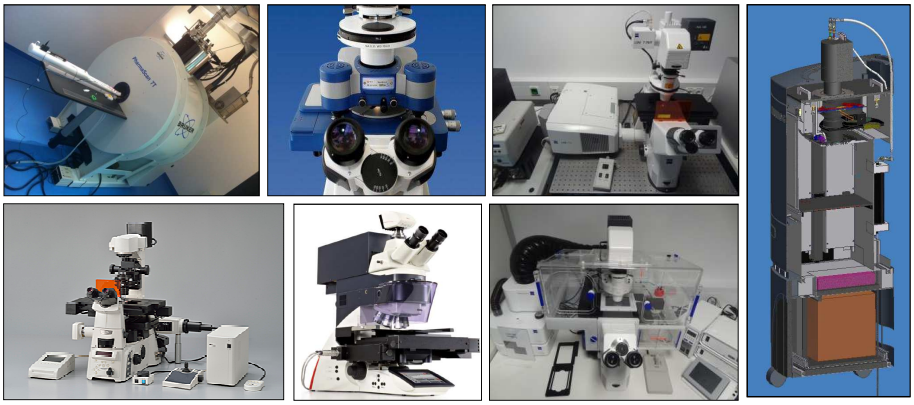
Fraunhofer IZI

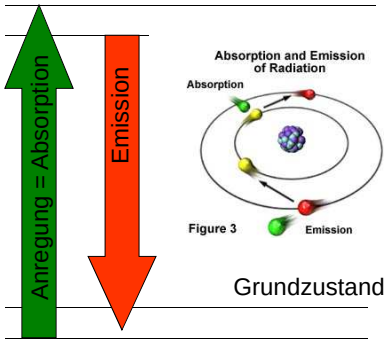
Experimental Imaging - Alexander Kranz, MD



Fraunhofer IZI

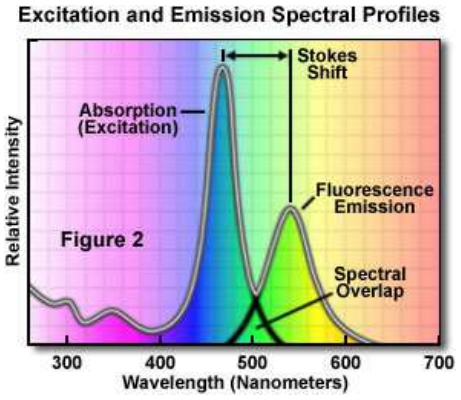
Basic Principle Fluorescence Imaging

Anregungszustand



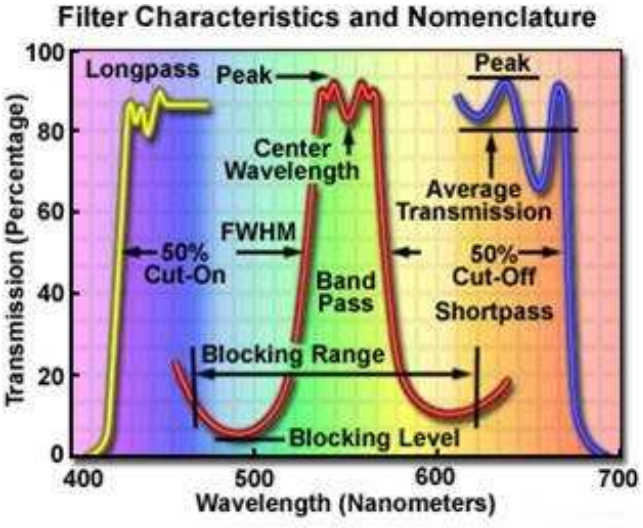
Grundzustand

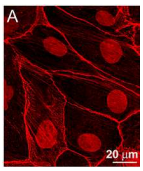
Jablonski Energiediagramm



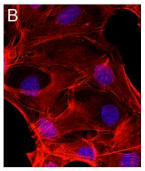
Farbstoff	Exzitation	Emission
DAPI	359	461
FITC	495	519
Alexa 488	499	520

Fraunhofer IZI

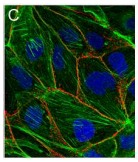




Excitation
543nm
Beamsplitter:
HFP 488/543
Emission:
560nm LP



Emission:
560-615 nm BP
650 nm LP

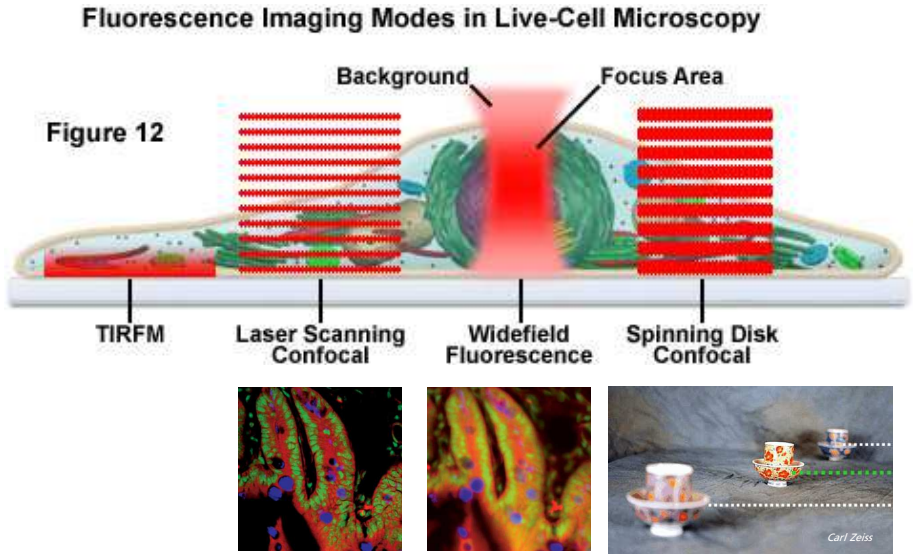


Emission:
Spectral 11nm
552-723nm

North et al., 2006

Ex/Em Filter must be selected to match your fluorochome combination

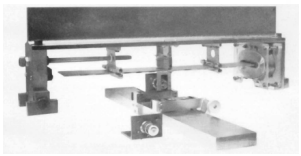
Fraunhofer IZI

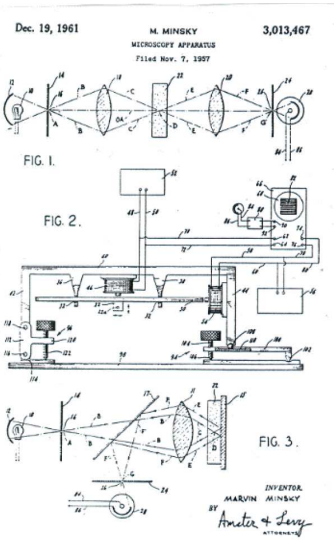


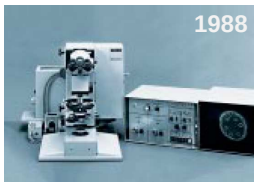
Fraunhofer IZI

Marvin Minsky

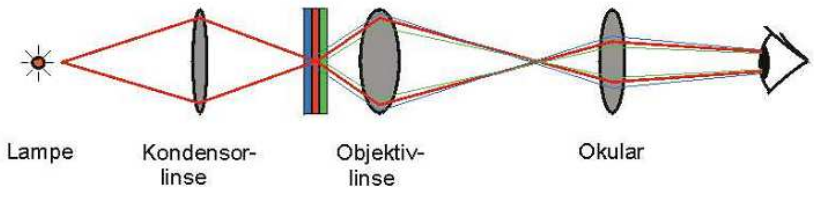
1955

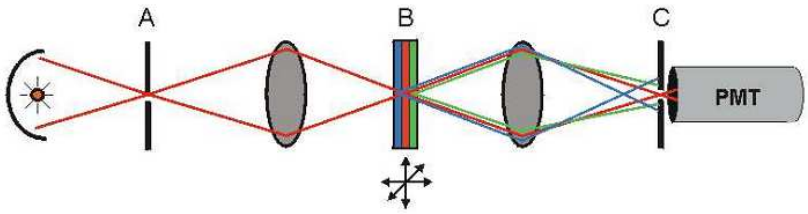






Fraunhofer IZI





Fraunhofer IZI

Composition

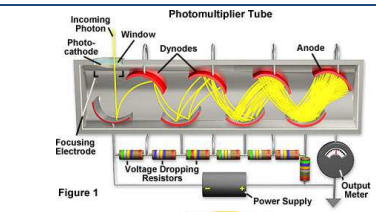
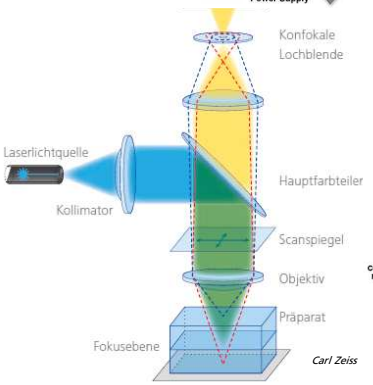
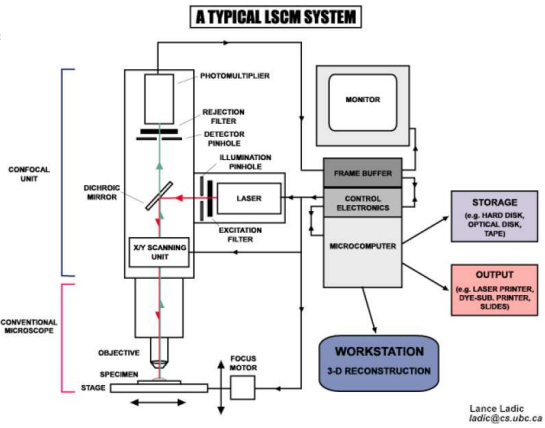


Figure 1

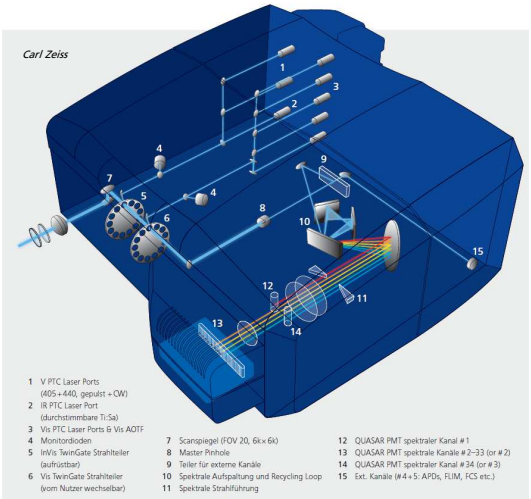




Lance Ladic
ladic@cs.ubc.ca

Fraunhofer IZI

Optical path



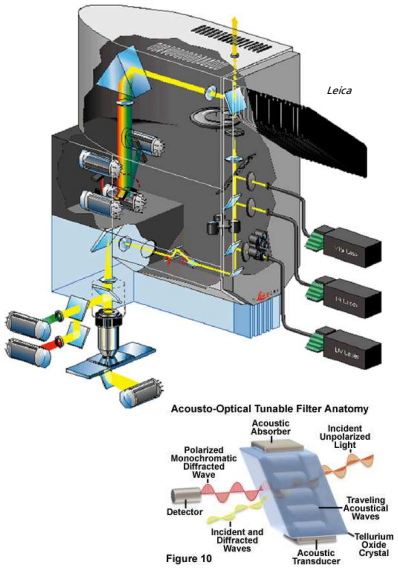
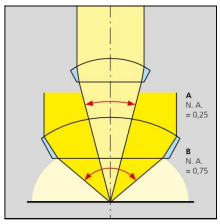
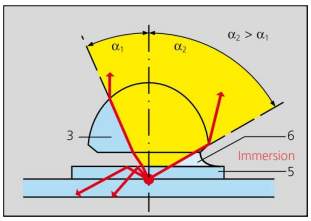


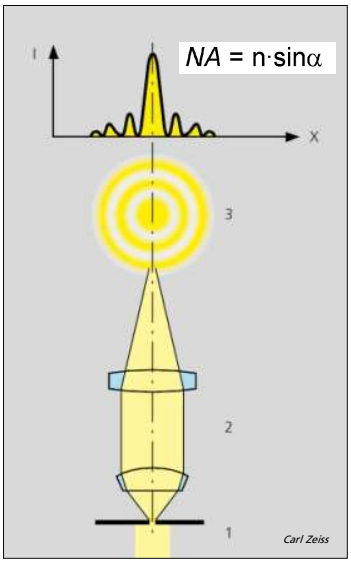
Figure 10

Fraunhofer IZI

Numeric aperature and resolution







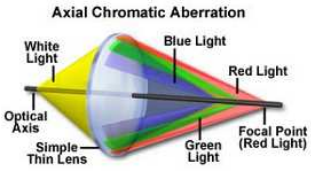
Öffnungwinkel des Objektivs
Brechnungsindex des Immersionsmediums (n)

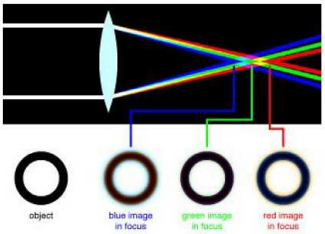
Definiert das Auflösungsvermögen
Kann in Luft NIE größer als 1 werden

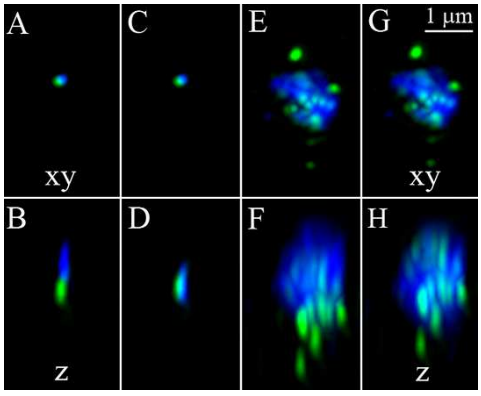
Ein einzelner Punkt wird als Airy Scheibe abgebildet

Fraunhofer IZI

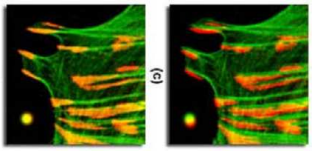
Chromatic aberration







North et al., 2006



Correction with color beat

Fraunhofer IZI

Spherical aberration

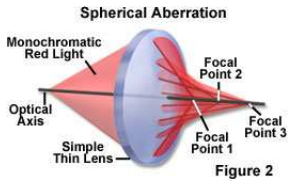
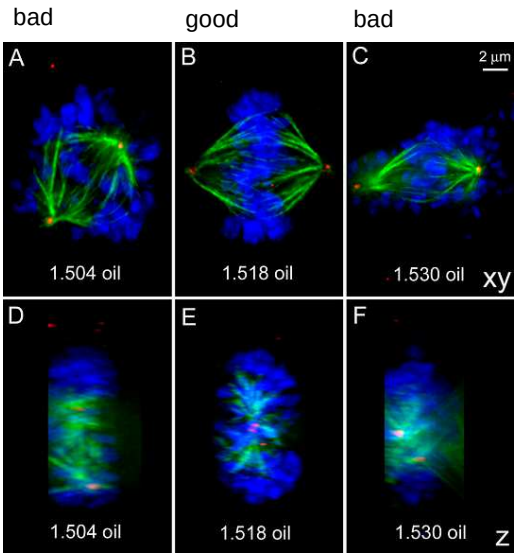


Figure 2

Refractive index mismatch =wrong immersion medium gives rise to geometrical aberrations

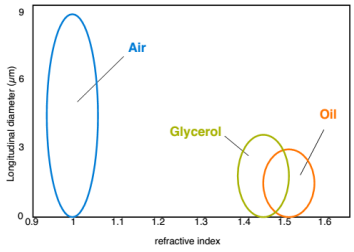


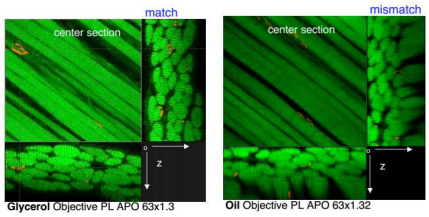
North et al., 2006

Fraunhofer IZI

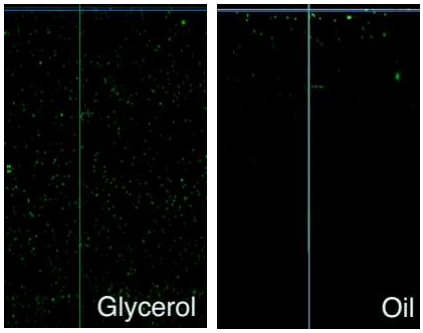
Mounting medium

Compound	Refractive Index	
Ethylenglycol	1,109	
Methanol	1,329	
Aqua	1,330	
Aceton	1,359	
Ethanol	1,361	
n-Hexan	1,375	
Isopropanol	1,378	
Glycerol in PBS pH7,4	1,385	40%
1-Propanol	1,385	
Moviol	1,389	
Glycerol in PBS pH7,4	1,394	50%
Isobutanol	1,396	
Ethylenglycolmonomethylether	1,402	
Glycerol in PBS pH7,4	1,405	60%
Diethylenglycoldiethylether	1,412	
Glycerol in PBS pH7,4	1,417	70%
Dioxan	1,422	
Glycerol in PBS pH7,4	1,425	80%
Glycerol in PBS pH7,4	1,435	90%
chloroform	1,448	

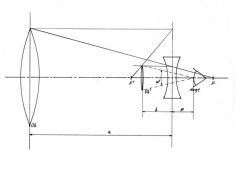


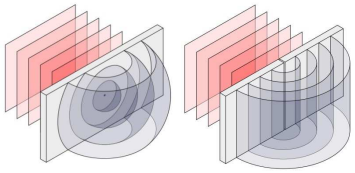


Glycerol Objective PL APO 63x1.3 **Oil** Objective PL APO 63x1.32



Fraunhofer IZI





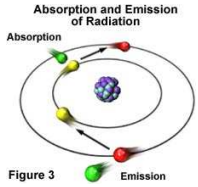


Figure 3

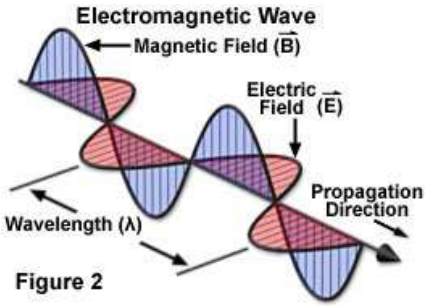


Figure 2

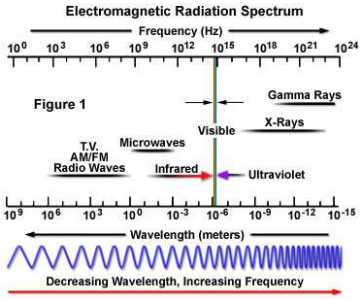


Figure 1

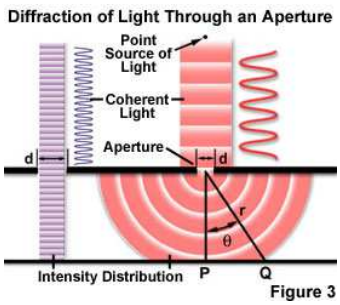


Figure 3

$$1AE = \frac{1,22\overline{\lambda}}{NA}$$ = Numerische Apertur

$$\overline{\lambda} = \sqrt{2}\,\frac{\lambda_{em}\lambda_{exc}}{\sqrt{\lambda_{em}^2 + \lambda_{exc}^2}}$$

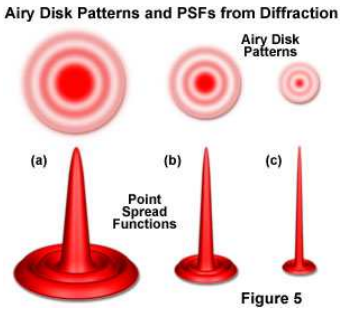
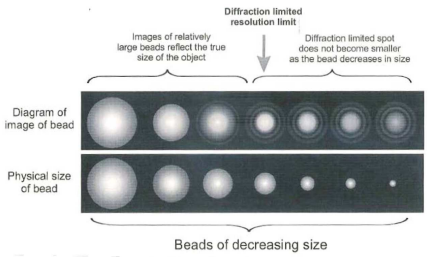
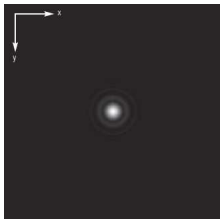


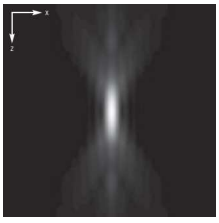
Figure 5



Point spread function

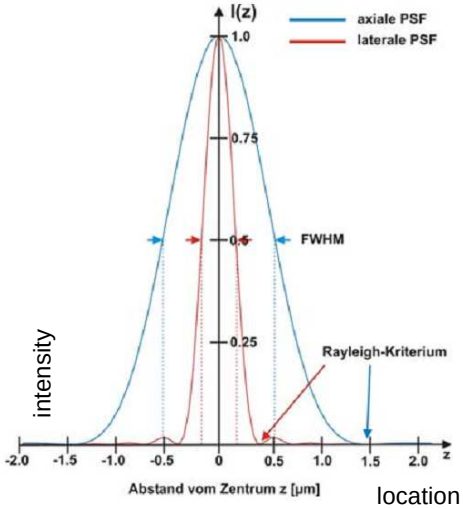


Laterale Projektion



Axiale Projektion

- Point Spread Function (PSF)
- Wenn Intensität auf die Hälfte des Zentralen Maximum abgesunken dann Halbwertsfläche
- Setzt sich aus axialen und lateralen Anteil zusammen: Halbwertsbreite =
- Full Width at Half Maximum (FWHM)

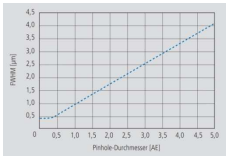


location

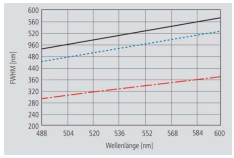
Fraunhofer IZI

FWHM characteristics

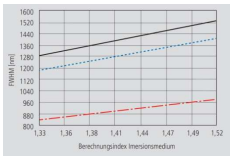
FWHM ist abhängig von:



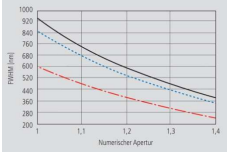
Blendengröße = Pinhole Durchmesser



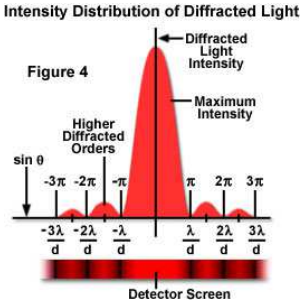
Wellenlänge des verwendeten Lichts



Brechungsindex Imersionsmedium



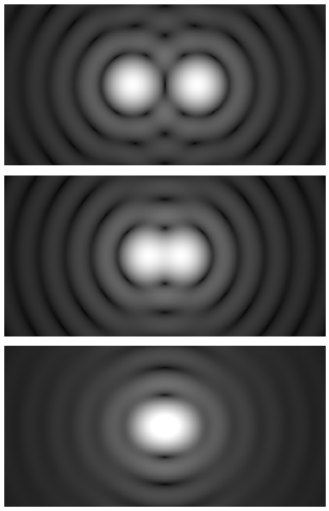
Numerische Apertur

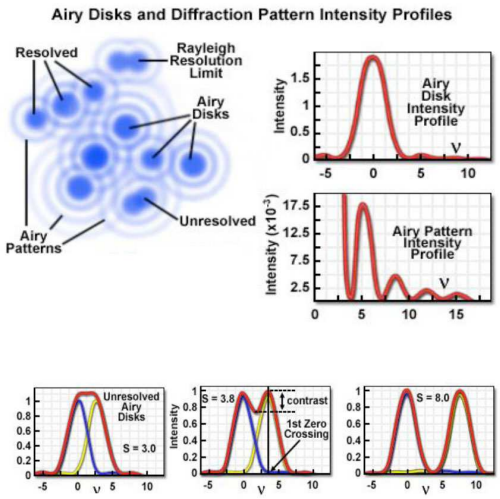


Carl Zeiss

Fraunhofer IZI

Angular resolution and Rayleigh criterion

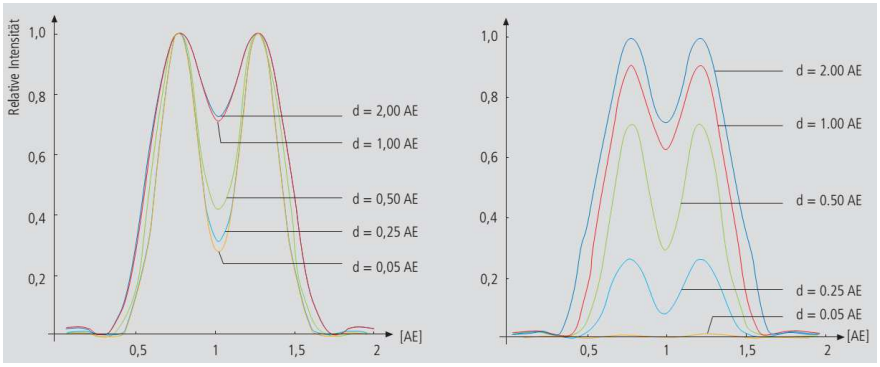


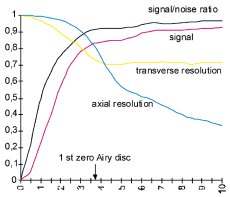


26.5% depression in brightness btw two maxima is giving the sensation of twoness

Fraunhofer IZI

Pinhole / Rayleigh unit and intensity





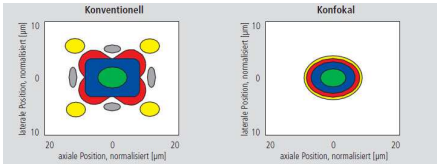
Small pinhole

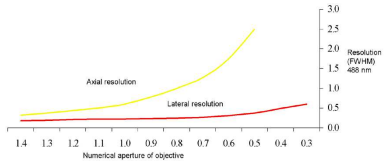
- thin optical section
- high z-resolution
- low signal strength

$$1RE = \frac{n \cdot \bar{\lambda}}{NA^2}$$

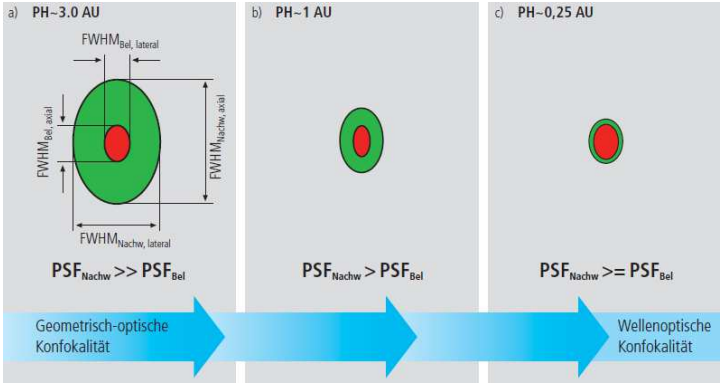
Fraunhofer IZI

Condition of confocality & resolving power





Carl Zeiss



Fraunhofer IZI

Resolution limits

Conventional Microscopy: $d = \frac{0{,}61 \cdot \lambda}{NA}$

d = optical resolution
NA = Numerical aperture
λ = Wavelenght of light

	Geometrisch optische Konfokalität	Wellenoptische Konfokalität
Laterales Auflösungs-vermögen	$\frac{0{,}51 \cdot \lambda_{em}}{NA}$	$\frac{0{,}37 \cdot \overline{\lambda}}{NA}$
Axiales Auflösungs-vermögen	$\frac{0{,}88 \cdot \lambda_{exc}}{n - \sqrt{n^2 - NA^2}}$	$\frac{0{,}64 \cdot \overline{\lambda}}{n - \sqrt{n^2 - NA^2}}$
Optische Schnittdicke	$\sqrt{\left(\frac{0{,}88 \cdot \lambda_{em}}{n - \sqrt{n^2 - NA^2}}\right)^2 + \left(\frac{\sqrt{2} \cdot n \cdot PH}{NA}\right)^2}$	$\frac{0{,}64 \cdot \overline{\lambda}}{n - \sqrt{n^2 - NA^2}}$

Geometry Of the Probing beam spot

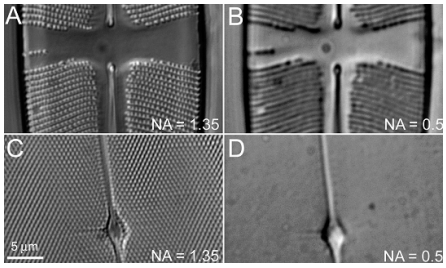
Pinhole size

Fraunhofer IZI

Numeric aperature and resolution

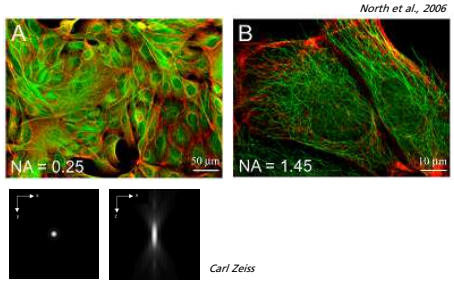
size varies: tissue / cellular / molecular - LSM limit

Animal cell: 10-30 µm	Nucleus: 3-10 µm	Microtubules: 25 nm
Antibody: 10-15 nm	GFP: 2-6 nm	Dye molecule: 1 nm



North et al., 2006

Light microscope best case scenario: 200 x 200 x 400 nm



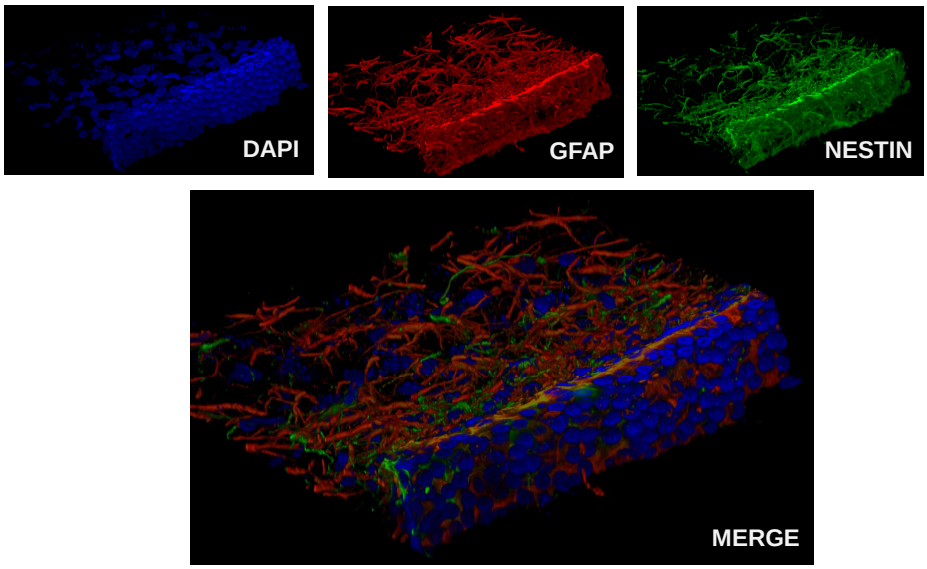
North et al., 2006

Carl Zeiss

Check the PSF of the Lens!

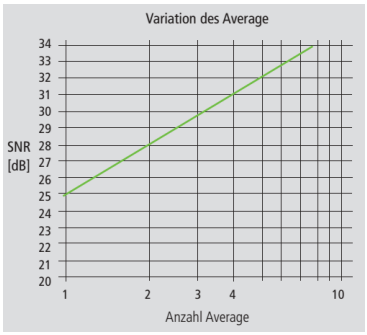
Fraunhofer IZI

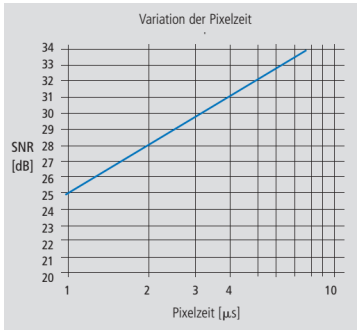
Example



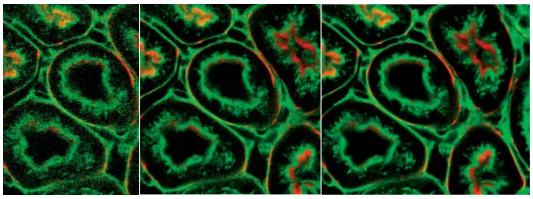
Fraunhofer IZI

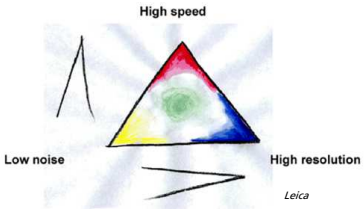
Quality intensification





Carl Zeiss

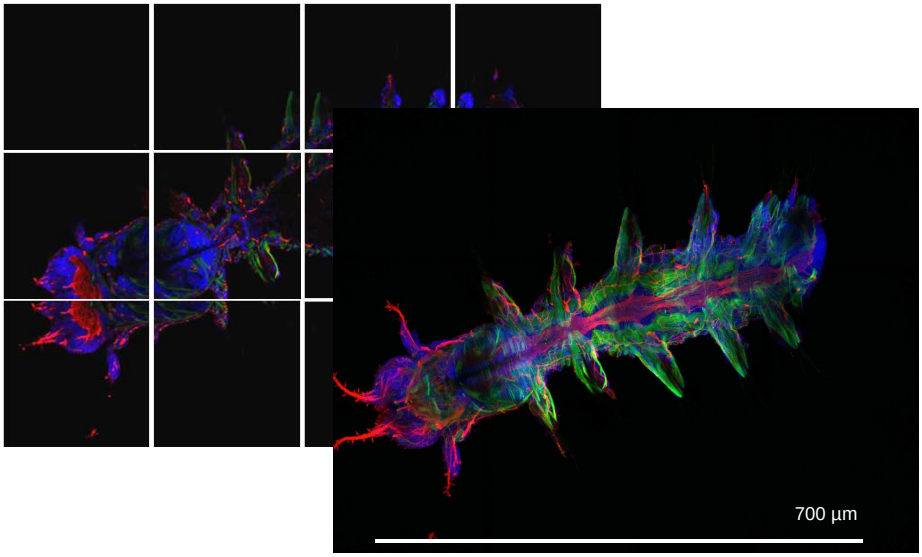




Leica

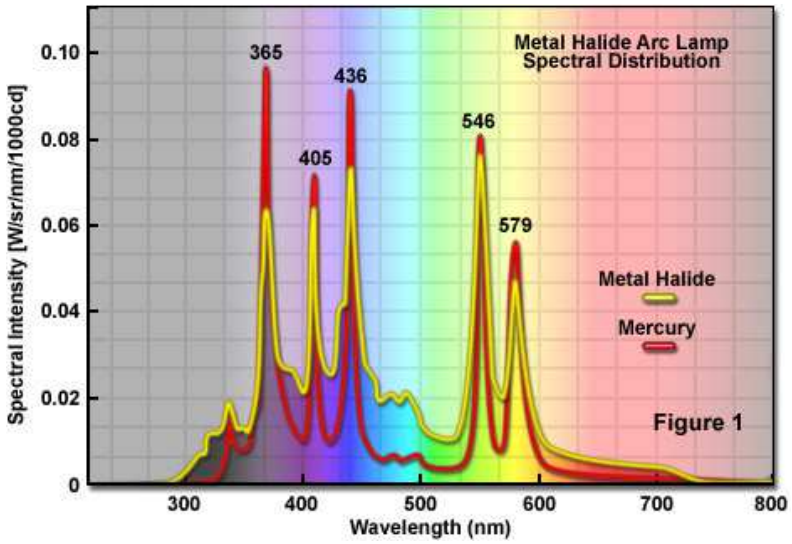
Fraunhofer IZI

Stiching



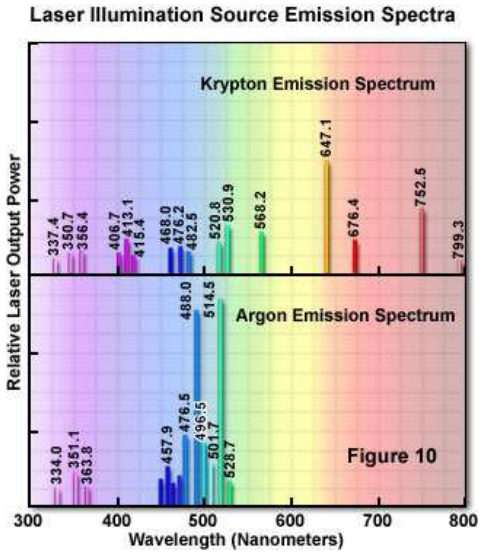
Fraunhofer IZI

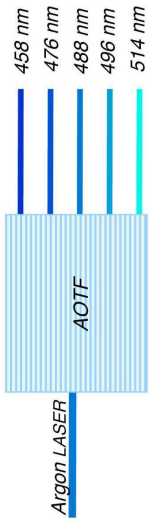
Lightsource Fluorescence



Fraunhofer IZI

Lightsource CLSM





Argon-Ionen Laser

λ=514nm / 488nm

Neodym-dotierter Yttrium-Aluminium-Granat-Laser

λ=532nm

Helium-Neon-Laser

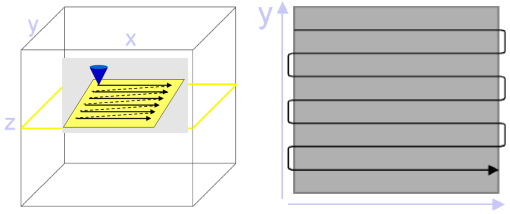
λ=543nm

Diodenlaser

λ=635nm

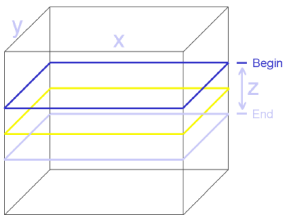
Fraunhofer IZI

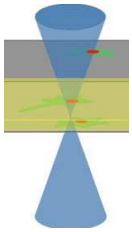
Scanning

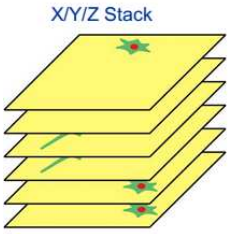


Beam scan:

- x/y: by galvanometer driven scan mirrors
- Z: sample is moved via galvanometer driven stage or electronic focus of the microscope

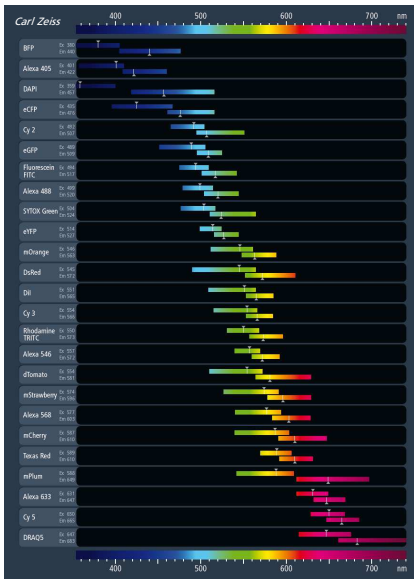


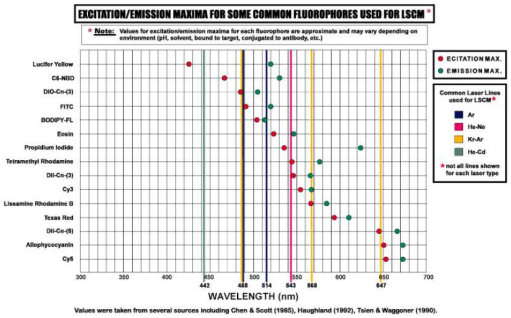




Fraunhofer IZI

Fluorescent Dyes and Proteins



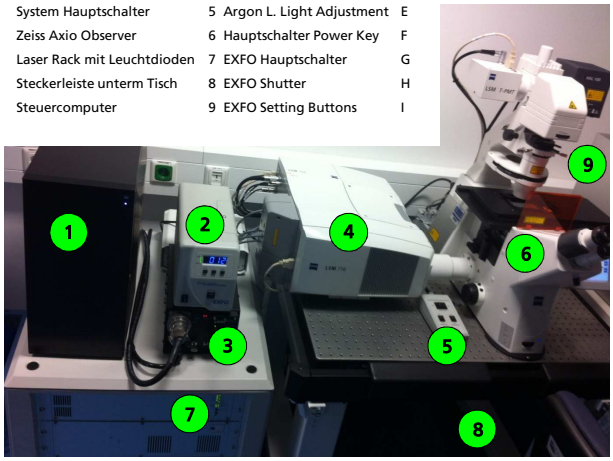


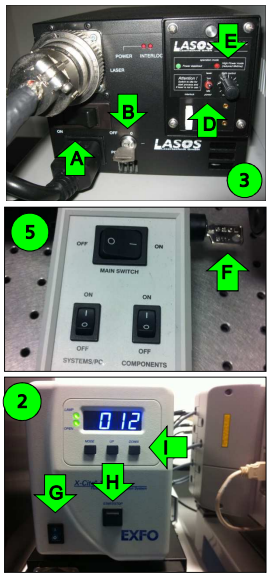
$$F = \sigma \cdot Qe \cdot I \quad [Photonen/sec]$$

Fraunhofer IZI

Fraunhofer IZI

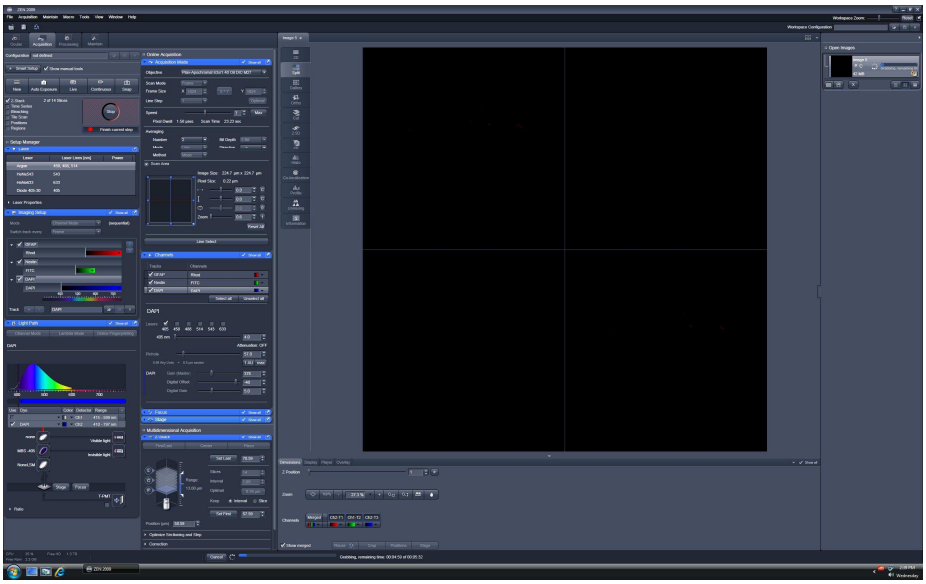
Server zur Datenverarbeitung	1	Argon L. Hauptschalter	A
EXFO Fluoreszenzlampe	2	Argon L. Power Key	B
Argon Laser	3	Argon L. Power LED	C
LSM Modul am rechten Port	4	Argon L. Idle/Run Switch	D
System Hauptschalter	5	Argon L. Light Adjustment	E
Zeiss Axio Observer	6	Hauptschalter Power Key	F
Laser Rack mit Leuchtdioden	7	EXFO Hauptschalter	G
Steckerleiste unterm Tisch	8	EXFO Shutter	H
Steuercomputer	9	EXFO Setting Buttons	I





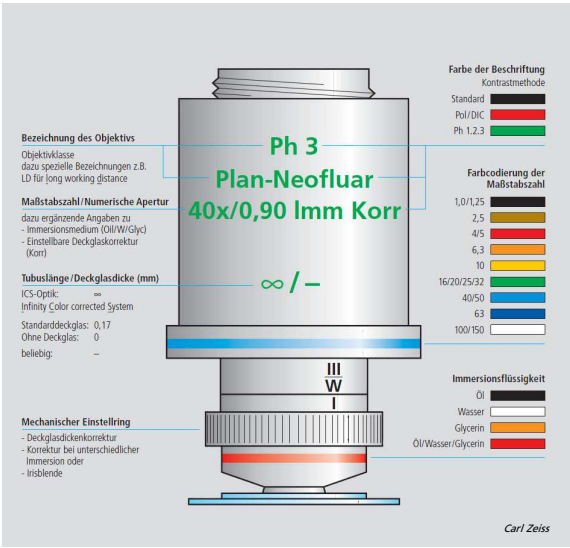
Fraunhofer IZI

Our system: ZEN Software



Fraunhofer IZI

Lenses



EC PLAN NEOFLUR

Korrektion 435nm – 670nm
3 Farb-Korrektion

10x 5.60mm 0.3NA T
20x T
40x 0.20mm 1.3NA ÖL DIC

PLAN APOCHROMAT

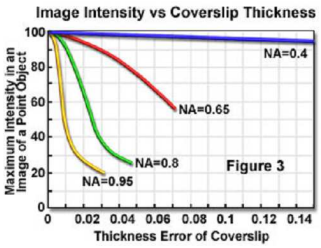
Korrektion 420nm – 670nm
4 Farb-Korrektion

63x 0.18mm 1.4NA ÖL DIC

405 458 488 514 543 633

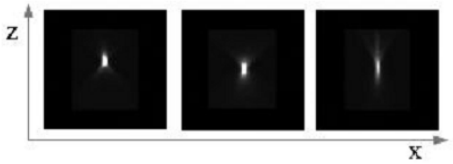
Fraunhofer IZI

The last 500 µm are important
Coverslip = 0.17 mm



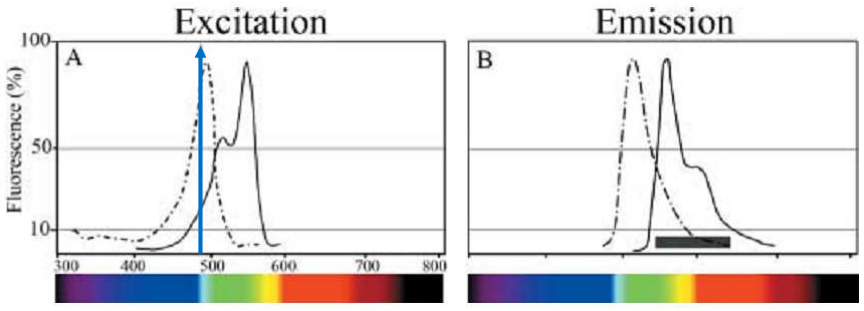
Performance Reduction with Coverslip Thickness Variation

NumericalAperture	0.01 mm Deviation	0.02 mm Deviation
0.30	none	none
0.45	none	none
0.70	2 percent	8 percent
0.85	19 percent	57 percent
0.95	55 percent	71 percent



coverslip thickness: <0.17 mm 0.17 mm >0.17 mm Diaz-Zamboni et al., 2008

Fraunhofer IZI



Bolte et al., 2006

FITC	490 nm max. Ex
Cy3	552 nm max. Ex
Laser	488nm

Cross-talk

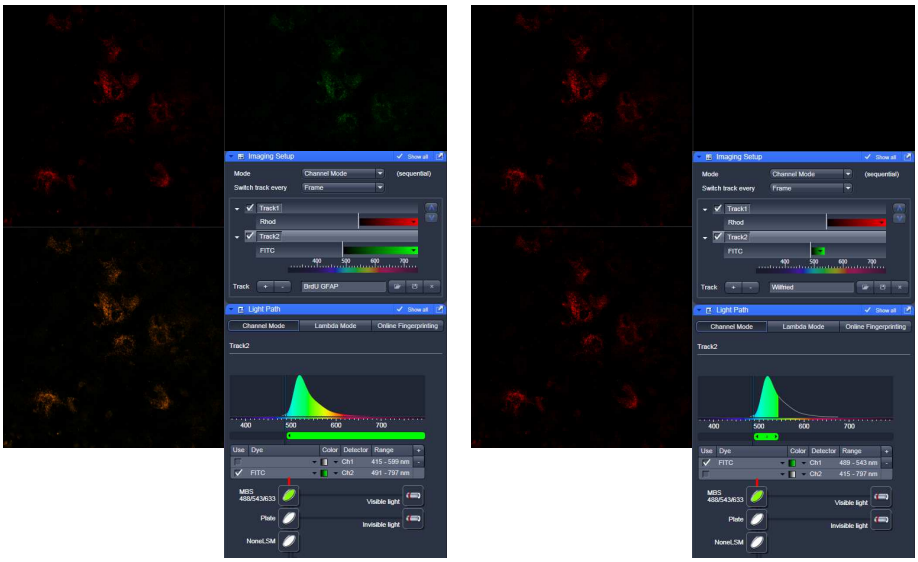
FITC	520 nm max. Emi
Cy3	570 nm max. Emi
PMT	Cy3 Detection

Bleed-through

Always use sequential acquisition!

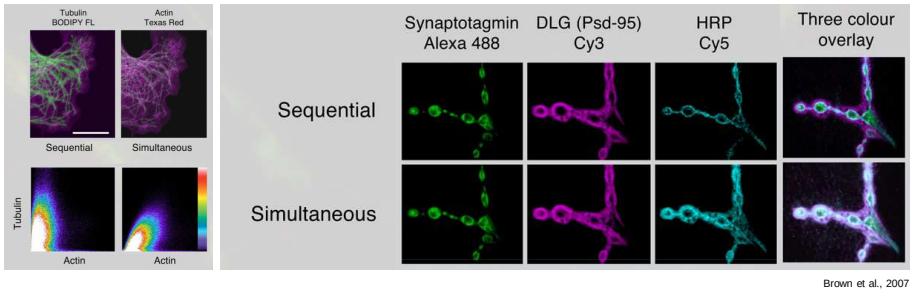
Fraunhofer IZI

Bleedthrough

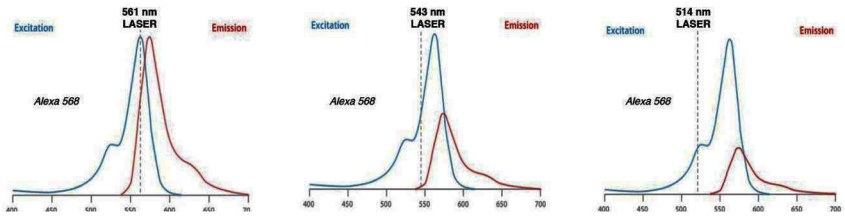


Fraunhofer IZI

Cross-Talk & Link between Excitation / Emmission

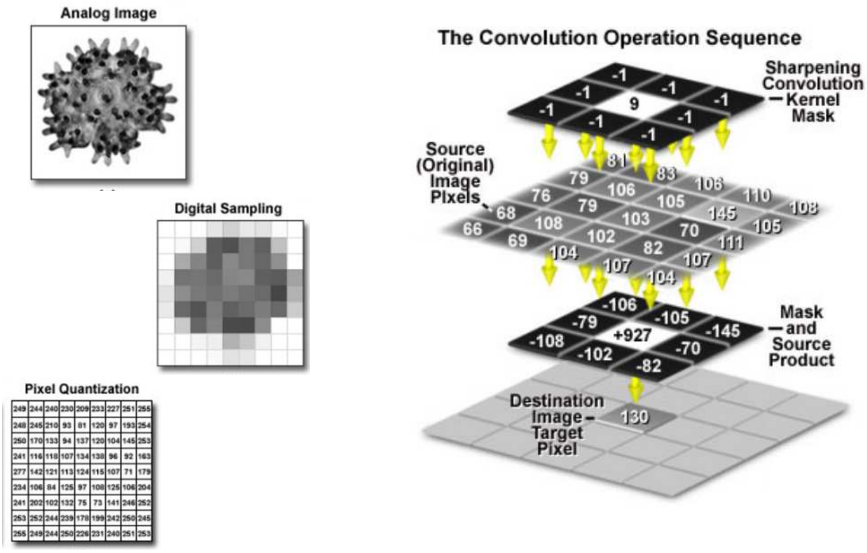


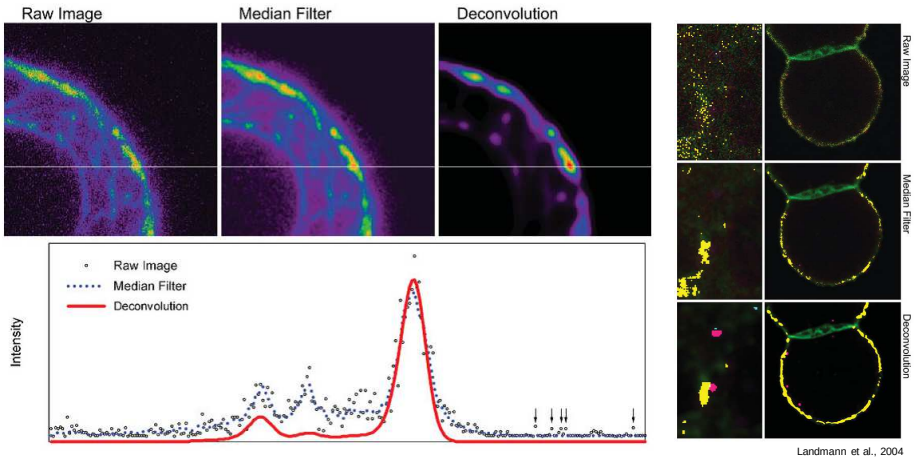
Brown et al., 2007



Remote excitation – Lower emission

Fraunhofer IZI





Landmann et al., 2004

Use Deconvolution

Calculation based on PSF of the lens

File format

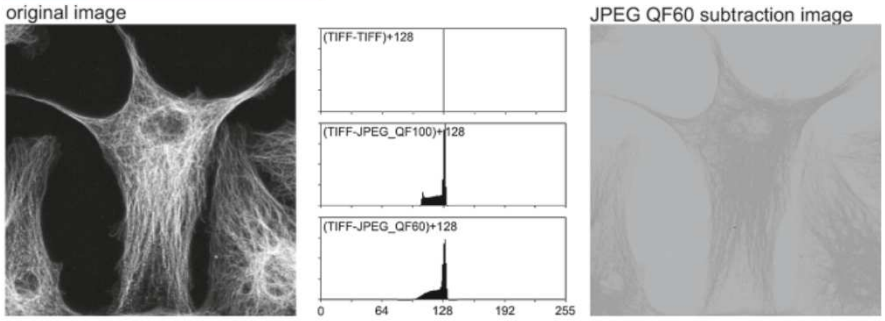
Digital File Format Memory Requirements

Pixel Dimensions	Grayscale (8-Bit)	Bitmap (24-Bit)	JPEG (24-Bit)	TIFF (24-Bit)
16 x 16	2k	2k	2k	2k
64 x 64	6k	13k	5k	13k
128 x 128	18k	49k	12k	49k
256 x 256	66k	193k	22k	193k
320 x 240	77k	226k	24k	226k
512 x 512	258k	769k	52k	770k
640 x 480	302k	901k	56k	902k
800 x 600	470k	1,407k	75k	1,408k
1024 x 768	770k	2,305k	104k	2,306k
1280 x 1024	1,282k	3,841k	147k	3,842k
1600 x 1200	1,877k	5,626k	161k	5,627k
2250 x 1800	3,960k	11,869k	276k	11,867k
3200 x 2560	8,002k	24,001k	458k	24,002k
3840 x 3072	11,522k	34,561k	611k	34,562k

JPEG = joint photographic experts group

TIFF = tagged image file format

Use proprietary formats of your vendor



Cromey et al., 2010

Fraunhofer IZI

Dynamic range and bleaching

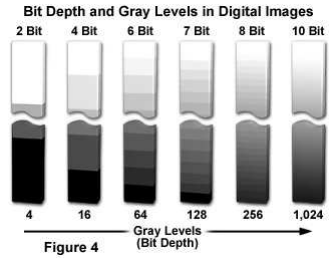
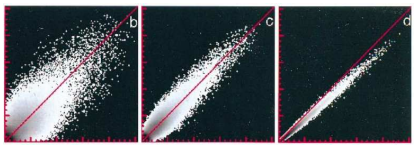
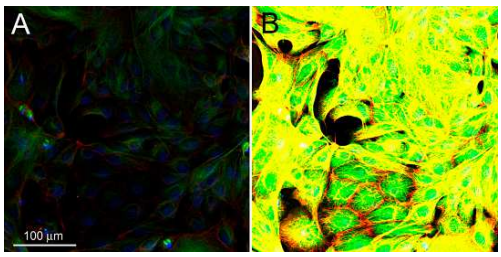


Figure 4



Demandolx et al., 1997

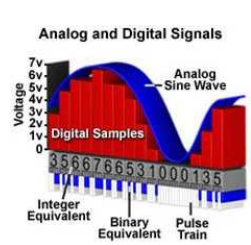
Dont bleach the area before imaging
-> bad S/N ratio

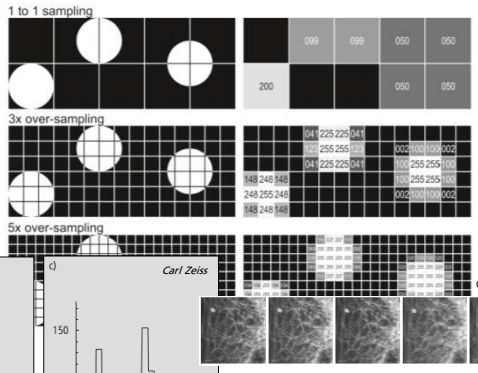


North et al., 2006

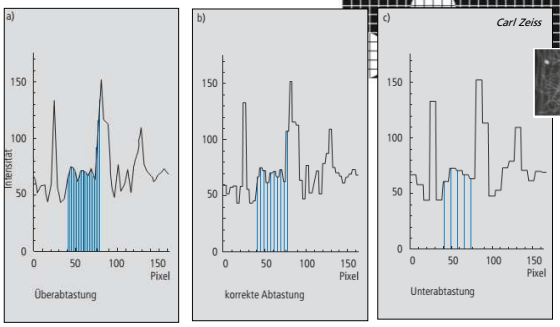
NA determines intensity
Avoid saturation! = data loss

Fraunhofer IZI





Cromey et al., 2010

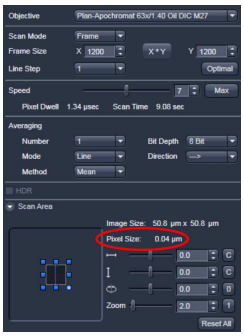


Nyquist Theorem

Optimale Schrittweite =

halbe Periodendauer des aufzulösenden Struktur-abstandes

Oversampling XY



Oversampling Z

Pinholes

1 Ary/ Channel

equal optical sections

Match all!

Table 1. The laws of Abbe and their effect on optical resolution and pixel sizes in wide-field and confocal microscopy.

	Wide-field		Confocal	
	Lateral resolution dx, y	Axial resolution dx, z	Lateral resolution dx, y	Axial resolution dx, z
Expression	$0.61\lambda_{em}/NA$	$2\lambda_{em}/NA^2$	$0.4\lambda_{em}/NA$	$1.4\lambda_{em}/NA^2$
Limit resolution of a 63×oil immersion objective with NA = 1.32 at λ_{em} = 500 nm	232 nm	574 nm	152 nm	402 nm
Minimal justified pixel size for this objective	101 nm	250 nm	66 nm	175 nm

Increasing the **brightness** shifts the entire intensity histogram to the right.
Increasing the **contrast** causes the intensity histogram to expand

Positive **gamma** (>1) increases the intensity of the mid-tones in the image more than the darker or lighter parts of the image.

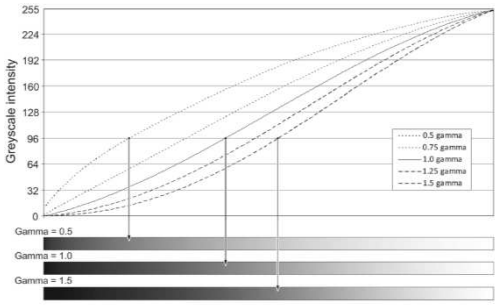
Because gamma adjustments are non-linear, they should be declared in the figure legend or the methods section of a paper.

Brightness ☺
Contrast ☺
Gamma ☹

Carefull with filters!

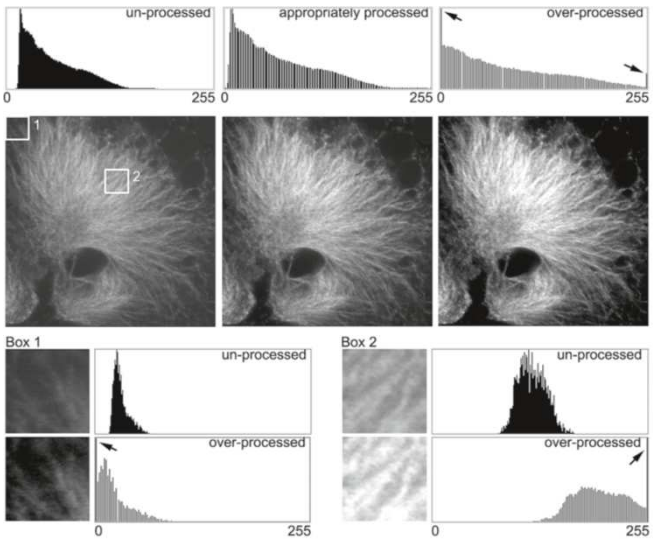
Change whole picture

No selective changes



Cromey et al., 2010

Fraunhofer IZI



Cromey et al., 2010

Fraunhofer IZI

Pixel binning: basic principle

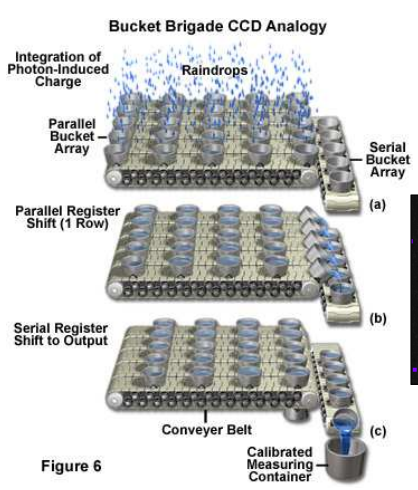
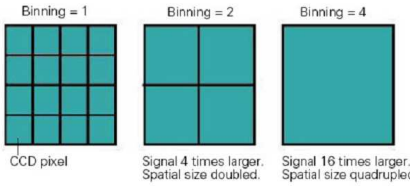
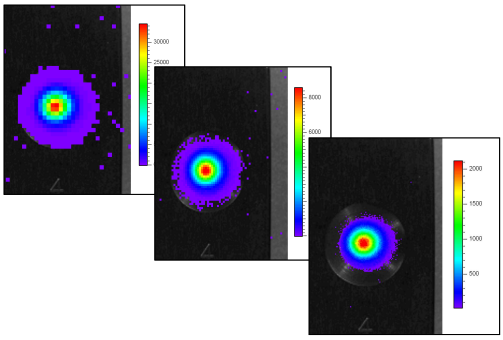


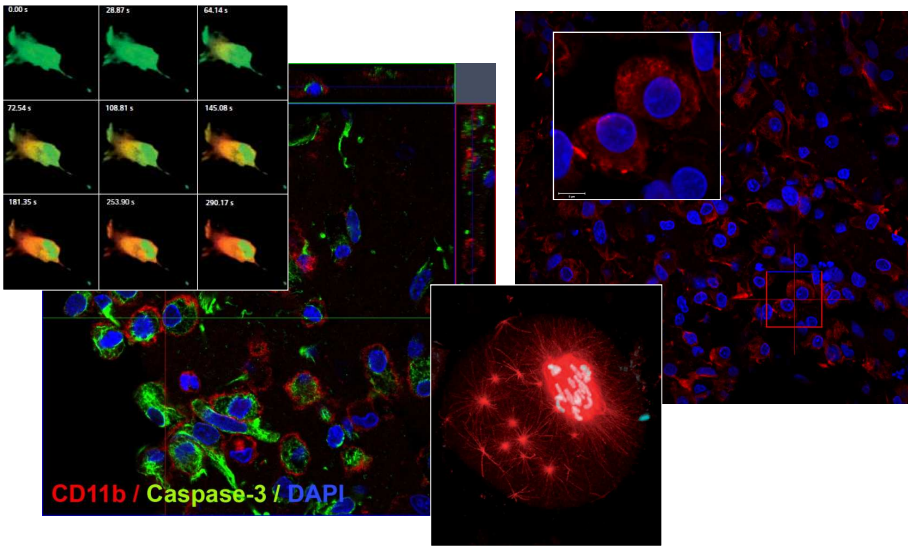
Figure 6





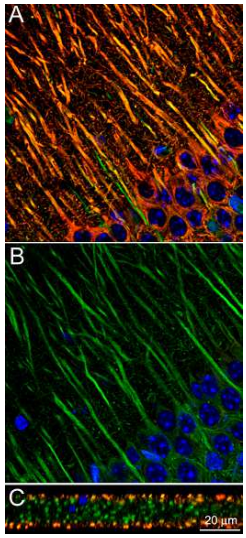
Fraunhofer IZI

Examples

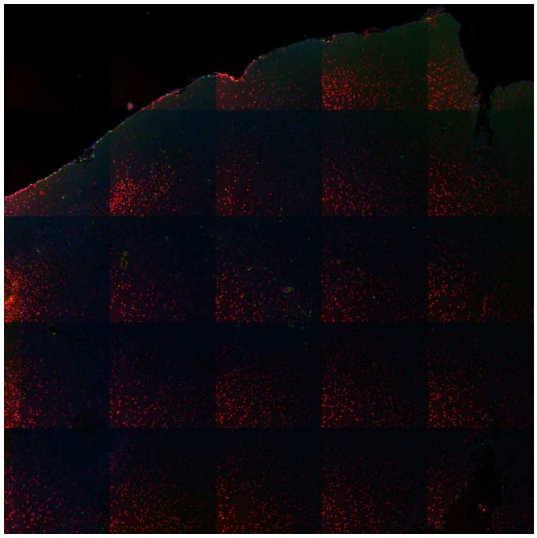


Fraunhofer IZI

Problems



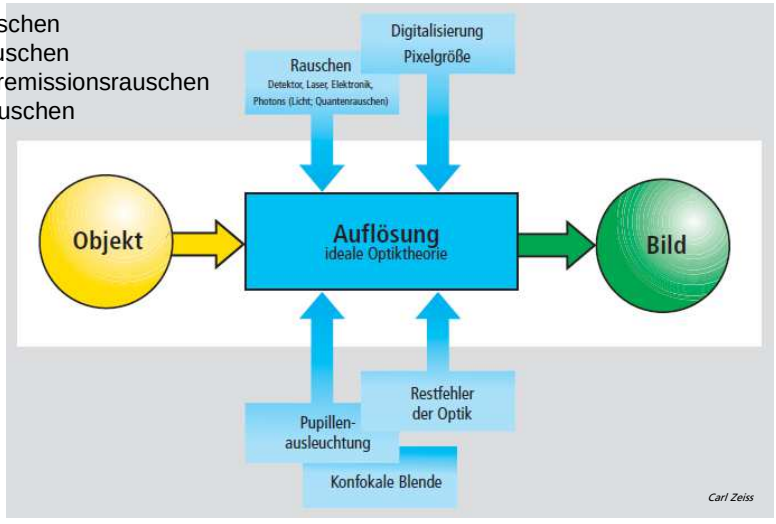
North et al., 2006



Fraunhofer IZI

Summary CLSM

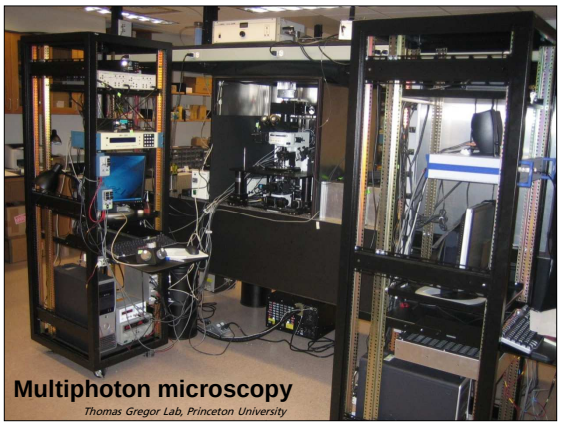
Laserrauschen
Schrotrauschen
Sekundäremissionsrauschen
Dunkelrauschen

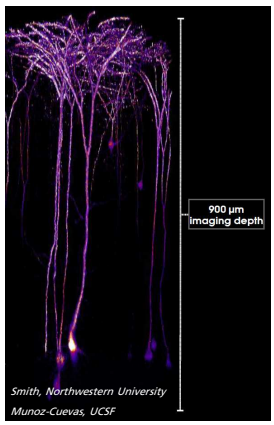


Fraunhofer IZI

Fraunhofer IZI

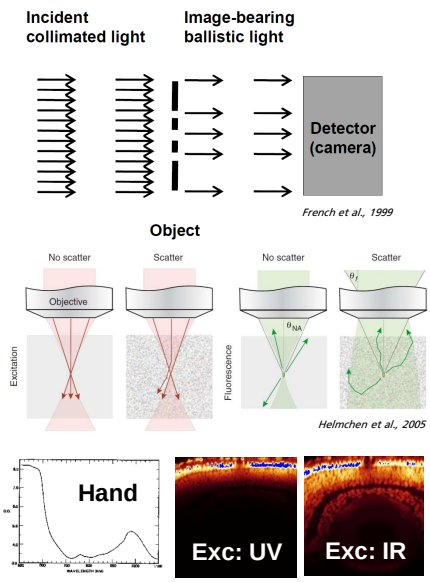
Experimental Imaging - Alexander Kranz, MD

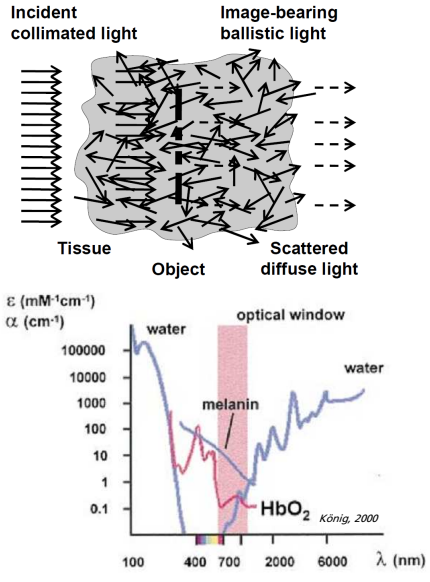




Fraunhofer IZI

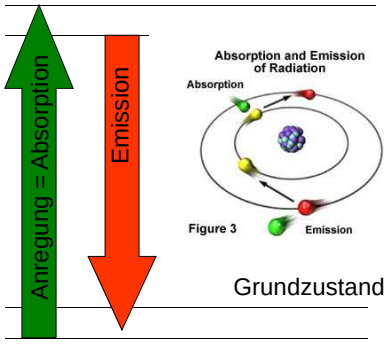
Light in tissue



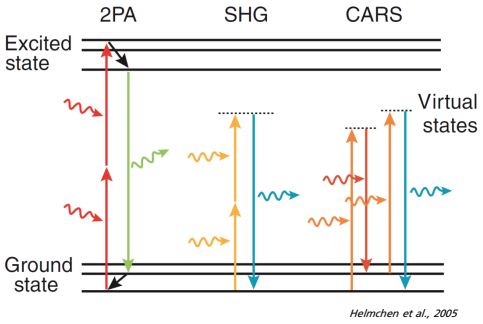


Fraunhofer IZI

Extension of Basic Fluorescence



Jablonski Energiediagramm



Simultaneous absorption virtual state:
Very short lifetime ~10^{-17} s

Requires high power: probability of excitation in focal plane resulting in a quadratic dependence on the light **intensity**

Fraunhofer IZI

Probability

In bright sunlight,
A molecule of rhodamine B

- absorbs one photon every second

- absorbs a photon pair every 10 million years

- no 3-photon absorption is expected throughout the entire age of the universe

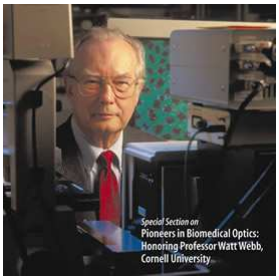
Fraunhofer IZI

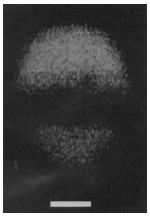
History of Multiphoton Microscopy

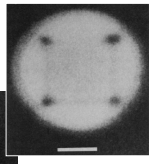
Göppert-Mayer
1931

Kaiser
1960

Denk & Webb
1990







Denk et al., 1990

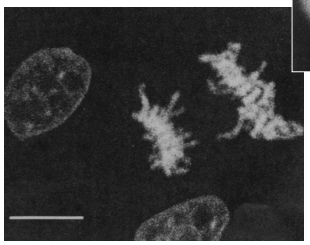
Über Elementarakte mit zwei Quantensprüngen
Von Maria Göppert-Mayer

(Göttinger Dissertation)

(Mit 5 Figuren)

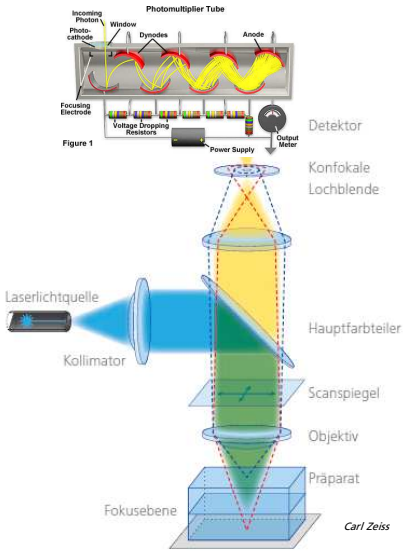
Einleitung

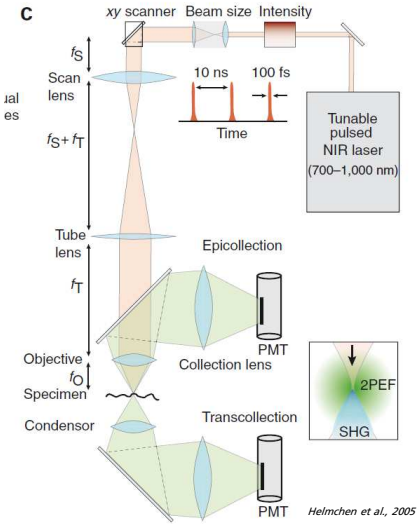
Der erste Teil dieser Arbeit beschäftigt sich mit dem Zusammenwirken zweier Lichtquanten in einem Elementarakt. Mit Hilfe der Diracschen Dispersionstheorie[1]) wird die Wahrscheinlichkeit eines dem Ramaneffekt analogen Prozesses, nämlich der Simultanemission zweier Lichtquanten, berechnet.



Fraunhofer IZI

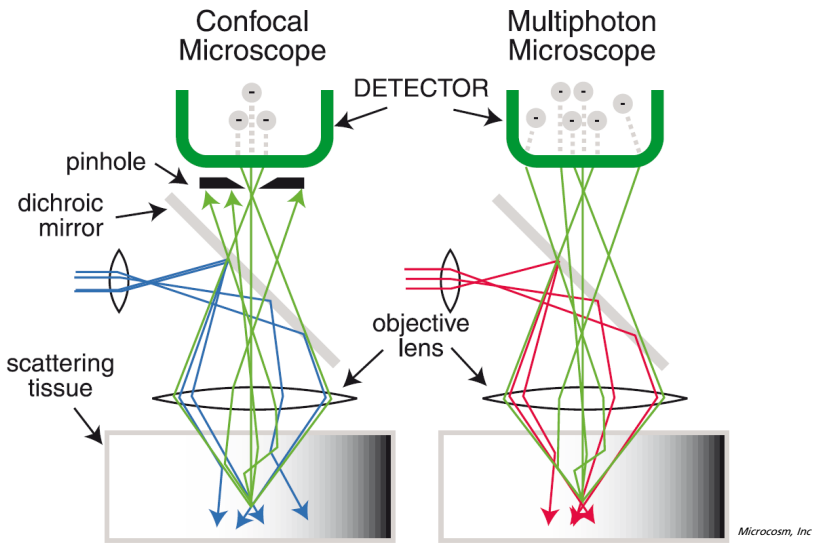
Comparison of structural composition





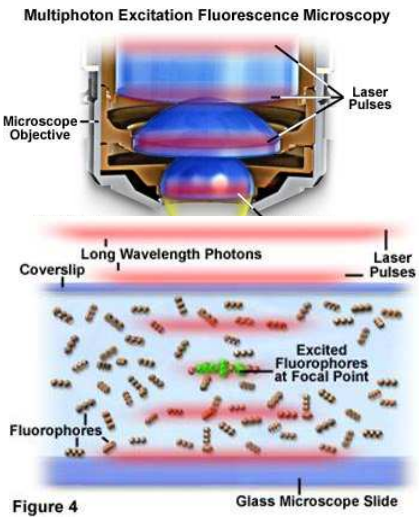
Fraunhofer IZI

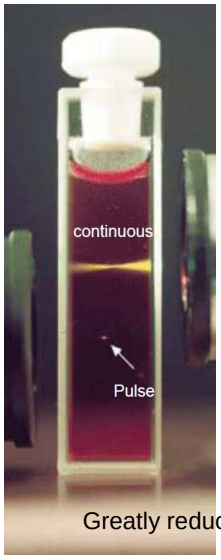
2PM: Intrinsic sectioning withou pinhole aperture

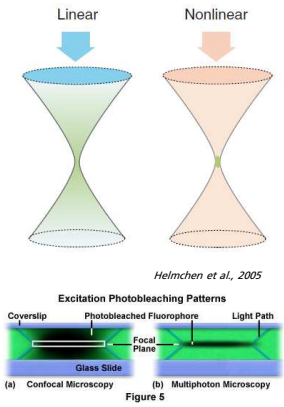


Fraunhofer IZI

Focal spot multiphoton microscopy







Greatly reduces out of plain bleaching

Oheim et al., 2006

Fraunhofer IZI

Laser Type (Spectral Region)	Wavelength(s) (Nanometers)
Argon Fluoride Excimer (UV)	193
Krypton Chloride Excimer (UV)	222
Helium Cadmium (UV, Visible)	325, 442
Argon (Visible)	488, 514
Copper Vapor (Visible)	510, 578
Nd:YAG Frequency Doubled (Visible)	532
Helium Neon (Visible, Near IR)	543, 594, 612, 633, 1150, 3390
Gold Vapor (Visible)	628
Rhodamine 6G Dye (Visible, Tunable)	570-650
Ruby (Visible)	694
Diode Semiconductor (Visible, Near IR)	630-1600
Ti:Sapphire (Visible - Near IR)	680-1130
Nd:YAG (Near IR)	1064
Erbium (Near IR)	1540
Hydrogen Fluoride (Near IR)	2600-3000
Carbon Dioxide (Far IR)	9600, 10600

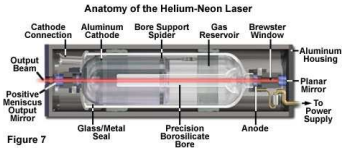


Figure 7

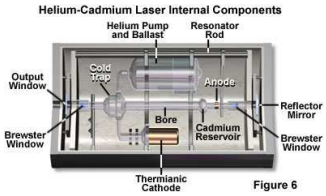
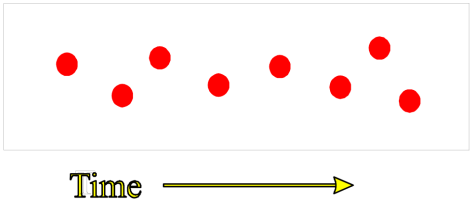
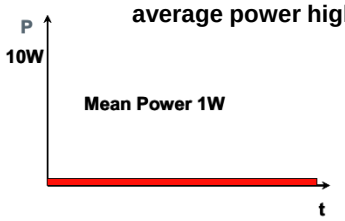


Figure 6

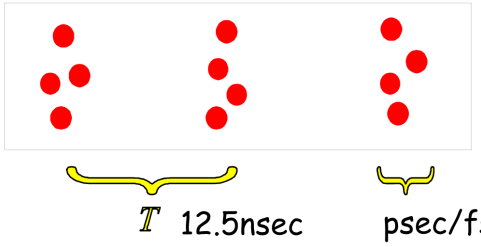
Fraunhofer IZI

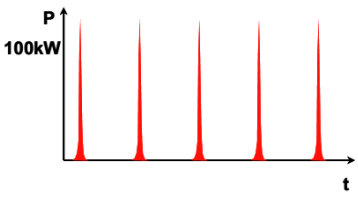
Continuous wave laser





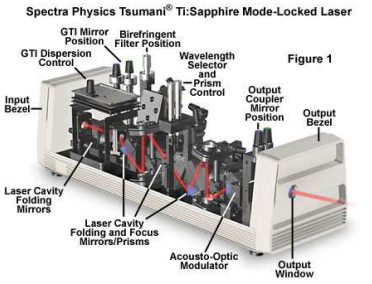
Pulsed laser

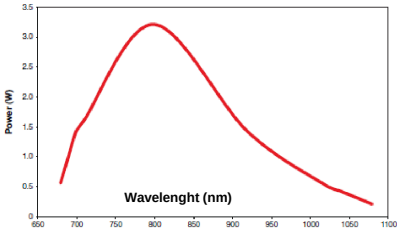




repetition rate in range of fluorescence life time

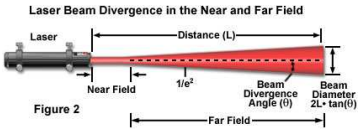
Fraunhofer IZI

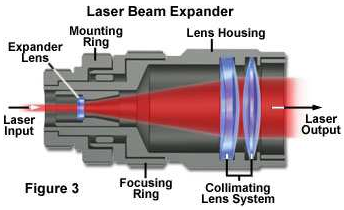




A laser for two photon microscopy:

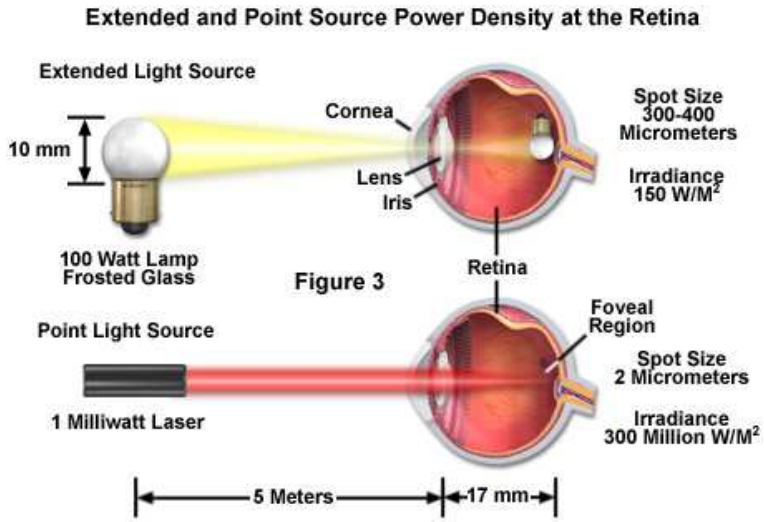
tuning range 690 to over 1050 nanometers
pulse widths ~ 100 femtoseconds
Pulse frequency 80 MHz
average power 2W





Most common MP laser is mode locked titanium sapphire (Ti:S), tunable 700-1000nm

Fraunhofer IZI



Fraunhofer IZI

Suitable dyes for Multiphoton

Fluorochrome	Absorption	Emission
Alexa Fluor 350	720-800	440
Alexa Fluor 488	720-800	515
Alexa Fluor 546	720-840	569
Alexa Fluor 568	720-840	596
Alexa Fluor 594	720-850	610
Alexa Fluor 633	720-900	647
AMCA	780-800	444
bis-MSB	680-750	420
Bodipy	900-950	512
Calcium Crimson	900	615
Calcium green	780-850	531
Cascade Blue	750-800	420
Coumarin 307	780-800	530
CY2	780-800	506
CY3	780	565, 615
CY5	780-820	670
Dansyl Hydrazine	700-750	440
DAPI, Hoechst	700-820	455, 478

Fluorochrome	Absorption	Emission
DiA	800-860	580
DID	780-820	670
DiO	780-830	510
eCFP	800-900	476
eGFP	820-950	509
eYFP	860-950	532
Fluorescein	780 - 820	519
Indo-1 free	690-720	490
Indo-1 Ca2+	690-720	400
Lucifer Yellow	860-890	533
Mito Tracker red	750-840	600
Nile Red	810	640
Oregon Green	780-860	526
Propidium Iodide (PI)	820-850	617
Rhodamin B	800-840	600
Rhodamine 123	780-860	550
Sytox Green	740-760 or 880-940	524
TRITC	800-840	572

Zeiss

Conventional dyes have a broad excitation spectrum when excited with pulsed NIR-L

Fraunhofer IZI

Excitation probability

$$n_a \approx \delta \left(\frac{P_{avg}^{\ 2}}{\tau f} \right) \left(\pi \frac{NA^{\ 2}}{hc\lambda} \right)^2$$

cross section = Wirkungsquerschnitt =
probability of a molecule to absorb
2 photons simultanously

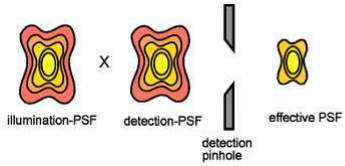
MP excitation favoured when:

- large cross section
- high laser peak power
- high NA objective lens
- low wavelenght
- short puls width and repetition time

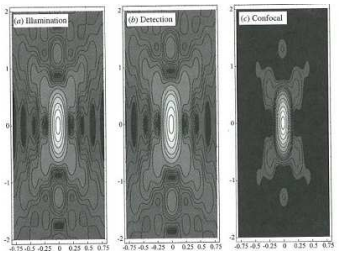
n_a: probability of excitation
δ: excitation cross section
P_{avg}: average power incident light (peak power)
τ: pulsewidth
f: repetition rate
***NA*: Numerical aperture**
***h*: Planck's constant**
***c*: Speed of light**
λ: Wavelength

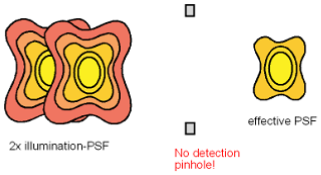
Fraunhofer IZI

Comparing confocal and multiphoton

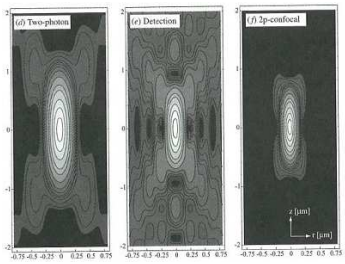


confocal microscopy



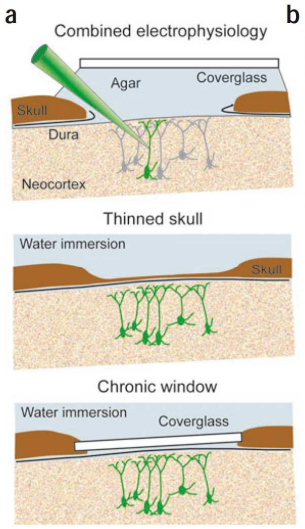


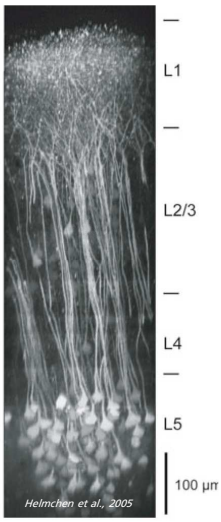
2P microscopy



Fraunhofer IZI

Maximum imaging depth





Available laser power

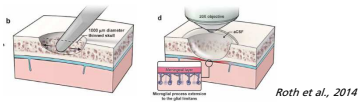
Scattering mean-free-path

Tissue properties

- Density properties
- Microvasculature organization
- Cell-body arrangement
- Collagen / myelin content

-Specimen age

-Collection efficiency



Layer V pyramidal cells in the visual cortex in a 2 month old Thyl-eGFP mouse

Fraunhofer IZI

Summary Multi Photon Microscopy

True „Molecular Imaging“ with single-molecule sensitivity

Wealth of indicators capable of specific targeting (dyes, SHG, quantum dots)

Sub-micron resolution

Optical sectioning in thick, turbid media and deep penetration

Wide variety of biological and clinical applications

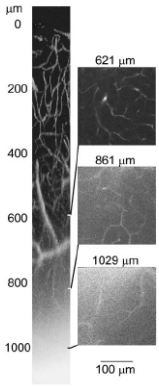
Near IR-light scatters less and is less toxic than blue light

Limited photobleaching and photodamage in the image plane

More efficient light collection (better looking images)

Unaffected by chromatic aberrations

High purchasing and operating cost of pulsed femtosecond IR lasers



Svoboda et al., 2006

Fraunhofer IZI

Source

http://www.microscopyu.com/

http://zeiss-campus.magnet.fsu.edu/

http://www.olympusmicro.com/

http://www.leica-microsystems.com/science-lab/

Fraunhofer IZI