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LETTER

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Observation of Frequency-Domain Fluorescence Anomalous Phase Advance Due to Dark-State Hysteresis

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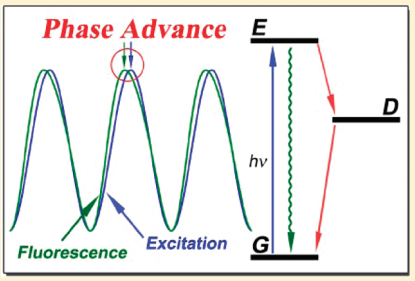
Supporting Information

ABSTRACT: Frequency-domain fluorescence spectroscopy, commonly referred to as phase fluorometry, is a classic approach to study the lifetime dynamics of fluorescent systems. Here we report an interesting phenomenon: unlike conventional fluorescence lifetime phase fluorometry in which the fluorescence trace always lags behind the modulated excitation source, the detected signal from certain fluorophores can actually exhibit fluorescence anomalous phase advance (FAPA) as if the fluorescence is emitted "ahead" of the source. FAPA is pronounced only within a range of modulation frequencies that are outside quasi-static and quasi-equilibrium conditions. We attribute FAPA to photoinduced dark state hysteresis, supported by both simulations of photodynamic transitions and experiments with dark-state promoters and quenchers. Being a fast and straightforward frequency-domain reporter, FAPA offers a unique and specific contrast mechanism for dark state dynamics sensing and imaging.



SECTION: Kinetics, Spectroscopy

Fluorescence spectroscopy is one of the most widely used spectroscopic tools. Time-domain and frequency-domain measurements are two complementary approaches to study the lifetime dynamics of fluorescent systems.[1−3] Whereas the time-domain measurement records the time-resolved fluorescence decay under a pulsed light source, the frequency-domain technique, commonly referred to as phase fluorometry, uses a sinusoidally modulated light source to excite the fluorophores. The resulting fluorescence signal trace, which is also modulated sinusoidally in time, will display a relative phase lag by $\varphi = \arctan(\omega \cdot \tau)$ and a concurrent reduction in modulation amplitude by $m = 1/(1 + (\omega \cdot \tau)^2)^{1/2}$ in which ω is the modulation frequency and τ is the fluorescence lifetime of the fluorophore. Both the fluorescence phase lag (i.e., negative phase compared with the laser modulation) and reduced modulation amplitude can be utilized to extract information about the fluorophore's excited-state lifetime in phase fluorometry. Because a variety of physical-chemical parameters, such as local viscosity, ion strength, solvent polarity, molecular binding, quenching, and energy transfer can affect the excited state lifetime τ, both time-domain and frequency-domain fluorescence lifetime spectroscopy and microscopy have seen widespread applications in chemistry, biology, and materials science.[1−8]

Here we report a novel spectroscopy phenomenon. We observed that the absolute phase differences between the emitted fluorescence traces and the sinusoidally modulated 445 nm excitation laser at 400 kHz are, surprisingly, positive (i.e., ahead of the laser modulation) for flavin adenine dinucleotide (FAD), Rhodamine 6G (Rh6G), and fluorescein isothiocyanate (FITC). Our experimental setup (Figure 1A) is a laser confocal fluorescence microscope configured for frequency-domain phase fluorometry. (See the Supporting Information.) As shown in Figure 1B, Rh6G exhibits a slightly positive (<1°) phase, FITC shows a moderately positive phase around 3°, and the phase of FAD is significantly in advance of the laser by almost 10°. Moreover, the phase advance is found to be a photoinduced effect: the positive phase shift of FAD increases with higher excitation power of the 445 nm laser, which is resonant with an absorption peak of FAD (Figure 1C). Phosphorescence is not detectable for these molecules that are in air-saturated aqueous solutions at room temperature because of the faster process of nonradiative quenching by triplet molecular oxygen than radiative phosphorescence.

Such a positive absolute phase appears as if fluorescence is emitted "ahead" of the sinusoidal excitation source. This effect is opposite to the conventional fluorescence lifetime picture that fluorescence emission should always lag behind the modulated excitation source, regardless of whether the excited state decay kinetics is single-exponential or multiexponential.[1−3] Moreover, the observed power dependence in Figure 1C is also inconsistent with fluorescence lifetime phase fluorometry, where the amount of the phase lag should be independent of the excitation intensity.[1−3] Therefore, we call our observation frequency-domain fluorescence anomalous phase advance (FAPA).

To account for the observed FAPA effect, we decided to investigate the consequence of the existence of a long-lived dark state D (whose fluorescence emission is extremely weak) based on the clue that the triplet-state quantum yield of FAD (∼10%[9])

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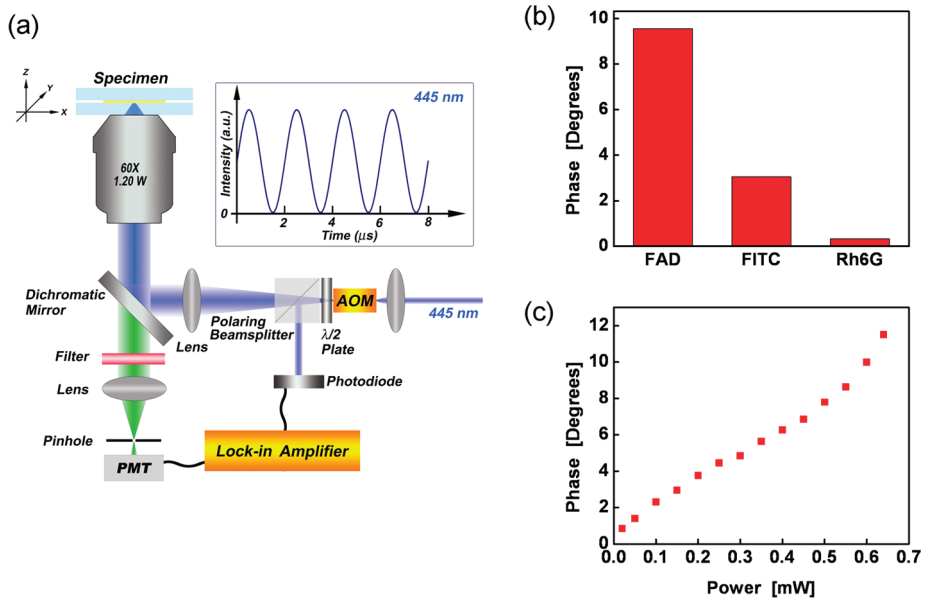

Figure 1. (A) Schematic of the confocal phase fluorometry setup. A lock-in amplifier is used to compute the phase difference between the collected fluorescence and the modulated excitation laser. Inset: a sinusoidally modulated laser excitation is used. (B) Positive phase relation of the fluorescence signals generated by FAD, FITC, and Rh6G with respect to the excitation laser modulated at 400 kHz. A peak power of 0.6 mW (600 kW/cm^2) is used at the laser focus. (C) Phase advance of FAD fluorescence increases with excitation power under a 400 kHz modulation frequency.

or FITC (~3%) is known to be higher than that of Rh6G (<1%) in aqueous solutions. Dark states of fluorescent systems have recently attracted considerable interest because of their important roles in many areas including super-resolution fluorescence imaging,[10−13] fluorescence photoblinking, and photobleaching of single fluorophores,[14,15] individual fluorescent proteins[16] and semiconductor nanoparticles,[17,18] transient state sensing,[19] and triplet-state relaxation microscopy.[20,21] To our knowledge, the influence of dark states presence in phase fluorometry has rarely been addressed in the literature.

To gain theoretical understanding, we first simulated the photodynamic transition process of fluorescent molecules for a three-state system consisting of a ground state *G*, an excited state *E*, and a dark state *D*, as shown in Figure 2A, under sinusoidally modulated laser excitation. (See the Supporting Information.) In the first case, which mimics the situation of Rh6G, the intersystem crossing rate from *E* to *D* is simply set to zero, and the resulting fluorescence trace (green trace in Figure 2B) is seen to travel almost exactly in phase with the laser. This is expected from conventional fluorescence lifetime phase fluorometry with a modulation frequency (500 kHz) being 500 times slower than the fluorescence lifetime (~4 ns) used in our simulation. The second case is set to mimic our experiment on FAD: the fluorescence lifetime is set to 4 ns, the dark-state relaxation rate is set to 2 μs^{-1}, the triplet-state quantum yield is set to 10%, and the optical excitation rate is set to 12 μs^{-1}, which is close to our experiment, where a 445 nm laser of 600 kW/cm^2 is resonant with FAD's absorption peak with an extinction coefficient of ~10^4 M^{-1} cm^{-1}. As shown by the red trace in Figure 2B, the FAPA effect emerges: a phase advance of 10.5° from the simulated trace is close to our experimental result of 9.5° on FAD. Note that unlike in fluorescence lifetime phase fluorometry, the phase shift in FAPA is exhibited only by the top of fluorescence trace, which is asymmetrically distorted.

We then investigate the modulation frequency dependence of the FAPA effect of FAD through both experiment and simulation. When the modulation frequency was varied from 100 kHz to 2 MHz, we observed in our experiments that there is a pronounced peak in the 300−500 kHz range, as shown in Figure 3A. Meanwhile, based on the simulated fluorescence trace, the resulting phase difference can be computed for each modulation frequency. The numerically simulated frequency response shown in Figure 3B also indicates the existence of a frequency peak. Therefore, both experiment and simulation confirm that the FAPA effect is pronounced only within a range of modulation frequencies. This is again different from standard fluorescence lifetime phase fluorometry. We note that the FAPA effect vanishes and the fluorescence phase turns negative at even higher modulation frequency (Supporting Information), as expected from conventional fluorescence lifetime phase fluorometry.

The nonmonotonic frequency response of the FAPA effect can be attributed to the modulation frequency being resonant with the intrinsic time scale of the dark state relaxation. On one hand, when the modulation frequency is too fast, which can be understood as a quasi-static limit, the fraction of the population trapped in the dark state will have no time to respond. This will result in very little modulation depth of the dark state population, as supported by the 10 MHz simulation shown in Figure 3C. On the other hand, when the modulation frequency is too slow, which can be considered as a quasi-equilibrium limit, the dark-state population can essentially reach equilibrium with the excitation intensity at any moment during the modulation cycle. This will lead to very little phase shift of the dark state population, as supported by the 10 kHz simulation shown in Figure 3C. Therefore, FAPA requires not only a pronounced modulation depth of the dark state population but also its appropriate dynamics.

The physical origin of the FAPA effect can be understood as follows. When the modulation frequency of the excitation light is chosen to be close to the intrinsic time scale of the dark state relaxation, fluorophores can be driven into or out of long-lived dark states with a nonvanishing rate at any moment of the modulation cycle. Mechanistically, the rising half of the sine laser

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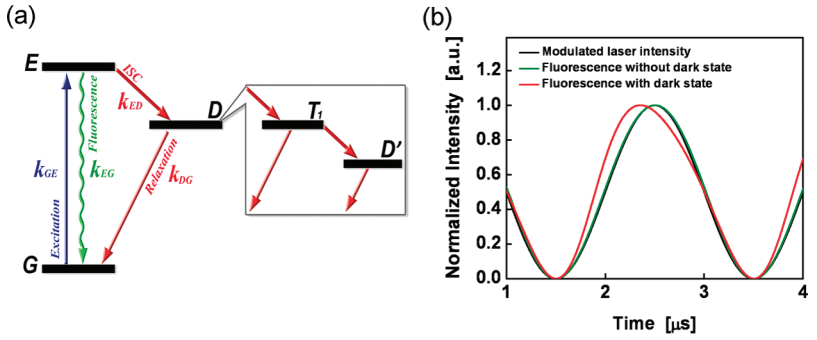

Figure 2. (A) Three-state quantum system of fluorophores consisting of a ground state G, an excited state E, and a dark state D. Photophyiscal parameters used are: $k_{GE} = 12\,\mu s^{-1}$ at the peak of the sine wave excitation, fluorescence emission rate $k_{EG} = 250\,\mu s^{-1}$, intersystem crossing rate $k_{ED} = 24\,\mu s^{-1}$, and dark-state relaxation rate $k_{DG} = 2\,\mu s^{-1}$. (B) Simulated time-dependent fluorescence signal traces in the absence of any dark states (green curve) and in the presence of a dark state (red curve) under a modulated laser excitation at 500 kHz (black curve). Unlike the green trace, which has no phase shift, the red trace appears ahead of the excitation by ~10.5°.

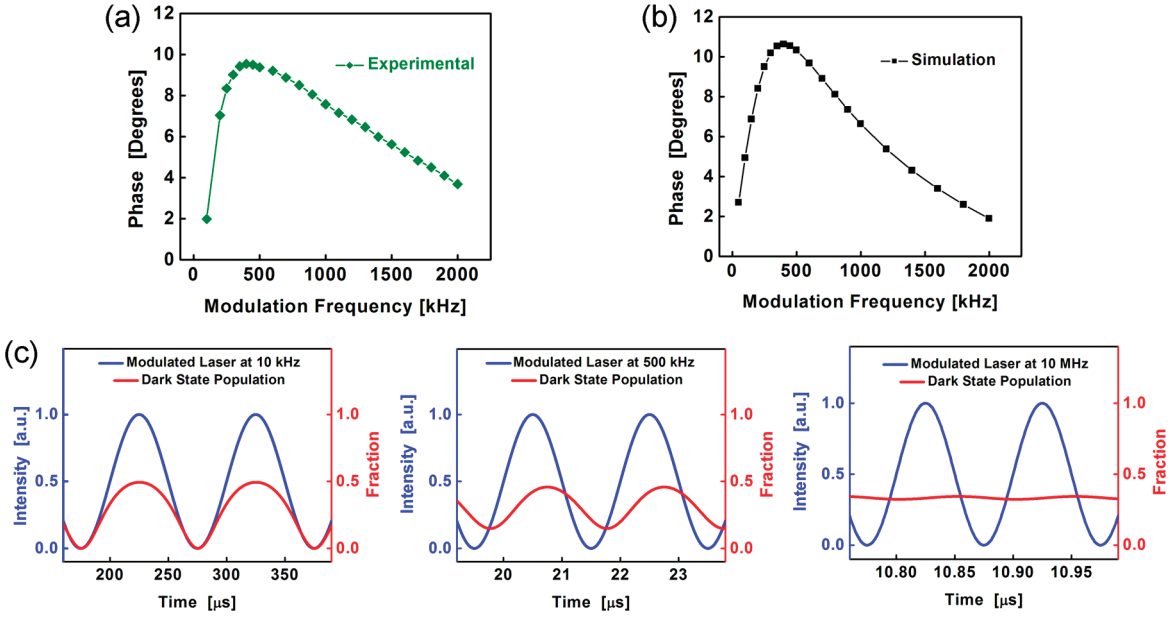

Figure 3. (A) Experimental FAPA modulation frequency response of FAD fluorescence (a peak intensity of 600 kW/cm^2 at the laser focus). A peak at ~400 kHz is evident. (B) Numerical simulation of the modulation frequency response of the FAPA effect. Parameters were identical to those used in the three-state model in Figure 2. (C) Simulated time-dependent dark-state populations at 10 kHz, 500 kHz, and 10 MHz laser modulation frequencies. Compared with the slow and fast limits, only the intermediate modulation frequency creates a dark-state population with both a sufficient modulation depth and an appreciable phase shift.

cycle not only generates fluorescence but also moves population into the dark state, which needs some finite time (similar to the laser modulation period) to return back to the bright excitation−emission cycle. As a result of dark-state hysteresis, the falling half of the sine laser cycle will excite relatively less population from the ground state and thus less fluorescence signal compared with the rising half of the laser cycle. This imbalance eventually will skew the resulting fluorescence waveform and create an apparent phase advance.

The triplet state is often regarded as the gateway to various subsequent radical states formed through charge transfer from or to the environment.[14,22] Therefore, we next explore the consequence of promoting and suppressing the triplet state population on the FAPA effect. A series of chemical species such as heavy-atom iodide, mercaptoethanol, and nitroxide radical were used.

Because of the strong spin−orbit coupling arising from the heavy atom effect, potassium iodide (KI) is known to accelerate otherwise forbidden and slow transitions of k_{ED} and k_{DG} between singlet and triplet states in both directions.[11,23] As shown in Figure 4A, adding KI to the FAD solution not only considerably decreases the absolute FAPA signal size but also shifts the modulation frequency peak from the original 400 kHz to 500 kHz to 1.5 MHz with increasing KI concentration. On the basis of the frequency response of the FAPA effect, as understood in Figure 3, this shift in the peak of the modulation frequency toward higher frequencies implies that KI has significantly shortened the triplet state lifetime of FAD. This trend would result in lower triplet-state population trapping and account for the observed weaker FAPA effect of FAD upon the addition of KI.

Interestingly, the addition of KI is found to have an opposite effect on Rh6G solution. As shown in Figure 4B, the addition of KI results in a noticeable increase in the FAPA phase and a concurrent shift in the peak of the modulation frequency response toward higher frequencies. We attribute this to the more dominant effect of KI in accelerating the intersystem crossing rate k_{ED} than in

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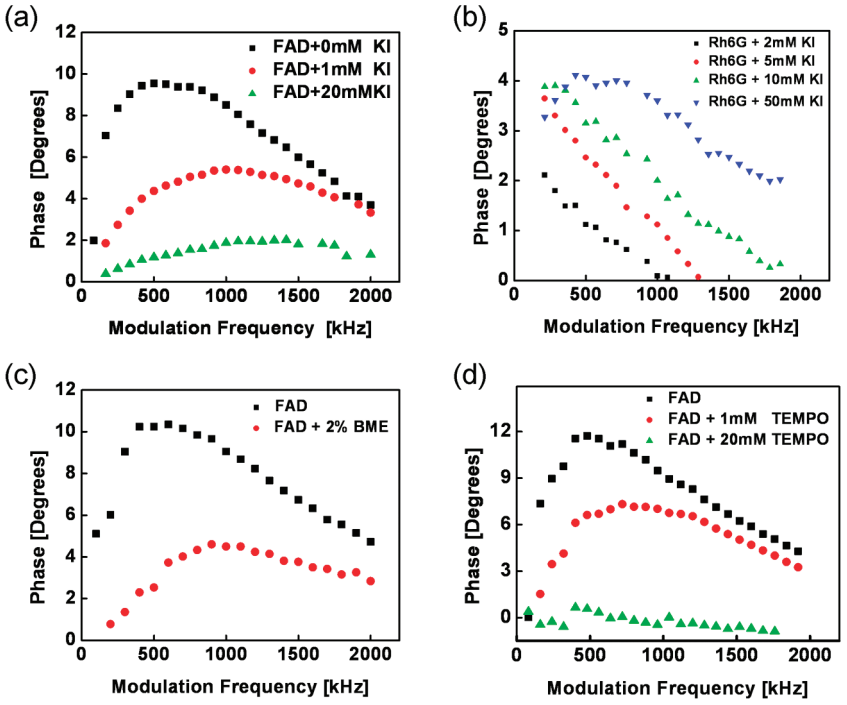

Figure 4. (A) FAPA frequency response of FAD with different added KI concentrations. Note both the amplitude attenuation and peak shift of FAPA curves with higher KI concentration. (B) Response of the FAPA phase of Rh6G with added KI. The progressive phase increase with added KI is interpreted as an acceleration of the intersystem crossing rate from *E* to *D*. (C) FAPA frequency response for FAD without and with 2% BME (v/v). BME is a known triplet quencher. (D) FAPA frequency response for FAD with added TEMPO, which is a stable free radical. In all (A−D), a peak intensity of 600 kW/cm^2 of the 455 nm laser is used at the focus.

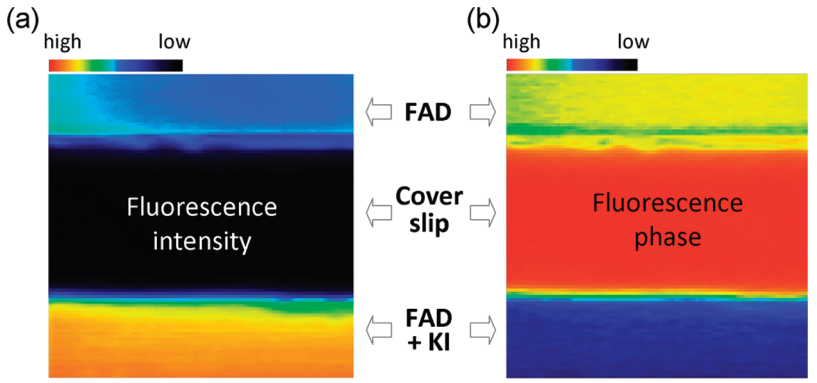

Figure 5. Microscopy images based on fluorescence intensity (A) and FAPA phase (B) from a sample made of 20 μM FAD (up) and 20 μM FAD + 20 mM KI (down) separated by a thin coverslip vertically mounted on a horizontal coverslip. The 200 × 200 μm images are composed of 100 × 100 pixels with a pixel dwell time of 3 ms. A peak intensity of 600 kW/cm^2 of the 445 nm excitation laser modulated at 400 kHz and a lock-in time constant of 3 ms were used. The coverslip gives a vanishing fluorescence intensity that results in phase overflow at the lock-in amplifier. Whereas the addition of 20 mM KI to FAD increases the time-averaged fluorescence intensity in (A), it significantly reduces the FAPA effect in (B).

accelerating the triplet-state relaxation rate k_{DG}. A similar effect of KI on promoting overall triplet state population in Rh6G has been previously observed using fluorescence correlation spectroscopy.[23,24]

We then test the effect of two other chemicals, β-mercaptoethanol (BME) and 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO), respectively. Addition of BME to FAD solution is found to suppress significantly the FAPA effect, as observed in Figure 4C. This is consistent with the fact that BME has commonly been used as a triplet-state quencher in in vitro single-molecule spectroscopy and imaging.[10,25] Being a stable free nitroxide radical, TEMPO can efficiently quench the triplet states of many molecules such as ketones and aromatic hydrocarbons.[26] As shown in Figure 4D, the addition of 1 mM TEMPO to FAD is found to decrease the FAPA signal and concurrently shift the frequency peak by over 200 kHz toward higher frequencies, indicative of a reduction in the triplet state lifetime. Taken together, all of the above tests of KI, BME, and TEMPO prove that the FAPA effect can indeed probe photophysical population and dynamics of the triplet (dark) states.

Finally, as shown in Figure 5, we demonstrate microscopic imaging with the FAPA effect as a contrast mechanism to emphasize that the FAPA signal could serve as a fast and straightforward reporter on the spatial variations of dark states' dynamics, which are otherwise difficult to probe. We believe that

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FAPA-based imaging is interesting because dark states can sense local environments differently than singlet states. For example, the fluorescence intensity image (Figure 5A) and the FAPA phase image (Figure 5B) display opposite contrasts between FAD and FAD/KI solutions: adding KI to FAD decreases FAPA magnitude and enhances overall fluorescence intensity by reducing dark state trapping but without affecting the fluorescence lifetime too much. Besides, it is known that physicochemical properties such as magnetic field[27] and oxygen concentration[28] have much stronger interactions with dark states than with fluorescent bright states.

Several fluorescence techniques have been developed to study dark states. Fluorescence correlation spectroscopy (FCS) and its modulated version (modulated FCS) have been proven to be a powerful technique for this task.[24,29] FAPA is radically different from FCS in that FAPA is a frequency-domain approach whereas FCS is a time-domain technique. Moreover, FAPA has the advantage of using the bright ensemble fluorescence signal as opposed to the weak single-molecule fluorescence in FCS, with the microsecond dark-state dynamics encoded in the ensemble fluorescence response. Therefore, the acquisition of the FAPA signal, which is achievable on a millisecond time scale, is much faster than building up an autocorrelation function, which normally takes 10−100 s. Most recently, Widengren et al. have developed interesting methods to analyze how the time-averaged fluorescence intensity changes under different laser modulation schemes such as varying pulse widths and pulse separation.[19] Our approach is conceptually different, intrinsically faster, and technically more convenient: the FAPA phase shift is computed "on the fly" as a single parameter specifically reporting on dark-state dynamics thanks to the phase-sensitive detection capability of the lock-in amplifier.

Being a fast frequency-domain reporter, the FAPA effect holds promise to enhance our understanding of dark-state dynamics in complex temporal and spatial environments. First, the FAPA effect can directly report on spatially varying differences in dark-state populations and lifetimes in biological specimens and in materials, as briefly demonstrated in Figure 5. The present study on the FAPA effect of FAD lays a foundation for future bioimaging, given the universal abundance of endogenous flavin proteins inside live cells and tissues. Second, the FAPA effect can detect temporally varying differences in dark-state dynamics on a fast (∼millisecond) time scale. Considering the growing interest in the physicochemical nature of dark states by many basic research areas including photobleaching, blinking, photoswitching mechanisms of organic fluorophores, fluorescent proteins and semiconductor nanoparticles,[10−21] we propose that the FAPA effect will potentially be of use in furthering these spectroscopy investigations.

To summarize, the FAPA effect reported and characterized here is the first frequency-domain fluorescence method to probe the dark state of fluorescent molecules. The effect itself 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.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details, additional data on modulation frequency dependence and details of numerical simulations. The material is available free of charge via the Internet http://pubs.acs.org.

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