Published in final edited form as:

Method Appl Fluoresc. 2013 September 1; 1(3): 037001. doi:10.1088/2050-6120/1/3/037001.

Extending single molecule fluorescence observation time by amplitude-modulated excitation

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Abstract

We present a hardware-based method that can improve single molecule fluorophore observation time by up to 1500% and super-localization by 47% for the experimental conditions used. The excitation was modulated using an acousto-optic modulator (AOM) synchronized to the data acquisition and inherent data conversion time of the detector. The observation time and precision in super-localization of four commonly used fluorophores were compared under modulated and traditional continuous excitation, including direct total internal reflectance excitation of Alexa 555 and Cy3, non-radiative Förster resonance energy transfer (FRET) excited Cy5, and direct epi-fluorescence wide field excitation of Rhodamine 6G. The proposed amplitude-modulated excitation does not perturb the chemical makeup of the system or sacrifice signal and is compatible with multiple types of fluorophores. Amplitude-modulated excitation has practical applications for any fluorescent study utilizing an instrumental setup with time-delayed detectors.

1. Introduction

The ability to observe single fluorescent molecules for extended periods of time is important for many different areas of research, such as quantifying nanometer-distanced structural information of protein dynamics [1-5] and nucleic acids [6-8] by Förster resonance energy transfer (FRET) [9], quantifying diffusion dynamics of molecular machines and biomolecules by single molecule tracking [10-15], and understanding cellular dynamics such as motor protein movement [16, 17] and membrane signaling [18]. Fluorescent molecular probes, while extremely useful due to their small size, high signal-to-noise ratio, and ease of use, are inherently limited by photobleaching. Photobleaching occurs when a fluorophore enters a permanent dark state due to chemical damage [19] and renders the probe useless for further observation. This is in contrast to bulky colloidal beads [20], quantum dots [21], large vesicles [22], or thousands of fluorophores [23] where photobleaching is not of concern.

Extending the time until photobleaching is therefore appealing when single molecule fluorophores are required. For example, in particle tracking, increased dye observation time increases the number of points in a trajectory, leading to a more accurate estimate of the diffusion coefficient [10]. In FRET, a prolonged observation time allows detection of state-to-state transitions in order to extract kinetic information [1, 4, 5]. For super-localization studies, an increase in observation time increases the number photons collected, which improves the precision of localization [24]. In fluorescence correlation spectroscopy, longer fluorophore observation times allows for decreases in experimental time [25].

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Supplementary data video description Video 1. Side-by-side comparison of directly excited Cy3 molecules with (left) traditional and (right) modulated excitation. Each image is 12.8 × 12.8 μm and every tenth frame is shown (1.6072 Hz).

Traditional approaches to reducing photobleaching have involved preventing photodynamic reactions in which a combination of light and oxygen interact with the fluorophore and cause oxidative damage. Oxygen can be removed by chemical means; commonly, enzymatic-based oxygen scavenging solutions remove molecular oxygen that either reacts as a free radical with the fluorophore or quenches excited fluorophores [26]. While these studies have offered a myriad of different solution cocktails [27-32] and protective strategies [33, 34], the compatibility of the chemicals used with the system of interest must be thoroughly investigated prior to experiments, and often the requisite pH and/or ionic conditions are incompatible with complex biological systems or *in vivo* studies. Another strategy to extend dye lifetime is to decrease excitation intensity. For most powers used in single molecule studies, the number of emitted photons per second increases linearly with incident intensity before reaching a saturating value [19, 35]. Reduced excitation intensity indeed extends observation time, but with the result of reduced signal-to-noise. Measuring at low signal-to-noise ratios can adversely affect identification and localization of single molecules. Finally, a synthetic approach to investigate new fluorophores with improved lifetimes is another method aimed at better fluorophore options in the future [36-38], but does not offer an immediate solution for non-synthetic labs that depend on commercially available fluorophores. Also, fluorophores often require specific functionalization, such as N-hydroxysuccinimide or hydrazide, for labeling applications that may not be available with newly discovered fluorophores.

Here we present a simple method to extend the observation time of fluorescent dyes that does not perturb the chemical makeup of the system or sacrifice signal, and is compatible with any existing fluorophore. This goal is achieved by modulating the excitation using an acousto-optic modulator (AOM) synchronized to the data acquisition rate of the detector. The hardware setup modulates the excitation in our fluorescent microscope that utilizes a detector with inherent down time in data acquisition due to data conversion and storage. The modulated laser prevents the unnecessary and unobservable excitation of a fluorophore that ordinarily occurs during the dead time of the detector. Four commonly used fluorophores were studied by wide field microscopy, including directly excited Alexa 555 and Cy3 and non-radiative FRET excited Cy5 in a total internal reflection fluorescence (TIRF) excitation geometry and directly excited Rhodamine 6G (R6G) in an epi-fluorescent setup. Improvements in dye observation time of up to 1500% and precision of super-localization of 47% were demonstrated with modulated excitation compared to traditional continuous excitation under the acquisition settings and powers used. The method described can be used in addition to standard chemical methods for improving dye photostability; can potentially be applied to a wide range of detection instrumentation including charge coupled devices, complementary metal-oxide-semiconductor (CMOS) cameras, or other time-delayed detectors; and is compatible with many different types of single fluorescent molecules, including those with or without oxygen scavengers.

2. Materials and methods

2.1 Single molecule sample preparation

Protocols have previously been described in Darugar, *et al.* [6]. All chemicals were received from Sigma Aldrich unless otherwise noted. Briefly, samples were prepared following standard streptavidinbiotin immobilization procedures. Glass coverslips were plasma cleaned and modified with a Vectabond (Vector Labs) surface according to manufacturer's protocol. The samples were functionalized with biotin polyethylene glycol (PEG) 5000-NHS ester (JenKem Technology) and methyloxypolyethylene glycol 5000 propionic acid N-succinimide ester (Fluka Analytical) at a 1:10 ratio. Custom hybriwell chambers (Grace Bio-

Labs, 43018C) were applied over the PEGylated surface to allow for injecting and flowing different solutions.

For Cy3 and Cy5 single molecule samples, transactivation response (TAR) DNA was used [6, 39, 40]. The PEGylated slides were treated with streptavidin (Invitrogen) and either labeled with purified Cy3 (donor)- and Cy3/Cy5 (donor/acceptor)-labeled biotinylated TAR-DNA (TriLink Biotechnologies). Alexa 555 samples were prepared by injecting 17 μ L of 50 pM Alexa 555 conjugated streptavidin (Invitrogen) diluted in a solution of 1x buffer (25 mM HEPES buffer, 400 mM NaCl) over the PEGylated surface for 10 s. Unbound molecules were rinsed out by injecting the chamber with 1x buffer. R6G samples were prepared by spin casting 1 nM R6G (Invitrogen) in 1% (wt/wt) poly(methyl methacrylate) (MicroChem) dissolved in toluene at 2500 rpm for 30 s on a KW-4A spin coater (SPI Supplies).

Cy3, Cy5 and Alexa 555 samples were imaged in the presence of reduction-oxidizing oxygen scavenging system prepared based on the work of Vogelsang, *et al.* [27]. The solution comprised of 1x buffer, 0.01% (w/w) glucose oxidase, 238 mM D-(+)-glucose, 0.05% (v/v) catalase, 1 mM L-ascorbic acid, 1 mM methyl viologen dichloride hydrate, and 2 mM MgCl_2 . The oxygen scavenger was flowed in the single molecule sample chamber at a rate of 1 μ L/min using a Kent Scientific Genie Plus flow system for dye lifetime studies.

2.2 Wide field microscope setup

A schematic of the instrument setup is shown in figure 1. A 532 nm solid state laser (Coherent, Compass 315M-100SL) was used for excitation. The beam was passed through an acousto-optic modulator (IntraAction, 402AF1) controlled by frequency generators (Fluke, 271-U 115V) and was expanded prior to focusing through a lens. The lens was on a translation stage that allowed the ability to change the focus to be either at the edge of a 1.45 NA, 100x, oil-immersion objective (Carl-Zeiss, alpha Plan-Fluar) for through-the-objective TIRF microscopy or at the center of the objective for epifluorescence wide field microscopy. The incident excitation intensity was $\sim 5 \text{ mW/cm}^2$ and the TIRF excitation penetrated a $1/e$ depth of $\sim 85 \text{ nm}$. Emission was collected in an epi-fluorescence setup and was separated from excitation with a dichroic mirror (Chroma, z532/633rpc) and notch filter (Kaiser, HNPF-532.0-1.0). Two dichroics (Chroma, T640lpxr) separated the emission and cross talk was reduced with bandpass filters (Chroma, ET685/70m and ET585/65m). The signal was detected on an electron-multiplied charge coupled device (EMCCD) (Andor, iXon 897) at an electron multiplying gain of 300 and integration time (t_{collect}) of either 30 ms for the TAR DNA and R6G or 100 ms for the Alexa 555 samples. Alexa 555 required increased integration time compared to the Cy and R6G samples due to the lower quantum yield in order to obtain an adequate signal-to-noise ratio for analysis.

2.3 Analysis method

Analysis was performed using MATLAB R2011b and OriginPro 8.6. Data series collected by the EMCCD were converted to a MATLAB compatible format as a series of two dimensional images. Molecules were identified from each image in a search for pixels above a threshold set as three standard deviations above the mean of a Gaussian fit to the intensities of all pixels within a single frame [41]. The three standard deviation threshold ensured 99.73% confidence that identified pixels were not due to background. The identified pixels were then fit with a two-dimensional Gaussian, an adequate approximation of the diffraction limited point spread function. Tolerances on the size and symmetry of the Gaussian fit were included to reject falsely identified pixels due to detector noise and non-resolvable agglomerations of single molecules. The number of molecules per frame and the

centroid position from the Gaussian fits were used in the reported results, as detailed later in this paper.

3. Results and discussion

3.1 Modulation scheme

Figure 2a shows a representative time trace of the detector collection in single molecule experiments that utilize an EMCCD or CMOS detectors. Two-dimensional detectors such as these have an inherent down time (t_{store}) needed to either vertically shift the collected signal of each pixel to the storage register and analogue-to-digital conversion of the data for EMCCD detectors or for analogue-to-digital conversion at each pixel with CMOS detectors. While the vertical shift time for a single pixel can be on the order of microseconds ($1.7 \mu\text{s}$ for the experiments here), the array of pixels (here being 512×512 pixels), leads to a t_{store} usually on the order of millisecond scale or higher to shift an entire frame for the most recent version of detectors [42, 43]. The time of t_{store} can rival that of the integration time of the detector (t_{collect}), such as with the Cy and R6G experiments in this work where $t_{\text{collect}} = 30 \text{ ms}$ and $t_{\text{store}} = 32 \text{ ms}$. Although ultra-fast detection using subsections of detector chips is a possible alternative to reduce t_{store} , spatial information must be sacrificed. As a result, we focus on a basic collection scheme that utilizes the entire detection chip.

In traditional single molecule experiments, the excitation laser is run continuously (figure 2b) throughout the entire data collection and storage process. During t_{store} , the fluorophore undergoes excitation and emission cycles that are not recorded by the detector. Fluorophores can undergo a limited number of excitation and emission cycles before photobleaching, commonly on the order of 10^5 - 10^6 cycles [19]. The cycles occurring during t_{store} contribute to this limited number, despite not adding any information to the data collected.

We introduced a new modulated excitation scheme (figure 2c) that extends the observation time of fluorophores by removing unnecessary excitation during t_{store} . The excitation was synchronized to detection using frequency generators and an AOM. In this work the EMCCD detector used had $t_{\text{store}} = 32 \text{ ms}$. Two frequency generators were used in our setup to be compatible with an existing alternating-laser excitation arrangement. The frequency generators were structured in a master-slave arrangement but use of a single frequency generator or a daisy-chain setup would be a viable alternative option. The master frequency generator was set at a frequency of ν_{rate} , or the corresponding frequency at which a single frame of data is collected and stored on the EMCCD ($\nu_{\text{rate}} = 16.072$ or 7.576 Hz for $t_{\text{collect}} = 30$ and 100 ms , respectively) and a low pulse symmetry (5%). A short pulse from the master frequency generator was sent both to the slave generator and EMCCD to synchronize the excitation and detection, respectively. The pulse sent to the slave generator triggered a series of pulses that were the same length as t_{collect} , here at a frequency of 1 kHz , symmetry of 90%, and a burst count of 30 or 100 bursts for 30 and 100 ms integration times, respectively. Because the bursts were at such a high frequency and symmetry it was equivalent to a continuous pulse over t_{collect} . The signal produced by the slave frequency generator was sent to the modulation driver of the AOM that gated the first order diffraction mode of the excitation laser “on” during t_{collect} and “off” during t_{store} . The gated excitation was synchronized to the detection by the pulse received by the EMCCD from the master frequency generator. Transistor-transistor logic reception of the pulse at the EMCCD triggered the collection of a frame at an integration time of t_{collect} as set in the detector software.

3.2 Study of dye observation times – Cy3, Cy5, Alexa555

Data of single molecule observation times were collected for direct TIRF excitation of Cy3 and Alexa 555, non-radiative FRET excited Cy5, and direct epi-fluorescent excitation of

R6G with the traditional and modulated excitation schemes. Figure 3 reports the percentage of single molecules emitting over time with respective dye observation times found by exponential fits. Single molecule statistics should be representative of bulk measurements, thus the time dependent intensity should follow a single exponential decay for a sample with a single fluorescent species present where the coefficient within the exponent is the dye observation time [19]. Video S1 shows two image series of the data analyzed in figure 3a in real time and as a side-by-side comparison to demonstrate the improvements modulated excitation offers in single molecule studies.

Improvements in dye observation time were found for each of the four different fluorophores studied, but to varying degrees. The directly TIRF excited fluorophores Cy3 and Alexa 555 had a more significant improvement, by 215 and 1500%, respectively, while the FRET-excited Cy5 and direct epi-fluorescent excited R6G had improvements of 119% and 97%, as listed in figure 3. Further shown dynamically in supplementary data video 1, single Cy3 molecules can be identified throughout the time series, up to 50 s, with modulated excitation while with traditional excitation, close to all of the molecules have photobleached by this time. Video 1 also shows a larger percentage of the single molecules photobleach with traditional excitation. The observed improvements depend on the time scale of the detection scheme and excitation intensity; therefore, larger improvements in observation time may be possible for other experimental setups and time scales.

These results show that amplitude modulation can improve the observation time in a variety of conditions, including different families of fluorophores (cyanine, Alexa, and rhodamine), different excitation schemes (direct TIRF, FRET TIRF, and direct epi-fluorescence), and different sample conditions (aqueous oxygen scavengers, polymer matrix). Importantly for the later, this demonstrates that our method can improve dye observation time for systems where oxygen scavengers are not compatible or may interfere with the experimental variables. For samples in which oxygen scavengers can be used, the extension in dye observation time is in addition to on top of those offered by the scavenging systems.

It would be expected that all of the dyes would have an improvement that was proportional to $t_{\text{store}}/t_{\text{cycle}}$ (51.6 and 24.2% for Cy3/Cy5/R6G and Alexa 555, respectively). A possible explanation for this discrepancy may be that the chemical composition of the solution varies with the exposure time leading to variation in photobleaching rate. As stated earlier, photobleaching occurs when oxidative reactions irreversibly damage a fluorophore. The oxidative chemical make-up of the system may vary over the course of the experiment [28], along with possible pH changes [26, 29] due to changes in the total exposure time of the solution to the excitation, the only variable within the study. Additionally, the photophysics of the dyes are complex and can vary based on intensity, pulsing, alternating of different wavelengths, and direct versus FRET excitation. It has been found that for FRET measurements on confocal setups, pulsing of the laser on the order of 10 MHz can actually decrease the lifetime of the acceptor [44, 45]; this is likely why we see the lower amount of improvement in the Cy5 studies presented. Discrepancy between our observation of an improved observation of Cy5 compared to the reported reduction in lifetime in Eggeling, *et al.* and Kong, *et al.* are likely due to the difference in the time scales of the pulsing. The resilience of fluorophores due to solution and photophysical conditions has been covered in the literature [26-32, 44-46].

3.3 Improved localization of single molecules with modulated excitation

The burgeoning field of super-resolution microscopy [47-52], where the resolution defeats the diffraction limit of light (~250 nm), depends in many instances on the super-localization of single fluorophores. Traditionally, a two-dimensional Gaussian fit to the emission point spread function yields the centroid location of the molecule. The standard deviation of the

Gaussian fit determines the precision of the localization and is inversely proportional to the square root of the number of photons collected [24]. As an example application of how modulated excitation can improve super-resolution measurements, the localization of single molecules with and without modulation was compared.

As reported in figure 4, amplitude-modulated excitation improves the precision of localization for Cy3 by 47% for the experimental conditions used. Figure 4 a and b reports the average two-dimensional Gaussian fit for 294 and 242 Cy3 molecules measured with traditional and amplitude-modulated excitation, respectively. Because our samples were static (sample drift < 1 nm), the location of single molecules was determined from a summed image of all the frames collected. Static samples are relevant to many super-resolution cell based studies, where cells are fixed with aldehyde derivatives to avoid any structural alterations [53]. This example is also relevant to stimulated emission depletion (STED) super-resolution microscopy, where the photon yield of stationary fluorophores within the excitation light influences the resolution [54]. Extended observation time also increases the number of localized points obtained over the course of individual frames, as shown in figure 4 c and d. On average for the 294 and 242 Cy3 molecules measured with traditional and amplitude-modulated excitation, 39 ± 6 and 114 ± 25 points were obtained per molecule, respectively. This relates to stochastic reconstruction microscopy (STORM), where the localization distribution is obtained from the total number of points obtained from a single molecule [55]. From the respective fits in figure 4 a and b, the standard error in localization was calculated by taking the product of the standard deviation in x and y (σ_x and σ_y determined from the Gaussian fit) divided by the square root of the amplitude of the fit. The error in localization was found to be 0.25 and 0.17 nm/photons^{1/2} for traditional and modulated excitation, respectively. While the reported values for both the traditional and modulated excitation have a very high precision and would be adequate for most experiments, the concept here could be applied to systems that suffer from much shorter lifetimes and lower quantum yields than the Cy3 example presented.

4. Conclusions

We report a hardware-based method that can improve dye observation time by up to 1500% and super-resolution localization by 47% for the presented experimental conditions by synchronizing the excitation and detection rates. This method has practical applications for any experiment where photobleaching is of concern and uses detectors with a data storage time-delay that interferes with collection. Because it is a hardware approach, the method is compatible with any chemical makeup of a sample, including different fluorophore and solution conditions. This concept can be easily implemented in any current setup using excitation modulation, such as acousto-optic or electro-optic modulators for alternating-laser excitation [56-59] and polarization-modulated studies [60, 61], or a high-speed shutter system, as long as modulation and data acquisition can be synchronized.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

L. Kisley is supported by the National Science Foundation Graduate Research Fellowship under Grant No. 0940902. C. Landes acknowledges the Welch Foundation [Grant C-1787], the National Science Foundation [Grants CBET-1133965 and CHE-1151647], and the National Institutes of Health [Grant GM94246-01A1]. Also, Dr. Stephan Link and his research group are to thank for helpful discussions.

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Figure 1. Schematic of wide field microscope, including AOM controlled by frequency generators (Freq. gen.) and EMCCD detector.

Figure 2. Relation of detection scheme of a typical EMCCD over time (a) compared to traditional (b) and modulated excitation (c).

Figure 3.

Percent of single molecules remaining over time with respective fits and observation times reported for (a) Cy3 donor-TAR DNA excited directly, (b) Cy5 donor/acceptor-TAR DNA excited non-radiatively by energy transfer via Cy3, (c) Alexa 555 excited directly by TIRF, and (d) direct excitation with an epi-fluorescent geometry of R6G. Each curve is the average of three 1000 frame trials.

Figure 4. Average two-dimensional Gaussian fit to summed single Cy3 molecule point spread functions and the respective relative standard error in localization for (a) traditional and (b) modulated excitation. Localized individual centroid locations from two-dimensional Gaussian fits in each frame for representative single Cy3 molecules for (c) traditional and (d) modulated excitation.