Electronic Supplementary Material (ESI) for Lab on a Chip.

Supplementary figures

Figure SI 1: Metal master mold and PDMS replica for micro-structured hydrogel molding

As described in the experimental section, the micro-structured hydrogel is molded on a PDMS mold fabricated thanks to a replica process using a micro-milled metal master mold. Pictures of these molds are presented in Figure SI 1.

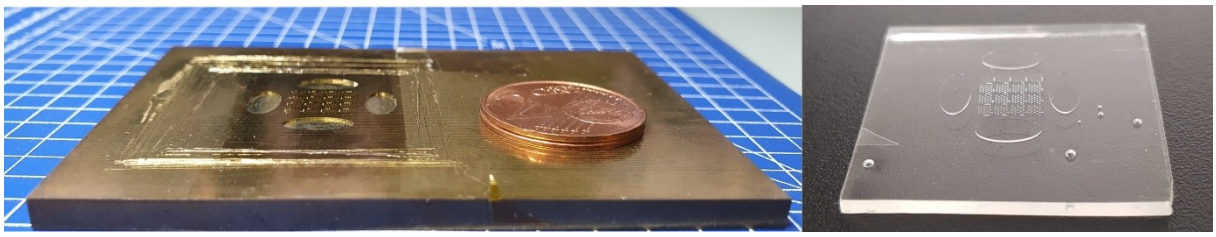


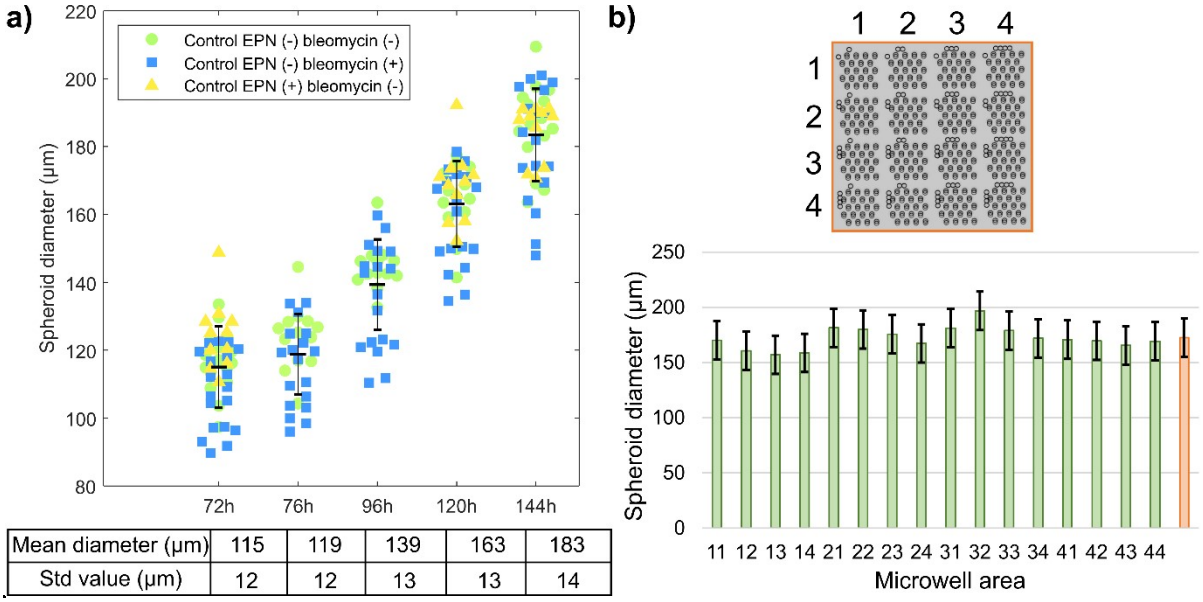
Figure SI 1: *Micro-milled metal master mold (left) and PDMS replica (right) for hydrogel molding.*

Figure SI 2: Study of spheroid size regularity

Diameter evolution was studied for 3 different groups of the same experiment, from 3 to 6 days after cell seeding (Figure SI 2a). Results show that the standard deviation is of 12 to 14 µm, which is inferior or of the same order of magnitude compared to other techniques of spheroid production, that are more labor-intensive. For example, B. Gao et al[1] obtained a deviation of around 15 µm and W. Zhang et al[2] around 30 to 40 µm, for the formation of their spheroids with hanging-drop method.

In addition, to ensure that there is no size deviation between the several areas of microwells composing the array (16 areas of 20 microwells), spheroid diameter was measured and compared between all areas. Figure SI 2b shows that the mean diameters of each area are comparable and close to the mean diameter of the whole array (orange bar), ensuring that there is no significant size deviation on the array. It can also be noted that the standard deviation obtained here (17 µm) is close to the values determined in Figure SI 2a.

Therefore, the method used here allows the production of regular spheroid, both on one array of microwells and between the arrays, which ensures a better reproducibility of the results and prevents to have biased results of EPN effect due to a large size distribution.



Mean diameter (µm)	115	119	139	163	183
Std value (µm)	12	12	13	13	14

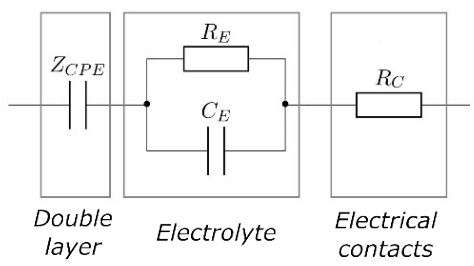
Figure SI 2: *(a) Evolution of HT29 cell spheroid diameter (N = 16/condition) of control groups from one experiment represented in Figure 6. Point = value for one spheroid imaged, Long horizontal line = mean, Small horizontal line = STD value, Green = healthy spheroids, Blue = bleomycin only, Yellow = EPN only. (b) Study of HT29 cell spheroids diameter regularity (mean +/- STD) on all the microwell areas of an array, one experiment. Green = Mean diameter on an area of 20 microwells, Orange = Mean diameter on all the array.*

Figure SI 3: Equivalent electrical circuit of the device and determination of the voltage drop in the microfluidic chamber

The equation given in Figure SI 3a describes the electrical potential inside the chamber as a function of the applied potential, determined thanks to the equivalent electrical circuit represented in Figure 2a.

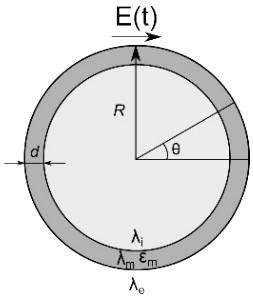
Concerning the evolution of the transmembrane potential (TMP), it was calculated using the equation given by T. Kotnik and D. Miklavcic[3], with properties (conductivity, permittivity) of HT29 cells from MyDEP software[4,5].

a)



$$U_e = U_{tot} \times \frac{Z_e}{Z_{tot}} = U_{tot} \times \frac{\frac{R_e}{1+j\omega R_e C_e}}{K(jw)^{-\beta} + \frac{R_e}{1+j\omega R_e C_e} + R_C}$$

b)



$$V = 1.5ER\cos\theta\frac{1}{1+j\omega\tau_m} \qquad \tau_m = \frac{Rc_m}{\frac{2\lambda_i\lambda_e}{\lambda_i+2\lambda_e} + \frac{R}{d}\lambda_m}$$

Figure SI 3: *(a) Equivalent electrical circuit of the device and equation of the electrical potential U_e in the chamber as function of the total applied potential U_{tot}. R_E = 245 Ω, C_E = 1,07.10^{-10} F, R_C = 43 Ω, K = 6,4.10^4 Ω, β = 0,9, ω = 10 kHz. (b) Characterization of the evolution of transmembrane potential (TMP) of cells with frequency. E = 800 V.cm^{-1}, R = 6,6 µm, θ = 0, λ_i = 0,182 S.m^{-1}, λ_e = 0,03 S.m^{-1}, λ_m = 1,091.10^{-4} S.m^{-1}, d = 5 nm, $c_m = \frac{\varepsilon_m}{d}$, ε_m = 6,8.10^{-11} F.m^{-1}, τ_m = 0.47 µs.*

Figure SI 4: Monitoring of medium exchange inside the microfluidic chamber with confocal microscopy

To confirm the kinetics of medium exchange inside the microfluidic chamber found with impedance measurements (Figure 3), confocal microscopy was done *in situ* to monitor the diffusion of a fluorescent solution in the hydrogel. A micro-structured agarose hydrogel was molded on a coverslip to allow direct observation with a 25X objective (NA 0.95) of the confocal microscope (Leica SP5). It was incubated in PBS before being mounted in the microsystem. A 0.05 mM FITC (fluorescein isothiocyanate) solution in PBS was injected in the microfluidic chamber with a 2.5 mL syringe and 21G needle by volume of 0.5 mL before the acquisition of a z-stack to image the whole thickness of the hydrogel (Δz = 20 µm, 1.5 min/stack). An additional acquisition was made after 5 min without any injection to determine if the fluorescence had reached a constant value. Fluorescence was then measured on the stacks with ImageJ software by defining a region of interest (ROI) on the confocal image of the hydrogel. Mean fluorescence on the whole thickness of the hydrogel imaged is represented in Figure SI 4. It is compared to the maximum intensity measured on a hydrogel incubated 24h in the same FITC solution, thus saturated in FITC solution. Results show a complete medium

diffusion inside the hydrogel in 10 min for 2.5 mL injected, which is comparable to what was observed with impedance measurements.

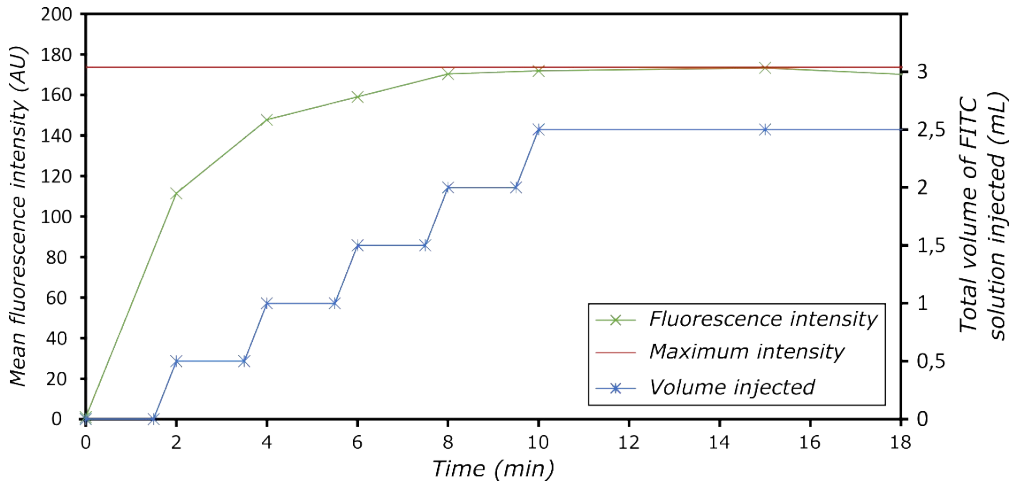


Figure SI 4: *Mean green fluorescence intensity on the whole thickness of hydrogel measured in situ with confocal microscopy (25X, NA 0.95) during injection of FITC solution in the microfluidic, containing the micro-structured hydrogel without cell spheroids. Red line = mean intensity measured on a hydrogel saturated 24h in FITC solution, Green line = FITC fluorescence intensity, Blue line = volume injected.*

Figure SI 5: Double use of propidium iodide (PI) as maker of cell death or to evaluate EPN efficiency

Taking advantage of the microsystem enabling easy observation with confocal microscopy, PI red fluorescence intensity was compared for dead control spheroids and electroporated spheroids, to show the double use of PI as a dead or electroporated cell marker. We also studied a control put in presence of PI without EPN. Due to the small size of the sample, the p-value was evaluated with the non-parametric Wilcoxon statistical test, allowing to test the hypothesis that two statistical series have close medians, at a threshold of 5%.

Results, presented in Figure SI 5, confirm that PI can have this dual use, as red fluorescence intensity measured on spheroids is significantly higher (more than 2.5 times and p-value < 2.10^{-4}) for dead spheroids than for electroporated ones. The other control, PI without EPN, is not represented here because no fluorescence could be measured, as expected due to the absence of permeabilization. Only one or two dead cells were visible for some spheroids. These results demonstrate that EPN conditions do not induce any cell toxicity right after EPN, as nearly no dead cells can be observed on electroporated spheroids (Figure SI 5b).

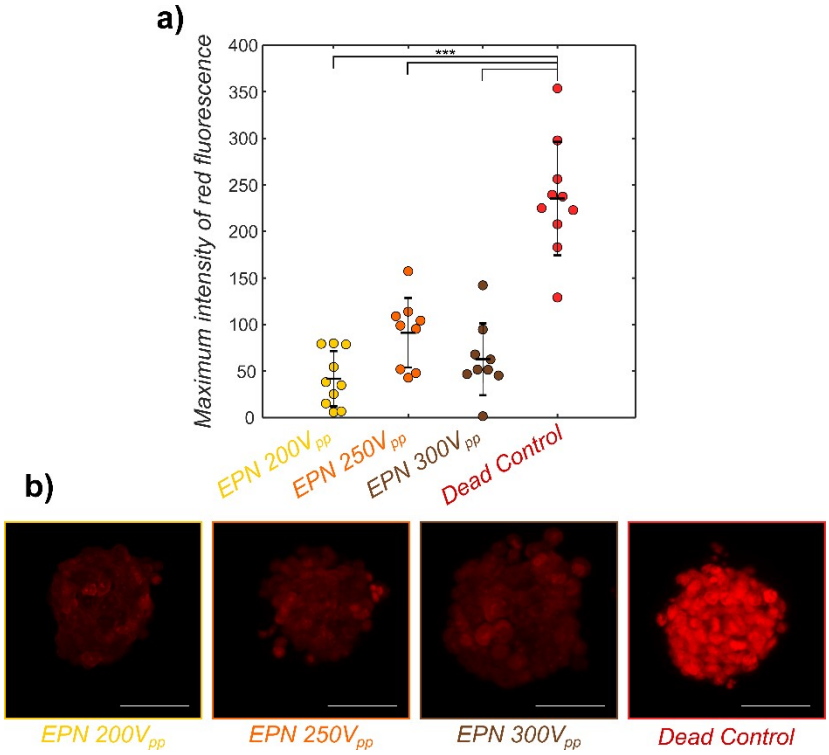


Figure SI 5: *(a) Maximum PI fluorescence intensity on dead HT29 cell spheroids (red) or HT29 cell spheroids electroporated in presence of PI (Yellow = 200 V_{pp}, Orange = 250 V_{pp}, Brown = 300V_{pp}), on fixed samples. Point = value for one spheroid imaged, Long horizontal line = mean, Small horizontal line = STD value, N = 10 spheroids/condition, one experiment, *** p < 0,001. (b) MIP of confocal microscope z-stack images (25X) of dead spheroids or spheroids electroporated in presence of PI, realized with the same acquisition and contrast parameters. Red = PI, Scale bar = 100 µm.*

Figure SI 6: Spheroid viability after EPN

Confocal images were realized on spheroids labelled with FDA and PI 2h after EPN. They were compared with images obtained on dead spheroids, killed by incubation 30 min in 70% ethanol. Quantification of red fluorescence on maximum intensity projection (MIP) shows that mortality due to the pulsed electric field apply is very low. As it can be seen in Figure SI 6a, there are only few dead cells. Figure SI 6b shows that cell mortality is below 3% if we compare red fluorescence intensity of electroporated spheroids to the one of dead spheroids, with less dead cells for the lower electric field intensity tested. It also shows that EPN is reversible, as no PI can enter into viable cells 2h after EPN.

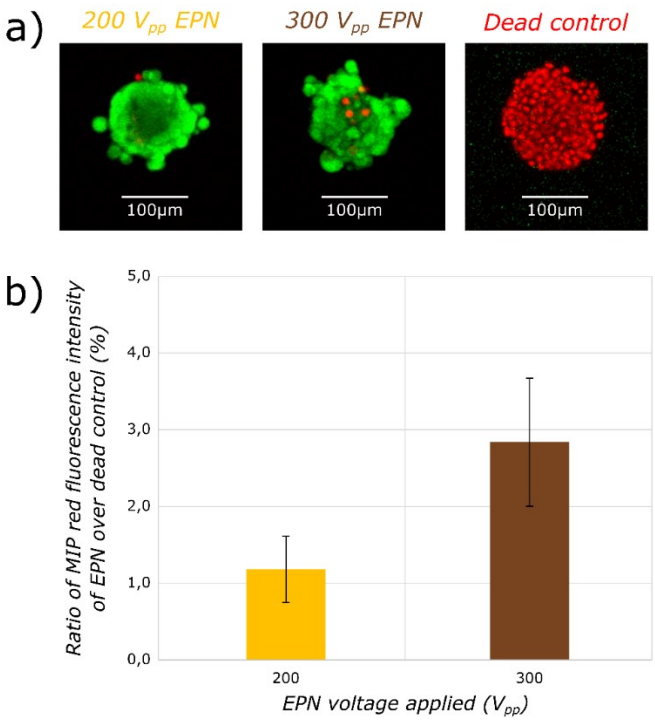


Figure SI 6: *(a) MIP merged confocal microscope images (10X, NA 0.3) of HT29 cell spheroids marked with FDA and PI 2h after EPN and dead control (cells killed with ethanol). Green = FDA, Red = PI. (b) Mean +/- STD value of the ratio of MIP red fluorescence intensity of EPN spheroids over the one for dead spheroids for the two EPN voltage tested. Yellow = 200 V_{pp}, Brown = 300 V_{pp}, N = [4 to 6] spheroids/condition, one experiment.*

Figure SI 7: Quantification of the proliferative layer within spheroids 3 days after experiment with or without EPN and/or anti-cancer treatment with bleomycin

In addition to Figure 6c, red fluorescence intensity, corresponding to EdU labelling of proliferative cells, and green fluorescence intensity, corresponding to NucGreen labelling of cell nuclei, were measured on confocal images, made 3 days after the experiment. Thanks to a Matlab routine, the Pearson correlation coefficient (PCC) between EdU and NucGreen was calculated and plotted as function of the distance from the periphery to the core of spheroids. It evaluates the colocalization of two fluorescent probes[6]. In this case, it enables to determine how deep inside the spheroids cells are in a proliferative state (PCC > 0.5)

Results confirm what is observed in Figure 6c, namely that the 3 control groups have similar proliferative layer: cells are on a proliferative state up to around 30% of the spheroid diameter (PCC > 0.5), which represents a few external proliferative layers (1 to 3) as it can be seen in Figure 6c. It shows that EPN only or bleomycin only has no significant effect on cell proliferation. On the contrary, for spheroids treated with EPN in presence of bleomycin, the Pearson coefficient is reduced by more than 70%, and stays below 0.2. It demonstrates the expected effect of bleomycin on electroporated cells: stopping cell proliferation in the entire spheroids.

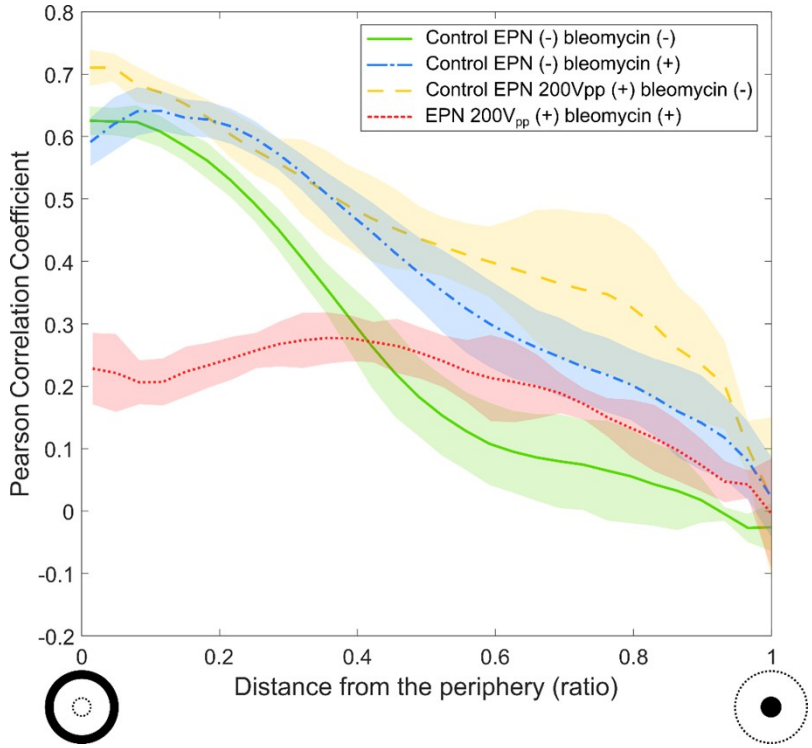


Figure SI 7: *Pearson correlation coefficient for HT29 cell spheroids stained with EdU and NucGreen 3 days after experiment with (+) and/or without (-) EPN and/or bleomycin as function of the distance from the periphery. Shaded area = SEM value, Green = healthy spheroids, Blue = bleomycin only, Yellow = EPN only, Red = EPN with bleomycin, N = [7 to 10] spheroids/condition, one experiment.*

References

1. B. Gao, C. Jing, K. Ng, B. Pingguan-Murphy and Q. Yang, *Acta Mech. Sin.*, 2019, **35**, 329–337.

2. W. Zhang, C. Li, B. C. Baguley, F. Zhou, W. Zhou, J. P. Shaw, Z. Wang, Z. Wu and J. Liu, *Anal. Biochem.*, 2016, **515**, 47–54.

3. T. Kotnik and D. Miklavcic, *IEEE Trans. Biomed. Eng.*, 2000, **47**, 1074–1081.

4. J. M. Huber, A. Amann, S. Koeck, E. Lorenz, J. M. Kelm, P. Obexer, H. Zwierzina and G. Gamerith, *J. Cancer Res. Clin. Oncol.*, 2016, **142**, 1955–1966.

5. J. Cottet, O. Fabregue, C. Berger, F. Buret, P. Renaud and M. Frénéa-Robin, MyDEP: a new computational tool for dielectric modeling of particles and cells, *Zenodo*, 2019.

6. K. W. Dunn, M. M. Kamocka and J. H. McDonald, *Am. J. Physiol.*, 2011, **300**, C723–C742.