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Periodic acceptor excitation spectroscopy of single molecules

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Abstract Alternating-laser excitation (ALEX) spectroscopy has recently been added to the single-molecule spectroscopy toolkit. ALEX monitors interaction and stoichiometry of biomolecules, reports on biomolecular structure by measuring accurate Förster resonance energy transfer (FRET) efficiencies, and allows sorting of subpopulations on the basis of stoichiometry and FRET. Here, we demonstrate that a simple combination of one continuous-wave donor-excitation laser and one directly modulated acceptor-excitation laser (Periodic Acceptor eXcitation) is sufficient to recapitulate the capabilities of ALEX while minimizing the cost and complexity associated with use of modulation techniques.

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Introduction

Alternating-laser excitation (ALEX) spectroscopy combined with single-molecule Förster resonance energy transfer (smFRET) is a powerful tool for measuring distances and stoichiometry in individual molecules and for fluorescence-aided molecular sorting (Kapanidis et al. 2004, 2005a). ALEX was first demonstrated by alternating a green and a red laser at the microsecond time scale (Kapanidis et al. 2004). Fluorescence emission from spectrally distinct fluorophores was recorded at two distinct wavelength ranges and further distinguished by the excitation period (donor or acceptor) resulting in four separate photon-streams, which were used for calculations of FRET efficiency E and stoichiometry S for each detected fluorescence burst (due to the transit of a single molecule). A similar approach uses pulsed lasers with an interleaved pulse sequence, giving access to the fluorescence lifetime of each fluorophore (Kukolka et al. 2006; Laurence et al. 2005; Muller et al. 2005; Ruttinger et al. 2006). ALEX has been implemented at different time scales (nanosecond: ns-ALEX, microsecond: μs-ALEX, and millisecond: ms-ALEX), in wide-field and point-detection schemes, and on diffusing and immobilized molecules (Kapanidis et al. 2005a), and has been combined with fluorescence correlation spectroscopy (Doose et al. 2005; Muller et al. 2005). Recently, ALEX has been extended to three excitation lasers and three fluoro-

phores (3-color ALEX), a format that identifies populations containing up to three fluorophores, and obtains structural information by measuring up to three distances within single molecules (Lee et al. 2007).

The advantages of ALEX over smFRET (Deniz et al. 1999; Ha 2001) or coincidence methods (Li et al. 2003; Schwille et al. 1997) have been demonstrated by measuring accurate-FRET efficiencies (Lee et al. 2005), and by studying protein–DNA interactions (Kapanidis et al. 2004), gene transcription mechanisms (Kapanidis et al. 2005b), and biopolymer flexibility (Laurence et al. 2005). ALEX is also useful for FRET-based studies of biomolecular dynamics, since it distinguishes between true conformational changes and fluorophore photophysics [such as photoinduced on/off switching of carbocyanine dyes (Bates et al. 2005; Heilemann et al. 2005), typical FRET acceptors].

Currently, implementing μs-ALEX requires (i) adding electro-optical modulators and the associated polarization optics to each excitation laser used (Kapanidis et al. 2004), or (ii) coupling several laser wavelengths to an acousto-optical modulator (Nir et al. 2006), or (iii) using directly modulatable lasers, equipment that can be costly and represent an additional level of complexity. To simplify the use of μs-ALEX and thus increase its appeal to a wider scientific community, we describe here a simplified version of μs-ALEX, in which we dispense of modulating the donor-excitation laser, dubbed PAX for Periodic Acceptor eXcitation. Using doubly labeled DNA oligonucleotides as standards, we demonstrate that PAX is equivalent to conventional μs-ALEX in terms of extraction of the stoichiometry S and FRET efficiency E parameters.

Materials and methods

Sample preparation

Fluorescently labeled double-stranded DNA fragments with 35 bases were used as in Kapanidis et al. (2004). For single-molecule measurements, DNAs were diluted in 10 mM HEPES-HCl, pH 7.0, 500 mM NaCl, 100 μg/ml BSA, 1 mM mercaptoethylamine, and 5% glycerol; the final concentration was ~100 pM.

Optical setup

All experiments were performed on a home-built confocal microscope as illustrated in Fig. 1. Two laser beams [from a 532 nm green Nd:YAG laser (Samba, Cobolt AB), and a 638 nm red diode laser (Cube, Coherent)] were coupled through a single-mode optical fiber (cut-off wavelength 488 nm, Thorlabs) into the optical path of an inverted microscope (IX71, Olympus). The red laser was modulated at 10 kHz using TTL signals. In PAX, the green laser was not modulated; in ALEX, the green laser was modulated using an electro-optical modulator (EOM) and a polarizer (P) (Kapanidis et al. 2004). Laser power was 300 and 80 μW for the green and red laser, respectively, measured without modulation at the objective nosepiece. The beams were combined by a dichroic mirror (DM1) and focused through an oil-immersion objective (OBJ) (UPlanSApo 60×, 1.35NA, Olympus) 30 μm in solution. Fluorescence from diffusing molecules was collected through the objective, separated from excitation by a dichroic mirror (DM2), spatially filtered [using a 50 μm pinhole (PH)], spectrally filtered [using a 585DF70 and a 650LP filter for the green and red channel, respectively, (F1) and (F2)] and detected using avalanche photodiodes [SPCM-AQR 14; Perkin Elmer, (APD1) and (APD2)]. Fluorescence bursts were identified and fluorescence contributions to the different channels computed. Background contributions were estimated from time traces of the four photon streams and subtracted from individual bursts for calculation of E and S values. Leakage and direct excitation were estimated from D-only and A-only samples, respectively. Only bursts with $F^{A_{\mathrm{em}}}_{A_{\mathrm{exc}}} > 30$ (for ALEX) and $\tilde{F}^{A_{\mathrm{em}}}_{A_{\mathrm{exc}}} > 30$ (for PAX) were included in analysis. E histograms were calculated from all bursts with $S >$ 0.3 to reject A-only bursts. S histograms were calculated from all detected bursts. The correction-detection factor γ was estimated to $\gamma \approx 1$ from a linear plot of $1/S$ as a function of E_{PR} (Eq. 6).

Theory

Use of conventional ALEX results in four distinct photon-emission streams, corresponding to four distinct photon counts for every fluorescence burst (the observable from a single molecule diffusing through the confocal observation volume). Including crosstalk terms, the four photon counts *for a single burst* are $F^{D_{em}}_{D_{exc}}$, $F^{A_{\mathrm{em}}}_{D_{\mathrm{exc}}}$, $F^{A_{\mathrm{em}}}_{A_{\mathrm{exc}}}$, and $F^{D_{\mathrm{em}}}_{A_{\mathrm{exc}}}$ [see Lee et al. (2005)]. For a typical FRET pair comprising a donor fluorophore (D) and a red-shifted acceptor fluorophore (A), the FRET signal is included in the $F^{A_{\mathrm{em}}}_{D_{\mathrm{exc}}}$ photon count, which also includes crosstalk terms such as the D-emission into the A-detection channel (D-leakage, Lk), and the A-emission due to A-direct-excitation at the D-excitation wavelength (A–direct-excitation, Dir) (Lee et al. 2005). The crosstalk photons need to be calculated and sub-

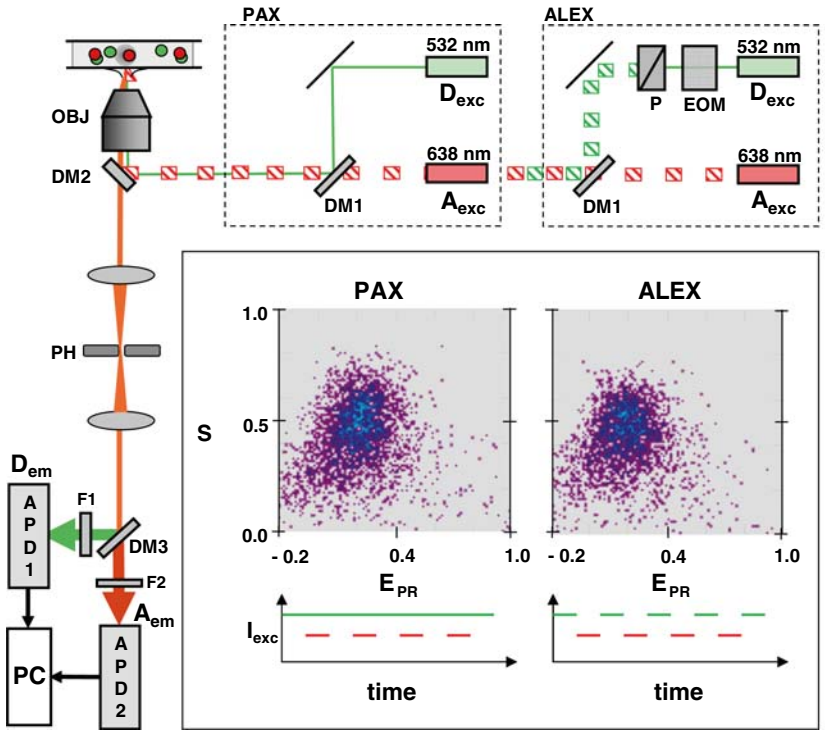

Fig. 1 Comparison of PAX and ALEX instrumentation and *E*–*S* histogram. The confocal setup with either PAX or ALEX excitation path is illustrated as described in "Materials and methods". *Box* E_{PR}-*S* histograms recorded using PAX (*left*) and ALEX (*right*) for a 20 bp *D*–*A* separation DNA

tracted for measuring accurate FRET from single molecules (Lee et al. 2005). The Lk crosstalk is defined as

$$\mathrm{Lk} = l\,F_{D_{\mathrm{exc}}}^{D_{\mathrm{em}}} \tag{1}$$

where l is the D-leakage coefficient, determined using the ratio $F_{D_{\mathrm{exc}}}^{A_{\mathrm{em}}} / F_{D_{\mathrm{exc}}}^{D_{\mathrm{em}}}$ as measured for D-only species. The Dir crosstalk is defined as $\mathrm{Dir} = d\,F_{A_{\mathrm{exc}}}^{A_{\mathrm{em}}}$, where d is the A-direct-excitation coefficient, and determined using the ratio $F_{D_{\mathrm{exc}}}^{A_{\mathrm{em}}} / F_{A_{\mathrm{exc}}}^{A_{\mathrm{em}}}$ as measured for A-only species. The FRET efficiency E for a single burst is defined as:

$$E = \frac{F^{\mathrm{FRET}}}{\gamma F_{D_{\mathrm{exc}}}^{D_{\mathrm{em}}} + F^{\mathrm{FRET}}}, \tag{2}$$

where $F^{\mathrm{FRET}} = F_{D_{\mathrm{exc}}}^{A_{\mathrm{em}}} - \mathrm{Lk} - \mathrm{Dir}$ and γ is a detection-correction factor that depends on the fluorophore quantum yields and the emission-channel detection efficiencies (Lee et al. 2005). The stoichiometry ratio S_γ for a single burst is defined as:

$$S_\gamma = \frac{\gamma F_{D_{\mathrm{exc}}}^{D_{\mathrm{em}}} + F^{\mathrm{FRET}}}{\gamma F_{D_{\mathrm{exc}}}^{D_{\mathrm{em}}} + F^{\mathrm{FRET}} + F_{A_{\mathrm{exc}}}^{A_{\mathrm{em}}}}. \tag{3}$$

Both E and S_γ are not directly observable in an experiment with unknown γ. We thus define the ratios E_{PR} and S as γ-independent experimental observables:

$$E_{\mathrm{PR}} = \frac{F^{\mathrm{FRET}}}{F_{D_{\mathrm{exc}}}^{D_{\mathrm{em}}} + F^{\mathrm{FRET}}}, \tag{4}$$

$$S = \frac{F_{D_{\mathrm{exc}}}^{D_{\mathrm{em}}} + F^{\mathrm{FRET}}}{F_{D_{\mathrm{exc}}}^{D_{\mathrm{em}}} + F^{\mathrm{FRET}} + F_{A_{\mathrm{exc}}}^{A_{\mathrm{em}}}}, \tag{5}$$

where S is a function dependent of E_{PR}; this relation is used to measure γ from experimental data (Lee et al. 2005):

$$1/S = 1 + \gamma\beta + \beta(1-\gamma)E_{\mathrm{PR}} = \Omega + \Sigma E_{\mathrm{PR}}, \tag{6}$$

with $\beta = \Omega + \Sigma - 1$ and $\gamma = (\Omega - 1)/(\Omega + \Sigma - 1)$ (Ω and Σ are the intercept and slope, respectively, of the linear relation in Eq. 6).

In PAX, the photon count $F_{A_{\mathrm{exc}}}^{A_{\mathrm{em}}}$ is not experimentally observable (due to continuous D-excitation). However, $F_{A_{\mathrm{exc}}}^{A_{\mathrm{em}}}$ can be calculated from the observed $F_{D_{\mathrm{exc}}+A_{\mathrm{exc}}}^{A_{\mathrm{em}}}$ (the photon count for A-emission under simultaneous D- and A-based excitation) and $F_{D_{\mathrm{exc}}}^{A_{\mathrm{em}}}$:

$$\tilde{F}_{A_{\mathrm{exc}}}^{A_{\mathrm{em}}} = F_{D_{\mathrm{exc}}+A_{\mathrm{exc}}}^{A_{\mathrm{em}}} - F_{D_{\mathrm{exc}}}^{A_{\mathrm{em}}} \tag{7}$$

where $\tilde{F}$ denotes a photon count measured indirectly; hereafter, the notation $\tilde{X}$ will refer to variables that depend on $\tilde{F}_{A_{\mathrm{exc}}}^{A_{\mathrm{em}}}$ (as in PAX) rather than on $F_{A_{\mathrm{exc}}}^{A_{\mathrm{em}}}$ (as in ALEX). Combining Eq. 4, 5 and 7 we find an expression for $E_{\mathrm{PR}}$and S based on PAX observables:

$$\tilde{E}_{\mathrm{PR}} = \frac{\tilde{F}^{\mathrm{FRET}}}{F_{D_{\mathrm{exc}}}^{D_{\mathrm{em}}} + \tilde{F}^{\mathrm{FRET}}}, \tag{8}$$

$$\tilde{S} = \frac{F_{D_{\mathrm{exc}}}^{D_{\mathrm{em}}} + \tilde{F}^{\mathrm{FRET}}}{F_{D_{\mathrm{exc}}}^{D_{\mathrm{em}}} + \tilde{F}^{\mathrm{FRET}} + F_{D_{\mathrm{exc}}+A_{\mathrm{exc}}}^{A_{\mathrm{em}}} - F_{D_{\mathrm{exc}}}^{A_{\mathrm{em}}}} = \frac{F_{D_{\mathrm{exc}}}^{D_{\mathrm{em}}} + \tilde{F}^{\mathrm{FRET}}}{F_{D_{\mathrm{exc}}}^{D_{\mathrm{em}}} + F_{D_{\mathrm{exc}}+A_{\mathrm{exc}}}^{A_{\mathrm{em}}} - \mathrm{Lk} - \tilde{\mathrm{D}}\mathrm{ir}}. \tag{9}$$

To accurately evaluate Eqs. 8, 9, we need to determine Lk and $\tilde{\mathrm{D}}$ir since $\tilde{F}^{\mathrm{FRET}} = F_{D_{\mathrm{exc}}}^{A_{\mathrm{em}}} - \mathrm{Lk} - \tilde{\mathrm{D}}\mathrm{ir}$. The leakage crosstalk is determined as described above, whereas to estimate the A-direct excitation, we use Eq. 7, replacing $F_{A_{\mathrm{exc}}}^{A_{\mathrm{em}}}$ with $\tilde{F}_{A_{\mathrm{exc}}}^{A_{\mathrm{em}}}$:

$$\tilde{\mathrm{D}}\mathrm{ir} = d\,\tilde{F}_{A_{\mathrm{exc}}}^{A_{\mathrm{em}}} = d\left(F_{D_{\mathrm{exc}}+A_{\mathrm{exc}}}^{A_{\mathrm{em}}} - F_{D_{\mathrm{exc}}}^{A_{\mathrm{em}}}\right), \tag{10}$$

where $d = F_{D_{\mathrm{exc}}}^{A_{\mathrm{em}}} \Big/ \left(F_{D_{\mathrm{exc}}+A_{\mathrm{exc}}}^{A_{\mathrm{em}}} - F_{D_{\mathrm{exc}}}^{A_{\mathrm{em}}}\right)$ is determined from A-only species.

Results

To compare PAX and ALEX, we collected data from three samples of 100 pM solutions of 35 bp dsDNA with D–A separations of 10, 20, and 30 bp [as in Kapanidis et al. (2004)]. We collected data in ALEX mode and computed E–S histograms as described (Lee et al. 2005). We repeated the measurement using PAX by modulating only the red laser (using identical excitation powers, measured without modulation). After identification of fluorescence bursts and background subtraction, we constructed E–S histograms (Fig. 1) for A-containing bursts (selected using thresholds of $F_{A_{\mathrm{exc}}}^{A_{\mathrm{em}}} > 30$ or $\tilde{F}_{A_{\mathrm{exc}}}^{A_{\mathrm{em}}} > 30$ for ALEX and PAX, respectively; this filtering removes D-only molecules). Leakage and direct excitation were estimated from D-only and A-only samples, respectively, to be l ~8% and d~10%. The detection-correction factor was $\gamma \sim 1$ (Eq. 6).

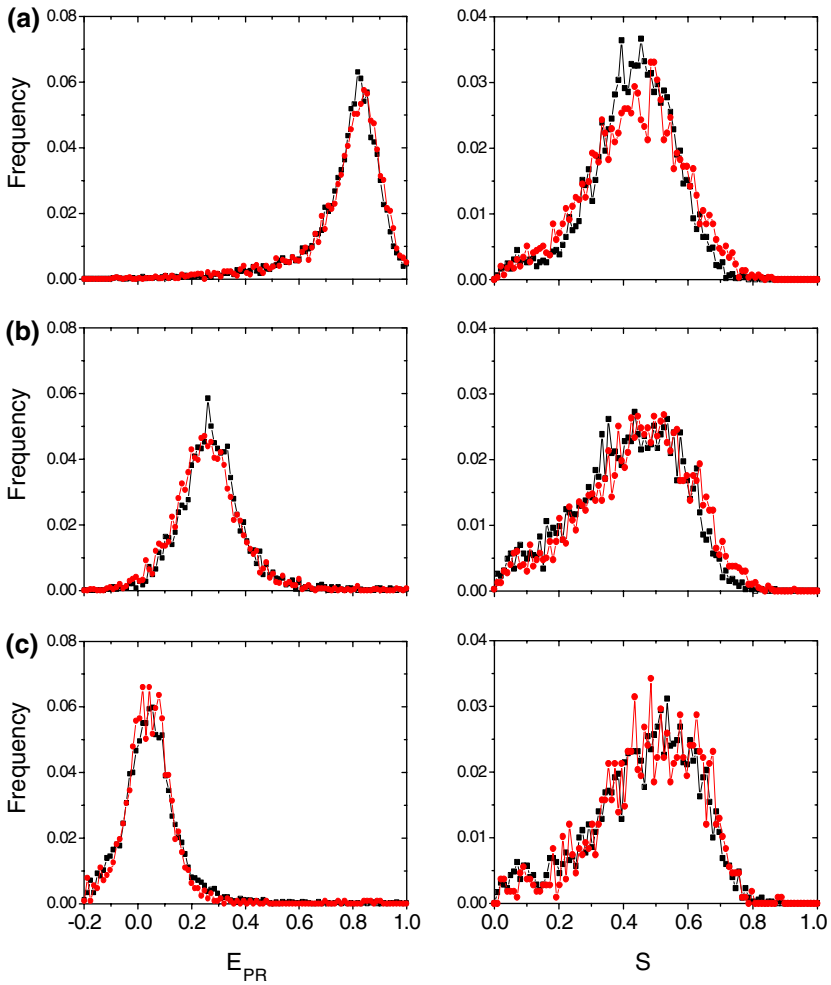

Fig. 2 Collapse of E_{PR}-S histograms for D–A labeled species on the E_{PR} (*left*) and S (*right*) axes. Each graph shows the E_{PR} or S distribution recorded using ALEX (*black*) and PAX (*red*) for DNA with D–A separation of 10 (**a**), 20 (**b**) and 30 (**c**) bp. E_{PR} histograms are calculated only for D–A molecules (using $S > 0.3$ to reject A-only bursts). S histograms were calculated from all bursts

Table 1 Means and standard deviations of E_{PR} and S distributions measured using ALEX and PAX

D–A separation (bp)	ALEX				PAX			
	E_{PR}		S		E_{PR}		S	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
10	0.773	0.144	0.428	0.130	0.776	0.151	0.433	0.147
20	0.275	0.123	0.414	0.160	0.261	0.127	0.441	0.164
30	0.052	0.117	0.462	0.163	0.046	0.107	0.477	0.157

Errors of the mean calculated from STD and the number of bursts for each measurement are smaller than 5×10^{-3} in all experiments

Our results establish that, for all DNAs, the 2D E_{PR}–S histograms obtained using ALEX are essentially identical to the ones using PAX (Fig. 1). The excellent agreement is also clear after inspecting the collapse of E_{PR} and S histograms for all DNAs (Fig. 2; Table 1 for means and standard deviations), where it is shown that the distribution of E and S is identical within error [noise contributions to the E histogram have been discussed in detail by Antonik et al. (2006) and Nir et al. (2006)]. This demonstrates that a combination of a modulated red laser with a continuous green laser (PAX) is sufficient to discriminate between D-only, D–A, and A-only molecules, and to extract FRET efficiencies identical to ALEX. The results also agree well with previously published experiments (Kapanidis et al. 2004; Lee et al. 2005) [note: E_{PR} differs from E_c in Kapanidis et al. (2004) in two ways: (i) we present mean values for E_{PR} whereas E_c represents the center of a fitted Gaussian distribution which slightly deviates at high E; (ii) correction for A-direct excitation was not included in E_c]. We conclude that PAX and ALEX are equivalent for determination of stoichiometry and FRET in a two-color sample.

Conclusion

In conclusion, we have shown that ALEX can be implemented using a simplified format with a directly modulated red laser and a continuous-wave green laser. The mean values and standard deviations of the S and E distributions recovered using PAX are essentially identical to those measured by ALEX with *two* alternating lasers. Hence, the sorting and resolving capability of PAX is sufficient for separating D–A labeled species with distinct FRET or distinct stoichiometry (e.g., D-only or A-only species). Extension to more excitation lasers, as in 3-color ALEX (Lee et al. 2007), is straightforward and would result in the saving of one laser alternation. The technique can be applied at any time scale (nanosecond, microsecond, millisecond, etc) and geometry (diffusing molecules, immobilized molecules, point detection or wide-field detection), as described in ref. (Kapanidis et al. 2005a). Overall, PAX enhances the ALEX methodology by substantially reducing the complexity and cost of instrumentation associated with ALEX.

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