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Can single molecule localization microscopy detect nanoclusters in T cells?

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Nanoclusters of cell surface receptors have been detected with single molecule localization microscopy (SMLM) and are thought to mediate signal transduction. Clustering of the T cell receptor (TCR), for example, was reported to control signalling efficiency and antigen discrimination. However, the ability to detect nanoclusters with SMLM has been questioned. Here, we review the detection limits of SMLM as defined by both the physical limits and data processing, as well as evidence for nanoclusters arising from complementary techniques. We conclude with an outlook of how future data analysis can reveal the implications of molecular self-organization for signalling.

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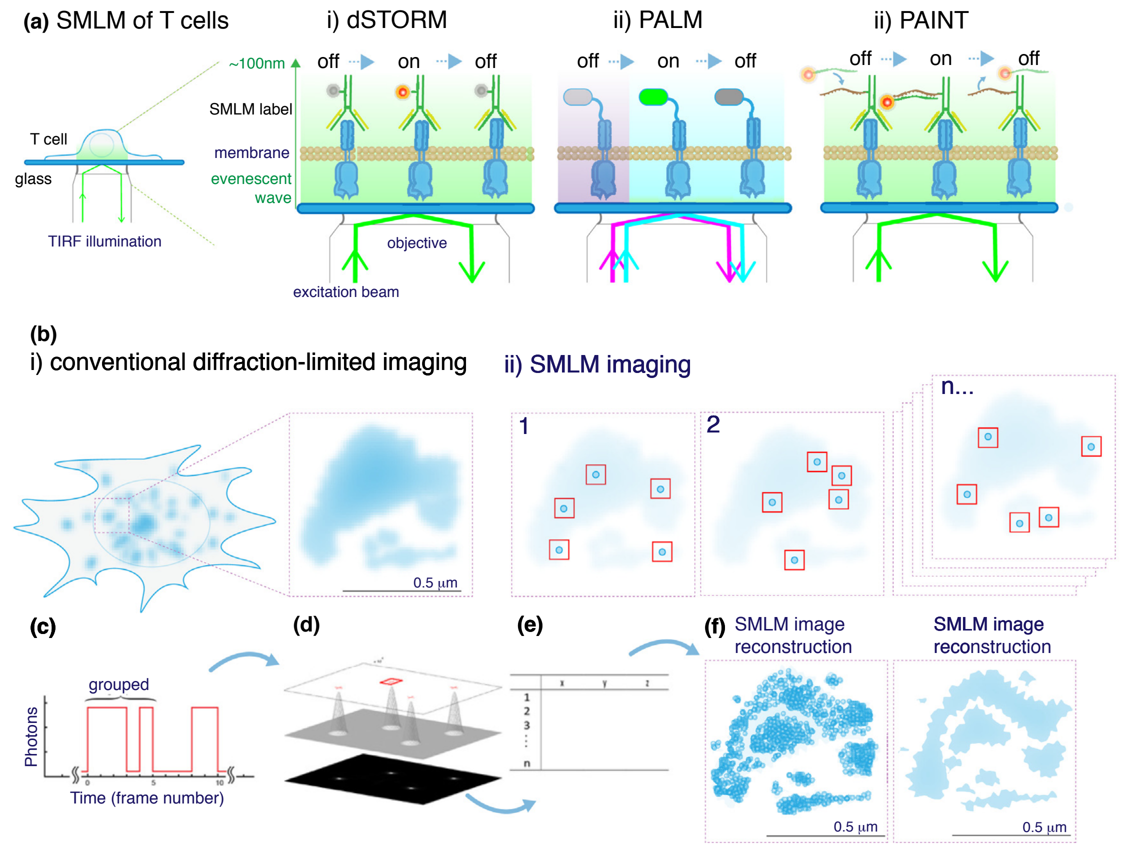
Introduction: localization microscopy for the detection of nanoclusters in T cells

A frontier in cell biology is to measure complex biological structures within the cell with nanometer resolution [1••]. Single molecule localization microscopy (SMLM also known as PALM, (d)STORM, PAINT etc., Figure 1) can map the position of individual genetically tagged or labelled molecules with 20–30 nm precision [1••,2,3]. One of the first applications of SMLM was to map the redistribution of T cell signalling proteins on membranes during T cell activation [4], a process thought to control signalling [5]. In T cells signalling begins with the recognition of peptides presented on major histocompatibility complex (pMHC) molecules by the T cell receptor (TCR)-CD3 complex. Binding between antigenic pMHC and TCR results in the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic tails of CD3 chains, a process called triggering, which induces downstream signalling culminating in the activation of effector functions and changes in cell fate. In activated cells, the TCR forms clusters that span the nanometer to micrometer scale (Box 1; Figure 2 and references therein). That clusters form after T cell activation is well documented, but there is currently debate about whether the TCR exists in pre-formed nanoclusters before encountering pMHC. Here, we review the detection and nature of nanoclusters revealed by SMLM data, and discuss some future directions in analysis methods that may bring more clarity to the field.

While the role of TCR clusters is not fully understood (and reviewed in depth elsewhere [6]), there is evidence that clustering can enhance avidity [7•,8], cooperative binding between TCR complexes [9•], and nanoscale segregation of TCR complexes from phosphatases [10•]. We found that in T cells stimulated with a high dose of pMHC or activating antibodies, TCRs were more likely to be signalling when they resided in dense nanoclusters compared to TCRs in less dense clusters or outside clusters in the same cell [11••].

Both the process of TCR cluster formation and the spatial relationship between TCRs across the nanometer and micrometer scales (i.e. single TCRs, dimers, nanoclusters, and microclusters) remain elusive. Single TCRs can diffuse freely in both resting and triggered cells, and pMHC engagement reduces TCR mobility [12] and increases nanocluster density [11••]. Concomitantly microclusters form at the cell periphery and are transported to the synapse center (Figure 2). Single, free TCRs are significantly more mobile than clusters and it is thought that nanoclusters and microclusters frequently exchange individual TCR–CD3 complexes. Consistent with this, reports based on electron microscopy [13,14], two-color coincidence detection microscopy [15] and biochemical methods [16] suggest 10–50% of TCR molecules reside in nanoclusters. Rather than being an all-or-none phenomenon, these nanoclusters likely exist in dynamic equilibrium with monomeric TCRs. Interestingly, a recent report using a synthetic TCR system suggests that new binding events were more likely to occur proximal to existing clusters or bound single TCRs [17•]. This suggests that TCR clustering over a variety of scales plays an important role in cooperative binding. It is

Figure 1



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Single Molecule Localization Microscopy (SMLM) imaging of T cell activation.
(a) It is common to adhere cells to a glass coverslip and conduct SMLM imaging under total internal reflection fluorescence (TIRF) illumination. The superior signal-to-noise ratio of TIRF microscopy makes it easier to detect blinking fluorophores and calculate the *x*–*y* coordinates. In SMLM, individual point emitters are transiently switched between two (or more) fluorescent states, typically ‘on’ and ‘off’, thus temporally segregating individual fluorophores so that multiple emitters are not present within the same diffraction-limited region. The intensity profile of the point emitter is captured with a camera and used to calculate the subdiffraction position of the emitting fluorophore (<250 nm *x*,*y*). SMLM modalities differ in the type of fluorophores used and how the temporal separation is achieved. For example, in i) dSTORM and ii) PALM synthetic and genetically encoded fluorophores are used, respectively, that are temporally separated via photo-activation, photo-switching or photo-conversion, while iii) PAINT techniques rely on transient binding-unbinding cycles to hold fluorophores within the illumination field long enough to localize.
(b) i) While conventional imaging simultaneously samples all emitters excited by the illumination field, ii) SMLM techniques restrict emission in each image frame to only a few stochastically occurring fluorescent events so that a large number of frames are collected to capture as many fluorescent events, that is, labelled species, as possible. As a result, two emitters that would be too close to resolve by diffraction-limited microscopy are discerned in different frames.
(c) Individual localizations from different frames can be grouped together into individual ‘molecules’. Typically, grouping requires user-defined threshold values, for example for the maximum number of ‘on’ frames that should be grouped together and the minimum number of ‘off’ frames to separate two molecules. However, defining these thresholds requires detailed knowledge of the photo-physics of the fluorophores and grouping of fluorophores with multiple reversible dark states often leads to counting errors.
(d) In SMLM, the intensity profile of point emitters is fitted to the point-spread function (PSF) of the microscope to obtain the molecular coordinates of the emitter and the corresponding localization precisions. Drift during the acquisition, instabilities within the microscope and read-out errors of the camera limit the localization precision to 20–30 nm.
(e) The molecular coordinates, or points, can be used to reconstruct an image for further analysis such as cluster analysis.
(f) A reconstructed image of the locations of molecules is generated for visual inspection. Faithful reconstruction of emitter position is dependent on the precision and accuracy with which single events can be localized, and thus influenced by the sample preparation, imaging, and post-acquisition analysis.

known that TCR clustering is more pronounced in resting antigen-experienced T cells than naïve T cells and so it is plausible that there is a link between the TCR clustering mechanism and the degree of antigen sensitivity [13].

In summary, the notion that single TCR–pMHC complexes may enhance proximal TCR–pMHC binding, and thereby nucleate nanocluster formation, is intriguing and warrants further examination.

Box 1 Nanoclusters, protein islands, domains and oligomers — what's in a name?

The term nanocluster can be difficult to decode in the literature, largely because there is no consensus definition. Here we adopt a conventional working definition for SMLM data summarized as: 'points that are grouped on a subdiffraction-limit scale (<~250 nm) to a greater extent than would be expected for a random distribution.' This definition, however, places no restriction on nanocluster size, density, or activity (i.e. triggered versus resting) and accordingly a wide range of TCR structures have been reported on the nanoscale by SMLM (Figure 2). Protein islands (35–70 nm [4]) and nanoclusters (200 nm–700 nm; [54]) have both been reported, along with nanoclusters of TCR-associated signalling proteins (≥200 nm [55]; ~50 nm; [56]) each with corresponding proposed molecular function. Interestingly, receptor nanoscale clustering is not restricted to T cells; the B-cell receptor and the mast cell FcεRI have also been shown to form nanoclusters by SMLM (though both are larger than that of the TCR; [6]) and clusters of molecules/complexes are observed across a wide variety of signalling pathways [57–59].

Experimental evidence for clusters in T cells also comes from other techniques. Lillemeier *et al.* used Transmission Electron Microscopy (TEM) and identified TCR 'domains' that were larger (40–300 nm) than clusters detected by PALM (35–70 nm) though both methods recorded similar numbers of TCRs per cluster (7–30 and 7–20 respectively) [4]. Other TEM reports confirm a combination of monomeric TCR complexes and oligomers of different sizes, ranging from 1 to >15 TCRs [13] and 1–10 TCRs [14], matching a single molecule study in live cells [15]. Similarly small TCR oligomers (bta) and monomers have also been detected by biochemical methods, including Blue Native (BN)-PAGE analysis, gel filtration, and co-immunopurification [16,60,61], suggesting that at least some TCR–TCR association is relatively strong and stable.

Taken together, rather than confound the definition of nanoclusters, these distinct but overlapping terms, (protein island, domain, oligomer) and experimental approaches (SMLM, TEM, Live-cell imaging and biochemistry), likely point towards a central concept — that nanoscale clustering of TCRs occurs across multiple scales and no one definition is likely to fit. Instead, considering a multiscale range of nanoclusters ranging from dimers to assemblies hundreds of nanometers in length, and accounting for experimental context, is likely a more useful perspective for investigating their function.

While it has been reported that nanoclusters concatenate to form microclusters containing 70–300 TCRs [4], the physical forces driving TCR cluster growth in activated T cells remain unclear. It can easily be conceptualized how various short-range and long-range 'attractive forces' translate individual ligand binding events into a spatial organization on different length scales. While the nature of such 'forces' is currently unspecified, a short-range force can be imagined as maintaining the proximity of two neighboring TCRs, leading to 'oligomers' such as dimers, trimers, tetramers and so on. Interactions within oligomers are generally regarded as stable and can be detected biochemically (Box 1) [16]. In contrast, long-range forces are processes that actively redistribute TCRs across the immunological synapse (Figure 2) and can involve actin retrograde flow and motor proteins. Thus the coalescence of individual receptors may lead to the dynamic formation of nanoclusters, which may in turn coalesce to form microclusters, resulting in superimposed spatial organizations on different length scales.

In the following section we consider the technical challenges in detecting such a dynamic and heterogeneous mixture of spatial organizations.

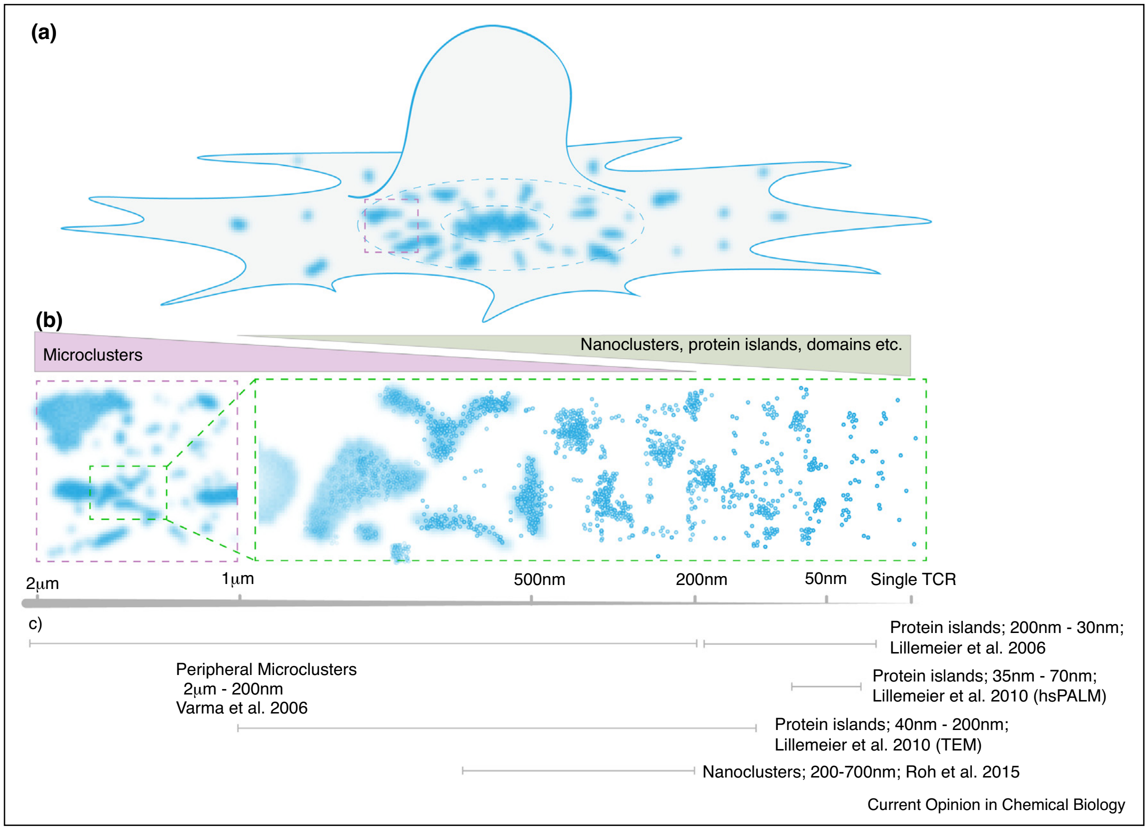
Technical obstacles in nanocluster detection: detection efficiency and counting variance

One of the advantages of SMLM is that each data set contains the position of many molecules of the same species. It therefore should be possible to detect the functional or organizational diversity within a single cell. In practice, however, the detection of rare events with SMLM is ultimately limited by the detection efficiency, which governs the probability that any given molecule will be detected. For the TCR this can be a limiting factor, since in T cells it is known that even a single antigen molecule can trigger an activation response [18]. Thus an open question for SMLM imaging is how likely it is that a single copy of an engaged and signalling receptor is captured in a sea of 50 000 inactive receptors.

Detection efficiency is governed both by the labelling efficiency and the photon number emitted from each fluorophore. Labelling efficiency can be maximized by using proteins that are genetically fused to fluorescent proteins, but even then the overall detection probability is generally <60% [19]. Photon number can be maximized by using organic dyes and grouping the fluorescent events that originated from the same, re-emitting fluorophore. However, re-exciting fluorophores and grouping can lead to both undercounting and overcounting errors. This is particularly the case for organic dyes as these dyes have multiple, reversible dark states and the blinking properties change during acquisition [3]. Hence even sophisticated simulations cannot fully define the parameters for grouping or assess the extent of overcounting and undercounting.

The complex photophysics which defines and limits detection efficiency also causes a large variance in the number of localizations per fluorophore [20••]. As such, small nanoclusters of <5 TCR–CD3 complexes or oligomers can often not be detected, and counting molecules is generally unreliable. This is evident when the stoichiometry of the TCR–CD3 complex was examined with single molecule techniques [21]. Despite the TCR complex containing two CD3ε subunits, the efficiency of detecting CD3ε colocalization using antibody labelling and single molecule colocalization (2.5–10%) or FRET techniques (FRET efficiency >0.2) was too low to detect dimers. It thus follows that absence of clear evidence for αβTCR dimers in single molecule imaging techniques is not equivalent to evidence for the absence of TCR–CD3 dimers. A similar problem arises in Rossboth *et al.* where small nanoclusters could not be

Figure 2



T cell activation induces a multi-scale spectrum of T cell receptor nanoclusters.
(a) A representation of an activated T cell on a coverslip surface presenting the canonical supramolecular molecular activation clusters (SMACs) of TCR (blue) including the peripheral (p)SMAC (outer ring) and central (c)SMAC (inner ring) of the immunological synapse. Separation between pSMAC and cSMAC typically occurs on the scale of 1–10 μm. The red box indicates an example region of interest elaborated on in subpanels **(b)** and **(c)**.
(b–c) TCR structures observed within the immunological synapse range from the peripheral microclusters at micrometer scale [65] to nanoclusters on the nanometer scale (b) and the approximate TCR cluster dimensions reported by microscopy experiments, referred to collectively here as nanoclusters (c) [4,54,66]. Nanocluster dimensions overlap with microclusters at the micrometer scale, and approach the predicted lateral scale of a single TCR complex (12–18 nm) at the nanometer scale, representing a multi-scale spectrum. Blue spots represent SMLM localizations.

detected yet it was concluded that no nanoclusters existed in resting T cells [22•].

The problem of imperfect detection efficiency and counting variance in dSTORM cannot be overcome by varying the density of labels or reducing the expression level [23]. In fact, by addressing the overcounting issue of re-excited molecules, it is possible that Baumgart *et al.* have gone too far in the other direction and massively undercounted molecules and thus nanoclusters. The problem with the varying labelling analysis is that it assumes that the underlying spatial organization, that is, the existence of one type of nanoclusters, is stable and uniform. This is clearly not the case when the expression levels are varied (as some structures only exist above a concentration threshold) but it is probably also not the case for TCR nanoclusters in T cells given the great natural diversity in cluster size, shape and molecular density that coexist within one cell. Not only is uniformity of architecture an important assumption for the analysis, but it also assumes that all antigen-binding sites are equally accessible. If there are steric problems with accessibility of certain sites, for example in clustered TCRs, then staining with subsaturating amounts of antibody will preferentially label more accessible sites and exacerbate labelling efficiency artefacts.

We took the opposite approach in Pageon *et al.* [11••]. Rather than grouping localizations into molecules, we analyzed the ungrouped localizations and therefore deliberately overcounted. This gave us a better chance of detecting signalling TCR–CD3 complexes that did not reside in clusters and indeed, we found a higher proportion of phosphorylated TCR–CD3 complexes outside clusters than in clusters with low molecular density. In our paper, and in many of the papers cited above, it was possible to draw conclusions by keeping the imaging and data processing conditions identical for all experiments and comparing spatial organizations within a cell or between cells, for example resting versus activated T cells.

To overcome the barriers just discussed, there are three major avenues for improvements in detection efficiency and counting variance: detection probes, algorithms and hardware. As these recent technological advances are refined and taken up into regular use, characterization of nanocluster spatial diversity will only improve. Firstly, the recent development of DNA-PAINT as a SMLM modality has permitted emitter blinking without manipulating dye photophysics. Instead, dye-labelled DNA molecules diffuse freely until they hybridize to complementary strands conjugated to various probes [24•,25,26]. Owing to the virtually inexhaustible supply of fluorophore, the capacity for multiplex imaging with one fluorescent channel [27], and the ability to distinguish *bona fide* hybridization events from background via spatial and temporal filtering of blinks, DNA-PAINT has the capacity for the long-term imaging (>100 000 frames) required to detect rare events otherwise missed by SMLM. As an example, DNA-PAINT has been applied to five EGF receptors in cancer cells revealing their non-homogenous distribution into ligand-sensitive clusters ranging from ~20 to 600 nm [28•]. This demonstrates the possible utility of DNA-PAINT in the characterization and detection of engaged and signalling TCRs, as well as the surrounding spatial organization.

The raw data arising from complex modes of data acquisition are a convolution of technical and biological signals and generally requires extensive computational processing to extract a 'pure' biological signal (or as close to pure as possible). In SMLM, the development of algorithms for the reliable extraction of localizations is still an active field of research. For example, a pre-processing method called HAWK has been proposed that lowers the density of emitters, in turn making localization identification more reliable [29•]. A new iterative approach called UNLOC for estimating particle locations has been suggested [30•] that can estimate the centers of overlapping point spread functions. There are at least two methods that propose to reconstruct a high-resolution image without explicitly calculating localizations. The first uses intensity gradients on a subpixel grid and temporal information to smooth out random intensity fluctuations [31]. The second uses a neural network and can deal with a high emitter density [32]. However, these image reconstruction techniques lack the desirable feature of molecule locations.

As important as pre-processing algorithms are, they are no substitute for better data acquisition with improved hardware. To date, commercial SMLM typically achieves a localization precision of 20–30 nm. At a high fluorophore density (i.e. where the fluorophores are within 30 nm of each other), it is thus challenging to assign emitted photons to the correct fluorophore. However, with a localization precision of 2–3 nm, it would not only be possible to distinguish closely spaced fluorophores but also improve detection efficiency as fewer photons are required to map the sharper diffraction profile of a single fluorophore. Excitingly, Hell and colleagues proposed a new imaging system, termed MINFLUX [33,34], that is a hybrid between localization microscopy and stimulated emission depletion (STED) and achieves 1–2 nm accuracy from only a few hundred photons.

From SMLM localizations to biological interpretation

Although the aim of preprocessing algorithms is to minimize technical artefacts and arrive at a pure biological signal, they generally do not help with biological interpretation. To take a specific example, the function of TCR clusters and how clusters emerge from individual TCRs remain open questions in T cell biology. A first step towards answering these questions using SMLM imaging data involves the clustering of either grouped or ungrouped localizations into discrete clusters. There are many clustering algorithms in common use [35–39], (Pike *et al.*, *bioRxiv* doi: https://doi.org/10.1101/400275), [40–42] and it is difficult to decide on the 'best' clustering algorithm for a particular biological question. Given that diversity in size, shape and density can be expected, combined with a large counting variance, imperfect detection efficiency and spurious localizations caused by non-specific binding, different clustering algorithms will give different output dependent on the algorithms' assumptions.

Furthermore, while data pre-processing and data analysis are often performed independently of each other, it should be kept in mind that all the preceding steps influence the final outcomes [45]. Surprisingly, the uncertainty that the data processing steps introduce is not currently carried through to the final results. Instead, once a fluorescent event is deemed to be a molecule, it is included in the final cluster analysis irrespectively of photon number, localization precision or accuracy of grouping parameters. Current algorithms typically present the number of molecules as though they were certain and report an error in their position rather than, for example, providing a confidence interval of the number of molecules per cluster. Thus there is scope for a more nuanced analysis pipeline in which points in the final image are weighted according to the sum of the uncertainties in each processing step, for example, via the localization count distribution [20••].

Another major aim for multicolor T cell imaging studies is to infer causal networks of molecular interactions from spatial patterns of molecular colocalizations or exclusions. In a recent example, Sherman *et al.* used three-color

SMLM data to search for synergistic interactions between molecules downstream of TCR activation [43]. However, investigating the co-clustering of multiple proteins produces more challenges than investigating a single protein. From a statistical perspective, multicolor SMLM images can be classed as a multitype spatial point pattern, which is under active research in other scientific disciplines [44].

In summary, there are a large number of analysis methods for clustering and colocalization and each algorithm is good at detecting specific types of structures or correlations in specific forms of data. However, heterogeneity in TCR organization within the same cell, and the nature of SMLM data, can present problems for the interpretation of results. In many cases existing algorithms oversimplify or fail to capture the underlying TCR biology. In the next section we elaborate on how approaches from other disciplines could be instructive for analyzing SMLM data.

Outlook — statistical approaches in other fields

A grand challenge in signalling is to understand how an ensemble of interacting molecules can self-organize to carry out biological function, and to predict how perturbations vary the associated biological function. Towards this aim, rigorous statistical descriptions of molecular spatial arrangements are necessary. In our view, this aspect of SMLM is in its infancy and there is enormous room for innovation. Fortunately, we can look to other scientific disciplines for inspiration. A better description of how TCRs are positioned with respect to each other will give insight into how the membrane keeps nanoclusters separate. To this end, we can adapt tools used to analyze the topology of bird flocks [46], information flows in animal groups [47] or competition between trees [48]. To better understand the dynamic process of cluster formation, we can look to statistical physics. For example, a study of colloidal liquids (specifically those with a short-range attraction and long-range repulsion) shows how the distribution of cluster sizes can be used to define the phase state, which has important implications for thermodynamic limits of cluster size [49]. Further examples can be found in Box 2. Another open question is how various spatial arrangement of receptors is associated with the likelihood that TCR phosphorylation will occur given a neighboring receptor is also phosphorylated. We can look to mathematical descriptions of other self-organizing systems such as the 'secrete-and-sense' mechanism [50], where geometry is used to describe the cells' degree of autonomy versus the degree of collectiveness. While the focus of this review is on TCR clustering, statistical methodology to describe molecular spatial organization will be applicable to a wide range of signalling pathways. Conversely, comparative analyses of disparate signalling pathways will highlight whether there are common information processing strategies playing out in the spatial domain.

Box 2 The physics of clustering

Not only can clusters be experimentally observed, but their very existence is also mathematically feasible through the emergent self-organizing properties of systems of interacting particles. A very small selection of examples follows. Das *et al.* simulated particles on a lattice and showed how positive feedback can lead to cluster formation [62]. Iron and Rumsey analyzed a spatial reaction-diffusion system and found that stable structures can form when the probability of free receptor binding is in a critical range — when too high or too low, uniform receptor concentrations result [63]. Ullrich *et al.* performed particle-based diffusion simulations of syntaxin on the neuronal membrane and showed that there is a fragile balance between syntaxins stored in large clusters versus free syntaxins or small clusters that are free to diffuse [64]. Importantly, with only assumptions about the interaction potential between particles and diffusion constants, it was found that dissociation rates per particle decreased with increasing size of the cluster, meaning that the smaller the cluster, the more transient it is. Although perhaps counter-intuitive, there is evidence that complex structures can arise through the random interactions of many particles, and that the type of structure can be tuned by varying the 'rules' of interaction. Although it is difficult to conclusively link computational models and experiment in a causative manner, further work in this field will help develop a conceptual understanding.

Multi-color analysis was briefly discussed in the previous section. However, it must be emphasized that concomitant with enhanced resolution new definitions of colocalization are needed. Overlapping signal makes sense when thinking about colocalization on the diffraction-limited scale (hundreds of nanometers), but as we near molecular scale resolution it is physically not possible for two molecules to occupy the same space and thus this definition is insufficient [3]. However, to pre-define colocalization distances may bias the analysis, and so innovation is also possible on this front. One idea would be to use a cross-nearest neighbor distance analysis. This was demonstrated previously to quantify the degree of segregation between two species with non-random distributions [51] and for conditional dependence of business types (e.g. newsstands often occur near train stations but not *vice versa*) [52], and recently extended to social geography [53].

In conclusion, proof-of-principle studies have indicated the enormous potential of SMLM to provide insights into the mechanisms of T cell signalling in intact cells and with improvements in hardware, labelling and detection efficiencies as well as in statistical analysis, SMLM may reveal the full diversity in TCR triggering mechanisms.

Conflict of interest statement

Nothing declared.

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