Correlative confocal fluorescence lifetime and Atomic Force Microscopy imaging by ISS and JPK

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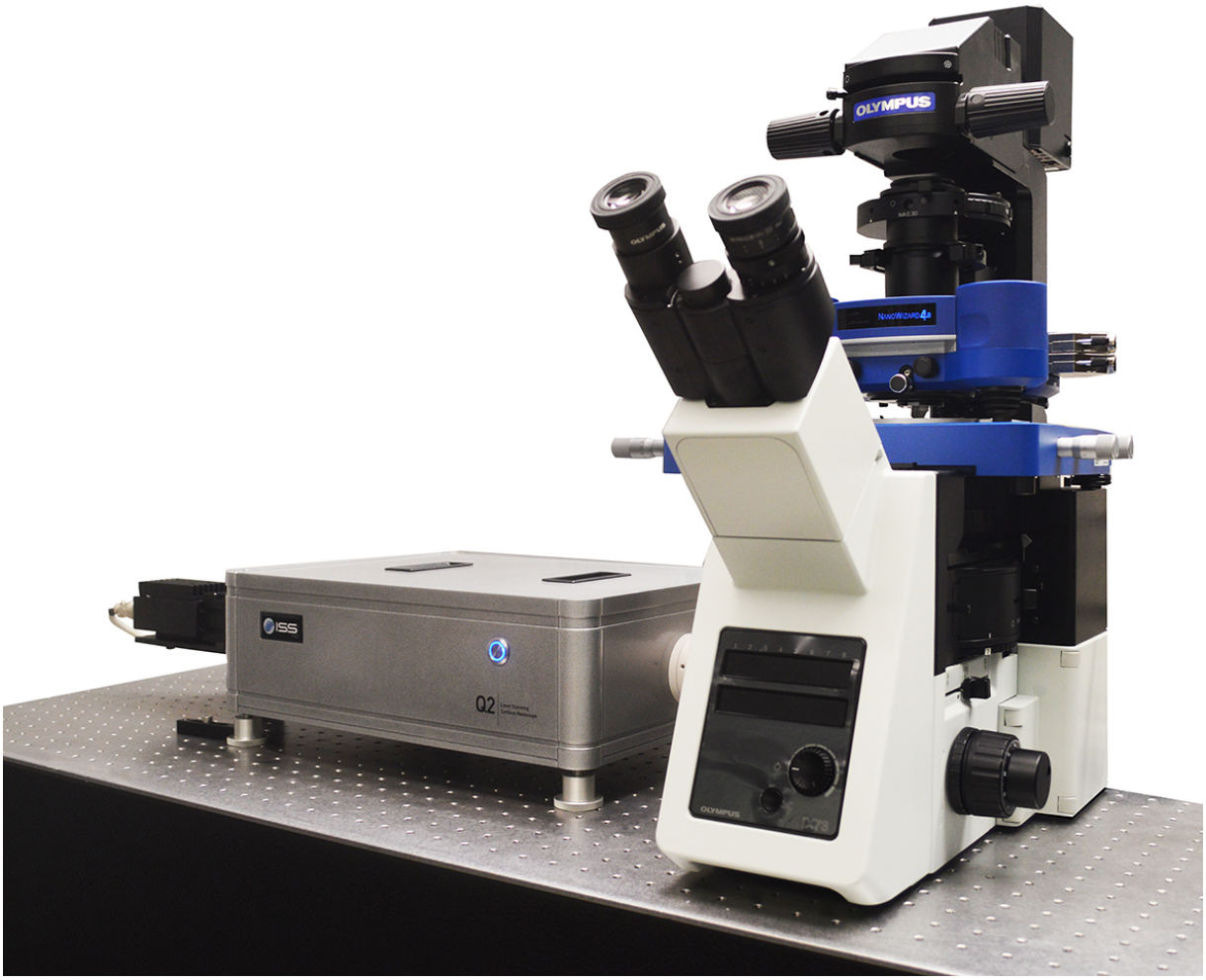

1. Introduction

Confocal microscopy is an advanced fluorescence microscopy technique that offers great axial resolutions for optical sectioning of a thick specimen; it has been routinely used in many research areas, especially in biological sciences [1]. In addition to the steady-state intensity information, ISS confocal microscopy instruments also provide the fluorescence lifetime information at each spatial (X, Y, Z) location, which can be combined with time to track the dynamic changes of a sample. Fluorescence lifetime is the average time a molecule spends in the excited state before returning to the ground state, typically with the emission of a photon, and it carries information about events in the probe's local microenvironment that affects the photo physical processes. By measuring the fluorescence lifetime, fluorescence lifetime imaging microscopy (FLIM) provides unique opportunities to many studies in chemistry, material and biological sciences [2]. A brief description of the ISS confocal FLIM capabilities is given in Section 2.

Atomic Force Microscopy (AFM) is well known as a multi-purpose tool for imaging a wide range of different samples with nanometer scale resolution in air and under controlled environmental conditions in liquid using forces ranging from several pN to nN. Topography, roughness, and mechanical properties are crucial parameters influencing biological processes such as cell adhesion/motility, morphology and mechanics as well as the development of stem/progenitor cells [3-7]. Modern operating modes like Quantitative Imaging (QI™) enable AFM not only to study sample morphology with nano-meter resolution, but also to map mechanical and adhesive properties of a sample in their native environment. The combination of these properties with ISS advanced optical microscopy technology creates a multi-parametric tool that allows for extensive characterization of specimens (ranging from single molecules to nanoparticles to complex systems like living cells [8]).

2. The confocal FLIM instrumentation by ISS

The Q2 and Alba instruments are designed and manufactured by ISS for a variety of quantitative confocal microscopy measurements, summarized in Table 1 (visit www.iss.com/microscopy for more details). A recent development of the Alba-STED instrument enables the dual-label time-resolved super-resolution microscopy imaging to ~ 50 nm using a single combination of excitation and STED lasers at a reduced STED laser power suitable for liver cells [9].

ISS provides both the time-correlated single photon counting (TCSPC) [10] and the digital frequency-domain (FastFLIM) [11] techniques for FLIM solutions. The classical TCSPC module offers a high temporal resolution (6.5 ps @ FWHM), however, has a long dead time (100 ~ 150 ns), limiting its photon counting capability. In contrast, FastFLIM is a novel digital technique and has the following features:

- FastFLIM has nearly no dead time, leaving the limitation only to the detector itself.
- FastFLIM has four independent data input channels for simultaneous time-resolved imaging.
- FastFLIM has a clock input and three clock outputs for synchronization with other devices such as lasers, optical modulators, the AFM data acquisition unit and etc.
- FastFLIM can measure a wide range of fluorescence and phosphorescence lifetimes (from hundreds of picoseconds to tens of milliseconds).

- FastFLIM data can be analyzed (in the ISS VistaVision software) by three different methods – the non-linear least square fitting of the decay in the time domain; the non-linear least square fitting of the phase delays and the demodulation ratios in the frequency domain; and the phasor plots [12-15].

Intensity and Lifetime Imaging

- Single-photon or multi-photon confocal imaging in combination of x, y, z and t
- Multi-well screening and tile imaging
- Fluorescence lifetime imaging by TCSPC or digital frequency-domain (FastFLIM)
- Phosphorescence Lifetime Imaging (PLIM)
- Steady-state and time-resolved anisotropy imaging

Fluorescence Fluctuations Spectroscopy

- Number & Brightness (N&B)
- RICS (Raster Imaging Correlation Spectroscopy)
- Fluorescence Correlation Spectroscopy (FCS)
- Fluorescence Cross-Correlation Spectroscopy (FCCS) with pulse interleaved excitation (PIE) [16]
- Photon Counting Histogram (PCH)
- Fluorescence Lifetime Correlation Spectroscopy (FLCS)
- Scanning FCS by orbit scanning

Single Molecule and Single Molecule FRET Imaging

- Burst Analysis; FRET efficiency vs. Stoichiometry determination with PIE
- Multi-parameter imaging & analysis (intensity, lifetime, diffusion time, rotation time)

3D Particle Tracking Trajectories

- Nanoimaging reconstruction with 20nm resolution

Pulsed STED Superresolution Imaging

- Time-resolved STED
- STED FLCS
- Dual-label STED

Table 1: Routine applications by ISS Alba and Q2, powered by the ISS 64-bit VistaVision software

Figure 1 shows examples of FLIM and PLIM measurements using TCSPC and FastFLIM techniques on Alba and Q2 systems.

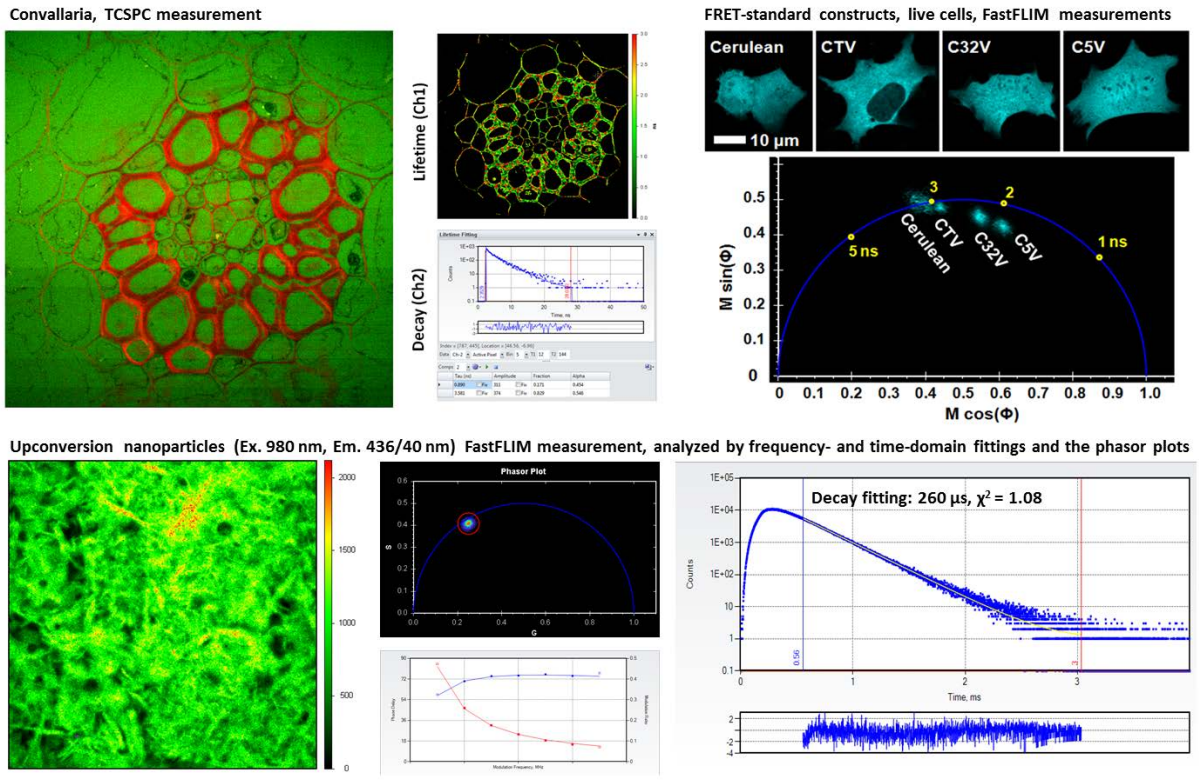

Figure 1: FLIM and PLIM measurements using TCSPC and FastFLIM techniques on Alba and Q2 systems.

The NanoWizard[R] AFM family from JPK Instruments

JPK Instruments develops and manufactures a wide range of force measurements tools, general imaging, and nanomechanical measurements based on Atomic Force Microscopy and Optical Tweezers. AFMs allow the probing and mapping of forces from a few pN to hundreds of nN which covers biological interactions from single molecule interactions to cell mechanics as shown in Figure 2 (left).

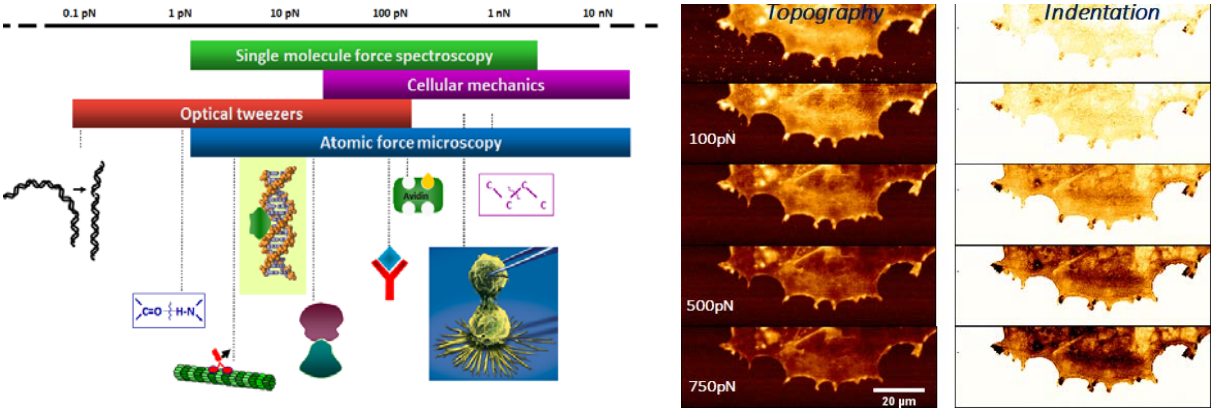

Figure 2: The diagram (Left) depicts the forces for biological process from molecular interactions to cell mechanics. JPK's AFMs and Optical Tweezers can address the range from a few pN to hundreds of nN. Shown on the right are data from living COS-7 cells from a force cube obtained using QI in a petri dish heater. The topographic and corresponding indentation images reveal useful information topography and mechanical properties of the cell.

The NanoWizard family is available with scan ranges up to 100x100x15um^3 and an additional CellHesion module with a z-range of >100um to enable cell interaction and tissue measurements. All AFMs are designed as tip-scanners allowing a wide variety of fluid cells, heaters and coolers, and electrical modules to be attached (visit usa.jpk.com for more details).

The correlative confocal FLIM-AFM measurement procedure

In ISS Alba or Q2 system, a sample is scanned by using galvo mirrors or a motorized XY stage (Piezo or linear encoded) or both; optical sectioning is achieved by moving the objective lens or the stage in Z with a Piezo or linear encoded motorized device. To avoid any perturbation to the sample, the XY scanning by galvo mirrors and the Z sectioning by moving the objective lens are preferred. This is important for integration with the JPK Nanowizard AFM system which images the sample by moving the AFM tip. The XY-scanning and Z-sectioning devices are synchronized to the data acquisition unit (TCSPC module or FastFLIM or both) to acquire fluorescence intensity and lifetime images. The typical detectors used in Alba or Q2 are GaAsP (H7422p) or Hybrid (R10467) photomultiplier tubes by Hamamatsu or single photon counting module avalanche photodiodes APDs by Excelitas.

Figure 3 shows the system diagram combining both ISS Q2 confocal FLIM and JPK NanoWizard AFM. The ISS Q2 nanoscope is coupled to an Olympus IX73 microscope and the NanoWizard is mounted on the microscope. The ISS 488-nm diode laser mounted on the ISS 3-diode laser launcher is delivered to Q2 via a polarization maintained (PM) fiber. The galvo mirrors are used for XY scan and a Z-Piezo device mounted on the objective lens is used for optical sectioning.

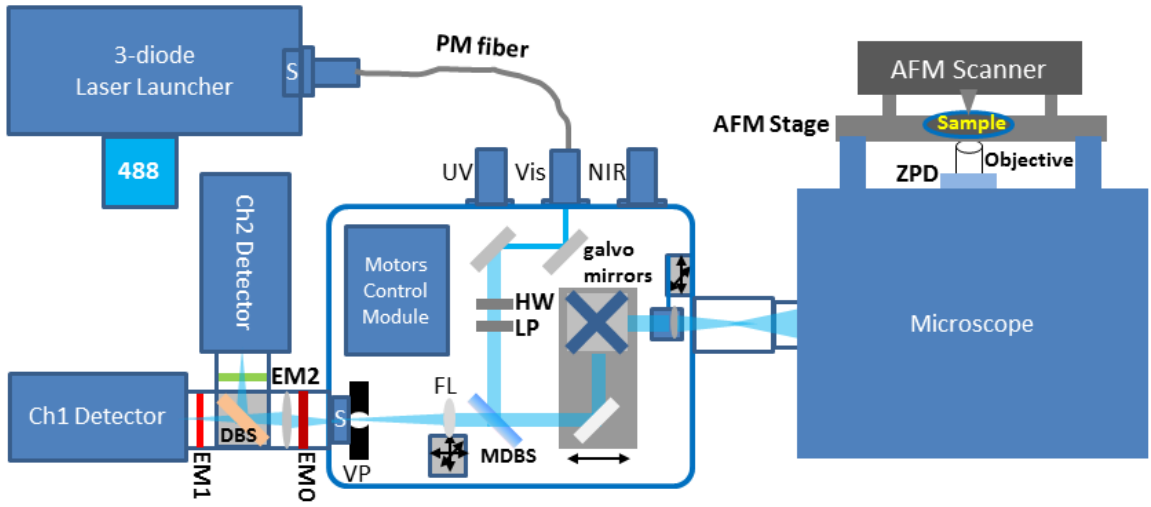

Figure 3: The system schematic combining ISS Q2 and JPK Nanowizard. HW: halfwave plate; LP: linear polarizer; MDBS: main dichroic beam splitter; VP: variable pinhole (motorized and continuously tunable from 20 to 1000 µm); S: Shutter; EM0, EM1, EM2: emission filters; DBS: dichroic beam splitter; ZPD: Z-Piezo device.

The **correlative confocal FLIM-AFM measurements acquisition procedure** goes as follows:

1. Place your sample on the stage.
2. Focus the sample with the objective lens through the eyepieces or the confocal live imaging.
3. Align the JPK Nanowizard AFM scanner with the ISS confocal FLIM system scanner. This is simply done by using the confocal scanner to acquire the images of the AFM tip at different positons controlled by the AFM scanner. When the tip is slightly above the engaged position, the image of the tip by reflecting the laser beam can be directly acquired by the confocal system.
   - The images of the tip at nine different positions are acquired by the confocal system by moving the AFM scanner (see Figure 4); nine TIF (default) images are saved by VistaVision.
   - The TIF images are opened by the direct overlay plugin in the JPK software; based on these images, an automated calibration routine will align the AFM and confocal data. Note that the images saved by VistaVision can be directly accessed by the JPK software.
4. Acquire the confocal lifetime image of your sample in VistaVision using Alba or Q2; when the lifetime image is saved, a TIF (default) image for the intensity information only is also saved.
5. Open the intensity image of your sample in the JPK software; now you have a fluorescent image as the background and you can zoom into any interesting feature for the AFM scan (see Figure 5).
6. Repeat step 4-5 to discover other fields of view of the same sample, or another sample. As long as the AFM scanner head is not removed from the microscope, it is not necessary to redo step 3.

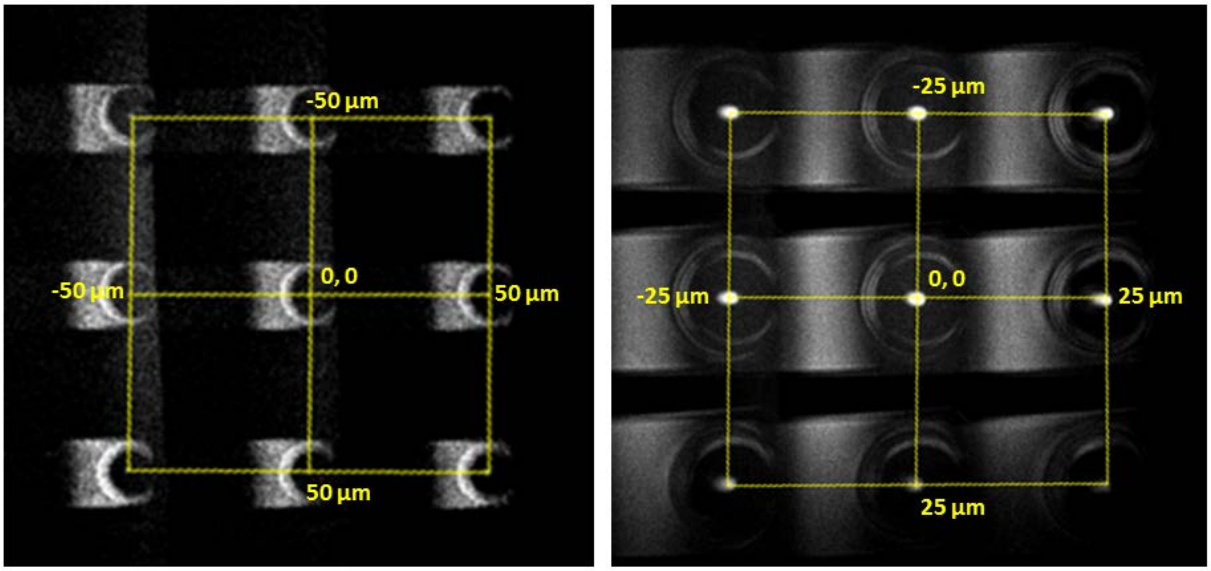

Figure 4: Overlay of the images of the Nanowizard tip at 9 different positions by Q2, using Olympus 20X / 0.75 NA (left) and 60X / 1.2 NA water (right) objective lenses.

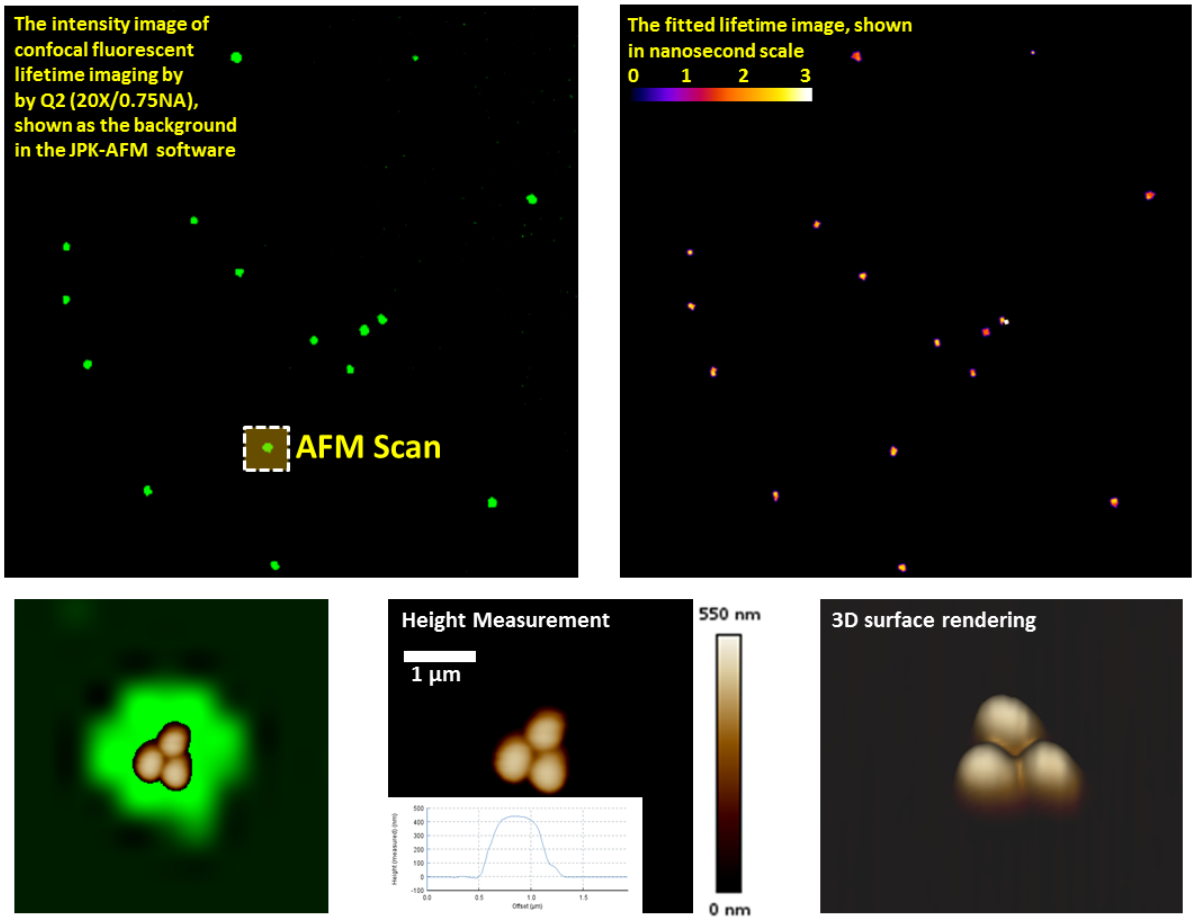

Figure 5: Correlative Confocal FLIM-AFM measurements of fluorescent beads.

Conclusions

Here, we describe a simple and robust approach for the correlative confocal FLIM-AFM measurements. This is achieved by a straightforward integration of ISS Q2 or Alba system with JPK AFM system, without any modification. Plus, simultaneous confocal lifetime and AFM imaging is allowed and fully tested.

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