PHOTONICS
# CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Contributors</td>
<td>ix</td>
</tr>
<tr>
<td>Preface</td>
<td>xiii</td>
</tr>
<tr>
<td>1 Fluorescence</td>
<td>1</td>
</tr>
<tr>
<td><em>David J. S. Birch, Yu Chen, and Olaf J. Rolinski</em></td>
<td></td>
</tr>
<tr>
<td>1.1 Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Spectra</td>
<td>2</td>
</tr>
<tr>
<td>1.3 Quantum Yield</td>
<td>6</td>
</tr>
<tr>
<td>1.4 Lifetime</td>
<td>12</td>
</tr>
<tr>
<td>1.5 Quenching</td>
<td>23</td>
</tr>
<tr>
<td>1.6 Anisotropy</td>
<td>30</td>
</tr>
<tr>
<td>1.7 Microscopy</td>
<td>38</td>
</tr>
<tr>
<td>1.8 Conclusions</td>
<td>48</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>48</td>
</tr>
<tr>
<td>References</td>
<td>49</td>
</tr>
<tr>
<td>2 Single-Molecule Detection and Spectroscopy</td>
<td>59</td>
</tr>
<tr>
<td><em>Michel Orrit</em></td>
<td></td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>59</td>
</tr>
<tr>
<td>2.2 Experimental Setups</td>
<td>62</td>
</tr>
<tr>
<td>2.3 Fluorescence Spectroscopy</td>
<td>66</td>
</tr>
<tr>
<td>2.4 Fluorescence Correlation Spectroscopy</td>
<td>72</td>
</tr>
<tr>
<td>2.5 Fluorescence Excitation Spectroscopy</td>
<td>78</td>
</tr>
</tbody>
</table>
7 Tissue Polarimetry 239
Alex Vitkin, Nirmalya Ghosh, and Antonello de Martino

7.1 Introduction, 239
7.2 Polarized Light Fundamentals, 240
7.3 Instrumentation, 266
7.4 Forward Modeling and Testing in Phantoms, 282
7.5 Applications, 297
7.6 Conclusions and Outlook, 313
References, 314

8 Optical Waveguide Biosensors 323
Daphné Duval and Laura M. Lechuga

8.1 Introduction, 323
8.2 Fundamentals of Label-Free Optical Waveguide Biosensing, 324
8.3 Surface Biofunctionalization, 328
8.4 Evaluation of Optical Biosensors, 331
8.5 Integrated Optical Waveguide-Based Biosensors, 334
8.6 Optical Fiber-Based Biosensors, 349
8.7 Lab-On-A-Chip Integration, 354
8.8 Summary, 357
References, 358

9 Light Propagation in Highly Scattering Turbid Media: Concepts, Techniques, and Biomedical Applications 367
R. R. Alfano, W. B. Wang, L. Wang, and S. K. Gayen

9.1 Introduction, 367
9.2 Physics Behind Optical Imaging Through a Highly Scattering Turbid Medium, 369
9.3 Study of Ballistic and Diffused Light Components, 378
9.4 Photon-Sorting Gates, 384
9.5 Transition From Ballistic to Diffuse Imaging in Turbid Media, 402
9.6 Conclusion, 404
Acknowledgments, 404
References, 404

10 Photodynamic Therapy 413
Rakkiyappan Chandran, Tyler G. St. Denis, Daniela Vecchio, Pinar Avci, Magesh Sadasivam, and Michael R. Hamblin

10.1 Historical Overview of PDT, 413
10.2 Introduction to PDT, 415
10.3 Photosensitizer Structure and Photophysical Properties, 418
10.4 Light Dosimetry and Photodynamic Therapy Light Sources, 422
10.5 Light-Based Strategies to Enhance PDT, 423
10.6 PDT Targeting and Nanotechnology, 425
10.7 PDT for Dermatology, 428
10.8 PDT for Oncology, 433
10.9 PDT for Infectious Disease, 435
10.10 PDT in Ophthalmology, 445
10.11 PDT and The Immune System, 446
10.12 Conclusion, 449
Acknowledgment, 449
References, 449

11 Imaging and Probing Cells Beyond the Optical Diffraction Limit 469
Mark Schüttpelz and Thomas Huser
11.1 The Quest for Achieving Optical Resolution Beyond ABBE’S Limit, 469
11.2 Stimulated Emission Depletion Microscopy, 474
11.3 Photoactivated Localization Microscopy and Stochastic Optical Reconstruction Microscopy, 477
11.4 Structured Illumination Microscopy, 483
11.5 Super-Resolution Optical Fluctuation Imaging and Other Approaches, 491
11.6 Outlook, 495
Acknowledgments, 496
References, 497

12 Technology 503
Ann E. Elsner and Christopher A. Clark
12.1 Basic Ocular Anatomy and Physiology, 503
12.2 Measurement Techniques, 514
12.3 Anterior Segment Diagnostics, Refractive Measurements, and Treatment, 522
12.4 Diagnostic Applications and Treatment of Posterior Segment, 529
References, 534

Index 543
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Since its inception, the term “photonics” has been applied to increasingly wide realms of application, with connotations that distinguish it from the broader-brush terms “optics” or “the science of light.” The briefest glance at the topics covered in these volumes shows that such applications now extend well beyond an obvious usage of the term to signify phenomena or mechanistic descriptions involving photons. Those who first coined the word partly intended it to convey an aspiration that new areas of science and technology, based on microscale optical elements, would one day develop into a comprehensive range of commercial applications as familiar and distinctive as electronics. The fulfilment of that hope is amply showcased in the four present volumes, whose purpose is to capture the range and extent of photonics science and technology.

It is interesting to reflect that in the early 1960s, the very first lasers were usually bench-top devices whose only function was to emit light. In the period of growth that followed, most technical effort was initially devoted to increasing laser stability and output levels, often with scant regard for possibilities that might be presented by truly photon-based processes at lower intensities. The first nonlinear optical processes were observed within a couple of years of the first laser development, while quantum optics at first grew slowly in the background, then began to flourish more spectacularly several years later. A case can be made that the term “photonics” itself first came into real prominence in 1982, when the trade publication that had previously been entitled Optical Spectra changed its name to Photonics Spectra. At that time the term still had an exotic and somewhat contrived ring to it, but it acquired a new respectability and wider acceptance with the publication of Bahaa Saleh and Malvin Teich’s definitive treatise, Fundamentals of Photonics, in 1991. With the passage of time, the increasing pace of development has been characterized by the striking
progress in miniaturization and integration of optical components, paving the way for fulfilment of the early promise. As the laser industry has evolved, parallel growth in the optical fiber industry has helped spur the continued push toward the long-sought goal of total integration in optical devices.

Throughout the commissioning, compiling, and editing that have led to the publication of these new volumes, it has been my delight and privilege to work with many of the world’s top scientists. The quality of the product attests to their commitment and willingness to devote precious time to writing chapters that glow with authoritative expertise. I also owe personal thanks to the ever-professional and dependable staff of Wiley, without whose support this project would never have come to fruition. It seems fitting that the culmination of all this work is a sequence of books published at the very dawning of the UNESCO International Year of Light. Photonics is shaping the world in which we live, more day by day, and is now ready to take its place alongside electronics, reshaping modern society as never before.

David L. Andrews

Norwich, U.K., July 2014
1

FLUORESCENCE

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1.1 INTRODUCTION

Within the wide range of spectroscopic techniques facilitated by photonics, fluorescence sits alongside the likes of spectrophotometry, Raman, FTIR, circular dichroism, and ultrafast in providing complementary and unique information. Although fluorescence can hardly be called a new phenomenon, there can be little doubt that it continues to facilitate many important new observations and techniques across a whole range of disciplines. Just as photonics has become an enabling technology so too fluorescence has become an enabling phenomenon. Fluorescence has made, and continues to make, particular impact in the biosciences and in healthcare. This has been dramatically demonstrated in recent years by the key role played by fluorescence in the complete sequencing of the human genome and in the displacement of radioactive markers by fluorescence probes in disease diagnostics. Underpinning the impact of fluorescence is a research base founded upon the fact that the nanosecond timescales and nanometer distances, in which the properties of fluorescence can be influenced, are ideally matched to many physiological processes and structures.

Originating from a spin-allowed singlet–singlet transition, fluorescence has a much higher quantum yield and is usually easier to study than its photophysical counterpart, phosphorescence, which involves a spin-forbidden triplet–singlet transition. Reflecting its more generic usage, and greater range of materials and conditions that facilitate fluorescence, here we concentrate on fluorescence rather than phosphorescence. Fluorescence is traditionally associated with aromatic molecules, of which there is a vast number, but recently there has been the emergence of a whole new
range of complementary luminescent nanoparticle emitters fabricated from semiconductors, gold and diamond.

When the readily accessible properties of fluorescence are combined with the high sensitivity afforded by photon counting photonics, fluorescence has enabled the ultimate limit of single molecule detection to be realized and this in turn is helping to open up new frontiers, such as molecular pathology, whereby metabolism, disease, and pharmacology can be studied at the most fundamental level. Taking the perspective of fluorescence as an enabling phenomenon, we have chosen in this chapter to survey the main techniques and measurements it “enables.” We cover spectra, quantum yield, lifetime, quenching, anisotropy, and microscopy, in each case citing topical review articles, many of the original references, underlying theory and modern day applications. The applications are also supported by descriptions of the context, theory, instrumentation, and techniques. Throughout we focus on the methods which are in most widespread use, while highlighting many of the most recent developments. There are already plenty of excellent general texts that survey fluorescence in the wider context of photophysics, and its related techniques, and in more depth than we do here [1–4]. Nevertheless, we hope our approach will provide a useful introduction for readers seeking to learn the basics through to the current state of the field by means of examples of what fluorescence might be able to do for them.

1.2 SPECTRA

Measuring absorption and fluorescence spectra is usually the first place to start in any fluorescence study. The origins of many of the fundamentals of fluorescence lie within spectra and although at times they might lack specificity, the importance of spectra in providing supporting information should not be overlooked when more advanced implementations of fluorescence are being undertaken.

1.2.1 Background and Theory

Fluorescence can be viewed as a multidimensional contour of intensity, wavelength, quantum yield, decay time, polarization, and position that together characterize the emitting species. Fluorescence spectra are today quite simple to measure and reveal information on the energy levels of a fluorophore, in terms of electronic (∼2–3 eV) and vibrational (∼0.01 eV) properties, that are superimposed on what is effectively a rotational continuum. All of these are capable of being influenced by the local environment, and hence, fluorescence spectra are not only a fingerprint of a molecule, but also can be used as a probe of local interactions.

The fluorescence spectroscopy of aromatic molecules is predominantly in the near ultraviolet (UV) to near-infrared (IR) (∼250–900 nm) as it is due to the excitation of weakly bound π-electrons rather than the more strongly bound σ-electrons. In general, where π-electron delocalization increases with the size of the molecule, the absorption and fluorescence spectra shift to longer wavelengths in the manner of a
FIGURE 1.1 Geometries for the measurement of (a) absorption ($I_0 - I$) and (b) fluorescence ($I_f$) spectra. (c) Simplified Jablonski energy level scheme for singlet states involved in fluorescence and (d) corresponding spectra measured in condensed media. PPO refers to the scintillator 2,5-diphenyloxazole, NATA is N-acetyl-l-tryptophanamide, a derivative of the fluorescent amino acid tryptophan, and HSA is the protein human serum albumin, which contains a single tryptophan.

particle in a box [2]. This can often lead to a useful intuitive expectation of where spectra occur for different molecules in respect to each other before any measurement is performed.

Figure 1.1 illustrates some of the basics of fluorescence spectroscopy. Unlike the measurement of absorption spectra, which by necessity requires the incident light and transmitted light to be detected in-line, fluorescence is usually detected off-axis in order to minimize the detection of the excitation light as this would otherwise swamp the much smaller fluorescence signal. In general, fluorescence is isotropic and is usually detected at 90° to the excitation as illustrated. The energy level scheme shown in Figure 1.1 just relates to the singlet manifold and is a simplified version of the Jablonski scheme [2]. The spectra shown illustrate the fact that in condensed media fluorescence occurs from the lowest vibrational level of the lowest electronic excited state $S_1$ to vibrational levels of the $S_0$ electronic ground state (Kasha’s rule [2]). Therefore, whereas absorption spectra contain information on the vibrational spacing of the excited state, fluorescence spectra contain information on the vibrational spacing of ground state. The fact that vibrational levels, although quantized, are not well resolved is due to the spectral smearing generated by rotational modes of lower energy. Taken together these properties result in fluorescence being
shifted to longer wavelengths as compared to absorption, the so-called Stokes shift, with both spectra often displaying mirror image symmetry across their overlap [1].

1.2.2 Experimental

Figure 1.2 shows the common L-format configuration of a fluorimeter for recording fluorescence spectra. It comprises a xenon source, excitation monochromator (e.g., Seya-Namioka geometry as shown here or Czerny-Turner), sample compartment, emission monochromator, and photomultiplier detector. Further optical components, either lenses or mirrors, for focusing are required in order to match the cone angles of the excitation and fluorescence to the monochromator \( f \) number. Polarizers, either dichroic for the visible or quartz prisms to extend down to the UV, are sometimes added (e.g., for use in anisotropy studies—see Section 1.6). Fluorescence spectra are usually corrected for the spectral response of the emission monochromator and detecting photomultiplier, by division of this combined instrumental function.
This configuration can also be used to record excitation spectra by keeping the monochromator tuned to an emission wavelength while scanning the excitation monochromator wavelength $\lambda$. For an optically dilute sample, the excitation spectrum is equivalent to the sample’s absorption spectrum. From the Beer–Lambert law,

$$I(\lambda) = I_0(\lambda) 10^{-\varepsilon(\lambda)cd},$$

where $I$ is the transmitted intensity after sample absorption, $I_0$ the incident intensity, $c$ the molar concentration in mol L$^{-1}$ (M), $d$ the sample path length in cm, and $\varepsilon(\lambda)$ the decadic molar extinction coefficient in mol$^{-1}$ L cm$^{-1}$, the ordinate in an absorption spectrum and the molecular fingerprint which is described by the spectral shape. $\varepsilon(\lambda)cd$ is defined as the optical density or absorbance of the sample.

In the limit of dilute solution, defined as $\varepsilon(\lambda)cd \ll 1$, and defining the fluorescence quantum yield $\Phi_f$ as the ratio of the rate of total fluorescence emission $I_f$ to the rate of absorption:

$$\Phi_f = \frac{I_f}{(I_0 - I)},$$

we obtain [5]

$$I_f = 2.303\Phi_f\varepsilon(\lambda)cd.$$

Because $\Phi_f$ is usually independent of excitation wavelength for aromatic molecules in condensed media, Eq. (1.3) shows how detecting fluorescence (even at only one emission wavelength), while scanning the excitation wavelength, leads to an excitation spectrum that is equivalent to the absorption spectrum for dilute solutions because $I_f \propto \varepsilon(\lambda)$. In the case of absorption, the spectrophotometer used to measure spectra automatically corrects for the spectral distribution of the light source, the spectral response of the monochromator and detecting photomultiplier, by dividing the two signals generated in a dual beam arrangement of sample and reference channels. By similar means fluorescence excitation spectra need to be corrected for the light source spectral output and the excitation monochromator spectral response. In this regard, rhodamine B in ethylene glycol has long been used as a quantum counter [6] as its fluorescence yield is independent of excitation wavelength in the range 220–600 nm and this overcomes the wavelength response of the photomultiplier. The limit of detection for fluorescence is orders of magnitude lower than that of absorption (e.g., $\sim 10^{-12}$ M compared to $10^{-8}$ M) because the latter ultimately relies upon measuring a small difference between two large signals in the sample and reference channel. Fluorescence on the other hand compares a signal with zero signal or low background, which is a much easier measurement.

### 1.2.3 Application Example—Melanin Spectra

In order to illustrate how absorption and fluorescence spectra often interplay in tandem, we consider the example of the auto-oxidation of 3,4-dihydroxy-\textit{l}-phenylalanine (\textit{l}-DOPA) to produce melanin (Fig. 1.3). \textit{l}-DOPA is a small molecule as aromatics go, but during its auto-oxidation a complex series of intermediaries are
FIGURE 1.3 (a) Absorption and (b) fluorescence spectra as eumelanin is polymerized from the auto-oxidation of an aqueous solution of L-DOPA at pH 12. The fluorescence excitation wavelength is 275 nm. (For a color version of this figure, see the color plate section.)

formed before an extended structure, believed to bear a close similarity to natural eumelanin, is formed [7].

Initially the phenyl ring of L-DOPA is seen to dominate the absorption spectrum with a peak occurring below 300 nm, similar to that for benzene, toluene, and the three fluorescent amino acids phenylalanine, tyrosine, and tryptophan. At these times, the fluorescence is quite strong. However, as time progresses, more extended structures are formed to give the characteristically broad band attenuation spectrum of melanin describing the photoprotective action of melanin and the 275-nm excitation generates less fluorescence as the excitation energy is dissipated nonradiatively [8]. Ideally it is preferable to use intrinsic fluorescence to report on structure. This is because it does not distort native structure as extrinsic fluorescence probes can do. However, complex heterostructures such as melanin usually contain multiple fluorophores and give a complex intrinsic fluorescence signature that is difficult to interpret. The same is true of the many types of protein that contain multiple fluorescent amino acids.

1.3 QUANTUM YIELD

Measuring absolute fluorescence quantum yield is not so common an objective these days since most of the fundamental processes of aromatic are well-established and the focus of fluorescence has shifted more toward biological molecules, where their natural environment is usually saturated with the quencher oxygen. However, recording relative changes is still very important in many studies.

1.3.1 Theory

Because the time-averaged rate of absorption must equal the total decay rate (otherwise energy would be piling up in a molecule), we can also express
Eq. (1.2) describing the fluorescence quantum yield $\Phi_f$ in terms of intramolecular processes as

$$\Phi_f = k_r / (k_r + k_{nr}),$$  \hspace{1cm} (1.4)$$

where $k_r$ is the radiative decay rate (units s$^{-1}$), $k_{nr}$ the nonradiative decay rate (composed of internal conversion to the ground state or intersystem crossing to the lowest triplet state), and their sum is the total decay rate. For this reason floppy molecules generally have a lower $\Phi_f$ than more rigid molecules. External interactions, for example, collisional quenching by oxygen, bring additional terms to the denominator and further reduce $\Phi_f$. For this reason, oxygen needs to be removed, usually by freeze-pump-thaw cycles or by nitrogen bubbling, for the unquenched $\Phi_f$ to be determined correctly. It will be seen that $\Phi_f$ can have a maximum value of 1. Unfortunately the simplicity of the definition of $\Phi_f$ belies the difficulty of determining it.

### 1.3.2 Experimental

It can be appreciated from Eq. (1.2) that, in principle, measuring the fluorescence quantum yield requires the determination of the number of photons absorbed and emitted due to fluorescence in a given time. This is a difficult requirement for which integrating spheres can be used. The sample is placed inside a reflective sphere with excitation and emission ports in an attempt to collect all the isotropic fluorescence over a $4\pi$ solid angle. Multiple reflections relay the fluorescence to the exit port. Both solids and solutions can be measured. Figure 1.4 shows one such configuration for an integrating sphere. Excitation and fluorescence rays are depicted and re-excitation by one of the fluorescence pathways shown illustrates a common source of error.

**FIGURE 1.4** (a) Typical integrating sphere light paths and (b) construction with sample loading mechanism displayed for cuvettes (top) and solid samples and powders (bottom). Photos courtesy of Horiba Scientific.
A baffle is usually incorporated to minimize reflections from the entrance to exit ports. Excitation light still needs to be spectrally filtered out after the exit port.

Integrating spheres have a long-established [9] and ongoing role to play in the quantification of standards, though they tend to be somewhat impractical and hence not in widespread use. Other absolute methods include calorimetry, photoacoustic spectroscopy actinometry, and thermal lensing. However, the usual recourse is to ratio the spectrally corrected fluorescence spectra of the unknown sample and a suitable reference standard of known quantum yield, that is, to determine a relative rather than an absolute quantum yield. A literature survey soon reveals that fluorescence quantum yields have often courted controversy over the years due to inconsistent measurements between different laboratories. Procedures for absolute and relative quantum yield measurements in solution are discussed in a recent article along with a comprehensive list of references on previous work and recommendations for standards (Table 1.1) [10].

The process by which relative quantum yields are determined involves first correcting the fluorescence spectrum for the spectral responses of the emission monochromator and detecting photomultiplier in order to obtain a true and undistorted fluorescence spectrum. For the sample S and reference standard R, of absorbance $A_S$ and $A_R$, and respective solvent refractive indices $n_S$ and $n_R$, we obtain

$$\Phi_{fs} = \frac{\Phi_{fr}I_{fs}(\lambda)A_Rn_S^2}{I_{fr}(\lambda)A_Sn_R^2},$$

(1.5)

where $I_{fs}(\lambda)$ and $I_{fr}(\lambda)$ refer to the integrated and spectrally corrected fluorescence spectra over wavelength for the sample and reference, respectively. Ideally the same solvent is used for both the sample and reference, but the $n^2$ ratio corrects for the effect of any solvent difference on the optical geometry over which fluorescence is collected [11]. Solvent refractive index also influences the fluorescence lifetime and the higher precision of lifetime measurement has proved useful in resolving seemingly conflicting results for quantum yield standards [12].

The accuracy in $\Phi_f$ is typically no better than 5–10% and such a spread in values is clearly evident in the literature [10]. This seems incongruous with modern day instrumentation, but in fact relative changes of either peak or integrated fluorescence, without measurement of the actual $\Phi_f$, or even spectral correction, are often adequate. The accurate determination of fluorescence quantum yield, like removing the quencher oxygen from solutions before measurement, is no longer of importance in many applications. This is illustrated by the following example.

### 1.3.3 Application Example—ThT Detection of Sheet Structure

Sheet structures occur in nature, for example, as stacks of graphite and $\beta$-sheets of protein or peptide that sometimes form fibrils [13]. Currently sheet structures are of considerable interest when synthesized as isolated monosheets in carbon nanotubes and graphene [14]. Thioflavin T (ThT) is a fluorescent dye (see Fig. 1.5) that has been
TABLE 1.1 Some fluorescence quantum yields ($\Phi_f$) with errors ($\Delta\Phi_f$) at 25°C that can be useful as standards for comparison and determining relative quantum yields

<table>
<thead>
<tr>
<th>Dye</th>
<th>Quinine sulfate</th>
<th>Coumarin 153</th>
<th>Fluorescein</th>
<th>Rhodamine 6G</th>
<th>Rhodamine 101</th>
<th>Oxazine 1</th>
<th>HITCI</th>
<th>IR125</th>
</tr>
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<tbody>
<tr>
<td>Solvent</td>
<td>0.105 M HClO₄</td>
<td>Ethanol</td>
<td>0.1 M NaOH</td>
<td>Ethanol</td>
<td>Ethanol</td>
<td>Ethanol</td>
<td>Ethanol</td>
<td>DMSO</td>
</tr>
<tr>
<td>$\Phi_f$</td>
<td>0.59</td>
<td>0.53</td>
<td>0.89</td>
<td>0.91</td>
<td>0.915</td>
<td>0.15</td>
<td>0.15</td>
<td>0.30</td>
</tr>
<tr>
<td>$\Delta\Phi_f$</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.028</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
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FIGURE 1.5  Increase in ThT fluorescence as beta sheets of Aβ are formed. The excitation wavelength is 450 nm and the fluorescence peak is at 480 nm. Reprinted from Reference 17 with permission from Elsevier.

widely used for revealing the formation of sheet structures [15], most notably when studying the aggregation of the peptide beta-amyloid (Aβ); the early oligomers of which are thought to be the precursor to Alzheimer’s disease. As already mentioned, floppy molecules generally have a low fluorescence yield due to having more favorable dissipative pathways other than fluorescence. Conversely rigidity imparts a higher fluorescence yield. ThT behaves as a molecular rotor and upon excitation undergoes intramolecular rotation to a low-fluorescent twisted intramolecular charge transfer (TICT) state [16]. This rotation is restricted between sheet structures, increasing the fluorescence quantum yield, which can thus be used as a probe of sheet formation. Figure 1.5 shows the peak wavelength and shape of the fluorescence spectrum of ThT in Aβ are constant as β-sheets are formed [17]. Hence it is perfectly workable to use the relative change in fluorescence quantum yield to monitor structural changes without actually having to put a number to Φf.

One of the limitations of using ThT to detect Aβ aggregation is that the early-stage oligomers, that are thought to disrupt neuronal cellular membranes and initiate Alzheimer’s disease, are not detected since only the β-sheets formed later lead to an increase in fluorescence. Changes in fluorescence lifetimes (to be discussed in the next section) have recently been shown to address this short-coming by using the intrinsic tyrosine fluorescence lifetime in the case of Aβ [17] and ThT fluorescence lifetime in the case of insulin fibrils [18].

In cases like eumelanin, where the intrinsic fluorescence is complex (see Fig. 1.3) due to the presence of multiple fluorophores, extrinsic probes with bespoke structural sensing characteristics like ThT come to the fore. Surprisingly, although the composition of melanin is well known to be largely dihydroxyindoles, its secondary structure, the minimum functional unit, and the very existence of a protomolecule
that can be replicated to form a secondary structure remains unclear despite decades of research with a whole gamut of techniques [7, 8]. The significance of not knowing the structure of eumelanin has bearing on a whole range of topics from bioelectronics (melanin is an efficient conductor and readily binds metal ions) to melanoma, the most virulent form of skin cancer. The likely structural steps in melanin synthesis are depicted in Figure 1.6.

Planar oligomeric structures of dihydroxyindoles are thought to form sheets that are bound together by π–π interactions. The oxidation of primary structures, either spontaneously or catalyzed, is generally thought to give rise to protomolecules (oligomers) composed of four or five monomers. These combine into planar sheets that stack due to π bonding, albeit less strongly bound than σ covalent bonds, thus accounting for eumelanin’s softness in the manner of graphite. The close packing, including π–π* interactions (~0.3–0.4 nm apart [8]), being consistent with eumelanin’s photo-protection via ultrafast nonradiative relaxation. The larger sheet structures have been proposed to form onion-like layers [19]. Recent fluorescence studies, based on the increase in ThT fluorescence quantum yield as eumelanin is formed by auto-oxidation of L-DOPA, support the notion of assembly via a protomolecule that subsequently forms sheets rather than assembly by monomer addition [20]. This is illustrated in Figure 1.7.
FIGURE 1.7 (a) Fluorescence spectrum of ThT, excited at 450 nm and peaking at 482 nm, superimposed on the intrinsic fluorescence background, as eumelanin is synthesized at pH 10 by the auto-oxidation of L-DOPA. (b) ThT fluorescence at 482 nm as the eumelanin synthesis progresses at pH 10 (∗), pH 10.8 (▲) and pH 11.4 (△). Reproduced from Reference 20 with permission from AIP Publishing LLC.

The melanin growth, as reflected by the increase in ThT fluorescence can be described using a classic sigmoidal function analogous to that encountered in amyloid fibrillation such as that of Aβ [21]:

\[ I_f(t) \sim I_{f0} + I_{\text{max}} / \{1 + \exp[-k_m(t - t_{1/2})]\}, \quad (1.6) \]

where \( I_f(t) \) is the fluorescence intensity and \( I_{f0} \) is the initial or background fluorescence intensity. The parameter \( I_{\text{max}} \) is the fluorescence maximum above the background, \( k_m \) is the rate of melanin formation, and \( t_{1/2} \) is the time when the fluorescence intensity has reached the half maximum value.

The very existence of the time-lag suggests that eumelanin is not formed by monomer addition, but rather it requires a protomolecule (or protomolecules) in a “binding ready” conformation to be preformed first. Moreover, the increase in ThT fluorescence in itself further supports the presence of sheet-like structures in eumelanin with the fluorescence no longer increasing when the eumelanin-forming reaction is complete.

1.4 LIFETIME

The fluorescence lifetime of a molecule is the average time it spends in the excited state before emitting a fluorescence photon. Typically in the nanosecond region, the fact that the fluorescence lifetime has a finite value is due to local perturbations and therein rests its usefulness as a probe on the angstrom scale.
Lifetime measurement has emerged in recent years as the most powerful and versatile technique in fluorescence spectroscopy. It can be performed in either the time or frequency domain. In comparison to spectra, quantum yield, and intensity, lifetime provides more kinetic information, a visual image that is easier to interpret, is analogous to a movie compared to a photo, resolves dynamics more closely, provides a more stringent test of kinetics models, and is easier to calibrate. Fluorescence lifetime is also making a mark beyond spectroscopy. For example, in sensing and fluorescence lifetime imaging microscopy (FLIM), to be discussed in Section 1.7.

1.4.1 Theory

The fluorescence lifetime \( \tau_f \) for aromatic fluorophores can be defined in terms of rate parameters, consistent with the definition for the fluorescence quantum yield \( \Phi_f \) (Eq. 1.4), as [2]

\[
\tau_f = \frac{1}{k_r + k_{nr}}. \tag{1.7}
\]

In the time domain, \( \tau_f \) can be conveniently visualized as it describes the fluorescence impulse response to \( \delta \)-function excitation at \( t = 0 \) given by

\[
i(t) = i(0) \exp\left(\frac{-t}{\tau_f}\right), \tag{1.8}
\]

and \( i(t) \) is the solution to the rate equation defining the decay of the concentration (population) of the \( S_1 \) singlet excited state \( M^* \), due to fluorescence emission:

\[
\frac{d[M^*]}{dt} = -(k_r + k_{nr})[M^*] = -\frac{[M^*]}{\tau_f} \tag{1.9}
\]

if multiple and noninteractive fluorescent emitters are present, the same treatment can be extended linearly by simply adding further exponential terms describing different lifetimes. The application of fluorescence lifetimes has benefitted greatly from the fact that most aromatic fluorophores possess a single exponential decay.

Combining Eqs. (1.4) and (1.7) we obtain

\[
\tau_f = \frac{\Phi_f}{k_r}. \tag{1.10}
\]

Equation (1.10) illustrates why the fluorescence lifetime can be used to monitor changes that influence fluorescence, as providing the radiative rate \( k_r \) remains constant, \( \tau_f \) offers equivalent information to \( \Phi_f \), is easier to measure, more precise and less prone to systematic error. Conversely if \( \Phi_f \) and \( \tau_f \) are measured, \( k_r \) and \( k_{nr} \) can be determined.
$k_r$ can be described by the Einstein coefficient $A$ and the transition dipole moment operator $M$ as [2]

$$k_r = \sum_m A u \rightarrow \text{Im} / <\psi^*_l/M/\psi_u> l^2$$

(1.11)

where, unlike for spectral lines in the simpler atomic case, $A$ has to be summed over the whole fluorescence spectrum attributed to the lowest vibrational level $u$ of the excited $S_1$ singlet state to the different vibrational levels $m$ of the singlet $S_0$ ground state.

The Strickler–Berg expression [22] and its variants [2] enable $k_r$ to be determined more easily by introducing the Einstein $B$ coefficient relating to the absorption spectrum:

$$k_r = 2.88 \times 10^{-9} n^2 <v_F^{-3}> A^{-1} \int \frac{\epsilon(v)dv}{v} ,$$

(1.12)

where $<v_F^{-3}> A^{-1}$ is the reciprocal of the mean value of $v_F^{-3}$ over the fluorescence spectrum ($v = 1/\lambda$) and $\int \frac{\epsilon(v)dv}{v}$ relates to the area under the absorption frequency spectrum.

The fluorescence lifetime dependence on $n^2$ via the Strickler–Berg equation has been shown to probe refractive index in cells using protein labeled with the green fluorescent protein [23].

Equation (1.12) can be approximated to

$$k_r \sim 10^{-4}/\varepsilon_{\text{max}}.$$

(1.13)

Given $\varepsilon_{\text{max}} \sim 10^5 \text{ mol}^{-1} \text{ cm}^{-1}$ for a strong absorber, Eq. (1.13) predicts that, in the absence of intramolecular nonradiative processes or intermolecular quenching, $\tau_f$ is $\sim 1$ ns. It should be cautioned that agreement between Eqs. (1.10) and (1.12) can be quite variable [24] due to potential sources of experimental error (see Section 1.3) and the presence of more complex photophysics, for example, quinine sulfate having a bi- not mono-exponential fluorescence lifetime decay [25]. Significant discrepancies between $k_r$ determined from Eq. (1.10) (which monitors the emitting state) and Eq. (1.12) (which monitors the absorbing state) have often been used to determine the presence of “hidden states,” such as those present in polyenes that are facilitated by intra-molecular twisting [26,27].

In the frequency domain, the equivalent expressions to Eq. (1.8) are

$$\tan \phi = -\omega \tau_f$$

(1.14)

$$m = 1/ (1 + \omega^2 \tau_f^2)^{1/2},$$

(1.15)

where $\omega$ is the modulation frequency (in rad s$^{-1}$) and $m$ a modulation factor that describes the relative amplitude of the fluorescence and excitation wave forms. The