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nsPEF dramatically affects peritoneal carcinomatosis tumors in a syngeneic orthotopic graft murine model

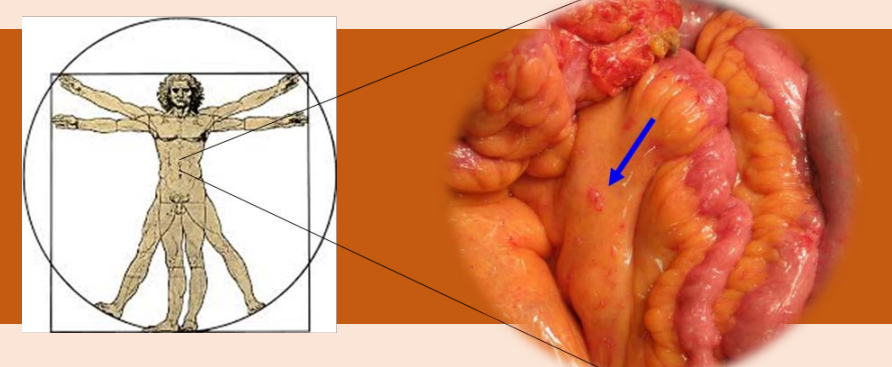
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Introduction



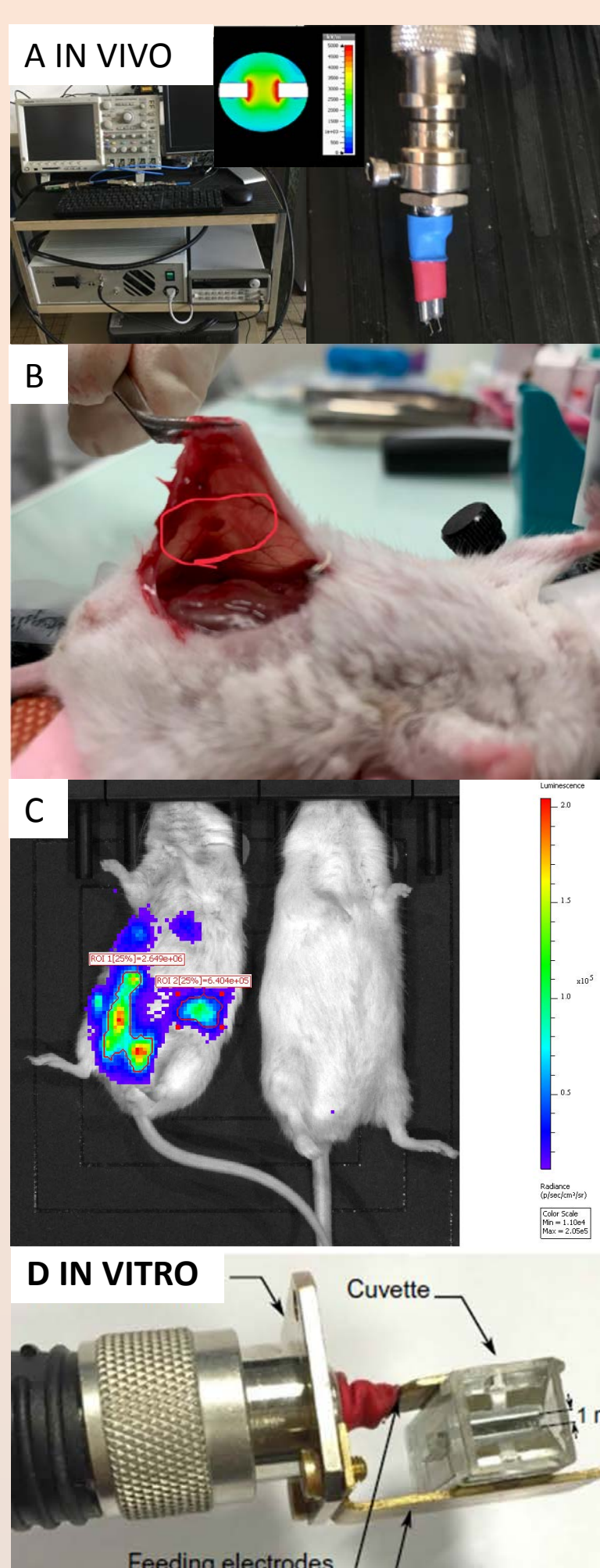
Peritoneal metastasis (PM) develops in 8.5–25% of patients with **colorectal cancer (CRC)** and is the second most frequent site for metastasis after the liver. It causes a high morbidity and mortality as the majority of patients presents an extensive PM (peritoneal carcinomatosis index (PCI) > 20).

Then, new multimodal therapeutic have emerged, as **high-intensity pulsed electric fields with nanosecond durations (3-300ns; nsPEFs)** for tumor ablation.

Animal models play an important role to study the development and progression of PM and to test innovative treatments. No study has compared the **different murine models** under the scope of medical practice, when it remains important to classify these animal models of limited or extensive PM from CRC. In fact, mice models of a limited PM (PCI < 20) is particularly interesting to test new treatments before the disease causes aggravate symptoms that are incompatible with animal ethics.

The aim of this study was to evaluate the implantation rate of **CT-26**, a type of murine colorectal cancerous cells, on the peritoneum site according to the injection site. Tumoral growth was then followed by bioluminescence, over 15 days post-grafting and nodules that have been exposed to 10 ns pulses were analyzed by histology. *In vitro* CT-26 cells were also analyzed by flow cytometry for their sensitivity to nsPEFs and chemotherapeutic drugs (Mitomycin, Oxypilatin): survival, calcium signaling and permeabilization. In parallel, peritoneal tumoral tissue were observed by multiphoton microscopy in order to evaluate the tumor microenvironment.

Methods



1. Cell culture - flow cytometry

CT-26 cells were grown at 37°C with 5% CO₂ in supplemented MEM and suspended at 1.2 million/ml in complete medium in a volume of 55 µl in a wideband electroporation cuvette containing complete medium (conductivity of 1.5 S.m⁻¹) and measured using a flow cytometer (Muse[®] Cell Analyzer, Millipore).

2. In vivo model

5-weeks old Balb-c mice (Charles Rivers, France) were grafted with firefly luciferase-expressing murine CT-26 colon carcinoma cells by intraperitoneal injection and followed the tumoral growth by bioluminescence over 15 days post-grafting. The injection volume was 40 µL with 30 000 cells. Mice were injected i.p. with 25 mg/kg of Xenolight D-Luciferin under isoflurane anesthesia and imaged with a CCD camera system (IVIS[®] Lumina, P. Elmer).

3. Exposure system

In vitro and *in vivo* nsPEF exposure setup are composed of an nsPEF generator, a digital phosphor oscilloscope (DPO), a high-voltage measurement device (tap-off), an electrode-based delivery system. The nsPEF generator (FPG 10-1NM-T, FID Technology, Germany) delivered 10ns pulses with amplitudes between 4.5kV and 10kV and with rise-times around 5ns. The numerical dosimetry of the nsPEF exposure setup was performed using numerical modeling and full-wave 3D simulations based on a discretization of Maxwell's equations in integral form.

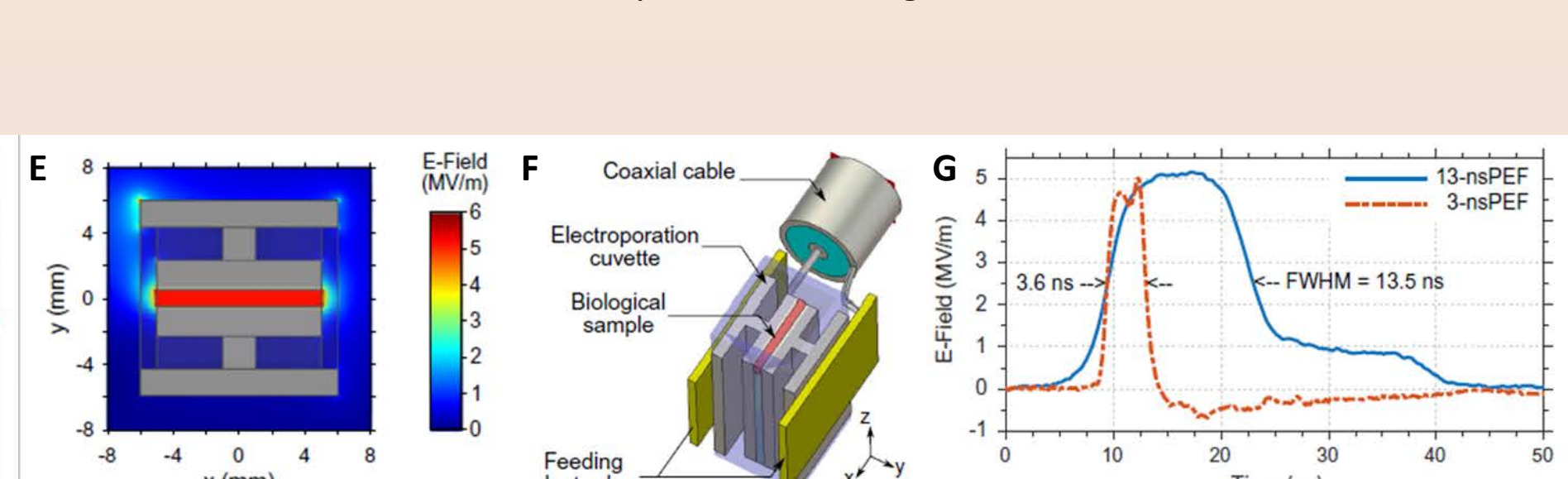


Fig 1. Experimental setup for *in vivo* (A-C) and *in vitro* exposure (D-G). *In vivo* electrodes has been designed (A) to expose tumoral nodules in the peritoneal site (B). Mouse tumor are tracked for growth and regression by bioluminescence (C). Electromagnetic field determination of the cuvette exposure system (D) by numerical modeling and full-wave 3D simulations based on a discretization of Maxwell's equations in integral form. The dosimetry of the *in vitro* delivery systems showed that the electric field in the central region between the electrodes had a good homogeneity, as based on 3D numerical simulations (E,F). The intensity of the electric field than can be delivered was around 50 kV/cm and the 10ns pulse had a 3.6ns rise time (G).

4. Multiphoton fluorescence and Histology

Tissues around the site of tumors (visceral peritoneum and parietal peritoneum) were fixed by 4% paraformaldehyde and embedded in paraffin. Sections (4 µm) were cut, stained with hematoxylin-eosin-safran (HES) and for CD3, CD4, CD8, Ki67.

A customized Olympus multiphoton microscope BX61WI/FV1200MPE with a 25X immersion objective (1.05NA, 2.0mm WD) was coupled with a tunable femtosecond Ti:Sapphire pulsed laser (Chameleon Ultra II, Coherent). Image stacks were acquired under 810nm excitation for second harmonic generation (SHG, collagen) and auto-fluorescence (elastin) wavelength with FluoView FV1200 software (v4.1.1.5, Olympus).

Results

Fig 2. All animals survived after the operative procedures (IV, SC, IP or A) and presented diverse and limited in size tumor nodules in the peritoneal cavity (A and B, example of peritoneum scratching from group A at day 15 postgrafting), HES staining of a colorectal localized nodule reveals an enrichment of CT26+ cells (B).

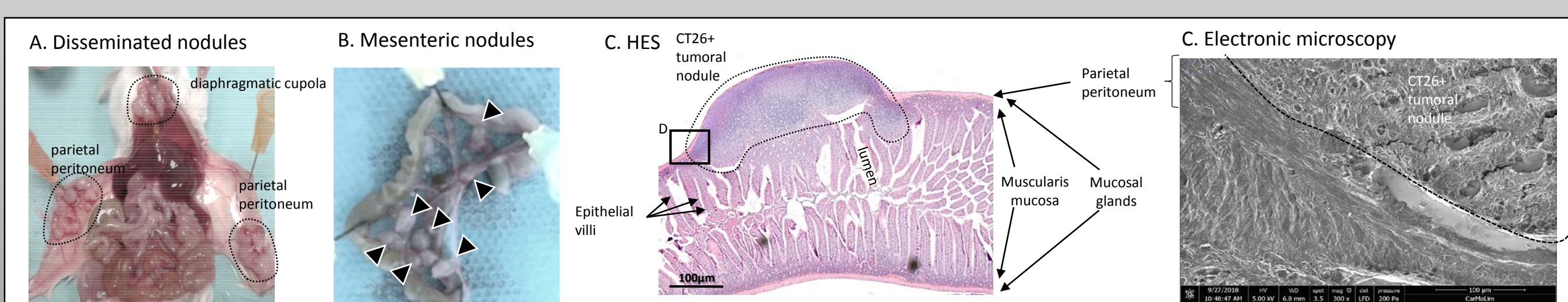


Fig 3. *In vivo* quantification of tumor growth by bioluminescence tracking of CT26 cells over 15 days reveals that the laparotomy surgery (group A : scratching of the peritoneum and intraperitoneal CT26 injection) presents a sufficient and adequate growth with a relevant Peritoneal carcinosis index (PCI)

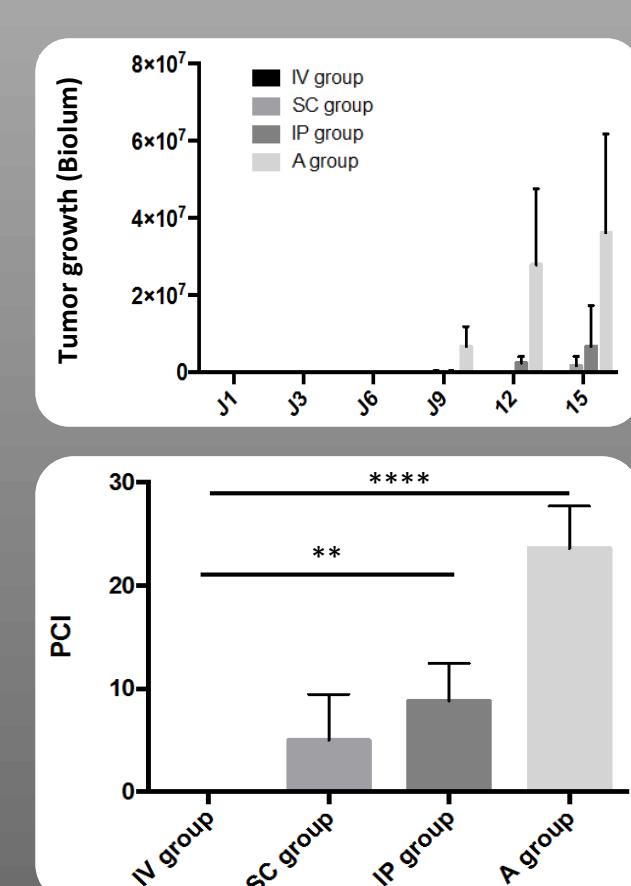


Fig 4. CRC nodules reveal high number of cells including Tumor infiltrating Lymphocytes (TILs) among a complex tumoral micro-environment (high vascularization, poor fibrosis, dense tissue). Scale bar in A applied to C,E,F,G and in B applied to D

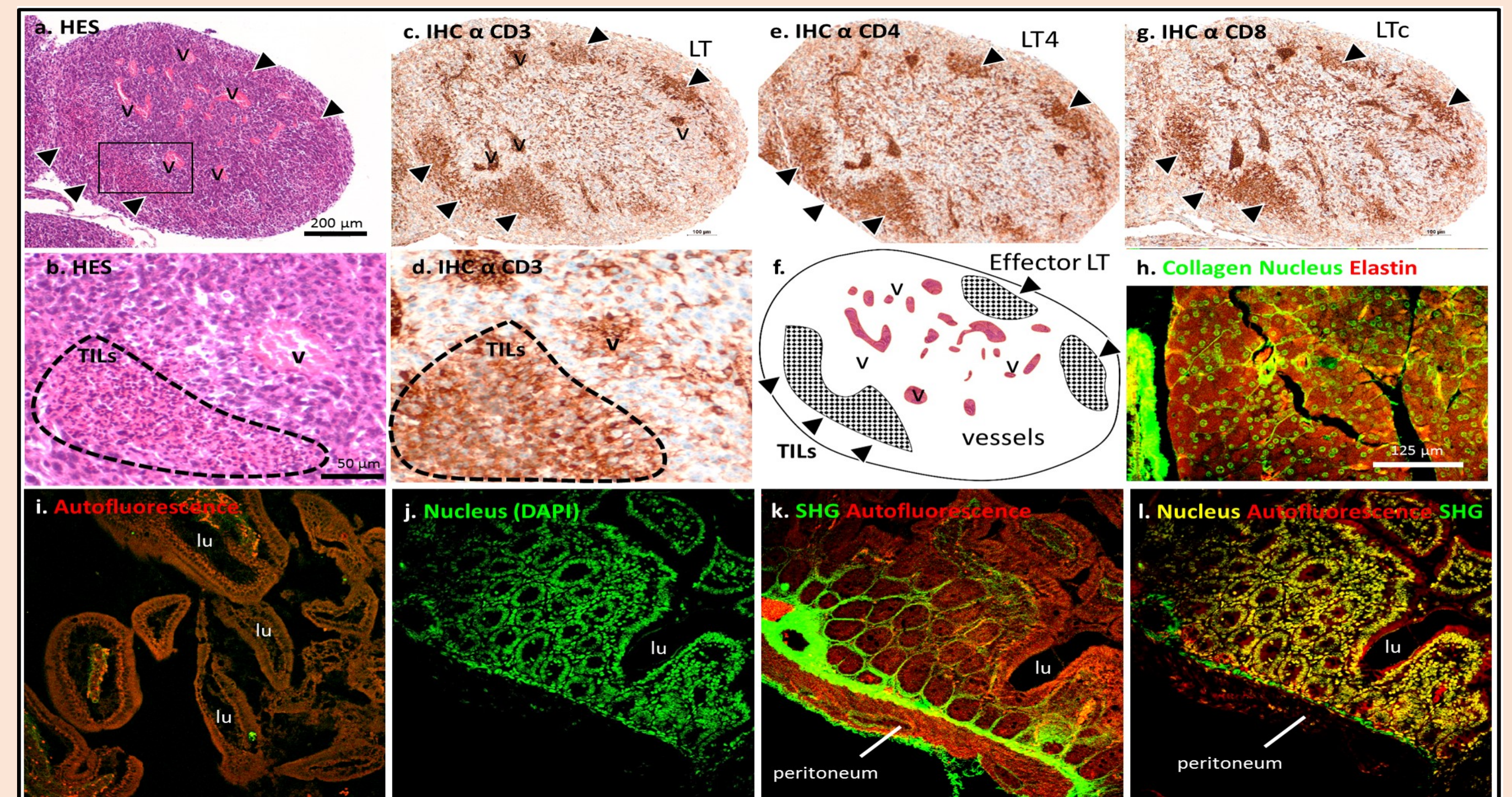


Fig 5. HES staining of microscopic tissue sections from nodules with no treatment, 10 pulses or 5000 pulses of 10 ns duration (biopsies at 3 days) reveals a strong histologic response when 5000 pulses are applied.

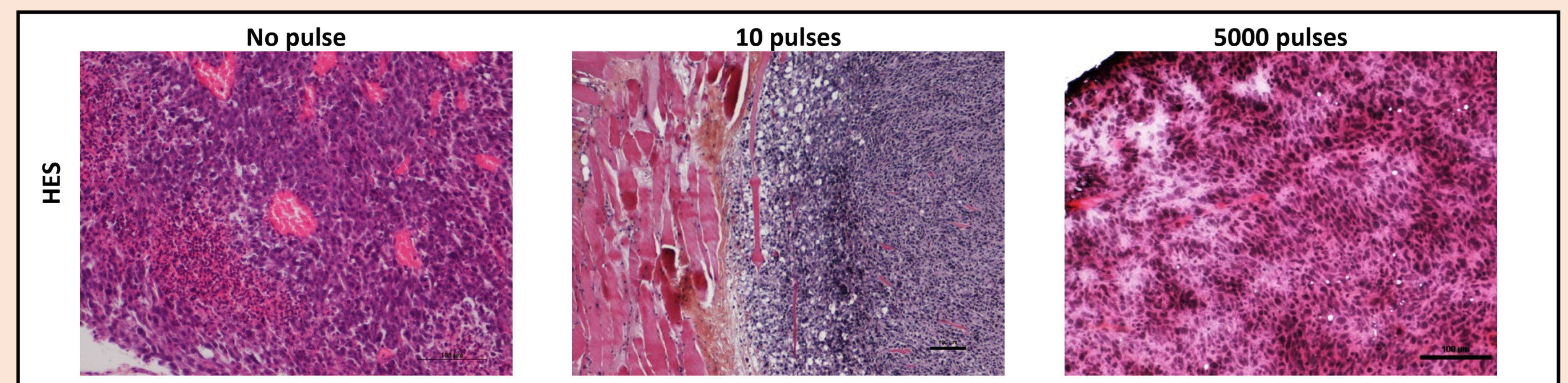


Fig 6. *In vitro* exposition of CT-26 cells to 10ns pulses induces immediate cell permeabilization and Ca²⁺ intracellular concentration under live confocal microscopy. (*** p<0.01). Experimental data are expressed as mean values ± SEM

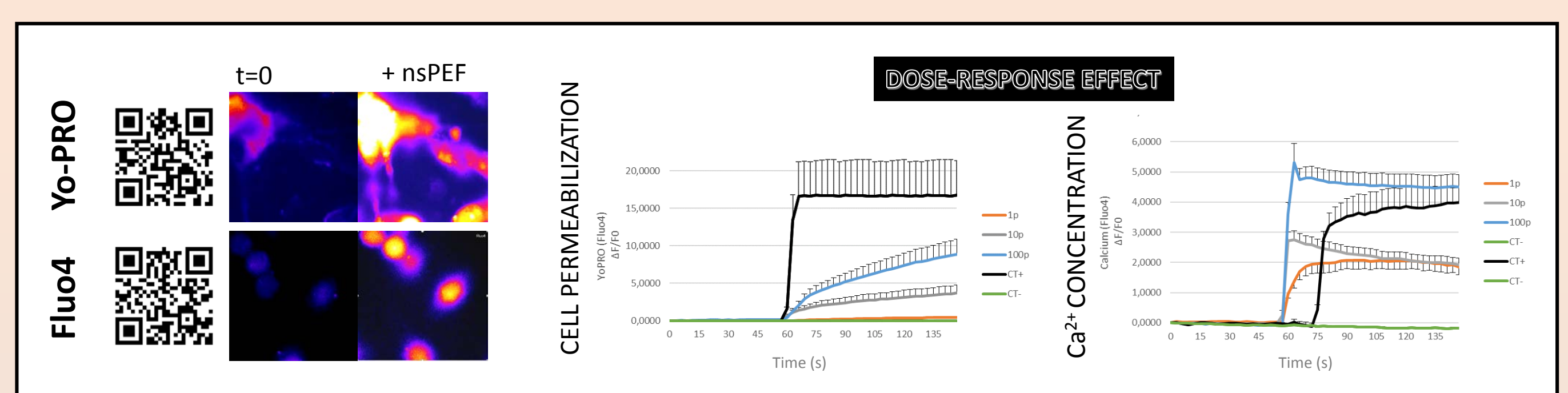
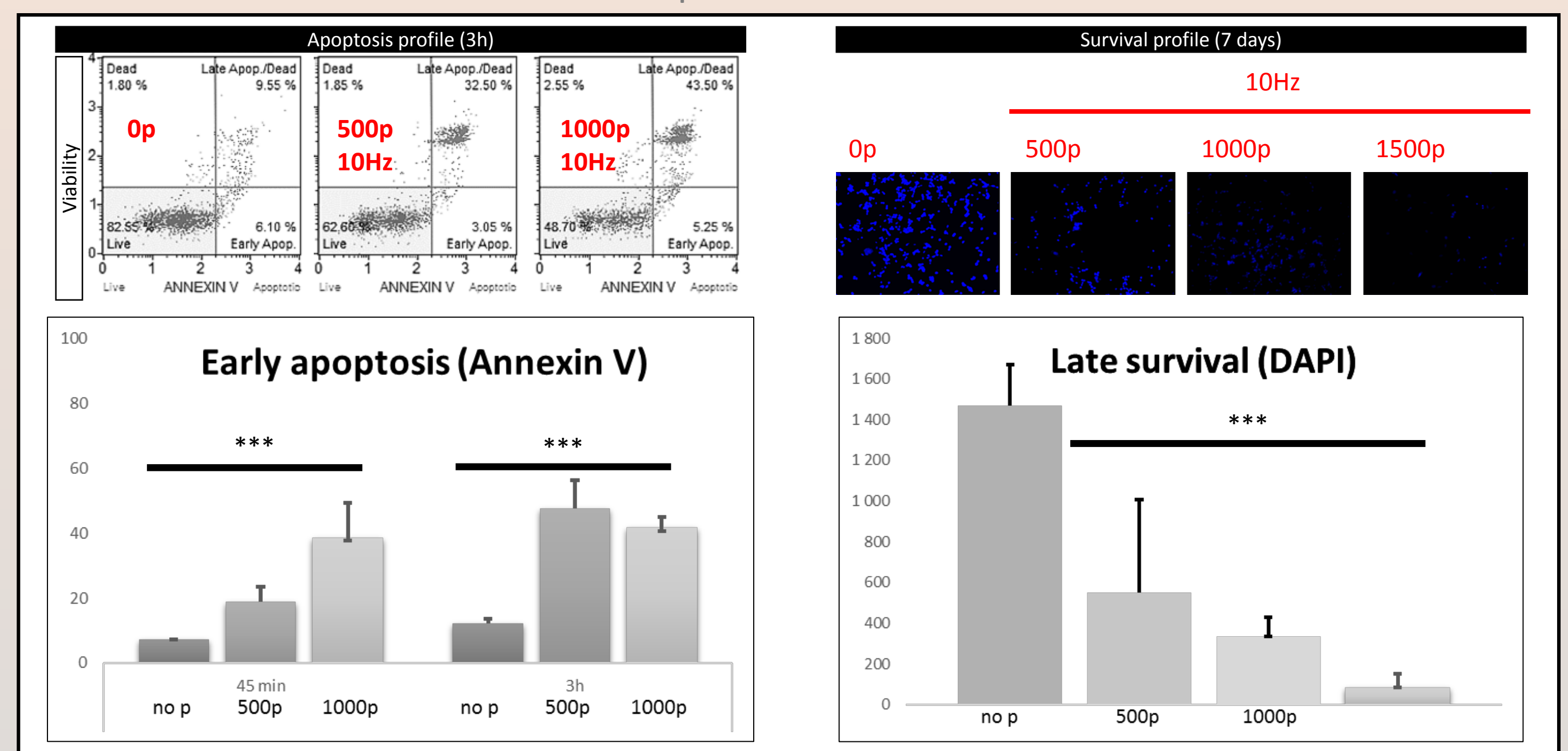
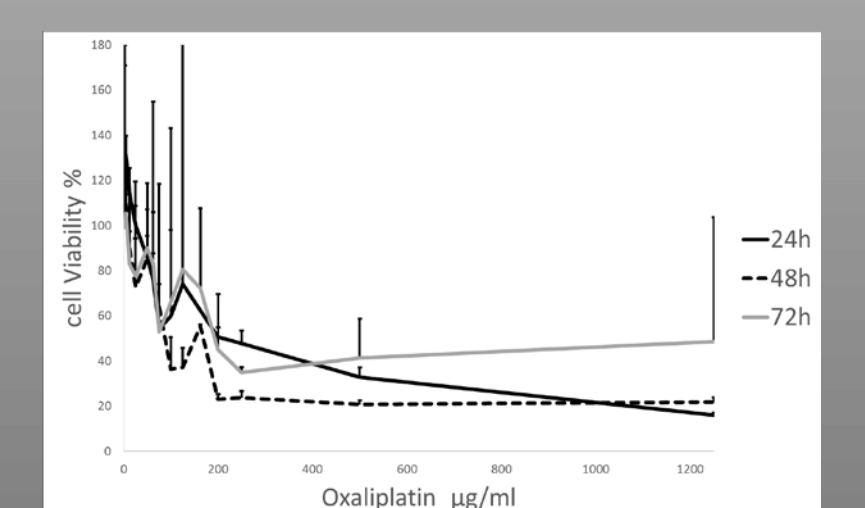
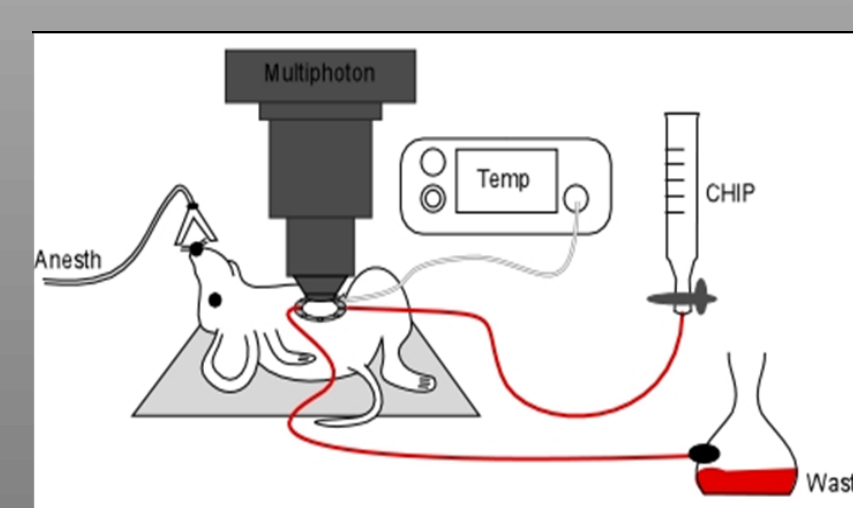


Fig 7. *In vitro* exposition of CT-26 cells to 10ns pulses induces cell death by apoptosis (A) and poor survival after 7 days post exposure (B). Three microscopic fields were arbitrarily chosen and the average DAPI+ cell number was determined (*** p<0.01). Experimental data are expressed as mean values ± SEM



Conclusions - Future studies

- We characterized *in vivo* the growth rate and dissemination of luc CT-26 tumors regarding injections sites to obtain a limited peritoneal carcinomatosis using peritoneal carcinomatosis index in mice (IPC < 10).
- Anatomic and histologic analysis corroborated the existence of various tumoral nodules with infiltrating immune T cells, and we described by multiphoton microscopy tumoral fibrosis (SHG) infiltrated tumoral cells, in comparison with non-pathologic peritoneum.
- Anatomic and histologic analysis by multiphoton microscopy showed atypical fibrosis (SHG) and infiltrated tumoral cells, in comparison with non-pathologic peritoneum.
- The dosimetry of the *in vivo* and *in vitro* delivery systems showed that the electric field in the central region between the electrodes had a **good homogeneity** (*in vitro* electroporation cuvette 60 kV/cm and *in vivo* bilelectrode 30-50 kV/cm).
- CT-26 cells are sensitive to 10 ns nsPEF *in vitro* and *in vivo*. Date are consistent with our previous published with a strong dose-dependent effect of nsPEF on cell viability, cell death (DAPI, Annexin V), calcium signaling (Fluo-4) and permeabilization (Yo-Pro). *In vivo* nsPEF exposure of nodules shows also dramatic cells death and proliferative inhibition.



- Then, *in vivo* electrodes have to be designed in order to facilitate the nsPEF exposure, for example using flexible electronic materials.
- nsPEF exposure can be studied in combination with HIPEC therapy to evaluate the anti-tumoral synergic effects and the immune response, with the addition of chemotherapeutic drugs like Mitomycin and Oxaliplatin.

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