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Membrane Protein Localization by SpecON

Axial Localization with Nanometer Precision Using Thin Metal-Dielectric Coatings

Spectrally coded optical nano-sectioning (**SpecON**) is a **high-resolution microscopy technique** that translates spatial (position) information of fluorescent markers into spectral (color) information providing a protein localization precision of up to 5-10 nm in live cells. The key element is a thin metal-dielectric coating on a microscope slide. The biocompatible design is such that the distance-dependent spectral "fingerprint" of fluorophores can be used to monitor their relative distance from the coating to study the positions and dynamics of key proteins in cell motility.

The Challenge of Imaging Proteins with Highest Accuracy

Precise protein localization within a submicron (evanescent) layer of excitation would be a powerful tool in the life sciences, particularly for high-resolution applications typically imaged in the popular Total Internal Reflection Fluorescence (TIRF) mode.

Most current approaches are either based on far field point spread function (PSF) engineering [1-3] or axial distance-dependent excitation and/or emission rate modifications [4, 5]. A common drawback, however, is their need to physically scan a sculptured point spread function in the axial dimension, which can make imaging slow and prone to photobleaching or both.

Imaging Principle of SpecON

An alternative approach for enhanced surface fluorescence imaging exploits the interactions of fluorophores with a dispersive reflecting structure to both constrain the effective excitation volume and modify the measureable emission spectra of the fluorophore. The key idea of SpecON is to utilize the position-dependent emission spectrum of fluorophores above a simple biocompatible metal-dielectric thin coating on a typical quartz slide (fig. 2A and B) to deduce the axial fluorophone distribution. The imaging configuration for SpecON (fig. 2) involves an objective lens with a high numerical aperture focusing at the interface of the cell membrane and coating. SpecON exhibits two desirable features: First, highlighting the

fluorescence of labeled membrane proteins in close vicinity to the coating (fig.

2A) and second, spectral nano-sectioning (fig. 2C-E). For the first feature the effective PSF is modified by the coating and on thus probes an axially constrained region above the substrate (fig. 2, A with and B without coating) in somewhat analogous manner to FLIC microscopy (2,3). For the second feature distinct spectral modifications are measured modifications here being defined relative to the fluorophore emission spectrum without the nanostructure. Figure 2C and D show an example of how at two lateral positions in a cell membrane with different axial fluorophore distributions different spectral modifications may be obtained. In this example the resulting data are fitted with a function describing the spectral modification from a normal axial density distribution (fig. 2E left), with the fitting parameters yielding an optimum mean (dashed vertical line, panel E, right) and spread (dotted horizontal line respectively) for the two locations.

In this way, the resulting spectral modification to the total measured fluorescence (fig. 2E, left) can be used to estimate the axial fluorophore distribution within distances of 10-150 nm above the substrate.

What causes the measurable spectral changes on the metal-dielectric coating? Such changes can be largely attributed to the spectral dependence of the reflection from such a nanostructure, yielding either red shifts or blue shifts in the measured emission spectrum depending on the distance. For the following experiments, metal and dielectric layers of approximately ~10 nm and ~7 nm thickness respectively are found to be most effective [6].

Applications and Data Analysis of SpecON

One possible application for SpecON is studying adhesion protein dynamics in cells, which are understood to be localized ~10-100 nm above the substrate-sample interface [7]. SpecON was performed in the geometry shown in figure 3A. A typical fluorescence images of a Alexa488-Paxillin stained 3T3 cell cultured on a coated substrate consisting of Quartz/Silver(12 nm)/Silicon-Nitride(7 nm) is shown in figure 3B. Similar to an image generated by TIRF Microscopy the SpecON image clearly shows a relative emission enhancement of the fluorescently labeled Paxillin in the near-field region above the coated substrate. Spectral analysis at six different locations of the Alexa488-Paxillin stained cell (indicated in 3B) yields the respective axial emitters distributions with the profiles shown in panel C (obtained by applying a Monte-Carlo algorithm) summarized in panel D. LAD_0 and LAD_1 represent the least and the second lowest least absolute deviations of these fits which serve as a measure of the certainty of the deduced axial emitter distribution.

SpecOn can be applied to live cells. In figure 4 we show a false color image of Paxillin-GFP transfected B16-fibroblasts (A-C) where the colors represent the calculated average distance of Paxillin-GFP from the substrate interface at each pixel. Panel A shows the overview; panels (B) and (C) show the depicted areas from (A). Also shown is the time evolution of the calculated substrate-Paxillin distance for the rear (D) and leading (E) edge of an individual cell. We observe periodic fluctuations in the axial Paxillin-GFP position with an average distance of approximately 40 nm for the leading edge, and approximately 60 nm for the rear edge, which is in good agreement with previous super-resolution studies [8,9].

SpecON also provides access to the axial dynamics of filopodia as recently shown in Actin-GFP transfected B16 Melanoma cells [6]. Filopodia often show fast formation and motion [10], so that studying their axial dynamics is challenging. Here, SpecON may lead to more reliable models of cell locomotion.

Despite the fact that SpecON is computationally more demanding than standard confocal or TIRF microscopy, and requires careful coating fabrication, we believe this young approach is highly promising for deciphering complex axial emitter density distributions by a single spectral measurement.

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