

Principles of the Detection of Single Molecules in Microscopy

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Abstract. Over the last decade, single molecule detection (SMD) of individual fluorescent chromophores has become a mature technique for spectroscopical and imaging purposes. In this contribution, the technical requirements for SMD concerning the fluorescent dyes as well as the microscopical setup are presented. In the last part, different criteria proving the detection of a single molecule emission are discussed.

Introduction

The fluorescence detection of single molecules in condensed phase has first been demonstrated more than a decade ago. First experiments have been performed at temperatures of liquid helium where single molecules can be selected due to their spectrally narrow, lifetime transform limited, zero-phonon lines [1,2]. Single molecule detection (SMD) has since become a valuable tool for biophysical research. Decisive for these applications was the visualization of single molecules even at room temperature single molecules with high sensitivity fluorescence microscopy [3]. Today, single molecule detection is either used as a spectroscopic tool or for the observation of movements of individual molecules even in living cells [4-6]. In this article, the requirements of a single molecule detection system based on the observation of single fluorescent dyes are sketched. Subsequently, the most important criteria which give evidence for the observation of single molecule are presented.

Fluorescent Dyes

Absorption and fluorescence. The photophysical behaviour of most fluorescent dyes can be described by a simplified Jablonski diagram with the singlet states S_0 , and S_1 and the triplet state T (Fig. 1).

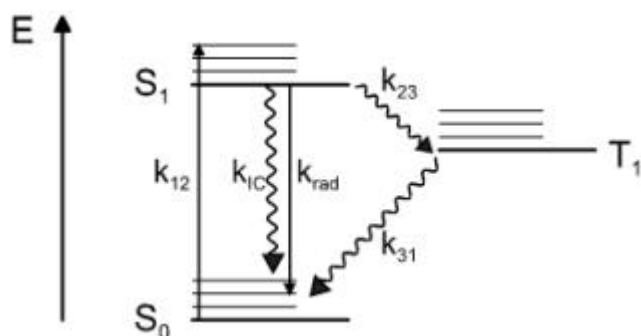


Fig. 1: Simplified Jablonski diagram of a dye molecule. The rate constants k_i are indicated. k_{21} as used in the text is the sum of $k_{IC} + k_{rad}$.

Excitation leads to a population transfer from the ground state S_0 to the first excited singlet state S_1 . This process with the rate constant k_{12} is dependent on the intensity I as well as on the absorption cross section (Eq. 1).

$$k_{12} = \sigma_{12}(\mathbf{n}) \cdot I_P \cdot \frac{1}{h\nu} \quad (1)$$

The absorption cross section σ is available from the extinction coefficient ϵ of the fluorescent dye. Typical maximum values for dye molecules which are appropriate for SMD are lying in the range between 1 and $4 \cdot 10^{-16}$ cm². Eq. 1 is correct as long as freely rotating molecules are investigated, however, when immobilized molecules are observed, up to threefold higher cross section have to be considered [7].

Several processes can take place from S_1 . Recovery of the ground state is achieved with the rate constant k_{21} via fluorescence emission or internal conversion (IC) in several nanoseconds. Although the fluorescence quantum yield Φ_{F1} is reduced by the latter, radiationless process, even fluorescent dyes like Cy5, which only possess moderate Φ_{F1} , can easily be detected. More problematic for SMD is the intersystem crossing (ISC) from S_1 to the triplet state T.

Triplet state and photostability. The extent of ISC influences the quality of SMD. Suitable fluorescent dyes exhibit ISC rate constants k_{23} which are well below 1% of the rate constant k_{21} . As the triplet state is rather long-lived (with the lifetime $\hat{\tau}_T$ in the μ s to ms-time range) compared to S_1 , saturation of the electronic system has to be taken into account. The emission rate constant k_{Em} , which indicates the yield of fluorescence photons from a single molecule, obeys a saturation behavior (Eq. 2).

$$k_{Em} = \frac{k_{12} \cdot \Phi_F}{1 + \frac{k_{12}}{k_S}} \quad ; \quad k_S = \frac{k_{21}}{C + 1} \quad (2)$$

The saturation rate constant k_S , which is equal to k_{21} in a two-level system, is reduced by $C+1$. C is the dimensionless product of the ISC-rate constant k_{23} and the lifetime of the triplet state $\hat{\tau}_T$. That the triplet state actually acts as a bottleneck, can be seen in SMD-experiments where the brightness of individual molecules is increased upon flushing with oxygen-rich atmosphere [8]. Oxygen is a well-known triplet quencher. However, this fluorescence enhancement occurs at the expense of the stability as the photochemical reaction of the triplet state with oxygen is supposed to destroy the fluorophore irreversibly [9]. In fact, the stability of fluorophores is the most crucial parameter in SMD. The quantum yield for photodestruction (photobleaching) is required to be much smaller than 10^{-5} [10]. Therefore, the population of the triplet state is detrimental in a two-fold way. On one hand, it leads to a reduction of the photon yield and while on the other hand the population of the triplet state is an important step in the photobleaching of the fluorophores.

Requirements for the setup

From the abovementioned estimation, one notices that typically not more than 10^8 fluorescence photons per second can be emitted from a single molecule due to saturation. This corresponds to an emitted power of less than 100 pW in the visible range of the spectrum. With this in mind, successful SMD poses three challenges: the delivery of sufficient excitation intensity, the removal of remnant excitation light and the efficient detection of the small number of fluorescence photons.

Excitation conditions. The intensity of the radiation is chosen so that a single molecule is excited 10^5 to 10^7 times per second. With a typical quantum yield for photobleaching, as stated above, the survival time of a single molecule is expected to be in the s-time range. The required excitation intensities are calculated from equation 1. Intensities of several kW/cm² are often used.

Light is best focussed by microscope objectives with a high numerical aperture (N.A.). Although several publications have shown that arc-lamps which distribute the power from the UV to the IR are bright enough for SMD [11,12], in most cases, lasers are favoured as excitation sources. As laser light can be focussed to a diffraction limited spot of a diameter of $\sim \lambda/2N.A.$, only several μ W of laser light are sufficient for SMD. For imaging of a larger area, proportional higher powers are needed.

Scattering, background and filtering. The fluorescence light stemming from a single molecule must be separated from reflected excitation light, elastically and inelastically scattered light, and from background fluorescence. The extent to which the reflected excitation light has to be rejected is determined by the ratio of the diffraction limited spot size to the absorption cross section of a single molecule. The factor is roughly 10^8 . This is achieved by epi-fluorescence excitation where the reflected light is $\sim 4\%$ of the illumination light for a glass-air interface, a dichroic mirror which has a high reflectivity ($>90\%$) for the excitation light and high transmittency ($\sim 90\%$) for the fluorescence light which is red-shifted to the excitation light (Fig. 2). Further suppression of up to 6 orders of magnitude is ensured by high performance bandpass filters. In the early days of SMD, colored glass filters were employed. These filters however exhibit autofluorescence, which is disadvantageous for SMD.

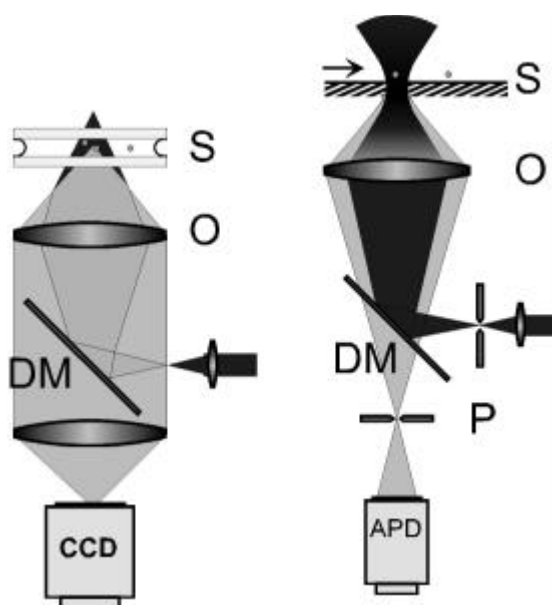


Fig. 2: Comparison of an imaging setup (left) with a sample scanning setup (right). Both microscope types are using epi-fluorescence excitation of the sample (S) via a dichroic mirror (DM) and a microscope objective (O). In wide-field imaging, a large sample area is imaged onto a CCD, whereas in confocal microscopy the fluorescence of a diffraction limited spot is detected by a point detector like an avalanche-photodiode (APD). An image is created e.g. by raster scanning the sample. In confocal microscopy, a better signal-to-background ratio is obtained because of the rejection of out-of-focus light by a pinhole (P).

More critical than the backscattered excitation light is light which is also wavelength shifted. One source is Raman scattered light. The cross section for this effect is for many media $\sim 10^{-28} \text{ cm}^2$ which is twelve orders of magnitude below a typical absorption cross section. Therefore, a single molecule can be detected in 10^{12} solvent molecules, which in water corresponds to a volume of $\sim 30 \text{ fl}$. Even smaller detection volumes of $\sim 1 \text{ fl}$ are realised in standard confocal microscopes. Further improvement of the signal-to-background is obtained by embedding single molecules polymer films of a few nm of thickness. The main source which might prevent SMD is the background contribution from weakly fluorescent impurities. A μM concentration of an impurity molecule which might have a $\ddot{O}_{\text{Fl}} \sim 10^{-3}$ easily exceeds the SMD-fluorescence. Consequently we exclusively utilize in our research solvents which are tested for their background fluorescence as are many HPLC-solvents. The background fluorescence is even stronger in living organisms where flavoproteins and NADH-related proteins are the main source for cellular blue-green autofluorescence [13]. These substances have their extinction bands in the blue-green part of the visible spectral region. Therefore, *in vivo* SMD experiments are facilitated when red fluorescent dyes are used.

Detection of the fluorescence of a single molecule. As a rough approximation, the fluorescence can be considered to be emitted isotropically. A large solid angle of this emission is collected with high N.A. objectives. The collection efficiency ζ_{coll} of objective lenses can be calculated from the aperture angle $\acute{\alpha}$ according to Eq. 3.

$$h_{Coll} = \frac{1}{2}(1 - \cos \alpha) \quad (3)$$

Losses of fluorescence light in the detection path are mainly due to interface reflections inside the objectives and of the filters. At last, the detection efficiency for the most widely used detectors lies in the range between 50 and 90%. The overall detection efficiency can be roughly estimated to be 3 to 10% and exact values are hard to measure [14]. When single molecules are located near an interface with different indices of refraction, one has to take into account that fluorescence photons are preferentially emitted into the medium with the higher index of refraction. This situation is further complicated, if polarization effects are considered [15,16].

Experimental setups. The benefits from the use of microscopic setup are threefold: high excitation efficiency, small detection volumes and good collection efficiency. Two general types of experimental realisation for SMD are nowadays established (Fig. 2). In „imaging“, an extended area, typically about several $100\mu\text{m}^2$, is imaged onto a charge coupled device (CCD) with a frame repetition frequency of up to 200 Hz. This method is used for the visualization of movements of individual molecules [6, 17]. If photophysical studies are the main interest, then „raster scanning“ of a sample area is preferred. In this case the use of point detectors like avalanche photodiodes (APDs) offers a higher time resolution than imaging detectors. However, in a typical probe scanning setup of this kind, the recording of an image of a larger sample area typically takes a minute. Further experimental options are discussed in ref. [18], which especially focuses on their use for SMD-studies of the widely used Green Fluorescent Protein.

Characterization of single molecule behaviour

When SMD experiments are performed, a main matter of concern is the verification that indeed the emission from only one single molecule is observed. In many cases, experimentalists rely on indirect phenomena like the expected brightness of a single molecule, the independence of the brightness of a single spot on dilution etc. There are however more direct criteria which can be consulted.

Antibunching. The most convincing experimental verification of the emission of a single molecule is this entirely quantum-optical effect [19,20]. Antibunching reflects the inability of an electronic two-level system to emit two photons at once. After the emission of a fluorescence photon, a new excitation process must take place before another fluorescence photon is emitted. The time spacing between both photons is therefore related to the fluorescence lifetime of the upper state. Antibunching is measured in a Hanbury-Brown and Twiss setup which consists of a start-stop experiment as in time-correlated single photon counting. Both the start and the stop event are triggered by fluorescence photons of the same molecule, each at a time. A normalized histogram of the measured time lags is shown in Fig. 3b.

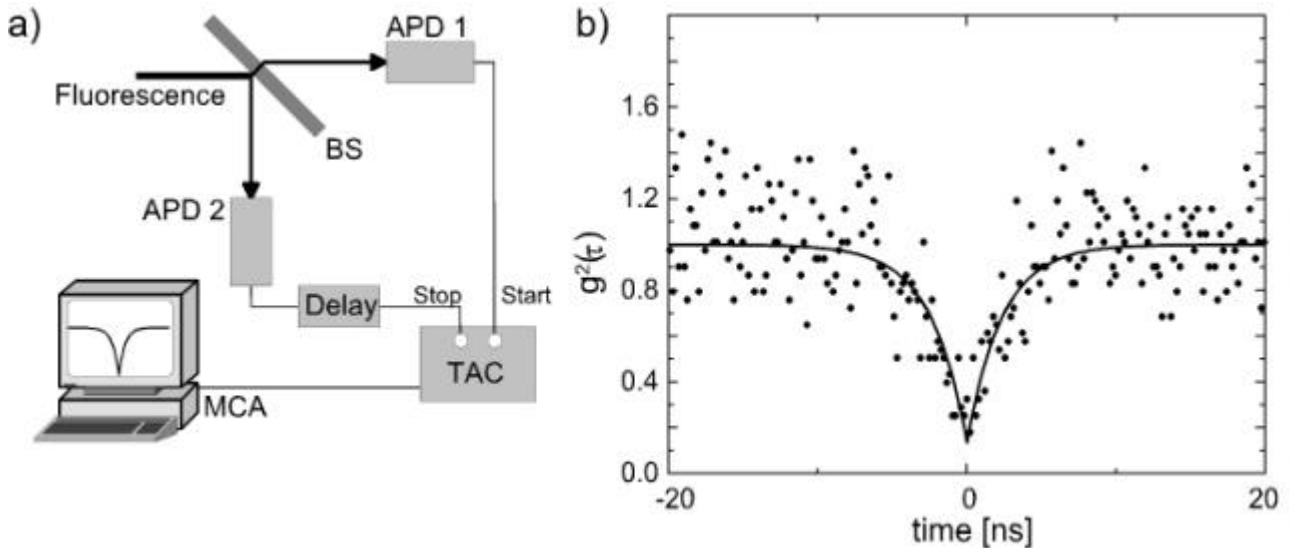


Fig. 3: Observation of antibunching (b) from single terrylene diimide (TDI) molecules in a Hanbury-Brown and Twiss experiment (a). In this start-stop-experiment, both the start and the stop signal are triggered by the detection of a fluorescence photon. Fig. 3b clearly shows the antibunching of photons emitted by a single TDI molecule for short times $t \approx 0$.

Although the observation of antibunching clearly indicates a single molecule, this proof is restricted to the most stable molecules.

Polarization dependent effects. The polarization properties of emitters immobilized in disordered samples provides another useful single molecule detection criterion. The transition dipole moment of a single chromophore is oriented along a certain molecular axis. If the molecules are immobilized, the transition dipole remains fixed in space. Excitation is only possible if the excitation polarisation is not perpendicular to this axis. The relative magnitude of the excitation efficiency is given by the square of the scalar product of the laser polarisation and the dipole moment (Eq. 4)

$$I_f \propto |\vec{\mu}_{12} \cdot \vec{E}|^2 \propto \cos^2 \theta \quad (4)$$

Rotation of the polarization about \hat{z} in time leads to a sinusoidal modulation of the emitted fluorescence light. This effect is not expected when an ensemble of molecules is excited. Here, due to the accidentally distributed orientation of many molecules, no modulation would be observed (Fig. 4).

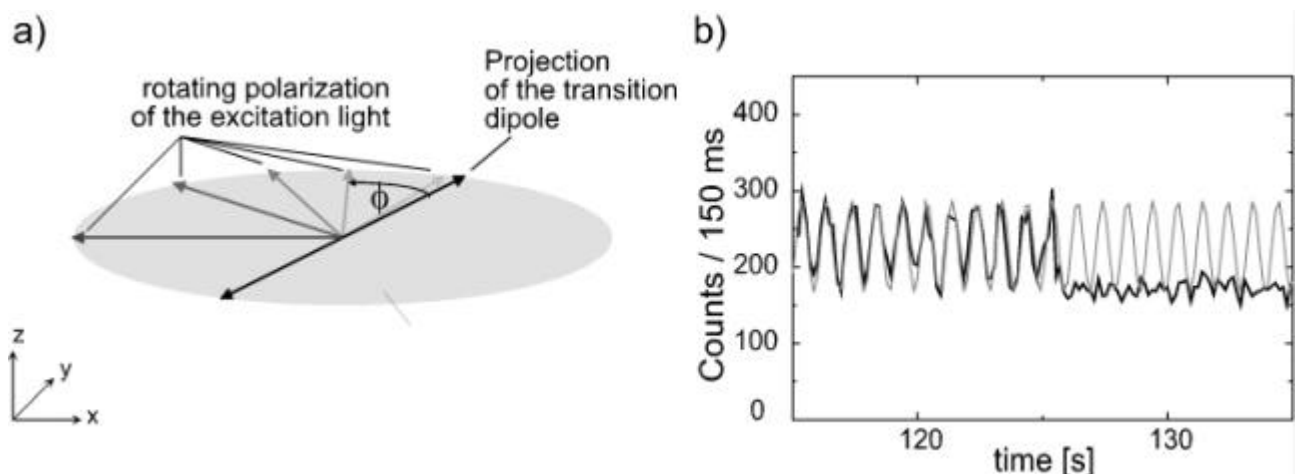


Fig. 4: Excitation anisotropy of a single molecule. The transition dipole of a single TDI molecule acts like a

polarizer on polarized light (a). The fluorescence of a single molecule is modulated by the rotation of the polarization of the excitation light (b). When the single emitter is bleached (at 125s), the background which is due to many impurity molecules, is isotropically excited.

On-off-dynamics. The fluorescence of a single molecule is not continuous but often interrupted by periods with no fluorescence (Fig. 5). These transitions are abrupt and might be due to e.g. intersystem crossing into the triplet state. Since no single-molecule luminescence is observed from this long-living state, it is called dark states. From histograms of the on and off-times, the rate constants k_{23} and k_{31} can be extracted [22]. In combination with time resolved lifetime measurements, a comprehensive photophysical characterisation of a single molecule is feasible. The easiest way to observe single molecule behaviour is to bleach it: the emission goes out in one step contrary to an ensemble where the fluorescence decreases exponentially.

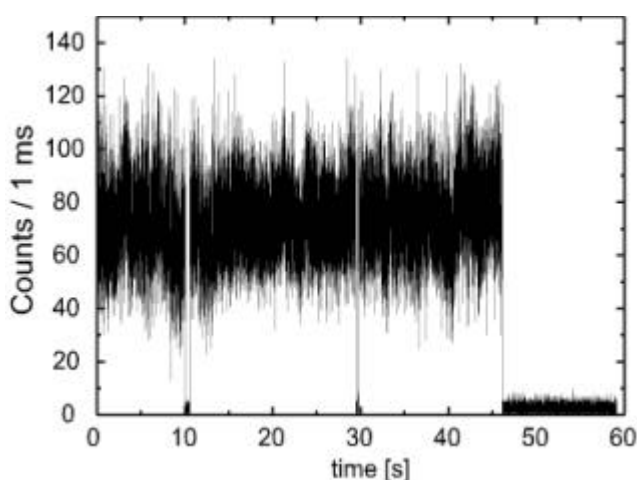


Fig. 5: A fluorescence time trace of a single (TDI) molecule. The fluorescence is two times interrupted before it bleaches completely at ~ 45 s. The strong fluorescence fluctuations are due to the population of the triplet state. In this example, the triplet lifetime has been determined to $\sim 30 \mu\text{s}$ and can therefore not be resolved in this representation. The typical on-time was $\sim 60 \mu\text{s}$ which corresponds to a quantum yield for intersystem crossing well below 0.1%. Without SMD, these values can not be determined.

Conclusion

SMD is nowadays a widely used method in many laboratories. With the use of home-built confocal microscopes and low-power lasers, an inexpensive realisation of an elementary SMD experiment is possible. If this experiment should be extended to in vivo measurements, most care must be expended to the purification of buffers and to the reduction of cellular autofluorescence. However, the use of excitation wavelengths in the red part of the optical spectrum also makes these challenges manageable. Due to the relative ease of the experimental setup, the ready availability of suitable, photostable and highly selective fluorophores, and the wealth of information obtainable from SMD experiments, the authors expect that use of SMD will further flourish and provide exciting new insights into the spectroscopical properties of fluorophores, but also into the biophysics of living organisms.

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