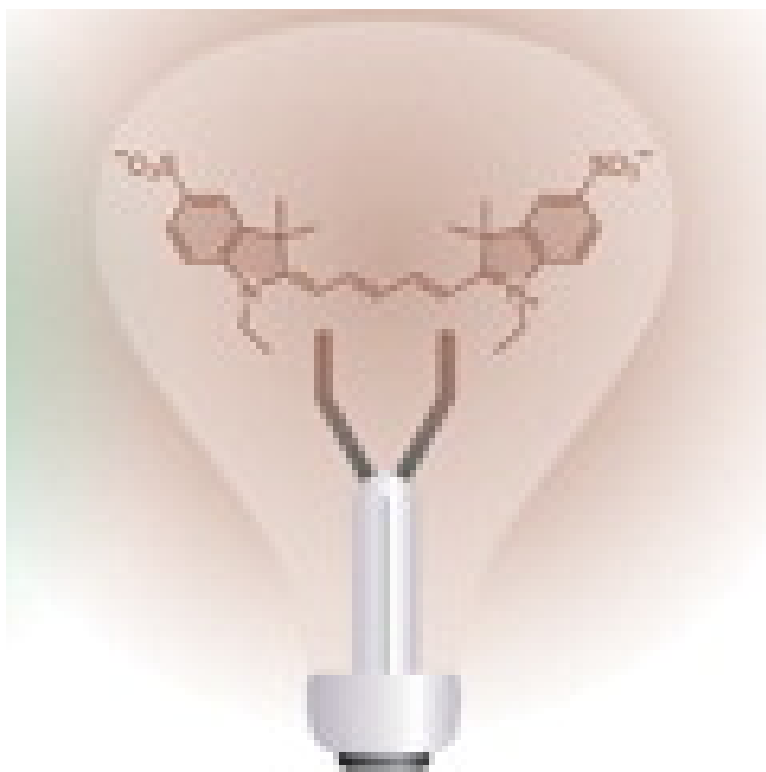


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Bright Ideas for Chemical Biology

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ABSTRACT Small-molecule fluorescent probes embody an essential facet of chemical biology. Although numerous compounds are known, the ensemble of fluorescent probes is based on a modest collection of modular “core” dyes. The elaboration of these dyes with diverse chemical moieties is enabling the precise interrogation of biochemical and biological systems. The importance of fluorescence-based technologies in chemical biology elicits a necessity to understand the major classes of small-molecule fluorophores. Here, we examine the chemical and photophysical properties of oft-used fluorophores and highlight classic and contemporary examples in which utility has been built upon these scaffolds.

Small fluorescent molecules are indispensable tools for chemical biology, being ubiquitous as biomolecular labels, enzyme substrates, environmental indicators, and cellular stains (1–12). Choosing a suitable fluorophore to visualize a biochemical or biological process can be daunting, given the countless molecules available either commercially (13) or through *de novo* design and synthesis. Fortunately, the plethora of fluorescent probes has an intrinsic modularity. Attachment of various reactive groups, substrate moieties, chelating components, and other chemical entities to a small number of “core” fluorophores gives rise to the ensemble of extant probes. Overall, these core fluorophores are well-established (9, 14), consisting of molecules with excellent spectral characteristics, high chemical stabilities, and facile syntheses. Probe selection and design can, therefore, be simplified by understanding the properties of these foundational fluorescent compounds.

In this Review, we trek along the electromagnetic spectrum and discuss the properties of the main classes of fluorescent molecules used in bioresearch. We also give examples of tools constructed from these fluorophores. We believe that comprehension of the strengths, weaknesses, and common uses of each dye class will equip the chemical biologist for expeditions to reveal new biochemical and biological phenomena.

A Brief History. The first well-defined small-molecule fluorophore was the natural product quinine (1), an important compound for both medicinal and organic chemistry (15). The visible emission from an aqueous quinine solution was reported by Herschel in 1845 (16). Stokes showed that this phenomenon was due to the absorption and then emission of light by quinine and coined the term “fluorescence” to describe this process (17). The importance of quinine as an antimalarial would later lead to an attempted synthesis by Perkin, starting from aniline derivatives. Of course, the total syn-

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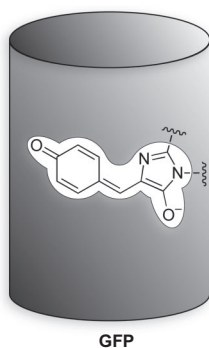
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thesis of quinine would tarry for many decades, realized in essence by Woodward and Doering and in practice by Stork (18). Instead, Perkin's fated synthetic route produced the first synthetic textile dye, mauvine, in 1865. Perkin's success in the commercialization of mauvine and other "aniline dyes" is often considered to be the birth of the modern chemical industry (15, 19). This achievement foreshadowed the discovery of many useful dye molecules, fluorescent and otherwise (20). These colored synthetic molecules were fodder for new biological experiments, and many found diagnostic or even clinical utility (21).

The intrinsic fluorescence of quinine also motivated the development of the fluorometer, which was needed to evaluate antimalarial drug cocktails during World War II (10). The commercialization of such instrumentation in the 1950s allowed increased use of fluorescence-based bioanalytical techniques (22). In the 1960s, the advent of the dye laser spurred much interest in the synthesis of novel or improved fluorescent molecules with desirable photophysical properties (23). Indeed, some structural permutations developed to enhance laser dyes persist in modern fluorescent bioprobes.

More recently, additional classes of fluorophores have joined the foray, including inorganic "quantum dots" and fluorescent proteins (9, 14). Although beyond the scope of this Review, GFP and its variants deserve special mention. These genetically encoded fluorophores are, in essence, small-molecule imidazolinone dyes embedded within a protein having a β -barrel tertiary structure (24, 25).



The dye is produced in an autocatalytic manner from native amino acid residues, its full maturation requiring molecular oxygen and producing an equivalent of hydrogen peroxide (26), a reactive oxygen species. The pro-

tein casing is essential, because the naked imidazolinone dye exhibits only meager fluorescence (27).

Mutagenesis has produced an assortment of fluorescent proteins with disparate chemical and spectral properties (28, 29) that enable, for example, impressive *in vivo* imaging experiments (30). Going forward, we expect small-molecule, inorganic, and proteinaceous fluorophores—each with particular benefits and drawbacks—to continue to facilitate both basic and applied research in chemical biology.

Fluorescence. The process of fluorescence is illustrated in the Jablonski diagram shown in Figure 1, panel a (10). Although this Review is focused on single-photon excitation processes, multiphoton excitation is also an important and vibrant field (31). The fluorescence process begins when a molecule in a singlet electronic ground state (S_0) absorbs a photon of suitable energy. This promotes an electron to higher energy orbitals, which relax quickly to the first singlet excited state (S_1). The decay of the excited state can occur with photon emission (*i.e.*, fluorescence) or in a nonradiative (NR) fashion. This NR "quenching" of the fluorophore excited state can occur through one of a variety of processes, including bond rotation or vibration, molecular collision (32), and photoinduced electron transfer (PeT) (33). The excited state can also undergo forbidden intersystem crossing (ITC) to the triplet excited state (T_1) and subsequent relaxation by either photon emission (*i.e.*, phosphorescence) or NR decay. ITC efficiency is increased by substitution with, or proximity to, atoms with high atomic number due to spin-orbit coupling—a phenomenon commonly termed the "heavy atom effect" (34). Another important pathway for decay of the singlet excited state involves FRET to an acceptor molecule. This process is distance-dependent and can be used as a "spectroscopic ruler" to measure the proximity of labeled entities (35).

A generic absorption/emission spectrum is shown in Figure 1, panel b. The maximal absorption (λ_{max}) is related to the energy between the S_0 and the higher energy levels. The absorptivity of a molecule at λ_{max} is given by the extinction coefficient (ϵ), defined by the Beer-Lambert-Bouguer law. The maximal emission wavelength (λ_{em}) is longer (*i.e.*, lower in energy) than λ_{max} because of energy losses by solvent reorganization or other processes (6). Stokes demonstrated this phenomenon by using a rudimentary filter set consisting of a stained glass window and a goblet of wine (17).

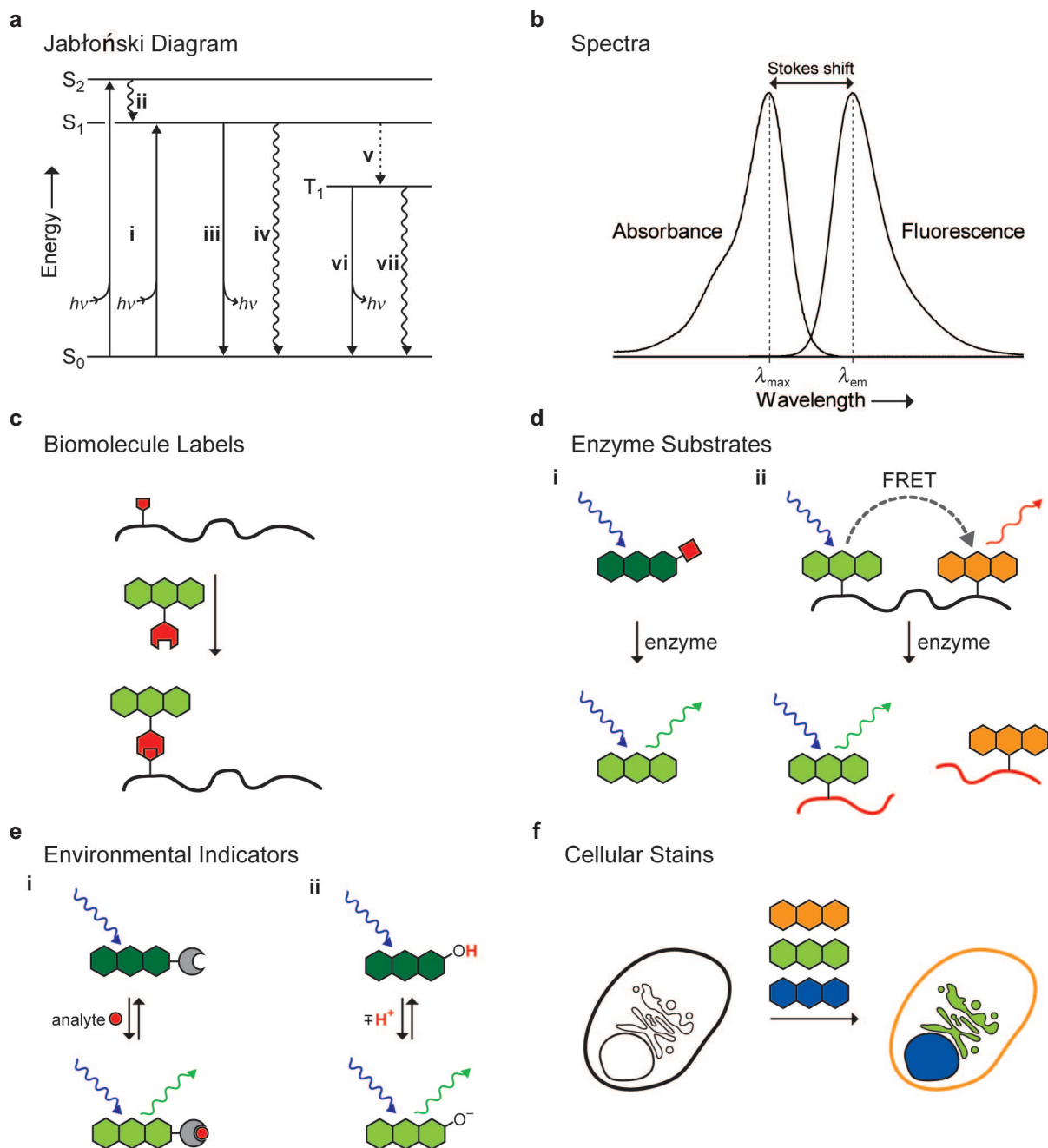


Figure 1. Photophysical concepts (a, b) and biological applications (c–f) of small-molecule fluorophores: a) Jablonski diagram (i) absorption of a photon gives an excited state, (ii) internal conversion to S_1 , (iii) fluorescence, (iv) nonradiative decay, (v) intersystem crossing to T_1 , (vi) phosphorescence, (vii) nonradiative decay; b) generic absorption and emission spectra; c) site-specific labeling of a biomolecule by an orthogonal reaction between two functional groups (red); d) enzyme substrates (i) enzyme-catalyzed removal of a blocking group (red) elicits a change in fluorescence, (ii) enzyme catalyzes the cleavage of a labeled biomolecule (red) and concomitant decrease in FRET e) environmental indicators (i) binding of an analyte (red) elicits a change in fluorescence, (ii) protonation of a fluorophore elicits a change in fluorescence; f) staining of subcellular domains by distinct fluorophores.

The difference between λ_{max} and λ_{em} is therefore termed the “Stokes shift”. Fluorophores with small Stokes shifts are susceptible to self-quenching *via* energy transfer, therefore limiting the number of labels that can be attached to a biomolecule (36). The lifetime of the excited state (τ) can range from 0.1 to >100 ns and is an important parameter for time-resolved measurements (37) and fluorescence polarization applications (38). Another critical property of a fluorophore is the quantum yield or quantum efficiency (Φ)—essentially the ratio of photons fluoresced to those absorbed.

Fluorophores are utilized in many ways, including as labels for biomolecules (Figure 1, panel c), enzyme substrates (Figure 1, panel d), environmental indicators (Figure 1, panel e), and cellular stains (Figure 1, panel f). The utility of a particular fluorophore is dictated by its specific chemical properties (*e.g.*, reactivity, lipophilicity, pK_a , stability) and photophysical properties (*e.g.*, λ_{max} , λ_{em} , ϵ , Φ , τ). A simple parameter for making meaningful comparisons between different fluorescent molecules is the product of the extinction coefficient and the quantum yield ($\epsilon \times \Phi$). This term is directly proportional to the brightness of the dye, accounting for both the amount of light absorbed and the quantum efficiency of the fluorophore. Accurate comparisons between dye molecules must include both of these parameters. A plot of $\epsilon \times \Phi$ versus λ_{max} for the major classes of biologically significant fluorescent dyes is shown in Figure 2. A list of the properties of these fluorophores can be found in Supplementary Table S1.

Classes of Fluorescent Dyes. *Endogenous Fluorophores.* Like quinine (1), many naturally occurring compounds exhibit measurable fluorescence (39). These include the aromatic amino acids, whose fluorescence properties were first described by Weber (40). Phenylalanine (2) and tyrosine (3) exhibit weak fluorescence under UV excitation wavelengths. Tryptophan (4) is the most fluorescent natural amino acid, with a λ_{max} of 280 nm, λ_{em} of 348 nm, extinction coefficient of $6.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, and a quantum yield of 0.13 (39). Tryptophan fluorescence is environmentally sensitive and has been used as an index for a variety of processes, including protein folding and ligand binding (41). Tryptophan can also be used in FRET applications (35) or serve as a quencher for a variety of fluorophores by PeT (42).

Other naturally occurring fluorophores include reduced nicotinamide cofactors (*e.g.*, NADH; 5) that show

measurable fluorescence with a $\lambda_{\text{max}}/\lambda_{\text{em}}$ of 340/435 nm (43). Flavins are also important intrinsic fluorophores, with flavin mononucleotide (FMN; 6) showing significant fluorescence with $\lambda_{\text{max}} = 450 \text{ nm}$, $\lambda_{\text{em}} = 530 \text{ nm}$, $\epsilon = 1.22 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, and $\Phi = 0.25$ (39, 44). Other native moieties are fluorescent, including porphyrins and pyridoxal derivatives (39). Collectively, endogenous fluorophores can give rise to “autofluorescence”, which can obfuscate desired signals from labeled entities in imaging and other *in cellulo* or *in vivo* experiments (45). Red-shifted dyes can circumvent this background problem, while allowing deeper tissue penetration (46). Long-wavelength excitation is also gentle to DNA, because nucleosides absorb at $\lambda_{\text{max}} \approx 260 \text{ nm}$ with $\epsilon \approx (7-15) \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (47).

Polycyclic Aromatics. Polycyclic aromatic compounds are a widely used subset of fluorescent dyes. In general, spectral properties correlate to size, and substitution on the abundant open valencies affords a variety of useful probes. A classic category of synthetic biomolecule labels is naphthalene derivatives. These include the amine-reactive 5-(dimethylamino)naphthalene-1-sulfonyl (dansyl) chloride (48) and other associated fluorophores (49, 50). Another related naphthalene derivative is 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS). Derivatives of this fluorophore, such as compound 7, exhibit a λ_{max} of 336 nm, λ_{em} of 520 nm, extinction coefficient of $6.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, and a quantum yield of 0.27 in water (51). EDANS remains in wide use, particularly in FRET-based experiments (52, 53). Naphthalene can be further elaborated to give 4-amino-3,6-disulfonylnaphthalimides (*e.g.*, compound 8) that absorb at 428 nm (54). These fluorophores bear the moniker “Lucifer yellow” and are useful polar tracers (13).

Pyrene-derived molecules also find use as probes. Derivatives of pyrene (9) show $\lambda_{\text{max}}/\lambda_{\text{em}}$ of 340/376 nm, $\epsilon = 4.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, and $\Phi = 0.75$ (13, 55). The environmental sensitivity of this fluorophore can be used to report on RNA folding (56). Pyrene also exhibits a long-lived excited state ($\tau > 100 \text{ ns}$). This long lifetime allows an excited

KEYWORDS

Fluorescence: A 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 consist of disparate chemical structures, including small molecules, proteins, and semiconductor beads

Extinction coefficient (ϵ): The absorptivity of a molecule at a given wavelength as defined by the Beer–Lambert–Bouguer law

Quantum yield (Φ): The ratio of photons fluoresced to photons absorbed

Stokes shift: The difference (in nm) between the absorption or excitation maximum (λ_{max}) and the emission maximum (λ_{em})

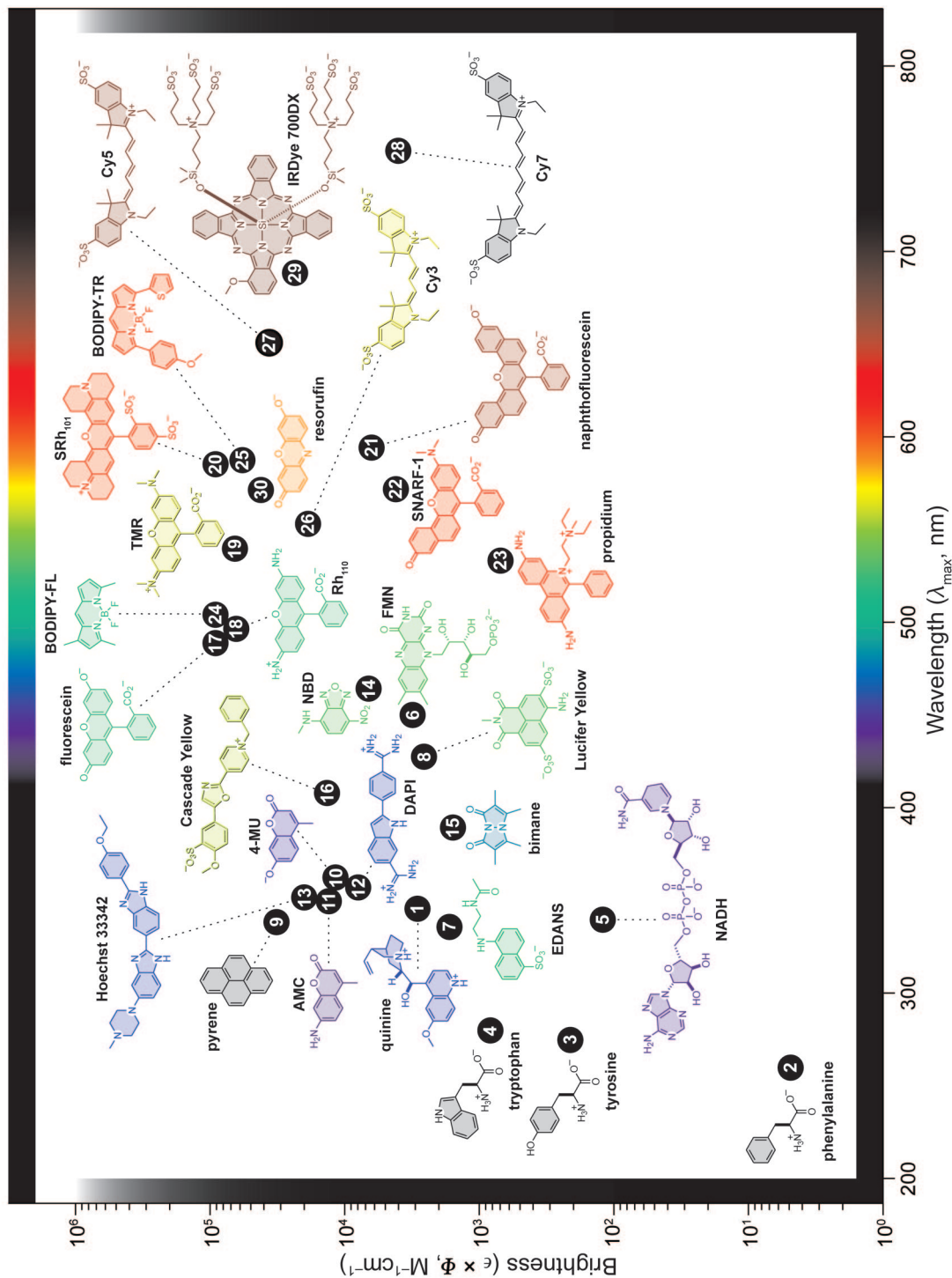


Figure 2. Plot of fluorophore brightness ($\epsilon \times \Phi$) vs the wavelength of maximum absorption (λ_{max}) for the major classes of fluorophores. The color of the structure indicates its wavelength of maximum emission (λ_{em}). For clarity, only the fluorophoric moiety of some molecules is shown.

pyrene molecule to associate with a pyrene in the ground state. The resulting excimer exhibits a bathochromic (*i.e.*, red) shift in fluorescence intensity ($\lambda_{em} \approx 490$ nm). This process can be used to measure important biomolecular processes, such as protein conformation (57). Sulfonation of pyrene elicits a bathochromic shift, affording useful compounds that are excited at >390 nm. These compounds include the pH probe 8-hydroxy-1,3,6-pyrenetrisulfonate (HPTS or pyranine) (58) and valuable sulfonated pyrene labels with high water solubility (13, 59).

Other polycyclic aromatic molecules are also sometimes used to construct useful fluorescent tools. Anthracene has been elaborated to prepare sensors for anions such as pyrophosphate (60). Perylene derivatives constitute another intriguing class of fluorophores that exhibit very high quantum yields in organic solvents (61) but require significant structural elaboration to become useful in water (62). Still another functional scaffold is coronene, which exhibits a long lifetime ($\tau \approx 200$ ns) that is useful in some time-resolved experiments (63).

Coumarins. Coumarins represent a broad class of natural products, pharmaceuticals, and fluorophores. Heteroatom substitution at position 7 of coumarin gives fluorescent molecules with UV or near-UV excitation wavelengths. A common example is 7-hydroxy-4-methylcoumarin (*i.e.*, 4-methylumbelliferone; 4-MU; **10**). Under basic conditions, the phenolate form of 4-MU ($pK_a = 7.8$) exhibits $\lambda_{max} = 360$ nm, $\lambda_{em} = 450$ nm, $\epsilon = 1.7 \times 10^3$ M⁻¹ cm⁻¹, and $\Phi = 0.63$ (64). The related 7-amino-4-methylcoumarin (AMC; **11**) displays similar spectral properties, which are constant above pH 5 (13). The large Stokes shift of coumarins is due in part to the significant change in dipole upon excitation and subsequent loss in energy by solvent reorganization (6).

Molecular probes built on the coumarin scaffold include useful biomolecular labels. Different reactive groups are compatible with this fluorophore and are typically attached at the 3 or 4 position of coumarin (13). The spectral characteristics of AMC can be tuned through different nitrogen substitution patterns (65). Still other substitutions (*e.g.*, fluorination or sulfonation) can yield coumarin dyes with desirable chemical properties, such as higher solubility in aqueous solution and lower sensitivity to pH (64, 66).

Coumarins are also useful for assembling enzyme substrates. Various derivatives of 7-hydroxycoumarin

can be used to assay an assortment of hydrolases (67, 68) and dealkylases (69). Peptidyl derivatives of AMC are widely used to measure protease activity (70). Microarrays of coumarin substrates have been built to examine protease specificities (71). AMC has also been elaborated to prepare substrates for other enzymes including deacetylases (72) and esterases (73).

Quinolines. The archetypal fluorophore quinine (**1**) is still employed as a fluorescence standard (74, 75). The 6-methoxyquinoline moiety can be alkylated, and the resulting quinolinium species is quenched collisionally by halide ions in solution. Several quinolinium compounds find use as indicators for chloride ion (76). The chelating properties of hydroxyquinoline derivatives have been exploited to create useful fluorescence-based kinase substrates (77) and fluorescent ion indicators (78).

Indoles and Imidizoles. The indole fluorophore has been elaborated beyond tryptophan to construct useful tools such as the calcium indicator Indo-1 (79). Another notable indole-based probe is 4',6-diamidino-2-phenylindole (DAPI; **12**), which binds in the minor groove of DNA (80). Because this binding is accompanied by a large increase in fluorescence, this molecule can be used to stain DNA for cellular imaging or other experiments (81).

The dibenzimidazole dyes originally developed by Hoechst AG are useful DNA-binding probes. Like DAPI, the Hoechst dyes bind in the minor groove of DNA and can be used for fluorescence microscopy and flow cytometry (1). Hoechst 33342 (**13**) is sufficiently cell-permeable for use in live cells (81). Unlike DAPI, the Hoechst dyes are quenched upon binding to DNA containing 5-bromo-2-deoxyuridine because of the heavy atom effect, thereby allowing cell-cycle analyses (82).

NBD. Another notable example of a small heterocyclic fluorophore is 4-nitrobenz-2-oxa-1,3-diazole (NBD) and other related benzoxadiazole compounds. Examples include the amine- or thiol-reactive NBD-Cl (83) and the thiol-reactive 7-chlorobenz-2-oxa-1,3-diazole-4-sulfonate (SBD-Cl) (84). Primary amine adducts of NBD-Cl (*e.g.*, com-

KEYWORDS

Quenching: Nonradiative relaxation of the fluorophore excited state due to molecular collision, energy transfer, intersystem crossing, or other processes

Förster resonance energy transfer (FRET): Distance-dependent, nonradiative energy transfer from the excited state of donor fluorophore to an acceptor dye

Fluorescent ion indicator: A fluorophore exhibiting fluorescence that is sensitive to ion concentration

Fluorogenic enzyme substrate: A compound in which enzymatic catalysis elicits a fluorescence increase

pound **14**) exhibit photophysical properties that belie the size of the molecule. Such derivatives emit in the green portion of the spectrum, with a $\lambda_{\text{max}} = 465$ nm, $\lambda_{\text{em}} = 535$ nm, $\epsilon = 2.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, and $\Phi = 0.3$ in MeOH (13). This lightweight fluorophore allows conjugates with small molecules, such as sugars, to retain biological activity (85). The environmentally sensitive fluorescence of NBD derivatives (86) can be exploited in a variety of ways, including the preparation of lipid probes (87) and novel kinase substrates (88).

Other UV-Excited Fluorophores. There are numerous examples of other small heterocyclic molecules as useful fluorescent probes. These include the 1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione (*i.e.*, bi-mane) structure (**15**) that exhibits moderate fluorescence with a $\lambda_{\text{max}} = 390$ nm, $\lambda_{\text{em}} = 482$ nm, and $\Phi = 0.3$ in aqueous solution (89). Halogenated versions of these fluorophores are useful thiol-reactive labels and can be used as fluorescent cross-linkers (90). Additional significant core dyes involve diaryloxazole structures, which can exhibit large Stokes shifts (91). This structure can be elaborated to yield useful organelle stains (92) and fluorescent labels (93). An example is the Cascade Yellow fluorophore (**16**), which shows $\lambda_{\text{max}} = 409$ nm, $\lambda_{\text{em}} = 558$ nm, $\epsilon = 2.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, and $\Phi = 0.56$ (13, 93).

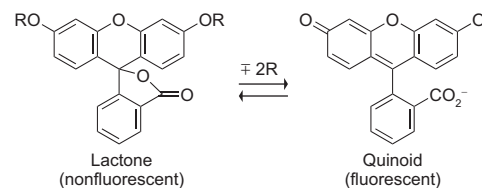
Fluorescein. The well-known xanthene dye fluorescein (**17**) was first synthesized by Baeyer in 1871 (94). Despite its antiquity, fluorescein remains one of the most widely utilized fluorophores in modern biochemical, biological, and medicinal research. Fluorescein exhibits several interesting (and underappreciated) properties in aqueous solution. For example, fluorescein can exist in seven prototropic forms, with the most biologically relevant molecular forms being the monoanion and the dianion that interchange with a $\text{p}K_{\text{a}} \approx 6.4$ (95). The dianion is the most fluorescent form with a λ_{max} of 490 nm, λ_{em} of 514 nm, extinction coefficient of $9.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, and a quantum yield of 0.95 (10, 13).

Fluorescein is an extremely versatile core dye. Fluorescein can be appended with reactive groups to yield important biomolecule labels (96). The structure of fluorescein can be modified further to tune properties such as $\text{p}K_{\text{a}}$ or wavelength. For example, 2',7'-difluorofluorescein (*i.e.*, Oregon green) is less basic ($\text{p}K_{\text{a}} = 4.6$) than fluorescein, maintains fluorescein-like wavelengths, and exhibits increased photostability relative to fluorescein (97). The addition of other substituents,

such as chloro groups, not only affects pH sensitivity (98) but also elicits a bathochromic shift in excitation wavelength. Examples include the traditional automated DNA sequencing dye 2',4,7,7'-tetrachlorofluorescein (TET), which exhibits a $\lambda_{\text{max}}/\lambda_{\text{em}}$ of 521/536 nm (13). Fluoresceins containing bromine or iodine substituents have red-shifted spectra and also exhibit significant intersystem crossing because of the heavy atom effect (99).

Fluorescein also serves as a scaffold for preparing indicator molecules. In particular, the pH sensitivity of fluorescein has been exploited to prepare small-molecule pH sensors (100). Changes in the $\text{p}K_{\text{a}}$ of fluorescein can be used as an index to report on the status of fluorescein-labeled biomolecules (95). Appending fluorescein with various chelating moieties affords sensors for biologically important ions. A most noteworthy example is the calcium indicator Fluo-3 developed by Tsien and coworkers (101), which can be used to measure calcium ion fluxes in live cells and is employed widely in high-throughput screening (102). Other notable examples of fluorescein-based indicators include compounds for detecting sodium (103), zinc (104), palladium (105), mercury(II) (106), and fluoride (107) ions, as well as clever nitric oxide sensors based on chelates with copper(II) (108).

Fluorescein exists in equilibrium between a “closed” lactone and an “open” quinoid form. Acylation or alkylation of the phenolic groups locks the molecule into the nonfluorescent lactone in an aqueous environment and serves as the basis for a variety of fluorogenic substrates for esterases, phosphatases, glycosylases, and other enzymes (109–112).



Fluorescein can also be “caged” with photolabile groups and unmasked by distinct wavelengths of light (113). Other substitutions can confer redox sensitivity to the fluorescein molecule (114). Appending fluorescein derivatives with electron-donating substituents on the pendant phenyl ring allows the construction of enzyme

substrates with only one substrate moiety. These Tokyo Green substrates show improved enzyme kinetics relative to disubstituted fluorescein substrates (115) and can be used for *in vivo* imaging (116).

Rhodamine. Isologues of fluorescein, the rhodamines are used widely as fluorophores. Some key characteristics of this dye class include low pH sensitivity and tunable spectral properties. Different *N*-alkyl substitution patterns on the rhodamine core can modify spectral characteristics. The simplest member of this class, rhodamine 110 (Rh₁₁₀; **18**), exhibits fluorescein-like spectral properties with $\lambda_{\text{max}} = 496 \text{ nm}$, $\lambda_{\text{em}} = 517 \text{ nm}$, $\epsilon = 7.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, and $\Phi = 0.92$ in aqueous solution (117). Substitution to tetramethylrhodamine (TMR; **19**) gives longer excitation and emission wavelengths ($\lambda_{\text{max}}/\lambda_{\text{em}}$ of 540/565 nm) but a lower quantum yield ($\Phi = 0.68$) (3). This lower quantum yield is likely due to decay of the excited state *via* rotation around the C–N bond (23). This undesirable decay process can be circumvented by freezing the C–N bond *via* appropriate substitution. Rhodamines containing rigid julolidine ring systems show higher quantum yields than do the unrestricted dyes (118) and exhibit longer excitation and emission wavelengths (13). Sulforhodamine 101 (SRh₁₀₁; **20**) is a julolidine-based dye that is common in bio research. Amine-reactive sulfonyl chloride derivatives of SRh₁₀₁ are sold under the trademark Texas red (13).

Rhodamine labels are often paired with fluorescein derivatives for FRET-based experiments because of efficient energy transfer between these xanthene compounds (13). Dye constructs containing both fluorescein and rhodamine moieties have proven useful for DNA sequencing. The fluorescein donor of these BigDye fluorophores can be excited by a single-wavelength light source, and the emission is dictated by the specific rhodamine derivative that serves as the FRET acceptor (119).

Rhodamines can also be used to assemble enzyme substrates. Acyl substitution of both the amino groups of a rhodamine locks the molecule into a nonfluorescent lactone form. As with fluorescein, this property can be exploited to prepare caged compounds (113) or fluorogenic molecules for enzymatic studies. Substrates based on Rh₁₁₀ for simple proteases were first described by Mangel in 1983 (120). More recent developments have centered on using Rh₁₁₀ to build useful caspase substrates to assay apoptosis (121). Rh₁₁₀-

based substrates have also been developed for phosphatases (122), esterases (117), and metal-ion catalysis in a cellular context (123).

Rhodamines have been used to build indicators for ions such as sodium (124) and calcium (*e.g.*, Rhod-2) (101). Other rhodamine derivatives have been assembled to detect reactive oxygen species in cells (125). Hybrid structures between fluorescein and rhodamine (*i.e.*, xanthene dyes with one oxygen and one nitrogen substituent) are termed “rhodols” and exhibit interesting spectral properties (126). The unique properties of these rhodol fluorophores can be harnessed to build probes such as ion indicators (127).

Naphthoxanthene Dyes. A notable modification to the fluorescein and rhodamine dyes is the introduction of a fused benzo ring into the xanthene structure. This modification elicits a severe bathochromic shift in excitation and emission wavelengths. A classic example is naphthofluorescein (**21**), which exhibits much longer wavelengths than does fluorescein ($\lambda_{\text{max}}/\lambda_{\text{em}}$ of 595/660 nm) under basic conditions (128). Unfortunately, the advantageous bathochromic shift is countered by an undesirable $\text{pK}_{\text{a}} = 8.0$ —well above the physiological pH—and a lower extinction coefficient ($\epsilon = 4.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) and quantum yield ($\Phi = 0.14$) (128). The poor fluorescence properties of naphthofluorescein limit the utility of this scaffold, though some useful derivatives have been reported (128–130).

Xanthene dyes that bear only one fused benzo ring display interesting spectral properties. Unlike the symmetrical fluoresceins and rhodamines, the resonance forms of these seminaphtho dyes are not equivalent and therefore exhibit dissimilar spectral properties. Thus, the asymmetry of the dye can be yoked to construct ratiometric fluorescent indicators. Probes from the seminaphthofluorescein (SNAFL) core include pH sensors (131) and other ion indicators (132). Rhodol-type seminaphthoxanthenes are also useful pH indicators (131, 133). One example is ratiometric pH sensor **22**, which bears the common name seminaphthorhodafuor-1 (SNARF-1). This compound displays a $\lambda_{\text{max}} = 573 \text{ nm}$, $\lambda_{\text{em}} = 631 \text{ nm}$, $\epsilon = 4.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, and $\Phi = 0.092$ at high pH values (131). Derivatives of dye **22** boast useful pK_{a} values ~ 7.5 , which can be tuned to lower values by fluorine substitution (133).

Phenanthridines. Phenanthridines derivatives are widely used DNA intercalators that exhibit higher fluorescence intensity upon binding to nucleic acids. Ex-

amples include the cationic dyes ethidium and propidium (**23**). In the presence of DNA, propidium presents $\lambda_{\text{max}} = 535 \text{ nm}$, $\lambda_{\text{em}} = 617 \text{ nm}$, $\epsilon = 5.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, and $\Phi = 0.13$ (13, 134). These values constitute a 20–30-fold increase in fluorescence relative to the free dye. The fixed ionic character of compound **23** limits passive diffusion through the intact membrane of living cells. Thus, propidium can be used to identify dead cells with compromised membranes (135).

BODIPY. The boron difluoride dipyrromethene (BODIPY) dye structure has been used to build a variety of useful fluorescent labels and other probes (136). Key features of this dye class are the insensitivity of the spectral properties to environment, the small Stokes shift, and the overall lipophilicity of the dye (13, 137). The core structure of BODIPY is somewhat base-sensitive, limiting its use in applications such as solid-phase peptide synthesis (138). The simplest BODIPY **24** shows fluorescein-like parameters with $\lambda_{\text{max}} = 505 \text{ nm}$, $\lambda_{\text{em}} = 511 \text{ nm}$, $\epsilon = 9.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, and $\Phi = 0.94$ and is commonly called BODIPY-FL (3, 13). Another important property of this class of dyes is the tunability of wavelength through appropriate substitution. BODIPY dyes can thus serve as surrogates for traditional dyes such as fluorescein, tetramethylrhodamine, and many others. One example is the BODIPY-TR fluorophore (**25**), which exhibits spectral properties similar to those of Texas red (*i.e.*, SRh₁₀₁; **20** (3, 13)).

The ensemble of probes built on the BODIPY scaffold is centered largely on fluorescent labels, but some indicators for ions and other molecules have been reported (103, 139). These fluorophores are particularly useful labels for fluorescence polarization techniques (140). The nonpolar character of BODIPY allows incorporation into lipophilic probes (137). Moreover, the small Stokes shift of BODIPY dyes causes efficient self-quenching of overlabeled biomolecules. This phenomenon can be utilized to create useful protease substrates, because proteolysis of densely labeled proteins leads to an increase in fluorescence intensity (141).

Cyanines. The term “cyanine dye” denotes a dye system with a polymethine chain between two nitrogens (*i.e.*, $\text{R}_2\text{N}-(\text{CH}=\text{CH})_n-\text{CH}=\text{N}^+\text{R}_2$). This dye system, which resembles the retinaldimine visual pigment of rhodopsin (142), has been the subject of many seminal studies on the molecular basis of color (143). Numerous cyanines and associated polymethine struc-

tures are useful as labels (144), DNA stains (134), and membrane potential sensors (145–147). Perhaps the most well-known cyanine dyes in modern bio research are the CyDye fluorophores, which are based on a sulfoindocyanine structure (148). These compounds are given common names according to the number of carbon atoms between the dihydroindole units. Cy3 (**26**) shows spectral characteristics that are comparable to TMR with $\lambda_{\text{max}} = 554 \text{ nm}$, $\lambda_{\text{em}} = 568 \text{ nm}$, $\epsilon = 1.3 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$, and $\Phi = 0.14$ in water. Cy5 (**27**) exhibits longer wavelengths with $\lambda_{\text{max}} = 652 \text{ nm}$, $\lambda_{\text{em}} = 672 \text{ nm}$, $\epsilon = 2.0 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$, and $\Phi = 0.18$. Longer cyanine constructs, such as Cy7 (**28**), exhibit a $\lambda_{\text{max}}/\lambda_{\text{em}}$ of 755/788 nm, albeit with a lower quantum yield ($\Phi = 0.02$) (2). Further elaboration of the cyanine core can provide control over wavelength. For example, introduction of a fused benzo ring in the dihydroindole moieties elicits a bathochromic shift of ~20–30 nm (149). This structural modification is designated with a “.5” suffix (*e.g.*, Cy5.5).

The CyDyes are useful biomolecular labels and are now the standard fluorophores for microarrays and many other analyses (14). CyDye pairs are also often used for FRET experiments (150) and can be utilized as photoswitchable probes for ultra-high-resolution imaging (151). A significant drawback to cyanine labels is the severe dependence of the fluorescence of their bioconjugates on the number of fluorophores per biomolecule. This phenomenon likely has several causes and can limit the utility of CyDye conjugates in some applications (152). Newer (albeit structurally mysterious) sulfonated cyanine dyes reportedly overcome this problem (153).

Phthalocyanines. The phthalocyanine structure serves as a scaffold for a variety of interesting compounds, from pigments to photosensitizers. Wavelength absorption and other properties can be tuned by structural modification or through substitution of metal centers (20). To prevent dye aggregation and facilitate water solubility, inclusion of numerous ionic substituents is necessary (154). A successful example of a phthalocyanine fluorescent label is IRDye 700DX (**29**), which shows $\lambda_{\text{max}} = 689 \text{ nm}$, $\lambda_{\text{em}} = 700 \text{ nm}$, $\epsilon = 1.7 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$, $\Phi = 0.14$, and excellent photostability (155).

Oxazines. Substituted oxazine compounds are useful fluorophores. Of particular importance is resorufin (**30**), whose anion exhibits $\lambda_{\text{max}} = 572 \text{ nm}$, $\lambda_{\text{em}} = 585$

nm, $\epsilon = 5.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, and quantum yield = 0.74. These attributes have some sensitivity to pH, because resorufin has a pK_a of 5.8 (156). Use of the resorufin scaffold to prepare fluorescent labels has been limited (157), though this dye has been used to construct fluorogenic molecules that are unmasked by various hydrolases (158–160) and cytochrome P450 enzymes (161).

Resorufin exhibits interesting redox properties. Oxidation to the *N*-oxide yields resazurin, which is only weakly fluorescent. Resazurin can be reduced to resorufin by biological reducing equivalents and, thus, has been used to assay cell viability (162). In addition, reduced versions of resorufin are nonfluorescent but can be oxidized to resorufin by hydrogen peroxide in the presence of horseradish peroxidase. These compounds are useful for the ELISA and other assays (163).

Other important oxazine dyes include cresyl violet, which can be elaborated to give substrates for proteases (164) and esterases (73). A key property of several oxazine fluorophores is their environmental sensi-

tivity. These compounds can be used to prepare useful compounds, such as labels to report on protein conformation (165).

Conclusions. Known small-molecule fluorophores have a wide range of spectral and chemical properties. Elaboration of these core structures has provided numerous probes for assaying biological systems. Nonetheless, extraordinary opportunities remain, because delving deeper into biochemical and biological phenomena will require ever more sophisticated and tailored probes. Scientists who straddle the fields of chemistry and biology are best equipped to fashion these tools and then wield them to illuminate otherwise inscrutable life processes.

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