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1 **Cold atmospheric plasma-induced acidification of tissue surface: visualization and**
2 **quantification using agarose gel models**

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10 **ABSTRACT**

11
12 The biological effects induced by cold atmospheric plasmas (CAPs) on human tissues are
13 mainly due to the production of reactive oxygen and nitrogen species (RONS). Some RONS
14 are also responsible for pH lowering of the treated medium. The CAP-induced acidification
15 has beneficial effect on biological tissues, contributing to the anti-bacterial effect and to the
16 healing improvement observed in treated wound. In this work we investigated the local
17 acidification induced by a helium CAP treatment using tissue models made of agarose gels
18 with adjusted pH around 7.4 to mimic generic organs or around 5.5 to simulate skin surface
19 pH. Using fluorescein as a pH-sensitive fluorescent marker, we developed a useful technique
20 to visualize and quantify the local acidification induced by CAP exposure of tissue surface.
21 The different capillaries used to produce the plasma jet, the treatment time, the initial pH of
22 the surface and the buffer capacity of the tissue model were shown to modulate both the size
23 of the impacted surface and the intensity of the pH decrease. The proposed technique can
24 be advantageous to study the acidifying effect induced by plasma. This method can help to
25 plan safe and controlled plasma treatments in order to avoid hyper-acidification of the tissue,
26 especially when a localized treatment is administered.

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29 **Keywords:** plasma, CAP, pH measurement, fluorescence, fluorescein, agarose model

30 Today cold plasmas generated at atmospheric pressure (CAPs) are extensively exploited for
31 biomedical applications [1, 2]. Dermocosmetic research is one of the most promising fields of
32 plasma medicine [3]. The encouraging results obtained in wound disinfection and healing
33 and the easy accessibility of this external organ, make the skin a privileged target for plasma
34 treatments [4]. In dermatology, CAPs have been already employed in clinical trials to treat
35 mainly infected wounds. Their use for cosmetic purposes is much more recent and still at the
36 research level. For this type of application, the safety of treatment conditions is primordial
37 and requires various *in vitro* studies before passing to *in vivo* treatments. Several models
38 have been proposed to mimic and study the skin properties *in vitro* [5]. Among these, gelatin
39 and agarose are the most used for plasma treatments [6, 7]. The biological effect of CAPs
40 reside mostly in RONS production. Quantification and distribution of these species inside the
41 target is crucial to plan the convenient plasma treatment. RONS detection in liquids is
42 commonly performed in liquids thanks to the plethora of commercially available probes.
43 Conversely, direct RONS quantification inside semi-solid targets like gelatin or agarose is
44 more complex. Kawasaki and collaborators employed the iodine test (KI-starch) to visualize
45 RONS generation in agarose upon CAP treatment [8]. Dobrynin and colleagues, used
46 agarose gel to visualize the penetration depth of plasma-generated hydrogen peroxide and,
47 in the same work, they used fluorescein added to agarose gel to demonstrate the plasma-
48 induce pH decrease inside the target [6]. CAP-induced acidification in media is a known
49 effect, the pH drop is being mainly due to the generation of acid species like nitrous and nitric
50 acid [9, 10]. The acidification degree depends on CAP parameters (frequency, gas flow rate
51 and exposure time) and on the buffer capacity of the medium. CAP treatment has been
52 shown to significantly increase acidification of skin [11]. Today, to measure skin pH, pH-
53 meters with flat electrodes are conveniently used [12]. Although these instruments give
54 precise measurements of pH, the recorded value is the average of the hydrogen ion
55 concentration on the whole surface underneath the electrode, i.e. at least one centimeter
56 square. When a tissue is treated with a plasma-jet, the CAP impacted zone is exposed to
57 the highest concentration of chemical species. In such “focalized” treatments, the pH on the
58 exposed area can dramatically decrease generating chemical burns and permanent
59 damages. Understanding how CAP generated species are delivered in an organ or in intact
60 skin is of a crucial importance in order to propose a safe treatment. In this work we used
61 agarose gels as models to mimic human tissues. Two models with different pH were used:
62 one having a pH around 7.4 to mimic most of human tissues and the other having a pH
63 around 5.5 to simulate the outer layers of the skin. In order to visualize and quantify the local
64 acidification induced by CAP in these models, we pre-mixed fluorescein to the agarose gels.
65 We took advantage of the fluorophore fluorescein for its well-known pH-dependent
66 fluorescence [13-15]. In fact, fluorescein coupled to gelatin was previously used to measure
67 localized acidification induced by cancer cells in Matrigel® [16]. Thanks to its very low
68 toxicity, fluorescein can also be used as an internal tracer in human body and has been
69 proposed for the non-invasive measurement of retinal pH [17]. Concerning plasma, an
70 attempt to qualitatively demonstrate the pH drop induced by CAP in fluorescein-containing
71 agarose was formerly done [6] but the precise extend of pH decrease and the impacted area
72 remain to be studied.

73 In this work, we used sodium fluorescein imbedded in a 2% agarose gel to mimic human
74 tissues. Agarose was dissolved either on a physiologic saline solution (NaCl 150mM) or in a
75 slightly buffered saline solution (NaCl 150 mM, HEPES 5.5 mM). The buffered or non-

76 buffered solutions were adjusted to different pH. Six solutions with pH ranging from 7.4 to 2.5
77 were used for establishment of the calibration curve. After adding agarose (2%) and
78 fluorescein (50 μ M) to each solution, the resulting mixtures were gently heated in a
79 microwave and poured in 6-well plates. The volume was calculated in order to get a 2 mm
80 thick gels in each well. The fluorescent gel at pH around 7.4 was used as a model for generic
81 organ while the gel pH 5.5 was used as a skin model [18]. Plasma treatment was carried out
82 using the previously described helium Plasma Gun device [19, 20] already assessed for *in*
83 *vivo* experiments [21].

84 To study the impact of the jet on the plasma exposed target, two different glass capillaries
85 were used: a straight capillary with an internal diameter of 4 mm and a capillary having a
86 tapered extremity with an inner diameter of 1.5 mm. The tip of the capillary was set at 10 mm
87 above the gel surface. Each treatment was performed for 120s or 300s, at a frequency of 1
88 kHz and a helium gas flow rate set at 0.5 L/min. For each experiment, three fluorescence
89 intensity images of the agarose-containing wells were acquired: before the treatment,
90 immediately after plasma exposure and 10 min after plasma exposure, using the Typhoon™
91 FLA 9500 biomolecular imager (GE Healthcare Life Science) equipped with a 473 nm (blue
92 LD laser) and a Y520 emission filter (wavelength range \geq 520 nm). High quality pictures
93 were acquired with a pixel size of 100 μ m. Images were exported and processed in ImageJ.
94 Real dimension of the 8-Bit grey scale images were assigned in mm units using the set scale
95 function. The background variations and the drift of fluorescence that can occur during the
96 three consecutive scans were measured and corrected. For a qualitative and visual analysis
97 of the acidification induced by plasma, images were converted in pseudocolors using the
98 ICA3 option in Lookup Tables. The fluorescence intensity of the 6 wells was quantified and
99 the measured integrated density values were assigned to the known pH values. A calibration
100 curve of the gray scale values vs pH was obtained using the roadboard function (Figure 1).
101 To measure pH evolution before and after the treatment in our models, a straight line
102 selection of 18-22 mm, passing through the center of the treated area, was manually traced.
103 A plot profile of fluorescence intensity showing pH variations along the traced line was
104 obtained. Data from each plot profile can be exported in excel or other spreadsheet software
105 for further analysis. In this paper we used the software GraphPad Prism version 6 and GIMP
106 for chart and figure design respectively.

107 The effect of CAP treatment on pH modulation was first studied in agarose gel without buffer
108 capacity. Non-buffered agar models mimicking generic organs (pH 7.4) or skin (pH 5.5) were
109 treated for 120s with plasma-gun using either a straight or a tapered capillary (Figure 2).
110 When a tapered capillary was used, the thin plasma jet (diameter < 1mm) induces a focalized
111 pH drop (Figure 2a) either in the generic organ (4 pH units) or in the skin model (3 pH units).
112 Interestingly, the pH dropped to a value around 3 either in the neutral or in the already acidic
113 model. It is known that during plasma treatment one of the acidic species produced is nitrous
114 acid. This weak acid has a pKa 3.16 [22]. We can speculate that the nitrous acid locally
115 produced creates a buffer at pH 3 keeping the pH more stable around this value when a
116 short treatment is administered. A similar pH drop, in a non-buffered saline, was already
117 showed by Hänisch et al; at this pH values, plasma treated solution has been shown to exert
118 a potent bactericidal effect [23]. When the straight capillary was used (Figure 2b), the larger
119 plasma jet led to a minor and uniform acidification in the generic organ and the skin model (1
120 pH units in both cases). In all the treatments, very slight pH variations were registered 10 min

121 after plasma exposure. We measured the diameter of the acidified surface generated with
122 the two capillaries. The skin model, exposed to the jet generated by the tapered capillary,
123 shows an acidified surface with a diameter 1.5 times larger than the one measured on the
124 generic organ model (12 mm vs 8 mm) and 2 times larger (12 mm vs 6 mm) when the
125 straight capillary is used. It appears that the acidic species spreads better in the already
126 acidic model.

127 The observed acidification could overestimate the real acidifying effect induced by CAP on
128 human tissues. In fact, human organs possess buffer systems that offset pH variations to
129 maintain physiological values [24]. The skin, in its outer layers possesses its own buffer
130 system [25]. In order to improve our models, we prepared fluorescein-containing gels with a
131 HEPES-based buffer system. In figure 3, buffered-fluorescent agarose models were
132 exposed to CAP using the tapered capillary. When the buffered gel was exposed for 120s to
133 plasma treatment, we measured a minor pH decrease respect the one measured previously
134 in the non-buffered agarose. The generic organ model (figure 3a) shows a drop of just 1.5 pH
135 units while the skin model decreases its pH of 1 pH units (fig. 3b) . A longer treatment of
136 300s results in a higher pH decrease in the generic organ model (2.5 pH units) and a weaker
137 decrease in the skin model (1.5 pH units). In figure 4, we exposed agarose models to
138 plasma, using a straight capillary. When the buffered-generic organ model was exposed to
139 the jet generated with this capillary, we observed almost no pH variation for the short
140 treatment of 120s and a very little pH drop, less than 0.5 pH units, for the longer treatment of
141 300s (Figure 4a). The treatment of the skin model using the straight capillary (Figure 4b)
142 results in a pH drop, around 0.5 and 1 pH units for short and long CAP treatment
143 respectively, the measured acidification appearing weaker compared to the one observed
144 using the tapered capillary. As observed in the non-buffered system, the treatment of the skin
145 model results in a wider acidified surface, on average 1.4 times larger (9.7 mm vs 6.7 mm).
146 In conclusion, the presented method permits not only to visualize in situ acidification but also
147 to quantify the pH changes, in the range of 2.5 to 7.4 pH units. Although the described
148 method can have some limitations and weakness, it offers the advantage of measuring the
149 pH on a very small spot. To date the precise measurement of these limited surfaces using
150 flat pH-meters is not possible. These pH-meters, whose smaller probe surface is around 1
151 cm², can only give a pH mean value of the whole covered surface. We demonstrated here
152 that this novel technique can help understanding the impact of a plasma treatment on a
153 biological tissue to better plan *in vivo* plasma therapy. In fact, these results show how
154 different parameters such as the capillary shape, the treatment time, the initial pH and the
155 buffer capacity of the tissue model can influence the plasma-induced acidification in a treated
156 tissue. Particularly, the diameter of the plasma jet was shown to induce an impact on the
157 acidic species distribution on the treated surface, suggesting that large jet can be used to
158 have a diffuse and uniform treatment while thin jets can be useful to focalize the treatment
159 without disturbing the surrounding tissue pH.

160

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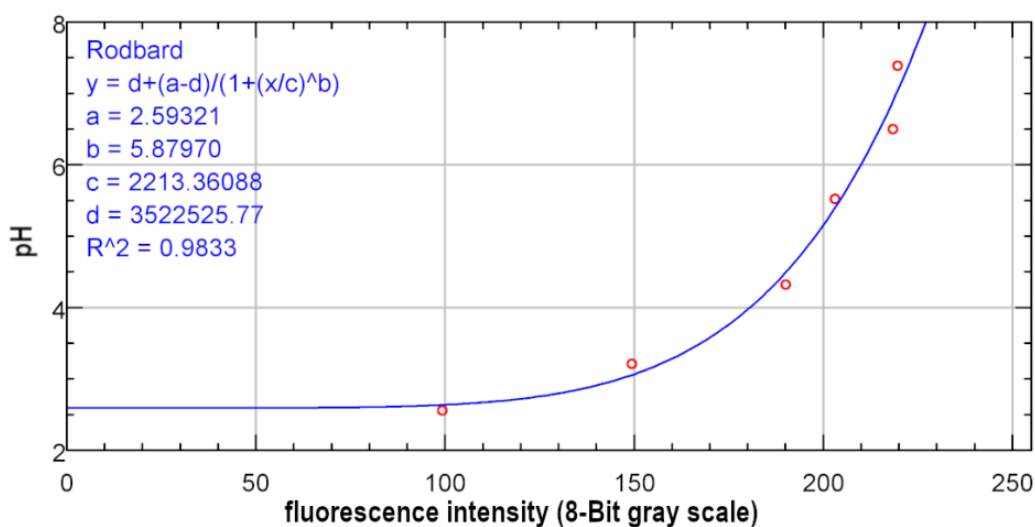
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FIGURES

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242 **Figure 1.** A typical roadboard calibration curve of fluorescein fluorescence intensity
 243 vs pH obtained in ImageJ.

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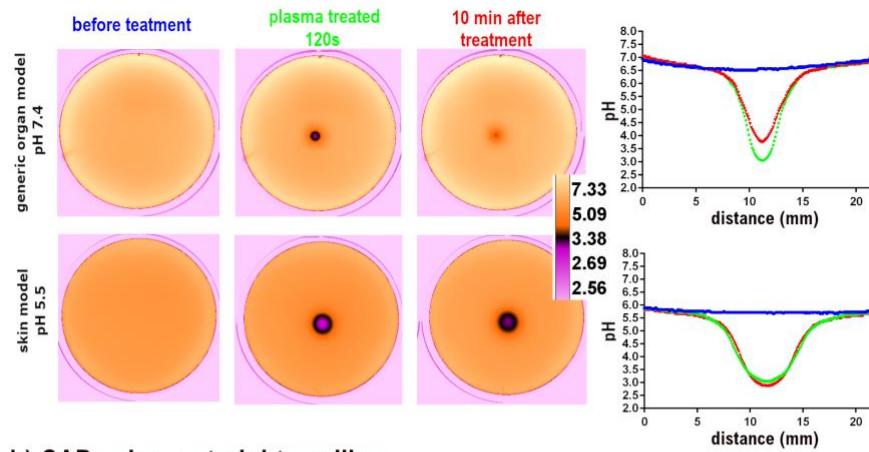
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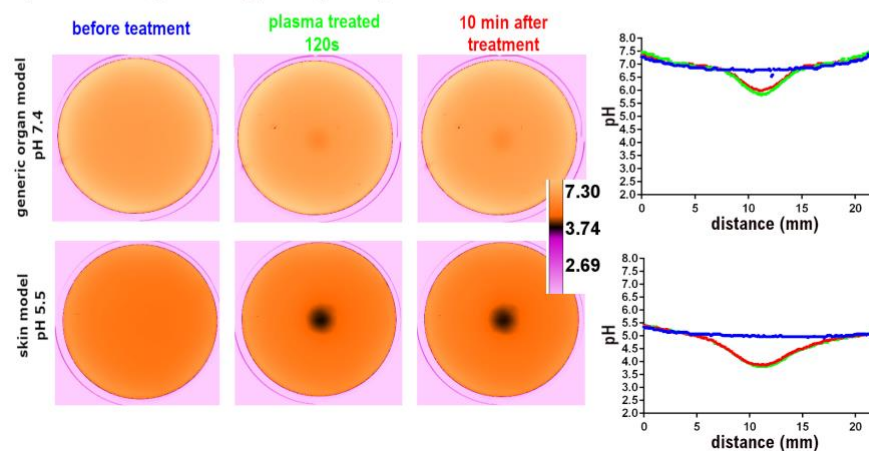
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a) CAP using a tapered capillary



b) CAP using a straight capillary



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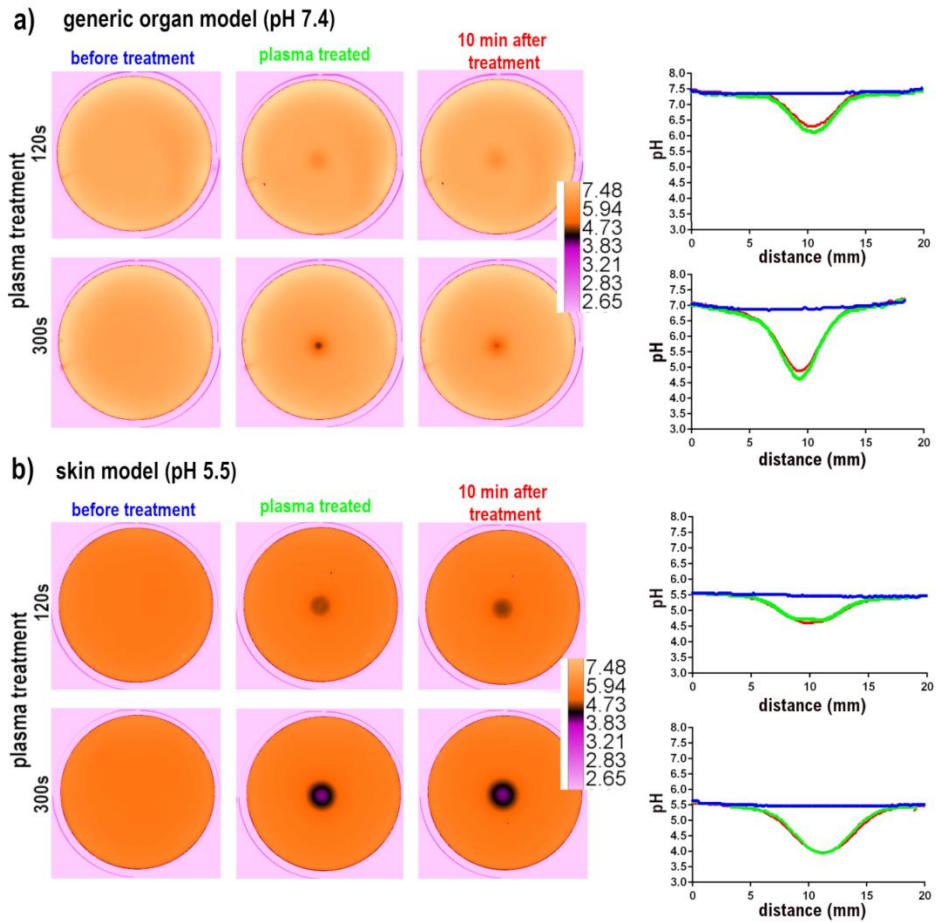
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252 **Figure 2.** Non-buffered fluorescent agarose gels, with initial physiological around 7.4 or 5.5, treated
253 with CAP using a tapered (a) or a straight capillary (b) to generate the plasma-jet. The fluorescence
254 emission of the agarose gel was acquired as 8-Bit gray scale images and converted in ICA3
255 pseudocolor to better visualize the acidified area, the pH pseudocolor calibration bar is shown on the
256 right of the images. On the right part of the figure the pH plots graphs measured before (blue trace),
257 immediately after plasma treatment (green trace) and 10 min after plasma treatment (red trace)

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CAP using a tapered capillary



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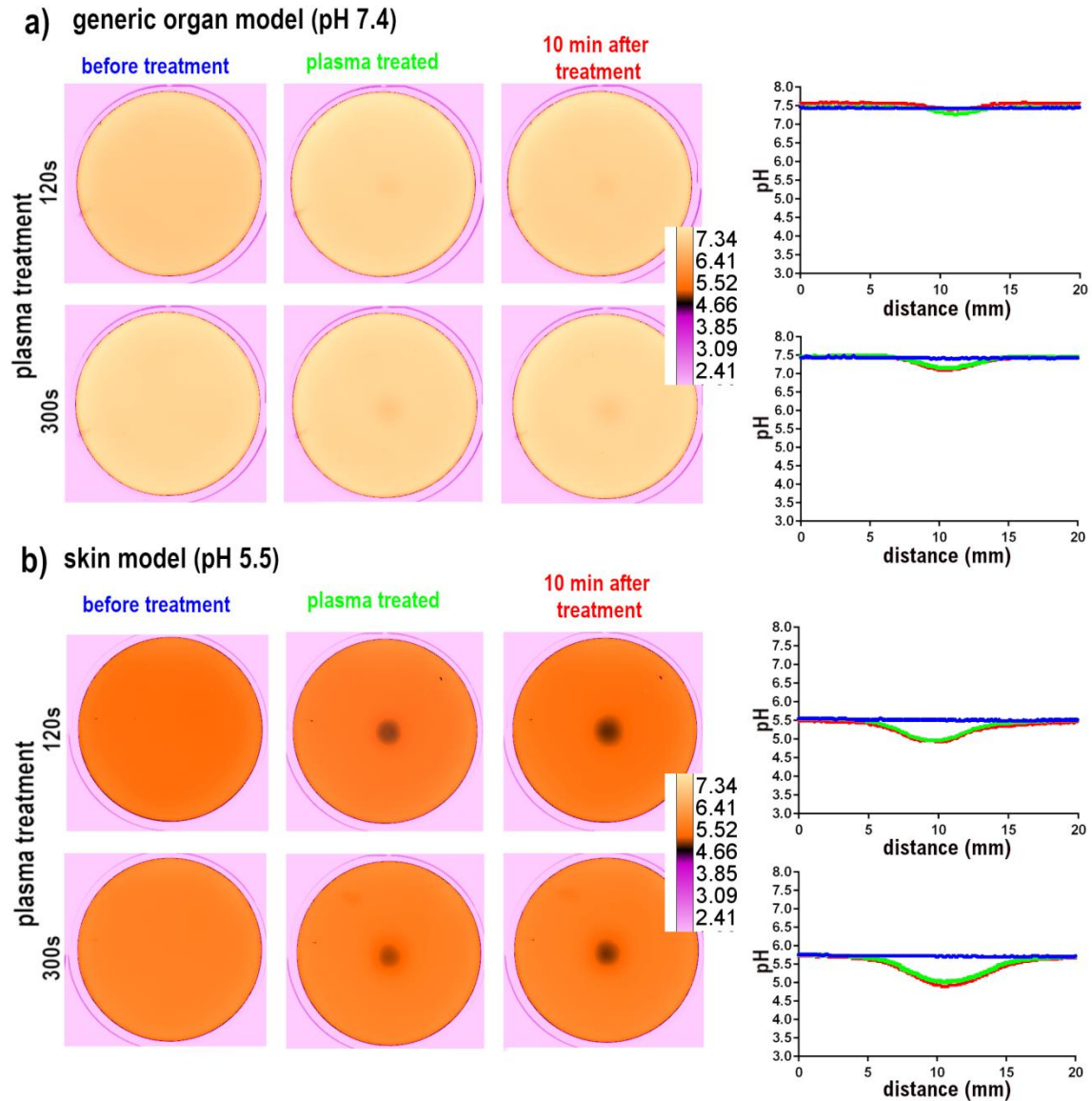
261

262 **Figure 3.** Buffered fluorescent agarose gels mimicking a generic organ pH (a) or skin (b) treated with
263 CAP for 120s or 300s using a tapered capillary to generate the plasma-jet. The fluorescence emission
264 of the agarose gel was acquired as 8-Bit gray scale images and converted in ICA3 pseudocolor to
265 better visualize the acidified area, the pH pseudocolor calibration bar is shown on the right of the
266 images. On the right part of the figure the pH plots graphs measured before (blue trace),
267 immediately after (green trace) and 10 min after plasma treatment (red trace)

268

269

CAP using a straight capillary



270

271 **Figure 4.** Buffered fluorescent agarose gels mimicking a generic organ pH (a) or skin (b) treated with
 272 CAP for 120s or 300s using a straight capillary to generate the plasma-jet. The fluorescence emission
 273 of the agarose gel was acquired as 8-Bit gray scale images and converted in ICA3 pseudocolor to
 274 better visualize the acidified area, the pH pseudocolor calibration bar is shown on the right of the
 275 images. On the right part of the figure the pH plots graphs measured before (blue trace), immediately
 276 after (green trace) and 10 min after plasma treatment (red trace)

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