Fluorescence, fluorochromes and confocal microscopy
Outline

Phenomics and microscopy
History of microscopes
Fluorescence and Fluorophores
Fluorescence Microscope
Confocal Microscope
Advanced Applications of Confocal Microscope
Plant phenomics, fluorescence and confocal microscopy

Location and expression level of a “gene of interest”

Leaf surface, epidermis morphology, stomata density

Cell division / cell elongation / cell differentiation
"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
History of Microscopy

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.
History of Microscopy

This middle seventeenth century version of the simple one-lens microscope uses a sliding rod to focus the specimen.

The Hooke design was a functional improvement over the traditional motif, and even included a lighting apparatus to aid in specimen illumination.
History of Microscopy

18th Century Microscopes

Cuff’s Microscope (circa mid 1700s)

Jones’ Most Improved Compound Microscope (circa late 1700s)
History of Microscopy

Hugh Powell Microscope (circa 1841-1862)

The "Monkey" Microscope (circa 1850)

19th Century Microscopes
History of Microscopy

Olympus Vanox Microscope (circa 1971)

Nikon Diaphot Inverted Tissue Culture Microscope (circa 1985)

20th Century Microscopes
Cell division cycle in wheat root tips

Fluorescence microscopy image of a dividing alfalfa (Medicago sativa) cell (Microtubules, chromosomes)
fluorescent minerals
the term fluorescence comes from the mineral "fluorite"

Fluorescence occurs when a molecule relaxes to its ground state following excitation.

Excitation: $S_0 + h\nu \rightarrow S_1$

Emission: $S_1 \rightarrow S_0 + h\nu$

Stoke’s shift
Detection of proteins by Immunofluorescence

Common Fluorochromes
FITC
Rhodamine
Texas Red
Cyanine dyes

AlexaFluor dyes
wide spectrum, stable brighter and bleach resistant
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
My phenomics project requires a fluorescent dye that….

… a fluorescent dye that specifically stains
leaf oils of Cannabis

… a fluorescent dye that specifically stains
the root hairs of Arabidopsis
Novel fluorescent chemical discovery through combinatorial chemistry
Discovery of novel live cell permeable fluorescent chemicals

14585 compounds > microarray scanner > confocal microscopy
Novel dyes to stain plant oil bodies in live cells
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.

<table>
<thead>
<tr>
<th>promoter</th>
<th>GFP</th>
<th>your favorite protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>promoter</td>
<td>your favorite protein</td>
<td>GFP</td>
</tr>
</tbody>
</table>

* Aequorea victoria
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 (near-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.
**Fluorescent Protein Color Variants**

**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)
The biggest advantage of using GFP is...
Fluorescence Microscope

upright

inverted
Fluorescence Microscope

- Objective lens
- Dichroic mirror
  Reflects shorter wavelength
- Filter that selects emission wavelength
- Filter that selects excitation wavelength

Specimen
Filter Sets of Fluorescence Microscopy
Confocal Laser Scanning Microscopy

Immature pollen and endothecium cells of *Tradiscantai virginiana*
Confocal Laser Scanning Microscopy
Optical Sectioning with confocal microscopy

conventional  |  confocal
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
A) Bleaching techniques:
FRAP, iFRAP, FLIP

**FRAP: Fluorescence Recovery After Photobleaching**

Selective Laser bleaching with Laser Scanning Confocal Microscope

Fluorescence recovery
FRAP: Protein Mobility Comparison

protein X

protein Y

A) Bleaching techniques
FRAP, iFRAP, FLIP
FRAP: Kinetics of Fluorescence Recovery

A) Bleaching techniques
FRAP, iFRAP, FLIP

FRAP: Kinetics of Fluorescence Recovery

- Immobile fraction
- Mobile fractions

Graph showing a plot of relative fluorescence intensity against time (sec). The graph illustrates the kinetics of fluorescence recovery after photobleaching (FRAP), with a marked half recovery time ($t_{1/2}$).
iFRAP: inverse FRAP

bleach everything else but the 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
A) Bleaching techniques
FRAP, iFRAP, FLIP

FLIP: Fluorescence Loss in Photobleaching

Depletion time (s)
Relative intensity

Histone2B

YFP

His2B

YFP
A) Bleaching techniques
FRAP, iFRAP, FLIP

FLIP: Depletion Comparison

![Graph showing depletion comparison for Your Protein 1 and Your Protein 2 over time.](image)
You can bleach with laser but, lasers can also be used to “activate” or “photoconvert” a fluorescent protein...

Activatable and Photoconvertable fluorescent proteins: Highlighters

- **Activation**
- **Color Conversion**
B) Photoconversion techniques

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

Measure diffusion of green before and after.
B) Photoconversion techniques
PA-GFP, Kaede/Kikume, PS-CFP, EosFP, DRONPA

fluorescent proteins from anemones and coral

cleavage of peptide backbone changes the chromophore
B) Photoconversion techniques
PA-GFP, Kaede/Kikume, PS-CFP, EosFP, DRONPA

Cyan colored before conversion turns green after intense violet illumination.
B) Photoconversion techniques
PA-GFP, Kaede/Kikume, PS-CFP, EosFP, DRONPA

Green colored before conversion turns red after intense violet illumination. Activated (red) EosFP, not activated (green) EosFP.
B) Photoconversion techniques
PA-GFP, Kaede/Kikume, PS-CFP, EosFP, DRONPA

Protonated and nonprotonated forms of the chromophore
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
C) Protein-protein Interactions
FRET, BiFC

- prt A
- prt B

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

Distance and Energy Transfer Efficiency

Distance (r, in Nanometers)

Energy Transfer Efficiency (Percent)

Förster Distance

R0

50 Percent Transfer Efficiency
BiFC: Bimolecular Fluorescence Complementation

GFP

my protein

your protein
BiFC: Bimolecular Fluorescence Complementation

BiFC is easier than FRET as it requires less complicated setup and equipment. However FRET is more suitable for reversible/dynamic interactions.
Future is Fluorescent!