Single-molecule detection with a two-photon fluorescence microscope with fast-scanning capabilities and polarization sensitivity

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We describe a laser-scanning two-photon fluorescence microscope that is capable of observing single molecules with excellent temporal resolution and three-dimensional spatial resolution. To demonstrate the capabilities of the instrument we present single-molecule fluorescence data obtained in several different scanning modes. In addition, a polarization-sensitive detection scheme can provide detailed three-dimensional information about the orientations of molecules in any of these scanning modes. © 1999 Optical Society of America

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Most fluorescence spectroscopic methods probe an ensemble of molecules to describe the average properties of a system. As with any average, information about individual local environments is lost. Single-molecule fluorescence detection allows individual members of an ensemble to be observed, so that detailed information about local environments can be obtained. Monitoring a molecule's behavior over time can then offer insights about changes in these local environments. However, a number of challenges must be overcome to meet this goal. In particular, observation of single-molecule dynamics requires high signal-to-noise ratios and fine spatial resolution as well as fast data-acquisition rates. Various excitation and detection schemes have been developed to address these needs.[1,2] For example, two-photon excitation (TPE) of fluorescence has proved to be a useful tool in single-molecule detection.[3–6] TPE offers the advantage of limited background fluorescence because the probability of excitation is appreciable only at the diffraction-limited focal point of the beam.[7] In addition, since Rayleigh and Raman scattering from the excitation source differ significantly in wavelength from the fluorescence emission, they can be filtered out readily. Finally, single-molecule detection by use of TPE can be achieved with different fluorescence microscopy schemes, including near-field,[5] far-field,[4] and confocal.

Combining TPE with the aforementioned requirements for detection and observation of single-molecule dynamics poses challenges to the experimentalist. Since TPE occurs in a small localized excitation volume, one must employ a scanning technique to acquire images. Futhermore, a highly efficient detector is required for observation of fluorescence from single molecules. The optimal detector is a single-photon-counting avalanche photodiode (APD), which can have a quantum efficiency of up to 70%, with an extremely low rate of dark counts. However, these detectors have small active areas (<0.01 mm^2). Since avoiding signal losses requires that the magnified fluorescence spot remain within the active area, stage scanning is often used to acquire images when APD's are employed. Although effective, stage scanning is slow and somewhat imprecise because a large specimen support must be translated. Alternatively, the excitation spot can be scanned rapidly with a galvanometer-driven $x-y$ scanning system. In this case the entire image area is magnified to a size larger than the active area of the APD. Consequently, laser scanning to date has precluded the use of APD's with a fast-scanning system.[8] Here we describe an apparatus that circumvents these difficulties, allowing rapid-scanning TPE with highly sensitive APD detection, such that fluorescence from single molecules can be observed readily and on a fast time scale. Our technique has a data-acquisition rate for single-molecule data that is comparable with that of the wide-field TPE scheme reported recently by Sonnleitner *et al.*[6] However, in contrast with the wide-field scheme, our technique allows for the rapid acquisition of images over large sample areas while retaining the full three-dimensional spatial resolution available from diffraction-limited multiphoton excitation. With the addition of a polarization-sensitive detection scheme, this instrument also allows us to obtain single-molecule orientational information.

Figure 1 shows the experimental setup. A home-built Ti:sapphire laser pumped by a 5-W argon-ion laser is the TPE source for our apparatus. The laser produces pulses that are 35 fs in duration before the beam's propagation through the microscope's optical path. The beam passes through a variable attenuator and then is incident on an $x-y$ mirror-scanning system modeled after one described previously.[9] The galvanometer-driven $x-y$ scanner (Cambridge Technology) has a maximum pixel scan rate of 50 kHz. The pixel size is determined by the angular deviation of the scanning mirrors at each step, and the ± 15 angular range of the scanner therefore allows a large area of the sample to be observed in a single image.

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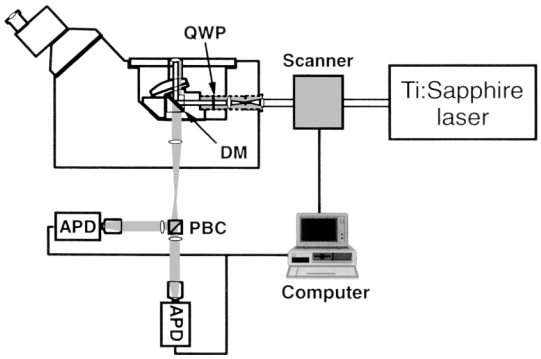

Fig. 1. Schematic diagram of the optical setup: DM, dichroic mirror; PBC, polarizing beam cube; QWP, quarter-wave plate.

The scanner is capable of rastering over a 256 × 256 pixel area in 0.524 s with a pixel residence time of 8 μs. Images with fewer pixels can be obtained correspondingly faster.

The scanning mirrors direct the beam through a 5× eyepiece and into the entrance port of a Zeiss Axiovert 100 inverted epifluorescence microscope. The beam is then polarized circularly by a quarter-wave plate, reflected by a dichroic mirror (Chroma Technology), and then focussed into the sample through a 40× Zeiss Fluar oil-immersion objective (numerical aperture, 1.3). Single-molecule fluorescence is collected by the same objective and then passes through the dichroic mirror. A polarizing beam cube positioned below the microscope's base port splits the fluorescence signal into two orthogonal polarizations so that orientational information about single molecules can be obtained. The image in each detection leg is demagnified to fit onto the active area of a single-photon-counting APD (EG&G SPCM-AG-161) by use of a tube lens and a long-working-distance 40× objective (Olympus LCPlanFl).

The apparatus is optimized by use of 15-μm fluorescent spheres (Molecular Probes). To compensate for chirp introduced by the optics in the experimental setup we minimize the TPE pulse width at the sample by maximizing the fluorescence intensity from a single sphere through adjustment of a pair of external fused-silica prisms. Single-sphere images are then used to determine the relative sensitivity of the two detection legs and to calibrate the physical size of an individual pixel.

Samples are prepared by spin coating a 10^{-11}-M solution of Rhodamine 6G in spectral-grade methanol onto the surface of a microscope coverslip that has been cleaned in the open flame of a Bunsen burner. The coverslip is affixed to a welled microscope slide and placed on the microscope stage. The sample is positioned electronically by a MAC 2002 (Ludl Electron Products), which is also used to control the focus of the microscope and to operate a shutter in the path of the excitation beam. Once the sample is in place, scans can be performed rapidly in zero, one, two, or three dimensions. We discuss all but the three-dimensional case here.

Figure 2 illustrates two-dimensional images acquired at different rates. The images shown, which are both 100 × 100 pixel squares taken from a 256 × 256 pixel scan, are the sum of the counts from both detectors. Image A was acquired in approximately 10 s at an excitation power of 3 mW. At this scan rate and excitation power, the molecules can be imaged several times before irreversible photobleaching occurs, permitting time-dependent studies of the dynamics of individual molecules. Image B was obtained in approximately 2 s at an excitation power of 15 mW. Although the signal-to-noise ratio decreases with increasing scan rate, the lower image shows that the molecules remain quite distinguishable at faster scan rates. The data-collection rate is thus limited primarily by the time required for one to obtain a good signal-to-noise ratio for a single pixel and by the number of pixels in an image, rather than by the rate at which a stage can be scanned.

Representative one-dimensional scans are shown in Fig. 3 for each individual detector and for the sum of both detectors. The lower half of Fig. 3 shows the integrated intensity profile for each molecule. A one-dimensional scan is produced by inhibition of the movement of one of the scanning mirrors, resulting in the repeated scanning of a single line of pixels. One-dimensional scans allow us to study the behavior of multiple isolated molecules on a time scale faster than is possible for two-dimensional scans. The scans in Fig. 3 demonstrate several different types of single-molecule dynamics. Molecules A, B, and C exhibit similar fluorescence dynamics, with each molecule yielding a fairly consistent amount of fluorescence

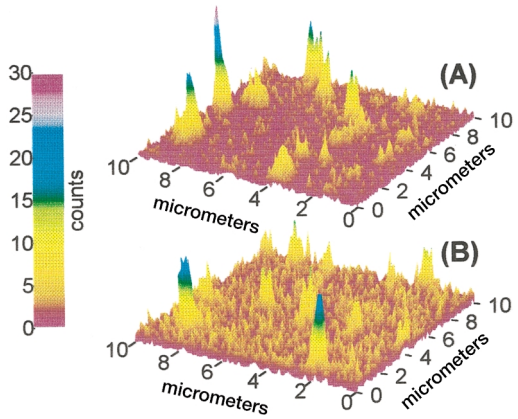

Fig. 2. Two-dimensional images taken at different scan rates and excitation powers: (A) 950 pixels/s at 3 mW, (B) 4750 pixels/s at 15 mW.

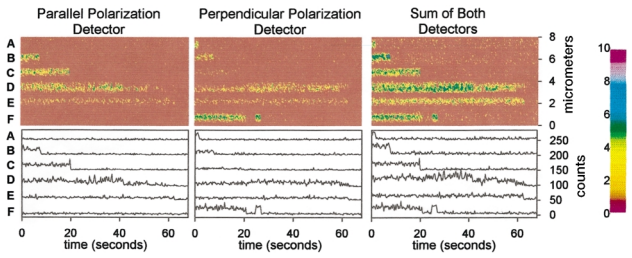

Fig. 3. Fig. 2. Composite images of one-dimensional scans. The top row is raw data, and the bottom row contains the integrated intensity profiles of the raw data. The integrated images are offset for clarity.

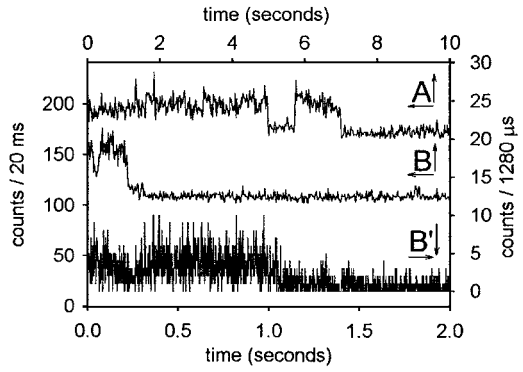

Fig. 4. Zero-dimensional time scans at different resolutions. (A) and (B) were binned at 128 time points/bin. (B′) is the first 2 s of (B) binned at eight time points/bin. The arrows accompanying each time scan indicate the appropriate time and counts axes.

per line before bleaching. Based on the signals from the individual detectors, it can be seen that molecules A and C are oriented predominantly along one polarization direction, whereas molecule B is oriented at an angle between the two polarizations. Molecule D is an example of a case in which the fluorescence intensity is highly variable in time. Although molecule E is in focus, it exhibits low fluorescence counts in both channels. As a result, we can conclude that the transition dipole of the molecule is oriented out of the plane of the coverslip, although the molecule can still be detected because of the high numerical aperture of our system. The last molecule shown (molecule F) exhibits fascinating dynamics. The molecule fluoresces initially for ~20 s and then turns off for approximately 5 s. The molecule again fluoresces for a few seconds before bleaching permanently. Note that if one collected the fluorescence without separating the light into the perpendicular polarizations, one could not say with certainty whether molecule F ceased to fluoresce or rotated out of the plane of the coverslip during the 5-s dead period. Our data allow us to rule out the latter possibility.

Figure 4 displays two samples of zero-dimensional data, in which the laser is focused onto a single molecule and data are collected as rapidly as possible (each time point is approximately 160 μs of fluorescence data). The data for molecules A and B are then binned, with each bin containing 128 time points. Each data point on the curve represents one bin, or approximately 20 ms of fluorescence. The images in Fig. 4, which are the sum of both polarization channels, display similar dynamics to those in Fig. 4, except that we have collected more data points in a shorter amount of time. Molecule A fluoresces for 10 s, turns off briefly, fluoresces again for 3 s, and then photobleaches permanently (similar to the behavior of molecule F in Fig. 3). The behavior of molecule B is also similar to the curves in Fig. 3. The bottom scan in Fig. 4 (labeled B′) is the first 2 s of data for molecule B binned instead at eight time points/bin (approximately 1 ms of fluorescence data per bin). This scan demonstrates that we are able to monitor single-molecule dynamics with good resolution on a very fast time scale.

In conclusion, we have demonstrated an apparatus that incorporates two-photon excitation, polarization sensitivity, and a novel detection system into a fast-scanning, far-field microscope that has the ability to monitor fluorescence from single molecules. Our instrument is capable of collecting single-molecule fluorescence images over a large sample area while retaining the full three-dimensional resolution benefits of multiphoton excitation. Data can be collected on a fast time scale in several different scanning modes and can provide detailed information on molecular orientation in any of these modes. This instrument will be valuable in numerous applications in chemistry, biology, physics, and other areas of science.

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