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Review

Enhancing single-molecule fluorescence with nanophotonics

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ABSTRACT

Single-molecule fluorescence spectroscopy has become an important research tool in the life sciences but a number of limitations hinder the widespread use as a standard technique. The limited dynamic concentration range is one of the major hurdles. Recent developments in the nanophotonic field promise to alleviate these restrictions to an extent that even low affinity biomolecular interactions can be studied. After motivating the need for nanophotonics we introduce the basic concepts of nanophotonic devices such as zero mode waveguides and nanoantennas. We highlight current applications and the future potential of nanophotonic approaches when combined with biological systems and single-molecule spectroscopy.

1. Introduction: challenges of single molecule fluorescence techniques

Biomolecular multi-component complexes are inherently dynamic and variable, fulfilling an amazing number of functions. Single-molecule techniques are unique in that they are able to reveal the complexity, dynamics, heterogeneity and variability of their structures, biochemical states, mobility and interactions. The degree of information can substantially exceed that of ensemble averaged values [1,2]. Especially, optical single-molecule techniques have evolved into a diversified tool-box that, with minimal invasiveness, visualizes molecular interactions, structures and their changes. These techniques report on positions of molecules (see e.g. localization based superresolution microscopy [3–5]), movements (single-molecule/particle tracking [6,7]) structures (FRET [8,9]), interactions (FRET, colocalization [10,11]), and their interactions with the environment (polarization assays, colors, fluorescence lifetime) [12]. With the single-molecule field well established [13], we examine the possible advancements of optical single-molecule detection. Especially in the light of recent developments we see the synergistic combination of nanophotonics with biomolecular single-molecule experiments as an exciting, emerging field whose potential we discuss in this minireview.

The classical biochemical and molecular biology toolbox comprises comparably simple techniques such as polymerase chain reaction (PCR or qPCR), electrophoretic mobility shift assays (EMSAs), western blots, molecular cloning, pulldown assays and cross-linking assays. These techniques are usually fast, and provide straightforward answers without much prior knowledge about the system of proteins and nucleic acids. Protocols for these techniques can be generalized and require only minor changes to adapt to specific situations, e.g. by choosing appropriate primer sequences. In contrast, more sophisticated biophysical techniques such as optical single-molecule detection but also atomic force microscopy (AFM), optical and magnetic tweezers, X-ray crystallography and NMR spectroscopy require more effort e.g. for sample preparation and instrumentation, or are expensive. Single-molecule experiments, for example, require substantial a priori knowledge about the biological system such as structural information of at least one of the proteins to place fluorescent labels. They require labelling, protein preparation and purification (although often the need for perfectly clean samples can be alleviated by single-molecule sorting [14]). For a single-molecule FRET experiment, further understanding of the system is required: how can the complex be assembled? What kind of conformational changes might happen? Finally, optical single-molecule techniques require a detailed technical experience about the choice of dyes, buffer conditions, data acquisition and analysis and so on. Seeing a simple band in a western blot seems so much easier! Accordingly, the gain in scientific knowledge with the aid of the biophysical technique has to compensate for the extra effort.

There is one important further factor preventing the broad study of biomolecular problems by optical single-molecule

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spectroscopy. Currently, optical single-molecule spectroscopy experiments are not generally applicable to each and every biological problem. One main underlying rationale for the limited applicability is the comparably small dynamic concentration range of single-molecule measurements from picomolar to lower nanomolar as described in the following.

Single-molecule measurements aim for the best signal-to-noise ratio so that the signal from an individual fluorescent molecule can be distinguished from the myriads of molecules around it. The main strategy is to decrease the observation volume in order to reduce the influence of scattering molecules, fluorescent impurities and the other molecules of interest in solution. This is why the most common single-molecule detection geometries in use are confocal microscopy with diffraction limited femtoliter observation volumes and total internal reflection microscopy that enables paralleled detection of many molecules. [15]. Still, a femtoliter observation volume hosts on average a single molecule at a concentration of about one nanomolar. At higher concentrations, it is likely that more than one molecule contribute to the signal and at much lower concentration it will take too much time for a molecule to enter the observation volume.

The picomolar to nanomolar concentration range is a serious issue for biomolecular single-molecule applications because it limits the range of accessible biomolecular interactions that can be studied with labeled constituents. This is because biological binding constants are generally in the millimolar to nanomolar regime [16]. With these binding constants the constituents will rather not bind in complexes but will remain isolated at the concentrations required for single-molecule detection so that the fraction of molecules in the complexes to be studied is likely too small to be detected against a background of unbound molecules. According to the law of mass action, the equilibrium could be driven towards the formation of complexes by increasing the educt concentration which is yet incompatible with the size of the observation volumina.

Hence, part of the solution to increase the applicability of single-molecule techniques for biological problems is the ability to carry out single-molecule measurements at biologically relevant micromolar concentrations without detecting the unbound, dye labeled species around. This will extend the scope of systems from a few rather stable complexes and sophisticated experimental strategies to more dynamic and flexible complexes studied with standardized protocols closer to the reality of biology in which constituents in complex biological machineries are in a dynamic exchange. It appears likely that nanophotonics will play an important role in extending these single-molecule capabilities.

2. The nanophotonic promises

Nanophotonics is an emerging field of physics that aims at controlling light on lengths scales smaller than the wavelength of light. For single-molecule experimenters who focus on biological questions nanophotonics holds two great promises that have started enabling more general single-molecule experiments with biomolecular systems in closer to real conditions [17–21].

First, nanophotonic structures can be used to decrease the available space for molecules and to confine light. The most prominent example is the so-called zeromode waveguide [22]. Zeromode waveguides are small holes in a metal film on a glass cover slip. For their production, commonly a metal film is evaporated to form a layer with thickness of more than 100 nm on glass cover slips and holes of ~40–300 nm diameters are produced in the film by nanotechnological procedures. A cross section is sketched in Fig. 1a. As the zeromode waveguides offer glass bottoms common binding chemistry can be applied [23]. The observation volume is reduced by two effects. Laterally, it is confined by the physical constraints of the metal walls. Axially, the light cannot propagate through these nanoapertures as the diameter is too small to sustain propagating electromagnetic modes. Instead, an evanescent excitation is restricted to the volume close to the glass surface leading to an overall reduction of the observation volume of the order of 100.000-fold [16]. In Fig. 1b the electric field within an aperture with a diameter of 50 nm and a thickness of 100 nm at a wavelength of 500 nm is simulated and illustrates the light confinement. Zeromode waveguides have demonstrated breakthrough applications especially for single-molecule real-time sequencing and for studies of the choreography of translation as more specifically discussed below [24,25]. For a long time, access to such structures was restricted to a few specialized labs with custom production. But with their recent commercial availability, zeromode waveguides can enter the main stream of biomolecular single-molecule research. Zeromode waveguides can, for example, be obtained from Pacific Biosciences.

On the other hand, nanophotonics offers a more intricate way of enhancing single-molecule fluorescence spectroscopy that has only recently become interesting for biomolecular applications. Besides confining the excitation volume to subwavelength dimensions, metallic (nano)structures as simple as single gold nanoparticles can strongly and diversely affect the fluorescence properties of dyes [26,27]. In a simple model, the fluorescence process can be described by the singlet ground state S_0, the first excited singlet state S_1 and the transitions between them comprising excitation, radiative and non-radiative rates (k_{ex}, k_r and k_{nr}, respectively) (Fig. 2a). As fluorescence microscopist with biological background one is used to the fact that dyes are not rock solid light bulbs but that they react to their environment [28]. Different dyes show different fluorescence quantum yields depending on physical parameters such as the local viscosity or the presence of quenching molecules. This variance of dyes manifested as a change in brightness in fluorescence images, FRET experiments or fluorescence assays (e.g. molecular beacons in quantitative PCR) is commonly related to a change of non-radiative processes, i.e. changes of the non-radiative rate k_{nr}. Nanophotonic interactions can, however, do much more. Nanophotonic structures can amplify the excitation intensity in extremely small, spatially defined spots through localized surface plasmons. This means that when a sample is evenly illuminated, molecules in nanophotonic hot-spots experience substantially stronger excitation light fields and can therefore appear much brighter (Fig. 2b). Pictorially, the nanophotonic structure collects the electromagnetic radiation like an antenna does for

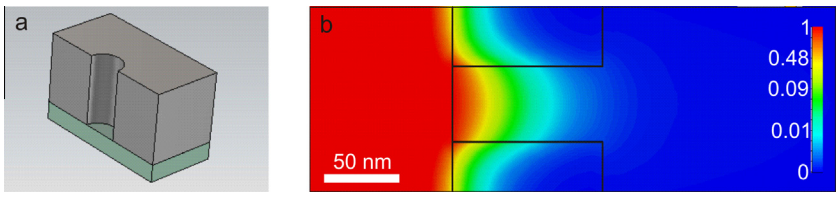

Fig. 1. (a) Sketch of the cross section of a zeromode waveguide (gray) on a glass cover slip (green). (b) Electric field simulation for an aperture with a diameter of 50 nm and a thickness of 100 nm at a wavelength of 500 nm. The color scale denotes the electric field intensity. The incident light cannot propagate through the aperture and it is confined to the volume close to the glass surface.