Published in final edited form as:

Mol Membr Biol. 2010 August ; 27(0): . doi:10.3109/09687688.2010.495353.

High plasma membrane lipid order imaged at the immunological synapse periphery in live T cells

Dylan M. Owen,

Chemical Biology Centre, Imperial College, London, UK

Stephane Oddos,

Chemical Biology Centre, Imperial College, London, UK

Sunil Kumar,

Chemical Biology Centre, Imperial College, London, UK

Daniel M. Davis,

Division of Cell and Molecular Biology, Imperial College, London, UK

Mark A.A. Neil,

Department of Physics, Imperial College, London, UK

Paul M.W. French,

Department of Physics, Imperial College, London, UK

Michael L. Dustin,

Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, USA

Anthony I. Magee, and

National Heart and Lung Institute, Imperial College, London, UK

Marek Cebecauer

National Heart and Lung Institute, Imperial College, London, UK

Dylan M. Owen: dylan.owen@unsw.edu.au; Stephane Oddos: stephane.oddos05@imperial.ac.uk; Sunil Kumar: sunil.kumar03@imperial.ac.uk; Daniel M. Davis: d.davis@imperial.ac.uk; Mark A.A. Neil: mark.neil@imperial.ac.uk; Paul M.W. French: paul.french@imperial.ac.uk; Michael L. Dustin: Michael.Dustin@med.nyu.edu; Anthony I. Magee: t.magee@imperial.ac.uk; Marek Cebecauer: m.cebcauer@imperial.ac.uk

Abstract

Cholesterol- and glycosphingolipid-enriched membrane lipid microdomains, frequently called lipid rafts, are thought to play an important role in the spatial and temporal organisation of immunological synapses. Higher ordering of lipid acyl chains was suggested for these entities and imaging of membrane order in living cells during activation can therefore help to understand the mechanisms responsible for the supramolecular organisation of molecules involved in the activation of T cells. Here, we employ the phase-sensitive membrane dye di-4-ANEPPDHQ together with a variety of spectrally-resolved microscopy techniques, including 2-channel ratiometric TIRF microscopy and fluorescence lifetime imaging, to characterize membrane order at the T cell immunological synapse at high spatial and temporal resolution in live cells at physiological temperature. We find that higher membrane order resides at the immunological synapse periphery where proximal signalling through the immunoreceptors and accessory proteins

in microclusters has previously been shown to take place. Observed spatial patterning of membrane order in the immunological synapse depends on active receptor signalling.

Keywords

lipid rafts; membrane lipid order; environment-sensitive fluorescent dye

Introduction

The immunological synapse (IS) is the junction formed between immune cells (e.g. T cells) and their targets (e.g. antigen-presenting cells, APCs) (1-3). It is formed during the process of immune cell activation and also during inhibition (4). Previously, it has been shown that within this junction, signalling and adhesion proteins connected to cell activation are patterned into a system of concentric Supra-Molecular Activation Clusters (SMACs) and mobile protein microclusters (1, 2, 5, 6). It was further demonstrated that the peripheral and distal regions of the synapse are important areas for adhesion and proximal signal transduction events such as protein tyrosine phosphorylation (6, 7). For the central area of the IS, the cSMAC, regulatory and signal enhancing roles have been proposed (7).

The appearance of certain proteins and lipids in specific areas of the membrane lead to the hypothesis that cholesterol- and sphingolipid-enriched, highly ordered membrane lipid microdomains (also known as lipid rafts) may play an important role in the organisation of molecules at the cell plasma membrane (8). Some proteins have been shown to accumulate in, and others to be excluded from, these entities using various 'raft markers' (9). Labelling rafts often includes multivalent cholera toxin: subunit B (CTxB) or antibodies, raising the possibility of aggregation of smaller structures. New nonintrusive sensing methods for membrane lipids' physicochemical properties are required for direct observation of these domains in living cells at physiological temperatures (10). Indirect measurements by Förster resonant energy transfer (11), fluorescence correlation spectroscopy (FCS) (12) and single particle tracking (13) demonstrated transient confinement of putative raft components in nanodomains in the plasma membrane of resting cells. In recent years the lipid raft (LR) hypothesis has then evolved into one with a more transient, dynamic picture; LRs are now thought to be small (10 – 200 nm), heterogeneous, dynamic domains that compartmentalize cellular processes and can be stabilized to form larger platforms (14). Data combining super-resolution imaging with FCS confirm this view (15). Experiments with artificial membranes have demonstrated that domains mimicking LRs in biological membranes have higher lipid ordering (liquid-ordered phase) than the surrounding 'non-raft' area (liquid-disordered phase) (16). Two strategies have been developed for imaging lipid ordering in cellular membranes using fluorescence; phase-partitioning dyes (17) and the environmentally sensitive fluorescent probes which can report on their local molecular surroundings (18, 19). The size and short average lifespan of membrane lipid microdomains poses a significant challenge to optical microscopy techniques which are limited in their spatial resolution and minimum image acquisition times. The use of a single environmentally-sensitive probe that can change its fluorescent properties depending on whether it is residing in ordered or disordered membranes is a potentially powerful approach for improving the spatio-temporal resolution of membrane studies. A prime example of this type of imaging is the use of the phase-sensitive, membrane-partitioning dye LAURDAN which shows a 50 nm shortening of its peak fluorescence emission wavelength between the liquid-disordered and liquid-ordered phases (20).

Membrane lipid microdomains have been implicated as having an important role in the organisation of signalling events at the IS of T cells (21, 22). Gaus and colleagues used

LAURDAN generalised polarisation (GP) values calculated from two spectral channels to image the distribution of membrane order in T cells. They found that higher order membrane regions accumulate in the contact site with T cells forming relatively diffuse peripheral ring in the IS (23). Subsequently, they used cholesterol analogue, 7-ketocholesterol (7KC), to disrupt membrane order and shown an impact on T cell activation (24). LAURDAN images in these works were acquired using fixed cells at 25°C with possible unphysiological effects on the highly dynamic membranes of living cells (25). In addition, two-photon excitation is required for LAURDAN imaging which results in a decrease in spatial resolution and, owing to the low two-photon excitation cross-section, longer acquisition times are required to achieve a given signal-to-noise level compared to single-photon-excited probes.

Recently, a new probe sensitive to membrane order, di-4-ANEPPDHQ, has been developed (26, 27) which can be excited in the blue spectral region with single-photon excitation providing increased spatial resolution and decreased acquisition times. More importantly, it allows the use of widefield illumination and detection thus opening the door to high-speed imaging and total internal reflection fluorescence (TIRF) microscopy. Di-4-ANEPPDHQ exhibits a 60 nm spectral blue-shift between the disordered and ordered lipid phases (27), is readily soluble in lipids and aqueous solutions and shows minimal fluorescence when not lipid-associated, presumably due to a high non-radiative fluorescence decay rate in aqueous environment. The combination of various imaging techniques together with the advantages of di-4-ANEPPDHQ allowed us to study the distribution of membrane lipid order in living cellular systems at physiological temperature and high spatial and temporal resolution, including in live primary T cells. These results add to the discussion of the role of membrane organisation in the activation processes of T cells.

Material and Methods

2-channel ratiometric TIRF microscopy

TIRF microscopy was performed on a custom-built microscope with excitation at 473 nm from a diode-pumped solid-state laser (Blues 50, Cobolt, Solna, Sweden) delivered via a single mode optical fiber (SMF) and a rotatable mirror to adjust the TIRF angle. Excitation is delivered into the back-port of an inverted epifluorescence microscope (IX71, Olympus, Tokyo, Japan) equipped with a X60, 1.45NA oil-immersion TIRF objective. Fluorescence is collected on an electron-multiplying CCD camera (EMCCD) (iXon, Andor, Belfast, UK) in the range 500–593 nm and 600–680 nm using a 2-channel imager (Dual-View, Optical Insights) (Fig. 1). Fluorescence above 680 nm was not collected due to high background fluorescence (Data not shown). Data were processed using custom software (LabVIEW, National Instruments, Austin, TX). Calculated GP values (Equation 1) for each pixel were then pseudo-coloured (from high membrane order – red, to low membrane order – blue; see colour bars for Figs. 2–5). The images were merged with the fluorescence intensity image such that the result displays both order and structural information. The sample was maintained at 37°C using a stage and objective heater (PeCon, Erbach, Germany). Quantification of image data was performed by measuring average GP values in the central region (defined from the centre to half way to the synapse edge) and the remaining peripheral region.

$$GP = \frac{I_{500-593} - I_{600-680}}{I_{500-593} + I_{600-680}} \quad \text{Equation 1}$$

Confocal microscopy

Single-photon excitation confocal microscopy was undertaken using an inverted, epi-fluorescence laser-scanning confocal microscope (TCS SP5, Leica Microsystems Ltd., Wetzlar, Germany) with a 63 $\times$, 1.25NA oil-immersion objective lens. Excitation was at 488 nm from an argon-ion laser. Fluorescence detection was in the wavelength bands 500–580 nm and 620–750 nm using internal photomultiplier tubes. Z-slices were acquired every 700 nm. GP values were calculated as for TIRF images. 3D reconstructions and *en face* views were generated using Volocity software (Improvision, Waltham, MA).

Fluorescence lifetime imaging

For FLIM, fluorescence was collected in the range 540–750 nm using a PMT and TCSPC electronics (SPC-830, Becker and Hickl GmbH, Berlin, Germany) with single-photon excitation at 460 nm from a frequency-doubled Ti:Sapphire laser (Tsunami, Spectra-Physics, Mountain View, CA). Fluorescence decays were fitted to a single-exponential function and pseudo-coloured as for the GP images (see *SI Text* for experimental details).

Optical tweezing

Cell and IS orientation by optical tweezing was performed using an infrared (980 nm) optical trapping beam as previously described (28). Confocal fluorescence imaging was then performed in the same way as for the 3D confocal microscopy.

Sample preparation

Jurkat T cells (clone E6.1) and Raji B cells were cultured in RPMI1640 medium (PAA, Pasching, Austria) supplemented with 10% foetal calf serum (FCS) at 37°C in a 5% CO₂ atmosphere. Raji B cells were loaded (or not) with the superantigen *Staphylococcal* enterotoxin E (SEE) for 90 min at 37°C. Human CD4⁺ T cells were isolated using a MACS kit for negative selection (Miltenyi Ltd) after separation of peripheral blood mononuclear cells on Ficoll gradient (GE Healthcare). One hour prior to imaging, the cells were resuspended in HEPES-buffered salt saline (HBSS) supplemented with 1mM Ca²⁺, 2mM Mg²⁺, 1% gelatine and 5 μ M di-4-ANEPPDHQ (Invitrogen, Carlsbad, CA). The use of serum-free medium containing gelatine dramatically reduces the fluorescence background from residual dye which would otherwise swamp the red wavelength channel (Figure S1).

Coverslip chambers (Lab-Tek, Brendale, Australia) coated with poly-L-lysine followed by 10 μ g/ml antibodies to CD3 (OKT3 mAb) in phosphate-buffer saline (PBS) and blocked by incubation at 37°C with PBS supplemented with 1% gelatin for 30 minutes were used for activatory surface stimulation of T cells.

Supported planar bilayers with GPI-anchored forms of I-E^k and ICAM-1 were prepared in FCS2 flow cells (Biopetech, Butler, USA) and used for stimulation of AND T cells as described before (2). The I-E^k molecules on the bilayers were loaded with 100 μ M MCC88-103 peptide in citrate buffer for 48 hr. The density of the GPI-anchored proteins in the bilayers was: I-E^k 20 sites/ μ m² and ICAM-1 150 sites/ μ m². (see *SI Text* for details).

For ordered phase disruption using 7KC, 15 mg/ml 7KC and cholesterol (both from Avanti Lipids, Alabaster, AL) in ethanol were combined in the ratio 2:1. Over 30 mins, these were then added to a solution of 50 mg/ml methyl- β -cyclodextrin (M β CD; Sigma-Aldrich, St. Louis, MO) in PBS at 80°C to a final sterol concentration of 1.5 mg/ml. 15 μ l of the lipid solution were then added to 1 ml of cell medium containing 5 $\times$ 10⁵ cells at 37°C for 30 minutes.

Results

IS are stable junctions between T cells and APCs that integrate signals and facilitate effector functions. To test whether the phase-sensitive dye, di-4-ANEPPDHQ, will allow us to image membrane order within this structure in real time we first incubated living T cells with APCs under physiological temperature of 37°C and applied conventional 2-colour confocal microscopy. We observed the accumulation of higher lipid order in the IS periphery and relatively lower order in the central region (Fig. 2c and f). Very low order was observed in intracellular membrane structures, as has been reported previously (29) (2b and e). These distributions agree well with previously published data acquired using LAURDAN and multi-photon microscopy on fixed cells at room temperature (23). Large macromolecular rearrangements in the IS and formation of peripheral adhesive ring and a central receptor-rich area in activated T cells were shown to be antigen signalling-dependent (1). We imaged the distribution of membrane order in T cells forming conjugates with APC in the absence of antigenic stimulus. Again, increased ordering of lipids was observed at the contact site between T cells and APC but little or no segregation into highly ordered peripheral ring and central area with relatively lower lipid ordering was observed (Fig. 2g-l).

Live cell imaging of T cell-APC conjugates is associated with a low probability that the IS will form in the imaging plane of the microscope thus requiring 3D z-stacks to be acquired (Cebecauer et al. in press). This limits spatial resolution as confocal microscopes typically have axial resolutions of around 700 nm and the 3-D rendering required to generate *en face* images of the IS further degrade the image quality. In order to overcome this limitation, we applied optical tweezers to reorientate the IS into the confocal imaging plane (28). This permits the IS to be directly imaged at the higher lateral resolution of approximately 200 nm. Again, a broad ring of high membrane order at the IS periphery was observed with decreased order towards the centre (Fig. 3a). This membrane order distribution, as shown in the GP value plot (Fig. 3b), was representative of n=8 cells.

As stated, conventional confocal fluorescence microscopy enables imaging of fluorescent objects in the focal plane with an axial resolution (sectioning strength) of around 700 nm. The signal detected may therefore include contributions from the plasma membrane and other intracellular structures within the focal volume. In order to overcome this constraint, we used 2-channel ratiometric TIRF microscopy (see *Materials and Methods* and Fig. 1) to image the membrane order of T cells activated on coverslips with immobilized anti-CD3 antibodies. Fig. 4a, b and c show a representative brightfield transmitted light image, TIRF intensity image and a membrane order (GP) map, respectively, of a live Jurkat T cell synapse imaged approximately 10 minutes after the contact with the activating surface. The cell shows a large, spread interface with the antibody-coated coverslip mimicking the IS formed by T cells spreading over the surface of an APC. A higher lipid order accumulation in the IS periphery with reduced order distributed towards the central region confirms the results observed during live imaging of T cells conjugated with APCs. As no 3D reconstruction is involved it is possible to perform accurate quantitative analysis of the data. Fig. 4h shows GP values from central and peripheral regions acquired from 20 individual cells ($p=0.10$) and a line profile of the GP values from one, representative case. The relatively small difference in GP values for central and peripheral regions of the IS is caused by contact shape variability and poorly defined border between these two regions.

It is known that cultured cells (e.g. Jurkat T cells) do not retain all the properties of primary cells. We therefore imaged the distribution of membrane order obtained from primary human CD4⁺ T cells activated on an anti-CD3 antibody-coated coverslip (Fig. 4d). The image shows a similar distribution of higher membrane order in the periphery with an

indication of the multi-focality observed in synapses formed between primary T cells and dendritic cells presenting the antigen on their surface (30).

The higher signal-to-noise level, lower background, increased sectioning strength and shorter image acquisition times of TIRF microscopy enabled us to observe some finer substructures within a larger field of high order at the IS periphery. Most strikingly, the peripheral ring of higher membrane order reproducibly showed discontinuities or small areas of lower order suggesting possible sub-compartmentalization of cellular membranes (Fig. 4c and d). As TIRF images can be acquired at high frame-rates, it is possible to obtain a time-course of the order distribution as a movie. Selected frames of a representative movie (Movie S1) are displayed in Fig. 4e. The general order distribution was observed to be stable over the imaged time-scale. However, we have been able to monitor points of lower order (blue) diffusing in the higher order background (Fig. 4e; arrow). Small mobile areas of high, intermediate and low order, which may represent different vesicular sub-synaptic structures, were observed in cells where plasma membrane di-4-ANEPPDHQ dye was reduced by a quick wash (Movies S2 and S3 and selected frames in Fig. 4f and g). We hypothesise that different vesicle populations may be important in trafficking of different membrane proteins, based on their affinity to membranes of various composition and lipid order. Of note, the resolution here is approximately $300 \times 300 \times 100$ nm (x, y, z) which is higher than has previously been possible when imaging membrane order.

Having established the capability to image membrane order at the IS at high spatial and temporal resolution, we investigated whether changes in membrane order, for example due to pharmacological treatment, can be investigated using our technique. The cholesterol analogue 7KC was previously described as an effective substance to reduce plasma membrane order of cultured T cells without significant effect on their viability and proximal signalling (24). As seen in Fig. 5 a and b (imaged by 2-channel ratiometric confocal microscopy), higher order of the plasma membrane was eliminated by 7KC pre-treatment of T cells. The effect on intracellular membranes was less dramatic but the order was still reduced, as expected. The cells maintained normal morphology even 90 minutes after treatment with 7KC. Using TIRF microscopy, the high membrane order ring observed in the IS periphery was absent in 7KC-treated cells activated on anti-CD3-coated coverslips (Fig. 5c and d) and quantitation supported the dramatic reduction of membrane order in the IS of 7KC-treated activated T cells (Fig. 4e). Reduced membrane order did not, however, affect the ability of T cells to spread over the activating surface.

Antibodies immobilized on the glass surface of coverslips are likely to reduce the diffusional behaviour of membrane proteins. Incorporating ligands for TCR (peptide-MHC) and adhesion molecules (ICAM-1) into supported planar lipid bilayers represents a more physiological activating surface for primary T cells (31). Supported bilayers allow ligands lateral freedom and therefore do not restrict mobility of membrane proteins on T cells. We found that di-4-ANEPPDHQ dye in medium shows high affinity for T cells but also for the planar bilayer. Standard 2-channel intensity imaging is not sensitive enough to image membrane order of T cells in the presence of a large disordered-phase background from DOPC in the planar bilayer. We have previously demonstrated that fluorescence lifetime imaging (FLIM) offers enhanced contrast when imaging di-4-ANEPPDHQ in live cells (29). We therefore applied FLIM using single-photon excitation and time-correlated single-photon counting (TCSPC) on a scanning confocal microscope to improve the contrast when imaging membrane order of activated T cells on supported planar bilayers. It should be noted that, due to the dynamic nature of primary T cell-bilayer synapses and the longer acquisition times required for FLIM, both the spatial and temporal resolution are reduced relative to the antibody-coated coverslip data (Fig. 4).

Fig. 6a-d show brightfield and FLIM images of primary T cell synapses formed on supported planar bilayers. The low order background (blue) is due to DOPC present in the bilayer, for which we found a similar fluorescence lifetime to previously published results (29). Despite the lower spatial and temporal resolution of the measurements, the observed order distribution at the synapse remains generally consistent with the previous results. We hypothesise that the discontinuous nature of the high order membrane regions around the periphery may represent membrane sub-compartmentalization. Treatment of primary murine T cells with 7KC (Fig. 6 e and f) disrupted higher order membrane regions that accumulated primarily in the periphery of the formed synapses. All these data support the high ordering of membrane lipids in the area of active signalling processes responsible for T cells stimulation.

Discussion

The formation of large assemblies composed of signalling molecules is thought to improve the efficiency and robustness of signalling processes in the crowded and dynamic environment of the cells (Cebecauer et al. *J Cell Science*; In press). Supramolecular activatory clusters and microclusters containing receptors and signalling proteins in the IS are essential for the initiation of signalling processes in T cells and activation of their effector function (32-34,1,2,5). The involvement of lipids in the formation and stabilisation of such assemblies has been suggested based on the presence of crucial cluster components in isolated detergent-resistant membranes (35-37) and on co-localisation studies of TCR complex constituents with LR markers in activated T cells (21). The concept of LRs importance for T cell activation has also been criticized and protein-protein interactions were suggested to be the sole force responsible for driving higher organisation of signalling molecules in the IS (38,39). Live cell imaging of membrane organisation in the IS and its dependence on lipid composition can therefore improve our understanding of molecular processes leading to the T cell activation.

Previous work suggested the polarised distribution of membrane order in T cells forming conjugates with APC in fixed cells at 25°C (20). Here we have imaged the distribution of membrane order at the IS at higher spatial and temporal resolution than has been achieved previously, and for the first time in living T cells at physiological temperature. This latter point is crucial because reducing the ambient temperature of cells even over a few degrees Celsius has been shown to induce membrane microdomain aggregation and T cell signalling (25). We applied a variety of fluorescence microscopy techniques including 3D 2-channel confocal imaging and optical tweezer-oriented live cell conjugates as well as 2-channel ratiometric TIRF microscopy using activating antibody-coated glass coverslips. In all cases tested, higher plasma membrane order was demonstrated to accumulate preferentially in the peripheral or distal regions of the synapse with lower lipid ordering in the central region. The observed distribution is in agreement with previously published data acquired at lower spatial and temporal resolution using the dye LAURDAN (23). Since similar membrane order distribution is observed with LAURDAN, a chemically different molecule, it is also possible to exclude potential artefactual protein-dye interactions. Further, high membrane order was removed by treatment with the cholesterol oxide, 7KC, indicating a cholesterol dependence rather than exclusively the effect of membrane proteins and their binding partners in the stabilization of higher order membranes.

We have observed partial accumulation of highly ordered membranes in the junction formed between T cells and APCs in the absence of antigen. Here, the increased lipid ordering is probably caused by the interactions of T cell adhesive receptors, such as CD2 and LFA-1 (in low-affinity states), and their ligands on the surface of the APC; CD58 and ICAM-1 (40). No large-scale segregation into high order peripheral ring and lower membrane order central

zone has been observed in the absence of the antigen. Such uniform distribution of membrane lipid order across the junction is similar to the previously reported random localisation of surface receptors (e.g. LFA-1 and TCR) in T cells forming conjugates with APC in the absence of antigen (1).

Previously, only steady-state images of membrane order distribution have been achieved. No changes in the overall general pattern of high membrane order forming peripheral ring of the IS with reduced lipid ordering in central zone have been detected over 1 minute using live cell imaging in TIRF mode. With this technique, it was possible to observe what we hypothesize to be sub-synaptic vesicles of low, medium and high membrane lipid order which have not been previously reported for any cellular system. The co-existence of sub-synaptic vesicles with various levels of membrane order is indicated in movies of cells where the plasma membrane di-4-ANEPPDHQ dye concentration was reduced by washing. Formation of sub-membranous vesicular structures of different properties supports the observation of the presence of proximal T cell signalling molecules, LAT and SLP-76, in non-overlapping, dynamic microclusters (M. Purbhoo and D.M.D., unpublished results).

We have succeeded in imaging membrane order in live primary CD4⁺ human T cells using 2-channel TIRF microscopy and have used di-4-ANEPPDHQ with FLIM to image membrane order at the primary murine T cell synapse formed with activating supported planar bilayers. These results represent a significant advance in terms of physiological relevance since many cultured cells of tumour origin have modified membrane lipid composition and planar bilayers represent a model for APCs with unrestricted mobility of ligand/receptor pairs therefore reducing the probability of increased membrane order due to restrictions caused by immobilized antibodies.

All presented data demonstrate formation of a wide ring of high membrane order at the IS periphery with reduced lipid ordering in the central region. The function of the central synaptic region, the cSMAC, remains controversial since fully functional synapses were demonstrated without any detectable cSMAC (41). On the other hand, regulatory and signal enhancing roles were proposed for the cSMAC based on data acquired using weak antigens (42). Since only relatively strong activatory signals were applied in our study, we cannot exclude the possible accumulation of more ordered membranes in the central region under different stimulatory conditions. The exact function of highly ordered membranes in peripheral and distal regions of the IS remains to be explored. The accumulation of integrins and associated molecules in the peripheral pSMAC supports its generally accepted adhesive function (1, 2). Formation of TCR microclusters required for T cell activation was demonstrated to occur primarily in the distal region (dSMAC) (6). We speculate that lipids forming higher order membrane environment may help the formation of larger proteinaceous domains (e.g. TCR microclusters) or platforms (e.g. integrins aggregating during the activation) by reducing molecular fluctuations within the plasma membrane. The latter is supported by the observation of higher membrane order in the junction between T cells and APCs in the absence of antigenic stimulus. Only weak adhesive interactions are expected to influence plasma membrane of T cells in the absence of TCR signalling (43).

The IS periphery is also a proximal signalling area (6). Many signalling proteins involved in immune cell activation were shown to contain post-translational modifications increasing their partitioning to highly ordered domains in membranes (44). Higher order membrane environments in the synapse periphery together with putative formation of vesicles with different lipid compositions and lipid order (Movies S1-S3) may present an elegant way of signalling regulation by compartmentalization of various signalling pathways into separate substructures.

The existence of small ($\varnothing < 40$ nm) membrane microdomains with increased order and therefore increased selective molecular confinement was observed in live cells indirectly by fluorescence correlation spectroscopy (12) and recently directly by super-resolution STED microscopy (15). Whether the observed lipid structure (higher membrane order peripheral ring) is formed by the aggregation of these nanoscopic lipid rafts as has been postulated (14) remains to be demonstrated. The small predicted size of LR in resting cells prohibits observation of their fusion in real time using diffraction limited imaging techniques.

Taken together, we believe that the methods used in this work have moved the limits of membrane order imaging towards higher spatial and temporal resolution and to live cell systems. The observation of high membrane order at the periphery and the presence of high and low order dynamic sub-synaptic structures poses important new questions for the role of membrane order in regulating T cell activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Rajat Varma and Etienne Joly for helpful suggestions and critical reading of the manuscript, and Chris Dunsby for helpful comments on the manuscript and assistance with optical tweezing. DMO, SK and SO acknowledge funding from the Engineering and Physical Sciences Research Council via the Chemical Biology Centre. We acknowledge Medical Research Council (MRC) grants G0700771 and G0100471 to AIM and National Institutes of Health grant AI43542 to MLD. DMD acknowledges funding from the MRC. PMWF and DMD acknowledge a Wolfson Royal Society Research Merit Award. We also acknowledge the Facility for Imaging by Light Microscopy at Imperial College.

References

1. Monks CRF, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-Dimensional Segregation of Supramolecular Activation Clusters in T Cells. *Nature*. 1998; 395(6697):82–86. [PubMed: 9738502]
2. Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML. The Immunological Synapse: A Molecular Machine Controlling T Cell Activation. *Science*. 1999; 285(5425):221–227. [PubMed: 10398592]
3. Bromley SK, Burack WR, Johnson KG, Somersalo K, Sims TN, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML. The Immunological Synapse. *Annu Rev Immunol*. 2001; 19(1):375–396. [PubMed: 11244041]
4. Davis DM, Chu I, Fassett M, Cohen GB, Mandelboim O, Strominger JL. The Human Natural Killer Cell Immune Synapse. *Proc Natl Acad Sci*. 1999; 96(26):15062–15067. [PubMed: 10611338]
5. Yokosuka T, Sakata-Sogawa K, Kobayashi W, Hiroshima M, Hashimoto-Tane A, Tokunaga M, Dustin ML, Saito T. Newly Generated T Cell Receptor Microclusters Initiate and Sustain T Cell Activation by Recruitment of Zap70 and Slp-76. *Nat Immunol*. 2005; 6(12):1253–1262. [PubMed: 16273097]
6. Varma R, Campi G, Yokosuka T, Saito T, Dustin ML. T Cell Receptor-Proximal Signals Are Sustained in Peripheral Microclusters and Terminated in the Central Supramolecular Activation Cluster. *Immunity*. 2006; 25(1):117–127. [PubMed: 16860761]
7. Lee KH, Dinner AR, Tu C, Campi G, Raychaudhuri S, Varma R, Sims TN, Burack WR, Wu H, Wang J, Kanagawa O, Markiewicz M, Allen PM, Dustin ML, Chakraborty AK, Shaw AS. The Immunological Synapse Balances T Cell Receptor Signaling and Degradation. *Science*. 2003; 302(5648):1218–1222. [PubMed: 14512504]
8. Simons K, Ikonen E. Functional Rafts in Cell Membranes. *Nature*. 1997; 387:569–572. [PubMed: 9177342]

9. Day CA, Kenworthy AK. Tracking Microdomain Dynamics in Cell Membranes. *Biochim Biophys Acta*. 2009; 1788(1):245–253. [PubMed: 19041847]
10. Cebeacauer M, Owen DM, Markiewicz A, Magee AI. Lipid Order and Molecular Assemblies in the Plasma Membrane of Eukaryotic Cells. *Biochem Soc Trans*. 2009; 37(5):1056–1060. [PubMed: 19754451]
11. Sharma P, Varma R, Sarasij RC, Ira, Gousset K, Krishnamoorthy G, Rao M, Mayor S. Nanoscale Organization of Multiple GPI-Anchored Proteins in Living Cell Membranes. *Cell*. 2004; 116(4): 577–589. [PubMed: 14980224]
12. Lenne PF, Wawrezynieck L, Conchonaud F, Wurtz O, Boned A, Guo XJ, Rigneault H, He HT, Marguet D. Dynamic Molecular Confinement in the Plasma Membrane by Microdomains and the Cytoskeleton Meshwork. *EMBO J*. 2006; 25:3245–3256. [PubMed: 16858413]
13. Suzuki KGN, Fujiwara TK, Sanematsu F, Iino R, Edidin M, Kusumi A. GPI-anchored receptor clusters transiently recruit Lyn and G{alpha} for temporary cluster immobilization and Lyn activation: single-molecule tracking study 1. *J Cell Biol*. 2007; 177(4):717–730. [PubMed: 17517964]
14. Pike LJ. Rafts Defined: A Report on the Keystone Symposium on Lipid Rafts and Cell Function. *J Lipid Res*. 2006; 47(7):1597–1598. [PubMed: 16645198]
15. Eggeling C, Ringemann C, Medda R, Schwarzmann G, Sandhoff K, Polyakova S, Belov VN, Hein B, Von Middendorff C, Schönl A, Hell SW. Direct Observation of the Nanoscale Dynamics of Membrane Lipids in a Living Cell. *Nature*. 2009; 457:1159–1163. [PubMed: 19098897]
16. Sengupta P, Baird B, Holowka D. Lipid Rafts, Fluid/Fluid Phase Separation, and Their Relevance to Plasma Membrane Structure and Function. *Semin Cell Dev Biol*. 2007; 18(5):583–590. [PubMed: 17764993]
17. Burack WR, Lee KH, Holdorf AD, Dustin ML, Shaw AS. Cutting Edge: Quantitative Imaging of Raft Accumulation in the Immunological Synapse. *J Immunol*. 2002; 169(6):2837–2841. [PubMed: 12218094]
18. Parasassi T, De Stasio G, d'Ubaldo A, Gratton E. Phase Fluctuation in Phospholipid Membranes Revealed by Laurdan Fluorescence. *Biophys J*. 1990; 57(6):1179–1186. [PubMed: 2393703]
19. Gaus K, Gratton E, Kable EPW, Jones AS, Gelissen I, Kritharides L, Jessup W. Visualizing Lipid Structure and Raft Domains in Living Cells with Two-Photon Microscopy. *Proc Natl Acad Sci*. 2003; 100(26):15554–15559. [PubMed: 14673117]
20. Gaus K, Zech T, Harder T. Visualizing Membrane Microdomains by Laurdan 2-Photon Microscopy. *Mol Membr Biol*. 2006; 23(1):41–48. [PubMed: 16611579]
21. Janes PW, Ley SC, Magee AI. Aggregation of Lipid Rafts Accompanies Signaling Via the T Cell Antigen Receptor. *J Cell Biol*. 1999; 147(2):447–461. [PubMed: 10525547]
22. Tavano R, Gri G, Molon B, Marinari B, Rudd CE, Tuosto L, Viola A. Cd28 and Lipid Rafts Coordinate Recruitment of Lck to the Immunological Synapse of Human T Lymphocytes. *J Immunol*. 2004; 173(9):5392–5397. [PubMed: 15494485]
23. Gaus K, Chklovskaya E, Fazekas de St Groth B, Jessup W, Harder T. Condensation of the Plasma Membrane at the Site of T Lymphocyte Activation. *J Cell Biol*. 2005; 171(1):121–131. [PubMed: 16203859]
24. Rentero C, Zech T, Quinn CM, Engelhardt K, Williamson D, Grewal T, Jessup W, Harder T, Gaus K. Functional Implications of Plasma Membrane Condensation for T Cell Activation. *PLoS ONE*. 2008; 3(5):e2262. [PubMed: 18509459]
25. Magee AI, Adler J, Parmryd I. Cold-Induced Coalescence of T-Cell Plasma Membrane Microdomains Activates Signalling Pathways. *J Cell Sci*. 2005; 118(14):3141–3151. [PubMed: 16014381]
26. Obaid AL, Loew LM, Wuskell JP, Salzberg BM. Novel naphthylstyryl-pyridinium potentiometric dyes offer advantages for neural network analysis. *J Neurosci Methods*. 2004; 134(2):179–190. [PubMed: 15003384]
27. Jin L, Millard AC, Wuskell JP, Dong X, Wu D, Clark AH, Loew LM. Characterization and Application of a New Optical Probe for Membrane Lipid Domains. *Biophys J*. 2006; 90(7):2563–2575. [PubMed: 16415047]

28. Oddos S, Dunsby C, Purbhoo MA, Chauveau A, Owen DM, Neil MAA, Davis DM, French PMW. High-Speed High-Resolution Imaging of Intercellular Immune Synapses Using Optical Tweezers. *Biophys J*. 2008; 95(10):L66–68. [PubMed: 18723590]
29. Owen DM, Lanigan PMP, Dunsby C, Munro I, Grant D, Neil MAA, French PMW, Magee AI. Fluorescence Lifetime Imaging Provides Enhanced Contrast When Imaging the Phase-Sensitive Dye Di-4-Anepdhq in Model Membranes and Live Cells. *Biophys J*. 2006; 90(11):L80–82. [PubMed: 16617080]
30. Brossard C, Feuillet V, Schmitt A, Randriamampita C, Romao M, Raposo G, Trautmann A. Multifocal Structure of the T Cell - Dendritic Cell Synapse. *Eur J Immunol*. 2005; 35(6):1741–1753. [PubMed: 15909310]
31. Groves JT, Dustin ML. Supported Planar Bilayers in Studies on Immune Cell Adhesion and Communication. *J Immunol Methods*. 2003; 278(1-2):19–32. [PubMed: 12957393]
32. Dustin ML, Olszowy MW, Holdorf AD, Li J, Bromley S, Desai N, Widder P, Rosenberger F, van der Merwe PA, Allen PM, Shaw AS. A Novel Adaptor Protein Orchestrates Receptor Patterning and Cytoskeletal Polarity in T Cell Contacts. *Cell*. 1998; 94:667–677. [PubMed: 9741631]
33. Bunnell SC, Hong DI, Kardon JR, Yamazaki T, McGlade CJ, Barr VA, Samelson LE. T Cell Receptor Ligation Induced the Formation of Dynamically Regulated Signalling Assemblies. *J Cell Biol*. 2002; 158(7):1263–1275. [PubMed: 12356870]
34. Campi G, Varma R, Dustin ML. Actin and Agonist MHC-Peptide Complex-Dependent T Cell Receptor Microclusters as Scaffolds for Signaling. *J Exp Med*. 2005; 202(8):1031–1036. [PubMed: 16216891]
35. Xavier R, Brennan T, Li Q, McCormack C, Seed B. Membrane Compartmentation is Required for Efficient T Cell Activation. *Immunity*. 1998; 8:723–732. [PubMed: 9655486]
36. Montixi C, Langlet C, Bernard AM, Thimonier J, Dubois C, Wurbel MA, Chauvin JP, Pierres M, He HT. Engagement of T Cell Receptor Triggers Its Recruitment to Low-Density Detergent-Insoluble Membrane Domains. *EMBO J*. 1998; 17(18):5334–5348. [PubMed: 9736612]
37. Drevot P, Langlet C, Guo XJ, Bernard AM, Colard O, Chauvin JP, Lasserre R, He HT. *EMBO J*. 2002; 21:1899–1908. [PubMed: 11953309]
38. Munro S. Lipid Rafts: Elusive or Illusive? *Cell*. 2003; 115(4):377–388. [PubMed: 14622593]
39. Douglass AD, Vale RD. Single-Molecule Microscopy Reveals Plasma Membrane Microdomains Created by Protein-Protein Networks that Exclude or Trap Signaling Molecules in T Cells. *Cell*. 2005; 121(6):937–950. [PubMed: 15960980]
40. Springer TA, Dustin ML, Kishimoto TK, Marlin SD. The Lymphocyte Function-Associated LFA-1, CD2 and LFA-3 Molecules: Cell Adhesion Receptors of the Immune System. *Annu Rev Immunol*. 1987; 5:223–252. [PubMed: 3109455]
41. Lee KH, Holdorf AD, Dustin ML, Chan AC, Allen PM, Shaw AS. T Cell Receptor Signaling Precedes Immunological Synapse Formation. *Science*. 2002; 295(5559):1539–1542. [PubMed: 11859198]
42. Cemerski S, Das J, Giurisato A, Markiewicz M, Allen PM, Chakraborty AK, Shaw AS. The Balance between T Cell Receptor Signaling and Degradation at the Center of the Immunological Synapse Is Determined by Antigen Quality. *Immunity*. 2008; 29(3):414–422. [PubMed: 18760640]
43. Dustin ML, Sanders ME, Shaw S, Springer TA. Purified Lymphocyte Function-Associated Antigen-3 binds to CD2 and Mediates Lymphocyte-T Adhesion. *J Exp Med*. 1987; 165(3):677–692. [PubMed: 3102676]
44. Lommerse PHM, Vastenhoud K, Pirinen NJ, Magee AI, Spaink HP, Schmidt T. Single-molecule diffusion reveals similar mobility for the Lck, H-Ras and K-Ras membrane anchors. *Biophys J*. 2006; 91(3):1090–1097. [PubMed: 16920696]

FIGURE 1.
 (a) Emission spectra of di-4-ANEPPDHQ in the ordered (green) and disordered (red) phases showing the wavelength ranges collected for each channel. (b) Schematic of the 2-channel ratiometric TIRF microscope.

FIGURE 2.

Membrane order at the synapse of live T cell-APC conjugates. (a, b and c) brightfield, GP image and 3D reconstruction showing en face view of the cell-cell interface (dashed line viewed from the direction of the arrow on b; APC position is indicated by dashed ring) of live di-4-ANEPPDHQ-stained Jurkat T cells forming synapses with unstained APCs (SEE-loaded Raji cells). (d, e and f) second example showing similar order distribution. APC (not shown) position is indicated with dashed circle and the other T cell is not shown for clarity (representative of n=XX). (g, h and i) and (j, k and l) show two examples of the synapses formed in the absence of antigenic stimulus (SEE; representative of n=16). The total acquisition time for the 3D stack was approximately 30 seconds with both images acquired approximately 10 minutes after synapse formation. In all images, red colour represents higher and green/blue lower order membranes. Scale bars 10 μ m.

FIGURE 3.

T cell synapse images using optical tweezers. (a) Activatory conjugate between Jurkat T cell and APC (as in Fig. 2 a) with the IS oriented in the focal plane using optical tweezers, showing a brightfield image, GP image and representative line profile across the synapse (b). Distributions representative of a total of $n = 8$ cases. Scale bar $10\ \mu\text{m}$.

FIGURE 4.

Order distribution at the T cell IS formed with activating antibody-coated glass coverslips. (a) Brightfield image showing the main cell body (outlined red) and the large synaptic interface (outlined yellow). (b) TIRF intensity image of the interface. (c) GP map of order at the synapse showing higher order (red) and lower order (green to blue) (representative of $n=20$). (d) Order map of the interface formed by a primary human T cell ($n = 6$). Presented images are of a T cell approximately 10 min after formation of contact with activatory surface. (e) GP value images showing dynamics of the observed order distribution (see also Supplementary Movie 1). (f and g) Images showing low- and high-order putative sub-synaptic vesicle (white arrows) diffusing in the plasma membrane background (see also Supplementary Movies 2 and 3). Acquisition time was 1 sec per frame. (h) Quantitative

analysis of GP values in the centre and periphery of Jurkat cell synapses (n=20, p=0.10) and a representative line profile across the contact site. Scale bars 5 μ m

FIGURE 5.

Effect of 7KC treatment on membrane order. (a and b) GP images of di-4-ANEPPDHQ-stained Jurkat T cells in suspension imaged using confocal microscopy without and after 7KC treatment, respectively. (c and d) TIRF GP images of the IS formed on an anti-CD3 antibody-coated coverslip ~10 min after contact formation without and after 7KC treatment, respectively. (e) Quantitation of membrane order in untreated and 7KC-treated T cell synapses (n=20) showing reduced order after 7KC treatment ($p < 0.001$). Scale bars 5 μm .

FIGURE 6.

Representative order distributions at the IS formed by primary murine T cells activated on supported planar bilayers (n=10). (a-d) Brightfield and FLIM images of di-4-ANEPPDHQ-stained primary T cells forming synapses on planar bilayers. (e and f) Brightfield and FLIM images of T cell synapses after treatment with 7KC. Scale bars 5 μm.