

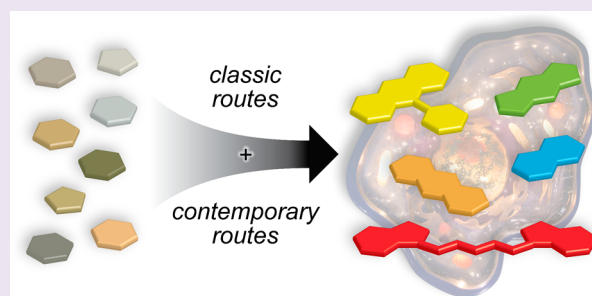
# Bright Building Blocks for Chemical Biology

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**ABSTRACT:** Small-molecule fluorophores manifest the ability of chemistry to solve problems in biology. As we noted in a previous review (Lavis, L. D.; Raines, R. T. *ACS Chem. Biol.* **2008**, *3*, 142–155), the extant collection of fluorescent probes is built on a modest set of “core” scaffolds that evolved during a century of academic and industrial research. Here, we survey traditional and modern synthetic routes to small-molecule fluorophores and highlight recent biological insights attained with customized fluorescent probes. Our intent is to inspire the design and creation of new high-precision tools that empower chemical biologists.



Advances in chemical biology often rely on small-molecule fluorophores with tailored chemical and optical properties.<sup>1–14</sup> Fulfilling the growing need for fluorescent probes requires an understanding of the chemistry that underlies their design and synthesis. Here, we review classic and contemporary synthetic routes to fluorescent dyes and highlight the exemplary use of sophisticated, custom-made probes.

As we noted in a previous review in this journal,<sup>3</sup> the vast collection of small-molecule fluorescent probes<sup>15</sup> is derived from a modest set of “core” scaffolds. These foundational molecules began to emerge during the 19th century. Indeed, the development of synthetic dyes birthed the modern chemical industry, as early synthetic methodology focused on organic colorants and their derivatives.<sup>16–18</sup> As the industry evolved, many companies shifted their efforts from dyes to drugs, leading synthetic chemists to focus on natural products and other potential pharmacological agents. Now, however, the ever-growing utility of small-molecule fluorophores in biology and biomedicine is providing a new impetus to apply synthetic chemistry to dyes. As detailed below, combining classic strategies for the synthesis of fluorophores with recent advances in synthetic methodology can lead to efficient routes to known fluorescent dyes and access to new ones.

## ■ FLUORESCENCE AND CHEMICAL STRUCTURE

The process of fluorescence is detailed in the stylized Jablonski diagram in Figure 1A.<sup>19</sup> Absorption of light by a molecule yields a singlet excited state ( $S_1$ ,  $S_2$ , etc.; Figure 1Ai). The key parameters used to describe this process are the absorption maxima ( $\lambda_{\text{max}}$ ) and the extinction coefficient at  $\lambda_{\text{max}}$  ( $\epsilon$ ). Upon excitation, energy loss occurs due to rapid relaxation to the first singlet excited state ( $S_1$ ) and reorganization of solvent molecules around the altered dipole of the excited state (Figure 1Aii). Fluorescence occurs when this excited state relaxes to the ground state ( $S_0$ ) through emission of a photon (Figure 1Aiii); each fluorophore has a characteristic emission maximum ( $\lambda_{\text{em}}$ ). Alternatively, the excited state can relax through nonradiative

processes due to bond rotation or photoinduced electron transfer (PeT, Figure 1Aiv), or Förster resonance energy transfer (FRET) to an acceptor molecule (Figure 1Av). In addition, intersystem crossing to the triplet state ( $T_1$ ) can occur (Figure 1Avi) with relaxation through radiative (Figure 1Avii) or nonradiative means (Figure 1Aviii). Photon emission from  $T_1$  is termed phosphorescence and usually exhibits significantly longer lifetimes and longer emission wavelengths than do fluorescent processes.

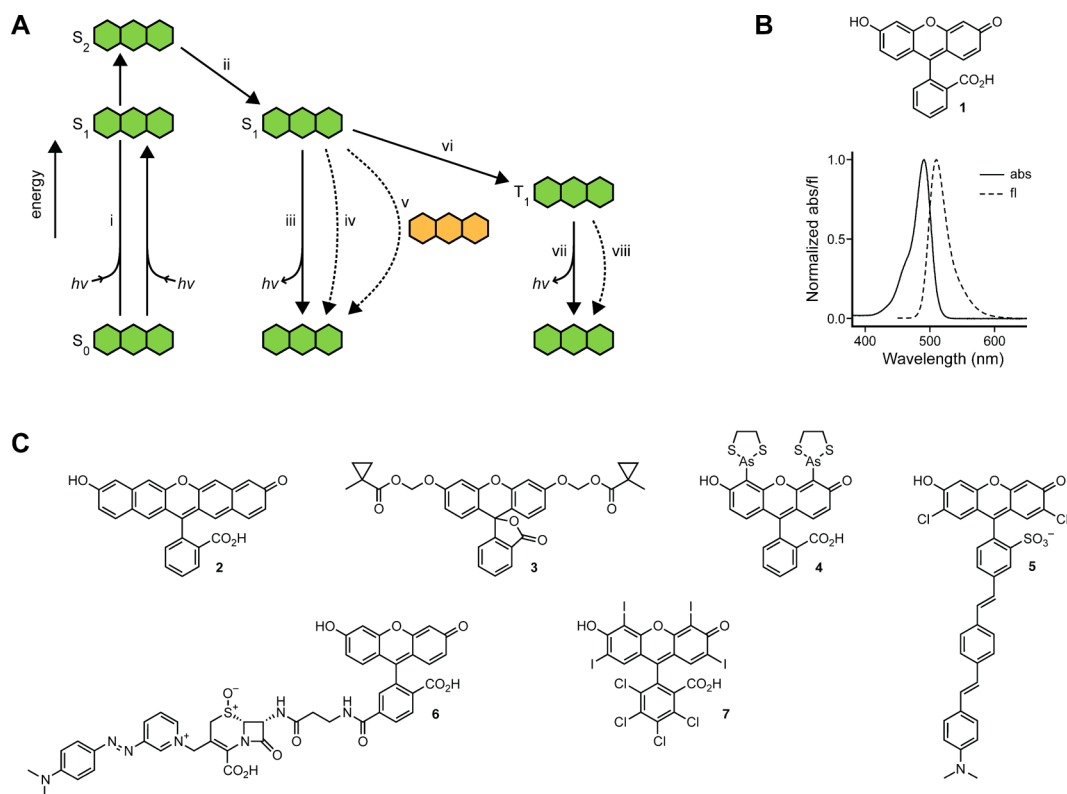
The consistent sequence of events leading to fluorescence emission endows fluorophores with several general properties. The absorbance and fluorescence emission spectra of a prototypical fluorophore, fluorescein (**1**), is shown in Figure 1B.<sup>20</sup> As a dianion, fluorescein absorbs blue light ( $\lambda_{\text{max}} = 491$  nm,  $\epsilon = 9.0 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ) and emits in the blue-green region of the spectrum ( $\lambda_{\text{em}} = 510$  nm). Typically, the energy loss stemming from relaxation to  $S_1$  and solvent reorganization around the excited molecule cause the emission wavelength to be longer than the absorption maximum. The difference in  $\lambda_{\text{max}}$  and  $\lambda_{\text{em}}$  is called the “Stokes shift” in homage to G. G. Stokes who elucidated the process of fluorescence in 1852.<sup>21</sup> Due to its symmetry, the fluorescein dianion undergoes only a modest change in dipole moment upon excitation, resulting in a small Stokes shift of 19 nm.

One notable exception to the  $\lambda_{\text{max}} < \lambda_{\text{em}}$  rule occurs under multiphoton excitation, in which two or more photons are absorbed simultaneously to yield the singlet excited state.<sup>22</sup> Fluorescein shows two-photon excitation peaks at 780 and 920 nm.<sup>23</sup> The fluorescence lifetime ( $\tau$ )—the average time between excitation and emission—is related to the relative rates of fluorescence and competing nonradiative processes, making this value important for fluorescence polarization and fluorescence lifetime measurements. The quantum yield value ( $\Phi$ ) denotes

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**Figure 1.** Photochemical concepts and examples of small-molecule fluorophores (A) Jablonski diagram showing (i) absorption of a photon to give an excited state, (ii) internal conversion to S<sub>1</sub>, (iii) fluorescence, (iv) nonradiative decay, (v) Förster resonance energy transfer (FRET), (vi) intersystem crossing to T<sub>1</sub>, (vii) phosphorescence, and (viii) nonradiative decay. (B) Absorption and emission spectra of the dianionic form of the prototypical fluorophore, fluorescein (1). (C) Fluorescein derivatives (2–7) with biological utility.

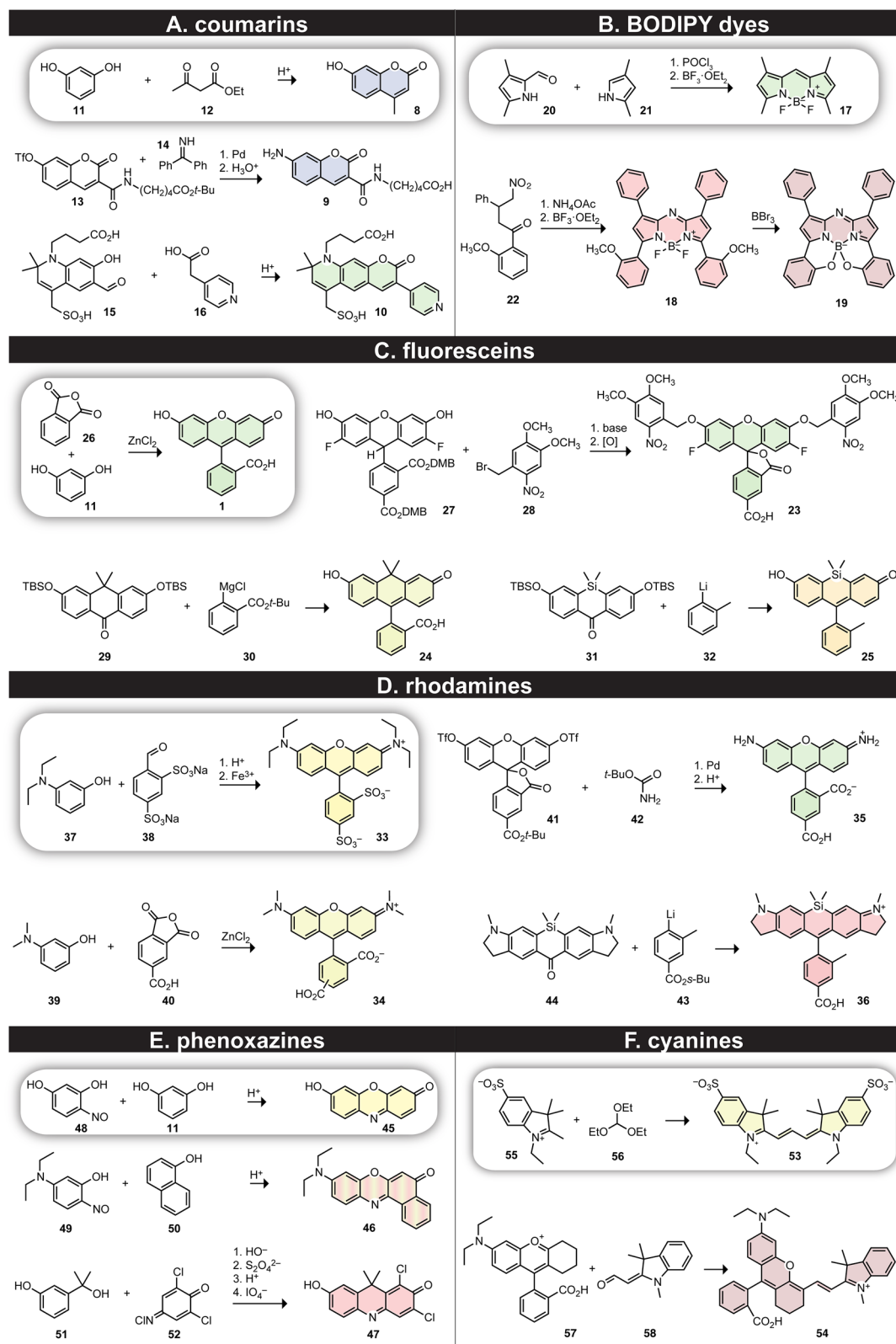
the quotient of photons emitted and photons absorbed. Fluorescein exhibits a lifetime of 4.16 ns (ref 24) and a quantum yield of 0.86,<sup>20</sup> indicative of a fluorescence process that is relatively efficient. A convenient measure of fluorophore brightness is the product of the extinction coefficient and quantum yield ( $\epsilon \times \Phi$ ); this value allows comparison of the photon yield of different classes of fluorophores under the same light intensity.<sup>3</sup> Another important parameter is photostability, which depends on the rate of photobleaching reactions that vary according to fluorophore structure and experimental conditions.

Each step in the fluorescence process can be modulated by synthetic organic chemistry. The fluorescent properties of fluorescein (1) can be tuned by structural modifications, as shown in Figure 1C. Dyes with red-shifted spectra are advantageous for biological imaging, as longer excitation wavelengths penetrate deeper into tissue and avoid eliciting cellular autofluorescence.<sup>4</sup> One method to increase absorption and fluorescence emission maxima is to extend conjugation within the dye. For example, the fusion of benzo rings to form naphthofluorescein 2 elicits a dramatic bathochromic shift with  $\lambda_{\text{max}}/\lambda_{\text{em}} = 708 \text{ nm}/790 \text{ nm}$ ,  $\epsilon = 5.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ , and  $\Phi = 0.0017$ .<sup>25</sup> Structural modifications that reduce the absorbance properties of the dye affect subsequent fluorescence properties. Attachment of blocking groups that are removable by light, enzymatic catalysis, or changes in chemical environment yield highly fluorogenic compounds that are useful for a variety of applications.<sup>12</sup> In compound 3, fluorescein is “masked” by O-alkylation with an acyloxymethyl (AM) ether, which locks the molecule in the nonfluorescent lactone form, thereby suppressing its fluorescence.<sup>26</sup> Fluorescein AM ethers are

chemically stable esterase substrates that can be used to probe esterase specificity with low background.<sup>27</sup> The  $\alpha$ -cyclopropyl esters in substrate 3 are hydrolyzed selectively by a specific exogenous esterase; this selective esterase–ester pair allows targeted delivery of small molecules to genetically defined cells.<sup>28</sup>

Structural changes can also affect nonradiative decay from the excited state. In general, changes in the rigidity of the molecule can change fluorescence by increasing nonradiative processes involving bond rotation. For example, the fluorescein moiety in the biarsenical fluorescein FAsH (4) exhibits low fluorescence in solution, due in part to rotation around the C–As bonds. Upon binding to a tetracysteine-containing helix in an engineered protein, FAsH is rigidified and exhibits a large increase in quantum yield with spectral properties similar to fluorescein ( $\lambda_{\text{max}}/\lambda_{\text{em}} = 508 \text{ nm}/530 \text{ nm}$ ).<sup>29</sup> Electron transfer to and from the excited state can also affect fluorescence. For example, inclusion of electron-rich “donor” substituents can affect fluorescence quantum yield by PeT. Upon excitation, an electron is transferred from the donor moiety to a vacant orbital in the ground state of a fluorophore. The ensuing charge-separated species then relaxes without emission of a photon.<sup>30</sup> This process has been exploited to create a myriad of small-molecule sensors for disparate analytes,<sup>1,31</sup> including a voltage sensor (5) based on a dichlorofluorescein linked to an electron-rich aniline via a phenylethene molecular wire. This compound embeds in cellular membranes and shows voltage-sensitive PeT with a 27% change in fluorescence intensity per 100 mV change in membrane potential.<sup>32</sup>

Close proximity of another dye can affect the quantum yield of a fluorescent molecule. FRET involves the direct transfer of



**Figure 2.** Useful synthetic routes to key small-molecule fluorophores: (A) coumarins, (B) BODIPY dyes, (C) fluoresceins, (D) rhodamines, (E) phenoxazines, and (F) cyanines. The classical route to each fluorophore is boxed. The color of a fluorophore structure indicates its wavelength of maximum emission ( $\lambda_{em}$ ).

energy from the excited state of the donor fluorophore to an acceptor molecule. This process is dependent on the distance between the two dyes and the overlap of the emission spectrum of the donor and the absorbance spectra of the acceptor.<sup>33</sup> A

sophisticated example of a system exploiting FRET is compound 6, which contains a fluorescein donor and azopyridine acceptor linked by a cephalosporin moiety.<sup>34</sup> In solution, the fluorescein moiety exhibits low fluorescence

intensity due to energy transfer to the nonfluorescent azopyridine “quencher”. Upon reaction with a variant of  $\beta$ -lactamase, the azopyridine is cleaved, resulting in a large increase in fluorescence. Importantly,  $\beta$ -lactamases can be engineered so that the fluorescein–cephalosporin conjugate remains linked covalently to the protein, a fluorogenic labeling reaction that is orthogonal to mammalian biochemistry.<sup>34</sup> Finally, the rate of intersystem crossing from the singlet to the triplet can be affected by structural changes. For example, the halogenated fluorescein derivative Rose Bengal (**7**) exhibits a low fluorescence quantum yield ( $\Phi = 0.018$ ) and a high intersystem crossing rate to the triplet state,<sup>35</sup> making Rose Bengal a potent singlet oxygen generator for a variety of applications.<sup>36</sup> Halogenation also causes a significant increase in absorption and fluorescence emission wavelengths ( $\lambda_{\text{max}}/\lambda_{\text{em}} = 548 \text{ nm}/566 \text{ nm}$ ),<sup>35</sup> showing that structural changes other than extending conjugation can alter the spectral properties of a dye.

Dyes **2–7** demonstrate how the different fluorescence properties of a simple fluorophore scaffold, fluorescein (**1**), can be modified to provide useful tools for chemical biology. Of course, the utility of fluorescent probes depends as well on their accessibility. We now survey the six major classes of small-molecule fluorophores—coumarins, boron dipyrromethene (BODIPY) dyes, fluoresceins, rhodamines, oxazines, and cyanines—and discuss proven synthetic strategies to build and modify their structures.

## ■ COUMARINS

The coumarin scaffold is a privileged structure in organic chemistry, occurring in numerous natural products and pharmacological agents.<sup>37</sup> Substitution at the 7-position with electron-donating groups yields highly fluorescent molecules. The prototypical coumarin fluorophore is 4-methyl-7-hydroxycoumarin (i.e., 4-methylumbelliferone, 4-MU, **8**; Figure 2A), which absorbs UV light ( $\lambda_{\text{abs}} = 360 \text{ nm}$ ,  $\epsilon = 1.7 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ ) and emits blue light ( $\lambda_{\text{em}} = 450 \text{ nm}$ ,  $\Phi = 0.63$ ).<sup>38</sup> This and related compounds are the basis for many fluorescent labels, fluorescent sensors, and fluorogenic enzyme substrates.<sup>39</sup> The  $\text{pK}_{\text{a}}$  of 4-MU is 7.8, making the molecule sensitive to changes in pH under physiological conditions. A common modification of the hydroxycoumarin scaffold is halogenation, which lowers the  $\text{pK}_{\text{a}}$  of the phenolic hydroxyl groups and thus limits pH-sensitivity.<sup>38</sup>

The 7-aminocoumarin scaffold is another common manifestation of this fluorophore class. Derivatives of the primary aniline 7-aminocoumarin, such as compound **9** ( $\lambda_{\text{max}}/\lambda_{\text{em}} = 380 \text{ nm}/444 \text{ nm}$ ,  $\epsilon = 1.8 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ ) do not exhibit significant pH sensitivity.<sup>40</sup> The carboxyl-containing linker makes the molecule a substrate for lipoic acid ligase, which can enable the site-specific labeling of proteins with synthetic fluorophores containing a short peptide tag.<sup>40,41</sup> *N*-Acylation of 7-aminocoumarins causes a hypsochromic shift and decrease in quantum yield, making *N*-acyl derivatives useful fluorogenic enzyme substrates.<sup>42</sup> *N*-Alkylation of the amino group elicits bathochromic shifts. For example, coumarin **10** exhibits relatively long wavelengths for this scaffold ( $\lambda_{\text{max}}/\lambda_{\text{em}} = 444 \text{ nm}/514 \text{ nm}$ ) due, in part, to the substitution on the aniline nitrogen. The extended conjugation in the dihydroquinoline moiety and the 3-pyridyl substituent also contribute to the useful spectral properties observed with this dye.<sup>43</sup>

Coumarins are often synthesized by the Pechmann condensation, which involves the acid-catalyzed reaction of a phenol and a  $\beta$ -carbonyl ester. The classic synthesis of **8**,

involving resorcinol (**11**) and ethyl acetoacetate (**12**), is shown in Figure 2A.<sup>44</sup> This condensation reaction gives high yields of 7-hydroxycoumarins and *N*-alkylated 7-aminocoumarins. The synthesis of dyes bearing primary anilines using this route is difficult, however, requiring the protection of the aniline nitrogen to achieve good conversion.<sup>45</sup> A recent advance in the chemistry of coumarin probes is the use of the Pd-catalyzed Buchwald–Hartwig cross-coupling approach to convert readily accessible 7-hydroxycoumarins to 7-aminocoumarins. Coumarin triflate **13** will react with benzophenone imine (**14**) in a palladium-catalyzed cross-coupling reaction, as shown in Figure 2A. Acid-catalyzed hydrolysis of the imine yields the desired 7-aminocoumarin in high yield.<sup>40</sup> Another method to prepare coumarins is a Knoevenagel condensation between a 2-formylphenol and appropriate  $\beta$ -substituted carboxylic acid or ester.<sup>46</sup> This reaction can be performed on highly functionalized molecules such as the reaction of dihydroquinoline **15** and 2-pyridylacetic acid **16** to afford highly soluble coumarin **10**.<sup>43</sup>

The flexible chemistry of coumarins continues to be exploited to prepare useful probes. For example, new fluorescent labels such as compounds **9** and **10** (Figure 2A) are useful in advanced imaging methods.<sup>40,43</sup> Fluorogenic coumarins also find broad use. Recent advances include new self-labeling substrates that release a highly reactive species upon enzymatic catalysis<sup>47</sup> and photoactivatable “caged” coumarins for examining gap junction coupling in animals.<sup>48</sup> Finally, coumarins are also effective photolabile groups themselves; extension of the conjugated structure<sup>49</sup> or use of thiocoumarins<sup>50</sup> affords red-shifted cages for releasing bioactive small molecules.

## ■ BODIPY

The boron dipyrromethene (BODIPY) dyes comprise a highly versatile class of fluorophores.<sup>51,52</sup> Characteristic properties of this scaffold include neutral charge, small Stokes shift, and insensitivity of fluorescence to environmental changes. The prototypical BODIPY dye is the tetramethyl compound **17**, which exhibits  $\lambda_{\text{max}}/\lambda_{\text{em}} = 505 \text{ nm}/516 \text{ nm}$  and  $\Phi = 0.80$ .<sup>53</sup> Derivatives of **17** are more photostable than fluorescein<sup>54</sup> and are especially useful where neutral charge and environmental insensitivity is advantageous (e.g., in lipid membranes).<sup>55</sup> BODIPY dyes are unusual in not containing exocyclic donor or acceptor groups, and thus can be modified at essentially any position to change spectral properties. For example, in fluorophore **18**, the bridging carbon has been replaced with nitrogen, and four aromatic substituents have been added. This highly substituted aza-BODIPY exhibits a dramatic bathochromic shift with  $\lambda_{\text{max}}/\lambda_{\text{em}} = 642 \text{ nm}/688 \text{ nm}$  and  $\Phi = 0.07$ .<sup>56</sup> In addition, changing the boron ligands can affect fluorescence properties. In compound **19**, the fluoro groups have been replaced with integrated phenol substituents, giving a further bathochromic shift ( $\lambda_{\text{max}}/\lambda_{\text{em}} = 728 \text{ nm}/746 \text{ nm}$ ) and a significant increase in quantum yield ( $\Phi = 0.51$ ) due to rigidification of the phenyl substituents.<sup>56</sup>

The original synthesis of BODIPY dye **17** involved a condensation reminiscent of a Vilsmeier–Haack reaction between 2-formyl-3,5-dimethylpyrrole (**20**) and 3,5-dimethylpyrrole (**21**) followed by treatment with  $\text{BF}_3$ .<sup>53</sup> This reaction enables the synthesis of a wide variety of asymmetric BODIPY derivatives and can be used to generate fluorophore libraries.<sup>57</sup> Symmetrical BODIPY dyes can be synthesized by condensation of two equivalents of pyrrole and an aldehyde to give



fluorophores with different functionality at the bridging carbon.<sup>58</sup> Aza-BODIPY dyes are synthesized using a different route with the pyrrole rings being formed *in situ*.<sup>59</sup> Fluorophore **18** can be synthesized from diarylpropanone **22** by reaction with ammonium acetate followed by treatment with  $\text{BF}_3$ , as shown in Figure 2B.<sup>56</sup> Transformation to **19** is achieved by demethylation with  $\text{BBr}_3$ .

The flexibility of the BODIPY scaffold has garnered intense synthetic investigation resulting in the development of numerous fluorescent dyes. Compounds **18** and **19** embody but two examples of how the spectral properties of these dyes can be modified through substitution. BODIPY dyes can be used to prepare bright bioorthogonal fluorogenic probes,<sup>60</sup> alcohol-reactive labels,<sup>61</sup> and efficient donors for FRET and bioluminescence resonance energy transfer (BRET).<sup>62</sup> In addition to creating new fluorescent labels, substitution with heavy halogens yields attractive singlet oxygen generators for photodynamic therapy.<sup>63</sup> PeT is also emerging as an effective way to modulate fluorescence intensity and produce functional fluorescent probes. A noteworthy BODIPY-based calcium indicator exhibits high sensitivity and can be attached to proteins.<sup>58</sup> More recently, photoactivatable BODIPY dyes have been introduced that rely on PeT to suppress fluorescence until activation with UV light.<sup>64</sup>

## ■ FLUORESCINS

First synthesized in 1871,<sup>65</sup> fluorescein (**1**) has become “the” ubiquitous small-molecule fluorophore, underlying countless industrial, medical, and scientific applications. Fluorescein endows automotive antifreeze with its “toxic” green color,<sup>66</sup> is an effective imaging agent for retinal angiography,<sup>67</sup> was used as a sea marker for capsule recovery by the National Aeronautics and Space Administration (NASA) during its Apollo missions,<sup>68</sup> and was the first antibody label for immunofluorescence microscopy.<sup>69</sup> As noted above, fluorescein is most fluorescent as the dianion (phenolic  $\text{pK}_a$  of 6.4, ref 70). As in the case of the hydroxycoumarins, substitution with fluoro or chloro groups causes a decrease in  $\text{pK}_a$ , generating dyes with less pH sensitivity and more photostability.<sup>71</sup>

A key property of fluorescein is an equilibrium between an “open,” fluorescent quinoid form and a “closed,” nonfluorescent lactone. Attaching blocking groups on the phenolic oxygens through ester or ether bonds can modulate this equilibrium; this strategy has been used to prepare fluorogenic substrates for many enzymes (e.g., compound **3**, Figure 1C).<sup>28</sup> In addition, this strategy can generate photolabile (“caged”) fluorophores, such as compound **23** (Figure 2C), which is a fluorinated fluorescein that is caged with 6-nitroveratryl groups.<sup>72</sup> Caged fluoresceins have been critical tools for elucidating the dynamics of cytoskeleton components,<sup>73</sup> and can be used for super-resolution localization microscopy.<sup>72</sup>

Although the fluorescence intensity of fluorescein can be controlled by structural changes,  $\lambda_{\text{max}}$  and  $\lambda_{\text{em}}$  are difficult to extend while preserving high brightness. For example, Rose Bengal (**7**, Figure 1C) does exhibit a red-shifted spectra ( $\lambda_{\text{max}}/\lambda_{\text{em}} = 548 \text{ nm}/566 \text{ nm}$ ) but at the expense of quantum yield ( $\Phi = 0.018$ ).<sup>35</sup> Instead, red-shifted fluorescein derivatives for use as labels have been prepared by introducing fused benzo rings (e.g., **2**, Figure 1C).<sup>25</sup> Naphthofluoresceins and seminaphthofluoresceins find use as cellular stains<sup>74</sup> and platforms for fluorescent indicators.<sup>75</sup>

Modification of the xanthene core of fluorescein has emerged as a useful method to shift the spectral properties of fluorescein.

Replacement of the xanthene oxygen with a *gem*-dimethyl group yields “carbofluorescein” (**24**), which exhibits a 53-nm bathochromic shift ( $\lambda_{\text{max}}/\lambda_{\text{em}} = 544 \text{ nm}/567 \text{ nm}$ ,  $\epsilon = 10.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ,  $\Phi = 0.62$ ).<sup>20</sup> Interestingly, the  $\text{pK}_a$  of carbofluorescein is 7.4, a full unit higher than that of fluorescein, demonstrating that this structural modification affects chemical as well as spectral properties. Likewise, incorporation of a dimethylsilyl group yields “silafluoresceins” such as **25**, which show an even larger red-shift relative to fluorescein ( $\lambda_{\text{max}}/\lambda_{\text{em}} = 582 \text{ nm}/598 \text{ nm}$ ,  $\epsilon = 11.0 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ,  $\Phi = 0.42$ ).<sup>76</sup>

The original synthesis of fluorescein involved the reaction of phthalic anhydride (**26**) with resorcinol (**11**) in a  $\text{ZnCl}_2$  melt at  $180^\circ\text{C}$  (Figure 2C).<sup>65</sup> More commonly, this condensation is performed in neat acid (e.g.,  $\text{CH}_3\text{SO}_3\text{H}$ ) at a more moderate temperature ( $\sim 85^\circ\text{C}$ ).<sup>70,71</sup> Use of phthalic anhydrides containing carboxyl or nitro substituents enables the synthesis of dyes with handles for bioconjugation on the pendant phenyl ring. These syntheses are not regioselective, yielding an isomeric mixture of products. Fortunately, the isomers are typically separable by crystallization, usually using diacetyl derivatives.<sup>69–71</sup> This facile and generalizable purification protocol makes pure isomers of fluorescein derivatives significantly more accessible (and less expensive) than analogous rhodamine derivatives (*vide infra*).

The open–closed equilibrium of fluorescein can be exploited to prepare a variety of fluorogenic compounds, as acylation or alkylation of the phenolic oxygens locks the molecule in the closed form. Removal of these blocking groups recapitulates the open form, causing a large increase in fluorescence. This strategy has been used to create a variety of enzyme substrates (e.g., **3**),<sup>26</sup> photoactivatable dyes (e.g., **23**),<sup>72</sup> and small-molecule sensors.<sup>9</sup> Fluorescein diethers can be deceptively difficult to synthesize, however, due to competing reactivity of the requisite *o*-carboxyl group on the pendant ring. This problem can be overcome by using reduced fluoresceins as synthetic intermediates. As shown in Figure 2C, the dihydrofluorescein **27** is first alkylated with 6-nitroveratryl bromide (**28**) followed by oxidation, which restores the fluorescein core with concomitant removal of the 2,4-dimethoxybenzyl (DMB) protecting groups. This sequence of reactions yields caged fluorescein **23** with a free carboxyl group for bioconjugation.<sup>72</sup> A similar sequence using reduced dye intermediates has been used to prepare fluorescein diether-based substrates for  $\beta$ -lactamase.<sup>77</sup>

The large bathochromic shifts in spectral properties observed for the carbofluorescein **24** and silafluorescein **25** stem from replacement of the xanthene oxygen with a *gem*-dimethyl or dimethylsilyl group. Although this type of modification is modest, it prevents the synthesis of carbo- and sila-fluoresceins using the acid-mediated condensation reaction typically used to prepare fluoresceins. Instead, these structures must be accessed by addition of a metalated aromatic group to an appropriately substituted benzophenone. For the synthesis of carbofluorescein, anthracenone **29** reacts with Grignard reagent **30** to yield fluorophore **24** after deprotection.<sup>20</sup> Silafluorescein **25** is prepared by reaction of silinone **31** with aryllithium species **32**.<sup>76</sup> We note that this strategy allows the synthesis of pure isomers, though the use of organometallics, especially aryllithium reagents, can require robust protection strategies for certain functional groups.

As detailed above (Figure 1C), the fluorescein scaffold continues to be used to build a variety of sophisticated probes for different applications. The recent advent of red-shifted

derivatives of fluorescein, such as **24** and **25**, will provide additional fodder for new fluorescent molecules. Already, these long-wavelength fluorophores have been used as the basis for new enzyme substrates<sup>20,76</sup> and ion indicators.<sup>78</sup> Improvements in the synthetic routes to carbofluoresceins and silarfluoresceins would facilitate the construction of additional red-shifted variants with masked or modulated fluorescent properties.

## ■ RHODAMINES

The amino counterparts of fluorescein, the rhodamines, were first described in the patent literature in the 1880s.<sup>79</sup> Rhodamines have certain advantages over fluoresceins, including lower pH-sensitivity, higher photostability, and tunable spectral properties.<sup>80</sup> The prototypical rhodamine is the tetraalkylated variant sulforhodamine B (i.e., lissamine rhodamine B or Kiton Red S, **33**, Figure 2D) first synthesized in 1887.<sup>79</sup> This fluorophore absorbs in the green and emits in the yellow ( $\lambda_{\text{max}}/\lambda_{\text{em}} = 565 \text{ nm}/586 \text{ nm}$ ,  $\epsilon = 8.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ).<sup>15</sup> Rhodamine **33** is a classic laser dye, and its sulfonyl chloride derivatives are used as amine-reactive fluorescent labels. A more common rhodamine label is tetramethylrhodamine **34**, which bears a carboxyl group on its phenyl ring that enables facile conjugation to biomolecules or attachment of ion-recognition motifs.<sup>81</sup> The change from sulfonates in dye **33** to carboxylates in fluorophore **34** also causes a slight decrease in  $\lambda_{\text{abs}}$  and  $\lambda_{\text{em}}$ ; tetramethylrhodamines such as **34** exhibit  $\lambda_{\text{max}}/\lambda_{\text{em}} = 548 \text{ nm}/572 \text{ nm}$ ,  $\epsilon = 7.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ , and  $\Phi = 0.45$ .<sup>20</sup>

In addition to modifications on the phenyl ring, changing the substituent patterns on the aniline nitrogens of rhodamine can modulate its spectral properties. Rhodamines devoid of *N*-alkyl groups exhibit a significant hypsochromic shift in spectral properties. For example, rhodamine 110 derivatives such as compound **35** show spectral properties that are similar to fluorescein ( $\lambda_{\text{max}}/\lambda_{\text{em}} = 496 \text{ nm}/517 \text{ nm}$ ,  $\epsilon = 7.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ , and  $\Phi = 0.88$ ).<sup>20,82</sup> Due to the high photostability and insensitivity to pH, this fluorophore and its derivatives find wide use as fluorescent labels.<sup>83</sup> Rhodamine 110 also serves as a scaffold for fluorogenic molecules. As in the case of fluoresceins, rhodamines that contain an *o*-carboxyl group on the pendant phenyl ring exist as an equilibrating mixture of the open fluorescent quinoid form and a closed nonfluorescent lactone form. Acylation of the nitrogens locks the molecule into a closed lactone form; removal of these groups causes a large increase in fluorescence. Thus, *N*-acyl rhodamine 110 derivatives are valuable fluorogenic compounds for measuring enzyme activity or serving as activatable labels for advanced fluorescence microscopy experiments.<sup>72,82,84,85</sup>

Also similar to fluoresceins, replacement of the xanthene oxygen with another atom, such as C, Si, Ge, or Te can modify spectral properties.<sup>20,86,87</sup> In particular, the carbon and silicon substitutions have provided useful fluorescent probes for biological imaging. Introduction of a quaternary carbon at this position yields "carborhodamines" that show a 55-nm bathochromic shift relative to isologous xanthene dyes.<sup>20</sup> Likewise, introduction of a silicon atom at this position gives "silarhodamines" that are red-shifted by ~90-nm relative to similarly substituted rhodamines. For example, compound **36** exhibits  $\lambda_{\text{max}}/\lambda_{\text{em}} = 691 \text{ nm}/712 \text{ nm}$ ,  $\epsilon = 1.0 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ , and  $\Phi = 0.12$ . This near-infrared absorbance maximum makes the dye suitable for the preparation of conjugates for *in vivo* imaging.<sup>88</sup>

The first reported synthesis of rhodamine used the acid-catalyzed condensation of aminophenol **37** with benzaldehyde

**38** followed by oxidation with  $\text{FeCl}_3$  to yield sulforhodamine B (**33**), as shown in Figure 2D.<sup>79</sup> A more common synthesis involves the condensation between a phthalic anhydride and 3-aminophenol to give a rhodamine dye that contains an *o*-carboxyl group on the pendant phenyl ring. For example, condensation of 3-(dimethylamino)phenol (**39**) and trimellitic anhydride (**40**) in  $\text{ZnCl}_2$  yields 5/6-carboxy-tetramethylrhodamine as a mixture of isomers.<sup>81</sup> (The numbering convention of xanthene dyes is based on fluoran.) Alternatively, such condensation reactions can be performed in propionic acid with catalytic  $\text{TsOH}$ .<sup>72</sup>

Although widely used, the condensation route has several limitations. First, this method depends on the availability of 3-aminophenol derivatives, limiting the functionality on the nitrogen atoms and the resulting xanthene ring system. Second, use of substituted phthalic anhydrides yields isomeric mixtures of rhodamines that are difficult to separate, requiring tedious chromatography. In rare cases, crystallization can be used to separate rhodamines<sup>89</sup> or their derivatives,<sup>72</sup> but this strategy is not generalizable. Thus, commercial rhodamine dyes are sold as isomeric mixtures; single isomers are available but expensive. Finally, some functional groups are incompatible with strongly acidic conditions, further restricting the reaction scope. For example, the synthesis of 5-carboxy-rhodamine 110 (**35**) using acid-catalyzed condensation is complicated not only by isomer formation but also by hydrolysis of the iminium dyes to form complex mixtures of rhodols and fluoresceins.<sup>90</sup>

Two strategies have emerged to circumvent the problems with classic rhodamine synthesis. Synthesis of rhodamine dyes directly from fluorescein triflates using the Buchwald–Hartwig cross-coupling reaction yields an array of diverse rhodamine dyes. For example, the synthesis of 5-carboxyrhodamine 110 (**35**) is accomplished by coupling 5-carboxyfluorescein bis-(triflate) **41** with *t*-butylcarbamate (**42**) and subsequent deprotection. This strategy leverages the ease of synthesizing isomerically pure fluoresceins as starting materials and can be extended to the synthesis of bright *N*-alkyl fluorophores and strongly absorbing *N*-aryl dyes. The cross-coupling approach also allows direct synthesis of fluorogenic *N*-acyl rhodamines, which are difficult to synthesize due to the insolubility of the free dye in organic solvents and the poor reactivity of the aniline nitrogen atoms on the cationic xanthylium ring.<sup>91</sup> This cross-coupling approach has been applied to transform carbofluoresceins such as **24** to diverse carborhodamines, enabling the preparation of red-shifted fluorescent dyes and fluorogenic compounds.<sup>20</sup> The other strategy to bypass the problems associated with condensation reactions is the addition of organometallic species to a fully *N*-alkylated diaminoxanthone. This reaction has been used to prepare a variety of rhodamine dyes and their isologues.<sup>92,93</sup> For example, silarhodamine **36** is synthesized by addition of lithiated benzoate **43** to diaminosilone **44**, followed by deprotection.<sup>88</sup>

The tunability of the rhodamine structure makes this dye class a useful scaffold for fluorescent probes. Accordingly, rhodamines remain the most useful basis for fluorescent labels, and the new carbo- and silarhodamine dyes enable sophisticated imaging experiments in cells and whole animals.<sup>20,87,88,93</sup> Synthesis of combinatorial libraries of rhodamine dyes and subsequent screening experiments have revealed cell type-specific fluorescent stains.<sup>94</sup> Incorporation of fluoro groups into rhodamines might improve photostability<sup>95</sup> and allows fluorous-phase separation of rhodamine conjugates.<sup>96</sup> Access to photoactivatable "caged" rhodamines<sup>97</sup>

and carborhodamines for super-resolution microscopy has been enhanced by the use of reduced rhodamines<sup>72</sup> or Pd-catalyzed cross-coupling.<sup>20,91</sup>

As with fluorescein, the open–closed equilibrium of rhodamine can be altered by structural perturbations. In addition to *N*-acylation, modification of the *ortho* substituent on the pendent phenyl ring also affects this equilibrium. For example, amidation of the *o*-carboxyl group causes the molecule to adopt the nonfluorescent lactam form, allowing the synthesis of photoactivatable molecules<sup>98</sup> and numerous reaction-based sensors.<sup>9,10</sup> Interestingly, the carbon- and silicon-containing rhodamine analogs have equilibria that are shifted toward the closed lactone form. This property increases the on/off contrast of fluorogenic derivatives<sup>20</sup> and endows these dyes and their conjugates with improved cell permeability.<sup>93</sup>

## ■ PHENOXAZINES AND ACRIDINONES

The phenoxazine dyes have a rich history as histochemical stains, laser dyes, and fluorescent probes.<sup>99</sup> Key attributes of this class of dyes are red-shifted spectra and sensitivity to the polarity of the medium. These dyes are structural analogs of the xanthene-based fluoresceins and rhodamines, being built from similar phenol and aniline building blocks. The oxazine nitrogen in the ring structure, however, elicits a large bathochromic shift. The simplest phenoxazine dye, resorufin (**45**, Figure 2E), shows  $\lambda_{\text{max}}/\lambda_{\text{em}} = 572 \text{ nm}/585 \text{ nm}$ ,  $\epsilon = 5.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ , and  $\Phi = 0.74$  as the anion. As with fluorescein, the fluorescence of resorufin is pH-sensitive with a  $\text{pK}_a$  of 5.8.<sup>100</sup> Alkylation or acylation of the phenol causes a severe reduction in quantum yield and a hypsochromic shift in  $\lambda_{\text{max}}$ . Thus, resorufin serves as a scaffold for a variety of enzyme substrates<sup>101</sup> and as a photoactivatable label.<sup>102</sup> In addition, the phenoxazine system undergoes facile redox chemistry; both reduced and oxidized forms are nonfluorescent. Reduced resorufin derivatives are useful substrates for peroxidases,<sup>15,103</sup> and resorufin *N*-oxide (i.e., resazurin) is a useful cell-viability stain that is reduced to fluorescent **45** inside living cells.<sup>104</sup>

Similar to case of the fluoresceins and rhodamines, further shifts in spectral properties can be achieved by incorporating dialkylamino substituents or fused benzo rings, or by modifying the heterocyclic oxygen. The asymmetric Nile Red (**46**) exhibits  $\lambda_{\text{max}}/\lambda_{\text{em}} = 591 \text{ nm}/657 \text{ nm}$  in aqueous solution that undergoes a dramatic hypsochromic shift in nonpolar solvents such as xylene, showing  $\lambda_{\text{max}}/\lambda_{\text{em}} = 523 \text{ nm}/565 \text{ nm}$  and a  $10^2$ -fold increase in fluorescence. This shift allows for the staining of lipid droplets in live cells<sup>105</sup> and super-resolution imaging of cellular membranes.<sup>106</sup> Another modification of resorufin is the replacement of the phenoxazine oxygen with a *gem*-dimethyl group. A key example of this strategy is 7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO, **47**), which displays  $\lambda_{\text{max}}/\lambda_{\text{em}} = 646 \text{ nm}/659 \text{ nm}$  and  $\epsilon = 5.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ , and a  $\text{pK}_a$  of 5.0.<sup>15,107,108</sup> As in the case of resorufin, alkylation of phenolic oxygen elicits a large change in fluorescence. DDAO has been used to prepare caged fluorophores<sup>108</sup> and substrates for  $\beta$ -galactosidase<sup>107</sup> and aryl sulfatases. Notably, the DDAO sulfatase substrate enables facile profiling of enzymatic activity in different strains of *Mycobacterium tuberculosis*.<sup>109</sup>

The standard syntheses of phenoxazine dyes involve acid-mediated condensation reactions of phenols and nitrosylated aromatic compounds. Resorufin (**45**) can be prepared by the reaction of 4-nitrosoresorcinol (**48**) and resorcinol (**11**) in  $\text{H}_2\text{SO}_4$  to yield the desired dye, as shown in Figure 2E.<sup>110,111</sup>

Similarly, reaction of nitrosylated 3-aminophenol **49** and 1-naphthol (**50**) yields Nile Red (**46**).<sup>99</sup> The synthesis of DDAO (**47**) uses a different strategy. Tertiary alcohol **51** and commercially available chloroamine **52** are first reacted under basic conditions to form the  $\text{C}_{\text{aryl}}\text{--N}$  bond, reduced to give a diphenyl amine, condensed with acid to form the acridine ring system, and then reoxidized to yield phenoxazine **47**.<sup>107</sup> This multistep synthetic protocol is indicative of the need for innovation with phenoxazine and related dyes, as new synthetic methodology could expand the utility of this interesting scaffold.

## ■ CYANINES

The cyanine dyes encompass a large and diverse class of molecules with the general structure  $\text{R}_2\text{N}-(\text{CH}=\text{CH})_n-\text{CH}=\text{N}^+\text{R}_2$ . General properties of the cyanine dyes include large extinction coefficient values and highly tunable structures. Prior to 1993, the utility of cyanines in biological research had been primarily as a stain for cellular membranes<sup>112</sup> or DNA.<sup>113</sup> The development of indocarbocyanine dyes bearing sulfonate groups for solubility and functional groups for bioconjugation (i.e., the “CyDyes”) brought this dye class into the realm of fluorescent labels for proteins and nucleic acids.<sup>114,115</sup> Cy3 (**53**, Figure 2F) exhibits  $\lambda_{\text{max}}/\lambda_{\text{em}} = 554 \text{ nm}/568 \text{ nm}$ ,  $\epsilon = 1.3 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ , and  $\Phi = 0.14$ ; these spectral properties are comparable to tetramethylrhodamine **34**. (The CyDye nomenclature is based on the number of carbons between the indoline moieties.) An increase or decrease in the length of the polymethine chain gives fluorophores with longer or shorter  $\lambda_{\text{max}}$  and  $\lambda_{\text{em}}$  values, respectively.<sup>116</sup>

Akin to the BODIPY scaffold, many cyanine dyes do not have exocyclic donor and acceptor groups. Thus, many strategies for modulating rhodamines and fluoresceins cannot be applied to this dye class. In one strategy to overcome this problem, cyanine–rhodamine fusions, such as compound **54**, have been shown to absorb at long wavelengths ( $\lambda_{\text{max}}/\lambda_{\text{em}} = 720 \text{ nm}/750 \text{ nm}$ ,  $\epsilon = 12.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ) and show good fluorescence properties ( $\lambda_{\text{em}} = 750 \text{ nm}$ ,  $\Phi = 0.30$ ). Importantly, this class of molecules can be modulated using strategies similar to those applied to fluorogenic rhodamines.<sup>117</sup> Analogous coumarin–cyanine<sup>118</sup> and fluorescein–cyanine<sup>119</sup> hybrid structures are also known.

The synthesis of cyanine dyes generally follows the addition of an aldehyde (or equivalent) to an activated carbon species such as a 2-alkylindolinium salt.<sup>120</sup> The synthesis of Cy3 (**53**) follows this general scheme and involves the condensation of indolinium salt **55** with triethyl orthoformate (**56**) to yield the symmetrical **53**,<sup>114</sup> as shown in Figure 2F. We note that this reaction has been used for nearly a century<sup>121</sup> and can now be performed using solid-phase synthesis techniques.<sup>122</sup> Similarly, the rhodamine–cyanine hybrid **54** is formed by condensation of chromenylum compound **57** with aldehyde **58**.

Cyanine dyes are used most often as biomolecular labels and as fluorogenic stains that show significant increase in fluorescence upon binding to a target protein or nucleic acid.<sup>15</sup> A hallmark of cyanine dyes is their excellent photostability. This resistance to photobleaching can be enhanced further by minor<sup>123</sup> or major<sup>124</sup> structural modifications. Other alterations can make cyanine fluorescence responsive to enzymatic activity or environmental changes. For example, substrates for *Escherichia coli* nitroreductase<sup>125</sup> and pH sensors<sup>126</sup> can be produced by exploiting particular modifications of the cyanine nitrogen atoms. Alterations in the



polymethine chain allow the construction of fluorescent indicators.<sup>127–129</sup> In addition, fluorogenic compounds can be based on FRET between two different cyanine dyes (e.g., Cy3 and Cy5).<sup>130</sup> Finally, cyanine dyes exhibit interesting blinking phenomena under reducing conditions,<sup>131,132</sup> making them useful for super-resolution microscopy experiments.<sup>133</sup>

## CONCLUSIONS AND FUTURE DIRECTIONS

The extant panoply of small-molecule fluorophores derives from an amalgamation of ancient and modern synthetic methodology. For some dye classes, such as the fluoresceins, the classic acid-mediated synthetic route remains advantageous due to readily available starting materials and facile purification protocols. For others, such as coumarins and rhodamines, modern organic reactions (e.g., transition metal-catalyzed cross-couplings) are advantageous for the expeditious synthesis of otherwise challenging compounds. New chemical methodology can also allow the synthesis of isologous structures, such as the carbo- and sila-rhodamines, which expand the accessible chemical and spectral properties available to established dye scaffolds. Sophisticated organic chemistry can also enable the functionalization of fluorescent dyes with chemical moieties that improve the function of these polycyclic aromatic groups in biological contexts, such as polyethylene glycol<sup>134</sup> or sulfonates.<sup>135</sup> Combinatorial synthetic routes extend the horizon still further.<sup>11</sup> Going forward, we expect innovations in organic chemistry to enable the synthesis of new fluorophore derivatives with enhanced properties.

In addition to improvements in synthetic organic chemistry, advances in molecular genetics expand the utility of small-molecule fluorophores in biological systems. The discovery and development of fluorescent proteins has revolutionized biological imaging, as genetically encoded fluorophores now supplement small-molecule fluorophores and indicators.<sup>136</sup> Genetic control also enables the expression of proteins<sup>13,137,138</sup> and nucleic acids<sup>139</sup> that can be labeled with specific small molecules, including dyes. These intersecting strategies will enable interrogations in the most complex of biological environments, such as intact tissue or whole animals, and will continue to shed light on the chemical underpinnings of biological processes.

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### Notes

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## KEYWORDS

**BODIPY:** a small-molecule fluorophore based on boron dipyrromethene and having versatile spectral properties tunable by substitution

**brightness:** the product of extinction coefficient and quantum yield ( $\epsilon \times \Phi$ ), which is a useful parameter for comparing different fluorophores

**coumarin:** a small-molecule fluorophore based on 2H-chromen-2-one that is used as a scaffold for UV-absorbing fluorogenic dyes

**cyanine:** a small-molecule fluorophore with the general structure:  $R_2N-(CH=CH)_n-CH=N^+R_2$  that is a useful scaffold for voltage indicators, nucleic acid stains, and biomolecule labels

**fluorescein:** a small-molecule fluorophore containing a 3,6-dihydroxyxanthene motif that was first synthesized by Adolf von Baeyer in 1871 and is in widespread use

**fluorescence:** a photophysical process involving (1) photon absorption by a fluorophore giving an excited state, and (2) relaxation of the excited state by emission of another photon

**fluorophore:** a fluorescent moiety that can arise from disparate chemical structures, including small molecules, proteins, metal chelates, and nanoparticles

**oxazine:** a red-shifted small-molecule fluorophore with a phenoxazine core that is typically substituted with hydroxyl or amino groups at the 3 and 7 positions

**rhodamine:** a small-molecule fluorophore based on 3,6-diaminoxanthene that is similar to fluorescein but has pH-independent fluorescence

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