Using EMCCD Cameras for high-resolution Photoswitching Microscopy

Application Note

Dr. Gerd Wiebusch, University Bielefeld

Introduction

We have developed and investigated a novel far-field microscopic technique named dSTORM (**d**irect **S**tochastic **O**ptical **R**econstruction **M**icroscopy) for multi-color high resolution fluorescence imaging of biological structures and cellular processes below the diffraction limit (~200 nm). This technology is based on optically switchable molecules, i. e. the reversible transition of fluorophores between a detectable fluorescent and a nonfluorescent (dark) state, and the application of highly sensitive EMCCD cameras (Electron Multiplying Charge Coupled Device), which detect single photons with nearly 100% efficiency.

We use standard organic fluorophores out of different spectral regions as for example ATTO 520, Alexa 568, Alexa 647, Cy 5 etc. in aqueous solution as photoswitches with laser excitation as they can be easily coupled to selected cellular components via functional groups. The reversible transition from the dark state into the fluorescent state is either light induced (one needs two laser wavelength) or can be controlled by the special chemical conditions in the aqueous buffer like oxygen concentration, redox properties etc. (one needs only one laser wavelength).

Wide-field fluorescence microscopy at the single molecule level can be used to reconstruct a superresolution image from thousands of single molecule localizations (by iterative switching of a subset of the fluorophores from the dark state to the fluorescent state with subsequent precise position localization of each individual fluorescent spot). Experimentally, a sample that has been densely labeled with the fluorescent photoswitch, is prepared such that most fluorophores are in the dark state, and only an optically resolvable subset of molecules, that have enough distance to allow their localization as single emitters, is fluorescent at a time. To ensure the isolation of the fluorescence emission of individual fluorophores in time the lifetime of the dark state should be substantially longer than the lifetime of the fluorescent state. The lifetime of the fluorescent state is mainly controlled by the intensity of the excitation laser for the chosen fluorophore whereas the lifetime of the dark state depends on the chemical conditions in the aqueous solution as mentioned above (for a more complete discussion see (1)).

The raw data in the dSTORM single-molecule technique consist of a stack of more than thousand individual frames, each containing diffraction-limited fluorescence images of single stochastically photoswitched molecules present in the sample. The emission profiles of single emitters (point spread function, PSF) are analyzed through the approximation with a Gaussian fit to determine their centers with high precision. After many repetitive cycles of photoactivation, localization and deactivation the resultant information is used to reconstruct a total high-resolution image of the positions of the molecules that break the diffraction barrier of light with an experimental resolution down to ~20 nm.

The resolution achievable in the dSTORM technique depends on the localization precision (how well the center of each PSF can be determined), and therefore on a high single-to-noise ratio in each image. This means: Maximizing the number of collected photons from each photoswitchable molecule and minimizing the background fluorescence and detector noise. The density of photoswitchable molecules in the sample must be suitable and the total number of emitted photons per molecule before photobleaching high (an intrinsic property of the molecule and the surrounding medium).

Experimental Setup

The experimental setup is shown schematically in figure 1.

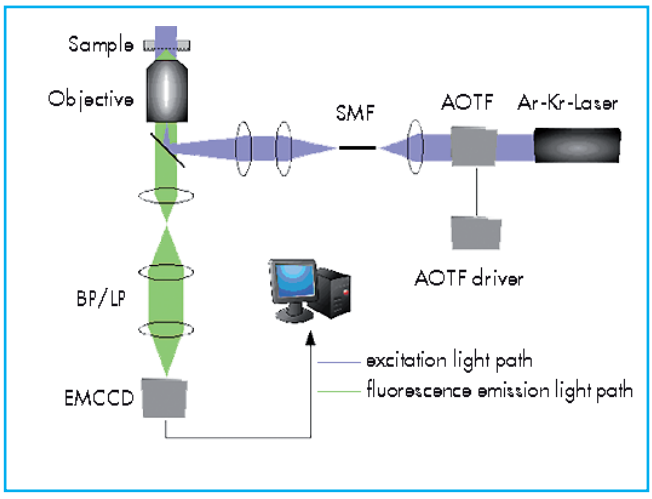

Fig. 1 Experimental setup

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Fluorescence imaging was performed on an inverted microscope (Olympus IX71) equipped with an oil-immersion objective with a high numerical aperture (PlanApo 60x, NA 1.45) for optimal photon collection efficiency. Wide-field fluorescence microscopy increases the acquisition speed because many molecules per frame can be imaged simultaneously. Working in the total internal reflection (TIRF) configuration is advantageous because of the low penetration depth into the sample leading to extreme rejection of background fluorescence and thus facilitating the detection of single fluorescent molecules.

For photoswitching we work with an Ar/Kr mixed gas laser (Coherent I70C) and an acusto optical tunable filter (AOTF) as a rapid switch to select the appropriate excitation wavelength and laser power for the individual fluorophore used. A single mode fiber (SMF) and a telescope system (beam expander) is used for coupling the excitation laser into the microscope and focusing it on the back focal plane of the objective (objective type TIRF).

Using appropriate bandpass and longpass filters (BP/LP) and dichroic beamsplitters the weak fluorescence emission was detected and imaged on an EMCCD camera. An additional telescope is employed before the camera for proper magnification. A fast computer with image acquisition software (Andor Solis) and large storage capacity is needed.

Typically several thousand photons can be detected from a single fluorophore per image. In each image fluorescent spots (PSF) are identified and fit to a Gaussian function to determine their center of mass with high accuracy. Summing up all individual localization events of the same fluorophore in a two dimensional histogram a localization accuracy of ~20 nm can be achieved. Around 10,000 frames at frame rates of typically 30 Hz were used to reconstruct a superresolution image, corresponding to measurement times of several minutes.

An important factor to detect and amplify the single-molecule fluorescence emission is the use of an electron multiplying cooled CCD (EMCCD) camera. We are working with iXon and iXon+ EMCCD cameras from Andor Technology with different chip and pixel sizes (models iXon DV887, iXon+ DU897, iXon+ DU888, iXon+ DU860). The chips of these cameras are back-illuminated leading to a quantum efficiency of ~90% across the visible spectrum. Employing an on-chip multiplication gain makes the camera capable of single-photon detection. The chip is cooled to -70 °C to reduce the dark noise in the sensor to a negligible value. Fast readout (10 MHz A/D rate per pixel) allows rapid imaging and reduce the time to generate a high resolution image.

In our standard setup we are working with an iXon+ DU897 DCS-BV EMCCD (512 x 512 pixel chip, 16 µm pixel size), operating in the frame-transfer mode and with 10 MHz/pixel readout rate. For the localization of single molecules it is important to match the pixel size of the EMCCD camera to the size of the point spread function (for a more complete discussion see (2)). A system magnification (objective and additional lenses) of ~200 leads to a total field of view of ~40 µm, corresponding to an effective pixel size of ~80 nm per pixel. An iXon+ DU860 (128 x128 pixel) is used for faster image tacking (500 Hz) with reduced field of view and an iXon+ DU888 (1024x1024 pixel) to get the largest field of view for imaging extended cellular structures.

Example

To demonstrate the potential of photoswitching microscopy for subdiffraction resolution fluorescence imaging of cellular structures we used fixed COS-7 cells (African green monkey kidney cells) and stained the microtubule network applying immunocytochemistry and antibody fragments labeled with the organic fluorophore Alexa 647. As can be seen in figure 2 photoswitching images show superior resolution as compared to conventional wide-field images of the microtubule network.

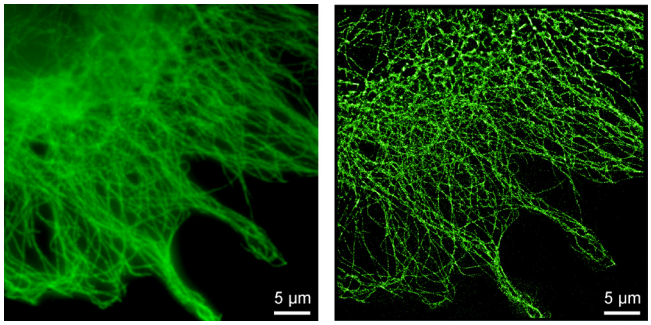

Fig. 2 Conventional wide-field image of the microtubule network (left side) Photoswitching microscopy image with subdiffraction resolution (right side)

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References

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[2] H. Shroff, H. White, and E. Betzig, Current protocols in Cell Biology 4.21, Dec. 2008

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