Chapter 9
Fluorescence

In the previous chapter, we learned about spectrophotometric detection for biosensor applications, namely, detection of light absorbance at a certain wavelength. This method works well for a variety of applications. Despite its popularity, it has some limitations. The target molecule must exhibit specific coloration(s), i.e., absorption peaks at certain specific wavelength(s). It is possible to use a combination of an enzyme and a substrate that will exhibit a specific coloration. An example of this would be glucose oxidase and benzidine in detecting glucose (will be discussed later in Chap. 12). However, such enzyme–substrate colorimetric assay is not always possible. Sometimes, when the target is a complicated biomolecule, such as a protein, virus, or even bacterium, this is not an easy task because such an enzymatic reaction is generally not possible.

A more generalized approach is to "label" the target molecule with a specific dye, and quantify the concentration of the dye. This dye can be conjugated directly to the target, but a more generalized approach is the use of a secondary bioreceptor that is conjugated with a dye. Figure 9.1 graphically illustrates this concept:

Bioreceptors (primary antibodies in Fig. 9.1) are pre-immobilized on a solid surface prior to the assay. A solution that may contain target molecules (antigen to the primary antibody in Fig. 9.1) is added. Upon washing, all unbound molecules are washed away. If dye (fluorescent dye in Fig. 9.1) is conjugated to the target prior to the assay, we simply need to monitor fluorescent emission coming from the solid surface. This monitoring confirms the existence of target molecules and possibly quantifies its concentration through light intensity measurement. However, this direct dye conjugation is impractical, as the other molecules in a test solution may also be conjugated with fluorescent dyes. Therefore, secondary antibodies, identical to the primary antibodies but pre-conjugated with fluorescent dyes, are added to the surface followed by washing. These secondary antibodies will bind to captured target on the surface if it is present. Again, measurement of fluorescent emission from the surface can confirm the existence of a target and/or quantifying its concentration. Other types of bioreceptors can also be used, including DNA/RNA and enzymes.

J.-Y. Yoon, *Introduction to Biosensors*, DOI 10.1007/978-3-319-27413-3_9

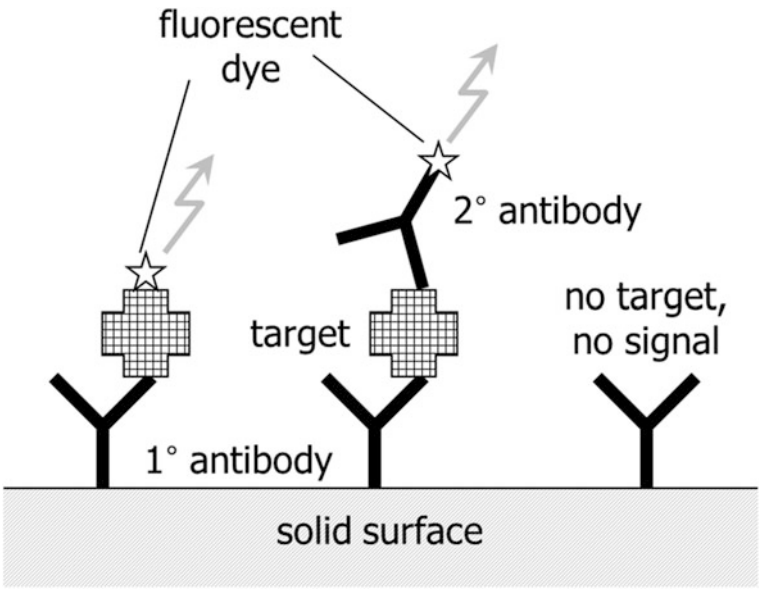


Fig. 9.1 Use of fluorescent dye in biosensing

Two different types of dyes have been used—*radioisotope* and *fluorescent dyes*. Radioisotopes are extremely powerful, as a tiny amount of them can exhibit significant magnitude of radioactive decay, leading to extreme sensitivity. Due to the strict regulation on the use of radioisotopes and the difficulty of their use, however, radioisotopes are losing their popularity. Fluorescent dyes have mostly replaced the applications of radioisotope labeling. Due to the recent advancements in optoelectronic components and devices, fluorescent dyes have become at least comparable to radioisotope dyes in terms of their sensitivity. Fluorescent dyes are not toxic, safe to use, and relatively easy to conjugate to bioreceptors for labeling.

9.1 Fluorescence

Before we begin learning about fluorescent dyes, we should first learn the definition of *fluorescence*, especially in comparison with absorption spectrophotometry. In absorption spectrophotometry, the wavelengths of incident light do not change when the light passes through a cuvette—only its intensity is attenuated at certain wavelengths. If the solute in a solution is *fluorescent* (i.e., fluorescent dyes), the color of emitted light from the cuvette is altered to the longer wavelength. This difference is schematically illustrated in Fig. 9.2.

An easy example of fluorescence is a *fluorescent lamp*, which has mostly replaced incandescent light bulbs in lighting industry (although fluorescent lamps are recently being challenged by LED lamps). In a fluorescent lamp, the tube is

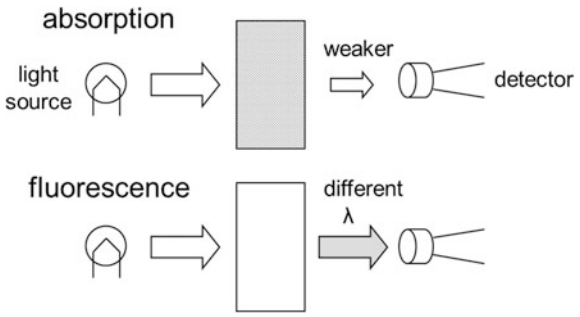


Fig. 9.2 Absorption versus fluorescence

charged with mercury vapor, which produces UV light upon applying electrical voltage. The inner surface of the tube is coated with fluorescent coatings, which absorb UV light and emit visible lights (excitation = UV, shorter wavelength; emission = visible, longer wavelength). The term fluorescence was derived from the mineral *fluorite*, which is largely calcium fluoride.

When molecules are exposed to light irradiation (exposure to photons), the energy carried by the photons is transferred to the electrons in the molecules, which moves them from the stable ground state to the unstable excited states. As these excited states are not preferable to the molecules, they want to lose this excess energy and return back to the ground state. This can happen by emitting the photons at the identical wavelength as that of initial light irradiation. When it occurs, it will look like nothing has ever happened. Molecules may also use this excess energy for molecular rotations and/or vibrations (internal energy U), or production of heat (Q). For most molecules, both happen at the same time, resulting in a light emission from the molecules that is attenuated, hence absorption. As certain molecules do convert more photons to U and Q at a specific wavelength than the other wavelengths, we should get different absorption intensities over a range of wavelengths, which is an absorption spectrum.

For the molecules that exhibit fluorescence, they do absorb more photons at a specific wavelength (e.g., for the mineral fluorite, the maximum absorption occurs at UV color). The excited electrons return back to the ground state mostly by emitting photons (but not U and Q), but the energy loss happens in two stages. A small amount of energy is lost in the first stage, which shifts the excited electrons to the less excited state (known as *Stokes shift*). Later, a large amount of energy is lost, returning the excited electrons back to the ground state. Therefore, the emitted light from the fluorescent molecule should carry less energy than the incident light. The energy of light, or more generally electromagnetic wave, is related to the wavelength by the following equation:

$$E = \frac{hc}{\lambda} \tag{9.1}$$

where

E = energy, h = Planck's constant = 6.6×10^{-34} J s,
c = speed of light = 3×10^{8} m s^{-1}, λ = wavelength.

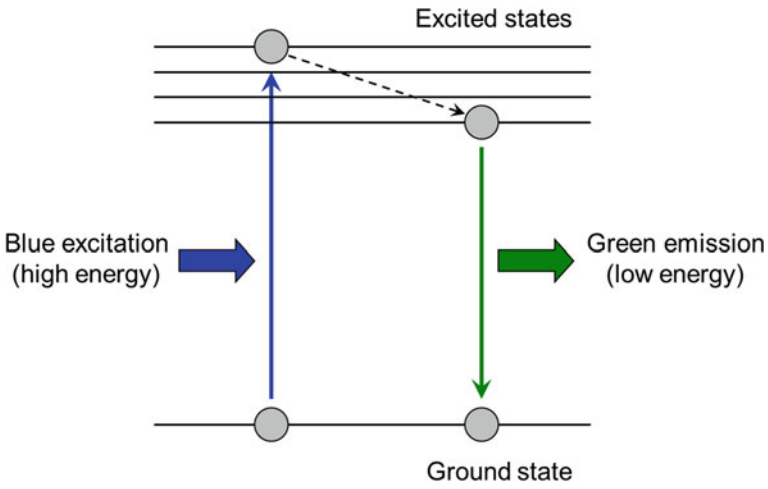
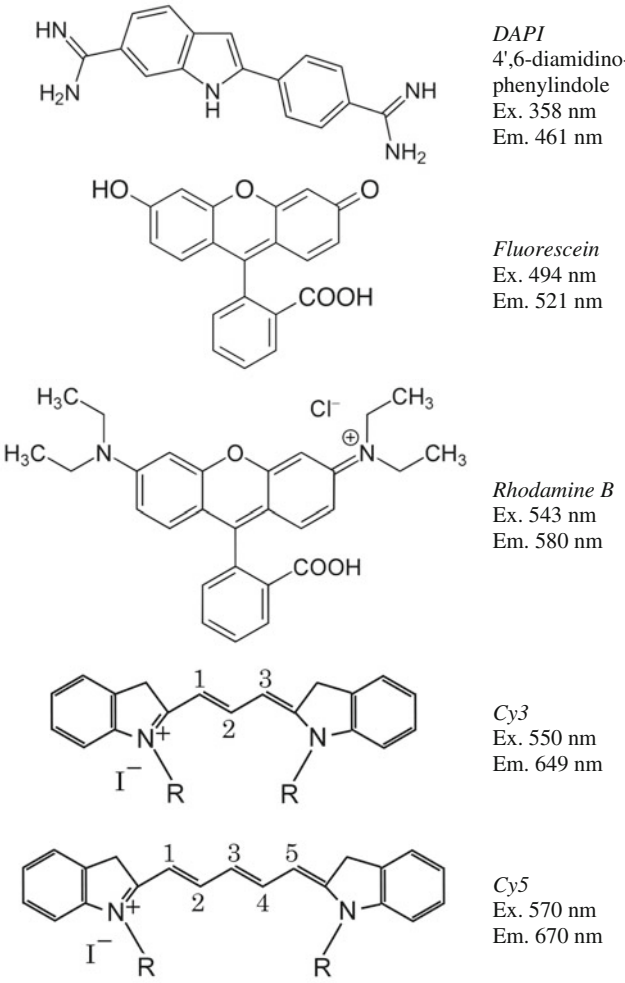


Fig. 9.3 Fluorescence

Therefore, the emitted light should have a longer wavelength than the incident (excited) light. The optimum wavelengths of excitation and emission vary by the type of fluorescent dyes. For the fluorescent lamp, excitation is UV and emission is visible light. For the example shown in Fig. 9.3, excitation is blue and emission is green.

9.2 Fluorescent Dyes

At this point you may wonder why fluorescent dyes are better than regular absorption dyes. This is due to the superior sensitivity of fluorescence photometry over absorption photometry. Pico- (10^{-12}) or even femtograms (10^{-15}) of fluorescent dyes per 1 mL solution can be detected, which is not possible with conventional absorption dyes. This superiority is due to the fact that many solvents (especially water) are "transparent" to the fluorescence measurement, as those solvents are not fluorescent. In absorption measurement, however, almost all solvents do attenuate some light (absorption by solvents), which creates unnecessary background noise and affects its sensitivity. Many different fluorescent dyes have been identified and used in numerous chemical and biological applications. The following list is just a small fraction of available fluorescent dyes (Fig. 9.4). All fluorescent dyes possess a couple of aromatic ring structure, which is primarily responsible for the loss of small energy shift (Stokes shift). You can also notice that the molecular size of a fluorescent dye is correlated with the wavelengths of excitation and emission maxima.



DAPI
4',6-diamidino-2-phenylindole
Ex. 358 nm
Em. 461 nm

Fluorescein
Ex. 494 nm
Em. 521 nm

Rhodamine B
Ex. 543 nm
Em. 580 nm

Cy3
Ex. 550 nm
Em. 649 nm

Cy5
Ex. 570 nm
Em. 670 nm

Fig. 9.4 Fluorescent dyes

Note that both fluorescein and rhodamine dyes are typically conjugated with isothiocyanate (–N=C=S) at the bottom aromatic ring to facilitate chemical conjugation to proteins, called fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC).

Fluorescent dyes have frequently been used in many life science applications. The most common example would be fluorescence microscopy. In *fluorescence microscopy*, we typically label two or three different portions of a target (typically cells) with two or three different fluorescent dyes. For the example shown in Fig. 9.5, three different fluorescent dyes are used—DAPI, FITC, and TRITC—each conjugated to different portions of a cell. The microscopic slide is first excited with

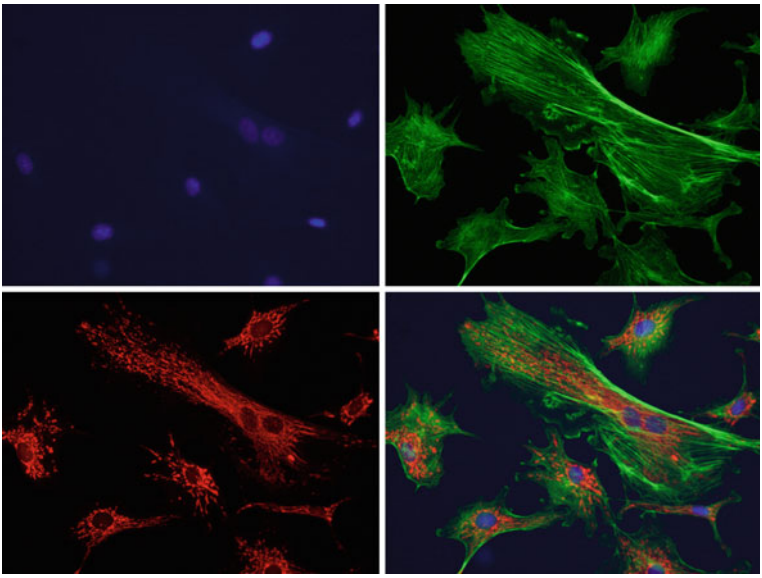


Fig. 9.5 The fluorescence microscopic images of a cell. Different portions of a cell are conjugated with different fluorescent dyes. Excitation/emission colors: UV-*blue* (*top left*), *blue-green* (*top right*), *green/red* (*bottom left*), and combined (*bottom right*)

UV for DAPI to emit blue light. This image is captured in black and white to maximize its resolution, as we already know its color. Next, the slide is excited with blue for FITC to emit green color. The image is again captured. Finally, the slide is excited with green for TRITC to emit yellow-red color. Again, the black and white image is captured. Computer software then assigns an appropriate pseudo-color to each black and white image, and superimposes all three images to obtain a nice image shown at the bottom right corner in Fig. 9.5.

To obtain fluorescence microscopic images, we need a fluorescence microscope (Fig. 9.6), which is essentially a regular light microscope equipped with a fluorescent light source, filter cube, and an appropriate computer software. A *filter cube* is an essential component in a fluorescence microscope, which delivers excitation light only to the specimen but not to the eyepiece or camera and emission light only to the eyepiece or camera.

Figure 9.7 shows two different versions of a fiber cube, one for an upright microscope and the other for an inverted microscope. In both cases, a dichroic mirror (also known as a beam-splitting mirror) reflects the short excitation light but lets the long emission light to pass through. For imaging with three different fluorescent dyes, we need three different filter cubes that can mechanically slide through a microscope horizontally. A fluorescent light source is also important to deliver just the color that is needed for excitation.

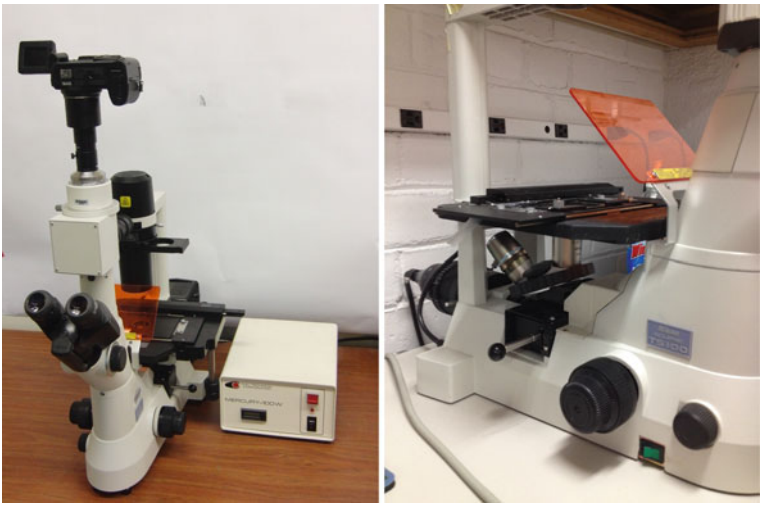


Fig. 9.6 Inverted fluorescence microscope showing the overall shape (*left*) and objective lenses/filter cubes (*right*)

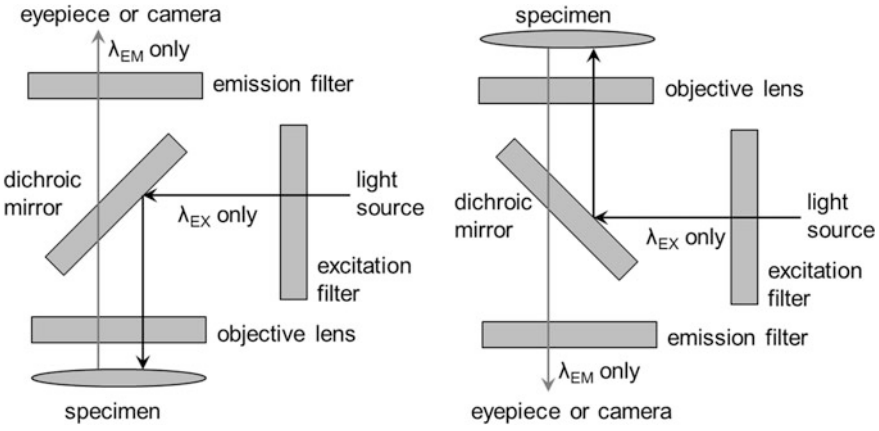


Fig. 9.7 Filter cubes for upright (*left*) and inverted (*right*) fluorescence microscopes

9.3 Advanced Fluorescent Dyes: GFP, SYBR, and QD

Fluorescent dyes typically need to be conjugated to either a target molecule or a bioreceptor (e.g., antibody) prior to the assay. There are several examples of "advanced" fluorescent dyes that do not require such pre-assay conjugation.

The first example is green fluorescent protein (GFP), which is actually a protein rather than a chemical. This protein exhibits green fluorescence, with excitations at 395 or 475 nm and emission at 509 nm. First isolated from the jellyfish, *Aequorea victoria*, GFP has frequently been used as a marker for gene expression. When a target gene is inserted into an organism, the gene for GFP is also inserted. If the gene insertion is successful, GFP will be synthesized, and green fluorescence can be observed.

The second example is *SYBR* family of dyes from molecular probes (the most well-known example is SYBR Green I). *SYBR Green I* itself is a cyanine dye (Fig. 9.8), whose structure is similar to Cy3 and Cy5 shown in Fig. 9.4. SYBR Green I has a strong, specific affinity to double-stranded DNA (dsDNA), and when it binds to dsDNA, it exhibits strong green fluorescence (excitation at 497 nm and emission 520 nm). SYBR Green I is frequently used to identify and quantify dsDNA in the sample, where it functions as both fluorescent dye and bioreceptor. SYBR Green I is particularly useful in monitoring the progress of polymerase chain reaction (PCR). PCR is the process of amplifying the amount of target DNA sequence from the sample, and the production of target dsDNA can easily be monitored by adding SYBR Green I dye to the PCR mixture. PCR assay with this fluorescence monitoring feature is known as real-time PCR or quantitative PCR (qPCR). PCR will be further discussed later in this textbook (Chap. 14).

There are other examples of DNA intercalating dye that can be used for DNA sensing and PCR. The most well-known example is *ethidium bromide* (Fig. 9.8), which has been known long before the introduction of SYBR family dyes. Ethidium bromide also intercalates the dsDNA and exhibits strong orange/yellow fluorescence (emission at 590 nm) with UV excitation (excitation at either 300 or 360 nm). Ethidium bromide has been used to identify and quantify dsDNA in *gel electrophoresis*, which has also been popularly used as an endpoint identification tool for conventional PCR assays.

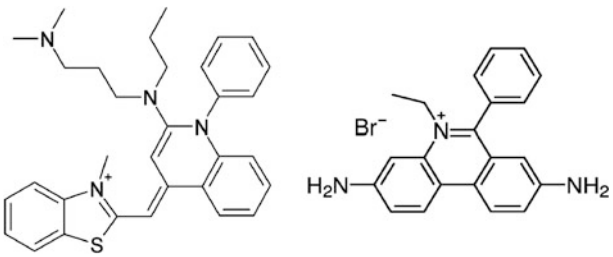


Fig. 9.8 DNA intercalating dyes: SYBR *Green* I (*left*, Ex. 497 nm, Em. 520 nm) and ethidium bromide (*right*, Ex. 300 or 360 nm, Em. 590 nm)

The last example of advanced fluorescent dyes is not about pre-assay conjugation, but rather about overcoming the disadvantage of fluorescent dyes: *photobleaching*. Continuous light exposure to fluorescent dyes leads to their destruction, thus fluorescent emission decays over time. To overcome this problem, a new type of fluorescent-like dye has been developed: *quantum dots*. Quantum dots are not fluorescent dyes, as they are essentially colloidal semiconductor nanocrystals. Despite this difference, they do absorb UV light (UV excitation) and emit visible light (visible emission), which is very similar to fluorescent dyes (hence, it is called *artificial fluorescence*). The emission wavelength becomes longer as the size of quantum dots gets bigger, although they are all excited by UV regardless of their size. Quantum dots are much brighter, yet exhibit much less photobleaching than fluorescent dyes, at a cost of possible toxicity to cells. Quantum dots will be further discussed in Chap. 15.

9.4 Autofluorescence

Autofluorescence is a naturally occurring fluorescence emission, mostly from biological samples such as cells and tissues. Since there exist so many different types of chemicals and biomolecules in cells and tissues, it is natural that some of them exhibit fluorescent characteristics. In fluorescence microscopy and fluorescence biosensing, this autofluorescence comes largely from sample matrices, such as blood, urine, saliva, cell culture, water, food, etc. Such autofluorescence from sample matrices can be quite problematic, since it obscures the true fluorescence signal from the target molecules. Examples of autofluorescence include nicotinamide adenine dinucleotide (NAD^+/NADH), Flavin adenine dinucleotide (FADH/$FADH_2$), urobilin, polymers, paper, chlorophyll, etc.

Nicotinamide adenine dinucleotide (NAD^+/NADH) from cell and tissue samples (Fig. 9.9): NAD^+/NADH is a common co-enzyme found in many types of cells, involved in several different redox reactions. Both NAD^+ (oxidized form) and NADH (reduced form) strongly absorb deep UV (peak at 260 nm), while only NADH shows blue fluorescence (emission peak at 460 nm). This unique distinction between NAD^+ and NADH has been utilized in many different biological assays (and subsequently in biosensing), although the presence of NADH in biological sample can cause some trouble in detecting other types of fluorescent molecules.

Flavin adenine dinucleotide (FAD^+/FADH/$FADH_2$) from cell and tissue samples (Fig. 9.9): FAD^+/FADH/$FADH_2$ is another common co-enzyme found in many types of cells, again involved in several different redox reactions. FAD^+ is the fully oxidized form, FADH is the partially reduced form, and $FADH_2$ is the fully reduced

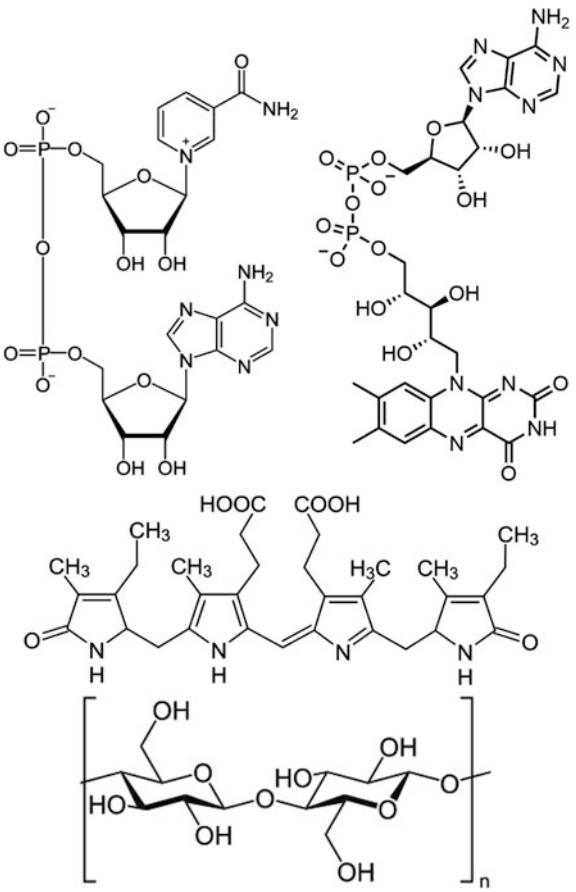


Fig. 9.9 Chemicals responsible for autofluorescence: NADH (*top left*) and FAD^+ (*top right*), urobilin (second row), and cellulose (third row)

form. All three forms strongly adsorb blue light (peak at 450 nm), and the oxidized FAD^+ emits green fluorescence (emission peak at 520 nm). Both NAD^+ and FAD^+ have been utilized in glucose sensing, which will be discussed later in Chap. 12.

Urobilin from urine samples (Fig. 9.9): This is the molecule responsible for yellow coloration and green fluorescence of urine. In the body, the aged red blood cells, which are essentially a sack containing hemoglobin (refer to the previous chapter), are cleared in spleen. The heme portion of hemoglobin is turned into bilirubin, and excreted as bile into the small intestine. The microbes in the large intestine further break the bilirubin into urobilinogen (colorless). Some of the urobilinogen is converted into sterobilin (brown color), responsible for the coloration of feces. Some of urobilinogen is reabsorbed back into the bloodstream,

oxidized to urobilin (yellow color), and excreted as urine, responsible for the coloration of urine. Urobilin strongly absorbs blue color (peak at 450 nm), resulting in yellow (=green + red) coloration. This blue absorption also leads to a small amount of green fluorescence (emission peak at 530 nm), again causing some troubles in biosensing from urine samples.

Polymers used for sensor platforms/substrates: Many polymeric materials are known to exhibit fluorescence upon UV irradiation. The emission wavelengths are quite broad, typically ranging from blue to green color. Since the polymers exist in varying chain lengths and different molecular conformations, several different modes of fluorescence can be found from a single polymeric sample, resulting in multiple peaks of fluorescent emissions.

Paper (*cellulose fibers*) from sensor platforms/substrates (Fig. 9.9): Like polymers, most papers (cellulose fibers) also exhibit blue to green fluorescence upon UV irradiation. Autofluorescence of polymers and papers is quite important in biosensing applications, since many biosensors are being developed using polymers or papers as a substrate material. For example, many glucose test strips (Chap. 12), lateral flow assays (Chap. 13), and lab-on-a-chip biosensors (Chap. 14) are being fabricated using either polymers or papers.

Chlorophyll from plant samples: Chlorophyll is the chemical responsible for the green coloration of many plants. Most chlorophylls (note that there exist several different forms of chlorophyll, while *chlorophyll a* being the most common and well-known) absorb blue and red colors, thus generating green coloration. In addition, chlorophyll exhibits red fluorescence, with a very strong peak at 670–680 nm (red) and the second one around 720–730 nm (far red). This red fluorescence must be considered in testing any plant samples, including algae, which has been a quite popular theme for bioenergy research.

9.5 Detection of Fluorescence

The main difference between spectrophotometry and fluorescence sensing is illustrated in Fig. 9.2: difference in wavelength (λ). Therefore, isolation of emission light from excitation light becomes critical in fluorescence sensing. In fluorescence microscopy, this isolation is achieved using a dichroic mirror, which reflects the excitation light and pass the emission light (Fig. 9.7). To prevent the excitation light leaking into the emission wavelength and the emission light leaking into the excitation wavelength, excitation filter and emission filter are also required. These *optical filters* are either *low-pass filters*, *high-pass filters*, or *band-pass filters*. Similar to op-amp filters, a low-pass filter passes the light whose wavelength is shorter than a certain wavelength, a high-pass filter longer than a certain wavelength, and a band-pass filter a small range of wavelengths. Same optical filters can be utilized in developing fluorescence-based sensors, in a layout similar to that of a fluorescence microscope.

In practical fluorescence sensing, simpler fluorescence sensing is possible using optical fibers, LEDs, and/or laser diodes. Excitation with an LED or a laser diode makes the excitation light monochromatic, eliminating the need for an excitation filter. Delivering this excitation using an optical fiber or a collimating lens make the excitation light delivered coherently to the sample. The excitation light that passes through the sample (i.e., transmitted light) can be detected at 0° (relative to the light source). The fluorescent emission light can be detected at any angle other than 0°, since the excitation beam is coherent (or concentrated to one direction). The most commonly used angle of fluorescent detection is either 180° (*back scatter*) or 90° (*side scatter*). As the excitation light is coherent, no excitation light can be detected at these angles (180° or 90°), eliminating the need for an emission filter as well as a dichroic mirror (or a dichroic filter).

Figures 9.10 and 9.11 show the schematic diagrams of these angled detection of fluorescence, for the case of a green fluorescent dye (excitation at 475 nm and emission at 530 nm). Figure 9.10 shows the schematic of 180° back scattering fluorescence detection, using a *reflection probe*, which we briefly discussed in the previous chapter (Chap. 8). The blue LED light (475 nm) is delivered through the core of the reflection probe, while the back scattered green fluorescence (530 nm) is picked up through the shell-side bundle of fibers, eventually delivered to the *miniature spectrometer*. If there is no reflection, this particular setup is quite easy-to-use and usable for various field applications. Unfortunately, reflections from the sample container as well as the liquid–air interface are quite common, as shown in the left side of Fig. 9.10, and the miniature spectrometer may pick up both excitation and emission lights. Therefore, the reflection probe must be dipped into the liquid sample, but not too close to the side walls or the bottom surface of the

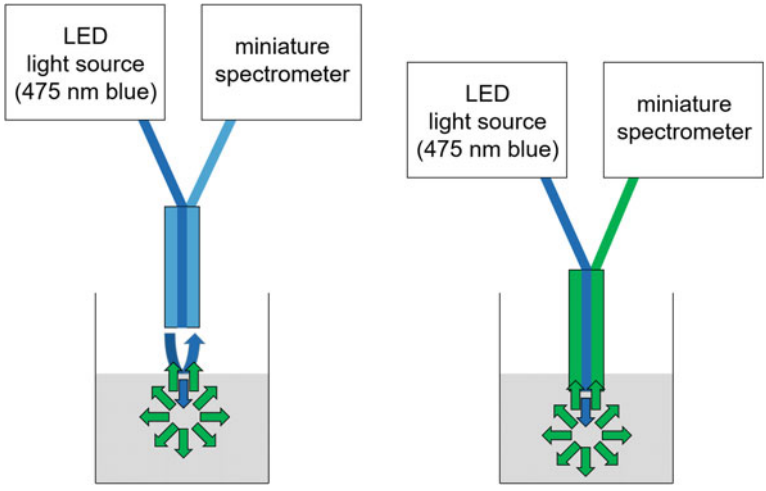


Fig. 9.10 A reflection probe captures green fluorescence from a liquid sample. Both excitation and emission lights are picked up in the *left* setup, due to the reflection at the air–liquid interface, while only emission light is picked up in the *right* setup with the probe dipped into the liquid sample

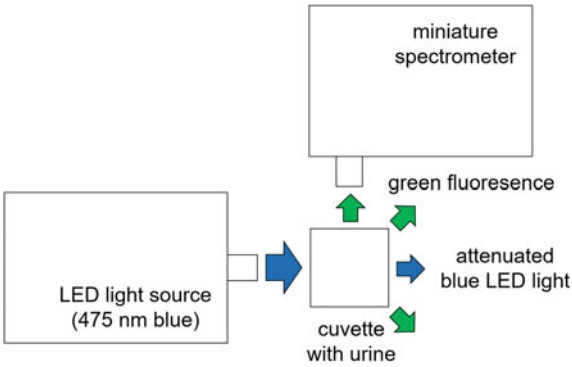


Fig. 9.11 An LED light source and a miniature spectrometer are aligned at 90° for side scatter fluorescence detection

sample container (e.g., beaker), as shown in the right side of Fig. 9.11. This may not be practical in using small sample containers such as centrifuge tubes or cuvettes.

Figure 9.11 shows the schematic of 90° side scattering fluorescence detection, which is more appropriate in dealing with small-sized sample containers (a cuvette in this case).

In the following two laboratory tasks, we will learn these two types of filter-free detection of fluorescence: 180° back scatter detection using a reflection probe for a fluorescein solution (Task 1) and 90° side scatter detection for human urine (Task 2). Other types of filtered or filter-free fluorescence detection are possible, e.g., utilizing laser diodes, quantum dots, smartphones, 30° forward scatter, 45° forward scatter, etc.

9.6 Laboratory Task 1: 180° Back Scatter Fluorescence Detection for Fluorescein

In this task, you will need the following:

- A miniature spectrometer (USB4000 or Flame from Ocean Optics) with appropriate software (OceanView™ from Ocean Optics)
- A desktop or laptop computer
- A reflection probe with optical fibers (R400-7-UV–VIS)
- A light source with blue LED (LS-450 from Ocean Optics)
- Electronic balance, weighing paper, laboratory spatula
- Distilled and/or deionized water
- Beakers
- A vortex mixer; A sonicator (optional)
- Fluorescein
- Latex gloves, delicate task wipers (Kimwipes®)

– Locate the LS-450 blue LED light source that produces either pulsed or continuous output at 475 nm (the blue region) for fluorescence measurements.

- The 475 nm blue LED is slightly off from the optimum excitation peak (494 nm), but it should still provide sufficient fluorescence signals. If possible, try to use a different LED that is closer to 494 nm.
- Insert the input leg of the optical fibers from the reflection probe into the light output port on the LS-450, hand-tighten only.
- Make sure to place the switch in the "off" position. Plug the 12 V DC/1.5 mA power supply into the LS-450 before plugging it into a 115 AC power outlet. Note: Do not plug in the serial port connector.
- Switch to continuous mode. The blue LED should light up.
- Dispense 40 mL of deionized or distilled water into a 50-mL glass beaker.
- Point the probe down at the water with the probe tip height set equal to the top of the beaker.
- Take the spectrum. Adjust the integration time to make the peak bigger or to prevent signal saturation. If the spectrum is noisy, you may wish to increase the scans to average to reduce the noise over time, or increase the boxcar width to smoothen the spectrum over the wavelengths. The blue illumination should be reflected at the air–water interface, and you should get a peak at 475 nm.
- You should also get multiple peaks from the fluorescent lights at the ceiling of your lab. Turn off the lab lights and check whether those peaks have disappeared.
- Dispense 40 mL of deionized or distilled water into a 50-mL glass beaker. Add fluorescein to a final concentration of 0.25 mg/mL.
- Cover the beaker with a sealing film (e.g., Parafilm), and vortex it for 1 min. Sonicate the solution as desired (optional), since the fluorescein has limited solubility in water.
- Point the probe down at the water with the probe tip height set equal to about 1 cm above the solution. Make sure that the probe tip is dry. Take the spectrum (Probe Dry). Most of your blue illuminations will be reflected at the air–water interface, with limited fluorescent emission coming from your solution. Therefore, you should have a bigger peak of blue (475 nm—reflection) and a smaller peak of green (525 nm—fluorescent emission) (Fig. 9.12).
- Now, immerse the probe 1 cm down from the air–water interface (Fig. 9.13). Take the spectrum (Probe Wet). Since there is no reflection at the air–water

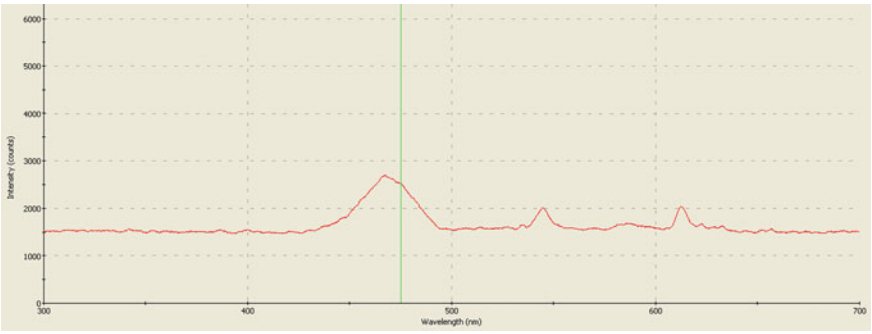


Fig. 9.12 Experimental data of Task 1—*blue* illumination (475 nm) with ambient light interference

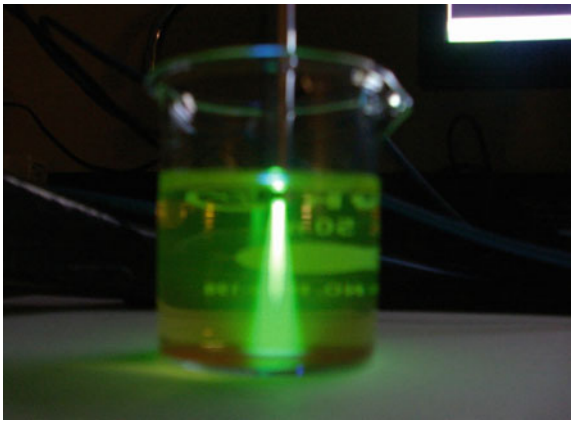


Fig. 9.13 *Blue* irradiation from the probe excites fluorescein to emit *green* color

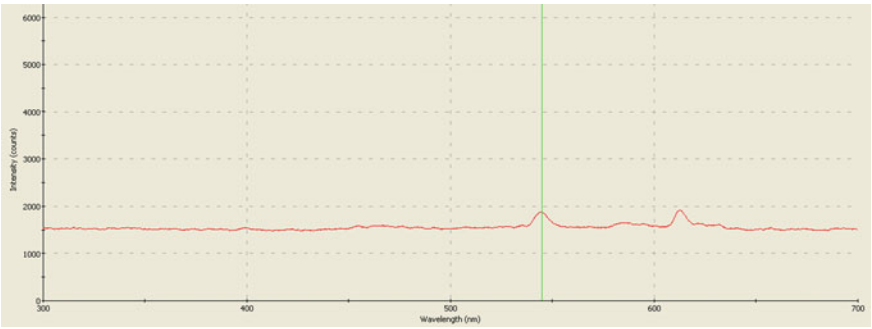


Fig. 9.14 Experimental data of Task 1—fluorescent emission (545 nm) with ambient light interference under "probe wet" condition. Note that fluorescent excitation (475 nm) cannot be seen

interface you should have only green fluorescent emission (525 nm) (Fig. 9.14). If you immerse your probe too deep, the probe starts to catch the reflection at the bottom of the beaker, and the blue peak may re-emerge.

– Repeat the Probe Dry and Probe Wet experiments with the lab lights turned off.

Alternative Task 1: Reflection Probe for Other Fluorescent Dyes

Repeat Task 1 with other fluorescent dyes, such as rhodamine. As the excitation wavelength of rhodamine is different from that of fluorescein, you should change the LED of the LS-450 light source.

Question 9.1

For a mixture of fluorescein and rhodamine solutions, roughly sketch the spectra with (a) blue light source (475 nm), (b) green light source (555 nm), and (c) incandescent light bulb. Assume "probe wet" condition (i.e., no reflection of excitation light).

9.7 Laboratory Task 2: 90° Side Scatter Fluorescence Detection for Urine

In addition to the materials and equipment required for Task 1, you will need:

- Human or animal urine samples
- Filter paper (optional)
- Cuvette

– As explained in Sect. 9.4, urobilin in urine is responsible for the yellow coloration of urine (absorbs blue but not green and red, resulting yellow coloration), as well as green fluorescence (excitation at 450 nm = blue and emission at 530 nm = green).
– The 475 nm blue LED is slightly off from the optimum excitation peak (450 nm) of urobilin, but it should still provide sufficient fluorescence signals. If possible, try to use a different LED that is closer to 450 nm.
– Fill the cuvette with various human or animal urine samples. For this laboratory exercise, a sterilized human urine sample from pooled human donors is used.
– Using a filter paper, filter this urine sample (optional).
– Align the LED light source and the miniature spectrometer at 90° to the cuvette, as shown in Figs. 9.11 and 9.15.

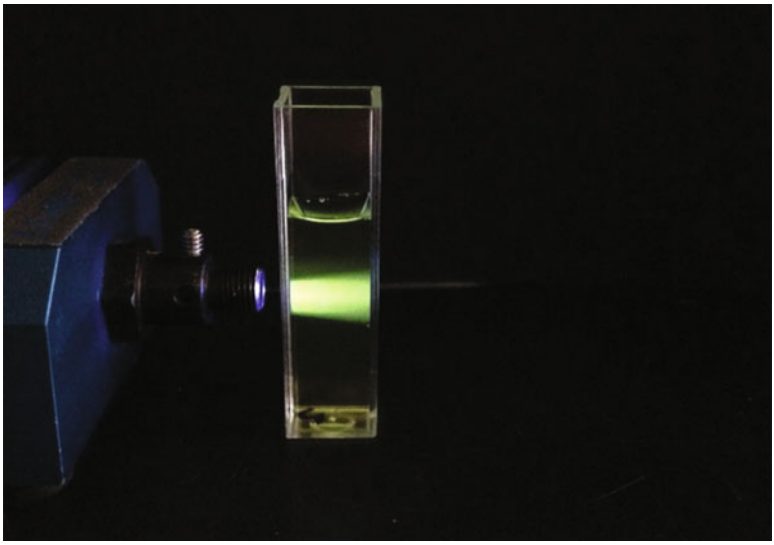


Fig. 9.15 *Blue* irradiation from the LED light source excites urine sample to emit *green* color

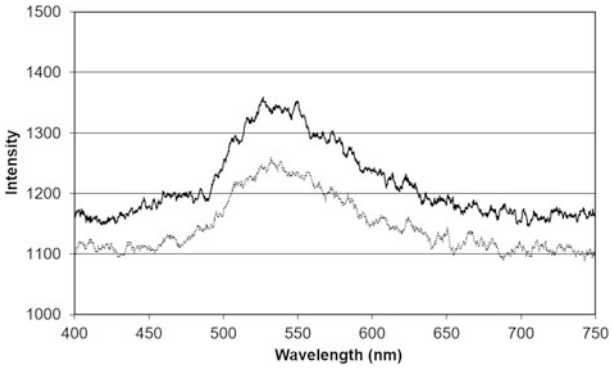


Fig. 9.16 Experimental data of Task 2—fluorescent emission (530 nm) of unfiltered urine (*solid line*) and filtered urine (*dashed line*)

– Measure fluorescent intensities at its peak (530 nm) (Fig. 9.16).
– Repeat the same experiment for filtered urine. Can you observe a decrease in fluorescence?

Alternative Task 2: LED–PD Circuit for Urine

Repeat Task 2 with the LED–PD circuit shown in Chap. 8 Task 2. Use blue LED as a light source (Fig. 9.17).

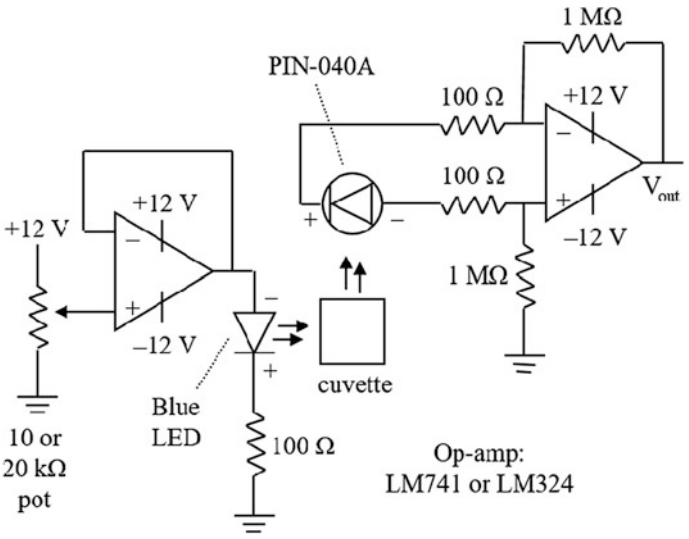


Fig. 9.17 Circuit diagram of Alternative Task 2

References and Further Readings

General

Demchenko A (2008) Introduction to fluorescence sensing, 1st edn. Springer, New York
Goldys E (2009) Fluorescence applications in biotechnology and life sciences, 1st edn. Wiley-Blackwell, Oxford
Lakowicz J (2006) Principles of fluorescence spectroscopy, 3rd edn. Springer, New York

Advanced Fluorescent Dyes (Sect. 9.3)

Molecular Probes® (2015) Qdot® Nanocrystal technology overview. http://www.lifetechnologies.com/us/en/home/brands/molecular-probes/key-molecular-probes-products/qdot/technology-overview.html

Autofluorescence (Sect. 9.4)

Cho S, Park TS, Nahapetian TG, Yoon J-Y (2015) Smartphone-based, sensitive μPAD detection of urinary tract infection and gonorrhea. Biosens Bioelectron 74:601–611
Cornelius CE (1980) Liver Function. In: Kaneko JJ (ed) Clinical biochemistry of domestic animals, 3rd edn. Academic Press, Burlington
Danielson TL, Rayson GD, Anderson DM, Estell R, Fredrickson EL, Green BS (2003) Impact of filter paper on fluorescence measurements of buffered saline filtrates. Talanta 59:601–604
Kerkhoff JF, Peters HJ (1968) A reproducible estimation of the urobilin concentration in urine by means of a modified schlesinger test. Clin Chim Acta 21:133–137
Lakowicz JR, Szmacinski H, Nowaczyk K, Johnson ML (1992) Fluorescence lifetime imaging of free and protein-bound NADH. Proc Natl Acad Sci USA 89:1271–1275
Nurmukhametov RN, Volkova LV, Kabanov SP (2006) Fluorescence and absorption of polystyrene exposed to UV laser radiation. J Appl Spectrosc 73:55–60
Plitt KF, Toner SD (1961) A study of the fluorescence of cellulosic polymers. J Appl Polym Sci 17:534–538
Pedrós R, Moya I, Goulas Y, Jacquemoud S (2008) Chlorophyll fluorescence emission spectrum inside a leaf. Photochem Photobiol Sci 7:498–502