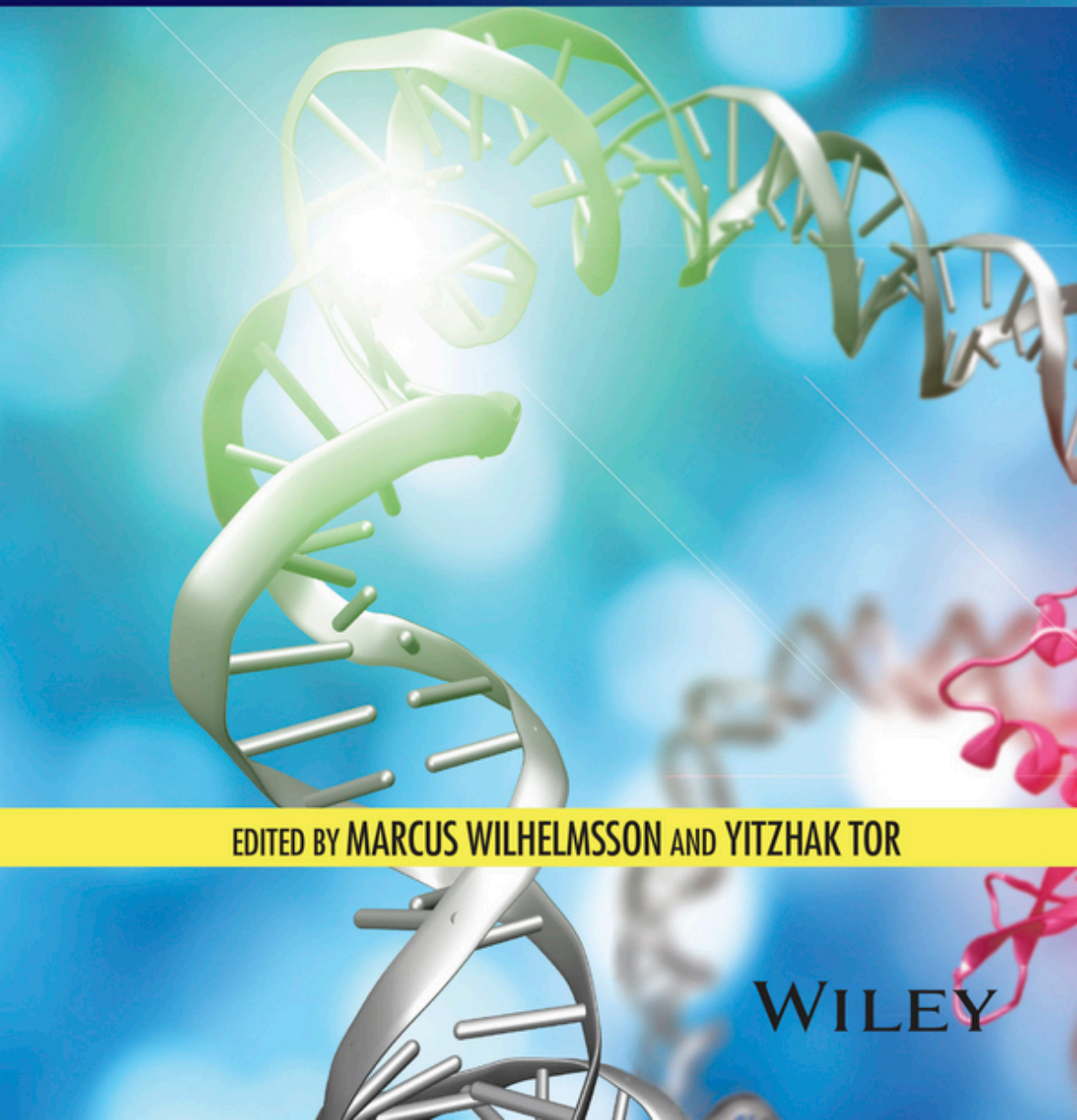
FLUORESCENT ANALOGS OF BIOMOLECULAR BUILDING BLOCKS

DESIGN AND APPLICATIONS



EDITED BY MARCUS WILHELMSSON AND YITZHAK TOR

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Design and Applications

Edited by

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PREFACE

Fluorescence spectroscopy, an established and highly sensitive analytical technique, has been extensively used by the scientific community for many years. For decades, however, the majority of users have relied on a limited number of established fluorophores, either naturally occurring or of synthetic origin. This has dramatically changed in recent years.

Major technological advances in fluorescence-based instrumentation and techniques, including single-molecule spectroscopy, have triggered a renewed interest in the synthesis and development of new fluorescent probes and labels. Two major paths have been taken that are fundamentally related to the above-mentioned two (i.e., of biosynthetic or synthetic origin) but differ in their accommodation of the challenges presented by modern techniques and contemporary scientific questions. A particularly intriguing and emerging area of research, which is highlighted in this book, is the fabrication of minimally perturbing fluorescent analogs of otherwise nonemissive biological building blocks, including amino acids, lipids, and nucleosides.

To share with the reader the renaissance in this field of fluorescent biomolecules and their building blocks, we open with a general and concise tutorial of fluorescence spectroscopy. As readers would appreciate, it is practically impossible to capture all the nuances associated with the development of new fluorescent probes in such a book. To partially correct for this "deficiency," the second chapter provides a condensed overview of naturally occurring and synthetic fluorescent biomolecular building blocks, addressing the core issues and key advances in this field. Selected topics are then elaborated on in individual chapters.

While most laboratories utilize steady state and perhaps basic time-resolved techniques, a great deal of information can be obtained from more sophisticated experiments. Albinsson and Nordén discuss the theory and applications

of polarized light spectroscopy-based techniques and their application for the study of biomolecules. Such experiments can be done in bulk solution as well as in microscopy and single-molecule modalities to provide information about the separation and orientation of chromophores.

Before moving on to discuss new synthetic chromophores in later chapters, we first cover fluorescent proteins as they have become the cornerstone of modern biophysics. Two main approaches are typically considered. One relies on the genetic expression of the classical green fluorescent protein and its variants, where the chromophore is generated from the spontaneous condensation of naturally occurring amino acids as discussed by Jung. A distinct approach, presented by Durkin and Budisa, relies on the incorporation of intrinsically fluorescent noncanonical amino acids by *in vitro* translation techniques, which exploit an expanded genetic code. Both techniques are extremely powerful and provide experimentalists with an enhanced toolbox of emissive proteins, but rely on rather sophisticated biochemical techniques for protein expression. A simplified approach is discussed by Armitage, where genetically encoded antibody fragments and fluorogenic dyes assemble noncovalently to form bright fluorescent complexes.

One element, distinguishing protein biochemists from the community interested in nucleic acids is that, unlike aromatic amino acids that are emissive, the canonical DNA and RNA nucleosides are all practically nonemissive. This has triggered rather extensive efforts aimed at the synthesis and implementation of fluorescent nucleoside analogs. Several approaches are covered here. Saito and Bag discuss diverse families of solvatochromic nucleosides produced by either covalently linking known chromophores to the native nucleosides or by conjugating additional aromatic rings to the native nucleobases. Chicas and Hudson specifically discuss fluorescent cytidine analogs, with emphasis on pyrrolo-C and its derivatives, both in the context of oligonucleotides and in PNAs. Sekine and coworkers elaborate on another family of pyrimidine analogs built around the pyrimidopyrimidoindole motif. While diverse applications have previously been reported, the authors focus here on the implementation of this responsive family of emissive C analogs within triple-stranded motifs. In contrast to the responsive families of fluorescent C analogs mentioned above, Wilhelmsson describes a family of minimally responsive chromophores, which makes them ideal for FRET studies. Well-matched FRET pairs, unique among nucleoside analogs, can then be used to accurately assess nucleobase–nucleobase distance and orientation, generating high-resolution 3-D structural information.

Although the birth of fluorescent nucleoside analogs as a field is frequently attributed to Stryer's 1969 disclosure of 2-aminopurine, an archetypical and extensively employed emissive nucleoside, the number of newly developed and useful purine analogs is substantially smaller compared to their pyrimidine counterparts. This is partially due to synthetic considerations but also likely reflects that modifying the purine core, unlike that of the pyrimidines, frequently hampers their WC and Hoogsteen pairing abilities as well their accommodation within higher structures. In this context, Luedtke describes useful 8-modified purine analogs, which are exploited for the study of G-quadruplexes without detrimental structural effects. Sinkeldam and Tor then discuss the design and implementation of minimally

perturbing yet responsive fluorescent nucleoside analogs, frequently referred to as isomorphic surrogates. Structural and functional elements imparting sensitivity to environmental factors (such as polarity, viscosity, and pH) are introduced into the nucleosidic skeleton with the smallest possible size and functional perturbation.

While all analogs described were designed to form WC pairs and be paired with their native complementary nucleobases, Hirao and coworkers discuss unnatural base pair systems, where both partners selectively recognize one another and discriminate against the canonical nucleobases. While some of the analogs made are in fact emissive, such selective pairing practically expands the genetic code and facilitates the incorporation of other bright fluorescent labels with high efficiency and selectivity. Deviating even further from the canonical structure of the native nucleosides, Crisalli and Kool replace the native heterocyclic nucleobases with aromatic fluorophores, while maintaining the phosphate–sugar backbone. Due to their chromophore–chromophore interactions, such DNA-like oligomers, coined fluorosides, display unique photophysical features and provide a fertile motif for the combinatorial discovery of new sensors and labels.

Similarly to the biomolecular building blocks of proteins and nucleic acids, the majority of membrane components are nonemissive. Designing emissive analogs to study these unique assemblies imposes certain structural and functional issues. Chattopadhyay and colleagues review several popular membrane probes and highlight their potential for extracting information on the environment, organization, and dynamics of membranes. Cebecauer and Šachl then take a rather comprehensive look at diverse fluorescent probes that have been developed to assess lipid phases and their separation, membrane viscosity, and curvature as well as pH and potential. They conclude by discussing future directions and cell biology questions that may be addressed in future using lipophilic fluorescent probes.

We conclude this book with a rather unique chapter discussing small fluorophores that don't serve as components of higher molecular weight biomolecules or assemblies. Wilson discusses the design and utility of fluorescent neurotransmitter analogs as tools for exploring neurotransmission and its regulation. Such analogs can be used to investigate receptors, enzymes, and transporters that interact with native neurotransmitters.

As most readers appreciate, contemporary fluorescence spectroscopy, with all its experimental variations, touches numerous and very diverse fields. Yet, with all the technological advances, in its most fundamental level, this amazing spectroscopy relies on the availability of suitably designed fluorescent probes. The creative and elegant approaches presented here highlight how judiciously designed and implemented fluorescence probes could significantly promote advances in biophysics, biochemistry, and structural biology. What is perhaps less obvious is that the design and implementation of such probes remains an empirical exercise. Our ability to predict the intricate photophysical features of designer probes and their response to diverse environmental effects is still rather primitive and, for the most part, qualitative. It is likely (and it is certainly our hope) that computational approaches developed in coming years will refine the experimentalists' approach, which frequently relies on trial and error. Nevertheless, as evidenced by two Nobel prizes awarded in recent years

(R. Y. Tsien, M. Chalfie, and O. Shimomura in 2008 and W. E. Moerner, S. W. Hell, and E. Betzig in 2014), fluorescence spectroscopy continues to pave the road forward in critical scientific disciplines. We hope that this book inspires the next generation of young scientists to dive into this fascinating field and spend their creative years ensuring that the future of this field remains bright and colorful!

Assembling such a collection of quality chapters, as any editor knows, takes far longer than originally expected and planned. It requires the ultimate cooperation of authors, reviewers, and publishers. We thank them all. We feel the end product is clearly worth the effort and wait.

Marcus Wilhelmsson, Chalmers University of Technology, Gothenburg, Sweden
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1

FLUORESCENCE SPECTROSCOPY

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1.1 FUNDAMENTALS OF FLUORESCENCE SPECTROSCOPY

Fluorescence spectroscopy is unique in its combination of sensitivity with experimental versatility. While all optical spectroscopy techniques benefit from the very short timescale of the photon absorption and emission sequence (Fig. 1.1), an additional and major advantage of fluorescence spectroscopy is the energy difference in the wavelength of excitation and emission. Unlike UV–vis or infrared spectroscopy, where the minimal loss of incident light intensity due to sample absorption is measured, fluorescence spectroscopy yields an energetically distinct signal, frequently remote from, and therefore free of interference by the excitation wavelength (Fig. 1.1).

In short, light of an appropriate energy, the excitation wavelength, elevates a chromophore to the Franck–Condon state, normally a higher vibrational level of S_1, S_2 or higher (S_n) within 10^{-15} s. This extremely fast process is followed by internal conversion (ic) and vibrational relaxation (vr) within 10^{-12}–10^{-10} s to the lowest vibronic and potentially emissive S_1 state. Due to these processes, there is an energy difference

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Edited by Marcus Wilhelmsson and Yitzhak Tor.

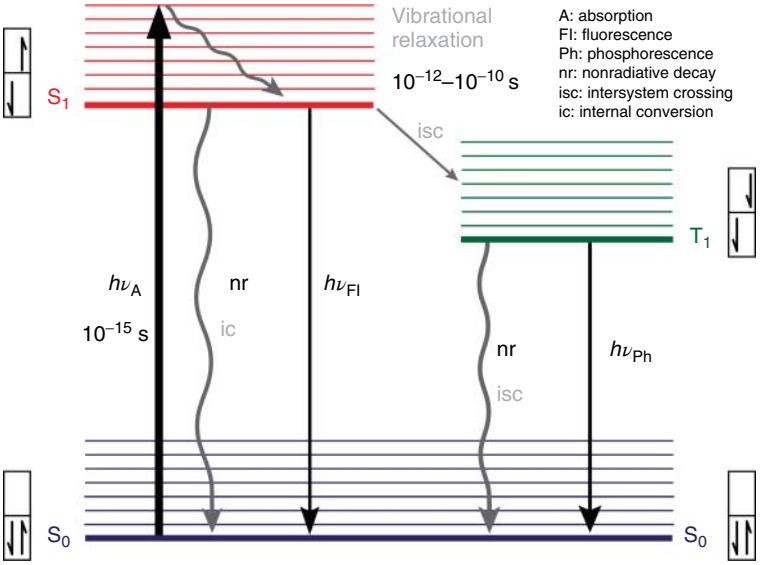

Figure 1.1 A simplified Jablonski diagram not including higher singlet excited states than S_1.

between the photons required for excitation and the photons emitted. This difference ($\nu_{abs} - \nu_{em}$), typically expressed in cm^{-1}, is called the Stokes shift and is an intrinsic property of a fluorophore in a given set of conditions. The excited state lifetime (τ), with typical values of 0.5–20 ns for organic fluorophores, is the result of the sum of all nonradiative (k_{nr}) and radiative (Γ) decay rates reflecting the processes returning the fluorophore to its ground state (Eq. 1.1).

$$\tau = \frac{1}{\Gamma + k_{nr}} \tag{1.1}$$

Several factors impact the potential utility of any fluorophore. The efficiency of the excitation process is dependent on the chromophore's molar absorptivity (ε), which itself is proportional to the cross section (σ). The efficiency of the emission process, the fluorescence quantum yield (Φ), reflects the fraction of emitted photons with respect to the absorbed ones. Expressed in rate constants, the quantum yield (Φ) is determined by the radiative rate constant (Γ) over the sum of the radiative (Γ) and all nonradiative (k_{nr}) rates (Eq. 1.2):

$$\Phi = \frac{\Gamma}{\Gamma + k_{nr}} \tag{1.2}$$

The combined efficiency of the excitation and emission is expressed by the brightness ($\varepsilon \times \Phi$), the product of molar absorptivity (ε), and fluorescence quantum

yield (Φ). Hence, poorly emissive fluorophores can still enjoy sufficient brightness if their low quantum yield is compensated by a high molar absorptivity. Or, *vice versa*, highly emissive fluorophores possessing high quantum yields can still suffer from low brightness due to a low molar absorptivity.

Note that a spin forbidden additional pathway, named intersystem crossing (isc), populates the much longer lived triplet (T_1) state. The generally slow radiative decay from T_1 to the ground state is known as phosphorescence and not further discussed here (Fig. 1.1).

The advent of relatively affordable, robust, yet sophisticated, benchtop fluorimeters in conjunction with the vast and growing number of commercially available fluorescent probes have contributed to the accessibility and popularity of fluorescence spectroscopy. It has become one of the most important analytical techniques for the *in vitro* study of biomolecules and *in vivo* cellular imaging, providing spatial and temporal information.[1,2] The "*in situ*" study of intricate and large biomolecules in their complex environment is further facilitated by exclusive excitation of fluorescent probes to minimize background emission. Provided noninterfering probes are used, the inherently nonperturbing fluorescence measurement delivers valuable insights into biomolecules in their native environments.

The fundamentals of excitation and emission, as depicted in the simplified Jablonski diagram, form the foundation of any fluorescence technique (Fig. 1.1). The versatility ranges from exotic one-of-a-kind studies requiring very sophisticated instrumentation to straightforward, but yet very informative, techniques available on most modern benchtop fluorimeters. The majority of techniques commonly used in the study of biomolecules fall in the latter category and are briefly discussed in the following section.[1,3–5] For more specialized fluorescence and microscopy techniques, the reader is recommended to turn to other chapters in this book or to journal articles focused on a certain technique.

1.2 COMMON FLUORESCENCE SPECTROSCOPY TECHNIQUES

1.2.1 Steady-State Fluorescence Spectroscopy

The quintessential fluorescence-based technique is steady-state fluorescence spectroscopy. The emission spectrum of a fluorophore is recorded upon excitation with a constant photon flux light source (e.g., a xenon arc lamp), typically at its absorption maximum or where it can be selectively excited if other chromophores are present. The fluorescence spectrum obtained provides the fluorophore's emission signature, its wavelength-dependent emission intensity, and emission maximum (see example in Fig. 1.2). Instrument settings and detector sensitivity aside, the emission intensity is dependent on the fluorescence quantum yield of the fluorophore and is proportional to its concentration provided sufficiently dilute samples are used (absorbance <0.05). The emission maximum is an inherent property of the fluorophore but could

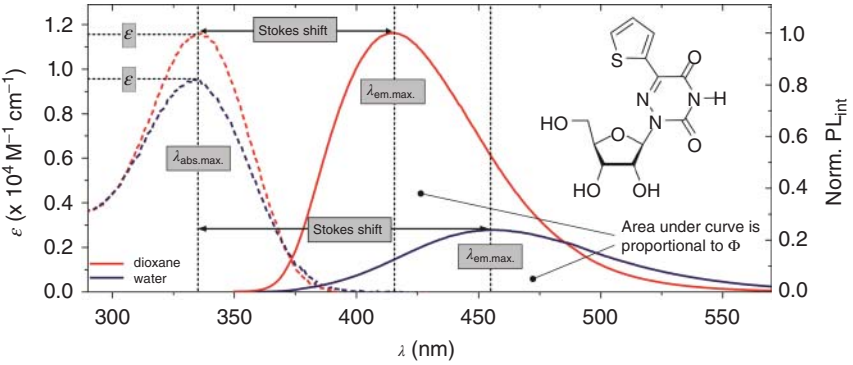

Figure 1.2 Absorption (dashed lines) and fluorescence (solid lines) spectra of 5-(thiophen-2-yl)-6-aza-uridine in water (black) and dioxane (gray). Annotations illustrate the most important parameters that can be obtained. The difference in Stokes shift in water and dioxane reveals the environmental polarity sensitivity of this isomorphic fluorescent nucleoside. *Note:* Stokes shifts are typically reported in energy units, commonly cm^{-1}.

be highly dependent on its immediate environment and subject to diverse effects (e.g., solvent polarity, viscosity, pH). The emission intensity measured in steady state can be used to estimate the fluorophore's quantum yield (Φ) using reference fluorophores with known quantum yields emitting at similar wavelengths as the fluorophore under investigation and the same instrument settings.[1]

Fluorophores possessing a different dipole moment in their excited state compared to the ground state frequently reveal sensitivity to environmental polarity. This behavior, termed solvatochromism, results from a solvent's ability to accommodate and thereby lower the fluorophore's excited state energy by solvent molecule rearrangement. By definition, a fluorophore is said to show positive solvatochromism if the emission maximum undergoes a bathochromic (to longer wavelength) shift upon increasing solvent polarity and negative solvatochromism if the emission maximum undergoes a hypsochromic (to shorter wavelength) shift. While potentially complex and subjected to artifacts, fluorogenic probes possessing such traits have been used to examine local polarity in biomolecules, including DNA,[6–10] proteins,[11–15] and membranes.[16–19]

Traditionally, polarity has been expressed using dielectric constants (ε), a parameter reflecting bulk property, and its derived orientational polarizability (Δf).[20,21] Newer microenvironmental polarity parameters (e.g., Reichardt's $E_T(30)$ scale), utilizing zwitterionic solvatochromic chromophores with a polarity-sensitive ground state and hence absorption maximum, enable polarity measurements on the molecular level.[22] This is especially relevant for probing biomolecular cavities, environments that deviate significantly in polarity from the aqueous bulk. In comparison to the dielectric constant and orientational polarizability, the $E_T(30)$ scale typically better describes changes in spectral phenomena, like Stokes shift, as a response to changing solvent polarity.[23]

1.2.2 Time-Resolved Fluorescence Spectroscopy

Despite the increased complexity and the sophisticated optics and electronics required, the additional layer of information obtained from time-resolved fluorescence experiments makes it complementary to steady-state spectroscopy. The informational content in a steady-state fluorescence spectrum is limited to an averaged emission profile of the entire population of excited fluorophores. Distinguishing between individual fluorophores in a heterogeneous sample and/or the same kind of fluorophore experiencing different local environments is therefore not possible. In such cases, time-resolved measurements are frequently invaluable. In its simplest form, a time-resolved fluorescence measurement gives a monoexponential decay curve from which the concentration-independent fluorescence lifetime can be calculated. This is an important parameter since it reflects the time available for a chromophore to diffuse or interact with its environment in its excited state. Hence, time-resolved fluorescence spectroscopy has the potential to provide insight into the excited state dynamics of a chromophore by comparing its lifetime under different experimental conditions to its natural lifetime (τ_n). The latter is the fluorescence lifetime (Eq. 1.1) in the absence of nonradiative processes (Eq. 1.3). Albeit complex, the radiative decay (Γ) rate can be calculated from the absorption spectrum, the molar absorptivity, and the emission spectrum of the chromophore.[1]

$$\tau_n = \frac{1}{\Gamma} \tag{1.3}$$

In most biophysical studies, where the binding, structure, and folding of biomolecules are studied, fluorescent probes could simultaneously exist in different environments. Each environment, bound/unbound, exposed to/shielded from solvent, likely has a unique influence on the fluorophore's excited state and is reflected by changes in emission maximum, quantum yield, and fluorescence lifetime. In contrast to steady-state fluorescence spectroscopy, time-resolved fluorescence analysis can facilitate the simultaneous analysis of multiple emissive states with overlapping spectral bands, each with its own fluorescence decay, by deconvolution of a sample's multiexponential decay curve. For example, the folding of an enzyme containing two emissive tryptophan residues might position each in a different local environment, a situation likely undistinguishable with steady-state fluorescence spectroscopy. A time-resolved fluorescence measurement, however, will likely give a biexponential intensity decay with a different contribution for each tryptophan residue. Changes in the relative contributions upon interaction of the enzyme with its substrate may reveal which tryptophan residue is most affected by the binding event, thereby revealing its proximity to the binding site.

Quenching experiments also greatly benefit from time-resolved fluorescence measurements by distinguishing between static (ground-state complex formation) and collisional (diffusion) quenching. In the former, the fluorescence lifetime is unaffected, whereas collisional quenching does affect the lifetime. Similarly, analysis of time-resolved fluorescence spectra, when applied to resonance energy transfer (RET) studies (*vide infra*), reveals whether all, or a subset of donors, engage in the RET process. See the following additional discussion.

1.2.3 Fluorescence Anisotropy

In most common solution phase fluorescence-based experiments, a fluorophore is excited with unpolarized light and the emission is measured without polarization. When a fluorophore is excited with polarized light, the emission remains polarized if the chromophore's Brownian motion, or tumbling, in the excited state prior to emissive decay to the ground state is slower than the excited state lifetime. A small molecule fluorophore in a nonviscous environment of ambient temperature typically has a tumbling rate faster than its fluorescence lifetime. Hence, if excited with polarized light under such conditions, the resulting emission will be completely depolarized and isotropic. If the fluorophore, however, is attached to a large (bio)molecule (e.g., a protein), or exposed to a highly viscous medium, the fluorophore's tumbling rate will slow down. The emission retains, at least in part, the polarized excitation if the tumbling rate is slower than the fluorescence lifetime. The extent of fluorescence polarization (P) is then calculated using Equation 1.4. Herein, $I_{\parallel}$ and $I_{\perp}$ stand for parallel and perpendicular polarized emission intensity, respectively.

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \tag{1.4}$$

Polarization (P) is interchangeable with anisotropy (r) (Eq. 1.5) since both are expressions of the same phenomenon.

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \tag{1.5}$$

To measure fluorescence anisotropy, excitation and emission polarizers have to be installed in a standard steady-state fluorescence spectrometry setup. In a tandem fluorescence experiment, a fluorophore is excited with vertically polarized light and the intensity of its vertically polarized emission is recorded. This is followed by a second vertically polarized excitation, but now the intensity of the horizontally polarized emission is recorded. To take the instrumental properties into account, one has to also measure horizontal excitation polarization combined with horizontal and vertical emission polarization, respectively (G-factor).[1] The fluorescence lifetime of the fluorophore plays a crucial role in the sensitivity of the fluorescence anisotropy experiment. In a biomolecular binding study, the fluorescence lifetime of the fluorophore needs to be sufficiently long to give a close-to-zero anisotropy when unbound. As a result, a significant drop in the tumbling rate due to binding to a much larger biomolecule (e.g., a protein) yields a maximum retention of polarization.

In addition to the aforementioned biomolecular binding studies, fluorescence anisotropy has found use in protein dynamics,[24,25] as well as in studying protein–protein[26] and protein–nucleic acid interactions.[27–29] Fluorescence anisotropy is also used in membrane fluidity and microviscosity studies,[30–32] and to determine aqueous bulk-membrane partition coefficients of fluorescent probes.[33] The fundamentals and various applications of fluorescence anisotropy including time-resolved fluorescence anisotropy have been the topic of selected recent reviews.[34,35]

1.2.4 Resonance Energy Transfer and Quenching

Steady-state fluorescence spectroscopy, time-resolved fluorescence spectroscopy, and fluorescence anisotropy, as described above, are typically, although not necessarily, concerned with monitoring a single fluorescent probe. Fluorescence techniques that exploit interactions between chromophores (such as a fluorophore and a quencher or a fluorophore and another distinct fluorophore) are extremely powerful and have been widely used in the study of biomolecules.

Valuable molecular information can be obtained from two commonly studied quenching mechanisms: dynamic and static. The former, also called collisional, quenching, is described by a linear relationship between the quenching effect and the quencher concentration as defined by the Stern–Volmer equation (Eq. 1.6),[36] and its modification, the Lehrer equation.[37] In Equation 1.6, $[Q]$ is the quencher concentration and F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively. The bimolecular quenching constant, fluorescence lifetime of the fluorophore in the absence of quencher, and the Stern–Volmer quenching constant are denoted by $k_q, \tau_0,$ and K_D, respectively.

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_D [Q] \tag{1.6}$$

Hence, the Stern–Volmer quenching constant is given by Equation 1.7.

$$K_D = k_q \tau_0 \tag{1.7}$$

Deviation from linearity implies the contribution of static quenching due to formation of a ground-state complex between the fluorophore and quencher, as stated by the Perrin model.[38] The utility of fluorescence quenching experiments is illustrated in selected recent reviews for multiple fields including the study of RNA folding, dynamics, and hydridization[39–41] protein folding, structure and dynamics,[42,43] protein–membrane interactions,[44–46] and membrane microdomains.[47–50] Nevertheless, extracting molecular information from fluorescence quenching studies can be challenging since apparent quenching can also result from unrelated technical issues, such as the sample's turbidity or high optical density.

The disadvantage associated with quenching experiments can be largely overcome by exploiting RET, a nonradiative process between two molecular entities typically referred to by donor and acceptor. The RET process is facilitated by either a Dexter or Förster mechanism. The Dexter mechanism requires orbital overlap and hence close proximity to the donor and the acceptor. In contrast, the Förster process, based on a dipole–dipole coupling between a donor and an acceptor, operates over larger distances and is viable when there is significant spectral overlap of donor emission with acceptor absorption. The size of biomolecules (30–60 Å)[1] is in the same range as the Förster critical distance of many D/A pairs, the distance at which the energy transfer efficiency is 50%. This makes Förster resonance energy transfer (FRET) broadly applicable for biomolecular studies. As in quenching studies, the emission of the donor fluorophore is quenched, but a sensitized acceptor emission at a longer wavelength is frequently observed.

Prior to any FRET experiments, the Förster distance (R_0) in Å, for the donor–acceptor pair used, must be calculated using values for donor quantum yield (Φ_D), spectral overlap of donor emission and acceptor absorption ($J(\lambda)$), relative orientation of donor and acceptor (κ^2), and the refractive index of the medium (n) (Eq. 1.8).

$$R_0 = 0.211(\kappa^2 n^{-4} \Phi_D J(\lambda))^{1/6} \tag{1.8}$$

The rate of the energy transfer process, $k_{ET}(r)$, can now be calculated based on the distance between the donor and the acceptor (r), the fluorescence lifetime of the donor in absence of the acceptor (τ_D), and the Förster distance (R_0) (Eq. 1.9).

$$k_{ET}(r) = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6 \tag{1.9}$$

The efficiency of the RET process can be expressed as the Förster distance (R_0) over the sum of the Förster distance (R_0) and the donor–acceptor distance (r) (Eq. 1.10).

$$E = \frac{R_0^6}{R_0^6 + r^6} \tag{1.10}$$

In general, $\kappa^2 = 2/3$, reflecting random interchromophore orientation, is used when calculating R_0. It must be noted, however, that in certain cases this assumption could be a crude oversimplification leading to false interpretations.[51] Moreover, by making the approximation that $\kappa^2 = 2/3$, the opportunity is lost to get orientational information about the system under study.[52] With the Förster distance (R_0), determined for the chosen FRET pair (Eq. 1.8), the FRET efficiency is strongly dependent on the distance between the donor and acceptor (Eq. 1.10). This enables calculation of the distance (r) between donor and acceptor sites on a macrobiomolecule or its complexes, provided the distance does not change during the excited state lifetime.[1] Therefore, the FRET phenomenon has been termed a "spectral ruler."[53,54]

FRET is not limited to distance measurements in biomolecules but can also be used in binding, folding, and hybridization studies. Because of its wide applicability, FRET measurements have been used, for example, in membrane research to study microdomain formation[47] and transmembrane peptides in surface-supported bilayers.[55] Applications of FRET in nucleic acid research have been widely described in selected reviews on structure, folding, hybridization, and dynamics of RNA[41,56–58] and the sequence-dependent structure, stability, and dynamics of nucleosomes.[59] FRET measurements have also been exploited to investigate protein folding, protein–protein interactions, and cellular signaling events in live cells.[42,60–62]

1.2.5 Fluorescence Microscopy and Single Molecule Spectroscopy

Advancement in instrumentation and increased availability of bright (and sometimes organelle specific dyes) fluorophores have led to increased sensitivity of

fluorescence-based spectroscopy techniques. Developments in cellular visualization include total internal reflection (TIRF), confocal, and two- or multiphoton fluorescence spectroscopy.[63–68] Another fairly recent development is fluorescence lifetime imaging microscopy (FLIM), where a fluorescent probe is used to stain a biological sample (e.g., a cell).[50,69,70] Image contrast is based on differences in fluorescence lifetime as a result of probe distribution over multiple unique locations (e.g., cellular components).

Further advancements in single photon excitation in the late 90s of the last century have led to single molecule spectroscopy, the ability to follow the emission of just one molecule at a time facilitated by optical "tweezers" or trapping.[71–74] The magnitude of this achievement is easily appreciated by the realization that a typical 1 mL, 1 μM fluorescent probe sample contains (1×10^{-9} mol fluorescent probe * 6.02×10^{23} (Avogadro's number)) $\sim 6 \times 10^{14}$ fluorescent molecules! As outlined in the previous section, the averaged emission profile of this unfathomable number of fluorescent probes is informative and sufficient for numerous studies. The ability to follow complex biological processes at the single molecule level, however, is greatly beneficial. An example of such a complex process is the conformational changes a ribosome undergoes during the translation of messenger RNA into proteins.[75] The development of single molecule spectroscopy has benefitted virtually all areas of biomolecular research as described in selected reviews.[42,76–83] The advent of single molecule spectroscopy was quickly exploited to enable single pair FRET studies.[72] Such studies proved instrumental in the areas of nucleic acid (DNA/RNA) structure, folding, and dynamics,[84–87] DNA–protein interactions,[88,89] and nucleosome conformations.[90,91] It must be noted here that the conjunction of the discovery[92] and development[93] of the highly emissive green fluorescent protein (GFP), with the advancement of single molecule spectroscopy forged one of the most useful tools in modern biology.[94]

1.2.6 Fluorescence-Based *in vivo* Imaging

Arguably, the pinnacle of fluorescence spectroscopy applications in the life sciences is fluorescence-based *in vivo* imaging. This is the most recent addition to invaluable existing imaging techniques including X-ray, positron emission tomography (PET), ultrasound, and magnetic resonance imaging (MRI). Besides potential cost reduction, development of fluorescence-based imaging techniques brings the advantage of improved resolution and contrast. An additional benefit of a fluorescence-based approach, which is lacking in established imaging techniques, is the potential of fluorescent probes to respond in real time to specific physiological changes.[95]

The majority of fluorescent probes absorbs and emits in the ultraviolet and visible domain of the electromagnetic spectrum. To efficiently penetrate through living tissue, avoiding absorption by water, lipids, as well as[96] oxy- and deoxyhemoglobin, light of near-infrared (NIR) wavelengths (700–1000 nm) is used.[97–99] Hence, ideal fluorescent probes for *in vivo* imaging combine a low-energy excitation wavelength with a large Stokes shift. Diverse examples of probes suitable for, but not limited to, *in vivo* use exist[100,101] and include modified nucleosides[102–104] and amino acids[105,106] in addition to dendrimers, nanoparticles, and quantum dots.[106,107] Most promising

is the development of (near)infrared fluorescent proteins (IFPs) with a recent example characterized by an excitation maximum of 684 nm ($\varepsilon > 90{,}000\ M^{-1}\ cm^{-1}$), emission maximum of 708 nm and a quantum yield of 0.07.[108] Fortunately, the long wavelength excitation required to excite NIR probes is deemed safe, making whole body fluorescence tomography an exciting prospect.[109] Alternatively, to minimize absorption by the surrounding tissue, suitable short wavelength absorbing fluorophores can be subjected to two- or multiphoton excitation using long-wavelength laser excitation.[110–113]

Besides probe development, technological improvements have also contributed to the advancement of fluorescence-based imaging techniques. For instance, differences in fluorescence lifetimes enabled isolation of probe emission from emission of the surrounding tissue.[114,115] The emergent field of NIR fluorescent probes and their *in vivo* imaging applications has been the topic of several reviews.[95,99,109,116–118]

1.3 SUMMARY AND PERSPECTIVE

In the preceding sections, the most commonly used fluorescence spectroscopy techniques are discussed. Their importance and applicability in research areas involving biomolecular building blocks is illustrated with selected examples. Due to the vast scope of fluorescence techniques available, this chapter cannot be comprehensive. Important developments not mentioned here include, for example, fluorescence correlation spectroscopy (FCS). This technique is based on fluctuations of fluorescently labeled compounds (e.g., biomolecular building blocks) in very small volumes. FCS is most useful in the study of dynamic molecular processes in living cells (e.g., diffusion, ligand–protein, protein–protein, and protein–DNA interactions).[119–122]

The next chapter discusses the fundamental features of the native fluorophores found in biomolecules, followed by a concise overview outlining the development of fluorescent analogs of fluorescent building blocks. Each of the following chapters discusses a specific use of such fluorescent analogs in conjunction with fluorescence spectroscopy. Together, these chapters illustrate not only the diversity in fluorescence techniques used but also the plethora of research areas that greatly benefit from it. Undoubtedly, the desire to explore new research areas has pushed the technological development of fluorescence instrumentation. These advancements, *vice versa*, enabled exploration of new scientific frontiers. Perhaps we find ourselves at the mere beginning with many exciting fluorescence-based discoveries ahead of us.

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2

NATURALLY OCCURRING AND SYNTHETIC FLUORESCENT BIOMOLECULAR BUILDING BLOCKS

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2.1 INTRODUCTION

Most common biomolecules and their building blocks lack appreciable emission. When emissive, as in the case of certain fluorescent amino acids (e.g., phenylalanine and tryptophan), their excitation and emission energies are relatively high and found in the UV range. As a result, their utility in biophysical studies, high-throughput assays and imaging applications can be rather limited. This has prompted the development of functional and emissive surrogates. This chapter, bridging our opening discussion and the more focused chapters to follow, concisely discusses the main contributions in this area.

Designer fluorescent probes should ideally resemble their natural counterparts in terms of their molecular size and shape, while retaining their inherent function. We refer to such probes as being isomorphic. This feature, of course, presents a fundamental predicament, as structural modifications aiming to alter the electronic features of a chromophore, inevitably also impact its basic physical properties as well as interactions with its environment and other biomolecules. Nevertheless, elegant advances have been made in this field. After we highlight the naturally occurring fluorescent

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biomolecular building blocks, we concisely summarize the main derivatives developed as emissive surrogates of the major families of biomolecular building blocks.

2.2 NATURALLY OCCURRING EMISSIVE BIOMOLECULAR BUILDING BLOCKS

Whether viewed from a utility or design perspective, it is most inspiring to take notice of the inherently fluorescent building blocks selected by Nature. It is probably safe to state that it was not their fluorescence but rather their structural properties that made them pass Nature's selection criteria. Regardless, their emissive properties are a fortunate coincidence that provide scientists with molecular tools to study the biomolecules containing such building blocks. Interestingly, each family of biomolecules (i.e., proteins, nucleic acids, and lipids) has at least one known naturally occurring emissive building block (Fig. 2.1).

Best known and frequently utilized are the protein building blocks tryptophan (**1**) and tyrosine (**2**) (Fig. 2.1, Table 2.1). The rather unfavorable fluorescence properties of phenylalanine (**3**) limit its use. Tyrosine, on the other hand, enjoys reasonable fluorescence quantum yield with a pH-sensitive emission maximum that shifts from 310 to 340 nm upon deprotonation. Tyrosine lacks sensitivity toward polarity, a trait for which tryptophan, besides its robust fluorescence quantum yield, is well known for.[8] Due to its relative lipophilic character, tryptophan is often buried inside the hydrophobic protein interior. Protein unfolding exposes tryptophan to the polar aqueous environment, causing a shift in its emission maximum from 309 to 355 nm.[1] As discussed in numerous reviews, besides folding/unfolding experiments, applications include protein dynamics and ligand binding.[1, 9–11]

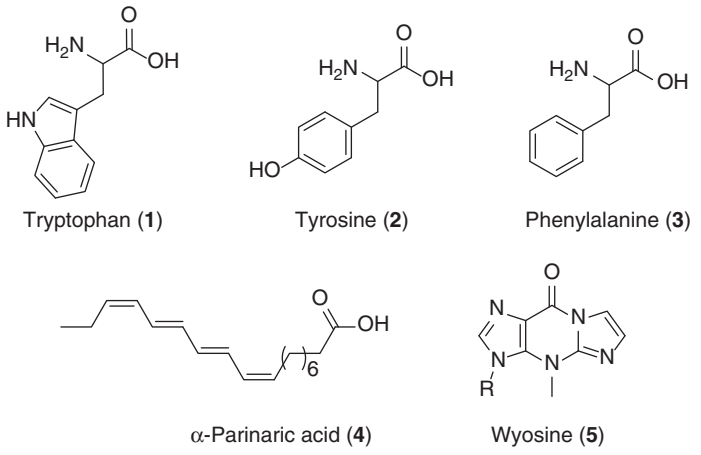

Figure 2.1 Naturally occurring fluorescent biomolecular building blocks.

TABLE 2.1 Selected Spectroscopic Properties of Naturally Occurring Fluorescent Biomolecular Building Blocks[a]

#	Name	Solvent	λ_{abs} (ε)	λ_{em}	Φ_{fl}	τ
1	Tryptophan	Buffer pH 7	279	355	0.01–0.4	
2	Tyrosine	Buffer pH 7	275	310[b]	0.14	3.3–3.8
3	Phenylalanine	Buffer pH 7	258	282	0.024	
4	α-Parinaric acid	Methanol	319, 304(79)	432	0.017	1.3
		Decane	321, 306(74)	432	0.054	5.2
5	Wyosine	Buffer pH 7	235(32), 295(7.4)[c]	450[d]	0.044[d]	

[a] Values for λ, and ε are given in nm and 10^3 M^{-1} cm^{-1}, respectively. Values for amino acids **1**, **2**, and **3** are obtained from different sources.[1–3]

[b] If deprotonated $\lambda_{em} = 340$ nm.[4]

[c] Spectral data, especially the long-wavelength absorption maximum, is pH sensitive.[5] Earlier findings for λ_{abs} in unbuffered water of 235 nm ($\varepsilon = 17.6$) and 294 ($\varepsilon = 4.2$) are seemingly contradicting with tabulated values.[6]

[d] Values for the nucleobase determined in aqueous 0.01 M Tris-HCl, containing 0.1 M NaCl, 10 mM Mg^{2+} at pH 7.5.[7]

A naturally occurring fluorescent membrane constituent is α-parinaric acid (**4**) (Fig. 2.2). This conjugated polyunsaturated fluorescent fatty acid was isolated for the first time from Parinari laurinum in 1933, and 20 years later identified as the (*Z*),(*E*),(*E*),(*Z*)-isomer.[12] Comparison of the spectral data in methanol and decane, two solvents of dramatically different polarity,[13] reveal almost identical absorption and emission maxima (Table 2.1). This can be attributed to the absence of a strong dipole moment in the ground and excited state as a result of the aliphatic hydrocarbons that cap the π-system. There are, however, significant differences in fluorescence quantum yield and fluorescence lifetime for the two solvents (Table 2.1).[12, 14] After treatment with iodine, the all trans-β-parinaric acid was obtained and spectroscopically characterized in the late 1970s,[15] followed by application as a fluorescent probe in the study of synthetic phospholipid membranes.[16]

Interestingly, adding limited amounts of polyunsaturated fatty acids can stabilize artificial phospholipid membranes, whereas larger amounts can destabilize them.[17] Other naturally occurring polyenes, although not native membrane constituents, that have been used in early membrane studies for their lipophilicity and emissive properties are retinol, retinal, and other cartenoids.[14, 18] For the same reasons, the macrolide antibiotics filipin and amphotericin also found use as fluorescent membrane probes.[19] The last two, however, are rather large, complex, and known to induce cell lyses.

The four canonical nucleobases that make up all nucleic acids virtually lack appreciable fluorescent properties with their high energy absorption, very low quantum yield, and short excited state lifetimes.[20–24] Despite numerous posttranscriptionally modified ribonucleosides,[25] the nucleic acids research field is perhaps the most deprived of naturally occurring fluorescent analogs of its building blocks. Yet, among them there is one, the fluorescent nucleoside wyosine (**5**) (Fig. 2.1) and its emissive derivatives wybutosine and wybutoxosine that share the same chromophore. Their

biosynthetic pathway was recently deciphered,[26] and their natural occurrence has been established in baker's yeast $tRNA^{Phe}$,[7, 27–29] Torula yeast,[30] rat and bovine liver,[31, 32] and plants.[33] Wyosine's glycosidic bond is exceptionally susceptible to hydrolysis, which proved to be a major hurdle in its isolation from natural sources.[6] Despite its potential, its chemical instability and lack of a Watson–Crick hydrogen bonding face likely explains why wyosine has not been recognized as a potential fluorescent nucleoside surrogate for the study of nucleic acids.

All naturally occurring fluorescent biomolecular building blocks share one critical structural commonality: an extended (aromatic) π-system (Fig. 2.1). They therefore offer inspiration and a blueprint for fluorescent probe designers. Introduction or extension of an existing π-system bathochromically shifts the absorption maximum allowing for a π–π* excitation, which is often followed by a radiative π*–π decay process. Hence, such a modification is an integral part of any design aimed to endow nonemissive natural biomolecular building blocks with appreciable fluorescence properties. Interestingly, the efficiency of the emissive process is strongly dependent on structural rigidity, nature of the substituents, and environmental factors (e.g., polarity, pH, viscosity). This provides probe designers with the opportunity to tailor the fluorescence response of the probe toward environmental characteristics.[34] For instance, sensitivity to pH can be controlled by inclusion or exclusion of basic or acidic sites.[35, 36] Introduction of a strong push–pull system by judicial placement of donor (electron releasing) and acceptor (electron withdrawing) moieties typically results in responsiveness to environmental polarity.[37–39] Sensitivity to viscosity, or molecular crowding, is virtually absent in the most rigid fluorophores that lack single-bond linkages between π-systems. Conversely, introduction of such a linkage constructs a "molecular rotor" and likely imparts a fluorescence probe with enhanced sensitivity to viscosity.[40] Besides the synthetic hurdles accompanied with such alterations, the real design challenge is to limit the structural modification to a minimum, often a prerequisite to ensure interchangeability of the natural building block with its fluorescent surrogate.

2.3 SYNTHETIC FLUORESCENT ANALOGS OF BIOMOLECULAR BUILDING BLOCKS

Due to the vast amount of scientific literature available, the following sections cannot and are not aimed to be comprehensive. Rather, they are intended to illustrate the various structural designs that have been explored to impart nonemissive natural biomolecular building blocks with desirable fluorescent properties. To this end, the scope, with a focus on isomorphicity, of fluorescent analogs of membrane constituents, amino acids, and nucleosides is given. Readers will, however, be directed to reviews that significantly elaborate on selected topics. The depicted structures herein, along with their tabulated basic spectral properties, allow for interesting comparison of their structure–photophysical properties relationship (SPPR).

2.3.1 Synthetic Emissive Analogs of Membranes Constituents

Biological membranes, crucial for sustaining cellular integrity and function, consist of a complex mixture of membrane constituents including proteins, phospholipids, and fatty acids. The amphiphilic nature of the last two constituents, a polar head group with a lipophilic tail, is the primary driving force that shapes the architecture of the biological membrane in aqueous environments.[41–43] Lipid bilayers are amenable to multiple kinds of fluorescent labeling as has been discussed in multiple reviews.[4, 44–52] Strategies can be categorized by polar head group labeling, apolar chain-end labeling, on- and in-chain labeling, and noncovalent labeling. The last approach typically exploits inherently lipophilic, and hence hydrophobic, dyes including 1-ethylpyrene,[53] diphenyl hexatriene (DPH) (**6**) (Fig. 2.2, Table 2.2),[60–62] methyl-9-anthroate,[63, 64] 4-(dicyanovinyl)julolidine,[65, 66] and steroidal skeletons resembling aminodesoxyequilenin.[67, 68] Exposed to an aqueous environment containing lipid bilayers, such dyes will either precipitate or dissolve in the apolar interior of the membrane according to a system-specific partition coefficient (K). Despite the presence of organized domains, for example, rafts and superlattices,[69, 70] the typical fluid nature of the membrane interior, however, provides ample mobility and thus limits knowledge and control over the dye's exact position. To improve its positioning, one of the benzene rings of diphenyl hexatriene (**6**) has been functionalized with the polar trimethylammonium to anchor it to the polar head group region.[71]

More common approaches exploit phospholipid head group, chain-end, or in- and on-chain functionalization, thereby effectively determining the probe's immediate environment and thus its applications. The lack of a suitable scaffold for synthetic extension or expansion to impart membrane constituents with fluorescence properties limits the strategy to the inclusion of known fluorophores. Located at the polar head group of a lipid, the fluorophore will be in immediate contact with the polar extracellular matrix allowing the probing of processes at the cell surface. A widely used example is the dansyl-labeled phosphatidyl ethanolamine (DPE) (**7**) (Fig. 2.2).[72] Its sensitivity to polarity was exploited for the study of protein–lipid interactions[73, 74] in addition to local polarity[72, 75] and fluidity[75] of biological membranes. Other probes in this category include phospholipid head groups labeled with coumarin,[76] nitrobenzoxadiazole,[77] or rhodamine B.[78]

To explore the interior of membranes, the chains of fatty acids and phospholipids have been functionalized with fluorophores. The on-chain approach is exemplified by 12-(9-anthroyloxy) stearic acid (12-AS) (**8**)[72] and its analog 12-(9-anthroyloxy) stearic acid anthraquinone.[63, 79] This design has the advantage that it allows for controlled positioning of the probe in proximity to or remote from the polar head group thereby facilitating the study of membrane polarity,[72] fluidity,[63, 79] and protein–lipid interactions.[80] Free rotation enabled by the linker, however, can complicate spectroscopic analysis, which likely explains the limited popularity of this approach. Conjugation of the fluorophore at the chain-end of a phospholipid or fatty acid positions it deep in the apolar interior of the membrane. An example of such a design

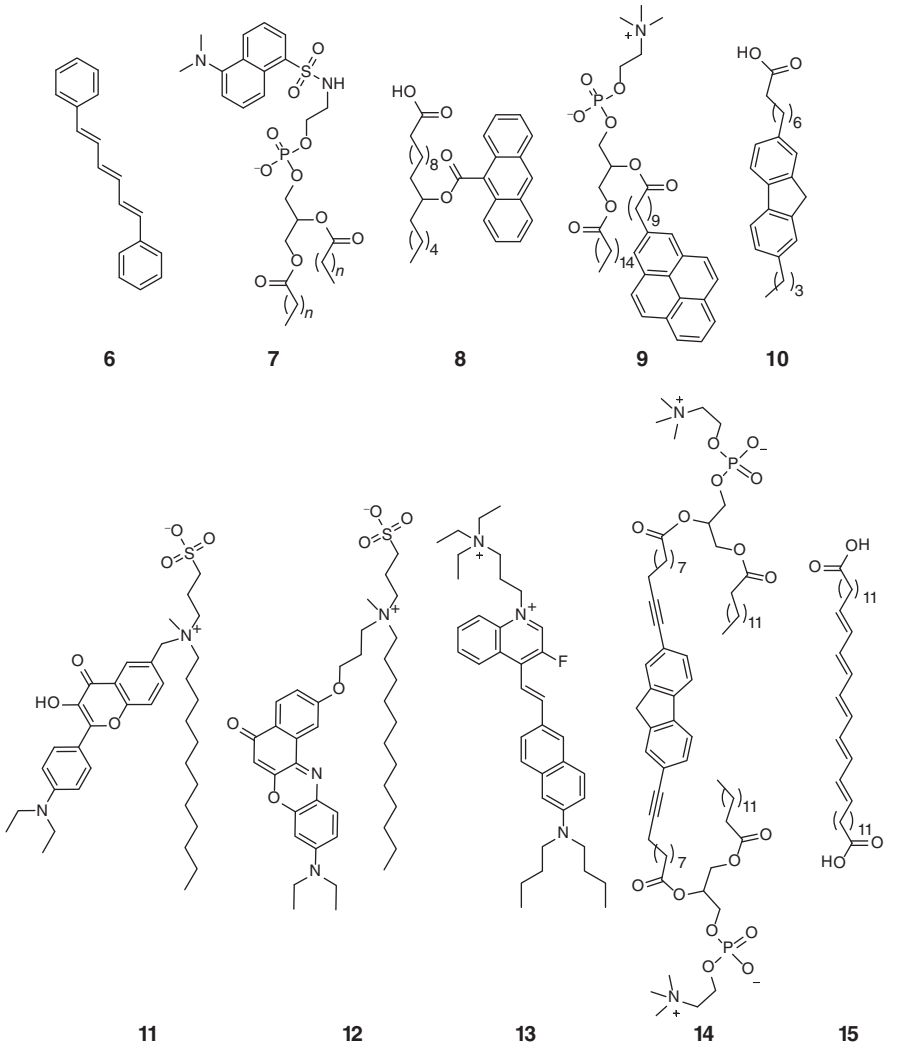

Figure 2.2 Examples of noncovalent, polar head group, on-chain, chain-end, and in-chain fluorescent membrane probes.

that enjoys very high fluorescence quantum yield is a pyrene chain-end labeled phosphatidylcholine (pyrene-PC) (**9**) (Fig. 2.2, Table 2.2).[53, 81, 82] This probe has been extensively used to study membrane fluidity,[81] the effect of cholesterol on membrane properties,[83] membrane microdomains,[84–86] protein–lipid interactions,[87] and membrane permeability.[88] This design strategy does, however, potentially suffers from "looping-back," where a probe aimed to be located at the inner membrane folds back closer to the polar head group due to the flexibility of the aliphatic chain.[89] This drawback can be overcome with an in-chain approach controlling positioning

TABLE 2.2 Spectroscopic Properties of Selected Synthetic Fluorescent Membrane Constituents[a]

#	Name	Solvent	λ_{abs} (ε)	λ_{em}	Φ_{fl}	τ
6	DPH[b]	Hexane	352, 370	430	0.64	15.7
7	DPE[c]	Methanol	346(3.6)	514		
8	12-AS	Methanol	362(7.8)	458	0.071	1.6
		Hexane		446		10.5
9	Pyrene-PC	Methanol	342(37)[d]	376[d]	0.65[e]	410[e]
10	C8A-FL-C4	Methanol	270(38), 297, 309	319	0.65	
11	3HF[f]	Ethanol	431	521/570		
		Hexane	396	423/554	0.14	
12	NR[g]	Buffer	521	657	0.002	
		Dioxane	526	592	0.74	
14	BAexFluorPC[h]	DMPC	308, 329	334		1.3
15	trans-PDA	Chloroform	353(92), 335(95), 320(60)	474	0.14[i]	

[a] Values for λ and ε are given in nm and 10^3 M^{-1} cm^{-1}, respectively.
[b] Data from Bachilo *et al.*[54] and Palmer *et al.*[55]
[c] Data from London *et al.*[92]
[d] Data from Sinkeldam *et al.*[50]
[e] Data in ethanol from Hermetter *et al.*[48]
[f] Data for 4-diethylamino-3-hydroxyflavone, λ_{em} data represent emission from normal and tautomeric state after ESIPT (N*/T*), respectively.[56, 57]
[g] Buffer is phosphate buffer of pH 7.4.
[h] Only a spectroscopic study in dimyristoylphosphatidylcholine (DMPC) vesicles is reported, λ_{abs} and λ_{em} are extracted from graphs, and only the most contributing τ is given.[58]
[i] Quantum yield in DMPC vesicles.[59]

of the fluorophore in the membrane by limiting its mobility. Hence, probes such as fluorene-labeled fatty acid (C8A-FL-C4) **10**[90, 91] (Fig. 2.2, Table 2.2) have been used in depth analysis of membranes with a focus on phospholipid topology[89, 92, 93] in addition to membrane localization and penetration of, for example, cholesterol[94–96] and membrane-bound proteins and peptides.[97]

Other examples of fluorescent aromatic hydrocarbons used include anthracene and ethynyl-extended anthracene,[98] and vinyl-extended dihydrophenanthrene.[99] Incorporation of 3-hydroxyflavone, 3HF, (**11**) and Nile Red, NR, (**12**)[100] illustrate the use of fluorescent aromatic heterocycles (Fig. 2.2, Table 2.2).[101, 102] The former, 3-hydroxyflavone (**11**), can undergo excited state intramolecular proton transfer (ESIPT)[56, 103] and is sensitive to surface charge and hydration.[101] Nile Red (**12**) is polarity sensitive as well but, in contrast to **11**, cannot undergo ESIPT due to the absence of acidic protons.[100] Both **11** and **12** bind specifically to the outer membrane leaflet, posses spectral properties sensitive to changes in lipid order, and can detect cellular apoptosis.[100–102] Like Nile Red (**12**), a recently reported family of environmentally sensitive quinolinium-based membrane probes show remarkable redshifted emission.[104] Among them, quinolinium **13** reveals a lipid order-dependent long-wavelength emission maximum ranging from ~660 to almost 700 nm with an emission profile tailing over 800 nm (Fig. 2.2).

Regardless of favorable spectroscopic properties, arguably the best position control of a membrane probe can be achieved using the symmetrical bolaamphiphile design where the in-chain fluorophore is anchored in the membrane by two polar head groups limiting both longitudinal and transverse maneuverability. This elaborated design is illustrated with the ethynyl-extended fluorene (**14**).[58] When it comes to isomorphic design principles, the most representative class of probes are the polyene membranes substituents. Their successful design is validated and strongly inspired by aforementioned naturally occurring α-parinaric acid (**4**) (Fig. 2.1). Superior to designs with bulky fluorophores, such polyene-containing phospholipids have been used as probes in live cells.[105] In all trans-pentanoic diacid (trans-PDA) (**15**), as in **14**, the bolaamphiphile design locks the polyene probe in place while its ultraslim fluorophore limits membrane perturbation to an absolute minimum (Fig. 2.2).[106] Its high molar absorptivity values, emission in the visible part of the electromagnetic spectrum, and sufficient quantum yield (Table 2.2) were used to study probe dynamics and fluidity of lipid bilayers.[59]

2.3.2 Synthetic Emissive Analogs of Amino Acids

Besides the emissive aromatic residues of tryptophan (**1**), tyrosine (**2**), and phenylalanine (**3**) (Fig. 2.1, Table 2.1), the naturally occurring amino acids have high-energy UV absorption maxima and lack fluorescence. Imparting the native amino acids with desirable fluorescence properties is a challenge since protein function relies heavily on correct folding thereby limiting the modifications that can be tolerated without detrimental structural or functional repercussions. Material-related applications have, however, exploited modified residues. Noncanonical amino acids[107] and their fluorescent amino acids substitutes have appeared in numerous overview articles.[50, 108–113]

Despite the already useful features of tryptophan (**1**) and tyrosine (**2**), a desirable enhancement of their spectral properties would include a redshifted absorption spectrum to allow for selective excitation, which is especially valuable in proteins that already contain one or more tryptophan residues. Synthetic derivatization of tryptophan's (**1**) aromatic core is a straightforward approach and has led to a variety of subtly modified tryptophan mimics, all sharing its shape and size but with altered spectroscopic properties.[50, 111, 114, 115] One notable modification is 7azaTrp (**16**) (Fig. 2.3, Table 2.3).[115, 121, 122] Replacing the benzene core in tryptophan for a pyridine core causes a notable redshift of the absorption and emission maximum to 291 and 391 nm, respectively. Despite the lower quantum yield compared to native tryptophan, 7azaTrp (**16**) has been incorporated in β-galactosidase,[123] membrane protein EIImtI,[115] and used for DNA–protein binding studies.[114] Tryptophan remains an inspiring starting point for new designs as is illustrated very recently with analogs comprised of an expanded ring system such as 1*H*-pyrrolo[3,2-*c*]isoquinoline. They possess a larger Stokes shifts and their absorption and emission maxima are distinct from tryptophan.[124]

Instead of exploiting tryptophan, a general strategy to impart amino acids with fluorescent properties is a straightforward attachment of known fluorophores. Side-chain modification gives access to virtually limitless number of fluorescent amino acids.[50]

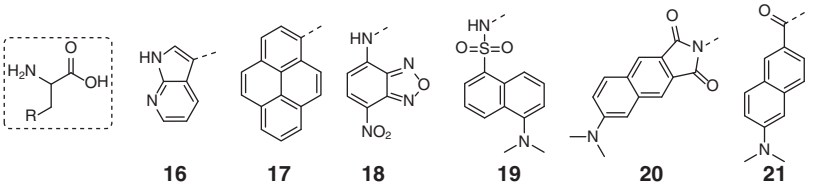

Figure 2.3 Examples of emissive amino acid analogs 7azaTrp (**16**), 1PyrAla (**17**), NBDAla (**18**), 51dansylAla (**19**), 6DMNA (**20**), and Aladan (**21**).

TABLE 2.3 Spectroscopic Properties of Selected Synthetic Fluorescent Amino Acids[a]

#	Name	Solvent	λ_{abs} (ε)	λ_{em}	Φ_{fl}	τ
16	7azaTrp	Water	291	391	0.01	1.24
		Methanol	297	366	0.01	
17	1PyrAla[b]	Ethanol	241(79.4), 272, 343	376	0.65	
18	NBDAla[c]	Ethanol	264, 330, 462(19.7)	532	0.38	
19	51DansylAla[d]	Methanol	335(4.0)	518	0.23	
		Dioxane	335(4.1)	479	0.54	
20	6DMNA[e]	Water	388	592	0.002	
		Dioxane	372	498	0.22	
21	Aladan[f]	Water	364(14.5)	531		
		Cyclohexane	342	401		

[a] Values for λ and ε are given in nm and 10^3 M^{-1} cm^{-1}.
[b] Values for pyrene.[48, 116]
[c] Data for 7-benzylamino-4-nitrobenz-2-oxa-1,3-diazole.[117]
[d] Values for 5-(dimethylamino)-*N*-methylnaphthalene-1-sulfonamide.[118]
[e] Values for model compound 6DMN-GlyOMe.[119]
[f] Values for Prodan.[120]

A plethora of derivatives have been reported, here exemplified by alanine mimic 1PyrAla (**17**)[125] (Fig. 2.3). Although analog **17** is endowed with the desirable high quantum yield of the hydrocarbon fluorophore pyrene, it lacks a significantly red-shifted emission maximum (Table 2.3).

Shifting the emission to lower energies can frequently be introduced by judicial positioning of an electron-releasing moiety (donor or D) and an electron-withdrawing moiety (acceptor or A) yielding chromophores that are often referred to as "charge transfer," "push–pull," or D–A chromophores. Importantly, this electronic feature, augmenting the chromophore's polarization, is typically accompanied by an enhanced sensitivity to environmental polarity, a general concept that has been widely applied in fluorescent probe design including fluorescent amino acids.[50, 113]

A classical example of such a chromophore is 4-amino-7-nitro-2,1,3-benzoxadiazole (NDB). Interestingly, compounds containing a nitro group are often assumed to be nonemissive, making the first report in the late 1960s of

the fluorescence properties of NBD containing glycine, with NBD attached to the *N*-terminus, a rare exception.[126] Almost a decade later, NBD alanine, also functionalized at the *N*-terminus, was subjected to rigorous investigation of its sensitivity to solvent polarity establishing that the quantum yield decreases, molar absorptivity increases, and both absorption and emission maximum undergo a redshift with increasing solvent polarity.[127] NBD's spectral responsiveness has led to applications in protein and membrane studies.[50] Much later, NBDAla (**18**) was developed, having alanine's methyl group replaced by NBD enabling substitution of alanine by its emissive surrogate **18**, enjoying a high fluorescence quantum yield and redshifted emission maximum in ethanol of 0.38 and 532 nm, respectively (Fig. 2.3, Table 2.3).[128] Similar to **18**, 51dansylAla (**19**), containing the well-known push–pull chromophore dansyl, also displays a significant polarity-dependent emission maximum while maintaining a robust quantum yield under common conditions (Fig. 2.3, Table 2.3).[123, 129] It has been used in folding/unfolding,[129] polarity,[130] and binding[131, 132] studies. Analogously, dansyl has also been exploited to impart lysine with emissive properties.[123]

Other related push–pull designs include 6DMNA (**20**)[119] and the Prodan-modified alanine Aladan (**21**)[133] (Fig. 2.3, Table 2.3). The former, 6DMNA (**20**), possessing a polarity-sensitive emission maximum ranging from 498 nm in dioxane to 592 nm in water, has been studied after incorporation in a central position in a hexapeptide[130] and in peptide–protein binding studies.[119] Prodan-containing Aladan (**21**), featuring a 130-nm difference in emission maximum going from apolar cyclohexane (401 nm) to polar water (531 nm), has been used to estimate local polarity in proteins[133] despite its alleged destabilizing effect.[134] It must be noted that the large polarity-dependent shift in emission maxima for Aladan (**21**) as well as 6DMNA (**20**) is also accompanied by significant shifts in absorption maximum thereby limiting the potential polarity sensitivity if expressed as a function of Stokes shift ($\nu_{abs} - \nu_{em}$). In contrast, polarity hardly has an effect on the absorption maximum of the dansyl chromophore (**19**). The polarity sensitivity of its emission maximum, however, is comparatively modest (Table 2.3).

It is important to note that even though the nomenclature of some of the aforementioned fluorescent amino acids implies that they are alanine mimics, their properties deviate significantly from the structural dimensions and polarity of the native amino acid. Hence, substitution of alanine for any of these emissive surrogates may adversely influence folding or stability upon incorporation into peptides or proteins, which in turn might affect the function. When isomorphicity is a prerequisite, the tryptophan mimics, here represented by 7azaTrp (**16**), are arguably among the most desirable fluorescent amino acid surrogates.

2.3.3 Synthetic Emissive Analogs of Nucleosides

Pioneering research in the 1960s and 1970s formed not only the foundation for modern fluorescent probe development for the study of membranes and proteins but also for the study of nucleosides. Merely 16 years after the unraveling of the double helix structure of DNA,[135] the inherent lack of fluorescent properties of the