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Spatial organization and signal transduction at intercellular junctions

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Abstract

The coordinated organization of cell membrane receptors into diverse micrometre-scale spatial patterns is emerging as an important theme of intercellular signalling, as exemplified by immunological synapses. Key characteristics of these patterns are that they transcend direct protein–protein interactions, emerge transiently and modulate signal transduction. Such cooperativity over multiple length scales presents new and intriguing challenges for the study and ultimate understanding of cellular signalling. As a result, new experimental strategies have emerged to manipulate the spatial organization of molecules inside living cells. The resulting spatial mutations yield insights into the interweaving of the spatial, mechanical and chemical aspects of intercellular signalling.

Cell-to-cell communication is mediated by various methods. Endocrine signals are secreted and reach distant target cells, paracrine signals are secreted and reach targets in the vicinity, and autocrine signals are secreted and received by the same cell. By contrast, in juxtacrine signalling, surfaces of interacting cells come into direct contact and receptor–ligand recognition at this interface triggers intracellular signalling. Cell–cell interactions involve multiple adhesion and signalling molecules, the collective behaviour of which regulates signal transduction[1]. Also intrinsic to juxtacrine signalling configurations are large physical constraints on molecular movement and assembly. Genetic and biochemical approaches have been invaluable in identifying the molecular components of signal transduction pathways in juxtacrine signalling and in characterizing the biochemical interactions among them. Despite this wealth of information, in many cases it remains impossible to describe the behaviour of a signalling system in terms of the individual molecular properties of its components. Protein–protein inter actions and the formation of molecular clusters are widely implicated in signal transduction and contribute to a first level of cooperativity[2–4]. Recently, the coordinated organization of cell membrane receptors into micrometre-scale patterns has emerged as a broadly important theme of intercellular signalling[1,5–9].

A paradigm for the interplay of spatial patterns and signal transduction is the junction between T cells and their target cells, termed the immunological synapse[8–13]. Spatial

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patterns of proteins at the cell–cell interface develop as hundreds of receptors recognize their cognate ligands on the apposed cell membrane. Multiple signalling and adhesion molecules also become organized into distinctive spatial patterns at the interface between the two cells[8,9,13,14] (FIG. 1). The patterns create long-range interactions and seem to have specific purposes in signal transduction[8,9]. They host the local enrichment or depletion of key signalling components, which can bias biochemical cascades towards different functional outcomes. For example, this can result in location-specific signalling of identical receptors. Recent evidence suggests that the spatial organization of the immunological synapse has an active role in regulating the signalling state of individual molecular components, and thus can alter long-term cell activation[15–17]. Various different spatial arrangements can occur between different types of immune cells and their respective targets, correlating with different functions[8,13]. The protein patterns are not static on the cell surface[18,19]. Instead, they evolve on the timescale of signalling, usually over the course of minutes, and can change depending on the cell signalling state[15,16]. Here, we highlight recent evidence suggesting that the spatial organization of proteins at cell–cell interfaces may be a widespread regulatory mechanism of intercellular signal transduction.

This Review focuses on the relationship between protein spatial organization and signalling in intercellular junctions, highlighting examples primarily from the immunological synapse. We discuss two structures that are largely responsible for physically controlling this spatial organization: the cell membrane and the actin cytoskeleton. Finally, we review emerging experimental strategies to study and manipulate spatial organization and signalling in living cells.

Micrometre-scale signalling patterns

At the front-line of adaptive immunity, T cells recognize pathogen-derived peptides on the surface of antigen presenting cells (APCs) at the immunological synapse[20]. Activation of T cells is triggered when T cell receptors (TCRs) recognize their ligand — major histocompatibility complexes (MHCs) displaying the appropriate antigenic peptide (pMHCs). Within 5 minutes of contact, pMHC–TCR complexes form molecular clusters containing tens to hundreds of molecules that are driven by the actin cytoskeleton from the periphery to the centre of the immunological synapse[21]. Concurrently, the adhesion molecule leukocyte function-associated antigen 1 (LFA1; also known as αLβ2 integrin), on the surface of T cells, ligates intercellular adhesion molecule 1 (ICAM1) on the APC. ICAM1 becomes enriched in a peripheral ring surrounding the central accumulation of TCR within 5 minutes of contact. The TCR and ICAM1 patterns can span the interface of the cell–cell contact zone, which is about 5–10 micrometres in diameter (FIG. 1). This organization was first seen more than a decade ago, triggering intense interest in its possible roles in T cell signalling[5,22]. Since then, the spatial and temporal complexity of immunological synapses has been explored by a combination of biochemical, genetic, imaging and patterning approaches. Many other signalling and adhesion molecules reorganize as well, and the patterns have functional consequences[8–10,12–14,23]. In light of this preponderance of observations, we argue that the consideration of spatial organization is indispensable to understanding signal transduction at this inter cellular junction.

Here, in an effort to highlight the best understood examples of how spatial organization can affect signalling, we focus on the classical protein pattern of the T cell immunological synapse: TCRs in the centre and adhesion molecules in a peripheral ring. Our discussion is not intended to be a comprehensive review of immunological synapse signalling, which is well described elsewhere[24–27]. It is important to note that a diversity of protein patterns besides the canonical one form for different subsets of T cells at different developmental stages and for other immune cell–cell interactions[8]. The developmental precursors of T

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cells, thymocytes, form multifocal TCR synapses[28]. Another subset of immune cells, natural killer (NK) cells, also form organized signalling junctions with their target cells[29–31]. Their receptors recognize MHCs on other cells, but trigger an inhibitory signal that protects the target cells from the cytotoxic activity of the NK cell. These receptors also reorganize into different patterns at the junction with the target cells: multifocal, homogeneous or homogeneous with regions in which other molecules are excluded. An intriguing configuration is the ring-like enrichment of the NK cell receptors around a central zone of ICAM1, which resembles an inverted T cell synapse[29,30]. The mechanisms of pattern establishment and control in NK cells seem to be different from those in T cells. NK cells do not require cytoskeleton- or ATP-dependent processes, and the pattern morphology depends on MHC surface density and the balance of activating and inhibitory receptors that are engaged[30–32]. The exact relationships between spatial organization and signalling in thymocyte and NK cell synapses are less understood than in T cells, but their variety underscores the diversity of protein patterns at intercellular junctions.

The signalling state is location-dependent

The immunological synapse provides several striking examples of how spatial patterns can influence signalling activity. Within five minutes of cell–cell contact, TCR clusters are transported by the actin cytoskeleton to a micrometre- scale zone at the centre of the contact interface[5]. It is well-established that TCRs in this central zone are dephosphorylated and internalized[5,23] (FIG. 1; FIG. 2a). TCR signalling is determined not just by engagement to its ligand pMHC but also by its spatial position[17,23,33]. Recently, the location-specific signalling state of TCRs at a late signalling stage (about an hour after contact) was addressed[16] (FIG. 2b). TCRs in an initial phosphorylation state are localized to the periphery of the immunological synapse, whereas TCRs in a terminal phosphorylation state are at the centre[15,16]. At both time points, the signalling state of TCRs is altered as a function of their location at the centre or the periphery of the cell–cell interface.

Patterns in immunological synapses are dynamic structures that are actively controlled by and able to adapt to signalling activity. For example, the strength of TCR activation can determine TCR organization and signalling. High stimulation of T cells (by many or strong TCR ligands) results in the aforementioned accumulation of TCRs at the centre of the cell–cell interface, where TCRs become dephosphorylated and internalized[23]. However, at low stimulation (by fewer or weaker TCR ligands), or at later time points of strong stimulation, there is evidence that the central zone assumes an opposite role and becomes the site of sustained phosphorylation[15,16] (FIG. 2). These opposing behaviours suggest that the central and peripheral zones do not have fixed roles as stimulatory or inhibitory regions. On the contrary, their specific effect on signalling depends on the overall signalling context. The immunological synapse is a “molecular machine controlling T cell activation” (REF. 5) that uses spatial organization to balance its signalling outcome[23].

Altered spatial organization and collective signalling

At high stimulation, as described above, TCRs are transported by the actin cytoskeleton to the centre of the immunological synapse, where they are down regulated. Because TCR deactivation coincides with TCR transport to the centre of the immunological synapse, it was initially unclear whether TCR activity is spatially or temporally regulated. This ambiguity was resolved by an experiment in which some TCR clusters were physically constrained in the periphery at all time points, including when they would otherwise be in the centre[17] (FIG. 3). The experimental strategy to achieve such selective control over TCR spatial organization is discussed later. The net result of these experiments was to reveal that at the same time point the peripheral TCR clusters remain phosphorylated while the central TCR clusters are dephosphorylated and inactivated. The simultaneous presence of signalling

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and non-signalling TCRs provides direct evidence that the TCR signalling state is influenced by its radial location irrespective of the time point of signalling. Furthermore, the altered TCR localization and signalling increased the T cell calcium flux, a downstream response that triggers transcription factor activation, and thus altered the signalling outcome of the whole T cell.

In the above example[17], the translocation of TCRs from the centre to the periphery was constrained by physical barriers (FIG. 3b; see also later section on experimental organization control). This perturbation allowed the comparison of biochemically identical cells, in which only the localization of a specific receptor is altered. We refer to these perturbations as 'spatial mutations'. The term 'mutation' refers originally to genetic alterations and here its definition is extended to include the alteration of spatial parameters.

The previous example illustrates how, at high activation, constrained TCR transport to the centre decreases TCR signalling downregulation. Conversely, at low stimulation, there is evidence that enhanced TCR transport to the centre boosts TCR signalling[15]. Immunofluorescence shows that the centre of the immunological synapse is the site of sustained signalling under low stimulatory conditions[15,16]. This observation raised the question whether enhancing TCR transport at such conditions would also alter T cell signalling. Indeed, artificially induced central accumulation of TCRs, in cases where it would not occur naturally, resulted in enhanced long-term cell signalling[16] (FIG. 3c,d).

The regulatory function of spatial organization is further exemplified by experiments that test the cellular response to spatially inhomogeneous stimuli. Besides physical barriers that alter the spatial reorganization of proteins driven by a cell[17], pre-patterned arrangements of different surface receptor ligands or antibodies can impose spatial organization on a cell. These are another version of our definition of spatial mutation, and experimental methodologies to achieve this are discussed later. In one striking example of this strategy, researchers recreated the pattern of the canonical immunological synapse, with TCRs in the centre surrounded by adhesion molecules, and successfully activated the T cells (see also later section on experimental organization control)[33]. More importantly, when the pre-programmed geometry differed from the natural pattern, T cell signalling was noticeably altered. Different geometrical orientations were tested: one of inversion, whereby TCRs are in a peripheral ring and adhesion molecules are in the centre, and another of breaking up the localization of TCRs into multiple foci. After several hours of activation by the different patterns, the secretion levels of a proliferative cytokine were similar. However, secretion of the targeted cytokine was decreased in cells responding to the inverted pattern.

The spatial organization of co-stimulatory molecules also affects signalling. Recent work using immobilized patterned antibodies to stimulate cells shows that T cells are maximally co-stimulated with CD28, but only if CD28 is in the periphery with respect to TCRs[34]. If CD28 is forced to the centre or CD28 and TCR colocalize, cell signalling decreases (see also later section on experimental manipulation of organization). In this case, TCR and CD28 signalling need to be spatially segregated, and the CD28 has to be peripheral to TCRs for optimal co-stimulation. This work uses fixed patterns that do not fully reflect the dynamic association of TCRs and CD28 in microclusters in the periphery and their accumulation in separate compartments in the centre of the immunological synapse[27,35]. However, it tests and emphasizes the difference in signalling in the two synapse zones and its effect on long term T cell activity.

Spatial organization in biochemical signalling cascades

Intracellular biochemical signalling cascades operate far from reaction equilibrium and homogeneity. As such, many simple chemical rules of thumb no longer apply. For example,

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classical chemical kinetic rate equations are predicated on the assumption that all species are fully mixed and, therefore, randomly distributed in solution. This is not true for any of the signalling systems we consider here, thus the measured kinetic rates for specific protein interactions in solution do not apply[36]. Although it is clear that the spatial organization of molecular components can bias reaction outcomes, specific and quantitative analysis of these effects is often quite difficult by human intuition alone[23,37–40]. Nevertheless, this seems to still be the predominant method used to deduce biological signalling mechanisms. However, computational modelling is gaining ground as an important aspect of the study and understanding of spatially regulated signalling cascades. In one example of this, a computational model was developed that specifically included the spatial position of reactants[23]. Simulations revealed how the synapse centre can be a site for both enhanced signalling and downregulation. Although the model is simplified and can not capture the full intricacy of T cell biology, it provided a substantial advance in the mechanistic understanding of TCR signalling regulation. Better and more inclusive models continue to emerge[37–41], as do more quantitative experiments to measure spatial organization and function in cellular signalling systems.

Another revealing example of the spatial sensitivity of a simple reaction network is the initiation of blood clotting, which involves a cascade of tens of biochemical reactions. Competition between catalytic product formation and diffusive mixing controls the progress of the whole signalling cascade and exhibits distinctive sensitivity to spatial organization. If the reactants are patterned on a surface, the initiation of the clotting reaction depends not on the overall amount of reactant but on the dimensions and spacing of regions of reactant[42,43]. This dependence can be reproduced in a synthetic signalling cascade that has similar competition between product formation and dispersion by diffusion. The *in vitro* sensitivity of the blood clotting reaction cascade to spatial organization is physiologically relevant *in vivo*. Bacteria in the blood stream release factors that can initiate blood clotting. In mice, clotting caused by bacterial infection occurs only in regions where bacteria are clustered and yield a high local concentration of reactant[44].

Cellular organization mechanisms

The physical mechanisms that establish and regulate the spatial organization of signalling molecules are equally as important as the chemical reactions themselves. Here, we highlight key roles of the cell membrane and the actin cytoskeleton.

Cell membrane

The cell membrane is a spatially heterogeneous yet liquid mixture of lipids, proteins and other molecules that provides the environment in which nearly all signal transduction processes occur. Protein–protein interactions in the membrane clearly have a central role in defining the assembly and composition of receptor signalling clusters. The lipid and cholesterol components of the cell membrane also exhibit clear miscibility phase separation *in vitro*, and this is thought to contribute to membrane organization *in vivo*[44]. Membrane domains formed by phase separation are widely referred to as lipid rafts. Although a significant role for lipid composition on membrane organization *in vivo* is almost a certainty, specifics of the raft hypothesis remain hotly debated. This subject is comprehensively reviewed elsewhere and is not discussed in detail here[45].

What is clear is that many signalling systems, such as through TCRs, signal from clusters that form dynamically in the cell membrane (probably through a combination of protein and lipid interactions). These clusters are sometimes referred to as rafts. What is not clear is to what degree the composition and structure of these clusters varies in time, from one to another and on the same cell under different signalling conditions. Indeed, much of the

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controversy surrounding the term ‘membrane raft’ stems from the difficulty in defining structures that are so dynamic and variable. There may well be certain characteristics common to many types of molecular clusters occurring in the cell membrane. From a signalling perspective, however, we suggest that any such commonality is not the most relevant feature. It is the specific functionality resulting from individual details of the composition and distribution of signalling clusters that we must elucidate in order to understand signalling mechanisms at the cell membrane.

Recent work has revealed a specific example of the functional organization of the membrane signalling proteins in T cells[46]. TCRs and LAT are organized in protein clusters (sometimes referred to as islands) of 7–30 molecules in quiescent cells. On activation, these clusters coalesce and form larger structures of a few hundred molecules that have been referred to as microclusters (all of these structures could perhaps be considered as different types of raft)[21]. This study combines three different experimental approaches — hsPALM, transmission electron microscopy (TEM) and dcFFCS — to observe different aspects of the protein cluster arrangements, yielding an unprecedented high-resolution and dynamic view of membrane organization. This observation of independent dynamic domains of TCRs and LAT and their concatenation on activation reveals an elegant choreography of membrane organization that evolves dynamically during signalling. We suggest that substantial breakthroughs in understanding signalling processes may be achieved by revealing the rich repertoire of membrane organizational states.

Juxtacrine signalling at intercellular junctions, such as the immunological synapse, leads to some interesting membrane mechanical effects that can also contribute to protein clustering[1,47]. The dissociation constant for an intermembrane protein complex is different from that in solution because the probability of interactions depends on the intermembrane separation and the protein surface density[46,48] (FIG. 4a). Although membranes are flexible, high bending is energetically unfavourable. In intercellular junctions, a protein-binding pair from opposite membranes can effectively prevent the interaction of a neighbouring protein-binding pair if the intermembrane spacings of the pairs are very different[49] (FIG. 4b) or can induce clustering among similar proteins[50]. An important consequence of this is that intermembrane protein binding can exhibit cooperative effects between remote pairs of proteins that are not in direct contact. The proximity of short and long intermembrane pairs requires that the membranes bend to accommodate the size difference. In the fluid environment of the cell membrane, this intermembrane size selection can lead to spatial segregation into patterns that guide signalling[37,49,51–53] (FIG. 4c). For example, experiments[54] have shown that protein intermembrane spacing is crucial for TCR signalling and it has been proposed that the exclusion of long phosphatases from TCR regions of short intermembrane spacing is the reason for TCR triggering[51,55]. Re-engineered pMHC–TCR complexes with elongated intermembrane spacing disrupt signalling in the T cell without altering the intrinsic complex interactions.

Actin cytoskeleton

The actin cytoskeleton has long been implicated in controlling the dynamic spatial organization of the immunological synapse. It mediates long-range interactions by physically transporting surface molecules, such as TCRs and the integrin LFA1, and directly interacts with the components of signalling cascades[21,25,55–57]. The actin cytoskeleton itself is a dynamic structure with a highly heterogeneous composition and mechanical properties. There are regions of lower or higher branching and different polymerization and depolymerization rates[53,56,58–60]. For example, in the immunological synapse, the outer periphery of the cell-to-cell interface displays a dense network of filamentous actin (F-actin), whereas the centre of the interface is either depleted of F-actin or F-actin is present at a lower density[24,56]. TCR signalling leads to the activation of multiple regulators of actin

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polymerization, such as Cdc42, WAVE2 (also known as WASF2), and Wiskott–Aldrich syndrome protein (WASP), which themselves can remodel the local cytoskeleton[55].

The regulation of protein transport is key to controlling the signalling activity of individual proteins and the whole cell. Adaptor molecules[25,58,61], such as talin for LFA1 (REF. 62), mediate selective interactions between signalling molecules and actin. As spatial organization varies with the overall signalling context of the cell, it is plausible that these adaptor molecules are not just constant links but are actively regulated. The adaptor proteins for TCR transport remain elusive. The ezrin, radixin and moesin (ERM) proteins, which can bind both TCRs (or other signalling molecules) and F-actin, are implicated but their role is still controversial and they do not seem to be sufficient for TCR transport[25,55].

The mechanisms by which the cytoskeleton physically and selectively transports molecules are under investigation. Although it is clear that receptor transport in the immunological synapse is driven by actin, the mechanism of differential protein sorting remains unresolved. TCRs and LFA1 are driven towards the centre of the immunological synapse, but their final destinations are micrometres apart[56,63] (FIG. 1). Recent experiments in which the clustering state of LFA1 was externally manipulated have begun to shed more light on such processing of actin-mediated sorting. LFA1 does not form the large clusters that TCRs do; however, its cluster size can be experimentally controlled through the bivalent and tetravalent antibody cross-linking of LFA1 or its ICAM1 ligand[63]. The higher the degree of cross-linking, the closer LFA1 is brought to the centre of the immunological synapse, where TCRs would be. With the tetravalent cross-linking, LFA1 reaches the centre of the immunological synapse, sharing it with the TCR clusters. This observation suggests that sorting of proteins in the immunological synapse can be accomplished by the regulation of cluster size and cluster interactions with the driving actin cytoskeleton (FIG. 4d).

The physical interaction of TCR clusters with actin is further revealed in experiments that alter the native path of TCR clusters to the centre of the immunological synapse[57]. The experimental strategy to introduce selective barriers to TCR transport is discussed later. When TCR clusters encounter a physical barrier *en route* to the centre, they do not always stop. If they approach a barrier at an angle with respect to the driving actin flow, they continue their motion tangential to the barrier. They remain driven by the actin cytoskeleton, although their apparent directions differ. This behaviour strongly suggests that multiple, dynamic and weak interactions couple TCR clusters to the moving actin flow, similar to a drag force. This mechanism allows TCR clusters to divert around obstacles and still be driven by actin without building up substantial elastic energy. The mechanism is also easily extended to sort many molecules on the cell surface.

Multiple length scales of spatial organization

So far we have primarily discussed micrometre-scale organization of proteins in the immunological synapse. At least for TCRs, this pattern is a result of the accumulation of multiple smaller clusters of tens to hundreds of molecules. Such clusters are observed for many other molecules in the immunological synapse, including LAT[64], ζ-chain-associated protein kinase 70 (ZAP70)[64], SH2 domain containing leukocyte protein of 76kDa (SLP76; also known as LCP2)[65], CD28 (REF. 35) and CD2 (REF. 53). Besides those seen in immune cells, smaller clusters have long been observed for many other membrane proteins, such as ErbB family receptors[66], and even for intracellular signalling molecules such as Ras[67]. A recent study of the ephrin type A receptor 2 (EPHA2) receptor Tyr kinase suggests that clustering and micrometre-scale spatial translocation of the receptor clusters can lead to unanticipated emergent properties such as mechanical-force sensing[68]. In chemotaxis, bacterial receptors also exist in an organized manner at the cell poles, with exponentially distributed cluster sizes from tens to thousands of receptors[69]. In these clusters, different

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receptors functionally cooperate[70,71]. Although in this review we focus on micrometre-scale organization, there is a continuum of protein spatial organizations, from molecular complexes to micrometre-scale domains that span the cell-to-cell interface[72]. We should anticipate that organization at all these different levels feeds back to regulate specific protein and collective cell signalling.

Experimental organization control

Studies of the role of spatial organization in signal transduction require experimental approaches that directly manipulate the spatial component of cellular signalling systems. In response to this need, several strategies have recently been introduced. These are based largely on surface chemistry and material fabrication techniques that are not typically included in a classical cell biology repertoire. A more detailed discussion of these methodologies as they apply to cell biology follows.

Hybrid live cell–supported bilayer junctions

Hybrid live cell–supported lipid membrane junctions can reconstitute much of the micrometre- and molecular-scale re organization that occurs in natural intercellular junctions. Aspects of the immunological and neuronal synapse can be reconstituted between live cells and synthetic surfaces[5,73] (FIG. 5a). Recently, a complex signalling interaction between the EPHA2 receptor Tyr kinase on live epithelial cells and its membrane surface ephrin A1 ligand in supported membranes has also been achieved[68]. The supported membrane is a continuous fluid lipid bilayer that forms by the spontaneous self-assembly of liposomes or proteoliposomes on clean glass surfaces[74]. Proteins with membrane anchors can be tethered to the bilayer and experience the free lateral mobility that is inherent to the native cell membrane[75,76]. Other advantages of this system are control over identity and quantity of the components[77]. Additionally, the well-defined planar interface facilitates high resolution imaging by fluorescence microscopy.

Solid-state structures patterned on the glass surface can act as barriers to the lateral diffusion and transport of lipids and proteins in the supported lipid bilayer[75,76,78] (FIG. 5b). As receptors engage their ligands in the bilayer, they are also selectively restricted by the pattern on the substrate. Crucially, only the proteins interacting directly with the membrane components are subjected to the constraints; the rest of the cell is free to rearrange. Different configurations of metal lines pre-patterned on the surface (for example, by electron beam lithography (BOX 1)) can alter the geometric pattern of cell surface receptors and associated signalling molecules[17,57,63] (FIG. 6a). A key feature of a supported membrane with patterns of mobility barriers is that molecular-level clustering is allowed to proceed naturally but large-scale organization is selectively under direct control. This allows analysis of large-scale spatial pattern and clustering effects without the side effects caused by the altered molecular-scale assembly of signalling complexes (which would occur on purely solid surfaces).

Box 1

Patterning strategies

Functionalized surface–live cell interactions are a powerful model system for the study of intercellular signalling. The surface can be patterned with regions of metals, proteins or lipid bilayer in different configurations by several methods. The different strategies vary in the size and precision of the features, the area that can be patterned and the ease of use[80,82].

Microfluidics

Several channels with different proteins flow unmixed over the surface and deposit juxtaposed patterns[83,84,107]. Pattern geometry is limited to permissible flow patterns but allows for multiple proteins to be co-patterned. The resolution is < 10 μm.

Photolithography

The surface is initially coated with a light-sensitive polymer. Illumination with visible or ultraviolet light through a patterned mask destroys the polymer and allows for metals or proteins to be deposited. After removal of the rest of the polymer, the rest of the surface can be filled with another protein or lipid bilayer[81,85]. The resolution is ~ 1μm.

Electron-beam lithography

Similar to photolithography, but an electron beam is used to draw patterns that destroy the polymer and create regions for differential deposition[17,78]. The resolution is ~ 20nm.

Microcontact printing and nanoimprinting

The surface is patterned, by either method, and physically etched according to the pattern to create a template or master three-dimensional (3D) surface[82,86]. The pattern can be further replicated by deposition of polydimethylsiloxane (PDMS), which solidifies according to the 3D surface. Either the template surface or the separated PDMS block are absorbed with protein and stamped on the target surface, thus maintaining the patterned configuration. Typically, nanoimprinting resembles embossing more than stamping, recreating a 3D effect in the target surface. The resolution for microcontact printing is ~ 1 μm and for nanoimprinting is < 20 nm.

Dip-pen nanolithography

An atomic force microscopy (AFM) tip loaded with a small molecule or a protein solution as ink is used to write on the surface[87]. Any pattern is possible, but deposition is serial. The resolution is < 50 nm.

Block co-polymer micelle nanolithography

Polymers self-assemble over large areas of the surface into regular patterns that are used as templates for further functionalization[88]. The resolution is < 20 nm.

Direct spatial pattern on solid surfaces

The geometry of the hybrid signalling junction can also be altered by direct patterning of proteins on solid surfaces (FIG. 5b; FIG. 6b,c). Strategies inspired by semiconductor fabrication and polymer chemistry have emerged to manipulate the spatial organization of molecules on the surface and therefore inside living cells[79–82] (BOX 1). The surface is patterned with subcellular features that localize cellular ligands in pre-set configurations (FIG. 5b). The pattern feature size, geometry, area patterned and diversity of proteins that can be patterned at the same time vary greatly between different techniques. The patterning strategies include microfluidics[83,84], photolithography[81,85], electron beam lithography, microcontact printing[86], nanoimprinting[82], dip-pen nanolithography[87] and block–co-polymer micelle nanolithography[88]. Some of these patterning strategies can be combined with supported fluid lipid bilayers on the same surface, providing both mobile and immobile stimuli[17,57,75,81,89–91]. The cell biological application of these techniques was originally pioneered for the study of cell adhesion and focal adhesion sites[80,92–94]. More recent developments have emphasized control of signalling specificity along with spatial organization[17,33,34,75,91,95,96]. Their application in cell studies meets some biology-inherent challenges. At the very least, patterns need to be biocompatible with subcellular and even molecular dimensions[17,33,34,93].

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In the studies of TCR localization to the centre or the periphery, antibodies to TCRs and adhesion molecules were patterned with features as small as a few micrometres[33]. The presentation of anti-TCR antibodies in a peripheral ring constrained TCR triggering and signalling to this specific peripheral configuration and prevented TCRs from forming a central cluster (FIG. 6b). In studies of CD28 co-stimulation, anti-TCR and anti-CD28 antibodies were added to the adhesion molecule-rich surface, thus offering a pre-set orientation for activated TCRs and CD28 (REF. 34) (FIG. 6c). Such inhomogeneous ligand presentations have revealed the importance of spatial organization to specific molecules or signalling pathways.

Control of spatial organization can also be achieved by the optical control of protein or activity. Light-sensitive[97–99] photo-switchable molecules[98,100,101] or local uncaging[102–104] combined with diffraction-limited lasers enable high spatial and temporal control over protein activity. Optically controlled uncaging of T cell-activating peptide can stimulate the cells with high temporal and possibly spatial resolution[102,103]. Both of these types of approach can cause spatially inhomogeneous activation of cells and, when combined with readouts of signalling[105,106], can elucidate mechanisms of the spatial control of signalling.

Conclusion

The immunological synapse provides vivid examples of how signalling reactions in biology use spatial organization on multiple length scales. The proteins in this intercellular junction are highly organized into micrometre-scale patterns that control signal transduction. The spatial organization of the interface determines the signalling state of individual molecules and the signalling state of a cell determines its specific spatial organization. The immunological synapse provides examples of how altered spatial organization changes the signalling outcome of a single molecule or the cell as a whole. Computational modelling and patterning of simple reaction networks illustrate how complex biochemical cascades depend strictly on the spatial organization of their components. We argue that spatial organization is an integral part of signal transduction regulation that is probably important for other juxtacrine signalling systems as well.

Spatial organization is physically controlled by the interplay between the cell membrane and the actin cytoskeleton. New experimental strategies have emerged that draw inspiration from fields such as semiconductor fabrication and polymer chemistry and apply these concepts to manipulate the spatial organization of molecules inside living cells. These approaches are becoming indispensable to understanding the control of spatial organization and exploring its role in signalling.

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Glossary

Cytokine A member of a large family of immunomodulating secreted proteins that interact with cellular receptors. Cytokine production results in the activation of an intracellular signalling cascade that commonly regulates processes such as inflammation

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Miscibility phase separation	The partitioning of lipid components (in the context of membranes) into domains that have different chemical compositions and physical properties
LAT	(Linker for activation of T cells). A transmembrane protein that on TCR activation becomes rapidly phosphorylated and binds multiple adaptor molecules and indirectly recruits others
hsPALM	(High speed photoactivated localization microscopy). A fluorescence imaging technique in which sequential activation, localization and bleaching of fluorescent reporter proteins yields an image with a resolution of a few tens of nanometers, well below the diffraction limit
dcFFCS	(Dual colour fluorescence cross-correlation spectroscopy). A technique that analyses the dynamics and association of two different diffusing fluorescent proteins
Chemotaxis	Directed cell movement according to chemical stimuli
Liposome or proteoliposome	A vesicle made of lipid bilayer in an aqueous environment. Membrane proteins can be incorporated in the bilayer
Photo-switchable molecule	A molecule with a functionality (ligand binding, conformational change or absorption spectrum) that is controlled by light and in some cases can be toggled on and off
Uncaging	The light-controlled release of a functional group that hides (cages) another functional group

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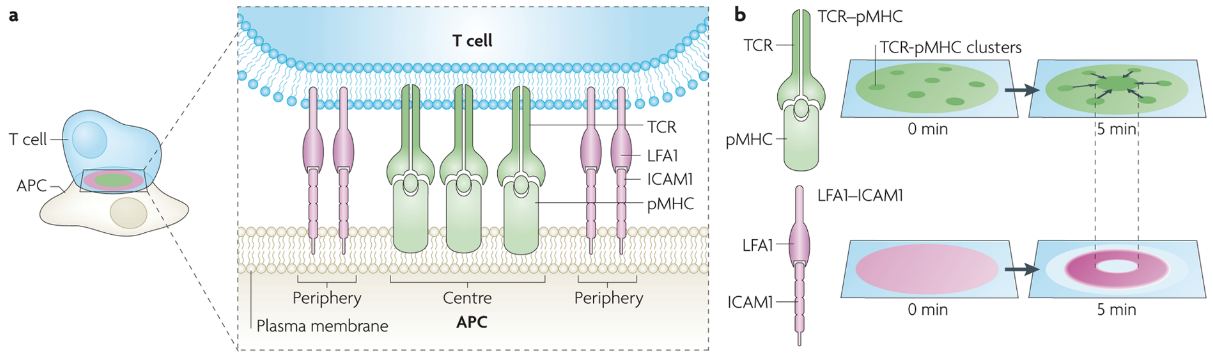

Figure 1. Micrometre-scale protein patterns in the immunological synapse

a. The intercellular junction between a T cell and an antigen presenting cell (APC) is known as the immunological synapse. Micrometre-scale protein patterns emerge at the interface between the two cells. A top down and *en face* view of the immunological synapse reveals highly organized, concentric protein regions. T cell receptors (TCRs) bound to major histocompatibility complexes displaying an antigenic peptide (pMHCs) localize at the central (green) region, and the T cell leukocyte function-associated antigen (LFA1; also known as αLβ2 integrin) bound to intercellular adhesion molecule 1 (ICAM1) localizes to the peripheral (purple) region. **b.** Formation of micrometre-scale patterns from the time point of contact with an activating APC. TCRs recognize pMHCs and form small clusters (dark green) that are driven by the actin cytoskeleton to the centre of the immunological synapse (top). After 5 minutes, most of the TCRs are in the central zone of the immunological synapse. The T cell integrin LFA1 recognizes ICAM1 and the conjugates form an enriched ring, peripheral to the TCR central zone (bottom).

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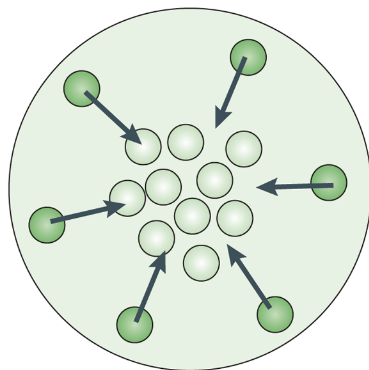

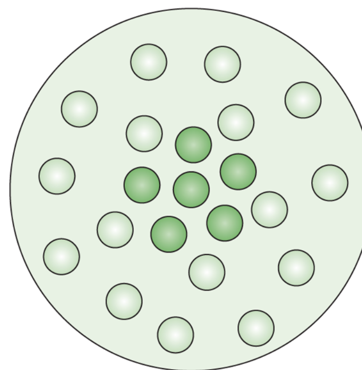

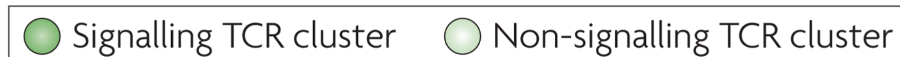

Figure 2. Signalling states are location-dependent in the immunological synapse
T cell receptors (TCRs) are distributed throughout the immunological synapse; however, their signalling state depends on their location and the time point from their contact with an antigen presenting cell (APC). **a.** At early signalling (< 20 minutes from APC contact), TCR clusters form and signal in the periphery[21]. These clusters are transported by the actin cytoskeleton to the centre, where they are downregulated[5,8,22,23]. **b.** At late signalling (> 40 minutes from APC contact), non-signalling (or low-signalling) TCR clusters are detected in the periphery. Signalling TCR clusters that are fully phosphorylated on all sites are seen in the centre[16].

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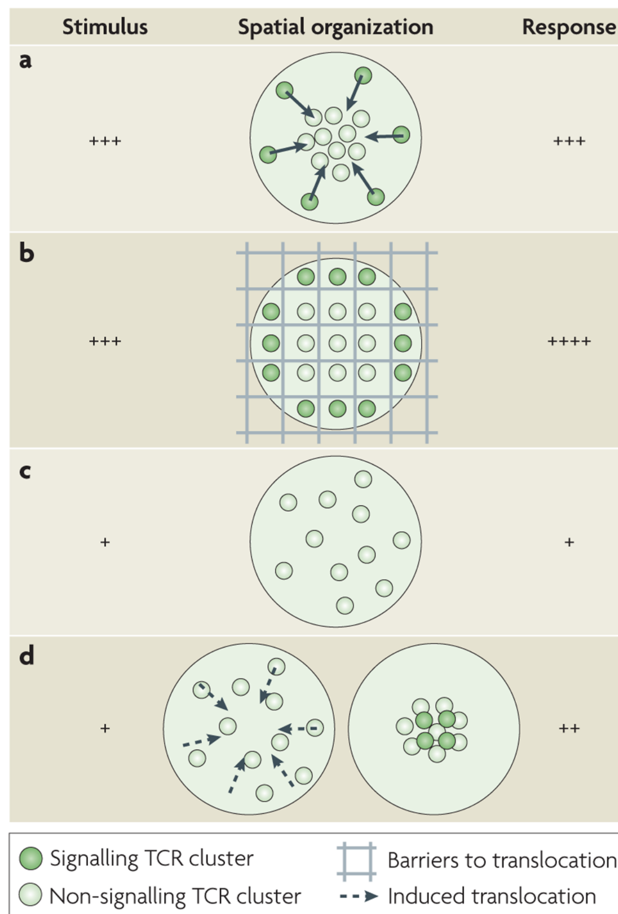

Figure 3. Spatial organization influences cell signalling in the immunological synapse

The spatial organization of signalling and non-signalling T cell receptors (TCRs) changes with different levels of cell stimulation and perturbing the spatial organization of TCRs by blocking their transport modifies the overall response of the cell. **a.** At high T cell activation (by strong or many agonists), signalling TCRs are located in the periphery and are transported to the centre, where they are downregulated. The T cell response is strong[5,8,22,23]. **b.** Physical barriers block the transport of TCRs to the centre at high T cell activation, and TCR clusters are constrained to the periphery, where they continue to signal. As a result, the T cell response is prolonged and higher than in part **a**[17]. **c.** At low T cell activation (by weak or few agonists), signalling TCRs are undetectable and non-signalling TCRs are in the periphery. The T cell response is weak. **d.** When TCR transport to the centre is artificially induced at low levels, TCR signalling can be detected in the centre and the response of the T cell is higher than in part **c**[16].

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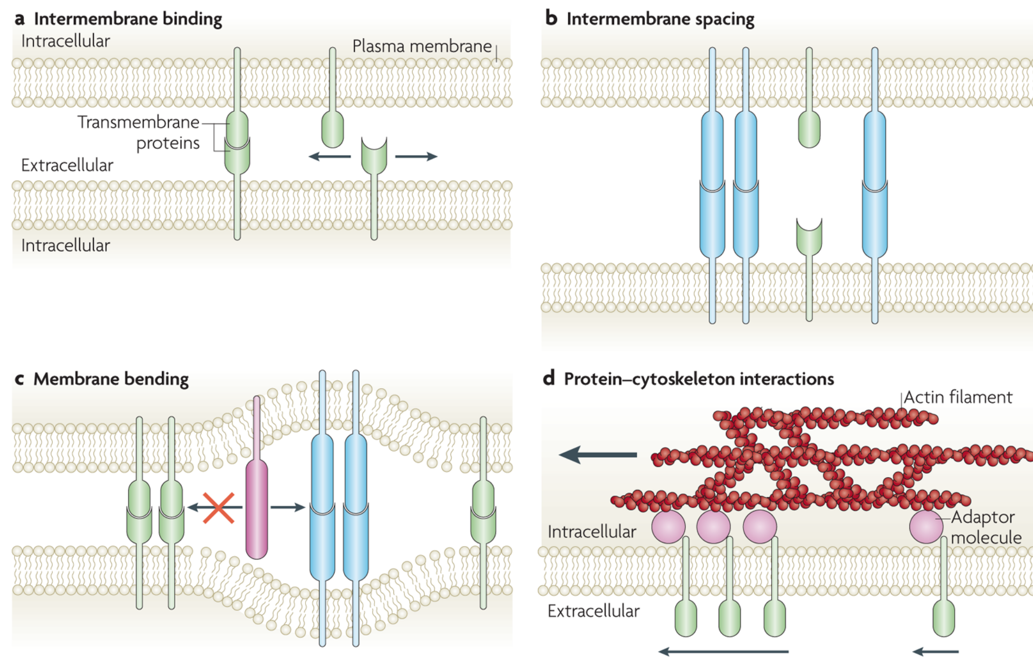

Figure 4. Cellular mechanisms controlling spatial organization in intercellular junctions

The interplay of the cell membrane and cytoskeleton at intercellular junctions yields short- and long-range spatial organizations of proteins, which transcend direct protein–protein interactions. **a.** The probability of a binding interaction between two membrane proteins is much higher when their orientation is pre-aligned by the membrane, compared to proteins in solution. Therefore, weak interactions are effectively strengthened. **b.** Binding across the intercellular space is governed by intermembrane spacing, which is determined by established protein-binding pairs. **c.** The binding of pairs that create different sizes of intermembrane spacing are segregated (blue versus green binding pairs) to minimize membrane bending. Additionally, large proteins (red) can enter wide intermembrane spacing regions, but are excluded from entering regions of tight intermembrane spacing. **d.** The moving actin cytoskeleton, through multiple weak associations with adaptor molecules, can selectively transport membrane molecules (green) and establish long-range protein organization. The force applied by actin can depend on protein cluster size[63].

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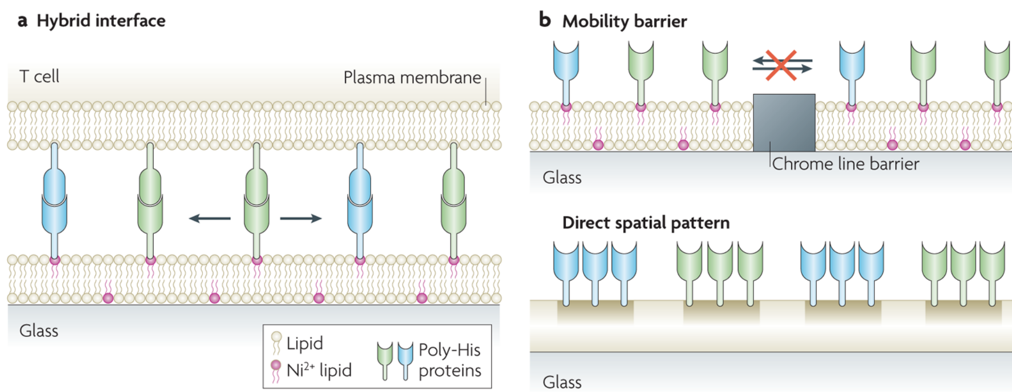

Figure 5. Experimental manipulation of spatial organization in intercellular junctions

a. Cell-to-cell interactions are reconstituted in hybrid interfaces between living cells and functionalized surfaces. The glass coverslip is functionalized with a supported lipid bilayer that is stably adhered to the surface, while exhibiting free diffusion (arrows) of its lipid components. Proteins are tethered to the fluid bilayer, for example by poly-histidine tags that bind Ni^{2+}-chelating lipids. Cells recognize their membrane-anchored ligands and can rearrange their organization. **b.** The surface can be patterned with subcellular features that alter the native spatial organization of membrane proteins. Chrome lines are barriers to lipid mobility and the transport of membrane-tethered proteins and any cell proteins engaged with them (top). They can restrict the reorganization of cell surface proteins initiated by the cell. Different proteins (green and blue) can be immobilized to the surface in any pre-set configuration, forcing cell ligands to also reorganize according to the presented arrangement (bottom).

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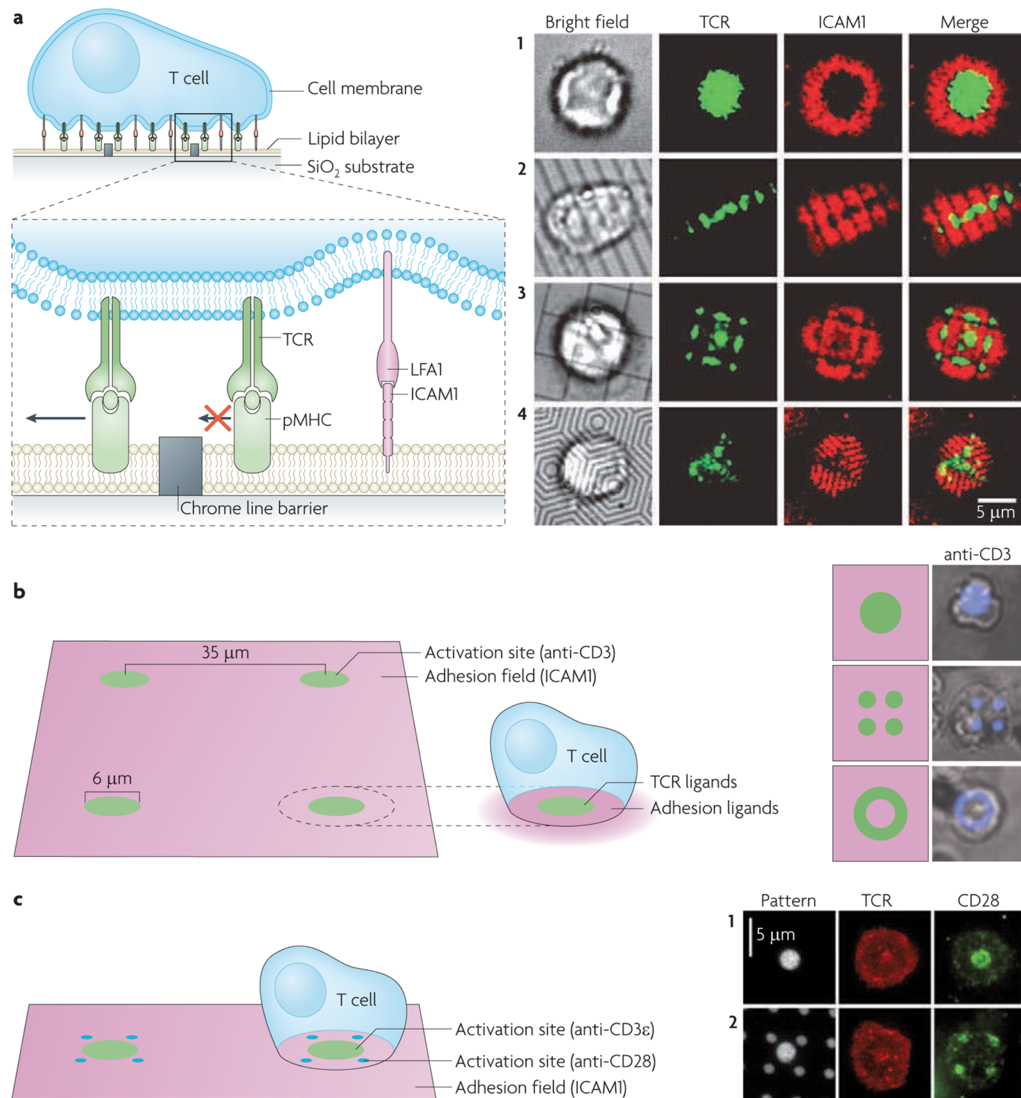

Figure 6. Immunological synapse spatial mutations

a. Physical barriers to protein transport on a fluid supported lipid bilayer. Thin chrome lines create barriers to the diffusion of bilayer-tethered proteins (such as major histocompatibility complexes displaying an antigenic peptide (pMHC)) and cellular proteins (such as T cell receptors (TCRs)) interacting with them (left). The spatial organization of the immunological synapse (TCRs (green) and intercellular adhesion molecule 1 (ICAM1; red)) without (1) and with (2–4) barriers of different geometries (right). **b.** Subcellular size protein patterns functionalized on a surface. A TCR-activating antibody (anti-CD3; green) and an adhesion molecule (ICAM1; purple) are patterned according to the immunological synapse pattern: anti-CD3 is central to the surrounding adhesion molecules (left). Anti-CD3, shown in schematics and cell overlays (in which anti-CD3 is blue) can be seen in a wild-type central zone pattern or in two variant patterns: multifocal and a peripheral ring (right). **c.** The subcellular pattern of a TCR-activating antibody (anti-CD3ε; green) and a co-stimulatory antibody (anti-CD28; blue) on an adhesion molecule (ICAM1; purple)-rich surface (left). Different patterns are tested for their effect on T cell activation: TCR and CD28 follow the pattern of anti-CD3 and anti-CD28 antibodies, respectively, which can be either co-localized or segregated. Images in part **a** are reproduced, with permission, from REF. 17 © (2005)

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