

Assessment of Oxidative Effects of Cold Atmospheric Plasma via Image Processing Techniques

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Abstract—Cold atmospheric plasma (CAP) produces reactive oxygen and nitrogen species (ROS/RNS) that induce oxidative modifications on microbial surfaces. This study quantitatively assessed CAP-induced oxidative effects on KI-starch agar using image processing techniques. Plasma treatments were applied with varying discharge gaps, duty cycles, frequencies, and treatment times, resulting in 648 images. From these images, 106 features related to color, texture, and frequency were extracted and analyzed using Pearson and Spearman correlations, multi-way ANOVA, and multicollinearity checks. Plasma treatment time emerged as the primary factor influencing visual features, while interactions among all plasma parameters were statistically significant ($p < 0.001$). A final set of 30 non-redundant features captured the key surface modifications, offering a reliable framework for predictive modeling. These results provide a quantitative understanding of plasma-induced oxidative effects and highlight the potential of image-based markers for optimizing plasma-based antimicrobial applications.

Index Terms—Cold Atmospheric Plasma, Image Processing, Antimicrobial Activity, Plasma Medicine, Statistical Analysis

I. INTRODUCTION

Cold Atmospheric Plasma (CAP) is a type of plasma generated by ionizing gases at low temperatures [1]. Its ability to operate at low temperatures enables its application without causing damage to living cells, tissues, and microorganisms [2]. The generation of active components such as reactive oxygen species (ROS), reactive nitrogen species (RNS), free radicals, ultraviolet light, and electric fields during plasma ionization significantly influences biological and chemical processes [3]. These components influence biological and

chemical processes, leading to structural changes in microorganisms, the development of antimicrobial effects, regulation of cell proliferation, and modifications of certain biological structures. Particularly, reactive species such as (Reactive Oxygen Nitrogen Species (RONS) can cause oxidative and nitrosative stress in cells, leading to cell membrane damage and disruption of metabolic functions [4].

One of the most significant benefits of CAP in biomedical applications is its strong antimicrobial properties. Studies have shown that CAP can effectively eliminate bacteria, viruses, and fungi [5], [6]. Furthermore, studies have shown that direct CAP applications subject many microorganisms, including biofilms, to powerful electrical fields that can damage their physicochemical structure [7]. Traditional sterilization and disinfection methods often become ineffective as microorganisms develop resistance over time. Moreover, many chemical disinfectants can lead to toxic side effects. CAP overcomes these issues with its broad-spectrum antimicrobial activity, providing sterilization without leaving residues on the treated surface [8].

Despite the widespread application of CAP, there is still a need to accurately determine the plasma parameters to optimize its antimicrobial efficacy. Therefore, this study aims to analyze the oxidative effects of CAP under different plasma conditions using image processing and statistical analysis methods to determine optimal treatment parameters. This study quantitatively reveals oxidative effects through image processing, thereby enabling future bacterial assays to establish a direct link between oxidative visualization and antimicrobial efficacy. To effectively assess the oxidative effects of CAP, a reliable visualization method is required. Among various techniques, the KI-starch agar method stands out for its ability to provide a rapid and direct assessment of plasma-generated

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reactive species [9], [10]. In this technique, reactive species produced by CAP oxidize the KI-starch complex, resulting in a visible color change in the agar medium. The intensity and spread of the color change may be directly related to parameters such as plasma treatment time, frequency, duty cycle value, and discharge gap. In this study, statistical analysis was performed to elucidate the correlation between color changes and plasma treatment parameters.

II. MATERIALS AND METHODS

A. Data Collection

1) *Preparation of Agar Medium and KI-Starch:* For the preparation of the agar medium, 3 grams of agar powder were mixed with 200 mL of deionized water (DIW). The mixture was then heated on a hot plate at 95-100 ° C and homogenized using a magnetic stirrer. The solution was cooled to 50-55°C. For the preparation of the KI-starch solution, 1.2 grams of starch (Sigma Aldrich) were gradually added to 200 mL of preheated DIW and mixed using a magnetic stirrer. Once homogenized, the solution was allowed to reach room temperature, after which 2.4 grams of Potassium Iodide (KI, TEKKIM) were added and the solution was homogenized again. The prepared KI-starch solution was mixed with the agar solution, which had been cooled to approximately 50°C, and the resulting mixture was poured into sterile Petri dishes at 15 mL per dish. The agar was then allowed to solidify at room temperature for 30-60 minutes.

2) *Plasma Device and Experimental Setup:* A custom-made plasma device was used to apply plasma treatments to the KI-Starch agars. The plasma was generated using a specially manufactured copper electrode in the Dielectric Barrier Discharge (DBD) method to ensure controlled plasma generation and uniform application. Four fundamental plasma parameters were examined: discharge gap (0.5,1,1.5 mm), duty cycle(40%, 60%, 80%, 100%), frequency (1, 2, 3 kHz), and treatment time(5-10-15-30-45-60 sec). Each parameter plays a determining role in the antimicrobial efficacy by influencing the intensity and distribution of reactive species generated by plasma.

3) *Imaging Setup:* Plasma treatment was applied to the KI-starch agar medium using the predefined experimental parameters. The ROS/RNS generated by the plasma application caused visible color changes on the agar surface. Images were captured using a fixed camera angle under uniform lighting conditions. Example of images shown in Fig. 1. All experiments were carried out in triplicate to ensure the reliability and reproducibility of the results. 648 agar image were collected from 216 different experimental combinations, each with 3 repetitions.

Images	Plasma Parameters
	Discharge gap: 1.5 mm Duty Cycle: 40% Frequency: 1000 Hz. Plasma Treatment Time: 5 sec
	Discharge gap: 1 mm Duty Cycle: 100% Frequency: 1000 Hz. Plasma Treatment Time: 5 sec
	Discharge gap: 1 mm Duty Cycle: 100% Frequency: 3000 Hz. Plasma Treatment Time: 15 sec
	Discharge gap: 1.5 mm Duty Cycle: 100% Frequency: 2000 Hz. Plasma Treatment Time: 60 sec

Fig. 1. Examples of obtained images.

B. Image Processing

1) *Preprocessing:* Raw images obtained from experimental studies were subjected to various preprocessing steps to make them suitable for analysis. First, to reduce the influence of irrelevant areas such as labels and background, and to standardize the image dimensions, all images were resized to 512×512 pixels. To focus on the central region where the plasma effect was most intense, a circular cropping was applied. In this process, a circular mask was created at the center of the image. To reduce color imbalances and lighting variations, the White Patch Retinex (WPR) method was employed. WPR is a color balancing and illumination correction technique that rescales the color channels based on the brightest (white) regions of the image [11]. This minimizes color distortions caused by lighting sources, enabling more consistent and accurate color analysis. To enhance image contrast, Contrast Limited Adaptive Histogram Equalization (CLAHE) was applied. CLAHE divides the image into small regions and enhances local contrast in each region, making plasma-induced color changes more distinguishable. It also prevents noise amplification caused by excessive contrast enhancement [12].

2) *Feature Extraction:* To numerically evaluate the effects of plasma treatment on the agar surface, multidimensional features based on color, texture, and frequency were extracted. To ensure that the analysis focused solely on plasma-affected regions, Region of Interest (ROI) masks were created for each image using alpha channel-based binary masking to exclude irrelevant areas. Regarding color features, statistical measures including mean, standard deviation, skewness, kurtosis, and entropy were calculated for the red, green, and blue channels of the RGB color space. To capture color variations more

accurately, RGB images were converted to HSV [13] and LAB color spaces. In the HSV space, mean, standard deviation, skewness, kurtosis, and entropy values were extracted from the hue, saturation, and value channels. Similarly, in the LAB space, these statistical features were computed for the lightness (L), green-red (A), and blue-yellow (B) channels. This comprehensive approach allowed a more detailed and reliable assessment of plasma-induced color changes. Additionally, a purple color ratio was calculated from the HSV space to quantify plasma-generated purple color variations. In order to quantify the relationships between different color channels, R/G and R/B ratios were calculated in RGB, S/V in HSV, and A/B in LAB. Intensity-based features were extracted from grayscale images, including fundamental statistics such as mean, standard deviation, skewness, kurtosis, and entropy, as well as histogram-based features including width, energy, entropy, and contrast. This enabled a detailed quantification of changes in intensity levels caused by plasma treatment. Texture features were derived using the Gray Level Co-occurrence Matrix (GLCM) method. GLCM models the spatial relationships between pixels to provide statistical texture parameters such as contrast, homogeneity, energy, correlation, and dissimilarity [14]. For frequency analysis, Fast Fourier Transform (FFT) was applied to examine structural regularities and periodicities induced by plasma on the surface. Mean and standard deviation values of the amplitude and phase spectra were calculated to numerically characterize frequency components.

3) *Dataset Construction:* All color, intensity, texture, and frequency features extracted from images were compiled into a dataset along with sample IDs. The target variable was defined as the purple area induced by plasma exposure ("Purple Area"), which was measured in mm² using ImageJ.

4) *Data Normalization:* Because the resulting features have different scales and distributions, they pose a risk of creating imbalances in the analyses. To address this issue and ensure comparability across features, all numerical data were normalized using the Z-score method. In this method, each feature was rescaled internally using its own mean and standard deviation. Identity information and output variables were excluded from this process.

C. Statistical Analysis

Statistical analyses were performed to investigate the relationships between plasma parameters and the numerical features extracted from KI-starch images. A feature-wise approach was applied, evaluating each feature individually against the plasma parameters (Discharge Gap, Duty Cycle, Frequency, and Plasma Treatment Time) to identify the most sensitive features while excluding irrelevant or noisy ones. Pearson and Spearman correlations were calculated to examine relationships between each parameter and feature, and those with significant correlations ($p < 0.05$) and strong associations ($|r| > 0.5, p < 0.01$) were selected for further analysis. Multi-way ANOVA was then used to assess both main and interaction effects, treating all parameters as cat-

egorical variables and applying a significance threshold of $p < 0.001$. Finally, highly correlated feature pairs ($|r| > 0.9$) were identified and one feature from each pair was removed, resulting in an independent, multicollinearity-free feature set suitable for further analysis.

III. RESULTS AND DISCUSSION

A. Correlation Analysis

Pearson and Spearman correlation coefficients were calculated to evaluate the relationships between plasma parameters and 106 extracted image features. After filtering by statistical significance ($p < 0.05$), 318 significant correlations were identified with Pearson and 272 with Spearman.

Among the parameters, Plasma Treatment Time consistently showed the strongest relationships with visual features. Notably, high correlations were observed for Mean R ($r_{\text{Pearson}} \approx -0.57$, $r_{\text{Spearman}} \approx -0.72$), Entropy R ($r_{\text{Pearson}} \approx -0.58$, $r_{\text{Spearman}} \approx -0.72$), and Skewness R ($r_{\text{Pearson}} \approx 0.56$, $r_{\text{Spearman}} \approx 0.72$). These results suggest that longer treatment reduces brightness and entropy while increasing asymmetry in pixel distributions, indicating systematic surface modifications.

Other parameters (Discharge Gap, Duty Cycle, Frequency) exhibited weaker but still significant effects, particularly on features related to texture complexity and distribution (e.g., negative correlation between Discharge Gap and Entropy R). Overall, the correlation analysis highlights treatment time as the dominant experimental factor influencing surface morphology.

TABLE I
NUMBER OF SIGNIFICANT FEATURES AND HIGHEST PEARSON AND SPEARMAN CORRELATIONS PER PARAMETER

Parameter	Number of Significant Features	r_{max} (Pearson)	r_{max} (Spearman)
Discharge Gap	85	0.304	0.314
Duty Cycle	91	0.210	0.212
Frequency	51	0.136	0.104
Plasma Treatment Time	101	0.704	0.822

B. Multi-Way ANOVA

The multi-way ANOVA analysis provided a comprehensive understanding of how plasma parameters influenced the extracted features. Examination of the main effects confirmed that each parameter individually contributed to significant variations in color, texture, histogram, and frequency-based descriptors. Among these, Treatment Time consistently emerged as the most dominant factor, producing strong and systematic changes across nearly all feature categories, particularly in intensity- and histogram-related measures.

However, the analysis also revealed that the plasma process cannot be adequately explained by single-parameter effects alone. The four-way interaction among Discharge Gap, Duty Cycle, Frequency, and Treatment Time was found to be highly significant ($p < 0.001$) and often produced stronger influences than the individual parameters themselves. This result highlights that the observed modifications on the agar surface are not merely additive effects of independent variables, but rather

the outcome of a synergistic and nonlinear interplay among all four plasma parameters.

For instance, while Treatment Time increased the purple color area in general, its magnitude and distribution were strongly dependent on the simultaneous configuration of Discharge Gap, Duty Cycle, and Frequency. In other words, a long treatment time could either amplify or diminish the plasma impact depending on how the other three parameters were combined. Such findings demonstrate that the plasma-surface interaction is inherently multifactorial, where the collective balance of gap distance, energy delivery, oscillation frequency, and exposure duration determines the resulting feature patterns.

This strong four-way interaction emphasizes that optimizing plasma treatment requires a holistic consideration of all parameters simultaneously, rather than adjusting them in isolation.

C. Multicollinearity

To prepare the extracted image features for potential machine learning applications, multicollinearity was carefully assessed among the statistically significant features. Feature pairs exhibiting very high correlations ($|r| > 0.99$) were considered redundant, and only one representative from each pair was retained. This process resulted in a final set of 30 non-redundant features, encompassing a broad spectrum of visual characteristics:

Color-based metrics: Mean R, Kurtosis R, Skewness B, Std H, Kurtosis H, Entropy H, Std S, Skewness S, Kurtosis S, Entropy S, Kurtosis B_lab, Entropy B_lab

Histogram-derived features: Hist_Width R, Hist_Width G, Hist_Width B, Hist_Width V, Hist_Width L, Hist_Energy H, Hist_Energy S, Hist_Energy B_lab, Hist_Contrast H, Hist_Contrast S, Hist_Contrast A

Texture descriptors: GLCM_Contrast, GLCM_Dissimilarity

Frequency-domain measures: FFT_Amplitude_Mean

Color ratios: A/B, Gray_Hist_Width

The resulting correlation matrix confirmed that no feature pair exceeded the multicollinearity threshold, ensuring sufficient independence among features. This carefully selected and diverse set provides a robust foundation for subsequent modeling and analysis while capturing the key visual characteristics influenced by plasma treatment.

IV. CONCLUSION

This study demonstrated that plasma applications induce significant and systematic changes in agar surface characteristics, as evidenced by both colorimetric and texture-based image features. Correlation analyses identified strong relationships between plasma treatment parameters—especially plasma treatment time—and key visual descriptors, while multi-way ANOVA confirmed that both main effects and the four-way interaction of Discharge Gap, Duty Cycle, Frequency, and Treatment Time significantly influenced color, texture, and frequency-based features. These findings highlight the multidimensional impact of plasma on surface properties,

providing quantitative insight into plasma-induced oxidative effects.

Although direct microbial inactivation was not measured, the results offer a robust foundation for future studies. Applying the same plasma parameters in bacterial assays could establish a link between oxidative visualization and antimicrobial efficacy, supporting the optimization of plasma treatment conditions.

Moreover, the curated set of 30 non-redundant image features provides a comprehensive quantitative framework for data-driven approaches. Machine learning models can utilize these descriptors to predict treatment outcomes, optimize parameters, or guide quality control in plasma-based applications.

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