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MOLECULAR SPECTROSCOPY AS A TOOL IN STUDYING BIOPHYSICAL PROBLEMS*

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A joint application of flow cytometry, a fast optical analysis of animal cells, and the Forster-type resonance energy transfer technique provided a suitable tool for the study of proximity relationships on the cell surface.

Fluorescence cell sorting and analysis made it possible to study the distribution pattern of cell membrane components such as the $H-2K^k$ antigen. The relative distribution of the concanavalin A receptor and the $H-2K^k$ antigen has also been studied.

The steady development of modern physical theories and experimental methods for the last 30 years has influenced biology to such an extent that nowadays we may speak of a revolutionary development of this science. Molecular spectroscopy has provided a particularly powerful means to study biophysical problems.

Three of the recently developed luminescence spectroscopic methods seem to be extremely versatile. These methods are in the chronological order of their introduction to biophysics: fluorescence activated cell sorting and analysis [1, 2, 3], fluorescence redistribution after photobleaching (FRAP) [4, 5] and time-dependent delayed fluorescence and phosphorescence spectroscopy [6, 7] at room temperature.

All these experimental techiques have contributed to the rapid development of cellular and molecular biophysics more than any other experimental technique so far, except for X-ray crystallography. These luminescence techniques also have the great advantage of enabling us to investigate living material, including living cells. If compared with some of the resonance spectroscopic methods, like NMR, ESR or Mössbauer spectroscopy, the latter have much lower sensitivity and demand more expensive hardware.

After the rapid and really spectacular success of biochemistry in studying biological material, its molecular compositions and chemical reactions, more and more information has been obtained indicating the importance of molecular dynamics in the living material. The rotational and translational mobility of macromolecules reflects the combined effects of size, shape, internal flexibility, environmental fluidity and stereochemical constraints, static and dynamic interaction, proximity relationships, etc. Luminescence techniques have opened up a new world, a time window

* Dedicated to Prof. I. Tarján on his 70th birthday.

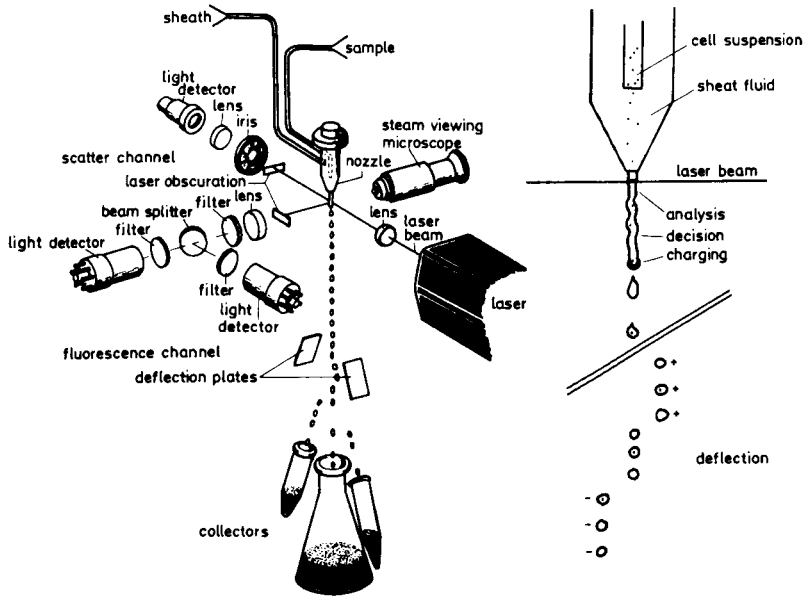

Fig. 1. Details of the optical interrogation and sorting of the flow cytometer, FACS III (Becton-Dickinson)

ranging from $10^{-9} - 10$ s, making the study of such motions and interactions possible. However, the statistical nature of the biological interaction, i.e. the slight individual differences if we study only a limited number of biological objects like cells, may misinform us regarding minor, although sometimes essential details.

In this communication we intend to deal briefly with fluorescence-activated cell sorting and analysis, with some references to the work carried out in our Laboratory. Fluorescence-activated cell sorting and analysis was the first method to give spectroscopic information about individual cells at a speed of $1—10^4$ cell/s. Fig. 1 shows the basic principle of the instrument. A cell suspension is driven through a capillary system. Suitable adjustment of the air pressure in the sample and sheath containers allow a sample of fluorescently tagged cells to be injected into the centre of the flowing stream of compatible sheath liquids, establishing a laminar coaxial flow within the nozzle-transducer assembly, until the thin fluid stream has a diameter comparable to the diameter of a single cell. A laser beam orthogonal to the cell stream is centred so that the scattered light and the fluorescence signals can be collected and centred on photosensitive detector systems, like photomultipliers or photodiodes. As Fig. 2 shows, the signals can be individually collected either by a microprocessor, or in a computer. This system allows data collection and analysis at high speed with great precision. The scattered light is proportional to the particle or cell size and shape and also viability [8, 9, 10]. The fluorescence signal (or signals) can give information on any of the cell constituents labeled specifically by fluorescence dyes. In the case of multiple

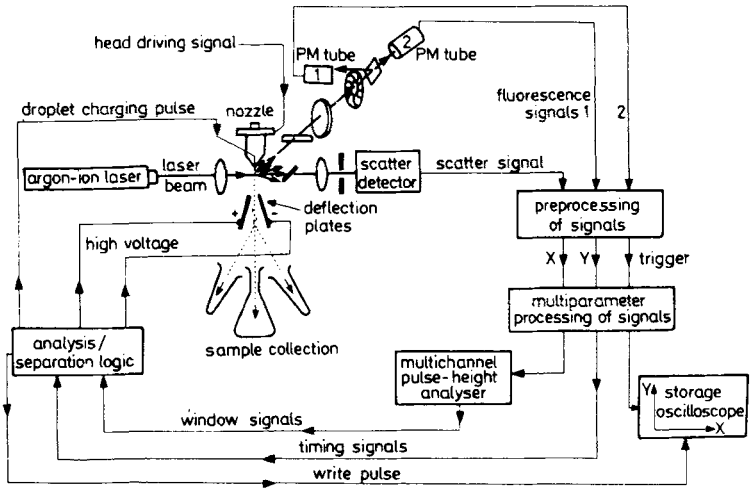

Fig. 2. Schematic block diagram of the signal processing of the flow cytometer, FACS III (Becton-Dickinson)

detection and double excitation, anisotropy data or resonance energy transfer processes can be traced equally well [11].

The immediate application of the high speed analysis of the cells, giving information on the cell size distribution, DNA and/or protein content, number and distribution of specifically labeled ligand binding sites, etc., is plausible in cell biology, hematology, immunology, gynecology, oncology, etc.

Naturally, analysis in itself, although very important, is still not the ultimate possibility of this system. A properly applied piezoelectric transducer is capable of presenting uniform droplet formation. If the analysis of the optical signals occurs at the unbroken jet of the fluid stream where the droplets are not yet formed, and the electronic analysis of the signals is fast enough compared to the flow, then applying a pulse of proper shape to the saline in the flowchamber at the time the wanted (analyzed) cell is about to be pinched off into a droplet, the cells giving certain signals can be separated. Generally three droplets are charged, making the separation of the cells much safer.

Figs 3 and 4 demonstrate some of our data on the DNA content of normal and Gross virus leukemic lymphocytes. The proportion of cells having large DNA content is higher in the case of leukemic lymphocytes than in the case of normal ones, i.e., the leukemic lymphocytes divide faster than normal cells. Other significant differences between normal and leukemic cells were found when samples stained with fluorescein diacetate (FDA) were analyzed in the flow cytometer (Figs. 5 and 6). FDA is hydrolysed inside the cells by esterase enzymes and the liberated fluorescein accumulates in the cells. The analysis of scattered light and fluorescence intensity distribution histograms of cells revealed that the fluorescein content of large leukemic cells is three times higher

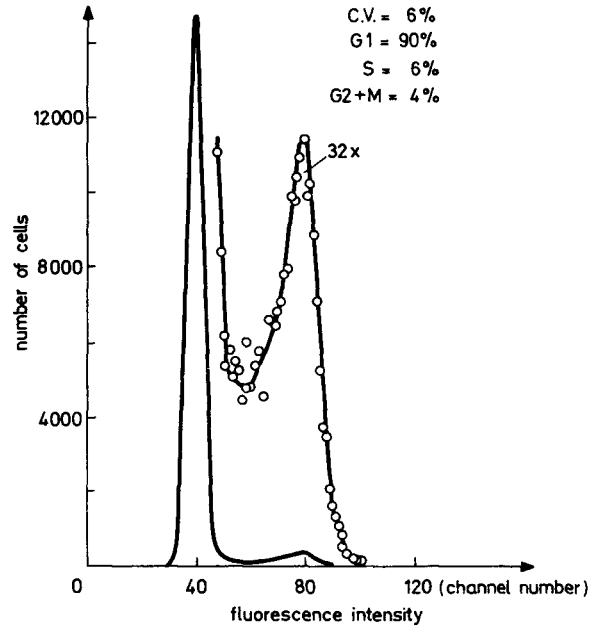

Fig. 3. Fluorescence intensity distribution histogram of normal mouse lymphocytes. The cells were fixed with alcohol and stained with Hoechst 33 342 dye which is specific for DNA. The cell cycle distribution of cells is shown in the figure

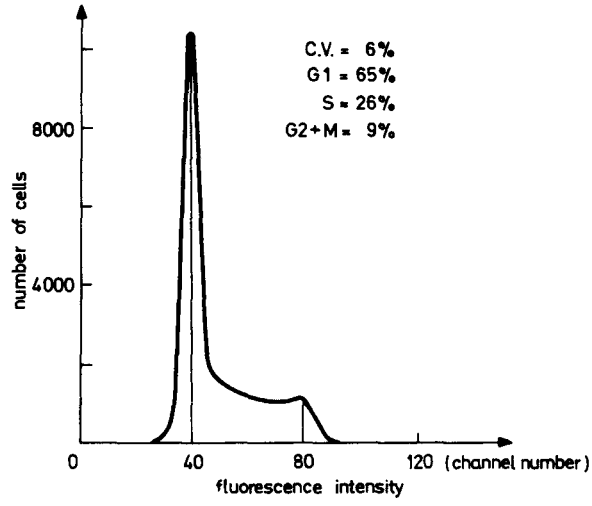

Fig. 4. Fluorescence intensity distribution histogram of leukemic mouse lymphocytes. The cells were fixed with alcohol and stained with Hoechst 33 342 dye which is specific for DNA. The cell cycle distribution of cells is shown in the figure

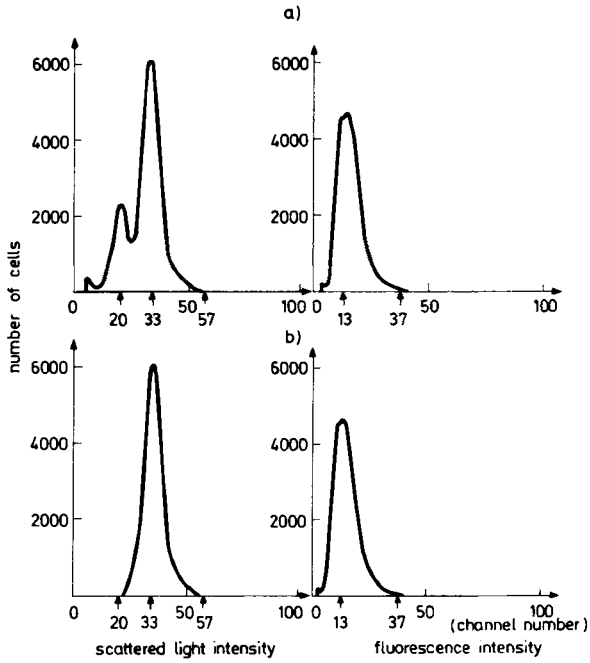

Fig. 5. Frequency distribution histograms of scattered light and fluorescence intensities of normal C3H/He-mg × AKR/F_1 hybrid mouse lymphocytes. For the analysis the lymphocytes were prepared from mesenteric lymph node. The distribution histograms were recorded in the 6th min of the FDA hydrolysis. Data collection was triggered with the scattered light (*A*) or with the fluorescence intensity (*B*). The first and second peaks of light scattering distribution histogram (*A*) belong to the red blood cells and normal lymphocytes, respectively

than that of the small, normal ones. The observed differences both in size and fluorescein content of normal and leukemic lymphocytes enable one to distinguish unequivocally between these cells [12, 13, 14].

Fig. 7 gives a more advanced version of a fluorescence flow cytometer compared to the system shown in Figs. 1 and 2. In this system the dual laser excitation provides a new possibility for the study of cell surface receptors and their proximity relationships. The binding sites for the ligands, Con *A* and anti-$H-2K^k$, on the cell surface of T-41 lymphoma cells were simultaneously labeled with fluorescein izothiocyanate (FITC) and tetramethyl rhodamine izothiocyanate (TRITC) conjugated ligands. The two dyes fulfil the Förster requirement for fluorescence energy transfer as the emission spectra of the donor dye (FITC) and the absorption spectra of the acceptor dye (TRITC) substantially overlap (Fig. 8).

The transfer efficiency of a Förster type fluorescence resonance energy transfer depends on the negative sixth power of the donor–acceptor distance (R) and parameter

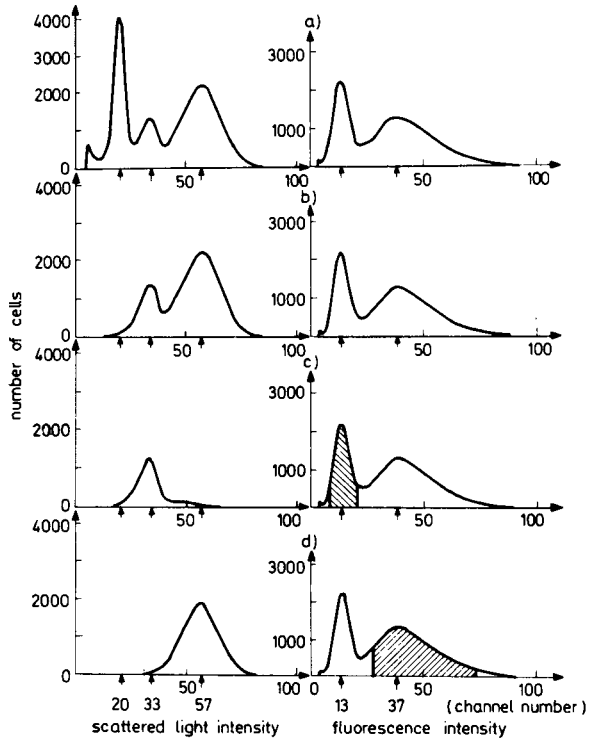

Fig. 6. Frequency distribution histograms of scattered light and fluorescence intensities of leukemic C3H/He-mg × AKR/F_1 hybrid mouse lymphocytes. For the analysis the lymphocytes were prepared from mesenteric lymph node. The distribution histograms were recorded in the 6th min of the FDA hydrolysis. Data collection was triggered with scattered light (*A*), fluorescence intensity (*B*), or the hatched region of fluorescence intensity (*C*, *D*) shown in the Figure. The first, second and third peaks of the light scattering distribution histogram (*A*) belong to the red blood cells, normal lymphocytes and the leukemic lymphocytes, respectively

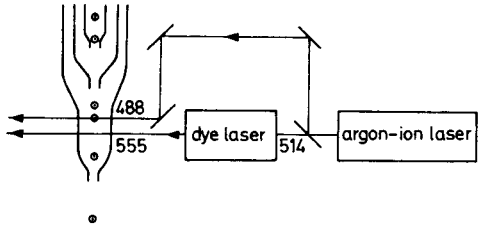

Fig. 7. Schematic diagram of a flow cytometer with dual laser excitation

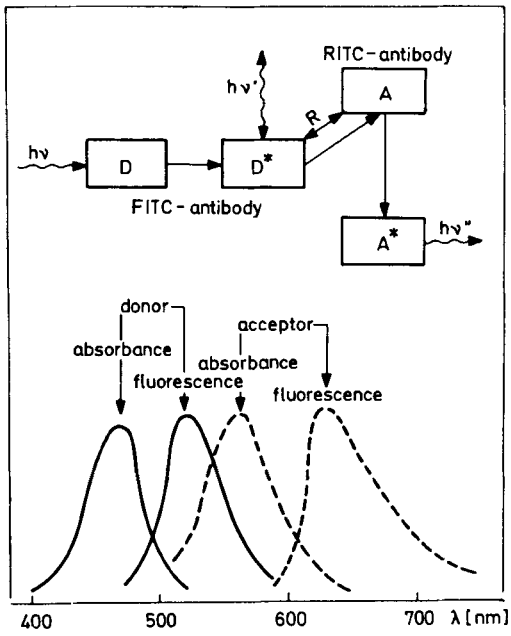

Fig. 8. Fluorescence excitation and emission spectra of FITC and TRITC conjugated ligands

R_0. R_0 is the distance between the donor and the acceptor where the probability of the emission of the excitation energy by the donor and acceptor is

$$ET = \frac{R_0^6}{R_0^6 + R^6}, \tag{1}$$

$$R_0^6 = 8.8 \cdot 10^{-25} \cdot Q_D \cdot \kappa^2 \cdot n^{-4} \cdot \int_0^\infty \varepsilon_A(\lambda) F_D(\lambda) \lambda^{-4} \, d\lambda \, \text{cm}^6,$$

where $\varepsilon_A(\lambda)$ is the molar extinction coefficient of the acceptor, $F_D(\lambda)$ is the normalized fluorescence spectrum of the donor, λ is the wavelength in cm, Q_D is the quantum yield of the donor, κ^2 is an orientation factor determined by the spatial relationships of the donor and acceptor, i.e. by the angle between their respective absorption and emission oscillators, and n is the refractive index of the medium. ET can efficiently be determined between donor and acceptor separated within 2—8 nm.

The transfer efficiency (ET) is related to the emission of the acceptor excited through the donor by the following expression:

$$ET = EA_D^A - \alpha ED_D^D - \beta EA_A^A. \tag{2}$$

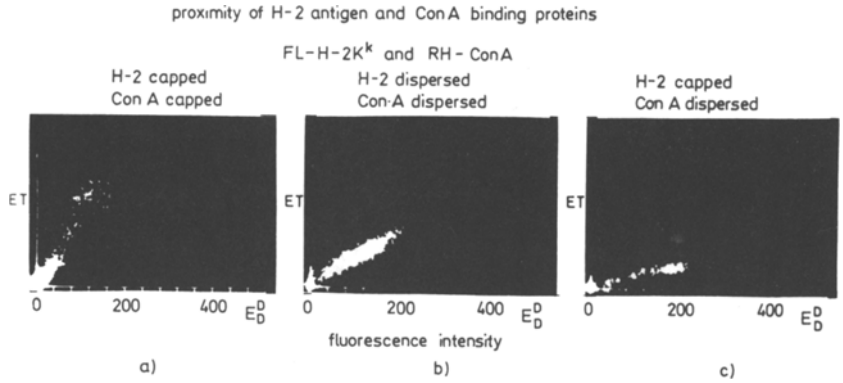

Fig. 9. Fluorescence resonance energy transfer measurements on single cell basis. The dots on photographs represent cells and the coordinates of dots correspond to parameters of cells shown on the $X(E_D^D)$ and $Y(ET)$ axis. The cells were stained with FITC conjugated Con *A* and TRITC conjugated anti-$H-2K^k$ immunoglobulin simultaneously

EA and *ED* represent the fluorescence intensity of the acceptor and donor, respectively. The superscripts and subscripts stand for donor and acceptor emission and excitation, respectively. *A* represents the emission or excitation of the acceptor, while *D* denotes the donor excitation and emission peaks. The emission of the donor is excited at the excitation site of the donors, detecting the emission at the donor emission site (the donor excitation is provided by the 488 nm argon-ion laser line, the emission is detected through a 515 nm narrow bandpass interference filter in front of the photomultiplier). ED_D^D will be proportional also to the "unwanted" emission of the donor at the acceptor emission site (>590 nm). EA_A^A, the emission of the acceptor excited at 555 and detected above 590 nm is proportional to the direct excitability of the acceptor at the excitation wavelength of the donor. The λ and β correcting factors, multiplied by the ED_D^D and EA_A^A and subtracted for the EA_D^A value result in a value directly proportional to the transfer efficiency. Fig. 9 shows a plot of our experiments where the proximity relationships of cell surface receptors like Con *A* and $H-2K^k$ are represented by the slope of the fitted curve. Con *A* is a protein of a molecular weight of 120 000, which binds specifically to a certain kind of oligosaccharide on the cell surface. $H-2K^k$ is an antigen determining the self (i.e. characterizing) of a mouse cell. This method recently developed by Drs T. M. and Donna Jovin has served as a tool to detect an uneven distribution between cell surface ligand binding sites at nm level [15, 16].

The number of Con *A* receptors renders it possible to have the fluorescein labeled proteins (FITC-Con *A*) at a distance from the rhodamine labeled other cell surface binding ligand, the anti-$H-2K^k$ antibody, so that it also fulfils the distance requirement of resonance energy transfer.

As Fig. 10 shows, certain cell surface elements may have a distribution having the specifically labeled ligand binding sites collected at one pole of the cell like a "cap". This

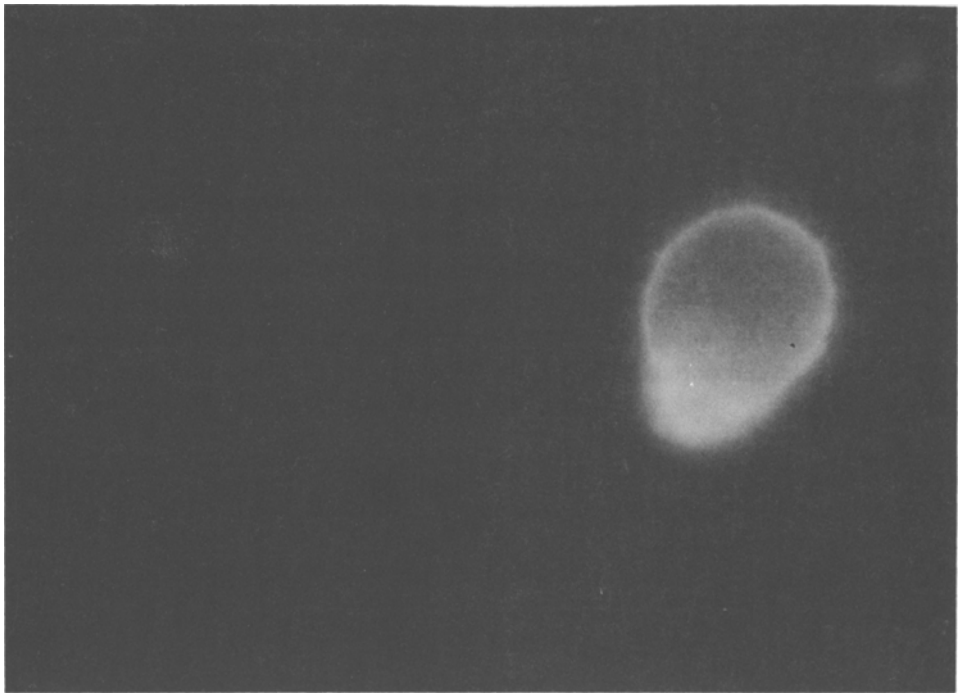

Fig. 10. Cell surface pattern of fluorescence of the same cell at green excitation (left — cap formation) and blue excitation (right — uniformly distributed fluorescence). The cells were labeled with FITC conjugated Con *A* and TRITC conjugated anti-$H-2K^k$ immunoglobulins

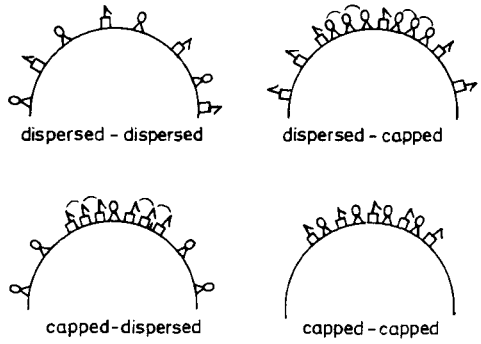

Fig. 11. Schematic drawings of the distribution of binding sites in dispersed and cap forming state on the cell surface

phenomenon, called capping, with quite a number of interesting cell-biological implications, is perfectly suitable to alter the relative distribution of the cell surface elements in case they are "capped" separately. Fig. 11 demonstrates a simplified scheme of the capping and also the disperse distribution of the ligands, and, consequently, the ligand binding sites.

Though the phenomenon outlined above has already been known for ten years, the exact biophysical investigation of the possible distribution patterns of cell surface receptors and other ligand binding sites is still missing [17]. The use of fluorescence energy transfer as a slide rule at molecular level, and the application of the cell sorter and analyzer giving individual information at single cell level, without losing the advantage of the cell populations, bring us really closer to a better understanding of cellular biophysics.

Acknowledgements

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A more detailed account on some of the experiments outlined here briefly will be published elsewhere.

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