Digital holographic microscopy evaluation of dynamic cell response to electroporation

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Abstract: Phase-derived parameters and time autocorrelation functions were used to analyze the behavior of murine B16 cells exposed to different amplitudes of electroporation pulses. Cells were observed using an off-axis digital holographic microscope equipped with a fast camera. Series of quantitative phase images of cells were reconstructed and further processed using MATLAB codes. Projected area, dry mass density, and entropy proved to be predictors for permeabilized cells that swell or collapse. Autocorrelation functions of phase fluctuations in different regions of the cell showed a good correlation with the local effectiveness of permeabilization.

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1. Introduction

Cell electroporation (EP) consists in controlled permeabilization of the plasma membrane by electric pulses. Various molecules (otherwise nonpermeant) may enter or exit the cytoplasm (e.g., DNA, cytotoxic drugs, polysaccharides [1]). Cellular EP has already proven valuable in various biomedical fields, as an adequate procedure for drug delivery (electrochemotherapy - standard clinical procedure for treating tumors [2]), for gene electrotransfer (vaccines, gene therapies in oncology [3]) and for cell fusion with many biotechnological applications [4]. Despite these various applications, the dynamics of the cellular response to electric pulses is only partially explored and our understanding is still limited. The experimental characterization of the cell membrane reorganization subsequent to pulses application is classically done by three categories of approaches:

i/ measuring electrical/dielectrical modifications induced by pulses [5,6],

ii/ studies of transmembrane transport (transport of fluorescent small molecules, plasmid or naked DNA coding for reporter genes, radioactive molecules [7],

iii/ monitoring of changes in the lipid bilayer packing [8].

The main limitation of most of these techniques is that they induce per se some perturbations (dielectric techniques expose cells to additional electric fields while transport and lipid packing techniques usually rely on using fluorescent labels or radioactive probes). There is thus a need of label free methods to assess the response of the living cells to EP pulses.
Digital Holographic Microscopy (DHM) is suitable for observations on living cells in their natural environment, providing quantitative phase images (QPIs) without using any staining or chemicals. DHM offers values of the phase shifts induced by the optical path length of the biological sample which is registered in each pixel of the holographic image [9]. The optical path length is proportional to the so called “dry mass” which is mostly determined by the protein content of the cell in the corresponding pixel [10]. Computation of the dry mass is possible due to the capability of DHM to deliver quantitative information with physical significance, in all three dimensions, using the reconstructed images from the holograms recorded in an interferometric setup [11]. Images from DHM support new computational approaches: automation of the analysis procedure based on Fourier space adaptive filtering process [12], investigation of the fractal properties of different blood cells [13], discrimination between normal and abnormal cells [14] and training of machine learning algorithms able to distinguish cancer cells [15].

During the last years DHM proved to be very useful to the biomedical domain. DHM presents a real clinical potential in identifying and counting tumor cells [16], evaluating the metastatic potential of cancer cells [17], monitoring the red blood cells geometric and chemical changes during storage [18], studying the dynamics of cellular necrosis [19]. Moreover, DHM showed its potential to witness the cellular uptake of nanographens for drug-delivery [20]. Very recently, holotomographic imaging was used to observe morphological changes of ovarian cancer cells subsequent to electrochemotherapy and chemotherapy [21]. In dynamic regime DHM may be used for bacteria tracking [22] and for monitoring cellular activity (e.g., neuronal networking activity [23] or cardiomyocytes dynamics [24]).

Being an imaging technique which does not imply scanning of the sample, DHM is adequate for studying fast processes [25–28]. As we have shown in a previous study [29], the method is also suitable for studying changes in the optical and shape-related characteristics of the cells after electroporation. Refractive index and cell height could be calculated in specifically defined cell areas and monitored at certain time moments after the application of EP pulses.

The present study introduces a method based on the autocorrelation functions applied on phase images, to characterize the fast dynamics of the cell response to EP pulses. All recordings were done during 90 seconds, with a time resolution of milliseconds. Attached cultured cells were electroporated during the acquisition of holograms. After QPIs reconstruction and cell segmentation, phase related parameters were calculated either on the whole cell or in certain regions of interest. The time evolution of these parameters was analyzed.

### 2. Materials and methods

#### 2.1. Cell preparation

B16F10 cells (murine melanoma line) were cultured in a humidified atmosphere containing 5% CO$_2$ at 37°C. B16F10 cell line represents a largely used aggressive melanoma model for in vitro studies regarding electroporation applied as permeabilization procedure for electrochemotherapy [30]. Cells were routinely cultured with Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, D5796) supplemented with 10% fetal bovine serum (Sigma-Aldrich, F7524). Cells were detached from the culture dish (Trypsin/EDTA, Sigma-Aldrich, T4174), seeded at low density ($2\times10^4$ cells/ml) on 2cm diameter round cover slips and kept (in Petri dishes) in incubator for 24h to achieve 30% confluence.

Cells were washed twice with 2ml PBS (phosphate buffer saline, 0.145M NaCl, 0.0027M KCl, 0.0081M Na$_2$HPO$_4$, 0.0015M KH$_2$PO$_4$, pH 7.4), then the cover slip was transferred to the electroporation chamber. 2ml electroporation buffer (300mM mannitol, pH 7.34, 310mOsm/kg, 15µS/cm) were added and the chamber was mounted on the DHM setup. Once the EP chamber was mounted on the microscope, a few seconds were needed to select the convenient observation field and to focus the image. Usually, it did not take more than 15-20 seconds.
Electroporation efficiency was verified in a separate experiment by adding Propidium Iodide (Fluka, 81845) in the electroporation buffer (25µM final concentration) and visualizing cells in fluorescence microscopy (porated cells show a red fluorescence). The percentage of electroporated cells was calculated as the ratio of the number of PI incorporating cells to the total number of cells observed in visible light (a minimum of 100 cells per pulses amplitude). In our experimental conditions, EP efficiency was above 90% (data not shown).

2.2. Cell electroporation and holograms acquisition

For EP pulses application, a pulse generator (ELECTRO cell β10, BetaTech, France) was integrated in an off-axis DHM setup with a Mach-Zehnder configuration [17]. The off-axis configuration was used because, although it requires a more complicated experimental setup, it presents the advantage of forming the +1 and −1 diffraction orders spatially separated. Thus, the resulted real reconstructed image is more accurate. The interferometer was equipped with a HeNe laser (λ=632.8 nm, 1.5mW), two Nikon objectives (40×, N.A. 0.85) in the reference and object beams to match both wavefront curvatures on the CCD sensor plane. The setup presented a lateral resolution of 900nm, and a vertical resolution of 10nm. At the interferometer entrance, the light intensity was chosen such as to obtain a good hologram contrast and to avoid the camera sensor saturation; an average light intensity of around 50µW/cm² onto the probe proved to be adequate.

Cells electroporation was performed using electrochemotherapy specific sequences of electric pulses with various amplitudes (4 bipolar pairs of rectangular pulses, duration: 100µs for positive and negative pulses, 5µs between them, frequency of pulses pairs: 1ms, amplitude: 0.6, 0.8 and 1kV/cm for both positive and negative waves). Pulses were applied using stainless steel parallel electrodes which were touching the glass bottom of the chamber in which the cover slip with cells was placed (Fig. 1).

![Fig. 1. Off-axis interferometer setup used for acquisition of holographic images of electroporated cells.](image)

For image acquisition a fast camera (FastCam SA1, 5400fps at 1024 × 1024, Photron, USA) was used. Holograms were acquired at a rate of 60fps, for 1.6s before and 90s after EP pulses delivery (EP pulses were delivered between frames 100 and 101); time series of 5400 frames were recorded.

Among all cells, those fulfilling the following criteria were chosen for acquiring holograms: i/ well-individualized cells, because they were supposedly exposed to homogeneous electric field and their images were easy to segment, ii/ the long axis of the cell was perpendicular to the electric field (parallel to the electrodes). 29 electroporated cells fulfilling both criteria were used for data computation, 5 cells were used as Controls (no EP pulses applied), each cell belonging to an independent experiment.
QPIs were reconstructed using Koala software [31]. From each experiment resulted a series of *.txt files (one file for each frame of the time series), containing the values of the phase shift for each image pixel.

2.3. Image processing

The image processing was performed under customized MATLAB codes and comprised the following steps (Fig. 2):

Correction for frames with outlier phase shift values. In each frame the minimum phase shift value (corresponding to the background) was subtracted from each pixel of that frame. To eliminate the frames with outlier phase shift values the following procedure was used: in a time series of QPIs, the mean phase shift of each frame was calculated, then the distribution of these means was computed. Frames with the mean phase shift below the first quartile \(-1.5 \times \text{interquartile range}\) or above the third quartile \(+1.5 \times \text{interquartile range}\), were excluded. The discarded frames were replaced by linear interpolations between the closest non-discarded frames in both directions in order to preserve temporal intervals and the numbers of frames.

Normalizing the phase shift values. All phase shift values in all frames were divided by the maximum value of the entire series of frames. Thus, all phase shift values are in the \((0; 1)\) range.

Cropping of images. The smallest rectangle containing the cell was cropped manually from the first frame and in all subsequent frames the same rectangle was automatically cropped.

Binarization. For each frame a mask was generated by binarizing the image using Otsu’s method. The largest connected component was kept, its holes (if any) were filled and connected components outside the largest one were discarded.

ROI selection. The masks were applied to the *.txt files of each respective frame, after they were cropped similar to the normalized files. Pixels inside the mask represent the region of interest (ROI) and were further used for analysis.

![Fig. 2. Example of image processing steps: a) reconstructed QPI of a cell; b) its mask after binarization and filling holes; c) the final segmented QPI of the cell. The mask was computed for each frame of the whole sequence of 5400 images.](image)

2.4. Computed parameters

To analyze the effects of EP, a set of parameters computed on the whole cell surface was used: projected area (PA), dry mass density (DMd) and entropy (En) [32,33]. To compare the parameters which depend on the cell size, their values were normalized to the average values before the pulse application. For a better visualization of the time evolution of the parameters, a 6Hz Low pass FFT filter was applied.
In each pixel of a QPI there is a phase shift value $\varphi(x,y)$ which depends on the cell height (along the propagation axis) and average refractive index in that pixel [34].

Projected area ($\mu m^2$) was calculated as the number of pixels inside the mask multiplied with the pixel size.

Dry mass density ($pg/\mu m^2$) at each pixel was calculated as:

$$DMd(x, y) = \frac{\lambda}{2\pi \alpha} \varphi(x, y)$$  \hspace{1cm} (1)

where $\lambda$ is the wavelength, $\alpha$ is the average refractive increment of protein (0.2ml/g) [35] and $\varphi(x, y)$ is the measured phase shift in the pixel of $(x, y)$ coordinates.

Entropy (En) was calculated as:

$$En = \sum \varphi(x, y) \ln \varphi(x, y)$$  \hspace{1cm} (2)

where the sum is extended over the entire cell.

On the 5400 QPIs series, time dependent autocorrelation functions of the phase shift fluctuations were computed (using a lag-time up to 900 frames), on regions of $3 \times 3$ pixels located as follows:

- one region is centered on the maximum phase shift point of the QPI (further called “cell center”, marked as CC in Fig. 3);

- other two regions were chosen on a direction along the electric field lines containing the CC point (dashed line in Fig. 3(a)), on each side of the CC, at coordinates presenting similar slope of the phase shift profile on that direction (further called “midpoints” and marked as MP1 and MP2 in Fig. 3) (slopes were computed using specific MATLAB codes).

Autocorrelations were calculated using MATLAB function xcorr. For graphical representation, autocorrelation functions were averaged for each region and each EP pulses amplitude; 5 to 10 cells were used for each average (Fig. 6). For quantitative analysis, single-exponential fit of individual autocorrelation functions was used to compute the initial values and relaxation times of the autocorrelation decays ($\tau^*$). These initial values and relaxation times were averaged for each region and EP pulses amplitude (Fig. 7). The cells electroporated with 1kV/cm which swelled or collapsed, were excluded.
3. Results and discussion

Exposure of a cell to EP pulses produces a permeabilization of the membrane lipid bilayer to molecules which otherwise are nonpermeant. Depending on the electric pulses characteristics and conditions of permeabilization (buffer conductivity, temperature, cell dimension, culture density, etc.) the exchange of molecules across the membrane may last various time intervals, finally the membrane recovering its status of impermeability [6,36–38]. In this situation, electroporation is called reversible, the cell keeping a good viability. If the permeability of the membrane lasts long enough to exceed the cellular capacity to recover, the process is called irreversible electroporation and leads to the cell disintegration [39]. It is considered that exchanges of molecules begin during the pulses application [7] and continues for milliseconds (in case of monoatomic ions) up to minutes and even hours, for larger molecules. Water molecules are the main and fastest elements crossing the permeabilized membrane [40]. During the pulses, the transport of charged particles is mainly electrophoretic, while after the pulses, the transport is diffusive, being driven by the electrochemical gradients [41]. One can expect that all these transport processes modify the optical properties of the cell, as well as the cell shape and size. DHM can be thus an appropriate technique for studying the kinetics of such processes at the single cell level, by analysis of QPIs.

QPIs of single cell, electroporated with different pulses amplitudes, were reconstructed and analyzed. Two approaches were used: 1/analyzing the parameters extracted from QPIs as global parameters computed on the whole cell, and 2/ analyzing the phase shift fluctuations computed in significant areas of interest within the cell image.

3.1. Time series of QPIs of electroporated cells

Figure 4 presents some of the QPI frames from the time series representative for cells which were electroporated. In the first three rows are shown the cells electroporated using pulses of 0.6kV/cm, 0.8kV/cm and 1.0kV/cm. In the last two rows, are presented the cells electroporated at 1.0kV/cm, which manifest exceptional behavior of swelling and collapse. This behavior is a consequence of the osmotic swelling of the cells (the fixed macromolecules in the cytosol are “attracting” the water from outside) and they eventually collapse due to a membrane breakdown.

The cells electroporated at 0.6 and 0.8kV/cm (pulses amplitudes characteristic for reversible electroporation) present no modifications of cell shape or significant variations of pixel intensities to be observed at naked eye inspection. However, when higher pulses amplitude is used (1.0kV/cm), in some cases, a major cellular damage is observed. Figure 4 (4th row) shows obvious shape modifications - the cell swells and its edges are fading - or, in other cases (Fig. 4 last row), the cell completely loses its content (the cell membrane breaks). A computational analysis of phase shift values which would “sense” the cell changes induced by EP pulses, would facilitate the access to quantitative estimation of these changes.

3.2. Global parameters

While the normalized PA of Controls remains approximately constant (black trace), for cells electroporated at 0.6 and 0.8kV/cm, PA slowly decreases (red and green traces, respectively); for 1.0kV/cm, the cells response is diverse: some cells present a decrease in PA (dark blue trace), while others have an important increase of PA (light blue traces) (Fig. 5(a)). Our interpretation of this behavior is the following: for cells electroporated with low EP fields and some of those exposed to 1.0kV/cm, due to the permeabilization of the cell membrane, mannitol and water are penetrating the cell in an attempt to compensate the osmotic imbalance for fixed cytoplasmic macromolecules, the cell will increase in height, which leads to a slight decrease of the projected area. At 1.0kV/cm, if the degree of permeabilization is higher, due to cellular particularities, the cell will swell in all dimensions and the PA will increase (light blue long trace). If the PA
increasing rate is very high, the cell collapses, probably due to their limited possibilities to compensate the membrane expansion (light blue short trace).

Dry mass is also sensitive to the application of EP pulses: shortly after the application of pulses, it decreases for all pulses amplitude (data not shown), except the case of swelling or collapsing cells, when dry mass increases. By consequence, DMd, as the ratio of dry mass to PA, remains quasi-constant, with the same notable exception of the swelling or collapsing cells (Fig. 5(b)). In the latter case, the PA increase due to water penetration prevails on the dry mass increase due to mannitol entering the cell; hence the ratio dry mass/PA decreases.

The global parameter called entropy (En) is a measure of the degree of homogeneity in term of phase shift values present within a cell QPI. As can be seen in Fig. 5(c), En remains unchanged no matter the applied voltage, with exception of cells electroporated with 1.0kV/cm which swell or collapse. These cells show a fast and pronounced increase of entropy immediately after the pulses application. The cells with the highest rate of entropy increase eventually collapse: the water penetrates the cell, the highest values of the phase shift disappear and the phase shifts distribution flattens.

Similar results were obtained by Calin et al. [29] in experiments performed in the same conditions of EP, the cells being monitored for longer period of time, but using a lower time resolution method. The PA parameter was decreasing in the first 2 minutes after EP, similarly to what have been obtained in the present work (the decreasing evolution of red, green and dark blue traces in Fig. 5(a)). The parameter named optical phase shift (OPS) in [29] is the equivalent to the DMd parameter in the present study and behaves similarly for the 1kV/cm electroporated cells (dark blue line in Fig. 5(b)): slightly decreases in the 90 seconds post-pulse period.
Fig. 5. Time evolution of several global parameters computed from QPIs. All parameters were normalized with respect to their initial values: (a) projected area (PA), (b) dry mass density (DMd), and (c) entropy (En) for Controls and cells exposed to different pulses amplitudes.

3.3. Autocorrelations

A qualitative analysis of the autocorrelation functions (Fig. 6) leads to the observation that for the CC, the curves are gathered in a closer bunch while for the MPs, the values of the autocorrelation functions are more dependent on the EP amplitudes.

By applying monoexponential fit on each autocorrelation function, initial values (at $\tau=0$) and relaxation times ($\tau^*$) were obtained; their averaged values are represented in Fig. 7.

The initial values are normalized (with respect to the corresponding values of the Controls) in order to better visualize their behavior as a function of EP amplitudes (Fig. 7(a)). One may see that these values decrease in all regions with increasing the EP amplitudes. For midpoint regions this behavior is more accentuated when the pulses amplitude is 0.8 or 1.0kV/cm.

The relaxation times (Fig. 7(b)) for the CC region are independent of the applied pulses amplitudes, while for the MP1 and MP2 regions, the relaxation times have higher values and decrease with the increasing EP amplitudes. To interpret our observations, some considerations regarding autocorrelation functions are needed.

In general, low relaxation times indicate a fast loss of the autocorrelation, which is associated to a behavior closer to a “random” fluctuation, while a high relaxation time, corresponding to a slow loss of the autocorrelation, implies that the signal fluctuates in a “more ordered” manner. The analysis of the fluctuation signals may be performed either by methods based on the fluctuations amplitude or by using the autocorrelation functions. There are reports about differences in fluctuations of the phase shift in different regions of a cell, depending on the orientation of the cellular membrane with respect to the optical axis of the microscope, by using the analysis of the fluctuations amplitude. For instance, Rappaz et al. [25] have observed, on red blood cells, significantly higher amplitudes of the phase shift fluctuations in regions where the membrane was “more parallel” to the optical axis, because in these regions, small lateral displacements of the cell
Fig. 6. Averaged time autocorrelation functions of the phase shift fluctuations for different EP amplitudes computed on the three selected areas: (a) central region (CC), (b) and (c) MP1 and MP2 (as defined in Fig. 3).

Fig. 7. Averaged parameters of the monoexponential fit of the autocorrelation functions represented vs. pulses amplitude for the cell center region (CC) and the two mid-points (MP1 and MP2): (a) the initial values, (b) the relaxation times.
membrane have a strong impact on the phase. Similarly, Yamauchi et al. [42] reported that, on attached MCF-7 breast cancer cells, higher amplitudes of fluctuations were observed in regions where the cell membrane general plane was closer to be parallel with respect to the optical axis. On the other hand, Monzel et al. [43] used the autocorrelation functions for analyzing membrane fluctuations of human red blood cells in experiments based on dynamic optical displacement spectroscopy; they found higher relaxation times of the autocorrelation function in regions where the membrane was bended, compared to regions where the membrane was flat, which is in good agreement with our observations on Control cells.

When comparing the relaxation time values for the CC and MPs regions, after the electric pulses application, one must consider that the effect of the electric field in the center of the cell is less important than the one in lateral regions, due to the orientation of the cell membrane general plane against the direction of the applied electric field: in the center, the membrane is almost “parallel”, while in MP1 and MP2 the membrane is almost “perpendicular” to the electric field direction (Fig. 3(b)). It is well documented [39] that in the latter case, the EP voltage induces a much higher transmembrane potential, the electropermeabilization is much more efficient and the disturbance of the membrane is expected to be more important. This situation is clearly observed in our experiments: the higher the pulses amplitude, the lower the relaxation times at MPs (Fig. 7(b)), tending to attain membrane fluctuations similar to those in the CC. The perturbation due to the electric pulses induces a degree of “randomness” in the membrane fluctuations in the MP regions.

The novelty of our method consists in using autocorrelation analysis on time-series of quantitative phase images obtained from cells which underwent a certain perturbation (in our case, application of EP pulses). There are previous studies employing DHM to monitor dynamics of cell growth [44], cells presenting spontaneous activity as response to temperature variation (cardiomyocytes [24]) or responding to various chemical stimuli (HEK cells or neurons [23]). These studies use different approaches for data processing (mainly based on spatiotemporal mean squared displacement of the phase shift) than in our work. On the other hand, membrane dynamics have been analyzed using autocorrelation functions, not in conjunction with DHM but with different experimental techniques (wide field [45] or confocal microscopy [43]).

4. Conclusions

The 90 seconds long evolution of the global cell parameters (PA, DMd and En) was an expected one in case of the cells electroporated with 0.6 and 0.8kV/cm: PA slightly decreased, DMd and En remained constant. When increasing the pulses amplitude to 1kV/cm, these parameters either kept the same behavior (for those cells which kept their integrity) or showed an exceptional evolution in the case of cells which presented an accentuated swelling or collapsed. In the latter case, the cells fate may thus be anticipated based on the high rate of evolution of these parameters within the first 10-15 seconds after pulses delivery. Further work is necessary to find out whether there is a threshold of the global parameters evolution rate which would discriminate between reversible and irreversible electroporation, or to correlate the evolution of global parameters with the cell size and its position relative to the electric field direction.

The analysis of time autocorrelation functions of the phase fluctuations is a novel approach in DHM as well as in the field of electroporation which allows to reveal details of cellular response to EP in different regions. It was found that the autocorrelation functions for those regions which are less prone to electropermeabilization, are not affected by the EP pulses while those corresponding to regions in which the membrane permeabilization is favored, are strongly modified by EP pulses.

The novelty of our method consists in using autocorrelation analysis on time-series of quantitative phase images obtained from cells which underwent a certain perturbation (in our case, application of EP pulses). Previous studies using DHM as a dynamic approach were applied on
cells in a stationary state, the cells being exposed to different conditions. In our study DHM was used to monitor the cellular “relaxation” subsequent to the stress generated by the electroporation pulses. To the best of our knowledge, it is the first time that the autocorrelation functions are used for analyzing QPIs of electroporated cells. Thus, the valuable advantage of DHM to capture fast processes may be fully exploited. Moreover, DHM showed to be a powerful technique for studying cell electroporation without any disturbance of the cell status.

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