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Near-field scanning optical microscopy in liquid for high resolution single molecule detection on dendritic cells

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Abstract Clustering of cell surface receptors into microdomains in the plasma membrane is an important mechanism for regulating cellular functions. Unfortunately, these domains are often too small to be resolved with conventional optical microscopy. Near-field scanning optical microscopy (NSOM) is a relatively new technique that combines ultra high optical resolution, down to 70 nm, with single molecule detection sensitivity. As such, the technique holds great potential for direct visualisation of domains at the cell surface. Yet, NSOM operation under liquid conditions is far from trivial. In this contribution, we show that the performance of NSOM can be extended to measurements in liquid environments using a diving bell concept. For the first time, individual fluorescent molecules on the membrane of cells in solution are imaged with a spatial resolution of 90 nm. Furthermore, using this technique we have been able to directly visualise nanometric sized domains of the C-type lectin DC-SIGN on the membrane of dendritic cells, both in air and in liquid.

Keywords: Near-field scanning optical microscopy; Single molecule detection; Sub-diffraction limit; Cell surface distribution; Microdomain; DC-SIGN

1. Introduction

The organisation of proteins into micro- or nanodomains in the cell membrane plays an important role in cellular function [1]. Resolving the exact size, structure and composition of these domains is important in order to understand their specific function. The combination of a high spatial resolution technique to resolve individual domains, together with single molecule detection sensitivity to determine the composition of the domain, is therefore required. Immunogold labelling in combination with transmission electron microscopy has the potential to offer both high resolution and insight into domain composition [2–4], however only very thin parts of a dried sample can be imaged. Furthermore, dehydrating cells results in compaction of the cell membrane, increasing the possibility to artificially induce protein aggregation, a clearly unwanted artefact when studying membrane domain organisation.

To date, fluorescence microscopy remains as the most widely used technique for live cell imaging. Membrane components can be directly visualised via specific antibodies or direct fusion with a green fluorescent protein (GFP) family member [5]. Single molecule detection on cell membranes has been demonstrated using confocal, widefield epifluorescence and total internal reflection microscopy [6]. In particular, the last two techniques have allowed the monitoring of protein dynamics revealing differences in the lateral diffusion of membrane proteins and strongly supporting the hypothesis of compartmentalisation within the cell membrane [7]. Unfortunately, these techniques are diffraction limited, with a resolution >300 nm for the visible regime, excluding direct visualisation of domains smaller than this value. Furthermore, the concentration of fluorescent molecules has to be reduced artificially in order to observe individuals.

There are two possible routes to break the diffraction limit. One way concerns the use of point spread function engineering [8], where stimulated emission depletion microscopy has already shown single molecule detection sensitivity [9]. Applicability of this technique at the single molecule level on cells and over the full visible spectrum is still awaiting. The second approach is near-field scanning optical microscopy (NSOM), where a subwavelength aperture probe is scanned in close proximity to the sample. So far, this is the only technique that combines surface sensitivity, single molecule detection, and nanometric (<90 nm) optical resolution together with simultaneous topographic information [10]. Therefore, NSOM is an ideal technique to study the organisation of the plasma membrane in detail [11], while taking full advantage of all available fluorescence labelling methods, including the use of GFPs.

The most relevant cell membrane studies using NSOM include localisation of host and malarial proteins on fixed mouse fibroblasts [12], the imaging of membrane lipids and proteins on fibroblast [13], the distribution of major histocompatibility complexes I and II [14] and visualisation of individual GFP fused to integrins on fibroblasts [15]. More recently, single

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Abbreviations: NSOM, near-field scanning optical microscopy; DC, dendritic cell; DC-SIGN, DC-specific ICAM-3 grabbing nonintegrin; PBS, phosphate buffered saline

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molecule studies on complete cells using NSOM have been performed (B.I. de Bakker, in preparation). However, all of these studies were performed on fixed and critical point dried samples mainly because of the difficulties in reliably regulating the distance between tip and the soft cell surface while operating in physiological buffers. Although important biological information can be extracted from NSOM on dry biological samples, these results are always subject to potential drying artefacts [16].

Recently, we have introduced a diving bell concept which enables easy and reliable regulation of the tip–sample distance in liquid environments. Interaction forces exerted on the sample are below 300 pN [17]. Here, we show for the first time detection of individual fluorescent molecules on a cell membrane in solution and with nanometric spatial resolution. To demonstrate the potential of the technique for cell membrane studies, we have investigated the spatial organisation of the transmembrane protein DC-SIGN on immature dendritic cells (imDC) in solution. ImDCs are crucial for our immune system, since they bind efficiently to pathogens via surface receptors. The recently identified surface receptor DC-SIGN is a C-type lectin exclusively expressed on DCs and plays important and distinct roles during the immune response [18,19]. Near-field fluorescence imaging in liquid shows distinct fluorescence spots of ~100 nm in diameter and different brightness spread randomly on the surface of the immature DC. These results on immature DCs in solution confirm our previous notion that DC-SIGN is clustered into nanometric sized domains on the membrane of critical point dried immature DC [4]. The sizes of the domains are ~100 nm, well below the diffraction limit of light, and thus not resolvable even with the best confocal microscope. Liquid operation of NSOM opens the way to directly visualise and quantify the size and composition of membrane domains, like lipid rafts [20], in solution.

2. Materials and methods

2.1. Technique: NSOM in liquid

The core of a NSOM is the fibre probe which is raster scanned over the sample surface. The obtainable lateral optical resolution is determined by the size of the aluminium coated aperture (typically ~90 nm) used to excite the sample. To obtain this resolution, the probe is kept in close proximity (<10 nm) to the sample surface using a piezo-electric force sensing element (tuning fork), oscillated at 32 kHz [21]. Height feedback is performed by keeping the phase difference between the driving excitation and the tuning fork signal constant [22,23]. In this way, optical and topographic maps of the surface are created simultaneously. To operate the NSOM in liquid, we use a diving bell concept [17] which ensures that the tuning fork is vibrating in air, while the tip is immersed in liquid. This enables reliable operation in liquid, while the force exerted on the sample is below 300 pN. Our home built combined confocal/NSOM microscope equipped with the tuning fork diving bell is schematically shown in Fig. 1.

The proteins of interest are fluorescently labelled with Alexa-647 (see Section 2.2) and excited using the 647 nm line of an argon/krypton-ion laser (CW, Spectra-Physics). In confocal mode, circularly polarised excitation light is reflected by a dichroic mirror (650 DRLP Omega Optical) and focused onto the sample using an oil immersion objective (100×, 1.3 NA). In NSOM mode, the sample is excited via the fibre probe (aperture ~90 nm). The emitted fluorescence is collected and spectrally separated from the excitation light using a 665 nm long pass filter. A polarising beam splitter is used to split the signal into two perpendicular polarisation components. Both signals are sent to photon counting avalanche photodiodes (APDs). The fluorescence images generated in this way reflect the in-plane orientation of each single fluorescent molecule.

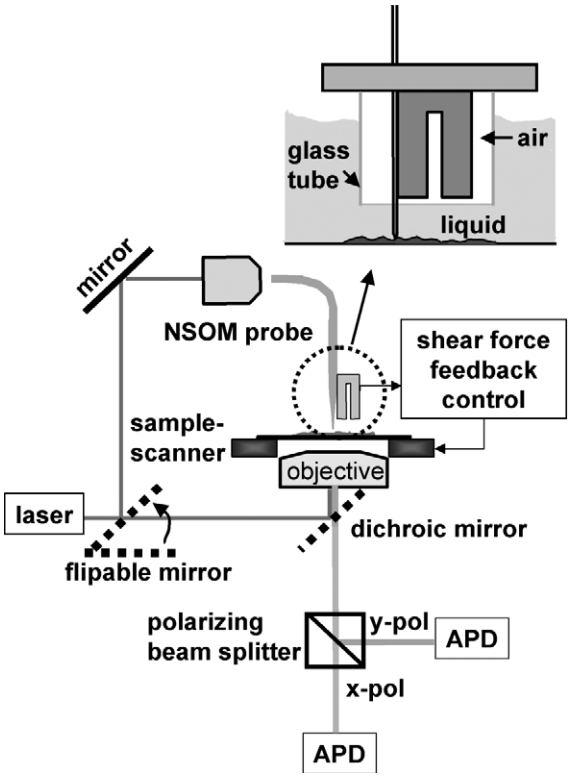

Fig. 1. Schematic diagram of the combined confocal/NSOM setup. The ipable mirror enables easy switching between the NSOM and confocal mode of operation. With the diving bell (see inset) only the tip is immersed in liquid, while the tuning fork sensor is vibrating in air [17].

2.2. Sample preparation

Immature DCs were cultured from healthy human blood monocytes in the presence of interleukin-4 and GM-CSF (500 and 800 units/ml, respectively) for six days to obtain immature DC [18]. The DCs were stretched on a poly-L-lysine coated coverslip for 1 h at 37 °C, rinsed three times with phosphate buffered saline (PBS) and fixed with 1% paraformaldehyde in PBS for 15 min at room temperature. The specimens were washed three times in PBA (PBS, containing 0.5% bovine serum albumin and 0.01% sodium azide). The cells were then incubated with primary monoclonal antibodies against DC-SIGN in PBA (10 μg/ml) for 60 min at room temperature. After three washing steps with PBS, a second incubation was performed for 60 min at room temperature, with an Alexa647 labeled goat antimouse IgG antibody (GαM-Alexa647) in PBA (10 μg/ml), allowing fluorescent detection of the anti-DC-SIGN antibody. Labeled samples were washed three times in PBS and post fixed in 1% paraformaldehyde. The wet samples were stored in PBS containing 1% paraformaldehyde. As a reference, critical point dried samples were also prepared, as described by Cambi et al. [4].

3. Results and discussion

3.1. NSOM versus confocal on dried immature DCs

To demonstrate the advantage of NSOM over confocal microscopy, we first examined the distribution of DC-SIGN on the membrane of a critical point dried immature DC in air. Some typical images are shown in Fig. 2. We select individual cells using a CCD camera and bright field illumination (Fig. 2A). The selected immature DCs are imaged in confocal mode with a typical scan size of 20×20 μm. A region of interest showing fluorescence contrast is selected for further confocal (Fig. 2B) and near-field investigation (Fig. 2D).

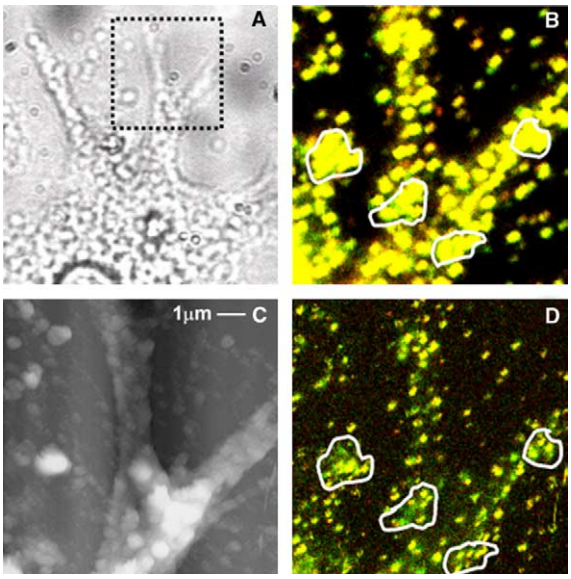

Fig. 2. A selected area of the dendritic cell of interest (A) is imaged in confocal mode (B). The same area is imaged with NSOM resulting in a topographic image (C) with a corresponding high resolution optical image (D). In the high resolution image individual "spots" can be resolved in the highlighted areas, whereas the same areas appear fully packed upon confocal investigation.

Simultaneous with the near-field optical information, we obtain topographic information as shown in Fig. 2C.

The fluorescence intensity in the confocal image is high, due to both high expression levels and close packing of DC-SIGN on the membrane. The resolvable fluorescent "spots", all have a similar diffraction-limited size of ~300 nm. Comparing the NSOM fluorescent image of the same area, it is clear that the high resolution enables the identification of individual "spots" in areas apparently fully packed upon confocal investigation. Furthermore, while the "spots" resolved in the confocal image vary only in intensity, the NSOM optical image reveals "spots" differing both in size and intensity. This observation is consistent with the work of Cambi et al. [4] and a detailed study using NSOM in air (B.I. de Bakker, in preparation), both working with critical point dried immature DCs.

3.2. NSOM on immature DCs in liquid

To investigate the organisation of DC-SIGN on immature DCs in solution, we have used the NSOM equipped with the diving bell for liquid operation. Just before imaging microscope slides containing the cells were washed with PBS, mounted in a liquid cell and covered with ~1 ml of PBS solution. In Fig. 3A, the resulting topography is shown. The line trace in Fig. 3B demonstrates that the feedback is reliable and stable, with the tip following the contours of the cell. Fine dendrites, with a height of 100 nm, are apparent in the topographic image at the cell edges, while the central part of the cell, where the nucleus resides, can reach several microns in height. This is consistent with measurements using tapping mode atomic force microscopy on living DCs (A. Cambi, unpublished observations). The appearance of the small dendrites in the topography also demonstrates the gentle imaging capabilities of the technique.

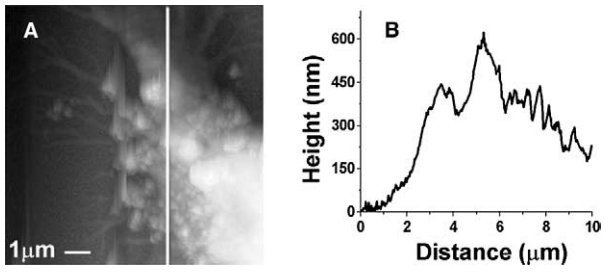

Fig. 3. The topography image (A) of an immature dendritic cell in buffer solution obtained with tuning fork shear force feedback. The white line in (A) indicates the position of the line trace as shown in (B).

After selecting a region of interest with confocal microscopy, a high resolution image of the same area is performed with NSOM. Fig. 4 shows the fluorescence image in confocal (4A) and NSOM (4B) modes. The colour in the optical images reflect the in-plane orientation of the emission dipole of the excited fluorophores. The colour scale ranges from red to green, reflecting a 90° change in in-plane orientation. A yellow colour corresponds to an equal amount of photons on both APDs reflecting either the presence of multiple emitters in the excited volume, or a single emitter with 45° in-plane or an out of plane orientation.

Comparing the confocal image in Fig. 4A with the NSOM optical image in Fig. 4B, the difference in lateral resolution is striking. In the confocal image, the dendritic cell appears to be completely covered with proteins. Individual molecules are only visible outside the cell region, due to unspecific binding of labelled antibodies to the substrate. In contrast, using near-field excitation we can not only resolve individual "spots" but

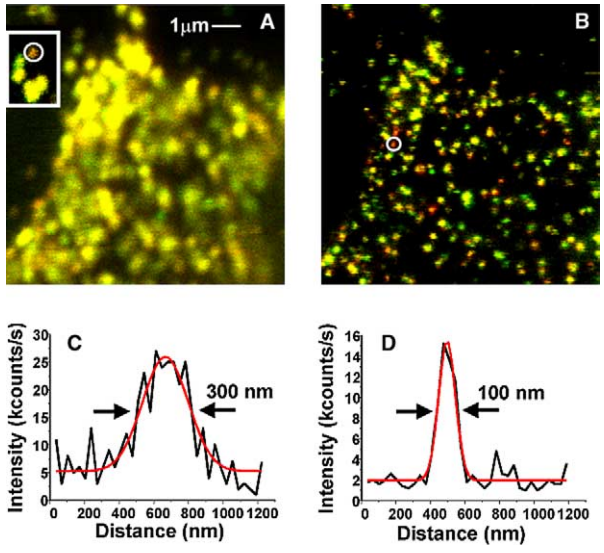

Fig. 4. Fluorescence image of a dendritic cell in buffer solution collected in confocal mode (A) and NSOM mode (B). The white circles indicate the position of the line traces through single molecules (recognised by their unique dipole emission, red or green colour) in (C) and (D). These line traces reflect the superior resolution of NSOM (100 nm) in (D) as compared to the diffraction limited confocal resolution (300 nm) in (C). The intensity scale is locally changed in the confocal image (indicated by the white box in (A)) in order to visualise the individual molecule.

also individual molecules on the cell surface. The presence of a well-defined polarised emission (colour of most spots is green or red) is indicative for unique dipole emission and thus single molecule detection. It is important to note that due to the smaller excitation volume of NSOM the contribution of the fluorescence background from the cytosol is approximately ten times lower as compared with confocal illumination.

The line traces through individual molecules in Fig. 4C (on the glass substrate) and Fig. 4D (on the cell membrane) demonstrate the superior resolution of NSOM (100 nm) with respect to the diffraction limited resolution of confocal microscopy. In both cases, the resolution is measured as the full width at half maximum of a Gaussian fit to the fluorescence profile. To our knowledge, these images show for the first time single molecule detection on a cell membrane in solution with nanometric resolution.

3.3. Size and intensity of domains on cells in liquid

Fig. 5A shows again a NSOM image of an imDC containing labelled DC-SIGN proteins. To obtain this image, we use an excitation intensity of 400 W cm^{-2} and an integration time per pixel of 10 ms, for the 256×256 pixel image. It is clear that the "spots" differ in brightness and size. The number of molecules in each fluorescent "spot" is related to its brightness. This brightness is determined by adding all photon counts within a contour of ~15% of the peak intensity and subtracting the background in the immediate vicinity of each "spot". The resulting brightness distribution for 87 domains is shown in Fig. 5B. The photons detected per domain range from less than 1 kCounts to almost 60 kCounts, while the photons being collected from a single molecule are approximately 0.5 kCounts. The broad and large deviation from the single molecule value indicates a wide spread in the number of Alexa647 molecules per spot, strongly supporting the notion that DC-SIGN is clustered into domains on the membrane of immature DCs in solution.

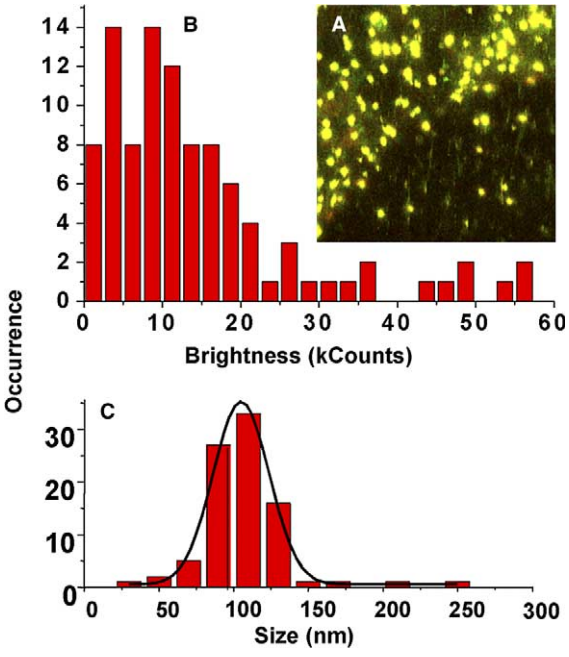

Fig. 5. (A) Near-field image of an imDC in buffer solution, excited at 400 W cm^{-2}. The bright "spots" in (A) vary in size and intensity. This is reflected by the intensity distribution in (B) and the size distribution in (C).

The size of every individual domain can be determined by fitting the measured intensity with a 2D Gaussian profile. The spot size is then defined as the full-width at half-maximum (FWHM) of the fit. The resulting size distribution for 87 "spots" is shown in Fig. 5C. The size distribution peaks at 105 nm indicate that measured average size of the domains is mainly determined by the size of the (90 nm) aperture and, therefore, well below the diffraction limit of light.

The biological role of DC-SIGN clustering is extensively discussed by Cambi et al. [4]. The organisation of DC-SIGN into domains resulted crucial for dendritic cells to regulate their virus binding capacities. Cambi et al. [4] observed DC-SIGN clustering by means of transmission electron microscopy. Here, we confirm that DC-SIGN is organised in sub-diffraction limit sized domains on the membrane of hydrated DCs. The size of the domains on DCs in solution is somewhat smaller than the domain sizes found on dehydrated DCs as reported by Cambi et al. [4] and work in preparation by B.I. de Bakker. This deviation is most probably due to the differences in Section 2.2.

4. Conclusions

In this work, we have demonstrated the detection of individual molecules on the membrane of cells in solution with nanometric optical resolution using NSOM. Furthermore, we have investigated the distribution of DC-SIGN in the membrane of whole, hydrated immature DCs in liquid. We found that DC-SIGN is organised in clusters, with a size of 100 nm or smaller and with a wide spread in molecules per domain, upto a factor of 60. These results are consistent with the observation of ~200 nm sized domains using TEM [4] and NSOM on dried DCs confirming that indeed DC-SIGN is organised into sub-diffraction limit sized domains in the membrane of imDCs.

The application of NSOM in solution will open the way to high resolution live cell imaging. As a scanning probe technique, NSOM is less suitable for monitoring fast lateral diffusion of membrane complexes, however its superb resolution in the z-direction should allow monitoring of exo- and endocytosis processes with high speed and sensitivity.

Currently, we are investigating the co-localisation between membrane proteins and lipids associated to lipid rafts [20]. Until now, possible association between lipid rafts and membrane proteins could only be studied using biochemical methods such as detergent extraction in combination with flotation assays or confocal techniques based on co-patching [24]. With the single molecule detection capabilities and high resolution of NSOM, we should be able to directly visualise lipid rafts and quantify their size and composition.

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