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Setting up and Calibration of Simultaneous Dual Color Wide Field Microscope for Single Molecule Imaging

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ABSTRACT

Single molecule spectroscopy is considered among the most important spectroscopic and microscopic analytical tools especially that follow the biological reactions. The usage of wide field microscope in single molecule spectroscopy is not something new but what we introduce is a simultaneous dual color imaging wide field microscope setup. The setup alignments tested, and all the species we used to prepare the sample were tested free from unwanted fluorescence. A real single molecule experiment was done using streptavidin stained with Atto655.The system is proved to be able to detect single molecules in two channels exited with different colors at the same time, and it is ready for the experiments.

Keywords: wide filed- single molecule imaging- streptavidin- Atto655

INTRODUCTION

At all times, Spectroscopy is one of the most important analytical techniques. Spectroscopy is one of the ancient scientific issues. It started from several hundreds of years even from the roman age, by discovering the rainbow emerging from a prism in front of the sun [1]. Later in the 17th Newton was from the first generations who established the first rules of spectroscopy; this was the first attempts in atomic spectroscopy [2]. Another important branch of spectroscopy is the molecular spectroscopy which started in the 1800 with the discovery of the IR range in the spectra of the sun through a prism by Sir William Herschel [3]. But the real start of the Infrared spectroscopy comes by employing the Fourier transformation, this step is the breakthrough of Fourier transformation infrared (FTIR) to determine the structure of the protein as one from the most important molecules for human [4]. The merging of molecular modeling with the experimental FTIR plays an important role in investigating the structure of the biological molecules like proteins and amino acids [5, 6].

The molecular relaxation in some substances gives luminescence [7]. The excitation- relaxation time is a finger print for the substance and called the life time [8, 9]. Scientists classified the different life time into two groups, the first called fluorescence and the second called phosphorescence [10]. In luminescent molecules with very fast life time up to few milliseconds [11, 12]are the fluorescent molecules, while for the longer life times are the phosphorescent molecules. In some cases the lifetime limits are overlapped with what is called long lifetime fluorescence or short life time phosphorescence [13].The way to differentiate between the fluorescence and phosphorescence is the electron spin multiplicity during the excitation-emission process, if it is the same we have fluorescence otherwise phosphorescence is the case. Normally if the relaxation takes place from the excited singlet state we have fluorescence however if the relaxation starts from the triplet state the luminescence is called phosphorescence [14].
Hirsschfeld et., al. Investigated and established the capability of an optical method to detect ultra-low concentration of biological samples especially those immersed samples in physiological buffer [15]. By the 7th decade of the last century they succeeded to detect single antibody labeled with around 100 dye molecules [16, 17].

The vast development of the single molecule spectroscopy especially that depending on laser as a source, and at room temperature techniques, offers advanced facilities for investigating macromolecules as individuals *in-vivo, in-vitro* even *in-situ* [18,19]. By the single molecule techniques one can follow surface tethered molecules or freely defused ones, this makes the follow-up of any biological interaction or any conformation of such molecule easy and even by doing some efforts, available [20, 21]. In addition we can investigate the molecular dynamics and the biological activity beyond the masking effect of the other surrounding molecules and the averaging over the ensemble [22, 23]. In the normal spectroscopic methods and techniques a molecular synchronization should be done, but by the single molecule spectroscopy we can know the time traces and the molecular arrangement over the sample and through the reaction time without a complicated analysis.

After the enhancement and development of the electronics and the new invention of novo detectors depending on new ideas, the usage of single molecule spectroscopy is not restricted to only the visible range spectrometers but also it makes use of Raman spectroscopy as a new single molecule tool. In this regime the surface enhanced Raman scattering (SERS) is the best from the Raman techniques which becomes a tool that could work under room temperature [24]. The single molecule SERS gives the opportunity to detect single nano-particles, like the scanning of only single silver nano-particles in a colloidal that contains vast heterogeneous molecules based on the special dependence on the molecular hydrodynamic radius [25].

An example of the singularity and advantage of the single molecule techniques over the conventional spectroscopic methods is the detection of single Rhodamine dye (R6G) attached to a nano-particle. By single molecule SERS we got signal enhancement in order of 10^{15} times of that registered by the traditional techniques [26].The detection of individual objects were not to happen without the development of new technologies of high sensitive detectors, that are the foundation of the charge coupled devices (CCDs) and the avalanche photo-diodes (APDs), the single photon avalanche diodes (SPADs) and the focal plan arrays (FPAs). All the mentioned detectors and more are used with microscopes in several optical setup like, wide field, total internal reflection (TIRF), confocal, and near field scanning microscope.

In this work a dual beam wide field microscope is built for single molecule fluorescence imaging and test experiments were carried out for all the constituents which form the sample environment. In addition test experiments and time series imaging for single molecule sample were also carried out.

MATERIALS AND METHODS

One of the challenges of setting up any single molecule technique is the presence of suitable, high signal to noise ratio and ultra-low dark current. For this purpose we purchased our back illuminated EM-CCD iXon Camera from Andor technology Ltd., Belfast, UK.

The microscope is one from the essential parts in the system; it must be suitable and convenient with the technique. While we pursue to establish an epifluorescence technique hence the inverted microscope is the only choice. We purchased an inverted microscope IX-83 equipped with oil immersed objective 100X, its numerical aperture 1.4. The microscope purchased from Olympus, Japan.

One from the most important parts in any spectroscopy setup is the excitation source. In this setup a laser source with Gaussian beam profile should be used, so we got an Argon laser from Innova C90-5 from Coherent CA-USA. While we develop a simultaneous dual beam system we need another source, mainly we can use the same argon laser that contains several lines but when we need a red laser we must use another laser source. For the red laser we used a He-Ne laser source.

The excitation beams are guided by reflecting mirrors to combine both of them using a dichroic mirror (the dichroic band determined according to the used laser lines).The dichroic mirror should be selected so that it reflects only the excitation lines with a narrow reflection band and transmit fluorescence from the sample. The reflected laser beams go through the objective to focus on the sample located on the microscope glass slide. Thermo Fisher microscope cover slips no.1 (thickness from 0.13 to 0.16 mm) has been used to be convenient with the microscope objective lens. The excited dyes make fluorescence emission in all directions, part from the emitted beam go through the objective lens and the parallel beam passes to the CCD through the microscope tube lens.

A specific emission filter, for every dye according to the emission spectra has been used to reduce any fluorescence from the background or any scattered beam. In the case of single laser beam or subsequent lasers, it is easy to install the emission filter in the microscope cube filter and exchange the emission filters by simply rotate the cube filter holder especially that the new generation of microscopes are motorized and this process could be done

electronically. But in the simultaneous dual laser beam setup we have to add an arrangement of other long path mirror and emission filters as shown in figure 1. The fluorescence beams should come side by side in front of the CCD camera sensor. Thermo Fisher-Invitrogen microspheres model Tetra Speck with diameter 100 nm has been used for testing the alignment.

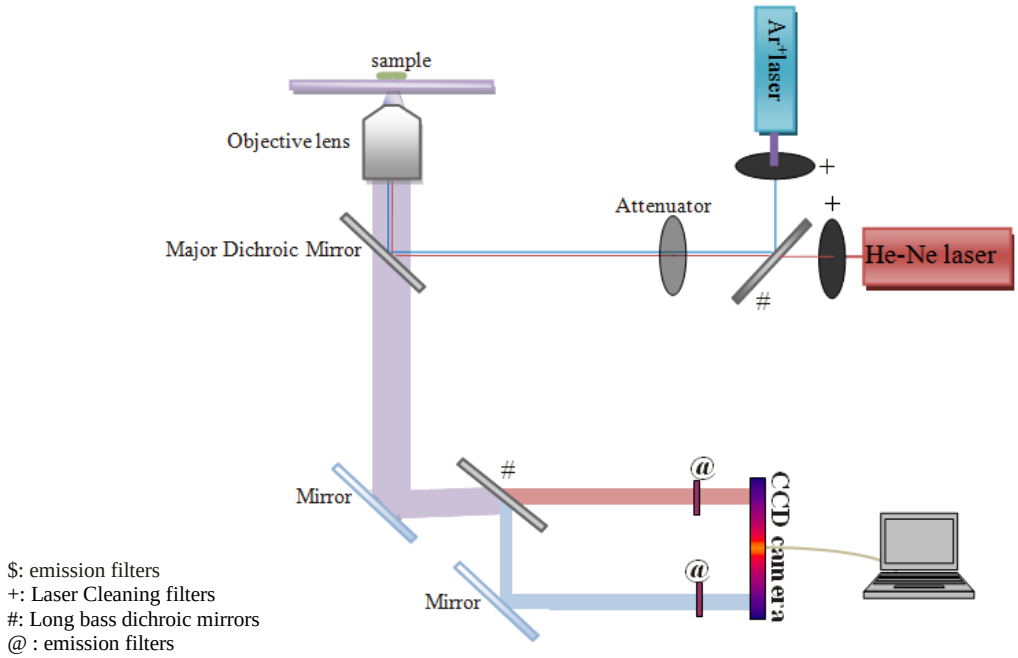

Figure 1: Simultaneous Dual color wide field microscope

RESULTS AND DISCUSSION

After setting up the system, the alignment was tested using microscope cover slip covered with tetra speck beads as shown in figure 2. The challenge with single molecule is that the signal comes from only single transmitter, this means that it is very weak.

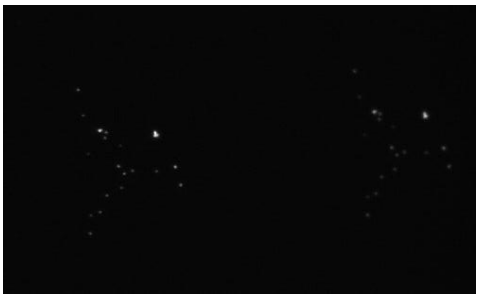

Figure 2 The tetra speck beads image through the red channel (to the right) and green channel (to the left)

For that reason and beside the appropriate setup with the most sensitive available detectors, all the reactants and the constituents used or fall in the objective focus must be tested from the auto fluorescent particles. Glass slides, all buffers used, water and objective lens oil were tested to insure that there are no any background signals and that the signal comes only from the sample.

Glass slides washed 10 times with methanol then 5 times with acetone in ultra sonic bath to remove any dirt or dust from the surface. The glass slides were mounted over the microscope and its upper surface get imaged after adding a drop of a mounting solution. Figure 3 show sample of non-autofluorescent glass slide and fluorescent ones.

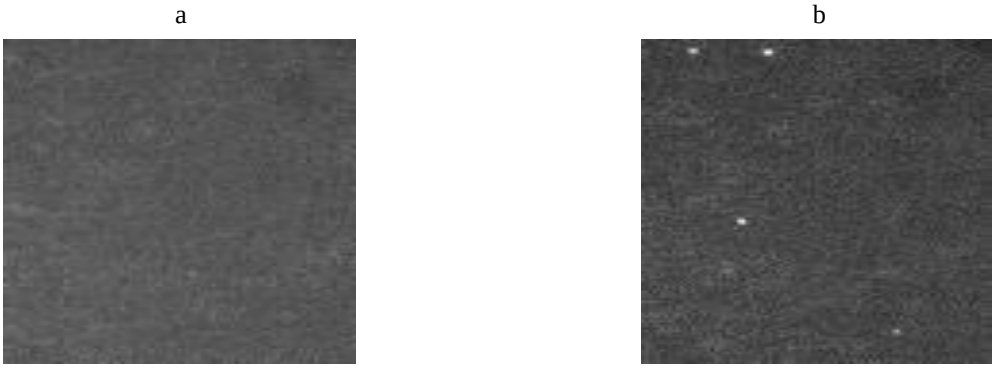

Figure 3 Glass slides test with only mounting solution a) non-autofluorescent glass slide. b) It is clear the bright spots resulting from the autofluorescent of the rare earth particles in the glass slide.

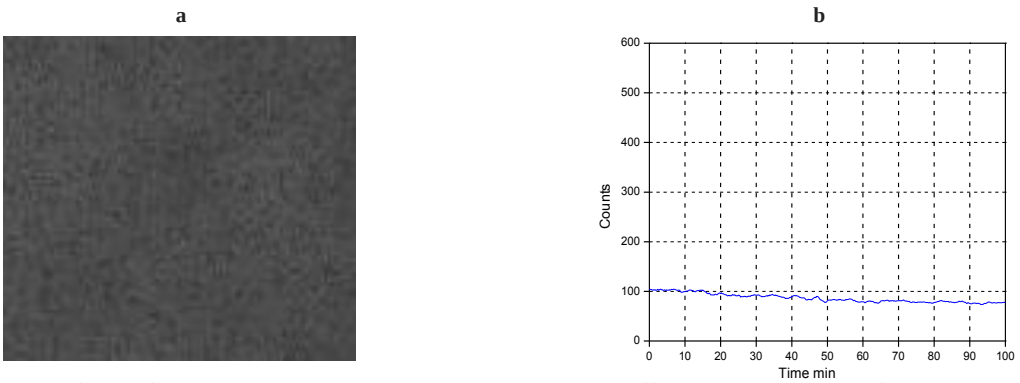

Figure 4 a) Image of drop of Milli Q water used in preparing the sample or the buffers. b) The time series of a point in image of only buffer solution.

Figure 5 Overlay of time series images of streptavidin labeled with Alexa633, measurement performed with an excitation wavelength of 632nm and an exposer time of 2s for every frame.

We did the same for the used buffer (the image is not presented) to be sure that nothing in the buffer is fluorescently active in the regime of our imaging, to know the level of background that permit to identify the signal in the real experiment as shown in figure 4b.

This test steps must be done every time especially for the buffer and the water used to insure that there is no microorganism growth in one from the constituents that may lead to unwanted fluorescence events. Moreover the glass slides should be tested one by one.

For the single molecule test we prepared a solution of 50 nM of streptavidin labeled with Atto655 purchased from Sigma-Aldrich GmbH, Steinheim, Germany in a 2.5ng/ml of Na_2CO_3. Normally the glass slide should be treated with biotinylated polyethylene glycol (PEG), but instead the glass slide cleaned and a 20 µl of the sample distribute gently on the top of glass slide swimming on the top of a cork in a very weak sonicator bath. This method is cheaper than biotinylated PEG and enough for this kind of experiments.

The image of Streptavidin lapelled with Atto 655 is shown in figure 5, the image cropped and enlarged 2 times to see only the active channel. The bright spots in figure 5 may represent either single molecule or more. For this reason and to be sure that we already have single molecules we did time series imaging, every single shot with exposer time 2ms and laser power 100 mW. Using image J software from National Instruments of Health, USA, we can overlay all the time series images and follow the intensity of every spot. The time series imaging with such high laser power causes bleaching for the fluorophore, if this bleaching went in single step to the back ground, this means that the spot represents single molecule. On the other hand, if the spot intensity decays to more than one step this means that we have aggregation of more than single molecule as shown for some spots in figures 6 and 7.

Figure 6 a, b and c show the intensity variation of only one spot along 100 seconds of illumination, in some spots the fluorescence intensity did not start from the zero time this might be because of the blinking of the dye that some time is consumed in the system adjustment, and the sharp focusing before starting the time series.

In figure 6d such spot is noticed which not blink in the beginning because blinking depends on the molecular dipole moment orientation. Figure 7 shows an example of spots that did not refer to single molecule, in figure 7a and b two steps decay is showed.

Figure 7b is an answer to the question if both dyes decay at the same time how can we determine if the spot refers to a single or two dyes? The answer is clear in the histogram that even if they will decay together they will not blink also together, in the histogram single dye blinked and the next fluoresces then both fluoresce and decayed together. Figure 7c shows an aggregation of more than molecule.

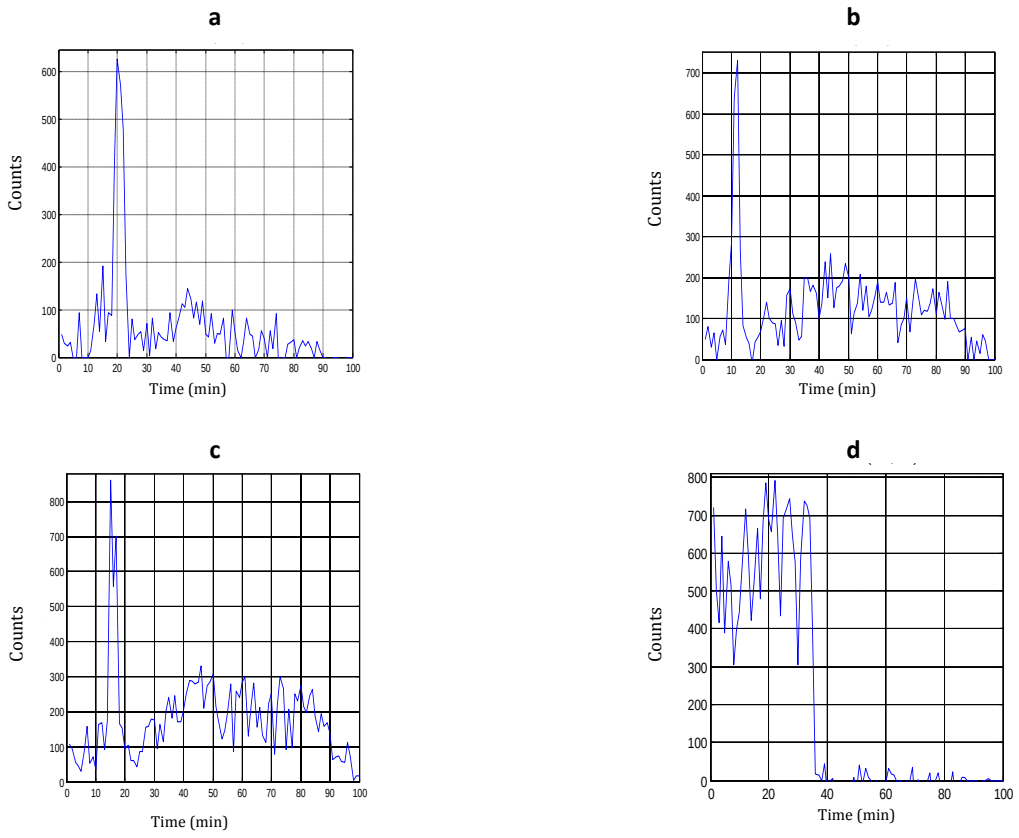

Figure 6: Photo-bleaching measurement for a streptavidin labeled with Alexa633. A time series of measurement performed with an excitation wavelength of 635nm and an exposer time of 2ms for every frame. Shows single step decay indicating a single molecule.

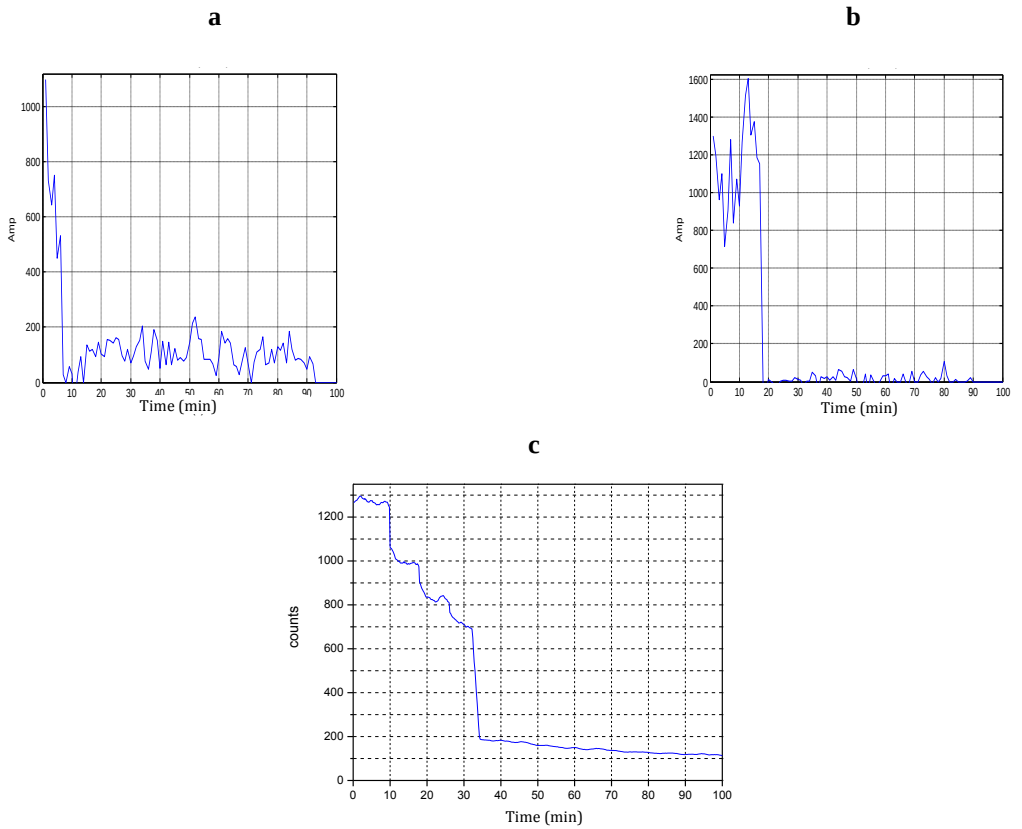

Figure 7: bleaching of single events in the overlaid time series images all show a)triple step decay representing 3 molecules in this position b) two step decay representing 2 molecules in this position c) multi-step decay representing aggregation of molecules in this position

CONCLUSION

Based upon the introduced technique and the test experiments, it is clear that single molecule spectroscopy is among the nascent tools in the analyses of biological interactions according to the following contradictory remarks:
The method used in glass slide preparation is efficient in this kind of experiments. The investigated system is now completely ready for single molecule imaging; on equal footing with other related methods according to the sample like photon induced electron transfer, and other convenient methods. The validity of the system to detect single molecules, in two channels simultaneously as seen in figure 2, has been verified.

For easier and faster imaging, the excitation beam has been modified by introducing an acoustic filter for imaging non-stable dye or biological sample.

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