FLUOROPHORES FOR SINGLE-MOLECULE IMAGING IN LIVING CELLS:
CHARACTERIZING AND OPTIMIZING DCDHF PHOTOPHYSICS

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FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

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March 2010
I certify that I have read this dissertation and that, in my opinion, it is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy.

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ABSTRACT

The number of reports per year on single-molecule imaging experiments has grown roughly exponentially since the first successful efforts to optically detect a single molecule were completed over two decades ago. Single-molecule spectroscopy has developed into a field that includes a wealth of experiments at room temperature and inside living cells. The fast growth of single-molecule biophysics has resulted from its benefits in probing heterogeneous populations, one molecule at a time, as well as from advances in microscopes and detectors.

There is a need for new fluorophores that can be used for single-molecule imaging in biological media, because imaging in cells and in organisms require emitters that are bright and photostable, red-shifted to avoid pumping cellular autofluorescence, and chemically and photophysically tunable. To this end, we have designed and characterized fluorescent probes based on a class of nonlinear-optical chromophores termed DCDHFs. This Dissertation describes various physical and optical studies on these emitters, from sensing local environment to photoactivation.

Chapter 1 is a general introduction to fluorescence and single-molecule spectroscopy and imaging. Single-molecule experiments in living cells are discussed and probes used for such experiments are summarized and compared. Chapter 2 explores the basic photophysics of the DCDHF fluorophores and some general methods of measuring relevant spectroscopic parameters, including photostability. Chapter 3 discusses the various approaches we have taken to modify particular properties by changing the fluorophore’s structure. We have redesigned the DCDHF fluorophore into a photoactivatable fluorogen—a chromophore that is nonfluorescent until converted to a fluorescent form using light—described in Chapter 4. Finally, a different, chemical route to fluorescence activation is presented in Chapter 5.

The remainder of the Dissertation is the Appendix and a full Bibliography. The Appendix includes a table of photophysical parameter for DCDHF fluorophore, various protocols used in the experiments discussed, MatLab codes, and NMR spectra.
Data, figures, tables, and excerpts are used with permission in this dissertation from the following publications:


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I joined the Moerner lab because W.E. seemed to run a fun and exciting research program, and I have not been disappointed. Other members of the Moerner Lab have been instrumental in my education and research. Kallie Willets mentored me when I first arrived at Stanford. Kallie was fun to work with and I am very grateful for the time and energy she dedicated to helping me get a solid footing in the lab by teaching me the right way to do things (and clean up afterwards).

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\[
\frac{\text{signal}}{\text{rms noise}} = \frac{R_{em} DT}{\sqrt{R_{em} DT + BT}} = \frac{\Phi_t \sigma_\lambda I_\lambda \left( \frac{\lambda}{hc} \right) D \sqrt{T}}{\sqrt{\Phi_t \sigma_\lambda I_\lambda \left( \frac{\lambda}{hc} \right) D + R_d I_d D + R_d}}
\]  
(1.1) ........................................7

\[
\tilde{\nu}_A - \tilde{\nu}_F = \frac{2}{hc} \Delta f \left( \frac{\mu_E - \mu_G}{a^3} \right) + \text{constant}
\]  
(2.1) ..................................................36

\[
P_N = m_{N/M}
\]  
(2.2) ........................................................................................................42

\[
N_{\text{photons}} = \frac{1}{D} N_{\text{detected}} = \frac{1}{D} \text{camera counts} = \frac{1}{\eta Q F_{\text{coll}} F_{\text{opt}} F_{\text{filter}} G_{EM} G_{A/D}} \ 	ext{camera counts}
\]  
(2.3) ........................................44

\[
\Phi_B = \frac{R_B}{R_{abs}} = \frac{1}{\tau_B R_{abs}} = \frac{1}{\tau_B \sigma_\lambda I_\lambda \left( \frac{\lambda}{hc} \right) \tau B \Phi_F = \Phi_F \frac{\Phi_B}{\Phi_F}
\]  
(2.4) ..................................................46

\[
\tau = f_1 \tau_1 + f_2 \tau_2 = \frac{\alpha_1 \tau_1^2 + \alpha_2 \tau_2^2}{\alpha_1 \tau_1 + \alpha_2 \tau_2}
\]  
(2.5) .................................................................46

\[
N_{\text{photons}} = N_{\text{abs}} \Phi_F = R_{abs} \tau_B \Phi_F = \sigma_\lambda I_\lambda \left( \frac{\lambda}{hc} \right) \tau_B \Phi_F = \Phi_F 
\]  
(2.6) ..................................................46

\[
R = \frac{I_{on}}{I_{off}} = \frac{n_{on} I_{off}}{I_{off}} = n_{on}
\]  
(4.1) ..............................................................110

\[
R_{\text{eff}} = \frac{S_{on}}{S_{off}} = \frac{n_{on} I_{on}}{n_{off} I_{off}} = \frac{p n_{off} I_{on}}{I_{on}} = p \frac{I_{on}}{I_{off}} = p R = p n_{off} = n_{on}
\]  
(4.2) ..................................................110

\[
R_{\text{eff, preact}} = \frac{S_{on}}{S_{off, preact}} = \frac{n_{on} I_{on}}{n_{off} I_{off} + q n_{off} I_{on}}
\]  
(4.3) ........................................................111

\[
= \frac{n_{on} I_{on}}{n_{off} I_{off} \left( 1 + \frac{q n_{off} I_{on}}{n_{off} I_{off}} \right)} = \frac{p R}{1 + q R}
\]  
(4.4) ........................................................111
\[ \Phi_p = \frac{R_p}{R_{\text{abs}}} = \frac{1}{\tau_p R_{\text{abs}}} = \frac{1}{\tau_p \sigma_\lambda I_\lambda \left( \frac{\lambda}{hc} \right)} \] (4.4) ..............................................................112
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1.

INTRODUCTION
1.1. BASICS OF OPTICAL SPECTROSCOPY OF MOLECULES

Spectroscopy is the study of how light interacts with matter as a function of wavelength or energy. When light impinges upon a molecule, the oscillating electromagnetic field can couple the ground state with higher energy state(s), resulting in an excited molecule. The excited molecule can then relax to the ground state radiatively (fluorescence or phosphorescence) or nonradiatively. Figure 1.1 depicts the energy levels and transitions that are typically relevant in fluorescence microscopy.

![Jablonski Diagram](image)

**Figure 1.1.** A Jablonski diagram depicting molecular energy levels and transitions. Solid arrows represent absorptive or emissive vertical (Franck-Condon) transitions; squiggly lines represent nonradiative transitions. VR is vibrational relaxation, or vibrational relaxation; ISC is intersystem crossing from singlet to triplet. Approximate timescales are as follows: absorption and fluorescence transitions are practically instantaneous (< fs); VR occurs over ps; average times in the excited electronic state before fluorescence emission are ns; ISC is a forbidden transition so phosphorescence is typically slow (μs–s or longer); for typical widefield SMS imaging, excitation times are on the order of one photon absorbed per 10 μs.

Using a semiclassical approach, with the light treated classically as an electromagnetic wave and the molecule treated quantum mechanically, it is possible to use time-dependent perturbation theory to calculate the (time-dependent) probability of the radiative transition. (This approximation holds for low light irradiance, as is the...
situation in nearly all laboratory conditions for fluorescence microscopy.) The probability that light will be absorbed to produce a molecule in an excited electronic state scales linearly with the absolute square of the transition dipole moment, the irradiance of the on-resonance light, and the time the molecule is exposed to the light.

The $x$ component of the transition dipole moment is $\sum_i x_i e \psi^0_{S0}$, where $\psi^0_{S0}$ is the sum of the $x$ coordinates of electrons in the molecule multiplied by the charge $e$, and $\psi^0_{S1}$ are the time-independent wavefunctions of the S0 ground and the S1 excited states. The transition dipole moment can be related to the classical oscillator strength, the Einstein coefficients, and selection rules.

Figure 1.2 depicts the potential-energy surfaces of the ground and excited states of a molecule, and the vibronic (i.e. electronic and vibrational) transitions between the two surfaces. A vertical arrow depicts a change in the energy with no change in the nuclear coordinates, or the structure of the molecule. This is described by the Born–Oppenheimer approximation, which assumes that the electronic and nuclear wavefunctions can be treated independently because electrons move and rearrange on a time scale much faster than the nuclei. The Franck–Condon principle holds that, upon (practically instantaneous) excitation, the molecule maintains its ground-state structure; the excited molecule can then relax from the Franck–Condon state to other lower energy excited-state structures via vibrational relaxation and nuclear rearrangement. In Figure 1.2, the Franck–Condon state is directly above the ground state, and its transition probability determined by the overlap integral between vibrational wavefunctions in the two electronic states. The practical outcomes of this principle are that molecules are more likely to absorb higher energy light (bluer) and emit photons with lower energy (redder), and that emission spectra are often mirror-images of each other. (For rigid aromatic molecules such at naphthalene, where the vibronic bands in the spectra can be resolved at room temperature in solution, the Franck–Condon principle also explains the relative heights of the different vibronic peaks.)
Although treated as delta functions in a zeroth-order approximation, absorption lineshapes are broadened by various mechanisms: homogeneous mechanisms affect all molecules in identical fashion, whereas inhomogeneous mechanisms are the outcome of differences among molecules. The most fundamental mechanism for fluorophores is lifetime (or uncertainty) broadening, which broadens the absorption profile of all molecules the same amount and is the direct outcome of the finite excited-state lifetime: the exponential decay of the excited-state population (or probability) yields a Lorentzian lineshape. Dephasing via collisions or phonons can also homogeneously broaden lineshapes. At room temperatures, the Doppler affect is a major source of inhomogeneous broadening: for a molecule moving at high speeds, the frequency of the incident light changes with direction and relative to the other molecules, broadening the width of the population. Other differences in local environment from molecule-to-molecule broaden the population width by shifting or splitting the absorption lines. On the single-molecule level (one focus of this Dissertation, described below), many of these inhomogeneous mechanisms (e.g. spatial fluctuations in matrix density, strains, stresses, local E fields, etc.) can be observed by measuring differences among individual molecules. In fact, early single-molecule experiments at
cryogenic temperatures relied on inhomogeneous broadening to spectrally isolate molecules, recording sharp peaks from a few molecules in the far-detuned wings of the population width.\textsuperscript{2}

For more information about photophysics, refer to the following books:


### 1.2. SINGLE-MOLECULE SPECTROSCOPY AND IMAGING (SMS)

#### 1.2.1. Brief History of Early Efforts in Single-Molecule Imaging and Biophysics

The optical absorption of single molecules was originally detected in solids at cryogenic temperatures by direct sensing of the absorbed light;\textsuperscript{3} subsequently, researchers detected optical absorption by measuring the fluorescence from single emitters under similar conditions.\textsuperscript{4} In the early experiments, optical saturation, spectral diffusion, photon antibunching, resonant Raman, electric field effects, and magnetic resonances of single molecules were observed.\textsuperscript{2} Optical detection of single molecules was eventually performed at room temperature from burst analysis in solution,\textsuperscript{5-7} in microdroplets,\textsuperscript{8} using near-field tips,\textsuperscript{9} and by 3D nanoscale tracking of single emitters in porous gels.\textsuperscript{10}

As single-molecule techniques addressed biologically relevant systems and samples at room temperature, biophysics quickly became an active target of single-molecule spectroscopy and imaging (SMS) research.\textsuperscript{2, 11, 12} Single copies of
fluorescent proteins (FPs) were imaged and the ability to control photoswitching was demonstrated, Förster-resonance-energy transfer (FRET) was observed on the single-pair level, the diffusion of single emitters was recorded in a phospholipid membrane, single motor proteins were imaged, and the nucleotide-dependent orientations of single kinesin motors were measured.

1.2.2. Motivations for Single-Molecule Imaging

The main reason for performing SMS is the ability to measure the full distribution of behavior instead of a single population average, thus exposing normally hidden heterogeneities in complex systems. A full distribution of an experimental parameter provides more information than the ensemble average; for instance, the shape of the distribution may be skewed or reveal multiple subpopulations, which may offer insight into underlying mechanisms. Each single molecule is a local reporter on the makeup and conditions of its immediate surroundings—its “nanoenvironment”—and thus acts as a readout of spatial heterogeneity of a sample. SMS also measures time-dependent processes that are not necessarily synchronized throughout the sample or population. For example, multiple catalytic states of an enzyme will be convolved with all the states of other copies in an ensemble, whereas a SMS experiment can measure uncorrelated stochastic transitions of a single enzyme. SMS also has the ability to observe intermediate states or rare events, given that the instruments have sufficient time resolution.

Because living systems are highly complex samples, with spatial and temporal heterogeneities that have biological relevance and with a wealth of processes that operate at the single-biomolecule level, SMS is a powerful tool to better understand the processes involved in life. Without needing to synchronize populations of biomolecules or cells, SMS is able to record the time evolution of these samples, for instance showing the sequence of events in a pathway. In many situations, fluctuations and rare events may be essential to biological function, making studying each single molecule that much more powerful. Finally, sparsely labeling a population of biomolecules (as is sufficient for many SMS experiments) reduces the chances that the
probe will interfere with the higher-level biology one is studying. For these reasons, SMS is quickly becoming a popular technique in biophysics and cell biology.

1.2.3. Key Requirements for Single-Molecule Detection

SMS traditionally requires the following: a transparent, nonfluorescent host matrix; molecules that are resolved by separating them in space (by more than the diffraction limit of ~200 nm), time, or wavelength; and probes that are efficient absorbers, highly fluorescent, and exceptionally photostable.

Most importantly, the signal-to-noise ratio (SNR) must be greater than one for a reasonable averaging time. The SNR for detecting one molecule in fluorescence spectroscopy is: \[ \text{SNR} = \frac{\text{signal}}{\text{rms noise}} = \frac{R_{em} DT}{\sqrt{R_{em} DT + BT}} = \frac{\Phi_F \sigma_\lambda I_\lambda \left( \frac{\lambda}{hc} \right) D \sqrt{T}}{\sqrt{\Phi_F \sigma_\lambda I_\lambda \left( \frac{\lambda}{hc} \right) D + R_b I_\lambda D + R_d}}, \] (1.1)

where \( R_{em} \) is the emission rate of the single molecule, \( \Phi_F \) is the fluorescence quantum yield of the fluorophore, \( \sigma_\lambda \) is the absorption cross-section at the excitation wavelength \( \lambda \), \( T \) is the detector counting interval (or binning time), \( I_\lambda \) is the excitation irradiance at the sample, \( h \) is Planck’s constant, \( c \) is the speed of light, \( B \) is the overall measured background count rate, \( R_b \) the irradiance-dependent background count rate from the sample, \( R_d \) is the dark-count rate from the detector, and \( D \) is the instrument-dependent collection factor which includes losses due to the microscope optics and filters as well as the detector quantum efficiency (see Chapter 2). Note that the Poisson statistics of measuring sparse photons from a single emitter results in the SNR improving with the square root of the binning time. The two limits are background limited (\( B \gg R_{em} \)) and shot-noise limited (\( B \ll R_{em} \)), the latter being the best possible scenario where the SNR increases as the square root of the irradiance. In general, fluorescence microscopy in living cells is in the background-limited regime (see below). Another measure is the signal-to-background ratio, or \( (R_{em} D + B)/B \). While this measure is independent of irradiance, it does not represent a fundamental detection criterion.
because it ignores the Poisson nature in the fluctuations of the signal and noise, and thus is less useful.

### 1.2.4. Instrumentation for Single-Molecule Detection

The instruments and methods used for SMS by detection of fluorescence have been extensively described and reviewed elsewhere; here, I summarize a few of the basic techniques.

**Microscope Configurations**

SMS experiments generally use inverted optical fluorescence microscopes, configured in either wide-field illumination or confocal (see Figure 1.3). The simplest wide-field method is epifluorescence, in which an expanded excitation beam is focused at the back focal plane of the objective, producing a collimated illumination beam at the sample. Fluorescence is collected through the same objective, filtered from any scattered excitation light using a dichroic mirror and long-pass or bandpass filters, and imaged onto a camera.

![Figure 1.3. Generalized microscope configurations for SMS in cells. From Lord et al.](image)

Because epifluorescence excites a large volume of sample, background signal from out-of-focus emitters can hinder imaging thick samples. Total-internal-reflection fluorescence (TIRF) imaging solves this problem by exciting only a thin slice of the sample nearest the coverslip. Excitation light is directed into the objective off center, causing the beam to totally internally reflect at the coverslip, subsequently producing a quickly decaying evanescent field up from the surface. The fluorescence excited by
the evanescent field is collected through the objective, filtered, and imaged using a camera. Because the evanescent field falls off exponentially within ~100 nm, TIRF is useful when the region of interest is very near the coverslip. A variation called quasi-TIRF (also referred to as “pseudo-” or “leaky-TIRF”) also sends in the excitation beam off-center, but not far enough for total-internal reflection; instead, a highly angled beam exits the objective and illuminates the sample in a slice thicker than TIRF but thinner than epifluorescence.

Another method to reduce out-of-focus fluorescence is confocal microscopy, which is a point-detection, scanning technique. A collimated excitation beam that slightly overfills the back aperture of the objective is directed into the microscope, producing a diffraction-limited spot at the sample. The confocal spot is scanned across the sample and emission is collected through the objective, filtered, focused through a pinhole (which rejects out-of-focus light), recollimated, then focused onto a point detector. Confocal imaging is not constrained to be near the coverslip, so can be used to image deeper into a sample or for three-dimensional scanning. The primary drawback of confocal is that it requires scanning the stage or the beam and a point detector, so multiple parts of the sample cannot be imaged simultaneously. This makes widefield configurations much more practical for single-particle tracking and imaging dynamic structures.

**Detectors**

In order to measure the stream of photons from a single emitter, a detector must exhibit low dark counts, high quantum efficiencies over a range of wavelengths, and low noise (from readout, electron multiplication, or analog-to-digital conversion). For details regarding detector types, characteristics and capabilities of different detectors, quantitative resolution and sensitivity parameters, sufficient signal-to-noise ratios for SMS detection, and other technical details see references 21, 23.

There are two classes of detectors for SMS, single-element or point detectors, and two-dimensional array detectors. Point detectors for confocal imaging include photomultiplier tubes (PMT), avalanche photodiodes (APD or SPAD), or hybrids thereof. While PMTs have large detection area (~1 cm²) and ps–ns time resolution,
APDs have higher quantum efficiencies and more easily detect single photons; moreover, APDs have very low dark counts, have faster time resolution, and output a digital signal that can easily be interfaced with a computer. The major drawbacks of APDs are (a) the small detection area, which makes aligning onto the sensor more difficult, and (b) the limited photon detection rates.

Wide-field imaging configurations allow the use of multidetector arrays or cameras, such as charge-coupled devices (CCD). Modern Si CCDs often include on-chip electron multiplication to increase sensitivity and reduce readout noise; moreover, frame-transfer technology permits faster imaging rates by performing the slow readout step on a separate, dark section of the chip. These cameras typically have quantum efficiencies >80% for the visible spectrum and frame-integration times of 10–100 ms, or faster for fewer pixels.

**Optics**

High quality lenses, mirrors, and filters are especially important for the ultrasensitive detection required for SMS. Immersion objectives with high numerical aperture (NA ~1.4) are necessary to collect as much of the emission as possible, but can complicate polarization. In addition, objectives for SMS should be achromatic and corrected for the coverslip material and thickness. Objectives specifically designed for TIRF are carefully designed to allow the excitation beam to be far off center.

Optical filters and dichroic mirrors must not fluoresce, must pass as much fluorescence as possible, and must reject all pump scattering and as much spurious signal as possible. Thus, long-pass filters must have sharp cut-on spectra, with optical density 6+ at shorter wavelengths. Bandpass filters can be helpful to remove longer-wavelength background fluorescence or excess Raman scattering from water, but it is important to overlay the filter’s transmission spectrum with the emission spectrum of the fluorescent label to avoid rejecting too much of the emission. Filters and lenses inside the microscope should be anti-reflection coated and aberration-corrected. For instance, thin dichroics can easily bend and distort the image; 2-mm or thicker dichroics are preferable.
Sources

Light sources for SMS are many, but are usually lasers. Single-frequency dye lasers were used in the early cryogenic SMS experiments, where finding the narrow absorbance peaks required tuning the excitation light. At room temperature, gas (e.g. argon-ion, helium-neon, etc.), diode, or solid-state lasers are typically used. While lasers are necessary for some techniques, epifluorescence excitation is possible using broadband sources such as arc lamps or light-emitting diodes. White-light or fiber lasers can also provide broadband light, produced by nonlinear optical effects when high-energy pulses (from a titanium-sapphire or other pulsed laser) are transmitted through special optical fibers. Broadband sources can be convenient because they are tunable to a range of colors, but are more difficult to filter than a monochromatic excitation source. In all cases, the excitation source should be filtered to reject unwanted leakage of the other colors or laser lines.

1.3. SMS IN LIVING CELLS

Single-molecule biophysics spans a range of experiments, from force studies of single macromolecules using tweezers or cantilevers to in vitro assays of fluorogenic enzymatic turnovers. For instance, by decorating a biomolecule with many copies of a probe, researchers have studied single DNA strands, membrane molecules, motors, and viruses. In this Dissertation, I focus instead on single-molecule spectroscopy and imaging experiments that measure the signal from each individual fluorescent label in living cells. Moreover, in the interest of space, I will not discuss the related area of fluorescence-correlation spectroscopy, although the method can probe the ensemble dynamics of single emitters and has been applied to living cells.
<table>
<thead>
<tr>
<th>Year</th>
<th>Milestone (Living Cells in Bold)</th>
<th>Researchers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1961</td>
<td>catalytic activity from one enzyme measured (by observing the build-up of fluorescence from a fluorgenic substrate)</td>
<td>Rotman</td>
<td>28</td>
</tr>
<tr>
<td>1981</td>
<td>single strands of DNA visualized (labeled with many fluorophores)</td>
<td>Yanagida, Chu</td>
<td>29, 30</td>
</tr>
<tr>
<td>1982</td>
<td>diffusion of single membrane-bound receptor molecules measured (labeled with many fluorophores)</td>
<td>W. Webb</td>
<td>31</td>
</tr>
<tr>
<td>1989–1990</td>
<td>first optical detection of single chromophore in condensed matter (cryogenic)</td>
<td>Moerner, Orrit</td>
<td>2-4</td>
</tr>
<tr>
<td>1990</td>
<td>action from single molecular motors recorded by observing motion of filaments (e.g. sliding assays)</td>
<td>Vale, Yanagida, Spudich</td>
<td>36</td>
</tr>
<tr>
<td>1990</td>
<td>room-temperature detection of single molecules from burst analysis in solution</td>
<td>Keller, Rigler, Zare</td>
<td>5-7</td>
</tr>
<tr>
<td>1991</td>
<td>first imaging of a single molecule (cryogenic)</td>
<td>Moerner</td>
<td>2, 37, 38</td>
</tr>
<tr>
<td>1992</td>
<td>elastic measurements of single strands of DNA using magnetic tweezers</td>
<td>Bustamante, Croquette, Bensimon</td>
<td>24, 26</td>
</tr>
<tr>
<td>1993</td>
<td>room-temperature SMS using near-field tips</td>
<td>Betzig</td>
<td>9</td>
</tr>
<tr>
<td>1995</td>
<td>single motor molecules imaged</td>
<td>Yanagida, Kinosita, Vale</td>
<td>16-18</td>
</tr>
<tr>
<td>1996</td>
<td>3D nanoscale tracking of single emitters, using TIRF evanescent field</td>
<td>Moerner</td>
<td>10</td>
</tr>
<tr>
<td>1996</td>
<td>first single-pair FRET measurements</td>
<td>Weiss, Ha</td>
<td>14</td>
</tr>
<tr>
<td>1996</td>
<td>diffusion of single emitters recorded in a phospholipid membrane</td>
<td>Schmidt, Schindler</td>
<td>15</td>
</tr>
<tr>
<td>1997</td>
<td>first SMS of fluorescent proteins</td>
<td>Moerner, Tsien, Vale</td>
<td>13, 39</td>
</tr>
<tr>
<td>1997</td>
<td>first room-temperature SMS example of controlled photoswitching, a fluorescent protein</td>
<td>Moerner, Tsien</td>
<td>13</td>
</tr>
<tr>
<td>1998</td>
<td>3D orientations of single molecules measured from dipole emission pattern (room-temperature)</td>
<td>Moerner</td>
<td>40</td>
</tr>
<tr>
<td>1998</td>
<td>3D super-resolution by spectral selection of single molecules (cryogenic)</td>
<td>Betzig, van Oijen, Schmidt, Moerner</td>
<td>37, 38, 41, 42</td>
</tr>
<tr>
<td>2000</td>
<td>spFRET measured in living cells</td>
<td>Yanagida, Kusumi, S. Webb</td>
<td>43-45</td>
</tr>
<tr>
<td>2000</td>
<td>3D tracking of single fluorophores in living cells</td>
<td>Schütz, Schindler</td>
<td>46</td>
</tr>
<tr>
<td>2000</td>
<td>transmembrane ion channels tracked</td>
<td>Schmidt, Schütz, Schindler</td>
<td>46, 47</td>
</tr>
<tr>
<td>2001</td>
<td>nucleotide-dependent orientations of single kinesin motors measured</td>
<td>Moerner, Goldstein</td>
<td>19</td>
</tr>
<tr>
<td>2001</td>
<td>binding kinetics to chemotactic receptors in the membrane observed</td>
<td>Yanagida</td>
<td>48</td>
</tr>
<tr>
<td>2001</td>
<td>infection pathway of singly-labeled viruses observed</td>
<td>Bräuchle</td>
<td>49</td>
</tr>
</tbody>
</table>
Studying living cells can be significantly more difficult than in vitro samples or fixed cells, because a living cell is a complex environment with elaborate interactions among components and cells exhibit continually changing states. Nevertheless, the reasons that make living cells tricky to study are fundamental characteristics of biology, and better understanding these attributes is critical to a deeper understanding of actual biological processes. See Table 1.1 for a selected timeline of SMS experiments with relevance to living cells.
1.3.1. Basic Requirements for SMS in Living Cells

SMS traditionally requires a transparent, nonfluorescent host matrix; molecules that are resolved by separating them in space (by more than the diffraction limit of ~200 nm), time, or wavelength; and probes that are efficient absorbers, highly fluorescent, and exceptionally photostable.

Imaging single emitters in living cells introduces further challenges. (1) In order to maintain low background counts, one must avoid cellular autofluorescence, the result of exciting endogenous cellular fluorophores (e.g. flavins, NADH, tryptophan). Autofluorescence can be avoided by selecting an imaging wavelength longer than about 500 nm, where biological fluorophores typically do not absorb, and by using cell growth media and imaging buffers that are free of fluorophores. (2) The cell membrane is a significant barrier to cell entry, because the probe must be able to pass through the hydrophobic lipid bilayer. Choosing a genetically expressed or membrane-permeable probe is critical for intracellular labeling, unless microinjection or electroporation is performed. (3) One of the primary challenges to live-cell imaging is specific labeling of predetermined sites on proteins or oligonucleotides. Due to the complex chemical environment inside cells, many bioconjugation reactions used in vitro (e.g. maleimide with cysteine or N-hydroxysuccinimide ester with lysine) are not sufficiently selective. Therefore, bioorthogonal labeling reactions are necessary for targeting organic fluorophores to biomolecules of interest in the cell. More commonly, researchers have relied on the genetic expression of FPs for single-molecule biophysics in living cells. (4) After an exogenous probe is targeted inside the cell, the difficulty of washing out unbound copies introduces further complications. Therefore, there must be a method to reject spurious signal from non-targeted fluorophores. For instance, this can be accomplished by employing fluorogenic labeling reactions or by adjusting the detector integration times to average out the signal (spread over many pixels) from quickly diffusing unbound fluorophores. (5) Finally, it is imperative that the experiment does not significantly interfere with the relevant biology of the cell. High labeling ratios, large probe size, photoradical production, and genetic manipulation can change phenotypes or even kill cells. Thus,
careful controls are necessary to ensure that the labeling technique, sample preparation, and imaging conditions do not alter the physiology of interest.

1.3.2. Probes for SMS in Cells

As FPs are powerful tools used extensively in biological imaging, it is not surprising that they are also important for live-cell SMS. To minimize background, longer-wavelength FPs (e.g. EYFP and other orange- or red-emitting FPs) are most desirable; therefore, there has been a sustained effort to tune FPs to longer wavelengths and to impart other beneficial qualities (e.g. photostability, brightness, monomeric or tandem dimers).

Table 1.2. Fluorophores for SMS in living cells.

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>Photoactivatable?</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPs</td>
<td>genetically expressed</td>
<td>less photostable (only ~100,000 photons emitted per molecule)</td>
<td>no</td>
<td>88, 90, 92, 94</td>
</tr>
<tr>
<td>GFP, EYFP</td>
<td></td>
<td>too blue</td>
<td>yes</td>
<td>13</td>
</tr>
<tr>
<td>Organic Fluorophores</td>
<td>small, tunable, photostable (millions of photons emitted per molecule)</td>
<td>challenging labeling</td>
<td></td>
<td>94</td>
</tr>
<tr>
<td>FlAsH/ReAsH, rhodamines, cyanine dyes, DCDHF</td>
<td>specific labeling, photostable, bright photostable, bright photostable, red, environmental sensing, enter cells</td>
<td>less photostable</td>
<td>no</td>
<td>86, 95, 97</td>
</tr>
<tr>
<td>QDs and Nanoparticles</td>
<td>photostable (tens of millions of photons or more emitted per particle)</td>
<td>large, cell entry, challenging labeling, blinky</td>
<td></td>
<td>100, 101</td>
</tr>
<tr>
<td>caged QDs, silver nanoclusters, photoswitching polymer nanoparticles, upconverting nanoparticles</td>
<td>photoactivatable, high cross-section</td>
<td>yes</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td></td>
<td>photoswitchable</td>
<td>yes</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td></td>
<td>very photostable, pumped in NIR</td>
<td>no</td>
<td>105</td>
<td></td>
</tr>
</tbody>
</table>

The major drawback to FPs is that they are generally an order of magnitude less photostable than many small organic fluorophores, which can emit millions of photons.
before photobleaching. Commonly used classes of organic fluorophores include the following: Cy3 and other carbocyanine dyes, rhodamines, fluoresceins, dicyanomethleydihydrofurans (DCDHFs), terylene and rylenes, etc.

Quantum dots (QDs) are semiconductor nanocrystals that photophysically act like fluorescent molecules for most purposes. Other nanoparticles are also used in imaging, such as scattering centers and nanoclusters. While QDs and nanoparticles are typically very photostable, they are much larger than organic fluorophores or FPs, their emission blinks in a complex way, and their large size may hinder motion of the analyte and obfuscate the true dynamics. However, this problem is mitigated by the fact that, at the low Reynolds numbers that these particles experience, drag forces are linear with velocity and radius (instead of scaling with the square of both, as they would at the high Reynolds numbers of everyday human experience). Unfortunately, QDs and nanoparticles are usually endocytosed and remain trapped in endosomes instead of entering the cytosol, so they have faced hurdles in targeting in live cells.

Finally, several groups have developed photoswitchable or photoactivatable probes, which are necessary for super-resolution microscopies, pulse-chase imaging, photoactivation–tracking experiments and more: FPs, Cy3-Cy5 pairs, DCDHFs, rhodamines, merocyanines, QDs, nanoparticles, and others. See Table 1.2 for more information about fluorescent probes used in live-cell SMS.

### 1.3.3. Labeling in Cells

There are several labeling strategies for live-cell imaging (see Table 1.3). Genetic expression of FPs directly provides specific labeling, and thus most live-cell SMS experiments have imaged FPs fused to proteins of interest. Targeting exogenous probes such as organic fluorophores or nanoparticles poses a significant challenge, but offers the benefits of more photostable or photochemically sophisticated fluorophores, as well as possible reduction in size and steric effects.
Table 1.3. Labeling approaches for SMS in living cells.

<table>
<thead>
<tr>
<th>Labeling Technique</th>
<th>Examples</th>
<th>Applicable Systems</th>
<th>Developments Needed</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic Expression of FPs</td>
<td>EYFP</td>
<td>live cells</td>
<td>increased photostability</td>
<td>88, 90, 92, 94</td>
</tr>
<tr>
<td>Enzymatic Tags</td>
<td>Halo, SNAP, BirA, Sfp–CoA</td>
<td>live cells</td>
<td>better washing, smaller fusion</td>
<td>85, 111</td>
</tr>
<tr>
<td>Genetic Peptide Tags</td>
<td>FlAsH</td>
<td>live cells, typically bulk imaging</td>
<td>reduce off-target labeling, strengthen binding</td>
<td>85, 86, 88, 111</td>
</tr>
<tr>
<td>Antibodies</td>
<td>biotin/streptavidin</td>
<td>fixed cells, surfaces of live cells</td>
<td>stronger binding, smaller size, expanded applications for live cells</td>
<td>85, 88, 111</td>
</tr>
<tr>
<td>Bioorthogonal Rxns</td>
<td>click</td>
<td>typically surfaces of live cells</td>
<td>simple expression of reactive tags</td>
<td>84</td>
</tr>
<tr>
<td>Nonspecific Labeling</td>
<td>maleimide, NHS ester</td>
<td>in vitro labeling of biomolecules</td>
<td>better routes to introduce exogenous molecules into cells</td>
<td></td>
</tr>
</tbody>
</table>

Strong but noncovalent binding to short peptide motifs has been demonstrated by Tsien and others using FlAsH-type fluorophores. Although ReAsH has been imaged on the single-molecule level in vitro, live-cell SMS imaging with FlAsH-type fluorophores has faced problems of off-target labeling and low photostability.

Irreversible bioconjugation of fluorophores with pendant chemical “tags” to engineered enzymes genetically fused to proteins of interest has the potential to compete with FP labeling. For instance, SNAP-tags and HaloTags are two such fusion enzymes, which react with synthetic benzylguanine and chloroalkane tags, respectively; after the reaction, the tag—and any probe linked to the tag—are covalently linked to the enzyme, and thus to the protein of interest. In another enzymatic strategy, Sfp-labeling uses a CoA-modified fluorophore and a peptide tag on the protein of interest, which are covalently coupled by an extrinsic enzyme.

1.3.4. Summary of SMS Imaging Studies in Living Cells

Early work imaging single molecules in living cells has been reviewed elsewhere. I will briefly touch on these earlier studies, which primarily involved tracking single proteins in cell membranes, but also summarize a wide range of more recent experiments (see Figure 1.4).
Molecules in Membranes

Fast CCD cameras have made it possible to image in widefield and track in real time single molecules moving in plasma membranes. The membrane of living cells was an early target of SMS, especially in attempts to observe the elusive lipid “rafts” or other structures and domains: by tracking single proteins or emitters, researchers probed tiny regions to search for heterogeneous dynamics. Kusumi et al. applied high-speed particle tracking, with frame rates as high as 40 kHz, to observe the movements and confinement of molecules in the plasma membrane. Hop diffusion between submicron regions was observed, hinting of a “picket fence” model of membrane rafts (i.e. that bulky transmembrane proteins are corralled by the membrane-associated cytoskeletal filaments).

A team led by Moerner and McConnell tracked fluorescently labeled transmembrane proteins and measured the large decrease in diffusion by depleting cholesterol. They also measured the diffusion dynamics of single small, lipid-like fluorophores embedded in the lipid bilayer. Originally, these studies indicated that the diffusion of the transmembrane protein called the major histocompatibility complex of type II and its glycolipid-anchored form was not altered after actin was depolymerized. Because actin provides the cytoskeletal structure, these results seemed to contradict the picket fence model. A more recent re-examination of the same system at higher time resolution by both groups revealed some evidence of membrane compartments. Nevertheless, the existence of lipid rafts in living cells is still contentious in the membrane community, and more studies will be necessary before any models are widely accepted.

Many biomedical researchers seek small proteins that assist cargo to enter cells, often drugs or genetic material that otherwise could not pass the plasma membrane. Lee et al. tracked single DCDHF-labeled polyarginines to determine possible entry mechanisms for cell-penetrating peptides. Quantitative analyses of the peptides interacting with the plasma membrane indicated that polyarginines enter the cell via multiple pathways or via a mechanism other than passive diffusion or endocytosis,
which may have implications in the biomedical uses of arginine-based cell-penetrating peptides and their cargo.

Figure 1.4. Scheme of some general approaches to SMS in live cells. From Lord et al.\textsuperscript{22}

Longer tracking times require emitters that are photostable for minutes, such as QDs or nanoparticles. Dahan et al.\textsuperscript{51} labeled glycine receptors in the membranes of living neurons, tracked for minutes and observed multiple domains of the cell surface, as single receptors diffused from synaptic regions to areas outside the synaptic membrane. They also recorded cell-entry events, confirmed later using electron microscopy. Given that QDs are large, the authors expressed concern that the size of the probe might have hindered motion of the receptors in the synapse; while such
hindrance could not be fully ruled out, a comparison with a relatively small antibody labeled with the organic fluorophore Cy5 revealed equal percentage of rapidly diffusion receptors with either label. Besides these concerns with the large size, the QD labels permitted long imaging times and their brightness resulted in higher-precision tracking.

Transmembrane ion channels are also an interesting target for SMS high-precision tracking. Harms et al.47 imaged ion channels labeled with FPs in live cells, measuring lateral diffusion, polarization, and stoichiometry of single proteins and aggregates. Schütz et al.46 localized in three dimensions single Cy5-labeled hongotoxin molecules, which bind with high affinity to potassium channels. These studies accessed information about the lateral, rotational, and axial motion of ion channels and associated toxins; they primarily served as proof-of-principle experiments, demonstrating the power of single-molecule tracking in multiple dimensions and with high precision.

There are numerous other proteins, structures, and processes in the plasma membrane that warrant further research. Ueda et al.48 studied how cells detect and move in chemical gradients: by labeling cyclic adenosine monophosphate (cAMP) with Cy3, they observed its binding kinetics to chemotactic receptors in different regions of the membrane of live Dictyostelium discoideum cells. For instance, the dissociation kinetics of cAMP were significantly altered in a mutant cell line lacking G protein, a molecular switch coupled to the receptor and involved in the chemotaxis signaling pathway. Other researchers have applied SMS to count the number of subunits in membrane-bound proteins by counting the number of photobleaching steps,80, 81 which is important for better understanding of protein-protein interactions and subunit assembly.

**Molecules in the Nucleus**

In eukaryotic cells, biology that occurs inside the nucleus is essential to cell processes. Nuclear pores are large protein complexes that form selective holes in the nuclear envelope, the double lipid bilayer that forms the nucleus. Nuclear pores allow the transport of RNA and proteins involved in gene replication between the cytoplasm...
and the nucleus. Given the essential role of the nuclear pore, understanding how single biomolecules interact with the complex would be valuable. Yang et al.\(^{64}\) imaged nuclear pore complexes in living HeLa cells, recording the trajectory of single copies of substrates (labeled with one or two Alexa-555 fluorophores) undergoing transport through the pores. They were able to construct a high-resolution map of the pores from such traces. Other researchers have performed more in-depth studies of the dwell times of single molecules interacting with nuclear pore complexes.\(^{116}\)

Because the nuclear envelope is an efficient barrier, introducing exogenous molecules into the nucleus can be challenging. In order to get around this problem, Knemeyer et al.\(^{117}\) microinjected directly into the nucleus fluorescent oligonucleotides, which hybridized with mRNA strands. The researchers then used a pulsed laser source and fluorescence-lifetime confocal imaging to separate the relevant signal from the autofluorescence background, which exhibited a shorter lifetime. Apparent blinks in the signal from a few spots offered some evidence that the researchers were indeed imaging single fluorophores. Although primarily a proof-of-principle study demonstrating the feasibility of both microinjection and lifetime-separated fluorescence imaging, it opens the doors for subsequent experiments to examine more complicated biology that occurs within the nucleus.

**Cytoskeletal Molecules**

Because of their small size and the relative lack of understanding of their structural components, prokaryotes are especially interesting for single-molecule imaging. A team led by Moerner, Shapiro, and McAdams has studied protein localization and movement in living cells of the bacteria *Caulobacter crescentus* using FP fusions as fluorescent labels.\(^{60-63}\) In a high-precision tracking study, they observed the movement of MreB proteins (an actin homolog).\(^{61}\) Protein motion was measured at two different time scales: the diffusion of free monomers of MreB was recorded with CCD integration times of 15 ms yielding diffusion coefficients on the order of 1 \(\mu\text{m}^2\text{ s}^{-1}\); using time-lapse imaging, the speed of the slower, directed “treadmilling” motion of labeled copies incorporated into the MreB filament was measured at approximately 6 nm s\(^{-1}\). (Treadmilling occurs when monomers add to one end of the filament while
the other end dissociates, resulting in a labeled segment moving through the relatively stationary filament.) Because this treadmilling motion was so slow, single fluorophores are likely to photobleach before a long enough trace is acquired. Instead, the motion was measured with time-lapse, using 100-ms integration times separated by up to 10 s of darkness. At these longer frame-integration times, signal from diffusing monomers was spread over many pixels and only recorded as background; light from a slowly moving copy in the filament was concentrated on a few pixels and appeared as signal above the background as a diffraction-limited spot. Tracing out these slowly moving spots revealed super-resolution maps of MreB filaments.

In a separate study, the same team visualized single copies of FP-labeled PopZ, a protein that anchors the chromosome origins, and its excursions between poles of *C. crescentus* cells. Most copies of the protein diffused throughout the cell, while some stopped moving after reaching a cell pole. Such dynamics corroborate a diffusion-and-capture model for PopZ localization at cell poles.

Other researchers have also used live-cell SMS to study protein localization in bacteria. Niu et al. photoactivated FPs and tracked single monomers of the cytoskeletal protein FtsZ, a homolog of tubulin, and imaged helical patterns of the polymerized form in *Escherichia coli* cells. They also found that monomeric FtsZ molecules moved throughout the entire cell and consistently exhibited anomalously slow diffusion at long time scales, suggesting that the monomers encounter barriers in the membrane or in the cell. These studies expanded the limited knowledge about bacteria structural and chromosomal organization, as well as explored the mechanisms of cell division.

**Trafficking of Single Molecules inside Cells**

Understanding how signaling molecules, cellular components, and viruses are trafficked in living cells is an important goal in biomedical imaging. A team led by Chu and Mobley labeled nerve growth factor (NGF) with single QDs and tracked their transport in the axons of living neurons, concluding that a single NGF is sufficient to initiate signaling. By observing individual endosomes being trafficked along the axon toward the cell body, they were able to record a variety of behaviors, such as
stop-and-go, short retrograde movement, and multiple endosomes pausing at the same location in an axon. Moreover, labeling with only a single QD offered information that would have been obscured with many labels: a majority of the endosomes contained only one single NGF–QD conjugate. This claim was made after observing a photoblinking signal, which is indicative of single emitters,\textsuperscript{37} it was further corroborated by mixing two colors of QDs and observing that most endosomes emitted only one color, which would be highly unlikely if each endosome contained many NGF–QD copies.

Seisenberger et al.\textsuperscript{49} observed the infection pathway of viruses singly labeled with Cy5 dyes in living HeLa cells, tracking the viruses as they interacted with the membrane, as they were endocytosed, and as motors directed them inside the cells. The SMS study revealed that the virus infected the cells in much less time than previously observed using bulk experiments, providing insight into the mechanisms that viruses employ to infect cells.

Because the density of macromolecules and cytoskeletal structures is much higher in cells than in the buffers used for in vitro assays, observing how biomolecular motors perform in the typical conditions inside living cells is of particular interest. Cai et al.\textsuperscript{74} studied single kinesin motors in COS mammalian cells, and correlated intensity fluctuations with physiological conditions. They measured the average speed and run length that individual motors, extracted from single-molecule traces. Pierobon et al.\textsuperscript{75} tracked single myosin motors labeled with QDs in living HeLa cells, measuring even slightly higher velocities than in vitro.\textsuperscript{51, 75} Because these parameters agree with bulk and in vitro assays, the researchers concluded that the molecular crowding within a living cell does not significantly hinder the transport speeds of those motor proteins.

**Gene Expression**

The Xie group has applied SMS to study gene expression in living *E. coli* bacteria cells,\textsuperscript{70, 71} summarized in a recent review.\textsuperscript{72} These studies explored the stochastic nature of gene expression and probed the dynamics of transcription. Moreover, by watching individual expression events in dividing cells, they were able to follow how events unfurl generations later.
In order to explore the full dynamics of the system, the researchers probed multiple time scales of protein motion (similar to the approach taken by the Moerner team\(^6\) described above). Static emitters were possible to detect above the autofluorescence of cells, but single proteins diffusing in the cytosol moved too quickly to be captured, blurring into background at even the fastest readout speeds of the CCD cameras. To image these fast-moving emitters, Xie et al. cleverly borrowed a concept from strobe photography: during each 100-ms integration time, a single bright 10-ms flash from the laser excited the sample; because the diffusing proteins did not move more than a couple pixels during the laser flash, they appeared as spot instead of a blur in the image. Xie et al. also used this stroboscopic time-lapse technique to image individual proteins diffusing very quickly on DNA, determining the diffusion coefficient by varying the stroboscopic exposure time from 10–100 ms and measuring the molecule’s displacement.\(^7\)

**spFRET**

Single-pair FRET (spFRET) has been used in a few studies to measure signaling interactions and protein conformations. Many novel observations would have been not possible without spFRET measurements, because the ensemble FRET value does not reveal the dynamics, stoichiometry, binding order, orientation, or temporal information that is observable using SMS.

For instance, using Cy3 and Cy5 fluorophores as the FRET donor and acceptor labels, Sako et al.\(^4\) observed epidermal growth factor (EGF) receptor signaling in living A431 mammalian cells. The early events in the signaling process include dimerization and autophosphorylation of the receptor. By tracking single EGFs labeled with Cy3 or Cy5, the researchers were able to use spFRET to detect when two copies of the EGF–receptor complex dimerized. They also imaged a Cy3-labeled antibody that binds only to phosphorylated receptors; because the antibody–Cy3 more often colocalized with EGF–Cy5 receptors that were twice as bright as other receptors, the authors determined that the receptor first dimerizes, then phosphorylation occurs after the dimer forms. They were also able to observe that binding of EGF to a dimer of
receptors is much stronger than the binding to a monomer, and that EGFs bind one at a time to the receptor dimer, instead of as a pair.

Other researchers explored more dimensions of the spFRET signal in order to separate the details of EGF binding and receptor dimerization. With a polarizer and a dichroic mirror, S. Webb et al.\textsuperscript{45} split the output of the microscope into four regions of the camera, simultaneously measuring the polarization and FRET signals from single EGFs labeled with Cy3 or Cy5. Live A431 cells were incubated with the labeled EGFs, which were allowed to bind to the receptors in the cell surface. FRET efficiency is a complex parameter that depends not only the proximity but also the orientation between the donor and acceptor molecules; by knowing the orientation of the two chromophores (from the polarization of the emission), the two factors in FRET efficiency can be decoupled. Indeed, the researchers observed some events where changes in the spFRET signal were the outcome of orientation changes and other events that resulted from changes in proximity.

Other signaling events have also been measured using spFRET. Murakoshi et al.\textsuperscript{44} applied the technique to observe the activity of Ras, a G protein that influences various signaling pathways in the cell. Because the precise transduction mechanism of the Ras signal switch is poorly understood, the ability to detect single Ras activating events with spFRET could be helpful. Cells that were engineered to express a Ras–FP were microinjected with guanosine triphosphate (GTP) labeled with a Bodipy organic fluorophore. The researchers monitored binding of the GTP–Bodipy to Ras–FP using the FRET signal from single pairs, and observed that Ras diffusion was subsequently suppressed. Such immobilization after binding events may reveal a larger complex Ras interacts with during signaling.

\section*{1.4. SUPER-RESOLUTION SMS IMAGING}

The spatial resolution of far-field optical microscopy is determined by the diffraction-limited size of the point-spread function. This limit—recognized by Abbe, Rayleigh, and others—means that photons from multiple emitters closer than about
half the wavelength of light used cannot be simultaneously resolved spatially when detected in the far-field. However, emitters can be differentiated by taking into account properties of the photons other than just their locations, such as time and wavelength, making the actual photophysics and photochemistry of the emitter more important.

1.4.1. History of Super-Resolution SMS Imaging

Early work in low-temperature SMS regularly resolved single emitters spaced much closer than the optical diffraction limit: by taking advantage of narrow absorption linewidths and tunable dye lasers, researchers spectrally separated molecules that were spatially close.\textsuperscript{37, 38, 41, 42} At biologically relevant temperatures, where linewidths are broad, color alone is insufficient to differentiate many molecules within a diffraction-limited region, and other parameters are necessary for super-resolution SMS. For instance, if a single molecule moves through a structure, localization of the molecule at each time point yields a superresolution image of the structure.\textsuperscript{61} Photoswitching offers a more generally applicable temporal control of the fluorescence from single molecules, once again giving researchers a property that could be harnessed for super-resolution imaging.

In 2006, three groups independently reported super-resolution imaging based on photoswitching/photoactivation of single molecules (termed PALM, STORM, and FPALM).\textsuperscript{67-69} Super-resolution images are constructed from rounds of photoactivating sparse subsets of a sample and localizing those single emitters with high precision, building up over time a final image with high spatial resolution. Most of the first efforts in super-resolution SMS imaging used nonbiological samples or cells that had been fixed by polymerizing molecules of the cytoplasm, primarily because each image requires hundreds of camera frames and many tens of seconds to acquire. Recently, however, advances in microscope setups and photoactivatable probes—as well as the careful selection of slowly changing (quasi-static) objects—has allowed several groups to obtain super-resolution images of structures and molecular interactions in living cells.
(Other super-resolution techniques, such as stimulated-emission-depletion and structured-illumination microscopies also take advantage of photophysics of fluorophores, as well as sophisticated optical setups, to measure super-resolution images and are applicable to living cells; however, because these techniques do not inherently require single-molecule detection, they will not be discussed here.)

1.4.2. Super-Resolution SMS Imaging of Living Cells

S. Hess et al. imaged at high resolution the membrane protein hemagglutinin in fixed and living fibroblast cells using a photoactivatable FP called PA-GFP. Hemagglutinin has been proposed to associate with nanometer-scale membrane rafts, and probing protein distributions at high resolution can shed light on raft content and structure. The images revealed irregular, extended clusters of hemagglutinin, thus undermining models of lipid rafts that predict smooth, rounded boundaries, as defined by fluid-fluid phase segregation. Moreover, this study found that fixed cells had quantitatively different protein distribution, confirming that fixing cells can cause nonbiological artifacts.

The team led by Betzig, H. Hess, and Lippincott-Schwartz studied dynamics in living COS-7 cells by combining super-resolution with single-particle tracking to image high-density trajectories of membrane proteins (Gag and VSVG) labeled with a FP called EosFP. Moreover, because many trajectories can be measured in one cell, a map of mobility was constructed for individual cells: clusters of slowly moving proteins were found among large regions of highly mobile molecules. Shroff et al. also imaged the changes in adhesion complex structures over several minutes in living NIH 3T3 cells labeled with EosFP. By obtaining super-resolution images in a time series, the researchers were able to display how the structures grow and changes as the cell moved.

The Moerner team imaged super-resolution structures in living bacteria C. crescentus. Bacteria pose a unique challenge for super-resolution fluorescence imaging: because of their tiny size, only a few diffraction-limited spots can fit within the cell before they are unresolvable. Moreover, cytoskeleton structures and protein localization is particularly of interest in C. crescentus, in order to help explain the
mechanisms of asymmetric cell division. In one study, a Cy3–Cy5 covalent heterodimer was synthesized and the outside of *C. crescentus* cells were coated with the photoswitching molecule. Super-resolution images of the spindle-like stalk were obtained. Because the Cy3/Cy5 photoswitching system requires the addition of thiol at high concentration, imaging using those fluorophores inside cells faces serious challenges, thus the first demonstration of the use of this fluorophore pair in a live cell was aimed at a bacterial extracellular stalk.

Therefore, a different approach was taken for imaging the internal cytoskeletal protein MreB in living *C. crescentus* using EYFP, which the Moerner lab demonstrated was a photoswitch over a decade ago. The integration time per CCD frame was chosen carefully so that MreB proteins incorporated in the cytoskeleton were imaged, but unbound monomers moved too fast to be captured. In addition, time-lapse imaging was employed in order fill in some gaps in the cytoskeleton structure. This approach was possible because MreB proteins treadmill along the polymerized structure, as discussed above.

### 1.5. OUTLOOK

While ensemble biochemistry and imaging experiments will always be fundamental to cell biology, SMS has proven itself over the last decade as an invaluable tool for probing heterogeneous populations, dynamics, stoichiometry, trafficking, and structure inside living cells. The future of live-cell SMS is flush with promise, including advances from super-resolution biophysics to controllable emitters, from high-sensitivity detection to fast integration times, from new optical techniques to advances in image processing.

There are limits to what we can learn about biology by studying only isolated cells; therefore, SMS in living systems is progressing toward more complex environments, including cell–cell interactions and whole-organism studies. For instance, researchers have recently begun imaging single molecules within tissues of living vertebrates. Moreover, interfacing living cells with tools such as supported
lipid bilayers may facilitate imaging cell–cell interactions and signaling pathways in conditions similar to those inside organisms.\textsuperscript{121}

Adaptive optics and wavefront engineering, the state-of-the-art in astronomy, are beginning to appear in cell imaging and SMS.\textsuperscript{122, 123} Wavefront correction in real time may be able to reduce aberrations from cells or media, but will require fast software feedback. In addition, custom shaping of the point-spread function (on the excitation or the detection side) will allow researchers to encode more information, such as axial position, into SMS images.\textsuperscript{123} Other advances in bulk biological microscopy, such as light-sheet illumination and nonlinear optics, will be applicable to SMS as the techniques and instrumentations are refined.\textsuperscript{124}

Super-resolution SMS techniques and single-molecule tracking in living cells will require faster, more sensitive cameras. Alternatively, faster confocal scanning techniques (such as the Nipkow spinning disk), if their optical throughput can be increased significantly, could offer video-rate imaging with the capability to reject out-of-focus background.\textsuperscript{125, 126} Super-resolution methods also need multicolor sources that switch between many colors quickly, are easy to use, can be effectively filter, and integrate into a conventional SMS microscope setup. For instance, sets of light-emitting diodes and/or tunable filters used in conjunction with lamps or white-light lasers could serve as multicolor sources.

Live-cell imaging and super-resolution SMS both are limited by probe photophysics and labeling techniques. Increasing localization precision and tracking times require probes with much higher photostability; super-resolution of dynamic structures will require photoswitches that cycle many times and emit several thousands of photons each cycle. Advances in SMS of living cells will require new and improved specific labeling methods that are bioorthogonal, fast, effective, and nonperturbing. Moreover, all super-resolution techniques require high-density specific labeling without altering phenotype.

Regardless of these challenges, SMS in living cells has potential to reveal a new and unexplored level of detail in biology and medicine.
1.6. SCOPE OF THE DISSERTATION

This Dissertation describes the work I have completed in Professor W.E. Moerner’s lab in the Department of Chemistry at Stanford University, since joining the lab in 2004. The remainder of this Dissertation will focus on the photophysics and applications of one class of single-molecule fluorophores, the DCDHF's. All molecules in this class were synthesized by students and postdocs in the laboratory of Professor Robert J. Twieg at Kent State University, with whom we have had fruitful and long-term interactions. I will describe the various physical and optical studies that I have performed on these emitters, from sensing local environment to photoactivation. Chapter 2 explores the basic photophysics of the DCDHF fluorophores and their methods of measurement, including photostability. (While general measurement and analysis methods are described in Chapter 2, specialized methods are described at the end of the corresponding section of the text.) In Chapter 3, I will discuss the various approaches we have taken to modify particular properties by changing the fluorophore's structure. We have redesigned the DCDHF fluorophore into a photoactivatable fluorogen—a chromophore that is nonfluorescent until converted to a fluorescent form using light—described in Chapter 4. A different, chemical route to fluorescence activation is presented in Chapter 5. The remainder of the Dissertation includes the Appendix and a full Bibliography.
2.

BASIC PHOTOPHYSICAL PROPERTIES OF THE DCDHF CLASS OF FLUOROPHORES AND METHODS OF MEASUREMENT
In this Chapter, I will introduce the DCDHF class of fluorophores and discuss their basic photophysical properties. For instance, the DCDHFs tend to be photostable single-molecule emitters, they exhibit solvatochromism and sensitivity to viscosity, and they can be imaged in living cells and other biological media. In this Chapter, I will discuss these basic properties as well as the general experimental methods I have used. In Chapter 3, I will go beyond these basic characteristics to explore the various structure–property relations we have tested with specific design.

2.1. THE DCDHF CLASS OF FLUOROPHORES

2.1.1. Nonlinear Optics and Push–Pull Chromophores

In the late 1980s and 1990s, the nonlinear optical properties of push–pull chromophores—structures containing electron donor and electron acceptor units separated by a π-electron-rich conjugated linker—were well documented.127-129 The 2-dicyanomethylene-3-cyano-2,5-dihydrofuran (DCDHF) unit is a useful π-accepting unit, and continues to be used in nonlinear applications, especially in electro-optic media.130 Chromophores containing a DCDHF acceptor and an amine donor, separated by a π-conjugated system (Figure 2.1), were also optimized for photorefractive polymer applications.131-133 In these materials, the linear polarizability anisotropy is very important to enhance the orientational photorefractive effect, and the push–pull character of the chromophore was intended to increase both the hyperpolarizability \( \beta \) and the ground state dipole moment \( \mu_G \) via the charge-transfer absorption and the asymmetric charge distribution, respectively. Increases in donor and acceptor strength also red-shift the absorption, lowering the energy required to produce an intramolecular charge-transfer upon photoexcitation.134

2.1.2. DCDHFs as Fluorophores

Most nonlinear optical chromophores are not known to be strong fluorescent emitters, especially because very strong charge transfer can produce large excited-state
structure distortions. In the case of photorefractive polymer materials, the dominant figure of merit is not fluorescence but $\mu^2(\Delta \alpha)$,\textsuperscript{135-137} where $\Delta \alpha$ is the polarizability anisotropy (the tensor that reports how easily the electron distribution of the molecule distorts in an applied electric field). Chromophores containing a carbocyanine structure were found to be optimal for such nonlinear optical applications.\textsuperscript{138}

![Figure 2.1. Schematic structure of the DCDHF fluorophores. The amine donor and DCDHF acceptor are connected by a $\pi$-conjugated linker. The R groups can be modified (usually without affecting the photophysics) in order to add reactive functionality or increased solubility. The naming scheme used in this Dissertation specifies the $\pi$ system: “DCDHF-(\pi unit closest to acceptor)…-(\pi unit closest to donor)” with the $\pi$ units denoted P = phenylene, V = vinyl, T = thiophene, N = naphthalene, A = anthracene; the amine donor is not specified because it is present in all structures. (See Appendix for structure drawings.)

In 2003, K.A. Willets and O. Ostroverkhova\textsuperscript{139} discovered that some chromophores designed for photorefractive polymers,\textsuperscript{140} the DCDHFs, also have high fluorescence quantum yields in glassy polymer films and that they emit millions of photons before photobleaching. This means that when the local environment inhibits certain intramolecular twists, the excited-state distortion is not so large as to prevent fluorescence. Since then, I have designed, interacted regarding the synthetic efforts of our synthetic collaborators, and characterized hundreds of members of the DCDHF class for application as fluorophores for single-molecule imaging. This Dissertation concentrates on molecules specifically optimized for potential use as cellular fluorescent labels.\textsuperscript{1, 87, 99, 139, 141-147}
2.2. DCDHF SOLVATOCHROMISM

DCDHF fluorophores exhibit two types of sensitivity to the local environment: solvatochromism (as a result of the charge-transfer character of the excitation) and viscosity dependence (due to suppression of bond twists that permit nonradiative decay pathways).\(^1\)

A simple consequence of the strong solvatochromism that DCDHFs exhibit is that the fluorescence is substantially red-shifted with respect to the excitation in biological media, making it easy to filter out background due to scatter and autofluorescence, which typically have smaller Stokes shift.\(^90\) Solvatochromism in other fluorophores has also been harnessed to report local hydrophobicity, because changes in polarity cause changes in the color or intensity of the emission signal.\(^148\) Moreover, the viscosity-dependent brightness that DCDHFs exhibit has a more direct advantage in background suppression: copies of DCDHF or DCDHF-labeled biomolecules not in a rigid region of interest are dim, and contribute much less to the fluorescence background. Fluorescence microscopy in the total-internal-reflection fluorescence (TIRF) mode is not always a viable option (e.g. if one needs to image away from the glass interface such as at the upper membrane of a cell or if the optical polarization of TIRF cannot be tolerated), and fluorescence readout only in certain regions of a cell permits low-background epifluorescence imaging of those regions.\(^54,\,82\)
Figure 2.2. Diagram representing the physical mechanism of solvatochromism. Upon absorption (or emission), the nuclear coordinates of the surrounding solvent molecules do not have time to rearrange; subsequently induced dipoles (from electronic reconfiguration in the solvent) stabilize the Franck–Condon state. The relatively long lifetime in the excited state permits solvent nuclear reorientation and increased solvation stabilization. Solvent molecules with larger permanent dipole moments will stabilize the excited state more than nonpolar solvents; therefore, solvents with higher polarity should cause an increase in the Stokes shift. In the Lippert–Mataga formalism, the high-frequency component of the polarizability (the term containing the index of refraction $n$ in Equation 2.1) is subtracted, because all states of the fluorophore are stabilized by the induced dipoles approximately equally.

Solvatochromism is an expected outcome of the push–pull character of donor–π–acceptor chromophores (Figure 2.2). Conventionally, the Lippert–Mataga equation\textsuperscript{149} (Equation 2.1) may be used to probe the influence of the solvent polarity on the Stokes shift. In most push–pull chromophores, the charge separation (i.e. dipole moment) is greater in the excited-state manifold. When such a charge-transfer fluorophore is
excited from the ground to excited state, the nearby solvent dipoles can reorient around the larger excited-state dipole moment, thus stabilizing the system and lowering the energy of the excited state (and simultaneously destabilizing the ground state). This effect becomes more pronounced as the solvent polarity is increased, and the Stokes shift increases in more polar solvents. In the Lippert–Mataga approximation,\textsuperscript{149} the orientation polarizability $\Delta f$ is used as a parameter to represent the degree of molecular rearrangement around a dipole in a continuous medium, thus leading to the observed dependence of the Stokes shift on the square of the dipole moment change:

$$\tilde{\nu}_A - \tilde{\nu}_F = \frac{2}{hc} \Delta f \frac{(\mu_E - \mu_G)^2}{a^3} + \text{constant} \quad (2.1)$$

where $\Delta f = \frac{\varepsilon_r - 1}{2\varepsilon_r + 1} - \frac{n^2 - 1}{2n^2 + 1}$

and $\tilde{\nu}_A$ and $\tilde{\nu}_F$ are the wavenumbers of the absorption and emission, $\mu_G$ and $\mu_E$ are the ground- and excited-state dipole moments, $a$ is the Onsager cavity radius (assumed to be 5 Å), $n$ is the refractive index of the solvent, $\varepsilon_r$ is the relative dielectric constant of the solvent, $h$ is Planck’s constant, and $c$ is the speed of light. The solvent parameter $\Delta f$ takes into account only the low-frequency polarizability (i.e. the slow rearrangement of nuclear coordinates of the solvent molecules), and removes the high-frequency polarizability component (from electron redistribution in the solvent, which occurs on the same time scale as photoinduced charge transfer in the fluorophore) from the total polarizability.\textsuperscript{149,150}
Figure 2.3 demonstrates the influence that the overall size of the π structure has on solvatochromism for a series of DCDHF fluorophores with different acene π units. Increasing the conjugation length (from a single phenyl group P to larger naphthalene N and anthracene A) between the longitudinally situated donor and acceptor increases the sensitivity of Stokes shift to solvent polarity. This trend can be explained by the fact that the charge redistribution in the excited state occurs over a greater distance in the cases of naphthalene and anthracene, thus resulting in a larger change in the dipole moment between the ground and excited state. (This of course assumes that the magnitude of charge displaced does not change. In push–pull chromophores, the dipole moment eventually plateaus as the π-system is made very large.151)
2.3. DCDHF SENSITIVITY TO VISCOSITY

The fluorescence quantum yield ($\Phi_F$) of DCDHFs increases in rigid environments, as recognized in the early work of K.A. Willets\(^1\) and demonstrated in Figure 2.4. A strong dependence of fluorescence quantum yield on viscosity of the solvent was also observed.\(^1\) The working model for this dependence is a twisted intramolecular charge transfer (TICT) state that opens a nonradiative relaxation channel.\(^{152}\) Quantum-mechanical calculations have indicated that the excited-state structure with the lowest energy involves an approximately 90° rotation of the dicyanomethylene group around the methylene bond (labeled $\delta$ in Figure 2.5).\(^1\) In viscous media, such structural isomerizations are hindered, decreasing the probability of entering the TICT state (the pathway on the right in Figure 2.5). Because the radiative decay rate remains relatively unchanged, the nonradiative pathway becomes kinetically less accessible and the fluorescence quantum yield increases. It is worth noting that this mechanism relies on local effects in the immediate proximity of the molecule, and the strong connection between fluorescence and viscosity observed for various protic solvents and methanol-glycerol mixtures may arise from networks of hydrogen bonds than can form around the acceptor portion of the molecule.

Fluorescence-lifetime imaging microscopy\(^{153,154}\) is a currently available imaging modality which could be performed using DCDHF molecules. In Chapter 3, I report that the fluorescence lifetimes of bulk DCDHF-N samples depend on the solvent or environment in which the fluorophore resides. The fluorescence lifetime from one-photon excitation increases from below the instrument response time in polar ethanol to a few nanoseconds in nonpolar toluene and even higher in a film of the glassy polymer PMMA; the fluorescence lifetime from two-photon excitation exhibits a similar increase going from solution to polymer. The increase in lifetime can be correlated to the increase in quantum yield and is most likely the result of suppressing the non-emissive TICT relaxation channel in toluene and PMMA. This property could be used to follow dynamic changes in local environment on the single-molecule level or to map different regions within a sample, using fluorescence lifetime as contrast.
Figure 2.4. DCDHF fluorophores have higher fluorescence quantum yields in solvents with higher viscosity or when rigidized. Equal concentrations of fluorophore in liquid and frozen solvent solutions illuminated by a handheld UV lamp (365 nm), with a 500 nm long-pass filter placed before the lens of a digital camera in order to remove scattered excitation light and record only the fluorescence. In the rigid environment of the frozen solvents, emission dramatically increases. This fluorescence jump upon increase in local rigidity is characteristic of the entire class of DCDHF fluorophores and occurs in a range of solvents. From Lord et al.144

Other such “molecular rotors” have been reported in the literature.155-157 Experiments using DCDHF in cells have demonstrated the utility of obtaining strong fluorescence only from relatively viscous local environments: e.g. when studying the plasma membrane of living cells, the observed emission signal is brightest from fluorophores located in the lipid bilayer.54, 82 Because molecules that are not in the membrane are dim, they do not contribute to the fluorescence background. Therefore, there is less need for washing away unbound fluorophores or for background-reducing microscopy configurations (e.g. TIRF or confocal).
Figure 2.5. Proposed photophysical mechanism accounting for the interplay between radiative and nonradiative pathways of DCDHF fluorophores. DCDHF-P in its excited state $S_1$ can relax either via channel I to $S_{1\phi}$ or via channel III to $S_{1\delta}$. In channel III, a twist around the methylene bond $\delta$ produces a stable TICT state ($S_{1\delta}$), from which the molecule can relax back nonradiatively to ground state $\Gamma_0$. In viscous media, this twist is hindered and relaxation to the relatively planar $S_{1\phi}$ state via channel I becomes more competitive so that fluorescence emission can compete with the nonradiative pathway; thus, the fluorescence quantum yield increases. From Willets et al.\textsuperscript{1} and Lord et al.\textsuperscript{87}
2.4. PHOTOSTABILITY OF DCDHFS

Inherent photostability is one of the most important parameters of a single-molecule fluorophore: the more excitation–emission cycles each molecule survives before it photobleaches, the more it reports on its location, environment, orientation, diffusion kinetics, or whatever parameter the experiment aims to measure.

<table>
<thead>
<tr>
<th>fluorophore</th>
<th>( \Phi_B (10^{-6}) )</th>
<th>( N_{\text{photons}} (10^6) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCDHF-A</td>
<td>1.7</td>
<td>2.2</td>
</tr>
<tr>
<td>DCDHF-V-P</td>
<td>2.8</td>
<td>~1.9</td>
</tr>
<tr>
<td>DCDHF-N</td>
<td>3.4</td>
<td>1.1</td>
</tr>
<tr>
<td>R6G</td>
<td>3.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Texas Red</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>DCDHF-P</td>
<td>6.6</td>
<td>2.4</td>
</tr>
<tr>
<td>fluorescein</td>
<td>63.5</td>
<td></td>
</tr>
</tbody>
</table>

\( \Phi_B \) in gelatin, \( N_{\text{photons}} \) in PMMA
average standard error of the mean is ~30%

Singlet molecular oxygen, produced when triplet oxygen interacts with a fluorophore trapped in its triplet state, is often blamed as the culprit in many photodestruction reactions.\(^{158-160}\) Therefore, various oxygen-scavenging systems are often employed in single-molecule biophysics experiments to extend the survival time of the fluorophore.\(^{161,162}\) However, molecular oxygen is a potent triplet quencher,\(^{163}\) because the molecular oxygen triplet ground state helps couple the fluorophore triplet state back to the ground singlet state (thus increasing the rate of reverse intersystem crossing). Therefore, removing oxygen from solution can result in dim, intermittent fluorescence because emitters become trapped in long-lived dark states. Careful experiments have demonstrated that removing oxygen can even reduce the total number of emitted photons while simultaneously impeding the emission rate.\(^{163-166}\) Because oxygen removal can generate inconsistent behavior from structure to structure, fluorophores with inherently robust photostability are always desirable.
Two fundamental measures of fluorophore photostability are the total number of photons emitted for a single molecule before it permanently photobleaches ($N_{\text{photons}}$) and the photobleaching quantum yield ($\Phi_B$), which is the probability of photobleaching (or at least disappearing) for each photon absorbed. These two parameters generally scale inversely to each other ($N_{\text{photons}} = \Phi_F/\Phi_B$), but can also be regarded as independent useful tests of photostability, measured using different experiments and assumptions.

In Table 2.1, measured values for $N_{\text{photons}}$ and $\Phi_B$ are reported for several DCDHFs, as well as for the standard fluorophores Rhodamine 6G, Texas Red, and fluorescein. Many DCDHFs exhibit comparable or superior photostability to Rhodamine 6G, which is a demonstrably good single-molecule emitter; moreover, all DCDHFs measured are at least an order of magnitude more photostable than fluorescein. The Appendix contains a table that also reports extinction coefficients, absorption and fluorescence emission maxima, and photostability parameters for many DCDHF structures.

### 2.4.1. Single-Molecule Measurement: Total Photons Emitted

For the $N_{\text{photons}}$ measurement, single-molecule time traces of emission from microscope movies are used to extract the total number of detected photons before photobleaching molecule-by-molecule, where all the photons (minus background) contributing to a single-molecule spot are spatially and temporally integrated. Results from hundreds of single molecules are histogrammed or plotted versus a probability distribution, and an average number of photons detected is extracted from an exponential fit and Equation 2.5 below.

For the latter approach, the probability distribution used is the following:

$$P_N = mN/M,$$

or the ratio of the number of bleached singles $m$ surviving after a given number of emission cycles $N$ to the total number of molecules $M$ in the measurement set. In other words, the probability for any value of photons emitted is determined by counting the number of single molecules that emitted that number of photons or more divided by the total number of molecules in the population: if 50 molecule emitted at
least 500,000 photons before photobleaching, and the other 150 bleached before emitting 500,000, the probability $P_{500,000} = m_{500,000}/M = 50/200 = 0.25$. The value of $P_N$ is plotted for each value of $N$, and fitted using exponentials (see Figure 2.6). This approach for determining average photons detected avoids any artifact from choice of bin size, and gives comparable results to histogramming. (In either case, the value of $N$ plotted may be the detected values, requiring the final average to be converted using Equation 2.3, or the values may be converted to emitted photons before plotting.)

$$f = a\cdot\exp(-b\cdot x) + c\cdot\exp(-d\cdot x)$$

$$a = 0.6902$$
$$b = 1.8610\times10^{-5}$$
$$c = 0.3892$$
$$d = 3.3409\times10^{-6}$$

$N_{\text{photons, detected}} = 240,000$

$N_{\text{photons, emitted}} = N_{\text{photons, detected}}/D = 240,000/0.1 = 2.3\times10^6$

**Figure 2.6.** An example of measuring total photons emitted imaging many single copies of a fluorophore in a PMMA polymer film. The values of the total counts before photobleaching (or photons, in this case, because I divided the counts by the camera gain before plotting) for all the molecules are ordered smallest to largest value of $N$; these are then plotted against a probability distribution. The probability distribution used is $P_N = m/M$, the ratio of the number of bleached singles $m$ surviving after a given number of emission cycles $N$ to the total number of molecules $M$ in the measurement set. In other words, the probability for any value of photons emitted is determined by the percent of single molecules that emitted that number of photons or more.
To convert from photons detected to photons emitted, the camera gain—both electron-multiplying gain $G_{EM}$ and conversion gain $G_{A/D}$ (defined as the number of analog-to-digital converter counts per photoelectron)—as well as collection efficiency $D$ of the microscopy setup must be measured. The collection efficiency is given by $D = \eta Q F_{coll} F_{opt} F_{filter}$, which is the product of the camera quantum efficiency $\eta Q$ of converting incident photons into photoelectrons, the angular collection factor $F_{coll}$ determined by the objective NA, the transmission factor through the objective and microscope optics $F_{opt}$, and the transmission factor through the various filters $F_{filter}$, respectively. $^{21}$ Typical values of $D$ are approximately 5–15% for epifluorescence; values for confocal are lower. The conversion from camera counts to total photons emitted is:

$$N_{\text{photons}} = \frac{1}{D} N_{\text{detected}} = \frac{1}{D} \frac{\text{camera counts}}{G_{EM} G_{A/D}} = \frac{\text{camera counts}}{\eta Q F_{coll} F_{opt} F_{filter} G_{EM} G_{A/D}}. \quad (2.3)$$

The gain values $G_{EM}$ and $G_{A/D}$ should be checked regularly, as they can change over months. $G_{EM}$ can be measured by simply imaging a sample of constant irradiance (and a completely dark sample) at many different EM gain values. The $G_{EM}$ value is calculated by subtracting the mean signals of the dark samples from the mean signals from the constant-irradiance sample, then taking the ratio of the mean dark-subtracted camera signal with EM gain on and the mean dark-subtracted signal with EM gain off. For the Andor iXon+, the measured gain was very similar to the software setting (e.g. 250 gain setting corresponded to a true EM gain of 225). The conversion gain $G_{A/D}$ can be calculated with various methods. A simple approach takes advantage of shot noise statistics and involves imaging a dim sample at various irradiances, plotting the variance of the image versus the mean signal at each intensity level, then extracting the conversion gain from the inverse of the slope of the resulting straight line. For details, see the supporting information of Thompson et al.$^{170}$

True photon-counting detectors such as avalanche photodiodes provide an alternative detection scheme which reduces error introduced by inaccuracies in camera gain values, but imaging with such detectors requires confocal scanning and measuring molecules one at a time.
Note that the $N_{\text{photons}}$ calculation does not depend on irradiance, wavelength, or extinction coefficient (unless the molecule is excited so strongly as to experience excitation from the first excited state to higher excited or triplet states that leads to premature bleaching, and this regime is to be avoided).

### 2.4.2. Bulk Measurement: Photobleaching Quantum Yield

A complementary ensemble measurement, the photobleaching quantum yield $\Phi_B$, defined as the probability of photobleaching per photon absorbed, can be extracted from study of a bulk sample. Plots of integrated, background-subtracted emission intensities versus time for bulk samples are fit using one or more exponentials, and the time constant $\tau_B$ is extracted from these photobleaching curves (see Figure 2.7).\(^9^3\)

**Figure 2.7.** Example bulk photobleaching curve of R6G in gelatin. At time = 0 s, the 488 nm laser begins illuminating the sample and the camera records the fading of fluorescence. The integrated counts are arbitrary units, because the photobleaching calculation uses the (weighted) decay constants. The three-exponential fit used here included a vertical offset from zero to account for background in the camera counts.
From these plots, the ratio of the bleaching rate \( R_B \) to the absorption rate \( R_{\text{abs}} \) can be calculated:

\[
\Phi_B = \frac{R_B}{R_{\text{abs}}} = \frac{1}{\tau_B R_{\text{abs}}} = \frac{1}{\tau_B \sigma_\lambda I_\lambda \left( \frac{\lambda}{hc} \right)},
\]

(2.4)

where \( \tau_B \) is the decay constant in the exponential fit to the decay curve, the absorption cross-section at the excitation wavelength \( \sigma_\lambda \) is related to the molar absorption coefficient \( \varepsilon_\lambda \) by the equation

\[
\sigma_\lambda = \ln(10)\varepsilon_\lambda / N_A = 2303\varepsilon_\lambda / N_A \approx 10^{-16} \text{ cm}^2
\]

for a good absorber, \( I_\lambda \) is the excitation irradiance at the sample, \( \lambda \) is the excitation wavelength, \( h \) is Planck’s constant, and \( c \) is the speed of light. If measured under similar conditions to a single-molecule imaging experiment but at approximately an order of magnitude higher concentration, \( \Phi_B \) is an independent check of the photostability: the smaller the value of \( \Phi_B \), the more slowly the fluorescence from sample fades—and less likely the fluorophore is to disappear or photobleach.

Bulk photobleaching decay curves are often not single exponential, and the average decay constant for a two-exponential fitting function,

\[
\sum_{i=1}^{n} \alpha_i e^{(-t/\tau_i)}
\]

is given by:

\[
\bar{\tau} = f_1\tau_1 + f_2\tau_2 = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2}{\alpha_1\tau_1 + \alpha_2\tau_2},
\]

(2.5)

where \( f_i = \alpha_i\tau_i / \sum_j \alpha_j\tau_j \) is the fractional area under the multi-exponential curve.\(^{149}\) (Some other authors use \( t_{90\%} \), the irradiation time in seconds for 90% conversion to product, as a more practical measure than the decay constant \( \bar{\tau} \); compare values carefully).

Connecting the two kinds of measurements, the bulk number of photons emitted can be calculated from the inverse of the photobleaching quantum yield and the fluorescence quantum yield \( \Phi_F \):

\[
N_{\text{photons}} = N_{\text{abs}} \Phi_F = R_{\text{abs}} \tau_B \Phi_F = \sigma_\lambda I_\lambda \left( \frac{\lambda}{hc} \right) \tau_B \Phi_F = \frac{\Phi_F}{\Phi_B}.
\]

(2.6)

This technique is independent of the photon losses in the microscope, so it is a separate corroboration of the single-molecule measurement. For instance, a bulk
measurement yielded $N_{\text{photons}} = 2.6 \times 10^6$ photons emitted per DCDHF-N molecule, comparable to the single-molecule value of $1.4 \times 10^6$. As an independent check of this method of calculating $N_{\text{photons}}$, I extracted the bulk value of number of photons emitted per rhodamine 6G molecule in PVA from bulk photobleaching and found $1.4 \times 10^6$, which is comparable to the published value of $1.9 \times 10^6$ photons emitted per Rhodamine 6G molecule in ethanol.\textsuperscript{95} Because of experimental variability (standard errors of the mean for $N_{\text{photons}}$ or $\Phi_B$ can be as high as 30%) and differences in fluorescence quantum yields among fluorophores, the two photostability measures may not be exactly inverse; however, trends in each measure should be similar among fluorophores.

2.5. CELL AND BIOLOGICAL IMAGING WITH DCDHFS

2.5.1. Screening in Cell-like Media

Imaging single molecules in living cells has strict criteria: not only must the emitter be bright and photostable, but also it must be red-shifted enough to avoid background from cellular autofluorescence from flavins and other endogenous fluorophores pumped at wavelengths shorter than approximately 500 nm,\textsuperscript{90} be water soluble and membrane-permeable, be washable after incubation, and be specifically targetable.

Quantifying photophysical parameters of single molecules in living cells is not always possible. A more practical approach is to screen fluorophores in cell-like media such as protein gels, agarose gels, or PVA aqueous films. For instance, Figure 2.8A shows single copies of acene DCDHF fluorophores embedded in gelatin, an aqueous protein gel. Photostability parameters ($N_{\text{photons}}$ or $\Phi_B$) measured in gelatin offer a better measure of how well the fluorophore will perform in cells than testing only in organic solvents and polymer films.

Nevertheless, the ultimate test of a fluorophore is whether it is visible on the single-molecule level in a living cell. Figure 2.8B–C shows different DCDHF
molecules in the plasma membrane of a living Chinese hamster ovary (CHO) cell. Although these are only qualitative measures, they are evidence for the utility of these fluorophores as cellular probes.

**Figure 2.8.** Examples of DCDHF fluorophores used in aqueous media and in cells. (A) Epifluorescence images of DCDHFs in gelatin. *(left)* Surface plot of emission from single DCDHF-N molecules in a gelatin film. *(right)* Single DCDHF-A molecules imaged in a gelatin film. From Lord et al.144 *(B)* Three CHO cells incubated with fluorogen DCDHF-V-P-azide (see Chapter 4) are bright after activation. The fluorophore DCDHF-V-P-amine lights up in the cells. False color: red is the white-light transmission image and green shows the fluorescence images, excited at 594 nm. Scalebar: 20 μm. *(inset)* Single molecules of activated DCDHF-V-P-amine in a cell under higher magnification. Background was subtracted and the image was smoothed with a 1×1-pixel Gaussian. Scalebar: 800 nm. From Lord et al.99
2.5.2. DCDHFs as Lipid Analogs

Quantifying the brightness and photostability of emitters in cells, while difficult, is ultimately necessary to confirm their efficacy. For instance, S. Nishimura measured the diffusion behavior of singles of seven DCDHF structures in the cell membrane, and found by measuring signal-to-background levels and total photons detected that DCDHF-N performed comparably to TRITC, a Rhodamine derivative.

![Figure 2.9. DCDHF lipid analogs and their spectra in ethanol. (A) Structures of DCDHF derivatives used as membrane probes and their names in this figure. (B) Normalized absorption spectra for the DCDHF derivatives shown in A. (C) Normalized emission spectra for the DCDHF derivatives shown in A. The excitation wavelengths used to obtain the emission spectra are 460 nm for ZL01_029Y, DCDHF-18, and ZL01_032Y, 488 nm for DCDHF-CF3, 514 nm for DCDHF-N-12, and 594 nm for TH-DCDHF-6V. DCDHF-N-6 and DCDHF-N-12 have identical spectra, so only one is shown. The fluorescence spectrum of DCDHF-CF3 exhibits a small shoulder, which could be attributed to poor solubility in ethanol. From Nishimura et al.]

For this experiment, DCDHF versions with long alkyl chains on the amine donor were synthesized by the Twieg group at Kent State in order to mimic the polar head and nonpolar tail of membrane lipids (Figure 2.1). These DCDHF lipid analogs insert well into the lipid bilayer, where higher viscosity brightens the fluorophore to the point that it is visible and trackable, even with EMCCD camera integration times as short as 15–30 ms (Figure 2.8B). Charged DCDHF versions exhibited slower
diffusion kinetics than the neutral versions, possibly indicating that they partitioned into different lipid environments, or possibly resulting from stronger association of neighboring lipid molecules around the additional charges.

**Methods for Cell Imaging**

Movies of acene DCDHFs in the plasma membrane (see Chapter 3) were recorded using a back-illuminated, frame-transfer, electron-multiplication Si EMCCD camera (Andor iXon or Roper Cascade). Samples were made by incorporating the probe into CHO cells as follows. A fluorophore film was made by drying 5 μL of an 80 μM solution of DCDHF-A in chloroform onto a glass coverslip. This film was reconstituted with 50 μL of ethanol, 4 μL of this ethanol solution was added to 400 μL of RPMI 1640 cell medium without phenol red (Gibco). The cells were incubated with 440 μL of ~80 nm final dye/RPMI solution for 20 min at 37°C. The solution was then aspirated and the cells were gently washed one time with RPMI 1640 medium which was then replaced by the CHO cell imaging buffer. (For further experimental details, components of the CHO imaging buffer, and imaging conditions for DCDHF-N, see reference 54.)

2.5.3. **Labeling Biomolecules with DCDHFs**

**Reactive Forms of DCDHFs**

Labeling biomolecules requires some type of reactivity. For instance, some experiments have used a DCDHF derivative with reactive maleimide functionality; N-hydroxysuccinimide (NHS) variants have also been prepared. In most cases, adding reactive functionality to DCDHFs via single-bonded alkyl attachments had no affect on the photophysics, because the added groups do not significantly interact electronically with the fluorophore’s charge-transfer absorption or other photophysical properties.

**Targeted Labeling with HaloTag**

For specific labeling in living cells, some targeting scheme is necessary (see Chapter 1). Here, I demonstrate enzymatic labeling with a HaloEnzyme fused to a protein of interest and a HaloTag on a DCDHF fluorophore (Figure 2.10A). The
HaloEnzyme recognizes and forms a covalent linkage to the HaloTag, thus creating a protein–HaloEnzyme–HaloTag–fluorophore covalent complex (Figure 2.10B). In this fashion, the fluorophore specifically labels the protein of interest.

![Image](image1.png)

**Figure 2.10.** Living *C. crescentus* cells expressing HaloEnzyme fused to the cytoskeletal protein FtsZ, labeled by incubating with a HaloTag version of DCDHF-V-PO-amine (in A). (B) The HaloEnzyme system is a modified haloalkane dehalogenase designed to covalently bind to synthetic HaloTag ligands (the chloroalkane tail on the fluorophore). (C–D) FtsZ is known to form a tight ring in the center of the cell; note the tight fluorescence localized at the ring in most of the cells. False color overlay: red is fluorescence; grey is white-light transmission image. The image in C is ~17×17 μm; the image in D is ~5×8 μm.

For example, experiments were performed to label the FtsZ cytoskeletal protein in bacteria. The protocol for labeling *C. crescentus* cells prepared by the Shapiro lab expressing FtsZ–HaloEnzyme with a HaloTag DCDHF is as follows. Cells were grown in PYE growth medium, then in M2G minimal-fluorescence buffer (see

51
Appendix), to which was added 0.3% xylose to induce expression of the HaloEnzyme version of FtsZ. After inducing for 1 h, the cells were centrifuged at 13.4 krpm for 60 s to pellet and rinsed with clean M2G buffer. To label with the fluorescent probe, 8% DMSO was added to the cells and the HaloTag DCDHF in DMSO was added very slowly to a final concentration of 1 nM. After 90 min incubation, cells were rinsed with clean M2G 3×, incubated for 30 min in clean M2G, then rinsed 6× more. Cells were then placed on an agarose pad and imaged (Figure 2.10).

Bioorthogonal Reactions with DCDHFs

I designed DCDHF derivatives that can participate in “click” (i.e. the copper-catalyzed reaction between an azide and an alkyne to produce a triazole) or other bioorthogonal reactions for cell labeling. See Chapter 5 for full details.

2.6. GENERAL METHODS FOR MEASURING DCDHF PHOTOPHYSICS

2.6.1. Bulk Solution Photophysical Characterization

Bulk solution absorption were acquired on a Perkin-Elmer Lambda 19 or Cary 6000i UV–vis spectrometer and emission spectra on a Horiba Fluoromax-2 or Fluorolog-3 fluorimeter, using a standard 1 cm path length, quartz cuvette. Fluorescence quantum yields were referenced against standards with known quantum yields, corrected for differences in optical density and solvent refractive index. For instance, DCDHF-P and DCDHF-N in toluene were measured against Rhodamine 6G in ethanol ($\Phi_F = 0.95$) and the quantum yield of DCDHF-A in toluene was measured against Texas Red in ethanol ($\Phi_F = 0.93$). Fluorophores in other solvents were generally measured against their own values in toluene. All quantitative measurements were done at low concentrations (absorbance values less than 0.2) to avoid any complications with dimer or aggregate formation. Extinction coefficients were measured from dilutions of solutions with known concentrations.
2.6.2. Bulk Photophysics in Polymer Films

For bulk spectra in polymer films, a small amount of highly concentrated dye solution was mixed into a 20% (by mass) solution of poly(methyl methacrylate) (PMMA, T_g = 105°C, MW = 75,000 g/mol, atactic, polydispersity ~2.8, PolySciences Inc.) in toluene, then the solution was spin-cast onto a glass slide producing a film 20μm thick as measured by ellipsometry. An option for making even thicker films is drop-casting the polymer solution onto a glass slide, adding enough to fill the slide but not so much that the surface tension breaks and the solution flows off the glass. For absorption spectra, the slide was placed orthogonal to the beam with an undoped film in the reference channel; for emission spectra, the slide was placed in an orientation so as to reject the scattered excitation light. Absorption and emission were measured at several locations throughout the film to compensate for macroscopic heterogeneity in film thickness and coverage, which otherwise would result in variations in absorbance and emission signals.

2.6.3. Measuring Bulk Fluorescence Lifetime

Fluorescence-lifetime experiments on the acene DCDHF (D, C, D, H, F) (see Chapter 3) were performed using a home-built scanning-stage confocal microscope based upon a commercial inverted microscope (Nikon TE300). Samples were excited using 532 nm illumination from an optical parametric oscillator (Coherent Mira-OPO) pumped by a mode-locked Ti:sapphire laser (Coherent Mira 900) with a repetition rate of 75 MHz and pulses 120 fs in duration; the Ti:sapphire was pumped by an Ar-ion laser (Coherent Innova 200). For two-photon fluorescence lifetime measurements, 800 nm light was used directly from the mode-locked Ti:sapphire laser. Emitted photons passed through a 545 nm dichroic and two 545 nm long-pass filters, and were detected with a single-photon avalanche photodiode (Micro Photon Devices PDM) equipped with a time-correlated single-photon counting data analysis board (PicoQuant TimeHarp200). The instrument-response function was recorded using scattered excitation light from a clean glass coverslip (the two long-pass filters were removed and OD filters were added to the beam path). The instrument-response function was deconvolved from the data, and then fit using a maximum-likelihood estimator.
2.6.4. Methods for Imaging Single-Molecules in Polymer Films

Samples for quantitative single-molecule studies were prepared in 1% (by mass) solutions of PMMA in distilled toluene doped with nanomolar fluorophore concentrations; these solutions were then spin-cast onto plasma-etched glass coverslips to produce films 30 nm thick as measured by ellipsometry. (The solvent distillation and plasma treatment of the substrates is done to remove spurious sources of fluorescence.) Samples were studied using a Nikon Diaphot 200 or Nikon IX71 inverted microscope in an epifluorescence configuration using one of the following lasers: 407 nm Kr-ion laser (Coherent Innova-301); 488 nm solid-state laser (Novalux Protera 488-20); the 514 nm line from an Ar-ion laser (Coherent Innova); 532 nm light from a continuous-wave doubled Nd:YAG laser (Spectra-Physics Millenia); or the 594 nm line from a HeNe laser (Meredith Instruments, 5 μW output). The irradiance of the excitation at the samples was between 0.25 and 2 kW/cm². The emission was collected through either a 60× or 100×, 1.4–1.45 N.A. oil-immersion objective, filtered using appropriate dichroic and long-pass filter to remove scattered excitation light, and imaged onto a back-illuminated, frame-transfer Si CCD camera (e.g. Roper Scientific MicroMAX or Andor iXon). Typical frame-integration times were 50–100 ms.

2.6.5. Methods for Photostability Measurements

SMS Photostability Measurements in PMMA

Sequences of single-molecule images (i.e. movies) were used to extract the total number of detected photons before photobleaching, where all the photons (minus background) contributing to a single-molecule spot were spatially and temporally integrated. For further details on the data analysis, refer to the Appendix.

Bulk Photostability Measurements in Gelatin

Samples for aqueous bulk photostability measurements and single-molecule images were prepared using 5% (by mass) gelatin (type A, Bloom ~200, MP
Biomedicals) in purified water. The gelatin solution was liquefied at 37°C. A small volume (<0.5 μL) of dye stock solution in dimethylsulfoxide was mixed with 10 μL gelatin, sandwiched between two glass coverslips, and allowed to gel at room temperature. Samples were imaged using the microscope and CCD camera setup described above.

2.7. CONCLUSION

The DCDHF's represent a broad and versatile class of fluorophore with applications in single-molecule imaging in living cells. We have designed and characterized many different DCDHF derivatives (the Appendix contains a table with many different structures and their photophysical properties). In general, the DCDHF's are photostable, exhibit sensitivity to their local environment, and can be imaged on the single-molecule level in biologically relevant media. In the next chapter, I will discuss the various approaches we have taken to modify the photophysics of DCDHF's using structure–property relationships.
3.

STRUCTURE–PROPERTY DESIGN OF DCDHF FLUOROPHORES
Because the DCDHF fluorophores are synthetically flexible—meaning that the basic physical characteristics discussed in Chapter 2 are maintained even with different π-conjugated linkers or different groups added to the donor or acceptor end—we have been able to explore the relationships between the structure of the compound and its photophysical properties. For instance, by modifying the π groups, we have changed the color of the fluorophores. By restricting twists of certain bonds in the structure, we can brighten the fluorescence. In this chapter, I will discuss the various structure–property relationships we have evaluated.

3.1. RED-SHIFTING DCDHFS USING ACENE GROUPS

One of the more straightforward structure–property relationships with DCDHFs and other push–pull chromophores is the red-shift that accompanies an increase in the length of the conjugated linker between the donor and acceptor. Assuming a particle-in-a-box model, increasing the length of the roughly linear push-pull DCDHF fluorophore should lower the energy and spacing between the ground and excited state, and thus produce red-shifted absorption. This can be seen, for example, in going from DCDHF-P to DCDHF-V-P: there is a 75 nm absorption red-shift (in toluene) with the addition of a vinyl group.
Figure 3.1. Normalized absorption and fluorescence emission spectra of DCDHF-P, DCDHF-N, and DCDHF-A in toluene (going from the short phenyl group to the longer naphthyl and anthryl). Absorption for DCDHF-N is at long enough wavelengths for excitation at 514 or 532 nm, thus avoiding much of the cellular autofluorescence; DCDHF-A can be excited at 532 or 594 nm. Note also the enhanced Stokes shift of DCDHF-A (104 nm) and DCDHF-N (53 nm) over that of DCDHF-P (21 nm). From Lord et al.144

Because cis-trans isomerism and bond twists involving the vinyl group can reduce the fluorescence quantum yield, it may be preferable to extend the conjugation using more rigid groups.144 For instance, replacing the phenyl group with a naphthalene or anthracene increases the absorption wavelength in toluene 40 nm for DCDHF-N and nearly 100 nm for DCDHF-A (see Figure 3.1). The table in the Appendix includes various other red DCDHF fluorophores with combinations of vinyl, phenyl, and thiophene linkers.
Figure 3.2. (A) Three acene DCDHF structures with increasing conjugation, and thus increasing absorption wavelength. (B) Demonstrating the quantum-yield dependence on viscosity: in rigidified frozen samples, the fluorescence emission increases drastically. The “6” in the names refer to the six carbon tails on the amine. From Lord et al.144

The derivatives DCDHF-N, which contains a 2,6-naphthyl linker between the donor and acceptor, and DCDHF-A, with a 2,6-anthryl linker (see Figure 3.2), not only maintain the solvatochromism and viscosity sensitivity characteristic of their phenyl-containing sister DCDHF-P but also can be excited at 514 nm, 532 nm, or longer (where cellular autofluorescence background is drastically suppressed).54, 90
Table 3.1. Spectral parameters of DCDHF-P, DCDHF-N, and DCDHF-A in a representative range of liquid solvents. From Lord et al.\textsuperscript{144}

<table>
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<th>solvent</th>
<th>(\Phi_F)</th>
<th>(\varepsilon_{\text{max}}) (M(^{-1}) cm(^{-1}))</th>
<th>(\lambda_{\text{abs}}^\text{max}) (nm)</th>
<th>(\lambda_{\text{em}}^\text{max}) (nm)</th>
<th>SM (N_{\text{photons}})</th>
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<tbody>
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<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td>486</td>
<td>507</td>
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<td></td>
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<tr>
<td>PMMA</td>
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<td>594</td>
<td>686</td>
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<td>35,000</td>
<td>585</td>
<td>689</td>
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</tr>
<tr>
<td>acetone</td>
<td>0.023</td>
<td>29,000</td>
<td>588</td>
<td>826</td>
<td></td>
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<tr>
<td>ethanol</td>
<td>0.013</td>
<td>29,000</td>
<td>602</td>
<td>826</td>
<td></td>
</tr>
<tr>
<td>water</td>
<td>&lt;0.001</td>
<td></td>
<td>~600</td>
<td>&gt;830</td>
<td></td>
</tr>
</tbody>
</table>

\(\Phi_F\) is the fluorescence quantum yield, \(\varepsilon_{\text{max}}\) is the maximum molar extinction coefficient, \(\lambda_{\text{abs}}^\text{max}\) and \(\lambda_{\text{em}}^\text{max}\) are the wavelengths of maximum absorption and emission, and SM \(N_{\text{photons}}\) is the average total number of photons emitted per molecule before photobleaching measured molecule-by-molecule.

The naphthyl DCDHF was used as a membrane probe: single copies were studied as lipid analogs to probe diffusion in the plasma membrane of eukaryotic cells.\textsuperscript{54} Here, I report the fundamental photophysical properties and environmental dependence of two acene DCDHFs in controlled conditions (e.g. in solvents with a range of polarities and in polymer films) in order to better judge and understand the full utility and potential of these probes.

### 3.1.1. Bulk Photophysical Properties of Acene DCDHFs

As mentioned above, a key goal of this work is to identify derivatives in the DCDHF class with long-wavelength absorption and emission where cellular autofluorescence is diminished. In most cases, pumping with wavelengths of 488 nm or shorter produces strong emission from essential cellular components such as flavins.\textsuperscript{90} As can be seen in Figure 3.1 and Table 3.1, in contrast to DCDHF-P, the new acene DCDHFs can be efficiently pumped using green or yellow light (e.g. 532 nm) and exhibit large Stokes shifts, further enabling filtering to reject pumping light. Moreover, a full study of DCDHF-N and DCDHF-A solvent photophysics reveals that the reporter functions discovered in DCDHF-P apply to the red-shifted dyes. Below I
discuss the properties of these fluorophores that are beneficial for cellular imaging (including turn-on fluorescence upon rigidization, strong polarity sensitivity of emission wavelength and fluorescence lifetime, and bright emission), which make the red DCDHFs useful for various imaging modalities. Methods for these studies are described in Chapter 2.

**Viscosity Sensitivity of Acene DCDHFs**

As is typical of many other DCDHF derivatives we have explored (see Chapter 2), the acene DCDHFs exhibit sensitivity to solvent viscosity: the fluorescence quantum yield increases with increasing rigidity of the host. For example, compare the quantum yields in solution and in polymer listed in Table 3.1 and note the increased brightness upon freezing solutions seen in Figure 3.2. The previously proposed mechanism for this viscosity-dependence involves a twist of the dicyanomethylene double bond, which introduces a nonradiative relaxation pathway via a TICT state (see Figure 2.5). Our own repetition of the original Hartee-Fock calculations on DCDHF-N showed no significant departure from previous results (data not shown). While these calculations suggest that the sensitivity to local environment originates from the same intramolecular twist, they do not provide any insight into the enhanced quantum yield of the acene dyes over DCDHF-P; however, a mechanism could be revealed by a full photophysical study of the relaxation pathways, including a more extensive computational study.
Figure 3.3. Environment-sensitivity spectroscopic data from Table 3.2 and Table 3.3. (A) Log of fluorescence quantum yield as a function of the log of viscosity (relative to water $\eta = 1.01$ cP) for DCDHF-N. The data for the fluorophore in alcohols are fit by a line with slope of 0.89 ($R^2 = 0.91$). The fluorophore does not exhibit this obvious trend in other solvents. (B) Lippert plot of emission Stokes shift versus the polarity parameter $\Delta f$ (see Chapter 2 for definition) for DCDHF-N in all solvents in Table 3.2 and DCDHF-A for a range of solvents. The data for DCDHF-N are fit to a line with slope of 6921 cm$^{-1}$ ($R^2 = 0.86$); the fit for the DCDHF-A data has a slope of 7757 cm$^{-1}$ ($R^2 = 0.88$). From Lord et al.¹⁴⁴

Viscosity sensitivity for DCDHF-N is quantified experimentally in the data in Table 3.2 and Figure 3.3A, which plots the logarithm of quantum yield versus the logarithm of solvent viscosity. Just as was previously seen with DCDHF-P,¹ the
alcohol solvents show a definite correlation between DCDHF-N emission probability and solvent viscosity, whereas other solvents do not exhibit a single trend. (As a side comment, note that the high quantum yield of DCDHF-N in toluene and benzene as compared to cyclohexane in Table 3.2 is observed to some degree in all DCDHF derivatives studied, and is likely due to some specific solvent interaction—possibly \(\pi\)–\(\pi\) stacking between the solvent and the fluorophore—that restricts intramolecular rotation pathways.)

Table 3.2. Solvatochromic properties of DCDHF-N.

<table>
<thead>
<tr>
<th>solvent</th>
<th>(\Phi_F)</th>
<th>(\lambda_{\text{abs}}^\text{max}) (nm)</th>
<th>(\lambda_{\text{em}}^\text{max}) (nm)</th>
<th>n</th>
<th>(\varepsilon_r)</th>
<th>(\Delta f)</th>
<th>(\eta) (cp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>0.003</td>
<td>550</td>
<td>675</td>
<td>1.3330</td>
<td>78.5</td>
<td>0.3193</td>
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</tr>
<tr>
<td>acetonitrile</td>
<td>0.014</td>
<td>543</td>
<td>664</td>
<td>1.3442</td>
<td>37.5</td>
<td>0.3053</td>
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<tr>
<td>acetone</td>
<td>0.015</td>
<td>533</td>
<td>660</td>
<td>1.3588</td>
<td>20.70</td>
<td>0.2843</td>
<td>0.316</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.015</td>
<td>549</td>
<td>675</td>
<td>1.4770</td>
<td>47.2</td>
<td>0.2640</td>
<td>1.98</td>
</tr>
<tr>
<td>THF</td>
<td>0.18</td>
<td>529</td>
<td>636</td>
<td>1.4050</td>
<td>7.52</td>
<td>0.2096</td>
<td>0.55</td>
</tr>
<tr>
<td>ethyl acetate</td>
<td>0.21</td>
<td>523</td>
<td>623</td>
<td>1.3723</td>
<td>6.02</td>
<td>0.1997</td>
<td>0.455</td>
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<tr>
<td>ethyl ether</td>
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<td>518</td>
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<td>0.2332</td>
</tr>
<tr>
<td>chloroform</td>
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<td>554</td>
<td>613</td>
<td>1.4459</td>
<td>4.806</td>
<td>0.1482</td>
<td>0.58</td>
</tr>
<tr>
<td>isopropyl ether</td>
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<tr>
<td>dipropylamine</td>
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<td>0.0826</td>
<td>0.53</td>
</tr>
<tr>
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<td>1.4010</td>
<td>2.42</td>
<td>0.0477</td>
<td>0.363</td>
</tr>
<tr>
<td>toluene</td>
<td>0.85</td>
<td>526</td>
<td>579</td>
<td>1.4961</td>
<td>2.379</td>
<td>0.0134</td>
<td>0.59</td>
</tr>
<tr>
<td>benzene</td>
<td>0.88</td>
<td>525</td>
<td>581</td>
<td>1.5011</td>
<td>2.284</td>
<td>0.0030</td>
<td>0.652</td>
</tr>
<tr>
<td>n-heptane</td>
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<td>537</td>
<td>1.3878</td>
<td>1.9</td>
<td>−0.0034</td>
<td>0.55</td>
</tr>
<tr>
<td>cyclohexane</td>
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<td>541</td>
<td>1.4266</td>
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<td>−0.0014</td>
<td>0.898</td>
</tr>
</tbody>
</table>

*Alcohols*

<table>
<thead>
<tr>
<th>solvent</th>
<th>(\Phi_F)</th>
<th>(\lambda_{\text{abs}}^\text{max}) (nm)</th>
<th>(\lambda_{\text{em}}^\text{max}) (nm)</th>
<th>n</th>
<th>(\varepsilon_r)</th>
<th>(\Delta f)</th>
<th>(\eta) (cp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol</td>
<td>0.013</td>
<td>540</td>
<td>660</td>
<td>1.3288</td>
<td>32.63</td>
<td>0.3084</td>
<td>0.597</td>
</tr>
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<td>1.3611</td>
<td>24.30</td>
<td>0.2886</td>
<td>1.2</td>
</tr>
<tr>
<td>2-propanol</td>
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<td>543</td>
<td>651</td>
<td>1.3776</td>
<td>18.3</td>
<td>0.2729</td>
<td>2.4</td>
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<td>1-butanol</td>
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<td>546</td>
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<td>2.948</td>
</tr>
<tr>
<td>benzyl alcohol</td>
<td>0.076</td>
<td>560</td>
<td>654</td>
<td>1.5396</td>
<td>13.1</td>
<td>0.2061</td>
<td>5.8</td>
</tr>
</tbody>
</table>

*Left columns* Measured properties of fluorophore: fluorescence quantum yield (\(\Phi_F\)) and absorption and emission wavelengths. *Right columns* Solvent properties reported in references 174, 175: relative dielectric constant (\(\varepsilon_r\)), index of refraction (n), orientation polarizability (\(\Delta f\)), and viscosity (\(\eta\)). The excitation wavelength was 488 nm. The \(\Phi_F\) of DCDHF-N in toluene referenced against Rhodamine 6G in ethanol (0.95); all other solvents measured against DCDHF-N in toluene. The rows are ordered by decreasing \(\Delta f\). From Lord et al.144

The small viscosity range in Figure 3.3A covers only one decade, so it does not represent the large increase in quantum yield that occurs in more rigid environments: the plot is only a small portion of the dynamic range. Figure 3.2 better illustrates the dramatic jump in brightness as a solution of DCDHFs in liquid solvent is frozen to
form a rigid environment. To partially quantify the full increase of fluorescence, I measured the quantum yield of DCDHF-N in a PMMA polymer film to be nearly unity, while that in ethanol is only 1.7% (see Table 3.1). A similar increase in the fluorescence is demonstrated for DCDHF-A in Figure 3.2 and Table 3.1. We attribute this jump in brightness to the drastic change in viscosity, given that PMMA is still quite polar, approximately equivalent to dimethylsulfoxide.\(^{176}\)

<table>
<thead>
<tr>
<th>Table 3.3. Solvatochromic properties of DCDHF-A</th>
<th>(\lambda_{\text{abs}}) max (nm)</th>
<th>(\lambda_{\text{em}}) max (nm)</th>
<th>(n)</th>
<th>(\varepsilon_r)</th>
<th>(\Delta f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>600</td>
<td>830</td>
<td>1.3330</td>
<td>78.5</td>
<td>0.3193</td>
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<tr>
<td>acetonitrile</td>
<td>586</td>
<td>801</td>
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<td>0.3053</td>
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<td>DMF</td>
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<td>804</td>
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<td>36.7</td>
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<tr>
<td>DMSO</td>
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<td>811</td>
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<td>0.2640</td>
</tr>
<tr>
<td>THF</td>
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<td>1.4050</td>
<td>7.52</td>
<td>0.2096</td>
</tr>
<tr>
<td>chloroform</td>
<td>626</td>
<td>743</td>
<td>1.4459</td>
<td>4.806</td>
<td>0.1482</td>
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<td>toluene</td>
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<td>1.4961</td>
<td>2.379</td>
<td>0.0134</td>
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<td>0.3084</td>
</tr>
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<td>774</td>
<td>1.4101</td>
<td>13.9</td>
<td>0.2493</td>
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</table>

(Solvent properties reported in references 174, 175: relative dielectric constant (\(\varepsilon_r\)), index of refraction (\(n\)), and orientation polarizability (\(\Delta f\)). The rows are ordered by decreasing \(\Delta f\). From Lord et al.\(^{144}\))

**Solvatochromism of Acene DCDHFs**

In solution, the acene fluorophores exhibit strong solvatochromism in addition to the viscosity sensitivity discussed above. Table 3.2 and Table 3.3 list absorption and emission maxima, fluorescence quantum yield, and molar absorption coefficient (\(\varepsilon_{\text{max}}\)) for DCDHF-N and DCDHF-A measured in various solvents. To explore the dependence of the Stokes shift on the solvent polarity, I used the Lippert–Mataga formalism for general solvent polarity effects (see Chapter 2).\(^{149}\) Recalling that DCDHFs have an asymmetric donor–acceptor motif, it is reasonable to expect changes in the molecular dipole moment upon excitation: the charge-transfer state can be stabilized by solvent dipoles rearranging around the excited fluorophore; thus, the extent of stabilization depends on the polarity of the solvent. The Lippert plots in
Figure 3.3B show the Stokes shift versus $\Delta f$, and from the slopes I calculated the change in the dipole moment upon excitation to be $\Delta \mu = |\mu_E - \mu_G| = 9.4$ D for DCDHF-N and 9.7 D for DCDHF-A. These values are significantly greater than the value of 4.4 D for DCDHF-P, in other words, the acene derivatives exhibit stronger sensitivity to solvent polarity than their phenyl sister. Moreover, because DCDHF-N and DCDHF-A exhibit red-shifts in emission of over 100 nm going from nonpolar to polar media (see Table 3.1), it should be possible to record emission spectra to harness this reporter function so as to monitor polarity changes in the immediate local environment of single molecules.

**Fluorescence Lifetime of Acene DCDHFs in Different Environments**

Fluorescence-lifetime imaging microscopy has been popularized as a means to detect local environmental differences, and it is not unexpected that this is another imaging modality available using DCDHF molecules. Table 3.4 shows that both the one-photon excited and two-photon excited (in brackets) fluorescence lifetimes of bulk DCDHF-N samples depend on the solvent or environment in which the fluorophore resides. The one-photon lifetime increases from below the instrument response time in polar ethanol to a few nanoseconds in nonpolar toluene and even higher in PMMA; the two-photon lifetime exhibits a similar increase going from solution to polymer. The increase in lifetime can be correlated to the increase in quantum yield and is most likely the result of suppressing the non-emissive TICT relaxation channel in toluene and PMMA. This property could be used to follow dynamic changes in local environment on the single-molecule level or to map different regions within a sample, using fluorescence lifetime as contrast.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\Phi_F$</th>
<th>$\tau_F$ (ns)</th>
<th>$\tau_F$ [TPE] (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ethanol</td>
<td>0.017</td>
<td>&lt; 0.22</td>
<td></td>
</tr>
<tr>
<td>toluene</td>
<td>0.85</td>
<td>2.7 [2.5]</td>
<td></td>
</tr>
<tr>
<td>PMMA</td>
<td>0.98</td>
<td>3.2 [2.9]</td>
<td></td>
</tr>
</tbody>
</table>

Values in brackets are fluorescence lifetimes with two-photon excitation. The lifetime in ethanol is shorter than the instrument response. Measured lifetime values are $\pm 0.1$ ns. From Lord et al.144
3.1.2. Photostability of Acene DCDHF

Single-Molecule Properties

A strong test of the utility of a fluorophore is its ability to be imaged at the single-molecule level, which requires bright fluorescence, weak coupling with dark states, and robust photostability. The acene DCDHF are superb single-molecule emitters: for example, single copies of DCDHF-A and DCDHF-N in films of aqueous protein gels are easily visible as shown in Figure 3.5, wide-field epifluorescence images of typical samples. The methods used to assess photostability are described in Chapter 2.

![Histogram of total photons detected from 193 different DCDHF-N molecules in a PMMA film. $N_{\text{photons, detected}}$ is the exponential parameter of a single-exponential fit (solid line). (Inset) The spatially integrated fluorescence intensity time trace of a representative individual molecule. The reported intensity is background-subtracted and converted to photons emitted. Emission terminates at 22 s due to photobleaching. Very few molecules exhibited any blinking on the 100-ms integration time scale of the measurement, which is consistent with other members of the class of DCDHF fluorophores. From Lord et al.144](image)

To better characterize the photostability of a single-molecule emitter using one simple parameter, I recorded the distribution of the number of photons emitted from
single fluorophores before photobleaching, \( N_{\text{photons}} \) (see Table 3.5). This parameter assesses a fundamental property of the emitter because it should not depend on excitation intensity (in the absence of saturation or nonlinear effects): if the pumping intensity is selected to produce a desired emission rate, this parameter assesses how long a molecule will survive on average. From wide-field epifluorescence movies, 193 DCDHF-N and 135 DCDHF-A single-molecule traces (similar to Figure 3.4, inset) were recorded, inspected for digital bleaching, background-subtracted, and integrated to build a distribution (Figure 3.4). These \( N_{\text{photons, detected}} \) distributions exhibit the expected exponential shape for a first-order photobleaching process, and single-exponential fits yield 130,000 photons detected per DCDHF-N molecule on average and 226,000 photons detected per DCDHF-A. Converting to the number emitted using the detection efficiency \( D \), I find \( N_{\text{photons}} = 1.4 \times 10^6 \) photons emitted per DCDHF-N molecule on average and \( 2.2 \times 10^6 \) per DCDHF-A, which approach the values of DCDHF-P and Rhodamine 6G (\( 2.4 \times 10^6 \) and \( 1.9 \times 10^6 \) photons emitted per molecule, respectively),\(^95\),\(^139\) both of which are demonstrably good single-molecule fluorophores.

**Bulk Photostability of Acene DCDHFs**

The single-molecule \( N_{\text{photons}} \) value for DCDHF-N is comparable to the value determined using the bulk photobleaching behavior of high-concentration samples of DCDHF-N in PMMA. A bulk measurement yielded \( N_{\text{photons}} = 2.6 \times 10^6 \) photons emitted per DCDHF-N molecule, comparable to the single-molecule value of \( 1.4 \times 10^6 \); if anything, the single-molecule value may be an underestimate. As an independent check of this method of calculating \( N_{\text{photons}} \), I measured the photons emitted per Rhodamine 6G molecule in polyvinyl alcohol to be \( 1.4 \times 10^6 \), which is comparable to the published value of \( 1.9 \times 10^6 \) photons emitted per Rhodamine 6G molecule in ethanol.\(^95\)

To compare the photostability of the acene DCDHFs to other commonly used cellular fluorophores in a cell-like model environment, I measured the photobleaching quantum yield (\( \Phi_B \)) in an aqueous protein gel. Our measured values for R6G and Texas Red \( \Phi_B \) are similar to their respective literature values in water.\(^95\) Average
values for all measured $\Phi_B$ are displayed in Table 3.5; the acene DCDHFs prove to be more photostable than Rhodamine 6G, Texas Red, and fluorescein in this aqueous environment.

<table>
<thead>
<tr>
<th>fluorophore</th>
<th>$\Phi_B$ ($10^{-6}$)</th>
<th>$N_{\text{photons}}$ ($10^6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCDHF-A</td>
<td>1.7</td>
<td>2.2</td>
</tr>
<tr>
<td>DCDHF-N</td>
<td>3.4</td>
<td>1.1</td>
</tr>
<tr>
<td>R6G</td>
<td>3.5</td>
<td>1.4</td>
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<tr>
<td>Texas Red</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>fluorescein</td>
<td>63.5</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.5. Photobleaching quantum yields and total photons emitted of acene DCDHFs and commercial fluorophores.

The acene DCDHFs not only are resilient to photobleaching, they also resist entering long-lived dark states: most DCDHF-N and DCDHF-A molecules exhibited little or no blinking on the time scale of our experiments (i.e. 100-ms integration time, movies lasting tens of seconds), as seen in the sample time-trace inset of Figure 3.4.

3.1.3. Cellular Imaging using Acene DCDHFs

The ultimate test of our fluorophores is whether they can be imaged on the single-molecule level in a cellular environment long enough to report on some process of interest. Recently, we successfully used several different DCDHF derivatives as fluorescent lipid analogs as tracers to probe cellular membrane dynamics.\textsuperscript{54} Single copies of the naphthyl derivative (both DCDHF-N and DCDHF-N-12, a version with longer alkyl chains on the amine donor) were visualized diffusing in the plasma membrane of mammalian CHO cells (Figure 3.5A). We were also able to image single copies of DCDHF-A in CHO cell cell membranes (Figure 3.5B).
Figure 3.5. Epifluorescence images of acene DCDHFs in living CHO cells. (A) Image of single DCDHF-N molecules diffusing in a region of a CHO plasma membrane, with 2×2 Gaussian smoothing. The excitation wavelength was 532 nm, the intensity at the sample was ~2 kW cm⁻², and the integration time was 15.4 ms per frame. (B) Surface plot of emission from single copies of DCDHF-A in a CHO cell membrane, with 3×3 Gaussian smoothing. The excitation wavelength was 594 nm, the intensity at the sample was ~0.75 kW cm⁻², and the integration time was 100 ms. From Lord et al.144

In the future, with further optimization of the DCDHF class, we hope to design additional high-performance reporter molecules capable of detection at the single-molecule level in cells; but already DCDHF-N emits as many photons and can be imaged with a signal-to-noise ratio comparable to that of a popular lipid-analog fluorophore, Trite-DHPE (a tetramethylrhodamine derivative), as reported in detail in reference 54.

3.1.4. Summary of Acene DCDHFs

This work has described two acene relatives of the earlier-reported DCDHF-P molecule, DCDHF-N and DCDHF-A, which exhibit strong fluorescence upon pumping at the longer wavelengths that are necessary for single-molecule imaging in cells. These two long-wavelength fluorophores also exhibit the strong emission and weak dark states which give rise to single-molecule properties approaching those of DCDHF-P and Rhodamine 6G when embedded in a PMMA matrix, and superior photostability in an aqueous protein environment.
Further experiments involving DCDHF-N and DCDHF-A can take advantage of other properties of the DCDHF class of fluorophores that these acene derivatives possess, such as measurable shifts in emission-wavelength or fluorescence-lifetime with variations in local polarity. Such experiments include protein labeling using maleimide covalent binding and FRET pairing with other fluorophores or other DCDHF derivatives. In some biological experiments, it may be necessary to impart better water solubility to these fluorophores, which can be achieved by adding alcohol, carboxylic acid, or sulfonic acid functional groups to the structure (either off the donor or acceptor). These first results—minimized background fluorescence from free fluorophores in solution, long-wavelength absorption and emission, high photostability in aqueous environments, and large solvatochromism—demonstrate that acene-linked DCDHF fluorophores are high-quality single-molecule emitters for cellular imaging.

3.2. RED-SHIFTING DCDHFS USING MULTIPLE AROMATIC GROUPS

Another approach for red-shifting the DCDHF optical response is using oligoaromatic conjugation. The more extensively conjugated bisaromatic systems DCDHF-P-P, DCDHF-P-T, DCDHF-T-P and DCDHF-T-T, are expected to show a bathochromic shift of the absorption and emission band. In addition, a trisaromatic dye and two bisaromatic dyes with an olefin unit were also synthesized for comparison (see Table 3.6).

The conjugated donor-\(\pi\)-acceptor push-pull chromophores with \(\pi\)-bridges of phenyl and thiophene rings and their combinations are widely recognized for applications in materials science because of their interesting and useful optoelectronic properties. Extension of the conjugation unit from one aromatic ring to two aromatic rings leads to a bathochromic shift for their charge-transfer electronic absorption. Chromophores bearing a benzo–1,3-dithiol-2-ylidene donor and
dicyanomethylene acceptor with a 4,4'-biphenyl instead of a 1,4-benzene push the absorption from the visible to the near-infrared.\textsuperscript{180} Since the thiophene ring has less resonance energy than benzene, it permits more effective $\pi$-electron delocalization.\textsuperscript{181-185} Bithiophene provides a pronounced red-shift compared with biphenyl indicative of enhanced electron transmission from the donor to the acceptor.\textsuperscript{186-191}

<table>
<thead>
<tr>
<th>(π)</th>
<th>$\epsilon_{\text{max}}$ (M$^{-1}$cm$^{-1}$)$^a$</th>
<th>$\lambda_{\text{abs}}$ (nm)$^a$</th>
<th>$\lambda_{\text{em}}$ (nm)$^a$</th>
<th>Stokes Shift (cm$^{-1}$)$^a$</th>
<th>$\lambda_{\text{ex}}$ (nm)$^a$</th>
<th>$\Phi_F$</th>
<th>Tolune {PMMA}</th>
<th>$N_{\text{photons}}$ $^b$</th>
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<tr>
<td>P</td>
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<td>486</td>
<td>505</td>
<td>774</td>
<td>470</td>
<td>0.044</td>
<td>{0.92}</td>
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<td>514</td>
<td>528</td>
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<td>488</td>
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<td>603</td>
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<td>709</td>
<td>4380</td>
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<td>0.34</td>
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<td>V-T-P</td>
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<td>611</td>
<td>723</td>
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<td>570</td>
<td>0.07</td>
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</tr>
<tr>
<td>V-T-T</td>
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<td>600</td>
<td>0.13</td>
<td>&gt;3.8x10$^4$</td>
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\textsuperscript{a} All the absorption and emission maxima were measured in toluene. \textsuperscript{b} For comparison, $N_{\text{photons}}$ for R6G = 1.4x10$^6$ photons per molecule (bulk measurement in PMMA). \textsuperscript{c} The quantum yield value reported before (10%) is less accurate and has been corrected here. \textsuperscript{d} These values were determined from bulk measurements of the dyes doped in PMMA films, excited at 633 nm. They are lower limits because the calculation uses the quantum yields in toluene, which are lower than those in PMMA; if the (correct) higher PMMA quantum yields were easily measurable, the $N_{\text{photons}}$ values would be higher (in a linear fashion). From Lu et al.\textsuperscript{147}

Here I describe the spectroscopic properties—including absorption, fluorescence, solvatochromism, and single-molecule imaging properties—of DCDHF dyes with an extended $\pi$-system comprised of combinations of benzene and thiophene rings. Most of these compounds have the desired combination of enhanced quantum yields with absorption and fluorescence at longer wavelengths that very effectively overcome the background autofluorescence seen in many biological samples. On the single-molecule level, this new group of dyes includes some of the brightest and most long-lived among the DCDHF class of single-molecule emitters. For many of these dyes in PMMA, single copies emit brightly for tens of seconds to minutes under typical epifluorescence imaging conditions (even without oxygen scavengers), emitting
several millions of photons before photobleaching. Moreover, as with other DCDHFs studied, these fluorophores seldom exhibit blinking on the time scale of our experiments (100-ms integration time). These DCDHF fluorophore compare favorably with other excellent single-molecule emitters, such as rhodamines and perylene diimides, which emit millions of photons.95, 96 Terrylene diimides can emit tens or even hundreds of millions of photons, but face significant solubility hurdles.192 Overall, these new DCDHF dyes show excellent promise as bright, long-lived red emitters—necessary criteria for single-molecule experiments in the cellular environment.

3.2.1. Absorption Spectra of Multiple-Aromatic DCDHFs

The absorption and other photophysical properties of the bisaromatic DCDHFs are all summarized in Table 3.6. All these dyes have the same dialkylamine donor and the same DCDHF acceptor and differ only in the π-conjugation linkage present. Thus, differences in the longest wavelength absorption band (the charge-transfer band) reflect only the composition of the long axis π-conjugation bridge of the molecules. A bathochromic shift results from addition of a second ring, either a thiophene or benzene, to the parent DCDHF-P or DCDHF-T. The absorption spectra of a set of four bisaromatic DCDHF chromophores in toluene are shown in Figure 3.6. Addition of a benzene ring to the single phenyl linkage in DCDHF-P led to a bathochromic shift of 20 nm (DCDHF-P-P), while a thiophene ring addition induced a shift of 105 nm (DCDHF-P-T) or 89 nm (DCDHF-T-P) depending on the position of the new ring. Similarly, an addition of a second phenyl ring to the thiophene linkage dye DCDHF-T led to a bathochromic shift of 77 nm (DCDHF-P-T) or 61 nm (DCDHF-T-P) and an additional thiophene ring for dye DCDHF-T gave a 120 nm (DCDHF-T-T) bathochromic shift. Overall, the most effective conjugation was observed with two thiophene rings. Comparison of the absorption maxima of the different DCDHF derivatives indicates that the charge-transfer properties can be effectively tuned by iterations of the π bridge.
The biphenyl linkage of DCDHF-P-P shows diminished π-overlap efficiency due to a confinement of π electrons associated with the high aromatic stabilization energy of the benzene ring and the potential barriers arising from the steric hindrance of the ortho hydrogens that inhibit the coplanarity between the two phenyl rings. In its X-ray structure, the dihedral angle \( \theta \) between the two aromatic (phenyl) rings is \(-19.8^\circ\), which is larger than that between thiophene and phenyl ring in compound DCDHF-P-T \((10.0^\circ)\). It is well known that the absorption wavelength increases as the number of phenyl units in the push-pull system increases, but this effect saturates (usually with the bi- or terphenyls); after saturation, adding more phenyl units eventually causes a hypsochromic shift. Adding a third phenyl ring does indeed cause a hypsochromic shift: the trisaromatic dye DCDHF-P-T-P has an additional phenyl ring compared to DCDHF-T-P, and the absorption maximum of DCDHF-P-T-P is 541 nm, 34 nm to the blue of DCDHF-T-P. This blue shift occurs because the additional phenyl ring disturbs the charge transfer since the whole molecule deviates even more from a planar structure, as shown from the dihedral angle \( \phi \) between thiophene and phenyl ring of \(11.8^\circ\) in Table 3.7. In contrast, the
introduction of thiophene in the $\pi$-conjugated system tolerates more thiophene units before the absorption red-shift saturates. This has been explained by the reduced aromaticity of the thiophene ring and relative ease of co-planarity for adjacent thiophene rings.\textsuperscript{193} As such, it is no surprise to observe that the absorption maxima of DCDHF-P-T and DCDHF-T-P fall in the range of the absorption maxima between DCDHF-P-P and DCDHF-T-T dyes. To some extent, DCDHF-P-T has better electronic push-pull character than DCDHF-T-P (the former’s Stoke shift is 16 nm longer), which can be explained from the calculated dihedral angles ($dr_2$) between phenyl and thiophene ring of these two molecules (Table 3.7). In DCDHF-T-T, the \textit{trans} conformation between two thiophene rings produces little steric interaction of hydrogens at the $\beta,\beta'$ position, thus a preferred coplanar structure is possible.\textsuperscript{194, 195} This is also consistent with dihedral angle calculations for this molecule in Table 3.7. Finally, and in contrast to the addition of a third aromatic ring, dyes DCDHF-V-T-P and DCDHF-V-T-T (with an additional vinyl unit on DCDHF-T-P and DCDHF-T-T between the aromatic core and the acceptor) continue to shift the absorption to the red significantly.

Dihedral angles from both of the available x-ray results and model calculations indicate that the rings are twisted, although the calculations may not always capture the correct direction or exact magnitude. Errors in calculating values for $da$ may be due to the use of a dimethylamino donor group in the calculation model rather than the dihexyl amino group found in the actual molecules. This analysis shows that the dihedral angle between aromatic rings ($dr_2$) is a better indicator of the degree of charge-transfer character than that between DCDHF ring and the adjacent ring ($dr_1$). Although these $dr_2$ values for each compound from X-ray results and model calculations are different, they have the same trends, with DCDHF-P-P having the largest dihedral angle and DCDHF-P-T having the smallest value.
Table 3.7. Calculated torsion angles between the carbon off the amine and the average plane of the ring adjacent to the amine (\(da\)), between the DCDHF and the adjacent ring (\(dr_1\)), between the first ring and its adjacent ring (\(dr_2\)), and between the second and third rings (\(dr_3\)), where applicable. Quantum-chemistry calculations are described in Section 3.2.5. Values in brackets are from available X-ray structures. From Lu et al.\(^{147}\)

<table>
<thead>
<tr>
<th>((\pi))</th>
<th>(da)</th>
<th>(dr_1)</th>
<th>(dr_2)</th>
<th>(dr_3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>0.5</td>
<td>9.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>8.5</td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-P</td>
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<td>0.0</td>
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</tr>
<tr>
<td>V-T</td>
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<td>−0.3</td>
<td>−0.2</td>
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</tr>
<tr>
<td>P-P</td>
<td>1.3 {-7.8}</td>
<td>13.9 {-2.2}</td>
<td>−27.2 {-23.0}</td>
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</tr>
<tr>
<td>P-T</td>
<td>10.0 {-3.2}</td>
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</tr>
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<tr>
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<td>−3.1 {1.0}</td>
<td>−172.2 {-162.3}</td>
<td>162.9 {-168.5}</td>
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3.2.2. Emission Spectra of Multiple-Aromatic DCDHFs

The emission maxima, Stokes shift and quantum yield of the DCDHF dyes measured in toluene are summarized in Table 3.6 while Figure 3.7 shows the normalized emission spectra of the bisaromatic DCDHF fluorophores in toluene. It is clear that the addition of an olefin to the single aromatic ring dye provides a significant bathochromic shift, which is accomplished with a sacrifice of quantum yield in solution (Table 3.6). On the contrary, introduction of the second aromatic ring, particularly a thiophene ring, affords not only a red-shift but also increased quantum yield (\(\Phi_F\)) in toluene compared with the original dyes with a single benzene or thiophene \(\pi\)-bridge. For example, addition of a benzene ring to the single phenyl linkage provides a small bathochromic shift of 22 nm from 486 nm for DCDHF-P to 506 nm for DCDHF-P-P, but this is accompanied by an order-of-magnitude increase in quantum yield (4.4% to 82%). If the second ring is a thiophene, both the absorption
wavelength and quantum yield are further enhanced. DCDHF-T-P has the highest quantum yield (74%) and DCDHF-T-T has the longest absorption wavelength and offers five times the quantum yield (50%) compared to the single thiophene dye DCDHF-T (11%). The trisaromatic DCDHF-P-T-P has a longer wavelength emission maximum than any of the bisaromatic dyes while its absorption maximum wavelength is less than any of the thiophene-containing dyes; this triaromatic possesses the highest Stokes shift (4380 cm$^{-1}$) amongst all the DCDHF dyes studied here. The bisaromatic-vinyl compounds DCDHF-V-T-P and DCDHF-V-T-T have longer wavelength emission maxima and higher quantum yields 7% (DCDHF-V-T-P), 13% (DCDHF-V-T-T) compared with DCDHF-V-P (2%) and DCDHF-V-T (2%), but the quantum yields are still much lower than their corresponding bisaromatic counterparts DCDHF-T-P and DCDHF-T-T.

![Emission spectra of some multiaromatic DCDHFs. From Lu et al.](image)

It is interesting to note that the dye DCDHF-P-P has the highest Stokes shift among all the bisaromatic dyes. The Stokes shift provides information about the excited state and it is quite possible that flattening (reduction of torsion angles) of the
excited state structure plays a role in this phenomenon. In the ground state, there exists a significant torsion angle between the two phenyl rings, which prevents the effective conjugation and results in a short-wavelength absorption. The very large Stokes shift is indicative of a large excited-state dipole moment and the change in electronic structure due to molecular flattening. The high Stokes shift for the trisaromatic DCDHF-P-T-P may also due to flattening in the excited state.

3.2.3. Solvatochromism of Multiple-Aromatic DCDHFs

These bisaromatic dye materials exhibit strong solvatochromism. Because the DCDHF dyes are donor–acceptor fluorophores, the dipole moment of the molecule is expected to increase upon excitation and charge transfer. The influence of the solvent polarity on the Stokes shift was explored with the Lippert–Mataga equation (Chapter 2), in which the fluorophore is modeled as a dipole located in a cavity in a continuous solvent-dipole environment. The polarity of the solvent is approximated using the orientation polarizability $\Delta f$, which represents how easily solvent molecules rearrange around a dipole in a continuous medium (see Figure 2.2).
Figure 3.8. Lippert-Mataga plots of molecules with multiple aromatic groups. The slopes for the fits are 9290 (R² = 0.70), 7517 (R² = 0.77), 7047 (R² = 0.83), and 2416 cm⁻¹ (R² = 0.56) for 5–8, respectively. The steeper the slope, the greater the excited-state charge transfer and solvatochromism is exhibited. From Lu et al.¹⁴⁷

Plots of the Stokes shift as a function of the solvent Δf for the bisaromatic dyes are shown in Figure 3.8. The absorption and emission wavelengths of bisaromatic dyes in solvents with a range of Δf (orientation polarizability) values are found in Table 3.8. The slopes for the fits are 9290, 7517, 7047, and 2416 cm⁻¹, respectively. The previously reported highest slope value for a DCDHF is 7757 cm⁻¹ for DCDHF-A, a red-emitting fluorophore with an anthracene core.¹⁴⁴ The Lippert–Mataga slope for DCDHF-P is only 1588 cm⁻¹. The steeper the slope, the greater the change in dipole moment upon photoexcitation Δμ and more sensitive solvatochromism is exhibited. The significant solvatochromism of the bisaromatic DCDHFs is not
surprising because the extended conjugation allows for more charge separation upon charge transfer in the excited state. Such large Stokes shifts can be usefully applied in practice by using emission filters with longer wavelength cutoff, further reducing background. Moreover, it is possible to use the changes in emission wavelength to probe local (i.e. nanometer-scale) polarity.

### Table 3.8: Solvatochromatic properties of some multiaromatic DCDHFs. From Lu et al.\textsuperscript{147}

<table>
<thead>
<tr>
<th>solvent</th>
<th>$\Delta f$</th>
<th>DCDHF-P-P</th>
<th>DCDHF-P-T</th>
<th>DCDHF-P-P</th>
<th>DCDHF-P-T</th>
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</thead>
<tbody>
<tr>
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<td>$\lambda_{abs}$</td>
<td>$\lambda_{em}$</td>
<td>$\lambda_{abs}$</td>
<td>$\lambda_{em}$</td>
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</tr>
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<td>594 710</td>
<td>666 740</td>
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</tbody>
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#### 3.2.4. Single-Molecule Imaging of Multiple-Aromatic DCDHFs

In the context of optical imaging, one rigorous test of the utility of a fluorophore is its ability to be successfully imaged at the single-molecule level, which requires strong fluorescence, weak coupling with dark states, and photostability. These bisaromatic DCDHF fluorophores are strong single-molecule emitters: single copies of DCDHF-P-T in PMMA are easily visible in an epifluorescence image of a typical sample (Figure 3.9). To better characterize the quality of these single-molecule emitters using one simple parameter, I recorded the distribution of the number of photons detected from single fluorophores before photobleaching, and converted this value to total photons emitted using the known losses in our collection system. The photon collection efficiency of our setup is $D = \eta_Q F_{\text{coll}} F_{\text{opt}} F_{\text{filter}}$, as described in Chapter 2. At the emission wavelengths, $\eta_Q = 85\%$ for our camera; the maximum possible $F_{\text{coll}}$ for our setup is $38\%$ for a single dipole emitter aligned horizontally; I
measured $F_{\text{opt}}$ for our setup to be 50%; and I measured $F_{\text{filter}}$ to be between 50–65% for the different emission ranges. After histogramming a single-molecule emitted photon distribution, a fit to one or two exponential decays yielded the average number of photons.

**Figure 3.9.** Epifluorescence image of single copies of DCDHF-P-T in PMMA. From Lu et al.\textsuperscript{147}

Values for total number of photons detected and total number of photons emitted $N_{\text{photons}}$ are reported in Table 3.6. (Some of the $N_{\text{photons}}$ values in Table 3.6 are measured from bulk samples in PMMA. This measurement used bleaching curves and the rate of photon absorption to estimate the total photons emitted. The equation for this calculation can be found in Chapter 2.) The $N_{\text{photons}}$ values for these derivatives are all high; in fact, some are higher than those for Rhodamine 6G (1.9×10\textsuperscript{6} photons emitted per molecule),\textsuperscript{95} which is a demonstrably good single-molecule fluorophore.
These red bisaromatic DCDHF emit millions of photons, without requiring rigorous removal of oxygen from the sample,\textsuperscript{162} and thus offer a potentially useful tool for high-resolution measurements of location or dynamics within living cells.

### 3.2.5. Quantum-Chemistry Calculations of Multiple-Aromatic DCDHF Fluorophores

Quantum-chemistry calculations were used to predict torsion angles in the multiple-aromatic DCDHFs (Table 3.7). Gaussian 03 was used to calculate the optimized ground-state geometries for various DCDHF derivatives containing multiple aromatic groups in the $\pi$ core of the fluorophore.\textsuperscript{199} In order to determine the best level of theory and optimal basis set for these molecules, I performed a series of systematic tests. I used the crystal structure of molecule DCDHF-P-T measured by the Twieg lab as a starting structure and ran ground-state geometry optimizations using HF, BLYP, and B3LYP; for each level of theory, I used 3-21G and 6-31G(d) basis sets. I also ran calculations using more elaborate basis sets (e.g. 6-31+G(d)) and/or higher levels of theory (e.g. MP2), but found only modest increases in accuracy at very high costs. Hartree-Fock theory, using either basis set, produces a structure that is qualitatively incorrect: the dihedral angles between the phenyl and thiophene rings are off by approximately 40 degrees. Density functional theory performed much better, and BLYP and B3LYP structures were all qualitatively comparable to each other and the known crystal structure. To quantify the errors, I compared each of the bond lengths, bond angles, and dihedral angles for each of optimized structures directly with the corresponding values in the known crystal structure using a home-written program. When ignoring errors in hydrogen dihedral angles—which should have minimal effect on the photophysics of the molecules—I determined that BLYP/6-31G outperformed the other viable combinations of basis set and level of theory; therefore, I used this combination to calculate the optimized geometries of other DCDHFs with multiple aromatic groups. In cases where there were conformers, I report the structures with the lowest calculated energy.
3.2.6. Summary

A group of new fluorescent dye materials for single-molecule imaging applications containing a DCDHF acceptor group and a combination of phenyl and thiophene π-conjugation have been characterized. These fluorophores with two aromatic rings providing conjugation linkage have absorption shifted to the red, generally exhibit significantly increased quantum yields, and show increased resistance to photobleaching in many cases. The thiophene-thiophene combination provided the longest absorption wavelength while the phenyl-thiophene gave the best quantum yield. Single-molecule analysis reveals that bisaromatic DCDHFs include some of the brightest and longest-lived emitters in this class of fluorophores studied so far. These red emitters are easily imaged and observed for minutes before photobleaching, making them attractive for applications in single-molecule biological and cellular studies.

3.3. RED-SHIFTING AND BRIGHTENING DCDHFS BY CONSTRAINING TWISTS

In the previous sections, I discussed two straightforward routes to red-shifting the absorption and emission of the DCDHF fluorophores. Here, I discuss an approach that we expected to increase the fluorescence quantum yield by restraining bond twists in the structure; in addition, this approach also generally red-shifted the dyes.

Although quantum-mechanical modeling revealed methylene-bond twists as one major source of viscosity sensing in DCDHFs, the various other twists in the molecule may also significantly contribute to lowering the fluorescence quantum yield in solution. Moreover, the methylene bond does not have synthetic “handles” with which to constrain rotations, because modifying the cyano groups would also drastically alter the photophysics.
Therefore, in order to experimentally test the influence of other bond twists on the fluorescence quantum yield, a series of fluorophores were synthesized with increasing constraint on the amine rotation produced by tetrahydroquinoline rings, and I performed optical and physical characterization of these molecules. Table 3.9 demonstrates that restraining the amine not only increases the fluorescence quantum yield but also slightly red-shifts the absorption and emission. However, the quantum yields of molecules in solution do not approach the values measured in the rigid polymer film, which indicates that the amine bond alone is not responsible for the viscosity sensing.

These experimental results do not entirely match the earlier calculations, and suggest that the TICT mechanism in DCDHFs is more complex than just one bond rotation. Further computational studies or ultrafast measurements might help clarify the exact mechanism of viscosity sensing of DCDHF fluorophores.
It is well-known that polar fluorescent dyes containing an electron-donating group (e.g. an amino group) and an electron withdrawing group often exhibit large Stokes shifts because of intramolecular charge-transfer states. In the case of an amine donor, replacing the typical dialkylamino groups by a cyclic tetrahydroquinoline group would be one way to shift the absorption and emission to longer wavelengths. Twisting about the amine–phenyl bond might also have an effect on the brightness of the emission.\textsuperscript{1} To test this idea, we explored several new DCDHF fluorophores with a tetrahydroquinoline ring in an attempt to improve the quantum yield and simultaneously shift the wavelength further to the red.

### 3.3.1. Photophysical Characteristics of the Constrained-Ring DCDHFs

The relevant physical properties of the different DCDHF dyes in toluene are summarized in Table 3.9. As can be seen, the absorption and emission wavelengths systematically increase as the nitrogen donor is constrained by one saturated ring and then by two saturated rings. In the group of benzene substrates with the presence of vinyl group (V-P or V-N), the absorption wavelength shifts to the red 19 nm with addition of one ring and 32 nm with addition of two rings while the emission wavelength shifts to the red 15 nm with one ring and 25 nm with two rings. In the group of benzene substrates without the presence of vinyl group (P or N), the emission shifts are slightly smaller, 10 and 22 nm for the addition of one ring and two rings, respectively. Also, constraining the twists of the amine donor has a substantial influence on the fluorescence quantum yield. It is increased almost five times by comparing DCDHF-P with DCDHF-P-(2rings) and twice by comparing DCDHF-V-P with DCDHF-V-P-(2rings).
The same trends were observed in more conjugated systems, as shown in Table 3.9. In the 2,6-substituted naphthalene systems, the emission wavelength increased upon constraining the amine twist (bathochromic shifts in the emission of 31 and 28 nm, respectively). The quantum yield was also enhanced; this is especially true for DCDHF-N, which exhibits an increase of almost two orders of magnitude. The 1,4-substituted system is more like the benzene systems, with 33 and 16 nm bathochromic shifts, in absorption and emission, respectively.
Table 3.9. Influence of constraining the amine from twisting on photophysical properties.

<table>
<thead>
<tr>
<th>Parent Compound</th>
<th>Number of constraining rings</th>
<th>( \varepsilon_{\text{max}} ) (M(^{-1}) cm(^{-1}))[^a]</th>
<th>( \lambda_{\text{abs}} ) (nm)[^b]</th>
<th>( \lambda_{\text{em}} ) (nm)[^b]</th>
<th>( \Phi_{\text{F in toluene}} ) {PMMA}</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCDHF-P</td>
<td>0</td>
<td>486</td>
<td>505</td>
<td>0.044 {0.92}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>495</td>
<td>515</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>503</td>
<td>527</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>DCDHF-V-P</td>
<td>0</td>
<td>562</td>
<td>603</td>
<td>0.02 {0.39}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>581</td>
<td>618</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>594</td>
<td>628</td>
<td>0.053</td>
<td></td>
</tr>
<tr>
<td>DCDHF-N</td>
<td>0</td>
<td>527</td>
<td>576</td>
<td>0.85 {0.98}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>545</td>
<td>607</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>DCDHF-V-N</td>
<td>0</td>
<td>574</td>
<td>671</td>
<td>0.0049</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>600</td>
<td>699</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>DCDHF-V-(1,4)N</td>
<td>0</td>
<td>538</td>
<td>661</td>
<td>0.0067</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>571</td>
<td>677</td>
<td>0.0096</td>
<td></td>
</tr>
</tbody>
</table>

\[^a\] In dichloromethane. \[^b\] In toluene. From Wang et al.\(^{143}\)

With the spectral data plotted, it is clear that the absorption and emission wavelengths both increase with the increasing constraint of the donor amine twist (Figure 3.10). A previous theoretical treatment of the electronic properties of the DCDHF dyes showed that amine twist had little effect on the ground- and excited-state energy surface and was not responsible for the large viscosity sensitivity.\(^1\) The experimental results here quantify the degree to which the exact structure of the donor group shifts the spectra.

### 3.3.2. Single-Molecule Imaging of Constrained-Ring DCDHFs

An important test for the utility of a fluorophore is its ability to be imaged at the single-molecule level, which requires strong fluorescence, weak coupling with dark states, and photostability. Importantly, single-molecule imaging of derivative DCDHF-P-(2rings) demonstrates that we maintained the favorable photophysical properties of the parent molecule as shown in Figure 3.9. To characterize the quality of a single-molecule emitter using one simple parameter, I report the total number of photons emitted from a single fluorophore before photobleaching. Measurements on 182 individual molecules yielded an average number of photons emitted \( N_{\text{photons}} = 1.1 \times 10^6 \) photons per molecule. This value is comparable with the values of
DCDHF-P and R6G (2.4×10^6 and 1.9×10^6 photons emitted per molecule, respectively), both of which are among the best single-molecule fluorophores.

![Figure 3.12](image-url)  
**Figure 3.12.** Epifluorescence image of single copies of DCDHF-P-(2rings) in a PMMA film imaged at 488 nm excitation. The surface was calculated from two position dimensions and pixel intensity of a Gaussian-smoothed image. From Wang et al.143

### 3.3.3. Summary of Constrained-Ring DCDHFs

The Twieg lab has synthesized and I have characterized several examples of DCDHF fluorophores with the amine donor either acyclic or constrained in one or two tetrahydroquinoline rings. Generally, inclusion of the donor in a ring annulated to the benzene or naphthalene aromatic π-core results in a bathochromic shift of absorption and emission accompanied by an increase in the quantum yield. Introduction of the first tetrahydroquinoline ring produces the largest effect, with a smaller effect produced by the second ring. Favorable performance in single-molecule fluorescence imaging is maintained for the newly synthesized derivatives.

Our previous calculations on the DCDHF parent molecule showed that furan-dicyanomethylene twists on the excited state manifold predominantly control the changes in emission observed in different hosts.1 The results of this section suggest that twists about the amine–benzene bond also noticeably affect the fluorescence emission. A more detailed solution-phase calculation including excited-state effects needs to be done in the future in order to confirm the exact mechanism.
3.4. INCREASING WATER SOLUBILITY OF DCDHFS
USING HYDROPHILIC GROUPS

The approaches I described in the previous sections of this chapter were successful at predicting how modifying the structure of DCDHF fluorophores affects their photophysics. However, photophysical parameters are not the only properties of fluorophores that dictate their utility. For instance, biophysical and cell-imaging experiments require fluorophores that can be used in aqueous environments, and most of the molecules described so far require relatively nonpolar solvents. Full water-solubility is not always necessary or desirable (e.g. fluorescent lipid analogs must have some hydrophobicity); however, many applications do require fluorophores that are soluble and photostable in water.

The original work on molecules in the DCDHF class was carried out in organic solvents, in which the fluorophores are very soluble. For single-molecule cell experiments, stock fluorophore solutions in DMSO or ethanol were diluted into the aqueous buffer; because of the very low dye concentrations, aggregation in water was not a problem. Nevertheless, in order to introduce true water solubility, synthetic efforts were undertaken to add alcohol, carboxylic acid, and sulfonic acid groups to two DCDHFs, and the optical properties were explored. As expected, sulfonic acid groups imparted the most solubility (up to \(10^4\) ppm). Significant water solubility was achieved without compromising desirable photophysical properties of the DCDHF class of fluorophores.

In this section, structures of interest with their names are shown in Table 3.10. To illustrate the problem, ws1 and ws2 have less than 0.001 ppm water solubility. With alkyl substituents at the various R positions, such molecules have an amphiphilic motif and label cell membranes easily. In order to prevent membrane binding and enable applications in the cellular cytosol, it is clear that one must develop water soluble forms. To explore the water solubility potential of these fluorophores, the Twieg lab modified the donor component with a variety of hydrophilic groups (alcohol, carboxylic acid and sulfonic acid), and their water solubility and photophysics in aqueous environments were determined.
Table 3.10. Photophysical properties and water solubility measurements of different DCDHF and DCDHF-V-P chromophores.

<table>
<thead>
<tr>
<th>type</th>
<th>#</th>
<th>structure</th>
<th>$\lambda_{\text{abs}}$</th>
<th>$\lambda_{\text{em}}$</th>
<th>$\Phi_F$</th>
<th>$\varepsilon_{\text{max}}$ (cm$^{-1}$ M$^{-1}$)</th>
<th>Solubility in water (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCDHF-P type</td>
<td>ws1</td>
<td><img src="image1" alt="ws1 structure" /></td>
<td>470$^a$</td>
<td>546</td>
<td>0.012</td>
<td>89,900</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>ws12</td>
<td><img src="image2" alt="ws12 structure" /></td>
<td>498</td>
<td>529</td>
<td>0.001</td>
<td>15,100</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>ws14</td>
<td><img src="image3" alt="ws14 structure" /></td>
<td>496$^b$</td>
<td>531</td>
<td>0.002</td>
<td>44,200</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>ws15</td>
<td><img src="image4" alt="ws15 structure" /></td>
<td>493</td>
<td>528</td>
<td>0.001</td>
<td>64,700</td>
<td>$1.50 \times 10^2$</td>
</tr>
<tr>
<td></td>
<td>ws16</td>
<td><img src="image5" alt="ws16 structure" /></td>
<td>500</td>
<td>526</td>
<td>0.001</td>
<td>37,300</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>ws17</td>
<td><img src="image6" alt="ws17 structure" /></td>
<td>489</td>
<td>529</td>
<td>0.002</td>
<td>31,500</td>
<td>$2.80 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>ws19</td>
<td><img src="image7" alt="ws19 structure" /></td>
<td>500</td>
<td>517</td>
<td>0.001</td>
<td>79,300</td>
<td>$&gt;2 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>ws21</td>
<td><img src="image8" alt="ws21 structure" /></td>
<td>496$^b$</td>
<td>–</td>
<td>–</td>
<td>65,800</td>
<td>$&gt;2 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>ws26</td>
<td><img src="image9" alt="ws26 structure" /></td>
<td>477</td>
<td>517</td>
<td>0.001</td>
<td>57,300</td>
<td>$7.30 \times 10^2$</td>
</tr>
</tbody>
</table>
### 3.4.1. Photophysical Properties of Water-Soluble DCDHFs

With these highly water soluble fluorophores available, our concerns about whether or not the presence of these hydrophilic groups will affect the photophysical properties of these fluorophores could be evaluated. To this end, we compared the fluorophores bearing different hydrophilic groups with their original low polarity analogs ws1 and ws2. Table 3.10 shows that within each group, DCDHF-P type and DCDHF-V-P type, the absorption and emission wavelengths are quite similar. The differences for fluorophores ws1, ws2, ws6 from their counterparts can be attributed to

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Absorption (nm)</th>
<th>Emission (nm)</th>
<th>Quantum Yield</th>
<th>Fluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCDHF-V-P type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ws27</td>
<td>480</td>
<td>520</td>
<td>0.004</td>
<td>42,700</td>
</tr>
<tr>
<td>DCDHF-V-P type</td>
<td>ws2</td>
<td>560$^a$</td>
<td>642</td>
<td>13,500</td>
</tr>
<tr>
<td>ws6</td>
<td>535$^a$</td>
<td>646</td>
<td>0.002</td>
<td>77,900</td>
</tr>
<tr>
<td>ws9</td>
<td>587$^b$</td>
<td>-</td>
<td>87,200</td>
<td>~0.01</td>
</tr>
<tr>
<td>ws24</td>
<td>585</td>
<td>639</td>
<td>0.01</td>
<td>37,000</td>
</tr>
</tbody>
</table>

*a: A small volume of ethanol stock solution added to cuvette of water.

*b: Aggregation observed in water.

From Wang et al.146
the addition of ethanol to the aqueous solution. A few other fluorophores in Table 3.10 (i.e. ws17, ws19, and ws27) exhibit somewhat blue-shifted absorption or emission relative to their counterparts. Given the amphiphilic character of these molecules, the anomalous photophysics may be due to the formation of aggregates or micelles in water at the relatively high concentrations required for bulk measurements. However, at the nanomolar concentrations actually used for single-molecule experiments, such effects are unlikely. For instance, although the fluorophores used in our earlier studies were not soluble in water at bulk concentrations, single molecules were observed diffusing in the cell membrane at the nanomolar regime. As such, this means that while we increased the water solubility of this family of fluorophores, the desirable spectroscopic properties were essentially unchanged.

![Image](image_url)

**Figure 3.13.** Fluorophore ws17 (left two vials) and ws24 (right two vials) dissolved in liquid water (vials L) and in solid ice (vials S) under irradiation with a UV lamp (a filter was used to block the scattered light). The fluorescence is significantly enhanced in the ice samples responding to the dramatic enhancement in viscosity. From Wang et al.146

This outcome is also confirmed by independent viscosity dependence experiments. Previously, in a mixed solvent of glycerol and methanol, the quantum yield of fluorophore ws1 was found to grow with the increase of the glycerol content. Here, two identical solutions of both ws17 and ws24 in water were prepared. For each pair, the sample on the right was frozen while the other sample remained at room temperature (Figure 3.13). Under illumination with a UV lamp, the liquid sample (left
of each pair) shows limited fluorescence while the frozen sample (right of each pair) is strongly emissive. Therefore, the viscosity dependence of the emission is maintained after addition of dicarboxylic acid and sulfonic acid. This suggests that our DCDHF fluorophores have the potential for application in a cellular environment beyond lipid-analog membrane probes.

3.4.2. Summary

We have designed a series of DCDHF fluorophores with a range of functional groups and modified their water solubility without compromising their photophysical properties. These results suggest that this class of fluorophores possess strong potential for a broad range of biological labeling applications. The methods applied here to solubilize the DCDHF fluorophores should also be applicable in other cases where water solubilization is required.

3.5. TUNING PHOTOPHYSICS WITH DCDHF DIMERS

Now that I have discussed the basic structure–property relationships we have explored with the DCDHF class of fluorophores, I will report on some more “sophisticated” photophysical and photochemical properties we have designed.

Physically associated dimers of DCDHFs have been used to demonstrate excitonic behavior. For instance, Conley et al.\textsuperscript{145} showed that two copies of a DCDHF fluorophore can self-quench (via an H-dimer, see Figure 3.14) when pushed together using DNA hybridization. Here, I discuss covalent homodimers of DCDHF compounds that are permanently maintained (via a cyclohexane ring, forming spiro dimers) in either an H- or J-dimer configuration (Figure 3.15). We designed these compounds in order to directly probe the relationship that alignment has on dimer photophysics.
Figure 3.14. The exciton model in spectroscopy. H-type dimers, in which the transition dipoles are parallel, are shown on the left. H-dimers light of higher energy, because radiative transitions involving the lower-energy state are highly unlikely. Fluorescence tends to take place from the lowest-energy excited electronic state (i.e. Kasha’s rule); therefore, H-dimers tend to exhibit quenched fluorescence because of the reduced transition dipole moment from that level. J-type dimer, in which the transition dipoles are in-line, exhibit a red-shift in the absorption and an increase in the transition dipole moment (and thus are more likely to absorb and emit light).

Chromophores very close together are predicted to exhibit spectral changes compared to isolated molecules, caused by coupled transition dipoles that introduce a splitting in the electronic states (Figure 3.14). These excitonic dimers, or excimers, can show color shifts and fluorescence quenching or enhancement. In general, when two chromophores align such that their transition dipoles are in-line (J-dimer, named after Edwin Jelley\textsuperscript{201}), the subsequent excimer fluoresces more strongly; when the molecules align to have their dipoles parallel (H-dimer), the fluorescence of excimer is quenched because the transition is prohibited (the effective transition dipole moment of the lowest state is near zero). In both cases, the absorption is predicted to be enhanced because the transition dipole moments sum, effectively doubling the absorption cross-section.
These predictions from the simple exciton model\textsuperscript{200} were confirmed in our optical experiments on several covalently linked spiro dimers prepared by the Twieg laboratory (Figure 3.15 and Table 3.11). Both spiro dimers exhibited enhanced molar absorbance values; the J-type spiro dimers were brighter and the H-dimers had their fluorescence quenched. The spectral shifts also follow the predictions: the absorption of the J-dimers is red-shifted and the H-dimers are blue-shifted. Both types of spiro dimers have larger molar absorbance values than their monomers.

I used quantum-mechanical calculations to predict the spectroscopic properties of the H- and J-dimers. Structures were calculated using DFT in Gaussian 03\textsuperscript{199} with BLYP/6-31G(d), then the transition energies were calculated using the semi-empirical ZINDO calculation and the keywords: \texttt{ZINDO(50-50 Root=1 NStates=10)}. As can be seen in Table 3.1, the trends in the calculated values of transition energy and strength qualitatively track with the measured values: H-dimers are blue-shifted and J-dimers are red-shifted; I calculated that both spiro types of dimer have approximately
double the transition dipole moment (i.e. oscillator strength or molar absorbance) compared to their monomers.

Table 3.11. Measured and calculated spectral properties of several dimers (measured in toluene)

<table>
<thead>
<tr>
<th>#</th>
<th>type</th>
<th>$\lambda_{\text{abs}}$ (nm)</th>
<th>$\Delta\lambda$ (nm)</th>
<th>$\varepsilon_{\text{max}}$ (M$^{-1}$ cm$^{-1}$)</th>
<th>QY</th>
<th>Zindo transition energy (nm)</th>
<th>oscillator strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>mono</td>
<td>486</td>
<td>0</td>
<td>67,500</td>
<td>0.04</td>
<td>423</td>
<td>1.21</td>
</tr>
<tr>
<td>0</td>
<td>J</td>
<td>501</td>
<td>+16</td>
<td>100,000</td>
<td>0.54</td>
<td>428</td>
<td>2.44</td>
</tr>
<tr>
<td>0</td>
<td>H</td>
<td>461</td>
<td>−24</td>
<td>123,000</td>
<td>0.03</td>
<td>410</td>
<td>2.20</td>
</tr>
<tr>
<td>1</td>
<td>mono</td>
<td>562</td>
<td>0</td>
<td>47,000</td>
<td>0.02</td>
<td>465</td>
<td>1.61</td>
</tr>
<tr>
<td>1</td>
<td>J</td>
<td>596</td>
<td>+31</td>
<td>154,000</td>
<td>0.36</td>
<td>480</td>
<td>2.80</td>
</tr>
<tr>
<td>1</td>
<td>H</td>
<td>542</td>
<td>−25</td>
<td>67,000</td>
<td>0.40</td>
<td>463</td>
<td>2.33</td>
</tr>
<tr>
<td>2</td>
<td>mono</td>
<td>602</td>
<td>0</td>
<td>35,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>J</td>
<td>671</td>
<td>+69</td>
<td>88,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>H</td>
<td>592</td>
<td>−10</td>
<td>133,000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

# = number of vinyl groups between the phenyl group and the furan (e.g. # = 0 for the structures shown in Figure 3.15).

$\Delta\lambda$ = the shift in absorption wavelength from the monomer.

*Italics are outputs from Gaussian calculations.*

### 3.6. DCDHFS THAT ARE DUAL-COLOR SINGLE-MOLECULE EMITTERS

Exciton dimers are one type of sophisticated fluorophore we have designed and characterized. However, some complicated properties of the DCDHFs were unexpected and have proven to be very interesting. For instance, one version of the DCDHF fluorophore exhibited dual-color emission. This section summarizes several experiments intended to try to understand this phenomenon.

#### 3.6.1. Observed Effect

**SMS**

When imaging single molecules of DCDHF-P-T immobilized in PMMA, we surprisingly noticed yellow singles in addition to the expected red. These emitters are so bright and long lived that I was able to image them without gain using a consumer digital single-lens reflex (SLR) camera (Figure 3.16).
In order to image multicolor single molecules using a scientific EMCCD camera, I used a dual-viewing system, which splits the emission image from the microscope into two color channels using a dichroic mirror; the two channels are directed to different regions on the CCD chip and recorded simultaneously. The filters were chosen to detect the range 550–600 nm in the short-wavelength path and the range 610–800 nm in the long wavelength path. The two color channels can be subsequently merged into a false-color image where the image from one channel is colored red and the other green; when both are present in the merge, the spot looks yellow (Figure 3.17).
Figure 3.17. False-color images based on two-color imaging microscopy, excitation 532 nm. (A–B) Frames from movies showing single molecules changing from green to red or from red to green, respectively. The frame number increases from left to right and from top to bottom. (C–D) Time stacks centered at the circles in A–B, respectively, demonstrating the green-to-red or red-to-green transition. However, this switching behavior is not seen in most copies of the fluorophore.

**Bulk Spectroscopy**

Bulk samples of DCDHF-P-T in solution (toluene or ethanol) and in PMMA exhibit photochemistry that most other DCDHFs do not: bulk samples bleach in only a few hours (they change from blue/purple to colorless) if exposed to bright light (Figure 3.18). Removing samples from room light or providing only a nitrogen atmosphere dramatically slows bleaching rate, indicating that both oxygen and light are required for the photochemical bleaching reaction to take place. This unique
bleaching behavior may be help elucidate the mechanism of the multicolor emission on the single-molecule level.

![Figure 3.18. Bulk sample photooxidation in (A) toluene and (B) PMMA films. New samples were blue, as were samples left in the dark; samples left in the light from a 5-W Xe lamp overnight bleached to yellow or colorless. Bubbling with nitrogen significantly reduced the bleaching rate in light, thus indicating a photooxidation mechanism. (C) Bulk spectra revealing multiple peaks after partial photooxidation.](image)

I was able to see bulk green emission in frozen toluene. In Figure 3.19, the two vials were exposed to light and air over time (following the arrows as time increases).
Initially, there was only red emission from both liquid and frozen samples; over time and light exposure, the liquid sample still emitted in the red—but with decreasing intensity—and the frozen sample emitted in the green. It is still unclear whether this is the same species as the singles that look yellow in PMMA—it may be that the yellow emission was composed of time-dependent switching between green and red.

**Figure 3.19.** Photobleached samples in toluene fluoresce in the green when frozen. Vials on the left are liquid and vials on the right are frozen. Illumination was performed using 365 nm light from a Hg lamp; a long-pass filter was placed between the sample and the camera to reject excitation light.

**Labeling Proteins**

We also observed that the maleimide version of DCDHF-P-T showed a blue-shifted shoulder in the bulk fluorescence spectra when bound to the chaperonin GroEL (Figure 3.20). The relative intensity of the blue-shifted shoulder changed at different stages of the GroEL catalytic cycle; this effect may allow ratiometric sensing of changes in protein structure or polarity, but will need further experiments to determine the mechanism of the change.
DCDHF-P-T on GroEL
Emission Spectra

Figure 3.20. Bulk fluorescence spectra of the dual-emitter bound to the chaperonin GroEL in buffer, excited at 532 nm. Emission spectra of DCDHF-P-T conjugated to GroEL (black curve), after adding substrate MDH (red), and then ATP (green). The relative intensity of the blue-shifted peak at ~630 nm changes at different stages of the GroEL cycle.

After observing dual emission in bulk, I decided to explore the possibility of imaging single molecules of DCDHF-labeled GroEL in agarose. With an oxygen-scavenging system (glucose, glucose oxidase, catalase, and \( \beta \)-mercaptoethanol) in the sample, I used the dual-viewer to measure two color channels and record single-molecule movies (Figure 3.21). This was a simple experiment to test whether dual-emitting singles of DCDHF-labeled GroEL are observable, which they are. The next step in this line of investigation would be to try to measure changes in the emission of the same single molecule upon addition of substrate and ATP.
Figure 3.21. Multicolor single molecules of DCDHF-P-T covalently attached to GroEL via a maleimide reactive group. The proteins were immobilized in agarose with oxygen scavenger added. Imaging was performed with 532 nm excitation and a dual-viewing system (a dichroic splits red and green emission onto different regions of the CCD). The two false-color images were superimposed, several frames were summed together, and the background was subtracted to produce this color image. Note: the labeling ratio on the protein is greater than unity.

3.6.2. Chemical Analysis

N.R. Conley and I performed separation and analysis experiments on bleached and partially bleached solutions of DCDHF-P-T.

From MS analysis of each band in the TLC from Figure 3.22B, I recorded the masses of the starting material (527 g mol$^{-1}$), a putative photooxidized species shown in Figure 3.23 (SM + 32 = 559 g mol$^{-1}$), and other putative photoproducts or contaminants. Band 2 in Figure 3.22B, the yellow emissive band, exhibited a significant proportion of the photooxidized species (559 g mol$^{-1}$), so I posit that this is the yellow species I observe in the SMS and bulk spectroscopy measurements.
Figure 3.22. (A) TLC plate of DCDHF-P-T and a bleached sample of the same. The plate was run with acetone and hexanes (from the bottom; the solvent front is about 60% of the way up), then imaged under 365 nm illumination. The starting material fluoresced red while the bleached material exhibited a yellow-emitting band. (B) A schematic of the several bands in a TLC plate of a partially bleached DCDHF-P-T sample. The colors correspond to the fluorescence under 365 nm illumination. Bands 1 and 2 are very dim; 3 and 4 look yellow under room lights. I performed MS analysis on each of these bands.

3.6.3. Possible Mechanisms

There are several reasonable mechanisms for dual emission. For instance, some chromophores exhibit emission from both a locally excited (LE) state as well as a red-shifted charge-transfer (CT) state. Single molecules exhibiting this behavior may switch between the LE and CT state, and thus may switch colors.\textsuperscript{202} One would expect reversibility on the single-molecule level (which I may have observed in a few cases, see Figure 3.17). In our case, this mechanism would indicate the DCDHF-P-T fluorophore emits from the TICT state, while other DCDHFs do not. The LE–CT mechanism could be tested by measuring the transient absorption spectra using a femtosecond laser and observing the two states and by observing different ratios of red and green single molecules in different media (e.g. PMMA vs PBMA polymer films).\textsuperscript{1}
Another possible mechanism is conformational isomerization of the fluorophores, with different conformers locked into place in the rigid polymer film.\(^{203-206}\) This mechanism could also be reversible and would depend on the rigidity of the matrix or the steric hindrance of the relevant twists in the compound (likely the phenyl–thiophene twist).

Dimers or aggregation are another possibility, with excimers or exciplexes emitting at a different color than the uncoupled individual molecules. However, because DCDHFs are generally very soluble in toluene and PMMA and because I did not observe exciplex emission at higher concentrations used for the bulk spectroscopy, this is an unlikely cause. Moreover, as seen in Figure 3.17, I observed both red-to-green and green-to-red transitions; instead, as one single molecule in the exciplex bleaches, one should observe only shifts from the exciplex-to-normal transitions.

Finally, I could be witnessing a photochemical change or degradation.\(^{210}\) This change could be reversible or irreversible and should be easy to observe on the ensemble level. The most obvious test is imaging single molecules of a new sample and an old or photooxidized sample and observing a change in the color of the

---

**Figure 3.23.** Possible photochemical mechanisms. (A) Possible photooxidation mechanism of bleaching and dual emission color. This oxygen addition has literature precedent.\(^ {207, 208}\) Moreover, the mass of the photooxidized structure on the right was found in the MS of a partially photobleached sample. (B) Possible rearrangement mechanism.\(^{209}\)

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103
emitters. Figure 3.23 displays two possible mechanisms for photochemical change in the structure, both from the literature.\textsuperscript{207-209} The photooxidation or photorearrangement product may be responsible for the unexpected blue-shifted emission of some of the single molecules and the new peaks that emerge in the bulk spectroscopy (Figure 3.18 and Figure 3.19).

I propose the photooxidation mechanism in Figure 3.23A, because solutions bubbled with nitrogen photobleached significantly slower than those exposed to oxygen. In addition, I observed a $+\text{O}_2$ mass in the MS of a photobleached sample. However, it is possible that the accelerated bulk photobleaching of DCDHF-P-T is a red herring and is not related to the multicolored singles I observed; therefore, more experiments are necessary.

**3.6.4. Summary**

If the exact mechanism of the dual emission from single molecules of DCDHF-P-T is determined, this property could be exploited for a readout of local environment. For instance, there is some indication that the emission color of DCDHF-P-T reports on the catalytic cycle of GroEL when the fluorophore is covalently attached to the chaperonin. Further investigation of this effect and other potential uses of the dual emitter are warranted.

**3.7. PHOTOCAGING FLUORESCENCE OF DCDHFS USING AN AZIDE**

While the dual-color photochemistry I describe in the previous section was unexpected, we have also introduced sophisticated color-changing photochemistry in the DCDHF class of fluorophores by design. For instance, by tuning the strength of the donor substituent in the DCDHF compounds, we have been able to significantly blue-shift the absorption peak of the chromophores and disrupt their fluorescence. This is an expected result of removing the red-shifted charge-transfer band by eliminating the push–pull character of the compound. If the disrupting substituent can
be photochemically converted back to an electron donor, the fluorescence could be photoactivatable. In fact, by replacing the amine with an azide, we have demonstrated such a new class of photoactivatable fluorophores.\textsuperscript{99, 211} A full discussion of the resulting azido fluorogens follows in Chapter 4.

### 3.8. CONCLUSION

We have demonstrated various ways in which modifying the chemical structure of the DCDHF fluorophores tunes their photophysical functions in predictable ways. For instance, it is possible to red-shift the absorption and emission from the fluorophores by extending the length of the $\pi$-conjugated linker between the amine donor and DCDHF acceptor groups, as expected from a simple particle-in-the-box model. We increased the fluorescence quantum yield by sterically restricting bond twists. We also increased water-solubility by added polar and charged groups to the basic structures. Finally, we tested excitonic dimer models by synthetically confining pairs of chromophores in different configurations. Some puzzles remain to be resolved in the dual-emission fluorophores. The synthetic flexibility of the DCDHF fluorophores, combined with these structure–property relationships, reveals the range of functions this class of fluorophores can have. Moreover, understanding the physical mechanism of the spectroscopic properties of these fluorophores has allowed us to introduce more sophisticated photophysics, such as photocaged fluorescence, which will be discussed in further detail in the following chapter.
4.

PHOTOACTIVATABLE AZIDO CHROMOPHORES
Recently, several groundbreaking advances in optical imaging have occurred, which allow imaging far beyond the diffraction limit of visible light without requiring placement of tips or apertures in contact with the specimen. Because optical microscopy is generally noninvasive, can record dynamical changes, and acts at a distance, this promises to be a revolution in optical microscopy. Some methods (STED, interference) require careful patterning of optical fields, while others rely on controlling the emission of single fluorophores, the primary interest here. Advances in super-resolution fluorescence imaging by controllable photoactivation of single-molecule emitters (e.g. PALM, FPALM, STORM) require new and optimized activatable fluorophores. If one single emitter is switched on at a time in a diffraction-limited region (~250 nm), its location can be determined well below the diffraction limit by fitting the point-spread function (i.e. image of the single molecule). A super-resolution image of a labeled complex structure can then be reconstructed from many successive rounds of weak photoactivation and fitting. For a description of the key method, see Figure 4.1.

**Figure 4.1.** Schematic showing the key idea of localization-based super-resolution microscopy of a structure. (A) It is not possible to resolve the underlying structure in a conventional widefield fluorescence image because the fluorescent labels are in high concentration and have overlapping point-spread functions. (B) Using controllable fluorophores, it is possible to turn on and image a sparse subset of molecules, which then can be localized with nanometer precision (black line is the underlying structure being sampled). Once the first subset of molecules photobleaches, another subset is turned on and localized. This process is repeated and the resulting localizations summed to give a super-resolution image of the underlying structure. From Thompson et al. 213
Several groups have been developing photoswitchable fluorescent proteins, \textsuperscript{13, 106, 107} organic fluorophores, \textsuperscript{79, 97-99, 211, 214, 215} and quantum dots\textsuperscript{102} in order to build the toolbox of controllable emitters.\textsuperscript{110} Recently, we reported a photoactivatable azido version of a push–pull fluorophore that contains a DCDHF moiety as a very strong electron-accepting group.\textsuperscript{99, 211} In addition to super-resolution imaging, the ability to photochemically control the fraction of emitting molecules has additional applications in pulse-chase experiments, single-molecule tracking, or in situations where the number of emitting molecules at a given time must be kept low.

### 4.1. STRATEGIES FOR DESIGNING AND CHARACTERIZING PHOTOACTIVATABLE FLUOROPHORES

Taking into account both the localization precision\textsuperscript{216, 217} and the Nyquist–Shannon sampling theorem,\textsuperscript{218, 219} the best emitters for photoactivation and localization-based super-resolution imaging will maximize the number of well-localized unique molecules per area per time.\textsuperscript{78} To achieve this, good photoactivatable fluorophores must turn on easily in high yield, be bright, emit many photons, densely label the sample, and have a high contrast between on and off states. In addition, the probe must be easily photoactivated to avoid cell damage from short-wavelength activating illumination.

#### 4.1.1. Photostability

One of the most important parameters for single-molecule fluorophores is the number of photons each molecule emits before photobleaching ($N_{\text{photons}}$). Scaling inversely with $N_{\text{photons}}$ is the photobleaching quantum yield ($\Phi_B$), or the probability of bleaching with each photon absorbed (see Chapter 2). A very low value of $\Phi_B$ corresponds to not only a long-lived fluorophore, but also to very high precision in localizing the point emitter, because the localization precision in many cases is given
by the usual diffraction limit (\(\sim \lambda/2\text{NA}\)) divided by the square root of the number of detected photons.\textsuperscript{216, 217}

### 4.1.2. Labeling Density

Besides the number of photons emitted, fluorophore labeling density is another important variable that determines the ultimate resolution. Because super-resolution imaging by switching point sources is effectively a sampling of the true underlying structure, there are well-known requirements on the labeling density (or spatial sampling frequency) for a correct reproduction of the structure from the samples at a given resolution. For instance, the Nyquist–Shannon sampling theorem\textsuperscript{63, 78, 218-220} requires that the fluorophores label the structure of interest at a frequency (number per spatial distance) that is at least double the desired resolution. For an ultimate resolution of \(\sim 20\) nm in two dimensions, this requirement means that there must at least one label every 10 nm on average, translating to hundreds of labels in each diffraction-limited area; for 3D imaging, this requirement increases to thousands or tens of thousands of labels per diffraction-limited volume.

### 4.1.3. Turn-On Ratio

The Nyquist–Shannon theorem basically requires that labels must actually be localized to the same average density as the labeling. This criterion adds a further restriction on the emitters in that the turn-on ratio (i.e. the contrast between the bright and dark states of the molecule) must be very high, lest the many weakly emitting “off” molecules in a diffraction-limited spot drown out the signal from the one “on” molecule. This section describes an accurate and experimentally convenient method for measuring the turn-on ratio of photoactivatable fluorophores. For bulk experiments, the fluorogens are doped into a film (e.g. polymer, gelatin, agarose) at approximately 1–2 orders of magnitude higher concentration than single-molecule experiments, but otherwise are imaged under similar conditions. This measurement assumes that one is working in a concentration regime where the emitters are dense enough to obtain a sufficient statistical sampling of the population but separated enough to avoid self-quenching or excimer behavior.
The goal of the experiment is to measure how many times brighter an activated molecule is than the preactivated fluorogen. Thus, the limit I care about is when the intensity from one bright molecule \( I_{on} \) equals the intensity from \( n_{off} \) dark fluorogens (i.e. when \( I_{on} = n_{off} I_{off} \)):

\[
R = \frac{I_{on}}{I_{off}} = \frac{n_{off} I_{off}}{I_{off}} = n_{off} .
\]  

(4.1)

Assuming that every dark molecule becomes fluorescent is rarely correct; to the contrary, \( n_{on} < n_{off} \) is the common situation. One could measure \( R \) by averaging over many single molecules; however, this would select only the fluorogens that become fluorescent, and the value would be artificially inflated.

Alternatively, it is more accurate to measure an effective turn-on ratio that takes into account the reaction yield. In a bulk experiment, the background-subtracted intensities can be integrated over a large region before activation (\( S_{off} \)) and after activation to steady-state (\( S_{on} \)). Not all copies of the fluorogen convert to the fluorescent species, as the simple ratio \( R \) assumes above; the overall reaction yield \( p \) is almost always less than unity. Therefore, the total number of emitters that will turn on is the reaction yield times the number of precursor molecules: \( n_{on} = p n_{off} \). The ratio of the background-subtracted signals in a bulk experiment gives the effective turn-on ratio \( R_{eff} \), which is the experimentally relevant parameter:

\[
R_{eff} = \frac{S_{on}}{S_{off}} = \frac{n_{on} I_{on}}{n_{off} I_{off}} = \frac{p n_{off} I_{on}}{p n_{off} I_{off}} = \frac{p I_{on}}{I_{off}} = p R = p n_{eff} = n_{on} .
\]  

(4.2)

The value \( R_{eff} \) corresponds directly to the maximum number of molecules that one could localize, \( n_{on} \), in a diffraction-limited spot before the aggregate signal (\( n_{off} I_{off} \)) of all the dark fluorogens required for that number of localizations equals the signal from one on molecule \( I_{on} \).

The measured value of \( R_{eff} \) should be considered a lower limit, because any molecules already in the “on” state before activation (preactivated molecules) contribute to the background signal in the frames before activation, thus lowering the measured value of the parameter. The percentage \( q \) of preactivated molecules should be kept low by protecting the fluorophore stock solution and samples from room lights.
and by pretreating the sample with the imaging wavelength to return preactivated molecules to the “off” state (prebleaching) the sample if possible. Regardless, some preactivation will inevitably occur. I can calculate the effect preactivation has on measuring $R_{\text{eff}}$ by including signal from preactivated molecules in the dark measurement:

$$
R_{\text{eff, preact}} = \frac{S_{\text{on}}}{S_{\text{off, preact}}} = \frac{n_{\text{on}} I_{\text{on}}}{n_{\text{off}} I_{\text{off}} + q n_{\text{off}} I_{\text{on}}}
$$

$$
= \frac{n_{\text{on}} I_{\text{on}}}{n_{\text{off}} I_{\text{off}} \left(1 + q n_{\text{off}} I_{\text{on}} / n_{\text{off}} I_{\text{off}}\right)} = \frac{p R}{1 + q R} = \frac{R_{\text{eff, true}}}{1 + q R}. \quad (4.3)
$$

Thus the multiplicative correction factor to convert from measured to true effective turn-on ratio is $(1 + qR)$. Even 0.1% preactivation could artificially deflate the measured value by half (assuming the $R = I_{\text{on}} / I_{\text{off}}$ of one isolated molecule is 1000). Therefore, minimizing preactivation (or, alternatively, maximizing prebleach) before measuring the effective turn-on ratio can increase the value of $R_{\text{eff}}$ such that it approaches the true ratio. However, prebleaching is not always an option—if the sample is highly light-sensitive—so $R_{\text{eff}}$ remains a practical measure of the lower limit for the turn-on ratio.

Resolution on the order of nanometers or tens of nanometers requires labels with densities of many thousands of localizations per $\mu$m$^2$ and therefore turn-on ratios in the hundreds or thousands. For example, if I assume the diffraction limit to be approximately 250 nm, the area of the diffraction-limited spot is about 50,000 nm$^2$. If $R_{\text{eff}} = 325$, there is a maximum of 325 localizations in each diffraction-limited spot, so the average distance between each localization is approximated by $\sqrt{50,000 \text{ nm}^2 / 325} = 12$ nm. The Nyquist–Shannon theorem\(^{218, 219}\) requires a sampling at least of twice the desired resolution, limiting the resolution to about 25 nm (in two dimensions). For three dimensions, the excitation volume in z is much larger than in x–y. Therefore, much higher contrast ratios and labeling densities are required for high-resolution imaging. For more details, see the supplemental material of Shroff et al.\(^{78}\)
4.1.4. Photoconversion Efficiency

Photoconversion from a precursor fluorogen to the emissive form can be monitored in bulk by measuring changes over time in absorbance or emission of the reactant or photoproduct of interest. The quantum yield of photoconversion $\Phi_P$ is defined as:

$$\Phi_P = \frac{R_p}{R_{abs}} = \frac{1}{\tau_p R_{abs}} = \frac{1}{\tau_p \sigma_\lambda I_\lambda \left(\frac{\lambda}{hc}\right)},$$

(4.4)

where $\tau_p$ is the decay constant in the exponential fit to the decay curve, the absorption cross-section $\sigma_\lambda$ at the activation wavelength, $I_\lambda$ is the irradiance at the sample, $\lambda$ is the activation wavelength, $h$ is Planck’s constant, and $c$ is the speed of light.

The higher the value of $\Phi_P$, the more the sensitive the fluorogen is to the activating light, so less potentially cell-damaging blue or UV irradiation is required to activate fluorescence. Note that $\Phi_P$ is the probability that the starting material will react for each photon absorbed. A fraction of those molecules then become fluorescent because the photoreaction yield to fluorescent product is usually less than unity.

Photophysical properties of various photoswitchable molecules extracted from the literature are shown in Table 4.1, along with values for a new class of photoactivatable molecules which will now be described. Here I use the term “photoactivation” to describe a molecule that is dark before being turned on in some way, while “photoswitching” also includes molecules whose emission may be turned on and off multiple times.
Table 4.1. Photophysical properties of various photoswitchable molecules, including whether the fluorophore can be cycled between bright and dark states multiple times, absorption and emission peaks, and molar absorption coefficient, fluorescence quantum yield, photoconversion quantum yield, turn-on ratio, photobleaching quantum yield, and total photons emitted. All values are reported for the photoconverted form except photoconversion quantum yield. The first three rows are fluorophores we have designed and characterized; the data for the other rows were extracted from the literature.

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>$\lambda_{\text{abs}}/\lambda_{\text{em}}$ (nm)</th>
<th>$\varepsilon_{\text{max}}$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi_F$</th>
<th>$\Phi_P$</th>
<th>Turn-on ratio$^{[b]}$</th>
<th>$\Phi_B { N_{\text{photons}} }$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCDHF-V-P-azide$^{[99, 211]}$</td>
<td>570/613</td>
<td>54,100</td>
<td>good</td>
<td>0.025–0.39$^{[c]}$</td>
<td>excellent</td>
<td>4.1×10^{-6}</td>
</tr>
<tr>
<td>DCDHF-V-PF$_4$-azide$^{[23, 211]}$</td>
<td>463/578</td>
<td>20,000</td>
<td>very good</td>
<td>0.0062</td>
<td>moderate</td>
<td>9.2×10^{-6}</td>
</tr>
<tr>
<td>DCM-azide$^{[211]}$</td>
<td>456/599</td>
<td>31,100</td>
<td>excellent</td>
<td>0.18</td>
<td>excellent</td>
<td>6.2×10^{-6}</td>
</tr>
<tr>
<td>Cy3/Cy5+thiol$^{[79]}$</td>
<td>647/662[a]</td>
<td>200,000</td>
<td>very good</td>
<td>0.18</td>
<td>excellent</td>
<td>$\leq$1000$^{[c]}$</td>
</tr>
<tr>
<td>PC-RhB$^{[94, 95, 97]}$</td>
<td>552/580[a]</td>
<td>110,000</td>
<td>moderate</td>
<td>0.65</td>
<td>moderate</td>
<td>$\leq$600,000$^{[c]}$</td>
</tr>
<tr>
<td>EYFP$^{[15, 53, 80, 94, 222-224]}$</td>
<td>514/527[a]</td>
<td>83,400</td>
<td>moderate</td>
<td>0.61</td>
<td>(1.6×10$^{-4}$)</td>
<td>5.5×10^{-3}</td>
</tr>
<tr>
<td>PAGFP$^{[90, 94, 225, 226]}$</td>
<td>504/517</td>
<td>17,400</td>
<td>moderate</td>
<td>0.79</td>
<td>moderate</td>
<td>$\leq$6×10^{-5}</td>
</tr>
<tr>
<td>mEosFP$^{[220, 225, 226]}$</td>
<td>559/581</td>
<td>37,000</td>
<td>good</td>
<td>0.62</td>
<td>very good</td>
<td>$3.0×10^{-5}$</td>
</tr>
<tr>
<td>PAmCherry1$^{[228]}$</td>
<td>564/595</td>
<td>18,000</td>
<td>moderate</td>
<td>0.46</td>
<td>(identical to</td>
<td>$21,000$</td>
</tr>
<tr>
<td>Dendra2$^{[228-230]}$</td>
<td>553/573</td>
<td>35,000</td>
<td>moderate</td>
<td>0.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kaede$^{[30, 231]}$</td>
<td>572/582</td>
<td>60,000</td>
<td>moderate</td>
<td>0.33</td>
<td>(28)</td>
<td></td>
</tr>
<tr>
<td>mOrange1$^{[228, 230, 231]}$</td>
<td>615/640</td>
<td>85,000</td>
<td>poor</td>
<td>0.85</td>
<td>(16)</td>
<td></td>
</tr>
<tr>
<td>Dronpa$^{[232, 234]}$</td>
<td>503/518[a]</td>
<td>95,000</td>
<td>very good</td>
<td>0.85</td>
<td>$\leq$3×10^{-5}</td>
<td></td>
</tr>
</tbody>
</table>

[a] Reversible photoswitch. Some fluorophores listed as not reversible may be, but have yet to be reported as such. [b] Ratio of the fluorescence after and before photoactivation (see definition above). Some papers report a “contrast ratio” of red to green fluorescence, which is the product of the fold increase in red fluorescence and fold decrease in the green fluorescence; therefore, those reported contrasts are many times higher than the turn-on ratio, which is the relevant parameter for super-resolution imaging. Other papers report “contrast ratios” without definition, so I cannot confidently compare these values directly to turn-on ratio. [c] DCDHF become brighter when rigidized.87, 142 [d] This range corresponds to $R_{\text{eff}}-R$. [e] In the SI of reference 221 is reported only 0.1% spontaneous turn-on at ideal conditions (e.g. very high thiol and oxygen-scavenger concentrations). This value does not take into account the inherent on-off ratio of a single Cy5, so it is an upper limit. [f] Value estimated from photoconversion wavelengths, intensities, times, and spectra reported previously.231
4.2. THE AZIDO PUSH–PULL CLASS OF PHOTOACTIVATABLE FLUOROBENS

For several years, we have been exploring the properties of push–pull fluorophores containing an amine donor covalently linked to an electron acceptor group, such as a DCDHF. Recently, we created a novel class of photoactivatable single-molecule fluorophores by replacing the amine with an azide, which is not a donor. With long-wavelength pumping, the azido fluorogenic molecules are dark, but applying low-intensity activating blue light photochemically converts the azide to an amine, which restores the donor–acceptor character, the red-shifted absorption, and the bright fluorescent emission (Figure 4.2).

4.2.1. Design of the Azido Fluorogens

Photoactivatable (or “photocaged”) donor–π–acceptor push–pull chromophores can be designed by disrupting the charge-transfer band, and therefore significantly blue-shifting the absorption to the extent that it is no longer resonant with the imaging laser. In these cases, photoactivation requires a photoreaction that converts the disrupting component to a substituent that is capable of restoring the charge-transfer band. For example, if the donor is removed in the fluorgenic form, photoactivation should produce a functional group which is capable of donating electrons into the chromophore’s conjugated network.

![Figure 4.2](image)

**Figure 4.2.** Photoconversion of dark azide-substituted fluorogens produce fluorescent amine-substituted fluorophores, which may involve insertion into C–H or C–C bonds. A dark photoproduct is also possible.
While amines are strong electron-donating substituents, azides are weakly electron-withdrawing (see Table 4.2).\textsuperscript{236} Recovering fluorescence from aryl azides is possible because they are known to be photolabile. The photochemistry of aryl azides has been studied extensively;\textsuperscript{237} the photoreaction most often reported involves the loss of dinitrogen and rearrangement to a seven-membered azepine heterocycle. However, electron-withdrawing substituents on the aromatic ring can stabilize the nitrene intermediate and promote formation of the amino functionality.\textsuperscript{238} Because push–pull chromophores inherently contain a strong electron-withdrawing substituent, an azido push–pull molecule should be more prone to photoconvert to the fluorescent amino version upon irradiation with activating light that is resonant with the blue-shifted absorption of the azido fluorogen.

| Table 4.2. Hammett substituent constants\textsuperscript{a} for relevant groups at para position |
|-------------------------------|--------|
| substituent                  | $\sigma_p$ |
| $-\text{N}_3$                 | 0.08   |
| $-\text{NH}_2$                | -0.66  |
| $-\text{NO}_2$                | 0.78   |
| $-\text{CN}$                  | 0.66   |
| $-\text{CH}═\text{C(CN)}_2$   | 0.84   |
| $-\text{CH(CN)}═\text{C(CN)}_2$ | 0.98  |
| $\text{DCDHF}\textsuperscript{b}$ | +      |

\textsuperscript{a} Positive values are electron-withdrawing substituents; negative are electron-donating. For more information, see section 8.3 of reference 239. Values are from reference 236. \textsuperscript{b} The Hammett values for the DCDHF acceptor moiety is unknown, but it is a known strong electron-accepting group; cyano, dicyanovinyl, and tricyanovinyl values are included for rough extrapolation.

Push–pull chromophores containing an electron donor, a conjugated network (\(\pi\)) and an electron acceptor have been explored for many years for nonlinear optics,\textsuperscript{127} photoinduced electron transfer,\textsuperscript{240} and photorefractivity,\textsuperscript{133} some molecules in this class were found to be good single-molecule labels as described in the preceding chapters.\textsuperscript{87, 139, 142, 144, 147} In our approach, a nonfluorescent, blue-shifted azide–\(\pi\)–acceptor fluorogen precursor is photoconverted to a fluorescent, red-shifted amine–\(\pi\)–acceptor fluorophore. In the fluorogen, the donor is absent, but the product fluorophore contains all three necessary components of the complete donor–\(\pi\)–acceptor push–pull chromophore (Figure 4.2). Because the azido fluorogens do not exhibit the red-shifted charge-transfer band typical of push–pull chromophores,\textsuperscript{134, 235}
they are not resonant with the wavelengths used to excite the amino version of the fluorophore (Figure 4.3 and Table 4.3), and are therefore dark.

C

RS01021 (DCDHF-P-azide) reaction with DTT in ethanol

no obvious reaction

- Col 1 vs A(R21E)
- Col 3 vs A(r21dtt1)
- Col 5 vs A(R21DTT5)
- ethanol
- +DTT
- 26min
Figure 4.3. (A) Absorption curves in ethanol (bubbled with N₂) showing photoactivation of DCDHF-V-P-azide over time to fluorescent product DCDHF-V-P-amine. Different colored curves represent 0, 10, 90, 150, 240, 300, 480, and 1320 seconds of illumination by 3.1 mW/cm² of diffuse 407 nm light. The sliding isosbestic point may indicate a build-up of reaction intermediates. Dashed line: absorbance of pure, synthesized DCDHF-V-P-amine. Inset: Fluorescence from 594 nm pumping. After activation (solid line), there is at least a 100-fold increase from DCDHF-V-P-azide (dotted line), which is practically nonemissive. From Lord et al.99 (B) Photoactivation kinetics from data in A. The total yield of the reaction ([DCDHF-V-P-amine]/[DCDHF-V-P-azide]) is 69%. Photoconversion data for DCDHF-V-P-azide were fit using two exponentials (τ = 7.4 and 291 s); data for DCDHF-V-P-amine were fit using one exponential (τ = 353 s). (C) DCDHF-P-azide does not react appreciably with dithiothreitol (DTT), which indicates that these aryl azides are bioorthogonal. (D) In very basic solution (pH 11), DCDHF-P-azide does change in the presence of high-concentration DTT.

In related work, Bouffard et al. designed a chemically caged DCDHF fluorophore in an effort to detect cysteine: an electron-withdrawing sulfonyl group
was added to the nitrogen and thus interfered with its donor capability until it was cleaved off by any cysteine, producing an amine capable of donating electrons. Budyka et al.\textsuperscript{243} have previously reported that an azido hemicyanine dye (similar to the azido stilbazolium reported below) undergoes conversion to an amine upon irradiation with visible light; however, the fluorescence properties of this dye were not reported.

4.2.2. Demonstrating Photoactivation with the Azido DCDHF

Azido DCDHF fluorogens can be activated to form bright fluorophores in a live cell environment. Figure 4.4 and Figure 4.5 show azido-DCDHF fluorogens activated with low amounts of blue light in live CHO cells. The resulting fluorophores are bright in the aqueous environment of the cell. In these figures, the cells were incubated with azido DCDHF fluorogens which penetrate the cell membrane and non-specifically label the interior of the cell.

The azido push–pull chromophores meet many of the critical requirements for super-resolution single-molecule imaging (Table 4.1): several emit millions of photons before irreversibly photobleaching, are photoconverted with high quantum efficiency, exhibit high turn-on ratios, and possess moderate molar absorption coefficients and quantum yields.
Azido DCDHFs are photoactivatable. In contrast to the amine group, the azide group is not electron donating; therefore, charge-transfer band of the chromophore is disrupted and the absorption is significantly blue-shifted, so it is no longer resonant with the imaging laser. Irradiance with low-intensity 407 nm light converts the azide to an amine, which repairs the donor-acceptor character of the fluorophore and returns the absorption and emission to longer wavelengths. (A) Three CHO cells incubated with the DCDHF-V-P-azide fluorogen are dark before activation. (B) The fluorophore lights up in the cells after activation with a 10-s flash of diffuse, low-irradiance (0.4 W cm⁻²) 407 nm light. The white-light transmission image is merged with the fluorescence images (white), excited at 594 nm (~1 kW cm⁻²). Scalebar: 20 μm. (C) Single molecules of the activated fluorophore in a cell under higher magnification. Scalebar: 800 nm. From Lord et al.²⁹

Figure 4.2 and Figure 4.13 display several possible photoconversion pathways. With the loss of N₂, the azide on DCDHF-V-P-azide can be photoconverted to a reactive nitrene. The nitrene can then convert into an amine (DCDHF-V-P-amine) or a nitro group (DCDHF-V-P-nitro); both these structures were actually isolated and characterized (see below).²⁹

Figure 4.4 and Figure 4.5 demonstrate the utility of the azido DCDHF as a fluorogen: before photoactivation, cells incubated with DCDHF-V-P-azide are dark; after photoactivation, the cells light up as DCDHF-V-P-azide converts to DCDHF-V-P-amine. The intensity of activating light required to generate fluorescent DCDHF molecules is very low, at least three orders of magnitude lower than the intensity of
the imaging light. This helps ensure that the high-energy blue or UV activating light does not kill the cells of interest or alter their morphology. Furthermore, photobleaching is three orders of magnitude less likely than photoconverting, so the activated fluorophore emits millions of photons before photobleaching (see Table 4.1).

Figure 4.5. (A) Two living CHO cells incubated with DCDHF-V-P-azide before activation and after a five-second flash of diffuse, moderate-irradiance (13 W cm⁻²) 407 nm light. The 594 nm light (500 W cm⁻²) for imaging was illuminating the sample the entire time, except for the brief period of 407 nm activation. False color: gray is the white-light transmission image and red the thresholded fluorescence images. Bar, 15 μm. (B) Snapshots from a movie of single molecules of DCDHF-V-PF₄-amine embedded in a PMMA film, immediately after photoactivation of the corresponding azide. The inset shows the same frame immediately before activation. Preactivated molecules were first prebleached using high-irradiance 514 nm light, and then the sample was imaged at a lower irradiance (2 kW cm⁻²). To activate, a 100-ms flash of low-irradiance 407 nm light (0.2 kW cm⁻²) was applied. Pseudocolor scale: the number of counts in this 100-ms frame ranges from 459 at the minimum pixel to 6644 at the brightest pixel (which corresponds to approximately 40–578 photons detected per pixel per 100 ms). Bars, 2 μm. From Lord et al.²¹¹

The photophysics of this azido DCDHF fluorogen are favorable compared to other popular photoswitchable or photoactivatable molecules: it is red-shifted, emits many photons, and requires only low doses of blue light (see Table 4.3). However, the disadvantages of the azido DCDHF system are: it is not genetically targeted, as fluorescent proteins are; DCDHFs with a primary amine exhibit more blinking than those with secondary or tertiary amines (see Figure 4.6); and the photoactivation is
irreversible (once the fluorophore photobleaches, it cannot be reactivated, which is possible in some photoswitches.\textsuperscript{79, 97, 98, 225, 233})

In some imaging scenarios, irreversibility is advantageous: because the millions of photons emitted are not spread over many activating cycles, the localization precision can be high for each molecule. (For static structures, reversible switching is not ideal, because reactivation of an emitter that has already been localized is superfluous and only adds complexity to any image reconstruction.) Blinking, however, is not particularly desirable, but it can sometimes be used to reduce the emitter concentrations in super-resolution microscopy methods.\textsuperscript{212} This issue may be addressed in future work by finding a DCDHF version that does not blink as a primary amine, or via fluorogenic photoaffinity labeling that produces the secondary amine (because DCDHFs with a secondary amine are less prone to blink). When combined with a targeting moiety that brings the molecule close to the location of interest, fluorogenic photoaffinity makes such targeting permanent, and thus could resolve two disadvantages of the azido DCDHF system simultaneously.
**Method for Preparing Samples for Live-Cell Microscopy**

Samples were studied using an Olympus IX71 inverted microscope in an epifluorescence configuration\(^2\) using 488 or 514 nm illumination from an Ar-ion laser (Coherent Innova) or the 594 nm line from a HeNe laser (Meredith Instruments, 5 μW output); the irradiance at the sample was typically 0.5–1.0 kW cm\(^{-2}\). The emission was collected through a 100×, 1.4 N.A. oil-immersion objective, filtered using appropriate dichroic and long-pass filters to remove scattered excitation light, and imaged onto an electron-multiplying Si EMCCD camera (Andor iXon+) with integration times of 20–100 ms. For details of CHO cell culture, see reference 245. CHO cells were plated on fibronectin-coated borosilicate chambered coverslips overnight prior to imaging. CHO cells were treated with 1 μM dye solution (1 mM dye stock in ethanol into growth medium) at 37 ºC for 1 hr, followed by extensive PBS buffer rinses to remove excess dye. Briefly, cells were imaged at 22 ºC in supplemented PBS buffer. That is, imaging was performed within 45 min after removing the cell tray from the 37 ºC incubator to ensure cell viability. No changes in cell morphology were observed after photoactivation. Moreover, previous studies using DCDHFs in living cells did not encounter complications with toxicity.\(^5\)

\(^{122}\)

### 4.3. GENERALITY OF THE AZIDO PUSH–PULL CLASS OF PHOTOACTIVATABLE FLUOROGENS

We also extended the concept of azido push–pull fluorogens to compounds beyond DCDHFs. The various azido push–pull chromophores collected in Figure 4.11 and Table 4.3 are all photoactivatable: upon pumping the blue-shifted absorption, the nonfluorescent azido fluorogens photoconvert to longer-wavelength amino versions that are fluorescent. For example, see spectra in Figure 4.7 for NBD-azide; the other fluorogens follow the same trend and spectra are collected in Figure 4.12. Although there are several photoproducts, N.R. Conley and I confirmed with DCDHF-V-P-azide that the fluorescent amino product of the photoreaction is dominant via HPLC–MS.
and NMR (see section below). For the other fluorogens, the presence of an amino fluorophore in the photoreaction mixture is corroborated by recovery of the long-wavelength push–pull absorption and emission spectra. (In the case of DCM-azide, the absorption spectra of the photoreaction mixture include peaks that obscure the amino species; nevertheless, fluorescence excitation spectra reveal only one fluorescing species, which overlaps with the absorption curve of the independently synthesized DCM-amine. See Figure 4.12.)

Figure 4.7. (left) Spectra of NBD-azide photoactivation. (See Figure 4.12 for spectra of the other fluorogens.) Because the azide cannot participate as a donor in a charge transfer, the fluorogen exhibits blue-shifted absorption ($\lambda_{\text{abs}} = 384 \text{ nm}$) with respect to its amine-donor sister ($\lambda_{\text{abs}} = 456 \text{ nm}$). Upon irradiation with UV light, the azido fluorogen (solid black curve) converts to the amino fluorophore (solid grey curves). Fluorescence (dotted red lines) excited at 440 nm increases significantly after photoconversion (right). Photoactivation of DCDHF-P-azide using a 385 nm flashlight (1.1 mW cm$^{-2}$). The short-wavelength azido absorption peak (triangles) disappears with time and the long-wavelength absorption peak (circles) corresponding to the fluorescent amino fluorophore grows in. The time constant ($t_p = 85 \text{ s}$) from the exponential fit of the disappearance of the azido fluorogen is used to calculate the photoconversion quantum yield ($\Phi_p$) in Table 4.3. From Lord et al.$^{211}$

The azido push–pull chromophores I present in this Dissertation meet many of the critical requirements for super-resolution microscopy: several are photoconverted with high efficiency without a catalyst, emit millions of photons before irreversibly
photobleaching, exhibit high turn-on ratios, and possess moderate molar absorption coefficients and quantum yields (see Table 4.3).

4.3.1. Photostability of Photoactivatable Fluorogens

I have reported previously that the DCDHF molecules emit millions of photons and therefore have low values for $\Phi_B$ (see Chapter 2), and this is also true of some of the new activated fluorophores reported here. These photostable emitters enable precise localization and sophisticated imaging schemes: I demonstrated previously that single molecules of DCDHF-V-PF$_4$-azide in PMMA can be photoactivated and localized to less than 20 nm standard deviation in all three dimensions; two molecules separated by 36 nm were resolved in three dimensions.$^{123}$

As shown in Table 4.3, adding electron-withdrawing fluorines to the aromatic group (DCDHF-V-PF-amine and DCDHF-V-PF$_4$-amine) had little effect on the photostability parameter $\Phi_B$. DCM-amine is also a strong single-molecule emitter, with a $\Phi_B$ comparable to most DCDHFs. DCM-amine has a higher fluorescence quantum yield in solution, and thus is more likely to be bright throughout a sample (not just in rigid environments, which is a feature of DCDHFs$^{87,142}$). While NBD-amine is brightly fluorescent in all environments, it is many times less photostable than DCDHFs. The stilbazolium-amine fluorophore is also not as photostable as the DCDHFs or even DCM-amine (a precise value was not determined).

Method for Sample Preparation for Microscopy

Samples for aqueous bulk photostability measurements and quantitative single-molecule measurements were prepared using 5–10% (by mass) gelatin (type A, Bloom 200, MP Biomedicals) or 1% poly(vinyl alcohol) (PVA, 72000 g mol$^{-1}$, Carl Roth Chemicals) in purified water. The gelatin solution was liquefied at 37 °C; then a small volume (<0.5 μL) of dye stock solution in ethanol was mixed with 10 μL gelatin, sandwiched between two Ar-plasma-etched glass coverslips, and allowed to gel at room temperature. Single-molecule samples for the movie were made in 1% poly(methyl methacrylate) (PMMA, $T_g = 105$ °C, MW = 75000 g mol$^{-1}$, atactic, polydispersity ~2.8, PolySciences Inc.) in toluene by mass. For polymer samples, a
small volume of stock dye solution was mixed into an aliquot of PVA in water or PMMA in toluene, then the mixture was spin-coated onto an Ar-plasma-etched glass coverslip at 3000 rpm for 30 s. Thick samples of PMMA were prepared by drop casting 200–400 μL of 10% (by mass) PMMA in toluene and allowing the film to dry for several hours.

### 4.3.2. Photoconversion Efficiency of the Azido Fluorogens

Figure 4.4 and Figure 4.5 show that DCDHF-V-P-azide was readily activated in living CHO cells without obvious photodamage. By adding four electron-withdrawing fluorines to the benzene ring, we were able to further stabilize the nitrene and increase Φ_P by more than a factor of two (compare DCDHF-V-P-azide and DCDHF-V-PF₄-azide in Table 4.3). Other acceptor groups and structures resulted in even higher Φ_P values; for instance, NBD-azide needs to absorb only a few photons on average before photoreacting. These high photoconversion efficiencies enable activation at lower intensities; thus, super-resolution imaging by photoactivation should achieve higher cycling rates without causing photodamage to living cells. A drawback of experiments taking advantage of these very high Φ_P values is that they require near complete darkness (or red lights) during sample preparation to prevent preactivation of the fluorogens. Stock fluorogen solutions in ethanol in the dark were resistant to thermal activation for several days or weeks (data not shown).

**Specific Methods for Bulk Solution Spectroscopy for Photoconversion**

Bulk solution absorption and emission spectra were acquired on a Perkin-Elmer Lambda 19 UV–vis spectrometer and a Horiba Fluoromax-2 fluorimeter using standard 1-cm path length, quartz cuvettes. Fluorescence quantum yields were referenced against standards with known quantum yields, corrected for differences in optical density and solvent refractive index. All quantitative measurements were performed at low concentrations (absorbance values less than 0.2) to avoid any complications with dimer or aggregate formation. Molar absorption coefficients were measured from dilutions of solutions with known concentrations.
Photoconversion in ethanol was performed using one of the following light sources: a 365 nm hand-held UV lamp (0.62 mW cm$^{-2}$); a 385 nm diode flashlight (1.1 mW cm$^{-2}$, see Figure S5 for spectrum); the 407 nm line from a Kr-ion laser (Coherent Innova-301, 3.1 mW cm$^{-2}$).

The overall chemical reaction yields to fluorescent product listed in Table 4.3 were measured from the absorbance values in the photoactivation spectra. Yield was defined by $\frac{[\text{amino}]}{[\text{azido}]} = \frac{(A_{\text{amino}}/\varepsilon_{\text{amino}})}{(A_{\text{azido}}/\varepsilon_{\text{azido}})}$, where $i$ and $f$ refer to initial and final values, respectively. In cases where other photoproducts contributed significant absorbance at the amino peak wavelength, the final absorbance value for the amino peak $A_{\text{amino},f}$ was corrected for this additional absorbance.

### 4.3.3. Turn-on Ratio of the Azido Fluorogens

To measure the effective turn-on ratio, the signal on the camera from many activated fluorophores in a PMMA film was divided by the signal from the same location in the sample before activation; the background intensity level measured in an undoped film was subtracted from both signals (see Figure 4.9). This measurement is experimentally relevant because it not only uses illumination intensity levels comparable to those used in actual imaging, but also considers the decrease in contrast due to a chemical reaction yield that is less than unity. The resulting effective turn-on ratio $R_{\text{eff}} = 325$ serves as a lower limit. Alternatively, the turn-on ratio $R = 1270$ measured on the single-molecule level is the upper limit on the ultimate labeling density. (For a full discussion of the meaning of the turn-on ratio, see above.)

#### Measuring the Turn-on Ratio

The simple turn-on ratio $R$ was measured on the single-molecule level as shown in Figure 4.8. The intensity from a single-molecule spots was spatially integrated and compared to the intensity from the same area immediately before activation. Both values were corrected for a general offset from dark counts and the background from the polymer matrix. For this measurement, the average turn-on ratio to be $R = 1270 \pm 500$. 

126
Figure 4.8. Measuring turn-on ratio $R$ on the single-molecule level. Two snapshots from a movie of single molecules of DCDHF-V-PF$_6$-amine embedded in a PMMA film immediately before (left) and after (right) photoactivation of the corresponding azide. Preactivated molecules were first prebleached using high-intensity 514 nm light, and then the sample was imaged at lower intensities (2 kW cm$^{-2}$). To activate, a 100-ms flash of low-intensity 407 nm light (0.2 kW cm$^{-2}$) was applied. The number of counts in this 100-ms frame ranges from 459 at the minimum pixel to 6644 at the brightest pixel (which corresponds to approximately 40–578 photons detected per pixel per 100 ms). Each frame is approximately 10×10 μm.

From Lord et al.$^{211}$

For the bulk experiments, the fluorogens were doped into a thick PMMA polymer film at approximately 1–2 orders of magnitude higher concentration than single-molecule experiments, but otherwise imaged under similar conditions. This approach assumes that I am working in concentration regime where emitters are dense enough to get a statistical sampling of the population but separated enough to avoid self-quenching or excimer behavior. From our previous experience with bulk and single-molecule samples of fluorophores in polymer films, I am confident that I am safely in this regime. The average effective turn-on ratio measured this way was $R_{\text{eff}} = 325 \pm 15$.

Because in some measurements the on and off signals fell outside the dynamic range of the CCD detector, it was necessary to use different intensities or gain levels for the dark and background measurements than for the fluorescent signal. This required that the intensities and gain levels were accurately measured and accounted for in the calculation of the turn-on ratio.
4.3.4. Intermediate Lifetime of the Azido Fluorogens

The lifetime of the nonfluorescent intermediate of DCDHF-V-PO-azide was measured immediately after the photoconversion. Imaging in the widefield epifluorescence microscope at 594 nm (∼1 kW cm⁻²), a sample in aqueous agarose gel was repeatedly photoactivated with 0.5 s of high-intensity 405 nm light (∼1 kW cm⁻²). These bright bursts of violet light both photobleached any existing fluorescent species and initiated the photoconversion of the azido species to fluorescent amino products, via a nonfluorescent intermediate (putatively a nitrene, but there may be additional intermediates requiring rearrangement before fluorescence appears).
Figure 4.10. Measuring the intermediate lifetime.

Multiple activation cycles were summed into one master curve (Figure 4.10). By fitting with a single exponential the increase of fluorescence over time after the photobleaching/photoactivating burst, the average lifetime of the intermediate was determined to be 1.84 s. This relatively long lifetime is likely the result of stabilizing the nitrene intermediate with electron-withdrawing substituents. Moreover, the long lifetime explains the sliding isosbestic point in Figure 4.3.

Presumably, this lifetime is dependent on the environment, solvent, and temperature; however, this was measured in an aqueous environment that is not dissimilar to the cell.
4.3.5. Structures and Spectral Properties of the Azido Fluorogens

DCDHF-P-azide

DCDHF-V-P-azide

DCDHF-V-PCN-azide

DCDHF-V-PO-azide

DCDHF-V-PF-azide

DCDHF-V-PF₄-azide

DCM-azide

stilbazolium-azide

NBD-azide

Figure 4.11. Structures of push–pull azido fluorogens.
Figure 4.12. Spectra of azido push–pull fluorogens, their photoreaction mixtures and fluorescence spectra of the azido fluorogens and amino photoproducts. From Lord et al.211

Table 4.3. Photophysical and photochemical parameters of various azido fluorogens (in ethanol unless otherwise stated)

<table>
<thead>
<tr>
<th>Azido Fluorogen</th>
<th>λ&lt;sub&gt;abs,azide&lt;/sub&gt; &lt;sup&gt;[a]&lt;/sup&gt;</th>
<th>λ&lt;sub&gt;abs,amine&lt;/sub&gt; &lt;sup&gt;[b]&lt;/sup&gt;</th>
<th>λ&lt;sub&gt;fl,amine&lt;/sub&gt; &lt;sup&gt;[c]&lt;/sup&gt;</th>
<th>Yield &lt;sup&gt;[d]&lt;/sup&gt;</th>
<th>Φ&lt;sub&gt;P&lt;/sub&gt; &lt;sup&gt;[f]&lt;/sup&gt;</th>
<th>Φ&lt;sub&gt;B&lt;/sub&gt; &lt;sup&gt;[h]&lt;/sup&gt; (10&lt;sup&gt;−6&lt;/sup&gt;)</th>
<th>in PVA</th>
</tr>
</thead>
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<tr>
<td>DCDHF-P-azide</td>
<td>379</td>
<td>475</td>
<td>496</td>
<td>24%</td>
<td>0.003</td>
<td>~0.08</td>
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</tr>
<tr>
<td>DCDHF-V-P-azide</td>
<td>424</td>
<td>570</td>
<td>613</td>
<td>65%</td>
<td>0.025</td>
<td>0.0059</td>
<td>4.1&lt;sup&gt;[k]&lt;/sup&gt;</td>
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<tr>
<td>DCDHF-V-PCN-azide</td>
<td>415</td>
<td>479</td>
<td>591</td>
<td>~50%</td>
<td>~0.09</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>DCDHF-V-PO-azide</td>
<td>443</td>
<td>572</td>
<td>627</td>
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<td>0.095</td>
<td></td>
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<td>{385}</td>
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<td>DCDHF-V-PF-azide</td>
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<td>DCDHF-V-PF&lt;sub&gt;4&lt;/sub&gt;-azide</td>
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<td>463</td>
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<td>0.017</td>
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<tr>
<td>DCM-azide</td>
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<td>456</td>
<td>599</td>
<td>&lt;30%</td>
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<td>0.085</td>
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<td>stilbazolium-azide</td>
<td>375</td>
<td>449</td>
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<td>~25%</td>
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<tr>
<td>NBD-azide</td>
<td>384</td>
<td>456</td>
<td>539</td>
<td>~50%</td>
<td>0.20</td>
<td>~0.2</td>
<td>100&lt;sup&gt;[l]&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>{14,400}</td>
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</table>

[a] Peak absorbance (nm) and molar absorption (M<sup>−1</sup> cm<sup>−1</sup>) for azido fluorogen, and [b] amino fluorophore. [c] Fluorescence peak (nm) of amino fluorophore. [d] Overall chemical reaction yield to the fluorescent amino product. [e] Fluorescence quantum yield of amino fluorophore. DCDHFs become much brighter in rigid environments.142 [f] Photoconversion quantum yield of azido fluorogens to any product. [g] Wavelength (nm) used to photoactivate azido fluorogens from one of the following light sources: a 365 nm hand-held UV lamp (0.62 mW cm<sup>−2</sup>); a 385 nm diode flashlight (1.1 mW cm<sup>−2</sup>); or the 407 nm line from a Kr-ion laser (Coherent Innova-301, 3.1 mW cm<sup>−2</sup>). [h] Photobleaching quantum yield of amino fluorophore. Fluorescein in gelatin is 64×10<sup>−6</sup>. [i] In dichloromethane. [j] In acetonitrile. [k] In gelatin. [l] In PMMA. From Lord et al.211
4.3.6. Chemical Analysis of Photoproducts of the Azido Fluorogens

For most of the azido fluorogens, I relied on UV–vis and fluorescence spectroscopy in order to identify the amine products, comparing the spectra of the photoconverted samples to the spectra of independently synthesized versions of the amino fluorophores. For the DCDHF-V-P-azide case, detailed chemical analysis was performed. Samples for these bulk chemical studies were photoconverted in ethanol, both with and without removing dissolved oxygen by bubbling N₂, and analyzed using NMR and HPLC–MS.

![Chemical structures and reactions](image)

**Figure 4.13.** Photoactivation reactions of the azido DCDHF fluorogen. Aryl azides are known to be photolabile: the loss of dinitrogen leaves a reactive nitrene intermediate, which can rearrange to form a seven-membered azepine heterocycle (not shown), an amine (DCDHF-V-P-amine, 2), or a nitro (DCDHF-V-P-nitro, 3) group. Compounds 1 and 3 are not fluorescent when pumped at long wavelengths, but photoproducts 2 and 4 are fluorescent. Compound 4 is hypothetical and the result of nitrene inserting into C–C or C–H bonds of a nearby biomolecule.

**Column Chromatography and NMR**

A solution of photoconverted DCDHF-V-P-azide in ethanol was separated on a TLC plate (1:3 acetone:dichloromethane) into two bands: a red band with lower Rₕ that was fluorescent under UV light (365 nm) and a yellow band with higher Rₕ that was nonemissive; the yellow band was not present when the solution of DCDHF-V-P-
azide was deoxygenated by bubbling N₂ before and during photoconversion. (Adequate separation was not achievable using dichloromethane and hexanes or dichloromethane alone; therefore, we resorted to acetone in the mobile-phase solvent mixture.)

For chromatography, the photoproducts were separated on a column using silica gel as the stationary phase and 2:1 hexanes:acetone as the mobile-phase solvent. Two bands were well separated: a yellow band of DCDHF-V-P-nitro eluted first, then a red band of DCDHF-V-P-amine eluted later (Figure 4.14).

NMR spectra of column-separated photoproducts confirm these assignments, as compared to pure, synthesized samples (although the yellow band was contaminated with some other minor photoproducts).²³⁸,²⁴⁶ DCDHF-V-P-azide: ¹H NMR (400 MHz, CDCl₃, δ): 7.65 (d, J = 8.4 Hz, Ar, 2H), 7.61 (d, J = 16 Hz, vinyl, 1H), 7.13 (d, J = 8.4
Hz, Ar, 2H), 6.97 (d, J = 16 Hz, vinyl, 1H), 1.80 (s, CH3, 6H). DCDHF-V-P-amine (photoconverted from DCDHF-V-P-azide, column separated): 1H NMR (400 MHz, CDCl3, δ): 7.58 (d, J = 16 Hz, vinyl, 1H), 7.50 (d, J = 8.4 Hz, Ar, 2H), 6.80 (d, J = 16 Hz, vinyl, 1H), 6.70 (d, J = 8.8 Hz, Ar, 2H), 4.39 (s, NH2, 2H), 1.76 (s, CH3, 6H). DCDHF-V-P-amine (pure synthesized independently): 1H NMR (500 MHz, CDCl3, δ): 7.58 (d, J = 16 Hz, vinyl, 1H), 7.50 (d, J = 8.5 Hz, Ar, 2H), 6.80 (d, J = 17 Hz, vinyl, 1H), 6.70 (d, J = 8.5 Hz, Ar, 2H), 4.39 (s, NH2, 2H), 1.76 (s, CH3, 6H). DCDHF-V-P-nitro (photoconverted from DCDHF-V-P-azide, crude, column enriched): 1H NMR (300 MHz, CDCl3, δ): 8.34 (d, J = 8.7 Hz, Ar), 7.80 (d, J = 8.4 Hz, Ar), 7.69 (d, J = 11 Hz, vinyl), 7.12 (d, J = 14 Hz, vinyl), 1.83 (s, CH3). DCDHF-V-P-nitro (pure synthesized independently): 1H NMR (400 MHz, CDCl3, δ): 8.34 (d, J = 8.8 Hz, Ar, 2H), 7.80 (d, J = 8.8 Hz, Ar, 2H), 7.68 (d, J = 16.8 Hz, vinyl, 1H), 7.12 (d, J = 16.4 Hz, vinyl, 1H), 1.83 (s, CH3, 6H).

**Purification of DCDHF-V-P-amine and DCDHF-V-P-nitro by Semi-Prep HPLC**

An ethanolic solution containing ~1 mg mL⁻¹ of DCDHF-V-P-azide was photoconverted using a 150-W Xe lamp for 5 min under air. Photoproducts DCDHF-V-P-amine and DCDHF-V-P-nitro were separated by HPLC on a Hypersil Hyper Prep 100 BDS–C18 column (10.0×250 mm) with linear gradient elution (5–100% acetonitrile over 25 min, 5 min hold at 100% acetonitrile; balance by volume, 0.1 M tetraethylammonium acetate buffer, pH 7.5; total flow rate, 4 mL min⁻¹). The UV–vis absorption spectrum of the column eluent was continuously monitored using a Shimadzu diode array detector (SPD–M10A). Under these conditions, compounds DCDHF-V-P-amine and DCDHF-V-P-nitro exhibited retention times of 20.9 and 22.5 min, respectively. No detectable DCDHF-V-P-azide (RT = 23.6 min) remained after photoactivation.

**HPLC–MS Characterization of Photoproducts**

Ethanolic solutions of DCDHF-V-P-azide were photoconverted using diffuse 407 nm laser light under nitrogen or air. The photoactivation products were analyzed by HPLC–MS (Waters 2795 Separations module with 2487 Dual λ Absorbance Detector;
Waters Micromass ZQ mass spectrometer). Gradient elution (2–95% acetonitrile with 0.1% formic acid over 20 min, 10 min hold at 95% acetonitrile/formic acid; balance by volume, water with 0.1% formic acid) through a C18 column (2.1×40 mm) was employed for the separation. The column eluent was subjected to electrospray ionization, and positive and negative ions with $m/z$ from 100–1000 amu were detected.

In the absence of oxygen, photoconversion of DCDHF-V-P-azide produced DCDHF-V-P-amine (RT = 11.36 min; ESI$: m/z = 301.7$, [M–H]$^-$; ESI$^+$: $m/z = 303.5$, [M+H]$^+$) as the only major photoproduct. A putative azo dimer (RT = 16.97 min; ESI$^-$: $m/z = 599.7$, [M–H]$^-$) was observed as a minor photoproduct.

In air, photoactivation of DCDHF-V-P-azide produced a mixture of DCDHF-V-P-amine (RT = 11.43 min; ESI$: m/z = 301.5$, [M–H]$^-$; ESI$^+$: $m/z = 303.4$, [M+H]$^+$) and DCDHF-V-P-nitro (RT = 12.99 min; ESI$: m/z = 331.5$, [M–H]$^-$, 315.5 [M–O–H]$^-$, 301.5 [M–2O–H]$^-$) as major products. After several days in air and room lights, an unidentified species believed to be generated from DCDHF-V-P-nitro formed in the solution (RT = 19.15 min; ESI$^-$: $m/z = 367.6$).

### 4.4. FLUOROGENIC PHOTOAFFINITY LABELING USING AZIDO PUSH–PULL FLUOROGENS

Besides super-resolution imaging, the azido push–pull fluorogens can be applied to other biological imaging schemes. For instance, the well-known photoaffinity labeling (PAL) scheme requires a chemically inert binding molecule that becomes reactive upon illumination; the reactive photoproduct forms a covalent bond to a biomolecule to which it is bound or near. This technique has been used to study small-molecule binding, binding-pocket chemistries, and protein-protein interactions. Aryl azides are common PAL tags, because the nitrene intermediate is reactive and long-lived (I measured a lifetime of nearly 2 s, see above).
Figure 4.15. (A) A schematic of nonspecific fluorogenic photoaffinity labeling (PAL) of whole cells. The nitrene intermediate resulting from the photoconversion of an azide to an amine is reactive enough to insert into bonds of nearby biomolecules. The reaction simultaneously turns-on fluorescence and covalently links the probe to the biomolecule. (B) Gel electrophoresis of CHO-cell lysate demonstrating fluorogenic PAL using NBD-azide. (Similar results were observed with DCDHF-P-azide, data not shown.) The left panel shows the stained protein, imaged with white light; the right panel is fluorescence from NBD-amine photochemically cross-linked to proteins, imaged using 488 nm. The left lane in the gel (+PAL) is protein covalently labeled with NBD-amine by PAL. The right lane (–PAL) is a control performed by mixing into the cell solution preactivated NBD-
amine, which is fluorescent but cannot participate in the covalent PAL bioconjugation reaction. The fluorescence signal in the control lane was significantly lower. The blurry fluorescence on the bottom of the gel is from the unbound dye at the front edge; equal brightness in both lanes indicates equal dye concentration in the PAL and control. From Lord et al.\textsuperscript{211}

Here, H.D. Lee and I demonstrate fluorogenic PAL: a dark precursor that can become both fluorescent and bioconjugated in one illumination step. Figure 4.15 shows proteins from CHO cells labeled with an azido push–pull fluorogen by fluorogenic PAL and purified by denaturing gel electrophoresis. Figure 4.16 demonstrates fluorogenic PAL using pure protein bovine serum albumen (BSA) in water. These experiments demonstrate that nonspecific fluorogenic PAL is possible; moreover, it should be possible to engineer a binding pocket for one of these azido push–pull fluorogens, increasing the PAL reaction yield and producing a targeted fluorogenic bioconjugation system. Additional targeting strategies to place the fluorogen at a position of interest can also be envisioned.

Some azide-based fluorogenic PAL ligands have been reported previously.\textsuperscript{251-255} In these earlier studies, the light required to excite fluorescence was in the high-energy ultraviolet (e.g. 280–350 nm), which prohibits ultrasensitive detection in living cells, because these short wavelengths pump cellular autofluorescence and cause cell damage. Moreover, photoconversion in these previous cases did not produce fluorophores photostable enough to be applied to single-molecule imaging. Our azido push–pull fluorogens can be activated and imaged using visible light, and several produce fluorophores that can be easily imaged on the single-molecule level.
4.4.1. Methods for Photoaffinity Labeling

Photoaffinity labeling (PAL) of azido fluorogens was performed on CHO cells. Under red lights, 20 μL of ~1 mM NBD-azide and DCDHF-P-azide stock solutions in ethanol (kept in the dark to prevent preactivation) were mixed with separate 200 μL aliquots of CHO cells suspended in PBS buffer. The mixed solution was photoactivated using a 365 nm handheld Hg UV lamp (0.4 mW cm⁻²) for 30 minutes. The nitrene intermediate inserted into bonds of accessible biomolecules. For a control, aliquots of the fluorogen stock solutions were preactivated before mixing with cells; the preactivated dye is unable to participate in the covalent PAL bioconjugating photoreaction because the nitrene does not survive for more than a few seconds (see above).

The CHO cells were then lysed using RIPA buffer, and passed through 200 μL pipette tip 50 times. The insoluble portion of the lysate was spun down at 4 °C, 14000
rpm and discarded. The soluble supernatant was mixed with SDS and heated to 95°C. The lysate was then separated on a 12% polyacrylamide SDS gel to separate the PAL fluorescence from unbound fluorophores. After the electrophoresis completed, the gel was destained using a 10% acetic acid solution in water and methanol. To image the gels, a GE Healthcare Typhoon 8600 scanner was used with 488 nm excitation, a 526 short-pass filter, and 600 PMT sensitivity setting. The protein in the gel was then stained with Coomassie Brilliant Blue and destained overnight. The Coomassie bands were imaged using white light (BioRad Gel Doc scanner with Universal Hood II).

4.5. CONCLUSION

A wide variety of azido fluorogens are photoactivatable to produce bright push–pull fluorophores, some of which emit millions of photons before photobleaching. For instance, the various DCDHF derivatives as well as DCM are high-quality single-molecule emitters given their photostability and brightness (especially in rigid environments). In addition, the azido push–pull molecules can participate in fluorogenic PAL reactions to label biomolecules covalently in situ. For cell imaging, these fluorogens will ultimately need to be targeted to biomolecules of interest, but in any case do not require the addition of other chemicals (e.g. catalysts, thiols, or oxygen scavengers). Azido push-pull fluorogens thus provide a useful new class of photoactivatable probes for a variety of applications in biological labeling and super-resolution imaging.
5.
BIOORTHOGONAL CONJUGATION REACTIONS WITH DCDHF FLUOROPHORES
When labeling biomolecules in living cells, chemical reactions involved in the bioconjugation must be bioorthogonal, or specific enough not to react with nontargeted biomolecules. The Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of an alkyne with an azide—the “click” reaction—is one such bioorthogonal reaction, because neither azides nor alkynes are typically found in cells. A serious drawback of the click reaction for bioconjugation is the need for the catalyst, which limits labeling to the exterior of cells because the copper compound is toxic. Furthermore, there is a need for fluorogenic bioorthogonal reactions, a probe that only becomes fluorescent when covalently attached to a target biomolecule. The benefit to this scheme is the reduced need for washing of cells to remove unbound fluorophores. With these motivations, we developed some fluorogenic and uncatalyzed bioorthogonal conjugation reactions using the DCDHF fluorophore as the scaffold.

5.1. CLICK REACTION WITH DCDHFS

Initially, we explored the possibility of clicking two parts of a DCDHF together to form the fluorophore: if the precursor halves, or “prefluorophores,” are nonfluorescent at the pumping wavelength for a fluorescent clicked product, then the reaction would be fluorogenic. This approach was taken in the literature with an azidocoumarin, and I theorized that a similar approach would work with a DCDHF containing an azide or alkyne group. The basic concept of the fluorogenic click reaction is similar to the concept behind the photoactivation of the azido fluorogens discussed in Chapter 4: because the prefluorophore does not contain a strong electron-donating group, the charge-transfer band should be absent, significantly blue-shifting the absorption; after the reaction, if the donor is present (see Chapter 4) or the conjugation of the compound is extended (see Chapter 3), the product should be red-shifted and fluorescent.
Figure 5.1. (A) Spectra and structures of a preDCDH (RS01039 or DCDHF-P-alkyne) and the red-shifted triazole products RS01086 and RS01065. (B) Absorption and fluorescence emission spectra of the preDCDH and triazole products in toluene, demonstrating increasingly red-shifted emission from a click reaction with the azidobenzene and

Andor, gain 100 (false color)

taken with digital camera (real color)
The original design of this scheme reacted a nonfluorescent preDCDHF (containing an acetylene group instead of an amine donor) with an azidobenzene or an azidobenzamine, thus generating a compound with extended conjugation and a donor. Preliminary spectra of the product and reactant offered evidence that the reaction is fluorogenic: the products (RS01086 and RS01065) of DCDHF-P-alkyne (RS01039) and azidobenzamine are dramatically red-shifted and fluorescent in toluene (Figure 5.1). In PMMA, single molecules of the products are multicolored and bright (Figure 5.1). With Randy Lowe in the Chidsey lab, I also clicked an alkyl azide to DCDHF-P-alkyne and saw multicolored singles (data not shown). It seems that, in some configurations, the triazole can act as an electron donor and allow long-wavelength emission from the click-generated fluorophore. While this approach shows potential, further optimization is needed to sufficiently differentiate the products from the prefluorophores. For instance, although the triazole unit is known to both act as an electron donor and as a conjugated group, it is unclear in the case of DCDHFs whether either the electron-donating strength of the triazole or its extent of conjugation would be sufficient for a significant change in emission after the click reaction. Moreover, the need for a copper catalyst reduces the applicability of this reaction in living cells.

5.2. THERMAL AZIDE-TO-AMINE REACTION WITH NORBORNENE

Strained alkenes and alkynes are known to participate in the click reaction with azides without the need for an external catalyst. With that in mind, N. Liu in the Twieg lab reacted an azido DCDHF with norbornene, which contains an alkene that is
activated from ring strain.\textsuperscript{258} We posited that the azido DCDHF would participate in a thermal version of the photoactivation discussed in Chapter 4.

\begin{center}
\textbf{A}
\end{center}

\begin{center}
\textbf{B}
\end{center}

\textbf{NL03006 & NL03010 absorption in CH2Cl2}
The reaction between an azido DCDHF and a norbornene did indeed produce a triazole in solution at room temperature, albeit very slowly. Moreover, we observed an unanticipated (yet fortuitous) rearrangement from the triazole to a secondary amine (Figure 5.2); in other words, a thermal azide-to-amine (ATA) reaction. The final product of the thermal ATA reaction is red-shifted from the azido starting material and fluorescent. This reaction therefore is an option for fluorogenic bioorthogonal conjugation: if a biomolecule is labeled with a norbornene, then an azido fluorogen is added to the cell, only those targeted biomolecules become fluorescent (see below).
A

\[
\text{NL03045A, NL03045B & NL03056 absorption in EtOH}
\]

B

\[
\text{NL03045A, NL03045B & NL03056 absorption in EtOH}
\]

\[
\text{Molar Absorptivity (L Mol}^{-1} \text{ cm}^{-1})
\]

\[
\text{Wavelength (nm)}
\]

\[
\text{NL03045A in ethanol} \quad \text{NL03045B in ethanol} \quad \text{NL03056 in EtOH}
\]
Unfortunately, the reaction between norbornene and DCDHF-P-azide or DCDHF-V-P-azide is very slow, taking months to go to completion. For applications in cell labeling, this slow reaction rate is unacceptable. To speed up the thermal ATA rate, we activated the azido fluorogen by adding fluorine substituents to the benzene ring, thus stabilizing the nitrene intermediate (Figure 5.3). The reaction between norbornene and DCDHF-V-PF₄-azide proceeded in minutes instead of months, a much more practical reaction rate. The downside of the fluorinated fluorogen is that the rearrangement product is an aziridine instead of a secondary amine, and thus does not exhibit as large a red-shift as expected.
A

strain-induced click

rearrangement

B

NBD-azide and Norbornene ATA Reaction in Water
Finally, we tested the thermal ATA reaction using a non-DCDHF fluorogen. Shown in Figure 5.4, we reacted the NBD-azide with norbornene, and again observed an accelerated thermal ATA reaction resulting in increased fluorescence. This fluorogen did form the secondary-amine product instead of the aziridine, and thus warrants further study.

5.2.1. Synthesis of a Norbornene–NHS Ester

For future labeling experiments, it may be valuable to (nonspecifically) attach a norbornene to a biomolecule. Therefore, N.R. Conley and I made a reactive version of norbornene, which contains an NHS ester group to label lysines (Figure 5.5).
The synthesis of a norbornene containing an NHS ester was reported in the literature (Figure 5.6). N.R. Conley and I followed the procedure to make a norbornene-NHS for bioconjugation to a protein: To a mixture of 6.9 g (33.4 mmol) dicyclohexylcarbodiimide (DCC), 3.586 g (31.2 mmol) N-hydroxysuccinimide (NHS), and 3.30 g (23.9 mmol) of 5-norbornene-2-carboxylic acid, 150 mL of tetrahydrofuran (THF) was added under a nitrogen atmosphere and stirred for 2 days. A white precipitate formed, which was filtered, and the filtrate was concentrated in a rotovap.

A TLC separation of the liquid filtrate revealed two components, and one main band that was fluorescent under 256 nm illumination. The filtrate was then purified by column chromatography using silica gel as the stationary phase and a mixture of 95% dichloromethane and 5% ethyl acetate as the mobile phase. The main band eluted second, then was concentrated on a rotovap. The resulting white precipitate was further purified by recrystallizing in hexanes and dichloromethane.

The final product was characterized with NMR and HPLC-MS, revealing two isomers (mostly exo). $^1$H NMR (300 MHz, CDCl$_3$, $\delta$): 6.25 (dt, 2H), 3.25 (q, 1H), 2.80

Figure 5.5. Bioconjugation of a norbornene-NHS with the lysine residue of a protein.

Figure 5.6. Reaction scheme to synthesize the norbornene-NHS.
5.3. CONCLUSION

We have explored two avenues to fluorogenic bioorthogonal conjugation onto biomolecules. In the first approach, we used the popular click reaction to add an electron donor to and extend the conjugation of a nonfluorescent compound, resulting in a red-shifted fluorophore. This reaction is one example of the concept of combining two prefluorophores together to form a fluorescent product. The click reaction requires a Cu(I) catalyst, that is both exogenous and toxic; therefore, this approach to fluorogenic bioorthogonal conjugation may be limited when trying to label living cells. A further issue with this approach arises from the fact that the final linkage produced is a triazole, whose conjugation properties are not as clearly defined as more aromatic linkers. This influences the design in ways that must be explored further.

In the second approach, an azide-to-amine (ATA) reaction occurs with only ambient thermal energy (no light supplied) between azido push–pull fluorogens and the strained alkene in norbornene. The reaction converts a blue-shifted and nonfluorescent starting material to a red-shifted fluorophore. The thermal ATA reaction I describe in this Chapter is applicable to a variety of the azido fluorogens discussed in Chapter 4. So far, we have been able to tune the starting materials to increase the time to complete the reaction from months to minutes, into a range that is applicable to cellular labeling. With further optimization, this reaction could prove a valuable approach to fluorogenic bioorthogonal labeling.
APPENDIX:
NOMENCLATURE
The acronym “DCDHF” stands for “dicyanomethylene dihydrofuran,” which refers to the electron-acceptor group 2-dicyanomethylene-3-cyano-2,5-dihydrofuran. “DCDHF” also stands for the entire donor–π–acceptor system, as in the “DCDHF fluorophore.” Although this may cause some mild confusion, the context generally reveals whether the term “DCDHF” refers to the acceptor group alone or the entire compound.

The naming scheme for fluorophores used herein specifies the system: DCDHF-(π unit closest to acceptor)-….-(π unit closest to donor). The π units are denoted P = phenylene, V = vinyl, T = thiophene, N = naphthalene, A = anthracene; the amine donor is generally not specified because it is present in most structures. The amine (or azide or nitro, etc) are explicitly included in the name in situations where not doing so would cause confusion.

Other acronyms and symbols that are used throughout include:

- β: hyperpolarizability
- ε: molar absorption coefficient
- η_Q: detector quantum efficiency
- λ: wavelength
- Δμ: change in dipole moment
- μ_E: excited-state dipole moment
- μ_G: ground-state dipole moment
- Φ_B: photobleaching quantum yield
- Φ_P: photoactivation quantum yield
- Φ_F: fluorescence quantum yield
- σ: absorption cross-section
- τ_F: fluorescence lifetime
- 3D: three-dimensional
- APD: avalanche photodiode
- ATA: azide-to-amine
- ATP: adenosine triphosphate
- B: overall measured background count rate
- BME: β-mercaptoethanol
- BMOI: biomolecule of interest
- BSA: bovine serum albumin
- CCD: charge-coupled device
- CoA: coenzyme A
- CHO: Chinese hamster ovary
- CT: charge transfer
- D: microscope collection efficiency
- DCC: dicyclohexylcarbodiimide
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCDHF</td>
<td>2-dicyanomethylene-3-cyano-2,5-dihydrofuran</td>
</tr>
<tr>
<td>DCM</td>
<td>2-(2,6-dimethyl-4H-pyran-4-ylidene)malononitrile</td>
</tr>
<tr>
<td>DFT</td>
<td>density-functional theory</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EMCCD</td>
<td>electron-multiplying charge-coupled device</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>$\Delta f$</td>
<td>orientation polarizability</td>
</tr>
<tr>
<td>$F_{\text{coll}}^\text{coll}$</td>
<td>angular collection factor of a microscope objective</td>
</tr>
<tr>
<td>$F_{\text{filter}}^\text{filter}$</td>
<td>transmission factor through optical filters</td>
</tr>
<tr>
<td>$F_{\text{opt}}^\text{opt}$</td>
<td>transmission factor through the objective and microscope optics</td>
</tr>
<tr>
<td>FCS</td>
<td>fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>FP</td>
<td>fluorescent protein</td>
</tr>
<tr>
<td>FPALM</td>
<td>fluorescence photoactivation localization microscopy</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster-resonance-energy transfer</td>
</tr>
<tr>
<td>$G_{\text{EM}}$</td>
<td>electron-multiplying gain</td>
</tr>
<tr>
<td>$G_{\text{A/D}}$</td>
<td>analog-to-digital conversion gain (counts per photoelectron)</td>
</tr>
<tr>
<td>$h$</td>
<td>Planck’s constant</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>$I_\lambda^\lambda$</td>
<td>excitation irradiance at the sample</td>
</tr>
<tr>
<td>IRF</td>
<td>instrument-response function</td>
</tr>
<tr>
<td>ISC</td>
<td>intersystem crossing</td>
</tr>
<tr>
<td>LE</td>
<td>locally excited</td>
</tr>
<tr>
<td>LED</td>
<td>light-emitting diode</td>
</tr>
<tr>
<td>M</td>
<td>molecular ion (i.e., without fragmentation)</td>
</tr>
<tr>
<td>$m/z$</td>
<td>mass-to-charge ratio</td>
</tr>
<tr>
<td>M2G</td>
<td>a minimal medium for cell culture</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NA</td>
<td>numerical aperture</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NBD</td>
<td>7-nitrobenzo[c][1,2,5]oxadiazol-4-amine</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>$N_{\text{detected}}$</td>
<td>average number of total photons detected per molecule</td>
</tr>
<tr>
<td>$N_{\text{photons}}$</td>
<td>average number of total photons emitted per molecule</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PALM</td>
<td>photoactivation localization microscopy</td>
</tr>
<tr>
<td>PBMA</td>
<td>poly(butyl methacrylate)</td>
</tr>
<tr>
<td>PMMA</td>
<td>poly(methyl methacrylate)</td>
</tr>
<tr>
<td>PMT</td>
<td>photomultiplier tube</td>
</tr>
<tr>
<td>POI</td>
<td>protein of interest</td>
</tr>
<tr>
<td>PSF</td>
<td>point-spread function</td>
</tr>
<tr>
<td>PVA</td>
<td>poly(vinyl alcohol)</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
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<tr>
<td>--------</td>
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</tr>
<tr>
<td>PYE</td>
<td>a cell-growth medium</td>
</tr>
<tr>
<td>QD</td>
<td>quantum dot</td>
</tr>
<tr>
<td>R6G</td>
<td>Rhodamine 6G</td>
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<td>$R_{abs}$</td>
<td>photon absorbance rate</td>
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<td>$R_b$</td>
<td>irradiance-dependent background count rate</td>
</tr>
<tr>
<td>$R_B$</td>
<td>photobleaching rate</td>
</tr>
<tr>
<td>$R_d$</td>
<td>dark count rate</td>
</tr>
<tr>
<td>$R_{em}$</td>
<td>photon emission rate</td>
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<td>RIPA</td>
<td>radio immunoprecipitation assay</td>
</tr>
<tr>
<td>rms</td>
<td>root-mean-squared</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SLR</td>
<td>single-lens reflex</td>
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<tr>
<td>SMS</td>
<td>single-molecule spectroscopy and imaging</td>
</tr>
<tr>
<td>SNR</td>
<td>signal-to-noise ratio</td>
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<tr>
<td>spFRET</td>
<td>single-pair FRET</td>
</tr>
<tr>
<td>STORM</td>
<td>stochastic optical reconstruction microscopy</td>
</tr>
<tr>
<td>T</td>
<td>detector time interval</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TICT</td>
<td>twisted intramolecular charge transfer</td>
</tr>
<tr>
<td>TIR</td>
<td>total-internal-reflection fluorescence</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>TPE</td>
<td>two-photon excitation</td>
</tr>
<tr>
<td>VR</td>
<td>vibrational relaxation</td>
</tr>
</tbody>
</table>
APPENDIX:
TABLE OF BASIC PHOTOPHYSICAL PARAMETERS
OF VARIOUS DCDHFS
Names, structures, and photophysical parameters of various DCDHFs and standard fluorophores. The structures are generalized: the methyl groups on the amine and dihydofuran ring may vary in length and composition; the data presented is representative of chromophores with varying R₁–R₄ groups, because varying the length of the alkyl chains has little effect on the photophysics. See text for details on photophysical parameters.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>$\lambda_{\text{abs}}$/λ&lt;sub&gt;em&lt;/sub&gt; {ε&lt;sub&gt;max&lt;/sub&gt;}</th>
<th>$\Phi_\text{F}$ in toluene {PMMA}</th>
<th>$\Phi_\text{B}$ in gelatin {PMMA} (×10⁻⁶)</th>
<th>$N_{\text{photons}}$ in PMMA (×10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCDHF-P</td>
<td><img src="structure1.png" alt="Structure" /></td>
<td>486/505 {71,000}</td>
<td>0.044</td>
<td>6.6</td>
<td>2.4</td>
</tr>
<tr>
<td>DCDHF-P-(1ring)</td>
<td><img src="structure2.png" alt="Structure" /></td>
<td>495/515 {95,900}&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCDHF-P-(2ring)</td>
<td><img src="structure3.png" alt="Structure" /></td>
<td>503/527 {90,400}&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCDHF-V-P</td>
<td><img src="structure4.png" alt="Structure" /></td>
<td>562/603 {45,500}</td>
<td>0.02</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>DCDHF-T</td>
<td><img src="structure5.png" alt="Structure" /></td>
<td>514/528 {100,000}</td>
<td>0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCDHF-V-T</td>
<td><img src="structure6.png" alt="Structure" /></td>
<td>614/646 {114,000}</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCDHF-N</td>
<td><img src="structure7.png" alt="Structure" /></td>
<td>526/579 {42,000}</td>
<td>0.85</td>
<td>3.4</td>
<td>1.1</td>
</tr>
<tr>
<td>DCDHF-V-N</td>
<td><img src="structure8.png" alt="Structure" /></td>
<td>574/671 {58,100}&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCDHF-V-(1,4)N</td>
<td><img src="structure9.png" alt="Structure" /></td>
<td>538/661 {22,200}&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCDHF-A</td>
<td><img src="structure10.png" alt="Structure" /></td>
<td>585/689 {35,000}</td>
<td>0.54</td>
<td>1.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Compound</td>
<td>Structure</td>
<td>λ&lt;sub&gt;max&lt;/sub&gt;/ε&lt;sub&gt;max&lt;/sub&gt;</td>
<td>λ&lt;sub&gt;em&lt;/sub&gt;/ε&lt;sub&gt;em&lt;/sub&gt;</td>
<td>λ&lt;sub&gt;em&lt;/sub&gt;/ε&lt;sub&gt;em&lt;/sub&gt;</td>
<td>λ&lt;sub&gt;em&lt;/sub&gt;/ε&lt;sub&gt;em&lt;/sub&gt;</td>
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<tr>
<td>---------------</td>
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<td>----------------</td>
<td>----------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>DCDHF-P-P</td>
<td>![Structure Image]</td>
<td>506/623{31,000}</td>
<td>0.82</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>DCDHF-T-T</td>
<td>![Structure Image]</td>
<td>634/679{71,800}</td>
<td>0.50</td>
<td>[2.1]</td>
<td></td>
</tr>
<tr>
<td>DCDHF-V-T-T</td>
<td>![Structure Image]</td>
<td>708/779{49,800}</td>
<td>0.13</td>
<td>[3.4]</td>
<td></td>
</tr>
<tr>
<td>DCDHF-P-T</td>
<td>![Structure Image]</td>
<td>591/663{44,000}</td>
<td>0.21</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>DCDHF-T-P</td>
<td>![Structure Image]</td>
<td>575/631{22,000}</td>
<td>0.74</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>DCDHF-V-T-P</td>
<td>![Structure Image]</td>
<td>611/723{47,300}</td>
<td>0.07</td>
<td>[0.13]</td>
<td></td>
</tr>
<tr>
<td>DCDHF-P-T-P</td>
<td>![Structure Image]</td>
<td>541/709{28,000}</td>
<td>0.34</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>DCDHF-TTT</td>
<td>![Structure Image]</td>
<td>612/645 &gt;0.44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCDHF-carbazole</td>
<td>![Structure Image]</td>
<td>580/621</td>
<td>0.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCDHF-V-carbazole</td>
<td>![Structure Image]</td>
<td>612/699</td>
<td>0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhodamine 6G</td>
<td>![Structure Image]</td>
<td>530/556&lt;sup&gt;[c]&lt;/sup&gt; {105,000&lt;sup&gt;[c]&lt;/sup&gt;}</td>
<td>0.95&lt;sup&gt;[c]&lt;/sup&gt;</td>
<td>3.5</td>
<td>1.4</td>
</tr>
<tr>
<td>fluorescein</td>
<td>![Structure Image]</td>
<td>483/515&lt;sup&gt;[c]&lt;/sup&gt; {92,300&lt;sup&gt;[c]&lt;/sup&gt;}</td>
<td>0.79&lt;sup&gt;[c]&lt;/sup&gt;</td>
<td>64</td>
<td></td>
</tr>
</tbody>
</table>

In toluene unless otherwise noted. [a] Absorption and emission maxima (nm) and molar absorption coefficient (M<sup>-1</sup> cm<sup>-1</sup>). [b] In dichloromethane. [c] In ethanol. Data from references 143, 144, 147.
APPENDIX:

VARIOUS PROTOCOLS FROM WEMPIRE WIKI
AGAROSEMOUNTINGMEDIUM

Prep

- put 0.15g agarose (SM low melting point agarose 24–28C mp) in 10 mL buffer in falcon tube
  - pour powder out of bottle, don't use spatula to weigh out
- heat to 80C in water bath and shake tube periodically to dissolve powder
- make 100-uL aliquots while solution still hot (change pipette tips frequently because of viscosity of agarose when cooling)
- let aliquots cool to room temperature and then store at –20C

Use

- heat aliquot(s) to 80C
  - re-aliquoting into sample-size aliquots at this stage may be helpful
- cool to and maintain at ~40C (37C)
  - check to make sure that sample components (ex: proteins) are stable at this temperature!
- add small amount of sample stock to make ~1 nM
- mix
- quickly put 10 uL onto glass coverslip and sandwich using another coverslip (don't want bubbles)
- refreeze aliquots you don't use

ALIGNINGMULTIPLEBEAMSWIDEFIELD

These are some guidelines for aligning multiple laser beams into one microscope (especially for widefield epifluorescence illumination). As of November 2007, this applies to 8b back and 9a back setups.

General rules

- The most important rule is to be considerate of your fellow users. Treat other people's beam paths as you would have them treat yours!
- Do not touch shared optics unless you're willing to realign all the beams that share those optics!
- If you grossly misalign an optic (i.e. remove a mirror), you have two options: (a) realign it well, or (b) leave it obviously misaligned (so that the next user knows
what the problem is and doesn't have to go hunting for a misalignment throughout the table). Either way, you should leave a note, send an email, or--if you dare--tell the next user to his or her face.

- When you have a question about anything or if you need an extra set of hands/eyes, ask. It's better to ask than apologize. Conversely, offer help when others need it: it will only make your life easier in the future!
- Keep all optics well labeled so that the beam paths that share each is obvious. Other labels, such as "DON'T TOUCH" or "adjust me" are helpful, too.
- Give yourself enough time before and after using the setup to complete any necessary realignments.

**Gross alignment (e.g. the first time or when you need to add a new line)**

1. Ensure that any new optics do not block existing lines.
2. Initially set up beam so that it starts at the same height as the widefield lens (and the other beams going into the 'scope) and is parallel to the table. (Tip: if you set up an iris on a post and post holder so that the beam passes through the center of the iris, you can move the iris around the table to check and adjust the height of the beam at subsequent locations in the beampath.) Also, start the beam parallel to one of the two horizontal axes of the table.
3. Use telescoping lenses to expand the beam. Do this by placing in the beam a short-focal-length lens and a long-focal-length lens, separated by the sum of their focal lengths. Note that, for optimal illumination, you want the beam size to be large enough to fill the back aperture of the objective. Conversely, a beam that is too large will be clipped on mirrors. Choose lenses that will give a collimated beam of about 0.75".
4. At each mirror, adjust the mirror so that the beam makes a right angle turn and maintains the same height. Place the next mirror so that the beam hits the center.
5. Use good bases and posts for important optics. Use magnetic bases for movable optics only (e.g. OD, etc.).

**Fine adjustments (e.g. day-to-day use and aligning)**

1. The best for aligning is a blank glass coverslip, with immersion oil, in focus.
2. Flip down the widefield lens (immediately before the microscope).
3. Remove the LP emission filter and make sure that you're on the correct dichroic setting.
4. **WARNING:** Add enough OD to the laser beam so that you don't harm your eyes if you look into the eyepieces. This will probably be more than OD 2. If you plan to image the spot onto the camera, you will need even more OD. Do not leave full power going into the 'scope with the LP missing; someone will hurt themselves. Always check the output of the eyepieces with your hand before you check with your eyes!
5. If well aligned, the confocal spot will appear in the center of the imaging region and will grow symmetrically when you change the focus.

6. If the beam is not aligned, then you will need to "walk" the beam using two mirrors. Use only mirrors designated to that beam; do not adjust mirrors that multiple beams share! The purpose of walking the beam is to move the beam in a systematic way so that not only the position but also the angle of the laser beam is correct. You may have your own careful method, but here is a suggestion:
   - First, note the location of the confocal spot in the camera (or eyepiece) imaging region.
   - To walk the beam, adjust one knob (i.e. vertical or horizontal) on the mirror furthest from the 'scope so that the confocal spot away from the center of the imaging region.
   - Using the analogous knob (i.e. V or H) on the closer mirror, correct the shape of the confocal spot. Note the new position of the spot; it should be closer to the center.
   - If the location is worse, then reverse your actions and continue with reverse walking.
   - Repeat with the other dimension (V or H). Now the spot should be in the center of the imaging region and be symmetric. Check the symmetry by changing the focus.
   - Bottom line: Use the far mirror to move the position of the spot (because it has a larger effect) and the near mirror to correct the shape of the spot. This way, you shouldn't walk off the mirrors.

7. With the confocal spot well aligned, flip up the widefield lens. Adjust the x, y, and z of the lens so that you have a collimated beam coming out of the objective, normal to the imaging plane.

8. Now return the LP emission filter (and remove some of the OD) and replace the blank coverslip with a test sample. (This may be a SM sample if you're confident, or a bulk sample to view the shape and position of your Gaussian widefield illumination.)

9. NB: If there's something strange (interference patterns, dramatic misalignment, etc.), be sure to check the basics: Are you using the correct dichroic? Is the coverslip in focus? Is there an emission filter in place? Is there a bubble in the oil? etc.

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**BACTERIAFIXATION**

(Suitable for Caulobacter Crescentus and E. Coli)

This protocol is sufficient for simple bacterial cell fixation (i.e. for visualization of fluorescent fusion proteins). If immunofluorescence or other labeling protocols are desired in conjunction to cell fixation, care need to be taken to remove as much of the
fixation solution as possible (see separate protocol). Also, please see separate protocol for mammalian cells.

1. Grow cells as usual in M2G. Harvest cells at ~ OD660 0.3–0.4.
2. Prepare mixture of 135 μL methanol in 5 mL cold 1× M2 salts (or 135 μL 37% formaldehyde in 5 mL cold 1× M2 salts).
3. Spin 5 mL cell culture in a 15-mL falcon tube (7830 rpm for 90s) at 4 °C. Remove supernatant.
4. Resuspend the cell pellet in the cold methanol or formaldehyde mixture. Be careful to pipet gently; no bubbles should form (this is particularly difficult with the formaldehyde).
5. Incubate cells in the formaldehyde or methanol solution at room temperature for 10 min, shaking gently once or twice to make sure that the cells stay suspended and do not pellet.
6. Incubate cells on ice for > 30 min.
7. Spin cells for 15 min at 7830 rpm at 4 °C to pellet.
8. Remove supernatant and resuspend in 5 mL chilled 1× PBS buffer (pH 7.4) or chilled 1× M2 salts. Spin for 15 min at 7830 rpm at 4 °C.
9. Repeat step 7 two more times for a total of three washes. The fixed cells can now be stored in the falcon tube for days. Prepare samples for imaging on agarose pads as usual.

CARYSPECTROMETER

Cary 6000i (department UV-vis):

1. In order to get trained, email Brett Carter (carterb@stanford.edu) and Steve Lynch (srlynch@stanford.edu). You will need to get your ID card “Track 3 encoded” in order to unlock the door.
2. Turn on instrument (with nothing in beam paths).
3. Start "Scan" Cary program.
4. Click "Setup"; choose range and click the "Baseline" radio button under corrections.
5. Insert two blanks and click "Baseline."
6. Run sample by clicking "Start." Chose a good filename and sample description.
7. You can change the cursor to track mode by clicking the button that looks like a cursor arrow. You can choose which runs to display by clicking on the button that looks like horizontal colored lines.
8. When finished, open the runs you want to use and save them as .csv files (be sure to click the radio button that means you only save that one visible run).
CAULOBACTER

Storing Aliquots

- After PYE growth and dilution into M2G, allow PYE solution to keep growing for the day until it's very dense
- Add 10% DMSO
- Aliquot into epi tubes and store at -80 C

Culturing

( HDL 4/10/09) What you would need for this is 1 frozen aliquot of the strain of caulobacter you need, a PYE plate that has the right antibiotics for your strain, a pipette, a pipette tip (you can also use a cell spreader, but we don't always have that in the lab.) Generally speaking, if you have a xylose-based induction system, you'd be using kanamycin, and if you are using a vanillate-based promoter, you'd use gentamycin.

1. Take the frozen aliquot in eppendorf tube out straight from the -80 degrees C freezer.
2. Open the cap of the eppendorf tube, and use the pipette and the pipette tip to scrape the surface of the frozen aliquot quickly.
3. Take the pipette tip, now with caulobacter, quickly spread onto the plate in a zigzag with a tight pitch. Do not puncture the gel.
4. Use the same pipette tip, draw lines that cross the zigzag
5. Repeat step 4, this time draw lines that cross the lines you drew in state 4.
6. Repeat step 5, this time draw lines that cross the lines drawn in step 5.
7. Leave the plate in room temp for 3 days before parafilmimg and placing into the refrigerator.

The point of step 3-7 is to ensure that you have well separated single colonies that you can pick from when you want to grow your cells in solution.

Growing in Solution

- From the petri dish (currently use one labeled "EYPF-MreB"), scoop one colony using a p20 tip
- Put tip into test tube with 5 mL of PYE growth medium with 1 uL kanamycin (5000×)
- After 12 hours (e.g. in the evening if you started growth in the morning), dilute 100-500 uL into 5 mL M2G buffer with 1 uL kanamycin
- The cells should be ready for dilution the next morning (not imaging, yet)
- Dilute 50 and 100 uL of those cells into two test tubes each with 5 mL M2G and 1 uL kanamycin for afternoon imaging
- Incubating and Washing Cells
- Add probe to test tube of cells
- Incubate for however long you want
• In a 1.5 mL epi tube, place 1 mL cells
• Spin tube down for 90 s at 10,000 rpm
• Cells should pellet. Pull off supernatant and replace with 1 mL M2G buffer
• Repeat

**Making Agarose Pads**

• Make pads well before you are ready to image
• Use two large (35×50) coverslips for each pad
• Heat 2% (by mass) agarose in M2G until just boiling (not boiling over!)
• Put 700 uL agarose onto coverslip and sandwich with the other
• Place in moist container
• When ready, pull off one coverslip, place 2 uL cells on agarose and sandwich with small etched coverslip
• Cut away excess agarose with razor blade and seal edges with wax

**Notes from Julie**

I'll add to the protocols when I have a chance, but for the time being, a few thoughts:
• Adding kanemycin isn't a general thing, it is specific to the cell strain that you are using (the MreB-EYFP cells are kanemycin resistant)
• When growing cells in test tubes, thinking about times is OK, but really the rule of thumb is that you want the cells to be between OD 0.1 and 0.4 for them to be in the log phase of their growth, so dilute according to that. It's worth using the UV-Vis to check OD the first few times so you know what that looks like. Not sure if I agree with the times that you wrote though overall - I think that if you start growth in PYE in the morning, they won't be concentrated enough to dilute in the afternoon unless you put cells in 1mL PYE instead of 5mL. And in the evening, I usually add 50-200 uL per 5 mL M2G, then in the morning, more like 500–1000uL cells per mL M2G.
• I use 1mL (not 700uL) when preparing agarose pads on the big coverslips.
• You can spin the cells down at 13.4 krpm (this is how So Yeon and Marcelle taught me to do it).
• Not sure if your protocol is wrong or if you're doing things differently, but I put cells on one side of the pad and cover them with a clean large coverslip, then put a small coverslip on the back for sealing purposes. This helps a lot with sample stability because the large coverslip covers the whole stage opening.
CLEANING OPTICAL FILTERS

From Omega website:

- All optical elements are delicate and should be handled as carefully as possible. The glass and anti-reflective (AR) coated surfaces will be damaged by any contact, especially if abrasive particles have come into contact with the surface. In most cases, it is best to leave minor debris on the surface.
- Use of oil-free dry air or nitrogen under moderate pressure is the best tool for removing excessive debris from an optical surface. In the case that the contamination is not dislodged by the flow of gas, please use the following protocol for cleaning the part:
  - Clean the part using an absorbent towel such as Kimwipes, not lens paper. Use enough toweling so that solvents do not dissolve oils from your hands which can make their way through the toweling onto the coated surface.
  - Wet the towel with an anhydrous reagent grade ethanol.
  - The use of powder-free gloves will help to keep fingerprints off the part while cleaning.
  - Drag the trailing edge of the ethanol soaked Kimwipe across the surface of the component, moving in a single direction. A minimal amount of pressure can be applied while wiping. However, too much pressure will damage the component.
  - If the surface requires additional cleaning, always switch to a new Kimwipe before repeating the process.
  - The purpose of the solvent is only to dissolve any adhesive contamination that is holding the debris on the surface. The towel needs to absorb both the excessive solvent and entrap the debris so that it can be removed from the surface. Surface coatings on interference filters and dichroics are typically less hard than the substrate. It is reasonable to expect that any cleaning will degrade the surface at an atomic level. Consideration should be given as to whether the contamination in question is more significant to the application than the damage that may result from cleaning the surface. In many cases, the AR coatings that are provided to give maximum light transmission amplify the appearance of contamination on the surface.

CLEANING SCINTILLATION VIALS

- Be sure to use caps with polyethylene cone (not metal film and cork).
- Rinse with SMS ethanol twice.
- Rinse with SMS toluene (or solvent to be used) twice.
- Flame if necessary.
- Plasma etch if necessary.
• Fill with SMS solvent.

CLEANING TOLUENE

Distill toluene until clean. The still is in the hood in room 5a.
Beware: toluene is flammable!
1. plug in heater and heat at 65V (should be already set). turn on cold water.
2. remove foil around reservoir. fill reservoir and leave uncapped until toluene boils.
3. allow refluxing (evaporation and condensation) for ~ 10 minutes.
4. flush out distillation tube.

EVAPORATOR

This is the metal evaporator/depositer that is in room 9a. It is currently broken: the cooling water for the deposition detector corroded some wires. Frank J. should know more about what it needs to get working again, or see the hanging file.
1. Turn off the vacuum pump and open the air line.
2. Load samples, lower bell, turn on vacuum pump, and achieve vacuum of ~10⁻⁶.
3. Turn on water (two red valves).
4. Turn on power (lower white switch).
5. Select correct source.
6. Turn on rotator (silver switch).
7. Turn on microprocessor computer (button).
8. Push “prog.”
9. Set deposition rate (2–3 Å/s or 1.0 Å/s for thin films).
10. Do others manually.
11. Set “new rate” the same as “dep rate.”
12. Set thickness (twice). Second one first (“thick spt”), then the first one (“final thickness”). I used 0.5 kÅ.
13. Set density (2.7 g cm⁻³ for Al).
14. Set z-ratio (1.080 for Al).
15. Push “prog” again to continue.
17. Be in remote mode (silver switch) and turn on the remote (a light comes on).
18. Turn dial to adjust current. First ramp, then wait, then deposit (~26–30 amps).
19. Watch dep rate and vacuum (starts degassing at @ 16–20 amps). If vacuum drops (increase pressure), stop increasing and let degas. Watch for drop in dep rate; if it jumps, then good (we want to be at ~1 Å/s). Slower rate is OK, it just takes longer.
20. When it reaches desired film thickness, ramp down the amperage and turn off the following: computer, power, remote, rotation.
21. Wait ~15 minutes.
22. Turn off pump and water and open air line.
23. When finished, close air line and pump back down. Be sure to wait to hear the turbo pump switch on.

**FLUOROLOGFLUORIMETER**

**Fluorolog-3 (department fluorimeter):**

1. In order to get trained, email Brett Carter (carterb@stanford.edu) and Steve Lynch (srlynch@stanford.edu). You will need to get your ID card “Track 3 encoded” in order to unlock the door.
2. If lamp has been off for >15 min, turn on main power (bottom switch) and lamp (top switch).
3. Flip switch on power strip over near CPU.
4. Turn on computer. Password = fluorolog3
5. Use the T detector (Em2) to go out to 1000 nm.
6. Set HV to 1450 V.
7. Make sure to use correction file: tcorrect. (Signals: T and Tc/R)
8. Linear range = 2×10^6 (raw data).
9. To convert (export) data to plain text (.prn), under “Arithmatic,” choose “Do Program.” Find “convert.ab” and convert all the files you need.
10. When finished, record use in logbook.

**GELATINMOUNTINGMEDIUM**

Gelatin is an aqueous protein. It makes a good peptide alternative to agarose.

- Make a 5% (g:g) solution of gelatin type A (Bloom 225) in water.
- Heat to 37 C.
- After it is well dissolved, you may want to make aliquots and freeze them. That way, you only have to thaw a few at a time and the entire stock will last longer.
- Store gelatin in −4 C freezer or it rots.
- In a small volume of gelatin (~20 uL), mix in sample.
- Deposit 10 uL dye-doped gelatin on glass coverslip.
- Place another coverslip to make sample sandwich.
- Let the sample cool to room temperature and the sample will gel.
HOUSEN2SYSTEM

Concept: The system (in room 6) works by having 4 cylinders connected to the main house line. Only two cylinders at a time provide gas (the primary at first, then the secondary when the primary pair is empty). The complex system is a series of one-way valves and gauges. Setting a pair of tanks to “Primary” simply sets the pressure from that half slightly higher than the other half, so the main line draws from those two first. A switch measures when the pressure falls to that set for the “Secondary” setting, and sends a signal to the light box in room 9b. For more info, please see the notes below and/or the manual (in room 6).

1. When the red light near the door in 9b (“Secondary Supply in Use”) illuminates, that means the two primary tanks are empty, and the line is now drawing from the secondary tanks.
2. Go to room 6. The two tanks that are currently set to “Primary” are empty.
3. Label those two as empty and exchange them with two full cylinders.
4. Connect the new cylinders to the system and open them.
5. Switch the primary tanks to “Secondary” and vice versa. Now the main line will draw from the partially empty tanks first (and the full ones when those are empty). Now the indicator light in room 9b should be green.
6. Log your actions on the logbook hanging on the wall.
7. Order two new Ni-T cylinders from Praxair.

Notes:

- If the bulbs go out in the indicator box in 9b, there are more bulbs inside the box.
- Please adjust only the following two sets of valves: the two “Primary/Secondary” levers and the two knob valves that isolate the cylinder pairs from the main system (they look just like the valves directly on the cylinders). Other adjustments (especially the two black regulators at the top of the system) will mess up the delicate balances in the system. It will make Sam very unhappy, because he spent two days carefully setting the pressures!
- Although there are one-way valves throughout the system, they will leak if you leave a line unconnected from a cylinder or uncapped. If you must leave a connector uncapped for more than a few minutes (while the cylinder is waiting for pickup, for instance), please isolate that half from the main system (close the knob valve on that side). But be sure to remember to reopen the valve when you reconnect the system!
- The pressure gauge on the left is inaccurate. Fortunately, you may ignore all the pressures. If you’re curious, the pressure of the main line is the gauge in the middle. Below it is a system pressure-release switch.
  If the house nitrogen is not going to be used for an extended period (such as over winter break), close all the cylinders: there may be some tiny leaks in the main line, and there’s no reason to waste gas.
IMAGEJ

This page should be devoted to instructions for using the ImageJ program for editing movies and images. It should include links, plugins, directions, etc. Some protocols may still deserve their own entry in the wiki (such as TotalPhotonsSM), but for those that don't, let's use this entry as a sort of depository and reference and linking page.

Website for ImageJ: http://rsb.info.nih.gov/ij/
There is also information and plugins on WEM11A: \wem11a\LAB\Experimental_Info\ImageJ

Protocols for Specific Uses:

Adding laser-color indicators
1. Open the movie of interest.
2. If necessary, convert the movie to a color (e.g. RGB, 8-bit color, etc.) under Image -> Type.
3. Split the movie into substacks in time of the different laser colors (e.g. frames 1-50 and 51–100 for green then red, respectively). You can do this using the Substack Maker plugin (see below).
4. Open the Multi Measure plugin.
5. Choose an ROI(s) and add it to the list by clicking the "Add" button in the Multi Measure window or by hitting the spacebar. This ROI will appear in the same place in all the substacks you have open, when you select a substack then click on the ROI name in the list.
6. Choose a color you want to fill the ROI, under Image -> Color -> Color Picker.
7. Fill the ROI you have selected by clicking the "Fill" button on the Multi Measure window. You will see a warning asking if you want to do the actions for the entire stack. Click "Yes" or you will just fill that one frame.
8. Repeat with the correct color(s) for each substack.
9. To recombine the substacks, use the Concatenate plugin. Be sure to check the "Keep Original Images" button, in case you screw up!
10. Now save as an AVI (if you want to use the movie in PowerPoint) or a TIFF.

Overlapping dual-viewer movies
1. Save movie as TIFF in WinView (or open in ImageJ using the SPE plugin).
2. Open .tif in ImageJ.
3. Open MultiMeasure (a ImageJ plugin) and create/open ROIs of the two colors.
4. Select one region, crop under Image -> crop, and save as ~_red.tif or ~_green.tif
5. Repeat steps 2&4 for other color.
6. Equalize contrast in each channel to make the background the same (or can adjust colors later). See "Setting the contrast..." instructions below.
8. Adjust contrast and brightness, save as ~_rg.tif, or as ~.avi
Measuring time traces or total photons from many single molecules (see entry TotalPhotonsSM)

Setting the contrast the same across several movies to compare

1. Open movies of interest.
2. Open Image -> Adjust -> Brightness/Contrast.
3. Find a level of brightness and contrast that looks good for the movie. Note the upper and lower limits.
4. Click the "Set" button and input the upper and lower limits that you liked. Click the "Propagate to all open images" button. Click OK.
5. Now all the movies should have the same contrast.
6. Repeat until the contrast on all movies it acceptable.

Flattening the image (from uneven illumination)

- For a qualitative image, it's probably fine to use the FFT function (which flattens using a Fourier-transform filter). Open Process -> FFT -> Bandpass Filter and experiment with the bandpass range.
- For more accurate flattening and background-subtraction, read this website (http://www.macbiophotonics.ca/ImageJ/image_intensity_proce.htm) about Intensity Processing and this one (http://imagejdocu.tudor.lu/imagej-documentation-wiki/how-to/how-to-correct-background-illumination-in-brightfield-microscopy) with more info about background correction.

Useful Plugins:

Multi Measure (http://www.optinav.com/Multi-Measure.htm) is a very helpful plugin that let's you measure and alter many ROIs through an entire stack. For instance, you can draw many ROIs around many single molecules, then measure the intensity of each ROI for each frame and export to a text file. (See an example here: TotalPhotonsSM.) Another use is to fill in one or two ROIs with color for all the frames of a movie (e.g. to show laser color alternating). You can apply the same ROIs to other movies of the same size and even save theROIs for later use.

Substack Maker (http://rsb.info.nih.gov/ij/plugins/substack-maker.html) lets you split movies up into many sections, which you can manipulate and save separately.

Concatenator (http://rsb.info.nih.gov/ij/plugins/concatenator.html) lets you concatenate (combine) two stacks of the same type and size. For more than two stacks, see theConcatenate plugin below.

Concatenate (http://rsb.info.nih.gov/ij/plugins/concatenate.html) lets you concatenate multiple (more than two) stacks of the same type and size.

Open_SIF (http://rsb.info.nih.gov/ij/plugins/open-sif.html) lets you open Andor SIF files. (Be sure to download the version of the plugin that lets you open new versions of the Andor software, called "Open_SIF.class", not the one called "OpenSIF_.class" listed on the page.)

Open SPE (http://rsb.info.nih.gov/ij/plugins/spe.html) and Save as SPE let you open and save as PI/Acton camera software files.

**Running Z Projector** ([http://valelab.ucsf.edu/~nico/IJplugins/Running_ZProjector.html](http://valelab.ucsf.edu/~nico/IJplugins/Running_ZProjector.html)) calculates a running average from the data in a stack. The images in layer 1–n (n is user-specified) are combined using the user specified method (average intensity, max intensity, or summed intensity), and this is repeated from image 2–(n+1), etc.

**RGB Gray Merge** ([http://rsbweb.nih.gov/ij/plugins/rgb-gray-merge.html](http://rsbweb.nih.gov/ij/plugins/rgb-gray-merge.html)) combines up to four 8-bit grayscale images or stacks (gray, red, green and blue) into one RGB image or stack.

**SpotTracker** ([http://bigwww.epfl.ch/sage/soft/spottracker/](http://bigwww.epfl.ch/sage/soft/spottracker/)) tracks one particle, with the option to first enhance spots.

**Particle Tracker** ([http://www.cbl.ethz.ch/Downloads/ParticleTracker](http://www.cbl.ethz.ch/Downloads/ParticleTracker)) tracks multiple particles.

**Slice Labeler** ([http://rsb.info.nih.gov/ij/plugins/slice-labeler.html](http://rsb.info.nih.gov/ij/plugins/slice-labeler.html)) adds a label in the top-left corner of each slice of a stack or to the location of any rectangular selection.

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**MIRAOPOLASER**

Instructions for using the OPO in room 8a (Coherent Mira):

1. If the OPO is not going to be used for a week or more, the LBO crystal is stored in a desiccator.
2. Open the cooling-water valves for the OPO.
3. Turn on OPO controller box.
4. Block beam with diffuser and flip mirror to let beam into OPO. CAUTION: beam from Mira Ti:Sapph laser will burn your hand!
5. Wait for the temperature of crystal to be stable.
6. Roughly adjust cavity length (knob in center on end of OPO) until light comes out.
7. Fine adjust cavity length (knob on controller) for both max power and symmetric pulse shape.
8. On readout: red line = spectrometer, blue line = autocorrelator (pulse width).
9. Tweak H and W mirrors on top ends of OPO for max power and symmetric pulse shape.
10. Changing wavelength: look at Mira-OPO curve and temp curve, change temp (Ts) on controller, change Mira, then repeat cavity adjustment. If decreased wavelength, increase cavity length.
MIRATISAPPHLASER

Coherent Mira (Ti-Sapp laser in room 8a):
1. If you’re not using the OPO, make sure the cooling water valves to the OPO are closed.
2. Turn on (smaller) chiller in room 17 (check water level).
3. Turn on Mira controller.
4. Open shutter of Innova Sabre laser and let light into Mira.
5. Wait 15 minutes.
6. Put power meter in Mira beam as a secondary reference.
7. Switch to CW mode to tweak.
8. Open slit (far left, bottom knob) all the way.
10. Adjust setting (check table of wavelength to setting) on monochrometer knob (chrome). CAUTION: don’t change more than 20–30 nm at a time, or aligning will be difficult.
11. Tweak P2 (H and V knobs on far right) for maximum power output.
12. Tweak M7 (H and V, third pair from right).
13. Center slit by closing slit until power drops, adjusting slit position, repeat until at least half power, then reopen slit.
14. Tweak BP2. Start cw, until power drop, then turn ccw until back to power, then 2 full turns further ccw.
15. Re-tweak P2 and M7.
16. Switch to ML.
17. Close slit until both CW and ML component are zero. Slowly open slit (continue although CW component appears, it will disappear by further opening of the slit). Mode-locked when there is no CW component shown (always go a little further to check, then reopen without losing mode-locking).
18. If mode-locking fails, reopen slit and repeat procedure without changing wavelength. Or go back to previous wavelength where mode-locking was achieved, adjust beam path to again achieve mode-locking, then move a smaller shift in wavelength.

Shutdown: Close shutter of Innova Sabre laser, turn off Mira controller, and turn off cooler after a few minutes.

ORIENTINGFILTERSCHROMA

Orienting optical filters (from the Chroma website):
• Proper orientation of the filter is necessary in order to minimize autofluorescence and maximize performance. There is a caret (arrow) located on the edge of each filter in order to aid orientation.
• Excitation (x) filters should be positioned with the arrow pointing toward the specimen, toward the inside of the cube, and away from the light source.
• Emission (m) filters should be placed with the arrow pointing toward the specimen, toward the inside of the cube, and away from the detector/eye.
• Dichroic mirrors should be mounted with the coated surface toward the light source, excitation filters, and the specimen. The dichroics either have an arrow on the side pointing to the coated side, or they are beveled on the coated side. The beveled side is the smaller surface.

**ORIENTINGFILTERSOMEGA**

From Omega:

In most applications, an interference filter should be placed with the most reflective, metallic looking surface toward the light source. The other surface usually can be distinguished by its more colored or opaque appearance. When oriented in this way, the thermal stress on the filter assembly is minimized. Spectral performance is unaffected by filter orientation. Typically, our filters are labeled with an arrow on the edge, indicating the direction of the light path.

**PLASMA-ETCHINGGLASSCOVERSLIPS**

Using the plasma etcher in room 9b is usually sufficient to clean glass coverslips for SMS imaging.
1. Use known clean slides. Take all slides from same side of package.
2. If necessary, place slides on lens-cleaning paper and put scratch on top surface (for focusing) with diamond-tipped pen. (Usually, it is easy enough to focus on junk on the surface in wide-field and using the spot in confocal.)
3. Place all slides in ceramic holder with top (scratch) facing same direction (i.e. towards “Coors”).
4. Plasma etch with Ar (purple glow) for 10 minutes. Keep gas flow low enough that it doesn’t blow the slides away!
5. Use slides immediately or within a few hours. After too long, the coverslips can collect fluorescent contaminants.
6. Don't re-etch slides: start over with new ones. Re-etching is not as effective and the glass can get very thin.
7. Always check a cleaned slide as a control to make sure glass is clean.
For fluorimetry, you can cut the slides very small (the diagonal of the cuvette holder) for right-angle detection. Alternatively, you can tape the slide to the front of the holder (facing the light source) and choose front-face detection. The department fluorimeter has FF detection option (turn the knob on the top of the instrument).

**Method 1: Drop Cast (more reliable)**

1. Cut a glass microscope slide approx 1×1"
2. Use PMMA:toluene 10–20% by mass
3. Mix in dye to PMMA solution
4. Carefully pipet approx. 200–500 uL onto the slide, filling the entire slide
5. Pipet more PMMA/dye until the desired thickness—the surface tension will keep the solution on the slide, and bulge
6. Remove any bubbles
7. Let the film dry

**Method 2: Spin Cast**

2. Cut glass microscope slides into approximately 1×1.5” rectangles.
3. Make a very concentrated stock dye solution in toluene.
4. In a epi tube, mix 10–20 uL stock dye solution with 200 uL PMMA:tol 20% (m/m) solution.
5. On the spin coater, set the acceleration to 10,000 and the spin speed to 1,000 rpm on the knob (digital reading will be something like 700 rpm). Set the spin time to 30 s.
6. Pipet 200 uL of the well mixed dye/PMMA:tol 20% solution onto the center of the slide and start the spinner.
7. Blank the UV-vis spectrometer with two slides coated with PMMA:tol 20%. Replace the front slide with a dye sample and take a spectrum.
8. For the fluorimeter, remove the cuvette stand and secure the slide holder (Thorlabs, part FP01) with double-sided tape on the bottom of the post holder. Turn off room lights and check that the excitation beam hits the slide and that the reflected beam does not hit the detection port. A long-pass filter can be taped to the detection port to be safe, but it is not necessary. (See p 39 of SJL’s notebook 2 for a diagram.)
9. Take several measurements of both reference and dye PMMA films. Find the maximum fluorescence emission; different areas of the film may exhibit drastic differences in emission. But be sure to maintain the same orientation of the slide holder for reference and sample.
10. When calculating the QY, be sure to include the ratio of ODs of the reference and sample, because you will not try to match them.
Making PMMA (SMS) sample for spin coating:
1. Place some amount of PMMA (directly from bottle) into clean (see above) vial.
2. Add 100 times mass in toluene.
3. Allow to sit overnight or until dissolved. Do not sonicate. Do not heat.
   • Note: For PVA, heating is necessary (see this link: http://www.polysciences.com/shop/product.asp?pf_id=15132&dept_id=300150) For 88% hydrolyzed, 85°C should be fine. But still don’t sonicate.
4. Dilute stock dye solution $10^3$ times (e.g. 1 uL stock into 1000 uL toluene). Mix.
5. Dilute solution from above another $10^3$ (e.g. 0.1 uL in 100 uL polymer stock solution). Mix.
6. Check concentration and adjust if needed.
7. Alternatively, measure concentration of stock dye solution and dilute to SM (nanomolar) concentration.
8. Clean chuck of spin coater with acetone before use.
9. Spin 80 uL of solution (e.g. 1% by mass PMMA in toluene) at 2500 rpm for 30 seconds (at "20" acceleration setting).
10. Clean chuck again with acetone to avoid clogging.

SURFACEPLOT3D

You can use Matlab to create a 3D surface plot from a 2D image.
1. In ImageJ, use the Gaussian filter to blur the image.
2. While in ImageJ, convert the file type to 16 bit. Save as a TIFF file.
3. Type \texttt{a = imread('file.tif');} and hit return.
4. Type \texttt{b = double(a);} and hit return.
5. If necessary, subtract the background by typing \texttt{c = b - mean(mean(b));} and hit return.
6. Type \texttt{surf(c); shading interp} and hit return.
7. You can turn off the axes with \texttt{axis off}
8. The standard color map is "Jet". Try \texttt{colormap(hsv)} or others!
**TOTALPHOTONSSM**

\[ N_{\text{photons,detected}} \textbf{Measurements} \]

**Winview:**
1. Use “subset” macro or “binning and skipping” (select UINT16) to split up movies
2. Save as .tiff (not 8 bit)
3. NB: It is possible to do all this in ImageJ using the SPE and substack maker plugins from the ImageJ website.

**Image J:**
1. Go to Analyze -> Set Measurements and make sure that the only option checked is “Integrated Density”
2. Use “Multi Measure” plugin
3. Circle molecule, hit CR (i.e. enter)
4. Repeat for all molecules
5. Select all ROIs and click “multi” to get z-slices
6. Save as .txt file
7. Optional: use “Z-Project” to help see where molecules are

**Matlab:**
1. Run `total_photons_jmulti_auto_GRAPHICAL_GUI.m`
2. Optional: run `histogram.m` and choose a good bin size

**SigmaPlot:**
1. Import data from histogram
2. Run regression wizard for exponential decay
3. Plot results

**Optional Alternative method after Matlab raw data:**

In the past, we plotted a histogram by binning the \( N_{\text{tot,detected}} \) values for each molecule and extracted a time constant from the exponential fit. The main drawback of this method is the bias in choosing a bin size. This choice is removed when you plot using an accumulated probability distribution. \( P = R_n \), where \( R_n \) is the ratio of the number of bleached singles at a certain number of photons \( n \) to total number of singles in the measurement set. (See Molski, A. Statistics of the bleaching number and the bleaching time in single-molecule fluorescence spectroscopy. J. Chem. Phys. 2001, 114(3), 1142–1147.)
1. In Excel or something, order the raw \( N_{\text{tot,detected}} \) values largest to smallest
2. For the y-axis, use a probability distribution: \( P = m/M, \) where \( m = 0, 1, 2, 3 \ldots M-1 \), and the total number of molecules analyzed is \( M \)
3. This should be an exponential distribution (or multiple exponentials), so extracting the time constant is the same as before

4. The basic idea is that you are asking the probability of emitting more than $N$ photons. For the largest value measured $N_{\text{max}}$, zero molecules emitted more than $N_{\text{max}}$, so the probability should be $0/M$. For the smallest value measured $N_{\text{min}}$, all molecules except one emitted more, so the probability is $(M-1)/M$. 
TOTAL PHOTON COUNTS FROM MOVIES

total_photons_jmulti_auto_GRAPHICAL_GUI.m

% 01/16/2008
% Sam Lord updated program so that it doesn't adjust for conversion
gain
% 02/09/2007
% Julie Biteen
% Modified from Sam's "total_photons_jmulti_auto.m"
% Substitutes the part marked "NEW GUI ... END NEW GUI" for the
commented-out part marked "OLD METHOD... END OLD METHOD"
%This program reads results from ImageJ, Multi Measure output. It
reads from each column another molecule, then moves to the next
file.
%This is modified from Kallie's program of March 27, 2002.
%This program finds the total number of photons each molecule emits.
First it allows the user to determine the threshold for when
the molecule is on or off. Then it subtracts off the
%background. Then it sums the intensities over all of the on times.
Finally, it multiplies
%the value it determines by the conversion gain of the camera to get
number of photons.
%clear all variables
clear all
photon_counter=1;
disp('This program does NOT assumes a set conversion gain, but it
does use an integration time of 100 ms');
%user inputs file directory; this is where the program will look for
files
pathname=input('Directory Path: ','s');
path(path,pathname);
files=dir(pathname);
fprintf(1,'\n');
fprintf(1,'For each of the upcoming graphs,\n');
fprintf(1,'click where you''d like the threshold (green line) to
be,\n');
fprintf(1,'and hit return once the threshold is OK.\n');
fprintf(1,'\n');
fprintf(1,'If you would like to skip a given molecule,\n');
fprintf(1,'click between the red and cyan lines,\n');
fprintf(1,'\n');
for f=3:length(files)
c=2; %this makes the program go through all the molecules in all the
columns
molecules = 1;
while  c < molecules+2;
a=dlmread(files(f).name,'\t', 1, 0); %tab delimited, starting at:
row1, col0
molecules = (size(a,2))-2; %determines number of rows of ASCII file
time=a(:,1);
time=time*0.1; %changes from frame number to time in seconds (assume
0.1 s integration)
brightness=a(:,c); %moves through columns until end, then goes to
next file
%set threshold to determine on/off times using method from erwin's
labview program
ordered_brightness=sort(brightness,1);
min_brightness=ordered_brightness(15); %makes sure blank frames don't
skew threshold
max_brightness=max(brightness);
difference=max_brightness-min_brightness;
standard_thresh=0.5; %adjustable parameter to determine
fraction_thresh
fraction_thresh=standard_thresh*difference; %fraction of max-min
current_thresh=fraction_thresh+min_brightness; %threshold value
%plot brightness and the threshold to determine whether the threshold
works
length_array=length(brightness);
thresh_array(1:length_array,1)=current_thresh;
% OLD METHOD
%proceed='n';
%while proceed=='n'
%plot(time,brightness,time,thresh_array)
%check threshold value with user before proceeding
%proceed=input('Threshold OK? (y/n/q): ','s');
%if proceed=='n'
%current_thresh = -1;
%while isempty( current_thresh ) || current_thresh < 0 %makes sure
doesn't quit if no number entered
%current_thresh=input('Input a new threshold value: ');
%end
thresh_array(1:length_array,1)=current_thresh;
elseif proceed=='q'
%disp('abort file:');
%disp(files(f).name);
%disp('molecule:');
%disp(c-1);
%NEW GUI
%Making some lines to click between to set proceed=='q'
min1_array(1:length_array,1)=min_brightness*0.99;
min2_array(1:length_array,1)=min_brightness*0.95;
proceed = 'n';
while proceed=='n'
    plot(time,brightness,time,thresh_array,time,min1_array,time,min2_array)
    %ginput enables you to select points from the figure using the mouse for cursor positioning
    [garbage,new_thresh]=ginput(1);
    %if you are happy with threshold, and hit return, ginput returns an empty matrix, i.e., length = 0
    if length(new_thresh)== 0
        proceed='y';
    elseif new_thresh < (min_brightness*0.99)
        proceed='q';
        fprintf(1, 'Aborted measurement on file %s, molecule number %d.
', files(f).name, c-1);
    else
        %normal situation - the place you clicked becomes the new threshold, and the next loop iteration replots accordingly.
        current_thresh=new_thresh;
        thresh_array(1:length_array,1)=current_thresh;
    end
end
%END NEW GUI
if proceed == 'q'
else
    spacer=1;
    indexer=1;
    %collect all the on time intensity data in one array and the off time background data in another
    for holder=1:length_array
        if brightness(holder)>=current_thresh
            intensity_array(indexer)=brightness(holder);  %creates array of all on times
            indexer=indexer+1;
        else
            %END OLD METHOD
            %NEW GUI

background_array(spacer)=brightness(holder); %collects all off time background values
spacer=spacer+1;
end
end
sum_BG_array=sum(background_array);
%sums all of the backgrounds
background=sum_BG_array/length(background_array);
%computes an average background
%subtract background from the intensity values
BG_subtracted_intensity=intensity_array-background;
%sum the intensity values to get the overall intensity of the molecule
intensity=sum(BG_subtracted_intensity);
%get the total number of photons by multiplying by the conversion gain and 1/QE
%conversion gain is 1.92 e/ct at 1 MHz, med
%quantum efficiency at ~535 nm is ~83%. But it is not in this calculation. QE should be included for total photons incident on camera, when you do:
%photons=(intensity*1.92)/0.83
%photons=(intensity*1.92);
photons=intensity;
%create a histogram of all of the molecules total photon counts;
photon_hist(photon_counter,1)=photons;
photon_counter=photon_counter+1;
clear intensity_array background_array BG_subtracted_intensity
end
clear time brightness thresh_array min1_array min2_array c=c+1;
end
end
string=input('save file for histogram as: ','s');
data=[photon_hist];
savename=[string,'.txt'];
save(savename,'data','-ascii');
COMPARE Z-MATRICES OF ELECTRONIC STRUCTURES FROM GAUSSIAN

comparezmats.m

% Sam Lord: sjlord@stanford.edu
% 5/17/06
% This program compares the z-matrices for multiple QC calculations using different levels of theory or different basis sets. It calculates differences in the bond lengths, angles, and dihedrals directly from a z-matrix from Molden. Be sure to use "molden -A 'structure'" when looking at a file; this prevents Molden from rewriting the z-matrix. Go into the Z-matrix Editor in Molden, click "US" and "Gaussian," then save the z-matrix with a good name (e.g. '~_in' or '~_out' for before and after "movie," respectively).

% Because I used the crystal structure as the starting z-matrix, I can compare the starting structure to the optimized structure (before and after pressing "movie" in Molden). This ensures that all the angles and dihedrals are defined the same (I was having trouble comparing the original crystal structure z-matrix to the calculation .log files' z-matrices, because things changed).

% Molden defines a dihedral of -190 as +170. In other words, I needed to correct these far negative angles to their corresponding positive angles (or the difference is 360 too big). So I take any angle over 180 and subtract it from 360.

clear all
% Input the initial z-matrix (e.g. '~_in') from Molden ("first") in for reference. Use the z-matrix that Molden outputs (e.g. '~_out') after you run "movie" in calculation. These read in the variables at the end of the z-matrices:
reference=dlmread('zl78y_newopt_blyp321g_zmat_in','','47,1);
calculation=dlmread('zl78y_newopt_blyp321g_zmat_out','','47,1);
difference_uncorr=abs(reference-calculation);
% This loop corrects for angles larger than 180:
i=1
while i<(length(difference_uncorr)+1)
    if difference_uncorr(i)>180
        differenceT(i)=360-difference_uncorr(i)
    elseif difference_uncorr(i)<=180
        differenceT(i)=difference_uncorr(i)
    end
    i=i+1
end
% Need the transform of the result of the above loop:
difference=differenceT'

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% This separates the three types of values by when in the column they appear:

diff_bondlengths=[difference(1); difference(2); difference(4); difference(7:3:end)]
diff_angles=[difference(3); difference(5); difference(8:3:end)]
diff_dihedrals=difference(6:3:end)
% Now copy the three columns of absolute differences and paste into %SigmaPlot

**HISTOGRAM DATA AND CHOOSE BIN SIZE**

**histogram.m**

% Sam Lord
% 11/29/05
% This is a program that reads in a single column of values, allows you to determine the number of bins, shows you a histogram and fit, then saves the histogram data (bins and frequency). It uses the hist function and the exp1 fitting procedure. This does not save any of the fitting data.

rawfile=input('raw data file (.txt): ','s');
rawdata=dlmread(rawfile);
proceed='n';
while proceed=='n' %this loop lets you change number of bins
nbins=input('number of bins: ');
[n,xout] = hist(rawdata,nbins);
m=[xout;n];
xy=m.:
xdata=xy(:,1);
ydata=xy(:,2);
[fresult,gof] = fit(xdata,ydata,'exp1')
%fresult,gof,output = fit(x,y,'exp1')
x=[min(xdata):100:max(xdata)];
predicted = fresult(xdata);
real = ydata;
residuals = predicted - real;
subplot (2,1,1); bar(xdata,ydata)
hold;
plot(x,fresult(x),'-r')
hold;
subplot (2,1,2); plot(xdata,residuals)
proceed=input('binning good? (y/n) ','s');
end
save=input('save file for histogram as (.txt): ','s');
%savename=[save,'.txt'];
savename=[save];
dlmwrite(savename, m.', ', '\t'); %transposes m and saves (tab delimited)
APPENDIX:
NMR AND MS SPECTRA
Azido Fluorogens

DCDF-V-P-azide

(400 MHz, CDCl3)


(DCDHF-V-P-azide
(400 MHz, CDCl₃)
DCDHF-V-P-amine (by photoconversion)

(400 MHz, CDCl₃)
DCDF-V-P-amine (by photoconversion) (400 MHz, CDCl₃)
DCDHF-V-P-amine (by photoconversion)

(400 MHz, CDCl₃)
DCDHF-V-P-amine (by photoconversion)

(400 MHz, CDCl₃)
DCDHF-V-P-amine (synthetic)

(500 MHz, CDCl₃)
DCDHF-V-P-amine (synthetic)
(500 MHz, CDCl₃)
DCDHF-V-P-amine (synthetic)

(500 MHz, CDCl₃)

DCDH-V-P-amine (synthetic)
DCDHF-V-P-amine (synthetic)

(500 MHz, CDCl₃)

DCDHF-V-P-amine (synthetic)
crude DCDHF-V-P-nitro (by photoconversion)

(300 MHz, CDCl3)
crude DCDHF-V-P-nitro (by photoconversion)
(400 MHz, CDCl₃)

DCDHF-V-P-nitro (synthetic)
DCDHF-V-P-nitro (synthetic)

(400 MHz, CDCl₃)

DCDHF-V-P-nitro (synthetic)
DCDHF-V-P-nitro (synthetic) (400 MHz, CDCl₃)
DCDHF-V-P-nitro (synthetic)
(400 MHz, CDCl₃)
Dual Emitter DCDHF-P-T

(see Figure 3.22 for which bands 1–4 denote)

various photoproducts

LordS_071001_18949 20 (0.956) Cm (17:25)1: Scan ES+

1.18e7

365.2

327.3

309.1

563.5 366.3

416.4 41.4

477.5 433.3

593.8 659.4 615.4

691.1

LordS_071001_18949 20 (0.973) Cm (16:26)2: Scan ES-

1.69e5

527.6

527.2

351.0

327.1

350.4

401.1

400.8

387.5

486.6 473.1

425.4

441.6

515.7

500.6

560.2

559.8

610.8

600.7

586.6

654.7

649.9

670.8

718.3

698.7

various photoproducts

LordS_071001_18949 20 (0.973) Cm (16:26)2: Scan ES-

1.69e5

527.6

527.2

351.0

327.1

350.4

401.1

400.8

387.5

486.6 473.1

425.4

441.6

515.7

500.6

560.2

559.8

610.8

600.7

586.6

654.7

649.9

670.8

718.3

698.7

various photoproducts

LordS_071001_18949 20 (0.973) Cm (16:26)2: Scan ES-

1.69e5

527.6

527.2

351.0

327.1

350.4

401.1

400.8

387.5

486.6 473.1

425.4

441.6

515.7

500.6

560.2

559.8

610.8

600.7

586.6

654.7

649.9

670.8

718.3

698.7

various photoproducts
78y + O2

Various photoproducts

208 peaks at 200 m/z in positive ESI mode.

sjl-3 and -4 both had very intense
sl-4 and sl-5 both had very intense peaks at 200 m/z in positive ESI mode.


218


226


