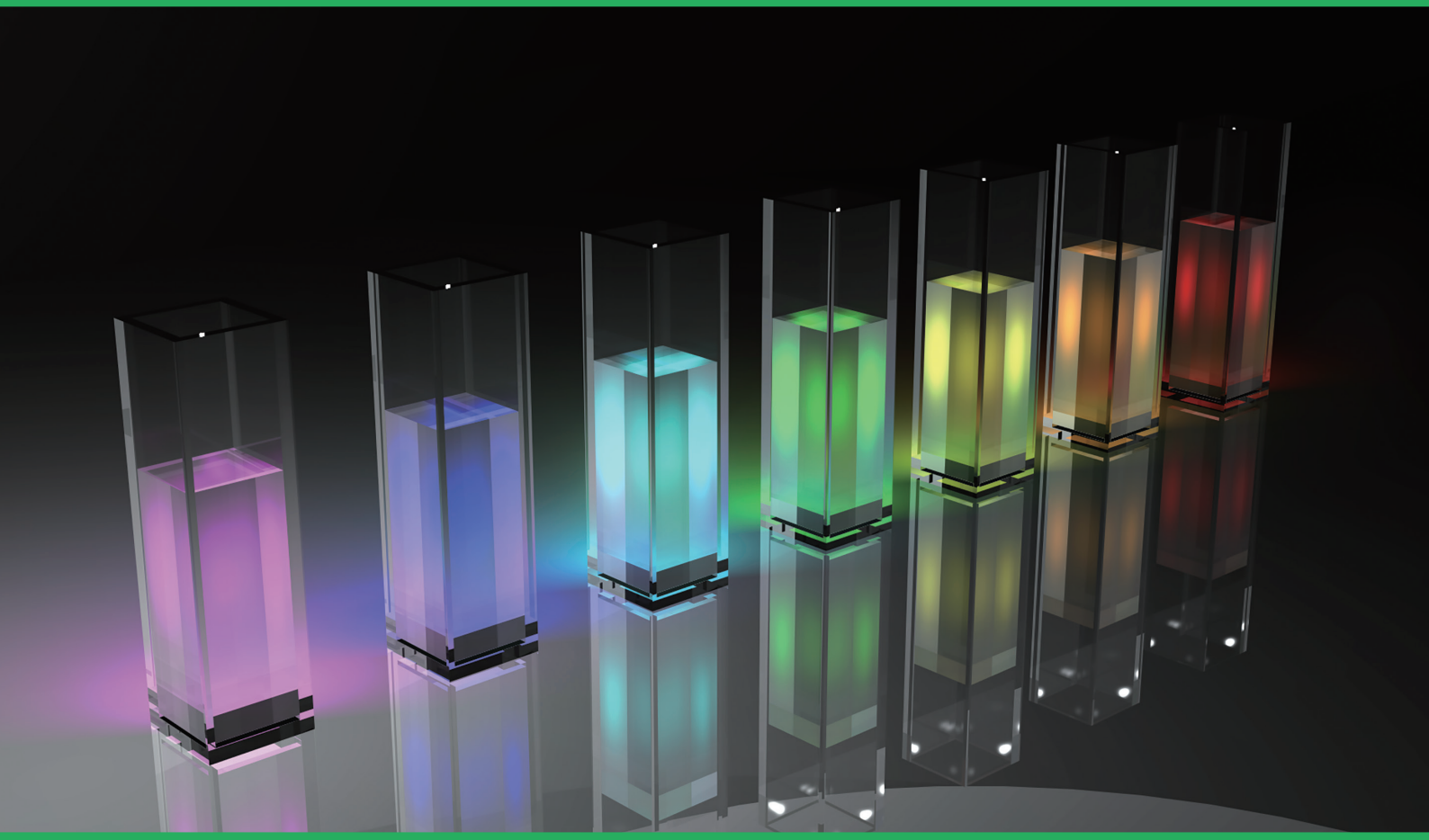
Application eBook

Fluorescence Spectroscopy



JASCO

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What is fluorescence?

Some material has the property that the light can be given by the absorbed energy. This phenomenon is generally called "luminescence". Table 1 shows the types of luminescence. Fluorescence is the one of photoluminescence, which is the light emission after the absorption of photon (ultraviolet/visible light).

Table 1. Types of luminescence

Type	Source	Example
Electroluminescence	Electric field	LED, organic EL, inorganic EL
Chemical luminescence	Chemical reaction, oxidation	White phosphorus (blue-green light emission), formaldehyde, paraldehyde with alkali/ethanol solution, oxidation of siloxene by Ce (IV)
Photoluminescence	UV rays, visible rays, infrared rays	Fluorescence and phosphorescence
Bioluminescence	Physiological absorption, enzymatic oxidation	Fireflies produce luminescence by catalytic oxidation of luciferase
Radioluminescence	α/β/γ rays, neutrons	Scintillator (NaI · TlI)
X-ray luminescence	X-ray	X-ray phosphor (e.g. ZnCdS: Ag, ZnS: Ag)
Cathodoluminescence	Electron beam	Phosphor, cathode ray tube for CRT, apatite
Thermoluminescence	Heat	Luminescence due to temperature rise (fluorite, sulfide phosphor)
Sonoluminescence	Sound waves, ultrasonic waves	Chemical reaction by the stimulation of sound (oxidation reaction of luminol)
Triboluminescence	Friction, strain and destruction	Crystal sugar, ZnS

Absorption and emission

The energy contained in a photon is expressed by $E = hv$ (E: energy, h: Planck's constant, v: frequency). When light hits a substance, its energy is absorbed by electrons, and the electronic configuration changes from the stable ground state to a higher energy excited state. Luminescence occurs when this energy is released in the form of light emission rather than molecular vibrations or heat generation. The electronic configuration then returns to the ground state (Figure 1). In many cases, part of the absorbed energy is emitted as thermal energy, so that the emitted light has a longer wavelength than the absorbed light (Stokes shift).

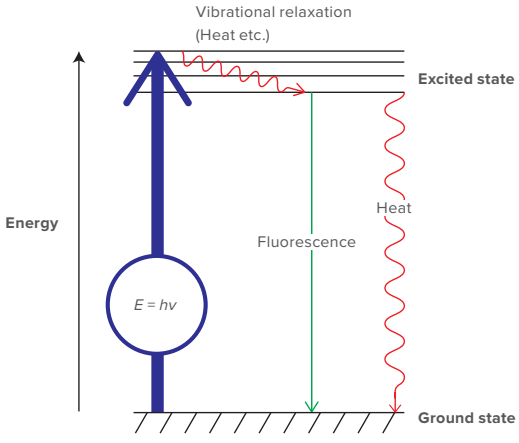


Figure 1. Jablonski diagram showing types of light emission

Excitation spectrum and fluorescence spectrum

In the excitation spectrum, the emission wavelength is fixed, and the excitation wavelength is scanned to measure the fluorescence intensity. The excitation wavelength that gives the maximum fluorescence intensity is the optimal excitation wavelength. The shape of the excitation spectrum is similar to that of the absorption spectrum (Figure 2), and the strongest fluorescence is also given at maximum absorption wavelength. It occurs because the amount of excitation light, which is absorbed at maximum absorption wavelength, is the largest, and the emitted energy when transferring from excited state to ground state is large.

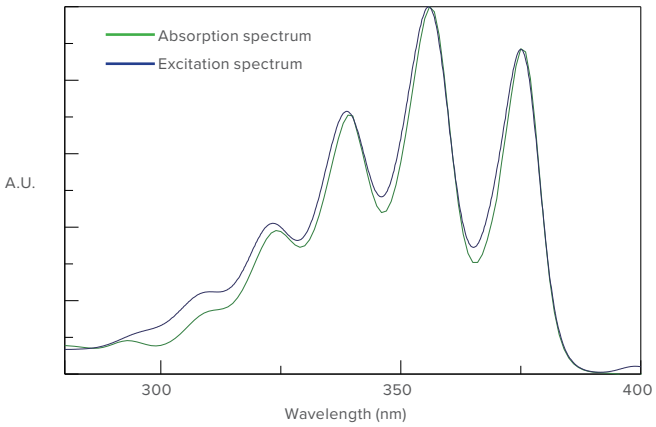


Figure 2. Absorption spectrum and excitation spectrum

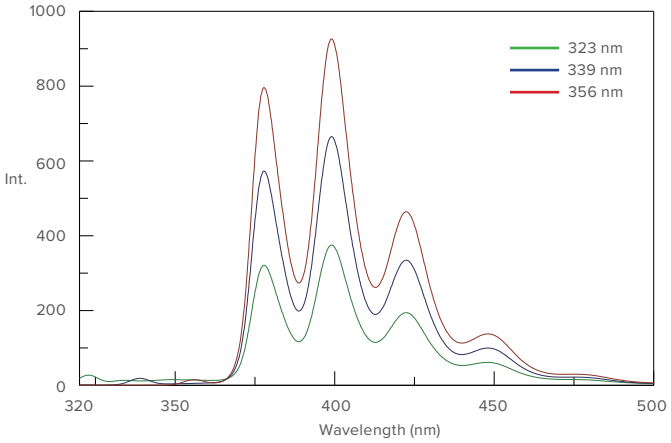


Figure 3. Fluorescence spectra at different excitation wavelengths

The fluorescence spectrum is acquired by fixing the excitation wavelength and scanning the emission wavelength. Since the fluorescence spectral profiles do not vary with excitation wavelength (Figure 3), the optimal excitation wavelength that gives the strongest fluorescence light should be used for the fluorescence measurement. The peaks of fluorescence spectrum appear at longer wavelength than the ones of absorption spectrum. Since the part of emitted energy is lost by heat etc, that phenomenon occurs.

Most of phosphor (benzene, anthracene, rhodamine 6G etc.) has the mirror image relationship between absorption and fluorescence spectra (Figure 4 - right) because the interval of vibrational level in its excited state is similar to the one in its ground state (Figure 4 - left). Sometimes, its mirror image relationship may not be satisfied due to the interaction between sample and solvent.

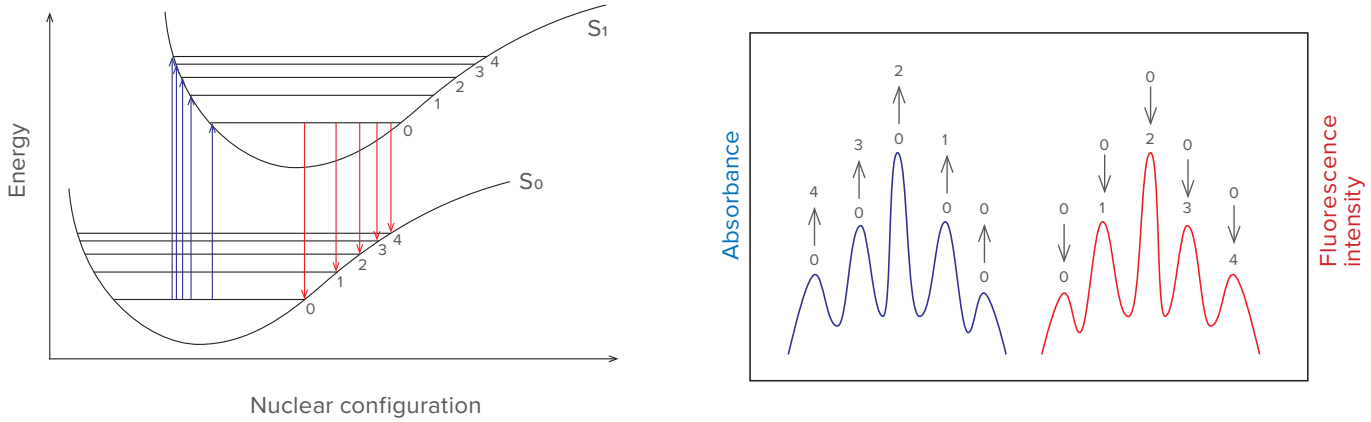


Figure 4. Vibration levels in excited and ground states (left), mirror image relationship between absorption and fluorescence spectra (right)

Advantages of fluorescence spectroscopy

Fluorescence spectroscopy offers the following advantages:

- High sensitivity

  Absorption spectroscopy detects decreases in incident light intensity, whereas fluorescence spectroscopy detects the intensity of emitted light. It is highly sensitive because it detects the difference from zero light intensity.

- High selectivity

  Molecular species that emits the fluorescence are limited. In addition, emission wavelength and excitation wavelength depend on the phosphor. Therefore, fluorescence spectroscopy is the analytical method with high selectivity. This advantage is used for the fluorescence labeling to monitor the target molecule by binding it to the fluorescent molecule.

- High environmental dependency

  The shape and intensity of the fluorescence spectrum are sensitive to the environment surrounding the fluorescent molecules (solution pH, temperature, solvent type, coexisting salt). This can be used to probe the surrounding environment.

Solutions by fluorescence spectroscopy

Fluorescence spectroscopy is applicable at various fields and purposes, and provides the breakthroughs as below.

Life science

- Sample concentration

  It indicates the concentration information of fluorescent in sample. It can also show the concentration of target molecules by specifically binding/interacting with fluorescent dyes (or fluorescent probes).

- Fluorescence anisotropy/Fluorescence polarization

  It indicates the degree how much the fluorescence light which is emitted from fluorescent is polarized. It can also detect the change of protein structure, the change of molecular weight at decomposition of biopolymer, and the interaction between biomolecules (protein-protein, antigen-antibody, DNA-protein, protein-protein).

- Melting temperature

  It indicates the temperature when the abundance ratio of native protein is equal to the one of denatured protein, and also indicates thermal stability of protein.

- Dissociation constant

  It represents the concentration ratio between the complex and its component molecules at chemical equilibrium. It can be used as the parameter of the affinity between protein and ligand.

Material science

- Fluorescencee property

  It shows specific excitation/emission information (wavelength and intensity) which the fluorescent has.

- Quantum yield

  It is the ratio between photons which is absorbed to sample and the ones which is emitted from sample. It can express the emission efficiency of fluorescent.

- Phosphorescence lifetime

  It is the time when the amount of fluorescent molecules in excited state is decreased to 1/e (approximately 37 %). It can provide the meaningful information about emission mechanism.

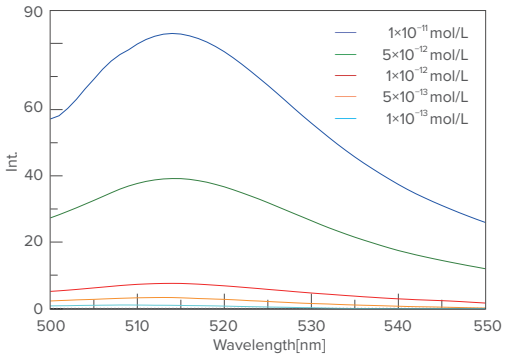
- Luminous color/Color rendering index

  Luminous color can be represented by quantifying the fluorescent's color. Color rendering index indicates the degree how much the color appearance of objects under the light source reproduces the one under sunlight.

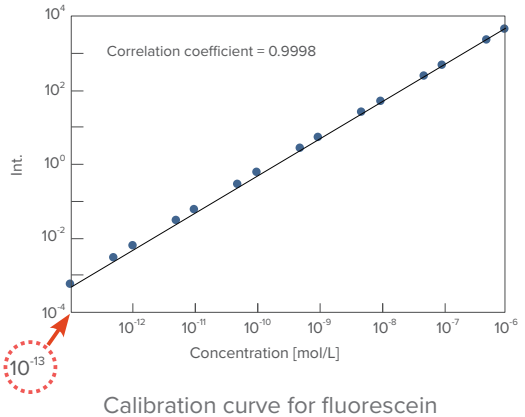
FP-8050 Series Spectrofluorometer

Outstanding sensitivity and linear dynamic range

The high-throughput optical system and low noise signal processing of the FP-8050 Series results in a high signal-to-noise (S/N) performance up to 8,500:1* (RMS). In addition, the dynamic range of FP-8050 Series has been developed to provide up to 7 orders of magnitude. As shown in the calibration curve for fluorescein, there is excellent linearity even for samples at very low concentrations.



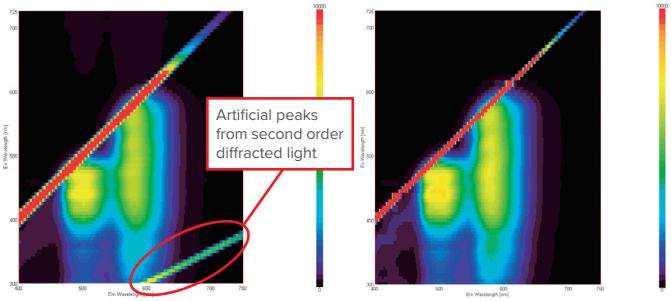
Fluorescein spectra for a range of concentrations



Calibration curve for fluorescein
(Auto Sensitivity Control System (SCS) ON)

Higher-order light cut-off filters

In order to remove peaks originating from higher-order diffracted light, cut-off filters appropriate to the measurement wavelength should be used. The models FP-8350, FP-8550, and FP-8650 include cut-off filters that are switched automatically as the wavelength range is set by the user.

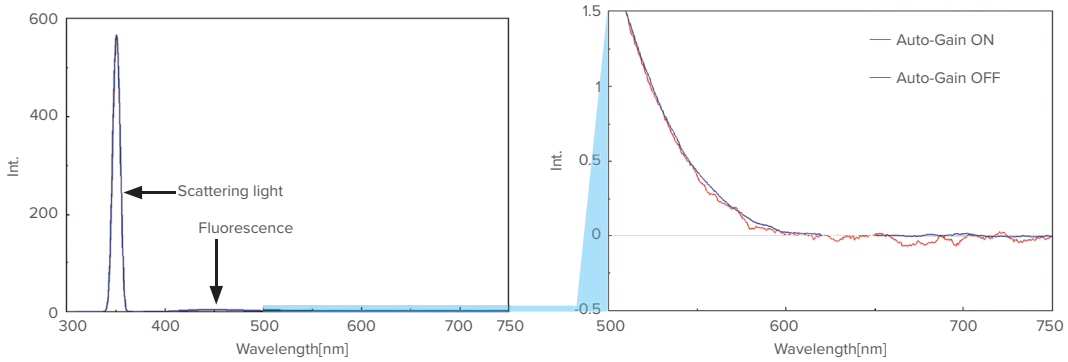


Without Cut-Off Filter With Cut-Off Filter

3D Spectra of Fluorescent Orange Color Plate

Auto-adjustment of detector gain and sensitivity

The FP-8050 Series includes both Auto-Gain and Auto Sensitivity Control System (SCS), which automatically adjust the detector gain and sensitivity for optimum measurement. Auto-Gain automatically adjusts the gain of the signal from the detector so that the S/N is optimized throughout the entire scan range for spectral measurement, and weak fluorescence peak shapes can be observed against scattered light with a high S/N. The Auto-SCS allows the user to create calibration curves for a wide concentration range without having to manually change the instrument sensitivity settings.



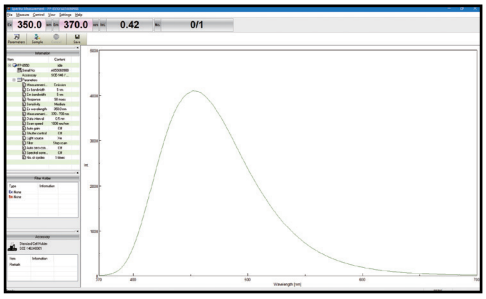
Fluorescence spectra of quinine sulfate solution

*Typical S/N for FP-8500 for water Raman, using on peak baseline noise.

Accurate spectral correction

Spectral correction should be made to any fluorometer for the measurement of accurate and reliable spectral data. All models in the FP-8050 Series can be spectrally corrected using a simplified procedure. JASCO has a range of calibration standards for spectral correction, including standard light sources and Rhodamine B.

Calibrated WI light source



User-friendly interface

A simple sequence of buttons guides the user through routine operation from measurement to saving data.

Enhanced measurement functions

- Saturation log (photometric value)
- Fluorescence Maxima search
- Automated shutter function (open/close)
- Self-motion auto-zero
- Simple parameters

Automatic Accessory Recognition

Accessories are automatically recognized when installed in the instrument. Spectra Manager™ logs the accessory name and serial number; this information is saved in the data file for a complete record of the measurement.

Daily monitoring of instrument performance

Validation program

The Validation program includes a full suite of tests to validate instrument performance. When executed, simple prompts guide the user on how to perform the tests. All instruments in the FP-8050 Series include a mercury lamp as standard for wavelength calibration.

Daily check program

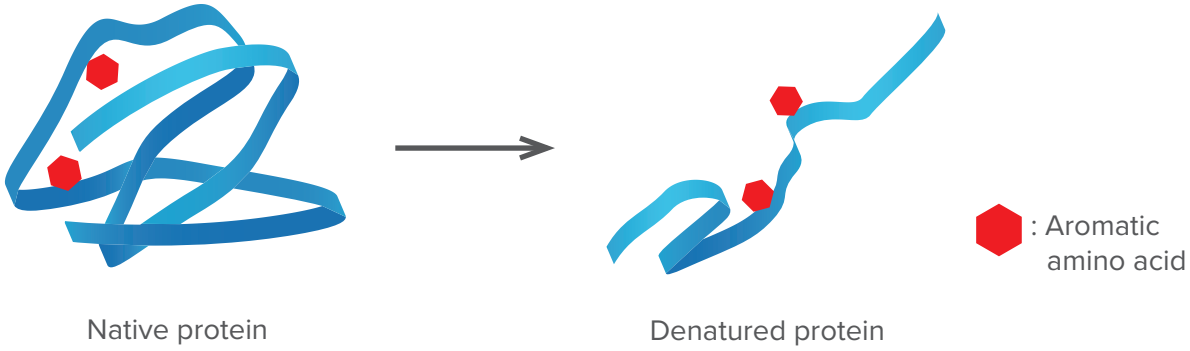
The Daily Check is a simple alternative to the Validation program; a less comprehensive performance check can be made at any time for continuous performance monitoring. After starting the Daily Check program, a timer is activated, and sample measurement is executed automatically after a predetermined stabilization time. The batch display lets the user check the cumulative data over a period of time.

Instrument activity log

The instrument activity log is a useful alternative to the paper log books found in multi-user facilities. A record is maintained of user, duration of operation and lamp usage.

Fluorescence measurement of heat-denatured protein

In order to evaluate the stability of protein, measuring the fluorescence that derives from aromatic amino acid (side-chain) is effective. Aromatic amino acids of protein are phenylalanine, tryptophan and tyrosine. They have the maximum fluorescence at 282 nm, 304 nm and 348 nm respectively (aqueous solution), and native protein has the maximum fluorescence at 340 nm because the fluorescence peaks of aromatic amino acids are stacked. Ramping the temperature of native protein, the high order structure varies by thermal denaturation. It means that the location of aromatic amino acid is changed from inside of protein to outside, and the maximum emission wavelength is shifted to long wavelength due to the change of the interaction between side-chains. Therefore, monitoring the maximum emission wavelength by changing the temperature or pH of protein enables to evaluate the structure stability of protein.



Native protein

Denatured protein

Since the structure stability of protein is directly linked to its functional stability, the thermal stability evaluation of protein by fluorescence measurement will be excellent solution at the following situations in R&D of antibody and diagnostic drugs.

- Stability comparison between before and after modified protein
- Stability comparison between original biological drug and biosimilar drug
- Investigation of drug storage condition

JASCO can provide the peltier cell holder, which can control the temperature of liquid sample with high accuracy. In addition, it is capable of the automatic measurement at a specific temperature and melting temperature (T_m) analysis.

Fluorescence Measurement of Heat-Denatured Lysozyme

Thermal denaturation of lysozyme which includes the tryptophan residue was evaluated.

[Sample]

- Lysozyme aqueous solution: 0.1 mg/mL

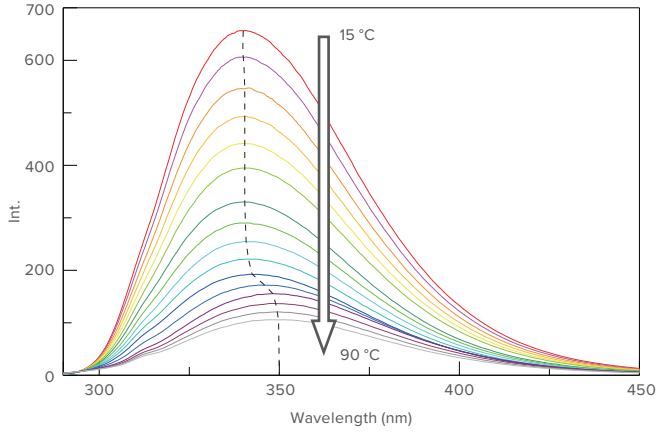
[Measurement parameters]

Bandwidth (excitation):	2.5 nm	Excitation wavelength:	280 nm
Bandwidth (emission):	5 nm	Wavelength range (emission):	290–450 nm
Response:	0.5 sec.	Data interval:	0.2 nm
Sensitivity:	Medium	Scanning speed:	200 nm/min.
Measurement start temperature:	15 °C	Temperature interval:	5 °C
Measurement end temperature:	90 °C	Temperature gradient:	1 °C/min.

- In order to make the sample temperature uniform, the measurement was performed during stirring the sample with stirrer.
- Spectra measurement was performed after 60 seconds from reaching to the setting temperature.

Figure 2a shows the changes of fluorescence spectra related to the temperature variations, and figure 2b shows the ratio between the fluorescence intensity at 340 nm and the one at 350 nm. As shown in figure 2a, the peak wavelength was shifted from 340 nm to 350 nm while the temperature was raised. Plotting the fluorescence intensity ratio (350 nm/340 nm) realized that its ratio was changed significantly at approximately 70 °C. These results indicates that the protein was denatured by raising temperature, and that tryptophan residue which was buried in protein was exposed to outside of protein. In addition, it estimated the melting temperature of lysozyme at approximately 70 °C.

a. Changes of fluorescence spectra



b. Temperature monitoring of fluorescence intensity ratio (350 nm/340 nm)

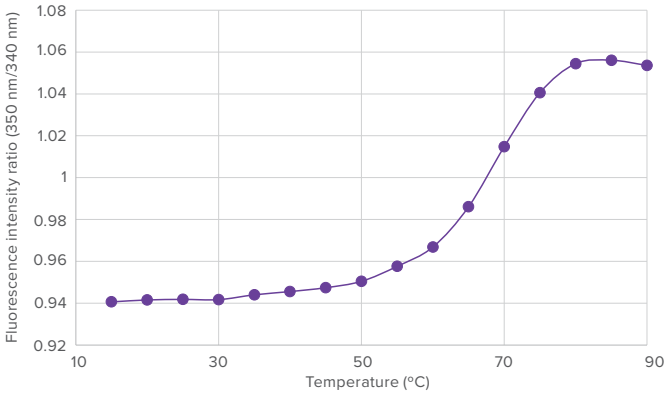


Figure 2. Measurement results

Monitoring of protein behavior by using FRET

FRET is the mechanism that the energy is transferred non-radiatively from an excited state donor to a neighbor acceptor (Figure 1). FRET is an abbreviation for Forester (or fluorescence) resonance energy transfer. Since FRET typically occurs when the distance between the donor and acceptor is 1–10 nm, it enables to monitor the structure change of protein and the interaction between specific proteins by binding donor and acceptor to the target protein.

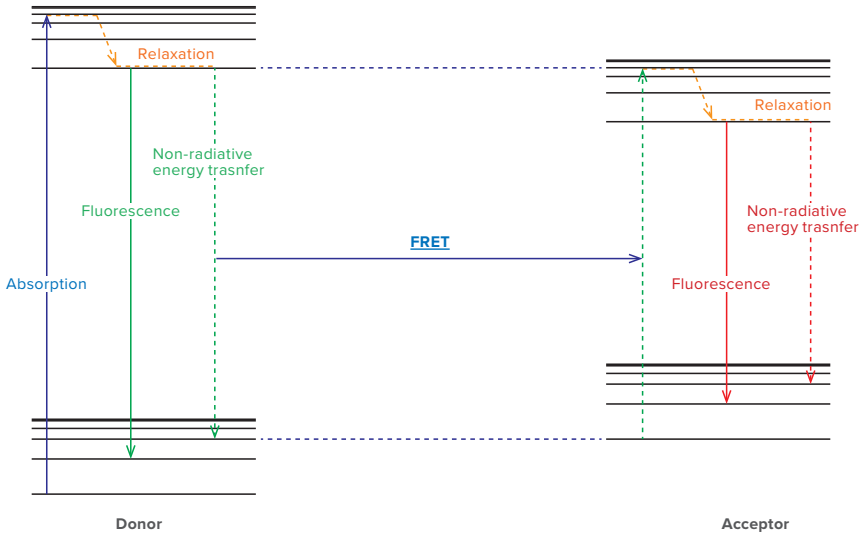


Figure 1. Energy transition in a FRET pair

FRET is monitored by the spectrofluorometer, which measures the fluorescence/quenching of acceptor or excited donor. FRET efficiency depends on the following factors (Figure 2).

1. Spectral overlap between the donor and acceptor

   As the overlap area of the donor's fluorescence spectrum and the acceptor's absorption spectrum is larger, FRET efficiency is higher.

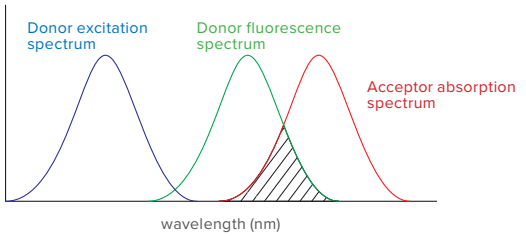
2. Distance between donor and acceptor

   FRET efficiency is inversely proportional to the six power of distance between the donor and acceptor.

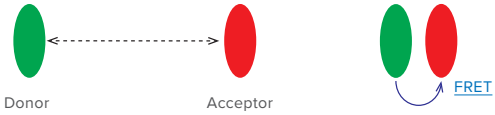
3. Orientation of donor and acceptor

   FRET efficiency is a maximum when the two dipole moments are parallel or anti-parallel to each other, and no energy transfer occurs when the dipole moments are perpendicular to each other.

1. Spectral overlap between donor and acceptor



2. Distance between donor and acceptor



3. Orientation of donor and acceptor

Figure 2. Factors that influence FRET

One of monitoring ways of protein behavior by using FRET is a cyan fluorescent protein (CFP) - yellow fluorescent protein (YFP) pair.

CFP and YFP, which are color variants of green fluorescent protein (GFP), are well-known as the fluorescent protein that FRET occurs. CFP absorbs excitation light of 433 nm, and produces fluorescence light of 476 nm. When CFP is close to YFP, the energy which CFP has absorbed is transferred to YFP by the interaction between CFP and YFP, and YFP produces fluorescence light of 527 nm (Figure 3) [1].

Its property can be applied to protein behavior monitoring by binding CFP and YFP to the target protein.

Fusing CFP and YFP to two different host proteins enables to monitor the protein-protein interactions (Figure 4. intermolecular FRET), and fusing CFP and YFP to two different sites in the same protein enables to monitor the protein conformational change (Figure 4. intramolecular FRET).

[1] Roger Y. Tsien 2009 Constructing and Exploiting the Fluorescent Protein Paintbox (Nobel Lecture). *Angew. Chem. Int. Ed.* **48**: 5612–5626

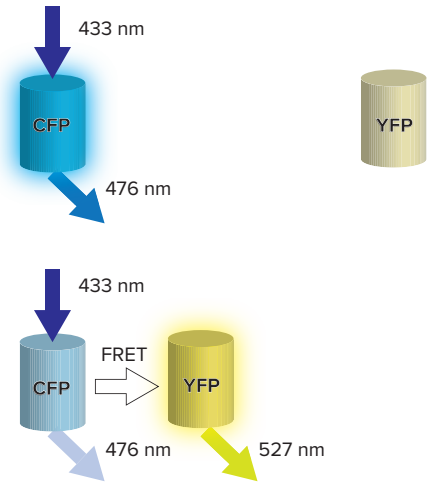


Figure 3. FRET between CFP and YFP

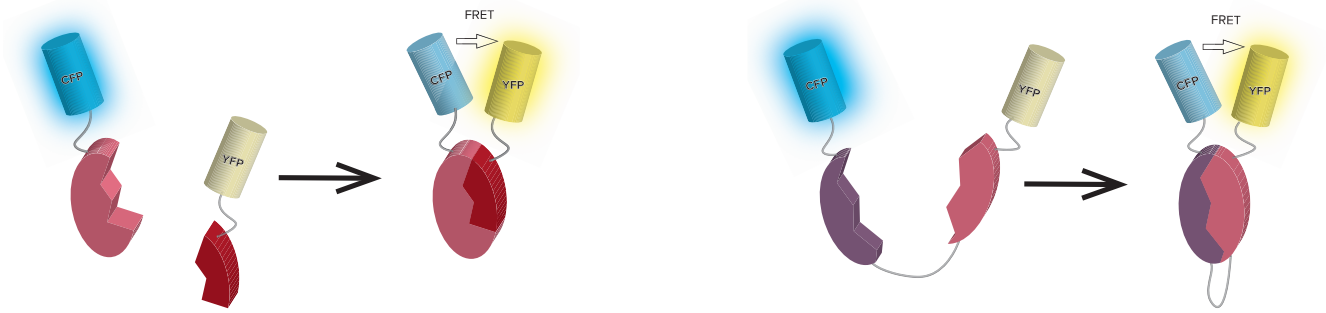


Figure 4. Intermolecular FRET (left) and Intramolecular FRET (right)

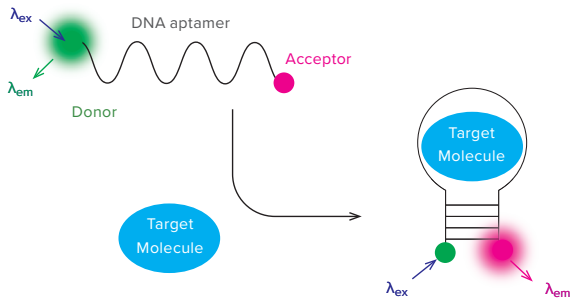


Figure 5. Schematic diagram of biosensor by using DNA aptamer

This property can be also applied to biosensor. One of its applications is the biosensor by using DNA aptamer, which has the capability to bind a specific molecule.

DNA aptamer, whose ends are fused donor/acceptor fluorescent molecules, can detect the target molecule because FRET occurs by changing its structure when binding DNA aptamer to target molecule (Figure 5).

JASCO's spectrofluorometer can detect FRET phenomenon with high sensitivity, and provide its useful information to the user.

Sensitivity evaluation of biosensor by using FRET

One of FRET application examples is the murine anti-TNT monoclonal antibody (anti-TNT mAb), which can form a complex with trinitrotoluene (TNT). Anti-TNT mAb absorbs the excitation light of 280 nm, and produces the fluorescence light of 340 nm. When anti-TNT mAb forms a complex with TNT, FRET is occurred between the anti-TNT mAb (donor) and TNT (acceptor). The energy is transferred from the anti-TNT mAb excited at 280 nm to the TNT, and the fluorescence at 340 nm from the anti-TNT mAb is quenched (Figure 1). By utilizing this interaction as biosensor, this article shows the evaluation result of anti-TNT mAb sensitivity against the aromatic nitro compound as well as TNT by using spectrofluorometer.

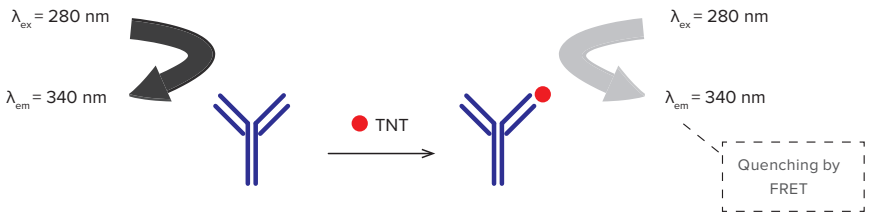


Figure 1. Schematic illustration of fluorescence quenching by FRET

[Reagents and samples]

- Murine anti-TNT monoclonal antibody (anti-TNT mAb): 1.6×10^{-7} mol/L[1]

- Aromatic nitro compounds

Trinitrotoluene (TNT):	4.4×10^{-8}, 4.4×10^{-7}, $4.4\times10^{-6.5}$, 4.4×10^{-6}, $4.4\times10^{-5.5}$, 4.4×10^{-5} mol/L
Trinitrobenzene (TNB):	4.7×10^{-8}, 4.7×10^{-7}, $4.7\times10^{-6.5}$, 4.7×10^{-6} mol/L
2,6-dinitrotoluene (2,6-DNT):	5.5×10^{-8}, 5.5×10^{-7}, 5.5×10^{-6}, 5.5×10^{-5}, 5.5×10^{-4} mol/L
2-Nitrotoluene (NT):	7.3×10^{-8}, 7.3×10^{-7}, 7.3×10^{-6}, 7.3×10^{-5}, 7.3×10^{-4} mol/L
Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX):	4.5×10^{-8}, 4.5×10^{-7}, 4.5×10^{-6}, 4.5×10^{-5}, 4.5×10^{-4} mol/L

- Solvent

  pH 7.4 phosphate buffer

[1] Satoko Suzuki, Toshifumi Uchiyama, Ken-ichi Akao, Koushi Nagamori, Hiroshi Nakayama and Yuji Ito, TNT sensing using non-labeling FRET, Japan-Taiwan Medical Spectroscopy International Symposium/14th Annual Meeting of the Japan Association of Medical Spectroscopy, AWAJI, JAPAN 2016

[Measurement flow]

4.4×10^{-8} mol/L TNT solution was added to 800 µL of 1.6×10^{-7} mol/L anti-TNT mAb, and the fluorescence measurement was performed. After measurement, the final concentration of TNT was increased step-by-step by adding each TNT solution (4.4×10^{-7} mol/L, 4.4×10^{-6} mol/L, $4.4\times10^{-5.5}$ mol/L and 4.4×10^{-5} mol/L), and fluorescence measurement of each different TNT concentration solution was performed.

Regarding the other aromatic nitro compounds, similar measurements were performed.

[Results]

The fluorescence spectra of mixture solution of anti-TNT mAb and TNT were shown in Figure 2. As the TNT concentration increases, the fluorescence intensity of anti-TNT mAb becomes weak. Similar measurements were performed using other aromatic nitro compounds as samples. The degree of progress of complex formation reaction was determined by the decrease of the fluorescence intensity at 340 nm. Figure 3 shows the relations between the dose of aromatic nitro compounds and the reaction ratio. From this curve, the half maximal inhibitory concentration (IC_{50}) was estimated (Table 1). This result shows that anti-TNT mAb has sensitivity to TNT, TNB, NT and 2,6-DNT, especially have high sensitivity to TNT and TNB whereas cannot detect RDX.

From these results, anti-TNT mAb is expected as high sensitive nitro compounds biosensor without using fluorescent label.

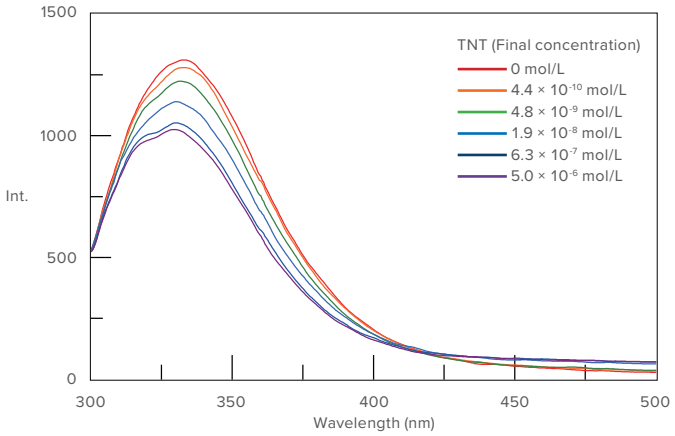
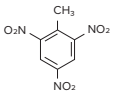
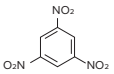
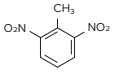
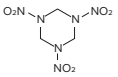


Figure 2. Dose-response curve of Anti-TNT mAb and aromatic nitro compounds

Table 1. Analysis of binding of different explosive compounds to anti-TNT

Name	Structure	IC_{50} (M)
TNT		4.0E-08
TNB		3.8E-08
2,6-DNT		7.9E-06
NT		3.6E-04
RDX		-

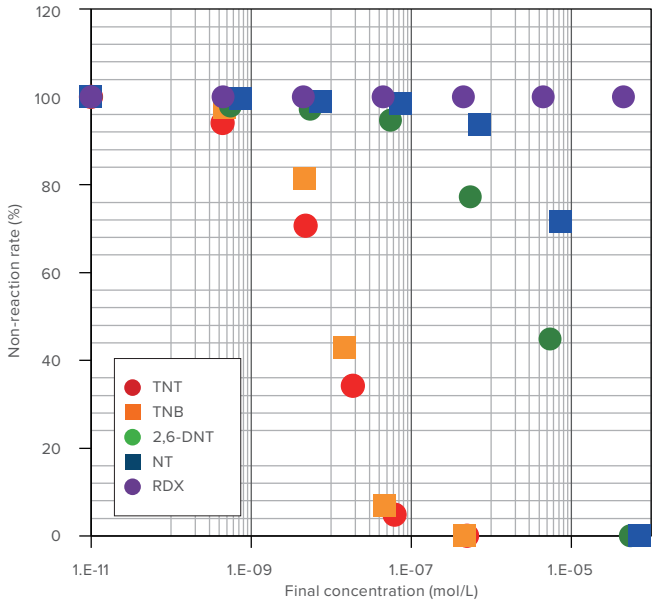


Figure 5. Dose-response curve of Anti-TNT mAb and aromatic nitro compounds

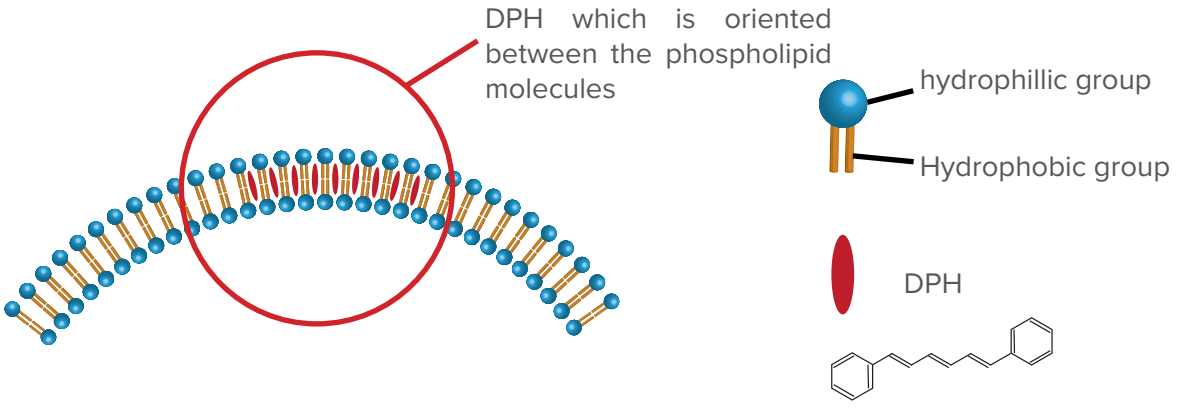
Fluorescence depolarization measurement

Fluorescence anisotropy (*r*) is the ratio of the polarized component to total intensity. It depends on the fluorescence lifetime (the time from exciting the phosphor to emitting the light), and depends on what degree its fluorescent does not move during the fluorescence lifetime and maintains the polarization when it is excited. Therefore, measuring its parameter (or fluorescence polarization) enables to know the degree of fluorescent's rotation, which depends on the molecular size, the viscosity around the molecule, the intermolecular interaction and so on. These techniques mainly apply to the following measurements of bio-sample.

- Size/conformation monitoring of biopolymer
- Evaluation of local structure in biopolymer
- Evaluation of inter-biomolecule interaction

This application note will demonstrate the use of the spectrofluorometer and its automatic polarizers to monitor the temperature-dependent polarization of diphenyl hexatriene (DPH) upon addition to a lipid bilayer.

DPH which is oriented between the phospholipid molecules has strong polarization property, and the status of biomembrane can be monitored by depolarization measurement.



[Measurement procedure]

(1) Calculation of G factor

Since the monochromator in emission side has polarization characteristics, G factor (*G*) for correcting the instrumental polarization artifacts should be calculated beforehand by using fluorescent dye for fluorescence depolarization measurement. The polarizer at excitation side should be installed to horizontal orientation (H), and then the intensity when the analyzer at emission side is set to vertical orientation (V) should be measured (I_{HV}), and the one when the analyzer is set to horizontal orientation (H) should be measured (I_{HH}). G factor is calculated as below.

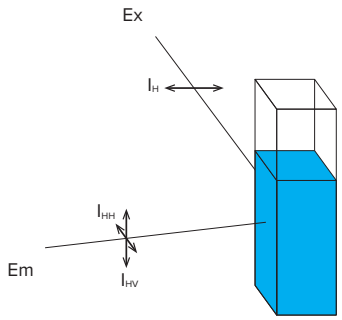


Figure 1. Measurement overview of G factor

G factor (*G*):

$$G = \frac{I_{HV}}{I_{HH}}$$

(2) Calculation of fluorescence anisotropy and fluorescence polarization

The polarizer at excitation side should be installed to vertical orientation (V), and then the intensity when the analyzer at emission side is set to vertical orientation (V) should be measured (*IVV*), and the one when the analyzer is set to horizontal orientation (H) should be measured (*IVH*). G factor is applied to IVH to perform the instrumental correction.

Total fluorescence intensity (*F*): $F = I_{VV} + 2G \cdot I_{VH}$

Fluorescence anisotropy (*r*): $r = \dfrac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2G \cdot I_{VH}}$

Fluorescence polarization (*P*): $P = \dfrac{I_{VV} - G \cdot I_{VH}}{I_{VV} + G \cdot I_{VH}}$

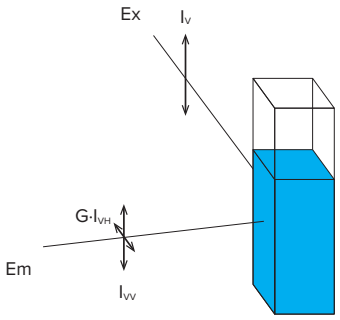


Figure 2. Measurement overview of fluorescence anisotropy and polarization

[Results]

G factor was measured by using mixture sample (liposome and DPH), and then temperature scan measurement was performed. DPH is located to the space between the lipid bilayers, and is oriented in a certain direction. Figure below shows the fluorescence anisotropy at each temperature, and indicates that the motion of DPH is confined at low temperature. In addition, it also shows that the depolarization occurs at approximately 40 °C. It means that the fluctuation of layer became large due to phase transition of lipid bilayer, and the motion of DPH became large.

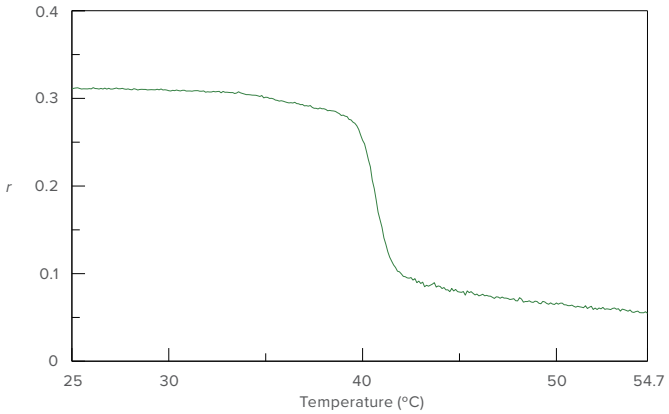


Figure 3. Fluorescence anisotropy at each temperature (DPH interacting with liposome)

[Samples]

- Phospholipid:130 µM
- DPH: 0.6 µM

[Measurement parameters]

Excitation wavelength:	357 nm
Emission wavelength:	430 nm
Bandwidth (excitation):	5 nm
Bandwidth (emission):	5 nm
Response:	2 sec.
Data interval:	0.1 °C
Ramping rate:	20 °C/hour

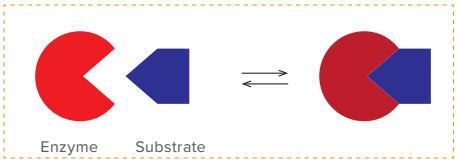
Enzyme kinetics probed by fluorescence spectroscopy

Enzyme activity is frequently investigated in the medicinal, biochemistry, and food science research fields to elucidate reaction rates and the affinity of enzyme-substrate interactions. There are some way to monitor the process of enzyme-substrate interactions, and spectrofluorometer is one of their effective tools.

JASCO offers the hardware/software for enzyme kinetics, which provides the reliable data. This section introduces how to obtain enzyme kinetic data.

Overview

One of the best-known models of enzyme kinetics is Michaelis-Menten kinetics. It is the reaction model that an enzyme (E) binds to a substrate (S) to form a complex (ES), which in turn releases a product (P) regenerating the original enzyme.



Enzyme (E) + Substrate(S) → Enzyme-Substrate Complex (ES) → Enzyme (E) + Product (P)

The initial velocity (v) of formation of P is represented as the following formula.

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \qquad (1)$$

This equation is called the Michaelis-Menten equation. Here, [S] is the substrate concentration. V_{max} represents the maximum reaction velocity at saturating substrate concentration, and K_m , which is called the Michaelis constant, is equal to the substrate concentration at which the reaction velocity is half of V_{max}. Plot v versus [S] based on the equation (1) is called Michaelis-Menten plot (figure 1), which enables to calculate the V_{max} and K_m.

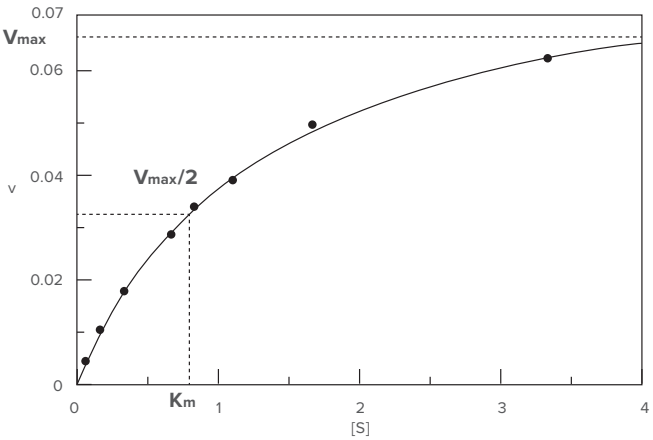


Figure 1. Michaelis-Menten plot

The high V_{max} means that the maximum activity of target enzyme is high. In addition, K_m indicates the affinity between enzyme and substrate. Low K_m means that the affinity between enzyme and substrate is high, and enzyme-substrate complex is formed quickly. On the other hand, high K_m means that the affinity between enzyme and substrate is low, and enzyme-substrate complex is formed slowly. V_{max} and K_m, can be calculated by the following procedure, which requires the analytical instrument such as the spectrofluorometer.

(1) Adds the substrate into enzyme solution, and perform time-course measurement, which monitors the fluorescence intensity of product or substrate.

(2) Obtains the initial velocity (v) by the slope at the start of reaction.

(3) Changes the substrate concentration, and obtains the initial velocity (v) through (1) and (2). And then, create [S] versus v plot.

Since obtaining V_{max} and K_m from Michaelis-Menten plot by eye makes the error easily, the modification of equation (1) is performed. The following methods are well-known as the modification method of Michaelis-Menten equation, and V_{max} and K_m can be obtained from slope and intercept of these plot.

Lineweaver-Burk plot: 1/[S] vs 1/*v*

$$\frac{1}{v} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \qquad (2)$$

Plot 1/*v* versus 1/[S] (Figure 2) based on the modified version of the Michaelis-Menten equation given above. Determine the slope (*a*) and the intercept (*b*) of the straight line using the least squares method and then calculate V_{max} and K_m using equations (3) and (4), respectively. This plot is often used for calculating V_{max} and K_m, and is effective at low substrate concentration range.

$$V_{max} = \frac{1}{b} \qquad (3)$$

$$K_m = a \times V_{max} \qquad (4)$$

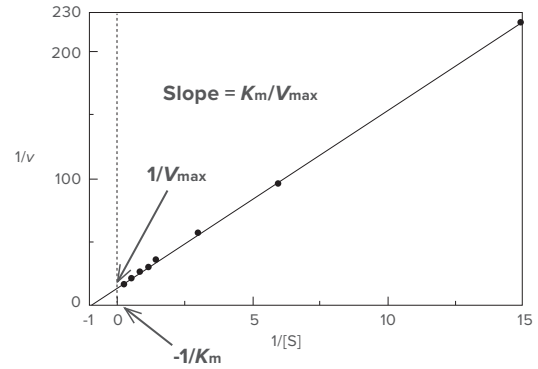


Figure 2. Lineweaver-Burk plot

Hofstee plot: [S] vs [S]/*v*

$$\frac{[S]}{v} = \frac{1}{V_{max}} \times [S] + \frac{K_m}{V_{max}} \qquad (5)$$

Plot [S]/*v* versus [S] (Figure 3) based on the modified version of the Michaelis-Menten equation given above. Determine the slope (*a*) and the intercept (*b*) of the straight line using the least squares method and then calculate V_{max} and K_m using equations (6) and (7), respectively. This plot is effective at high substrate concentration range.

$$V_{max} = \frac{1}{a} \qquad (6)$$

$$K_m = b \times V_{max} \qquad (7)$$

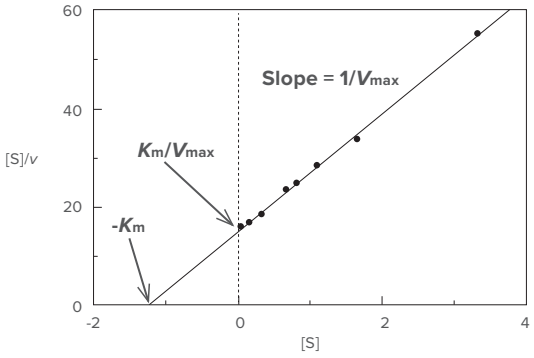


Figure 3. Hofstee plot

Eadie plot: *v*/[S] vs *v*

$$v = -K_m \times \frac{v}{[S]} + V_{max} \qquad (8)$$

Plot *v* versus *v*/[S] (Figure 4) based on the modified version of the Michaelis-Menten equation shown above. Determine the slope (*a*) and the intercept (*b*) of the straight line using the least squares method and then calculate V_{max} and K_m using equations (9) and (10), respectively. This plot is effective at wide range of substrate concentration.

$$V_{max} = b \qquad (9)$$

$$K_m = -a \qquad (10)$$

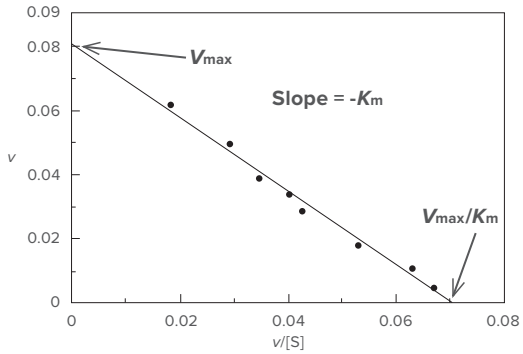


Figure 4. Eadie plot

Measurement of trypsin activity using fluorescence peptide substrate

Proteases are responsible for breaking down biological molecules into smaller polypeptide chains through hydrolysis. Upon hydrolysis of the substrate (methylcoumarin-amide [MCA]), 7-amido-4-methylcoumarin (AMC) and a water-bound trypsin peptide are produced. Trypsin is a protease that is commonly used in assays to determine the enzymatic activity of a molecule. After cleavage of the substrate via hydrolysis, the trypsin activity can be measured by monitoring the fluorescence intensity at 440 nm of the isolated product, AMC as a function of time. This section shows the example of trypsin activity evaluation by fluorescence peptide MCA substrate.

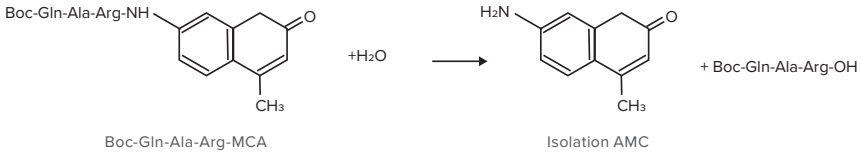


Figure 1. The hydrolysis reaction of MCA by protease

[Samples]

- Intensity standardization sample: 50 µmol/L AMC solution
- Enzyme solution: 10 nmol/L Trypsin bovine pancreas typeVIII
  50 mmol/L Tris-HCl
  0.15 mol/L NaCl
  1.0 mmol/L $CaCl_2$
  0.1 mg/mL BSA
- Substrate solution: Boc-Gln-Ala-Arg-MCA solution
  (Last concentration: 0.5, 1, 2.5, 5, 10, 20, 40 µmol/L)

Concentration for adjustment (µmol/L)	Last concentration (µmol/L)
240	40.0
120	20.0
60	10.0
30	5.0
15	2.5
6	1.0
3	0.5

[Measurements]

1) Fluorescence-spectrum measurement of AMC

Excitation and the Fluorescence spectrum of 50 µmol/L AMC were measured (Figure 2). It turns out that the maximum emission wavelength from this result is 440 nm.

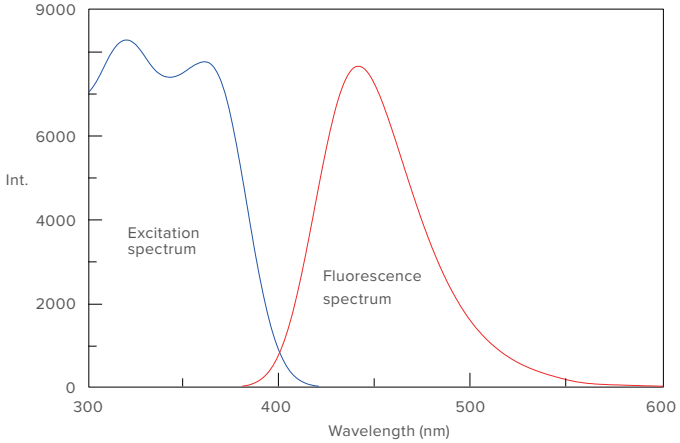


Figure 2. Excitation/Fluorescence spectra of AMC

[Measurement parameters]

Excitation wavelength:	360 nm (fluorescence spectrum)
Emission wavelength:	440 nm (excitation spectrum)
Bandwidth (excitation):	5 nm
Bandwidth (emission):	10 nm
Scanning speed:	500 nm/min.
Data interval:	1 nm
Response:	0.5 sec.
Sensitivity:	200 V

2) Vertical axis switching (into concentration from fluorescence intensity)

Intensity standardization was performed in order to change the vertical axis into the numerical value equivalent to concentration.

50 µmol/L AMC solution of 0.5 mL is dropped at enzyme solution 2.5 mL. Fluorescence intensity of last concentration of 8.333 µmol/L AMC solution was set to 8333 µmol/L AMC solution.

3) Enzyme activity measurement

Substrate solution 0.5 mL of each concentration was dropped at enzyme solution 2.5 mL, and time course measurement of the fluorescence intensity of the isolation AMC was performed to it. The results are shown in Figure 3.

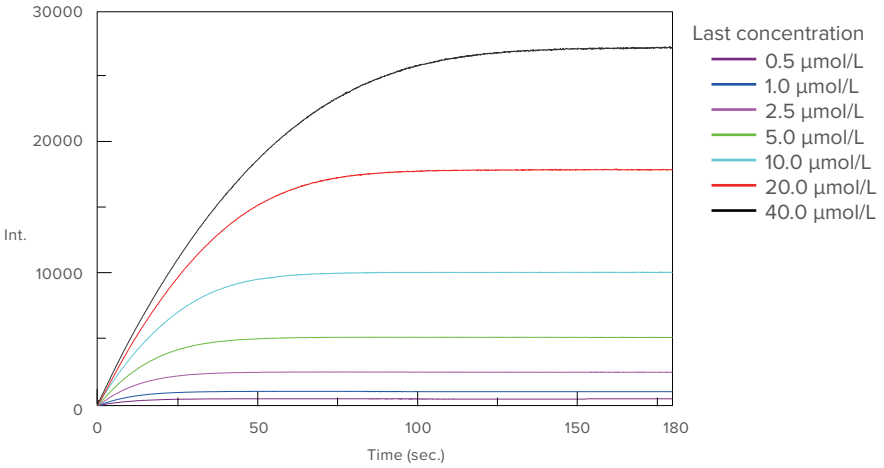


Figure 3 Monitoring results of an enzyme reaction process

[Measurement parameters]

Excitation wavelength:	360 nm
Emission wavelength:	440 nm
Bandwidth (excitation):	5 nm
Bandwidth (emission):	10 nm
Data interval:	0.1 sec.
Response:	0.1 sec.
Sensitivity:	200 V

[Analysis]

Each initial velocity was calculated from inclination of the time variation data of each substrate concentration, and the Lineweaver-Burk plot was performed (Figure 4). K_m=5.99 and V_{max}=35270 nmol/L·min^{-1} were obtained from this result.

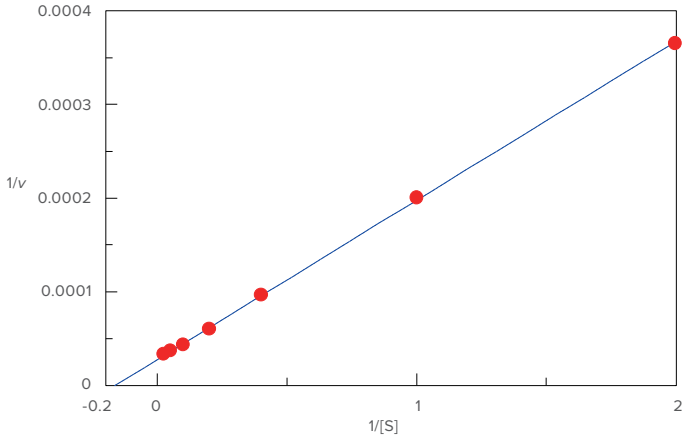


Figure 4. Lineweaver-Burk plot

[Calculation results]

Calculation method: Lineweaver-Burk Plot

V_{max}: 35270

K_m: 5.99

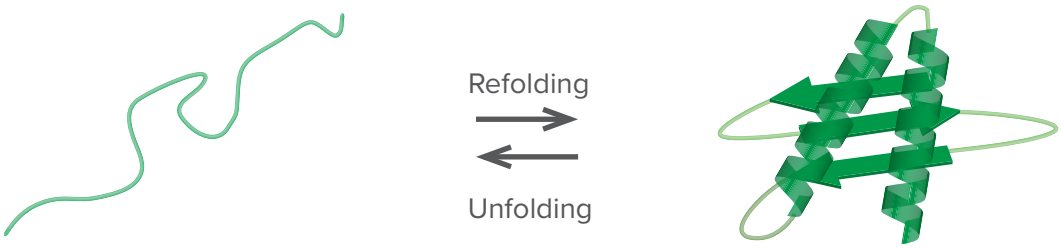
$1/v = 0.000170 \cdot 1/[S] + 0.0000284$

Reaction process monitoring by fluorescence stopped-flow system

Stopped-flow is the technique for monitoring the fast chemical reaction on millisecond time scale. After mixing more than two solutions quickly, it monitors the reaction process through spectroscopies (fluorescence, circular dichroism or absorption). The measurement examples by using stopped-flow system are as below.

Synthesis of organic/inorganic compound
- Property evaluation of novel metal complex and organic compound (porphyrins, fullerenes etc.)
- Reaction process analysis of active oxygen, free radical, and anti-oxidative substance
- Drug development, polymerization reaction

Biochemistry
- Analysis of enzyme catalyst reaction and folding reaction process of protein
- Analysis of interaction between ligand and protein/DNA
- Reaction evaluation of artificial heme protein



Denatured state　　Native state

JASCO's stopped-flow system gives the time course data with high accuracy, and can calculate the reaction rate by fitting the following reaction rate equation to the obtained data.

1-step reaction

A → B　　$Y(t) = a \cdot exp\left(-\frac{t}{\tau}\right)$　　(1)

2-step reaction

A → B → C　　$Y(t) = a \cdot exp\left(-\frac{t}{\tau_1}\right) + b \cdot exp\left(-\frac{t}{\tau_2}\right)$　　(2)

$Y(t)$: measured value
t: time
τ, τ_1, τ_2: time constants
a, b: constants

Acid unfolding of horse cytochrome C measured with a fluorescence stopped-flow system

The fluorescence characteristics of the tryptophan residue in proteins will vary depending on the structures surrounding the residue. This characteristic of cytochrome C is derived from the tryptophan in the residue position 59. The natural state of this tryptophan residue is so close to the Heme iron residue that the fluorescence is quenched by nonradiative energy transfer to the Heme iron. When Cytochrome C is denatured by an acid, the distance between the tryptophan and Heme iron changes and the fluorescence intensity enlarges.

This section introduces the measurement example of the change in fluorescence intensity by the acid denaturation of Cytochrome C as measured by the JASCO stopped-flow measurement system.

As shown in figure 1, the fluorescence intensity has been changed extremely according to the acid denaturation of Cytochrome C. The calculated range was 35 to 5000 msec and a 2-step reaction mechanism was applied for the calculation. The calculated results show an excellent fit to the experimental data, and the reaction rate constants of first step and second step are 433 msec. and 3611 msec., respectively.

[Sample]

- Syringe 1: 10mL, 0.5 mg/mL Cytochrome C
- Syringe 2: 10mL, 0.1 N sulfuric acid

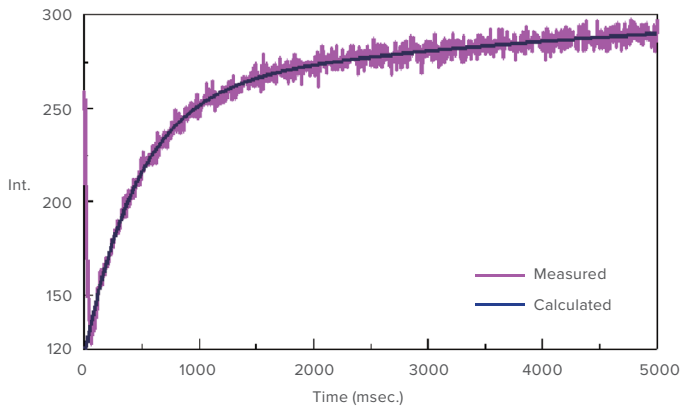


Figure 1. Fluorescence intensity monitoring for the denaturation of Cytochrome C

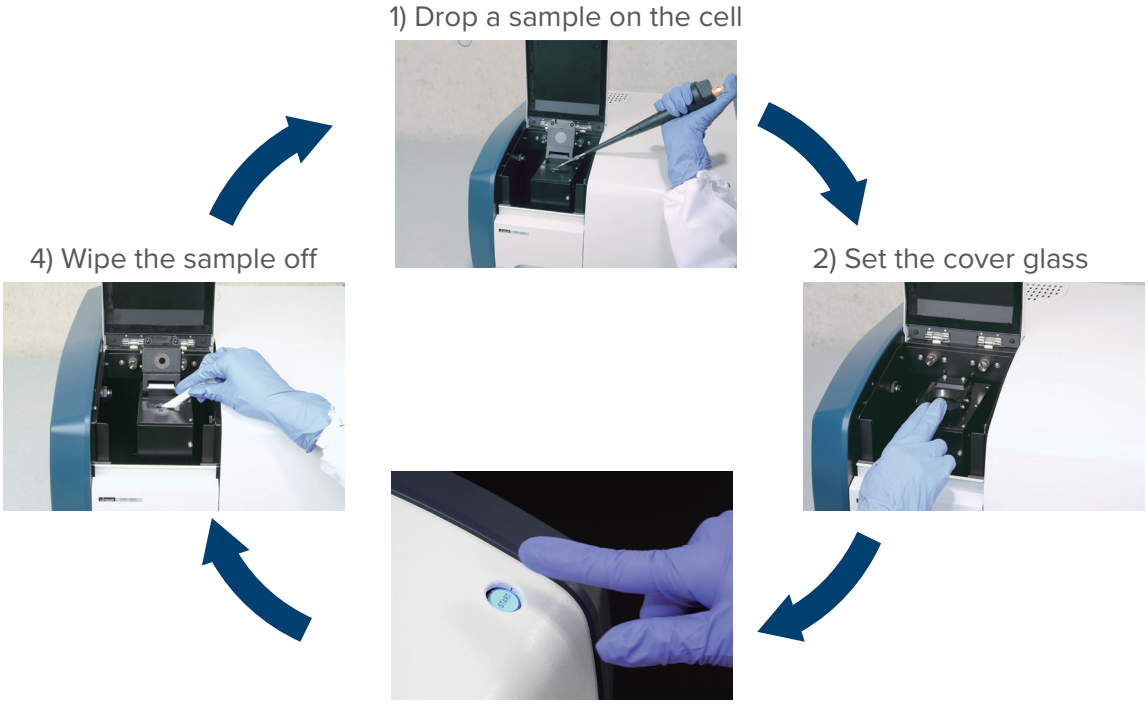
[Measurement parameters]

Excitation wavelength:	280 nm
Emission wavelength:	340 nm
Bandwidth (excitation):	5 nm
Bandwidth (emission):	5 nm
Data interval:	5 msec.
Response:	2 sec.
Sensitivity:	Manual
Accumulation:	4
Flow time:	35 msec.
Mixing ratio:	S1 : S2 = 1 : 1
Flow volume:	S1, S2: 100 µL
Start data acquisition:	35 msec. before flow time ends

Calculation range:	35–5000 msec.
Reaction rate formula:	$Y(t) = -142.6 \times \exp(-t/432.8) + -47.7112 \times \exp(-t/3611)$
Step 1 time constant:	432.8 msec.
Step 1 rate constant:	0.002310 msec.$^{-1}$
Step 2 time constant:	3611 msec.
Step 2 rate constant:	0.0002769 msec.$^{-1}$

Quantitative analysis of λDNA using One drop accessory

One drop attachment is a microsampling accessory that enables fluorescence measurements to be obtained with only 5 µL of sample. It is the most suitable and effective for quantitative analysis of multiple samples such as fluorescently-labeled DNA and various kinds of fluorescent dye.



This section demonstrates the use of the One Drop accessory to obtain fluorescence data and create a calibration curve of λDNA labeled with PicoGreen®.

[Results]

The fluorescence spectra of λDNA for a variety of concentrations is shown in Figure 1, and indicates that the maximum emission wavelength is 523 nm.

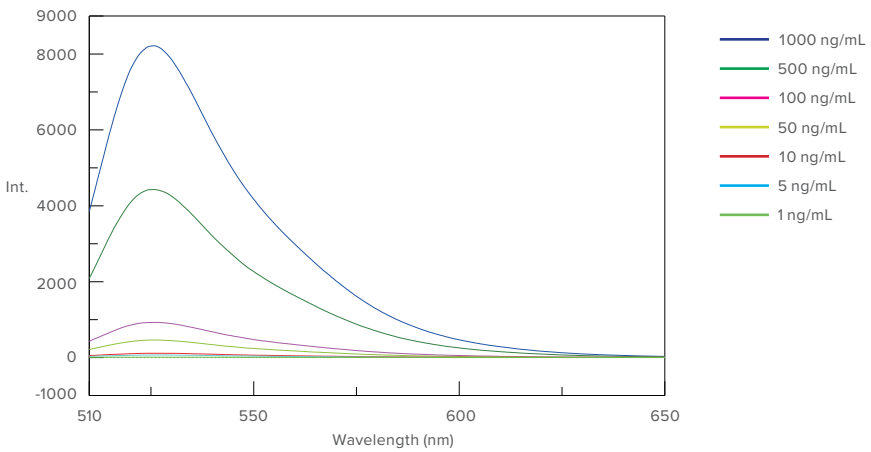


Figure 1. Fluorescence spectra of λDNA labeled with PicoGreen®

The fluorescence intensity at the maximum emission wavelength (523 nm) was then measured five times for each sample to ensure measurement reproducibility. Table 1 shows these measurement reproducibility results for each sample concentration.

Table 1. Measurement reproducibility results

Conc. (ng/mL)	1	2	3	4	5	Ave.	S.D.	C.V (%)
0	53.3	52.4	55.9	53.1	55.2	54.0	1.49	2.8
1	63.6	68.1	65.9	66.5	65.1	65.8	1.68	2.6
5	110.3	106.7	105.1	104.0	110.0	107.2	2.86	2.7
10	157.6	155.5	156.1	153.0	151.7	154.8	2.39	1.5
50	447.1	465.3	460.2	455.8	469.0	459.5	8.56	1.9
100	865.9	856.9	848.3	850.6	853.9	855.1	6.86	0.8
500	3842.7	3831.0	3858.0	3828.9	3811.0	3834.3	17.42	0.5
1000	7766.2	7992.1	7925.3	7972.8	7805.7	7892.4	101.15	1.3

[Measurement parameters]

Measurement mode:	Emission		
Excitation wavelength:	480 nm	Emssion wavelength:	523 nm
Bandwidth (excitation):	20 nm	Bandwidth (emission):	20 nm
Response:	1 sec.	Sensitivity:	620 V

A calibration curve was then created using the average value for each concentration from the reproducibility results in Table 1 and is shown in Figure 2. The correlation coefficient is 0.9999 and the standard error is 6.819, indicating a good fit.

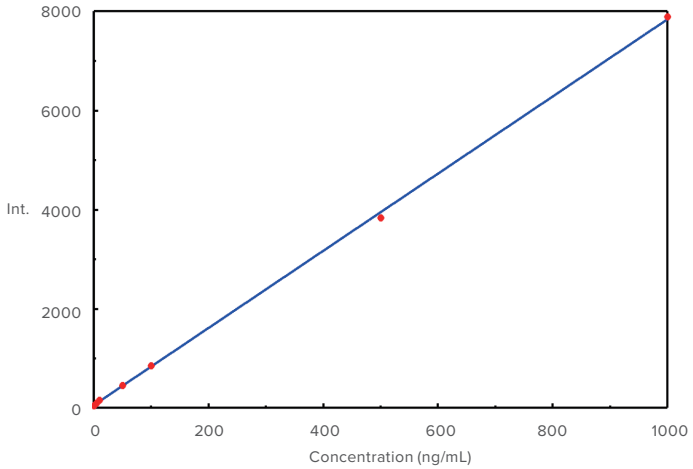


Figure 2. Calibration curve of λDNA labeled with PicoGreen®

Calibration curve equation :	Int.= 7.780 × Conc.+ 57.5211
Correlation coefficient :	0.9999
Standard error :	6.819

Fluorescence quantum yield measurement

Fluorescence quantum yield is defined as the ratio of the number of photons emitted from sample as fluorescence to the number of photons in the excitation light absorbed. Absolute method and relative method are known as measuring methods.

Relative method

Relative method can calculate quantum yield of unknown sample by comparing the intensity of standard fluorescence with unknown sample, and accordingly, the relative method is easier to get the results of quantum yield.

[Calculation method]

In order to calculate relative quantum yield, the following parameters are required.

(1)Quantum yield of standard sample: Φ_{st}

(2)Absorbances at excitation wavelength for the standard sample (st) and the unknown sample (x): A_{st}, A_x

(3)Areas of fluorescence spectrum with spectral correction for the standard sample (st) and the unknown sample (x): F_{st}, F_x

(4)Average refractive index values in the wavelength range to calculate the area of fluorescence spectrum for the standard sample (st) and the unknown sample (x): n_{st}, n_x
*) Its information is required when the solvent of the standard sample is different from that of unknown sample

(5)Dilution ratio for the standard sample (st) and the unknown sample (x) respectively: D_{st}, D_x
***)** Its information is required when the standard or unknown samples for fluorescence spectrum measurement are diluted

By using the parameters above, relative quantum yield of unknown sample, Φ_x is shown by the following equation.

$$\emptyset_x = \emptyset_{st} \cdot \left(\frac{A_{st}}{A_x}\right) \cdot \left(\frac{F_x}{F_{st}}\right) \cdot \left(\frac{{n_x}^2}{{n_{st}}^2}\right) \cdot \left(\frac{D_x}{D_{st}}\right) \qquad (1)$$

Absolute method

The absolute method allows to detect all the fluorescence from the sample and integrates using integrating sphere. Therefore, its method enables more accurate quantum yield measurement.

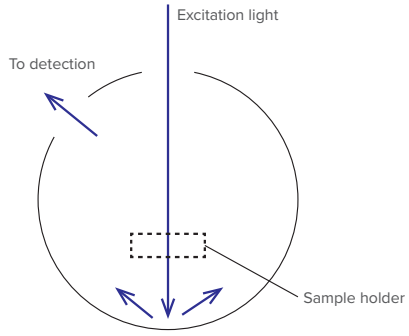
[Measurement method]

(1)Measuring incident light

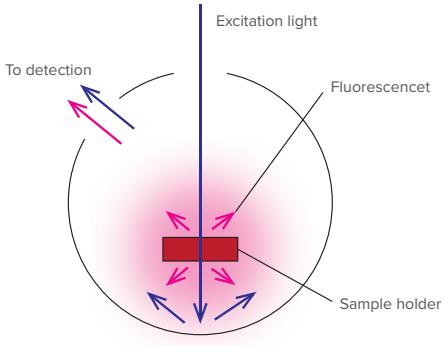
Set the cell with solvent on the sample cell holder in integrating sphere, and measure spectrum of incident light (In case of the solid sample, confirm nothing is set on the sample cell holder in the integrating sphere, and measure spectrum of the incident light). Obtained peak area is defined as area from incident light, S_0 (equivalent number of photons in the incident light).

(2)Measuring sample

Set the sample on the sample holder, and measure scattering and fluorescence spectra of the sample. Obtained excitation wavelength peak area is defined as area scattered from sample, S_1 (equivalent number of photons which were not absorbed), and peak area in the emission wavelength range is defined as area emitted from sample, S_2.



Measuring incident light

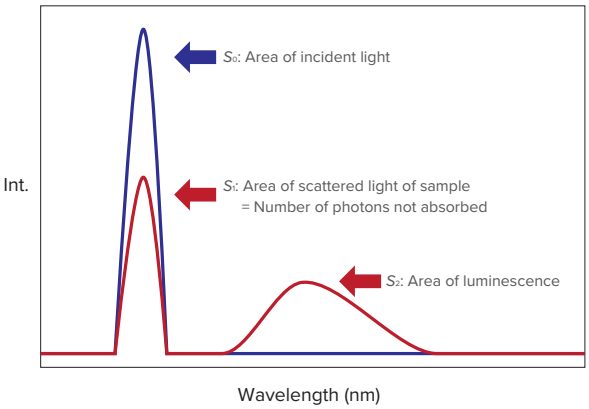


Measuring sample

(3) Calculating quantum yield

Calculate in accordance with the following.

- Sample absorption (%) = $(S_0 - S_1)/S_0 \times 100$
- External quantum yield (%) = $S_2/S_0 \times 100$
- Internal quantum yield (%) = $S_2/(S_0 - S_1) \times 100$



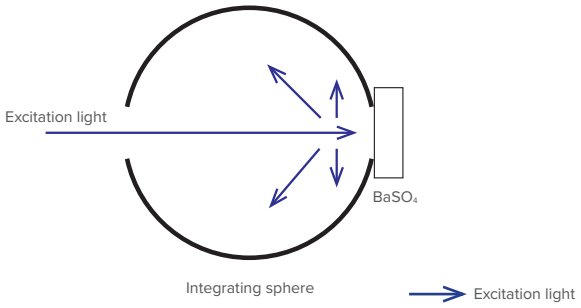
JASCO can provide the fluorescence quantum yield measurement system (relative method or absolute method), and also propose the proper solution to meet the user's requirement.

Internal quantum efficiency measurement of phosphor powders

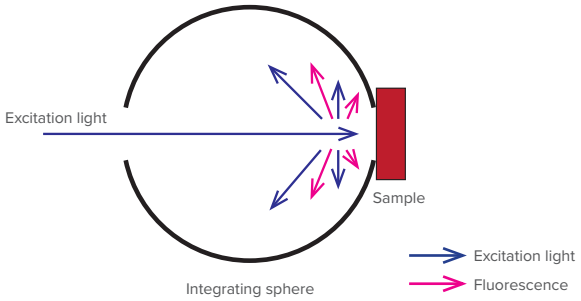
Some white LEDs consist of two types of materials: a luminescent diode that emits a blue light in the near-UV region, and a phosphor that emits visible light from the absorption to the near-UV region. Determining a phosphor's internal quantum efficiency is an important parameter in evaluating the emission efficiency of a white LED, and the standards about its evaluation method (JIS R1697/ISO 20351) have been published. This section illustrates the measurement reproducibility of the internal quantum efficiency of two phosphor powders from the evaluation of a white LED by using the dedicated system which is compliant with JIS R 1697 /ISO 20351.

[Measurement procedure]

1) Fill $BaSO_4$ into the cell, and measure the incident light spectrum.



2) Fill the sample into the cell, and measure the spectrum that includes excitation/scattering light and fluorescence.



3) Perform the spectrum correction by using calibrated light source, and apply it to measured spectra.

[Samples]

- Yellow phosphor, red phosphor

[Measurement parameters]

Excitation wavelength:	455 nm
Measurement range:	380–780 nm
Bandwidth (excitation):	5 nm
Bandwidth (emission):	5 nm
Scanning speed:	500 nm/min.
Response:	0.1 sec.
Sensitivity:	355 V

[Results]

To confirm reproducibility, five repeat measurements were made for each sample (by refilling the sample cell). High reproducibility was obtained for both phosphor measurements as indicated by the difference between the maximum and minimum values of the internal quantum efficiency (within 1.5% with a coefficient of variance of 0.6%).

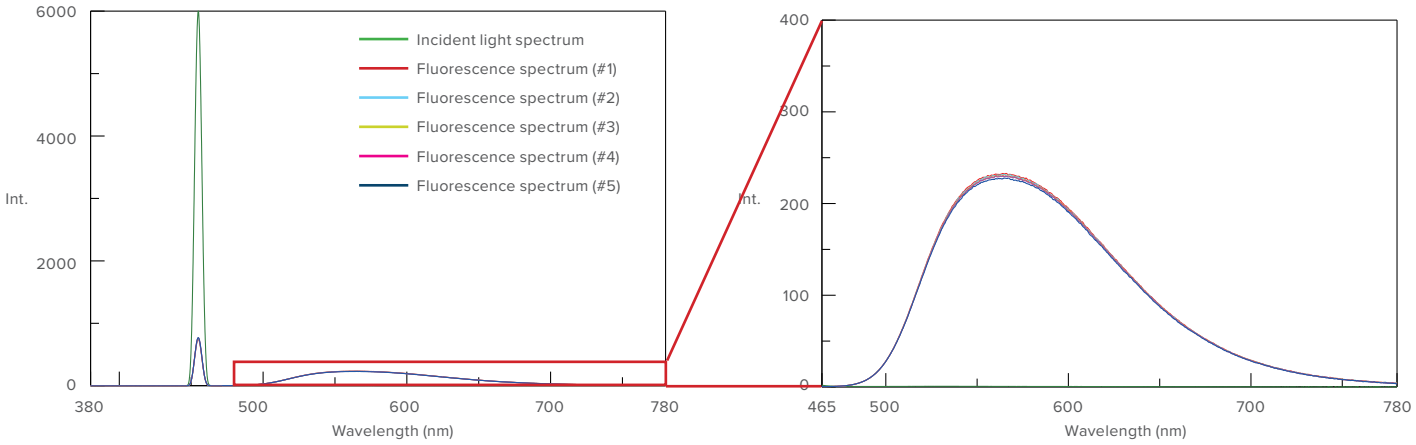


Figure 1. Fluorescence spectra of yellow phosphor

Table 1. Calculated results of internal quantum efficiency (yellow phosphor)

	1	2	3	4	5	Ave.	S.D.	C.V.
Light volume absorbed by sample	87.6 %	87.4 %	87.3 %	87.2 %	87.1 %	87.3 %	0.19	0.22
External quantum efficiency	80.7 %	80.3 %	79.9 %	79.7 %	79.0 %	79.9 %	0.64	0.80
Internal quantum efficiency	92.1 %	91.9 %	91.6 %	91.4 %	90.7 %	91.5 %	0.54	0.59

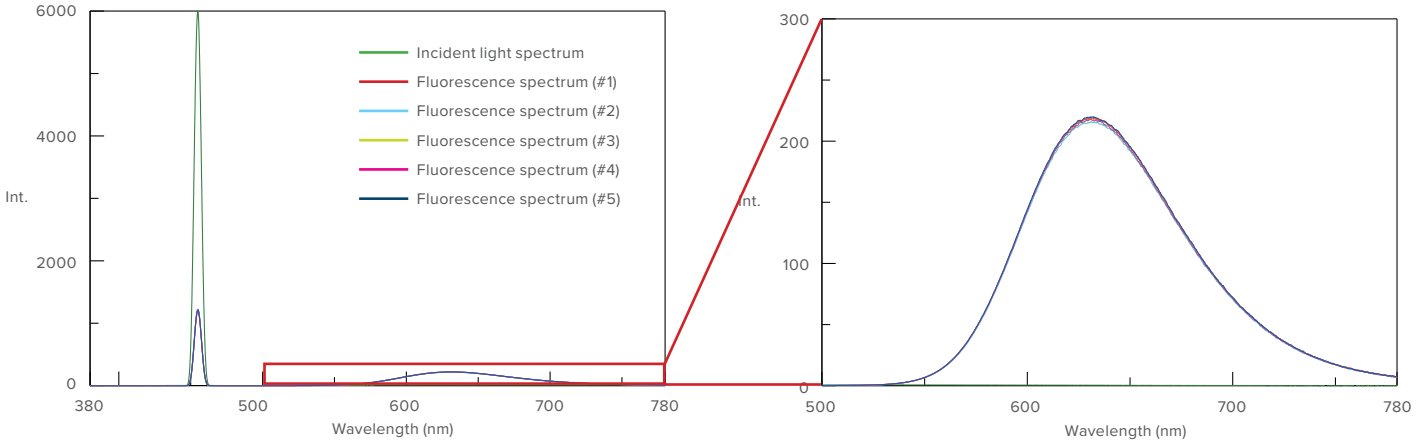


Figure 2. Fluorescence spectra of red phosphor

Table 2. Calculated results of internal quantum efficiency (red phosphor)

	1	2	3	4	5	Ave.	S.D.	C.V.
Light volume absorbed by sample	78.2 %	77.6 %	77.9 %	77.8 %	77.9 %	77.9 %	0.22	0.28
External quantum efficiency	65.8 %	64.9 %	65.9 %	65.4 %	66.1 %	65.6 %	0.48	0.73
Internal quantum efficiency	84.1 %	83.6 %	84.6 %	84.1 %	84.9 %	84.3 %	0.50	0.60

Phosphorescence measurement

Light emission as a result of photo-excitation can occur partially in the form of fluorescence and partially as phosphorescence. Phosphorescence occurs when an intersystem crossing occurs, and electrons cannot return directly to the ground state, but instead make a transition to an intermediate state. They then relax slowly to the ground state, resulting in phosphorescence. Fluorescence occurs on a timescale of nanoseconds, whereas phosphorescence can continue for much longer periods. When electronic transitions occur in a substance, electron pairs generally have opposite spins (Pauli's exclusion principle). However, when an intersystem crossing occurs, the spins are the same. Since this is a forbidden state, relaxation to the ground state is slow.

When measuring phosphorescence substance by spectrofluorometer, the sample is irradiated with excitation light, and then the light after cutting the excitation light should be observed.

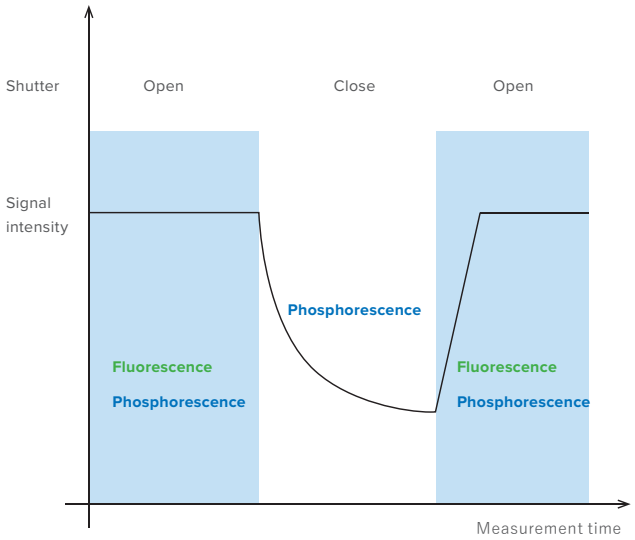


Figure 2. Observed signal when opening/closing the shutter

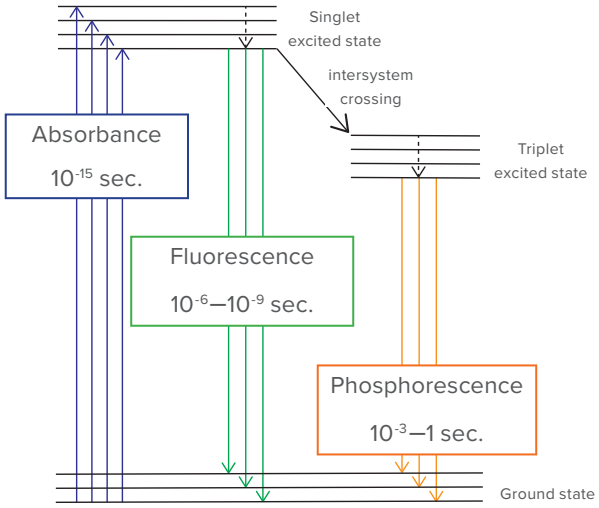


Figure 1. Phosphorescence due to intersystem crossing

Since fluorescence and phosphorescence are mixed while the sample is irradiated with excitation light, the phosphorescence only cannot be observed. Therefore, utilizing the property that lifetime of phosphorescence is longer than the one of fluorescence, cutting the excitation light enables to observe phosphorescence only.

JASCO's spectrofluorometer has the shutter at the monochromator of excitation side, which enables to measure the phosphorescence. Figure 2 shows the signal of fluorescence/phosphorescence when opening/closing the shutter. When opening the shutter, the sample is irradiated with excitation light, and the observed signal is mixture of fluorescence and phosphorescence. When closing the shutter, the phosphorescence only is observed.

This section shows the evaluation examples of phosphorescence.

Phosphorescence quantum yield measurement

Phosphorescent compounds are frequently used as the luminescent layer in organic electro-luminescent devices. Quantum yield measurement of phosphorescence substances is required for developing such materials. JASCO developed a new dedicated system for calculating quantum yield from the measured phosphorescence spectra at 77K. A dedicated system was evaluated by using benzophenone which is a representative phosphorescence substance. Figure 3 illustrates the measured spectra and Table 1 shows the calculation results of quantum yield (φ). The calculated phosphorescence quantum yield was 0.93 that corresponds well with the literature-based value of 0.9[1].

[1] The chemical society of Japan, Courses in Experimental Chemistry 3 basic physical chemistry, Maruzen ISBN: 4-621-07303-6

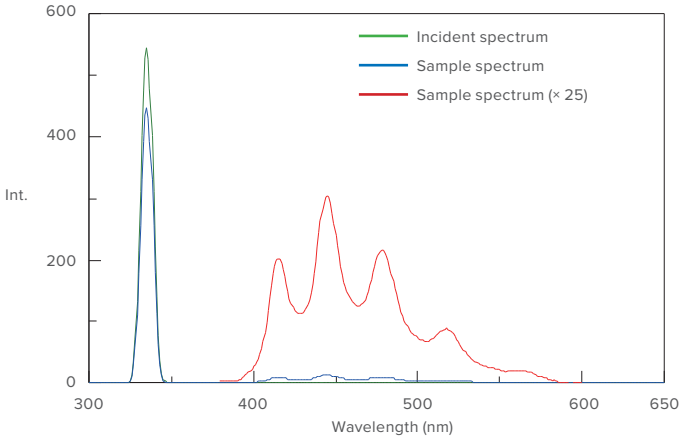


Figure 3. Spectra of benzophenone

Table 1. Phosphorescence quantum yield of benzophenone

	Int.
Incident photon number: S_0	4954.3
Photon number unabsorbed by sample: S_1	4074.0
Photon number emitted by sample: S_2	819.3
Quantum yield [Measured]	0.93
Quantum yield [Literature]	0.90

Detection of Phosphorescence from Singlet Oxygen using NIR Spectrofluorometer

Singlet oxygen is highly active and destroys biological molecules. Recently, its use for destroying cancer cells or bacteria has been researched. Herein, this section shows measurements of phosphorescence at 1270 nm emitted by singlet oxygen using a near-infrared spectrofluorometer with Eosin Y as a photosensitizer.

The excitation-emission matrix measurement of singlet oxygen reveals a suitable excitation wavelength as well as whether the singlet oxygen has been generated. Near-infrared spectrofluorometer is effective tool for research of singlet oxygen.

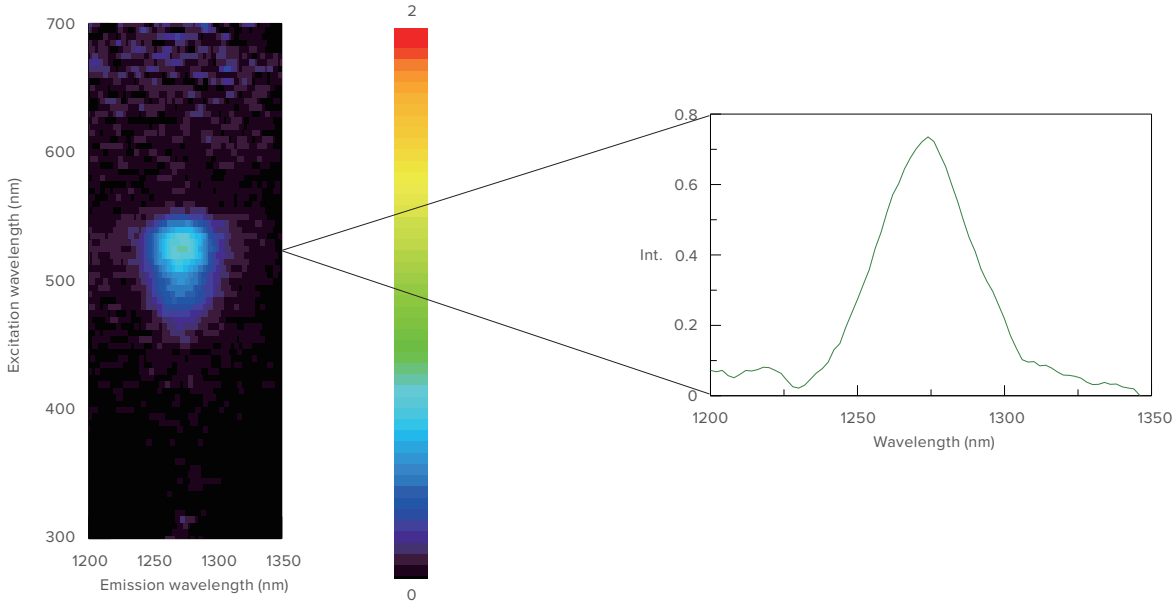


Figure 4. Excitation-emission matrix and fluorescence spectra (excitation wavelength: 525 nm) of singlet oxygen.

Acknowledgment

Special thanks to associate professor Takunori Harada (Department of Integrated Science and Technology, Faculty of Science and Technology, Division of Applied Chemistry, Oita University) who provided the sample.

Remote fluorescence measurement using optical fiber

JASCO has various accessories for supporting the user's measurement, and these accessories can be mounted to spectrofluorometer easily. One of JASCO's accessories is optical fiber interface, which enables the fluorescence measurement by holding the probe close to or immersed in the sample. A spectrofluorometer with optical fiber can be used for a variety of measurements, such as when the sample is larger than the sample chamber, to follow an in-situ reaction, or for measurement in hazardous environments, such as high/low temperature and high pressure. This section shows the measurement example by using spectrofluorometer with optical fiber.

[Measurement]

Fluorescence measurements of fluorescent reflector (red/yellow) were performed by using spectrofluorometer, optical fiber and dark chamber. The spectral correction was also performed by using the reflected light of the calibrated WI lamp from standard white plate.

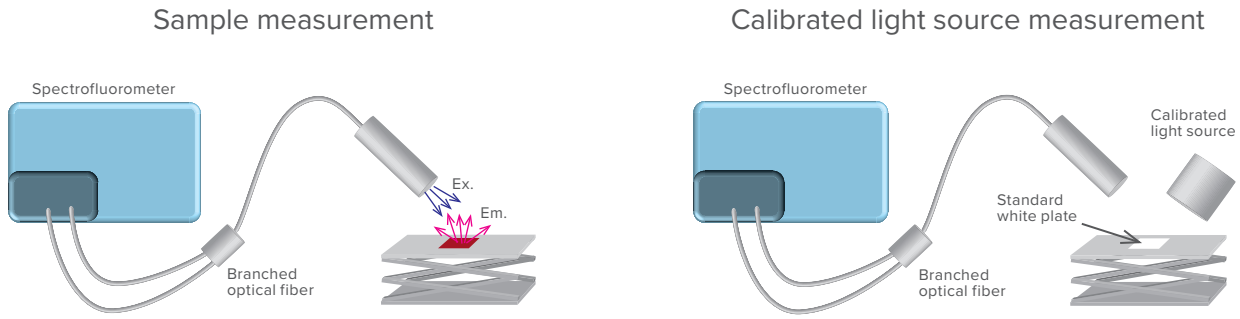


Figure 1. Overview of measurement system

Figures 2 shows the measurement results. The profiles of the spectra of each colored sample measured using both the optical fiber and film holder were consistent with each other.

This system is effective in the case such as the measurement of a sample which is larger than sample chamber and the measurement under the severe environment.

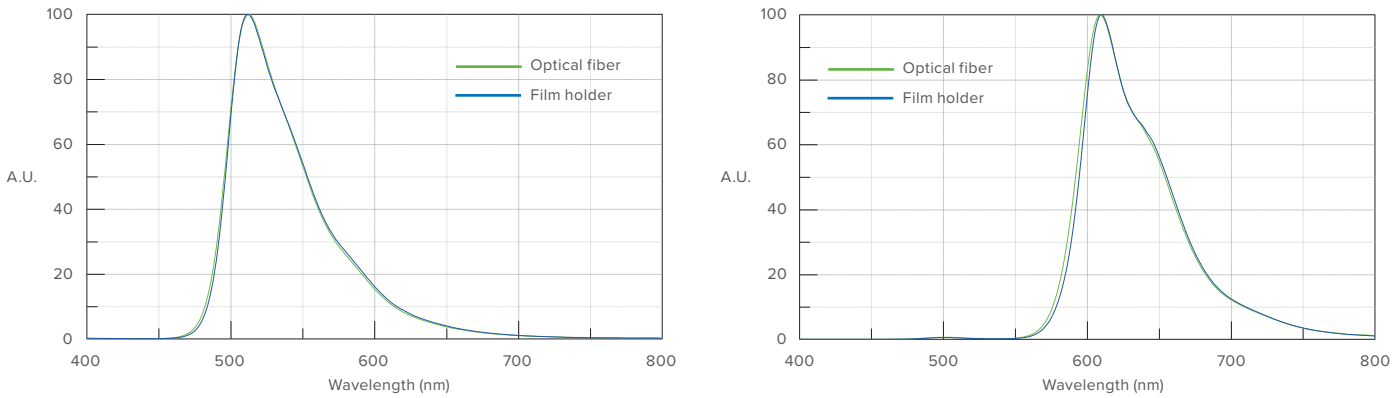
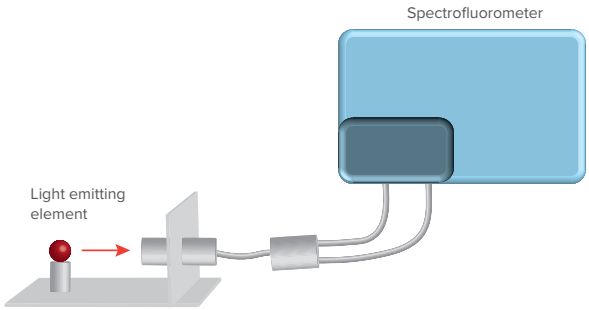
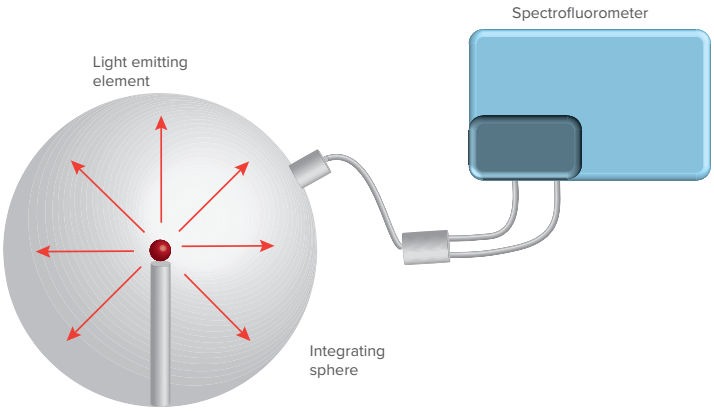


Figure 2. Peak normalized fluorescence spectrum of the yellow fluorescent reflector (left) and the red fluorescent reflector (right), excitation wavelength: 310 nm

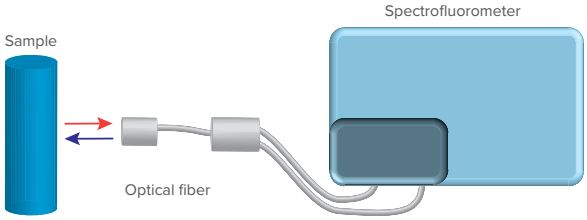
The solution examples by using optical fiber are shown as below.



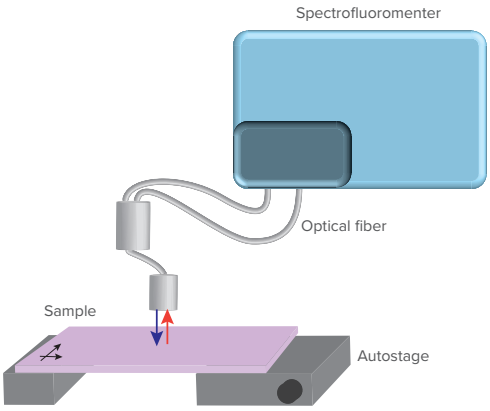
Lamp evaluation (oriented type)



Lamp evaluation (Omnidirectional type)

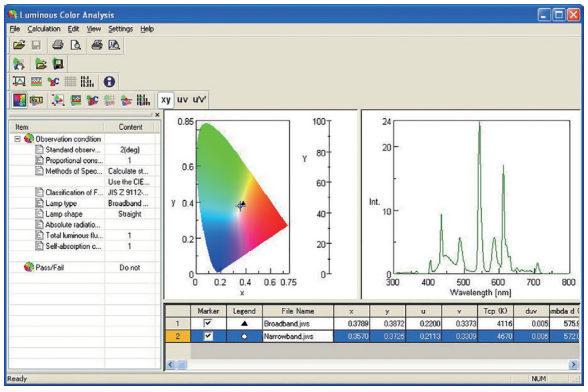


In-situ measurement



Mapping measurement

In addition, JASCO can provide the useful optional software to convert the luminous/rendering color into numerical values and to evaluate them quantitatively. It has the calculation function of each color model which CIE (International Commission on Illumination) has published, and is also compliant with JIS regulation (color specification). Since it includes pass/fail evaluation function, it will be powerful tool to manage the luminous color of product.

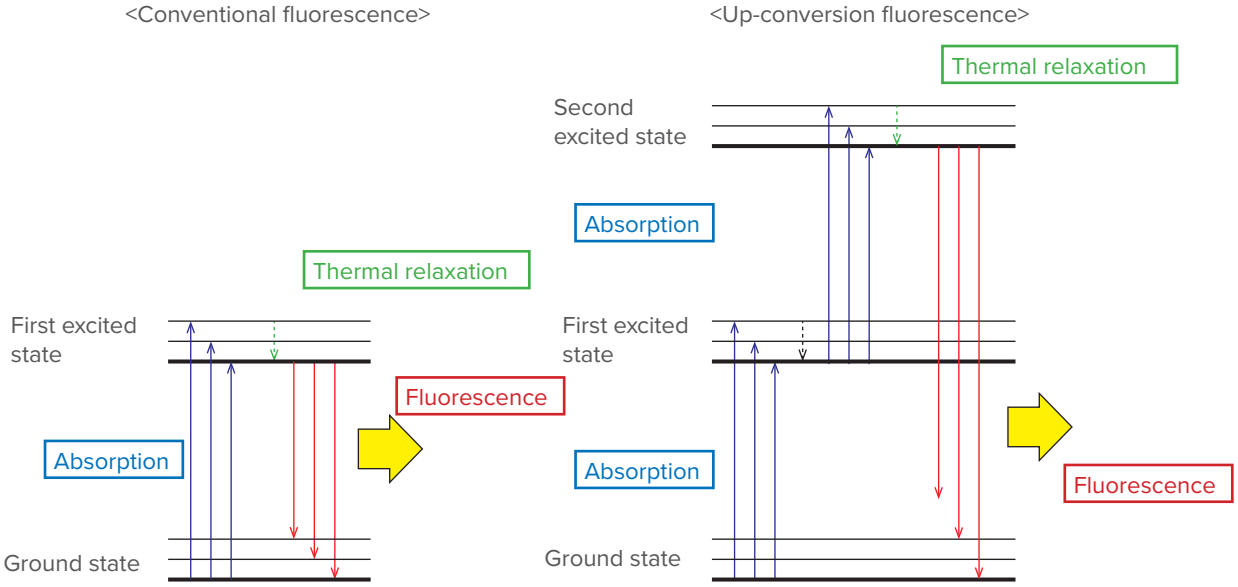


[Luminous Color Analysis] program

Evaluation of up-conversion phosphor

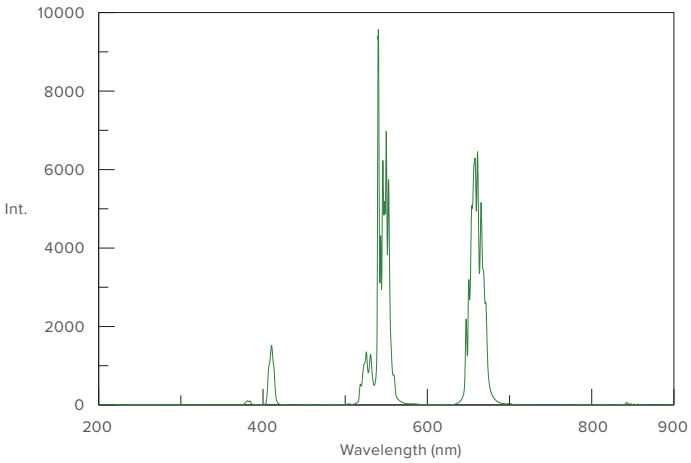
Generally, fluorescence appears on the long wavelength side, which means that its energy is lower than the one of excitation light (the light absorbed by the sample). On the other hand, in the case of up-converting phosphor, the energy of fluorescence is higher than the one of excitation light because two or more photons involves its reaction, and the fluorescence appears on the short wavelength side. This is particularly useful for evaluating labels used for biological imaging, materials in infrared solar cells and development of inks for anti-counterfeiting.

JASCO has developed the up-conversion measurement system, which can detect very small fluorescence by using laser. This section shows the measurement examples of the up-conversion phosphors.



Mechanism of up-conversion fluorescence

The fluorescence at visible light region could be observed when irradiating 980 nm light which the human's eye cannot observe.



Our up-conversion fluorescence measurement system with 980 nm near-IR laser could observe the up-conversion fluorescence.

Up-conversion quantum yield measurement of phosphors with heavy rare earth element

[Samples]

- YTa_7O_{19}: Er_{10}, Yb_{40}

[Measurement parameters]

Excitation wavelength:	980 nm
Bandwidth (emission):	5 nm
Scanning speed:	1000 nm/min.
Data interval:	0.2 nm
Response:	0.2 sec.
Laser output:	150 mW

Each sample was measured three times to evaluate the measurement reproducibility. The three measurements are overlaid and shown in different colors in Figures 1. Table 1 shows the quantum yield measurement result. This result demonstrates that the up-conversion system can evaluate quantum yields at levels of less than 1%. Since the quantum yield of up-conversion phosphor is generally very low, combining laser source with spectrofluorometer enables to observe very weak fluorescence. This dedicated system is expected to be effective tools for studying the excitation process of up-conversion phosphor.

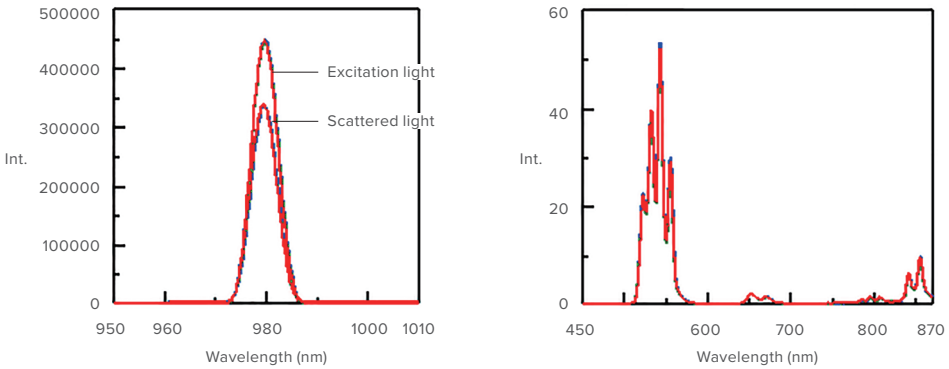


Figure 1. The scattered light spectra and the fluorescence spectra (Blue: first, Green: second, Red: third)

Table 1. Result of internal quantum yield

	Area of Excitation light	Area of Scattered light	Area of Absorption	Area of Fluorescence	Sample absorbance (%)	Internal quantum yield (%)
1	2.80E+06	2.14E+06	663270	1453.86	23.69	0.22
2	2.73E+06	2.14E+06	587840	1378.84	21.57	0.23
3	2.83E+06	2.14E+06	687620	1433.80	24.28	0.21

Acknowledgment

Data courtesy of Dr. Kouji Tomita, Department of Chemistry, School of Science, Tokai University

Evaluation of environmental water using PARAFAC

Recently, excitation-emission matrix (EEM) measurements and parallel factor (PARAFAC) analyses have been widely used for evaluating the quality of environmental water, such as in rivers and lakes.

Such water often contains compounds such as humic acid, tryptophan, and artificial chemicals. Therefore, multiple overlapping peaks appear in the EEM. PARAFAC is a very powerful analysis method in such situations and can separate the principal components of EEM. It can be used to predict the compounds present by comparing with published EEM. It can also be used for quantitative analysis.

In this section, a PARAFAC analysis of EEM for river water is described.

[Samples]

30 water samples (3 samples each at 10 locations shown in figure)

[Measurement parameters]

Excitation range:	250–450 nm
Emission range:	260–700 nm
Bandwidth (excitation):	5 nm
Bandwidth (emission):	5 nm
Excitation step:	5 nm
Scanning speed:	1000 nm/min.
Response:	50 msec.
Sensitivity:	Medium

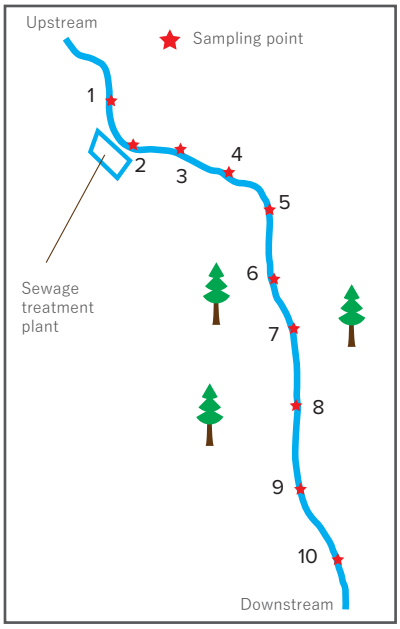


Figure 1 Schematic diagram of sampling points

[Results]

Representative EEM for each sampling point are shown in figure 2.

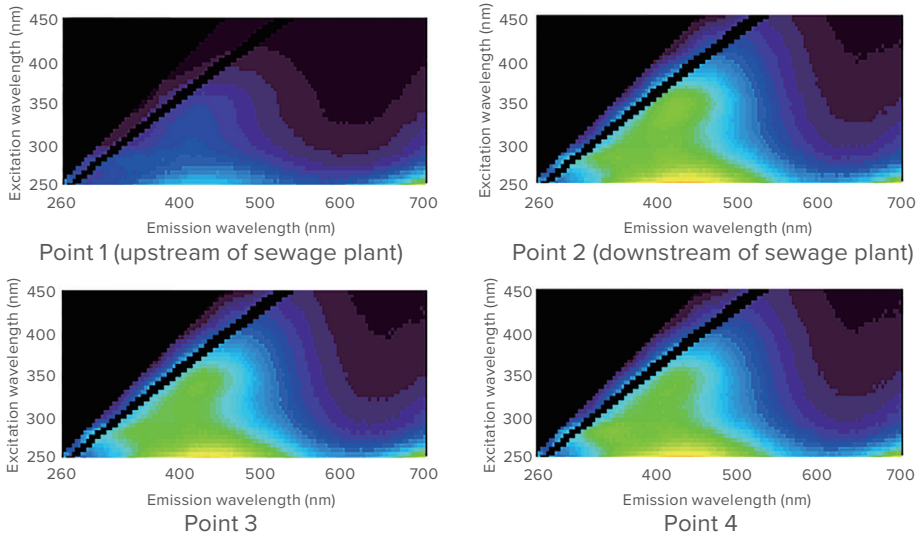


Figure 2. EEM for each sampling point

A PARAFAC analysis was applied to 30 sets of EEM. When the number of components was set to 4, the results of PARAFAC analysis were as shown in figure 3.

Based on the literature[1], components 1 and 3 appear to be humic acid-like compounds and component 2 appears to be tryptophan. Component 4 is a residual error. Score plots for the different components are shown below.

[1] Chen. W. *et.al. Environ. Sci. Tech.* **2003**, 37, 5701-5710.

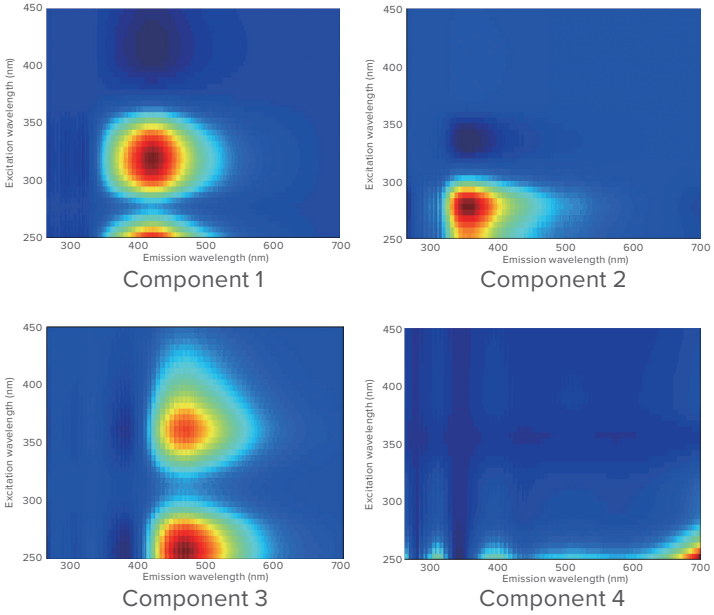


Figure 3. Components determined by PARAFAC (assuming 4 components)

Clustering based on the sampling points can be seen. The results indicate a sudden increase (about 2.5 to 3 times) in the concentration of humic acid-like compounds and tryptophan downstream of the sewage treatment plant, but then no rapid increase between points 2 and 5.

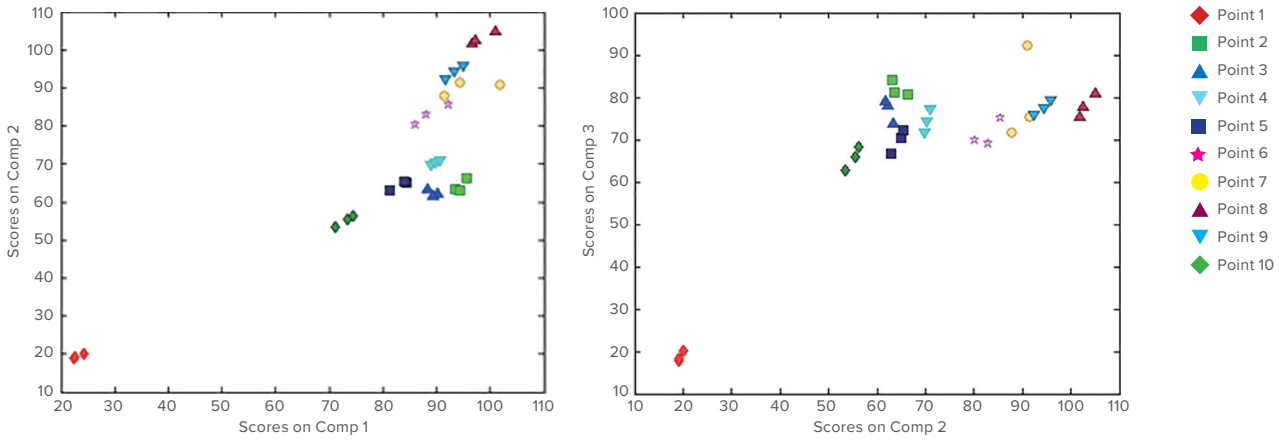


Figure 4. Score plots

Acknowledgment

Special thanks to Prof. K. Sankoda (Saitama University) who provided the sample.

High-Speed Excitation Emission Matrix Measurement of Olive Oil

Recently, Excitation-Emission Matrix (EEM) is drawing attention as the method for determining the type, quality, and geographical origin of food products. To firmly establish EEM as a reliable method for this type of analysis, more references are required, and therefore many more samples should be measured (from hundreds to thousands).

In order to increase sample throughput, a system has been developed for high-speed EEM measurement using the spectrofluorometer coupled with a multi-channel spectral detector. To demonstrate the ease-of-use of this high-speed measurement system, this application note reports the results for the measurement of a variety of olive oil samples. In addition, this note shows the use of EEM interpretation for characterizing the olive oil samples.

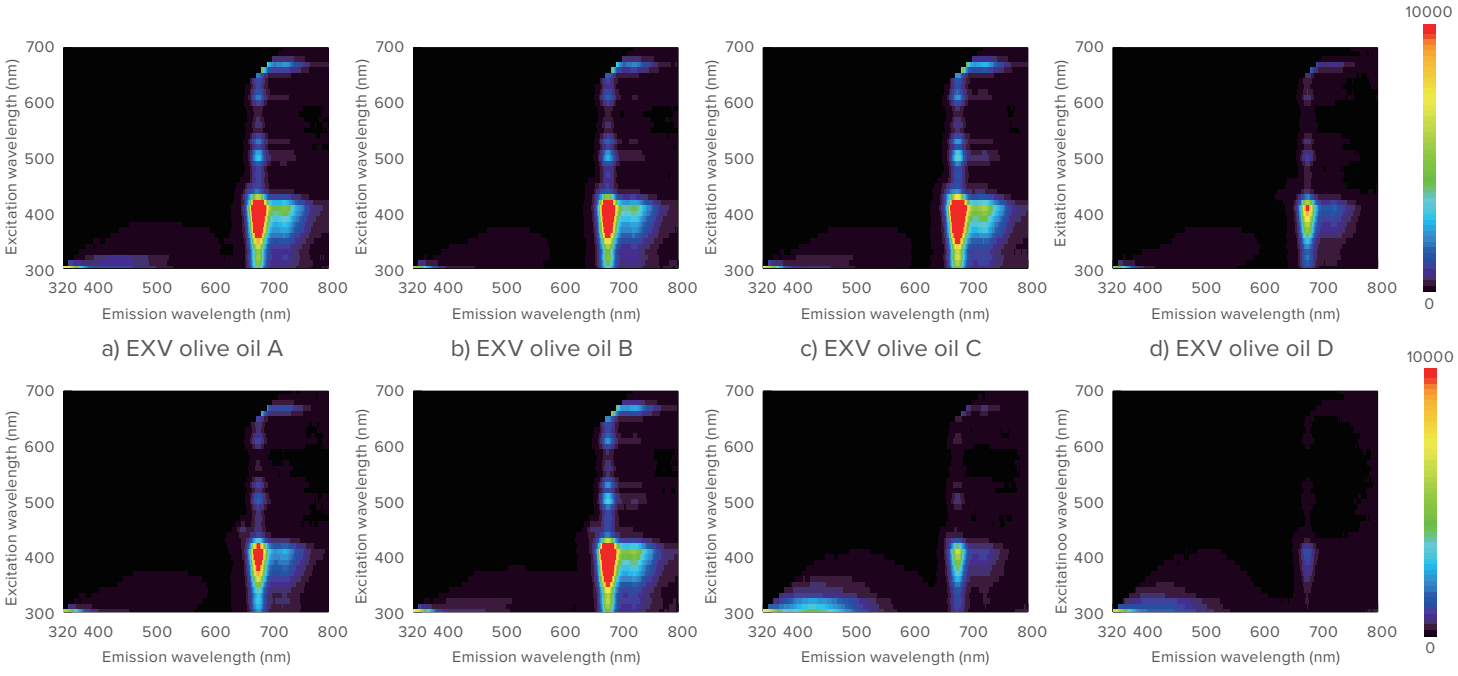
[Samples]

Solutions of extra virgin (A, B, C, D, E, and F) and pure (G and H) olive oil were diluted in hexane at a 1:350 ratio to obtain an absorbance less than 0.02 A.U.

[Results]

EEM after processing are shown in Figure 1. The peak locations in Figure 1 and literature* were used to estimate the components of the samples and are the following: oxidation product (Excitation: 300 to 400 nm, Emission: 320 to 500 nm) and chlorophyll (Excitation: 300 to 700 nm, Emission: 650 to 800 nm).

*Kongbonga YGM, Ghalila H, Onana MB, Majdi Y, Lakhdar ZB, Mezlini H and Ghalila SS, *Food and Nutrition Sciences*, **2**, 692-699, (2011).



a) EXV olive oil A b) EXV olive oil B c) EXV olive oil C d) EXV olive oil D

e) EXV olive oil E f) EXV olive oil F g) Pure olive oil G h) Pure olive oil H

Figure 1. EEM of olive oil samples

Parallel factor analysis (PARAFAC) was performed on the EEM and the number of component spectra was set to 2. Figure 2 shows the component EEM calculated by PARAFAC. The first component EEM is chlorophyll and the second is an oxidation product.

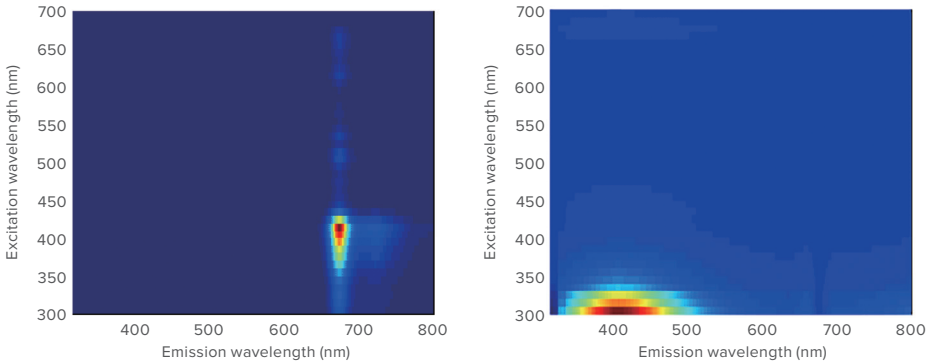


Figure 2. First (left) and second (right) component EEM calculated by PARAFAC.

Figure 3 shows the score plot for the first component (chlorophyll) and the second component (an oxidation product). Regarding the distribution in the extra virgin olive oil, the score for the first component is large while the second is small. However, the distribution in the pure olive oil shows the score of first component is small while the second is large. This trend is related to the production process of the olive oil. Extra virgin olive oil is produced by squeezing and filtering the fruit of the olive without any chemical treatment process, and the acidity of the olive oil is less than 0.8%. On the other hand, pure olive oil is a blend of virgin olive oil and refined olive oil, so the acidity of pure olive oil is less than 1%. In addition, the chlorophyll content of pure olive oil is small because it is reduced during the production process for refining the olive oil.

These results demonstrate JASCO's high-speed fluorescence measurement system and the use of PARAFAC analysis for the interpretation of EEM. PARAFAC was used to extract the component EEM to estimate the compounds from the component EEM. The score plot calculated by PARAFAC can provide information about the characteristics of a sample and is useful for determining sample type and quality.

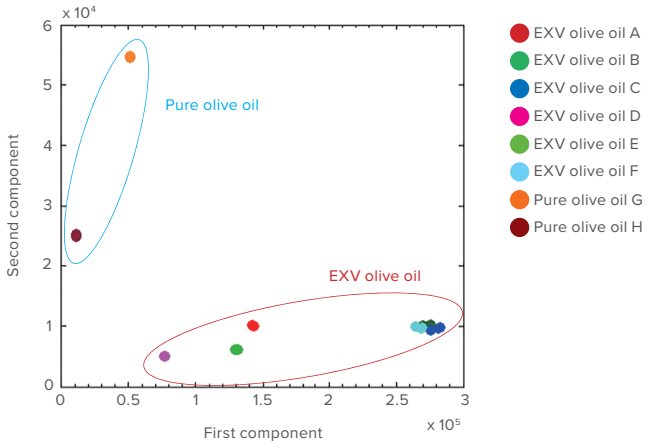


Figure 3. Score plot of the first and second components analyzed by PARAFAC

Petroleum product assessment by counmarin analysis

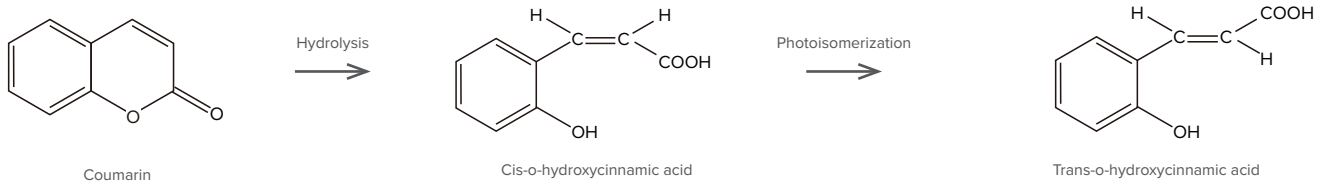
To prevent the production of illegal light diesel oil, which contains kerosene or heavy oil, 1 ppm of coumarin is added to either the kerosene or a heavy oil as a discriminator. The analysis procedure for determining the discriminator and its mixing concentration is standardized by the Japan Petroleum Institute and uses a spectrofluorometer to determine concentration (JPI-5S-71-2010, Petroleum products - Determination of coumarin - Fluorescence spectrometry). Coumarin analysis method is as below;

- Conventional method (B method); usage of reagents is large, and separatory funnel is required for preparation.
- Simplified quantitative method (A method); flat bottom test tube with screw cap is used, and usage of reagents is small.
- Simplified qualitative method: fluorescence colorimeter is used for its method, which examines whether light diesel oil includes coumarin additive oil.

JASCO can provide dedicated coumarin measurement system, which can determine the coumarin concentration without feeling the stress. This section shows the measurement procedure of A method by using dedicated coumarin measurement system

[Principle]

Coumarin is hydrolyzed in alkaline solution and becomes cis-O-hydroxycinnamic acid. The cis-O-hydroxycinnamic acid is then isomerized by ultraviolet radiation and converted to trans-O- hydroxycinnamic acid. Since trans-O-hydroxycinnamic acid fluoresces, the product can be quantified using fluorescence spectroscopy.



Coumarin | Cis-o-hydroxycinnamic acid | Trans-o-hydroxycinnamic acid

Figure 1. Hydrolysis and photoisomerization of coumarin.

[Sample preparation]

(1) Alkaline solution

Adds 10 g of sodium hydroxide and 20 g of sodium nitrate to 100 mL volumetric flask, fills ultrapure water to the calibration line. After dissolving, stores its solution in a polyethylene vessel.

(2) Alcohol solution

Mixes 80 mL of 1-butanol and 60 mL of ethanol.

(3) Coumarin standard stock solution (10,000 mg/L)

Adds 1 g of coumarin to 100 mL volumetric flask, and fills toluene to the calibration line.

(4) Coumarin standard solution (100 mg/L)

Adds 5 mL of coumarin standard stock solution to 500 mL volumetric flask, and fills n-dodecane to the calibration line.

(5) Coumarin standard solution (1 mg/L)

Adds 5 mL of coumarin standard solution (100 mg/L) to 500 mL volumetric flask, and fills n-dodecane to the calibration line.

[Measurement procedure]

The standard samples are mixed in test tubes, according to the specified ratios in Table 1. Shaking the samples, the coumarin is extracted in the alkaline solution, and hydrolyzed. The photoisomerization reaction is induced by irradiating the sample using a spectrofluorometer and an excitation wavelength of 360 nm. The fluorescence intensity is detected at 500 nm and used to generate a calibration curve.

Table 1. Mixing ratios of the standard solution

Additive Concentration (%)	Coumarin Solution (1 mg/L) (mL)	n-dodecane (mL)	Alkaline Solution (mL)	Alcohol Solution (mL)
0.0	0.0	7.0	5.0	8.0
10.0	0.1	6.9	5.0	8.0
40.0	0.4	6.6	5.0	8.0
80.0	0.8	6.2	5.0	8.0
120.0	1.2	5.8	5.0	8.0

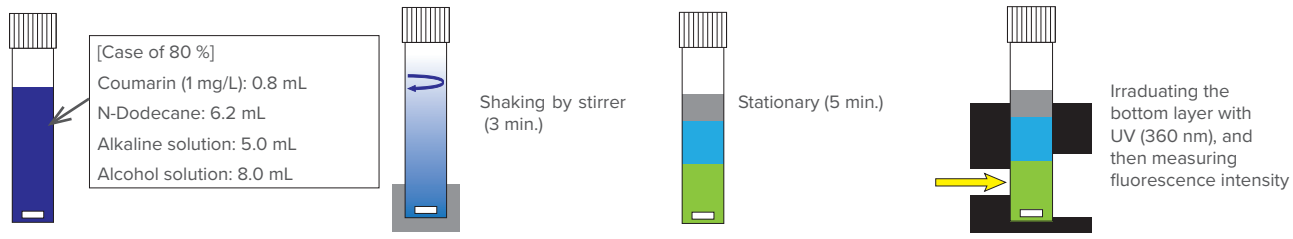
(1) Adds the sample, n-dodecane, alkaline solution, alcohol solution and stirrer bar to the test tube.

(2) Shakes the sample by the stirrer (240 r.p.m., 3 minutes). The coumarin is extracted in the alkaline solution, and hydrolyzed.

(3) After shaking, the sample is kept stationary for 5 minutes to allow for separation of the solution layers (top: light diesel oil, middle: alcohol, bottom; alkaline).

(4) Mounts the test tube to the spectrofluorometer. Stirring the bottom layer (alkaline solution), irradiates its solution with UV light (360 nm) for 5 minutes so that the photoisomerization reaction is induced. After photoisomerization, stops stirring, and measures the fluorescence intensity (excitation wavelength: 360 nm, emission wavelength: 500 nm). And then, generates the calibration curve by using obtained values.

(5) When examining the unknown sample, adds 1 mL of unknown sample, 6 mL of n-dodecane, 5 mL of alkaline solution and 8 mL of alcohol solution to the test tube. Performs the similar processing, and measures the fluorescence intensity (excitation wavelength: 360 nm, emission wavelength: 500 nm). Calculates the coumarin mixing ratio by using calibration curve.



JASCO CORPORATION
2967-5, Ishikawa-machi, Hachioji-shi, Tokyo 192-8537 Japan
Tel: +81-42-649-5177 Fax:+81-42-646-4515 Web: www.jasco.co.jp
Japan

JASCO INTERNATIONAL CO., LTD.
11-10, Myojin-cho 1-chome, Hachioji-shi, Tokyo 192-0046, Japan
Tel: +81-42-649-3247 Fax: +81-42-649-3518 Web: www.jascoint.co.jp/english/
Australia, Hong Kong, India, Indonesia, Korea, Malaysia, New Zealand, Pakistan,
Philippines, Russia and CIS countries, Singapore, Taiwan, Thailand, Vietnam

JASCO INCORPORATED
28600 Mary' s Court, Easton, Maryland 21601, U.S.A.
Tel: +1-410-822-1220 Fax: +1-410-822-7526 Web: www.jascoinc.com
Argentina, Bolivia, Brazil, Canada, Chile, Colombia, Costa Rica, Guatemala, Mexico,
Paraguay, Peru, Puerto Rico, United States of America, Uruguay, Venezuela

JASCO EUROPE S.R.L.
Via Luigi Cadorna 1, 23894 Cremella (LC), Italy
Tel: +39-039-9215811 Fax: +39-039-9215835 Web: www.jasco-europe.com
JASCO Deutschland www.jasco.de | **JASCO UK** www.jasco.co.uk | **JASCO France** www.jascofrance.fr
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JASCO CHINA (SHANGHAI) CO., LTD.
Room No.D, 10F, World Plaza, 855 Pudong South Road, Pudong New Area, Shanghai, China
Tel: +86-21-6888-7871 Fax: +86-21-6888-7879 Web: www.jasco-global.com
China

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