



Article

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

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Abstract

Cold atmospheric plasma (CAP) has emerged as a promising anticancer approach because of its ability to selectively eliminate malignant cells. Among the proposed mechanisms of this selectivity, the Bauer theory emphasizes the synergistic action of plasma-derived hydrogen peroxide (H₂O₂) and nitrite (NO₂⁻), leading to the transient generation of primary singlet oxygen (¹O₂). This early event inactivates membrane-bound catalase, allowing tumor cell-derived H₂O₂ and peroxynitrite to initiate a self-amplifying cycle that produces secondary ¹O₂, as a hallmark of CAP selectivity. To test this hypothesis, in this work, we monitored extracellular dissolved oxygen (DO) dynamics in HT-29 colorectal cancer cells treated with an argon plasma jet using time-resolved phosphorescence lifetime spectroscopy. Temporal variations in DO likely reflect the cumulative effect of rapid ¹O₂ production and its reactions with cells. A delayed surge in extracellular ¹O₂ was observed specifically in dying cancer cells within the 10–20 min window predicted by the model. Intracellular ROS imaging confirmed a strong correlation between intracellular ROS, extracellular ¹O₂ dynamics, and viability loss. Together, these results provide mechanistic validation of Bauer's redox model and suggest that early oxygen dynamics after CAP exposure can serve as predictive markers for treatment efficacy in plasma or photodynamic therapies.

Keywords: cold atmospheric plasma; selectivity; Bauer theory; singlet oxygen; colorectal cancer cells; time-resolved oxygen dynamics

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1. Introduction

Cold atmospheric plasma (CAP) has emerged as a promising modality for cancer therapy in oncological therapeutics [1–3]. The antitumor effects of plasma are mediated largely through the generation of reactive oxygen and nitrogen species (RONS), which perturb redox homeostasis and trigger programmed cell death pathways [4–6]. A defining hallmark of CAP in cancer therapy is its remarkable selectivity, whereby malignant cells are preferentially eliminated while healthy cells are largely spared [4,5,7]. Multiple mechanisms have been proposed to explain this effect, yet across these hypotheses a unifying theme is the central role of plasma-generated RONS [8,9]. Cancer cells, in comparison to their normal counterparts, typically maintain a higher basal oxidative stress and weaker antioxidant defenses, making them especially vulnerable to further ROS/RNS challenges [3,10–12]. Differences in plasma membrane composition—such as lower cholesterol content—enhance permeability to oxidants; overexpression of aquaporins, particularly AQP3 and AQP8, facilitates rapid uptake of hydrogen peroxide (H_2O_2) [13–17]; altered ion channel activity and mitochondrial dysfunction amplify intracellular oxidative stress; and features of the tumor microenvironment, including acidic pH and hypoxia, modulate RONS stability and reactivity [16,17]. While each of these factors contributes to differential sensitivity, it is the dynamics of RONS generation, accumulation, and signaling that are consistently regarded as the decisive trigger of selective cytotoxicity [18–21].

Among mechanistic frameworks, the Bauer theory provides a particularly detailed, time-resolved account of how RONS drive plasma selectivity [22,23]. According to this model, immediately after plasma exposure, extracellular long-lived oxidants such as H_2O_2 and nitrite (NO_2^-) react to form a short-lived burst of singlet oxygen ($^1\text{O}_2$). This initial $^1\text{O}_2$ pulse partially inactivates membrane-bound catalase, a critical enzyme shielding cancer cells by decomposing H_2O_2 and peroxynitrite (ONOO^-) [22]. Once catalase protection is impaired, accumulating extracellular H_2O_2 and ONOO^- can react with endogenously produced superoxide (O_2^-) from NOX1 activity at the tumor cell surface, fueling a self-amplifying cycle of secondary $^1\text{O}_2$ production [24–26]. This feedback loop further disables catalase, intensifies oxidative stress, and ultimately drives malignant cells into apoptosis or necrosis [10,20]. Temporally, the initial $^1\text{O}_2$ consumption and generation occur within seconds, while the exponential secondary production emerges over the next 10–20 min, coinciding with irreversible oxidative damage and cell death [23,25,26]. This dynamic RONS-centered mechanism exemplifies how plasma oncology exploits cancer cells' intrinsic redox vulnerabilities to achieve therapeutic selectivity [1,5,22,24].

The selective cytotoxicity of CAP toward cancer cells is largely attributed to RONS, yet the precise mechanisms remain under investigation [3,5,8]. For the first time in the context of non-thermal plasma cancer cell treatment, this work aimed to employ a time-resolved phosphorescence sensor for DO to experimentally validate Bauer's hypothesis regarding the selective cytotoxic action of $^1\text{O}_2$. Specifically, we investigated for the first time, to the best of our knowledge, the dual mechanism by which cancer cells not only succumb to plasma-induced oxidative stress but also actively amplify the propagation of cytotoxic oxidants, thereby contributing to their own selective destruction. To directly evaluate the Bauer theory, we tracked the estimated extracellular singlet oxygen $^1\text{O}_2$ in HT-29 colorectal cancer cells treated by Argon plasma jet with varying viability, high, intermediate, and low metabolic activity ($\text{MTT} >50\%$ vs. $<50\%$), as well as in the presence and absence of cells. To this end, although the lifetime of $^1\text{O}_2$ in aqueous systems is limited to the microsecond range, its highly reactive interactions with membrane lipids led to the formation of peroxidized intermediates and the subsequent regeneration of ground state O_2 . This cycle can influence the net concentration of molecular oxygen detected by the DO probe, implying that the observed DO dynamics represented an integrated signature of $^1\text{O}_2$ activity. To correlate extracellular DO consumption parameters with intracellular ROS

and cell viability, intracellular ROS imaging with DCFH-DA, an intracellular fluorescence probe, has been employed not only to track intercellular ROS level but also to artifacts such as pH changes.

2. Materials and Methods

(A) Cold Plasma Jet Device

CAP device used in this study was designed for the treatment of HT-29 colorectal cancer cells. Developed in-house, the device consisted of a quartz dielectric tube with an inner diameter of 3 mm and an outer diameter of 5 mm. A stainless-steel inner electrode (1.5 mm in diameter) was centrally positioned within the tube, while a grounded stainless-steel outer electrode was cylindrically arranged around the tube outlet. Unlike conventional designs, the quartz tube was not enclosed in a Teflon body, resulting in an open-structured configuration.

Plasma generation was powered by a high-voltage, kilohertz-frequency sinusoidal power supply (0–20 kV, up to 20 kHz; Basafan Co., Tehran, Iran), allowing for precise control of the operating parameters to maintain a stable and biologically compatible plasma discharge. Argon (Ar) was used as the working gas, with its flow rate regulated at 3.5 standard liters per minute (SLM) using a Breezens mass flow controller.

(B) Cell preparation

I. HT- Cell line 29

The human colorectal adenocarcinoma cell line HT-29 was obtained from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). These cells exhibit an epithelial-like polygonal morphology, grow in adherent clusters, and can develop gland-like structures under certain conditions[12,27]. In high-glucose media, HT-29 cells display enhanced proliferation, whereas in the presence of differentiation-inducing agents such as sodium butyrate or under glucose limitation, they can acquire enterocyte-like features, including mucus production and expression of intestinal enzymes [7,9]. Due to these characteristics, HT-29 cells serve as a well-established model for investigating colorectal cancer biology, therapeutic responses, and plasma-induced cellular effects [6,8,15]. The typical morphology of HT-29 cells observed under an optical microscope is presented in Figure 1.

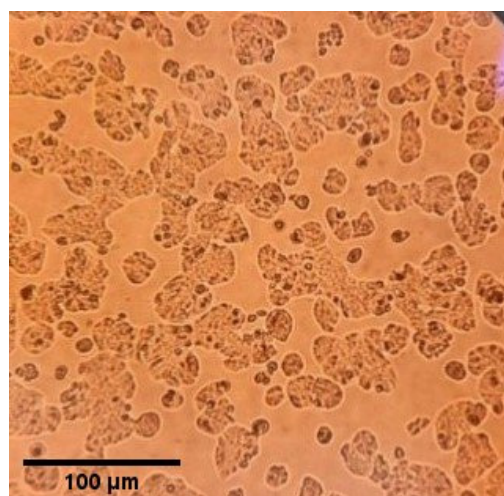


Figure 1. Optical micrograph of HT-29 colorectal adenocarcinoma cells under standard culture conditions showing polygonal epithelial morphology.

I. Cell Culture Conditions and Medium

HT-29 cells were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM; 4.5 g/L glucose; Bio Idea, Tehran, Iran) supplemented with 10% fetal bovine serum (FBS; DNA BioTech, Tehran, Iran) and 0.5% penicillin–streptomycin to prevent bacterial contamination. Cultures were incubated in a humidified incubator at 37 °C with 5% CO₂ and 99% relative humidity.

Subculturing was performed when cells reached approximately 80% confluence. Cells were detached using 0.25% trypsin–EDTA, neutralized with complete medium, and reseeded at appropriate densities. Centrifugation at 1200 rpm for 5 min at 22 °C was used to collect cells before reseeding. All procedures were performed under sterile conditions using a laminar flow hood.

(C) Intracellular ROS Detection Using DCFH-DA Probe

2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich, St. Louis, MO, USA) is a widely used fluorescent probe for the detection of intracellular reactive oxygen species (ROS) in cells and tissues [13,14]. DCFH-DA is a non-fluorescent, cell-permeable compound that is hydrolyzed by intracellular esterases to DCFH, which remains trapped inside the cell. Upon oxidation by ROS, including hydrogen peroxide (H₂O₂), superoxide (O₂^{•−}), and hydroxyl radicals (•OH), DCFH is converted into Dichlorofluorescein (DCF)—a highly fluorescent molecule [9,28,29].

The fluorescence intensity of DCF is directly proportional to the intracellular ROS levels and can be measured using fluorescence microscopy or spectrofluorometry, providing a sensitive and quantitative assessment of oxidative stress [8]. DCFH-DA offers high specificity and sensitivity for ROS detection and has been extensively used in various cell types and tissues [6,22].

However, extraneous chemical reactions in the culture medium or excessively high ROS levels may affect probe performance and lead to inaccurate results [30–32]. In this study, DCFH-DA (Sigma-Aldrich, St. Louis, MI, USA) was applied to HT-29 cells to monitor intracellular ROS generation following cold atmospheric plasma treatment.

(D) Fluorescence Microscopy

Intracellular ROS levels were assessed using a Zeiss AxioLab fluorescence microscope (Zeiss, Göttingen, Germany). In this study, DCFH-DA-loaded HT-29 cells were imaged to visualize ROS generation. Images were captured with the digital camera and analyzed using standard image analysis software, version 1.51n, to quantify fluorescence intensity and ROS localization. This approach provided a sensitive and reliable method for monitoring cellular oxidative responses following plasma treatment [8,14].

(E) Real-time DO measurements

I. Optical Oxygen Sensor

Photonics-based oxygen sensors are advanced tools for monitoring oxygen levels in biological and industrial environments [33,34]. Time-resolved phosphorescence lifetime spectroscopy enables precise measurement of oxygen concentration by analyzing the decay of phosphorescence from metal-complex DO probes such as platinum, ruthenium, or palladium [26,33]. These probes, embedded in an oxygen-permeable polymer matrix, emit luminescence with micro- to millisecond lifetimes upon optical excitation.

In this study, the sensor utilized a high-power blue LED (~410 nm) as a cost-effective alternative to nanosecond lasers, illuminating the oxygen-sensitive film to produce red emission proportional to DO. The emitted light is captured by a photodiode, processed, and transmitted to a computer for continuous measurements (~2.9 s per reading). Integration with fluorescence imaging allows spatial mapping of oxygen distribution in living

cells, including cancer models [11,19]. Polydimethylsiloxane (PDMS) was used to stabilize the sensor in culture wells, providing optical transparency and mechanical durability [7].

I. Sensing Mechanism of the Photonic Oxygen Sensor

The active sensor region contains a metal–organic complex in a highly permeable polymer matrix, allowing oxygen to diffuse and interact with the dye [18,20]. Oxygen quenching reduces the phosphorescence lifetime, while oxygen depletion maximizes it. The photodetector records these changes, enabling quantitative determination of oxygen concentration [33,34].

Time-resolved measurements capture luminescence decay over micro- to millisecond timescales, enhancing sensitivity and accuracy [9]. Incorporating nanostructured matrices can further improve performance, while combining with imaging techniques enables real-time spatial monitoring of oxygen in biological systems [2,6].

I. Calibration of the Optical Oxygen Sensor

Calibration ensures sensor accuracy by correlating luminescence lifetime with known oxygen concentrations [33]. A HANNA Edge HI 2020 electrochemical oxygen meter with a Clark electrode was used as a reference. Then, 100% saturation was established using a KCl solution, while 0% saturation was achieved with a deoxygenated solution prepared with sodium sulfate and cobalt chloride under nitrogen bubbling [33].

Each sensor was fixed in a temperature-controlled water chamber and PL lifetime readings were collected in triplicate. Data were linearized to obtain calibration slopes and coefficients, which were used to convert luminescence lifetime measurements into oxygen concentrations [33,34]. This procedure ensures reliable real-time monitoring of dissolved oxygen in experimental systems. A schematic overview of the integrated plasma treatment and optical sensing configuration is shown in Figure 2.

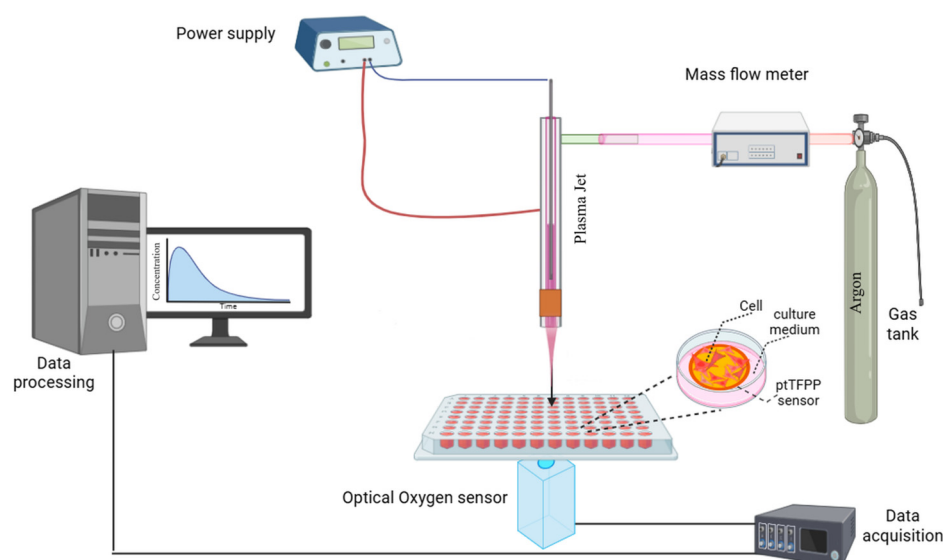


Figure 2. Schematic representation of the cold atmospheric plasma (CAP) treatment and sensing setup. The system integrates an argon-based plasma jet directed toward a well plate containing either HT-29 cells or culture medium. A PtTFPP-based optical oxygen sensor is embedded at the bottom of the well, coupled with a photonic probe for real-time detection of phosphorescence lifetime changes, enabling dynamic monitoring of dissolved oxygen (DO) during plasma exposure.

*(F) Cell Seeding**I. Cell Seeding on Plate*

HT-29 cells were used to assess cell viability and proliferation. Following trypsinization and counting, cells were uniformly seeded into 96-well plates at a density of 4×10^4 cells per well (200 μ L per well). Cells were allowed to attach and grow as a monolayer on the plate surface. Plates were incubated under standard conditions (37 °C, 5% CO₂) for 24–48 h until the desired confluence was reached.

For oxygen measurements, two oxygen-sensitive sensors were used. A 6-well plate was employed, and one sensor was fixed at the bottom of two wells. Once cells reached optimal density, they were seeded directly onto the sensors. After sufficient cell attachment and growth, oxygen measurements were performed.

II. Cell Seeding on Petri Dish and Coverslip

To prepare HT-29 cells, standard protocols of cell culture were followed [2]. Cells were seeded in 3 cm Petri dishes for fluorescence microscopy studies. Coverslips were used to facilitate imaging and stabilize cells [11].

To enhance cell adhesion, coverslips were coated with gelatin. A stock gelatin solution was prepared by dissolving 10 mg gelatin in 2 mL phosphate buffer, followed by homogenization. For working solution, 20 μ L of stock was diluted in 4.5 mL phosphate buffer. A volume of 1.5 mL of the working solution was added uniformly to each coverslip in the Petri dish, ensuring even distribution and avoiding air bubbles. Coated dishes were placed in a laminar flow hood for 30 min, with prior UV sterilization for 10 min to reduce microbial contamination.

Cells were harvested using trypsin, centrifuged, and resuspended in complete medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. A total of 10^4 cells per dish were seeded onto the gelatin-coated coverslips. Dishes were incubated at 37 °C with 5% CO₂, providing optimal conditions for cell adhesion, growth, and proliferation. After the incubation period, cells were ready for treatment with the CAP.

(G) Viability Assessment

The viability and proliferation of HT-29 colorectal cancer cells following CAP treatment were assessed using the MTT colorimetric assay. Cells were seeded in 96-well plates at 200 μ L per well and incubated under standard conditions (37 °C, 5% CO₂) for 24–48 h to reach appropriate confluence.

Plasma treatment was performed using an argon-based cold plasma jet, positioned at a fixed distance of 7 mm from the cells for 60 s. A total of 23 voltage-frequency combinations within the operational range of a kilohertz-frequency plasma source were applied. After treatment, cells were incubated for an additional 24 h before performing the MTT assay.

Metabolically active cells converted MTT into formazan crystals, which were subsequently solubilized using dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using a microplate reader. Cell viability (%) was calculated relative to untreated controls using Equation (1):

$$\left(\frac{\text{Absorbance of treated well}}{\text{Absorbance of control well}} \right) \times 100 = (\%) \text{Cell Viability} \quad (1)$$

*(H) Plasma treatment**I. MTT Assessment*

The MTT assay is based on the reduction in MTT (a yellow tetrazolium salt) to purple formazan crystals by mitochondrial dehydrogenase enzymes in metabolically active cells. After incubation, the formazan was solubilized in DMSO, and absorbance was measured

at 570 nm using a microplate reader. Viability was calculated as a percentage relative to the untreated control cells.

Cell viability and proliferation of HT-29 cells following plasma treatment were evaluated using the MTT colorimetric assay. Cells were seeded to reach appropriate confluence, and 23 voltage-frequency combinations within the optimal range of an argon-based CAP with a kilohertz-frequency power supply were applied. Plasma was delivered at a fixed distance of 7 mm from the cells for 60 s at a gas flow rate of 3.5 SLM.

In the 96-well plate, six wells were used as controls (three without argon gas and three with argon gas). Each treatment condition was repeated three times to ensure reproducibility. Immediately after plasma exposure, the plate was sealed with parafilm and incubated for 24 h to allow cells to respond to the treatment. Subsequently, the MTT assay was performed. Only metabolically active cells reduce MTT to formazan, providing a quantitative measure of viable cells.

II. DCFH and pH Treatment

For intracellular reactive oxygen species (ROS) assessment, a DCFH-DA probe was prepared by diluting a stock solution (0.9 mg/mL in DMSO) in a 1:20 ratio, followed by a second 1:20 dilution, yielding the working solution. A volume of 150 µL of the diluted probe was transferred to microtubes for experiments.

Plasma treatment was applied under six voltage-frequency conditions selected based on prior MTT results. After each plasma exposure, samples containing DCFH-DA were observed under a fluorescence microscope, and emission spectra were recorded using a spectrometer. Each experiment included a control group (no plasma) and was repeated three times for accuracy.

Simultaneously, the effect of plasma on solution pH was assessed. For each voltage-frequency condition and for a control, 1 mL of DCFH-DA working solution was placed in a Petri dish and exposed to the plasma jet at a 7 mm distance and 3.5 SLM for 60 s. The pH of the solution was immediately measured using a pH meter. All measurements were conducted in triplicate. The results were reported in Supplementary Materials.

III. Intracellular ROS

HT-29 colorectal cancer cells were prepared for exposure to the CAP jet by seeding them in 3 cm Petri dishes containing gelatin-coated glass coverslips to enhance cell adhesion and facilitate subsequent fluorescence imaging. Gelatin coating was prepared by dissolving 10 mg gelatin in 2 mL PBS. For the working solution, 20 µL of stock was diluted in 4980 µL PBS, and 1.5 mL of this solution was added to each coverslip. Coated dishes were incubated under a laminar flow hood for 30 min after UV sterilization for 10 min.

Cells were trypsinized, centrifuged, and resuspended in fresh complete DMEM (supplemented with 10% FBS and 1% penicillin-streptomycin). Approximately 10^4 cells per dish were seeded in 2 mL medium and incubated at 37 °C with 5% CO₂ until they adhered and reached suitable confluence for plasma treatment.

Plasma treatment was applied after 24–48 h of incubation. Cells were exposed to an argon-based cold plasma jet positioned 7 mm above the sample, with a 3.5 SLM gas flow for 60 s. Seven voltage-frequency combinations were selected based on prior MTT results, including three inducing apoptotic responses and four associated with proliferation. Each plasma condition was repeated four times with 1 h intervals between treatments to ensure reproducibility. Untreated cells were maintained as controls.

Intracellular ROS generation was assessed 24 h post-treatment using DCFH-DA fluorescence staining. Treated and control cells were imaged with a fluorescence microscope, and emission spectra were recorded to quantify ROS levels.

(I) AO/PI Dual Staining for Apoptosis Detection

To determine whether CAP-induced cytotoxicity in HT-29 colorectal cancer cells was caused by apoptotic cell death, acridine orange and propidium iodide (AO/PI) dual staining was performed. This method helps distinguish viable cells from early apoptotic, late apoptotic, and necrotic cells by assessing membrane integrity and nuclear morphology.

HT-29 cells were seeded in 3 cm Petri dishes containing gelatin-coated coverslips and treated with CAP. After plasma exposure the cells were incubated for 24 h, rinsed twice with PBS, and stained with a working solution of AO and PI (10 µg/mL each in PBS). The staining solution was applied for 5 min at room temperature in the dark. Cells were immediately imaged using a fluorescence microscope with the appropriate filter sets. Acridine orange passes through intact membranes and stains viable and early apoptotic nuclei green. Propidium iodide labels late apoptotic and necrotic nuclei red once membrane integrity is lost. Morphological signs of apoptosis such as chromatin condensation, membrane blebbing, and nuclear fragmentation were examined [35].

AO/PI findings were evaluated together with MTT viability results, intracellular ROS imaging (DCFH fluorescence), and extracellular oxygen dynamics. This combined analysis helped identify the dominant form of CAP-induced cell death and determine whether oxidative stress preferentially activated apoptotic pathways in each treatment condition.

(J) Data analysis

Quantitative analysis of intracellular ROS levels was performed using ImageJ software, version 1.51n. Fluorescence microscopy images of cells stained with DCFH-DA were converted to grayscale, with background subtraction applied to remove non-specific signals. Regions of interest (ROIs) were manually selected to measure the mean fluorescence intensity for each sample. Fluorescence values were normalized to the untreated control group to account for baseline variations and facilitate comparative analysis across experimental conditions.

All numerical data, including fluorescence intensities and viability percentages, were analyzed and visualized using OriginLab software, version 10.1.0.178, which was used to generate bar graphs, error bars ($\pm$ standard deviation), and comparative visualizations.

(K) Statistical analysis of data

I. Statistical analysis of intracellular ROS data

Statistical assumptions

- Data were first normalized to the mean of the control group.
- Normality: assumed due to small sample size ($n = 3$) and absence of extreme outliers.
- Homogeneity of variance: visually comparable variance across groups.
- One-way ANOVA followed by Tukey's post hoc test was applied.

A one-way analysis of variance (ANOVA) revealed a statistically significant effect of plasma treatment condition on normalized signal intensity ($F(3,8) = 110.77$, $p < 0.001$). Post hoc multiple comparisons using Tukey's test demonstrated that the T2 condition differed significantly from all other groups, while T1 and T3 did not differ significantly from each other. These results confirm a treatment-dependent modulation consistent with the selected viability regimes.

II. pH measurements (Supplementary Data)

Tukey post hoc summary

- All treated groups significantly differ from reference ($p < 0.01$);
- Progressive decrease in pH with treatment intensity;
- No variance inflation observed.

One-way ANOVA demonstrated a statistically significant effect of treatment condition on pH values ($F(6, 14) = 33.20$, $p < 0.001$). Tukey's multiple comparison test confirmed

that all treatment groups differed significantly from the reference condition, indicating a systematic, treatment-dependent acidification of the medium.

III. H₂O₂ titration experiments (Supplementary Data)

Tukey post hoc outcomes

- 0.5–1.5 mM H₂O₂ groups significantly higher than reference ($p < 0.001$);
- ≥ 2 mM groups not significantly different from reference;
- Indicates nonlinear, concentration-dependent behavior.

One-way ANOVA showed a significant effect of hydrogen peroxide concentration on emission intensity ($F(7, 16) = 36.41$, $p < 0.001$). Tukey's post hoc test revealed that intermediate H₂O₂ concentrations (0.5–1.5 mM) produced significantly higher signals compared to the reference, whereas higher concentrations did not, indicating a non-monotonic concentration–response relationship.

IV. Statistical analysis of extracellular oxygen dynamic parameters under different plasma regimes

One-way ANOVA followed by Tukey's post hoc test was applied to compare plasma regimes (T1: 13 kV–6 kHz, T2: 12 kV–7 kHz, T3: 12 kV–8 kHz). All parameters exhibited statistically significant differences among plasma conditions ($p < 0.001$).

3. Results

(A) MTT-Assay

The cytotoxic effect of CAP on HT29 cells was first quantified using the MTT assay. As shown in Figure 3a, plasma exposure led to a voltage–frequency-dependent reduction in cell viability after 24 h.

Among the 23 tested cases, three representative tests were selected for further intracellular ROS analysis; (i) a test in which dead cells outnumbered viable cells (strong cytotoxicity induction), (ii) a test in which viable cells predominated and only a minor fraction underwent cytotoxicity (moderate cytotoxicity), and (iii) a test in which viable and apoptotic cells were present in nearly equal proportions (~50% viability, intermediate cytotoxicity) (Figure 3b).

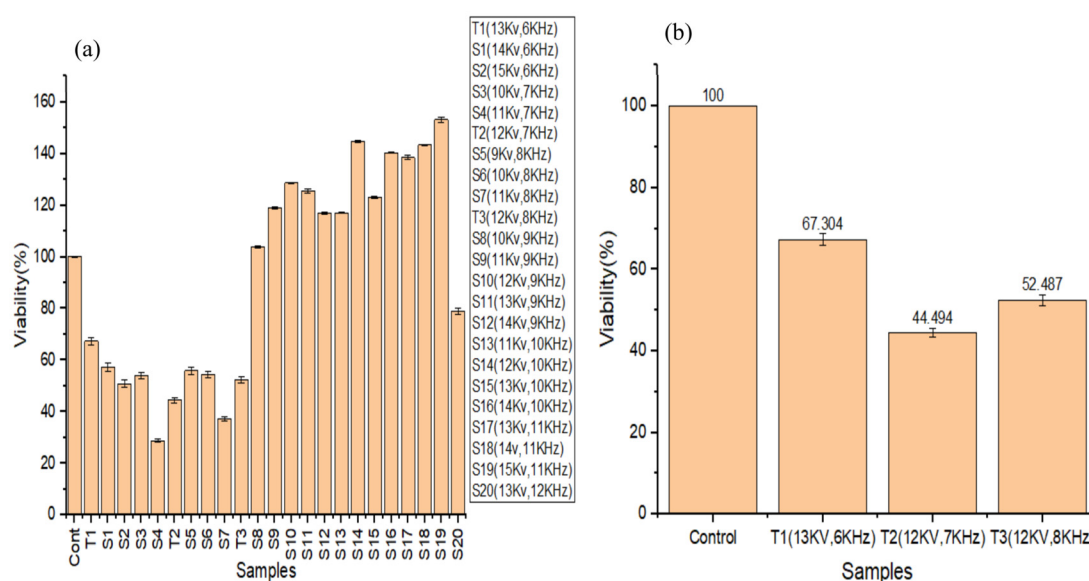


Figure 3. (a) Voltage–frequency-dependent viability of HT-29 cells 24 h post-CAP treatment (MTT assay). (b) Comparison of cell viability under three selected plasma regimes (12 kV–7 kHz, 13 kV–6 kHz, 12 kV–8 kHz) relative to controls.

Table 1 summarizes the viability outcomes of the selected cases relative to the untreated control.

Table 1. HT29 viability after 24 h CAP treatment under three selected plasma regimes (12 kV–7 kHz, 13 kV–6 kHz, 12 kV–8 kHz).

Condition	Voltage–Frequency	Relative Viability (% of Control)	Biological Outcome
T ₁	13 kV–6 kHz	>50% (above IC ₅₀)	Moderate cytotoxicity
T ₂	12 kV–7 kHz	<50% (below IC ₅₀)	Strong cytotoxicity induction
T ₃	12 kV–8 kHz	~50%	Intermediate cytotoxicity

These three tests were subsequently applied for intracellular ROS measurements using the DCFH probe and for extracellular oxygen dynamic experiments, enabling direct correlation between cytotoxicity, intercellular ROS production, and extracellular oxygen dynamics.

(B) AO/PI Dual Staining Confirms the Apoptotic Nature of CAP-Induced Cell Death

To evaluate whether CAP-induced cytotoxicity was mainly apoptotic, AO/PI staining was performed 24 h after exposure for the three selected plasma conditions (T₁, T₂ and T₃). The fluorescence patterns, shown in Figure 4, closely reflected the cell-viability trends observed in the MTT assay.

T₁ (13 kV at 6 kHz; moderate cytotoxicity)

In T₁ most cells displayed green AO staining and normal nuclear morphology, which indicates preserved membrane integrity. A smaller subset of cells showed yellow to orange fluorescence, consistent with early apoptosis. Only a few cells were PI-positive. These AO/PI observations aligned well with the moderate intracellular ROS increase detected in this group. Overall T₁ triggered only partial apoptosis and most cells remained viable.

T₂ (12 kV at 7 kHz; strong cytotoxicity)

In the T₂ condition most cells showed bright orange to red nuclei, indicating loss of membrane integrity and chromatin condensation, which are characteristic of late-stage apoptosis. Nuclear fragmentation and membrane blebbing were also common. Only a small number of cells retained the green AO signal. These observations were consistent with the high intracellular ROS levels measured in this group. Altogether the AO/PI results confirmed that T₂ predominantly induced apoptotic cell death.

T₃ (12 kV at 8 kHz; intermediate cytotoxicity, ~50% viability)

The T₃ condition produced a mixed population of viable and apoptotic cells. Roughly half of the cells exhibited yellow to orange fluorescence, indicating progression from early to late apoptosis, while the remaining cells retained a green signal. This pattern matched the approximately fifty percent viability measured in the MTT assay. The AO/PI findings were also consistent with intermediate levels of intracellular ROS. These combined results confirm that T₃ led to an intermediate apoptotic response.

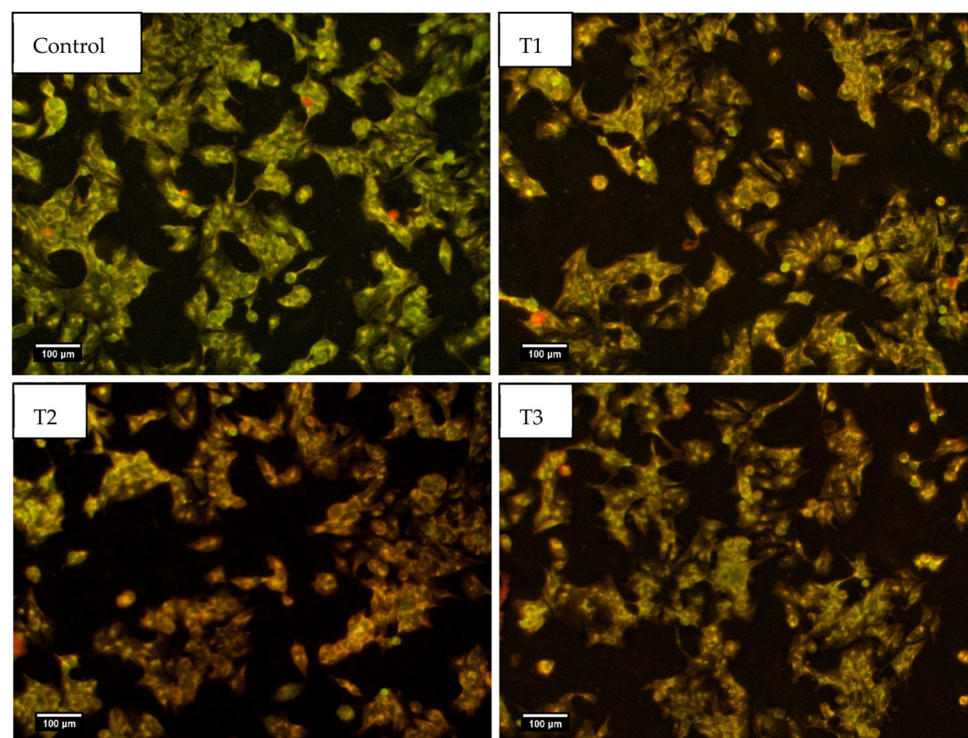


Figure 4. AO/PI staining of HT-29 cells 24 h after CAP treatment. T1 shows predominantly viable, green-stained cells (moderate cytotoxicity), T2 displays strong red/orange PI-positive staining indicative of late apoptosis (high cytotoxicity), and T3 presents a mixed green/orange population consistent with intermediate apoptotic response.

(C) Intracellular ROS level (Fluorescence Imaging)

To directly assess intracellular ROS dynamics following CAP exposure, HT-29 cells were incubated with the DCFH-DA probe and imaged using fluorescence microscopy. Upon oxidation by ROS, non-fluorescent DCFH is converted to fluorescent DCF, allowing semi-quantitative evaluation of intracellular ROS through fluorescence intensity. Representative fluorescence images are shown in Figure 5, corresponding to three selected plasma regimes (12 kV–7 kHz, 13 kV–6 kHz, and 12 kV–8 kHz) at multiple time points: before CAP exposure, immediately after treatment, and 24 h post-treatment. In all cases, CAP exposure resulted in enhanced intracellular fluorescence compared to control, confirming the generation of ROS inside the cells.

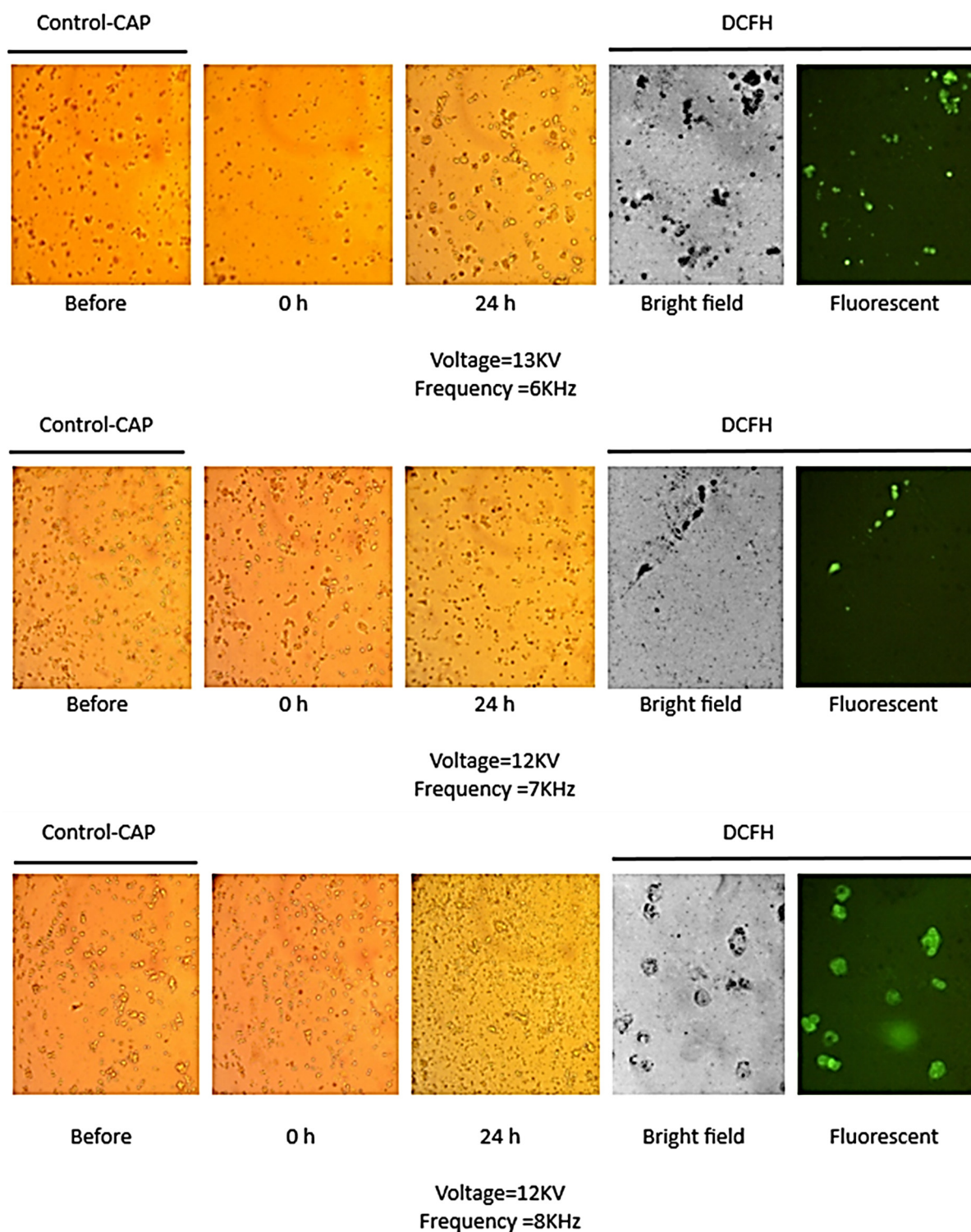


Figure 5. Fluorescence and bright-field microscopy images of DCFH-DA-stained HT-29 cells before, immediately after, and 24 h post-CAP treatment under three plasma regimes (13 kV–6 kHz, 12 kV–7 kHz, 12 kV–8 kHz). Enhanced green fluorescence indicates intracellular ROS accumulation after CAP treatment.

Quantitative image analysis using ImageJ revealed distinct fluorescence intensity profiles (Figure 6).

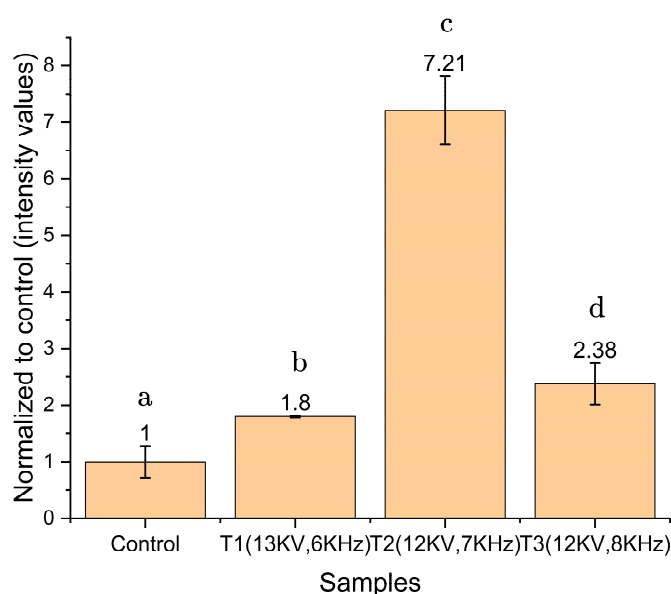


Figure 6. Quantitative analysis of intracellular ROS bar graph of normalized DCFH fluorescence intensity in HT-29 cells under three selected voltage–frequency conditions compared to untreated controls, highlighting the correlation between ROS levels and CAP-induced cytotoxicity. Groups marked with the same letter are not significantly different from each other ($p > 0.05$). Groups marked with different letters are significantly different from each other ($p \leq 0.001$).

At 12 kV–7 kHz (T_2), corresponding to the strongest apoptotic effect in MTT, intracellular fluorescence intensity increased sharply and remained elevated at 24 h, consistent with sustained ROS accumulation leading to apoptosis. In contrast, at 13 kV–6 kHz (T_1), the fluorescence signal showed a moderate increase, indicating moderate ROS generation and partial cytotoxicity. Finally, at 12 kV–8 kHz (T_3), intracellular fluorescence intensity was intermediate between T_1 and T_2 , consistent with ~50% viability observed in the MTT assay. This indicates that viable and apoptotic cells were present in nearly equal proportions, representing a balanced state with intermediate ROS generation and cytotoxicity.

Together, these results establish a clear correlation between intracellular ROS levels and the biological outcomes of CAP treatment. High ROS accumulation (T_2) aligned with strong death induction, while moderate or intermediate ROS levels (T_1 and T_3) corresponded to partial cytotoxicity.

(D) Extracellular DO Dynamics

Extracellular oxygen dynamics were investigated using time-resolved PL spectroscopy [36]. Two calibrated optical sensors were employed to monitor oxygen quenching signals before and after CAP treatment, enabling reconstruction of both oxygen concentration and luminescence lifetime profiles.

Figure 7a–c present the scatter plots of luminescence lifetime and corresponding oxygen concentration in a cell-free culture medium while Figure 7d–f show the same parameters in the presence of HT-29 cells. In the cell-free culture medium, since only one sensor was used, the PL lifetimes are enough to compare the results. In both cases, CAP exposure caused a decrement in oxygen concentration right after treatment, reflecting strong ROS generation and oxygen consumption. By time, the balance of the consumption of the plasma-derived ROS and the generation of the oxygen results in a steady state level for

dissolved oxygen concentration close to that before plasma exposure in the presence of HT29 cells.

To quantify extracellular oxygen dynamics, two parameters were defined:

Maximum oxygen depletion (Max Activity): the difference between lowest oxygen concentration reached right after exposing CAP and the stable oxygen concentration level before plasma treatment, reflecting the maximum extracellular oxygen consumption and ROS formation.

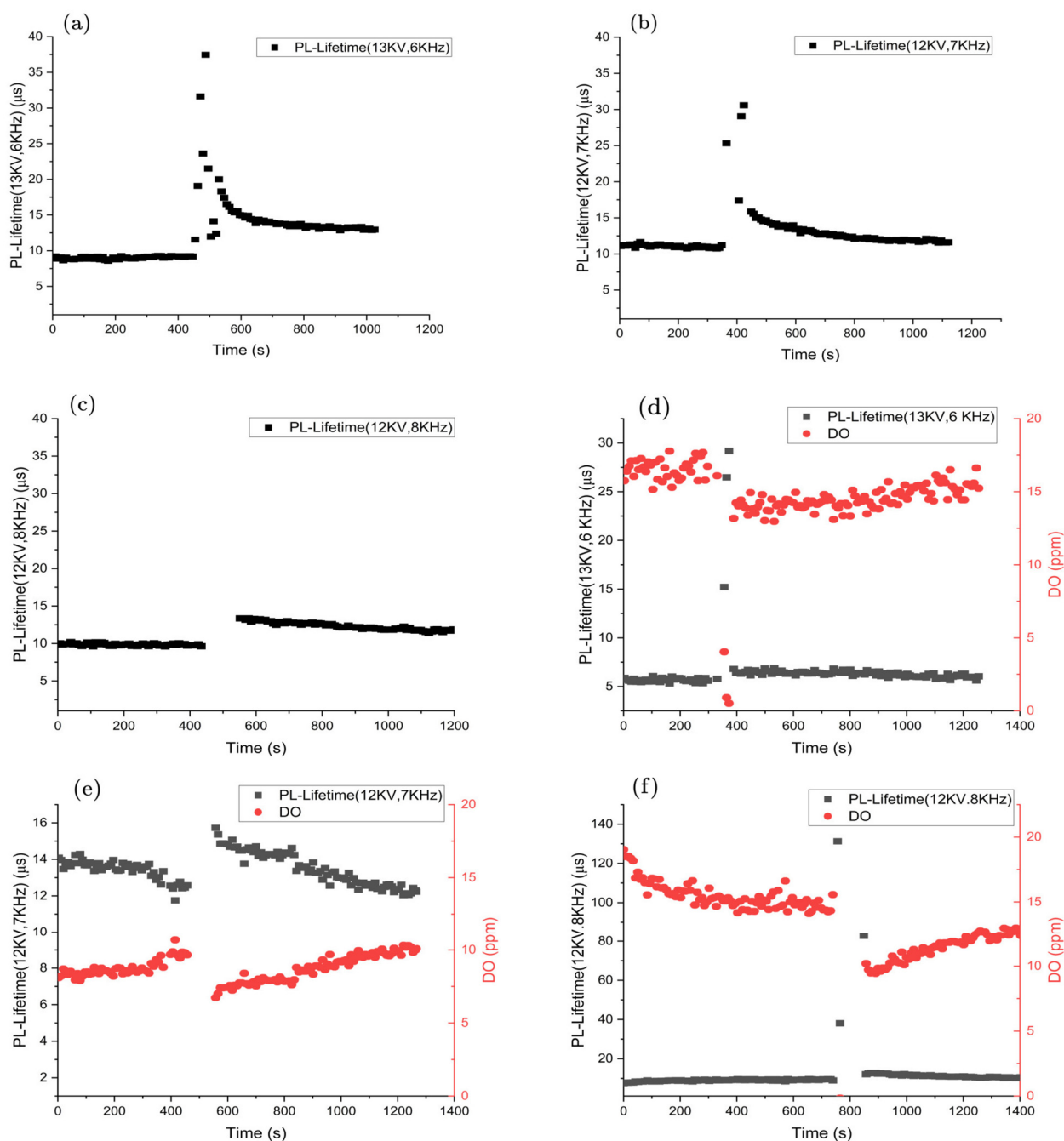


Figure 7. Oxygen kinetics recorded by time-resolved phosphorescence in (a–c) cell-free DMEM and (d–f) medium containing HT-29 cells after CAP exposure. Scatter plots show PL lifetime (black) versus oxygen concentration (red), illustrating strong oxygen depletion in cell-free medium and partial restoration by cellular oxygen release.

The rate of oxygen production in the medium (WIDTH): the spread of lifetime/concentration curves, reflecting the rate and dynamics of oxygen production (broader width = faster rate, narrower width = slower rate). In this regard, the time to reach half of the maximum oxygen depletion (Max Activity) defined as WIDTH. The two parameters (Max Activity and WIDTH) are shown in Figure 8.

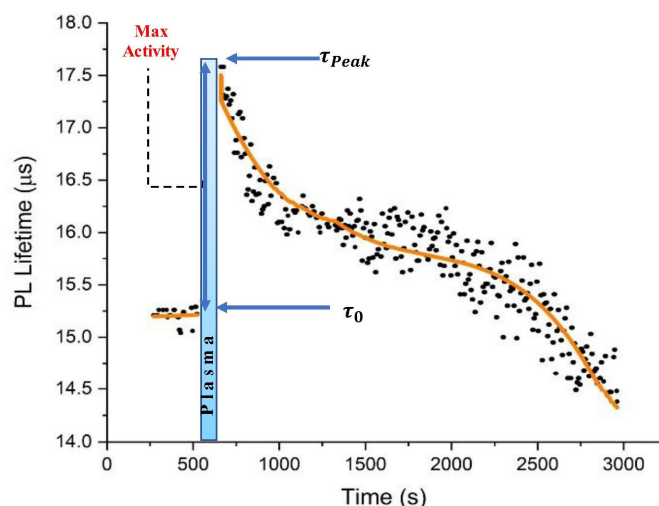


Figure 8. Distribution width of oxygen dynamics. Bar graph comparing the time span of oxygen depletion/recovery phases (WIDTH) in cell-containing media under three plasma regimes, illustrating faster oxygen dynamics in apoptotic conditions and slower kinetics in viable conditions.

Oxygen time-traces were recorded in parallel for all three cases in cell-free medium and in medium containing HT-29 cells and two defined parameters—maximum oxygen depletion (MAX Activity) and the WIDTH—were extracted, as seen in Figure 9.

To establish a comprehensive picture of CAP-induced oxidative stress, extracellular oxygen dynamics are compared with intracellular ROS levels (DCFH fluorescence) and cell viability (MTT assay). In the first step, how the defined extracellular oxygen parameters related to MTT results was examined. In the cell-containing condition, MAX values showed a considerable correlation with cell survival. Cases with higher viability exhibited deeper oxygen depletion, which is consistent with the metabolic activity of the viable cells, consuming oxygen in the minutes following CAP exposure. WIDTH provided complementary and more interesting information: in apoptotic conditions, WIDTH became narrower. In T₂, where viability was low (most of the cells in the apoptotic phase), this narrowing reflected accelerated oxygen turnover driven by apoptotic activity. In T₁, where more cells remained viable, WIDTH broadened showing that surviving cells slowed the oxygen kinetics by continuing to consume oxygen. However, it is important to note that in the cell-free measurements, MAX and WIDTH graph behavior showed no consistent relationship with the trend of corresponding MTT results, underscoring the strong link between oxygen dynamics and cell fate.

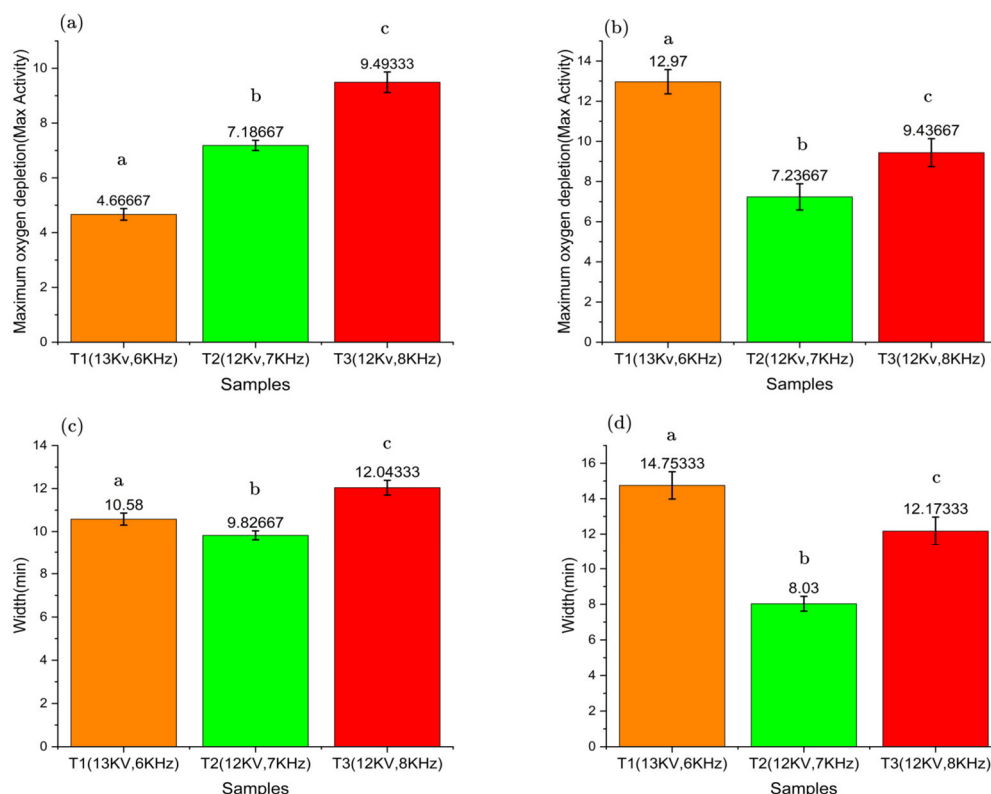


Figure 9. Quantitative comparison of extracellular oxygen dynamic parameters following CAP treatment under three plasma regimes: T1 (13 kV–6 kHz), T2 (12 kV–7 kHz), and T3 (12 kV–8 kHz). (a) Maximum oxygen depletion (MAX) in cell-free medium; (b) MAX in cell-containing medium; (c) WIDTH of oxygen depletion–recovery dynamics in cell-free medium; (d) WIDTH in cell-containing medium. Groups marked with the same letter are not significantly different from each other ($p > 0.05$). Groups marked with different letters are significantly different from each other ($p \leq 0.001$).

In the next step, the three cases are directly compared with one another. In the cell-free medium, MAX values varied only slightly between plasma settings (Figure 9a), and WIDTH remained nearly constant at around ten minutes across all cases (Figure 9c). This confirmed that plasma chemistry alone dictates the initial oxygen depletion while the culture medium under various exposing conditions does not have a considerable effect on the rate of the oxygen generation after treatment. In contrast, the cell-containing group displayed pronounced differences. T₂ showed a relatively small MAX, while T₁ exhibited the deepest MAX (Figure 9b), indicating that viable cells consumed oxygen on top of the plasma-driven effect. T₃ was intermediate, with MAX values close to those in the cell-free condition. When looking at WIDTH (Figure 9d), T₂ had the narrowest profile (around eight minutes), T₁ the broadest (about fourteen minutes), and T₃ again resembled the cell-free condition. These results show that both MAX and WIDTH diverge strongly between cases with cells phases.

In the last step, we compared each test in the presence and absence of cells. In T₂, MAX was essentially unchanged between conditions, showing that the initial depletion was plasma-driven, while WIDTH decreased from 9.8 min without cells to 8.0 min with cells, reflecting accelerated oxygen generation rate by apoptotic cells. In T₁, MAX became deeper when cells were present, reflecting oxygen consumption by metabolic activity of the viable cells, while WIDTH broadened from ten to fourteen min, consistent with viable cells prolonging the dynamics. In T₃, both MAX and WIDTH were similar in the presence

and absence of cells, indicating a balance between oxygen release from apoptotic cells and oxygen consumption by viable cells.

Taken together, the results establish that MAX is a marker of the primary, plasma-driven depletion of oxygen, while WIDTH serves as a sensitive indicator of secondary, cell-mediated oxygen dynamics. When considered alongside MTT viability data, the two parameters provide a predictive framework: strong apoptosis is characterized by reduced viability, high intracellular ROS, small MAX, and narrow WIDTH; moderate cytotoxicity is marked by preserved viability, deep MAX, and broad WIDTH; and intermediate outcomes fall between these extremes, as summarized in Table 2. Thus, oxygen dynamics recorded within the first twenty minutes after CAP exposure provide an early predictor of the long-term fate of tumor cells.

Table 2. Summary of key parameters across experimental conditions.

Condition (Voltage–Frequency)	MTT Viability (24 h)	Intracellular ROS (DCFH)	Extracellular Oxygen (max)	Extracellular Oxygen (width)	Interpretation
12 kV–7 kHz (T ₂)	<IC ₅₀ (strong cytotoxicity)	High	Smaller (less depletion, due to O ₂ release by apoptotic cells)	Narrower (faster oxygen dynamics)	Oxidative stress shifted inside cells; extracellular depletion compensated by cellular O ₂ release.
13 kV–6 kHz (T ₁)	>IC ₅₀ (moderate cytotoxicity)	Moderate	Larger (more depletion)	Broader (slower kinetics)	ROS partially intracellular, extracellular depletion reflects direct CAP–medium interaction.
12 kV–8 kHz (T ₃)	~50% intermediate cytotoxicity)	Low/near baseline	Similar with and without cells (balanced depletion)	Unchanged (balanced kinetics)	Apoptotic cells release O ₂ while viable cells consume it; opposing effects cancel each other, leading to an intermediate state.

4. Discussion

In this work, by combining several approaches including MTT viability assays, intracellular ROS imaging, AO/PI staining, and time-resolved extracellular oxygen monitoring, we were able to create a multi-layered picture of how plasma reshapes the oxidative environment of cancer cells.

To establish a comparative framework linking plasma parameters to cellular fate, we exposed HT-29 colorectal cancer cells to a series of voltage–frequency combinations of the argon cold atmospheric plasma jet and classified the responses based on post-treatment viability MTT results. Among twenty-three tested regimes, three representative conditions were selected to define distinct biological outcomes: one dominated by cell survival, one by death, and one displaying a balanced intermediate state. Specifically, treatments at 13 kV–6 kHz produced the highest survival fraction (>50% viability), 12 kV–8 kHz resulted in approximately equal proportions of viable and death cells (~50% viability), and 12 kV–7 kHz induced pronounced death (<50% viability). These three cases served as the core experimental groups for the next analyses.

Thereafter to determine whether the viability outcomes observed in the MTT assay indeed reflected apoptotic cell death, we quantified intracellular ROS using the fluorescence-based DCFH-DA assay. For probe validation (in the Supplementary Materials), we first characterized the optical response of DCFH-DA by recording its fluorescence emission spectra under plasma exposure and by titrating with increasing concentrations of hydrogen peroxide (H₂O₂). These calibration experiments revealed a clear dependence of probe fluorescence on both ROS concentration and medium pH, confirming that the probe

reliably reports oxidative stress within the physiological pH range of living cells (see Supplementary Materials).

Subsequently, intracellular ROS imaging demonstrated a marked, voltage-frequency-dependent rise in fluorescence intensity that closely mirrored the MTT-derived viability trends. In particular, plasma conditions associated with reduced viability (12 kV–7 kHz) produced the strongest intracellular ROS accumulation [11,13,21], whereas higher-survival regimes (13 kV–6 kHz) exhibited only moderate increases.

Our combined analysis that integrates MTT viability measurements, intracellular ROS imaging, real-time extracellular oxygen dynamics, and AO/PI apoptosis staining shows that CAP mainly induces apoptotic cell death in HT-29 colorectal cancer cells. The AO/PI assay demonstrated that treatment conditions producing strong intracellular ROS generation, particularly the 12 kV–7 kHz regime, led to classical apoptotic features such as chromatin condensation, membrane blebbing, and nuclear fragmentation. These observations indicate that oxidative stress drives cells toward a programmed apoptotic pathway rather than necrotic breakdown. The AO/PI results also supported the oxygen-dynamic signatures identified earlier in the study. Taken together, these findings provide multilevel evidence that CAP induces apoptosis through ROS-dependent mechanisms and that early changes in oxygen dynamics within the first 20 min after treatment can serve as predictive indicators of long-term cell viability. Integrating biochemical markers such as AO/PI with biophysical oxygen-dynamic measurements offers a coherent mechanistic framework for redox-guided optimization of plasma-based cancer therapy.

To obtain a clearer picture of the selective processes underlying plasma-induced cell death, we continuously monitored the temporal dynamics of dissolved oxygen in the extracellular culture medium for several hours following plasma treatment. Real-time luminescence oximetry revealed that oxygen concentration dropped sharply immediately after exposure, reflecting plasma-driven oxidative reactions, and then gradually recovered toward baseline. To quantitatively describe these dynamics, two parameters were defined: MAX, the minimum oxygen concentration reached after treatment, and WIDTH, the timespan of oxygen depletion and recovery [33,34]. In cell-free medium, both parameters remained relatively constant across plasma regimes, indicating a purely physicochemical response. However, in the presence of cells, distinct patterns emerged, shallower MAX values and narrower WIDTH profiles were associated with strongly apoptotic conditions, while deeper MAX and broader WIDTH reflected higher metabolic activity and cell survival [3]. These trends demonstrate that oxygen dynamics encode information about the cellular response to plasma: the initial depletion phase mirrors the primary plasma-induced oxidative input, whereas the recovery dynamics reflect secondary, cell-mediated processes [18]. Our data provide experimental support consistent with this mechanistic framework. Within our measurements, the parameter MAX reflects the primary plasma-induced oxidative input, while WIDTH captures the secondary, cell-driven amplification phase [8].

At the same time, comparison of intracellular and extracellular outcomes revealed an intriguing redox redistribution [5]. The redox redistribution summarized in Figure 10 illustrates this reciprocal relationship between intracellular ROS buildup and extracellular oxygen depletion. Strong apoptosis correlated with high intracellular ROS accumulation but with weaker extracellular oxygen depletion [9,11]. This apparent paradox reflects the physiological collapse of apoptotic cells: once respiration and membrane integrity are lost, extracellular oxygen consumption decreases while intracellular oxidative stress intensifies through mitochondrial dysfunction and secondary oxidant formation [28,37]. Conversely, viable cells sustain respiration, producing deeper extracellular oxygen depletion but milder intracellular ROS buildup [21]. This selective redistribution of oxidative burden explains how cold plasma induces apoptosis through internal ROS amplification rather

than external oxidative excess. Importantly, this mechanism also suggests why CAP treatments tend to spare healthy cells: apoptotic cancer cells act as localized sinks of oxidative stress, confining damage within malignant populations.

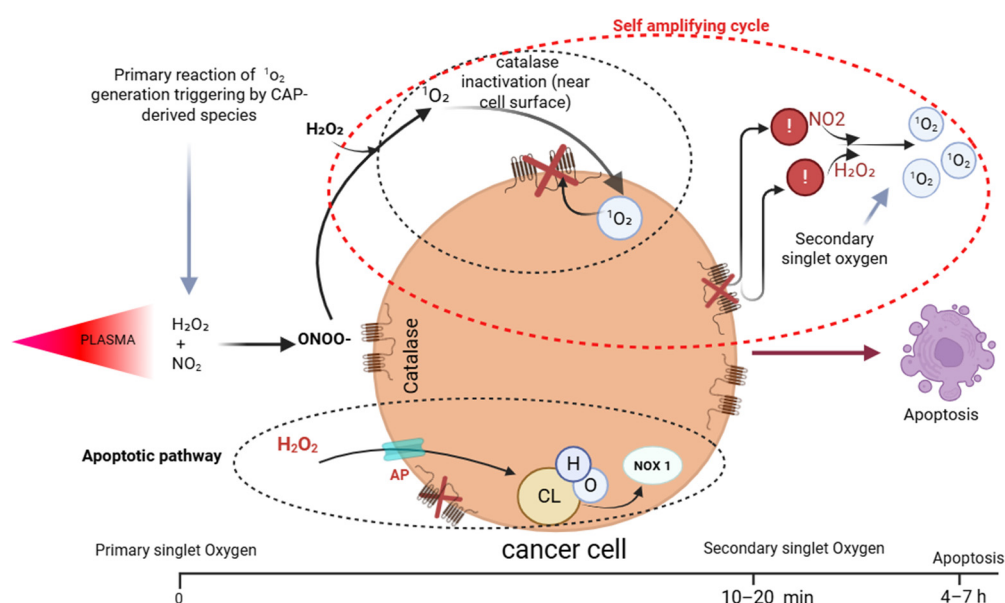


Figure 10. Comparative schematic summarizing redox redistribution after CAP exposure: strong apoptosis correlates with high intracellular ROS and weak extracellular O_2 depletion, whereas viable cells exhibit the opposite pattern.

5. Conclusions

In this work, combining several approaches including MTT viability assays, intracellular ROS imaging, and time-resolved extracellular oxygen monitoring, we were able to create a multi-layered picture of how plasma reshapes the oxidative environment of cancer cells. Our findings provide experimental validation of Bauer's redox model of plasma mode of action [25,27], while also establishing a predictive framework based on oxygen dynamics recorded within the first 20 min post-CAP exposure. This model holds potential for forecasting long-term cell fate in plasma therapy and even photodynamic therapy [1,38].

The surge of intracellular ROS observed is consistent with the activation of classical apoptotic pathways—namely, mitochondrial dysfunction, cytochrome c release, and caspase activation [9,28,37]. Taken together, these findings confirm that the cytotoxicity observed in MTT assays stems primarily from oxidative stress-induced apoptosis, rather than necrosis.

Importantly, the characteristic WIDTH patterns observed within the first 20 min after treatment provided an early predictor of the eventual cytotoxic outcome. This aligns with the temporal dynamics of Bauer's model, where secondary 1O_2 amplification peaks within 10–20 min, offering a window for real-time assessment of treatment efficacy [23,26,27].

In accordance with Bauer's hypothesis on the selective apoptotic response to plasma treatment, the observed temporal variations in dissolved oxygen are plausibly linked to secondary singlet oxygen 1O_2 formation. Although 1O_2 itself has a microsecond lifetime and cannot be directly detected through DO measurements, its rapid reactions with cellular lipids and biomolecules yield stable oxidation products and regenerate ground-state O_2 , thereby modulating the measurable stable oxygen in the medium. Consequently, the recorded DO dynamics likely reflect the cumulative impact of transient 1O_2 generation and decay processes within the cell culture environment.

According to Bauer's two-step redox theory, plasma first introduces long-lived oxidants such as hydrogen peroxide (H_2O_2) and nitrite (NO_2^-), which interact to generate primary singlet oxygen ($^1\text{O}_2$) and transiently inactivate membrane-bound catalase [1,27]. Once this enzymatic protection is weakened, cancer cells themselves become active participants in a self-amplifying oxidative loop, where endogenous superoxide and peroxy-nitrite reactions drive the secondary wave of singlet oxygen production [5,22].

The narrower WIDTH observed under cytotoxic conditions precisely mirrors the accelerated secondary ROS dynamics predicted by Bauer's model, representing a clear, time-resolved signature of singlet oxygen amplification [28]. Moreover, the ability of these oxygen-dynamic parameters to predict cell fate within the first 20 min after plasma exposure corroborates Bauer's emphasis on transient, redox-driven events as the decisive trigger of selective oxidative cytotoxicity [26]. Together, these findings transform the Bauer model from a theoretical framework into a quantitatively verified mechanism, firmly anchoring it within the experimental landscape of plasma oncology [21].

Beyond mechanistic insight, the early predictive capability of oxygen dynamics offers a promising translational avenue for adaptive, redox-guided plasma therapy, enabling the real-time adjustment of treatment parameters to maximize selectivity and minimize collateral effects.

6. Limitations and Perspectives

While this work represents, to the best of our knowledge, the first experimental validation of Bauer's theory, a few limitations and perspectives can be envisioned.

This study was conducted *in vitro*, and *in vivo* tumor tissues will present additional complexity. Vascular oxygen supply, extracellular matrix properties, and immune responses may all influence CAP effects [19,20]. Moreover, plasma treatment has been shown to induce oxygen-level modulation in mouse and human tissues, which can be monitored in real time using ruthenium-based fiber-optic sensors (i.e., Oxylight system) [37,39]. The integration of such *in vivo* oxygenation monitoring with the time-resolved extracellular oxygen measurements implemented in the present study could provide a coherent multimodal platform for controlled and feedback-guided plasma antitumor therapy. Future experiments should therefore include ROS scavengers and caspase or necroptosis inhibitors to further elucidate the involved death pathways, as well as *in vivo* validation to determine whether MAX and WIDTH parameters can predict tumor response under physiologically relevant oxygenation dynamics.

The validation of the experimental method proposed in this work should be extended with other non-thermal plasma sources and other plasma delivery protocols, including so-called direct treatment, as well as plasma conditioned solution exposure. The full validity of the time resolved phosphorescence DO sensor based on dissolved oxygen measurement but not on the challenging direct quantification of singlet oxygen to validate Bauer's model is one of the limitations of this study. Future investigations may try to perform spin trap electron paramagnetic resonance to confirm the experimental demonstration. Nevertheless, the easy implementation and time resolved nature of the DO probe are considerable advantages supporting its validation by the number of groups involved in plasma medicine studies.

Finally, as one of the key hallmarks for cancer therapy is the selectivity of the therapeutic protocol, the exposure of both healthy and cancer cells would be highly relevant to confirm the cell involvement in the ROS-based treatment.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antiox15020209/s1>. 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 H₂O₂ concentration demonstrating ROS-driven oxidation and pH-dependent response. Statistical significance compared to the reference case is indicated as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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