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Two-dimensional receptor patterns in the plasma membrane of cells. A critical evaluation of their identification, origin and information content

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Abstract

A concise review is presented on the nature, possible origin and functional significance of cell surface receptor patterns in the plasma membrane of lymphoid cells. A special emphasize has been laid on the available methodological approaches, their individual virtues and sources of errors. Fluorescence energy transfer is one of the oldest available means for studying non-randomized co-distribution patterns of cell surface receptors. A detailed and critical description is given on the generation of two-dimensional cell surface receptor patterns based on pair-wise energy transfer measurements. A second hierarchical-level of receptor clusters have been described by electron and scanning force microscopies after immuno-gold-labeling of distinct receptor kinds. The origin of these receptor islands at a nanometer scale and island groups at a higher hierarchical (μm) level, has been explained mostly by detergent insoluble glycolipid-enriched complexes known as rafts, or detergent insoluble glycolipids (DIGs). These rafts are the most-likely organizational forces behind at least some kind of receptor clustering [K. Simons et al., Nature 387 (1997) 569]. These models, which have great significance in trans-membrane signaling and intra-membrane and intracellular trafficking, are accentuating the necessity to revisit the Singer–Nicolson fluid mosaic membrane model and substitute the free protein diffusion with a restricted diffusion concept [S.J. Singer et al., Science 175 (1972) 720].

Keywords: Receptor patterns; Cell-surface organization; Fluorescence energy transfer; Scanning force microscopy; Electron microscopy

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1. Introduction

Receptor patterns, made from MHC class I and II molecules, tetraspan proteins, IL-2 receptor subunits, the erbB family molecules and in some restricted cases the transferrin receptor, have been identified in the plasma membrane of different cell lines, most of them from lymphoid lineage [2–13]. On the one hand, the quasi-permanent assembly of these molecules has been in variance of the most important paradigm of the Singer–Nicolson fluid mosaic membrane model, namely the unlimited free diffusion of cell surface protein elements in the lipid bilayer. There is no doubt about the importance and significance of the Singer–Nicolson model, however, new data demand a revisiting it. Similar conclusions have been drawn by several authors from a variety of experimental data suggesting restricted diffusion of cell surface molecules [7,14–18]. On the other hand, the origin of these supramolecular structures was hardly explained by the unifying force of their ligands. With some exceptions like that of the EGF receptor, there was not enough support and evidence obtained that these were organized by cytoskeletal elements or some direct molecular forces between the members of such homo- and hetero-associates [19–21]. Fluorescence resonance energy transfer (FRET), has been the oldest physical technology, apart from the chemical cross-linking, to present evidence for the existence of such supramolecular formations [22–25]. The advent of single particle tracking, atomic force and near-field scanning optical microscopies revealed the existence of restricted diffusion of otherwise mobile cell surface molecules and as a new aspect of their assemblies the two distinct hierarchical levels of non-random receptor co-distribution patterns were introduced [26–31]. The first level was undoubtedly the 2–10-nm distance relationship limited homo- and hetero-associations, while the second level was discovered when immuno-gold beads with diameters in the more than 10-nm level also showed a non-random (i.e. non-Poissonian) distribution in the cell membrane [7,8,20,32]. The new paradigm, an explanation for the existence of such oligomerization has come from inside the cell in a literary sense. Simons et al. [1] and others provided ample evidence on the existence of lipid rafts, composed of sphingolipid and cholesterol. These structural organization forms may be instrumental not only for the maintenance of the first hierarchical level of homo- and hetero-associates of apparently unrelated receptor patterns at a nanometer scale, but also on a higher hierarchical level.

In this paper we attempt to present a brief cross-section of methods and data on the problems, which have particular importance in enlightening some problems of immunology, in particular, and cell-to-cell communication and cell physiology, in general. A special emphasis will be put on the analysis of generation and physiological meaning of two-dimensional receptor patterns based on pair-wise fluorescence energy transfer measurements between associated receptors.

2. Analysis of the methodological approaches in study cell surface non-random homo- and hetero-associations

The methods for studying receptor assemblies in the plasma membranes may vary from semiquantitative approximations to highly sophisticated, practically single molecular investigations [33–41].

Fluorescence energy transfer, i.e. energy tunneling between suitably selected fluorescence donor–acceptor pairs is a very widely applied method, which is due to its relative simplicity and the good targeting possibility of the selected dye molecules to their destiny by monoclonal antibodies or their fragments ([42], for reviews see [7,12]). The difficulties to transform the measured transfer efficiency values to intermolecular distances are known from the work of Dale et al. [43,44]. Nevertheless, the existence of energy transfer signals between two receptor populations on a single cell or determined over a larger cell population in a flow cytometer undoubtedly reports that a significant amount of the two receptor kinds are in each others molecular vicinity. However, it demands rather complicated,

sometimes fluorescence lifetime resolved measurements, or, e.g. long-range electron transfer to determine the degree of oligomerization [45–53].

Immuno-gold beads of different sizes, which were applied sequentially on different receptors show that such hetero-association of receptors is rarely complete and there are non-associated fractions as well [32]. The immuno-gold bead-defined distribution patterns represent a second, higher hierarchical level of receptor co-clustering from several hundred nanometers to micrometer levels, in contrast to the FRET-defined smaller receptor islands between 2 and 10 nm [26].

3. Generations of two-dimensional receptor patterns from pair-wise FRET measurements

FRET measurements are dependent on distance relations of the actual fluorescence donor and acceptor pairs as described by the equations below.

$$E = R_0^6/(R_0^6 + R^6), \quad (1)$$

where E is the percentage of the transferred energy from the fluorescence donor to the acceptor dye, R_0 is a spectroscopic parameter, defining the distance between donor and acceptor, when the probability of the transfer of the donor-absorbed energy to the acceptor, or its emission by the donor is 50%, and R is the actual distance in three-dimensions between the emission dipole of the donor dye and absorption dipole of fluorescence acceptor dye.

Let's assume that the emission dipole of a donor molecule and the absorption dipole of an acceptor molecule, can be considered as free rotors. In this case the κ^2 factor, the critical orientation-angle-derived parameter can be considered as an average for random orientation and it can be reduced from a value between 0 and 4, to 0.66 or near to that numerical factor [54,55]. Thus, generally we can conclude that in a favorable case the FRET efficiency, i.e. what percentage of the energy absorbed by the donors is transferred to the nearby acceptor molecule, will depend largely on the (approx. 2–10 nm) distance relationship between the donor and acceptor molecules [7,43,44,56,57]. When we measure flow cytometric energy transfer, we can obtain data between distance relationships of A and B receptor populations, which is averaged for each cell and can be measured on a cell-by-cell basis [58–61]. If we intend to get a general map, from data on the distance relationship among A, B, C, D...., etc., receptors or other cell surface molecules measured at a pair-wise fashion, it can be constructed as follows [8,20,61,62].

1. Measure the proximity between each possible pair of the receptors in question, determining FRET efficiency data preferentially from the lower density population as a donor, towards the higher density population as an acceptor. If the density of the X and Y populations is approximately identical, it is advised to measure FRET from both directions. This can be a general rule, as well, unless some of the receptor densities are not too low to get energy transfer from the higher density population to the lower one. These data carry the proximity information of the pair-wise densities between all variations of the existing receptors.
2. Tabulating these data we have the means to generate a two-dimensional model on the relative average distance of these receptors. Accepting that higher FRET efficiency means closer steric distance and applying the 'triangulation' method of geographical map makers the following way, one can get an average relative distance map of the receptor molecules [7]. The almost universally present MHC class I and II molecules — which have also reasonable high densities — can be used as a corner stone for the maps to be generated. The distances of the MHC class I molecule heavy-chain and light-chain from the MHC class II molecule determines a triangle (Fig. 1). The next receptor can be localized on this map, through determining its distances from all of the above three points. It determines its unambiguous, yet uncor-

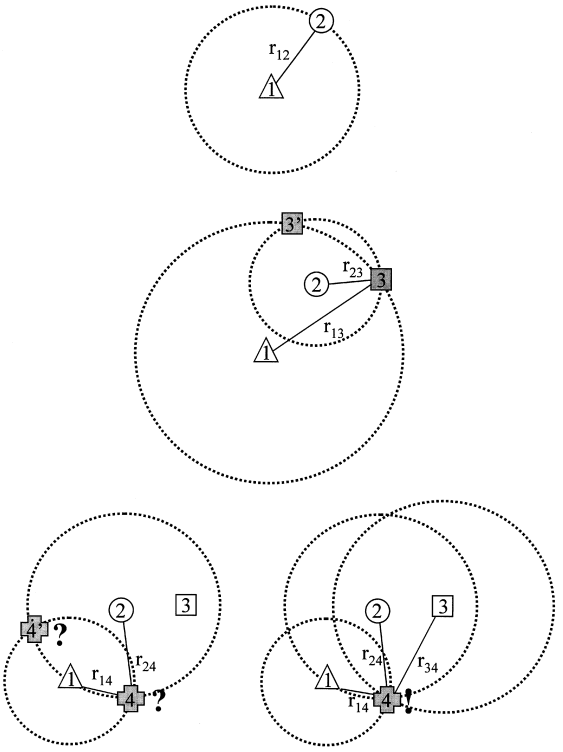

Fig. 1. Derivation of two-dimensional receptor maps of individual receptors from FRET distance data measured in a pair-wise fashion between donor and acceptor labeled receptors. An epitope having a distance from the other as determined by FRET can be localized around the perimeter of a circle. If we have a third epitope it has two localization possibilities, the intersections of the circles, which represent their distances from the above two epitopes. These have a mirror-symmetry. Any of those can be selected as a starting triangle. The localization of the fourth point can be determined from the distances of the above three points. Any further point can be positioned in two-dimension based on the distances from three points of the earlier defined epitopes.

rected (e.g. for labeling ratios with fluorescence dyes) and apparent average position on the map. The fifth receptor can also be localized through determining its distances from any three points the above four steric points. If mirror symmetrical positioning problem arises one more distance measurement between another (the fourth) point can help (Fig. 1). This may occur, when one receptor density is significantly different from the others, and this procedure can easily be continued.

The map evidently has several sources and possibilities of distortion:

1. We speak about distances between fluorescent donor–acceptor pairs, which are generally bound to the pre-selected receptors through relatively large monoclonal antibodies. Using Fab fragments of monoclonal antibodies, whenever it is feasible helps to decrease ambiguities. Recently, we tried to combine flow cytometric energy transfer data and available crystallographic parameters on generation of dimeric models of MHC class I molecules with fixed attached Fab fragments. It was interesting to learn that FRET data between the plane of the membrane and distinct parts of the molecules resulted in a number of acceptable and unlikely models. Higher oligomers provided an even less number of allowed steric positioning within FRET distance (Gáspár et al., submitted).
2. The localization of the epitope for the antibody on the targeted cell surface molecules — in most cases — is unknown. This could be embarrassing for determining proximity between lengthy (e.g. cellular adhesion molecules) and much shorter (MHC class I, II and T cell receptor, etc.) molecules. Despite an accurate measurement of a three-dimensional distance, its two-dimensional projection may not be correct. Nevertheless, if the above projection is reproducible, it carries structural information.
3. A geometrical targeting antibody model of Kubitscheck et al. [63] helps to decrease the problems, but cannot eliminate all of them. The most often experienced facts are that different monoclonal-antibody-aliquot labeled separately, with dyes provide us with very similar (or near identical) FRET data. This fact suggests (although indirectly) that the same lysyl-ε-amino-groups could be responsible for binding the dye molecules. This is especially likely at approximately 1:1 dye to Fab labeling ratio. Direct determinations of amino-acid sequences, which can preferentially bind dye molecules, were carried out in separate labeling experiments. The immedi-

ate environments of the dye-labeled lysyl-ε-amino groups showed very similar amino acid patterns (unpublished observations), again providing evidence that energy transfer distance relationships are rather reliable parameters as determined in a flow cytometer over a large cell population.

4. We determine an average energy transfer value for each cell over a large (statistically convenient) cell population in a flow cytometer, yet the receptors have different densities. This means that the patterns, generated by the above-described way through triangulation, represent the maximum receptor occurrence rate in such a receptor micro-island. On the other hand receptor populations may have only a fraction of them to be co-clustered with other receptor kinds.
5. The localization of the dye molecules on the Fab fragment is also rarely known, but as we seen above, the binding sites tend to be identical. This is a valuable asset, when the number of the dye molecules on the Fab fragments can be kept at the 1:1 ratio. Despite all the above sources of distortion and convolution possibility, a list that is far from being complete, the method is very robust. Data are extremely reproducible and distances between receptor population A and B are generally close, or near identical after changing the site of donor and acceptor molecules and determining the same proximity between B and A, or using antibodies labeled in different aliquot with the same ratio of fluorescence dyes. Receptor patterns could be very closely identical in the plasma membrane of distinct cell lines, as well. However, they could be different concerning their homo-association patterns, with some possibilities for functional significance. Recently, it has been found that otherwise identical receptor patterns of three distinct T lymphoma lines differed only in one minor detail. One cell line stringently demanded interleukin-2 (IL-2, a cellular growth hormone) in cell culture. The other two cell lines were growing without adding this specific molecule. The minor difference in the receptor patterns was that the IL-2 receptor's α-subunit was monomeric in the membrane of the IL-2-dependent cell line and was dimeric on the other two cell lines growing independently of IL-2. There is no evidence for a direct causal relationship between the difference in receptor homo-associations and the growing properties, however, this difference in the receptor pattern may have its functional significance (Matkó et al., submitted). Enrichment of the plasma membrane with cholesterol or at least partial removal of it may change the structure of cell surface patterns [64] (Matkó et al., submitted).

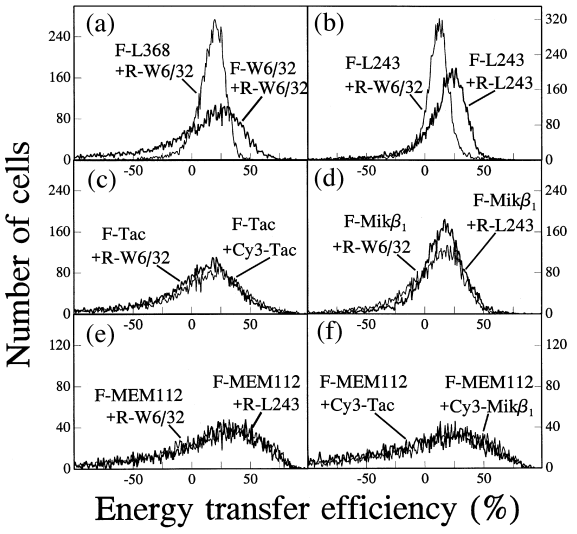

Fig. 2. FRET distribution patterns over cell populations. The distance determinations can de derived from the median of these direct FRET distributions of two epitopes over a cell population. The curves were derived from pair-wise FRET measurements between monoclonal antibody targeted and donor- and acceptor-dye-labeled cell surface receptors of lymphoid cell lines as described in [7].

In Figs. 2 and 3 we can see FRET derived energy transfer distribution data in the form of histograms and two-dimensional receptor patterns on Kit 225 IG3 cell line, which are very much identical to those on the Kit 225 K6 and the HUT-102 T lymphoma lines.

We can conclude that two-dimensional receptor patterns, despite undoubted distortion and convolution problems, carry important informa-

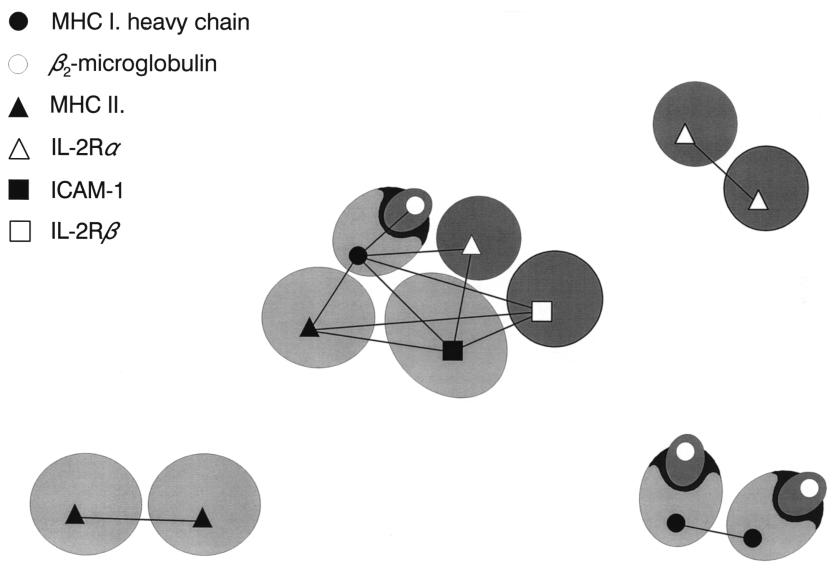

Fig. 3. Two-dimensional receptor patterns of six distinct epitope-sites of individual receptors at the surface of a lymphoma line (Kit 225 IG3). These formations, where the circled territories are only symbolic and distances between their geometrical centers carry information on distance relationships, show the relative distribution patterns of the selected cell surface receptors as obtained from the triangulation methodology described in the text to Fig. 1. See the analysis for more detailed justifications for their existence and meaning in the article.

tion on the cell-surface receptor distribution. The cause of particular changes cannot always be identified. Nevertheless, we can postulate that if we observe homo- or hetero-association, or their changes by FRET, they must occur at a significant ratio of the receptor population, otherwise we could not be able to 'see' them. The lack of energy transfer between two receptor kinds does not necessarily mean the complete lack of, e.g. homo-associations. Binding even the shorter Fab fragments — not even mentioning the much bulkier whole antibodies — can position the fluorescence labels on the opposite sides of a hetero- or homo-dimer. In such a way the dyes can be localized beyond the Förster distance, i.e. the upper limit of the dipole–dipole proximity that is obligatory to observe FRET.

The determination of relative distances, in a pair-wise fashion between receptors, led to the discovery that non-random distribution means that receptor proximities follow a repeated pattern in the plasma membrane. In this quasi-periodic distribution the stoichiometric receptor complexes are also repeated and their proximity relationships are very similar or even identical.

Fluorescence energy transfer measurements make possible the determination of these quasi-permanent receptor structures (which must also have an average lifetime, regulated by the exo- and endocytotic processes), and a repeated 'triangulation' of proximities makes possible two-dimensional receptor pattern generation.

We haven't discussed the so-called photobleaching energy transfer method separately, which can be very efficient in microscopes. The reason was that this method had very similar problems as those outlined for flow cytometric energy transfer [65–70]. The physical basis of the photobleaching-FRET technique is that the bleaching-time of the donor dye increases in the presence of the acceptor. The phenomenon has its origin in the fact that efficiency of the photochemical destruction of a dye is directly proportional with the time spent in the excited state. The presence of the acceptor decreases the efficiency of the photo-destruction of the donor, since the donor population is tunneling (a part) of its absorbed energy to the acceptor population. The applicability of this method in a microscope promotes selecting different areas of the cell surface,

cell interior, or even intercellular encounters as targets for FRET, i.e. proximity measurements [49,50,71]. Lifetime resolved energy transfer also contributes to the variety of available methods. The handicap of these microscope-based specialties is the lack of good statistics as compared with the abundance of available data from flow cytometry. On the other hand, basic cell physiological phenomena can be studied among better-controlled conditions by photobleaching energy transfer [7].

4. Electron microscopy and distinct versions of scanning force microscopy can cover a second hierarchical level of receptor distributions.

An independent methodical approach of the above outlined non-random receptor clustering in the plasma membrane could be obtained by applying electron and/or scanning force microscopy. Application of immuno-gold beads may solve the 'visibility' and identification of the receptors on individual cell surfaces.

In 1995 an attempt to identify individual receptors on lymphocyte surfaces led to the discovery of two distinct hierarchical levels of receptor distribution [20]. Cell surface receptor distribution patterns were studied, but the applied scanning (atomic) force microscopy could not resolve such cell surface elements on the highly corrugated plasma membrane, not even mentioning the receptor-identification problems. Applying immuno-gold beads to label MHC class I receptors in the plasma membrane of T lymphocytes we found a several hundred-nanometer scale pattern of their distribution.

The cell surface receptors can have random and non-random fashion distribution in the plasma membrane. Thus, the next step is to determine the difference between random and non-random distribution patterns. Chandrashekar [72] solved the problem for identifying clusters of stars by mathematical methods. His analysis shows that any randomly scattered distribution pattern of a large number of entities has to obey a Poissonian distribution law as described here:

$$P_{(k)} = (e^{-\lambda} \cdot \lambda^{k})/k!. \quad (2)$$

In which, k is the number of receptors (stars, etc.) which are supposedly non-randomly co-clustered in a pre-selected compartment and λ is the ratio of the number of the receptors and the compartments, if both numbers are very large and e is the symbol for the base number of natural logarithm.

The comparison of theoretically and experimentally obtained immuno-gold bead patterns allowed us to make distinctions between random and non-random distributions of the indirectly identified receptors by the gold beads in electron or scanning force microscopic pictures. The size of the beads allowed us to assume that more than one receptor can be accommodated under a single bead. However, one does not know, how many receptors have been covered by a single bead. It obviously depends on the size of the bead, the density and size of the receptor(s) and their homo- or hetero-clustering. This 'handicap' was utilized by Jenei et al. [32] to determine the ratio of the hetero-clustering between MHC class I and II type cell surface receptors. The comparison of the density of receptor A labeled with a 30-nm immuno-gold bead with that of receptor B labeled with 15-nm-diameter gold beads sequentially, targeting them to MHC class I and II molecules, respectively, provided a good approximation of the ratio of co-clustering receptor A with B, and B with A. This model can reasonably handle hetero-associations of receptors, but it is not applicable for studying homo-associations.

The simultaneous investigation of identically prepared aliquot of samples by atomic force, or scanning near-field optical microscopy provides us with possibilities to support electron microscopic pictures under even nearly physiological conditions [20,30–32]. Scanning force microscopy (SFM) is a generic name for a number of non-optical and optical microscopy. Atomic force microscopy (AFM) uses a very sharp and hard tip, made mostly from Si_3N_4, and the diameter of the tip at the contact place varies between 2 and 10 nm. Interactive forces between the surface and the tip generate signals — usually through a movement monitored by laser light and with a split diode as

sensor — which can serve as a feed back signal to hold the tip at a constant force or a constant height above the surface. The scanning of the sample in X and Y directions may be convoluted by the fact that corrugations of the surface make contact first with the side (shoulder) of the tip. However, the measurements in the Z direction are really very accurate even at a nanometer level. The scanning near-field optical microscopy (SNOM) eliminated the optical elements in order to overcome the limitations in resolution imposed by the Abbe-principle valid for optical lenses. A tiny hole–aperture, optical fiber tip, is scanning the surface at a very close distance. The surface is illuminated through a hole (fiber, aperture), where the spot-size is smaller, than the wavelength of the applied light. Relevant feedback signals prevent the breaking of the tip during the scanning and the collected light carry information on the surface corrugations and fluorescence signals, respectively. The optical signaling helps the identification of cell surface elements. The resolution power of the AFM is higher, but the identification with SNOM the fluorescently labeled cell surface elements is easier.

5. Methods to study cell surface dynamics.

Single particle tracking, fluorescence recovery after photo-bleaching, rotational relaxation studies based on phosphorescence anisotropy measurements, scanning force microscopies, monitoring the ever present membrane potential and ion-channel activities and their changes and application of a variety and multitude of optical tweezers and their combination with lasers, are among the more frequently used, leading biophysical techniques to study dynamics of cell membrane parameters [15,27–29,38–40,73–77]. It had been shown that changes in molecular conformations could also be monitored by intramolecular FRET measurements on cell surface molecules in a flow cytometer [78]. Tsong and Astumian [79] and Gonzales and Tsien [80] summarized the effects of membrane potential changes on the molecular parameters, which are perpendicular to the plane of the membrane and by scaling these changes they could even measure membrane potential.

Despite all efforts so far to quantitate receptor assemblies and their interactions and correlation with transmembrane signaling the available experimental data had not provided direct proofs on the number of interacting molecular partners, which can elicit physiological responses. The question, e.g. how many receptors are necessary to initiate a T cell mediated cytotoxic interaction, is not satisfactorily answered yet. Contradictory data are available on the mono-, di-, or tetrameric activation of immune receptors. Delon et al. [81] postulated that monomeric MHC class I molecules can be satisfactory for T cell activation. Boniface et al. [82] found that at least tetrameric assemblies of MHC class II molecules are the most effective for signal transduction and in their case, monomeric (soluble) molecules fail to transmit signals. Lanzavecchia et al. [83] presented important molecular biological evidence that a single MHC molecule with a foreign peptide was enough for transmembrane activation of nearly 200 T cell receptors. However, either the systems are far from providing a physiological environment, or the evidence is very indirect. A recent paper of Xavier et al. [84] also emphasized the necessity of membrane compartmentation in an effective T cell activation. The ‘joy of aggregation’, as Metzger [85] pointed out not so long ago, and ever-accumulating evidence support the view that efficient and timely cell activation demand a multitude of receptor–receptor interactions. This general rule does not exclude that under specific conditions on particular cell lines a one-to-one molecular interaction can be sufficient to evoke a functional response. Homo- and hetero-associations of receptors even if they are above the minimum requirement of signal processing, very likely amplify, ‘confirm’ and speed up signals and signal processing, which results in a more effective triggering of intracellular events. This fact emphasizes the importance to increase our knowledge on dynamic, physical events in the cell membranes.

Taken together this mini-review had tried to sum up a cross-section of some biophysical experimental methods, which may help to understand

the nature of membrane organizations. These data together with those dynamic methodical procedures having the capacity to investigate membrane or intracellular mobility even at a single molecular level can provide us with a rather complicated picture on cell physiology. Intra-membrane structures, which are embedded into the communication center of the cell, are rather crowded and not independently moving much as we believed earlier. The existence of sphingolipid and cholesterol rafts can provide a good explanation for the receptor assemblies. Ligands-specific and non-specific triggers of transmembrane signaling events may utilize these apparently unrelated receptor vicinities, enforced by molecular structures. The role of such physical parameters as ion traffic and changes in the membrane potential, which can change large conformations in a specific and non-specific way is acknowledged, but is far from being understood. The restricted diffusion model in the plasma membranes offers us a nice theater to work on at a single molecular, receptor assembly, or even whole cell level. The detailed meanings of signals for specific transport processes yet have to be decoded for the plasma membrane, for the cytosol and the nuclear envelope. Next steps will follow at the intercellular realm and in the higher degree living organs and multi-cellular structures.

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