Electronic Supplementary Material (ESI) for Nanoscale.

The T cell receptor displays lateral signal propagation involving non-engaged receptors

Daniel J. Nieves[1,3#*], Elvis Pandzic[2#], Sachith D. Gunasinghe[1], Jesse Goyette[1], Dylan M. Owen[3], J. Justin Gooding[4] & Katharina Gaus[1]

[1] EMBL Australia Node in Single Molecule Science, School of Medical Sciences and the ARC Centre of Excellence in Advanced Molecular Imaging, University of New South Wales, Sydney, Australia.

[2] Katharina Gaus Light Microscopy Facility, Mark Wainwright Analytical Centre, University of New South Wales, Sydney, Australia

[3] Institute of Immunology and Immunotherapy, School of Mathematics and Centre of Membrane Proteins and Receptors (COMPARE), University of Birmingham, Birmingham, UK.

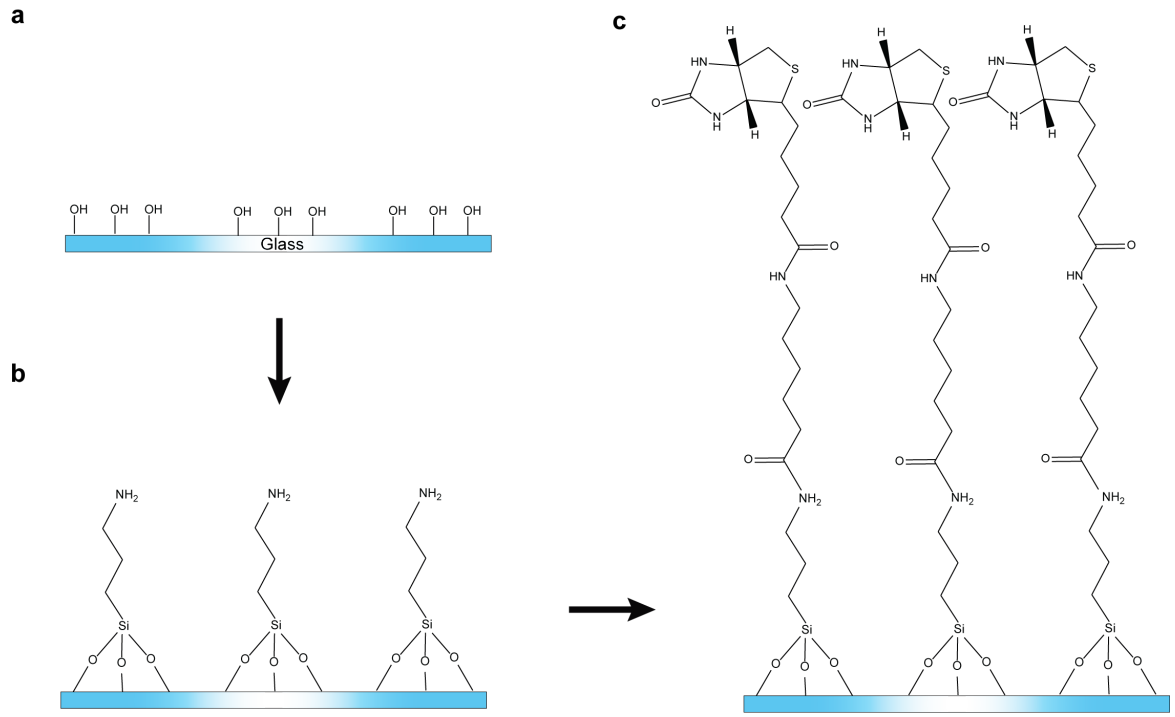
[4] School of Chemistry and Centre for NanoMedicine, University of New South Wales, Sydney, Australia

[#]Contributed equally.

*Corresponding lead contact: d.j.nieves@bham.ac.uk

Supplementary Information

SUPPLEMENTARY FIGURES



Scheme S1. Covalent modification of glass surfaces with biotin for immobilization of streptavidin coated polystyrene particles. The steps are: (**a**) Glass coverslips were cleaned with ethanol sonication, water sonication, acetone rinse, and a final water sonication before being dried thoroughly with N_2 gas. The glass was then air plasma cleaned to generate highly hydrophilic surfaces for silane modification. (**b**) The glass was reacted immediately with (3-aminopropyl)trimethoxysilane for 1 h, after which the surfaces were rinsed thoroughly with absolute ethanol and then water. N_2 gas used to remove large water droplets, and then the surfaces were annealed at 100°C. (**c**) After annealing, surfaces were cooled to room temperature and immediately reacted with an excess of freshly prepared biotinamidohexanoic acid 3-sulfo-N-hydroxysuccinimide ester for 1 h. Then, the surfaces were thoroughly rinsed with water, and ready for further modification with streptavidin-bearing polystyrene particles.

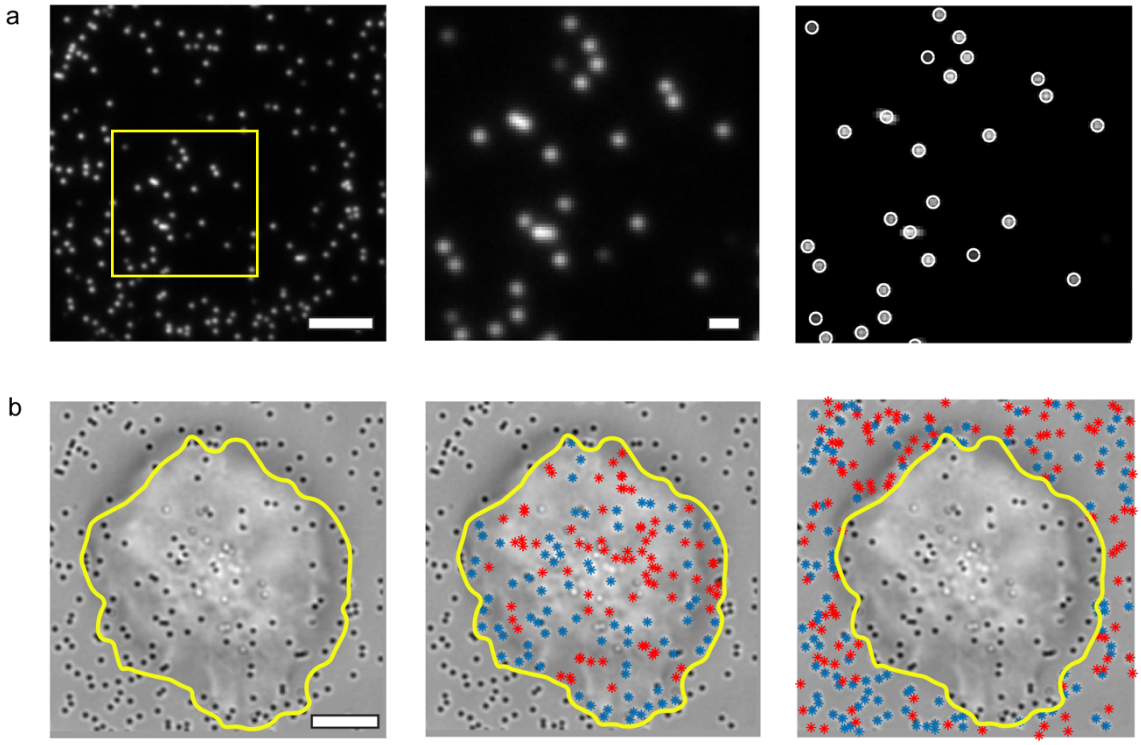


Fig.S1: SMLM analysis at sites of activating and non-activating sites. (**a**) To extract the density of TCR signaling protein at the activating site from SMLM images, the functionalized nanoparticles were co-labelled with biotinylated anti-CD3ε and biotin-FITC, allowing TIRF imaging of the activating site position (left: scale bar = 5 µm; middle: zoomed region of yellow box, scale bar = 500 nm). The position of the activating sites was then extracted by Gaussian fitting of band-passed FITC TIRF images (right) and rendered with a 340 nm diameter region of interest (ROI, white circles). (**b**) Cell contour (yellow) is extracted from the bright field image (right; scale bar = 5 µm) and the activating site positions (blue stars) within the cell contour **(**middle**)** or outside the contour (left) are segmented. For comparison, the same number of randomly generated non-activating sites was selected in areas where there are no particles (red stars). The activating and non-activating ROIs inside and outside the cell contour are then used to extract the density of SMLM localizations at these sites.

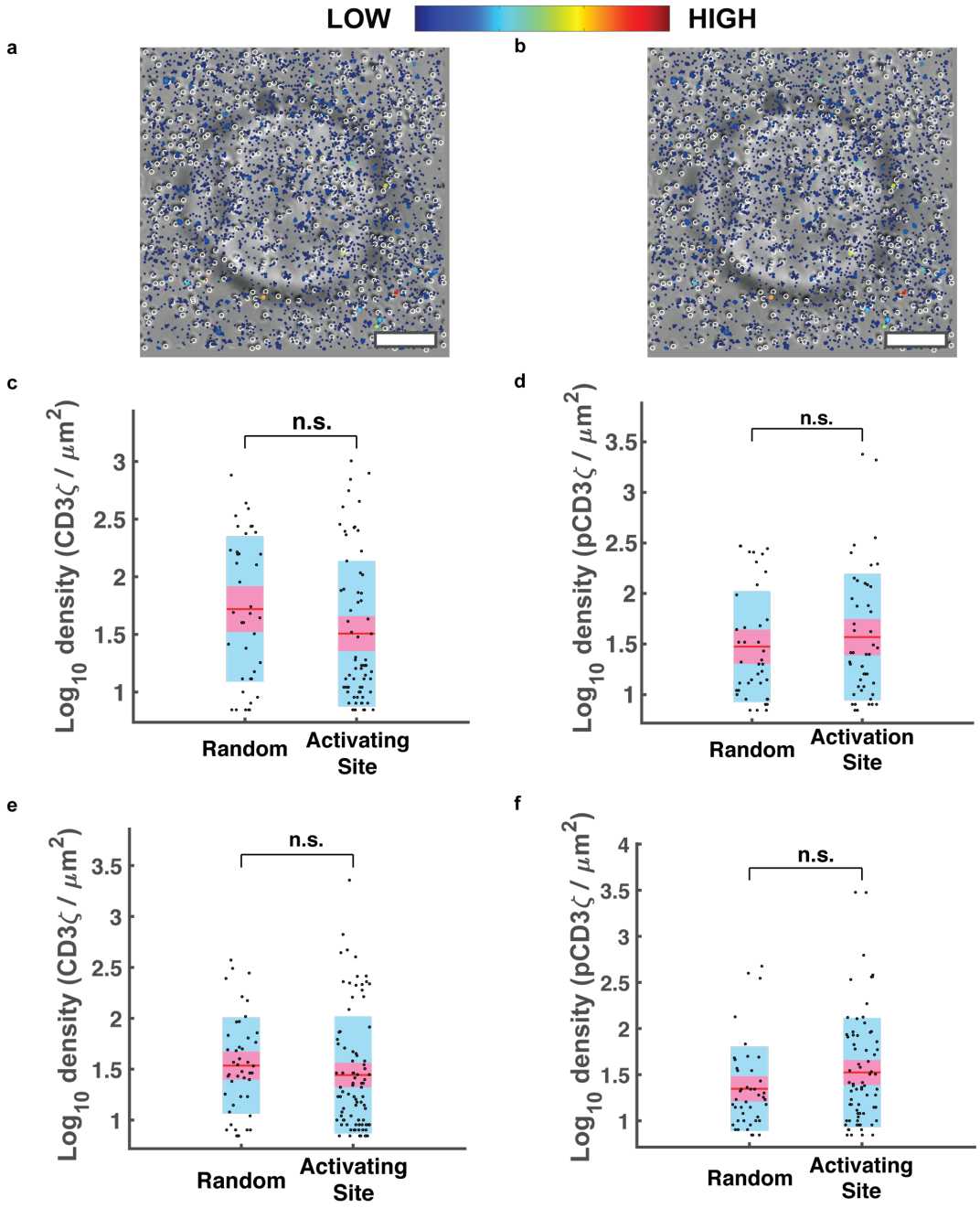


Fig.S2: Nanoparticles without activating ligands did not lead to enrichment of CD3ζ and pCD3ζ. (**a-b**) Jurkat T cells were incubated for 5 min at 37°C with surfaces modified with nanoparticles that did not contain TCR ligands. Surfaces were additionally coated with anti-CD90 antibodies to promote cell adhesion. Cells were fixed, stained for CD3ζ (a) or phosphorylated CD3ζ (b) and imaged with SMLM. Color scale indicates density as determined by local point pattern analysis. (**c-f**) Peak density of CD3ζ (c, e) and phosphorylated CD3ζ (pCD3ζ) activating site and randomly selected non-activating sites underneath the cell (c, e) and outside of the cell (e, f). Black circles indicate data from one activating/non-activating site, red lines are means, cyan boxes indicate standard deviation and magenta boxes 95% confidence intervals. n.s. = not significant ($P > 0.05$, one-way ANOVA). Particles numbers were n = 68 (c), n = 91 (d), n = 48 (e), n = 75 (f).

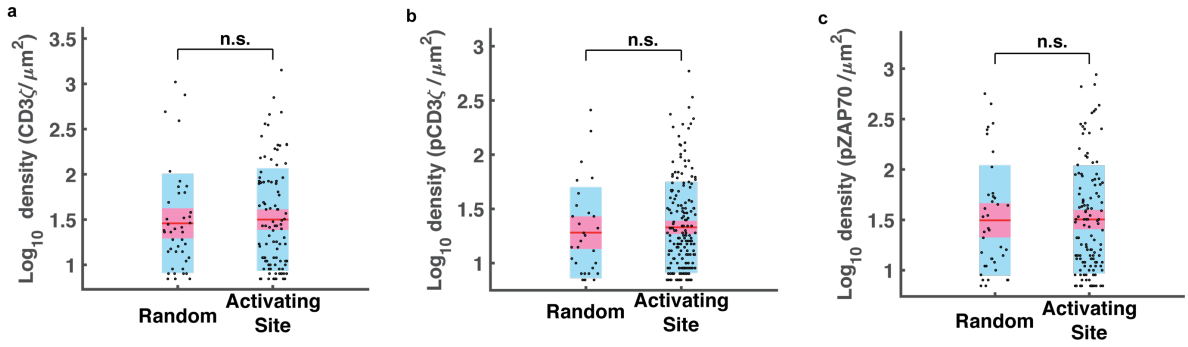


Fig.S3: Peak densities of CD3ζ, phosphorylated CD3ζ (pCD3ζ) and phosphorylated ZAP70 (pZAP70) outside the cell at activating and randomly sampled regions. (**a-c**) Peak density of CD3ζ (a), pCD3ζ (b) and pZAP70 (c) obtained from SMLM images at activating site and randomly selected non-activating sites outside of the cell. Black circles indicate data from one activating/non-activating site, red lines are means, cyan boxes indicate standard deviations and magenta boxes 95% confidence intervals. n.s. = not significant (P > 0.05, one-way ANOVA). Particles numbers were n = 98 (a), n = 181 (b), n = 122 (c).

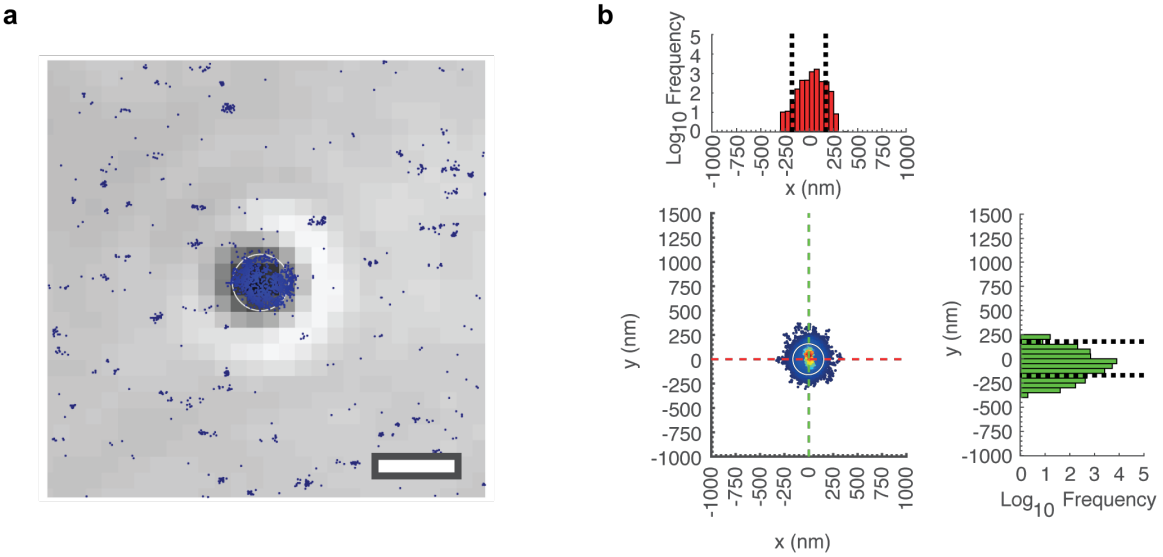


Fig.S4: TCR ligands did not reach beyond the activation site of the nanoparticle. To measure the reach length of the immobilized ligands, activating surfaces with nanoparticle functionalized with anti-CD3ε antibodies were stained with secondary antibodies (anti-mouse IgG) conjugated to Alexa Fluor 647 and imaged by SMLM. (**a**) SMLM image of the secondary antibody. Scale bar = 500 nm (**b**) Aligned and merged distributions of the secondary antibody localization; pseudo-coloring indicates density (blue to red is low to high). Average nanoparticle size and standard deviation are indicated with red and green dashed circles, respectively. Distributions along the major axis (green) and minor axis (red) are also shown with black dashed lines indicate boundary of the nanoparticles. Data are from 2 images and n = 196 particles.

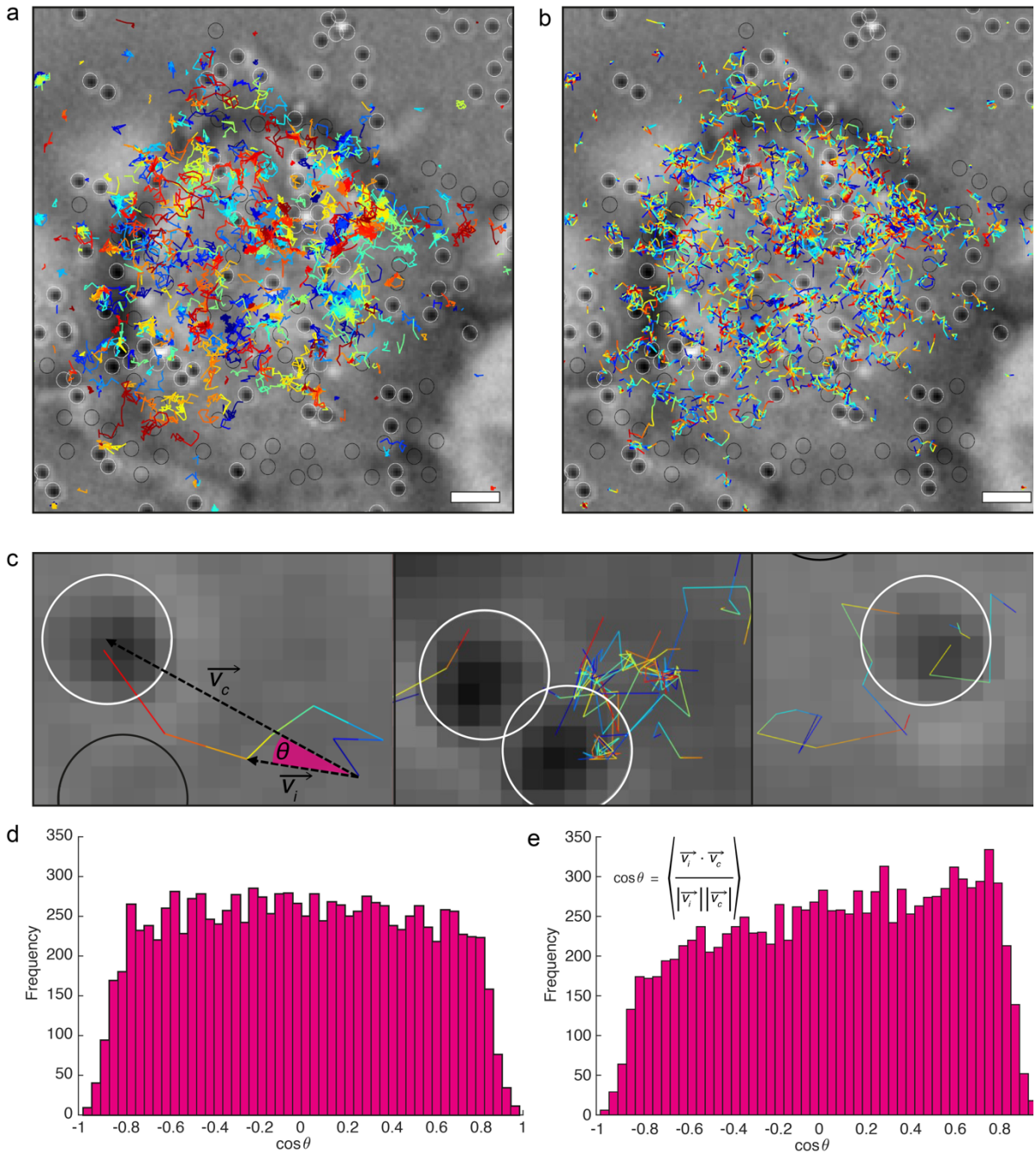


Fig.S5. CD3ζ preferentially diffuses towards anti-CD3ε activating nanoparticle sites. CD3ζ was genetically tagged with PAmCherry and expressed in Jurkat cells deficient in CD3ζ. This allowed single molecule tracking of CD3ζ in the membrane of live Jurkat cells activated on the nanoparticle surface. Single molecule trajectories of CD3ζ-PAmCherry superimposed over the bright field image of nanoparticle surface and Jurkat cell, with the position of the activating sites (white circles), and sites without ligand (black circles). Single molecule trajectories are colour-coded; a) a different colour for each trajectory, b) colour represents time from beginning to end of trajectory (blue – beginning, red -end). Scale bar – 2 µm. c) Zooms of trajectories and activating particles from b). Single CD3ζ-PAmCherry trajectories were analysed to determine their angle of movement, with respect to the centre of the nearest particle and the start of the trajectory (blue), for each point of the trajectory (*cos* $\boldsymbol{\theta}$). Trajectories, and thus CD3ζ-PAmCherry, that move towards the particles will on average have a positive mean *cos* $\boldsymbol{\theta}$. d) Average *cos* $\boldsymbol{\theta}$ for trajectories with respect to non-

activating virtual particles, i.e., regions of the cell not in contact with the anti-CD3ε activating nanoparticle sites. e) Average *cos* $\boldsymbol{\theta}$ for trajectories with respect to the anti-CD3ε activating nanoparticle sites.

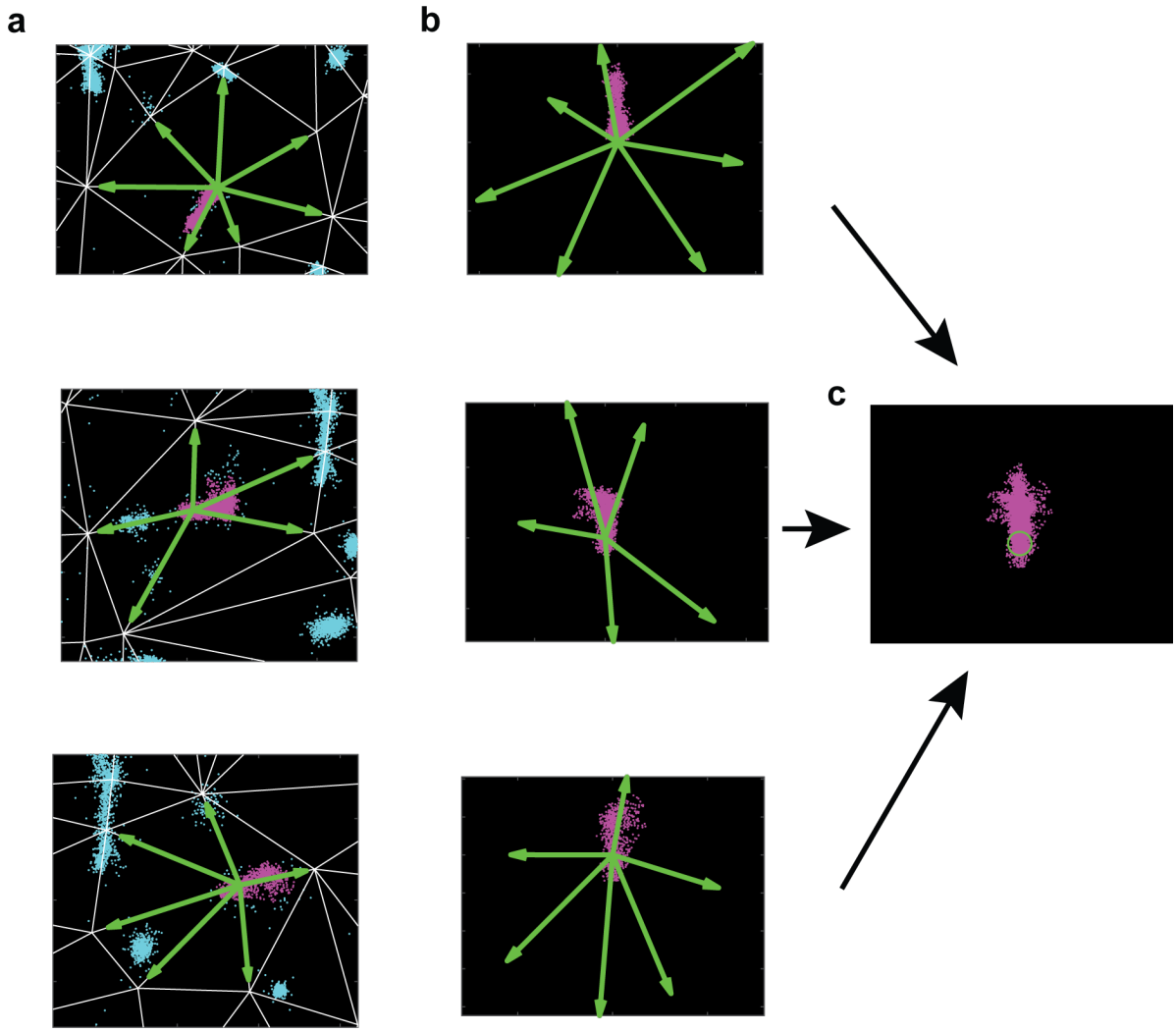


Fig.S6: Generation of 2D cluster extension maps from SMLM images to quantify signal propagation beyond activation site. SMLM image regions of three different nanoparticles from the same image, containing single molecule localizations of CD3ζ (magenta and cyan). (**a**) The positions of activating particles were first determined by Delaunay triangulation (white lines) and the closest particles to the one being analyzed marked with green arrows. (**b**) The CD3ζ clusters belonging to the particle of interest were segmented (magenta) from other clusters (cyan) using DBSCAN and rotated, together with the position vectors of the neighboring particles (green arrows) so that its major axis of the protein cluster is perpendicular to the x direction. (**c**) Aligned clusters are merged to cluster map of the 2D density. The position of the activating particle is indicated by the green circle.

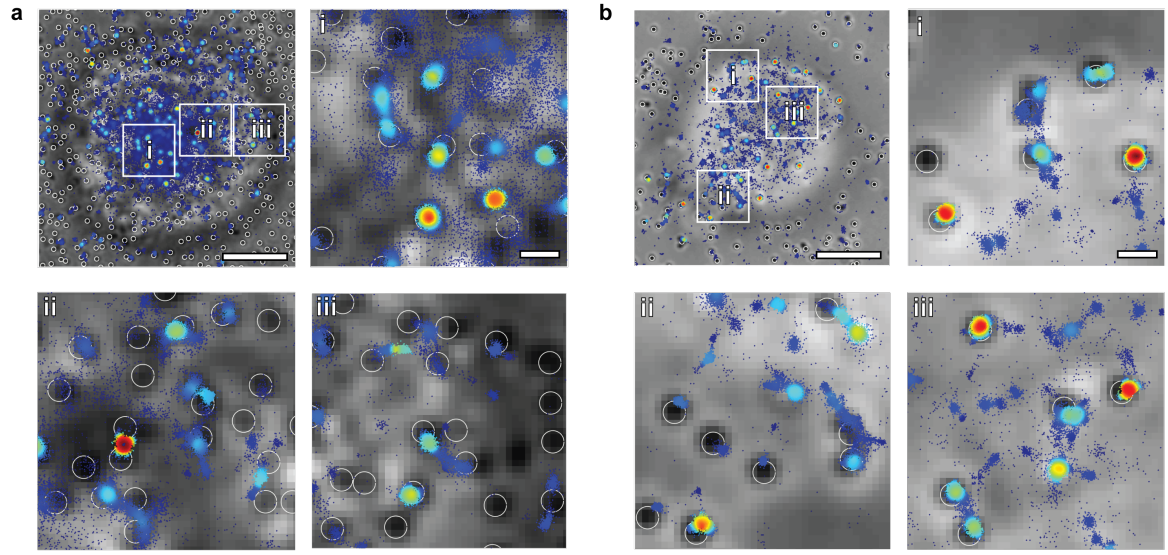


Fig.S7: Lateral propagation of TCR signaling could bridge distant activation sites c. (**a-b**) SMLM images of CD3ζ (a) and pCD3ζ (b), pseudo-colored according to density (blue to green indicates low to high) in Jurkat T cells activated on surfaces with nanoparticles (white circles) functionalized with anti-CD3ε antibodies. dSTORM data was superimposed over brightfield image of cells and surface. (i-iii). Highlighted regions (white square in a-b) where CD3ζ and pCD3ζ clusters spread to neighboring activation sites, essentially forming long-range bridges. Scale bars = 5 µm in (a-b) and scale bars = 500 nm for (i-iii).

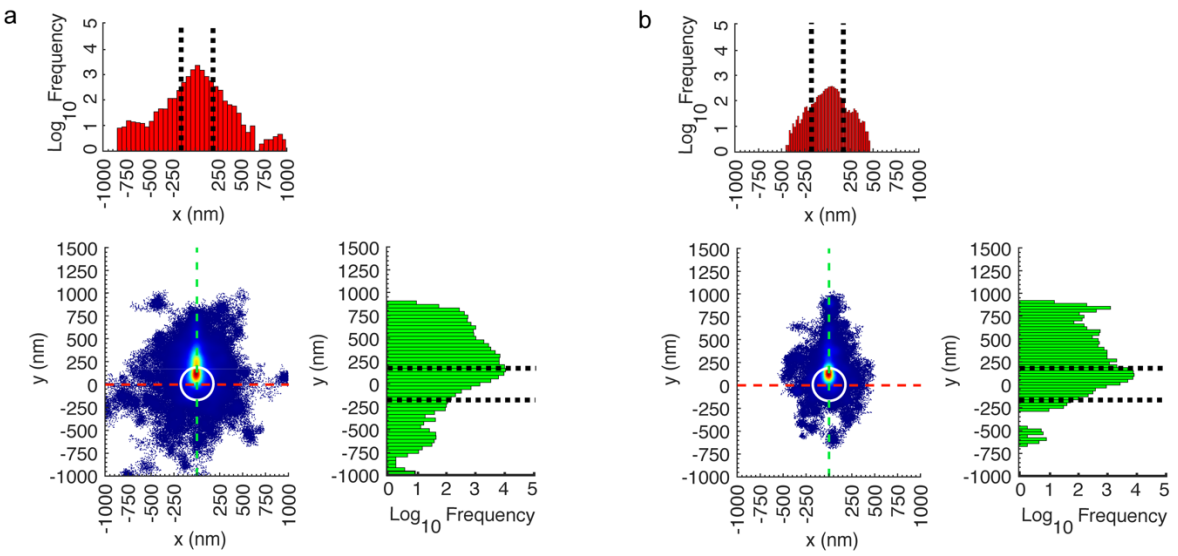


Fig.S8: Density distributions of tdSH2-ZAP70-EGFP PAINT and TCR-AF647 dSTORM at activation sites. Aligned and merged distributions of (a)tdSH-ZAP70-EGFP and (b) CD3ζ localizations relative to the center of activating nanoparticles (white circles) in Jurkat cells along the major axis (green) and minor axis (red). Dashed black lines indicate boundary of the nanoparticles

Supplementary Note 1: Calculation of the upper bound of biotinylated anti-CD3 per particle.

According to the manufacturers (Spherotech Inc.) the binding biotinylated antibody binding capacity for 1 mg of 4 μm Spherotech Streptavidin particles is typically 5-10 μg (3.33×10^{-11} to 6.67×10^{-11} mols, or 2.007×10^{13} to 4.02×10^{13} antibodies) depending on the number of biotins per antibody. Using the density of polystyrene (1.055 g/cm^3) and the volume of a 4 μm particle (3.35×10^{-11} cm^3) to calculate the number of particles in 1 mg (28285847 particles). The number of antibodies a single 4 μm Spherotech Streptavidin particle can bind is 709677 to 1419353 antibodies. Given the surface area of a 4 μm particle is equal to 50.3 μm^2, then the number of antibodies that can be bound on the particle per μm^2 is 14118 to 28237 antibodies. This means that for these particles we will have 1 antibody per 35 to 71 nm^2.

The density estimates of antibody/nm^2 calculated were then used to calculate the maximal number of antibodies bound by the 340 nm streptavidin nanoparticles. The surface area of a 340 nm nanoparticle is 0.36 um^2 = 36 nm^2. Thus, these particles have a binding range of 5127 to 10254 antibodies per particle.

In our experiments, we used a mixture of biotin-FITC and biotinylated anti-CD3 antibody, at final concentrations of 1 ng/mL (4.1×10^{-7} M) and 2.5 ug/mL (1.67×10^{-8} M), respectively. Thus, our biotinylated reagents had a molar ratio of antibody:FITC equal to 1:24.6. Given this will lessen the amount of antibody per particle, this molar ratio is used to scale the approximate number of antibodies per particle to 209 - 418.

It should be noted that our biotin antibody:FITC mixture is added after the particle are attached to the biotin cover glass surface (see **Scheme1** and **Materials and Methods**). Therefore, part of the surface of the nanoparticle, that bound to the surface or close to the surface, will be inaccessible by the biotin antibody:FITC, therefore the number of antibodies per particle will likely be lower than this estimate. Values are reported to at least 2 significant figures.

Movie Captions

Movie.S1: Exemplar single particle tracking of CD3ζ-PAmCherry. CD3ζ knock-out Jurkat cells transfected with CD3ζ-PAmCherry are deposited onto the activating particle surface (bright field image, left), activating particles are the dark circular features. Distributions of CD3ζ-PAmCherry and diffusion of single CD3ζ-PAmCherry are observed (middle). Overlay of brightfield image and single particle tracking time series