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Single-molecule imaging of suspended T cells

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Single-molecule light-sheet imaging of  
suspended T cells

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## Abstract

Adaptive immune responses are initiated by triggering of the T-cell receptor (TCR). Total internal reflection fluorescence microscopy (TIRFM)-based single-molecule imaging at coverslip/basal cell interfaces is commonly used to study this process. These experiments have suggested, unexpectedly, that the diffusional behaviour and organisation of signaling proteins and receptors may be constrained prior to activation. However, it is unclear to what extent the molecular behaviour and cell state is affected by the imaging conditions, *i.e.* by the presence of a supporting surface. In this study, we implemented single-molecule light-sheet microscopy (smLSM), which enables single receptors to be directly visualized at any plane in a cell, to study protein dynamics and organization in live, resting T-cells. The light sheet enabled acquisition of high quality single-molecule fluorescence images, comparable to that of TIRFM. By comparing the apical and basal surfaces of surface-contacting T cells using smLSM, we found that most coated-glass surfaces and supported lipid-bilayers (SLBs) profoundly affected the diffusion of membrane proteins (TCR and CD45), and that all the surfaces induced calcium influx to various degrees. Of the surfaces studied, T-cells interacting with ICAM-1-presenting SLBs were the least perturbed, but not to the extent of T cells suspended in agarose. Our results suggest that, for studying resting T-cells, surfaces are best avoided.

## Introduction

T-cell activation requires the engagement of membrane receptors by their ligands, which leads to downstream signaling (1) and activation of the adaptive immune response. This process is believed to depend especially on the dynamics (2) and spatial organization (3) of a few, key membrane proteins, including the T-cell receptor (TCR), which recognizes antigens leading to the signaling cascade that drives the immune response (2), and CD45, a protein tyrosine phosphatase that controls activation *via* dephosphorylation of the TCR and the *Src* kinases that phosphorylate the receptor, including *Lck* (4). While important aspects of the activation mechanism have been investigated using single-molecule fluorescence imaging (1, 5–7), revealing unexpected phenomena such as the formation of protein nanoclusters (8, 9), and TCR triggering elicited by the passive reorganization of proteins at “close contacts” (10), the organization and dynamics of proteins in non-activated or ‘resting’ T cells are less well described. Their elucidation is vital to determine the initial state from which receptor triggering is initiated and hence to completely understand the mechanisms responsible for T-cell activation. It is therefore important to develop new biophysical methods for imaging T cells as close to their resting state as possible.

The homopolymer poly L-lysine (PLL) has been widely used as a surface coating to facilitate the imaging of T cells that were presumed to be resting using TIRFM (9, 11–20). However, given that the contact of T cells with a PLL-coated surface is known to induce partial immobilization of the TCR (21) the possibility arises that the resting state of a T cell is perturbed under these conditions. We recently showed that *bona fide* TCR triggering is induced by the spatial reorganization of surface receptors on the plasma membrane when T cells contact protein-coated glass surfaces lacking TCR ligands by altering the phosphorylation state of the TCR at the single receptor level (10, 22). In these experiments and others (23, 24), non-interacting proteins, such as non-specific immunoglobulin (IgG), were used in attempts to passivate the glass surface. (10). Supported lipid bilayers (SLBs) have been used to create more physiological surfaces (22, 25), which typically requires the use of adhesion molecules to anchor the cells to the surface for imaging. However, even the disruption of the highly dynamic and ruffled surface of T cells (26) when they adhere to lipid bilayers represents a potentially significant perturbation of the cells’ physiology, which could be related to integrin out-to-in signaling known to take place on SLBs (27).

Furthermore, we have shown that ligand-independent triggering can occur on SLBs when contact is mediated with small, non-signaling adhesion molecules only (10). Given these uncertainties, there is a need to understand the extent to which surface contact *per se* affects the dynamics and spatial organization of single receptors at cell-glass interfaces, *i.e.* at the basal plane characterized using TIRFM, versus those less likely to be perturbed, *e.g.* receptors at the apical surface imaged using other approaches.

In recent years new techniques have been developed that can image individual membrane proteins away from the coverslip interface (28). For example, single-molecule light-sheet microscopy (smLSM) has been used to monitor the reorganization of the TCR during T-cell activation at subdiffraction resolution (29). Here, we apply smLSM to study the dynamics and organization of two well-characterized (30) and critically important (5) surface proteins, TCR and CD45 in Jurkat T cells. We show that all commonly used strategies for representing resting T cells on surfaces, such as PLL, passivation and SLBs either fail to immobilize cells for imaging or perturb membrane protein dynamics, cause CD45 exclusion, and induce calcium signaling. Our results suggest that truly resting T-cells may have to be imaged away from surfaces altogether. We achieve this by using smLSM to image cells suspended in a gel, establishing a platform for single-molecule imaging of live, resting T-cells.

## Materials and Methods

Full description of the methods can be found in the supplemental information

### Cell culture and labeling

TCR and CD45 proteins in a Jurkat T cell line were labelled using fragment antigen-binding fragments UCHT1 (TCR) and Gap8.3 (CD45) respectively, labelled with Alexa Fluor 488 (SI).

### Single-molecule imaging

**TIRFM** Through-objective TIRFM was performed at room temperature (20 °C) using a 488 nm fibre-coupled diode laser and a 100x 1.49 NA objective lens, with images being captured on an EMCCD camera at a frame rate of 20 Hz (SI).

**smLSM** A secondary perpendicular objective lens was used to introduce a light sheet, created using cylindrical lenses. TIRFM and smLSM could be switched between using a reversible mirror for direct comparison. A custom made sample chamber was constructed to allow the light sheet to enter the sample with minimal aberrations (SI and Fig. S2). The thickness of the sheet was measured to be 1.6 µm by measuring the PSF of 100 nm beads (SI and Fig. S3). Experiments were carried out at room temperature (20 °C).

### Single-molecule tracking

**Spot detection** A custom made Matlab script (31) was used to detect localizations with a signal to noise ratio above 3 (SI). Visualization in Fig. 3 was performed with the Trackmate plugin for ImageJ (32).

**MSD analysis** The ensemble MSD was constructed for each cell and the first 5 points (250 ms) of the curve were fitted to determine the diffusion coefficient (SI).

**JD analysis** Jump distance analysis (31) was applied to investigate heterogeneities in the diffusion coefficient distribution. The probability distribution  $P(r^2, \Delta t)$  of the squared distance,  $r^2$ , travelled in one time step,  $\Delta t$ , was fitted using

$$P(r^2, \Delta t) = \sum_{j=1}^m \frac{f_j}{4D_j \Delta t} e^{-\frac{r^2}{4D_j \Delta t}}$$

where  $f$  is the fraction corresponding to population  $j$  and  $m$  is the number of populations. JD distributions can be fitted with more than one population, but this is not always appropriate. A two-component fit to a single diffusing population,  $D$ , would result in  $D_1 < D < D_2$ . In this work, the purpose of the JD analysis is to identify an immobile population. JD analysis of fixed cells indicate that an immobile population will have a diffusion coefficient of 0.012 µm<sup>2</sup>/s, while the two-component fit to this data, incorrectly results in  $D_1 = 0.005$  and  $D_2 = 0.025$ . We chose the following criteria for using two-component fits based on the fixed data:  $D_1 < 0.03$  and  $D_2 > 0.03$ . This avoids fitting two components simply due to splitting a single population into two and instead necessitates both immobile and mobile populations.

## Results

### Off-surface single-molecule fluorescence imaging of T-cell membrane proteins using smLSM

smLSM was implemented using a bespoke instrument (Fig. S1-2) described in the Materials and Methods, and shown schematically in Fig. 1A. The axially thin light sheet (1.6  $\mu\text{m}$  FWHM, see SI and Fig. S3) enables single-molecule imaging by reducing unwanted background fluorescence due to only a single plane of the cell being illuminated. We used smLSM to perform single-molecule imaging of the fluorescently labelled membrane proteins TCR and CD45 in live Jurkat T-cells. We imaged the TCR, labelled with Alexa Fluor 488-tagged (degree of labeling  $\sim 2$ ) fragment antigen-binding fragments (Fab), on the apical surface of T cells resting on coated coverslips (Fig. 1B). We first compared the performance of the light sheet to standard epifluorescence (EpiFL) illumination (Fig. S4-5), at similar laser power densities (0.1  $\text{kW}/\text{cm}^2$ ) to those typically used in experiments (video S1). smLSM provided improved contrast, and facilitated accurate identification of single molecules compared to using EpiFL illumination. To quantify this difference, we imaged the same three cells using both techniques. Tracking of single molecules was done as described in Materials and methods. As expected, the light sheet gives a substantial increase in contrast of tracked particles (median signal-to-noise ratio: EpiFL - 3.4, smLSM - 5.9, Fig. S4-5). This is a clear demonstration of a system wherein EpiFL illumination is prone to inaccurate tracking, as results were highly dependent on analysis parameters (see SI for comparison), and where smLSM is required to successfully identify most molecules. We therefore conclude that the instrument is a suitable platform for quantitative single-molecule diffusion studies.

We also compared smLSM performance to TIRFM. We could observe stepwise photobleaching (Fig. S6A) using both TIRFM and smLSM, indicating that we were tracking single Fabs. Fig. 1B shows a schematic representation of a T cell attached to a PLL-coated coverslip, which pulls the cell membrane towards the coverslip. As shown, four excitation conditions (1-4) were explored. In the first three, the light sheet was used to image the top (1), middle (2) or bottom (3) of the same cell. In the final case, the bottom of the cell was imaged using 'standard' TIRFM (4). Sample images acquired under these conditions, with TCR labelled using Alexa Fluor 488-Fab, are shown in Fig. 1C (also see video S2). These images were all taken using the same cell within 1 minute and the excitation power density was kept low (0.1  $\text{kW}/\text{cm}^2$ ) to minimize photobleaching. Because the PLL appeared to immobilize the TCR, the same proteins could be identified (white arrows in Fig. 1C, conditions 3 and 4) using both techniques, albeit with a larger autofluorescence background in condition 3. The light sheet enables acquisition of high quality single-molecule fluorescence images, comparable to that of TIRFM.

### **PLL completely immobilizes T-cell membrane proteins on glass surfaces**

The application of smLSM enables precise measurements of the mobility of membrane proteins far above the coverslip, where they are unlikely to be affected by interactions with surfaces. This can be compared with results obtained for the same cell probed at the cell/coverslip interface using TIRFM, allowing the influence of the surface on the mobility of proteins to be established. The diffusion coefficient of the TCR on PLL-coated coverslips was measured at room temperature using TIRFM (Fig. 2A-D) at a power density of (0.5  $\text{kW}/\text{cm}^2$ ). Example ensemble MSD curves and diffusion coefficient histograms are shown in Fig. S7. Compared to an expected value of between 0.06  $\mu\text{m}^2/\text{s}$  at 37  $^\circ\text{C}$  (8), determined by fluorescence correlation spectroscopy, and 0.12  $\mu\text{m}^2/\text{s}$  at room temperature (33), measured by fluorescence recovery after photobleaching, the positively charged coating completely immobilized the TCR ( $D = 0.005 \pm 0.005 \mu\text{m}^2/\text{s}$ ; Fig. 2C and video S3). Here we define 'immobile' as anything below 0.01  $\mu\text{m}^2/\text{s}$ , based on diffusion measurements of TCR in fixed cells on PLL ( $D = 0.005 \pm 0.004 \mu\text{m}^2/\text{s}$ , 10 cells). Note the discrepancy in literature values for TCR diffusion, which is higher at lower temperature. This highlights the variation in measurements based on techniques, suggesting that orthogonal measurements using novel techniques such as smLSM are needed. When measurements were taken on the apical surface of cells on PLL (corresponding to position 1 in Fig. 1B-C) using smLSM (Fig. 2F-H), the diffusion coefficient was significantly larger ( $D = 0.093 \pm 0.038 \mu\text{m}^2/\text{s}$ ; Fig. 2E, Fig. 2J and video S4), which is within the range of literature values (8, 33), indicating that the observed immobilization was caused by surface interactions. The immobilization of TCR by PLL could also be observed when we imaged the TCR using a HaloTag fusion protein labelled with silicon rhodamine using TIRFM (video S5), which means that the observed effect is not caused by the presence of the Fabs. However, we saw more TCR using smLSM than with TIRFM when imaging the same cell (video S5) and some TCRs were now mobile, suggesting that the HaloTag labelling protocol resulted in intracellular staining. We therefore chose to label proteins using Fabs. We also measured the diffusion coefficient of CD45 (Fig. 2O), which has a significantly larger extracellular domain than the TCR ( $\sim 21 \text{ nm}$  versus  $\sim 8 \text{ nm}$ ; 10). It appeared initially as though CD45, unlike the TCR, retained some mobility on PLL. However, visual inspection of Fig. 2K-M and video S6 suggested that CD45 was mostly excluded from glass-cell interface, likely because of the large extracellular domain, and that the CD45 located inside the interface remained immobile, like the TCR.

### **TCR and CD45 are mobile on free surfaces**

While the T-cell proteins were mobile at the apical surface of cells sitting on PLL, it is still possible that the interaction with the coated coverslip could alter the state of the cell, as happens during activation (29), which might manifest in a change in mobility of the proteins at the apical surface. We therefore also investigated the apical surface of cells resting on non-specific bovine IgG and cells suspended in agarose away from the surface, wherein the environmental influence was expected to be near-minimal. In agarose, a standard triggering assay (34) based on the release of intracellular calcium indicated that the cells could be triggered normally when suspended in the gel (Fig. S8) by the addition of OKT3, implying that the cells were resting. In these conditions, the diffusion coefficient of the TCR ( $D = 0.068 \pm 0.023 \mu\text{m}^2/\text{s}$ ) (Fig. 2J and video S4) was not statistically different from that on the apical surface of the cell resting on the PLL ( $P = 0.06$ ) and IgG ( $P = 0.23$ ) coated coverslips. Similar results were obtained for CD45 (see Table S1 and video S7). We therefore conclude that the presence of PLL completely alters protein diffusional properties at the basal cell surface (imaged by TIRFM), while the diffusion properties were unaffected on the apical surface (imaged by smLSM).

### Protein immobilization by non-specific IgG on glass surfaces is size-dependent

We also investigated the mobility of TCR and CD45 on the basal surface of T cells sitting on non-specific bovine IgG. While this is presumed to be a 'passive' surface that should show minimal interaction with proteins, previous measurements showed that this surface induces the local depletion of CD45 from regions of cellular interaction with the surface that we have called 'close-contacts' (10). TIRFM-based single-molecule measurements showed here that the overall diffusion of the TCR near the interface (Fig. 2E) was not significantly influenced by the presence of the surface, as it was similar to that at the apical surface ( $D = 0.068 \pm 0.021 \mu\text{m}^2/\text{s}$ ,  $P = 0.04$ ). It should be noted, however, that the cells did not attach as vigorously to IgG-coated glass as they did to PLL, and the TCR moved in and out of the depth of field, consistent with the formation of undulating and/or discontinuous close-contacts. It is possible that the bulk of the TCRs were free to diffuse because they were located outside of close-contacts (Fig. 3G). In contrast to the TCR, the CD45 diffusion coefficient on the IgG-coated glass surface was significantly smaller than that on the apical surface (Fig. 2O) measured by smLSM, consistent with CD45 interacting with the IgG-coated surface at a longer distance than the TCR. The sample data in Fig. 3A (also see video S8) shows that mobile CD45 moves axially (based on the change in intensity) until it finally becomes static, presumably when it interacts with the surface (highest intensity). The spatial distribution of the mobile and immobile states was observable using the tracking analysis (Fig. 3B and video S6).

We further investigated the distribution of mobile and immobile states using jump distance (JD) analysis, which has been shown to be superior to MSD analysis when comparing small changes in diffusion coefficients (31), especially for short track lengths. More importantly, JD is compatible with tracks that undergo changes in mobility, as we have shown in Fig. 3A, and enables extracting mobile and immobile populations that might not show up in MSD analysis. Fig. 4A shows the JD distributions for the TCR and CD45 on the apical and basal surfaces of T cells resting on IgG-coated coverslips imaged by smLSM and TIRFM respectively. On the apical surface, the fitted distributions were well described ( $R^2 > 0.9$ ) by a single diffusing population (see Table S2 for all conditions). The resulting diffusion coefficients were similar to those determined by MSD analysis (Table S1). This was not the case at the cell/IgG-interface imaged using TIRFM, where we observed shifts in the histogram that required two-component fits. This was most likely caused by the proteins interacting with the surface coating. JD analysis revealed that  $75 \pm 14\%$  of the TCR population had a similar diffusion coefficient to that on the apical surface ( $D = 0.09 \pm 0.02 \mu\text{m}^2/\text{s}$ ) while the rest was immobile (Fig. 3E). We define 'immobile' as anything below  $0.01 \mu\text{m}^2/\text{s}$ , based on diffusion measurements of TCR in fixed cells on PLL ( $D = 0.01 \mu\text{m}^2/\text{s}$ , 10 cells) that agree well with the fitted immobile populations (Fig. S9). This artificial motion appears due to sampling noise and potentially mechanical vibration, causing fixed molecules to appear as having an apparent diffusion coefficient. CD45 on the other hand had a larger immobile population of  $38 \pm 14\%$  (Fig. 3C), likely due to more extensive interactions of its larger extracellular domain with the surface. However, for the mobile fraction ( $D = 0.06 \pm 0.04 \mu\text{m}^2/\text{s}$ ), the diffusion coefficient of CD45 was slower than that on the apical surface, which we also observed with MSD analysis (Fig. 2O). The cartoon in Fig. 4B summarizes the interactions observed on PLL and IgG. The Fabs increase the molecular weight of TCR and CD45 by  $\sim 10\%$  and  $\sim 20\%$  respectively. We do not expect the Fabs to influence the interactions between the studied proteins and the tested coatings because they bind near the membrane. UCHT-1 binds the two CD3- $\epsilon$  subunits that are part of the TCR complex (35). Gap 8.3 binds some part of the CD45d1-d4 region of CD45 (36), which does not include the 8-21 nm long mucin-like region of CD45.

### Most coated glass surfaces induce calcium signaling in T cells unlike suspension in agarose

Having observed perturbation of TCR and CD45 dynamics by PLL- and IgG-coated coverslips, we attempted to find a better coating for studying resting T cells. Fibronectin-coated glass is reported to not induce T-cell activation (37), and would enable TIRFM imaging. We found that membrane proteins on fibronectin (TCR:  $D =$

0.058±0.013  $\mu\text{m}^2/\text{s}$ ; CD45:  $D = 0.057 \pm 0.019 \mu\text{m}^2/\text{s}$ ) (video S9) were mobile unlike on PLL, while providing a suitable interface for imaging unlike IgG. Also, CD45 appeared to be moving freely within the probed interface (video S9). JD analysis showed that 25-30% of proteins on fibronectin were immobilized (Table S2), whereas most proteins remained mobile in agarose. These results indicated that the influence of fibronectin on TCR and CD45 dynamics were minimal compared to PLL, pointing to the possibility that T cells may have a resting phenotype in this condition. However, the morphology of the cells was altered, presumably due to adhesion of membrane integrins to the fibronectin (38). We applied a calcium release assay using Fluo-4 (34) to determine whether IgG and fibronectin are indeed non-perturbative and found that, while superior to the PLL and IgG coating in terms of its effects on TCR and CD45 diffusion, both IgG and fibronectin still caused a significant fraction of the cells to activate (Fig. 5G), compared to the agarose gel. Although the time taken for calcium release to occur on PLL, IgG and fibronectin appears longer (Fig. 6H) than that observed on OKT3-coated coverslips (77±21 s), the results were not statistically different. In terms of our choice of calcium assay, it could be argued that ratiometric calcium sensors such as Fura-2 are superior for monitoring subtle changes. However, we typically observed ~4-fold increases in calcium, which was much larger than observed for cells that did not activate (Fig. S10). We could clearly see the characteristic calcium response observed during T-cell activation on all surfaces, showing that Fluo-4 is a suitable calcium probe for these experiments as reported previously (34, 39).

We then proceeded to investigate the possibility of using SLBs, due to their ability to maintain membrane fluidity and their prevalent use in activation studies. Mobile anchors were required to adhere cells to the bilayers to facilitate imaging as cells interacting with neat SLBs were found to roll around on the surface. Two anchors were tested: (i) His-tagged chimeric protein comprising the extracellular domains rCD45rCD2 (estimated gap size of approximately 29 nm), which could bind to Jurkat T cells that were transfected with a non-signaling mutant form of rat CD48 (T92A) that binds rat CD2 with a comparable affinity to the human CD2-CD58 pair (~20  $\mu\text{M}$ ) and (ii) His-tagged ICAM-1 (estimated gap size of 40 nm), which would bind to LFA-1 integrin present on Jurkat T cells. The calcium assay showed that SLBs with rCD45rCD2 interacting with Jurkat (+CD48) resulted in significant calcium release while the ICAM-1 SLBs greatly reduced calcium signaling (Fig. 5G), although not to the extent of the agarose gel. We also measured the diffusion of TCR and CD45 on rCD45rCD2 SLBs (TCR:  $D = 0.044 \pm 0.014 \mu\text{m}^2/\text{s}$ ; CD45:  $D = 0.064 \pm 0.013 \mu\text{m}^2/\text{s}$ ), which was very similar to that on fibronectin. JD analysis showed that 23-29% of proteins were immobile (Table S2). We did not investigate ICAM-1 SLBs as we found that cells were highly mobile

### Reorganization of CD45 upon interaction with coated glass surfaces

As membrane protein dynamics was preserved on fibronectin and presumably on SLBs we sought to understand possible causes of the observed calcium signaling. Previous studies (10) have suggested that CD45 can be excluded on glass coated with PLL, non-specific IgG and by SLBs carrying mobile anchors, leading to calcium signaling and ZAP70 recruitment. We applied two-colour TIRFM imaging of CD45, labelled with saturating levels of Alexa Fluor 488-Fab, and the membrane stain CellMask Deep Red to monitor CD45 exclusion, relative to the membrane, by coated glass surfaces (Fig. 6 and Fig. S11). In all cases where we observed significant calcium release (PLL, IgG, fibronectin and rCD45rCD2 SLBs), we also noticed exclusion of CD45 relative to the membrane dye within only 5 minutes of interaction. Note that the exclusion zones for cells interacting with IgG were often difficult to resolve because of the minimal adhesion. Compared to ICAM-1 SLBs we could however see rings of CD45 (yellow highlights in Fig. S11 that are characteristic of exclusion (10). On SLBs with ICAM-1 we found that the cells were mobile (video S10), such that the coating is likely to maintain cells in their resting state but complicates the study of membrane protein dynamics and organization. smLSM imaging of CD45 and CellMask at the basal (bottom) plane of cells suspended in agarose (Fig. 6) showed minimal CD45 exclusion compared to cells interacting with coated surfaces. We verified that this observation was not simply a result of the imaging modality by directly comparing basal imaging of the CD45 distribution with TIRFM and smLSM (Fig. S12A) and by imaging the basal and apical surfaces of the same cell (Fig. S12B), which clearly showed that smLSM is capable of visualizing exclusion. To confirm that CD45 exclusion occurred relative to TCR, we also imaged TCR labeled with Alexa Fluor 488-Fab under saturating conditions. On PLL we could observe TCR throughout the contact area but the distribution was inhomogeneous unlike the membrane stain, indicative of clustering of TCR (Fig. S13). However, we do not suggest that these are TCR microclusters as the distribution was completely static and we could not see any centripetal motion of clusters (video S11).

Table 1: Summary of perturbations by coated glass surfaces. PLL-coated glass completely immobilizes TCR and CD45 proteins in proximity to the surface. On IgG, the proteins are only partially immobilized, with CD45 having a larger immobile population. On the apical surface, both proteins diffuse freely independently of sitting on coated coverslips or being suspended in agarose. Fibronectin and rCD45rCD2 SLBs minimally influence protein dynamics compared to PLL, while providing a suitable TIRFM interface, but these coatings influence cell morphology, induce calcium signaling and exclude CD45. SLBs with ICAM-1 induce minimal calcium flux and CD45 exclusion but does not immobilize cells, which complicates imaging. Agarose suspension maintains protein mobility and organization while inducing minimum calcium signaling.

	PLL (TIRFM)	IgG (TIRFM)	Fibronectin (TIRFM)	rCD45rCD2 bilayer (TIRFM)	ICAM-1 bilayer (TIRFM)	Agarose (smLSM)
<b>TCR diffusion</b>	Immobile	75 % mobile 25 % immobile	75 % mobile 25 % immobile	71 % mobile 29 % immobile	-	Freely diffusing
<b>CD45 diffusion</b>	55 % excluded 45 % immobile	62 % mobile 38 % immobile	70 % mobile 30 % immobile	77 % mobile 23 % immobile	-	Freely diffusing
<b>Calcium signaling</b>	54.0 % 139 s	53.7 % 130 s	44.0 % 147 s	48.7 % 112 s	15.0 % 194 s	2.5 % 131 s
<b>CD45 exclusion</b>	Yes	Yes	Yes	Yes	No	No
<b>Cell mobility</b>	Immobilized	Immobilized	Immobilized	Immobilized	Mobile	Immobilized

## Discussion

The observations made by investigating the diffusional properties and organization of the TCR and CD45 using smLSM and TIRFM are summarized in Table 1.

Both the TCR and CD45 were largely completely immobilized by contact with PLL-coated glass. Previously, the TCR was shown to be partially immobilized on PLL using confocal FRAP (21). Given the larger probe volume of confocal microscopy, it seems likely that the mobile population observed in those experiments was not associated with the cell surface. Single-molecule fluorescence measurements have also indicated immobilization of amoebal proteins by glass (40), and of human proteins by PLL (41). Despite this, PLL has been a popular choice for studying 'resting' T-cells using TIRFM (9, 11–16, 18, 19, 23). The immobilization caused by PLL is likely explained by its electrostatic properties. The polyelectrolyte pulls the cell membrane towards the coverslip, creating a large interface that is tightly bound to the surface. We observed, using both diffusion analysis and imaging, that the tightly bound surface dramatically excludes CD45. Cells also adhered to lipid bilayers presenting nickel cations only, which also led to CD45 exclusion (Fig. S14). This effect could be suppressed by removing the nickelated lipids (or by blocking with 1% BSA), whereupon the cells would 'tumble' along the bilayer. These observations suggest that charge alone, in the form of PLL or nickelated lipids, suffices to elicit strong T-cell adhesion.

On glass surfaces coated with passivating proteins (e.g. bovine IgG), a large fraction of the T-cell proteins remained mobile, further confirming that the major effects of PLL are due to its charge. The largest difference between the two membrane proteins we studied on T cells interacting with IgG-coated glass, was that there was a smaller fraction of mobile CD45 than TCR molecules. For use in diffusion-based studies, passivating coatings such as IgG appear to be less perturbative than PLL, but present their own problems insofar as (i) the discontinuous contacts that form allow proteins to move in and out of focus, and (ii) the effects of the surface on mobility become dependent on the size of the extracellular domains of the proteins. Nevertheless, IgG also immobilized a significant fraction of TCR molecules and induced the local exclusion of CD45.

The differences in the behavior of the TCR and CD45 on PLL and IgG can be explained by considering that the TCR has a smaller extracellular domain than CD45: i.e. 8 nm versus 21 nm, respectively (10). The Fabs used in this work each bind close to the membrane, and are therefore unlikely to affect surface interactions. A cartoon depicting the effects of the two surfaces on the proteins is shown in Fig. 3G. The TCR exhibits a mostly homogenous distribution over the cell surface when the cell membrane binds tightly to the PLL-coated coverslip (Fig. 3G bottom) whereas CD45 is excluded, with only a few immobilized proteins remaining inside the contact. On IgG-coated glass, there is much less exclusion of either protein as the weak interaction between the cell membrane and the surface appears to create pockets where the proteins can diffuse freely (Fig. 3G top). However, the large extracellular domain of CD45 makes it more likely to interact with the surface, as revealed by the observation that the immobile CD45 population is larger than that for the TCR.

The finding that PLL induces calcium influx in Jurkat cells is not completely surprising given that the strong adhesion of the cells to PLL-coated surfaces leads to CD45 exclusion (Fig. 6, Fig. S11, video S12), which suffices to initiate signaling (10). The extent to which an immortal cell line like Jurkat is representative of primary cells is, however, questionable. While continuously cycling cells probably cannot be considered to be truly 'resting', we would nevertheless expect primary cells to exhibit similar immobilisation, exclusion and calcium

responses on PLL given that similar effects are observed when they interact with IgG-coated coverslips and adhesion molecule-presenting bilayers (10). Contrary to the interactions we observed here for Jurkat T cells on PLL-coated glass, the effects of apparently less adhesive coatings like IgG (video S12), fibronectin and rCD45rCD2 SLBs on signaling can perhaps be explained in terms of the behavior of T-cell microvilli (26, 42, 43). These actin-rich protrusions continually push against surfaces in their effort to search for antigens. It is possible that the microvilli push with sufficient force to exclude CD45, thereby potentiating calcium signaling, even for relatively deep polymeric coatings like fibronectin (~25 nm, (44)). For ICAM-1 coated glass the relatively large gap between the cell and the surface (~40 nm) combined with both the and incomplete attachment and fluidity of the bilayer, might prevent microvilli from forming (large- or stable-enough) CD45 exclusion zones that can initiate calcium signaling. For agarose gel, the elasticity is comparable to the cell membrane (~38 kPa (45) vs. 5-11 kPa (46)), which might be less perturbative in response to the forces and motion exerted by the microvilli. Whether or not these processes in turn affect global protein mobility is unclear, although activation is associated with large-scale reorganization and clustering of the TCR (29). As shown here, diffusion on the apical surface was largely unaffected by surface coating, suggesting that surfaces mostly induce local rather than global changes in mobility on the time scales studied here. Nevertheless, the immobilization, slowing down and exclusion of proteins can influence signaling (10), making it important to be able to visualize molecular behavior in unperturbed cells, e.g. suspended in agarose above the surface and visualized using smLSM, as done here.

Fibronectin was found to be only slightly superior to both PLL and IgG in terms of its influence on TCR and CD45 dynamics. The kinase Lck, which has previously been shown to reside in nanoclusters on PLL-coated surfaces (9), is unclustered on fibronectin (37). However, we observed CD45 exclusion and calcium signaling on fibronectin-coated glass, indicating that this surface is also unsuitable for imaging resting T-cells. SLBs presenting the mobile adhesion protein rCD45rCD2 also caused partial immobilization of proteins, CD45 exclusion and calcium signaling, despite it likely creating a large gap between the surface and the cell (~29 nm). Therefore, even the use of large mobile anchors fails to secure the resting state. We found that the least perturbative surfaces comprised SLBs presenting ICAM-1. This coating has been used for numerous activation studies (22, 47, 48) and our results here suggest that the coating is minimally perturbative. However, the high mobility of cells on ICAM-1 coated SLBs, presumably due to the lack of calcium signaling, complicates its use for imaging resting T cells. Although it is conceivable that glass surfaces could be optimized in some way to prevent calcium release in the future, we suggest that for now cells should ideally be studied in suspension if the aim of the experiment is to image live resting lymphocytes.

In our analysis, we considered the diffusion of membrane proteins to be two-dimensional along the surface of an idealized sphere, such that the images acquired using the light sheet represent a two-dimensional projection of the spherical surface. Neglecting effects of the projection causes at most a 35 % reduction in the perceived diffusion coefficient at the equator of the sphere (49), proved via simulations (SI and Fig. S15). Furthermore, the surface can effectively be approximated by a plane as only the top ~1-2  $\mu\text{m}$  of the cell is imaged by the light sheet. However, this treatment neglects the fact that cell membranes are highly irregular. This is particularly true for T cells, which feature actin-rich structures such as microvilli and podosomes (26, 42). Therefore, proteins diffuse perpendicularly to the membrane surface, causing the observed diffusion to be slower than the actual value (50). We could observe this irregular structure using smLSM by imaging at the central position of the cell (Fig. S16). Three-dimensional tracking methods (51) could be used to resolve this problem and to measure the true mobility of membrane proteins on unperturbed surfaces, which we have begun to apply to T cells (52).

In conclusion, we have shown that membrane protein dynamics and organization are greatly influenced by interactions with surfaces used to immobilize cells for imaging, leading to calcium signaling. These findings suggest that T cells immobilized on coated coverslips will be perturbed from their resting state. If the goal is to image resting T cells, which is crucial to detect the early events that lead to T-cell activation, surfaces should ideally be avoided altogether. Single-molecule LSM of cells suspended in agarose gels allows single-molecule imaging to be performed on live, resting T-cells, and should be broadly applicable to other cell types.

## Author contributions

A.P., J.M., A.M.S., S.D., D.K. and S.F.L. designed research. A.P. implemented the light-sheet microscope and performed diffusion and imaging experiments. A.P., J.M. and A.M.S. performed calcium experiments. A.P. and A.R.C. analyzed data. A.P., K.K. and A.H.L. prepared and performed bilayer experiments. A.P., S.D., D.K. and S.F.L. wrote the manuscript with input from all authors.

## Supporting Citations

References (53–59) appear in the Supporting Material.

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### Captions:

**Fig. 1: Single-molecule imaging of membrane proteins on the apical surface of living T cells.** A: Schematic depicting smLSM geometry. B: Schematic of experiment showing how scanning of the light sheet enables sectioning of the T cell. The numbers correspond to four distinct cases in C. C: The same T cell was imaged at the apical (1), central (2) and basal (3) planes using smLSM, and at the basal plane using TIRFM (4). The gray dotted circle represents the size of the cell after spreading on a PLL-coated coverslip. The arrows in 3-4 highlight the same molecules imaged using smLSM (3) and TIRFM (4). The scale bar is 5  $\mu\text{m}$ .

**Fig. 2: PLL immobilizes TCR and CD45.** (A,F,K): Sample single-molecule images of T-cell proteins on the basal (A,K) and apical (F) surfaces. The scale bar is 5  $\mu\text{m}$ . The TIRFM data appears to be saturated because it has been contrast-matched with the smLSM data. (B,G,L): Schematics of the excitation schemes corresponding to A, F and K. (C,H,M): Tracking results from A, F and K. The color of each track corresponds to the diffusion coefficient determined by MSD analysis. The scale bar is 5  $\mu\text{m}$ . (D,I,N): Close-up of tracks from C, H and M. The scale bar is 300 nm. (E,J,O): Mean diffusion coefficient of TCR interacting with various surfaces. The diffusion coefficient was determined for each cell using the ensemble average MSD curve.  $n$  is the number of cells and  $t$  is the number of tracks for each dataset. The error bar represents the standard deviation of the cell to cell variation. The P-value corresponds to the result of an unpaired two-sample t-test.

**Fig. 3: T-cell membrane proteins interact even with non-specific IgG.** A: Sample TIRFM data showing how initially mobile CD45 above the surface sits down on IgG, becoming immobile. The blue track corresponds to the local 11 tracks from -5 to 5 frames. The yellow circle indicates the molecule. The scale bar is 1  $\mu\text{m}$ . Visualization was performed with the Trackmate plugin for ImageJ. B: Sample TIRFM frame (left) of CD45 on IgG with corresponding analyzed tracks (right) colored by diffusion coefficient (see color bar). The scale bar is 5  $\mu\text{m}$ . The dotted circle represents the size of the cell-IgG interface.

**Fig. 4: (A): Normalised jump distance distributions for T cells interacting with IgG for data taken on the apical (smLSM) and basal (TIRFM) surfaces.** Histograms were fitted with single or dual distributions as described in materials and methods. The black line shows the sum of all fitted distributions and the red and blue lines correspond to the slow and fast distributions respectively. The dashed lines indicate the peak of each distribution and the corresponding diffusion coefficient in  $\mu\text{m}^2/\text{s}$  is given above each line. B: Cartoon model of the influence of surface interactions on protein mobility. Only the extracellular domains of the proteins are to scale, while other dimensions have been exaggerated for illustrative purposes. The podosomes of the T cell loosely sit on the non-specific IgG surface (top), creating pockets in which proteins remain mobile (indicated by arrows). CD45 with its large extracellular domain is more likely to interact with the surface. The PLL surface (bottom) tightly binds the cell, excluding CD45 and immobilizing proteins (indicated by \*) within the cell-PLL interface. The insets show cartoons of the cell topology.

**Fig. 5: Surface-induced calcium flux varies greatly between supposedly resting surfaces.** (A-B): Low magnification (15 $\times$ ) epifluorescence image of Fluo-4-labelled Jurkat CD48+ T cells landing on an OKT3-coated coverslip at two time points. The arrows indicate individual cells that are initially weakly fluorescent (A), but upon activation undergo calcium release (B). The scale bar is 20  $\mu\text{m}$ . (C-D): Fluorescence image of Fluo-4-labelled T cells suspended in agarose. Cells do not experience an increase in calcium as they remain resting in the gel. Out-of-focus cells appear larger and dimmer than others due to the three-dimensional distribution in the gel. E: Representative examples from A and B of activating cells. F: Representative examples of cells remaining resting when suspended in an agarose gel. G: Fraction of cells activating within 5 minutes on supposedly resting surfaces.  $n$  represents the number of cells. Data was taken from 3 independent experiments for each condition. H: Average time taken for activation for all activated cells. \* $P < 0.01$ , \*\* $P < 0.001$ , n.s.  $P > 0.05$ , one-way ANOVA and Tukey's post hoc comparison. In H no significance ( $P < 0.05$ ) was found except for between OKT3 and ICAM-1 bilayer as reported.

**Fig. 6: Supposedly resting surfaces alter the organization of CD45.** Two-colour TIRFM imaging of CellMask Deep Red membrane stain shows the distribution and topology of the cell membrane for Jurkat CD48+ cells upon interaction with various environments for 5 minutes. CD45 was labeled with saturating levels of Alexa Fluor 488-Fabs. The inset depicts a schematic of the cell morphology. Imaging was done in TIRFM mode except for the agarose case, where smLSM was used. The rCD45rCD2 SLB has labelled mobile anchors, which somewhat disguises the membrane stain. The CD45 distribution shows large scale reorganization compared to the membrane stain for PLL, IgG, fibronectin and the rCD45rCD2 bilayer. The scale bar is 5  $\mu\text{m}$ .











