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Subdiffraction fluorescence imaging of biomolecular structure and distributions with quantum dots

Meike Heidbreder [a], Ulrike Endesfelder [a], Sebastian van de Linde [a], Simon Hennig [a], Darius Widera [b], Barbara Kaltschmidt [c], Christian Kaltschmidt [b], Mike Heilemann [a,*]

[a] Applied Laser Physics and Spectroscopy, Physics Department, Bielefeld University, Universitätsstrasse 25, 33615 Bielefeld, Germany
[b] Cell Biology, Bielefeld University, Universitätsstrasse 25, 33615 Bielefeld, Germany
[c] Molecular Neurobiology, Biology Department, Bielefeld University, Universitätsstrasse 25, 33615 Bielefeld, Germany

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ABSTRACT

We introduce semiconductor quantum dot-based fluorescence imaging with ~2-fold increased optical resolution in three dimensions as a method that allows both studying cellular structures and spatial organization of biomolecules in membranes and subcellular organelles. Target biomolecules are labelled with quantum dots via immunocytochemistry. The resolution enhancement is achieved by three-photon absorption of quantum dots and subsequent fluorescence emission from a higher-order excitonic state. Different from conventional multiphoton microscopy, this approach can be realized on any confocal microscope without the need for pulsed excitation light. We demonstrate quantum dot triexciton imaging (QDTI) of the microtubule network of U373 cells, 3D imaging of TNF receptor 2 on the plasma membrane of HeLa cells, and multicolor 3D imaging of mitochondrial cytochrome *c* oxidase and actin in COS-7 cells.

1. Introduction

Fluorescence microscopy is a versatile tool in biomedical research as it is noninvasive, specific, very sensitive, and compatible to experiments in living cells, tissue, or even organisms. In the past decades, a variety of fluorescence microscopes became commercially available that are facile to use and easily accessible for nonexperts, such that fluorescence microscopy has entered many research areas. However, as any lens-based light microscopy technique, fluorescence microscopy is limited in its spatial resolution to about 200 nm in the focal plane and >500 nm along the optical axis. In the recent past, a new branch of fluorescence microscopy emerged that developed strategies to bypass the inherent resolution limit of light microscopy [1,2], motivated by the need to perform microscopy at biomolecular length scales of some nanometres and to achieve even near-molecular optical resolution [1].

A large set of fluorescence methods that achieve superior optical resolution have been developed in the past years. These methods are different in the underlying principle used to generate a super-resolution image, in the achievable spatial resolution and the demand for specific labels [1–4]. Some methods have demonstrated to work in living cells [3,5] or even at video rate [6,7,24]. It is, however, the technical complexity and mathematical demand, together with expensive instrumentation, that often obviates a general use of these methods in everyday research. This is particularly true in research areas that would profit the most from imaging techniques with superior resolution and usually are nonspecialists in advanced and complex microscopy, i.e., biomedical research.

Very recently, we have introduced a method that enables ~2-fold optical resolution in 3D using semiconductor quantum dots as fluorescent labels [8]. This method can be implemented on any confocal microscopic system that is equipped with continuous-wave excitation in a spectral region of 450 nm to 550 nm, e.g., argon ion laser sources. The underlying physical phenomenon that finally leads to the resolution enhancement is a multiphoton absorption of semiconductor quantum dots. Specifically, a triple excitonic (triexcitonic) state is generated upon recombination emits blue-shifted fluorescence light, which is well separable from the “conventional” emission signal of the quantum dots. Different from conventional two- or multiphoton microscopy where virtual levels come into play and require the use of infrared excitation light and short laser pulses to provide the necessary intensity within the short lifetime of the virtual states, multiexcitons in quantum dots can be excited subsequently and with visible light. Furthermore, and again different from the very short-lived virtual levels in multiphoton microscopy, the individual excitons exhibit lifetimes that are long enough that

* Corresponding author. Applied Laser Physics and Laser Spectroscopy, Bielefeld University, Universitätsstr. 25, 33615 Bielefeld, Germany. Fax: +49 521 106 2958.
E-mail address: heileman@physik.uni-bielefeld.de (M. Heilemann).
URL: http://www.physik.uni-bielefeld.de/mh (M. Heilemann).

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conventional continuous-wave laser sources instead of short-pulsed (and expensive) laser sources can be used. In summary, the beauty of the concept lies in its very easy implementation on conventional microscopes, literally requiring a simple change in filter settings to achieve the resolution enhancement.

Here, we apply quantum dot triexciton imaging (QDTI) [8] to study the biomolecular structure and spatial organization in cells with subdiffraction spatial resolution. The benefit of using this concept lies in both the superior spatial resolution in 3D as well as the optical sectioning along the *z*-axis, which significantly reduces out-of-focus light. We demonstrate 3D imaging of proteins organized on cell membranes or subcellular organelles and simple approaches for quantitative analysis of protein distributions. Finally, we demonstrate how QDTI can be combined with organic fluorophores for multicolor confocal microscopy.

2. Experimental

2.1. Cell culture

U373 cells (human, glioblastoma–astrocytoma), HeLa cells (human, epithelial carcinoma), and COS-7 cells (derivative of the simian CV1) were grown in DMEM/F12 (High-Glucose; Gibco) containing 10% fetal calf serum (Gibco). Fresh medium was provided every second day. All cultures were incubated at 37 °C and 5% CO_2. For staining experiments, cells were trypsinized and transferred into Lab-Tek chamber slides (Nunc) and grown at 37 °C and 5% CO_2 until adhesion was re-established.

2.2. Fixation

Cells were fixed with 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS; Fluka) for 10 minutes at 37 °C. Cells were then washed with PBS and permeabilised with PBS containing 0.05% Triton X-100 (Sigma) for 10 minutes, then blocked with PBS containing 5% bovine serum albumin (BSA; Sigma) or 5% normal goat serum (NGS; Sigma) for 60 minutes at 37 °C. After washing with PBS containing 0.05% Triton X-100, cells were stained with antibodies.

2.3. TNFR2 and microtubule antibody stain

TNFR2 receptors were stained using anti-TNFR2 antibody (mouse anti-human; Axxora) or microtubules with anti-β-tubulin antibody (mouse anti-human; Invitrogen) for 1 hour. Cells were washed with PBS and then stained with QDot655 (goat anti mouse $F(ab')_2$; Invitrogen) at room temperature overnight. After washing with PBS containing 0.05% Triton X-100, nuclei were stained with Sytox Blue nucleic acid stain (Invitrogen) for 20 minutes. After washing with PBS, cells were embedded in Mowiol (Sigma) and stored at room temperature.

2.4. Cytochrome c oxidase antibody and actin stain

To stain cytochrome *c* oxidase of mitochondria, COS-7 cells were incubated with anti-OxPhos Complex IV subunit I antibody (mouse monoclonal; Invitrogen) for 1 hour. Actin was stained with Alexa Fluor 488 phalloidin (Invitrogen) together with the first antibody.

Afterwards cells were stained overnight at 4 °C with secondary antibodies carrying QDot655 (goat anti mouse $F(ab')_2$; Invitrogen). Three washing steps using PBS containing 0.1% vol./vol. Tween 20 (Sigma) were performed after each staining step. The cells were kept in PBS and were measured directly after the last staining step.

2.5. QDTI imaging

QDTI imaging was realized using a commercial confocal laser scanning microscope (LSM 710; Carl Zeiss, Jena, Germany) and analyzed using the ZEN2008 software (Carl Zeiss, Jena, Germany). Excitation wavelengths used were 488 nm and 458 nm, and emission windows were set at 580 to 620 nm (triexciton channel) and 650 to 720 nm (monoexciton and biexciton channels). Spectral separation on two detectors (photomultiplier tubes) was achieved by selecting a dichroic beam splitter (633 nm), and both detectors were operated at the same gain. Excitation powers were set to reach equal fluorescence signals on both detectors to ensure best comparability in terms of resolution increase. Typically, a 10-fold higher intensity was used for triexciton imaging. Pixel sizes of 40 to 100 nm (lateral) and 150 to 250 nm (axial) were used, and the pinhole diameter was set to 40 to 65 µm.

2.6. Image analysis

Image analysis was performed using ImageJ (NIH) and the "3D objects counter" plug-in. The algorithm was first tested on individual quantum dots adsorbed on glass and imaged in the triexciton channel. The approximate dimension of the point-spread function (PSF) under different imaging conditions (pixel size, pinhole, excitation intensity) was determined, and parameters to discern close-by quantum dots were estimated. Cell images were then analyzed using suitable values for intensity threshold and appropriate spot size (matching the experimental PSF of QDTI).

3. Results and discussion

Fluorescence microscopy of quantum dot-labelled biomolecules was performed with commercially available QDot655 (Invitrogen) on

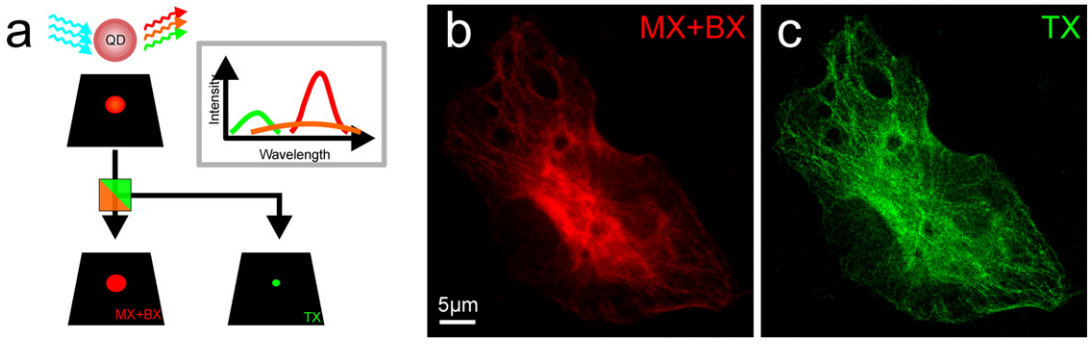

Fig. 1. (a) QDTI is realized by exciting semiconductor quantum dots QDot655 with 488 nm. As a consequence of multiexciton generation, three excitonic states with different fluorescence emission characteristics are generated. The emission of the triexciton is blue-shifted and can be spectrally separated. As a consequence of the three-photon absorption, the size of the point-spread function (PSF) is reduced ~2-fold, a measure of resolution enhancement. (b, c) Confocal fluorescence images of U373 cells, microtubule network stained by immunofluorescence using secondary antibodies labelled with QDot655. The triexciton channel (c) shows an increase in resolution and less contribution of out-of-focus light (optical sectioning).