Chapter 13
UV Fluorescence Detection and Spectroscopy in Chemistry and Life Sciences

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Abstract Fluorescence techniques are nondestructive analytical methods used in a wide range of applications. Since many fluorophores of interest can be excited with UV light and nowadays compact solid-state UV emitters are available, UV fluorescence methods are emerging. The chapter will give a survey of both fundamentals and applications of fluorescence in chemistry and life sciences emphasizing actual and potential applications of solid-state UV emitters. Particular attention is drawn to the use of autofluorescence spectroscopy for the detection of microorganisms and as diagnostic method for skin diseases.

13.1 Introduction

Fluorescence techniques, spanning fluorescence spectroscopy, fluorescence sensing, imaging, and microscopy are nondestructive optical, analytical methods. They are nowadays increasingly used in numerous scientific disciplines such as molecular biology, biophysics and chemistry, clinical diagnosis, and analytical and environmental chemistry. Besides their widespread use in fundamental and applied research, substantially efforts have been made in commercializing new fluorescence-based analytical devices. In the field of genomics, proteomics and

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M. Kneissl and J. Rass (eds.), *III-Nitride Ultraviolet Emitters*,
Springer Series in Materials Science 227, DOI 10.1007/978-3-319-24100-5_13

drug discovery fluorescence detection systems are indispensable tools enabling high-throughput screenings.

The increased use of fluorescence techniques is greatly enhanced by the commercial availability and further improvement of instrumentation such as compact (laser) light sources, highly sensitive detector arrays, or increasingly efficient sensor and imaging electronics.

Due to the strong influence of the surrounding medium on the fluorescence emission, fluorescent molecules are intensely used as probes for analyte recognition or investigation of chemical and biological systems. Many new fluorescent probes and reporters with selectivity for specific microenvironmental properties have been designed and developed, leading to an enhanced employment of fluorescence techniques in clinical diagnostics and environmental monitoring. Probing inside living cells is another broad field of application in the life sciences benefiting from the availability of novel and highly specialized fluorescent probes.

Besides the development and application of synthetic fluorescent probes specifically labeling (bio-)molecules or biological structures, label-free autofluorescence spectroscopy, making use of intrinsic fluorescent molecular species, is gaining more and more importance in biological cell imaging, tissue diagnostics, microorganism detection, and proteomics. Many of these intrinsic fluorophores, such as aminoacids, pyridine nucleotides, or collagen can be excited by UV light, making this a promising field for application of solid-state UV emitters.

Numerous monographs, handbooks, and reviews are available in the field of fluorescence reflecting its high relevance for a broad field of application (see e.g., [1–8]). Fluorescence detection and spectroscopy of natural and artificial fluorophores in general cover the UV, VIS, and IR spectral range. Due to the broad availability of powerful and compact (laser) light sources in the VIS and NIR, established fluorescence techniques and probes are still focusing on this spectral range. Especially in the context of bioimaging and genomics, fluorescence instrumentations and probes are designed to circumvent short-wavelength light-induced autofluorescence emission potentially disturbing the fluorescence signal of the specific probe (usually emitting in the VIS or NIR spectral range). Nevertheless, many natural and synthetic fluorophores can be excited with UV light. Thus, the scope and merit of this chapter is a survey of the extremely broad field of fluorescence emphasizing actual and potential applications of solid-state UV emitters. It starts with a short compendium of the basic phenomena and instrumental aspects of fluorescence techniques as well as an overview of UV excitable fluorophores (Sect. 13.2). The following Sect. 13.3 covers applications of fluorescence spectroscopy as analytical technique in lab-based organic, analytical and environmental chemistry, and biochemistry. In Sect. 13.4, fluorescent chemical sensing for analyte recognition and monitoring, imaging and detection of biological molecules, structures and cell functions as well as genomic and proteomic studies is introduced. The detection of living microorganisms as special biological analytes of increasing concern is issued separately in Sect. 13.5. Finally, in Sect. 13.6 fluorescence diagnosis of skin and tissue diseases is discussed with special emphasis on multiple wavelengths autofluorescence spectroscopy for skin cancer diagnosis.

13.2 Fundamentals and Apparative Aspects of Fluorescence Detection and Spectroscopy

Luminescence in general is the deactivation of electronically excited states by emission of light. Photoluminescence as an absorption-induced luminescence is subdivided into fluorescence and phosphorescence taking into account the spin multiplicity of the molecule. A molecule that shows fluorescence is called a fluorophore. A common tool to visualize the different processes and energy levels involved in the absorption and emission of light is the Jablonski diagram given in Fig. 13.1 which was first proposed by Professor Alexander Jablonski in 1935 [9]. It illustrates spectra and kinetics of fluorescence, phosphorescence, and delayed fluorescence.

In 1852, Sir George Gabriel Stokes coined the term fluorescence for the effect of UV-induced colored light emission of certain substances and formulated the so-called Stoke's law [10]. It says that the wavelength of a fluorescent light is always greater than the wavelength of the exciting light. This is usually observed for molecules in solution with one photon absorption. The distance between absorption and emission maximum is called the Stokes shift and is commonly expressed in wavenumbers (Fig. 13.2). In general, the Stokes shift is caused by thermalization of vibrational energy of the excited and the ground state but it is also influenced by solvent polarity, complex formation, excited-state reactions, and/or energy transfer. Luminescence whose radiation is located in a spectral region of shorter wavelengths than that of the exciting radiation is called anti-Stokes luminescence. In Raman spectroscopy, the appearance of (inelastically scattered) anti-Stokes radiation is common. In contrast, anti-Stokes luminescence is a less common effect and can only be observed as a result of sophisticated processes like multiphoton absorption [11], phonon-assisted photoluminescence upconversion (e.g., in semiconductor nanostructures) [12], or plasmon enhancement [13].

An important parameter of a photophysical or photochemical process is its quantum yield or quantum efficiency which is equal to the number of photons undergoing the process in relation to the total number of photons absorbed. The

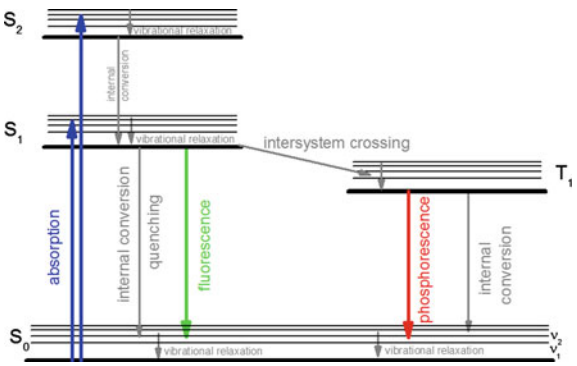

Fig. 13.1 Jablonski diagram

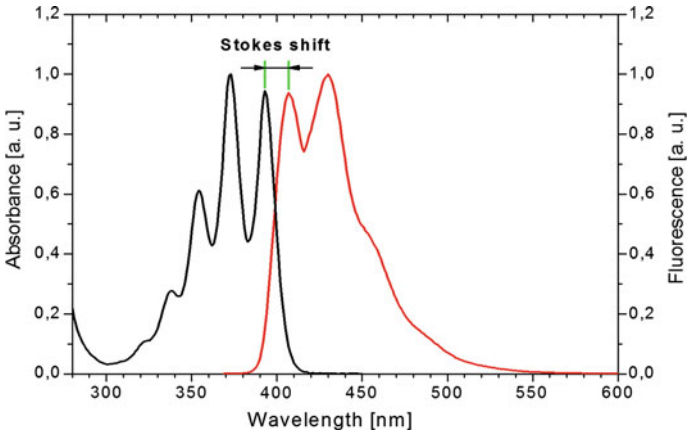

Fig. 13.2 Absorption (*black*) and emission (*red*) spectra of 9, 10-Diphenylanthracene in Cyclohexane (color online)

sum of quantum yields of all possible processes is equal 1. The fluorescence quantum yield Φ_F is the ratio between the number of fluorescence photons emitted and the number of photons absorbed. It provides information of competing non-radiative processes leading to depletion of the excited singlet state S_1 (see Fig. 13.1), like internal conversion (IC), intersystem crossing (ISC), intramolecular charge transfer, conformational changes, and interaction in the excited state with other molecules (electron and proton transfer, energy transfer, excimer, or exciplex formation). Φ_F can be determined experimentally by absolute measurements or relative to a fluorescent standard with a known Φ_F. In [14], different experimental procedures and techniques for the determination of Φ_F are described and compared. Both Φ_F and shape of the emission spectrum are independent of the excitation wavelength. The natural or intrinsic fluorescence lifetime (τ_0) denotes the mean time a molecule spends in the excited state if fluorescence is the only deactivation process. It is a theoretical value that cannot be determined experimentally due to competing processes like IC, ISC, or chemical reactions decreasing the experimentally accessible fluorescence lifetime, the latter referred to as excited-state fluorescence lifetime (τ_{ex}).

The interactions of a fluorescent molecule with its local environment (e.g., solvent, solid surface, organized media) are usually reflected in the spectral appearance and shape of the absorption and emission bands, the fluorescence quantum yield as well as the lifetimes of the excited-state molecules. The solvent-dependence of the position of emission bands is commonly included in the term solvatochromism [15] covering physical, chemical, temperature, and surface effects [16]. Physical effects are caused by dipole–dipole interactions of solute and solvent. In more polar environments, polar fluorophores will relax to a lower vibrational energy state before emission, resulting in emission at lower energies, i.e., longer wavelengths. Chemical effects are intermolecular hydrogen bonding,

proton or electron transfer (e.g., pH), external heavy-atom effects, and complex formation. The temperature of the environment can affect the luminescence behavior directly through temperature dependence of competing nonradiative processes and indirectly through temperature-dependent chemical phenomena like ground state complex formation, hydrogen bonding, or diffusion-controlled reactions (quenching). Further, if two electronically excited states of the solute are of very similar energy, a thermal promotion of the molecule to the upper state can occur. Metallic surfaces may induce an increase or decrease of radiative decay and resonance energy transfer rates of adsorbed fluorophores [1]. In metal or surface-enhanced fluorescence, near-field coupling between the fluorophore and surface modes of the metal plays a crucial role [17].

Reactions of a fluorescent molecule from the excited state S_1 such as proton or electron transfer, energy transfer, complex formation, or collisions with other molecules may lead to a depletion of the excited state by radiationless processes and thus to a decrease in fluorescence intensity and fluorescence lifetime referred to as quenching. The formation of nonfluorescent ground state complexes is called static quenching. For dynamic or collisional quenching, a key requirement is that the quencher must diffuse to the fluorophore during the lifetime of the excited state in order to cause a decrease in fluorescence intensity. It does not require spectral overlap and occurs only when the fluorophore and quencher are in molecular contact (within $\sim$5 Å) [1]. The decrease in fluorescence intensity due to quenching can be expressed by

$$F_0/F = 1 + K_{SV}[Q]$$

where F is the fluorescence intensity in the presence of quencher, F_0 is the intensity in the absence of quencher, $[Q]$ is the quencher concentration, and K_{SV} is the Stern–Volmer quenching constant. K_{SV} is a general term that may refer to either dynamic or static quenching processes. For static and dynamic quenching, the effect of quencher concentration on fluorescence lifetime is different. For static quenching, τ_0/τ is equal to 1 and for dynamic quenching $\tau_0/\tau = F_0/F$. Some examples of typical quenchers are molecular oxygen (quenching almost all fluorophores), aromatic and aliphatic amines (quenching unsubstituted hydrocarbons), and bromide and iodide (quenching through spin–orbit interaction).

Resonance energy transfer (RET, also FRET for Förster or fluorescence resonance energy transfer) is a through space transfer of excitation energy from a donor fluorophore to an acceptor molecule within a distance of 10–100 Å. The donor and acceptor must be spectrally related (donor emission is absorbed by the acceptor) and the donor and acceptor transition dipole orientations must be approximately parallel. By RET to an acceptor (which does not necessarily be fluorescent itself), the detectable emission of light by the donor fluorophore is quenched. This phenomenon is intensively used in biosensing and bioimaging (see Sect. 13.4). The distance at which 50 % of excited donors are deactivated by RET is defined as the Förster radius R_0 with its magnitude depending on the spectral properties of the donor and acceptor dyes.

The extent to which a fluorophore rotates during the excited-state lifetime τ_{ex} determines its polarization or anisotropy. These are both expressions for the same phenomenon. Fluorescence anisotropy (r) and polarization (P) are defined by

$$r = (I_v - I_h)/(I_v + 2I_h) \quad \text{and} \quad P = (I_v - I_h)/(I_v + I_h)$$

where I_v and I_h are the fluorescence intensities of the vertically (v) and horizontally (h) polarized emission, when the sample is excited with vertically polarized light [1]. Anisotropy measurements are widely used in biochemical applications (size and shape of proteins, local viscosity, or rigidity of various molecular environments).

Luminophores (fluorophores and phosphors) can be found in all kinds of compounds (inorganic, organic, and organometallic). The class of inorganic luminophores [4] includes

- transition metal and rare earth cations,
- uranyl ion,
- metal doped glasses,
- crystals and nanocrystals (Quantum dots) of semiconductor materials,
- metal ion doped crystals (e.g., ruby, beryl),
- trapped electron–hole pairs and oxygen-related lattice imperfections in crystals (e.g., silicates), and
- gases (e.g., SO_2, NO).

Organic luminophores are usually divided into natural (intrinsic) and synthetic fluorophores [18, 19]. Natural fluorophores absorbing in the UV are as follows:

- amino acids (tryptophane, tyrosine, phenylalanine);
- pyridine nucleotides (NADH and NADPH), flavins, and vitamins;
- cellulose, chitin, lignin, sporopollenin, collagen, and elastin;
- suberin and cutin;
- chlorophyll and phaeopigments, flavonoids, and alkaloids; and
- DNA/RNA and humic-like substances.

An overview of natural fluorophores relevant for characterization of human tissue can be found in Table 13.1 of Sect. 13.6. Synthetic organic luminophores absorbing in the UV spectral range are as follows:

- aromatic and polycyclic aromatic hydrocarbons (pyrene, anthracene, phenanthrene, benzene, benzo[a]anthracene, chrysene),
- naphthalene derivatives (dansyl chloride, EDANS), pyrene derivatives (Cascade Blue, Alexa Fluor 405 Dye, 1-Pyrenesulfonyl Chloride, HPTS),
- coumarines (Alexa Fluor 350 Dye, AMCA-X, DMACA, Marina Blue),
- quinolines (quinine sulfate),
- indoles, imidizoles (DAPI, Hoechst 33342), and
- other small heterocyclic molecules (Bimane, Lumazine, Carbazole, dyes with diaryloxazole structures).

Table 13.1 Fluorophores in human tissue [141, 142]

Fluorophore	Excitation (nm)	Fluorescence (nm)
Amino acids		
Phenylalanine	260	280
Tyrosine	275	300
Tryptophan	275	350
	280	350
Structural proteins		
Collagen	340–360	395–450
	320–350	400–440
Elastin	360	410
	290–325	340, 400
Collagen cross links	380–420	440–500
Elastin cross links	320–360, 400	480–520
Keratin	450–470	500–530
Enzymes and co-enzymes		
NADH	350	460
	290, 350–370	440–460
NADPH	340	460
FAD, flavins	450	520
	450	500–540
Vitamins		
Vitamin A	327	510
Vitamin D	390	480
Vitamin K	335	480
Vitamin B_6	320–340	400–425
Lipids		
Lipofuscin	340–390	430–460, 540
Ceroid	340–395	430–460, 540
Porphyrins		
	405	630
	400–450, 630	635–690, 704

Further, another class of fluorophores is the organometallic compounds covering complexes of ruthenium, rhenium, platinum, rare earth metals, or lanthanide ions, with most of them absorbing in the UV.

In principle, several parameters of fluorescence can be recorded. These are the intensity at given excitation and emission wavelengths, in dependence of the emission (fluorescence emission spectrum) or excitation wavelength (fluorescence excitation spectrum) or in a scanning mode with constant differences between excitation and emission wavelength (synchronous spectrum). The fluorescence intensity can also be recorded at vertical and horizontal polarizations from which emission anisotropy or polarization can be calculated. The fluorescence intensity

depends on the absorption coefficient of the system at the excitation wavelength as well as from the excitation intensity (until saturation). A further parameter is the fluorescence lifetime. Combining several emission spectra at a range of excitation wavelength gives a so-called excitation-emission matrix (EEM), fluorescent landscape, or total luminescence. An EEM is often used for the characterization of complex multicomponent fluorescent systems like intact food systems or dissolved organic matter in natural water (see Sect. 13.3) and may serve as a unique fingerprint for the characterized sample. In most cases, the complex data are analyzed by multivariate and multiway methods (chemometrics) [20]. Spatial resolution like in fluorescence imaging microscopy, (microarray) scanners, or multiplate readers adds another dimension to fluorescence measurements (see Sect. 13.4).

Instrumentation for fluorescence measurement usually requires the selection of excitation and emission wavelength by monochromators or filters. Usually, the radiation source is positioned at a 90° angle from the emission detector. In some cases (highly concentrated, opaque, or solid samples), the excitation light is focused to the front surface of the sample and then fluorescence emission is collected from the same region under a 22.5° angle (sometimes also 30° or 60°) to minimize reflected and scattered light (front face technique). Commonly used radiation sources in steady-state spectrofluorometers are high-pressure Xenon arc lamps. For special cases, Mercury lamps, Quartz–Tungsten Halogen lamps, LEDs, Lasers, or Laser Diodes are also used. A detailed description and evaluation of light sources and other fluorometer components is given by Lakowicz [1]. Almost all modern spectrofluorometers use photomultiplier tubes (PMTs) as detectors. Nevertheless, for spectral ranges where photomultipliers are unavailable or too expensive analogous detectors like InGaAs, InAs, or InSb photodiodes are applied. Charge-coupled devices (CCDs) enable a more compact design of spectrometers.

Information about the dynamics of excited states is essential in understanding photophysical, photochemical, and photobiological processes. For time-resolved fluorescence measurements, two complementary techniques are used, the time-domain method and the frequency-domain or phase-modulation method. In time-domain fluorescence, emission is recorded as a function of time after exciting the sample by a short pulse of light. In frequency-domain method, the sample is excited by a modulated source of light. The fluorescence emitted by the sample has a similar waveform, but is modulated and phase-shifted from the excitation curve. Both modulation (M) and phase shift (φ) are determined by the lifetime of the sample emission which reversely can be calculated from the observed M and φ [1, 4, 20]. While for frequency domain continuous or pulsed sources are suitable, time domain requires pulsed sources. Lasers and laser diodes are frequently used but also the application of UV LEDs is reported [21–23]. Possible ways to record time-domain data are streak cameras or boxcar integrators, but most instruments are based on the time-correlated single photon counting (TCSPC) method with a PMT or an Avalanche photodiode (APD) as detector [24, 25]. Since its first application for the measurement of luminescence decay curves of molecules in solution TCSPC today is used in very sophisticated areas like time-resolved laser scanning microscopy (fluorescence lifetime imaging microscopy FLIM, FLIM in combination with

FRET…), diffuse optical tomography, autofluorescence of tissue, fluorescence correlation spectroscopy, or time-resolved single-molecule spectroscopy, recent applications are described in detail by Becker [26].

13.3 Fluorescence in Lab-Based Instrumental Analysis

Since about 10 % of all existing molecules are fluorescent itself and many more can be modified by association with fluorescent probes, fluorescence spectroscopy, and fluorescence detection in general, they have found widespread application in organic, analytical, and environmental chemistry as well as biochemistry [27]. Especially in organic chemistry and related fields, steady-state and time-resolved fluorescence methods are extensively used to investigate structure–property relationships, effects of the chemical environment (e.g., solvent viscosity or pH) on structure, and properties of a molecule of interest as well as its electronic structure and excited-state or charge-transfer dynamics. At this point, it is also noteworthy that the latter application is not only limited to organic matter. In solid-state physics, photoluminescence spectroscopy (PL) is increasingly used to study the electronic structure and dynamics of inorganic (especially semiconducting nano-) materials, and contributes to a fundamental understanding of the electronic processes governing the catalytic activity or (opto-) electronic property of a material [28, 29].

In combination with separation methods such as high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), or size exclusion chromatography (SEC), fluorescence spectroscopy is a powerful method in instrumental analysis, where it is routinely used to identify organochemical, biochemical, and pharmaceutical substances with very low thresholds of concentration [27, 30, 31]. Hereby, usually a conventional or laser-induced fluorescence (LIF) flow cell is installed at the outlet of the column of the separation system. The feasibility of UV LED-induced fluorescence detection has also been demonstrated [32–34]. Typically, for strong UV absorbing materials the fluorescence sensitivity is 10–1000 times higher than that of UV absorption, making fluorescence the most sensitive among the existing modern HPLC detection methods. Using modern microfluidic devices and advanced instrumentation, it is even possible to detect the presence of a single analyte molecule in sample volumes down to a few nanoliters [30]. Besides sensitivity, another advantage of fluorescence detection is its high selectivity especially in combination with specific fluorescent indicators linked to analytes in the sample (see Sect. 13.4).

One common application of fluorescence in environmental chemistry is the measurement of carcinogenic polycyclic aromatic hydrocarbons (PAH, e.g., anthracene and perylene) in natural or drinking water by HPLC [27]. In food analysis, HPLC combined with fluorescence helps to study toxins such as aflatoxins (a carcinogenic contaminant present in certain batches of grain cereals), pathogenic microbes, fat oxidation, vitamins, amino acids, and also enzyme activity [35]. In the EEM mode, fluorescence spectroscopy alone can be used for the analysis of

complex multicomponent fluorescent food systems [36] (e.g., natural olive oils [37]) and human or animal blood serum [38, 39].

Fluorescence coupled with separation methods is also routinely used in forensics for the detection of drugs and poison (see Sect. 13.7).

Besides its extensive use in fundamental research and analytical chemistry, fluorescence spectroscopy has also gained importance in some industrial fields of applications from which one is the analysis of fluorescent and phosphorescent dyes and pigments, covering inorganic phosphors for lightning application or optical brighteners on textiles and papers. The latter are also known as blancophores, which are stilbene or triazine derivates absorbing in the UV and emitting in the blue spectral range, resulting in a color impression of "whiter than white" [40]. Another industrial application is the detection of PAHs in crude oil exploration [41]. Fluorescence is also used in authentication and counterfeit detection, whereby bank notes, passports, credit cards, or goods are equipped with special marks made of fluorescent inks that become visible under UV light excitation. Several counterfeit detection systems utilizing UV LEDs emitting in the UVA spectral range are patented and already commercialized (e.g., [42]).

Also, the identification of fluorescence signatures as evidence of organic material possibly present in soil or rocks on the mars surface has been considered [43]. For this purpose, the utility of including a 365 nm LED or a 375 nm laser diode in the panoramic camera system of the ExoMars rover to be launched by the European Space Agency in 2018 has been examined, which would allow to monitor rover drill cuttings optically for aromatic hydrocarbons.

Another emerging field of application is the characterization of organic matter in aerosols or natural and drinking water using fluorescence detection directly without HPLC fractionation [44–48]. In the EEM of a water sample, different fractions of dissolved organic matter, like protein-related amino acids, humic or fulvic acids, and pigments from algae or bacteria, always fluoresce in the same sector and thus can be separated solely by means of data analysis (Fig. 13.3).

Besides natural organic matter, anthropogenic water pollutants such as dyestuffs from textile or paper industry can be identified by means of fluorescence. One approach for water quality monitoring is to measure the complete EEM of a water sample and to subsequently analyze the 2D spectra in a standardized manner using multivariate data analysis [49]. For a rapid surveillance of water quality at the point of care, an economic fluorimetric detection system is conceivable which utilizes several LEDs and photodiodes with band-pass filters (both in the UV–VIS spectral range) that are properly selected according to the excitation and emission maxima of the various fractions of interest [50].

Many techniques in analytical and environmental chemistry as well as biochemistry are based on fluorometric detection systems, usually with both single excitation and emission wavelengths, to qualitatively or quantitatively determine the presence of a target analyte that is either intrinsically fluorescent or made fluorescent by fluorescence derivatization. In the latter case, a fluorescent probe is bound to the analyte by chemical reaction, akin to the procedure employed in colorimetry. For the determination of metal cations for example, chelates are

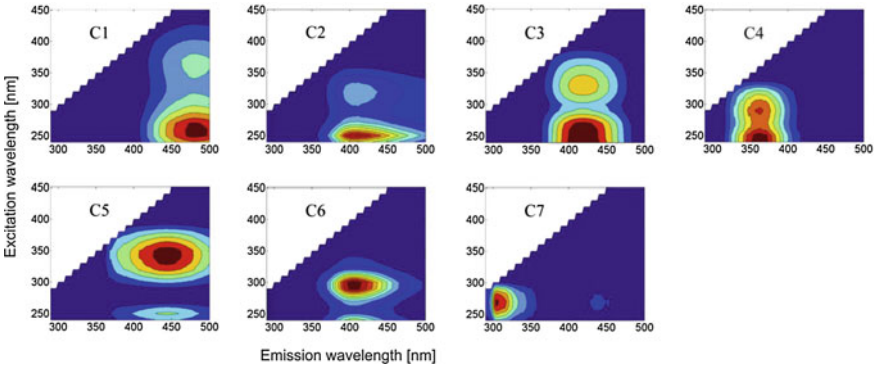

Fig. 13.3 Typical regions in the EEMs of natural water samples (*C1* terrestrial humic substances, *C2* terrestrial/anthropogenic humic substances, *C3* marine and terrestrial humic substances, *C4* amino acids (free or protein bound), *C5* terrestrial humic substances, *C6* marine and terrestrial humic substances, *C7* amino acids (free or protein bound). Reprinted with permission from [48], Copyright 2011, Elsevier

created with oxine (8-hydroxyquinoline), alizarine or benzoin, and afterward extracted by organic solvents [27]. Another example is the quantification of proteins in biological samples making use of the intrinsic protein fluorescence [51].

In the last decades, large efforts have been made to bring fluorometric detection systems out of the lab to the point of interest, where they can be routinely used—ideally by non-specialized personnel—as chemical sensors for a variety of applications. This will be issued in the following section.

13.4 Fluorescence Chemical Sensing for Environmental Monitoring and Bioanalytics

In the last four decades, the field of fluorescent chemical sensing has emerged substantially, due to the need of identification and monitoring potentially harmful substances and pollutants in the environment, including the concern about chemical and biological terrorism. Other important fields of application of fluorescent chemical sensing are bioanalytics, drug development, clinical diagnostics, and food quality surveillance. The field of fluorescent chemical sensing and biosensing is an active area of research and there are already numerous monographs and comprehensive reviews covering many aspects from fundamentals to special issues concerning new classes of fluorescent probes or immobilization [1, 3, 6, 52–55]. This section will be focused on fluorescent chemical sensing for recognition, monitoring, and imaging of (bio-) chemical analytes as well as genomic and proteomic studies, whereas the autofluorescence-based detection of living microorganisms and characterization of skin will be specially issued in two following sections.

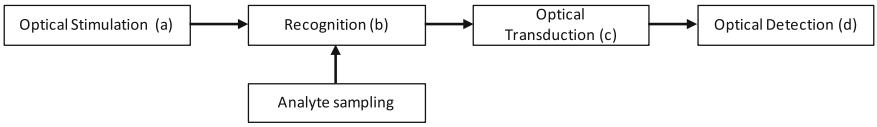

Fig. 13.4 General scheme of a fluorescence chemical sensor

A fluorescence chemical sensor consists of four main components which are a light source (a) to optically stimulate a recognition element (b) specifically interacting with an analyte, an optical transduction element (c) converting the physical or chemical response of the recognition element to a fluorescence optical output signal, and an optical detection system (d) (see Fig. 13.4).

Recognition elements in use are as manifold as analytes to detect ranging from cation, anion, or functional group-specific chemical receptors over enzymes, antibodies, proteins (lectins or avidins), neuroreceptors to DNA, or even living microorganisms.

In special cases, the recognition and transduction element is one chemical species and analyte binding to it results in generation of or change in fluorescence. Examples are dehydrogenase enzyme-catalyzed substrate reactions producing NADH, which can be followed by monitoring the NADH fluorescence with excitation and emission at around 350 nm and 450 nm, respectively [6, 56, 57]:

$$\text{Substrate} + \text{NAD}^+\,(\text{non-fluorescent}) \rightarrow \text{Product} + \text{NADH}\;(\text{fluorescent})$$

Such enzyme-based biosensors are used for the detection of chemical and biochemical analytes, for example, Ethanol or L-Leucine [56, 57].

Functional fluorescent dyes are one main class of transduction elements for fluorescence chemical sensor systems. The toolbox of fluorophores for this application is spanning small organic dyes, metal ligand complexes like metalloporphyrins, transition metal and lanthanide complexes, inorganic quantum dots (QDs) with size-dependent optical properties, carbon, silicon, and luminescent metal nanoparticles as well as inorganic upconversion phosphors. Especially, the use of functional inorganic nanomaterials as a new generation of fluorescent reporters is one of the fastest growing research fields [51].

Another broad class of transduction elements are genetically encoded fluorescent proteins, from which the green fluorescent protein (GFP) is the most prominent. The excitation maxima of the (unmodified) GFP fluorophore lie at 395 nm and 475 nm, whereas the emission maximum is at 509 nm [58]. GFP and alike can be expressed artificially by many cells and organisms. They can be fused specifically to other proteins or host genes, which makes same a valuable tool for investigations of individual cells and biological samples in general [58–60].

In recent decades, the development and application of bioassay systems based on genetically engineered whole cells has been addressed by many researchers [61]. Starting from this, also biosensor systems with whole-cell reporters have been developed and tested as alternative analytical tools. Especially, whole-cell bacterial

biosensors have been considered for testing various effects of water contaminants, such as genotoxicity, cytotoxicity or the potential of membrane, oxidative, or protein damage [61]. In fluorescent biosensors, the reporter bacteria are genetically engineered by fusing of the *gfp* gene reporters to an inducible gene promoter. Due to its stability, sensitivity, and convenient fluorescence detectability GFP, encoded by *gfp* gene, is one of the most popular tools for biosensing [62]. The fluorescence output signal intensity serves as measure for GFP production as a result of analyte-induced gene expression [63]. A disadvantage of GFP as biological transduction element in whole-cell biosensors is the delay between its production and fluorescence emission, which makes it impractical for real-time monitoring applications [63]. Whole-cell-based fluorescent biosensors have been developed and field-tested to measure the relative bioavailability of petroleum product-related aromatic compounds, such as benzene, toluene, ethylbenzene, and xylenes (BTEX) in contaminated water and soil samples [64, 65] or arsenic and other trace amounts in drinking water [66].

In principle, any phenomenon inducing a change in intensity, wavelength, anisotropy, or lifetime of a fluorophore can be used for chemical sensing. The most direct method of chemical sensing is a change in fluorescence intensity of the transducer in response to an analyte. This happens due to a process called collisional quenching in which the analyte influences the optical output intensity (or lifetime) of the fluorophore when it comes into proximity. Examples of fluorescence chemical sensors based on collisional quenching are sensors for oxygen (based on long lifetime metal ligand complexes like $[Ru(Ph_2phen)_3]^{2+}$ absorbing at 450 nm) [67, 68], chloride (based on quinolines absorbing in the UV spectral range of 318–366 nm) [69], sulfur dioxide, or chlorinated hydrocarbons [70, 71].

Fluorescence resonance energy transfer (FRET) is another and perhaps the most valuable mechanism for fluorescence chemical sensing. Although UV excitable fluorophores have been used, most strategies for pH, pCO_2, glucose, or ion sensing based on FRET are focussing on fluorescence excitation in the visible wavelength region in order to circumvent photobleaching or unwanted autofluorescence [72].

For pH and ion sensing, especially for Ca^{2+}, Mg^{2+}, Na^+, and K^+ sensing inside living cells or in body fluid samples, also special wavelength ratiometric fluorescent indicators have been developed (e.g., [73, 74]). The key characteristic of these indicators is a wavelength shift in the fluorescence spectrum occurring upon binding with the ion. The shift may occur in either the excitation or the emission spectrum. Measurement is achieved using two excitation wavelengths (in case of dual excitation indicators) or two detection ranges (dual emission indicators). For the indicator Fura-2, e.g., in the absence of Ca^{2+}, the excitation maximum is at 372 nm shifting to 340 nm when bound to Ca^{2+} [75]. Another ionic species of interest in chemical sensing, especially for applications in imaging cellular systems, are metal ions, such as Zn^{2+} for which also UV excitable fluorescent probes, e.g., based on naphthalimide or quinoline derivates as fluorophores, have been developed [76, 77].

The simplest way to set up a fluorescent chemical sensing system is to mix the target analyte with appropriate amounts of the recognition and transduction

molecular or cellular species in the liquid phase in vitro. The reaction vessel or assay is placed in a fluorescence optical detection system with light source and fluorescence detection channel adjusted to the excitation and emission wavelength of the transducer. This is routinely done in bioanalytics where multiplex analysis based on microplates in 96-, 384-well (or even more) format, according liquid handling systems and plate readers are the state-of-the-art [6].

Especially for field-testing applications as well as high-throughput analysis, liquid handling is sometimes impractical and solid-phase sensors and arrays have to be utilized. In this context, a critical aspect is the immobilization of the recognition and transduction element in order to homogenize and increase its local concentration in the sensor or array. Accessibility by external reagents and in the case of living microorganisms as sensing elements also biocompatibility are further issues of concern. Thus many research activities are focused on immobilization, and as a result various physical and chemical strategies, such as adsorption, crosslinking, covalent binding entrapment, Langmuir–Blodgett deposition, or sol–gel entrapment, have been developed and successfully applied [78–81].

During the past 30 years, fluorescence detection methods have extensively been applied in bioanalytical assays for analyte recognition, DNA sequencing, detection of DNA hybridization, of polymerase chain reaction products and for protein profiling, and determination of protein function [6, 82]. Since fluorescence is a highly sensitive and less restricting method than detection by radioactive labeling in this field, many sophisticated assays, e.g., in association with electrophoretic separation techniques (gel blotting, 2D gel electrophoresis), multiwell plates, or microarrays, as well as adapted and automated fluorescence readout systems have been developed.

With enzyme-linked immunosorbent assays (ELISA) proteins (e.g., antigenes), viruses, hormones, toxines, or pesticides can be detected in a liquid sample. In ELISA, an enzyme-linked antibody binds specifically to the analyte (antigene) and the occurence of an enzyme-catalyzed reaction serves as a measure for the analyte, whereby the enzyme reaction rate can be quantified by means of a fluorescent substrate, which is consumed in the reaction [1].

Since its commercial availability in 1996, DNA microarrays, also commonly known as biochips, are nowadays routinely used in genomics for monitoring the expression of more than thousand of genes using just a single microscopic slide. Microarray technology can be regarded as an extension of biosensing techniques since it utilizes a slide-spotted, micropatterned array of biosensors allowing rapid and simultaneous probing of a large number of bioassemblies [6]. It is of considerable value to high-throughput analysis, e.g., for sequencing of the human genom, where huge numbers of genes and their products need to be characterized [83].

Since the intrinsic DNA fluorescence is almost neglectable (nucleic acid), staining is mandatory [84]. Typical fluorescent nucleic acid probes used in microarray technology are excited in the visible spectral range but if beneficial for some reasons also UV excitable probes like Hoechst 33342 or DAPI can be applied. State-of-the-art microarray readers are based on laser excitation and emission filter. By means of image analysis, signal intensities for each dye at each microarray spot

are determined and the data is analyzed by cluster analysis [6]. Especially for the analysis of multicolor microarrays as well as for a better background discrimination, hyperspectral microarray scanner technology, where the spectrally resolved fluorescence emission of each spot is analyzed, is beneficial [85].

In recent times, the focus of biology has shifted from genomics to proteomics where proteins are cataloged and analyzed aiming at a detailed understanding of biological complexity and functions [6]. Besides pharmacoproteomics, dealing with the mechanistic basis of drug action and toxicity proteins can also serve as new biomarkers for medical diagnosis. In two general areas of proteomics, high-throughput techniques are mandatory, which are protein profiling and determination of protein function. Although protein chip technology is not as well developed as gene chip technology, their improvement is inevitable [6]. Analogous to DNA microarray in a protein microarray, a glass slide is spotted with thousands of protein probes. Subsequently, a biological sample is spread over the slide and binding at any spot is detected. Fluorescence detection is the widely used method [86]. Since labeling sample proteins with a fluorescent reporter (such as fluorescent proteins or BODIPY dyes) bears the risk that the fluorophore binding may alter the ability of the protein to interact with the immobilized capture agent, protein UV absorbance and intrinsic autofluorescence excited in the UV are promising methods of detection in the field [87–90].

Fluorescence microscopy is a well-established technique in the life sciences enabling optical (bio-) imaging. Numerous monographs, handbooks, and reviews are available covering this field (e.g., [1, 6, 91–94]). One advantage of fluorescence as optical contrast method in microscopy is its very high signal-to-noise ratio that allows to distinguish spatial distributions of molecular species of low concentration. Both exogeneous fluorescence, where species of interest in the specimen (cell, tissue or gel) are labeled with a fluorophore, and endogenous fluorescence (autofluorescence), allowing for label-free bioimaging, are used in fluorescence microscopy [6]. Especially, the availability of numerous, specifically engineered fluorescent reporters for labeling different parts and structures of cells or probing different cell functions, in combination with fluorescence microscopic imaging has had a major impact in biology and medicine [6, 91, 95]. Usually, these fluorescent reporters are designed to absorb and emit at wavelengths in the VIS or NIR in order to suppress cell or tissue autofluorescence background (mainly excited in the UV or blue spectral range) potentially disturbing the fluorescence emission signal of the reporter. Nevertheless, UV autofluorescence as contrast method enabling label-free imaging of intrinsic biomarkers such as aminoacids, (co-)enzymes, lipids, or structure proteins is gaining more and more importance in the field [96].

Conventional or wide field fluorescence microscopes widely used today are mainly epifluorescence microscopes, where excitation light illumination of the object or specimen is realized through the same objective lens collecting the fluorescence emission signal for imaging [93]. A dichroic beam splitter, transmitting or reflecting light depending on its wavelength, is implemented to separate the excitation from the fluorescence light.

Historically, the most prevalent fluorescence excitation sources have been Mercury-vapor high-pressure arc lamps (commonly referred to as HBO lamps) but also Metal Halide lamps have been employed [93]. A new development in conventional fluorescence microscopy is the application of LEDs as excitation light sources [92]. LED modules for microscopy now are commercially available from several companies allowing LED illumination at wavelengths in the UV (starting at 255 nm) and VIS spectral range, characterized by compact size, low power consumption, fast switching and adjusting properties (instantly illumination at full intensity), and other advantages inherent to state-of-the-art LED technology [92].

Besides conventional microscopy, LEDs are also promising light sources for prism-based total internal reflection fluorescence microscopy (TIRFM) [92]. In TIRFM, a prism is placed on (and optically coupled to) a cover slip, acting as (total internal reflection based) optical waveguide when collimated (LED-) light is directed onto the prism under a proper incidence angle (Fig. 13.5a). If the object below is intentionally kept in watery low refractive index medium and not optically coupled to the cover slip, an exponentially decaying evanescent wave extends into the specimen perpendicular to the glass/water interface. By this, the fluorescence emission of a thin optical slice of the specimen can be imaged with very low background noise. Inspired by TIRFM and first demonstrated by Ely Silk in 2002 [97], a simple illumination scheme in conventional fluorescence microscopy is to place a prism laterally onto the glass slide support and to immerse the specimen in a mounting medium optically coupled to the glass slide, whereby the excitation light passes through the whole specimen exciting the fluorophores homogeneously (Fig. 13.5b). As a possible application of a cheap and compact LED-based fluorescence microscope, screening of patient`s septum for tuberculosis in developing countries has been officially recommended by the WHO [98].

Since with LEDs the excitation energy can be controlled without noticeable changes of the spectral emission characteristics and furthermore fast switching even in the nanosecond range is possible, they are also beneficial as illumination light

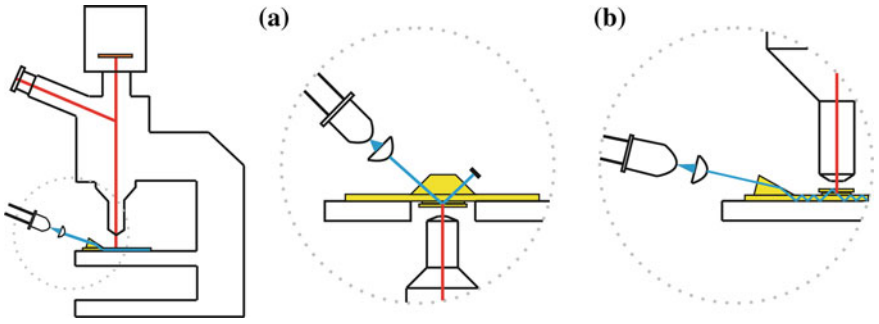

Fig. 13.5 Bright-field fluorescence microscopy setup with LED illumination either based on **a** a TIRFM setup with a prism placed over the observation area onto the microscopic slide (shown here for an inverted microscope setup) or **b** coupled into the side of the microscopic slide via a coupling prism (according to [92])

sources for two specialized variants of fluorescence microscopy, namely fluorescence resonance energy transfer microscopy (FRETM) and fluorescence lifetime imaging microscopy (FLIM). For a qualitative and relative tracking of the FRET process and when only a single FRET polypeptide construct is examined, acquisition of both the donor and acceptor emission at a single excitation wavelength is sufficient. In this case, the spectral contamination, i.e., the contribution to the FRET signal due to donor emission into the acceptor channel and the excitation of acceptor molecules by the donor excitation wavelength (spectral overlap), is constant. However, for quantitative assays or when the spectral contamination varies due to uncertain conditions of the surrounding media (e.g., fluorophore attachment to proteins with local concentration variations throughout a cell), multiple acquisitions are necessary. Here, LEDs are (low cost) alternatives to laser excitation light sources, enabling fast multiple acquisitions, especially when rapid FRET changes are expected in a cellular assay [92]. Another parameter, especially useful for quantitative FRET analysis but also for label-free imaging of intrinsic fluorophores is the fluorescence lifetime [99]. In FLIM, the fluorescence lifetime of a fluorophore is determined with spatial resolution. Lifetimes of most fluorophores typically used in cell biology are in the order of nanoseconds, implying acquisition accuracy in the picosecond range. As already discussed in Sect. 13.2, two principles, time-domain techniques, namely TCSPC and time-gating fluorometry and frequency-domain fluorometry can be applied for assessing fluorescence lifetime, based on either pulsing or modulating the LED emission. Some solutions, also covering the UV spectral range down to 255 nm, are already commercially available [92].

The development of confocal microscopy has further expanded the application of fluorescence microscopy in bioimaging [100]. It is based on an optical system, where a front and a back side focus are conjugated allowing only light from a selected plane of a (moveable) sample to reach the detector. Laser scanning microscopy (LSM) is a variant of confocal microscopy, where the light beam is scanned over the sample by means of movable mirrors, allowing fast optical sectioning of biological specimen even in vivo. Another technique based on confocal microscopy is fluorescence correlation spectroscopy (FCS). It is a powerful method for analyzing dynamics, local concentration, and photophysics of single molecules both in vitro and in vivo [8]. In FCS fluctuations of the fluorescence signal intensity, the mean values are measured and analyzed in a small (confocal) detection volume of the sample.

Both TIRFM and confocal microscopy (also in combination with FCS) can be applied for single molecule detection (SMD) where the size of the detection volume is reduced to less than one femtoliter [8]. Currently, in confocal microscopy and especially in FCS, only lasers are used as excitation light sources [1].

A well-established application of conventional fluorescence microscopy is fluorescence in situ hybridization (FISH), where the distribution of nucleic acids in tissue, cells, or chromosomes is analyzed in situ. It relies on DNA or RNA

specifically labeled with fluorescent probes and their computerized microscopic imaging with sensitive CCD detection [101]. Also, approaches based on probes bearing a mixture of fluorophores have been developed (termed as multicolor FISH and spectral karyotyping), which allow the identification of all 24 chromosomes within one sample [102, 103].

Flow cytometry is an optical method routinely used in both bioanalytics and medical diagnosis whereby cells are suspended in a stream of fluid and passed by a detection apparatus. It is employed in cell counting, cell sorting (fluorescence-activated cell sorting (FACS), which includes the ability of the instrument to sort cells according to specific measurement criteria), and biomarker detection since it allows simultaneous multiparametric analysis of physical and chemical characteristics of up to thousands of cells per second. Both flow cytometry and optical microscopy perform similar functions with the difference that in (confocal) microscopy, the (laser) light beam moves to analyze cells, whereas in flow cytometry the cells are moving (flowing). Typical flow cytometers are based on one or more laser sources for fluorescence excitation [6]. A wide variety of fluorochromes are employed in flow cytometry and the number is still growing driven by specific demands of new applications of flow cytometry [6]. Typical fluorochromes excited in the UV spectral range are coumarins and Cascade Blue for application in immunophenotyping, Hoechst 33342 and DAPI for DNA analysis and chromosome staining, and Indo1 for calcium flux measurements.

As main clinical application of flow cytometry, immunophenotyping basically deals with classification of white blood cells according to their surface antigen characteristics, which can then be used as a profile for a specific disease or malignancy. Measurement of the DNA count is another major application of flow cytometry and is applied to identify cell abnormality which involves genetic changes.

Monolithic integration of both optical and electronic components in general offers the possibility to miniaturize flow cytometers. As in fluorescence microscopy, flow cytometry platform design and versatility is benefiting from the availability of new, compact, and inexpensive (laser) diode emitters replacing bulky and inefficient gas lasers [6]. A particular configuration based on UV LED excited, time-gated fluorescence has already been proposed (Fig. 13.6) [104]. Significant progress has also been made in the area of microfluidics [105, 106]. Put together these two developments provide a good basis to produce flow cytometer-on-a-chip, expanding their versatility and application [6].

Imaging flow cytometry in general combines the statistical power and sensitivity of flow cytometry with spatial resolution and quantitative morphology of digital microscopy. The combination may be beneficial for various clinical applications since cells can be imaged and analyzed directly in bodily fluids. In this context, a compact cell-phone-enabled optofluidic imaging flow cytometer has already been introduced [107].

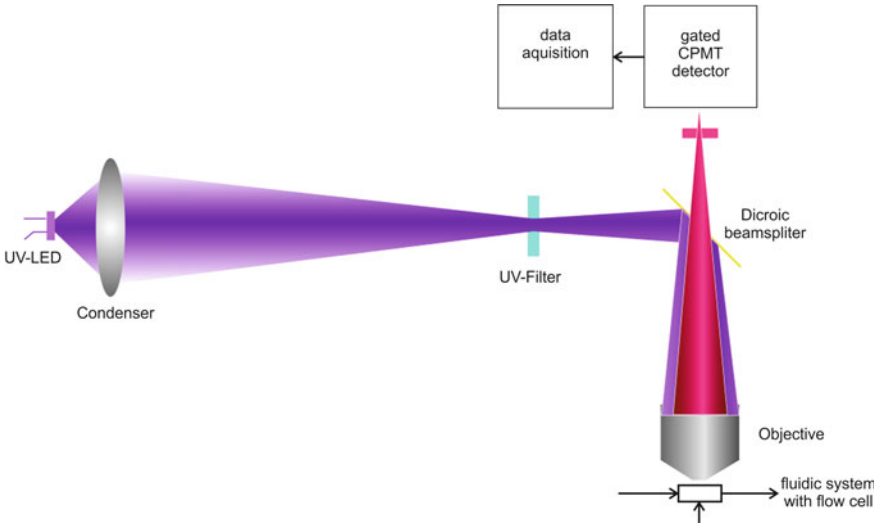

Fig. 13.6 Setup of a UV LED-based time-gated luminescence flow cytometer; *CPMT* channel photomultiplier tube (according to [104])

13.5 Detection of Microorganisms Using Autofluorescence

Microorganisms can be found everywhere: deep inside rocks, below the seafloor, and even in the most hostile environments like poles and deserts. Types that can cause diseases in a host are known as pathogens and their detection is of vital importance for health and safety reasons. Main areas of interest in the health care sector are the food industry, clinical diagnosis, and water or environment quality control [108].

Microorganisms can be divided into prokaryotes, namely bacteria and archaea, and eukaryotes, like fungi and algae. The classification of viruses as microorganisms is under debate, depending on their definition as living or nonliving. Some examples of bacterial pathogens of concern in food industry are *Salmonella*, *Listeria*, *Escherichia coli,* and *Campylobacter* [109]. Healthcare-associated pathogens, like Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile,* can be responsible for patients acquiring an infection in hospitals.

Various methods for the detection and analysis of microorganisms are in use. They comprise classical as well as modern, biosensor-based techniques. It is dependent on the application whether it is sufficient to just verify the presence of a minimal amount of biomass in an abiotic environment or to know the exact quantity of a specific pathogen on a given biological surface. All detection methods operate somewhere within this range.

Culture methods, which are based on the multiplication of the target organism on a growing medium, followed by visual colony counting, provide a reference. The

main disadvantage of these methods is the time that is needed for incubation which can be several days [108]. Commercially available so-called rapid detection methods such as ELISA, lateral-flow dipstick (LFD), and polymerase chain reaction (PCR) have significantly reduced the time and effort of the analysis of food products, but they still rely on a time-demanding enrichment step [109]. Other drawbacks are the costs for equipment and reagents especially for molecular biological methods.

A comparably new approach for microbial detection is biosensors, incorporating a target microorganism, a bio-specific recognition system, and a physicochemical transducer. Transduction methods in use also include fluorescence optical detection (see also Sect. 13.4). Biosensors can be miniaturized and automated, having the potential to both shorten the time of analysis and to provide selectivity and sensitivity comparable to established methods at a fraction of the cost [109].

Fluorescence methods are also suitable for directly monitoring the fluorescence of cellular components of microorganisms, and their metabolic states (live, dead, spores) [110]. Both extrinsic and intrinsic fluorescence can be applied for sensing purpose. Usually, the emission of exogenous synthetic fluorescent reporters, specifically labeling a target analyte characteristic for a microorganism to detect, is relatively strong and mainly excited in the visible spectral region.

Fluorescence detection methods that do not apply fluorescent reporters to microorganisms make use of the intrinsic or autofluorescence, sometimes also referred to as reagent-free identification. It is caused by fluorophores which exist as a natural component or product of a microorganism under examination. Using intrinsic fluorescence for detection and identification of microorganisms allows for real-time measurements in a noninvasive, contact-free manner rendering sample preparation unnecessary [111]. Only a few methods like flow cytometry are able to detect and differentiate viable but nonculturable (VBNC) bacteria. Using intrinsic fluorescence spectroscopy, it has been shown that this is possible without incubation even in the presence of culturable bacteria [112]. Furthermore, cellular fluorescence may also be used to determine the metabolic state of living cells. It allows their classification into three groups corresponding to the three main phases of the growth profile, i.e., lag phase, exponential phase, and stationary phase [112].

Many fluorescent biomolecules in microorganisms can be excited in the UV spectral range between 220 nm and 360 nm (Fig. 13.7). Proteins, which are an integral part of all organisms, obtain their fluorescent characteristics from aromatic amino acids (AAA) like tryptophan. Other important sources of fluorescence are the pyridine nucleotides NADH and NADPH in their reduced form, which are enzymatic cofactors appearing in all organisms. This is also true for flavines, such as flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) fluorescing in their oxidized form and some vitamin B6 compounds. Cell wall components like chitin (fungi, algae) are also a source of strong intrinsic fluorescence as well as dipicolinic acid (DPA) in the presence of calcium ions (spores). A comprehensive overview of relevant fluorophores and their optical parameters is provided by Pöhlker et al. [18].

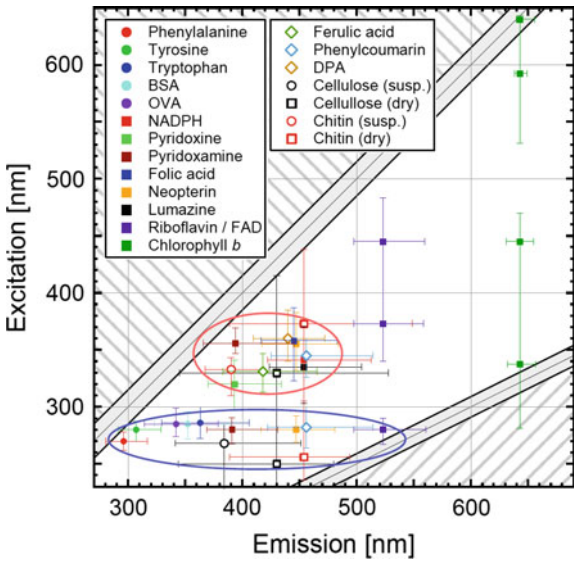

Fig. 13.7 Conceptual EEM displaying excitation and emission ranges of various biological fluorophores (*BSA* bovine serum albumin; *OVA* ovalbumin; *NADPH* nicotinamide adenine dinucleotide phosphate; *FAD* flavin adenine dinucleotide; *DPA* dipicolinic acid). The *red* and *blue* ovals depict "emission hotspots" where the modes of many fluorophores cluster. Reprinted with permission from [18], Copyright 2012, Copernicus Publications

For detection of the intrinsic fluorescence signal of relevant fluorophores, it is often beneficial to acquire with spectral resolution, because several biological fluorophores present very close emission spectra that become difficult to discriminate [96]. The main challenge in using intrinsic fluorescence for microbial detection is that the spectral characteristics of a fluorophore are highly sensitive to its local environment, such as binding of ligands, protein–protein association, pH, hydrophilicity, or lipophilicity. Therefore, the detailed knowledge of how these factors influence the fluorescence signal is essential [96].

As described above, fast and noninvasive methods for the detection of microorganisms would be particularly useful in decontamination tasks, medical assays, or forensic investigations. Depending on the location of the microbial load detection methods for surfaces, fluids, and air can be distinguished.

Surface fluorescence detection of microorganisms can be of different complexities at distinct substrate materials like glass, metal, plastics, cloth, foods, or even living tissue. Several variants of a real-time in situ detection system have been build up using filtered LEDs for the visible (455–635 nm) and the UV (365 nm, 1 mW each) spectral range as excitation sources (see Fig. 13.8) [111, 113, 114]. The UV diodes were used mainly to excite NADH and DPA fluorescence within an area of 1 cm^2. Nearly, hemispherical collection has been applied using parabolic-type reflectors to gather 90 % of the fluorescence [114]. An aspheric lens is another

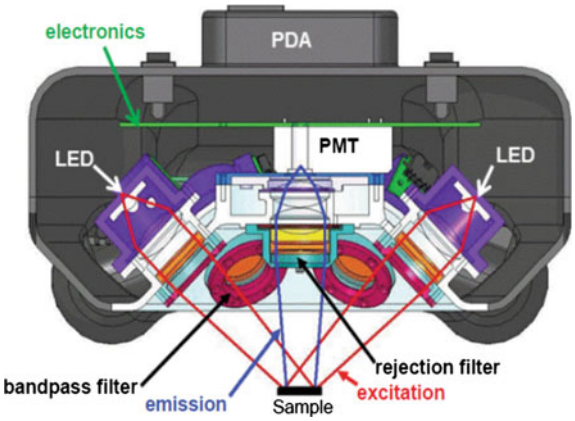

Fig. 13.8 Schematic diagram of a hand-held instrument for detection and quantification of microorganisms based on the intrinsic fluorescence, excitation by visible and UV LEDs, detection of the filtered fluorescence emission by photomultiplier tube (*PDA* personal digital assistant). Reprinted with permission from [111], Copyright 2012, Elsevier

effective way to collect emission from the spot using standard components [113]. With these systems, detection limits as low as 10 microbial cells per cm^2 could be provided on abiotic surfaces. Because of their sensitivity, photomultiplier tubes (PMTs) are often used for the detection of the band-pass filtered emission. Elimination of the excitation signal is done by rejection filters in front of the detector. Using amplitude-modulated light sources further improves the sensitivity and allowed for detecting microbial concentrations that vary by up to nine orders of magnitude [111]. A hemispheric detection geometry has also been accomplished using optical fibers while exciting a biofilm under seawater with a 280 nm LED source (600 μW, area 0.55 cm^2) [115]. Here, bacterial proteins were detected by their tryptophan fluorescence signal to a detection limit of 4×10^3 cells per cm^2 for an artificial biofilm. Fluorescence of surface contamination by clinically relevant bacteria has been investigated using excitation wavelengths of 280–800 nm with a commercial fluorescence spectrometer [116]. Measurements of the spatial distribution of microorganisms on surfaces have also been done using fluorescence imaging methods. A deep UV scanning microscope equipped with hollow-cathode lasers of 224 nm (HeAg) and 249 nm (NeCu) has been used for imaging bacterial fluorescence on opaque mineral and metal surfaces with a spatial resolution of 300 nm providing single cell sensitivity [117]. Another device uses an imaging spectrograph and line scanning to acquire images. Excitation of bacteria relevant in the food industry is done by a UVA lamp at 365 nm [118].

LEDs for the visible and the UV (365 nm) have been used to excite the intrinsic fluorescence of bacteria, fungi, and other microorganisms for monitoring of drinking water. With an instrument specifically designed for this purpose, a detection limit of 50 bacterial cells per liter was achieved [110]. A similar excitation source has been used by a related device, which could detect 10 microbial cells per

mL [111]. Both acquire band-pass filtered fluorescence with a PMT. A UV LED-based multi-wavelength fluorimeter system is described in [119] covering the wavelength range from 250 nm to 375 nm to excite autofluorescence of several microorganisms. The system has a turn-on time of 2–3 ns, internal modulation up to 300 MHz, and can operate in a continuous wave or pulsed mode [119]. Further studies based on non-LED UV light sources are also reported. To excite cultured cells and tissues over a wide range of 180–600 nm, monochromatized synchrotron radiation has been used [96]. Most widespread for investigation of microbial fluorescence is the use of commercial fluorescence spectrometers together with quartz cuvettes for sampling. They have been used to detect and differentiate bacteria like *Escherichia coli*, *Salmonella,* and *Campylobacter*, which are relevant for the food industry. Here, concentrations down to 10^3 cells per mL could be measured at an excitation of 200–400 nm and using principal component analysis (PCA) for data analysis [120]. Excitations of 250 nm for AAA and nucleic acids (NA), 316 nm for NADH and 380 nm for FAD allowed a discrimination of lactic acid bacteria at the genus, species, and subspecies levels [121]. An excitation of 250–340 nm was used to detect the fluorescence of tryptophan and NADH in bacterial cells and their comparison through PCA and factorial discriminant analysis (FDA) [122]. The fluorescence signals of tryptophan at an excitation of 280 nm and of NADH at 350 nm have been used to investigate the metabolism and the growth of cells while chemically restraining them [123]. A method for rapid identification of the clinically significant species *Escherichia coli*, *Enterococcus faecalis,* and *Staphylococcus aureus* has been shown using excitation wavelengths of 330–510 nm. The PCA technique applied to the fluorescence spectra showed that bacterial species could be identified with sensitivity and specificity higher than 90 % and in less than 10 min [124]. There is a certain overlap between the described cuvette-based methods and flow cytometry (see Sect. 13.4). The latter is a common tool in cell biology for rapid analysis of large populations of cells where fluorescence detection is well established. Here also intrinsic fluorescence of cellular molecules can be used beside extrinsic fluorescent stains or antibody probes [116].

The detection of intrinsic fluorescence is also applied to atmospheric aerosol particles, which include small pieces of biological materials and microorganisms (see Fig. 13.9) [18, 125]. Linear arrays of UV LEDs exciting at 290 nm for tryptophan and 340 nm for NADH have been applied to identify bacterial spores even during their time-of-flight [126]. The detection was provided by a UV transmission grating to spectrally disperse the light onto a 32-anode PMT achieving a detection limit of 350 µM of NADH. Using the excitation range of 210–419 nm of an optical parametric oscillator AAA could be identified as dominant features of the aerosols investigated [127]. The fluorescence detection of flavines, NAD(P)H, and tryptophan was accomplished with a parabolic collection mirror, a spectrograph, and an image-intensified CCD camera.

Altogether, the application of UV LED and LD sources is not yet widespread in fluorescence-based detection of microorganisms. However, as soon as emitters with

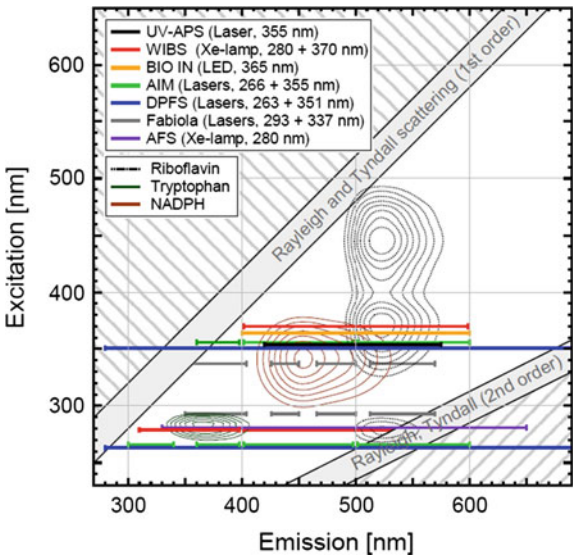

Fig. 13.9 Conceptual EEM displaying contour lines for the fluorophores tryptophan, NADPH, and riboflavin and operational ranges of selected bioaerosol detectors, represented by *horizontal colored lines*. Length of individual *lines* indicates measured emission band for a certain excitation wavelength shown as sharp line for purpose of clarity. Single-wavelength detectors are represented by *one line*, dual-wavelength detectors by *two lines*. Reprinted with permission from [18], Copyright 2012, Copernicus Publications

adequate performance are available at low cost it is very likely that they enable further improvement and development of effective and compact tools in this field.

13.6 Fluorescence in Medical Diagnosis of Skin Diseases

Fluorescence detection as a tissue diagnostic procedure is applied in a wide range of medical fields such as dermatology, dentistry, ophthalmology, laryngology, gastroenterology, neurology, or oncology to name a few [128–134]. This section will focus on fluorescence diagnosis in the field of dermatology, especially on skin cancer diagnosis based on UV autofluorescence excitation and spectroscopy.

In fluorescence diagnosis, it is to be distinguished between autofluorescence and exogenous fluorescence. For exogenous fluorescence, a fluorescent substance such as δ-aminolevulinic acid (ALA) or methylester aminolevulinate (MAL) is applied either topically in the form of a cream or systemically in the form of a drug given prior to the measurement. ALA attaches itself to healthy tissue and to pathological changes, in most cases cancer-affected tissue. In healthy tissue, ALA decomposes into nonfluorescent substances, whereas in damaged tissue, protoporphyrin IX (PpIX) is formed which can be excited to fluorescence by UV irradiation in the

UVA spectral range [135]. In the case of autofluorescence, endogenous fluorophores, such as NAD(P)H or tryptophan, are excited by UV irradiation circumventing the administration of auxiliary substances.

One of the first UV radiation sources for fluorescence excitation was the Woods lamp that had been invented by Robert W. Wood in 1903. Its first use in dermatology was reported in 1925 when it had been applied to detect fungal infections of the scalp [136]. UV radiation in the range of 340–400 nm is generated by a high-pressure mercury arc that passes an optical filter, the so-called Woods filter. It is nowadays primarily used for nonspectrally resolved fluorescence imaging, in order to visualize the borders of carcinogenic lesions. As the radiation intensity of the Woods lamp is low, dark rooms and extended observation periods including eye adaptations are required [137]. With the availability of UV LEDs, this imaging method can be improved by arranging UV LED-based ring lights around a camera objective enabling a more compact design and its adaptation to digital imaging systems [135].

For the noninvasive detection of skin cancer image processing systems based on RGB cameras are state-of-the-art. In part, this applies also to multispectral cameras which often cover also the NIR spectral range [138, 139]. Although image processing systems are very reliable, precancerosis lesions are often difficult to detect and to discriminate with this technology.

Autofluorescence spectroscopy based on excitation of intrinsic dermal fluorophores holds the potential to overcome the limitations of image processing for skin cancer diagnosis [140]. Typical dermal fluorophores and their excitation and emission wavelengths are listed in Table 13.1. The amino acids are generally excited in the shortwave UV range (260–295 nm) and exhibit their fluorescence still in the UVA range. In this context, tryptophan has been attributed an essential role in diagnostics. Structural proteins such as elastin and collagen are important fluorophores of the dermis. A prominent representative of the enzymes and co-enzymes is the NADH/NAD+ system contributing to autofluorescence only in its oxidized form. Some vitamins and lipids as well as porphyrins (emitting in the red spectral range) also show autofluorescence [141].

Before addressing the differentiation of benign from malignant tissue in dermatology, some studies investigating the influence of aging, proliferation, and photoaging on the autofluorescence emission will be discussed. One important finding was that with increasing aging tryptophan decreases and could be absent at high age [143]. Collagen decreases as well, whereas the autofluorescence of elastin increases. This is in agreement with the changes in the collagen/elastin index SAAID which is measured by multiphoton microscopy to evaluate the age of the skin [144]. Proliferation as observed after tape stripping increases the tryptophan autofluorescence that thus can be used as a marker for proliferation [145]. This also explains the increase in the tryptophan autofluorescence in psoriatics [146]. The porphyrin fluorescence is increased in these patients, too [142, 147]. Tryptophan autofluorescence also increases at extended UV exposure which may be due to an intensified epithelial growth. However, the collagen fluorescence is decaying at chronic UV exposure [148] and can, therefore, be used as a marker for photoaging.

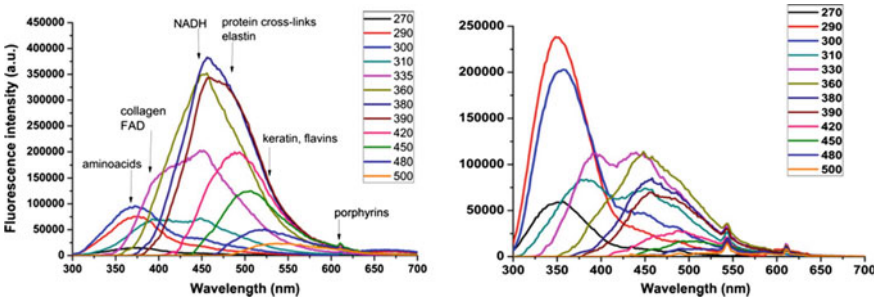

Fig. 13.10 Normal skin (*left*) and BCC (*right*) autofluorescence spectra at different excitation wavelengths (from 270 nm to 500 nm) and identification of the major endogenous fluorescence compounds observed. Spectra of BCC are recorded from an excised lesion. Reprinted with permission from [140], Copyright 2014, IEEE Photonics Society

Furthermore, DeAraujo et al. used laser-excited autofluorescence for discriminating between various types of human pathogenic fungi inducing several skin infections [149].

Dermatological tumor diagnostics by fluorescence spectroscopy has been applied in numerous studies for many years [140]. Nonmelanoma skin cancer (NMSC) lesions are suitable targets for fluorescence spectroscopy-aided diagnosis. NMSC usually covers basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) tumors. These lesions are among the most common cancers affecting Caucasians. BCC occasionally grows aggressively causing tissue destruction and it is often recurring after initial treatment. SCC, the second most widely spread cutaneous tumor, is associated with a risk of metastasis and is usually more aggressive and difficult to treat compared to BCC lesions [150]. The problem in diagnosis of skin cancer is the large variety of malignant forms of skin tumors. There are several subtypes, for example, BCC and SCC and a variety of benign and dysplastic forms with differences in optical and autofluorescence properties.

The autofluorescence spectra of normal tissue are shown in Fig. 13.10 left, at different excitation wavelengths. Borisova et al. [140] measured pure samples of collagen type I, porphyrin, and keratin and evaluated the other compounds in the spectra using the knowledge about excitation and emission maxima cited in the literature and Table 13.1. The amino acid fluorescence appears in the UV, collagen, and FAD at around 400 nm. NADH is the strongest fluorophore with emission at around 450 nm followed by elastin and keratin with green fluorescence. The autofluorescence of porphyrin shows a low-intensity signal at wavelengths above 600 nm. On the right side of Fig. 13.10, the autofluorescence spectra for early BCC tumor are shown at different excitation wavelengths. The most pronounced difference are the overall intensities of the fluorescence spectra which are lower for BCC compared to normal skin. This observation has frequently been reported in the literature [142, 151]. A crucial parameter for the diagnosis is the spectral shift of the autofluorescence. While the intensity doubles in the 320–360 nm range, it drops by a factor of 3 in the 390–460 nm range. This could be due to the changes in the

optical parameters within the tumor and to the increasing thickness of the tissue. The autofluorescence of the porphyrins is also carefully observed as it increases in higher-graded tumors. Autofluorescence diagnostics of malignant melanoma (MM) is hampered by the high absorption and reabsorption of the radiation. Moreover, it is limited to the skin surface due to the low penetration depth. Nevertheless, MM leads to a decay of the NADH fluorescence [152].

Existing studies had been focused on intensity changes in the autofluorescence, also in comparison to normal skin [151, 153–157]. Some researchers linked autofluorescence measurements with diffuse reflectance spectroscopy [158–161]. Wollina et al. could demonstrate that the diffuse reflectance-corrected autofluorescence of NADH, excited by a UV LED, permits BCC to be distinguished from actinic keratosis (AK) [162]. Other researchers complemented their autofluorescence experiments by time-resolved measurements [151].

Borisova et al. performed autofluorescence investigations on 536 patients affected by BCC (137 lesions), SCC (29), MM (41), benign skin lesions (194), keratoacanthoma (11), and dysplastic naevi (124) [140]. In addition to the autofluorescence, the diffuse reflectance of the skin was measured. The spectra were interpreted according to Savelieva et al. [163]. For clinical diagnostics, the spectral shape must be included into the analysis, and thus the autofluorescence analysis was made using excitation wavelengths of 365 nm and 405 nm, extracting 23 spectral parameters, intensity levels, specific minima and maxima, intensity ratios, slopes of the spectra in given ranges, etc. The obtained results permitted the development of a multispectral discrimination algorithm. In most of the BCC lesions (80 %), strong decreases in autofluorescence were observed compared to normal tissue. In case of SCC higher autofluorescence intensities occured. Also, the stage of the tumor changes the behavior of the spectra. In early stages of BCC, the intensity is reduced without changing the spectral form. In their advanced stage, BCC exhibits pronounced fluorescence maxima in the green-red spectral range, related to endogenous porphyrins [140, 164]. For a better specification, the type and melanin content of the skin has to be taken into account, too. It strongly affects the spectra and is an important part of the algorithm. Multispectral analysis provides high sensitivity >99.1 %, specificity >90.5 %, and diagnostic accuracy >93.3 % d for NMSC versus benign, MM versus benign, and MM versus NMSC [140].

Besides direct excitation in the UV spectral range, also two photon techniques are available making use of a femtosecond laser that is tunable from 690 nm to 1000 nm. They are based on two NIR photons exciting the fluorescence of the endogenous fluorophores at one single spot in the tissue [165]. The excitation at only one spot yields a high spatial resolution permitting cellular structures in the skin to be imaged up to approximately 200 µm in depth at high resolution. While NADH is the most important fluorophore, FAD, elastin, and collagen contribute also to the image acquisition. As the multiphoton technique is also capable of determining the fluorescence lifetime, the fluorophores can be differentiated even further. Taking into account that most fluorophores, for instance NADH, are metabolically active substances, possible changes in these substances can be utilized for wound healing or tumor diagnostics [166, 167].

13.7 Summary and Outlook

Many natural and synthetic fluorophores can be excited with UV light, and therefore numerous applications of UV fluorescence detection and spectroscopy are already existent. Since for sensing purpose low or medium power LED modules are usually sufficient, almost all of these applications are benefiting from the availability of solid-state UV emitters replacing bulky and inefficient lamps or lasers. Their compact size, adjustable wavelength, low power consumption, fast switching, and adjusting properties (just to name a few of the advantages inherent to state-of-the-art LED technology) not only enables miniaturization and thus point-of-care testing and analysis but also leads to methodologically new and promising approaches in clinical diagnosis and (environmental) chemical sensing. Especially when the autofluorescence of a multicomponent target is analyzed, UV LED technology is almost without alternative since it easily provides multiple wavelengths for fluorescence excitation of the ensemble of fluorophores that is either characteristically changing due to a malignancy or characteristic for the target to detect.

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