Basic Fluorescence Instrumentation

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Slide acknowledgements Dr. Theodore Hazlett, Dr. Joachim Müller
Create Fluorescence Contrast

Bright robust dyes

Quantum dots

Functionalized Nanoparticles
Fluorometry

Collecting Spectra, Polarization, Kinetics, Lifetimes …

- Instrument functioning and ageing
- Verify sample identity and integrity
- Verify optimum excitation and emission wavelengths
- Verify levels of scattered excitation and Raman signals
- Impurities in solvent, buffer or sample
- Preparation and validation for FCS, Lifetime and FLIM …

- Elucidate solvent, temperature, pH, aggregation effects … surfaces, films, substrates, molecule orientation …
Fluorometers

ISS PC1 (ISS Inc., Champaign, IL, USA)

Fluorolog-3 (Jobin Yvon Inc, Edison, NJ, USA)

QuantaMaster (OBB Sales, London, Ontario N6E 2S8)
More Examples of Fluorescence Based Instrumentation

Tecan ULTRA Evolution Plate Reader (Tecan Trading AG, Männedorf / Zürich, Switzerland)

Zeiss LSM 510 META Optical Confocal Microscope (Carl Zeiss AG, Jena, Germany)

Becton Dickinson BD FACSCanto Fluorescence Assisted Cell Sorter (FACS)
Main Fluorometer Components

Light Source

Computer

Excitation Wavelength Selection

Excitation Polarizer

Sample

Emission Polarizer

Emission Wavelength Selection

Detector

Note: Polarizers can slide in and out of the optical path
Fluorometer Components

Light Source
Sample Compartment
Detectors
Wavelength Selection
Polarizers
Computer & Software
ISS (Champaign, IL, USA) PC1 Fluorometer

Standard Light Source:
Xenon Arc Lamp
or
Light Emitting Diode (LED)
Light Sources
Lamp Light Sources: Arc Lamps (1)

1. Xenon Arc Lamp

- Lamp Emission Spectra:
  - UV
  - Visible
  - Ozone Free

- 15 kW Xenon arc lamp

2. High Pressure Mercury Lamps

- (High Intensities but concentrated in specific lines)

http://microscopy.fsu.edu/primer/anatomy/lightsources
Lamp Light Sources: Arc Lamps (2)

3. Mercury-Xenon Arc Lamp (greater intensities in the UV)

ARC LAMP ISSUES:
- Lifetime
- Stability (flicker + drifts)
- Safety
  - high internal gas pressures (potential eye damage)
  - hot
  - never stare into burning lamp
  - do not touch with bare hands (fingerprints on quartz lamp envelope)

LAMP HOUSING + OPTICS:

Conventional OR Compact

http://microscopy.fsu.edu/primer/anatomy/lightsources
4. Tungsten-Halogen Lamps

A Tungsten-Halogen lamp with a filter (arrow) to remove UV light.

The color temperature varies with the applied voltage (average values range from about 2200 K to 3400 K).
5. Light Emitting Diodes (LEDs)

Spectra for blue, yellow-green, and red LEDs. **FWHM** spectral bandwidth is approximately 25 nm for all three colors.

<table>
<thead>
<tr>
<th>Lamp</th>
<th>Luminous Flux (Lumens)</th>
<th>Spectral Irradiance (Milliwatt/Square Meter/Nanometer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBO 100 Watts</td>
<td>2200</td>
<td>30 (350-700 nm)</td>
</tr>
<tr>
<td>XBO 75 Watts</td>
<td>1000</td>
<td>7 (350-700 nm)</td>
</tr>
<tr>
<td>Tungsten 100 Watts</td>
<td>2800</td>
<td>&lt; 1 (350-700 nm)</td>
</tr>
<tr>
<td>LED (Blue, 450 nm)</td>
<td>160</td>
<td>6</td>
</tr>
</tbody>
</table>

White LED: typical emission spectrum
5. Light Emitting Diodes (LEDs)

Wavelengths from 260 nm to 2400 nm

Deep – UV LEDs $\lambda \approx 260$ nm

Near UV LED

[Diagram showing various wavelengths and LED types]
Many Wavelengths (nm) Available:

262, 266, 349, 351, 355, 375, 405, 415, 430, 440, 447, 473, 488, 523, 527, 532, 542, 555, 561, 584-593, 638, 655, 658, 671, 685, 785, 808, 852, 946, 980, 1047, 1053, 1064, 1080, 1313-1342, 1444, 1550

(DPSS) Diode-pumped solid state laser
Supercontinuum White Light

Ultrashort pulsed light focused into photonic crystal fiber

Photonic crystal fiber optic

SC-450
Supercontinuum Fibre Laser System

Relative intensity [dB]

Coupled average power: 67 mW
Wavelength: 800 nm
Pulse duration: 50 fs
Rep. Rate: 79 MHz
Detectors
Conversion of Light into an Electrical Signal

Non-Imaging Detector:
Photomultiplier (PMT)

Imaging Detector:
Microchannel Plate (MCP) PMT

MCP & Electronics
(ISS Inc. Champaign, IL USA)
The Classic PMT Design
End-On Tube

- Photocathode
- Vacuum Inside
- Dynodes
- Anode
- Window
- Constant Voltage (use of a Zener Diode)
- High Voltage Supply (-1000 to -2000 V)
- Resistor series (voltage divider)
- Capacitor series (current source)
- Ground
The Detector Dark Signal

Photocathode

Vacuum

Dynodes

Anode

Shutter Blocking All Light Access

Constant Voltage (use of a Zener Diode)

High Voltage Supply (-1000 to -2000 V)

Resister series (voltage divider)

Capacitor series (current source)

Ground

Vacuum

Shutter Blocking All Light Access

Constant Voltage (use of a Zener Diode)

High Voltage Supply (-1000 to -2000 V)

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Ground

Shutter Blocking All Light Access

Constant Voltage (use of a Zener Diode)

High Voltage Supply (-1000 to -2000 V)

Resister series (voltage divider)

Capacitor series (current source)

Ground
Photon Counting (Digital) and Analog Detection

Primary Advantages:
1. Sensitivity (high signal/noise)
2. Increased measurement stability
3. Digital signals

Photon Counting:
- Constant High Voltage Supply
- Discriminator Sets Level
- TTL Output (1 photon = 1 pulse)
- Counts / Time Unit
- Computer

Primary Advantage:
1. Broad dynamic range
2. Adjustable range

Analog:
- Variable Voltage Supply
- Anode Current
- Pulse averaging
Hamamatsu R928 PMT Family
Side-On Tube

Quartz Window with Photocathode Beneath

Wavelength Dependent Quantum Efficiency
Hamamatsu H7422P-40 PMT

40% Quantum Efficiency
300 – 720 nm GaAsP spectral response
Time resolution 150 – 250 psec
After-pulsing at highest gain

Fig. 13: Sensitivity of different photocathodes [34]
PMT Quantum Efficiencies

Cathode Material

Envelope Window Material
PMT Geometries

Side-On PMT

Opaque photocathode
Slightly enhanced quantum efficiency
Faster response time (compact design)
Less affected by a magnetic field

Head-On PMT

Semitransparent Photocathode
Smaller afterpulsing
Count rate linearity better
Better spatial uniformity

Figure 3: Common Photomultiplier Dynode Chain Configurations
Avalanche Photodiodes (APDs)

APD for analog detection

The silicon avalanche photodiode (Si APD) has a fast time response and high sensitivity in the near infrared region. APDs are available with active areas from 0.2 mm to 5.0 mm in diameter and low dark currents (selectable). Photo courtesy of Hamamatsu

APD for photon counting

Single photon counting module (SPCM) from Perkin-Elmer

70% Quantum Efficiency
Wavelength Selection

- Fixed Optical Filters
- Tunable Optical Filters
- Monochromators
Optical Filter Channel

Diagram showing the flow of light through the filter channel, with labels for PMT, LAMP, and SAMPLE.
Long Pass Optical Filters Based on Absorption of Light

Spectral Shape
Thickness
Physical Shape
but also possibly
Substrate Fluorescence (!?)

UV 36 HOYA
HOYA Y-46
Hoya O54
HOYA O-58
HOYA R62
Better Optical Filter Types…

<table>
<thead>
<tr>
<th>Filter Type</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broad Bandpass Filter</td>
<td>Hoya U330</td>
</tr>
<tr>
<td>Interference Filters</td>
<td>Chroma Technologies</td>
</tr>
<tr>
<td>Neutral Density Filter</td>
<td>Coherent Lasers OD 0.3</td>
</tr>
</tbody>
</table>

- Transmission (%)
- Wavelength (nm)
Instrument Validation through Fluorescent Standards

Filterwheels

- HBO 103 Hg Lamp
- Excitation Filter
- Dichroic Mirror
- Emission / Barrier Filter
- Specimen

Adjustable excitation wavelength
Tunable Optical Filters

Liquid Crystal Filters:
An electrically controlled liquid crystal elements to select a specific visible wavelength of light for transmission through the filter at the exclusion of all others.

AO Tunable Filters:
The AOTF range of acousto-optic (AO) devices are solid state optical filters. The wavelength of the diffracted light is selected according to the frequency of the RF drive signal.
1. Slit Width (mm) is the dimension of the slits.

2. Bandpass is the FWHM at the selected wavelength.

3. The dispersion is the factor to convert slit width to bandpass.
The Inside a Monochromator: Tunable Wavelengths

- Zero Order (acts like a mirror)
- Nth Order (spectral distribution)
- Grating
- 1st Order spectrum

MIRRORS
Order of Diffraction

0-th order (acts like a mirror)

1-st order

2-nd order
Higher Order Light Diffraction & Spectral Features

Emission Scan:
Excitation 300 nm
Glycogen in PBS

- Excitation (Rayleigh) Scatter (300 nm)
- Water RAMAN (334 nm)
- 2nd Order RAMAN (668 nm)
- 2nd Order Scatter (600 nm)

Fluorescent Contaminants

Spectrum 1
Spectrum 2
Energy for the OH stretch vibrational mode in water (expressed in inverse wavenumbers): 3200 cm\(^{-1}\)

Simple formula to calculate the wavelength of the Raman peak:

1. Insert the excitation wavelength (e.g. 490 nm) in the following equation:

\[
\frac{10^7}{\frac{10^7}{490} - 3200} = 581 \text{ nm}
\]

2. The result specifies the position of the Raman peak in nanometers (i.e. the Raman peak of water is located at 581 nm for this excitation wavelength of 490 nm.)
Changing the Bandpass

1. Drop in intensity
2. Narrowing of the spectral selection

@ Fixed Excitation Bandpass = 4.25 nm

Changing the Emission Bandpass

Collected on a SPEX Fluoromax - 2
Monochromator Polarization Bias

Tungsten Lamp Profile Collected on an SLM Fluorometer

Wood’s Anomaly

No Polarizer

Parallel Emission

Perpendicular Emission

Fluorescence vs. Wavelength

250 to 800 nm

Technical vs. Absolute spectra


for more on the correction of (emission) spectra
Correction of Emission Spectra

ANS Emission Spectrum, no polarizer

ANS Emission Spectrum, parallel polarizer

from Jameson et. al., Methods in Enzymology, 360:1
Excitation Correction
The Instrument Quantum Counter

Common Quantum Counters (optimal range) *

- Rhodamine B (220 - 600 nm)
- Fluorescein (240 - 400 nm)
- Quinine Sulfate (220 - 340 nm)

Linearity of Rhodamine as a quantum counter

The maximum inner filter effect needed!

Polarizers
Two UV selected calcite prisms are assembled with an intervening air space. The calcite prism is birefringent and cut so that only one polarization component continues straight through the prisms. The spectral range of this polarizer is from 250 to 2300 nm. At 250 nm there is approximately 50% transmittance.
Filter Choice For Steady-State as well as Time-Resolved Polarization Measurements

Make sure absolutely no scattered excitation light is detected!

An inserted emission filter should block the excitation very well

Why?

\[
P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}
\]

Scattered excitation light influences \( I_{\parallel} \)
Sample Optimization
Signal Attenuation of the Excitation Light

**PMT Saturation**

**Fluorescence vs. Signal**

- Reduced emission intensity:
  1. ND Filters
  2. Narrow monochromator slit widths
  3. Move off absorbance peak
Concentration

Attenuation of the Excitation Light through Absorbance

Sample concentration & the *inner filter effect*

Rhodamine B in ethanol

Correct Optical Density (OD)

The Second Half of the *Inner Filter Effect*: Attenuation of the Emission Signal

Absorbance Spectrum

(1) Spectral Shift
(2) Change in Spectral Shape
Spectroscopy Cuvettes
Handling Highly Absorbing Solutions

Use smaller optical pathlengths for excitation and emission

Quartz/Optical Glass/Plastic Cells with Caps / Stoppers

O.D >> 0.04

Excitation

Emission Path Length

Filter Detector

Excitation Path Length (1 cm)

4 Position Turret
SPEX Fluoromax-2, Jobin-Yvon
Front Face Detection

Triangular Cells

Excitation

Filter Detector

Reflected Excitation & Emission

Thin Cells & Special Compartments

IBH, Glasgow G3 8JU
United Kingdom

Excitation

Emission

Mirror

Haemoglobin sample

Absorbance Measurements

[1] Adapted from Gryczynski, Lubkowski, & Bucci Methods of Enz. 278: 538
Lifetime Instrumentation

\[ 50.0\% \times \exp\left(-\frac{t}{0.14 \text{ ns}}\right) + 50.0\% \times \exp\left(-\frac{t}{3.98 \text{ ns}}\right) \]

- Time, ns
- Frequency (MHz)
- Phase (degrees)
- Magnitude
Light Sources for Decay Acquisition: Frequency and Time Domain Measurements

Pulsed Light Sources (frequency & pulse widths)

**Mode-Locked Lasers**
- ND:YAG (76 MHz) (150 ps)
- Pumped Dye Lasers (4 MHz Cavity Dumped, 10-15 ps)
- Ti:Sapphire lasers (80 MHz, 150 fs)
- Mode-locked Argon Ion lasers

**Directly Modulated Light Sources**
- Diode Lasers (short pulses in ps range, & can be modulated by synthesizer)
- LEDs (directly modulated via synthesizer, 1 ns, 20 MHz)

**Flash Lamps**
- Thyratron-gated nanosecond flash lamp (PTI), 25 KHz, 1.6 ns
- Coaxial nanosecond flashlamp (IBH), 10Hz-100kHz, 0.6 ns
The Pockels Cell is an electro-optic device that uses the birefringent properties of calcite crystals to alter the beam path of polarized light.

In applying RF power, the index of refraction is changed and the beam exiting the side emission port (0 polarized) is enhanced or attenuated. In applying RF the output light becomes modulated.
Time Correlated Single Photon Counting

- Pulsed Light Source
- Sample Compartment
  - Filter or Monochromator
    - Neutral density (reduce to one photon/pulse)
- Photon Counting PMT
- Timing Electronics or 2nd PMT
- Constant Fraction Discriminator
- Time-to-Amplitude Converter (TAC)
- Multichannel Analyzer

**Instrument Considerations**

- Excitation pulse width
- Excitation pulse frequency
- Timing accuracy
- Detector response time (PMT 0.2-0.9 ns; MCP 0.15 to 0.03 ns)
(1) The pulse width and instrument response times determine the time resolution.

(2) The pulse frequency also influences the time window. An 80 MHz pulse frequency (Ti:Sapphire laser) would deliver a pulse every 12.5 ns and the pulses would interfere with photons arriving later than the 12.5 ns time.
There is still a polarization bias due to the geometry of our excitation and collection (even without a monochromator)!!

**Corrective polarizer settings**

An intuitive argument:

\[
\begin{align*}
[1] &= I_0 + I_{90} \\
[2] &= I_0 + I_{90} \\
[3] &= I_0 + I_{90} \\
[4] &= I_0 + I_{90} \\
[5] &= 2 \times I_{90} \\
[6] &= 2 \times I_{90} \\
\text{Total} &= 4 \times I_0 + 8 \times I_{90}
\end{align*}
\]

The total intensity is proportional to:

\[I_0 + 2 \times I_{90}\]

Setting the **excitation angle to 0°** and the **emission polarizer to 54.7°** the proper weighting of the vectors is achieved.*

\[\sin^2 54.7° = 2/3\]

Frequency Domain Fluorometry

CW Light Source → Sample Compartment → Filter or Monochromator → Analog PMTs (can also be done with photon counting)

RF

S1 = n MHz
S2 = n MHz + 800 Hz

Sine-Wave Signal Generators / Synthesizers S1 and S2

Pockels Cell

Reference

Turret

Digital Acquisition Electronics

Similar instrument considerations as with TCSPC

Computer Driven Controls

Digital PMTs

RF

Signal

Locking Signal
### Instrument Validation through Fluorescent Standards

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>( t ) (ns) (^{a})</th>
<th>( \lambda_{ex} ) (nm)</th>
<th>( \lambda_{em} ) (nm)</th>
<th>( d )</th>
<th>( e )</th>
</tr>
</thead>
<tbody>
<tr>
<td>NATA</td>
<td>Water</td>
<td>3.04 ± 0.04</td>
<td>295–325</td>
<td>325–415</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Anthracene</td>
<td>Methanol</td>
<td>5.1 ± 0.3</td>
<td>300–330</td>
<td>380–442</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Cyclohexane</td>
<td>5.3 ± 0.2</td>
<td>295–325</td>
<td>345–442</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>9-Cyanoanthracene</td>
<td>Methanol</td>
<td>16.5 ± 0.5</td>
<td>295–325</td>
<td>370–442</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Cyclohexane</td>
<td>12.4 ± 0.5</td>
<td>295–325</td>
<td>345–380</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Erythrosin B</td>
<td>Water</td>
<td>0.089 ± 0.002</td>
<td>488, 514, 568</td>
<td>515–575</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>0.48 ± 0.02</td>
<td>488, 514</td>
<td>515–560</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>9-Methylcarbazole</td>
<td>Cyclohexane</td>
<td>14.4 ± 0.4</td>
<td>295–325</td>
<td>360–400</td>
<td>5</td>
<td>4</td>
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<tr>
<td>DPA</td>
<td>Methanol</td>
<td>8.7 ± 0.5</td>
<td>295–344</td>
<td>370–475</td>
<td>7</td>
<td>7</td>
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<tr>
<td></td>
<td>Cyclohexane</td>
<td>7.3 ± 0.5</td>
<td>295–344</td>
<td>345–480</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>PPO</td>
<td>Methanol</td>
<td>1.64 ± 0.04</td>
<td>295–330</td>
<td>345–425</td>
<td>7</td>
<td>7</td>
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<tr>
<td></td>
<td>Cyclohexane</td>
<td>1.35 ± 0.03</td>
<td>295–325</td>
<td>345–425</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>POPOP</td>
<td>Cyclohexane</td>
<td>1.13 ± 0.05</td>
<td>295–325</td>
<td>380–450</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Rhodamine B</td>
<td>Water</td>
<td>1.71 ± 0.07</td>
<td>488–514</td>
<td>515–630</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>2.53 ± 0.08</td>
<td>295, 488, 514</td>
<td>515–630</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Rubrene</td>
<td>Methanol</td>
<td>9.8 ± 0.3</td>
<td>300, 330</td>
<td>530–590</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>SPA</td>
<td>Water</td>
<td>31.2 ± 0.4</td>
<td>300–330</td>
<td>370–510</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>( p )-Terphenyl</td>
<td>Methanol</td>
<td>1.16 ± 0.08</td>
<td>284–315</td>
<td>330–380</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Cyclohexane</td>
<td>0.99 ± 0.03</td>
<td>295–315</td>
<td>330–390</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^{a}\) Data collected by N. Boens and M. Ameloot.

\(^{b}\) Abbreviations used: NATA: N-acetyl-L-tryptophanamide, DPA: 9,10-diphenylanthracene, POPOP: 1,4-bis[5-phenylloxazol-2-yl]benzene, PPO: 2,5-diphenyloxazole, SPA: N-(3-sulfolpropyl)acridinium. All solutions are deoxygenated by repetitive freeze–pump–thaw cycles or by bubbling \( N_2 \) or \( Ar \) through the solutions.

\(^{c}\) The quoted errors are sample standard deviations.

\[^{d}\] Number of lifetime data measured.

\(^{e}\) Number of lifetime data used in the calculation of the mean lifetime \( t \) and its standard deviation \( s \). The difference between columns \( d \) and \( e \) gives the number of outliers.

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Thank you