Advanced Microscopy

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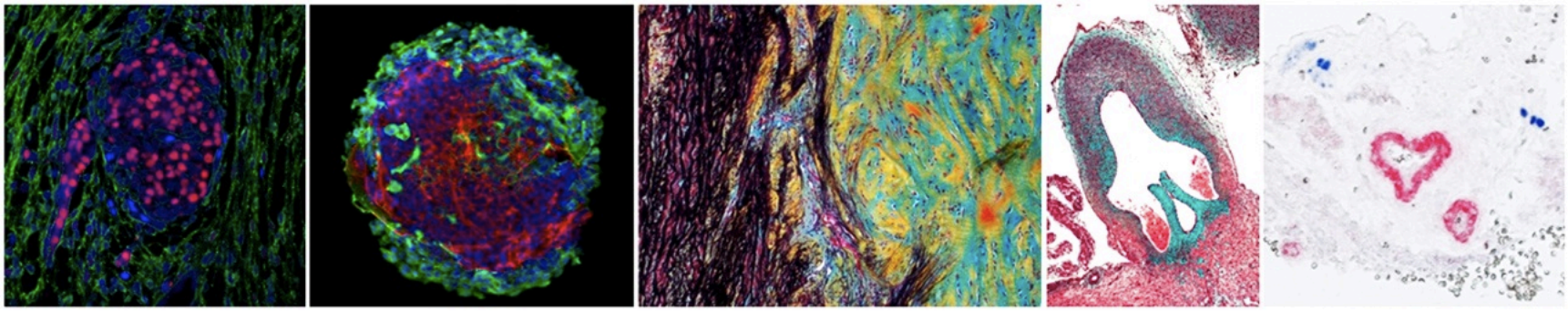


Advanced Microscopy



Introduction

Basics of light microscopy

- Resolution, NA, refractive index, objectives, contrast

Fluorescence Microscopy

- Fluorophores and fluorescence
- Wide-field and confocal fluorescence microscopy

Fluorescence Life time imaging microscopy (FLIM)

History of microscopy

Microscope in 1872

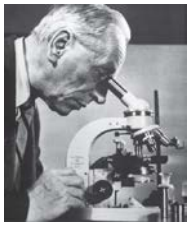














$$d = \frac{\lambda}{2n\sin\alpha}$$



Laser scanning microscope, today







History of microscopy

Microscope in 1872



Schott



Abbe



Zeiss



Laser scanning microscope, today



Fraunhofer



Köhler



Zernike



$$d = \frac{\lambda}{2n\sin\alpha}$$

Stokes



Göppert-Mayer



Förster



What does microscopy mean?

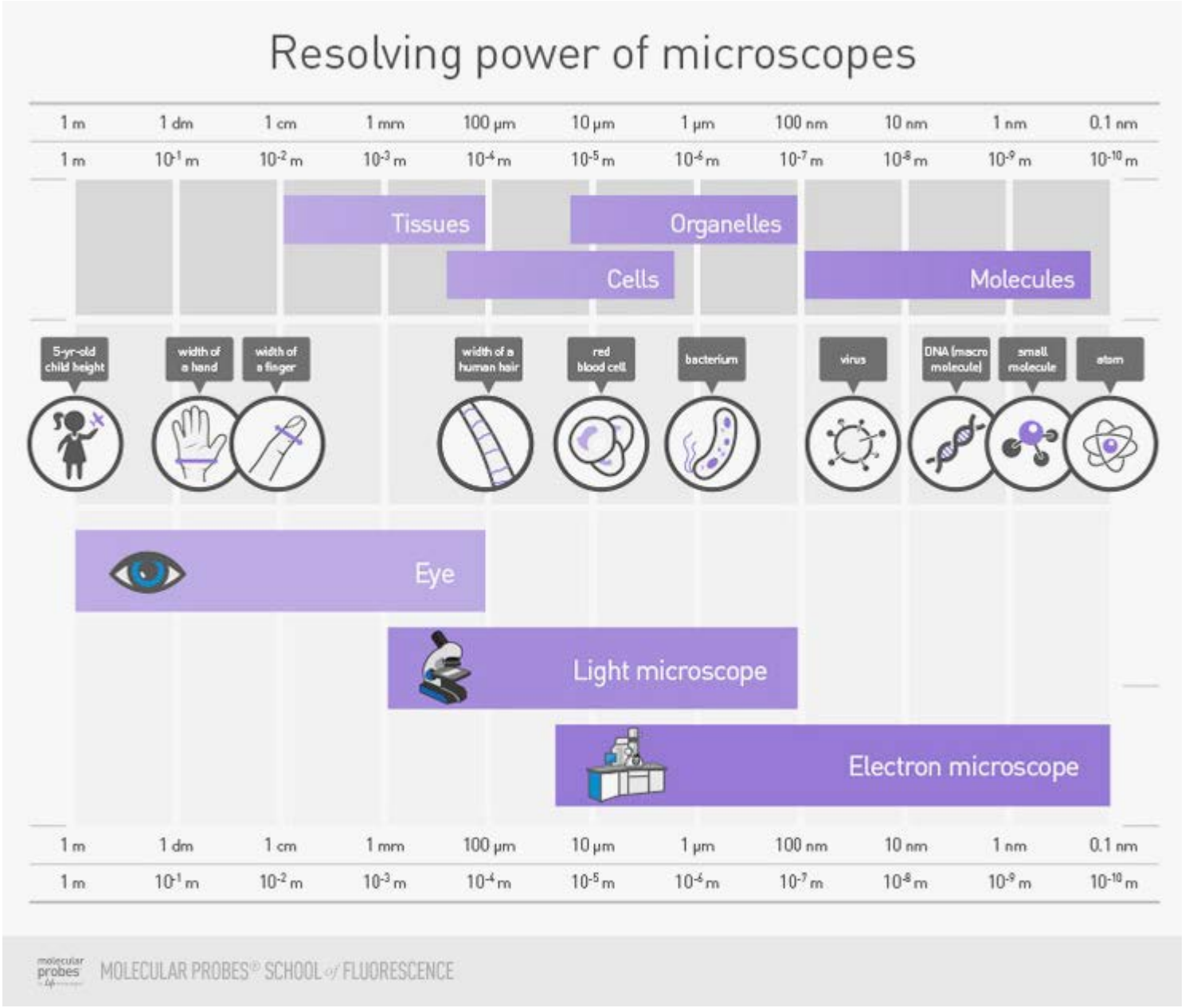
- Microscope (from the ancient Greek)

  Mikrós = "small"

  Skopeîn = "to look" or "see“

- Generating a larger image of something small
- Stepwise and multiple magnification
- Basic principle: objective generates a magnified intermediate image, which is again magnified by the tube lens

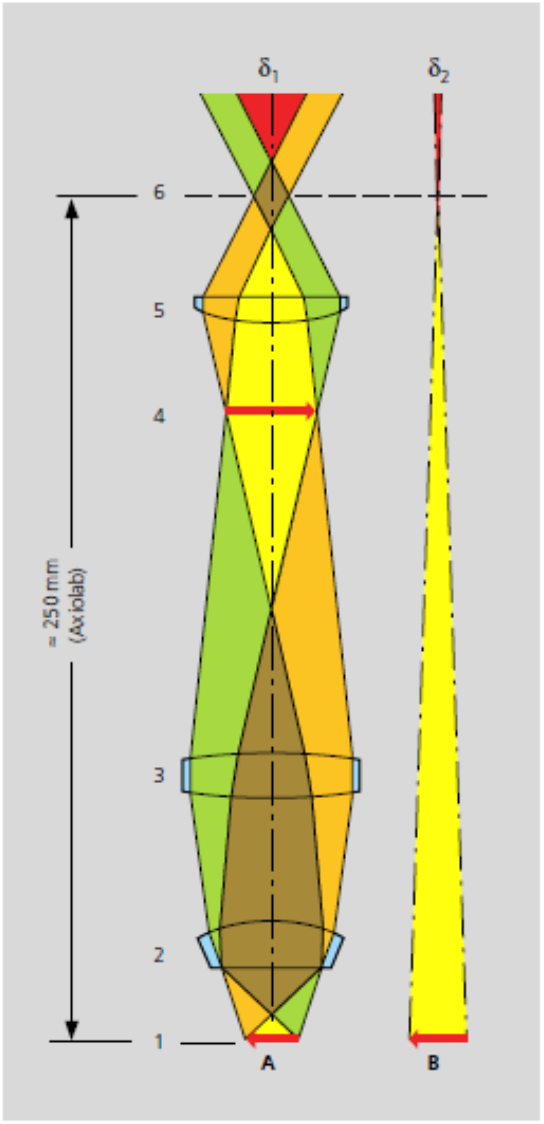




https://www.thermofisher.com/ca/en/home/life-science/cell-analysis/cell-analysis-learning-center/molecular-probes-school-of-fluorescence/fundamentals-of-fluorescence-microscopy/epifluorescence-microscope-basics.html

Basic light pathway

1. Object
2. Objective
3. Tube lens
4. Magnified intermediate image
5. Eyepiece (ocular)
6. Eye



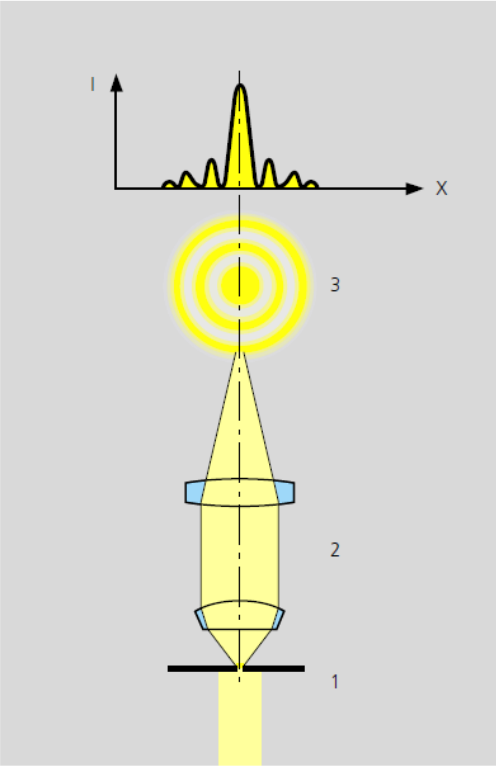
Zeiss: Microscopy from the very beginning

Magnification:

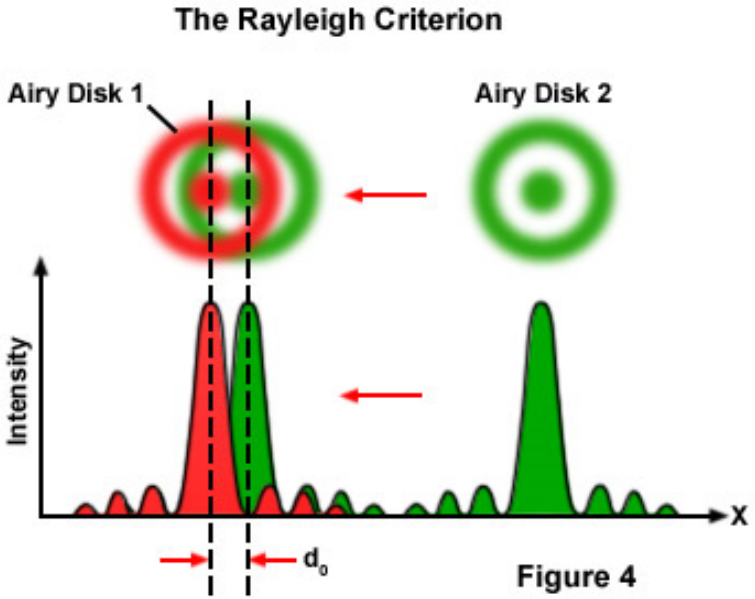
$M_{microscope} = M_{objective} \times M_{ocular}$

Resolution

- Objective and tube lens image an ‘airy disc’



Zeiss: Microscopy from the very beginning

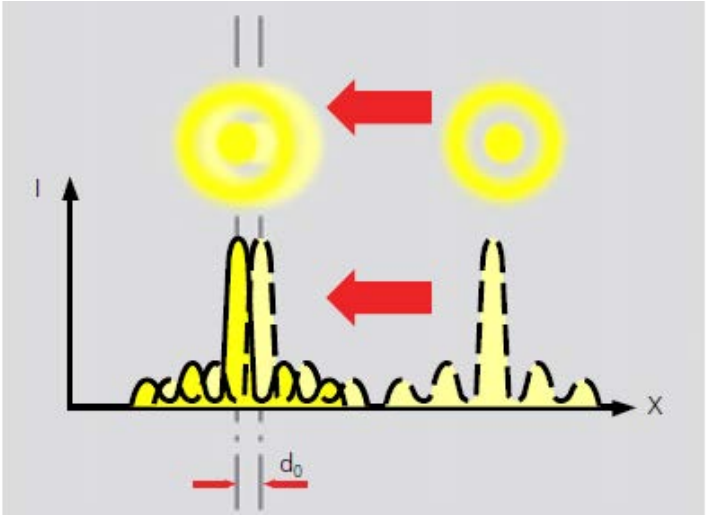


1 Object
2 Objective and tube lens
3 Diffraction rings =“airy disk”

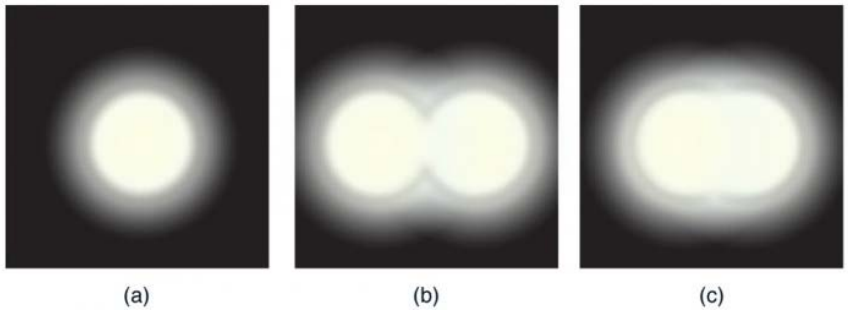
Resolution



- d_0 = distance at which two points are seen separable



Zeiss: Microscopy from the very beginning



https://courses.lumenlearning.com/austincc-physics2/chapter/27-6-limits-of-resolution-the-rayleigh-criterion/

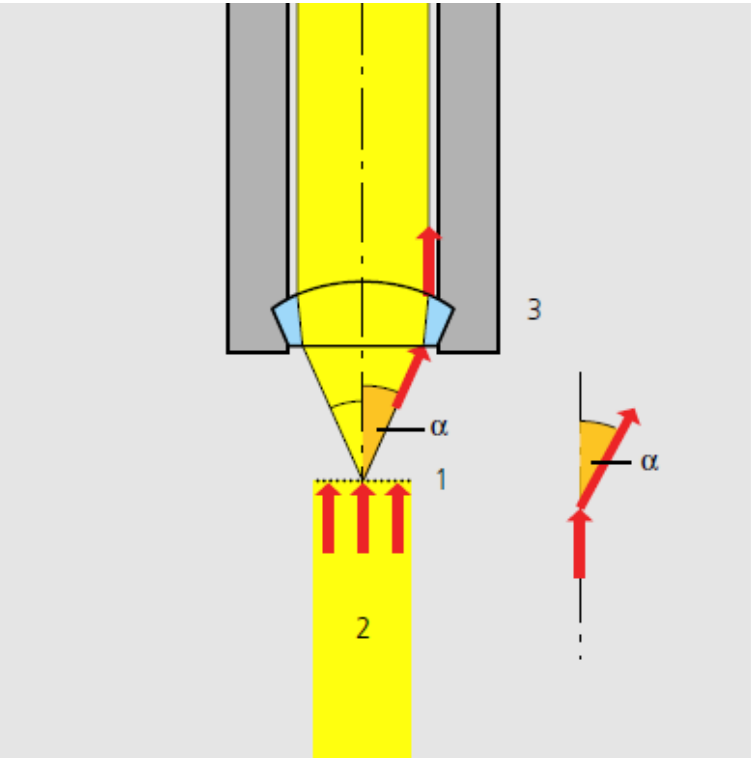
Abbe formula:

$$d_0 = \frac{1.22\lambda}{N.A_{obj.} + N.A._{Cond}} \quad \text{or more simply } d_0 = \frac{\lambda}{2N.A}$$

λ = wavelength of light, e.g. 550 nm (green)

=> The resolution is dependent on the N.A.

Numerical Aperture (N.A.)



Zeiss: Microscopy from the very beginning

1 Object
2 Incident light
3 Objective

- N.A. = “opening” of an objective
- The smaller the object the more light is diffracted
- To collect sharp images from small objects, the objective needs to collect as much diffracted light as possible

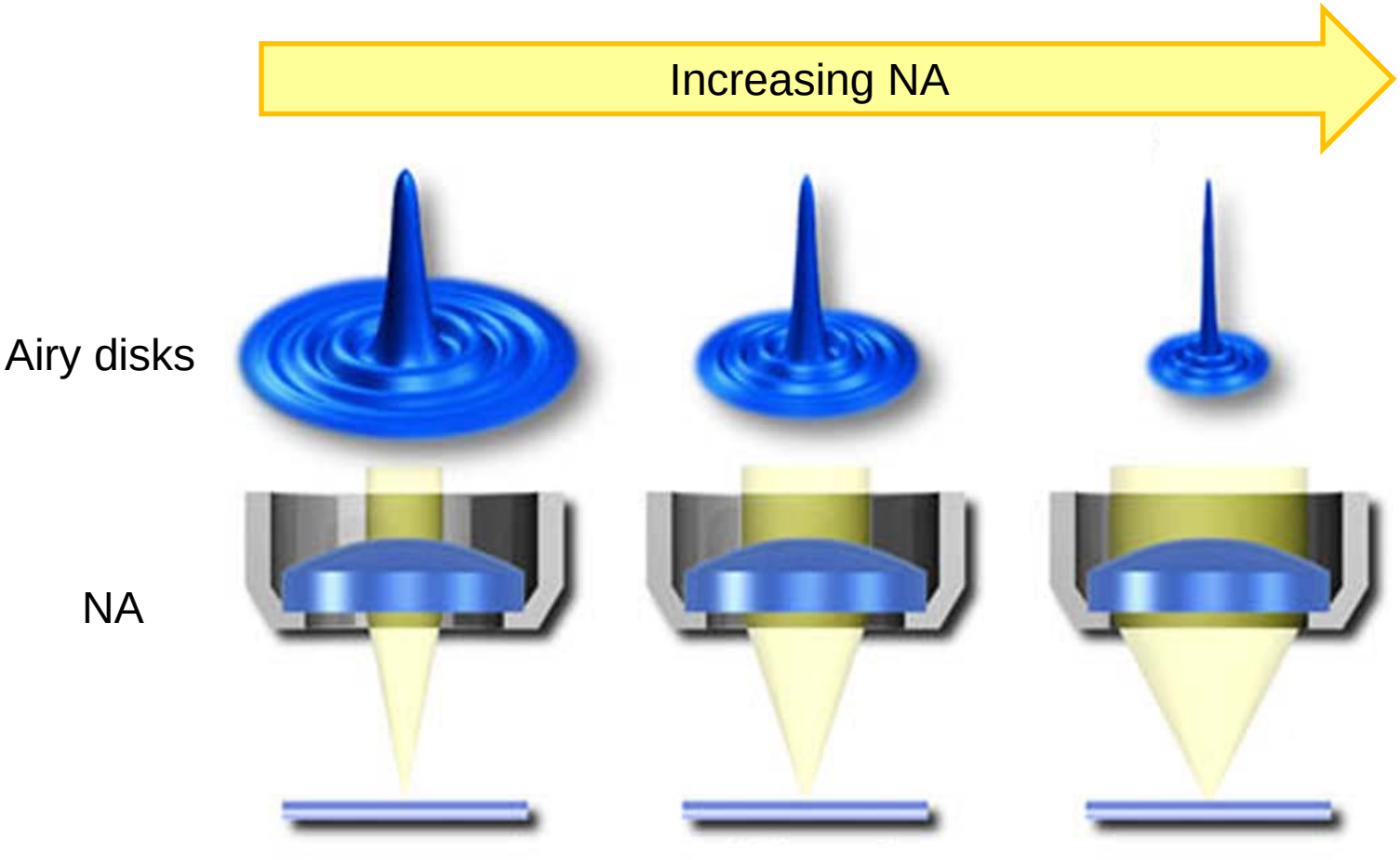
Numerical Aperture = N.A. = n · sin α

α is half the opening angle of the objective.

n is the refractive index of the immersion medium used between the objective and the object.

(n = 1 for air; n = 1.51 for oil or glass)

Resolution

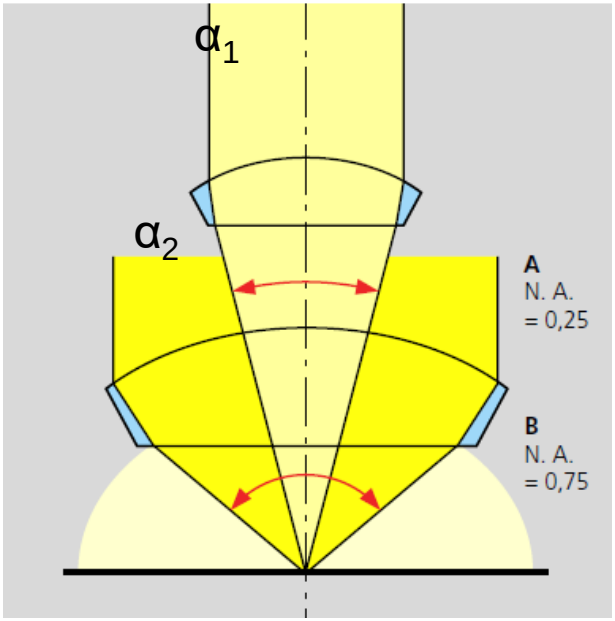


http://www.olympusmicro.com/primer/anatomy/numaperture.html

Increasing the resolution

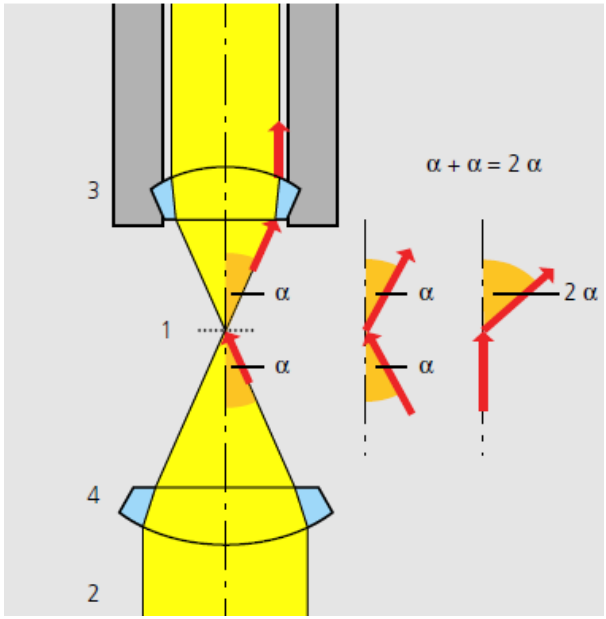
$$d_0 = \frac{\lambda}{2N.A}$$

Increase N.A. of the objective



Zeiss: Microscopy from the very beginning

Condenser lense (4) with the same N.A. as the objective

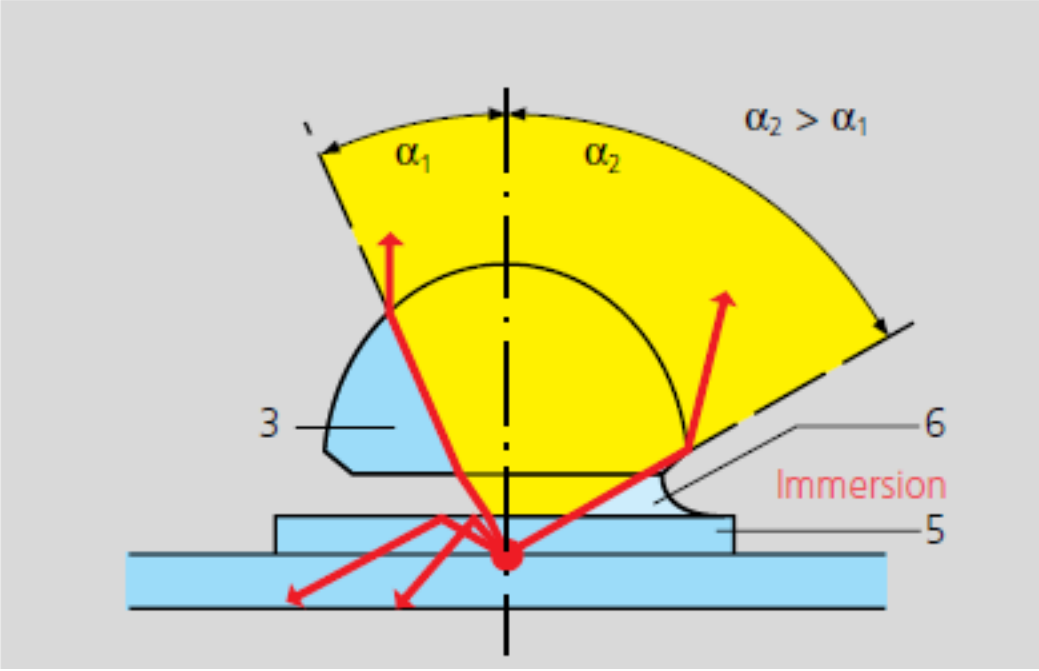


Zeiss: Microscopy from the very beginning

Increasing the resolution

- N.A. is dependent on the refractive index n
- Increasing n due to reduction of the reflections on the microscopic devices

  => more light reaches the objective



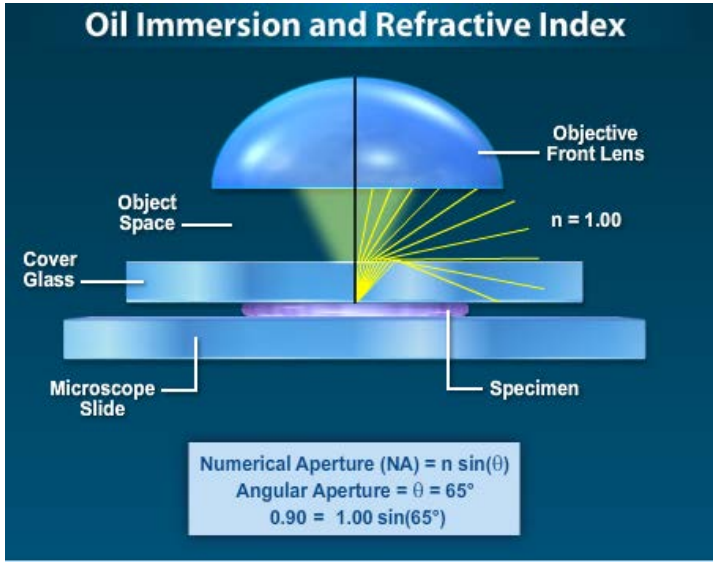
3 Objective
5 Cover slip
6 Immersion liquid

Zeiss: Microscopy from the very beginning

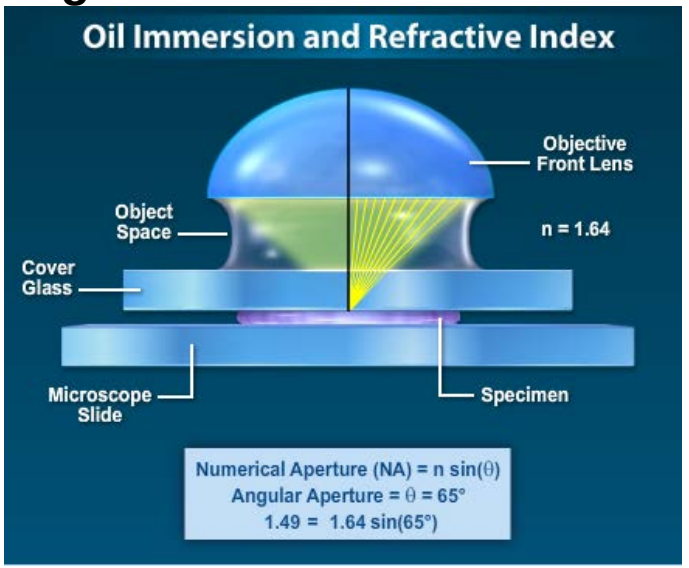
Refractive index

Refractive index n is a value that describes the speed of light in a second medium compared to light speed in vacuum

Low refractive index



High refractive index



http://zeiss-campus.magnet.fsu.edu/tutorials/basics/oilimmersionrefractiveindex/index.html

Refractive indices n of immersion liquids

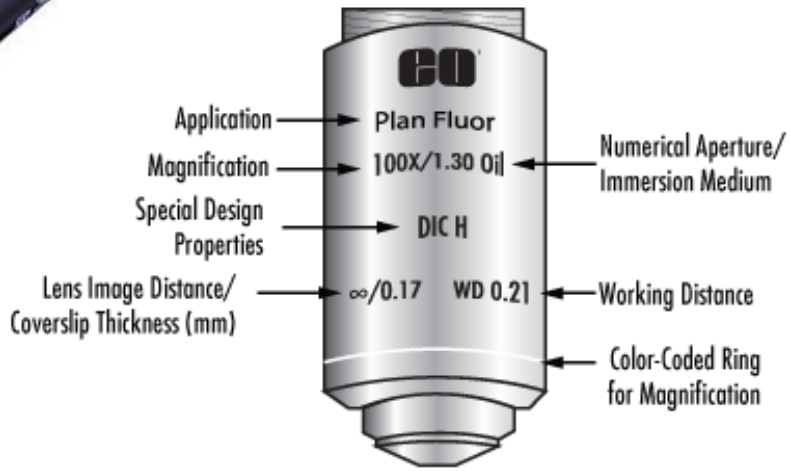
Air	= 1.00
Water	= 1.33
Glycerol	= 1.47
Oil	= 1.57

Objectives





Romeis: Mikroskopische Technik

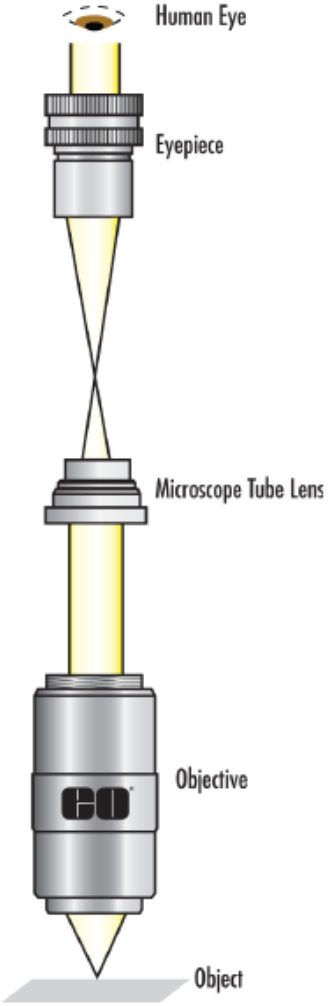


Magnification	1X	2X	3X	4X	10X	20X	40X	60X	100X
Color Code	Black	Gray	Red	Yellow	Green	Light Blue	Light Blue	Dark Blue	White

www.edmundoptics.de/technical-resources-center/microscopy/understanding-microscopes-and-objectives/

Infinity-corrected Objectives

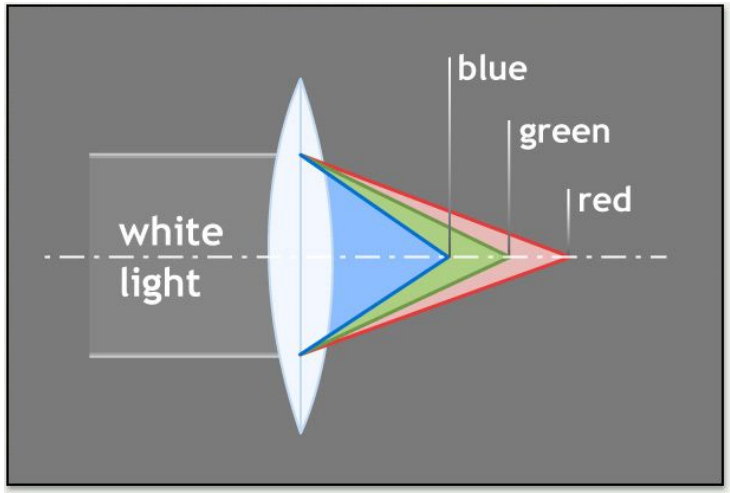




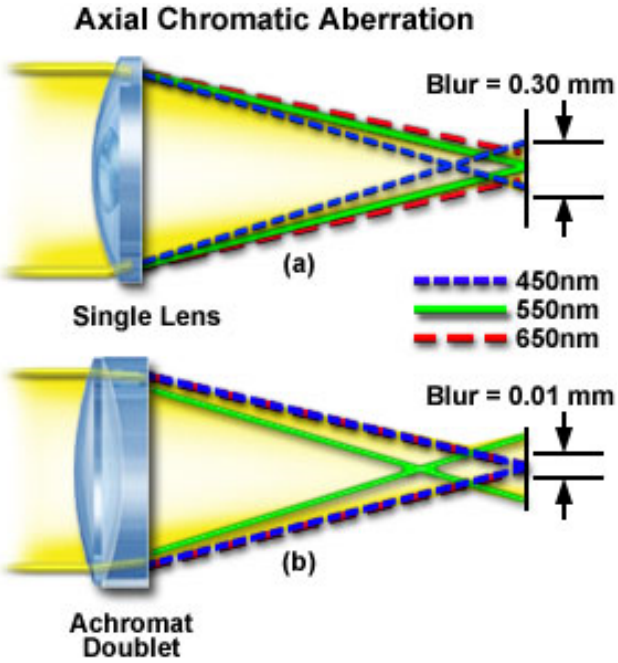
www.edmundoptics.de/technical-resources-center/microscopy/understanding-microscopes-and-objectives/

Chromatic aberration

- Failure of the lens to focus all colors on the same point, due to a different refractive index for different light wavelengths
- Achromat is corrected for blue and red light
- Apochromat is additionally corrected for green light



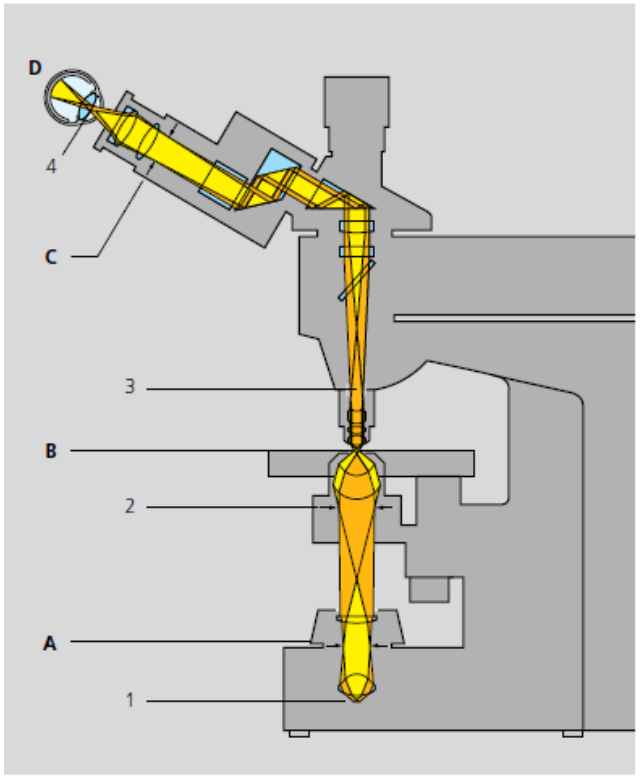
http://laserpointerforums.com/f54/what-difference-beam-diameter-red-green-blue-lasers-52298.html



http://www.microscopyu.com/tutorials/java/aberrations/chromatic/

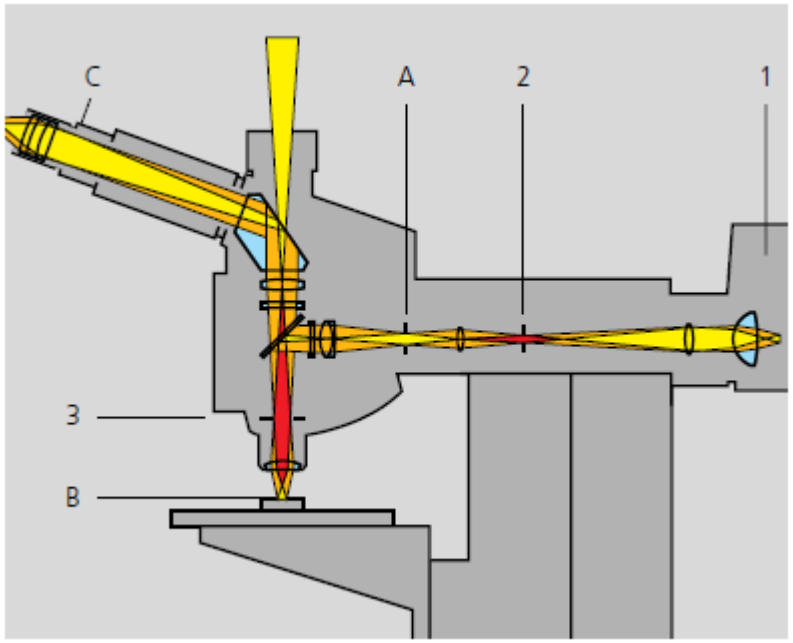
Light pathways

Transmission light microscopy



Zeiss: Microscopy from the very beginning

Reflected light microscopy



Zeiss: Microscopy from the very beginning

Two groups of optical planes:

1-4: = beam path of the pupil, determines the resolution
A-D: = image forming beam path

Image Contrast

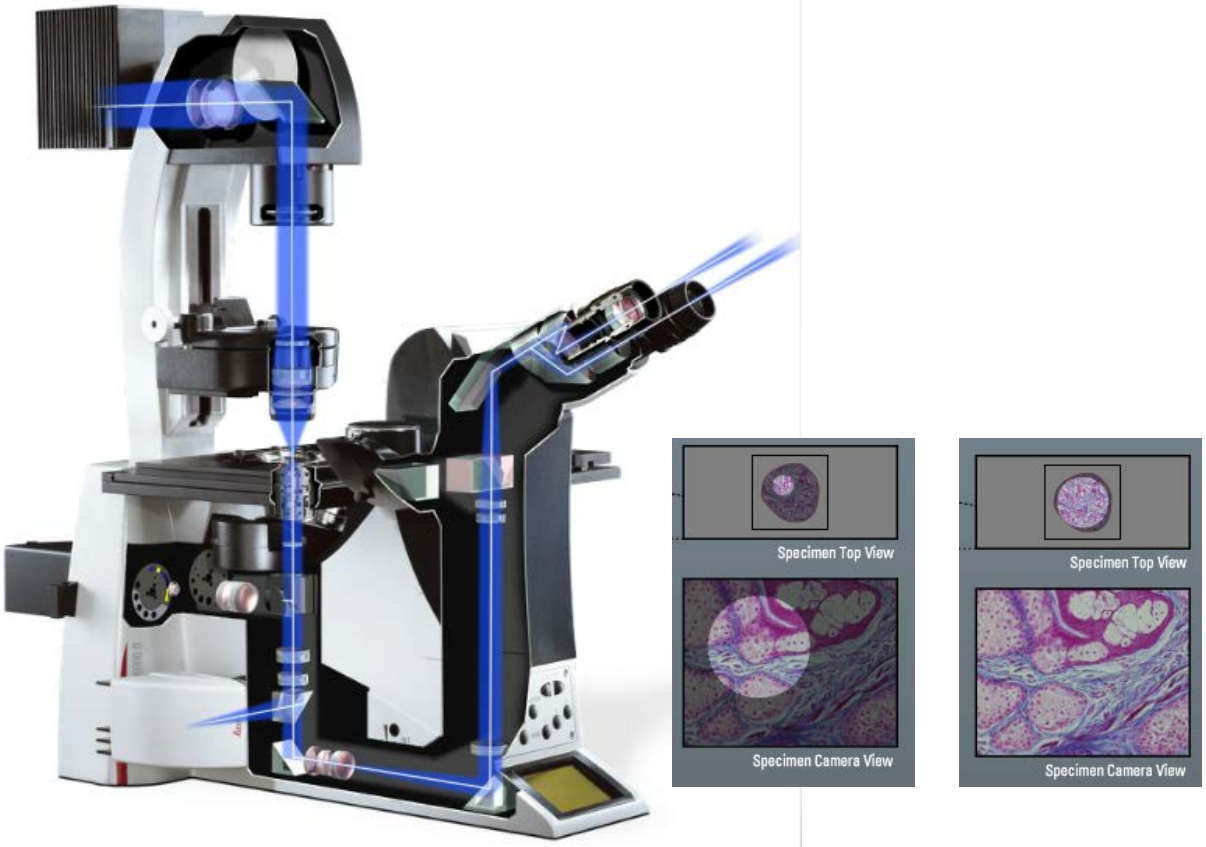
- Contrast is the difference between the signal in one pixel and that in another that conveys information about the shape of the specimen or the difference in signal intensity between various parts of an image or between details of interest and “background”

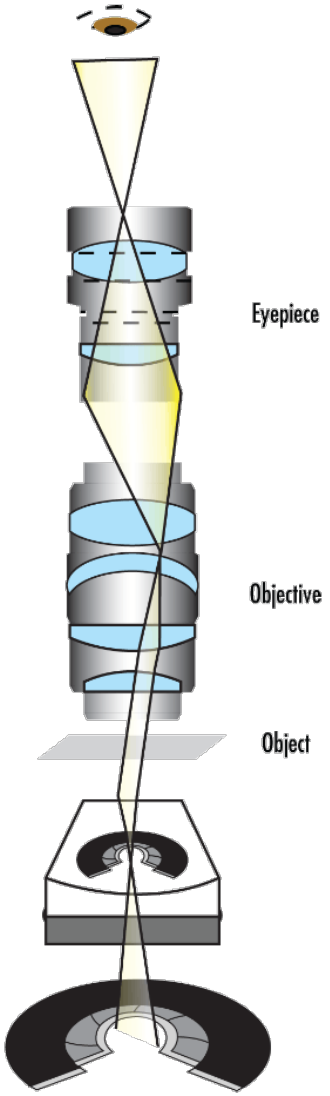
- Various physical and digital “filters” can be used to select specific signals

- Contrast enhancing techniques in optical light microscopy
  - Köhler illumination
  - Phase contrast
  - Differential interference contrast (DIC)
  - ….

Köhler illumination

The illumination of the object is limited to the observed area





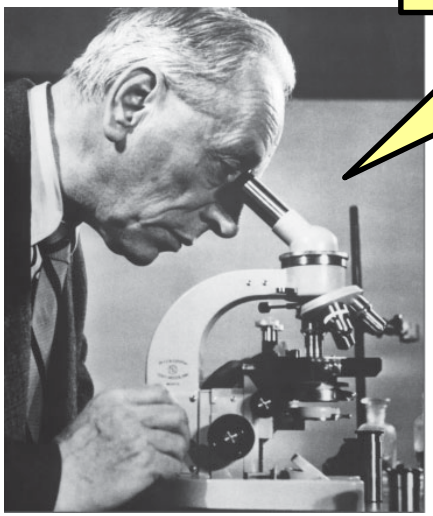
http://www.leica-microsystems.com/science-lab/koehler-illumination-step-by-step-guide-to-optimal-illumination-of-specimen/

www.edmundoptics.de/technical-resources-center/microscopy/understanding-microscopes-and-objectives/

Phase contrast

"Phase contrast was not discovered while I was working with a microscope, but originated in my interest in diffraction gratings."

Dr. Zernike



Murphy: Fundamentals of light microscopy and electronic imaging

bright field

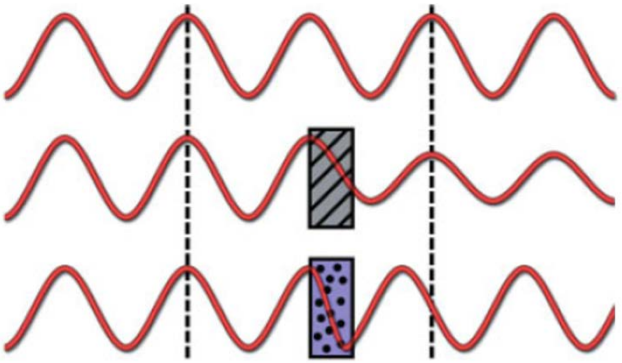


phase contrast



Phase contrast

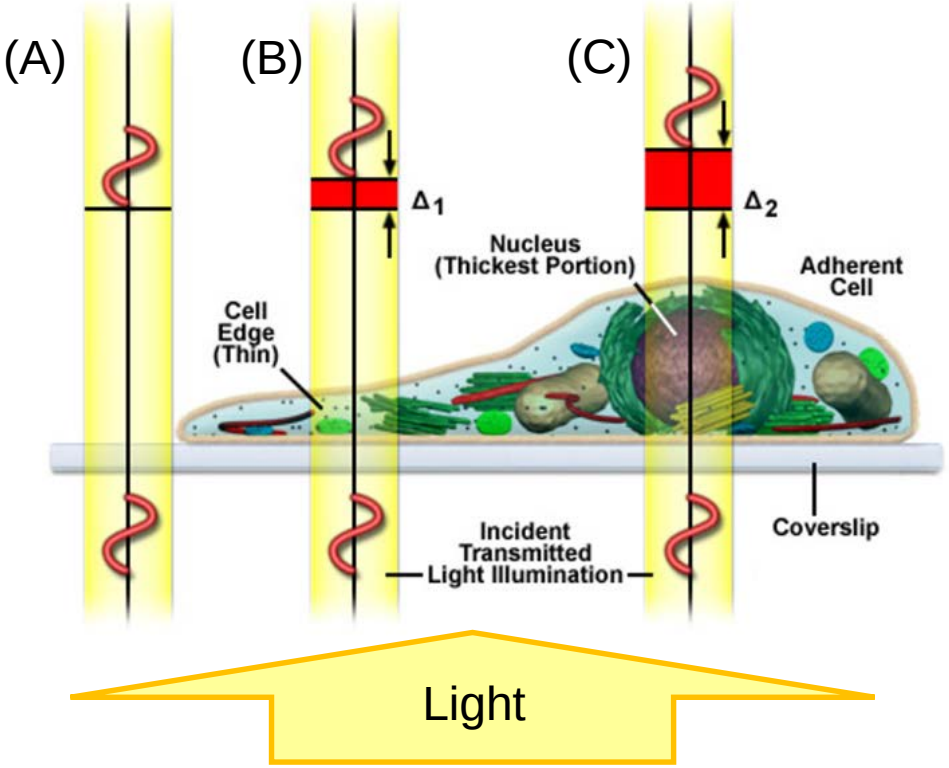
- Small differences between the refractive indices of the aqueous solution (A) the cell periphery (B) and the nucleus (C)
- Shift of light rays are translated into intensity shifts



Amplitude shift

Phase shift

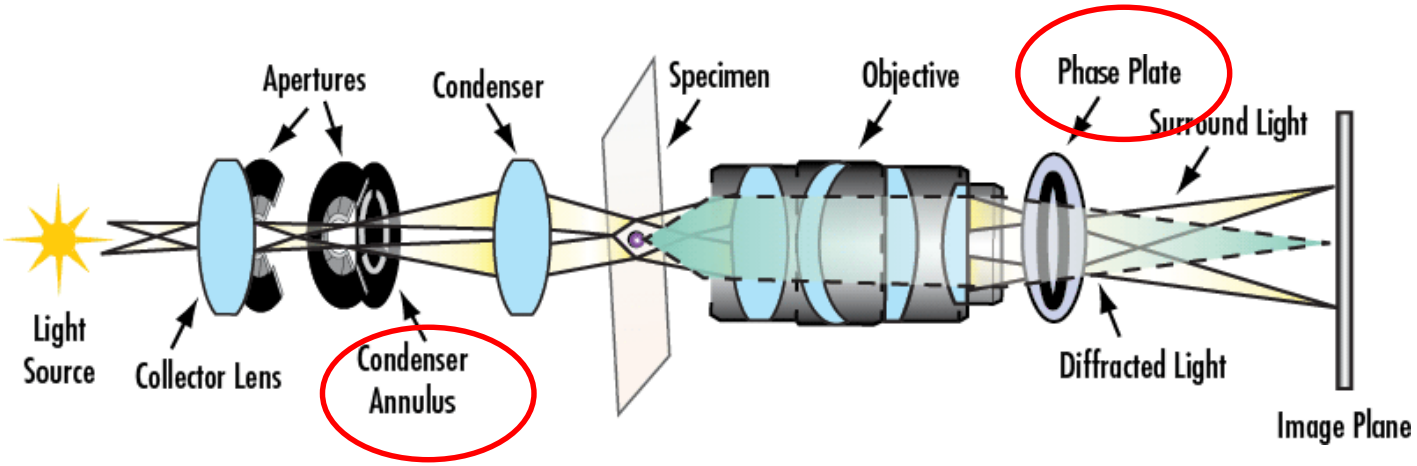
Murphy: Fundamentals of light microscopy and electronic imaging



http://zeiss-campus.magnet.fsu.edu/articles/basics/contrast.html

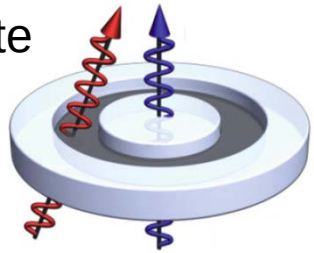
Phase contrast

- Condenser annulus: Opaque plate that illuminates the specimens in defocused parallel light ray
- Phase plate: Alters the phase and amplitude of the light



http://www.edmundoptics.de/technical-resources-center/microscopy/optical-microscopy-application-phase-contrast/

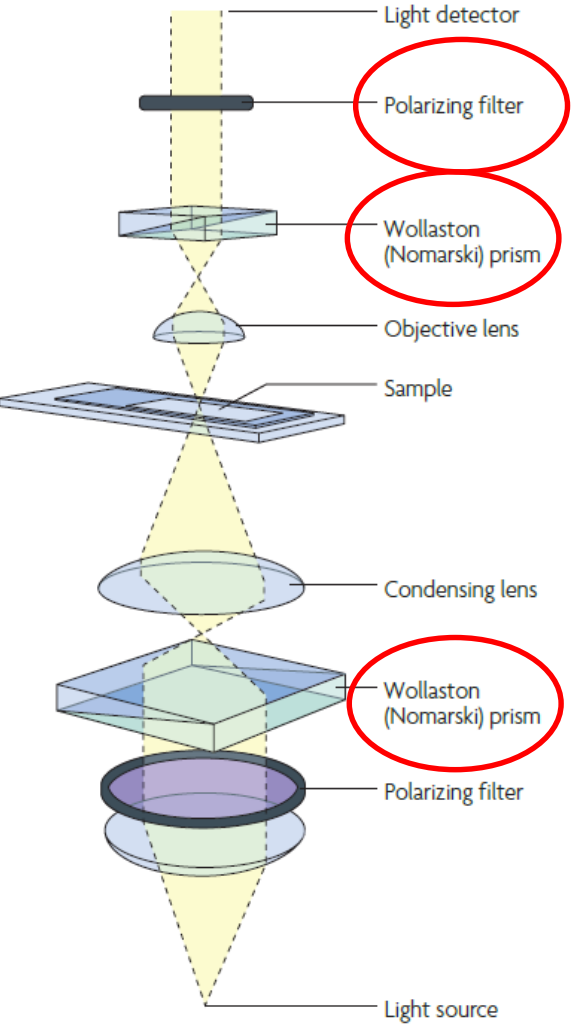
Phase plate



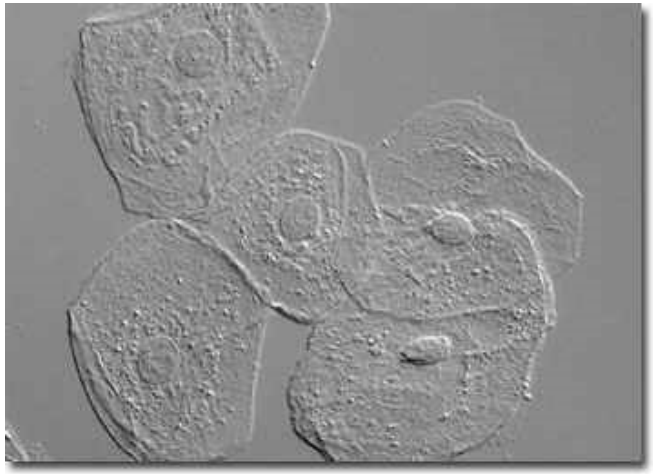
Amplitude of cell-surrounding light is reduced

Murphy: Fundamentals of light microscopy and electronic imaging

Differential interference contrast (DIC)



http://www.nature.com/milestones/light -microscopy

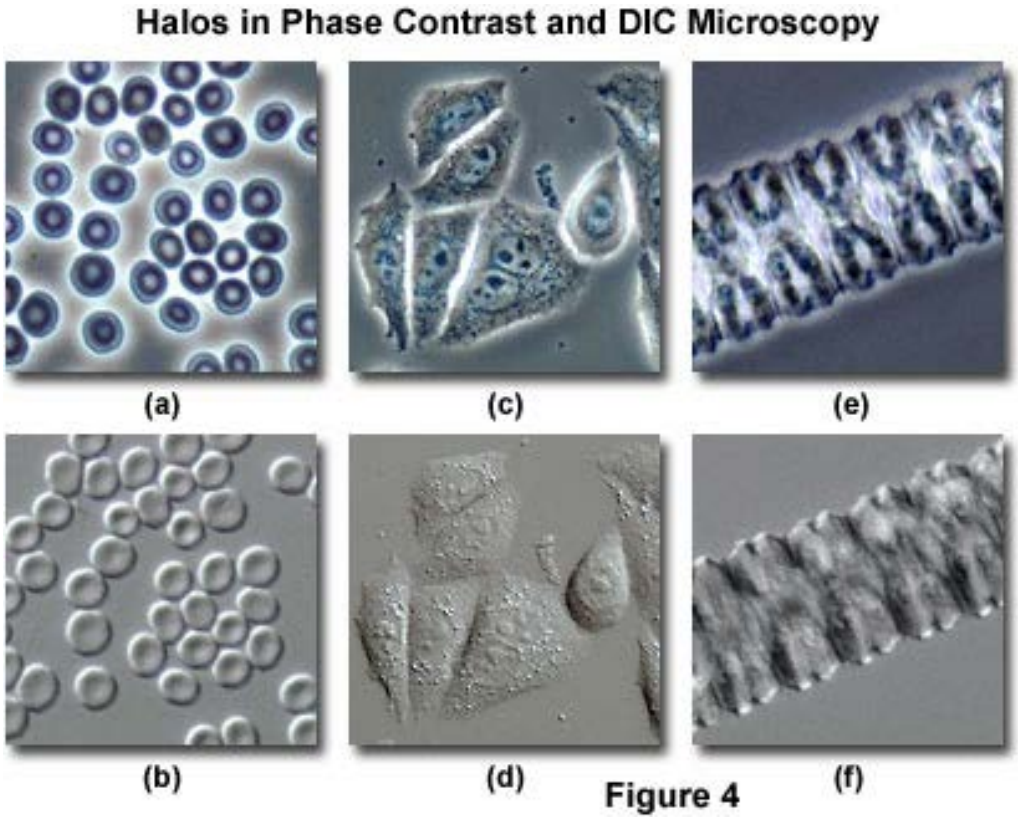


http://micro.magnet.fsu.edu/primer/techniques/dic/dicgallery/cheekcellssmall.html

- Use of polarized light
- Wollaston prism separates the light into two sister beams
- Sister beams pass the specimens (phase shifts occurs)
- Second prim fuses the beams and phase shifts were translated into amplitude shift
- Pseudo three-dimensional relief

DIC vs. Phase contrast

- Complementary techniques capable of producing high contrast images of transparent biological phases



https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/dic/dicphasecomparison/

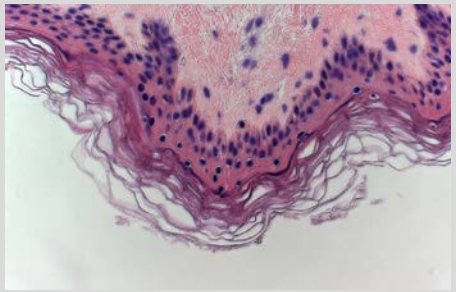
Limitations of light microscopy

- Resolution of light microscopy is limited due to the wavelength of the light source and N.A. of objective and condenser
- Optical contrasting methods resolve limited biological information

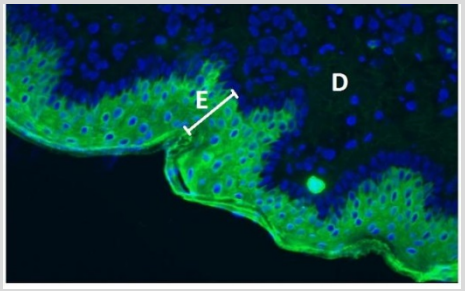
Dyes are needed to visualize specific molecules and structures in biological specimens

Histological dyes

Eosin
Hematoxylin



Fluorescent dyes



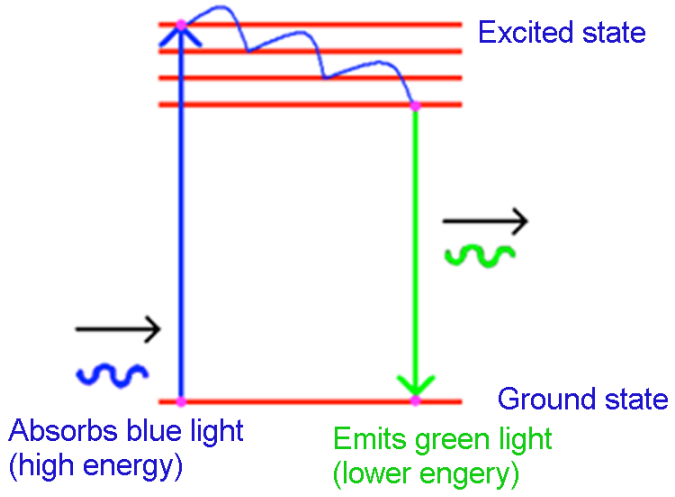
Keratin K10
DAPI

http://wahoo.nsm.umass.edu/histology/content/fluorescent-image-skin

Fluorescence Microscopy

Principle of fluorescence

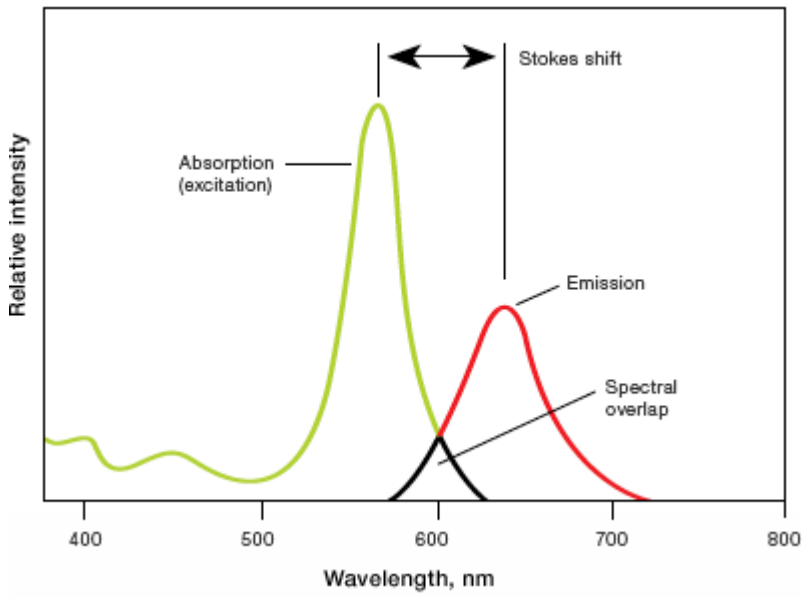
- Luminescent process in which molecules emit light
- Fluorophores absorb light of one wavelength and emit (fluoresce) light of another wavelength
- Fluorescence light is emitted rapidly during relaxation of excited molecules
- Random process



http://www.st-andrews.ac.uk/seeinglife/science/imaging/fluorescence/fluorescence.html

Principle of fluorescence

- “Stokes shift” describes the wavelength shift from excitation maximum to emission maximum
- “More or less all living samples contain material that is fluorescent by nature, a phenomenon called autofluorescence” (R. Borlinghaus)



Absorption and emission spectrum are specific for a fluorophore

http://www.bio-rad.com/evportal/de/DE/evolutionPortal.portal?_nfpb=true&_pageLabel=SolutionsLandingPage&catID=LUSQ6KKG4

Fluorophores



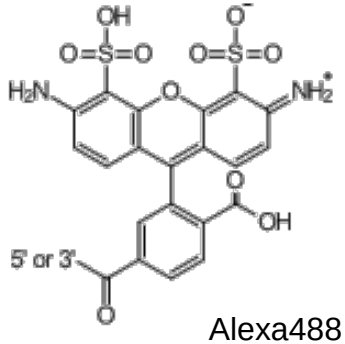
Green fluorescent protein (GFP)

Fluorescence Intensity F:

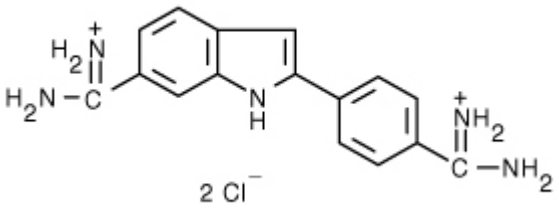
$$F = \sigma * Q * I$$

Q = Quantum yield
I = Excitation light intensity
σ = Molecular absorption cross section

Fluorophore	Quantum yield	λ_{Ex}	λ_{Em}
GFP	0.6	488	535
YFP	0.61	520	542
DsRed	0.79	558	583
Alexa488	0.81	488	535
DAPI	0.58	358	421



Alexa488



4',6-diamidino-2-phenylindole (DAPI)

Fluorophores

Fluorescence life-time

- Every fluorescent molecule has an average time that it spends in the excited state followed after excitation
- Decay of fluorescence intensity after excitation can be measured
- The average time is referred as the fluorescent life time τ

Quenching

- Reversible loss of fluorescence e.g. due to non-radiative energy transfer

Photobleaching

- Irreversible reactions that lead to loss of fluorescence
- Oxygen contributes to photobleaching
- Photobleaching is dependent on molecular environment

Key characteristics of fluorescent probes and specimens

Property	Definition/impact
Optical properties	
Excitation spectrum	Wavelength dependence of the ability of light to induce fluorescence emission of a chromophore
Extinction coefficient ε_λ ($M^{-1}\,cm^{-1}$)	Quantity characterizing a fluorophore's ability to absorb photons at a particular wavelength λ
Emission spectrum	Wavelength dependence of the emitted fluorescence
Fluorescence quantum yield (QY)	Ratio between the number of photons emitted and the number of photons absorbed
Molecular brightness	Product QY $\times$ ε quantifies the relative rate of photon emission from different fluorophores under identical excitation conditions
Photostability	Resistance to photobleaching, quantified by the quantum yield of photobleaching, that is, ratio of the number of photobleached molecules to the total number of photons absorbed in a sample during the same time interval
Physicochemical properties	
Size	Most often, the smaller the better
Material	May affect chemical stability, toxicity, options to functionalize the probe
Solubility	Most biological applications require aqueous solvents
Cytotoxicity	Effect on sample viability
Phototoxicity	Effect on sample viability
Conjugation chemistry	Determines labeling target
Localization of target	Affects the choice of conjugation
Metabolism of target	Determines temporal stability of labeled construct in live cells

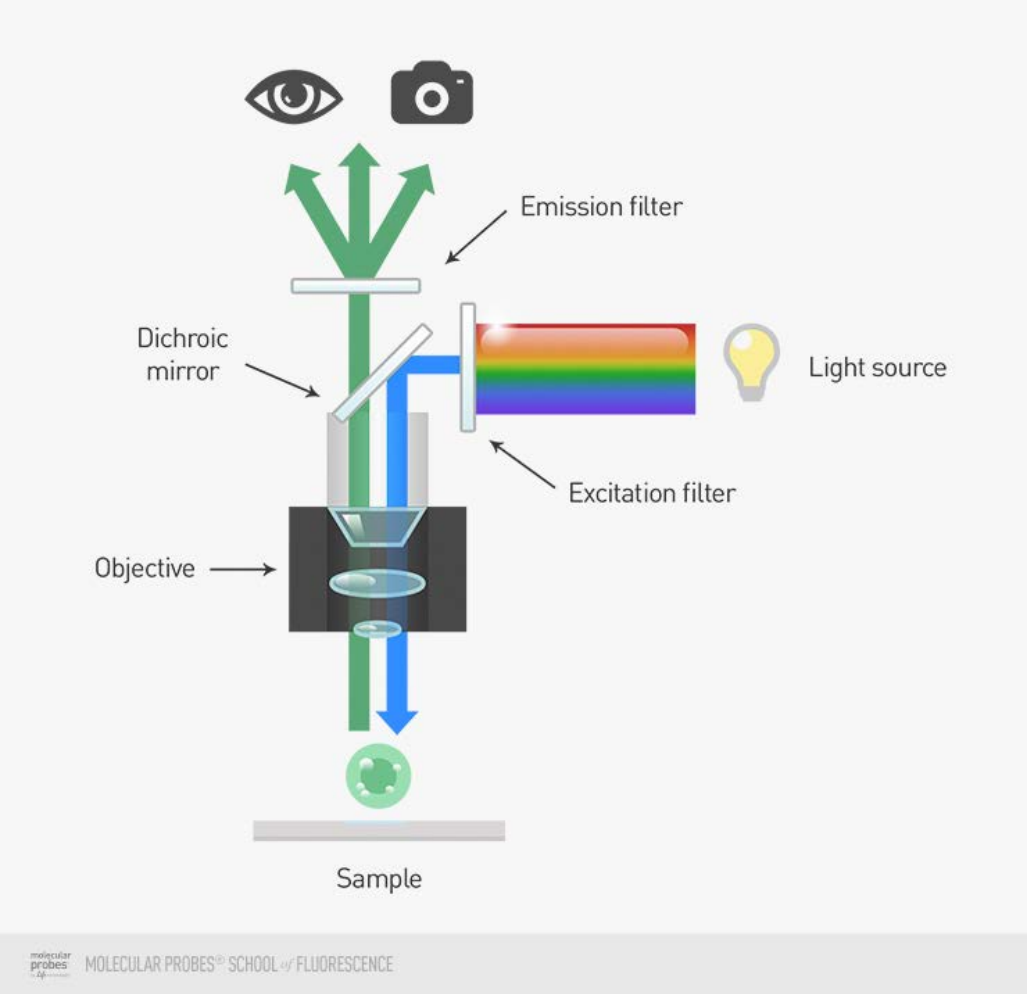
Kubitscheck: Fluorescence Microscopy: From Principles to Biological Applications

Fluorescent dyes: Examples

Dye	Absorption max [nm]	Emission max [nm]
DAPI	358	461
FITC	495	519
GFP (wildtyp)	395, 475	510
Cy3	547	561
Cy5	647	665
Alexa Fluor 488	495	519
Alexa Fluor 568	578	603
Texas Red	595	615
Rhodamin Red	570	590

Basic light path: Wide-field fluorescence microscopy

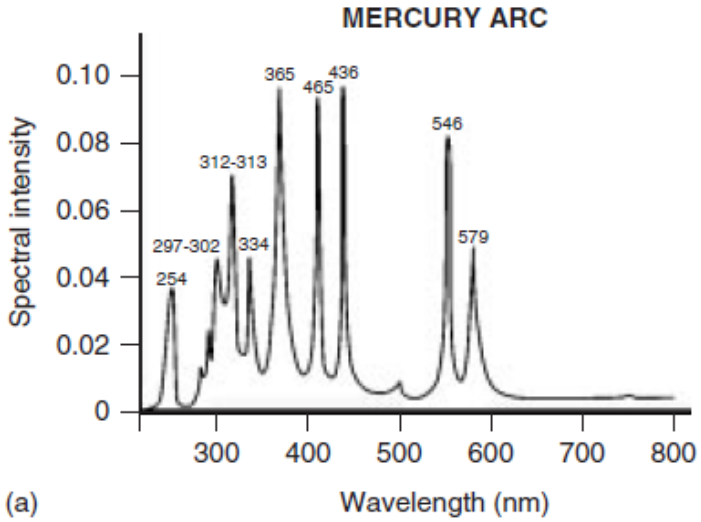
- Dichroic mirror reflects blue (UV) light
- Longer wavelength can pass the mirror

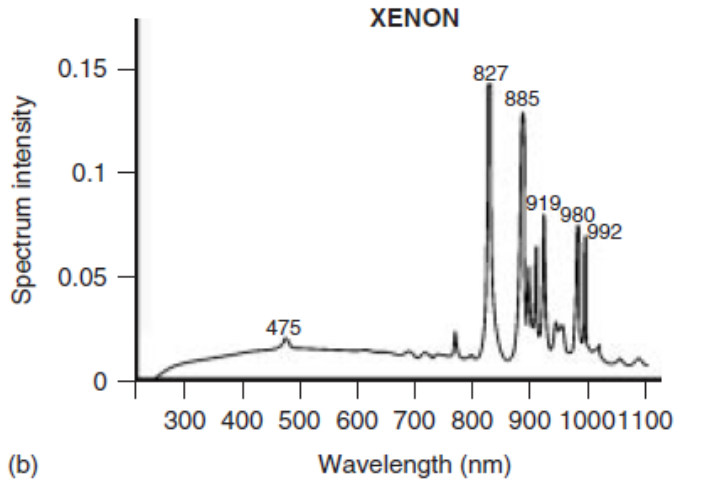


https://www.thermofisher.com/ca/en/home/life-science/cell-analysis/cell-analysis-learning-center/molecular-probes-school-of-fluorescence/fundamentals-of-fluorescence-microscopy/epifluorescence-microscope-basics.html

Light sources

- **Broad UV/visible light spectrum**
  - Mercury arc lamp
  - Xenon burner
- **Monochromatic Laser light**
  - Gas lasers
  - Solid state laser
  - Diode lasers





Kubitscheck, Fluorescence Microscopy: From Principles to Biological Applications

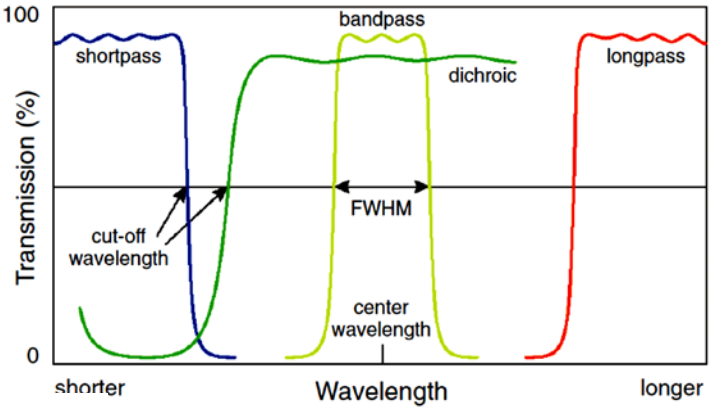
Fluorescence filter units

- **Shortpass filter**

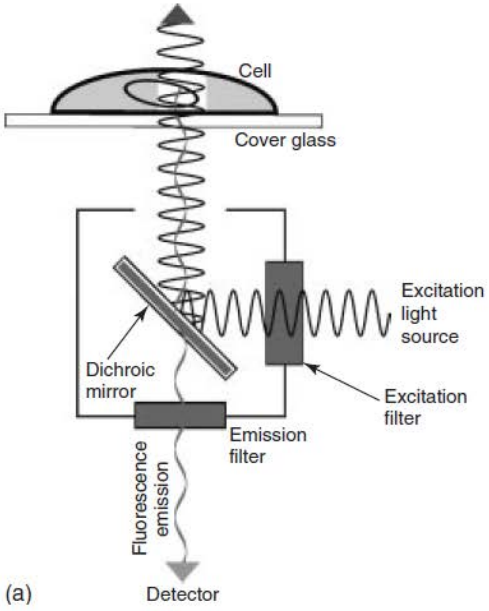
  Transmits light only below a certain wavelength
- **Bandpass filter**

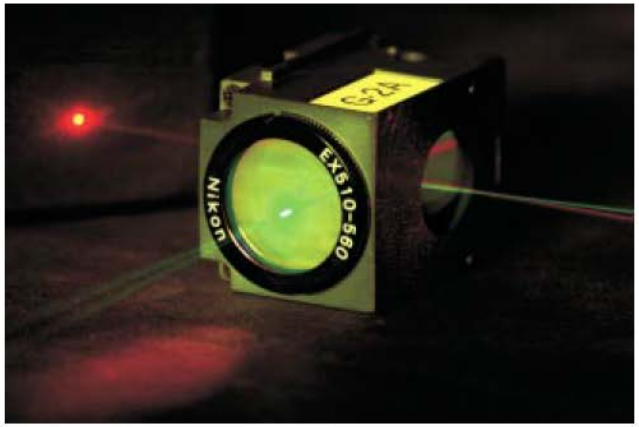
  Transmits light only in a specific segment
- **Longpass filter**

  Cuts at defined wavelength



Pawley: Biological confocal microscopy

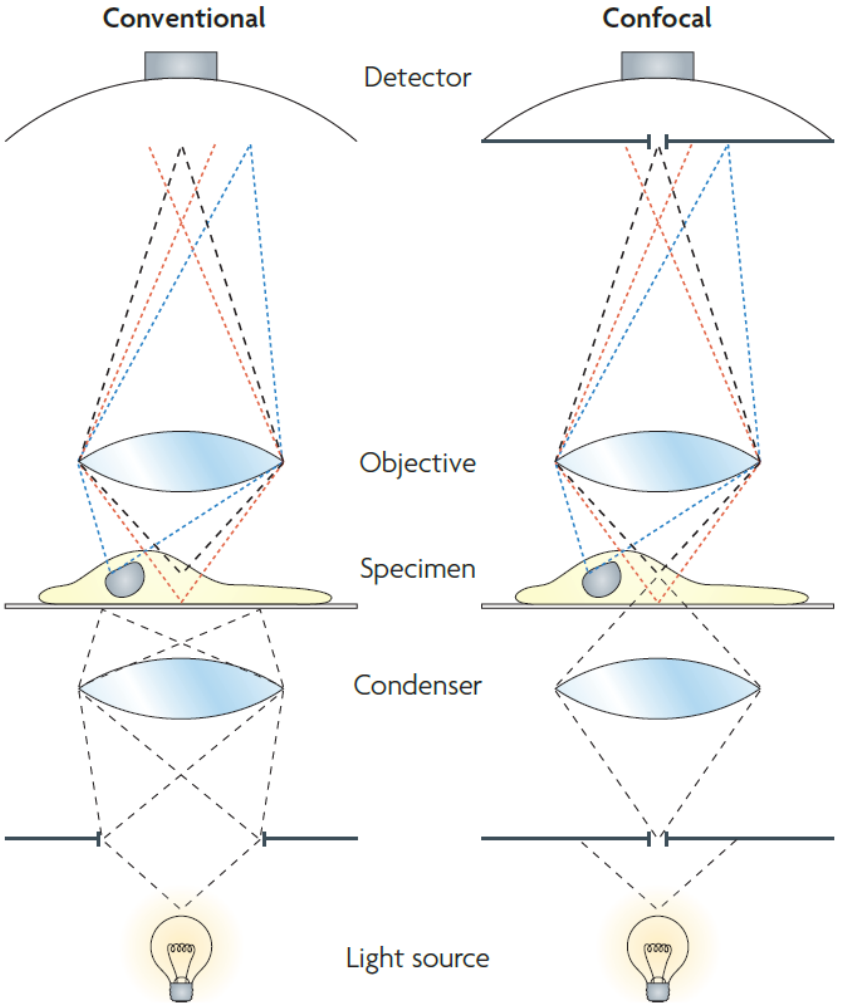




Kubitscheck, Fluorescence Microscopy: From Principles to Biological Applications

Set-up of fluorescence microscopes

Fluorescent light is detected from all focal planes



Pinhole

Fluorescent light emitted from one focal spot is detected

Pinhole

http://www.nature.com/milestones/light -microscopy

Confocale laser scanning micoscopy

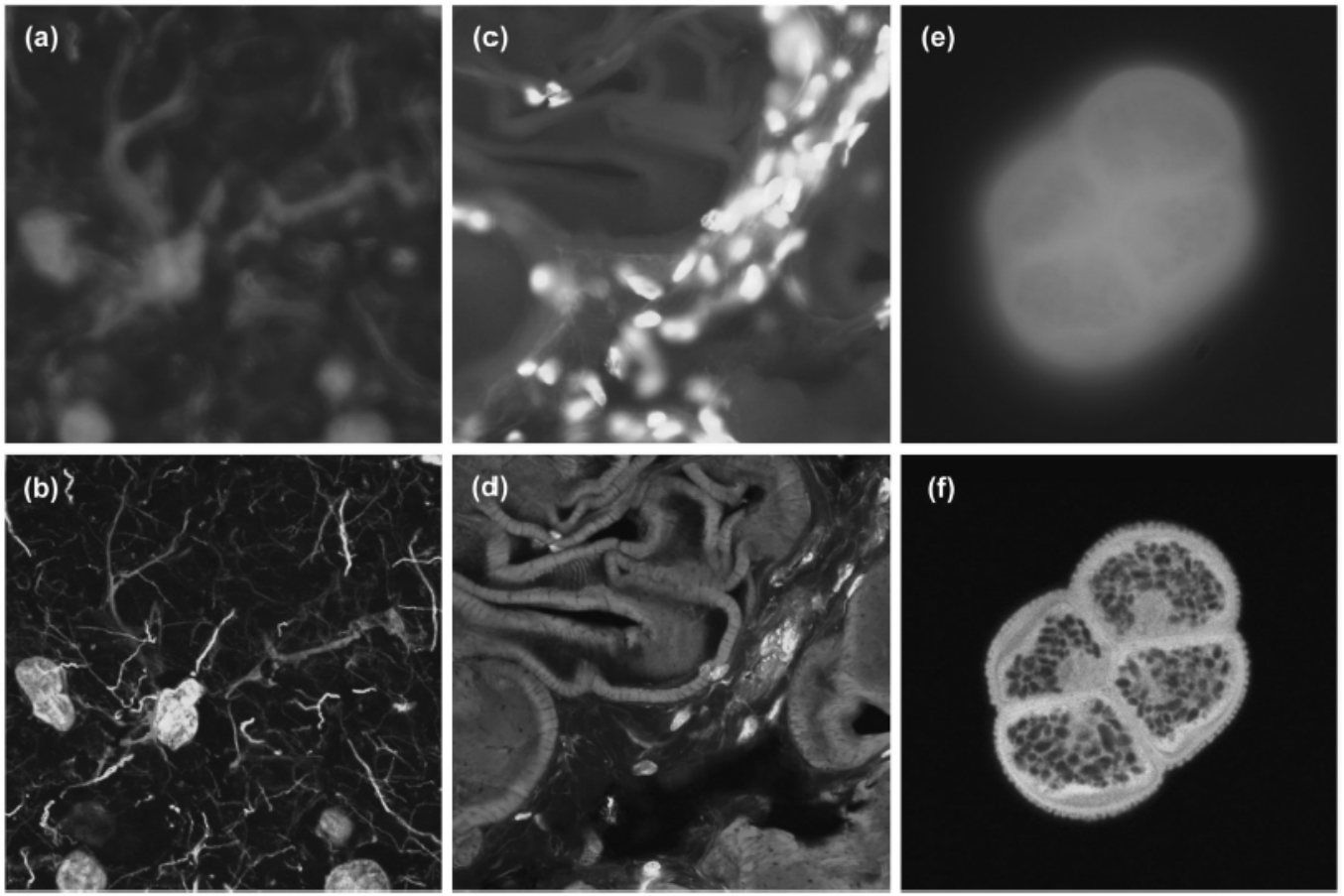
Thick section of mouse hippocampus

Thick section of rat smooth muscle

Autofluorescence of pollen grain

Widefield microscopy

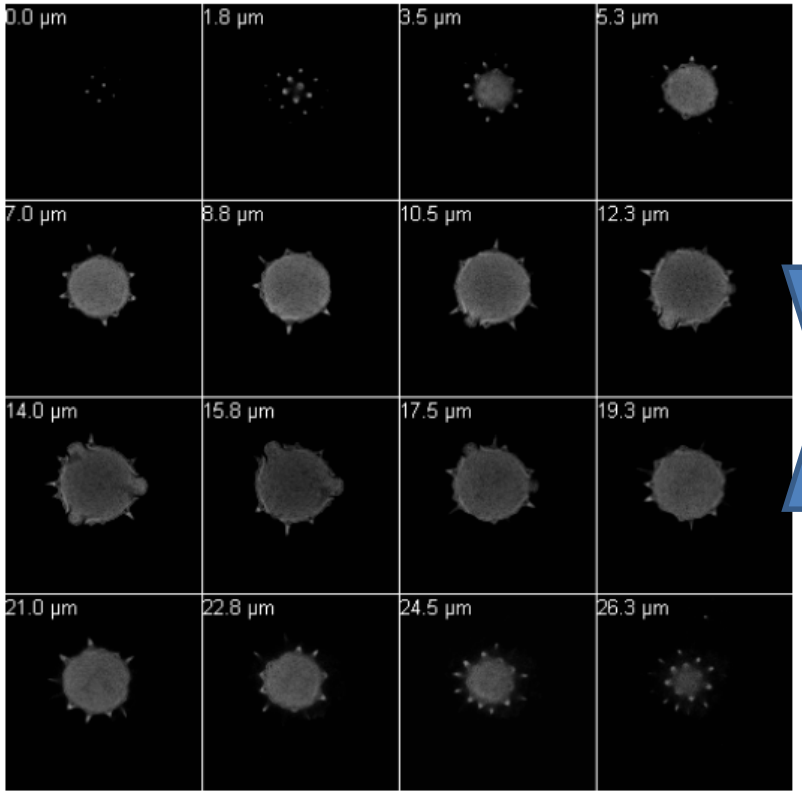
Confocal laser scanning



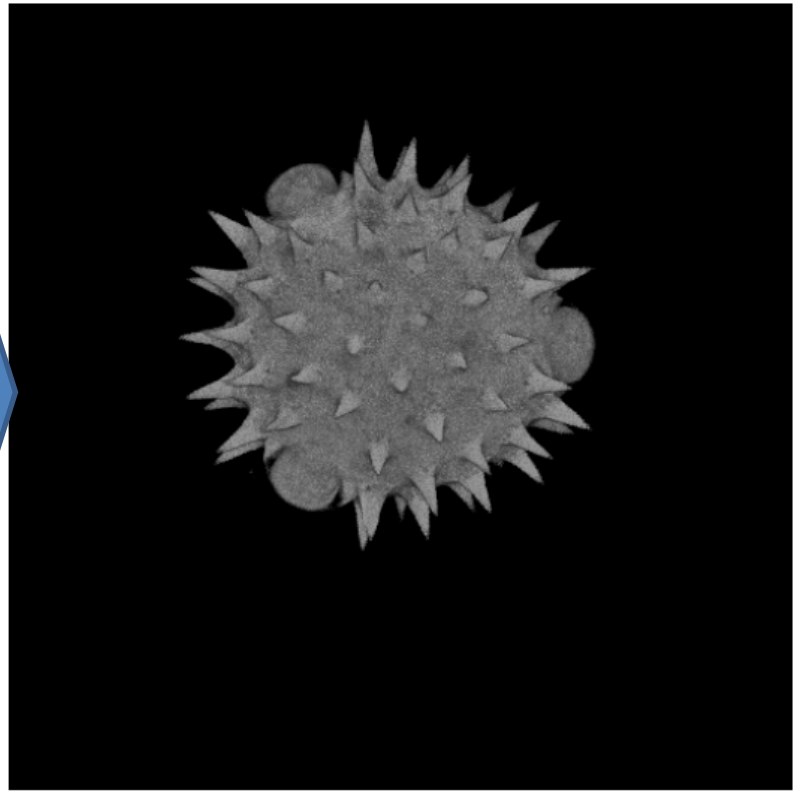
Claxton, Confocal microscopy

Confocal laser scanning micoscopy

Single stacks with variations in z-direction



3D Projection

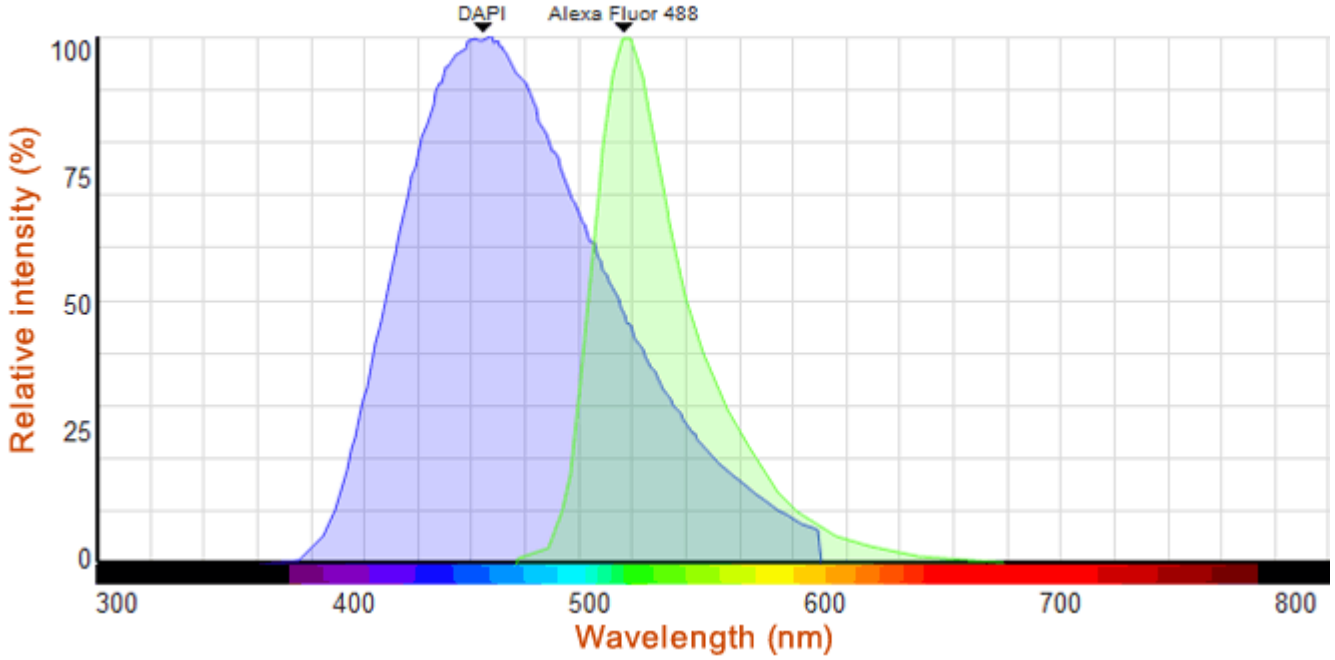


Zeiss brochure: Confocal laser scanning microscopy

Simultaneous Fluorescence Imaging

- When two (or more) fluorescent probes are used within the same sample, it is possible that the emission spectra of the two probes overlap

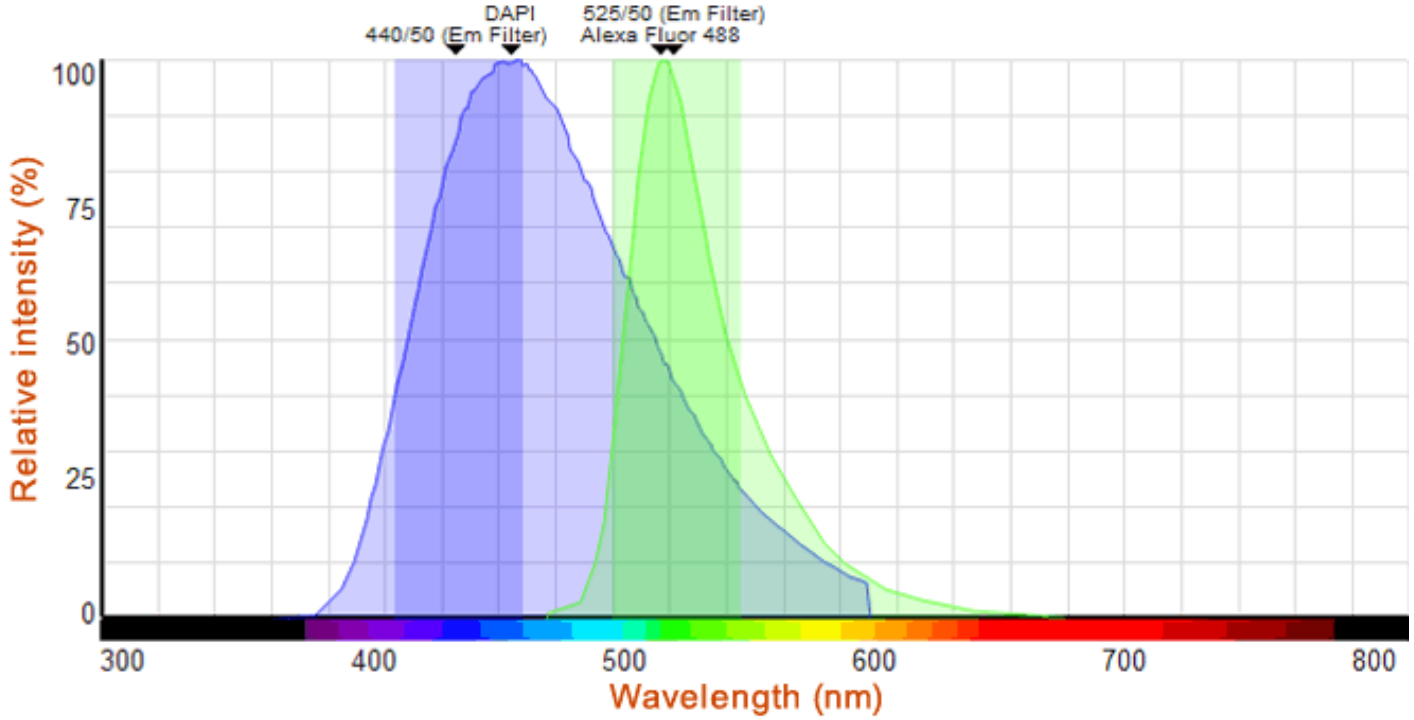
Emission spectra for DAPI (blue) and AF488 (green)



http://www.ammrf.org.au/myscope/confocal/confocal/image/seqsim/

Simultaneous Fluorescence Imaging

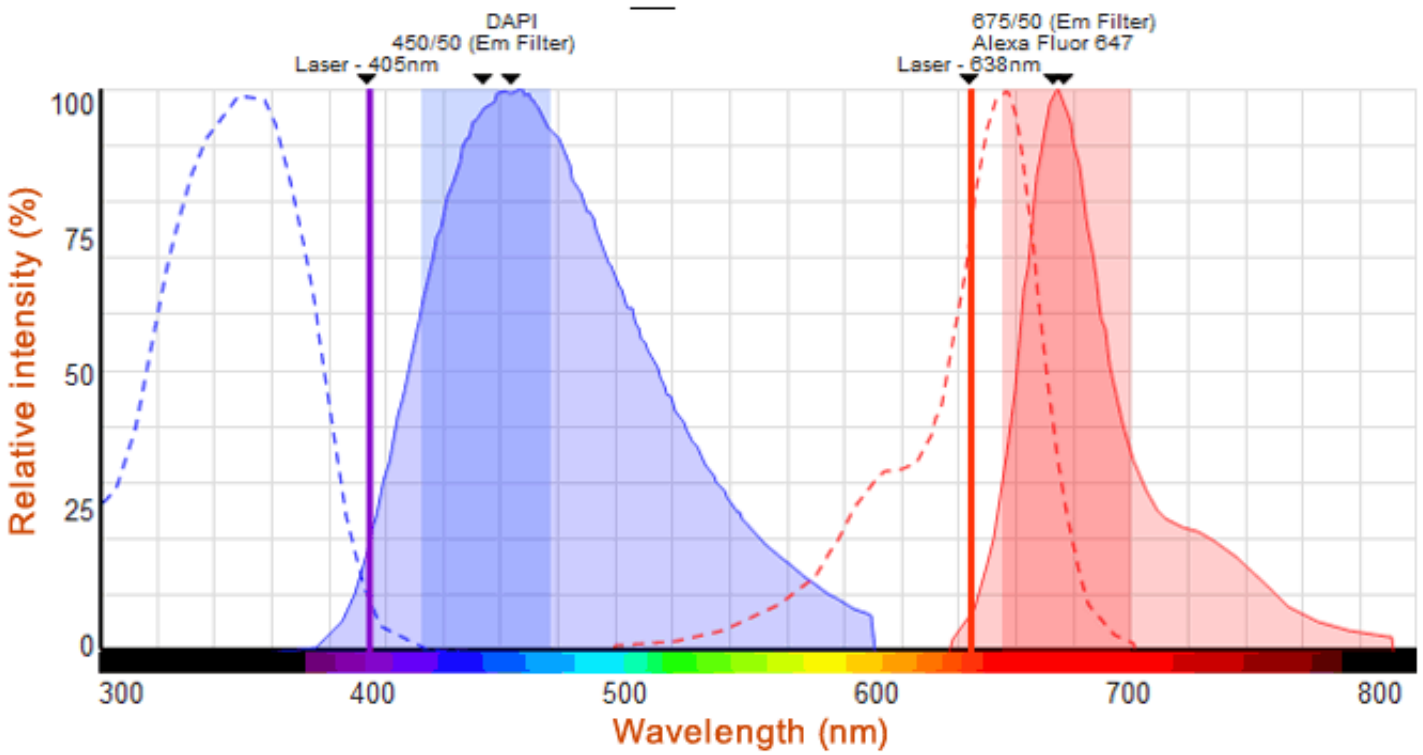
- Some of the fluorescence emission from both DAPI and AF488 will pass through the 525/50nm filter
- About 30% of the total signal detected in the second detector will originate from DAPI



http://www.ammrf.org.au/myscope/confocal/confocal/image/seqsim/

Simultaneous Fluorescence Imaging

- Simultaneous imaging of DAPI and AF647
- Minimal cross-excitation or emission of the two fluorophores
- No spectral bleedthrough



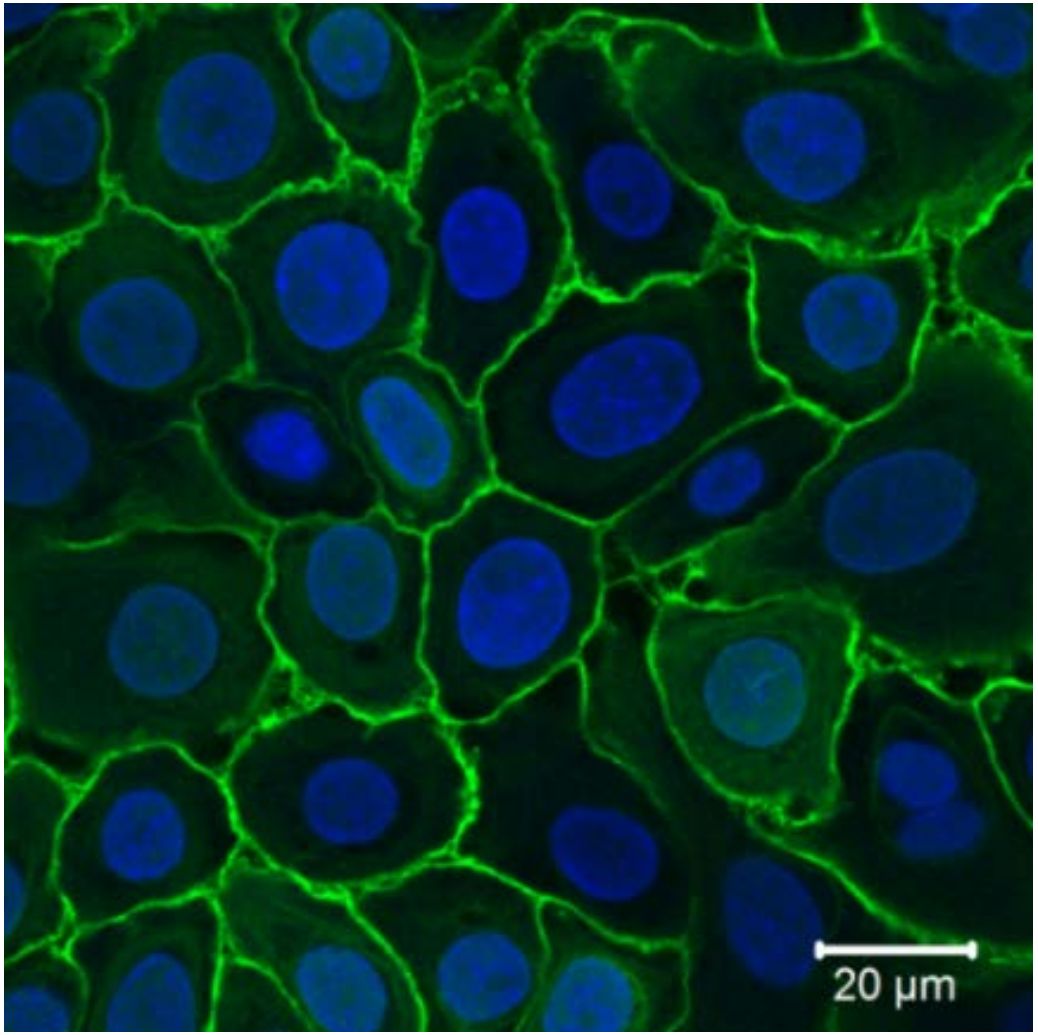
http://www.ammrf.org.au/myscope/confocal/confocal/image/seqsim/

How to get fluorescence into the specimens?

- Fluorescent dyes bind to specific cellular structures (nuclei, mitochondria)
- Antibodies conjugated to fluorophores
- Cells / animals are genetically modified to express fluorescent proteins e.g. green fluorescent protein (GFP)
- Use of intrinsic fluorescence (autofluoresecence and multiphoton imaging)

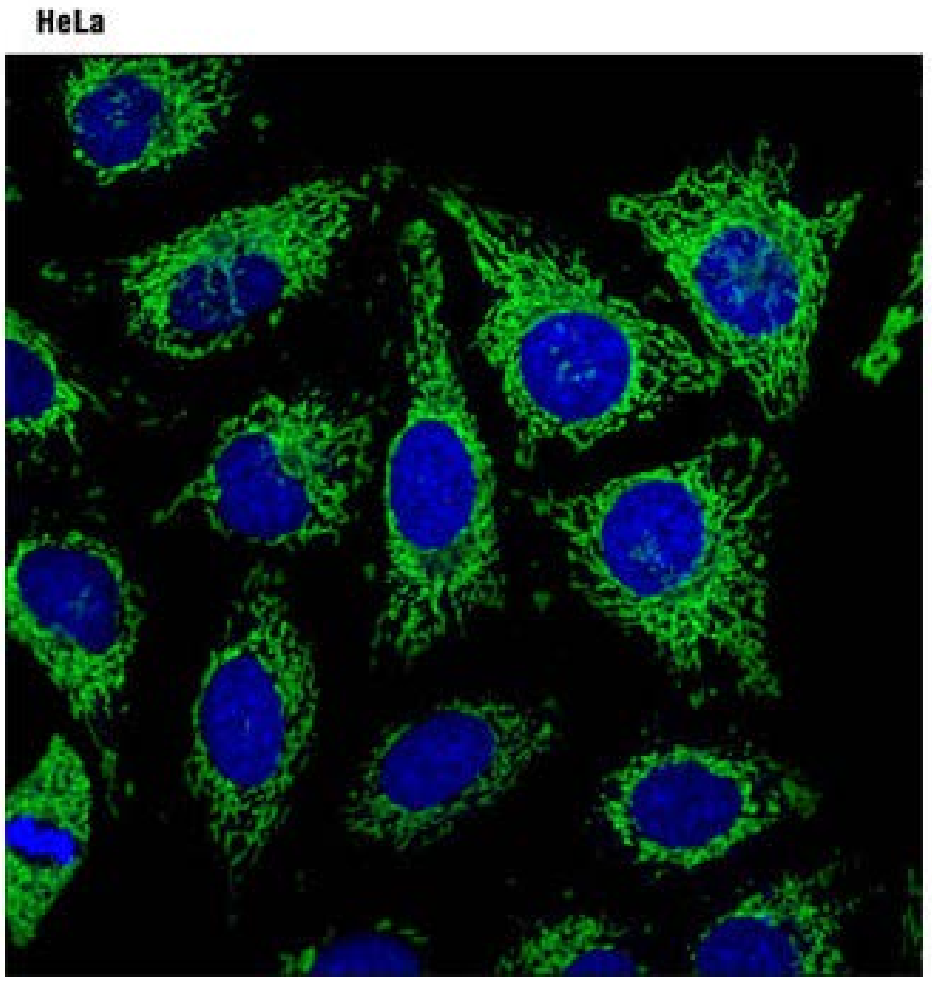
Fluorescent dyes bind to specific cellular structures

- DAPI
- Cell membrane



Fluorescent dyes bind to specific cellular structures

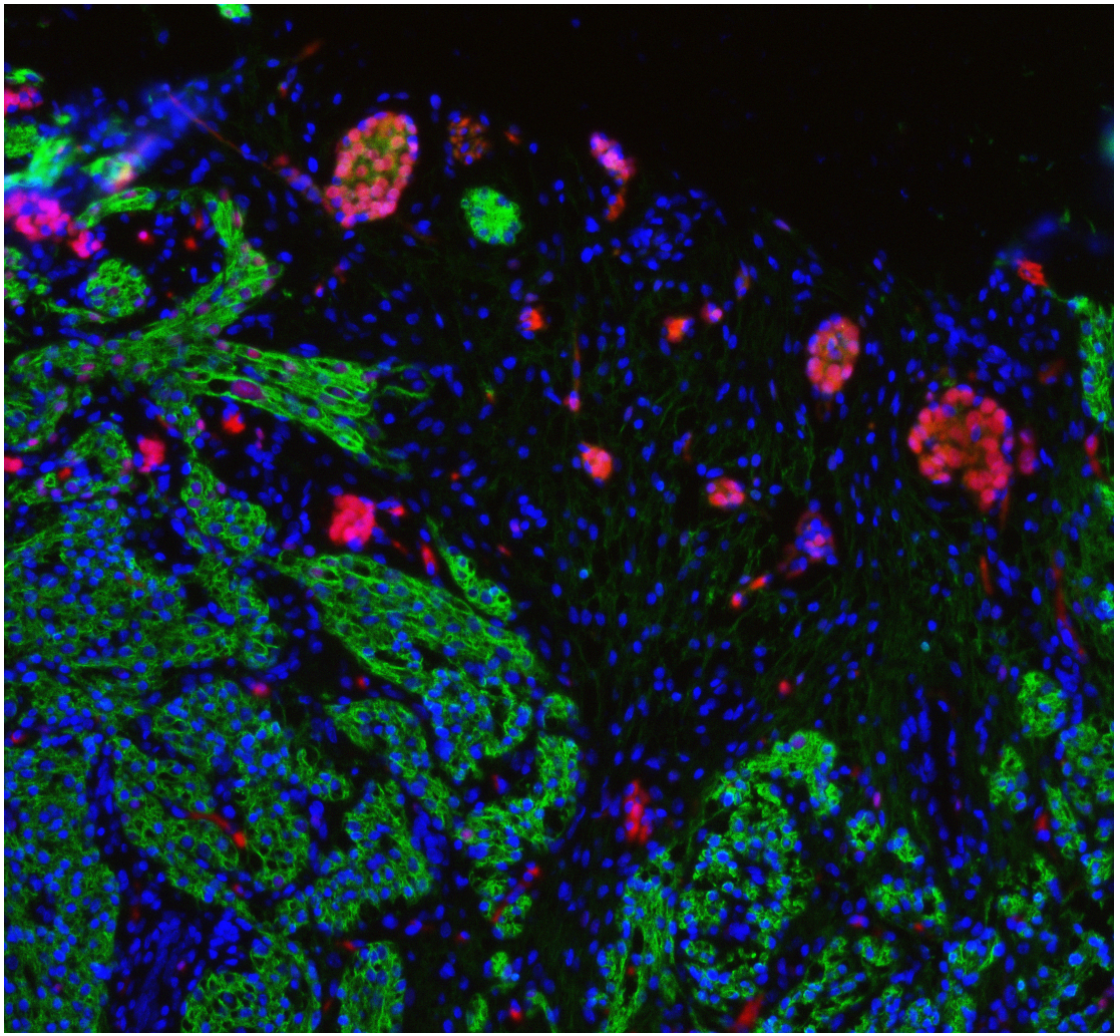
- DAPI
- MitoTracker



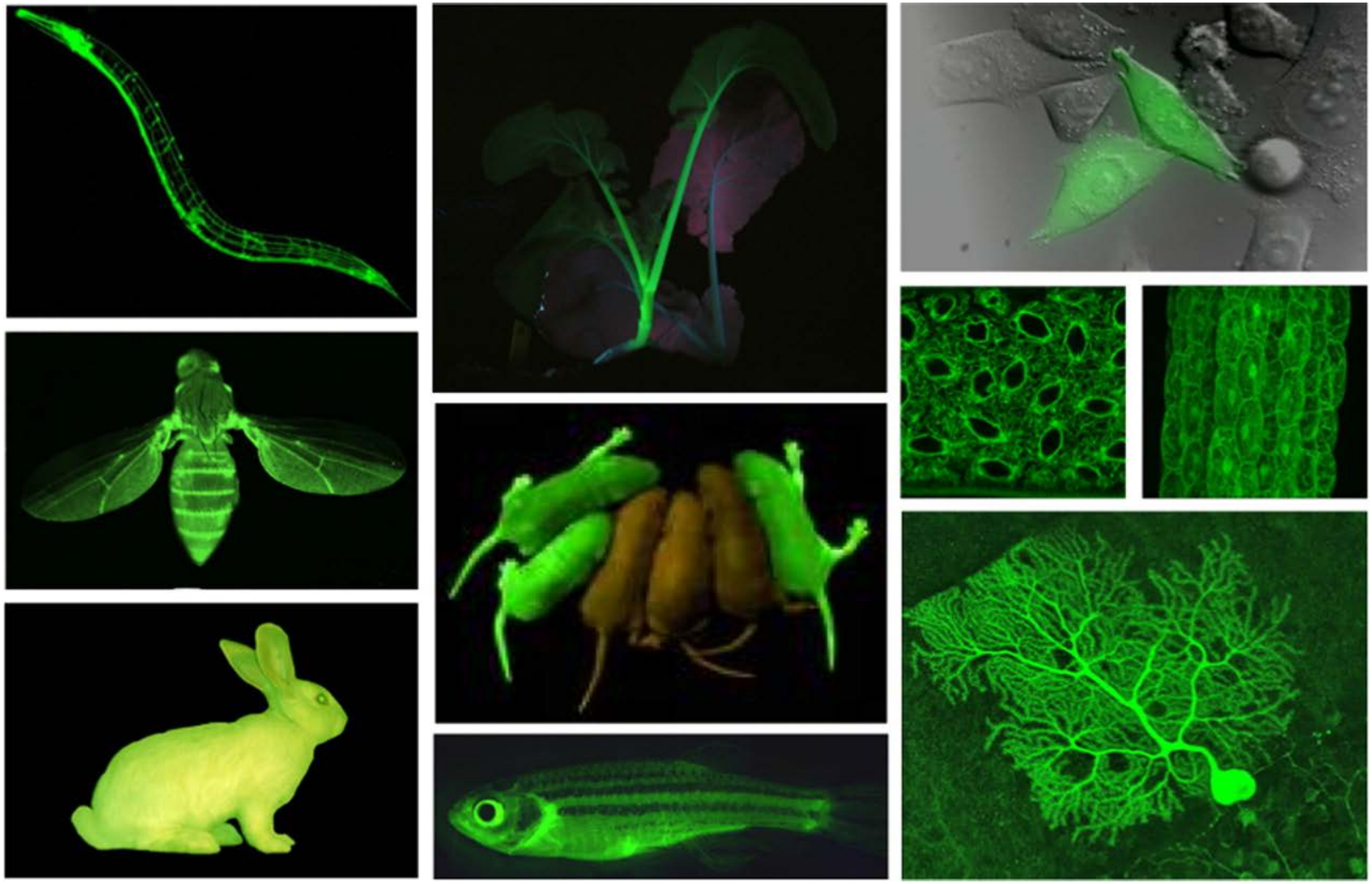
https://www.cellsignal.com/products/buffers-dyes/mitotracker-green-fm/9074

Antibodies conjugated to fluorophores

Stem cell niches



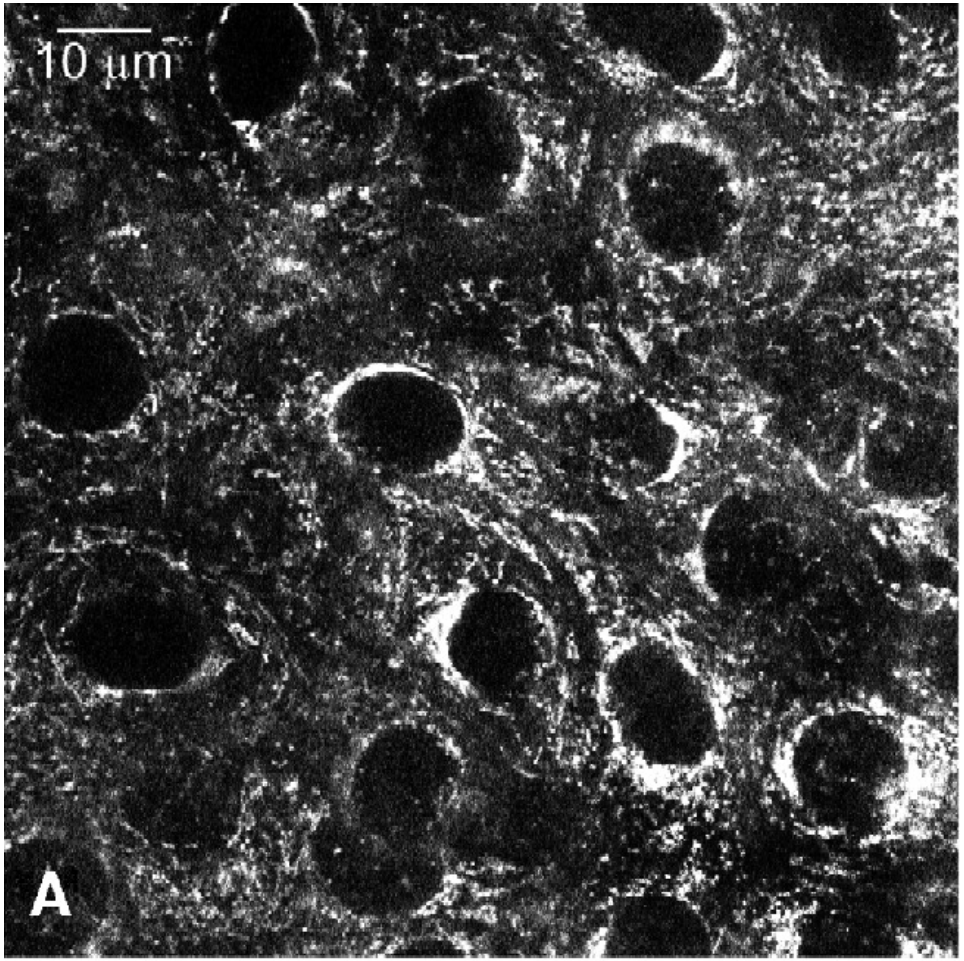
Cells / animals are genetically modified to express GFP



http://www.pnas.org/content/106/25/10073/F10.expansion.html

Intrinsic fluorescence

Multiphoton Imaging
Excitation of NADP(H)
λ_{ex}=720 in dermal cells



Schenke-Layland, Adv. Drug Del Reviews, 2008

Limitations of fluorescence microscope

Wide-field

- Thickness of specimens
- No z resolution
- Information about colocalization is insufficient
- Use of multiple dyes is limited
- Dyes with similar excitation and emission ranges cannot be discriminated
- Bleaching of fluorophores

Confocal

- Dyes with similar excitation and emission ranges cannot be discriminated with standard detectors
- Simultaneous imaging of several fluorescent probes is limited
- Issues due to autofluorescence within the specimens
- Required time for imaging of thick tissue (several minutes)
- Bleaching of fluorophores
- Quantification of fluorescence signal is limited

Special fluorescence imaging techniques

Fluorescence bleaching

- Fluorescence recovery after photobleaching (FRAP)
- Fluorescence loss in photobleaching (FLIP)
- Fluorescence correlation spectroscopy (FCS)

Quenching

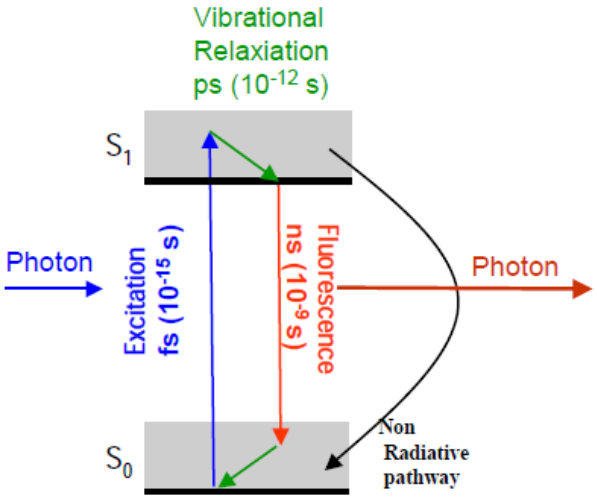
- Förster-Resonance Energy Transfer (FRET)

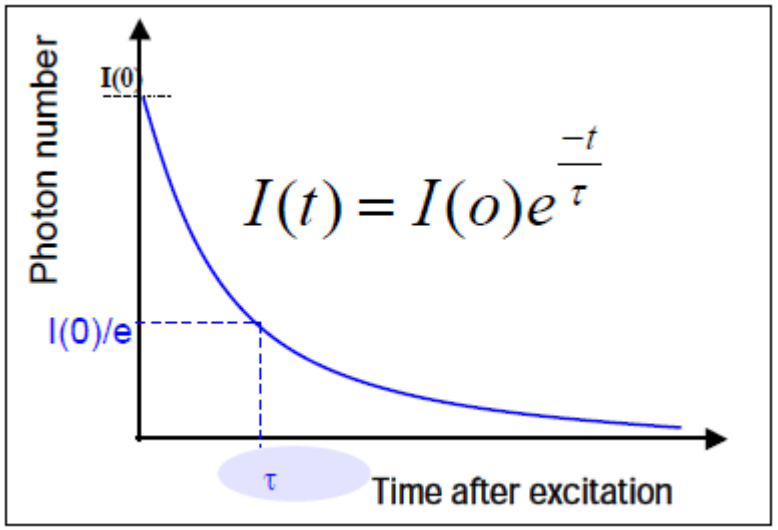
Fluorescence life-time imaging (FLIM)

Fluorescence Lifetime Imaging (FLIM)

Fluorescence Life-time Imaging (FLIM)

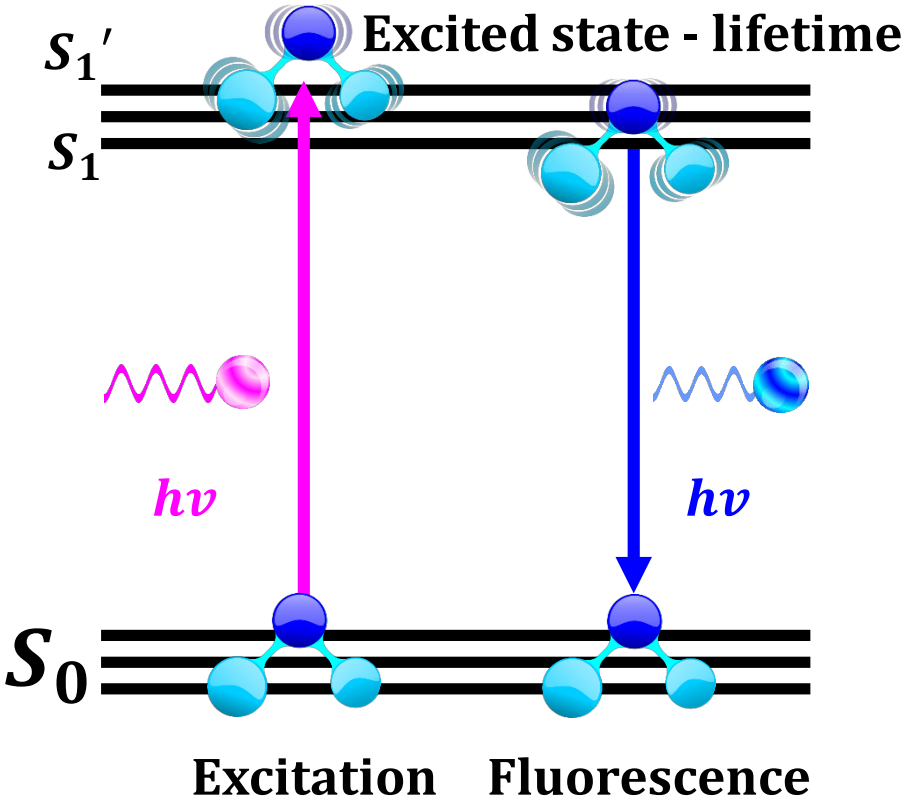
- Every fluorescent molecule has an average time that it spends in the excited state followed after excitation
- This average time is referred as the fluorescent life time τ
- Decay after excitation is an exponential curve
- Fluorescence lifetime imaging uses contrast is based upon the fluorescence lifetimes



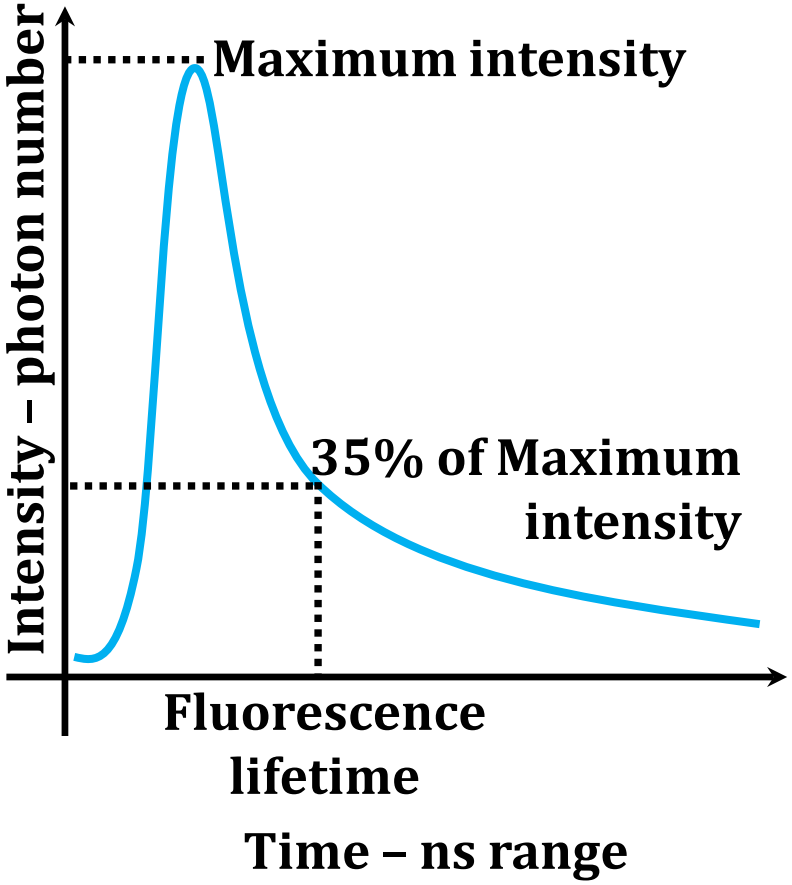


http://www.biology.uiowa.edu/microscopy/pdf/FLIM_Basics_iowa.pdf

Fluorescence lifetime

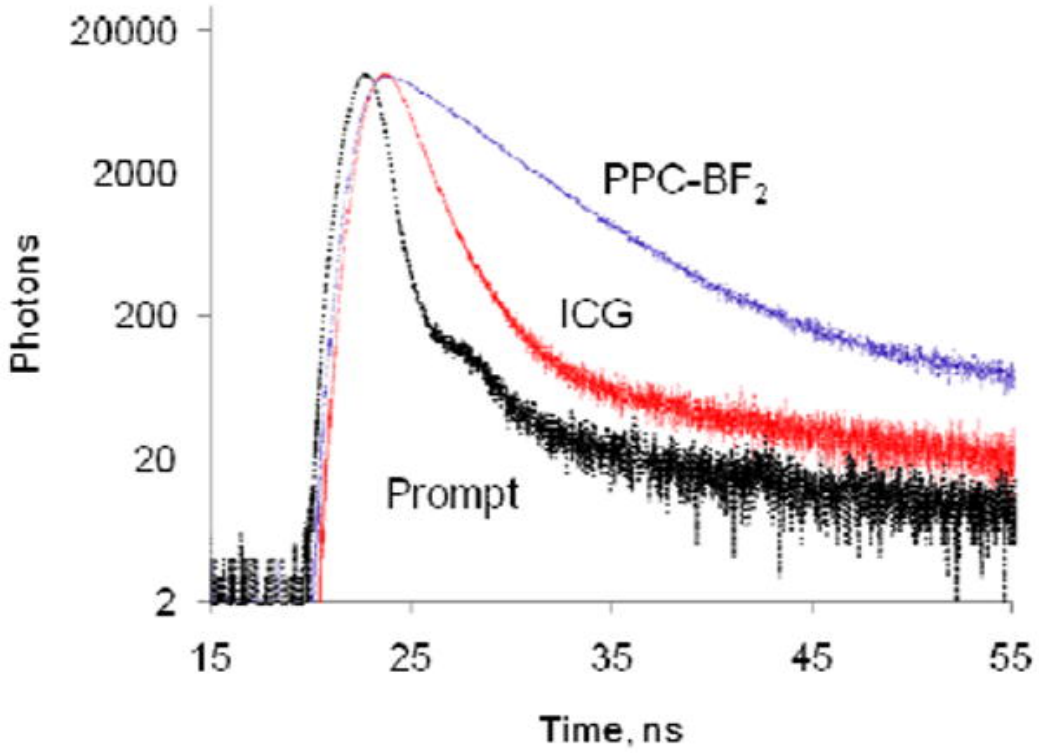


Simplified Jablonski energy diagram



Fluorescence decay curves

- ICG and Pyrrolopyrrole cyanine -BF2 (PPC) in DMSO
- Excitation at 773 nm and emission at 820 nm
- Prompt corresponds to the instrument response with no fluorophore present



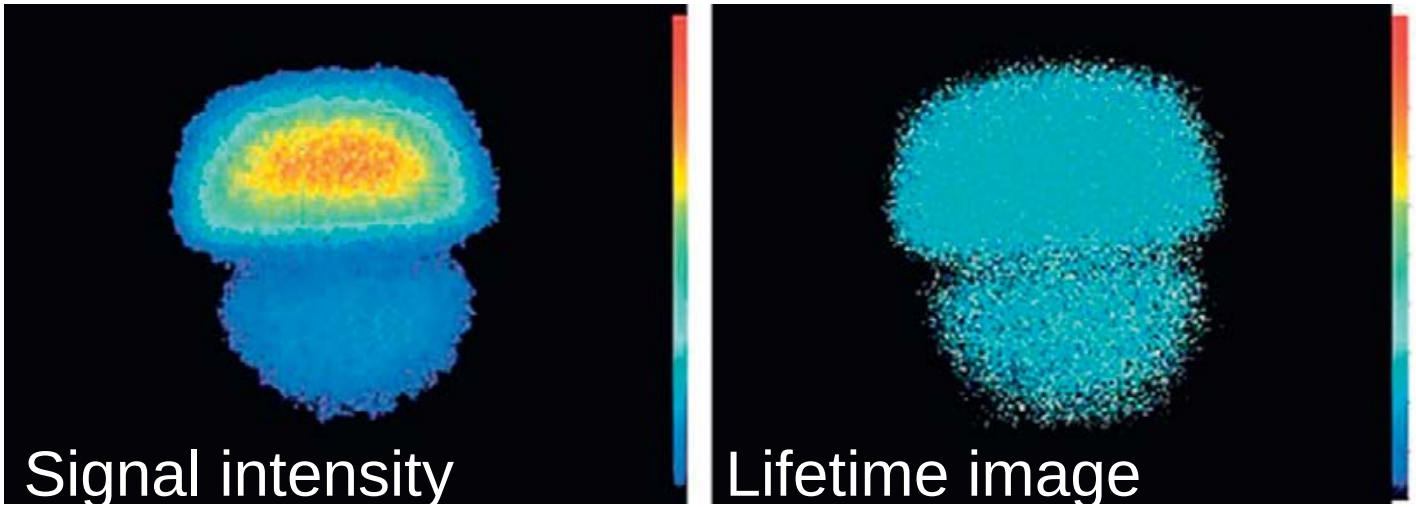
Berezin MY, Achilefu S. *Chemical reviews*. 2010

Advantages of FLIM

FLIM is insensitive to a variety of intensity-based artifacts:

- Variations in excitation source intensity
- Detection gain settings
- Optical loss in the optical path
- Sample variation in sample fluorophore concentration
- Photobleaching
- Microscope focusing

Intensity-independent fluorophore lifetimes recovered by FLIM



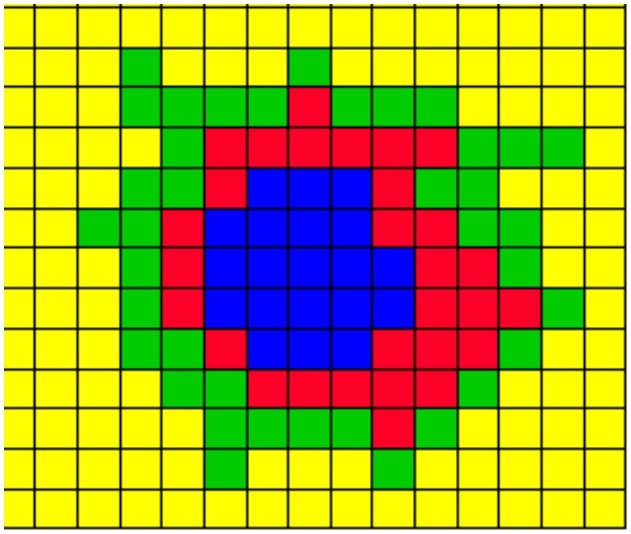
Chen et al. Methods in Cell Biol. 2013

Fluorescence Life-time Imaging (FLIM)

- τ_f is specific for a fluorophore
- Based on τ_f multiple dyes can be distinguished in one channel at high detector sensitivity

FLIM can be used for:

- Ion concentration imaging
- Chemical microenvironment (pH, protein-binding…)
- Quantitative fluorescence measurements



Fluorescence lifetime image

Wavelength -color lock-up table

τ_1 τ_2 τ_3 τ_4

http://www.biology.uiowa.edu/microscopy/pdf/FLIM_Basics_iowa.pdf

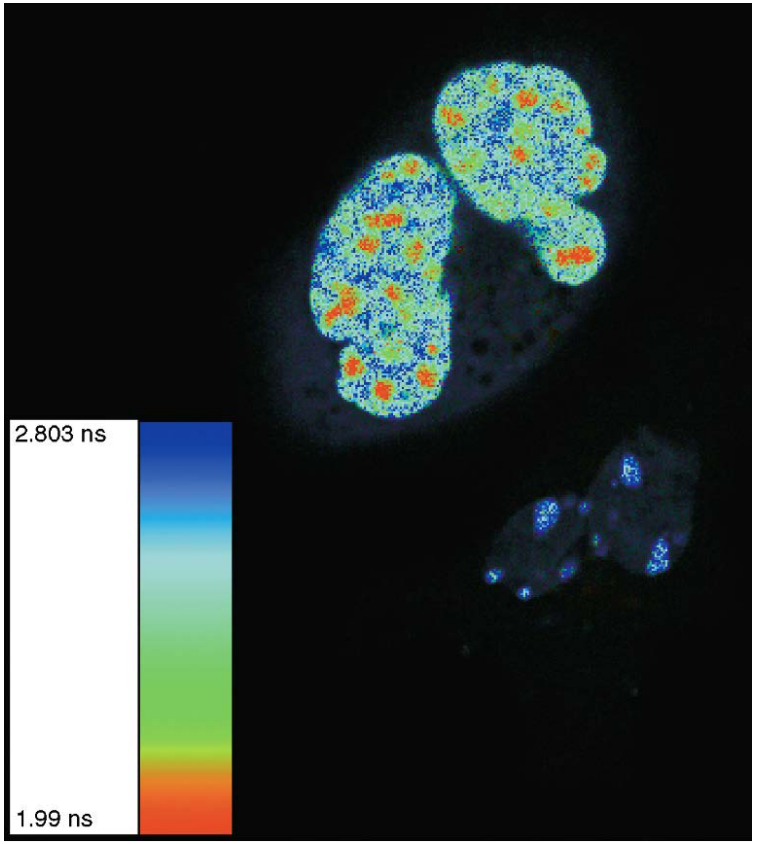
FLIM: Detection modes

Frequency-domain FLIM

- Measurement of the phase shift of fluorescent light with respect to the phase of a modulated excitation source

Time-domain FLIM

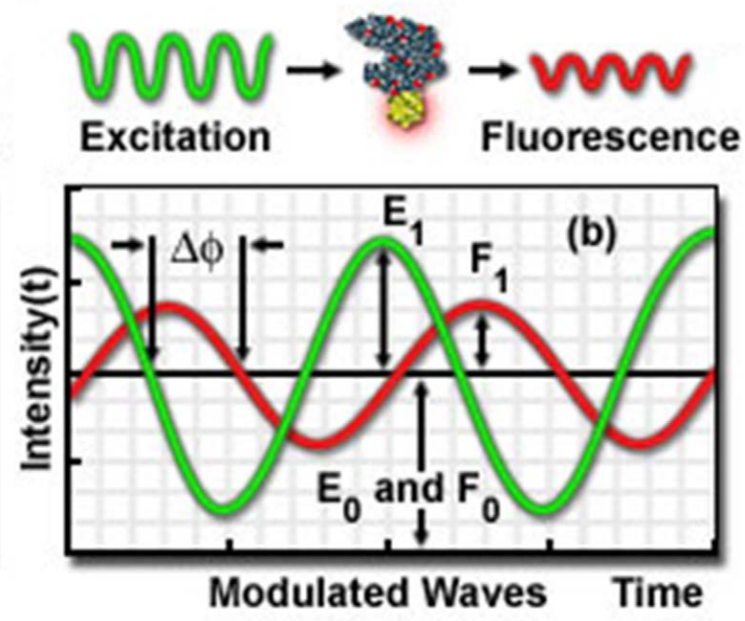
- Measurement of the time dependency of fluorescence intensity with respect to a short excitation pulse.



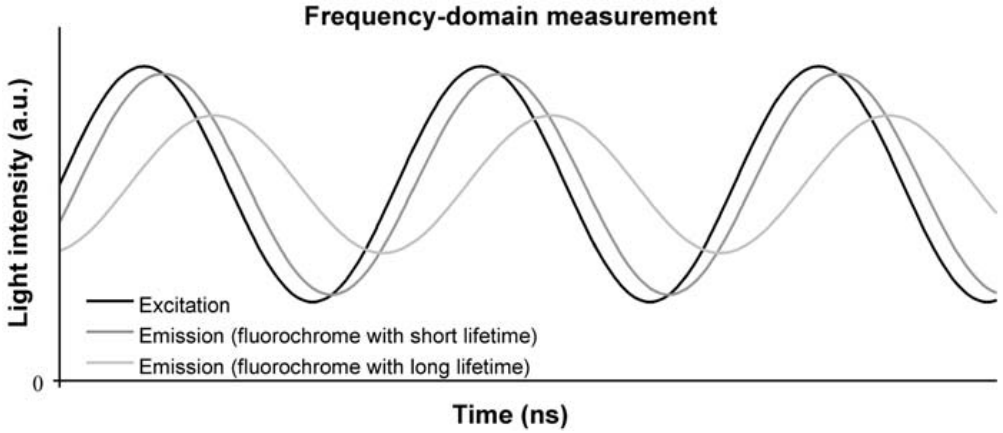
Pawley: Biological confocal microscopy

Frequency-domain FLIM

- Commonly used with wide-field microscopy
- Phase-modulated light source
- Emission is delayed relative to the excitation wave
- Phase shift Δϕ is measured



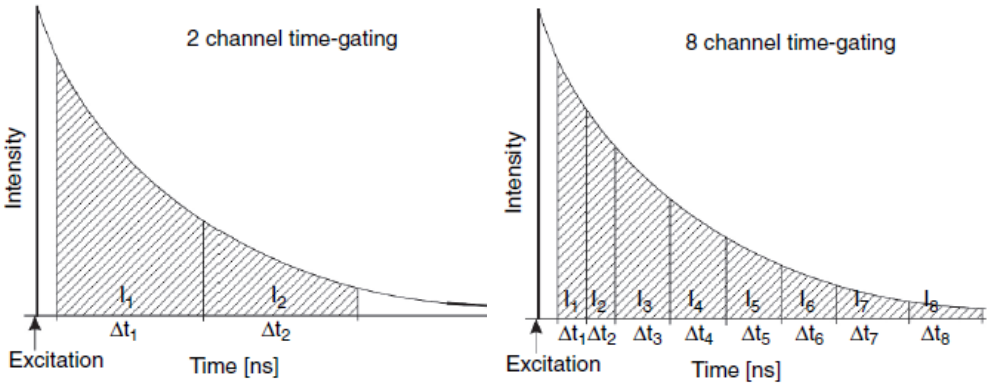
http://www.olympusfluoview.com/applications/flimintro.html



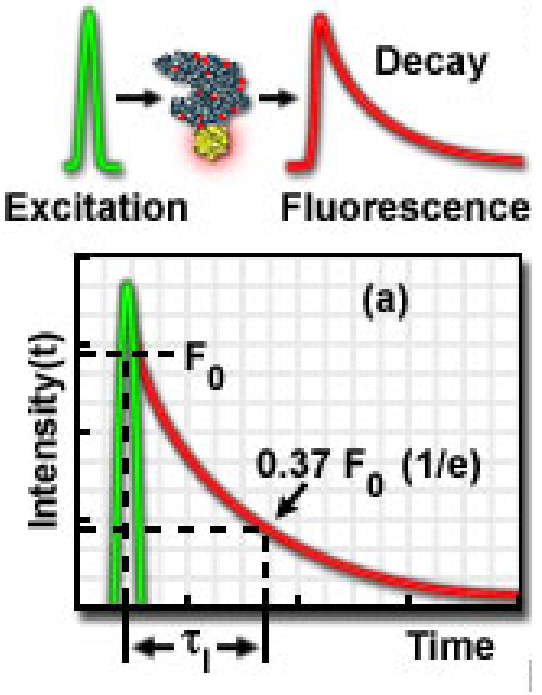
Van Munster, 2005

Time-domain FLIM

- Use of a pulsed light source
- Time correlated single photon counting (TCSPC)
- Or time gating



Pawley: Biological confocal microscopy



http://www.olympusfluoview.com/applications/flimintro.html

TABLE 27.2. Comparison of Different Lifetime Imaging Methods

			Phase Fluorometry	
	Time Gating	TCSPC	Pulsed	Sinusoidal
Shortest τ_f	0.2–0.5 ns	0.1 ns	0.2–0.5 ns	0.2–0.5 ns
Sensitive to bleaching	No	No	Yes	Yes
Photon economy	Good	Good	Good	Poor
Duty cycle	<0.1	<0.1	<0.1	50%
Acquisition time per pixel	$>10^{-5}$ s	$>10^{-4}$ s	$>10^{-5}$ s	$>10^{-4}$ s
Costs	Moderate/high	Moderate/high	Moderate/high	Low

Pawley: Biological confocal microscopy

Multi-FLIM Labeling and Segmentation

Determination of six different fluorescent life times by TCSPC

LY = Luciferase Yellow decay times of 4.27 and 4.95 ns

NYO	=	2.8 ns
FITC	=	3.1 ns
BB	=	3.2 ns
Nile Red (NR)	=	3.6 ns
Benzopyrylene	=	10.8 ns

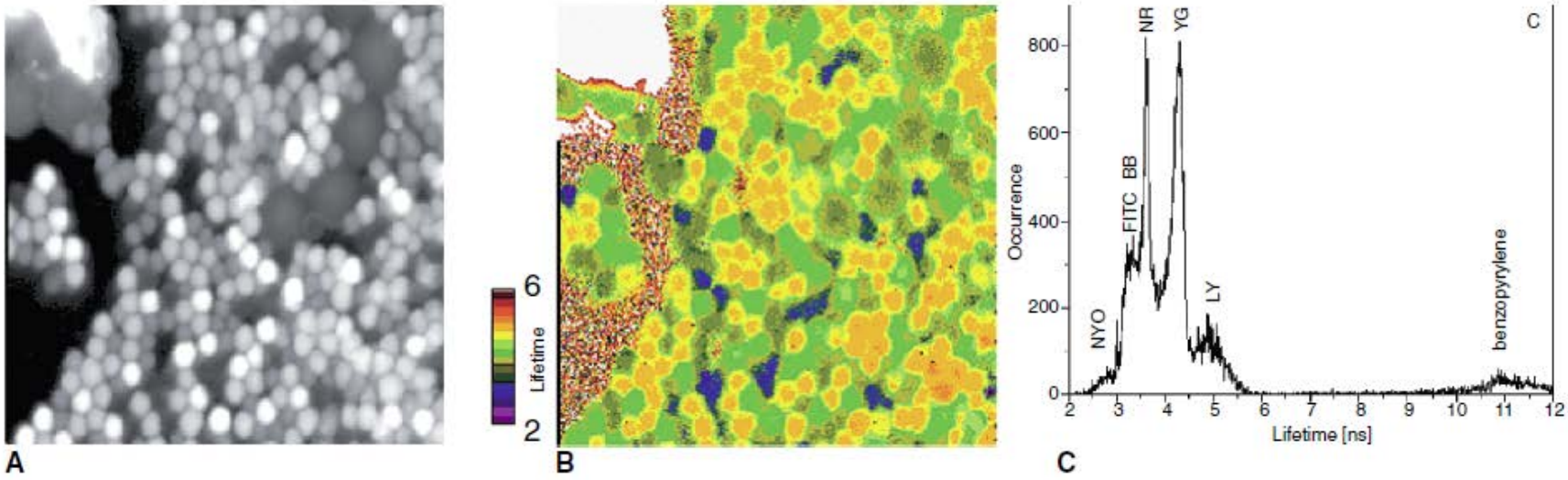
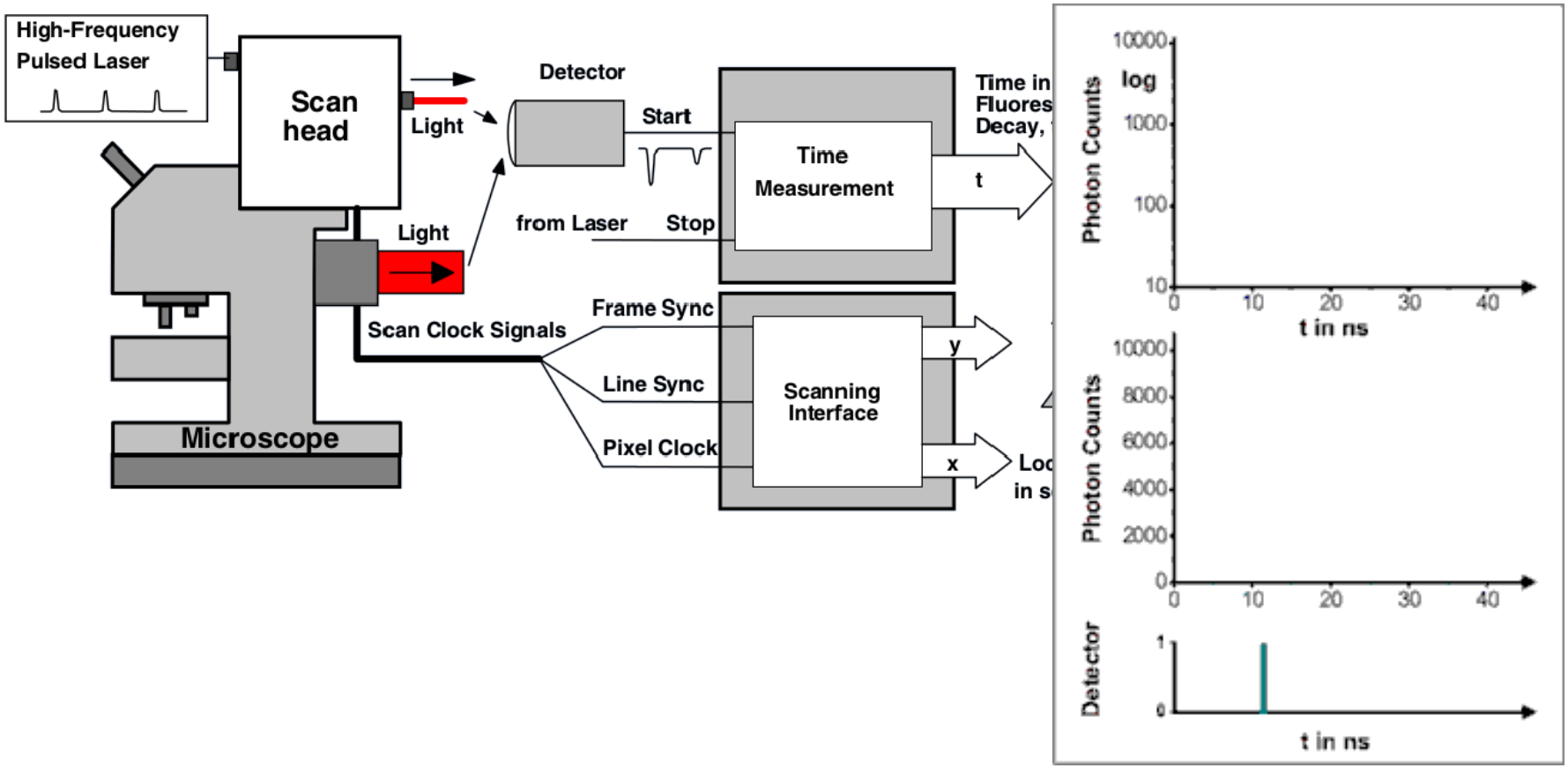


FIGURE 27.8. (A) Intensity image of a specimen containing a number of different fluorescent beads and crystals. (B) Corresponding fluorescence lifetime image. (C) The histogram of fluorescence lifetimes.

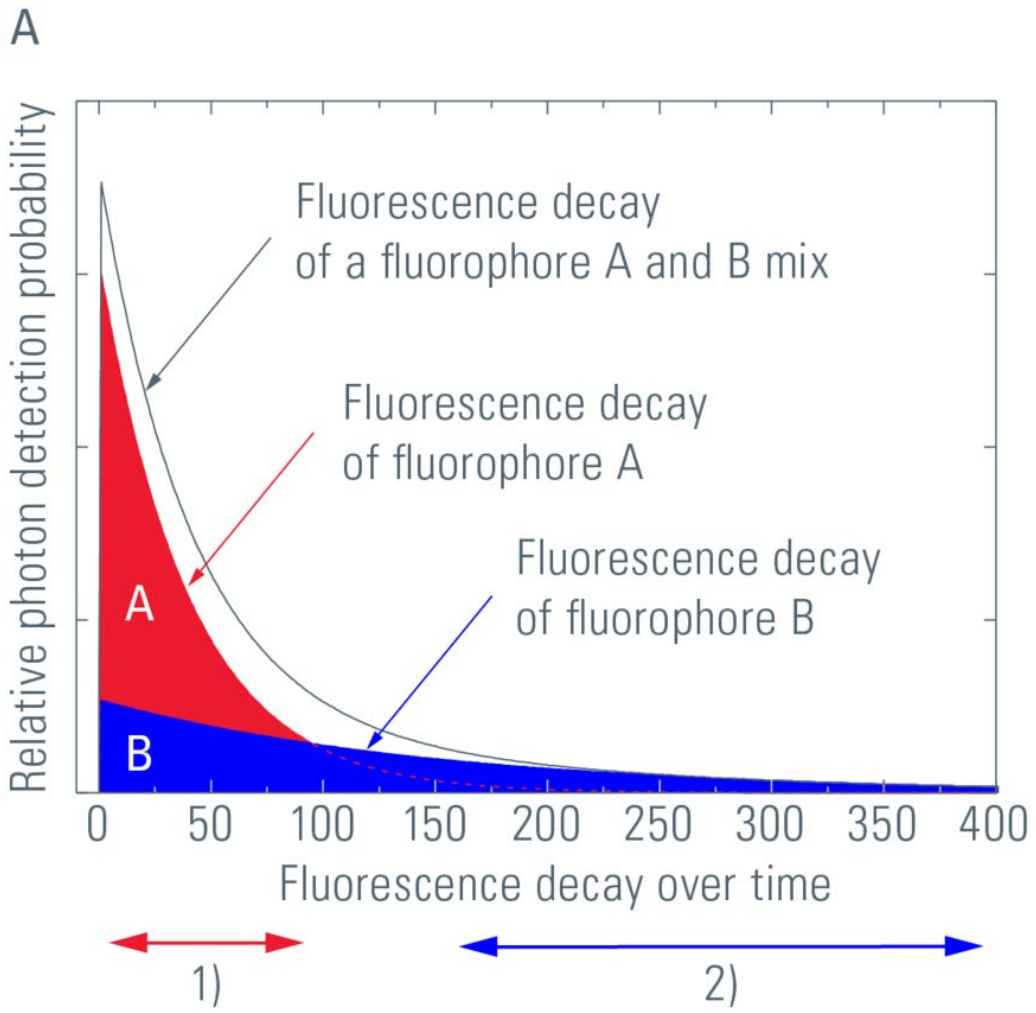
Pawley: Biological confocal microscopy

Fluorescence lifetime imaging microscopy (FLIM)



Becker, Wolfgang: The bh TCSPC handbook. 6th. Becker & Hickl GmbH, 2015

Time-domain FLIM



Fluorescence lifetime imaging microscopy (FLIM)

Fluorophore	Solvent	Excitation max [nm]	Emission max [nm]	Lifetime [ns]
Endogenous				
Tryptophan	Water	295	353	3.1
Tyrosine	Water	275	304	3.6
Phenylalanine	Water	260	282	6.8
NADH	Water	350	460	0.4
FAD	Water	450	525	4.7
FMN	Water	450	525	2.3
Exogenous				
ATT0 565	Water	561	585	3.4
Rhodamine B	Water	562	583	1.68
Cy3	PBS	548	562	0.3
Cy5	PBS	646	664	1
DAPI	TRIS/EDTA	341	496	0.16
FITC	PB pH 7.8	494	518	4.1
Fluorescein	PB pH 7.5	495	517	4
GFP	Buffer pH 8	498	516	3.2
Hoechst	TRIS/EDTA	336	471	0.35
Lucifer yellow	Water	428	535	5.7

We can excite

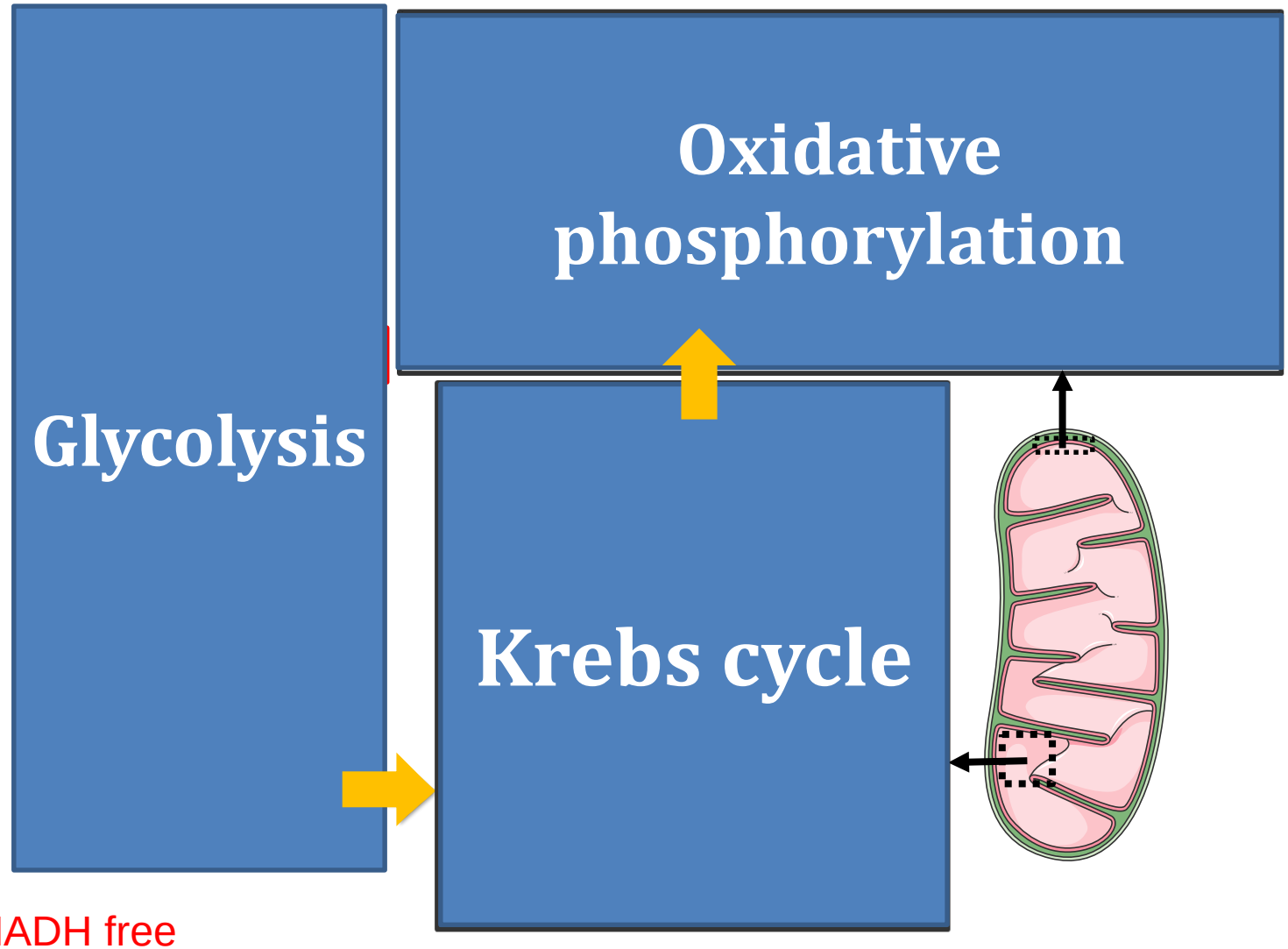
Chen, L.-C., Lloyd 3rd, W. R., Chang, C.-W., Sud, D. & Mycek, M.-A. Fluorescence lifetime imaging microscopy for quantitative biological imaging. Methods Cell Biol

Intrinsic Fluorophores

Fluorophore	Excitation λ (nm)	Emission λ (nm)	Fluorescence Lifetime τ (ns)
Keratin		525	
NAD(P)H	340	450–470	0.3–0.6*
Flavins	370, 450	530	5.2
Melanin	UV/VIS	440, 520, 575	0.2/1.9/7.9
Elastin	300–340	420–460	0.2–0.4/0.4–2.5
Collagen	300–340	420–460	0.2–0.4/0.4–2.5
SHG	720–960	360–480	0
Lipofuscin	UV/VIS	570–590	
PPIX	405 . . . 630	635, 710	11

Schenke-Layland, J. Biophotonics 2008

FLIM of NADH – ATP production

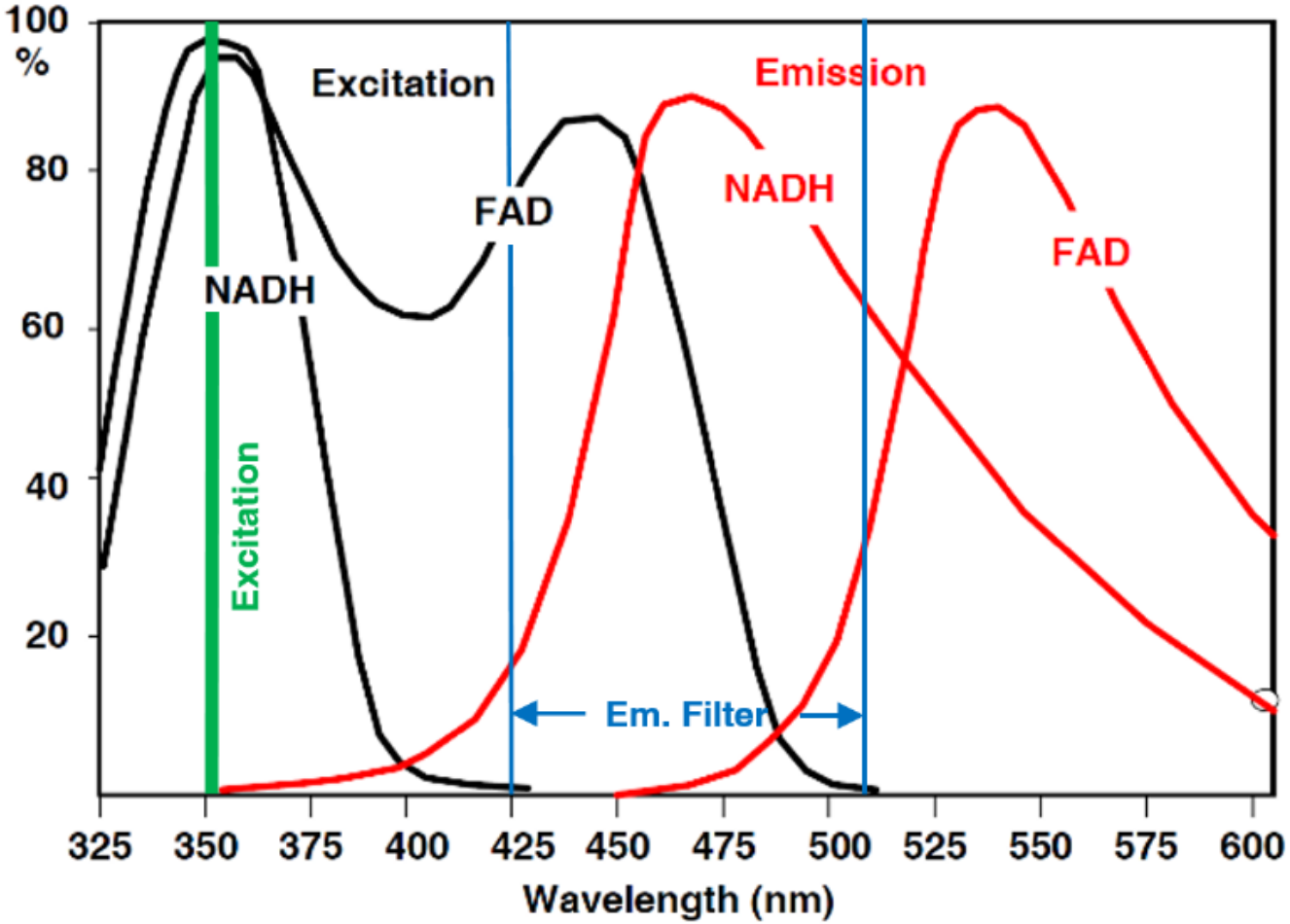


NADH free
NADH protein bound

NAD(P)H

- Reduced Nicotinamide adenine dinucleotide (phosphate)
- Important cofactor in cell metabolism
- Free NAD(P)H: short lifetime τ_1
- Protein-bound NAD(P)H: long lifetime τ_2
- Typical values: τ_1~ 700 ps and τ_2 ~ 2600 ps
- Hypothesis: lifetime and contribution changes with disease

FLIM of NADH



Becker, Wolfgang: The bh TCSPC handbook. 6th. Becker & Hickl GmbH, 2015

For NAD(P)H:

$$F(t) = \alpha_1 \exp\left(-\frac{t}{\tau_1}\right) + \alpha_2 \exp\left(-\frac{t}{\tau_2}\right)$$

$$\tau_m = \alpha_1 \tau_1 + \alpha_2 \tau_2$$

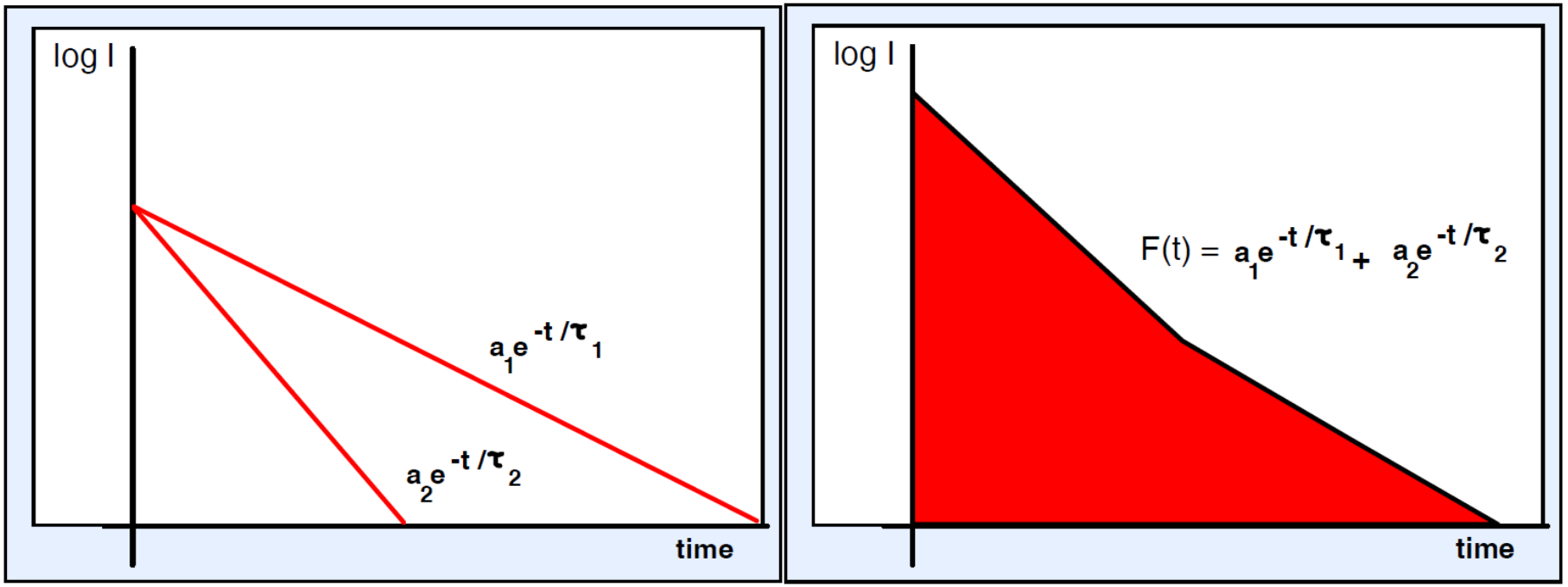
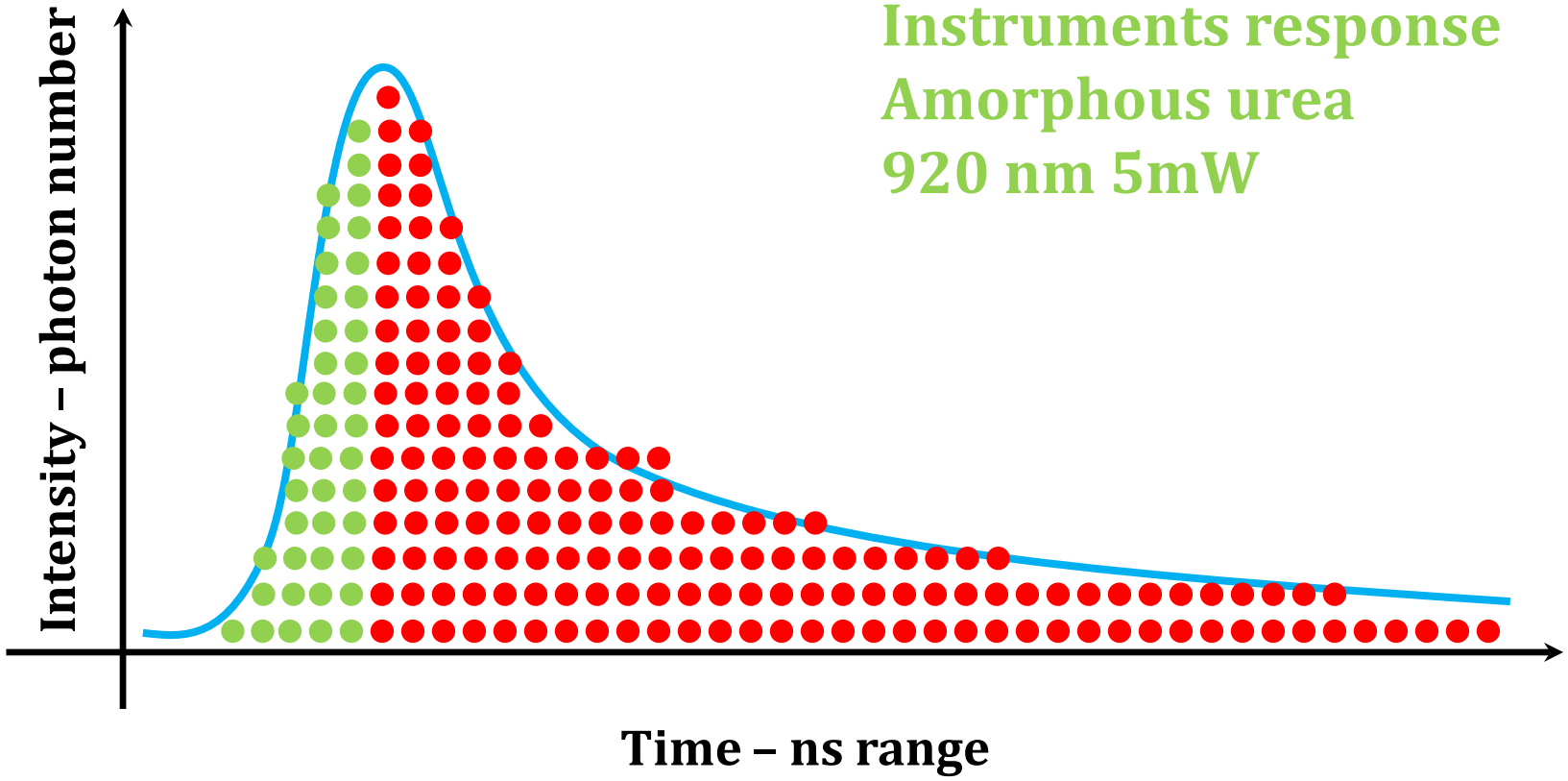


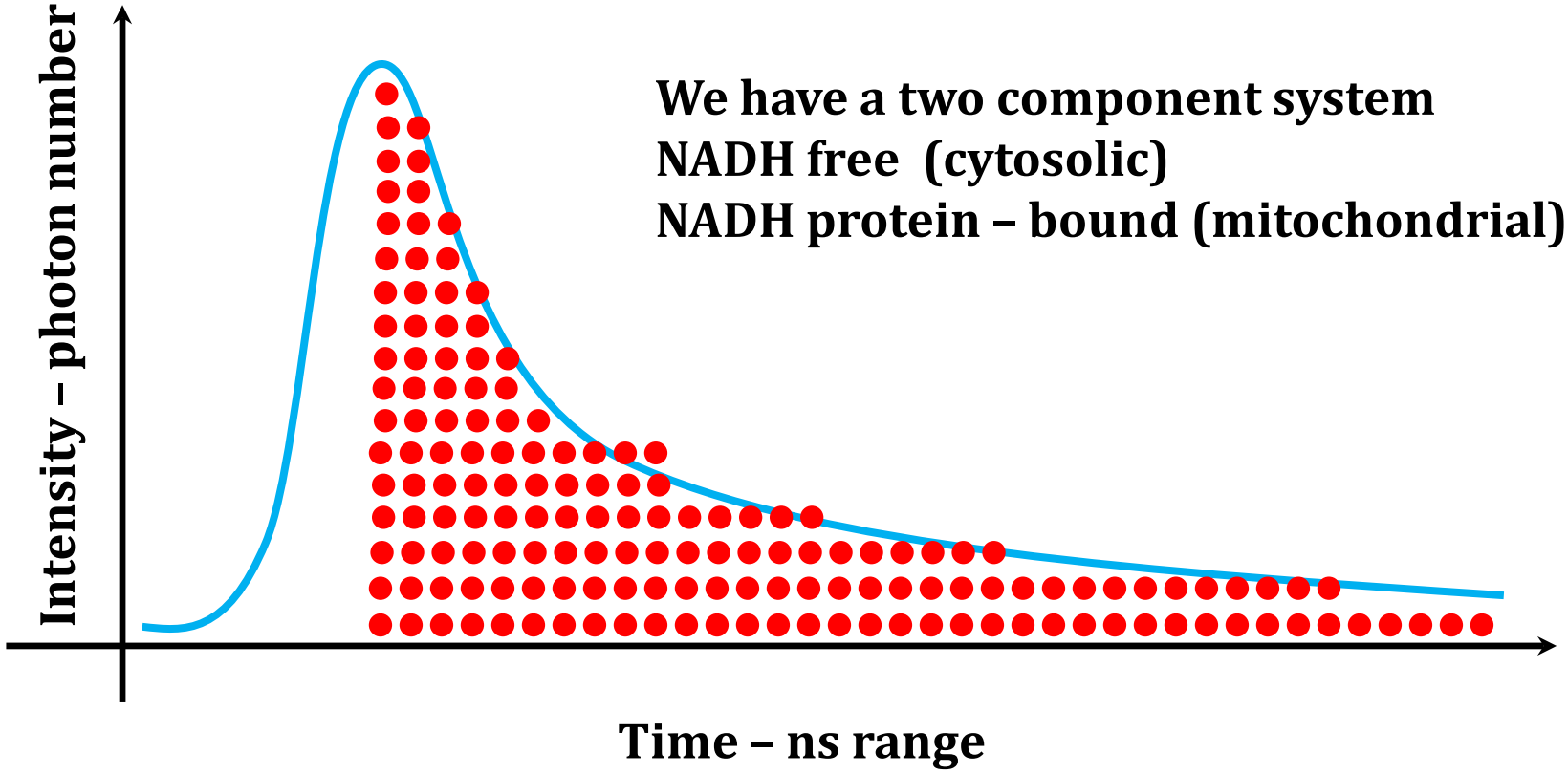
Figure 1: Sum of two exponential decays showing different lifetimes

Source: SPCImage 5.0 manual

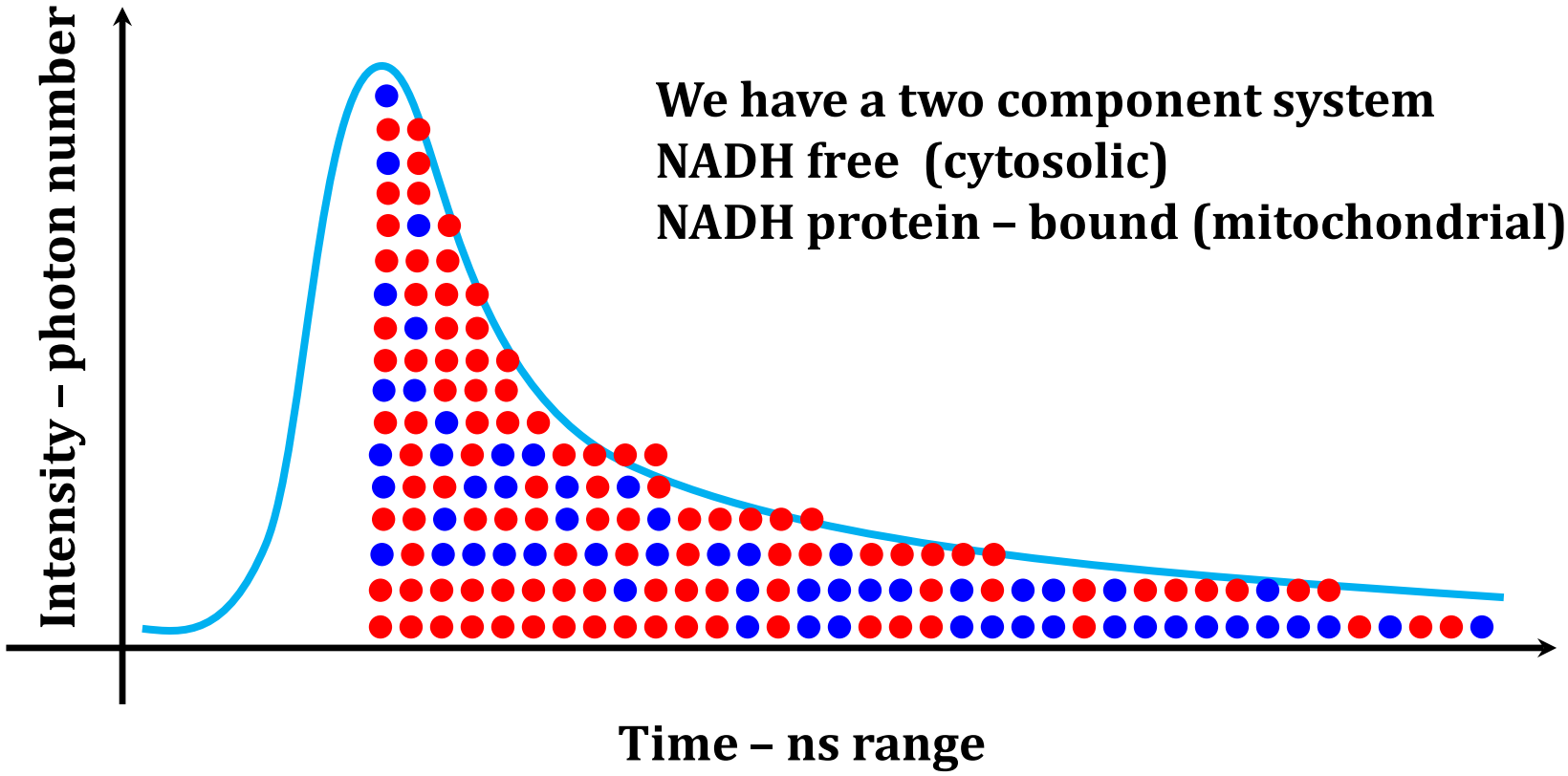
Multiexponential decay fitting



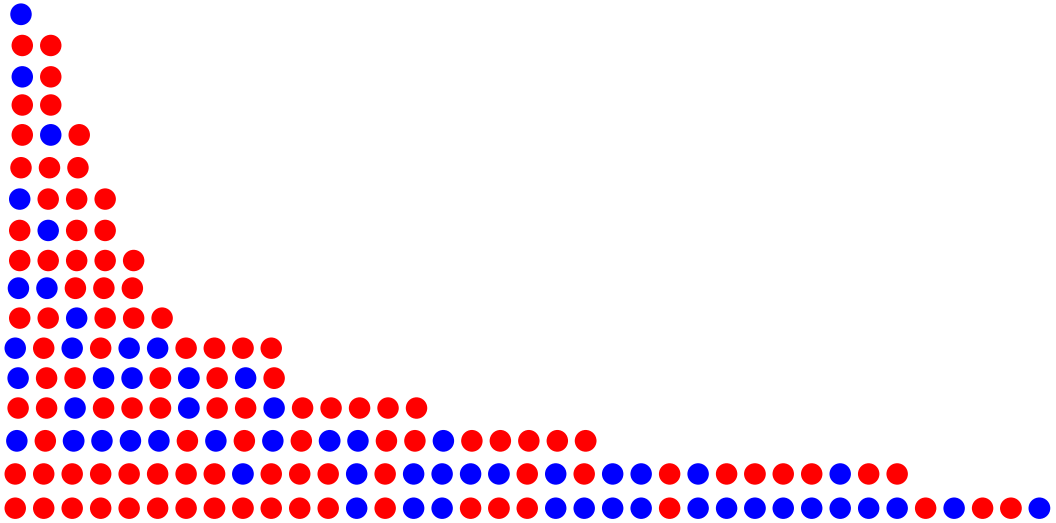
Multiexponential decay fitting



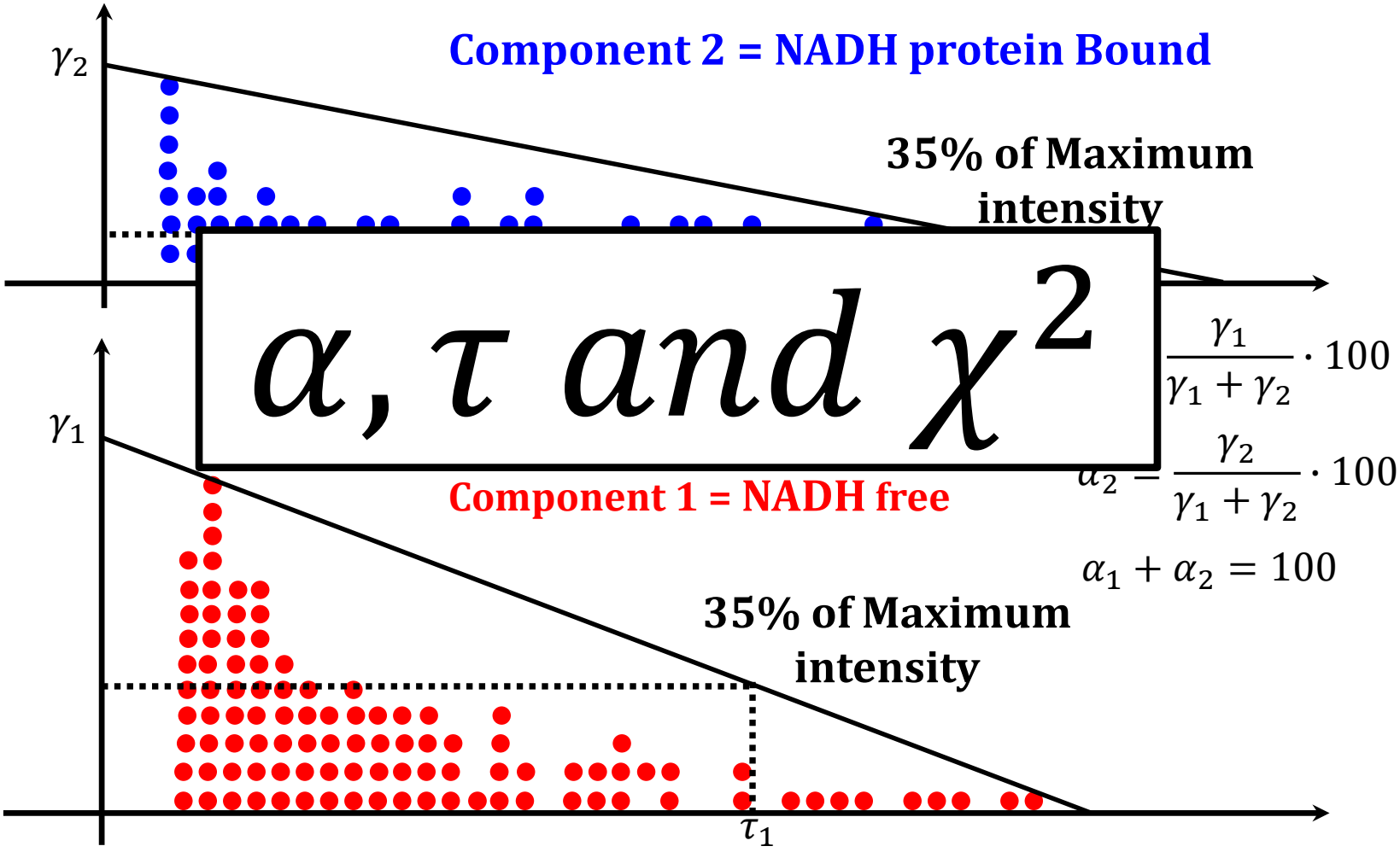
Multiexponential decay fitting



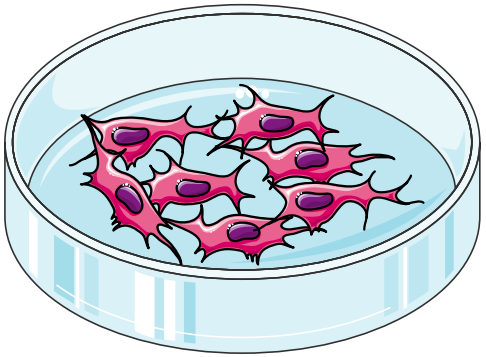
Multiexponential decay fitting



Multiexponential decay fitting



Experimental setup



Human dermal fibroblast (hDFs)

FLIM measurements

Potassium cyanide (KCN)

Hydrogen peroxide (H_2O_2)

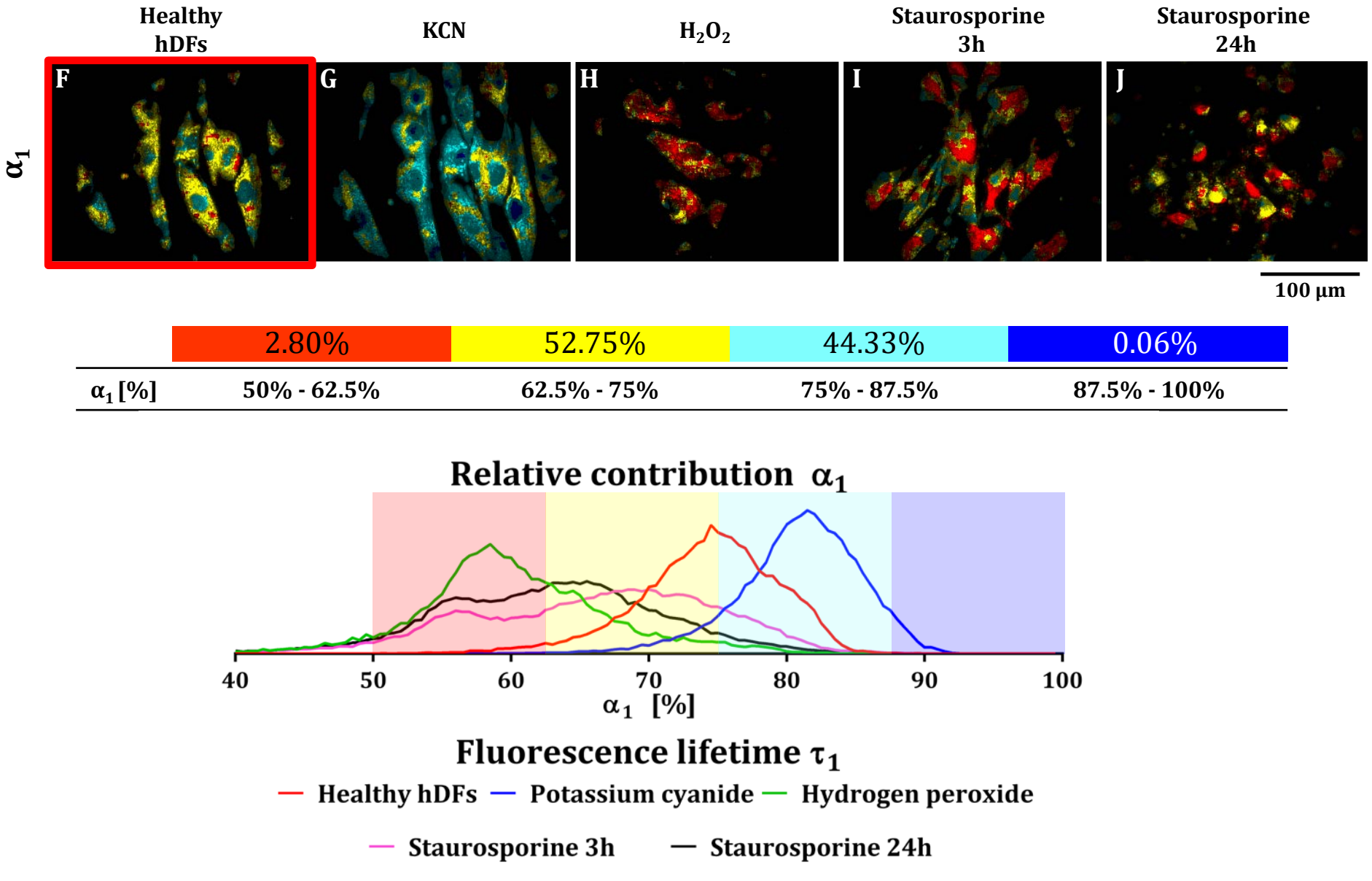
Staurosporine

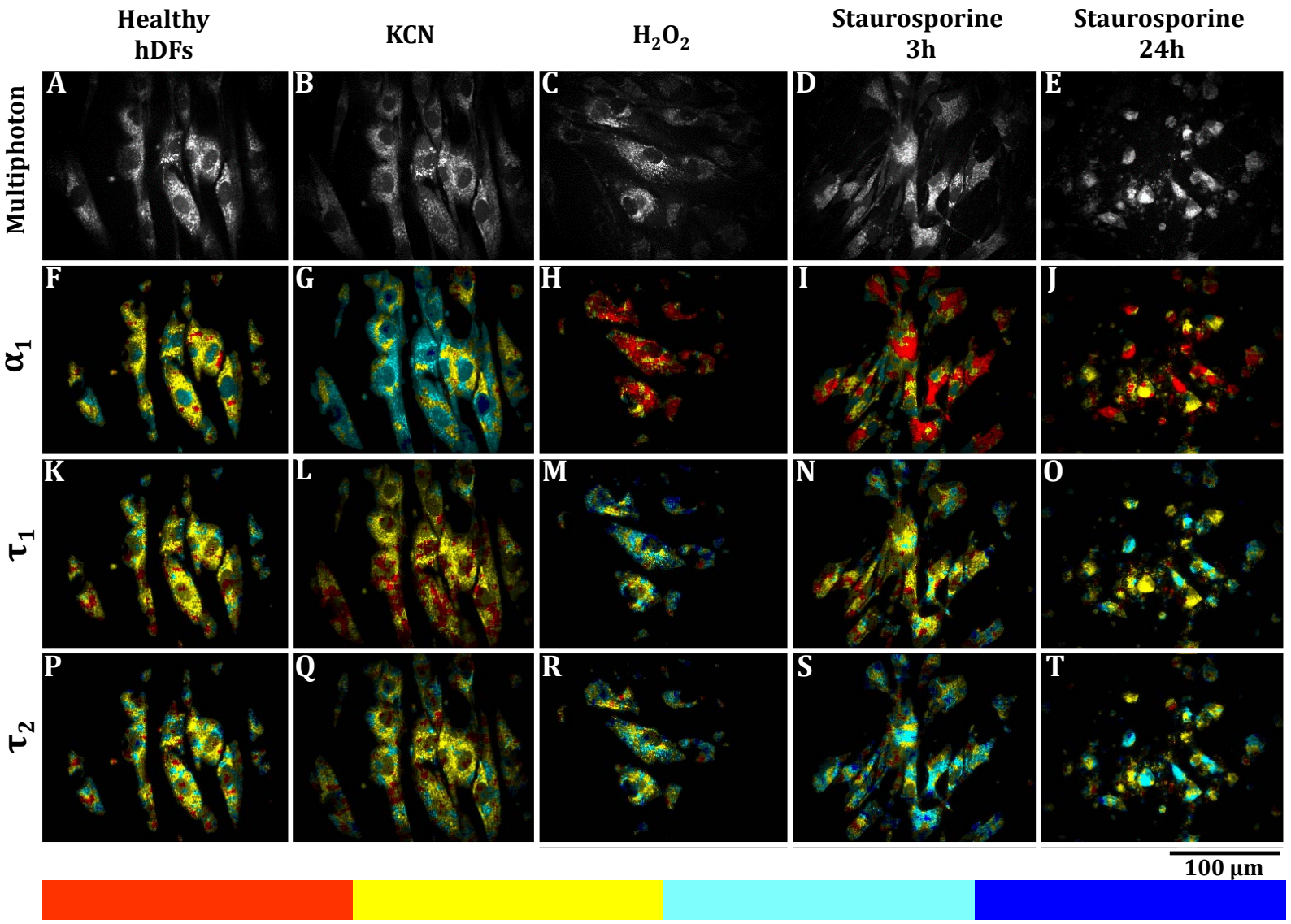
3 h

24 h

FLIM measurements

MTS test

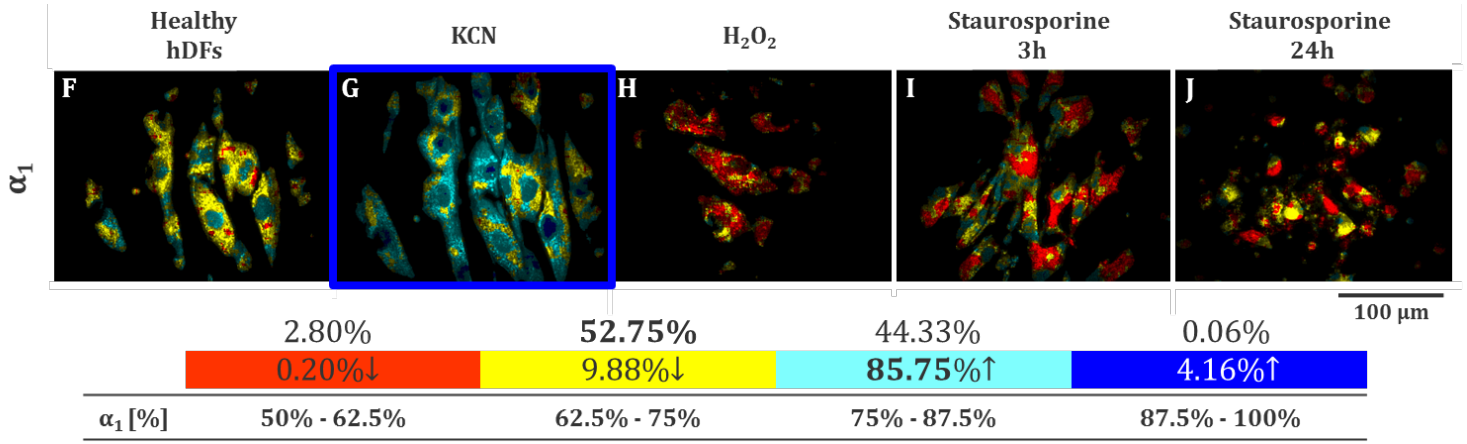

Healthy hDFs
KCN
H_2O_2
Staurosporine 3h
Staurosporine 24h
F
G
H
I
J
α_1
100 µm
2.80%
52.75%
44.33%
0.06%
α_1 [%]
50% - 62.5%
62.5% - 75%
75% - 87.5%
87.5% - 100%
Relative contribution α_1
40
50
60
70
80
90
100
α_1 [%]
Fluorescence lifetime τ_1
Healthy hDFs
Potassium cyanide
Hydrogen peroxide
Staurosporine 3h
Staurosporine 24h

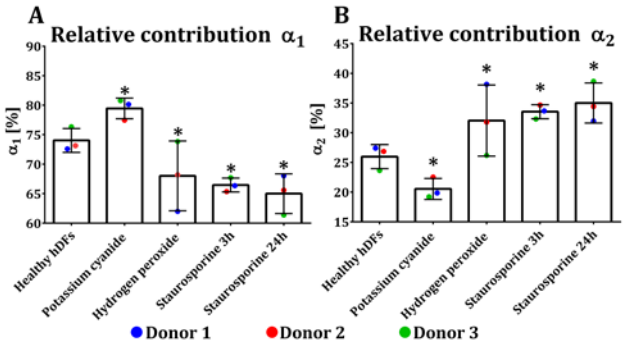


α_1 [%]	50% - 62.5%	62.5% - 75%	75% - 87.5%	87.5% - 100%
τ_1 [ns]	0.25 – 0.48	0.48 – 0.72	0.72 – 0.96	0.96 – 1.2
τ_2 [ns]	2.0 – 2.5	2.5 – 3.0	3.0 – 3.5	3.5 – 4.0

FLIM parameters enable the metabolic evaluation of the inquired specimen

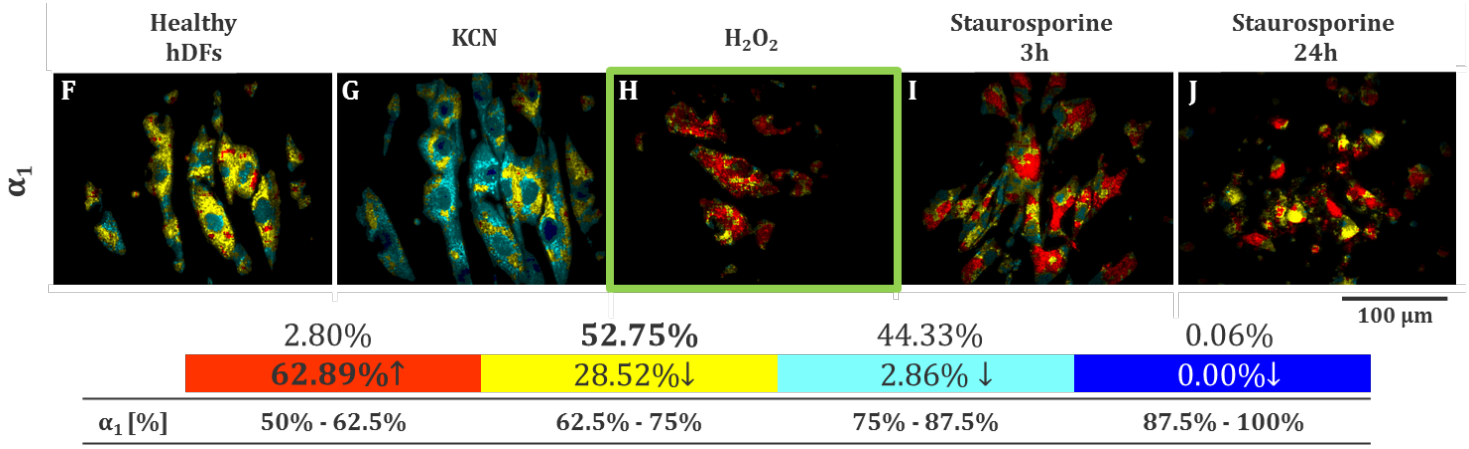
- Oxidative phosphorylation inhibition translates into an increment on the relative contribution of free NADH.

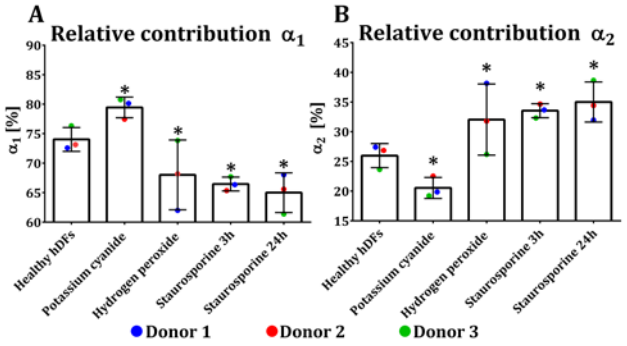




FLIM parameters enable the metabolic evaluation of the inquired specimen

- Glycolysis inhibition results in a reduction of the relative contribution of free NADH.





Summary FLIM

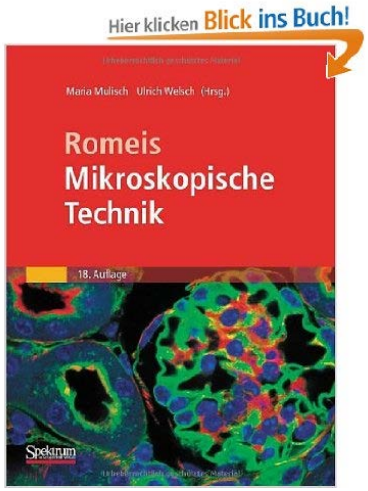
- Intensity-independent information from fluorescent molecules
- Imaging of protein-bound and free metabolites

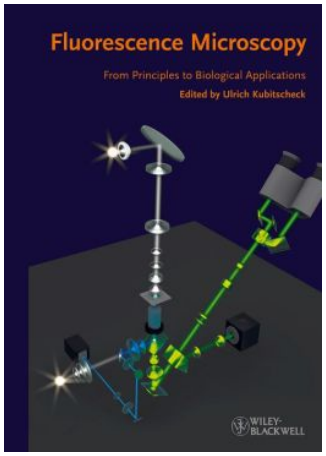
-> Label-free Metabolic profiling of cells

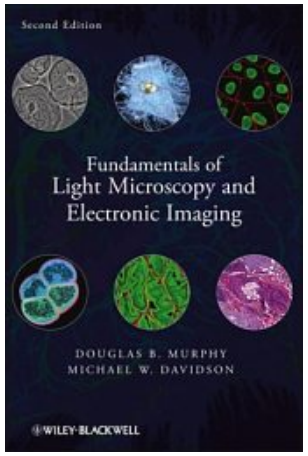
- Sensitive to the fluorophores microenvironment

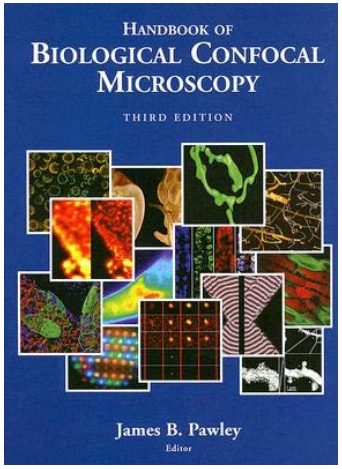
-> new ways for life scientists to detect, visualize, and investigate structure and function in biological systems

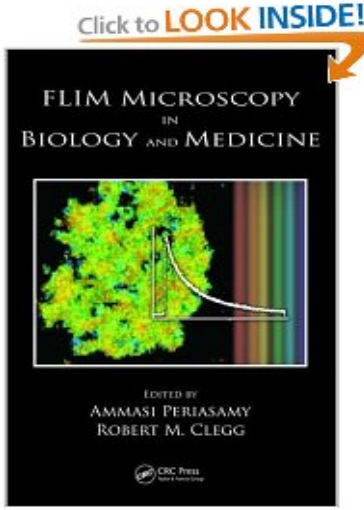
Literature Books











- Romeis: Mikroskopische Technik, ISBN: 978-3-8274-1676-6
- Kubitscheck: Fluorescence Microscopy: From Principles to Biological Applications, Print ISBN: 9783527329229
- Murphy: Fundamentals of Light Microscopy and Electronic Imaging, ISBN Print ISBN: 9780471692140
- Pawley: Handbook Of Biological Confocal Microscopy, ISBN 978-0-387-45524-2
- Periasamy: FLIM microscopy in medicine and biology, ISBN: 978-1420078909

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- http://www.zeiss.de/C1256B5E0051569F/EmbedTitelIntern/Microscopy_from_the_very_beginning/$File/Microscopy_from_the_very_beginning.pdf (24.05.2013)
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- http://zeiss-campus.magnet.fsu.edu/referencelibrary/pdfs/ZeissConfocalPrinciples.pdf (12.07.2013)

Leica tutorials

- http://www.leica-microsystems.com/science-lab/ (24.05.2013)

Other websites

- www.edmundoptics.de/technical-resources-center/microscopy (24.05.2013)

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