The Investigation of the Empiric Fluorescence Concentration Depolarization Relation and Its Realization in the Evaluation of Fluorophors Concentration and Membrane Potential

This study deals with the investigation of the dependency of fluorescence polarization (FP) upon potential dye concentration in order to locally and temporarily monitor changes of mitochondrial membrane potential (MMP) via FP measurements.

Shortcomings in the evaluation of fluorophor concentration via fluorescence intensity are reviewed and an alternative approach, based on phenomenon of concentration depolarization of fluorescence (CDF), is proposed for the first time.

The phenomenon of CDF or self-depolarization has gone unresolved till today, even though it was reported already in the 20s and 30s of the last century. The most reliable experimental study of the phenomenon was published by Ph&Sv in 1940. It covers more than a three concentration decade of four fluorophors and has been accepted as fundamental. Therefore, a significant part of the present study is based on these measurements. As, to date, there is no single exact theory which predicts the dependency of FP upon fluorophor concentration, we deliberately chose to follow the experimental approach of Ph&Sv for both experimental and analytical investigation in this study. Based on the result obtained by analyzing our own results, as well as others' published results, a new empiric formula was proposed to describe CDF, based on Perrin's equation:

$$P_{origin}(C) = \frac{1}{a + (\mathrm{b} \cdot C)^n}$$

According to the proposed approach, CDF is non-linearly dependent on concentration. Furthermore, in a confined region we call the Linear Dynamic Range (LDR), this dependency can be expressed as a linear equation:

$$P_{ip}(\tilde{C}) = d - e\tilde{C}$$

Three approaches; numerical, geometric and analytical were applied in order to characterize this expression in numerous aspects.

Furthermore, this expression was utilized to convert the Nernst equation from a logarithmic concentration law, which describes the voltage across a membrane (membrane potential - MP) - $\Delta\psi_{MP}$ into a simple and user-friendly linear dependency of $\Delta\psi_{MP}$ upon the fluorescence polarization:

$$\Delta\psi_{MP} = \frac{R \cdot T \cdot \ln 10}{z \cdot F \cdot e}\left[P_{out}(C) - P_{in}(C)\right]$$

The proposed linear FP-based Nernst equation is valid for concentrations in the LDR. Thus, in order to make use of it, a novel method for the evaluation of cellular and subcellular fluorophor concentration was developed.

Employing this method, it was found that the mitochondrial averaged TMRM concentration is actually the mid value of the LDR measured for TMRM solutions. This clearly justifies the use of the linearized Nernst equation in evaluating MMP in U937 cells treated with and without CCCP, and similar biological assays.

This study is comprised of five Chapters.

Chapter 1 – Background: It shortly reviews the relevant physical and biophysical basis for this study. This includes principals of fluorescence, methods for measuring membrane potential and some biological aspects regarding the mitochondrion as well.

Chapter 2 – Theory: A critical review of the most established publications of CDF are given, and the numerical examination of their results as well. This is followed by proposing a novel approach in exploring the empirical aspect of CDF which is then thoroughly investigated and numerically experimented. The outcomes of this effort are numerous. For instance, the parameterization of Perrin's and Ph&S equations, the LDR, linearization of $P(C)$, development of the mathematical expressions for the errors ε ,

ξ and of $\tilde{C}_0$ - the mid value of the LDR, and the limits of the LDR under allowed error, etc. Finally, the FP based - linearization of the exponential Nernst law is presented.

Chapter 3 – Material and Methods: Lists the materials used and their sources. It describes experimental methods and measurement systems used in this work as well.

Chapter 4 – Measurement of fluorescence solution: Data of $P(C)$ of Fluorescein, Rhodamine 123 and Tetramethyl Rhodamine Methyl Ester in glycerin-PBS solutions, for the case of $T/\eta \neq 0$, are given.

Chapter 5 – Measurement of intact living cells: An experimental justification for the use of the linear FP-based Nernst equation in the evaluation of MMP, is given. This is done by showing that the assessed mitochondrial averaged TMRM concentration is actually the mid value of the LDR measured for TMRM solutions. This is followed by a series of mitochondria membrane potential measurements, which were realized on 50 single U937 cells, employing the developed linear FP-based Nernst equation.