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Supplemental Information

Single-Molecule Light-Sheet Imaging of Suspended T Cells

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Materials and methods

Cell culture and labeling

Jurkat T cell line (Clone E6-1, ATCC® TIB-152™) were grown in RPMI medium supplemented with 10% FCS, 1% HEPES buffer (1M), 1% sodium pyruvate (100mM), 2% L-glutamine (200mM) and 1% penicillin-streptomycin.

For diffusion measurements, Jurkat T cells were labelled using fragment antigen-binding fragments (Fabs) obtained from mouse anti-human antibodies (UCHT-1 and gap 8.3). Approximately 10^6 cells were incubated with UCHT1 (CD3e) or Gap8.3 (CD45) Alexa Fluor 488-labeled Fabs (degree of labelling ~ 2, determined by UV-Vis spectroscopy) diluted in 1 ml of PBS at a final concentration of 1 nM (TCR) or 10 nM (CD45) at 4 °C for 30 minutes. These concentrations were determined by titration to find a suitable density of well-separated individual fluorophores. A higher concentration was used for CD45 due to a significant amount of proteins being excluded from the cell-glass interface. After incubation cells were washed three times with PBS (P4417, Sigma-Aldrich). Proteins were also labelled with HaloTag® in a separate experiment, but this approach was discounted due to intracellular labelling (see video S10).

For bilayer experiments, Jurkat T cell line expressing the transmembrane-anchored, non-signaling, T92A-mutated form of rat CD48 was prepared as previously described (1). The following mobile anchors were attached to the bilayers. DNA encoding the human ICAM-1 extracellular region (residues 1–480, UniProtKB P05362) and the chimeric protein comprising rat CD45 D1-D4 (residues 194-546, UniProtKB P04157) and rat CD2 extracellular region (residues 1-202, UniProtKB P08921) were ligated into pHR vector, double His_6 tags were encoded at their 3′ end. ICAM-1 and rCD45-rCD2 were expressed transiently in 293T cells. Proteins were purified using size-exclusion chromatography.

For two-colour imaging of CD45 exclusion, approximately 10^6 Jurkat CD48+ T cells were labelled with the Gap8.3 fabs at a final concentration of 1 µM in 1ml PBS at 4 °C for 30 minutes. Labelled cells were then incubated with 50 ng/ml CellMask Deep Red Plasma membrane Stain (C100046) for 2 minutes at room temperature (20 °C). After labelling, cells were washed three times with PBS.

TIRFM

Experiments were performed on a bespoke instrument (Fig. S1 for a schematic of the setup). A fibre-coupled 488 nm diode laser (15 mW iFLEX-2000, Qioptic) was used for all tracking experiments and an excitation filter (FF01-488/6-25, Semrock) was used to spectrally filter and remove unwanted residual wavelengths other than those at 488nm. The circularly polarized laser beam was collimated and expanded 2.3× using a Galilean beam expander to slightly overfill the back aperture of a 100×, 1.49 numerical aperture (NA) oil immersion objective lens (MRD01991, Nikon). This was used for excitation and acquisition and housed in the microscope (Eclipse Ti-U, Nikon). Total internal reflection was achieved by reflecting the laser off a quad-edge laser-flat dichroic mirror (Di01-R405/488/532/635-25x36, Semrock) and focusing the laser at the back focal plane of the objective, off axis, such that the emergent beam at the sample interface was near-collimated and incident at an angle greater than the critical angle, Θ_c ~ 67 °, for a glass/water interface. This generated a ~40 µm diameter TIR footprint with power densities in the range 0.01-0.1 kW/cm^2 at the coverslip, accounting for ~4-fold near field enhancement at the sample plane (2). The setup could also be used in epifluorescence (EpiFL) mode by focusing the laser at the back focal plane of the objective on axis. The internal magnification of the microscope was used to achieve a final magnification of 150×. The fluorescence emission was filtered using a bandpass emission filter (#67-031, Edmund Optics), and focused onto an EMCCD camera (Evolve 512 Delta, Photometrics). Image stacks of 100 frames were acquired using an exposure time of 33 ms, an electron multiplying (EM) gain of 250 and a frame rate of 20.4 Hz.

Glass coverslips were plasma cleaned for 30 minutes and then coated with solutions of 0.01 % (w/v) poly-L-lysine (PLL) (150-300 kDa, P4832, Sigma-Aldrich), 0.1 mg/ml bovine IgG (I5506, Sigma-Aldrich) or 0.1 mg/ml fibronectin (F0895-1MG, Sigma-Aldrich) for 30 minutes. The coated coverslips were then washed three times with PBS, and 100 µl of 10^4/ml labelled cells in PBS was added to the coverslip. Experiments were performed at 20°C and all cells were imaged within 10 minutes of sitting down on the coverslip.

For two-colour experiments a multichannel imaging system (DV2, Photometrics) was mounted in front of the EMCCD. A fibre-coupled 637 nm diode laser (20 mW iFLEX-2000, Qioptic) was used to excite the cell membrane stain along with additional excitation (FF01-640/14-25, Semrock) and emission (#67-038, Edmund Optics) filters. 100 µl of 10^4/ml membrane- and CD45-labelled cells in PBS was added to the SLB-coated coverslips and were then imaged.

Experiments were also done on fixed cells to establish ‘immobile’ cutoffs for MSD and JD analysis. The TCR was labelled with Alexa Fluor 488 Fabs as described previously. These cells were allowed to settle on PLL-coated

coverslips for 10 minutes after which they were fixed with a solution of 4% paraformaldehyde and 0.2 % glutaraldehyde in PBS. After 30 minutes of fixation, cells were washed 3x with PBS and were then imaged.

smLSM

The optical setup for smLSM (Fig. S1 and Fig. S2 for a schematic of the setup), optimised for single-molecule fluorescence detection, used a perpendicular secondary objective lens (10× long working distance WD = 33.5 mm, NA = 0.28, Mitutoyo) to introduce a thin sheet of excitation light into the sample (see Fig. 1A and Fig. S1). The collimated beam previously used for TIR excitation was instead directed into the secondary objective using a reversible mirror, such that the two illumination modes could easily be switched in-between. The light focused by the secondary objective was slightly defocused in the sample plane dimension (Fig. S1) using two cylindrical lenses, creating a wide sheet of light. Acquisition parameters was the same as for TIRFM and the power density was matched by achieving the same signal above background at the coverslip interface as in TIRFM experiments. The secondary objective was tilted by 5 ° to minimize scattering caused by light passing through other cells (Fig. 1A). The position of the light sheet was controlled by varying the position of the light entering the secondary objective using a mirror. The focal point of the sheet was controlled by mounting the secondary objective onto a translational stage.

A custom made sample chamber was designed to create a flat interface through which the light sheet could enter the sample (Fig. S1 and Fig. S2). The chamber was constructed by attaching two coverslips to each other at an 85 ° angle using a putty-like pressure-sensitive adhesive (Fig. S2B). A hydrophobic barrier pen (QVC0500, eBioscience) (blue trace in Fig. S2B) was used to create a border for the liquid sample to eliminate leakage. Unlike manufactured sample chambers (3), the coverslip chambers are very affordable. Unlike microcapillaries (4), the coverslips ensure complete light collection by the high NA acquisition objective and provides physical access, which is useful for adding cells or potentially implementing nanocapillaries for manipulation and injection (5).

A schematic of the complete optical setup is shown in Fig. S1. Fig. S2A shows a photo of the setup. A counterweight in the form of a stainless steel nut is used to hold the sample chamber (Fig. S2B) down into the chamber holder (KM100C, Thorlabs). The chamber holder sits on a three axis piezo stage (P-611.3S Nanocube, Physik Instrumente), which can be used to manipulate the sample and scan the light sheet.

Light sheet characterisation

The point spread function of the light-sheet was evaluated by scanning diffraction-limited fluorescent beads suspended in a gel (6) which simulate a photo-stable point source. 1 µl of 100 nm TetraSpeck beads (T-7279, Thermo Fisher) were added to a 1 ml solution of 0.5 % Phytagel (P8169, Sigma-Aldrich) and 0.1 % $MgSO_4$ in Milli-Q water (18.3 MΩ/cm). The piezo stage was used to scan the sample over 10 µm in steps of 100 nm (see Fig. S3A). A 0.25 numerical aperture, 10x objective lens (FN22, Olympus) was used to image the beads due to its large depth of field. A Matlab (MathWorks, R2015a) script was used to fit three-dimensional Gaussian distributions to the acquired image stacks. The thickness of the sheet, as a function of axial distance along the light-sheet propagation direction, could be fitted (Fig. S3B) to determine the minimum beam waist of the sheet (ω_0 = 550 nm, full width at half maximum, FWHM = 1.3 µm) and the Rayleigh length (10 µm). The thickness of the sheet is significantly smaller than that achieved using HILO excitation (FWHM = 6 µm (7) at 10 µm above the surface) and this implementation of the light sheet has a Rayleigh length well-suited to imaging T cells, which are about 10 µm in diameter.

EpiFL and smLMS comparison

Significant improvement in signal-to-noise-ratio (SNR) could be achieved by applying smLSM (Fig. S4). Note that this does not correspond to single-molecule SNR as the fabs have multiple labels (degree-of-labelling ~ 2). However, as the same individual cells are being compared, we are able to determine the relative improvements offered by smLSM. Because of the SNR improvement longer tracks (mean track length: EpiFL – 14.7, smLSM – 21.9) were obtained. To provide a fair comparison the excitation power was kept similar in both conditions, which can be seen in the measured intensities (Fig. S5A). The reduction in background noise is also shown (Fig. S5B). Despite the improvement in signal a similar number of tracks were identified for the two imaging methods (Total tracks: EpiFL – 143, LSM - 132). This is caused by the significant noise and background present when imaging using EpiFL imaging (mean background (photons/pixel/frame): EpiFL – 25.1, smLSM – 6.5) resulting in numerous inaccurate localizations. Due to these localizations the diffusion coefficient determined by MSD analysis was different for the two methods (EpiFL - D = 0.115 $\mu m^2/s$, smLSM - D = 0.066 $\mu m^2/s$. This can be resolved by applying a larger SNR threshold (SNR=3: EpiFL - D = 0.063 $\mu m^2/s$, D = 0.063 $\mu m^2/s$) which also results in a significantly lower amount of tracks in the case of EpiFL (Total tracks: EpiFL – 72, smLSM - 116). The localization precision was also determined using the Peak Fit plugin for Imagej. The precision is similar in both cases (Median precision:

EpiFL – 23.0 nm, smLSM – 21.1 nm), but again there were more localizations in the smLSM case due to the improved SNR (Localizations: EpiFL – 1071, smLSM – 2769). Note that the uncertainty in localization shifts the MSD curve, which is taken into account when fitting the MSD. Since we use the ensemble MSD curve for each cell, the uncertainty introduced by the localization error is minimal, as evidenced by our ability to measure diffusion coefficients below 0.01 $\mu m^2/s$. We also compared the localization precision for smLSM with TIRFM as shown in Fig. S6B.

Single-molecule imaging on the free surfaces of cells

For smLSM imaging of cells, the custom made sample chamber coverslips were coated with PLL and IgG as was described for TIRFM. A lower density of cells (10^3/ml) was used to minimize scattering caused by the light sheet passing through cells, which is not an issue when using TIRFM imaging. Experiments were performed at room temperature and all cells were imaged within 10 minutes of sitting down on the coverslip.

Measurements were also performed on cells suspended in gels, where potential effects induced by surface interactions could be eliminated. Low melting point agarose (A9414, Sigma-Aldrich) was dissolved in PBS by heating at a concentration of 2 % (w/v). The agarose solution was then placed in a heated water bath (37 °C). A 10 µl suspension of labelled cells was mixed with 100 µl of the agarose solution, and the mixture was finally pipetted onto the sample chamber where it gelled within a few seconds. While the gelation temperature (26-30 °C) of the low-melt agarose used is below body temperature the melting point is much higher (<65 °C), such that brief gelation at room temperature creates a gel that is stable at 37 °C. It would thus be possible to use the developed platform at physiological temperatures.

SLB preparation

SLBs were prepared as described previously (1). Glass coverslips were cleaned for one hour using piranha solution (3:1 sulfuric acid/ hydrogen peroxide). After rinsing the slides with Milli-Q water (18.3 MΩ/cm) the slides were plasma cleaned for 30 minutes and a silicon well (Grace Bio-Labs) was attached to each slide. 20 µl of lipid vesicle solution consisting of 1 mg/ml of 95% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 5% DGS-NTA(Ni) (Avanti Polar Lipids, Alabaster USA), which binds to His-tags, was added to each PBS-filled silicone well and was incubated for 30 minutes. Wells were washed three times with PBS solution and 20 µl of protein solution, either His-rCD45rCD2 (labelled with Alexa Fluor 647) or His-ICAM1, at 30 µg/ml were added to each well. After one hour of incubation at room temperature the wells were washed three times with PBS. We also prepared some SLBs without any nickelated lipids and some with nickelated lipids that were blocked with 1% BSA in PBS for 15 minutes followed by washing 3 times in PBS, to study the effect of charge on calcium release.

Calcium triggering assay

We slightly modified a standard calcium triggering assay used for identifying T-cell activation (8). Jurkat T cells were labelled with the calcium sensitive dye Fluo-4 (F14201, Thermo Fisher Scientific). Approximately 10^6 cells were incubated in 1 ml of cell culture medium with Fluo-4 at a final concentration of 5 nM 37 °C for 30 minutes. After incubation cells were washed and resuspended in 0.1 ml of cell culture medium (for agarose) or PBS (for coated glass surfaces) with 2.5 mM probenecid (P36400, Thermo Fisher Scientific). To study activation in agarose, the cell suspension was mixed in equal amounts with 2 % agarose and the final solution was deposited on a cover slip. To study activation on coatings, the cell suspension was directly deposited on coated coverslips.

Cells were imaged in EpiFL mode with a 0.25 numerical aperture, 10× objective lens (FN22, Olympus), 1.5× internal magnification. A low power density of 1 W/cm^2 was used to avoid laser-induced activation of T cells, which could be observed at higher powers. Time lapse sequences were taken using an exposure time of 100 ms and a cycle time of 1 s. TTL modulation of the laser was used to minimize the effect of excitation on activation. For cells in agarose, 1 µl of 25 µg/ml OKT3 (produced in the Davis lab) was added to the gel to artificially activate the cells. Given the large pore size of the agarose gel the OKT3 can diffuse towards the cells readily.

Calcium triggering was analysed by tracking cells and monitoring intensity changes over time. A difference of Gaussians filter (pixel radii 1 and 5) was applied to remove noise and extract signal over background. The intensity for each cell was averaged in a 3x3 matrix centered on the peak intensity. The baseline intensity was determined by the threshold used in the tracking algorithm. This was confirmed to coincide with cells falling onto the surface. We then considered cells to be triggered when the peak intensity reached more than 2.5 times the baseline intensity. This threshold was based on the typical noise level for non-activating cells (Fig S9) and the typical increase in fluorescence on OKT3-coated surfaces (Fig S9).

Single-molecule tracking

A Matlab script was used for single-molecule detection, tracking and diffusion analysis (9). A bandpass filter was applied to raw images, which enabled identification of local maxima. A signal-to-noise ratio (SNR) threshold of 3 was then applied to these local maxima to select likely single-molecule localizations. The noise for each localization was calculated using the intensities of the pixels in a shell around the maxima. Pixels overlapping with other maxima were excluded from the SNR calculation. Selected localizations were linked together to form tracks (10). Only tracks longer than 4 frames were used for diffusion analysis.

Mean square displacement (MSD) analysis

The MSD (9) for each track was calculated. To determine the diffusion coefficient, an ensemble MSD curve was constructed by averaging the MSD of each track. The first 5 points of the ensemble MSD curve were then fitted using $MSD = 4Dt + 4\sigma^2$, where t is time and σ is localization precision, to determine the diffusion coefficient, D, for two-dimensional diffusion over the membrane (10).

Simulations of 2D plane approximation

The diffusion of 3000 randomly distributed particles about a 100 pixel diameter spherical surface was simulated over 300 time steps using Matlab (see end of SI for code). The diffusion coefficient was chosen such that most particles travel less than a pixel in a single time step. For each pixel, the instantaneous diffusion coefficient was determined by averaging the jump distances of each particle that travelled through that pixel. A comparison was made between the known great circle distance (in 3D) and the 'perceived' 2D distance that would be projected to and recorded on the image plane by a detector. The reduction in diffusion coefficient due to the geometry of the sphere is shown in Fig. S15A. A profile of this reduction along the centre of the sphere is shown in Fig. S15B, which indicates that the maximum reduction in perceived diffusion coefficient occurs at the equator of the sphere where there is a 33 % reduction. If a 2 μm thick light sheet images the top of a T-cell, the radius of the 2D projection of the excited volume would be 3 μm for a 10 μm diameter T cell (corresponding to pixels 20-80 in Fig. S15B). In this case the maximum reduction in perceived jump distance is only 10%. This simulation neglects any membrane disarrangement or surface ruffles and assumes a perfectly spherical cell, nonetheless it demonstrates that the spherical geometry of the cell does not radically alter the recorded 2D diffusion coefficient as measured by both MSD and jump-distance analysis.

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Supplementary tables

Table S1: Ensemble MSD diffusion cofficient measurements. D – diffusion coefficient, n – number of cells, t – number of tracks, t_L – mean track length. The error indicates the standard deviation of the cell-cell variation. TIRFM experiments probe the cell-glass interfaces whereas smLSM and agarose experiments probe the free cell surface.

		TCR				CD45 (μm²/s)			
		D (μm²/s)	n	t	t_L	D (μm²s⁻¹)	n	t	t_L
TIRFM	PLL	0.005±0.005	13	1040	31.5	0.033±0.014	16	1136	21.5
	IgG	0.068±0.021	15	965	19.0	0.040±0.012	14	567	17.7
smLSM	PLL	0.093±0.038	13	557	18.1	0.072±0.029	11	344	17.2
	IgG	0.079±0.023	10	422	18.7	0.095±0.020	11	367	15.8
Agarose (smLSM)		0.068±0.023	13	572	16.2	0.081±0.022	11	462	16.3
Fibronectin (TIRFM)		0.058±0.013	17	4836	19.3	0.057±0.019	12	1477	25.8
rCD45rCD2 bilayer (TIRFM)		0.044±0.014	14	1351	12.0	0.064±0.013	16	2758	14.6
Fixed cells (TIRFM)		0.005±0.004	11	8539	23.5	-	-	-	-

Table S2: Jump distance analysis of single-molecule experiments. D – diffusion coefficient, R^2 – goodness of fit, D_j – diffusion coefficient of population j, F_j – fraction of popluation j. Bold data indicates chosen fitting model based on $D_1 < 0.03$ and $D_2 > 0.03$.

		TCR						CD45					
		1 pop		2 pop				1 pop		2 pop			
		D	R^2	D_1	D_2	F_2	R^2	D	R^2	D_1	D_2	F_2	R^2
TIRFM	PLL	**0.007**	**0.97**	0.004	0.015	0.50	0.99	0.013	0.90	**0.006**	**0.041**	**0.55**	**0.99**
	IgG	0.052	0.90	**0.013**	**0.086**	**0.75**	**0.98**	0.027	0.90	**0.010**	**0.063**	**0.62**	**0.98**
smLSM	PLL	**0.085**	**0.96**	0.054	0.169	0.48	0.99	**0.080**	**0.92**	0.031	0.132	0.70	0.97
	IgG	**0.085**	**0.96**	0.039	0.127	0.69	0.98	**0.110**	**0.94**	0.053	0.178	0.66	0.97
Agarose (smLSM)		**0.097**	**0.94**	0.054	0.185	0.56	0.98	**0.093**	**0.94**	0.038	0.146	0.71	0.98
Fibronectin (TIRFM)		0.049	0.94	**0.015**	**0.076**	**0.75**	**0.99**	0.050	0.95	**0.020**	**0.082**	**0.70**	**0.99**
rCD45rCD2 bilayer (TIRFM)		0.032	0.92	**0.01**	**0.057**	**0.71**	**0.99**	0.042	0.90	**0.01**	**0.067**	**0.77**	**0.99**
Fixed cells (TIRFM)		**0.012**	**0.95**	0.005	0.025	0.61	0.99	-	-	**-**	**-**	**-**	**-**

Supporting videos

All movies show raw and unprocessed data. Contrast and brightness settings are identical across environments within each video.

Video S1: TCR imaged on the same cell using Epifluorescence (left) and smLSM (right). The scale bar is 5 µm and the frame rate is 20 Hz.

Video S2: smLSM and TIRFM imaging of the TCR in a single cell. The schematic on the top indicates the exciation method and position of the light sheet, which is scanned from bottom to the top of the cell. TCR is immobile at the basal surface and mobile on the apical surface. The scale bar is 5 µm and the frame rate is 20 Hz.

Video S3: Sample TIRFM videos of TCR in cells resting on PLL (left) or IgG (mid) compared with imaging in agarose (right). The scale bar is 5 µm and the frame rate is 20 Hz.

Video S4: Sample smLSM videos of TCR on the apical surface of 3 different T cells resting on either PLL (left) or IgG (mid) and suspended in agarose (right). The scale bar is 5 µm and the frame rate is 20 Hz.

Video S5: TIRFM (left) and smLSM (right) video of TCR in Jurkat T cells resting on PLL. The TCR is expressed with a HaloTag protein that is labelled with Silicon Rhodamine.

Video S6: Sample TIRFM videos of CD45 in cells resting on PLL (left) or IgG (mid) compared with imaging in agarose (right). The scale bar is 5 µm and the frame rate is 20 Hz.

Video S7: Sample smLSM videos of CD45 on the apical surface of 3 different T cells resting on either PLL (left) or IgG (mid) and suspended in agarose (right). The scale bar is 5 µm and the frame rate is 20 Hz.

Video S8: TIRFM imaging of a single CD45 protein in a T cell resting on IgG. The protein is initially dim and mobile. As it interacts with the surface (becomes brighter) it becomes immobilized. The tracks represent +-5 frames. The scale bar is 1 µm and the frame rate is 20 Hz. Visualization was performed with the Trackmate plugin for ImageJ.

Video S9: Sample TIRFM videos of TCR (left) and CD45 (right) in cells resting on fibronectin. The scale bar is 5 µm and the frame rate is 20 Hz.

Video S10: TIRFM video of membrane stain CellMask Deep Red showing how T-cell microvilli move around on ICAM-1 SLBs. The scale bar is 2 µm, the exposure time is 600 ms, the frame rate is 1 Hz and the playback rate is 6x real time.

Video S11: TIRFM video of Jurkat T cells spreading on PLL. TCR is labelled with Alexa Fluor 488-Fabs under saturating conditions. The scale bar is 2 µm, the exposure time is 100 ms, the frame rate is 1 Hz and the playback rate is 10x real time.

Video S12: Two-colour TIRFM videos of Jurkat T cells spreading on various surfaces. CD45 is labelled with Alexa Fluor 488-Fabs under saturating conditions and the membrane is labelled with CellMask Deep Red. The scale bar is 5 µm, the exposure time is 200 ms, the frame rate is 1 Hz and the playback rate is 10x real time.

Simulation code

```
clear all
parts = 3000;   %Number of particles
r = 50;         %Radius of sphere
frames = 300;   %Number of timesteps

%Initialize positions
x = zeros(parts,1);
y = zeros(parts,1);
z = ones(parts,1)*r;

% Prebake - Evenly distributes particles on sphere surface
for i = 1:100
    for j = 1:parts
        rotot = (rand(1,3)-0.5)/1;
        M = rot3(rotot(1),rotot(2),rotot(3));
        res = [x(j),y(j),z(j)]*M;

        x(j) = res(1);
        y(j) = res(2);
        z(j) = res(3);
    end
end

% Simulate
nDis = zeros(r*2);  %Number of particles travelled through nDis(x,y)
dis = zeros(r*2);   %2D distance at dis(x,y)
disGC = zeros(r*2); %Great circle distance at disGC(x,y)
for i = 1:frames
    for j = 1:parts
        rotot = (rand(1,3)-0.5)/10;             %Set random rotation vector
        M = rot3(rotot(1),rotot(2),rotot(3));   %Determine rotation matrix
        res = [x(j),y(j),z(j)]*M;               %Apply rotation
        d(i,j) = sqrt( (x(j)-res(1))^2+(y(j)-res(2))^2 );   %Calculate jump distance
        Pd = [x(j),y(j),z(j)];  %Starting position
        Ps = res';              %Post rotation position
        rd(i,j) =  r*atan2(norm(cross(Pd,Ps)),dot(Pd,Ps));  %Calculate angle between Pd and Ps

        xPos = floor(x(j))+r+1; %Find nearest integer position
        yPos = floor(y(j))+r+1; %Find nearest integer position
        nDis(xPos,yPos) = nDis(xPos,yPos)+1;    %Count local number of particles
        dis(xPos,yPos) = dis(xPos,yPos)*(nDis(xPos,yPos)-
1)/nDis(xPos,yPos)+d(i,j)/nDis(xPos,yPos);         %2D jump distance
        disGC(xPos,yPos) = disGC(xPos,yPos)*(nDis(xPos,yPos)-
1)/nDis(xPos,yPos)+rd(i,j)/nDis(xPos,yPos);    %Real jump distance
        x(j) = res(1);  %Update positions
        y(j) = res(2);
        z(j) = res(3);
    end
    i   %Display current frame
end

%Plot results
subplot(1,2,1)
imagesc(dis./disGC);
colorbar
subplot(1,2,2)
plot(1:100,dis(50,:)./disGC(50,:));

%Function for applying spherical rotation
function M = rot3(roll,pitch,yaw)
    rM=[1    0           0;
        0    cos(roll)   sin(roll);
        0    -sin(roll)  cos(roll)];
    pM=[cos(pitch)      0       -sin(pitch);
        0               1       0;
        sin(pitch)      0       cos(pitch)];
    yM=[cos(yaw)        sin(yaw)        0;
        -sin(yaw)       cos(yaw)        0;
        0               0               1];
    M = rM*pM*yM;
end
```