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# Colour-coded nanoscale calibration and optical quantification of axial fluorophore position

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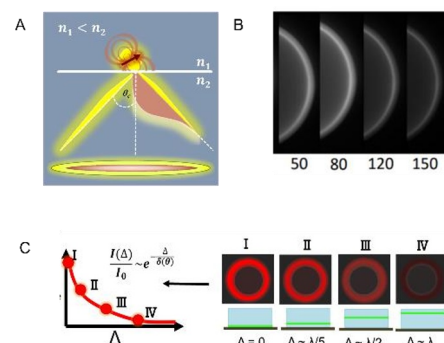
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**Abstract.** Total internal reflection fluorescence (TIRF) has come of age, but a reliable and easy-to-use tool for calibrating evanescent-wave penetration depths is missing. We provide a test-sample for TIRF and other axial super-resolution microscopies for emitter axial calibration. Our originality is that nanometer(nm) distances along the microscope's optical axis are color-encoded in the form of a multi-layered multi-colored transparent sandwich. Emitter layers are excited by the same laser but they emit in different colors. Layers are deposited in a controlled manner onto a glass substrate and protected with a non-fluorescent polymer. Decoding the penetration depth of the exciting evanescent field, by spectrally unmixing of multi-colored samples is presented as well. Our slide can serve as a test sample for quantifying TIRF, but also as an axial ruler for nm-axial distance measurements in single-molecule localization microscopies, supercritical-angle fluorescence, and related super-resolution.

Fluorescence microscopies offering a surface selectivity, like total internal reflection fluorescence (TIRF) microscopy or supercritical-angle fluorescence (SAF) microscopy[1-3] allow to selectively visualize the near-membrane space of live cells while minimally interfering with biological function. By suppressing fluorescence excitation (TIRF) or detection (SAF) from deeper sample regions, they provide the contrast for selectively imaging individual small organelles or even single molecules in live cells. Both techniques can be combined for better surface selectivity.[4]

However, a major problem with near-surface fluorescence intensities is that they are notoriously difficult to interpret in quantitative terms, as reviewed recently by us.[2] While the penetration depth of the near-field exciting TIRF can - in principle - be calculated from wavelength of light, the refractive-index ratio of the substrate and sample, and the incidence angle of the incoming laser beam, neither the beam angle nor the sample index are known with much precision in a typical biological experiment. Additionally, in a real-life experiment, the near-field decay deviates from the expected single exponential, due to optical aberrations, scattering and imperfections, particularly in the mostly used objective-type TIRF geometry, where a high-NA objective is used to guide the laser beam to the reflecting interface. Thus, the precise relationship between TIRF intensities and axial fluorophore distances is generally unknown. This is problematic, as many TIRF applications like axial super-resolution imaging, but also co-localization studies, Förster resonance energy transfer

(FRET), near-membrane fluorescence recovery after photobleaching (FRAP), near-membrane photo-chemical uncaging or photoactivation/switching as well as single-particle tracking (SPT) all require the quantitative interpretation of near-surface fluorescence in terms of axial distance (or, equivalently, illuminated volume).



**Fig. 1.** A. an illustration of an emission of a dipole in proximity to the interface. The emission is highly depended on its axial position, at axial distances of  $\Delta < \lambda$  the fluorescence emission is at super critical angles (SAF), above the critical angle,  $\Theta_c$ . B. a series of measurements of samples, in which the fluorescence layers is at different axial position as is indicated below (from 50nm to 150 nm). The emission is clearly decreases with axial distance. C. expected emission patterns of fluorescence layers at different axial positions from the surface. Theoretically the emission should decay exponentially.

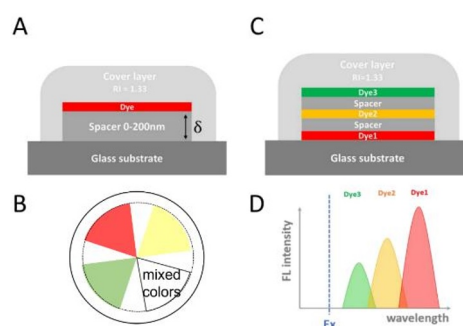
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Quantifying inhomogeneous optical wave fields on a sub-wavelength length-scale is a daunting task. Various approaches and calibration procedures have been devised,[2] but neither a common standard has emerged, nor has any manufacturer implemented a reliable solution for directly measuring the beam angle and the depth penetration of the evanescent wave on a given microscope. Existing techniques are laborious, they use home-made samples and often probe only a single location in the illuminated field of view. Axial (z-) measurements reported in the literature are, in fact, often based on a theoretical, *calculated* penetration depth

Herein we present a standardized nano fabrication sample together with an axial fluorophore distance calibration technique for axial nano-metrology measurements (see Fig. 1).[4]

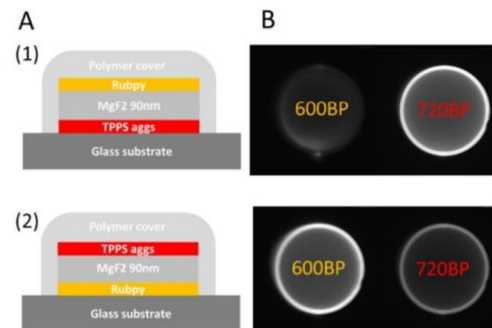
Our samples are based on thin layers and their assembly to sandwiches alternating a transparent spacer layer (with refractive index similar to that of a biological cell) of precisely controlled nm-thickness, a 2-5 nm thin emitter layer and a  $\mu\text{m}$ -thick capping layer of the same polymer.[4] We also developed a technique for producing homogenous, flat and bright emitter layers over large surface areas. Using several of these sandwiches featuring the emitter at different axial distance, one can precisely recover axial fluorophore distances based on the radiation pattern imaged in the back-focal plane (BFP) of the objective as is shown in Fig. 1.

A drawback of this procedure is that it requires a sample change to measure several sandwiches. The current research aims at multiplexing the measurement by producing multi-color, multi-layered sandwiches, from which the penetration depth of the evanescent field exciting TIRF is recovered in a single spectral measurement, followed by color unmixing as is presented in Fig. 2.



**Fig. 2.** A schematic representation of a single-color layer calibration sample. A dye layer (red) is deposited on a nanometric transparent spacer layer (grey) of thickness  $\delta$  and capped with a transparent polymer having the refractive index of water (light grey). B, multiplexing dye layers colour-encodes depth. C, pizza-slice like arrangement of different pure and mixed color segments on a round microscope glass substrate (borosilicate microscope coverglass). D, expected intensity modulation and color mix upon evanescent-wave (TIRF) excitation at 488 nm: the closer the dye to the interface, the more it contributes.

Fig.3. A shows an example samples containing two-colors, multi-layered sandwiches. The BFP imaging of those samples as is presented in Fig.3. b. reveals the penetration depth of the exciting evanescent field from the known axial fluorophore distances in the sample and the measured mixed color. One can clearly see that the respective dye layer closer to the interface is dominating the fluorescence emission, as expected from the near-field confinement of the evanescent wave and vice versa.



**Fig. 3.** A *dual-colour sandwich*. A, schematic representation of 2 complementary dual-color sandwiches featuring a yellow- (Rubpy) and red-emitting (TPPS) fluorophore emitter layer, separated by a 90-nm thin spacer layer and covered with My-133-MC polymer as a protective layer. B, Backfocal plane (BFP), fluorescence images of these samples, taken through the bandpass filters as indicated, upon 488-nm evanescent-wave excitation. Note the clear intensity differences: the respective dye layer closer to the reflecting interface is dominating the fluorescence emission, as expected from the near-field confinement of the evanescent wave. In the lower case (TPPS aggregates on top), the long-tailed emission of Rubpy results in some spectral cross-talk, indicating the need for spectral unmixing.

Further studies will be needed to decode the penetration depth of the exciting evanescent field, by spectrally unmixing the measured color vector and by calculating the relative contribution of the respective dye layers located at different axial distances, as a function of the penetration depth of the optical near field.

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