A signal analysis based identification of protein's intracellular translocations via fluorescence polarization

In recent years, extensive investigations have been performed using live cell models in culture, both in basic research and drug development. Currently, the principal analytical technique is based on Image Analysis (IA) of cells subject to diverse physiological and pathological conditions. Nevertheless, IA is a very complex, subjective and slow process.

In contrast, in the course of the present study, I have developed a method based on Signal Analysis (SA) in order to identify biological processes in living cells generally, and changes in the sub-cellular location of proteins (translocation), induced by physiological or pathological conditions, in particular. The transition form Image Analysis to Signal Analysis is essential and can, for the first time, enable the estimation of the occurrence and/or non-occurrence of translocation in a High Throughput Screening (HTS) mode.

The proposed system is based on the following premises: the translocation of proteins during physiological and/or pathological conditions, normally involves local changes in the protein concentrations. Hence, if the proteins inherently possess a fluorescing compound, the concentration of the later will change accordingly and can affect its spectroscopic properties. The latter effects are, for the most part, detectable and therefore can provide a means to ascertain the event of intracellular protein translocation and perhaps even offer an instrument to trace such translocations. In this regard, two types of changes can be most conveniently measured after an increase in probe concentrations: the probe fluorescence intensity and fluorescence polarization.

In the present work, I focused on the translocations yielding protein concentrations high enough to enable Homo-Resonance Energy Transfer (homoRET) between accompanying probes.

To improve the probability of such energy transfers, I searched for a fluorescent probe having the following properties: as large as possible an overlap between the absorption and emission spectra of the probe, high quantum efficiency in the intracellular medium generally and in the environment of target proteins particularly, high specificity and effectiveness of binding to target proteins, low probe leakage from the cell, and fluorescence polarization that is insensitive to the host medium.

In view of these, I selected the fluorescent protein eGFP. The overlap between its absorption and emission spectra is high, the protein is suited for cell investigations and can be specifically attached to every target protein, and therefore it does not leak from the cell.

In order to confirm these properties, I carefully investigated the degree of sensitivity of the fluorescent protein eGFP to environmental factors, such as: viscosity, pH, etc. I found that, while these factors greatly influence fluorescence intensity measurements, fluorescence life time as well as rotational correlation time of eGFP, their effect on fluorescence polarization is marginal up to negligible.

Having confirmed these properties for free eGFP, I proceeded to measure intracellular eGFP. The work was performed on HEK 293 cells that express the fluorescent complex eGFP-PKCδ. The biological model involved the induction of translocation by C2-Ceramide. This leads to the translocation of the complex from the cytoplasm to the Golgi apparatus.

Initially, I confirmed the possibility that the cellular protein concentration is high enough to allow HomoRET. Briefly, I showed that the fluorescence polarization of the intracellular complex is lower than that of the same complex outside the cell in aqueous environment.

Using unique analytical tools that I have developed for this research, I found that in the cells where translocation occurred, there was an inverse correlation between fluorescence intensity and fluorescence polarization – starting from a definite fluorescence intensity value and further on. Figure A demonstrates this phenomenon, showing a representative, real time measurement of a cell where translocation took place after C2-ceramide exposure.

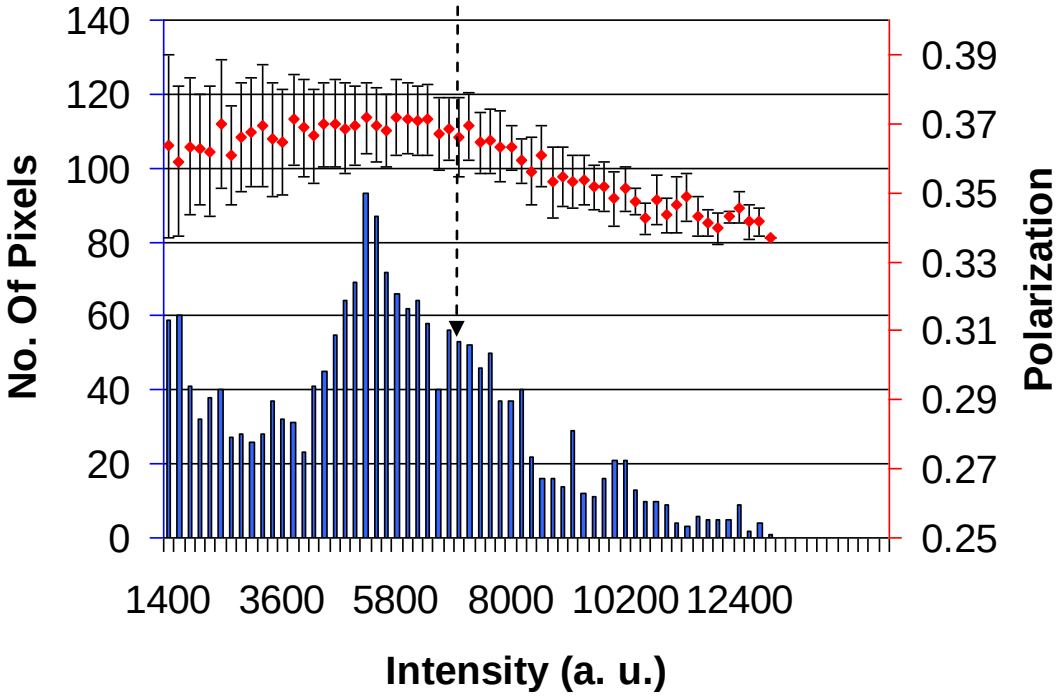

Figure A: Fluorescence intensity histogram (blue columns) obtained from a fluorescing cell after translocation induction. For each histogram column, average fluorescence polarization (red rhombuses) and standard deviations (bars) were calculated.

After obtaining the images of cell fluorescence intensity and calculating fluorescence polarization for each pixel, polarization values were grouped according to the intensity respective to each pixel. Such grouping yielded the results shown above in Figure A. As it can be seen, the blue columns represent the frequency of pixels in the cell according to their intensity. For each column in a given intensity area, I calculated the average polarization values relative to the corresponding pixels. Average polarization values are represented by the red rhombuses and the standard deviations of these polarization values are depicted by the bars. The figure demonstrates that in the intensity area left to the dashed vertical line, there is no correlation between the intensities (the abscissa) and polarization values. In other words, while polarization behaves as a constant value in this area, the intensity rises. This is not the case regarding the intensity to the right of the crossing line. Starting from that point on, there is an observable inverse correlation between the polarization and intensity values. The higher the intensity, the gradually lower is the degree of polarization. This result indicates the possibility that, starting from the point where the

derivative of the polarization curve becomes negative, there is a significantly enhanced probability for HomoRET occurrence.

To quantify this result, I used Pearson's correlation. First, there was calculated the correlation between the intensity and polarization for each cell in which translocation occurred and/or not. In the second stage, the average correlation and its standard deviation were calculated for each group. The latter results are shown in Figure B. The pair of columns to the left show that before the translocation induction the average correlation between the intensity and polarization (as obtained in over 50 cells) tends to zero. On the other hand, after translocation induction (the pair of columns to the right), very notably, the average correlation between the intensity and polarization is indeed high and negative (for the cells that underwent translocation) and marginal in the control group (in the cells that did not undergo translocation).

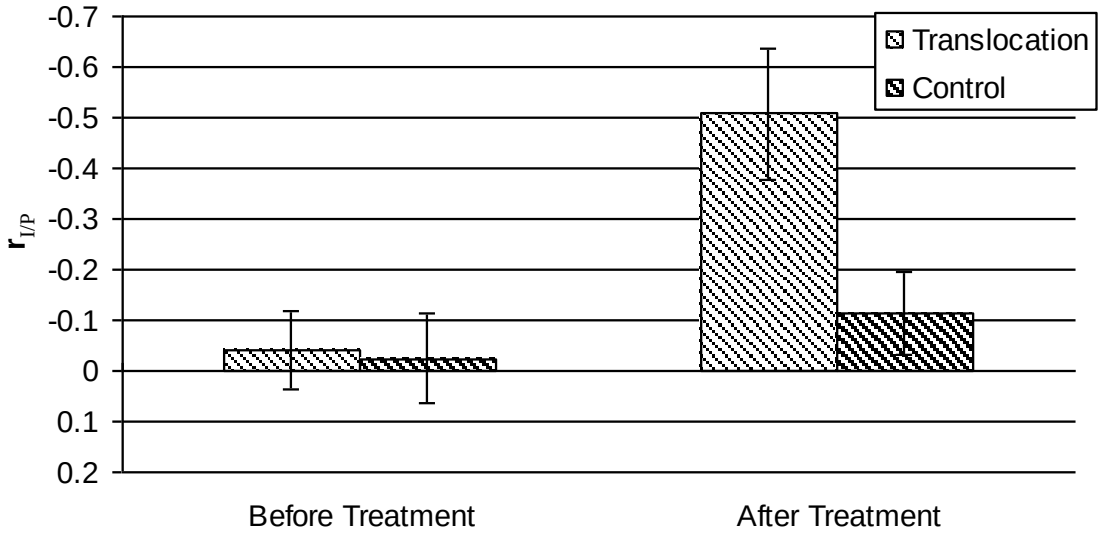

Figure B. The Correlation ($r_{I/P}$) between fluorescence intensity and polarization measure, averaged over 50 cells, before and after the induction of translocation. Differences between $r_{F/P}$ values obtained before and after induction of translocation are noticeable.

Using the above devolved methodology, I succeeded to describe, for the first time, objective, inherent and highly statistically significant parameters for a rapid detection (by signal analysis) of cells in which translocation occurred out of the entire cell sample. The results of such rapid, automatic detection are presented in Figure C. Utilizing the above-said objective parameters, an algorithm was constructed enabling

to discriminate between cells in which translocation took place (red circumferences) and those where it did not occur (white circumferences).

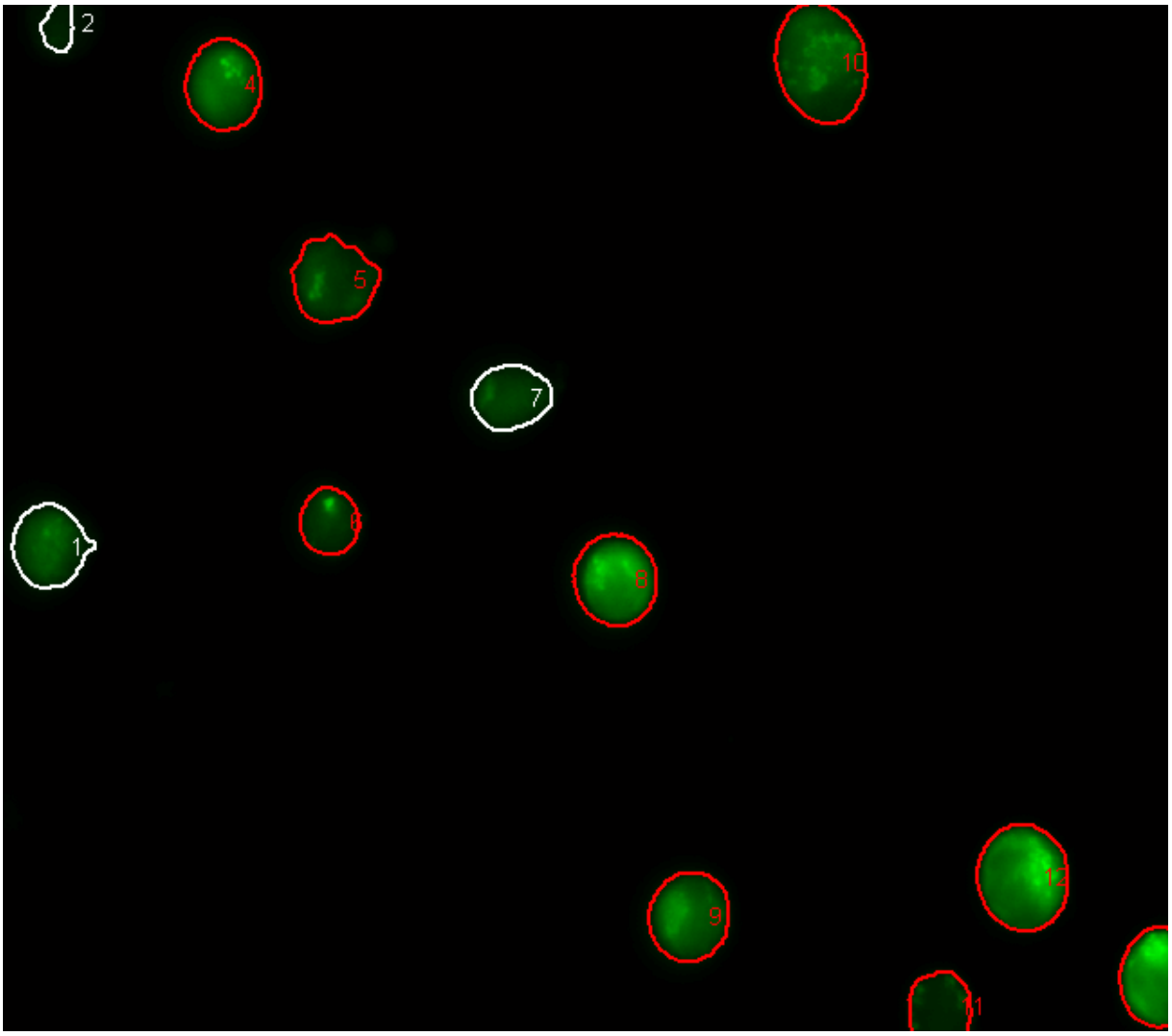

Figure 3. Fluorescence intensity map, with cells marked by the present correlation algorithm. Circumferences' colors represent each cell group: red – cells that underwent translocation, white – cells in which translocation did not take place.

Figure C above presents the fluorescence intensity map for cells identified by the algorithm that I constructed. It can be seen that, apart from 3 cells marked by the white circumference, in all the other cells translocation took place and was identified.

It should be noted that the capability presented in this work is currently unavailable. Therefore, it is believed that the present work may have a significant impact for drug discovery and development, with special reference to those methodologies that are based on the detection of the effects of drug candidates on eGFP-associated cDNA libraries.