

Supplementary for

Time-Resolved Oxygen Dynamics Reveals Redox-Selective Apoptosis Induced by Cold Atmospheric Plasma in HT-29 Colorectal Cancer Cells

Validation of the DCFH Probe

Since pH strongly influences the stability and reactivity of plasma-generated reactive oxygen and nitrogen species (ROS/RNS), pH monitoring was included as a critical parameter. Acidification during CAP exposure is primarily attributed to the formation of nitric and nitrous acids and can modulate oxidative stress responses in cells [1].

pH values of the culture medium and probe solutions were measured immediately before and after plasma treatment using a calibrated digital pH meter. Calibration was performed with standard buffer solutions (pH 4.0, 7.0, and 10.0). Measurements were taken in triplicate for each condition, and mean values were used for analysis. These data were used to correlate physicochemical changes induced by CAP with corresponding biological responses.

DCFH-DA is cell-permeable and non-fluorescent; after enzymatic deacetylation by intracellular esterase, it forms DCFH, which can be oxidized by ROS to fluorescent DCF (green emission, ~530 nm). To validate the applicability of DCFH-DA as a probe for intracellular ROS detection, we examined its optical response under various ROS conditions. In this regard, two complementary approaches were employed.

- (i) UV–Vis’s emission spectroscopy of DCFH solutions subjected to plasma treatment:

A four-channel Avantes optical spectrometer (Avantes, Netherlands) was employed to determine the intercellular ROS, through photoluminescence spectroscopy (PL). The visible spectral range (400–700 nm) is particularly relevant for monitoring the excitation and emission of the DCFH-DA probe, with characteristic wavelengths around 490 nm for excitation and 520 nm for emission, enabling sensitive detection of intracellular ROS fluorescence[2].

As illustrated in Figure S1, CAP progressively decreased pH compared with the untreated control, due to the formation of oxide plasma-derived species (H_2O_2 , HNO_2 , HNO_3). Measurements across six treatment conditions (T1–T6) revealed a gradual decline from pH 7.3 (reference) to ~7 (T6). On the other hand, CAP exposure induced notable changes in the physicochemical properties of the DCFH solution.

UV–Vis’s emission and fluorescence spectroscopy confirmed that these pH alterations significantly influenced the probe’s optical response (Figures S1.b and S1.c). Emission spectra showed spectral

changes consistent with oxidation of DCFH to DCF. However, fluorescence intensity did not exhibit a simple monotonic increase with expected ROS levels: under lower pH conditions, fluorescence decreased even when ROS generation was expected to rise. This suggests that low pH medium by plasma treatment strongly modulates probe response, as it was expected.

- (ii) Fluorescence measurements upon external addition of H_2O_2 at different concentrations (Figure S1.c).

To investigate the response of the DCFH-DA probe to varying concentrations of a stable oxygen species like H_2O_2 , we generated a line chart illustrating the fluorescence intensity changes of DCFH-DA in response to varying H_2O_2 concentrations. This demonstrated ROS-driven oxidation and pH-dependent fluorescence behavior.

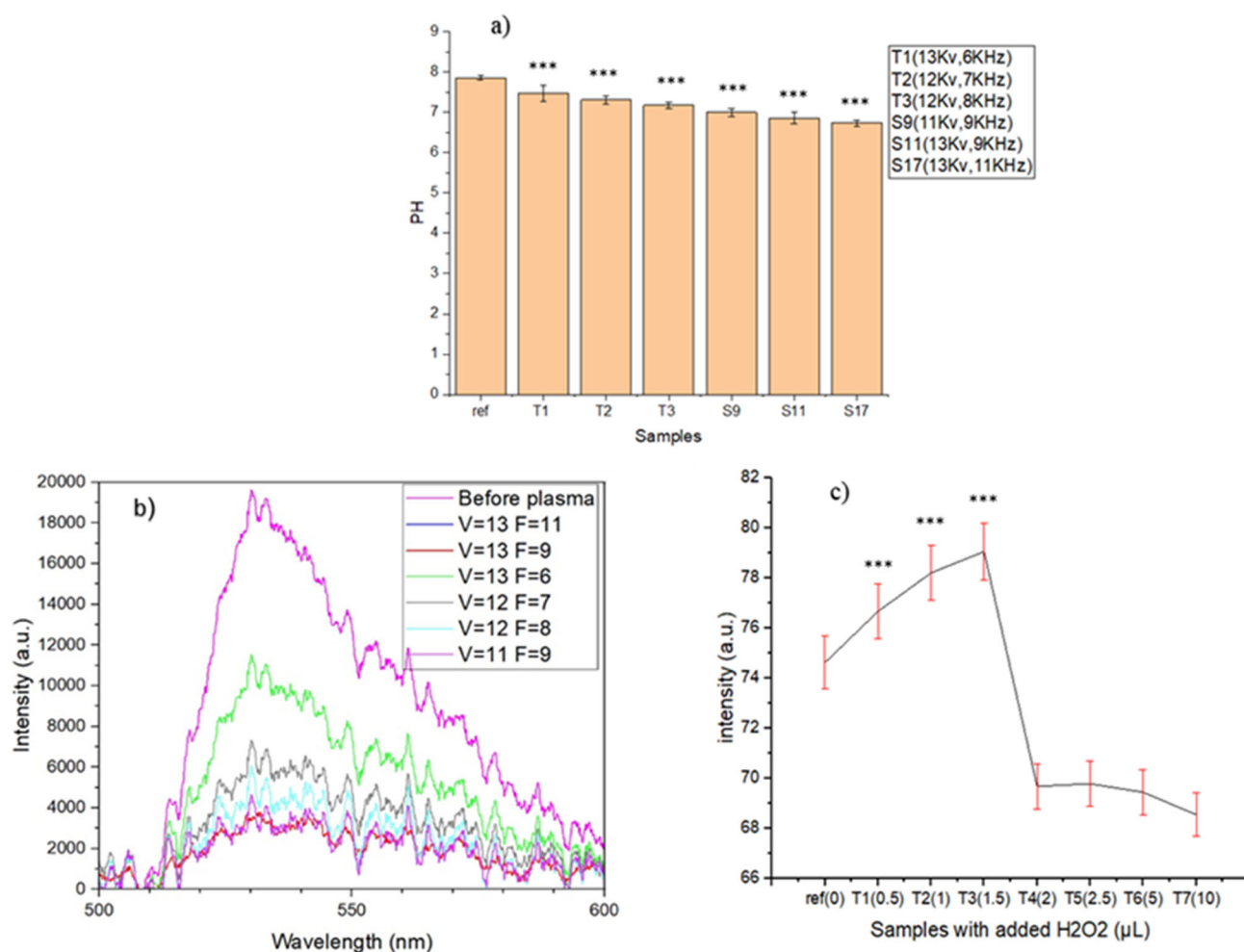
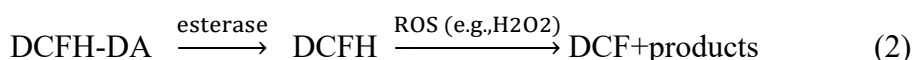


Figure S1. Optical characterization of DCFH-DA probe under CAP exposure: (a) pH variation in DCFH-DA solution across six plasma conditions (T1–T6); (b) UV-Vis emission spectra showing oxidation to fluorescent DCF; (c) Fluorescence intensity versus H_2O_2 concentration demonstrating ROS-driven oxidation and pH-dependent response. Statistical significance compared to the reference case is indicated as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

As be seen in Figure S1.c, at low concentrations of H₂O₂ (0.5–1.5 μL), the fluorescence intensity of DCF increased markedly, consistent with enhanced ROS-driven probe oxidation. In alkaline states, this response is favorable as the probe's deacetylation and subsequent oxidation by ROS (e.g., H₂O₂) proceed efficiently:



In contrast, at higher concentrations in acidic states or phases, the fluorescence intensity decreased. This decline was explained by pH monitoring: higher concentrations of H₂O₂ shifted the medium toward acidity, destabilizing H₂O₂ and accelerating its decomposition into H₂O, thereby lowering the effective ROS available for DCFH oxidation. The relevant decomposition reactions in acidic conditions are:



It must be mentioned that, in our future analysis, since the intracellular environment is mildly alkaline, an increase in fluorescence intensity more directly reflects increased intracellular ROS production [3, 4] .

References:

- [1] Wang and Joseph. Quantifying cellular oxidative stress by Dichlorofluorescein assay using microplate reader. *Free Radical Biology and Medicine*. ;27 (5–6) :612–616. doi:10.1016/S0891-5849(99)00107-0
- [2] Kalyanaraman, Darley-Usmar, Davies, Dennery, Forman, Grisham, et al. Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. *Free Radical Biology and Medicine*. ;52 :1–6. doi:10.1016/j.freeradbiomed.2011.09.030