Alternating-Laser Excitation of Single Molecules

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ABSTRACT

Single-molecule fluorescence spectroscopy addresses biological mechanisms and enables ultrasensitive diagnostics. We describe a new family of single-molecule fluorescence methods that uses alternating-laser excitation (ALEX) of diffusing or immobilized biomolecules to study their structure, interactions, and dynamics. This is accomplished using ratios that report on the distance between and the stoichiometry of fluorophores attached to the molecules of interest. The principle of alternation is compatible with several time scales, allowing monitoring of fast dynamics or simultaneous monitoring of a large number of individual molecules.

INTRODUCTION

Room-temperature, real-time observation of individual molecules is revolutionizing several areas of physics, chemistry, and biology by eliminating the ensemble averaging present in conventional analysis and by uncovering static and dynamic heterogeneities. Single-molecule methods monitor dynamics of biomolecules under equilibrium and complex reaction kinetics under nonequilibrium conditions, capturing views that are inaccessible to ensemble methods owing to the lack of synchronization of biomolecules.

Single-molecule detection is characterized by three major attributes. First, the properties of ensembles of billions of molecules can be described reliably by observations of a few single molecules (due to ergodicity). Second, single-molecule observations carry far more information than do ensembles (when ergodicity breaks down). Third, the amount of sample necessary to capture the new information is miniscule (<10^-16 mol), several orders of magnitude lower than the amount needed for ensemble methods. Such properties are well aligned with modern science, which moves toward smaller scales, higher throughputs, and higher-complexity systems, the descriptions of which require monitoring of several observables. However, to mine the wealth of information displayed by single molecules, one has to pose the “right” questions to the molecules; in the case of single-molecule fluorescence spectroscopy (SMFS), this requirement led to novel fluorescent probes and labeling strategies and complex excitation/detection schemes. SMFSs are applied to either diffusing or immobilized molecules, accessing different time scales of information. Several excellent reviews exist on many aspects of SMFSs.

Here, we describe a new family of fluorescence methods that uses alternating-laser excitation of single molecules to study their structure, interactions, and dynamics through measurements of distances and stoichiometries.

CLIMBING THE LADDER OF COMPLEXITY: MORE EXCITATIONS, FLUOROPHORES, AND EMISSIONS

The pioneers of SMFS were concerned with the milestones of detecting a single molecule and a single fluorophore. The first milestone was reached in 1976 by Hirschfeld, who detected single antibodies carrying ~100 fluorophores. Fifteen years later, the Keller group at Los Alamos, driven by single-molecule DNA sequencing, detected single fluorophores in solution at room temperature (cryogenic conditions).
detection of single molecules will not be discussed here). Room temperature observations of immobilized single fluorophores were followed by near- and far-field microscopies, with applications in biomolecular systems (reviewed in ref 4). Early methods employed a single excitation wavelength and a single emission wavelength to interrogate molecules labeled with a single fluorophore, reporting on fluorophore localization on a surface, fluorescence lifetime, diffusion constants, or changes in quantum yield.

Steps toward higher complexity introduced a second detection channel. The added channel detected a second polarization component or a different wavelength, reporting on the location and rotational freedom of fluorophores or on spectral jumps due to transient changes in emission spectra (reviewed in ref 4).

Soon thereafter, a second fluorophore was introduced; the presence of two fluorophores within a certain distance allowed interprobe distance measurements, either through high-resolution localization of the two fluorophores or through fluorescence resonance energy transfer (FRET; also known as Förster resonance energy transfer). In both cases, the use of single-wavelength excitation was maintained (Figure 1). An application of the single-excitation/multiple-emissions concept used semiconductor nanocrystals for ultrahigh-resolution colocalization,18 since nanocrystals of different sizes combine overlapping, wide, high cross-section excitation bands with distinct, sharp emission bands (Figure 1A). Thus, nanocrystals of distinct emission bands were excited and detected simultaneously, enabling multicolor imaging.19 A similar concept is employed for two-photon excitation of fluorophores.20 Unlike the absorption spectrum for single-photon excitation (narrow and symmetric to the emission spectrum), two-photon excitation spectra are relatively wide and independent of emission wavelength (Figure 1B), permitting simultaneous excitation of distinct-emission fluorophores by a single far-red or infrared laser.

A breakthrough in our ability to monitor structure and interactions occurred in 1996, when FRET between a single pair of fluorophores (single-pair FRET; spFRET) was demonstrated. FRET is the nonradiative transfer of excitation energy from a fluorophore serving as a donor (D) to a complementary fluorophore serving as an acceptor (A). FRET acts as a “spectroscopic ruler” for the 1−10 nm scale, since FRET efficiency, $E$, is a sensitive function of interprobe distance $R$ (specifically, $E = \frac{1}{[1 + (R/R_0)^6]}$, where $R_0$ is a constant that equals the interprobe distance at $E = 50\%$). Initial work was followed by far-field observations, both for molecules immobilized on surfaces21 and for those diffusing in solution.22 The development of spFRET was a pivotal moment for single-molecule detection; it paved the way for studying macromolecular structure and interactions and has been used to study the dynamics of proteins, nucleic acids, and their complexes,2,12 mainly by observing distance changes and their kinetics.

However, during mechanistic analysis of transcription complexes by our group, it became apparent that spFRET was not a general platform for quantitative analysis of structure, due to numerous corrections required to measure FRET accurately and to the presence of species that obscure FRET measurements when $R > 6−8$ nm. Moreover, spFRET is not a general platform for quantitative analysis of interactions. For example, for the interaction $M^A + L^D \rightleftharpoons M^A L^D$ (where $M^A$ is an acceptor-labeled macromolecule and $L^D$ is a donor-labeled ligand), spFRET cannot quantitate the major species involved owing to several factors: First, spFRET yields a measurable signal only when D–A distances in the $M^A L^D$ complex are sufficiently short ($R_{D-A} < 6−8$ nm) to distinguish complexes from free $L^D$ species. Second, inactive states of acceptors result in $M^A L^D$ species that exhibit D-only characteristics,22 leading to apparent increases in free $L^D$ species. Finally, no $M^A$ species are detected, and complexes with stoichiometries other than 1:1 (e.g., $M^A[L^D]_2$) cannot be identified as such by spFRET. These limitations, compounded with substoichiometric labeling, photophysics, photobleaching, and aggregation/dissociation phenomena, have limited the implementation of spFRET and called for additional observables to improve its resolving capability.

All methods described up to this point use single excitation sources, mainly to avoid chromatic aberrations.

**FIGURE 1.** Single- and alternating-laser excitation approaches to SMFS: (A) single-laser excitation of fluorophores with wide overlapping excitation spectra and separable emission spectra; (B) single-laser excitation of fluorophores with wide overlapping two-photon excitation spectra and separable emission spectra; (C) single-laser excitation of fluorophores participating in FRET in which the donor is excited directly by the laser and emits at the D-emission wavelength whereas part of the D-excitation energy is transferred to the acceptor, which emits at wavelengths longer than the donor; (D) alternating-laser excitation of fluorophores able to participate in FRET. For 50% of the time, the donor is excited by the D-excitation laser; in the case of FRET, there is acceptor emission. For the rest of the time, the acceptor is excited by the A-excitation laser, resulting in A-emission. Duty cycles can vary.
Alternating-Laser Excitation of Single Molecules

To develop a general FRET-based method for monitoring structure and interactions simultaneously, we reasoned that alternation between two excitation wavelengths accesses structural information using a donor-excitation wavelength and interaction information using both the donor- and acceptor-excitation wavelength. Thus, we introduced alternating-laser excitation (ALEX) spectroscopy, a method that obtains D-excitation- and A-excitation-based observables for each single molecule by rapid switching between excitations (Figure 2). Alternating excitation was used previously for immobilized molecules but only as a qualitative check of acceptor excitation. In conclusion, proximity between a FRET donor and acceptor is necessary for distance analysis but insufficient for interaction analysis; on the other hand, coincidence of a FRET donor and acceptor in the absence of FRET is necessary for interaction analysis but generates no distance constraints.

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Alternating excitation recovers distinct emission signatures (Figure 3A) by calculating two fluorescence ratios: the FRET efficiency, $E$, which reports on donor-acceptor distance, and the distance-independent ratio, $S$, which reports on donor-acceptor relative stoichiometry:

$$E = \frac{F_{\text{D}^\text{Aem}}}{F_{\text{D}^\text{Aexc}}} / \left(\frac{F_{\text{D}^\text{Dem}}}{F_{\text{D}^\text{Dexc}}} + \gamma F_{\text{D}^\text{Dexc}}\right)$$

$$S = F_{\text{D}^\text{Aem}} / \left(F_{\text{D}^\text{Aexc}} + F_{\text{D}^\text{Aexc}}\right)$$

where $F_{\text{D}^\text{Dexc}}$ is D-excitation-based D-emission, $F_{\text{D}^\text{Dem}}$ is D-excitation-based A-emission, $F_{\text{D}^\text{Aexc}}$ is a sum of D-excitation-based emissions, $F_{\text{D}^\text{Aexc}}$ is a sum of A-excitation-based emissions (Figure 2B), and $\gamma$ is a detection-correction factor. All emissions refer to single molecules.

Microsecond ALEX ($\mu$s-ALEX): Structure and Interactions of Diffusing Molecules

We applied ALEX to diffusing molecules using microsecond time scale alternation ($\mu$s-ALEX; Figure 3) using DNA
fragments as D-only controls, A-only controls, and D−A species with various interprobe distances. On E−S histograms, D-only species appeared as species with low E and high S, whereas A-only species appeared as species with low S. In contrast, all D−A species displayed intermediate S and variable E (due to different D−A distance); the latter observation demonstrated the independence of E and S.

Since ALEX identifies D-only, A-only, and D−A species, it is suitable for analysis of interactions. In a binding equilibrium, D-only and A-only species represent free interactants and D−A represents a complex (Figure 4A); equilibrium binding and kinetic rate constants can be measured by counting molecules, due to the linear relation between observed molecules and concentration (for subnanomolar concentrations). For an interaction of a macromolecule M with a ligand L (Figure 4A), which has an equilibrium dissociation constant $K_d$, the fraction of bound macromolecules (fractional occupancy $ı$) at ligand concentration $[L]$ is $ı = [ML]/([ML] + [M]) = [L]/([L] + K_d)$, transformed to

$$\theta = \frac{[D−A]/([D−A] + [A-only])}{[D-only]/([D-only] + K_d)}$$

Measuring $\theta$ at several ligand concentrations $[L]_0$ allows calculation of $K_d$; $\theta$ can also be used to evaluate association/dissociation kinetics.
We studied the interaction of catabolite activator protein (CAP) with DNA as a model for protein–nucleic acid interactions by monitoring the interaction of D-labeled CAP (as “ligand” CAPD; Figure 4D) with its preferred DNA site (as “macromolecule” DNA) with or without cyclic AMP (cAMP). With cAMP, CAP-DNA complexes were detected as D-A species (Figure 4C). CAP-DNA complexes displayed an E distribution with high E values (due to DNA bending toward CAP) and considerable heterogeneity (due to two possible D-labeling sites on CAP [Figure 4D] and interconversion between complexes with variable DNA bending). Without cAMP, few complexes were formed. We then assessed equilibrium binding by titrating DNA with CAP (Figure 4E). With cAMP, $K_d \approx 32$ pM; without cAMP, CAP binds to DNA >150-fold more weakly ($K_d > 5$ nM). Our analysis was in excellent agreement with ensemble assays.

**Accurate FRET Determination within Single Molecules Using ALEX**

Since diffusion spFRET combines high sensitivity, the absence of surface-induced perturbations, and the ability to identify subpopulations, it is suitable for structural analysis of biomolecules with substantial heterogeneity. We demonstrated previously that diffusion spFRET can be used to measure distances within DNA. However, due to limitations, spFRET had been confined to qualitative studies of structure and structural changes and to a FRET range between 40% and 100%.

**FIGURE 4.** Analysis of protein–DNA interactions using ALEX-FAMS. Panel A shows analysis of interactions. Using a labeled macromolecule (MA; A-only species) and a labeled ligand (LD; D-only species), we can monitor the formation of complex MA LD (D–A species) on the E–S histogram by monitoring occupancy, $\theta$, at a ligand concentration, [LD]. Panel B shows the E–S histogram for DNA, Panel C shows the E–S histogram for DNA, CAP, and cAMP. Panel D shows the CAP–DNA complex and labeling scheme. The acceptor was on DNA, and the donor on either of two possible sites on CAP (arrows); labeling efficiency was kept low, so most D–A species have one donor. The low labeling efficiency does not affect the determination of the binding constant of the interaction; it only changes the maximum occupancy, $\theta_{\text{max}}$. Panel E shows titrations of DNA with CAP in the presence of cAMP ( ● , tight interaction) or in the absence of cAMP ( ○ , weak interaction).
We recently used \(\mu s\)-ALEX to measure accurate FRET and distances from single molecules.\(^{28}\) Using ALEX to sort species based on donor–acceptor distance and stoichiometry, we determined the correction factors needed for accurate FRET measurements. D-only species provide D-leakage factor \(l\) (which accounts for D-emission into the A-detection channel), A-only species provide A-direct-excitation factor \(d\) (which accounts for A-emission into the A-detection channel due to A-direct-excitation by \(D_{\text{exc}}\), and not due to FRET), and two or more D–A species with large difference in \(E\) provide the \(\gamma\) factor.

\(\mu s\)-ALEX Studies of Gene Transcription

Since ALEX monitors structure and interactions simultaneously, it can dissect biological processes orchestrated by coordinated changes in distance, stoichiometry, or both, such as binding events followed by conformational changes, translocation events on linear tracks culminating in dissociation of components, assembly/disassembly pathways, and template-directed oligomerization events.

We used ALEX to address questions about gene transcription, during which genes are copied to messenger RNA by the enzyme RNA polymerase (RNAP) (Kapanidis et al., manuscript in preparation). We first investigated the fate of initiation protein \(\sigma^{70}\) during the transition from transcription initiation to elongation, a transition known as promoter escape (where a promoter is a special DNA sequence that precedes genes).\(^{30}\) Protein \(\sigma^{70}\) is required for RNAP binding to promoters, for opening double-stranded DNA to expose genetic information, and for disengaging RNAP from promoter DNA during escape. The textbook version of transcription predicts that 100% of \(\sigma^{70}\) dissociates after escape, but recent studies revealed \(\sigma^{70}\)-containing elongation complexes.\(^{30}\)

An ALEX assay that addresses \(\sigma^{70}\) release uses a labeling scheme wherein a donor is placed on \(\sigma^{70}\) near the RNAP leading edge and an acceptor is placed at the downstream end of DNA and at a distance from the donor that results in low FRET; this scheme results in distinct outcomes for the two models describing the fate of \(\sigma^{70}\) upon escape (Figure 6A). Upon formation of the open RNAP\(\sigma^{70}\)-DNA complex (a complex wherein RNAP interacts with partly single-stranded promoter DNA and where no transcription occurs), two populations are seen (Figure 6B, left): free DNA (low-S species) and open complex (intermediate-S, low-E species). Under conditions that allow the formation of the first stable elongation complex by the large majority of open complexes,\(^{30}\) no change is seen in the ratio of free DNA to RNAP\(\sigma^{70}\)-DNA complex (monitored by the \(S\) histogram), showing no dissociation of \(\sigma^{70}\) upon escape (Figure 6B, right). However, as seen in the \(E\) histogram for the RNAP\(\sigma^{70}\)-DNA complex, the donor–acceptor distance has decreased (compared with open complex), consistent with downstream translocation of RNAP\(\sigma^{70}\) on DNA. Using ALEX, we measured the content of \(\sigma^{70}\) in early and mature elongation complexes and studied their structure, stability, and dynamics, providing strong support for the proposal that \(\sigma^{70}\) release upon escape is not obligatory.

\(\mu s\)-ALEX: Sorting Photophysics from Dynamics

To study dynamics in nonequilibrium systems, extended observations of individual molecules are necessary,
achieved through simultaneous observation of multiple surface-immobilized molecules within a field of view. This is accomplished using total internal reflection (TIR) microscopy, which combines evanescent-wave excitation within a thin layer from a surface and wide-field detection by an ultrasensitive camera. However, single-excitation TIR suffers from limitations similar to those described earlier: the presence of artifactual D-only species, the inability to detect D-only species when R
\text{D} \nonumber \text{A} \nonumber \text{R}
, the inability to detect A-only species, and complex photophysics.

To overcome such limitations, we combined ALEX with TIR microscopy in an approach termed as millisecond-ALEX (ms-ALEX) microscopy. The term millisecond refers to both detection and excitation time scales; it is, however, set by the frame rate of the camera, which is limited by the signal-to-noise ratio achieved using fluorophores compatible with single-molecule detection. The alternating-excitation source was formed as in \mu s-ALEX and sent to a prism-based TIR microscope. Donor and acceptor fluorescence was detected using dual-view detection (four emission intensities per molecule; Figure 7A), and ratios E and S were calculated for each molecule and at each alternation period.

ms-ALEX was tested on surface-immobilized transcription complexes, achieving specific immobilization by preparation of biotinylated complexes, surface modification with biotin–poly(ethylene glycol) and streptavidin, incubation of complexes with the modified surface, and wash of unbound material. After analyzing minute-long movies, we constructed E–S histograms that resolve D-only (high-S, low-E) and D–A (intermediate-S) species (Figure 7B). Additional thresholds (ratios or fluorescence intensities) select species further; for example, D–A species can be selected using the sum of F_Dex and F_Aex intensities (Figure 7C).

Thresholding can also be used for one-dimensional E or S histograms. For example, the E histogram for D-only and D–A complexes essentially reconstructs the outcome of single-laser excitation TIR microscopy (Figure 7D). Thresholding removes all D-only species, permitting E measurements even at low FRET (Figure 7E; E \approx 0.18) and extending the range of accessible distances.

FIGURE 6. Application of ALEX to \sigma_70 release upon promoter escape. Panel A presents predictions of models regarding \sigma_70 release. Release of \sigma_70 from an RNAP–DNA complex results in dissociation of a low-E D–A species to free \sigma_70 (D-only) and to a \sigma-free RNAP–DNA elongation complex (A-only). In contrast, retention of \sigma_70 converts a low-E* D–A species to a high-E* D–A species. Panel B presents E–S histograms for open and elongation complexes for promoter lacUV5 (E* is a modified expression of E). Species with 0.45 < S < 0.8 (D–A species; in black rectangle) correspond to \sigma_70-containing complexes with DNA, while species with S < 0.3 (A-only species) do not contain \sigma_70. The D–A species and A-only species are clearly separated on the S histogram. Since the S distribution remains unchanged upon formation of the first stable elongation complex, \sigma_70 is retained upon escape.
FIGURE 7. ms-ALEX microscopy. Panel A shows fluorescence from multiple immobilized complexes obtained by alternation between donor excitation (left) and acceptor excitation (right). Encircled molecules represent the main species. Panel B shows the $E$-$S$ histogram of a low-FRET sample containing all species. Panel C shows the $E$-$S$ histogram of the sample in panel B after only the D–A species are retained. Panel D shows the $E$-histogram for single-laser excitation TIR, showing overlap of D-only and D–A species. Panel E shows the $E$-histogram for ms-ALEX microscopy, after D–A species are selected (as in panel C). Panel F shows fluorescence-intensity time traces for a high-$E$ complex, showing that increased $f_{Aex}^{Dem}$ is due to acceptor blinking. Panel G shows FRET traces calculated from panel F. top, $E$ calculated using the complete timetrace; bottom, $E$ values after the time points with active acceptor are selected, thus eliminating changes in $E$ due to acceptor blinking.
Importantly, ms-ALEX identifies and removes photophysics (e.g., fluorophore blinking) from fluorescence-intensity time traces for individual molecules (Figure 7F). Anticorrelated intensities \( f_{D_{ax}}^{D_{ax}} \) and \( f_{A_{ax}}^{A_{ax}} \) (Figure 7F, green and red curves) are used routinely in single-excitation TIR to identify FRET changes due to dynamics that change interprobe distances. However, processes that render a FRET acceptor unable to accept energy or to emit photons produce a similar observable: a transient decrease in apparent \( E \) (Figure 7F; low-\( E \) state in Figure 7G, top). In ns-ALEX, direct observation of the acceptor state using \( A_{ex} \) excitation (\( f_{A_{ax}}^{A_{ax}} \) signal; Figure 7F, black curve) shows clearly that the specific FRET change is due to acceptor blinking. Thus, by selecting parts of the time trace wherein the acceptor is active (using a \( f_{A_{ax}}^{A_{ax}} \) threshold), we can generate blinking-free \( E \) traces (Figure 7G, bottom), allowing conformational transitions to be observed without complications. Using this “photophysics sorting”, we studied dynamics during promoter escape by RNApol.

Similar analysis that monitors transient \( S \) changes can detect multiround loading/unloading of biomolecules on immobilized templates.

**Nanosecond-ALEX: Searching for Fast Dynamics**

Interlacing two pulsed lasers (\( D_{exc} \) and \( A_{exc} \); picosecond pulses) with a fixed time delay between pulses achieves alternating-laser excitation while monitoring donor and acceptor fluorescence lifetimes (nanosecond time scale). Two time measurements are made for each photon: the time since the experiment began (macrotime \( t \)); Figure 8A) and the time delay between a \( D_{exc} \) pulse and a photon (microtime \( r \); Figure 8B). The macrotime is used to identify single-molecule transits across the observation volume and to form correlations and time traces; the microtime is used to determine fluorescence lifetime and assign laser excitation to a photon (by dividing photons into groups detected after \( D_{exc} \) and after \( A_{exc} \); Figure 8B) (Lawrence et al., submitted for publication).

ALEX at the nanosecond time scale (nanosecond-ALEX or ns-ALEX) creates new capabilities for single-molecule FRET. For example, ns-ALEX allows distance measurements without detection corrections, since lifetime measurements measure FRET using only donor lifetime in the presence \( (t^D) \) and absence \( (t^D) \) of acceptor \( (E = 1 - (t^D/r^D)) \). Since such measurements involve a single fluorophore and detector, no detection correction is necessary.

Nanosecond-ALEX can scrutinize the rotational freedom of fluorophores, setting limits for the extent that probe orientation affects a distance measurement. (FRET, in addition to its distance dependence, depends on the relative orientation of D-emission and A-excitation dipoles,\(^{35}\) compounded in orientation factor \( \kappa^2 \), which equals \( \frac{2}{3} \) for free rotation of donor and acceptor.\(^{36}\) Characterization of the rotational freedom is necessary for reliable distance measurements and performed using steady-state and time-resolved fluorescence anisotropy.\(^{37}\) In ns-ALEX, we use linearly polarized excitation and divide the fluorescence emission by polarization, as well as by spectrum, thus performing time-resolved anisotropy and FRET measurements. Lifetime measurements measure the decay of anisotropy over the 0.1–10 ns time scale, directly evaluating the rotational freedom of the fluorophores.

Lifetime measurements improve distance measurements by recovering distances that fluctuate on timescales slower than fluorescence lifetime. FRET shortens donor lifetime \( t^D \) according to \( t^D = t^D (1 - E) \). For a single interprobe distance, the donor-intensity decay is described by a single (shortened) lifetime. For multiple distances, one expects multiple donor lifetimes, which are difficult to recover solely from donor-intensity decays. Global analysis of donor-intensity and acceptor-intensity decays improves the accuracy of recovered distance distributions, since acceptor-intensity decays emphasize short distances.
(high $E$; short $r^D$), whereas donor-intensity decays emphasize long distances (low $E$; long $r^D$). Distance fluctuations on the time scale of fluorescence lifetime narrow the observed distance distributions.  

FRET-based analysis of distance distributions using ensemble time-resolved fluorescence is obscured by the presence of multiple species, such as folded and unfolded proteins, or incomplete labeling. ns-ALEX uses single-molecule sorting to select each species (Figure 8C), obtaining lifetime histograms that include photons from only one species. Fluorescence-intensity decay analysis is typically performed for all photons from each species, since it is difficult to perform using the few photons (<100) detected from a single molecule. For each sample, diffusing molecules are sorted into species on $E$–$S$ histograms (Figure 8C), and fluorescence-decay plots are generated for the photons from each species (Figure 8D–G). ns-ALEX recovers all the parameters needed to describe donor- and acceptor-intensity decays. For instance, when the intensity after $D_{exc}$ and $A_{exc}$ for A-only species (Figure 8E) is compared, direct acceptor excitation by $D_{exc}$ (A-direct excitation) is quantified. Donor-emission into the acceptor-detection channel (D-leakage) is similarly quantified using D-only species (Figure 8D). These corrections, along with the intrinsic acceptor lifetime after $A_{exc}$, quantify the crosstalk contributions of D-leakage and A-direct excitation, constraining the fitting of the acceptor decays after the $D_{exc}$ pulse.

The end result of ns-ALEX is the simultaneous recovery of distance distributions, orientational dynamics, and accurate distances, opening new avenues for studying biomolecular structure, dynamics, and interactions.

Conclusion and Outlook
Alternating-laser excitation spectroscopy adds new tools to the single-molecule toolbox. ALEX-based sorting analyzes complex samples without strict requirements for high purity, optimal distance range, or high concentration, facilitating reagent preparation. ALEX offers the ability to perform sophisticated experiments in solution, enabling direct comparisons between ensemble and single-molecule assays and obviating immobilization.

Apart from the advantage of ALEX over spFRET in monitoring interactions without FRET, a salient feature of ALEX is the improved distance measurement within single molecules. Since SMFS sensitivity is high (requiring 6–10 orders of magnitude less material than crystallography or NMR), the accurate distance capability of ALEX (with fluorophores having adequate rotational freedom), combined with rapid preparation of small amounts of labeled biomolecules, can lead to solution-based, low-resolution structures of biomolecules and their complexes, as done using ensemble-FRET and distance-restrained docking.  

Such an approach is suitable for large, multicomponent, heterogeneous complexes of cellular machinery (especially if inaccessible to conventional structural biology), and it will benefit from existing crystallographic or NMR-based structures.

The flexibility of ALEX leaves ample room for expansion. An extension of µs-ALEX adds a third fluorophore, excitation source, and detected-emission range (three-color ALEX). For example, an SMFS-compatible, fluorescent-like fluorophore complements well the common FRET pair of tetramethylrhodamine and Cy5. We demonstrated the feasibility of three-color ALEX using triply labeled DNA (N.L., A.K., and S.W., manuscript in preparation). Multicolor ALEX allows simultaneous measurements of several distances within a single molecule, allowing triangulation and construction of structural models. It also enables real-time, multiperspective views of biomachinery, since monitoring multiple distances and stoichiometries within a complex (such as the replisome or the ribosome) will detail the extent, kinetics, and sequence of conformational changes and assembly/disassembly events that define mechanisms of action.

ALEX can be extended to study fluorescent species in concentrations higher than 1 nM. This can be achieved by combining ALEX with correlation analysis on sorted species, recovering fluctuation time scales and associated amplitudes. Methods that confine excitation volume will greatly increase the upper concentration limit of ALEX. The concentration limitation (general for SMFS) can be solved by using nonfluorescent analytes, which can be present at any concentration and can monitor their effect by observing the status of the labeled species (present in picomolar concentrations).

Other extensions combine ALEX with physical sorting (purifying species of interest by on-line detection of distance and stoichiometry, followed by amplification or analysis) and with observations of interactions/dynamics in living cells (pending advances in site-specific labeling). Finally, ALEX-based high-throughput bioassays will result in robust biotechnology applications, such as diagnostic and drug-discovery assays.

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