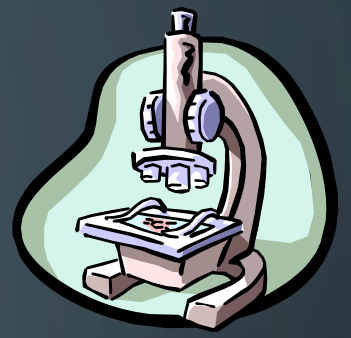
2021/2022 - Light microscopy in Cellular Biology



Fluorescence Microscopy

part2

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FLUORESCENCE A SMALL SUMMARY

- What is it?
- Where does it come from?
- Advantages
- Disadvantages

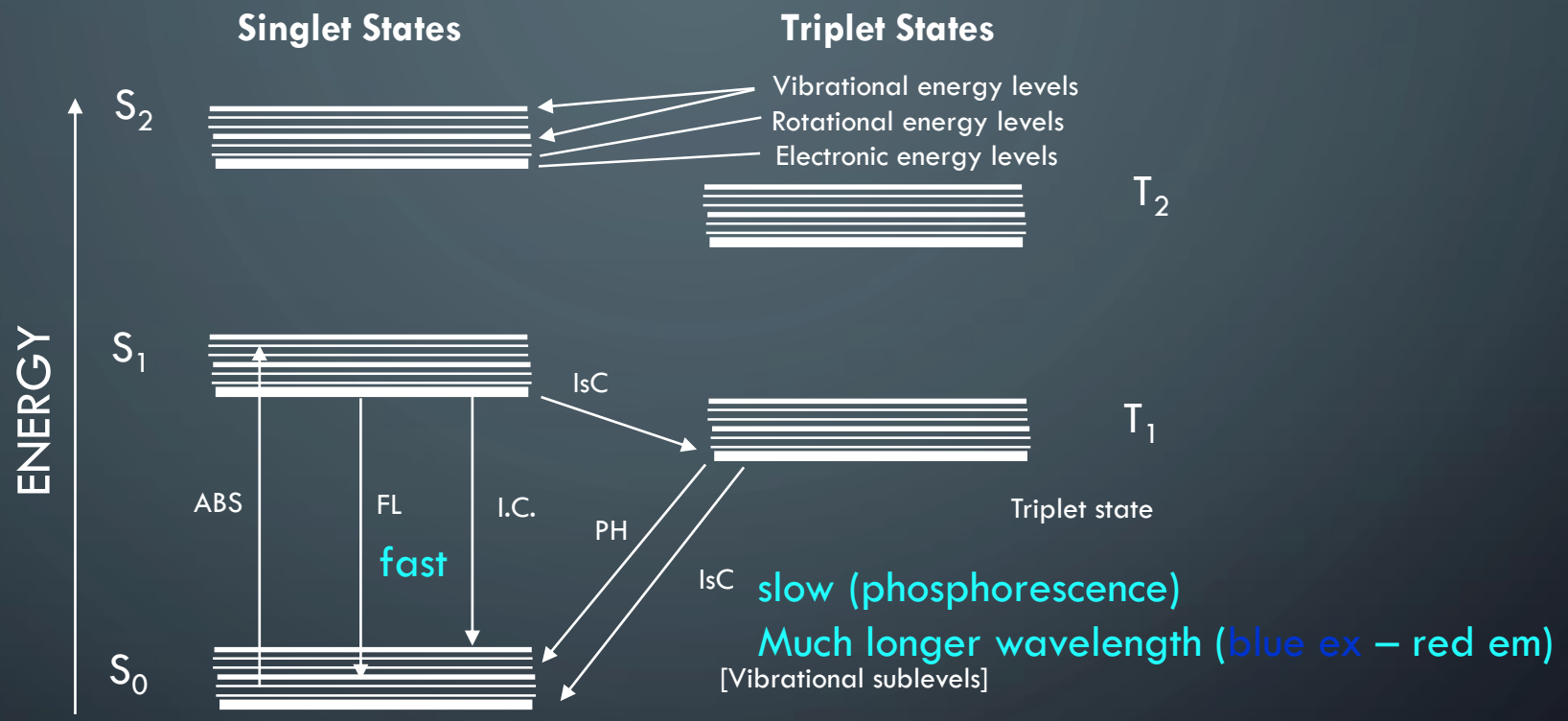
FLUORESCENCE

- Chromophores are components of molecules which absorb light
- e.g. from protein most fluorescence results from the indole ring of tryptophan residue
- They are generally aromatic rings



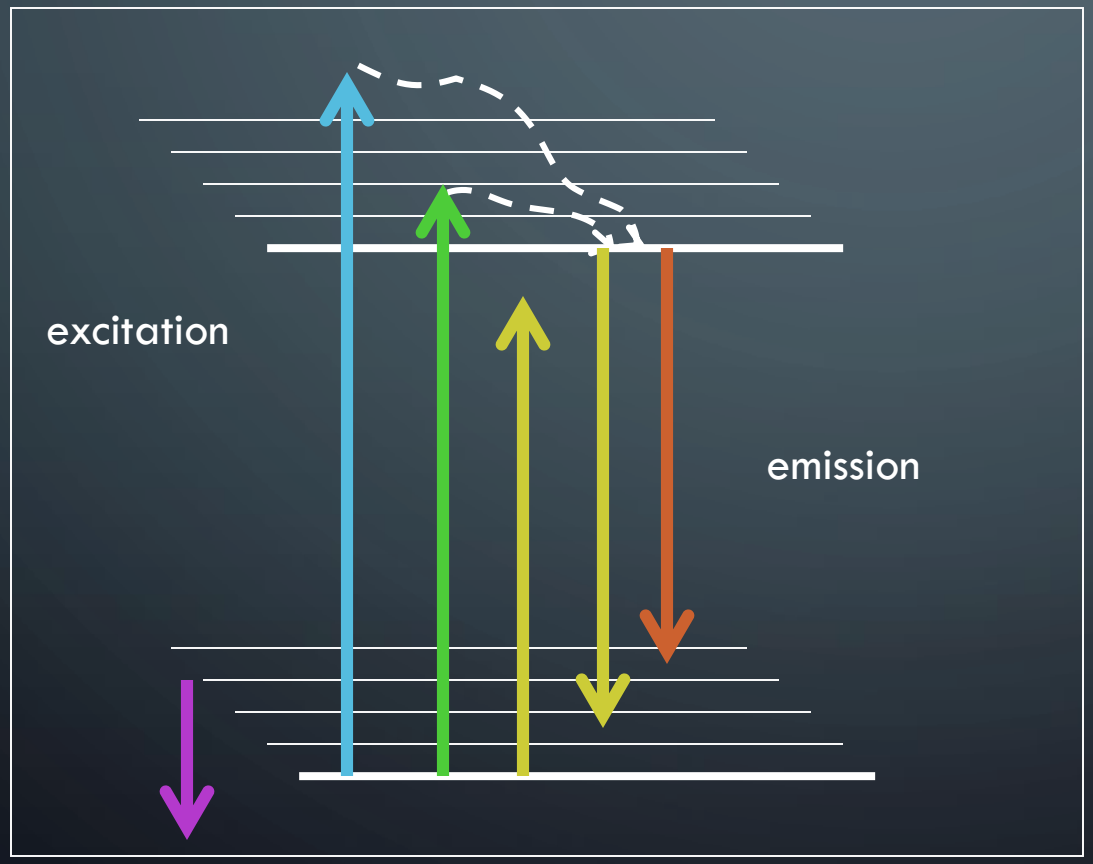
FLUORESCENCE

Jablonski Diagram



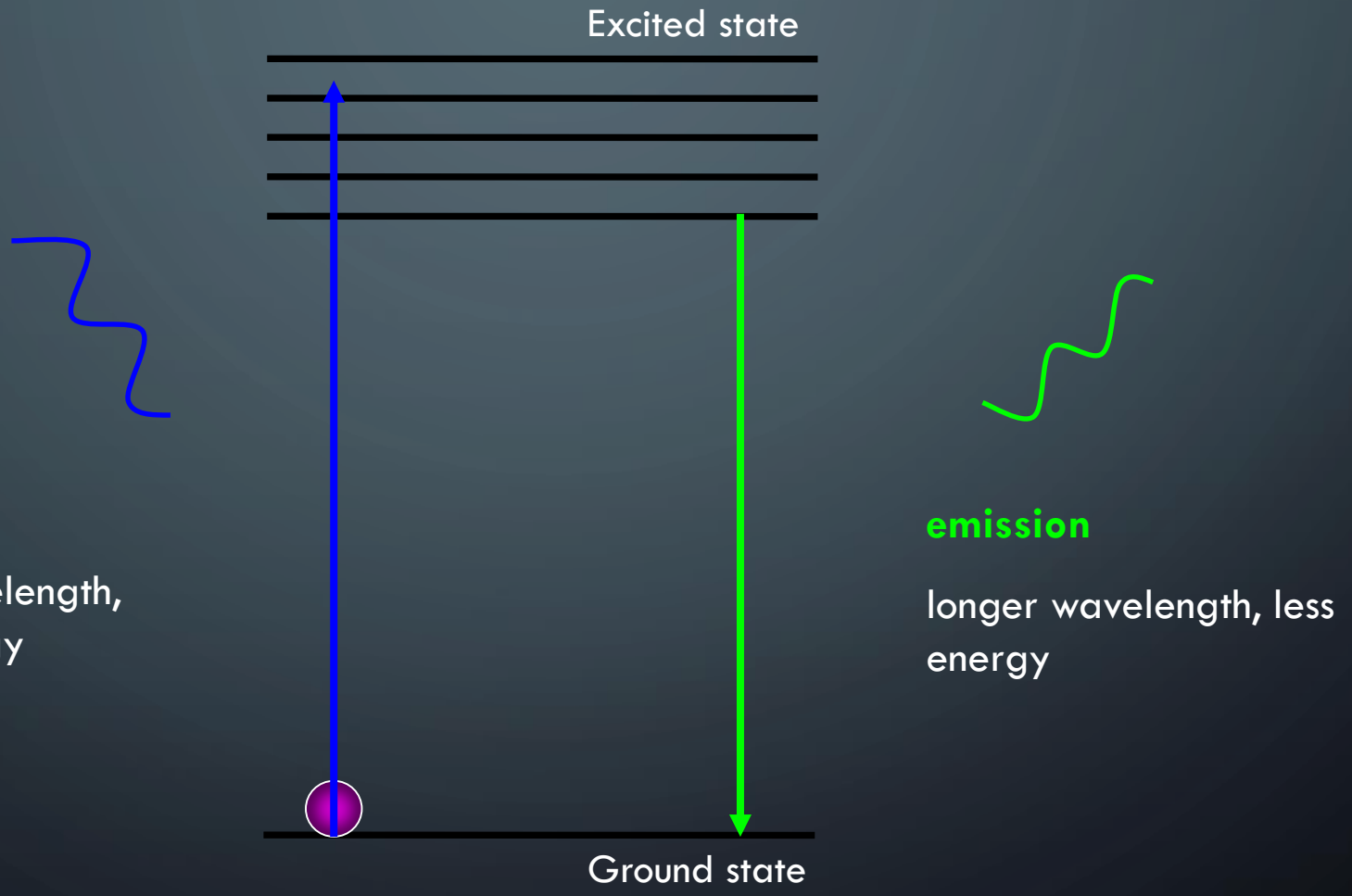
ABS - Absorbance S 0.1.2 - Singlet Electronic Energy Levels
FL - Fluorescence T 1,2 - Corresponding Triplet States
I.C.- Nonradiative Internal Conversion IsC - Intersystem Crossing PH - Phosphorescence

FLUORESCENCE MICROSCOPY: BASICS OF THEORY



- Absorbance spectrum limits excitation.
- Energy states limit excitation
- Molecule returns to lowest vibrational state emitting heat
- Light is emitted on return to ground state

FLUORESCENCE



excitation

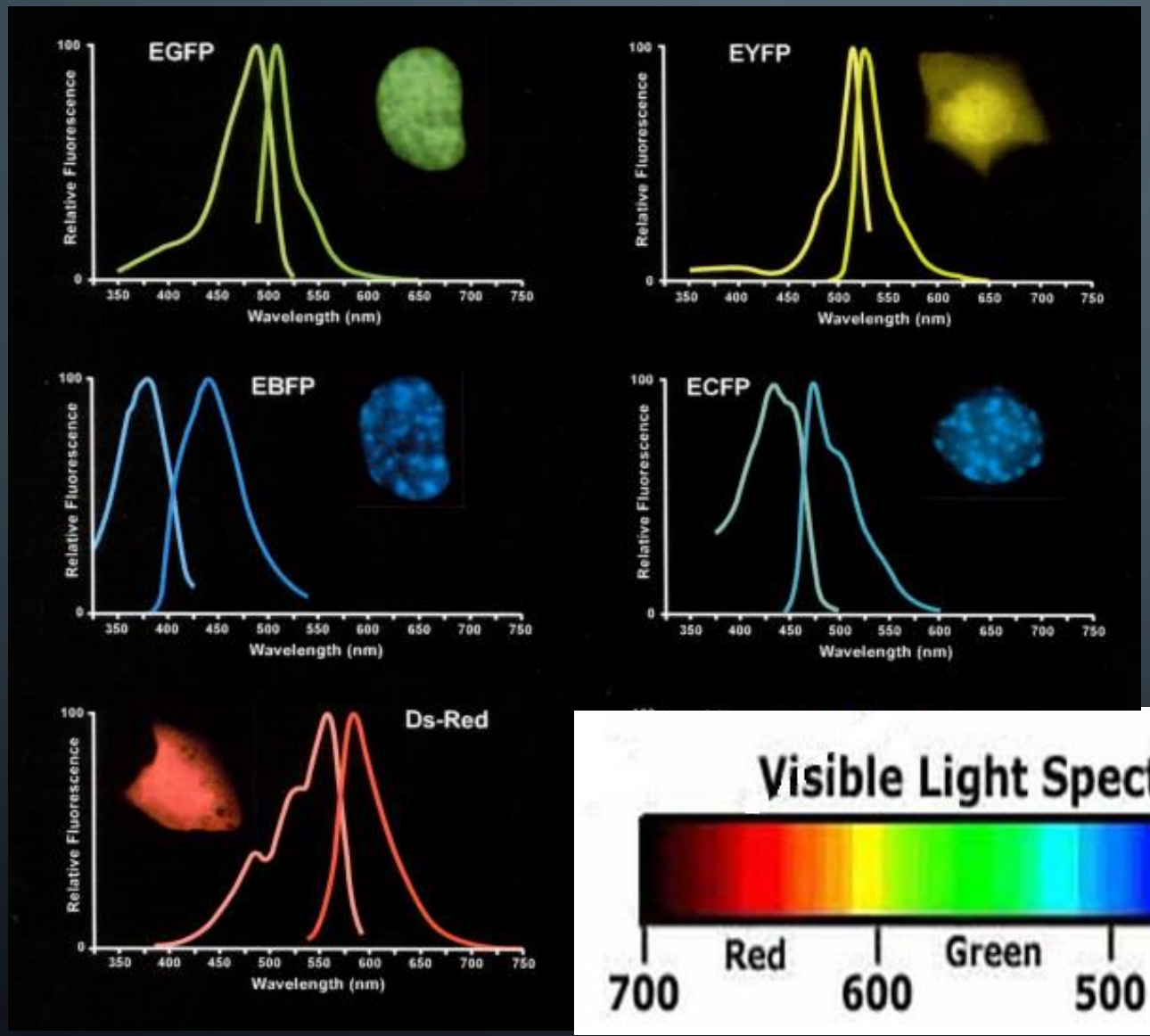
shorter wavelength, higher energy

emission

longer wavelength, less energy

→ **Stoke's shift**

Fluorophores (Fluorochromes, chromophores)

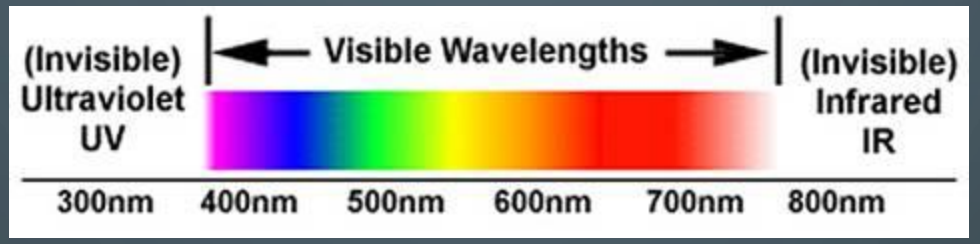


- Special molecular structure
- Aromatic systems (PI-systems) and metal complexes (with transition metals)
- characteristic excitation and emission spectra

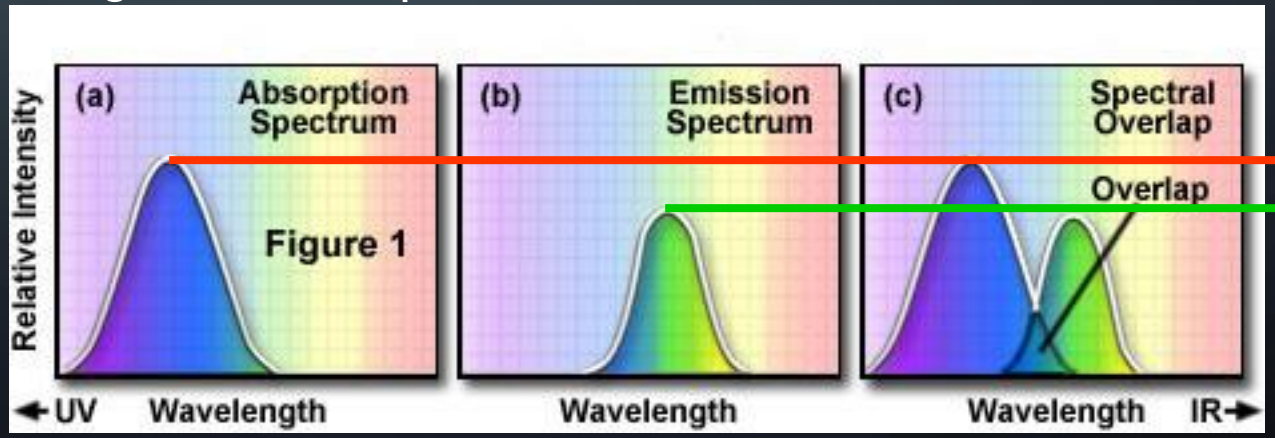
STOKE'S SHIFT

✓The emission spectrum of an excited fluorophore is usually shifted to longer wavelengths when compared to the absorption or excitation spectrum

Excitation 495 nm
Emission: 520 nm

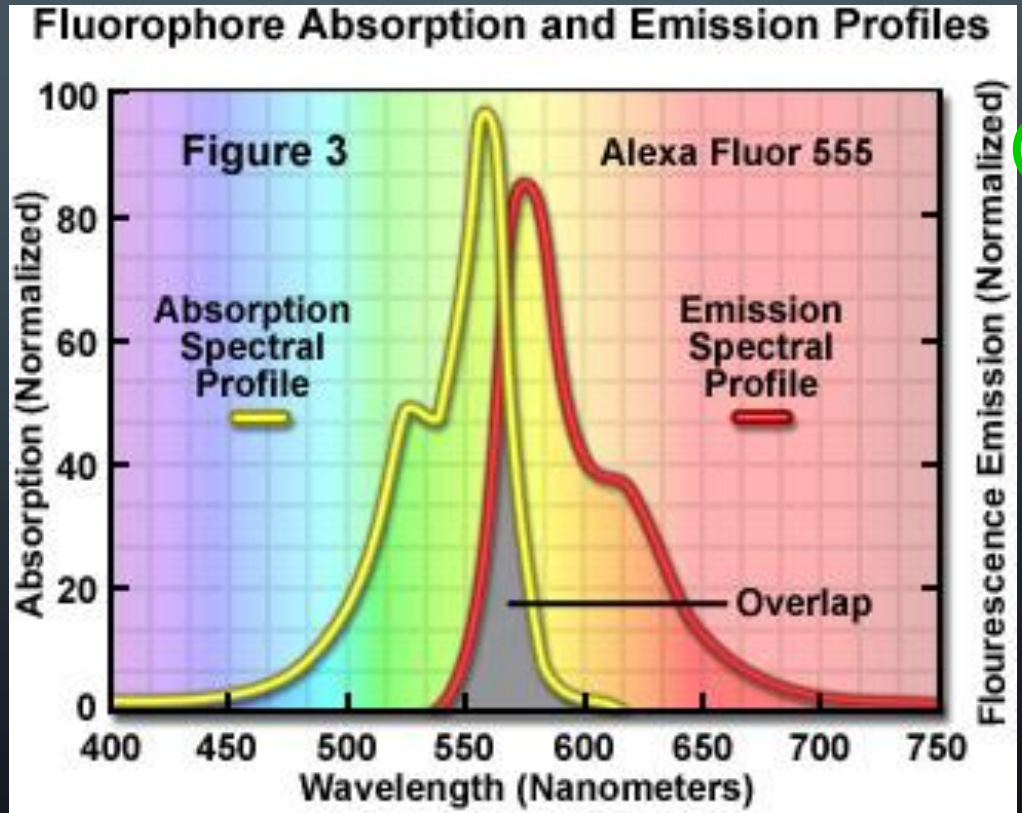


- The intensity of the fluorescence is very weak in comparison with the excitation light (10^{-3} to 10^{-5}).
- The emitted light re-radiates spherically in all directions.
- Dark background is required to enhance resolution.

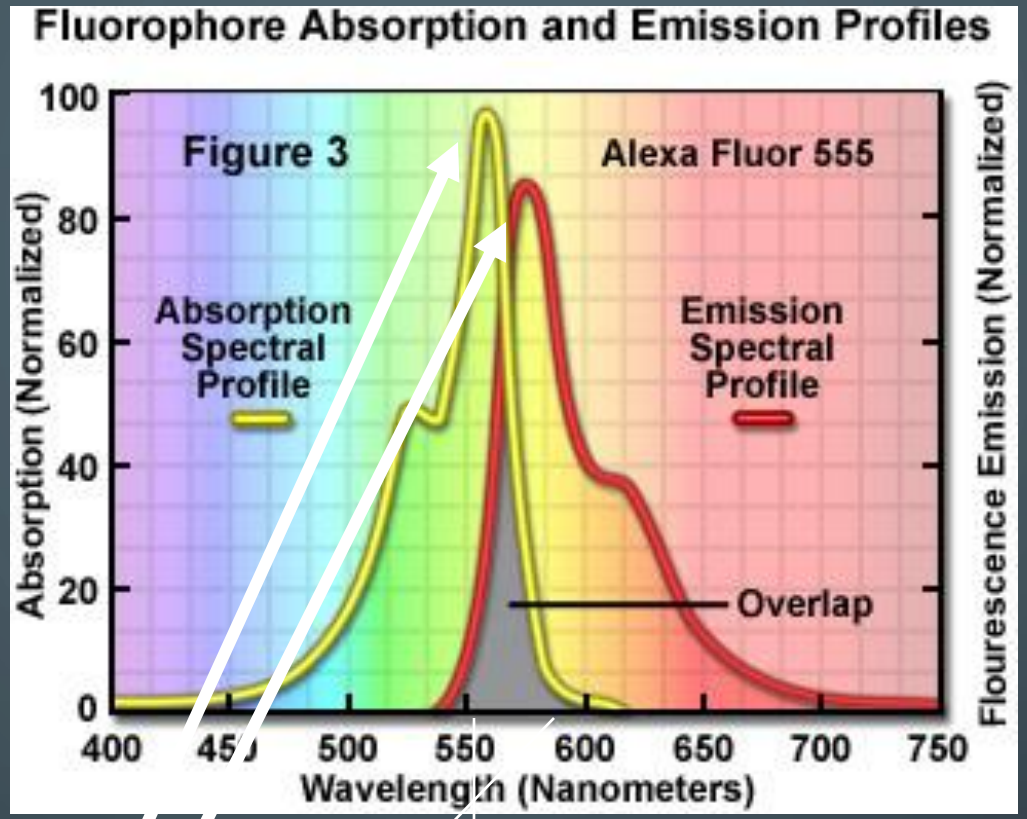


STOKE'S SHIFT

✓As Stokes' shift values increase, it becomes easier to separate excitation from emission light through the use of fluorescence filter combinations.

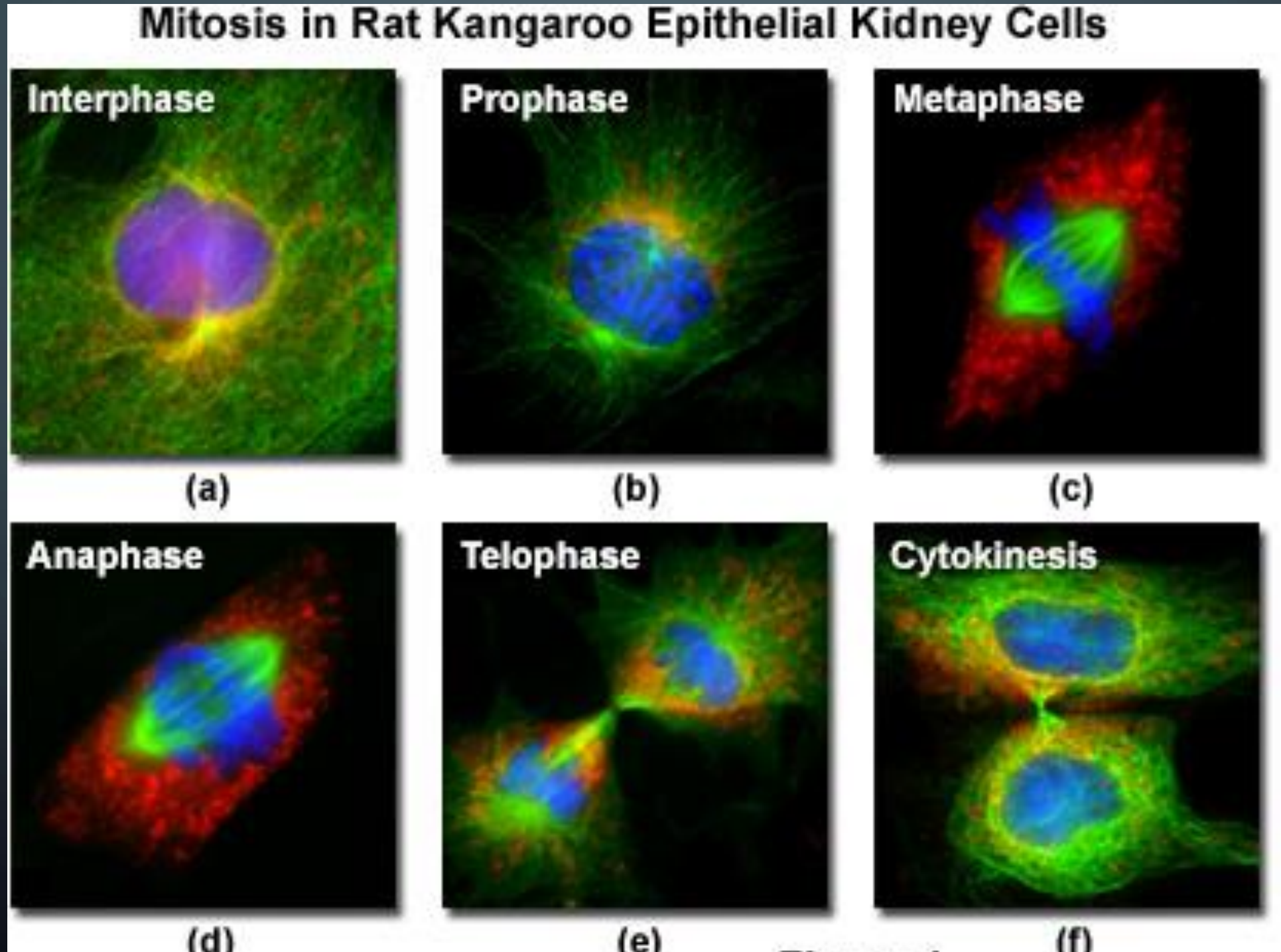


Remember Dichoric Mirror ???

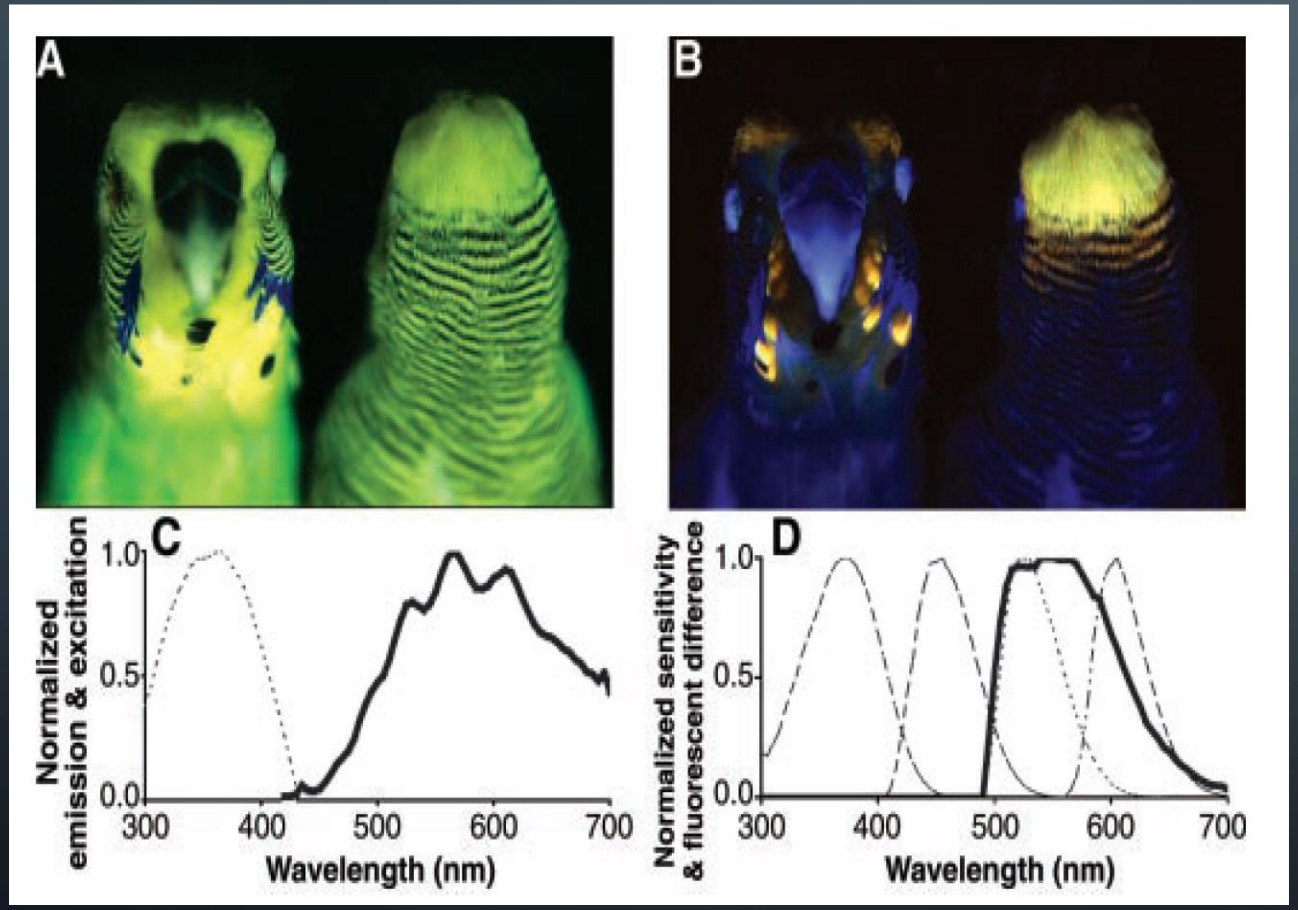


Data for Alexa Fluor 555

- absorbs light in the yellow-green region
- produces yellow-orange emission
- to achieve maximum fluorescence intensity
  - a fluorophore is usually excited at wavelengths near or at the peak of the excitation curve,
  - And detected at widest possible range of emission wavelengths that include the emission peak

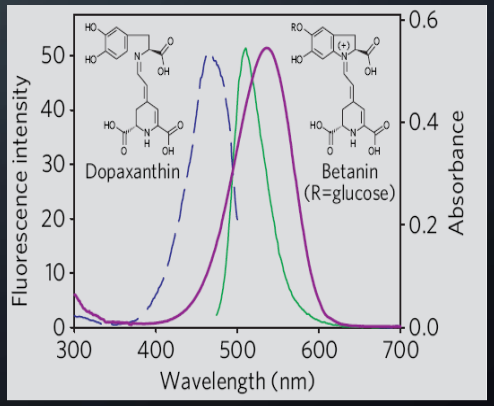

Mitosis in Rat Kangaroo Epithelial Kidney Cells
Interphase
Prophase
Metaphase
(a)
(b)
(c)
Anaphase
Telophase
Cytokinesis
(d)
(e)
(f)

NATURAL FLUORESCENCE









PARAMETERS

- Extinction Coefficient
  - ε refers to a single wavelength (usually the absorption maximum)
- Quantum Yield
  - Q_f is a measure of the integrated photon emission over the fluorophore spectral band
- At sub-saturation excitation rates, fluorescence intensity is proportional to the product of ε and Q_f

$$\phi = \frac{\text{Number of emitted photons}}{\text{Number of absorbed photons}}$$

- Lifetime 1 –10x10^{-9}secs (1-10 ns)

FLUORESCENCE

Stokes Shift

- is the energy difference between the lowest energy peak of absorbance and the highest energy of emission

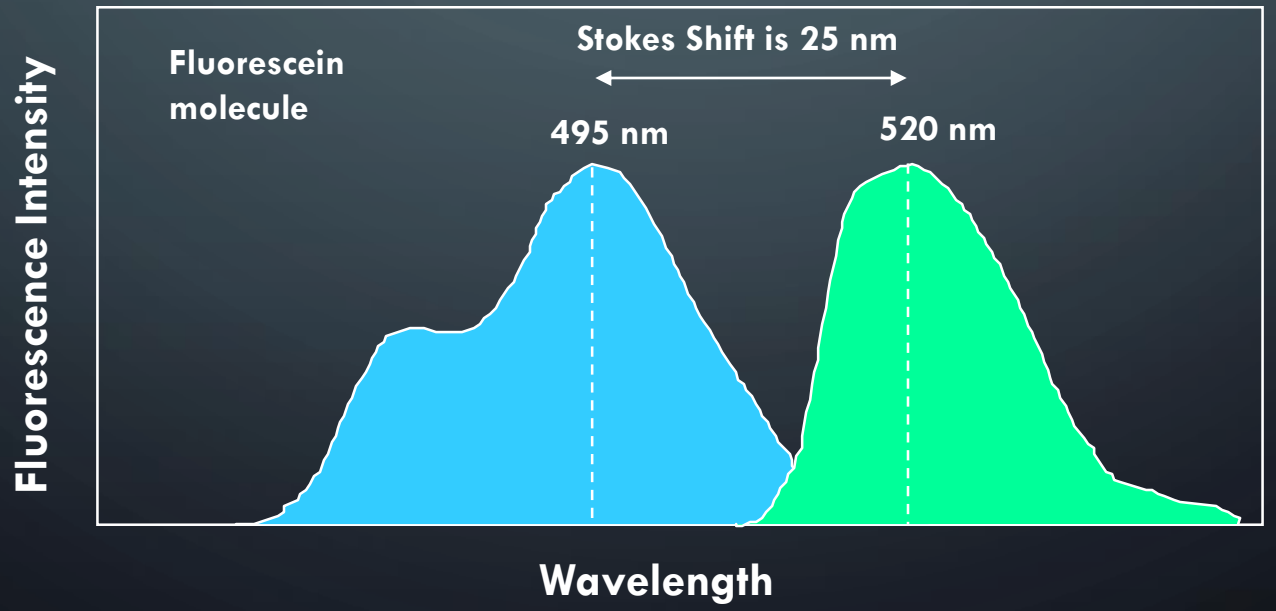
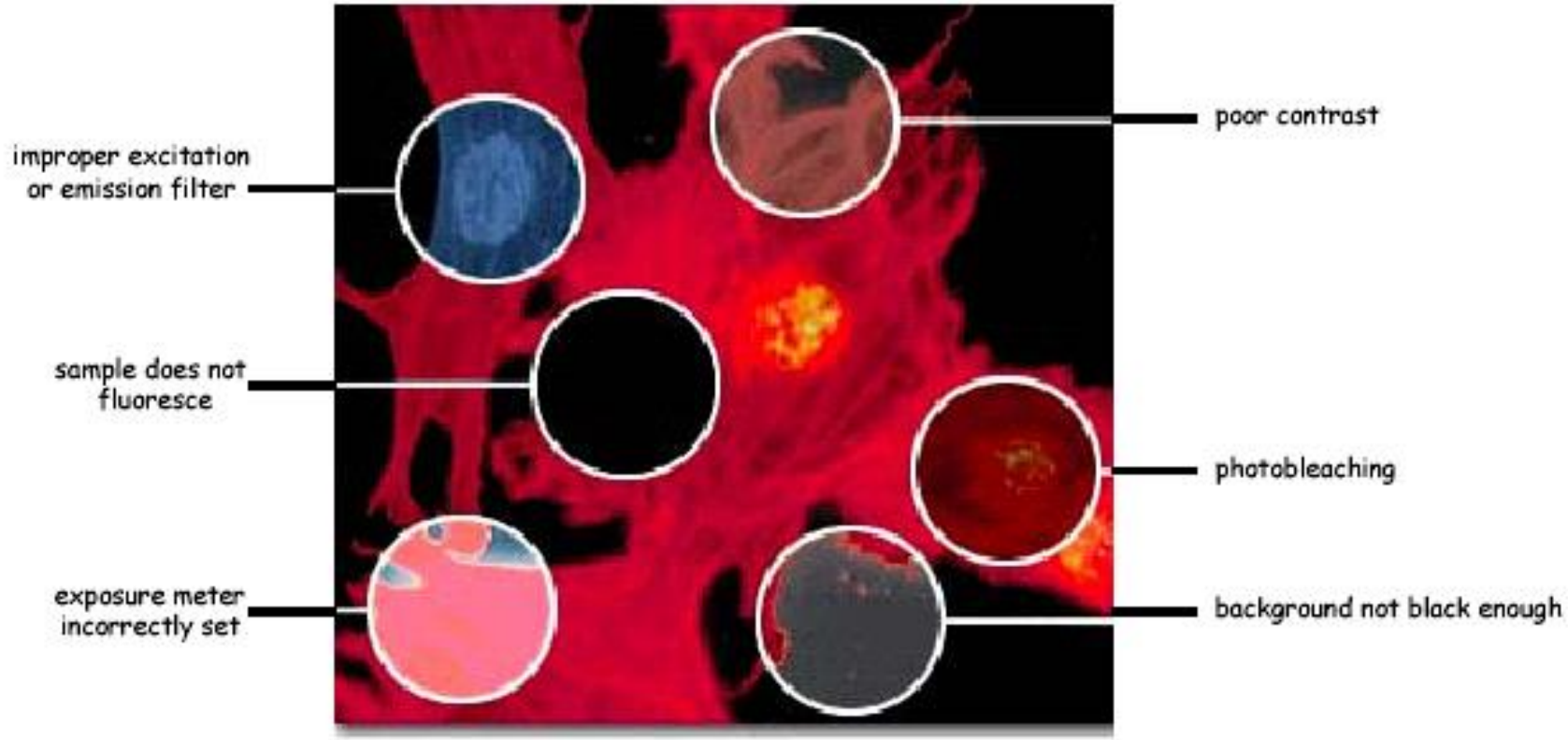


Figure 3: Problems with Fluorescence microscopy



FLUORESCENCE EXCITATION SPECTRA

Intensity

related to the **probability** of the event

Wavelength

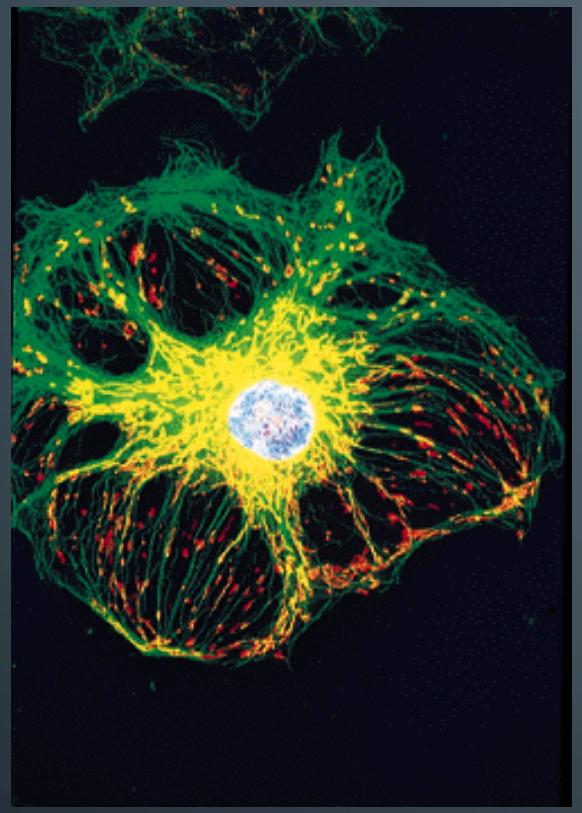
the **energy** of the light absorbed or emitted

FLUORESCENCE

The **longer** the wavelength the **lower** the energy

The **shorter** the wavelength the **higher** the energy
e.g. UV light from sun causes the sunburn
not the red visible light

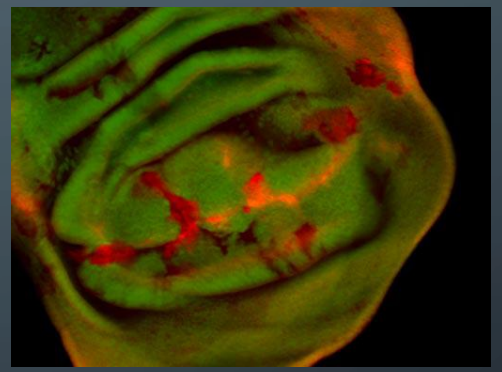
MULTICHANNEL FLUORESCENCE LABELLING



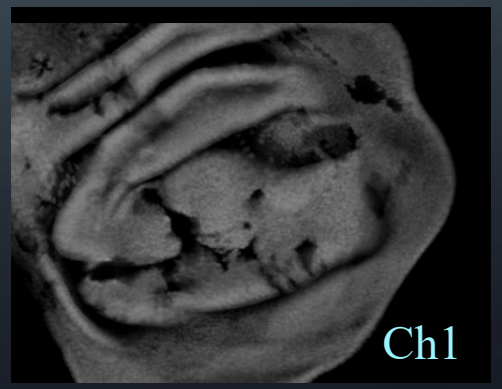
Arterial edothelial cell
Ch1(Green) FITC Tubulin
Ch2(Red) mitotracker
Ch3(Blue) DAPI

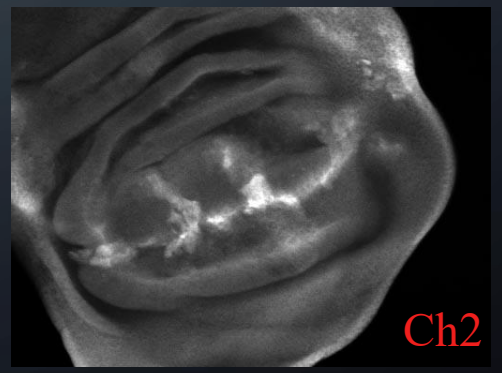
- Direct coupling to macromolecules
- Fluorescent dyes and substrates
- Fluorescent fusion proteins
- Fluorescent Antibodies

Ch1(Green)
UBI-GFP



Ch2(Red)
Texas Red
anti-rabbit
& Rabbit anti-
BGal





PHOTOBLEACHING

- Defined as the irreversible destruction of an excited fluorophore (discussed in later lecture)
- Methods for countering photobleaching
  - Scan for shorter times
  - Use high magnification, high NA objective
  - Use wide emission filters
  - Reduce excitation intensity
  - Use "antifade" reagents (not compatible with viable cells)

QUENCHING

Not a chemical process

Dynamic quenching =- Collisional process usually controlled by mutual diffusion
Typical quenchers – oxygen
Aliphatic and aromatic amines (IK, NO2, CHCl3)

Static Quenching
Formation of ground state complex between the fluorophores and quencher with a non-fluorescent complex (temperature dependent – if you have higher quencher ground state complex is less likely and therefore less quenching

ANTIFADE AGENTS

- Many quenchers act by reducing oxygen concentration to prevent formation of singlet oxygen
- Satisfactory for fixed samples but not live cells!
- Antioxidents such as propyl gallate, hydroquinone, p-phenylenediamine are used
- Reduce O_2 concentration or use singlet oxygen quenchers such as carotenoids (50 mM crocetin or etretinate in cell cultures); ascorbate, imidazole, histidine, cysteamine, reduced glutathione, uric acid, trolox (vitamin E analogue)

PROBES FOR PROTEINS

Probe	Excitation	Emission
FITC	488	525
PE	488	575
APC	630	650
PerCP™	488	680
Cascade Blue	360	450
Coumerin-phalloidin	350	450
Texas Red™	610	630
Tetramethylrhodamine-amines	550	575
CY3 (indotrimethinecyanines)	540	575
CY5 (indopentamethinecyanines)	640	670

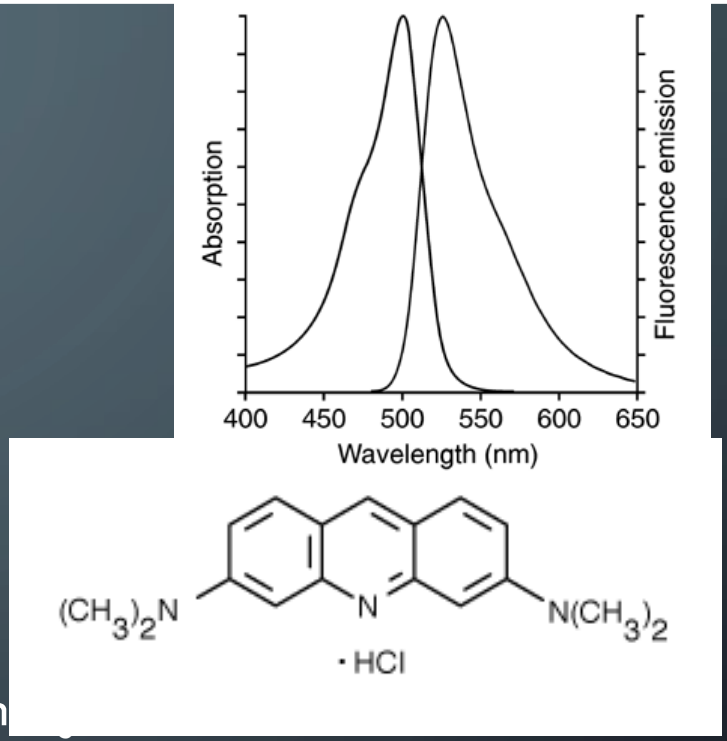
PROBES FOR NUCLEIC ACIDS

- Hoechst 33342 (AT rich) (uv) 346
- DAPI (uv) 359 461
- POPO-1 434 456
- YOYO-1 491 509
- Acridine Orange (RNA) 460
- Acridine Orange (DNA) 502 6
- Thiazole Orange (vis) 509 525
- TOTO-1 514 3
- Ethidium Bromide 526 4
- PI (uv/vis) 536 620
- 7-Aminoactinomycin D (7AAD) 555



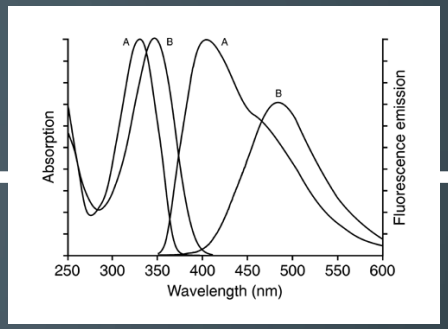
DNA PROBES

- AO
  - Metachromatic dye
    - concentration dependent emission
    - double stranded NA - Green
    - single stranded NA - Red
- AT/GC binding dyes
  - AT rich: DAPI, Hoechst, quinacrine
  - GC rich: antibiotics bleomycin, chromamycin olivomycin, rhodamine 800



PROBES FOR IONS

Indo-1



• INDO-1	E_x350	E_m405/480
• QUIN-2	E_x350	E_m490
• Fluo-3	E_x488	E_m525
• Fura -2	E_x330/360	E_m510

INDO-1: 1H-Indole-6-carboxylic acid, 2-[4-[bis[2-[(acetyloxy)methoxy]-2- oxoethyl]amino]-3-[2-[2-[bis[2- [(acetyloxy)methoxy]-2-oxoetyl]amino]-5- methylphenoxy]ethoxy]phenyl]-, (acetyloxy)methyl ester [$C_{47}H_{51}N_3O_{22}$] (just in case you want to know….!!)

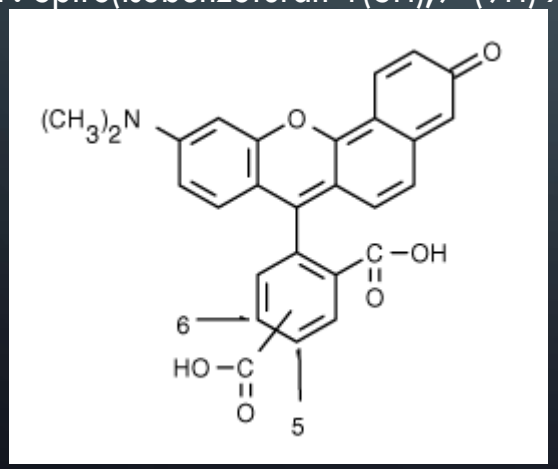
FLUO-3: Glycine, N-[4-[6-[(acetyloxy)methoxy]-2,7- dichloro-3-oxo-3H-xanthen-9-yl]-2-[2-[2-[bis[2-[(acetyloxy)methoxy]-2- oxyethyl]amino]-5- methylphenoxy]ethoxy]phenyl]-N-[2-[(acetyloxy)methoxy]-2-oxyethyl]-, (acetyloxy)methyl ester

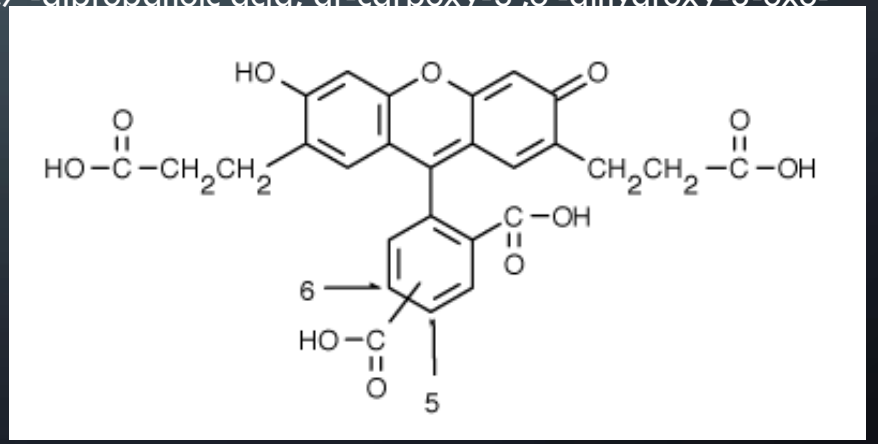
PH SENSITIVE INDICATORS

Probe	Excitation	Emission
• SNARF-1 $C_{27}H_{19}NO_6$	488	575
• BCECF $C_{27}H_{20}O_{11}$	488	525/620
	440/488	525

SNARF-1: Benzenedicarboxylic acid, 2(or 4)-[10-(dimethylamino)-3-oxo-3H- benzo[c]xanthene-7-yl]-

BCECF: Spiro(isobenzofuran-1(3H),9'-(9H) xanthene)-2',7'-dipropanoic acid, ar-carboxy-3',6'-dihydroxy-3-oxo-

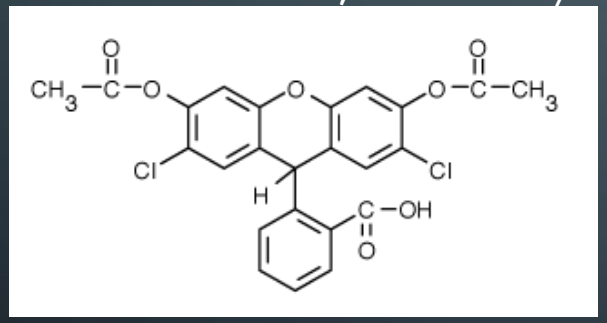


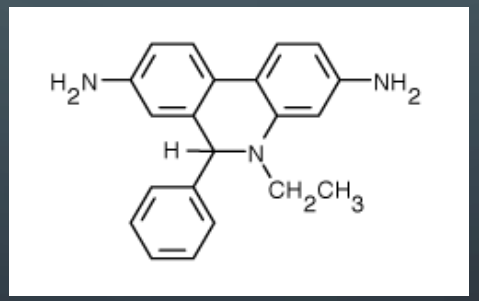


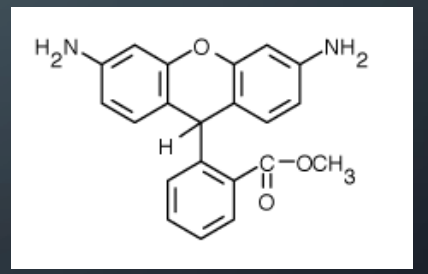
PROBES FOR OXIDATION STATES

Probe	Oxidant	Excitation	Emission
• DCFH-DA	(H_2O_2)	488	525
• HE	(O_2^-)	488	590
• DHR 123	(H_2O_2)	488	525

DCFH-DA: 2',7'-dichlorodihydrofluorescein diacetate (2',7'-dichlorofluorescin diacetate; H2DCFDA)







$C_{24}H_{16}C_{l2}O_7$ DCFH-DA - dichlorofluorescin diacetate

$C_{21}H_{21}N_3$ HE - hydroethidine 3,8-Phenanthridinediamine, 5-ethyl-5,6-dihydro-6-phenyl-

$C_{21}H_{18}N_2O_3$ DHR-123 - dihydrorhodamine 123 Benzoic acid, 2-(3,6-diamino-9H-xanthene-9-yl)-, methyl ester

SPECIFIC ORGANELLE PROBES

Probe	Site	Excitation	Emission
BODIPY	GOLGI	505	511
NBD	GOLGI	488	525
DPH	LIPID	350	420
TMA-DPH	LIPID	350	420
RHODAMINE 123	MITOCHONDRIA	488	525
DIO	LIPID	488	500
DII-CN-(5)	LIPID	550	565
DIO-CN-(3)	LIPID	488	500

BODIPY - borate-dipyrromethene complexes
DPH – diphenylhexatriene
NBD - nitrobenzoxadiazole
TMA - trimethylammonium

OTHER PROBES OF INTEREST

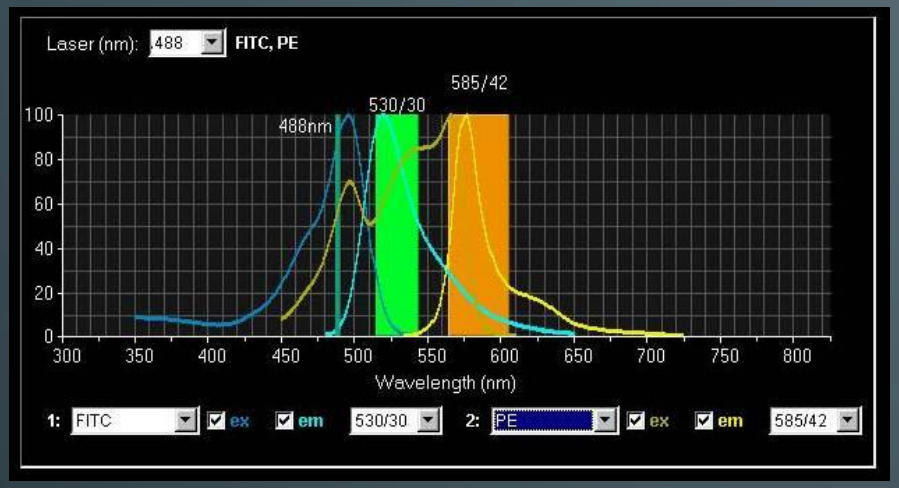
- GFP - *Green Fluorescent Protein*
  - GFP is from the chemiluminescent jellyfish *Aequorea victoria*
  - excitation maxima at 395 and 470 nm (quantum efficiency is 0.8) Peak emission at 509 nm
  - contains a p-hydroxybenzylidene-imidazolone chromophore generated by oxidation of the Ser-Tyr-Gly at positions 65-67 of the primary sequence
  - Major application is as a reporter gene for assay of promoter activity
  - requires no added substrates

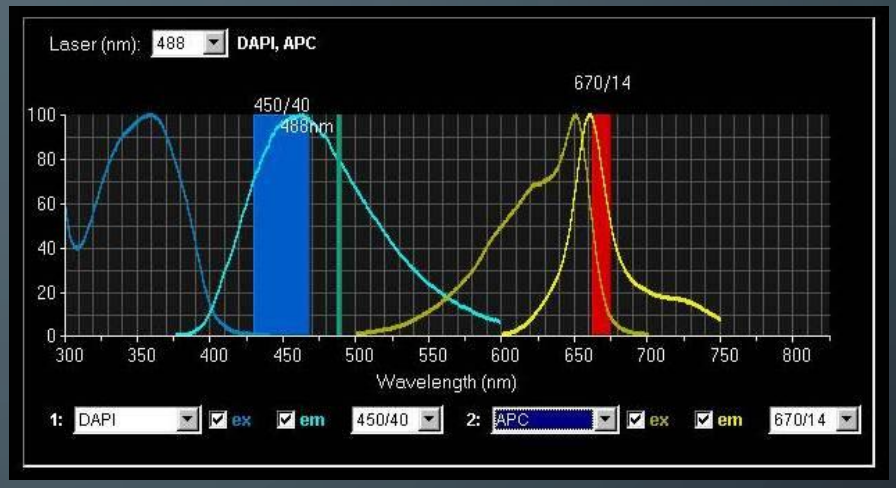
MULTIPLE EMISSIONS

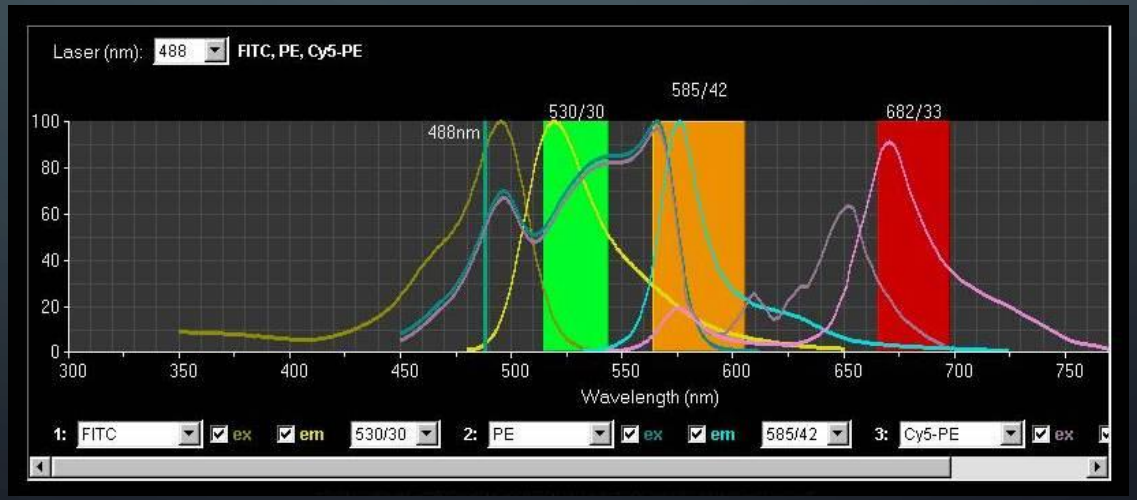
- Many possibilities for using multiple probes with a single excitation
- Multiple excitation lines are possible
- Combination of multiple excitation lines or probes that have same excitation and quite different emissions
  - e.g. Calcein AM and Ethidium (**ex 488 nm**)
  - emissions **530 nm** and **617 nm**

FILTER COMBINATIONS

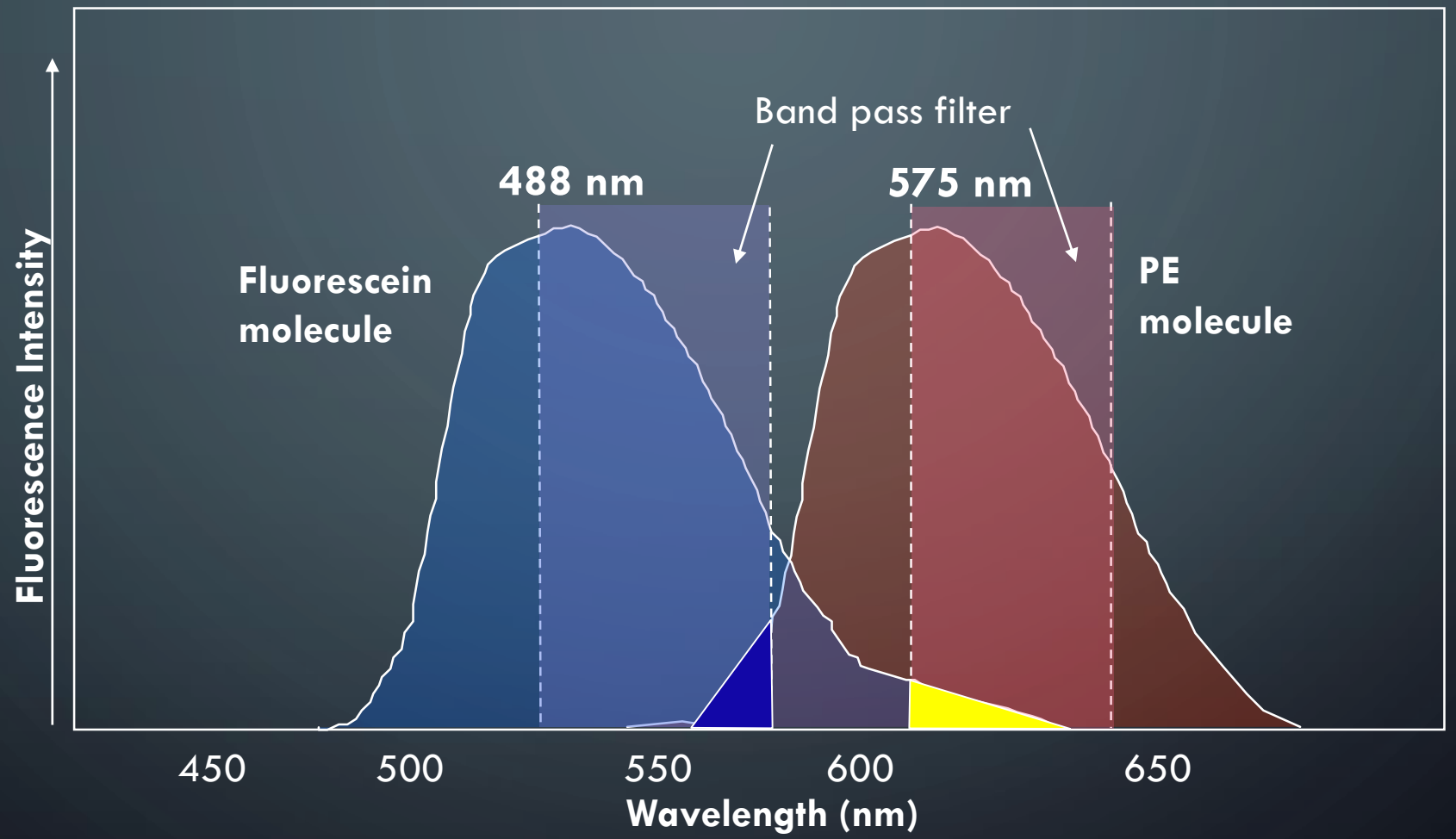
- The band width of the filter will change the intensity of the measurement







FLUORESCENCE OVERLAP



Overlap of FITC fluorescence in PE PMT

Overlap of PE fluorescence in FITC PMT

Fluorescence microscopy

– Principle and practical consideration

Fluorescence microscopy

Excites and observe fluorescent molecules

The most commonly used microscopy

High resolution, sensitive with low background, multi-channel…

comes with variations (fancy names).

deconvolution, OMX, deltavision
confocal, spinning disc, two photon
TIRF, FRAP, FRET, FLIM, iFRAP, FCS …

PALM, STED, STORM, SIM, (super-resolution)

still in development

What can you do with a fluorescence microscope?

For example:

Determine the localisation of specific (multiple) proteins

Determine the shape of organs, cells, intracellular structures

Examine the dynamics of proteins

Study protein interactions or protein conformation

Examine the ion concetration etc.

can observe in live cells

FLUORESCENCE TECHNIQUES

SPECIAL APPLICATIONS:

- FRET and FLIM
- FRAP and photoactivation
- TIRF

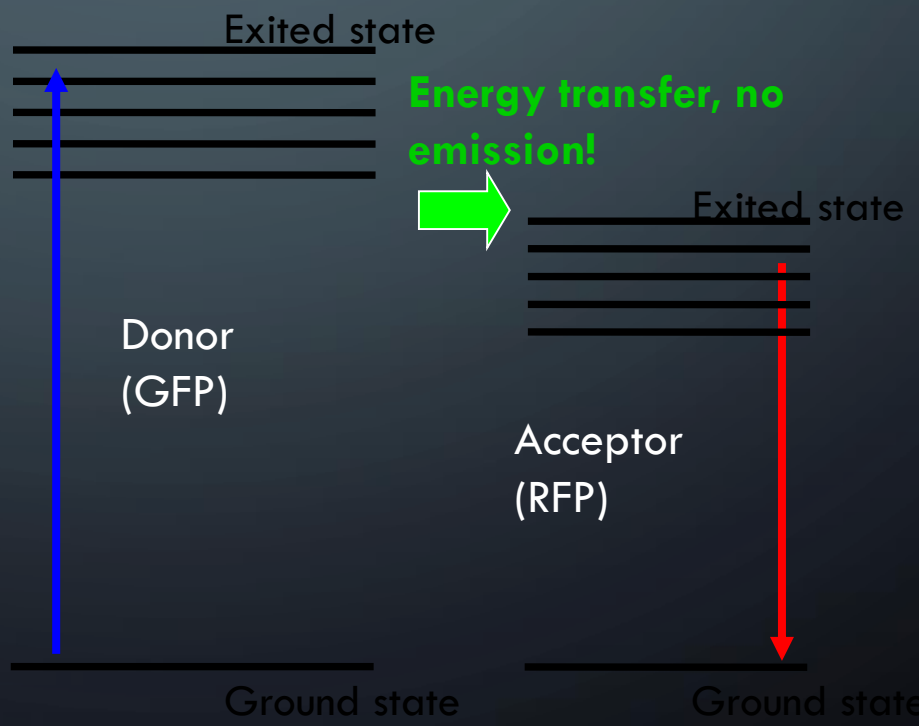
FRET (FLUORESCENCE RESONANCE ENERGY TRANSFER)

- method to investigate molecular interactions
- **Principle:** a close acceptor molecule can take the excitation energy from the donor (distance ca 1-10 nm)

No FRET



FRET situation: Excitation of the donor (GFP) but **emission** comes from the acceptor (RFP)



FRET

ways to measure:

- Acceptor emission

  Detect the emission of the acceptor after excitation of the donor, e.g. excite GFP with 488 but detect RFP at 610 (GFP emission at 520)

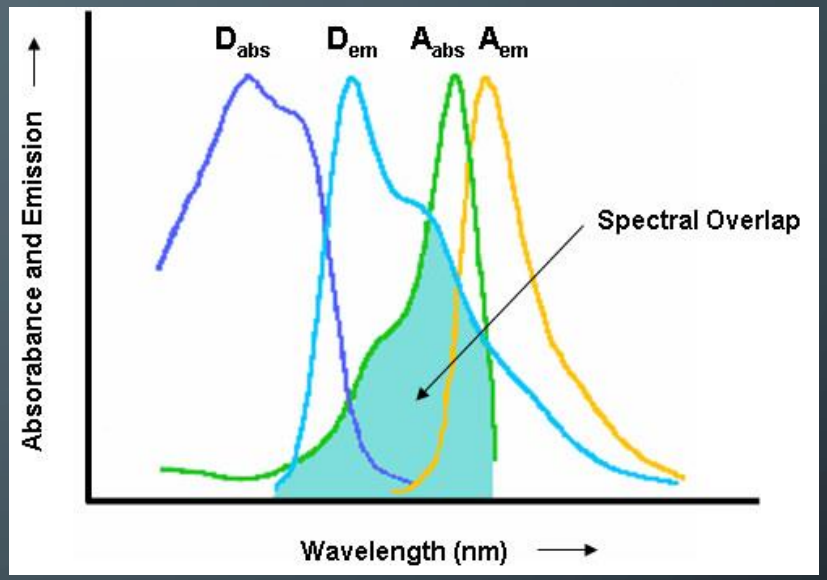
- Donor emission after acceptor bleaching take image of donor, then bleach acceptor (with acceptor excitation wavelength - RFP:580nm), take another image of donor → should be brighter!

FRET

You need:

- a suitable FRET pair

(with overlapping excitation/emission curves)



Disadvantages:

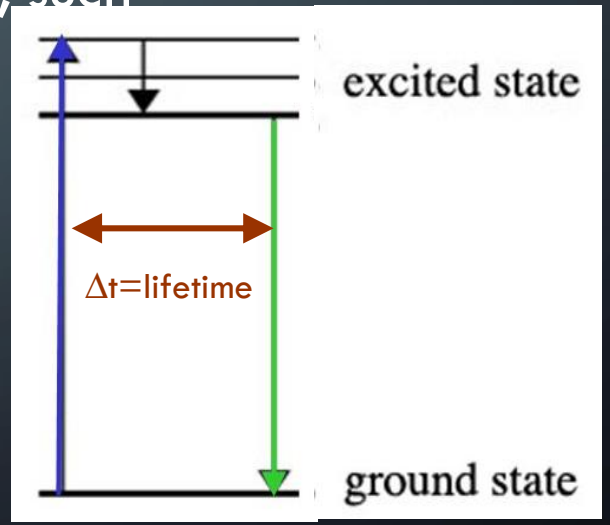
- Bleed through (because of overlapping spectra)

→Limitation of techniques (filters etc)

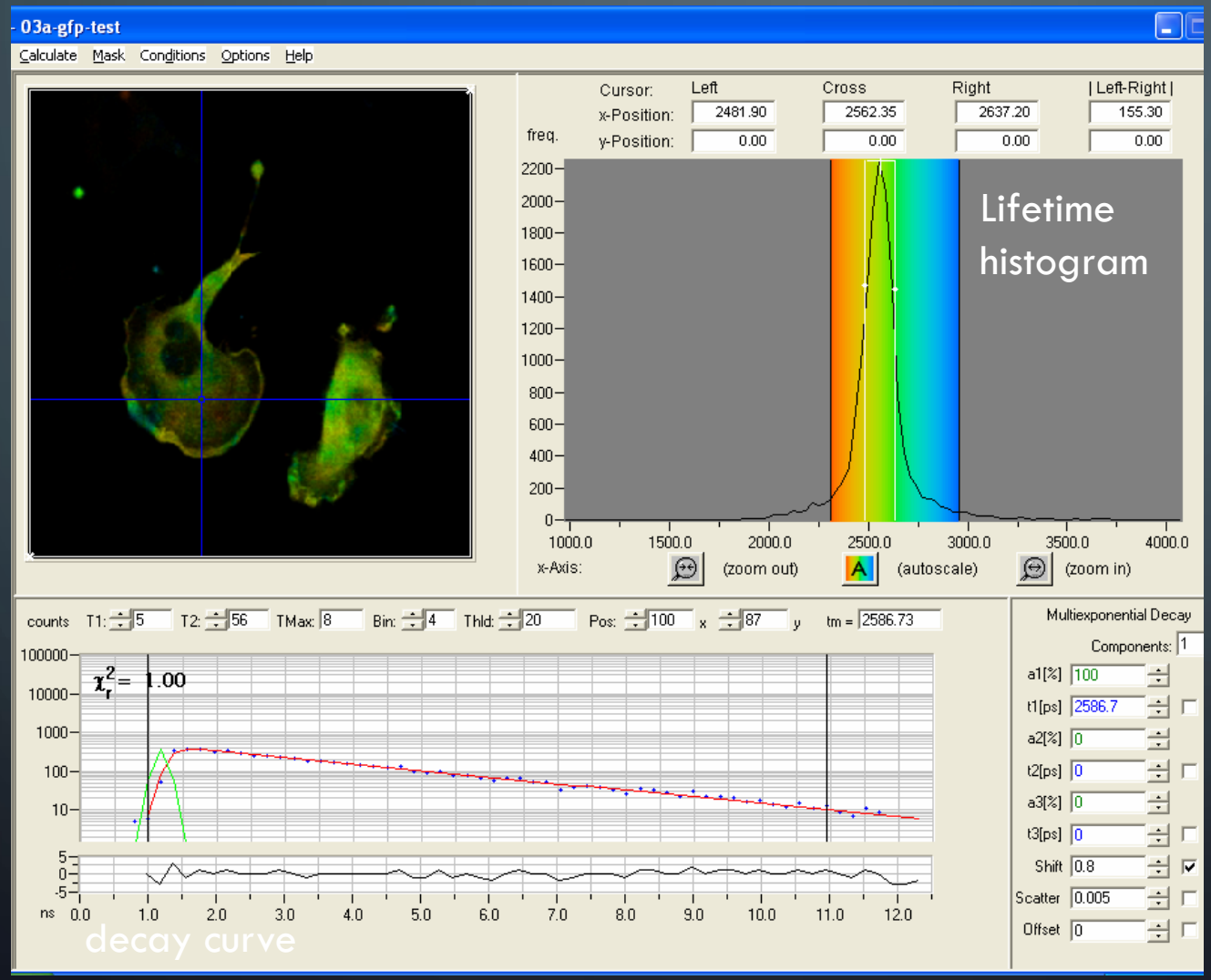
- Photobleaching only with fixed samples
- Intensity depends on concentrations etc

FLIM
(FLUORESCENCE LIFETIME IMAGING MICROSCOPY)

- measures the lifetime of the excited state (delay between excitation and emission)
- every fluorophore has a unique natural lifetime
- lifetime can be changed by the environment, such as:
  - ✓ Ion concentration
  - ✓ Oxygen concentration
  - ✓ pH
  - ✓ **Protein-protein interactions**



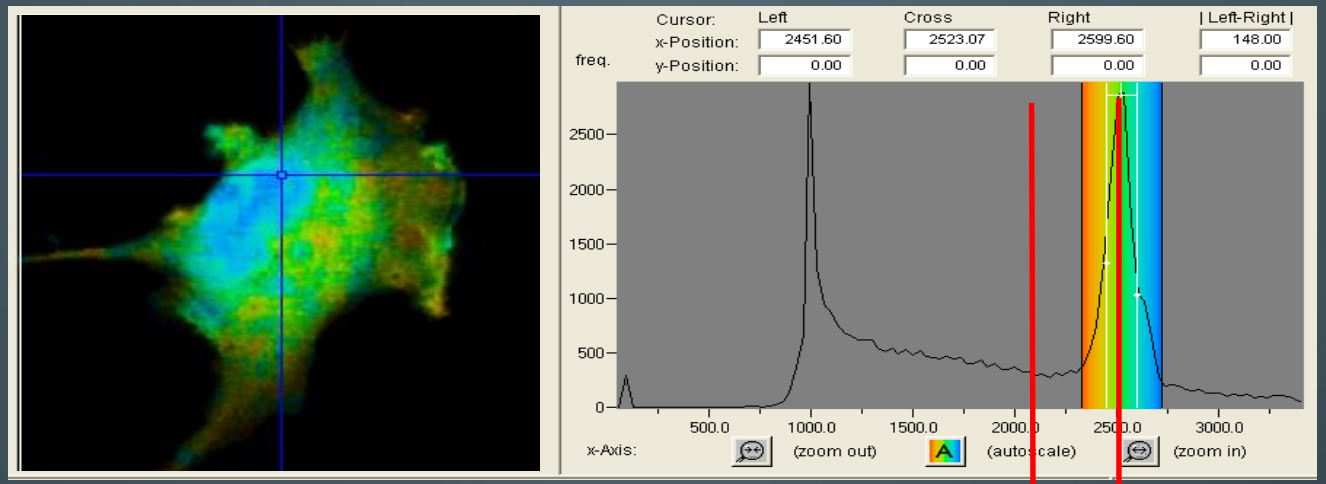
FLIM



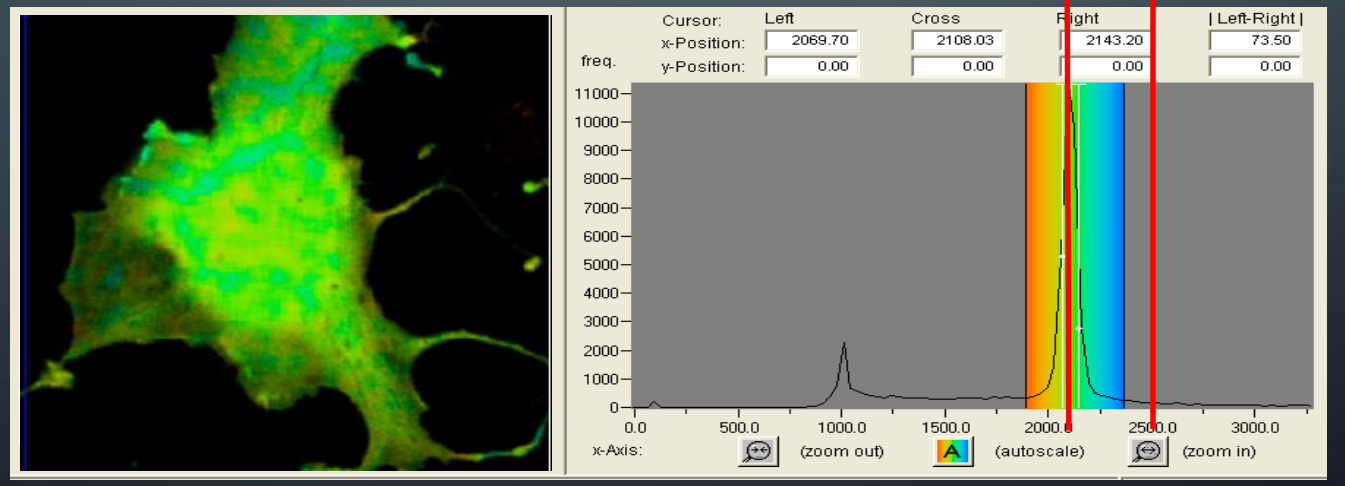
Excitation of many electrons at the same time → count the different times when they are falling back down (i.e. photons are emitted)

lifetime = ½ of all electrons are fallen back

EXAMPLE OF FLIM-FRET MEASUREMENT



GFP expressed in COS 1 cell: average lifetime of 2523 ps



fused GFP-RFP expressed in COS 1 cell: average lifetime of 2108 ps

FLIM

You still need: a suitable FRET-pair with the right orientation of the π-orbitals

➔ Interaction of proteins is not enough, because fluorophores have to be close enough and in the right orientation!

Use of FLIM: measurements of concentration changes (Ca2+), pH change etc, Protein interactions

→ *FRET: Leica confocal 2 or Olympus FV 1000*

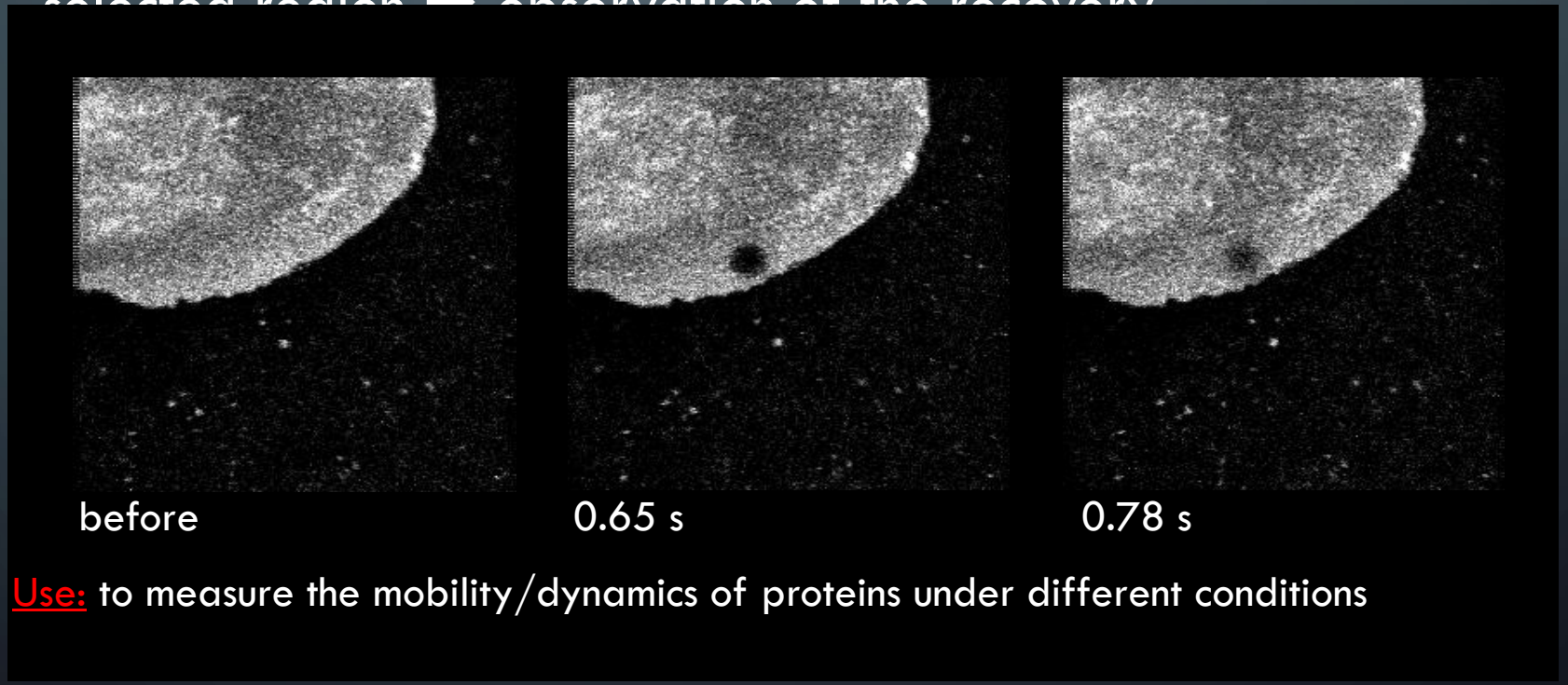
→ *FLIM: Leica confocal 1 and soon LIFA system from Lambert Instruments*

SPECIAL APPLICATIONS:

- FRET and FLIM
- **FRAP and photoactivation**
- TIRF

FRAP (FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING)

- Intense illumination with 405 laser bleaches the sample within the selected region → observation of the recovery

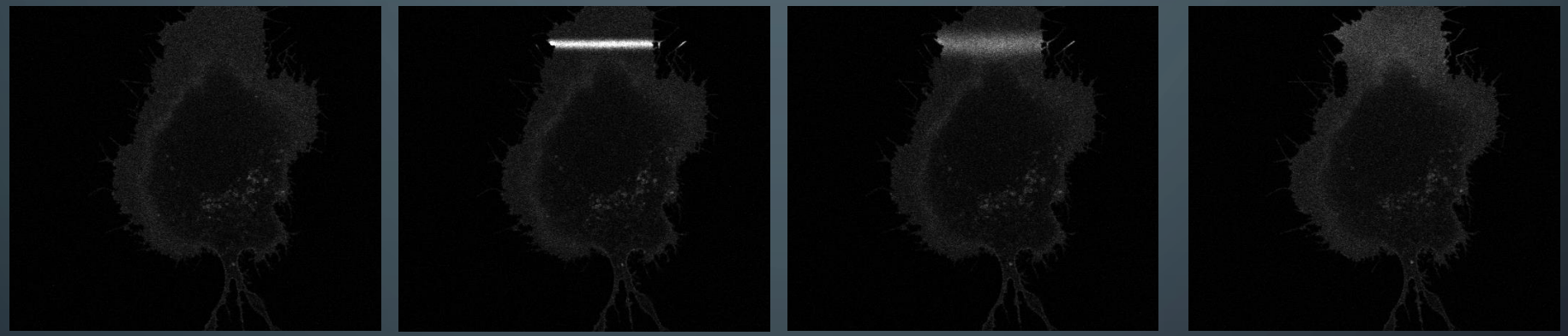


Use: to measure the mobility/dynamics of proteins under different conditions

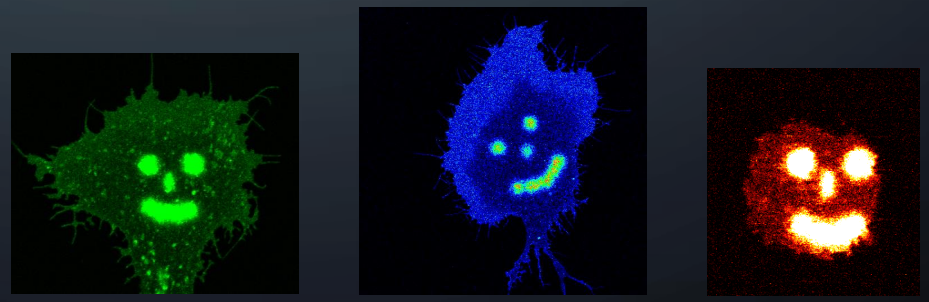
→ *Olympus FV 1000*

PHOTOACTIVATION

- Fluorophore only becomes active (= fluorescent) if excited (e.g. with 405 laser) due to structural change



Pictures taken from a activation movie: activation of a line trough the lamellipodia of the cell, activated GFP_F diffuses quickly

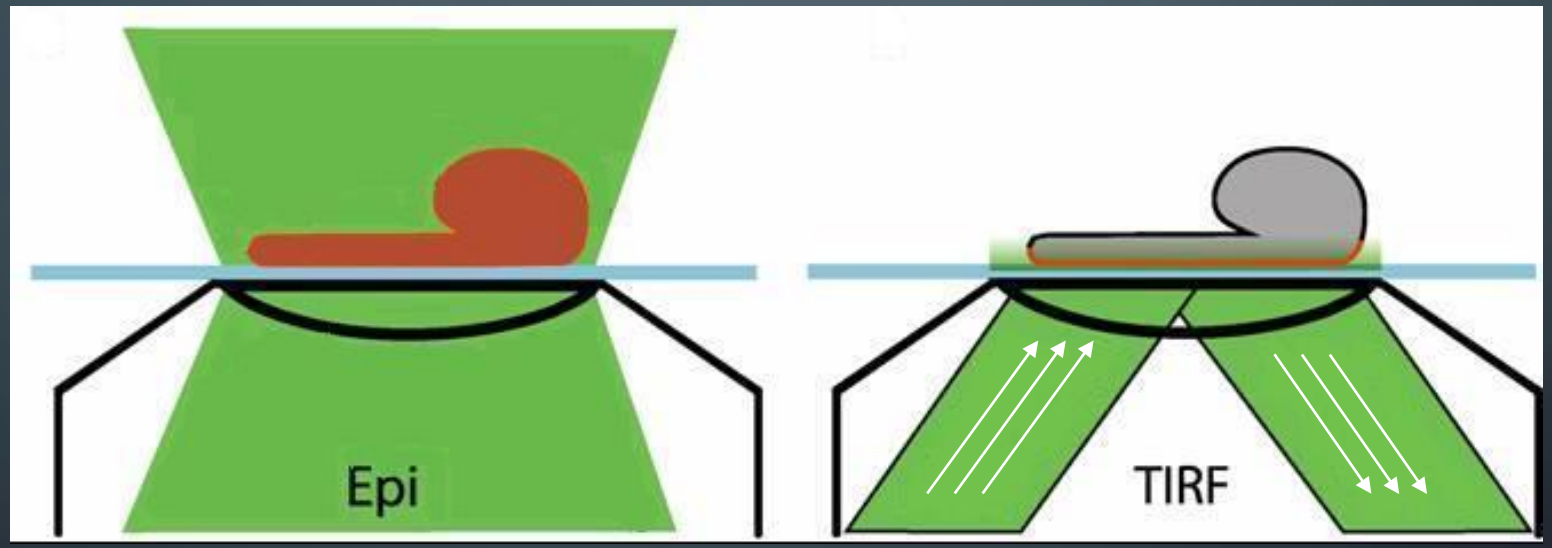


→ *Olympus FV 1000*

SPECIAL APPLICATIONS:

- FRET and FLIM
- FRAP and photoactivation
- **TIRF**

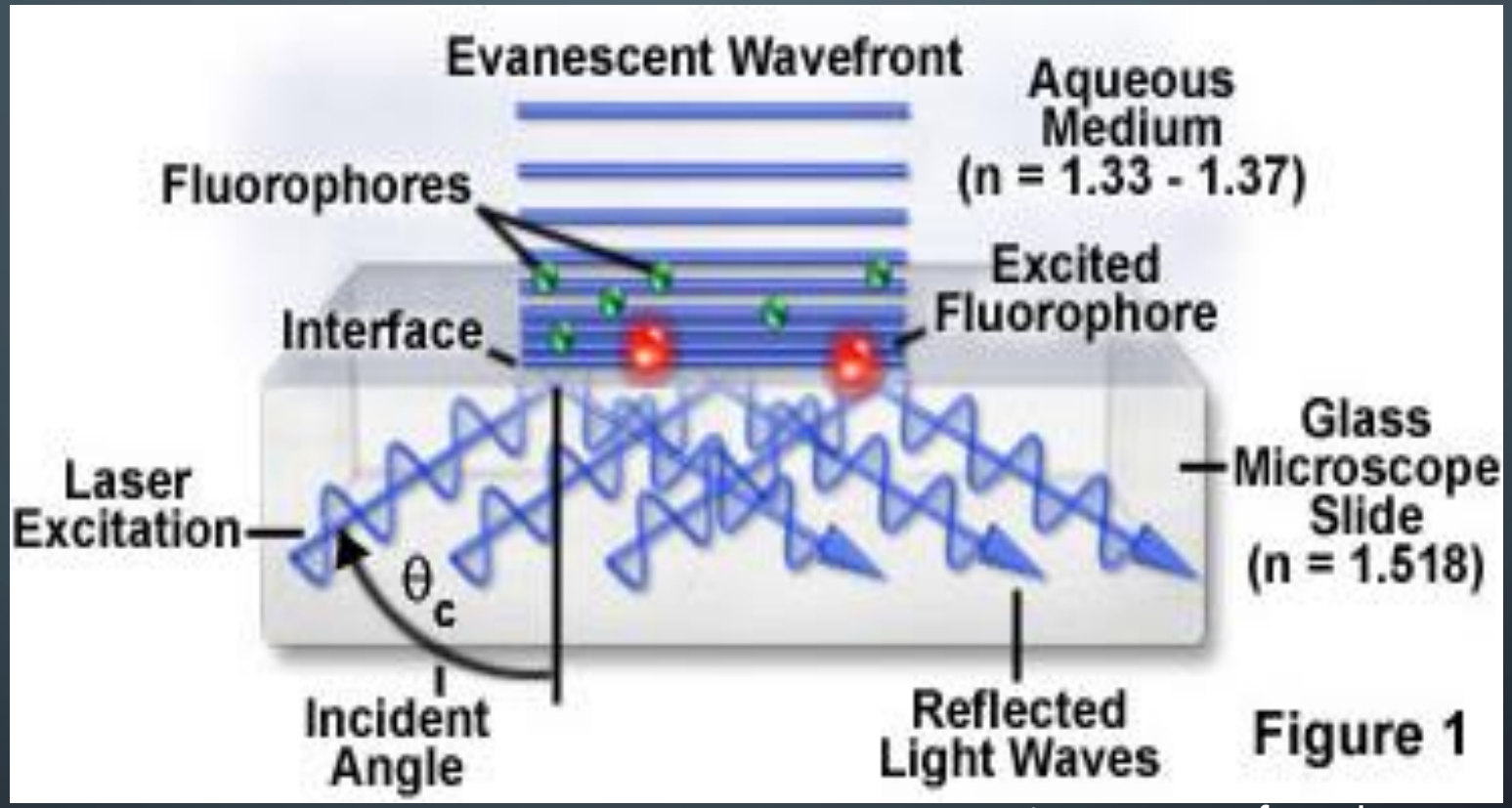
TIRF (TOTAL INTERNAL REFLECTION FLUORESCENCE)



You need:

- TIRF objectives with high NA
- TIRF condensor, where you are able to change the angle of illumination
- Glass coverslips

TIRF



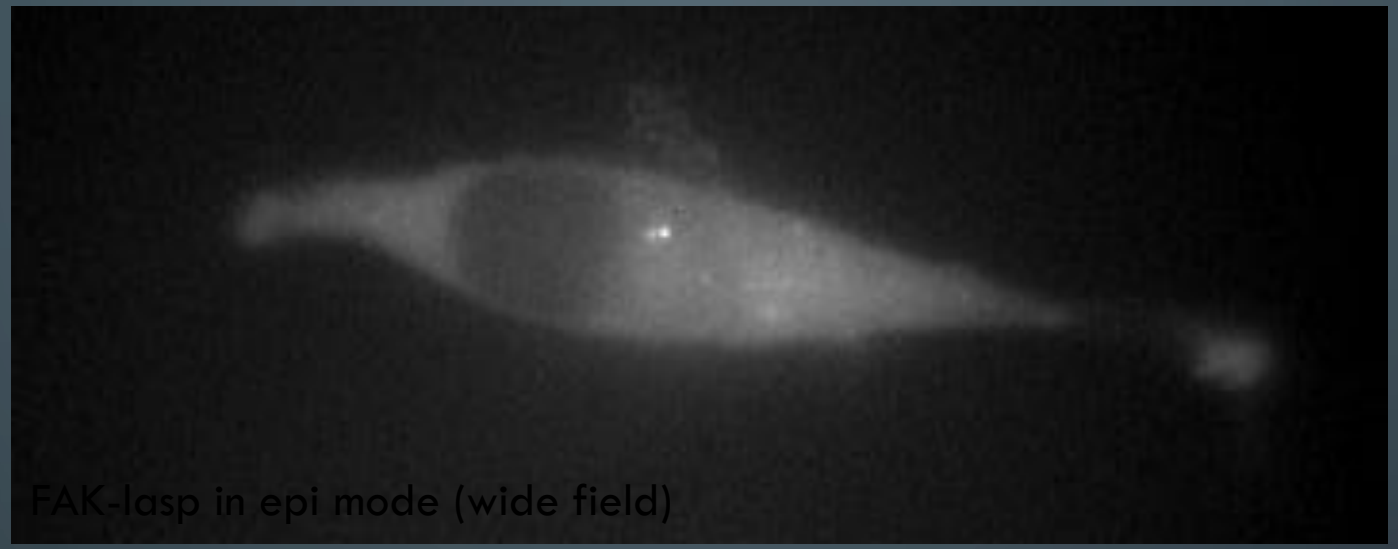
micro.magnet.fsu.edu

Result: very thin section at the bottom of the sample → 150-200nm

Use: to study membrane dynamics (endocytosis, focal adhesions, receptor binding)

→ *Nikon TE 2000*

TIRF VS EPI



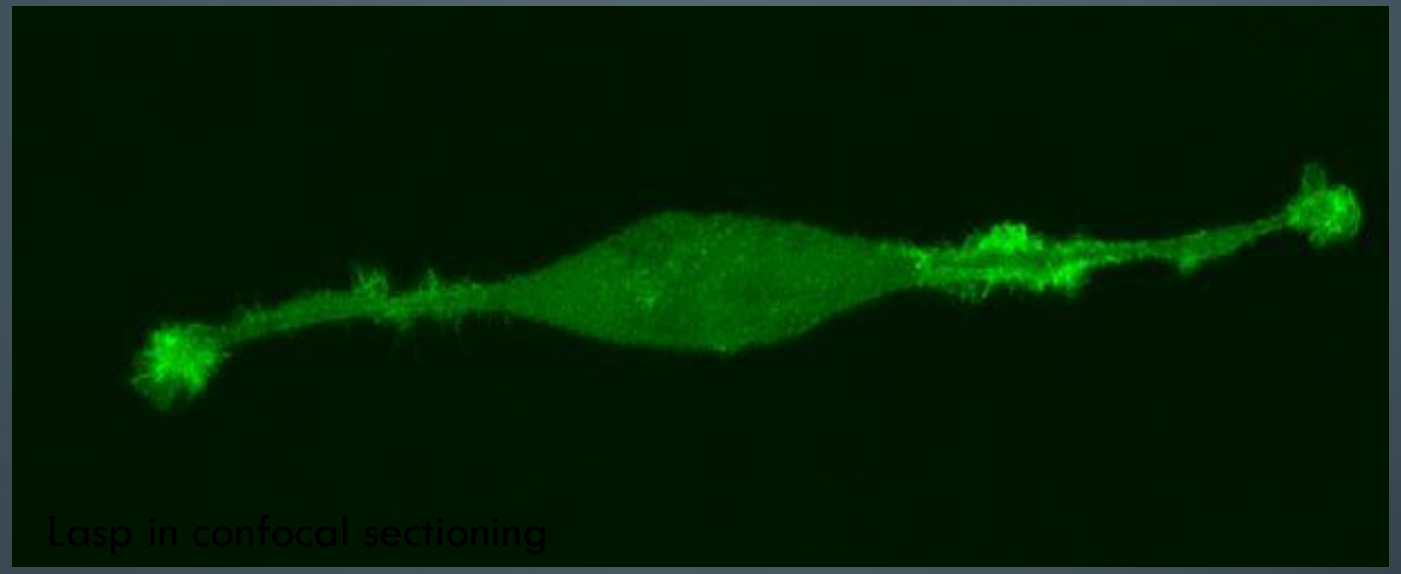
FAK-lasp in epi mode (wide field)

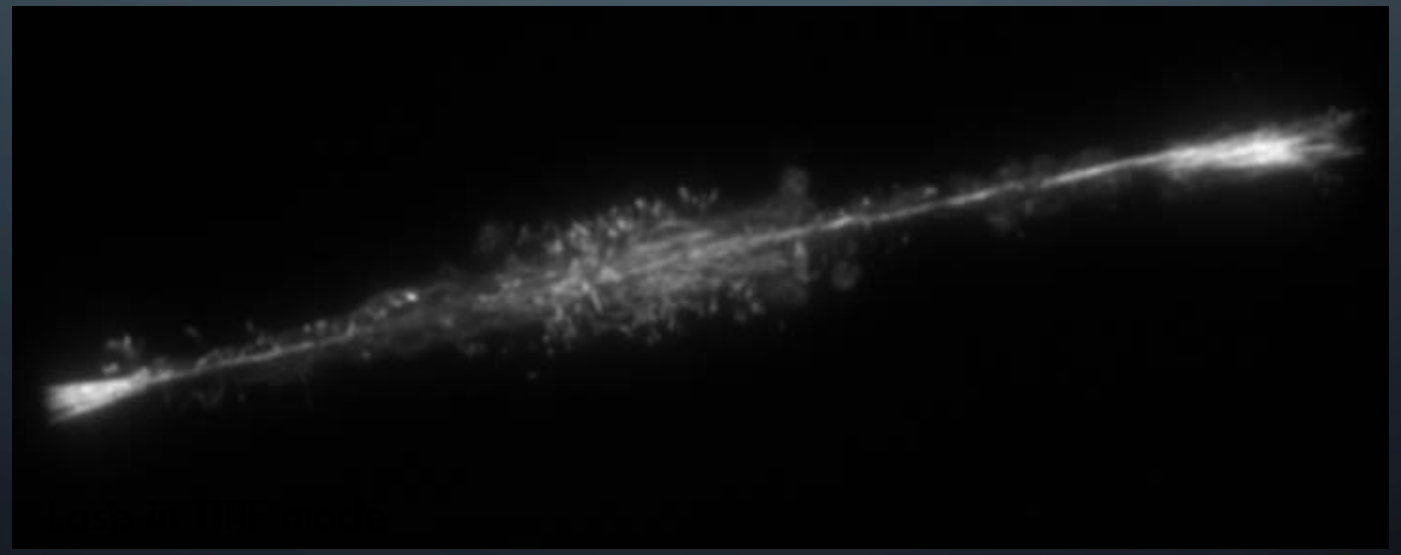


FAK-lasp in tirf mode (wide field)

Heather Spence, R10

TIRF VS EPI





Heather Spence, R10

SUMMARY/COMPARISON

method	excitation	detection	sectioning	use
Wide field	Whole sample	Whole sample	No sectioning	Simple fluorescence samples
confocal	Whole sample	One z-plane	350-500nm	High contrast images, optical sectioning
2-Photon	One z-plane	One z-plane	500-700nm	Deep tissue imaging, optical sectioning
FLIM/FRET				Protein interactions
FRAP + photoactivation	405 laser (UV)			dynamics/mobility
TIRF	Only bottom plane	Only bottom plane	150-200nm	Membrane dynamics