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Determination of Resting Membrane Potential of Individual Neuroblastoma Cells (IMR-32) Using a Potentiometric Dye (TMRM) and Confocal Microscopy

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The potentiometric dye, Tetramethylrhodamine methyl ester (TMRM) has been extensively used with fluorometry or optical microscopy to evaluate the electric potential across plasma or mitochondrial membranes. We present here a TMRM confocal microscopy-based potential measurement technique. Corrections are introduced to minimize nonspecific dye binding and insensitivity to low background levels. We have used this technique to compare the resting membrane potential of proliferating and differentiated human neuroblastoma cells (IMR-32).

KEY WORDS: Resting membrane potential; Tetramethylrhodamine ester; confocal microscopy; neuroblastoma; IMR-32.

INTRODUCTION

The resting membrane potential (V_m) is a critical property of neuronal cells. Maintenance of an appropriate V_m is vital for maintaining physiological functions such as action potential propagation, cell signaling and ion channel gating. V_m can be measured by traditional patch clamp methods or by fluorescence spectroscopic techniques. Fluorescence techniques have gained popularity over the past decade due to the development of fluorescent dyes with improved sensitivity and biocompatibility in addition to the emergence of spectroscopic and microscopic instruments for signal identification and quantification [1]. There are a variety of V_m-sensitive fluorescent dyes, of which the oxonols [2,3] and rhodamine derivatives [4,5] are the most widely used. We have previously evaluated the V_m of neuroblastoma cell lines using oxonol dyes with flow cytometry [3,6]. However, due to interactions between oxonols and the potassium ionophore, valinomycin, it was not possible to correct for oxonol nonspecific binding. The results were only useful for qualitative comparison purposes of large populations of cells. On the contrary, rhodamine derivatives do not interact with valinomycin; cells can be depolarized to correct for the errors introduced by non-specific binding. Additionally, it is possible to determine individual cell V_m via microscopic techniques, since an individual cell can be visually tracked throughout the entire measurement and correction process.

Confocal laser scanning microscopy is an ideal candidate for V_m measurement because the out-of-focus blurring is essentially absent from confocal images, which enhances the quality and/or resolution of the images obtained. However, due to current confocal microscopy instrumentation and the nature of Nernstian dyes, the quantitative determination of V_m can be accomplished only after a series of corrections. In this paper, we describe such corrections for a Nernstian dye; Tetramethylrhodamine methyl ester (TMRM) used with confocal laser scanning microscopy for determining the V_m of proliferating and differentiated human neuroblastoma cells (IMR-32).

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MATERIALS AND METHODS

V_m Measurement Principle

TMRM is a lipophilic potentiometric dye. After loading, it undergoes a re-distribution across plasma membrane of individual cells following the Nernst equation:

$$V_m = -2.3\left(\frac{RT}{ZF}\right)\log_{10}\left(\frac{D_{in}}{D_{out}}\right) \quad (1)$$

where V_m is the resting membrane potential in mV; Z is the number of charge on TMRM ion, which is equal to 1; F is the Faraday's constant, R is the ideal gas constant; T is the absolute temperature and D_{in} and D_{out} represent the concentration of the dye at the intracellular and extracellular side of the plasma membrane, respectively. With confocal optics, the concentration of the dye, D, is proportional to the fluorescent intensity F. Therefore, at room temperature, the above equation can be rewritten in such a way that V_m is a sole function of the ratio of intracellular fluorescent intensity to extracellular fluorescent intensity, F_{in}/F_{out}:

$$V_m = -58\log_{10}\left(\frac{F_{in}}{F_{out}}\right),\ \text{mV} \quad (2)$$

Ideally, V_m can simply be determined through measurement of intracellular and extracellular fluorescent intensities after dye loading. However, both the dye and the imaging system have limitations that call for corrections in order to obtain accurate V_m values.

Most potentiometric dyes exhibit a non-potential dependent affinity for cell components. Although TMRM is among those exhibiting the lowest non-specific affinity, the interference is too significant to be ignored. Without correction the V_m value can be overestimated by at least 20% [7]. To address this problem, cells were depolarized to $V_m = 0$ through incubation with the potassium ionophore, valinomycin. The ratio of intracellular fluorescent intensity to extracellular fluorescent intensity in the completely depolarized state, F_{in_free}/F_{out_free}, was measured and used to correct for the membrane potential value obtained from Eq. (2):

$$V_m = -58\log_{10}\left(\frac{F_{in}\cdot F_{out\_free}}{F_{out}\cdot F_{in\_free}}\right),\ \text{mV} \quad (3)$$

The fluorescent intensity was captured by a photomultiplier tube, which re-creates the image by converting the light signal into a raster scanning pattern with each pixel in the image ranging from 0 to 255 artificial fluorescent units. Regions of interest (ROIs) were defined via the image processor's cursor controls. An ROI was defined inside individual cells within the cytosolic or nuclear area where the non-specific binding is lowest. Extracellular area contained a large pool of evenly distributed dye and ROIs were defined in regions free of cells and any glowing debris. Typically, the black and gain level of the imaging system were set in such a way that the intracellular fluorescent intensity was well below the maximum value for an accurate measurement. Under such circumstances the extracellular fluorescent intensities F_{out} and F_{out_free} were very low and beyond the sensitivity of the detection devices, thus introducing a large error in the calculated V_m value. This problem has not been addressed by other investigators, who have described similar methods [8,9]. We address this problem herein by using a more powerful excitation light to elevate the extracellular fluorescent intensities to a value that can be accurately quantified. Thus the measurement of intracellular and extracellular fluorescent intensities were physically separated with the latter being excited with 10-fold power achieved by adjusting the excitation neutral density filter from 10 to 100%. It has been shown that when the concentration of the fluorescent dye is low, the fluorescent intensity F is a function of the power of excitation beam P_0:

$$F = 2.3K'AP_0 \quad (4)$$

where K' is a constant depending on the quantum efficiency of the fluorescence and A is the absorbance of medium the excitation beam traverses. Based on Beer's Law, K' and A are fixed for the same dish of cells. Therefore, the fluorescent intensity resulting from with 100% illumination is 10 times that with 10% illumination.

Combining Eqs. (3) and (4), the V_m after correcting for both limitations from the dye and the imaging system came to:

$$V_m = -58\log_{10}\left(\frac{F_{in}^{10\%}\cdot\frac{F_{out\_free}^{100\%}}{10}}{\frac{F_{out}^{100\%}}{10}\cdot F_{in\_free}^{10\%}}\right) = -58\log_{10}\left(\frac{F_{in}^{10\%}\cdot F_{out\_free}^{100\%}}{F_{out}^{100\%}\cdot F_{in\_free}^{10\%}}\right),\ \text{mV} \quad (5)$$

When all parameters in Eq. (5) were measured, an additional petri dish filled with HEPES buffered saline was required to acquire the background values, B, at equivalent black and gain settings under two different excitation powers. To yield true values, all gray level data were corrected by deducting background values. The final working equation for determining the resting membrane potential of individual cells was:

$$V_m = -58\log_{10} \times\left[\frac{\left(F_{in}^{10\%}-B^{10\%}\right)\cdot\left(F_{out\_free}^{100\%}-B^{100\%}\right)}{\left(F_{out}^{100\%}-B^{100\%}\right)\cdot\left(F_{in\_free}^{10\%}-B^{10\%}\right)}\right],\ \text{mV} \quad (6)$$

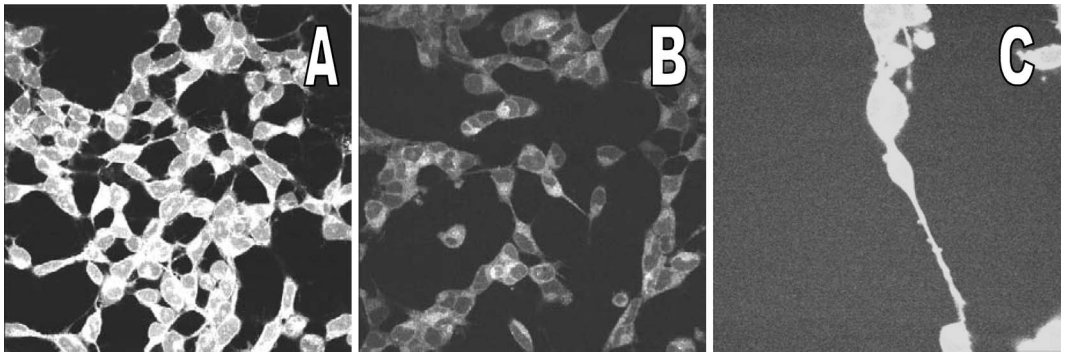

Fig. 1. Confocal images of IMR-32 cells loaded with 0.5 μM TMRM. Nuclear regions exhibited the lowest and more uniform fluorescence and therefore were chosen for resting membrane potential quantification. Non-depolarized cells (A) exhibited stronger intracellular fluorescence than depolarized cells (B), reflecting significant non-voltage-dependent TMRM binding. Excitation with stronger power saturated the inracellular fluorescence (C), but allowed for accurate measurement of extracellular fluorescence intensity.

Cell Culture, Dye Loading and V_m Determination

The IMR-32 neuroblastoma cell line was obtained from ATCC and was routinely cultured in 75-cm^2 tissue culture flask (Costar, Cambridge, MA) with 25 mL growth medium at 37°C in a 10% CO_2 humidified atmosphere. The growth medium was made with Eagle Minimum Essential Medium (MEM) containing 10% heat inactivated fetal bovine serum (FBS), 2.2 g/L sodium bicarbonate, 2 mM L-glutamine and 1 mM sodium pyruvate [9]. Growth medium was replaced every other day. At 75% confluence, cells were split 1:2 through detachment via 0.1% trypsin, pelleting and re-suspension. To induce differentiation, cells were harvested and re-plated on No. 1.5 glass coverslips embedded in a 35-mm petri dish (MatTek Co., Ashland, MA) at 5×10^5 cells per coverslip. Two days after plating, growth medium was replaced with 1 mL differentiation medium comprised of MEM with 5% heat inactivated fetal bovine serum, 2.2 g/L sodium bicarbonate, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM dibutyryl cAMP and 2.5 μM 5-bromodeoxyuridine [10,11]. Differentiation medium was changed on a daily basis. For TMRM staining, cells plated on No. 1.5 glass coverslip were washed with HEPES buffered saline (HBS) twice and incubated with 2 mL HBS containing 0.5 μM TMRM for 20 min at 37°C in humidified incubator with 10% CO_2.

A confocal imaging system (PCM-2000, Nikon, Melville, NY) equipped with high-performance detection and control electronics. The imaging system was linked to an inverted microscope (TE300, Nikon), equipped with a X60 Apochromat, oil-immersion, high-numerical aperture (1.40) objective lens. The imaging system's helium/neon mixed gas laser (GreenHeNe) was used to excite the fluorescent dye. Individual cells were positioned with inverted bright-field microscope light path. Cells were then scanned at 548 nm with the GreenHeNe laser along the z-axis (optical slices) covering the entire height of the sample to select the slice offering the greatest cytoplasm area. The resulting fluorescent signals were captured through a 565 nm long-pass filter (BA1, Nikon, Melville, NY) by a photomultiplier detector. An imaging processing software, SimplePCI (Compix Inc. Cranberry Township, PA), was used to select ROIs and determine the mean gray level in ROIs.

RESULTS AND DISCUSSION

Typical confocal images of dye-loaded cells are shown in Fig. 1. As shown in Fig. 1A, TMRM stained the cell cytosol as well as intracellular organelles. The nuclear region was stained to the least extent, suggesting that the area demonstrated the lowest non-potential dependence binding [7]. Therefore, the measurement of intracellular fluorescence intensity $F_{\text{in}}^{10\%}$ was made at these relatively darker and uniformly stained regions. Assessment of non-potential dependent binding $F_{\text{in\_free}}^{10\%}$ was made on the same regions of cells incubated with 0.5 μM TMRM dye in depolarizing HEPES buffered saline (HBS) containing 130 mM KCl, 5.5 m NaCl and 1 μM valinomycin for 10 min (12) (Fig. 1B). Extracellular fluorescence, $F_{\text{out}}^{100\%}$ and $F_{\text{out\_free}}^{100\%}$, which are considerably weaker than intracellular fluorescence, were measured with elevated excitation power from regions free of cells and any glowing debris (Fig. 1C). The resting membrane potential of individual cells were obtained by plugging these measurement values into Eq. 6.

V_m histograms from undifferentiated (day 2) and differentiated (day 13) IMR-32 cells are presented in

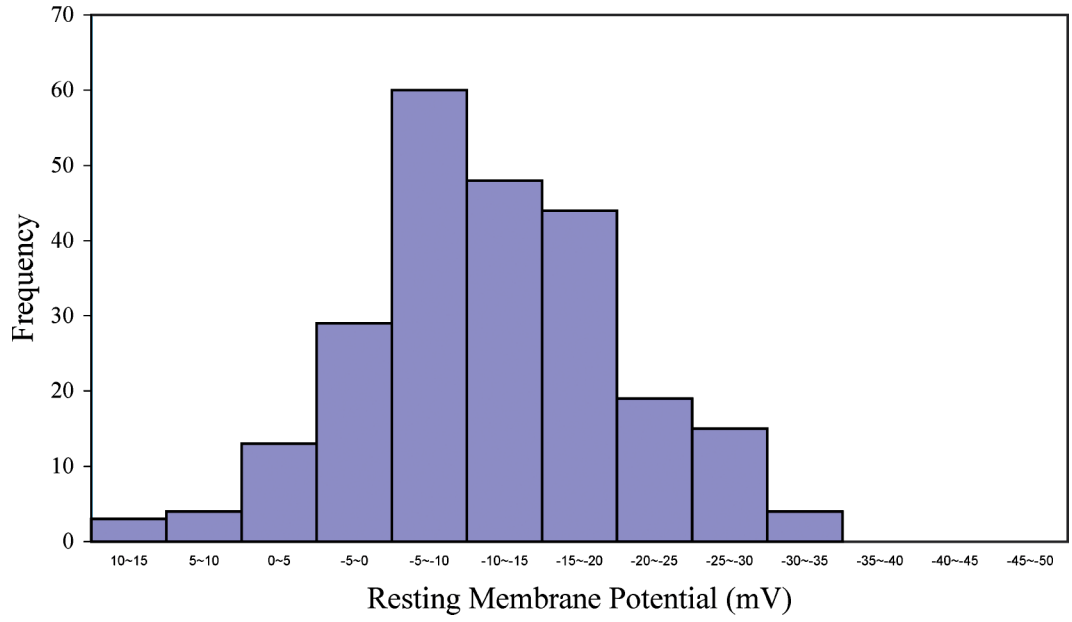

Fig. 2. Histogram of the resting membrane potential of non-differentiated (Day 2) IMR-32 neuroblastoma cells ($n = 240$). Data were obtained by confocal microscopy.

Figs. 2 and 3, respectively. The nonparametric Two-sample Kolmogorov-Smirnov test was used to test whether the V_m distributions were identical between cells in different stages. The test was based on the null hypothesis H_0: the two histograms are identically distributed versus the alternative hypothesis H_1: the two histograms are not identically distributed, and H_0 is rejected at 5% level.

The p-values for comparison between day 2 (undifferentiated) and day 13 (differentiated) cells (0.9992) suggested that there was no significant V_m development for differentiated cells. However, comparisons of the mean V_m values for day 2 cells (-11.37 mV) versus day 13 cells (-18.68 mV) revealed a hyperpolarization following differentiation that is too small to be detected by the non-parametric test. This finding is consistent with results

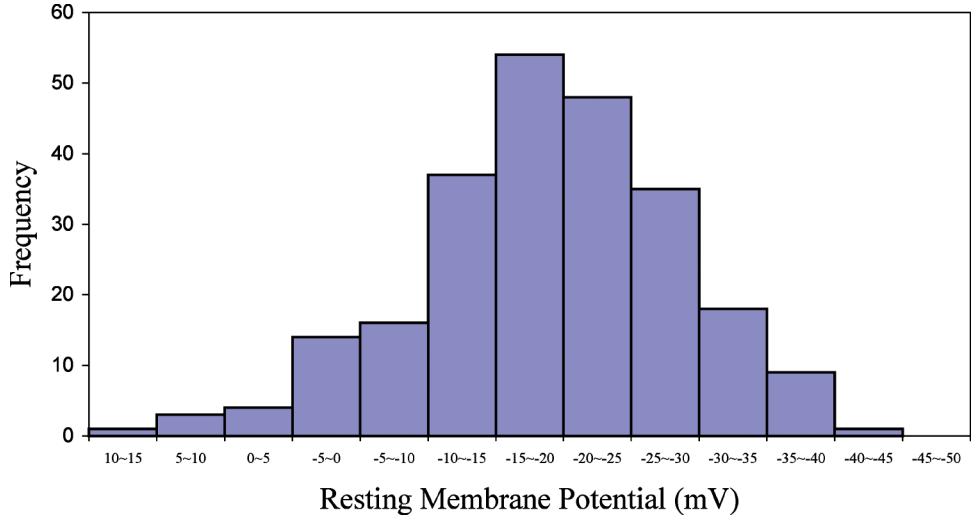

Fig. 3. Histogram of the resting membrane potential of differentiated cells (Day 13) IMR-32 neuroblastoma cells ($n = 240$). Data were obtained by confocal microscopy.

from a previous study in our laboratory from cells differentiated with 10 μM 5-bromodeoxyuridine [3]. This study showed that the IMR-32 cell line does not develop a V_m typical of excitable neurons (-60 to -90 mV). Since V_m arises due to maintenance of different ionic concentrations across the cell membrane (mainly potassium), further investigation into absence of V_m development in IMR-32 cells may be directed at the expression of potassium channels. The method has successfully used in collagen-immobilized neuroblastoma cell V_m measurement [13].

In conclusion, the application of fluorescent potentiometric dyes coupled with confocal optics, if adequately controlled and corrected, offers a convenient alternative to microelectrode-based techniques for quantitative determination of V_m at the single cell level.

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