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# Characterization of intracellular reactive species production stimulated by cold atmospheric pressure plasma irradiation

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## Abstract

Recent progress in biological applications of cold atmospheric pressure plasmas (CAP) shows that various biological effects are mainly due to intracellular oxidative reactions induced by reactive oxygen and nitrogen species (RONS) produced by CAP. For scientific and practical aspects, intracellular RONS production characteristics stimulated by CAP treatment have to be elucidated. We focused on the difference in intracellular RONS production between CAP irradiation to cell suspension and incubation of cells with CAP-irradiated buffer solution. Flow cytometric assays using RONS-reactive probes demonstrated that CAP irradiation to a buffer solution containing A549 cells enhanced the intracellular RONS level. The incubation of the cells with the CAP-irradiated buffer solution also increased the RONS level. Furthermore, the contribution of hydrogen peroxide to the intracellular RONS production was investigated. As a result, injecting a concentrated $H_2O_2$ solution with a needle demonstrated that a highly concentrated region of hydrogen peroxide that would be formed by CAP irradiation contributed to enhancing intracellular RONS production.

**Keywords:** Plasma medicine, atmospheric pressure plasma jet, intracellular reactive species, oxidative stress.

## 1. Introduction

Cold atmospheric plasma (CAP) has been recently studied for medical applications [1, 2]. For example, CAP treatment shows antitumor effects *in vitro* and *in vivo* [3, 4]. Appropriate CAP treatment induces the selective cell death on cancer cells compared with normal cells [5]. Furthermore, cell culture medium irradiated with CAP induces the selective cancer cell death, similar to direct CAP irradiation [6]. Therefore, many studies have investigated molecular mechanisms. In particular, apoptotic cell death stimulated by CAP treatment was well studied. One of the possible predominant triggers in the apoptotic cancer cell death is DNA damage [7, 8]. We also reported that CAP treatment induces strand breaks and chemical modification in genomic DNA [9]. Recent progress in biomedical applications of CAP, such as cancer therapy, shows that the biological effects are due to oxidative reactions caused by reactive oxygen and nitrogen species (RONS) [10, 11]. Therefore, RONS production in aqueous media and living cells have been investigated to characterize the CAP treatments.

For the above reasons, we focused on intracellular RONS production stimulated by CAP treatment. Intracellular RONS level can be evaluated by cell-permeable RONS-reactive fluorescent probes. We used a general oxidative stress fluorescence probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-$H_2$DCFDA). Nonfluorescent CM-$H_2$DCFDA can penetrate the cell and is deacetylated by cellular esterase to 2',7'-dichlorodihydrofluorescein ($H_2$DCF). The chloromethyl derivative has improved cell retention. In the presence of RONS, $H_2$DCF is rapidly oxidized to 2',7'-dichlorofluorescein (DCF), which is highly fluorescent, with excitation and emission wavelengths of 498 and 522 nm, respectively. $H_2$DCF reacts with superoxide, hydrogen peroxide ($H_2O_2$), hydroxyl radical, singlet oxygen, hypochlorite, nitric oxide, peroxynitrite, and alkylperoxyl radical [12]. Although the probe does not distinguish individual types of RONS well, the fluorescence intensity indicates the intracellular RONS level. This measurement is convenient and straightforward; therefore, many previous studies adopted this probe to evaluate the intracellular RONS level.

For example, the condition of CAP treatments, such as plasma irradiation time [9, 13-18], carrier gas (argon or helium) [19], addition of oxygen [20], type of medium [21], and addition of antioxidants [22, 23], affect the intracellular RONS level after the treatment. The cell cycle dependency [17], the difference between tumor cells and normal cells [24], 2D and 3D cell culture [16], and the effect of ascorbate were also investigated [25]. The plasma-activated medium also elevates the intracellular RONS level [26, 27], and the addition of antioxidant reagents suppressed the RONS level [28]. Furthermore, comparison with hydrogen peroxide and radiation [29] and a combination of CAP and hyperthermia [30] were reported. Although many previous studies investigated the RONS production in the plasma-irradiated cells as described above, the comparison of direct and indirect plasma irradiation was not well studied yet. Especially, the difference between the intracellular RONS production rate of direct and indirect CAP treatment could be important information in this field. Further investigation of the role of hydrogen peroxide in the intracellular RONS production stimulated by direct and indirect treatments is also needed.

In this study, A549 cells, which are a human lung cancer cell line, were used. The RONS-reactive fluorescence probe CM-$H_2$DCFDA was loaded into A549 cells before CAP treatment. The probe-loaded A549 cells were suspended in Dulbecco's modified phosphate-buffered saline without magnesium chloride and calcium chloride (D-PBS (−)). The cell suspension was then irradiated with helium atmospheric pressure plasma jet (APPJ). After CAP treatment, the intracellular RONS level was measured by flow cytometry. Flow cytometry assays were also performed after incubation following CAP treatment to investigate the intracellular RONS level changes stimulated by indirect CAP treatment. Furthermore, a similar experiment with high concentration hydrogen peroxide injection to the cell suspension was performed to simulate the locally high concentration region of hydrogen peroxide that would be formed by CAP irradiation.

## 2. Materials and methods

### 2.1 Cell culture

The experimental procedure and apparatus were followed by the previous paper with some modification [9]. The human lung tumor cell line A549 (RIKEN BRC) was used in this study. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine (FUJIFILM Wako Pure Chemical), 10% fetal bovine serum (FBS, One Shot fetal bovine serum, Thermo Fisher Scientific), and penicillin/streptomycin (PS) (FUJIFILM Wako Pure Chemical) at 37°C in 5% $CO_2$. Cells at 70-80% confluence in T75 flasks were treated with 0.25% trypsin-EDTA (FUJIFILM Wako Pure Chemical), harvested by centrifugation, and suspended in D-PBS (−) (FUJIFILM Wako Pure Chemical).

### 2.2 Atmospheric pressure plasma source

Fig. 1 shows the experimental setup for APPJ irradiation. The APPJ consisted of a quartz glass tube with an inner/outer diameter 2.1/2.6 mm, with two copper tape electrodes (10 mm width) spaced 5 mm apart. One electrode was powered (10 $kV_{0\text{-}p}$ sinusoidal voltage at 17 kHz) and the other was grounded. The distance between the grounded electrode and the nozzle was set to 10 mm. The glass tube was fixed in a plastic syringe filled with insulating oil. Helium was used as a carrier gas at 1.5 L $min^{-1}$.

### 2.3 Plasma treatment and detection of intracellular RONS

Reactive species in the plasma-irradiated cells were analyzed with a general oxidative stress fluorescence probe CM-$H_2$DCFDA (Thermo Scientific). Before plasma irradiation, A549 cells suspended in D-PBS (−) were incubated with 10 μM CM-$H_2$DCFDA for 30 minutes at 37°C. The stained cells were then centrifuged at 400×g for 5 minutes at 4°C, resuspended in D-PBS (−), and the cell concentration was adjusted to $1.0\times10^5$ cell $mL^{-1}$. One milliliter of the cell suspension in one well of a 24-well tissue culture test plate was irradiated with the APPJ for 60-180 seconds. The gap between the nozzle and the surface of the cell suspension was set to 10-40 mm. All APPJ irradiations were carried out at room temperature. After plasma irradiation of the cell suspension, detection of intracellular reactive species was performed with flow cytometry with a CytoFLEX flow cytometer (Beckman Coulter).

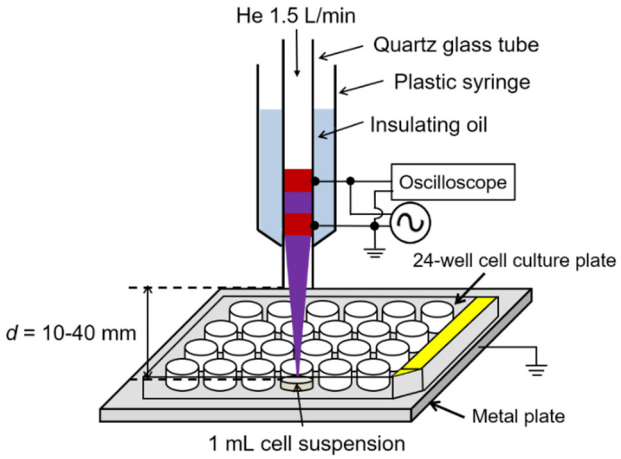


Fig.1. Schematic illustration of an APPJ irradiation apparatus.

## 3. Results

Prior to investigating the intracellular RONS production characteristics between direct and indirect CAP treatment, we performed similar experiments with previous studies to confirm the validity of the experimental procedure. The result is shown in Fig. 2. Fig. 2A shows typical flow cytometry histograms. The histograms are representative of three independent experiments. CAP treatment resulted in the increases in DCF fluorescence intensity compared with the untreated control. Longer CAP treatment time showed larger increases in the intensity of fluorescence. The relative DCF fluorescence intensity compared with the untreated control was obtained by dividing the median DCF fluorescence intensity by that of the untreated control using these histograms. Fig. 2B shows the relative DCF fluorescence intensity. 180 s of helium gas irradiation, as a negative control, did not show an increase in the relative DCF fluorescence intensity. In contrast, CAP treatment showed a statistically significant DCF fluorescence increase. The relative DCF fluorescence intensity gradually increased with the CAP treatment time. Fig. 2C shows the relative DCF fluorescence intensity at various distances from the nozzle to the liquid surface. The CAP treatment time was set to 120 seconds. 20 mm of the distance resulted in the largest fluorescence increase. Besides, 30 mm of the distance showed a significantly smaller fluorescence increase compared with 20 mm. No significant change in the relative DCF fluorescence intensity was not observed at 40 mm of the distance compared with 30 mm.

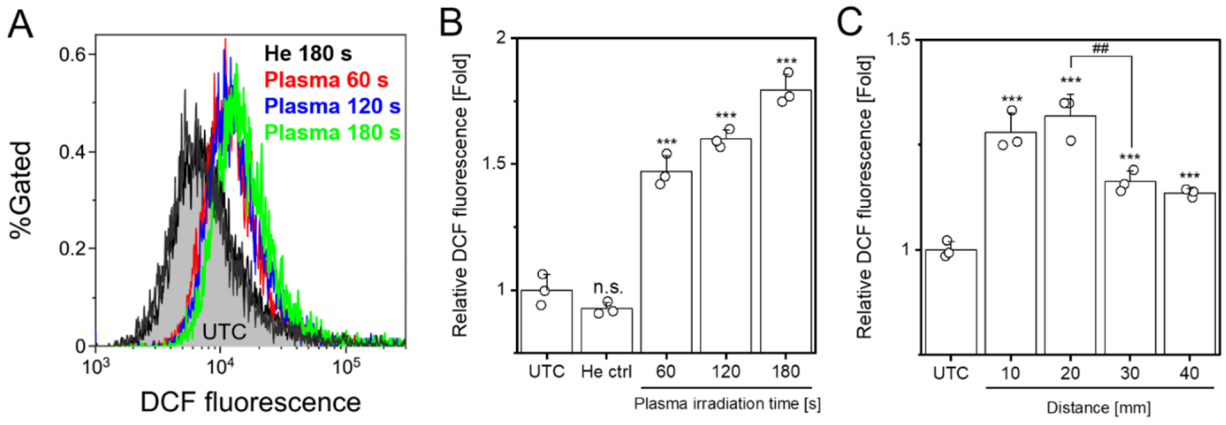


Fig.2. Flow cytometry analysis of intracellular RONS immediately after CAP treatment. (A) Typical flow cytometry histograms. The flow cytometry histogram of the untreated control (UTC) is filled gray. (B) Median DCF fluorescence intensity normalized to UTC. "He ctrl" indicates a control experiment, in which the cell suspension was exposed to helium gas flow for 180 s. (C) Relative Median DCF fluorescence intensity at various distances from the nozzle to the liquid surface. Data are expressed as the mean ± standard deviation (SD) of triplicate measurements. Statistical significance was recognized at $^{***}p < 0.001$ vs. UTC and $^{\#\#}p < 0.01$ as determined with the Student's *t*-test. n.s.: not significant ($p > 0.05$ vs. UTC) (A) and (B): The gap between the nozzle and the surface of the cell suspension was kept at 10 mm. (C): CAP treatment time was set to 120 s.

After the flow cytometric analysis following the 180 s of CAP treatment, the cell suspension was incubated at 37°C for the designated time to investigate the intracellular RONS level changes stimulated by indirect CAP treatment. Fig. 3A shows typical flow cytometry histograms. Incubation following the 180 s of CAP treatment resulted in the increases in DCF fluorescence intensity. Fig. 3B shows the relative DCF fluorescence intensity compared with the untreated control. Incubation with the CAP-treated medium increased in the relative DCF fluorescence intensity. However, 60 minutes of the incubation did not show a statistically significant increase in the relative DCF fluorescence intensity. As shown in Fig. 3A, the flow cytometry histogram of the 60 minutes of the incubation time seems to be two peaks. We assumed that these two peaks attributed to a different population in cell health, so we performed dot plot analysis. Fig. 3C shows a dot plot of forward scatter (FSC), representing cell size, vs. DCF fluorescence. The dot plot of the 60 minutes of the incubation, as shown in Fig. 3C-4, seems to be two cell populations. The cell population with higher DCF fluorescence intensity showed more than two times higher than that of the 30 minutes of incubation. The other cell population showed smaller DCF fluorescence intensity than the 30 minutes of incubation, and the cell population showed a slightly smaller FSC than the others. Fig. 3D shows representative flow cytometry histograms of FSC. The 60 minutes of incubation time increased the population of smaller FSC compared with shorter incubation time.

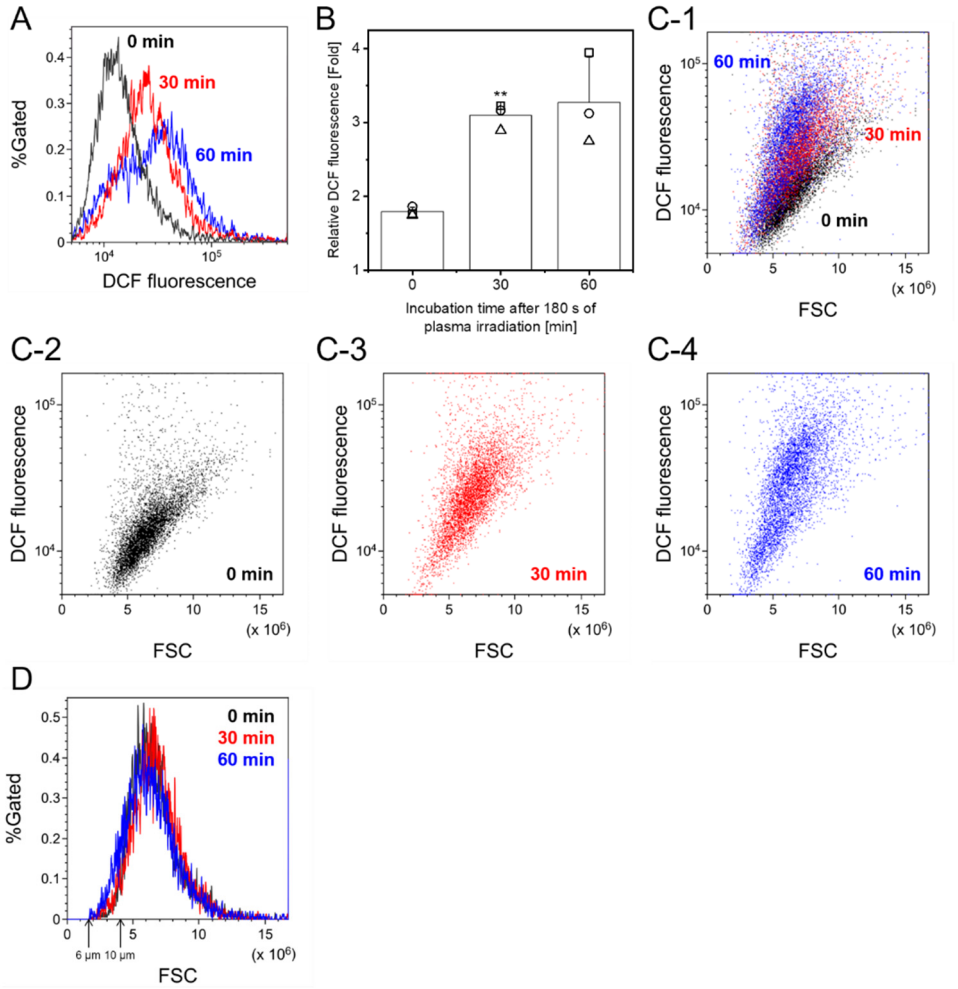


Fig.3. Flow cytometry analysis of intracellular RONS after incubation following 180 s of CAP treatment, as shown in Fig. 2. The incubation time was set to 0, 30, and 60 minutes. The gap between the nozzle and the surface of the cell suspension was set to 10 mm. (A) Typical flow cytometry histograms. (B) Median DCF fluorescence intensity normalized to UTC. Data are expressed as the mean±SD of triplicate measurements. Statistical significance was recognized at $^{**}p < 0.01$ vs. without incubation after CAP treatment as determined with the paired t-test. The same symbol indicates the same CAP treatment. (C) Dot plot analysis (*x*-axis: forward scatter (FSC), *y*-axis: DCF fluorescence intensity). The dot plots of overlay (C-1), 0 minute (C-2), 30 minutes (C-3), and 60 minutes (C-4) of the incubation time after CAP treatment are shown. (D) Representative flow cytometry histograms of FSC. The incubation time after CAP treatment is indicated. The correlation between FSC and cell size was determined using Flow Cytometry Size Calibration Kit (Invitrogen).

Fig. 3 suggests that long-lived RONS in the CAP-treated cell suspension stimulated the intracellular RONS production; therefore, we considered the contribution of hydrogen peroxide in the intracellular RONS production stimulated by direct and indirect CAP treatment. The previous investigation demonstrated that 180 seconds of He APPJ irradiation generated ca. 250 μM of $H_2O_2$ in D-PBS(−) [9]. Ma et al. previously demonstrated that the preincubation of cells with catalase (CAT) suppressed the intracellular RONS level [13]. In this study, we conducted the intracellular RONS assessment of the cells treated with CAP and the CAP-irradiated buffer solution. In addition, 10 μg $mL^{-1}$ of CAT was added to the cell suspension before CAP treatment, and then He APPJ was irradiated to the cell suspension for 120 seconds. The CAT concentration was determined by CAP treatment to CAT solution (See ***Supporting Information***). Furthermore, the APPJ was also irradiated to 1 mL of D-PBS (−) under the same irradiation condition, and then $1.0\times10^5$ A549 cells were suspended in the CAP-irradiated buffer with or without CAT for 120 seconds. Fig. 4A shows the relative DCF fluorescence intensity for each treatment. 120 seconds of CAP treatment showed a significant increase in median DCF fluorescence intensity, as same as Fig. 2. CAT addition did not show the difference in the median DCF fluorescence intensity by comparing that without CAT. The relative DCF fluorescence intensity of the cells treated with the CAP-irradiated buffer solution for 120 seconds did not change compared to the untreated control. CAT addition to CAP-irradiated buffer solution also showed no significant change in the DCF fluorescence intensity.

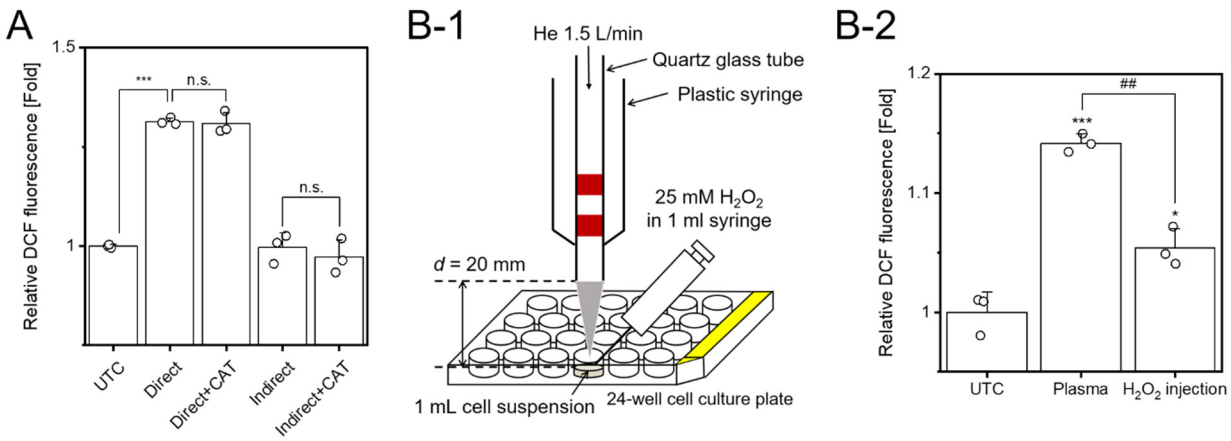


Fig. 4. Intracellular RONS production stimulated by direct and indirect CAP treatment. (A) Median DCF fluorescence intensity normalized to UTC. "Direct" indicates CAP irradiation to cell suspension for 120 seconds. "Direct+CAT" indicates the CAP treatment with 10 μg $mL^{-1}$ catalase. "Indirect" indicates 120 seconds of the treatment with CAP-irradiated buffer. "Indirect+CAT" indicates the indirect treatment with 10 μg $mL^{-1}$ CAT. The gap between the nozzle and the surface of the cell suspension was set to 20 mm. (B) Hydrogen peroxide injection to simulate the locally high concentration region of hydrogen peroxide that would be formed by CAP irradiation. (B-1) Experimental setup. (B-2) Median DCF fluorescence intensity normalized to UTC. (A, B-2) Data are expressed as the mean±SD of triplicate measurements. Statistical significance was recognized at $^{*}p < 0.05$, $^{***}p < 0.001$ vs. UTC, and $^{\#\#}p < 0.01$ as determined with the Student's *t*-test.

From Figs. 3 and 4A, we considered that the difference between direct and indirect CAP treatments attributed to two-dimensional (2D) RONS concentration distribution during the direct CAP treatment. Kawasaki et al. reported the 2D distribution visualization of the RONS after passing through water using a KI-starch gel reagent [31, 32]. Therefore, the relationship between the intracellular RONS production and RONS distribution in a liquid must be clarified. The primary long-lived species is hydrogen peroxide; therefore, we assumed that a locally high concentration region of hydrogen peroxide that would be formed by CAP irradiation affects the intracellular RONS production. Therefore, hydrogen peroxide injection was performed to simulate the locally high concentration region of hydrogen peroxide. Fig. 4B-1 shows the experimental setup. The experimental setup is the same as Fig. 1, but APPJ was not generated. Instead of APPJ, 25 mM $H_2O_2$ in D-PBS (−) was injected using a 1 mL syringe (Gastight syringe 1001RN, Hamilton) and a syringe pump (Baby Bee Syringe Drive, Model MD-1001, BASi) driven by the controller (Model MD-1020, BASi). The injection speed and the injection time were set to 5 μL $min^{-1}$ and two minutes, respectively; therefore, the injection volume was 10 μL, and the final concentration was 250 μM. Fig. 4B-2 shows the relative DCF fluorescence

intensity for each experiment. Although the $H_2O_2$ injection could not simulate the CAP treatment completely, the $H_2O_2$ injection significantly increased the median DCF fluorescence intensity, comparing the untreated control.

## 4 Discussion

We performed the detection of intracellular RONS after CAP treatment. Table 1 summarizes the CAP treatment condition performed in this study. In this study, the general oxidative stress fluorescence probe CM-$H_2$DCFDA was used. This probe can penetrate the cell and is deacetylated by cellular esterase. The deacetylated probe cannot penetrate the cell membrane. Therefore, the DCF fluorescence increase indicates the intracellular RONS production or the influx of RONS into the cell. Fig. 2 shows that CAP treatment resulted in a significant change in the flow cytometric profile relative to the controls (UTC and helium gas 180 seconds), suggesting that the intracellular RONS level was increased with increasing the irradiation time. We have confirmed that our CAP treatment produced hydroxyl radicals, superoxide, hydrogen peroxide, nitrite, and nitrate in D-PBS (−) [9]. Both short-lived and long-lived RONS may have affected the increase in the intracellular RONS level.

Table 1. CAP treatment condition performed in this study

| | Plasma irradiation time [s] | Distance from the nozzle to the liquid surface [mm] | Incubation time following the plasma irradiation [min] | Irradiation target |
|---|---|---|---|---|
| Fig. 2A, B | 60, 120, 180 | 10 | 0 | Cell suspension |
| Fig. 2C | 180 | 10, 20, 30, 40 | 0 | Cell suspension |
| Fig. 3 | 180 | 10 | 0, 30, 60 | Cell suspension |
| Fig. 4 | 120 | 20 | 0 | Cell suspension D-PBS (−) |

Fig. 2C shows the intracellular RONS production characteristics of the gap between the plasma nozzle and the liquid surface. In this study, the plasma plume length was typically about 20 mm in atmospheric air, and the gap between the plasma nozzle and the liquid surface was 10-40 mm. Therefore, the APPJ contacted the liquid surface when the gap was set to 10 or 20 mm. When the gap was kept at 30 and 40 mm, the APPJ did not contact the liquid surface. In Fig. 2C, 30 mm of the distance showed a significantly smaller fluorescence increase compared with 20 mm. No significant change in the relative DCF fluorescence intensity was not observed at 40 mm of the distance compared with 30 mm. The plasma irradiation in contact with the liquid surface proved to be more effective in inducing an enhancement of the intracellular RONS level.

Fig. 3 also showed the increase of the RONS level stimulated by incubation with a CAP-treated buffer solution. Since the lifetime of hydroxyl radicals and superoxide is very short, the CAP-treated buffer solution should not have contained these short-lived RONS. Our previous experiments demonstrated that approximately 250 μM $H_2O_2$, 5 μM nitrite, and 19 μM nitrate were produced in one milliliter D-PBS (−) during 180 seconds of plasma irradiation [9]. These long-lived RONS in the plasma-irradiated liquid may have likely increased the RONS level observed after the incubation following CAP treatment.

By comparing Figs. 2B and 3B, 180 seconds of direct CAP treatment stimulated higher RONS production than 30 minutes of indirect CAP treatment. This simple comparison of treatment times suggests that direct CAP treatment may significantly affect RONS generation than indirect CAP treatment. In other words, direct CAP treatment has a larger intracellular RONS production rate than indirect CAP treatment. In Fig 3B, on the other hand, 60 minutes of indirect CAP treatment seems to stimulate similar RONS production with 180 seconds of direct CAP treatment; however, Fig. 3C-4 showed that there were two cell populations. The cell population with larger DCF fluorescence intensity showed a larger RONS level than 180 seconds of direct CAP treatment.

In Fig. 3C, the cells after 60 minutes of incubation following CAP treatment showed smaller FSC than the others. A change in FSC is a direct measure of cell size; a decrease in FSC indicates a decrease in cell size [33]. Therefore, Fig. 3C suggests that the cells started to shrink during the incubation. Cell shrinkage is one of the typical morphological features of apoptotic cell death [34]. However, our previous investigation showed

that 180 seconds of CAP treatment with similar experimental parameters did not induce a significant cell death 24 hours after the treatment. In this previous experiment, the cells were re-suspended into a cell culture medium, which was not irradiated to CAP [9]. Although further investigations should be required to conclude that the cell shrinkage was related to CAP treatment induced apoptotic cell death, the incubation following CAP treatment may have induced apoptotic cell death.

Fig. 4 shows the comparison of the intracellular RONS production between direct and indirect CAP treatment. CAP irradiation to the cell suspension for 120 seconds resulted in a significant increase in the intracellular RONS level; however, the incubation of cells with the CAP-irradiated buffer solution for 120 seconds did not increase the RONS level. On the other hand, Fig. 3 indicated that the plasma-irradiated liquid enhanced the intracellular RONS level after incubating cells with CAP-irradiated buffer for 30 minutes. As mentioned above, the indirect CAP treatment has a lower intracellular RONS production rate than the direct CAP treatment. These results suggest that 120 seconds of the incubation of cells with the CAP-irradiated buffer, shown in Fig. 4A, was too short to enhance the intracellular RONS level in this study.

As mentioned above, we measured $H_2O_2$ concentration in D-PBS (−) after 30, 60, and 180 seconds of the irradiation in our previous experiment [9]. The $H_2O_2$ concentration was proportional to the irradiation time, so we estimated $H_2O_2$ concentration after 120 seconds of the irradiation as 185 μM from the slope. Therefore, the CAP-irradiated buffer solution in this study may have contained 185 μM $H_2O_2$, shown in Fig. 4A. Although plasma irradiation, CAT treatment, and RONS detection conditions were different, Ma et al. reported that hydrogen peroxide played a major factor in intracellular RONS production [13]. Hydrogen peroxide possibly penetrates the cell membrane through aquaporins. Aquaporins (AQPs) modulate intracellular hydrogen peroxide flux [35, 36]. Yan et al. demonstrated that silencing the expression of AQP8 in glioblastoma cells suppressed the increase in the intracellular RONS level stimulated by the incubation with the CAP-irradiated medium [37]. Furthermore, the hydrogen peroxide crosses the cell membrane and reacts with iron and possibly copper ions to form hydroxyl radicals. Therefore, we investigated the contribution of $H_2O_2$ to intracellular RONS production. As shown in Fig. 4A, however, CAT addition to the target solution did not significantly change the intracellular RONS level in direct and indirect CAP treatments. Catalase can decompose hydrogen peroxide immediately, but only if the hydrogen peroxide concentration is optimal for the catalase concentration. Therefore, we assumed that CAP treatment formed the locally high concentration region of hydrogen peroxide. Although a KI-starch reagent cannot identify the reacted RONS, in fact, the 2D distribution visualization using a KI-starch gel reagent showed the local supply of the RONS after passing through water [31, 32]. In addition, a computational investigation also showed the steep concentration gradient of $H_2O_2$ [38]. Furthermore, we confirmed the generation of hydroxyl radicals [9], and they can form $H_2O_2$ with a high rate constant. Although there is no direct evidence of the existence of a high concentration $H_2O_2$ region, for the above reasons, the locally concentrated region was simulated by the injection of 25 mM $H_2O_2$ solution. As shown in Fig. 4B, the $H_2O_2$ injection significantly increased the median DCF fluorescence intensity, comparing the untreated control. This result suggests that the locally high concentration region of hydrogen peroxide contributed to enhancing the intracellular RONS production. However, the $H_2O_2$ injection could not completely simulate the CAP treatment. The CAP irradiation may have formed a more highly concentrated region. Alternatively, the other factors, i.e., short-lived reactive species, would have contributed to the intracellular RONS production. This issue could be investigated in future work.

## 5. Conclusion

Flow cytometric assays using RONS-reactive probes were performed to investigate the intracellular RONS production stimulated by CAP treatments. The He APPJ irradiation to a buffer solution containing A549 cells enhanced the intracellular RONS level. The incubation of the cells with the CAP-irradiated buffer solution also increased the RONS level. The direct CAP treatment showed a more significant effect on intracellular RONS production than the indirect CAP treatment by comparing the treatment time. The locally high concentration region of hydrogen peroxide was simulated by a needle connected to a syringe containing $H_2O_2$. We demonstrated that a highly concentrated region contributed to enhancing intracellular RONS production.

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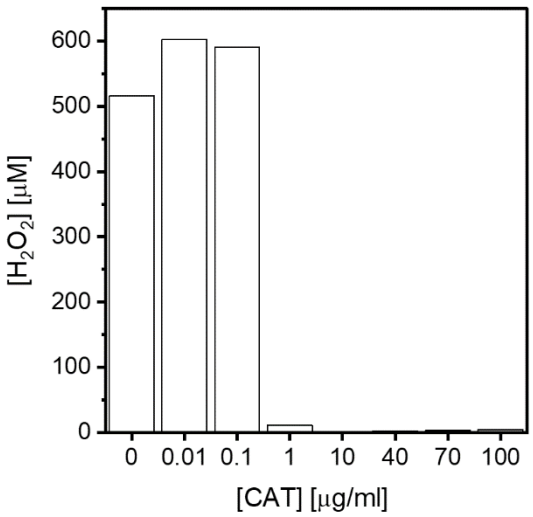
## Supporting Information

Prior to investigating the effect of catalase (CAT) addition on hydrogen peroxide ($H_2O_2$) production during CAP treatment, $H_2O_2$ concentration in CAP-treated sodium phosphate buffer solution with or without catalase was determined. 100 µl of 50 mM sodium phosphate buffer (pH 7.4) with 0-100 µg $mL^{-1}$ catalase (FUJIFILM Wako Pure Chemical) was added to one well of the 96-well plate. CAP treatment time was set to 180 seconds. After CAP treatment, $H_2O_2$ concentration was determined by Pierce Quantitative peroxide Assay Kits (aqueous-compatible formulation) (Thermo Scientific) and a multimode microplate reader (Varioskan, Thermo Scientific).




Supplementary Fig. 1. $H_2O_2$ concentration in CAT solution after 180 seconds of CAP treatment.