MULTIPHOTON-EXCITED FLUORESCENCE IMAGING AND CORRELATION OF SINGLE QUANTUM DOTS USING A COMBINED APPROACH OF LASER SCANNING MICROSCOPY AND FCS

N. Opitz, M. Kahms, T. Mitroshina, B. Oeke and J. Kuhlmann

Max-Planck-Inst. für molekulare Physiologie, D 44227 Dortmund / Germany

norbert.opitz@mpi-dortmund.mpg.de

Abstract: Single quantum dot detection sensitivity can be demonstrated with high spatio-temporal resolution using a combined approach of Fluorescence Correlation Spectroscopy (FCS) , Multi-Channel Scaling (MCS) and Nonlinear (multiphoton-excited) Laser Scanning Microscopy (NLSM) demonstrating the unique opportunities of multi-modality nonlinear laser microscopy in conjunction with quantum dots for probing complex biological systems at the single protein level.

Introduction

Fluorescing semiconductor nanocrystals (so-called quantum dots) are of particular importance in both cellular and molecular physiology due to their outstanding optical and biological features such as e.g. brightness, photostability, potential for wavelength-multiplexing, biocompatibility and low toxicity. However, despite the fascinating results of biological applications recently reported [1,2,3,4,5,6], these investigations are up to now all restricted to the conventional (linear) single-photon excitation regime.

Hence, we performed first nonlinear-microscopy based experiments using an ultrafast mode-locked Ti-Sap-Laser with high repetition rate (76 MHz) and short pulse width (about 200 fs) in order to explore the potential and peculiarities of quantum dots in conjunction with different microscopical techniques such as fluorescence correlation spectroscopy (FCS), multi-channel scaling (MCS) and spatially-resolved intracellular imaging (NLSM).

Materials and Methods

Investigations were performed with quantum dots (streptavidin coated cadmium selenide (core) / zinc sulfide (shell) nanocrystals (Qdot_605 Streptavidin Conjugate), purchased from Quantum Dot Corp., Hayward (CA) with a main emission band at around 610 nm (+- 12 nm , FWHM) and a fluorescence quantum efficiency of ca. 0.5. These quantum dots were dissolved in 50 mM borate buffer , pH 8.3 , containing 0.05 % sodium azide and 2 % BSA.

Results

Fig.1 shows the fluorescence count rate of a 0.2 nM solution of quantum dots excited with 820 nm (average laser power 7.5 mW, emission < 700nm) with an average count rate of about 100 kHz along with photon bursts of up to 500 kHz when single quantum dots diffuse into the optically confined 2hν-excitation volume (order of femtoliter). The corresponding auto-correlation curve is depicted in Fig.2 along with a best fit according to a single species model demonstrating relatively huge deviations which may be attributed to the Gaussian-size-distribution of the nanoparticles.
An average diffusion coefficient can be estimated from the mean residence time of quantum dots (5.04 ms) to about D = 7.8 * 10 (-8) cm^2/s. This diffusion coefficient is about a factor 40 smaller than those measured for conventional fluorophores (e.g. Rhodamine Green).

Fig.3 displays a multichannel scaler (MCS) trace of the same solution of QDs, i.e. a fluorescence time trace monitored with high temporal resolution of 0.41 ms (bin width) demonstrating that only a low background count rate of ca. 10 kHz can be found in the absence of QDs, whereas drastic increases of the count rate can be observed when QDs diffuse into the 2hν-excitation volume.

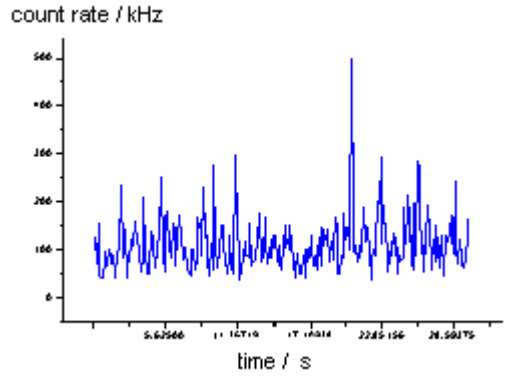

Figure 1: Fluorescence count rate of a 0.2nM solution of quantum dots excited with 820 nm (average laser power 7.5 mW, emission < 700 nm).

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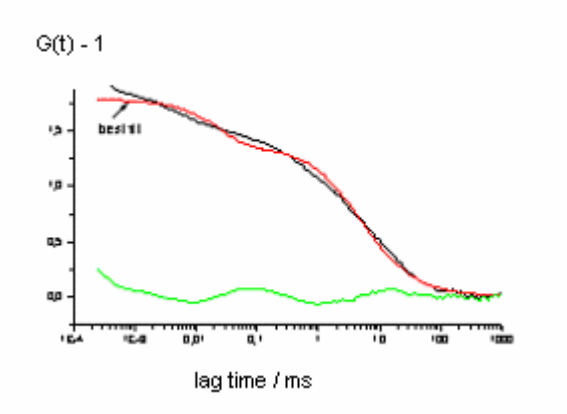

Figure 2: Corresponding auto-correlation curve to Fig.1 along with a best fit according to a single species model.

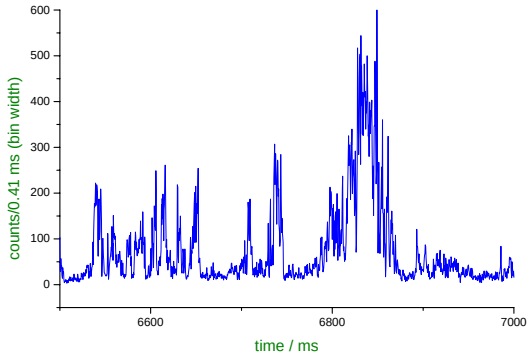

Figure 3: Multichannel scaler (MCS) trace of a 0.2 nM solution of QDs (bin width 0.41 ms).

Using an excitation wavelength range from about 760 nm to 840 nm we find exceptionally high two-photon absorption cross-sections and fluorescence quantum yields resulting in FCS count rates of up to 340 kHz per single quantum dot (which is about a factor 30 higher than those of conventional fluorophores). As a consequence, highly diluted solutions of quantum dots (even down to the sub-picomolar range) can still be analysed with high temporal resolution (Fig. 4).

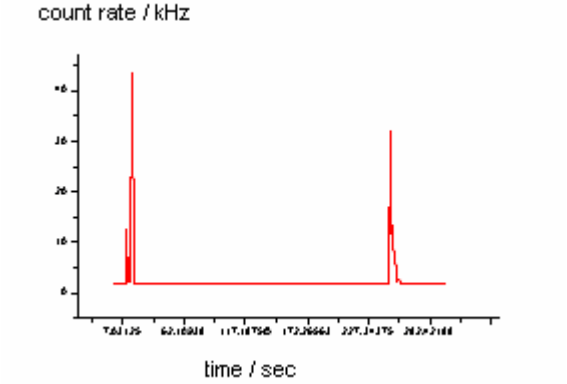

Figure4: Count rate of a 0.2 pM solution of QDs.

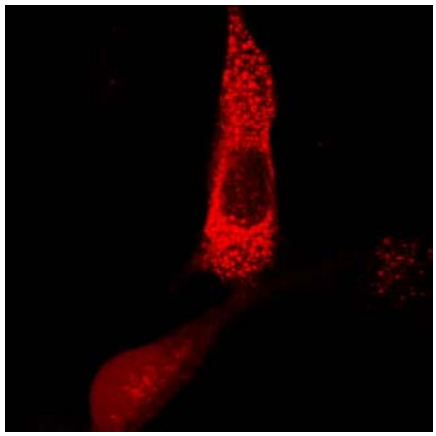

Figure 5: 2-photon excited fluorescence image of MDCK cells microinjected with a few nl of a 0.2 nM QD-solution.

Fig.5 shows a 2-photon excited fluorescence image of MDCK cells microinjected with few nl of a 0.2 nM solution of QDs. As can be recognized, agglomerations of QDs can be observed probably due to intracellular instabilities of the streptavidin coated QD suspension and non-specific intracellular interactions with cytoplasmic constituents.

Finally, we succeeded in single quantum dot imaging and tracking via Total Internal Reflection Fluorescence Microscopy (TIRFM) utilizing a novel back-illuminated Electron Multiplying CCD-Camera (EMCCD-Camera) with fast acquisition rate and ultimate sensitivity. TIRF-microscopy is a powerful method to observe and measure membrane associated processes in living cells and even single molecule dynamics. In our setup TIRF illumination of the probe is achieved via a high numerical aperture objective (Zeiss, Plan Fluar 100*, NA 1.45, Oil) (see Fig.6) and can be combined with wide field fluorescence illumination utilizing the TILL TIRF dual port epifluorescence condenser (Fig. 7) coupled to a Zeiss microscope (Axiovert 135 TV). Fig. 8 displays the overall setup of the TIRF microscope.

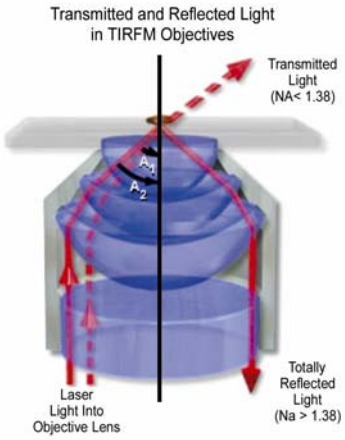

Figure 6: Radiation pathway within an objective-type TIRF-microscope

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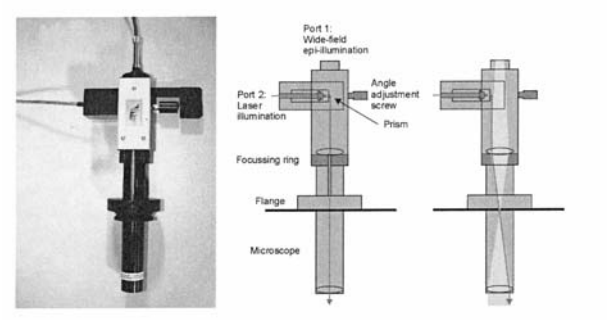


Figure 7: TILL-TIRF dual port epifluorescence condenser

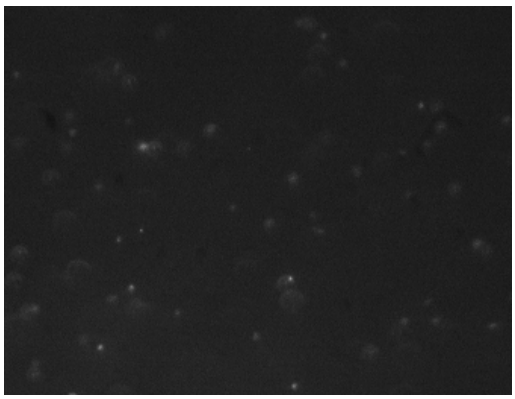
For challenging low-light imaging applications we use ANDOR's Ixon DV 887 back illuminated Electron Multiplying CCD camera (Fig. 9). The EMCCD-camera has single photon detection capability without an image intensifier, combined with greater than 90 % quantum efficiency of a back-illuminated sensor. Containing a 512* 512 Frame Transfer CCD sensor , it enables charge to be multiplied on the sensor before it is read out. The EMCCD gain of the camera can be varied from unity up to a thousand times directly through the software. The system offers up to 10 MHz pixel read out rate, for both EMCCD and conventional amplifier outputs and benefits from minimized dark current with unequalled thermoelectric cooling down to –90°C.

Fig. 10a+b show as an example the improved imaging conditions of the TIRF mode as compared to conventional brightfield illumination using fluorescent beads of about 100 nm diameter. As can be recognized, the TIRF mode shows a considerably enhanced contrast since signals from the bulk solution are eliminated. Fig. 11 shows a TIRF image of single quantum dots utilizing the EMCCD camera with a gain of about 400.

Figure 8: Overall setup of the EMCCD-coupled TIRF-microscope.

Figure 9: Andor's IXON DV 887 back-illuminated Electron Multiplying CCD-camera.

We also could demonstrate the potential of the EMCCD-coupled TIRF-microscope for single quantum dot tracking thanks to the fast image acquisition rate of up to 35 Hz for full frames (512*512 pixel). In addition, we observed the typical blinking behaviour of single nanocrystals. Finally, Fig. 12 shows the image of a single enlarged quantum dot representing in fact the Point Spread Function (PSF) of the overall microscopical set-up.



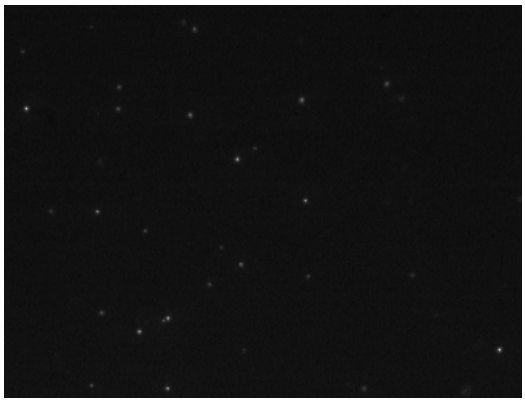


Figure 10a+b: Improved imaging conditions of the TIRF-microscope mode as compared to conventional bright field illumination using fluorescent beads of about 100 nm diameter.

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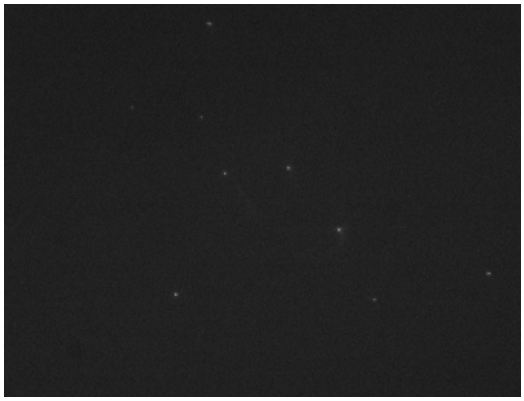

Figure 11: TIRF-microscopic image of single quantum dots

Conclusion

The above results and additional optical features (e.g. possibility for single wavelength excitation of differently sized and, thus, emission-wavelength tuned quantum dots) suggest a particular 2hν-advantage of quantum dots for dual-color fluorescence cross-correlation measurements, since only a single excitation wavelength can be used to simultaneously excite different quantum dots with high absorption cross-sections resulting in very favourable signal-to-noise ratios and the reduction of adverse effects caused by chromatic aberration.

Moreover, first investigations concerning intracellular nonlinear imaging of quantum dots inspire for e.g. real-time observation of ligand-receptor interaction and of molecular trafficking (via individual imaging and tracking of quantum dot labelled proteins) within single cells (under physiological conditions) due to the exceptional brightness and photostability of fluorescing semiconductor nanocrystals.

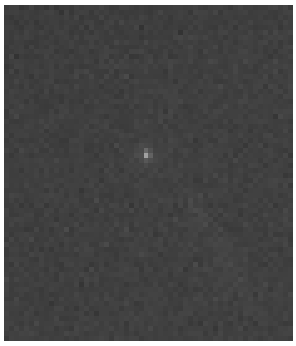

Figure 12: Image of a single enlarged quantum dot representing the Point Spread Function (PSF) of the microscope.

Finally, the investigations demonstrate the inherent biological potential of fluorescing semiconductor nanocrystals in conjunction with EMCCD-coupled TIRF-microscopy for e.g. real-time observation of ligand-receptor interactions or of molecular trafficking by monitoring the movement of individual proteins (quantum dot labeled) inside plasma membranes of living cells with utmost sensitivity and photostability as well as fast acqusition rates. In our actual approach we utilize the setup to analyze GFP-tagged membrane anchored proteins in artificial and cellular plasma membranes.

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