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Wide-field time-resolved fluorescence anisotropy imaging (TR-FAIM): Imaging the rotational mobility of a fluorophore

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We report a picosecond time-gated fluorescence lifetime imaging (FLIM) system extended to perform time-resolved fluorescence anisotropy imaging (TR-FAIM). Upon excitation with linearly polarized laser pulses, the parallel and perpendicular components of the fluorescence emission from a sample are imaged simultaneously using a polarization-resolved imager. The imaging technique presented here quantitatively reports the rotational mobility of a fluorophore as it varies according to the local environment. In a single acquisition run it yields maps of both rotational correlation time and fluorescence lifetime as they vary across a sample. TR-FAIM has been applied to imaging standard multiwell plate samples of rhodamine 6G dissolved in methanol, ethylene glycol, trimethylene glycol, and glycerol. The observed rotational correlation times and fluorescence lifetimes, which report the local viscosity and refractive index of the local rhodamine 6G environment, respectively, are in good agreement with previously published single point measurements. By considering the linear dependence of the rotational correlation time on viscosity up to 20 cP, we are able to obtain a two-dimensional viscosity map. Wide-field maps of rotational correlation time, and therefore viscosity, have been obtained. This illustrates the potential to image the local viscosity and fluorescence lifetime distributions of fluorophore tagged proteins in cells.

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I. INTRODUCTION

Fluorescence imaging is an increasingly important tool in the biological and physical sciences. The fluorescence emission can be characterized by lifetime, wavelength, or polarization to provide structural and functional information. The fluorescence lifetime τ can report on the type of fluorophore, its orientation, its interaction with other molecules¹ or

the properties of its environment, including the refractive index^{2–6} and the viscosity.⁷ While some molecules exhibit a viscosity dependent fluorescence lifetime due to the competition of nonradiative de-excitation pathways such as internal twisting with radiative de-excitation,⁸ in general the fluorescence lifetime is independent of solvent viscosity. However, the local viscosity has a strong influence on the rotational mobility of the fluorophore in its environment and this can be determined by measuring the time-dependent polarization anisotropy of the fluorescence. After excitation with a linearly polarized light pulse, the initially polarized fluorescence emission rapidly depolarizes due to rotational diffusion of the fluorophore.^{9,10} The rotational diffusion of the

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probe during the fluorescence lifetime τ of the excited state can be characterized by the rotational correlation time θ , which is the average time for molecular tumbling. θ depends on the viscosity η of the solvent and the size and shape of the rotating molecule (Debye–Stokes–Einstein hydrodynamic model),¹¹

$$\theta = \frac{4\pi a^3 \eta}{3kT}, \quad (1)$$

where a is the apparent molecular radius of the rotating molecule, k is the Boltzmann constant, and T is the temperature.

Time-resolved single-point cuvette studies of rotational diffusion of molecules have been used to measure the internal viscosities of membranes, to elucidate binding or denaturation of molecules [through a change of the apparent molecular radius in Eq. (1)] and to provide clues to their relative location.⁹ Using the fluorescence probe DPH in cell membranes, measurements of fluorescence lifetimes together with the steady-state anisotropy provided early evidence of heterogeneity in cell membranes.¹² Dayel *et al.* studied the diffusion of green fluorescent protein in the endoplasmic reticulum of cells by comparing *rotational* diffusion times obtained by time-resolved anisotropy measurements with *translational* diffusion coefficients obtained by fluorescence recovery after photobleaching.¹³ Time-resolved fluorescence anisotropy measurements have also been used to study sol-gel particle growth as an alternative to scattering techniques,¹⁴ and also to elucidate information about pores in sol-gels.¹⁵

Although single-point time-resolved anisotropy measurements of heterogeneous samples, such as cells, can provide very useful information on the torsional dynamics of cell constituents,¹⁶ the extension of this technique to two dimensions is not well established. We note that steady-state two-dimensional (2D) fluorescence anisotropy imaging (FAIM) was demonstrated by Gough and Taylor and also by Dix and Verkman, who applied it to the study of living cells.¹⁷ However, there is only one report of time-resolved fluorescence anisotropy imaging: Buehler *et al.*¹⁸ resolved the ratio of the parallel and perpendicular fluorescence polarization components temporally and spatially by measuring the stimulated emission induced by a probe pulse whose polarization was rotated with respect to the excitation pulse. Their elegant quasiconfocal scanning microscopy technique requires the consecutive acquisition of the image sets corresponding to the perpendicular and parallel fluorescence components. (After the submission of this article, Clayton *et al.* reported the use of frequency-domain fluorescence lifetime imaging (FLIM) for time-resolved fluorescence anisotropy imaging, where the images at different polarizations are similarly acquired separately.¹⁹) Consecutive acquisition is vulnerable to errors arising from biophysical or biochemical changes and movement of the sample between the acquisition of the two images, or to instrumental drift and other effects, such as photobleaching. In single-point time-resolved measurements, the simultaneous acquisition of the parallel and perpendicular component of the fluorescence emission has long been used, either by using an optical or electronic delay to collect the different components in the same multi-

channel analyzer (MCA) time range,^{20,21} or by sending them to different MCA segments using a router.²² We present here a wide-field time-resolved fluorescence imaging system that acquires two polarization-resolved images simultaneously, effectively realizing time-resolved fluorescence anisotropy imaging (TR-FAIM). This is achieved by incorporating a polarization-resolved imager (PRI), which simultaneously acquires images polarized parallel and perpendicular to the excitation beam, into our wide-field time-gated FLIM system. We note a similar approach to acquire steady-state anisotropy images of single molecules has been realized by simultaneous acquisition of both polarization components using a Wollaston prism polarizer.²³

The TR-FAIM extension of FLIM yields not only fluorescence lifetime maps, but also rotational correlation time maps and maps of the initial polarization anisotropy. To demonstrate this technique we have applied it to the simultaneous measurement of the rotational correlation times and fluorescence lifetimes of rhodamine 6G in solvents of different viscosity and different refractive index. Introducing a position- and time-dependent G factor, we are able to correct for spatially dependent detector sensitivity and depolarization in the imaging system.

II. THEORETICAL BACKGROUND

The time-resolved fluorescence anisotropy $r(t)$ is defined as

$$r(t) = \frac{I_{\parallel}(t) - GI_{\perp}(t)}{I_{\parallel}(t) + 2GI_{\perp}(t)} = \frac{D(t)}{F(t)}, \quad (2)$$

where $I_{\parallel}(t)$ and $I_{\perp}(t)$ are the fluorescence intensity decays parallel and perpendicular to the polarization of the excitation, respectively. G accounts for the different transmission efficiencies of the two components. Assuming a freely rotating spherical or oblate fluorophore, $r(t)$ decays monoexponentially.¹⁰ The time dependence of the difference decay $D(t)$ is the product of the intensity decay of the excited state (assumed to be mono-exponential in this case) and the anisotropy decay

$$D(t) = F(t) \cdot r(t) = I_0 \exp\left(-\frac{t}{\tau}\right) \cdot r_0 \exp\left(-\frac{t}{\theta}\right) \quad (3)$$

and for the perpendicular and parallel components we obtain

$$I_{\perp}(t) = \frac{1}{3} I_0 \exp\left(-\frac{t}{\tau}\right) \cdot \left[1 - r_0 \exp\left(-\frac{t}{\theta}\right)\right], \quad (4a)$$

$$I_{\parallel}(t) = \frac{1}{3} I_0 \exp\left(-\frac{t}{\tau}\right) \cdot \left[1 + 2r_0 \exp\left(-\frac{t}{\theta}\right)\right]. \quad (4b)$$

From these equations, it is clear that a measurement of the fluorescent lifetime of the two components is affected by the rotational depolarization, as depicted schematically in Fig. 1. The initial anisotropy r_0 of a fluorophore is given by²⁴

$$r_0 = \frac{2k}{2k+3} \left(\frac{3 \cos^2 \alpha - 1}{2} \right), \quad (5)$$

where α is the angle between the absorption and emission dipole moments of the molecule and k the number of photons

FIG. 1. Schematic of the fluorescence decay after pulsed excitation in the presence of polarization anisotropy. While the parallel ($I_{||}$) and perpendicular ($I_{\perp}$) components clearly deviate from a single exponential decay, the total fluorescence $F = I_{||} + 2I_{\perp}$ (dotted line) as well as the fluorescence measured at the magic angle of 54.75° , which eliminates bias due to polarization effects, (dashed line) remain unaffected.

creating the excited state. The absorption probability is proportional to $\cos^2 \alpha$ and for single photon excitation ($k=1$) and molecules with $\alpha=0$, the maximum value for the initial anisotropy is 0.4.

Note that Eq. (3) strictly holds only for spherical and oblate molecules. For randomly shaped molecules with three different free axes of rotation, five rotational correlation times are theoretically possible. However, no more than three have experimentally been resolved.^{10,25} In addition, a hindered rotation of the fluorophore, for example in lipid bilayers or cell membranes, leads to an additional term r_∞ in Eq. (3)

$$r(t) = (r_0 - r_\infty) \exp\left(-\frac{t}{\theta}\right) + r_\infty, \quad (6)$$

resulting in the anisotropy not decaying to zero.²⁶ Apart from rotational molecular mobility, scattered light, radiative, and nonradiative energy transfer can also lead to fluorescence depolarization. The use of highly diluted samples ensures these effects are minimized.

III. EXPERIMENT

A. Instrumental setup

The experimental apparatus and the data acquisition scheme for simultaneous TR-FAIM and FLIM are shown in Fig. 2. The excitation source is an argon-ion laser pumped mode-locked Ti:sapphire laser (Spectra-Physics, Tsunami). Its output is frequency doubled in a BBO crystal to produce a 0.2 nJ, 100 fs wide-field excitation source operating at 80 MHz repetition rate, that can be tuned from 415 to 490 nm. The orientation of the linearly polarized output can be altered with a ($\lambda/2$) half waveplate. Since the polarization change induced by the waveplate is wavelength dependent, a sheet polarizer is placed behind it in order to ensure linearly polarized excitation of the sample. This is of particular importance when the same waveplate is used for different wavelengths. Note that, in this setup, we cannot use a rotating diffuser to destroy the spatial coherence of the laser beam since this would scramble the polarization orientation of the laser light. The laser beam enters the inverted microscope, being reflected towards the sample by a dichroic beamsplitter and a 45° mirror (not shown), thus providing a wide-field excitation of the sample.

The fluorescence generated in the sample is imaged by two lenses of comparable focal length ($\approx 1:1$ imaging system) onto the entrance plane (image plane 1) of a commercially available polarization-resolved imager (Optical Insights, Dual-View Micro-ImagerTM with a polarization/anisotropy filter cube). A polarizing beamsplitter then divides the fluorescence into the vertical and horizontal polarization components to produce a composite output (image plane 2) comprising two spatially identical images of the sample that differ only in their polarization content. A gated optical image intensifier (GOI) (Kentech Instruments Ltd., GOI/HRI), placed at image plane 2, produces a time-gated (full width half maximum ≈ 400 ps) fluorescence intensity image on the phosphor screen that is relayed to an 8-bit intensified Charge

FIG. 2. (Color) (a) Experimental setup of the wide-field time-domain TR-FAIM and FLIM instrument. The PRI containing a polarizing beamsplitter is situated directly in front of the GOI, the output phosphor screen of which is imaged with a CCD camera. The PRI splits a single image (here, a single square well of a multi-well plate) in image plane 1 into two spatially identical images differing only by their polarization (image plane 2), which are thus recorded simultaneously. (b) A series of such polarization-resolved fluorescence intensity image pairs are acquired at a range of delays after the excitation pulse, thereby sampling the fluorescence decay profiles.

Coupled Device (CCD) camera (Photonic Science, ISIS 3), with an image size of 576×576 pixels. A series of images is acquired at varying delays after excitation to sample the fluorescence decay. Without the polarization components, this system is similar to the FLIM system we reported earlier,²⁷ which can provide FLIM maps with an update time of only 3 s and resolve lifetime differences of <10 ps.²⁸

B. Data acquisition and image registration

Fluorescence decay data containing information about the fluorescence lifetime and polarization anisotropy of the sample are obtained by acquiring a series of time-gated fluorescence intensity images at a range of time delays after excitation (starting at 400 ps after the fluorescence peak in order to avoid the need for deconvolution). This essentially corresponds to the simultaneous acquisition of $I_{\parallel}(t)$ and $I_{\perp}(t)$. As shown in Fig. 2(a), for a vertically polarized excitation beam, the left part of the composite image, detecting the vertically polarized component, corresponds to the parallel component $I_{VV}(t)$, and the right part, detecting the horizontally polarized component, corresponds to the perpendicular component $I_{VH}(t)$. For a horizontally polarized beam, this reverses and the left subimage detects the perpendicular component $I_{HV}(t)$, whereas the right subimage detects the parallel component $I_{HH}(t)$. To obtain the results shown here, we acquired 15 time-gated images at different delay positions, each one being the average of 50 video-rate images. The total acquisition time was 77 s. This could be speeded up by a factor of 10 by reducing the number of time gates and also by increasing the numerical aperture (NA) of the imaging system (currently 0.045), thus increasing the signal and therefore requiring fewer averages.

Before a fitting algorithm can be applied, the left and right subimages have to be spatially overlapped for all time-gated images to enable calculation of Eq. (2). This requires that the two subimages have to be adjusted precisely in terms of their relative size, position, and rotation with respect to each other (image registration). Without registration, the parallel component of one location would be related to the perpendicular component of a different location. The registration has been performed with commercially available software (Optical Insights, M $\acute{e}$ langeTM imaging and processing software) that accounts for lateral displacement, rotation and magnification of one subimage with respect to the other.

C. Data analysis

After image registration, the polarization anisotropy can, in principle, be calculated for each time-gated image according to Eq. (2), using M $\acute{e}$ langeTM. However, we found that a more precise analysis requires the experimental determination and subsequent mathematical incorporation of the G factor, which in our case turns out to be position- and time-dependent rather than a constant number, as discussed below (see also the Appendix for details of the derivation of the G factor). For this reason, we used a custom written LABVIEW program (National Instruments) to calculate and incorporate the $G(x, y, t)$ factor and subsequently calculate the time series of polarization anisotropy images $r(t)$ as well as the

time series of the total fluorescence decay $F(t)$. Finally, a custom written LABVIEW program has been used to fit a single-exponential decay to $r(t)$ yielding the characteristic rotational correlation time θ . In the same way the fluorescence lifetime τ , unperturbed by the rotational diffusion, can be obtained by fitting a single exponential decay to $F(t)$.

D. Sample preparation and sample holder

The samples were prepared by placing rhodamine 6G (BDH Chemical LTD, Poole, UK) in a flask and adding ethanol (BDH, AnalaR[®] grade), ethylene glycol (Aldrich), or trimethylene glycol (Aldrich) to obtain a concentration of 10^{-5} M. The rhodamine 6G in glycerol (spectrophotometric grade, Aldrich) sample was prepared by dissolving rhodamine 6G in a small amount of acetone (Aldrich, spectrophotometric grade), which was deposited in a flask and evaporated. This leaves the inside of the flask coated with a thin layer of rhodamine 6G; glycerol is simply added and heated gently to dissolve the rhodamine 6G in it. The refractive index of the solvents was measured with a standard refractometer at 589 nm and their viscosity with an Ostwald viscometer relative to the viscosity of ethylene glycol, which is known to be 19.5 cP at 20 °C.^{29–31} All measurements, including lifetime and anisotropy measurements, were made at a temperature of 20 ± 2 °C. The samples were placed in a standard multiwell plate, which consisted of a rectangular array of wells, each having a lateral dimension of 4 mm $\times$ 4 mm and a maximum capacity of 100 μ l, with a transparent plastic base. The well plate was then placed onto the inverted microscope and imaged at $1 \times$ magnification.

IV. RESULTS AND DISCUSSION

A. FLIM maps at parallel and perpendicular emission polarization

To verify that our FLIM system is sensitive to rotational depolarization, we measured the extensively characterized dye rhodamine 6G in solvents of different viscosity. Figure 3(a) shows the first time-gated fluorescence intensity image of the two emission components of two wells filled with rhodamine 6G dissolved in methanol and ethylene glycol excited with linearly polarized laser pulses at $\lambda_{\text{ex}} = 490$ nm. While rhodamine 6G in methanol shows similar intensities for both polarization components, rhodamine 6G in ethylene glycol shows a more intense parallel component. Ethylene glycol has a sufficiently high viscosity that the initial polarization anisotropy depolarises slowly enough to be resolved. From a series of these time-gated intensity images, at different delays with respect to the excitation pulse, a FLIM map [Fig. 3(b)] can be obtained. The false color scale represents the apparent fluorescence lifetime when the decays are fitted to a single exponential decay. The top well, with rhodamine 6G in methanol, yields a very similar apparent fluorescence lifetime for both parallel and perpendicular detection. The bottom well with rhodamine 6G in ethylene glycol, however, does appear to yield a shorter lifetime for the parallel component and a longer lifetime for the perpendicular one. This is due to the rotational depolarization of the fluorescence decay, c.f. Eqs. (4a) and (4b). An analysis of the fluorescence

FIG. 3. (Color) First time-gated fluorescence intensity images (a), (c) and FLIM maps (b), (d) of two wells of a multiwell plate filled with rhodamine 6G in methanol (top row of each image) and ethylene glycol (bottom row of each image) at $\lambda_{\text{ex}} = 490$ nm. The false color scale of the FLIM maps represents the apparent fluorescence lifetime values. (a), (b) Vertically polarized excitation: The left half of the images is the parallel emission ($\parallel$) of the two wells; the right half is the perpendicular emission ($\perp$). (c), (d) Horizontally polarized excitation: The left half is the perpendicular emission; the right half is the parallel emission. (e) Representative single pixel decays of each well for vertical excitation. The rotational depolarization in methanol is much faster than the fluorescence decay. In ethylene glycol it occurs over a similar time scale and therefore leads to noticeably different decays for the parallel and perpendicular polarization of the emission [c.f. Fig. 1 and Eqs. (4a) and (4b)].

decay of representative single pixels of the image in Fig. 3(b) is shown in Fig. 3(e). In ethylene glycol, the effect of the polarization–depolarization of the fluorescence can clearly be seen and is in agreement with the description in Fig. 1. In methanol, the fluorescence decays of the two components are very similar [Fig. 3(e)], due to the rapid rotational depolarization caused by the low viscosity of the solvent [cf. Eq. (1)]. The fluorescence emission is depolarized within a period (≈ 100 ps) that is 40 times shorter than the fluorescence lifetime (≈ 4 ns) and this can be difficult to resolve. The dynamic range of motions measurable by anisotropy decay is limited by the fluorescence lifetime; typically correlation times between 0.1 and 10 times the fluorescence lifetime, or 30 times using constrained analysis, may be resolved.^{32,33}

To verify this interpretation, we rotated the polarization of the excitation beam by 90° . For both the time-gated intensity image [Figs. 3(c)] and the FLIM map [Fig. 3(d)], we find the expected inversion of the effect observed in Figs. 3(a) and 3(b), confirming that our time-gated FLIM system with polarization discrimination is sensitive to the rotational depolarization of the emitted fluorescence.

B. Polarization leakage and G-factor correction

To calculate correct rotational correlation time maps and fluorescence lifetime maps, the different transmission efficiencies for the parallel and perpendicular polarizations have to be taken into account using the G factor in Eq. (2). In experimental setups using monochromators, often $G \gg 1$ whereas in setups based on dichroic beamsplitters and filters

as in our case, $G \approx 1$.⁹ Nevertheless, our particular setup based on a polarization-resolved imager uses a polarizing beamsplitter that has a considerable leakage of approximately 10% of the wrong polarization component over the wavelength range from 460 to 660 nm. Fortunately, this 10% leakage is symmetric and can therefore be easily accounted for using the following equations:

$$I_{\parallel, \text{exp. corr.}} = \frac{9I_{\parallel, \text{exp.}} - I_{\perp, \text{exp.}}}{8}, \quad (7a)$$

$$I_{\perp, \text{exp. corr.}} = \frac{9I_{\perp, \text{exp.}} - I_{\parallel, \text{exp.}}}{8}. \quad (7b)$$

Having corrected for the polarization leakage, we can now calculate the anisotropy decay $r(t)$ and the total fluorescence decay $F(t)$ according to

$$r(t) = \frac{I_{\parallel, \text{exp. corr.}}(t) - GI_{\perp, \text{exp. corr.}}(t)}{I_{\parallel, \text{exp. corr.}}(t) + 2GI_{\perp, \text{exp. corr.}}(t)}, \quad (8)$$

$$F(t) = I_{\parallel, \text{exp. corr.}}(t) + 2GI_{\perp, \text{exp. corr.}}(t). \quad (9)$$

The G factor corrects for the optical transmission differences for the two polarization components in the remaining optics, which are expected to be very small.^{16,34} Nevertheless, interpreting G as position- and time-dependent, i.e., $G(x, y, t)$, rather than constant, permits corrections for spatially dependent depolarization due to the objective lens and spatially dependent sensitivity of different detector pixels or imperfect polarizer alignment. For these reasons, $G(x, y, t)$ must be measured experimentally. Unlike the L-format and

T-format configurations that are commonly used in single-point measurements, where the detection is collected at right angles to the excitation, in our case the detection is collinear. $G(x,y,t)$ must hence be determined bearing in mind the geometry of our set up, as schematically depicted in Fig. 10 (Appendix). As derived in the Appendix we obtain the following relation for $G(x,y,t)$ of a collinear excitation and fluorescence emission setup:

$$G(x,y,t) = \sqrt{\frac{I_{VV}(x,y,t) I_{HV}(x,y,t)}{I_{VH}(x,y,t) I_{HH}(x,y,t)}}. \quad (10)$$

$G(x,y,t)$ can thus be obtained by recording two time series of fluorescence intensity images at vertical and horizontal excitation and extracting the four resulting half images. A similar formula has recently been derived by different authors.^{16,34} Calculating $G(x,y,t)$ according to Eq. (10) can be considered as global analysis. Originally, global analysis was introduced to increase the statistical accuracy by increasing the number of measurements leading to the same result.³⁵ Global analysis has recently been used to reduce spatial fluctuations in the FLIM maps by setting certain fitting parameters to be equal for all pixels in an image.³⁶ In our case, this corresponds to the two measurements necessary to determine $G(x,y,t)$. Both of these measurements must lead to the same result, i.e., the anisotropy at each temporal position, which is the starting point of the derivation of Eq. (10) (see Appendix).

Figure 4(a) shows $G(x,y,t)$ at the first time gate ($t=0$) calculated from the vertical and horizontal excitation measurements shown in Fig. 2, using Eq. (10). It is clear that $G(x,y,t)$ is not a constant number but depends on the spatial position. Only at the locations of the two wells is $G(x,y,t)$ valid; the borders should be ignored since there is no fluorescence and therefore no G factor defined. A G image is calculated for each time gate and Fig. 4(b) shows the time evolution of the spatially averaged values of the top and bottom well of the G image. In all cases the G values are close to 1, as expected for a system composed of components that alter the state of polarization only slightly.⁹ Nevertheless, two interesting observations can be made. First, for the top well we find $G < 1$, whereas for the bottom well $G > 1$. This means that the vertical component is transmitted or detected preferentially in the upper part, and the horizontal component in the lower part, of our imaging system. This behavior is observed for all three acquisition runs on different samples [different symbols in Fig. 4(b)], which demonstrates that this is not a coincidence or a sample dependent effect, but instead caused either by a spatial variation of the detector sensitivity or of the optical transmission for the two components. The second observation is that the bottom well shows a time dependence, whereas the upper well does not. Apparent time dependencies of the G factor in L-format single-point measurements can be attributed to a misalignment of polarizers, but they could in our case also be caused by a spatial variation of the background level of the detector.

For comparison, we have also calculated $G(x,y,t)$ for the set of measurements we performed at 415 nm excitation, under slightly different alignment conditions. Both the

FIG. 4. $G(x,y,t)$ calculated from vertical and horizontal excitation measurements using Eq. (10). (a) shows the G image at the fluorescence peak at $\lambda_{\text{ex}} = 490$ nm from the results shown in Fig. 3. (b) shows the time evolution of the spatially averaged top well and bottom well of the G image, the different symbols account for the fact that three acquisition runs were made, using different samples to demonstrate the reproducibility. (c) is as (a), but for a corresponding sample with $\lambda_{\text{ex}} = 415$ nm. (d) is as (b), but with $\lambda_{\text{ex}} = 415$ nm.

$G(x,y)$ image [Fig. 4(c)] and the $G(t)$ curves [Fig. 4(d)], are similar to the measurements at 490 nm, but shifted to lower G values and have a different time dependence. We attribute this to small differences in the alignment of the polarizer or in the background level. Nevertheless, one has to keep in mind the original purpose of $G(x,y,t)$ as outlined before, i.e., to correct for the above mentioned artefacts. Therefore it does not matter if we obtain slightly different results for different alignments because $G(x,y,t)$ is merely a monitor of the artefacts it corrects for.

An additional consideration has to be made when using high NA objectives to collect the fluorescence. Although this is not the case in our current 1:1 imaging setup, it will be of importance once we apply the technique to anisotropy imaging of cells. Axelrod,³⁷ and later Ha *et al.*³⁸ derived formulae to account for the effect that high NA lenses “see around” the fluorophore and therefore collect all three emission components I_x , I_y , I_z with a certain weighting for a given analyzer position instead of only the component that corresponds to the analyzer position. Tramier *et al.* were able to verify Axelrod’s formulas experimentally in rhodamine 6G, where they observed a significant decrease of r_0 with increasing NA of the objective lens, while finding the fluorescence lifetime and the rotational correlation time unchanged.¹⁶

FIG. 5. (Color) TR-FAIM results of two wells filled with rhodamine 6G in methanol (top) and ethylene glycol (bottom). (a) Anisotropy $r(t_1)$ 400 ps after the excitation pulse, showing strong anisotropy in ethylene glycol and apparently low anisotropy in the sample in methanol since it has already decayed almost entirely. (b) The map of the rotational correlation time θ in a false color scale from 0 to 10 ns. White pixels represent θ values beyond the color scale, whereas black pixels represent nonconverging fits, caused by the low $r(t_1)$ in methanol. The correlation time scale can be converted directly into a viscosity scale ranging from 0 to 55 cP, assuming the validity of the hydrodynamic model in Eq. (1). (c) The anisotropy decays averaged over the individual wells and extending the fit to $t=0$, thus yielding approximate r_0 values even without deconvolution. For the sample in methanol, r_0 has been set equal to the one obtained in ethylene glycol in order to enable an approximate determination of θ .

C. TR-FAIM maps: Influence of the viscosity on the rotational correlation time

Images of the anisotropy in each pixel of the sample are obtained by calculating the anisotropy, according to Eqs. (7a), (7b), (8), and (10), for each time-gated fluorescence intensity image. Figure 5(a) shows a map of the anisotropy $r(t_1)$ at the first time gate after the excitation pulse, which corresponds approximately to a map of the initial anisotropy r_0 . A mask derived from the total fluorescence image $F(t_1)$ (see Sec. IV D) has been applied to the map, thus limiting it to the region of fluorescence, in order to eliminate the random noise outside the regions of fluorescence where anisotropy is not defined. While rhodamine 6G in ethylene glycol shows significant anisotropy with an average value of $r(t_1) = 0.30$ close to the maximum value $r_0 = 0.4$ for single photon excitation [cf. Eq. (5)], a very small anisotropy of $r(t_1) = 0.015$ can be seen for rhodamine 6G in methanol. For rhodamine 6G in methanol, $\theta \ll \tau$, making it inherently dif-

ficult to resolve these fast correlation times³² (~ 100 ps in methanol at 20 °C).³⁹ A single exponential fit is then applied for each image pixel, yielding the rotational correlation time θ . Figure 5(b) shows the resulting θ map. A relatively homogeneous rotational correlation time distribution around 5 ns is observed for the well filled with rhodamine 6G in ethylene glycol. Overall, much shorter approximate values for θ , although very noisy due to the low value of $r(t_1)$, are obtained in methanol. In both cases, the average value of the fit offset was found to be approximately zero, as expected for freely rotating molecules ($r_\infty = 0$). To reduce the number of fitting parameters and increase the statistical accuracy, we fixed the offset to zero and refitted the data.

The initial motivation of this study was to use anisotropy measurements to image the environment of a fluorophore. Equation (1) enables the direct determination of the viscosity of the solvent if the volume of the fluorophore is known. Porter *et al.* reported a theoretical value for the apparent molecular radius of 0.56 nm for rhodamine 6G, obtained from theoretical rotation times for an oblate rotator with semi-axes of 0.7 and 0.2 nm.³⁹ Using this value, we are able to convert the time scale in the θ map into a viscosity scale, effectively obtaining a η map, as indicated in Fig. 5(b).

Figure 5(c) shows the average anisotropy decay for both wells. The values of r_0 were determined by extrapolating the decays back to the temporal position of the excitation pulse, i.e., 400 ps before the first time-gated image. For rhodamine 6G in ethylene glycol we obtain $r_0 = 0.324$, which is in agreement with the values reported by other authors at similar excitation wavelengths.^{16,40} For θ we obtain 4.92 ns, which is consistent with the value reported by Bauer and Balter.^{41,42} These authors have measured θ for two excitation wavelengths (458 nm and 515 nm) at different temperatures. Whereas the dependence of θ on the excitation wavelength appears to be negligible, they observe a very strong dependence on the temperature via the change in the viscosity of ethylene glycol near room temperature. In particular, they obtain values for θ of 2.4 ns (40 °C), 4.6 ns (20 °C), and 15.2 ns (0 °C). This observation may not only explain the small difference from our result, which has been obtained at a lab temperature of 20 ± 2 °C, but also the often claimed deviation of ethylene glycol from the prediction given in Eq. (1). Several authors have measured rhodamine 6G in ethylene glycol, obtaining widely different results.^{30,31,40,42} The decay curve of the sample in methanol [Fig. 5(c)] is almost within the noise due to $\theta \ll \tau$ and can only be fitted by limiting the number of fitting parameters. Apart from fixing the offset and t_0 , it is also necessary to fix the initial anisotropy r_0 to the same value as we found for ethylene glycol, which is a reasonable assumption since r_0 should be solvent-independent. By doing this, an approximate rotational correlation time value of 0.13 ns is obtained, which is consistent with the values obtained by a variety of authors.^{21,39,43}

We have also performed additional TR-FAIM measurements of rhodamine 6G in other solvents. Figure 6 shows the resulting θ maps; each of them having a relatively high viscous solvent in the bottom well [in increasing order of viscosity: (a) ethylene glycol, (b) trimethylene glycol and (c) glycerol] and methanol as a reference in the top well. The

FIG. 6. (Color) Rotational correlation time maps of two wells filled with rhodamine 6G in different solvents. The rotational correlation time is represented by a color scale from purple (0 ns) to red (20 ns). In all cases the solvent in the top well is methanol, whereas the solvent in the bottom well is of higher viscosity: (a) ethylene glycol, (b) trimethylene glycol, and (c) glycerol. The increase of θ with solvent viscosity is evident and can also be seen in the histogram of the rotational correlation times (d).

histogram of the rotational correlation times for the three viscous solvents is given in (d). From these images and the rotational correlation time histogram, the increase in rotational correlation time with solvent viscosity is evident and thus the rotational mobility of the probe in its environment yields contrast. Figure 7 shows a plot of the rotational cor-

FIG. 7. Viscosity dependence of the rotational correlation time; extracted from TR-FAIM maps of rhodamine 6G in solvents with different viscosity. Also plotted are values obtained by other authors (see Refs. 30, 31, and 42).

relation times determined by us using TR-FAIM and by others using single-point methods as a function of viscosity. The straight line corresponds to the calculated rotational correlation time using Eq. (1) and an apparent radius of 0.56 nm for rhodamine 6G.³⁹ We find our data in excellent agreement with the calculation in the low viscosity regime (0.6–20 cP), which has already been studied systematically by Chuang and Eisenthal.³⁰ Interestingly, their only value that deviates from the calculation is for ethylene glycol, which the authors attribute to solvent–solvent aggregation due to hydrogen bonding. In contrast, our data for this solvent at both excitation wavelengths, and the values reported by Bauer and Balter,⁴² follow the predicted linear behavior. It is only at considerably higher viscosities that we observe a deviation from the model, in reasonable agreement with the data obtained by Rice and Kenney-Wallace using mixtures of ethylene glycol and glycerol.³¹ This deviation from the model is most likely because in this viscosity range the solvent molecules can no longer be considered small compared to rhodamine 6G. Hence, the Debye–Stokes–Einstein hydrodynamic model,¹¹ upon which Eq. (1) is based, no longer applies.

The initial anisotropy r_0 values determined from our measurements agree with those reported previously. Whereas a value close to the theoretical maximum of 0.4 is expected for excitation near the absorption maximum of the molecule (540 nm), a strong decrease in r_0 has been reported by Heiss *et al.*⁴⁰ for much shorter excitation wavelengths, in good agreement with our measurements at 415 and 490 nm. Lower values, and even negative values, of r_0 can occur when the absorption and emission dipoles are not parallel [see Eq. (5)].

D. FLIM maps: Influence of the refractive index on the fluorescence lifetime

An additional output of TR-FAIM is that it directly provides maps of the lifetime of the total fluorescence $F(t)$, unperturbed by the polarization anisotropy and thus equivalent to measurements at the magic angle where the emission polarizer is set to 54.75° . This eliminates bias due to polarization effects, as the perpendicular component $I_\perp(t)$, weighted by $\sin^2 54.75^\circ = 2/3$, is twice as strong as the parallel component $I_\parallel(t)$, weighted by $\cos^2 54.75^\circ = 1/3$, such that the fluorescence decay is $1/3 I_\parallel(t) + 2/3 I_\perp(t)$ [see Eqs. (4a) and (4b)].⁹ Images of the total fluorescence in each pixel of the sample are obtained when calculating $F(t)$ according to Eqs. (7a), (7b), (9), and (10) for each time-gated fluorescence intensity image. A single exponential fit is then applied for each image pixel, yielding the fluorescence lifetime τ unperturbed by rotational depolarization. Figure 9 shows the corresponding FLIM maps of two wells containing rhodamine 6G in methanol (top well) and ethylene glycol (bottom well), as obtained by vertical excitation [Fig. 9(a)] and horizontal excitation [Fig. 9(b)]. Both measurements, as expected, show the same result in terms of lifetime contrast between the two solvents as well as homogeneous lifetime values within each well, yielding average values for vertical excitation of $\tau = 4.17 \pm 0.09$ ns in methanol and $\tau = 3.67 \pm 0.07$ ns in ethylene glycol and for horizontal excitation τ

FIG. 8. Plot of the inverse of the average fluorescence lifetime values vs the square of the refractive index, extracted from FLIM maps of rhodamine 6G in solvents with different refractive index. Also plotted are values obtained by other authors (Refs. 4 and 49). The solid line gives the best fit to the data.

$=4.20 \pm 0.08$ ns in methanol and $\tau = 3.78 \pm 0.06$ ns in ethylene glycol. The error values quoted refer to the standard deviation of the average lifetime within each well.

The reason for the lifetime contrast between rhodamine 6G in different solvents is, in this case, not their differing

FIG. 9. (Color) FLIM maps of two wells with rhodamine 6G in methanol (top) and ethylene glycol (bottom) for (a) vertical excitation and (b) horizontal excitation. (c) The fluorescence decays averaged over the individual well areas of (b).

viscosity. Only in fluorophores whose chemical structure allows nonradiative energy relaxation mechanisms through internal twisting, is the fluorescence lifetime affected by the solvent viscosity, hindering this relaxation mechanism and therefore leading to an increase in fluorescence lifetime with viscosity.⁴⁴ Since we observe a decrease in fluorescence lifetime with a highly viscous solvent such as ethylene glycol, the lifetime contrast in Fig. 9 cannot be caused by the viscosity. In any case, the chemical structure of rhodamine 6G does not allow relaxation through internal twisting. The natural radiative lifetime τ_0 depends on the refractive index of the surrounding medium due to its polarizability. The Strickler–Berg formula relates τ_0 of a fluorophore to its absorption and emission spectra and predicts a dependence on the refractive index.⁴⁵ This also applies for the fluorescence lifetime τ , which has been verified experimentally by single-point fluorescence lifetime measurements of dyes,^{2–4} tryptophan,⁶ and by single-point and FLIM measurements of the green fluorescent protein.^{5,46} This dependence of τ on the refractive index of its environment is a fundamental behavior that applies to all fluorophores, irrespective of their structure or their environment.¹ The reason why this dependence is often overlooked is that spectral shifts may mask it and different quenching effects frequently dominate, in particular interaction with other fluorophores. Although the exact relation between τ and n can be rather complex as has been demonstrated by Shibuya⁴⁷ and, recently, by Topygin *et al.*,⁶ an approximation is given by $\tau^{-1} \sim n^2$. In Fig. 8, we have plotted the inverse of the average τ values as a function of the square of the refractive index, including values obtained by other authors in single-point measurements. The good agreement between previous results and ours confirms that our FLIM system in TR-FAIM configuration is capable of performing simultaneously precise imaging measurements of the fluorescence lifetime in addition to the polarization anisotropy measurements discussed above. The observed linear behavior confirms the validity of our approximation and opens the way to quantify the refractive index of the environment by means of the fluorescence lifetime.

The combined FLIM and TR-FAIM technique presented here has been shown to yield simultaneously 2D maps of the rotational correlation time θ and the fluorescence lifetime τ of the sample. The particular advantage of our approach is the fact that the images of the perpendicular and parallel components are acquired simultaneously in a wide-field imaging modality. This makes our technique considerably less vulnerable to instrumental drifts, photobleaching, and movement of the sample during acquisition. Introducing a position- and time-dependent, rather than constant, G factor, we are able to correct for spatially dependent depolarization due to the objective lens and spatially dependent sensitivity of different detector pixels. In our study of the fluorescence decay dynamics of the dye rhodamine 6G we have been able to identify, separate, and quantify the dependence of the rotational correlation time on the viscosity, as well as the dependence of the fluorescence lifetime on the refractive index of the solvent. There is excellent agreement between the results reported by other authors using single-point measurements and our TR-FAIM system. Parameter constraints en-

FIG. 10. The polarization of the fluorescence emission for different setups. (a) The geometry of an L-format setup for horizontal and vertical excitation. Note that for horizontal polarization of the excitation beam, the emission polarization is perpendicular for both horizontal and vertical emission polarizer settings, which allows the G factor to be determined as the ratio of both measured intensities (see Refs. 9 and 10). (b) The geometry for a collinear setup where the rotation of the polarization of the excitation also results in a rotation of the polarization of the emission. The determination of the G factor is therefore different.

able estimations of the rotational correlation time down to almost 100 ps.

Relying on parallel pixel acquisition of both polarization components, the limiting speed factor of our system is currently the data processing of the acquired images. Nevertheless, the spatial overlapping of the two polarization images can now be done in real-time during acquisition using software (Mélange™). In addition, a significant increase in speed can be achieved by visual inspection of the video rate fluorescence intensity image (being directly related to r_0), and the rapidly calculated polarization-sensitive FLIM maps (being directly related to θ and τ). It would be beneficial to use this fast FLIM mode for guidance, which can provide FLIM maps with an update time of between a few seconds and up to 1 min, and use the detailed analysis of the TR-FAIM mode only after having found the region of interest.

In addition to the inherent difficulty of resolving correlation times that are either much longer or much shorter than the fluorescent lifetime, the relatively wide gate width of our detection system (400 ps) has the effect that the fluorescence anisotropy of rhodamine 6G in methanol has almost completely decayed within the first time gate. This limited temporal resolution could, in principle, be increased significantly by means of deconvolution of the decay with the instrumental response. This would enable the resolution of the fast rotational correlation times found in nonviscous liquids much better than currently achieved and we hope to incorporate deconvolution into our data analysis soon. The extension of the work presented here to the imaging of entire multiwell plates⁴⁸ or to microscopy at high magnifications is straightforward. We believe that the TR-FAIM technique we present in this work will have a dramatic impact on studies of biologically relevant samples, enabling, for instance, the imaging of local microviscosity, binding events, and refractive index distributions in live cells expressing fluorophores such as green fluorescent protein.

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APPENDIX

In order to calculate the G factor to account for different transmission and detection efficiencies for the parallel and perpendicular polarization, the collinear geometry of our set up must be taken into account, as schematically depicted in Fig. 10. The intensity of the parallel component is given by

$$I_{\parallel}(t) = \frac{I_{HH}(t)}{kS_H} = \frac{I_{VV}(t)}{kS_V}, \quad (\text{A1})$$

where k is a proportionality factor, and S_H and S_V the sensitivities of the horizontal and vertical detection channels. The intensity of the perpendicular component is given by

$$I_{\perp}(t) = \frac{I_{VH}(t)}{kS_H} = \frac{I_{HV}(t)}{kS_V}. \quad (\text{A2})$$

For vertical excitation, Eqs. (A1) and (A2) yield the following expression:

$$\frac{I_{\parallel}(t)}{I_{\perp}(t)} = \frac{I_{VV}(t)S_H}{I_{VH}(t)S_V} = \frac{I_{VV}(t)}{I_{VH}(t)} G^{-1}, \quad (\text{A3})$$

where $G = S_V/S_H$. For horizontal excitation, we obtain this expression

$$\frac{I_{\parallel}(t)}{I_{\perp}(t)} = \frac{I_{HH}(t)S_V}{I_{HV}(t)S_H} = \frac{I_{HH}(t)}{I_{HV}(t)} G. \quad (\text{A4})$$

Hence, the G factor correction as applied to the time-resolved anisotropy equation Eq. (8) is different for the two polarization orientations of the excitation beam. For vertical excitation polarization

$$r_V(t) = \frac{I_{VV}(t) - GI_{VH}(t)}{I_{VV}(t) + 2GI_{VH}(t)}, \quad (\text{A5})$$

whereas for the horizontal excitation

$$r_H(t) = \frac{I_{HH}(t) - G^{-1}I_{HV}(t)}{I_{HH}(t) + 2G^{-1}I_{HV}(t)}. \quad (\text{A6})$$

As this point, we make the assumption that the anisotropy is a property of the sample, irrespective of the instrumental setup used to measure it. Thus, if the polarization of the excitation is rotated by 90°, the time-resolved fluorescence anisotropy must remain the same, i.e.,

$$r_V(t) = r_H(t). \quad (\text{A7})$$

Any differences must be due to the G factor, which accounts for the transmission and detection efficiencies of the optics for vertical and horizontally polarized light for every image pixel ($G(x,y)$). Although this derivation yields a time-independent G factor, we experimentally find it to be time dependent. This may be caused by different background lev-

els in the two subimages or by misalignment of the polarizer. A more comprehensive treatment would be required to explicitly derive this dependence. By allowing the G factor to also be position dependent, it accounts for spatially dependent depolarization and detector sensitivity. Rearrangement of Eqs. (A5) and (A6) yields the explicit expression for $G(x,y,t)$ of a collinear excitation and fluorescence emission setup [Eq. (10)].

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