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Frequency-domain phase fluorometry in the presence of dark states: A numerical study

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ABSTRACT

Fluorescence anomalous phase advance (FAPA) is a newly discovered spectroscopy phenomenon: instead of lagging behind the modulated light, fluorescence signal can exhibit FAPA as if it precedes the excitation source in time. While FAPA offers a promising technique for probing dark state lifetime, the underlying mechanism is not fully elucidated. Herein we investigate frequency-domain phase fluorometry as a result of intricate interplay between a short-lived fluorescent state and a long-lived dark state. In particular, the quantitative dependence on modulation frequency, excitation intensity, nonradiative decay, intersystem crossing and dark-state lifetime are explored respectively. A comprehensive view of phase fluorometry emerges consequently.

1. Introduction

Fluorescence spectroscopy is one of the most widely used spectroscopic tools in various areas of science and technology [1,2]. Unlike fluorescence intensity, the fluorescence lifetime of the fluorophore reflects its intrinsic photophysical properties that are independent of fluorophore concentration, photobleaching and excitation condition. More importantly, fluorescence lifetime is extremely sensitive to the surrounding environment including molecular binding, excited-state quenching, local viscosity, solvent polarity, ion strength, refractive index and energy transfer [3–6]. As such, complementary to the popular fluorescence-intensity-based measurements, fluorescence lifetime spectroscopy has played a unique and important role in providing valuable information about the local physicochemical environment of fluorophores [1–8]. For example, fluorescence lifetime imaging microscopy (FLIM) has flourished as a powerful tool in optical imaging [4,5], and fluorescence lifetime of individual fluorophore/protein complex has been monitored in real time to reveal conformational fluctuation dynamics [8].

Commonly, there exist two distinct approaches to measure the fluorescence lifetime: the time domain and the frequency domain methods. The time domain measurement makes use of a train of short pulses of light (normally femtoseconds or picoseconds) to excite the sample repeatedly and then records the time-dependent fluorescence decay profile that follows the pulsed excitation, from which the fluorescence lifetime can be deduced. To the contrary, in frequency domain fluorescence lifetime measurement, commonly referred to as phase fluorometry, the sample is excited by a sinusoidally modulated continuous wave light source and the resulting modulated fluorescence is subsequently monitored and analyzed. The present study mainly deals with the frequency domain phenomenon.

The conventional wisdom about phase fluorometry is that, owing to the finite and non-vanishing fluorescent lifetime τ of the fluorophore, the modulated fluorescence emission exhibits a reduced modulation depth and is always delayed in time relative to the original excitation. Quantitatively, the reduced modulation depth

$$m = 1/\sqrt{(1+\omega^2\tau^2)} \tag{1}$$

and the delayed phase angle

$$\phi = \arctan(\omega\tau) \tag{2}$$

where ω is the circular modulation frequency in radians per second. Both m and ϕ contain the lifetime information about the excited state of the sample [1]. Obviously, a high modulation frequency will lead to a more negative phase delay that is closer to $-90°$, whereas a slow modulation will result in a small but still negative phase delay according to Eq. (2). It is crucial to note that under no circumstances could the phase shift become positive in the conventional phase fluorometry.

Most recently, our group reported an experimental observation of frequency-domain fluorescence anomalous phase advance (FAPA) [9], which is opposite to the expected phase delay in conventional fluorescence lifetime phase fluorometry. When fluorescent molecules flavin adenine dinucleotide (FAD), Rhodamine 6G (Rh6G) and fluorescein isothiocyanate (FITC) are excited by a sinusoidally modulated laser around MHz, instead of detecting a negative phase shift between the emitted fluorescence and the

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excitation laser, the measured phase shift is actually positive which suggests that the fluorescence is emitted "ahead" of the laser source. Further experiment showed that FAPA is pronounced only within a narrow range of modulation frequencies that are outside quasi-static and quasi-equilibrium conditions [9].

FAPA is believed to be induced by the dynamical hysteresis of long-lived dark state of fluorescent molecules, as the FAPA signal is found to be strongly dependent on the concentrations of a series of known triplet state quenchers and promoters [9]. Dark state, which universally exists for almost all fluorescent molecules, refers to the long-lived non-fluorescent state of fluorophores [10]. It is interesting to note that dark states of fluorescent molecules have recently attracted considerable attentions in a number of microscopy applications. First, the dark state lifetime is typically much longer than the fluorescence lifetime, rendering it to be highly sensitive to a certain class of weak environmental properties [11]. Second, by exploiting the stochastic switching or blinking of fluorophores or fluorescent proteins, fluorescence imaging can circumvent the diffraction limit in terms of spatial resolution [12–15]. Third, triplet relaxation microscopy leads to a major increase in total fluorescence signal and photo-stability of fluorescent molecules under imaging [16,17]. Considering FAPA is tightly related to the dark states of fluorescent molecules, FAPA provides a promising technique to study the dark states dynamics and also imaging dark states lifetime distributions in complex biological samples [9].

Although our earlier experimental report provides evidence of the photo-physical effects of dark states underlying FAPA, the coherent picture and the quantitative characterization are not available. In particular, under the interplay between the fluorescent state and the dark state, how the phase fluorometry behaves in the entire modulation frequency range, how the phase shift turns over from the conventional negative value to the positive, how the conventional phase delay and the new FAPA are different from each other, and how phase fluorometry can be used to probe the changes of the relevant photophysical rates are still unknown. In this study, we seek to comprehensively investigate the role of dark state in phase fluorometry, which to our best knowledge has not been studied in the literature before. Various aspects of phase fluorometry will be separately examined including the dependence on light modulation frequency, excitation light intensity, nonradiative decay rate, intersystem crossing rate and dark-state lifetime, respectively.

2. Method

We use a simplified three-level kinetic model: the ground state (G), the singlet excited state (E) and the dark state (D) as shown in Fig. 1. The dynamic transitions of molecular population on these three states under excitation of a sinusoidally modulated laser could be described by the following system of ordinary differential equations:

$$N_G(t) + N_E(t) + N_D(t) = 1; \tag{3}$$

$$\frac{dN_G(t)}{d(t)} = -k_{GE}(t)N_G(t) + (k_{EG} + k'_{EG})N_E(t) + k_{DG}N_D(t); \tag{4}$$

$$\frac{dN_E(t)}{dt} = k_{GE}(t)N_G(t) - (k_{EG} + k'_{EG})N_E(t) - k_{ED}N_E(t); \tag{5}$$

$$\frac{dN_D(t)}{dt} = k_{ED}N_E(t) - k_{DG}N_D(t); \tag{6}$$

$$k_{GE}(t) = \frac{I_0(\sin(2\pi\nu t) + 1)\lambda\sigma}{hc} \tag{7}$$

where $N_G(t)$, $N_E(t)$, $N_D(t)$ represent the time-dependent probability of fluorescent molecules in the ground state, the singlet excited state and the dark state, respectively, $k_{GE}(t)$ denotes the time-dependent rate constant of the optical excitation process due to the sinusoidally modulated excitation laser intensity at focal point $I(t) = I_0(\sin(2\pi\nu t) + 1)$ where ν is the modulation frequency of laser in Hz, k_{EG}, k'_{EG}, k_{ED}, and k_{DG} are rate constants of fluorescence emission, non-radiative decay from fluorescent state, the intersystem crossing and dark state relaxation, respectively. In the present work, the wavelength of the excitation laser, λ, is set to 445 nm, and molecular absorption cross section, σ, is set to 1×10^{-17}cm^2, which are the parameters for FAD.

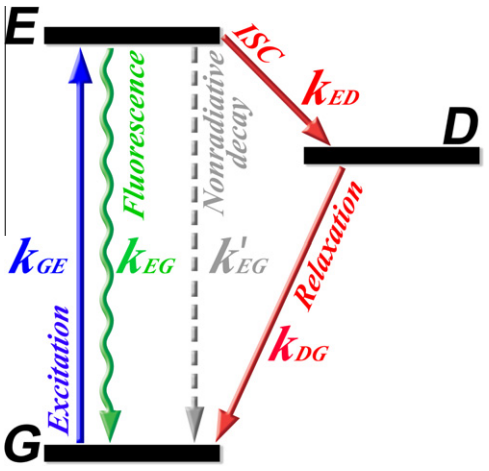

Fig. 1. A simplified three-level energy diagram. G represents the ground state; E represents the excited state; D represents the dark state. k_{GE}, k_{EG}, k'_{EG}, k_{ED}, k_{DG} are the excitation rate constant, the fluorescent rate constant, the nonradiative decay rate constant, the intersystem crossing rate constant and the dark-state relaxation rate constant, respectively. Stimulated emission from E is not considered as biological fluorophores often have large Stokes shifts.

Matlab is used to simulate the progression of the dynamic transitions governed by the above systems equation, as there is no closed-form analytical solution to the above system of equations. The time step of the progression is chosen to be 0.1 ns, which is much shorter than any of the involved transition rates. Based on the simulated fluorescence trance, $N_E(t)$, the phase shift of the emitted fluorescence signal with respect to the modulated laser can be numerically calculated. We first multiply $N_E(t)$ with the reference signal ($I = I_0 \sin(2\pi\nu t)$), and then sum up their product to obtain the in-phase component X. Similarly, $N_E(t)$ is also multiplied with another reference signal $I = I_0 \sin(2\pi\nu t + \pi/2)$ that is 90° out of phase with the excitation laser, and their product is also summed up to produce the out-of-phase component Y. The final phase shift between the simulated fluorescence trace with respect to the excitation laser is computed as

$$\phi = \arctan(Y/X) \tag{8}$$

The above numerical recipe is identical to the procedure how a dual-phase lock-in amplifier extracts the phase shift in the phase fluorometry experiments.

3. Results

To have a global view of phase fluorometry, the phase shift is calculated as a function of a broad range of modulation frequencies (from 50 kHz to 325 MHz) of the excitation source with and without the involvement of a dark state. As shown in Fig. 2A, the phase shift curve from a three-level kinetic model in the presence of a dark state is not exactly the same as the one produced by the conventional two-level model in the absence of a dark state. The net difference between the above two phase curves, which is due to the pure contribution from a dark state, is depicted in Fig. 2B. In the high frequency region, these two curves are identical to each

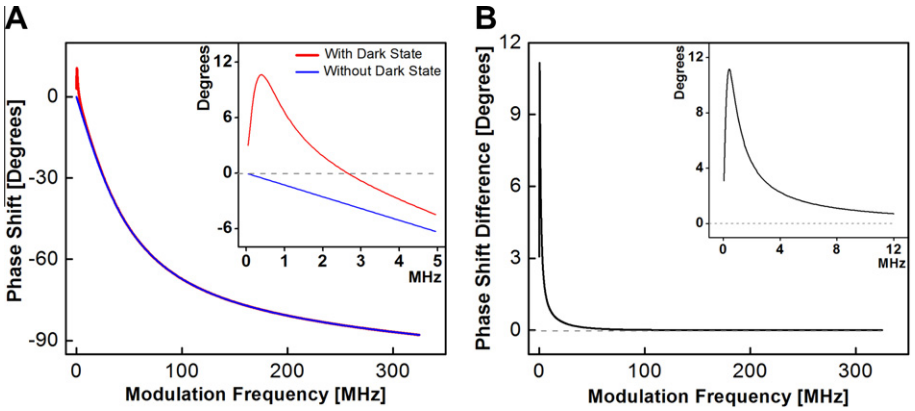

Fig. 2. (A) Modulation frequency response of fluorescence phase shift based on a two-level kinetic model (blue curve, without dark state, $I_0 = 500$ kW/cm^2, $k_{EG} = 271$ μs^{-1}, $k'_{EG} = 0$ μs^{-1}) and a three-level kinetic model (red curve, with dark state, $I_0 = 500$ kW/cm^2, $k_{EG} = 247$ μs^{-1}, $k'_{EG} = 0$ μs^{-1}, $k_{ED} = 24$ μs^{-1}, $k_{DG} = 2$ μs^{-1}). k_{EG} of the two-level model is set to equal to $k_{EG} + k_{ED}$ of the three-level model to make sure both excited singlet states have the same fluorescence lifetime. Inset is the zoom-in portion of the initial part of the curve at low frequencies. (B) Net difference of the phase shifts between kinetic models with and without dark states. Inset is the zoom-in portion of the beginning part of the curve. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

other: both of them are gradually approaching the expected −90° when the modulation frequency is high enough. Thus, the dark state has little effect on the fluorescence phase shift when the modulation frequency is high enough at which the conventional phase fluorometry picture is accurate. However, in the lower frequency region, although the conventional two-state model predicts that the phase shift would vanish gradually, the phase curve in the presence of dark states shows a pronounced positive peak above zero. This exactly reproduces the observed phenomenon of FAPA: the emitted fluorescence trace precedes instead of lagging behind the excitation laser in a narrow range of slow modulation frequency!

Dynamical hysteresis of dark states is suggested to underlie the above frequency response and resonance of FAPA [9]. To further understand the mechanism, the dark state population traces, together with the corresponding fluorescence intensity traces, are simulated under different laser modulation frequencies. Through these graphs in Fig. 3, the fluorescence traces experience an obviously non-monotonic change of phase shift as the modulation frequency varies. The phase goes from −67° at 100 MHz to −11° at 10 MHz, to 0.37° at 2.5 MHz and peaks around 10° at 0.5 MHz. When the frequency keeps slowing down, the fluorescence phase advance gradually attenuates to 4.6° at 0.1 MHz and becomes almost in phase with the laser at 10 kHz. We now examine the concurrent dynamics of dark states during cycles of modulations. When the laser is modulated at very high frequencies, the dark state population of fluorescent molecules is essentially constant in time because the laser modulation is too fast for the long-lived dark state to respond. At this quasi-static condition, the final phase is solely determined by fluorescence lifetime as normal. However, when the modulation frequency reduces to a point where the dark state could catch up, FAPA effect starts to appear. The rising half of

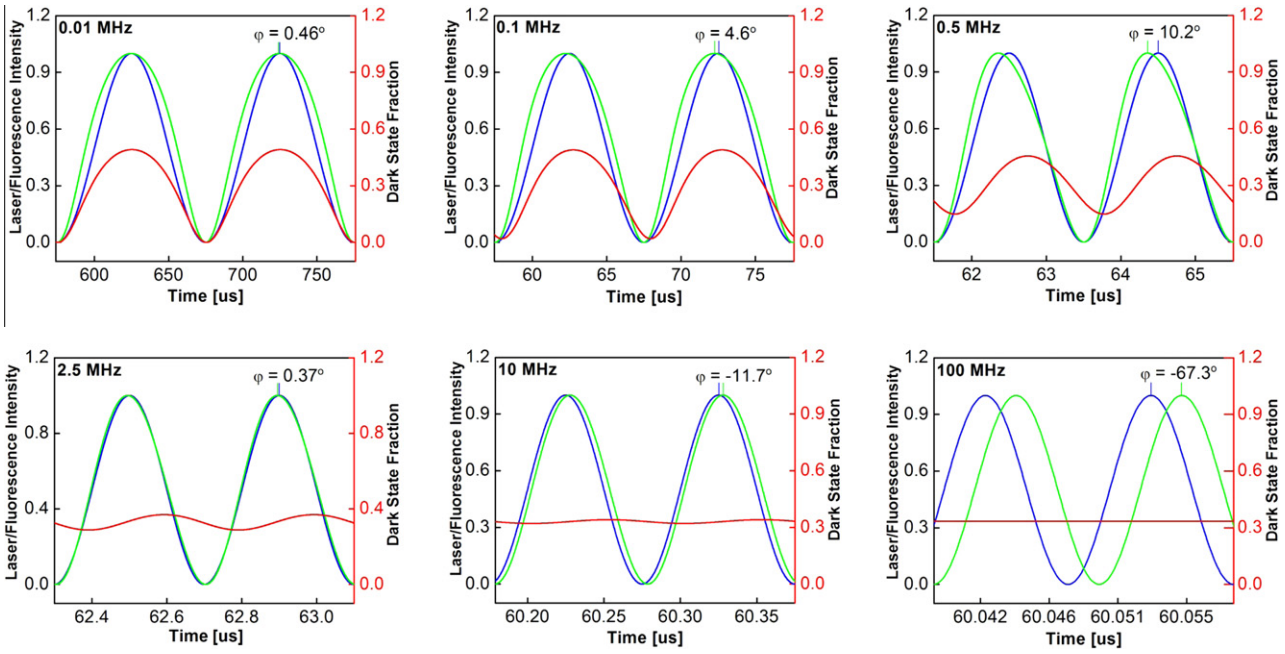

Fig. 3. Time-dependent fluorescence traces (green curves) and dark-state populations (red curves) generated from a three-level kinetic model excited by a laser (blue curves) modulated at frequencies of 0.01 MHz, 0.1 MHz, 0.5 MHz, 2.5 MHz, 10 MHz and 100 MHz ($I_0 = 500$ kW/cm^2, $k_{EG} = 247$ μs^{-1}, $k'_{EG} = 0$ μs^{-1}, $k_{ED} = 24$ μs^{-1}, $k_{DG} = 2$ μs^{-1}). The intensities of laser excitation and fluorescence emission have been self-normalized to unity, whereas the dark-state population reflects the absolute value. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the modulated laser cycle pumps the molecules into the dark states. Due to the long-lived characteristics of the dark state, the falling half of the modulated laser cycle will see fewer molecules in the ground state and result in less fluorescence. Thus, the dark-state hysteresis causes asymmetrical ground-state population which consequently skews the emitted fluorescence trace in time and creates an anomalous phase advance. We call this dark state resonance condition. Eventually, when the modulation frequency is much slower than the dark-state relaxation, the dark-state population will quickly adjust to the laser intensity variations at every moment. At this quasi-equilibrium condition, the fluorescence trace is in phase with the modulated laser trace. Therefore, the dark state will strongly modify phase fluorometry only in a dynamical resonant condition that is outside both quasi-static and quasi-equilibrium conditions, which might partially be the reason why FAPA was not reported until very recently.

FAPA effect originated from dark state hysteresis is essentially a nonlinear optical spectroscopy effect, deviated from conventional fluorescence lifetime phase fluorometry. In Fig. 4, under different excitation laser intensities, phase curves behave radically differently in the high and low modulation frequency ranges. In the high modulation frequency range (i.e., quasi-static condition for dark states), phase curves almost overlap with each other under different excitation laser intensities. This is predicted by Eq. (2), as the fluorescence lifetime is independent of the excitation intensities. In the intermediate modulation frequency range (i.e., dark state resonance region), however, the phase curves show strong dependence on the laser intensity. When the laser intensity is low enough (<10 kW/cm^2), the positive phase peak is barely visible. When the laser intensity increases, more molecules will be driven and trapped in the dark state, causing the fluorescence wave form to be skewed more and thus a stronger FAPA effect. The nonlinear nature of FAPA described here is closely related to the saturation of the fluorescence emission which has been employed for super-resolution fluorescence microscopy [18,19].

Although FAPA cannot be accounted for by fluorescent singlet states, excited-state lifetime of the singlet state can surprisingly have an effect on the magnitude of FAPA. We study the full frequency range of phase curves by tuning the nonradiative decay rate k'_{EG} from the excited state E as shown by Fig. 5, a faster

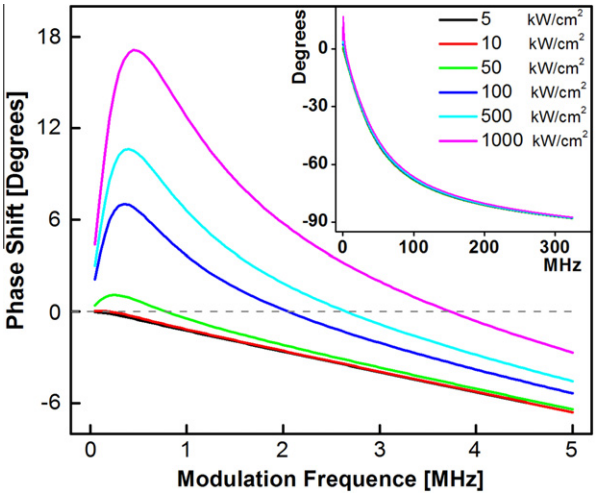

Fig. 4. Modulation frequency response of the fluorescence phase shift generated from a three-level kinetic model under different excitation laser intensities ($k_{EG} = 247\ \mu s^{-1}$, $k'_{EG} = 0\ \mu s^{-1}$, $k_{ED} = 24\ \mu s^{-1}$, $k_{DG} = 2\ \mu s^{-1}$). The modulation frequency has been extended over to 325 MHz in the inset. While the phase delay is independent of the excitation intensity in the high modulation frequency range, FAPA increases with laser power in the low modulation frequency range.

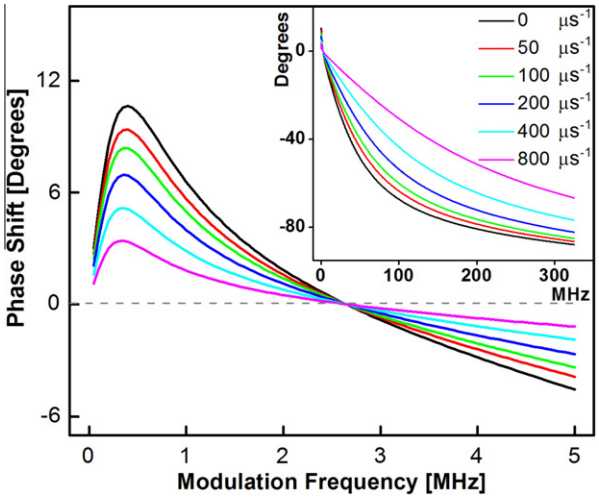

Fig. 5. Modulation frequency response of the fluorescence phase shift generated by a three-level kinetic model with different nonradiative decay rates ($I_0 = 500$ kW/cm^2, $k_{EG} = 247\ \mu s^{-1}$, $k_{ED} = 24\ \mu s^{-1}$, $k_{DG} = 2\ \mu s^{-1}$). Note that all the curves cross the same zero point. The modulation frequency has been extended to 325 MHz in the inset. Both the phase delay in the high frequency range and the phase advance in the low frequency range show clear dependence on the nonradiative decay rates.

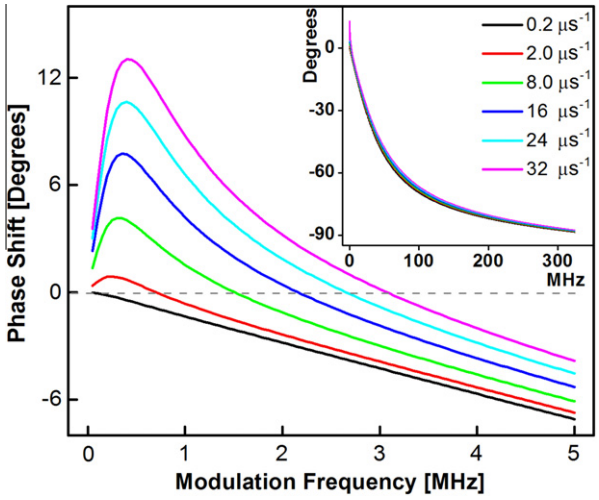

Fig. 6. Modulation frequency response of the fluorescence phase shift generated from a three-level kinetic model with different intersystem crossing rate constants ($I_0 = 500$ kW/cm^2, $k_{EG} = 247\ \mu s^{-1}$, $k'_{EG} = 0\ \mu s^{-1}$, $k_{DG} = 2\ \mu s^{-1}$). The modulation frequency has been extended to 325 MHz in the inset. FAPA in the low modulation frequency range increases along with the intersystem crossing rates, while the phase delays in the high frequency range are essentially non-variant.

nonradiative decay rate constant reduces the phase difference between the fluorescence and the laser for both the positive and negative values of phase shift across the entire frequency range. In the high modulation frequency range at which FAPA vanishes, a faster nonradiative decay reduces the fluorescence lifetime of excited state, which would lead to a smaller magnitude of phase delay, as predicted from Eq. (2). In the intermediate modulation frequency range at which FAPA is dominant, a faster nonradiative decay leads to an effectively smaller brunching ratio for molecules going into the dark state via intersystem crossing, which therefore attenuates the FAPA effect. Therefore, despite of the distinct underlying photophysical mechanism, the FAPA effect can also probe the alterations of fluorescence lifetime given all the other parameters stay the same, just like the conventional phase fluorometry.

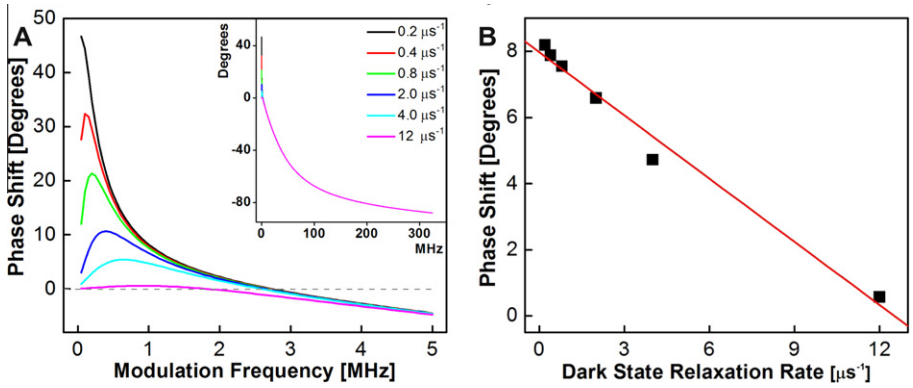

Fig. 7. (A) Modulation frequency response of the fluorescence phase shift generated from a three-level kinetic model with different dark state relaxation rate constants ($I_0 = 500$ kW/cm^2, $k_{EG} = 247$ μs^{-1}, $k'_{EG} = 0$ μs^{-1}, $k_{ED} = 24$ μs^{-1}). The modulation frequency has been extended to 325 MHz in the inset. While all phase delay curves overlap with each other in high frequency range (>5 MHz), FAPA peaks not only attenuate in amplitude but also shift to higher frequencies with the increasing dark state relaxation rates. (B) Scatchard plot between dark state relaxation rates and phase shifts under the same 1 MHz modulation frequency.

Intersystem crossing rate has a strong influence on the magnitude of FAPA. As shown in Fig. 6, when the intersystem crossing rate of fluorescent molecules increases, the FAPA peak becomes much more pronounced, as a larger intersystem crossing rate means a higher quantum yield of dark state formation and the subsequent trapping. We believe that the varying intersystem system crossing rate (i.e., the dark state quantum yield) is mainly the reason why FAD, Rh6G, and FITC exhibit different degrees of FAPA under the same excitation condition as observed experimentally [9]. Note that the phase delay curves in the high modulation frequency region show some slight difference when the intersystem crossing rate changes, which is due to the small variations of the resulting fluorescence lifetime. Hence, FAPA is significantly more sensitive than the conventional phase delay to the change of intersystem crossing rate.

Finally, we investigate how the dark state relaxation rate could affect phase fluorometry. Due to the presence of dark state quenchers and promoters, the relaxation rate of dark state can be modified significantly. First, it is worthy noting that, all the curves in the high modulation frequency region completely overlap with each other despite of the different dark state relaxation rates (the inset of Fig. 7A). This observation is consistent with our previous insight that the dark state has little effect on phase curves at high laser modulation frequencies. Second, as shown in Fig. 7A, the shorter dark state lifetime traps fewer molecules effectively and hence leads to lower amplitude of FAPA peak. When the modulation frequency is fixed at 1 MHz, as shown in Fig. 7B, the phase shift value bears an empirical linear relationship with respect to the dark state relaxation rate. This offers a Stern–Volmer-like equation relating the measurable FAPA effect to the concentration of dark state quenchers. Third, more interestingly, the shorter dark state lifetime also corresponds to the "horizontal" shift of the peak position to a higher modulation frequency, which has been observed experimentally when triplet state quencher is added to FAD solution [9]. Such a shift arises because the "horizontal" frequency position of the FAPA peak is an indicator to the time scale of the dark state relaxation, as implied by our earlier discussion on dynamical resonant condition. Therefore, when the concentration of the dark state quencher increases, the dark state will exhibit a shorter lifetime, which would lead to a FAPA curve that peaks at a higher modulation frequency.

4. Conclusion

Dark states appear to enrich the behaviors of the classical frequency-domain phase fluorometry. In the present study, we have systematically investigated various aspects of phase fluorometry in the presence of dark states, in light of the recently observed fluorescence anomalous phase advance (FAPA). It is shown that FAPA exists in a narrow range of modulation frequency that is resonant with the intrinsic time scale of dark-state lifetime, outside both the quasi-static and the quasi-equilibrium conditions of dark state dynamics. Unlike the conventional phase fluorometry picture, FAPA effect is strongly dependent onto the excitation laser intensity. While nonradiative decay from fluorescent states can affect both FAPA and conventional fluorescence lifetime phase delay, the intersystem crossing rate and dark-state relaxation rate can only be probed by FAPA exclusively. Thus, FAPA effect offers a specific, fast, and convenient reporter for sensing and imaging dark-state population and dynamics, which could be complementary to the widely used fluorescence lifetime spectroscopy and microscopy.

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