MRC Laboratory of Molecular Biology

1. Labelling for Fluorescence Microscopy

2. Förster Resonance Energy Transfer

3. Light Sheet Microscopy

Ben Sutcliffe
- Light Microscopy Facility -
28th January 2020

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1. Labelling for Fluorescence Microscopy

2. Förster Resonance Energy Transfer

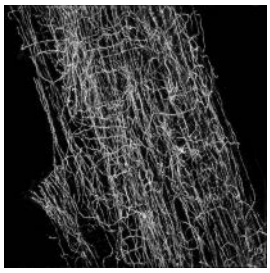
3. Light Sheet Microscopy

Ben Sutcliffe
- Light Microscopy Facility -
31st January 2019

2

Why fluorescently label biomolecules?

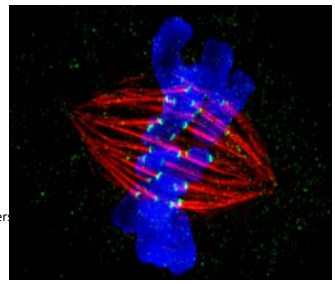
- Specificity and Sensitivity.



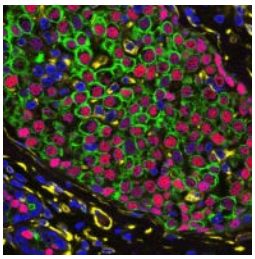
Microtubule labelling with EMTB-3xGFP in contracted heart muscle myocyte

Lab of Ben Prosser, University of Pennsylvania (http://hosting.med.upenn.edu/prosserlab/)
Robison, P., Science. 2016

- Ease of multiplexing.



Chromatin (blue), Mitotic spindle (red), Kinetochore (green).
Image by Jane Stout, Indiana Univers



Breast cancer section.
DAPI (Blue)
Cytokeratin 8/18 (Green)
Vimentin (Yellow)
ER (Red)

Robertson et al., BMC Cell Biology, 2008

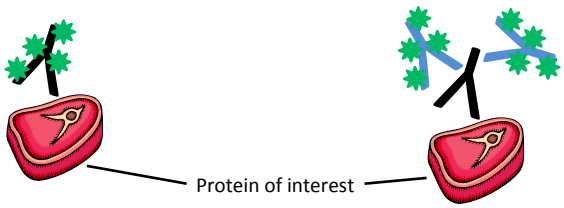
- Localisation and interaction

3

How?
- Immunofluorescence (IF)

- Immunohistochemistry (**tissues**) and immunocytochemistry (**cultured cells**).
- Requires antibody directed against your protein of interest – **mono**clonal or **poly**clonal.
- Fixation method important determinant of antibody recognition.
- Permeabilisation necessary if target protein is intracellular.

Direct **Indirect**



- More certainty of specificity.
- Quicker staining.

- Signal amplification.
- Colour flexibility.
- More antibodies = more controls.

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Chemical Fixation

- The ideal fixation method should immobilize cellular macromolecules, whilst retaining cellular/ subcellular architecture and permitting access of antibodies to their target antigens.

	Organic solvents	Cross-linkers
Examples	Alcohols, acetone.	Paraformaldehyde, glutaraldehyde, glyoxal
Action	Remove lipids and dehydrate cells, thereby precipitating proteins and exposing epitopes.	Chemically interlinks cellular contents.
Advantages	Good antibody accessibility and epitope presentation.	Good retention of subcellular architecture.
Disadvantages	Cell shrinkage renders spatial information inaccurate.	Permeabilisation with detergents or organic solvents required for antibody access to intracellular antigens.

- The most suitable fixation method will depend on what you are trying to detect and where it is.
- Antibody datasheets should give information on compatible fixation methods for epitope presentation. If your antibody is not commercial then you should test a range of conditions.

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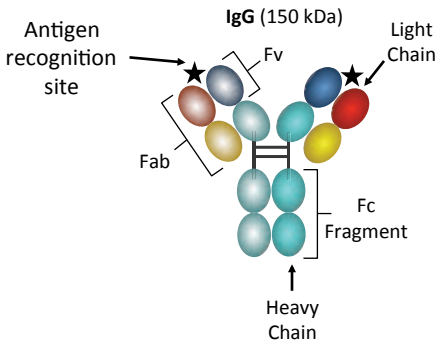
Antibodies

Conventional Antibody and Variants:

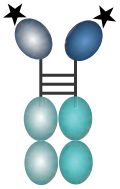


Fab (50 kDa)

ScFv (25 kDa)

- Better access to antigen.
- Labelling density high and label size small – Ideal for super-resolution microscopy.

Camelid IgG (95kDa)



Nanobody (12-15 kDa ~2nm)

Heavy Chain Antibody and Nanobody:

Fab = antibody binding fragment
Fv = variable fragment
Fc = constant fragment
ScFv = single chain variable fragment

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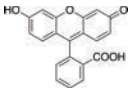
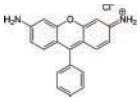
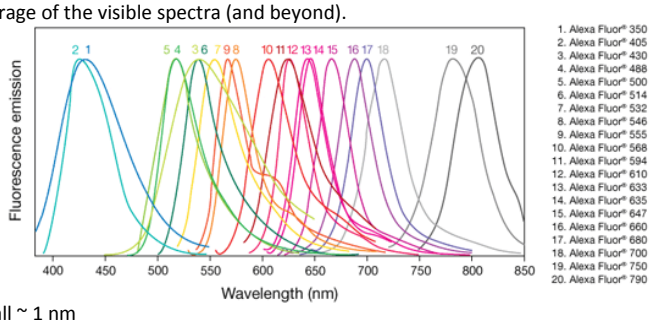
Labelling Antibodies - Organic Dyes

- First generation dyes:
  - Fluorescein (Green): low photostability and pH sensitivity.
  - Rhodamine (Orange-Red): low water solubility and dimerization-induced fluorescence quenching.





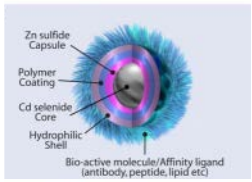
- Modern dyes (developed in the last 15-20 years) tend to have much better spectroscopic and biochemical properties e.g. boratedipyrromethene (BODIPY), Cyanine (CY), ATTO and Alexa fluor series.
- Full coverage of the visible spectra (and beyond).



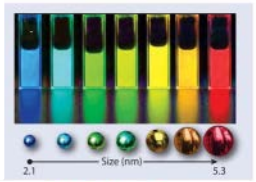
- Very small ~ 1 nm

Images from Wikipedia and Invitrogen websites.

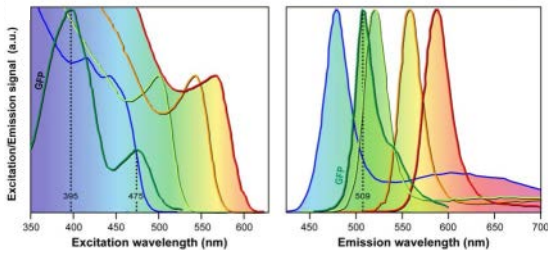
Quantum Dots



- Inorganic fluorescent **nanoparticles** (2-50 nm).
- Conjugation to biomolecules determines specificity e.g. streptavidin or antibodies.
- **Very bright** and highly **photostable**.



- Large absorption range combined with narrow emission spectrum – Ideal for multiplexing.
- **Electron-dense** and can therefore be used for correlated fluorescence and electron microscopy.
- Functionalization can significantly increase size (up to 100 nm), which can affect access to confined compartments e.g. vesicles or synapses.
- May exhibit **blinking** and high propensity for **aggregation**.

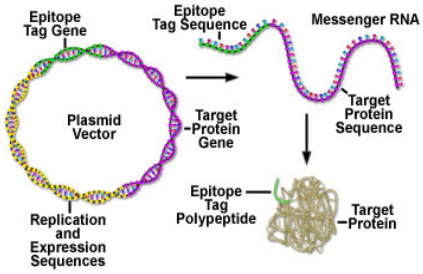


Ishikawa-Ankerhold, Ankerhold and Drummen Molecules, 2012

No Antibody…Use an Epitope Tag

What if you don't have an antibody directed against your protein of interest that works well for IF staining?

One solution is to express your protein **fused** to a short **epitope tag** for which high affinity antibodies are commercially available.



- Examples of epitope tags include:

- **Flag-tag** (sequence - DYKDDDDK): The first epitope tag to be published and therefore patented by Sigma-Aldrich. A designed peptide.

- **HA-tag** (sequence - YPYDVPDYA): Fragment 108-106 of human influenza hemagglutinin, which is the antigenic glycoprotein responsible for the binding of flu virus to the surface of cells.

- **V5-tag** (sequence – GKPIPNPLLGLDST): Derived from a small epitope (Pk) present on the P and V proteins of simian virus 5 (SV5).

- **Myc-tag** (sequence - EQKLISEEDL): Fragment of the myc transcription factor and proto-oncogene.

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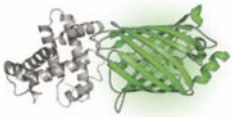
Labelling Without Antibodies

Fluorescently label the protein directly

Genetic fusion with fluorescent protein



Genetic encoded non-fluorescent tags

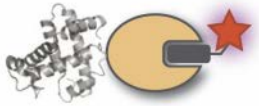
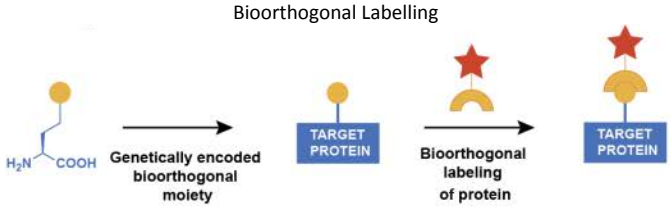


Image modified from Romanini and Cornish, Nature Chemistry, 2012

Bioorthogonal Labelling



Davis and Chin, Nature Reviews Molecular Cell Biology, 2012

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In Vitro labelling of reactive groups

- It is possible to **covalently** link fluorophores to **purified** proteins using reactive derivatives of organic fluorophores that selectively bind to **functional groups** present in the target protein.
- Common reactive groups include:

- **Isothiocyanate derivatives** such as fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) react with primary amines e.g. **lysine** sidechains.
- **Succinimidyl esters** such as NHS-fluorescein/rhodamine also react with **primary amines** but are preferred to isothiocyanates due to **greater specificity** and more **stable** linkage.
- **Maleimide-activated** fluorophores such as fluorescein-5-maleimide react with the **cysteine** side chain –SH group.

Warnings:

- Over labelling can cause protein precipitation – optimisation of conditions is required.
- Following the labelling reaction, it is necessary to remove any unreacted fluorophore. This is usually accomplished by size exclusion chromatography.

Usage:

- Labelled protein can be used for **cuvette-based fluorescence** measurements, ***in vitro* reconstruction** experiments or introduced into living cells by **microinjection**.

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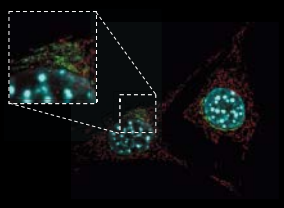
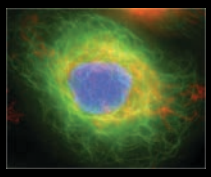
Cellular compartment dyes

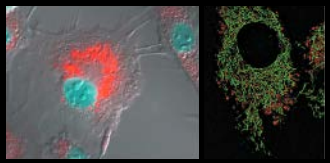


Fluorescent Ceramide Analogs
Golgi
various colours, live/fixed cell
(ThermoFisher.com)

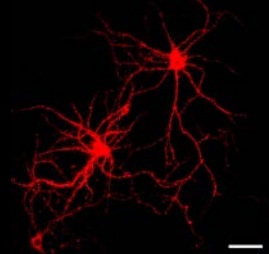


Tubulin Tracker™
Tubulin, available in various colours, live cell
(ThermoFisher.com)

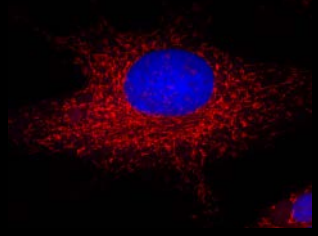
ER-tracker
Endoplasmic reticulum, various colours, live cell
(ThermoFisher.com)



Lysotracker
Lysosomes, live/fixed cell
(Thermofisher.com)



DiI - carbocyanine plasma membrane dye.
Fixed Cultured Primary hippocampal neurons.
(Cheng, C., et al, 2014 Front Neuroanat)



Mitotracker
Mitochondria, live/fixed cell
(Image: Ben Sutcliffe)

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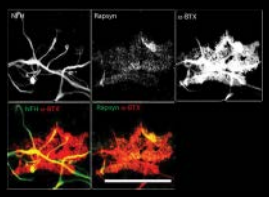
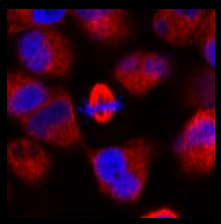
High affinity natural interactions

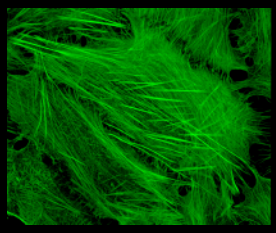


Alpha-BTX – AChR (synapses)
(Vilmont et al Development 2016)

Vitamin binding – Streptavidin-Biotin
(signal boosting strategy)



Paclitaxel derivatives – microtubles
(SiR-Tubulin, Spirochome.com)



Phalloidin conjugates – actin
(Wikipedia.org, image: Howard Vindin)

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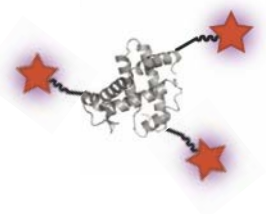
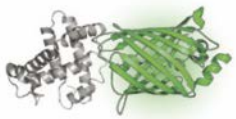
Labelling Without Antibodies

Fluorescently label the protein directly



Genetic fusion with fluorescent protein



Genetic encoded non-fluorescent tags

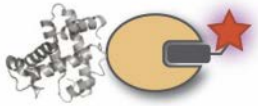
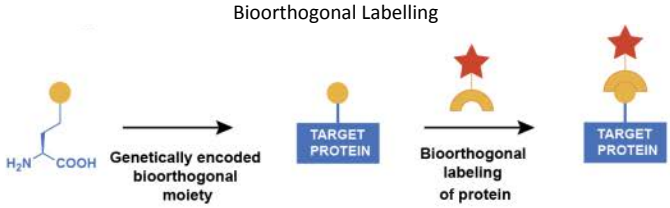


Image modified from Romanini and Cornish, Nature Chemistry, 2012

Bioorthogonal Labelling



Davis and Chin, Nature Reviews Molecular Cell Biology, 2012

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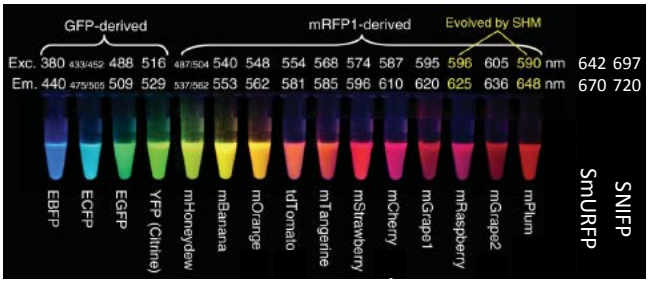
Fluorescent Proteins (FPs)

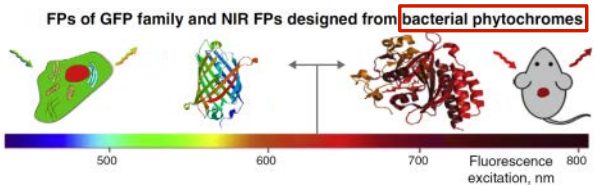
- Genetically encoded fluorescent tag (~27-35 kDa) - perfect specificity.
- Transfection, electroporation, binary systems
  - Wild type and tagged protein present (perhaps not ideal)
    - CRISPR/Cas9



Tsien, FEBS Letters, 2005, Rodriguez, Nat Met, 2016

FPbase

https://www.fpbase.org/



Current Opinion in Chemical Biology, 2015 and Shiro Lab at RIKEN, Japan

Aequorea victoria jellyfish.
GFP (www.dailyuw.com)

Discosoma sp.
mRFP (www.coralbiome.com)

Rhodopseudomonas palustris
NIR FPs (www.phylomedb.org)

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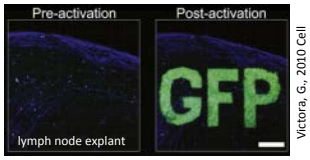
Optical Highlighter FPs

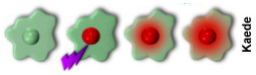
- Photoactivatable FPs:



Victora, G., 2010 Cell

- Start non-fluorescent (dark) and can be switched on by illumination at a specified wavelength e.g. PAGFP, PATagRFP and PAmCherry.

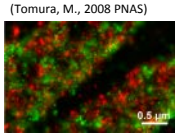
- Photoconvertible FPs:



Newborn mouse, Kaede in all cells. UV turns cells Green, so can be tracked (Tomura, M., 2008 PNAS)

- Emission spectra can be shifted (usually to longer wavelength) by illumination at a specified wavelength e.g. Kaede, Eos and Dendra (all green-to-red).

- Photoswitchable FPs:



PALM image of Paxillin-Dronpa in red
H. Shroff, H. Hess, Janelia Farm, USA.

- Emission spectra can be reversibly switched off and on by illumination at specified wavelength(s) e.g. Dronpa (green).

- IrisFP is a photoconvertible/switchable FP derived from Eos. Like Eos it is irreversibly converted from green-to-red. However, in its red form it can also be cycled between on and off states.

➔ Optical highlighter FPs are ideal tools for investigating protein dynamics and PALM (Jon's talk Thursday)

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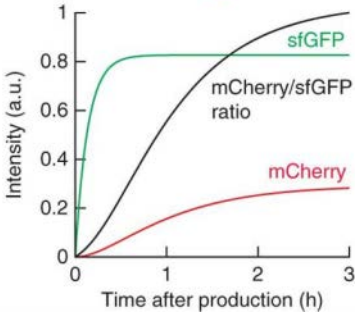
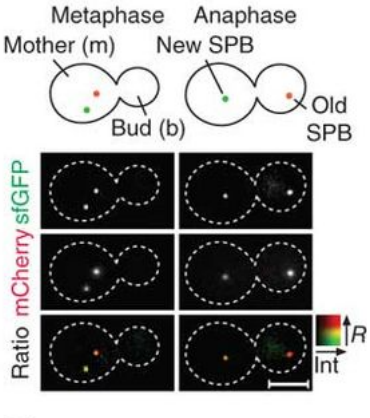
Schematic modified from Olympus website.

Tandem Fluorescent timer proteins

- Can be used to assess aging of a protein pool
- Works by calculating ratio of fluorescence between **fast** and **slow** maturing FPs





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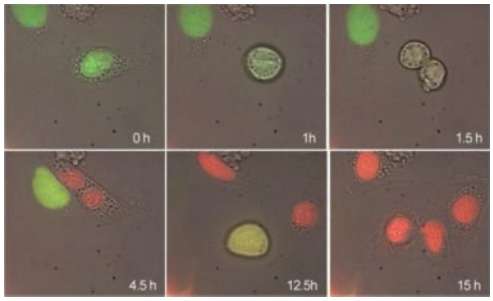
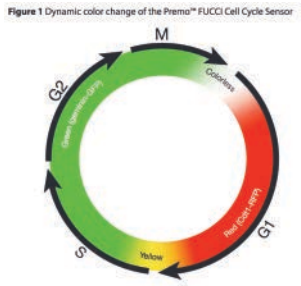
Khmelinskii, A. et al, 2012 Nat Biotechnol

Cell Cycle labelling





Premo™ FUCCI Cell Cycle Sensor
Green (G2/M) to Red (G1) to Yellow (S phase)
ThermoFisher
https://www.thermofisher.com/order/catalog/product/P36237

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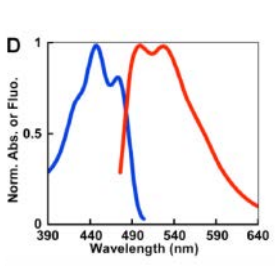
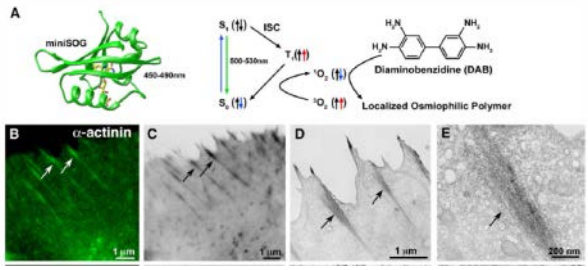
Correlative Fluorescence and EM – MiniSog

- Protein tag - **Mini Singlet Oxygen Generator** (MiniSOG) – 15.5 kDa fluorescent flavoprotein engineered from Arabidopsis phototropin 2.
- Illumination of MiniSOG generates sufficient singlet oxygen to locally catalyse the polymerization of diaminobenzidine (DAB) into an insoluble osmiophilic reaction product resolvable by EM.
- Permits the use of strong aldehyde fixation, which optimally preserves ultrastructure but can hinder access to antibodies and gold nanoparticles. Also doesn't require permeabilisation.
- Shown to work in mammalian cells, intact nematodes, Drosophila and rodents.

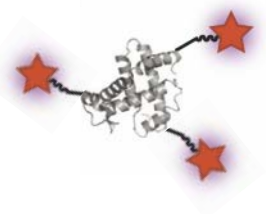




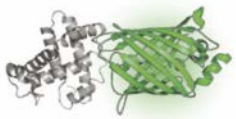
Images from Shu *et al.*, PLoS Biology, 2011

Labelling Without Antibodies

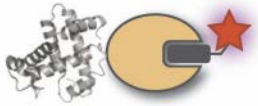
Fluorescently label the protein directly



Genetic fusion with fluorescent protein

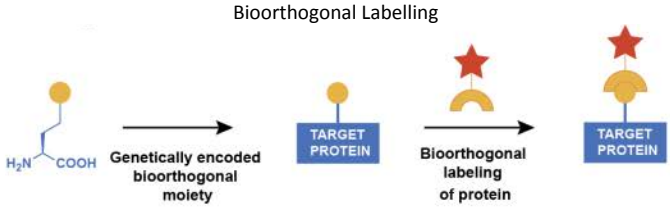


Genetic encoded non-fluorescent tags



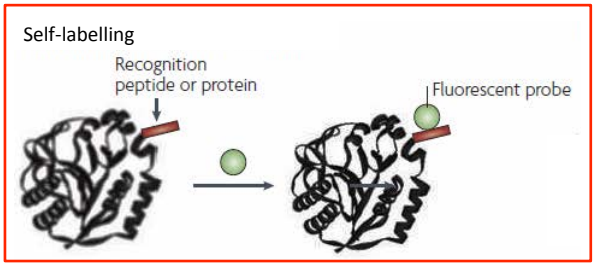
Images modified from Romanini and Cornish, Nature Chemistry, 2012

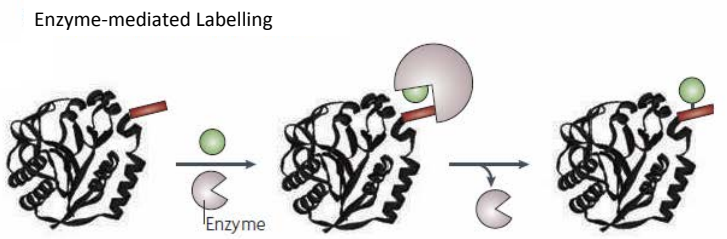
Bioorthogonal Labelling



Davis and Chin, Nature Reviews Molecular Cell Biology, 2012

Targeting Small-Molecule Probes to Genetically Encoded Tags





Fernandez-Suarez and Ting, Nature Reviews Molecular Cell Biology, 2008

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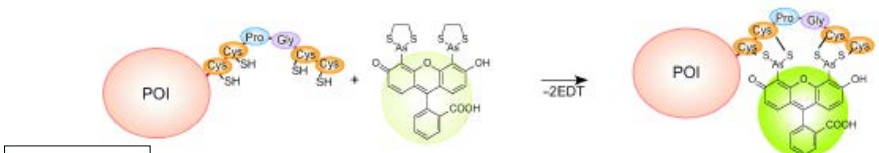
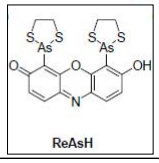
FlAsH/ReAsH (Invitrogen)

- FlAsH was the first example of a **chemical surrogate** to fluorescent proteins for labelling proteins in living cells with organic fluorophores.
- FLasH and ReAsH are **membrane permeable** derivatives of fluorescein (green) and resorufin (red).
- They are initially nonfluorescent, but become fluorescent when they bind to recombinant proteins containing a **tetracysteine** tag (CCXXCC).
- **Non-specific labelling** of thiol-rich biomolecules e.g. cysteine rich proteins.





Soh, Sensors, 2008

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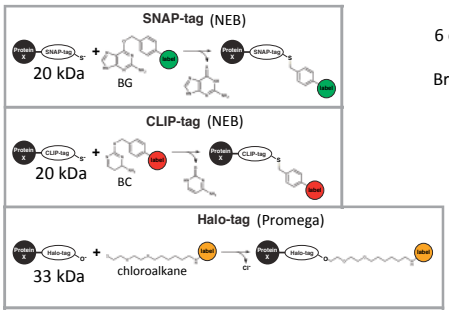
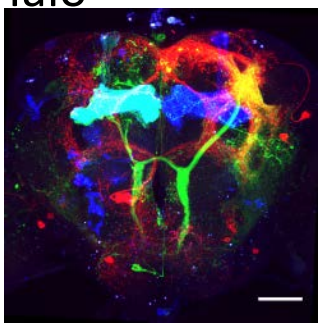
Chemical Labelling

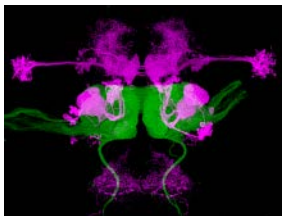
SNAP, CLIP and Halo



6 channel chemical labelling using Brainbow approach



- In fixed tissue (slices or whole organs) these tags allow for very rapid staining, in the order of 10 minutes vs 2 days for antibody labelling.



Orthogonal labelling of different cell populations

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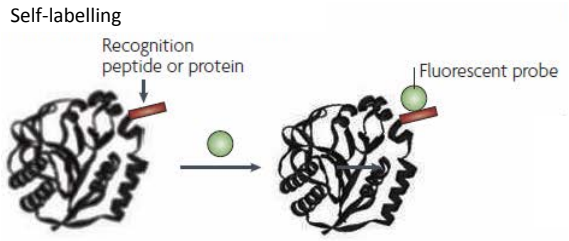
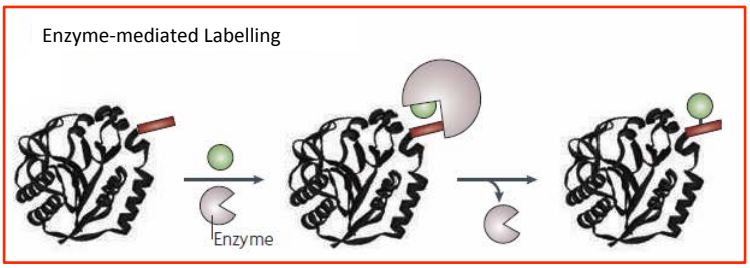
Targeting Small-Molecule Probes to Genetically Encoded Tags

Self-labelling





Fernandez-Suarez and Ting, Nature Reviews Molecular Cell Biology, 2008

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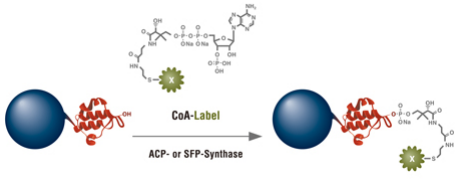
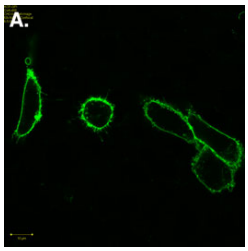
ACP- and MCP-tags (NEB)

- NEB also sell ACP- and MCP-tags, which are **smaller** than SNAP/CLIP/Halo-tags at 9 kDa but require an additional **enzyme** to **catalyse** the labelling step.
- The ACP/MCP-tag substrates are **non-cell-permeable** and are therefore only suitable for the labelling of surface proteins.
- The same fluorescently-conjugated substrates will label both ACP- and MCP-tags.
- **Specificity** is determined by the **synthase** used for labelling. *However*, while ACP-Synthase will modify predominantly the ACP-tag, **SFP-Synthase** will label **both** ACP- and MCP-tags. Therefore, **<u>sequential</u>** incubation is required for dual-colour labelling.



Enzymatic Tag					
	Applications	NEB #	Excitation*	Emission*†	Size
ACP/MCP tag	Non-cell-permeable				
	CoA 488	#S9348S	506	526	50 nmol
	CoA 547	#S9349S	554	568	50 nmol
	CoA 647	#S9350S	660	673	50 nmol



Images from NEB website.

Other Genetic Tag-Fluorescent Ligand-Based Labelling Strategies

Method	Tag	Tag size (aa)	Substrate derivative	Enzyme used	Covalent attachment	Commercial availability
Self-labeling						
Tetraserine	SSPGSS	6–10	Bisboronic acid		Yes	
Tetracysteine	CCPGC	6–12	Biarsenical		Yes	Invitrogen (Lumio™)
SNAP-tag®	hAGT	182	Benzylguanine		Yes	New England Biolabs
CLIP-tag™	hAGT	182	Benzylcytosine		Yes	New England Biolabs
HaloTag®	DhaA	296	Chloroalkane		Yes	Promega
TMP-tag	eDHFR	157	TMP		No	Active Motif (LigandLink™)
A-TMP-tag	eDHFR:L28C	157	A-TMP		Yes	
Enzyme-mediated labeling						
ACP-tag	ACP	77	CoA	AcpS or Sfp	Yes	New England Biolabs
MCP-tag	ACP:D36T/D39G	77	CoA	Sfp	Yes	New England Biolabs
A1	GDSLDMLEWSLM	12	CoA	AcpS	Yes	
S6	GDSLSWLLRLLN	12	CoA	Sfp	Yes	
Biotin ligase	GLNIFEAQKIEH	15	Biotin, ketobiotin	BirA	Yes	
Lipoic acid ligase	GFEIDKVWYDLDA	12–17	Lipoic acid	LplA	Yes	
PRIME	GFEIDKVWYDLDA	13	7-hydroxycoumarin	LplA:W37V	Yes	
SorTag	LPXTG	6	Gly_3	Sortase A	Yes	

Harald Tschesche *et al.*, Methods in Protein Biochemistry, 2011

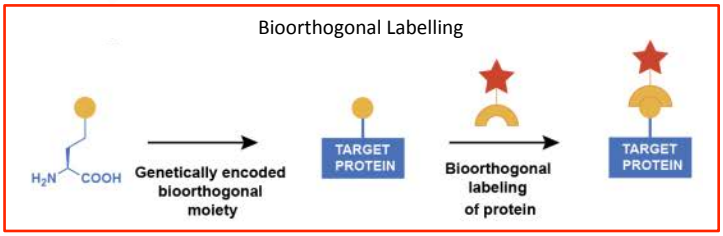
Labelling Without Antibodies

Fluorescently label the protein directly

Genetic fusion with fluorescent protein

Genetic fusion with a non-fluorescent peptide/protein that binds a fluorescent ligand

Image modified from Romanini and Cornish, Nature Chemistry, 2012



Davis and Chin, Nature Reviews Molecular Cell Biology, 2012

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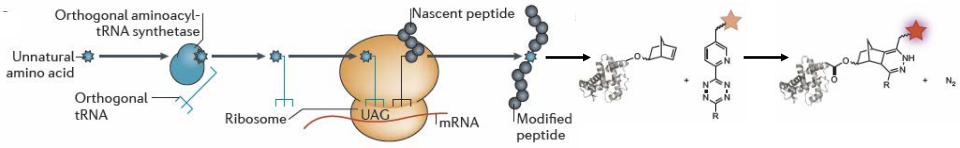
Bioorthogonal Labelling

- Bioorthogonal reactions between two functional groups that do not react with biological molecules **but** selectively react with each other e.g. tetrazines and strained alkenes/alkynes.
- Genetic code expansion and reprogramming have facilitated the site-specific incorporation of **unnatural amino acids** bearing bioorthogonal functional groups into proteins in bacteria, mammalian cells and animals.
- Incorporating unnatural amino acids requires orthogonal **aminoacyl-tRNA synthetase/tRNA pair**.



Davis and Chin, Nature Reviews Molecular Cell Biology, 2012

Romanini and Cornish, Nature Chemistry, 2012

- Tetrazine linker quenches fluorescence of some fluorophores prior to reaction with the unnatural amino acid, thereby reducing background fluorescence.
- Protein expression can be tightly controlled by regulating amino acid availability.

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Summary

Labelling for Fluorescence Microscopy

- You can do multiplexing with fluorescent labelling.
- Immunofluorescence provides us with specificity and sensitivity (fixed samples).
- Chosen **Fixation** method is important.
- There are other techniques available that allow **live cell** imaging - FPs.
- You can also do **rapid** chemical labelling in thick tissues.

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1. Labelling for Fluorescence Microscopy

2. **F**örster **R**esonance **E**nergy **T**ransfer (**F**luorescence **R**esonance **E**nergy **T**ransfer) **FRET**

3. Light Sheet Microscopy

Mathias Pasche
- Light Microscopy Facility -

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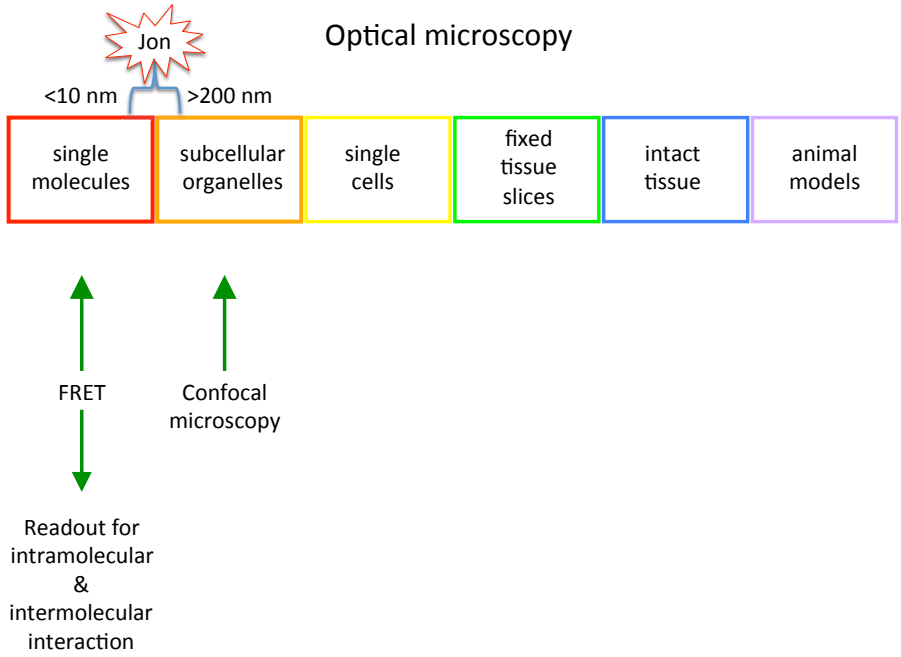

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FRET

Förster **R**esonance **E**nergy **T**ransfer
or
Fluorescence **R**esonance **E**nergy **T**ransfer

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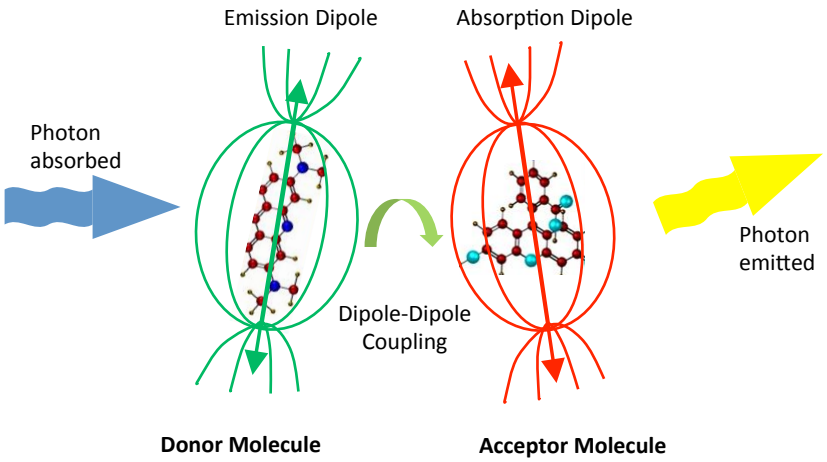
Resonance Energy Transfer



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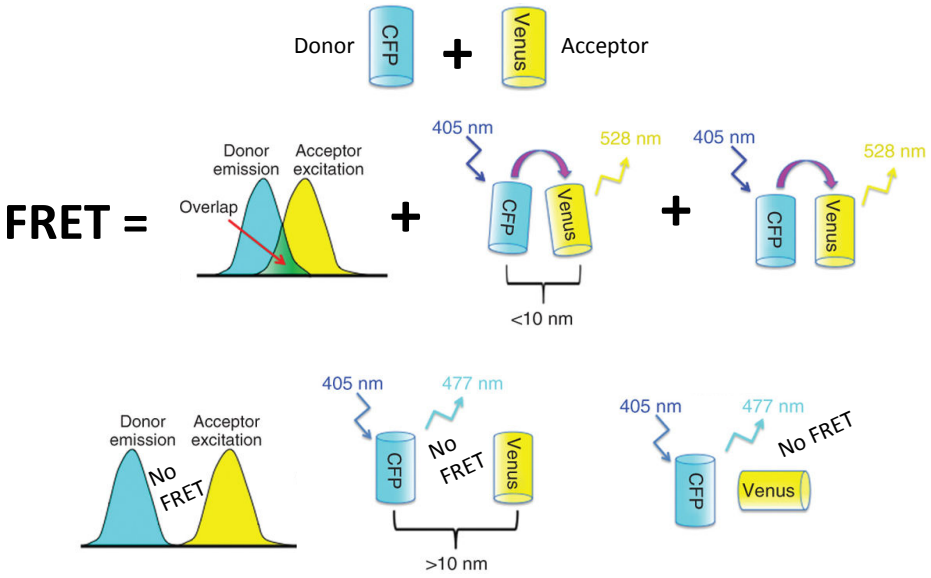
FRET requirements



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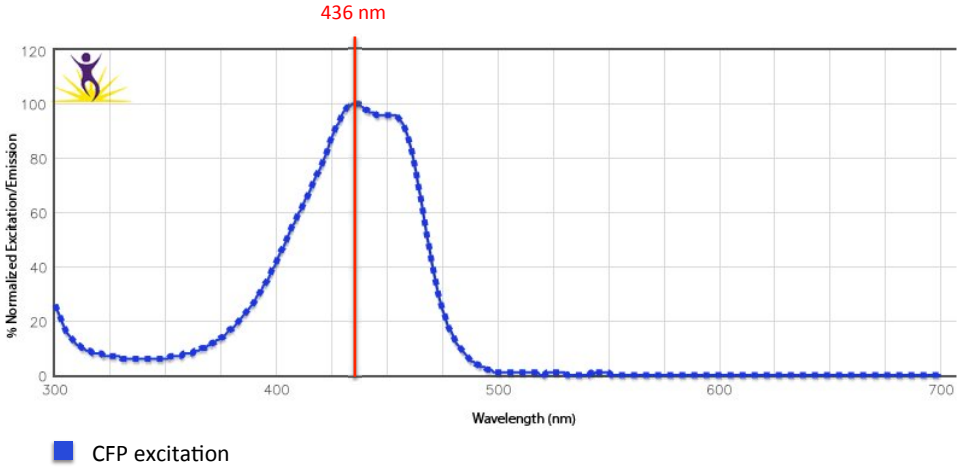
Adapted from Broussard et al., Nature Protocols (2013)

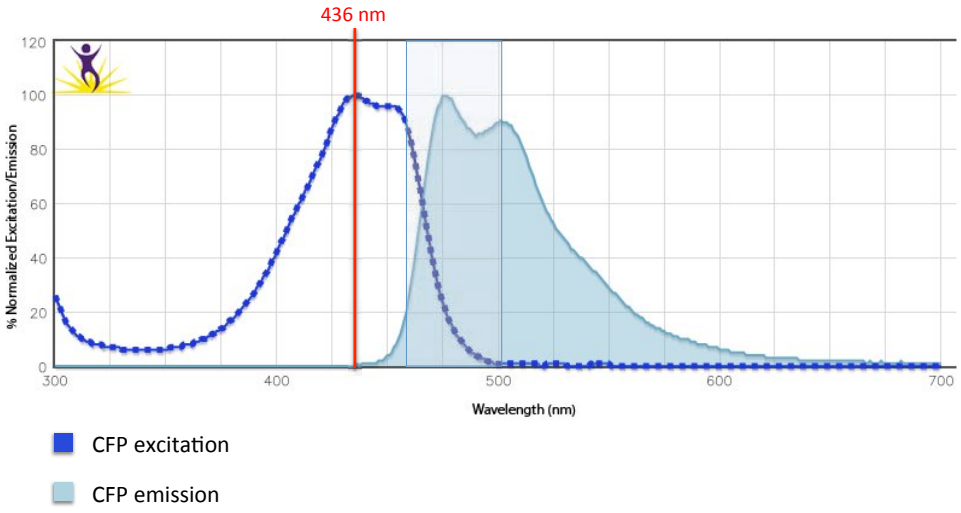
Spectral characteristics of Resonance for FRET: CFP-to-YFP



35

https://www.biolegend.com/spectraanalyzer

Spectral characteristics of Resonance for FRET: CFP-to-YFP



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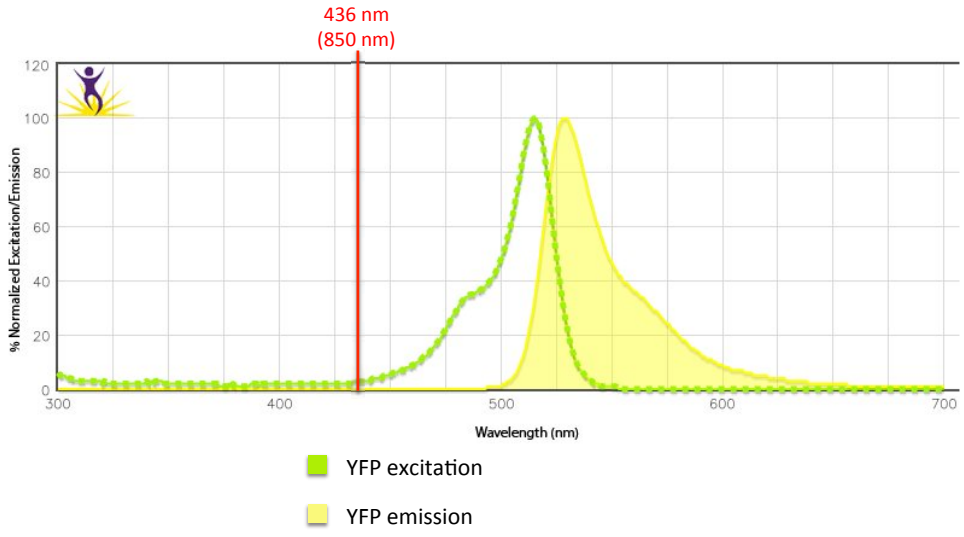
https://www.biolegend.com/spectraanalyzer

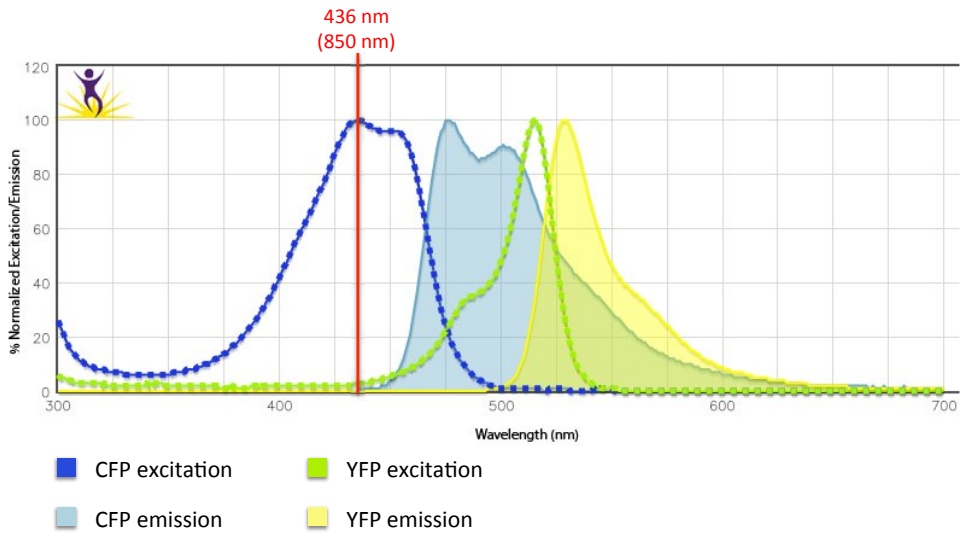
Spectral characteristics of Resonance for FRET: CFP-to-YFP



37

https://www.biolegend.com/spectraanalyzer

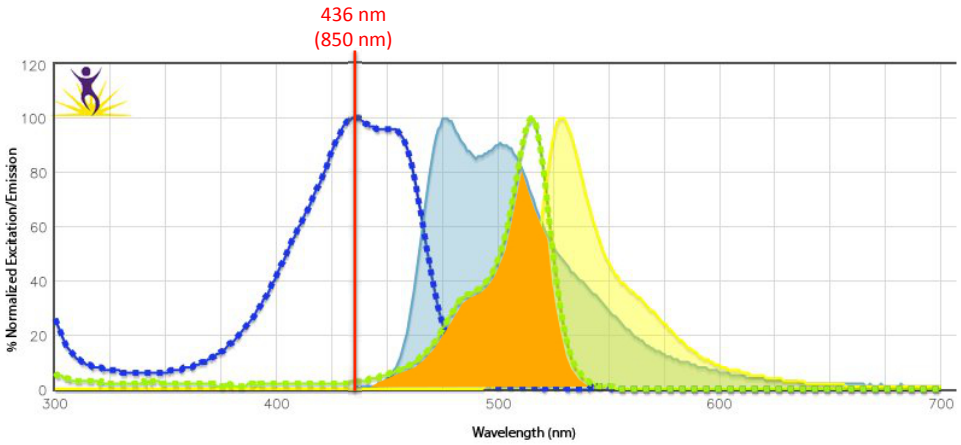
Spectral characteristics of Resonance for FRET: CFP-to-YFP



38

https://www.biolegend.com/spectraanalyzer

Spectral characteristics of Resonance for FRET: CFP-to-YFP



CFP excitation
CFP emission
YFP excitation
YFP emission

Overlap between CFP emission and YFP excitation (shaded region) leads to efficient FRET interaction.

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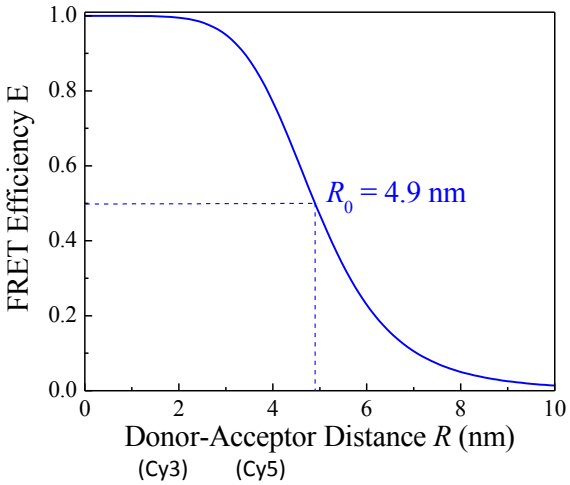
https://www.biolegend.com/spectraanalyzer

Spatial Dependency of FRET efficiency

$$E = \frac{\text{number of quanta transferred from D to A}}{\text{number of quanta absorbed by D}} = \frac{1}{1+(r/R_0)^6}$$



r = D and A separation distance

R_0 = Förster distance parameter calculated from spectroscopic parameters & dipole orientations of D and A.

At r = R_0, FRET is 50% efficient.

FRET AS A MOLECULAR RULER

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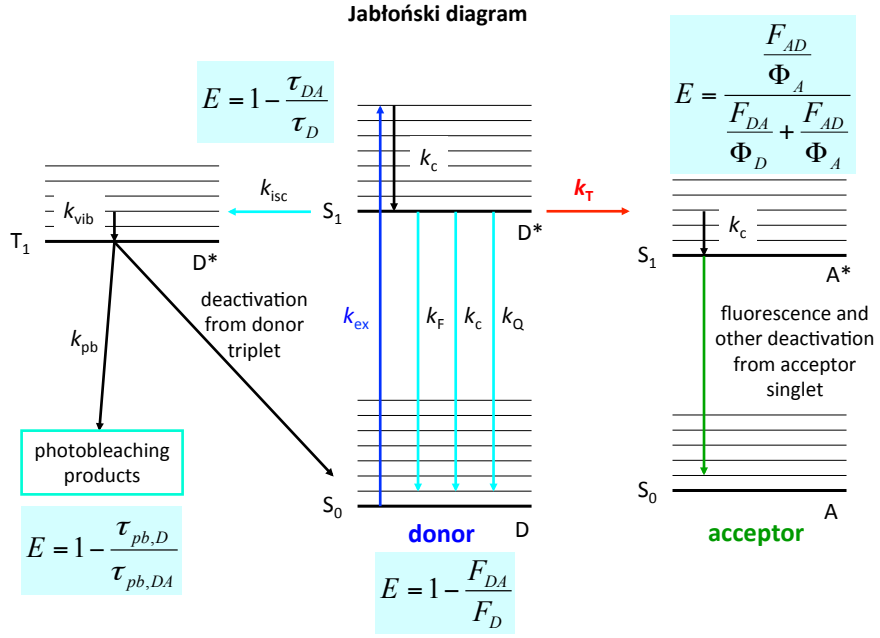
FRET Spectroscopy

Jabłoński diagram



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FRET Methods

- Acceptor photobleaching
- Sensitised emission
- Spectral imaging - unmixing
- FRET - Fluorescence Lifetime Imaging Microscopy (FLIM)

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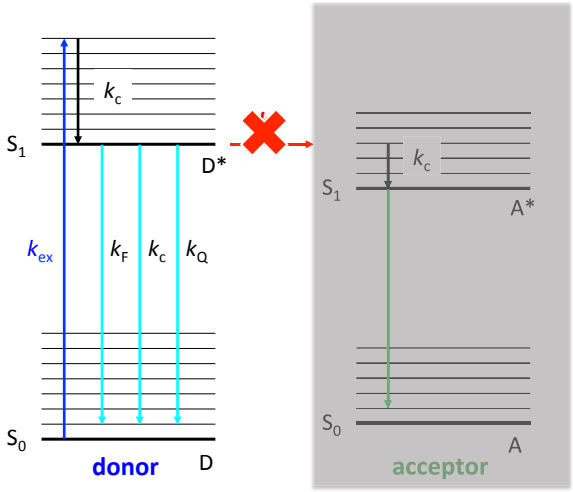
Acceptor Photobleaching

$$E = 1 - \frac{F_{DA}}{F_D}$$



- Easiest way to obtain FRET measurements
- Could be measured with a non-fluorescent acceptor
- Mainly for fixed samples
- Only measuring the Donor fluorescence

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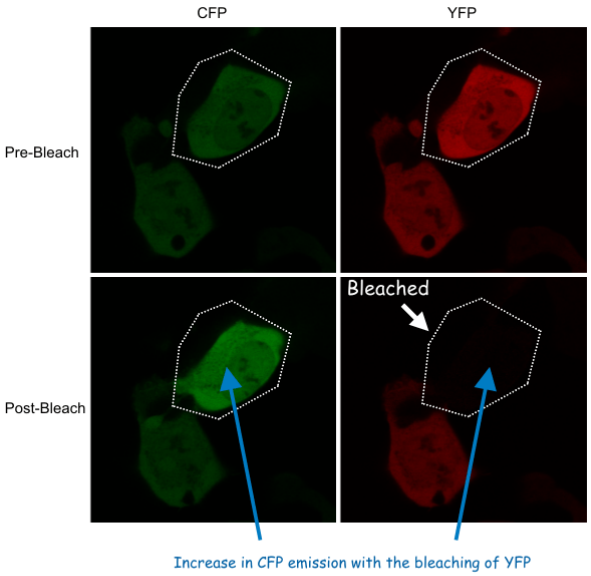
Acceptor Photobleaching



Confirms FRET

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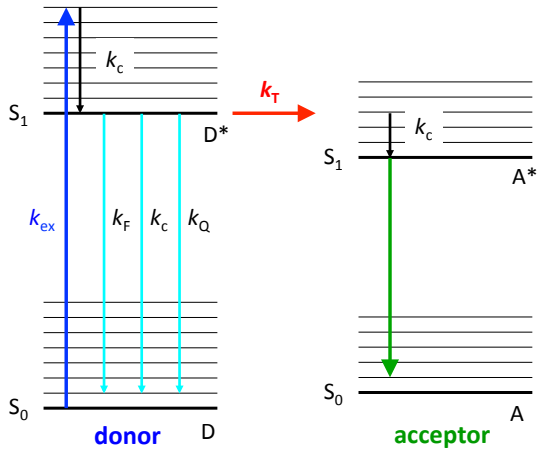
Adapted from www.robarts.ca

Sensitised Emission

$$E = \frac{\frac{F_{AD}}{\Phi_A}}{\frac{F_{DA}}{\Phi_D} + \frac{F_{AD}}{\Phi_A}}$$

- Measuring **acceptor emission** caused by FRET
- Could be done on a regular **widefield** microscope with only **3 different filter cubes:**
  - Donor emission.
  - Acceptor emission.
  - Donor excited Acceptor emission.
- Live-cell applicable
- Requires plenty of controls
- Susceptible to **artefacts** resulting from **crosstalk**



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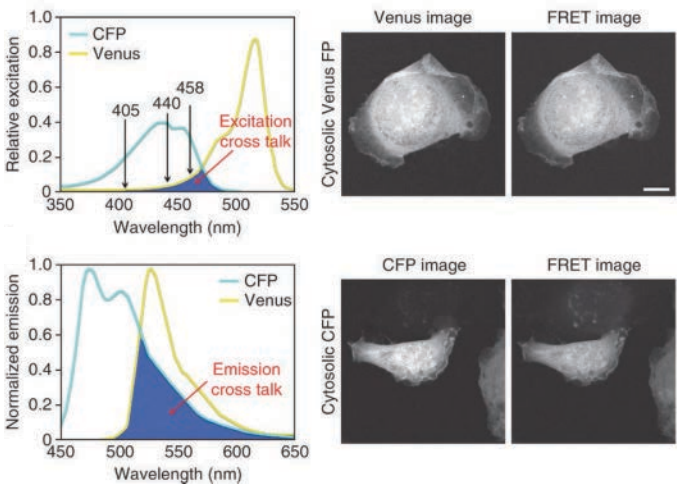
Crosstalk issues:

Overlapping Spectra



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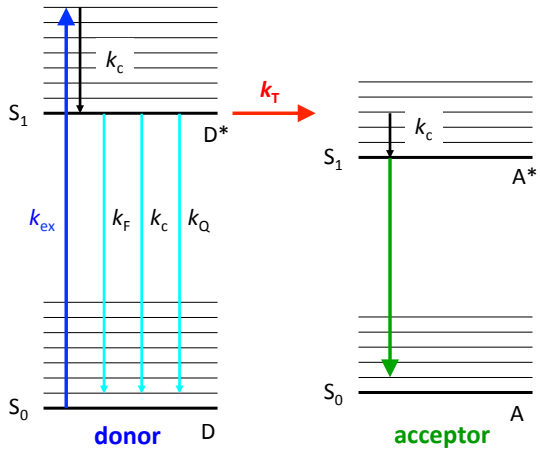
Adapted from Broussard et al., Nature Protocols (2013)

Spectral imaging – Unmixing

Dealing with crosstalk

$$E = \frac{\frac{F_{AD}}{\Phi_A}}{\frac{F_{DA}}{\Phi_D} + \frac{F_{AD}}{\Phi_A}}$$



- Measuring the **entire emission spectrum** using a spectral detector (Zeiss 710 upright 780, 780 UV and 880)
- **Quick**, because of simultaneous scanning
- Allows **live cell imaging**
- Requires plenty of controls
- Sorts out problems with autofluorescence

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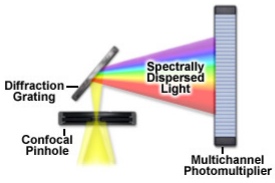
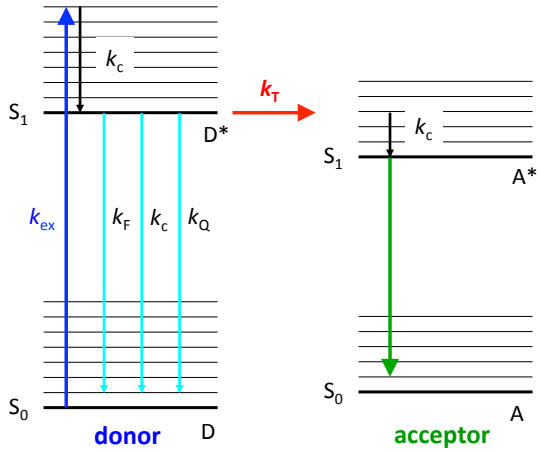
Spectral imaging – Unmixing

Dealing with crosstalk





- Measuring the **entire emission spectrum** using a spectral detector (Zeiss 710 upright 780, 780 UV and 880)
- **Quick**, because of simultaneous scanning
- Allows **live cell imaging**
- Requires plenty of controls
- Sorts out problems with autofluorescence

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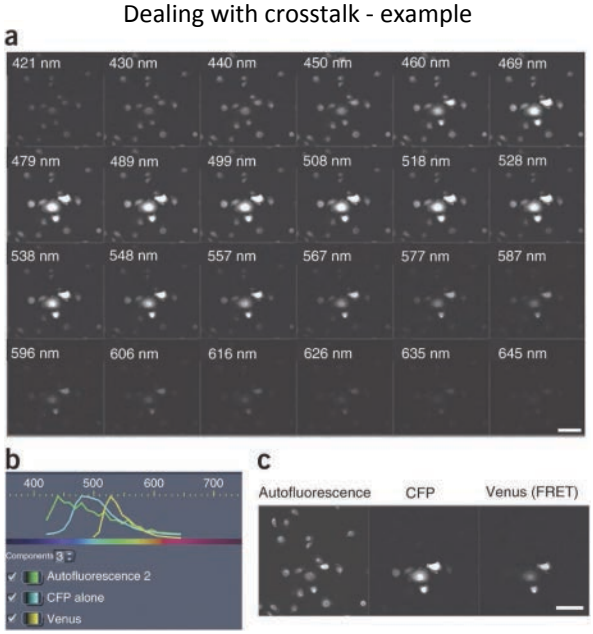
Image from Zeiss Online Campus

Spectral imaging – Unmixing

Dealing with crosstalk - example



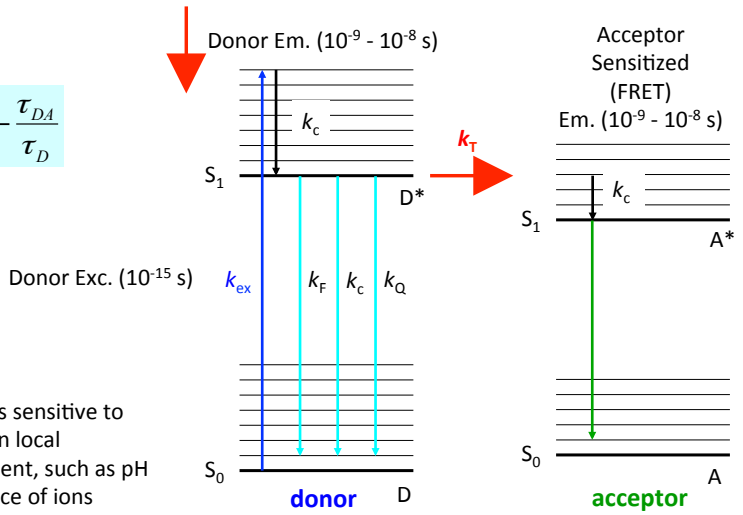
Reference spectra

Unmixed Result pixel by pixel

Adapted from Broussard et al., Nature Protocols (2013)

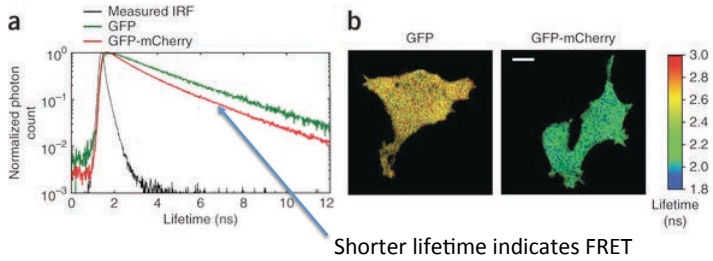
FRET – Fluorescence Lifetime Imaging Microscopy (FLIM)

$$E = 1 - \frac{\tau_{DA}}{\tau_D}$$



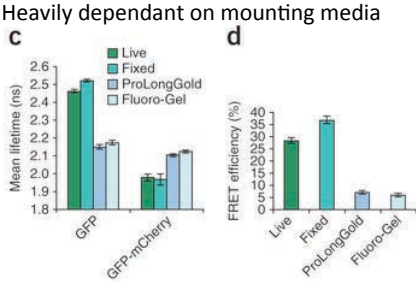
- Lifetime is sensitive to changes in local environment, such as pH or presence of ions
- … AND crucially to the presence of an acceptor molecule

FRET FLIM



Shorter lifetime indicates FRET

Heavily dependant on mounting media



$$E = 1 - \frac{\tau_{DA}}{\tau_D}$$

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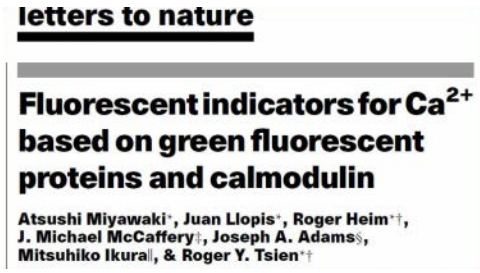
Adapted from Broussard et al., Nature Protocols (2013)

Using FRET to produce indicators



letters to nature

Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin

Atsushi Miyawaki*, Juan Llopis*, Roger Heim*†, J. Michael McCaffery‡, Joseph A. Adams§, Mitsuhiko Ikura‖, & Roger Y. Tsien*†

NATURE | VOL 388 | 28 AUGUST 1997

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Yellow Cameleon – a chimera

Ca^{2+}-binding protein
Calmodulin

Calmodulin-binding domain of
Myosin light chain kinase

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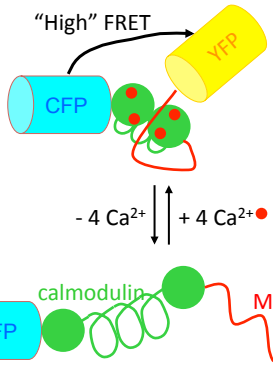
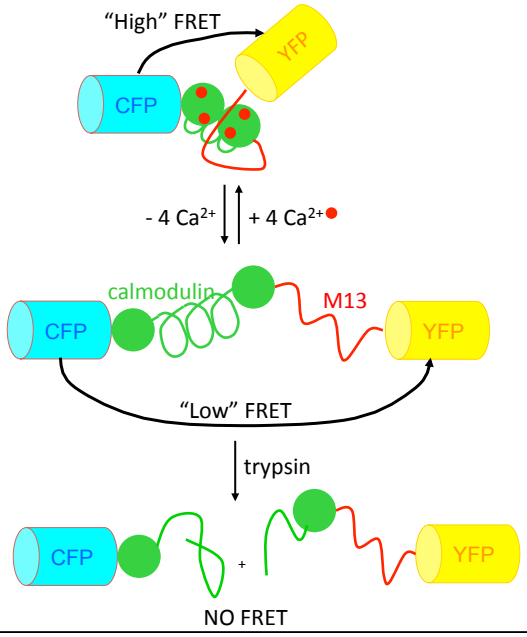
Yellow Cameleon



54

Yellow Cameleon



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Neuron, Vol. 26, 583–594, June, 2000, Copyright ©2000 by Cell Press

Optical Imaging of Calcium Transients in Neurons and Pharyngeal Muscle of *C. elegans*

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Pierre Vincent,§# Roger Y. Tsien,‡§
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*Department of Biology
†Biomedical Sciences Graduate Program
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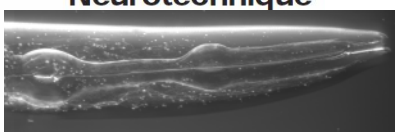
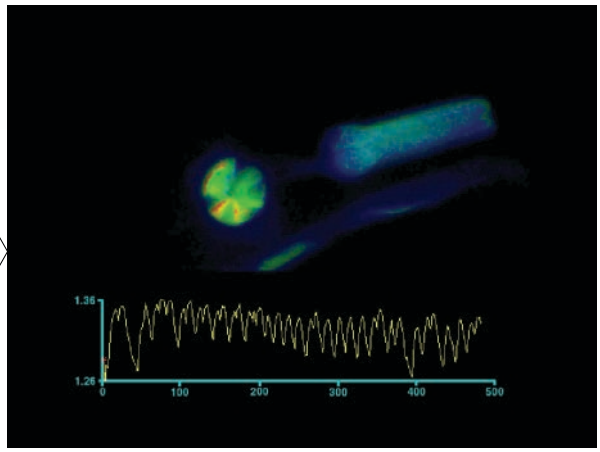
Neurotechnique



Cameleon FRET indicator showing Ca^{2+} transients in the *C. elegans* pharynx



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Movie from W. Schafer, LMB

Correction or measurement	Sample	Acceptor Photo-bleaching	Sensitized emission	Spectral Imaging	Fluorescence Lifetime
Autofluorescence	Unlabeled Cells	Yes	Yes	Yes	Yes
CFP emission crosstalk, CFP spectra, determine CFP lifetime	CFP alone	No	Yes	Yes	Yes
Venus excitation crosstalk, Venus spectra	Venus alone	No	Yes	Yes	No
Negative FRET control	Unlinked CFP & Venus	Yes	Recommended	Recommended	Recommended
Positive FRET control, determine CFP FRET lifetime	Linked CFP & Venus	Recommended	Recommended	Recommended	Recommended
Sample of interest	CFP & Venus experimental sample	Yes	Yes	Yes	Yes
Shows high FRET, short CFP lifetime	CFP & Venus Positive biological control	Recommended	Recommended	Recommended	Recommended
Shows low FRET, long CFP lifetime	CFP & Venus Negative biological control	Recommended	Recommended	Recommended	Recommended

Adapted from Broussard et al., Nature Protocols (2013)

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Summary

Fluorescence **R**esonance **E**nergy **T**ransfer

You can use FRET to measure interactions below the diffraction limit.

You can use it as a molecular ruler

It is useful for bio-sensors

But you must do all the controls!

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1. Protein Labelling for Fluorescence Microscopy

2. Förster Resonance Energy Transfer

3. Light Sheet Microscopy

Ben Sutcliffe
- Light Microscopy Facility -

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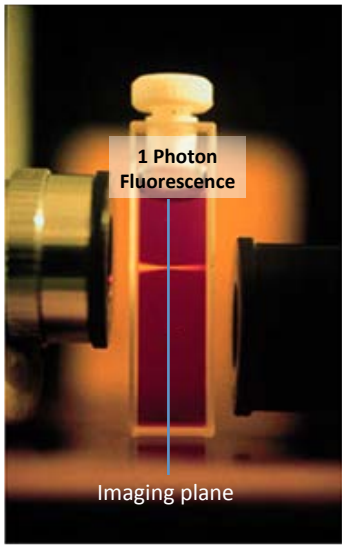
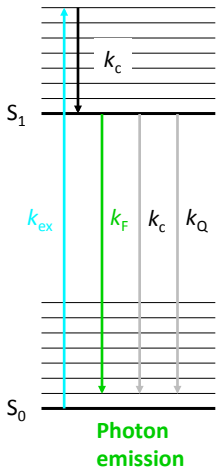
Laser Scanning Microscopy



Brad Amos, MRC

Excitation with:
1 High Energy Photon



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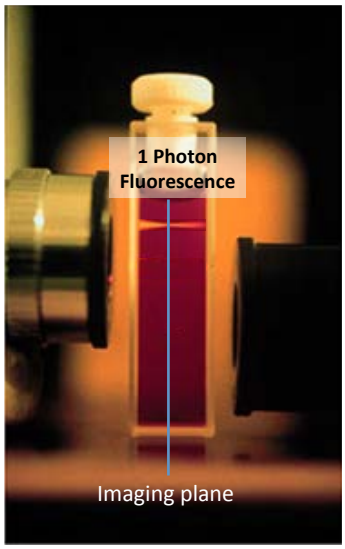
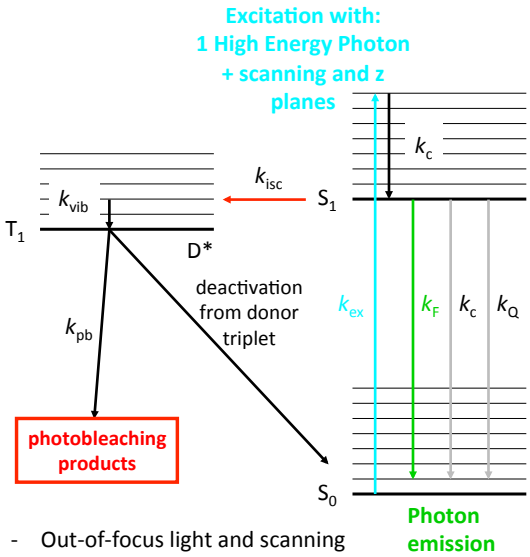
Another route to exit the excited state = Bleaching!



Brad Amos, MRC



- Out-of-focus light and scanning increase the chance to end up in a photo bleached state dramatically.

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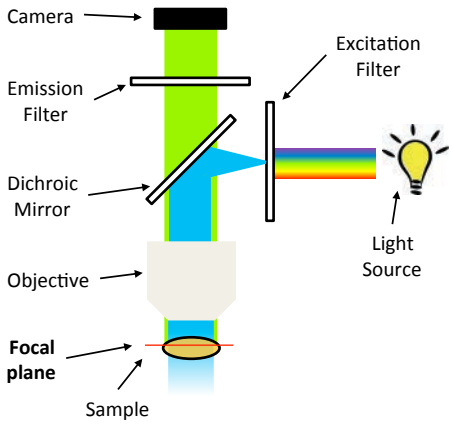
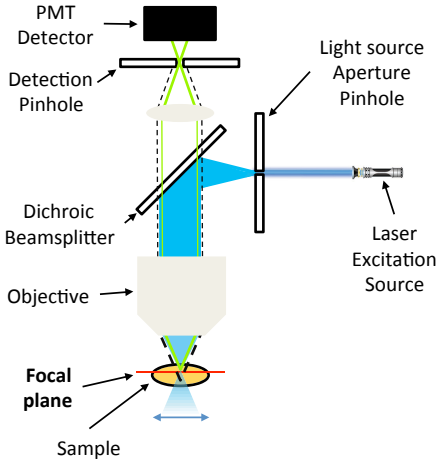
Widefield v Confocal





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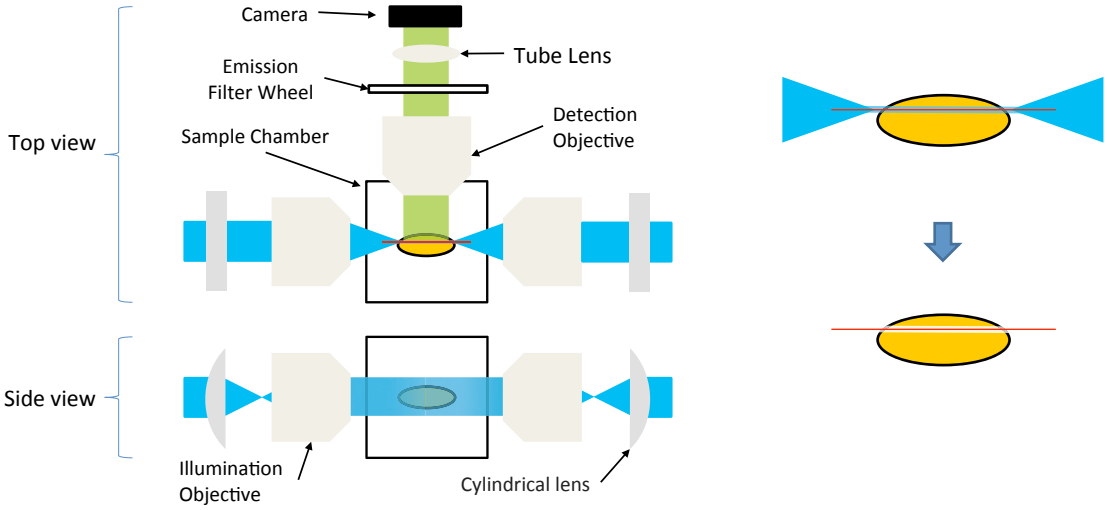
mSPIM at the LMB:

Gentle imaging for your live samples



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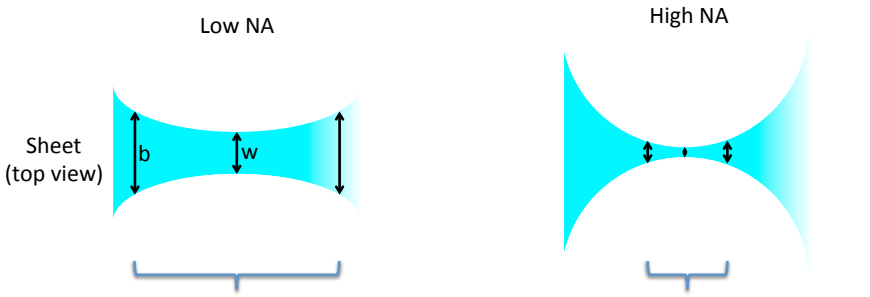
Effect of changing the Numerical Aperture of the Illumination lens



Wide Field of View = thicker sheet

Narrow Field of View = thinner sheet

- Smaller field of view enables the use of a thinner sheet

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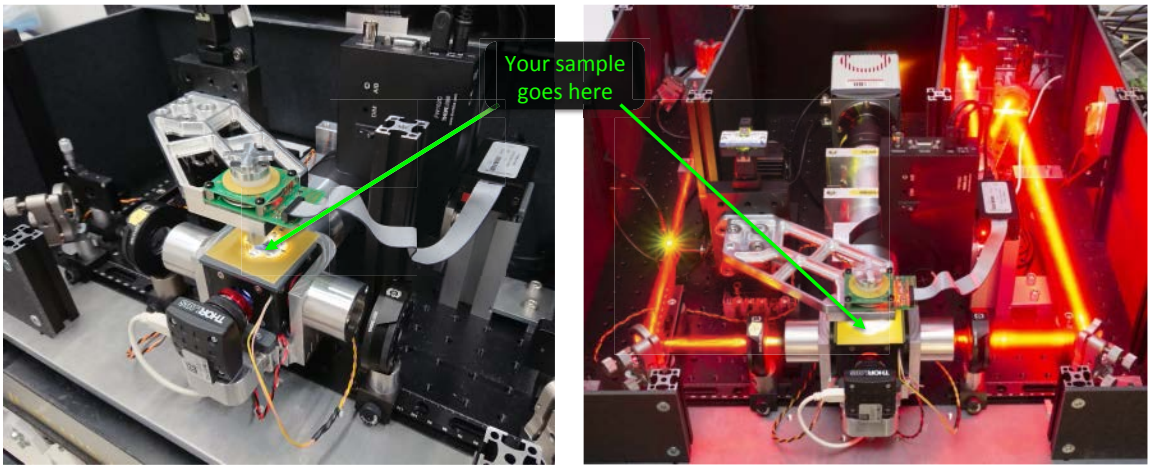
The mSPIM (Selective Plane Illumination Microscope)



- Based on the OpenSPIM.org design
- 2-sided illumination = more even illumination & fewer artefacts
- Temperature controlled

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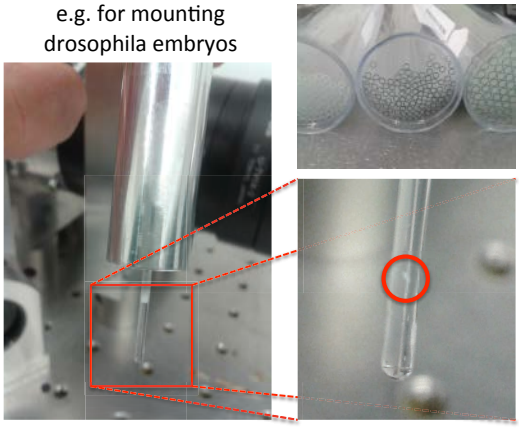
Modified from the initial microscope previously built by Mathias (alumni) with Adam Fowle (Workshop)

Mounting techniques
Various sample holders

- Mounting e.g. in 1% low melting point agarose, but other options possible

Capillaries with plungers
e.g. for mounting
drosophila embryos



Cup-shaped mould & sample
carrier for e.g. oocytes

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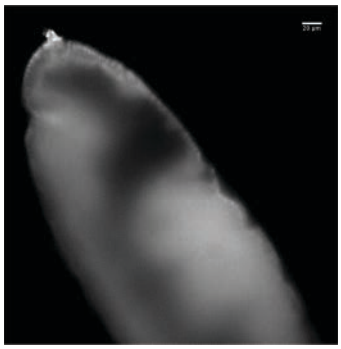
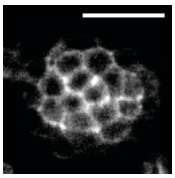
Real life samples

- Fixed drosophila embryo expressing Rho-Kinase-C-GFP (Röper lab)
  - Individual z-planes (spaced 3 µm)





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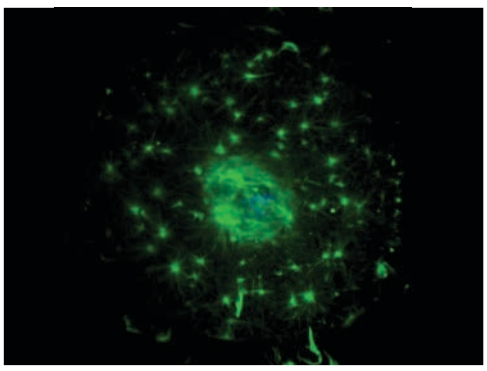
Sample kindly provided by Dr. Clara Sidor

Real life samples

- Fixed mouse oocyte (Schuh lab)
  - Tubulin – Alexa488 & Hoechst



140 µm

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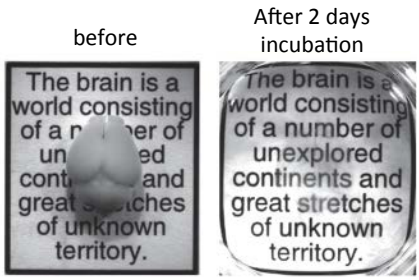
Sample kindly provided by Dr. Zuzana Holubcova

Overcoming Scatter:
Tissue clearing

- Various reasons for scatter:
  - Lipids
  - Pigments
- Eliminating/Minimize scatter allows to image deeper in the sample – similar goal as with 2P microscopy
- Tissue Clearing (e.g. seeDB, Clarity, RIMS, Cubic, RapiClear)
- Potentially useful for other microscopy techniques



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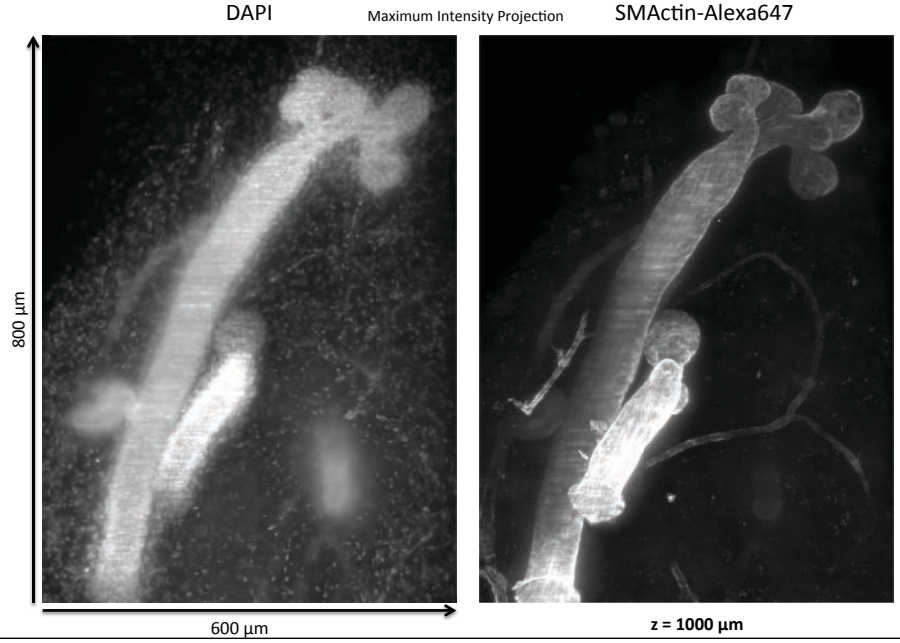
Murine mammary gland tissue – cleared (Cubic2)



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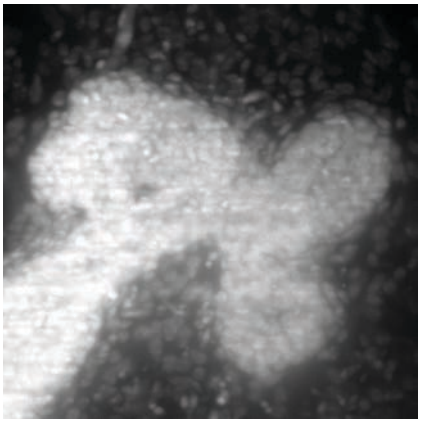
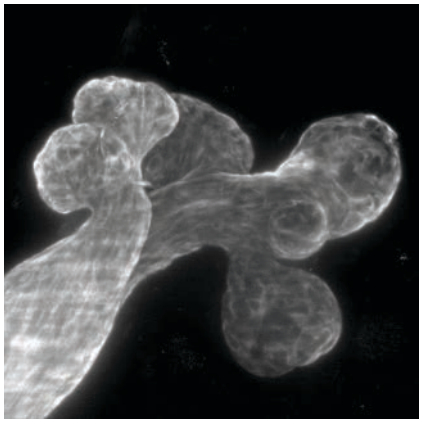
Murine mammary gland tissue - cleared

DAPI



SMActin-Alexa647



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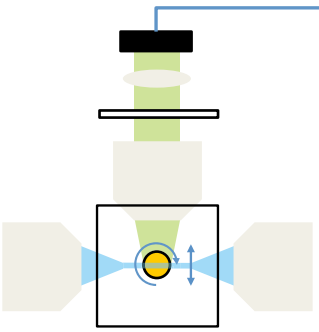
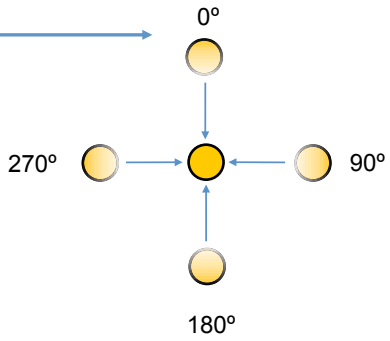
Overcoming Scatter
Multiview Imaging and Reconstruction





Two software options here at the LMB:

- Bead or Segmentation based Fusion in **Fiji**
- Image intensity profile based Fusion in **Huygens**

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Real life samples

- Live imaging of Drosophila Salivary gland development
  - 4 hour time-lapse (images every 5 minutes)
  - 5 views over 360 degrees

Fusion of Single time point

Fusion of all timepoints
Viewed from one angle

3D render of all timepoints

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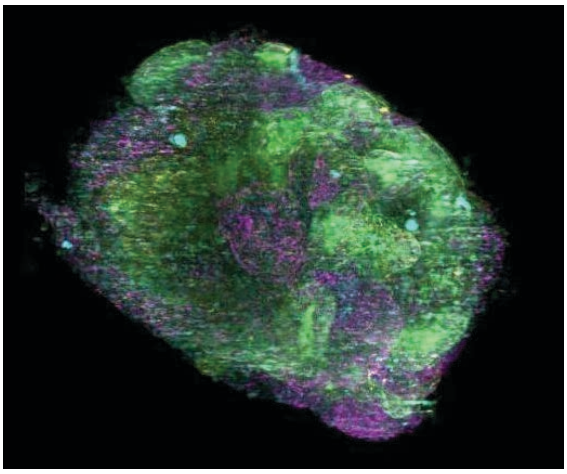
Sample kindly provided by Annabel May (Röper group)

Real life samples

Fixed kidney organoid



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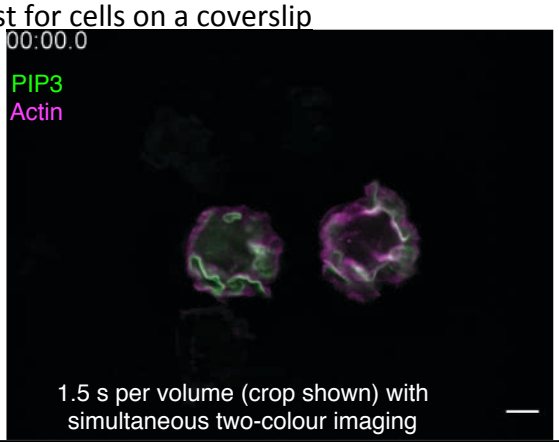
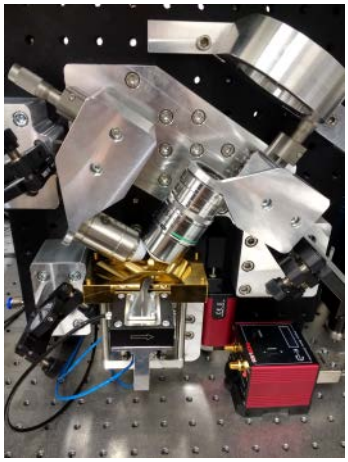
Sample kindly provided by John-Poul Ng Blichfeldt (Röper group)

Sub-cellular Light Sheet

- Built by James Manton
- Achieves the same imaging quality/speed as the Lattice Light Sheet only much simpler
- Best for cells on a coverslip





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Summary

Light Sheet Microscopy

- Allows you to do live cell imaging for much longer due to **less bleaching**.
- Enables you to look at larger samples much **faster** that with other methods.
- Multiview imaging allows more isotropic resolution
- Clearing tissue allows deeper imaging.

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Thank you for your attention!

Any questions?

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