Parametric optimization of electric field strength for cancer electrochemotherapy on a chip-based model

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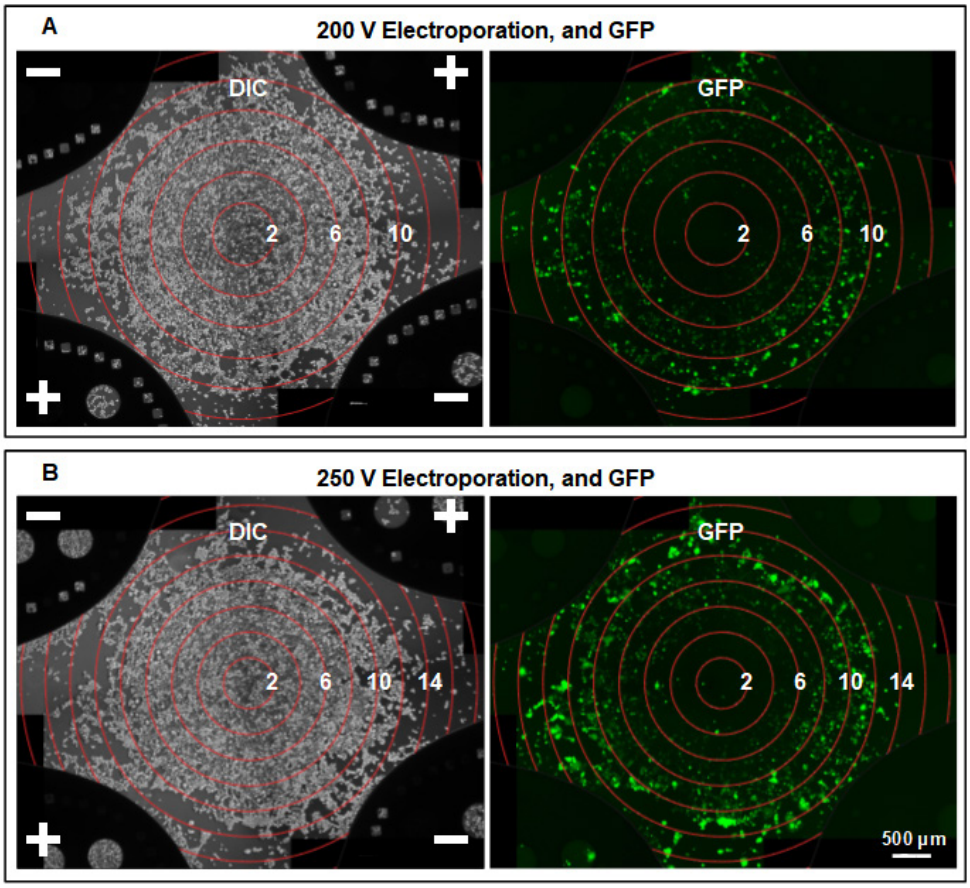
Supplementary Figure S1

In situ monitoring the DNA expression on four-leaf micro-electrode chip (F-MEC).

The bright field images (left) and fluorescent images (right) of GFP-expressed HEK-293 cells 48 hours after performing electroporation with four-electrode stimulation mode. The conditions are: (A) 200 V voltage, 3 square pulses, 100 μs pulse duration; (B) 250 V voltage, 3 pulses, 100μs pulse duration. In each image, the electric field contours were marked by red rings, the Arabic numbers on curves represent the contour values (unit: $\times 10^4$ V/m).

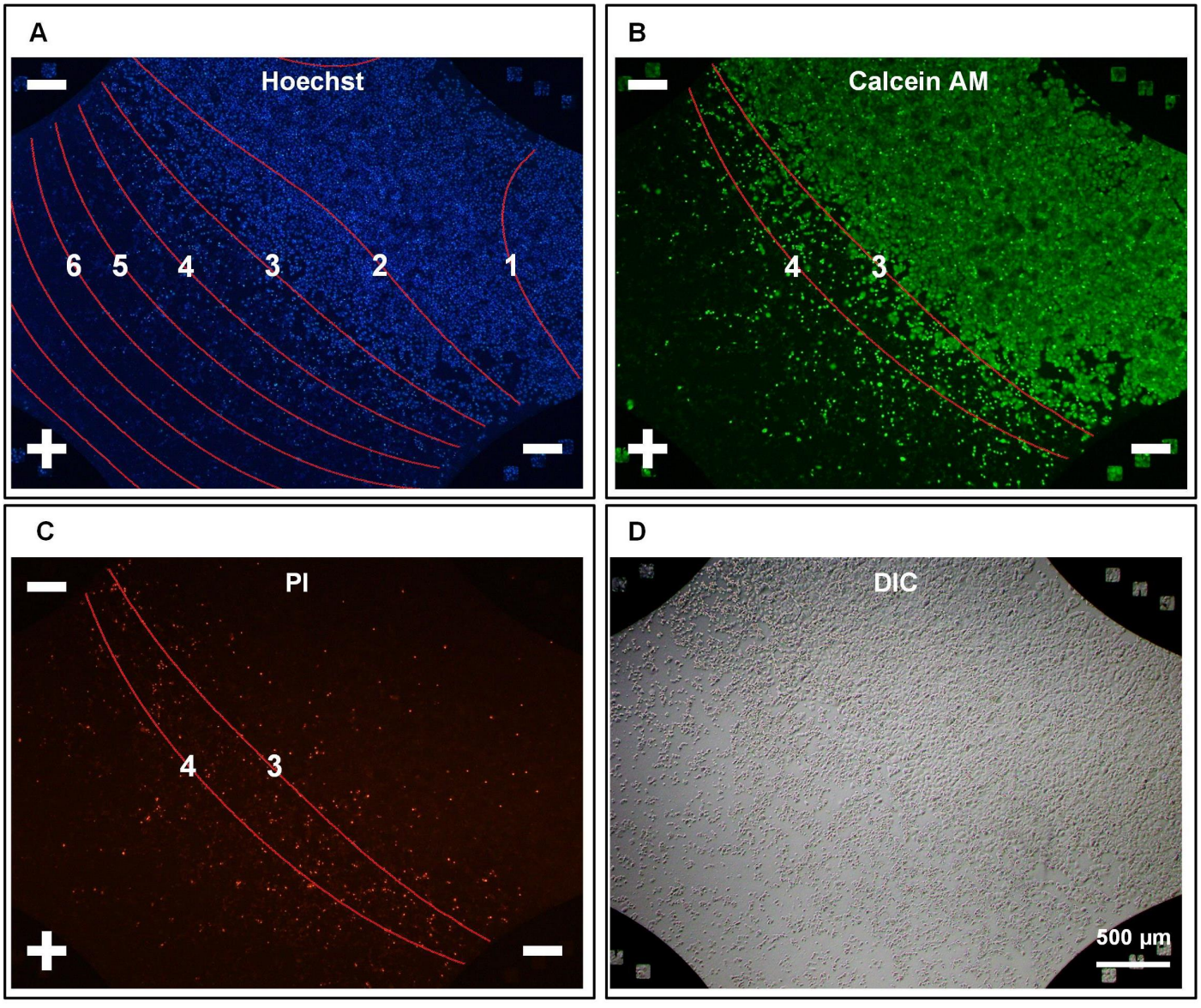


A GFP (pEGFP-C3) plasmid which encoded green fluorescence protein was used to illustrate the electroporation-mediated gene expression on HEK-293 cells. We cultured cells on the surface of F-MEC, and then applied a voltage on F-MEC to generate a linearly varied electrical field in which all cells were exposed. By *in situ* observing all electroporated cells, we found some cells were dead because of the excessive electrical field strength (higher than 1.2×10^5 V/m), some cells remained alive but not GFP-expressed because the electrical field was insufficient (lower than 6×10^4 V/m), and the rest of cells were with both high viability and efficient GFP expression. Since the positions of all cells directly indicated the electrical field strength applied on cells, by determine the position of well-electroporated cells, we could easily obtain the corresponding optimal electrical field strength.

Supplementary Figure S2

Analyzing cell existence/survival/death.

48 hours after being treated with ECT (detailed protocols were listed in Experimental section), MCF-7 cells were washed with 1×PBS buffer three times and stained with 1 mL cell culture medium which contained 0.3 μL Hoechst 33342 (Sigma-Aldrich), 0.3 μL Calcein AM (Sigma-Aldrich) and 1 μL propidium iodide (PI, Sigma-Aldrich). After being incubated for 10 minutes, cells were fluorescently imaged by microscope (IX73, Olympus, Japan). Hoechst (A), Calcein AM (B) and Propidium Iodide (C) were used to indicate cell existence, survival and death, respectively. (D) is the bright field image. The conditions are: 150 μg/mL bleomycin, 8 square electrical pulses, 150 V voltage, 100 μs pulse duration, 1 s interval. In (A), (B) and (C), the electric field contours were marked by red rings, the Arabic numbers on curves represent the contour values (unit: 10^4 V/m).



The results demonstrated that in the scenario of our chip-based study, solo Hoechst staining provided us a boundary of cell existence, and this very boundary also clearly differentiated cell survival and death, as:

1) The distribution of living cells

Above figure simultaneously exhibits patterns of cell existences (blue), viable cells (green) and dead cells (red). In Hoechst staining (blue), an obvious boundary districting cell existence and detachment was located between 3×104 and 4×104 V/m contours. In the upper right area of the boundary, the overwhelming majority of attached cells are alive (green, Calcein AM staining). On the other side, scattered green spots existed in the lower left area of the boundary. However, many of these spots also exhibited PI staining (red), hinting the breakdown of cell membrane and the coming cell death. Overall, almost all living cells located in the upper right area of the boundary.

2) The distribution of dead cells

The overwhelming majority of scattered cell located in lower left area of the boundary were dead, while almost no dead cells in the upper right area of the boundary.

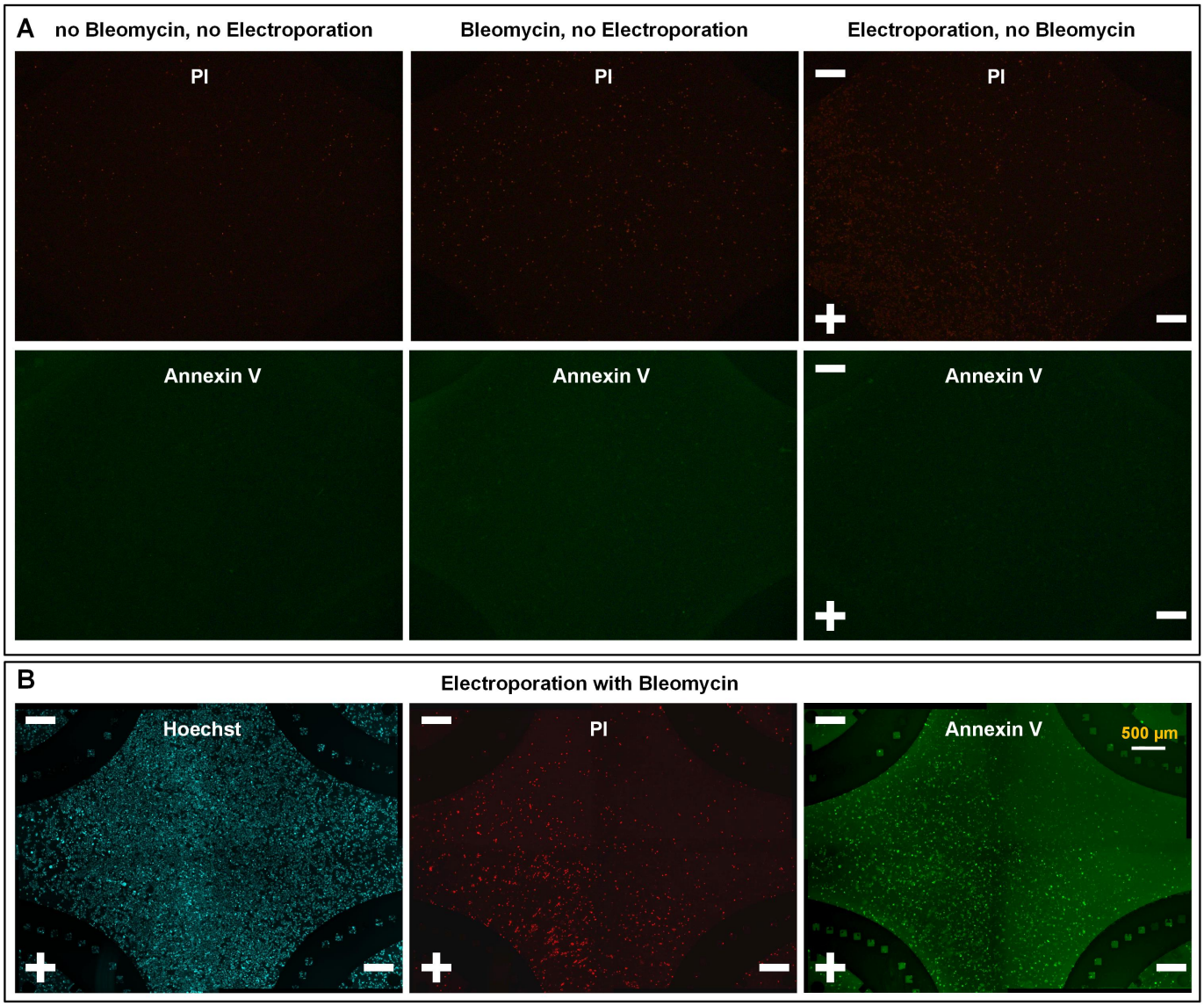
Overall, we believe that compared with staining both cell death and survival by two different dyes, which increases the complexities of experiment, imaging and image analysis, solely staining cell existences by only Hoechst is a better method which is easy, reliable and efficient.

Supplementary Figure S3

In situ monitoring the cell apoptosis on four-leaf micro-electrode chip (F-MEC).

(A) Controls: Neither propidum iodide nor Annexin V successfully stained cells which were separately treated with bleomycin (middle), electroporation (right) or neither of them (left)

(B) The Hoechst staining image (left), the PI staining image (middle) and the Annexin V staining image (right) of MCF-7 cells, 12 hours after electroporation-mediated electrochemotherapy



After being delivered into cell interior, bleomycin acts as an enzyme creating single and double strand DNA breaks (1). Cell death due to bleomycin happens in the following two ways:

1) If several millions of bleomycin molecules were delivered, tumor cells were killed within a few minutes to few hours, due to pseudoapoptosis, where bleomycin short-circuited the apoptosis pathway by creating the fragmentation of characteristic DNA (1-3).

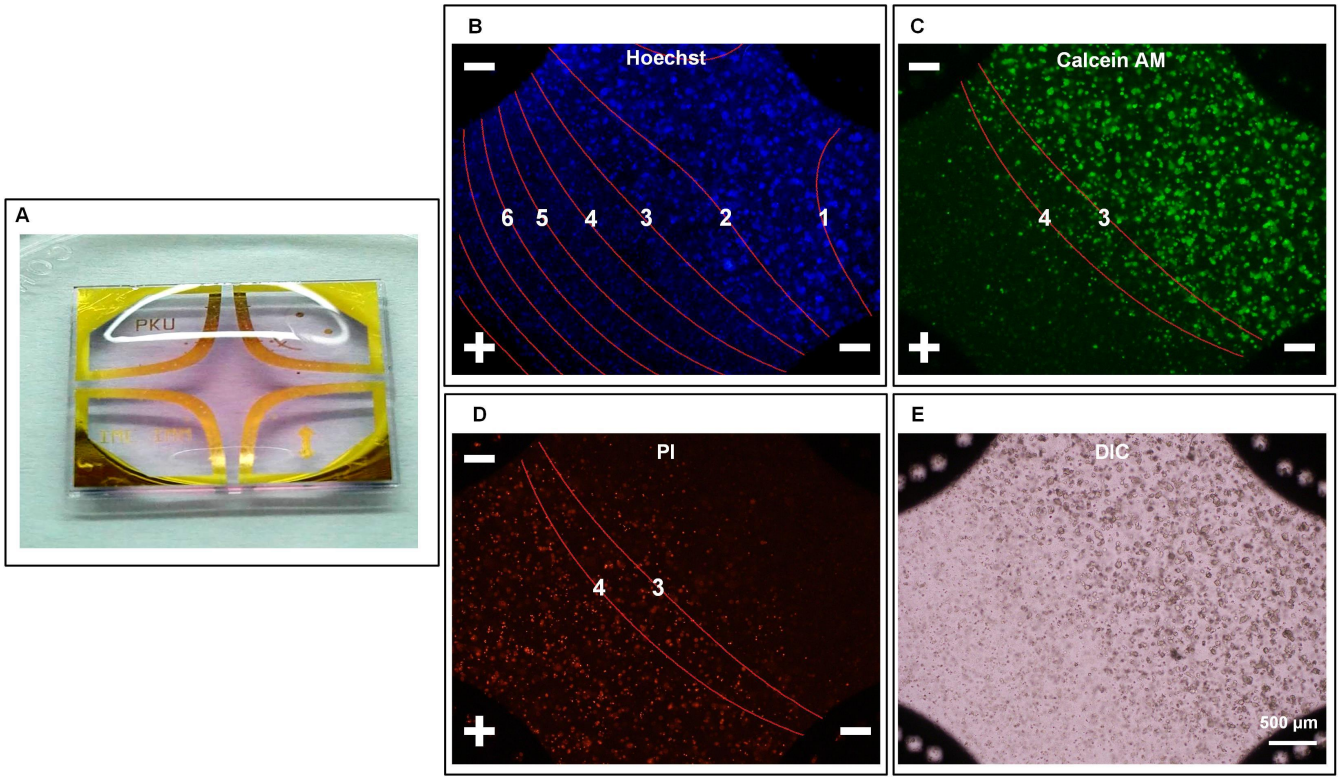
2) If only a few thousands of bleomycin molecules were introduced into tumor cells, the cells were arrested in the G2–M phase. The cells then died in a very slow process which lasted for about three doubling times (3).

For the rest of images in this study, cells were overserved 48 hours after electrochemotherapy, in this period, both rapid pseudoapoptosis and slow cell apoptosis were completed. Above images reveals the MCF-7 cell responses 12 hours after electroporation-mediated electrochemotherapy. According to the Hoechst staining image, there was no significant difference between dead and survival region since the lysis of tumor cells had not manifested at that time yet. Tumor cells which were killed within a few minutes were determined by PI staining. And cells in the intermediate stages of apoptosis, which meant in a slow dying process rather than being killed immediately by electrochemotherapy, were determined by Annexin V. The results revealed two different ways of tumor cell death due to bleomycin.

Supplementary Figure S4

Cell clusters on F-MEC

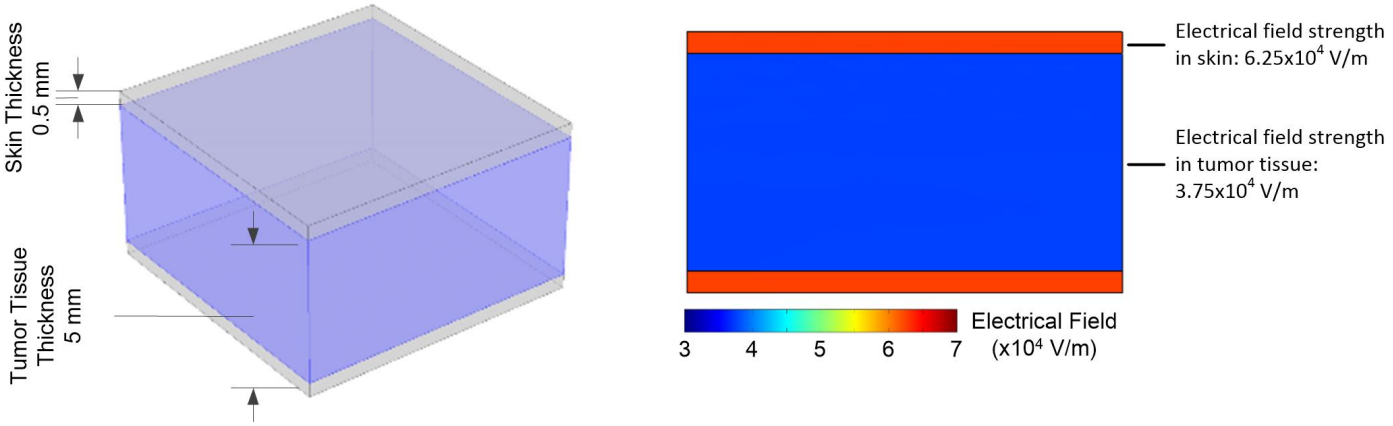
1.6×10^5 MCF-7 cells were cultured in 100 μL Matrigel to form a cell cluster on F-MEC (A). 48 hours after being treated with ECT, cells were fluorescently stained and imaged. Hoechst (B), Calcein AM (C) and Propidium Iodide (D) were used to indicate cell existence, survival and death, respectively. (E) is the bright field image. The conditions are: 150 μg/mL bleomycin, 8 square electrical pulses, 200 V voltage, 100 μs pulse duration, 1 s interval. In (B) (C) and (D), the electric field contours were marked by red rings, the Arabic numbers on curves represent the contour values (unit: 10^4 V/m).



Before constructing cell clusters on F-MEC, Matrigel matrix (Corning, USA) was thawed at 4°C overnight. MCF-7 cells were harvested and resuspended to a density of 3.2×10^6 cells/mL. For each F-MEC, 50 μL cell suspension (1.6×10^5 cells) and 50 μL Matrigel matrix were mixed and added on F-MEC immediately. The F-MEC was then incubated at 37°C for 30 minutes until Matrigel matrix gelled. Then, 1 mL DMEM was dropped on each F-MEC for overnight culture. 48 hours after being treated with ECT (detailed protocols were listed in Experimental section), cells were stained by Hoechst, Calcein AM and PI, before being imaged. Compared with cell monolayer, cell clusters exhibited a similar pattern of cell existence/survival/death. The boundary districting cell live and death was located between 3×10^4 and 4×10^4 V/m contours. These proof-of-concept results demonstrate that F-MEC is compatible with culturing cell spheroids/clusters which are helpful for mimicking complex tumor environment.

Supplementary Figure S5

Calculation of electrical field distribution

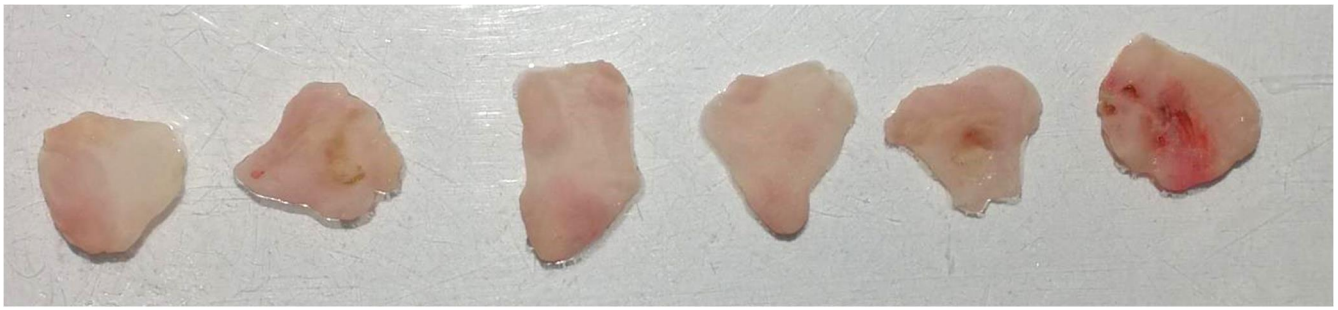


The *in vitro* studies revealed that the optimal electrical field range of ECT was between 2.7×10^4 and 3.5×10^4 V/m. Since the parallel plate electrodes were attached on the skin of tumor tissue while *in vivo* verifying the optimal electrical filed range, we calculated the electric field distribution in both skin and tumor tissue to exclude the influences of skin. As shown in above simulation (by Comsol Ver. 5.0), the conductivities of skin and tumor tissue were respectively set as 0.05 and 0.03 S/m, the thickness of skin and tumor tissue were respectively set as 0.5 and 5 mm. While applying 250 V on skin surface, the electric field strength on the tumor tissue was 3.75×10^4 V/m. Likewise, the relationships between voltages applied on the skin surface and the electric field strengths on tumor tissue were listed as the following table.

Voltage (V)	400	300	250	200	150	100	50
Electric Field Strength ($\times10^4$ V/m)	6	4.5	3.75	3	2.25	1.5	0.75

Supplementary Figure S6

Skin damages



24 hours after ECT, the mice were sacrificed and the electrically stimulated skins were harvested for imaging. Compared with lower voltage (200 V), 300 V brought slight burn marks which would recover in 48 hours. 400 V voltage resulted in severe damage which would not recover during the whole experimental period.

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