Ph.D. THESIS

Data processing software for the detection of cell surface protein patterns

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1. INTRODUCTION

The cell membrane of eukaryote cells is the barrier between the extra- and intracellular space and it provides interface for several signal transduction and metabolic processes. The mastery of the structure and functionality of cell membrane is indispensable to understand several biological processes.

The research field of our department is the investigation of the proximity of cell membrane components (lipids) on different hierarchical levels, with biochemical and biophysical means. The chemical compounds of the cell membrane can be studied quantitatively and qualitatively by biochemical techniques. Nowadays it is proved that most of membrane processes requires the colocalization of certain membrane components. Several receptors (interleukin receptor, IL-2R, immune synapse) formed by more than one subunits, that together are able to bind their ligand and perform a downstream signal. Hence the investigation of the proximity of membrane components proximity is essential to understand cell membrane related biological processes.

Fluorescence resonance energy transfer (FRET) is a spectroscopic method to detect the distance relationship of fluorescently labeled molecules. In recent years, numerous FRET based techniques have been developed for flow cytometry, microscopy and SNOM. The FRET process is a dipole-dipole interaction in which an excited donor fluorophore transfers its energy to an acceptor molecule in close vicinity (1-10 nm) in a non-radiative way. The main application of FRET as a spectroscopic ruler is based on that the rate of energy transfer depends on the inverse sixth power of the distance between the two interacting molecules. The energy transfer efficiency can be determined from the change of the various physical parameters (intensity, photobleaching lifetime, fluorescence lifetime etc.) of the fluorophore molecules, which are related to the distance between the donor (fluorophore gives the energy) and acceptor (accept the energy) molecules.

Fluorescence activated cell sorting and analysis (FACS) or flow cytometry is a solution for high-speed quantitative analysis (including FRET measurment) of cell populations on a cell-by-cell basis. In such an instrument, cells flow through a nozzle or flow cell and are illuminated with focused laser light. For each cell, optical signals, light-scatter and fluorescence arising from specific labels are detected and subsequently digitized. The central dogma of flow cytometry states that the intensity of the detected optical parameter is proportional to the respective cellular parameter.

The common way to measure FRET processes is via the decrease of donor fluorescence in the presence of an acceptor, which can be accompanied by an increase of fluorescence of the acceptor (if the acceptor is a fluorescent molecule). One of its disadvantages is that the measured signal gives average information from the whole cell.

The photobleaching FRET is one of the microscopic FRET techniques. Upon continuous excitation of the donor molecules, their fluorescence intensity decreases due to photobleaching, which is an irreversible photochemical degradation process. For a double-labeled sample, i.e. with both donor and acceptor molecules present, energy transfer between the fluorophores opens an additional relaxation pathway for the excited donor molecules. Thus the decline in the number of donor excited states by photobleaching takes longer. To determine the transfer efficiency (E), two series of images should be recorded, one from the sample labeled solely with donor, and one from a sample double-labeled by donor and acceptor. The photobleaching decay curves can be obtained from the image series by fitting exponential function to each pixel series. The time when the initial fluorescence intensity decreases to $1/e$ part of its initial value is defined as the photobleaching time constant. The energy transfer efficiency can be calculated from the bleaching time constants of the donor-only labeled and donor-acceptor double-labeled samples. The inhomogeneity and the microcolonies of membrane components can be studied by this technique and its resolution is limited by the resolving power of the microscope.

The immunogold labeling method, that uses antibodies conjugated gold particles, is a powerful technique in biological research to study ultrastructural localization of various antigens on the cell surface. Applying the appropriate statistics and stereological tools on the digitalized TEM (transmission electron microscopy) or AFM (atomic force microscopy) images, several characteristics of the distribution of antigens (correlation and cross-correlation) can be studied.

The above described three methods vary in technique and methodology as well as the yielded biological information, but all of them require robust hardware and software system for data acquisition and analysis. Nowadays both processes are computer aided and the commercial instruments are distributed with complete data acquisition and analysis software packages. Generally the hardware configuration of the instrument does not need to be altered when a slightly modified method should be introduced, but the analysis process can be altered especially. Accordingly new routines, function, graphic user

interface are needed, because often the accuracy of the measurement is hindered by the time consuming analysis process.

Our purpose was to reinstate the lack of analysis programs, in the case of the above-mentioned three methods, to improve the analysis programs of these existing techniques, to deliver a simplified and more efficient way for data analysis. The extensive knowledge of the theoretical background of the biological techniques, that are investigated, is negligible in the case of such kind of development. Our developed software packages are already tested and introduced, and the publications, listed in the Other Publications chapter, are prepared with the help of our programs.

Flow cytometric fluorescence resonance energy transfer (FCET)

The determination of energy transfer by the FCET method is based on the measurement of the fluorescence intensity changes of the fluorophore molecules. When energy transfer occurs, the quantum yield of the donor molecules will be smaller due to the additional pathway for relaxation and the fluorescence intensity of the donor molecules is decreased. On the other hand, in the presence of the excited donor molecules the amount of acceptor molecules in the excited state is increased due to energy transfer, which is called sensitized emission.

The FCET method is a solution for high-speed quantitative analysis of cell populations on a cell-by-cell basis. With this technique several optical signals, light-scatter and fluorescence arising from specific labels, can be detected for each cells and subsequently digitized. Many parameters can be calculated from the measured data including energy transfer efficiency on a cell-by-cell basis. However the yielded information is an average parameter of each cell, therefore the spatial heterogeneities of the measured parameters on the cell surface can not be studied by this technique.

Photobleaching fluorescence resonance energy transfer (pbFRET)

The photobleaching fluorescence resonance energy transfer (pbFRET) technique is one of the several microscopic adaptations of FRET. The determination of the energy transfer efficiency is based on photobleaching time constant of the fluorophore. The

photobleaching process of the fluorophore can be detected by a fluorescence microscope. During this time course several images can be acquired and digitalized to record the decrease of the fluorescence intensity.

The number of images to be collected depends on the bleaching time constant of the fluorophore and the energy transfer efficiency. The higher the bleaching time constant, the longer illumination time required for bleaching the same amount of donor molecules. The occurrence of energy transfer also slows down the bleaching process, since it opens an additional competitive way for relaxation of excited state donor molecules, and thus the mean bleaching time constant of donor fluorophore is increased as well.

The photobleaching decay curves can be obtained from the image series by fitting double-exponential function to each pixel series. The time when the initial fluorescence intensity decreases to *1/e* part of its initial value is defined as the photobleaching time constant. The initial intensity weighted mean photobleaching time constant value can be calculated from the parameters of the best fitting exponential function ($<\tau>$). The average energy transfer efficiency value can be calculated from the mean photobleaching time constant values of the donor-only and donor-acceptor double-labeled samples.

Immonogold labeling, analysis of receptor point patterns

Although with the FRET method it is possible to study the proximity of molecules on a molecular level (1-10 nm) and using different concentrations of fluorofores higher order associations can also be studied, it is difficult to investigate the membrane components on higher organization level and the size of homo- and heteroclusters. Immunogold labeling is a specific and sensitive tool for detection of various cell surface antigens. The studied proteins are labeled using antibodies conjugated with gold particles and ultrathin sections less than 100 nm of the samples are used to detect and visualize them by transmission electron microscopy. In a more complicated system, double immunogold labeling using gold particles of two different sizes can be used for an assessment of spatial relationships, colocalization, or mutual exclusion of different membrane proteins. Applying the appropriate statistical or stereological tools (estimation of the first- and second-order properties of the immunogold labeled proteins distribution (point process)) the higher order organization of the membrane components can be studied.

The first-order property of the point process describes how the density varies in the area. If the density ($\lambda(x, y)$) is constant, then it is location independent and the process is homogeneous or stationary, otherwise inhomogeneous. Therefore the probability to find a given number of points in an elementary area follows the Poisson distribution with λ parameter. To analyze the spatial distribution of the immunogold labeled proteins the position (x, y coordinates) of the gold particles has to be determined. Then the sample area is divided to unit cells, and the frequency of the cells is plotted as a function of its number of gold particles. If the distribution of the studied proteins is random, then the fitted Poissonian suites well. In the case of significant difference the distribution of the immunogold labeled proteins is not random i.e. some kind of driving force is acting between the studied membrane components.

The second-order properties refer to the spatial patterns in subregions of the mapped sample area. Analysis of second-order properties includes the Ripley`s K function and the estimation of K^{-1} and PCF (pair correlation function) functions.

2. SPECIFIC AIMS

We intended to develop analysis software for each method or rather to complete the available software packages with other method specific routines.

We had the following aims during our investigations:

- The commercially available flow cytometers are designed for routine clinical diagnosis, since their software package contains all the program components necessary to perform the measurement and data analysis, but they are not feasible for energy transfer calculation on cell-by-cell basis. Our aim was to develop a software that provides a multi-document user interface with a tool for defining arithmetic equations on the measured cell parameters.
- The analysis of photobleaching image sequences requires specific software to determine the photobleaching time constant values on a pixel-by-pixel basis, which is not commercially available. Therefore our aim was to develop a software package that provides assistance for the data analysis and the presentation of the results.
- To analyse the images of immunogold labeled samples taken by transmission electron microscopy, we developed a program to determine the position of the gold particles on the mapped area, and to store their coordinates, according the size of particle, in the appropriate file formats for further statistical analysis.

3. MATHERIALS AND METHODES

Cells, antibodies and dyes used in FCET measurements

N87 gastric tumor cell line (American Type Culture Collection (Rockville, MD)) was used for the FCET measurements, and was grown in RPMI 1640 medium containing 10 % heat-inactivated FCS, 2 mM L-glutamine and 50 µg/ml gentamycin in humidified air containing 5 % CO_2 at 37 °C. Two epitopes of ErbB2 receptors were labeled 4D5 (Trastuzumab, Herceptin™) and 2C4 (Pertuzumab, Omnitarg™) -Fabs. The Fab fragments of antibodies were labeled with Cy3 and Cy5 dyes.

Freshly harvested cells were washed twice in ice-cold phosphate buffered saline (PBS; pH 7.4), then 1 million cells were suspended in 50 µl PBS. Cells were incubated for 30 minutes on ice in dark with conjugates of Fab fragments in saturating concentrations. For the double-labeling of FRET samples, the two fluorescently conjugated Fabs were added simultaneously to the cells. Then cells were washed twice in ice-cold PBS and fixed with 1 % formaldehyde and PBS.

Cells, antibodies and dyes used in pbFRET measurements

The EBV-transformed human JY B lymphoblastoid cell line, was grown in RPMI 1640 medium containing 10 % heat-inactivated FCS, 2 mM L-glutamine and 50 µg/ml gentamycin in humidified air containing 5 % CO_2 at 37 °C.

The antibodies W6/32 (IgG2aκ) specific for the heavy chain of MHC I (HLA-A,B,C), L368 (IgG1κ) binding β2-microglobulin (the light chain of MHC I) and L243 (IgG2a) specific for MHC class II (HLA-DR) were prepared from hybridoma supernatants. Aliquots of purified mAbs were conjugated with 6-(fluorescein-5-carboxamido) hexanoic acid succinimidylester (SFX) or 6-(tetramethyl-rhodamine-5-(and-6)-carboxamido) hexanoic acid succinimidyl ester (TAMRAX, Molecular Probes, USA). Labeling ratios were determined by a spectrophotometer. Cells were washed twice and suspended in phosphate buffered saline (PBS; pH 7.4) at concentration 0.5-1×106 cells/50 µl and were incubated with saturating amounts of SFX- and/or TAMRAX-conjugated mAbs for 45 minutes on ice. Thereafter cells were washed twice in PBS and fixed with 1 % formaldehyde in PBS on ice for 30 minutes. During labeling special care was taken to keep the cells at ice cold temperature to avoid induced aggregation of cell surface molecules.

Cells, antibodies used in Immunogold labeling

Jurkat TagC15 cells were cultured in RPMI 1640 medium containing 10 % heat-inactivated FCS, 2 mM L-glutamine and 50 µg/ml gentamycin in humidified air containing 5 % CO_2 at 37 °C. Transfected cells were washed twice with Hanks' solution and labeled with anti-FLAG M2 primary antibody (mouse anti-human, Sigma, Budapest) on ice for 40 min. The cell suspension was washed twice and labeled with polyclonal secondary antibodies conjugated with 10 nm gold beads (AuroProbe EM 10 nm, Amersham Pharmacia). Cells were fixed with 1% paraformaldehyde for 1 h and 2% glutaraldehyde in 0.1 M Sorensen's buffer at pH 7.2 (overnight) and prepared for transmission electron microscopy.

Software development environments

The FCET analysis (ReFlex) and the electron microscopic image analysis (ClickOnGold) programs were developed in Borland Delphi Studio (Borland Software Corporation, Scotts Walley CA, USA). We have chosen this developer package because it supports Object Oriented Programming and contains several components, including TreeView, and StringGrid that are utilized in our programs. On the other hand it has an easy-to-use developer interface, which provides several tools (object inspector, property editor) to aid designing the GUI for the program.

The pbFRET image series analysis software was developed in National Instrument LabVIEW 6.1 (National Instruments Corporation, Austin TX). We have chosen this software because of its powerful graphical development environment, which provides several objects (graph controls, image display control) to aid designing the program, and it contains also several mathematical VIs and routines. Originally the LabVIEW graphical programming language was designed for engineers, because of its easy to use interface and the simplified programming theory, but recently the language is powerful enough to develop robust software.

The software packages were developed for Windows platform, and were tested on Windows9X, WindowsNT, Windows2000 and WindowsXP operating systems.

4. RESULTS AND DISCUSSIONS

Computer program for determining fluorescence resonance energy transfer efficiency from flow cytometric data, determination of the proximity of the ErbB2 receptor epitopes

The calculation of FRET and AFRET (autofluorescence corrected FRET) on a cell-by-cell basis should be assisted by powerful data processing software, because of the high amount of data and the relatively tedious calculations. The program should be able to open the flow cytometry standard file (FCS), to perform the appropriate calculations, and it has to provide a graphic interface and specific tools for the user to perform the gating (selection) of the population of interest.

Since this technique is used for solving several biological problems in our department and by our collaborators in everyday practice, our aim was to satisfy the novel demands. We developed a program to calculate fluorescence resonance energy transfer efficiency values, which is able to handle list mode flow cytometry standard (FCS) files. The program has a multiple document interface, which allows for the user to analyse FCS files simultaneously. This program generates standard plots of one- and two-parameter histograms on linear or logarithmic scales. Graphical gating tools help to select the populations of interest and to restrict the calculation of energy transfer values to them. Since the calculations could be different for various methods, the software has an equation editor module, where specific equations can be defined on the measured and calculated parameters.

The following experiments were performed to demonstrate the differences between the mean energy transfer efficiency values calculated with different methods implemented in our software. Two epitopes of ErbB2 receptors were labeled on the N87 gastric tumor cell line with 4D5 and 2C4 Fabs. The Fab fragments of antibodies were labeled with Cy3 (donor) and Cy5 (acceptor) dyes. We measured the fluorescence intensities in the appropriate channels on a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

The easiest way to determine the energy transfer efficiency by is the quenching calculation, which determines an average FRET efficiency for the whole cell population. For the calculation one has to determine the average fluorescence intensity of the donor

and acceptor labeled sample, measured in the donor channel, and the average fluorescence intensity of the donor-only labeled sample, measured in the same channel, and finally the ratio of the two average values has to be subtracted from one. This calculation can be performed with any commercially available program which can handle the FCS files. However, in the case of cell-by-cell based calculation of energy transfer efficiency values we get a cell-by-cell distribution of E values rather than a single population mean, and the mean E value can be evaluated by applying the appropriate statistic.

Our software was used for the determination of the FCET and AFCET values. According to our results using the *E(quenching)* calculation method, the E value (0.145) is significantly different from those of the other two calculations ($mean(E_{FRET}) = 0.282$; $mean(E_{AFRET}) = 0.253$). The explanation for this difference is that the *E(quenching)* value is calculated from the mean intensities.

The differences between the E values calculated with the FCET and AFCET methods are not so significant, but the coefficient of variance of the AFCET histogram is smaller (Figure 4 - 1, black histogram) because of the cell-by-cell autofluorescence correction. If the studied membrane components have a low expression level, the fluorescence signal can be comparable to the autofluorescence signal, and the cell-by-cell correction for autofluorescence can generate more realistic FRET distributions with less variance.

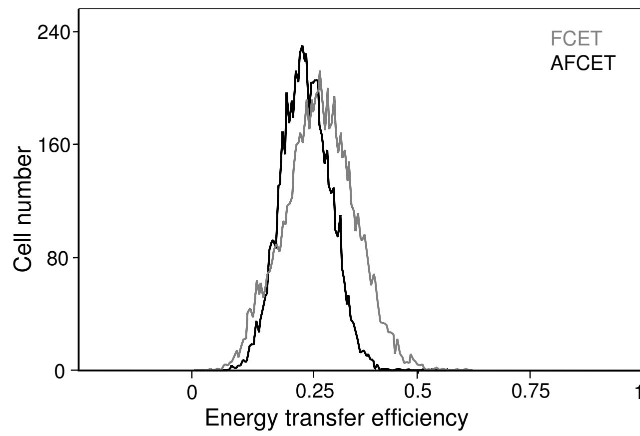

Figure 4 - 1: Energy transfer histograms. The distributions of energy transfer efficiency values are displayed on the graph. Values were calculated with AFCET (black curve) and FCET (gray curve) methods on a cell-by-cell basis. The coefficient of variance of the AFCET histogram is smaller than the variance of the FCET histogram, because the correction for autofluorescence was carried out on a cell-by-cell basis in the former case.

Computer program for determining fluorescence resonance energy transfer efficiency from photobleaching image series, determination of the proximity of the MHC I and II molecules subunits

Fluorescence activated cell sorting and analysis (FACS) or flow cytometry is one solution for high-speed quantitative analysis of cell. With this technique cell populations can be studied and evaluated relatively fast and the energy transfer efficiency values can be determined on a cell-by-cell basis. Because of the high number of cells, it can provide a relatively good statistics, but the energy transfer values are averaged for each cell, thus no information is available about the diversity of these values on the cell surface of single cells.

To gain subcellular information from the cell one of the available techniques is the photobleaching resonance energy transfer (pbFRET). To determine the transfer efficiency (E) two series of images should be recorded using fluorescence microscope with digital camera or PMT, one from the sample labeled solely with donor, and one from a sample double-labeled with donor and acceptor. The number of images to be collected depends on the bleaching time constant of the fluorophore, the intensity of the excitation light and the energy transfer efficiency. The photobleaching decay curves can be obtained from the image series by fitting exponential function to each pixel series. The photobleaching time constant can be determined from the parameters of the fitted curve and the average energy transfer efficiency value is calculated from the photobleaching time constants of the differently labeled samples.

Several commercially available programs have the options to record and display bleaching image series and to generate bleaching curves from the recorded images, but they do not support curve fitting on a pixel-by-pixel basis, just single curve fitting is possible. Because of the high number of pixels (in general image size 512 × 512 pixel) and the time-consuming calculations, this procedure should be assisted by a powerful image processing software. We developed a program to calculate fluorescence resonance energy transfer efficiency from raw image file series. The program automatically generates the bleaching decay curves from the image series and fits them with optional single, double or triple exponential functions. The bleaching time constant values can be displayed and saved in color-coded images (`tau map`) and histograms.

In order to demonstrate the functionality of the program, FRET efficiencies between the two chains (β2m, heavy chain) of MHC I as well as between MHC I (β2m) and MHC II were determined at the surface of JY human B lymphoma cells. Proteins were targeted by SFX- and TAMRAX-conjugated monoclonal antibodies serving as donor and acceptor, respectively.

FRET efficiencies were determined by using the following samples:

1, JY cells labeled with SFX-L368 (targeting β2m, donor)

2, JY cells simultaneously labeled with SFX-L368 and TAMRAX-W6/32 (targeting β2m and MHC I heavy chain, respectively; donor + acceptor)

3, JY cells labeled with both SFX-L368 and TAMRAX-L243 (targeting β2m and MHC II, respectively; donor + acceptor)

4, JY cells labeled with TAMRAX-L243 (binding MHC II; acceptor-only labeled)

5, unlabeled JY cells

Since the photobleaching process is highly sensitive to the oxygen concentration, experiments were performed on the mixture of donor-only and double-labeled samples, to ensure the same level of dissolved oxygen. In order to distinguish donor-only and double-labeled cells, an additional image was recorded in the acceptor channel (λ_{ex} = 543 nm, λ_{em} > 580 nm) at the end of each experiment. The sample labeled with TAMRAX-L243 alone (acceptor-only labeled sample) was used to check whether the intensity of the acceptor is affected upon donor excitation. If the acceptor is also bleached upon the donor photobleaching, it loses its ability to accept energy from the excited donors and as a result the bleaching time constant decreases. The fifth sample was used as a background control.

The samples were measured on a Zeiss LSM 510 microscope (Lasers: Ar ion at 488 for donor excitation, He-Ne at 543 nm for acceptor excitation), and image series were converted to raw format and analyzed with our software. Four image series of 2-3 cells were taken from each sample (~ 10 cells/sample). We determined 600-1500 photobleaching time constants (`tau`) per cell, i.e. the mean time constants were calculated from approximately seven thousand data for each sample. The resulting distribution histograms of "tau" values for the double-labeled samples are shown in Figure 4 - 2.

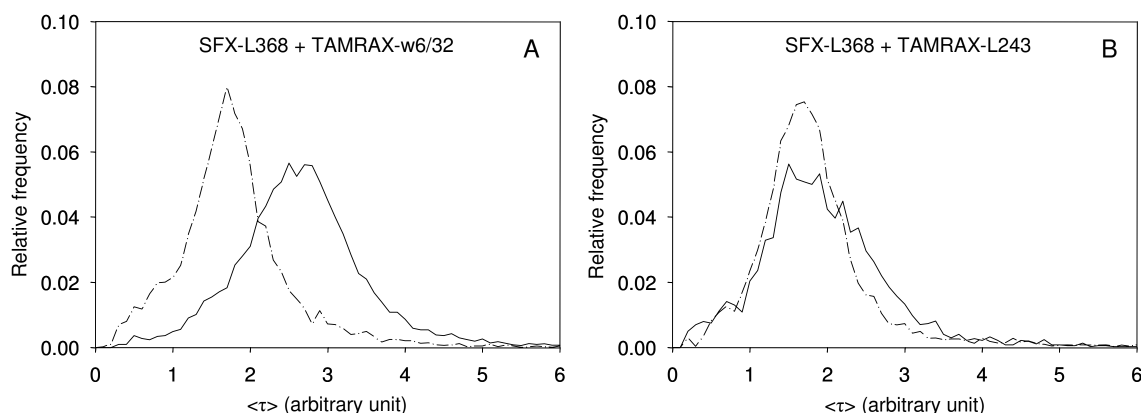

Figure 4 - 2. Results of the representative pbFRET experiments. The two chains of MHC I were targeted by SFX-L368 and TAMRAX-W6/32 mAbs, respectively, whereas MHC II was labeled by TAMRAX-L243 mAb. SFX was used as donor and TAMRAX as acceptor. Photobleaching experiments were performed on the mixture of donor-only and double-labeled cells. The presence of FRET resulted in the shift of the distribution histograms of double-labeled cells (solid lines) toward the higher values in comparison with those of donor-only labeled cells (dashed-dotted lines). In the case of A the shift is more prominent, than in the case of B, i.e. the two chains of MHC I molecules are in closer proximity than β2m and MHC II, thus the probability of FRET is higher.

The mean values of photobleaching time constants for donor-only labeled cells (sample #1) were independent of whether they were determined in the mixture of samples #1 and #2 or that of #1 and #3: the calculated values are 1.69 ± 0.35 and 1.65 ± 0.48, respectively. In the case of the double-labeled cells the mean constants for samples #2 and #3 were 2.62 ± 0.56 and 1.78 ± 0.56, respectively. FRET efficiencies for the SFX-L368/TAMRAX-W6/32 (β2m – MHC I heavy chain) and for the SFX-L368/TAMRAX-L243 (β2m – MHC II) pairs are 35.5% and 6.9%, respectively.

Electron microscopic image processing software, analysis of the distribution of Kv1.3 ion channels

One possible method is the electron microscopic immunogold labeling technique to study the cell surface proteins distribution on a higher organization level. The cell surface proteins can be detected after immunogold labeling with antibodies, conjugated with high electron density colloidal gold particles, by transmission electron microscopy. The gold particles appear as small dark spots on the digitalized images, which cell surface pattern and distribution can be studied applying the appropriate spatial distribution analysis.

Since the software for statistical analysis of point processes according to second-order properties was our disposal (offered by Pavel Hozák), but the image analysis program to determine the position and the size of gold particles was missing. Therefore we developed a software is able to determine the position of gold particles on digitalized images, as well as to analyse the first-order properties of their distribution.

We investigated the distribution of Kv1.3 ion channels on the cell surface of Jurkat cells according to first- and second-order properties of their distribution. The image coordinates and the size of the gold particles were determined by our program. Applying the appropriate grid size (120×120 pixels, approx. 5 particles/cell) the cells gold number histogram can be determined and fitted (Figure 4 - 3.).

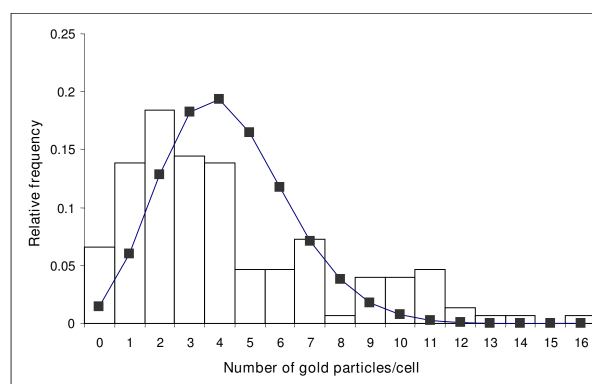

Figure 4 - 3.: Statistical analysis of the distribution of the immunogold labeled Kv1.3 channels. TEM pictures were digitized, and our software was used to determine the pixel coordinates of the gold beads. Grids (120×120 pixels) were superimposed on the picture, and the number of particles per grid element was determined. Frequency histograms were generated, and the corresponding Poisson distributions —■— were superimposed on the histograms. χ^2 test indicates that the observed distribution are significantly different from the stochastic Poisson distributions ($P < 0.05$).

The none-randomness of the distribution of channels is confirmed by the first-order property analysis, since the cells gold number histogram significantly differs from the fitted Poisson function, which was generated from the average gold count (λ = 4.25) of the cells.

The distribution of the channels can be also studied by its second-order properties, estimating the Ripley`s K function, K^{-1} function or the PCF (Pair Correlation Function). To estimate the K function of the point process we have to determine the average number of gold particles around each particle in a range with r radius, and this have to be divided by the average surface density of them. If the point process is completely random then $K(r) = \pi r^2$ for any r value, but if the K value is smaller than the points are aggregated otherwise dispersed.

To estimate the PCF we have to determine the K^{-1} function that is the integral of the PCF at r_1, r_2 interval. The K^{-1} function is estimated like the K function, but in this case the number of points is weighted by the points distance. The PCF can be calculated from the K^{-1} as its derivative for a given ($r, r + \Delta r$) interval.

To study the aggregate level of the channels the second-order properties of the distribution were determined, the K-1 and the PCF were estimated (Figure 4 - 4.).

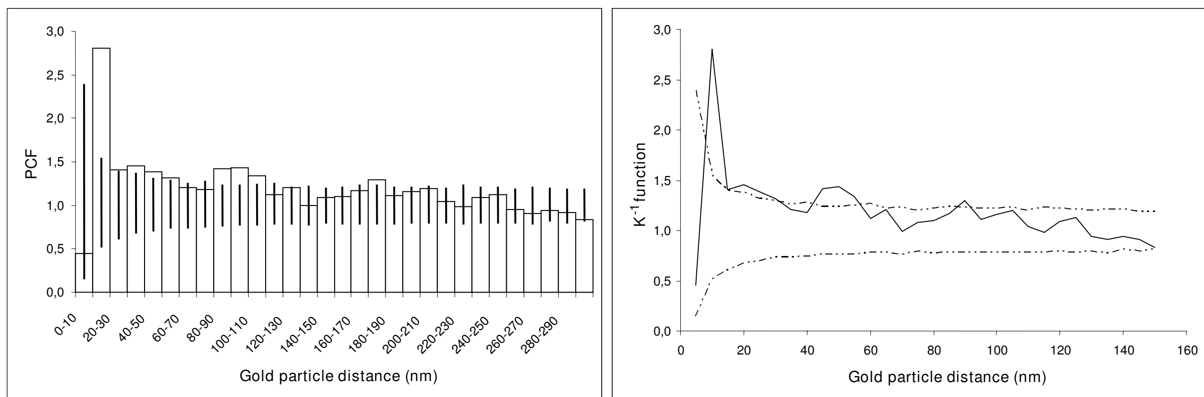

Figure 4 - 4.: Second-order analysis of the distribution of the immunogold labeled Kv1.3 channels, estimation of the PCF and K^{-1} functions. The Kv1.3 ion channels form aggregates at the surface of the Jurkat cells. In 0-10 nm range the estimated values of both functions are very low, which can be explained by the size (10 nm gold beads) of the gold particles (mutual exclusion), because their effective centers cannot be arbitrarily close together. In 20-30 nm range the high estimated values can be explained by the tension forces during the drying process.
The 95% confidence interval for the K^{-1} function was determined by Monte-Carlo simulation, which upper and lower bounds are displayed with dotted line on the left graph.

The PCF and K-1 functions significantly differ from the values of the completely spatial randomness in the ranges 40-60, and 80-100 nm, and therefore the distribution of the Kv1.3 ion channels is not stochastic, there is some kind of driving force, which leads to form aggregates at the cell surface.

5. SUMMARY

Our aims were to develop analysis software for three biophysical techniques to investigate the proximity relationships of cell surface components or rather to complete the available software packages with other method specific routines.

Our results are the follows:

- We developed software with multiple document interface, which is capable to handle and analyze several FCS files parallel, and that is able to interpret the user defined arithmetic expressions on a cell-by-cell basis. The program has an interface which aids the user to arrange the measured FCS file in a well perspicuous order, and the visualization and representation of the results. We determined the energy transfer efficiency values on N87 gastric tumor cells with three formerly developed calculations methods (FCET, AFCET, quenching) to present the functionality of our software.
- We developed a software package for analysis of photobleaching image sequences, to simplify the analysis and the presentation of the results. We investigated the proximity of the subunits of MHC I and II molecules in the cell surface of JY cells using our program to analyze the photobleaching image series.
- We developed a program to determine the position of immunogold labeled antigens in electron microscopic digital images and we performed the spatial distribution analysis of the Kv1.3 ion channels in the surface of Jurkat cells by the investigation of the first- and second-order properties of their point process.

6. PUBLICATIONS

The thesis is based on the following publications

1. Szentesi G, Horváth G, Bori I, Vámosi G, Szőllősi J, Gáspár R, Damjanovich S, Jenei A, Mátyus L. Computer program for determining fluorescence resonance energy transfer efficiency from flow cytometric data on a cell-by-cell basis. Comput. Meth. Prog. Bio. 2004;75:201-211.

IF: 0.686 (JCR 2004)

2. Szentesi G, Vereb G, Horváth G, Bodnár A, Fábián A, Matkó J, Gáspár R, Damjanovich S, Mátyus L, Jenei A. Computer program for analyzing donor photobleaching FRET image series. Cytometry Part A 2005;67A:119-128.

IF: 2.698 (JCR 2004)

Other publications

1. Matkó J, Bodnár A, Vereb G, Bene L, Vámosi G, Szentesi G, Szőllősi J, Gáspár R, Horejsi V, Waldmann TA, Damjanovich S. GPI-microdomains (membrane rafts) and signaling of the multi-chain interleukin-2 receptor in human lymphoma/leukemia T cell lines. Eur. J. Biochem. 2002;269:1199-1208.

IF: 3.260 (JCR 2004)

2. Panyi G, Bagdány M, Bodnár A, Vámosi G, Szentesi G, Jenei A, Mátyus L, Varga S, Waldmann TA, Gáspár R, Damjanovich S. Colocalization and nonrandom distribution of Kv1.3 potassium channels and CD3 molecules in the plasma membrane of human T lymphocytes. P. Natl. Acad. Sci. USA 2003;100:2592-2597.

IF: 10.452 (JCR 2004)

3. Bene L, Szentesi G, Mátyus L, Gáspár R, Damjanovich S. Nanoparticle energy transfer on the cell surface. J. Mol. Recognit. 2004;18(3):236-253.

IF: 1.859 (JCR 2004)

4. Bene L, Szőllősi J, Szentesi G, Damjanovich L, Gáspár R, Waldmann TA, Damjanovich S. Detection of receptor trimers on the cell surface by flow cytometric fluorescence energy homotransfer measurements. BBA-Mol. Cell. Res. 2005;1744:176-198.

IF: 3.482 (JCR 2004)

5. Horváth G, Petrás M, Szentesi G, Fábián A, Park JW, Vereb G, Szőllősi J. Selecting the right fluorophores and flow cytometer for fluorescence resonance energy transfer measurements. Cytometry Part A 2005;65A:148-157.

IF: 2.698 (JCR 2004)