

Using Single Molecule Microscopy Technique to Achieve Elongated Time-Period Blinking using Total Internal Reflection Technique

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Abstract

Recent advances in field of optical microscopy have made it possible to detect image and obtain spectroscopic data from individual molecules, revealing information about population distributions (both static and dynamic) as well as photophysical information. As higher levels of time resolution become available layers of dynamic information are uncovered, elucidating the fine detail of chemical reaction normally hidden within the ensemble. Single molecule (SM) techniques are especially relevant to macromolecular biochemistry as chemical processes occurring within large molecules are often too complicated to study in the ensemble.

Single Molecule Fluorescence Microscopy (SMFM) is achieved using coherent laser light to excite the fluorophore to a singlet excited state, from which it can relax rapidly. However this can be followed by either absorption of additional photons, eventually lead to an ionised or "photo-bleached" state, or spin-inversion and non-radiative intersystem crossing to a triplet state. The excitation volume has been reduced to achieve elongated time-period blinking using Total Internal Reflection.

Keywords: Quantum dot, Total Internal Reflection TIR, Single molecule SM, SMFM, Blinking

الخلاصة

ان التطورات الحديثة في مجال المجهر الضوئي جعلت من الممكن الكشف عن الصورة والحصول على البيانات الطيفية من الجزيئات الفردية، وكشف عن المعلومات حول توزيعات الكثافة (السكونية والدينامية على حد سواء)، بالإضافة الى المعلومات الإضافية عن الفيزياء الضوئية Photophysical. كما ان زمن التحليل يتواجد في المستويات العليا وطبقات عن المعلومات الحيوية تكون غيرمتاحة، توضيح التفاصيل الدقيقة للتفاعل كيميائي عادة مخفية. التقنيات ذات الصلة بالجزيء الواحد (SM) والمتعلقة بالكيمياء والعمليات الكيميائية التي تحدث داخل الجزيئات الكبيرة غالبا ما تكون معقدة للغاية للدراسة.

مجهر جزيء المضان واحد (SMFM) والذي يتحقق باستخدام ضوء الليزر لإثارة fluorophore إلى الحالة المثارة، والذي يمكن ان يعاني الاسترخاء بسرعة. ولكن هذا يمكن اتباعها من قبل أي من امتصاص الفوتونات إضافية، تؤدي في نهاية المطاف إلى حالة "الصورة ابيض" مؤقتة أو، أو تدور، وانعكاس معبر intersystem غير الإشعاعي إلى حالة الثلاثي. تم تخفيض حجم الإثارة لتحقيق ممدود الفترة الزمنية للومض باستخدام الانعكاس الداخلي الكامل.

الكلمات المفتاحية: كمية النقطة، الانعكاس الداخلي الكامل، الجزيء الواحد، مجهر جزيء المضان الواحد، ومض.

Introduction

Prevalent throughout teaching and published works of chemistry and biology are examples of reactions and processes occurring on an atomic/molecular scale. Despite these neat stoichiometric representations, relatively little is known about the individual dynamic processes that lead to reaction as most structural and kinetic information is garnered by measurement of ensemble averages. Direct observation of structural artefacts in materials and molecules has been made possible by techniques such as tunnelling electron microscopy (TEM) and atomic force microscopy (AFM) [Xie, 1998]. Though these techniques can image individual molecules (Fig.1). They are unable to provide spectroscopic or dynamic data [Selvin, 2008]. Recent advances in the field of optical microscopy have made it possible to detect [Moerner, 1989, Tittel *et al.*, 1997], image [Pinaud *et al.*, 2006, Kukura *et al.*, 2009] and obtain

spectroscopic data [Ha *et al.*, 1996, Lu *et al.*,1997] from individual molecules, revealing information about population distributions (both static [Ha *et al.*,1996, Lu *et al.*,1997] and dynamic [Schmidt *et al.*,1995, Geva *et al.*,1998]) as well as photophysical information [Wilfried *et al.*,2002, Lee and Osborne, 2007].

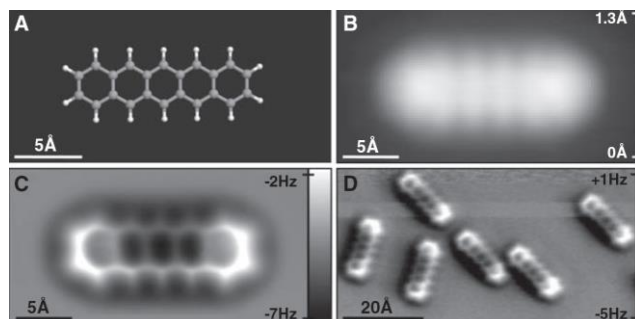


Fig.1 Pentacene imaged using AFM (Gross *et al.*, Science, 2007)

As higher levels of time resolution become available, layers of dynamic information are uncovered, elucidating the fine detail of chemical reaction normally hidden within the ensemble [Xie, 1998]. Single molecule (SM) techniques are especially relevant to macromolecular biochemistry as chemical processes occurring within large molecules are often too complicated to study in the ensemble [Selvin, 2008].

SM optical microscopy utilises fluorescent species, single molecule fluorescence microscopy (SMFM) measures the radiative emission of a fluorophore relaxing from an excited state to its ground state. In SMFM this is achieved using coherent laser light to excite the fluorophore to a singlet excited state, from which it can relax rapidly. However, this can be followed by either absorption of additional photons, eventually lead to an ionised or “photo-bleached” state, or spin-inversion and non-radiative intersystem crossing to a triplet state. Relaxation from this triplet state (phosphorescence) is spin-forbidden and therefore occurs much more slowly than fluorescence. The cycle of excitation/emission of a fluorophore can be seen in figure 2 [Davies, 2007].

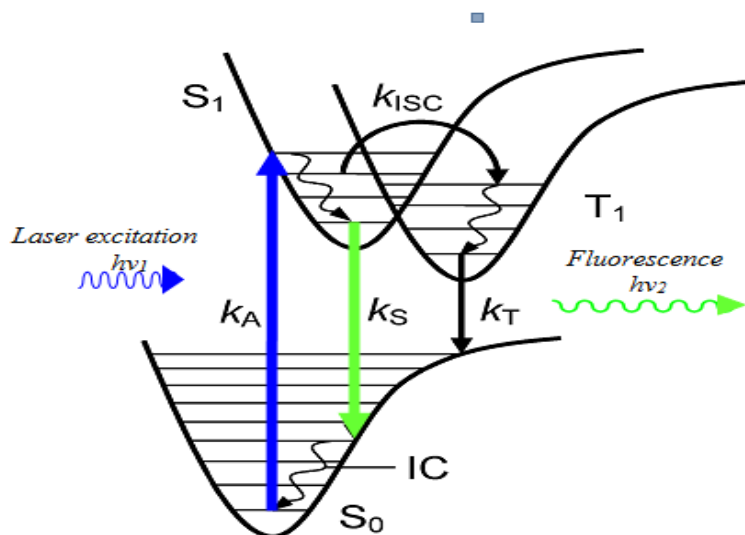


Fig. 2 Cycle of a fluorophore. $h\nu_1$ is absorbed, causing an electron to be promoted to an excited state Decay from excited state occurs either by direct emission (fluorescence) or intersystem crossing followed by phosphorescent decay from triplet state. Both routes produce emitted radiation that is Stokes shifted from the laser excitation wavelength due to rapid internal conversion [Davies, 2007].

Molecules, even those considered macroscopic in chemical terms are extremely small. A fluorophore with a high quantum yield (**QY** – ratio of photons absorbed to photons emitted) may emit strongly in the ensemble but produce an intrinsically weak signal at the single molecule level, while typically possessing a finite photochemical lifetime of approximately 10^6 cycles [Davies, 2007]. SM microscopy samples require very low concentrations to achieve spatial resolution of individual fluorophores which can lead to fluorescence signal becoming buried in background signal – noise. Noise can be divided into two forms; electronic and chemical.

Electronic noise, such as shot noise (caused by random fluctuations in charge) and thermal noise (caused by equilibrium fluctuations in charge) occurs within all electronic devices. Dark current occurs specifically within photo-sensitive detectors, mainly due to thermal activity. Thermal noise and dark current are both temperature dependent and as such can be significantly minimised by intense cooling of electronic equipment.

Chemical noise is due to intrinsic properties of both the fluorophore under investigation and the solvent or matrix which it resides within. Sources of chemical noise include elastic (Rayleigh) and inelastic (Raman) scattering and auto fluorescence (intrinsic fluorescence from solvent molecules or the fluorophore, a particular problem when probing biological macromolecules) [Selvin, 2008; Lakowicz, 2006].

Intrinsic fluorescence is dealt with by using fluorescent tags emitting in regions that are spectrally (Stokes) shifted from wavelength ranges identified as problem areas. Stokes shifting is also being utilised to greatly reduce interference from Rayleigh scatter of the excitation wavelength. As mentioned above, fluorescence wavelengths are red-shifted with respect to excitation wavelengths. With careful use of equipment including dichroic mirrors and long/band-pass filters, scattered laser light can be spectrally and spatially filtered from the signal, preventing saturation of the detector with unwanted wavelengths.

Raman scatter can be both Stokes and Anti-Stokes shifted and as such can overlap with emitted wavelengths from the fluorophore [Selvin, 2008; Lakowicz, 2006]. Use of time-gating equipment can differentiate effectively instantaneous scattering events from the longer fluorescence lifetimes, however the most effective method of reducing signal from inelastic scatter is severe constraint of the excitation volume, reducing the ratio of solvent molecules to fluorophores.

Different types of fluorescence microscopy approach volume reduction in different ways. For example, **confocal microscopy (CM)** confines the illuminated sample area in the xy plane (where the z axis is defined as the direction of the laser beam) by tightly focusing the beam, while allowing greater freedom along the z axis [Lakowicz, 2006]. This type of confinement is particularly suited to diffusion-type studies, where free molecules in solution can be studied moving in and out of the excitation volume, providing dynamic information. Conversely, **total internal reflection fluorescence microscopy (TIRFM)** is highly suited to molecules that can be immobilised on a substrate, as it confines the illuminated volume along the z axis; allowing a wide field to be studied (the TIR footprint) while still reducing the excitation volume to picolitres. This is achieved by an artefact of total internal reflection.

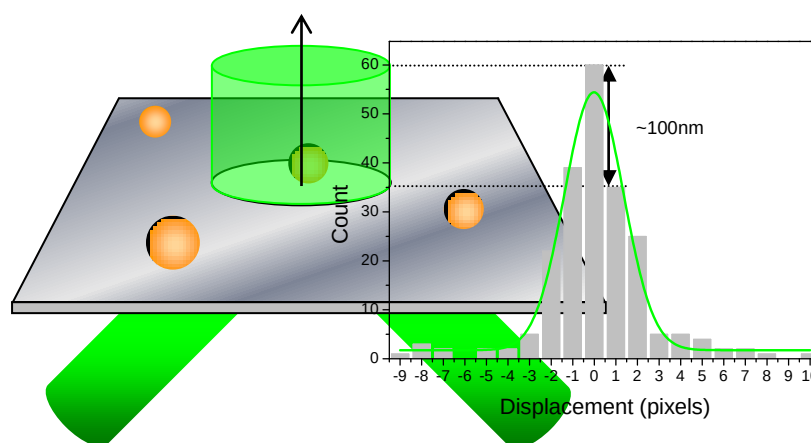


Fig. 3 Schematic representation of system undergoing total internal reflection. Incident radiation approaches interface between glass coverslip and aqueous sample containing fluorophores at critical angle and is totally reflected, propagating an evanescent wave. A wide field of illumination is achieved in a thin optical slice, exciting fluorophores contained within a picolitre excitation volume

The evanescent wave is a standing wave of infinite period, propagated when light is totally reflected at the interface of two media with sufficiently mismatched refractive indices. The wave penetrates ~100 nm into the sample and decays exponentially in intensity with distance from the interface:

$$E(z) = E(0) e^{(-z/d)} \quad \text{Eq 1}$$

Where:

$E(z)$ = Energy at distance z normal to the interface, $E(0)$ = Energy at the interface, d = penetration depth of wave into sample. d is defined as the distance into the sample that $E(z)$ has reduced to $(1/e)E(0)$ and is related by the following equation to:

$$d = \lambda(i)/4\pi \times (n(1)^2 \sin^2 \theta(1) - n(2)^2)^{-1/2} \quad \text{Eq 2}$$

λ_i = the wavelength of the incident illumination θ = the incident angle $n(1)$ and $n(2)$ = the refractive indices of the media at the interface (in the case of fig 3, the coverslip is defined as having refractive index $n(1)$ and the sample has refractive index $n(2)$).

Requirements for TIR: Light approaches the interface at incident angle θ . Above and at a critical angle θ_c (dependent on the media either side of the interface) the incident beam is reflected by the interface, back into the first medium. For total internal reflection to occur and an evanescent wave propagated, there must be a reduction in refractive index (n) across the interface between slide and sample (n_2 to n_1) [Alivisatos,1996]. The relationship between refractive indices and critical angle is described by **Snell's Law**:

$$\theta_c = \sin^{-1} (n_1/n_2) \quad \text{Eq 3.}$$

As example that is shown in figure 4, where n_2 = glass n_1 = water, the critical angle can be calculated as 60.1° .

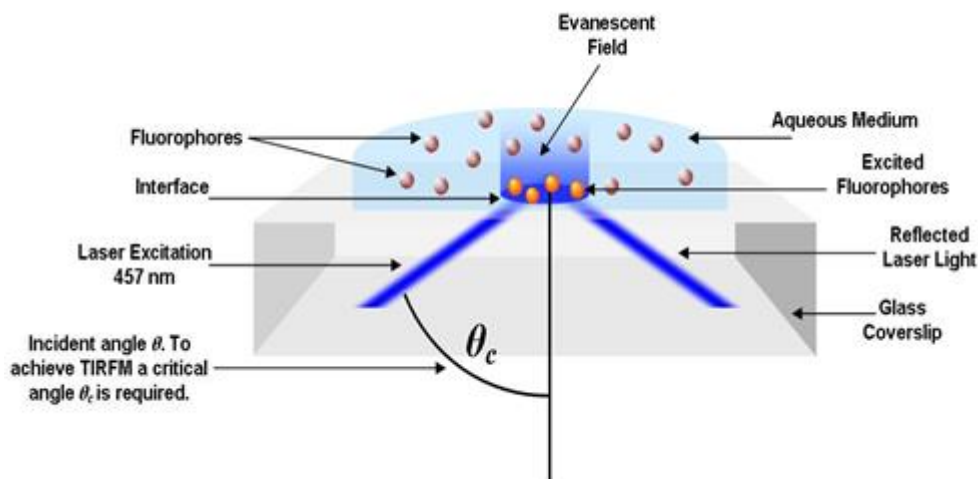


Fig. 4 Schematic representation of TIR showing refractive indices of glass coverslip and sample medium, as required for calculation of critical angle from Snell's law.

Experimental work

TIR microscope setup is used to produce quality data. The incident beam must be clean (having a Gaussian distribution with no high-frequency noise), circularly polarised (due to the dependence of photon absorption on correct dipole orientation, in TIRM molecules are not free to rotate and therefore show a distribution of orientations) and collimated (producing a beam of consistent width). Optical equipment used for this purpose is illustrated in figure 5. Once the laser beam (of appropriate wavelength to excite the fluorophore under study) has passed through the optical track it enters the microscope. Coherent laser light is produced by a Beamlok 2080 Argon/Krypton gas phase laser at a power of less than 40 mW. On leaving the laser, the beam is plane polarised and can feature unwanted high frequency noise,

undesirable features for SM microscopy. Fluorophore absorption of photons is dependent on dipole orientation, meaning that only a small proportion of molecules correctly aligned would be excited by plane polarised light. To obtain high quality results, a degree of beam attenuation is required before the microscope.

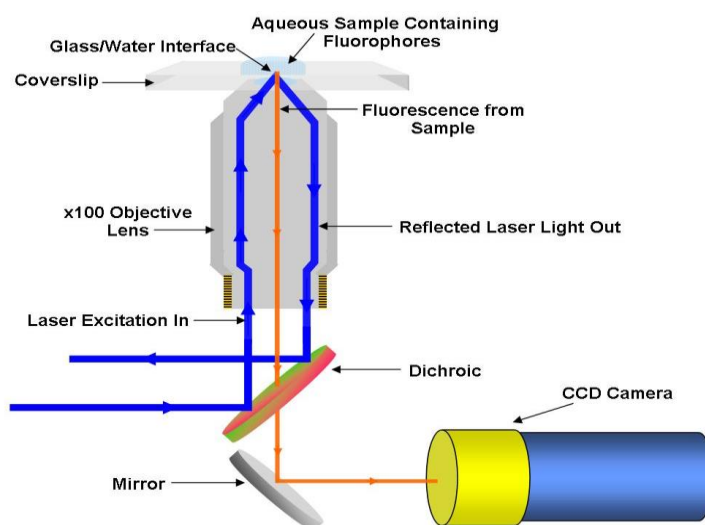


Fig.5 Optical track for TIRF microscopy. Coherent light is produced by an Ar/Kr laser, spatially filtered to produce a clean and columnated Gaussian beam, circularly polarised and attenuated using neutral density filters before being sent to the microscope. A part of the beam is split from the main path and tightly focused before recombining with the main beam for the purposes of automatically focusing the microscope.

Novel Autofocus Track

Presented study here is a novel method of autofocussing SM images during the course of experiments. A weak beam is split from the main TIR beam and passed through a zoom lens consisting of a matched pair of lenses to form a collimated beam with a much smaller cross section than the TIR beam. This is capable of being directed independently of the main beam, allowing it to be passed through the microscope slide at an angle as shown in figure 6. A macro in image processing software Image Scion tracks and records movement of the illuminated spot as the automated stage moves in the z direction and checks the spot position during image recording experiments, driving the automated stage to bring the image back into focus as required. Finer control of focus is achieved by a smaller illumination spot and a longer path across the xy plane.

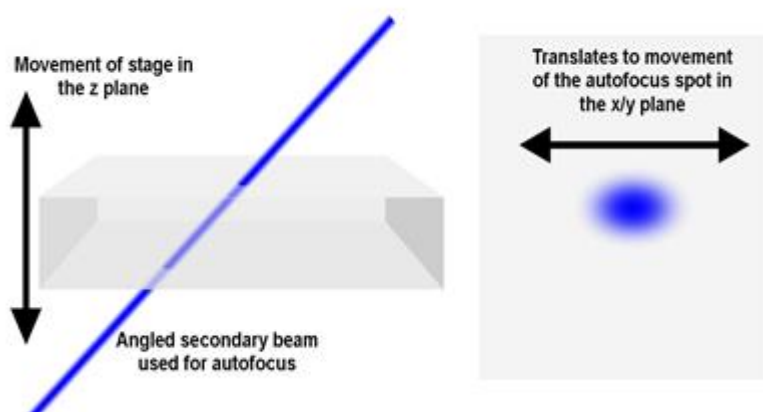


Fig. 6 Novel autofocus setup. A tightly focused beam secondary to the TIR beam penetrates the glass slide at an angle. Movement of the spot from this beam is recorded by image capturing software and used to drive the automated microscope stage allowing automatic fine focus.

Results and Discussion

Fluorescence Intermittency of a Single Quantum Dot

Streptavidin coated CdSe/ZnS QDs emitting at 650 nm were commercially sourced from Invitrogen and diluted in 0.1 M PBS buffer to a concentration of 1/10000 of the stock solution. These were investigated under TIR using excitation radiation from an Ar/Kr laser lasing at 488 nm with an illumination intensity of approximately $1.8 \times 10^3 \text{ W/cm}^2$. Image Scion was used to record images of the QDs and Image J was used to process the images, using a simple macro to obtain pixel intensities from a greyscale image. These were plotted as a function of time to demonstrate the stochastic nature of fluorescence intermittency at three orders of magnitude in time as shown in figure 7.

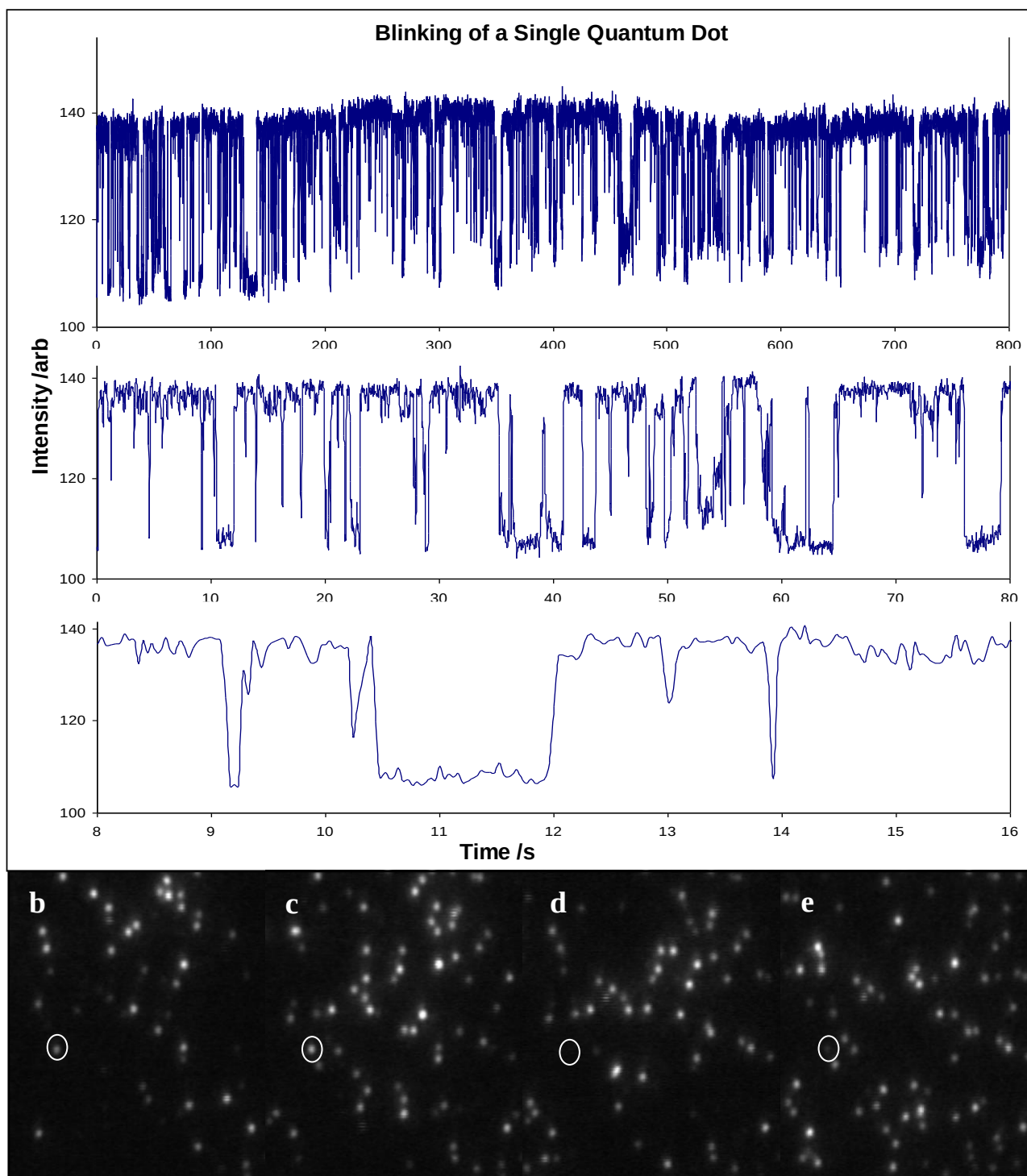


Fig. 7 Images and data obtained under TIR of commercially sourced, streptavidin-coated QD650. (a) shows 8, 80 and 800 seconds of light intensity produced by a single quantum dot. Highly binary fluctuations can be seen that occur regularly over a long time period. (b) through e show raw image data collected from the TIRF microscope taken at 23.12 s, 255.24 s, 363.40 s and 724.00 s respectively. The QD processed in (a) is indicated.

Conclusions

Single molecule (**SM**) techniques are especially relevant to macromolecular biochemistry as chemical processes occurring within large molecules are often too complicated to study in the ensemble. Single Molecule Fluorescence Microscopy (**SMFM**) is achieved using coherent laser light to excite the fluorophore to a singlet excited state, from which it can relax rapidly. However this can be followed by either absorption of additional photons, eventually lead to an ionised or “photo-bleached” state, or spin-inversion and non-radiative intersystem crossing to a triplet state. The excitation volume has been reduced to achieve elongated time-period blinking using Total Internal Reflection.

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