



HAL
open science

Analysis of In Vitro Cell Viability Approaches to Provide Early Efficacy Prediction of Electrochemotherapy Treatments

Anne Calvel, Alexia de Caro, Olivia Peytral-Rieu, Camille Gironde, Christophe Furger, David Dubuc, Katia Grenier, Marie-Pierre Rols

► **To cite this version:**

Anne Calvel, Alexia de Caro, Olivia Peytral-Rieu, Camille Gironde, Christophe Furger, et al.. Analysis of In Vitro Cell Viability Approaches to Provide Early Efficacy Prediction of Electrochemotherapy Treatments. *IEEE Journal of Electromagnetics, RF and Microwaves in Medicine and Biology*, inPress, pp.1-9. 10.1109/JERM.2024.3379012 . hal-04650849v2

HAL Id: hal-04650849

<https://hal.science/hal-04650849v2>

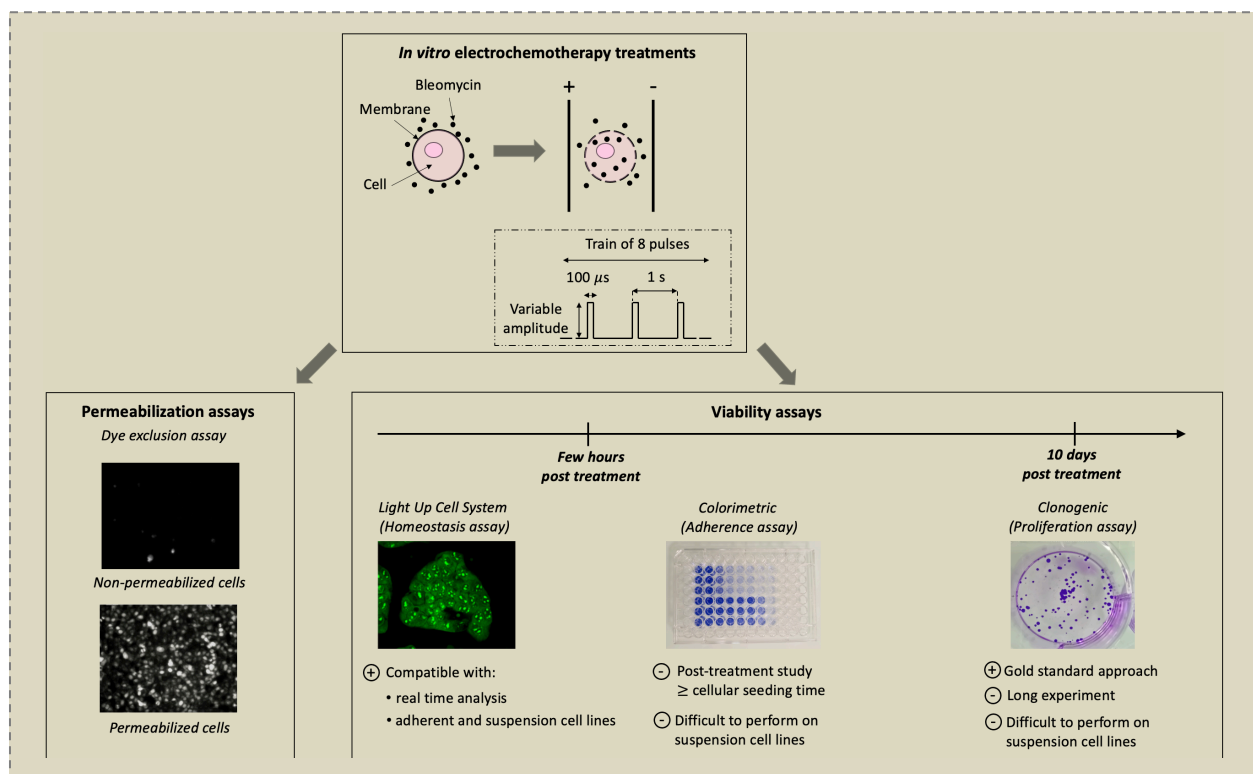
Submitted on 17 Jul 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Analysis of *In Vitro* Cell Viability Approaches to Provide Early Efficacy Prediction of Electrochemotherapy Treatments

Anne Calvel, *Student Member, IEEE*, Alexia de Caro, Olivia Peytral-Rieu, *Member, IEEE*, Camille Gironde, Christophe Furger, David Dubuc, *Member, IEEE*, Katia Grenier, *Member, IEEE*, Marie-Pierre Rols



Evaluation of the effectiveness of electrochemotherapy treatments by performing cells' permeabilization and viability assays. Comparison of the approaches toward the early prediction of cells response.

Take-Home Messages

- Cancer cell permeabilization and viability are evaluated after electrochemotherapy treatments, the latter aiming at enhancing the uptake of cytotoxic molecules into the cells.
- Different viability assays, including an innovative one, are applied and compared in terms of precocity of the cellular response.
- Electrochemotherapy treatment reveals similar tendency after evaluation of viability with the different approaches performed 2, 24, 48 hours or 10 days post-treatment.
- Whereas the clonogenic assay requests an experimental duration of ten days, the colorimetric assay and the Light Up Cell System technique are both convenient for studying cell viability in a couple of hours, while the second and recent approach also maintains cells alive and is compatible with non-adherent cell lines.

Analysis of *In Vitro* Cell Viability Approaches to Provide Early Efficacy Prediction of Electrochemotherapy Treatments

Anne Calvel, *Student Member, IEEE*, Alexia de Caro, Olivia Peytral-Rieu, *Member, IEEE*, Camille Gironde, Christophe Furger, David Dubuc, *Member, IEEE*, Katia Grenier, *Member, IEEE*, Marie-Pierre Rols

Abstract Among all the cancer treatments developed, electrochemotherapy has shown great promise in recent decades. This approach combines the local delivery of electric pulses with the administration of poorly-permeant cytotoxic agents. We aim to investigate the effects of electrochemotherapy treatments and predict their impacts on cell viability, especially at the earliest stage. We explore different approaches to evaluate cell viability, involving time periods from several days to few hours post treatment. Besides commonly-used approaches such as clonogenic and colorimetric assays, we investigate an innovative viability assay, the Light Up Cell System assay, and compare these methods. Even if the conducted viability assays demonstrate the interest of using electric fields to enhance the cytotoxic agent penetration into cells and potentiate their effects, our study demonstrates that the colorimetric and Light Up Cell System assays can predict the cell response to electrochemotherapy treatment as early as 2 hours post-treatment, whereas the gold standard for assessing cell viability, the clonogenic assay, necessitates 10 days of experimentation. Moreover, the Light Up Cell System assay seems particularly interesting, as it provides similar results to the well-established colorimetric technique while offering the advantages of maintaining cells alive and being suitable for the study of non-adherent cell lines.

Keywords — Electrochemotherapy, electric fields, cell permeabilization, LUCS, colorimetric, clonogenic assays.

I. INTRODUCTION

ELECTROCHEMOTHERAPY (ECT) is a promising approach to enhance the cytotoxic effect of exogenous molecules. Based on ElectroPoration (EP), ECT consists in applying, locally at the tumor sites, pulsed electric fields to transiently permeabilize cell membranes in order to facilitate the uptake into cells of non-permeant or poorly-permeant molecules, such as bleomycin [1] and cisplatin [2], which are systemically or intratumorally injected [3]. ECT is currently used in more than 160 European clinical centers [4] [5]. In the treatment of solid tumors, ECT shows encouraging results, with an objective response rate of about 80% after a single treatment [3] [6]. The standard treatment is well defined in the European Standard

Operating Procedure on Electrochemotherapy (ESOPE). Nevertheless, fundamental and preclinical research devoted to electrochemotherapy is still ongoing, with new drugs being tested and new sets of electric pulses being explored for effective tissue EP [4] [7] [8].

Unfortunately, in 20% of cases, the ECT treatments are not effective enough to lead to remission, underscoring the challenges of predicting treatment outcomes [9] and the interests of tackling these challenges [10] [11]. Being able to predict as early as possible the response to treatments in order to assess their efficacy could then help to increase the effectiveness of ECT treatments and make ECT more widespread. Performing *in vitro* and *in vivo* studies for anticipating the response to treatments would make it possible to adjust the electrical parameters and chemical concentrations to be delivered, aiming toward personalized medicine [12] [13] [2] [14].

We present *in vitro* studies as they are adapted to rapidly obtain the cellular response to treatments [15], aiming ultimately to predict the effectiveness of treatments on cells directly collected from patients. Such 2D studies may be expanded to 3D models [16] that are relevant models for better mimicking *in vivo* reality, as they enable the simulation of the heterogeneity of the tumor microenvironment as well as cellular interactions [17] [18].

The intensity of the electric field is a crucial parameter for triggering EP, as demonstrated by Schwan's equation [19]. Beyond a certain threshold, called the

This article is an expanded paper presented at the IEEE IMBioC 2023, Leuven, Belgium. This work was supported by the Université Fédérale Toulouse Midi-Pyrénées and Région Occitanie.

A. Calvel, O. Peytral-Rieu, D. Dubuc and K. Grenier are with the National Scientific Research Center, Laboratory of Analysis and Architecture of Systems, 31400 Toulouse, France (phone: +33 5 61 33 62 00; e-mails: acalvel@laas.fr; opeytral@laas.fr; dubuc@laas.fr; grenier@laas.fr). O. Peytral-Rieu, D. Dubuc are also with the University of Toulouse, France.

C. Gironde, C. Furger are with the Anti Oxidant Power company, Toulouse, France (cgironde@laas.fr; cfurger@laas.fr).

A. Calvel, A. de Caro and M-P. Rols are with the Institute of Pharmacology and Structural Biology, 31077, Toulouse, France (phone: +33 5 61 17 59 00; e-mails: anne.calvel@ipbs.fr; alexia.de-caro@ipbs.fr; marie-pierre.rols@ipbs.fr).

permeabilization threshold, EP can lead to reversible [20] or irreversible [21] membrane permeabilization. After a reversible EP, the membranes pores reseal, leading to cell survival, in contrast to irreversible EP in which cell membranes remain permanently damaged, resulting in cell death (see Fig.1). Both reversible and irreversible EP are used in clinical practice: ECT [3] and gene therapy [22] are based on the reversible EP, while tumor ablation uses the irreversible EP [23] [24] [25].

Conducting *in vitro* assays is essential to understand the occurrence or non-occurrence of the resealing of plasma membranes [26].

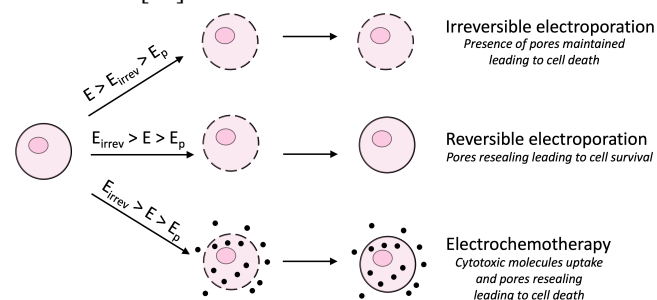


Fig 1. Schematic diagram illustrating reversible electroporation, irreversible electroporation and electrochemotherapy.

Quantifying the state of permeabilization of cells regarding the different electric field strengths applied helps identifying the most appropriate field intensities to achieve the intended outcome while minimizing side effects and preserving the integrity of healthy cells. Such a study can be carried out using permeabilization assays, which consist in monitoring the uptake of non-permeant exogenous molecules into cells [26].

In addition, quantifying cell death that is induced by the treatments helps to assess their effectiveness. There are a number of cell viability detection methods which range from the simplest dye exclusion assay to highly complex analysis [26] [27]. The speed, cost, efficiency, reliability and complexity of equipment all play a role in determining the choice of the assay to use.

Cell viability measures the proportion of live cells within a population. Healthy cells actively proliferate whereas growth-arrested, senescent and dead or dying cells do not. Cell proliferation is a relevant indicator of cell viability; it reflects to an increase in cell number due to cell division. Cell proliferation assays are therefore a useful tool for assessing cell viability by providing a readout on the number of actively dividing cells.

Among the existing assays, clonogenic assays are the gold standard to study cell viability. Such assays evaluate the ability of individual cells to proliferate and form colonies [28]. However, clonogenic assays require a long time to set up (several days) as it is necessary to wait for the cells to divide over several generations. It is therefore important to be able to find complementary assays, or even alternatives, that can predict cell death in a shorter time.

Colorimetric assays are a well-established technique that reflect the state of cell proliferation by measuring the

proportion of cells that have adhered to the culture plate [29]. These assays are based on the use of dyes, such as crystal violet (CV) dye, that enter into the cells and bind to DNA and proteins. The intensity of CV staining directly depends on the number of cells. However, when the treatments are carried out on freshly trypsinized cells, it is then necessary to wait until the healthy cells have adhered once again to the culture plate (which takes a few hours). Moreover, colorimetric assays can hardly be applied to non-adherent cell lines.

In addition to these traditional viability study techniques, we were interested in an innovant and attractive approach, Light Up Cell System (LUCS) method. It was developed as a homeostasis assay for cytotoxicity application [30] [31]. LUCS assay is based on the concomitant presence inside living cells of a biosensor, Thiazole Orange (TO), and an illumination resulting in a fluorescent signal. When TO is added in the culture medium, it enters the cells and intercalates with nucleic acids but is quickly removed from viable cells by efflux transport proteins, resulting in a low fluorescence level. Application of light (470 nm, 240 mJ/cm²) triggers TO photoactivation that generates Reactive Oxygen Species (ROS). The presence of ROS alters efflux transport proteins or other cell functions, leading in a massive entry of TO into cells and an increase of fluorescence emission. However, for altered cells, efflux transport proteins of TO enter the cell even before illumination. Thus, by comparing the fluorescence ratios of TO before and after illumination, cell homeostasis level can be deduced. LUCS has been developed to allow independent analysis of cell number, using a ratio mode. LUCS offers the advantage of being able to be used directly after treatment, for all types of cell line. The cells remain alive throughout the study, enabling kinetic monitoring.

In the current study, we aim at promptly predicting the cellular response to EP and ECT treatments. We therefore propose to compare different cell viability techniques at various time periods after treatment: (i) LUCS assays and (ii) colorimetric assays, both performed a few hours after treatment (2, 24 or 48 hours later) and (iii) clonogenic assays, performed 10 days after treatment. They are applied to investigate the impact of electric field intensities on HCT-116 cells, human colorectal carcinoma cells. Cell permeabilization and cell viability, when EP treatments are performed, is studied and extended to investigate the synergistic effect of EP with a drug commonly used in clinical practice, bleomycin. The latter induces free radicals, resulting in DNA double strand breaks and cell death [32]. The effect of bleomycin results in a cell growth inhibition after one or two cell generations.

II. MATERIAL AND METHODS

A. Solutions and reagents

Gibco DMEM (high glucose, GlutaMAX supplement and pyruvate), fetal bovine serum (FBS) (Hyclone), pen-strep solution (100X) (Gibco), 0.05% Trypsin-EDTA (Hyclone),

Gibco DPBS without Calcium and Magnesium (1X), propidium iodide are purchased from Thermo Fisher Scientific. DPBS with Calcium and Magnesium (1X) is purchased from Eurobio Scientific. HCT-116 cell line is purchased from the American Type Cell Collection (ATCC® CCL-247™). CV, propidium iodide (PI) and the electropulsation buffer components (10 mM K_2HPO_4/KH_2PO_4 , 1 mM $MgCl_2$, 250 mM of sucrose) are purchased from Sigma Aldrich. Bleomycin is purchased from MedChemExpress. Acetic acid is purchased from Carlo Erba Reagents. LUCS kit assay (NeoStress) is purchased from Clinisciences.

B. Cell culture

HCT-116 (passages 10-30) cells are cultured at 37°C under 5% CO_2 in DMEM medium supplemented with 20% of FBS and 1% pen-strep solution. Cells are subcultured twice a week at a final density of 15 000 cells/cm².

C. Electric pulses delivery

All electric pulses are delivered in an electropulsation buffer with a low conductivity (0.17 S/m) to limit Joule effect. The electric pulses are applied through flat and parallel stainless-steel electrodes, with an inter-electrode distance of 4 mm. For each condition, including control ones, 100 μ L of cells in suspension is placed between the electrodes, with concentrations of 3.10^6 cells/mL for colorimetric, LUCS and permeabilization assays, and concentrations of 150,000 cells/mL for clonogenic assay.

For both permeabilization and viability assays, the applied electric pulses consist of a train of 8 pulses of 100 μ s width at a frequency of 1 Hz. These parameters are commonly used in clinical practice [5]. Intensities ranging from 0 to 2.5 kV/cm are studied. The electric pulses are delivered by an electropulsator (Jouan GHT 1287) and their shapes are checked with an oscilloscope (Tektronix TDS 2012C), as shown in Fig. 2.

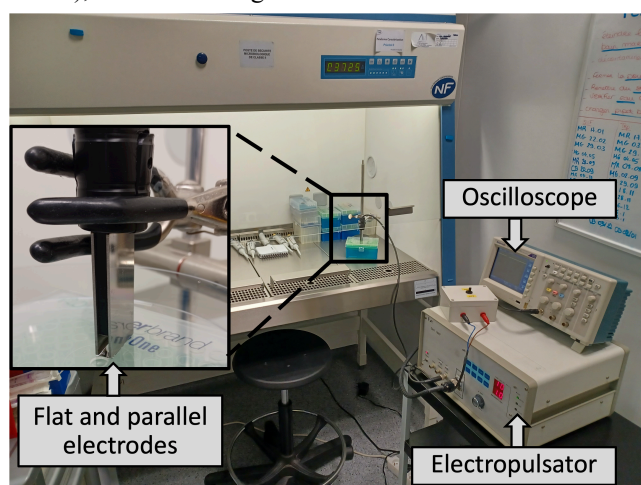


Fig 2. Set up for EP and ECT treatments on HCT-116 cells in suspension. Electric pulses are delivered through flat and parallel electrodes by an electropulsator. The shape of the pulses is checked with an oscilloscope.

D. Biological assays for the evaluation of cells permeabilization and viability

Permeabilization assay

PI is a non-permeant molecule used as a biomarker to assess cell permeabilization. Once cell membrane is damaged, PI binds to double stranded DNA and its quantum yield is enhanced 20-30-fold.

To assess cell permeabilization, PI is added in the cell suspension at a final concentration of 50 μ M prior to the application of electric pulses. Directly after the EP treatment, the entire cell suspension is collected and kept in ice until performing flow cytometry (BD LSRFortessa X-20) analysis over 20,000 cells. The percentage of permeabilized cells as well as the fluorescence intensity are determined. The obtained results are presented in section III. A. (see Fig. 3).

Short term viability assays: LUCS and colorimetric assays

In order to determine a suitable bleomycin concentration for the ECT treatments, we performed a dose-response of bleomycin on cells with LUCS assays. Bleomycin at concentrations ranging from 0 nM to 0.5 mM is obtained with a serial dilution factor of 2 or 10. Bleomycin at the desired concentration was added in 100 μ L cell suspensions. The cells were then seeded in 96-well plates at a final density of 15,000 cells/well in 100 μ L of culture medium. In order to perform LUCS assays, TO is added in each well at a final concentration of 0.8 μ M. Fluorescence level is measured during 30 mins using Varioskan Flash plate reader (from Thermo-Fisher) set up at 505/535 nm (excitation/emission wavelength). All the plates are then illuminated 3 times with a LED (excitation at 465 nm, energy density: 240 mJ/cm², frequency: 10 kHz, time: 10 s), each illumination being followed by a reading. Once these studies had been carried out, we selected a bleomycin concentration of 250 nM for the ECT treatments (see section III. B). Few minutes after the application of the electric pulses, the cell suspension is collected and is placed in a well of a 96-well plate after performing an intermediate dilution to achieve a final concentration of 15,000 cells/well in 100 μ L of culture medium. Cells are then incubated at 37°C under 5% CO_2 . For short-term viability assays, we aim at studying durations corresponding to 1 or even 2 cell generations times, as well as shorter times to get closer to real time analyses. Therefore, cells are removed from the incubator either 2, 24 or 48 hours after the received treatment. LUCS assays are carried out and followed by colorimetric assays on the same culture plate. To perform colorimetric assays, the culture medium is removed and a rinse with DPBS with calcium and magnesium is performed. CV (0.1% in aqueous solution) is added, a 15-minute agitation is carried out and rinses with DPBS containing calcium and magnesium ions are done. Acetic acid (10% in H_2O) is then added, leading to cell lysis. A reading of the optical density is finally made with a spectrometer (Varioskan Flash) at a 595 nm wavelength.

Long term viability assays: clonogenic assays

ECT treatments consist in adding bleomycin in the cell suspension at a final concentration of 250 nM. Electric pulses with an intensity ranging from 0 kV/cm to 2.5 kV/cm are then delivered. Few minutes after the treatment, the entire cell suspension is collected and an intermediate dilution in culture medium is performed to reach a concentration of 2,500 cells/mL, in a final volume of 4 mL. A successive dilution in culture medium is performed to reach a final concentration of 250 cells/mL, and cells are seeded in 6-well plates with a final density of 500 cells/well, in a final volume of 2 mL. Cells are incubated at 37°C under 5% CO₂. For one of the conducted experiments, a change of culture medium was carried out 24 hours post treatment, in order to mimic the physiological elimination of the cytotoxic drugs injected in the patients [33]. After 10 days, culture medium is removed and a rinse with DPBS with calcium and magnesium is performed. CV (0.1% in aqueous solution) is added, a 15-minute agitation is carried out and rinses with DPBS with calcium and magnesium are done. A count of the number of clones is finally realized.

III. RESULTS

A. Permeabilization assays

Permeabilization is quantified by the uptake of PI into the cells. The uptake of the fluorescent dye is indeed the signature of the permeabilization of the plasma cell membrane. We submitted the cells to increasing values of electric field strengths and determined the permeabilization (i.e. the percentage of cells that become fluorescent) and its efficiency (i.e. the amount of fluorescent dye incorporated into the permeabilized cells). The results are presented in Fig.3.

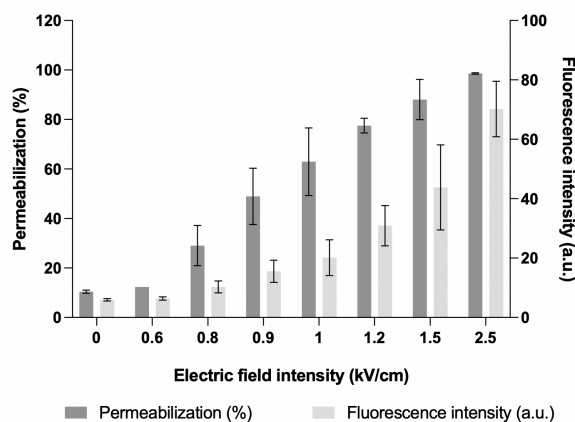


Fig 3. Effect of the electric field intensity (0-2.5 kV/cm) on the permeabilization of HCT-116 cells. Percentage of permeabilization (dark gray) and fluorescence intensity (light gray) obtained by evaluating the uptake of PI (50 μ M) into cells by flow cytometry. For all conditions, two batches are studied. This study is representative of two independent experiments.

For electric field intensities below 0.8 kV/cm, the obtained permeabilized cell population rate (<11%), correspond to naturally damaged or dead cells. Cell permeabilization only occurs for higher electric field

strengths, and increasing the applied electric field intensity leads to an increase in the number of permeabilized cells (and in the fluorescence intensity), which is in line with Schwan's equation and in agreement with the literature [34]. A significant increase in the number of permeabilized cells is obtained for an intensity of 0.9 kV/cm, where half of the cell population has been permeabilized. Intensities higher than this value should be applied in order to achieve effective ECT treatments. Electric field strengths of 2.5 kV/cm allow to permeabilize the entire cell population (98.6%).

Fluorescence intensity reflects the influx of PI into cells. A good correlation is obtained between the percentage of permeabilization and the fluorescence intensity, as the number of molecules entering the cells increases with the intensity of the electric field. The obtained results can be explained by the fact that the electric field strengths control the permeabilized region of the cell surface [20].

In order to determine whether the applied electric fields lead to reversible or irreversible EP, viability studies must be carried out (see sections III.C, III.D and III.E).

B. Dose-response of bleomycin

The selection of the bleomycin concentration used for ECT treatments is based on a dose-response for concentrations ranging from 0 to 0.5 mM (see Fig.4).

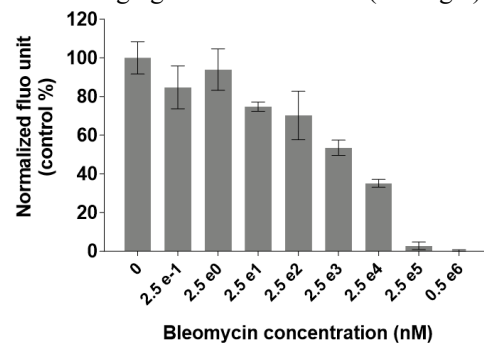


Fig 4. HCT-116 dose-response to bleomycin (concentrations ranging from 0 to 0.5 mM). HCT-116 cell viability percentage obtained by measuring LUCS ratios 48h after treatment. For all conditions, three batches are studied.

Bleomycin concentrations comprised between 0 and 250 nM had no important impact on cell viability (<30% of mortality), at the opposite of concentrations of more than 25 μ M which had a drastic effect (>65% of mortality).

In our study, we wanted to highlight the enhancement of drugs delivery into cells with EP and accentuate the expected effect on cell death for the detection at an early stage of cell viability. This is why we selected for the ECT treatments a bleomycin concentration that is 5 times higher than what is commonly used for *in vitro* studies [14], while not inducing a significant cell death. All ECT treatments have therefore been performed by using bleomycin at a 250 nM concentration.

C. Clonogenic assays

Ten days after treatment, cells were stained with CV, that allowed to easily determine the number of clones. Fig.5

shows an example of the clones obtained for different field intensities (0, 1 and 2.5 kV/cm), with (second row) and without (first row) addition of bleomycin (250 nM). The number of clones obtained was ~300 for the control condition (0 kV/cm, 0 nM). It is reduced to 176 when electric field alone is applied at 1 kV/cm and to ~100 when bleomycin alone is administered. The combination of electric field applied at 1 kV/cm and bleomycin at 250 nM allowed to drastically reduce the number of clones to 12, showing their synergistic effect.

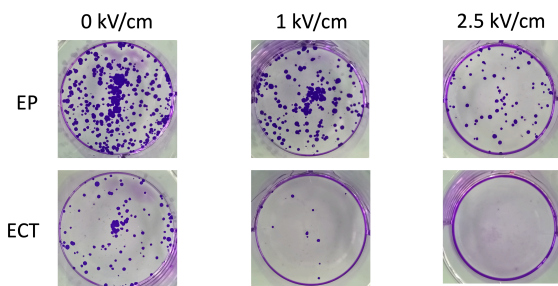


Fig 5. HCT-116 clones survival obtained by staining with CV dye. The first row corresponds to clones that were formed 10 days after EP treatment (application of an electric field with intensities ranging from 0 to 2.5 kV/cm). The second row corresponds to clones that were formed 10 days after ECT treatment (combination of the application of an electric field with intensities ranging from 0 to 2.5 kV/cm and the administration of 250 nM bleomycin).

Fig.6 shows the percentage of the clones obtained for all the electric field intensities tested, when combined or not to bleomycin.

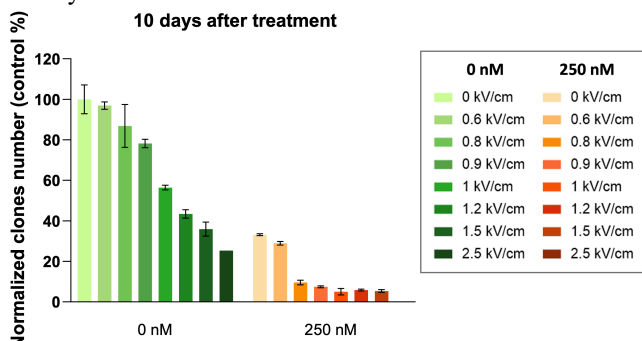


Fig 6. Percentage of HCT-116 clones obtained 10 days after different treatments. No change of culture medium is performed during the incubation time. In green is presented the viability rate after EP treatment (i.e. after application of an electric field only) and in orange is presented the viability rate after ECT treatment (i.e. after the combination of the application of an electric field and the administration of 250 nM of bleomycin). For all conditions, two batches are studied. The obtained results are representative of two independent studies.

EP treatments (i.e. only the electric field is applied) can have a direct impact on cell survival: intensities greater than 1 kV/cm severely alter cell integrity, leading to the mortality of more than a half of the cells.

Administration of bleomycin alone led to high cell mortality (~67%). This high rate can be explained by the fact that the incubation time for clonogenic assays (i.e. 10 days) is quite long and no change of culture medium is made during this period. *In vivo*, the cytotoxic drugs injected in the patients before ECT treatment (bleomycin or cisplatin) are eliminated [33].

However, the effect of the electric fields on the uptake of bleomycin into cells is noticeable. In particular, cell viability is dramatically reduced when applying 1 kV/cm electric fields. At 2.5 kV/cm, all the cells were dead.

To better mimic reality, we performed complementary clonogenic assay involving a change of the culture medium performed 24h after treatment in order to get rid of bleomycin. Fig.7 shows the corresponding percentage of clones that are obtained.

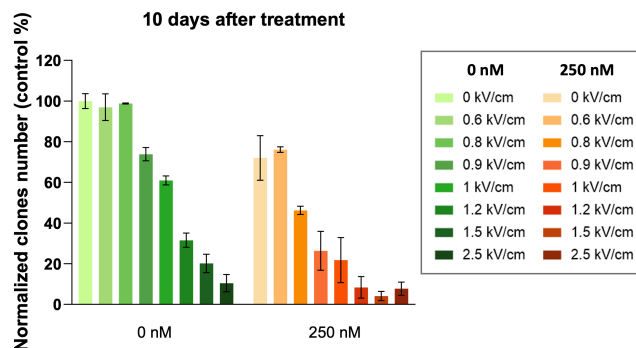


Fig 7. Percentage of HCT-116 clones obtained 10 days after different treatments. A change of culture medium is performed 24h post treatment. In green is presented the viability rate after EP treatment (i.e. after application of an electric field only) and in orange is presented the viability rate after ECT treatment (i.e. after the combination of the application of an electric field and the administration of 250 nM of bleomycin). For all conditions, two batches are studied.

If cell viability is better preserved when bleomycin is eliminated after 24 hours (~28% of cell mortality induced in the case of bleomycin alone), the synergistic effect of the electric fields and bleomycin is well observable (~39% of cell death when electric field alone is applied with an intensity of 1 kV/cm, against ~78% of cell mortality induced in the case of ECT treatment at the same electric field strength).

The aim being to be able to predict the long-term response of cells over short periods of time, we then used other cell viability techniques.

D. Colorimetric assays

Optical density of CV staining can be measured after cell lysis for the different treatment conditions as shown in Fig. 8.

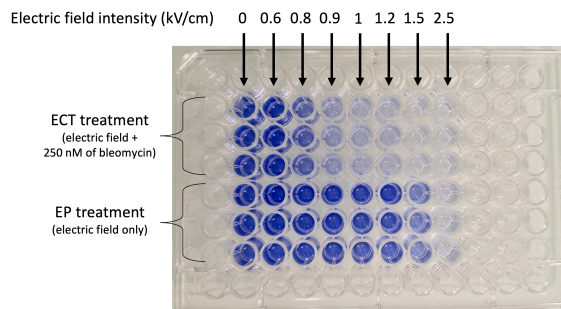


Fig 8. Example of colorimetric assay performed 48h after EP and ECT treatment. Each column corresponds to a different electric field intensity condition (kV/cm). The first three lines correspond to a triplica of ECT treatments (combination of the application of an electric field and the administration of 250 nM of bleomycin). The last three lines correspond to a triplica of EP treatments (application of an electric field only).

Strong dye colors are the signature of a high cell population, while weak colors reveal that the applied treatments have altered cell proliferation. Columns correspond to different electric field strengths, ranging from 0 to 2.5 kV/cm. The first three lines correspond to the obtained cell population 48 hours after ECT treatments. The last three lines correspond to the obtained cell population 48 hours after EP treatments.

The results of colorimetric assays performed 2h, 24h and 48h after EP and ECT treatments are shown in Fig.9.

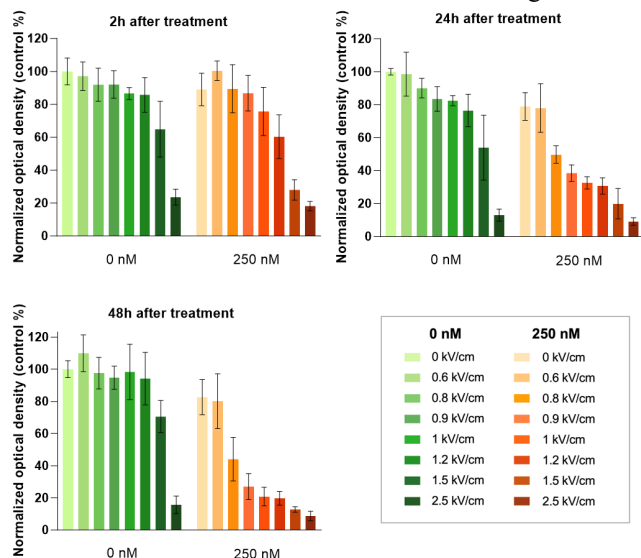


Fig 9. HCT-116 cell viability percentage obtained by measuring optical density 2, 24 and 48h after different treatments. In green is presented the viability rate after EP treatment (i.e. after application of an electric field only) and in orange is presented the viability rate after ECT treatment (i.e. after the combination of the application of an electric field and the administration of 250 nM of bleomycin). For all conditions, three batches are studied and three independent experiments are performed.

In line with the intensity of the CV dye (see Fig.8), the higher the field strength, the lower the optical density value. This reflects an increase in the mortality rate with the electric field intensity, in particular for electric field values higher than 1.2 kV/cm, where there is a dramatic drop showing the direct effect of electric pulses on cell viability.

Similar viability trends are obtained for the different studied times. Bleomycin alone has no significant effect. The combined effect of pulsed electric field and bleomycin on cells is only slightly visible 2 hours after the treatment but clearly observed 24 hours and pronounced 48 hours after ECT treatment, which is in agreement with the fact that the drug acts during the G2 phase of the cell cycle (that is around 20 hours).

These assays highlight the impact of the electric field alone (green) on cell viability. Electric field strengths of 2.5 kV/cm led to a mortality rate of almost 80% as early as 2 hours after treatment.

The efficacy of ECT treatments (orange) is highlighted for electric field strengths greater than the permeabilization threshold: a much higher mortality rate is obtained after ECT treatments in comparison to when bleomycin alone is administrated. When cells are only exposed to bleomycin

(250 nM), there is no significant impact on their viability (~11%, ~21% and ~17% of cell death is obtained 2h, 24h and 48h after treatment, respectively). However, exposing cells to pulsed electric fields under the same conditions enhances drugs uptake. Indeed, the application of electric field at 1 kV/cm potentiates the effect of bleomycin by increasing cell mortality to ~24%, ~67% and ~79%, respectively 2h, 24h and 48h after treatment.

The obtained results 48h post-treatment are consistent with the ones obtained for clonogenic assay when a change of culture medium is performed.

E. LUCS assays

The last tested viability assay corresponds to the LUCS method. The profiles as well as the percentage values obtained by performing LUCS assays are presented in Fig.10.

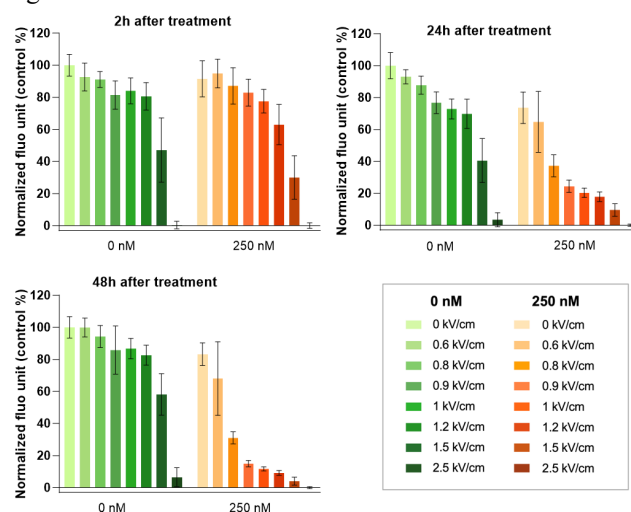


Fig 10. HCT-116 cell viability percentage obtained by measuring LUCS ratios 2, 24 and 48h after different treatments. In green is presented the viability rate after EP treatment (i.e. after application of an electric field only) and in orange is presented the viability rate after ECT treatment (i.e. after the combination of the application of an electric field and the administration of 250 nM of bleomycin). For all conditions, three batches are studied and three independent experiments are performed.

The induced cell mortality 2h, 24h and 48h after ECT treatments at 1kV/cm is ~22%, ~80% and ~88% respectively, while it was only ~8%, ~26% and ~16% 2h, 24h and 48h after the bleomycin alone was injected. It is important to note that electric fields at 1 kV/cm do not affect cell viability in the short term, with 13% mortality 48 hours after treatment.

These results are similar to those obtained by performing colorimetric assays, as Pearson $r > 0.98$ (performed on GraphPad Prism) among the studied times. This strengthens the reliability of the previous obtained results with colorimetric assays.

These studies validate the multiplexing capability of LUCS technology as well as the fact that this technique does not induce short-term cell death. Indeed, the cells studied with the colorimetric tests were studied beforehand with the LUCS technology, and the results obtained show a

good correlation between the two techniques.

IV. DISCUSSIONS

Clonogenic assay is known to be a viability method giving reliable and representative results, as far as long-term eradication is required for cancer treatment. However, this method is time consuming, with an incubation time of 10 days to reach suitable clone sizes. With the perspective to develop *in vitro* tests, to define optimum parameters for personalized ECT treatment, this time is too long. The bleomycin concentration selected in this study (250 nM) appears to be too high for long-term *in vitro* studies to achieve significant contrasts among the different applied treatments. For such a high concentration, a change of culture medium is required to overcome the cytotoxic impact of bleomycin when it remains in prolonged contact with the cells. Even if the number of repetitions in this study is sufficient, the testing of statistical significance has not been performed at this stage.

Compared to clonogenic assays, short-term viability assays can be performed on 96 well-plates, requiring lower quantities of biological material.

Cells colorimetric and LUCS assays allow to predict cell behavior in a shorter time compared to clonogenic assay, and the results obtained between these two methods are comparable. They are also easy to perform. However, regarding the colorimetric technique, errors can be more easily produced with different rinses and pipetting that are carried out. Furthermore, colorimetric assays can hardly be applied to suspension cell lines.

In comparison to colorimetric assay, LUCS assay offers the significant advantages of using cells that remain alive during the entire process. The cells are directly treated in the culture medium, avoiding possible biases related to rinsing and allowing the cells to remain in favorable conditions. LUCS is suitable for multiplexing and can be used on non-adherent cell lines.

These first studies are encouraging as they highlight that it is possible to predict the long-term cellular response over short periods, from 2 hours after treatment. Moreover, it also validates the use of LUCS method to assess cell viability in short times, paving the way for real-time *in situ* studies.

V. CONCLUSIONS

All the permeabilization and viability assays after EP allow to define the best conditions for ECT efficiency. The optimum electric field intensities to be used for ECT are those preliminary defined for EP, leading to a high rate of permeabilization with no severe effect on cell viability. In our present work, these electric field strengths range from 0.8 to 1 kV/cm.

Both permeabilization and viability assays allow to select the best suitable electric field parameters for effective treatments. In this study, electric field strengths of 1 kV/cm permeabilize more than 60% of the cell population while

maintaining cell integrity (around 90% 48h after treatment and more than 50% 10 days after treatment). Delivering electric pulses of such intensity highly potentiates the cytotoxic effect of bleomycin.

This study allowed to conduct *in vitro* assays to evaluate the effectiveness of ECT treatments by different viability techniques. If clonogenic assay is considered the gold standard method, short-term viability assays can predict cell behavior following the received treatments. Colorimetric and LUCS methods provide representative results since a good correlation is obtained between them. Because of its many advantages, the LUCS method seems particularly interesting to use.

ACKNOWLEDGMENT

The authors would like to thank Jelena Kolosnjaj-Tabi for her constructive criticism of the manuscript.

REFERENCES

- [1] L. M. Mir, M. Belehradec, C. Domenge, S. Orłowski, B. Poddevin, J. Belehradec Jr, G. Schwaab, B. Luboinski and C. Paolett, Electrochemotherapy, a novel antitumor treatment: first clinical trial, C. R. Acad. Sci. Paris, t. 313 Série III (1991) 613-618.
- [2] G. Sersa, M. Cemazar, and D. Miklavcic, Antitumor Effectiveness of Electrochemotherapy with as-Diamminedichloroplatinum (II) in Mice, Cancer Research 55 (1995) 3450-3455.
- [3] J. Gehl, G. Sersa, L. W. Matthiessen, T. Muir, D. Soden, A. Occhini, P. Quaglino, P. Curatolo, L. G. Campana, C. Kunte, A. J. P. Clover, G. Bertino, V. Farricha, J. Odili, K. Dahlstrom, M. Benazzo, L. M. Mir, Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases, Acta Oncol. 57 (7) (2018) 874–882.
- [4] M. Cemazar, G. Sersa, Recent advances in electrochemotherapy, Bioelectricity 1 (4) (2019) 204–213. doi:10.1089/bioe.2019.0028.
- [5] M. Marty et al., “Electrochemotherapy – an easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: Results of ESOPE (european standard operating procedures of electrochemotherapy) study,” European Journal of Cancer Supplements, vol. 4, no. 11, pp. 3–13, Nov. 2006.
- [6] G. Di Monta, C. Caracò, E. Simeone, A. M. Grimaldi, U. Marone, M. Di Marzo, V. Vanella, L. Festino, M. Palla, S. Mori, N. Mozzillo, P. A. Ascierto, Electrochemotherapy efficacy evaluation for treatment of locally advanced stage III cutaneous squamous cell carcinoma: a 22-cases retrospective analysis, J. Transl. Med. 15 (1) (Dec. 2017).
- [7] H. Falk, L. W. Matthiessen, G. Wooler, J. Gehl, Calcium electroporation for treatment of cutaneous metastases; a randomized double-blinded phase II study, comparing the effect of calcium electroporation with electrochemotherapy, Acta Oncol. 57 (3) (2018) 311–319.
- [8] N. Rembiałkowska, V. Novickij, E. Radzevičiūtė-Valčiukė, E. Mickevičiūtė, A. Gajewska-Naryniecka, J. Kulbacka, Susceptibility of various human cancer cell lines to nanosecond and microsecond range electrochemotherapy: Feasibility of multi-drug cocktails, Int. J. Pharm. 646 (123485) (2023) 123485.
- [9] D. Miklavcic, R. V. Davalos, Electrochemotherapy (ECT) and irreversible electroporation (IRE) -advanced techniques for treating deep-seated tumors based on electroporation, Biomed. Eng. Online 14 Suppl3 (Suppl 3) (2015) 11.12.
- [10] G. Sersa, K. Ursic, M. Cemazar, R. Heller, M. Bosnjak, L. G. Campana, Biological factors of the tumour response to electrochemotherapy: Review of the evidence and a research roadmap, Eur. J. Surg. Oncol. 47 (8) (2021) 1836–1846.
- [11] L. Bigi, G. Galdo, A. M. Cesinaro, C. Vaschieri, A. Marconi, C. Pincelli, F. Fantini, Electrochemotherapy induces apoptotic death in melanoma metastases: a histologic and immunohistochemical investigation, Clin. Cosmet. Investig. Dermatol. 9 (2016) 451–459.
- [12] J. O. Larkin, C. G. Collins, S. Aarons, M. Tangney, M. Whelan, S. O'Reilly, O. Breathnach, D. M. Soden, G. C. O'Sullivan,

- Electrochemotherapy: aspects of preclinical development and early clinical experience, *Ann. Surg.* 245 (3) (2007) 469–479.
- [13] L. Gibot, L. Wasungu, J. Teissié, M.-P. Rols, Antitumor drug delivery in multicellular spheroids by electroporation, *Journal of Controlled Release* 167 (2013) 138–147.
- [14] T. García-Sánchez, I. Leray, M. Ronchetti, R. Cadossi, L. M. Mir, Impact of the number of electric pulses on cell electrochemotherapy in vitro: Limits of linearity and saturation, *Bioelectrochemistry* 129 (2019) 218–227.
- [15] S. Orlowski, J. Belehradek, C. Paoletti, L. M. Mir, Transient electroporation of cells in culture, *Biochemical Pharmacology* 37 (24) (1988) 4727–4733.
- [16] M. N. Zakelj, A. Prevc, S. Kranjc, M. Cemazar, V. Todorovic, M. Savarin, J. Scancar, T. Kosjek, B. Groselj, P. Strojjan, G. Sersa, Electrochemotherapy of radioresistant head and neck squamous cell carcinoma cells and tumor xenografts, *Oncol. Rep.* 41 (3) (2019) 1658–1668.
- [17] A. de Caro, E. Bellard, J. Kolosnjaj-Tabi, M. Golzio, M.-P. Rols, Gene electrotransfer efficiency in 2D and 3D cancer cell models using different electroporation protocols: A comparative study, *Pharmaceutics* 15 (3) (Mar. 2023).
- [18] P. Bregigeon, C. Rivière, L. Franqueville, C. Vollaire, J. Marchalot, M. Frénéa-Robin, Integrated platform for culture, observation, and parallelized electroporation of spheroids, *Lab Chip* 22 (13) (2022) 2489–2501.
- [19] H. P. Schwan, Electrical properties of tissue and cell suspensions, in: *Advances in Biological and Medical Physics*, Elsevier, 1957, pp. 147–209. doi:10.1016/b978-1-4832-3111-2.50008-0.
- [20] M.-P. Rols, Electroporation, a physical method for the delivery of therapeutic molecules into cells, *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1758 (3) (2006) 423–428. doi:10.1016/j.bbame.2006.01.005.
- [21] R. Davalos, L. M. Mir and B. Rubinsky, Tissue Ablation with Irreversible Electroporation, *Annals of Biomedical Engineering* 33 (2005) 223–231.
- [22] M. Golzio, M.P. Rols, J. Teissié, In vitro and in vivo electric field-mediated permeabilization, gene transfer, and expression, *Methods* 33 (2004) 126–135.
- [23] B. Al-Sakere, F. André, C. Bernat, E. Connault, P. Opolon, R. V. Davalos, B. Rubinsky, L. M. Mir, Tumor ablation with irreversible electroporation, *PLoS ONE* 2 (11) (2007) e1135. doi:10.1371/journal.pone.0001135.
- [24] J.-P. Tasu, D. Tougeron, M.-P. Rols, Irreversible electroporation and electrochemotherapy in oncology: State of the art, *Diagn. Interv. Imaging* 103 (11) (2022) 499–509.13.
- [25] P. A. Garcia, R. V. Davalos, D. Miklavcic, A numerical investigation of the electric and thermal cell kill distributions in electroporation-based therapies in tissue, *PLoS ONE* 9 (8) (2014) e103083. doi:10.1371/journal.pone.0103083.
- [26] T. Batista Napotnik, D. Miklavčić, In vitro electroporation detection methods – an overview, *Bioelectrochemistry* 120 (2018) 166–182. doi:10.1016/j.bioelechem.2017.12.005.
- [27] S. Šatkauskas, B. Jakštys, P. Ruzgys, M. Jakutavičiūtė, Different cell viability assays following electroporation in vitro, in: *Handbook of Electroporation*, Springer International Publishing, Cham, 2016, pp. 1–14.
- [28] T. T. Puck, P. I. Marcus, Action of x-rays on mammalian cells, *J. Exp. Med.* 103 (5) (1956) 653–666.
- [29] B. Gabriel, J. Teissie, Control by electrical parameters of short and long-term cell death resulting from electroporation of Chinese hamster ovary cells. *Biochim Biophys Acta* 1266 (1995) 171–178.
- [30] S. Derick, C. Gironde, P. Perio, K. Reybier, F. Nepveu, A. Jauneau, C. Furger, LUCS (light-up cell system), a universal high throughput assay for homeostasis evaluation in live cells, *Scientific Reports* 7 (1) (dec 2017). doi:10.1038/s41598-017-18211-2.
- [31] C. Gironde, C. Dufour, C. Furger, Use of LUCS (light-up cell system) as an alternative live cell method to predict human acute oral toxicity, *Toxicology Reports* 7 (2020) 403–412. doi:10.1016/j.toxrep.2020.02.010.
- [32] J. Chen, M. K. Ghorai, G. Kenney, J. Stubbe, Mechanistic studies on bleomycin-mediated DNA damage: multiple binding modes can result in double-stranded DNA cleavage, *Nucleic Acids Research* 36 (11) (2008) 3781–3790. doi:10.1093/nar/gkn302.

[33] W. E. Evans, G. C. Yee, W. R. Crom, C. B. Pratt, A. A. Green, Clinical pharmacology of bleomycin and cisplatin, *Head amp; Neck Surgery* 4 (2) (1981) 98–110. doi:10.1002/hed.2890040204.

[34] M. Rols, J. Teissié, Electroporation of mammalian cells. quantitative analysis of the phenomenon, *Biophysical Journal* 58 (5) (1990) 1089–1098. doi:10.1016/s0006-3495(90)82451-6.



A. Calvel (S'22) received M.S. degree in Biophysics from Université Toulouse-III-Paul-Sabatier, Toulouse, France, in 2021; Engineering Degree in physics from the Institut National des Sciences Appliquées de Toulouse, Toulouse, France, in 2021. She is currently a PhD Student with the Laboratory of Analysis and Architecture of System, National Scientific Research Center (LAAS-CNRS), Toulouse and with the Institute of Pharmacology and Structural Biology, National Scientific Research Center (IPBS-CNRS), Toulouse, France. Her scientific research interests include the use of microwave based dielectric spectroscopy for the study of electroporation of single cells.



A. de Caro received M.S. degree in Biochemistry and Molecular Biology from Université Toulouse-III-Paul-Sabatier, Toulouse, France, in 2021. She is currently a PhD Student in the Institut of Pharmacology and Structural Biology (IPBS-CNRS), Toulouse in Cellular Biophysic team. Her scientific research interests include the study of electroporation coupled with cytotoxic drugs on cancer cell models' viability to improve current treatment of electrochemotherapy for skin and deep-seated tumors.



O. Peytral-Rieu (S'20, M'23) received M.S. degree in translational medicinal chemistry from University of Montpellier, Montpellier, France, in 2019; Engineering Degree in chemistry, biology and health from the Engineering Chemistry School of Montpellier, Montpellier, France, in 2019. She received the Ph.D. degree in Electromagnetic, Hyper Frequency systems from the University of Toulouse, Toulouse, France, in 2022. She is currently an associate professor with the Laboratory of Analysis and Architecture of System, National Scientific Research Center, Toulouse. Her current research interests include the use of microwave based dielectric spectroscopy for the study and the characterization of 3D biological samples. Dr. Peytral-Rieu is a member of the IEEE Microwave Theory and Technology Society (IEEE MTT-S), and member of the European Microwave Association (EuMA).



C. Gironde received Biotechnology degree from the University Toulouse III Paul Sabatier, Toulouse, France, in 2011. She worked on the development of an allergy diagnostic microarray at UMR152 Pharma DEV, Toulouse, from 2012 to 2014. In 2014, she joined LED company as part of a joint research program with N2IS research group at the Laboratory of Analysis and Architecture of System, National Scientific Research Center (LAAS-CNRS), in Toulouse. She is currently working as a research technician specialising in cell biology at Anti Oxidant Power (AOP), developing new tests and managing research and development programs in partnership with MH2F research group at LAAS-CNRS. More recently, she has been involved in setting up the LUCS (Light Up Cell System) technology for new applications such as measuring the effect after cell electrolocation or characterising microtissues.



C. Furger received his M.S. at Université Paris Cité in 1991 and his PhD in cell biology at Sorbonne Université (Paris) in 1995. He was a post-doctoral fellow in cancer research at the Garvan Institute of Medical Research (GIMR) in Sydney, Australia (1995-1998) before joining the IPBS-CNRS lab in Toulouse (1999-2004). In parallel to his academic activities, he is the founder of two biotech start-up companies: NovaLeads (CEO 2000-2006 and CSO 2006-2012) and AOP (CEO 2017-present), and he was also in charge of

developing the biotech branch of the L.E.D. company (2013-2017). His main research interests focus on cell biology, measure of cytotoxicity, assessment of redox status and more recently on the development of a new generation of assays dedicated to understanding the mode of action of antioxidants in living cells. He is the author of a scientific reference book on cell biology: "Live Cell Assays - From Research to Regulatory Applications". John Wiley & Sons Eds (New York, USA), 248 pp, July 2016.



D. Dubuc (S'99, M'03) received the Agregation degree from the Ecole Normale Supérieure de Cachan, Paris, France, in 1996, and the M.S. and Ph.D. degrees in electrical engineering from the University of Toulouse, Toulouse, France, in 1997 and 2001, respectively. From 2002 to 2013, he was an Associate Professor with the University of Toulouse, and a researcher with the Laboratory of Analysis and Architecture of System part of National Scientific Research Center (LAAS-

CNRS), Toulouse, France. From 2007 to 2009, he was a Visiting Senior Researcher with the Laboratory of Integrated Micromechatronic Systems (LIMMS-CNRS)/Institute of Industrial Science (IIS), University of Tokyo, Tokyo, Japan. Since 2013, he is Professor at the University of Toulouse. His research interests include the development of microwave circuits integrated with microtechnologies and their application to wireless telecommunication and biology. He has participated or organized several international events, including GAAS 2005 and the 2020 MTT-S International Microwave Biomedical Conference (IMBioC 2020), where he acted as Topical Committee Chair. Prof. Dubuc is a member of the IEEE Microwave Theory and Technology Society (IEEE MTT-S), and member of the European Microwave Association (EuMA). He has authored and co-authored over 200 technical conference, letter, journal papers and book chapters, holds 3 patents and is involved in the spreading of microwave techniques within industrial products.



Katia Grenier (S'99, M'03) received her M.S. and Ph.D. degrees in electrical engineering from the University of Toulouse, Toulouse, France, in 1997 and 2000, respectively. After a Postdoctoral Fellowship at Agere Systems (Bell Labs) in 2001, she joined the Laboratory of Analysis and Architecture of System of the National Scientific Research Center (LAAS-CNRS), Toulouse, France and was engaged in RF MEMS circuits on silicon. From 2007 to 2009, she was with the Laboratory

for Integrated Micromechatronic Systems of CNRS (LIMMS-CNRS)/Institute of Industrial Science (IIS), University of Tokyo, Tokyo, Japan, where she was engaged in launching research activities on miniature microwave-based biosensors. Her research interests in LAAS-CNRS are now focused on the development of microwave-based systems for biological and medical sensing applications as well as for evaluating electromagnetic waves effects on living organisms. Dr. Grenier is a member of the Technical Committee dedicated to Biological Effects and Medical Applications of the IEEE Microwave Theory and Techniques Society and of the European Microwave Association. She serves as Program Committee member of several conferences including the IEEE MTT-S International Microwave Symposium and the European Microwave Week and chaired the IEEE BioWireless 2015 & 2016, the IEEE International Microwave BioMedical Conference 2020 and the Executive Committee chair of the IEEE International Microwave Bio Conference (IMBioC) until 2020. She is also involved in Women in Microwave events' organization. She has authored and co-authored over 220 technical conference, letter, journal papers and book chapters, holds 4

patents and is involved in the spreading of microwave techniques within industrial products.



Marie-Pierre Rols received a Master's degree in Biochemistry, a Ph.D. in Cell Biophysics and the Habilitation à Diriger les Recherches from the Paul Sabatier University of Toulouse in 1984, 1989 and 1995, respectively. She is currently Director of Research at the IPBS-CNRS laboratory in Toulouse, Secretary of the French Society for Nanomedicine (SFNano), Board member of the Bio Electrochemical Society (BES), and Council member in charge of "biological sciences" of the

International Society for Electroporation-based Therapies and Treatments (ISEBTT). Her main research interests lie in the fields of membrane electroporation in vitro and in vivo. In the last years, she has focused on the mechanism of DNA electrotransfer. Marie-Pierre Rols is the author of more than 200 articles in peer-reviewed journals. In 1993 she received the Galvani Prize of the Bioelectrochemical Society, in 2006 a joined prize of the Midi-Pyrénées region, and in 2022 the Franck Reidy Award. She has been member of the management committee of the COST TD1104 - European network for development of electroporation-based technologies and treatments (EP4Bio2Med).