Spatial information on receptor clustering in flow cytometry using FRET

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Introduction

Flow cytometry is commonly used to detect the overall fluorescent signal of a whole cell and thus to analyze whether or not certain target molecules are present on the cell. However, no information on the distribution or clustering of the molecules of interest can be gained from these signals. Yet, the clustering of receptors or the interaction of proteins plays a key role in almost all biological functions.
Therefore, plenty of research efforts are spent in this field. Conventional investigation methods, like two-hybrid systems, are usually very laborious and highly time consuming. Foerster resonance energy transfer (FRET)[1] is often used to identify protein-protein interactions by confocal microscopy[2].
However, this technique requires expert knowledge and produces vast amounts of data. In order to overcome these limitations, we developed a FRET-based flow cytometry analysis program, which automatically provides spatial information about protein interactions on the cell[3].

Methods

1 Automatic FRET measurement

The FRET Express Mode Program automatically measures and determines the FRET efficiency on a cell-by-cell basis on the MACSQuant® Analyzer 10.

Four cell samples are required to accurately determine FRET efficiency:

1. **Blank**
   Subtraction of background fluorescence
2./3. **Donor only** and **Acceptor only**
   Determination of cross-talk between the donor and acceptor channels
4. **FRET sample**
   Calculation of FRET efficiency in the sample

The controls and FRET samples are measured and gated for the target populations by the program. Briefly, for the determination of the FRET efficiency, relative changes in donor and acceptor fluorescence intensities are registered. Based on this and taking into account the results obtained from the control samples, the rate of transferred energy is then calculated accurately by the FRET Express Mode Program in a fully automated manner.

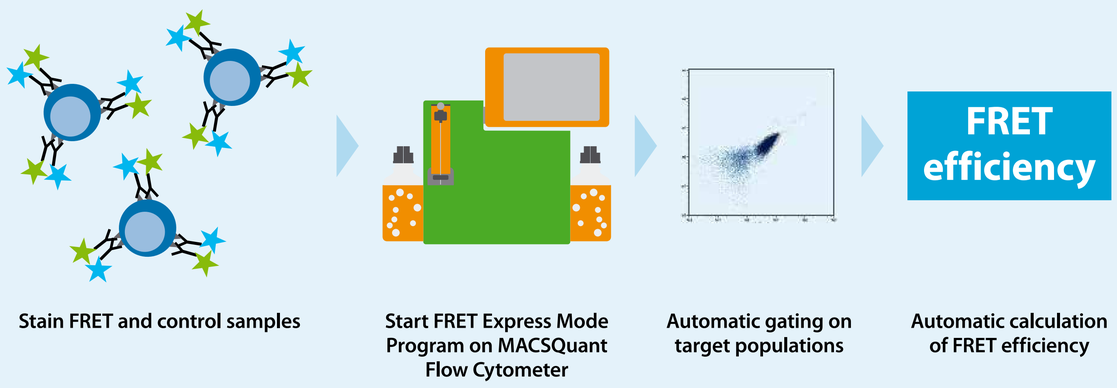

Figure 1

Results

1 Validation of program functionality

We validated the sensitivity and accuracy of the FRET program using FRET standard beads with defined linker lengths between the fluorochromes. FRET efficiency readouts generated automatically by the program were compared to established non-automated FRET calculations. The high correlation between the two methods (R2 = 0.95) proved the functionality of the FRET program.

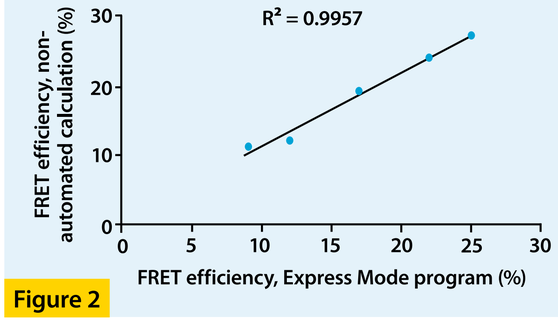

Figure 2

2 Program application: Analysis of CD3-CD4 FRET on T cells

The FRET program was applied to measure the dynamic clustering of CD3 and CD4 receptors on the cell surface after T cell activation. Here, PBMC were isolated from buffy coat and the T cells were activated using 1 µg staphylococcal enterotoxin B (SEB). **(A)** The analysis using the FRET program showed that the FRET efficiency significantly increased after a 2-minute incubation with SEB and decreased again 5 hours after activation. **(B)** The clustering could be visualized using confocal microscopy (CM). Cells were stained with CD3-FITC and CD4-APC. For the non-activated cells, CD3 and CD4 receptors were distributed equally on the cell surface. After a 2-minute activation with SEB, small activation clusters of CD3 and CD4, the so-called microclusters, began to form. After five hours of incubation, large clusters of CD3 had formed[4]. **(C)** The interaction between CD3 and CD4 was validated using a colocalization analysis tool for CM. The normalized colocalization data generated by CM are highly similar to the data resulting from the FRET analysis program. However, it is obvious that for non-activated cells the colocalization coefficient is significantly higher than the FRET efficiency, which is due to the lower resolution of the confocal microscope.

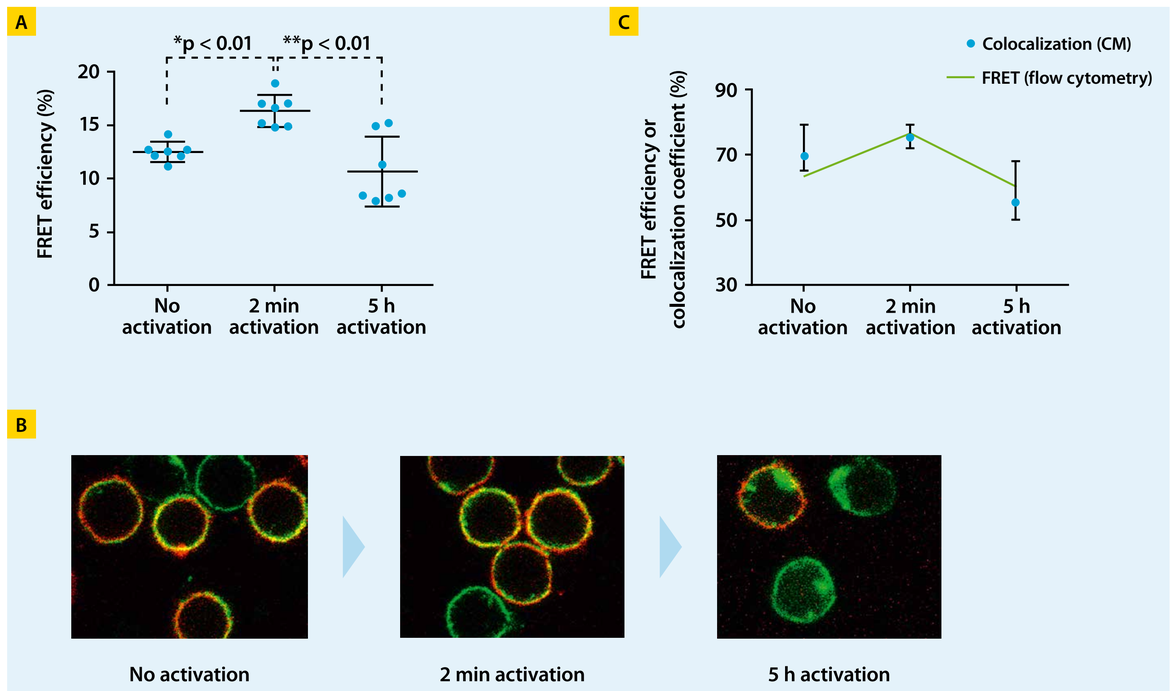

Figure 3

3 Program application: Analysis of CD3 homoclustering on T cells

The FRET program enabled us to analyze homoclustering of the CD3 receptor after T cell activation. A single epitope of CD3 was stained with either CD3-VioBlue® or CD3-FITC, and cells were activated with SEB for 2 minutes. T cell activation led to a significant increase in FRET efficiency due to CD3 cluster formation[4]. T cell activation could be inhibited by pre-incubating SEB with an anti-SEB blocking antibody **(A)** before adding SEB to the cells and was reduced by adding SEB and its blocking antibody simultaneously **(B)**.

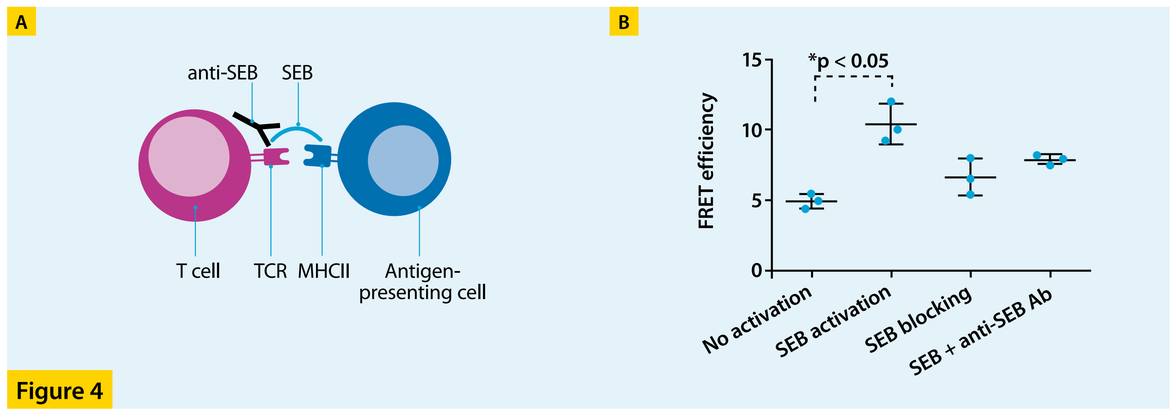

Figure 4

Conclusion

We developed a program which enables the automated flow cytometric measurement of FRET in living cells. This so-called FRET Express Mode program allows for the detection of protein-protein interactions on cells. The program overcomes certain limitations of conventional flow cytometry, as it not only provides information on whether or not a particular molecule is present on a cell, but also automatically analyzes its spatial distribution and interaction with other molecules.
Therefore, the FRET Express Mode Program will simplify the analysis of biologically relevant changes within a cell.

References

1. Förster, T. (1948) Annals of Physics 2: 55–75.
2. Nagy, P. *et al.* (1998) Eur. Biophys. 27: 377–389.
3. Stryer, L. *et al.* (1978) Annu. Rev. Biochem. 47: 819–846.
4. Huppa, J.B. *et al.* (2010) Nature 18: 963–967.