COST EMF - MED (Action BM1309):
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STSM Report:

Role of frequency spectrum of complex signals: an experimental study on cells

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Abstract:

In this STSM, the response of different cell types (DC3F, SHSY-5Y, HaMSC) to a set of different pulsed stimulations was analyzed looking at calcium fluxes which are a fundamental second messenger in cells. The idea is to understand if any correlation between the frequency content of the stimulation and the calcium responses can be established. I also investigated if spreading the pulse dose can be a way to sensitize the cells, enabling a higher permeabilization and hence to get augmented calcium fluxes. Experiments were conducted using fluorescence microscopy and acquiring florescence outcomes in real-time using a ratiometric dye for calcium detection. Cell were pulsed within coplanar waveguide microelectrodes integrated into the microscope stage.

A. Purpose of the STSM

In attempting to establish new therapeutic benefits from the application of electric or electromagnetic fields, to consolidate or to optimize various treatments efficacy, diverse time domain signals (trains of electric/electromagnetic pulses of different shapes) are often applied both *in vitro and in vivo*. Examples of these therapies are the transcranial magnetic stimulation, the deep brain stimulation, as well as the electrochemoterapy, the electroablation of tumors and the neuronal stimulation by short electric pulses. Some authors [*Pakhomova et al., PloseONE, 2011*] observed, looking at the uptake of specific fluorescent dyes, cells "sensitization" after the application of electric pulses if the delivered dose is split in a few fractions. The mechanism of this phenomenon is still debated and even if a number of different parameters were investigated so far, the full comprehension of the phenomenon is still mostly unclear. Evidently cell sensitization to the electromagnetic field application can have a significant impact in therapeutic applications as a way to reduce the delivered dose to get a desired effect. Therefore, there is an obvious need of further research attempting to understand basic mechanisms to appropriately use this effect in emergent medical applications as for example cancer ablation or neuronal stimulation mediated by electric fields.

The purpose of this STSM was to investigate the phenomenon of cell sensitization translating the problem from the "time domain" into "the frequency domain". Indeed, when a train of pulses is applied to a biological target, depending on the inter-pulse intervals (repetition frequency) and the delay between bursts of pulses, the associated frequency spectrum changes. So, the STSM aimed to understand whether cell sensitization can be

correlated with the frequency content of a delivered train of pulses and if this observable can be used to optimize the intended effects.

B. Work Description

To asses this goal, a wide-band matched coplanar waveguide was used in combination with fluorescence microscopy to detect calcium fluxes of cells under electric stimulation (i.e. cells electropermeabilization detected by the penetration of calcium in the cells). The benefit of the use a coplanar waveguide structure is its wide frequency band, thus allowing to test very short electric signals, in the nanosecond time scale, in a very efficient way. The setup has been largely tested and its operational efficacy is proved as well as its biocompatibility with cells in real-time experiments [*Garcia-Sanchez et al., BBA Biomembranes 2018*]. In Fig. 1 the coplanar electrodes are shown with the support used to integrate the device in the inverted microscope stage. During STSM experiments, I used electric pulses lasting 10 ns and different stimulation patters. Globally 24 and 12 pulses were delivered arranged in a single or in multiple fractions for a total of eight different stimulation modalities (due to confidentiality reasons the used patters cannot be fully disclosed). The two different total pulse numbers were taken into account in order to be sure to not expose cells to a high dose that would be able to cause cell membrane irreversible electro-permeabilization.

The monitoring of calcium fluxes is an interesting observable, as calcium has a determinant role in a number neurological degenerative dysfunctions and the potentiality of its regulation can have a great impact in therapeutic applications mediated by the electric fields. In this regards, I exposed to different train of pulses, neuroblastoma cell line (SHSY-5Y) as a model to mimic neuronal-like cells.

At the same time, I also exposed stem cells, i.e. human mesenchymal stem cells (haMSCs), to investigate their response to calcium after electrical stimulation. These stem cells are interesting due to their potential use in regenerative medicine.

Finally, I also exposed a third cell type, Chinese hamster fibroblast DC-3F, as these cells are very well known in the host laboratory. DC-3F are very robust cell and are fast to grow, thus they represented a very good model for setting the initial experimental protocol.

Therefore, the work included experiments on these three cell types using electric signals presenting different low and high frequency contents. This choice was performed in order to establish a relation with the calcium fluxes detected in real-time. The FURA-2 ratiometric fluorescence dye was used as a marker of calcium fluxes due to its sensitivity and to the possibility, after calibration, to correlate florescence intensities to effective calcium concentrations released in the cells.

Sham exposures were performed during each set of experiments. For each cell type, the experiments were repeated in triplicate.

At the host laboratory specific equipment to perform these real-time experiments is available : a 10 ns pulse generators (Fid Technology) which was triggered by an external HP voltage generator to produce the different stimulation patters, presented in Fig. 1. The generation chain was automatized through a LabVIEW program (Fig. 1). Experiments were performed integrating the coplanar electrode in the stage of an inverted epifluorescence microscope (Zeiss, Axiovert 100) as presented in Fig. 1. Fluorescence was detected using time lapse acquisition (total acquisition time 11 min, 1 minute before exposure and 10 minutes after pulses delivery) with a fast CDD camera. The protocol for cell staining using the FURA-2 was established during the first days of the STSM together with the use, at a suitable concentration, of an aduvant agent (Pluronic) added to cells to facilitate FURA-2 entry. Also the microscope filters (at 340 and 380 nm) as well as acquisition time where set during the first days of the STSM and used during the whole experimental period. Cell culturing and concentration for the experiments were also defined rigorously. Globally the amount of work produced has been much more than the foreseen one in the proposal of this STSM.

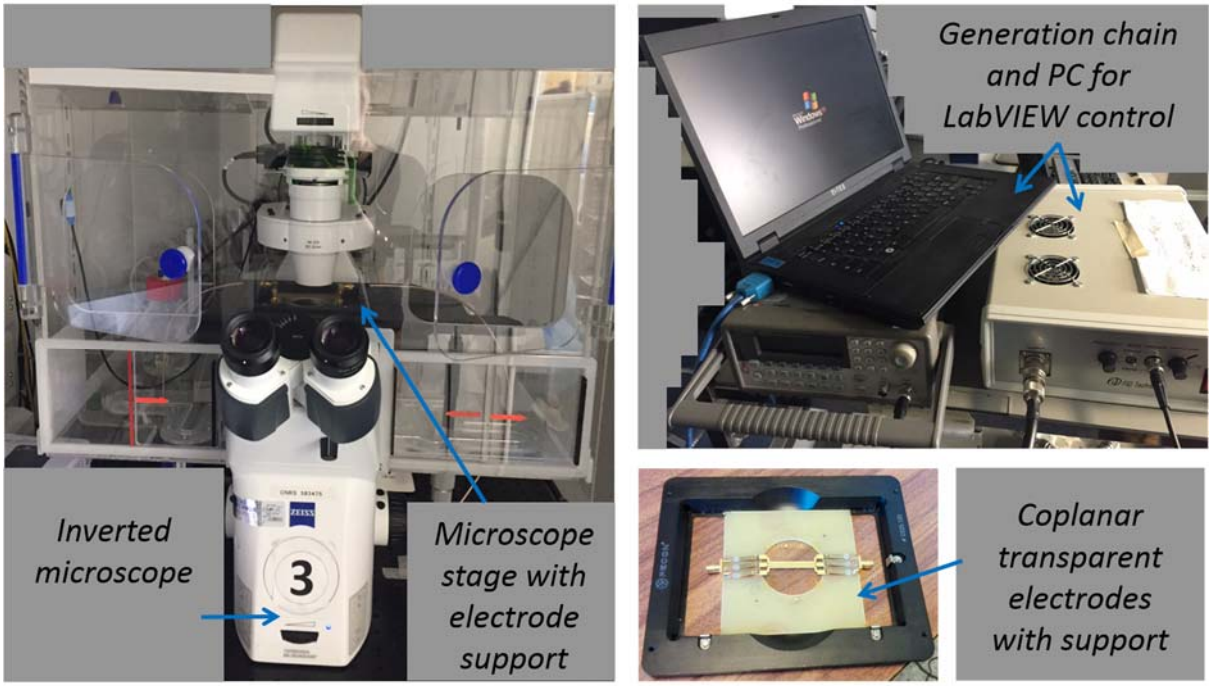

Fig. 1: the experimental setup is composed by an inverted microscope, the coplanar electrodes support, and the generation chain automatized using a LabVIEW program. The coplanar electrodes used over the transparent substrate are also shown.

C. Results

The main results of the STSM can be summarized in 3 points: 1) the definition of the Ca response during and after electric pulse stimulation (i.e. assessment of cell sensitization), 2) the assessment of cell depend thresholds for the effect, 3) the quantification of the fluorescence levels in dependence on the cell type.

Looking at the first point, the obtained results show that there is no sensitization of the cells caused by the delivery of multiple fractions of pulses when the global pulse number is maintained constant. Lower levels of fluorescence were achieved when 12 pulses (instead of 24) are delivered, as expected. At the end of the exposure, when repeated fractions of pulses were delivered, the final fluorescence reached the same or a lower level obtained by delivering the same number of pulses in a single burst. An example of this kind of results is presented in Fig. 2 for SHSY-5Y cells for some stimulation patterns. So, these results seem to demonstrate that there is no correlation between the cell response and the frequency content of the applied stimulation. Cell sensitization observed by other groups looking, for example at cell viability, was achieved using different type of electric pulses and on a different cell type. I was not able to reproduce the sensitization phenomenon for cell calcium fluxes in our investigated cells.

Higher Ca fluxes seemed associated to the increase of the repetition frequency of the pulse trains. This effect was visible for the stimulation patterns of 24 pulses, while no substantial difference was achieved for the stimulation patterns of 12 pulses comparing the two different tested repetition frequencies of 1 and 100 Hz.

Concerning the point 2, it was possible to assess the threshold for cell Ca release. This threshold was higher for HaMSC compared to DC-3F and SHSY-5Y cells. Specifically, the SHSY-5Y cells seem the more sensitive to pulses stimulation, may be due to their neuronal nature and the fact that Ca is a strong regulator in neuronal-like cells.

For the point 3, it was observed that the reached Ca levels are different for the same stimulation modality on the different tested cells. This observation implies that cell permeabilization reached different levels in the different cells independently on their dimensions. Indeed, HaMSC which are the biggest cells resulted to have the lower Ca uptake release. This effect can be also associated not only to the permeabilization level of the

membranes, but also to the level of their oxidation and the implication of the membrane voltage gated channels in regulating Ca influx from the outside to the cell inside. Since the experiments where performed in the presence of external Ca ions, at the moment, it is not possible to conclude about the implication of internal cell organelles as the endoplasmic reticulum in regulating the Ca release. During the STSM, the method of data elaboration has been setup combining software for image treatment and Matlab routines. At the moment the final statistical analysis is still in progress as a great number of cells (> 20) have been analyzed during each experiments. To summarize a total of 72 independent experiments were performed analyzing globally more than 1440 cells.

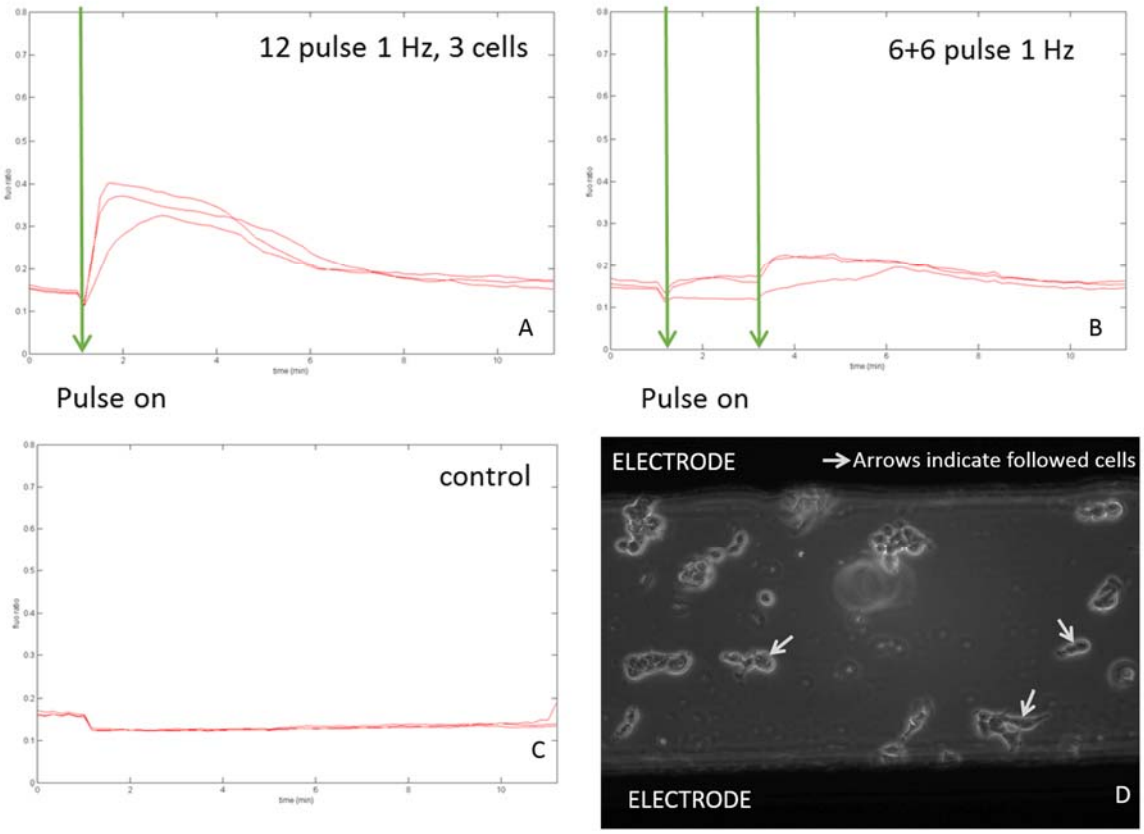

Fig. 2: fluorescence ratio as a function of the time for different stimulations modalities, 12 pulses at 1Hz panel A, 6+6 pulses delivered with a delay of two minutes in panel B, the control in panel C. In panel D the phase contrast image of cells at the beginning of the experiment is shown.

D. Future collaboration with host institution

This STMS reinforced the historical collaboration between the CNRS UMR 8203 Gustave Roussy and ENEA SSPT Division of Health Protection Technologies. The ENEA Bioelectromagnetic Group studies from long time the behavior of SHSY-5Y under electric and magnetic stimulations. The CNRS UMR 8203 are, on the other side, specialists in the application of electric pulses to haMSC for regenerative purposes.

This collaboration shed light on the role of electric field frequency in stimulation of cells establishing a relation between the frequency content of a signal and calcium fluxes.

These results seem also particularly interesting in the light of common applications to grants at the European level. The two labs are now involved in preparing a FET-OPEN proposal involving MSC and Ca fluxes. The data of this STSM are extremely interesting as a support of this UE proposal.

Future experiments are planned to assess the Ca response in the absence of the external Ca and in the presence of different types of molecules with specific actions on cell responses related to Ca or to cell membranes permeabilization.

E. Expected Publications

The material, which is very interesting, will be published in a good journal as BBA general subject. Due to the great amount of work, the different analyzed cell types and a number of different stimulation modalities, it seems even possible to report these results in multiple journal publications of high quality (i.e. Scientific Reports, Frontier). This material will be also presented in next conferences oriented to BioEM applications in medicine and biology (as EMF-MED 2018 organized by the COST Action BM1309 in Split in September 2018).

F. Other Comments

No further comments are present.

Confirmation by the host institution of the successful execution of the STSM:

We confirm that Caterina Merla has performed the research work as described above.

L. M. Mir

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