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Highly Efficient Detection of Single Fluorophores in Blood Serum Samples with High Autofluorescence

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Abstract

Single molecule detection (SMD) is usually performed on surface-immobilized molecules with diffraction-limited observation volumes, typically with confocal optics to suppress background from the sample and instrument. In this paper we consider the more difficult task of detecting single fluorophores in the presence of a substantial fluorescence background. We determined that for a readily accessible macroscopic observation volume of 1 pL that the background from undiluted blood serum was approximately equal to 2700 Cy5 molecules, and the background from whole blood equal to about 14 000 Cy5 molecules in whole blood. These high backgrounds appear to preclude the possibility of SMD of Cy5 molecules. However, we show that the signal-to-noise ratio (SNR) in high background samples can be increased dramatically by reduction of the observed volume. We were able to detect single surface-bound Cy5-labeled DNA (Cy5-DNA) oligomers in diluted blood serum with an SNR near 40. We also examined freely diffusing Cy5-DNA in blood serum. The data showed that single Cy5-DNA molecules could be detected even in the undiluted serum. We further investigated the SNR on silver island films. We found that the fluorescence signal was greatly enhanced in the presence of metallic nanostructures showing a larger SNR in the application tested. These results suggest the possibility of clinical assays based on SMD in blood serum and possibly whole blood. Increased SNR near metallic nanostructure could probably overcome the need for diffraction-limited volumes and enhance our ability to do *in situ* SMD.

INTRODUCTION

There are constant efforts to increase the sensitivity and speed of assays for clinically relevant analytes and other biomolecules such as nucleic acids and diagnostic protein antigens. Many of these methods rely on PCR or ELISA amplification which requires complex sample handling (1). The use of PCR and ELISA shows that amplification methods are still needed to detect moderately low copies of analyte molecules. The detection and characterization of a single molecule represents the ultimate goal in ultrasensitive analysis (2–6). Single molecules are commonly observed using diffraction-limited confocal microscopy (7–11) and fluorescence correlation spectroscopy (12–14). Single molecule detection (SMD) is often performed on surface-bound molecules to restrict the motion and allow observation of a diffraction-limited area volume containing the fluorophore (15–17). Therefore, the size of the volume element imposes experimental constraints for SMD. Nowadays, it is commonly accepted that in the case of fluorescent SMD the probed volume has to be about 1 fL, which is equivalent to a cube of 1 μm dimension on each side. Restriction of the volume is needed to avoid overwhelming signal from background emission and Raman scatter (18,19). The sample, substrate and optics are selected for low background emission.

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For any fluorescence method, an intrinsic fluorescence signal has to be discriminated from background signals including a scattered light. As the fluorescence emitted by a single molecule is extremely weak, reduction of the background signals is essential for the detection of a single molecule. As a result, SMD is not presently used for high sensitivity detection. Single molecules are observed but not usually counted. It is considerably easier to find and measure single immobilized molecules than to count the number of molecules in a sample. SMD requires the use of complex fluorescence excitation and detection schemes that isolate one or just a few molecules in a complex. As a result, SMD is not widely used for bioassays containing a large number of individual fluorophores and is typically not a tool for high sensitivity assays. In this work, we demonstrate that SMD is possible even in the presence of undiluted serum. The ability to detect single target molecules under practical conditions would have produced implications for medical diagnostics and detection of biohazard organisms.

Metallic nanostructures offer an alternative to increase the brightness of single fluorophores which may allow SMD with less sophisticated instruments. Gold and silver metallic nanostructures have attracted much attention because of their size and shape-related optical properties, which differ significantly from those of bulk metals (20,21). The effects of metallic nanostructures on the spectral properties of fluorophores have been reported recently (22–25). We have intensively studied the interactions of fluorophores with metallic surfaces and particles (26–28). These effects appear to be the result of through-space electromagnetic interactions among the optical fields, the nearby molecules and the electronic plasma resonances localized on the metal surface. These effects can increase the field incident on the fluorophore and cause the changes in quantum yields, lifetime and photostability. Thus, the use of metallic nanostructure to enhance probe fluorescence has great potential for applications in the fields of medical diagnostics and biotechnology which have high background emission.

MATERIALS AND METHODS

Sample preparations

All oligonucleotides were obtained from the Biopolymer Core Facility at the University of Maryland School of Medicine. Nanopure water purified using Millipore Milli-Q gradient system was used for all experiments. All other compounds were purchased from Sigma-Aldrich and used as received. The labeled oligomers with Cy5 on the 5' ends were obtained from the Biopolymer Core Facility of the University of Maryland, Baltimore. The coverslips used in the experiments were first soaked in a 10:1 (vol:vol) mixture of concentrated H₂SO₄ and 30% H₂O₂ overnight, extensively rinsed with water, sonicated in absolute ethanol for 2 min and dried with air stream. The purity was checked by fluorescence measurements at single molecule levels.

Raw blood and serum obtained from healthy mice were diluted to 10% of the original concentration with ultrapure water. Silver island films (SIFs) were deposited on cleaned glass coverslips by reduction of silver nitrate as reported previously (26–28). The formed SIFs are greenish and nonreflective. Only one side of each slide was coated with SIF. The particles are typically 100–500 nm across and 70 nm high covering about 20% of the surface.

Confocal microscopy

Single-molecule images were collected on a scanning confocal microscopy (MicroTime 200; PicoQuant). A single mode pulsed laser diode (635 nm, 100 ps, 40 MHz) (PDL800; PicoQuant) was used as the excitation light. The collimated laser beam was spectrally filtered by an excitation filter (D637/10, Chroma) before directing into an inverted microscope (IX 71; Olympus). Individual images were recorded at 150 × 150 pixels using a 100×/1.3 NA oil immersion objective. The fluorescence that passed a dichroic mirror (Q655LP; Chroma) was

focused onto a $75\text{ }\mu\text{m}$ pinhole for spatial filtering to reject out-of-focus signals and then reached the single photon avalanche diode (SPCM-AQR-14; Perkin Elmer, Inc.). The excitation power into the microscope was maintained at less than $2\text{ }\mu\text{W}$. Time-dependent fluorescence data were collected with a dwell time of 50 ms. The data were stored in the time-tagged-time-resolved mode, which allows recording every detected photon with its individual timing information. The high numerical aperture apochromatic oil immersion objective along with the $75\text{ }\mu\text{m}$ confocal pinhole resulted in a confocal detection volume of 0.5 fL . The probe concentrations used in the experiments are about 1 nM .

Bulk fluorescence measurements

Emission spectra from DNA-SIF layers and DNA layers were collected in front-face geometry on an SLM 8000 spectrofluorometer with 635 nm excitation from a xenon lamp.

RESULTS AND DISCUSSION

Estimation of autofluorescence from blood and serum

We examined the possibility of SMD in samples with high autofluorescence. We collected ensemble emission spectra of Cy5 in serum samples to determine the intensity of the autofluorescence compared to single Cy5 molecules (data not shown). We found that the background from serum is equivalent to about 4.5 nM Cy5 molecules and the background from blood is equivalent to about a 26 nM Cy5 probe concentration. If we assume that the observed volume with our moderately focused optics was 1 pL , a cube $10\text{ }\mu\text{m}$ on each side, then the background from blood and serum is equal to 2700 and 14 000 Cy5 molecules, respectively. Then, for the observed volume of 1 pL , the signal-to-noise ratio (SNR) of a single Cy5 molecule in serum is $1/(2500)$ and the SNR in blood is about $1/(14\text{ }000)$ (Fig. 1). As a result, it is essentially impossible to detect Cy5 molecules in serum or blood using macroscopic observation volumes.

The situation improves dramatically if the observed volume can be restricted around the fluorophore. If the observed volume is reduced to the diffraction-limited volume near 1 fL , the SNR can be enhanced to a value of 0.37 in serum and of 12 in blood, respectively. If the observed volume can be decreased still further, as is possible using metallic nanostructures, then the SNR for a single Cy5 molecule in serum and blood can be near 100. Of course the increase in SNR is the result of knowing the location of the fluorophore and where to locate the observed volume. Such localized observation is not practical for bioassays using macroscopic samples. These calculations predict that in SMD experiments with a diffraction-limited volume the emission from a Cy5 molecule would have a brightness equal to the background and would thus be detectable.

In addition to SNR we also need to consider the number of analyte molecules flowing through the observed volume. We assumed a spatial flow rate of $10\text{ }\mu\text{m s}^{-1}$ which is easily obtained using modest pressures. We assumed an analyte concentration of 0.2 pM , which is a typical clinical decision point. At this flow rate and concentration the numbers of molecules flowing through the observation volume are $100\text{ }000\text{ s}^{-1}$ for $(10\text{ }\mu\text{m})^3$, 1000 s^{-1} for $(\text{ }\mu\text{m})^3$, 27 s^{-1} for $(0.3\text{ }\mu\text{m})^3$ and 1.0 s^{-1} for $(0.1\text{ }\mu\text{m})^3$. These calculations show that an adequate number of analyte molecules will pass through the observed volume. There are several considerations which make the situation even more favorable than shown in Fig. 1. The blood or serum samples will probably be diluted with reagents. The dilution factor can easily be 10-fold, resulting in a 10-fold increase in the SNRs. Additionally, even in the smallest volume of $(0.1\text{ }\mu\text{m})^3$ an average of 60 molecules will pass in a minute.

Effect of the observed volume on the SNR

We examined Cy5-DNA molecules immobilized on glass slides to test the effect of the observation volume on single molecule detectability. We changed the observed volume (area) by defocusing the objective in our single molecule instrument. Determination of the confocal volume was based on the imaging of fluorescent beads (100 nm diameter). Subresolution beads were treated as a point source and were used to scan the confocal volume. These measurements yielded an approximate lateral radius of $0.32\ \mu\text{m}$ when the sample is in focus. Figure 2 shows images of the Cy5-DNA probe-alone (1 nM) spin coated on a glass slide. The fact that single molecules are being observed is proved by the single-step photobleaching events. Individual molecules suffer photobleaching as evidenced by the discrete drop in fluorescence intensity to the background level, providing strong evidence that single dye molecules had been observed. Other analyses provide the criteria to assure the observed fluorescence signals are truly single-molecule events and are not artifacts such as contaminants scattering. These criteria can be summarized as: (1) The spot size is in the same order as the diffraction-limited size of the laser focus in the confocal setup. (2) The signal intensity is consistent with that expected for single-molecule emission considering the properties of the molecule, the excitation intensity, and the efficiency of optical filters. (3) The density of the spots is proportional to the concentration of dye in the solution (29). During the study, we selected the molecules in as unbiased a fashion as possible. The general appearance of the time traces depicted is representative of the overall trend observed from more than 50 single molecules in each environment. Among 85% of time traces collected for these investigated molecules clearly show one-step photobleaching, corresponding to the typical behavior expected for a single molecule (Fig. 2). The intensity is fairly constant until it drops abruptly to the background level in a single step. Theoretically, for epi-illumination with confocal detection, tight spatial filtering of the collected fluorescence can restrict the full height of the probe volume to $<1\ \mu\text{m}$. High numerical aperture microscope objectives easily focus light to wavelength dimensions, which for 600 nm light would be about 0.22 fL. When the sample is in focus the SNR is 70. The SNR is decreased modestly to 20 as the sample is defocused by $1\ \mu\text{m}$.

We then examined probe molecules spin coated on glass with 10-fold diluted fresh mouse serum (Fig. 3). When the sample is in focus an SNR of 40 is obtained. This is comparable to the SNR of 37 predicted in Fig. 1 for undiluted serum. We regarded this as good agreement between the macroscopic and SMD experiments. As the sample is defocused by $1\ \mu\text{m}$ (an approximate lateral radius of $0.82\ \mu\text{m}$), the SNR decreased dramatically to 1.5. This result shows that SMD in serum cannot be achieved without a diffraction-limited volume, which makes it difficult to use SMD for routine bioassays.

The previous experiment was for Cy5 immobilized on a surface. An assay based on SMD in serum or blood would most likely need to be performed with freely diffusing molecules in solution. To model this situation we performed fluorescence intensity distribution analysis measurements for Cy5 in blood serum. As the goal was to detect the Cy5 molecules, the emphasis will be on the changes in brightness in different environments. The data presented are in terms of brightness as pulse intensity histograms. The burst size histogram shows the frequency of a certain number of events/bin. In Fig. 4, the intensity distributions for serum solutions containing Cy5 and pure serum solutions are depicted. The convolution of two different densities is observed. In both 10-fold diluted serum and undiluted serum there is a significant shift in the histogram to higher intensities in the presence of Cy5. We believe these higher intensity bursts are due to Cy5 molecules passing through the observed volume. Additionally, we observed higher event frequencies at lower count rates in the presence of Cy5 molecule in serum samples. The possible origins could arise from possible conformation states of Cy5 molecules and the existence of various nanoenvironments. The latter could be the results of the interactions occurring between the serum components and the dye molecules.

Nevertheless, the results indicate that we could detect the freely diffusing Cy5 molecules in 10-fold diluted serum and even in undiluted serum.

It is interesting to consider why single fluorophore can be detected in such high background samples. We believe detection is possible because the background is due to a large number of weakly emitted species, which individually are much less bright than Cy5. In the single molecule images the bright Cy5 molecules are visible above the diffuse background. Similarly, in intensity distribution histograms, the intensity bursts due to the Cy5 molecule are larger than for the more weakly emitting species.

Effects of SIFs on SMD SNR

In order to observe single fluorophores the incident intensities must be high to obtain high rates of excitation and emission. As a result single fluorophores can be observed for only a short period of time, typically seconds, prior to photobleaching. Additionally, observation of single fluorophores is usually accomplished using diffraction-limited volumes. Otherwise the single emission is overwhelmed from background due to autofluorescence or Raman scattering. Metal-enhanced fluorescence can thus be useful in SMD by increasing the photostability and brightness of fluorophores. We examined Cy5-DNA in serum which was spin-coated on a silver particle substrate (Fig. 5). In fact, different sites on the silvered surface in which the probe molecules were immobilized are moderately varied. For example, fluorescence can be partly quenched if the Cy5 molecule is close to the metallic nanostructure. In contrast, when the attached fluorophore is at a certain distance from the metallic particle, both the incident field and radiated field may be enhanced, thereby tending to produce increased fluorescence; it would be much more stable than the emission of the molecule directly deposited on glass. As was discussed previously, we only consider those bright spots which show single-step photobleaching. The first striking observation is that the single molecule count rates are 10-fold larger than those found in the absence of silver particles. A second important observation is that the SNR is greatly improved over that focused for Cy5 in serum without silver particles, despite the fact that the autofluorescence background has also been amplified to some extent. When the sample is in focus an SNR of 150 is obtained. This is much higher than the SNR depicted in Fig. 3, which was observed on inert glass substrate with a value close to 40. As the sample is defocused by 1 μm along the optical axis, the SNR decreased noticeably to 15, which is still compatible to the SNR value of Cy5 in pure water. The result indicates that SMD in serum could be achieved with a larger than diffraction-limited volume in the presence of metallic nanostructures. The increase in SNR suggests that the Cy5 emission is enhanced by the silver particle to a larger extent than the background emission.

CONCLUSION

At first glance it is difficult to understand how a single fluorophore can be detected in the presence of an overwhelming amount of background, even with a small observed volume. Detection of the fluorophore must be a result of the different nature of its emission compared to the background. It is likely that the background is due to a large number of emitting species which are individually much less bright than the individual Cy5 molecules. Hence the intensity due to the background does not contribute high intensity fluctuations as does Cy5. Similarly, the background emission from the slides spin-coated with serum diffuses across the entire area and is less bright at any particular spot. In the surface-immobilized samples, with excitation focus larger than diffraction-limited volume, the bright Cy5 molecules are still visible above the background on the metallic substrate. With the metallic structure, the SNR of Cy5 in serum is increased four- to 10-fold when staying focused. This implies the possibility of detecting single fluorophores in larger focusing volume in high background samples. In summary, these results suggest the use of bioassays based on SMD in biological samples with high background

emission. The effects also imply potential applications to increase detectability of single molecules bound to surfaces which contain metallic structures, for either biophysical studies or high sensitive assay. However, the applications of fluorophore-metal interactions will require more defined nanostructures, which can be accomplished by batch methods (*e.g.* spatial control provided by lithographic technologies) with the desired optical properties.

Acknowledgments

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Figure 1.

Calculated signal-to-noise (background) ratio for single molecules in media within limited observation volumes.

Figure 2.
Single molecule images of Cy5 (1 nM) spin-coated on glass using pure water.

Figure 3. Single molecule images of 1 nM Cy5 in 10-fold diluted mouse serum spin-coated on glass slides.

Figure 4. Fluorescence intensity distribution histograms of Cy5 (1 nM) in 10-fold diluted (a) and undiluted serum (b).

Figure 5.
Single molecule images of 1 nM Cy5 in 10-fold diluted mouse serum spin-coated on silver island films