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Cold atmospheric plasma in cancer therapy[a)]

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Recent progress in atmospheric plasmas has led to the creation of cold plasmas with ion temperature close to room temperature. This paper outlines recent progress in understanding of cold plasma physics as well as application of cold atmospheric plasma (CAP) in cancer therapy. Varieties of novel plasma diagnostic techniques were developed recently in a quest to understand physics of CAP. It was established that the streamer head charge is about 10^8 electrons, the electrical field in the head vicinity is about 10^7 V/m, and the electron density of the streamer column is about 10^{19} m^{-3}. Both *in-vitro* and *in-vivo* studies of CAP action on cancer were performed. It was shown that the cold plasma application selectively eradicates cancer cells *in-vitro* without damaging normal cells and significantly reduces tumor size *in-vivo*. Studies indicate that the mechanism of action of cold plasma on cancer cells is related to generation of reactive oxygen species with possible induction of the apoptosis pathway. It is also shown that the cancer cells are more susceptible to the effects of CAP because a greater percentage of cells are in the *S* phase of the cell cycle. © *2013 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4801516]

I. INTRODUCTION

Nowadays, cold non-thermal atmospheric plasmas have tremendous applications in biomedical engineering.[1] Cold plasma can potentially offer a minimally-invasive surgery option that allows specific cell removal without influencing the whole tissue. Conventional laser surgery is based on thermal interaction and leads to accidental cell death, i.e., necrosis and may cause permanent tissue damage. In contrast, cold non-thermal plasma interaction with tissue may allow specific cell removal without necrosis.[2] In particular, these interactions include cell detachment without affecting cell viability, controllable cell death, etc. It can be used also for cosmetic methods of regenerating the reticular architecture of the dermis. The aim of plasma interaction with tissue is not to denature the tissue, but rather to operate below the threshold of thermal damage and to induce chemically-specific response or modification. In particular, presence of the plasma can promote chemical reactions that would have the desired effect. Chemical reactions can be promoted by tuning the pressure, gas composition, and power. Thus, it is important to find plasma conditions that produce the desired effect on tissue without thermal treatment.

It should be pointed out that earlier applications of plasma in medicine relied mainly on the thermal effects of plasma.[3] Heat and high temperature have been utilized in medicine for a long time for the purpose of tissue removal, sterilization, and cauterization. One of the successful applications of thermal plasma is argon plasma coagulation (APC) in which highly conductive plasma allows passing a current through the tissue.[4] APC is being used to cut tissue and, in particular in endoscopic applications.[5]

Stoffels *et al.*[6] studied the plasma needle device and demonstrated the promising potential of the cold plasma in biomedical applications. They demonstrated that the cold plasma can interact with organic materials without causing thermal/electric damage to the surface. These earlier results suggested that cold plasmas have great potential in biomedical applications. This understanding motivates the development of a variety of reliable and user-friendly plasma sources. Laroussi and Lu described the operation of a cold plasma plume using helium as the carrier gas.[7] They demonstrated that the plasma plume can be touched by bare hands and can come in contact with skin and dental gums without causing any heating or painful sensation. The device that later received the name "plasma pencil" was further characterized by Laroussi *et al.*[8] A non-equilibrium plasma plume with lengths of 4 and 11 cm was generated by Lu *et al.*[9,10] A similar plasma source was described by Kolb *et al.*[11] They demonstrated that yeast grown on agar can be eradicated with a treatment lasting only a few seconds. Fridman *et al.* demonstrated that cold plasmas can promote blood coagulation and tissue sterilization.[12] It was shown previously that thermal plasma treatment is very beneficial in terms of blood coagulation and sterilization, but it induces significant damage. On the contrary, non-thermal cold plasmas can lead to the same result without any side effects associated with thermal plasmas.

There is some controversy in the literature with respect to the mechanism of plasma-cell interaction. Some authors are of the opinion that ion species play the most important

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role in plasma-cell interactions by triggering intracellular biochemistry.[13–15] On the other hand, the same and other authors suggested that neutral species play the primary role in some plasma-cell interaction pathways.[16] It was suggested that various plasma effects are highly selective and that different species can have either "plasma killing" (such as O) or "plasma healing" (such as NO) effects.[17] The roles of other species such as O_3, OH, etc., are not clear.

In summary, recent studies of cold atmospheric plasmas have shown great potential for the use in biomedical applications. Their distinguished physical and chemical properties are defined by the uniqueness of the non-thermal non-equilibrium plasmas. Depending on their configuration they can be used in the following areas: wound healing, skin diseases, hospital hygiene, sterilization, antifungal treatments, dental care, cosmetics targeted cell/tissue removal, and cancer.[18–22] In addition, studies of the impact of cold plasma on cell motility have been conducted.[23,24] It was shown that cold plasma leads to decrease in the cell motility.

Very recent research demonstrated great potential of cold plasma treatment in cancer therapy.[25–27] These studies demonstrated that (a) cold plasma application selectively eradicates cancer cells *in vitro* with a lesser effect on normal cells; and (b) significantly reduces tumor size *in vivo*. It was shown that reactive oxygen species (ROS) metabolism and oxidative stress responsive genes are deregulated.[24] Overall cold plasma has demonstrated intriguing potential for cancer treatment. Further progress is dependent on creating new devices that can enhance the established cold atmospheric plasma (CAP) effect, and understanding the underlying mechanism of plasma action on cells.

The variety of different effects of plasma can be explained by their complex chemical composition and variations in the way that CAP is generated. In fact, CAP is a cocktail containing variety of reactive oxygen species, reactive nitrogen species, charge particles, UV, etc. This variety of species leads to the variety of effects mentioned above. In general, the CAP sources can be classified into three major groups according to the principal mechanism of generation and application:

(a) *Direct plasmas* employ living tissue or organs as one of the electrodes, and thus, living tissue directly participates in the active discharge plasma processes. Some current may flow through living tissue in the form of small conduction current, displacement current, or both. Conduction current has to be limited to avoid any thermal effects or electrical stimulation of the muscles. The dielectric barrier discharges (DBD) are typical example of direct plasma sources.[3]

(b) *Indirect plasmas* are produced between two electrodes and are then transported to the area of application entrained in a gas flow. There is great variety of different configuration of indirect plasmas sources exists in the size, type of gas and power. They range from very narrow "plasma needles" to larger "plasma torches."[8,21,28,29]

(c) *Hybrid plasmas* that combine the production technique of direct plasma with the current-free property of indirect plasma; which is achieved by introducing a grounded wire mesh electrode, which has much smaller electrical resistance than the skin—so that practically all the current passes through the wire mesh. One of the best examples is the plasma dispenser/"HandPlaSter."[30]

II. STATE OF THE ART MODELING OF THE COLD PLASMA JETS

The state of the art numerical modeling of cold atmospheric plasmas for medical applications is mainly limited to the study of the "plasma needle" described by Stoffels *et al.*[2] The first numerical study of this device was performed by Brok *et al.*[31] The device was studied using a time-dependant, 2D fluid model based on the diffusion equation. The code included air chemistry, and modeled a large number of helium and nitrogen species. The code considered a wide range of chemical reactions, including excitations, ionization, disassociation, and recombination. To account for deviation from the Maxwellian distribution, the authors used a Boltzmann solver to tabulate the transport coefficients and reaction rates as a function of electron energy. A similar approach was taken by Sakiyama and Graves.[32,33] These authors also used a 2D fluid model based on the diffusion equation to study the plasma needle. However, this code implemented the fine element method in order to accurately capture the needle geometry. An even more limited set exists for codes utilizing a particle-based approach. One example of this approach is a work by Shi *et al.*[34] Here, the authors used the particle-in-cell method coupled with Monte Carlo collisions to model a pulse-induced discharge between two parallel plates. This model accounted for the avalanche ionization; however, air chemistry was neglected. Feasibility of particle-in-cell modeling was performed by Hong *et al.*[35] by comparison with fluid simulations. It was found that electron energy distribution is strongly non-equilibrium especially near the sheath region. It follows from this analysis that a kinetic treatment is needed to capture the electron distribution function. A hybrid approach for the atmospheric pressure discharge was recently presented by Iza *et al.*[36] A kinetic approach is used to simulate pulse-on phase of plasma while fluid approach is used to simulate multiple pulses. Only very recently models of the atmospheric plasma jets were developed.[37] A numerical study of ionization waves propagating through a circuitous capillary channel and impinging upon a target, in the context of remote delivery of plasma species for biomedical applications, has been conducted.[38] Unlike the plasma bullets in open configurations, the ionization wave fronts in the capillary channel are followed by an extended tail of high electron temperature and ionization up to several centimeters long.[39]

It should be pointed out that, by large existing models were not validated by direct comparison with experiments. Only some integrated CAP properties such as plasma shape, ionization wave speed, etc., were compared with the experiment. The main reason is the absence of detailed experimental measurements of the atmospheric plasmas. This paper presents recent measurements of the CAP using multiple diagnostics including optical emission

spectroscopy, microwave scattering, fast imaging, Rogovsky coil, and DC scatterer.

III. COLD PLASMA JET DIAGNOSTICS

In this section, we describe the various diagnostic tools that were recently developed aiming at detailed characterization of the atmospheric plasmas.

The non-equilibrium plasma device studied is shown in Fig. 1.[23,39,40] It consists of non-conducting discharge tube equipped with pair of electrodes. One electrode is a metal wire placed on the tube's axis ending just few centimeters before the tube's exit. This electrode has electrical insulation along its entire length except for the end face which directly touches the plasmas. The second electrode is a metal ring attached to the discharge tube from the outside and isolated from contact with the plasma column by the tube's walls. The discharge is driven by AC high voltage with discharge voltage, U_d, of about several kVs and a frequency of about 15–30 kHz. Helium flow is supplied through the tube. The discharge has two different spatial regions. The main discharge region is located entirely inside the discharge tube (plasma column is extended from the central electrode and ends at about ring electrode), while part of the discharge is extended from the tube in a form of well-collimated plasma jet having typical length of approximately several centimeters. This extending part of the discharge is usually referred to as a non-equilibrium cold plasma jet.[38,39,41–44]

A. Electrical parameters of the discharge

Electrical parameters of the non-equilibrium plasma sources such as discharge voltage and current can be measured using conventional current and high voltage probes.[38,39] These measurements indicate that the discharge is not sustained continuously and represents a series of elementary discharge events (1 event per period of the driving high voltage) characterized by amplitudes of current bursts of about 10 mA and decay times of few μs as shown in Fig. 2(a). The main part of discharge current is circulated via the circuit central electrode → main discharge column (inside the tube) → ring electrode. However, a relatively small portion of the current (5%–10%) is extending out of the discharge tube, flowing along the plasma jet and then being collected by the ring electrode. Temporal evolution of the current flowing through the jet measured using Rogowski coil is shown in Fig. 2(a).

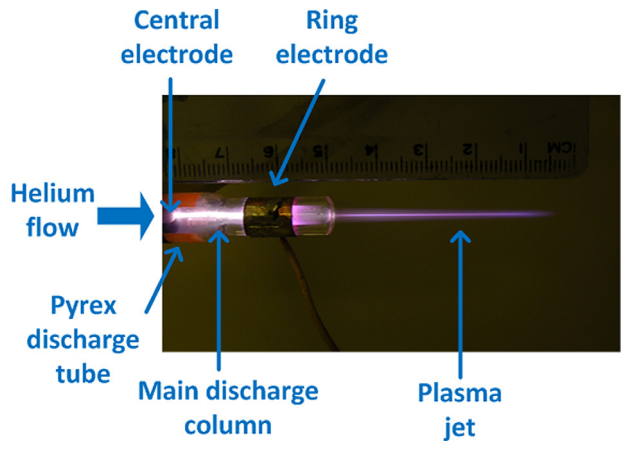

FIG. 1. Photo of the non-equilibrium plasma device.

B. Optical diagnostics

Diagnostic tools that have been traditionally employed for characterization of the cold atmospheric plasmas include intensified charge-coupled device (ICCD) cameras and optical emission spectroscopy.[40–43,45,46] Such studies confirmed the non-continuous nature of the discharge and traced in great detail temporal and spatial dynamics of the plasma jet. It was shown that the plasma jet represents a series of breakdown events developing once per period of the discharge driving high voltage immediately after ignition of the main discharge. Each breakdown event represents propagation of the ionization front starting at the main discharge and propagating several centimeters along the straight line outside the tube as shown in Fig. 2(b).[44] Velocities of ionization front propagation were found to be in the range from 10^6–10^8 cm/s depending on discharge parameters. The ionization front typically slows down along the propagation path and for lower driving voltage amplitudes.[44] The composition of ionized and excited species in jet by means of optical emission spectroscopy was investigated in number of papers.[9,10,45,46] Strong spectral lines corresponded to excited and ionized states of oxygen, nitrogen, and hydroxyl radicals were observed along with He lines. The temperature was estimated from the comparison of measured and simulated emission spectra of nitrogen second positive system, and was found to be close to the room temperature.

C. Plasma density measurements

New methodology for the plasma density diagnostics in the non-equilibrium atmospheric plasma jet was recently demonstrated.[39,40] This approach utilizes the measurements of radiation pattern scattered from plasmas being irradiated with microwaves. The concept of this method was first proposed theoretically by Shneider and Miles,[47] and then successfully implemented experimentally in studies of laser-induced avalanche ionization in air, resonance-enhanced multiphoton ionization in argon and non-thermal atmospheric plasma jets.[39,40,48,49] In the Rayleigh regime (when skin layer depth—δ_{skin} and wavelength of microwave radiation $\lambda >$ transversal size of plasma column), the electric field amplitude of the scattered wave (E_s) is proportional to the conductivity (σ) and scatterer volume (V): $E_s \propto \sigma V$. Absolute plasma density measurements can be conducted after calibration of the Rayleigh Microwave Scattering (RMS) system with dielectric scatterers with known physical properties.[40]

The RMS system utilized two microwave horns for radiation and detection of microwave signal. 12.6 GHz microwave radiation with linear polarization along the plasma column. The temporal evolutions of average plasma density are presented in Fig. 3 for driving discharge voltage amplitude of about 2.2 kV. It was observed that the plasma density typical values are in the order of 10^{13} cm^{-3} and decay times

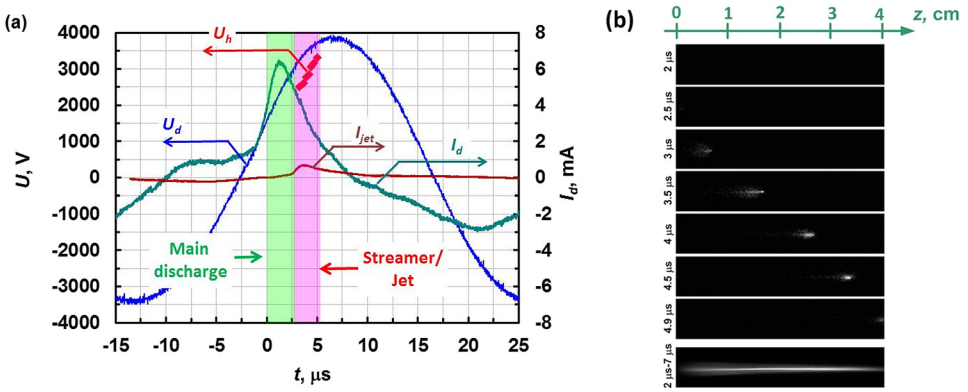

FIG. 2. (a) Electrical parameters of the discharge and (b) typical photographs indicting propagation of ionization front.

of the plasma column are few μs. More details on the RMS system and plasma density measurements can be found elsewhere.[39,40]

D. Plasma potential measurements

Very recently a simple method for measuring the electrical potential of streamer head was proposed.[45] The method is based upon stopping ("scattering") of streamer propagation by means of externally created electric potential. The external electric potential was created using a metal ring with an electric potential applied to it. Since the electric field around the streamer head can be approximately expressed as $(U_h - U_r)/2r_s$,[50] where U_h—potential of streamer head in the absence of the ring, U_r—ring potential, and r_s—streamer channel radius, the ring potential can disturb the electric field around the streamer and even stop its further propagation when the condition of $U_r = U_h$ is satisfied. Therefore, streamer head potential can be determined from measurements of the threshold potential required to stop the streamer propagation. Fig. 4 presents the temporal evolution of streamer head potential $U_h(t)$ for the amplitude of driving high voltage 2.6 kV, superimposed with the temporal evolution of voltage applied to the discharge electrodes. It was observed that streamer head potential was close to the central electrode potential (within 10%–15%). This experiment indicates that the electrode potential is transferred to the streamer head along the streamer column to which it is attached with no significant voltage drop. Measurement of the streamer head potential allows the determination of a number of key streamer parameters such as head charge ($1–2 \times 10^8$ electrons), electrical field in the head vicinity (about 100 kV/cm), average conductivity ($3–7 \times 10^{-3}$ $\Omega^{-1}\,cm^{-1}$), and plasma density of the streamer column ($1–2 \times 10^{13}\,cm^{-3}$).

Typical parameters of the discharge and the CAP jet measured by employing the diagnostic array described above are presented in Table I. It should be noted that this table represents most complete data set to date that characterizes CAP.

IV. PLASMA INTERACTION WITH CANCER CELLS

One of the most promising applications of CAP in medicine is related to cancer therapy. In this section, we describe some recent results of both *in-vitro* and *in-vivo* applications of CAP in cancer therapy. To this end cold plasma effect on various cancer cell lines was studied in order to determine efficacy of CAP in cancer therapy. The list of cancer cell lines considered includes lung, bladder, skin, head and neck, brain, etc.[26]

In-vitro studies of lung cancer revealed a strong selective effect of the cold plasma treatment, resulting in 60%–70% of SW900 cancer cells detaching from the plate in the zone treated with plasma, while no detachment was observed in the treated zone for the normal human bronchial epithelial (NHBE) cells under same treatment

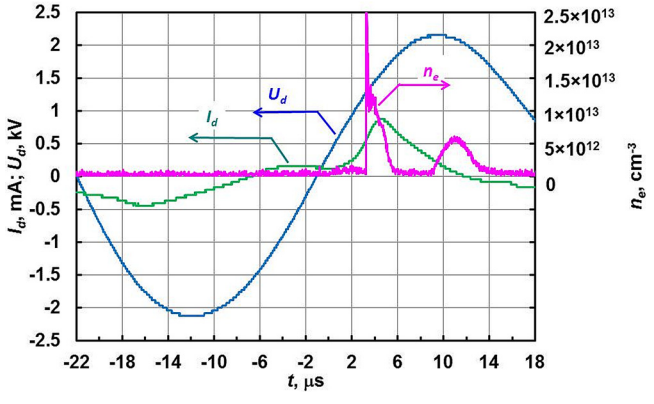

FIG. 3. Temporal evolution of average plasma density in atmospheric plasma jet for $U_{HV} = 2.2$ kV.

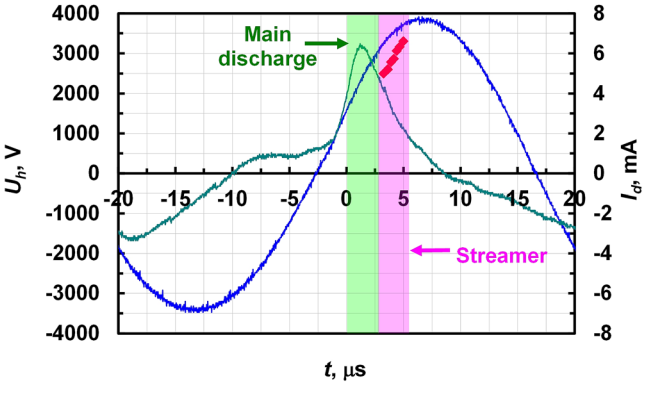

FIG. 4. Temporal evolution of discharge current (I_d), discharge voltage (U_d), and streamer head potential (U_h). Potential of streamer head is close to potential of central electrode and following its temporal behavior in all cases. This indicates that voltage drops potential of central electrode is transferred to the streamer head without significant drops.

TABLE I. Typical discharge and plasma parameters of the CAP.

Discharge current	10 mA
Discharge voltage	2–5 kV
Plasma jet current	<1 mA
Plasma decay time	Few μs
Streamer length	4–5 cm
Streamer radius	3×10^{-2} cm
Streamer head charge	$1–2 \times 10^{8}$ electrons
Speed of ionization front	$1.5–2 \times 10^{6}$ cm/s
Average conductivity in the streamer channel	$3–7 \times 10^{-3}\ \Omega^{-1}\,\text{cm}^{-1}$
Average plasma density in the streamer channel	$1–2 \times 10^{13}\,\text{cm}^{-3}$

conditions.[26] Plasma treatment leads to a significant reduction in SW900 cell count, while NHBE cell count is practically unchanged.

Furthermore, quantitative studies of CAP selectivity were performed. Both fibroblast and neuroblastoma cells were treated with the cold plasma device for 0, 30, 60, and 120 s. Annexin V and 7-AAD staining were performed for flow cytometry analysis at 24 and 48 h after treatment.[26] As seen in Figure 5, a clear-dose response to cold plasma treatment is seen in the neuroblastoma cells at both 24 and 48 h, while the treated fibroblast cells do not differ from control at either 24 or 48 h. These findings suggest that the cold plasma jet has a more selective effect on neuroblastoma (cancer) cells than that on fibroblast (normal) cells.[26]

V. *IN VIVO* STUDIES. CAP TREATMENT OF TUMOR

In order to determine the cold plasma effect *in-vivo*, we applied the cold plasma jet to nude mice bearing subcutaneous bladder cancer tumors (SCaBER). We examined the mouse skin after cold plasma treatment and did not see any damage to the skin after 2 to 5 min of treatment. Tumor models treated by cold plasma are shown in Figure 6.

One can see that the single plasma treatment leads to tumor ablation with neighboring tumors unaffected. These experiments were performed on 10 mice with the same outcome.[26] We found that tumors of about 5 mm in diameter are ablated after 2 min of single time plasma treatment [see Figure 6], while larger tumors decreased in size. Interestingly, ablated tumors did not grow back while partially affected tumors started growing back a week after treatment, although they did not reach the original size even 3 weeks after treatment.

VI. TARGETING THE CANCER CELL CYCLE BY COLD ATMOSPHERIC PLASMA

In this section, we describe possible mechanisms behind the observed CAP selectivity towards the cancer cells. It will

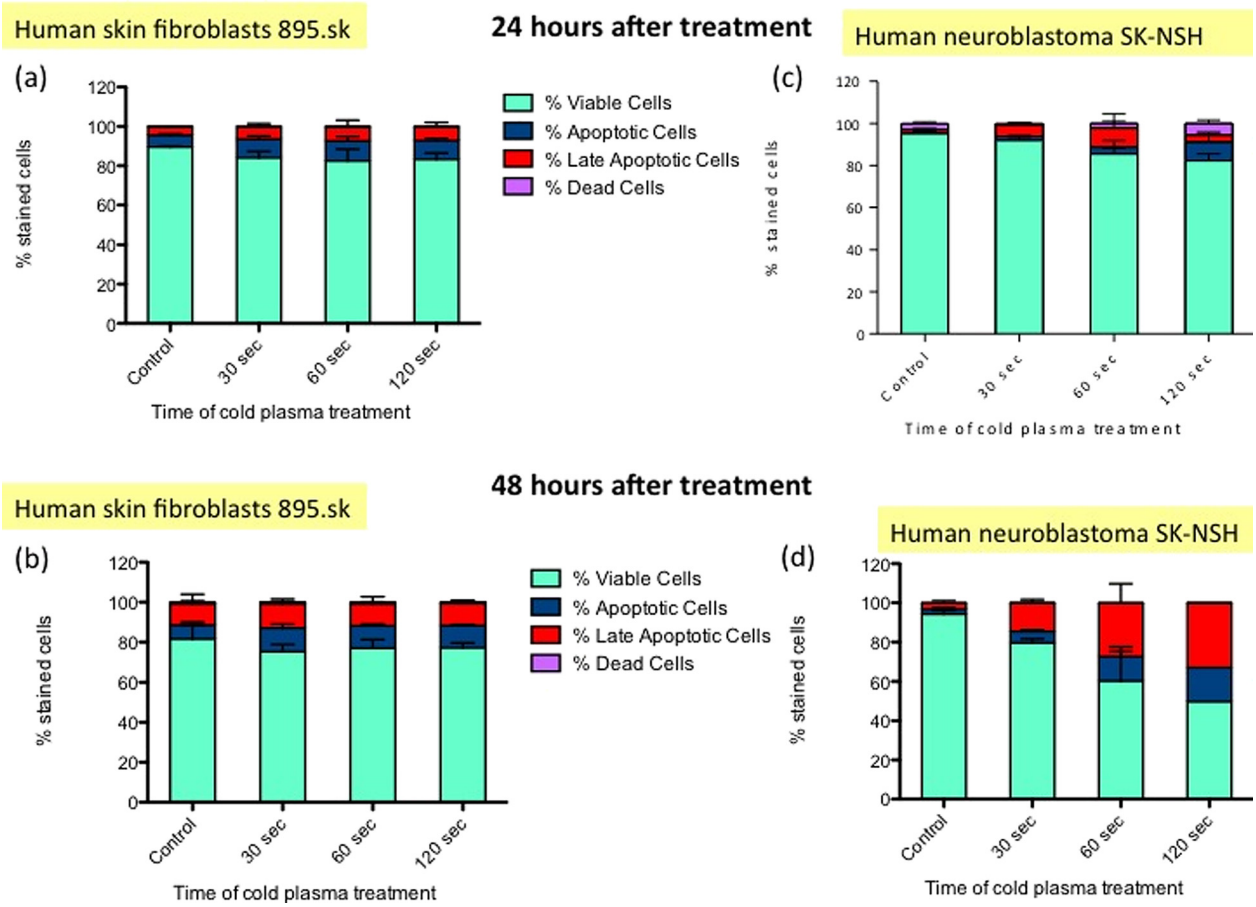

FIG. 5. Selectivity effect of plasma treatment: fibroblast cells treated with the cold plasma device for 0, 30, 60, and 120 s. (a) 24 h; (b) 48 h Neuroblastoma; (c) 24 h; (d) 48 h. Annexin V and 7-AAD staining were performed for flow cytometry analysis at 24 and 48 h after treatment. Four-quadrant analysis of the results characterizes the cells as viable (unstained), apoptotic (Annexin V positive), late-apoptotic (double positive), and dead (7-AAD positive).

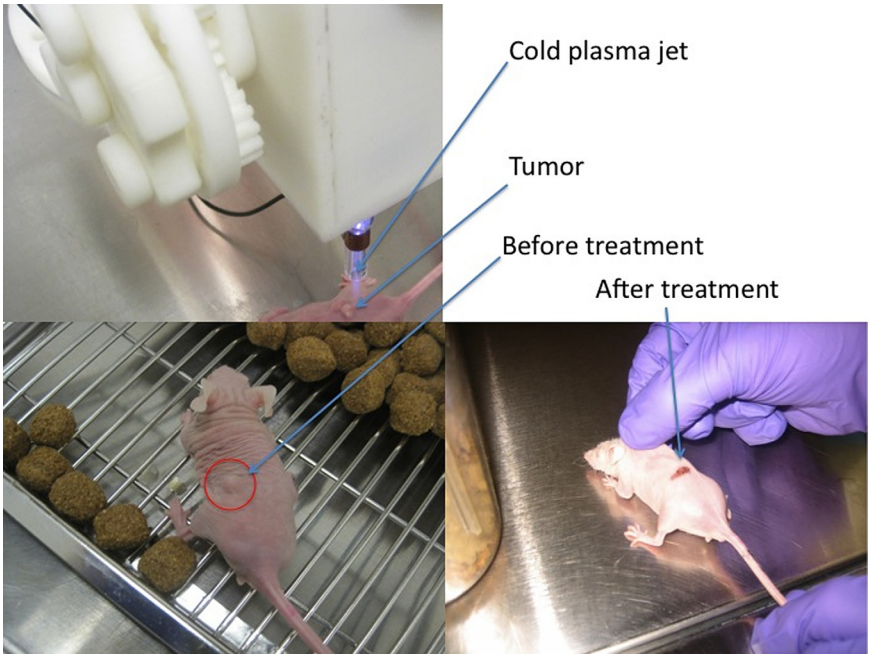

FIG. 6. (a) Cold plasma device; (b) typical image of mice with a single tumor before and approximately 1 week after treatment.

be shown that it is based on the hypothesis that cancer cells are more susceptible to the effects of CAP because a greater percentage of cells are in the S phase of the cell cycle. To test the validity of this hypothesis the normal cells derived from mouse skin as well as two mouse skin cancer cell lines will be treated by CAP.[51]

The cell cycle describes the different steps that take place as a cell proliferates. When cells proliferate, they move progressively from G1-phase to S-phase (synthesis of DNA), G2-phase, and then M-phase (mitotic phase) where they divide; and than revert back to G1—phase after division. Cells in G1-phase may enter G0-phase, quiescent state. Between S and G2 and between G2 and M phases there are what have been called "checkpoints"—natural mechanism that controls cell progression through the cell cycle: verification whether the processes at each phase of the cell cycle have been accurately completed before progression into the next phase. One of the hallmarks of cancer is the deregulation of the molecular mechanisms controlling the cell cycle.[52]

To this end the CAP effect on the cell cycle of normal, 308 and PAM212 (both 308 and PAM212 are cancer cell lines) cells was studied. Figures 7(a), 7(d), and 7(g) are bright-field images with 10× magnification of wild type keratinocytes, 308 and PAM212 cells morphology, respectively. In order to determine the nature of CAP effect on the cell cycle an analysis of the cell cycle was carried out. Cells were treated with the nucleotide analog EdU that is incorporated into DNA as it is being replicated. Flow cytometry was used to assess the number of cells in the three distinct phases of the cell cycle (G0/G1, S, and G2/M) simultaneously for all three cell types as described in Ref. 52. The data are shown in Figure 7. The colored diagrams in the up-right corner of Figures 7(a), 7(d), and 7(g) represent the typical distribution of the cells within the cell cycle from the experimental data for not-treated cells; the red arrows indicates the general trend of cancer cells to have more cells in S-phase phase, thus indicating that the timing of the cell cycle is different for the chosen cells. Results in Figures 7(b) and 7(c); 7(e) and 7(f); 7(h) and 7(i) are presented at 24 h after the cells were CAP treated for 60 s compared to the control (untreated) cells. As expected, fewer normal cells were in S phase (∼10%) compared to the two cancer cell lines (transformed cells are highly proliferative): ∼50% for 308 cells and ∼45% for PAM212 cells. No increase in the fraction of cells in the S-phase after CAP treatment was observed for the three cell types: their number either remained the same or decreased. However, we did observe an increase in the standard deviation value range of CAP treated cells in the S-phase of around ∼20% for all three cell types suggesting that not all cells within the population of cells responded in the same way to CAP treatment. While there is no significant difference in the numbers of cells in the S phase of the cell cycle, one can see that the number of cells in the G2/M fraction increased by ∼25% for normal cells and 2- to 3-fold for transformed cells (indicated with red arrows). One can see that the increase in the fraction of cells at the G2/M phase of the cell cycle is accompanied a decrease in the number of cells in the G0/G1 fraction.[51]

VII. CONCLUDING REMARKS

In summary, this paper overviews cold atmospheric plasma physics and application in cancer therapy. Varieties of diagnostics tools were applied to the cold plasmas including fast imaging, optical spectroscopy, microwave scattering, and potential measurements based on DC scatterer. These measurements revealed that the streamer head charge is about $1–2 \times 10^8$ electrons, the electrical field in the head vicinity is about 100 kV/cm, the average conductivity of plasma jet is $3–7 \times 10^{-3}$ $W^{-1}\,cm^{-1}$ and the plasma density of the streamer column is about $1–2 \times 10^{13}\,cm^{-3}$.

A recent pilot study demonstrated new *in vitro* and *in vivo* responses of cancer cells upon treatment with cold

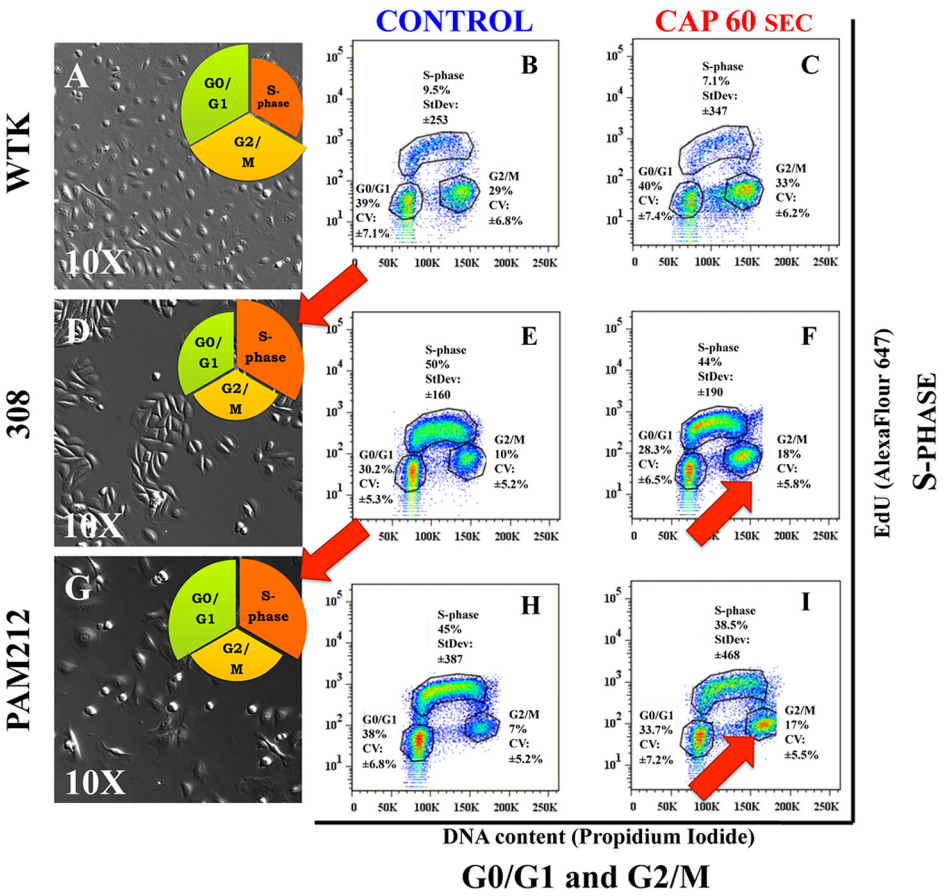

FIG. 7. The cell population's distribution during the cell cycle after CAP treatment. ((a)–(c)) Wild type keratinocytes, ((d)–(f)) papilloma (308) cells, and ((g)–(I)) carcinoma (PAM 212) cells are shown. ((a),(d),(g)) Bright-field images of WTK, epidermal papilloma (308 cells), and epidermal carcinoma (PAM212 cells) cells are shown with a magnification of 10×. The schematic diagrams in the up-right corner represent the typical distributions of the cell populations inside the cell cycle. The detailed studies of the cell cycles for control and cells treated with CAP for 60 s in 24 h after treatment are shown. The correlation between DNA content (propidium iodide) and DNA-replicating cells (EdU-component) is shown for: ((b),(c))—normal cells (WTK); ((e),(f))—papilloma cells (308 cells); and ((h),(i))—carcinoma cells (PAM212 cells) in ∼24 h after CAP treatment. CV was used to characterize the propidium iodide data (linear scale) and s.d. was used for EdU-data (log scale) in each phase. Fractions of the number of cells in each cell cycle phase are shown in percents. The data are shown for ∼25 000 cells for each experimental condition. The experiments were repeated 2–3 times.

plasma jets. These very surprising results suggest that the cold plasma jet can selectively ablate some cancer cells (lung, melanoma, head and neck, brain, and bladder), while leaving their corresponding normal cells essentially unaffected. Such selective effect of cold plasma on different cell types suggest that it is possible to find the right conditions with plasma treatment affecting only cancer cells, while leaving normal cells essentially unharmed. It was shown that mid-sized tumors in nude mice were destroyed after a 2 min single time treatment by cold plasma without thermal damage. Finally, it was found that selectivity might be associated with the CAP effect on the cell cycle of the cancer cells. It is also shown that the cancer cells are more susceptible to the effects of CAP because a greater percentage of cells are in the S phase.

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