PALMIRA

Novel nanoscopy technique – imaging organelles at 40 nm

Light microscopy has always been suffering from the limit imposed by the Abbes law which manifested itself as presence of Airy disks. These structures defined the resolution of any optical systems regardless of imaging modality. For all users of microscopy systems it meant that image objects separated by distance roughly equal to half the wavelength of light used for imaging, coalesced into one diffuse spot. Until recently, the Abbe limit seemed to be the ultimate barrier in optical resolution therefore all images obtained from high performance light microscopes were irrevocably diffraction-limited. All finer structures in low sub-micrometre space were impossible to render without major distortions. To address this problem several novel imaging techniques have been developed to overcome the Abbe limit and to open up new avenues of research at nanometre scale.

One of these single-molecule approaches has led to the development of nanoscopic techniques based on continuous rather than pulsed molecular switching of fluorophores used as fluorescent markers. Groups lead by Dr. Alexander Egner and Prof. Stefan Hell based at Max-Planck Institute for biophysical chemistry in Goettingen have demonstrated that nanoscale resolution in far-field fluorescence microscopy was possible by using reversible photoswitching and localization of individual fluorophores. Their experiments were based on the premise of very fast recording from the interior of fixed cells using photactivable molecular markers. These single molecules were then visualised by asynchronously recording the photon bursts of individual molecular switching cycles. Using this technique it has been possible to image subcellular assemblies of microtubules at 40 nm resolution. This method, stemming from original PALM described by the Betzig group at Janelia Farm, is referred to as PALM with independently running acquisition, or PALMIRA.

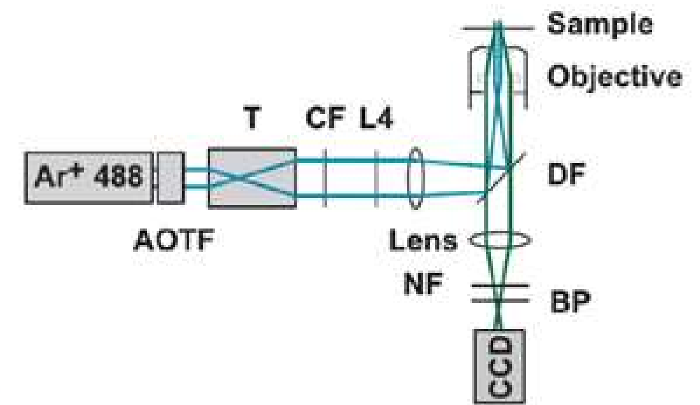

Figure 1: PALMIRA setup including gas laser with an AOTF, optical components of the light path e.g.telescope (T), beam splitter (DF) and Andor iXon3 860 EMCCD camera.

A PALMIRA setup is very similar to wide field microscope but it uses gas laser instead of an incandescent or halogen lamp to deliver intense, coherent light needed for sample excitation (Figure 1).

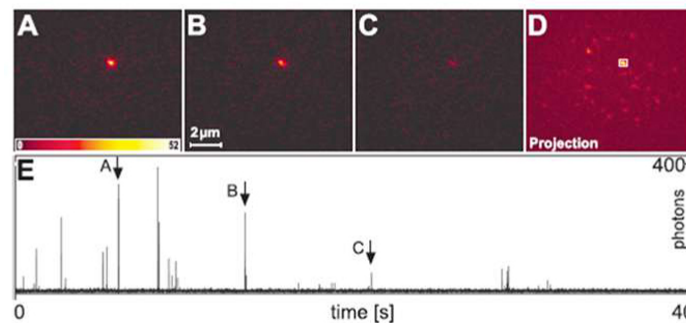

Figure 2: Imaging single fluorophore molecules on PALMIRA setup. Three recordings of a single molecule are shown in (A-C) and the maximum projection from the whole series of 20000 image frames is displayed in (D). The object marked in (D) is most likely a single molecule as its concentration was only 1.27 nM. Panel (E) shows the time trace integrated over the 4x4 pixels marked in (D).

Before the main biological sample can be imaged certain calibration measurements have to be done to find out the systems PSF and to make sure that single molecules emitted the expected amount of light needed for analyses (Figure 2).

Three typical recordings of a sparse sample of the reversible photoswitchable protein are shown below. They were acquired at a frame rate of 500 Hz. At this rather high frequency Andor iXon3 860 camera operated at 97% duty cycle which is 515 full frame images per second. The excellent signal-to-noise ratio of the data is evident in the time trace (Fig. 2 E) where the root-mean-square of the combined background and readout noise typically averaged to be less than 1 photon/pixel. Molecular switching off as well as its bleaching are stochastic processes therefore it is unknown whether the molecule shown here was in its dark state or bleached after the recording.

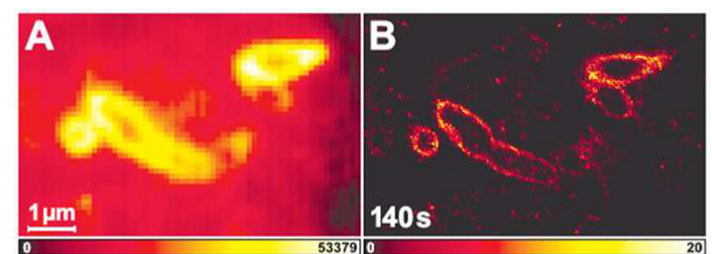

Figure 3: Individual 200 nm thick cells of E. coli imaged with conventional (A) and PALMIRA (B) mode. It took 140 seconds to record image in PALMIRA.

After initial calibration steps were complete it became possible to use this set of data in a localisation algorithm applied to the bulk of final experimental images obtained from various cells including bacterium E. coli and PtK2 line. Both, mammalian and bacterial cells were fixed with cold methanol or subjected to cryopreparation and imaged in conventional or PALMIRA mode (Figures 3 and 4).

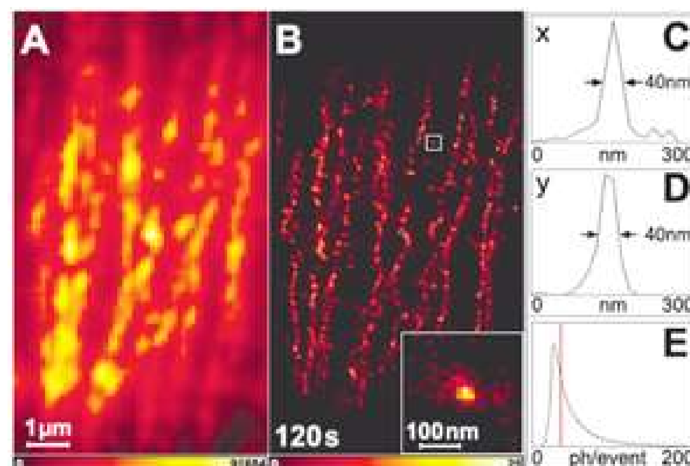

Figure 3: Individual 200 nm thick cells of E. coli imaged with conventional (A) and PALMIRA (B) mode. It took 140 seconds to record image in PALMIRA.

PALMIRA clearly demonstrates the big potential of photoswitching and localisation techniques for biological imaging. Having pushed the diffraction limit from ~200 nm to 40 nm; solutions like PALM, STORM and PALMIRA will be very attractive alternatives to complex STED microscopy systems. Furthermore

PALMIRA can take advantage of the faster frame rates of the iXon Ultra 897 and the new, 'Optically Centred Cropped Mode' ideal for super resolution techniques and capable of delivering faster acquisition bridging the gap between relatively slow PALM and STORM and real-time / video rate techniques of wide field and confocal microscopy.

Andor Solutions for PALMIRA

For this particular study the researchers used iXon3 860 EMCCD for its speed and sensitivity. Andor have recently added te iXon Ultra 897 to its range of EMCCD cameras. The iXon Ultra 897 works well for super resolution techniques where the electron multiplication can be utilised for weakly fluorescent samples and the fast frame rates with the "Optical Centred Crop Sensor" mode allow the capture of dynamic events. Facilitated by a fundamental redesign, the new iXon Ultra platform takes the popular back-illuminated 512 x 512 frame transfer sensor and overclocks readout to 17 MHz, pushing speed performance to an outstanding 56 fps (full frame), whilst maintaining quantitative stability throughout. The significant speed boost offered in the iXon Ultra 897 facilitates a new level of temporal resolution to be attained, ideal for speed challenged applications such as live super resolution techniques.