Red light, green light: probing single molecules using alternating-laser excitation

Yusdi Santoso, Ling Chin Hwang, Ludovic Le Reste and Achillefs N. Kapanidis

Abstract

Single-molecule fluorescence methods, particularly single-molecule FRET (fluorescence resonance energy transfer), have provided novel insights into the structure, interactions and dynamics of biological systems. ALEX (alternating-laser excitation) spectroscopy is a new method that extends single-molecule FRET by providing simultaneous information about structure and stoichiometry; this new information allows the detection of interactions in the absence of FRET and extends the dynamic range of distance measurements that are accessible through FRET. In the present article, we discuss combinations of ALEX with confocal microscopy for studying in-solution and in-gel molecules; we also discuss combining ALEX with TIRF (total internal reflection fluorescence) for studying surface-immobilized molecules. We also highlight applications of ALEX to the study of protein–nucleic acid interactions.

Introduction

The last 15 years in biochemistry and biophysics have been marked by the rising popularity of single-molecule methods as versatile, sensitive and direct methods for studying structure, interactions and dynamics [1–8]. Single-molecule methods are still technically demanding; however, when implemented successfully, they uncover information usually hidden in conventional ensemble measurements. For example, single-molecule methods yield distributions of an observable (e.g. the proximity of two DNA sites within a transient complex or dwell times at a certain state during a pathway), not just the average, thus uncovering heterogeneity of static or dynamic nature usually inaccessible to ensemble methods. Single-molecule methods also provide real-time trajectories of reactions performed by individual molecules, thus observing directly the presence and lifetime of intermediates without the need to synchronize the reaction in question. In the present article, we discuss ALEX (alternating-laser excitation) spectroscopy, a new single-molecule fluorescence method that interrogates structure and interactions within protein–nucleic acid complexes.

ALEX is a method that builds on single-molecule measurements of FRET (fluorescence resonance energy transfer). FRET is a photophysical interaction (a non-radiative transfer of excited-state energy between two fluorescent probes) that reports on the proximity of two sites within a biomolecule or a complex of biomolecules. The two sites are usually modified by two different probes that act as the FRET donor and acceptor. Owing to its sensitivity to the donor–acceptor distance, FRET has been used as a ‘spectroscopic ruler’ for measuring distances within the 2–10 nm range [9]. FRET between a single pair of fluorophores was first demonstrated in 1996 [10]. Since then, single-molecule FRET has been developed further and applied to many biomolecular systems and interactions, with particular success in exploring protein–nucleic acid interactions, protein folding and dynamics, and nucleic acid folding and dynamics [1–5,11–26].

Single-molecule FRET experiments in solution typically employ dilute solutions of labelled biomolecules [27]. When a labelled biomolecule diffuses through a femtolitre-sized observation volume formed by a focused laser beam, the laser light excites the donor fluorophore. If the acceptor is within a distance that allows FRET to occur, the donor transfers energy to the acceptor and causes the acceptor to emit fluorescence; light emitted from the donor and the acceptor is then detected on separate detectors. The apparent FRET efficiency for a single molecule is given as the ratio of the number of photons detected on the acceptor channel (i.e. photons emitted mainly due to FRET) over the total number of detected photons. This information is summarized in one-dimensional FRET histograms (Figure 1A) that report on biomolecular structure.

Often, single-molecule FRET is limited by its limited dynamic range for distance measurements and by complex photophysics of the fluorophores used. For instance, it is difficult to differentiate between low FRET species (with donor–acceptor distance longer than 6–8 nm) and species that contain only donor (D-only species, arising due to free components or photodestruction of the acceptor; Figure 1A [27]). Moreover, complexes with stoichiometries other than 1:1 cannot be identified easily using single-molecule FRET. These limitations are more pronounced when working with single diffusing molecules.

Key words: alternating-laser excitation (ALEX), biomolecular interaction, fluorescence resonance energy transfer (FRET), protein–DNA interaction, single-molecule fluorescence, total internal reflection fluorescence (TIRF).

Abbreviations used: ALEX, alternating-laser excitation; 3c-ALEX, three-colour ALEX; FRET, fluorescence resonance energy transfer; TIRF, total internal reflection fluorescence.

1 To whom correspondence should be addressed (email a.kapanidis1@physics.ox.ac.uk).
Figure 1 | ALEX spectroscopy of single molecules
(A) One-dimensional $E$ histogram from a typical single-molecule FRET experiment. D-only indicates molecules containing only the donor label; A-only indicates molecules containing only the acceptor label (not visible using single-molecule FRET); D-A indicates molecules containing both the donor and the acceptor. D-only molecules and molecules with large donor-acceptor distance (>8 nm) are grouped together at low $E$. (B) Two-dimensional $E$-S histogram obtained using ALEX. Ratio $S$ reports the presence of donor and/or acceptor. Only D-A molecules are selected (orange box), and their $E$ values are plotted on a one-dimensional $E$ histogram. (C) A single molecule diffusing through the confocal volume is excited by alternating green and red lasers to produce a characteristic photon burst. (D) Photons are grouped into four channels depending on the laser excitation ($D_{\text{exc}}$ or $A_{\text{exc}}$) and the emission detector ($D_{\text{em}}$ or $A_{\text{em}}$). After bursts due to single molecules are identified, the photons from various channels are counted for each molecule. $f_{\text{Dem}}^{D_{\text{exc}}}$, total photons collected at X-emission channel due to Y-excitation. $E$ and $S$ can then be calculated according to eqns (1) and (2) (see the main text); the photon count $f_{\text{Dem}}^{A_{\text{exc}}}$ is not considered since it is negligible.

Concept of ALEX spectroscopy

ALEX spectroscopy [28,29] extends single-molecule FRET by providing direct information on the presence and state of both donor and acceptor fluorophores. This information is summarized in two-dimensional histograms of FRET and relative fluorophore stoichiometry (Figure 1B), which report on biomolecular structure and stoichiometry respectively.

In essence, ALEX enables ‘virtual’ sorting of fluorescent molecules by separating singly labelled species from species that contain both fluorophores (e.g. protein–DNA complexes formed between singly labelled proteins and singly labelled DNA).

ALEX is achieved by alternating between donor and acceptor excitation lasers for sample illumination (Figure 1C) and obtaining donor-excitation and acceptor-excitation-based
ALEX on diffusing molecules in free solution and in gels

Single molecules diffusing in free solution produce characteristic fluorescence bursts as they traverse the observation volume (Figure 2A, middle panel); the typical timescale for each transit for a small protein is \( \sim 1 \) ms. Since the detection time of each photon is recorded, search algorithms can be used to identify bursts corresponding to single molecules. Various statistics are computed for every molecule (e.g. photons detected in the donor channel, apparent FRET efficiency) and are used for calculating FRET and stoichiometry ratios; these ratios are plotted on two-dimensional \( E-S \) histograms that permit virtual sorting of molecular subpopulations (Figure 2A, right-hand panel). Accurate FRET efficiencies can be recovered after applying corrections for spectral cross-talks and instrumental factors [30].

Virtual sorting through ALEX does not always eliminate the need for purification. For example, some reactions that form specific protein–DNA complexes also form aggregates or non-specific complexes. If not removed, these contaminating species can complicate ALEX measurements in solution. In such cases, confocal ALEX can be combined with gel-purification steps. If the species of interest can be separated in non-denaturing polyacrylamide gels, the band containing the complex of interest can be excised and examined directly using confocal ALEX (Y. Santoso and A.N. Kapanidis, unpublished work). This purification step removes unwanted contaminants and enriches the sample for the molecules of interest.

The presence of the gel matrix also slows down diffusion of molecules through the confocal volume. Slowly diffusing molecules are excited for a longer time inside the confocal volume, allowing more photons to be collected per molecule; the increase in photon count improves the photon statistics, narrowing the width of \( E \) distribution since the contribution of statistical noise decreases [31,32]. Nevertheless, confinement of molecules in-gel does not change the characteristic mean values of \( E \) and \( S \), hence facilitating direct comparison with ALEX measurement in solution (Y. Santoso and A.N. Kapanidis, unpublished work).

ALEX on surface-immobilized molecules

Combination of ALEX with TIRF microscopy is a powerful way to observe dynamics of surface-immobilized molecules in real time. This method exploits the phenomenon of total internal reflection, which occurs when light passes into a lower refractive index medium at an angle larger than the critical angle, creating an evanescent wave that decays exponentially away from the interface [33]. TIRF selectively illuminates only a thin slice (\( \sim 100 \) nm) over the glass/solution interface, hence reducing the fluorescence background from open solution above the surface. The illuminated area is imaged using an ultrasensitive camera that simultaneously detects many molecules in the field of view over the course of the experiment (Figure 2B, left-hand panel) [34]. When
coupled with surface immobilization, TIRF measurements track the time trajectory of the fluorescence emitted from many molecules in parallel, thus offering an attractive method for following dynamics during a non-equilibrium reaction (Figure 2B, middle panel). A TIRF set-up can also be coupled with a liquid-delivery system to exchange reagents and wash unbound species. This has been used for ‘walking’ reactions by adding and removing reagents in sequence [35,36].

Single-excitation TIRF–FRET has enabled elegant experiments that allowed remarkable insight into molecular mechanisms, especially in transactions of proteins and nucleic acids [12,14,16,22,26,35–39]. However, such experiments are limited by the dynamic range of FRET; moreover, it is sometimes difficult to separate FRET fluctuations due to fluorophore photophysics (e.g. one induced due to transient formation of non-absorbing non-emitting states of fluorophores) from FRET fluctuations because of conformational changes that alter the donor–acceptor distance [29]. To overcome such limitations, Margeat et al. [40] combined ALEX with TIRF microscopy. As with its confocal counterpart, TIRF–ALEX reports on the acceptor state and presence through the stoichiometry ratio \( S \), and permits virtual sorting through the two-dimensional \( E–S \) histogram (Figure 2B, right-hand panel). In other cases, the presence of acceptor blinking can be first checked using ALEX; if blinking is absent, then any FRET fluctuations can be attributed to structural changes [13,41].

### Application of ALEX for biomolecular interactions

There is a wide range of mechanistic questions that are addressable using ALEX spectroscopy (Figure 3). Some questions involve the detection of conformational changes: in essence, do two molecular parts move relative to each other during a certain process, and, if they do, what is the nature (e.g. rotation, translation) and direction of motion? There are three types of distance that are typically monitored: intra-protein distances, intra-nucleic acid distances (Figure 3C) and protein–nucleic acid distances (Figure 3A). Moreover, changes in molecular stoichiometry due to an assembly or dissociation process can be observed using changes in stoichiometry \( S \). For example, an assay for protein translocation on a nucleic acid involves placing a FRET...
**Figure 3** | ALEX applications on protein interactions with nucleic acids

(A) Left: acceptor-labelled DNA. Middle: protein binding forms a donor–acceptor complex (intermediate S). Right: protein translocation on DNA increases FRET efficiency (higher E). (B) Left: a short double-labelled single-stranded DNA fragment results in high E (short end-to-end distance due to short persistence length) and intermediate S. Middle: hybridization with complementary strand forms double-stranded DNA (dsDNA) and increases donor-acceptor distance (decreases E). Right: enzymatic cleavage of dsDNA releases two half-sites that diffuse separately in solution (intermediate S population disappears). (C) Left: donor–acceptor DNA. Middle: interaction with protein decreases donor-acceptor distance. Right: a conformational change in the DNA decreases the separation between donor and acceptor (increase in E). (D) DNA-binding proteins are detected through molecular coincidence of two half-sites. Left: without protein, the two half-sites interact only transiently (no intermediate S). Right: added proteins bind stably to transiently assembled full sites, giving rise to donor-acceptor species (intermediate S). A, acceptor; D, donor.

acceptor at the downstream end of the double-stranded nucleic acid and a FRET donor on the protein. When the protein interacts with the nucleic acid, the interaction manifests as a change in stoichiometry (molecules converting from the acceptor-only into the donor-acceptor population), whereas subsequent translocation is observed by an increase in FRET efficiency (Figure 3A; see [42] for a published example). Using similar observables, one can observe nucleic acid hybridization and subsequent site-specific nucleic acid cleavage (Figure 3B); protein-induced DNA bending and compaction (Figure 3C; see [43] for a published example); and protein-dependent DNA assembly (Figure 3D; K. Lymperopoulos, M. Heilemann, L. Hwang, R. Crawford and A.N. Kapanidis, unpublished work).
The addition of a third excitation laser, a third fluorophore and a third detection-wavelength range can expand the two-colour ALEX into a method known as three-colour ALEX (3c-ALEX) [44,45]. The 3c-ALEX method monitors FRET for every fluorophore combination (for blue–green, blue–red and green–red FRET) as well as relative stoichiometry of each fluorophore. This additional capability allows interactions among three different components to be monitored simultaneously in one experiment, a property that can be used to probe simultaneous distance measurement [44] and multi-component assembly pathways [46].

Conclusions and outlook
ALEX spectroscopy adds new and important capabilities to the single-molecule toolbox. ALEX-based sorting analyses complex samples without strict requirements for high purity, optimal distance range or high concentration, facilitating reagent preparation. ALEX is compatible with in-solution and in-gel confocal measurements as well as with on-surface TIRF experiments. The stoichiometry ratio $S$ provides a handle for detecting molecular coincidence independently of FRET. Additionally, $S$ separates donor-only molecules from donor–acceptor molecules with large donor–acceptor separation, thus extending the working range of single-molecule FRET.

TIRF–ALEX combines the attractive features of TIRF with the ability to determine stoichiometry, thus distinguishing fluorescence signals due to fluorophore photophysics from signals due to conformational changes, and detecting species with long donor–acceptor distances. The timescales accessible to TIRF measurements (~10 ms) are currently restricted by the limited number of detected photons and the photostability of the fluorophores (seconds to minutes). Recent advances in camera technology, coupled with use of buffer additives [47] and more photostable fluorophores (e.g. colloidal quantum dots), will expand the accessible timescale, from hundreds of microseconds (limited by the photon count and the camera sensitivity), to tens of minutes (limited by photobleaching).

Extensions of ALEX in the nanosecond timescale have given rise to the methods of nanosecond-ALEX [48] and pulsed interleaved excitation [49]. Both methods use time-resolved fluorescence lifetime measurements to probe fast conformational dynamics and recover distances that fluctuate on timescales similar to or longer than fluorescence lifetime; these methods also enable the use of fluorescence-fluctuation analysis. The end result of nanosecond-alternation is a wealth of information on distance distributions and orientational dynamics, which opens new avenues for studying biomolecular structure, dynamics and interactions.

Finally, excitation-confinement devices, such as zero-mode waveguides [50], nanopipettes [51] and nanofluidics [52], can be used to extend the working concentration of solution-based ALEX from the 10–200 pM range to the 1–1000 nM range. The ability to work with higher concentrations of labelled molecules will be helpful for studying proteins with moderate affinity for their interacting partners, and will increase the appeal and usefulness of the ALEX-based family of single-molecule fluorescence methods.

We thank Mike Heilmann and Soeren Doose for their invaluable help in establishing ALEX spectroscopy at Oxford Physics and the rest of the members of the Gene Machines group for helpful discussions and experimental support. We also thank the groups of Richard Ebright [HHMI (Howard Hughes Medical Institute)/Rutgers University, New Brunswick, NJ, U.S.A.], Shimon Weiss [UCLA (University of California Los Angeles), Los Angeles, CA, U.S.A.] and Ted Laurence (Lawrence Livermore National Laboratory, Livermore, CA, U.S.A.) for a fruitful collaboration on transcription-related projects, and Joanne Tang for pre-submission editorial assistance. Funding for our research on the development and application of ALEX methods was provided by the U.K. Interdisciplinary Research Collaboration in Bionanotechnology, the European Union (MIRG-CT-2005-031079), the EPSRC (Engineering and Physical Sciences Research Council) (EP/D058775) and the National Institutes of Health (NIH R01 GM069709). Y.S. was funded by Nanyang Technological University, Singapore.

References


Received 7 April 2008 doi:10.1042/BST0360738