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IMAGING BIOLOGICAL MOLECULES WITH SINGLE MOLECULE SENSITIVITY USING NEAR-FIELD SCANNING OPTICAL MICROSCOPY

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ABSTRACT

We have developed a near-field scanning optical microscope with the sensitivity to detect single fluorescent molecules. Our microscope is based on scanning a sample under a tapered and metal coated fiber optic probe and has an illumination-aperture diameter as small as 100 nm. The microscope simultaneously acquires a shear force (height) image with a height noise of ~ 1 nm. We have used this system to demonstrate the detection of single molecules of Rhodamine-6G on silica. In this paper, we explore the use of NSOM for investigations of biological molecules. We have prepared and imaged double-stranded DNA intercalated with thiazole orange homodimer (TOTO); single chromosomes stained with propidium iodide; and β-phycoerythrin proteins on dry, borosilicate-glass surfaces. At very dilute coverages, isolated fluorescent spots are observed for the un-intercalated TOTO dye and for β-phycoerythrin. These fluorescent spots exhibit emission intensity fluctuations and abrupt bleaching transitions, similar to the intensity behavior observed previously for single Rhodamine 6G molecules on silica.

INTRODUCTION

We are investigating the application of near-field scanning optical microscopy (NSOM) to imaging molecules of biological interest. We have built an NSOM with lateral resolution better than the diffraction limit. The microscope also has the sensitivity to locate the positions of single fluorescent dye molecules on the surface of a transparent sample.[1,2] NSOM is a scanned probe microscopy, where a tiny light source is held in close proximity to a sample surface.[3] The probes are fabricated by tapering and metal coating single mode optical fibers, while leaving a small aperture in the metal at the tip.[4] The light source in NSOM is effectively a small aperture in an opaque metal film illuminated with laser light.

The small aperture size (~ 100 nm) allows molecules to be excited with high irradiance (0.1 to 1 kW/cm^2 without significantly heating the tip) with low power (nanowatts). Most of the background scattered light is from the optical fiber and tip structure. Low power results in low background scattered light and low background noise. Since the background level is small, single molecule fluorescence signals are observed above the noise in the background under ambient conditions.

The high resolution and sensitivity inherent in NSOM has been used, for example, to image the locations of multiple fluorophores attached to chromosomes[5]; single fluorescent allophycocyanin protein trimers[6] and the light harvesting complexes[7] involved in photosynthetic systems; and fluorescently labeled actin filaments in fixed cells[8]. In this paper, we present preparation methods and images for several different types of biological molecules on dry cover slips: fluorescently labeled hamster chromosomes; thiazole-orange-homodimer (TOTO) stained DNA

stretched out on a glass surface; and a protein, β-phycoerythrin, present in the photosynthetic complexes of algae.

Optical near-field measurements on single fluorophores produce results that are qualitatively different than measurements on many molecules.[1,2,9-13] Single molecules are observed as isolated spots in fluorescence images of low coverage samples. We have located single Rhodamine 6G (R6G) molecules on bare silica substrates in air with ~ 100 nm spatial resolution. By positioning an NSOM probe over single R6G molecules, we have observed photobleaching of individual R6G molecules.[1] Single molecules on silica bleach abruptly and display fluorescence intensity fluctuations instead of fading gradually.[1,11] In this paper, we find that low coverage samples of TOTO dye and β-phycoerythrin produce isolated fluorescent spots. Results of the time dependent intensity measurements on fluorescent spots observed in samples prepared with TOTO stain, and β-phycoerythrin are presented in the remainder of this paper. These spots display intensity fluctuations and photobleach abruptly, as well. This behavior is emerging as a ubiquitous feature of fluorescent samples at very low coverages using NSOM.

EXPERIMENTAL DETAILS

Details for the preparation of submonolayer coverages of R6G on silica glass can be found in previous work.[1,2] The method involves spin coating and evaporation of an R6G-methanol solution on cleaned silica or borosilicate surfaces.

β–phycoerythrin was deposited on cover slips in a similar manner. Borosilicate cover slips (Corning 0211 glass) were cleaned in NaOH and water, and dried in air. An ammonium sulfate solution of β–phycoerythrin (Molecular Probes, Inc.) was dialyzed against an aqueous solution of phosphate buffered saline (PBS) to exchange the buffers. The β–phycoerythrin/PBS solution was then spin coated on the cover slips at a concentration of $3x10^{-8}$ M. This solution was dried also between a slide and cover slip while observing the bulk fluorescence under an epi-fluorescence microscope. The fluorescence was irreversibly reduced upon drying. The cause of the modification is not known. In the rest of this paper, β–phycoerythrin spin coated and dried on cover slips will be referred to as drying-modified β–phycoerythrin.

Samples of DNA on cleaned cover slips were prepared as follows. Streptavidin was diluted in carbonate buffer and dried on one surface. Excess carbonate and streptavidin were removed by carefully rinsing the surface with pure water. The DNA (λ phage DNA, 48.5 kbp, 16 μm long, labeled at both ends with biotin from New England BioLabs) was stained with an intercalating dye, thiazole orange homodimer (TOTO-1, Molecular Probes, Inc.), using standard protocols.[14,15] The stained-DNA solution was applied to the streptavidin coated cover slips and allowed to incubate at room temperature for 30 minutes. The samples were observed while wet using conventional epifluorescence microscopy to ensure that binding of the biotin to streptavidin had occurred. In most cases, single DNA ends were bound to the glass. The DNA was then stretched out on the glass surface by either allowing the liquid to evaporate or mechanically removing the cover slip from a slide. During the drying

process, the tethered end of the DNA remained fixed, the free end remained in solution, and the DNA stretched out as the liquid meniscus passed over the surface.[16]

Samples of chromosomes appropriate for imaging in a scanned probe microscope were prepared as follows. Hamster-human hybrid cells containing human chromosomes 4 and 8 (cell line UV-20HL-21-27, courtesy of Larry Thompson at Lawrence Livermore National Lab) were grown in tissue culture flasks. The cell growth cycles were synchronized with colcemid to produce metaphase cells. Chromosomes were then isolated from the suspended metaphase cells according to the Aten procedure[17], which preferentially stains the chromosomes with propidium iodide over other cell extracts. Droplets of chromosome solution were placed on clean cover slips, and placed in a humid atmosphere to prevent drying. After an hour, the chromosomes settled onto the cover slip at the bottom of the droplet. The remaining suspension of buffer and cellular debris was then slowly exchanged with pure water by repeated dilution via pipetting to and from the droplet, with flow rates slow enough to prevent resuspending the chromosomes. Then the samples were dried in air. Samples prepared in this way prevented smearing of the chromosomes onto the cover slips and their surfaces were exposed to air without an over layer of cellular debris.

Our near-field microscope[1,2] is similar to those described previously.[3,4] The probes were tapered single mode optical fibers, that had been coated with an opaque film of aluminum such that a small optical aperture remained at the tip.[1-4] For these experiments, 0.1 to 100 nW of continuous wave argon ion laser light (514.5 nm wavelength) emerged from the tip, and this was used to excite fluorescence from

objects on the surface. Fluorescence was detected using photon counting techniques while scanning the sample under the probe to produce the optical image. A technique known as shear force microscopy (SFM)[3] was used to hold the sample close to the tip (< 10 nm) while imaging. SFM measures surface-height images; we obtained height images concurrently with fluorescence images.

RESULTS

A fluorescence excitation image of Rhodamine 6G on silica taken in air at room temperature is shown in Fig. 1. The image is 2.6x2.7 μm^2 in size. Each fluorescent spot shows the location of a single R6G molecule on the silica surface. The spots have a full width at half maximum (FWHM) diameter of 100 nm. An individual dye molecule at room temperature has an optical cross section with a lateral extent of < 1 nm, i.e., the molecule is excited by the optical electric field in a region much smaller than the spot size in Fig. 1. Since fluorescent molecules can be thought of as a point probes or detectors of the electric field distribution at the end face of an NSOM probe,[9] they can be used to determining the approximate size of the near-field aperture. We estimate the illumination aperture diameter of the probe used to image Fig. 1 to be ~ 100 nm. This spot size is smaller than the diffraction limit for a far-field focused laser beam using a high numerical-aperture lens (~ $\lambda/2$ = 257 nm).

Figure 2 shows the photobleaching behavior of single R6Gs. These traces were obtained with the NSOM tip held in position above single fluorescence spots such as those in Fig. 1. The traces in Fig. 2 (a)-(d) are for single molecules, and traces (e) and (f) are for dark regions of a sample (no R6G molecules). Traces (e) and (f)

show the background level, and the level of systematic fluctuations in this experiment. For the single molecules in traces (a)-(d), the emission rate starts out above the background, fluctuates for a time on the order of a minute, and then drops to the background. Images taken tens of minutes after such experiments show that the fluorescence does not recover. This form of photo-induced bleaching is very different from bulk measurements with many molecules, where a gradual fading of the signal is observed. Here, a single molecule is repeatedly cycled through its ground and excited states, giving rise to long periods of approximately constant fluorescence. During one of the optical cycles the molecule undergoes a photo-induced transformation (possibly photochemical) and stops fluorescing.[1] When a large number of molecules are bleached together, the photon shot noise in the total signal is larger than the signal from a single molecule, and individual bleaching events are not observable. Abrupt changes in intensity, and other long term variations are observed in the fluorescence signals in Fig. 2 (traces a through d) before the final bleaching transition. There has been speculation on the origin of the fluctuations,[1,11] but direct evidence linking them to a specific microscopic processes has not been reported. Again, these fluctuations cannot be observed above the shot noise in the signal from many molecules.

The high spatial resolution in NSOM is useful for imaging heterogeneous samples and can be used to study biological molecules. Figure 3 shows an example of a hamster chromosome stained with propidium iodide. The image on the left is the fluorescence excitation image, and the image on the right is the shear force, or height image. The chromosome is 6 μm long, each chromatid arm is 2 μm wide, and the

height is 1.3 μm. Carefully preparing the samples with the method described above prevents the chromosomes from being spread out and flattened on the surface. High spatial frequencies are observed in the optical image, and may be due in part to variations in dye incorporation near the surface of the chromosome. Dye within the chromosome at depths deeper than the near-field extent (further than the aperture diameter) is effectively "out-of-focus", and will contribute a more slowly varying background. One is cautioned that height variations of the tip above the surface as it scans across steep edges will contribute high spatial frequencies to the optical image, as well. Fluorescence in-situ hybridization (FISH) chemistry is a method for attachment of fluorescent probes to specific locations along DNA. Recently, FISH labeled chromosomes were imaged using AFM and NSOM.[5] Chromosomes subjected to FISH chemistry were found to be flatten to < 100 nm in thickness. Presumably the protein scaffolding was disrupted. Since FISH labeled chromosomes were thinner, the portion of a chromosome beneath an NSOM probe was entirely within the near-field (i.e. in focus).[5]

Fluorescence excitation images for TOTO-stained λ-DNA stretched out on a cover slip are shown in Fig. 4. The image on the left is 14x14 μm^2 in size. The locations of many individual DNA strands are detected by the fluorescence excited from the intercalated TOTO dye. The strands appear to be ~ 150 nm wide, narrower than the diffraction limit. (The physical width of the DNA is ~ 2 nm). The region within the white box in (Fig. 4 (a)) was scanned to show the L shaped strand in more detail (Fig. 4 (b)). The fluorescence is not uniform along the length of the strand. This could be due to several effects: non-uniform staining, energy transfer effects, or simply

break-up and relaxation of the strand after deposition on the glass surface. In addition to the DNA strands, there are fluorescent spots in the background. Control samples prepared by spin coating TOTO dye without DNA do not have the stringy DNA features. The fluorescent spots are due to the un-intercalated dye deposited on the glass. The spot density is an order of magnitude lower than compared with spin coating of R6G, but the intensities are comparable to single R6G molecules. It is possible that the individual spots in Fig. 4 are from aggregates of between 10 and 30 TOTO molecules, and may not be from single TOTO fluorophores.

Figure 5 shows the photobleaching behavior the individual fluorescence spots on TOTO control samples (without DNA). As observed for the single R6G molecules, these spots display an abrupt bleaching transition to the background level. Intensity fluctuations are observed also, and the rate of fluctuations appears to be much higher than for R6G.

Images of dilute samples of β-phycoerythrin spin coated on cover slips contain isolated fluorescent spots, as well. Photobleaching of these fluorescent spots is shown in Fig. 6. β-phycoerythrin is a protein with 34 fluorophores contained in a volume approximately 10 nm in extent.[18] The fluorophores are spaced closely enough that electronic excitation energy is rapidly transported throughout the system.[18] When β-phycoerythrin was spin coated and dried on a glass surface, the fluorescence intensity was observed to decrease significantly with the result that the individual spots observed in NSOM images were comparable in brightness to single R6G. β-phycoerythrin in solution are 25 times brighter than individual R6G.

Possibilities for the reduced emission intensity include increased quenching of fluorescence within intact β-phycoerythrin or disruption of the protein into smaller fragments. In any case, the bleaching of these single spots occurs as an abrupt, final drop to the to the background level. In the longer time traces in Fig. 6, intensity fluctuations are observed also.

SUMMARY

We have investigated the preparation and imaging of several types of biological molecules using NSOM. A method for preparing chromosomes that preserves their gross structure is presented. Near-field imaging of fluorescently labeled DNA stretched out on a surface is presented. Dilute samples of drying-modified β-phycoerythrin produce images with individual fluorescent spots.

In all cases where the samples are dilute, isolated fluorescent spots are observed. R6G, TOTO, and drying-modified β-phycoerythrin all have this feature. For R6G, control experiments were performed to be sure the spots were from R6G and not from a residual impurity in the sample preparation.[1,2] The fluorescence intensity was recorded with time by positioning the NSOM tip over the individual spots. In all of these systems, abrupt photobleaching and intensity fluctuations were observed. The R6G system has been studied more extensively, and strong evidence has been presented[1,2] that the individual fluorescent spots observed in NSOM are due to individual R6G molecules. Abrupt photobleaching occurs for a single fluorophore as follows: during optical cycling through the ground and excited states, the molecule fluoresces with approximately constant mean rate, and with low probability during a

single optical cycle undergoes a photo-induced transformation to a non-fluorescent condition. For the newer systems, TOTO and β-phycoerythrin, it is not yet known if the single fluorescent spots are due to single fluorophores or aggregates of molecules. If they are small aggregates, the abrupt bleaching could occur when one of the molecules in the aggregate undergoes a photo-induced transformation to a strongly non-radiative species, which becomes a trap and sink of the excitation energy.[19] The fluorescence of the entire aggregate then would be quenched. Photobleaching of much larger aggregates (~ 10 μm in extent), known as J-aggregates, has been studied in NSOM.[20] The bleaching is localized to small regions of the aggregate, on-the-order of both the NSOM aperture diameter and the migration length of the excitons (shared electronic excitations within aggregates). The abrupt photobleaching behavior is not observable in bulk measurements. Despite the unknown number of fluorophores in the fluorescent spots for TOTO and β-phycoerythrin, this very different intensity versus time behavior highlights one of the important aspects of studying matter in the highly divided limit; qualitatively different phenomena are observed at the single particle level that are not observable in ensemble measurements.

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FIGURE CAPTIONS

Figure 1. Fluorescence excitation image showing the locations of single Rhodamine–6G molecules on a silica surface. The image size is 2.6x2.7 μm^2. The fluorescent spots can be viewed as images of the 100 nm NSOM aperture as detected by single point like R6G molecules.

Figure 2. Fluorescence intensity versus time for single Rhodamine 6G molecules. Traces (a)-(d) were taken on single spots such as those in Fig. 1, and traces (e) and (f) are on dark regions. Each traces are offset by multiples of $1.5x10^4$ counts/sec. The power out of the near-field tip was 0.1 nW.

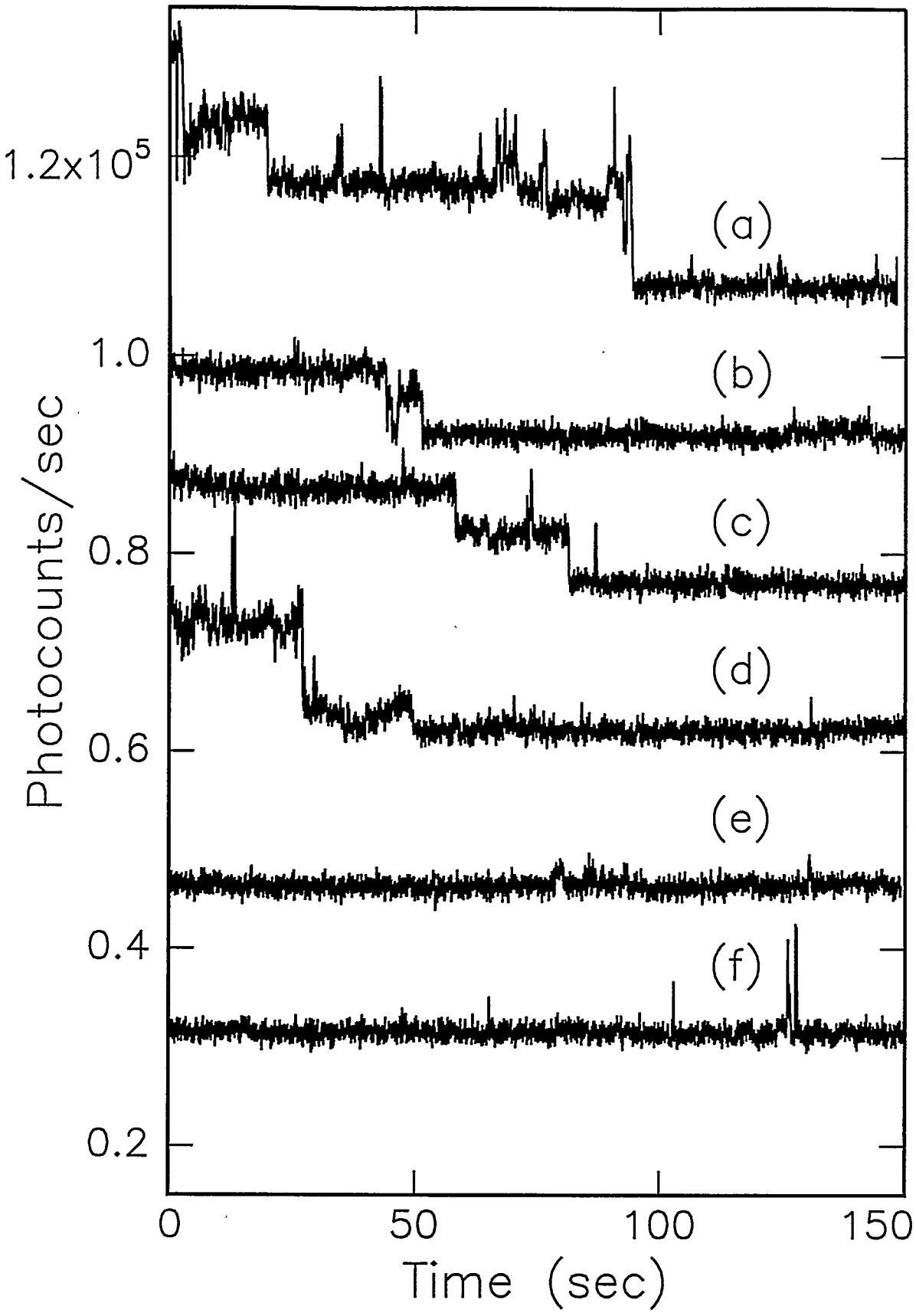
Figure 3. Near-field and shear force images of a single hamster chromosome. (a) is the fluorescence excited from the propidium iodide stained chromosome and (b) is the height image obtained concurrently with (a). The height image is displayed in a derivative format. The image is 8.5x8.5 μm^2 in size.

Figure 4. Near-field fluorescence excitation images of individual λ-DNA strands stretched out on borosilicate glass surface. The fluorescence is excited from a dye intercalated into the DNA (TOTO-1). The single spots in the background are from un-intercalated TOTO dye deposited on the glass. The image on the left is ~ 14x14 μm^2 in size, and the image on the left is ~ 5x5 μm^2 in size.

Figure 5. Photobleaching behavior of individual fluorescent spots for TOTO-1 dye on borosilicate. The traces are offset by multiples of 1.1×10^3 photocounts/50 msec.

Figure 6. Photobleaching behavior of drying-modified β-phycoerythrin. The traces are offset by multiples of 3.5×10^3 photocounts/sec

Fig. 1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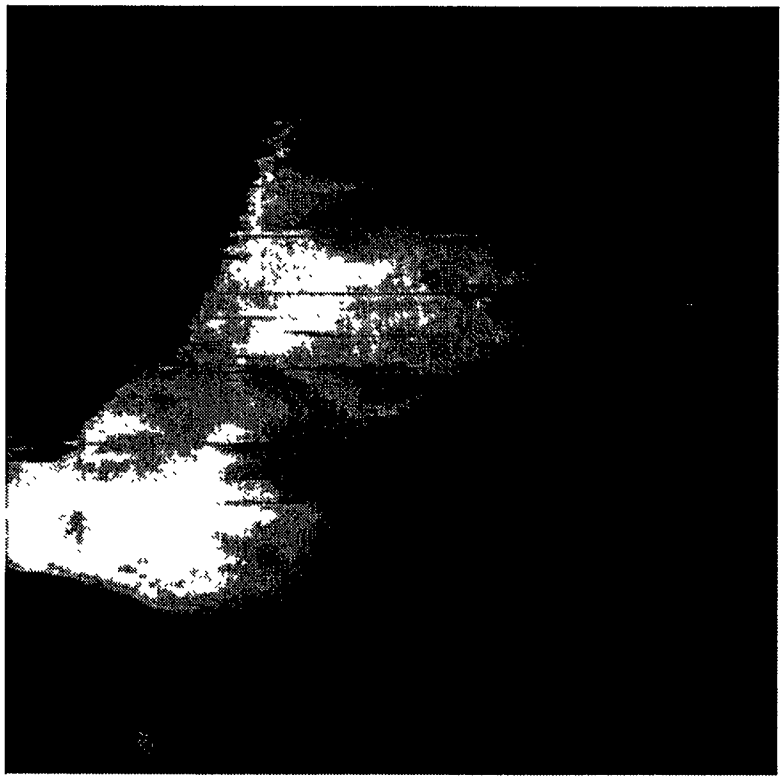
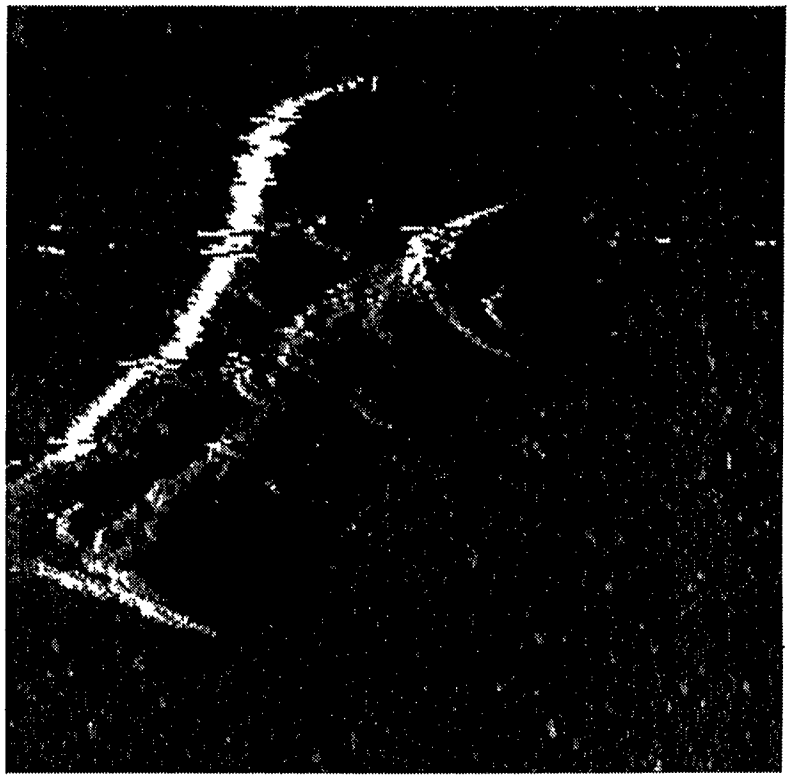
0.6
0.4
0.2
Photocounts/sec
(a)
(b)
(c)
(d)
(e)
(f)
0
50
100
150
Time (sec)

Fig. 2

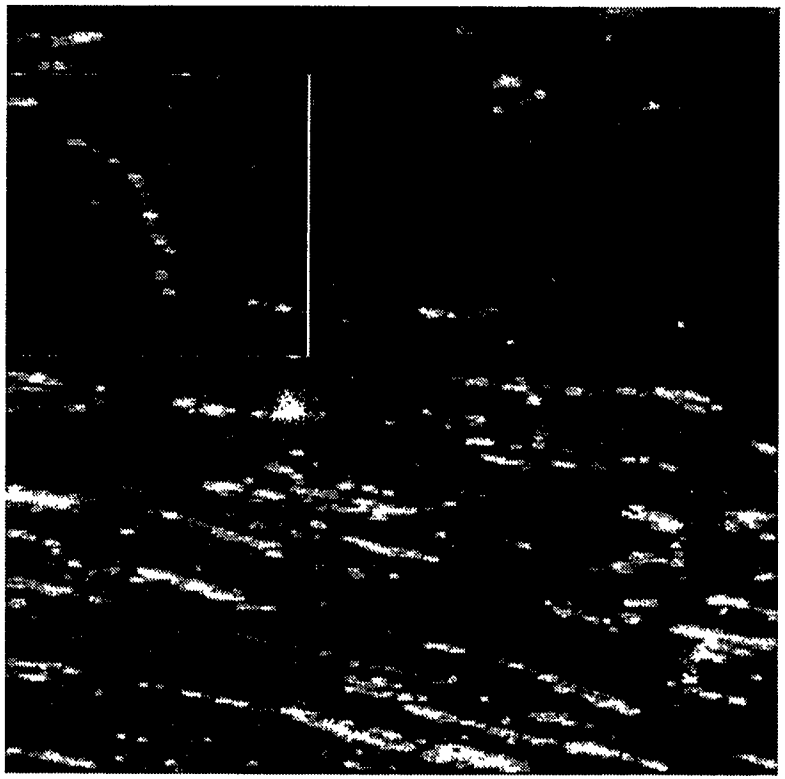


(a)



(b)

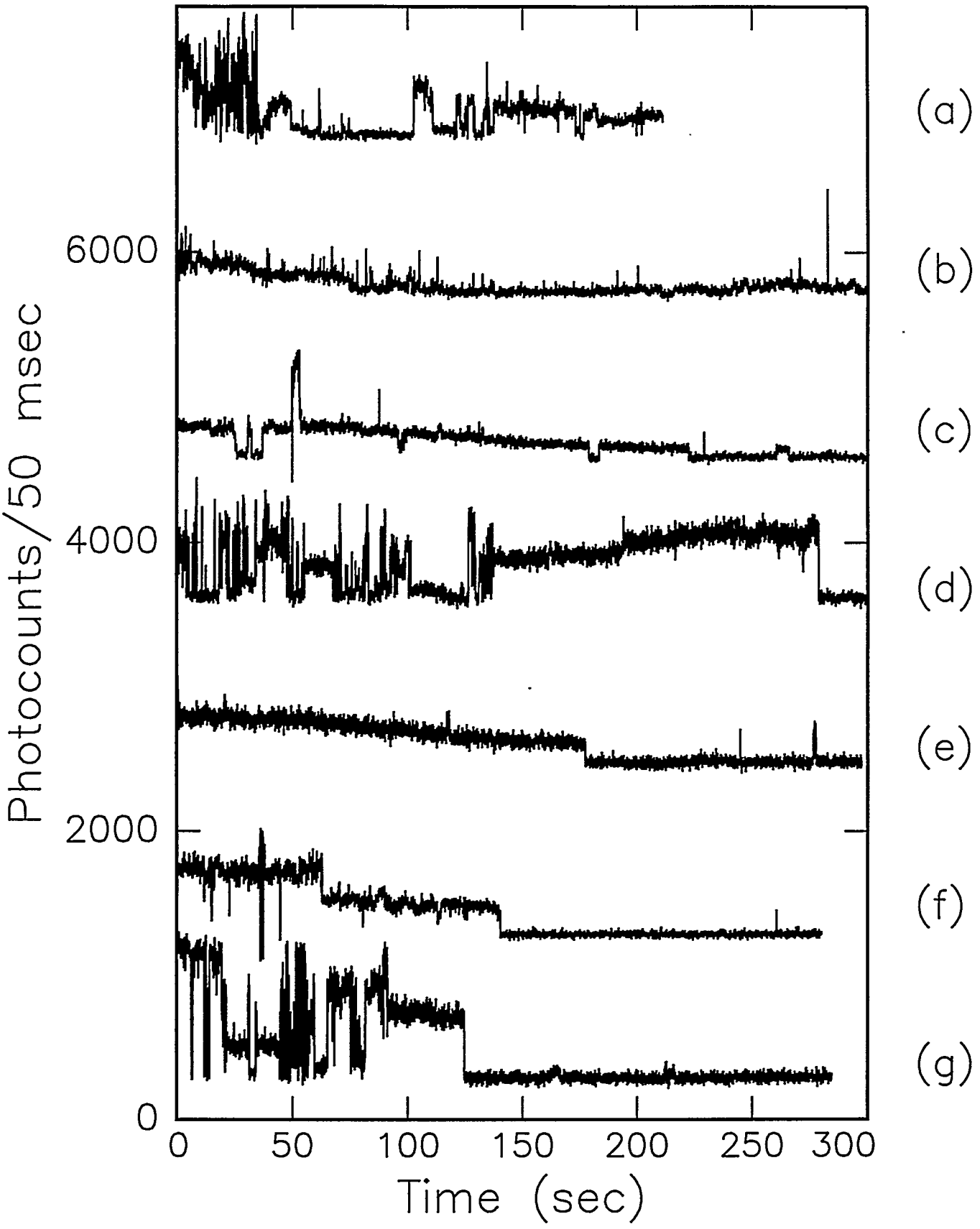
Fig 2



(a)

(b)

Fig. 4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0
50
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(c)
(d)
(e)
(f)
(g)

Fig. 5

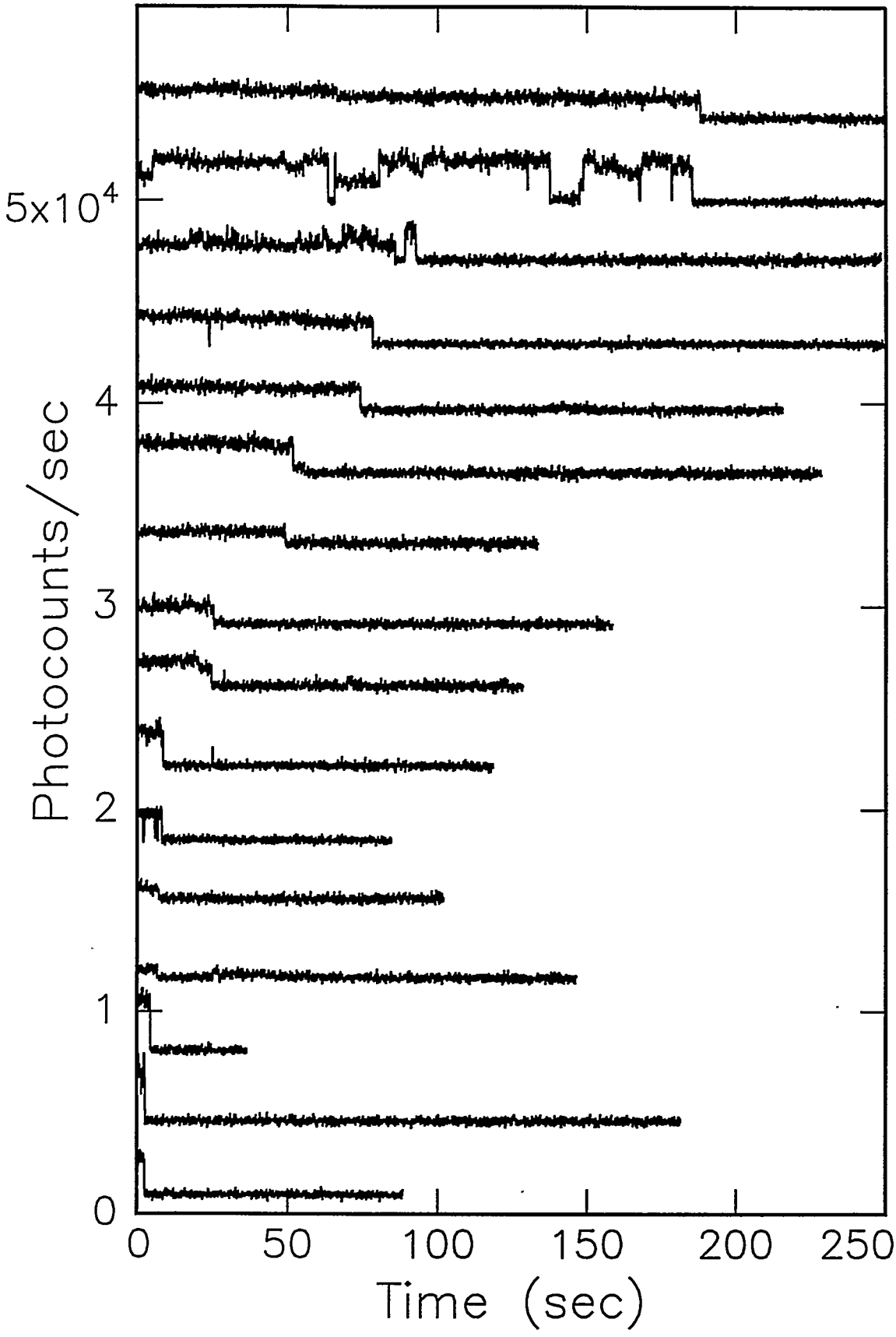

Fig. 6