Bioelectrochemistry 103 (2015) 39–43

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

# Image processing for non-ratiometric measurement of membrane voltage using fluorescent reporters and pulsed laser illumination

Aude Silve *, Sarah Rocke, Wolfgang Frey *

*Institute for Pulsed Power and Microwave Technology, Karlsruhe Institute of Technology, Hermann-von-Helmholtz-Platz 1, Eggenstein-Leopoldshafen 76344, Germany*

ARTICLE INFO

*Article history:*
Received 16 January 2014
Accepted 9 July 2014
Available online 23 July 2014

*Keywords:*
Transmembrane voltage
Transmembrane potential
ANNINE-6
Image processing
Intensity fluctuations

ABSTRACT

The measurement of transmembrane voltages induced by pulsed electric field exposure can be achieved by using fluorescent dyes like ANNINE-6. Such approach requires a quantitative determination of the fluorescence intensity along the cell's membrane by image processing. When high temporal resolution is required, the illumination source is frequently a dye-laser which causes high fluctuations in the intensity of illumination which in turn affects the fluorescence intensity and thus the quality of the results. We propose an image processing technique that allows to overcome the fluctuations and to produce quantitative data. It uses the optical background noise as a correcting factor. Standard deviation in the fluctuations is thus efficiently reduced by at least a factor of 2.5. Additionally we draw attention to the fact that the parasitic component of the laser radiation (ASE) can also suppress fluctuations although it deteriorates wavelength precision.

© 2014 Elsevier B.V. All rights reserved.

## 1. Introduction

Electrical signals such as transmembrane voltages (TMVs) are essential to cells and living organisms in general. They are known to play an important role in development, regeneration and cancer growth [1,2]. Most recent discoveries on the role of transmembrane voltage were made possible by the use of fluorescent reporters [3,4]. When driven beyond physiological values, TMV can also act as physical stress and modify properties of cell plasma membrane. This can be achieved by applying an external electric field. Indeed, when a biological cell is submitted to a uniform external electric field, a locally non-uniform transmembrane voltage is induced along the membrane. This can be theoretically predicted by solving the Laplace equation [5–7] and was experimentally verified using fluorescent voltage reporters inserted into the membrane [8–11]. The supraphysiological transmembrane voltages resulting from pulsed electric field exposure trigger an increase of cell membrane permeability, a phenomenon known as electropermeabilization (also known as electroporation) [12].

Since it has first been established [13,14] electropermeabilization has been intensively studied and has led to the development of several applications for example in oncology [15,16] or in the food-industry [17, 18]. Traditionally the electric field was generated by applying electric pulses with durations of a few µs up to a few ms. More recently, cells have been challenged with much shorter electric pulses with durations of a few ns. Such short electric pulses also affect the permeability of cell plasma membrane [19–23] and additionally can induce intracellular effects [24,25]. They are also known to modulate TMV of cells. This can be qualitatively observed by using fluorescent voltage reporters and an experimental setup suitable for observing temporal variations in the nanosecond range. Such setups, using nanosecond lasers as illumination sources have already been established for TMV dynamic measurements [26,11]. In these experiments, the TMP measurements are non-ratiometric and the quantitative measurement of the intensity change related to the TMV which is induced by the electric field therefore requires a precise comparison of the level of fluorescence originating from two subsequent images of the same cell (see Fig. 1). The first image is acquired without electric field and provides the azimuthal fluorescence distribution $F_0$ and the second one gives information about the fluorescence F during the electric pulse. Usually the ratio of the two fluorescence signals $F/F_0$ along the membrane is then converted in a TMV value using the appropriate calibration curve of the fluorescence reporter [11]. We do not discuss the calibration in this paper but will focus on image acquisition and processing for exact determination of fluorescence ratios $F/F_0$. In particular, we draw attention to fluctuations of illumination intensity from image to image.

In experiments which require low temporal resolution (in the microsecond range), illumination can be continuously performed with fluorescent lamps which are sufficiently stable from image to image. However, the new setups that have been developed to achieve temporal resolution in the nanosecond range use pulsed laser light as an illumination source. In such a setup, the temporal resolution of the measurement is equal to the duration of the exciting laser pulse. With a standard nanosecond dye laser (which are most commonly used), it is about 3 ns, which is particularly well suited to observe TMV evolution

* Corresponding authors.
*E-mail addresses:* aude.silve@kit.edu (A. Silve), wolfgang.frey@kit.edu (W. Frey).

http://dx.doi.org/10.1016/j.bioelechem.2014.07.004
1567-5394/© 2014 Elsevier B.V. All rights reserved.

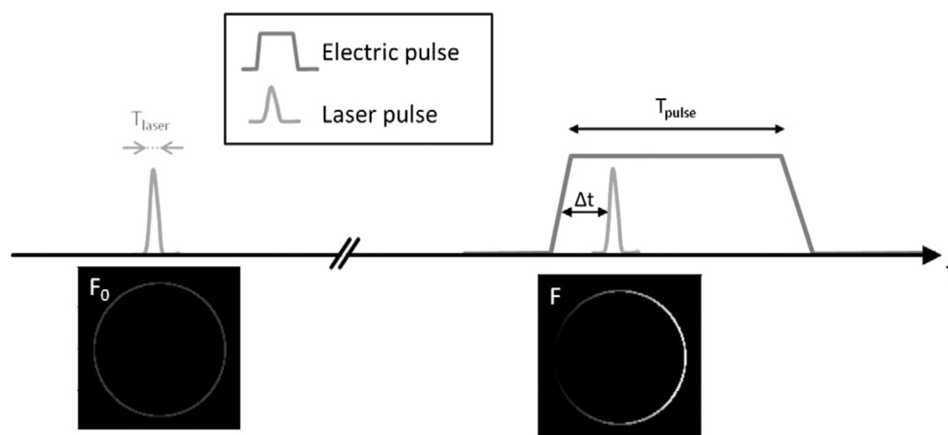


**Fig. 1.** Principle of an experiment for measurement of TMV. A first image is acquired previous to pulse exposure. It provides the basal fluorescent level $F_0$ along the membrane. A second image is acquired during (or after) pulse delivery. It provides the fluorescent level $F$, representative for the effect of the electric field pulse. The ratio of the two images can be analyzed to provide qualitative and quantitative information on the modulation of the TMV by the pulsed electric field.

during electric pulses with durations on the 100 ns time scale. In this type of laser, the lasing medium is an organic dye dissolved in a liquid solvent (dye solution). The main benefit of such lasers is to allow adjustment of wavelength. Moreover, a very narrow bandwidth of the laser pulse can easily be obtained by tuning the oscillator grating. The major drawback is that nanosecond dye lasers are well known to have fluctuating output energy from pulse to pulse. If this fluctuation is not corrected, it results in distortion of results.

## 2. Material and methods

### 2.1. Cell culture

Chinese hamster lung cell line – DC-3F – was grown in MEM — Minimum Essential Medium with the addition of 10% fetal bovine serum and supplemented with antibiotics (500 U/mL penicillin and 500 µg/mL streptomycin). The cells were maintained in a humidified atmosphere at 37 °C and 5% $CO_2$. Cells were routinely passed every 2 days. All medium and products for cell culture were purchased from Life technologies.

### 2.2. Preparation of ANNINE-6

ANNINE-6 (Sensitive Dyes, Germany) was dissolved at 0.5 mg/mL in 20% Pluronic F-127 DMSO (Molecular Probes, CA, USA) and sonicated for 5 min [27]. The stock solution was kept at −20 °C.

### 2.3. Cell staining

After trypsinization of exponentially growing cells and inactivation of the trypsin (ref 25300054 Technologies Gmb — VWR) with complete medium, the cells were centrifuged for 5 min at 400 *g* at 4 °C. The cell pellet was resuspended in ice cold PBS buffer (14200083 Technologies Gmb — VWR) and centrifuged with the same settings. The cell pellet was resuspended in ice cold PBS buffer at $2.5 \cdot 10^6$ cells/mL. 300 µL of the cell suspension was supplemented with 10 µL of ANNINE6 stock solution (final ANNINE6 concentration: 28 µM) and left for 7 min at 4 °C. After the waiting time the stained cells were centrifuged and the cell pellet was resuspended in 800 µL of ice cold PBS buffer and kept on ice.

### 2.4. Imaging setup

Stained cells were illuminated with a 5 ns (full width half maximum) laser pulse delivered by a Nd:YAG pumped dye laser (Narrowscan, Radiant Dyes Laser Accessories GmbH, Wermelskirchen, Germany). To provide the excitation wavelength of 468 nm, Coumarin 47 dissolved in ethanol (concentration: 0.25 g/mL) was used as an active laser medium in the dye laser. The laser pulse was coupled into the upright fluorescence microscope (Axioplan, Zeiss, Jena, Germany) via a fiber optic cable. After passing a laser clean-up filter (D470/40) and a dichroïc mirror (490 DCXR), the light was partially focused on the cells. The fluorescence light from the cells was acquired with a 63× objective (Zeiss, LD Plan Neofluar). It passed an emission filter (HQ 610/100) and was recorded by a cooled 12-bit iCCD camera (PCO DiCAM Pro, PCO AG, Kelheim, Germany).

### 2.5. Exposure to pulsed electric field

A few µL of stained cells solution were transferred into a 'microgap' which consists in two stainless steel electrodes 300 µm apart (and directly connected to the generator) that had been glued on a microscope slide. The glue layer was thinner than 5 µm, which permits a uniform distribution of the electric field to cells adjacent to the bottom of the microgap [28]. The thickness of the electrodes was 100 µm. This microgap was placed into a gap receptacle fixed to the stage of the microscope. A coverslip was placed over the gap and sealed with paraffin oil to avoid cell movement by evaporation of the buffer. Note that the microgap was used in all experiments described in the present paper, even when no electric pulse was applied, in order to insure constant illumination conditions.

## 3. Image processing method

### 3.1. Measurement of fluorescence value

All images are corrected for electronic noise first. The electronic noise is acquired separately with camera shutter closed. It is perfectly stable from one image to another and stable with time as expected from a camera cooled at −12 °C. An average value of electronic noise was computed by averaging all pixels and this value was subtracted from all pixel values of the images analyzed subsequently. One should note that in principle, the electronic noise can also be removed pixel by pixel (for each pixel of an image being analyzed, the electronic noise of this precise pixel can be subtracted). However, since the electronic noise was extremely flat among pixels, subtracting individual pixel values had the same impact as subtracting an average value from all pixels.

After removal of electronic noise, the average optical noise and the signal from cell membrane are extracted (Fig. 2). The average optical background value N is obtained by averaging pixel values on a large area (at least ten cell areas) far away (at least two cell distances) from

Download English Version:

https://daneshyari.com/en/article/1267899

Download Persian Version:

https://daneshyari.com/article/1267899

Daneshyari.com