Tracking sub-microscopic protein organisation at the plasma membrane of live cells using triple-colour super-resolution microscopy

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INTRODUCTION
Intercellular communication via signalling across the plasma membrane is a fundamental process throughout biology - from the immune response through to cancer and development – which requires control and regulation on many different levels. At the plasma membrane, it is widely known that features of the actin cytoskeleton and clathrin-coated pits play an important role in regulating signal transduction. In fact, spatiotemporal organisation of proteins into functional units known as microcompartments is thought to be important, with such membrane features shown to contribute to the transient assembly of nanoscopic heterogeneities in receptor organisation (ranging from 20–200 nm across) [1-4]. This is also important for cytokine signalling, where the signals originating from the interferon-α/β cell surface receptor (IFNAR) probably depend on co-organisation within the membrane. On ligand binding, the subunits of this receptor (IFNAR1 and IFNAR2) must associate in order to trigger a downstream response via the JAK/STAT pathway.

Observing this spatiotemporal organisation is an enormous experimental challenge. Electron microscopy may achieve the required resolution, but merely providing a fixed “snapshot” of the membrane fails to capture its highly dynamic nature. We therefore rely on in-vivo imaging, utilising the powerful technique of total internal reflection fluorescence microscopy (TIRFM). Based on the effect of total internal reflection at the glass-medium interface, fluorescence excitation in the sample is achieved by an evanescent wave instead of direct illumination. The exponential decay of the evanescent field illumination intensity restricts fluorescent excitation to a thin region extending 100–200 nm beyond the surface of the cover glass. Delivering images with such high axial resolution allows us to specifically observe processes occurring at the plasma membrane in detail. As an optical technique, however, the performance of TIRFM is limited by a resolution determined by the diffraction limit of light (approximately 200 nm), which means that sub-microscopic features of membrane organisation with key relevance for cell signalling lie beyond the reach of conventional light microscopy.

SUPER-RESOLUTION MICROSCOPY
Recent advances in super-resolution (SR) fluorescence microscopy technologies have managed to overcome this resolution limit [4], opening up many new avenues for exploring in-vivo protein dynamics. Relying on a variety of principles, one collection of techniques takes advantage of sequential activation and time-resolved localisation of photoswitchable or photoblinking fluorophores. At any one moment, a small subset of fluorophores is activated into a fluorescent state, each of which can be localised with precision far beyond the diffraction limit. After numerous cycles of capturing subsets of individual fluorophores, a high-resolution image is constructed from all sequentially detected molecules that could not be resolved if fluorescing simultaneously, enabling visualisation of nanoscale processes within living cells.

In our laboratory at the University of Osnabrück, we have established a novel triple-colour SR microscopy approach for observing membrane dynamics in live cells [5]. Combining two SR microscopy techniques allowed us to simultaneously observe both the cytoskeleton and receptor organisation, with an average resolution of 25 nm. In order to map the location of the IFNAR1 and IFNAR2 cell surface receptor subunits, we employed fluorescence photoactivation localisation microscopy (FPALM), whilst visualising the cytoskeleton at the plasma membrane using direct stochastic optical reconstruction microscopy (dSTORM). Although dSTORM and FPALM techniques both share the same principle, based on the time-resolved localisation of...
super-resolution microscopy

STORM

...of the actin cytoskeleton, we developed (HTL). To establish efficient labelling covalently linked to the HaloTag ligand system was chosen, and ATTO655 was employed for its application in live-cell imaging, having been demonstrated [11-13]. A key challenge for its application in live-cell imaging, however, is the specific labelling of target proteins in the cytoplasm. For fast and efficient exchange of photoactivatable green fluorescent protein (PAGFP) or orange fluorescent protein (PAtagRFP). In contrast, dSTORM exploits the spontaneous photo-switching of fluorescent dyes such as ATTO655 in the reducing environment of the cytosol, thus providing a third colour for live-cell triple colour SR imaging.

MATERIALS AND METHODS

To detect the multiple fluorescent signals originating from both dSTORM and FPALM at the plasma membrane, we employed TIRFM for SR imaging on three colour channels simultaneously. Detecting fluorescence from single molecules demands an exceptionally high signal-to-noise ratio, with maximum light yield and minimal background. We found that the optical design of our microscopy platform based on the Olympus xcellence cell^tirf system achieves minimal photon loss whilst retaining uniform sample illumination, even with the high laser powers required for localisation microscopy. Using our triple-colour SR approach, time-lapse imaging revealed rapid nanoscale dynamics of both the cytoskeleton and the two cell surface cytokine receptor subunits IFNAR1 and IFNAR2 in live cells under physiological conditions.

TRACKING CYTOKINE RECEPTOR LOCALISATION USING FPALM

Various photoactivatable and photoswitchable fluorescent proteins have been employed successfully for FPALM [6-10]. Multicolour FPALM imaging, however, is currently limited by the choice of photoactivatable proteins. Successful combination of PAGFP and PAtagRFP for dual-colour SR imaging has been demonstrated [10], which we employed for labelling IFNAR1 and IFNAR2, respectively.

RESOLVING THE CYTOSKELETON USING DSTORM

Overcoming the limitation of spectral overlap, we combined dual-colour FPALM imaging with dSTORM to observe either the cortical actin cytoskeleton, or clathrin-coated pits at the plasma membrane. We used the red fluorescent dye ATTO655, which is particularly susceptible to photooxidation, and for which efficient photoflipping in the cytoplasm of living cells has previously been demonstrated [11-13]. A key challenge for its application in live-cell imaging, however, is the specific labelling of target proteins in the cytoplasm. For fast and efficient exchange of photoactivatable green fluorescent protein (PAGFP) or orange fluorescent protein (PAtagRFP). In contrast, dSTORM exploits the spontaneous photo-switching of fluorescent dyes such as ATTO655 in the reducing environment of the cytosol, thus providing a third colour for live-cell triple colour SR imaging.

Figure 2

Resolving the actin cytoskeleton at the plasma membrane in vivo with dSTORM super-resolution microscopy. Images captured with TIRFM using the Olympus xcellence cell^tirf system. (a) Live HeLa cells stably transfected with Lifeact–GFP–HaloTag after staining with HTL–ATTO655. Much greater detail is resolved between the GFP and ATTO655 channels. (b, c) Zooming in on the marked region of interest (ROI), comparing dSTORM imaging (red) with the diffraction-limited GFP channel (green; scale bar: 2 µm). (b, c) Zooming in on the marked region of interest (ROI), comparing dSTORM imaging (red) with the diffraction-limited GFP channel (green). (a) Live HeLa cells stably transfected with Lifeact–GFP–HaloTag after staining with HTL–ATTO655. Much greater detail is resolved between the GFP and ATTO655 channels. (b, c) Zooming in on the marked region of interest (ROI), comparing dSTORM imaging (red) with the diffraction-limited GFP channel (green; scale bar: 2 µm). (d,e) Cross-sections through actin filaments shown in panels c and b, respectively (dSTORM: red line; GFP: green line). [5]
resulting from dSTORM imaging can be seen in Figure 3a. By applying time-lapse SR image analysis, clathrin-mediated endocytosis events could be discerned on the nanoscale (Figure 3b).

TRIPLE-COLOUR SR IMAGING
For triple-colour SR imaging, IFNAR1-PAGFP and IFNAR2-PAtagRFP were transiently expressed together with either CLC or lifeact fused to the HaloTag. After staining with HTL–ATTO655, we then acquired triple-colour images using TIRFM on the Olympus xcellence cell^tirf system.

An Olympus UAPO 150x TIRFM objective (NA 1.45, working distance 0.08 mm) was used for achieving maximum power density required for dSTORM. Images were acquired with an Andor Technology Xon DU897D EMCCD camera at 512×512 pixel resolution and at a frame rate of 30 Hz.

With repeated cycles of photoactivation at λ = 405 nm, and sequential excitation at λ = 488, 568, and 647 nm, we captured the IFNAR1-PAGFP, IFNAR2-PAtagRFP and the ATTO655 signals simultaneously on all three channels.

RESULTS
Developing a triple-colour SR imaging technique allowed us to visualise the sub-microscopic organisation of the type I interferon receptor in the context of the actin structure in live cells, achieving a resolution of 25 nm on all colour channels.

PROTEIN MICROCOMPARTMENTS
Using dual-colour FPALM imaged with TIRFM, our study initially revealed a heterogeneous distribution of both IFNAR1 and IFNAR2 receptor subunits, with partial co-clustering of both subunits occurring within sub-microscopic domains (Figures 4 a, b).

To probe this distribution further and investigate the role of the membrane, CLC–HaloTag or Lifeact-GFP-HaloTag were transiently co-expressed alongside the receptors. While only weak co-localisation with CLC–ATTO655 was found, we did instead observe distinct nanostructured co-organisation of the receptors and the actin cytoskeleton (Figure 4c).

Strikingly, IFNAR1 and IFNAR2 frequently co-localised at the periphery of actin structures (Figure 4d), suggesting an interaction with actin-associated proteins. Interestingly, cytoskeletal association has previously been suggested to be involved in type IFN receptor activation [14], but further experiments are needed to characterise these interactions in more detail.

BEYOND THE PLASMA MEMBRANE
We conducted our studies in the HeLa cell plasma membrane, looking at how its organisation coincides with receptor
localisation. Complementary research has shown that dynamic sub-microscopic organisation also plays a role within mitochondrial membranes [15]. At the University of Osnabrück, Professor Karin Busch and her group employed SR microscopy with a similar HaloTag system, and have also discovered dynamic microcompartmentation within the mitochondrial inner membrane. By simultaneous analysis of protein diffusion, they found this nanoscale organisation restricted the diffusion of protein complexes such as ATP synthase [15].

CONCLUSIONS

With submicroscopic membrane organisation found to be important in both regulating the transduction of cellular signalling cascades on the outside of the cell, as well as protein diffusion within organellar membranes, there is a clear niche for live-cell SR microscopy. Using advanced microscopy equipment such as our TIRF system has allowed us to push the boundaries of resolution, and establish a novel SR imaging approach so that we may truly understand the functional role of nanoscale membrane organisation and dynamics.

REFERENCES

Professor Jacob Piehler received his PhD from the University of Tübingen. During his postdoctoral research at the Weizmann Institute in Israel, he studied interferon interaction with IFNAR2, and as independent group leader at the Goethe-University, Frankfurt, he established techniques for investigating cytokine receptor assembly. Appointed as a Professor of Biophysics at the University of Osnabrück in 2008, he continues his interest in the structure, dynamics and signalling of cytokine receptors.

**Abstract**

Cell signalling is regulated by submicroscopic features of the plasma membrane, including the actin cytoskeleton and clathrin-coated pits. Total internal reflection microscopy is ideal for studying this, but resolution is limited to approximately 200 nm. This article explains how, at the University of Osnabrück, we have established a triple-colour super-resolution imaging approach to attain an average resolution of just 25 nm. Combining direct stochastic optical reconstruction microscopy (dSTORM) with fluorescence photoactivation localisation microscopy (FPALM), we revealed rapid nanoscale organisation and dynamics of both the cytoskeleton and two cell surface receptor subunits in vivo.

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