Parallel two channel near and far field fluorescence microscopy

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ABSTRACT

A two channel fluorescence microscopy technique for surface-generated fluorescence is presented. The realized fluorescence microscope allows high resolution imaging of aqueous samples. The core element of the instrument is a parabolic mirror objective used to collect the fluorescence at large surface angles above the critical angle of the water/glass interface. An aspheric lens, incorporated into the solid parabolic element, gives diffraction limited laser focusing and collects the fluorescence at low angles with respect to the optical axis. By separated collection of the fluorescence emitted into supercritical and sub critical angles, two detection volumes strongly differing in their axial resolution are generated at the surface of a glass cover slip. The collection of supercritical angle fluorescence (SAF) results in a strict surface confinement of the detection volume whereas collecting below the critical angle allows gathering the fluorescence emitted several microns deep inside the sample.

Keywords: geometrical optics, fluorescence microscopy, confocal microscopy, total internal reflection, single molecule

1 INTRODUCTION

Confocal microscopy is a widely used technique for single molecule detection due to its excellent signal to background ratio and the high count rate gathered per molecule. A well established confocal method to study biomolecular interactions is solution is fluorescence correlation spectroscopy. However, many bioanalytical applications such as heterogeneous immunoassays, DNA assays and cell studies on solid substrates involve the fluorescence detection in close proximity to interfaces. On this account, total internal reflection (TIRF) microscopy has been established for investigating surface fluorescence without background interference from unbound, freely diffusing molecules in solution. In this technique the illumination above the critical angle produces an evanescent field that expands into the sample volume up to several hundreds nanometers. Fluorescence imaging at water/glass interface has been obtained using prism-type [1-2] and objective-type [3-4] TIRF microscopy. One impediment for combining TIRF excitation with confocal optics is that microscopes objectives of high numerical apertures do not usually perform submicron focusing of the illumination at the surface incident at supercritical angles [5]. As a consequence, the illumination of a large surface area can be unfavorable for some applications because it can increase the photo-bleaching process substantially. To achieve a TIRF excitation of submicrometer size, an optical system is required that works at diffraction limited performance, even at very large illumination angles. On this account, we describe a parabolic mirror objective (PMO) as diffraction-limited high-aperture optics, as required in scanning confocal fluorescence TIRF microscopy. The objective performs as an efficient fluorescence collection optical element at the water/glass interface and simultaneously in the solution. Thus, the PMO objective opens the possibility of recording diffraction limited fluorescence images of the surface and in the bulk solution at the same time. This powerful alternative to TIRF, we refer to as the supercritical angle fluorescence (SAF) collection method [6].

2 SUPERCRITICAL ANGLE FLUORESCENCE EMISSION

In contrast to high angle illumination like in the objective-type TIRF microscopy, the presented optical geometry achieves its surface confinement by collecting the fluorescence at very high angles exceeding the critical angle of total internal reflection (~61° for water/glass interface). The significant surface selectivity is obtained on the basis of dipole emission properties near a dielectric interface [7]. The angular distribution of a dipole radiation near the interface is a superposition of the traveling and evanescent waves. Propagating waves can be recorded in the far field under an angle of at most the critical angle. When the emitting dipole is located in the close proximity of a dielectric interface, the near field evanescent waves can couple to the medium with higher refractive index at angles above the critical angle. This supercritical angle fluorescence emission mode can be transformed into propagating waves using the PMO objective, and subsequently recorded in the far field. Figure 1 shows the emission direction of fluorophores with isotropically oriented dipole moments at the water/glass interface, which is in biological microscopy the case of highest practical relevance. The calculations of the angular distribution of radiation for emitters located directly at the surface and located at a surface distance equal to a third of the emission wavelength are depicted. It is obvious that only surface-bound emitters radiate substantially energy above the

critical angle and amounts to ~34% of the dipole emission radiation in all directions. As the emitting dipole diffuses from the dielectric interface its radiant emission into the glass subsurface is limited to angles at most the critical angle. Thus, it is possible to design a parabolic interface to detect only the supercritical angle emission. Simultaneously, using a small aspheric lens incorporated into the parabola element a sub-critical angle excitation and detection becomes possible.

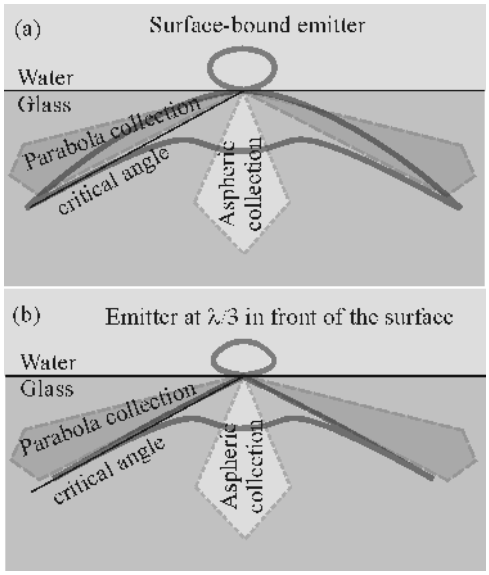

Figure 1. Polar plots of the emission direction of isotropically orientated fluorophores (bold line), (a) a surface distance of zero, (b) a surface distance equal to a third of the wavelength. The dashed line shows the collection angles of the two detection channels (see text).

2.1 Parabolic mirror objective description

A parabolic mirror (PMO) made from Zeonex (refractive index=1.5247) with a focal length of 7.1 mm and an external diameter of 47.5 mm was used. The outer diameter of the mirror limits the minimum angle collection to 62°, which is above the critical angle, where as an opaque aperture beneath the PMO limits the fluorescence collection under 75° (see Fig. 2). The limit imposed by the opaque disk facilitates a drastic reduction of the background as only 2% of the total fluorescence is emitted above 75°. The PMO redirects the near field emitted into the lower half-space in a parallel beam via total internal reflection at the parabola/air interface. Subsequently, the supercritical emitted fluorescence is refocused using a large aperture lens onto the sensitive area of a confocal detector (parabolic channel). As an excitation path, the laser beam is focused at the sample surface by an aspheric lens which is embedded in the parabolic mirror. The same aspheric lens is used to collect the sub critical fluorescence in a range of angles between 0° and 24° (0.62 numerical aperture) which amounts to ~6% of the overall emitted fluorescence (aspheric channel). The back reflex of the aspheric lens gives the focus position at the water/glass interface using a CCD camera. In other words, the aspheric channel resembles the normal confocal geometry whereas the parabolic channel acts as an inverted confocal TIRF [8].

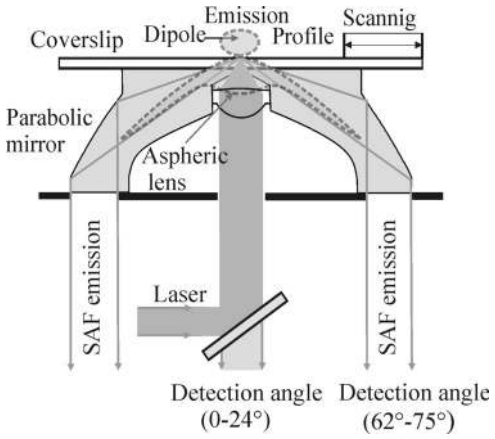

Figure 2: Schematic representation of the parabolic mirror objective with the two detection channels (parabolic and aspheric) used for super- and sub-critical angle geometry.

Scanning images sample was achieved by sliding the cover slip mounted on a metallic holder with a microscope stage over the front face of the PMO. All optical components are mounted below the cover slip leaving a free access to the sample from above.

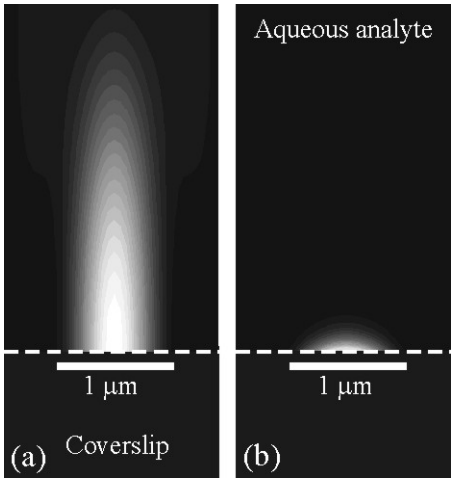

Figure 3. Detection volumes: (a) low angle collection (~2.5 μm), and (b) high angle collection (~130 nm).

Due to a significant contact area between the cover slip and the upper surface of the PMO via the microscope oil film, the capillary forces keep the vertical position of the glass cover slip constant for sizeable lateral movements. Thus, scanning large areas is possible without a dynamic focusing control of the objective lens as is often the case in confocal microscopy. Figure 3 shows the spatial collection efficiency for both detection channels calculated by ray tracing simulation. The two fluorescence optical detection channels have detection volumes of completely different

shape along the optical axis. The aspheric channel (Fig. 3a) gives access to the light emission from deep inside the analyte solution whereas the parabolic channel (Fig. 3b) acquires the emission of fluorophores located very close to the glass/water interface. Therefore, the PMO can give information of surface-bound and freely diffusing fluorescent molecules simultaneously. The $1/e^2$ intensity drop off in the axial direction was calculated for the aspheric channel to be ~2.5 μm, and for the parabolic channel ~130 nm, correspondingly.

The fluorescence detection efficiency of the system depends on the fraction of the emitted light in the range of captured angles and the transmission efficiency of the other optical elements employed in the system. Hence, for the parabolic channel the detection efficiency is found to be 16%, while for the inner aspheric channel the overall detection efficiency amounts to approximately 2.5 %.

2.2 Parabolic mirror objective scanning confocal microscopy

We used a low concentration solution of fluorescent beads (20 nm diameter) to investigate experimentally the imaging performance of the two channels microscope. Figure 4 (panels a, b) shows scanning confocal intensity image of fluorescent beads solution (10 μl) on a glass cover slip, recorded with both channels, simultaneously. The surface was scanned from top to bottom and from left to right with a spatial resolution of 156 nm per pixel. The scanning area was 75x75 μm with 1 ms intensity integration time per pixel.

The panel (a) in Figure 4 was recorded with aspheric channel and panel (b) with parabolic channel, respectively. Due to different detection volumes along the optical axis, striking different images of the same area are shown for the two detection channels (see Fig. 3). The aspheric channel shows fluorescence image of beads adsorbed at the surface glass coverslip (round spots) and, bright vertical lines attributed to freely diffusing beads or larger cluster beads into the detection volume. On the contrary, the parabolic channel, i.e. supercritical angle detection, shows only the image of beads adsorbed on the glass surface and remarkably rejects the fluorescence contribution of the diffusing particles. A signal-to-background ratio of up to 55 was measured for the parabolic channel while a value of 10 was obtained for the aspheric channel, respectively. Thus the signal-to-background ratio measured was in very good agreement with the calculated value of five between the two detection channels. The intensity difference between the two images is mainly due to a much higher light collection efficiency of the parabolic mirror at the glass/water interfaces. Thus, the surface bound fluorophores can be detected with a high signal-to-background ratio without interferences of fluorescence from diffusing molecules in bulk solution.

The lateral resolution of the microscope is limited by the size of the laser focus at the glass/water interface and should be identical for both detection channels. Consequently, the focusing optical performance of the aspheric lens objective is the limiting factor for the lateral resolution assuming the fact that its back aperture plane is overfilled with a well collimated Gaussian beam. A Gaussian fit of one bead image gives a full-width at the half-maximum (FWHM) of 510±5 nm in the horizontal plane for the aspheric inner channel, and 515±5 nm for the parabolic channel, respectively. Compared with conventional through-objective TIRF systems, the SAF microscope produces a significantly smaller detection volume for fluorescent analyte molecules.

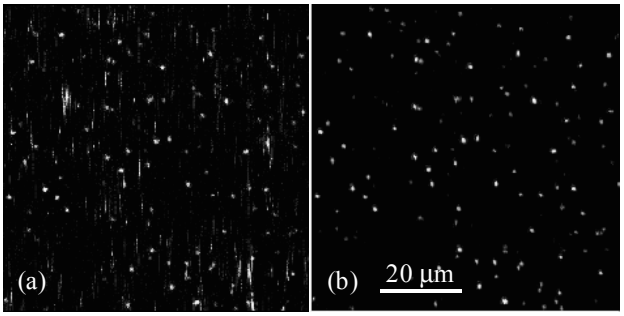

Figure 4. Fluorescence intensity image of beads in solution (75x75 μm). (a) aspheric channel and (b) parabollic channel.

To determine the sensitivity of the PMO objective for detecting single molecules in solution or adsorbed on the surface, a test sample of α-endorphin-Cy5 peptide conjugate (2 nM concentration, 20 μl) conjugate has been used. Figure 5a shows bright spots attributed to single endorphin-dye conjugate molecules nonspecifically adsorbed on the glass surface. Scanning image was obtained with 1ms integration time per pixel and 156 nm spatial resolution. The single molecule image exhibits diffraction limited spots with a signal-to-background ratio up to 25. Some pixels image appear dark within the bright spots, specifically for single molecules that undergo blinking or single stop photo bleaching which is a clear criterion for single-chromophore systems. The freely diffusion molecules do not affect the scanned image in the parabolic channel as their fluorescence contribution to the detected intensity is merely at the background level. On the other hand, the image recorded with the aspheric channel that extends deeper into the solution is saturated at nanomolar concentration (data not shown). Figure 5b shows single molecule bursts of peptide-dye conjugates resembling a hindered diffusing at the interface. Data collected in this manner allows the observation of characteristic photon bursts of single molecules diffusing through an open volume or non-specifically attached on the glass surface. As a consequence, due to the small detection volume of the parabolic channel very confined at the water/glass interface, the short temporal bursts exhibited in Figure 5b are most likely from single peptide-dye conjugates undergoing reversible adsorption on the glass

surface. At nanomolar concentrations the fluorescence detected with the inner aspheric channel resemble fluctuation due to more than one molecules diffusing into the detection volume. However, single molecule can be detected also with the inner channel at lower concentration as mentioned in ref 9.

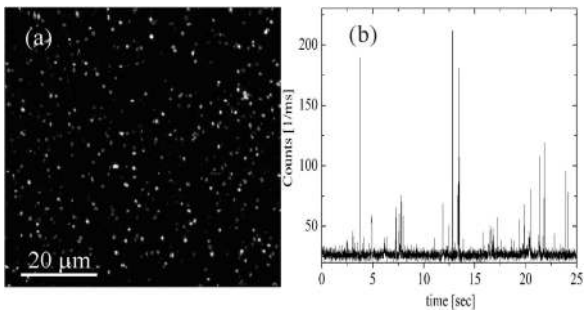

Figure 5. Single endorphin-dye conjugate detected with the parabolic channel. Panel (a) shows peptide molecules in aqueous solution adsorbed on a glass surface. Panel (b) shows single molecule bursts of peptides-dye conjugate resembling a hindered diffusing at the interface.

2.3 Conclusion

We shortly described the design and construction of a new confocal fluorescence microscope as an alternative to the widely used objective-type TIRF systems. The supercritical angle detection technique is advantageous over epifluorescence microscopes when it is applied to the detection in complex environments where one encounters background fluorophores at distances greater then the evanescent depth. The possibility to illuminate the sample at low surface angles offers a simultaneous detection in the analyte solution. Such excitation approach is technically easier and gives an improvement over objective-type TIRF which requires very high angles for excitation. In practice for example, with a 1.4 numerical aperture objective only 2.8% of its aperture can be used for TIRF illumination. Hence, correctly coupling the laser into the objective back focal plane is technically challenging. On the other hand, high index glass cover slips and special microscope oil are required for the 1.65 numerical aperture objectives. The two channels microscope described uses only disposables currently used in fluorescence microscopy, like common cover slips and regular microscope oil. Furthermore, it is difficult to produce an excitation spot of submicron dimensions using various TIRF methods, whereas with the geometry presented diffraction limited resolution is straight forward. So far, we have only used a commercially available aspheric lens with a numerical aperture of 0.62 for sample illumination. Consequently, the focusing performance of the aspheric lens is the limiting factor for the optical resolution of the microscope and the detection efficiency of the inner channel. A numerical aperture of the aspheric lens objective higher than 1.0 will give a better volume restriction (~1 fl or less), optical resolution, and a superior collection efficiency for the inner channel which is imperative in the case of single molecule detection. Currently, we are exploring a smaller geometry of the PMO explained that will fit in any turret of normal epifluorescence microscopes. The implementation to any epi-fluorescence geometries will be straightforward without any modification in the frame of commercial microscopes. The only technical approach needed is the separation of the two detection channels after the dichroic cube. This modification can be performed at the exit side fluorescence ports of any up-right microscope by using a 6 mm diameter mirror fixed on the center of an emission filter or a glass window for example. Such small mirrors with an elliptical top surface for 90° reflection are already commercially available. Thus, two perpendicularly emission paths outside the microscope main frame can be obtained easily. By detecting the volume and surface generated fluorescence simultaneously can be of a great benefit in cell biology for example when one encounters fluorophores in the membrane and deeper inside the cell. An excellent signal-to-background ratio at moderate illumination intensity, high resolution imaging, radical reduction of the detection volume along the optical axis, easy handling and stability, make the two channel fluorescence microscope a nice technique for surface fluorescence measurements down to the single molecule level.

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