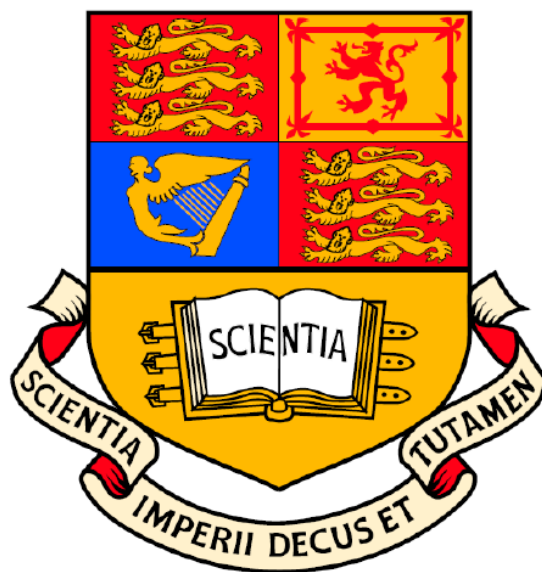




High-Resolution Imaging of Natural Killer Cell Immunological Synapses

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Abstract

The first observations of the immunological synapse have demonstrated that immune-cell signalling *in situ* does not simply depend on protein structures and signalling pathways but also on temporal and spatial coordinates. With the advent of new live-cell, three-dimensional fluorescence microscopy techniques our understanding of the relationship between the formation of the immunological synapse and the development of an immune response has been greatly improved.

Using artificial activating substrates as surrogate target cells or antigen presenting cells in conventional microscopes has so far been the state-of-the-art to obtain high-resolution images of immunological synapses. However, such artificial substrates may not fully recapitulate the complexity of intercellular interactions. Newly developed super-resolution techniques are very promising, but they remain inadequate for live-cell imaging. Technical improvements are therefore crucially needed to address these bottlenecks and improve our understanding of immune-cell signalling.

In this report we achieve high-speed high-resolution imaging of live intercellular immunological synapses by combining confocal microscopy with optical tweezers. We design, build and demonstrate the performance and flexibility of the instrument by imaging a variety of molecules at T cell and NK cell synapses.

NKG2D is an important receptor that allows NK cells to recognise and kill tumour cells. Due to the lack of suitable imaging technology, NKG2D signalling at the synapse remains unclear. We specifically use our new instrument to gain further understanding of NKG2D signalling, signal integration, and NKG2D-mediated cytotoxicity. For the first time at live intercellular NK-cell immunological synapses, we describe the formation and the dynamics of NKG2D microclusters. We show that these microclusters actively signal and that they coalesce around a secretory domain through which lytic secretions are delivered. Importantly, these results suggest that the physical distribution of NKG2D at the immunological synapse may play an important role in directing lytic-secretion delivery at the NK cell synapse.

Author declaration

All the work presented in this thesis is my own with the following exceptions:

- The TIRF microscope used in Chapter 3 was built with Dylan Owen.
- The T cells pcDNA3.1 constructs in Chapter 4 were generated by Marco Purbhoo.
- Some T cell samples in Chapter 4 were provided by Marco Purbhoo.
- Some of the 3D reconstructions of T cell synapses in Chapter 4 were performed by Marco Purbhoo.
- The VAV1-GFP and Grb2-mCherry constructs and the cells expressing these constructs in Chapter 5 were generated by Philipp Eissmann.
- The transfection of NKG2D-mCherry into Jurkat T cells used in Chapter 5 was performed by Philipp Eissmann.
- The generation of 721.221/MICA Cw4 in Chapter 5 was performed by Philipp Eissmann.
- The DAP10-GFP and DAP10(Y85F)-GFP constructs and the cells expressing these constructs in Chapter 5 were generated by Anne Chauveau.

Stephane Oddos

To my family

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List of acronyms

ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
aNKIS	Activating NK cell synapse
APC	Antigen presenting cell
APD	Avalanche photodiode
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
CCD	Charge-coupled device
c-SMAC/p-SMAC	Central/peripheral supramolecular activation cluster
CW	Continuous wave
DAP10	DNAX-activating protein of 10 kDa
DC	Dendritic cell
DIC	Differential interference contrast
eGFP	Enhanced green fluorescent protein
FACS	Fluorescence-Activated Cell Sorting
FasL	Fas ligand
FCS	Fluorescence correlation microscopy
FCS	Foetal calf serum
FP	Fluorescent protein
FPALM	Fluorescence photo-activated localization microscopy
FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence resonance energy transfer
GADS	GRB2-related adapter protein
GFP	Green fluorescent protein
GPI	Glycosyl phosphatidylinositol
Grb2	Growth factor receptor-bound protein 2
HCMV	Human Cytomegalovirus
HLA	Human leucocyte antigen
hsPALM	High-speed photo-activated localization microscopy
ICAM1	Inter-Cellular Adhesion Molecule 1
IFN- γ	Interferon- γ
iPALM	interferometry photo-activated localization microscopy
IR	Infrared
ITAM	Immunoreceptor tyrosine-based activation motifs
ITIM	Immunoreceptor tyrosine-based inhibitory motifs
KIR	Killer cell immunoglobulin-like receptor
LAT	Linker of activated T cells

Lck	Leukocyte-specific protein tyrosine kinase
LFA-1	Lymphocyte function-associated antigen-1
mAb	Monoclonal antibody
MCS	Multiple cloning site
MHC	Major histocompatibility complex APC
MICA/MICB	MHC class I polypeptide-related sequence A/B
MTOC	Microtubule organizing centre
NA	Numerical aperture
NADH	Nicotinamide adenine dinucleotide
NCR	Natural cytotoxicity receptor
NK	Natural Killer
PALM	Photo-activated localization microscopy
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinases
PKC	Protein kinase C
PLC	Phospholipase C
pMHC	Peptide major histocompatibility complex APC
PMT	Photomultiplier tube
psCFP	Photoswitchable cyan fluorescent protein
PSF	Point spread function
Rab	Ras-related protein
RNA	Ribonucleic acid
SEE	Staphylococcal enterotoxin E
SH2	Src homology 2
SHP-1/SHP-2	SRC homology 2-containing protein tyrosine phosphatase-1/2
SLM	Spatial light modulator
SLP-76	SH2 domain containing leukocyte protein of 76 kDa
STED	Stimulated emission depletion
STORM	Stochastic optical reconstruction microscopy
Syc	Spleen tyrosine kinase
TCR	T cell receptor
TIRF	Total internal reflection
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
ULBP	UL16 binding protein
UV	Ultra violet
WF	Wide field
YFP	Yellow fluorescent protein
ZAP70	Zeta-chain-associated protein kinase of 70 kDa

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Chapter I: Thesis aims and overview

The aim of the multidisciplinary project described in this report was to develop a high-speed high-resolution imaging technique for the study of the immunological synapse. This was particularly undertaken to improve our understanding of the molecular mechanisms that allow certain cells of the immune system, Natural Killer (NK) cells, to recognise and kill virally infected cells and tumour cells.

Chapter II is an introduction to NK cells and their role in immunity. This chapter introduces the concept of immunological synapse which is formed by immune cells with other cells. Through the notable examples of immunological synapses formed by T cells and NK cells, this chapter discusses the importance of the formation of immunological synapses in the build-up of an immune response.

Chapter III is a critical review of imaging techniques according to their spatio-temporal resolution and range of applications. After an introduction on fluorescence and fluorescence imaging, current and developing microscopy techniques are discussed, with an emphasis on their suitability to imaging immunological synapses formed with live cells.

Chapter IV is the description of the development of a new imaging technique combining cell micromanipulation using optical tweezers and confocal microscopy to image immunological synapses between two live cells. This chapter discusses the merits of this new technique in terms of spatio-temporal resolution, range of applications and physiological relevance by imaging the distribution of different signalling proteins at immunological synapses in a variety of cells and conditions.

Chapter V introduces NKG2D - an important receptor expressed by NK cells - which recognises proteins expressed by tumours. This chapter then describes how the newly developed technique was used to investigate NKG2D signalling at the intercellular activating NK cell immunological synapse.

Chapter VI is a summary and a discussion of the main results presented in the report.

Chapter II: The Natural Killer cell immunological synapse

Natural Killer cells

Natural Killer (NK) cells were discovered when researchers isolated lymphocytes unexpectedly exhibiting cytotoxic activity against tumour cells in the absence of prior immunisation [1, 2]. NK cells are large granular lymphocytes accounting for 5-10% of peripheral blood lymphocytes which can both influence innate and adaptive immunity through their cytotoxic effector and cytokine/chemokine secretor functions [3]. However, a recent study also shows that NK cells can be trained - becoming more protective against infection after initial exposure - which was previously only thought to be a feature of cells of the adaptive immune system [4].

NK cells actively participate in the innate immune response by secreting interferon such as IFN- γ which can kill infected cells and has inhibitory effects on the proliferation of tumour cells [5], and by interacting and activating macrophages [6] and dendritic cells [7]. NK cells can also secrete a range of other cytokines [8] and chemokines that can help recruit other immune cells to build up an immune response when needed [9].

NK cytotoxicity is mainly mediated by the release of large granules containing perforin and granzymes at the contact with their target cell [10]. NK cells can also engage target cell death in a perforin-independent manner by releasing members of the Tumour Necrosis Factor (TNF) family, such as TNF- α , TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) [11, 12].

NK cells are potent killers of various pathogens such as virally infected cells, microbial pathogens and tumours, and discriminate healthy cells from transformed and virally infected cells according to 'missing-self' recognition. Missing-self recognition refers to the ability of NK cells to detect a downregulation, or loss, of MHC class I expression at the surface of a target cell [13]. NK cells possess a large number of germ-line encoded receptors that allow them to perform this task. These receptors are termed either inhibitory or activating receptors. Inhibitory receptors can recognise MHC class I on the surface of target cells [14] while activating receptors can recognise a variety of ligands normally expressed by transformed or virally infected cells [3, 15, 16]. In general, both types of ligands are expressed by target cells. NK cell activity is therefore tightly regulated by a complex integration of activating and inhibitory signals that allow them to decide to kill or to spare a given target cell [17]. How these receptors interact with each other at the molecular level to lead to the proper immune outcome is still largely unknown.

Inhibitory receptors

Inhibitory receptors include human killer immunoglobulin-like receptors (KIR) [18, 19], leucocyte immunoglobulin-like receptors (LIR, ILT and LILR) [20, 21], and CD94/NKG2 receptors [22, 23]. While KIR and LIR are immunoglobulin-like type-I transmembrane receptors, CD94/NKG2 receptors are lectin-like type II transmembrane proteins. Inhibitory KIR can contain either two or three Immunoglobulin-like extracellular domains (KIR2D and KIR3D respectively) and long cytoplasmic domains (e.g. KIR2DL). The expression of an array of inhibitory receptors allows NK cells to recognise many types of MHC class I molecules (Table 1).

Receptor	Signalling	Ligands
ILT2/LIR1	ITIM	HLA-A, B, C, E, F, G
KIR3DL3	ITIM	?
KIR2DL3	ITIM	HLA-C
KIR2DL2	ITIM	HLA-C
KIR2DL1	ITIM	HLA-C
KIR3DL1	ITIM	HLA-B
CD94/NKG2A	ITIM	HLA-E

Table 1: Major inhibitory receptors with corresponding signalling pathway and ligands [24].

Activating receptors

NK cells possess a very wide array of activating receptors allowing them to recognise a variety of targets. The multi subunit immune recognition complex CD16 (FcγRIIIA) receptor allows NK cells to lyse antibody-coated opsonised target cells, a mechanism known as antibody-dependent cell-mediated cytotoxicity (ADCC) [25]. The lectin-like type II NKG2D receptor recognises self-proteins that are upregulated when cells are generally transformed, stressed or virally-infected such as MHC class I chain-related A chain (MICA), MHC class I chain-related B chain (MICB) [26] and the UL-16 binding proteins (ULBPs) [27]. Importantly, NKG2D signalling can trigger cell lysis by overriding inhibitory signalling [27], a mechanism that is involved in lysis of tumour and virally infected cells [28]. Other activating receptors expressed by NK cells include the relatively recently discovered 'natural cytotoxicity receptors' (NCRs) NKp30 [29], NKp44 [30] and NKp46 [31]. Ligands for NCRs remain elusive. While a ligand expressed on tumour cells - B7-H6 - has been found for NKp30 [32], physiological cellular ligands still have yet to be identified for NKp44 and NKp46. Interestingly, it has

been shown that NKp44 and NKp46 can bind the hemagglutinins of influenza virus in vitro [33, 34] while NKp30 does not.

Inhibitory signalling pathway

Downstream signalling of most inhibitory receptors is mediated by conserved sequences within their cytoplasmic tail or associated adaptor protein. Such sequences consist of one or more immunoreceptor tyrosine-based inhibitory motif (ITIMs). An ITIM motif contains the consensus sequence V/I/LxYxxL/V where x is any amino-acid. Upon receptor-ligand ligation, the tyrosines within ITIMs get phosphorylated by Src kinases [35] which then recruits protein tyrosine phosphatases such as Src homology 2 containing protein phosphatase-1 or -2 (SHP-1 or SHP-2) [14]. By immunoprecipitating phosphorylated proteins or protein complexes in presence and absence of inhibition, signalling molecules such as ZAP70 [35], PLC- γ [36], SLP-76 [37] and VAV1 [38] have been identified as targets of NK cell inhibition. Interestingly, inhibition is not only obtained through dephosphorylation of downstream molecules. A recent study showed KIR activation leads to increased phosphorylation of the Crk adaptor [39]. However, how inhibition of activating signals is orchestrated at the molecular level of the synapse still has to be elucidated

Activating signalling pathway

Most NK cell activating receptors - such as NCRs, CD16 (mediating ADCC) and CD94/NKG2 - share a common signalling pathway with T and B cells. These receptors signal through an immunoreceptor tyrosine-based activation motif (ITAM), consisting of the consensus sequence YxxL(x)₆₋₈YxxL, where x is any amino acid [40]. Such receptors lack cytoplasmic signalling domains and instead couple with

adaptors proteins containing the ITAM [41]. Human CD16, NKp30 and NKp46 couple with homodimers or heterodimers of CD3 ζ and Fc ϵ R γ , while NKp44 and CD94/NKG2 couple with a DAP12 homodimer [42-44].

In humans NKG2D is expressed on the surface of NK cells by coupling to a DAP10 transmembrane adaptor [45]. The final complex is a hexameric structure formed of two NKG2D molecules each coupled with two DAP10 adaptors [46]. Unlike other signalling adaptor proteins, DAP10 does not possess a classical ITAM motif but instead features a stimulatory YIMN motif [45]. Like ITAM receptors, DAP10 gets phosphorylated by a Src family kinase (e.g. Lck) upon ligation of NKG2D with one of its ligands. Unlike ITAM receptors, however, DAP10 activation does not recruit, nor phosphorylate ZAP70 or LAT proteins [47]. Instead DAP10 signals through two distinct signalling pathways, the DAP10-PI3K pathway and the DAP10-Grb2 pathway, with the latter signalling through VAV1, PLC- γ 2 and SLP-76. Both pathways are required for NK cell effector functions [48] .

NKG2D activation has different outcomes depending on the stimulation state of NK cells. NKG2D is only a costimulatory receptor for freshly isolated NK cells. In this context, NKG2D activation alone is not sufficient for cytotoxicity, proliferation and TNF- α /IFN- γ production. However, NKG2D activation enhances these effector functions when other activating receptors are triggered [49]. With activated NK cells (in the presence of IL-2 or IL-15 for example) NKG2D activation alone is sufficient to trigger NK cell effector functions [50].

The immunological synapse

Progress in fluorescence microscopy and fluorescence labelling of molecular components in cells have initiated a revolution. In particular, the discovery of the formation of an immunological synapse has demonstrated that immune cell signalling *in situ* does not only depend on protein structures, but also on temporal and spatial coordinates. In this report the immunological synapse is therefore defined as the interface between two contacting cells - at least one of them being an immune cell - where signalling events resulting from the cellular contact depend on temporal and/or spatial coordinates. What follows is a review of the studies that show that the formation of an immunological synapse is important for T cells and NK cells to achieve their effector functions.

Structure of immunological synapses

The T cell immunological synapse

T cells are part of the adaptive immune system. Antigen (Ag)-specific immune responses are elicited when T cells that express a specific T cell receptor (TCR) interact with Ag-presenting cells (APCs) that bear a cognate Ag peptide-MHC (pMHC). In the case of CD4+ or CD8+ T cells, this interaction leads to T cell activation or target cell lysis respectively, with the formation of an immunological synapse playing a central role for such T cell functions.

CD4+ (helper) T cell immunological synapses

The immunological synapse was originally found at the interface between CD4+ helper T cells and an APC (B cell) [51], or planar bilayers mimicking an APC [52]. In the first of these two studies, imaging of fixed immunolabelled conjugates showed an accumulation of signalling molecules, such as TCR

and PKC- θ , in a central cluster surrounded by a ring of the adhesion molecules LFA-1 and Talin. This organisation was mirrored by respective ligands on the APC, with pMHC at the centre segregated from ICAM-1 at the periphery. These micrometre-scaled domains were termed supramolecular activation clusters 'SMACS', with c-SMAC referring to the central cluster, and p-SMAC to the peripheral cluster.

The second study used live T cells interacting with planar bilayers in which fluorescent labelled pMHC and ICAM-1 were inserted and imaged. Compared to the previous study, it was then possible to image the immunological synapse formation dynamically at high-resolution. This revealed that ICAM-1 initially accumulated in a central area surrounded by a ring of TCRs within 30 s. However this structure dramatically inverted after 5 min - consistently to what had been observed between cell-cell conjugates - and was sustained as such for several hours.

T cell/APC interactions require prolonged signalling to achieve T cell priming [53]. First studies about the immunological synapse therefore originally supported the idea that c-SMACs could provide enhanced signalling by concentrating receptors and ligands. However, later studies - using more advanced low-background microscopy techniques - have suggested that T cell signalling could be initiated not through micron-scaled accumulation as originally thought, but rather in macromolecular assemblies termed microclusters [54-59]. Essential membrane-proximal signalling molecules such as TCRs, CD3, ZAP70 and SLP-76 were shown to accumulate in microclusters containing hundreds of each of these molecules upon T cell contact with the activating substrate. Importantly, phosphotyrosine-specific immunostainings showed these microclusters could signal. After maximum spreading, microclusters translocated towards the centre of the synapse. Crucially, kinases and adaptors dissociated from TCRs as they moved towards the centre such that the c-SMAC contained TCR-only microclusters therefore terminating signalling. In agreement with this loss of downstream signalling molecules, no phosphotyrosine could be detected in centrally-accumulated microclusters.

The formation and the importance of c-SMACs as described using these highly simplified models remain highly controversial and seem to very much depend on the cellular context. A study imaging TCR at the synapse formed by live T cells interacting with different APCs illustrated that the morphology of the synapse depends on whether the APC is a B cell or an DC, with the absence of c-SMAC formation in the latter case [60]. Through the imaging of thymocytes interacting with activating lipid bilayers [61] or stromal cells [62] it was also shown that no c-SMAC was formed during thymocyte activation and negative selection. Furthermore, imaging of cells that could not form c-SMACs was combined with computer simulations to demonstrate that the c-SMAC was the site for enhanced receptor triggering and degradation [63]. However this result was counterbalanced by a recent study that demonstrated sustained signalling transduction could happen in the c-SMAC in presence of weak affinity TCR-pMHC complexes [64].

The sometimes contradictory results of these studies show the difficulty of delineating a generalised morphology and role for the different compartments of the CD4+ T cell immunological synapse. This is mainly due to the use of models that differs between studies (for instance the use of cell-cell conjugates or activating planar bilayers, or the choice of APC) as well as experimental limitations (most studies are carried out *in vitro*). It is therefore clear that the immunological synapse is a complex entity that is highly sensitive to its environment.

CD8+ (cytotoxic) T cell immunological synapses

Immunological synapses formed by cytotoxic T cells have been shown to be similar to that formed by CD4+ T cells, with TCRs, PKC- θ and Lck accumulating at the centre, surrounded by a ring of LFA-1 and Talin [65, 66]. In addition to this it has been shown that c-SMACs formed by CD8+ T cells were composed of an extra domain for the secretion of cytolytic molecules adjacent to, and distinct from, signalling molecules [66]. However, by correlating T cell cytotoxicity to quantitative analysis of fluorescently labelled pMHC complexes, it was demonstrated that CD8+ T cells can elicit killing in the

absence of c-SMAC formation [67]. The role of the formation of the immunological synapse for T cell cytotoxicity thus remains unclear.

The NK cell immunological synapse

The immunological synapse between NK cells and target cells is the site of complex protein interactions that are important for determining the outcome of the NK cell mediated immune response. When the target cell is healthy (i.e. MHC class I positive) inhibitory signals dominate with the interaction giving rise to the so-called inhibitory synapse, sparing the target cell. In the opposite case the interaction leads to the lysis of the target cell and is termed an activating immunological synapse [68]. Here we will see that different immunological synapses form depending on the nature of interaction between NK and target cells.

The activating NK cell immunological synapse

The formation of the activating NK immunological synapse happens in sequential steps that have distinct cytoskeletal requirements [68-71]. The first step consists of initial engagement of receptors and adhesion molecules e.g. LFA-1 and Mac-1, with rapid segregation of adhesion molecules at the p-SMAC and receptors at the c-SMAC. A variety of downstream signalling molecules such as Src, VAV1, LAT, SLP-76, ZAP70 and PLC are then recruited, triggering actin polymerisation and accumulation at the p-SMAC [68-70, 72]. The resulting tighter conjugation produces further and prolonged receptor accumulation and improved synergy at the c-SMAC, which in turn induces signal amplification [69]. Importantly, this major actin rearrangement is absent during inhibitory interactions, with disruption of this mechanism impairing NK cell cytotoxicity [73]. Sustained receptor signalling then initiates signalling pathways which lead to the polarisation of the microtubule organising centre (MTOC) and subsequent recruitment of perforin and granzyme-containing granules at the immunological synapse [74-76]. The protein machinery involved in lytic

granule delivery and exocytosis at the synapse, in particular the role of the cytoskeletal proteins, is still ill-defined. Similarly, the molecular mechanisms driving the disassembly of the synapse after target cell death has yet to be elucidated [68]. In addition, most of these discoveries have been made using fluorescence immunostaining of fixed cells at different time points, and with poor spatial resolution. It is important to note that progress has been made by a recent study using high-resolution live-cell imaging of NK cells interacting with an activating lipid bilayer to show that adhesion molecules are essential to direct secretions at the synapse [77].

The inhibitory NK cell immunological synapse

The role of the inhibitory immunological synapse is to prevent unwanted killing of healthy cells. As with the activating synapse, the initial stage of the inhibitory synapse involves adhesion molecules as well as inhibitory receptors being recruited to the interface [78]. Importantly, activating receptors can also initially accumulate at the inhibitory synapse [79]. In this case however, signalling through inhibitory receptors such as NKG2A and KIR results in the activation of protein tyrosine phosphatases SHP-1 and SHP-2 and adaptor Crk that impairs actin rearrangements [39, 72, 80-83]. Further signalling through increased clustering of activating receptors is therefore prevented, resulting in the target being spared.

Importantly, the formation of the inhibitory immunological synapse remains independent of cytoskeleton rearrangements, lipid raft formation or ATP, and is driven by thermodynamic processes [78, 82, 84-87]. Ligand binding alone is sufficient to induce clustering and signalling of inhibitory receptors [86]. However, the inhibitory synapse is a very dynamic structure: with time, inhibitory receptors dispersed throughout the synapse form distinct clusters while LFA-1 accumulates at the centre and segregates from receptors [78, 85, 88]. KIR/MHC class I patterns change overtime and correlate with the density of MHC class I and inhibitory receptors [85, 88]. These patterns reflect the functional consequences of receptor clustering as a certain level of MHC class I expression is necessary for inhibition of cytotoxicity. Interestingly though, only a small fraction of these receptors

get phosphorylated in small microdomains suggesting that actual inhibition of activation signals could be targeted to increase its efficiency [89].

Functions of the immunological synapse

The importance of synapse formation as an essential mechanism orchestrating intermolecular interactions is now widely accepted. Twelve years after the first observation of the immunological synapse however, its exact functions remain elusive. Here we present a few of the functions for which experimental support is strong.

Stop signal

It has been formulated that the formation of a symmetrical synapse would cancel out forces produced by the centripetal actin flow and would therefore arrest T cell mobility on its APC [90]. This supports the idea that the formation of a synapse would provide a stop signal allowing proper T cell-APC signal integration and therefore optimal T cell activation.

Similar observations have been made about the NK cell synapse; it has been shown that NK cells in presence of an activating substrate form symmetrical synapses and show limited mobility, while NK cells in presence of an inhibitory substrate form asymmetrical synapses and remain mobile [91]. This crucially optimises NK cell effector functions, as it allows NK cells to integrate signals from a maximum number of potential targets.

Directing secretion

One of the first and most widely recognised roles for the synapse is directing secretion. This function is crucial for immune effector response, as it allows CD8⁺ T cells or NK cells to effectively concentrate secretions to the conjugated cells while keeping neighbouring cells unaffected. Using live cell imaging, directional secretion towards the synapse of lytic granules has been observed with T cells [66, 92, 93] and NK cells [77] and directed secretions of some cytokines has been shown with T cells [92].

Balancing signalling

It has been shown that cells that cannot form a proper immunological synapse are hypersensitive to Ag, resulting in increased proliferation and cytokine secretion [63]. Therefore, proper orchestration of intermolecular interactions by the immunological synapse appears to be essential in balancing signalling to prevent overstimulation of T cells.

Depending on the strength of the stimulus, it has been shown that TCRs get phosphorylated preferentially either at the periphery (strong stimulation) or at the centre (weak stimulation) of the synapse [64]. The strength of the cellular response is correlated to the spatiotemporal shape of the synapse, with interference of this shape resulting in altered cellular responses [94]. Another striking example of the importance of the synaptic environment for signalling molecules is the fact that the kinetic and affinity properties of the pMHC-TCR complex dramatically differ between measurements *in vitro* (using surface plasmon resonance) and measurements *in situ* (using quantitative fluorescence imaging at the synapse formed between a T cell and a activating bilayer) [95].

Molecular mechanisms for the formation of the immunological synapse

Here, we highlight the main known molecular mechanisms that drive the formation of the immunological synapse. It is likely that the formation of a synapse is the result of a complex combination of many of these mechanisms.

Membrane microdomains

Lipid rafts are membrane microdomains formed by phase separation of cholesterol-rich domains from the rest of the membrane that selectively associate with proteins through non-covalent interactions. Although their nature is still very much debated, such membrane microdomains are likely to be critical in driving protein clustering and subsequent signalling [96]. A recent study suggests that protein islands pre-exist in the cell membrane prior to signalling. Using three concurrent techniques (high-speed PALM (hsPALM), electron microscopy, and Fluorescence Correlation Microscopy (FCS)), the authors show that TCRs and LAT are organised in such islands containing 7-30 molecules in quiescent cells, and that they coalesce upon signalling [97]. Such pre-existing islands are thus likely to be important for the formation of larger protein clusters and subsequent signalling. However the exact origin of these protein islands and how they relate to lipid rafts remain unknown.

Protein sorting by size

Cellular membranes are flexible, but high bending is not energetically favourable. Therefore, at intercellular junctions, a given protein binding pair can prevent the binding of another pair if the intercellular space required by each of them is too different. In the fluid membrane, the intermembrane space can thus lead to size selection and spatial segregation as shown by *in silico* models [98]. This mechanism has been shown to be critical for TCR signalling as varying the size of pMHC controls both spatial segregation and signalling of TCRs, while chemical pMHC-TCR binding is not directly affected [99].

Actin cytoskeleton

TCR signalling activates many proteins regulating actin polymerisation [100]. At the synapse, F-actin is densely present in the periphery, while it is depleted at the centre, and physically transports proteins such as TCRs and LFA-1 [101].

While TCRs are driven towards the c-SMAC, LFA-1 remains at the periphery. The sorting mechanism that could explain this difference remains elusive. A recent study manipulating the clustering state of LFA-1 has shown that bivalent or tetravalent clustering of LFA-1 obtained by antibody cross-linking would bring LFA-1 to the centre of the synapse where TCRs are normally segregated [102], suggesting that actin-driven protein sorting at the immunological synapse depends at least on their clustering state.

Several studies have introduced physical barriers in the activating substrate so TCRs cannot diffuse freely [103]. Whilst the centripetal actin flow is not affected by the presence of such barriers, clusters of TCRs that encounter a barrier do not stop, but instead continue moving tangentially to the barrier. This is evidence of dynamic TCR-F-actin interactions analogue to a 'molecular clutch'.

Conclusion

Since first observations, the immunological synapse has been extensively studied and our knowledge of the spatio-temporal molecular mechanisms leading to the activation of the effector functions of certain important lymphocytes has greatly improved.

The system that has proved most useful so far to generate informative images of immunological synapses has been the combination of activating lipid bilayers and TIRF microscopy. Critically, this technology has revealed the presence of protein islands on the surface of quiescent cells and the formation of larger protein microclusters upon receptor activation.

Despite undeniable merits, such activating substrates do not recapitulate the complexity of an antigen presenting cell or target cell and some results cannot be easily reconciled with imaging studies that use real cell-cell conjugates. Although it would provide a much more physiologically relevant model to study immune cell signalling, imaging immunological synapses at high-speed and high-resolution between two live immune cells has been difficult due to the absence of adequate imaging technology.

Chapter III: Three- dimensional fluorescence microscopy

Fluorescence imaging

Fluorescence imaging has revolutionised biology with its ability to produce movies and images of live cells with high molecular specificity, low background and minimal invasiveness. Here we review what fluorescence is and how it can be described, as well as the photophysical properties of fluorophores that are important for fluorescence imaging. We then review the main imaging techniques that are used in conjunction with fluorescence imaging to achieve three-dimensional imaging of cells. Finally we discuss the most promising super-resolution techniques under development.

The photophysical process of fluorescence

Fluorescence is one of the phenomena that involve the emission of a photon by matter upon absorption of a higher-energy photon. It is therefore a photophysical process, which can be conveniently described using the electronic energy levels of the molecule of interest. In that model, fluorescence can be defined as the radiative (i.e. emitting a photon) relaxation of an electron from a higher electronic state to a lower one with unchanged angular momentum. If there is a change in angular momentum between these two electronic states, the phenomenon is termed phosphorescence. Fluorescence and phosphorescence can be grouped together under the term luminescence [104]. Jablonski diagrams schematically represent some of the electronic states, energy levels and electronic transitions of a given fluorophore that are necessary to describe such photophysical processes (Figure 1).

In resting molecules, electrons populate the ground state S_0 . Typically, at equilibrium, electrons populate the lowest vibrational energy level of a given electronic state. When electrons in S_0 are excited with an energy that is at least equal to the relevant energy gap, they are promoted to the excited states S_1 or S_2 . Excitation can occur in different ways: the simplest way is the absorption of a photon whose energy is greater than or equal to the energy gap between S_0 and S_1 or S_0 and S_2 . Multiple photons of lower energy that arrive at the molecule in a sufficiently short time (roughly the time it takes for photons to travel the diameter of the molecule) can also be simultaneously absorbed as long as their total combined energy fulfils the same criteria. This is termed multi-photon absorption, and possesses a specific advantage: two-photon excitation is used for microscopy *in vivo* as scattering and absorption of longer wavelengths in deep tissues is normally reduced [105]. Once in vibrational states of S_1 or S_2 , electrons will thermalise rapidly to reach an equilibrium state in which most of the population is in the lowest energy level of S_1 , either by vibrational relaxation (going down the vibrational levels of a given state) or internal conversion (going from S_2 to S_1 in a non-radiative fashion). The corresponding loss of energy is transferred to the solvent as heat.

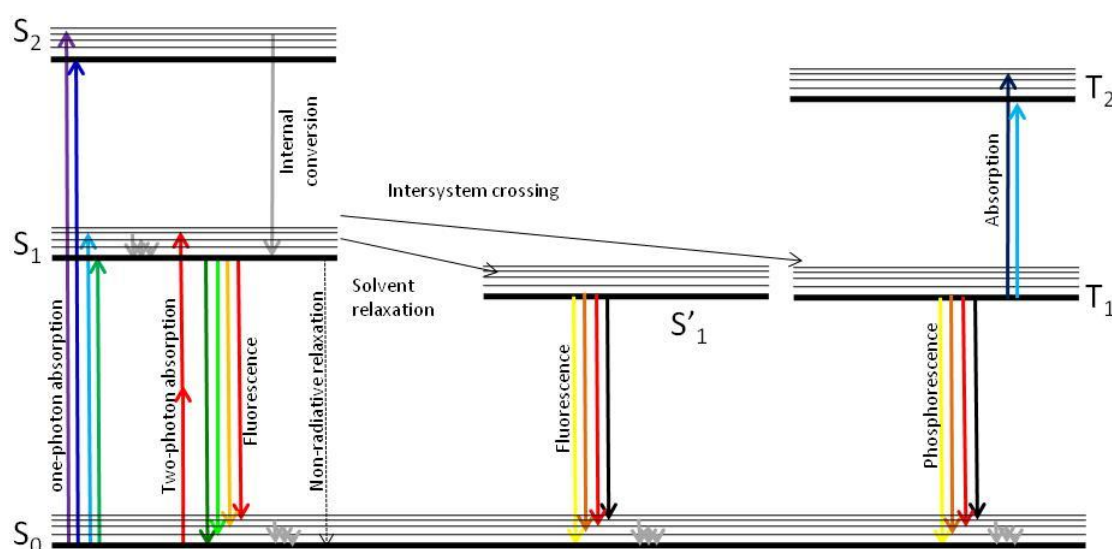


Figure 1: Jablonski diagram representing some of the electronic transitions that can be used to describe photophysical processes such as fluorescence.

Fluorescence can then occur through radiative relaxation of excited electrons from S_1 to S_0 . Alternative routes such as intersystem crossing (transition from S_1 and T_1 with change of electronic spin) and or non-radiative decay (transition from S_1 to S_0 without the emission of a photon) can also occur. A Jablonski diagram (Figure 1) shows a few of the transitions that a given electron can undergo after it reaches an excited state. The relative probability of each of these competitive events to occur is reflected in their corresponding transition rates (Table 2).

Process	Transition	Timescales
absorption	$h\nu + S_0 \rightarrow S_1^*$	10^{-15} s
Internal conversion	$X_Y^* \rightarrow X_Y + \Delta E$, where $X = T$ or S and $Y = 0, 1$ or 2	10^{-13} s
Fluorescence	$S_1 \rightarrow S_0^* + h\nu$	10^{-9} s
Non-radiative relaxation	$S_1 \rightarrow S_0 + \Delta E$	10^{-9} s
Intersystem-crossing	$S_1 \rightarrow T_1^* + \Delta E$	10^{-7} s
Phosphorescence	$T_1 \rightarrow S_0^* + h\nu$	10^{-6} s

Table 2: Time scales associated to various electronic transitions [106]. ΔE represents non-radiative energy, * represents vibrational energy.

Properties of fluorescence emission

Fluorescence is a relatively complex photophysical process and some of its properties can therefore alter the interpretation of a fluorescence image. Here we explain how fluorescence intensity, absorption and emission spectra, lifetime, and polarisation anisotropy can affect imaging.

Intensity

In the linear regime, fluorescence intensity is proportional to the fluorophore concentration, illumination power, and fluorophore quantum efficiency. The quantum efficiency of a fluorophore is

given by the ratio of the number of emitted photons per unit time to the number of absorbed photons per unit time. In a simple two-level system (S_0 and S_1) in the steady-state regime, the quantum efficiency (η) is given by the ratio of the radiative decay rate (k_r) to the total decay rate given by the sum of radiative and non-radiative decay rates (k_r and k_{nr}) (Equation 1).

$$\eta = \frac{N_e}{N_a} = \frac{k_r}{k_r + k_{nr}}$$

Equation 1: Quantum efficiency of a fluorophore as a function of decay rates in a two-level electronic system. η is the quantum efficiency, N_a is the number of absorbed photons/s, N_e is the number of emitted photons/s, k_r is the radiative decay rate, k_{nr} is the non-radiative decay rate.

Local excitation power and fluorophore quantum efficiency can be influenced in some cases by local factors such as sample density, molecular environment [107] or dipole orientation [104]. When those can be assumed to be uniform across the field of view, fluorescence intensity can be a direct representation of fluorophore concentration (at least in arbitrary units).

Under high-power illumination, fluorescence emission reaches a plateau: a phenomenon termed saturation. This happens when most of the molecules are in the excited state due to a surplus of excitation photons and this limits the amount of light that can be extracted from a given sample. It is also associated with increased photobleaching which will be discussed later on in this section.

Wavelength

The wavelength of an emitted photon is directly proportional to the energy gap between the initial and final energy levels (Equation 2).

$$\lambda = \frac{hc}{\Delta E}$$

Equation 2: Emission/absorption wavelength associated to electronic transitions as a function of the energy gap between these transitions. λ is the wavelength of the emitted photon, h is the Planck constant, c is the light velocity in the void and ΔE is the absolute value of the energy gap.

The fluorescence emission spectrum of a molecule is therefore directly related to its electronic structure. In principle, discrete energy levels should give rise to sharp peaks in absorption and emission spectrum. In condensed liquid or solid matter however, these spectra are smoothly broadened [104].

The wavelength that is required for a photon to excite an electron (photon absorption) follows the same conditions as photon emission (Equation 2). Because of the loss of energy due to the relaxation of vibrational states between photon absorption and fluorescence emission, the energy of the emitted photon is less than that of the absorbed one, thus producing a red-shift between the absorption and the emission spectra. This shift is known as the Stokes shift [108]. The presence of a Stokes shift is crucial for fluorescence imaging as it allows excitation and emission photons to be spectrally separated, typically using dichroic beam-splitters.

Lifetime

As shown by Jablonski diagrams and more specifically by the decay rates of the different energy levels, fluorescence emission is not an instantaneous process. The fluorescence lifetime (τ) is the average time an excited electron remains in the excited state before returning to the ground state. The fluorescence lifetime can therefore be expressed as a function of decay rates (Equation 3).

$$\tau = \frac{1}{k_r + k_{nr}}$$

Equation 3: Fluorescence lifetime as a function of decay rates. τ is the fluorescence lifetime of fluorophore in a two-level electronic system, k_r is the radiative decay rate, k_{nr} is the non-radiative decay rate.

A set of fluorophores therefore behaves as a modulation filter with a specific transfer function in the frequency domain. Such fluorophores produce a broadened pulse in response to a short pulse of light which is the interpretation of the same phenomenon in the time domain (Equation 4).

$$I(t) = I(0)e^{-t/\tau}$$

Equation 4: Fluorophore pulse response in a two-level electronic system. I is the fluorescence intensity from a large population of a given fluorophore, t is time, τ is the fluorescence lifetime.

Fluorescence lifetime is specific to a given fluorophore species, but is also affected by a variety of environmental factors, such as temperature or energy transfer and can therefore be used to map these across the sample [109]. The fluorescence lifetime impacts saturation excitation intensity, since molecules do not decay instantaneously to the ground state.

Polarisation

A fluorescent molecule can be considered as an electronic dipole that absorbs and emits light along preferred polarisation directions, depending on the orientation of the dipole. The degree of ‘polarisation’ of fluorescence is called fluorescence anisotropy and depends on dipole motion between absorption and subsequent emission of radiation. Fluorescence anisotropy can give

information of molecular orientation and rotational mobility [106]. In some cases where laser polarisation is fixed and dipole orientations are stationary but show non-uniform orientations across an image, the resulting fluorescence intensity is no longer proportional to the number of molecules within the image. Such situations should therefore be avoided for quantitative imaging.

Fluorescent probes and their properties

An incredibly large range of fluorescent probes now allow researchers to label almost any molecular species with high specificity. Here we review and compare the different labelling methods available.

Intrinsic fluorescence

A fluorophore is a molecule that emits fluorescence upon appropriate excitation. Intrinsic fluorophores are naturally present in some biological samples. In that case, their emission is termed autofluorescence. Examples of autofluorescent molecules include reduced nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), and collagen. Although somehow convenient – no further labelling is required - autofluorescence imaging suffers from relatively low excitation efficiency and often requires the use of expensive, biologically harmful UV sources and optics. Moreover it can be rather unspecific as different molecular species can fluoresce at the same time [110]. For these reasons, extrinsic fluorescent labelling is widely preferred for most biology applications, and in that case background autofluorescence is kept to a minimum.

Extrinsic fluorescence

Intrinsic fluorophores generally have a very large energy gap between the highest populated orbital and the lowest non-populated one which is why they often require high-energy UV photons to be excited. This energy gap can be lowered by delocalising the electrons across as many atomic orbitals as possible which can be done by increasing the number of pi-electrons (i.e. conjugated bonds) in molecules. In general, the more alternated conjugated bond/single bond motifs a molecule possesses, the further red-shifted its excitation and emission spectra will be [106]. By changing their chemical structures, it is therefore possible to design a range of fluorophores with specific spectral properties enabling the matching of excitation spectra with commonly available laser lines and optimising their brightness and resistance to photobleaching [111-113]. A huge range of fluorophores is now available to specifically label a given molecular species or even single molecules, therefore allowing them to be imaged with great specificity and contrast.

Dyes

Fluorescent dyes are generally designed to specifically stain a given molecular species and are therefore useful to localise their presence, for instance in different cellular compartments. However, such dyes generally do not stain one given molecule specifically and are therefore inappropriate for tracking. Examples of cell dyes include DRAQ5, Hoechst 33258, DAPI for DNA, and DiO, Dil, di-4-ANEPPDHQ for lipids.

Some dyes are also designed as sensors. For instance di-4-ANEPPDHQ shifts emission spectra depending on the order of the lipid phase it is in [114] whereas the brightness of Fluo-4 increases with Ca^{2+} concentration.

Immunofluorescence labelling

Small, bright and stable fluorophores that do not have any specific binding properties such as Alexa, Cyanine and Atto dyes can generally be used to label molecules using immunofluorescence labelling

[115]. This technique consists of conjugating one of these fluorophores to an antibody that is specific to a given molecule. Although highly specific, this technique generally requires samples to be permeabilised and fixed which is most often incompatible with live-cell imaging.

Fusion proteins

Fusion proteins have revolutionised many fields of Biology with their ability to label proteins of interest specifically and individually. While immunofluorescence labelling usually requires cells to be fixed, fusion proteins can be imaged in live cells. This is done by fusing the gene of the fluorescent protein to that of the protein of interest [116]. This technique is a powerful tool as it allows one protein to be covalently labelled by one fluorophore after ribosomal transcription and translation. It also means proteins can be tracked in real-time in live cells. The range of applications is therefore immense.

The first fluorescent protein - the Green Fluorescent Protein (GFP) - was discovered in the South Pacific jellyfish *Aequorea Victoria* [117, 118] and its structure was elucidated later on [119-121]. These studies show that the GFP chromophore is protected by a 4 nm-long surrounding β -barrel isolating it from the environment and making it less sensitive to photobleaching. The excitation maximum of GFP is 488 nm with an emission maximum at 508 nm. The full width at half maximum of the emission spectrum is around 50 nm but tails off beyond 600 nm and is skewed towards the red part of the spectrum.

GFP-variants have then been continuously engineered by mutating the gene to enhance its properties (e.g. enhanced GFP (eGFP)) or shift its excitation and emission spectra (Yellow Fluorescent Protein (YFP) and Cyan Fluorescent Protein (CFP)). This technique, combined with the discovery of orange to near infra-red fluorescent proteins [122], now provides us with a range of robust fluorescent proteins covering the whole visible spectrum [111, 123].

Quantum dots

Quantum dots are nanometre-sized semiconductor structures that can bind specific proteins by using antibody coatings. Their main advantage is that their emission spectra is much narrower than that of typical fluorescent proteins (30 nm typical full width at half maximum and not skewed towards longer wavelength, unlike most of fluorescent proteins and organic dyes) making spectral separation of a large number of them relatively easy. They are also brighter and almost insensitive to bleaching. However, they remain quite large (typically tens of nanometres; orders of magnitude larger than GFP) which can significantly disrupt protein functions [124, 125].

Photostability and phototoxicity

The number of excitation/emission cycles that a fluorophore can undergo is in general limited to 10^3 – 10^6 before it goes dark, which therefore restricts the number of photons that can be collected from fluorescent samples. This phenomenon is called photobleaching and the resistance of a given fluorophore to photobleaching is termed photostability. Using a high NA objective, only around 10^3 photons can be detected using a standard microscope, which means important applications such as single-molecule imaging can be difficult in high background environments. Photostability is therefore crucial for fluorescence imaging and is often a major motivation to engineer new versions of fluorescent proteins [123].

It is believed that photobleaching is due to interactions between fluorophores excited in a long-lived triplet state with nearby O_2 . This would give rise to highly reactive O_2 radicals, followed by an irreversible reaction destroying the fluorescent properties of the fluorophore [126].

Besides limiting the number of photons available, photobleaching is often accompanied by the creation of phototoxic chemical species, which is particularly critical for live samples. Photobleaching

depends on excitation power and this dependence is non-linear with high powers [127]. It is therefore desirable to keep illumination power at a minimum at all times.

Optical microscopy

The aim of optical microscopy is to perform fast high-resolution imaging of biological samples, preferably in three-dimensions. In this report we will review the main optical microscopy techniques and discuss them in terms of spatial resolution in all three dimensions, temporal resolution and suitability for biological imaging.

Microscopy spatial and temporal resolution

The aim of a fluorescent microscope is to form magnified images of fluorescent samples at the highest resolution possible. The resolution of a microscope is its ability to form separate images of two neighbouring objects. In a fluorescence microscope, the resolution of a given image is dictated by both the point-spread function of the microscope and the signal-to-background ratio (including noise). The signal-to-background ratio of an image mainly depends on sample properties (e.g. brightness) and therefore varies between acquisitions. The point-spread function of the microscope is a fundamental limit due to the diffraction of light by the optics of the microscope. The point-spread function of a microscope is the smallest image of a point object that a microscope can form.

It is equivalent to the transfer function in the Fourier space and therefore relates to the ability of a given microscope to image spatial frequencies contained in the object. For a Bessel function, the diffraction limited lateral resolution of a lens is given by the Rayleigh criterion (Equation 5) [128].

$$\Delta x = \Delta y = \frac{0.61\lambda}{NA}$$

Equation 5: Size of the PSF in the focal plane of a diffraction-limited lens as a function of wavelength and NA. Δx is the diffraction-limited lateral resolution along the x axis, Δy is the diffraction-limited lateral resolution along the y axis, λ is the working wavelength, NA is the lens numerical aperture of the lens in the relevant space.

For a high NA microscope objective at 500 nm, the diffraction limited lateral resolution is therefore typically around 250 nm.

The sectioning power of a microscope is its ability to reject out-of-focus light which therefore directly relates to its ability to form a three-dimensional image of a sample. A wide-field microscope does not have any sectioning strength, as the amount of energy reaching the detector from a given xy plane does not depend on its axis position z. In a confocal microscope, the combination of selective illumination and selective detection produces three-dimensional sectional power i.e. the amount of energy reaching the detector from a given xy plane decreases with the distance from the focal plane, resulting in sectioning strength (also termed axial resolution) (Equation 6) [129]. The sectioning strength of a confocal microscope is of the order of 1µm for high NA microscope, larger than the lateral resolution. The point-spread function of a confocal microscope is therefore highly anisotropic.

$$\Delta z = \frac{1.5n\lambda}{NA^2}$$

Equation 6: Size of the PSF along the optical axis of a diffraction-limited lens as a function of wavelength, NA and refraction index of the propagation space. Δz is the diffraction-limited axial resolution along the z axis, λ is the working wavelength, NA is the lens numerical aperture in the object space, n is the refraction index in the relevant space.

Wide-field microscopy

The simplest version of the fluorescence microscope is the epifluorescence microscope. Typically, the sample is uniformly illuminated by collimated light at the excitation wavelength. Uniform illumination of the sample is usually achieved through Kohler illumination [130]. In this case, an image of an incoherent light source (e.g. Hg lamp) is produced in the back aperture of the objective lens using a collecting and a condensing lens. An aperture stop and a field stop allow the illumination to be adjusted in the sample plane. The fluorescence signal from the sample is then collected and projected on a wide-field detector to form a fluorescence image. In most cases, both sample illumination and fluorescence signal collection are conveniently performed using the same objective lens. A dichroic beam splitter is used to separate the excitation light from the fluorescence emission.

Epifluorescence microscopes are usually very simple to use. However they cannot perform three-dimensional imaging due to the absence of sectioning strength, which results in background coming from the out-of-focus planes of the sample (Figure 2).

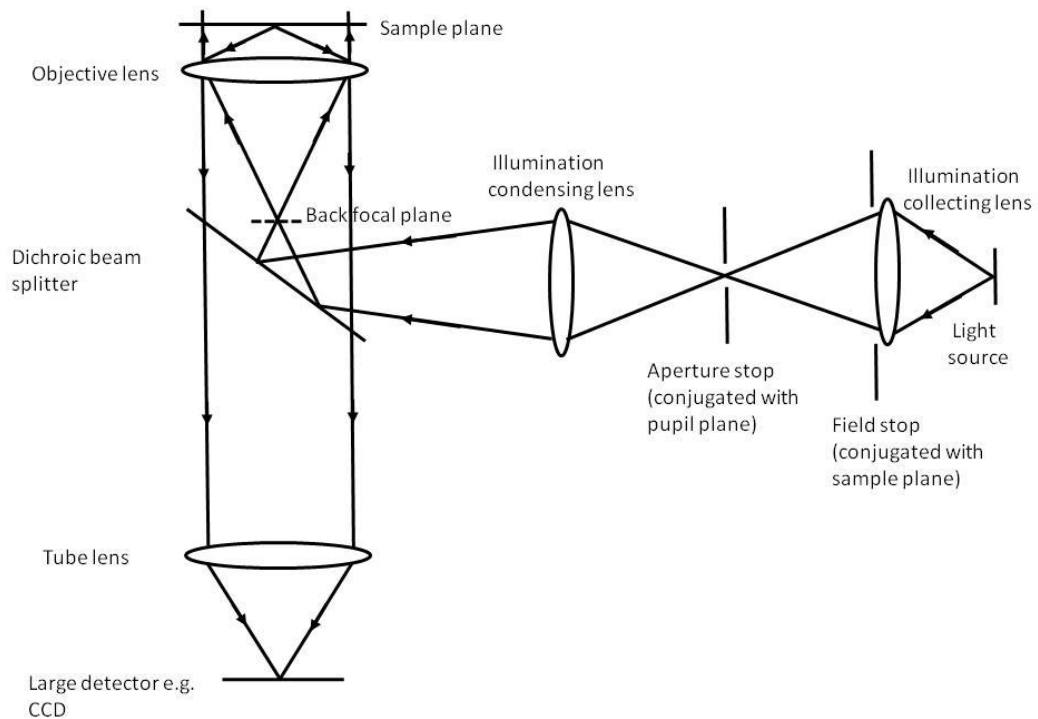


Figure 2: Schematic representation of a fluorescence wide-field microscope. The sample is uniformly illuminated by a source at the excitation wavelength. The emission coming from the sample is then imaged onto a wide-field detector, producing a fluorescence image of the sample.

Sectioned microscopy techniques

Confocal

The most common sectioned microscope today is the laser scanning confocal microscope. The principle of the confocal microscope was proposed in 1943 [131] and the instrument was patented in 1961 [132]. Successive technical improvements such as laser illumination [133] and beam scanning [134] have produced one of the most widely used commercial instruments in biomedical laboratories [135-137]. In a typical confocal microscope, fluorescence is excited by a point

illumination (laser source) that is scanned across the sample, and detected by a point detector (in general a pinhole in front of a photomultiplier tube or avalanche photodiode) (Figure 3). As the sampling point is scanned across the sample by the scanning mirrors in a given lateral plane, a computer reconstructs and displays the associated 2D image in real time. A dichroic beam-splitter is usually used to separate the excitation from the emission light.

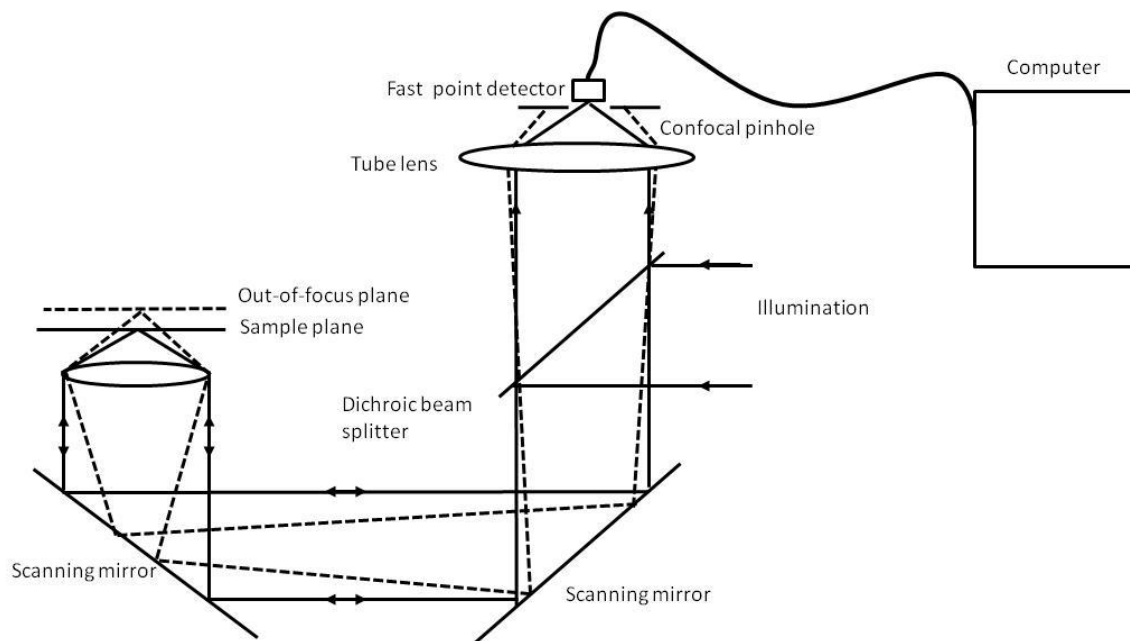


Figure 3: Schematic representation of a confocal microscope. The illumination is focused on the sample to create a excitation spot. The fluorescence coming from this spot is then collected onto a point detector through a confocal pinhole. The spot is then scanned across the sample to recreate a fluorescence image of the sample. The combination of point excitation and point detection (produced by the presence of the confocal pinhole) results in the rejection of the signal coming form out-of-focus planes (dotted lines).

The combination of selective illumination and detection results in the rejection of the out-of-focus light emitted by the sample. Therefore, three-dimensional images of fluorescent samples can be reconstructed from z-stacks of 2D confocal images. A standard confocal microscope can perform diffraction-limited imaging, and, equipped with a high-NA lens lateral and axial resolutions are about 250 nm and 1 μm respectively. Using a very small pinhole also produces a slight improvement in

lateral resolution up to factor 1.4 - but at the cost of collecting less light [135]. Most recent systems are now equipped with resonant scanners allowing video-rate imaging. Multi-colour imaging is also relatively easy due to the use of a point detector. Sophisticated systems allowing collecting up to thirty-two spectral channels independently are now commercially available.

Other sectioning techniques

Swept source microscopes

To produce a two-dimensional image, the illumination spot of a confocal microscope has to be scanned across the whole field of view, which limits the frame rate and means only one sampling point is acquired at once. Systems equipped with resonant scanners are now able to achieve very high scanning speeds.

Interesting alternatives to laser-scanning confocal microscopes are Nipkow-disk microscopes [138] and line-scanning microscopes [139] which are often termed as ‘parallelised’ confocal or swept source microscopes. The basic principle of such microscopes is to acquire several confocal points at the same time therefore allowing higher frame rates to be achieved. In the case of the spinning-disk microscope, small microlenses on a disk focus an incident beam through thousands of pinholes on a spinning disk to produce thousands of illumination spots similar to confocal spots. Fluorescence is collected through the pinholes on the spinning disk and the whole field of view is then imaged by rotating the disk. In the line-scanning microscope an illumination line acts as a line of confocal spots and is scanned across the sample, with the signal being collected through a slit rather than a pinhole.

These systems provide a trade-off between acquisition speed and signal-to-background ratio. If the confocal spots are too closely spaced, cross-talk between parallelised spots reduces the sectioning strength of these systems compared to that of a single spot-scanning microscope [139, 140].

TIRF microscopy

A total internal reflection (TIRF) microscope is a wide-field microscope whose illumination is at the critical angle such that illumination light gets totally reflected at the glass coverslip-aqueous sample interface. No direct excitation light reaches the sample, but instead an evanescent wave is produced (Figure 4).

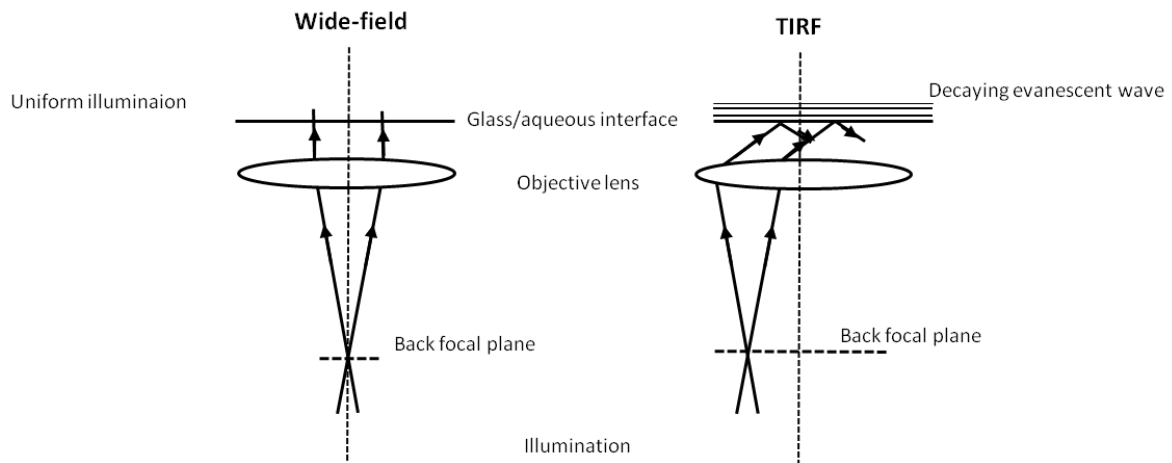


Figure 4: Comparison of the illumination schemes between a wide-field (left) and TIRF microscopes (right). In a wide-field microscope, the illumination is collimated along the optical axis. In a TIRF microscope, illumination is displaced in the back-focal plane of the objective such that the rays coming out of the objective are from the edge of the pupil. In a high-NA objective these rays are hitting the glass-water interface at the critical angle resulting in their being totally internally reflected at this interface. This generates a rapidly-decaying evanescent exciting the sample within a very limited depth which drastically limits the generation of out-of-focus background light.

This evanescent wave typically only penetrates and excites the sample within 100 -130 nm at the coverslip (depending on wavelength, objective NA and refraction indices), thus producing a sectioning strength far superior to that of a confocal microscope ($\sim 1\text{ }\mu\text{m}$ for a high-NA objective). Although it cannot perform three-dimensional sectioned imaging as only the plane next to the coverglass can be illuminated in TIRF mode, this technique is ideally suited to perform low-background imaging of parts of the sample that are next to the glass coverslip and is often exploited for single molecule imaging [141-143]. A notable example of the relevance of TIRF microscopy is the

imaging of membrane-proximal events happening at the immunological synapse between a cell and an artificial substrate such as antibody coated slides or glass-supported lipid bilayers. We built a TIRF microscope to image such events and data acquired in this context will be shown in this introductory chapter as an example.

Determination of the cytoplasmic and membrane-bound distributions of signalling molecules by comparing TIRF and wide-field images

Background

Signalling at the T cell synapse upon engagement of TCR is initiated by the kinases Lck and ZAP70 which phosphorylates the adaptors LAT and SLP-76. Recent live-cell imaging studies have shown that activation of T cells on activating substrates triggers the formation of signalling microclusters containing TCR, ZAP70, SLP-76 and LAT. While high-resolution imaging has revealed the role of these microclusters in initiating and sustaining signalling, the dynamics of crucial interactions between protein microclusters is still unclear, mainly due to the lack of sufficiently fast multicolour imaging.

Using high-speed multicolour imaging of T cells interacting with anti-CD3 activating coated slides, we set out to investigate the dynamics of the interactions of microclusters of various signalling molecules such as TCR, LAT, GADS, ZAP70 and SLP-76. The work presented here is part of a study on the role of the endosomal network in T cell signalling [59].

Using Jurkat T cells transfected with relevant fluorescently tagged signalling proteins, this study shows that GADS, SLP-76 and LAT form dynamic microclusters upon T cell activation. Strikingly, while SLP-76 and GADS clusters consistently overlapped, LAT formed distinct clusters. Movies of synapses show that LAT clusters moved faster than SLP-76 clusters and interacted with them in a transient manner. By staining cells with antibodies specific for phospho-LAT, the study demonstrates that LAT is phosphorylated at the site of contact between LAT and SLP-76 clusters. Dwelling time of LAT

clusters was signalling-dependent as mutated forms of LAT that were unable to interact with SLP-76/GADS exhibited shorter dwell times. On antibody-coated slides, microclusters of TCRs appeared stationary, which is expected using this model. Consistently with previous studies, clusters of ZAP70 overlapped with clusters of TCRs. Surprisingly, movies of synapses show that the movement of LAT was restricted by the ZAP70 clusters.

LAT primarily exists in the plasma membrane, but it has been shown by others that a significant proportion of LAT also exists in intracellular vesicles in resting and activated T cells. Using internalised membrane dye staining for vesicles and fluorescently labelled Rab8 and Rab7, we surprisingly observed that a significant proportion of the LAT involved in the signalling interactions mentioned above is actually found in vesicles. This was not the case with SLP-76 or ZAP70.

Next we set out to test the localisation of SLP-76, ZAP70 and LAT with regards to the plasma membrane. The sectioning strength of a confocal microscope is not sufficient to discriminate between molecules located next to the plasma membrane and those contained in sub-synaptic vesicles, away from the interface. Using TIRF microscopy, the illumination depth can be limited to 100-130 nm from the glass interface (to be compared with the WF depth of focus up to 1 μ m). When using antibody-coated slides as activating substrates (the substrate and the plasma membrane are around 70 nm deep), TIRF illumination excites fluorophores ~50 nm away from the plasma membrane of the cells, as opposed to ~900 nm using a confocal microscope, therefore allowing only membrane molecules to be detected. Therefore, comparing TIRF and WF images of the same cell, it is possible to compare the membrane and cytoplasmic distributions of a given protein (Figure 5).

While comparing TIRF and WF images is a powerful method to investigate protein localisation, two limitations should be considered. Because background is generally higher in WF than in TIRF (especially in our case when dealing with cytoplasmic molecules), some of the faint features will not be detectable in WF. This is not an issue for qualitative interpretation of the data. Also, TIRF images are not strictly imaging membrane-bound molecules as the illumination still penetrates the

cytoplasm. However, the extent of the “contamination” of membrane bound proteins by cytoplasmic molecules in TIRF images is much less than it would be with a confocal microscope.

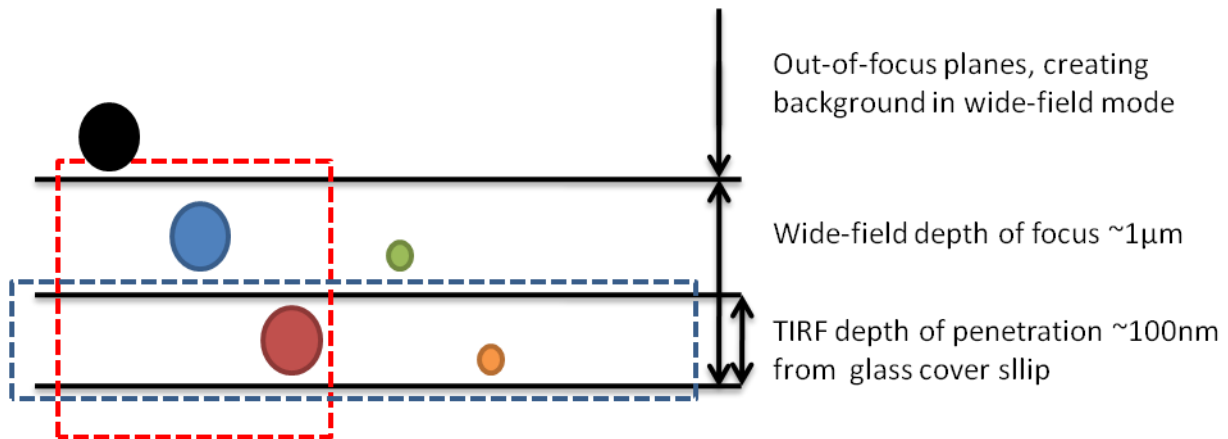


Figure 5: Comparison of TIRF and wide-Field images to discriminate cytoplasmic proteins from membrane-bound proteins. In TIRF mode, only molecules close to the interface will be detected (dashed blue box). In wide-field mode, cytoplasmic molecules will be detected while dimmer entities might not be detected (dashed red box). Dim entities (below wide-field background but detectable in TIRF) are represented by small circles, bright entities (e.g. above wide-field background) are represented by large circles.

Results

We compared images of fixed LAT, ZAP70 or SLP-76 transfectants acquired sequentially in TIRF and WF modes on anti-CD3-coated slides, all showing clear protein clustering. With LAT transfectants, a significant amount of clusters that are not detected by TIRF are detected using WF illumination. However, ~ 90% of WF SLP-76 and ZAP70 clusters co-localised with those detected in TIRF (Figure 6). Using a confocal microscope, all clusters seen using TIRF and WF illumination would appear on the same image (with the exception of very dim entities since TIRF microscopy has a higher signal-background ratio than confocal microscopy), thus making it impossible to discriminate between them.

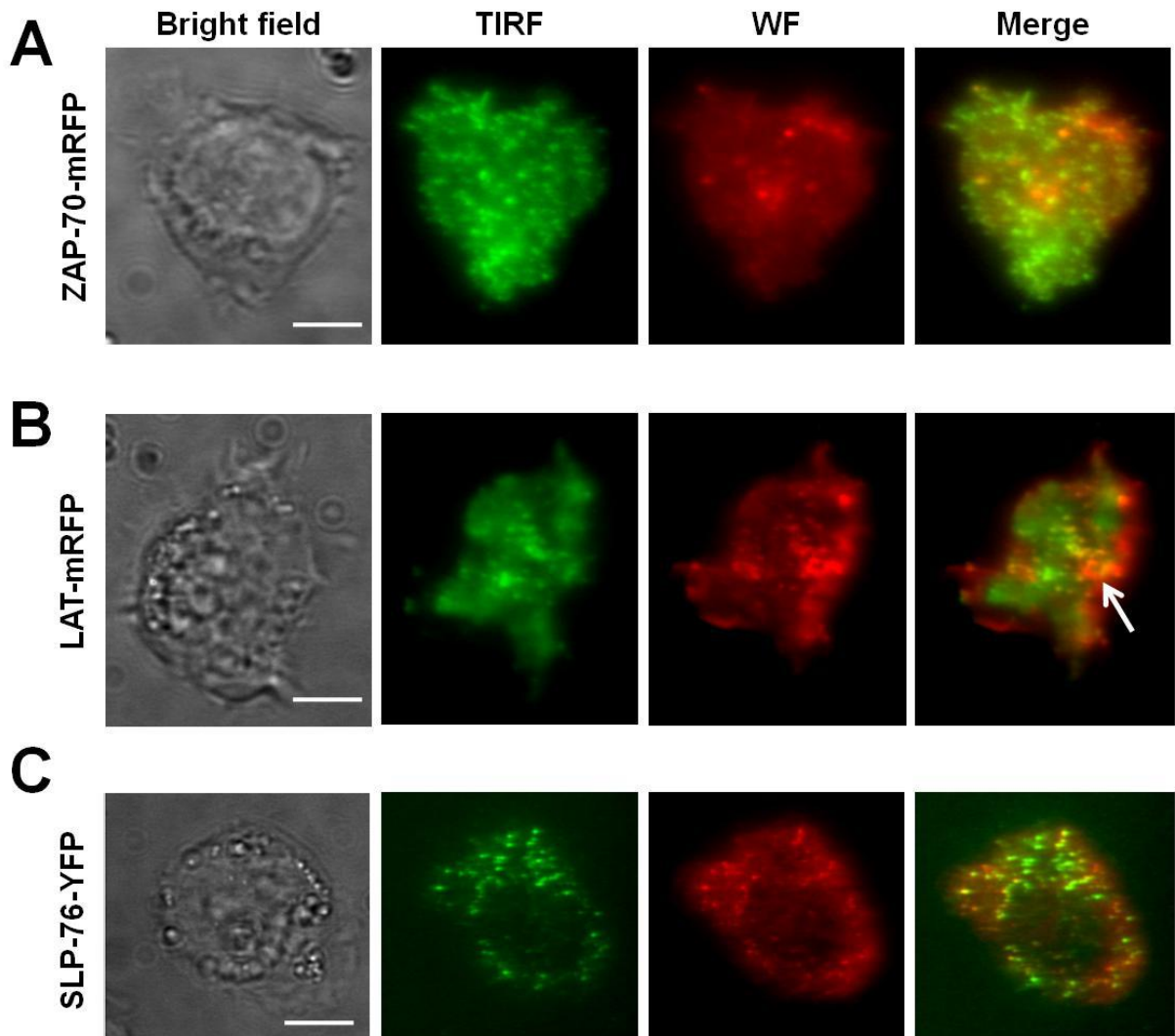


Figure 6: Comparison of TIRF (green) and wide-field (red) images of synapses formed between Jurkat T cells transfected with ZAP70-mRFP (A), LAT-mRFP (B) or SLP76-YFP (C) and activating anti-CD3 coated slides. Unlike ZAP70 and SLP-76 which are mainly detected at the membrane, a significant proportion of total LAT is detected in the wide-field image only, showing the presence of a pool of vesicular LAT away from the plasma membrane. Scale bar, 10 μ m.

Conclusion

Comparing TIRF and WF images shows that while SLP-76 and ZAP70 is mostly located within membrane-bound and/or membrane proximal clusters, a high amount of LAT is not membrane bound, which is consistent with the fact that some LAT colocalises with internalised membrane dye stainings, Rab8 and Rab7.

FRET microscopy

FRET (Fluorescence Resonance Energy Transfer) microscopy allows probing very short intermolecular interactions between two fluorophores. FRET is a distance-dependent physical process by which energy is non-radiatively transferred from a donor fluorophore to an acceptor. As the efficiency of the dipole-dipole energy transfer is dependent on the inverse sixth power of the distance between the two fluorophores, FRET microscopy is sensitive to intermolecular interactions within the 1-10 nm range, and is therefore ideally suited for investigating a variety of biological phenomena such as inter- or intramolecular changes in fixed or live samples [144, 145]. Quantitative measurement of the energy transfer between the donor and the acceptor is usually done either spectrally (e.g. by measuring intensity shifts in the donor and acceptor's respective spectral channels) or by measuring fluorescence lifetime shifts (e.g. by measuring a decrease in donor's lifetime). Although technically more complex, the latter is often preferred as it gives access to an absolute energy transfer measurement [146].

Recently, FRET was used to image the distribution of the activated form of KIR at the inhibitory NK cell immunological synapse. The authors of the study reported FRET between KIR-GFP (donor) and an anti-phosphotyrosine antibodies conjugated with the dye Cy3 (acceptor) by measuring GFP's lifetime across the synapse [89]. A decrease of the GFP lifetime was shown to occur in discrete regions. The reduction in lifetime was shown to correspond to a significant energy transfer to the Cy3, indicating that the distribution of phosphorylated KIR was formed in distinct microclusters.

Super-resolution microscopy techniques

The so-called super-resolution techniques break the diffraction barrier by exploiting the presence of dark and bright states in fluorescent markers by exploiting their saturation regime [149] or by exciting them sequentially either spatially [147] or temporally [148]. Due to these photophysical properties, the fluorescence microscope is in principle capable of unlimited “resolution” which, in this context, means that fluorophores can theoretically be located with infinite accuracy (as opposed to true imaging resolution). Although still in their infancy, three of these techniques, reviewed here, are currently serious candidates to become the new standard for live-cell imaging in the future.

PALM & STORM

Principle and tools

Photoactivated Localisation Microscopy (PALM) [150], Fluorescence Photoactivated Localisation Microscopy (FPALM) [151] and Stochastic Optical Reconstruction Microscopy (STORM) [152] are super-resolution imaging techniques. The principle is to switch photoswitchable fluorescent markers from a non fluorescent to a fluorescent state by exposure to their activation wavelength. While STED relies on point-spread function engineering, here, image resolution below the diffraction limit is achieved by extensive post-acquisition image processing.

PALM and STORM rely on the concept that an isolated emitter, although producing a diffraction limited spot on a detector, can be more precisely localised by finding the position of the centroid of the corresponding spot in the image [153]. Using this concept, nanometre-scaled localisation can be achieved, although the presence of background generally limits the accuracy to a few tens of nanometres.

Aided by the development of photo-activatable or photo-switchable fluorophores, PALM, STORM and FPALM have extended this concept to densely populated samples. Specifically, acquiring a full super-resolution image consists of many successive cycles during which non-overlapping single fluorophores are activated at once by keeping the activating beam adequately low. These fluorophores are then imaged until they are bleached or deactivated, and the process is repeated until the positions of enough molecules within the sample have been determined. Compiling these single elements together finally gives rise to the final image featuring a resolution exceeding the diffraction limit. Specifically, lateral resolution down to ~18 nm has been demonstrated [152].

Compared to STED, PALM and STORM are technically more straightforward and thus easier to implement as no specialised equipment other than a fluorescence microscope, CW lasers and sensitive CCD cameras is required. Also, labelling is somewhat easier as a number of photoactivatable organic dyes (e.g. caged rhodamine [150] and fluorescein [154]) and proteins (e.g. Dronpa [155] and its mutants including rsFastLime [156, 157], photoactivatable-GFP [158], photo-switchable-CFP [159], EosFP [160]) have been developed.

The process of image reconstruction is similar to signal digitalisation, where a complex signal/image, is replaced by a series of simple, perfectly 'determined' elements (e.g. bits). The need for subsequent image processing is a downside of PALM/STORM compared to STED as dim features in the image might be attributed to background and therefore discarded. As a result of this, the final, background-free image might not be a linear representation of the original object.

PALM/STORM, as originally described, did not seem to be the best candidate for super-resolution live-cell imaging. Indeed, it was plagued by extremely slow acquisition times exceeding several hours and was unable to probe samples in three dimensions as only TIRF microscopy could achieve sufficiently low background to allow accurate localisation of single molecules. However, many important improvements have been made, making it much more appealing.

Multicolour imaging

To achieve multicolour imaging, Bates *et al.* have created a palette of photoswitchable probes, each consisting of a coupled dye pair: a 'reporter' dye that can be imaged and an 'activator' dye that serves to activate the reporter when excited [161, 162]. This method, based on photoswitchable probes, is particularly powerful, as combinatorial pairing of three activators with distinct absorption wavelengths with three reporters with distinct emission spectra results in a total of nine distinguishable probes. Using three different activators paired with the same reporter (therefore with no need for any spectrally resolved detection), three-colour imaging has been demonstrated in fixed cells with a resolution of ~25 nm.

Sectioned PALM, STORM & iPALM

Recently, Huang *et al.* have demonstrated three-dimensional molecule localisation by introducing some astigmatism into the imaging path (using a weak cylindrical lens) so that the ellipticity of the image of each fluorophore can be used as an accurate measure of the distance of the fluorophore from the focal plane [163]. Three-dimensional organisation of microtubules in cells could be determined with a lateral resolution of ~25 nm while achieving an axial resolution of ~50 nm over a range of 600 nm. A recent interesting study also achieved three-dimensional sub-20 nm PALM imaging in all directions using two opposing objectives to perform multiphase interferometry [164].

Live-cell PALM/STORM

The development of bright photo-switchable FPs such as EosFP has allowed live-cell imaging as well as single-particle tracking to be performed using fluorophore localisation techniques, achieving a lateral resolution down to 60 nm [165-168]. With frame rates limited to a few frames per minute, this technique is inadequate for fast molecular processes and remains much slower than video-rate STED.

STED

Principle and tools

The resolution of a point-scanning microscope, such as a confocal or two-photon microscope, is directly dependent on the size of the illumination volume, since neighbouring fluorophores that are inside the same illumination volume cannot be distinguished. Thus, the concept underlying Stimulated-Emission Depletion (STED) microscopy is to superimpose a red-shifted, generally doughnut-shaped STED beam onto the usual focused excitation beam to quench the fluorescence outside a small spot using stimulated depletion [169]. The stimulated photons are discarded away from the detection path, as is the STED beam.

Scanning this small effective excitation spot across the sample yields a subdiffraction fluorescence image. In theory, the gain in resolution is unlimited as long as saturated depletion is achieved. However in practice this process strongly depends on the properties (e.g. photostability) of the fluorophores that are employed. It also depends on how well the concept can be technically implemented (e.g. shape of the STED beam and synchronisation of the STED and excitation beams), albeit simplified implementations employing CW beams have been demonstrated [170]. So far,

spatial resolutions down to 16-50 nm have been demonstrated with either single molecules, nanoparticles or biological systems [147].

Despite the high level of technical complexity that characterises STED microscopes, it is interesting to note that this technique has already proved to be useful to tackle a variety of biological issues for which increased resolution was critical [171-175]. The most impressive discovery was the observation of lipid nanodomains similar to lipid rafts using a combination of STED and FCS [176]. This study shows that some lipids are transiently trapped into cholesterol-mediated domains that are less than 20 nm in diameter, an observation that would have proved impossible using diffraction limited methods.

Live-cell FP-based STED

Due to the powerful STED beam (typically 100 times as powerful as the illumination beam), STED is often affected by increased photobleaching rates thus restricting the range of fluorophores that can be used to bright, robust dyes. However, fluorescent protein-based STED has been achieved with GFP [177] and YFP [178], the latter being in live cells. Here, specific illumination schemes were used to prevent the population of electronic states responsible for increased bleaching [179].

Multicolour STED

Single colour STED is already a complex technique due to the need for an additional radiation to achieve depletion. This being said, two-colour STED imaging has already been successfully demonstrated. Although the technique was first affected by unequal resolution between the green (30 nm) and the red (60 nm) channels [180], it was rapidly improved to offer a 30 nm resolution for both channels [181].

IsoSTED

In normal STED, the doughnut beam only depletes fluorescence in 2D (xy plane), but not axially. Therefore STED often requires using two-dimensional samples, as background coming from the non-depleted axial part of the point-spread function would impair the resolution improvement. To circumvent this problem, recent major improvements of STED include the generation of a fully isotropic (spherical) illumination spot of 45 nm in diameter by using a combination of two independent STED beams (for axial and lateral depletion) in a 4π microscope. This technique is termed isoSTED [182]. It is also the first ever generated spherical optical PSF.

Video-rate STED

An impressive study has recently managed to perform live-cell STED imaging at an impressive 28-frames/s video rate [183]. To allow this, the resolution improvement was slightly compromised to 68 nm and the field of view was reduced to 2.5 μm x 1.8 μm . However, impressive movies of fluorescently labelled synaptic vesicles inside neuronal axons could be achieved.

Structured Illumination

Principle and tools

The diffraction resolution limit in a light microscope specifies that the maximum spatial frequency that can be imaged is given by $k_{max} = \frac{2NA}{\lambda}$ where λ is the observation wavelength. In a 2D frequency space, this limit, which is the counterpart of the diffraction limited spot, defines an observable region of radius k_{max} . Outside this region, spatial frequencies get strongly attenuated or rejected and are therefore absent in the final image. Illuminating the sample using structured illumination e.g. a

sinusoidal pattern of frequency k_{ill} produces moiré fringes, thus translating the observable region by k_{ill} in the frequency space. Because it is then necessary to unmix partially overlapping frequency regions produced by each harmonic, a phase shift is introduced to reconstruct the final sub-diffraction image. In the case of linear sinusoidal structured illumination, a three-step phase shift is therefore necessary to unmix the three harmonics. Rotating the grid a few times to cover a circular frequency space is also necessary [149]. Two-fold increase in resolution is achieved using linear structured illumination as the maximum k_{ill} the sample can be illuminated with is dictated by the diffraction limit.

Non-linear multicolour structured illumination

It is not possible to increase the fundamental spatial frequency of the illumination pattern as this is limited by the diffraction limit. However, due to the fact that fluorophore intensity responds non-linearly at high excitation power, it is possible to increase the number of harmonics by saturating the fluorescent sample. In that case, the resolution can be in principle unlimited (as the number of harmonics can be arbitrarily increased). In practice, the resolution will be limited by signal-to-noise ratios and photostability. The cost of increasing the number of harmonics is that the number of phase shifts and grid rotations that have to be performed increases by the same number.

Using this technique, subdiffraction lateral resolution below 50 nm has already been performed [149]. Multi-colour, three-dimensional imaging with two-fold improvement both laterally and axially has also been recently demonstrated [184] .

Conclusion

High-resolution high-speed imaging of immunological synapses is generally achieved by using TIRF microscopy to image the interaction between an immune cell and an activating substrate mimicking a target cell or an antigen presenting cells. Although this approach has allowed researchers to produce very informative live images, it remains impossible to image immunological synapses between two live cells at high-resolution.

The recent advent of many super-resolution imaging techniques is very promising and is a potential solution to this problem. However these techniques remain in their infancy and their generalisation proves difficult. PALM and STORM techniques are still quite slow and are therefore unable to provide sufficient temporal resolution to describe the dynamics of signalling proteins at the immunological synapse. The extensive image processing that is needed after image acquisition should also be performed carefully, especially when it comes to discriminating background from actual signal, so the reconstructed super-resolved image is a faithful representation of the sample. Structured illumination is, in principle, relatively simple too, but in practice it remains prone to alignment and photobleaching artefacts, and the illumination powers and acquisition times that are needed are incompatible with live cell imaging. STED seems to be the most promising candidate, but it lacks of flexibility severely restricts its range of applications.

With all of these techniques, especially PALM/STORM and STED, the major issue remains the fact that most often they require highly specialised samples to actually achieve super-resolved imaging. For instance 2D samples are most often needed as the background coming from out-of-focus planes cancels any resolution gain. This issue comes from the fact that sectioning in general remains diffraction-limited only, even with so-called 3D techniques such as 3D STORM or iPALM. In most studies, therefore, 2D or specially designed 'non-flat' 2D samples (then called 3D) are used to

demonstrate the techniques. The only technique so far where real background discrimination matches lateral resolution is isoSTED. However it remains significantly complex.

In their current form, these techniques are therefore not yet suitable to perform high-speed and high-resolution imaging of intercellular immunological synapses.

Chapter IV: Development of a microscopy technique for high-speed and high- resolution imaging of intercellular immunological synapses

Introduction: Optical trapping

Current imaging techniques for immunological synapses can only achieve diffraction-limited imaging with artificial activating substrates as surrogate target cells or APCs. Improvements brought by new super-resolution techniques, often providing spatial resolution down to a few nanometres, are very promising. However, despite immense progress since they were first introduced, STED remains positively complex, which limits its application, while PALM, STORM and FPALM are still too slow to image protein dynamics at the synapse. Structured illumination remains highly sensitive to noise and has not yet been demonstrated with live cells.

The idea behind this project is that spatial and temporal resolution while imaging intercellular immunological synapses in a confocal microscope is limited by the fact that the cell-cell interface is lying in a plane that is perpendicular to the focal plane of the microscope (Figure 7)

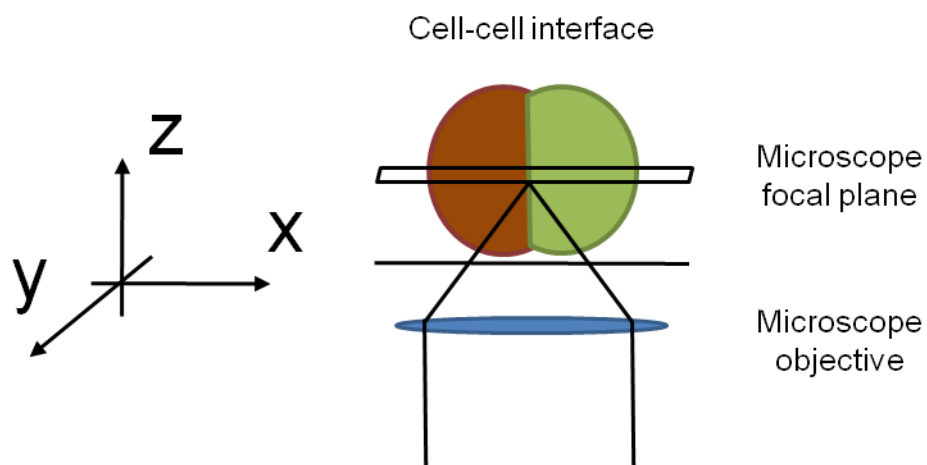


Figure 7: Diagram showing the geometrical arrangement of a cell-cell interface with regards to the focal plane of a microscope objective. In that configuration, the plane of the immunological synapse is perpendicular to the focal plane of the microscope objective.

The ratio between Equation 6 and Equation 5 (see page 51) is around 2 to 3 which means that the PSF of a confocal microscope is about 2 to 3 times as large along the optical (z) axis than it is within the focal plane (xy) (Figure 8). Thus, normal confocal imaging of the synapse can only yield micron-scaled resolution. The need to use 3-D rendering and interpretation to reconstruct the immunological synapse in the yz plane also deteriorates the final reconstructed image and makes accurate quantification difficult. In addition, this method only updates at a few frames per minute due to the need to acquire a z stack at each time point.

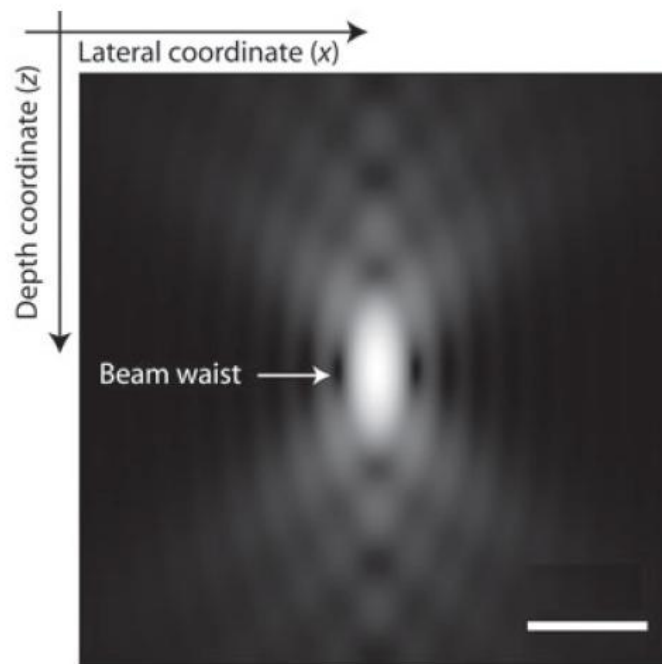


Figure 8: Shape of the point spread function of a confocal microscope 100x 1.4 oil immersion NA at 530 nm. The point spread function of a microscope objective is elongated along the optical axis resulting in poorer resolution in that direction. Scale bar, 500 nm. Adapted from [185].

Manipulation of cell conjugates into an imaging configuration such that the intercellular contact lies *en face* to the imaging plane would allow this interface to be imaged at the maximum speed and resolution allowed by the confocal microscope.

Optical trapping was first introduced by Arthur Ashkin and Steven Chu at AT&T Bell laboratories in 1986 [186]. Most of today's optical trapping set-ups are still derived from the same principle, where a trapping force is exerted on small objects by a tightly focussed beam.

Although the theory of optical trapping is still unclear when it comes to trapping objects whose size is of the order of the wavelength of the trapping radiation, it is more straightforward for objects that are either much smaller or bigger than this scale. In response to the electric field, small objects compared to the radiation wavelength develop an electric dipole moment. This dipole is then attracted towards high intensity electric fields. Large objects, such as the cells we use to study the immunological synapse, fall into the ray optics regime and can be described as refracting objects [187]. As a result of this refraction, momentum is transferred from the photons to the trapped object. This transfer of momentum draws the object towards the light focus (Figure 9), creating an optical gradient force that competes with the radiation pressure coming from the absorption of photons by the object, whose momentum transfer then propels the object along the photon flux away from the focus. To overcome this radiation pressure force, the optical gradient force has therefore to be large enough, which is usually achieved by generating a sharply focussed spot using high NA aberration corrected lenses.

These forces are up to 100 pN but can be orders of magnitude smaller [188-190] and can be applied on objects as small as 5 nm [191, 192] which is for instance ideally suited for the study of molecular motors. Optical trapping has been extremely useful to measure forces exerted by molecular motors such as ribosomes during RNA transcription, myosin or kinesin, showing that these molecular motors are not only used for cell motility and chromosome sorting but also for intercellular signalling and gene transcription. It has also been used to measure the viscoelastic properties of DNA, chromatin

and chromosomes, protein fibres and cellular membranes and study the optically guided neuronal growth [193-198].

The advent of phase-only spatial light modulators (SLM) has allowed researchers to generate optical tweezers of virtually any shape. This can be done in a holographic manner where the phase pattern imprinted on the SLM is projected into the back pupil plane of the objective which then operates a Fourier transform of the pupil wavefront [199]. This method requires the mathematical computation of the holographic pattern in the pupil plane that corresponds to the optical trap in the image plane. This can be circumvented by using a phase contrast filter whose role is to transform the phase pattern on the SLM directly into an intensity pattern in a plane conjugated to the image plane of the objective [200].

Optical trapping requires a focussed laser beam of sufficient power to achieve trapping. This can be detrimental for living organisms. Although local heating can be an issue when optical tweezers are used [201], it has been shown that using an infrared beam e.g. at 980 nm significantly reduces these effects [202].

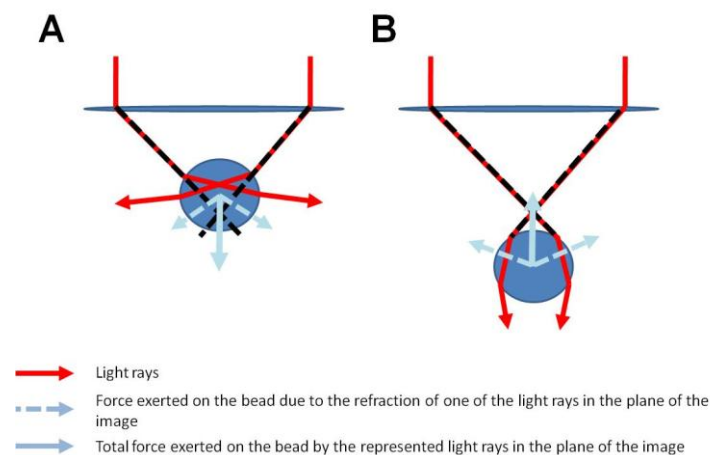


Figure 9: Ray optics description of the gradient force in optical traps for a transparent bead. Gradient forces (blue arrows) result in a stable three-dimensional trap. When the particle experiences a converging beam i.e. upstream of the beam focus (A), the net force (bold blue arrows) pushes the particle towards the focus (intersection of black dotted lines). When the particle experiences a diverging beam (B) i.e. downstream of the beam focus, the net force pulls the particle back towards the focus. The bold blue arrows represent forces applied on the object that result from light refraction (shown in red) and subsequent momentum transfer.

The aim of the first part of the project is to implement optical tweezers in a commercial confocal microscope to improve the imaging conditions of intercellular synapses (Figure 10). In such a configuration, live movies of the immunological synapse could be obtained in a series of xy frames as opposed to a series of z stacks. The gain in time resolution would therefore be of a factor 20-40, while spatial resolution would be improved by a factor 5.

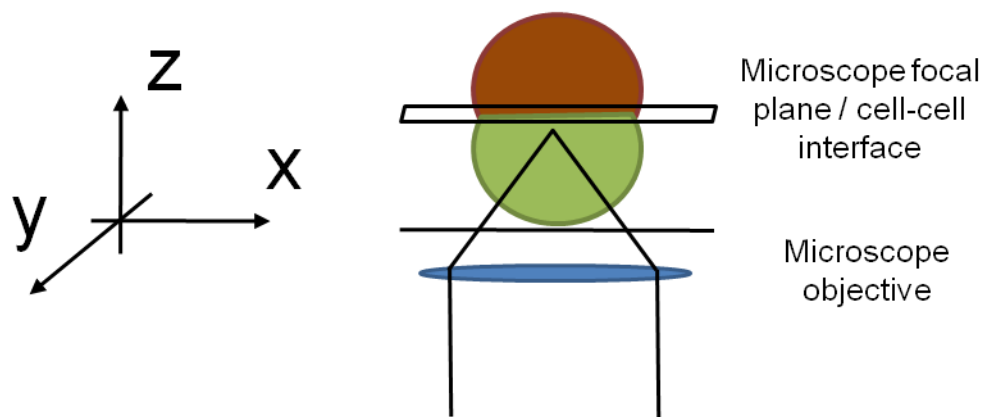


Figure 10: Diagram showing the geometrical arrangement of a cell-cell interface with regards to the focal plane of a microscope objective after manipulation of cell conjugates using optical tweezers. In that configuration, the plane of the synapse is parallel to the focal plane of the microscope objective.

Material and methods

Cells

Cell culture

Cells were cultured in R10 medium (RPMI 1640 medium supplemented with 10% FCS, 100 µg/ml Penicillin-Streptomycin and L-glutamine (Invitrogen)) at 37°C in a 5% CO₂ atmosphere. Cells were

passaged at a ratio of 10:1 or 10:2 every 3 days. Experiments were carried out the day after passaging.

Immune cell lines

For experiments with T cells, the immortal T cell line Jurkat E6.1 was used. For experiments with NK cells, we used the NK cell line YTS stably expressing the fusion protein KIR2DL1-GFP.

Target cell lines

For experiments with T cells we used Raji B cells pulsed with Staphylococcal Enterotoxin E (SEE) super-antigen. Cells were incubated with SEE (100 ng/mL) for at least 45 min at 37°C, 5% CO₂. After incubation, cells were centrifuged twice for 5 minutes and resuspended in R10 medium to remove excess SEE. For experiments with NK cells, the B cell line 721.221 stably expressing HLA-Cw6 was used.

Membrane and vesicle staining

To stain the cells with the membrane-partitioning dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD) (Molecular Probes), $\sim 10^6$ cells were incubated for 7 minutes at 37°C in a 5% CO₂ atmosphere in 1 μ L of R10 media with 1-3 μ L of DiD. Cells were then centrifuged at 1200 rpm for 5 minutes and resuspended in R10 media. To mark vesicles, the cells were used 4 hours after staining to allow internalisation of the dye.

Fusion proteins and construct sequences

Protein sequences were amplified by PCR from cDNA and c-terminally tagged with fluorescent protein by ligating in-frame into pcDNA3.1 vectors. This was done by Marco A Purbhoo.

Transfection method

Jurkat T cells were transiently transfected using microporation. Just before transfection, $\sim 3 \times 10^6$ cells were washed and resuspended in 30 μ L of the supplied electrolyte resuspension buffer. The microporator (Digital Bio Technology) settings for electric pulses were 1380 mV/30 ms/1 pulse as

indicated in the manufacturer's online database. Cells were then resuspended in R10 media with no antibiotics and were imaged 16 hours after transfection. The transfection efficiency was found to be around 20%.

Sample preparation

Imaging was performed in 8-well-chambered cover slides (Lab-Tek). Wells were first incubated for 5 minutes with FCS which was then removed. Wells were then loaded with ~ 200 μ L of the cell suspension. For optimal results, cells were diluted down to 10^5 cells/mL just before imaging to obtain a sparse distribution of cells in the field of view. Before imaging, T cells were incubated with APC for 20-30 minutes to cause them to form mature immune synapses. NK cells and their targets were then imaged promptly and those forming synapses were then trapped and imaged.

Cell fixation

For experiments with fixed T cells, Jurkat T cells were incubated with APCs (ratio 1:1) for 10 to 12 min until they form conjugates. After washing cells were resuspended in fixing solution, 2% PFA in PBS) and incubated 1 to 2 min at 37 °C. After washing cells were the resuspended in a quenching solution (50 mM NH_4Cl , 20 mM glycine in PBS), incubated for 5 mins at room temperature, washed again and finally resuspended in PBS before imaging.

Optical-tweezer set up

Confocal microscope

The optical tweezers were coupled into the non-descanned port of a commercial confocal microscope with galvanometric scanners (TCS SP5, Leica Microsystems Ltd). A 63 $\times$, 1.25NA oil-immersion objective was used.

Source characteristics

To generate the optical tweezers, a fibre-coupled continuous-wave (CW) single-mode solid-state laser diode emitting at 980 nm was used (LC96, Bookham Inc). The maximum output power of this source is 600 mW. The NA of the output fibre is 0.13. The diode was mounted into a Peltier cooled mount (Thorlabs Ltd) allowing connections for the temperature controller and driving current controller.

IR/visible dichroic characteristics

To couple the IR light used to trap the cells in the confocal microscope we used a dichroic beam splitter with a cut-off wavelength around 900nm (Chroma Technology Corp).

Tweezer optics

The output beam from the laser diode was collimated using an IR-coated lens (diameter = 16 mm, f = 25 mm, Comar) to produce a collimated beam of diameter $\sim$ 6.5 mm. To produce two independent, actuable traps, the beam were separated into two beams using a polarising beam splitter cube (bandwidth = 900-1300 nm, dimension = 25.4 mm, Newport Corporation). Actuation of the beams was performed using IR-coated steering mirrors (diameter = 25.4 mm, bandwidth = 750-1100 nm, Thorlabs). Before coupling into the microscope, the two beams were recombined using a similar polarising beam-splitter cube. To avoid vignetting, the microscope-objective pupil was imaged on the actuating mirrors using a pair of coated relay lenses in a 4f configuration (diameter = 25 mm, f =

200 mm and $f = 160$ mm, Comar). These lenses also expanded the beam by a factor 1.25, bringing the beam diameter to ~ 8.2 mm to slightly overfill the pupil of the microscope objective (Figure 11).

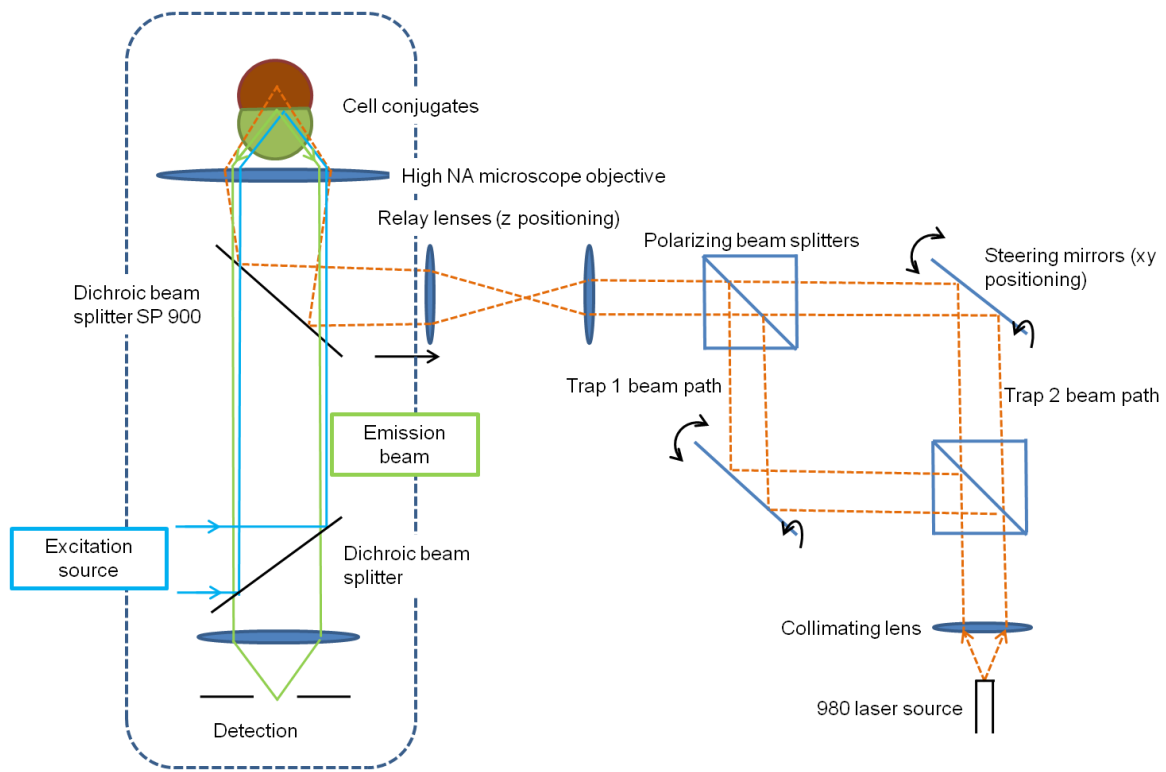


Figure 11: Schematic representation of the optical-tweezer set-up. The beam generating the optical tweezers (orange dotted line) is coupled into a commercial confocal microscope (represented by the elements enclosed within the dotted box). Relay lenses are used to conjugate the pupil (the back aperture of the objective) onto other limiting apertures in the system (here the steering mirrors) allowing the traps to be actuated across the whole field of view without significant loss of power.

Cell manipulation

Cells were manipulated in the xy plane using the steering mirrors. Manipulation of cells along the z axis was possible by defocusing the two relay lenses and thus producing a diverging wavefront in the pupil plane of the microscope objective. To rotate the cell conjugates, the power required was less than 30 mW at the back pupil plane for a few seconds. Once the cells were in the right configuration, less than 15 mW was needed to keep them balanced. Detrimental effects of such powers at this wavelength are negligible [202].

Image processing

ImageJ

Image processing was performed using the freeware package ImageJ (NIH). Image brightness and contrast were adjusted to maximise image clarity. For oversampled images, Fourier filtering could be used to remove residual noise.

Volocity

Volocity (Improvision) was used to produce 3D reconstructions and *en face* views of cell conjugates.

Volocity was also used to measure filopodia lengths and synapse areas.

Results

Development of the instrument

Optical tweezers were implemented in a commercial confocal microscope designed for live-cell imaging. They were designed to allow three-dimensional manipulation of live-cell conjugates while performing confocal imaging.

In general, 1-10 mW in the back aperture of a high NA microscope objective is necessary to trap cells. IR light is also preferable for cell trapping for two reasons: cells are mostly transparent in that spectral region and are therefore less damaged by thermal effects during trapping, and IR light can be easily decoupled from the visible spectrum using an appropriate set of filters, ensuring that the

use of the optical trap would not restrict the range of fluorescent tags that could be imaged in the visible range. A single-mode laser diode emitting at 980 nm with a maximum output power of 600 mW was coupled into the microscope and focused into its focal plane to generate optical traps (damage to cells at this particular wavelength is known to be minimal [202]). Two independently actuatable traps were produced using two polarising beam splitters (Figure 11).

Trapped cells can be positioned in all three dimensions using steering mirrors (along x and y axis) and relay lenses (along z axis). This can be done across the entire field of view thanks to adequate pupil relay (Figure 12 and Supplementary movie 1). Importantly, no restrictions on the initial imaging performance of the microscope are introduced while optical tweezers are being used.

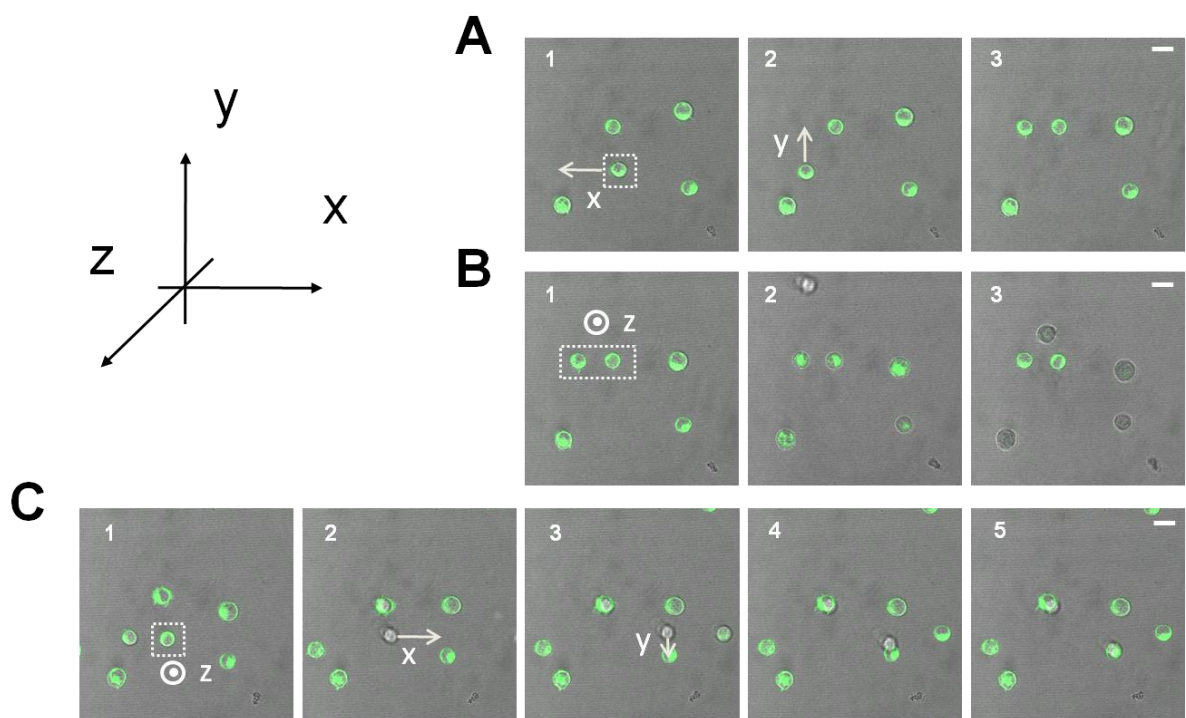


Figure 12: Cell manipulation in three dimensions while fluorescence confocal imaging is performed. T cells are fluorescently stained with a membrane dye. (A) x and y positioning. (B) z positioning. (C) x,y and z positioning combined, showing that the range of movement is sufficient to place one cell on top on the other. Scale bar, 10 μm . (Supplementary movie 1).

Next we set out to test if our set up could be used to arrange cell conjugates in a vertical configuration (Figure 10). To do this, cell conjugates were trapped after they formed, and the trap focus was then moved above the imaging plane. Due to gravity, cell conjugates usually arrange themselves in a vertical configuration (Figure 13 and Supplementary movie 2). Some suspension cells can slightly adhere to the bottom of the imaging chambers (Labtek) and can then be difficult to trap. In such cases, the imaging chamber was coated with FCS for a few minutes prior to cell imaging to block residual charges and reduce cell adhesion to the bottom of the chamber.

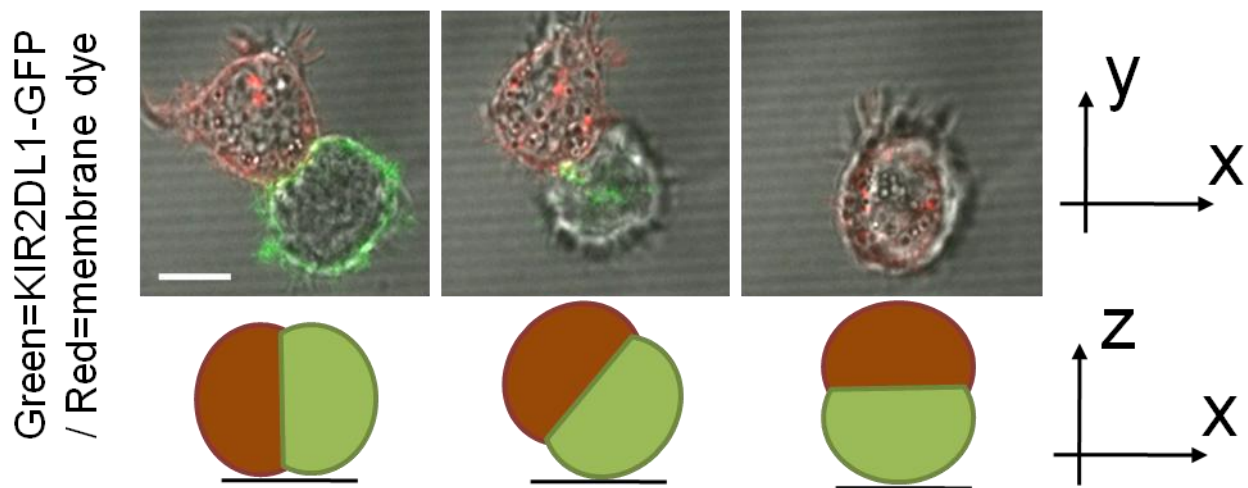


Figure 13: Manipulation of cell conjugates using optical tweezers to arrange the synapse into an *en face* imaging configuration in a confocal microscope. Using optical tweezers, the red cell is moved on top of the green cell. Green cell, YTS/KIR2DL1-GFP. Red cell, 721.221/Cw6 stained with DiD. Scale bar, 10 μm . (Supplementary movie 2).

The z position of the traps is set relatively to the focal plane of the objective by moving the relay lenses along the optical axis. Therefore, when the objective is moved up or down to image different horizontal planes within a trapped object, the traps will move by the same amount, and so will the trapped object if it is in suspension which means the same plane within the object will remain in the focal plane. Wavefront engineering has allowed absolute z-positioning of the traps by compensating for the movement of the microscope objective in real time [203, 204]. If these approaches can be

useful to image objects or cells trapped in suspension, they add unnecessary complexity when the sample lies on a coverslip such as cell conjugates forming a synapse. In that latter case, after initial manipulation, the power of the trapping beam can be set high enough to keep the cells in the desired configuration, but not so high that they would not move along the z axis when the user refocuses. Using this simple method, any plane can be imaged in vertically oriented conjugates (Figure 14 & Supplementary movie 3).

Importantly, this technique does not introduce any restrictions on the range of fluorophores that can be normally imaged in a given microscope which makes it a very versatile and flexible tool. In principle, it can also be combined to one of the super-resolution techniques mentioned before such as STED.

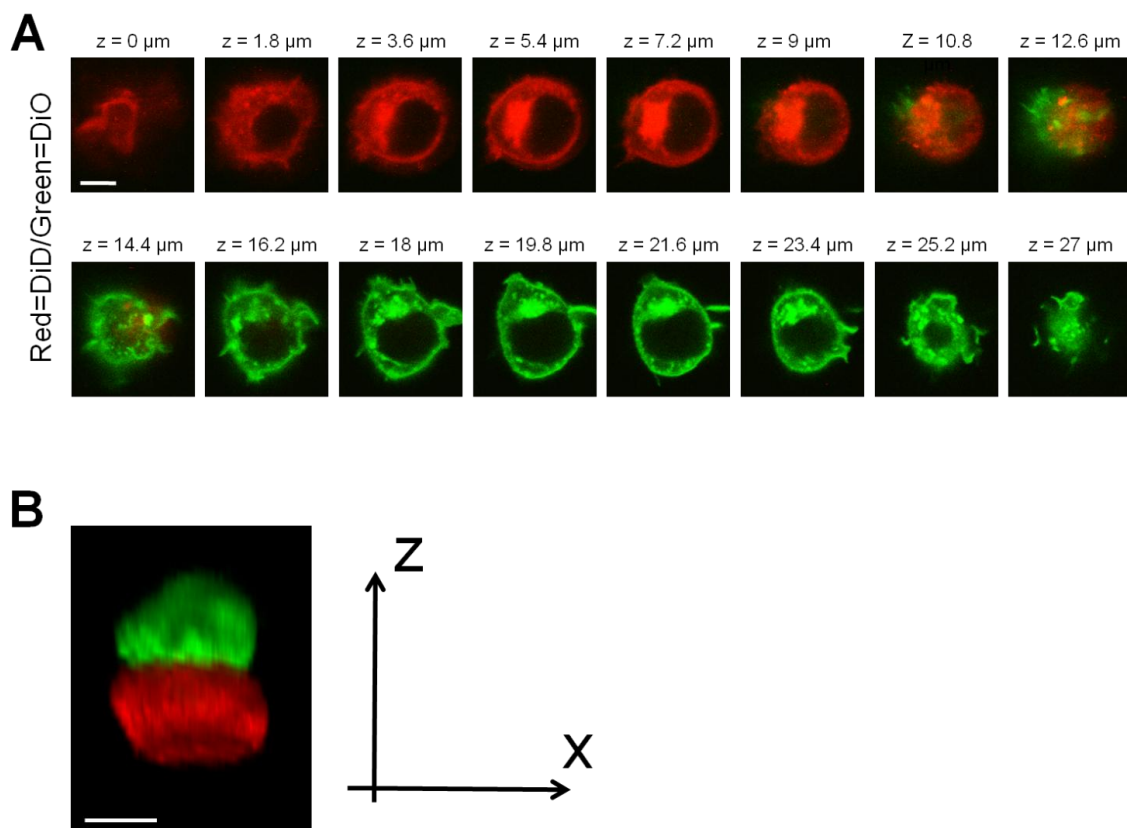


Figure 14: Confocal stack acquired through a vertically oriented cell conjugated. (A) z-stack through vertically-oriented optically trapped cell conjugates. Jurkat T cells are stained with spectrally distinct membrane dyes (Red=DiD, Green=DiO). Any plane within the conjugates can be focused on and imaged while cells are being trapped. (B) 3D reconstruction from optical sections in (A). Scale bars, 5 μm. (Supplementary movie 3).

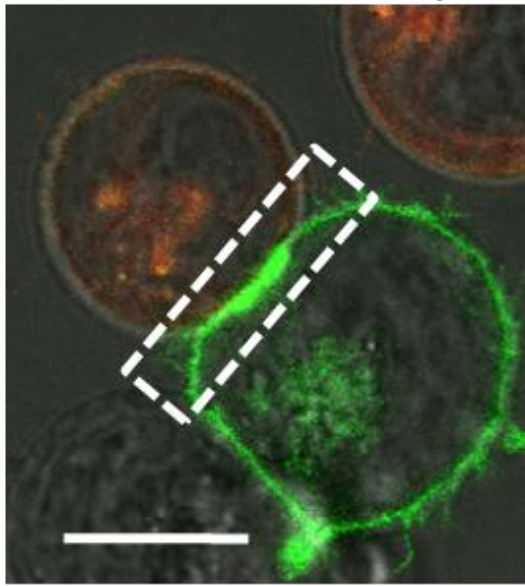
Demonstration of the instrument on T cell and NK cell immunological synapses

We showed that cell conjugates could be manipulated using optical tweezers so that the cell-cell interface lies *en face* to the imaging plane of a fluorescence confocal microscope. In this section we demonstrate that this technique can be used to image intercellular immunological synapses at high speed and high resolution. We imaged inhibitory receptors at the NK cell immunological synapse, T-cell receptor, proximal signalling molecules and intracellular vesicles at the T cell/APC immunological synapse with unprecedented temporal and spatial resolution.

Imaging of the inhibitory receptor KIR2DL1 at the human inhibitory NK cell synapse

We first imaged the supramolecular distribution of the inhibitory receptor KIR2DL1-GFP at the human inhibitory NK cell immunological synapse. From previous studies, it is known that KIR accumulates at the inhibitory NK cell synapse [78, 85, 88]. Using a well-characterised system - YTS/KIR2DL1-GFP as NK cells expressing KIR2DL1-GFP and 721.221/Cw6 as targets expressing a ligand for KIR - we imaged the distribution of KIR2DL1 at the immunological synapse formed between YTS/KIR2DL1-GFP and 721.221/Cw6 cells [205] using a three-dimensional reconstruction of a confocal stack of 40 slices across the synapse sampling at every 380 nm (Figure 15). The acquisition of a typical z-stack requires around 30 s so the final image exhibits an acceptable signal-to-noise ratio. As expected, on the 3D reconstruction, only micron-scaled details such as the central accumulation of KIR can be seen.

Green=KIR2DL1-GFP /
Red=membrane dye



3D reconstruction of
KIR2DL1-GFP

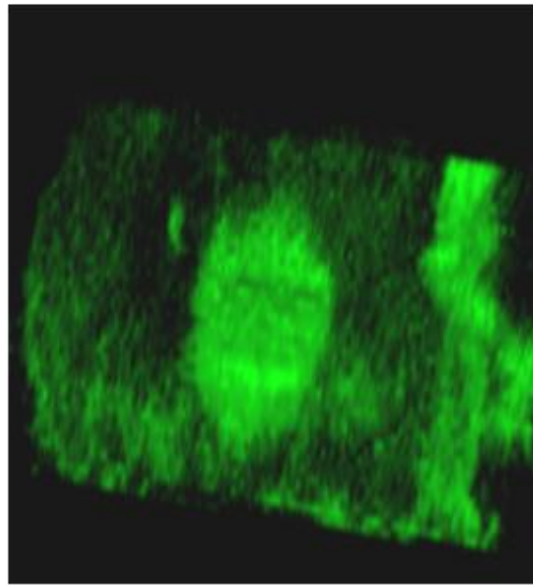


Figure 15: Conventional z-stack imaging and 3D reconstruction of a synapse from 40 slices. Between 30 s to 60 s were necessary to acquire a full z-stack (1 frame). The synapse shown is an inhibitory synapse formed between a NK cell expressing a GFP-labelled inhibitory receptor (YTS/KIR2DL-GFP, green) and a target cell expressing a ligand for this receptor (721.221/Cw6, red). The accumulation of KIR2DL1-GFP is present at the cell-cell interface (dotted box). After 3D reconstruction, the accumulation of the receptor at the centre of the synapse can be observed. Scale bar, 10 μm .

Conjugates were then oriented into an *en face* configuration using the optical traps. Thus we realised high spatial resolution (250 nm), high-speed (>1 frame per second) imaging of the immune synapse between live cell conjugates. In agreement with previous studies [88], we observed that the central accumulation of KIR2DL1 featured small areas in which the receptor was excluded (Figure 16 and Supplementary movie 4).

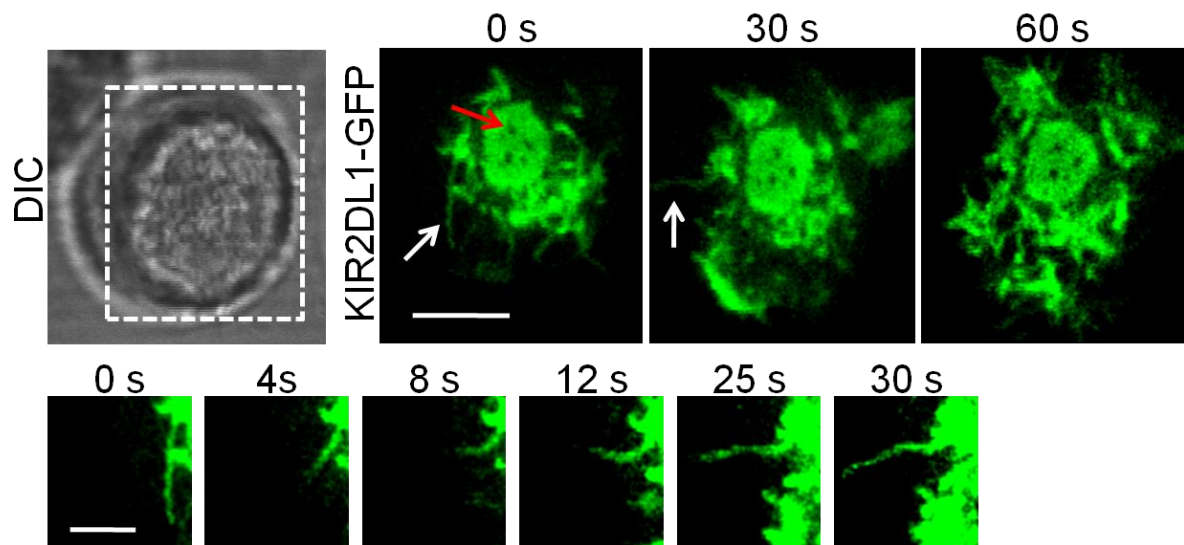


Figure 16: Top: high-speed, high-resolution imaging of a synapse between YTS/KIR2DL1-GFP and 721.221/Cw6 (as in Figure 15) using optical tweezers. Left: DIC image showing the vertical arrangement of the cells. Right: time-lapse live-cell fluorescence imaging showing the presence of small central exclusion zones (red arrow) and filopodial-like protrusions (white arrow) that were not observable using conventional 3D confocal imaging (Figure 15). Scale bar, 5 μm . Bottom: time series showing the highly active nature of filopodial extensions around the immune synapse. Scale bar, 2 μm . (Supplementary movie 4).

Importantly, new dynamic features of the inhibitory NK cell synapse that were not previously observable were also revealed. Our new approach allowed us to witness, for the first time at intercellular synapses, the formation of this central accumulation from small clusters of KIR2DL1 that moved to the centre from the synapse periphery (Figure 17). Surprisingly, numerous long (up to 12 μm) and highly dynamic protrusions were also detected within and around the distal regions of the immune synapse (Figure 16). Such structures are known to be important for cell adhesion and environmental probing [206]. Therefore, their highly motile nature may play a role in initiating or sustaining signalling, perhaps by allowing NK cells to probe large areas of the interface and augment the number of receptor/ligand binding events at the synapse.

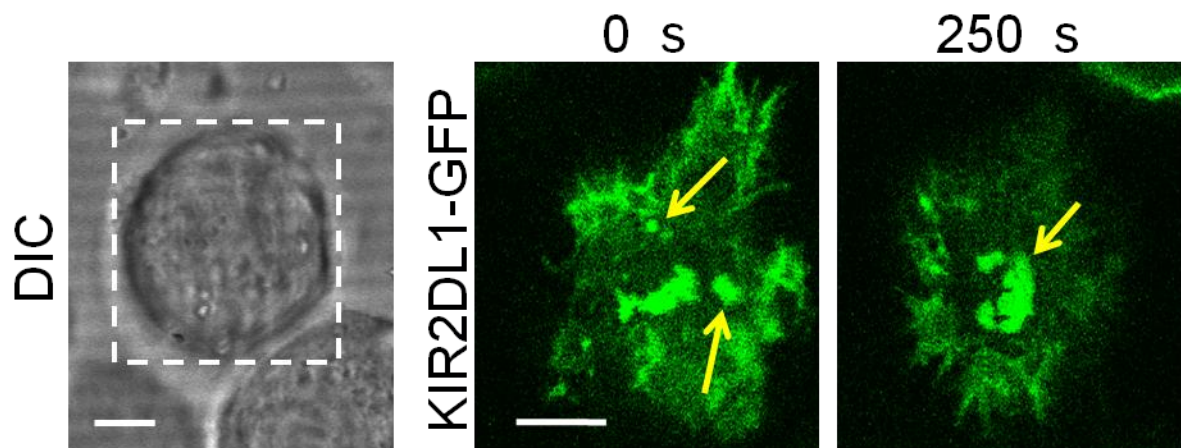


Figure 17: High-resolution imaging using optical tweezers and confocal microscopy of inhibitory synapses between YTS/KIR2DL1-GFP and 721.221/Cw6 shows the formation of the central accumulation of KIR2DL1 from peripheral clusters that coalesce (yellow arrows). Left: DIC image. Right: selected fluorescence frames from the KIR2DL1-GFP channel. Scale bars, 5 μm .

High-resolution imaging of T cell receptor and signalling proximal molecules at the T cell/APC immunological synapse

As described earlier in this report, T cell activation is accompanied by micron-scale segregation of proteins at the T cell/APC interface as demonstrated by early imaging studies [51, 52]. To date, however, the highest-resolution images have been obtained using artificial activating substrates [207-209]. Importantly, these recent improvements in imaging resolution have revealed that small protein microclusters are important for TCR signalling.

For this work, we first imaged the distribution of T cell receptors at an intercellular immunological synapse at high-speed and high-resolution after manipulation with optical tweezers. We then

investigated the supramolecular organisation of the fluorescently-tagged ZAP70, SLP-76, GADS and LAT proteins as well as interactions of SLP-76 with sub-synaptic vesicles.

T cell receptor imaging

We imaged the distribution of T cell receptors at intercellular synapses at high speed and high resolution using optical tweezers. We incubated Jurkat T cells expressing the YFP-tagged protein CD3 ζ together with APCs (Raji B cells pulsed with super-antigen) until they formed mature synapses. As expected, CD3 ζ accumulated at the centre of the synapse (Figure 18A and Supplementary movie 5). Numerous discrete clusters containing CD3 ζ were also detected in the periphery of the synapse, consistent with recent studies showing microclusters of T cell receptors that form in synapses with artificial planar stimuli previously identified as the main site for T cell signal transduction [54, 55, 207]. Strikingly, CD3 ζ present in the central accumulation appeared distributed in a ring, mostly excluded from the very centre of the synapse, consistent with this area being the site from which CD3 ζ is internalised [63]. Also, increased spatial and temporal resolution allowed CD3 ζ clusters to be studied between live conjugates. Tracking of these clusters revealed that trajectories were not necessarily centripetal as described in previous studies using planar-bilayer based stimuli (Figure 18B and C), with 30–40% of the clusters moving away from the centre to the synapse periphery. Due to an increased signal/noise ratio, quantitative analysis of cluster fluorescence intensity could also be undertaken to investigate their composition (Figure 18D). This size distribution exhibited a wide range, with some clusters being up to five times as large as the smallest detectable ones.

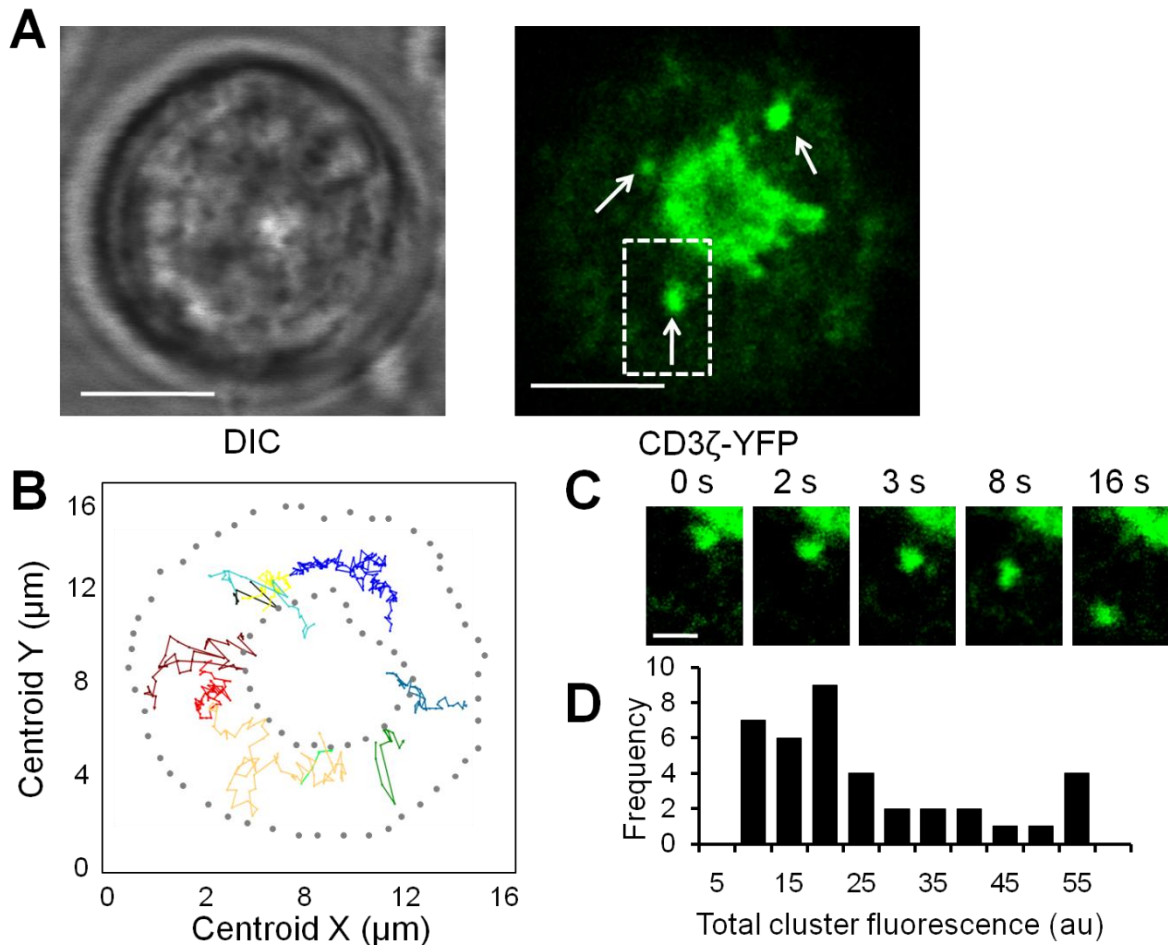


Figure 18: High-resolution, high-speed imaging of CD3ζ-YFP at the intercellular T cell/APC immunological synapse using optical tweezers. (A) Representative image of CD3ζ at the mature T cell/APC immunological synapse (examples of discrete clusters are shown by arrows). Left: DIC image. Right: fluorescence channel (CD3ζ-YFP). Scale bar, 5 μm. (B) Tracks followed by 10 representative clusters of CD3ζ-YFP within the synapse shown in A. Dotted lines highlight the limits of the centre and periphery of the synapse. (C) Example of high-speed imaging revealing the movement of a protein cluster from the centre to the periphery of the synapse. Scale bar, 2 μm. (D) Distribution of the fluorescence intensity of 40 CD3ζ clusters. (Supplementary movie 5).

Proximal signalling molecules: ZAP70, SLP-76, LAT and GADS at the T cell/APC

immunological synapse

Upon recognition of pMHC molecules by TCR, engagement of co-receptor CD4 activates Lck, which then phosphorylates ITAM motifs present on CD3γ, CD3δ, CD3ε and CD3ζ. ZAP70 then binds to CD3ζ's fully phosphorylated ITAMs via its two SH2 domains. Lck activates ZAP70, which in turn phosphorylates the adaptors LAT and SLP-76, the latter associating with GADS (Figure 19). The work

presented here was performed is part of a larger study on the role the endosomal network in T cell signalling [59].

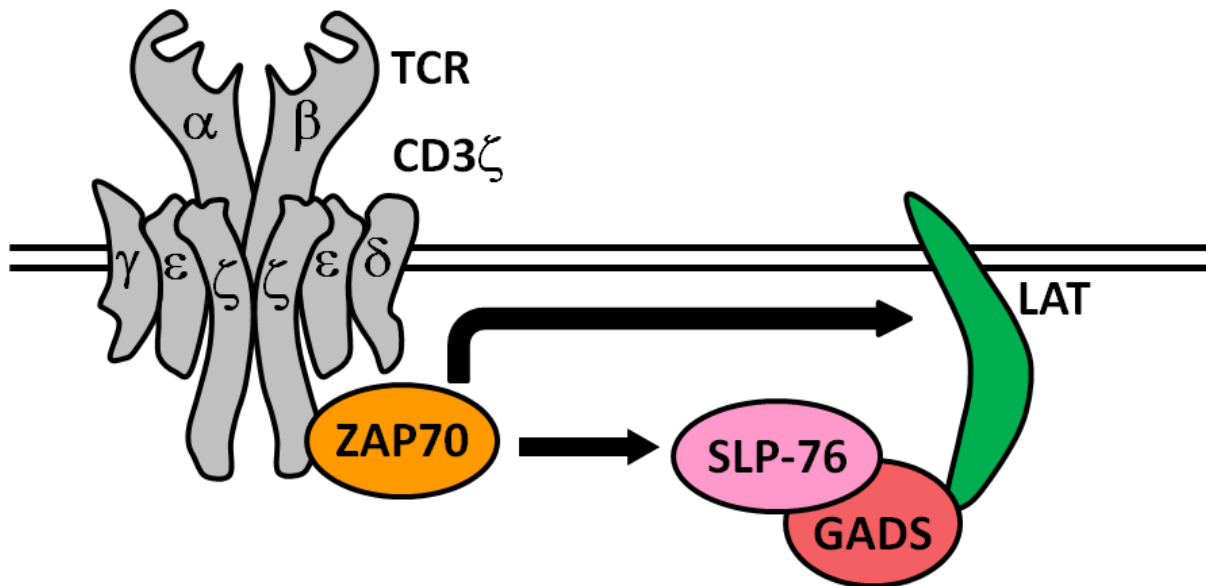


Figure 19: Simplified schematic of the first steps of the T cell activation signalling pathway. Upon TCR activation, ZAP70 binds CD3 ζ and activates LAT/GADS and SLP-76. The black lines represent the plasma membrane.

First we imaged SLP-76-YFP together with either ZAP70, LAT or GADS-mCherry (Figure 20, Figure 21 and Figure 22 respectively). This revealed that SLP-76 formed clusters distinct from those formed by ZAP70 and LAT at intercellular immunological synapses (Figure 20 and Figure 21). Also, SLP-76 and GADS consistently co-localised, therefore agreeing with biochemical data as well as imaging on antibody-coated slides (Figure 22). Comparisons with conventional 3D reconstructions show that this latter technique does not provide sufficient spatial resolution to distinguish such small protein clusters (top rows in Figure 20, Figure 21 and Figure 22).

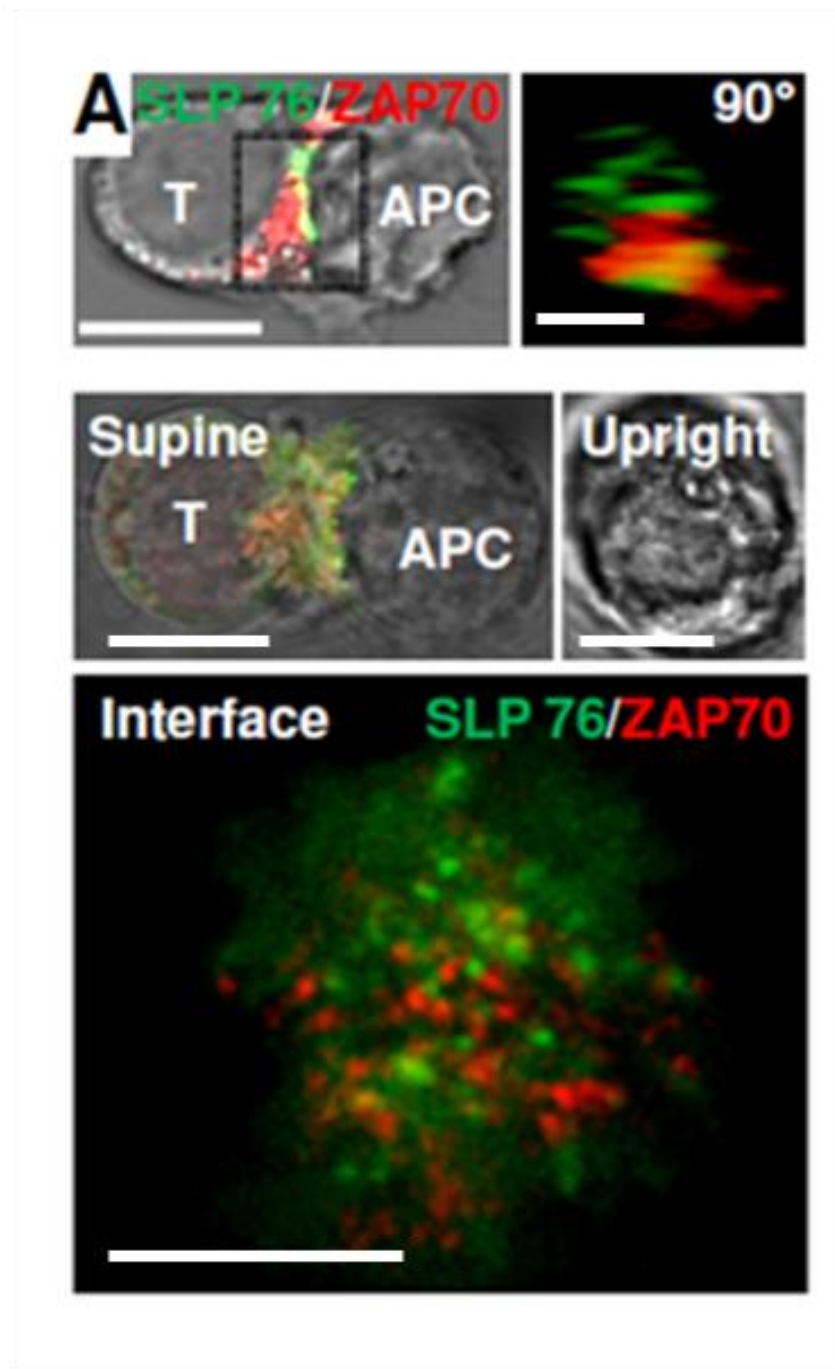


Figure 20: Two-colour fixed-cell imaging of proximal signalling molecules SLP-76-YFP and ZAP70-mCherry at the T cell/APC immunological synapse. Top row: image of the synapse obtained using conventional 3D reconstruction from 50 confocal slices. Bottom: side-view of cell conjugate and high-resolution image of the synapse using optical tweezers showing that SLP-76 and ZAP70 accumulate in distinct microclusters. Scale bars, 10 μm .

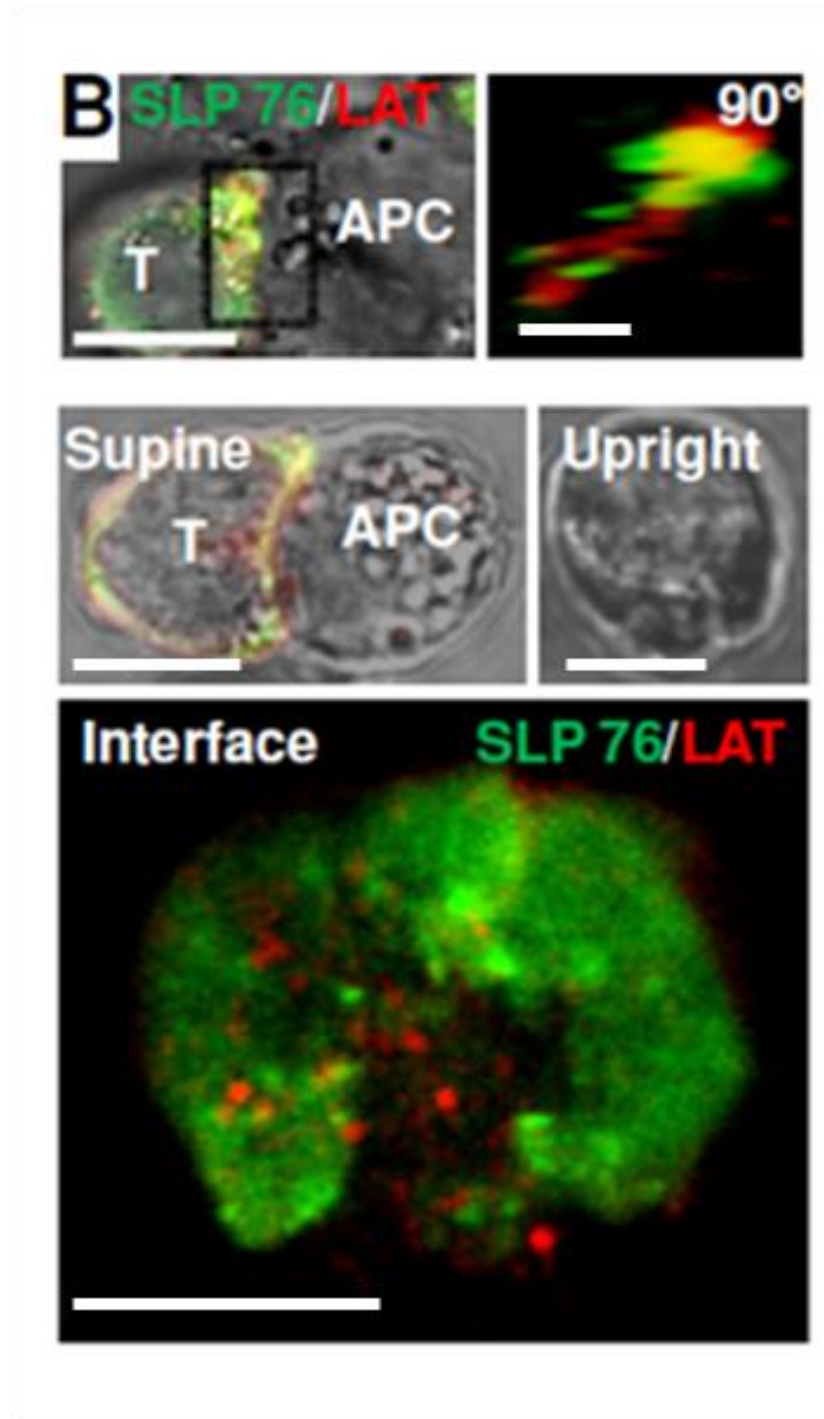


Figure 21: Two-colour fixed-cell imaging of proximal signalling molecules SLP-76-YFP and LAT-mCherry at the T cell/APC immunological synapse. Top row: image of the synapse obtained using conventional 3D reconstruction from 50 confocal slices. Bottom: side-view of cell conjugate and high-resolution image of the synapse using optical tweezers showing that SLP-76 and LAT accumulate in distinct microclusters. Scale bars, 10 μm .

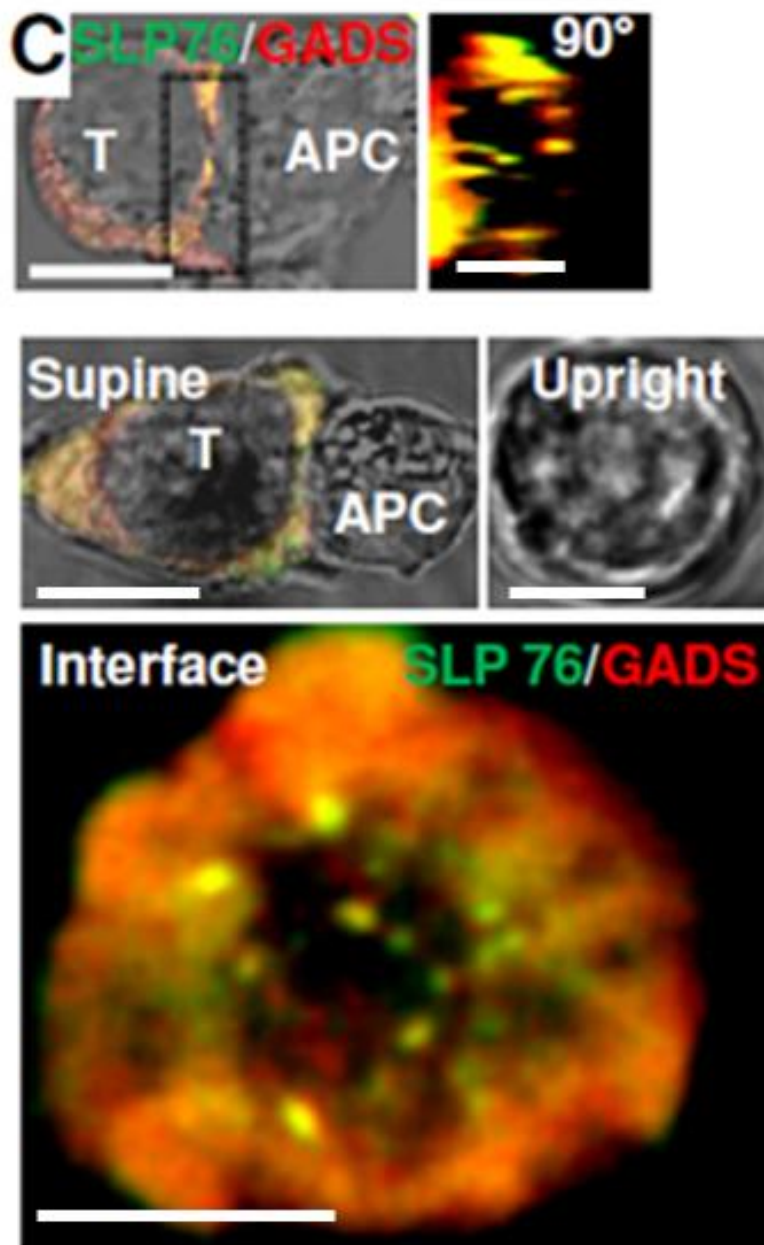


Figure 22: Two-colour fixed-cell imaging of proximal signalling molecules SLP-76-YFP and GADS-mCherry at the T cell/APC immune synapse. Top row: image of the synapse obtained using conventional 3D reconstruction from 50 confocal slices. Bottom: side-view of cell conjugate and high-resolution image of the synapse using optical tweezers showing that SLP-76 and GADS accumulate in the same microclusters. Scale bars, 10 μm .

The role of the endosomal network in T cell signalling has been very little studied, mainly because the resolution provided by existing techniques was insufficient to be able to image the movement of small and fast moving vesicular compartments near the synapse. We stained vesicles in SLP-76-YFP transfectants and imaged the immunological synapse formed with super antigen-pulsed Rajis (Figure 23A). Strikingly, many vesicles could be seen to interact with SLP-76 microclusters for up to 10 s (Figure 23B and Supplementary movie 6). Also, vesicles exhibited much reduced speeds during contact with a slow-moving SLP-76 cluster than when moving freely (Figure 24).

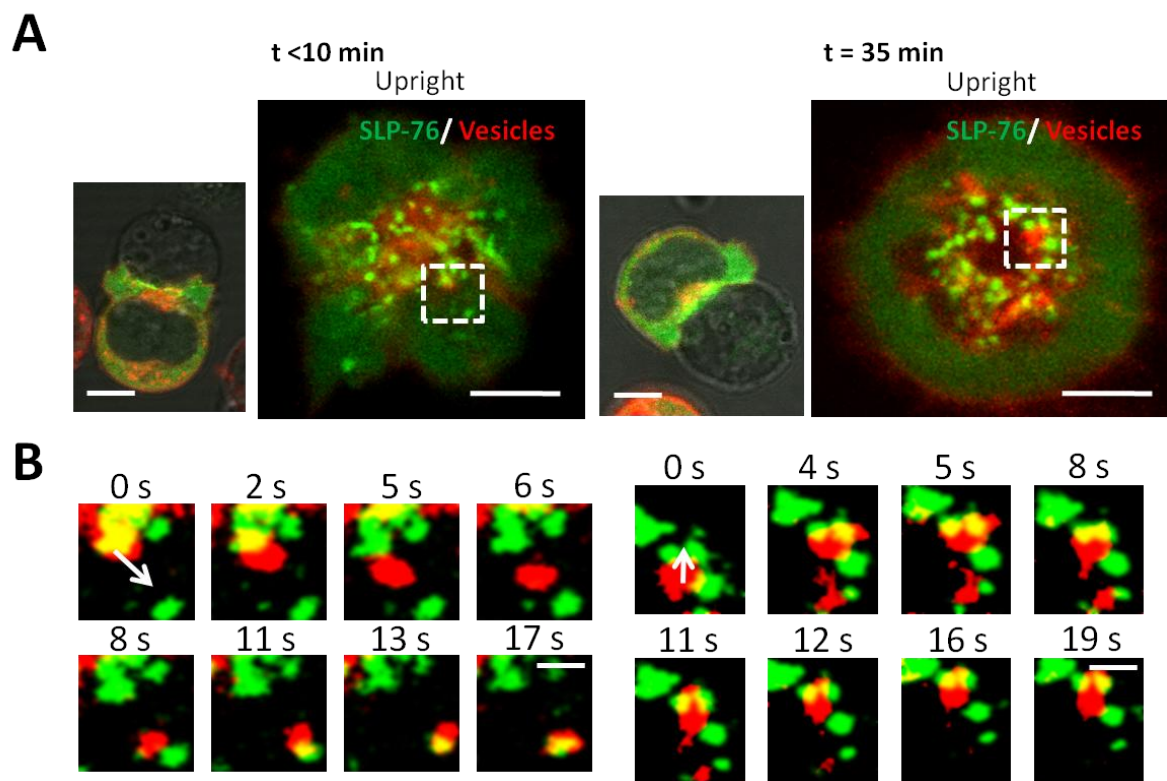


Figure 23: High-resolution live cell imaging of the T cell/APC synapse reveals SLP-76 interacts with sub-synaptic vesicles. (A) Side view and upright (i.e. using optical tweezers) imaging of SLP-76-YFP (green) and vesicles marked by internalised DiD (red) at t=10 min and t=35 min. (B) Examples of vesicle/SLP-76 interaction. White arrows show vesicles' initial movement direction. Insets are from dotted windows in (A). Scale bars, 10 μ m. (Supplementary movie 6).

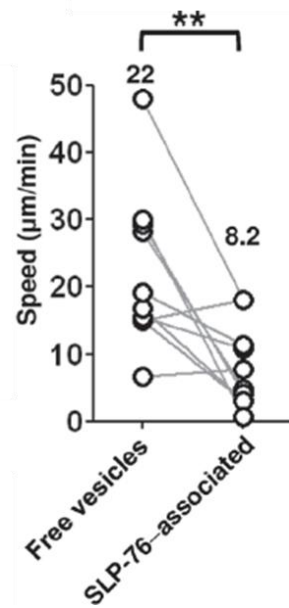


Figure 24: Comparison of the speed of free vesicles with SLP-76-associated vesicles. Vesicles dramatically slow down when contacting slow-moving SLP-76 clusters

Conclusion

In this chapter, we described the development of an instrument that is designed to perform fluorescence imaging of immunological synapses by combining confocal microscopy with optical tweezers. Compared to state-of-the-art techniques that use artificial target cells or APC, our system allows high-speed high-resolution imaging of immunological synapses to be performed in largely improved physiological conditions and with optimum flexibility: firstly, live conjugates can be used; secondly, cells can be imaged in normal culture medium; thirdly, using the tweezers does not affect the performance of the confocal microscope, i.e. it does not restrict the range of fluorophores that

could normally be used and finally there is no need for time-consuming sample preparation prior to imaging e.g. regular imaging chambers can be used.

We demonstrated the versatility of our instrument by imaging membrane receptors and intracellular signalling molecules at various live as well as fixed intercellular immunological synapses, with unprecedented temporal and spatial resolution. We first imaged the inhibitory receptor KIR at the live inhibitory NK cell/target cell synapse. In agreement with previous studies, we observed that the central accumulation of KIR featured small areas in which the receptor was excluded. Our new approach allowed us to observe, for first time at intercellular synapses, the formation of this central accumulation from small clusters of KIR that moved to the centre from the synapse periphery. Numerous long and highly dynamic protrusions were also detected within and around the distal regions of the synapse.

We then applied our system to the T cell/APC immunological synapse. When we imaged TCRs, an accumulation could be observed in the central region, while small clusters of TCRs formed in the periphery. Tracking of these clusters showed that their trajectories were not necessarily centripetal as described in previous studies using planar lipid bilayers as surrogate APC. Then, as part of a wider work about the role of sub-synaptic vesicles in T cell signalling [59], we imaged the intracellular signalling molecules ZAP70, LAT, SLP-76 and GADS in two-colour in fixed cells using optical tweezers. This revealed that SLP-76 formed clusters distinct from ZAP70 and LAT clusters, but associated with GADS. We then performed live-cell two-colour imaging with the tweezers that showed that SLP-76 microclusters formed at the intracellular immunological synapse transiently interacted with sub-synaptic vesicles.

Chapter V: Imaging NKG2D signalling at the activating NK cell immunological synapse

Introduction: Role of NKG2D and its ligands in immunity

NKG2D is one of the best characterised activating immunoreceptors which are associated to a response against distressed cells. Here we discuss why expression of NKG2D by NK cells is important in fighting tumours as well as infected cells.

Expression pattern of NKG2D

NKG2D was initially discovered in NK cells and T cells [210] and within humans, most NK cells and CD8⁺ T cells express NKG2D. In NK cells, NKG2D is upregulated by exposure to IL-15 [211], while most peripheral blood CD8⁺ T cells express NKG2D before activation [26, 212]. NKG2D is also expressed in peripheral blood and intraepithelial $\gamma\delta$ T cells [26, 213], but is not detected on LPS-activated human macrophages [214] and CD4⁺ T cells [26].

Ligands for NKG2D: structure and binding

The first protein that was found to bind NKG2D was MICA (MHC class-I-chain-related protein A), a soluble form of a non-classical class I molecule encoded in the MHC [26]. MICA was found to bind some lymphocytes, and the antibody developed to block the interaction was found to bind NKG2D. Subsequently, MICB, a protein close to MICA was found to bind NKG2D too. Other proteins binding to the UL16 protein of human cytomegalovirus (HCMV), were found to be ligands for NKG2D, and were called UL16-binding proteins (ULBPs) [27]. These ligands exhibit distinct structures and generally high affinity with NKG2D relative to other immunoreceptor/ligand interactions [215-217]. Structural analysis showed that MICA and ULBPs have similar structures with $\alpha 1$ and $\alpha 2$ domains similar to MHC class I molecules. Unlike MHC class I, however, the groove is closed and does not allow peptide binding. Interestingly, all ligands interact the same way with NKG2D, as the receptor amino-acids

that control the binding are the same and several contact residues are conserved between these ligands. There are some notable differences in structure between these ligands such as the presence of an $\alpha 3$ -like domain in MICA that is absent in ULBPs and the fact that some ULBPs are linked to the membrane by GPI anchors whereas other ULBPs, MICA and MICB are type-I transmembrane proteins.

Expression patterns of NKG2D ligands

A lot remains unknown about the expression patterns of such ligands. The current hypothesis is that their expression levels remain low on normal cells but get upregulated in pathological conditions [218]. Thus, MICA and MICB were only found to be expressed by intestinal epithelial cells [219]. Many tumour cell lines and primary tumours express MICA and MICB as a result of the activation of the heat shock transcription elements in the corresponding gene promoter, a mechanism which is involved in cell transformation [219]. Cell-surface expression of ULBPs by normal cells is generally low, although the level of corresponding mRNA, unlike MICA and MICB's mRNA, is present at notable levels in some normal cells [27]. Again, unlike MICA and MICB, the expression of ULBPs is not upregulated by heat-shock elements. Importantly, the expression of all NKG2D ligands is upregulated upon cell infection [212, 220].

Stimulation of NK cells through NKG2D

The activation of NKG2D has different outcomes depending on the stimulation state of NK cells. For freshly isolated NK cells, NKG2D is only a costimulatory receptor. In this context, NKG2D activation alone is not sufficient for cytotoxicity, proliferation and production of TNF- α and IFN- γ , but NKG2D activation enhances these effector functions when other activating receptors are triggered [49]. NKG2D activation alone is sufficient to trigger NK cell effector functions with activated NK cells [50].

Signalling pathways

In humans, NKG2D is expressed on the surface of NK cells by coupling to a DAP10 transmembrane adaptor [45]. The final complex is a hexameric structure formed of two NKG2D molecules each

coupled with two DAP10 adaptors [46]. Unlike other signalling adaptor proteins, DAP10 does not possess a classical ITAM motif but instead features a stimulatory YIMN motif [45]. Like ITAM receptors, DAP10 undergoes phosphorylation by a Src family kinase e.g. Lck upon ligation of NKG2D with one of its ligands. Unlike ITAM receptors however, DAP10 activation does not recruit nor phosphorylate ZAP70 or LAT proteins [47]. Instead, DAP10 signals through two distinct signalling pathways, the DAP10-PI3K pathway and the DAP10-Grb2 pathway, the latter signalling through VAV1, PLC- γ 2 and SLP-76. Both pathways are required for NK cell effector functions [48].

Role of NKG2D in tumour surveillance

As shown by blocking antibodies and transfection experiments, the expression of NKG2D ligands markedly increases the sensitivity of tumour cells *in vitro* [26, 221, 222]. The expression of NKG2D ligands *in vivo* also induces destruction of tissue [223]. However, as evidenced by the expression of these ligands by tumour cell lines [222, 224], tumours have evolved escape mechanisms to the expression of NKG2D ligands such as ligand downregulation [223] and NKG2D desensitisation by the production of high-levels of soluble MICA by some tumours [225, 226].

Due to its importance in cancer immunity, it is essential to understand NKG2D signalling not only in terms of protein structure and signalling pathways, but also *in situ*, at the synapse. Integration of activating signals by NK cells and subsequent lytic granule delivery are still extremely poorly understood, partly due to the lack of imaging techniques providing sufficient resolution of intercellular synapses [14, 68, 227, 228]. To address these issues, we use the high-resolution imaging technique presented in the previous chapter to investigate NKG2D signalling at the intercellular immunological synapse. First, we will image how NKG2D clusters and signals at the activating NK cell synapse. We will then investigate how signalling is integrated by the downstream signalling molecules DAP10, Grb2 and VAV1. Effects of inhibitory signalling on NKG2D signalling itself and NKG2D-mediated lytic granule delivery will also be examined.

Material and Methods

Cells

Cell types

An NKL cell line was used to provide NK cell effectors. This cell line was established from the peripheral blood of a patient with CD3⁻CD16⁺CD56⁺ large granular lymphocyte leukemia. NKL cells can mediate natural killing and antibody-dependent cellular cytotoxicity and exhibit proliferative responses similar to normal CD16⁺CD56^{dim} NK cells. NKL are morphologically close to normal activated NK cells [229].

Daudi cells were used as target cells. Daudi cells are a Burkitt's lymphoma B cell line that lacks expression of β 2-microglobulin. Expression of MHC class I can be rescued by transfecting Daudi cells to express β 2-microglobulin [230]. Here we used Daudi cells expressing MICA with or without MHC class I [231].

721.221 were also used as target cells in some experiments 721.221 cells are a MHC class I-negative human B-lymphoblastoid cell line [232].

Cell culture

NKL cells were cultured in R10 medium (RPMI 1640 medium supplemented with 10% FCS, 100 μ g/ml Penicillin-Streptomycin and L-glutamine (Invitrogen)) and 100 U/mL of recombinant IL-2 (Roche) at 37°C in a 5% CO₂ atmosphere. Cells were passaged at a ratio of 10:1 or 10:2 every 3 days. 722.221 cells were cultured in R10 media. Daudi cells were cultured in R20 medium. Experiments were carried out the day after passaging.

Phenotyping of cells

For flow cytometry analysis, FACS Calibur flow cytometers (BD Biosciences) were used. To stain the cells, around 200-300 μ L of cell culture were washed in a 96-well plate (centrifugation at 4 °C, 1300 rpm). Cells were then incubated in FACS buffer (PBS supplemented with 3% BSA and 0.02% sodium azide) containing the primary antibody at a concentration of 10 μ g/mL in a 96-well plate for 45 min on ice. Cells were then washed with FACS buffer twice and then incubated with the labeled secondary antibody in the dark. Cells were then washed and resuspended in 200 μ L of FACS buffer and taken to the flow cytometer. Analysis was done using FlowJo (Tree Star) by gating on live cells.

NKL cells were transfected with various combinations of NKG2D-GFP, KIR2DL1-GFP or/and KIR2DL1-mCherry. The transfectants generated here are NKL/NKG2D-GFP, NKL/KIR2DL1-GFP, NKL/NKG2D-GFP KIR2DL1-mCherry. 721.221 expressing HLA-Cw4 were obtained from a previous study [78] and then transfected to express MICA. Daudi cells expressing MICA and MHC class I were obtained from a previous study [231] and sorted by flow cytometry to obtain Daudi/MICA and Daudi/MICA MHC class I populations.

Antibodies

Table 3 lists antibodies used for FACS and imaging.

Antibody	Imaging (I) or FACS (F)	Origin	Concentration	Species
α-NKG2D	F	R&D systems	10 μ g/mL	mouse
α-KIR2DL1 (EB6)	F	Immunotech	10 μ g/mL	mouse
α-MHC Class I (W6/32)	F	eBiosciences	10 μ g/mL	mouse
Microtubule anti-α-tubuline (DM1A)	I	Sigma-Aldrich	10 μ g/mL	mouse
α-Perforin (δG9)	I	BD Biosciences	10 μ g/mL	mouse
α-Phosphotyrosine (4G10)	I	Upstate	10 μ g/mL	mouse
MICA (159227)	I	R&D systems	10 μ g/mL	mouse
α-human Cy5 conjugated Affiniy pure Goat antimouse IgG	F & I	Jackson ImmunoResearch	10 μ g/mL	goat
Alexa-633 conjugated Phalloidin (α-F-actin)	I	Molecular Probes	10 μ g/mL	

Table 3: list of primary and secondary antibodies used for surface staining (FACS) and imaging.

Phenotyping of NKL/NKG2D-GFP

Analysis of NKG2D surface expression by flow cytometry on NKL cells shows that NKL cells express NKG2D endogenously. In NKL/NKG2D-GFP transfectants, NKG2D surface staining linearly correlates with GFP signal, therefore demonstrating expression of NKG2D-GFP at the membrane (Figure 25).

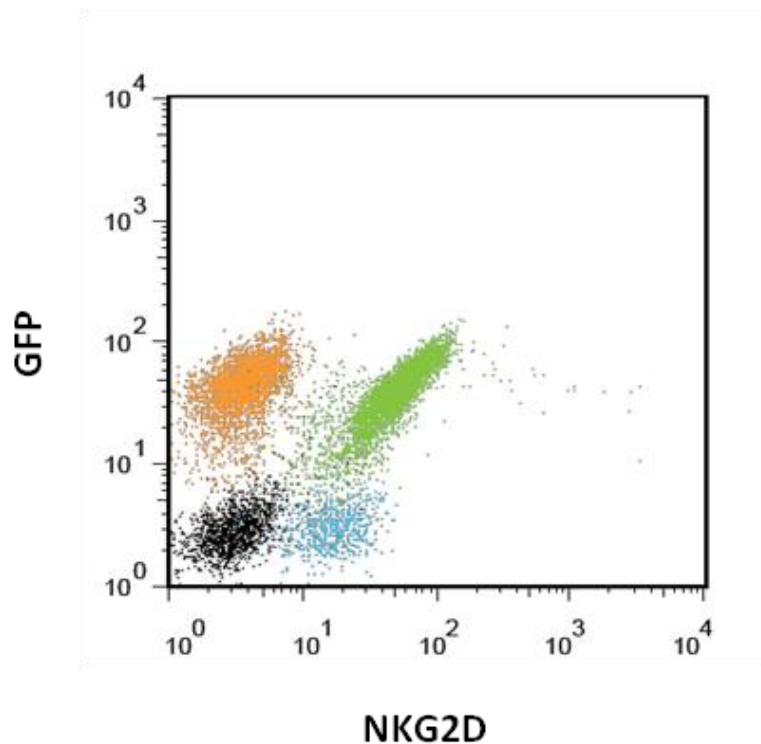


Figure 25: Cell surface expression of NKG2D correlated with GFP fluorescence in NKL and NKL/NKG2D-GFP. Total cell surface expression of NKG2D on NKL (blue) and NKL/NKG2D-GFP (green) shown relative to isotype control on NKL (black) and NKL/NKG2D-GFP (orange) assessed in live cells using flow cytometry. The expression of NKG2D expression was assessed using mAb staining.

Phenotyping of NKL/KIR2DL1-GFP

Analysis of KIR2DL1 surface expression by flow cytometry on NKL cells shows that NKL cells do not express KIR2DL1 endogenously. In NKL/KIR2DL1-GFP transfectants, KIR2DL1 surface staining linearly correlates with GFP signal, therefore demonstrating expression of KIR2DL1-GFP at the membrane (Figure 26). Remaining uncorrelated signal comes from presence of intracellular GFP, probably from recycling and degradation processes.

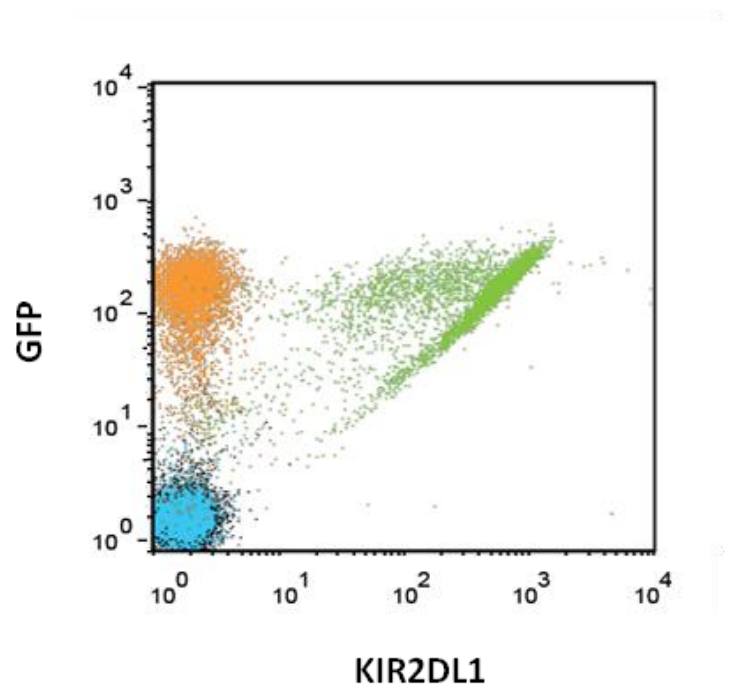


Figure 26: Cell surface expression of KIR2DL1 correlated with GFP fluorescence in NKL and NKL/KIR2DL1-GFP. Total cell surface expression of KIR2DL1 on NKL (blue) and NKL/KIR2DL1-GFP (green) shown relatively to isotype control on NKL (black) and NKL/KIR2DL1-GFP (orange) assessed in live cells using flow cytometry. The expression of KIR2DL1 expression was assessed using mAb staining.

Phenotyping of NKL/NKG2D-GFP KIR2DL1-mCherry

Analysis of NKG2D and KIR2DL1 surface expressions by flow cytometry on NKL and NKL/NKG2D-GFP KIR2DL1-mCherry double transfectants show that both fusion proteins are expressed at the surface and are consistent with what could be seen with single transfectants (Figure 27).

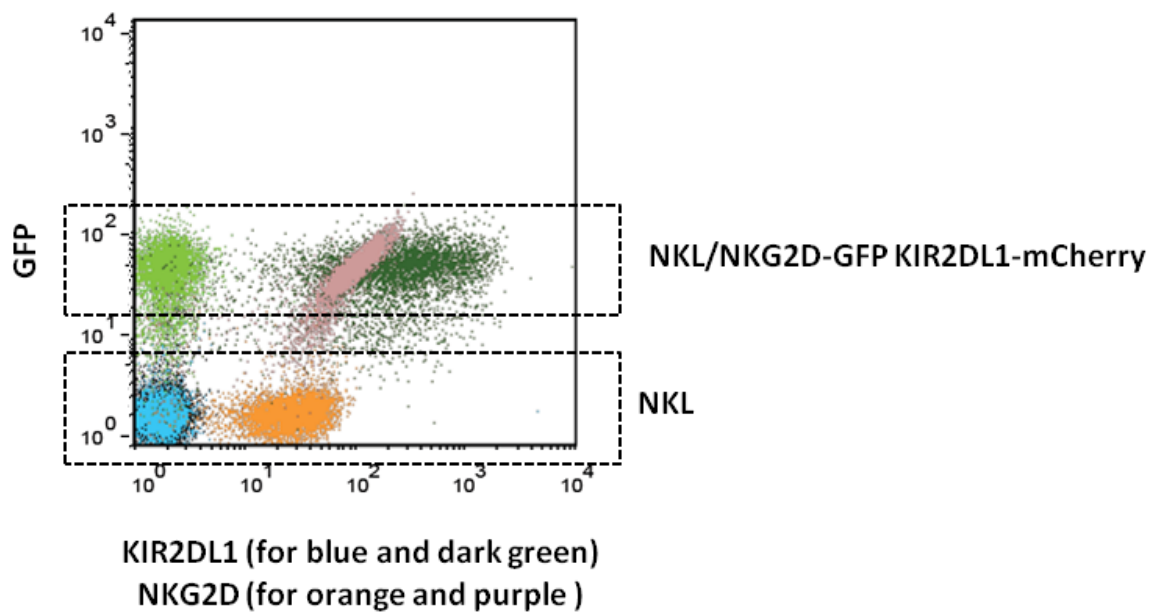


Figure 27: Cell surface expression of KIR2DL1 and NKG2D correlated with GFP fluorescence in NKL and NKL/NKG2D-GFP KIR2DL1-mCherry. Total cell surface expression of KIR2DL1 on NKL (blue) and NKL/NKG2D-GFP KIR2DL1-mCherry (dark green) shown relatively to isotype control on NKL (black) and NKL/NKG2D-GFP KIR2DL1-mCherry (light green) assessed in live cells using flow cytometry. Total cell surface expression of NKG2D on NKL (orange) and NKL/NKG2D-GFP KIR2DL1-mCherry (purple) shown relatively to isotype control (black) and unstained NKL/NKG2D-GFP KIR2DL1-mCherry (light green) assessed in live cells using flow cytometry. The expressions of NKG2D and KIR2DL1 expression were assessed using mAb staining.

Phenotyping of Daudi/MICA and Daudi/MICA MHC class I

Analysis of MICA and MHC class I surface expression by flow cytometry on Daudi/MICA and Daudi/MICA β 2m cells show that Daudi/MICA express MICA but not MHC class I while Daudi/MICA β 2m express both (Figure28).

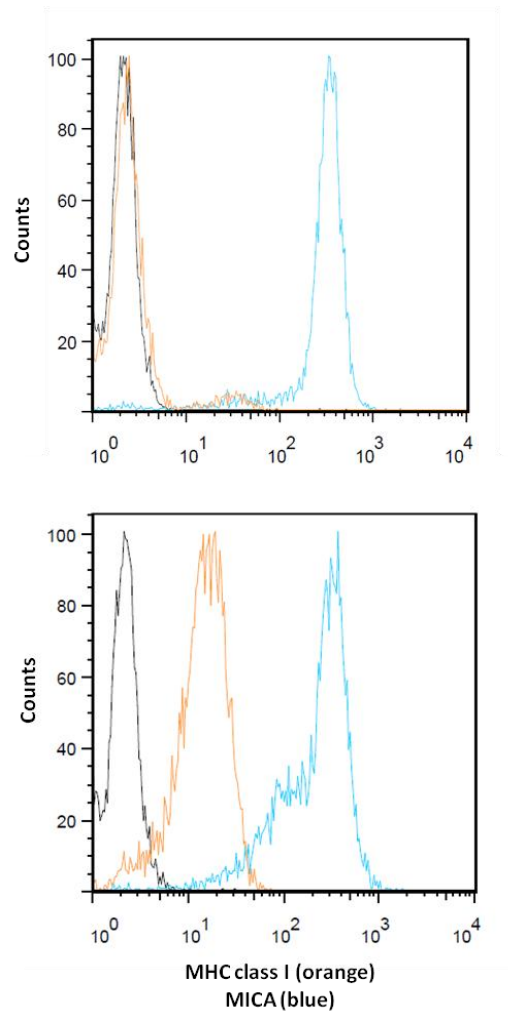


Figure 28: Cell surface expression of MHC class I and MICA on Daudi/MICA and Daudi/MICA β 2m. Cell surface expression of MICA (blue) and MHC class I (orange) on Daudi/MICA (top) and Daudi/MICA β 2m (bottom) shown relatively to isotype control (black) assessed in live cells using flow cytometry. The expressions of MHC class I and MICA were assessed using mAb staining.

Phenotyping of 721.221/MICA HLA-Cw4

Analysis of MICA and MHC Class I surface expressions by flow cytometry on 721.221/MICA HLA-Cw4 cells show that these cells express both MICA and MHC class I (Figure 29).

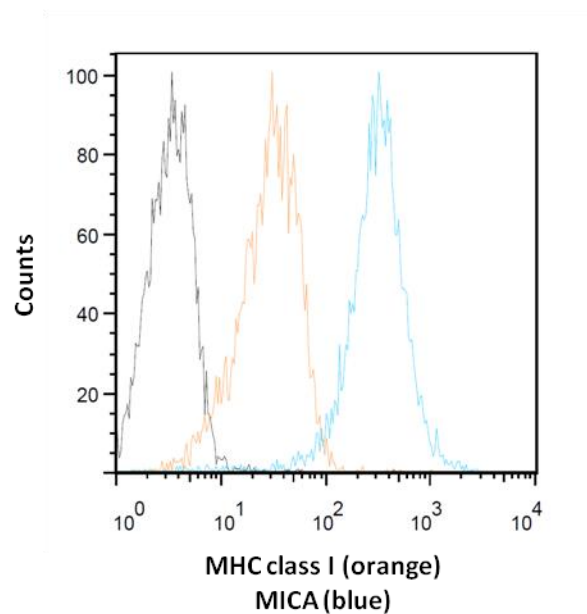


Figure 29: Cell surface expression of MHC class I and MICA on 721.221/MICA Cw4. Cell surface expression of MICA (blue) and MHC class I (orange) on 721.221/MICA Cw4 shown relatively to isotype control (black) assessed in live cells using flow cytometry. The expressions of MHC class I and MICA were assessed using mAb staining.

DNA Cloning

NKG2D-GFP and NKG2D-mCherry cloning strategies

A DNA fragment containing the NKG2D gene was obtained through PCR amplification of NKL cDNA. NKG2D is a C-type lectin and a type II transmembrane protein which means GFP had to be attached the N-terminus of NKG2D so the extracellular part of the protein can bind ligands.

First, NKG2D was tagged with GFP by inserting the NKG2D gene after the 3' end of the GFP gene in the pcDNA3.1 vector (Invitrogen). The gene coding for NKG2D-GFP was then subcloned into the PINCO vector as a BamHI-NotI fragment (Figure 30).

This was done by using the BsrGI restriction site that is present at the end of GFP as well as GFP-derived fluorescent proteins, and the NotI restriction site of the multiple cloning site (MCS) of the vector.

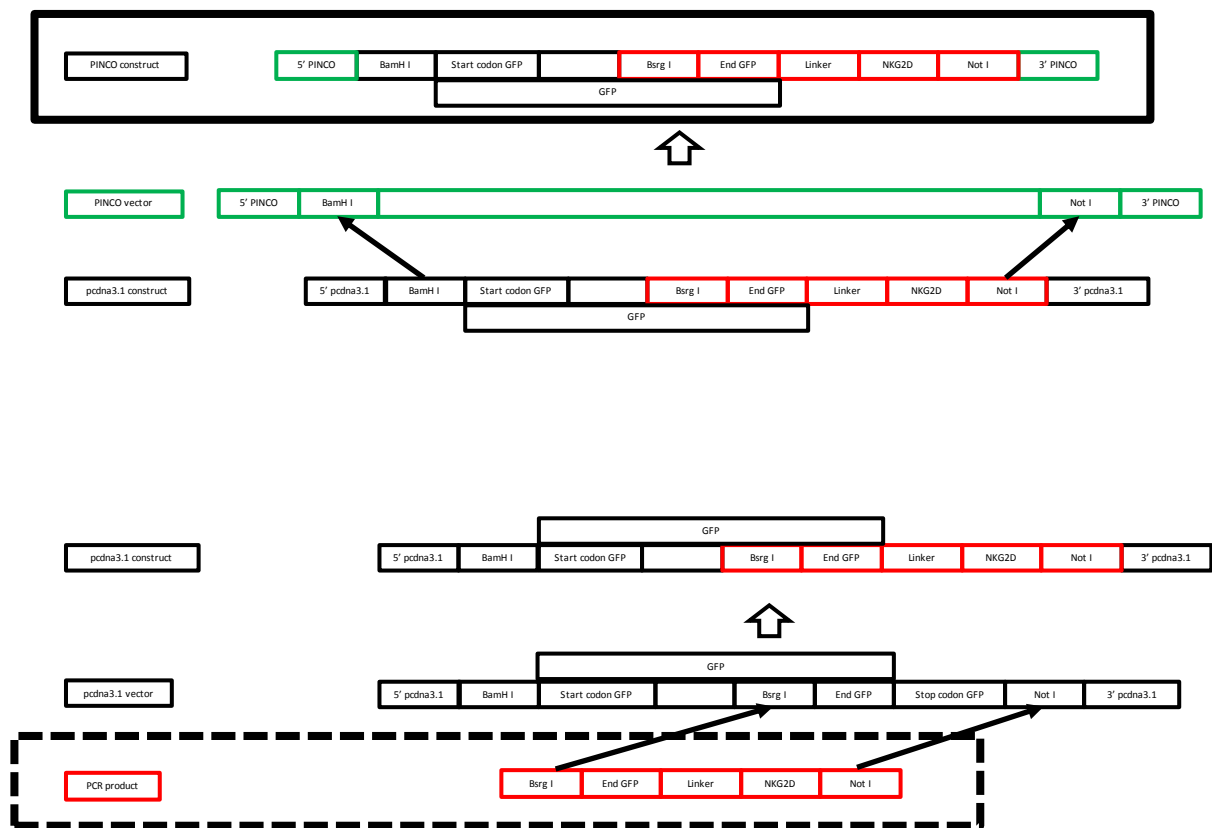


Figure 30: Cloning of the NKG2D gene into retroviral PINCO vector. A PCR fragment coding for NKG2D and the linker was inserted into the pcDNA3.1 vector as BsrGI-NotI insert. The fragment coding for NKG2D-GFP was then subcloned into the PINCO vector as a BamHI-NotI insert. Red boxes are from the PCR fragment. Black boxes are from the pcDNA3.1 vector. Green boxes are from the PINCO vector.

The forward primer was therefore designed to include the BsrGI-containing end of GFP (without stop codon), a long linker (GGGCGGGC) and the first 16 bases of the NKG2D gene (Figure 31).

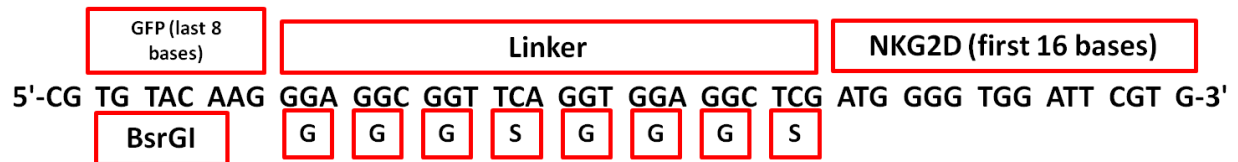


Figure 31: Map and sequence of forward primer for cloning of NKG2D into PINCO vector with GFP attached at the N-terminus of NKG2D.

The reverse primer consisted of the last bases of the NKG2D gene followed by the NotI restriction site (Figure 32).

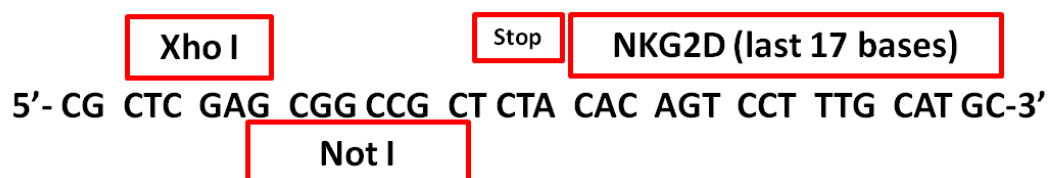


Figure 32: Map and sequence of the reverse primer for cloning of NKG2D into PINCO vector with GFP attached at the N-terminus of NKG2D.

DNA sequence after PCR was fully checked once inserted into pGEMTeasy vector (Promega) after PCR amplification. The NKG2D-mCherry was obtained by exchanging GFP by mCherry (obtained from pcDNA3.1-mCherry vector, using a BamHI – BsrGI digestion) in the pcDNA3.1 construct before subcloning the NKG2D-mCherry construct into the PINCO vector.

KIR2DL1-GFP and KIR2DL1-mCherry cloning strategies

The original pcDNA3.1 KIR2DL1-Citrine construct was obtained from a previous study [89]. The KIR2DL1 gene was first inserted into the pEGFPN1 vector (Clontech) using HindIII-BamHI restrictions sites. Then the KIR2DL1-GFP gene was transferred into the PINCO vector using XhoI-NotI restriction sites (Figure 33).

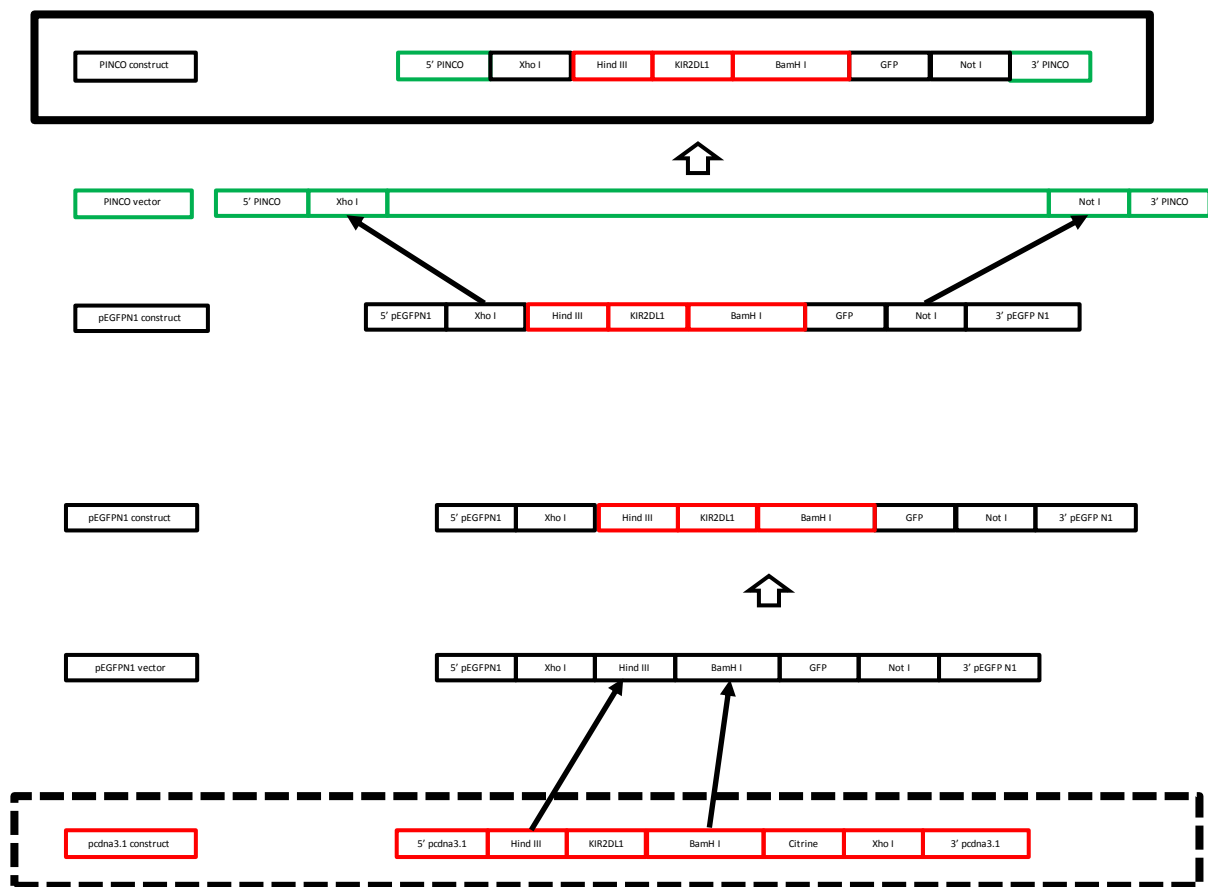


Figure 33: Cloning of the KIR2DL1 gene into retroviral PINCO vector. The fragment coding for KIR2DL1 was first subcloned from a pcDNA3.1. construct into the pEGFPN1 vector as a HindIII-BamHI insert. The fragment coding for KIR2DL1-GFP was then subcloned from the pEGFPN1 vector into the PINCO vector as a XhoI-NotI insert. Red boxes are from the pcDNA3.1 construct. Black boxes are from the pEGFPN1 vector. Green boxes are from the PINCO vector.

The KIR2DL1-mCherry was obtained by exchanging GFP by mCherry (obtained from pcDNA3.1-mCherry vector, using a BamHI – NotI digestion) in the pEGFPN1 construct before subcloning the KIR2DL1-mCherry construct into the PINCO vector.

PCR

PCR mixes were prepared using the Expand high-fidelity PCR system, dNTP kits (Roche) as specified by manufacturer's recommendations. PCR were performed using a PCR machine (Biorad) programmed as follows:

Initial denaturation (5 min, 94°C)

30 cycles of:

- Denaturation (30s min, 94°C)
- Annealing (30s, 57°C)
- Elongation (1 min, 72°C)

Final elongation (5 min, 72°C)

Final hold (4°C)

After PCR, PCR products were amplified by inserting them in PGEMTeasy vector (Promega).

Digestions

All single and double digestions were carried out for two hours at 37°C using the following mix:

- Enzyme 1 (NEB): 1 µL
- Enzyme 2 (NEB): 1 µL
- 10x Buffer (NEB): 4 µL
- DNA : 1 to 4 µL of dissolved DNA depending on concentration
- Water was added up to a total final volume of 40 µL

Transformation

Competent bacteria were incubated with plasmid for 20 min on ice, after which Super Optimal Broth with Catabolite repression (SOC) media was added. The mixture was then incubated at 37°C for 1 hr. The mixture was then spread on a selective Agar plate (32 g/L LB Agar in water, Sigma) with the appropriate selection antibiotic (50-100 µg/mL). Plates were then incubates overnight at 37°C.

Inoculation

Selection-resistant colonies were then inoculated using LB Broth medium (20g/L LB Broth medium in water, Sigma) overnight at 37°C with the appropriate selection antibiotic (50-100 µg/mL).

DNA Miniprep and Maxiprep

After sufficient growth of transformed bacteria, plasmid DNA was extracted using Miniprep or Maxiprep kits (Qiagen) according to manufacturer's recommendations.

Other transfectants

NKL/DAP10-GFP and NKL/DAP10(Y85F)-GFP were made by Anne Chauveau, and NKL/VAV1-GFP Grb2-mCherry by Philipp Eissmann as published previously [233]. 721.221/MICA HLA-Cw4 were generated by Philipp Eissmann.

Stable transfections

Protocol

The amphotropic packaging cell line Phoenix was transfected with PINCO-NKG2D-GFP, PINCO-NKG2D-mCherry, PINCO-KIR2DL1-GFP and PINCO-KIR2DL1-mCherry plasmid using Lipofectamin LTX (Invitrogen). Viral supernatant was collected 24 and 48 h after transfection. For infection, 5×10^5 /mL NKL cells were subjected to three sequential centrifugations (45 min, $725 \times g$) in the

presence of the viral supernatant. Cells expressing GFP or mCherry were enriched by flow cytometry one week later [234].

Killing assays

Killing assays were performed using a standard ^{35}S methionine assay. On day 1 target cells were washed three times with and resuspended in R10 medium lacking methionine and incubated overnight with ^{35}S methionine (1 μL / 10^6 cells). On day 2 target (T) cells were washed three times in R10 media and resuspended at 10^5 cells/mL. Effector cells (E) were washed once and resuspended in R10 media at 2×10^6 cells/mL. Cells were then incubated together for 5 hours in a V-bottomed plate at 20:1, 10:1 and 5:1 E:T ratios, each of them in triplicate. Spontaneous release (SR) was assessed by using a well containing target cells only. Total release (TR) was assessed by mixing target cells with 0.1M NaOH. At the end of the five-hour incubation cells were span down for 10 min at 4°C and radioactivity-containing supernatant was retrieved and incubated with the scintillating organic solvent Microscint 40 (PerkinElmer, USA) overnight at room temperature in an Optiplat (PerkinElmer, USA). On day 3 the Optiplates were read out using a Top Count PerkinElmer NXT.

The percentatage of lysed cells for a given set of effector and target cells was calculated as shown by Equation 7.

$$\text{Lysis (effector, target)} = \frac{\text{Count(effector, target)} - \text{Count(SR)}}{\text{Count(TR)} - \text{Count(SR)}}$$

Equation 7: Calculation of the percentage of lysed target cells by the effector cells during the killing assay as a function of the counts obtained for the effector/target well, the total release (TR) well, and the spontaneous release (SR) well.

Imaging

Microscope and tweezers

Two equivalent set-ups were used. The first set-up is the custom-made one described in Chapter III. The second set-up was made of a single-spot E3300 commercial optical tweezer generated by a 1070nm solid-state laser (Elliott Scientific, UK) coupled into the non-descanned port of a commercial confocal microscope with resonant scanners (TCS SP5, Leica Microsystems Ltd). Here a 63×, 1.2NA water-immersion objective was used. Apart from these differences, the two set-ups were identical and imaging was performed in the same manner.

Imaging at $t=0$

Orientating conjugates and imaging them once they are formed (Figure 34) means the first instants of synapse formation cannot be imaged. For instance, it is impossible to assess the precise timing of protein microcluster formation with regards to initial cell-cell contacts.

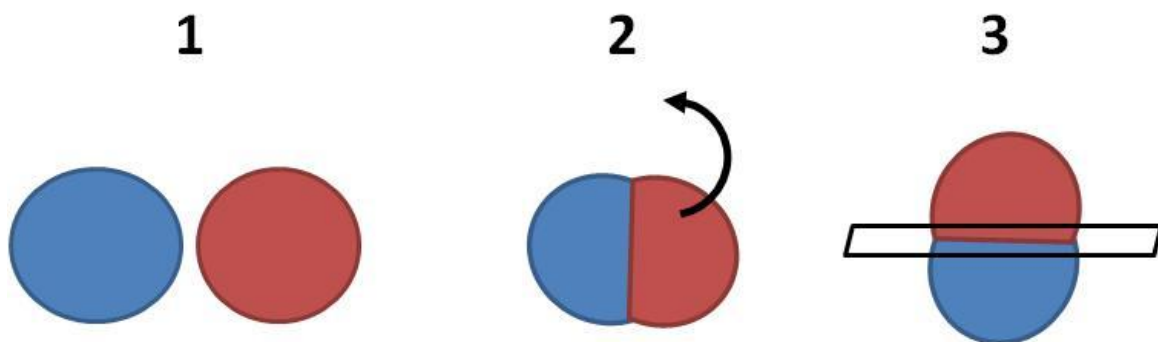


Figure 34: Schematic representation of cell manipulation not allowing high-resolution imaging to be started simultaneously to conjugate formation. The cell conjugate is manipulated and the synapse is imaged after the conjugate is formed.

We circumvented this downside by trapping a target cell and bringing it on top of an effector cell (Figure 35). This allows high-resolution imaging of the synapse to be started simultaneously to the initial cell-cell contact.

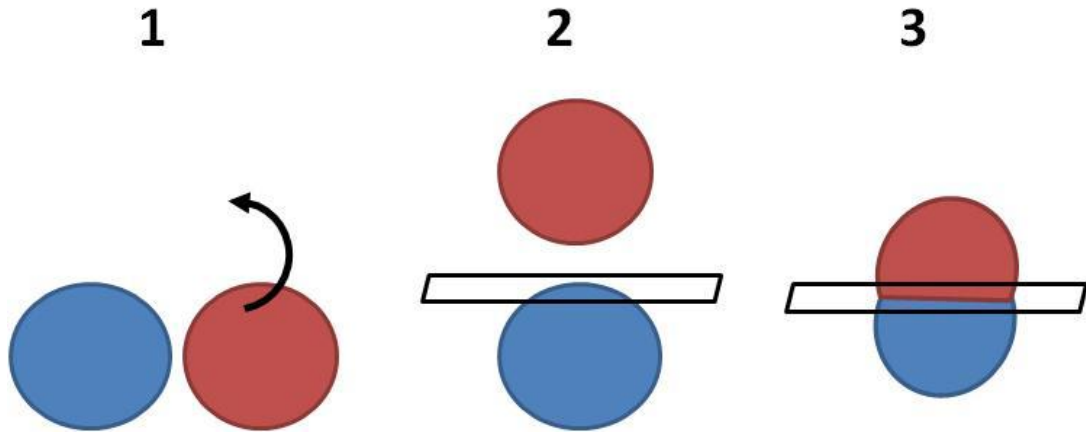


Figure 35: Schematic representation of cell manipulation allowing high-resolution imaging to be started simultaneously to conjugate formation. The cell conjugate is manipulated and the synapse is imaged before the conjugate is formed.

Pearson's correlation coefficient calculation

To quantitatively analyse the degree of correlation of two channels, background was first removed from both channels by subtracting signal detected within the dimmest part of the contact area from the rest of the image. Both channels were then normalised to avoid artefacts due to differences in dynamic range. Pearson's correlation coefficients (R_r) were then calculated using ImageJ as in Equation 8.

$$R_r = \frac{\sum_i (R_i - \bar{R}) \times (G_i - \bar{G})}{\sqrt{\sum_i (R_i - \bar{R})^2} \times \sqrt{\sum_i (G_i - \bar{G})^2}}$$

Equation 8: Pearson's correlation coefficient R_r as a function of pixel values in the red (R) and green channels (G). i is the pixel index and is summed over all pixels in the image. R_i is the intensity of pixel i in the red channel, G_i is the intensity of pixel i in the green channel.

Values for R_r range from -1 (anticorrelation) to 1 (total correlation). A value of 0 means absence of correlation between the two channels.

Cell conjugate immunostaining

Fixation

Effector and target cells were mixed 1:1 in a V-bottomed 96-well plate and incubated together for 12 min at 37°C 5% CO₂. After incubation cells were washed twice in PBS (centrifugation 1200 rpm for 2 min) to remove FCS. Cells were then resuspended in 100 µL PBS to break potential clumps. 100 µL of 4% PFA in PBS was then added, bringing the final concentration to 2%. Cells were then incubated for 45 min at room temperature and finally washed three times in PBS.

Permeabilisation

Cells were incubated in Triton X-100 (Sigma) 0.05% in PBS for 2 min and then washed in PBS.

Blocking and immunostaining

Cells were first blocked in blocking buffer (5% BSA in PBS) for 1 h at 4°C. They were then incubated with the primary antibody at 10 µg/mL for 1 hr at 4°C. Cells were then washed twice with blocking buffer, incubated with the secondary antibody (1 hr, 4°C in the dark) and finally washed twice with PBS and resuspended in PBS before imaging.

Imaging reagents

LysoTracker

To stain lytic granules, NKL cells were incubated with 100 nM LysoTracker Red DND 99 (Molecular Probes) for 2 hr at 37°C in pre-warmed R10 medium and then washed three times in PBS and resuspended in R10 medium before imaging.

Sytox blue

To image cell death, cells were imaged in presence of 0.5 µM Sytox-blue (Invitrogen).

Functionality of the NKG2D construct

The NKG2D construct that we used here was generated specifically for this study. It was therefore necessary to show the fusion protein resulting of the expression of the DNA construct was able to signal normally. Images of NKL/NKG2D-GFP and cell surface expression analysis of NKG2D-GFP by flow cytometry show that NKG2D-GFP is expressed in the plasma membrane which means it is able to couple to endogenous DAP10 (Figure 25 and Figure 38). We also need to ensure that the presence of GFP at the N-terminus of NKG2D does not alter DAP10 signalling. The expression of NKG2D-GFP in NKL/NKG2D-GFP is five times higher (Figure 25) than that of endogenous NKG2D. This means that a response of NKL/NKG2D-GFP to MICA-expressing targets will be mostly mediated through NKG2D-GFP as opposed to endogenous NKG2D. Here we perform a lysis assay and a spreading assay with NKL/NKG2D-GFP. Then, to rule out the role of endogenous NKG2D, we perform an assay in a cell line that does not express this receptor endogeneously.

Lysis assay

To test if the expression NKG2D-GFP induces any detrimental effects on the cytotoxicity of NKL cells, we performed lysis assays against Daudi/MICA. The results show that the cytotoxicity of NKL/NKG2D-GFP is preserved (Figure 36).

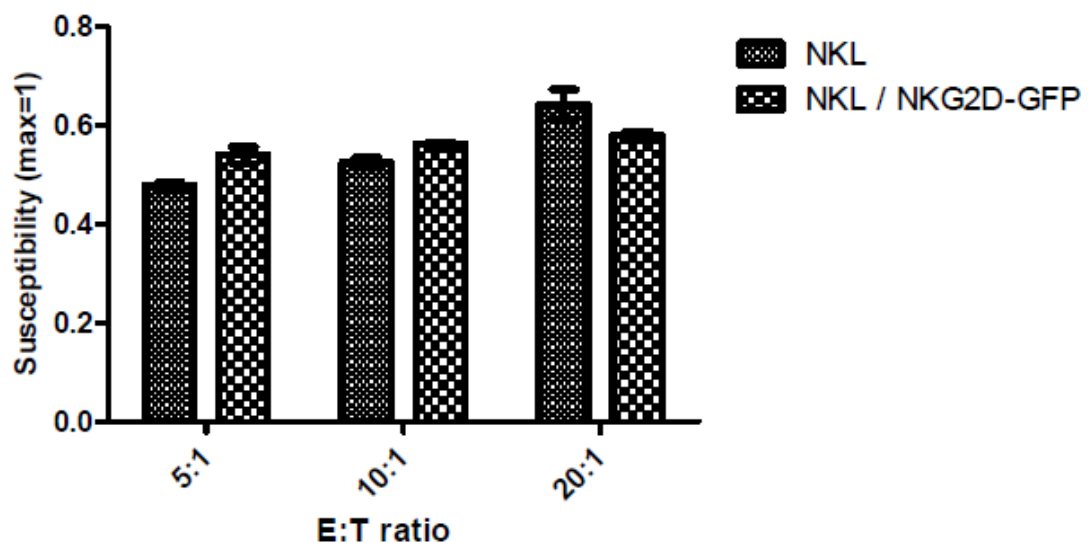


Figure 36: Expression of NKG2D-GFP by NKL cells does not significantly alter their killing function compared to wild-type NKL. Susceptibility of Daudi/MICA by NKL and NKL/NKG2D-GFP. Effector cells and target cells were use at various effector/target (E:T) ratios.

Signal transduction assay

NKG2D does not have a signalling intracellular domain but binds with DAP10 which can propagate signal upon NKG2D ligation with its ligand. A deficiency of either of these two molecules will therefore prevent signalling from the complex. We had to hand NK cells expressing a mutated version of DAP10-GFP (referred to as DAP10(Y85F)-GFP) where the signalling Tyrosine (Y) at position 85 had been replaced by a Phenylalanine, preventing subsequent signalling downstream of the NKG2D-DAP10 complex. The study where this construct is published also shows that NKL transfected with DAP10(Y85F)-GFP are dominant negative as they become unable to perform lysis of target cells,

while the normal DAP10-GFP construct signals normally and leads to normal effector lysis upon NKG2D ligation [233]. Live cell imaging of conjugates formed between NKL/DAP10-GFP and NKL/DAP10(Y85F)-GFP shows NKL/DAP10-GFP cells spread on their targets while NKL/DAP10(Y85F)-GFP cells are unable to do so. However initial clustering of NKG2D-DAP10 at the cell-cell interface can be still observed with NKL/DAP10(Y85F)-GFP. This shows that initial NKG2D-DAP10 clustering at the synapse is signalling independent while full spreading of the NK cell on its target is signalling dependent (Figure 37).

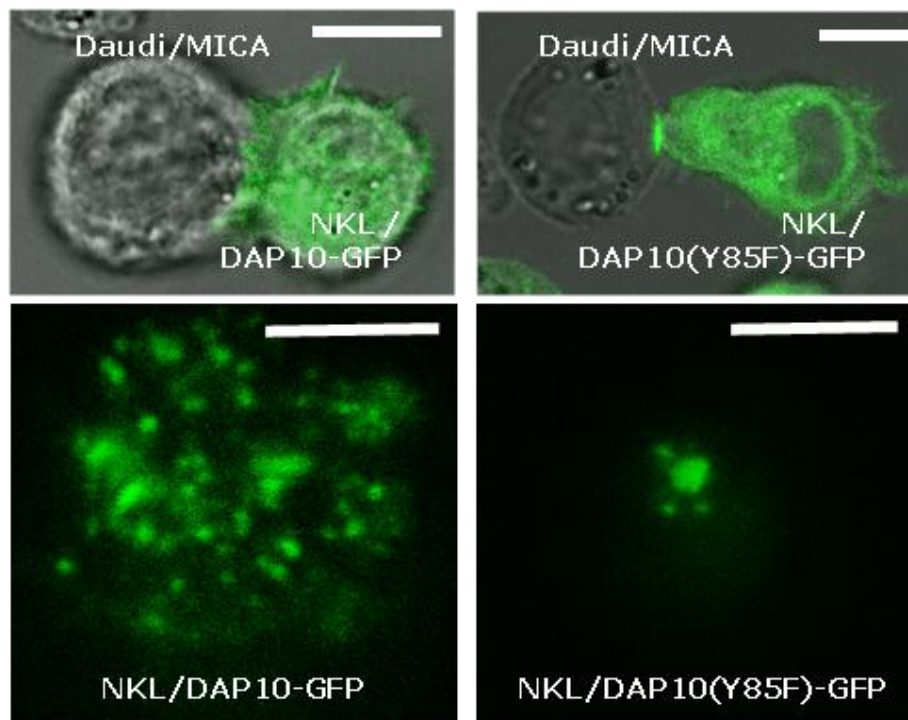


Figure 37: Impaired NKG2D/DAP10 signalling prevents NKL from spreading on their targets. Conjugation assay between Daudi/MICA (targets) and NKL/DAP10-GFP (left) or NKL/DAP10(Y85F)-GFP (right). Top row: side view confocal image. Bottom row: high-resolution image of the synapse using tweezers. Scale bars, 10 μ m. n>10.

To test our NKG2D-GFP construct, we performed time-lapsed imaging of the formation of conjugates between NKL/NKG2D-GFP and Daudi/MICA. Contrary to NKL/DAP10(Y85F)-GFP cells, NKL/NKG2D-

GFP cells were able to spread on their targets, demonstrating the construct is able to signal as expected and could therefore be considered fully functional (Figure 38).

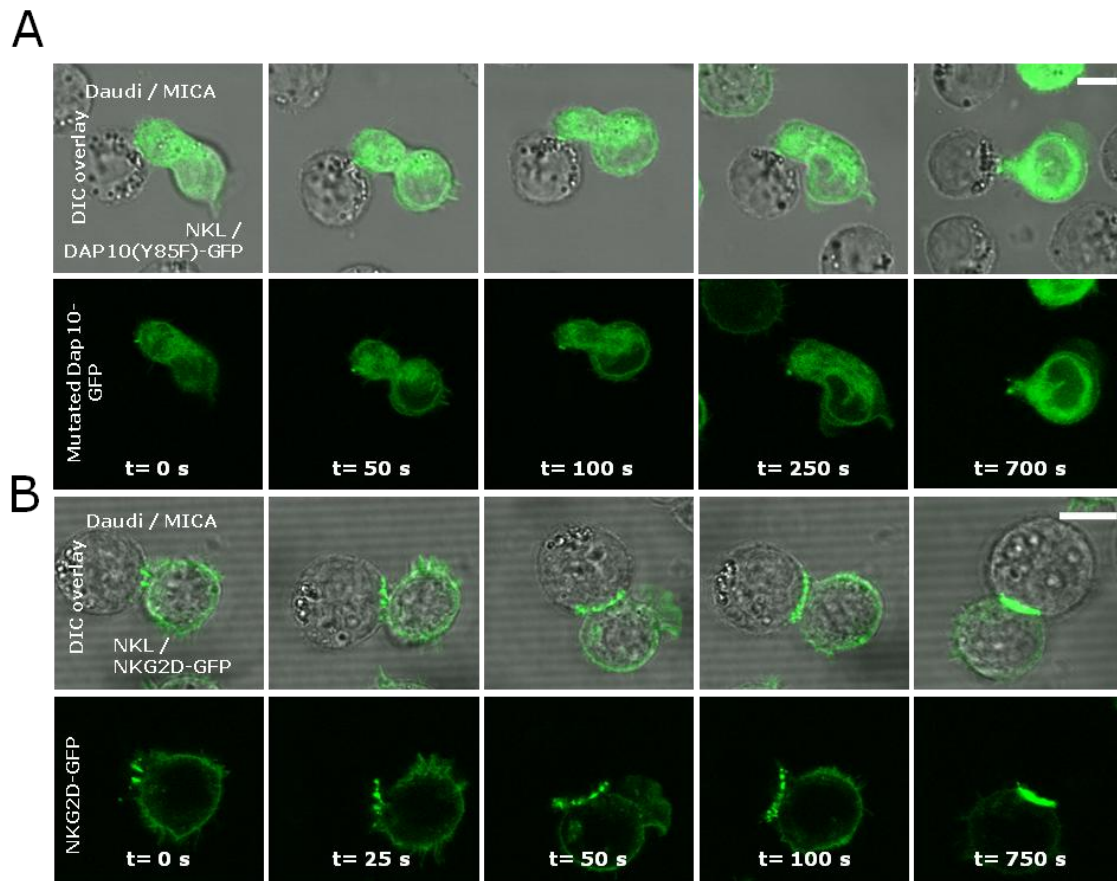


Figure 38: NKG2D-GFP mediated signalling in NKL/NKG2D-GFP is functional as NKL/NKG2D-GFP fully spread on their targets unlike signalling deficient NKL/DAP10(Y85F)-GFP. Top rows: time-lapse movie of conjugate formation between NKL/DAP10(Y85F)-GFP and Daudi/MICA. Bottom rows: time-lapse movie of conjugate formation between NKL/NKG2D-GFP and Daudi/MICA. Scale bars, 10 μ m. n>10.

Functionality of the NKG2D construct in Jurkat T cells

As said before, the expression of NKG2D-GFP in NKL/NKG2D-GFP is five times higher than that of endogenous NKG2D. This means that a response of NKL/NKG2D-GFP to MICA-expressing targets is

mostly mediated through NKG2D-GFP as opposed to endogenous NKG2D. Based on this assumption, the two previous assays are sufficient to conclude that the construct is fully functional. However, to definitively rule out the possible role of endogenous NKG2D, it was necessary to ensure the construct would work in a cell line that does not express NKG2D endogenously, to this end we used Jurkat T cells. Conveniently, Jurkat T cells express DAP10 [45] which allows NKG2D to be expressed at the surface in transfected cells (Figure 39).

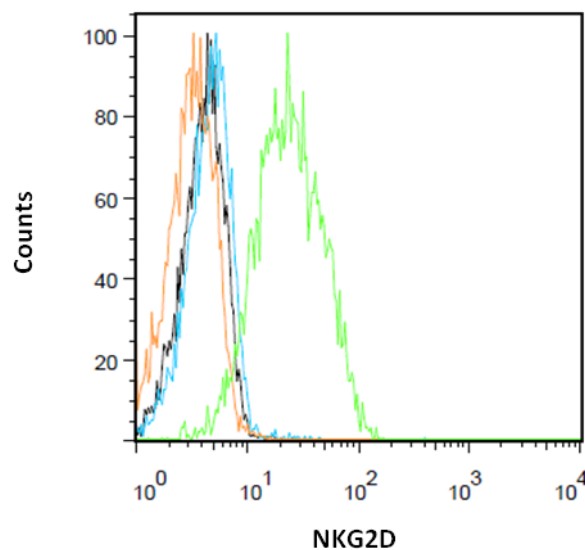


Figure 39: Cell surface expression of NKG2D on Jurkat and Jurkat/NKG2D-mCherry. Cell surface expression of NKG2D on Jurkat (blue) Jurkat/NKG2D-mCherry (green) shown relative to isotype control on Jurkat (black) and Jurkat/NKG2D-mCherry (orange) assessed in live cells using flow cytometry. The expression of NKG2D was assessed using mAb staining.

Microscopy analysis shows that when Jurkat T cells express NKG2D-mCherry, NKG2D-mCherry accumulate at the synapse with Daudi/MICA and that Jurkat T cells fully spread on their targets (Figure 40).

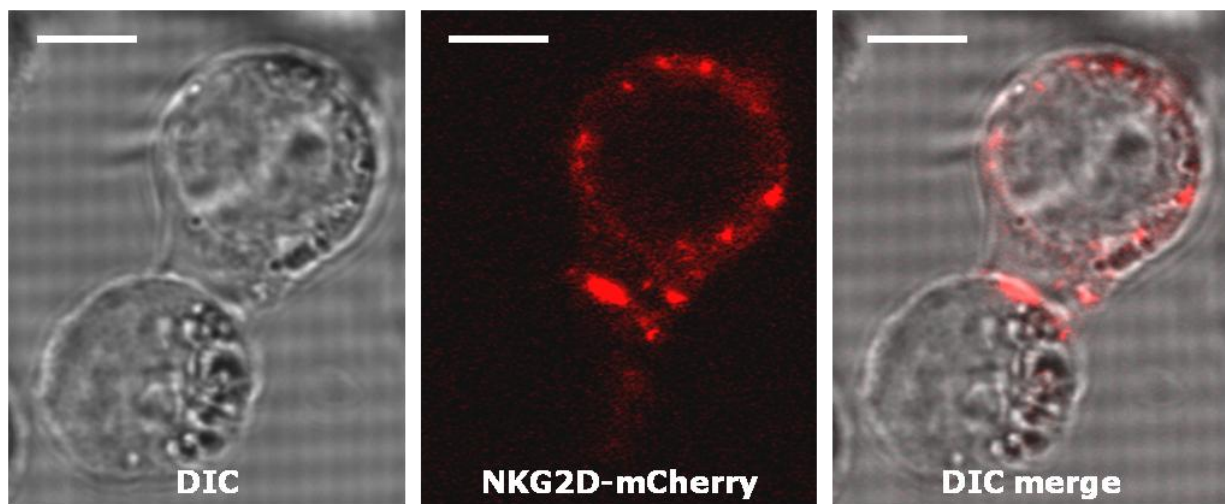


Figure 40: NKG2D-mCherry-mediated signalling is functional in Jurkat T cells as Jurkat/NKG2D-mCherry spread on Daudi/MICA and NKG2D-mCherry accumulates at the cell-cell interface. Formation of conjugates between Jurkat/NKG2D-mCherry and Daudi/MICA. Scale bar, 10 μ m. n>10.

To conclude, these data show that the extracellular part of the fusion protein (C-terminus) recognises MICA, that the intracellular part (N-terminus) binds DAP10, and that the construct is able to propagate signalling.

Results

NKG2D readily forms dynamic microclusters upon activation

First we set out to image NKG2D clustering at the activating NK cell synapse. Expression of MICA on the surface of a cell line that is initially not susceptible to NKL/NKG2D-GFP triggers NKL/NKG2D-GFP cytotoxicity (Figure 41). Therefore, NKG2D becomes activated upon contact between NKL/NKG2D-GFP and Daudi/MICA.

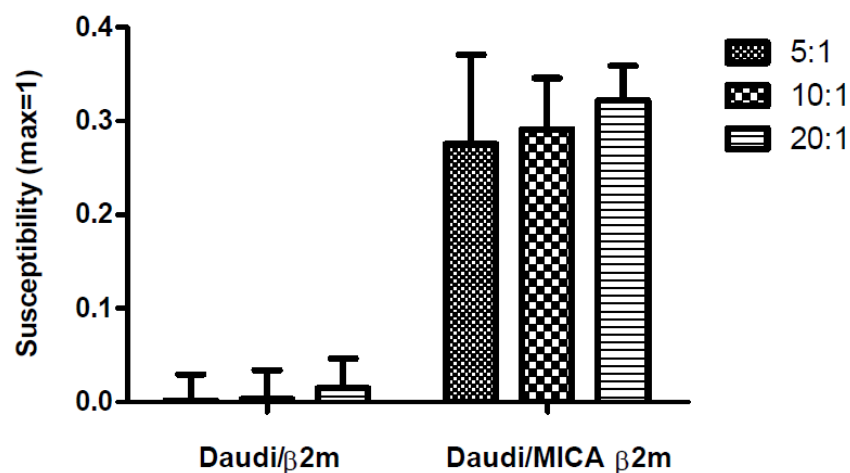


Figure 41: MICA expression on target cells triggers NKL/NKG2D-GFP NKG2D-mediated cytotoxicity. Susceptibility of Daudi/ β2m and Daudi/MICA β2m by NKL/NKG2D-GFP. Effector cells and target cells were use at various effector/target (E:T) ratios.

To image the distribution of NKG2D at the activating synapse upon its activation by target cells expressing MICA, we incubated NKL/NKG2D-GFP with Daudi/MICA and performed time-lapse live-

cell imaging. NKG2D-GFP could be seen to accumulate within seconds at the interface between the NK cells and their targets and this enrichment was maintained for the whole duration of the intercellular contact (Figure 42). Such movies also show NK cells spread on their targets until they reach a maximum, before eventually contracting. These morphological changes have been well described by others and are not the object of our study [91].

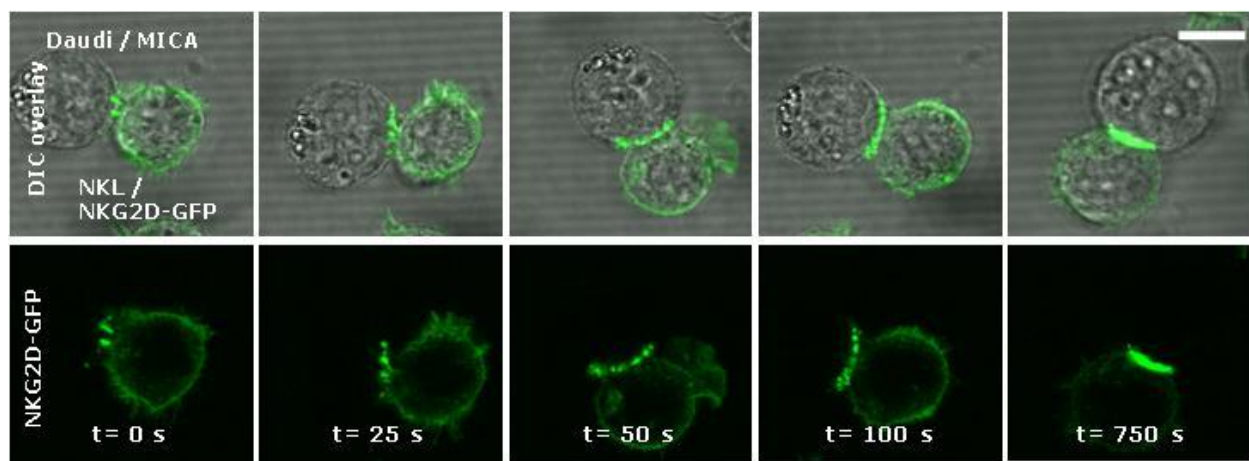


Figure 42: NKG2D accumulates at the activating NK cell immunological synapse. Time-lapse imaging of the formation of the synapse between NKL/NKG2D-GFP and Daudi/MICA. Top row: overlay of DIC image and NKG2D-GFP. Bottom row: NKG2D-GFP fluorescence channel. Scale bar, 10 μ m. n>20.

It can be difficult to assess if and when the target cell is dead as morphological modifications are not always obvious. To confirm that target cell death occurs during such NKG2D-mediated interactions, time lapse imaging of live cells was performed using Sytox blue (Invitrogen) which undergoes a 500-fold increase in brightness when it binds nucleic acids inside compromised cells. Imaging with Sytox Blue confirmed target cells underwent apoptosis during the interaction with NKL/NKG2D-GFP (Figure 43).

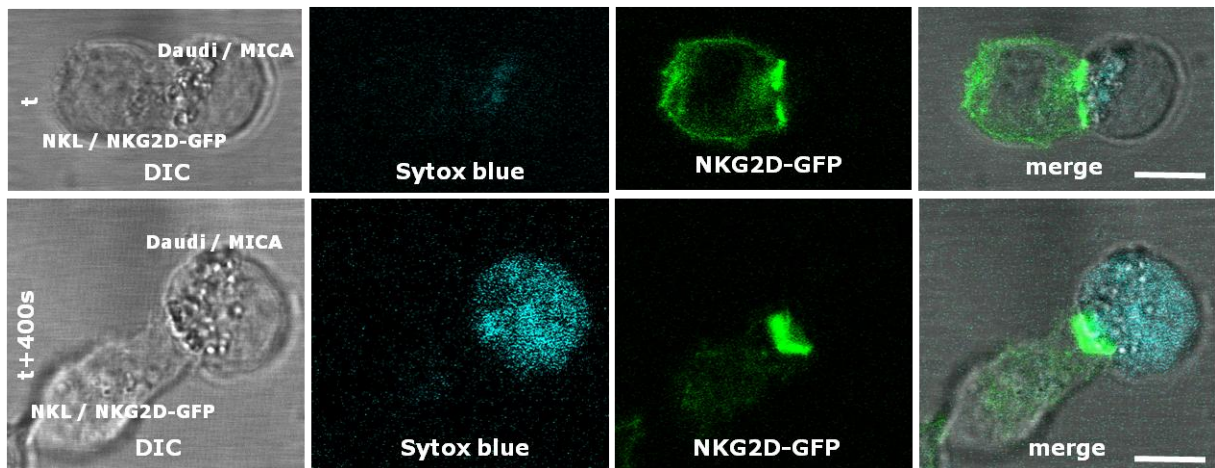


Figure 43: The NKG2D-mediated activating interaction leads to the lysis of Daudi/MICA by NKL/NKG2D-GFP. Time lapse live cell imaging of NKL/NKG2D-GFP and Daudi/MICA. Sytox Blue (blue fluorescence channel) in the media reports cell death. Top row: beginning of the cell-cell interaction NKG2D-GFP accumulates at the synapse. Bottom row: death of the target cell as shown by the increase in the brightness of Sytox Blue. Scale bars, 10 μ m. n>10.

Next we set out to image NKG2D clustering at the activating NK cell synapse with live conjugates at high speed and with high resolution, through the use of the optical tweezer technique described in the previous chapter. First, our aim was to acquire long-lasting time lapse movies (over 3 minutes), beginning from the initial time of contact between the target cell and NK cell and covering most of the interaction until target lysis. To capture the initial contact point we optically trapped and dropped a target cell on top of a randomly chosen NK cell. To prevent excessive bleaching of GFP, laser illumination was kept low and we ensured there was no oversampling as the aim here is to define a general trend in the clustering pattern of NKG2D at the synapse. Movies from cell-cell contact could be recorded for at least 4 to 5 minutes, which was sufficient as NKG2D clustering reached a stationary final phase in most viewed conjugates by 3 to 4 minutes.

Strikingly, NKG2D accumulated in microclusters immediately after initial cell-cell contact, although NKG2D clustering is absent at the surface of non interacting NK cells, at least within the resolution of the confocal microscope (~ 250 nm). Also, microclusters of NKG2D were continuously being formed during the spreading of the NK cell as the contact area expands (Figure 44). After this spreading

phase where microclusters are being generated, subsequent clustering of NKG2D can follow two distinct patterns: either absence of movement, and therefore of further clustering of the NKG2D microclusters formed during the initial phase. This type of behaviour was observed in 33% of conjugates (n=50) (Figure 44 bottom). Or, an active movement of microclusters formed during the initial spreading phase (67% of conjugates, n=50), resulting in active interactions between initially distinct microclusters and in their fusing into a ring pattern. (Figure 44 top).

Next, we set out to further characterise the behaviour of microclusters in terms of trajectories and speed. Movies with higher signal-to-noise ratio (smaller pixel size and higher excitation power) were acquired so dimmer structures could be observed. Also, to limit bleaching and allow higher frame rates, imaging was performed from the end of the initial spreading phase (Figure 45 & Supplementary movie 7). Such movies show the presence of discrete microclusters spread across the large contact area. Then, these clusters move erratically to fuse into a sharp ring-shaped pattern as described before. This ring-shaped pattern is stationary for as long as photobleaching allowed imaging to be performed (>4mins).

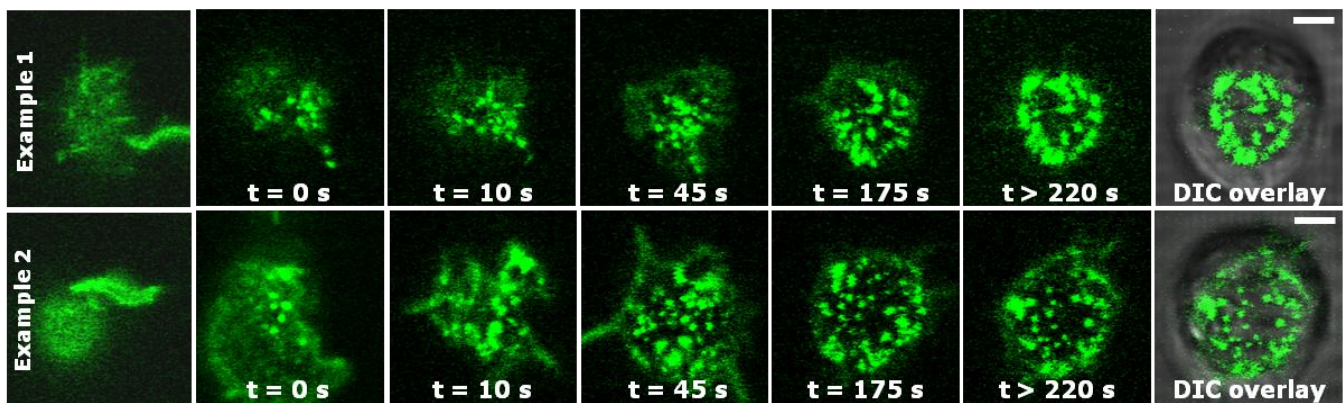


Figure 44: NKG2D readily clusters at the NK cell activating synapse upon cell-cell contact and accumulates in a ring (example 1, 66% of cases) or remains in discrete clusters (example 2, 34% of cases). High-resolution time-lapse live-cell imaging of the immunological synapse formed between NKL/NKG2D-GFP and Daudi/MICA, NKG2D-GFP fluorescence channels. In both cases, the target cell was deposited on top of the NK cell as described in Methods. The untimed frames are images of the surface of the NK cells before contact with their target cell. Scale bars, 10 μ m. n>50.

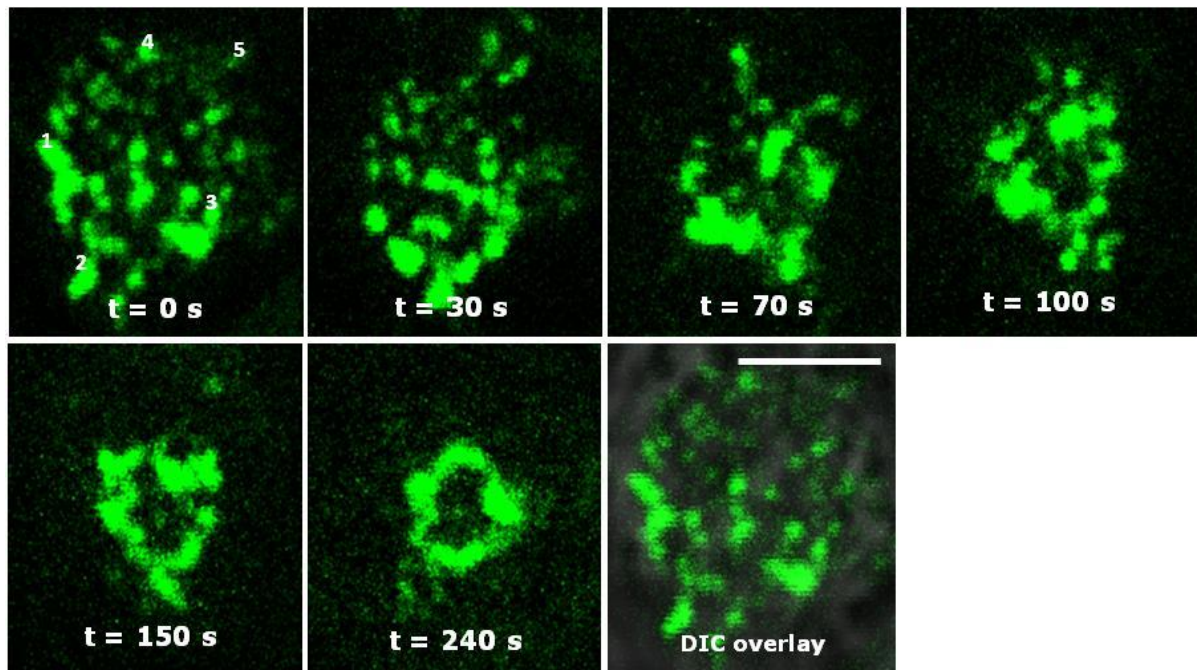


Figure 45: NKG2D clusters dynamically accumulate into a central ring at the activating NK cell immunological synapse. High-speed high-resolution time-lapse live-cell imaging of a synapse between NKL/NKG2D-GFP and Daudi/MICA, NKG2D-GFP fluorescence channel. Scale bar, 10 μm . (Supplementary movie 7).

Using this representative high-resolution movie, we first quantified the trajectories of NKG2D microclusters (Figure 46). We selected five clusters that were evenly distributed around the contact area. Each cluster was tracked for 170 s, the duration of time that it took the central ring to be clearly defined. After that time point, further tracking of these clusters became irrelevant as most clusters had fused into the ring-shaped pattern. During optical trapping, cell conjugates can be affected by random translational movement due to local flow. The contribution of this movement was removed so tracks would only account for microcluster movement within the synapse. This was achieved by recording the movement of a fixed point in the synapse. Rotational movement was negligible in the example shown.

Strikingly, the trajectories of NKG2D microclusters at the activating NK cell synapse are very different from what has been reported for T cell microclusters in previous studies (Figure 46) [55]. Like their T cell counterparts, NKG2D microclusters moved from the periphery to the centre, therefore exhibiting a certain centripetal component in their behaviour as each of these clusters finishes in the final central ring. However, their movement was much more erratic and complex than that observed with TCR on bilayers.

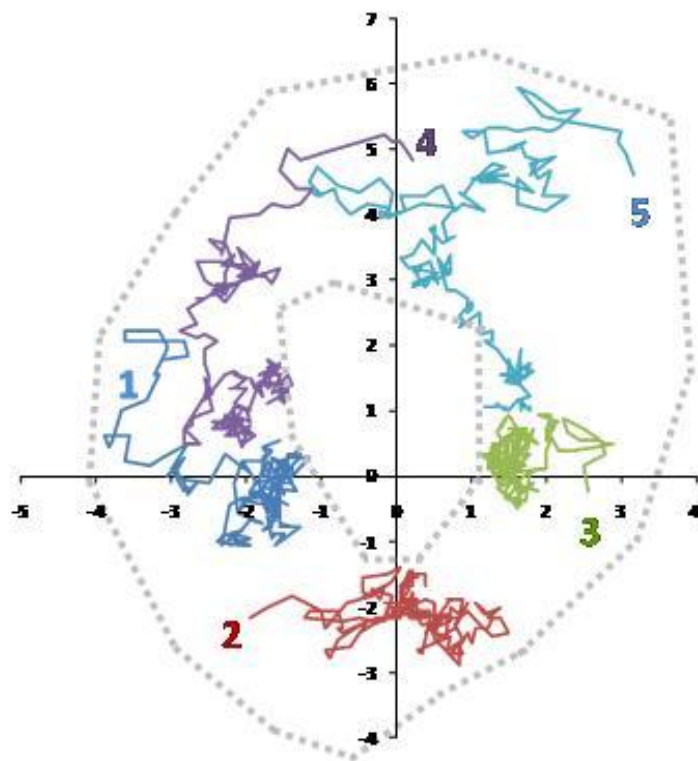


Figure 46: NKG2D microclusters follow erratic trajectories at the activating NK cell immunological synapse. Tracks of clusters numbered in Figure 45 during 170 s from the beginning of the movie ($t=0$). Tracks begin at the track-end next to the track number. Axes are in μm .

Next, the instantaneous speed distribution of NKG2D clusters was extracted from the track plot. The frame rate of the movie was 1 frame per second. As the smallest displacement that can be seen between two frames is equal to the microscope resolution (i.e. 250 nm), the accuracy on the instantaneous speed is 250 nm/s. Unlike what has been observed by others with T cells on bilayers, the instantaneous speed distribution exhibits an inhomogeneous distribution, with speed sometimes exceeding 500 nm/s (Figure 47).

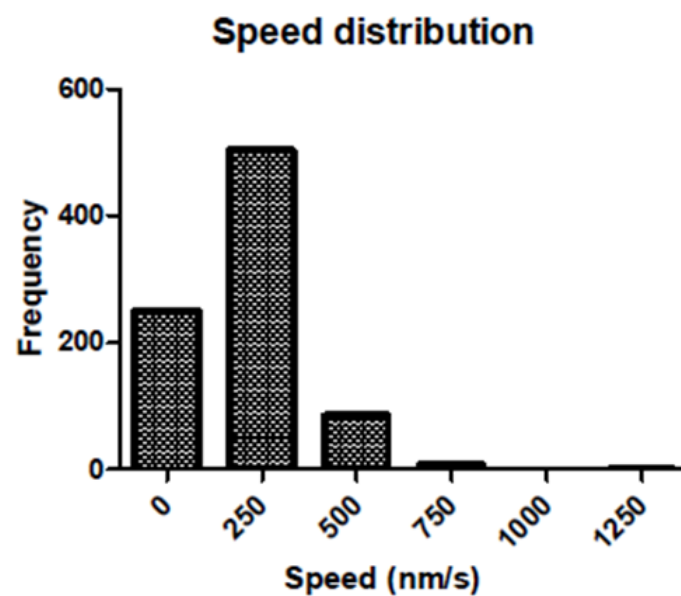


Figure 47: NKG2D clusters at the activating NK cell synapse exhibit a large speed distribution. Distribution histogram of instantaneous (between two consecutive frames) speeds extracted from movie shown in Figure 45.

NKG2D microclusters continuously signal upon activation

We have seen that microclusters of NKG2D readily form at the immunological synapse upon contact between NK cells and target cells expressing MICA. These structures are highly dynamic, as we have also observed that in a majority of cases, these microclusters accumulate into a final ring-shaped pattern. Next we set out to determine whether these structures can signal, and therefore if they are important for triggering NK cell cytotoxicity. NKL/NKG2D-GFP were incubated with Daudi/MICA between 12 and 30 min in a 96-well plates. Cell conjugates were then fixed, permeabilised and stained using a phosphotyrosine (pY) specific mouse antibody. A secondary goat anti-mouse Cy5 antibody was then used as a fluorescent marker.

Because it is difficult to control the exact time between initial cell contact and fixation, conjugates were divided after imaging into two categories according to what was observed with live conjugates. The first category consists of early synapses, when the NK cell is spread on its target and microclusters are still discrete and spread over the whole contact surface. The second category consists of late synapses, when the NK cell has contracted and extensive clustering of NKG2D has taken place. It was reported in T cell studies with activating bilayers that the activation state of TCR microclusters was highly dependent on the location of these microclusters in the synapse [207], as pY could be detected in newly generated peripheral microclusters which remain activated until they reach the centre of the synapse. Similarly, here we set out to investigate whether NKG2D signalling is different for early and late synapses. It is interesting to note that while NKG2D accumulated in a sharp ring-shaped pattern with live cells, such sharp patterns were very rarely observed with fixed conjugates which most often exhibited a large central cluster instead. Most likely, this notable

difference could be due to the fixation reagent which may slightly affect the morphology of synapses.

Overlap between clusters of NKG2D-GFP and anti-pY-Cy5 immunostaining was consistently observed (Figure 48). Unlike T cells on bilayers, there was no distinction between small/big clusters or peripheral/central clusters. Most of the microclusters of NKG2D-GFP exhibited significant detectable overlap with anti-pY-Cy5 fluorescence in early synapses i.e. when the NK cell is fully spread on its target (Figure 48 top rows). At later stages i.e. after the NK cell has contracted, when most of the NKG2D-GFP has clustered in a much bigger single cluster at the centre of the synapse, a clear overlap can still be seen between NKG2D-GFP clustering and anti-pY-Cy5 staining (Figure 48 bottom row). The average correlation coefficients calculated over 50 cells are high ($R_r=0.7\pm1$), therefore confirming that NKG2D clusters at the synapse continuously signal.

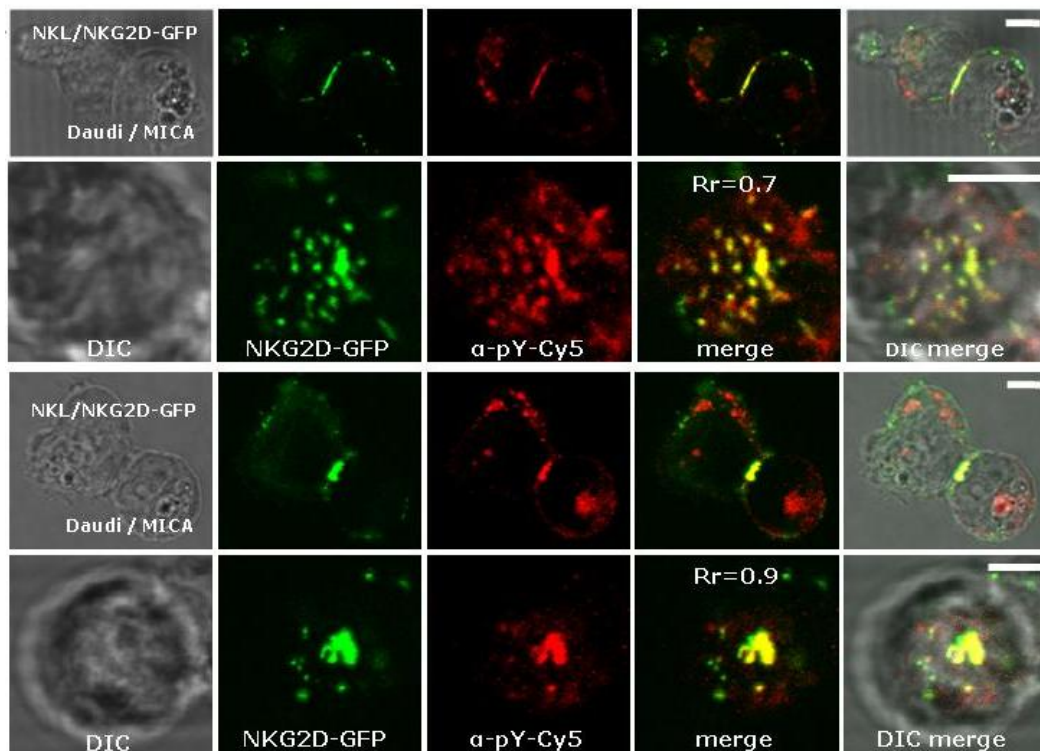


Figure 48: Clusters of NKG2D continuously signal at the activating NK cell immunological synapse. High-resolution fixed-cell imaging of synapses formed between NKL/NKG2D-GFP and Daudi/MICA. Conjugates were stained for pY (red). Top rows: early synapse. Bottom rows: late synapse. Side view images of conjugates before optical trapping are provided for each condition. Scale bars, 10 μ m. $n>50$.

Signalling of NKG2D clusters is independent of inhibitory signalling

We have seen that NKG2D forms dynamic, signalling clusters at the activating NK cell synapse. These clusters may therefore play a role in triggering NK cell cytotoxicity and subsequent killing of MICA expressing cells. NK cells discriminate healthy cells from tumour or virally-infected cells by integrating activating and inhibitory signals. Therefore it can be hypothesised that inhibitory signalling would affect NKG2D-mediated activating signalling at some level. Possible effects of inhibitory signalling would be for instance to prevent NKG2D from clustering, from being phosphorylated (via DAP10), or by preventing signalling from downstream signalling molecules. Some of these hypotheses will be tested here by comparing synapses formed between NK cells and target cells expressing MICA alone, and target cells expressing MICA and MHC class I, the role of MHC class I being to trigger some inhibitory signalling. We used Daudi/MICA as MICA-expressing targets and Daudi/ β 2m MICA as MICA + MHC class I expressing targets (Figure 28).

Expression of MHC class I on target cells does not necessarily lead to inhibitory signalling e.g. if the NK cells does not have matching receptors for the particular MHC class I expressed on targets. Lysis assays were performed to ensure that expression of MHC class I on Daudi cells does lead to a decrease in killing by NKL cells. Such assays show that the susceptibility of Daudi/MICA β 2m by NKL/NKG2D-GFP is reduced by around 50% in comparison with Daudi/MICA, therefore providing a system with a clear difference between the two conditions (Figure 49).

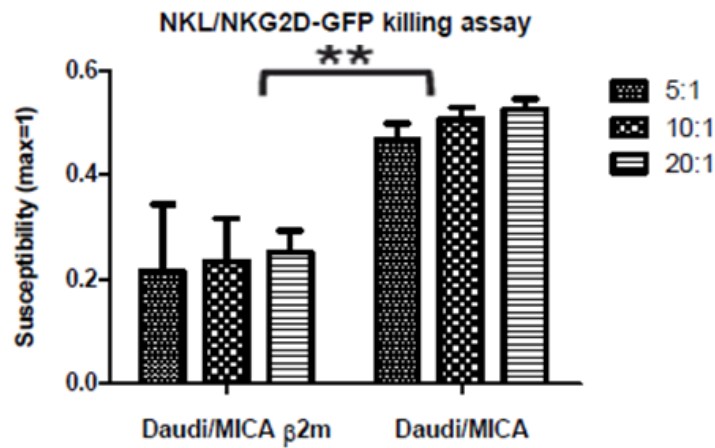


Figure 49: Expression of MHC class I by target cells significantly impairs susceptibility of NKL/NKG2D-GFP. Susceptibility of Daudi/MICA and Daudi/MICA β2m by NKL/NKG2D-GFP. Effector cells and target cells were used at various effector/target (E:T) ratios. Statistical test, Student's t test between Daudi/MICA and Daudi/MICA β2m (E:T ratio=10:1), $p < 0.01$.

MHC class I – mediated inhibitory signalling does not prevent NKG2D clustering at the synapse

First, we tested whether MHC class I expression by target cells affected clustering of NKG2D. Live-cell imaging of conjugates between NKL/NKG2D-GFP cells and Daudi/ MICA β2m was performed and showed NKG2D was still accumulating in microclusters at the synapse in presence of MHC class I (Figure 50).

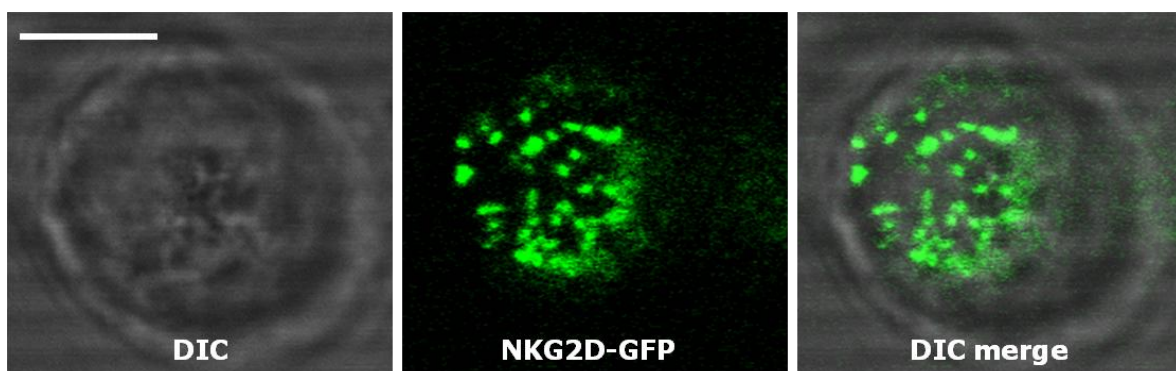


Figure 50: NKG2D forms microclusters at the NK cell synapse in presence of MHC class I inhibition. High-resolution live-cell imaging of NK cell immunological synapses between NKL/NKG2D-GFP and Daudi/MICA β2m. Scale bar, 10 μm. $n > 20$.

Signalling by NKG2D clusters is not prevented by inhibitory signalling

The next hypothesis we tested here is whether inhibitory signalling can prevent phosphorylation of NKG2D/DAP10 clusters. Conjugates between NKL/NKG2D-GFP and Daudi/MICA or Daudi/ MICA β 2m were fixed after 12 mins of co-incubation. They were then stained for pY. High-resolution images of conjugates were then acquired in both conditions. These images exhibited a consistent overlap between clusters of NKG2D and pY staining in both conditions (Figure 51).

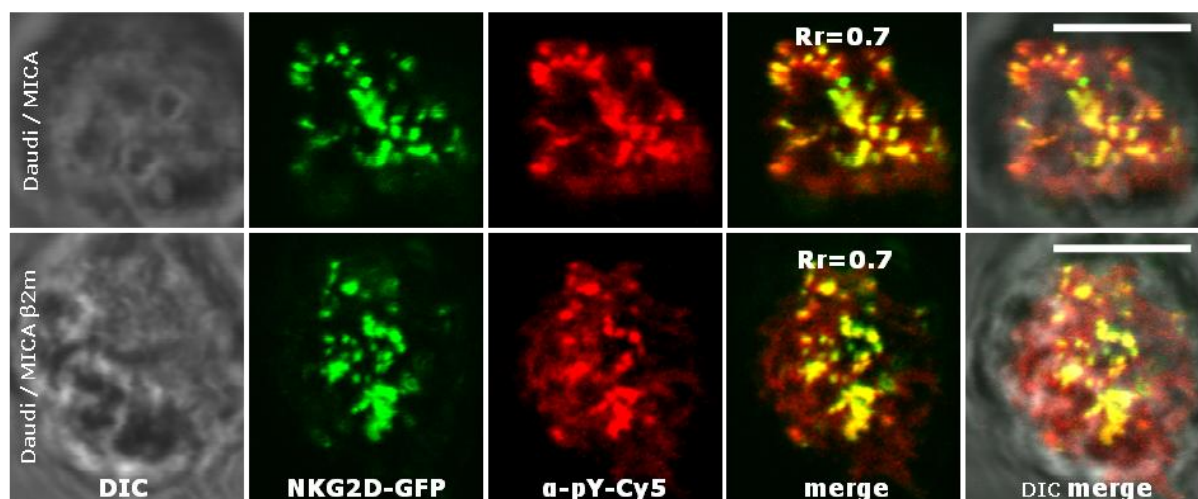


Figure 51: Expression of MHC class I by target cells does not alter the correlation of the localisation of pY with NKG2D clusters. High-resolution fixed-cell imaging of synapses formed between NKL/NKG2D-GFP and Daudi/MICA (top) or Daudi MICA β 2m (bottom). Conjugates were stained for pY (red). Scale bars, 10 μ m. n>20.

To better characterise the overlap between NKG2D clusters and pY, we measured their Pearson's correlation coefficients for over 20 conjugates in presence and in absence of MHC class I (Figure 52). Statistical analysis of these distributions shows the difference between the two distributions is not statistically significant. Therefore, localisation correlation between NKG2D and pY is not affected by MHC class I-mediated inhibitory signalling in the system used.

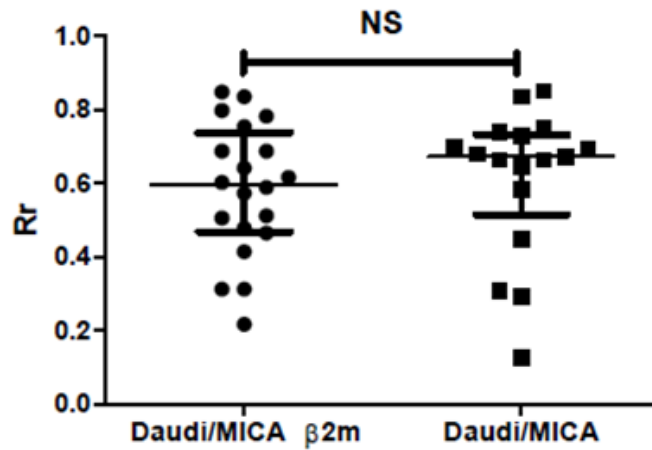


Figure 52: Expression of MHC class I by target cells does not alter the correlation of the localisation of pY with regards to NKG2D clusters. Pearson's correlation coefficient of NKG2D-GFP and pY calculated from high-resolution images (Figure 51) for conjugates formed by NKL/NKG2D-GFP and Daudi/MICA or Daudi/MICA β 2m.

NKG2D and KIR2DL1 accumulate in the same central cluster at the NK cell synapse

Due to the absence of high-resolution imaging, little is known about the interactions of inhibitory and activating receptors at the NK cell synapse. This interaction is crucial as it allows NK cell to integrate signals from target cells and therefore controls the outcome of the cell-cell interaction. We imaged conjugates between NKL/NKG2D-GFP KIR2DL1-mCherry with 721.221/MICA HLA-Cw4 as targets expressing ligands for both NKG2D and KIR2DL1. Interestingly, KIR2DL1 and NKG2D accumulated in the centre of the synapse, which could provide a way for KIR2DL1 to inhibit activating signalling by putting in close proximity phosphatases and ITAM motifs (Figure 53).

No further work was done with this target system (721.221 cells), as conjugate formation with these cells was rare. Several attempts to transfect Daudi cells expressing MICA with a ligand for KIR2DL1 were made but none succeeded.

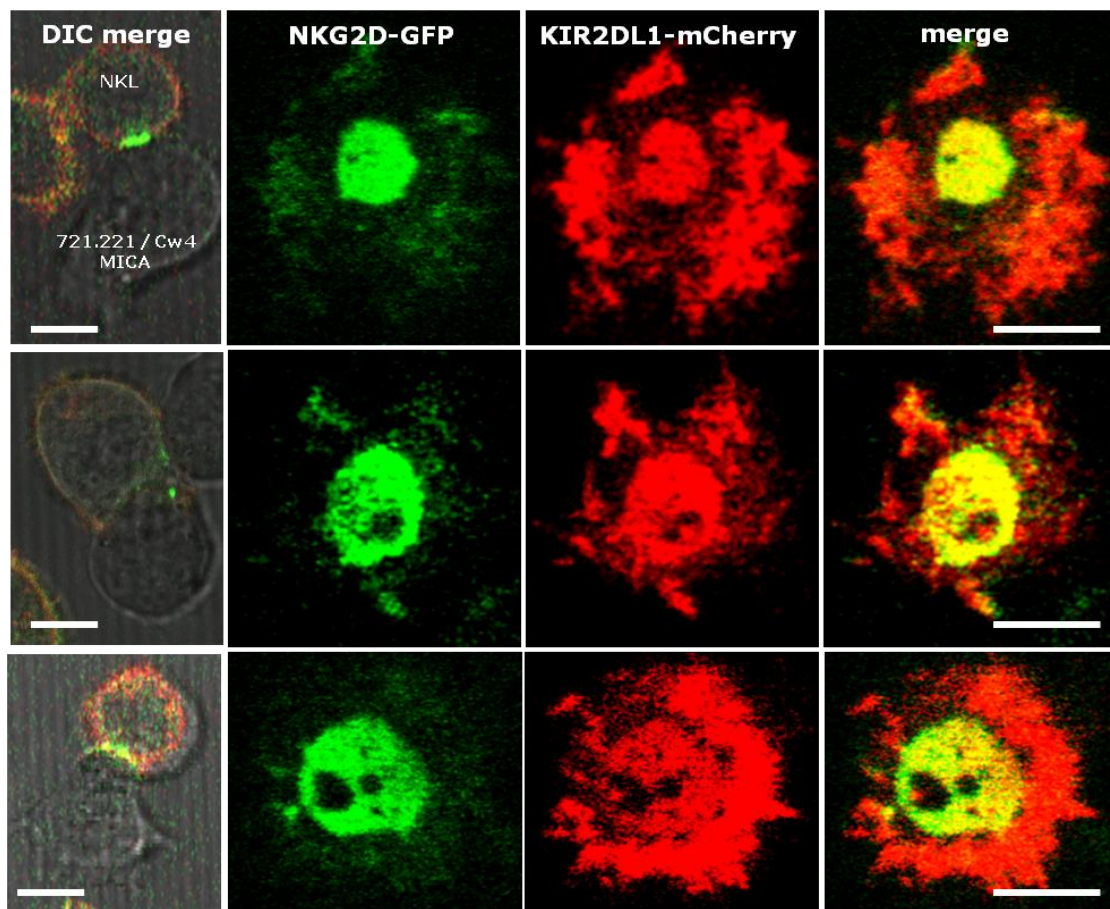


Figure 53: NKG2D and KIR2DL1 accumulate in the same central cluster at the NK cell synapse. Three examples of high-resolution live-cell images of synapse formed between NKL/NKG2D-GFP KIR2DL1-mCherry and 721.221/MICA HLA-Cw4. For each example side view images are provided (first column). Scale bars, 10 μm .

Clustering of downstream signalling molecules DAP10, Grb2, VAV1 upon NKG2D activation

Upon NKG2D ligation Grb2 binds phosphorylated DAP10. VAV1 in turns binds Grb2 directly. The Grb2-VAV1 intermediate then phosphorylates PLC- γ 2 and SLP-76. This DAP10-Grb2 pathway is required for optimum NK cell cytotoxicity [50]. We know that NKG2D forms dynamic signalling clusters upon activation but how downstream signalling molecules integrate and propagate this signal remains unknown. Here we use high-resolution imaging to investigate how these molecules cluster at the synapse. We also test whether they accumulate in the same clusters or if they interact through transient interactions.

DAP10 and NKG2D accumulate in the same microclusters

Structural studies have shown that NKG2D and DAP10 form a hexameric complex comprising two NKG2D molecules, each coupled to a DAP10 homodimer through charged amino-acids [46]. However, no imaging has been done to investigate the interaction of NKG2D and DAP10 at the synapse.

We imaged conjugates formed between NKL/DAP10-GFP NKG2D-mCherry and Daudi/MICA. Interestingly, almost perfect overlap could be seen between the two channels, confirming that

DAP10 and NKG2D do not segregate at the synapse, at least within the resolution of a confocal microscope (Figure 54).

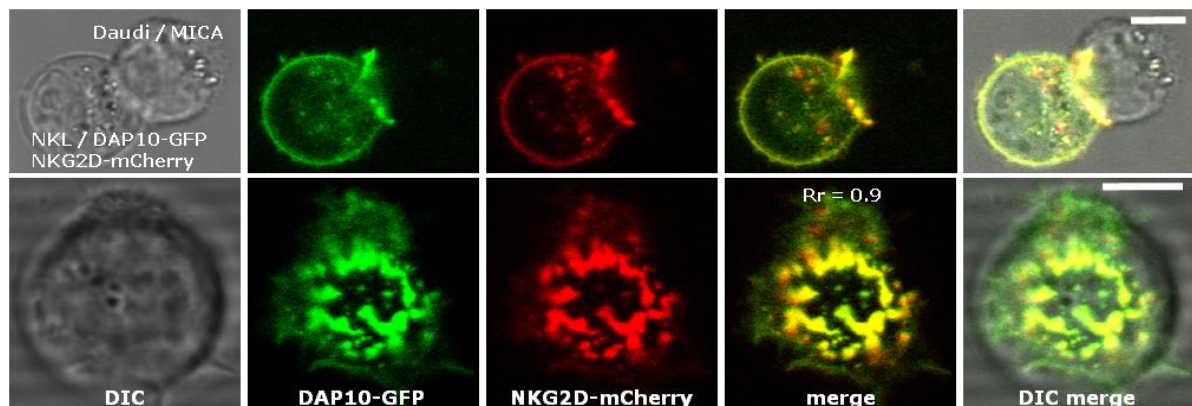


Figure 54: DAP10 and NKG2D accumulate in the same clusters at the activating NK cell immunological synapse. High-resolution live-cell images of a synapse formed between NKL/DAP10-GFP NKG2D-mCherry and Daudi/MICA. The side view images of the conjugate before optical trapping is provided (top row). Scale bars, 10 μ m. n>20.

Quantitative Pearson's correlation analysis of conjugates showed a very high degree of correlation between DAP10-GFP and NKG2D-mCherry, with median R_r equal to 0.9 ± 1 (Figure 55). These coefficients were calculated using randomly chosen conjugates featuring early and late synapses. Therefore the conclusion drawn here does not depend on the contact duration of the conjugates.

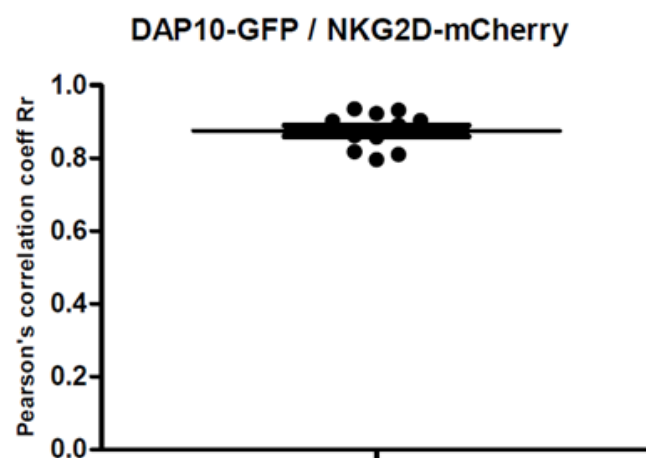


Figure 55: DAP10 and NKG2D accumulate in the same clusters at the activating NK cell immunological synapse. Pearson's correlation coefficient of DAP10-GFP and NKG2D-mCherry calculated from high-resolution images of conjugates formed by NKL/DAP10-GFP NKG2D-mCherry and Daudi/MICA.

VAV1 and Grb2 accumulate in the same microclusters

Upon NKG2D-mediated NK cell activation, Grb2 binds phosphorylated DAP10. VAV1 then binds directly Grb2. However it is unknown how the interaction between VAV1 and Grb2 propagates signalling upon activation i.e. if they cluster and how these clusters would interact. Here we perform high-resolution imaging of conjugates formed between NKL/VAV1-GFP Grb2-mCherry and Daudi/MICA. Strikingly, both VAV1 and Grb2 formed microclusters at the activating synapse and these clusters clearly overlapped and remained so during the whole interaction (Figure 56). Individual Grb2/VAV1 microclusters converged into a central ring, reminiscent of what we previously observed with NKG2D/DAP10 microclusters.

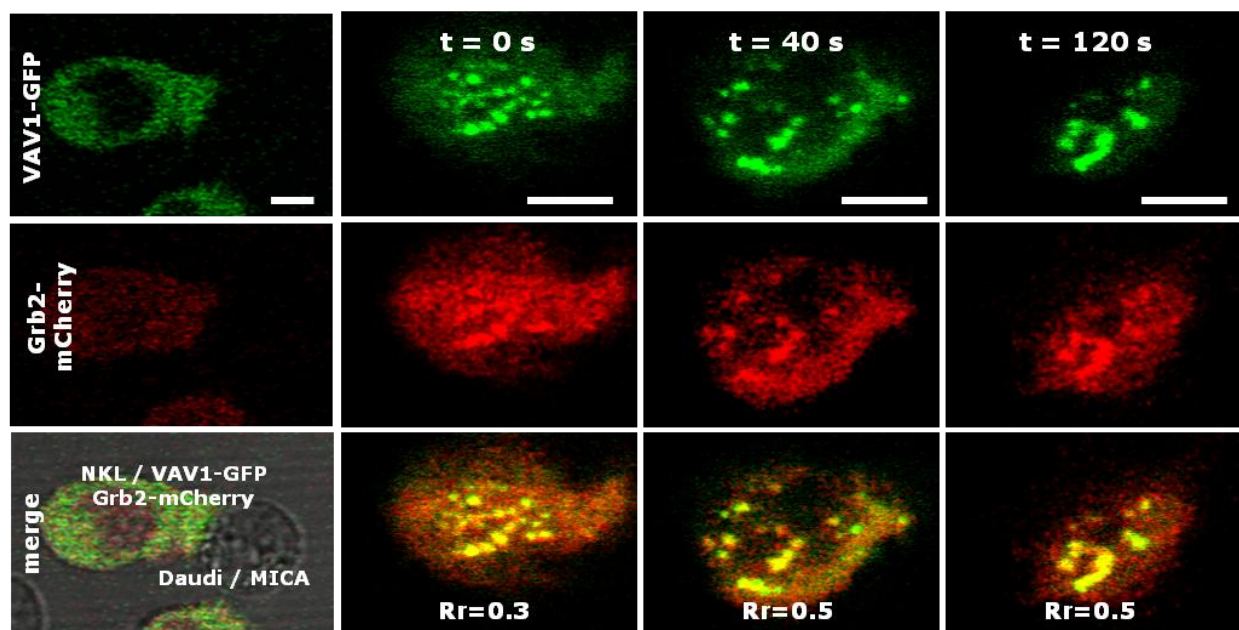


Figure 56: Grb2 and VAV1 accumulate in the same clusters at the activating NK cell immunological synapse. High-resolution live cell images of a synapse formed between NKL/VAV1-GFP Grb2-mCherry and Daudi/MICA. The side view images of the conjugate before optical trapping is provided (first row). Scale bars, 10 μm . $n > 10$.

Time-lapse imaging of Grb2/VAV1 clusters could be difficult due to the background produced by the presence of non-recruited cytoplasmic VAV1-GFP and Grb2-mCherry. Such structures therefore appeared often quite dim compared to the rest of the cell. Photobleaching would also worsen this, especially with mCherry. A high signal-to-background version of the final ring can be seen in Figure 57.

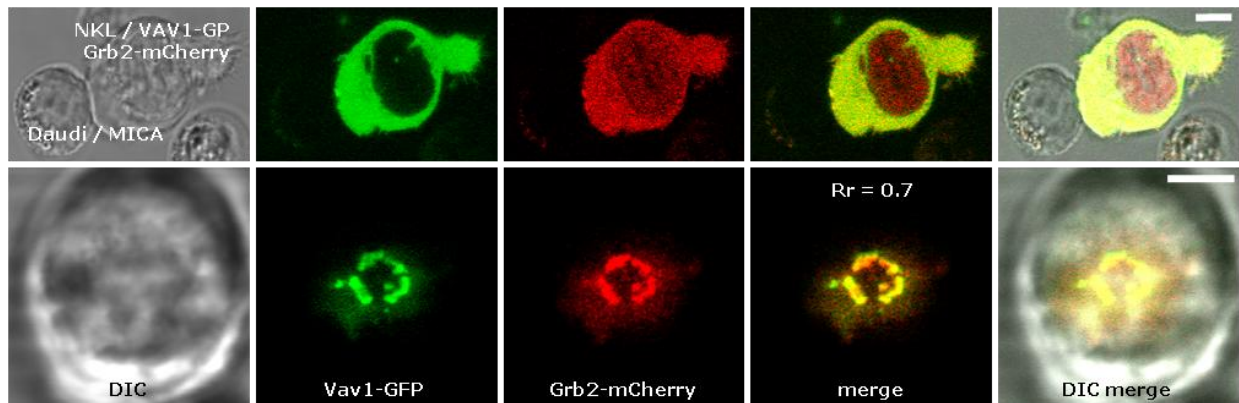


Figure 57: Grb2 and VAV1 form a ring-shaped cluster at the activating NK cell immunological synapse. High-resolution live-cell images of a synapse formed between NKL/VAV1-GFP Grb2-mCherry and Daudi/MICA. The side view images of the conjugate before optical trapping is provided (top row). Scale bars, 10 μ m. n>25.

Pearson's correlation analysis of randomly selected conjugates also confirms Grb2/VAV1 integrate activating signals through stable interactions, with R_r equal to 0.65 ± 1 (Figure 58).

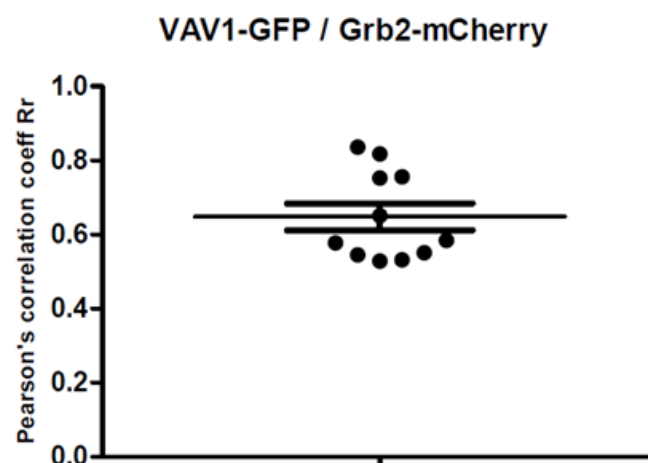


Figure 58: Grb2 and VAV1 accumulate in the same clusters at the activating NK cell immunological synapse. Pearson's correlation coefficient of VAV1-GFP and Grb2-mCherry calculated from high-resolution images of conjugates formed between NKL VAV1-GFP Grb2-mCherry and Daudi/MICA.

NKG2D microclusters coalesce around a secretory domain

Lytic granule delivery is a critical process in NK cell cytotoxicity as it controls one of the most important effector functions of NK cells. This process has to be highly directional so bystander cells are not affected by NK cell degranulation. However, very little has been done to visualise the spatio-temporal delivery of lytic granules at the NK cell synapse between live cells at high speed with high resolution. We have observed that NKG2D mediated signalling triggered the formation of NKG2D microclusters, and that these clusters eventually accumulated into a ring-shaped pattern. We have also seen that these clusters are consistently phosphorylated, both in discrete early microclusters as well as in late larger ring-shaped clusters. Another hypothesis for the role of this ring is directing lytic granules towards the target cell. To test this, we performed high-resolution imaging of activating synapses formed between NKL/NKG2D-GFP and Daudi/MICA to visualise lytic granule delivery in NK cells.

First we set out to image lytic granules at the synapse between live cells. Lytic granules were stained using LysoTracker Red (Invitrogen) which stains acidic intracellular vesicles. NK cells were then mixed into the imaging chamber with target cells, and imaged when conjugates were formed.

Initially, synapses only showed the presence of microclusters of NKG2D similar to what we observed previously, with only residual detection of LysoTracker signal (Figure 59). Strikingly, upon formation of the ring of NKG2D, strong LysoTracker signal was detected. Also, LysoTracker signal was restrained in a central patch that was enclosed within the central ring of NKG2D. It is likely that the phase during which LysoTracker can be detected is a degranulation phase.

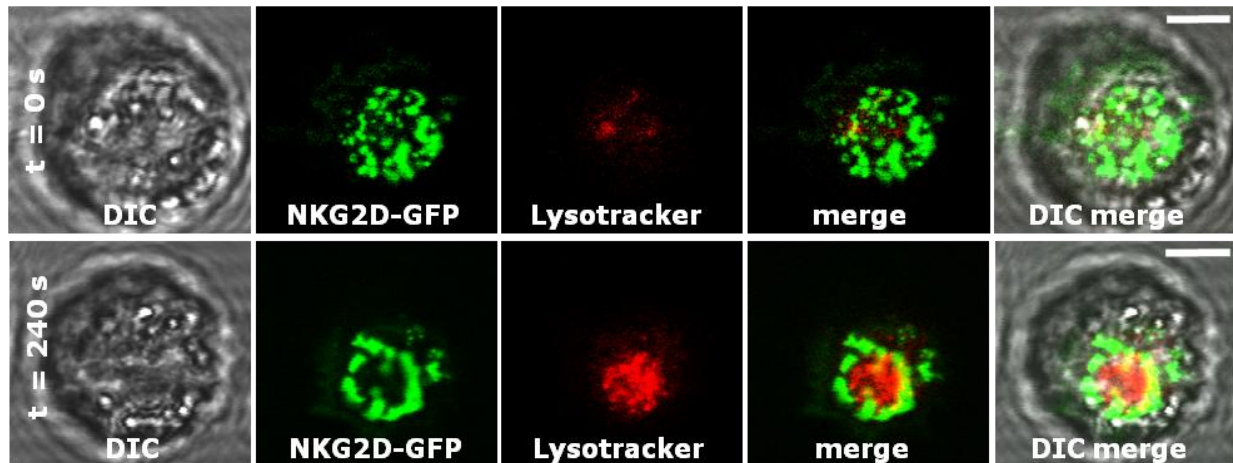


Figure 59: NKG2D clusters coalesce into a secretory domain for lytic granules at the activating NK cell immunological synapse. High-speed high-resolution time-lapse live-cell imaging of a synapse formed between NKL/NKG2D-GFP and Daudi/MICA. Lytic granules in NKL/NKG2D-GFP are stained with Lysotracker (red). Top row: early phase of the synapse. Bottom row: later phase of the synapse where NKG2D is clustered into a ring. Scale bar, 10 μm . n=3.

Lysotracker Red is used in major studies on lytic secretion of lymphocytes [66]. However, the spectrum of this acidic-compartment dye might be broader than just specific lytic granules. We immunostained conjugates between NKL/NKG2D-GFP and Daudi/MICA for perforin. Perforin is a cytolytic protein found in the lytic granules of NK cells and T cells, alongside other proteins such as Granzyme B and LAMP1 (CD107a). Upon degranulation, perforin forms a pore in the plasma membrane of the target cell by inserting itself into it. Images of fixed conjugates stained for perforin clearly show a similar pattern to what could be observed using Lysotracker, with synaptic perforin detected within the centre of a ring of NKG2D-GFP (Figure 60). The ring of NKG2D might not appear as sharp as what can be seen with live cells. Again, fixation seems to slightly affect the morphology of the synapse.

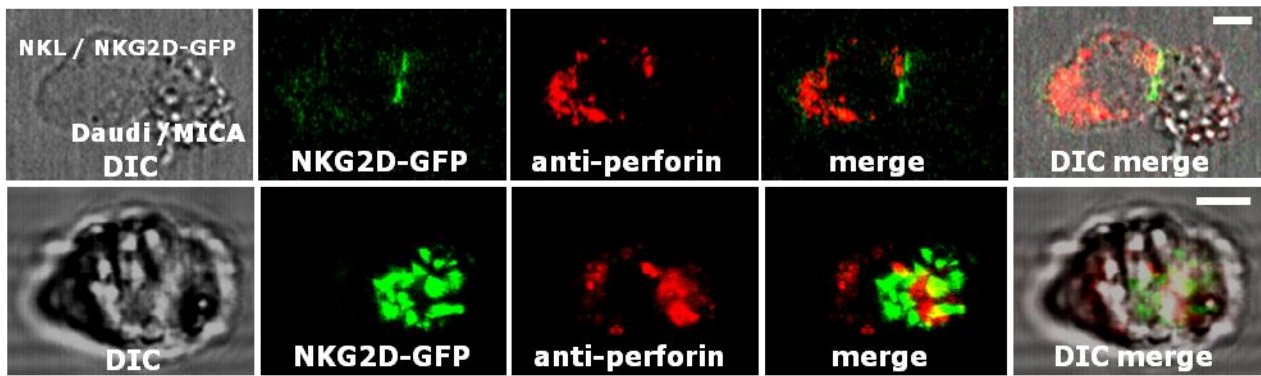


Figure 60: NKG2D clusters coalesce into a secretory domain for lytic granules at the activating NK cell immunological synapse. High-resolution fixed-cell imaging of a synapse formed between NKL/NKG2D-GFP and Daudi/MICA. Lytic granules in NKL/NKG2D-GFP are stained with an anti-perforin immunostaining (red). The side view images of the conjugate is also provided. Scale bars, 10 μ m. n=3.

Figure 59 and Figure 60 demonstrate that lytic granules are recruited towards the target cell and are confined within the NKG2D ring. However, they are not sufficient to show that these lytic granules are being degranulated and therefore delivered into the target cell. It is therefore necessary to show that granules can pass across the synapse. To test this, we imaged conjugates formed between Daudi/MICA and NKL/NKG2D-GFP loaded with LysoTracker and monitored the transfer of lytic granules from the NK cell to the target cell using side view confocal images of conjugates (Figure 61). Imaging showed that LysoTracker-stained compartments can pass across the hole in the centre of the NKG2D accumulation at the interface between the two cells. As NKG2D is bound in the membrane of the NK cell, a particle that goes past this barrier is being delivered into the conjugated cell. It is also interesting to note that during that process the target cell shows clear morphological signs of apoptosis. Together, these data demonstrate that the ring of NKG2D that is observed at the synapse is correlated with directional secretion of lytic granules.

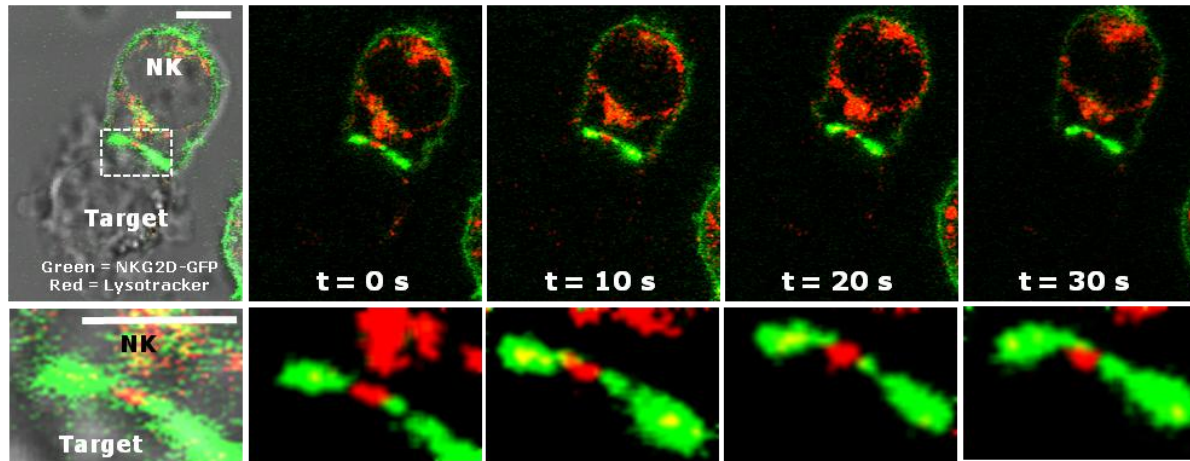


Figure 61: Lytic granules are released through the ring of NKG2D at the activating NK cell synapse. Time-lapse live-cell side-view imaging of a synapse formed between NKL/NKG2D-GFP and Daudi/MICA. Lytic granules in NKL/NKG2D-GFP are stained with Lysotracker (red). The bottom row is the enlarged inset (dotted-line box). Scale bars, 10 μm . $n=3$.

Chapter VI: Summary,

Discussion and Outlook

Confocal microscopy and optical tweezer to achieve high-speed, high-resolution of immunological synapses

The first aim of this multidisciplinary project was to design and develop a new imaging technique that could perform high-speed and diffraction-limited imaging of intercellular immunological synapses. Prior to the start of this project, no existing technique could achieve this without compromising the current range of applications of the microscope. To meet these requirements, we combined optical tweezers in a confocal microscope to micromanipulate cell conjugates. When an immunological synapse is formed between two cells in an imaging well or on a coverslip, it normally lies in the yz plane of the microscope objective, i.e. perpendicularly to the focal plane. The spatial resolution in that plane is only about 1 μm . Also, the need to acquire a full confocal z-stack to generate a single frame means that it can only update at a few frames per minute. Using optical tweezers, we were able to manipulate conjugated cells to orientate the immunological synapse in the focal plane. This allowed imaging the whole synapse formed between two live cells in a single confocal image. Compared to the acquisition of confocal z-stacks, this method improves the spatial resolution at which the synapse is imaged by a factor of 5, and increases the frame rates by around 20 to 50 fold. Also, quantitative fluorescence imaging at the intercellular synapse is made possible due to the isotropic shape of the PSF in the xy plane. We demonstrate the versatility of this instrument by imaging a range of immunological synapses using different cells, proteins, labels and conditions and showed that this method does not restrict the range of applications of the confocal microscope. We imaged the distribution of KIR2DL1-GFP at live inhibitory NK cell synapses, which

exhibited features that had not been accessible before. For the first time at high speed and with high resolution, we imaged TCRs and intracellular signalling molecules such as ZAP70, SLP-76, GADS and LAT with a range of labels and conditions. Finally we performed two-colour live-cell imaging to study the interactions of cytoplasmic vesicles with SLP-76 at the T cell/APC synapse to demonstrate the role of the endosomal network in T cell signalling.

Despite the advances brought by our approach, other existing or developing techniques still have some advantages. Imaging an immune cell interacting with an artificial activating substrate mimicking a target cell or an APC using TIRF microscopy remains an extremely popular technique. Since it is restricted to the glass-media interface, TIRF microscopy does not have the ability to image synapses between two real cells, which limits the physiological relevance of the technique for the immunological synapse. Despite this, the combination of TIRF with activating substrates is an invaluable quantitative tool. Due to better z-sectioning, TIRF microscopy is in general much more sensitive than confocal microscopy allowing, for example, single-molecule imaging to be performed. In addition, a vast number of alterations can be made on the activating substrate such as the addition of diffusion barriers which are useful for investigating the functions and the formation of the immunological synapse but are not easily achievable on a real cell [235]. These two techniques, tweezer/confocal and artificial substrates/TIRF therefore remain complementary.

In future, scientists will attempt to perform super-resolved imaging of the immunological synapse using STED or PALM/STORM. Such images would give invaluable information about the molecular interactions controlling immune cell functions. Due to experimental complexity and to the need of specially designed samples, the range of applications of these techniques remains extremely limited. Some groups, however, have already managed to provide new insights into the immunological synapse using PALM on artificial activating substrate [97]. Ultimately, technical progress should aim at both better spatial resolution and greater physiological relevance. If imaging the synapse *in vivo* with nanometre resolution is a long shot, the next achievable and reasonable step would be to

produce super-resolved images of synapses between two cells. This could be achieved by combining optical tweezer manipulation in a STED microscope which, in its 'simplest' doughnut form, provides nanometre resolution in the xy plane. Improvement in terms of labelling brightness and sensitivity are still needed to achieve this goal, but nonetheless it remains a distinct possibility that this may be reached in the near future.

High-resolution imaging of NKG2D signalling at the activating NK cell synapse

Next we set out to investigate NKG2D signalling and clustering at the activating NK cell synapse using our novel high-resolution imaging technique. NKG2D is an important receptor expressed by NK cells that allows them to recognise and kill tumours and virally-infected cells. Although the nature of the signalling molecules downstream of NKG2D are well-known, the lack of suitable imaging technology has hampered the study of NKG2D *in situ*. Therefore, the aim of this project was to describe the dynamics of the clustering of NKG2D and downstream signalling molecules such as DAP10, Grb2, VAV1 at intercellular immunological synapses. Furthermore we wished to investigate the mechanisms controlling this clustering from the first NK cell/target cell contact until degranulation and target cell lysis. The cellular system that we employed consisted of NKL cells - a NK cell line transfected with fluorescently labelled NKG2D, DAP10, Grb2 and/or VAV1 - as effector cells, and the Daudi cells - a B cell line - expressing MICA as target cells. These particular cells were chosen because NKG2D-mediated conjugation of NKL with Daudi/MICA and subsequent lysis proved to be very efficient, therefore they serve as a good model.

The imaging method, as described in the previous section, does not allow the synapse to be imaged from the initial cell-cell contact since cell manipulation is carried out once the conjugates are formed. To be able to address this issue, target cells were trapped and deposited on top of NK cells. In that case imaging of the synapse is started before any intercellular contact, giving access to the first instants of the formation of the synapse.

We set out to image the dynamics of the distribution of NKG2D at the NK cell activating synapse between NKL/NKG2D-GFP and Daudi/MICA. Upon cell-cell contact, cells formed clear conjugates, with NK cells fully spreading on their targets. NKG2D-GFP accumulated at the synapse and this accumulation was sustained during the whole cell-cell contact, until lysis of the target cell. Using the optical tweezers to image synapses at high-resolution showed that NKG2D was homogeneously distributed at the surface of unconjugated cells and readily formed discrete clusters upon cell-cell contact. Intriguingly, we showed that these clusters dynamically fused to form a central ring of NKG2D – a previously unknown property of NKG2D signalling at the synapse. Tracking of these clusters showed a surprisingly erratic behaviour in contrast to observations made with T cells on bilayers [55].

Further experiments would improve our understanding of the mechanisms driving NKG2D clustering at the activating NK cell synapse. The distribution of NKG2D-GFP appeared homogenous at the surface of quiescent NK cells. However, others showed that TCRs in T cells accumulate in preformed nanometre-scaled clusters in resting cells [97]. Such ‘islands’ of NKG2D cannot be detected with our system, and therefore their existence cannot be ruled out. Preformed clusters of NKG2D could explain why clusters of NKG2D can be seen to form almost spontaneously upon cell-cell contact, and detecting these would require the implementation of a super-resolved imaging technique such as PALM or STORM which would undoubtedly be easier in a TIRF-based system. Full quantifications of the rate of formation of NKG2D clusters and the number of molecules that are present in each clusters would be extremely useful pieces of information about what is needed to trigger NK cell activation. However our system is not the best suited for such studies. Ideally, this would be done in primary cells that do not overexpress signalling proteins, as protein overexpression and the type of cell line being used are likely to alter the number of proteins present in a given microcluster. Since transfection of primary cells can be difficult and might lead to overexpression, using non-blocking fluorescently labelled Fab fragments would be a good solution. The error on the measurements introduced by the presence of background might also be higher than with studies using TIRF

microscopy. The mechanisms driving the formation of the ring remain mainly unknown to us. Possible mechanisms may include a combination of f-actin-mediated driving force and sorting by protein size. Such hypothesis could be tested in our system. Finally, it would be worth studying the disassembly of the synapse and investigate what happens to ligated receptors after termination of the cell-cell contact. However, in our system, we need to prevent the cells from attaching to the coverslip to be able to trap them. NK cells therefore do not have any substrate to exert traction on and cannot move away from their targets. A system that would specifically allow trapping of the target cell while allowing the NK cell to attach to the substrate would be well suited for such a study.

Fluorescent pY immunostaining of conjugates formed between NKL/NKG2D-GFP and Daudi/MICA showed that the pY staining consistently correlated with the presence of NKG2D clusters. This showed that the clusters of NKG2D that are formed at the synapse contain activated proteins. Unlike T cells on bilayers [55], this was the case for both early and late synapses – with early and late synapses being discriminated on the basis of their morphology. In early synapses the NK cell is fully spread on its target while it undergoes a contraction phase at later stages [91]. Having access to more accurate time points would certainly be useful. However, due to the staining procedure, it is difficult to determine precisely the time of fixation with regards to the initial cell-cell contact. Also, the anti-pY primary antibody used here is not specific to any protein. It is therefore not possible to determine which one of the signalling molecules is actually phosphorylated within the microclusters. Generating specific antibodies for a given phosphorylated protein would be extremely useful in this respect and would allow the estimation of the relative proportion of different signalling molecules in a given microcluster. Another interesting approach would be to use FRET to report on the proximity of the fluorescently labelled proteins with the pY antibody but this method requires a thorough experimental design, robust analysis and controls [89]. Finally, it would be extremely useful to determine how many of DAP10 molecules need to be activated to trigger NK cell cytotoxicity. An

approach using quantitative fluorescence imaging correlated with cell activation similar to that developed for T cells could be considered [67].

Next we investigated how inhibitory signalling would affect either the recruitment, the clustering or the signalling of NKG2D itself at the synapse. We used Daudi/MICA β 2m which express MHC class I in addition to MICA. Lysis assays of Daudi/MICA and Daudi/MICA β 2m by NKL/NKG2D-GFP showed that susceptibility by NKL/NKG2D-GFP was markedly reduced in the presence of MHC class I. However, comparison of high-resolution images of synapses formed between NKL/NKG2D-GFP and Daudi/MICA β 2m showed the presence of MHC class I on target cells does not prevent NKG2D from forming signalling clusters.

These results are particularly interesting, as they give further insights about how NK cells integrate activating and inhibitory signals at the synapse. Further experiments would help clarify these mechanisms. As said before, it would be useful to have access to pY antibodies specific to a given protein. In this case, it would be particularly important to quantitatively assess if ITIM motifs are phosphorylated in each microcluster, relatively to phosphorylated DAP10 molecules. Importantly, our interpretation relied on the correlation of the analysis of whole batches of cells (lysis assays, flow cytometry) with individual cell analysis (imaging). A more robust approach would be to monitor or control inhibition precisely for each conjugate. A first approach would be to express both fluorescently labelled activating and inhibitory receptors in NK cells. This would allow interactions between these receptors to be imaged in live cells and would provide important information on the dynamics of these interactions. Photochemical approaches similar to photocleavable peptides developed for T cells [236] would for instance allow spatial and temporal control of NK cell inhibition in live cells.

Downstream signalling molecules such as DAP10, Grb2 and VAV1 propagate signalling downstream of NKG2D upon NKG2D activation. We next set out to investigate the distribution and the clustering of these signalling molecules in live cells as well as their interactions with each other. To investigate the interactions of NKG2D with DAP10 and the interactions of Grb2 with VAV1, two-colour imaging was performed. Imaging of NKL/DAP10-GFP NKG2D-mCherry against Daudi/MICA showed that NKG2D and DAP10 consistently accumulated in the same microclusters. This was expected as DAP10 and NKG2D associate in a hexameric complex through charged interactions [46]. However, an interesting hypothesis to test would be the interchange of DAP10 molecules between such complexes. Our experimental set up does not provide sufficient resolution or sensitivity to resolve such events. Using techniques and tools such as single-molecule imaging, photoactivatable labels, Fluorescence Recovery After Photobleaching (FRAP) or super-resolved imaging could quite easily provide an answer to this.

Next, we imaged interactions with Grb2 and VAV1 at the NK cell synapse, using NKL transfected with VAV1-GFP and Grb2-mCherry. Surprisingly, these two molecules were accumulating in the same microclusters at all times during cell-cell contacts. It was also interesting to see that the microclusters formed by these molecules eventually formed a ring in the same manner than NKG2D and DAP10. The next step would be to investigate the mechanisms that hold VAV1 and Grb2 together as well as the mechanisms that could possibly keep them separated to prevent the propagation of activated signals e.g. inhibitory signals.

Previous studies on T cells showed the presence of a secretory domain within the c-SMAC, surrounded by the p-SMAC which is made of adhesion molecules [66]. Similarly, we set out to determine the relationship between the ring formed by NKG2D and the lytic secretions. Using live cell markers for lytic granules with NKL/NKG2D-GFP conjugated with Daudi/MICA, live-cell imaging showed that NKG2D clusters coalesce around a secretory domain through which lytic secretions are delivered. Although they remain extremely useful to track lytic secretions in live cells, such live cell

markers may also label other acidic compartments. This spatial arrangement i.e. secretory domain surrounded by a ring of NKG2D was confirmed in fixed cells by a more specific anti-perforin fluorescence immunostaining. For the first time these data suggest that receptor patterning at the synapse might be physically important to direct lytic granules towards the target cell. However, further experiments are needed to establish such causality. Possible experiments to address this question could include using activating bilayer substrates with diffusion barriers that would prevent the formation of a ring without stopping the formation of NKG2D clusters and test if this disturbs the secretory domain.

Interestingly, these results give rise to more exciting questions than they answer. Because the task is just as much about dealing with new Biology as new technology, there is no doubt that answering them will require an integrated approach combining Engineering, Physics, Chemistry and Biology.

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