

SCIENCE IMAGING SYSTEMS

Application Note

No. 1

Fundamentals of Fluorometric Analysis

FLA-2000

Introduction

FUJI PHOTO FILM CO., LTD. has introduced fluorescence image analyzer FLA-2000, which is suitable for fluorescence measurement and radioisotopic measurement using the IP method. This Application Note is issued to facilitate the best possible use of FLA-2000.

FLA-2000 is equipped with two laser light sources: a newly developed SHG laser with a wavelength of 473nm and a He-Ne laser with a wavelength of 633 nm.

An example of measurement possibilities is EtBr or SYBR® Green at 473nm in the direct staining of gel electrophoresis. Excitation of SYPRO® Orange at 473nm is optimal for various proteins. AttoPhos™ can be used as substrate of ALP in immunochemical detection of Southern blotting membrane.

This pamphlet introduces the basic concepts of the fluorescence and fluorometric analysis methods. We will provide detailed information in later issues. To issue even better information, we would greatly appreciate your opinions and impressions on the use of FLA-2000.

SHG(Secondary Harmonic Generation)

This technology halves the wavelength of laser light using a special wavelength conversion crystal.

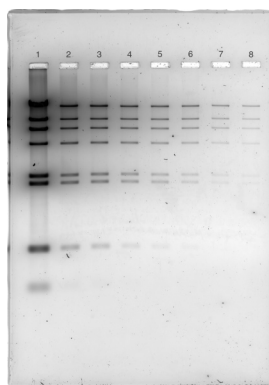
A laser diode and this crystal are assembled in a compact body.

ALP(Alkaline Phosphatase)

This is an enzyme that hydrolyzes phosphate moiety in alkaline solution.

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SYBR® Green stained gel (details; last page)

Summary

- Fluorescence emission wavelength is longer than its excitation wavelength.
- SYBR® Green is suitable for DNA detection in gels.
- SYPRO® Orange is recommended for protein detection.
- AttoPhos™ is used as an ALP label in immunochemical detection.

1 Principles of Fluorescence

Light : Color and Wavelength

Light is expressed in terms of wavelength and is classified as ultraviolet, visible and infrared light. The wavelength of ultraviolet light is 1-400nm, visible light is 380-800nm and infrared light is 800nm-1000nm.

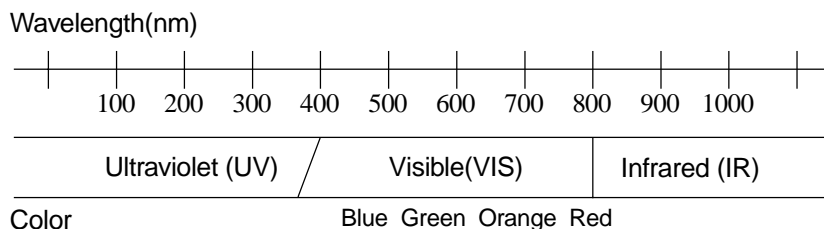


Fig.1-1

Wavelength

Light is characterized by its wavelength. As the light speed is constant, the relationship between wavelength and frequency is as follows.

Wavelength x Frequency = The speed of light (Constant)

Also the energy (E) is related to frequency by the following equation.

$$E = h\nu$$

(h: Planck constant, ν : frequency)

It can be said that shorter wavelength has higher frequency and greater energy.

Fig.1-1 Color and Wavelength

Visible light is an octave from 400nm to 800nm.

Fluorescence

A fluorescent compound absorbs ultraviolet and visible light and its molecules reach an excited state. The phenomenon of light emission during the process of returning to the ground state is called fluorescence.

Excited State

Excitation is the process of a molecule's transition from its normal low energy state to a high energy state.

Ground State

This is molecule's normal, low energy state. It comprises many energy levels, which absorb various wavelengths.

Principles of Fluorescence

- (1) Molecules enter an excited state after absorbing ultraviolet or visible light. Molecules reach an unstable state of high energy. (Excited singlet state: S_1)
- (2) Electrons lose excessive energy as vibration energy and reach the lowest level of excited singlet state.
- (3) Fluorescence occurs when electrons lose energy and reach the ground state.

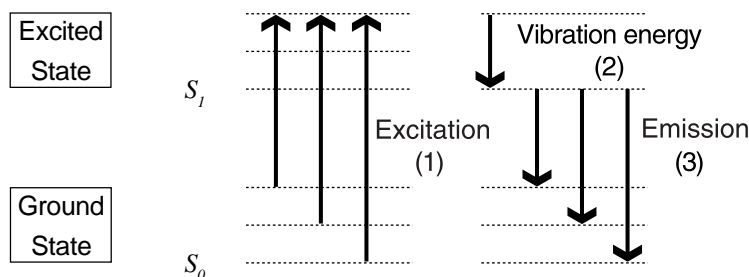


Fig.1-2

Fig.1-2 Principles of Fluorescence

A fluorescent molecule is excited from ground state to excited state. After losing part of its energy as vibration energy, it returns to its ground state by emitting fluorescence.

■ Relationship between Excitation and Emission Spectrum

The wavelength of emission spectrum shifts to the longer side of the excitation spectrum (Stokes' Law). This Stokes Shift is bigger when loss of energy by the vibration of electrons is larger.

Fluorescence analysis is easily performed when this Stokes Shift is large enough to separate the excitation and emission spectra.

■ Fluorescence Intensity

Fluorescence intensity is determined by the following five factors.

1. Strength of the incident light, I_0

The stronger the light, the higher the fluorescence intensity.

2. Quantum efficiency of the fluorophore, ϕ

Quantum efficiency is the ratio of photons emitted to photons absorbed.

Quantum efficiency = Emitted photons/Absorbed photons

Fluorescence becomes stronger as fluorescent quantum efficiency approaches 1.0.

3. Molar absorption coefficient, ϵ

This expresses the absorption of the fluorescent molecule in terms of incident light. Theoretical maximum is around 10^5 .

4. Concentration of the fluorescent molecule, C

Fluorescence intensity is proportional to the concentration in the range of lower densities. However, for high concentrations, the proportional relationship disappears. This quenching occurs mainly because the incident light is absorbed in the light path.

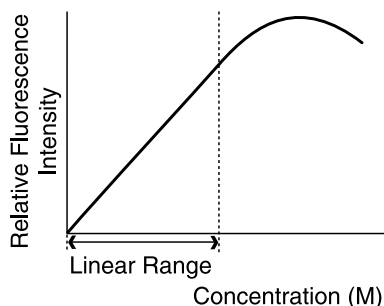


Fig.1-3

5. Sample thickness and length of the incident light, ℓ

Summarizing the above relationships, fluorescence intensity is expressed by the following equation.

$$F \propto I_0 \cdot \phi \cdot \epsilon \cdot C \cdot \ell$$

Vibrational Energy

This energy originates from molecular vibration. The energy (E) is expressed by the following equation.

$$E = h\nu \left\{ v_a + \frac{1}{2} \right\}$$

(ν : frequency, v_a : vibrational quantum number, h : Planck constant)

Quenching

This is the reduction in fluorescence intensity when the sample concentration is high.

Fig.1-3 Proportionality of Fluorescence Intensity

Lower density range is suitable for the quantitation. At higher densities, the incident light is absorbed and the light path becomes shorter.

2 Fluorescence Spectrum

Excitation light is necessary for fluorescence. The energy of the excitation light is absorbed by the fluorescent molecule and a portion of the energy is emitted as light. This reflects the energy state of electrons of the fluorescent molecule. Variations in the energy state of electrons cause variations in wavelength of excitation and emission, which is observed as spectrum.

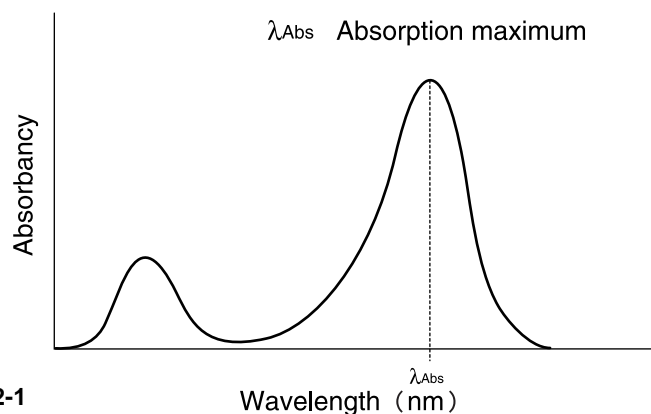


Fig.2-1

Fig.2-1 Example of an Absorbance Spectrum

The absorbance spectrum of a fluorescent molecule is influenced by various factors, such as pH, buffer constituents, coexistence of DNA, protein or organic solvents.

Theoretically, the maximum excitation wavelength equals the maximum absorbance wavelength.

Optimal excitation/emission wavelength has often been published. The excitation wavelength is expressed as Abs, λ_{Abs} , λ_{Ex} . The emission wavelength is expressed by λ_{Em} . To observe an excitation spectrum by spectro-photofluorometer, excitation wavelength should be scanned from 200nm upwards while observing at maximum emission wavelength.

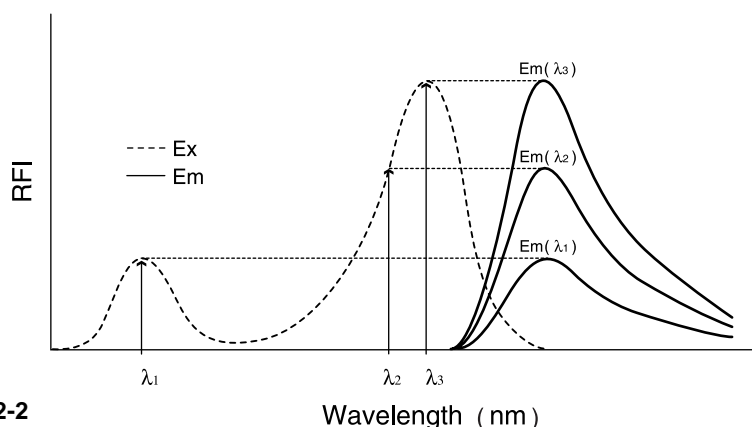


Fig.2-2

The shape of excitation spectrum is similar to the absorption spectrum. The wavelength of maximum emission is constant even when the excitation wavelength varies.

Abs

Absorbance

λ

Wavelength

Ex

Excitation

Em

Emission

RFI

Relative fluorescence intensity

Fig.2-2 An Example of Excitation/Emission Spectra

Excitation Spectrum:

The emission wavelength is fixed to λ_{Em} and fluorescence intensity is plotted against each the excitation wavelength.

The excitation wavelength is always shorter than that of emission.

Emission Spectrum:

The excitation wavelength is fixed to λ_{Ex} and fluorescence intensity is plotted against each the emission wavelength.

The emission wavelength is always longer and the spectrum is on the right side.

3 DNA Detection in Gels

Direct fluorescence staining of DNA in gels is a well-established method. Fluorescence enhancement occurs by intercalation of this stain to dsDNA. This method can detect a wide range of DNAs and RNAs.

EtBr

From the early days of DNA research, EtBr was known to emit red fluorescence when mixed with DNA.

This occurs because EtBr intercalates with DNA. However, fluorescence is emitted by double-stranded DNA, single-stranded DNA and RNA. Destaining procedure is inevitable to reduce the background fluorescence caused by interaction between gel and EtBr. In handling EtBr, gloves and other protective clothing must be worn to avoid danger of its mutagenicity.

After use, gels and waste solution should be processed with sodium hypochlorite or activated charcoal.

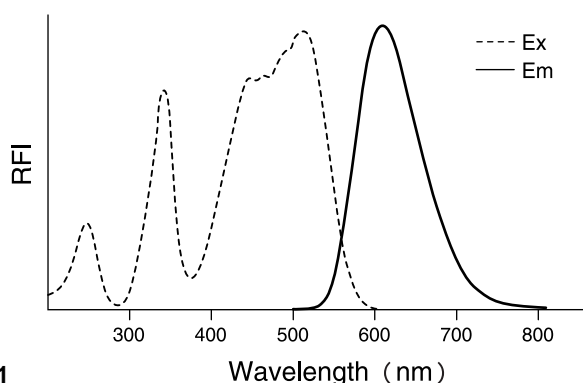


Fig.3-1

SYBR® Green

It is said that the fluorescence intensity of DNA detection by SYBR® Green is ten times more than that of EtBr. Moreover, low background simplifies the destaining procedure. It is also said that Ames testing shows low mutagenicity of SYBR® Green.

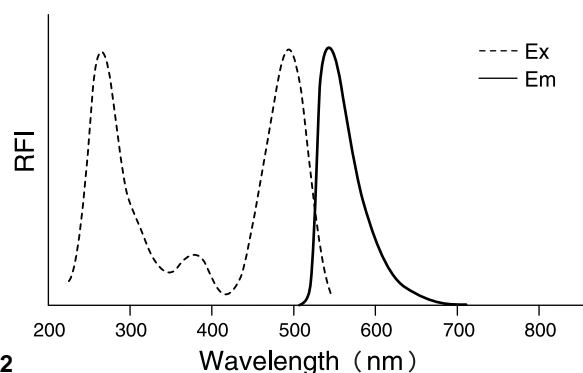


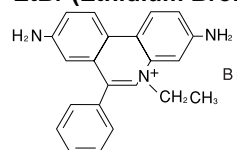
Fig.3-2

Intercalation DNA

The fluorescent stain is trapped in the base pair of DNA and the fluorescence intensity is enhanced.



EtBr (Ethidium Bromide)



EtBr is used to confirm the electrophoresis patterns of DNA. The gel is gently shaken in 0.01% EtBr in pH8.0 TAE or TBE buffer solution for 10 to 60 minutes, followed by observation on UV transilluminator at 254nm or 312 nm.

Single-stranded DNA

When double-stranded DNA is treated with strong alkaline solution or heat, it cleaves into two single-strand DNAs.

Fig.3-1 Excitation/Emission Spectra of EtBr-DNA (uncorrected*)

SYBR® Green

Two types of SYBR® Green are known. SYBR® Green I is for dsDNA and SYBR® Green II is for ssDNA and RNA.

Optimal excitation/emission is 497nm/520nm.

Fig.3-2 Ex/Em Spectra of SYBR® Green-DNA (uncorrected*)

*The shape of excitation/emission spectra are influenced by characteristics of the light source used in the spectrophotofluorometer. Correction of the spectrum by standard material is often done in this field. However, in this Application Note, uncorrected spectra taken by Hitachi F-4500 spectrophotofluorometer is used.

4 Protein Detection in Gels

The most common method of protein detection in gels has been CBB or silver stain. Fluorometric detection of protein, especially in gel electrophoresis, was not performed until when newly developed SYPRO® Orange was introduced recently.

■ CBB

Coomassie brilliant blue is the most commonly used blue stain for protein in gel. Destaining procedure is necessary because of high background.

■ Silver Stain

This has been the most sensitive staining method, but it is time-consuming.

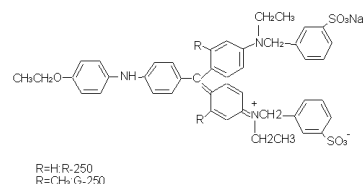
■ Fluorescamine

This fluorescent reagent for amines reacts with proteins as well. However, it is unstable in water so application to gel is not appropriate.

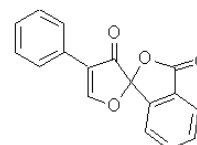
■ SYPRO® Orange

Recently, SYPRO® Orange was introduced as a fluorescent stain for protein in gels. SYPRO® Orange fluoresces with various proteins and shows stable fluorescence for a long time. Low background enables short post-washing time after staining. All these features show the superiority of SYPRO® Orange for detection of gel electrophoresis. Further, SYPRO® Orange stain does not interfere with immunochemical reactions on the Western blotted membrane. For this purpose, running buffer was used to dilute SYPRO® Orange instead of 7.5 % acetate solution. Excitation maximum of SYPRO® Orange is nearly the same as 473 nm wavelength of SHG laser used in FLA2000, which is highly favorable for high sensitive detection by this staining method.

CBB (Coomassie Brilliant Blue)



Fluorescamine



Fluorescamine itself is non-fluorescent, but after reaction with amines, a strongly fluorescent compound is formed.

SYPRO® Orange

Optimum excitation/emission wavelengths are 472nm/570nm.

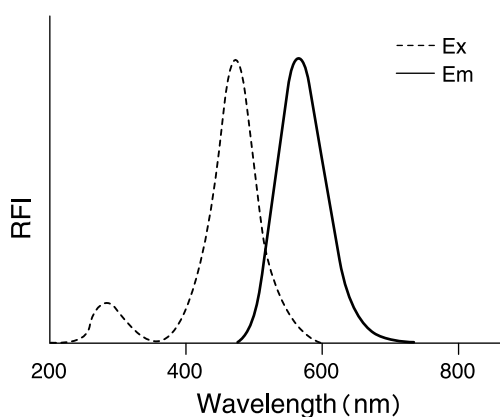


Fig.4-1

Fig.4-1 Excitation/Emission Spectra of SYPRO® Orange-Protein

5 Enzyme Amplified Fluorescence

Detection of specific DNA or protein on the blotted membrane requires high sensitivity.

Radioisotope (RI) has long been the most sensitive label. Recently, enzyme immunoassay technique has been successfully applied to blotted membranes. Fluorescent substrate and enzymatic amplification of product generation enable sensitivity comparable to the RI method.

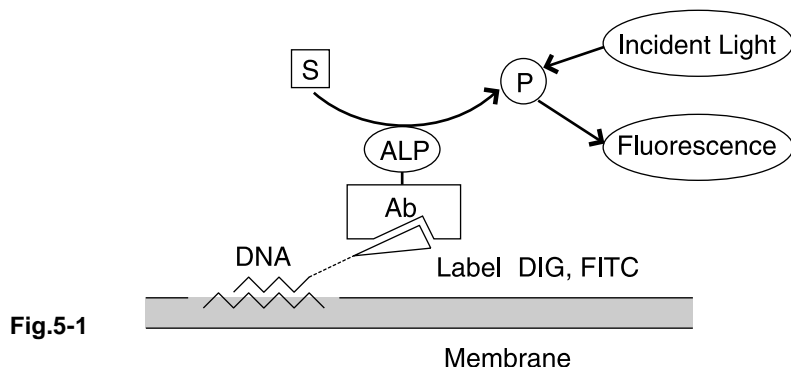


Fig.5-1

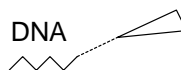
Fig.5-1 Enzyme Amplified Fluorescent Method

DIG	:	Digoxigenin
FITC	:	Fluorescein isothiocyanate
Ab	:	Antibody
ALP	:	Alkaline Phosphatase
S	:	Substrate*
P	:	Product(Fluorescent)
Light	:	Laser light source for FLA-2000
Fluorescence:	Emission from the fluorophore	

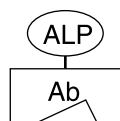
*The substrate differs from a chemiluminescent method. If CDP-Star® is used as the substrate for ALP, it becomes a chemiluminescent method.

The following reagents are used for detection of Southern blotted membrane.

- (1) A complimentary DNA fragment labeled with a small molecule such as FITC or DIG.



- (2) An ALP tagged antibody to FITC or DIG.



- (3) A fluorescent substrate for ALP such as AttoPhos®.



A 473 nm laser is used for excitation in FLA2000.

AttoPhos™

A fluorescent substrate for ALP. This substrate has weak fluorescence (Ex/Em = 420/560 nm) in pH9.5 solution. After enzyme reaction, a strong fluorescent product (Ex/Em = 482/560 nm) is formed.

6 References

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(1) Gel : 1% Agarose, 1 x TAE

(2) Standard sample :

Lane-1 : λ DNA/*Hind* III ; 1 μ g Lane-5 : λ DNA/*Hind* III ; 10ng

Lane-2 : λ DNA/*Hind* III ; 100ng Lane-6 : λ DNA/*Hind* III ; 5ng

Lane-3 : λ DNA/*Hind* III ; 50ng Lane-7 : λ DNA/*Hind* III ; 2ng

Lane-4 : λ DNA/*Hind* III ; 20ng Lane-8 : λ DNA/*Hind* III ; 1ng

(3) Electrophoresis

50 volts constant in 1 x TAE buffer.

(4) Stain

Two hours by 0.01% SYBR[®] Green I solution in 1 x TAE.

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(Fuji Photo Film Co., Ltd.)

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March 1998



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