Single-Molecule Fluorophores as Environmental Nanoprobes

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The DCDHF dyes are under development as a promising new class of single-molecule fluorophores. The utility of these fluorophores is derived from a range of attributes including their synthetic accessibility, structural versatility and photostability, all permitting a range of practical labelling applications. Their polarity- and viscosity-dependent emission, required for their application as local environment nanoprobes, is of particular interest. A wide range of DCDHF chromophores with different π-systems have been prepared for control of absorption and emission characteristics. Structure-function analysis has been performed in conjunction with theoretical evaluations of these chromophores.

Key words: Single molecule, DCDHF fluorophore, environmental sensitivity.

I. Introduction

Single-molecule detection techniques have undergone intensive development for both spectroscopic characterization and for imaging of individual fluorescent molecules.[1-8] Contemporary single-molecule techniques offer several advantages in comparison with more conventional imaging and spectroscopic methods. First, the observation of individual molecules removes the ensemble-averaging characteristics encountered in bulk experiments and permits the extraction of extra information which, in turn, may reveal otherwise hidden processes.[9-13] For example, in a molecular beacon study[8], if some of the beacons exist in a closed state while the remaining beacons exist in an open state then an ensemble characterization will only reveal some average properties of the fluorescence which are of little value. However, a single-molecule imaging study of the same beacon system, wherein the open and closed beacons are observed individually, would permit the construction of a frequency histogram for the actual distributions of both closed and open beacon states. A second benefit of single-molecule techniques involves removal of the need for synchronization of many single molecules undergoing a time-dependent process. Thus, single-molecule techniques could provide a more detailed picture of some biological process so that the exact mechanism could be elucidated.[2] Third, and more specifically, the observation of single molecules offers the opportunity to collect information about the nanoscale environment around an individual probe molecule. Thus, a molecule may report on its unique local environment by a change in its fluorescence intensity (related to changes of the lifetime of the states from which fluorescence occurs) and/or the energy of the fluorescent photons (related to the changes in the relative energies of the various electronic states involved). The environmental influence might come from a variety of sources including the surrounding viscosity and polarity and single-molecule probes are particularly useful for the analysis of inhomogeneities in such systems. A straightforward example is found in the analysis of single-molecule chromophores in glassy polymer matrices wherein information about the distribution of local free volume is revealed instead of just the average free volume (as is experimentally reflected in the distributions of lifetimes of the exited state responsible for fluorescence).[14]

Very generally, single-molecule imaging should be very difficult to achieve since it demands the detection of emission from an individual source above any background signals in a system swamped with an overwhelming number of host molecules as well as impurities in the focal volume containing the molecule of interest. There are instrumental challenges as well, including photon losses from filters and optical components, scattered light at the incident wavelength and detector dark current. Fortunately, the detection of single molecules can be greatly simplified by utilization of fluorescence and, as a result, more routine optical techniques become relevant and sufficient. Here the photons resulting from a fluorescence process are shifted to a longer wavelength relative to the absorption (the Stokes shift) and pump wavelength and are thus much more easily discriminated against the background. In order to increase the contrast of the fluorescence signal to background, one must also strive to improve the photophysical properties of the fluorophore so that a signal-to-noise ratio (SNR) for single-molecule signal is sufficient to obtain enough fluorescence information over a reasonable collection time.

Single-molecule techniques continue to evolve and have become increasingly widespread and sophisticated. For example, two-photon pumping can successfully be utilized on the single-molecule level for fluorophores with sufficient two-photon cross sections.[15] Single-molecule detection is also polarization sensitive and can even observe the orientations of suitable optically anisotropic molecules relative to an established experimental frame of reference.[16] A particularly exciting recent development involves new superresolution techniques that may offer the opportunity to examine single molecules in the sub diffraction limited regime.[17,18]

Fluorophores useful for applications in single-molecule studies must have a large absorption cross section so as to absorb excitation light efficiently, must have weak bottlenecks into dark states (such as triplet states), should have a high fluorescence quantum yield to emit fluorescence efficiently (or, in special cases, a variable quantum yield might be desirable), and finally, must have high photostability so that sufficient emitted photons are collected before the fluorophore ultimately photobleaches. At room temperature, these requirements have already been fulfilled by fluorescent labels based on laser dyes (such as rhodamines, cyanines, oxazines, etc) as applied to many biological applications and also by derivatives of some rigid polynuclear aromatic hydrocarbons such as terrylene or perylene. Other classes of fluorescent substances that have proven useful for single-molecule imaging are derived from naturally occurring proteins (GFP, the green fluorescent proteins, as well as their engineered derivatives)[19] and from quantum dots.[8] Even color center defects have been utilized as "single-molecule" sources.[20]

II. Experimental results

As single-molecule techniques become more sophisticated, fluorophores are now required that are not only suitable for imaging the localization of the dye but are also able to offer additional beneficial properties and reporting function. Since an important goal of single-molecule spectroscopy is to collect additional information from the fluorescence emission, fluorophores sensitive to their local environment may also serve as nanoscale reporters for information about their immediate environment. Also, fluorophores with good synthetic flexibility would allow introduction of a wider range of functional groups, and thus may be utilized in many different applications requiring covalent attachment or other specific interactions. Many of these demands are met by the DCDHF fluorophores. Hereafter, we concentrate on a few of these requirements in more detail. More specifically, we will concentrate on representative structure variations, which influence the quantum yield and wavelength of fluorescence and, in turn, influence their nano-reporter function.

The general structure of a DCDHF dye is found in Fig 1. The individual chromophore (the electronic/photonic active part) is found within the ellipse and is comprised of an R_1,R_2-disubstituted amine donor, a π-link (some combination of carbocyclic or heterocyclic aromatic rings and alkenes) and the DCDHF (dicyanomethylene dihydrofuran) ring with three acceptor cyano groups and additional R_3, R_4 groups. The various combinations of the amine donor, the π-system and the acceptor have primary influence on the electronic, absorption and emission properties of the fluorophore. The donor part of the molecule is relatively electron rich (the nitrogen has a pair of electrons in an available lone pair) and the acceptor part of the molecule is relatively electron deficient (it contains heteroatoms with hybridizations and configurations that attract and stabilize electron density). The FG_1 through FG_n are additional functional groups attached to the chromophore at various locations (in the R_1, R_2 donor tails, on the π-core or in the R_3 or R_4 substitutents of the acceptor). These FGs tend to serve two main roles. First, the modifying FG will influence the lipophilicity of the chromophore, i.e., where it will tend to distribute itself in an environment with varying polarity. For example, the FG may contain polar or even ionic sites (alcohols, carboxylic acids, sulfonic acids, etc.), which tend to enhance miscibility of the dye in an aqueous environment or the FG could be just simple long aliphatic tails, which would enhance miscibility of the dye in a nonpolar environment, as in a membrane.[37] Second, the FG may be reactive groups (maleimide, succinimide ester, etc.) permitting covalent attachment of the DCDHF fluorophore to biomolecules or other substrates. As an additional level of complexity, the system may exist as a monomer (m=1 and then with no need for an X linking entity) or a dimer (m=2) with X as some linking structure which organizes the individual chromophores spatially (control of orientation and distance with implications on energy transfer interactions of the individual chromophores). There are still more complex systems, which are not

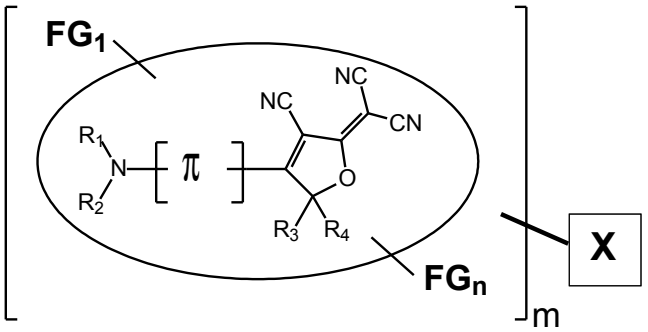


Fig. 1 The General Structure Features of a DCDHF Chromophore.

adequately represented by the simple cartoon in Fig 1. One such dimer system is a molecular beacon in which the "X" component might be a very complicated biological system such as a peptide or nucleic acid. Here the fluorophores terminating such a system may be identical but often they must be different and with their respective electronic properties tuned for highly specific interactions (such as FRET, Fluorescence Resonance Energy Transfer, etc.).

This DCDHF-type chromophores were first synthesized for electro-optical applications (here they are sometimes called TCF dyes)[21] and have been subsequently applied in a number of different fields including as photorefractives and media for THZ generation.[22-28] Many of the DCDHF chromophores exhibit the interesting ancilliary property of monolithic glass formation and it was in the course of the studies of the photorefractive properties of these chromophores that their attractive fluorescence properties were first manifested. Subsequent studies to date have revealed that this family of fluorophores can also serve as a single-molecule imaging dye.[14,30-32]

A general synthetic route for DCDHF dyes is shown in Fig. 2. For fluorophores with only a single phenyl ring in the π-system (5), the synthesis can be accomplished via two different methods. The α-ketol was synthesized by the

reaction of 4-fluoro- or 4-dialkylaminophenyl magnesium bromide with the trimethylsilyl protected acetone cyanohydrin. Malononitrile was then condensed with this α-ketol to form the DCDHF acceptor ring (from which the name of the dye class containing this ring is derived). In the case when R is a fluorine atom (4), an aromatic nucleophilic substitution reaction is used to install the disubstituted amine. For the DCDHF fluorophore with a styrene-conjugated system (7), intermediate 6 was synthesized via an improved method, in which the commercial 3-hydroxy-3-methyl-2-butanone was condensed with malononitrile in dry pyridine. The resulting heterocycle 6 was then reacted with a variety of 4-disubstituted aminobenzylaldehydes to give different DCDHF-V fluorophores (7).

Because the DCDHF fluorophores are constructed from a push-pull combination of the disubstituted amine donor and the potent dicyanomethylenedihydrofuran (DCDHF) acceptor they often possess a large ground dipole moment (calculated to be about 11 Debye for DCDHF-6, R_1=R_2=*n*-hexyl, R_3=R_4=CH_3, n=0, m=1). This large ground state dipole moment, which can couple and align with an applied external field, is a prerequisite for their original interest as electro-optic chromophores. Most fluorescent dyes are not highly polar like this but they are certainly not unprecedented either (e.g., Prodan[33] and DCM[34] are such dipolar dyes which have found application in both photonic and biological applications).

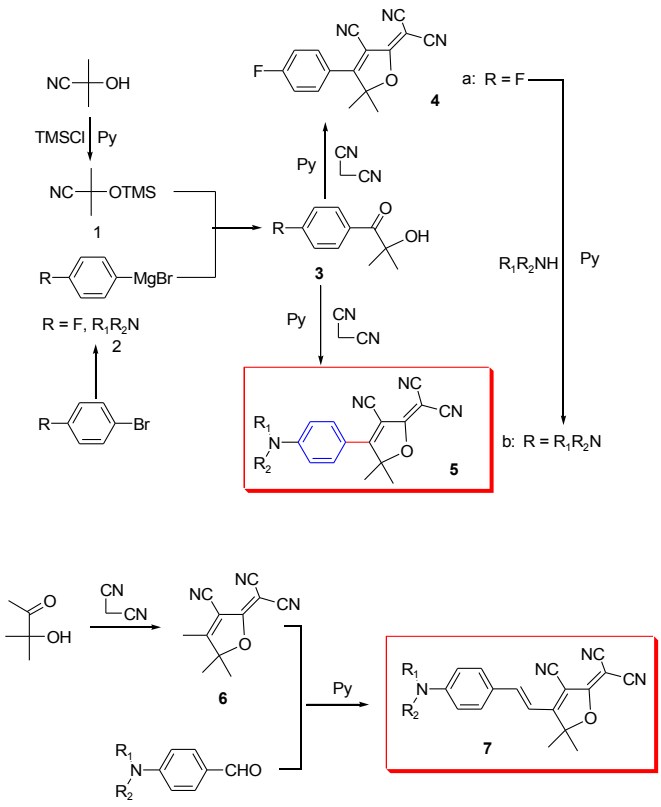


Fig. 2: General synthesis routes for the DCDHF fluorophores with either a single phenyl ring (5) or a styrene (7) as the π-system.

The polarity of the DCDHF dyes also enhances some valuable physical properties relevant to their applications as environmental reporters. The solvatochromism of polar dyes is an indication of their optical nonlinearity and is also a valuable probe of environmental activity since the solvent also influences the fluorescence wavelength. A twisted intramolecular charge transfer (TICT) state appears to be operating in the emission process and the fluorescence is expected to vary in solvents with different polarity. The bulk spectroscopic studies were performed on DCDHF-6 dissolved in a variety of solvents, both protic and aprotic.[14] Fig. 3 shows that both the absorption (top) and emission (bottom) spectra become red shifted as the DCDHF-6 is introduced into more polar solvents (from toluene to DMSO). Meanwhile, the overall intensity (quantum yield) is also strongly affected by the identity of the solvent with toluene producing the strongest emission. As a result, this family of fluorophores can serve well as a reporter for the polarity of the local environment.

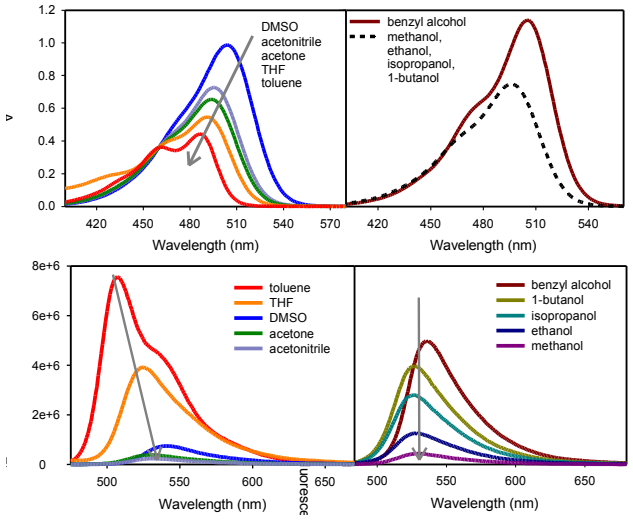


Fig. 3: Absorption and emission spectra of DCDHF-6 in different solvents. Very generally, the DCDHFs possess positive solvatochromism (a long wavelength shift in absorption as solvent polarity increases) with a corresponding decrease in fluorescence quantum yield as solvent polarity increases.

Another characteristic property for many DCDHF fluorophores is their solvent viscosity dependent fluorescence quantum yield.[14] A simple experiment demonstrating the viscosity response involves dissolution of DCDHF-6 in mixtures of methanol and glycerol in different ratios. Glycerol is about 2000 times more viscous than methanol and within in the range of compositions studied (0.5-15 cP) the quantum yield changed by more than an order of magnitude with a linear relationship between log (quantum yield) vs. log of (viscosity).[14]

III. Modification of DCDHF Properties

In order to rationalize the observed environmental spectroscopic sensitivity of the DCDHF molecules, calculations were performed to compare with the experiments described above.[14] There are three possible sites for rotation

that might contribute to a TICT (Twisted Intramolecular Charge Transfer) state: the amine-aryl twist (α), the aryl-dihydrofuran twist (β) and the dicyano-dihydrofuran twist (δ).

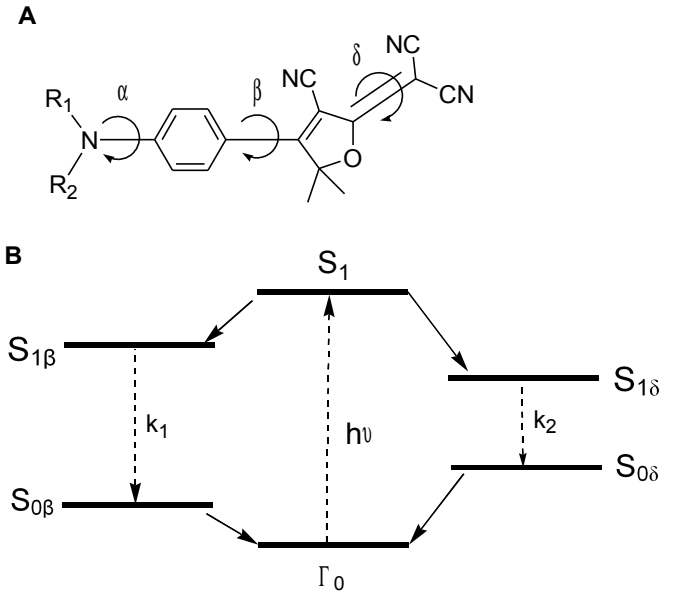


Fig. 4: (A) Possible dihedral twists in a DCDHF fluorophore. Note that the bond orders shown in the figure represent just one limiting resonance form (at all of the the sites of rotation indicated, the single bonds possess double bond character and the double bonds have single bond character); (B) Proposed energy level scheme and transitions to account for the photophysics of DCDHF fluorophores (see reference 14 for details).

The preliminary calculations indicated that the amine-aryl rotation □ has less influence on the energy of excited state while the aryl-furan and dicyano-furan rotations would lower the energy of the excited state. So, the following mechanism (Fig. 4B) was proposed: the molecule is excited by light from ground state (Γ_0) into a Frank-Condon state S_1 without a geometry change (Frank-Condon Rule). Given sufficient time, the molecule can relax into one of the two excited states through a change in δ or a change in β. From either of these two excited states, the molecule can then relax back to the ground state, reversibly changing the geometry back to the original form (Γ_0). So, there are two main pathways to relax the excited state. And the calculations indicate that the $S_{1\square}$ state has lower energy than the $S_{1\square}$ state

On the other hand, when the energy gap between $S_{1\delta}$ and $S_{0\delta}$ becomes so small that internal conversion will take the place of fluorescence emission (k_2), it could result in a fluorescence loss. The calculated energy gap also confirms that the energy of fluorescence detected is more similar to the energy gap between $S_{1\beta}$ and $S_{0\beta}$. So, the observation that the quantum yields of this family of fluorophores are polarity dependent can be explained in this way: If the solvent favors the nonradiative $S_{1\delta}$ state, the fluorescence quantum yield of the molecule will be quite low. Likewise, if the rotation of the dicyano group is slowed or prevented in a particular environment, the quantum yield will be larger. Thus enters the role of more viscous solvents, which slow down this rotation.

The absorption and emission properties of the DCDHF dyes have been finessed to a significant degree. As an example of the level of tuning that has transpired, consider the influence of donor connectivity modification. Control of rotation about "□" is feasible but has not yet been accomplished and control or rotation about "δ" may not be feasible by any reasonable structure modification. While the preliminary calculations did not identify a significant influence on the photophysical properties for rotation at site "α" a contribution from this site has been observed experimentally. Modifications to control rotation about "□" proved to be relatively straightforward and we have prepared a series of molecules in which the rotation here is essentially turned off by inclusion of the amine donor in one or two rings.

The first series of styrene DCDHFs with zero, one or two tetrahydroquinoline rings shown in Fig. 5 has been synthesized. Basically it follows the same synthetic protocol in Fig. 2. Different benzaldehydes (8 and 9) were made by Vilsmeier reaction of julolidine and alkylated tetrahydroquinolines. Together with commercially available 4-N,N-diethylaminobenzylaldehyde, they were condensed with previously made intermediate 6 to give our desired fluorophores (11 and 12).

Synthesis routes to the series of the phenyl DCDHF with no, one or two tetrahydroquinoline rings are shown in Fig. 6. Fluorophore 13 was made through the same intermediate 4 in Fig 2. Fluorophore 18 and 19 were prepared by bromination of 1-hexyl-1,2,3,4-tetrahydro-quinoline and julolidine followed by lithiation and trapping with protected cyano acetonhydrin 1 to make the respective 4-amino substituted α-ketols. Condensation of the resulting ketols with malononitrile gave the desired DCDHF dyes directly.

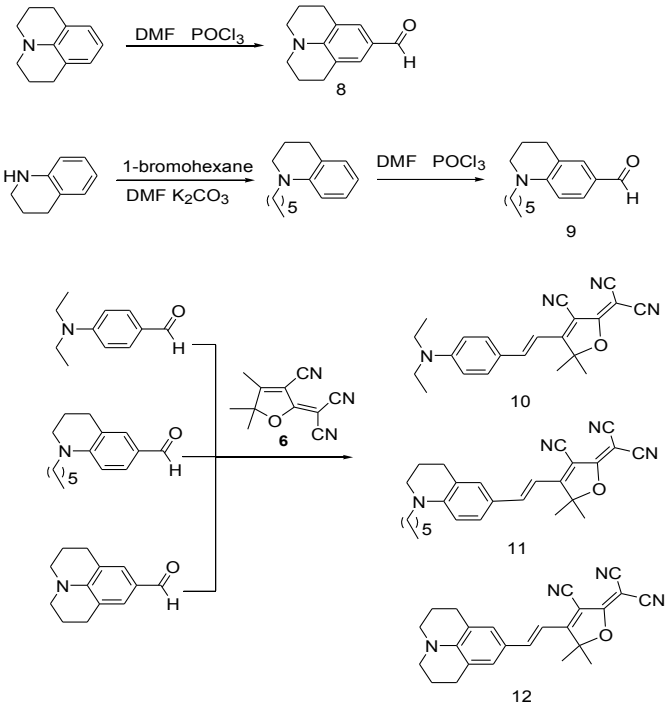


Fig. 5: The synthesis of styrene DCDHF w/o tetrahydroquinoline rings.

The relevant physical properties of the different DCDHF dyes in toluene are summarized in Table 1. It is obvious that the maxima of the absorption and emission wavelengths systematically increase as the nitrogen donor initially is acyclic and then is constrained by one ring and then two rings.

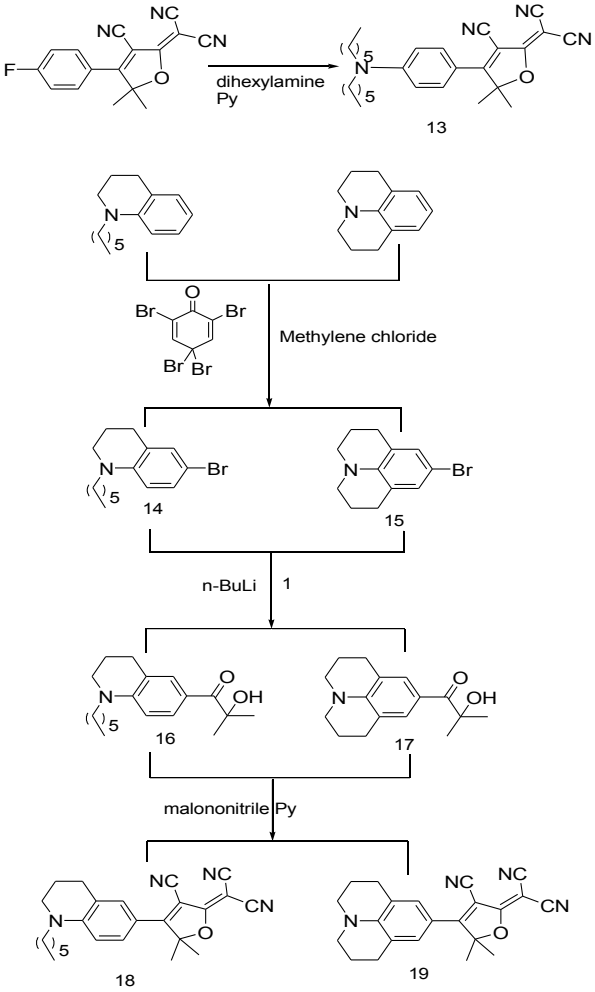


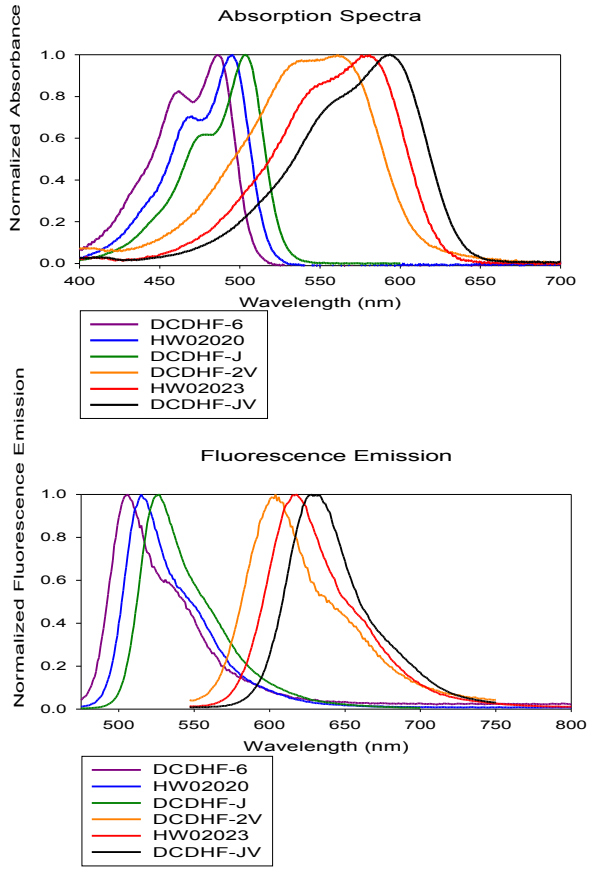
Fig. 6: Synthesis of phenyl DCDHF w/o tetrahydroquinoline rings

Table 1 Characterization of DCDHF dyes w/o tetrahydroquinoline rings (all values in toluene) respectively. The inclusion of the donor nitrogen in the rings also has a substantial influence on the fluorescence quantum yield, which is increased almost five times from 10 vs. 12 and twice from 13 vs. 19.

Normalized absorption and emission wavelengths are plotted

Group	Entry	λ_{abs}^{max}	λ_{em}^{max} (Stokes shift)	Φ_F toluene (PMMA)
	10	562	603 (41)	0.02 (0.39)
A	11	581	618 (37)	0.028
	12	594	628 (34)	0.053
	13	486	505 (19)	0.044 (0.92)
B	18	495	515 (20)	0.10
	19	503	527 (24)	0.21

state effects needs to be done in the future in order to confirm the exact mechanisms operating in the DCDHF fluorophores. In any case, the addition of tetrahydroquinoline rings provides one nice method to shift the absorption and emission to longer wavelengths. This red shift is due to both holding the amine donor into a more favorable configuration for electron donation and the addition of the inductively donating alkyl groups from the rings.



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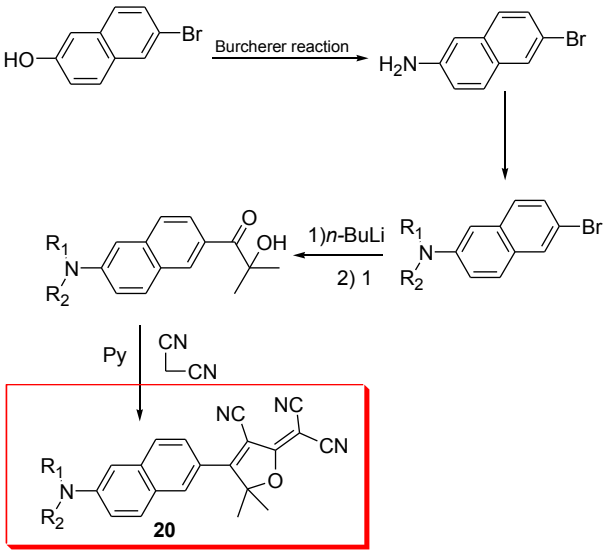

Fig. 8: Synthesis route for DCDHF fluorophores with naphthalene π-system.

The synthesis of DCDHF fluorophores with naphthalene rings starts with Bucherer reaction of 6-bromo-2-napthol. The free amine is alkylated with different R_1, R_2 groups and the bromo end undergoes lithiation and attacks the protected cyano acetohydrin (1) to give the desired α-hydroxyketone, which, in turn, condenses with malononitrile to obtain the target fluorophore 20. (Fig. 8) Compared with DCDHF fluorophores with phenyl rings (DCDHF-6, absorption at 486nm and emission at 505nm in toluene), this naphthalene based fluorophore (20: R_1= R_2= *n*-hexyl), with absorption at 547 nm and emission at 576 nm in toluene, is above the excitation range of flavins but its emission could overlap with that of flavins.

To further optimize the operational wavelengths, the aromatic core needs to be pushed still further and so the corresponding anthracene derivatives were examined. The synthesis began with commercially available 2,6-diaminoanthraquinone. However, all attempts to selectively modify only one of the two amines were unsuccessful. (Fig. 9) An attempt to carry out a selective Sandmeyer reaction (conversion of amine to bromide) on only one amine group failed at least in part due to solubility problems, producing mainly dibromide 24 and no monobromide 25 was

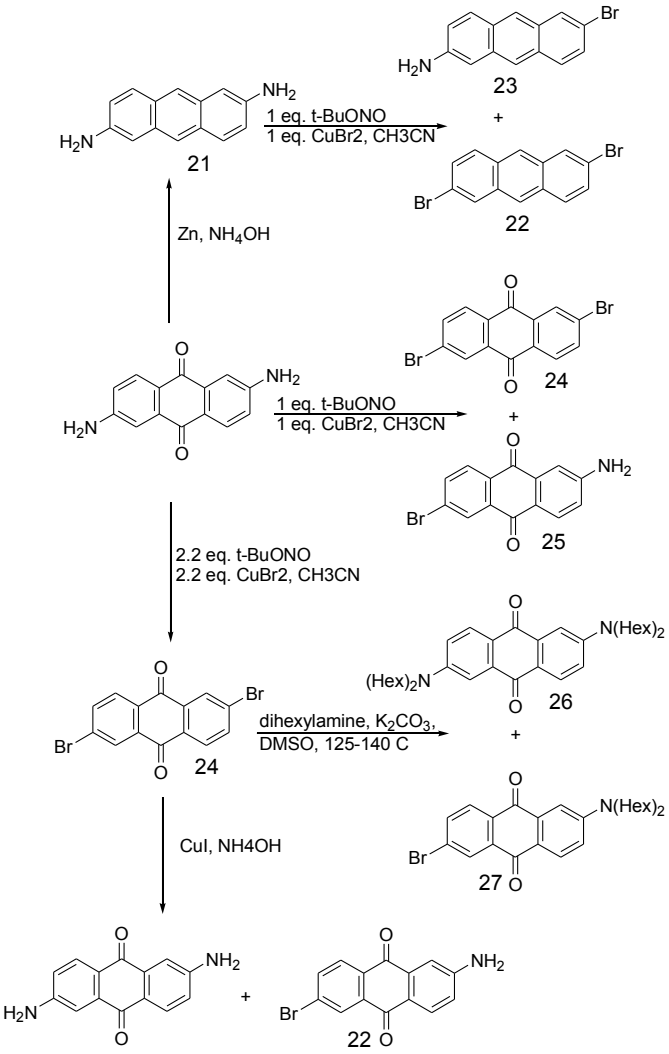

Fig. 9: Unsuccessful approaches attempted for the preparation of some asymmetric 2,6-disubstituted anthraquinones.

isolated. We then reduced the anthraquinone to diaminoanthracene (21) and tried to chemically differentiate these two amines. Using the approach to convert only one of the amino groups to a bromide by using one equivalent or less of tert-butyl nitrite and cupric bromide resulted in only a mixture of 22 and the starting material. Direct nucleophilic aromatic substitution of 24 with a secondary amine usually afforded an inseparable mixture. For example, reaction of 24 with dihexylamine in the presence of K_2CO_3 in DMSO afforded a mixture containing less than 5% of the desired 2-bromo-6-dihexylaminoanthraquinone (27) along with 2,6-bisdihexylaminoanthraquinone (26). This mixture was too difficult to separate for any practical application. Amination of 24 with CuI catalyst and an excess of ammonium hydroxide under pressure afforded only the starting amine and no asymmetric product was obtained.

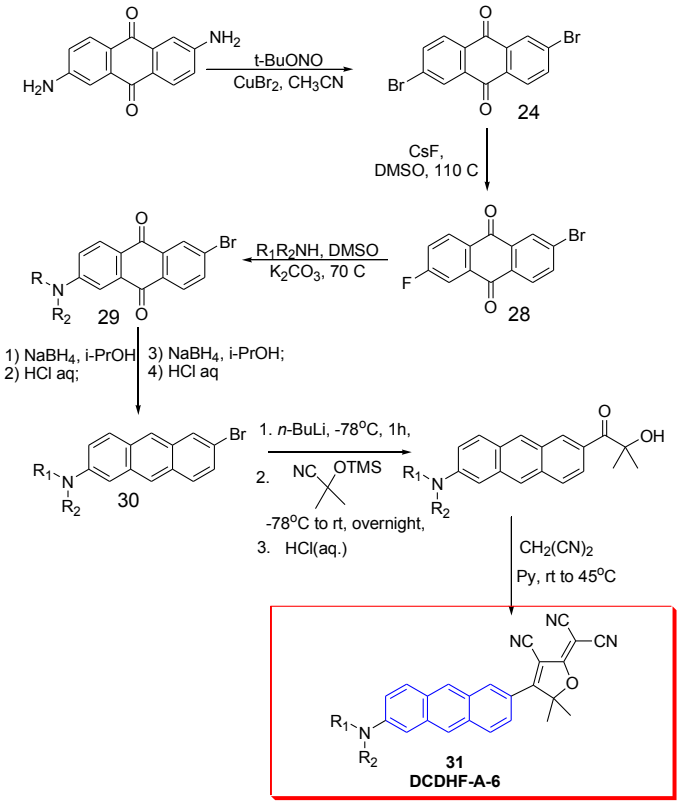

Fig. 10: Overall synthesis route for DCDHF fluorophores with an anthracene core.

Since the low solubilities of both 2,6-diaminoanthraquinone and anthracene (21) may play a key role in these failed attempts, we considered the possibility that a solution to break this symmetry should involve an intermediate that has similar or less solubility or reactivity than the starting substrate, so as to avoid preferential reactivity for the monofunctionalized product. So, 2-bromo-6-fluoroanthraquinone was considered because it is reported that 5,6-dibromo-1,2-acenaphthenequinone was successfully converted to 5,6-difluoro-1,2-acenaphthenequinone by reaction with CsF in anhydrous DMSO.[36] If 2-bromo-6-fluoroanthaquinone (28) has solubility comparable to 24 and if the fluorine could be preferentially replaced by the dialkylamino functionality via aromatic nucleophilic substitution then an appropriate unsymmetrical intermediate would be available. With 1.0 eq of CsF in DMSO at 110°C, about 45% of the starting dibromide (24) was converted to 2-bromo-6-fluoroanthraquinone (28) accompanied by about 10% of 2,6-difluoroanthaquinone, which is pretty promising. After several attempts, we found that treatment of 24 with 1.3 eq of CsF in anhydrous DMSO at 110°C gave about 2:1 ratio of 28 and 2,6-difluoroanthaquinone as detected by GC-MS (60% 2-bromo-6-fluoroanthraquinone, 30% 2,6-difluoroanthaquinone and the remainder was the starting 24). The halogen exchange reaction mixture in DMSO was directly treated with disubstituted amine and potassium carbonate at 70°C to give, after chromatography, 2-bromo-6-disubstituted aminoanthranquinone (30) in overall 18% yield for the two steps. After that, 30 was lithiated with *n*-BuLi at –78°C over 1 h and trapped with the TMS protected acetone cyanohydrin. The resulting α-siloxyimine intermediate was hydrolyzed to afford the crude α-ketol, which was used directly in the DCDHF ring preparation and thus providing the desired fluorophore 31 (Fig. 10).

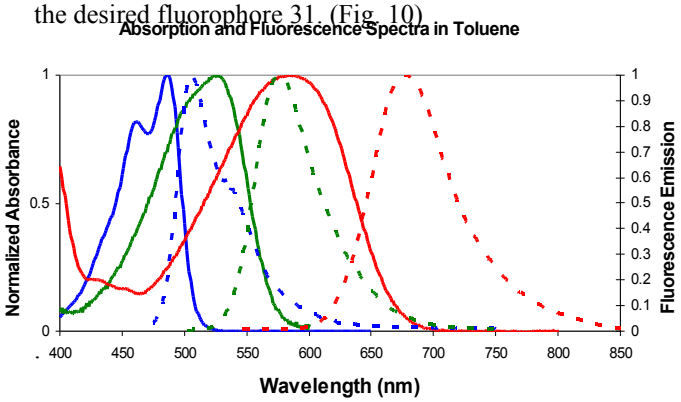

Fig. 11: Normalized absorption (solid lines) and emission (dotted lines) spectra of DCHDF fluorophores (R_1=R_2= n-hexyl) with the series of benzene, naphthalene and anthracene □-systems. (left to right; 13, 20, 31). Data in Table 2 is relevant to this plot.

The absorption and emission wavelengths of DCDHF fluorophores containing the benzene, naphthalene and the anthracene rings are plotted in Fig. 11. The solid lines represent absorption spectra and the dotted lines represent emission spectra. It is clear that both absorption and emission wavelengths are systematically red-shifted as the □-system is changed from phenyl to naphthalene to anthracene. The emission wavelength is shifted substantially—by almost 400 nm. 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 competing emission from cellular components. This newly synthesized fluorophores 31 (DCDHF-A-6), with absorption at 585 nm and emission at 689 nm in toluene (Table 1), can be efficiently pumped using green or yellow light and exhibit large Stokes shifts, further enabling filtering to reject pumping light.

Detailed photophysical properties for the fluorophores 13, 20 and 31 are listed in Table 2. Apparently, with the increase of absorption and emission wavelength from the phenyl derivative to the naphthalene derivative and thence to the anthracene derivative, the polarity dependence and viscosity dependence of this family of fluorophores are all well maintained. From the less polar solvent toluene to more polar solvent acetone, emission spectra of all three fluorophores are red-shifted and their quantum yields are lowered. From less rigid organic solvents to more rigid polymer (PMMA), the quantum yields of all three fluorophores are dramatically increased.

Other structures can also be employed to enhance the wavelength response in the DCDHF class. For example, the inclusion of multiple rings in the □-system has been examined. While outside the scope of detailed discussion here the inclusion of multiple thiophene rings into the □-system has been adopted from electrooptical materials and this structure change has produced fluorophores that emit in

the near infrared. Also beyond the scope of detailed discussion here is the growing array of reactive functional groups which have been successfully introduced into these dyes: *N*-hydroxy succinimide ester was introduced into the fluorophores to attach to different functionalized ologonucleotides; maleimide was introduced into the fluorophores to label different thiol containing peptides or proteins; and AM (acetoxy methyl) protected APTRA (o-aminophenol-*N,N,O*-triacetic acid group has been introduced to detect different ions.

Table 2. Spectral parameters of fluorophores 13, 20 and 31 in a representative range of liquid solvents and also in PMMA. The π-core homologation structure modification has pushed the fluorescence of these dyes into the near infrared.

	solvent	Φ_F	λ_{abs}^{max} (nm)	λ_{em}^{max} (nm)
13	PMMA	0.94		
	toluene	0.044	486	507
	acetone	0.0041	494	531
	ethanol	0.0066	469	548
20	PMMA	0.98	534	609
	toluene	0.85	546	579
	acetone	0.015	533	660
	ethanol	0.017	543	657
31	PMMA	0.71	594	686
	toluene	0.54	585	689
	acetone	0.043	588	846
	ethanol	0.013	604	846

V. Conclusions

In summary, a group of novel single-molecule imaging fluorophores, the DCDHF family, has been discovered and successfully imaged at the single-molecule level. Their polarity dependent absorption and emission wavelengths, and viscosity dependent quantum yields have provided additional benefits as polarity and viscosity reporters for their local environment. A mechanism for this dependence is proposed together with addition of tetrahydroquinoline rings to the molecule to identify the photophysical property related rotations at the amine donor part of the molecule. The high synthetic flexibility allows introduction of different functional groups into the fluorophore to achieve different goals: addition of different conjugation systems for optimal absorption and emission wavelengths; addition of different bioactive functional groups for different organelles labeling; and addition of different functional groups to detect different ions.

Acknowledgements

This work has been supported by National Institutes of Health grant Grant No. 1P20-HG003638, Department of Energy Grant No. DE FG02-04ER63777 and the Ohio Board of Regents. We also acknowledge contributions to the work described here by earlier collaborators at Kent State University and also collaborators at Stanford University who are named in the subsequent references.

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