High-Speed High-Resolution Imaging of Intercellular Immune Synapses Using Optical Tweezers

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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.

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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.

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

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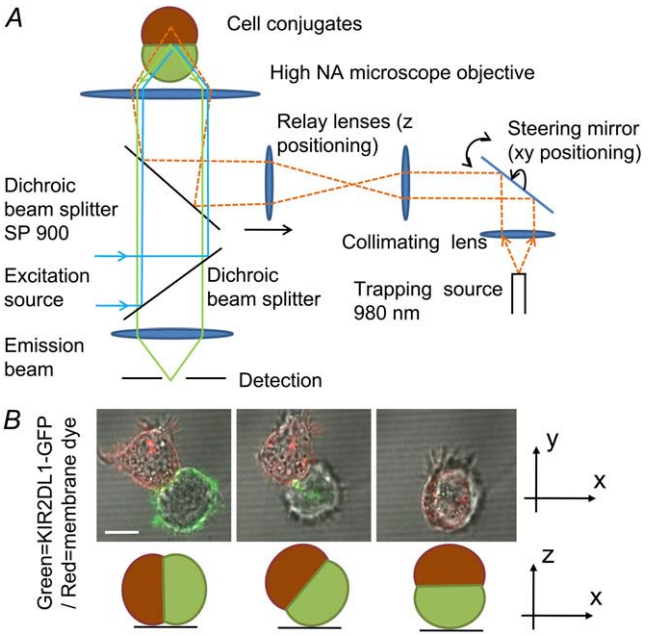

FIGURE 1 Optical tweezers in a confocal microscope. (*A*) Simplified schematic of the setup to allow 3D manipulation of live-cell conjugates and confocal fluorescence imaging. (*B*) Fluorescence and transmitted light images of a target (*red*) and effector cell (*green*) synapse being manipulated into an imaging configuration such that the intercellular contact lies en face to the imaging plane. Scale bar, 10 μm.

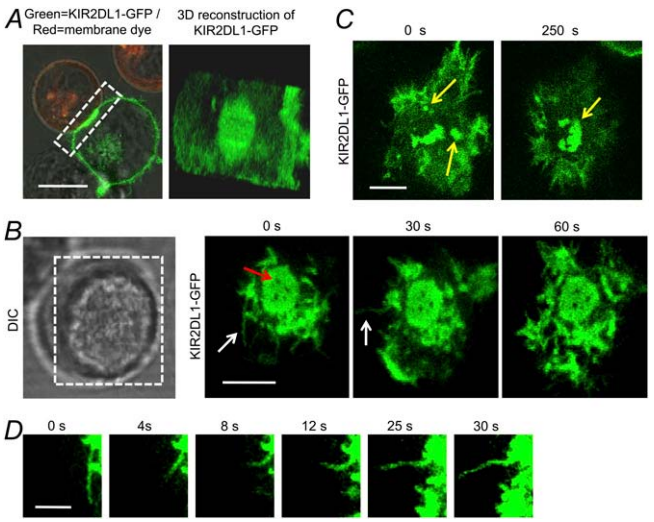

FIGURE 2 Imaging of the inhibitory KIR2DL1 receptor at the NK cell inhibitory immune synapse (*A*) Conventional *z*-stack imaging and 3D reconstruction of the synapse. Scale bar, 10 μm. (*B*) High-speed, high-resolution imaging showing the presence of small central exclusion zones (*red arrow*) and filopodial-like protrusions (*white arrow*). Scale bar, 5 μm. (*C*) Formation of the central accumulation of KIR2DL1 from peripheral clusters that coalesce (*yellow arrows*). Scale bar, 5 μm. (*D*) Time series showing the highly active nature of filopodial extensions around the immune synapse (protrusion highlighted in *B*). Scale bar, 2 μm. Data shown are representative of >20 images. Time reference is from the beginning of experiments.

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 (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). Although normal confocal fluorescence imaging of such cells requires at least 30 s to acquire a full three-dimensional (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, to our knowledge for the first time at intercellular synapses, the formation of this central accumulation from small clusters of KIR2DL1 that moved to the center 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 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 center 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 micro-clusters of T cell receptors that form in synapses with

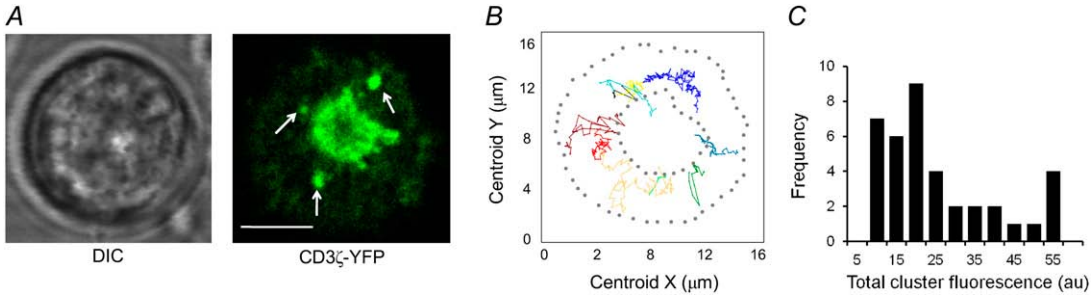

FIGURE 3 High-resolution, high-speed imaging of CD3ζ at the intercellular T cell/APC immune synapse. (*A*) Representative image of CD3ζ at the mature T cell/APC immune synapse (examples of discrete clusters are shown by arrows). Scale bar, 5 μm. (*B*) Tracks followed by 10 representative clusters of CD3ζ within the immune synapse shown in *A*. Dotted lines highlight the limits of the center and periphery of the synapse. (*C*) Distribution of the fluorescence intensity of 40 CD3ζ clusters. $n > 5$.

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 center 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 center to the synapse periphery. Due to an increased signal/noise ratio, quantitative analysis of cluster fluorescence intensity could also be undertaken to investigate their composition (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 of the supplemental files associated with this article, visit www.biophysj.org.

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