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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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9

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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28  
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 $\mu$ 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  $\mu$ 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 cm<sup>2</sup>, 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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- 173 1. Laroussi, M. and X. Lu, *Room-temperature atmospheric pressure plasma plume for*  
174 *biomedical applications*. Applied Physics Letters, 2005. **87**(11): p. 113902.
- 175 2. Kong, M.G., et al., *Plasma medicine: an introductory review*. New Journal of Physics,  
176 2009. **11**(11): p. 115012.
- 177 3. Heinlin, J., et al., *Plasma applications in medicine with a special focus on*  
178 *dermatology*. J Eur Acad Dermatol Venereol, 2011. **25**(1): p. 1-11.
- 179 4. Haertel, B., et al., *Non-thermal atmospheric-pressure plasma possible application in*  
180 *wound healing*. Biomol Ther (Seoul), 2014. **22**(6): p. 477-90.
- 181 5. Dąbrowska, A.K., et al., *Materials used to simulate physical properties of human skin*.  
182 *Skin Research and Technology*, 2016. **22**(1): p. 3-14.
- 183 6. Dobrynin, D., et al., *Deep Penetration into Tissues of Reactive Oxygen Species*  
184 *Generated in Floating-Electrode Dielectric Barrier Discharge (FE-DBD): An *In**  
185 *Vitro* Agarose Gel Model Mimicking an Open Wound. 2012. **2**(1-3): p. 71-83.
- 186 7. Szili, E.J., et al., *Tracking the Penetration of Plasma Reactive Species in Tissue*  
187 *Models*. Trends in Biotechnology, 2018. **36**(6): p. 594-602.
- 188 8. Kawasaki, T., et al., *Visualization of the Distribution of Oxidizing Substances in an*  
189 *Atmospheric Pressure Plasma Jet*. IEEE Transactions on Plasma Science, 2014.  
190 **42**(10): p. 2482-2483.
- 191 9. Chen, C.-W., H.-M. Lee, and M.-B. Chang, *Influence of pH on inactivation of aquatic*  
192 *microorganism with a gas-liquid pulsed electrical discharge*. Journal of Electrostatics,  
193 2009. **67**(4): p. 703-708.
- 194 10. Oehmigen, K., et al., *The Role of Acidification for Antimicrobial Activity of*  
195 *Atmospheric Pressure Plasma in Liquids*. Plasma Processes and Polymers, 2010.  
196 **7**(3-4): p. 250-257.
- 197 11. Heuer, K., et al., *The topical use of non-thermal dielectric barrier discharge (DBD):*  
198 *nitric oxide related effects on human skin*. Nitric Oxide, 2015. **44**: p. 52-60.
- 199 12. du Plessis, J.L., A.B. Stefaniak, and K.P. Wilhelm, *Measurement of Skin Surface pH*.  
200 *Curr Probl Dermatol*, 2018. **54**((pH of the Skin: Issues and Challenges)): p. 19-25.
- 201 13. Emmart, E.W., *Observations on the absorption spectra of fluorescein, fluorescein*  
202 *derivatives and conjugates*. Archives of Biochemistry and Biophysics, 1958. **73**(1): p.  
203 1-8.
- 204 14. Leonhardt, H., L. Gordon, and R. Livingston, *Acid-base equilibriums of fluorescein*  
205 *and 2',7'-dichlorofluorescein in their ground and fluorescent states*. The Journal of  
206 *Physical Chemistry*, 1971. **75**(2): p. 245-249.
- 207 15. Martin, M.M. and L. Lindqvist, *The pH dependence of fluorescein fluorescence*.  
208 *Journal of Luminescence*, 1975. **10**(6): p. 381-390.
- 209 16. Busco, G., et al., *NHE1 promotes invadopodial ECM proteolysis through acidification*  
210 *of the peri-invadopodial space*. FASEB J, 2010. **24**(10): p. 3903-15.
- 211 17. Martin, H., et al., *Sodium fluorescein as a retinal pH indicator?* Physiological  
212 *Measurement*, 2005. **26**(4): p. N9.
- 213 18. Mauro, T., et al., *Barrier recovery is impeded at neutral pH, independent of ionic*  
214 *effects: implications for extracellular lipid processing*. Archives of Dermatological  
215 *Research*, 1998. **290**(4): p. 215-222.
- 216 19. Robert, E., et al., *Characterization of pulsed atmospheric-pressure plasma streams*  
217 *(PAPS) generated by a plasma gun*. Plasma Sources Science and Technology, 2012.  
218 **21**(3): p. 034017.

- 219 20. Busco, G., et al., *Changes in Oxygen Level Upon Cold Plasma Treatments: Consequences for RONS Production* IEEE Trans Radiat Plasma Med Sci., 2018. 220 **2**(2): p. 147-152. 221
- 222 21. Collet, G., et al., *Plasma jet-induced tissue oxygenation: potentialities for new 223 therapeutic strategies*. Plasma Sources Science and Technology, 2014. **23**(1): p. 224 012005. 225
- 226 22. da Silva, G., E.M. Kennedy, and B.Z. Dlugogorski, *Ab Initio Procedure for Aqueous- 227 Phase pKa Calculation: The Acidity of Nitrous Acid*. The Journal of Physical 228 Chemistry A, 2006. **110**(39): p. 11371-11376.
- 229 23. Hänsch, M.A.C., et al., *Analysis of antibacterial efficacy of plasma-treated sodium 230 chloride solutions*. Journal of Physics D: Applied Physics, 2015. **48**(45): p. 454001
- 231 24. Hamm, L.L., N. Nakhoul, and K.S. Hering-Smith, *Acid-Base Homeostasis*. Clinical 232 journal of the American Society of Nephrology : CJASN, 2015. **10**(12): p. 2232-2242.
- 233 25. Zhai, H., et al., *Measuring human skin buffering capacity: an in vitro model*. Skin 234 Research and Technology, 2009. **15**(4): p. 470-475.

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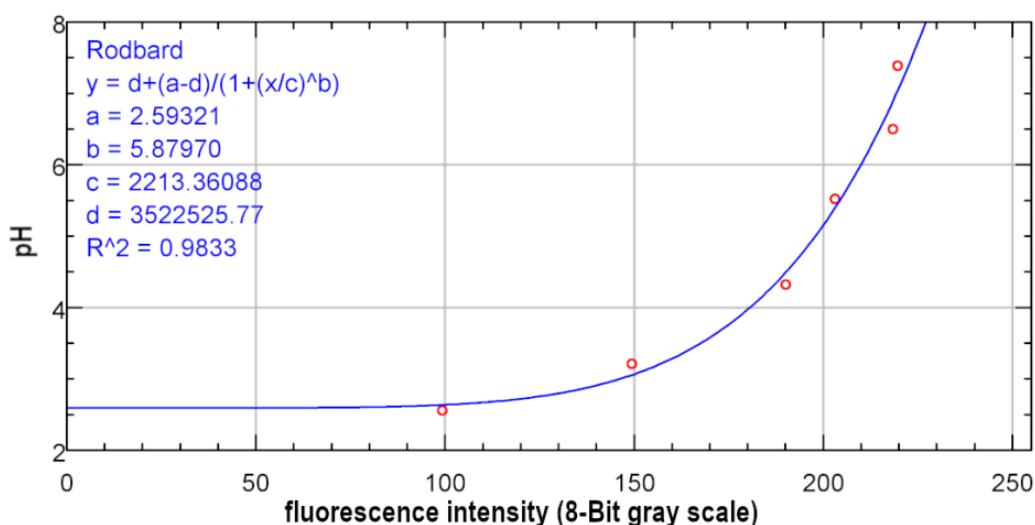
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## FIGURES

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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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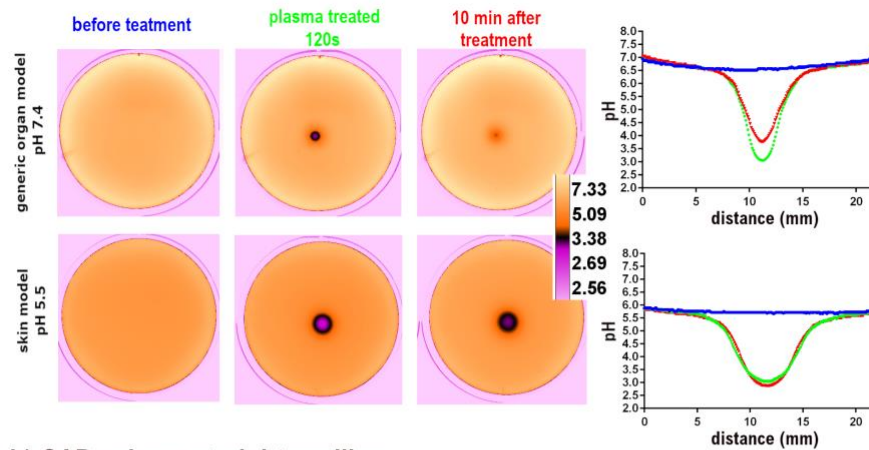
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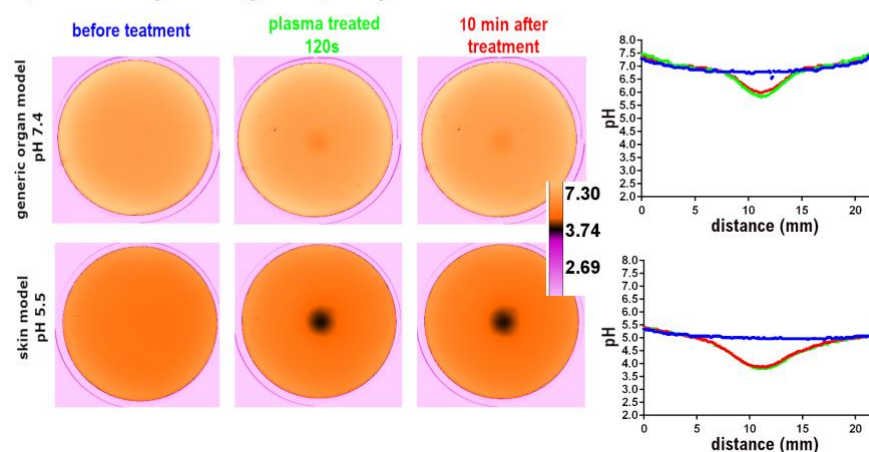
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a) CAP using a tapered capillary



b) CAP using a straight capillary



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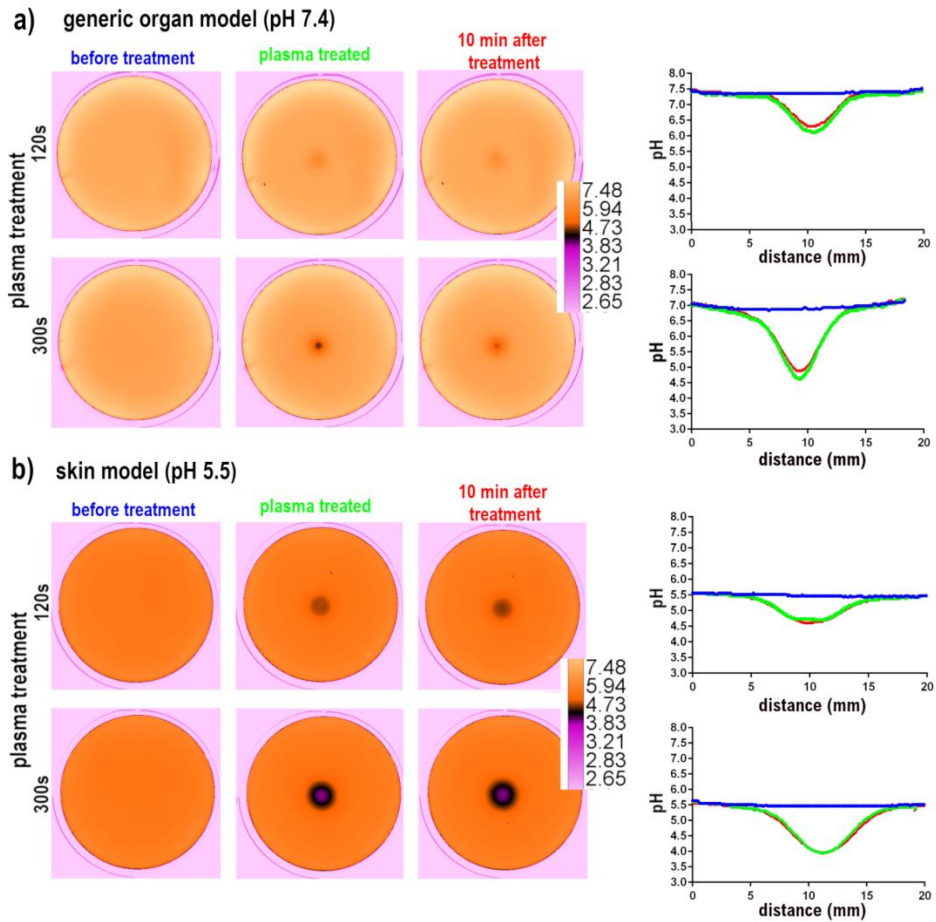
252 **Figure 2.** Non-buffered fluorescent agarose gels, with initial physiological around 7.4 or 5.5, treated  
253 with CAP using a tapered (a) or a straight capillary (b) to generate the plasma-jet. The fluorescence  
254 emission of the agarose gel was acquired as 8-Bit gray scale images and converted 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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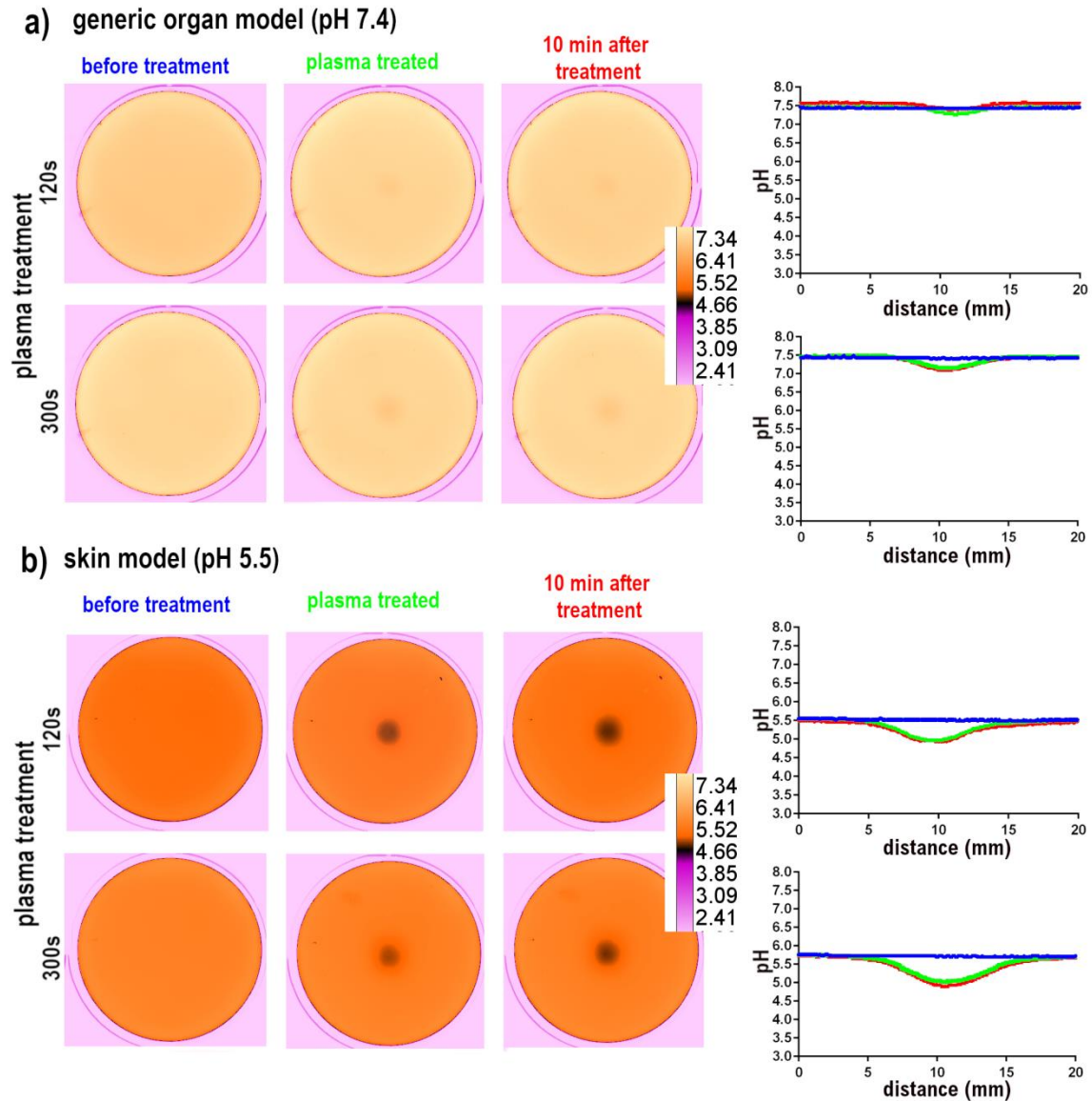
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262 **Figure 3.** Buffered fluorescent agarose gels mimicking a generic organ pH (a) or skin (b) treated with  
263 CAP for 120s or 300s using a tapered capillary to generate the plasma-jet. The fluorescence emission  
264 of the agarose gel was acquired 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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