Nonlinear Optical Chromophores as Nanoscale Emitters for Single-Molecule Spectroscopy

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ABSTRACT

Fluorescence imaging of single molecules at room temperature is a powerful technique for studying complex condensed phase systems and revealing structure and dynamics hidden by ensemble measurements. Successful single-molecule spectroscopic experiments rely upon strong emitters that can be detected at the level of individual copies above the relevant background signals. This Account discusses a class of nonlinear optical chromophores that not only are well-suited for single-molecule imaging but also offer additional beneficial properties such as a significant ground-state dipole moment, moderate hyperpolarizability, and sensitivity to local environment. An overview of the photophysical properties of several members of this class of molecules as well as a mechanism to help understand the environmental sensitivity is presented. Some preliminary applications of the chromophores as single-molecule reporters in cellular and polymer systems are discussed, along with detection of the emitters by two-photon fluorescence.

I. Introduction

For more than a decade, scientists around the world have been performing optical spectroscopy and microscopy on single reporter molecules in liquid, polymeric, crystalline, or biological environments.[1−14] Single-molecule fluorescence experiments require an optically transparent host containing exactly one highly fluorescent molecule of interest in the probed volume. Such studies have provided insight into a variety of complex condensed-phase phenomena, revealing processes and dynamics that are obscured by typical ensemble measurements. Because a typical single fluorophore is only a few nanometers in size, it is able in many cases to sense its local environment on a very small scale; in particular, the molecule can report on its immediate environment through changes in its fluorescence properties such as intensity, polarization, lifetime, or wavelength.[15−18]

Single-molecule fluorescence has been a valuable tool for experiments ranging from biophysics to polymer science. For example, single-molecule techniques have led to a better understanding of the mechanism by which molecular motors such as kinesin achieve motility along microtubules.[18−20] By labeling an individual kinesin motor with a fluorescent rhodamine dye and following the orientation of the probe, researchers correlated the motion of the motor with the presence of various nucleotides. Single-molecule imaging has also been used to study the diffusion of membrane-associated class II major histocompatibility complex (MHC) proteins in Chinese hamster ovary (CHO) cells.[21] Using a protocol that labels an individual MHC protein with a single Cy5 fluorophore, the authors followed single proteins as they diffused in the cell membrane and analyzed the motion in detail. Individual fluorescent molecules have also been used to study dynamics in polymer hosts, reporting on the fluctuations in the local environment. Deschenes and van den Bout[22] used polarization microscopy to study the rotation of single Rhodamine 6G dopant molecules in a poly(methacrylate) film and found that each molecule sampled several rotational time scales over the course of the measurement, switching rapidly from one to the next. Bartko and Dickson[23] have used advanced optical imaging methods to follow three-dimensional orientations of single molecules in polymers. Bowden et al. have studied butadiene polymers with single fluorescent perylene dyes covalently attached at the center of the polymer chain and followed their rotational motion in a poly(methyl methacrylate) host.[24] The variety of behaviors observed in the

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polymer hosts reflects the underlying heterogeneity intrinsic to these systems.

Each of the experiments cited above (and many others too numerous to cite here) relies upon a highly fluorescent reporter molecule that can provide information about its environment. For successful detection of a single copy above background signals, the fluorescent reporter must possess a combination of properties: strong absorption, high fluorescence quantum yield, weak bottlenecks into dark states (such as triplets), and high photostability. Fluorophores that not only meet these stringent demands for single-molecule studies but also offer additional, more specialized properties would be of particular interest. For example, fluorophores that are sensitive to their local environment or offer the synthetic flexibility to tune specific reporter properties will have widespread applications in both biological and polymer experiments. Thus, the development of new fluorescent dyes for use in single-molecule imaging is an important challenge due to the numerous problems in biomolecular and materials science that benefit from insight at the level of individual molecules.

Recent work in the Moerner laboratory has focused on a new class of fluorophores that meet the strict demands for single-molecule imaging and offer additional interesting properties such as a large ground-state dipole moment (μ_g), moderate hyperpolarizability (β), and sensitivity to the rigidity of the local environment.[25] The molecules within this class all contain an amine donor and a dicyanodihydrofuran (DCDHF) acceptor linked by a conjugated unit (benzene, thiophene, styrene, etc.). These molecules are named the DCDHF fluorophores after the acceptor unit[26] (for examples, see Figure 2). The DCDHF molecules are examples of nonlinear optical chromophores possessing large second-order nonlinearity by virtue of the asymmetric donor−acceptor structure and high degree of conjugation.[27−30] Nonlinear optical chromophores in polymers have received much attention from the chemical community in recent decades owing to the utility of such materials for second-harmonic generation, electrooptic phase modulation, and many other potential applications.[31−34] Originally designed to deliver high polarizability anisotropy and μ_g for photorefractive polymer applications,[35−37] the DCDHF fluorophores are also surprisingly well-suited for single-molecule fluorescence applications. This Account will provide an overview of the photophysical properties of several members of the DCDHF class as well as a proposed mechanism to understand the environmental sensitivity of the dyes. Some preliminary applications of the DCDHFs as single-molecule reporters will also be discussed, along with detection of the molecules by two-photon excited fluorescence.

II. DCDHF Characteristics and Photophysics

To probe a single molecule, a light beam (typically a laser) is used to pump a strongly allowed optical transition of the molecule of interest, and this absorption is sensed through the detection of fluorescence emission. Guaranteeing that only one molecule of interest is in resonance with the excitation light is generally achieved by a combination of dilution and tightly focused excitation as illustrated in Figure 1. In the left side of the figure, the schematic drawing shows only one reporter molecule in the focal volume of the excitation light, resulting in fluorescence emission only from this individual molecule. Visualizing the emission from a single molecule can be accomplished using several different experimental techniques including confocal microscopy, wide-field epi-illumination, total-internal-reflection (TIR), and near-field optical scanning microscopy (NSOM); these techniques have been described in detail elsewhere.[38] The right side of Figure 1 shows individual DCDHF-6 molecules (structure in the inset) in a poly(methyl methacrylate) (PMMA) film, imaged with wide-field epi-illumination fluorescence microscopy. This image shows the high signal-to-noise and signal-to-background ratios that are possible using a good single-molecule fluorophore in an optically clear polymer film.

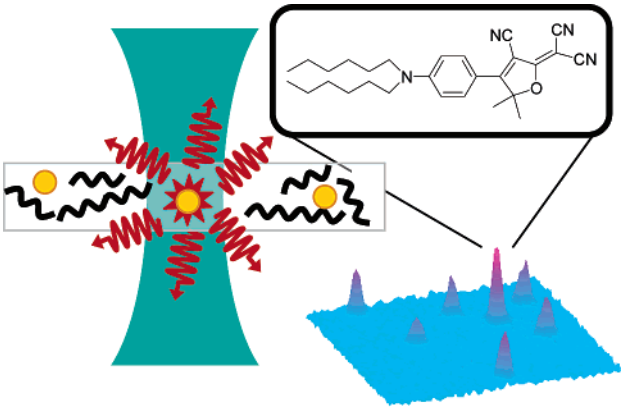

FIGURE 1. Schematic (left) of a focused optical beam pumping a resonant single molecule in a polymer film and image (right) of emission from single DCDHF-6 molecules in a PMMA film optically excited by a 488 nm laser beam. The area shown is a 4.8 μm × 4.8 μm region of the sample.

The generic structure of a DCDHF fluorophore is shown in Figure 2, along with seven representative molecules from the class. There are multiple sites for synthetic modification, and choosing different substituents at sites R_1−R_4 as well as the conjugated linker or aromatic (Ar) between the amine and dihydrofuran will tune the properties of the molecules. For example, Figure 3 shows the absorption and emission spectra of molecules **1**−**6** in toluene. These spectra span the visible region with absorption maxima ranging from 486 to 614 nm, and the spectral shifts are the result of well-known variations in the charge-transfer character of the absorption determined by the strength of the donor and acceptor and the length of the conjugated network. The bluest absorbing variants of the DCDHF class (**1** and **2**) have a phenyl ring connecting the amine and dihydrofuran; replacing this with either a thiophene (**4**), styryl (**5**), or thiophene-vinyl (**6**) shifts the spectra progressively toward the red. The substitution of the inductively withdrawing trifluoromethyl group for a methyl group on the dihydrofuran ring (**3**) also

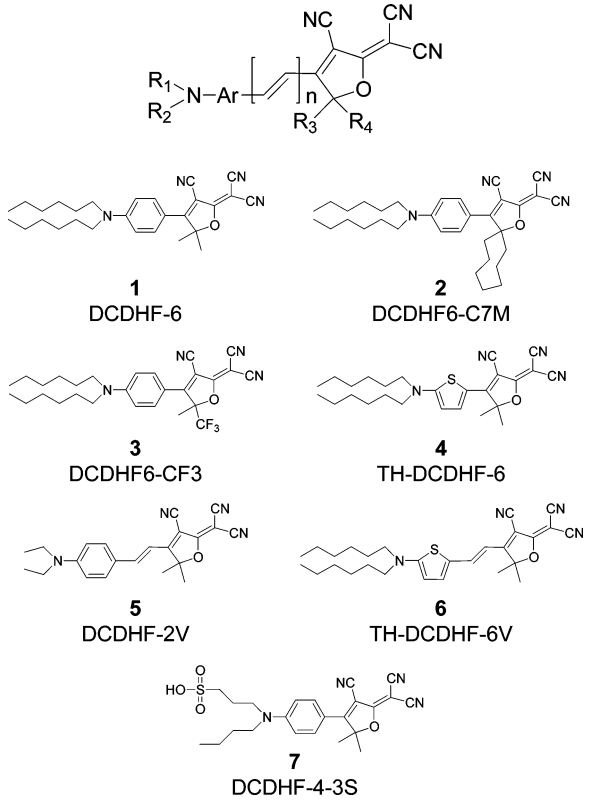

FIGURE 2. Generic structure of a DCDHF molecule (top), containing an amine donor linked through a conjugated unit to a dicyanodihydrofuran acceptor. Structures and informal names of seven members of the DCDHF class of molecules are shown in the lower part of the figure.

red-shifts the absorption and emission. Moreover, functional groups can be added to modify other physical properties including the solubility of the molecules. The sulfonic acid group on the amine tail of molecule **7** produces water solubility, while the spectral properties remain similar to those of the non-water-soluble analogue **1**.

Table 1 summarizes the spectral properties and photophysical measurements of molecules **1**−**6** in toluene.[25,39] The values for Rhodamine 6G, a common fluorophore used in single-molecule imaging, are also included in the table for comparison.[40] The measured molar extinction coefficients compare quite favorably with the rhodamine dye. However, the fluorescence quantum yields (Φ_F) of the DCDHFs in toluene, which reflect the probability that the absorption of a photon will result in the subsequent emission of a fluorescent photon, are moderate and well below the 95% value for Rhodamine 6G. In our early studies of these molecules, this was a surprising result considering the strong single-molecule fluorescence observed for the dyes in polymer films (as in Figure 1).

To resolve this issue, the Φ_F values for DCDHFs doped into spin-coated polymer films were carefully determined. The resulting values are reported in parentheses in Table 1 and are significantly enhanced in the polymer matrix over solution. Further studies in a variety of solvents revealed that the fluorescence quantum yields of the DCDHF dyes are strongly dependent on the identity of

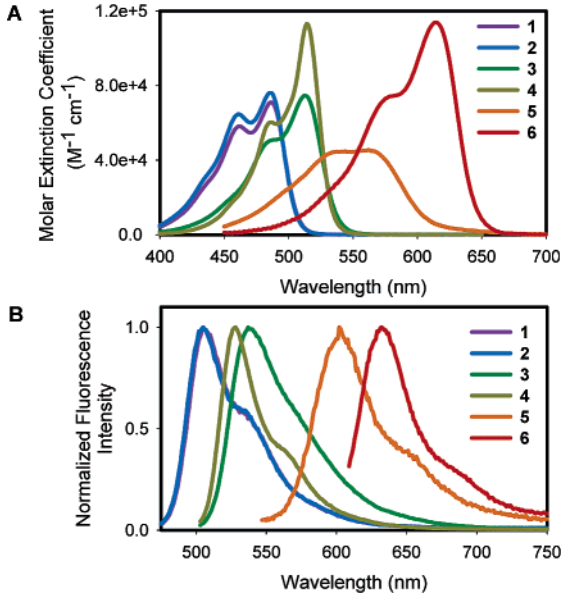

FIGURE 3. Absorption (A, in units of molar extinction coefficient) and emission (B) spectra of six DCDHF fluorophores in toluene. Compounds **1**−**2** were excited at 460 nm, **3**−**4** at 488 nm, **5** at 532 nm, and **6** at 594 nm.

Table 1. Photophysical Parameters the DCDHF Fluorophores

	λ_{abs}^{max} (nm)	λ_{em}^{max} (nm)	ϵ_{max} (M^{-1} cm^{-1})	Φ_F[a]	Φ_B[b] (10^{-7})	N_{tot}[c] (10^4)	μ_g (10^{-30} C m)
1	486	505	71 000	0.10 (0.92)	8.9	25	38
2	486	505	76 000	0.10 (0.95)	7.5	23	36
3	512	537	75 000	0.17 (0.71)	8.3	30	40
4	514	528	100 000	0.11 (0.73)	14	9.1	31
5	562	603	45 500	0.02 (0.39)	12	8.7	35
6	614	646	114 000	0.02	71	2.8	37
R[d]	530	556	105 000	0.95	5	$190\eta_c$[e]	*f*

[a] Values on the left are for dyes in toluene; the following reference dyes and excitation wavelengths were used: **1** and **2**, fluorescein in water, 460 nm; **3** and **4**, R6G in ethanol, 488 nm; **5**, Texas Red in ethanol, 532 nm; **6**, Cy5 in water, 594 nm. Values in parentheses are for dyes in a solid PMMA matrix referenced against KF241, a perylene derivative, in PMMA ($\Phi_F = 0.95$) and pumped at 460 nm for **1** and **2** and 488 nm for **3**−**5**. [b] **1**−**4** were measured at 488 nm, **5** and **6** at 594 nm. [c] **1**−**5** were measured at 488 nm; **6** was measured at 594 nm. [d] **R** = R6G in ethanol.[40] [e] Collection efficiency, η_c, was not reported in ref 40. [f] Cation.

their environment.[41] Results demonstrating the effect of the environment on the strength of fluorescence as well as the possible origin of this effect will be discussed below.

The photobleaching quantum yield (Φ_B), which reflects the photostability of the molecule, was determined for bulk DCDHF samples in PMMA films. A representative normalized bleaching curve for **1** at 0.5 kW cm^{-2} is shown in the inset of Figure 4A and demonstrates how the fluorescence intensity of the bulk sample decays over time. To fit the bleaching curves, a double exponential decay function plus an offset was required for all samples and excitation intensities. With the use of the initial slope of the fits (i.e., at $t = 0$), the (initial) bleaching rates as a function of excitation rate were calculated, and the results for **1** are shown in the body of Figure 4A. The photobleaching quantum yield was determined from the slope

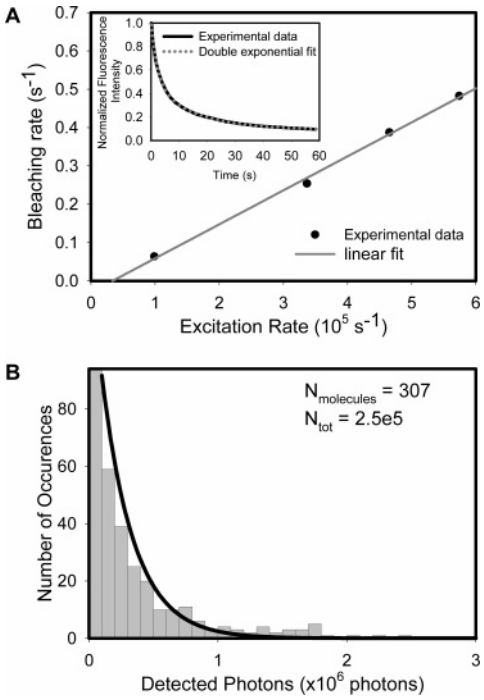

FIGURE 4. Panel A shows the ensemble bleaching rate of **1** in PMMA as a function of the excitation rate as measured with epi-illumination. A linear fit to the data is also shown. The inset shows the normalized ensemble bleaching curve of **1** in PMMA at 0.5 kW cm^{-2}. Experimental data (black line) is fit to a double exponential decay with offset (gray dashed line). Panel B shows a histogram of the total number of detected photons from 307 individual molecules of **1** in PMMA. N_{tot} is the exponential mean of a single-exponential fit (solid line).

of the best-fit line relating the bleaching rate to the excitation rate. The Φ_B values reported in Table 1 are quite small, particularly for **1**−**3**, and are easily comparable to the value for Rhodamine 6G.

Single-molecule concentration samples of the DCDHFs in PMMA were used and the number of (detected) photons emitted by each individual molecule before photobleaching were counted to determine the total number of (detected) photons (N_{tot}). A histogram of the number of detected photons for at least 100 molecules was constructed and then fit to a single-exponential decay function as shown in Figure 4B for **1**. The exponential decay parameters of the fitting functions are reported as N_{tot} in Table 1 for each of the DCDHFs. These values reflect only the number of detected photons and do not include losses due to filters and microscope optics. Dividing by these loss factors brings the total number of emitted photons into better agreement with the inverse of Φ_B as expected. For example, for **1**, about 50% of the emission is collected through the microscope objective[38] and 41% is collected through the filters, yielding a total number of emitted photons of $\sim 1.2 \times 10^6$, a value that compares quite favorably to other single-molecule fluorophores, such as Rhodamine 6G as reported in the table. Moreover, the DCDHFs demonstrate impressive photostability in PMMA, with no blinking observed in 85% of the single molecules on the 100 ms time scale of the measurement.

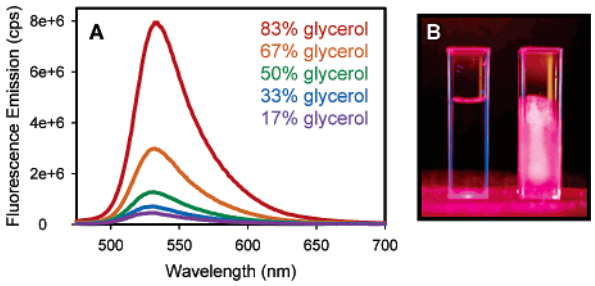

FIGURE 5. Fluorescence emission (A) of **1** in glycerol/methanol solutions of increasing viscosity, λ_{exc} = 460 nm (reprinted from ref 41 with permission. Copyright 2004 American Chemical Society) and (B) samples of **7** in water (left) and ice (right) illuminated with a UV lamp recorded with a conventional color camera.

III. Environmental Sensitivity

The DCDHF molecules can report environmental effects through their fluorescence intensity as mentioned above. Stimulated by the near order-of-magnitude improvement in quantum yield in the PMMA film compared to toluene solution, a relationship between fluorescence quantum yield and local viscosity or environmental rigidity was proposed.[25] Figure 5 provides two demonstrations of this relationship. In Figure 5A, DCDHF-6 was added to solutions of glycerol/methanol to determine the effect of viscosity on the bulk fluorescence. As the figure shows, the fluorescence emission increases as the concentration of glycerol—and thus the viscosity of the solution—increases. Figure 5B demonstrates the effect of local rigidity on DCDHF fluorescence. Two identical solutions of **7** in water were prepared, and then one was frozen while the other remained at room temperature. Under illumination with a UV lamp, the liquid sample (left) shows limited fluorescence while the frozen sample (right) is strongly emissive. Both experiments suggest that more rigid or viscous environments enhance the fluorescence quantum yield of the DCDHF dyes.

Because the property of environmentally sensitive fluorescence has considerable potential for use in single-molecule experiments, it is interesting to consider the origin of the effect. To address this, electronic structure calculations were performed on a DCDHF derivative analogous to **1** except a dimethylamine replaces the dihexylamine donor group.[41] Hypothesizing that an intramolecular twist was responsible for the environmentally sensitive fluorescence, we examined the effect of three specific intramolecular rotations on the ground and excited-state energies of the molecule: the amine−phenyl twist (α), the phenyl−dihydrofuran twist (ϕ), and the dicyano−dihydrofuran twist (δ) defined in Figure 6A.

Using quantum chemistry methods described elsewhere,[41,42] we calculated optimized geometries of the ground and excited states. The minimum energy ground-state structure, denoted Γ_0, is shown in Figure 6A and has planar amine−phenyl and dicyano−dihydrofuran groups but a twisted phenyl−dihydrofuran bond. Excited-state geometry optimizations revealed two local energetic minima in the first excited state, one in which the phenyl−dihydrofuran bond becomes more planar and the other

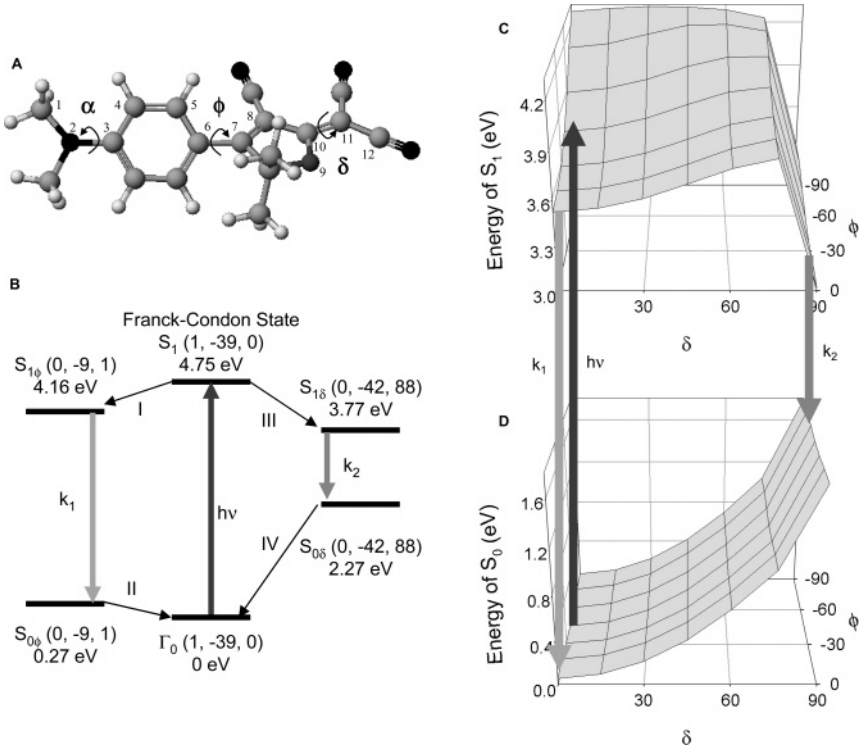

FIGURE 6. Panel A shows the structure of DCDHF-1 with intramolecular twists around the amine−phenyl, phenyl−dihydrofuran, and dicyano−dihydrofuran bonds labeled as α, ϕ, and δ, respectively. The molecule is shown in the Γ_0 configuration where $\alpha = 1°$, $\phi = -39°$, and $\delta = 0°$. Panel B shows the proposed photophysical mechanism based on the various energy levels involved in the DCDHF-1 electronic structure. Two first excited-state minima exist for this molecule (see text), allowing two different paths for relaxation. Panels C and D show potential energy surfaces of the first excited state (C) and ground state (D) of DCDHF-1 as a function of both the phenyl−dihydrofuran and dicyano−dihydrofuran dihedral angles. Arrows corresponding to $h\nu$, k_1, and k_2 from panel B are also included. Reprinted from ref 41 with permission. Copyright 2004 American Chemical Society.

in which the dicyanomethylene twists 90° with respect to the dihydrofuran ring. The two states are named $S_{1\phi}$ and $S_{1\delta}$, respectively. Changes in the amine−phenyl angle produced no energetic minima.

From these results, the mechanism shown in Figure 6B was proposed to describe the spectroscopic behavior.[41] The molecule is initially optically excited from the Γ_0 state into the Franck−Condon state, from which there are two possible relaxation pathways. In the first, the phenyl−dihydrofuran bond twists (path I) until it reaches the $S_{1\phi}$ geometry; from there, the molecule relaxes back to the ground state ($S_{0\phi}$) with rate constant k_1. The second relaxation pathway involves twisting the dicyano−dihydrofuran bond to reach the $S_{1\delta}$ state (path III) followed by relaxation to the ground state ($S_{0\delta}$) with rate k_2. Once in the ground state, the molecule can return to the Γ_0 geometry. This mechanism was developed further by calculating potential energy surfaces as a function of both ϕ and δ for the ground and first excited states as shown in Figure 6 panels D and C, respectively.[41] In these calculations, an activation barrier from the Franck−Condon state to the $S_{1\delta}$ state (path III) appears. Additional calculations reveal that this energy barrier changes in the presence of a simulated solvent, suggesting that the accessibility of this state may change in different environments.[41]

Although the calculated energy difference between $S_{1\phi}$ and $S_{0\phi}$ is larger than the experimental emission energy, this overestimate is not unexpected at this level of theory,[42] and we postulate that this transition is radiative. We also propose that the transition between $S_{1\delta}$ and $S_{0\delta}$ is nonradiative to remain consistent with emission spectra as well as experimentally measured differences in the ground- and excited-state dipole moments.[41] The presence of both radiative and nonradiative excited state minima is compatible with our experimentally observed environmentally sensitive fluorescence quantum yield. Because the twist leading to nonradiative relaxation has a solvent-dependent energy barrier, the DCDHF fluorescence quantum yield varies with local environment as the $S_{1\delta}$ state becomes more or less accessible. Thus, if the solvent favors the nonradiative $S_{1\delta}$ state, the fluorescence quantum yield of the molecule will be quite low; conversely, if the rotation of the dicyanomethylene group is slowed or prevented in a particular environment, the quantum yield will be much higher. In all cases, these proposals based on simulations are useful and should be explored in future experimental and theoretical studies of these fluorophores.

IV. Potential Applications

Lifetime Measurements. The reporter properties of the DCDHF fluorophores can be exploited in single-molecule experiments to reveal inherent differences in local environments or to monitor time-dependent changes in the host. Because the fluorescence quantum yield is related

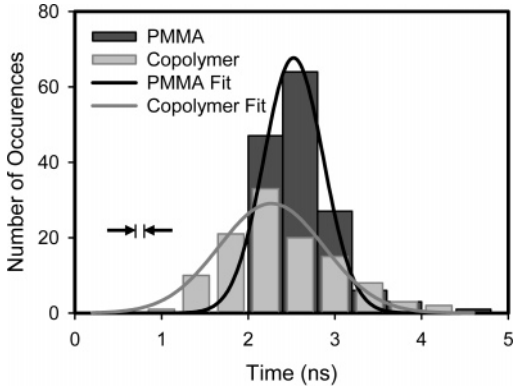

FIGURE 7. Histogram of single-molecule fluorescence lifetimes of DCDHF-6 in the butyl methacrylate/isobutyl methacrylate copolymer (light gray) and in PMMA (dark gray). For both data sets, 400 ps bins are used. Data are fit to Gaussian distributions. The error of individual determinations of lifetime is shown with the arrows. Reprinted from ref 41 with permission. Copyright 2004 American Chemical Society.

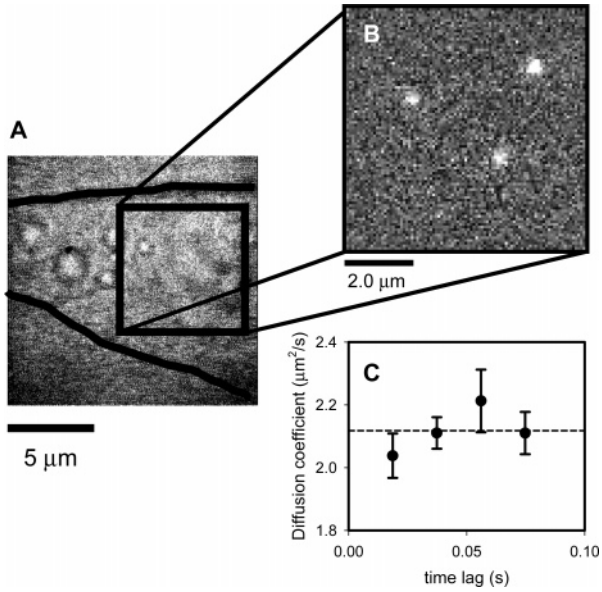

FIGURE 8. White-light transmission image (A) of a CHO cell showing the sketched cell edges and the region of interest, epifluorescence image (B) of single molecules of **3** in the upper membrane of the cell excited at 532 nm with an 18 ms integration time per frame, and apparent diffusion coefficients (C) plotted as a function of time lag for **3** in the cell membrane. The dashed line represents the mean value of the diffusion coefficient, $D = 2.1\ \mu m^2\ s^{-1}$. For experimental methods, see ref 21.

to the excited-state lifetime (τ_F) of a molecule ($\Phi_F = \tau_F/\tau_{rad}$ with τ_{rad} the radiative lifetime),[43] the τ_F value should also provide a sensitive measure of the local environment. Using conventional time-correlated single-photon counting (TCSPC) methods to record delay times between excitation by a pulsed laser source and emission from a single molecule,[43] the excited-state lifetimes of the DCDHF-6 molecules in different hosts can be measured. In toluene, where the measured quantum yield was quite low, the bulk excited-state lifetime was limited by the response time of the instrument (<400 ps); on the other hand, in PMMA where the quantum yield was near unity, the lifetime was several nanoseconds.[39,41]

Bulk studies of DCDHF-6 in different methacrylate polymer hosts revealed that the average excited-state lifetime of the molecule increased with increasing glass transition temperature (T_g) of the host. However, these studies do not directly yield insight into the overall heterogeneity of the environment, since only the average value of the lifetime is measured. For single-molecule experiments, dilute samples of DCDHF-6 were prepared in PMMA ($T_g \approx 120$ °C) and a (*n*-butyl methacrylate)/(isobutyl methacrylate) copolymer ($T_g \approx 35$ °C). Figure 7 shows histograms of the distribution of single-molecule lifetimes in the two different hosts, as well as fits to a Gaussian function. Consistent with the bulk results, the average lifetime in the copolymer is shorter than that in the PMMA. The widths of both distributions, however, are greater than the statistically expected measurement error of 100 ps, which can be attributed to the microscopic heterogeneity of the polymer films. The distribution of lifetimes is broader in the copolymer host, suggesting that a greater variety of microscopic local environments exist in this matrix. Recent work has shown that the lifetime of a twisted perylene derivative reports on the free volume of its host polymer matrix;[44] this idea may also apply to the DCDHF results. The local free volume of the copolymer is expected to be higher than that of the PMMA,[44] and thus our results suggest that the free volume is not only larger but also more broadly distributed in the copolymer. This example shows that these dyes may prove useful for sensing dynamic changes in local environment, simply by monitoring changes in the excited state lifetime of an individual molecule as its host environment is perturbed. Such measurements are useful not only for studying polymers but for any situation in which dynamic changes in environment are expected.

Biological Imaging. Many types of dynamic environments exist in biological systems, where a wealth of time-dependent processes and heterogeneous environments can be found. While synthetic modifications to optimize interaction of the DCDHF dyes with specific biological targets are underway, the fluorophores in their current form have already proven useful in the study of cellular membranes. As a result of the polar dicyanodihydrofuran headgroup and the nonpolar hydrocarbon tails on the amine, the DCDHF dyes can act as highly fluorescent lipid analogues. Using egg phosphatidylcholine vesicles to deliver the fluorophores to a cell membrane,[45] we have successfully inserted single-molecule concentrations of DCDHF probes into the plasma membrane of Chinese hamster ovary (CHO) cells. To illustrate, part A of Figure 8 shows a white-light transmission image of a CHO cell (typical dimensions $\sim 10\ \mu m \times 25\ \mu m$ in the image plane and $\sim 5\ \mu m$ thick). Figure 8B shows a wide-field fluorescence image of single molecules of **3** in the upper membrane of this CHO cell. Each molecule is rapidly diffusing in the membrane, and by following its position frame by frame and calculating its mean-squared-displacement as a function of time lag, one can calculate an average diffusion coefficient for the DCDHF molecules in

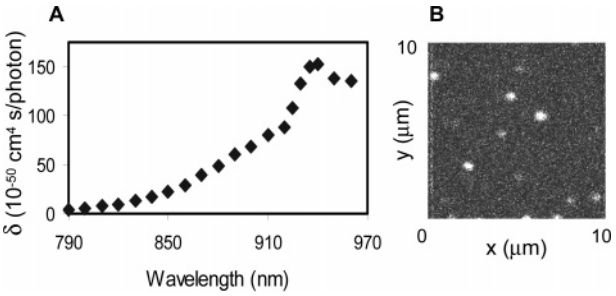

FIGURE 9. Two-photon absorption cross-section, δ, vs wavelength (A) for DCDHF-6 in PMMA and single-molecule two-photon fluorescence image (B) of DCDHF-6 in PMMA acquired with confocal microscopy. An average incident power of 157 μW at $\lambda = 930$ nm, linearly polarized, was used for excitation.

the membrane,[21] as shown in Figure 8C. The observed diffusion coefficient of 2.1 $\mu m^2\ s^{-1}$ is consistent with measured diffusion coefficients of other fluorescent lipid analogues such as Cy5-labeled DOPE,[45] which suggests that the DCDHF molecules are integrated into the lipid bilayer. The compatibility of the DCDHF dyes with cells as well as the synthetic flexibility to add reactive functional groups for site-specific labeling should allow the DCDHF fluorophores to be useful for a variety of in vitro and in vivo biological labeling experiments.

Two-Photon Fluorescence. One of the challenges of labeling live cells with single-molecule reporters is the large autofluorescence background, which can overwhelm the relatively small fluorescence signal from single-molecule labels. One solution to this problem involves long-wavelength emitters, and the DCDHF molecules allow for this possibility. Alternatively, many researchers have turned to two-photon fluorescence to avoid the naturally occurring background.[46,47] In these experiments, two (typically infrared) photons of frequency ω are used to excite the molecule rather than a single photon with the optically resonant frequency 2ω. While this reduces optical excitation of the autofluorescent species in the cell, two-photon absorption is a low probability event, typically requiring excitation with ~100 fs laser pulses. For example, the two-photon absorption cross section of rhodamine 6G in methanol, considered a fairly strong dye for two-photon applications, is 134 GM (where 1 GM = 10^{-50} cm^4 s $photon^{-1}$).[48] The optical nonlinearity of the DCDHF dyes suggests that they too may be useful two-photon emitters, and our recent measurements find two-photon cross sections reaching more than 150 GM for DCDHF-6.[49] Figure 9A shows the DCDHF-6 two-photon absorption cross section in PMMA as a function of pump wavelength. The spectrum peaks at 945 nm and approximately mirrors the shape of the single-photon absorption spectrum with double the wavelength. As with many two-photon dyes,[48,49] the spectrum is blue-shifted by more than 40 nm relative to the expectation from single-photon absorption. In Figure 9B, a single-molecule sample of DCDHF-6 in PMMA is pumped with 930 nm pulses from a mode-locked Ti:sapphire laser, and fluorescence from individual molecules is easily observed. The ability to observe single molecules of DCDHF-6 using two-photon fluorescence expands the range of these dyes, suggesting that they will be useful in applications where autofluorescence provides excessive background.

V. Conclusions and Future Outlook

Due to their favorable photophysical characteristics, as well as their unique properties, the DCDHF fluorophores are a valuable addition to the current library of single-molecule fluorophores. With the synthetic flexibility of the dye, a number of derivatives are available with absorption and emission spanning the visible range. The dyes meet the strict requirements for single-molecule imaging, offering low photobleaching quantum yields, a high total number of photons, and large molar extinction coefficients. Moreover, the DCDHF dyes also offer several additional properties that may prove beneficial for new and exciting single-molecule experiments. The dependence of the fluorescence quantum yield, and thus the excited-state lifetime, on the local environment is one such property and makes these dyes useful as probes of local environments. To explain this environmental sensitivity, a mechanism in which an intramolecular twist leads to nonradiative relaxation from the excited state has been proposed. This mechanism suggests that synthetic modifications to the DCDHF molecules can be made without sacrificing the environmental sensitivity; similarly, introducing bulky groups that limit intramolecular rotation might reduce this environmental sensitivity. The DCDHF molecules also possess a large ground-state electric dipole moment (Table 1) but are uncharged and thus do not require a counterion. Further, these dyes have a moderate hyperpolarizibility (β) that may lead to interesting nonlinear optical phenomena.[33]

Single-molecule experiments in both biological and polymer environments have been successfully performed using various DCDHF derivatives. The fluorophores have been used in polymer hosts to report on local free volume through differences in the excited-state lifetime. The dyes have also been incorporated into the membranes of live cells for use as lipid analogues, and single copies can be observed diffusing in the cell membrane. Two-photon fluorescence has been observed for single DCDHF fluorophores.

In future studies, the DCDHF fluorophores can be considered for situations where the molecule is specifically attached to a biomolecule or polymer or binds strongly to a biomolecule. When the molecule is located in a relatively rigid environment, the emission would be much brighter compared to the case where the molecule is unbound or is in a flexible local environment. Another class of studies can take advantage of the ground-state dipole moment; in particular, one would expect to be able to turn the molecule using an external electric field or a strong, time-varying local electric field. Finally, the optical nonlinearity of the molecule is a third local reporter variable that is waiting to be explored.

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