Fluorescence, Fluorescent microscopy and probes

Sir John Frederick William Herschel, 1854: Though perfectly transparent and colorless when held between the eye and the light, or a white object, it yet exhibits in certain aspects, and under certain incidences of the light, an extremely vivid and beautiful celestial blue colour, which, from the circumstances of its occurrence, would seem to originate in those strata which the light first penetrates the liquid....
Stokes shift

A matter of time

Absorption coefficient: frequency of absorption of photons

QE: ratio of absorbed and emitted photons

Brightness: determined by absorption coefficient and QE

Saturation

Multi-photon excitation

Brad Amos, MRC, Cambridge
Polarization/Anisotropy

Why use Fluorescence probes?
- Sensitivity
- Specificity
- Analysis of location and quantity of a single component in a complex mixture
- Detection of small quantities of fluorophores and fluorescent objects below the resolution limit
- Environmental sensitivity
- Does not rely on physical properties of the specimen for contrast generation

Commonly Used Light Sources for Fluorescence Microscopy
- Mercury arc lamp
- Xenon arc lamp
- Metal Halide doped Hg-Xe Arc
- LASERs (most often used for confocal, TIRF)
**Light Sources: Mercury & Xenon Arc Lamps**

- Halogens decrease carbon deposits and slow deterioration of electrodes
- X-cite illuminator uses liquid light guide; minimal heat transfer

**Metal Halide doped Hg-Xe Arc**

<table>
<thead>
<tr>
<th>Laser lines</th>
<th><a href="http://www.repairfaq.org/Sam/lasersani.htm">http://www.repairfaq.org/Sam/lasersani.htm</a></th>
</tr>
</thead>
</table>

**Relative Output of X-Cite Metal Halide vs. HBO 100**

- Argon
- HeNe
- Krypton
Filters

- Need to reject excitation light completely
- Need to be transparent for emitted light
- Need to match spectra of dyes

Spectra of dyes:

www.zeiss.com/micro
probes.invitrogen.com/resources/spectraviewer/
www.mcb.arizona.edu/ipc/fret/

Interference Filter Design

One cavity

- Incident Light
- Reflected: Destructive Interference
- Transmitted: Constructive Interference
- Transparent layer
- Semi-reflective coatings

Filter Terminology

Center wavelength / Full bandwidth
- 525/50
- 525
- 525LP
Choose filters that maximize excitation and emission

Choose filters that separate fluorophores

Two different UV filter sets

Filter cube (after Ploem)
Faster Wavelength Selection: Multiple Band Pass Filters & Filter Wheel(s)

Multiple Band Pass Emission Filter or 2nd Filter Wheel

Multiple Band Pass Dichroic Mirror

Excitation Filter Wheel

Specimen

J.C. Waters

Multiple Band Pass Filters

Faster Wavelength Selection: Multiple Band Pass Filters & Filter Wheel(s)

Multiple Band Pass Emission Filter or 2nd Filter Wheel

Multiple Band Pass Dichroic Mirror

Excitation Filter Wheel

Specimen

J.C. Waters

Multiple band pass filters

T (%) vs Wavelength (nm)

Excitation
Dichroic
Emission

J.C. Waters

Light Source

Excitation filter

Dichroic mirror

Emission filter

Barrier filter

3

2

1

J.C. Waters

Dichroic Mirror

Excitation Filter

Emission Filter

Barrier Filter
Types of fluorescent probes

- Immunofluorescence
- Fluorescent small molecules that bind specific cellular structures
  - DNA intercalating dyes (DAPI)
- Fluorescently labeled small molecules that bind specific cellular structures
  - Fluorescent phalloidin or taxol
- Fluorescently labeled proteins
- Fluorescent proteins (GFP)
- Genetically encoded tags binding fluorescent small molecules

The ‘classic’ dyes...

The Alexa Series Emission Spectra

Conjugation of organic dyes

Chemistry/Method
Amino groups (lysine): succinimidyl ester or isothiocyanate

Example:
Dynan driven gliding of microtubules labelled with TMR on lysine residues.

Targets
- Antibodies: direct/indirect labeling (Label density)
- Proteins: labeling site unspecific
- Small molecules, i.e. phalloidin, taxol
Immunofluorescence

Direct

Indirect

Quantum dots

• nanometre-scale crystals composed of atoms of an inorganic semiconductor material

Small dyes targeting specific cellular targets

<table>
<thead>
<tr>
<th>Dye</th>
<th>Target</th>
<th>Enhancement</th>
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<tbody>
<tr>
<td>DAPI</td>
<td></td>
<td></td>
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<tr>
<td>Hoechst 33258</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td></td>
<td></td>
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<tr>
<td>Mitotracker</td>
<td>Oxidized in mitochondria in fluorescent compound</td>
<td>~20 fold enhancement</td>
</tr>
</tbody>
</table>

GFP-discovery

O. Shimomura
D.C. Prasher
M. Chalfie

http://www.connoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm
GFP structure

Other genetically encoded tags
Biarsenical-tetracysteine labeling

HaloTag
- Catalytically inactive mutant of a hydrolase that efficiently forms a covalent bond with HaloTag ligand (Promega)
Quenching and FRET

Evidence for single dye pair FRET
When Cy5 bleaches, Cy3 emission recovers

Use FRET as molecular ruler
\[ E = \frac{R_0^6}{R_0^6 + R^6} \]

FRAP

Ellenberg et al., 1997 (Lippincott-Schwartz lab)
(Fluorescence Recovery After Photobleaching)
FLIP

Lippincott-Schwartz lab
(Fluorescence Loss in Photobleaching)

Photo-activation (PA-GFP)

Excitation spectrum before (filled), and after (open) photoactivation

Photo-activation (PA-GFP)

Koehler illumination

Figure 5
Lab samples:

- Drosophila S2 cells with various GFP-fusion proteins
- Stained tissue culture cells (multi-channel fluorescence)
- Fluorescent beads (visualize point spread function, registration shift)

THANKS!

Jennifer Waters
www.micro.magnet.fsu.edu
www.mcb.arizona.edu/ipc/fret/