Advanced fluorescence microscopy techniques

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Lecture Outline

History of microscopes
Fluorescence and Fluorochromes
Fluorescence Microscope
Confocal Microscope
Applications of Confocal Microscope
"Emeralds are usually concave so that they may concentrate the visual rays. The Emperor Nero used to watch in an Emerald the gladiatorial combats."

Pliny the Elder 23-79 A.D
The first known compound microscope, made by Zacharias and Hans Janssen in the 1590's.

Antoni van Leeuwenhoek was an amateur Dutch scientist who was granted for his discoveries in microscopy and high quality, but crude optical microscopes.
The Robert Hooke’s design was a functional improvement over the traditional motif, and even included a lighting apparatus to aid in specimen illumination.

Hooke’s microscope, from an engraving in *Micrographia.*

Public domain, Wikipedia
History of Microscopy

18th Century Microscopes

Edal Anton Lefterov, Wikipedia
History of Microscopy

19th Century
**History of Microscopy**

*20th Century Microscopes*
History of Microscopy

21st Century Microscopes
fluorescent minerals
Fluorescence and Fluorophores

the term fluorescence comes from the mineral “fluorite”
Fluorescence occurs when a molecule relaxes to its ground state following excitation.

Excitation: \( S_0 + hv \rightarrow S_1 \)

Emission: \( S_1 \rightarrow S_0 + hv \)

Stokes’s shift
Detection of proteins by Immunofluorescence

Common Fluorochromes
FITC
Rhodamine
Texas Red
Cyanine dyes

Novel AlexaFluor dyes
wide spectrum, stable
brighter and bleach resistant

Maria Francia & Boris Striepen, University of Georgia, Wikipedia

Tag
Secondary Antibody
Primary Antibody
Antigen
Tissue
Slide

Jakodak, Wikipedia
Staining Organelles with Fluorochromes

Nucleus
- DAPI
- Hoechst dyes
- Ethidium Bromide
- Propidium Iodide
- Acridine Orange

Mitochondria
- Mitotracker
- Mitofluor dyes
- Nonyl acridine orange

Golgi/ER
- ER tracker
- fluorescent Ceramide
- fluorescent Sphingomyosin

Lysozme
- Lysotracker
What if my research project requires a fluorescent dye that... specifically stains lung cancer cells?
Novel fluorescent chemical discovery through combinatorial chemistry
Discovery of novel live cell permeable fluorescent chemicals

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<th>Red</th>
<th>Far-red</th>
<th>Bf</th>
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14585 compounds > microarray scanner > confocal microscopy
Novel dyes to stain plant oil bodies in live cells

B2

C6

Ferhan Ayaydin and Soujanya Kuntam
Green Fluorescent Protein: GFP

GFP is a small protein (27 kD) and the DNA sequences coding for GFP can be manipulated by recombinant DNA technology to create gene fusion.
The GFP chromophore consists of a cyclic tripeptide derived from Ser-Tyr-Gly at positions 65–67 in the protein and is only fluorescent when embedded within the fully folded, complete GFP molecule.

- EGFP: Ser65 to Thr mutation (UV to blue excitation)

- Nascent GFP is not fluorescent, since chromophore formation occurs post-translationally. The chromophore is formed by a cyclization reaction and an oxidation step at Tyr66 that requires molecular oxygen.
**YELLOW Fluorescent Protein (YFP)**
(Thr 203 to Tyr)

Citrine variant is very bright relative to EYFP and has been demonstrated to be much more resistant to photobleaching, acidic pH, and other environmental effects.

Another derivative, named **Venus**, is the fastest maturing and one of the brightest yellow variant.

**CYAN Fluorescent Protein (CFP)**
(Tyr66 to Tryptophan)

**BLUE Fluorescent Protein (BFP)**
(Tyr66 to His)

**RED Fluorescent Protein (RFP)**
A San Diego beach scene drawn with an eight color palette of bacterial colonies expressing fluorescent proteins derived from GFP and the red-fluorescent coral protein dsRed. The colors include BFP, mTFP1, Emerald, Citrine, mOrange, mApple, mCherry and mGrape.
The biggest advantage of using GFP is...
Fluorescence Microscope

upright

inverted

Masur, Wikipedia

Nuno Nogueira, Wikipedia
Fluorescence Microscope

Diagram showing the components of a fluorescence microscope: light source, excitation filter, dichroic mirror, emission filter, objective, ocular, detector, and specimen.
Mercury Arc Lamp

UV

IR

filter block

DETECTOR
Filter sets used in fluorescence microscopy

- Excitation filter
- Dichoric mirror
- Emission filter
- Filter cube
- Light coming from excitation light source
- Light to detector
Confocal laser scanning microscopy
Optical sectioning with confocal microscope
Advanced Applications of Confocal Microscopy
Protein Dynamics and Interaction

A) Bleaching techniques
FRAP, iFRAP, FLIP

B) Photoconversion techniques
PA-GFP, Kaede/Kikume, PS-CFP, EosFP, DRONPA

C) Protein-protein Interactions
FRET, BiFC
FRAP: Fluorescence Recovery After Photobleaching

Selective Laser bleaching with Laser Scanning Confocal Microscope

Fluorescence recovery
A) Bleaching techniques
FRAP, iFRAP, FLIP

FRAP: Protein Mobility Comparison

protein X                               protein Y

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A) Bleaching techniques
FRAP, iFRAP, FLIP

FRAP: Kinetics of Fluorescence Recovery

- Relative fluorescence intensity vs. time (sec)
- Half recovery: $t_{1/2}$
- Immobile fraction
- Mobile fractions
A) Bleaching techniques

FRAP, iFRAP, FLIP

Diffusion-limited fluorescence recovery

\[ f(t) = e^{-2\tau_D/t} (I_0(2\tau_D/t) + I_1(2\tau_D/t)) \]

Reaction-limited recovery

\[ f(t) = 1 - e^{-k_{off}t} \]
A) Bleaching techniques
FRAP, iFRAP, FLIP

iFRAP: Inverse FRAP

Region of interest (ROI)
bleaching laser
prebleach                     bleach                       recovery
(outside of region of interest)

dissociation parameters of molecules can be measured
A) Bleaching techniques
FRAP, iFRAP, FLIP

FLIP: Fluorescence Loss In Photobleaching

Successive Laser Bleaching

YFP

His2B

pre-bleach 1.5s 10s 40s 80s 120s

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A) Bleaching techniques
FRAP, iFRAP, FLIP

FLIP: Fluorescence Loss in Photobleaching

\[ I_{rel} = \frac{T_0 I_t}{T_t I_0} \]
A) Bleaching techniques
FRAP, iFRAP, FLIP

FLIP: Depletion Comparison

Relative intensity

depletion time (s)

Your Protein 1
Your Protein 2
You can bleach with laser but, lasers can also be used to “activate” or “photoconvert” a fluorescent protein...

Part B
Activatable and Photoconvertable fluorescent proteins: Highlighters

activation

color conversion
# Photoconversion techniques

PA-GFP, Kaede/Kikume, PS-CFP, EosFP, DRONPA

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<th>PAFP</th>
<th>Absorbance$_1$ (nm)</th>
<th>Emission$_1$ (nm)</th>
<th>Absorbance$_2$ (nm)</th>
<th>Emission$_2$ (nm)</th>
<th>Photoconversion wavelength</th>
<th>Reversibility</th>
<th>Brightness$_1^*$</th>
<th>Brightness$_2^*$</th>
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<td>518</td>
<td>572</td>
<td>560</td>
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<td>none</td>
<td>2.64X</td>
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<td>Eos (protein)</td>
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<td>516</td>
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<td>?</td>
<td>490 nm</td>
<td>reversible, 390 nm</td>
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<td>580</td>
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<td>550 nm</td>
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<td>583</td>
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<td>green</td>
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*Brightness values are relative to EGFP.
C) Protein-protein Interactions
FRET, BiFC

FRET: Fluorescence Resonance Energy Transfer

energy transfer in a non-radiative fashion, through long-range dipole-dipole interactions (e.g. tuning forks)

distance should be 10nm or less

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C) Protein-protein Interactions
FRET, BiFC

protein-protein interactions

protein conformation changes

FRET pairs
CFP/YFP
GFP/RFP
NewFP/NewestFP
C) Protein-protein Interactions
FRET, BiFC

(YFP) conjugated protein is in close proximity
(YFP) conjugated protein is distant

FRET Efficiency, Förster distance
C) Protein-protein Interactions

FRET, BiFC

BiFC: Bimolecular Fluorescence Complementation
Discussion:

What happens if we use half GFP, half CFP and half YFP?

Multicolor Complementation
Is that possible?
The Future is Fluorescent!
Thank you for your attention!

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