Basics of Fluorescence Photometer

Hitachi High-Technologies Corporations

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Spectrofluorophotometer

1. What is Fluorescence?

1.1 When are you excited? (Excitation spectrum)

Please score your excitement in the following scenes.

1) Riding on a roller coaster.

2) Driving through a mountain pass.

3) Watching a soccer game in a stadium.

Persons A, B, and C answered these questions as follows.

	A	B	C
Roller coaster	70	90	30
Driving a car	80	30	40
Watch a soccer game	60	60	90

This shows the characters of A, B, and C well, doesn't it?

More generally, a person is excited when stimulated. However, it can be said that different persons are more easily excited by different kinds of stimulation. A third person can observe another person's personality by observing his excitement when stimulated.

How can it be noticed that a person is excited? The answer is simple. Various phenomena, such as an elevated heart rate, sweating, appearing flushed, crying, and shouting appear when a person is excited. It is sufficient only to observe any one of them.

Now, let's change the subject to actual fluorophotometry.

Specimens handled by fluorophotometry are too excited by stimulation. Different specimens (materials) are excited by very different stimulation. Excited specimens exhibit various phenomena which prove that they are excited.

For example, they emit light. If they emit strongly, it means they are very excited, but if they emit weakly, it means that they are only little excited.

By the way, what is the source of stimulation for a specimen? In the fluoroscopic method, light irradiation is used as a means to stimulate the specimen. Stimulation varies by changing the wavelength (color) of the irradiated light.

Based on the above, let's rewrite the opening questionnaire for fluorophotometry.

Please score the excitement (luminescence intensity) of each specimen in the following scenes.

1) Exposed to purple light.
2) Exposed to blue light.
3) Exposed to green light.

The answers (luminescence intensity) to these questions for Specimens A, B, and C were as follows.

Light to expose	Specimen A	Specimen B	Specimen C
Purple	70	90	30
Blue	80	30	40
Green	60	60	90

This shows characters of Specimens A, B, and C well, doesn't it?

In general, specimens handled by fluorophotometry will be excited to emit light when they are exposed to light. However, it can be said that different specimens are more likely to be excited (to emit) by light of different wavelengths. We can see the character of a certain specimen by observing its emission when irradiated with light of various wavelengths.

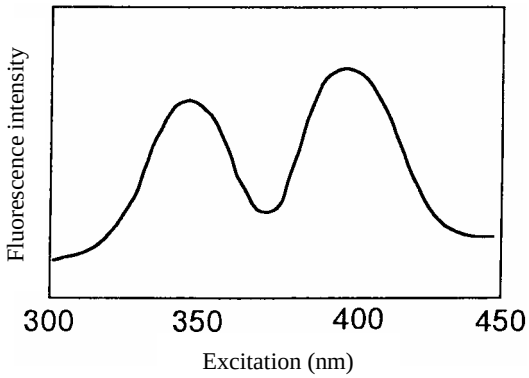
By the way,

State where the specimen is excited: ..... Excited state
Light used for excitation: ..... Excitation light
Emission after excitation: ..... Fluorescence
Answers to the questionnaire: ..... Excitation spectrum

With our eyes, we can distinguish excitation light only by using the names of colors such as purple, blue, and green. In fluorophotometry and spectral analysis, these colors of light are distinguished by wavelength (unit: nm, nanometer, 1 nm = 1 / billion m). Although it greatly depends on an observer's eyes, it is said that it is sufficient to observe only one phenomenon to confirm the excitement mentioned in the previous section.

Purple: 400 - 420 nm
Blue: 450 - 500 nm
Green: 500 - 550 nm

The shape of a general excitation spectrum is as follows. The illustrated specimen emits fluorescence most strongly when excited (stimulated) by light at 400 nm (thus, being excited to emit light) and also emits fluorescence considerably, although less when excited with an excitation light of 350 nm. A more detailed measurement example is provided in the Appendix.



1.2 What happens after excitement? (Fluorescence spectrum)

In the previous section, we explained that it is sufficient only to observe one phenomenon to confirm that a person is excited. However, let's talk about the key to determine the character of a specimen in more detail.
Please answer questionnaire, once again.
Please score the following items, assuming that you are excited.

1) Sweating
2) Flushed
3) Have a racing pulse

A, B and C answered to the questions as follows.

	A	B	C
Sweating	30	40	80
Flushed	60	80	40
Excited	90	60	50

The results show the characteristics of A, B, and C respectively.
Information about what happens after excitement expresses their characteristics (for example, C is a great sweater). A third person observes what happens to the excited person as a clue for knowing his personality.

As mentioned before, the emission after excitement is measured in fluorophotometry. Fluorescent emission can be distinguished by its color (wavelength), just as a person can be distinguished by his/her physical response. The above questionnaire is rewritten for fluorophotometry, as follows.

Please score how the excited specimen emits light (fluorescent light) of the following colors.

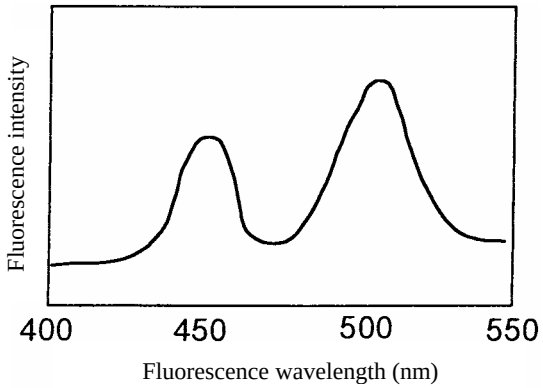
1) Emits yellow fluorescence.
2) Emits orange fluorescence.
3) Emits red fluorescence.

The answers to these questions for Specimens A, B, and C were as follows.

	Specimen A	Specimen B	Specimen C
(1) Yellow	30	40	80
(2) Orange	60	80	40
(3) Red	90	60	50

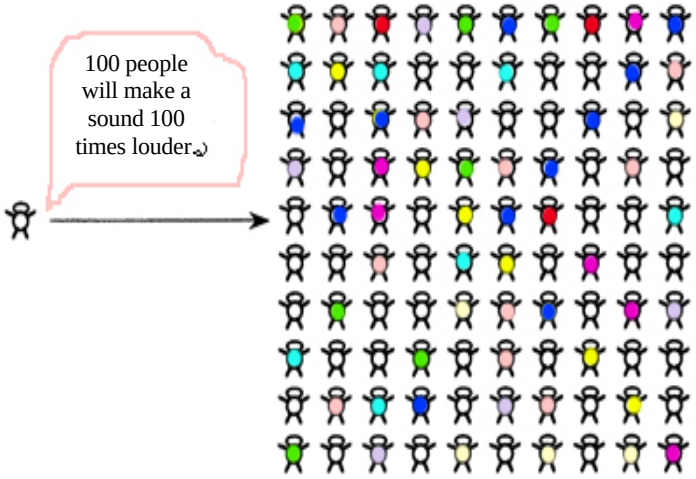
These results represent the characteristics of Specimens A, B and C, well.
The information about the color (wavelength) of the fluorescent light emitted by the excited specimen expresses the characteristics of the specimen, as well. A third person observes the wavelength of the fluorescent light emitted by the excited specimen as a clue for knowing the characteristics of the specimen.

The result of the above questionnaire is called a fluorescence spectrum. The figure on the right shows the shape of a general fluorescence spectrum. When the specimen is excited, it emits fluorescent light with wavelength of 500 nm most strongly, and 450 nm fluorescent light less, but considerably. A more detailed measurement example is presented in the Appendix.



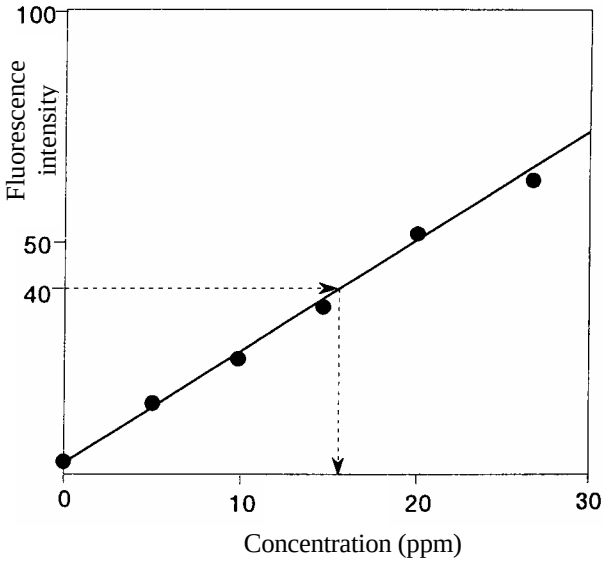
1.3 What if there are many human clones? (Quantitative measurement)

The next scenario is from science fiction. Suppose that it were possible to make many human clones (duplicated humans). Let's say that 100 clones were made of a person who would shout unconsciously when excited. What happens if the clones are gathered in one room and stimulated to be excited?
If they are perfect clones, a scream 100 times louder than that of one person will resound all over the room.
If the loudness of one person's shout is measured beforehand, and the stimulation is provided in a room where an unknown number of clones are present, the number of clones in the room can be figured out by measuring the total loudness of the shout.



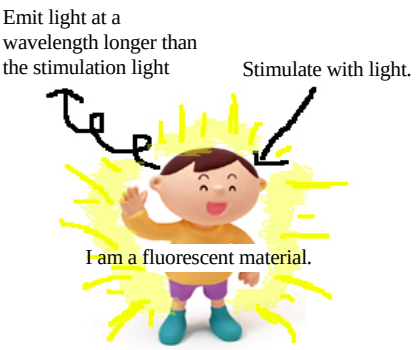
The quantitative operation which measures the concentration of a specimen by the fluoroscopic method is very similar to this.
If there are several specimens with the same components in which only the concentration varies, the fluorescence intensity becomes small (for a shout, a small sound volume) if the specimens are dilute (in the case of human clones, there are only a few), and the fluorescence intensity becomes large (a larger sound volume) if the specimens are dense (if there is a large number).

Concentration of standard sample (ppm)	Fluorescence intensity
00	05.0
05	15.0
10	25.0
15	35.0
20	45.0
25	55.0



A set of specimens (standard sample) with known concentrations are prepared to measure their fluorescence intensity. The results are plotted to obtain a calibration curve. After a calibration curve has been obtained, a specimen with unknown concentration can be measured.
For example, suppose the fluorescence intensity of the specimen is 40. By consulting the calibration curve shown in the right-hand figure, the concentration of the specimen can be estimated at 16 ppm.

What concentration range can actually be measured?
Based on the theory described above, it would seem that it is possible to measure even the most concentrated specimens, but that's not true. If a specimen is too dense, the fluorescence emitted from a certain part of the sample cell may be absorbed by its surroundings (which may distort the shape of the excitation/fluorescence spectrum), or the excitation light may not reach sufficiently deep into the sample cell, leading to decrease in fluorescence. Of course, if it is too dilute, fluorescence will be too weak to detect. In both cases, the measurable concentration range greatly depends on a measured object.

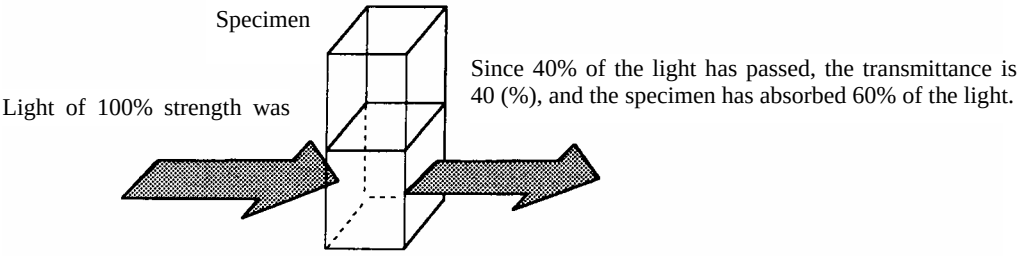


1.4 What's the difference from absorptiometry?

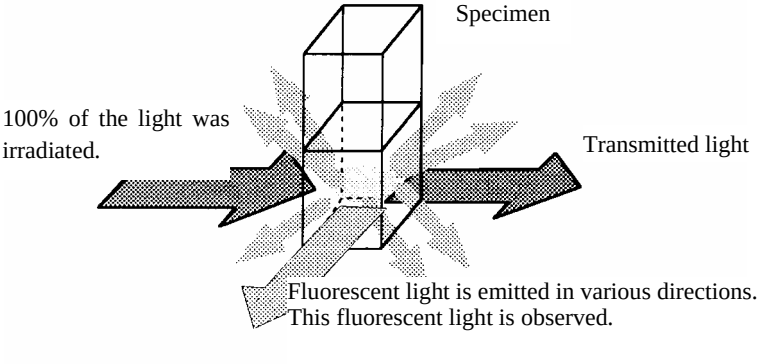
1.4.1 Wavelength to measure

In both absorptiometry, using an ultraviolet-visible spectrophotometer and fluorometry using a spectrofluorophotometer, measurement is carried out by irradiating light of various wavelengths onto the specimen. In this sense, they are very similar in terms of the configurations of the hardware of the systems. They are also similar in that the horizontal axis of plotted spectra is wavelength.
However, there is a definite difference between the measurement methods.

Absorptiometry measures how much of the irradiated light is absorbed by the specimen. Since the light, except for the absorbed portion passes through the specimen, the quantity of absorbed light can be estimated by measuring the intensity of the passed light (measurement of transmittance and reflectivity).
Please note that decreases in the irradiated light are observed while the wavelengths of the irradiated light and observed light are the same.



On the other hand, in observing emission in fluorometry, the irradiated light and the observed light are different; that is, they have different wavelengths.



Now let's consider this in more detail.
There are the following relations between wavelength of light and the energy that one photon of the light has.

Wavelength: Short ⟷ Long

Energy: Large ⟷ Small

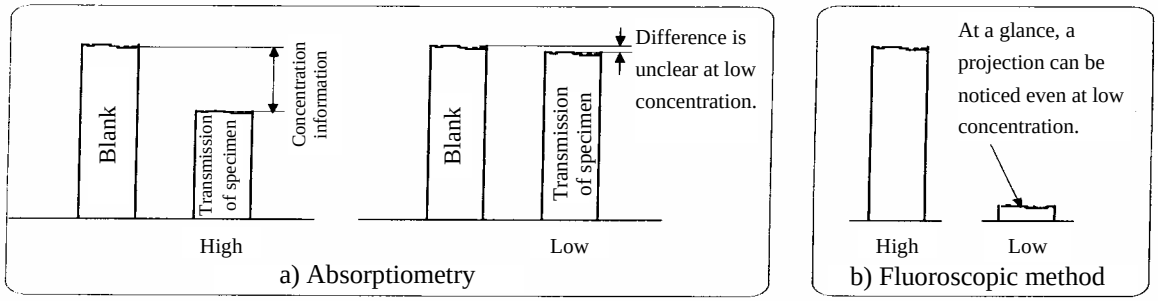
In fluorescence, the energy of the excitation light is absorbed, and then the energy is transformed into a different form of light energy (fluorescent light), which is then emitted. However, some energy can be lost in the conversion process. All the energy of the absorbed excitation light cannot be converted into fluorescence. Since there is some loss, the fluorescence energy will be smaller than the absorbed energy.
Therefore, the wavelength of the fluorescent light is always greater than the wavelength of the exciting light (Stokes's law).

In the figure, the fluorescent light is detected at a right angle to the excitation light. In particular, if it were detected in the direction the irradiated light passes, as in absorptiometry, the irradiated excitation light would be detected, as well.
As mentioned before, since the fluorescent light, but not the excitation light, is detected in the fluoroscopic method, it is not desirable that the excitation light enters the detection system.

So, the fluorescent light is detected in a perpendicular direction (at a right angle), at which the excitation light may enter the detection system the least. It can be observed at a right angle, since fluorescent light is usually emitted isotropically (in all directions).
Nevertheless, some of the excitation light enters the fluorescent light detection system, because of various irregular reflection events.

1.4.2 High sensitivity

In comparing quantitative measurements by the absorptiometry and the fluoroscopic methods, a specimen as dilute as 1/1000 of that measurable by absoptiometry can generally be measured by the fluoroscopic method. It is said that the detection limit of the fluoroscopic method is lower by three orders of magnitude.
Why is this?
From a glance at the following figure, it seems that the difference in the bar chart heights of the transmission of the blank and the specimen (information on concentration) at low-concentration by absorptiometry is clearer. However, the difference can easily be noticed at low concentrations in the fluoroscopic method, even when low bar charts are compared, when no light is emitted.
The fluoroscopic method is advantageous at low-concentrations.



It is as follows in theory.
Let's consider a dilute specimen with a transmittance of 99%. Although any measurement is accompanied by an error, we assume it to be 0.1% in this case. In normal absorptiometry, the quantity of transmitted light is measured compared with a blank (concentration of 0). Since error factors affect both the blank and the specimen by the same ratio,

Transmittance of the blank	100.0±0.1%
Transmittance of the specimen	99.0±0.1%

The difference between them (value related to the concentration of the specimen) is 1.0±0.2%

Since the error in the concentration measurement is 0.2%, while the difference of transmittance is 1.0%, it will include an error of 20%. On the other hand, since the difference with the zero level often corresponds to the concentration of the specimen in the fluoroscopic method,

Output signal level at specimen measuremen	100.0±0.1%,
Value corresponding to the blank	0.0±0.1% and

The difference between them (value related to the concentration of the specimen) is 100.0±0.2%.

The fluoroscopic method is very advantageous at lower concentrations, since the percentage error does not depend on the concentration of the specimen. In absorptiometry, the point where the concentration information (difference between the transmissions of the blank and the specimen), indicated by the arrow head in the figure becomes comparable to the noise level is the detection limit (0.2% here).
Since the amount of fluorescence itself is the concentration information (not the difference, as in absorptiometry) in the fluoroscopic method, if the fluorescent light is emitted it is possible to detect it by amplifying an electric signal.

Comparison of the detection limits of the spectrophotometer and spectrofluorophotometer systems (concentration units: μg/mL)

Materials	Spectrophotometer Absorbancy 0.001	Spectrofluorophotometer Detection limit
Benzene	1×10^{-3}	1×10^{-3}
Anthracene	2×10^{-3}	5×10^{-4}
Quinine sulfate	2.3×10^{-2}	1×10^{-5}
Riboflavin	1.9×10^{-2}	1×10^{-5}
Rhodamine B	8.2×10^{-3}	1×10^{-6}
Chlorophyll	5×10^{-1}	5×10^{-3}

Note: Spectrofluorophotometer model 650-10S was used. The detection limit was defined at a point where the fluorescent signal becomes twice as high as the background.

1.4.3 Large amount of information.

In the fluoroscopic method, there are two kinds of wavelengths: an excitation wavelength and a fluorescence wavelength. In a spectrum measurement, there are thus two kinds of spectra: an excitation spectrum and a fluorescence spectrum. In absorptiometry, there is only an absorption spectrum. The fluoroscopic method provides more information on the same specimen than the absorptiometry.
By the way, an excitation spectrum is used to determine the most effective excitation wavelength to induce fluorescence.
The energy of the excitation light has to be absorbed to induce fluorescence. It is natural to expect that if more is absorbed, more fluorescence will be generated. That is, an excitation spectrum turns out to be a spectrum very similar to the absorption spectrum of absorptiometry.

1.4.4 Shoot at one component

Assume that two components A and B were mixed in a sample cell. If their absorption wavelengths were almost the same .....
In absorptiometry, they cannot be undistinguished. However, in the fluoroscopic method, if the fluorescence wavelengths are different, even though the absorption wavelengths are the same, several components can easily be distinguished (although the excitation spectrum of each component is almost the same). One component out of many can be selectively measured.

1.4.5 Weak point of the fluoroscopic method

So far, the fluoroscopic method has been described as if it is very excellent compared with absorptiometry.
However, the fluoroscopic method has some weak points, as well.

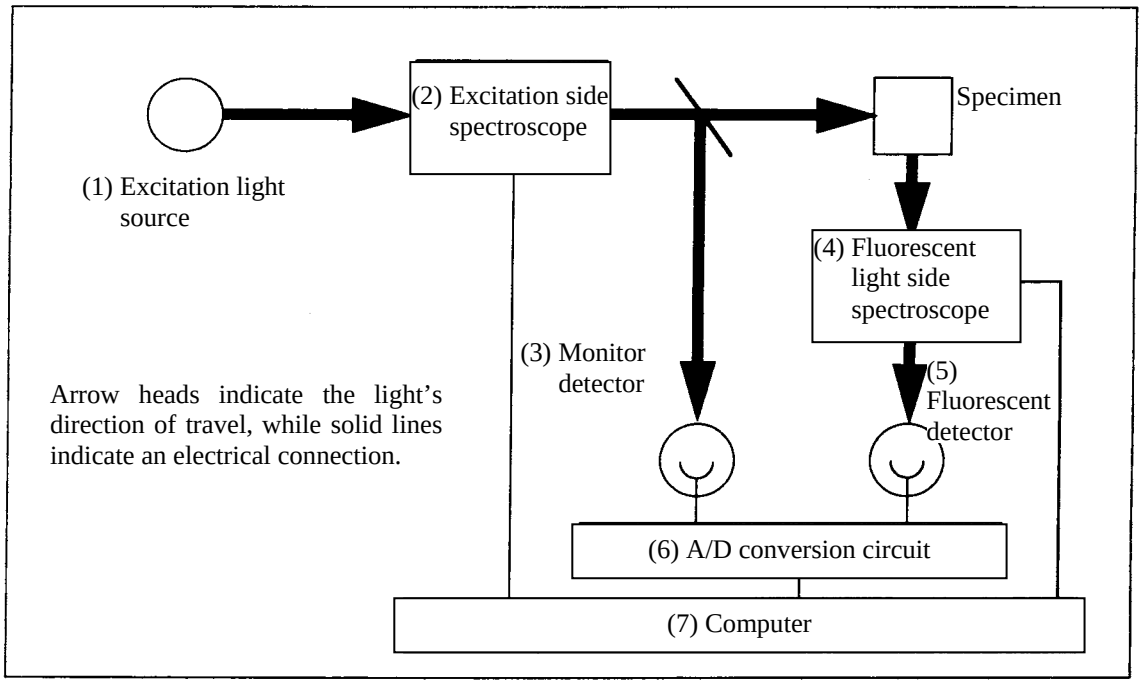
1) Most natural materials emit fluorescence very scarcely, even when exposed to excitation light of various wavelengths. Components which can be measured are thus limited, compared with absorptiometry. When working with a material which does not emit fluorescence, it may become measurable after pretreatment. However, the pretreatment is often complicated.
2) The results of measurement in absorptiometry are universal. Any system of any manufacturer may provide the same value, in principle.
   However, in the fluoroscopic method, the fluorescence intensity is a relative intensity, which has no units. For example, even systems of the same model of the same manufacturer may often provide different results due to individual differences in the detectors, etc. Even if the same specimen is measured, the luminescence intensity varies, say by 100 or 50 (although the shape of the spectrum does not change). Needless to say, the same will be true with other manufacturers' systems.

2. Mechanism of the Spectrofluorophotometer

2.1 General mechanism

The block diagram below shows a general spectrofluorophotometer.

(1) The excitation source is a light source for providing excitation light to irradiate a specimen, and a xenon lamp is usually used.



White light (light composed of various wavelengths) emitted from the light source enters an excitation side spectroscope (2). While measuring an excitation spectrum, this excitation side spectroscope (2) is moved to successively change the wavelength used to irradiate the specimen (this is called scanning or wavelength scanning). In contrast, when measuring a fluorescence spectrum, a specific wavelength is selected and fixed in the excitation side spectroscope.

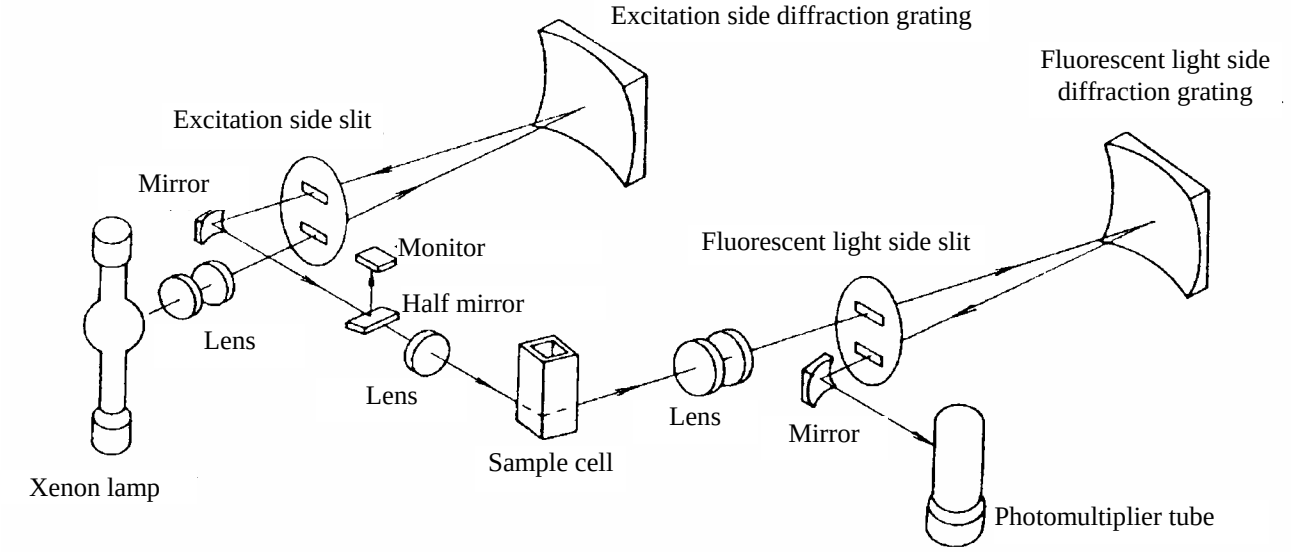
The light emitted from the excitation side spectroscope travels towards the specimen to excite it. Along the way, a half-mirror is used to split the light beam (diagonal line in the figure above) so that a portion also travels to the monitor detector (3). The monitor detector monitors the intensity of the excitation light irradiating the specimen. Generally, a photoelectric tube (phototube), a photodiode, a photomultiplier tube (also called a photomultiplier or photomul), etc are used.

When the excitation light reaches the specimen, the specimen is excited to emit fluorescent light. The emitted fluorescent light enters a fluorescent light side spectroscope (4) (although omitted in the diagram, this light is condensed by a lens).
When measuring the excitation spectrum, a specific wavelength is selected and fixed in the fluorescent light side spectroscope (4). When measuring a fluorescence spectrum, the fluorescent light side spectroscope is moved to measure the wavelengths of the emitted fluorescent light.

The fluorescent light leaving the fluorescent light side spectroscope enters the fluorescence detector (5). Generally, a photomultiplier tube is used. A fluorescent light detector converts the fluorescent light into an analog electric signal which is converted into a digital signal by an A/D conversion circuit (6).
A computer (7) controls wavelength scanning and digital signal processing.

2.2 Components to be used

The following is an optical system diagram of a spectrofluorophotometer.
Although detailed descriptions are omitted here, it is constituted of many optical devices like this.



2.3 Precautions in comparing performance

Performance specifications of a system are described in the last page of its catalog. Meanings of terms used here and how to read numbers are explained.

1) Sensitivity: An item a user cares the most. This is an index indicating how smooth data with little noise is obtained, and it can be said that the larger the index the better the system is. It is also called S/N (signal/noise). However, the number varies a lot with a measurement condition of sensitivity. It is not meaningful to compare without confirming that conditions such as a slit (bandpass) and a response (time constant) are the same. Some manufacturers report sensitivity measured on advantageous conditions in catalogs.

2) Light source: Manufacturers with some exceptions adopt 150 W xenon lamps for excitation sources. With an ozoneless lamp which offers very weak light of wavelength less than 220 nm, it is impossible to use light less than 220 nm as excitation light.

3) Measurement wavelength range: the wider the more versatile.

4) Slit: Also called bandpass. It represents the width of the slit at the inlet/outlet of a spectroscope. If the slit width is large, data with relatively lower noise (sensitive) can be obtained since the amount of light increases, however, minute peaks of a spectrum cannot be determined. If it is narrow, the minute peaks of the spectrum can be determined, however, light decreases leading to noisy data.

   Although it is greatly dependent on a specimen, since priority is given to that there is little noise when using a spectrofluorophotometer for quantifying a dilute specimen etc., it is not necessary to adhere to narrow slit width.

5) Response: If a large response is chosen, a spectrum becomes smooth, however, the shape will become blunt. When measuring excitation/fluorescence spectrum, optimal response should be selected depending on the scanning speed. Even if response is small (fast), noise will increase or a spectrum will be distorted if the response suitable for scanning speed is not chosen.

6) Others: Special measurement such as three-dimensional measurement and phosphorescence measurement may be possible using an optional software, some models can manage in standard.

3. Advanced Measurement

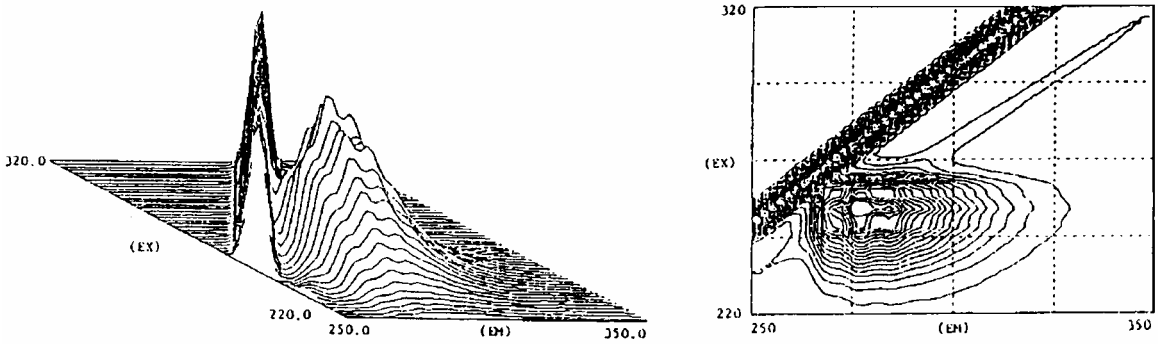
3.1 Spectrum correction

As described in “Differences from Absorptiometry” and “Weak point of fluoroscopic method”, the output data and shape is expected to vary with the system. If the same system model is used, the shape of the spectrum will remain the same, while only the data will differ between experiments. However, if different system models are used, even the shapes of the obtained spectra may differ. This is due to the specific characteristics of the optical system of each model. In a spectrum correction, the system characteristics (instrumental function) are measured in advance, and then used to process the output data for balance.

Some Hitachi spectrofluorophotometers have a function which measures the instrumental function using a standard. In this case, in the excitation side correction, the standard is rhodamine B. In the fluorescent light side short wavelength, it is a diffusion component. An optional substandard light source is used for fluorescent light side long wavelengths.

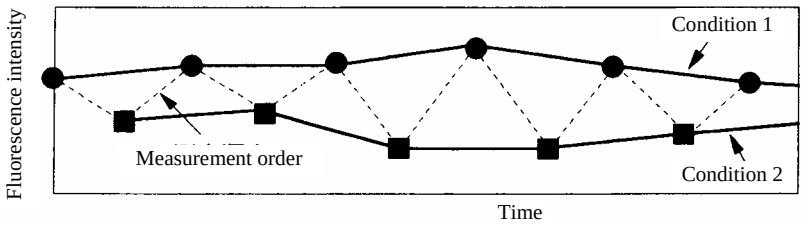
3.2 Three-dimensional measurement

An excitation spectrum is obtained by scanning the excitation wavelength, and a fluorescence spectrum is obtained by scanning the fluorescence wavelength. The lower left figure shows many spectra obtained by repeatedly measuring fluorescence spectra while changing the excitation wavelength. The lower right figure is a contour representation of the lower left figure. Suchh a three dimensional spectrum carries all of the information of tens of fluorescence spectra and excitation spectra, conveniently expressing a large amount information.



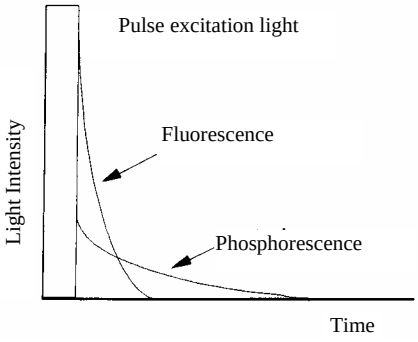
3.3 Intracellular calcium ion concentration measurement

The intracellular calcium ion concentration can be measured using the fluoroscopic method. The time variation of the output is measured while successively applying several wavelength conditions, allowing the concentration to be calculated.



3.4 Phosphorescence measurement

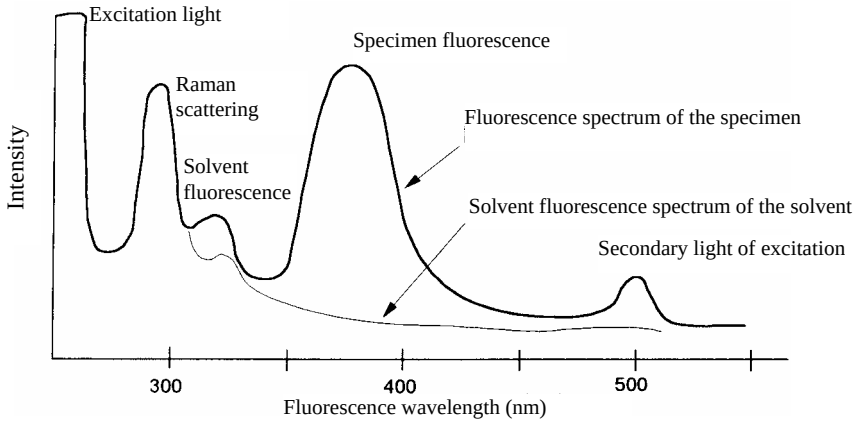
In a normal spectrofluorophotometer, a specimen is continuously irradiated with excitation light. If irradiated with a pulse (i.e., a flash of light), the fluorescence will disappear within the order of tens of nanoseconds (1 ns = 10^{-9} s), leaving phosphorescence, which continues to gleam for several milliseconds to several seconds.
In some models, a continuous excitation may convert into a light pulse with a chopper, to allow measurement of the phosphorescence component of the sample’s response.



Appendix

A. Measurement example for a fluorescence spectrum

In a measurement, various components, including Raman scattered light, the fluorescent light of both the solvent and specimen, and secondary light of excitation are mixed with the excitation light.



B. Glossary

1)	Sensitivity	Used to indicate system performance. Generally, this quantity is expressed by a signal to noise ration.
2)	Xenon lamp	Light source used for excitation
3)	Fluorescent light	Light emitted by the excited specimen.
4)	Unit of fluorescence intensity	Relative intensity; no units
5)	Fluorescent detector	Detector for measuring the intensity of emission. A photomultiplier tube (photomul) is commonly used.
6)	Fluorescence spectrum	Spectrum obtained by measuring the fluorescence wavelength
7)	Detection limit	Concentration of the most dilute specimen which can be measured. This limit depends on the specimen.
8)	Calibration curve	Graph for converting fluorescence intensity into concentration.
9)	Photomultiplier tube	→ fluorescence detector
10)	Measurement of intracellular Ca^{2+} concentration	One example of the application of the fluoroscopic method. The time changes at two or more wavelengths are measured simultaneously.
11)	Three-dimensional measurement	A fluorometry technique using three axes: excitation wavelength, fluorescence wavelength, and fluorescence intensity. The output data contains much information.
12)	Scanning	The successive shifting of the wavelength of a spectroscope to obtain a spectrum, or the resulting measurement.
13)	Stokes' Law	The principle that the fluorescence wavelength is longer than the excitation wavelength.
14)	Spectrum correction	Measurement with processing to compensate for the characteristics of the specific system used.
15)	Slit	The slit at the entrance of the spectroscope. This determines the resolution of the spectrum.
16)	Instrumental function	The characteristics of the specific system
17)	Secondary light	Light which appears at a wavelength twice that of the light entering spectroscope. For example, if light at a wavelength of 300 nm enters the spectroscope, secondary light will appear at 600 nm.

18) White light	Light with various wavelength components (sunlight or light of a xenon lamp).
19) Wavelength	Number corresponding to the color (energy) of light; commonly expressed in nm (nanometer) nm.
20) Wavelength scanning	→ Scanning
21) Bandpass	→ Resolution
22) Resolution	Indicatedsthe most narrow peak that can be measured.
23) Raman scattering	A type of scattered light which appears when materials are irradiated.
24) Phosphorescence	A type of emission. Even if the excitation light is cut off, the light due to phosphorescence will gleam longer than the fluorescent light.
25) Excitation source	→ Xenon lamp
26) Excited state	State in which a specimen has absorbed energy.
27) Excitation spectrum	Spectrum obtained by scanning the wavelength of the excitation light. This spectrum resembles an absorption spectrum.
28) Response	Index which weakens the output data. A number suitable for the scanning speed must be chose.
29) Excitation light	Light used to excite a specimen.