Fluorescence is the molecular absorption of light energy at one wavelength and its nearly instantaneous re-emission at another, usually longer, wavelength. Some molecules fluoresce naturally and others can be modified to make fluorescent compounds.

Fluorescent compounds have two characteristic spectra: an excitation spectrum (the wavelength and amount of light absorbed) and an emission spectrum (the wavelength and amount of light emitted). These spectra are often referred to as a compound's fluorescence signature or fingerprint. No two compounds have the same fluorescence signature. It is this principle that makes fluorometry a highly specific analytical technique.

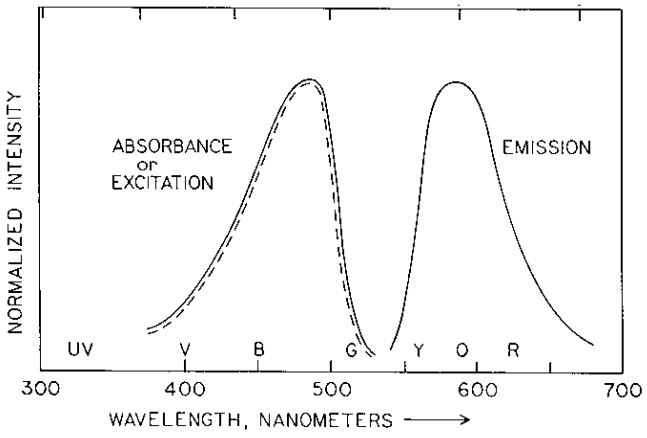
Fluorometry is the measurement of fluorescence. The instrument used to measure fluorescence is called a fluorometer or fluorimeter. A fluorometer generates the wavelength of light required to excite the analyte of interest; it selectively transmits the wavelength of light emitted, then it measures the intensity of the emitted light. The emitted light is proportional to the concentration of the analyte being measured (up to a maximum concentration). Fluorometers employ monochromators (a spectrofluorometer), optical filters (a filter fluorometer), or narrow band light sources like LED's or lasers to select excitation and emission wavelengths.

Fluorometry is chosen for its extraordinary sensitivity, high specificity, simplicity, and low cost as compared to other analytical techniques. Fluorometry is ordinarily 1000-fold more sensitive than absorbance measurements. It is a widely accepted and powerful technique that is used for a variety of environmental, industrial, and biotechnology applications. It is a valuable analytical tool for both quantitative and qualitative analysis.

1. FLUORESCENCE THEORY

Two excellent textbooks covering the details of fluorescence spectroscopy are: *Principles of Fluorescence Spectroscopy* by Joseph R. Lakowicz[1] and *Practical Fluorescence* by George G. Guilbault.[2] In these books, Lakowicz and Guilbault describe a number of different fluorescence phenomena. For the instruments manufactured by Turner Designs, the fluorescence normally observed in solution is known as Stokes fluorescence.

Stokes fluorescence is the reemission of longer wavelength (lower frequency) photons (energy) by a molecule that has absorbed photons of shorter wavelengths (higher frequency). Both absorption and radiation (emission) of energy are unique characteristics of a particular molecule (structure) during the fluorescence process. Light is absorbed by molecules in about 10^{-15} seconds which causes electrons to become excited to a higher electronic state. The electrons remain in the excited state for about 10^{-8} seconds then, assuming all of the excess energy is not lost by collisions with other molecules, the electron returns to the ground state. Energy is emitted during the electrons' return to their ground state. Emitted light is always a longer wavelength than the absorbed light due to limited energy loss by the molecule prior to emission.[3]



← **Figure 1** shows a representative excitation (absorbance) and emission spectrum.[4]

2. ADVANTAGES OF FLUORESCENCE

2.1 Sensitivity: Limits of detection depend to a large extent on the properties of the sample being measured. Detectability to parts per billion or even parts per trillion is common for most analytes. This extraordinary sensitivity allows the reliable detection of fluorescent materials (chlorophyll, aromatic hydrocarbons, etc.) using small sample sizes. Also, field studies can be performed in open waters without sample treatment. Fluorometers achieve 1,000 to 500,000 times better limits of detection as compared to spectrophotometers.

2.2 Specificity: Spectrophotometers merely measure absorbed light.[5] Spectrophotometric techniques are prone to interference problems because many materials absorb light, making it difficult to isolate the targeted analyte in a complex matrix. Fluorometers are highly specific and less susceptible to interferences because fewer materials absorb and also emit light (fluoresce). And, if non-target compounds do absorb and emit light, it is rare that they will emit the same wavelength of light as target compounds.

2.3 Wide Concentration Range: Fluorescence output is linear to sample concentration over a very broad range. Fluorometry can be used over three to six decades of concentration without sample dilution or modification of the sample cell.

2.4 Simplicity and Speed: Fluorometry is a relatively simple analytical technique. Fluorometry's sensitivity and specificity reduce or eliminate the sample preparation procedures often required to concentrate analytes or remove interferences from samples prior to analysis. This reduction in or elimination of sample preparation time not only simplifies, but also expedites the analysis.

2.5 Low Cost: Reagent and instrumentation costs are low when compared to many other analytical techniques, such as gas chromatography and HPLC. Reagent costs are low because, due to the high sensitivity of fluorometers, less reagent can be used. And, small laboratory filter fluorometers can now be purchased for less than $3,000 USD.

3. INSTRUMENTATION

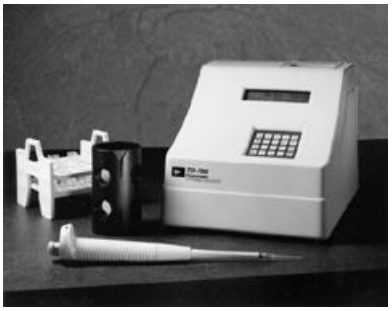
3.1 Instruments to Measure Fluorescence

There are two primary kinds of instruments that measure fluorescence: filter fluorometers and spectrofluorometers.

3.11. Filter Fluorometer. A filter fluorometer measures the ability of a sample to absorb light at one wavelength and emit light at a longer wavelength. A filter fluorometer is a good choice when sensitive quantitative measurements are desired for specific compounds. The comparative ease of handling and low cost make filter fluorometers ideal for dedicated and routine measurements. A fluorometer provides a relative measurement and can be calibrated with a known concentration standard or correlated to standard laboratory methods to produce quantitative measurements.

The TD-360 Mini-Fluorometer

The 10-AU-005-CE Field Fluorometer



The TD-700 Laboratory Fluorometer

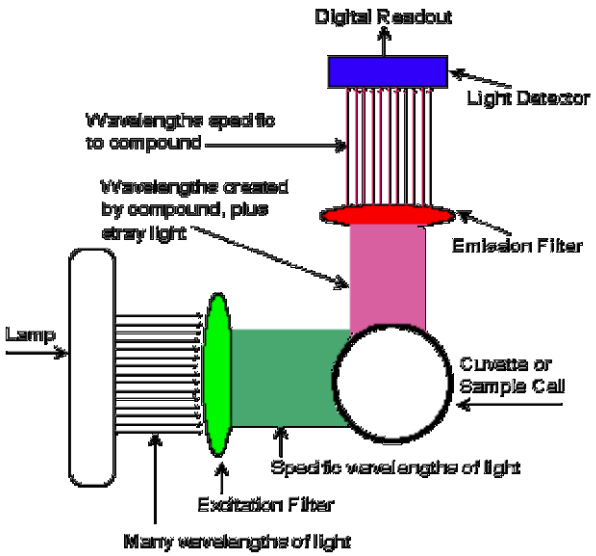
3.12. A spectrofluorometer uses an excitation monochromator (device which includes a wavelength-dispersing component as opposed to a filter) and an emission monochromator. Resolution is obtained with changeable fixed slits. The advantage of spectrofluorometers is that they allow for varying wavelength selection; the operator can scan a substance over a range of wavelengths. (For more details regarding spectrofluorometers, see Guilbault[6] and Lakowicz[7].) The disadvantage of spectrofluorometers is that they are often several times as costly as filter fluorometers and can only provide moderate sensitivity and specificity in comparison. If funds are available, optimal sensitivity and specificity can be obtained with a research-grade spectrofluorometer. Such instruments frequently have monochromators with continuous variable slits and a broad wavelength range (200 - 1000 nm). Some have dual excitation or dual emission monochromators for increasing sensitivity and reducing stray light. Data acquisition and manipulation must be mediated by a computer.[8]

3.2 How a Filter Fluorometer Works

A fixed or filter fluorometer uses optical filters to provide specific excitation and emission wavelengths. To measure different substances, most filter fluorometers allow the user to mechanically change to different optical filter configurations. A filter fluorometer is commonly used for quantitative analysis where sensitivity is a major factor.

A filter fluorometer works as follows: The light source sends out light in the excitation wavelength range of the compound to be measured. The light passes through an excitation filter, which transmits wavelengths specific to the excitation spectrum of the compound and blocks other wavelengths. The light passes through and excites the sample, and the light emitted by the sample passes through the emission filter (which is at a right angle to the exciting light to minimize light scatter). The emission filter further screens the light, the emitted light is measured by the detector, and the fluorescence value is displayed on the instrument.

Figure 2 illustrates the key components of a filter fluorometer: light source/lamp; excitation and emission filters; sample cell/cuvette; and light detector. →



3.21 Light Source: The lamp or light source provides the energy that excites the compound of interest by emitting light. Light sources include xenon lamps, high pressure mercury vapor lamps, xenon-mercury arc lamps,[9] lasers, and LED's. Lamps emit a broad range of light; more wavelengths than those required to excite the compound. Lasers and LED's emit more specific wavelengths.

Xenon lamps are very versatile and powerful, providing light output from 190-1200 nm.

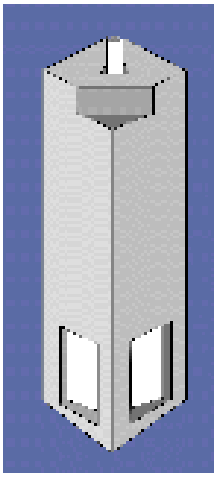
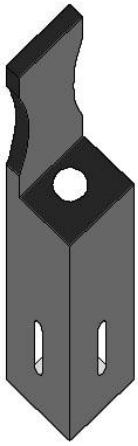
Mercury vapor lamps are usually more intense than xenon lamps, but the intensity is concentrated in wavelengths of the Hg spectrum. Various fluorescent phosphors are used to coat the lamps to provide the desired wavelength of exciting light. In general, these lamps are long-lasting.

Lasers. Convenient and inexpensive tunable lasers have long been sought for spectroscopic uses, including absorption, laser-induced fluorescence, Raman-scattering, high-resolution atomic spectroscopy, laser cooling, and environmental monitoring. The wide tuning ranges of external-cavity diode lasers provide a variety of wavelengths and their narrow linewidth with continuous tunability leads to high resolution scanning capability.

LED's. The latest revolutions in LED technology have just begun with the introduction of LED's based on AlInGaP and InGaN. The AlInGaP LED's offer better efficiencies than filtered incandescent light bulbs in the yellow through red portions of the spectrum. The InGaN LED's offer higher efficiencies than filtered incandescent light bulbs in the blue to green portions of the spectrum. Continuous advances in the performance of LED's have opened up a host of new applications for these solid-state light sources.

3.22 Excitation Filter: The excitation filter is used to screen out the wavelengths of light not absorbed by the compound being measured. This filter allows a selected band of light energy to pass through and excite the sample; it blocks other wavelengths, especially those in the emission spectrum. (Refer to Section 3.3 on Optical Filters.)

*3.23 Sample Cell/Cuvette***:** The sample cell or cuvette holds the sample of interest. The cuvette material must allow the compound's absorption and emission light energy to pass through. Also, the size of the sample cell affects the measurement. The greater the pathlength (or diameter) of the cell, the lower the concentration that can be read. Fluorometers commonly hold 10 mm square cuvettes, and/or 13 mm or 25 mm test tubes. Adaptors are available for 9µl capillary tubes and 100 µl minicells for small volumes. Cuvettes are made from borosilicate or quartz glass as well as various plastics that can pass the selected wavelengths of light.

	
Capillary Tube Adaptor	Minicell Adaptor

Fluorometers are also available for flow-through studies, where samples are pumped through a flow cell in the instrument's sample chamber. This allows for continuous, on-line monitoring of samples. Flow cells, too, are available in various diameters, and made of borosilicate or quartz glass. The TD-4100 On-Line Monitor does not have a glass flow cell. Aromatic hydrocarbons are detected in a stream of water which falls through an open chamber. This flow cell is referred to as a falling stream flow cell.

	
Model 10-AU One Piece Flow Cell	TD-4100 Falling Stream Flow Cell

3.24 Emission Filter: Stray light such as Rayleigh and Raman scatter is also emitted from the sample. In addition, stray background light may be present that has not passed through the sample. The emission filter screens out these components, allowing primarily wavelengths of light specific to the compound to pass through. (Refer to Section 3.3 on Optical Filters.)

3.25 Light Detector. The light detector is most often a photomultiplier tube, though photodiodes are increasingly being used. The light passing through the emission filter is detected by the photomultiplier or photodiode. The light intensity, which is directly proportional (linear) to the compound's concentration, is registered as a digital readout.

A photomultiplier tube (PMT) contains a material which creates an anode current proportional to light intensity. Typically, a chain of 6-12 dynodes, which amplify (multiply) the current, are present. High voltage is supplied to the PMT, which determines the intensity of the signal and also affects the noise. The higher the high voltage, the more sensitive the instrument and the greater the noise. Adjusting the operating level or sensitivity of a fluorometer involves finding the right balance between sensitivity and noise. Most PMT's used in fluorometers are sensitive to light in the 300 - 600 nm range; special red-sensitive PMT's are available, which provide sensitivity above 600 nm (necessary for applications such as chlorophyll measurement).[10] PMT's also give off some dark current (current present even when no light is on the PMT). In addition, temperature affects a PMT, as does aging over a period of time. Thus, for stability of readings, it is important when purchasing a fluorometer, to find out if the fluorometer is closed-loop (with stabilizing reference circuitry built in) or open-loop. Refer to Section 3.5 for details.

"A silicon photodiode (PD) converts incident light into an electric current. Two electrically dissimilar semiconductor layers create a potential barrier, usually a PN or NP junction. Incident photons with energy greater than or equal to the bandgap of approximately 1.12 eV create electron-hole pairs. Pairs produced within a diffusion length of the depletion region will eventually be separated by the electric field, producing a current in the external circuit as carriers drift across the depletion layer."[11] A photodiode is better suited to applications with low to moderate light levels in the ultraviolet and near-infrared range. Since it is small and rugged, with low power consumption, it is useful for compact and field instrumentation.

3.3 Optical Filters

Optical filters are chosen to be optimal for each application, cost effective, and durable. Filters are used to selectively pass a portion of the ultraviolet or visible spectrum.

In combination with a light source, the excitation filter allows only light which excites the molecule of interest to strike the sample. The emission filter allows the fluorescence from the sample to pass to the detector and blocks stray light

from the light source or interfering components in the sample. The reference filter is used in the reference path of the Turner Designs 10-AU series fluorometers and is a factor in determining the basic operating level of the instrument.

Filters can be used alone or in combination to select the desired spectral band. Optical filters obey the Bouguer-Lambert Law, which states that the spectral transmittance of two or more optical filters used simultaneously is equal to the product of the spectral transmittance of each filter.[12]

Filters with four types of spectral characteristics are used in Turner Designs fluorometers: broad or narrow bandpass (Figure 3), sharpcut (Figure 4), and neutral density.

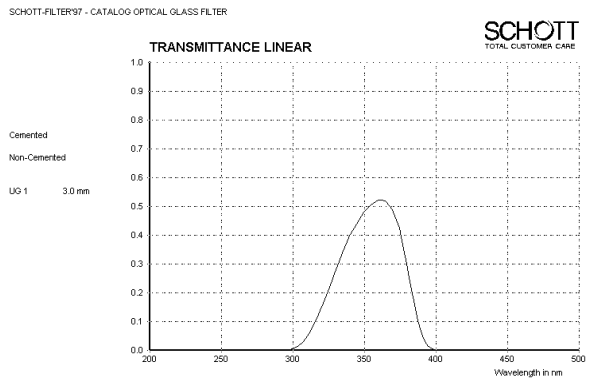


Figure 3. Bandpass Filter.

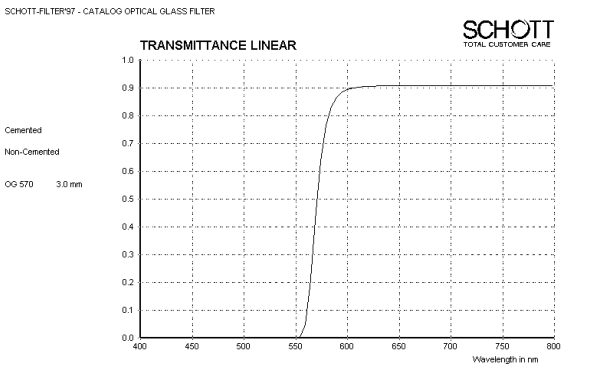


Figure 4. Sharpcut Filter

- **Broadband** filters pass a broad band of light. For instance, a broadband filter may transmit light from 300 - 400 nm, but block light with wavelengths shorter than 300 and longer than 400.

- **Narrowband** filters pass a narrow band of light (as little as 1 nm). For example, a 436 nm filter with a bandpass of 10 nm, will pass light from 431 - 441 nm (5 nm on either side of 436 nm).

- **Sharpcut** or edge filters are used to block light that is longer or shorter than a nominal wavelength. A 450 nm long-wave filter will allow transmission of light that is longer than 450 nm, but it will block light that is shorter than 450 nm. A 450 nm short-wave filter will transmit light that is shorter than 450 nm and block light that is longer than 450 nm.

- **Neutral density** filters, primarily used as reference filters, can be used to decrease the transmitted light across a very broad spectrum. For instance, a neutral density filter can be used to decrease the total light transmission by a factor of 10 or 100.

Three types of optical filters are used in Turner Designs Fluorometers: Optical Glass, Interference, and Gel Wratten.

3.31 *Optical Glass Filters.* Optical glass filters are made from glass that absorbs specific wavelengths of the spectrum. They are relatively inexpensive and are very durable under most conditions. Both bandpass, sharpcut, and neutral density filters are available in optical glass. However, the choice of filter glasses is limited. The amount of transmission and band width is dependent on the glass thickness.

Glass filters can be used for years or decades under most conditions. However, it is important to store optical glass filters in a stable environment, if not installed in the instrument, as their performance can be affected by the following factors:

- Thermal shock caused by a rapid temperature change.

- Solarization caused by prolonged exposure to ultraviolet light (can cause an increase in absorption, decrease in transmission).

- Exposure to high humidity or corrosive environments (can cause 'spotting' or 'staining', which changes the surface, resulting in increased light scattering off the surface and decreased transmission through the glass.)[13]

3.32 *Interference Filters*. In terms of spectral characteristics, interference filters can have broad or narrow bandpasses or can be sharpcut filters. Interference filters used in Turner Designs fluorometers are primarily narrow bandpass. Interference filters are made by coating optical glass with two thin films of reflecting material separated by an even-order spacer layer. The central wavelength and bandwidth of the filter can be controlled by varying the thickness of the spacer layer and/or the number of reflecting layers. To ensure out-of-band blocking -- blocking undesirable wavelengths of light -- an additional blocking component is added. While the additional blocking eliminates out-of-band light transmission and decreases background noise, it also decreases the overall light transmission through the filter which decreases the fluorescent signal. Interference filters typically permit 10 to 70% light transmission. The minimum specified transmission depends on the transmitted wavelength and bandwidth.

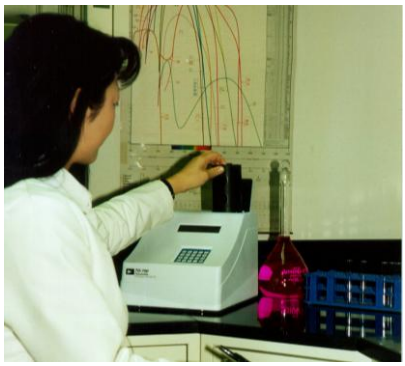
Interference filters are affected by temperature. The center wavelength will shift linearly with, and in the direction of, changes in temperature. For example, the temperature coefficient for a 400 nm filter is about 0.015 nm/°C. The center wavelength and maximum transmission of interference filters can drift with age, especially under conditions of high humidity and variable temperatures.[13] Good quality filters are hermetically-sealed to mitigate the affects of aging. Hermetically-sealed filters are guaranteed for one year; we have found that under good ambient conditions, such as in a laboratory, the filters show minimum signs of aging after two years or more.

A new interference filter usually has a uniformly dark side and a uniformly reflective or mirrored side. To protect the filter from heat and light, the reflective side should always face the light source. A filter that is affected by age and humidity will show discoloration around the outside diameter, this discoloration will move toward the center of the filter with time and additional damage. A symptom of aging is a significantly decreased maximum transmission which results in less sensitivity for a fluorescent assay. The recommended operating conditions for interference filters is -40°C to +70°C, and a maximum temperature change of 5°C/minute.[14]

The transmittance characteristics of filters can be checked for signs of aging using an absorption spectrophotometer.

3.33 *Gel Wratten Filters.* Gel Wratten filters can have broad or narrow bandpasses or can be sharpcut filters. Gelatin filters are made by dissolving specific organic dyes into liquid gelatin. The gelatin is coated onto prepared glass and when dry, it is stripped off the glass and coated with lacquer. Each filter is standardized for spectral transmittance and total transmittance. At Turner Designs, the gelatin filter is placed between two pieces of glass or in combination with other filters for use in the fluorometer. Like dyes in other applications, the spectral characteristics of the dyes used in filters may change depending on the dye used, age, and exposure to heat and light. Gelatin filters should be kept cool, dry and should not be subjected to temperatures higher than 50°C.[15] Most of the gelatin filters used by Turner Designs have been found to be stable under test conditions, which include up to two weeks of continuous exposure to several light sources.

3.4 Choosing between Field, Laboratory, and On-line Fluorometers

		
Field Fluorometer	Laboratory Fluorometer	On-Line Fluorometer

When selecting a fluorometer, there are several factors to consider:

- Will it be used in the field in harsh conditions or in the lab or other protected environment?
- Is long-term stability necessary? For example, is frequent calibration practical? And is the instrument going to be used for on-line monitoring?
- Will the instrument be used for discrete sampling or flow-though studies or both?
- Is battery operation required?
- Is portability required?
- What are the data collection requirements?
- Is temperature compensation for changes in sample temperature required?
- Is 4-20 mA signal output required?

Since fluorescence is a flexible technology, consider future applications to be undertaken as well as current needs when selecting a fluorometer.

3.5 Comparison of Fluorometers: Sensitivity, Dynamic Range, and Stability

When selecting a filter fluorometer for your study, sensitivity, dynamic range, and stability are important instrument factors to consider.

3.51 Sensitivity: Signal-to-Noise and Signal-to-Blank. Sensitivity of a fluorometer refers to the minimum detectable quantity of a compound of interest under specified instrument conditions. It is related to two factors: signal-to-noise and signal-to-blank. Practically, sensitivity means the minimum concentration that can be measured above background fluorescence of the interferences. Note that when comparing two instruments for sensitivity, absolute numbers are meaningless. One cannot simply read a sample and blank in two instruments and say the instrument with the “highest” numbers is more sensitive.

Signal-to-noise. Signal refers to the reading of light passed through a sample. Noise refers to the output from the instrument’s electronics, which is present whether or not sample is being read. Instrument noise can be seen by

placing a black/opaque sample, which allows no light (signal) into the instrument and looking at the readout. The signal-to-noise ratio can be improved either by increasing the signal or reducing instrument noise. The user can increase signal by using a larger diameter cuvette or increasing slit/window width. The manufacturer can reduce noise by installing a higher-quality, "less noisy" PMT and electronics. Note that merely increasing the instrument's operating level (sensitivity) will provide a larger signal, but will probably also increase instrument noise.

Signal-to-blank. This is related to signal-to-noise but not the same. Signal refers to the reading of a sample. Blank refers to the matrix liquid containing none of the compound to be measured and scattered light. Signal-to-blank ratio can be determined by measuring blank against sample concentration and determining the ratio. Signal-to-blank ratio can be improved by employing better optics for the specific chemistry. For example, reduce the amount of light scatter (light that hits the detector that has NOT passed through the sample or blank) with a narrower slit (light attenuator, especially on the emission side); or use optical filters with spectra and bandwidths more specific to the compound to be measured. Square vs. round cuvettes may even improve signal-to-blank. Note that merely increasing the instrument's operating level (amount of light to the sample and sensitivity of the detector and the electronics) will NOT improve signal-to-blank (and it may increase instrument noise). It will simply give larger numbers for both signal and blank with little change in the ratio between them. To improve signal-to-blank, the amount of stray light (light that has not passed through the sample; light not due to fluorescence of the compound) must be reduced.

A comparison of minimum detection limits among fluorometers is often made by using quinine sulfate or some other stable compound as a reference standard. This can work well in many cases, provided the instruments are properly and "equivalently" set up and operated. The standard must be pure and properly diluted and stable. Cuvettes must be clean and properly handled. (Note that at very low levels, quinine is not linear.[16] Currently, there are better compounds to use.[17])

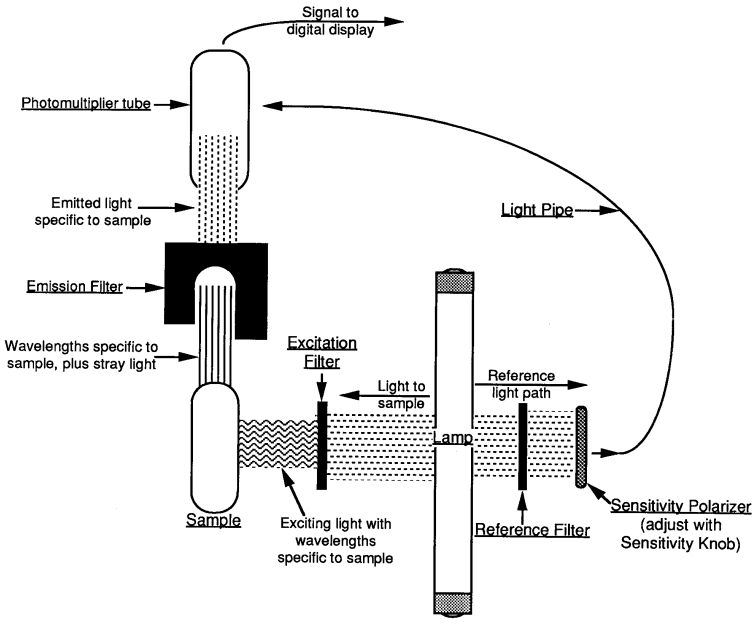
3.52 Dynamic Range. Dynamic range refers to the range of concentrations an instrument can read, from the minimum to the maximum detectable. The minimum detectable concentration is determined by signal-to-noise and signal-to-blank ratios. The maximum detectable concentration is determined by the compound's chemistry and by factors such as instrument sensitivity ranges, optical pathlength, specificity of optical filters, etc. Some instruments with a wide dynamic range, like the Turner Designs 10-AU series, can read compounds over a range of 5,000,000 to 1 when optimally set.

3.53 Stability: Closed-Loop vs. Open-Loop Fluorometer. Creating an electronically stable fluorometer is especially important for insuring that it produces consistent analytical results over long periods of time.

An **open-loop** fluorometer is one in which there is no correction for electronic sources of instability. This means that changes in components like the PMT and lamp (due to aging, temperature changes, etc.) are not compensated for by the instrument. These changes can cause errors in readings which necessitate frequent recalibration.

A **closed-loop fluorometer** compensates for electronic drift in the instrument. This means that if the lamp or PMT changes its performance, the signal output will remain stable. Accordingly, readings change only with changes in sample concentration. The fluorometer does this by correcting for any drift resulting from variations in lamp intensity and/or PMT sensitivity (see Figure 5). Readings are compared and realigned with very stable reference circuitry resulting in excellent stability of readings over time. Closed-loop instruments, like the TD-4100 and 10-AU-005-CE allow for long-term studies, less frequent calibration, and accurate on-line monitoring.

Figure 5 →



4. VARIABLES OF FLUORESCENCE

4.1 Linearity, Quenching, Turbidity, Bubbles in the Sample, pH, and Photochemical Decay.

4.11 Linearity. Fluorescence intensity is typically directly proportional (linear) to concentration. There are, however, factors that affect this linear relationship. When concentration is too high, light cannot pass through the sample to cause excitation; thus very high concentrations can have very low fluorescence (concentration quenching; see section 4.12). At intermediate concentrations, the surface portion of sample nearest the light absorbs so much light that little is available for the rest of the sample; thus the readings will not be linear, though they will be within the range of a calibration curve.[18] See Figure 6.[19]

The linearity of a sample is related to many factors, including the chemical composition of the sample and the pathlength the light must travel (thus, the diameter of the cuvette or sample holder is a factor). An unknown sample should always be tested for linearity. Because instrument factors also affect linearity, samples must be tested on the specific instrument to be used in the study; if cuvette size is changed or optical filters are changed to different wavelengths, linearity must be tested. To test for linearity, simply take a reading for a high concentration of the sample; dilute by a factor (1:1, 1:10, etc.); and take a reading for the dilute sample. If it is linear, the reading will go down by the same factor as the dilution. If it is non-linear, the reading will most likely increase as it is diluted. If it is in the range for a calibration curve, the reading will go down, but not as much as expected by the dilution. Although a calibration curve for a specific application may prove to be non-linear, if the curve is reproducibly accurate, then the calibration is accurate.

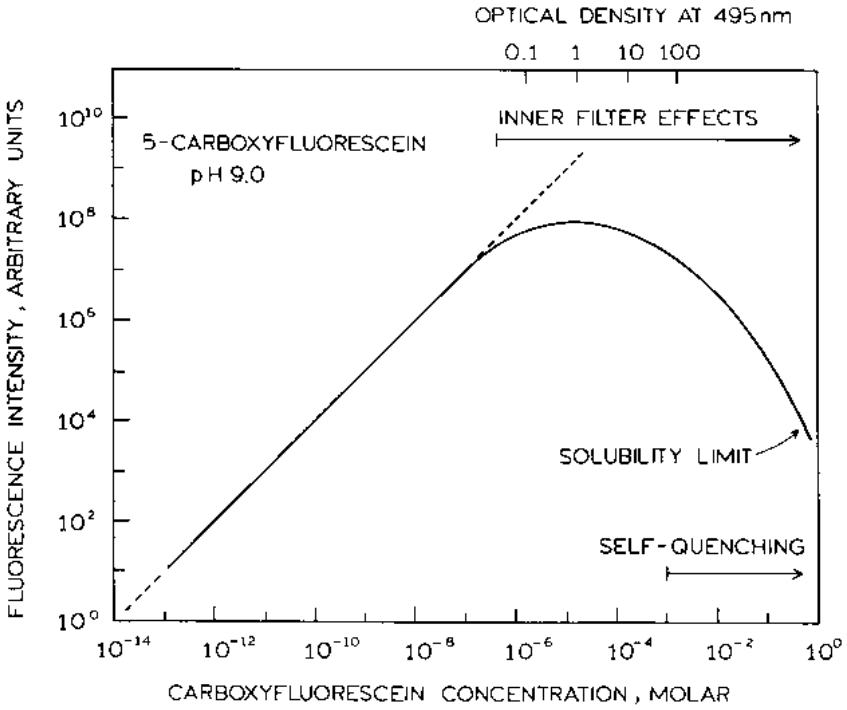


Figure 6.

For example, Rhodamine WT, a fluorescent tracer widely used in environmental studies, is linear to about 100 parts per billion (ppb) active ingredient using the Turner Designs 10-AU-005-CE fluorometer with the 25 mm cuvette. It is in the range for a calibration curve to about 500 ppb active ingredient.

4.12 Quenching. The term “quenching” refers to many factors that reduce, or quench fluorescence. Quenching as it relates to linearity is discussed in section 4.11. Temperature quenching is discussed in section 4.2. Factors that influence quenching can be controlled through instrument design and proper method development.

Fluorescence can be lost by:[20]

1. Collisional quenching involving collisions with other molecules that result in the loss of excitation energy as heat instead of as emitted light. This process is always present to some extent in solution samples; species that are particularly efficient in inducing the process are referred to as collisional quenchers (e.g. iodide ions, molecular oxygen, nitroxide radical).

2. Static quenching. Interaction of the fluorophore with the quencher forms a stable non-fluorescent complex. Since this complex typically has a different absorption spectrum from the fluorophore, presence of an absorption change is diagnostic of this type of quenching (by comparison, collisional quenching is a transient excited state interaction and so does not affect the absorption spectrum). A special case of static quenching is self-quenching, where fluorophore and quencher are the same species. Self-quenching is particularly evident in concentrated solutions of tracer dyes.

3. Resonance energy transfer. Like collisional quenching, this is an excited state interaction but the two participating molecules do not have to collide - it can occur over distances of 100 angstroms or more. One dye

(the donor) is excited by absorption of a photon, but instead of emitting a fluorescence photon, the excitation is transferred by electronic coupling to an acceptor molecule. The result of this exchange is an excited acceptor and a ground state donor. In signal terms the result is either fluorescence at longer wavelength (compared to the spectrum of the donor alone) or no fluorescence, depending on whether the acceptor is itself fluorescent or non-fluorescent.

4. Inner filter effect. This is a measurement artifact as opposed to a quenching process. It occurs in samples with very high absorbance (the absorbance can be due to the fluorophore itself or other absorbing components of the sample - it doesn't matter which). Under these conditions, all the incident exciting light is absorbed at the front face of the sample. In a conventional 90 degree detection geometry, the excitation light cannot penetrate deeply enough to the point at which the detection optics are focused. The result is the detected fluorescence decreases as the sample absorbance increases. As implied, the magnitude of inner filter effect depends on the geometrical relationship between the excitation and emission detection paths and on the thickness of the sample. To avoid inner filter effects (and other artifacts), it is generally advisable for the sample absorbance measured at the excitation wavelength to not exceed 0.1.

Quenching factors are one reason why it is very important to treat standards, blanks, and samples in exactly the same manner. Thus, for the best accuracy, prepare all solutions in the same manner, using the same reagents and matrix solution and the same preparation techniques; measure at the same temperature after the same amount of time. Good laboratory techniques will go a long way toward producing reliable results.

4.13 Turbidity. Fluorescence measurements are significantly more immune to the effects of turbidity compared to absorption techniques like UV/VIS spectrophotometers. Turbidity is often viewed as an impurity or background in a solution (i.e., suspended solid like silt). If the interfering substance is reflective, turbidity can create light scatter and readings will increase. If the interfering substance absorbs light, fluorescence will be reduced. If the interfering substance does not absorb light, however, then the fluorescence readings will not be effected unless there is so much turbidity that the emitted light cannot penetrate the water. In extreme cases of turbidity, samples can be filtered to minimize these effects. For various applications, turbidity can easily be corrected for in the field.[21] Keep in mind that these interferences can in effect be “canceled out” by using the same matrix water for standards, blanks, and samples and treating them exactly in the same manner.

4.14 *Bubbles in the Sample*. Since fluorescence is such a sensitive measurement, and since accuracy of readings depend on light exciting the molecules of the compound, bubbles in the sample can result in erratic or fluctuating readings. In cuvettes, if bubbles are suspected, wait for them to settle. In continuous flow systems, take care not to introduce bubbles by avoiding turbulent waters and water too near the pump; or employ a filtering system or bubble trap. Sample intake should be through the bottom and outflow through the top. In a falling stream flow cell, bubbles are not an issue as they are part of the background measurement.

4.15 pH. Fluorescence can be affected by pH. Yet, in field studies of water, this is rarely an issue. Where it is an issue, the usual rule applies: use the same matrix water for standards, blanks, and samples and treat them in exactly the same manner. If working with pH-dependent solutions, read solutions at different pH’s to determine affects on fluorescence.

In certain studies, pH factors can be an advantage. Spectral interferences can be minimized in analytical fluorescence spectrometry by adjustment of pH prior to measurement. The pH dependence of probe molecules has been used to determine the pH of intact and cured cells.[22]

4.16 Photochemical Decay. Many fluorescent molecules can be bleached or destroyed by light (fading of dyes in the sun). Ultraviolet light, especially, can cause certain molecules to break down. Fluorescence readings decrease as the molecules are destroyed. Rate of destruction varies depending upon environmental factors, including temperature. Fluorescein, for example, is destroyed rapidly in sunlight. Rhodamine WT, however, is adequately stable for field studies.[23] For chlorophyll measurements, samples and standards need to be kept in the dark until read. All flow measurements should employ opaque delivery hose to minimize photochemical interferences. Glass provides shielding from laboratory light and ultraviolet light; the extent of shielding varies depending on the type of glass.

4.2 Temperature

Fluorescence is affected by changes in temperature. As temperature increases, fluorescence decreases. Guilbault suggests that this is due to an increase of molecular motion with increasing temperature, which results in more molecular collisions and subsequent loss of energy.[24] The temperature coefficient varies depending upon the compound being measured. Using a filter fluorometer, this problem is easily resolved by measuring standards, blanks, and samples at the same temperature. For flow through studies, the researcher can set up a system to measure the temperature of samples, then compensate readings later using a spreadsheet program. Some field fluorometers like the Turner Designs 10-AU-005-CE offer a temperature compensation option, where the fluorometer measures sample temperature, then automatically compensates readings for changes in temperature.

4.3 Cuvette Size (Pathlength); Flow Cell Fouling

4.31 Cuvette Size. Linearity and detection limits are affected by cuvette size (pathlength). In general, the greater the diameter of the cuvette, the lower the upper end of the linear range, and the lower the limits of detection. For example, using the Turner Designs 10-AU-005-CE with the 25 mm cuvette, Rhodamine WT is linear to 100 ppb with detection limits in the range of 0.01 ppb in DI water. When the 13 mm cuvette is used, Rhodamine WT is linear to 400 ppb with detection limits of 0.04 ppb in DI water.

The range of concentrations to be measured and the detection limits can be optimized by selecting the appropriate size cuvette. This can be especially effective for measuring substances such as aromatic hydrocarbons. Readings can be obtained in the linear range by the appropriate selection of cuvette diameter (3 mm for example) and/or by offsetting the light path slightly or employing a light attenuator (smaller window or slit).

Note that sample volume is not as important as pathlength or diameter of the cuvette. Thus, for certain applications like DNA quantitation where sample volumes are low, special low-volume cuvettes with appropriate sample windows are available.

4.32 Flow Cell Fouling. Fouling of the flow cell will result in lower, inaccurate readings over time. This comes about when the glass or quartz cuvette becomes coated as sample passes through. How long this takes depends on the quality and temperature of the sample water. There are ways to minimize this problem, including regular cleaning of the flow cell and recalibration of the instrument; or employing a filtering system where biomass is a problem. The Turner Designs TD-4100 employs a non-fouling, falling stream detection system that does not use a glass flowcell, and therefore does not require cleaning.

5. CALIBRATION AND STANDARDS

5.1 Calibration of a Filter Fluorometer

Filter fluorometers can be calibrated by a number of different techniques. The most common calibration of a filter fluorometer consists of compensating for blank (solution containing zero concentration of the substance to be read) and adjusting the instrument to reflect a known concentration of sample (the standard). Filter fluorometers can also be calibrated by correlating their raw fluorescence signal compared to a standard laboratory method like EPA method 413.1.

Fluorescence is a relative measurement and the optics and electronics of each instrument vary, certainly from manufacturer to manufacturer, but also among instruments from the same manufacturer. A fluorometer must be calibrated and recalibrated whenever the optics or filters are changed.

Fluorescence is subject to temperature and other environmental effects; it is important to calibrate the fluorometer in conditions as close as possible to the actual conditions for your study. Recall that fluorescence is an extremely sensitive measurement. Sample readings are only as accurate as the standard and blank used to calibrate the instrument. It is important to be rigorous in laboratory procedures, such as cleaning labware and carefully preparing standards.

Instrument stability is critical to accurate readings. If frequent calibration is impractical or for long-term studies or on-line monitoring, a fluorometer with stable reference circuitry (closed-loop) like the Turner Designs 10-AU or TD-4100 should be used. See section 3.53.

5.2 Standards

5.21 Standards. A blank should be obtained before measuring sample. The blank is water or matrix liquid for the sample, taken before any of the substance to be measured has been added. This liquid should be used as the make-up liquid for standards and to set the instrument's reading to zero.

Normally, the standard will be a known concentration of the material to be quantitated. For single-point calibrations, and where a known concentration is needed, it is best to choose a standard with a concentration approximately 80% of the highest concentration to be read. A less concentrated standard may be used, but a higher concentration will provide greater accuracy.

If concentrations above the linear range are to be read, several standards should be used so a calibration curve may be prepared. For example, if the substance is linear to 250 ppm and measurable with a calibration curve to 1000 ppm, you might calibrate with a 200 ppm standard and take readings at 500, 750, and 1000 ppm for a calibration curve.

In some cases, calibration with a standard of known concentration is not necessary. In procedures such as *in vivo* chlorophyll or certain flow measurements, instrument sensitivity will be set with an unknown concentration. For *in vivo* chlorophyll measurements, for example, in most cases the "standard" will be an unknown sample from the body of water under investigation. During the sensitivity-setting procedure, while the unknown is running through the fluorometer, you should take a grab sample of the unknown immediately after it passes through the flow cell. This grab sample will be extracted later and the actual chlorophyll concentration determined. The researcher will then use a ratio calculation method to compare *in vivo* sample readings with extracted readings to determine the actual concentration of field samples.

Where the instrument has more than one range or "door" factor, many researchers will want to check for range-to-range correlation. Some fluorometers, such as the Turner Designs 10-AU are designed with excellent range-to-range correlation, making this kind of comparison unnecessary in most cases.

The stability of the standard is an important issue for accurate readings. Some substances, such as Rhodamine WT are stable for months. Others, such as chlorophyll will become inaccurate in as little as a few hours when exposed to light or temperature changes. Properly store standards to ensure stability or make new ones just prior to calibration. In some cases, purchasing premade standards is a practical and economical option. Contact Turner Designs for information about extracted chlorophyll and Rhodamine WT standards.

5.22 Secondary Standards. The stability and accuracy of standards is a critical factor in fluorescence measurements. In cases such as chlorophyll measurements, where standards are not stable over the long term, a stable secondary standard can considerably simplify procedures, is relatively inexpensive, and can provide confidence in the accuracy of readings.

A secondary standard is basically a substance that fluoresces at wavelengths similar to the substance being measured. Turner Designs, for example, offers secondary standards for many applications that are stable for years with no special storage requirements. Secondary standards are used in place of the primary standard after the instrument has been calibrated at least once with the primary standard.

For example, if measuring chlorophyll, the researcher would first obtain or prepare accurate chlorophyll standards and calibrate with these primary standards. (Premade, ready-to-use chlorophyll standards in 90% acetone are available from companies such as Turner Designs. Chlorophyll *a* in a dry powder ready for diluting in 90% acetone or methanol is available from chemical companies such as Sigma or Fluka.) The researcher would then read the secondary standard and record the concentration as compared to the primary standard. After this, the secondary standard could be used in place of the primary standard when calibrating.

Another advantage to a stable secondary standard is as a check on instrument stability. A secondary standard can be read periodically, and if the reading has not changed from the last calibration, the researcher can proceed with

confidence that the instrument is providing accurate readings. If the reading has changed significantly, simply recalibrate using the secondary standard. A secondary standard is especially useful for instruments that lack reference circuitry. A secondary standard greatly minimizes the need to constantly prepare fresh standards, which is expensive and time-consuming, as well as potentially introducing error from contaminated or improperly prepared standards.

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