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Identification of immobile single molecules using polarization-modulated asynchronous TDI-mode scanning

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Abstract

We report the development of a data acquisition method for identifying single molecules on large surfaces with simultaneous characterization of their absorption dipole. The method is based on a previously described device for microarray readout at single molecule sensitivity¹. Here, we introduced asynchronous time delay and integration- (TDI-) mode imaging to record also the time course of fluorescence signals: the images thus contain both spatial and temporal information. We demonstrate the principle by modulating the signals via rotating excitation polarization, which allows for discriminating static absorption dipoles against multiple or freely rotating single absorption dipoles. Experiments on BSA carrying different numbers of fluorophores demonstrate the feasibility of the method. Protein species with average labeling degree of 0.55 and 2.89 fluorophores per protein can be readily distinguished.

Keywords

single molecule fluorescence; microarrays; polarization

Functionalized surfaces have become a paramount instrument for global analysis of complex biological samples². In particular, DNA microarrays have turned into standard tools for expression analysis in molecular biology and genomic research, but also for pharmacogenomics, infectious and genetic disease and cancer diagnostics³. In addition, the growing interest in functional protein microarrays has driven the development of sophisticated technologies for arraying hundreds of functionally active proteins on biochip surfaces^{4, 5, 6}. In essence, global analysis provides information on the protein or RNA expression patterns, yet it requires rather large amounts of sample material for unambiguous analysis.

We recently combined microarray technology with single molecule detection⁷ in order to increase sensitivity in expression profiling to the ultimate limit^{8, 9}. In this case, expression levels were inferred from counting each bound molecule on the microarray spots¹⁰. Yet,

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single molecule analysis not only improved the assay sensitivity, it opens up new types of information, which were not accessible in conventional ensemble-based read-out¹¹. As examples, the presence of different sequence motifs¹² or repeats¹³, and the degree of cDNA labeling⁹ have been addressed based on single molecule analysis of surface bound nucleic acids. Even more so, surface-bound DNA can be sequenced based on single molecule analysis of single base extension^{14, 15, 16}.

Single fluorophore sensitivity, however, implies seeing also impurities within the sample and requires careful lab handling and data interpretation. To reliably identify single molecule fluorescence, researchers have developed several methods that are based on the access to spectroscopic parameters of these molecules. For example, photon anti-bunching^{17, 18} – the anti-correlated emission of photons within the fluorescence lifetime – has been used to proof fluorescence emission from DsRed sub-units¹⁹. In other approaches, the signal obtained from single molecules within a given time and excitation intensity – the characteristic molecular brightness – was utilized to characterize the composition of RNA polymerase-DNA transcription complexes²⁰ and to determine the number of subunits in functioning bacterial flagellar motors by observing step-wise photobleaching²¹. Polarization dependent absorption of light has been utilized to gain insights into the rotational behaviour of single molecules²². Image acquisition using defocused optics has been used to characterize the orientation of single molecule emission dipoles in 3 dimensions, which in principal allows for single fluorophore identification²³. In this manuscript, we exploited the modulation depth of the fluorescence signal upon rotating the excitation polarization, which – due to the known modulation frequency – represents a very robust readout parameter even in the presence of highly fluctuating signals.

Time delay and integration- (TDI-) mode was applied for sensitive fluorescence imaging in capillary columns²⁴, DNA sequencing blots²⁵, microfluidic channels²⁶, and on biochips⁹. In this mode the sample and the charges on the camera chip synchronously, thereby enabling the recording of arbitrarily long image stripes; henceforward, we refer to this operation mode as synchronous TDI-mode imaging. We have shown that imaging close to the optical diffraction limit becomes possible when the synchronization is adjusted appropriately¹. If the scanning speed of the sample is slower or faster than the line transfer on the camera chip, however, signals originating from point light sources get stretched along the scanning axis. In this asynchronous TDI-mode operation, the scanning direction encodes both spatial and temporal information. In this paper, we demonstrate the feasibility of this operation mode to discriminate single molecule emitters from aggregates containing multiple fluorophores.

Asynchronicity is characterized by the speed ratio between sample (v_{sample}) and image movement (v_{image}). In this study, we set $v_{image} = 50 \text{ lines} / s$ on the camera chip and $v_{sample} = 4 \mu\text{m} / s$ or $v_{sample} = 5 \mu\text{m} / s$ (only for Fig. 1b) in object space; the following estimation is provided for $v_{sample} = 4 \mu\text{m} / s$. When imaging a diffraction-limited object via asynchronous

TDI-mode, the length of the peak is given by $l_{peak} = \left| \frac{v_{image} \delta_{\perp}}{v_{sample} M} - 1 \right| \cdot \min(l_{ex}, l_{CCD})$, with $\delta_{\perp} = 20 \mu\text{m}$ the physical extension of a pixel perpendicular to the scanning direction, and $M = 100$ the optical magnification. Note that we neglected the extension of the point-spread

function here. Apparently, the peak length is limited either by the extension of the excitation field, l_{ex} , or by the size of the camera chip l_{CCD} ; we used here $l_{ex} \sim l_{CCD} = 100 \text{ pixel}$. In a real life experiment photobleaching may terminate the fluorescence even earlier, thereby shortening the peak length. Due to the asynchronicity, the pixel extension along the scanning axis, $\delta_{||}$, gets distorted: two points separated by 1 pixel without scanning will have a distance

of $\delta_{||} = \frac{v_{image}}{v_{sample} M} \cdot \delta_{\perp} = 2.5 \text{ pixel}$ in asynchronous TDI-mode scanning. The temporal resolution of the system depends on the difference between v_{sample} and v_{image} and is

characterized by the time per pixel $t_{pix} = 1 / \left| \frac{M v_{sample}}{\delta_{\perp}} - v_{image} \right| = 33 \text{ ms/pixel}$. Naturally, high difference in velocities increases the temporal resolution, yet at the expense of signal brightness per pixel and consequentially of the obtainable signal-to-noise ratio (SNR)²⁷. To give an estimation, SNR scales with $SNR \propto \text{signal} \cdot (l_{peak})^{-0.5}$. Thus, a peak stretched by a factor of 100 compared to a diffraction-limited signal requires a 10-fold higher signal for obtaining the same SNR.

The instrument is based on a setup described in detail previously^{1, 9, 28, 29}. In brief, the 647nm line of a Kr⁺-ion laser (Innova 301, Coherent, Santa Clara, California, USA) was coupled into the epi-port of an inverted fluorescence microscope (Axiovert 200M, Zeiss, Oberkochen, Germany). The profile and divergence of the laser beam were controlled using two cylindrical telescopes and a field stop, which allowed us to adjust the spot size in x- and y-direction, and to limit the excitation to the field of view of the camera. Fluorescence light was collected using a 100× oil immersion objective (α -Fluar, NA=1.45, Zeiss) and imaged onto a back-illuminated CCD camera (NTE/CCD-1340/100-EMB, Princeton Instruments, Trenton, New Jersey, USA). A motorized scanning stage (Scan IM 120×100, Märzhäuser, Wetzlar, Germany) allowed precise positioning and movement of samples during signal acquisition. For large area imaging we continuously moved the sample over the field of view, and simultaneously read out the camera synchronously or asynchronously in time delay and integration (TDI)-mode¹. We used a combination of a quarter wave-plate (Multiple-Order Quartz Wave Plate, $\lambda/4$ Retardation, Newport, Irvine, California, USA) and a linear polarizer (Polarcor™ Linear Polarizer, Newport, Irvine, California, USA) to generate linear polarized light at arbitrary polarization angle. The linear polarizer was mounted on a custom-built rotation stage which allowed to rotate the laser polarization with a frequency $f_{ex} = 2 \text{ Hz}$.

In this study we used fluorescent bovine serum albumin (BSA) as sample. 2mg BSA (Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in 1 ml of 0.2 M sodium hydrogen carbonate buffer (sodiumhydrogencarbonat, Merck, Germany) at pH 8.3. NHS-functionalized Atto647-dye (ATTO-TEC, Siegen, Germany) was dissolved in 1 mg/ml of amine-free DMSO prior to labeling. The degree of labeling was adjusted by tuning the molar dye-protein-ratio and the reaction time (15 min) at room temperature. To remove free dye molecules, a two-step purification was performed. First we purified the samples with PD-10 columns and pre-estimated the labeling ratio with a UV-Vis spectrophotometer (Helios α , Thermo Fisher Scientific Inc., Maryland, USA). Second, the eluate was HPLC-purified using Superdex S200 columns and stored in Phosphate Buffered Saline (PBS) at 4°C. Two samples were prepared with a molar ratio of 1:1 and 1:5 BSA vs. Atto647-NHS yielding an

effective degree of labeling of 0.55 and 2.89 fluorophores per BSA, respectively; in the following, we denote the two samples as BSA_{low} and BSA_{high}, respectively. The labeling degree was determined via the UV-Vis spectrophotometer.

Glass cover slips (Menzel, Braunschweig, Germany) were thoroughly cleaned using piranha solution (hydrogen peroxide (H₂O₂) and sulfuric acid (H₂SO₄) in a proportion of 3:7) for at least 2 hours and adjacently rinsed with distilled water. After cleaning, 50 µl Secure Seal Hybridization chambers (Sigma-Aldrich Co., article number C0975) were adhesively bound to the glass slides. Surfaces containing single molecule signals of either BSA_{high} or BSA_{low} at low surface density were prepared by incubating the glass slides with diluted protein solution for 10 minutes, and subsequently flushing with PBS buffer. The BSA concentration was adjusted such that an average surface density of ~1 molecule per 100 µm² was achieved. To prevent bleaching and blinking of fluorophores the chambers were filled with PBS buffer containing 4.5 mg/ml glucose, 36 mg/ml catalase, 216 mg/ml glucose oxidase and 1% 2-mercaptoethanol³⁰.

In principle, the obtainable information on single molecule dynamics could be utilized in various contexts: for example, the photobleaching time of dyes is easily determinable by measuring I_{peak} ; also photoblinking can be addressed by analyzing the modulation of the brightness. An exemplary image is shown in Fig. 1. For this, we immobilized fluorescently labeled BSA_{low} on a glass coverslip, and recorded the sample via conventional synchronous TDI-mode (Fig. 1a) and asynchronous TDI-mode (Fig. 1b). The asynchronously recorded scan reveals several single molecule traces, with fluctuations of the fluorescence signal, indicative of photoblinking of the Atto647 dye.

In this study, we show that by modulating the excitation polarization during a scan we can discriminate single immobilized emitters from diffraction-limited clusters containing multiple weak emitters, or from rotating molecules. The fluorescence signal of a single absorption dipole excited by linear polarized light is described by $I(\phi, \theta) = I_0 \cdot \cos^2(\phi) \cdot \cos(\theta)$ (Fig. 2a). Rotating excitation polarization (frequency f_{ex}) therefore results in an fluorescence intensity modulation with frequency $f = 2 \cdot f_{ex}$. Note that for freely rotating dipoles (e.g. a fluorophore bound to a diffusing protein) and for particles with many randomly oriented absorption dipoles, no such fluorescence modulation would be present. Fig. 1b & c show a comparison of the same BSA_{low} sample, recorded with the polarizer rotation stage switched off (b) and on (c). The asynchronous TDI-mode acquisition without rotating polarizer yields stretched peaks. When additionally modulating the linear polarization with $f_{ex}=2$ Hz, a periodic intensity modulation along single molecule traces becomes clearly observable for BSA_{low} (Fig. 1c) but is not present for BSA_{high} (Fig. 1d). Fig. 3 shows examples of raw intensity traces obtained for BSA_{low} (a) and BSA_{high} (b). The obtained modulation frequency $f \sim 4$ Hz of the fluorescence signal agrees well with the theoretical estimation.

For data analysis, single molecule signals were manually located on the fluorescence images; corresponding sub-images were generated and individually analyzed. Fourier filtering (4 Hz) of the raw data allowed for reliably localizing putative extrema. The resulting positions were used to determine the intensity values at the local minima in the raw data. For each stretched peak the average intensity values of local minima $\overline{I_{min}}$ and

background $\overline{I_{bg}}$ (see Fig. 2b) were determined and used to calculate $I_{valley} = \overline{I_{min}} - \overline{I_{bg}}$. The resulting distributions of I_{valley} for BSA_{low} and BSA_{high} are shown as probability density functions in Fig. 4a and b, respectively.

We next wanted to test whether the obtained distributions of I_{valley} agree with theoretical predictions, taking into account the degree of labeling for $BSA_{low}=0.55$ dyes per protein and $BSA_{high}=2.89$ dyes per protein. To evaluate the agreement between the experimental results and a model we performed statistical testing against a comprehensive set of Monte Carlo simulations³¹. We simulated the excitation of immobile BSA molecules, which were assumed to be labeled stochastically following a Poisson distribution and used the two-sample Kolmogorov-Smirnov hypothesis test as a non-parametric test. As an output the test yields the p-value, which is a measure of the statistical difference of the two distributions. The p-value quantifies the extremeness of a randomly drawn sample by specifying the probability of obtaining a sample at least as extreme as the one which was actually observed, assuming that the null-hypothesis H_0 is true. If we specify a significance level α such that H_0 will be accepted for $\alpha < p\text{-value}$ and rejected for $\alpha > p\text{-value}$, then α defines the probability of falsely rejecting H_0 . By calculating the p-value between the measured data set X and the different Monte Carlos simulations $Y(\lambda)$, we get an estimate of the parameter settings which likely lead to the observed data and of those parameters which would rarely lead to data as extreme as observed.

For the Monte Carlo simulations, we constructed a large set (10000) of dipoles equally distributed in angle space. The laser excitation has been modeled as a rotating field with ϕ the angle between excitation and dipole vector and θ the angle between dipole vector and surface normal (Fig. 2a). The intensity for each dipole-angle combination was calculated using $I(\phi, \theta) = I_0 \cdot \cos^2(\phi) \cdot \cos(\theta)$ with I_0 set to the measured peak values 109 counts and 141 counts for BSA_{low} and BSA_{high} , respectively. To each value $I(\phi, \theta)$ the corresponding Poisson noise $\sigma_{Poisson}(I(\phi, \theta))$ and the measured background noise

$\sigma_{background} = \exp\left(-\frac{(x - \mu)^2}{2\sigma^2}\right)$ with $\mu = 100$ counts background offset and $\sigma = 38$ counts background noise was added. Molecules containing multiple labels were simulated by adding n signals prior to noise addition. To account for stochastic labeling of proteins, n was distributed according to a Poisson process with expectation value λ ranging from 0.02 to 4 in steps of 0.02.

The resulting p-values obtained for BSA_{low} and BSA_{high} are shown in Fig. 4c. Clear and well separated maxima for both degrees of labeling are visible in the p-value spectra indicating that the two BSA populations can be well discriminated. Note that unlabeled BSA molecules cannot be observed by fluorescence microscopy, but yield contributions to the average degree of labeling determined by UV-Vis spectroscopy. We thus calculated the average degree of labeling of those BSA molecules which actually carried a label, assuming Poisson labeling statistics, and indicated these values by straight lines in Fig. 4c

($BSA_{low}^{app}=1.3$ and $BSA_{high}^{app}=3.1$). The best fit between data and model is close to the expected λ values, yet slightly below. Residual photobleaching of dyes prior to the experiment may have reduced the active fluorophores, thus accounting for this effect.

In a real life experiment, however, the task is typically inverse: from a single modulation pattern measured, the researcher wants to find out whether it originates from a single or from multiple absorption dipoles. This task can be approached using Bayes' theorem: the conditional probability $p(n|I_{\text{valley}})$ of observing a molecule carrying n dyes when measuring

a particular I_{valley} is given by $p(n|I_{\text{valley}}) = p(I_{\text{valley}}|n) / \sum_{n=1 \dots \sim 10} p(I_{\text{valley}}|n)$. In Fig. 5 we show the dependence of $p(n|I_{\text{valley}})$ on n for various I_{valley} . Assuming $I_{\text{valley}} = 10$ counts (the experimentally determined average value for BSA_{low}) we estimate a probability of 0.54 for observing a single absorber (filled stars). A slightly increased $I_{\text{valley}} = 30$ counts (squares) would yield a reduced probability of 0.21, showing the discrimination power against higher labeled molecules.

In summary, we presented a method that allows determining the dipole characteristics of fluorescent particles. The deliberately introduced asynchronicity between sample and image movement in the TDI-mode imaging adds a temporal resolution to the acquired images. Fluorescence is excited using linear polarized laser light whose polarization is rotated during the measurement. The obtained images contain spatial and temporal information on the fluorescent particles within a single image. Analysis of the modulation depth of resulting fluorescence traces enabled the characterization/discrimination of two differently labeled BSA populations. This proof-of-concept experiment shows the capability of discriminating single labeled molecules against molecules carrying two or more labels.

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Figure 1.

Fluorescence images of BSA_{low} (**a-c**) and BSA_{high} (**d**). (**a**) shows a synchronous TDI-mode image where the individual molecules are visible as diffraction limited peaks with high signal-to-noise ratio. (**b**) Asynchronous TDI-scan where individual diffraction limited objects are imaged as stretched peaks. Intensity fluctuations along the peaks can be attributed to photoblinking of the Atto647 dye molecules. (**c+d**) Polarization-modulated asynchronous TDI-mode images. While BSA_{low} molecules (**c**) show a strong fluorescence modulation and no signal between the maxima, BSA_{high} molecules (**d**) are characterized by a low modulation and significant fluorescence signal also between adjacent maxima. Images in (**b**) and (**c+d**) were acquired with $v_{\text{sample}} = 5 \mu\text{m} / \text{s}$ and $v_{\text{sample}} = 4 \mu\text{m} / \text{s}$, respectively.

Figure 2.

(a) Absorption dipole $\vec{d}$ and excitation vector $\vec{p}$ in the presented experimental configuration with θ characterizing the orientation of the dipole with respect to the xy-plane (elevation) and φ the angle between excitation vector and projection of the absorption dipole in the xy-plane (azimuth). (b) The emission intensity I is given by $I = I_0 \cdot \cos^2 \varphi \cdot \cos \theta$ with I_0 the intensity for parallel/anti-parallel orientation of $\vec{d}$ and $\vec{p}$. Readout parameter are the intensity of a local minimum $I_{\min}$ and the background signal I_{bg} . The offset for each stretched peak is calculated as $I_{valley} = \overline{I_{\min}} - \overline{I_{bg}}$.

Figure 3.

Fluorescence traces of single BSA molecules acquired in polarization-modulated asynchronous TDI-mode scanning. The solid lines show traces of an individual BSA_{low} (a) and BSA_{high} (b) molecule; the dashed lines show the background signal next to the stretched peaks. The determined modulation frequency f_{fl} of ~ 4 Hz agrees well to the expected value for a 2 Hz polarizer frequency f_{ex} . The average modulation depth I_{valley} is clearly different for the two BSA species.

Figure 4.

Probability analysis of the degree of labelling. **(a)** and **(b)** show the probability distributions of I_{valley} for BSA_{low} and BSA_{high}, respectively. In both cases, the probability density functions of measured data (black dots) agree well with Monte Carlo simulations (gray line). Note the pronounced tail towards higher valley intensities for strong labeled molecules. **(c)** The degree of labeling was estimated by comparison with simulated data via the Kolmogorov-Smirnov test. Simulations were performed with different labeling degrees λ (Poissonian distribution of labeling). Experiments on BSA_{low} (gray line) and BSA_{high} (black

line) were analyzed. The dotted horizontal line indicates the significance level of 0.05. Dashed vertical lines show the λ values calculated – based on the UV-VIS spectroscopy measurements – for the actually labeled fractions of $BSA_{low}^{app}=1.3$ (gray) and $BSA_{high}^{app}=3.1$ (black). The analysis reveals distinct maxima for the two differently labeled molecule species with maximum p-values of ~ 0.14 ($\lambda = 0.98$) and ~ 0.13 ($\lambda = 2.38$) for BSA_{low} and BSA_{high} , respectively.

Figure 5.

Probability for observing molecules carrying 1 to 8 fluorophores when measuring a particular I_{valley} of 0 counts (circles), 10 counts (filled stars; experimentally determined value for BSA_{low}), 30 counts (squares), or 50 counts (triangles); I_0 was set to the measured peak value of 109 counts for BSA_{low} . For increasing values of I_{valley} the labeling distribution shifts towards higher numbers of dyes per molecule n reaching 0 % singles at $I_{valley} = 50$ counts.