螢光分光光譜儀

Instrumentation of Spectrofluorometers

美商亞洲瓦里安科技股份有限公司台灣分公司

資深應用工程師　丁原石 0910665965

Agilent Technologies

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Interactions between electromagnetic waves and your samples

$E = h\nu$

- The internal energy of a molecule:

  $E_{total} = E_{electronic} + E_{vibrational} + E_{rotational}$

- UV-Vis , Fluorescence & Phosphorescence spectra of most molecules consists of broad bands
  - Related to combination of (Electronic + Vibrational + Rotational) transitions

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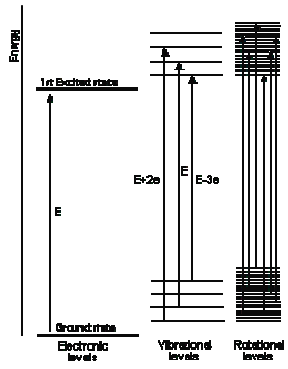
Energy levels of molecular

Fine Structure Considered



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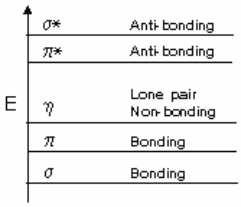
Organic Compounds
Energy and molecular transition of UV-Vis



σ to σ*,

n to σ*

n to π*

π to π*.

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What is Luminescence?

- ‘ an emission of light occurring at a temperature below that of incandescent bodies. It is distinct from incandescence, in which materials emit light as a result of their high temperature.‘

'*Luminescence comes about through the ability of certain substances to absorb light of relatively high frequency and re-emit it in installments of discrete lower frequencies (longer wavelength) '*

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Luminescence

Luminescence 為由物質所放射 (Emit)產生的，他是電子由激發態 （ Excited State) 以光 （ Photon) 的形式釋放出能量，回到基態（Ground State)時所產生的。

一般來說，包含下列三種

- Molecular fluorescence spectroscopy
- Molecular phosphorescence spectroscopy
- Chemiluminescence spectroscopy

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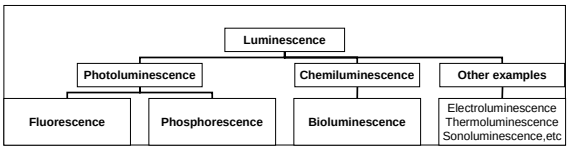
Types of luminescence



- For photoluminescence, the energy is provided by the absorption of IR, visible or ultraviolet light.
- Bioluminescence is the name given to chemiluminescent reactions that occur in living organisms.

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Types of emission processes

- Photoluminescence
  - excitation by absorption of light
    - fluorescence, lifetime 10^{-11} to 10^{-7} secs
    - phosphorescence, lifetime 10^{-3} to 10^{2} secs
- Chemiluminescence
  - excitation by chemical reaction
  - bioluminescence is a chemiluminescent reaction in a biological system

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去活化過程種類

1.Vibrational Relaxation：震動緩解 (in the same electronic state level)
2. External Conversion：外轉換，可能涉及激發態分子與溶劑或其他物質相互作用和能量轉移
3. Internal Conversion：內轉換，無輻射轉移至低能量電子態 (to lower electronic level)
4. Intersystem Crossing：系統間跨越，激發態電子自旋被反轉
5. 螢光
6. 磷光

} Radiative Transitions

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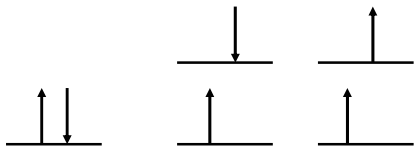
Electronic states



ground singlet state | excited singlet state | excited triplet state

Singlet state: All electrons in the molecule are spin-paired
Triplet state: One set of electron spins is unpaired

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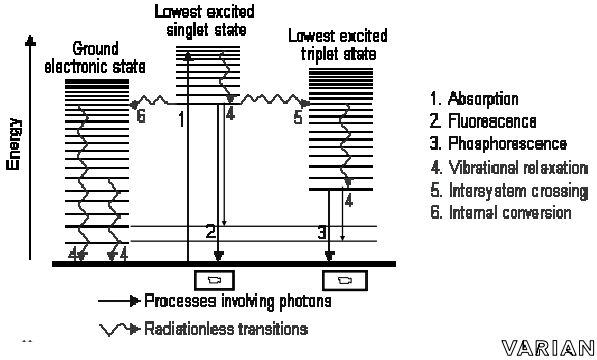
Possible physical process following absorption of a photon by a molecule



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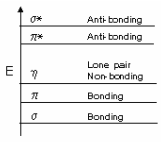
何種分子可能具有強螢光?

- 相當大的共軛分子結構
  Fairly large conjugate sysytem
  - Delocalized π electron
- 剛性之分子結構 Rigid structure
  - Reduce interaction with medium
- 平面分子結構 Planar



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Cary Eclipse

螢光分光光譜儀設計

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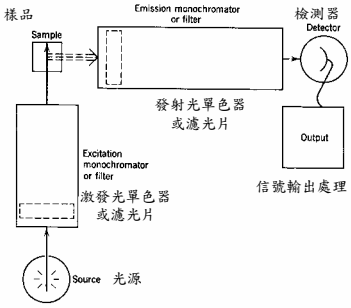
Essential component of fluorometer or spectrofluorometer



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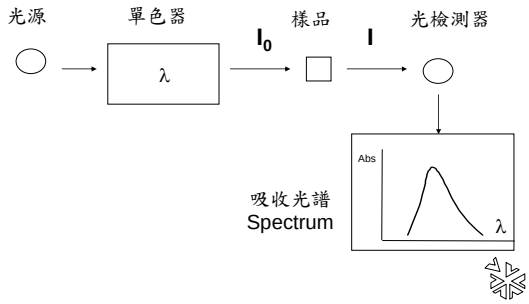
吸收光譜儀 (UV-Vis) Absorption spectrophotometer



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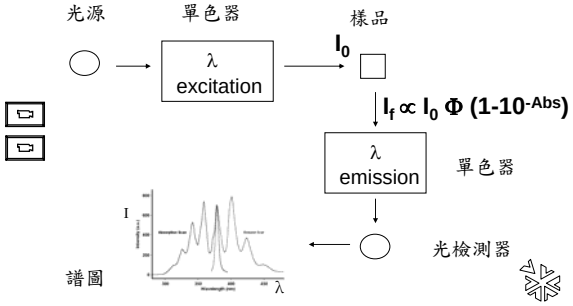
螢光分光光譜儀 Fluorescence spectrophotometer



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Cary Eclipse

- Measurement of:
  - Fluorescence螢光
  - Phosphorescence 磷光
  - Bioluminescence生物/化學冷光
- Wide range of accessories

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Cary Eclipse

主件Main components

- 燈源Lamp Module
- 激發光單色器Excitation Monochromator
- 參比檢出器Reference Detector
- 樣品室Sample compartment
- 發射光單色器Emission Monochromator
- 發射光檢出器Emission Detector
- 電子零件Electronics

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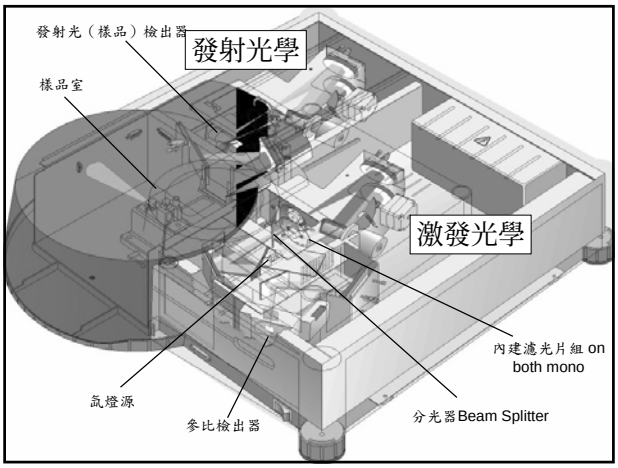
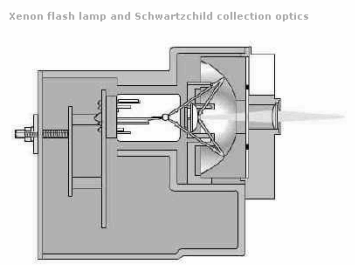


Schwarzschild collection optics Xe Lamp



極佳之靈敏度及低信號噪音
Excellent sensitivity and low signal noise

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The power of Xenon!

- 獨特之光免疫功能，檢測不用關蓋！

  Unique room light immunity for ***Fluorescence*** samples provides greater flexibility in measuring a wider range of samples.

  - Dark current correction (before sample measurement)
    - Ratio result =(sample PMT-dark)/(ref PMT-dark) x 1000
    - This is not applicable to
      - PHOSPHORESCENCE
      - BIOLUMINESCENCE

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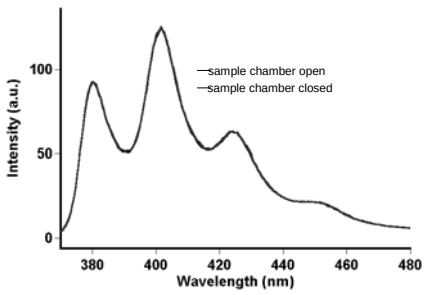
Cary Eclipse – Anthracene

室內光免疫功能room light immunity



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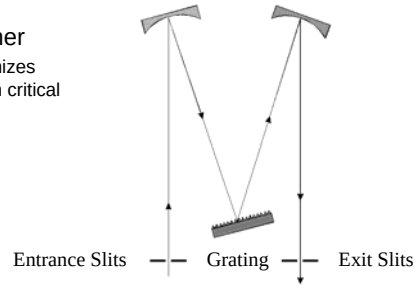
Monochromator 單色光器

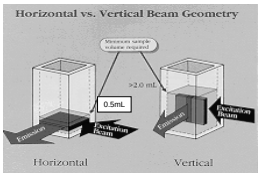
- Czerny- Turner
  - Design minimizes aberrations in critical applications



單光器Monochromators

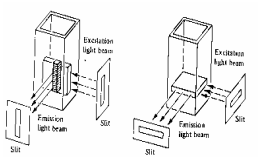
特點Feature

- Czerny Turner design
- 六種可選式狹縫
  - 1.5, 2.5, 5, 10, 20, 10 round
- **Horizontal slits**



優點Benefit

- 較小樣品體積
  - 1cm cuvette , sample量<0.5 mL
- Increased S/N
  - 擴大了檢測區域(region of viewing)
  - 增加發光信号度比垂直狹縫提高 5~30倍



Cary Eclipse design

- Detectors: 2 R928 photomultiplier tubes
  - large dynamic range (400-1000 Volts)
  - red-sensitive - Included as standard
- Revolutionary new electronics
  - Capture a phosphorescence data point every microsecond (10^{-6}s)

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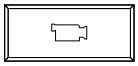
Cary Eclipse
儀器部件



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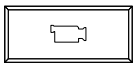
Cary Eclipse optics tour



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應用模式
Instrument Function Modes

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Scan
光譜掃瞄

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螢光光譜

一般會產生螢光物質有兩種特性光譜：

- 激發光譜 Excitation spectrum:
  wavelength and amount of light absorbed
- 發射光譜Emission spectrum :
  wavelength and amount of light emitted

⇒ Fluorescence Signature or Fingerprint
幾乎沒有兩種物質有相同之Fluorescence Signature

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Eclipse 有兩組光學系統

- 可單獨掃瞄其中之一光學
  - 固定激發光學，掃瞄發射光學
    - 結果為螢光發射光譜
      - often just referred to as the emission spectrum or the fluorescence spectrum
  - 固定發射光學，掃瞄激發光學
    - 結果為螢光激發光譜
- 可以同時掃瞄兩組光學
  - 通常固定兩組光學之波長差異
    - Synchronous scan

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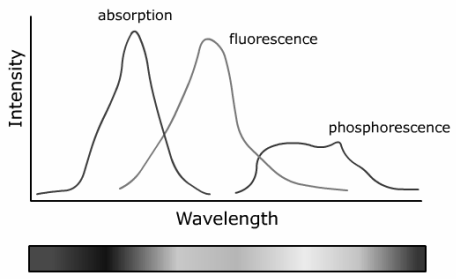
Absorption, fluorescence,and phosphorescence spectra



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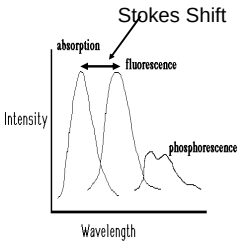
Stokes Shift

- 激發與發射光譜波峰之nm距離
  The difference in nanometers between the peak excitation and emission wavelengths of a fluorescent species.
- 表示在螢光發射前激發態生命期中之能量損耗
  This indicated the energy dissipated during the lifetime of the excited state before fluorescence.



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從發射光譜可以得到之訊息?

- 為分子結構及物質存在環境之特徵
  Is characteristic of molecular structure and environment of material
- 螢光發射光譜與激發波長無關，因為發射均由最低激發態產出
  The shape of the fluorescence spectrum is independent of the excitation wavelength because the emission always originates from the lowest excited state.
- 螢光光譜常與吸收光譜為"鏡像"
  The fluorescence spectrum is often a "mirror image" of the absorption spectrum.

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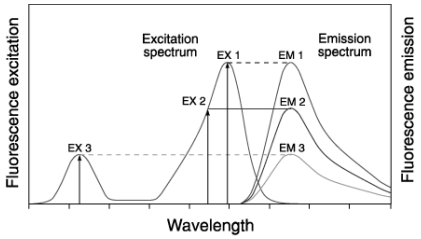
激發波長 vs. 發射波長



Excitation of a fluorophore at three different wavelengths (**EX 1, EX 2, EX 3**) does not change the emission profile but does produce variations in fluorescence emission intensity (**EM 1, EM 2, EM 3**) that correspond to the amplitude of the excitation spectrum.

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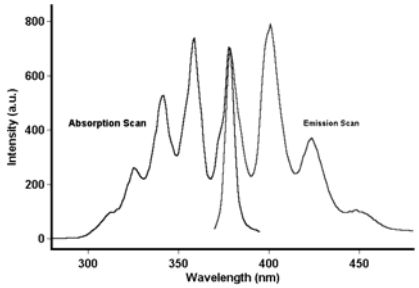
舉例：Absorption and Emission of Anthracene



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從激發光譜可以得到之訊息?

- 不同激發光波長造成之在特定發射波長強度圖
  Is a plot of intensity at a particular emission wavelength against the wavelengths of the exciting light.
- 尋找最佳激發波長使之於所選發射波長產生最佳螢光
  When collecting an excitation spectrum you are asking the question "where do I have to excite this system to see fluorescence at my chosen emission wavelength ?"

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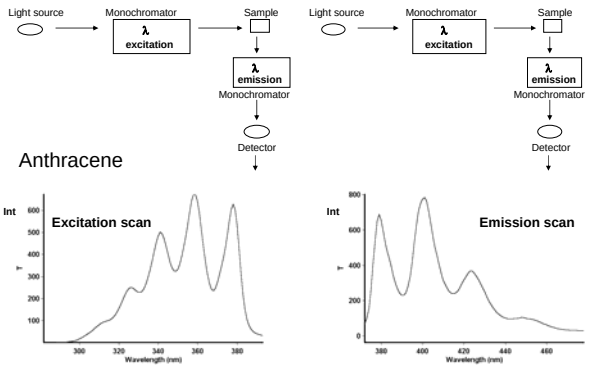
Excitation and Emission spectra



同時掃瞄Synchronous scans

- Generally plot intensity vs ex. mono. WL
- 可能有助於複雜混合物之光譜分離
  Permit separation of complex mixtures such as crude oil (after appropriate dilution)
- Spectra have unusual shape

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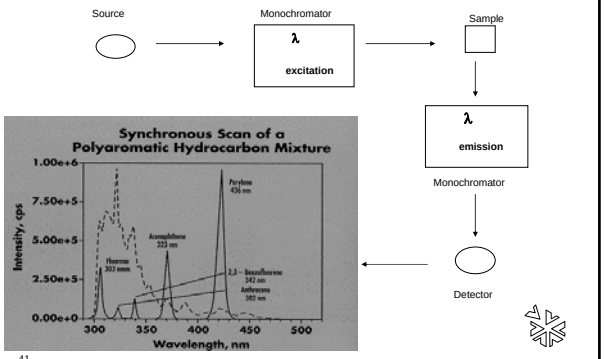
synchronous scan例子



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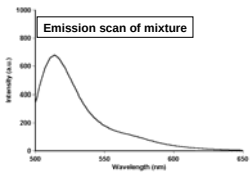
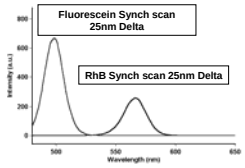
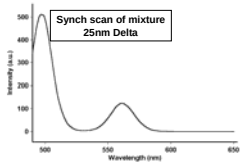
Synchronous scans

(Fluorescein + RhB mixture)







Artifacts in a fluorescence spectrum

Scattering

- 雷禮散射 Rayleigh scatter
  - 激發光之散射scatter of the excitation light
  - peak at the excitation wavelength
- 拉曼散射 Raman scatter
  - 溶劑造成之散射scatter due to solvent
  - 波峰出現於距激發波長固定能量位置（與溶劑相關）peak at a fixed energy from the excitation wavelength (solvent dependent)
- 倍頻 2nd Order
  - Scatter of higher order excitation light
  - 激發波長倍數位置peak at multiple of excitation wavelength

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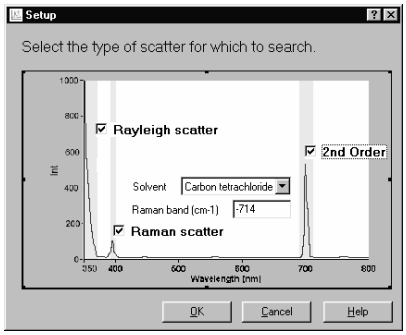
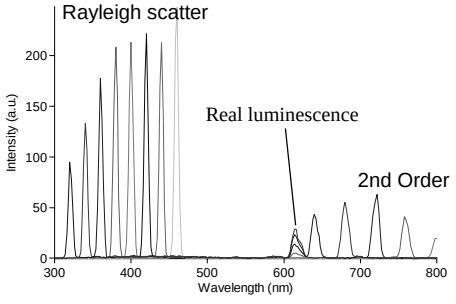
軟體自動判別
Software detects scattering artifacts..



散射 Scattering….



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拉曼散射 Raman Scattering

- During the Rayleigh scattering process, some of the incident energy can be abstracted and converted into vibrational and rotational energy. The resulting energy scattered is therefore of lower energy and longer wavelength than the incident radiation.
- The amount of energy abstracted is always constant ,Raman bands appear separated from the incident radiation by the same frequency difference.

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拉曼散射位置

Solvent	Position of Raman bands when excited at selected wavelength			
	Excitation wavelength 313 nm	Excitation wavelength 366 nm	Excitation wavelength 405 nm	Excitation wavelength 436 nm
Water	350	418	469	511
Acetonitrile	340	406	457	504
Cyclohexane	344	409	458	499
Chloroform	346	411	461	502

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Question!

- 若拉曼band正好與螢光發射光重疊影響，該怎麼辦？
  What will you do if the Raman band of the solvent coincides with the fluorescence emission of the solute?

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Types of collection modes

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Types of collection modes

- Fluorescence (lifetime 10^{-11} to 10^{-7} sec)
  - Generate emission with light source on
  - Measure the emission with the light source on

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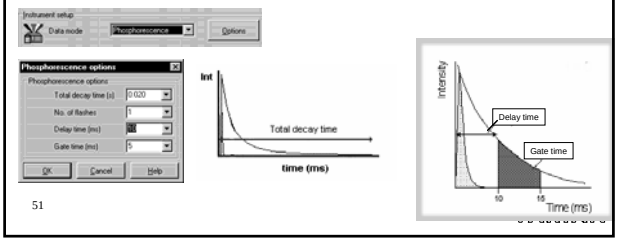
Types of collection modes

- Phosphorescence (lifetime 10^{-3} to 10^{2} sec)
  - Generate emission with light source on
  - Measure emission with light source off



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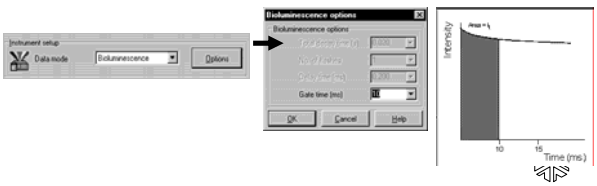
Types of collection modes

- Chemi-/Bio-luminescence
  - Generate emission with light source off
  - Measure emission with light source off



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Types of collection modes

- Usefulness of the “No. of flashes” parameter

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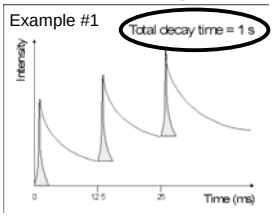
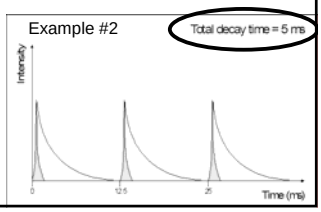
How many flashes should I perform?

Example #1



Example #2



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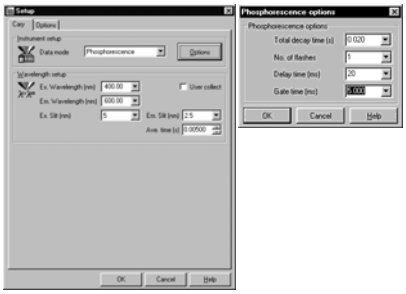
Phosphorescence data mode

- Single Reads example
  - Simple Reads, Advanced Reads, Concentration applications



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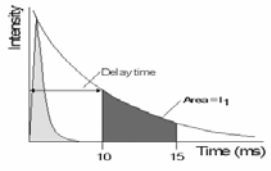
Ave. time = Gate time

Total Decay Time = 20ms
Delay Time =10ms
Ave time = Gate time = 5ms



Intensity = I_1

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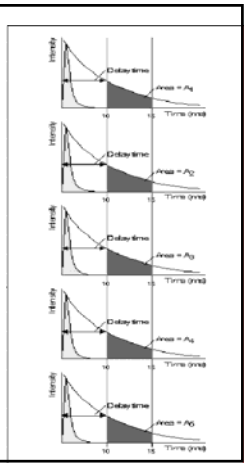
Ave. time = 5 x Gate time

Total Decay Time = 20ms
Delay Time =10ms
Gate Time = 5ms

Ave Time = 25ms

Ave Time/Gate Time = 5

Intensity $= \frac{A_1 + A_2 + A_3 + A_4 + A_5}{5}$



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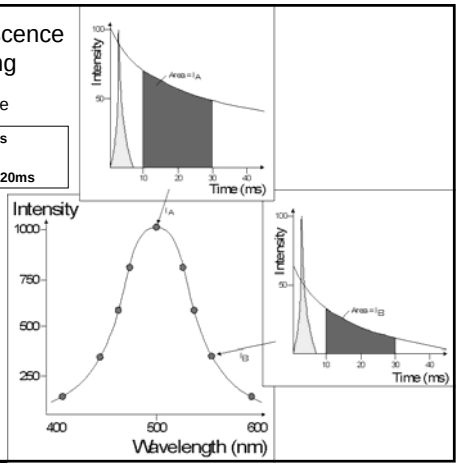
Phosphorescence scanning

Ave time = Gate time

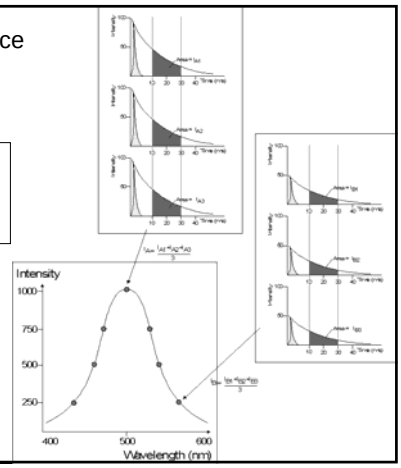
Total decay time = 40ms
Delay Time = 10ms
Ave time = Gate time = 20ms



Phosphorescence scanning

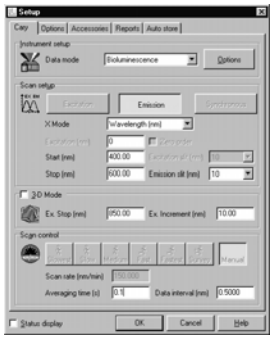
Ave time = 3 x Gate time

Total decay time = 40ms
Delay Time = 10ms
Gate time = 20ms
Ave time = 60ms



Bio-Chemi/luminescence data mode

- Scanning examples



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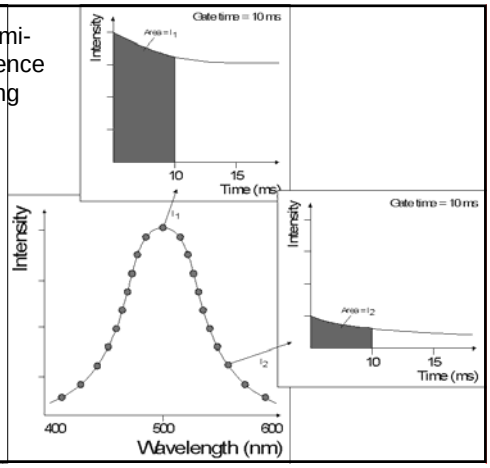
Bio/Chemi-luminescence scanning



Time dependent measurements

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Time dependent measurements

- 固定激發波長及發射波長
- 量測以時間為參數之信號強度

Measure the intensity as a function of time

- 動力學 Kinetics
  - 通常由於化學反應造成之強度改變change in intensity usually due to chemical reaction
  - time scale of seconds to minutes to hours
- 生命期 Lifetimes (phosphorescence)
  - 通常由於單一分子機制造成之強度改變change in intensity usually due to uni-molecular processes
  - time scale of time scale of ms to sec

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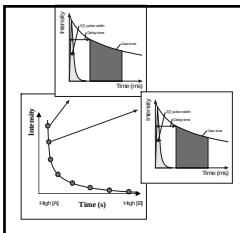

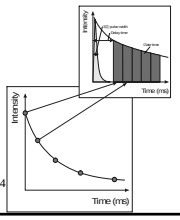

Kinetics Vs Lifetimes

Kinetics

A + B → C

A + Excitation → A*

A* → A + Emission

Lifetimes

A + Excitation → A*

A* → A + Emission

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Kinetics application

- Measure the increase or decrease in emission intensity as a function of time.
  - Fluorescence
  - Phosphorescence
  - Chemi/Bio-luminescence
- Obtain an Intensity vs time plot
- Fit the data with either a Zero, First or Second order equation

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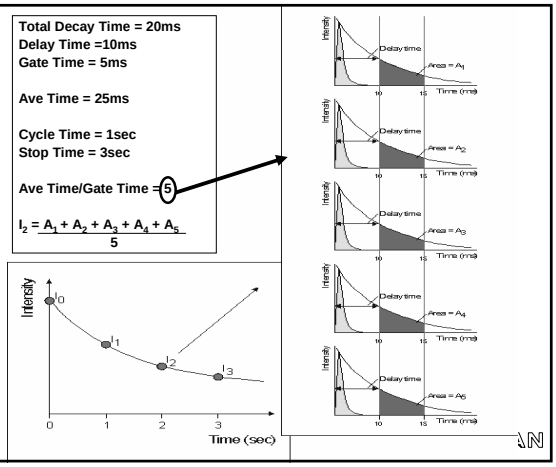
Total Decay Time = 20ms
Delay Time =10ms
Gate Time = 5ms

Ave Time = 25ms

Cycle Time = 1sec
Stop Time = 3sec

Ave Time/Gate Time = 5

$I_2 = \frac{A_1 + A_2 + A_3 + A_4 + A_5}{5}$



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Lifetimes application

- Time scales
  - Phosphorescence (μs to sec)
    - 1 μs data interval capabilities
    - Advanced Cary Eclipse electronics enables data to be collected 'real time' in most cases
  - Fluorescence (nanoseconds)
    - High end Fluorometers ONLY, not Cary Eclipse
    - Laser-based systems

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Lifetimes

- Definition of Lifetime (Decay time)
  - the average amount of time a molecule remains in the excited state following excitation
- Lifetimes (time resolved)

A + Excitation → A*

A* → A + Emission

  - Time based measurement like Kinetics
  - change in intensity usually due to uni-molecular processes

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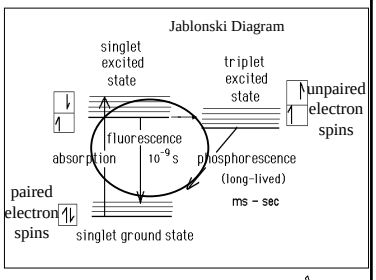
Lifetimes

- Why is this important?
  - Characteristic of a particular molecule under certain conditions
  - Solvent and temp effects
- Applications
  - Protein labelling
  - e.g. Eu labelling
    - long fluorescence lifetime



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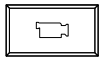
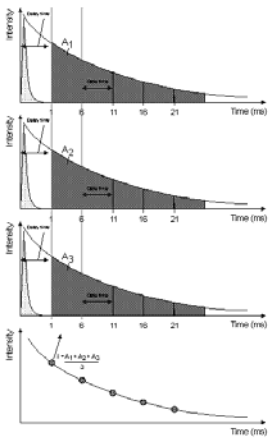
Phosphorescence lifetimes





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螢光定量
Quantitative fluorescence

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濃度效應Concentration Effects

- fluorescence intensity is proportional to input light intensity *at low concentrations*
- at high concentrations other effects come into play
  - self (concentration) quenching
  - energy transfer
  - formation of new species after absorption
- "inner filter" effect
- collection geometry - e.g. front face illumination (triangular cells)

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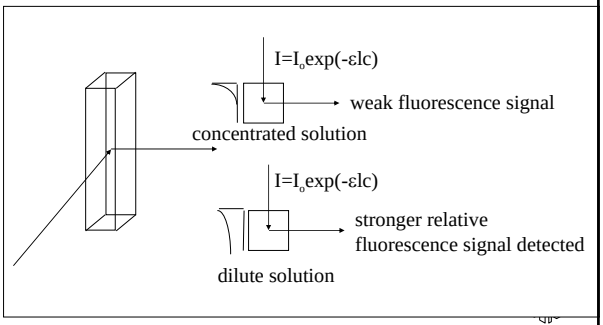
"Inner Filter" Effect



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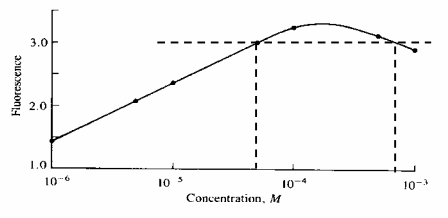
Inner filter effect

Coenzyme NADH in D.I. water



Linear range from 10^{-8} to 10^{-4} M

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其他影響定量準確性的因素
Other factors affecting quantitative accuracy

- 溫度效應Temperature effect
  - 改變碰撞次數Change number of collisions
- 溶劑效應
  - 改變黏稠度Change viscosity
- 酸鹼度效應pH effects
  - Change intensity and spectral characteristics of fluorescence
- Quenching(猝滅)

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極稀濃度溶液之考量
Working with dilute solutions

- 吸附 Adsorption
- 吸收槽之材質及品質Cuvette material and quality
- 溶劑及試劑
  - e.g. solvents should not stored in plastic containers
- 光分解Photo-decomposition
- 氧化
  - e.g. the presence of O_2 or traces of peroxide
- 其他污染
  - Stopcock grease
  - The growth of micro-organisms in buffer….
  - Filter (phenols present from the original wood)
  - ……

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Quantitation summary

- 螢光強度僅於低濃度時正比於物質濃度.
- 務必考慮系統線性範圍
- Typically C_{max}= 0.05/εl where ε is the molar extinction coefficient at the excitation wavelength, and l is the pathlength.

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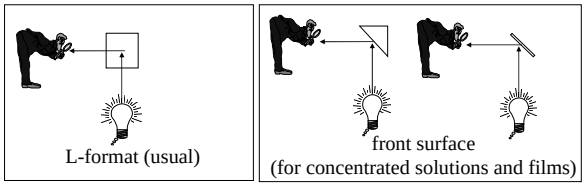
高濃度之定量
Measuring concentrated solutions



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Temperature dependent measurements – BioMelt™ Package

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Thermal Analysis

- DNA work being investigated using fluorescence
  - Higher sensitivity
  - Use Fluorescence Resonance Energy Transfer (FRET)
- Characterization of DNA melting curves
  - Drug diagnostics
  - Thermodynamic properties of DNA
    - attach various groups to DNA molecule
- Accessories
  - 4 cell peltier accessory
  - Temperature controller
  - Temperature probe

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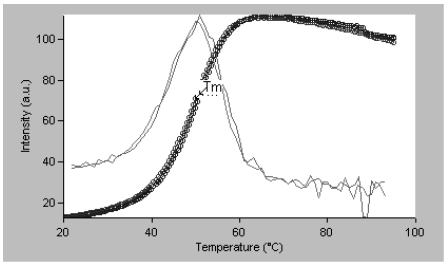
Example

Tm value using 9mer DABCYL labelled PNA probe with 5' 6-carboxyfluorescein labelled DNA.



Details refer to *Varian Fluorescence At Work* No.008.

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