High-speed high-resolution imaging of intercellular immune synapses using optical tweezers

Stephane Oddos*, Christopher Dunsby†, Marco A. Purbhoor‡, Anne Chauveau‡, Dylan M. Owen*, Mark A. A. Neil†, Daniel M. Davis‡ and Paul M. W. French†

‡Chemical Biology Centre; †Department of Physics; ‡Division of Cell and Molecular Biology, Imperial College London, UK.

ABSTRACT Imaging in any plane other than horizontal in a microscope typically requires a reconstruction from multiple optical slices that significantly decreases the spatial and temporal resolution that can be achieved. This can limit the precision with which molecular events can be detected, for example, at intercellular contacts. This has been a major issue for the imaging of immune synapses between live cells, which has generally required the reconstruction of en face intercellular synapses, yielding spatial resolution significantly above the diffraction limit and updating at only a few frames per minute. Strategies to address this issue have usually involved using artificial activating substrates such as antibody-coated slides or supported planar lipid bilayers, but synapses with these surrogate stimuli may not wholly resemble immune synapses between two cells. Here, we combine optical tweezers and confocal microscopy to realize generally applicable, high-speed, high-resolution imaging of almost any arbitrary plane of interest. Applied to imaging immune synapses in live-cell conjugates, this has enabled the characterization of complex behavior of highly-dynamic clusters of T cell receptors at the T cell / antigen-presenting cell intercellular immune synapse and revealed the presence of numerous, highly-dynamic long receptor-rich filopodial structures within inhibitory Natural Killer cell immune synapses.

Address reprint requests and inquiries to Daniel M. Davis, Tel.: +44-207-594-5420; Fax: +44-207-594-3044; E-mail: d.davis@imperial.ac.uk and Paul M. W. French, Tel.: +44207-594-7706; Fax: +44-207-594-7714; E-mail: paul.french@imperial.ac.uk.

Imaging where and when molecular interactions occur has a major role to play in contemporary cell biology. However, many intercellular interactions such as neuronal (1), immune (2,3) or virological (4) synapses, as well as interactions between cells and pathogens such as at the initiation of phagocytosis (5), typically require imaging planes out of the focal plane of the microscope. This is generally achieved using optically-sectioning microscopes to acquire image stacks and subsequently reconstructing the desired optical slices through the sample. Unfortunately this approach is slow and can usually only provide micron-scale spatial resolution, limiting the prospects for live imaging of the molecular events underlying such cellular interactions.

Here, we consider the specific example of the immune synapse, which is the interface formed between immune cells and target cells or antigen-presenting cells (APC), commonly observed in many immune effectors such as NK cells (6), cytolytic T cells (7) or B cells (8). The immune synapse is characterized by dynamic, synchronized rearrangements of proteins into areas termed supramolecular activation clusters (SMACs) sometimes arranged in a prototypical ‘bulls-eye’ configuration (2,3). Strategies to improve the imaging resolution have involved using horizontally orientated synapses between immune cells and artificial substrates mimicking target cells, such as antibody-coated glass slides (9) or supported planar lipid bilayers with anchored ligand proteins (10). However, such artificial substrates may not recapitulate some of the complexities in intercellular interactions.

FIGURE 1 Optical tweezers in a confocal microscope. (A) Simplified schematic of the setup to allow 3D manipulation of live-cell conjugates and confocal fluorescence imaging. (B) Fluorescence and transmitted light images of a target (red) and effector cell (green) synapse being manipulated into an imaging configuration such that the intercellular contact lies en face to the imaging plane. Scale bar, 10 µm.
Optical trapping has now become a common and useful tool in cell biology, allowing manipulations of live cells and small objects in all dimensions. Here, we demonstrate a simple experimental approach to image biologically relevant live-cell interfaces with the high frame rate and high spatial resolution currently only realized by techniques using artificial synapses. By using optical tweezers to manipulate live cell conjugates and orientate the synapse in the imaging plane of a confocal laser scanning fluorescence microscope, we have imaged protein organization at live NK cell/target cell and T cell/APC immune synapses with high speed and high resolution.

To implement optical tweezers, an optical trapping laser beam from a laser diode emitting at 980 nm was introduced into a commercially available laser scanning confocal fluorescence microscope (Fig. 1 A and see Supplementary Material, Data S1, for experimental details). This beam permitted us to manipulate cells in three dimensions while performing full confocal imaging with negligible loss of fluorescence signal. Using 980 nm light for the trapping beam results in low detrimental effects on live cells and this optical-tweezer system did not introduce any restriction on the range of fluorophores that could be imaged. Using steering mirrors and relay lenses, precise three-dimensional positioning of the traps could be achieved. Thus, horizontal orientation of the immune synapse was performed by trapping cell conjugates and then lifting the trap focus above the imaging focal plane as shown in Fig. 1 B. (see also Movie S1). Trapping power was then adjusted to prevent any movement of the vertically-oriented conjugates when the objective was moved along the z axis, allowing any planes parallel to the cell-cell interface to be imaged at full speed and high resolution (see Data S1).

To demonstrate our approach, we first imaged the supramolecular organization of the inhibitory NK cell human immune synapse. We incubated YTS NK cells expressing the GFP-tagged inhibitory receptor KIR2DL1 together with target cells (721.221) expressing the class I MHC ligand (HLA-Cw6) for KIR2DL1 (see Data S1 for details on cell lines), known to form a well-characterized inhibitory NK cell immune synapse (6,11). While normal confocal fluorescence imaging of such cells requires at least 30 s to acquire a full 3D data set with submicron axial resolution (Fig. 2 A), our set-up was only limited by the microscope scanning speed in the plane of focus. Thus we realized high spatial resolution (~250 nm), high speed (>1 frame per second) imaging of the immune synapse between live cell conjugates (Fig. 2 B-D and Movie S2), which revealed two new dynamic features of the inhibitory NK cell immune synapse. In agreement with previous studies (12), we observed that the central accumulation of KIR2DL1 featured small areas in which the receptor was excluded (Fig. 2 B and Data S1). However, our new approach allowed us to observe, 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 (Fig. 2 C). Surprisingly, numerous long (up to 12 µm) and highly dynamic protrusions were also detected within and around the distal regions of the immune synapse (Fig. 2, B and D, and Data S1). Such structures are known to be important for cell adhesion and environment probing (13). Therefore, their highly motile nature may play a role in initiating or sustaining signaling perhaps by allowing NK cells to probe large areas of the interface and augment the number of receptor/ligand binding events at the synapse.

The T cell/APC immune synapse has been extensively studied but, to date, the highest-resolution images have been obtained using artificial activating substrates (14). Here, we imaged the distribution of T cell receptors (TCRs) at an intercellular immune synapse at high-speed and high-resolution using optical tweezers. We incubated Jurkat T cells expressing the YFP-tagged protein CD3ζ together with APCs (Raji B cells pulsed with super-antigen) until they formed mature immune synapses. As expected, CD3ζ segregated to the centre of the synapse (Fig. 3 A and Movie S3). Numerous discrete clusters containing CD3ζ were also detected in the periphery of the synapse, consistent with recent studies showing microclusters of TCR that form in synapses with artificial planar stimuli previously identified as the main site for T cell signal transduction (14,15). Strikingly, CD3ζ 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 CD3ζ is internalized (16). Also, increased spatial and temporal resolution allowed CD3ζ 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 (Fig. 3 B), with 30-40% of the clusters moving away from the centre to the synapse periphery. Due to an increased signal-to-noise ratio, quantitative analysis of cluster fluorescence intensity could also be undertaken to investigate their composition (Fig. 3 C). This size distribution exhibited a wide range, with some clusters being up to five times as large as the smallest detectable ones.

In summary, we have demonstrated that high-speed, diffraction-limited resolution imaging of live cell-cell interactions can be realized using a relatively simple combination of optical tweezers and confocal microscopy. Due to its flexibility, this generally applicable method can provide new opportunities to extend our understanding of molecular mechanisms underlying the immune synapse as well as other cell-cell interactions.

SUPPLEMENTARY MATERIAL
To view all the supplement files associated with this article, visit www.biophysj.org.

ACKNOWLEDGEMENTS
This work was supported by the Biotechnology and Biological Sciences Research Council, the Department of Trade and Industry, a Lister Institute Research Prize, the Medical Research Council and the Royal Society. SO and DMO acknowledge PhD studentships with the Chemical Biology Centre funded by the Engineering and Physical Sciences Research Council. We apologize to authors whose relevant papers we could not cite due to space limitations.

REFERENCES AND FOOTNOTES


Supplementary Material

High-speed high-resolution imaging of intercellular immune synapses using optical tweezers

Stephane Oddos*, Christopher Dunsby†, Marco A. Purbhoo‡, Anne Chauveau‡, Dylan M. Owen*, Mark A. A. Neil†, Daniel M. Davis‡ and Paul M. W. French‡

*Chemical Biology Centre; †Department of Physics; ‡Division of Cell and Molecular Biology, Imperial College London, London SW7 2AZ, UK.

Corresponding authors: Daniel M. Davis, Tel.: +44-207-594-5420; Fax: +44-207-594-3044; E-mail: d.davis@imperial.ac.uk and Paul M. W. French, Tel.: +44207-594-7706; Fax: +44-207-594-7714; E-mail: paul.french@imperial.ac.uk.
Supporting Figures

**Figure S1** Z-stack of a cell pair held vertically by the optical tweezers and imaging of the interface.

**Figure S2** Top: Filopodia length in the plane of the inhibitory NK-cell Bottom: Numerous filopodial protrusions at the NK cell immune synapse human immune synapse.

**Figure S3** Number of sustained (>1min) exclusion areas within the central accumulation at the inhibitory immune synapse.

Supplementary Methods

Movies

**Movie S1** Time-lapse movie of the cells shown in Figure 1

**Movie S2** Time-lapse movie of the cell shown in Figure 2

**Movie S3** Time-lapse movie of the cell shown in Figure 3
Figure S1

A

Red = DiD

Green = DiO

Z = 0 μm
Z = 1.8 μm
Z = 3.6 μm
Z = 5.4 μm
Z = 7.2 μm
Z = 9 μm
Z = 10.8 μm
Z = 12.6 μm
Z = 14.4 μm
Z = 16.2 μm
Z = 18 μm
Z = 19.8 μm
Z = 21.6 μm
Z = 23.4 μm
Z = 25.2 μm
Z = 27 μm

B

X

Z
Figure S2

Histogram showing the frequency of filopodia lengths in μm. The x-axis represents filopodia length in μm, ranging from 2 to 13 μm. The y-axis represents frequency, with bars indicating the number of occurrences for each length interval. The image below the histogram shows a representative fluorescent image of cells with filopodia, with a scale bar included.
Figure S3
SUPPORTING FIGURE LEGENDS

Figure S1

Z-stack of a cell pair held vertically by the optical tweezers and imaging of the interface. One cell is stained with the membrane dye DiD (red), the other with the membrane dye DiO (green). (A) Confocal z-stack (20 steps) across the cell pair. The interface between the two cells is where both green and red appear on the same image (here at z = 12.6 μm). (B) 3D reconstruction of the z-stack in A showing the vertical arrangement of the cell pair. Scale bar, 5 μm.

Figure S2

Top: Filopodia length in the plane of the inhibitory NK-cell human immune synapse (YTS/KIR2DL1-GFP and 721.221/Cw6). A total number of 50 filopodia were analysed (using 3 representative cells over time-series of several minutes each). The indicated numbers refer to the length of the filopodia within the optical plane of the immune synapse. Only clear protrusions (i.e. > 2 μm) were considered. The average length of filopodia was ~ 6 μm. Bottom: Numerous filopodial protrusions at the NK cell immune synapse. The image has been saturated to highlight these dim structures. Scale bar, 5 μm.

Figure S3

Number of sustained (>1 min) exclusion areas within the central accumulation at the inhibitory immune synapse (YTS/KIR2DL1-GFP and 721.221/Cw6). Total number of analysed cells, 26.
SUPPLEMENTARY METHODS

Cells

- **Cell culture:** Cells were cultured in R10 media (RPMI1640 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 (green fluorescent protein) (1).

- **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 at 1200 rpm for 5 minutes and resuspended in R10 media to remove excess SEE. For experiments with NK cells, the B cell line 721.221 stably expressing MHC class-1-Cw6 was used (2).

- **Membrane dye staining:** to stain the cells with the membrane-partitioning dye 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiD) (Molecular Probes), ~10⁶ cells were incubated for 7 minutes at 37°C in a 5% CO₂ atmosphere in 1 μL of R10 media with 1-3 μL of DiD. Cells were then centrifuged at 1200 rpm for 5 minutes and resuspended in R10 media.
• **Fusion proteins and construct sequences:** CD3ζ and KIR2DL1 were amplified by PCR from cDNA and c-terminally tagged with fluorescent protein by ligating in-frame into pcDNA3.1 vectors containing Citrine and eGFP respectively.

• **Transfection method:** Jurkat T cells were transiently transfected using microporation. Just before transfection, ~3×10⁶ cells were washed and resuspended in 30 μL 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 loaded with ~ 200 μL of the cell suspension. For optimal results, cells were diluted down to 10⁵ 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 for them to form mature immune synapses. NK cells and their targets were imaged immediately and those forming obvious synapses were then trapped and imaged.
Optical-tweezer set up

- Full set up for two traps/optical elements

**Optical-tweezer main elements for two-actuable traps**

- **Source characteristics.** To generate the optical tweezers, a fibre-coupled continuous-wave (CW) 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. The diode power was controlled using the following calibration curve (provided by the manufacturer):
Power/driving current calibration curve of the tweezer source

- **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). The transmission curve of the dichroic filter is the following (provided by the manufacturer):

Transmission curve of the IR/visible dichroic
- **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 ~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.

- **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 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.
Imaging

- **Confocal microscope:** the optical tweezers were coupled into the non-descanned port of a commercial confocal microscope with galvanoetric scanners (TCS SP5, Leica Microsystems Ltd). A 63×, 1.25NA oil-immersion objective was used.

- **Detector-bandwidth:** detection was performed using an internal photomultiplier tube (PMT) of the confocal microscope. The detection bandwidth was 500-600 nm for YFP and GFP. For DiD, the detection bandwidth was 640-700 nm. The photomultiplier gain voltage was generally set to 1000 V. An internal 715 nm short-pass filter was used to block reflections from the trapping beam reaching the detectors.

- **Excitation laser:** fluorophore excitation was performed using the internal lasers of the confocal microscope. To excite YFP and GFP we used the 488 nm line of an Ar⁺ laser. To excite DiD we used the 633 nm line of a HeNe laser. Driving current was set at 40% of the maximum. Laser attenuation for the 633 nm line was in general set between 80-90%. Laser attenuation for the 488 nm line was generally set between 40-60%. Adjustment of the laser power during the course of the experiment could occasionally be performed to compensate for fluorophore photobleaching. The laser power could be increased to allow small and dim structures to be seen when necessary while allowing brighter structures to saturate the detector.

- **Frame rate and size:** The scanning rate was generally set at the fastest allowed by the galvanometric-scanners (400 Hz line-scanning rate). For a 512x512 pixel frame, this resulted in a frame rate of 1 frame per second. To achieve faster frame rates, the frame size could be reduced to 256×256 pixels, with no loss of resolution (the voxel size
remained below the maximum diffraction limited size) as the field of view was reduced to image only one pair of cell conjugates.

**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 occasionally 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.

**References**
