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Super-resolution characterization of TCR-dependent signaling clusters

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Summary

Multi-molecular signaling complexes drive the earliest events of immune cell activation via immunoreceptors with unexplained specificity and speed. Fluorescence microscopy has shown that these complexes form microclusters at the plasma membrane of activated T cells upon engagement of their antigen receptors (TCRs). Although crucial for cell function, much remains to be learned about the molecular content, fine structure, formation mechanisms, and function of these microclusters. Recent advancements in super-resolution microscopy have enabled the study of signaling microclusters at the single molecule level with resolution down to ~20nm. These techniques have now helped to characterize the size distributions of signaling clusters at the plasma membrane of intact cells and to shed light on the formation mechanisms that govern their assembly. Surprisingly, dynamic and functional nanostructures have been identified within the signaling clusters. We expect that these novel methodologies, combined with older techniques, will shed new light on the nature of signaling clusters and their critical role in T-cell activation.

Keywords

T-cell activation; microclusters; super-resolution microscopy; nanostructure; single molecule microscopy

Introduction

T-cell activation is an essential component of the adaptive immune response, mediating many responses including cytokine and antibody production, killing of infected or transformed cells, and tolerance to self-antigens. Loss of T-cell function results in severe immunodeficiency, while inappropriate T-cell activation can lead to autoimmune diseases (1–2). Proper T-cell activation depends in turn on cellular responses initiated by the T-cell antigen receptor (TCR). For the most common classes of T cells, activation begins when the antigen-binding α and β subunits of the TCR engage an antigen presented by a major histocompatibility complex (MHC) molecule on the surface of an antigen-presenting cell (APC) and initiate an intracellular signaling cascade (3–4). Only three to 10 activated receptors are needed to generate a complete cellular response (5). A number of factors including receptor-peptide affinity, the number of engaged receptors, and the presence of co-receptors all modulate downstream TCR signals leading to differing final outcomes such as proliferation, differentiation, or cell death. Because of the central importance of the TCR, understanding signaling mediated by this receptor has been a goal of immunological research for decades. Many of the components of this signal transduction cascade have been

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identified, while imaging techniques have provided unique insights into the organization of these molecules following TCR engagement. Currently, advanced imaging techniques are being used to understand TCR activation in molecular detail.

Many of the components required for TCR signaling have been identified using biochemical and genetic techniques. The TCR is a multi-subunit receptor without intrinsic kinase activity. Signaling starts with the phosphorylation of the cytoplasmic subunits of the TCR by Src family protein tyrosine kinases (PTKs), Lck or Fyn (3). Another PTK, ζ -associated protein of 70 kDa (ZAP-70), then binds to the phosphorylated ζ and CD3 chains, is activated by Lck phosphorylation, and in turn phosphorylates the adapter protein, linker for activation of T cells (LAT), and other substrates. The phosphorylation of LAT creates new binding sites allowing for the recruitment of another major adapter, Src homology 2 (SH2) domain-containing protein of 76 kDa (SLP-76), which also becomes phosphorylated on tyrosine residues (6). These two adapters serve as scaffolds for the binding of many other essential molecules that are required for T-cell activation (Fig. 1), including phospholipase C- γ 1 (PLC- γ 1), which regulates calcium signaling and diacylglycerol production (7).

Some cellular responses, such as a rise in cytosolic calcium, begin within seconds of TCR engagement, showing the rapid transmission of signals from the receptor to effector molecules. Light microscopy studies have led to the current consensus that this rapid signaling begins in microclusters of molecules recruited to activated, phosphorylated TCRs. TCR clusters were first visualized in 1999 by Grakoui *et al.* (8), who observed small peripheral clusters containing TCRs conjugated to fluorescent protein tags in T cells activated by MHC-antigen complexes presented in a lipid bilayer. The Samelson laboratory used stimulation with immobilized antibodies to show that many components of the TCR signaling cascade were rapidly recruited to visible clusters and that these structures were the sites of activation of critical signaling molecules (9) (Fig. 2A, B). Live cell imaging of T cells showed that the rise in cytosolic calcium is simultaneous with formation of a single visible microcluster (9). Since then, these micron-sized entities have been studied in a variety of model systems (10–11) and visualized in T-cell conjugates with APCs (12). Microclusters appear to be a general phenomenon and have been also reported in cytotoxic T cells and B cells (13). Studies using anti-CD3 coated coverslips to engage the TCR demonstrated that most of the downstream components of the signaling pathway including Lck, ZAP-70, LAT, SLP-76, Grb2, Gads, Nck, the Wiskott-Adlrich syndrome protein (WASP), PLC- γ 1, Vav, and c-cbl are all rapidly recruited to the first detectable TCR microclusters (9) (Fig. 2A,B). Other studies using lipid bilayers for activation confirmed these results and showed that the molecules in microclusters dynamically sorted into immune synapses (10). It has been clearly established that peripheral microclusters are responsible for sustained signals, as they contain phosphorylated forms of most signaling molecules and defects in cluster formation and persistence are associated with abrogated T-cell activation (14–16).

There are many unanswered questions regarding how a signaling cascade develops around a single receptor or adapter molecule within large microclusters, which can contain over one hundred TCR molecules (11). There could be considerable heterogeneity in individual TCR and LAT-based complexes. For example, Grb2, which binds many different molecules via its SH3 domain, can bind to three different phosphorylation sites on LAT, but these are the same sites that bind GRAP and Gads (17). Are different LAT molecules nucleating different signaling complexes within microclusters depending on which adapter binds to these tyrosines? Diffraction limited light microscopy cannot answer these questions. The stoichiometry of various components and the molecular architecture of the signaling complexes also remain uncertain. A variety of biophysical and biochemical techniques have been used to estimate the binding affinities and stoichiometry of signaling components, but

there are often differences between *in vitro* and *in vivo* binding. For instance, in the case of T cells, it has been shown that both VAV1 and Nck can bind to phosphorylated SLP-76 peptides directly *in vitro*, but Nck is required for *in vivo* interaction of VAV1 with SLP-76. In addition, it appears that two Nck-VAV1 dimers may bind to each SLP-76 molecule (18), but no direct imaging has been able to show how these molecules are arranged in the cell. Moreover, there is considerable evidence that the assembly of signaling complexes is highly cooperative. Using SLP-76 as an example, SLP-76 is recruited to LAT via Gads binding but PLC- γ 1, VAV1, and Itk are also required to maintain a stable complex (19–21). It has been suggested that the development of microclusters requires the cross linking of LAT molecules by Grb2 and SOS (22) and that interactions with the cytoskeleton are also required.

These complex structures are not static; real time imaging techniques have demonstrated the dynamic nature and changing composition of microclusters. On lipid bilayers or in APC-T cell conjugates, TCR microclusters move to the center of the cell (8, 12), while on antibody-coated coverslips, clusters containing LAT and SLP-76 rapidly translocate to near the microtubule organizing center (9, 23). The nature of these moving clusters is not well-defined. Although SLP-76 and LAT are internalized into vesicles (23–24), TCR microclusters may be moving along the cell surface (25–26). The composition of microclusters also changes rapidly. With activating molecules immobilized on a fixed surface, many components including Grb2, Gads, and c-Cbl are only transiently seen in microclusters (9, 27). Moreover, ZAP-70 and SLP-76 appear to move differently from the TCR in lipid bilayer systems (11). Finally, photobleaching experiments have shown that even molecules which appear to be static, such as that fraction of ZAP-70 bound to the TCR are in fact rapidly exchanging with cytosolic molecules, so the whole microcluster is continually reforming with new molecules. In the case of ZAP-70, the exchange rate changes with time, so that younger microclusters exchange rapidly, while older clusters are more static (9). In addition, it has been shown that LAT is mobile and exchanges between clusters (28). The structural events that underlie these changes in behavior are not known, nor do we understand how molecules exchange places in a structure that requires the cooperative binding of several different proteins. Observations based on a limited resolution of structures containing thousands of molecules cannot answer these important questions about the nature of signaling microclusters.

To answer these outstanding questions, we need to visualize signaling complexes in sufficient detail to define the structural arrangement of individual molecules and observe how molecules and molecular complexes form clusters. We would also like to be able to watch the development of structure within signaling complexes over time so that we can understand the fine details of molecular rearrangement as different molecules arrive and depart from microclusters. New super-resolution techniques are now being used to examine microclusters in greater detail. In this review, we discuss the results of several recent studies, which used super-resolution light microscopy to image microclusters in T cells at the level of single molecules. New data have provided insights into the clustering of molecules in quiescent cells along with estimates of the size of both TCR- and LAT-based signaling clusters. Importantly, some of these studies showed an unexpected, functional nanoscale organization of molecules within such complexes and nanoclusters.

Controversy over cluster size

The signaling clusters that are the focus of interest of this review have been studied extensively, but no consensus has been reached on the physical size of the minimal signaling complex in T cells. Various research techniques, such as biochemistry and imaging, each bearing unique advantages and drawbacks, have been used to examine molecular clustering

at the plasma membrane (PM), but they have generated a wide range of proposed sizes for TCR-based microclusters. To some extent, this is due to the fact that the resolution limit of the methods used affects the perceived size of the complexes. Indeed, many techniques lack the resolution needed to accurately sample a signaling complex if it is composed of only a few molecules. The microclusters observed by diffraction limited microscopy are approximately 200 to 500 nm in diameter (9–10, 12, 29) (Fig. 2A), but this size is clearly limited by the inability to resolve small fluorescent structures with visible light. In contrast, Schamel *et al.* (30) showed using biochemistry (Blue Native gels) that the TCR resides in monomers and clusters as small as dimers or trimers in unactivated cells (Table 1, discussed later). Upon TCR stimulation, these clusters grew in size but still consisted of a majority of monomers and small clusters (30). Immunogold staining and electron microscopy (EM) of ripped membrane sheets served to confirm that result (30). However, other EM studies showed that many immune receptors are clustered before activation (31) with cluster size increasing under various stimulating conditions (27). Overall, these studies have indicated that many molecules at the PM reside in small clusters or 'protein islands' and that effector molecules and receptors can be sorted into distinct nanoscale domains (32). Common to all studies is a significant increase in clustering of TCR and LAT upon TCR stimulation. Nonetheless, marked differences in cluster sizes result from using different techniques: from microclusters seen by diffraction limited microscopy, to protein islands visualized by TEM, to the dominance of monomers and dimers by biochemistry and fluorescence cross-correlation spectroscopy (FCCS). While it was hoped that super-resolution visualization would resolve this question, different studies continue to produce different estimates of the size of signaling microclusters (33–34).

Imaging clusters beyond the diffraction limit of light

In 1873, Ernst Abbe postulated that the lateral resolution of light microscopy is restricted to about half of the wavelength used for imaging (35). Recently, multiple methods have been developed to increase the resolution of light microscopy beyond the diffraction limit and are commonly known as super resolution microscopy (36). To study microclusters beyond the diffraction limit of light, we first utilized STED imaging (37). In this technique, resolution is improved by reducing the size of the excitation area. A high intensity laser of an appropriate wavelength for the fluorophore to be imaged is focused to produce a doughnut-shaped spot that induces stimulated emission from illuminated fluorophores, thus depleting any activated molecules and preventing fluorescence. This beam is aligned with a normal excitation laser to produce a very small effective excitation area in the center of the combined beams. The size of the excitation area can be decreased by increasing the intensity of the depleting beam, but STED images generally have a resolution limit of about 80 nm (38). This technique has been used extensively in neuroscience research, particularly for studying synaptic vesicle trafficking.

With this technique we observed microclusters stained with anti-phosphotyrosine primary antibodies and Alexa 647-labeled secondary antibodies at the PM of Jurkat T cells activated by contact with a stimulating coverslip. We observed that the microclusters detected by confocal microscopy were composed of still smaller clusters (Fig. 2C,D). What appeared to be a single microcluster by diffraction-limited light microscopy was instead a blurred image of multiple smaller clusters. However, the apparent cluster size was still limited by the resolution limit of the STED system (~80nm). With this technique, we could not determine if the smaller clusters were composed of even smaller entities. Indeed, it requires single molecule resolution or sensitivity to identify monomers or even molecular aggregates of dimers and trimers within a microcluster. STED images also contained small scattered clusters that were not part of a well-defined microcluster. Since LAT microclusters account for only a small fraction of the total LAT fluorescence at the cell surface as measured by

either confocal or TIRF microscopy (7), the existence of these small aggregates suggested that molecules not confined to microclusters might also be involved in T-cell activation. These observations emphasized the need to characterize the role and organization of all of the LAT molecules at the PM including those that are not in microclusters.

A single molecule view of signaling clusters

Several studies have used imaging methods based on the stochastic identification of single molecules to look at microclusters (36). Such techniques include photoactivated localization microscopy (PALM) (39) and stochastic optical reconstruction microscopy (STORM) (40). Both PALM and STORM techniques use photoswitchable or photoactivatable probes to control the fluorescent emission so that very few molecules are visible at any time. Each fluorophore can then be precisely localized from the Gaussian curve of detected photons produced by diffraction-limited optics. In PALM imaging, fluorescent proteins that change color (photoconversion), that change from inactive to fluorescent (photoactivation), or that turn on and off (photoswitching) are used as probes, while in STORM imaging, dye pairs are used as an optical switch. In a variation of STORM imaging, fluorescent molecules are directly toggled between inactive and active states with a high intensity laser (dSTORM) (41).

We used PALM microscopy to study the organization of several molecules in Jurkat T cells activated by contact with anti-CD3 ϵ coated coverslips, with a primary focus on LAT-based complexes. Results were confirmed with fixed T cells from mouse lymph nodes as well as PALM imaging of living Jurkat T cells. We measured the probability density of locating individual LAT-PAmCherry molecules with a precision of about 20 nm. LAT clustering was analyzed using pair correlation functions (PCF). We found that LAT was significantly preclustered in the inactivated state, while TCR activation caused an increase in the extent of clustering and a slight increase in cluster size. Under both conditions, the majority of LAT molecules resided in small nanoclusters containing only a few detectable molecules, generally less than three. Similar results were seen for the TCR, imaged using TCR ζ -PAmCherry.

Other studies that used super resolution light microscopy or EM to examine microclusters have reached a wide variety of conclusions (Table 1). The Gaus group (42) used dSTORM to examine the organization of phosphorylated LAT using anti-LAT p191 in Jurkat T cells activated by contact with anti-TCR coated coverslips for 10 min. This study identified clusters of phosphorylated LAT that were unresolved in diffraction-limited TIRF images as well as microclusters that were in fact made up of smaller entities. In a subsequent study, they identified continuous size distributions of LAT clusters by PALM imaging and Ripley K statistics. Consistent with our data, they identified significant clustering from 0 to 300nm in both resting and activated T cells. However, based on combined imaging of LAT with PALM and LATp191 with dSTORM, they concluded that only clusters of ~80 molecules consist of phosphorylated LAT and that these structures are intracellular vesicles.

Lillemeier *et al.* (33) used a variation of PALM to examine the organization of TCR ζ -photo-switchable cyan fluorescent protein 2 (PSCFP2) and LAT-PSCFP2 in ripped membrane sheets from T cells activated by contact with a stimulatory coverslip. They identified some clustering of molecules in quiescent cells with increased clustering observed after activation. TEM studies of the membranes also showed clusters of immunogold labeling that increased in size after activation for both TCR and LAT. In this study, microclusters appeared to be concatemers of multiple probability density peaks, representing distinct and stable domains of TCR and LAT that show little or no colocalization, although TCR and LAT concatemers became adjacent upon TCR activation. Both PALM and TEM data indicated that these

domains were 35–75 nm wide and up to 300 nm in their longest dimension, similar to the dimensions of a small microcluster. We discuss the data from this study and that of the Gaus group below.

Hsu *et al.*(43) performed PALM imaging of ZAP-70-tdEos, LAT-Dronpa, and SLP-76-Dronpa in Jurkat T cells. They found an average domain size of ~140 nm for ZAP-70, ~310 nm for LAT, and ~230 nm for SLP-76 in cells activated for 5 min with immobilized ligands, although they also observed larger clusters of LAT (390 nm) and SLP-76 (300 nm) in cells activated for shorter times (43). However, these clusters should all be clearly visible as distinct entities in STED images. Instead, we saw smaller clusters whose true size was probably less than 100nm in either dimension. Moreover, dcFCCS of live T cells on lipid bilayers showed a high fraction of mobile molecules, mostly monomers (33). While one explanation is that these molecules move between stable larger domains, another possibility is just that many TCR and LAT molecules exist as very small monomer, dimer, and trimer groups in the PM.

Toward a 'unified view' of molecular clustering at the PM

Single molecule imaging of molecular clustering at the PM of T cells, has not been able to resolve controversy over the cluster sizes as discussed above but has added new concepts such as protein islands and nanoclusters to the ongoing discussion in the field. Considering the wide range of results and views on molecular clustering at the PM of T cells, we discuss this issue in more detail. First, we recognize that part of the confusion over cluster size is due to imprecision of language, as the word cluster can refer to several different physical entities. Also, as discussed above, different imaging modalities give rise to apparent clusters at different length scales. Therefore, we start our discussion by revisiting the concept of molecular clustering, defining clustering as any distribution that shows significantly higher proximity of molecules relative to random placement at a region of interest. We aim to distinguish different levels of molecular patterning at the PM. We then examine some related results and possible models of molecular clustering at the PM. Finally, we offer a model of clustering over wide size distributions as a possible solution to this controversy.

Multiple levels of molecular clustering

Some apparent clusters, or non-random distributions of surface proteins, are caused by irregularities in the cell surface. At larger length scales around 200nm, the PM is not uniform. This is especially true for T cells approaching an APC or stimulating surface with protrusions and lamellae. Such lamellae confine and could enrich molecules in an area as the intercellular contact forms. Furthermore, even when the cell spreads, membrane ruffling continues to contribute to heterogeneity in the pattern of the intercellular contact. It is therefore expected that patterns derived from the architecture of the cell surface will result in non-random placement of various molecules at the PM of cells. Such heterogeneity can be referred to as 'first-order' clustering. Indeed, membrane patches seem to confine LAT clusters and pattern them at large distance scales (Fig. 2). That this is a general effect can be determined by imaging an independent PM protein that does not bind to any of the components of the TCR signal transduction cascade. In our studies (34), we used TAC (the α -chain of the IL-2 receptor) for this purpose. Both LAT and TAC appeared as clusters marking the entire lamella where it first contacted an activating coverslip. As the cells spread, the TAC molecules dispersed while LAT molecules remained in place, resulting in bright LAT fluorescence at the sites of first contact. Thus, the overall shape of many LAT microclusters is determined by the shape of the lamella that first encountered the activating surface.

Models for molecular clustering at the PM

To gain further insight into the nature of TCR and LAT clusters, we summarize in Table 1 the results of the data discussed above and related data from multiple studies using various research techniques. We note that several models regarding the size distribution of PM molecules of resting cells can be suggested based on these results: (i) Molecules at the PM of resting cells are largely or solely monomeric. This model is supported by biophysical work (44) and PALM imaging (45). Alternatively, (ii) Molecules are preclustered at the PM of resting cells and reside in well-defined domains or protein islands. This model is supported by TEM (27) and PALM (33). (iii) Molecules exist in a distribution of sizes, from monomers, dimers, and trimers to oligomers or domains of significant size. This model is supported by biochemistry (30, 46), PALM (34), and TEM (31, 47–48). Upon cell activation, all studies show an increase in the size of clustering. However, for most cases, the size increase is modest. This moderate change in clustering size is surprising, considering the diffraction-limited microscopy view that large microclusters are required for signaling (10–11). Moreover, an increase in cluster size has been correlated with gain of function of the molecules in many studies. Increased clustering has recently been related to the increased sensitivity of previously stimulated and memory T cells when compared to their naive counterparts (46). Nevertheless, it is still unclear how cluster size relates to molecular function. For example, in the case of B cells, the view of an increase in clustering coincident with a gain of function has been challenged by the Reth group (49), who showed that the disintegration of BCR clusters into monomers upon cell activation enables their function. We discuss this issue in the context of T cells below.

Resolving the controversy: distributions of molecular clustering at the PM

Bias in the interpretation of size distributions can easily be introduced by constraints on the methods used or during data analysis and interpretation. Differences in the statistical analysis methods used may account, in part, for the described differences in molecular clustering as captured by single molecule imaging techniques (33–34, 50). These statistical methods include Ripley's K and L statistics (51), the pair correlation function (PCF) (52–53), Hopkins, and nearest neighbor techniques (51). For instance, using Ripley's K statistics to identify clusters sizes tends to emphasize larger clusters than the PCF statistics and is more sensitive to the first-order clustering due to lamellar patterning of the PM (34, 53). In addition, the smallest structures can be easily disregarded as background by simple thresholding of images or identified molecules. It is also preferable to avoid averaging to observe the heterogeneity of clustering states and sizes. Finally, reporting just the average sizes of aggregates can be misleading, because one cannot know *a priori* which species dominate the cluster size-distribution and the possible heterogeneous and size-dependent functional role of such species.

Considering on one hand the different models for molecular clustering and on the other hand the different methods applied for their study, we propose a simple explanation for the apparent size disparities (Fig. 3). Using PALM microscopy and a minimal bias for the identification of molecular clusters, we observe that molecules can form aggregates of a wide range of sizes that are better described as (discrete) size distributions (30, 34). Importantly, these size distributions are skewed towards (i.e. dominated by) very small structures, namely monomers, dimers, and trimers. We refer to these structures as nanoclusters. The size distributions of molecular clusters undergo a moderate shift towards larger clusters, yet are still dominated by the smallest nanoclusters.

Our conjecture is that many different molecules likely reside at the PM in distributions of cluster sizes, although dominated by monomers, dimers, and very small nanoclusters. We hypothesize that different studies of molecular clustering at the PM of T cells have captured

or emphasized certain portions of these distributions, based on the sensitivities of the methods used and of related analyses. Thus awareness of the broad distributions of clusters sizes at the PM may resolve the controversy over the sizes of molecular clusters in resting activated T cells. How the size and molecular content of these clusters (nano or micro) relate to function remains an active area of research.

Mechanisms of protein clustering at the PM

We have proposed that molecules at the PM reside in a distribution of clusters sizes. However, this leaves the question about what forces hold the LAT molecules in place in those clusters that are triggered by TCR stimulation, that contain a wide range of signaling molecules and that generate the first cellular responses (54). The formation mechanisms of these TCR-dependent clusters remains unclear (55). Possible mechanisms for TCR cluster formation include activation-dependent recruitment into lipid rafts (56) and intracellular protein-protein interactions. For example, B-cell receptor clustering has been shown to depend on intermolecular interactions of its C μ 4 domain upon binding of monomeric cognate antigens (57). Both TCR and BCR clustering depend on the actin cytoskeleton, as the disruption of actin polymerization resulted in smaller and fewer TCR clusters at the plasma membrane (10–11).

Similarly, mechanisms of LAT clusters have been suggested to include lipid rafts (58) and protein-protein interactions (22). The Vale group has studied this problem by measuring the mobility of two LAT mutants at the PM using single molecule tracking. LAT variants that cannot be incorporated into clusters should show increased mobility, while variants that are trapped in clusters would show the same mobility as wildtype LAT (WT LAT) molecules. They observed that a mutant, which lacks two juxtamembrane cysteines (LAT-2CA) and abrogates LAT binding to lipids, had a mobility similar to WT LAT, indicating that it is trapped in LAT clusters (28). In contrast, a mutant that cannot participate in phosphorylation-dependent protein-protein interactions because all nine tyrosines in the cytoplasmic tail were replaced with phenylalanines (LAT 9YF) did show increased mobility, suggesting that it is not able to form clusters. They concluded that protein-protein interactions play a primary role in nucleating LAT clusters.

In our two-color PALM study, we used bivariate correlation statistics to test whether similar mutants mixed randomly with WT LAT molecules. In cells expressing both WT LAT conjugated to Dronpa and WT LAT conjugated to PAmCherry, these molecules showed homogeneous mixing and were both incorporated into nanoclusters. However, the extent of mixing of either LAT-2CA or LAT-4YF mutants with WT LAT was substantially decreased, showing that neither mutant was recruited to WT LAT nanoclusters. Importantly, in contrast to the single particle tracking method used by Douglass *et al.* (28), PALM allowed us to directly visualize the sorting of the WT and mutants of LAT into distinct clusters. Thus, in contrast to their conclusion, we suggest that formation of LAT-based signaling clusters requires both protein-protein and protein-lipid interactions, although we cannot rule out the possibility that the effects of the LAT-2CA mutant were caused by a lack of disulfide bond formation rather than protein-lipid interactions.

Molecular interactions and functionality of nanoclusters

Molecular clustering is commonly found in many signaling systems, including receptor tyrosine kinases (59), immune receptors (31, 57), and membrane proteins (60) (Table 1). From a cellular perspective, it is an efficient and flexible way of increasing the local concentration of molecules, such as enzymes and substrates, thus enabling the rapid and controllable propagation of biochemical reactions in the cell. Indeed, microclusters are considered the sites of early T-cell activation (61). Having shown that nanoclusters actually

dominate the size distribution of LAT clustering raises the question of whether these constructs are functional. Namely, can they propagate the TCR signal downstream or is recruitment into larger microclusters required to transmit some or even all of the signals? We describe below our method for resolving molecular interactions in nanoclusters as a means to address this question. We then describe the sequence of interactions and the nanoscale organization of molecules that lead the TCR signal via ZAP-70 to LAT, the interactions of LAT with its downstream effectors Grb2 and PLC- γ 1, and the interactions of LAT with the adapter SLP-76. Sections below detail various aspects of nanoscale organization.

Two color PALM: resolving molecular interactions in nanoclusters

In our studies, we used two-color PALM to image the interactions between the TCR and LAT as well as a set of downstream effectors in the intact PM of cells spread on stimulating coverslips. This method allows visualization of two different individual molecules and detection of their interactions within nanoscale clusters. However, due to the resolution limit of ~ 20 nm and the inability to measure all of the molecules present, the interaction between the imaged molecules is determined by statistical methods, whose description follows. The proximity of the identified molecules was analyzed using a bivariate pair correlation function (PCF). The bivariate PCF of the experimental samples can then be compared to a 'random labeling' model that serves as a null hypothesis. The random labeling model is represented by PCFs obtained from random placement of the two labels at the identified positions of the molecules. Nineteen random sets were generated by a Monte Carlo simulation and were plotted to generate PCF curves that correspond to random association of the two species. If the molecules randomly mixed within clusters, the bivariate PCF of the sample would lie within the two plots showing the 95% confidence interval generated by the random simulations. If the bivariate PCF lies above this confidence interval, the two molecules are attracted to each other beyond the random mixing model. Alternatively, if the bivariate PCF curve lies below the confidence interval, the molecules undergo some mutual repulsion. It is important to note that the interpretation of the two-color PALM results and bivariate PCF in terms of molecular interactions strongly relies on other data that indicated the direct binding of two molecular species, such as immunoprecipitation (IP) (7) and FRET (18, 20, 62). In fact, these methods complement each other, as IP and FRET average binding events over many complexes, whereas two color PALM provides information on single molecules but not on direct molecular interactions.

Nanoscale patterns of molecular interactions downstream of the TCR

Using two-color PALM and bivariate PCF statistics, we found that the TCR and ZAP-70 mixed randomly within TCR clusters of activated cells. This result is consistent with extensive biochemical data showing ZAP-70 binding to activated TCR. The mixing of LAT and ZAP-70 was only partial, suggesting that ZAP-70 resides more stably on the TCR. We further detected partial mixing between the TCR and LAT that decreased with TCR activation, yet was localized to specific domains of significant mixing (Fig. 4A,B). Since the TCR chains were immobilized by our coverslip imaging assay and since LAT remained mobile, we proposed a model whereby LAT is phosphorylated by TCR-bound ZAP-70. Our observation of the mixing between TCR and LAT can be explained by the breakdown of their microclusters into nanoclusters that can efficiently mix in specific places.

Imaging LAT and Grb2, we found that Grb2 mixed randomly within LAT clusters after TCR activation. Since most LAT clustering molecules are found in nanoclusters, we deduced that LAT nanoclusters are efficiently phosphorylated and are primed for the recruitment of downstream effectors such as SOS-1,2 or Cbl through the binding of Grb2 to its phosphotyrosines (22). In our hands, a second effector, PLC- γ 1, demonstrated a slightly

more complicated recruitment pattern to LAT clusters. PLC- γ 1 and LAT showed variable mixing levels with different samples falling both below and above the 95% confidence interval of random mixing. Such variability likely indicates the stochastic process of cluster formation and the cooperative recruitment of downstream proteins. Overall, PLC- γ 1 recruitment was very efficient and averaged close to random mixing levels.

Given these recruitment patterns of ZAP-70 to TCR and either Grb2 or PLC- γ 1 to LAT, a question can be raised whether all signaling molecules show efficient recruitment to clusters of scaffold proteins and receptors at the PM that approximate random mixing. By itself, the stochastic process of binding of effectors to scaffold proteins offers no explanation of any type of non-random patterning. With this question in mind, the recruitment pattern of SLP-76 to LAT has been very surprising. By bivariate PCF analysis, SLP-76 seemed to be recruited to LAT clusters with random mixing up to a length scale of ~ 160 nm. At shorter length scale, the two molecules seemed to separate, as the bivariate PCF curve fell below the 95% confidence interval of random mixing (Fig. 4D). Taking a closer look at larger clusters at the PM of the cell, we found that SLP-76 was enriched at the periphery of LAT clusters (Fig. 4C, insets).

Super resolution imaging has thus far enabled the identification of multiple distinct nanoscale patterns involved in T-cell activation: (i) the dynamic segregation of TCR clusters from LAT clusters upon TCR cell stimulation (33–34) with partial mixing of the two in isolated regions (34), (ii) the efficient and cluster-size-independent recruitment of Grb2 and PLC- γ 1 to LAT, and (iii) the nanostructure of LAT signaling complexes with the recruitment of SLP-76 to the periphery of LAT (34). Importantly, some of these surprising patterns could not be identified by previous techniques such as biochemistry, diffraction-limited microscopy, and even EM.

Possible mechanisms for the nanoscale patterning of signaling complexes at the PM

There are two long-standing theories on membrane organization that might also help explain the segregation of TCR and LAT clusters. It has been proposed that structures in the actin cytoskeleton partition the membrane into 230 nm domains (63) and micron-sized domains have been imaged using GFP-actin (64). BCR diffusion is limited by both ezrin and actin structures in quiescent cells and disruption of the actin structure induce a cellular calcium response (65–67). Nonetheless, it is not clear how the actin cytoskeleton could sequester clusters containing different proteins into different domains, nor is it clear that the size of the domains constrained by actin are small enough to account for the segregation of small nanoclusters. The lipid raft hypothesis proposes that differing affinities for liquid-ordered and disordered domains in the PM could separate proteins. Since LAT prefers liquid-ordered lipid domains while TCR prefers liquid-disordered domains, this would provide a mechanism to separate the two. Recently, a combination of STED and fluorescence correlation spectroscopy has shown that sphingolipids and glycosylphosphatidyl-inositol anchored proteins are transiently trapped in very small cholesterol-rich domains (68), which are consistent with small lipid rafts in the PM. However, it is not known if nanoscale lipid domains are capable of maintaining protein nanostructure. Neither explanation is entirely satisfactory, and the mechanisms that give rise to TCR and LAT patterning are still undetermined.

The recruitment of Grb2 and PLC- γ 1 to LAT nucleated clusters has been well described via multiple biochemical and imaging studies (7, 15, 20). It is thus not surprising that the two molecules were recruited efficiently to LAT clusters (34). Grb2 seemed to bind LAT homogeneously suggesting that no other interaction was needed for its recruitment to

phosphorylated LAT. The recruitment pattern of PLC- γ 1 to LAT seemed to be less homogeneous and likely requires cooperative interactions with additional signaling molecules (20).

In contrast to the recruitment patterns of Grb2 and PLC- γ 1 to LAT, the recruitment pattern of SLP-76 to the periphery of LAT clusters was surprising and could not be predicted by previous data. Several mechanisms might be considered regarding the formation of this nanostructure. One possible reason for the patterned recruitment of SLP-76 to LAT could be the linking of LAT to the actin cytoskeleton via interactions with Nck, Vav-1, and Itk (62). Indeed, others have seen a possible tie between nanoclusters and the actin cytoskeleton, where interactions with actin appeared to distort the shape of ZAP-70, LAT, and SLP-76 clusters (43). A second reason might be related to steric occlusion of the potentially bulky complexes of SLP-76 and associated molecules from the potentially dense core of LAT clusters. Although complete understanding of the formation mechanism of this nanostructure awaits further studies, we used two approaches to study the possible role of actin in the nanoscale organization of LAT and SLP-76. We first abrogated the binding of SLP-76 to its downstream partners, Nck, Vav-1, and Itk by a mutation of three relevant tyrosines (SLP-76-3YF), thus blocking its physical connection to actin (15, 34). We also used the drug Latrunculin A to abrogate actin polymerization. Both approaches resulted in the disruption of the LAT/SLP-76 nanostructure, showing the dependence of this structure on the linkage of LAT and SLP-76 complexes to polymerized actin.

The significance of nanoscale organization of signaling complexes

The dynamic nanoscale organization of TCR and LAT offers an appealing mechanism for controlled T-cell activation. By two-color PALM imaging, we showed that the kinase ZAP-70 mixes more efficiently with TCR clusters than with LAT clusters. Since ZAP-70 is the primary mediator of the signal from the TCR to LAT (69), we conjecture that LAT is most efficiently activated by TCR-bound ZAP-70 at the identified nanoscale domains of high mixing between the TCR and LAT (Figs 4 and 5). LAT can then serve to activate the cell after its exchange between additional microclusters throughout the plasma membrane (28). We thus call these domains 'hot spots' for T-cell activation. Such a mechanism would be much more efficient in the controlled activation of the cell than the proposal that LAT and TCR clusters come close upon TCR activation, yet remain segregated (33, 70). In contrast, Lillemeirer *et al.* saw no mixing of LAT and TCR ζ in either quiescent or stimulated cells, as LAT and TCR ζ concatamers came close but did not mix upon TCR activation (33).

The dominance of nanoclusters in the molecular clustering distributions at the PM of T cells raises several questions: are nanoclusters viable for signaling? Our data suggest that TCR and LAT nanoclusters do transmit signals, as they recruit their effectors, ZAP-70 and Grb2 efficiently, in a manner that seems independent of cluster size. This is in contrast to an earlier conclusion that large domains, at least 35 nm wide and up to 300 nm in length, are the smallest activation unit (33). This point needs to be resolved to understand the importance of nanostructure in signaling cascades. If nanoclusters are signaling entities, is there a distinctive functional role to microclusters that nanoclusters cannot perform, or are microclusters merely the relatively more rare occurrences of large aggregates of LAT nanoclusters? We still cannot say whether some molecules can bind only larger clusters and hope that future studies will be able to address this question.

It should be noted here that some studies looking at late time points after TCR stimulation have concluded that LAT microclusters are manifestations of vesicles in close proximity to the PM (50, 71). Such an interpretation argues against the functional role of nanoclusters in

signaling. However, vesicles containing internalized LAT generally appear well after TCR activation, while LAT-dependent triggering of T-cell signaling and downstream readouts such as calcium flux typically begin within seconds of receptor engagement. Moreover, many microclusters tend to fade and disappear within 3 to 5 minutes (9), and internalization of vesicles is concomitant with the loss of microclusters (23–24). Therefore, we suggest that the vesicles observed in this study after many minutes of TCR stimulation may not have much relevance to the initial events of signal transduction.

In contrast to the nanoscale organization patterns described above, the functional role of the nanostructure of LAT and SLP-76 seems less intuitive. One clue for such a functional role relates to the dynamics of SLP-76 at the PM of activated cells, where SLP-76 clusters are generated at the perimeter of spread cells and translocate via endocytosis towards the microtubule organizing center on microtubules (9, 23). By live cell imaging, the SLP-76-3YF mutant that abrogates its connection to polymerized actin also disrupted translocation. Thus, abrogating the SLP-76 and LAT nanostructure correlated with the loss of proper regulation of SLP-76 clusters. In multiple different ways, nanoscale organization and patterning at the PM appears to be crucial for proper T-cell activation and function.

Conclusions and perspective

Recent advances in optical microscopy, including super resolution methods such as PALM and STORM, have enabled the study of signaling complexes downstream of the TCR in intact PM of spreading cells with single molecule resolution. These methods, combined with statistical analyses of point patterns now offer a way to look closer into individual signaling complexes and clusters. Such studies have resulted in novel insights into the content and structure of signaling clusters downstream of the TCR and LAT. We now appreciate that signaling clusters appear in a wide distribution of sizes, from monomers, dimers, and trimers, to larger clusters including 'protein islands'. Nanostructures are likely components of microclusters but can also exist outside of microclusters. There is wide agreement that a basal level of clustering exists, with an increase in cluster sizes upon TCR stimulation, although some studies have not observed basal clustering (44). We found that the smallest clusters are efficiently phosphorylated and primed for signaling, although it is unclear whether all of the effectors can bind to these structures. At least one component of the complexes, the critical adapter SLP-76, demonstrated a surprising nanostructure within LAT clusters, as it is recruited to their periphery.

Having identified intriguing and intricate properties of signaling complexes and clusters, we now face new challenges. For instance, signaling complexes and clusters are dynamic structures that form and disappear in a time frame of seconds and recruit, release, and exchange molecules in fractions of a second (28, 72). Single molecule super-resolution techniques have been generally notorious for their slowness, with a typical acquisition time of a few minutes per image. Although fast single-color acquisition has been demonstrated for T cells (33–34), it would be of great interest to follow the dynamics of these processes in living cells and in multiple colors. Recent advances in single molecule labeling, imaging, and tracking offer hope that such processes can be resolved in the coming years.

Signaling complexes are heterogeneous multi-molecular structures that can involve complex molecular processes, including cooperativity and binding competition. To directly resolve the content and binding mechanisms of signaling complexes one would need single molecule imaging with at least three colors. Again, multicolor imaging has been demonstrated for STORM and PALM, with some constraints. Expanding these capabilities will enable a more complete characterization of the intricate structure and protein interactions within signaling complexes and clusters.

Studies involving the imaging of clusters often involve artificial interfaces between cells and functionalized coverslips (54). Although interfaces between T cells and various model activation surfaces mimic many of the phenotypic structures of the T cell/APC interface in diffraction-limited images, it is not clear whether artificial substrates affect the nanoscale organization of signaling clusters. For instance, James *et al.* (44) have shown that such interfaces restrict molecular mobility and promote artificial aggregation at the interface, in comparison to the apical sides of the cells. Ideally, one would like to study signaling complexes and clusters in real interfaces between immune cells and APCs.

Beyond the ability to image signaling complexes in single molecule detail, there is a pressing need for a framework of methods of statistical analysis that can describe, resolve, and quantify the properties of signaling complexes that emerge from these new data. Related methods have been developed in fields that deal with point patterns, including astronomy, social studies, and environmental studies. Such methods could be adopted and shared between the fields. Importantly, there is clear need to move beyond second-order statistics (pair correlation and Ripley's functions) that cannot capture structural properties such as morphology and topology. In addition, these methods cannot readily be expanded to treat tri-molecular interactions, such as the ones involved in cooperativity and binding competition.

We expect that advances in super-resolution imaging will continue to provide new and exciting data on critical features of T-cell responses and mechanisms of their activation. Already we have begun to see how signaling molecules are arranged into distinct nanostructures that appear to be necessary for proper signal transduction. There is reason to believe that nanostructure will be an important feature in many kinds of signaling complexes particularly those nucleated by other receptors. As more structures are understood at the single molecule level, many outstanding questions will be answered and new and unanticipated discoveries will be made.

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References

1. Goodnow CC, Sprent J, Fazekas de St Groth B, Vinuesa CG. Cellular and genetic mechanisms of self tolerance and autoimmunity. *Nature*. 2005; 435:590–597. [PubMed: 15931211]
2. Wilkinson B, Downey JS, Rudd CE. T-cell signalling and immune system disorders. *Expert Rev Mol Med*. 2005; 7:1–29. [PubMed: 16364187]
3. Samelson LE. Signal transduction mediated by the T cell antigen receptor: The role of adapter proteins. *Annual Review of Immunology*. 2002; 20:371–394.
4. Samelson, LE.; Shaw, AS. Immunoreceptor signaling : a subject collection from Cold Spring Harbor perspectives in biology. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, N.Y.: 2011.
5. Purbhoo MA, Irvine DJ, Huppa JB, Davis MM. T cell killing does not require the formation of a stable mature immunological synapse. *Nat Immunol*. 2004; 5:524–530. [PubMed: 15048111]
6. Zhang W, Sloan-Lancaster J, Kitchen J, Tribble RP, Samelson LE. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell*. 1998; 92:83–92. [PubMed: 9489702]
7. Zhang WG, Tribble RP, Zhu MH, Liu SK, McGlade J, Samelson LE. Association of Grb2 Gads, and phospholipase C-gamma 1 with phosphorylated LAT tyrosine residues - Effect of LAT tyrosine mutations on T cell antigen receptor-mediated signaling. *Journal of Biological Chemistry*. 2000; 275:23355–23361. [PubMed: 10811803]

8. Grakoui A, et al. The immunological synapse: A molecular machine controlling T cell activation. *Science*. 1999; 285:221–227. [PubMed: 10398592]
9. Bunnell SC, et al. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *Journal of Cell Biology*. 2002; 158:1263–1275. [PubMed: 12356870]
10. Campi G, Varma R, Dustin ML. Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. *J Exp Med*. 2005; 202:1031–1036. [PubMed: 16216891]
11. Yokosuka T, et al. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nature Immunology*. 2005; 6:1253–1262. [PubMed: 16273097]
12. Krummel MF, Sjaastad MD, Wulfig C, Davis MM. Differential clustering of CD4 and CD3zeta during T cell recognition. *Science*. 2000; 289:1349–1352. [PubMed: 10958781]
13. Harwood NE, Batista FD. Early events in B cell activation. *Annu Rev Immunol*. 2010; 28:185–210. [PubMed: 20192804]
14. Lee KH, Holdorf AD, Dustin ML, Chan AC, Allen PM, Shaw AS. T cell receptor signaling precedes immunological synapse formation. *Science*. 2002; 295:1539–1542. [PubMed: 11859198]
15. Bunnell SC, et al. Persistence of cooperatively stabilized signaling clusters drives T-cell activation. *Mol Cell Biol*. 2006; 26:7155–7166. [PubMed: 16980618]
16. Varma R, Campi G, Yokosuka T, Saito T, Dustin ML. T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. *Immunity*. 2006; 25:117–127. [PubMed: 16860761]
17. Balagopalan L, Coussens NP, Sherman E, Samelson LE, Sommers CL. The LAT story: a tale of cooperativity, coordination, and choreography. *Cold Spring Harb Perspect Biol*. 2010; 2:a005512. [PubMed: 20610546]
18. Barda-Saad M, et al. Cooperative interactions at the SLP-76 complex are critical for actin polymerization. *EMBO J*. 2010; 29:2315–2328. [PubMed: 20562827]
19. Dombroski D, et al. Kinase-independent functions for Itk in TCR-induced regulation of Vav and the actin cytoskeleton. *J Immunol*. 2005; 174:1385–1392. [PubMed: 15661896]
20. Braiman A, Barda-Saad M, Sommers CL, Samelson LE. Recruitment and activation of PLC gamma 1 in T cells: a new insight into old domains. *Embo Journal*. 2006; 25:774–784. [PubMed: 16467851]
21. Wu JN, Koretzky GA. The SLP-76 family of adapter proteins. *Semin Immunol*. 2004; 16:379–393. [PubMed: 15541653]
22. Houtman JC, et al. Oligomerization of signaling complexes by the multipoint binding of GRB2 to both LAT and SOS1. *Nat Struct Mol Biol*. 2006; 13:798–805. [PubMed: 16906159]
23. Barr VA, et al. T-cell antigen receptor-induced signaling complexes: Internalization via a cholesterol-dependent endocytic pathway. *Traffic*. 2006; 7:1143–1162. [PubMed: 16919152]
24. Balagopalan L, et al. c-Cbl-mediated regulation of LAT-nucleated signaling complexes. *Mol Cell Biol*. 2007; 27:8622–8636. [PubMed: 17938199]
25. Carrizosa E, et al. Hematopoietic lineage cell-specific protein 1 is recruited to the immunological synapse by IL-2-inducible T cell kinase and regulates phospholipase Cgamma1 Microcluster dynamics during T cell spreading. *J Immunol*. 2009; 183:7352–7361. [PubMed: 19917685]
26. Hashimoto-Tane A, et al. Dynein-driven transport of T cell receptor microclusters regulates immune synapse formation and T cell activation. *Immunity*. 2011; 34:919–931. [PubMed: 21703543]
27. Lillemeier BF, Pfeiffer JR, Surviladze Z, Wilson BS, Davis MM. Plasma membrane-associated proteins are clustered into islands attached to the cytoskeleton. *Proc Natl Acad Sci USA*. 2006; 103:18992–18997. [PubMed: 17146050]
28. Douglass AD, Vale RD. Single-molecule microscopy reveals plasma membrane microdomains created by protein-protein networks that exclude or trap signaling molecules in T cells. *Cell*. 2005; 121:937–950. [PubMed: 15960980]
29. Harder T, Kuhn M. Selective accumulation of raft-associated membrane protein LAT in T cell receptor signaling assemblies. *J Cell Biol*. 2000; 151:199–207. [PubMed: 11038169]

30. Schamel WWA, et al. Coexistence of multivalent and monovalent TCRs explains high sensitivity and wide range of response. *J Exp Med*. 2005; 202:493–503. [PubMed: 16087711]
31. Wilson BS, Pfeiffer JR, Surviladze Z, Gaudet EA, Oliver JM. High resolution mapping of mast cell membranes reveals primary and secondary domains of Fc epsilon RI and LAT. *Journal of Cell Biology*. 2001; 154:645–658. [PubMed: 11489921]
32. Wilson BS, et al. Markers for detergent-resistant lipid rafts occupy distinct and dynamic domains in native membranes. *Mol Biol Cell*. 2004; 15:2580–2592. [PubMed: 15034144]
33. Lillemeier BF, Mortelmaier MA, Forstner MB, Huppa JB, Groves JT, Davis MM. TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation. *Nat Immunol*. 2010; 11:90–96. [PubMed: 20010844]
34. Sherman E, et al. Functional nanoscale organization of signaling molecules downstream of the T cell antigen receptor. *Immunity*. 2011; 35:705–720. [PubMed: 22055681]
35. Born, M.; Wolf, E. Principles of optics : electromagnetic theory of propagation, interference and diffraction of light. 7th expanded ed. Cambridge University Press; Cambridge: 1999.
36. Huang B, Bates M, Zhuang X. Super-resolution fluorescence microscopy. *Annu Rev Biochem*. 2009; 78:993–1016. [PubMed: 19489737]
37. Hell SW, Wichmann J. Breaking the diffraction resolution limit by stimulated-emission - stimulated-emission-depletion fluorescence microscopy. *Optics Letters*. 1994; 19:780–782. [PubMed: 19844443]
38. Klar TA, Engel E, Hell SW. Breaking Abbe's diffraction resolution limit in fluorescence microscopy with stimulated emission depletion beams of various shapes. *Phys Rev E Stat Nonlin Soft Matter Phys*. 2001; 64:066613. [PubMed: 11736302]
39. Betzig E, et al. Imaging intracellular fluorescent proteins at nanometer resolution. *Science*. 2006; 313:1642–1645. [PubMed: 16902090]
40. Rust MJ, Bates M, Zhuang X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat Methods*. 2006; 3:793–795. [PubMed: 16896339]
41. van de Linde S, Heilemann M, Sauer M. Live-Cell Super-Resolution Imaging with Synthetic Fluorophores. *Annu Rev Phys Chem*. 2012; 63:519–540. [PubMed: 22404589]
42. Owen DM, et al. PALM imaging and cluster analysis of protein heterogeneity at the cell surface. *J Biophotonics*. 2010; 3:446–454. [PubMed: 20148419]
43. Hsu CJ, Baumgart T. Spatial association of signaling proteins and F-actin effects on cluster assembly analyzed via photoactivation localization microscopy in T cells. *PLoS One*. 2011; 6:e23586. [PubMed: 21887278]
44. James JR, et al. The T cell receptor triggering apparatus is composed of monovalent or monomeric proteins. *J Biol Chem*. 2011; 286:31993–32001. [PubMed: 21757710]
45. Sengupta P, Jovanovic-Talisman T, Skoko D, Renz M, Veatch SL, Lippincott-Schwartz J. Probing protein heterogeneity in the plasma membrane using PALM and pair correlation analysis. *Nat Methods*. 2011; 8:969–975. [PubMed: 21926998]
46. Kumar R, et al. Increased Sensitivity of Antigen-Experienced T Cells through the Enrichment of Oligomeric T Cell Receptor Complexes. *Immunity*. 2011; 35:375–387. [PubMed: 21903423]
47. Prior IA, Muncke C, Parton RG, Hancock JF. Direct visualization of Ras proteins in spatially distinct cell surface microdomains. *J Cell Biol*. 2003; 160:165–170. [PubMed: 12527752]
48. Yang S, et al. Mapping ErbB receptors on breast cancer cell membranes during signal transduction. *J Cell Sci*. 2007; 120:2763–2773. [PubMed: 17652160]
49. Yang J, Reth M. Oligomeric organization of the B-cell antigen receptor on resting cells. *Nature*. 2010; 467:465–469. [PubMed: 20818374]
50. Williamson DJ, et al. Pre-existing clusters of the adaptor Lat do not participate in early T cell signaling events. *Nat Immunol*. 2011; 12:655–662. [PubMed: 21642986]
51. Zhang J, Leiderman K, Pfeiffer JR, Wilson BS, Oliver JM, Steinberg SL. Characterizing the topography of membrane receptors and signaling molecules from spatial patterns obtained using nanometer-scale electron-dense probes and electron microscopy. *Micron*. 2006; 37:14–34. [PubMed: 16081296]

52. Stoyan, D.; Stoyan, H. *Methods of geometrical statistics*. John Wiley & Sons; Philadelphia: 1994. *Fractals, Random Shapes and Point Fields*.
53. Wiegand T, Moloney KA. Rings, circles, and null-models for point pattern analysis in ecology. *Oikos*. 2004; 104:209–229.
54. Balagopalan L, Sherman E, Barr VA, Samelson LE. Imaging techniques for assaying lymphocyte activation in action. *Nat Rev Immunol*. 2011; 11:21–33. * - Equal contribution. [PubMed: 21179118]
55. van der Merwe PA, Dushek O. Mechanisms for T cell receptor triggering. *Nat Rev Immunol*. 2011; 11:47–55. [PubMed: 21127503]
56. Dykstra M, Cherukuri A, Sohn HW, Tzeng SJ, Pierce SK. Location is everything: Lipid rafts and immune cell signaling. *Annu Rev Immunol*. 2003; 21:457–481. [PubMed: 12615889]
57. Tolar P, Hanna J, Krueger PD, Pierce SK. The constant region of the membrane immunoglobulin mediates B cell-receptor clustering and signaling in response to membrane antigens. *Immunity*. 2009; 30:44–55. [PubMed: 19135393]
58. Zhang WG, Tribble RP, Samelson LE. LAT palmitoylation: Its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. *Immunity*. 1998; 9:239–246. [PubMed: 9729044]
59. Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell*. 2000; 103:211–225. [PubMed: 11057895]
60. Henis YI, Hancock JF, Prior IA. Ras acylation, compartmentalization and signaling nanoclusters (Review). *Mol Membrane Biol*. 2009; 26:80–92.
61. Yokosuka T, et al. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nat Immunol*. 2005; 6:1253–1262. [PubMed: 16273097]
62. Barda-Saad M, Braiman A, Titerence R, Bunnell SC, Barr VA, Samelson LE. Dynamic molecular interactions linking the T cell antigen receptor to the actin cytoskeleton. *Nat Immunol*. 2005; 6:80–89. [PubMed: 15558067]
63. Kusumi A, et al. Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules. *Annu Rev Biophys Biomol Struct*. 2005; 34:351–378. [PubMed: 15869394]
64. Andrews NL, et al. Actin restricts FcεRI diffusion and facilitates antigen-induced receptor immobilization. *Nat Cell Biol*. 2008; 10:955–963. [PubMed: 18641640]
65. Treanor B, Depoil D, Bruckbauer A, Batista FD. Dynamic cortical actin remodeling by ERM proteins controls BCR microcluster organization and integrity. *J Exp Med*. 208:1055–1068. [PubMed: 21482698]
66. Batista FD, Treanor B, Harwood NE. Visualizing a role for the actin cytoskeleton in the regulation of B-cell activation. *Immunol Rev*. 237:191–204. [PubMed: 20727037]
67. Treanor B, et al. The membrane skeleton controls diffusion dynamics and signaling through the B cell receptor. *Immunity*. 2010; 32:187–199. [PubMed: 20171124]
68. Eggeling C, et al. Direct observation of the nanoscale dynamics of membrane lipids in a living cell. *Nature*. 2009; 457:1159–U1121. [PubMed: 19098897]
69. Wang H, et al. ZAP-70: an essential kinase in T-cell signaling. *Cold Spring Harb Perspect Biol*. 2010; 2:a002279. [PubMed: 20452964]
70. van der Merwe PA, Dunne PD, Klenerman D, Davis SJ. Taking T cells beyond the diffraction limit. *Nat Immunol*. 2010; 11:51–52. [PubMed: 20016513]
71. Purbhoo MA, et al. Dynamics of subsynaptic vesicles and surface microclusters at the immunological synapse. *Sci Signal*. 2010; 3:ra36. [PubMed: 20460647]
72. Huse M, et al. Spatial and temporal dynamics of T cell receptor signaling with a photoactivatable agonist. *Immunity*. 2007; 27:76–88. [PubMed: 17629516]
73. Tolar P, Sohn HW, Pierce SK. The initiation of antigen-induced B cell antigen receptor signaling viewed in living cells by fluorescence resonance energy transfer. *Nat Immunol*. 2005; 6:1168–1176. [PubMed: 16200067]

74. Wilson BS, Pfeiffer JR, Oliver JM. Observing FcεpsilonRI signaling from the inside of the mast cell membrane. *J Cell Biol.* 2000; 149:1131–1142. [PubMed: 10831616]

Fig. 1. Signaling complexes drive early T-cell activation

T-cell activation is initiated by the T-cell antigen receptor recognition of its peptide-MHC ligand on the surface of antigen-presenting cells (APCs). Following antigen recognition and costimulatory signaling, the intracellular chains of the TCR become phosphorylated within immunoreceptor tyrosine-based activation motifs (ITAMs), ZAP-70 is recruited and activated, and it then phosphorylates tyrosine residues on the Linker for activation of T cells (LAT); a protein that partitions into liquid-ordered domains of the membrane (indicated by boxes around the bilayer). These tyrosines then serve as docking sites for multiple adapter and effector proteins, thus triggering multiple cell functions.

Fig. 2. Signaling complexes reside in clusters smaller than the diffraction limit of light
 Confocal images of Jurkat E6.1 cells spread on (A) TCR stimulating (α CD3-coated) and (B) TCR non-stimulating (α CD45-coated) coverslips. The cells were stained with primary proteins against phosphotyrosines (pY), TCR, and Grb2 followed by Alexa dye conjugated secondary antibodies. LAT was directly conjugated with the photoactivatable fluorescent protein Dronpa. Images were acquired using a Zeiss 510Meta microscope and a 63X, 1.4 NA objective. Bars – 5 μ m. (C,D) Images of Jurkat E6.1 cells spread on TCR stimulating (α CD3-coated) The cells were stained with primary proteins against phosphotyrosines (pY) followed by secondary antibody conjugated to Alexa-647. Images were acquired using a Leica STED microscope. (C) Confocal mode. (D) STED mode. Arrowheads highlight areas of interest: top-a small cluster not visible in the confocal mode, middle-a diffraction limited cluster resolved into 2 smaller entities and bottom-a diffraction limited cluster resolved into 3 smaller entities.

Fig. 3. A 'unified view' of molecular clustering at the PM

We propose a model in which some membrane molecules such as the TCR and LAT cluster in a broad distribution of sizes. The distribution is strongly skewed towards monomers and very small clusters, e.g. dimers and trimers. Studies using different experimental methods and analyses have captured or emphasized only parts of such distributions due to inherent limitations in their sensitivities. This experimental variability has led to multiple views on the scale and significance of molecular clustering, as reflected by the breadth of terms that describe clusters at different scales.

Fig. 4. PALM imaging reveals nanostructures of signaling complexes

(A) Two-color PALM image of a Jurkat E6.1 cell on a TCR stimulating coverslip. The cell expressed LAT-Dronpa and TCRζ-PAmCherry. Bar - 2 μm. (B) Bivariate pair correlation function [PCF or $g_{12}(r)$] of the identified molecules within a study region of the cell. (C) Two-color PALM image of a Jurkat E6.1 cell on a TCR stimulating coverslip. The cell expressed LAT-Dronpa and SLP-76-PAmCherry. Bar - 2 μm. (D) Bivariate pair correlation function (PCF) of the identified molecules within a study region of the cell.

Fig. 5. T-cell activation is driven by the dynamic nanoscale organization of signaling clusters
 (A) TCR and LAT reside in small nanoclusters within lamellae. As a lamella touches the stimulatory surface, small signaling clusters composed of nanoclusters form at the point of contact. Phosphorylated TCR chains recruit ZAP-70, which in turn serve to phosphorylate proximal LAT nanoclusters at 'hot spots'. LAT then recruits multiple effector proteins in a size-independent fashion. As lamellae settle onto the surface, activated nanoclusters are confined within the contact area. Activated molecules are exchanged between nanoclusters.
 (B) The nanostructure of LAT nucleated clusters. The effector proteins Grb2 and PLC- γ 1 mix homogeneously within LAT clusters; however, the adapter protein SLP-76 is preferentially recruited to the periphery of LAT clusters, where it likely mediates interactions of the clusters with the actin cytoskeleton.

Table 1

Protein nanoclusters at the plasma membrane of cells

Protein ^a	Size (non-activated) ^b [no. of molecules] [nm]	Size (activated) [no. of molecules] [nm]	Related clustered proteins under study	Cell type	Method ^c	Ref.
TCR	(1),2,3,4...10+	Monomers and oligomers		2B4	TEM, BN-PAGE	(30)
TCR	1		CD4, CD45, CD3	JRT3	BRET, TCCD	(44)
TCR	7-20 35-70;40-300nm	7-30 35-70;40-300nm	LAT	5C.C7 LN T cells (MCC-1-E ^k)	PALM (fixed, live), TEM, FCCS	(33)
TCR	(1)-15+	(1-6)-15+		PBTs, naive and memory mouse T cells	TEM, BN-PAGE	(46)
LAT	(1-3)-20+	1-(20+)	FceRI, PLC-γ1.2, Gab2, PI3K,	RBL-2H3 (Mast) cells	TEM	(31)
LAT	7-20 35-70;40-300nm	7-30 35-70;40-300nm		5C.C7 LN T cells (MCC-1-E ^k)	PALM (fixed, live), TEM, FCCS	(33)
LAT	(2),3,4...10 ^d (0-20)-300nm	(2),3,4...50 ^d (0-20)-300nm	TCR, ZAP-70, LAT mutants, Grb2, PLCγ1, SLP-76	Jurkat E6.1, PBT	PALM (fixed, live)	(34)
LAT	~60 0-(120)-300nm	~80 0-(120)-300nm	LAT mutants	JCaM2	PALM	(50)
LAT	(1-3)-9+ (0-60)-180nm		VSVG, GPI, Lyn	COS-7	PALM	(45)
Additional examples						
BCR	1	Oligomers		J558L mouse cell line	FRET	(73)
BCR	Oligomers	1		S2 Drosophila Schneider cells	BiFC	(49)
FceRI	(1-3)-10+	1-(20+)	Lyn, Syk, Clathrin coated pits	RBL-2H3	TEM	(74)
ErbB2	1-(3-15)-50+ 10-170nm		ErbB3,4,EGFR, ShcP85,STAT5, PI3K	SKBR3		(48)
thi.tK (Ras)		6 12-20nm	GPI, EGFR, Angiotensin II receptor	BHK	TEM	(47)

Comments:

^aProtein acronyms: TCR, T-cell antigen receptor; LAT, linker for activation of T cells; BCR, B-cell receptor

^b() denotes the mode of a size-distribution;... - denotes a decaying size distribution; x - y - denotes a range

^cMethods' acronyms: BN-PAGE - Blue Native polyacrylamide gel electrophoresis; TEM - Transmission electron microscopy; BRET, bioluminescence resonance energy transfer; TCCD, two-color coincidence detection; PALM, photoactivated localization microscopy; FCCS, fluorescence cross correlation microscopy; FRET, fluorescence resonance energy transfer; BiFC, bifluorescence complementation

^dThe distribution did not include monomers