# Exposition of Cells in Suspension using Nanosecond Duration Electric Pulses - Detection of Permeabilisation by Cloning Efficiency Tests: Results and Artifacts.

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## Abstract

Intense (several MV/m) nanosecond duration electric pulses can induce damages on cells external membrane which can be detected by direct cell killing or by the uptake of non permeant molecules. We detail here the methodology to carry out those experiments, drawing the attention to exposure artifacts that demonstrate the importance of the implementation of a metrology specific to the ultrashort pulses.

## 1. Introduction

Electropermeabilisation is a well know word that describes how electric pulses can induce reversible or irreversible defects in a cell membrane thus allowing direct access to the cell inside. This technique is nowadays routinely used in research laboratories to transfect DNA into bacteria or mammalian cells. It has even been taken to the clinic to treat cancer by combining electric pulses to the use of a cytotoxic drug like bleomycin [1 2] (the procedure is known in the clinic under the term electrochemotherapy). In the traditional electropermeabilisation, pulses parameters can be chosen over a wide range and still lead to successful permeabilisation of cells. However, a consensus more or less came out and the most common protocol is to use between 2 and 8 pulses of 100 µs duration (which are referred to as micropulses), 1200 V/cm magnitude and to apply them with a repetition rate of 1 Hz.

More recently, people have started to investigate the effect of shorter pulses of only a few nanoseconds duration which are referred to under the term nanopulses. It is generally agreed that in order to observe any effect of those pulses, field magnitude must be raised to at least 20 kV/cm. Interestingly, different types of effects on biological cells have been observed already. Among them, we can separate between internal effects like liberation of calcium [3-5] and effects on the external membrane of the cell which leads to permeabilisation. One of the questions that emerge from these observations is whether the mechanisms that induce permeabilisation are the same when 100 µs or 10 ns pulses are applied. A possible approach to investigate this question is to perform cloning efficiency tests (eventually with bleomycin) to detect both irreversible and reversible permeabilisation. We detail here how to perform such experiments when dealing with nanopulses. Some preliminary results regarding the role of the number of pulses or the magnitude of the electric field are presented.

## 2. Materials and Methods

### 2.1 Cell culture and Cloning efficacy tests

Cell culture : chinese hamster lung cell line - DC-3F- was grown in MEM - Minimum Essential Medium (GIBCO Laboratories, Cergy-Pontoise, France, 31095) with addition of 10% fetal bovine serum (GIBCO Laboratories, Cergy-Pontoise, France, 10091-148) and supplemented by antibiotics. The cells were maintained in a humidified atmosphere at 37°C and 5% $CO_2$.

Cloning efficacy tests : after trypsinization of exponentially growing cells and inactivation of trypsin by complete medium, cells were centrifuged for 10 minutes at 1000 rpm and resuspended in ice-cooled S-MEM at a density of 5 x $10^6$ cells/ml (S-MEM, Minimum Essential Medium, GIBCO Laboratories, Cergy-Pontoise, France, 11380037). 90µl of the monodispersed cell suspension were mixed with 10µl of relevant bleomycin solutions at a 10-fold concentration (300nM) and 20µl of S-MEM. The suitable volume of the mixture was immediately deposited between the two electrodes (1 mm apart) and subjected to the electric treatment. After delivery of the electric pulses, cells were kept for 10 min at room temperature and then diluted in complete medium and seeded in triplicate in complete culture medium (250 cells/cell culture dish, 60 mm in diameter) for the cloning efficacy test. After 5 days

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maintained in a humidified atmosphere at 37°C, colonies were fixed with 5% formaldehyde, stained with crystal violet and counted.

## 2.2 Nanosecond Pulse Generator

A commercially available generator (FID GmbH, Model FPG 10-ISM10, Burbach, Germany) with an output impedance of 50 Ohm (50 Ω) was used to treat the cells. It generates trapezoidal monopolar pulses of 10 ns half width. The magnitude goes from 3kV to 8kV. The rising edge, fixed by the semiconductors commutation lasts between 0.4 and 0.6 ns. The falling edge lasts between 1 and 1.5 ns.

In order to measure the electric field exactly applied on the cells, a D-dot sensor was inserted in the ground electrode holding the cuvette [6]. This self-manufactured sensor showed a good band path up to 2 GHz which is largely above the maximum frequency content of the applied pulses. Integration of the signal from the sensor was made numerically under Matlab.

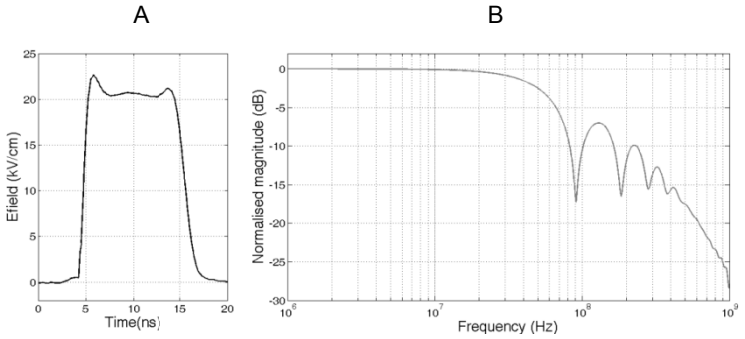


Fig. 1: A - Example of a typical pulse applied during cloning efficiency experiments. The measurement was made using a D-dot sensor. Integration of the signal from the D-dot was made numerically with Matlab. B –Normalized frequency content of the pulse.

## 2.3 Adjustment of the pulses applied

Cells were pulsed in conventional electroporation cuvettes purchased from VWR. The distance between the electrodes was one millimeter. The cuvette was connected to the coaxial cable coming out of the generator thanks to two large copper plate electrodes soldered on a N-connector. Naturally, adaptation between the 50 Ohm generator and cable and the biological load cannot be fully respected. Indeed, the cuvette containing the biological medium has an impedance dependent on the frequency. Up to about 100 MHz, the medium has a conductive behavior which will impose the magnitude of our pulses (Fig. 2 B-C). Above, the dielectric behavior of water appears. Since it is already at high frequencies, it barely slows down slightly the rising and falling fronts. Even higher, stray elements and in particular stray inductances appear (Fig. 2 A) but they are not a real issue according to the frequency contain of the pulses used in this study. According to this, and in order to get around the magnitude limitation of our generator, volumes were constantly adjusted in order to impose the desired final voltage.

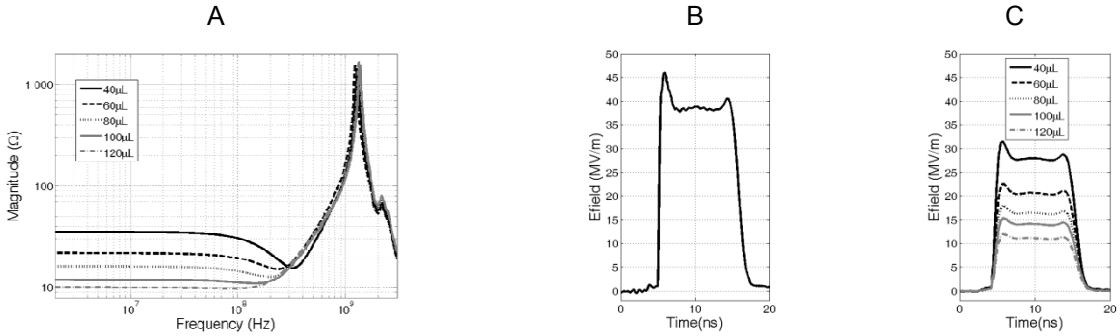


Fig. 2 : A- Impedance of cuvettes containing different volumes of SMEM. B –Measurement of the pulse applied on an adapted load. C- Measurements of the resulting pulses applied on the cuvettes for different volumes of medium.

## 3. Effect of the number of pulses and magnitude

Some preliminary experiments were carried out to find out whether or not nanosecond pulses could directly induce death. The magnitude of the pulses was fixed at 40 kV/cm, the repetition rate at 10 Hz and an increasing number of pulses were applied (Fig. 3 A). Cloning efficiency tests showed that almost no death was induced by the pulses alone if less than 100 pulses were applied. The application of 500 pulses however was sufficient to kill 50 % of the cells. The addition of bleomycin in the pulsing medium (S-MEM) lowered the number of pulses needed to kill cells showing that the defects induced in the membrane can be temporary. If the number of pulses is then fixed at 300 with a repetition rate of 10 Hz, the minimum required electric field can be evaluated (Fig. 3B). It appears that with 300 pulses, no direct killing is observed for magnitudes bellow 30 kV/cm while 50 % of cells can get killed if the magnitude is increased to 50 kV/cm.

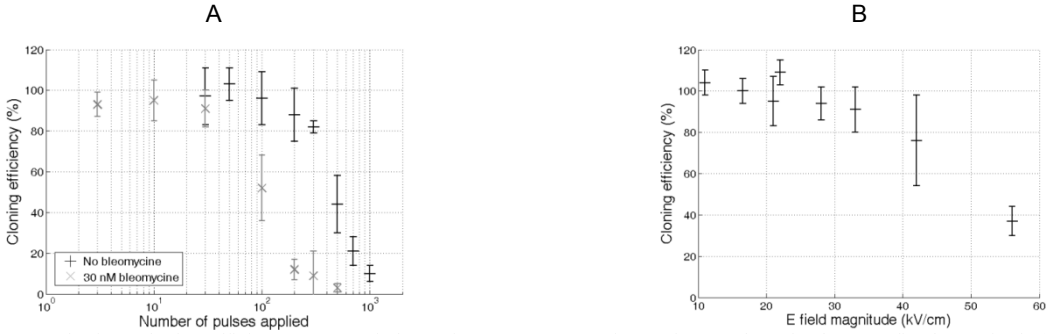


Fig. 3 : A - Results from cloning efficiency tests carried out after exposure to an increasing number of pulses at a fixed magnitude (40 kV/cm). Experiments were performed either with just standard medium (+) or with 30 nM bleomycine (x)
B – Results from cloning efficiency tests carried out after exposure to an increasing electric field magnitude. The number of pulses was fixed at 300 and the repetition rate at 10 Hz. Experiments were performed in standard medium.

## 4. Artifacts due to temperature increase at high repetition frequencies

Cuvettes containing 120 µl of pure medium were submitted to 2000 pulses applied with repetition frequencies of 10 Hz, 100 Hz or 1 kHz. The magnitude was initially adjusted to reach 10 kV/cm. The figure below (Fig. 4) shows the chronological evolution of the pulse magnitude for the different repetition frequencies.

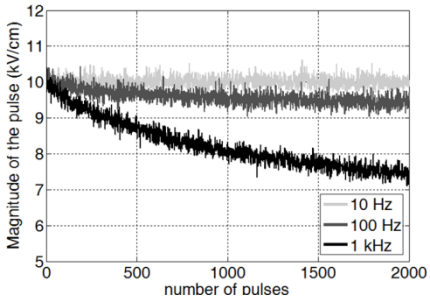


Fig. 4: Evolution of the pulse magnitude due to temperature increase when a high number of pulses are applied. Different frequency rates are presented.

It appears that the magnitude of the pulse applied can rapidly change depending on the repetition frequency used. The higher the repetition rate, the higher the magnitude drop. It turns out that even at not very high magnitudes; heating is induced in the biological medium. This heating however is not high enough to induce biological damage. However it induces a change in the conductivity of the medium and thus a change in the magnitude. Naturally, this phenomenon becomes even more critical at higher frequency rates. This kind of artifacts does not happen with microsecond pulses as a change in conductivity would not change the voltage applied when using any commercially available voltage pulse generator. Experiments on biological cells comparing the effects of 300 pulses applied at different repetition rate tend to show that higher repetition rates are slightly less efficient (data not shown) which might be linked to this temperature increase.

In all cases, constant measurement of the applied voltage can be considered as a reliable way of controlling any global warming of the sample. Indeed, the temperature dependence of ionic solutions like cells medium is about 2%/°C [7] which can easily be detected. Still, it is important to point that only global warming can be detected by such method and that it is very likely to have local heating in the solution before it can be globally detected.

## 5. Conclusions

Cloning efficacy test turns out to be a sufficiently sensitive method to detect both irreversible (cell killing by nanosecond pulse alone) and reversible permeabilisation (cell killing in the presence of bleomycine only). We believe this technique is very powerful since many cells are exposed simultaneously, providing thus a statistical effect regardless of cells individual size or state. It can moreover be used to compare permeabilisation obtained by classical microsecond pulses and it also constitutes a first good step before experiments *in vivo*. As it is shown above, not all experiments can be carried out without inducing artifacts. Heating can happen easily when repetition rates are increased. Moreover, impedance matching is not possible on the whole frequency content of the pulses. Parameters like the type of medium or the volume of solution used play a key role in the impedance matching and changing it can result in a huge change of pulse magnitude. As a consequence, we believe that constant monitoring is not optional.

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