FLUORESCENCE MICROSCOPY

Internal course 2014
January 14th
FLUORESCENCE MICROSCOPY

Why do we need it?
UNSTAINED SPECIMEN

Missing specificity
DIFFERENT STAINING STRATEGIES

Histological stain (Absorption) like e.g H&E staining (Hematoxilin and Eosin staining)

Fluorescent dyes: Sensitivity
FLUORESCENCE MICROSCOPY

Basic principles
ORIGIN OF FLUORESCENCE
ABSORPTION

![Graph showing absorption spectra for CY3 and CY5 dyes.](image)

- **Red line**: CY3
- **Green line**: CY5

The graph plots the absorption coefficient ($10^{-3} \varepsilon$) against wavelength (nm). The absorption peaks for CY3 and CY5 are observed at different wavelengths, indicating their distinct absorption properties.

**Molecular structures**

- **CY3**
- **CY5**

These structures illustrate the chemical makeup of the dyes, highlighting their functional groups and the positions of the absorption maxima.
FLUORESCENCE ENERGY DIAGRAM

Jablonski Diagram (very simplified)
Excitation and emission spectra are not discrete.
Scattered excitation light can be efficiently separated from fluorescence
The profile of the emission spectra are independent of the excitation wavelength.
1. Excitation $10^{-15}$ s
2. Internal conversion $10^{-12}$ s
3. Solvent relaxation $10^{-11}$ s
4. Fluorescence $10^{-9}$ s
5. Intersystem crossing $10^{-9}$ s
6. Phosphorescence $10^{-3}$ s
BLEACHING

Bleaching is irreversible (=fluorophore is destroyed)
Bleaching is dependent on the excitation power
Bleaching can also cause photodamage
FLUORESCENCE MICROSCOPY

Detection
In order to detect the fluorescence at 10% background, the excitation light must be removed or attenuated by a factor up to $\approx 10^{11}$. The ratio of excitation light to fluorescent light (IE/IFL) is:

- $IE/IFL = 10^4$ for strong fluorescence
- $IE/IFL = 10^{10}$ for weak fluorescence (e.g., in situ hybrid.)
EPIFLUORESCENCE

Sample

Objective

Excitation Light
EPIFLUORESCENCE

Sample

Objective

Back-scattered excitation light: IE/100

Fluorescence
EPIFLUORESCENCE

Excitation Light

Dichroic mirror
(passes green but reflects blue light)

Sample

Objective
EPIFLUORESCENCE

Sample

Back-scattered excitation light
IE/100

Objective

Dichroic mirror
(passes green but reflects blue light)

Fluorescence

Detector
EPIFLUORESCENCE
REAL WORLD

Sample

Objective

Back-scattered excitation light
IE/100

Dichroic mirror
(passes green but reflects blue light)

Fluorescence

Back-scattered excitation light
IE/10,000

Detector
EPIFLUORESCENCE
REAL WORLD

Sample

Objective

Dichroic mirror
(passes green but reflects blue light)

Fluorescence

Emission filter
(passes fluorescence but not back-scattered excitation light)

Detector

Back-scattered excitation light IE/100

Back-scattered excitation light IE/10,000

Back-scattered excitation light IE/10^{11}
EPIFLUORESCENCE

Sample

Objective

Scattered light

Dichroic mirror

Excitation Filterwheel

Alexa 488

Emission Filterwheel

Detector

488nm

520nm

HBO

488nm
DOUBLE FLUORESCENCE

Sample

Objective

Scattered light

Double dichroic mirror

(λ1 = 505nm + λ2 = 560nm)

Excitation Filterwheel (Bandpass)

Alexa 488

Emission Filterwheel (Bandpass)

Detector

HBO

488nm

520nm
DOUBLE FLUORESCENCE

Sample

Objective

Scattered light

Double dichroic mirror

Excitation Filterwheel (Bandpass)

Alexa 555

Detector

Emission Filterwheel (Bandpass, Longpass)

HBO

550nm

590nm
FLUORESCENCE MICROSCOPY

Implementation
IMPLEMENTATION OF EPIFLUORESCENCE
IMPLEMENTATION OF EPIFLUORESCENCE
TYPICAL FILTER PROFILES

Interference Filters for Fluorescence Microscopy

(a) Optical Block
- Rack Mounting Flange
- Retainer
- Barrier Filter
- From Illuminator
- Dichromatic Mirror
- Excitation Filter
- Fluorescence Filter Block

(b) Transmission (Percentage)
- DAPI-TRITC
- EX
- EM
- DM
- Wavelength (Nanometers)

400 500 600 700
FILTER CHARACTERISTICS AND NOMENCLATURE
ANATOMY OF AN INTERFERENCE FILTER
SINGLE COLOR DETECTION

Use longpass filter in the emission!
Bandpass emission filters are necessary in multicolor imaging.
FLUORESCENCE MICROSCOPY

Excitation
Why do we need fluorescence microscopy?

Basics about fluorescence

Fluorescence detection

Fluorescent excitation

Fluorescent dyes and staining procedures

Applications
SPECTRUM OF A MERCURY ARC LAMP

⇒ Ideal for excitation of GFP2, CFP and DsRed imaging but less convenient for EGFP
SPECTRUM OF A XENON ARC LAMP
LIGHT EMITTING DIODES (LED)

**Pro:**
- long lifetime
- Fast switching
- No unwanted excitation

**Contra:**
- flexibility
- intensity

<table>
<thead>
<tr>
<th>Color Name</th>
<th>Wavelength (Nanometers)</th>
<th>Semiconduct or Composition</th>
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</thead>
<tbody>
<tr>
<td>Infrared</td>
<td>880</td>
<td>GaAlAs/GaAs</td>
</tr>
<tr>
<td>Ultra Red</td>
<td>660</td>
<td>GaAlAs/GaAlAs</td>
</tr>
<tr>
<td>Super Red</td>
<td>633</td>
<td>AlGaInP</td>
</tr>
<tr>
<td>Super Orange</td>
<td>612</td>
<td>AlGaInP</td>
</tr>
<tr>
<td>Orange</td>
<td>605</td>
<td>GaAsP/GaP</td>
</tr>
<tr>
<td>Yellow</td>
<td>585</td>
<td>GaAsP/GaP</td>
</tr>
<tr>
<td>Incandescent White</td>
<td>4500K (CT)</td>
<td>InGaN/SiC</td>
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<tr>
<td>Pale White</td>
<td>6500K (CT)</td>
<td>InGaN/SiC</td>
</tr>
<tr>
<td>Cool White</td>
<td>8000K (CT)</td>
<td>InGaN/SiC</td>
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<tr>
<td>Pure Green</td>
<td>555</td>
<td>GaP/GaP</td>
</tr>
<tr>
<td>Super Blue</td>
<td>470</td>
<td>GaN/SiC</td>
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<tr>
<td>Blue Violet</td>
<td>430</td>
<td>GaN/SiC</td>
</tr>
<tr>
<td>Ultraviolet</td>
<td>395</td>
<td>InGaN/SiC</td>
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</table>
LIGHT SOURCES

Halogen lamp
- Continuous spectrum: depends on temperature
- For 3400K maximum at 900 nm
- Lower intensity at shorter wavelengths
- Very strong in IR

Mercury Lamp (HBO)
- Most of intensity in near UV
- Spectrum has a line structure
- Lines at 313, 334, 365, 406, 435, 546, and 578 nm

Xenon lamp (XBO)
- Even intensity across the visible spectrum
- Has relatively low intensity in UV
- Strong in IR

Metal halide lamp (Hg, I, Br)
- Stronger intensity between lines
- Stable output over short period of time
- Lifetime up to 5 times longer
FLUORESCENCE MICROSCOPY

Staining strategies
LABELLING CELLULAR COMPONENTS

Organelle specific dyes/molecules:
- Nucleus: DAPI, Hoechst.
- Mitochondria: DiOC₆, MitotrackerRed/Green
- Endoplasmic Reticulum: DiOC₆, ER tracker
- Golgi Apparatus: BODIPY FL

Advantage: easy to use, availability
Disadvantage: limited specificity
SMALL MOLECULE STAINING

Mitotracker

LysoSensor

DAPI
ANTIBODIES AS PROBES

Advantages: high-specificity
Disadvantages: availability
Major limitation: Targeting in live cells.
GREEN FLUORESCENT PROTEIN

Aequorea victoria (Jellyfish)

Chemistry Nobel price 2008

Osamu Shimomura
Martin Chalfie
Roger Y. Tsien
GREEN FLUORESCENT PROTEIN

The fluorescent protein palette: tools for cellular imaging.
Richard N. Day and Michael W. Davidson
APPLICATIONS OF FLUORESCENT PROTEINS (FP)

Table 1 | Properties of the best FP variants

<table>
<thead>
<tr>
<th>Class</th>
<th>Protein</th>
<th>Source laboratory (references)</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
<th>Brightness</th>
<th>Photostability</th>
<th>pKa</th>
<th>Oligomerization</th>
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<tbody>
<tr>
<td>Red</td>
<td>mCherry</td>
<td>Tsien (4)</td>
<td>587</td>
<td>580</td>
<td>6.3</td>
<td>&lt;4.5</td>
<td>Monomer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tdTomato</td>
<td>Tsien (4)</td>
<td>554</td>
<td>561</td>
<td>9.6</td>
<td>4.7</td>
<td>Tandem dimer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mStrawberry</td>
<td>Tsien (4)</td>
<td>574</td>
<td>606</td>
<td>28</td>
<td>&lt;4.5</td>
<td>Monomer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mScarlet</td>
<td>Evrogen</td>
<td>594</td>
<td>610</td>
<td>6.9</td>
<td>5.0</td>
<td>Dimer</td>
<td></td>
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<tr>
<td></td>
<td>DsRed-monomer</td>
<td>Clontech</td>
<td>556</td>
<td>556</td>
<td>3.5</td>
<td>4.5</td>
<td>Monomer</td>
<td></td>
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<tr>
<td>Orange</td>
<td>mOrange</td>
<td>Tsien (4)</td>
<td>548</td>
<td>552</td>
<td>49</td>
<td>5.0</td>
<td>Monomer</td>
<td></td>
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<tr>
<td></td>
<td>mK</td>
<td>MBL. Intl. (10)</td>
<td>548</td>
<td>559</td>
<td>31</td>
<td>5.0</td>
<td>Monomer</td>
<td></td>
</tr>
<tr>
<td>Yellow-green</td>
<td>mDitirme</td>
<td>Tsien (16,25)</td>
<td>516</td>
<td>529</td>
<td>59</td>
<td>5.7</td>
<td>Monomer</td>
<td></td>
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<tr>
<td></td>
<td>Venus</td>
<td>Miyawaki (1)</td>
<td>515</td>
<td>528</td>
<td>53</td>
<td>5.0</td>
<td>Weak dimer</td>
<td></td>
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<tr>
<td></td>
<td>YPet</td>
<td>Daugherty (2)</td>
<td>517</td>
<td>529</td>
<td>80</td>
<td>5.6</td>
<td>Weak dimer</td>
<td></td>
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<tr>
<td></td>
<td>mEYFP</td>
<td>Invitrogen (18)</td>
<td>514</td>
<td>527</td>
<td>51</td>
<td>6.0</td>
<td>Weak dimer</td>
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<tr>
<td>Green</td>
<td>Emerald</td>
<td>Invitrogen (10)</td>
<td>487</td>
<td>500</td>
<td>30</td>
<td>6.0</td>
<td>Weak dimer</td>
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<tr>
<td></td>
<td>EGFP</td>
<td>Clontech</td>
<td>498</td>
<td>507</td>
<td>34</td>
<td>5.0</td>
<td>Weak dimer</td>
<td></td>
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<tr>
<td>Cyan</td>
<td>GyPet</td>
<td>Daugherty (2)</td>
<td>435</td>
<td>477</td>
<td>18</td>
<td>6.0</td>
<td>Weak dimer</td>
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<tr>
<td></td>
<td>mCitrine</td>
<td>Tsien (22)</td>
<td>433</td>
<td>475</td>
<td>13</td>
<td>4.7</td>
<td>Monomer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cerulean</td>
<td>Pison (3)</td>
<td>433</td>
<td>475</td>
<td>27</td>
<td>4.7</td>
<td>Weak dimer</td>
<td></td>
</tr>
<tr>
<td>UV-excitable green</td>
<td>Cerulean</td>
<td>Grisebach (9)</td>
<td>350</td>
<td>311</td>
<td>25</td>
<td>4.7</td>
<td>Weak dimer</td>
<td></td>
</tr>
</tbody>
</table>

FLUORESCENT PROTEINS

The fluorescent protein palette: tools for cellular imaging.
TAG TECHNOLOGY

FlAsH; ReAsH
TMP-tag
SNAP-tag
HaloTag
Coumarine ligase
Flouresceine, Oxazine
Flourescein, Atto655
Tetramethylrhodamine (TMR), Atto633
TMR
Coumarin

LABELLING STRATEGIES
SUMMARY

Fixed specimens
  • Small, organelle specific molecules
    • Pro: availability, stability
    • Con: low specificity
  • Antibody labeled with fluorophore
    • Pro: flexibility, specificity
    • Con: complex production, stability

Living specimens
  • Small, organelle specific molecules
    • Pro: ease of use,
    • Con: Toxicity, permeability, specificity
  • Fluorescent Proteins
    • Pro: intrinsic labeling, known stoichiometry, flexibility
    • Con: slow, genetic tools
**FLUOROPHORES**

**BRIGHTNESS DEFINITION**

- $\varepsilon$: molar decadic extinction coefficient
  
  $$E = \varepsilon(\lambda) \times c \times d$$

  $$E = -\lg T$$

- $\text{QE}$: Quantum efficiency

  Brightness: $B = \text{QE} \times \varepsilon(\lambda)$
FLUOROPHORES
BRIGHTNESS DEFINITION
# FLOUROPHORES COMPARISON

<table>
<thead>
<tr>
<th>Dye Class</th>
<th>Examples</th>
<th>Brightness</th>
<th>Environment Sensitivity</th>
<th>Photo-stability</th>
<th>Bio-compatibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarin</td>
<td>AlexaFluor350</td>
<td>+</td>
<td>++++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>FITC</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>BOPIDY</td>
<td>BOPIDY FL</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++++</td>
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<tr>
<td>AlexaFluor</td>
<td>AlexaFluor488</td>
<td>++++</td>
<td>++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Quantum Dots</td>
<td>QDot605</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Cyanines</td>
<td>CY3</td>
<td>+++</td>
<td>++</td>
<td>++++</td>
<td>+++</td>
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<tr>
<td>Styryl Dyes</td>
<td>FM 1-43</td>
<td>++</td>
<td>++++</td>
<td>++</td>
<td>++</td>
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<td>Rhodamines</td>
<td>TRITC</td>
<td>+++</td>
<td>+++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>Fluorescent Proteins</td>
<td>EGFP</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++++</td>
</tr>
</tbody>
</table>

Table modified from: J. B Pawley, Handbook of Confocal Microscopy, 3<sup>rd</sup> edition, p. 355
Organelles and molecules can be labeled by:

- Organelle and protein specific fluorescent stains (e.g. DAPI).
- Labeling of antibodies/proteins with fluorophores.
- Autofluorescent proteins (e.g. GFPs).

Live cell imaging:

- FP (Fluorescent proteins, e.g. GFP) are the method of choice to label proteins or organelles.
- Injection of labeled antibodies is possible.
- Organelle specific stains like e.g. DAPI can be toxic for the cell.
FLUORESCENCE MICROSCOPY

Resolution
Shortest distance between two points on a specimen that can still be distinguished by the observer or camera as separate entities.

**Lateral resolution**

\[ \delta^R = 0.61 \frac{\lambda}{NA} \]

**Axial resolution**

\[ \delta^z = 2 \frac{\lambda n}{NA^2} \]

\(\lambda=540 \text{ nm}, \ NA=1.4, \ n=1.52: \) 235 nm - lateral, 838 nm - axial
POINT SPREAD FUNCTION

NA=0.8

NA=1.4
LOW PASS FILTERING

Object

Image

NA=0.8

NA=1.4
LIGHT MICROSCOPY IN LIFE SCIENCE

Lateral resolution

\[ d_{\text{min}} = \frac{\lambda_0}{2 \cdot n \cdot \sin \Theta} \]

\[ NA = n \cdot \sin \Theta \]

\[ r_{\text{Airy}} = 0.61 \frac{\lambda_0}{NA_{\text{obj}}} \]

Light Microscopy

Atom Molecule
Proteins RNA

Organelles Cells Worm Housefly Homo sapiens

1 Å 1 nm 10⁻¹⁰ m 10⁻⁹ m
1 Å

1 μm 1 mm 1 cm 1 m 10⁻⁶ m 10⁻³ m 10⁻² m

Light microscopy resolution limit

Lord Rayleigh
Ernst Abbe
SUMMARY

• Epifluorescence microscopy set-up is very sensitive.

• Ideal excitation light sources should fit the dyes in use.

• Specificity (molecules can be specifically labelled)

• Sensitivity (single molecule detection is possible)

• Fluorescence can report on the environment of the labelled molecule
MORE ABOUT FLUORESCENCE MICROSCOPY

1. Lecture
   Biomicroscopy I + II, Prof. Theo Lasser, EPFL

2. Books
   a) Principle of fluorescence spectroscopy,

3. Internet
   a) http://micro.magnet.fsu.edu
   b) Web sites of microscope manufactures
      Leica
      Nikon
      Olympus
      Zeiss

4. BIOp
   EPFL, SV-AI 0241, SV-AI 0140
   http://biop.epfl.ch/