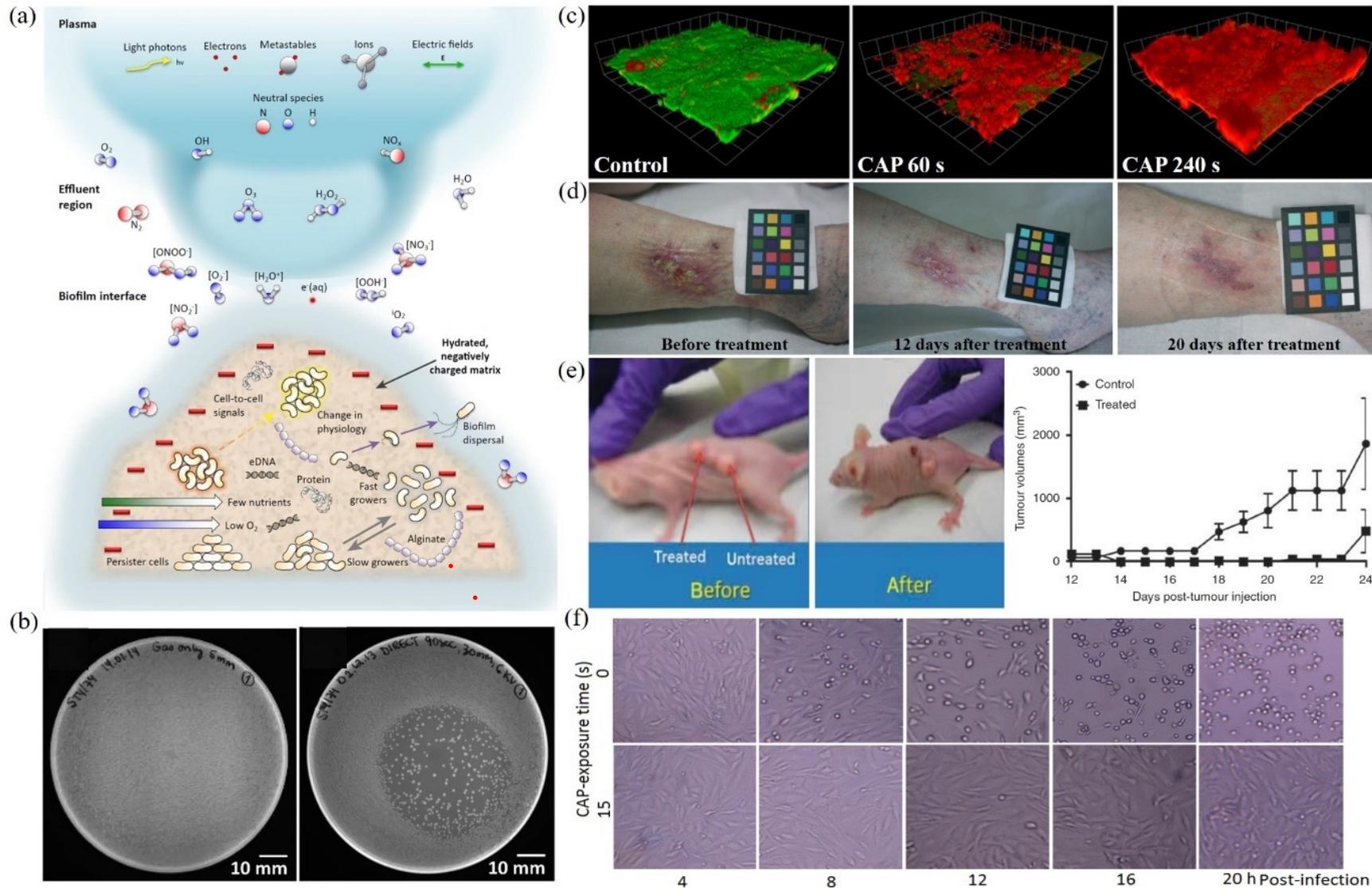
George Washington University, Washington, DC, United States, 20052.

June 22nd, 2021.

Outlines

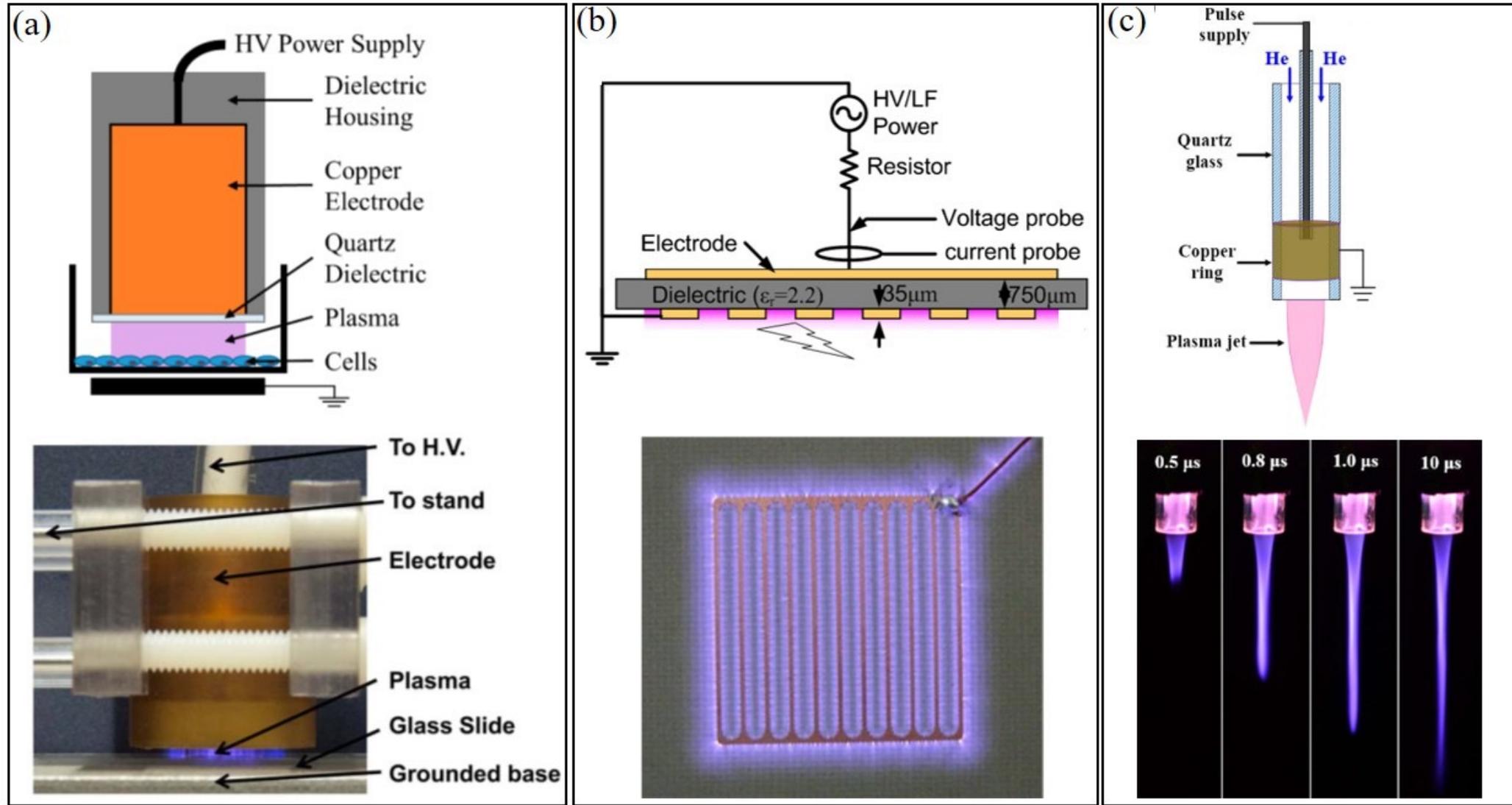
- General picture of biological killing by CAP.
- Killing mammalian (cancer) cells by physical factors in CAP.
- Activation/sensitization of cancer cells to drugs by CAP.
- Summary.

Plasma Medicine: a Glance



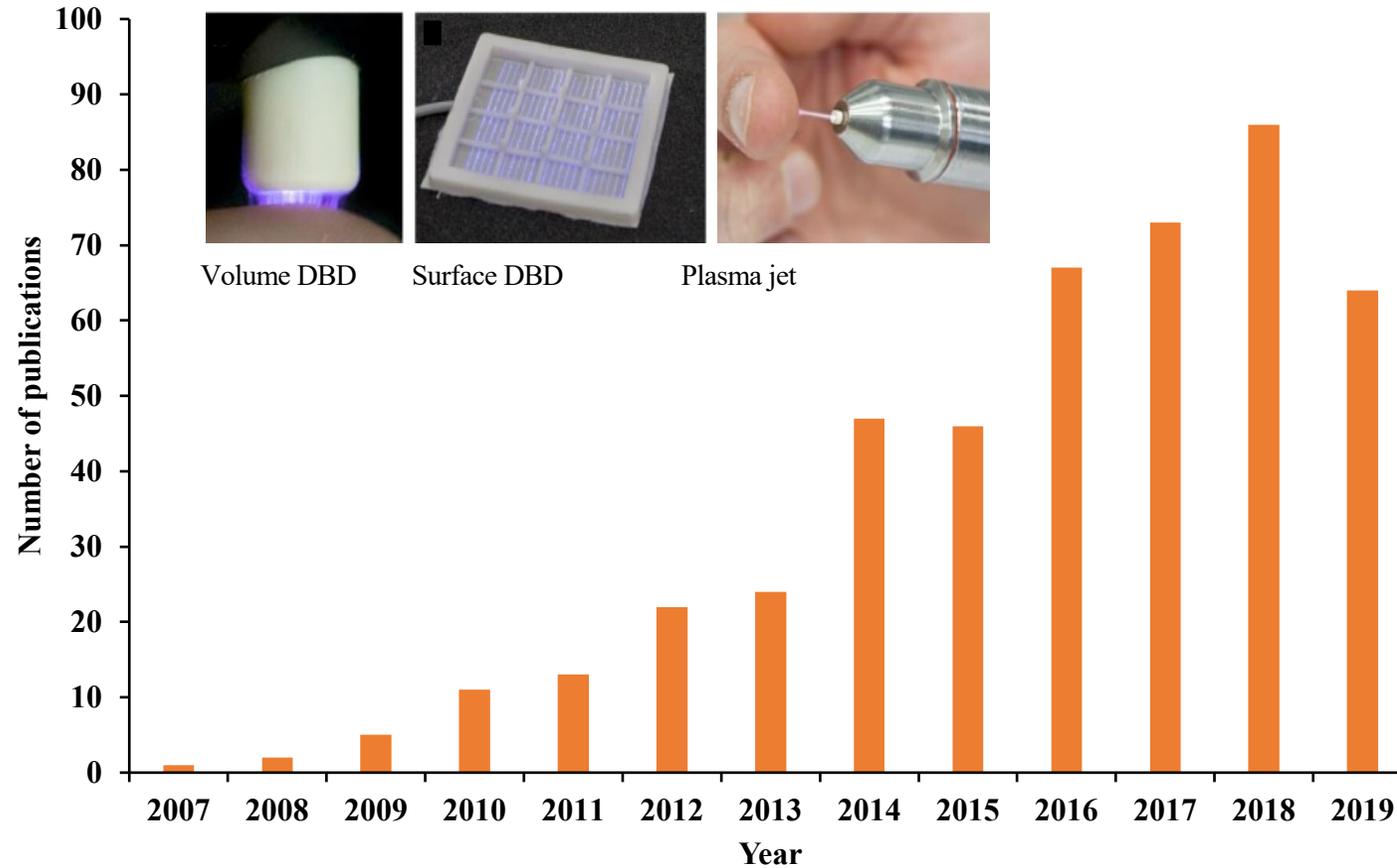
(a) Biological effect of CAP on biofilm. (Gilmore, B.F., et al. 2018, Trends in Biotechnology, 36.6, 627-638) (b) Growth inhibition of CAP source on bacteria (*S. Typhimurium*) grown on LB agar plates: control (left) and DBD treatment (right). (Angela, P.M., et al. 2016, Scientific Reports, 6, 35646) (c) Confocal scanning laser microscopic imaging of strong bacterial death in the CAP-treated *P. aeruginosa* biofilm grown on polycarbonate coupons. Live cells and dead cells were present in green and red, respectively. (Alkawareek, M.Y., et al. 2012, PloS One, 7.8, e44289) (d) The improved wound healing by CAP treatment. (Heinlin, J., et al. 2011, Journal of the European Academy of Dermatology and Venereology, 25.1, 1-11) (e) Tumor inhibition effect of CAP (Keidar, M., et al. 2011, British Journal of Cancer, 105.9, 1295-1301) (f) Inactivation of feline calicivirus and the inhibited infectivity on its host Crandell-Reese feline kidney cells by CAP treatment. (Aboubakr, H.A., et al. 2009, PloS One, 13.3, e0194618)

Typical CAP Sources in Plasma Medicine



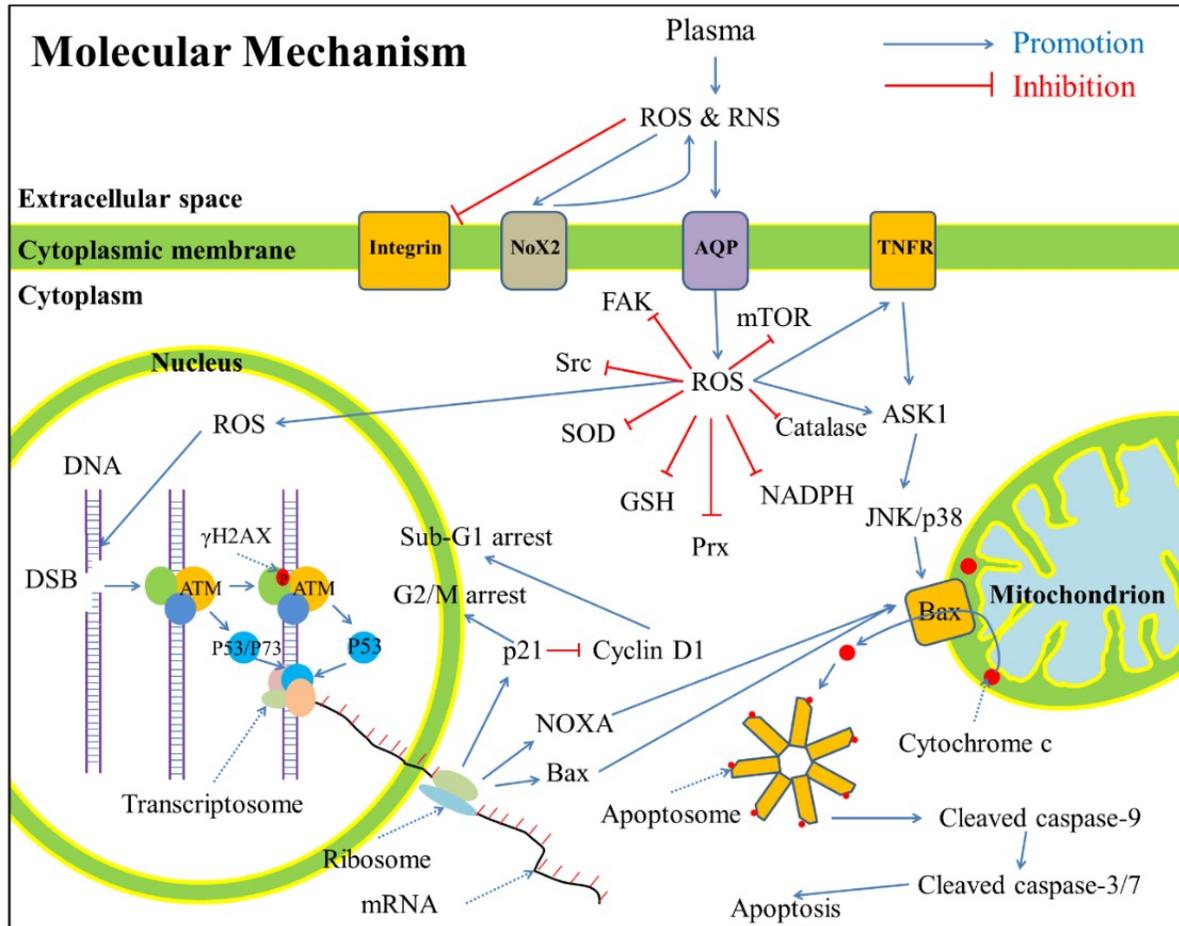
(a) A volume DBD source. (Lin, A., et al. International Journal of Molecular Sciences, 18.5, 966 (2017) and Kalghatgi, S., et al. PloS one, 6.1, e16270 (2011).) (b) A surface DBD source. (Kim, G.J., et al. Applied Physics Letters. 96, 021502, (2010).) (c) A CAP jet source. The length of bulk CAP can be modulated by controlling signals such as pulse width (μs). (Liu, Z., et al. Physics of Plasmas, 25.1, 013528 (2018).)

Cancer Treatment: Publication Number versus Year

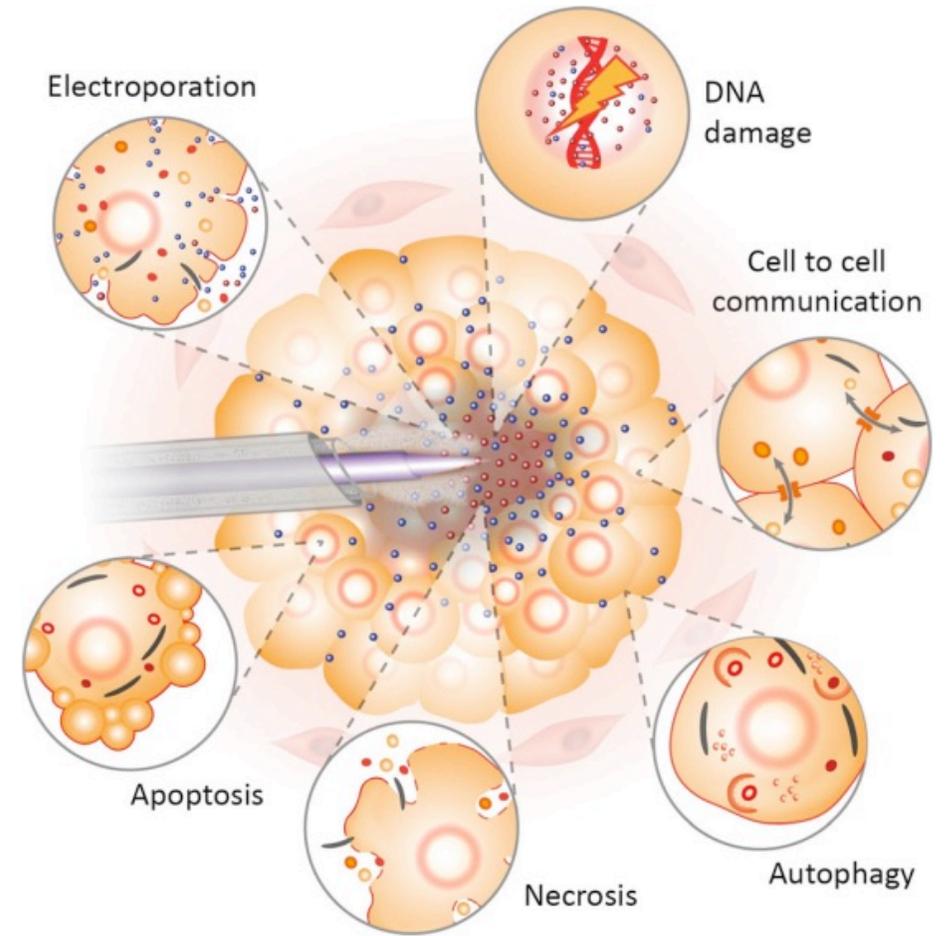


Anti-cancer Mechanism *in vitro*

(a)

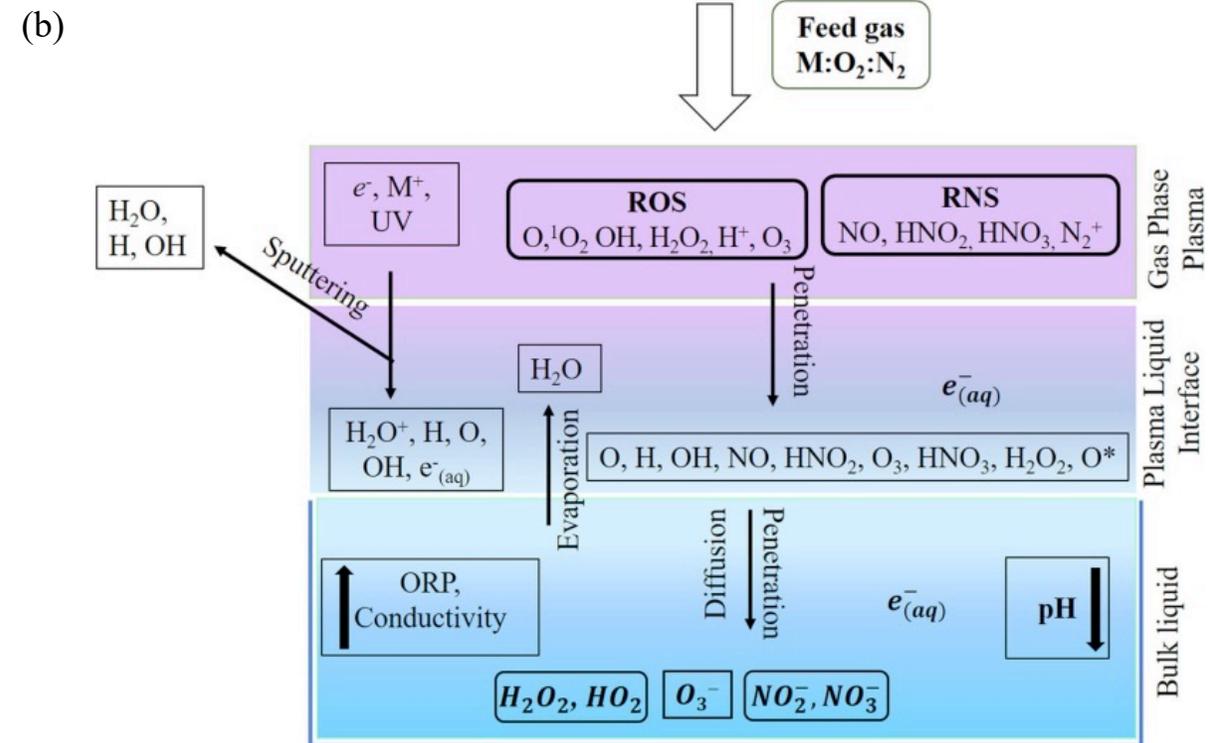
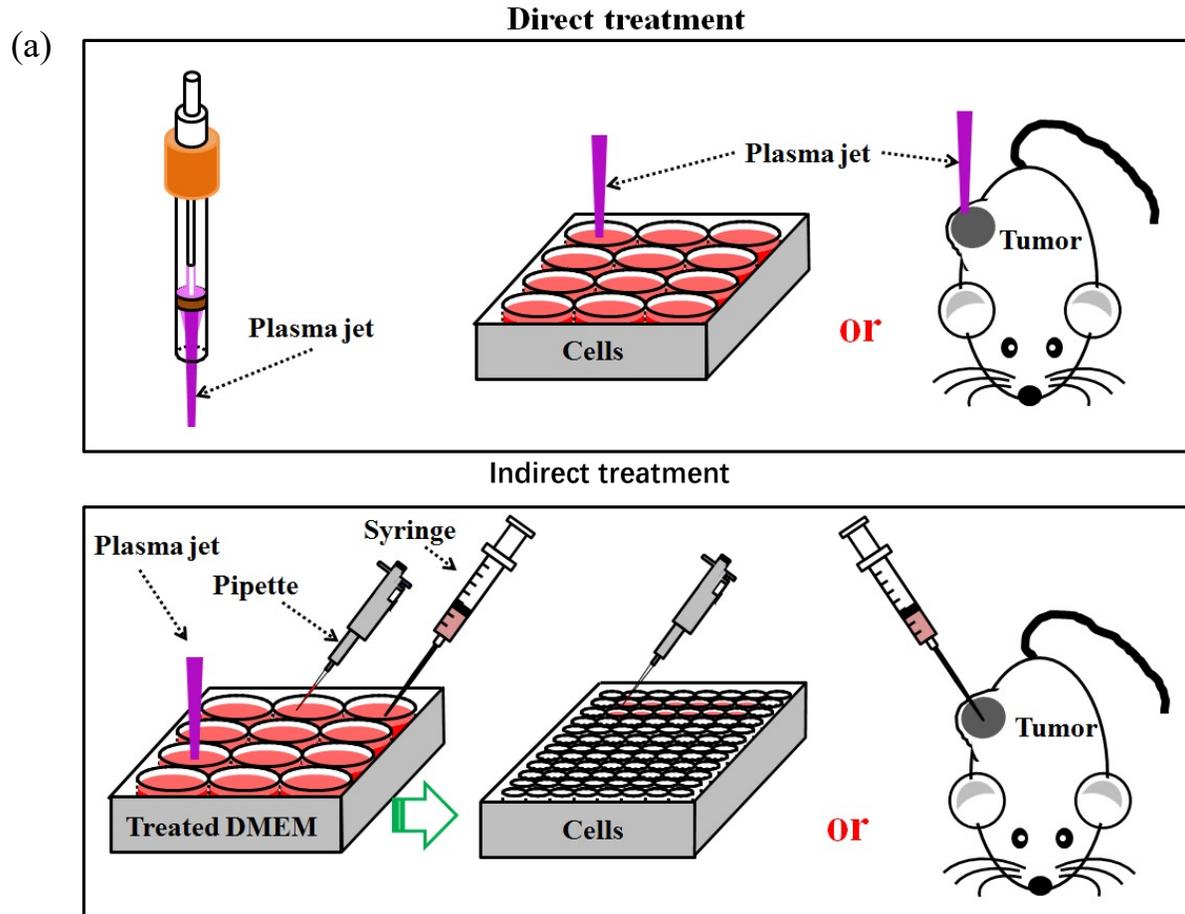


(b)

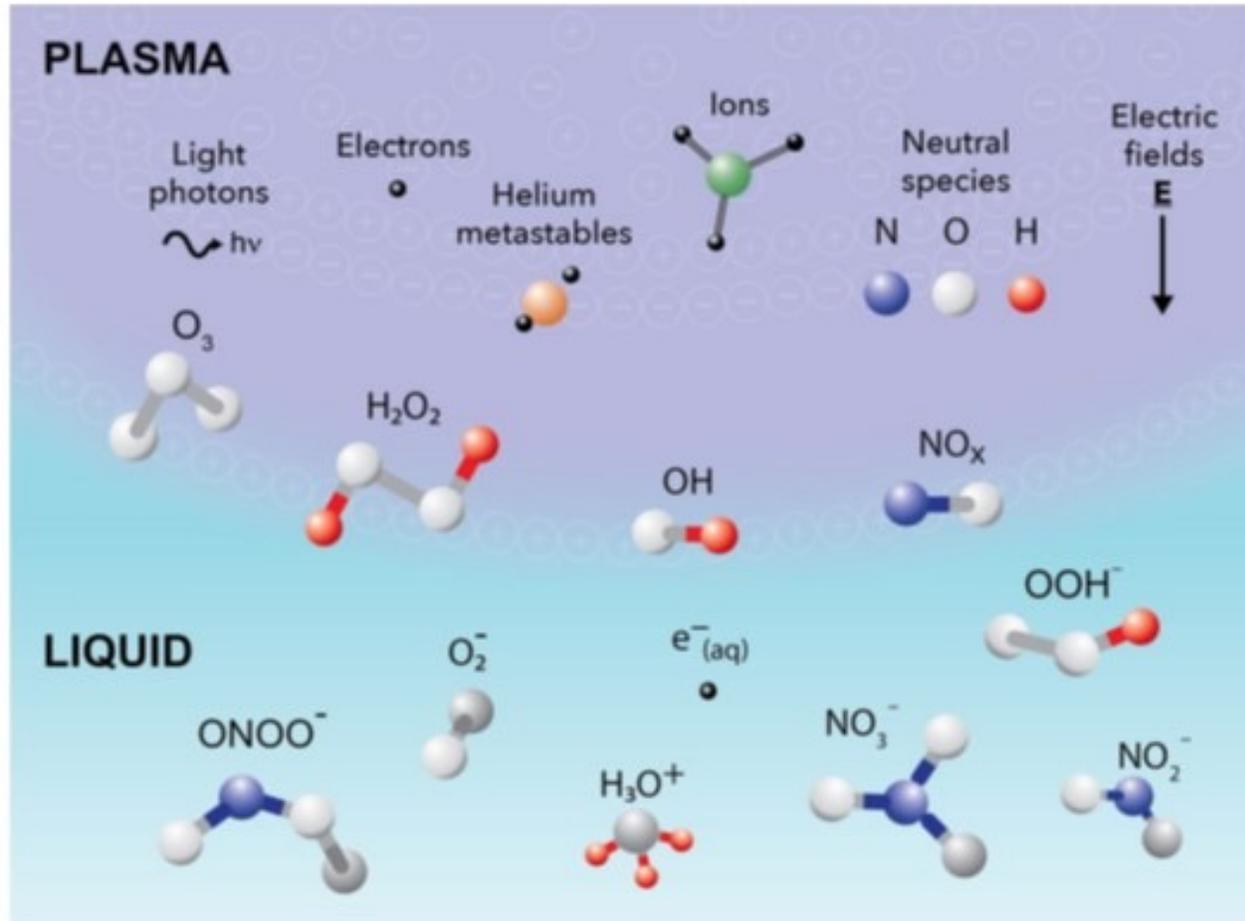


Malyavko, A., et al., 2020. Materials Advances, 1(6), pp.1494-1505.

Direct and Indirect, Dominant Roles of Reactive Species



Components in CAP



A schematic illustration of the typical component of CAP and reactive species formed in the CAP-treated liquid (Hirst, A.M., et al., 2016. Tumor Biology, 37(6), pp.7021-7031.)

What is the physical effect of CAP on mammalian cells?

No effect or it can be only observed under specific conditions?

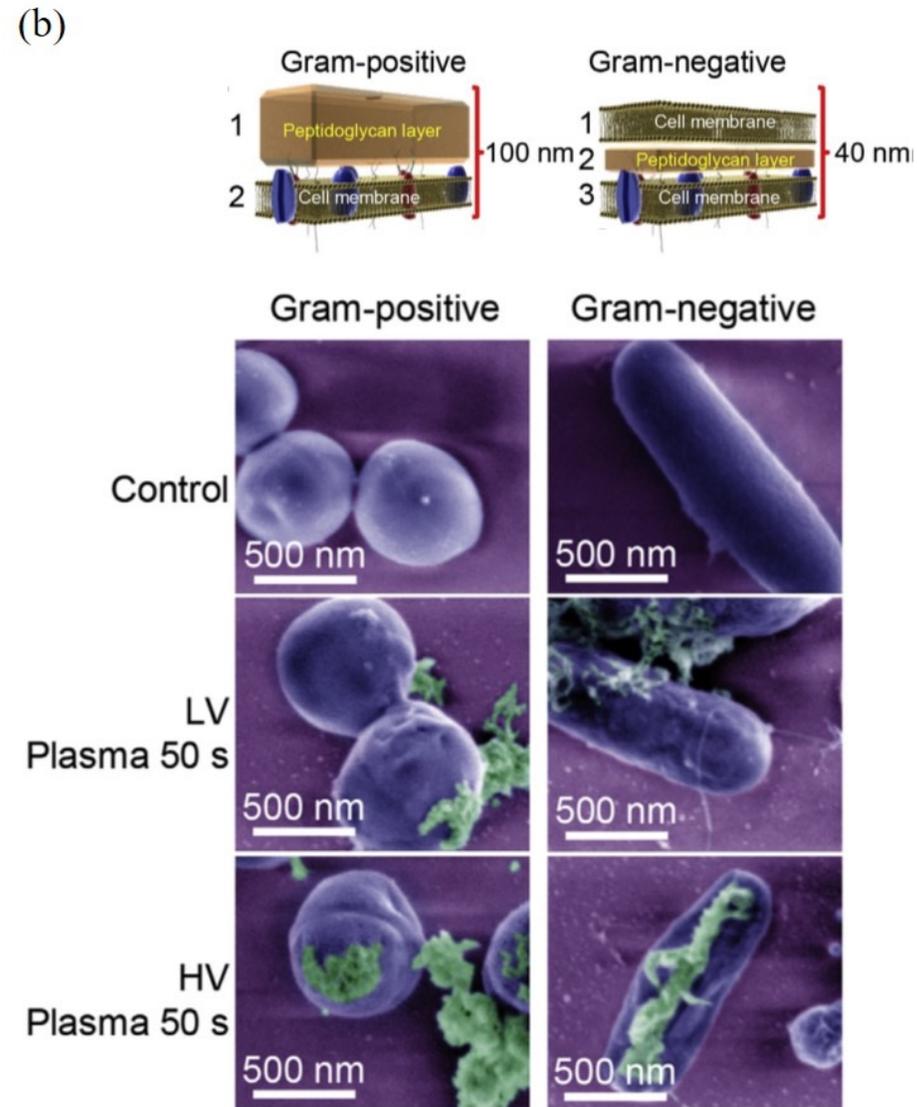
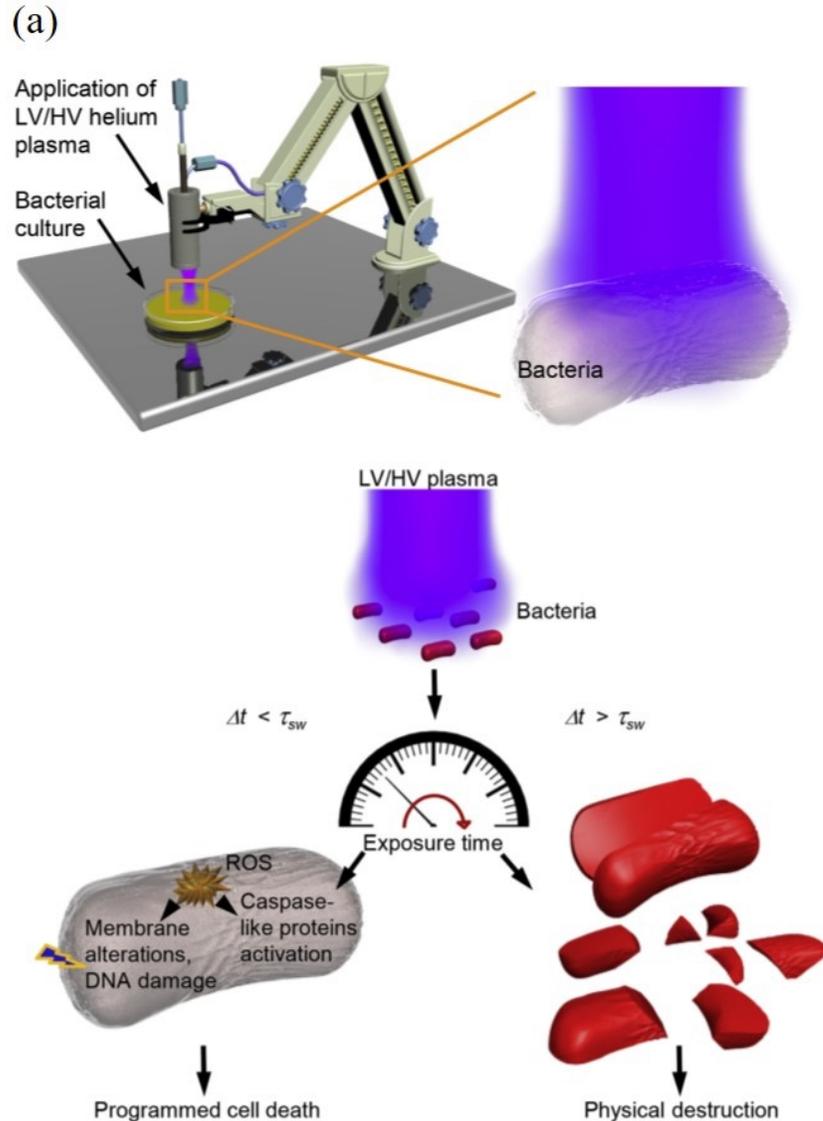
It has not been observed in anti-cancer studies until very recently.

However,

physical effect has been widely (or claimed to be) observed in anti-germ studies.

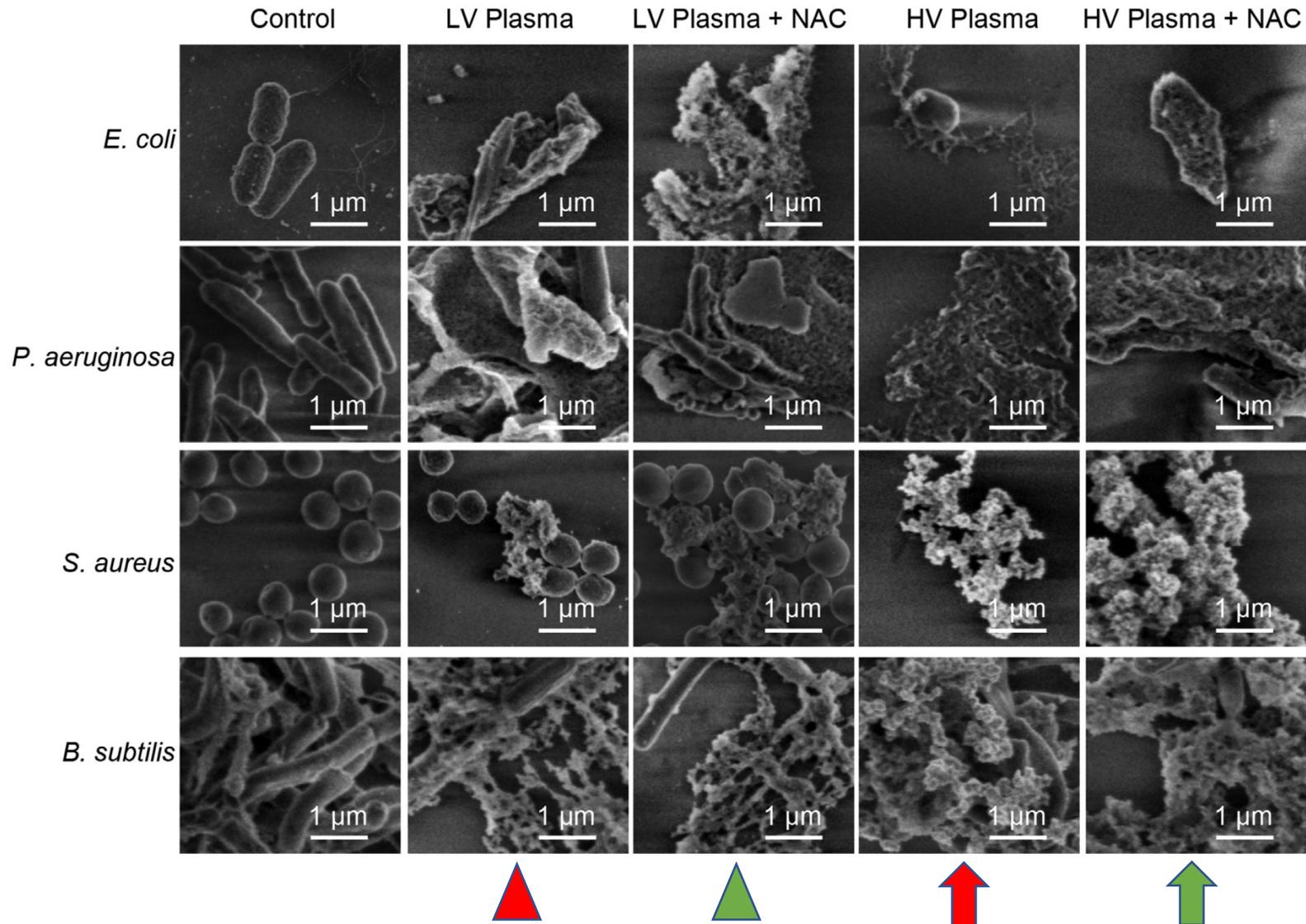
Why ??

Bacterial Death by CAP Treatment: widely observed structural damage on cell wall.



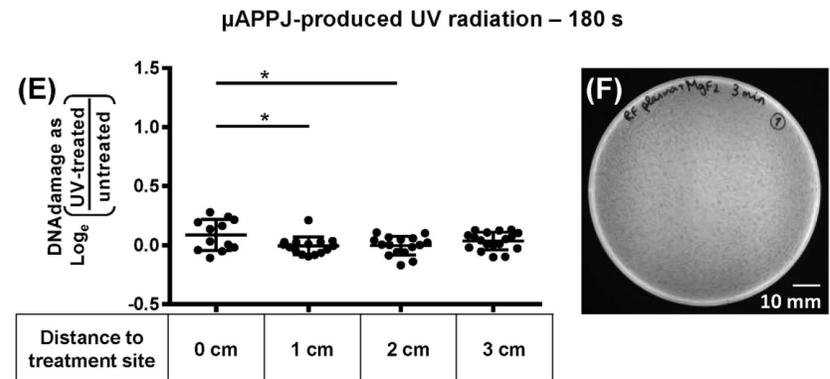
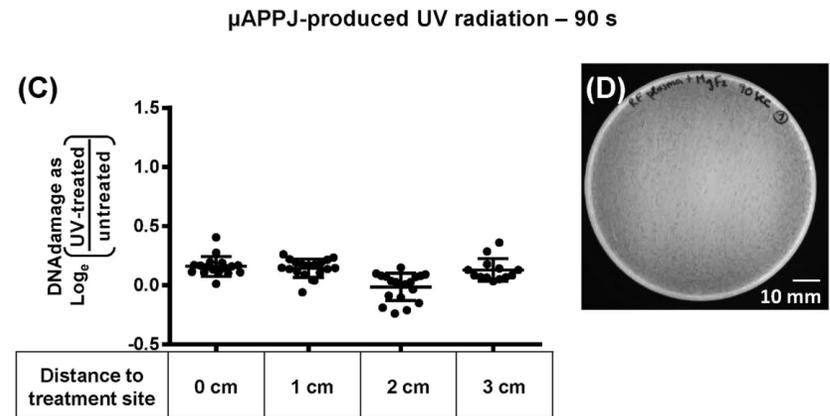
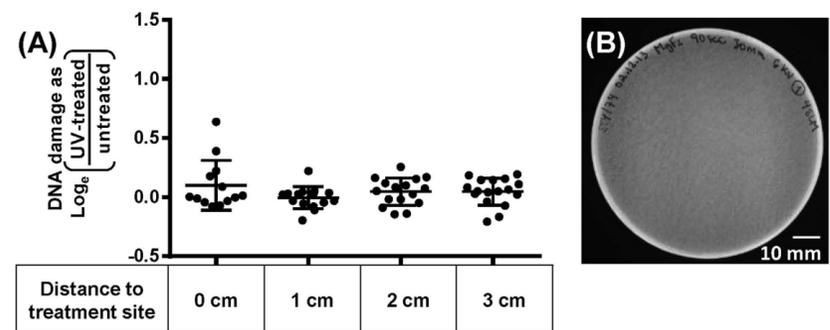
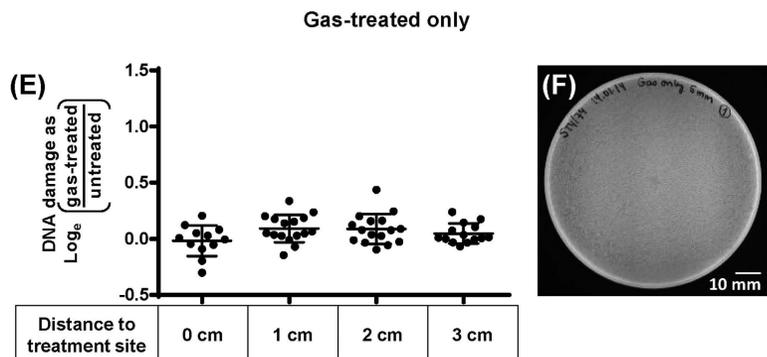
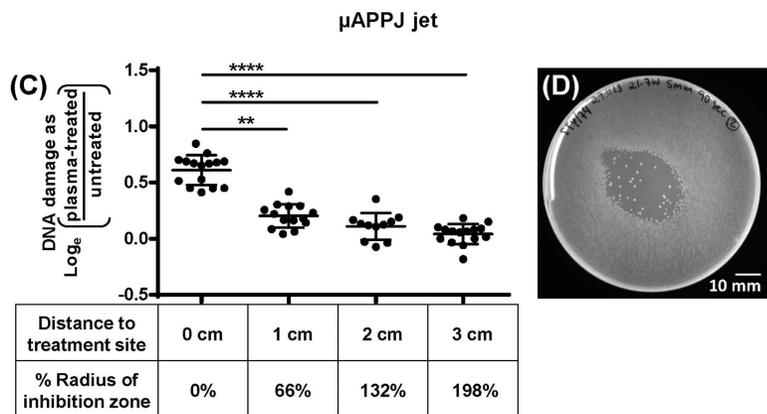
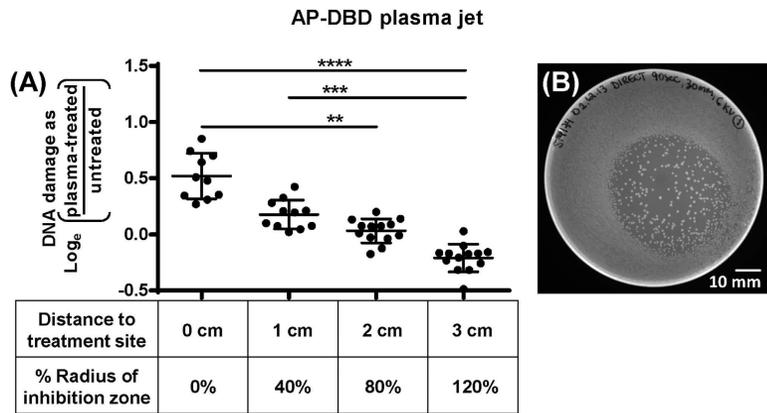
(a) The interplay of physical destruction and biological cell death upon CAP treatment. (b) False-colored Scanning electron microscope (SEM) images of bacteria exposed to CAP with high discharge voltage (HV) and low discharge voltage (LV). (Lunov, Oleg, et al. *Biomaterials*. 82 (2016): 71-83.)

ROS Scavenger Did not Inhibit the Anti-germ Effect



ROS scavenger N-acetyl-L-cysteine (NAC, 5 mg/ml) did not prevent physical disruption induced by non-thermal plasma. SEM of untreated (control) and bacterial strains after 60 s exposure with either HV or LV plasma. Lunov, O., et al. 2016. Biomaterials, 82, pp.71-83.

UV's Role in Anti-germ Effect: no impact.



Cell Wall vs. Cell Membrane

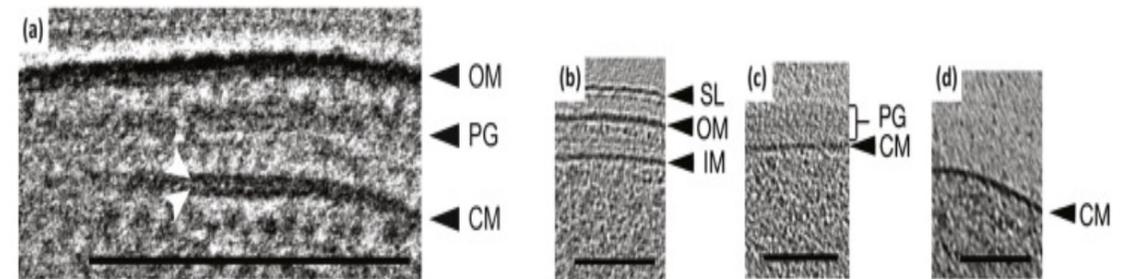
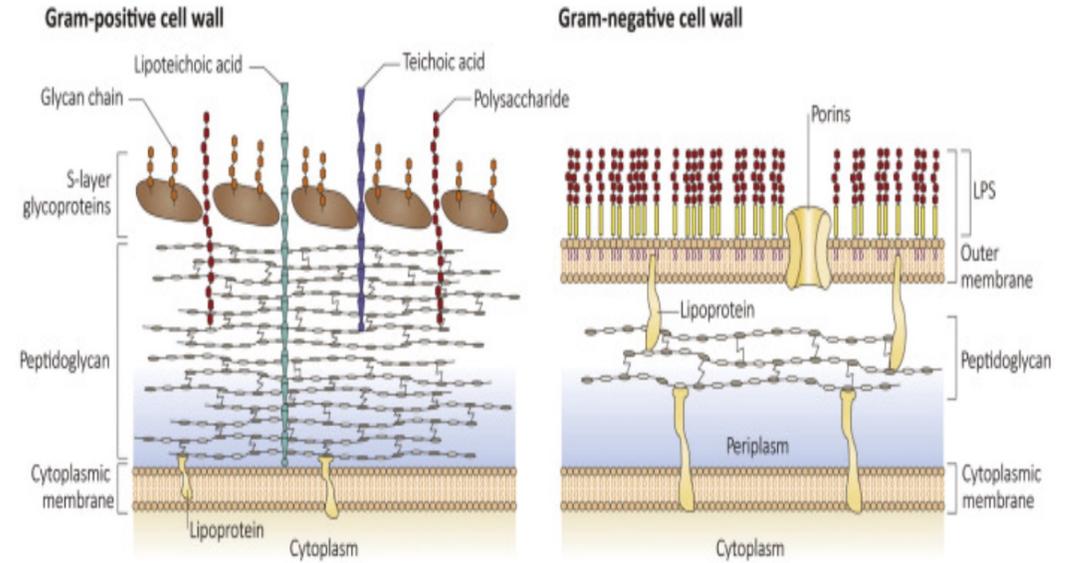
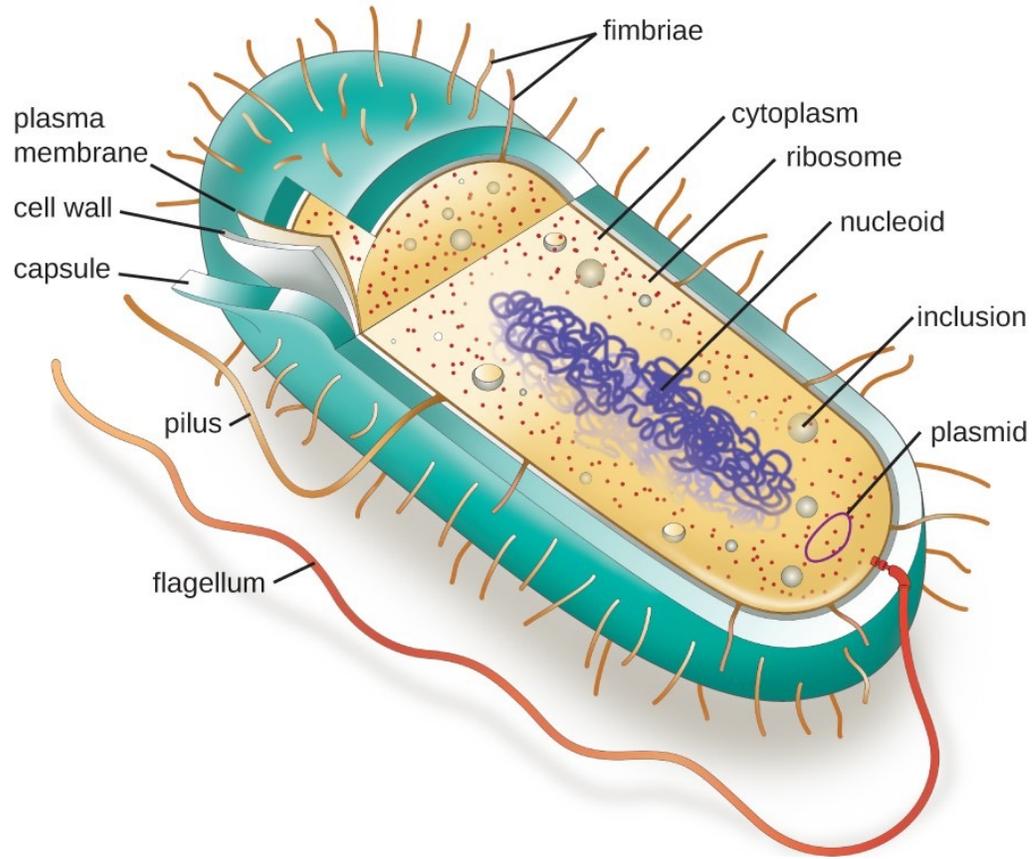
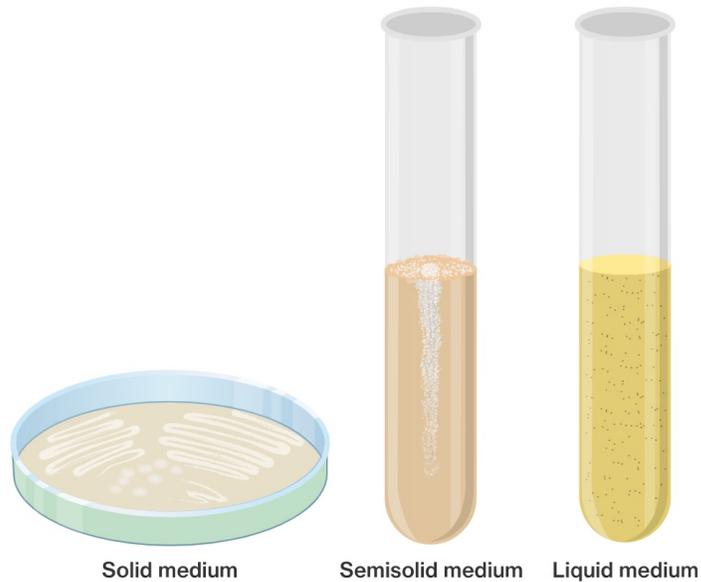


Fig. 1. (Upper panel) Schematic side view of cell envelopes of exemplarily Gram-negative and -positive bacterial cells. LPS, Lipopolysaccharides. (Lower panel) Electron micrographs show the architecture of a Gram-negative cell wall without S-layer showing the two leaflets of the cytoplasmic membrane (white arrows): (a) *Vibrio cholera*; a Gram-negative envelope with S-layer: (b) *Caulobacter crescentus*; Gram-positive cell wall: (c) *Listeria monocytogenes*; cell-wall less: (d) *Mycoplasma pneumoniae*. IM, inner membrane; OM, outer membrane; PG, peptidoglycan; SL, S-Layer; CM, cytoplasmic membrane; LPS, Lipopolysaccharides. Scale bars, 100 nm.

Solid Medium was Widely used in Bacterial Culture

Types of Growth Media



Solid medium

Semisolid medium

Liquid medium

Microbes can be grown in liquid, semisolid, or solid media, depending on the traits of the microbe.

<https://www.coursehero.com/sg/microbiology/culturing-techniques/>



Bacillus subtilis(3)



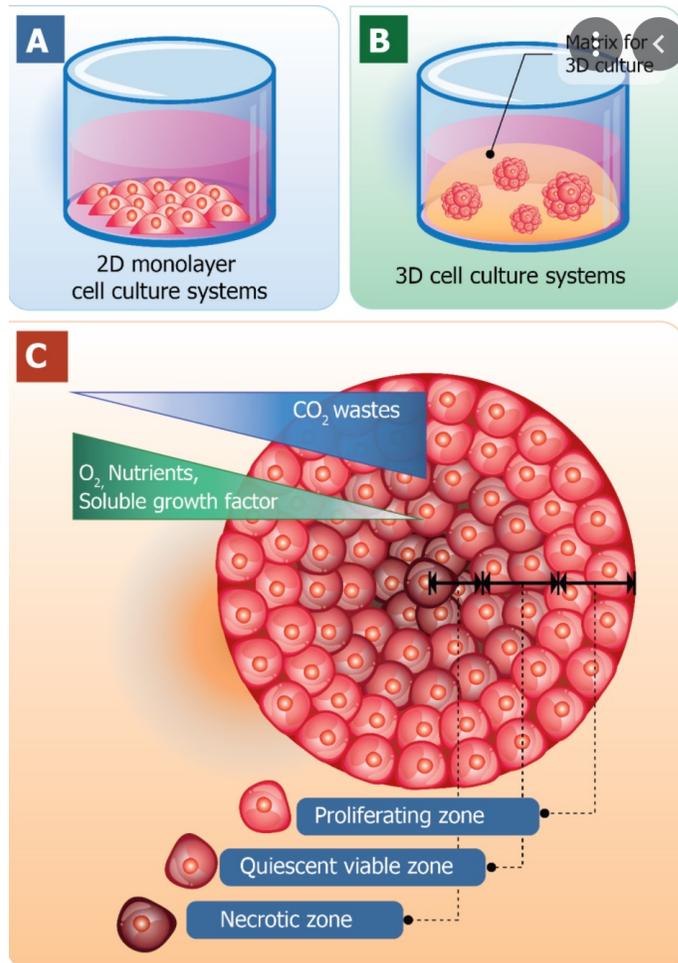
Candida Albicans is a type of yeast that can grow on the surface of skin(7)



Round yeast colonies(8)

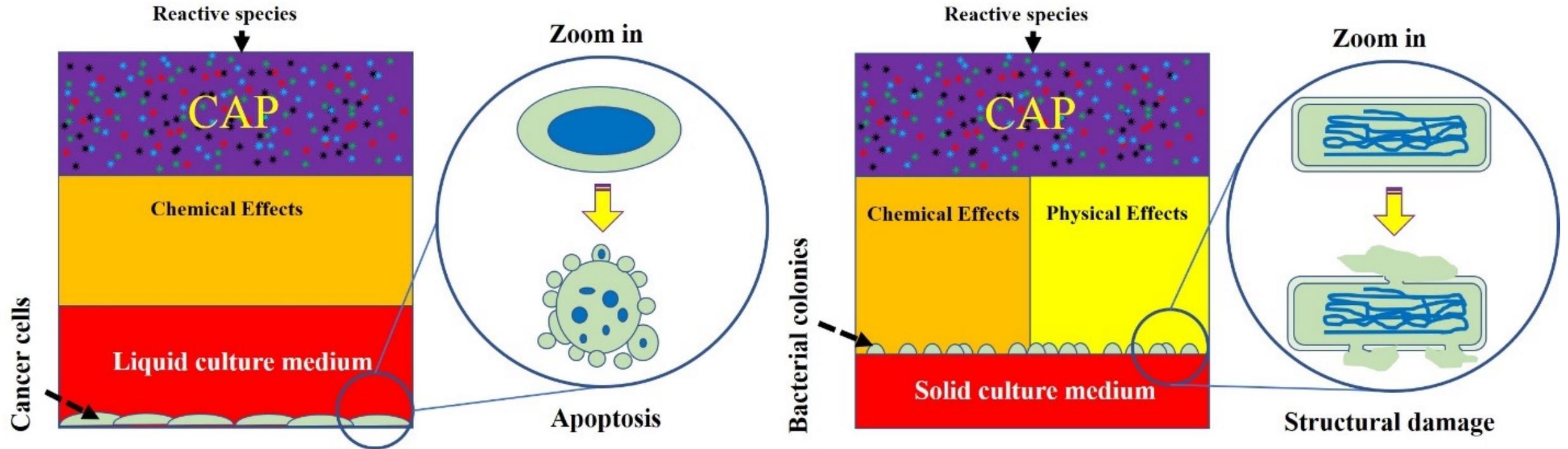
<https://www.sciencebuddies.org/science-fair-projects/references/interpreting-agar-plates>

Only Liquid Medium was used in Cancer Cells' Culture



<https://www.alamy.com/pipette-adding-sample-to-stem-cell-cultures-growing-in-pots-used-to-image65458433.html>

Comparison between Anti-cancer and Anti-germ

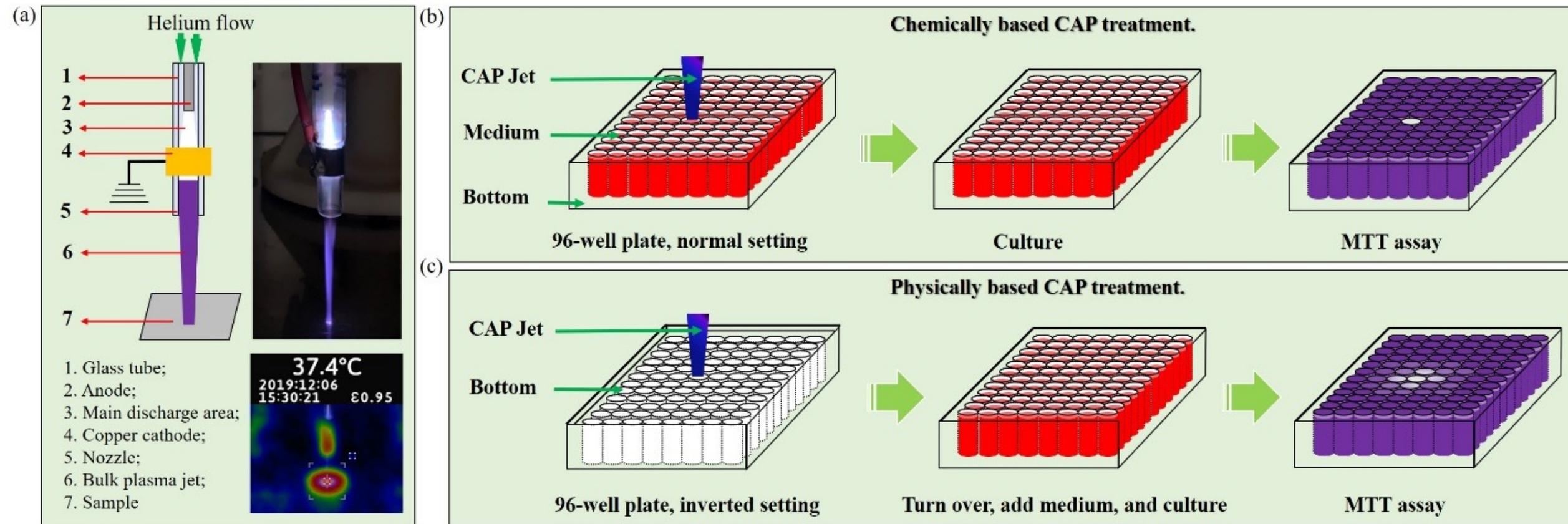


A schematic illustration of typical CAP treatment on cancer cells and bacteria *in vitro*. In most experimental setups *in vitro*, cancer cells or mammalian cells were immersed in a layer of medium during direct CAP treatment. In contrast, many bacterial cells were directly exposed to bulk CAP because solid culture medium was widely used in many cases. Such a different experimental tradition may naturally filter the physical effectors of CAP in the studies involving liquid culture medium.

Outlines

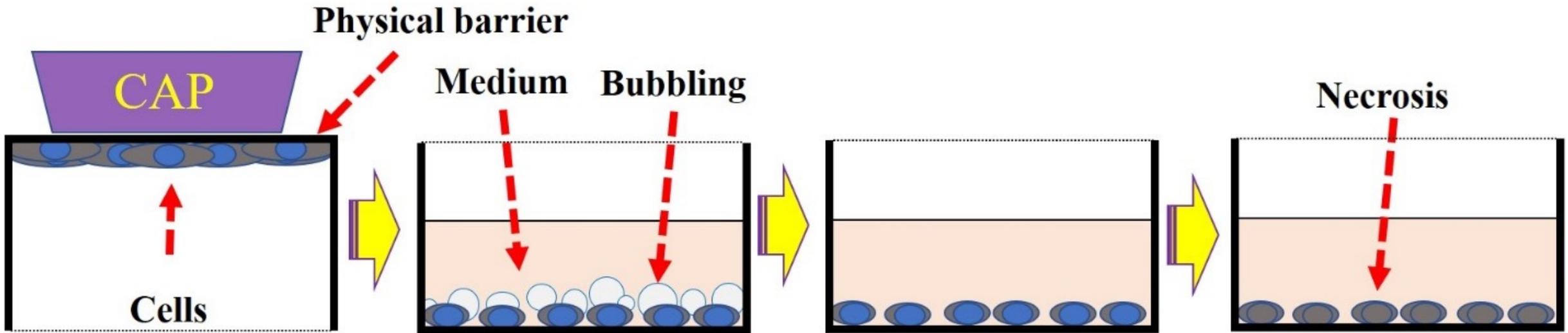
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Treatment on 96-well Plates



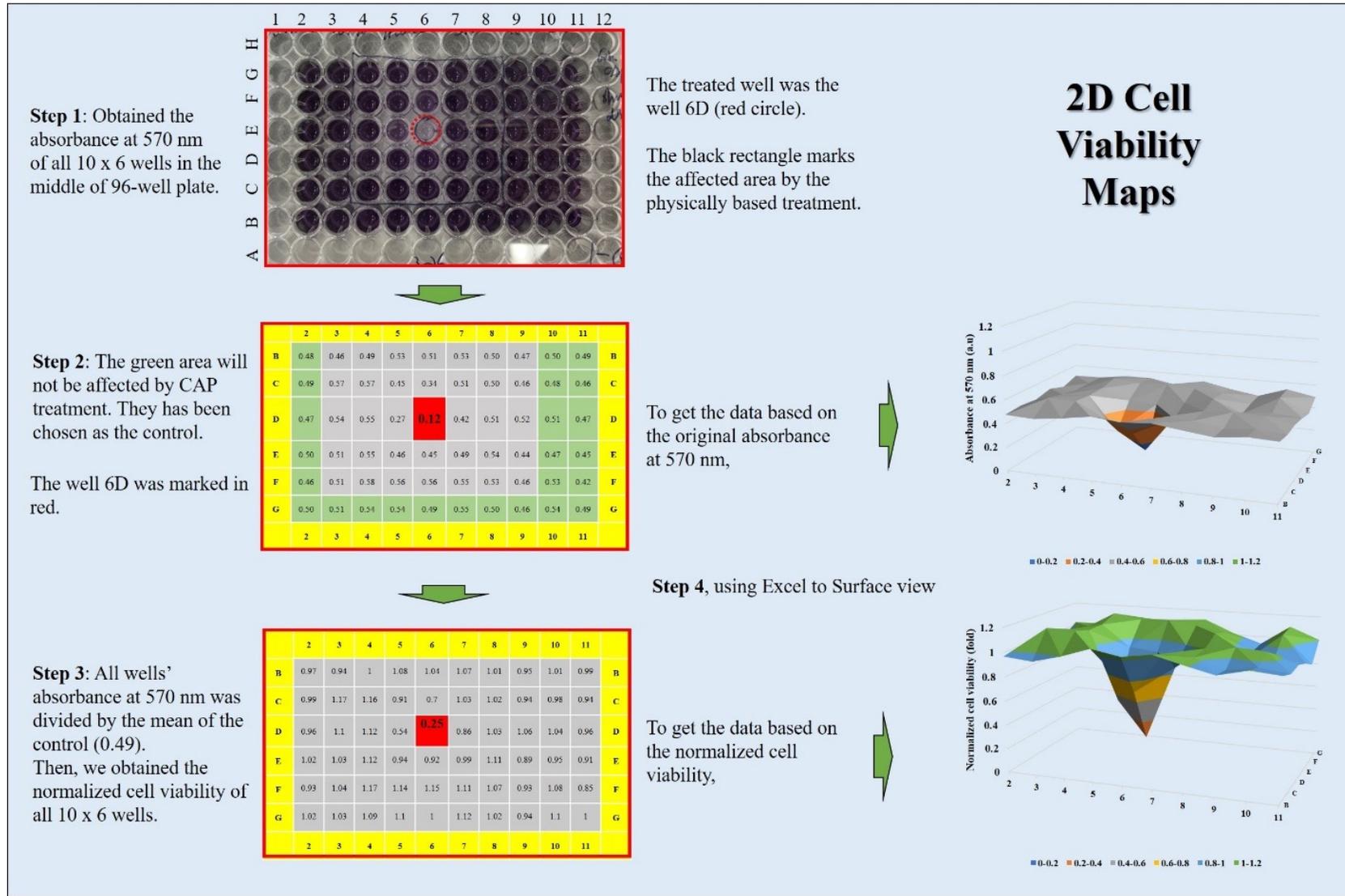
(a) Schematic illustration and photo of CAP jet. Discharge occurred between a coaxial stainless anode and another copper ring grounded cathode. Helium as the carrying gas take the ionized gas out the nozzle with a flow rate around 1.5 lpm. The diameter of glass nozzle was 4.5 mm. An infrared photo (FLUKE Visual IR Thermometer) was shown in bottom panel. In infrared photo, the CAP jet treated the bottom of a 35-mm dish. Yan, D., et al., 2020. *Scientific Reports*, 10, p.11788.

A Way to Reveal Physical Effect



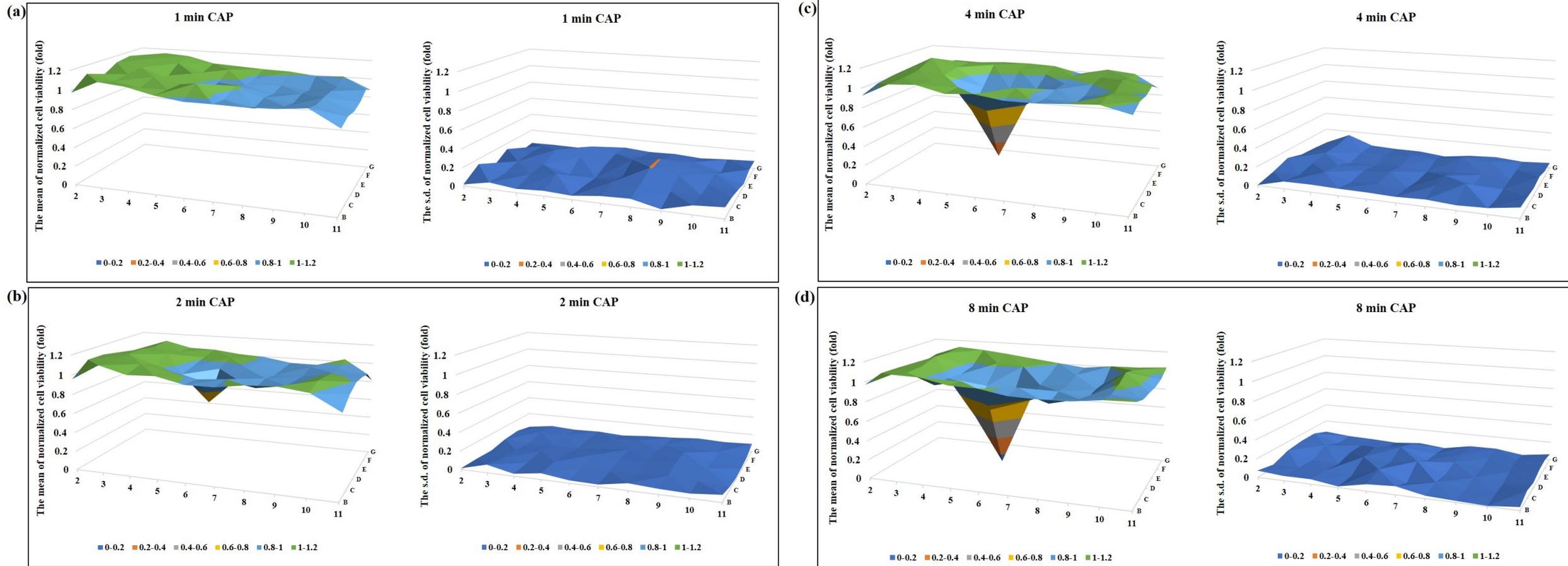
Schematic illustration of a physically-based CAP treatment *in vitro*. Here, the feature of necrosis has been used to represent the cell death pathway observed in the references. The quick bubbling and the quick cytosolic aggregation are two key features of the physically-based cell death.

3D Cell Viability Maps for 96-well Plates

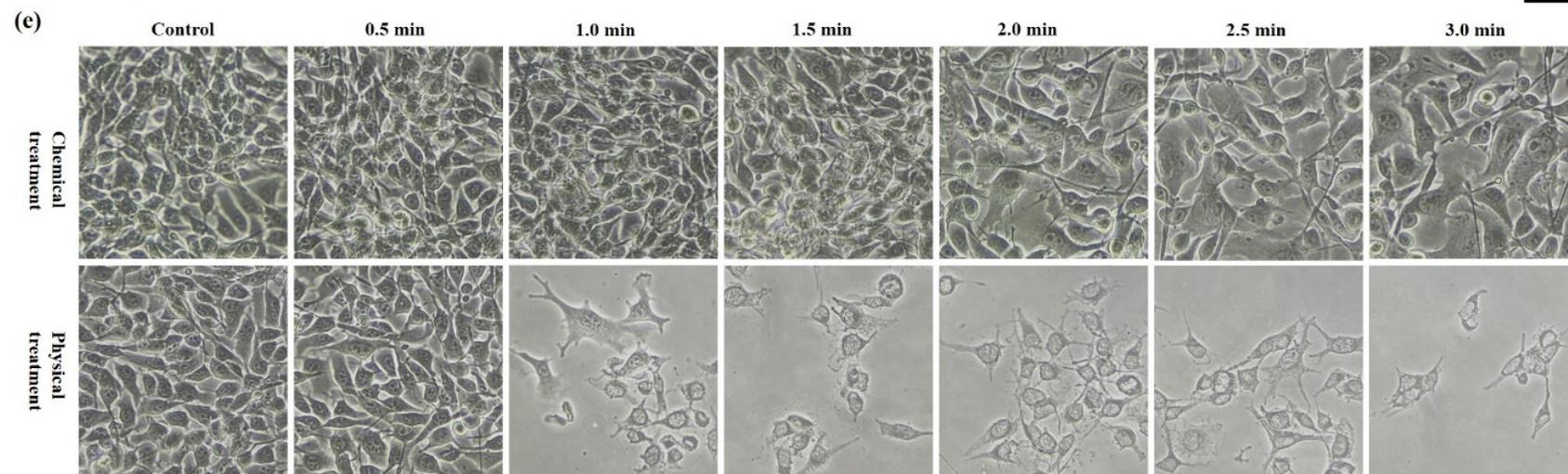
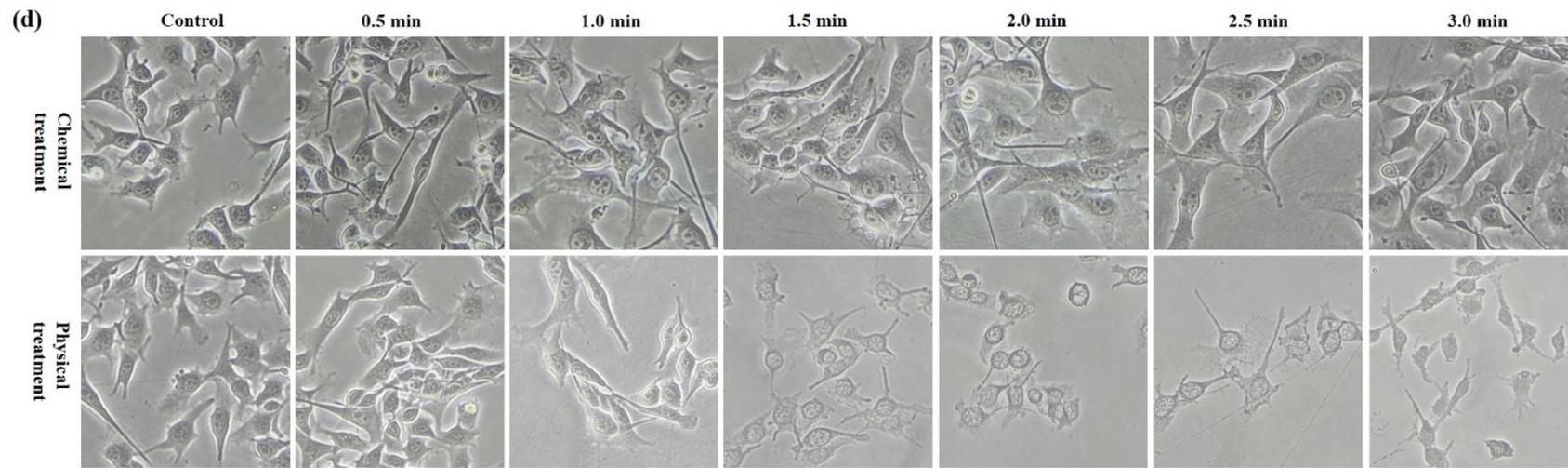
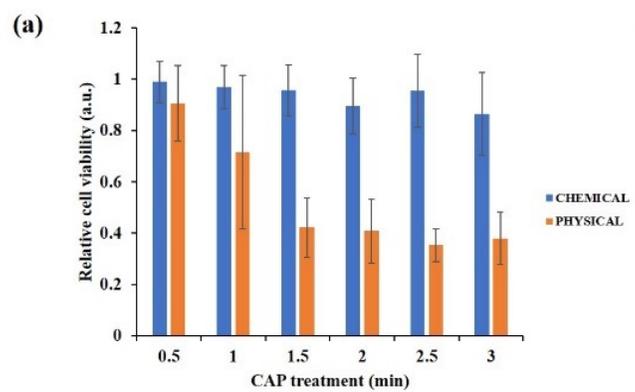


To obtain the map reflecting the normalized data, the original data would be divided by the mean value of control group. The data used to draw 3D maps was the mean of repeated tests. Each well's data were based on the mean value of the corresponding well's data in different repeats. The standard deviation (s.d.) of each well was also obtained, if needed, they could be shown in 3D maps based on s.d. Yan, D., et al., 2020. *Scientific Reports*, 10, p.11788.

Example: Physically-based Treatment



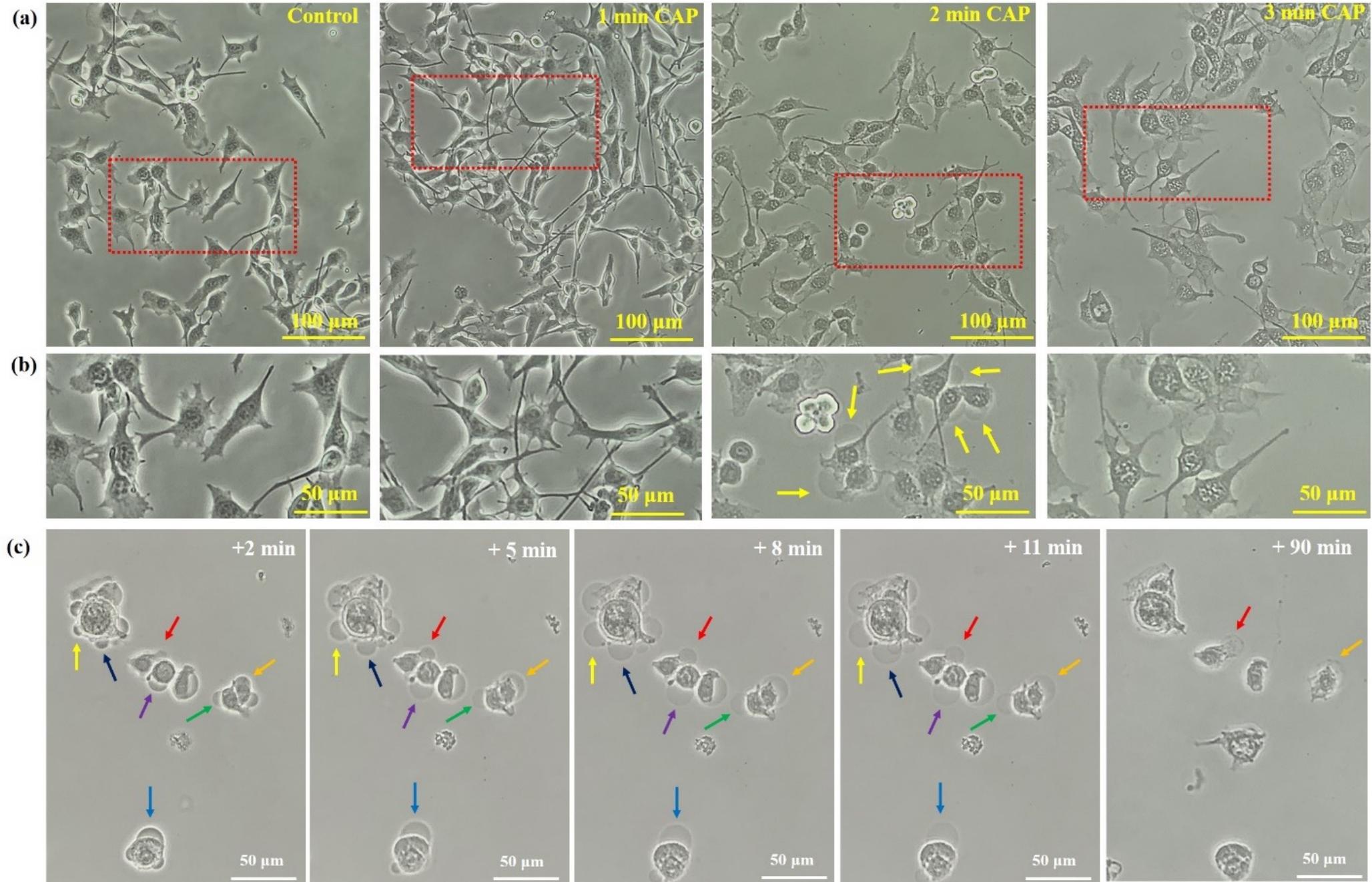
Physically-based CAP treatment effectively inhibited the growth of glioblastoma cells. CAP treatment was performed for 1 min (a), 2 min (b), 4 min (c), and 8 min (d). Treatment was performed on the bottom of 96-well plate, targeting the well 6D. For each case, 100 μL /well of U87MG (12×10^4 cell/mL) were seeded in 96-well plate and cultured for 7 h before treatment. Cells were cultured for 3 days before final MTT assay. In each case, mean value and s.d. of the normalized cell viability were presented in the left panel and the right panel, respectively. Yan, D., et al., 2020. *Scientific Reports*, 10, p.11788.



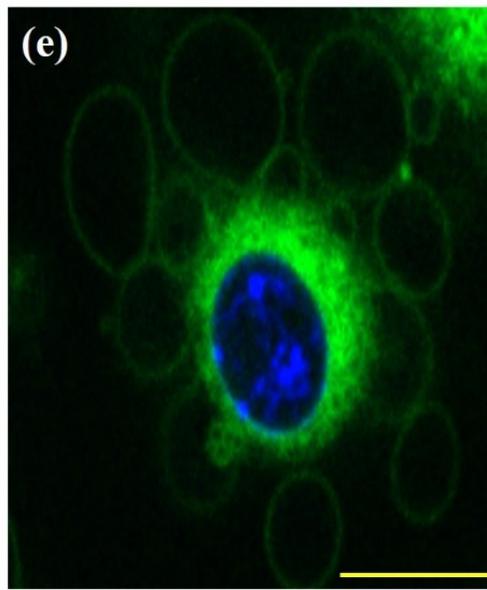
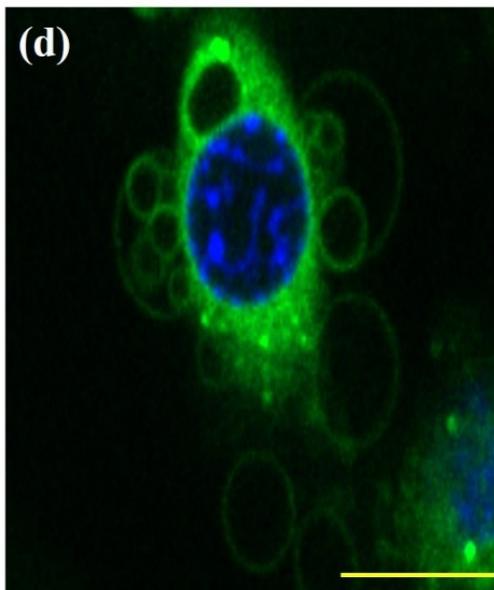
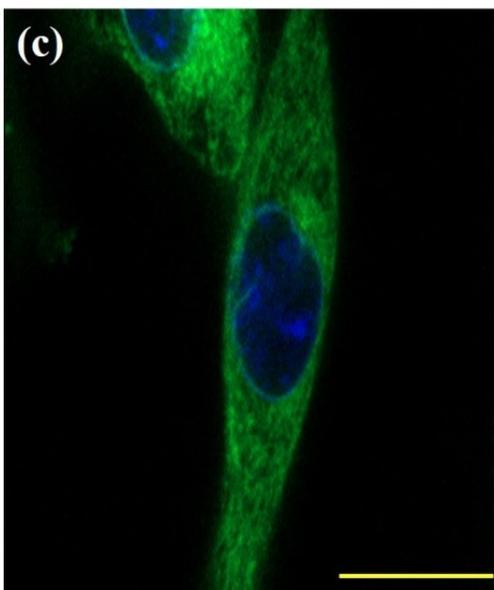
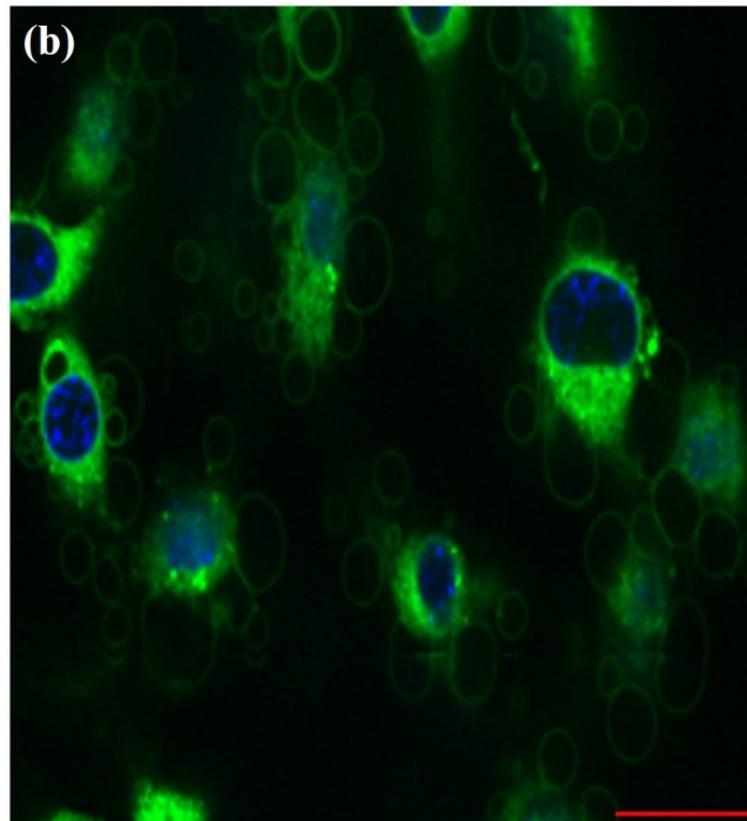
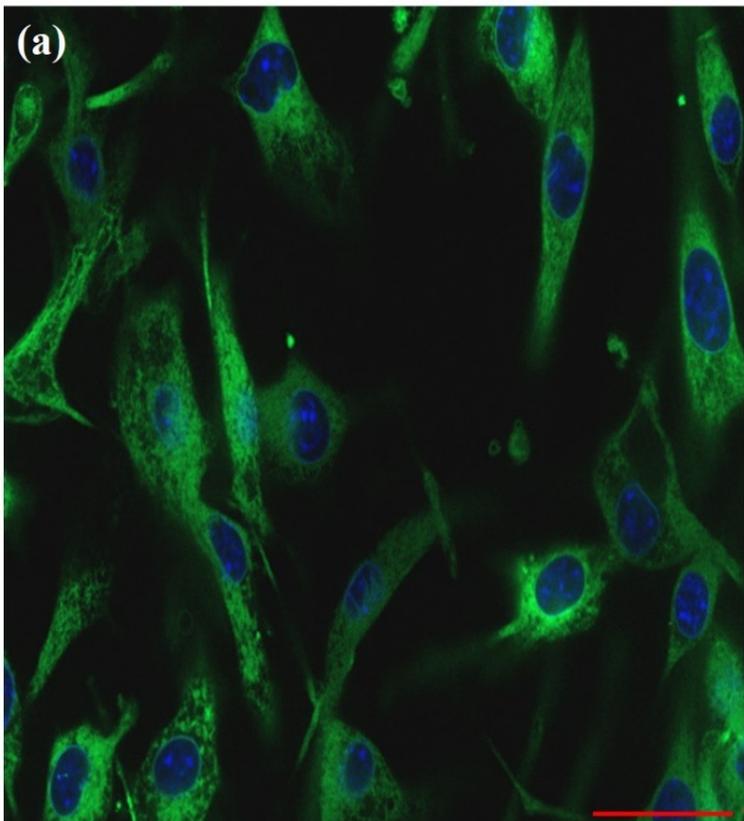
B16F10 melanoma cells

Yan, D., et al., 2020. ACS Applied Materials & Interfaces, 12(31), pp.34548-34563.

What Happened to Cells ?

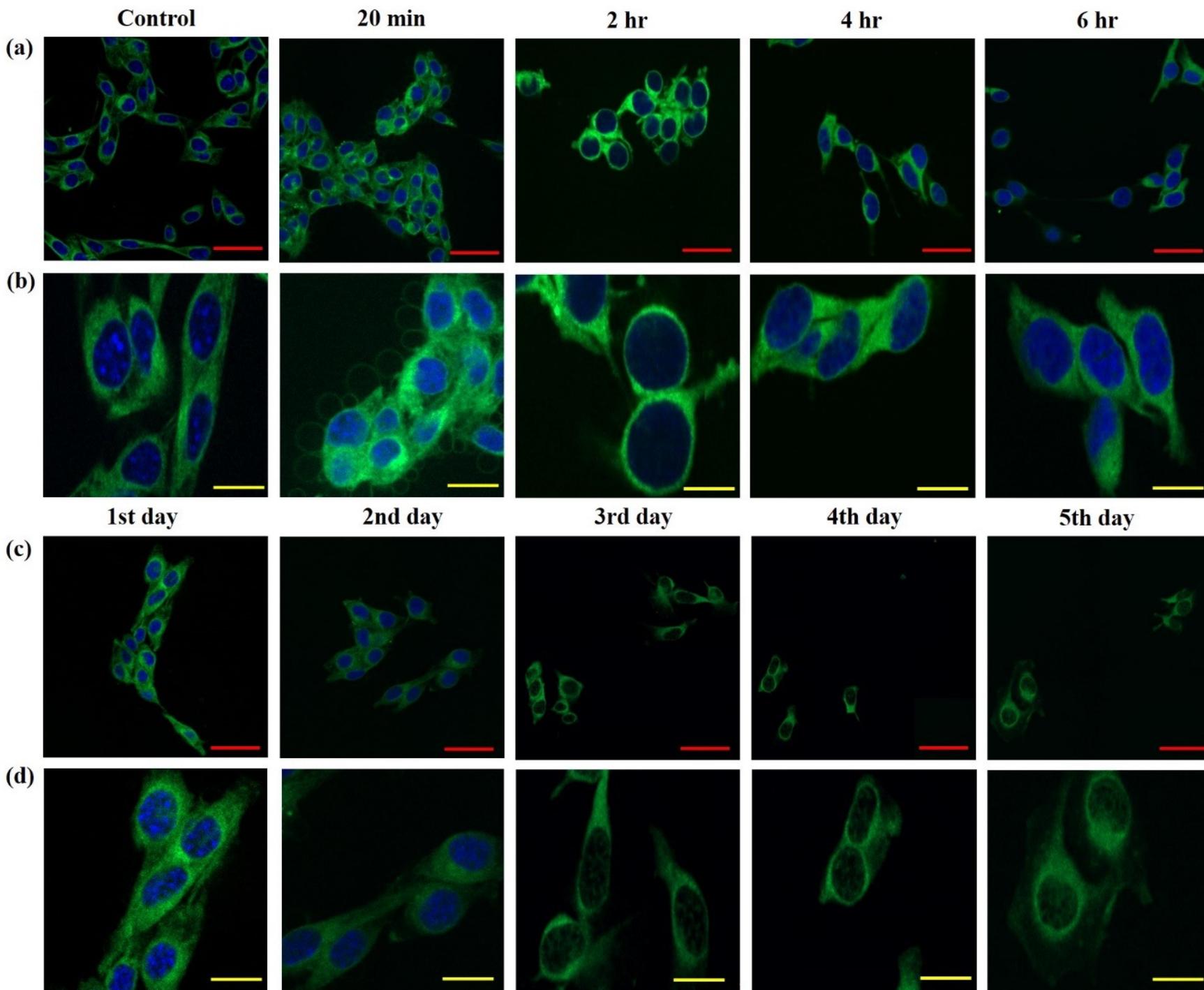


Yan, D., et al. ACS Applied Materials & Interfaces, 12(31), pp.34548-34563.



Live fluorescence imaging for the cellular change at 20 min after the physically based CAP treatment. (a) Control before the treatment. (b) 20 minutes after the treatment. (c) Zoomed-in photo of the control. (d) Example of the bubbling on a single cell. The growth of the small bubbles is illustrated in Video S1. (e) Another example of the bubbling on a single cell. The microtubules and DNA (nucleus) are shown in green and blue, respectively. The scale bars are 40 μm (red) and 20 μm (yellow). All images were processed using ImageJ software.

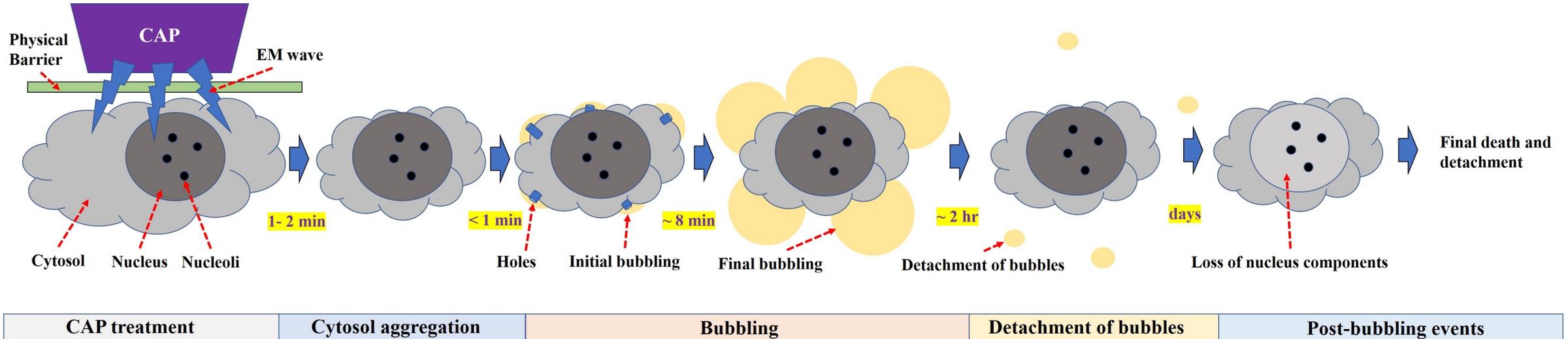
Yan, D., et al. 2020. ACS Applied Materials & Interfaces, 12(31), pp.34548-34563.



Fluorescence imaging of the physically based CAP-treated B16F10 cells over a long timescale. (a) Control before the treatment and the treated cells were observed 20 min, 2 h, 4 h, and 6 h after the treatment. (b) Zoomed-in photos. (c) CAP-treated cells were observed 1, 2, 3, 4, and 5 days after the treatment. (d) Zoomed-in photos. The microtubules and DNA (nucleus) are shown in green and blue, respectively. The gap between the nozzle and the bottom surface was 27 mm. The flow rate was 1.53 lpm. The scale bars are 40 μm (red) and 20 μm (yellow). All images were processed using ImageJ software.

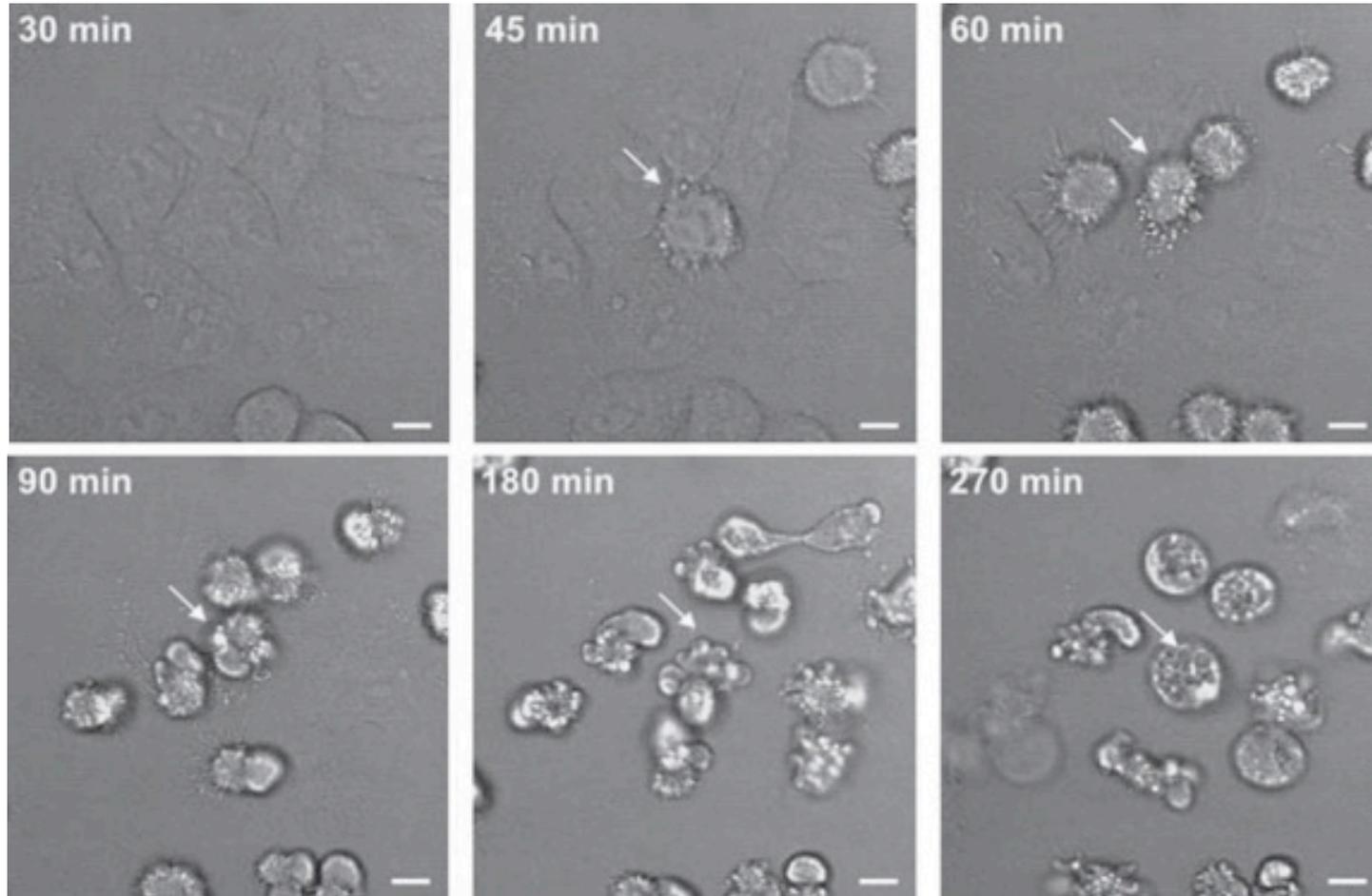
Yan, D., et al. ACS Applied Materials & Interfaces, 12(31), pp.34548-34563.

Whole Process of Physically Triggered Cell Death: a Necrosis



It has main four stages: cytosol aggregation, bubbling, detachment of bubbles, as well as postbubbling events. The first two stages are quick cellular response, totally lasting around 10 min. The detachment of bubbles may last more than 1 -2 hr. In contrast, the post-bubbling events may last days. The bubbling may be a process that the cells drastically lose it water components. The main cytosol shape will not change after the first stage, which gives cells an appearance as having been 'fixed' after the treatment. Such a 'fix shape' will be kept over days until the final cell death or detachment. (Yan, D., et al. (2020). ACS Applied Materials & Interfaces, 12(31), 34548-34563.)

Apoptosis Lasts Hours



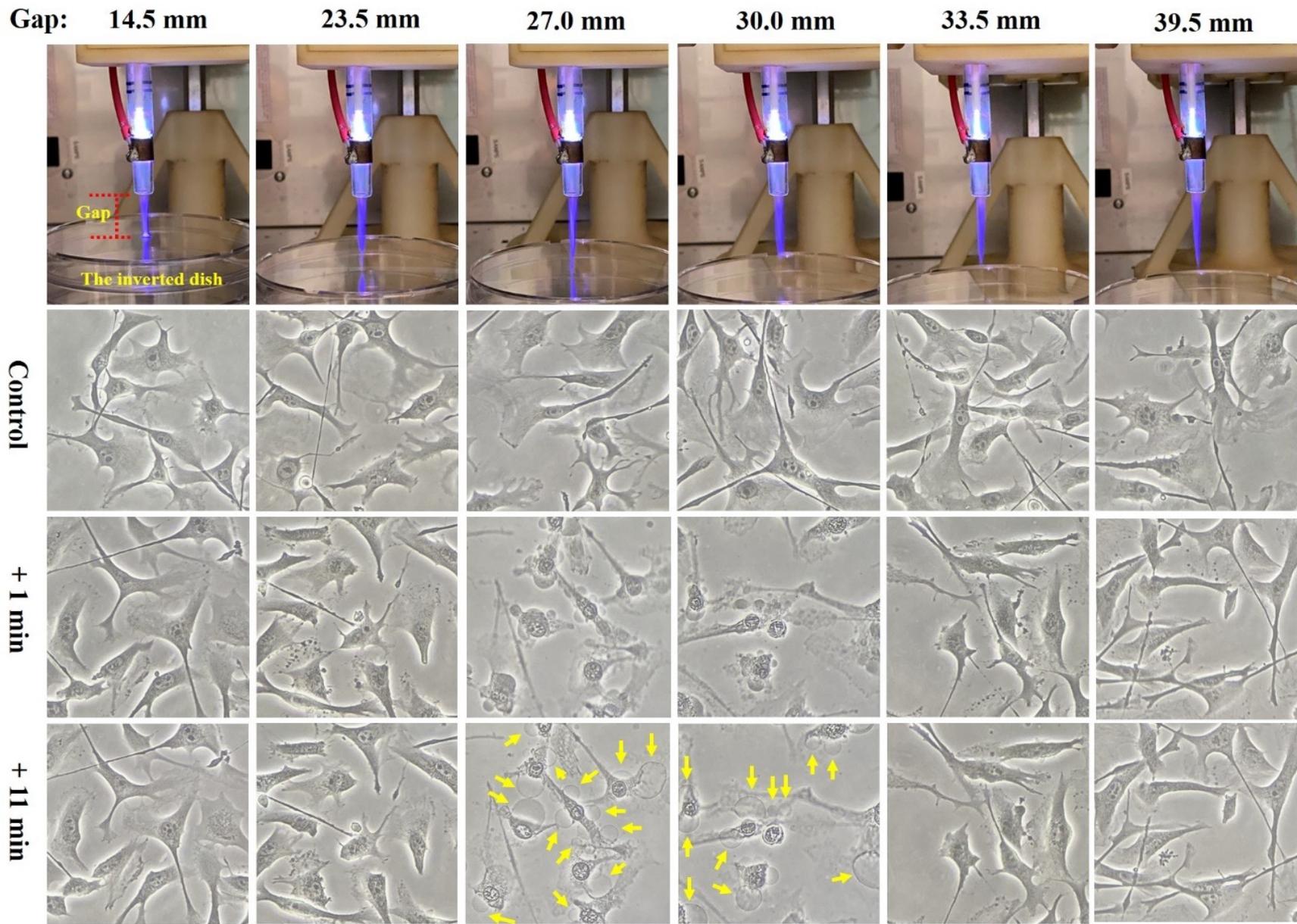
Apoptotic cell death of HeLa-CD95 cell lines upon stimulation with 1000 ng/ml anti-APO-1. Different HeLa-CD95 cell lines were stimulated with 1000 ng/ml anti-APO-1 and subjected to time-lapse microscopy. Apoptosis was detected based on morphological characteristics associated with apoptotic cell death, including condensation of the cytoplasm, membrane blebbing, and formation of apoptotic bodies. White arrows indicate a cell that shows typical characteristics of apoptotic cell death, that is, condensation of the cytosol (45 and 60 min), membrane blebbing (180 min), and formation of apoptotic bodies (270 min). Kober, A. M. M., et al. "Caspase-8 activity has an essential role in CD95/Fas-mediated MAPK activation." *Cell death & disease* 2.10 (2011): e212-e212.

Physical effect may only be observed under some specific conditions.

If it is so easily to be observed by a simple treatment, it should be observed years or a decade ago.

Details are the key !!

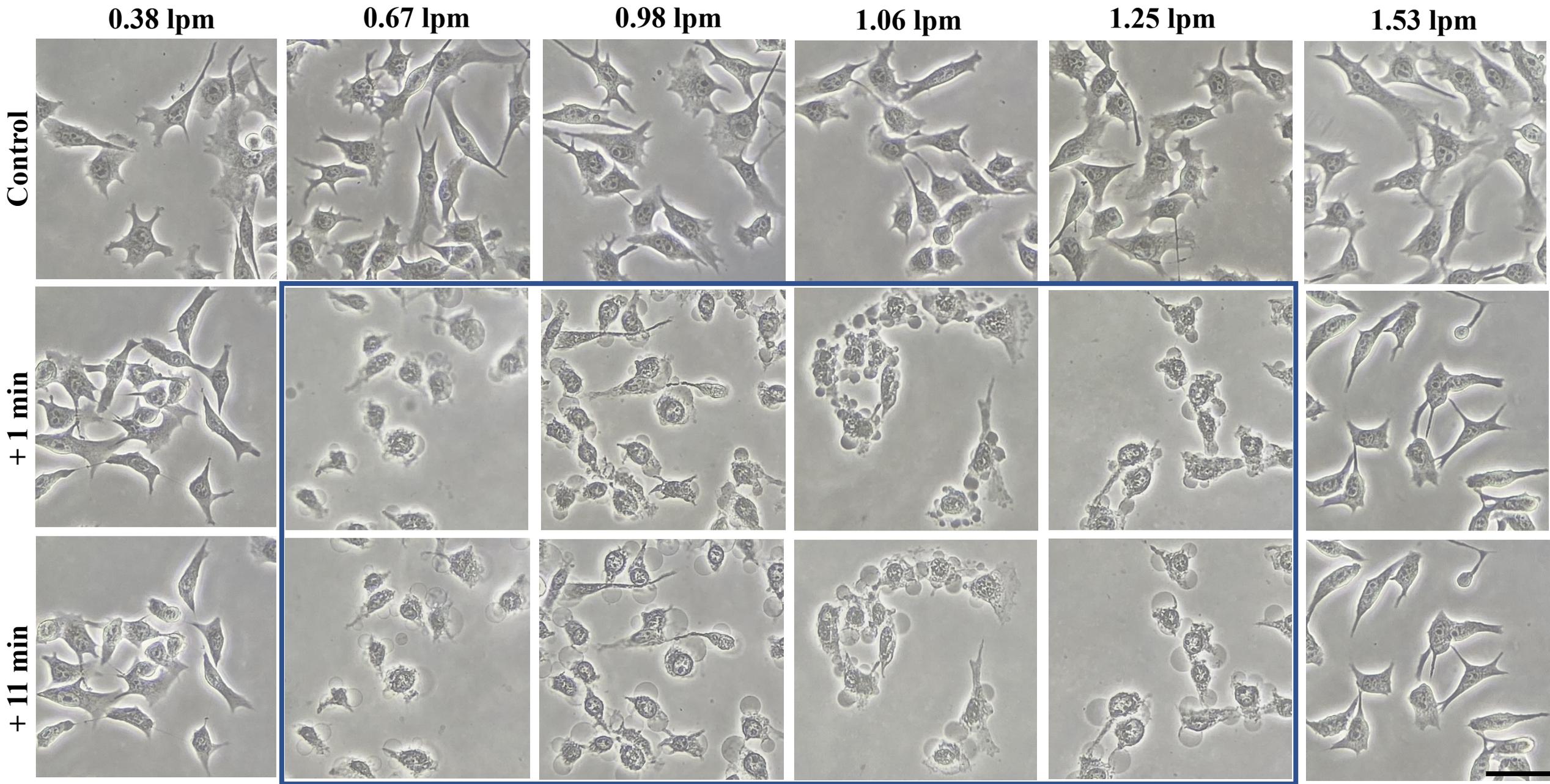
Gap between Nozzle and Target is Important



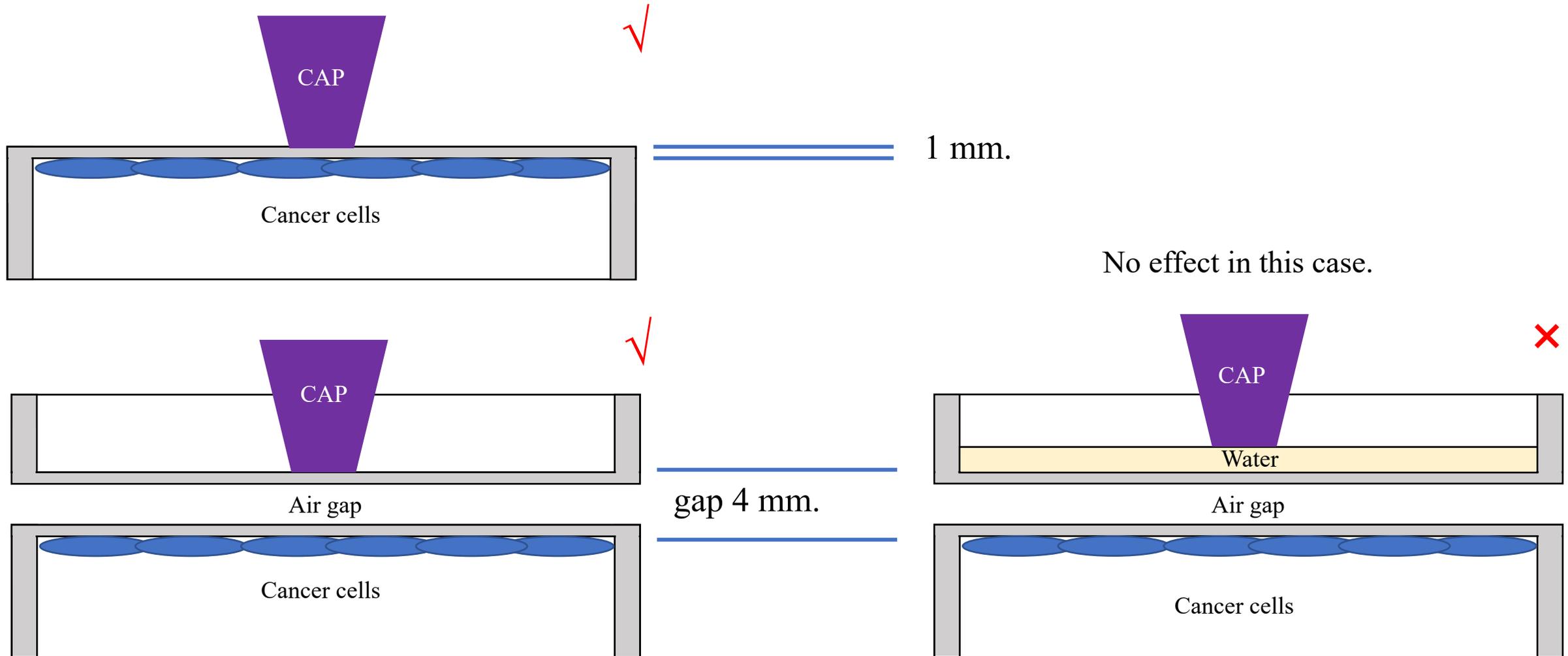
Bubbling occurs when the CAP source and the bottom surface of an inverted dish has a moderate gap. Neither too small nor too long gap will trigger bubbling. + x min means that the photo was taken at x min after the CAP treatment. Because the initial bubbles were not clear, only the bubbles after 11 min of growth were marked by yellow arrows. The scale bar was 50 μm (black).

Yan, D., et al. ACS Applied Materials & Interfaces, 12(31), pp.34548-34563.

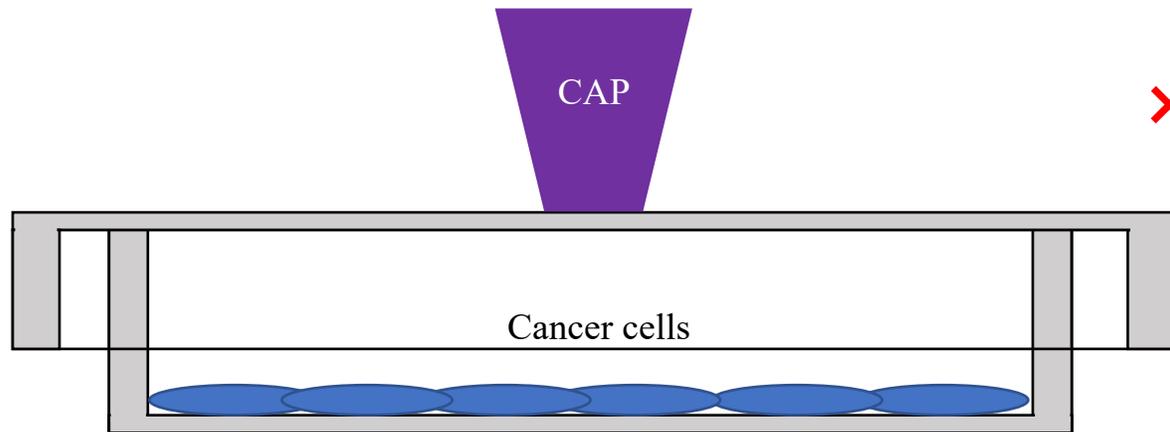
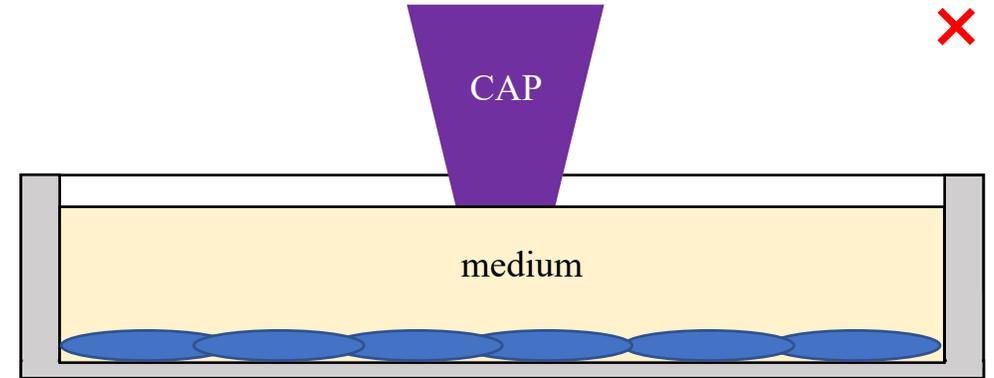
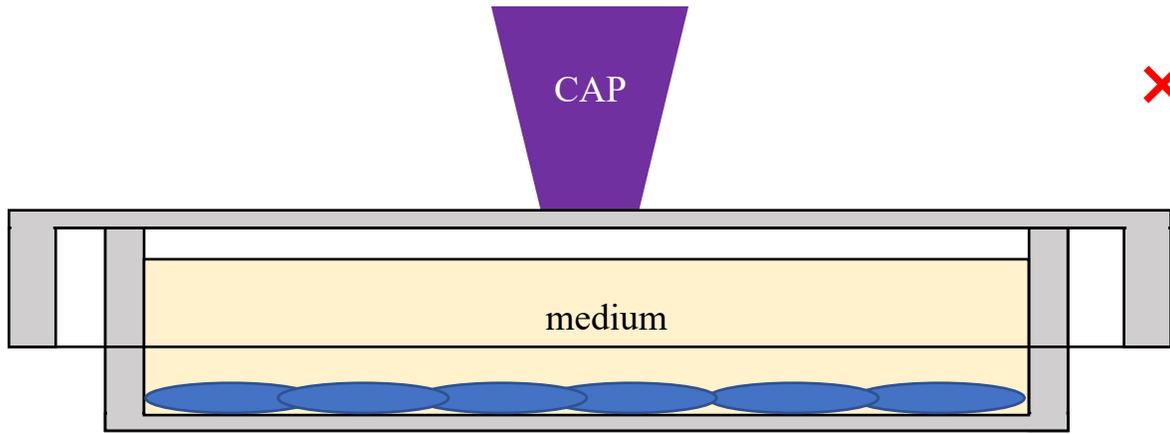
Flow rate of helium is Important



You may observe physical effect by following designs.

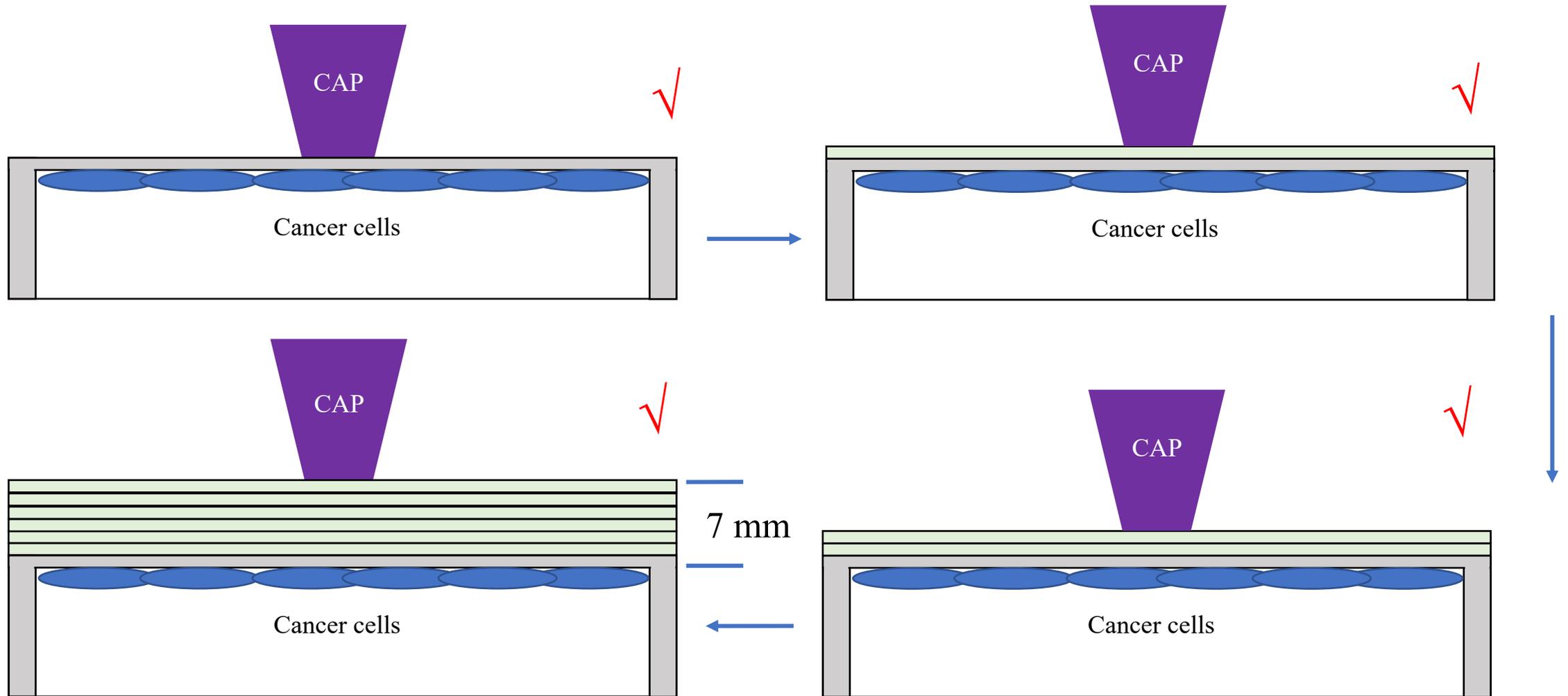


You may not observe physical effect by following designs.



gap: too large (19 mm for 12-well plate).

6 layers of glass slides will not completely block physical effect.

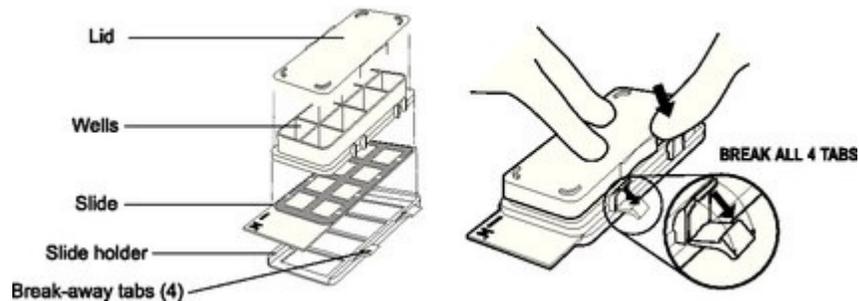


Another Example based on Cell Culture Slides.



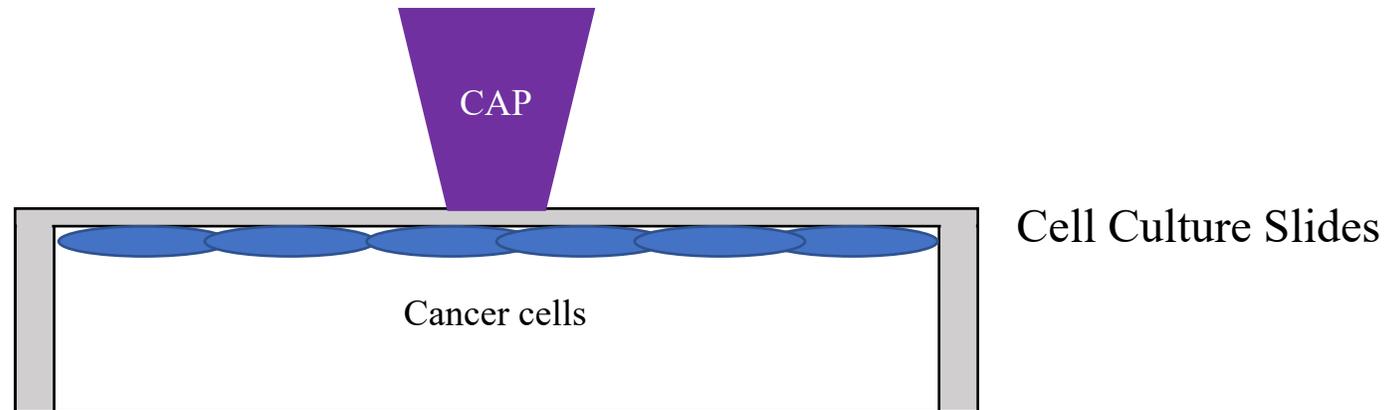
https://www.stellarscientific.com/cell-culture-chamber-slide-8-wells-polystyrene-chamber-glass-slide-sterile-6-pk-12-case/?gclid=CjwKCAjw8cCGBhB6EiwAgOReyy3fJx2rLTb3WDYTymRs7Wn-EafF45wCXN0yk1P6BdmNhWbm0oIBoCffoQAvD_BwE

<https://www.mattek.com/store/4-well-chambered-cell-culture-slide-case/>

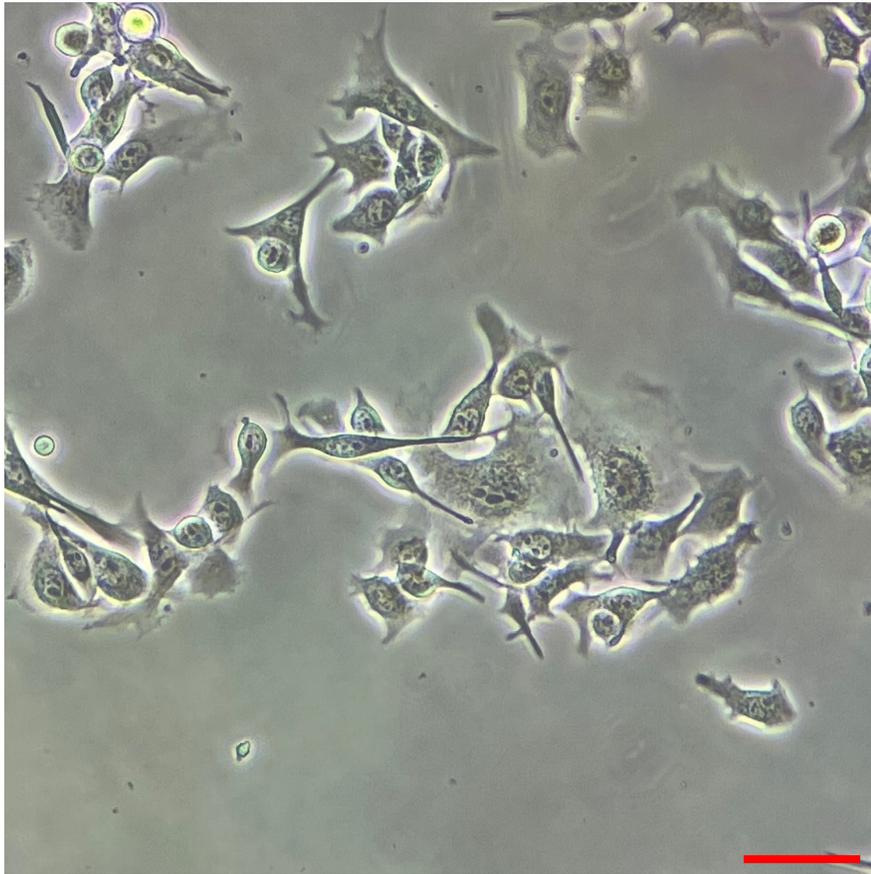


Cell Culture Slides: disassemble and re-assemble

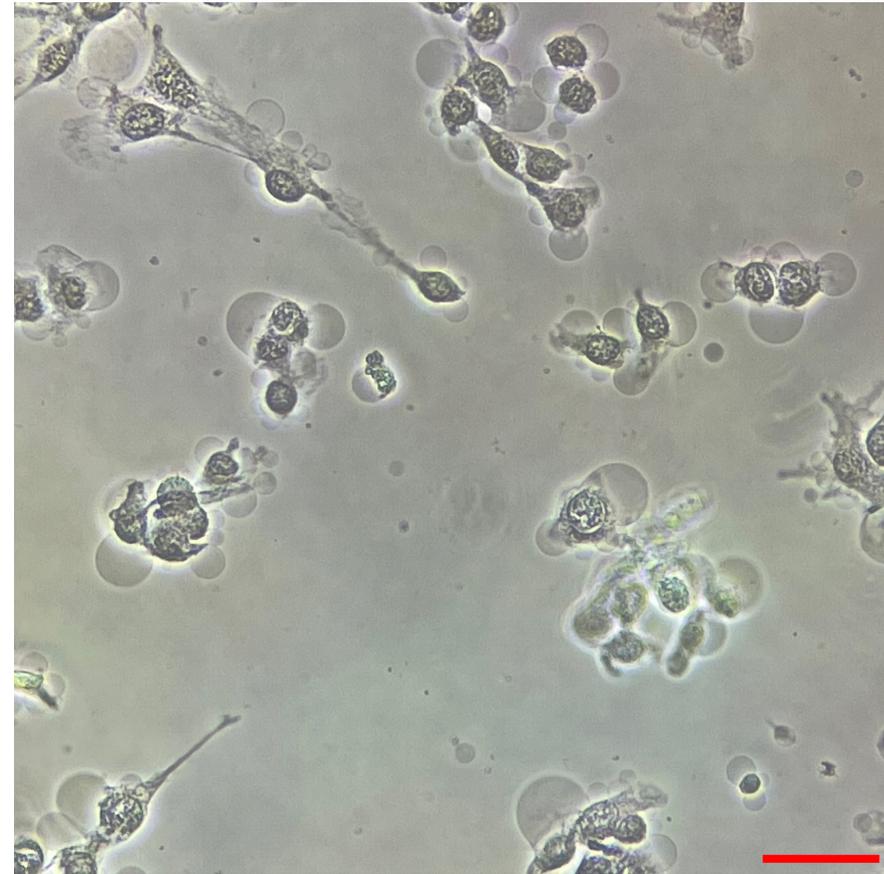
You can observe physical effect by following designs.



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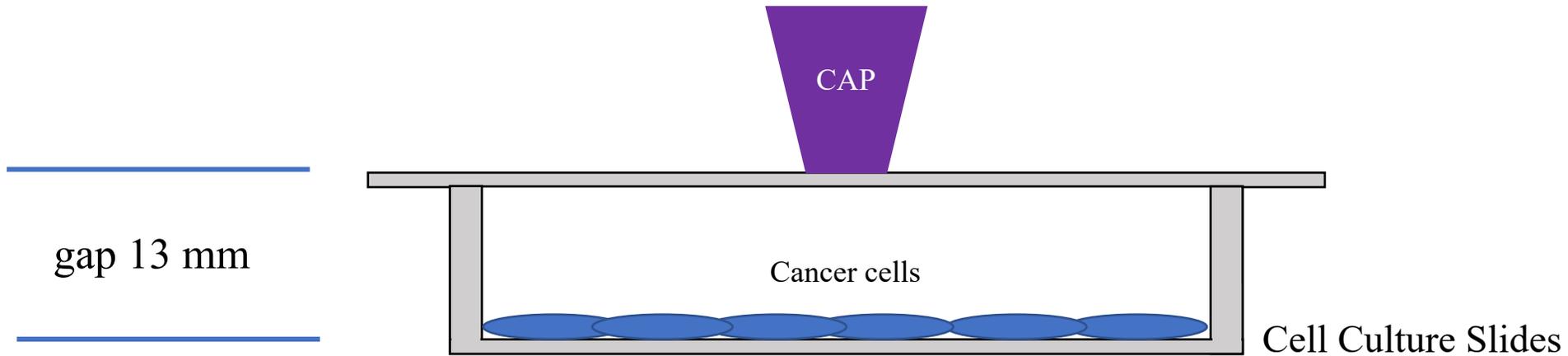
Control



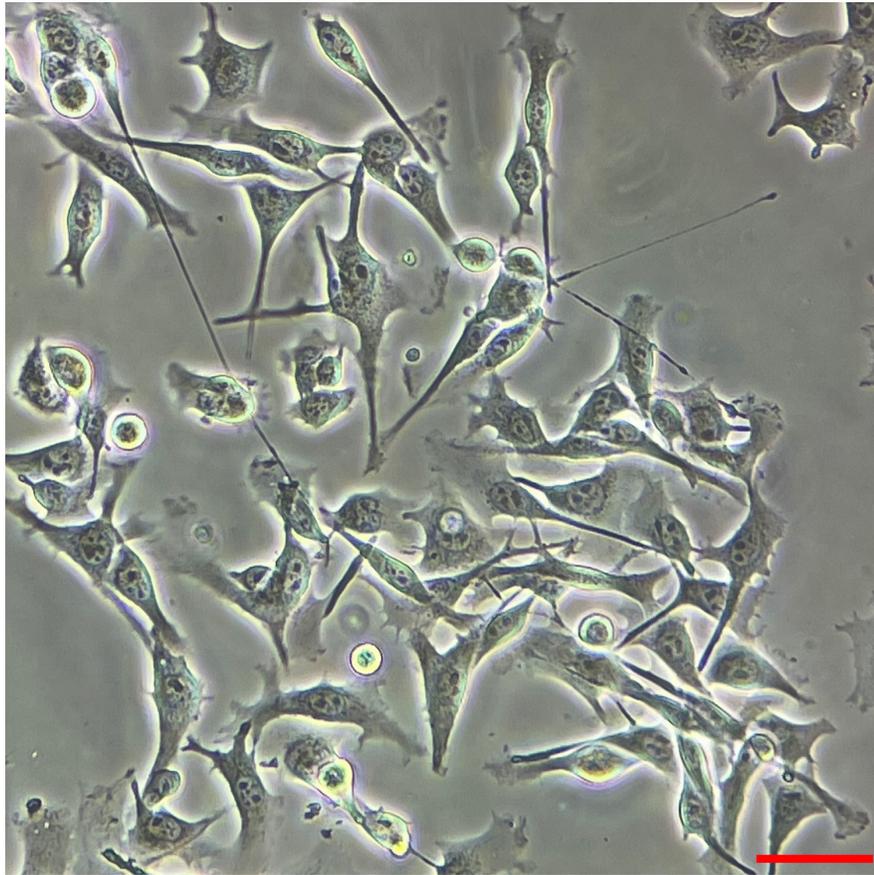
8 min after a CAP treatment

Scale bar: 50 μm

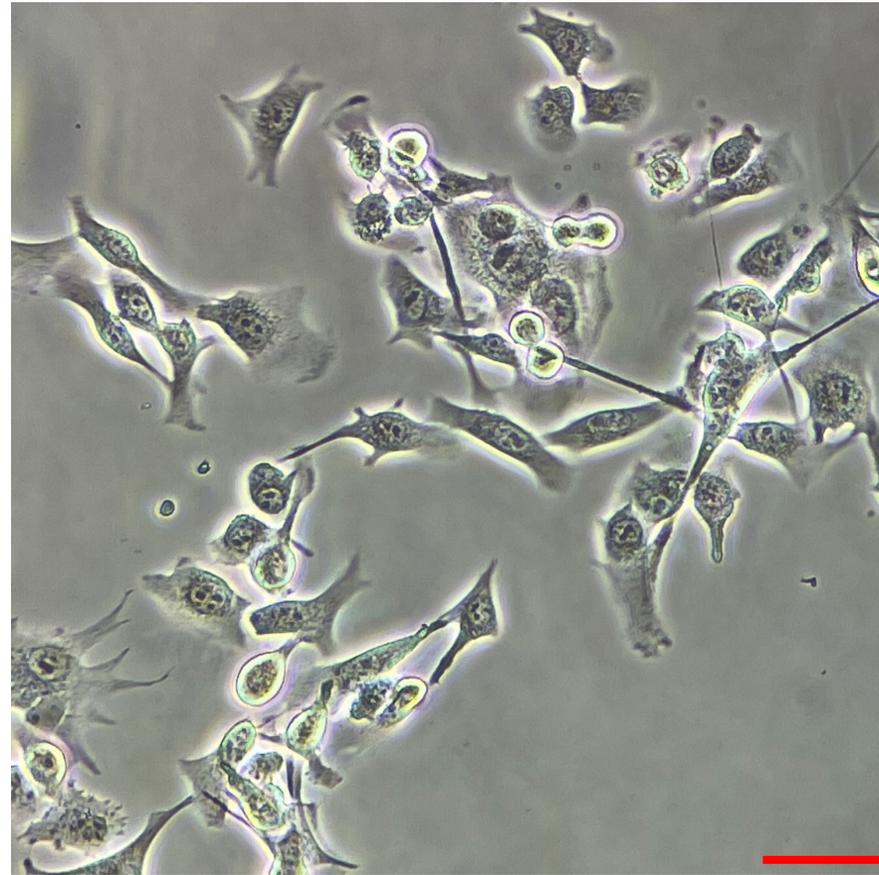
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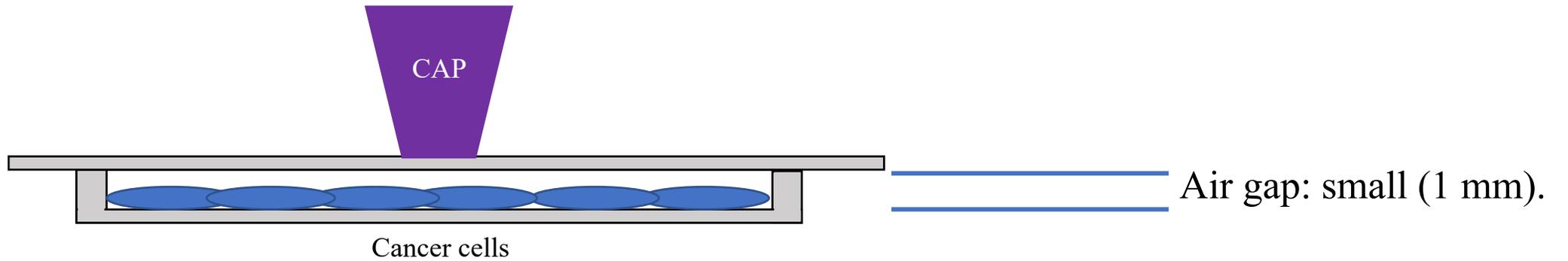
Control



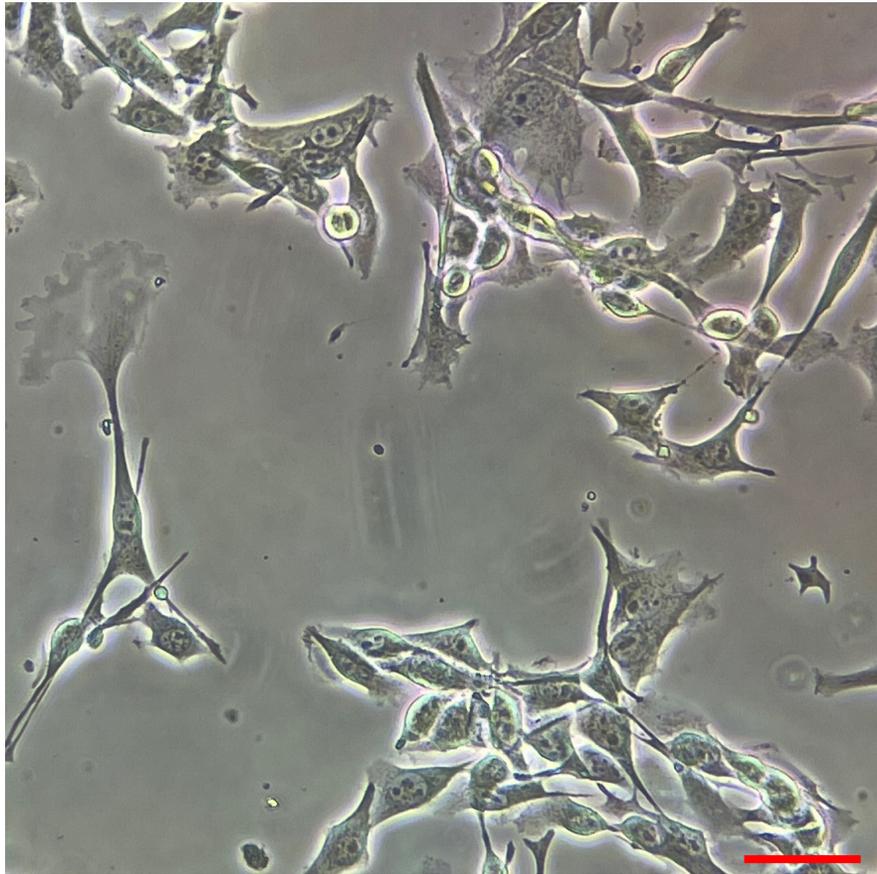
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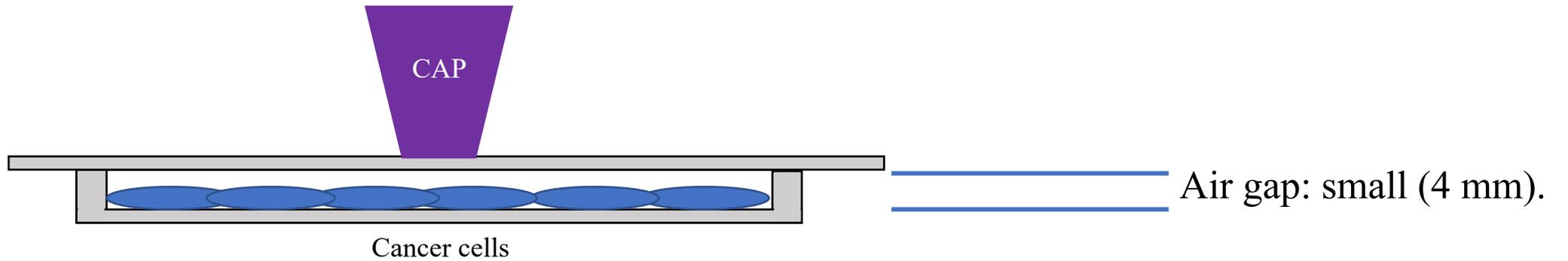
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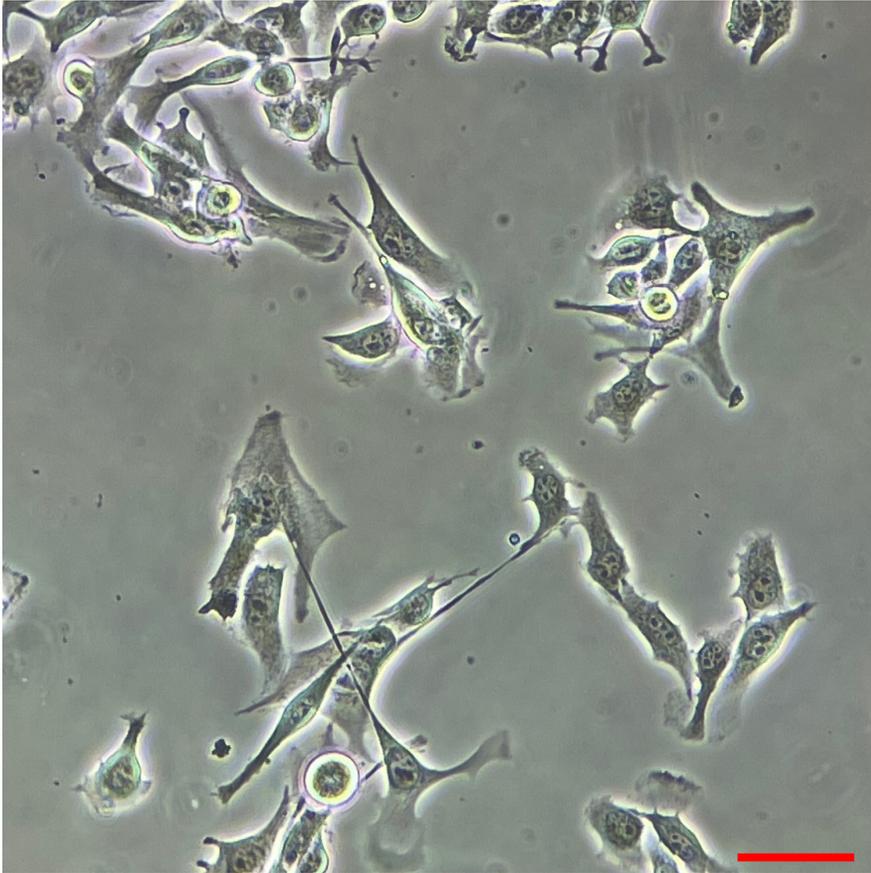
8 min after a CAP treatment

Scale bar: 50 μm

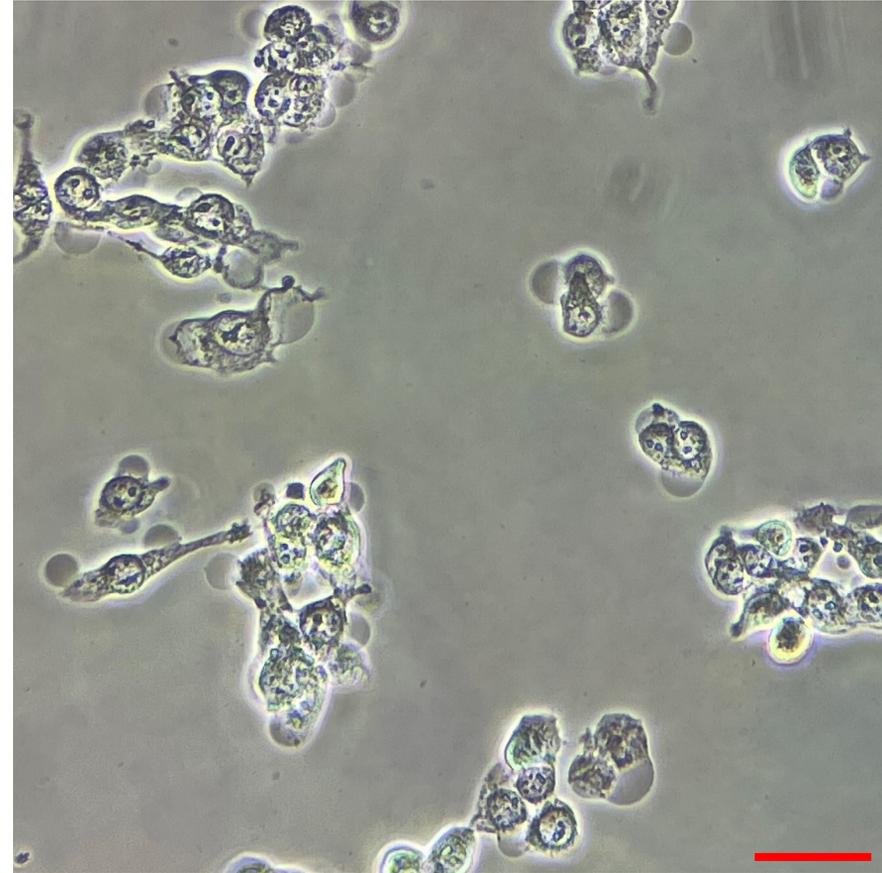
You may observe physical effect by following designs.



You may observe physical effect by following designs.



Control

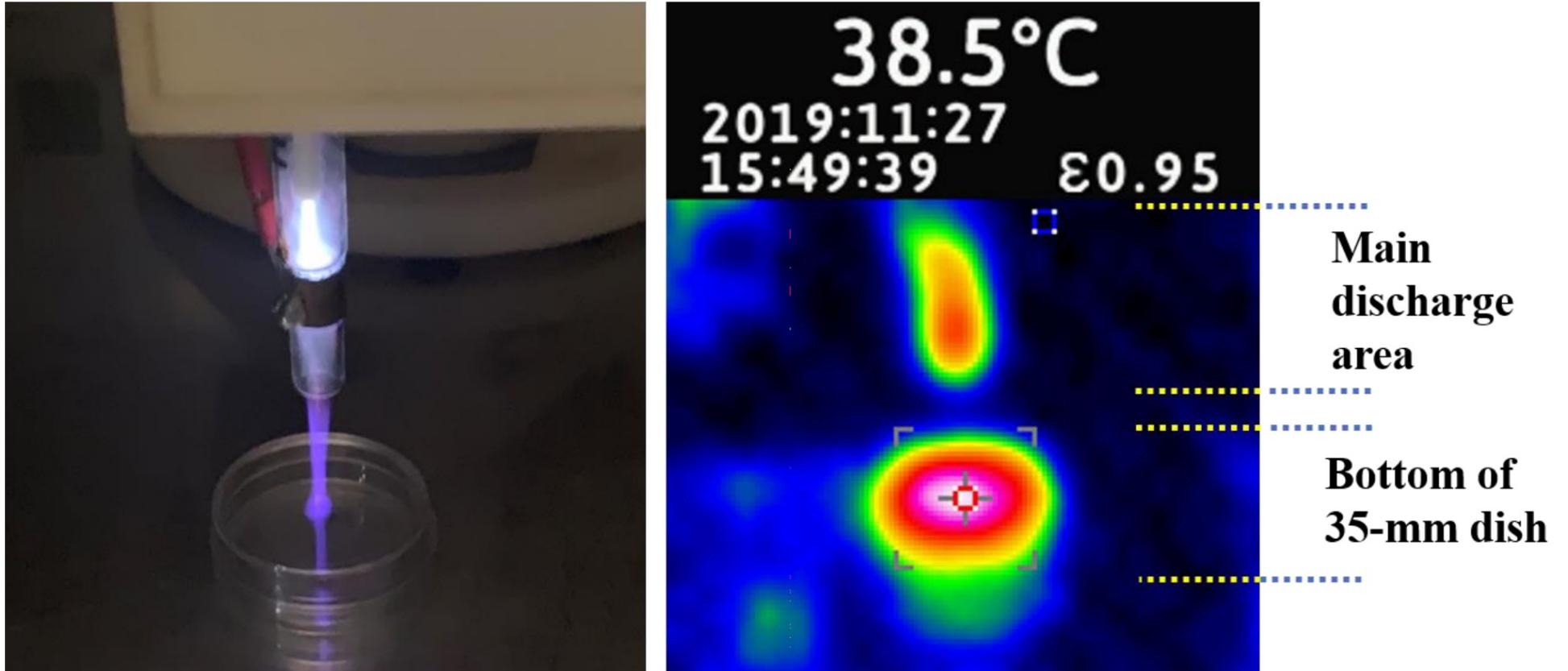


8 min after a CAP treatment

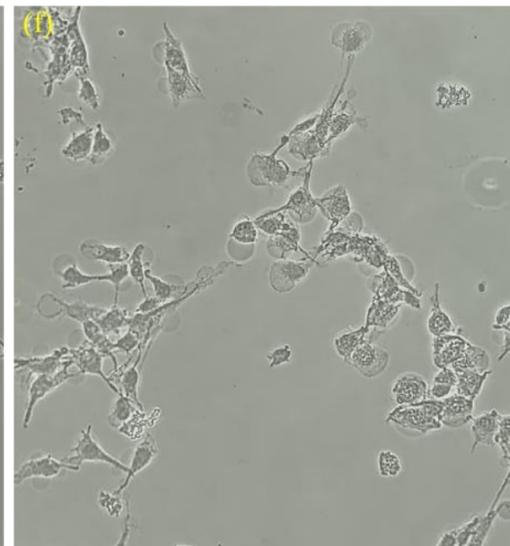
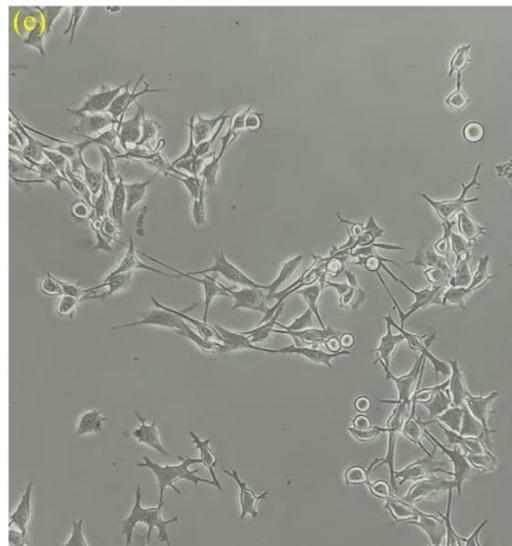
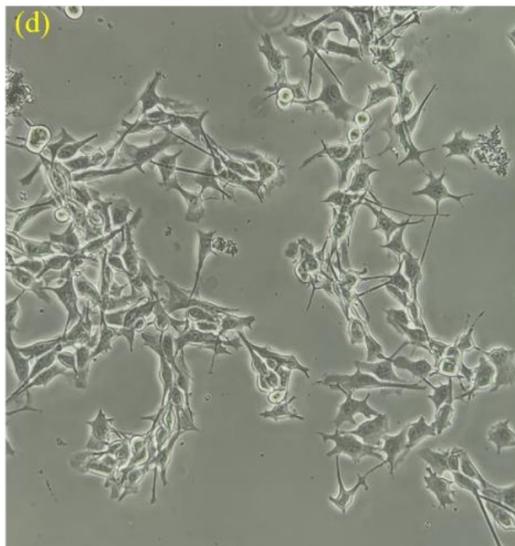
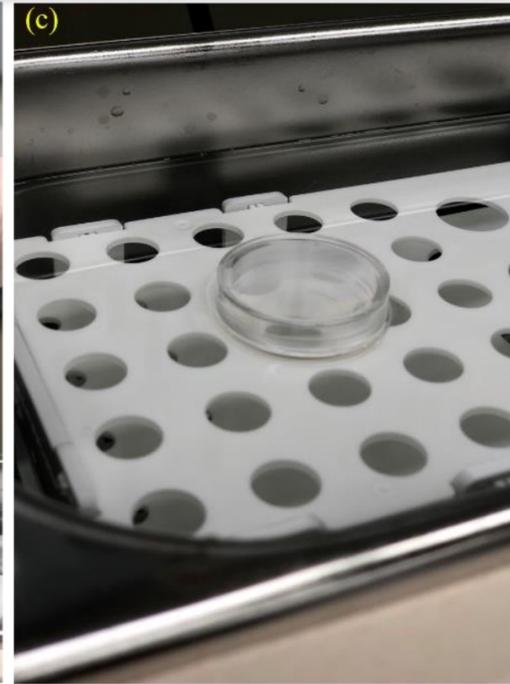
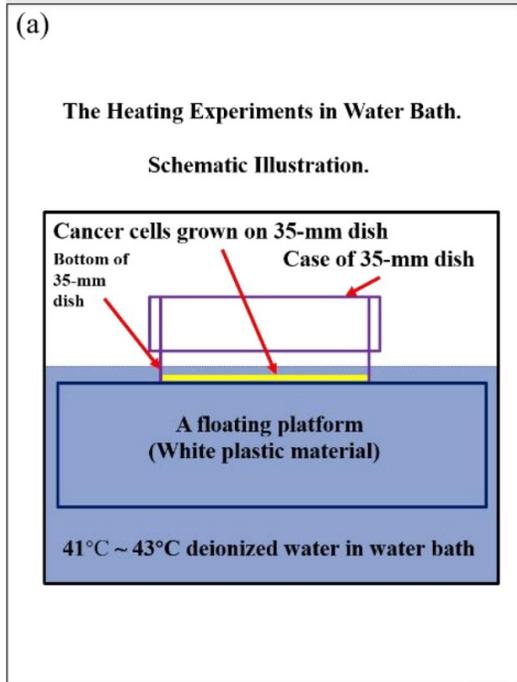
Scale bar: 50 μm

Preliminary discussion on mechanism

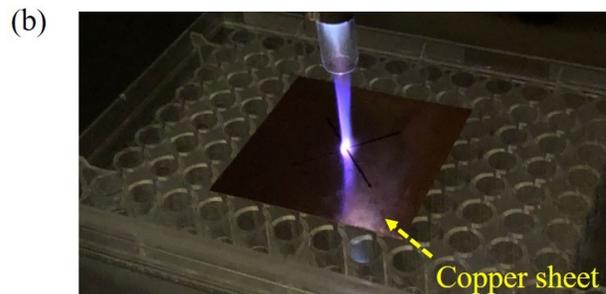
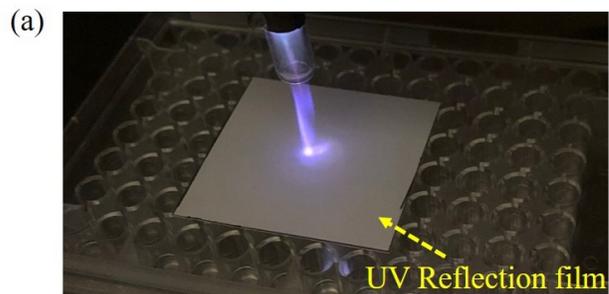
Heating Effect is Weak



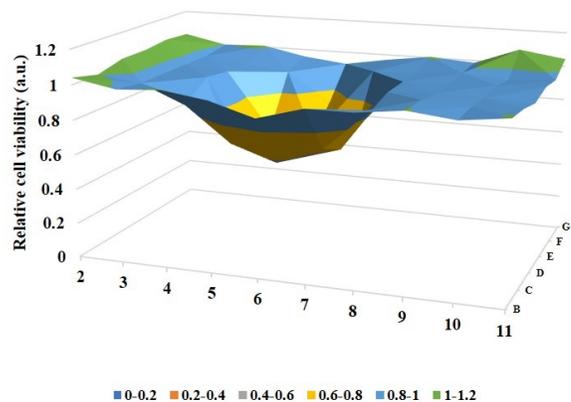
Temperature on the bottom of 35 mm dish after a 2 min of CAP treatment. Left panel is the optical photo of the CAP treatment on the bottom of a 35-mm dish. Right panel is the infrared photo taken by using FLUKE Visual IR Thermometer with a far model. The temperature has been measured for 4 times. The mean temperature was $38.5^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$. The temperature shown in the right panel was the value collected in one test. The gap between the nozzle and the surface of bottom of dish was 19.8 mm. Yan, D., et al. ACS Applied Materials & Interfaces, 12(31), 34548.



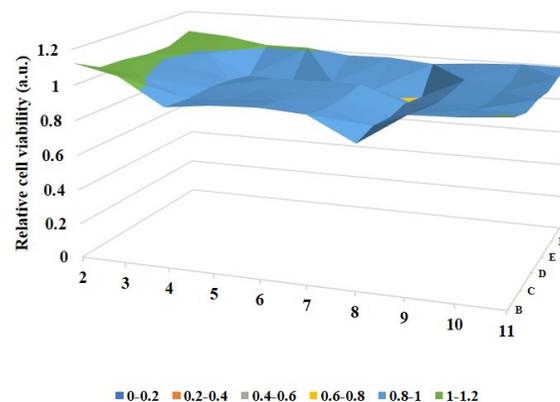
The heating experiment in the water bath (41°C ~ 43°C) will not cause the same cell death due to CAP treatment (2 min). (a) The schematic illustration of heating experiment. The bottom of the dish was immersed in the water with a temperature 41°C ~ 43°C. The medium was removed before the experiment. (b) The photo of the heating experiment in water bath. The temperature shown on the thermometer (ThermoPro TP01A, USA) was 41.0°C. (c) The floating of the 35-mm dish on a white floating platform. (d) The microscopic image (Nikon TS100 inverted phase contrast microscope) of the control group. (e) The microscopic image of the cells after a 2 S-12 min of heating experiment. (f) The microscopic image of the cancer cells after a 2 min of the physical CAP treatment. (e) and (f) were taken 11 min after the corresponding experiments. Yan, D., et al. ACS Applied Materials & Interfaces, 12(31), 34548.



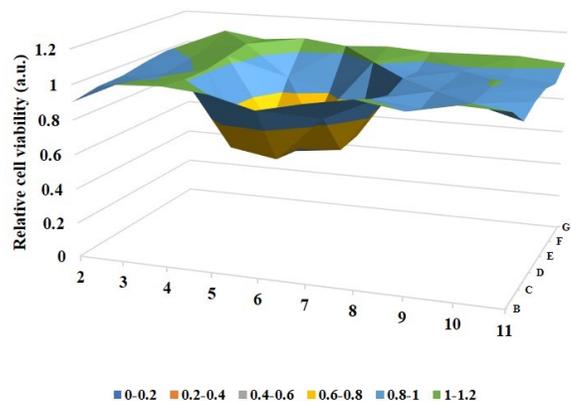
(c) UV reflection film, 5 x 5 wells size



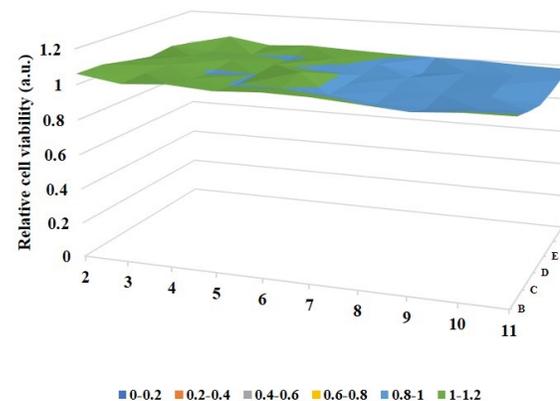
(d) Copper sheet, 5 x 5 wells size



(e) UV reflection film, 10 x 6 wells size



(f) Copper sheet, 10 x 6 wells size



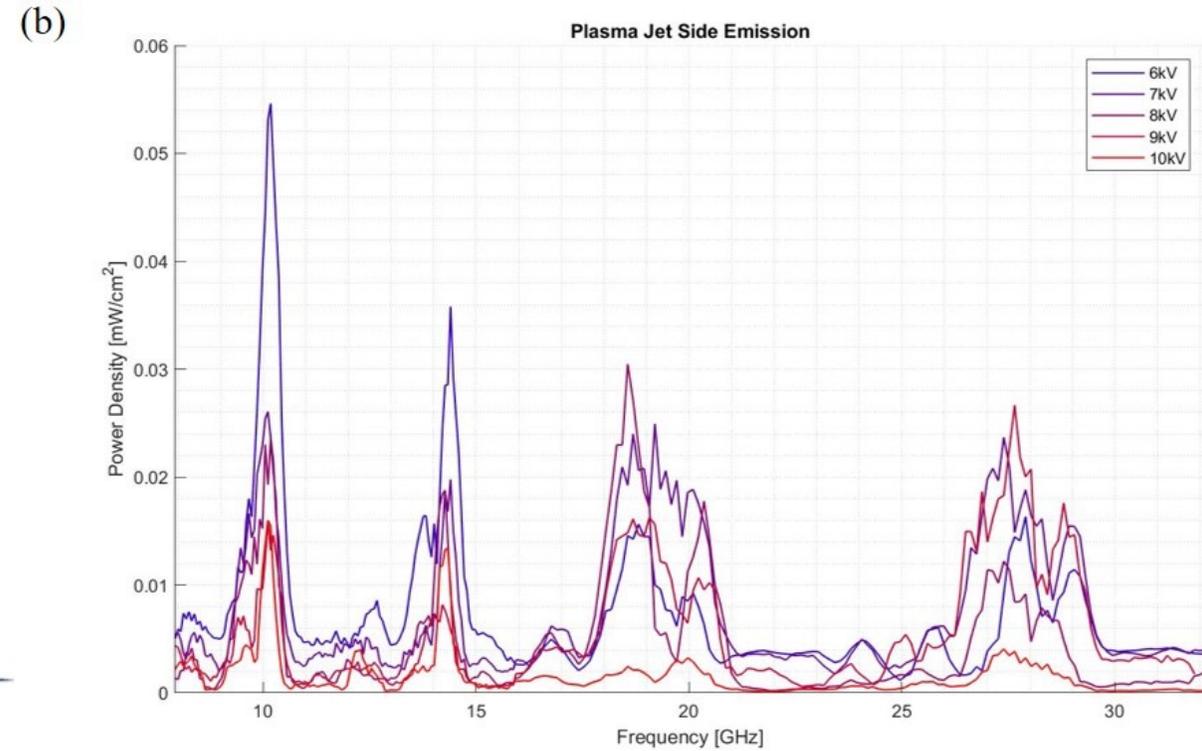
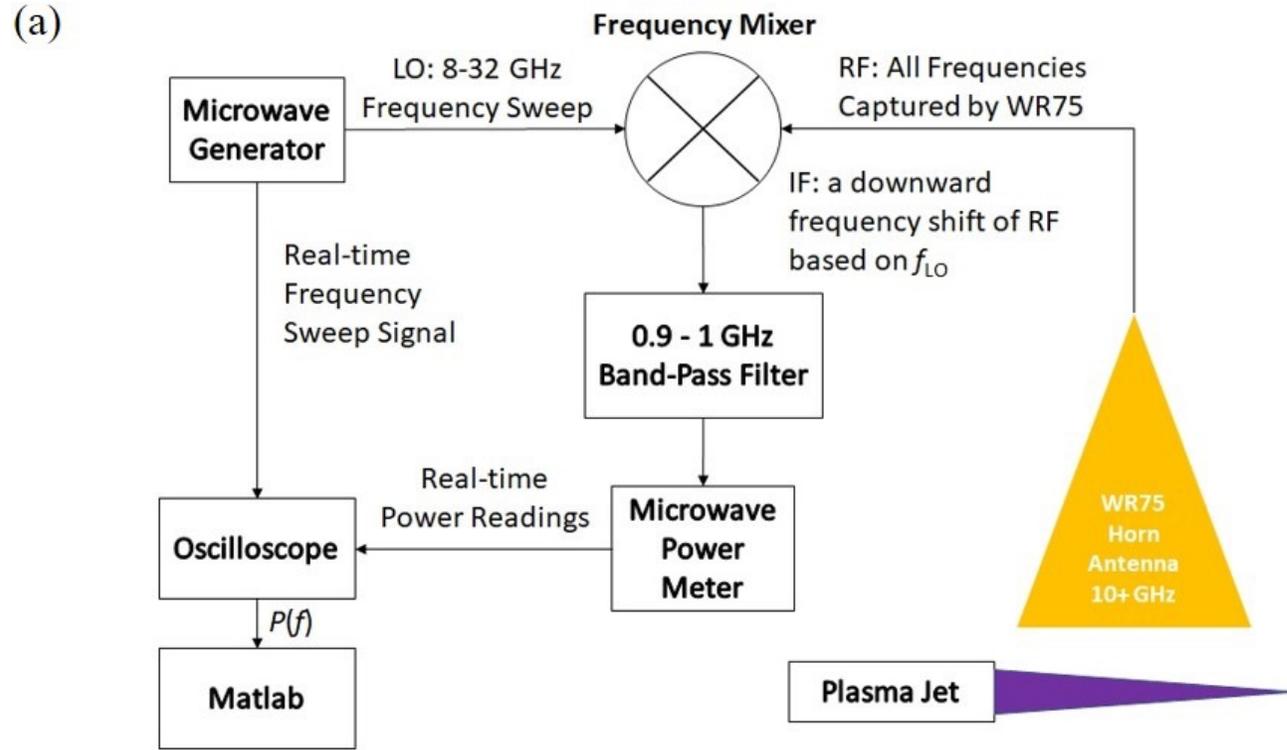
Melanoma Cells B16F10

Physically based anti-melanoma effects can only be blocked by a copper sheet rather than by a UV reflection film. (a, b) Photos of the physically based CAP treatments on the bottom of 96-well plates covered by the UV reflection film and copper sheet are shown in panels a and b, respectively.

Here, we just used the UV reflection film or the copper sheet with a size of 5×5 wells as an example. (c-f) Corresponding 2D cell viability is shown in panels c-f.

Yan, D., et al.. ACS Applied Materials & Interfaces, 12(31), pp.34548-34563.

Electromagnetic (EM) Emission from CAP jet



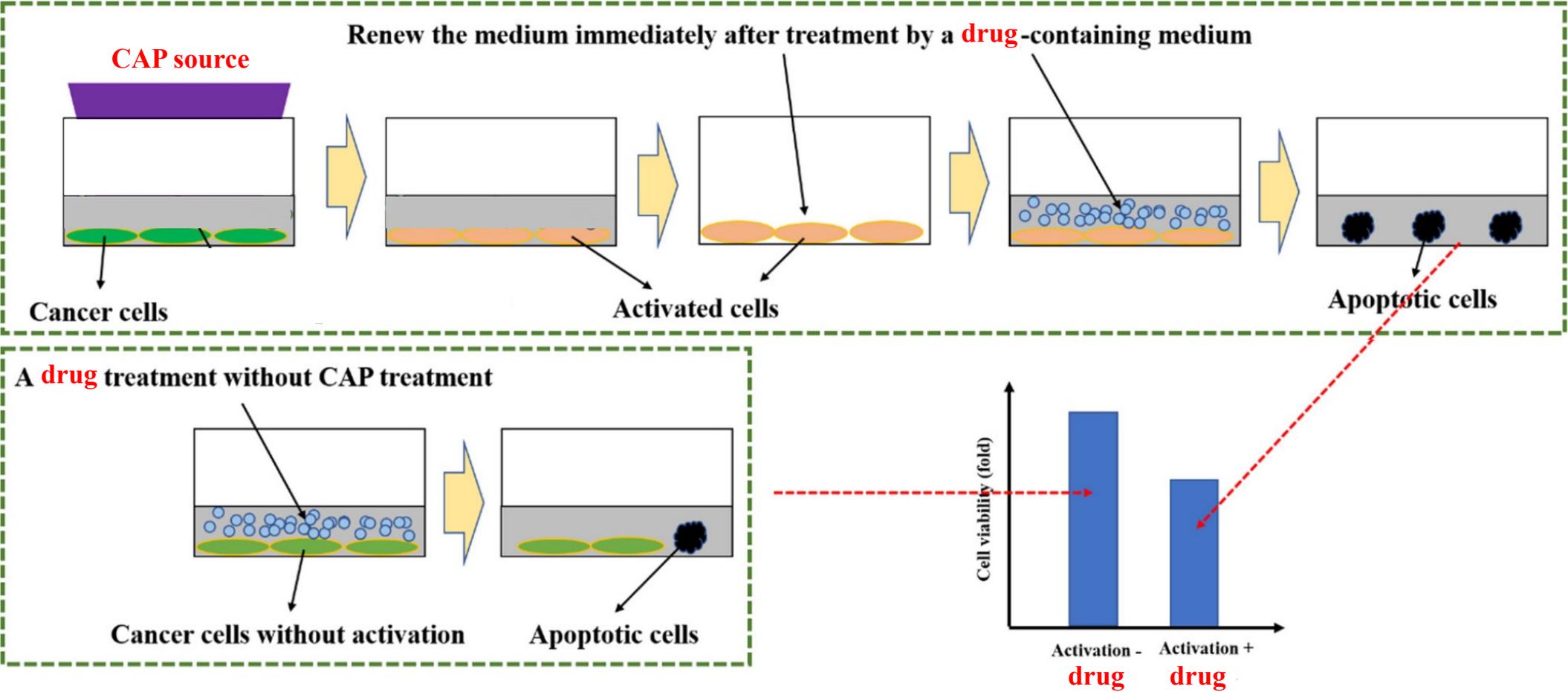
Microwave emission : due to plasma oscillation

EM emission of the CAP jet under different discharge voltages (pk-pk). (a) Heterodyne setup for radiofrequency (RF) power spectrum measurement. (b) RF spectra of the CAP jet over 8–32 GHz. Yan, D., et al.. ACS Applied Materials & Interfaces, 12(31), 34548.

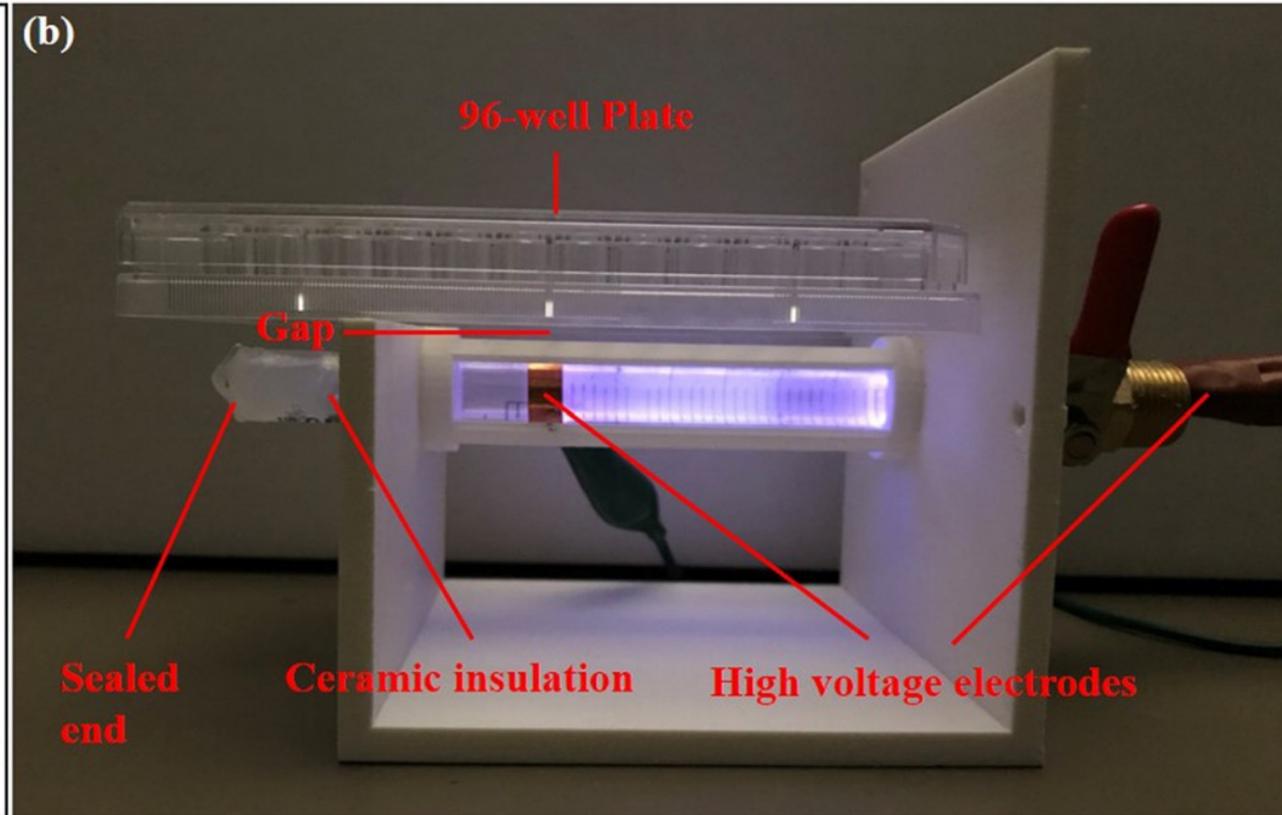
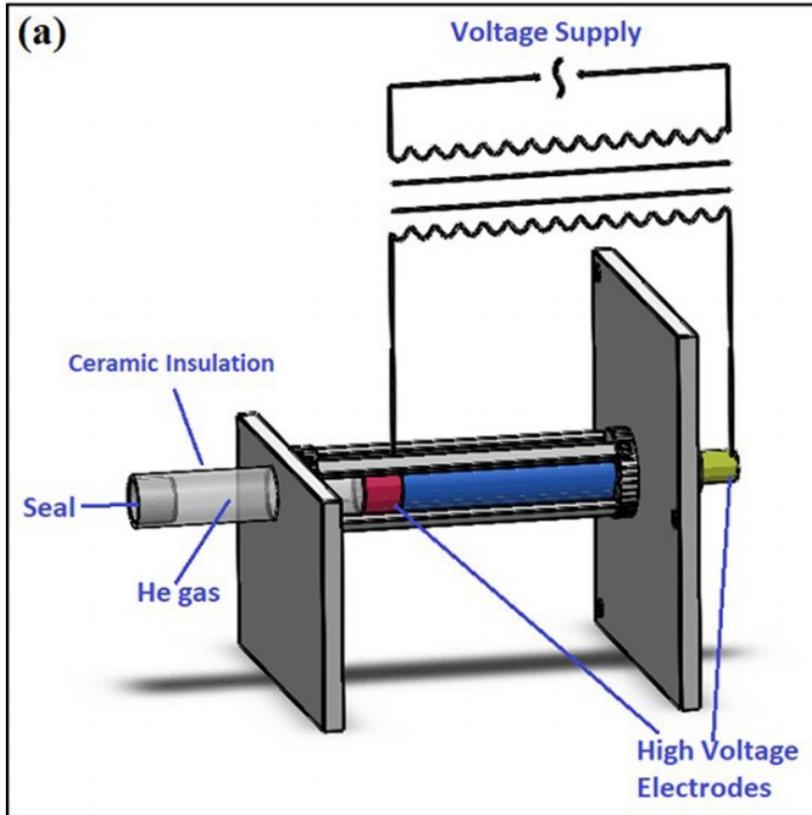
Outlines

- General picture of biological killing by CAP.
- Killing mammalian (cancer) cells by physical factors in CAP.
- **Activation/sensitization of cancer cells to drugs by CAP.**
- Summary.

Basic Strategy to Demonstrate Activation/Sensitization



Discharge Tube



Helium gas discharge tube. (a) Schematic illustration of setup; (b) Discharge tube in operation. Yao, X., et al., 2020. Sensitization of glioblastoma cells to temozolomide by a helium gas discharge tube. *Physics of Plasmas*, 27(11), p.114502.

Heating and UV Effect are Weak

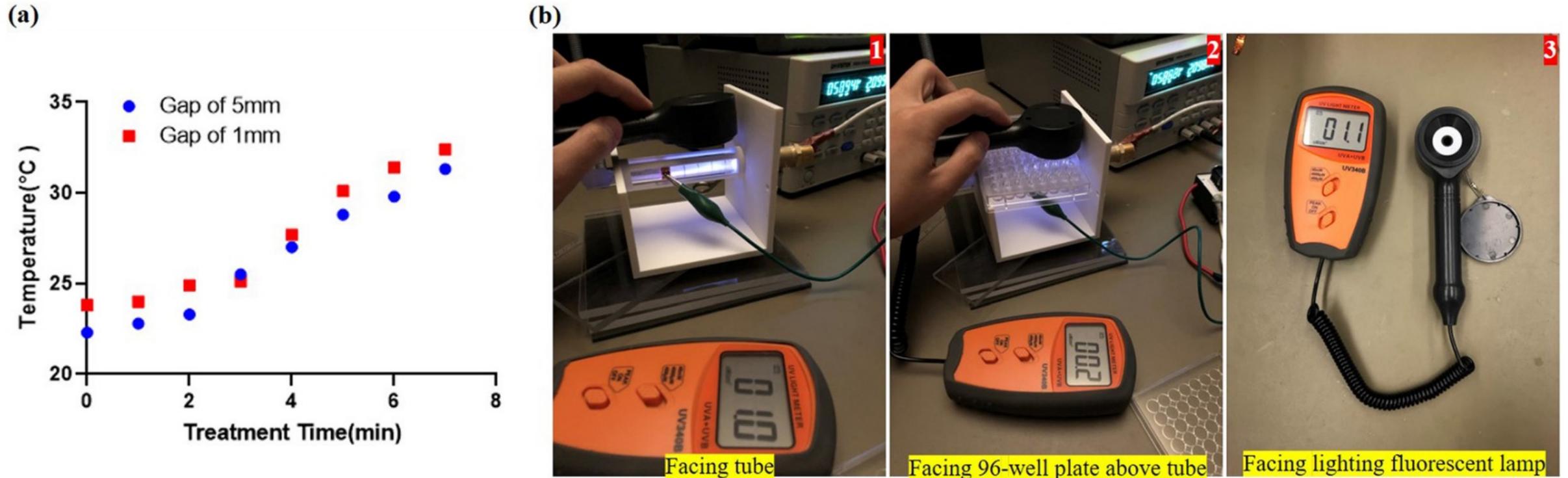
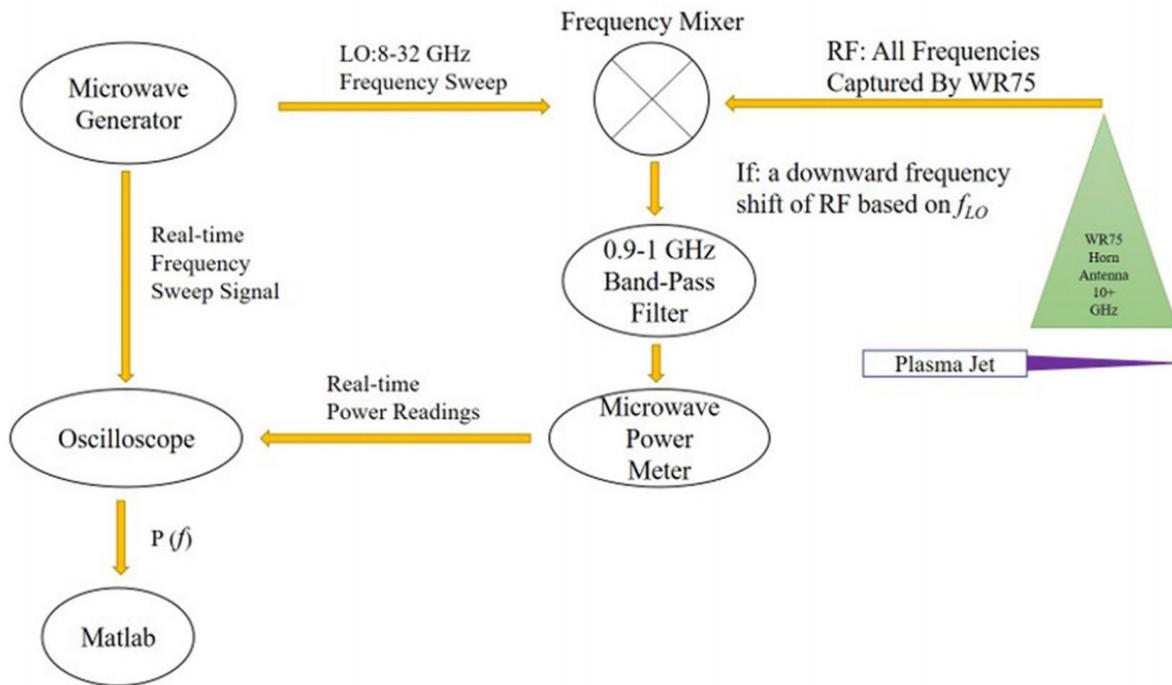


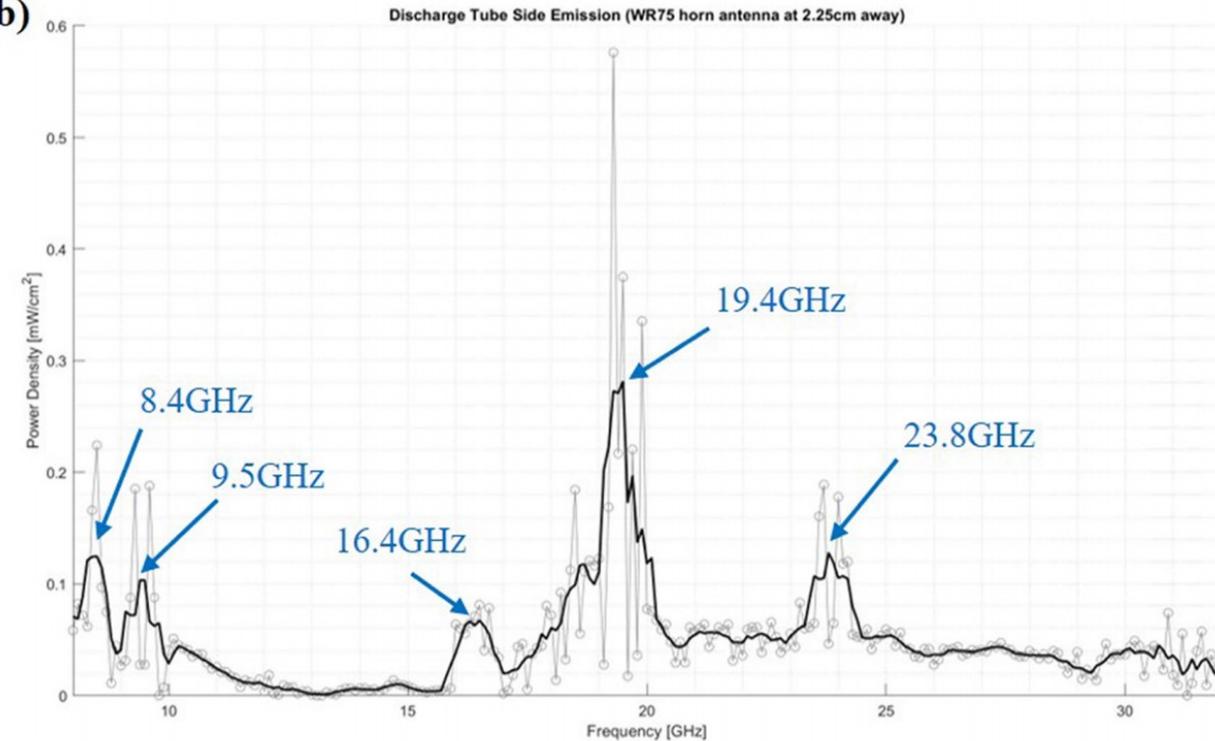
FIG. 2. Heating effect and UV irradiation of discharge tube. (a) The temperature of the treated plate. An HT-02D Handheld Digital IR Infrared Thermal Imaging Camera Thermometer was used for the thermal imaging. (b) The UV intensity measured by a UV radiometer (UV340B, KONGZIR). From left to right, the UV intensity from the discharge tube, the UV intensity after the blockage of 96-well plate, and the UV intensity of lighting at lab was $1.0 \mu\text{W}/\text{cm}^2$, $0.2 \mu\text{W}/\text{cm}^2$, and $1.1 \mu\text{W}/\text{cm}^2$, respectively.

EM Emission from Discharge Tube

(a)



(b)



EM spectrum of discharge tube. (a) Schematic illustration of heterodyne setup for RF power spectrum measurement. (b) An electromagnetic spectrum measurement of the ionized helium in the discharge tube. There are five main peaks (8.4 GHz, 9.5 GHz, 16.4 GHz, 19.4 GHz, and 23.8 GHz) in microwave power spectrum. Yao, X., et al. 2020. Sensitization of glioblastoma cells to temozolomide by a helium gas discharge tube. Yao, et al. *Physics of Plasmas*, 27(11), p.114502.

Temozolomide (TMZ): a widely used drug for glioblastoma therapy.

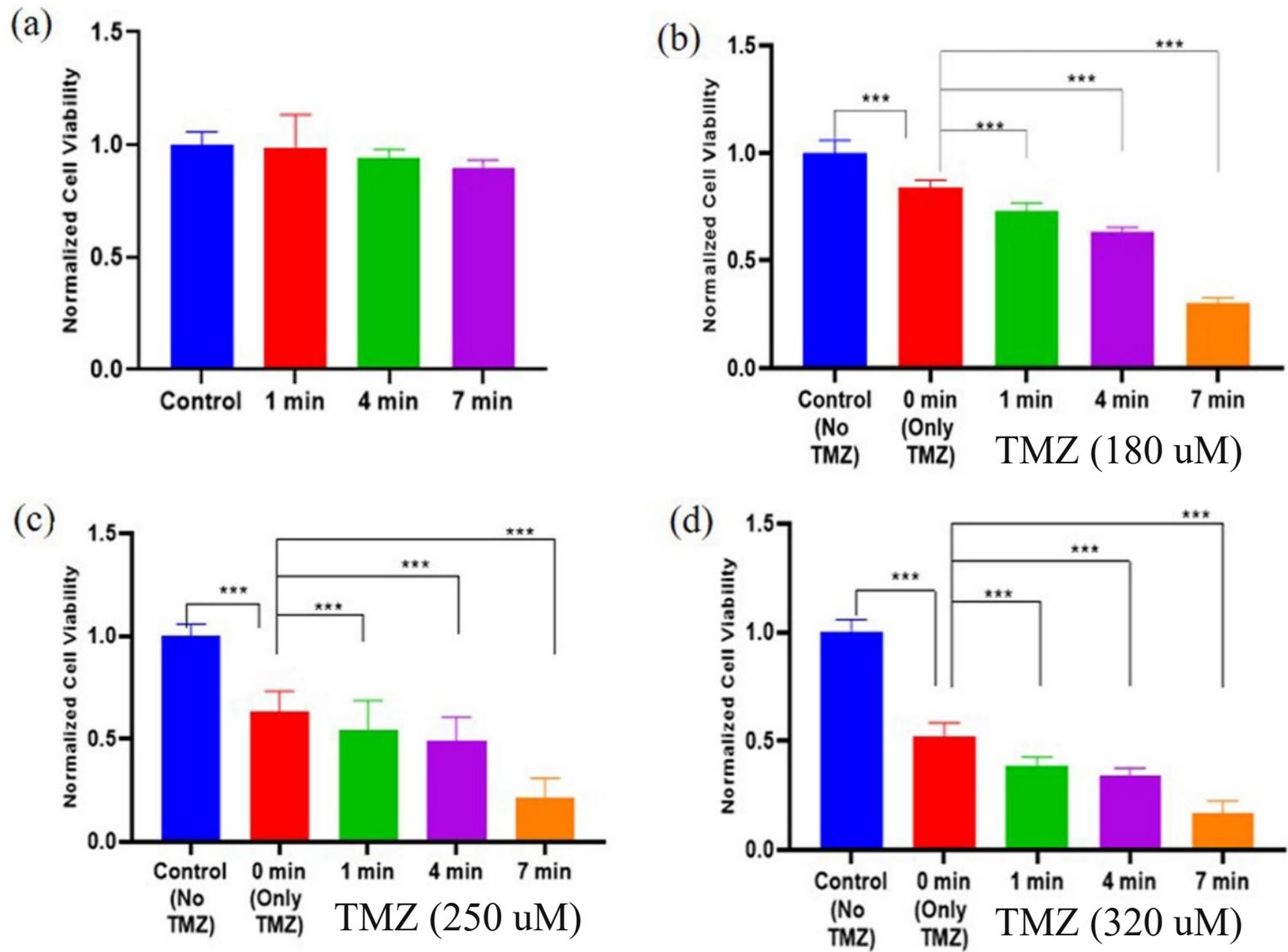


FIG. 4. The sensitization of the glioblastoma cells to the cytotoxicity of TMZ by the discharge tube. (a) The impact of discharge tube on U87MG cells. (b) The cytotoxicity of TMZ (180 μ M) on the sensitized U87MG cells. (c) The cytotoxicity of TMZ (250 μ M) on the sensitized U87MG cells. (d) The cytotoxicity of TMZ (320 μ M) on the sensitized U87MG cells. (e) The case with a larger gap (5 mm) with TMZ (250 μ M). (f) The cytotoxicity of TMZ (250 μ M) on the sensitized A172 cells. The gap was 1 mm for all figures

Weak Effect on Normal Cells

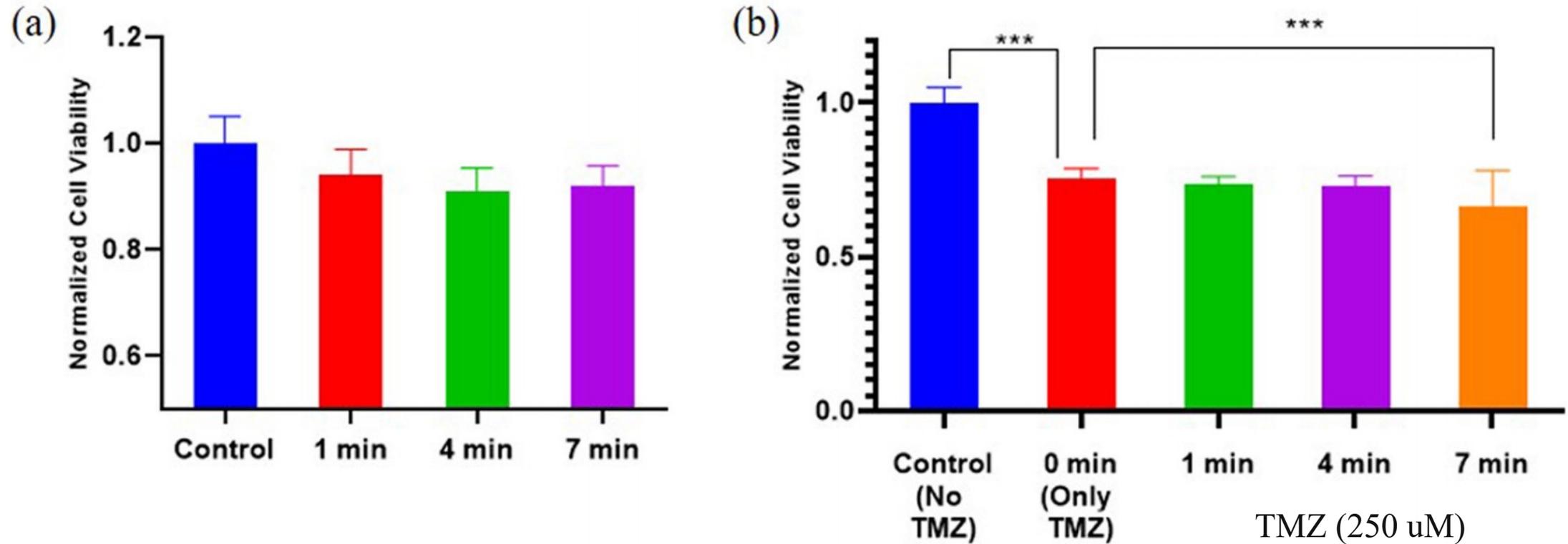


FIG. 5. The limited side effect on normal astrocyte cell line hTERT/E6/E7. (a) The discharge tube treatment alone. (b) The cytotoxicity of TMZ (250 μ M) on the tube-treated cells. The gap was 1 mm. (Student's t-test, $p < 0.05$, ***)

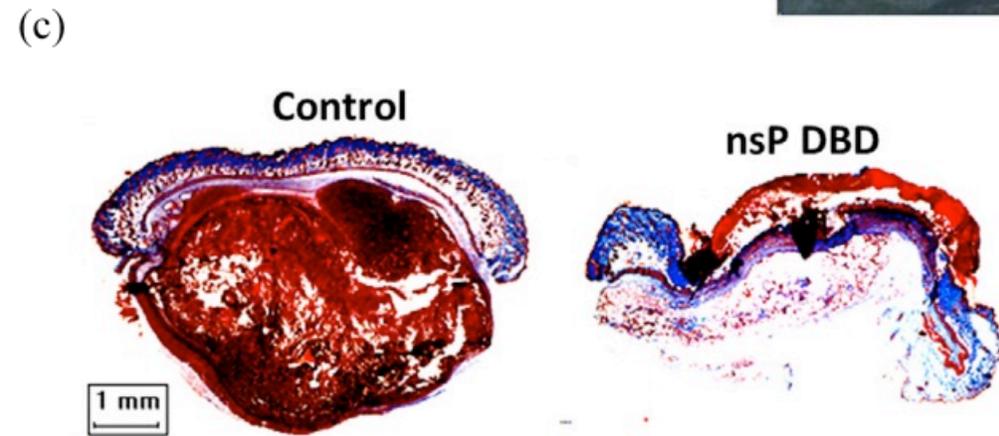
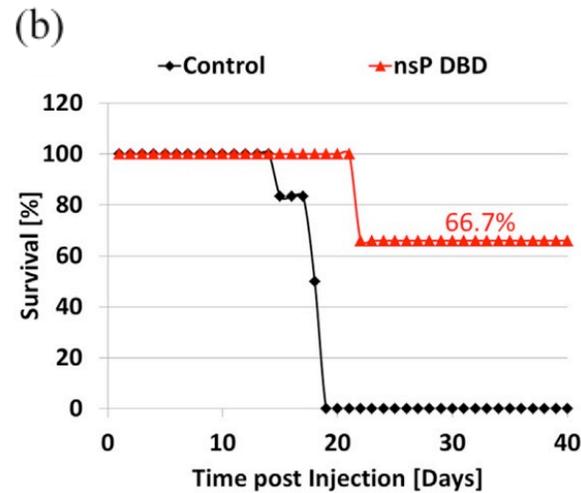
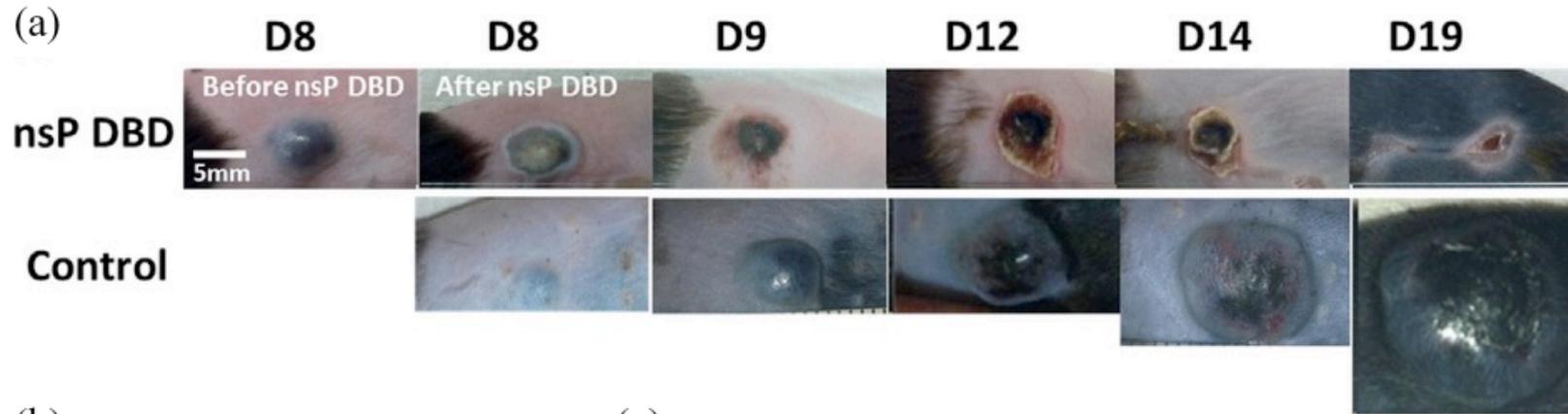
Outlines

- General picture of biological killing by CAP.
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- **Summary.**

Summary

1. Both chemical and physical components in CAP play some specific role to cause the cell death, not only cancer cells but also bacteria and others.
2. Physical factors have potential non-invasive nature, which can penetrate some barriers to cells. Concentration of reactive species is the core of chemically-based treatment. In contrast, the relative position between sources and targets may be a key factor in term of physical factors.
3. Sensitization of cancer cells to drugs by physically-based CAP treatment or sources is a potential new direction. Sensitization alone will not cause killing effect to protect normal tissues or cells
4. We need to think about a basic question in a practical way: how to use CAP in real clinical application? The answer to this core question will guide the further research in plasma medicine.

Anti-cancer *in vivo*: Eradication by a Treatment above the Skin



The eradication of melanoma tumor in mice using a nsPDBD source. (a) B16 Melanoma cells were injected on the rear flank of C57BL/6 mice. After 8 days, the tumor was treated a single time for 7 min with nsP DBD at 236 Hz and 33.6 kV. (b) Survival for nsP DBD treated tumors (red triangle) and control untreated tumors (black diamond) as a function of time post-injection. (c) The trichrome staining of nsP DBD treated tumor (left top, D22 post-injection) and control tumor (left bottom, D19 post injection). Histology of the nsP DBD treated tumor showed red skin staining confirming scab formation but no visible tumor below epithelium. (Natalie, C., et al. Plasma Processes and Polymers, 12.12, 1400–1409 (2015)).

Acknowledgements

I would like thank the great help from my mentor Dr. Michael Keidar and our team's hard-working and warm collaboration.

This work was supported by the National Science Foundation Grant 1747760. I also greatly appreciated the support from NSF.

Thanks for your attention !