Fluorophore discrimination by tracing quantum interference in fluorescence microscopy

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(Received 30 May 2010; published 21 January 2011)

We show fluorescence-detected quantum interference in a microscope setup and demonstrate selective enhancement or suppression of fluorophores using femtosecond pulse-pair excitation with periodic modulation of the interpulse phase.

DOI: 10.1103/PhysRevA.83.015402 PACS number(s): 33.80.Wz, 87.64.kv, 87.64.M−, 82.53.−k

I. INTRODUCTION

In fluorescence microscopy, simultaneous excitation of commonly employed fluorophores demands selective enhancement or suppression of fluorescence for fluorophore discrimination whenever required. The study and control of ultrafast fluorescence dynamics for such discrimination [1,2] in fluorescence microscopy [3,4] has gained interest in recent years as one of the promising applications of quantum (coherent) control methods by exploiting the phase coherence of a broadband ultrafast laser pulse through laser-pulse shaping [5]. Equivalent to pulse shaping, control over the time delay between a pair of pulses [6–8] (or a pulse train [9]) with definite interpulse phase relation can manipulate the excited-state population and hence the resulting fluorescence interferogram, hereafter called the quantum interference (QI) signal. Statistical analysis of the QI signal from pulse-pair excitation with indefinite phase relation (i.e., randomly phased or phase-randomized pulse-pairs) can furnish information on coherent dynamics [10,11]. One can also realize coherent dynamics in a much simpler way by observing the high-frequency QI signal within the pulse-pair temporal overlapping zone (away from the zero-delay i.e. strong overlapping region) by periodically modulating the interpulse phase. This approach has been utilized in controlled gas-phase molecular-fragmentation experiments [12] and studied in detail taking into account broadening of the spectral line shape as well as pulse profile, which is crucial in a condensed-phase environment [13,14]. In this Brief Report, we describe solution-phase fluorophore discrimination based on high-frequency QI observation and discuss its possible implementation in fluorescence microscopy. It is noteworthy to mention here that similar selective fluorescence suppression under pulse-pair and pulse-train excitation can also be rendered by exploiting dynamics beyond the coherence time scale, which has been explored by our group in recent times (using a two-photon pump followed by a one-photon stimulated emission scheme) and reviewed elsewhere [15].

II. METHODOLOGY

For an isolated diatomic molecule (having just one vibrational motion), electronic excitation by an ultrafast laser pulse (having a broad frequency bandwidth) couples many vibrational eigenstates of the molecule leading to the creation of a nonstationary vibrational wave packet in the electronic excited state. In addition, excitation by another time-delayed identical pulse creates another wave packet that interferes with the preceding one, depending on the interpulse delay and phase [6–8,16]. Such coherent nuclear wave-packet dynamics is known as vibrational wave-packet interferometry (WPI). Since fluorescence is proportional to the excited-state population, a constructive interference between these wave packets (when the relative phase between the pulse-pairs is zero) leads to an enhanced fluorescence, while a destructive interference (when there is a $\pm\pi$ phase difference between the pulse-pairs) leads to a decreased fluorescence. Fluorescence emission from the excited state is thus modulated with the interpulse delay while maintaining a definite (0 or $\pm\pi$) interpulse phase relation (known as phase-locked spontaneous light emission, or PLSLE [17]). An equivalent description is given based on spectral interferometry (SI) [18], where a pair of pulses (or a pulse train) also modulates the spectrum of a single pulse owing to Fourier transform and, by changing the interpulse delay and phase, the pulse spectrum may be tuned to perfectly match or mismatch with the vibronic absorption lines of the molecule leading to enhanced or suppressed absorption (equivalent to constructive or destructive WPI in the time-domain description) [19]. This holds for a collection of molecules too, provided the ensemble coherence is maintained (for example, in low-temperature dilute ensembles created by supersonic molecular-beam methods) [20]. Also, this description is applicable to any type of wave packet, e.g., electronic wave packets in atoms and molecules.

In such experiments, the QI signal consists of a slow modulation (due to vibrational WPI) on top of a high-frequency oscillation (known as Ramsey fringes [21]); the gradual decay of the WPI amplitudes (for successive vibrations) is due to loss of initial phase coherence. Since the time delay between pulse-pairs (which corresponds to the time delay between the center of two pulse envelopes) is directly related to the relative phase between them (with respect to a reference carrier-frequency oscillation) [22], one can extract coherent dynamical information by decoupling these two effects, e.g., by "active phase locking" where pulse-pairs are generated and combined in an interferometer and delay steps between the pairs are chosen in a way such that one particular relative phase is sampled [6,7]. One can also generate phase-locked pulse-pairs in a pulse shaper [23,24] where either one pulse is delayed in discrete steps as before (in which the carrier-envelope phase

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remains constant) or only the envelope of one pulse is delayed continuously maintaining the same relative phase (for which the carrier-envelope phase must vary) [22]. For periodic phase modulation, the relative phase is varied as the delay between the pairs is continuously changed; fine temporal resolution and robust phase stability ($\sim\lambda/100$ or better) are necessary when it is necessary to retrieve complete phase information of the signal by heterodyne detection as is done with "passive phase locking" (where phase-locked pulse-pairs, generated by diffractive optics, travel through common optical components for robust phase stability and fine temporal delay is maintained, e.g., using glass wedges) [25,26]. An alternative way to retrieve coherent dynamics is by making use of the statistical variance of interferometric noise following randomly phased pulse-pair excitation (known as coherence observation by interference noise, or COIN) [9,10]. In contrast, here we report selective enhancement or suppression of fluorophores based on direct observation of the QI signal only [12–14], without any deliberate attempt to unravel pure coherent dynamics.

Fluorescence-detected vibrational WPI has been studied for diatomic molecules in detail, particularly for dihalogens (or interhalogens) [5–7,16,27] or alkali metals [28,29] in the gas or vapor phase, as well as for dihalogens in inert gas matrices [18], since rapid vibrational dephasing for polyatomic molecules with higher degrees of motion obscures the observation of WPI effects. Also the theoretical treatments [6–8,10,11,16,27–29] considered only a short-pulse duration limit, i.e., when the pulse spectrum fully covers the linewidth of absorption. In the condensed phase, environment-mediated decoherence has made quantum control strategies a challenging field where homogeneous broadening (due to dynamic solvent fluctuations) and inhomogeneous broadening (due to static solvent structural inhomogeneity) of spectral line shapes require nonlinear optical techniques (e.g., photon echo spectroscopy) to separate each contribution [30]. In a recent experiment, Brinks *et al.* implemented a single-molecule fluorescence-detection technique to disentangle this spectral inhomogeneity under phase-locked pulse-pair excitation [31]. Taking into account the spectral line shape of absorption (which turns out to be a Voigt profile when both the homogeneous contribution with Lorentzian line shape as well as the inhomogeneous contribution with Gaussian line shape are present) and laser pulse (Gaussian profile), the QI signal under periodically phase-modulated pulse-pair excitation has shown to be varied for two different scenarios [13]: (1) if the pulse spectral width is at least an order of magnitude larger than the absorption linewidth, one expects the fringe frequency to be the inverse of the frequency width of the absorption profile and (2) at the other extreme, if a narrow pulse spectrum excites a subpopulation of the broad inhomogeneous absorption line shape, then the temporal fringe oscillations occur at a pulse carrier-wave frequency analogous to ultrafast hole-burning experiments [9,32]. For an intermediate situation (which happens to be the case in our experiment), the QI signal is neither given by the inverse of the spectral width nor follows the optical field oscillations [13,14]; for partial pulse-pair overlapping zone the QI signal differs from optical field oscillations due to nuclear dynamics i.e. vibrational WPI also [12]. Thus one expects an interesting fluorescence-detected QI signal which varies from one molecule to another, thereby paving the way for fluorophore discrimination based on interpulse delay only. Note that one reason for deliberately exploring the two-pulse overlapping zone was that, beyond this zone, the QI signal often dies out due to rapid solvent-mediated decoherence.

III. EXPERIMENT

The schematic of the set up is shown in Fig. 1(a). In our experiment, we used a 532-nm-pumped (second harmonic of a Nd:vanadate laser, Verdi 5, Coherent), home-built, mode-locked Ti:sapphire oscillator (MTS Mini laser kit, KM Labs), producing as short as ~20 fs (transform-limited width) pulses centered on 800 nm at 94 MHz repetition rate. The laser beam was sent to a Mach-Zehnder interferometer where two retroreflecting mirrors were placed, one in each arm. One of these mirrors was mounted on a mechanical stage (UE1724SR) driven by a motion controller (ESP300, Newport) that served as the fine delay line ($\sim\lambda/23$ resolution, equivalent to ~0.1 fs). The combined beam was frequency doubled by a β-barium borate (BBO) crystal (1 mm thick, Castech) and the resulting blue light was spectrally filtered using an infrared cutoff filter before being sent to the scan head of a commercial confocal fluorescence microscope (FV300/IX71, Olympus). An oil-immersion objective (UPlanApoN 1.4 NA, Olympus) focused the beam onto the sample (methanol solution of rhodamine-6G or basic fluorescein, each of 10^{-3} M) placed at the microscope stage. Fluorescence from each dye was collected by the same

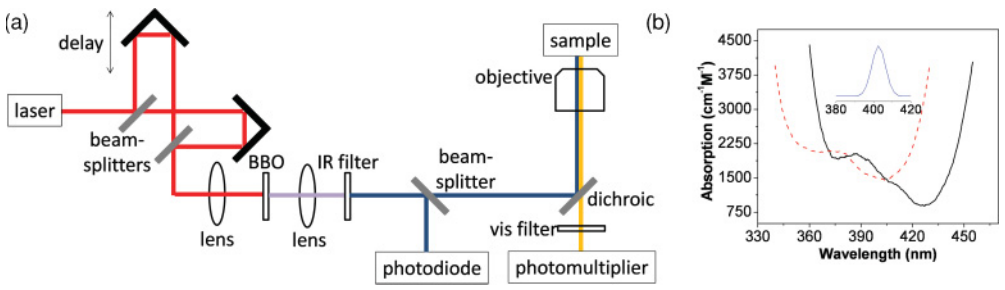

FIG. 1. (Color online) (a) Experimental setup; the scanning mirrors and confocal detection schemes are not shown for simplification. (b) Relevant absorption spectral profiles (lines) of rhodamine-6G in ethanol (solid black) and fluorescein in basic ethanol (dashed red); data were taken from the web resource for spectral database [34] and presented after smoothing over adjacent points. The inset shows the pulse spectra (solid blue).

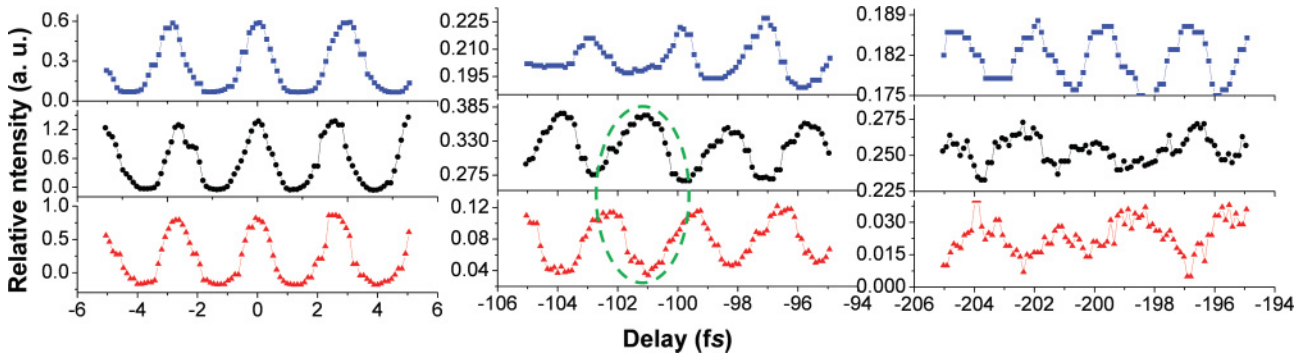

FIG. 2. (Color online) Fluorescence interferogram (QI signal) for rhodamine-6G (middle panel, black circles) and fluorescein (lower panel, red triangles) compared with optical field oscillation (upper panel, blue squares) for three different pulse-pair delay regions: ∼0 fs delay (left panel), ∼−100 fs delay (middle panel), and ∼−200 fs delay (right panel). The concurrent enhancement of rhodamine-6G fluorescence and suppression of fluorescein fluorescence at a definite interpulse time delay is also marked (dashed green oval).

microscope objective ("epi-fluorescence" collection) and sent to a photomultiplier (Hamamatsu) before it was separated from the excitation beam by a dichroic beam splitter and a long-pass (510 IF) filter (to ensure that no residual excitation light was present); all collection optics were inside the scan head and thus the total fluorescence (⩾510 nm) was collected. The photomultiplier output from the scan head was fed into a 600 MHz 10 GS/s digital oscilloscope (Wave Runner 64Xi, LeCroy) connected to the motion controller and interfaced with a personal computer using a GPIB card (National Instruments). For data acquisition, we used LABVIEW programming. Interferometric autocorrelation traces were measured using a large-area (1×1 cm^2) silicon photodiode (PDA100A, Thorlabs) connected to the same oscilloscope. The oscilloscope sampling time was chosen in such a way that phase stability was maintained over data acquisition time to fully recover the oscillatory QI signal (as well as fringe-resolved autocorrelation trace); however, phase stability could not be maintained over an image frame acquisition time (>1 s) for microscopy application (discussed below).

IV. RESULTS AND DISCUSSION

The relevant regions of absorption spectra of rhodamine-6G and fluorescein are shown in Fig. 1(b) [33,34]. The photophysical properties of rhodamine-6G are well studied [35]; between two dominant absorption peaks, it has a broad absorption centered on ∼385 nm. This electronic state has weak one-photon absorption (which becomes a dominant absorbing state under two-photon excitation). Similar is the case for fluorescein for which the absorption is centered on ∼370 nm. The pulse spectrum is centered on ∼402 nm with ∼7.3 nm bandwidth (full width at half maximum), which is smaller than the inhomogeneously broadened absorption spectra.

The fluorescence interferogram (QI signal) for each fluorophore is shown in Fig. 2 along with the interferometric autocorrelation trace (optical field oscillations). We zoomed into regions of strong, moderate, and weak pulse-pulse temporal overlapping zones. Near pulse-pair delay of zero, the QI signal exactly follows the optical field oscillation; at further delay times (around ±200 fs delay), the QI signal fades out indicating rapid loss of phase coherence in the condensed phase. However, for intermediate delay, the fluorescence oscillations completely differ from interferometric autocorrelation trace and, at some specific pulse-pair delay, the fluorescence intensities of rhodamine-6G and fluorescein completely go out of phase, i.e., they are anticorrelated. Thus, just by precisely controlling the interpulse delay, it is possible to enhance the fluorescence emission from one particular fluorophore while suppressing it from the other one, and this is of the utmost importance regarding selective excitation in fluorescence microscopy. Since the pulse excites only a subpopulation of the inhomogeneously broadened electronic state, we hypothesize that the QI signal results due to vibrational WPI also [12], in addition to phase relaxation [13,14], which are molecule specific and thus lead to the discrimination. However, we do not rule out other (e.g., nonlinear) effects.

Thus we show a simple method of quantum (coherent) control for fluorophore discrimination. As a rational extension of this approach to image biological specimens, we used confocal fluorescence laser-scanning microscopy where the specimen is scanned point by point to construct a two-dimensional image frame and each point is illuminated by a train of pulse-pairs over a certain time window (corresponding to the laser dwell time on each sample point). For a given time delay between the pulse-pairs an image frame is recorded and the time delay is varied for successive image frames; thus a series of image frames may be constructed as a function of the temporal delay which captures the QI signal with spatial resolution. However, although fine temporal resolution led us to follow variation of the QI signal and capture interesting ultrafast dynamics buried within the pulse-pulse interference zone, robust phase stability could not be maintained over the time period of image-frame acquisition (noted by fluorescence fluctuations over time for a given delay). Combining phase stability over longer time with fluorescence microscopy is presently being pursued in the authors' laboratory.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge Jahan Dawlaty for an illuminating discussion. We thank Wellcome Trust Foundation (UK) and DST (India) for financial support. A.K.D. and D.R. also thank CSIR (India) for financial support.

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