LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

BIOMEDICAL CENTER
CORE FACILITY BIOIMAGING

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Core Facility Bioimaging at the Biomedical Center

Fundamentals of Advanced Light Microscopy

Walter Brendel Zentrum München

BIN LMU BioImaging Network Munich

Topics today

- Basic Microscope Optics for Fluorescence Microscopy (refraction, compound microscope, aberrations, diffraction, resolution)

Break

- Principles of Fluorescence and Fluorescence Microscopy
- Image processing and image presentation
- Basics of Digital Imaging

Topics next week

- Confocal Microscopy (point scanners)
- How to record a good image
- Deconvolution

Break

- Multi-photon: fluorescence, SHG and THG
- Superresolution microscopy: STED and other approaches

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Basic Microscope Optics

for fluorescence microscopy

What are the numbers on objectives?

Example:
160/0.17
40/0.70

The viewing angle (Sehwinkel)

- Essential for the size of the image on the retina and thus the resolution of an object is the viewing angle

Increasing the resolution

- Decreasing the distance between the object and the eye: the viewing angle is enlarged.
  Problem: between 20-10 cm a limit is reached: the image becomes blurry (+ headache)

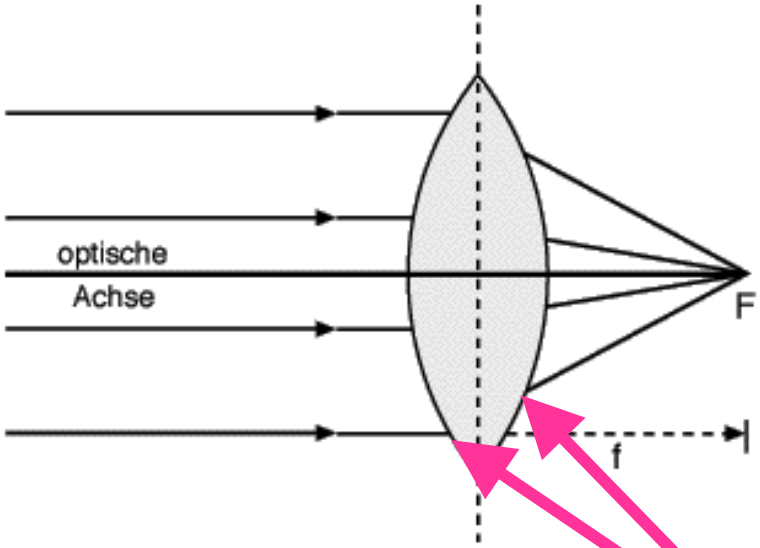
Maximal resolution: 0.15 – 0.3 mm.

How can we magnify?

- How does a glass lens work?

How can we magnify with a glass lens?

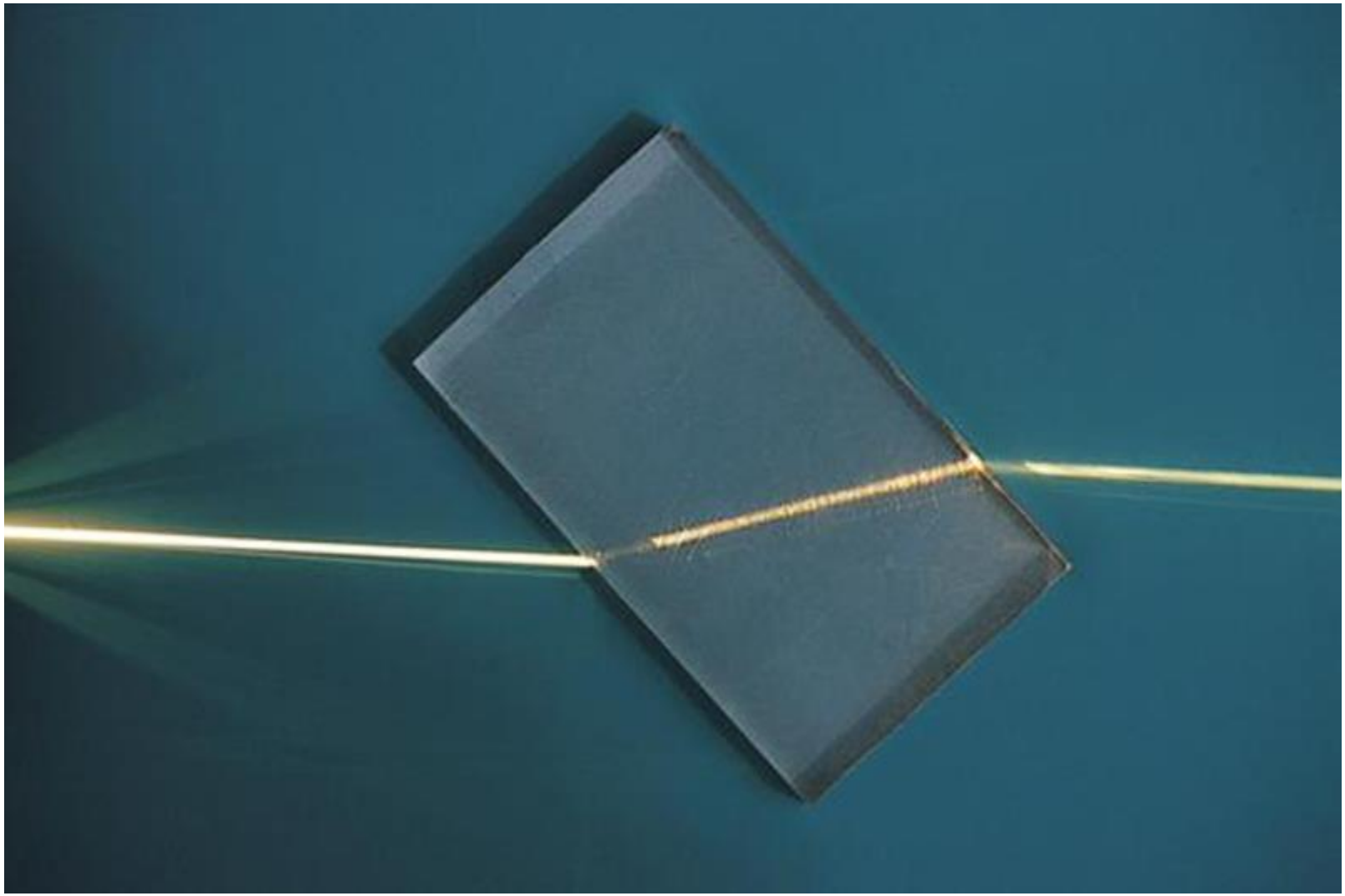
- What a collecting lens does:



What happens here?

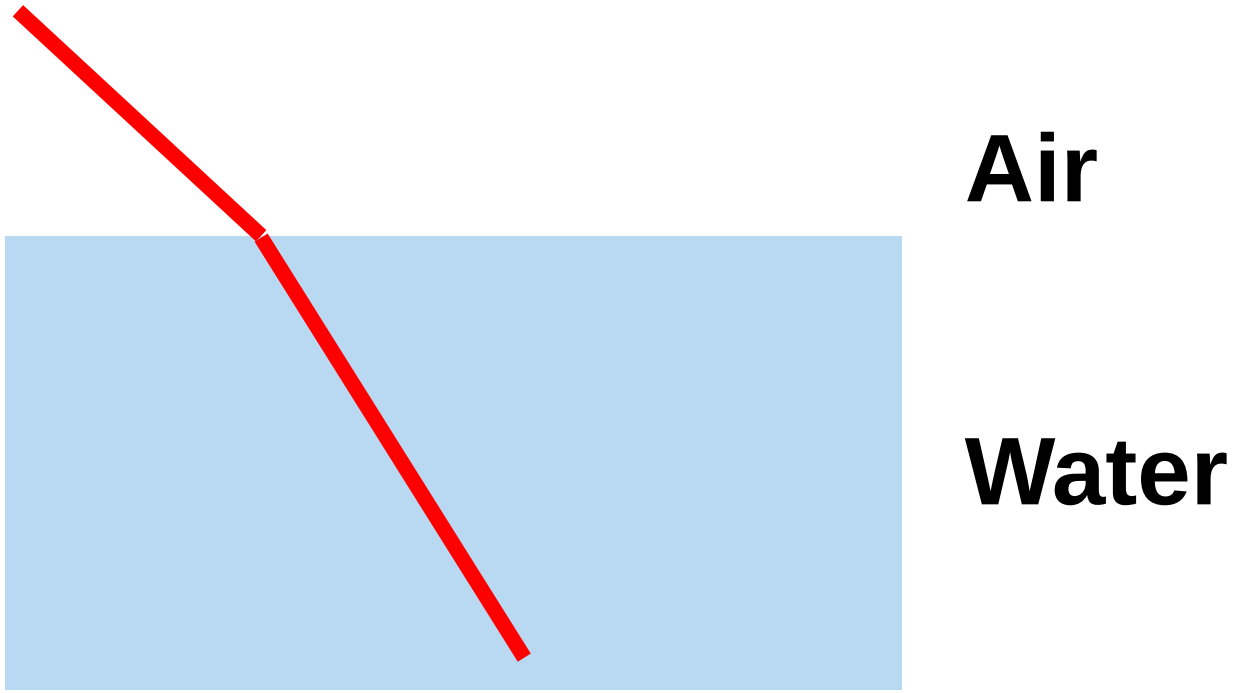
http://m.i4o.de/augen-abb07.gif

Refraction (Licht-Brechung)

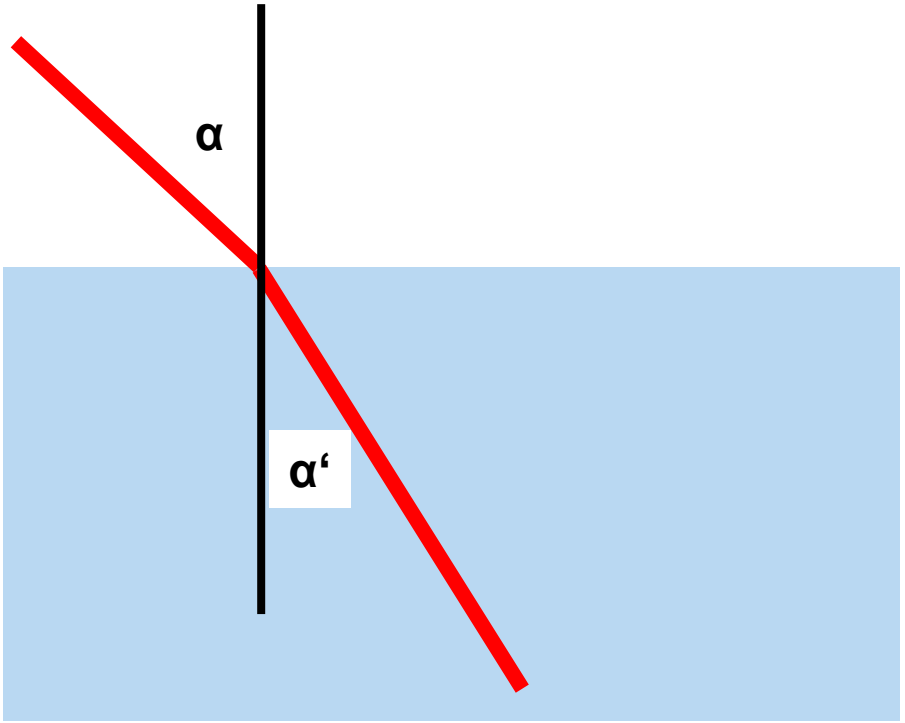


Source: https://en.wikipedia.org/wiki/File:Refraction_photo.png Public Domain.

Refraction scheme

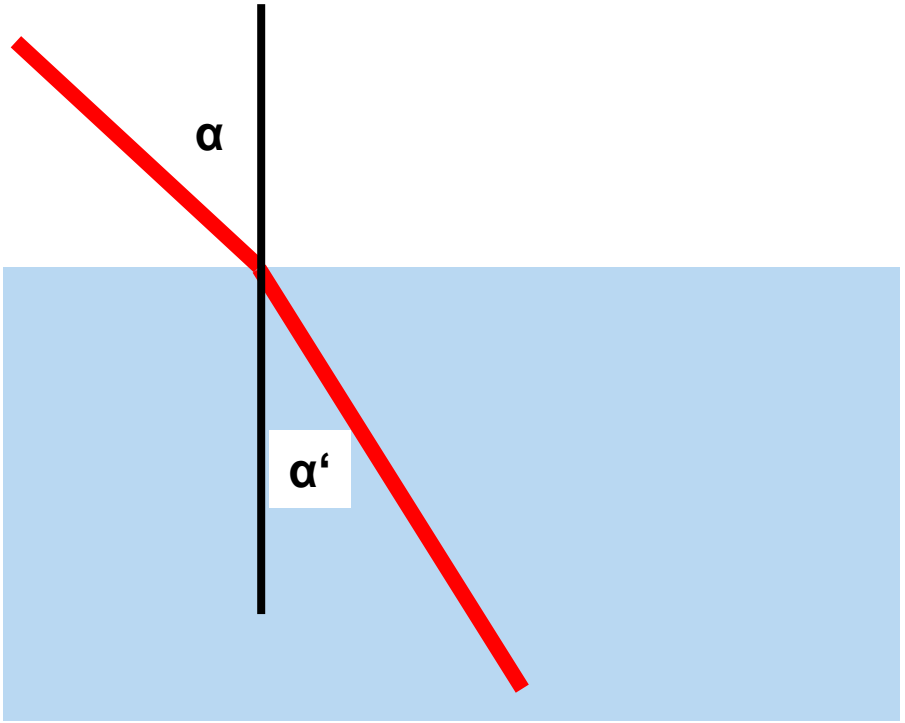


Refraction scheme



$$\frac{\sin\alpha}{\sin\alpha'} = n = \text{refractive index} = \text{Ri}$$

Refraction scheme



Air (Vacuum)

Water

$$\frac{\sin\alpha}{\sin\alpha'} = n = \text{refractive index} = \text{Ri}$$

Example:

$$\frac{\sin\alpha}{\sin\alpha'} = \frac{\sin 50}{\sin 35} = \frac{0.766}{0.573} = 1.33$$

Note:

$$\frac{\sin \alpha}{\sin \alpha'} = n$$

is valid only for refraction from vacuum (or air) in an optically denser material.

For refraction from any material in any other, e.g. from water into glass, the following applies:

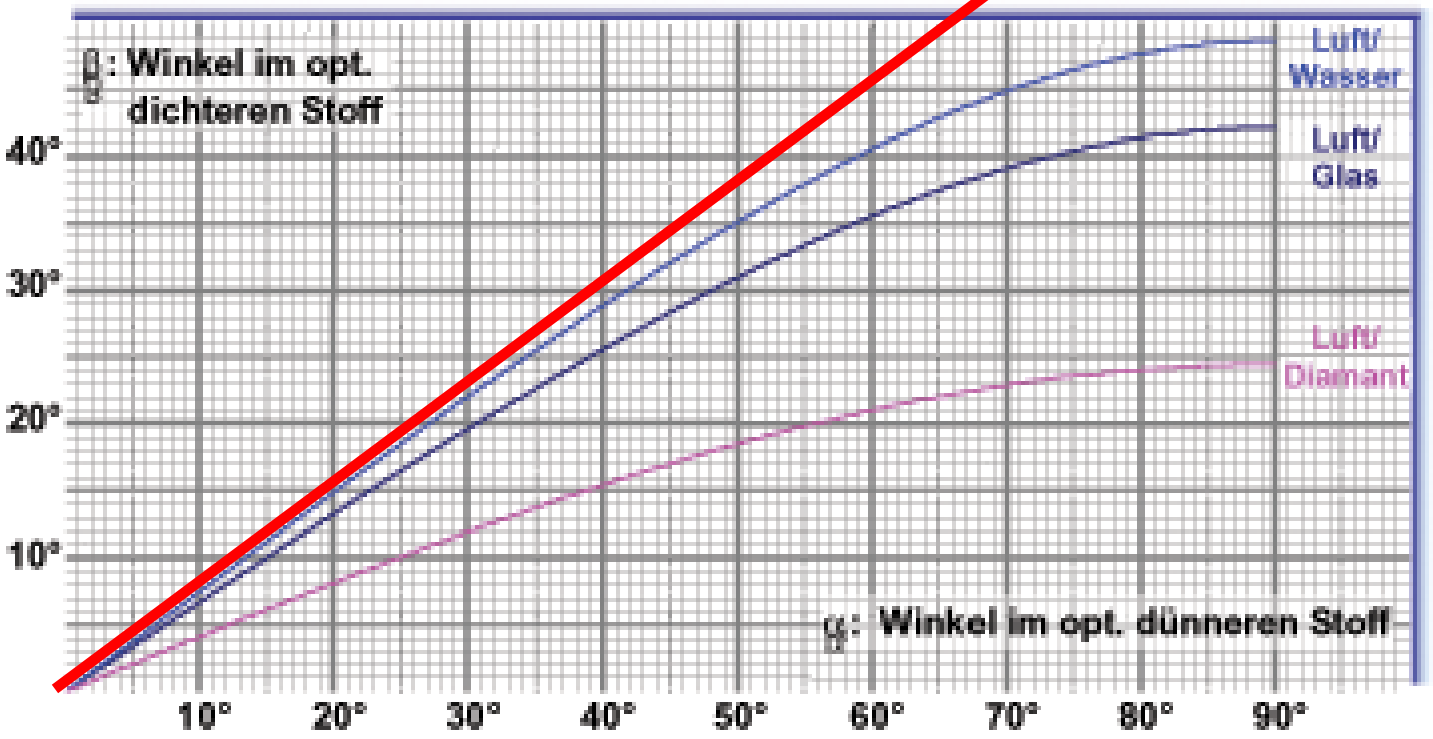
$$\frac{\sin \alpha_1}{\sin \alpha_2} = \frac{n_2}{n_1}$$

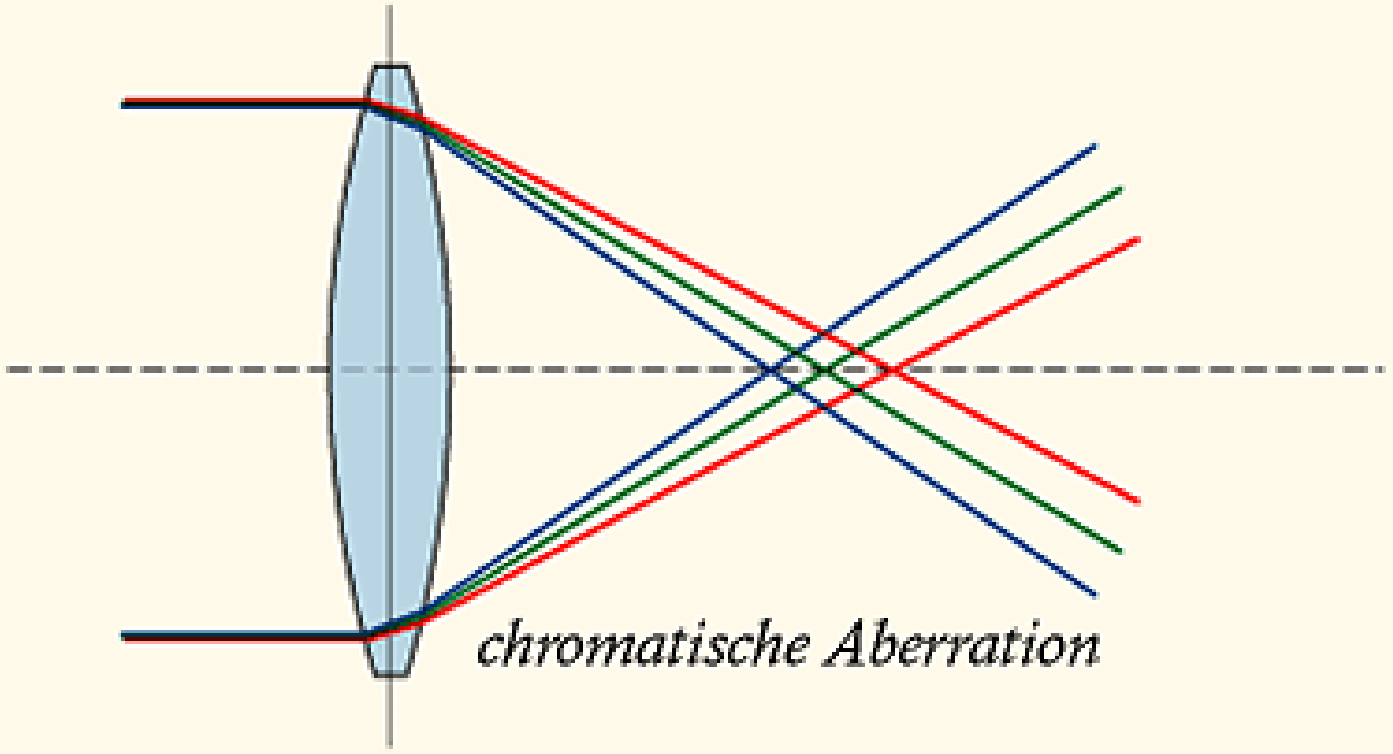
or:

$$\sin \alpha_1 \bullet n_1 = \sin \alpha_2 \bullet n_2$$

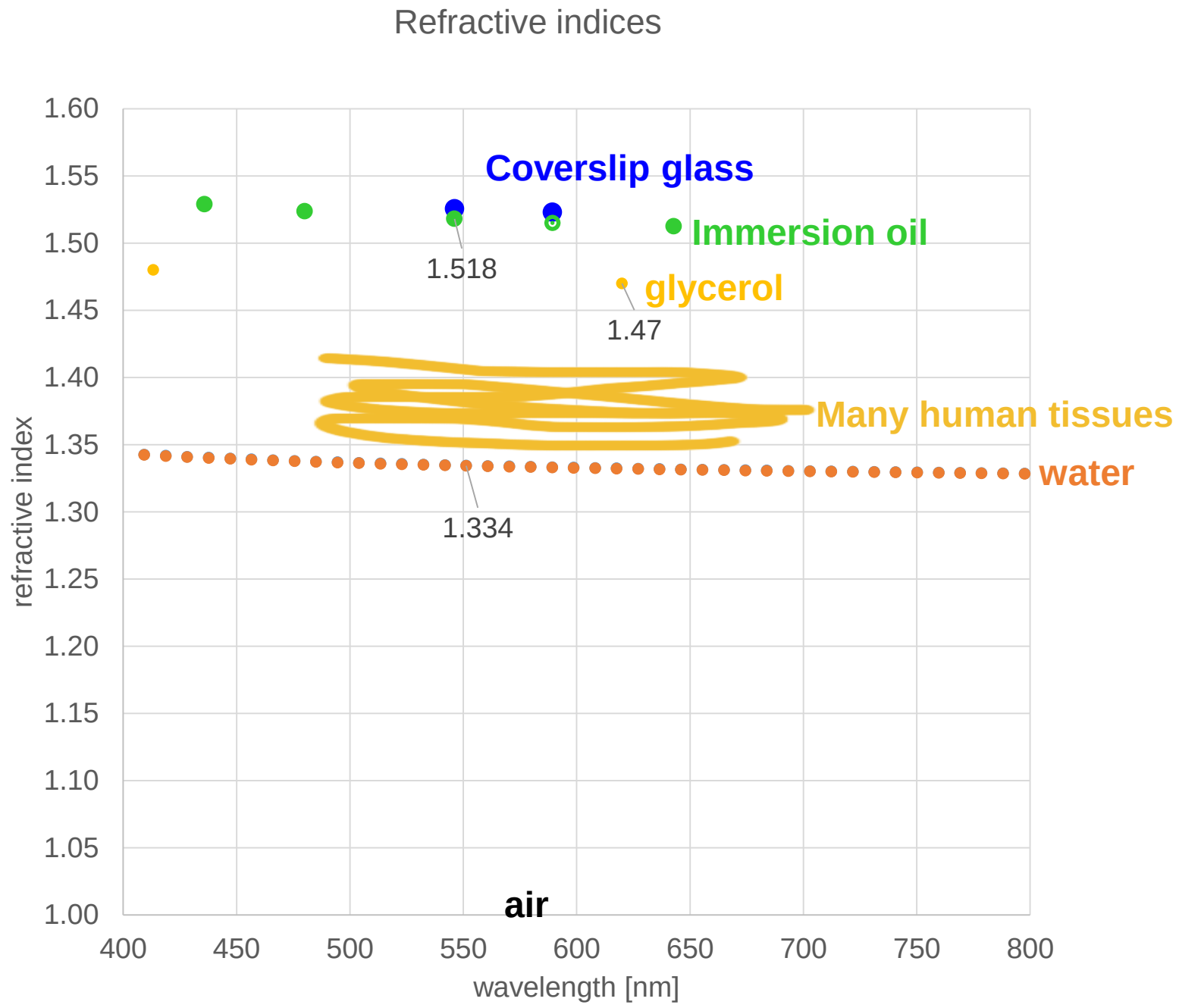
Refraction

Das Brechungsverhalten ist abhängig von der Stoffkombination an der Grenzfläche:

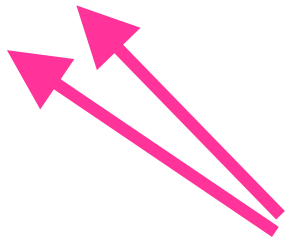




http://de.wikipedia.org/wiki/Abbildungsfehler



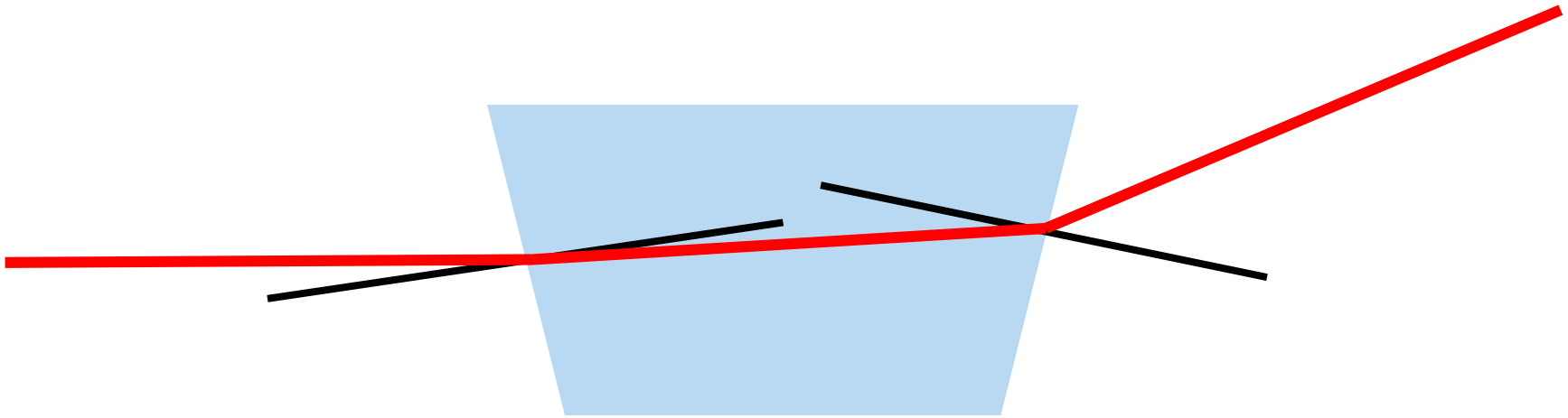
Data from http://refractiveindex.info



What happens here?

http://m.i4o.de/augen-abb07.gif

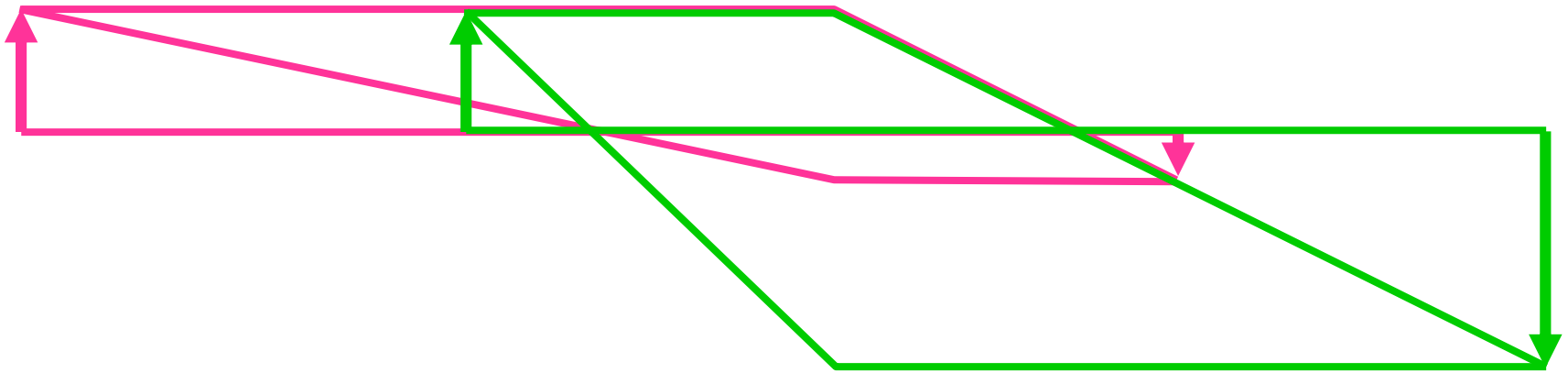
- Collecting lens: refraction in detail: $\alpha_{Air} > \alpha'_{glass}$



How does a collecting lens create an image?

Case 1: object is more than 2 focal lengths away: a smaller, inverted **real** image (Photo objective)

Case 2: Object less than 2x f but more than 1x f away: magnifyed, inverted **real** image (Projector-Objective). Inversion of case 1.

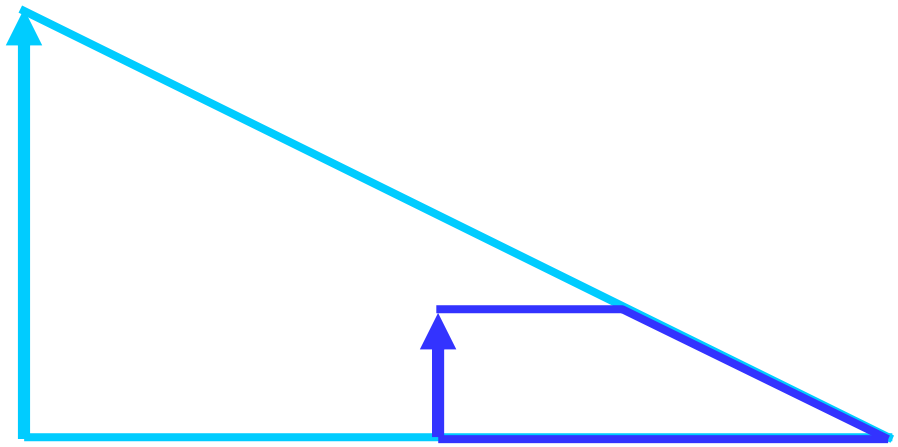


Das Lichtmikroskop, D. Gerlach, Thieme Verlag Stuttgart, Abb. 5

Case 1: object is more than 2 focal lengths away: a smaller, inverted **real** image (Photo objective)

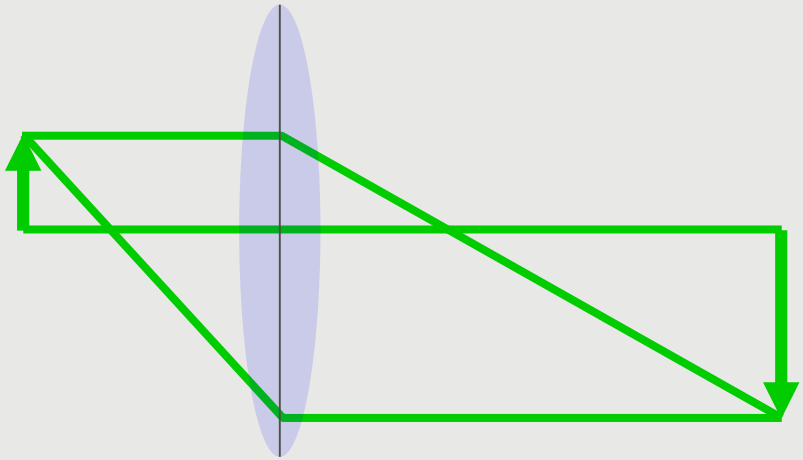
Case 2: Object less than 2x f but more than 1x f away: magnifyed, inverted **real** image (Projector-Objective). Inversion of case 1.

Case 3 (magnifying lens): Object within focal length: Magnified, upright **virtual** image. Lenses with high magnification (small and round) where called „simple microscope“.



Das Lichtmikroskop, D. Gerlach, Thieme Verlag Stuttgart, Abb. 5

The compound microscope

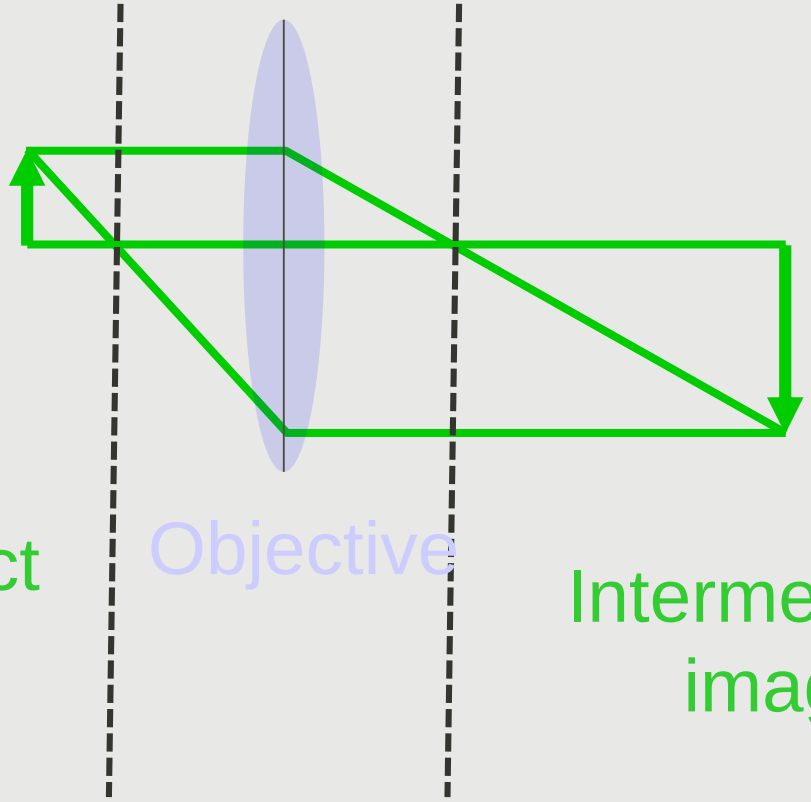


Object

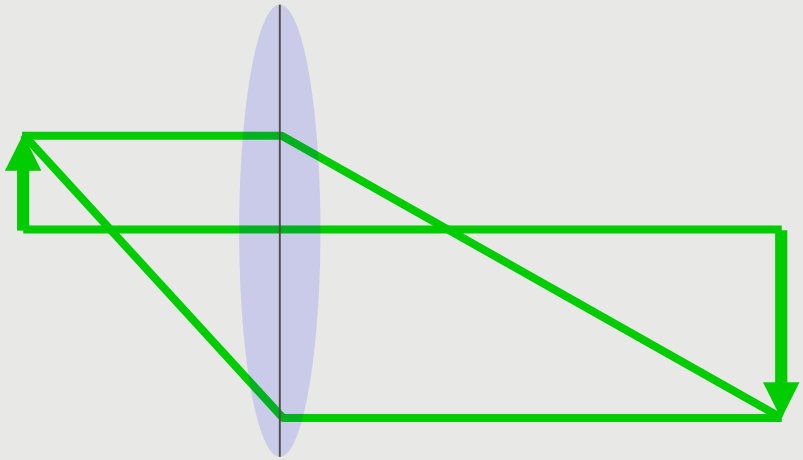
Objective

Intermediate image

The compound microscope

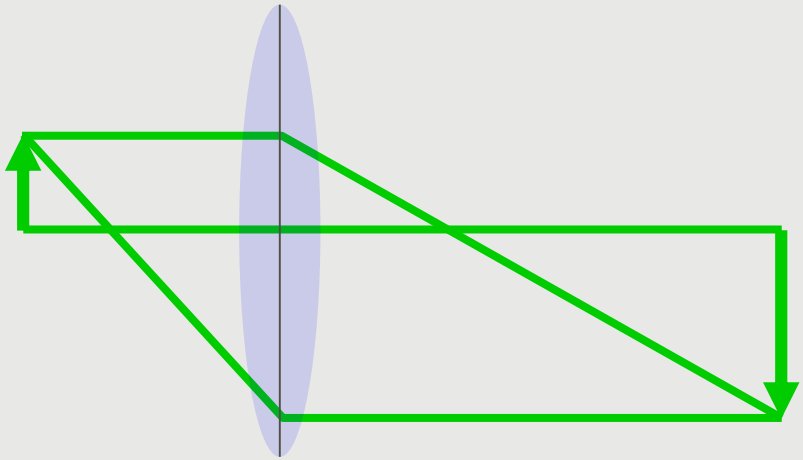


The compound microscope

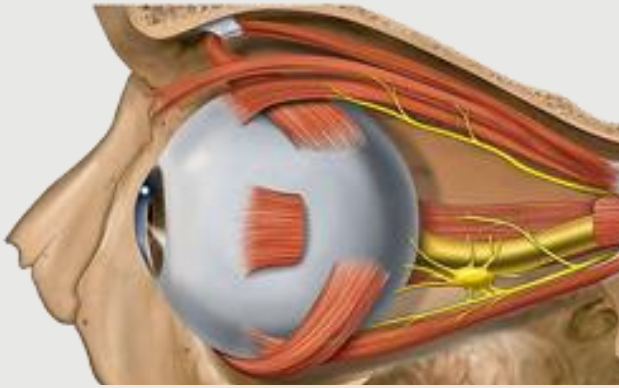


Intermediate image

The compound microscope

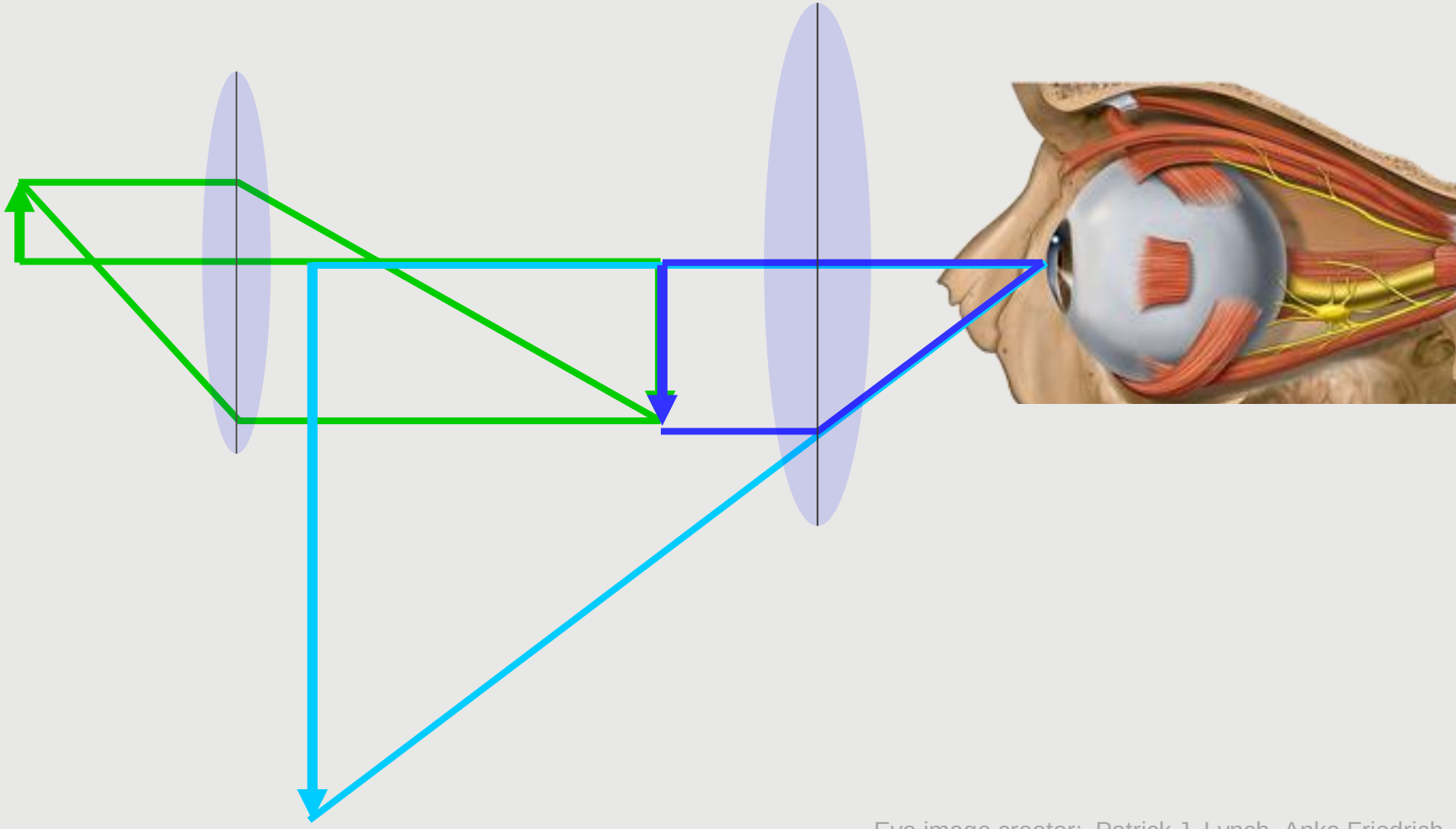


eye piece



Eye image creator: Patrick J. Lynch, Anka Friedrich
https://commons.wikimedia.org/wiki/File:Lateral_orbit_nerves_chngd.jpg

The compound microscope



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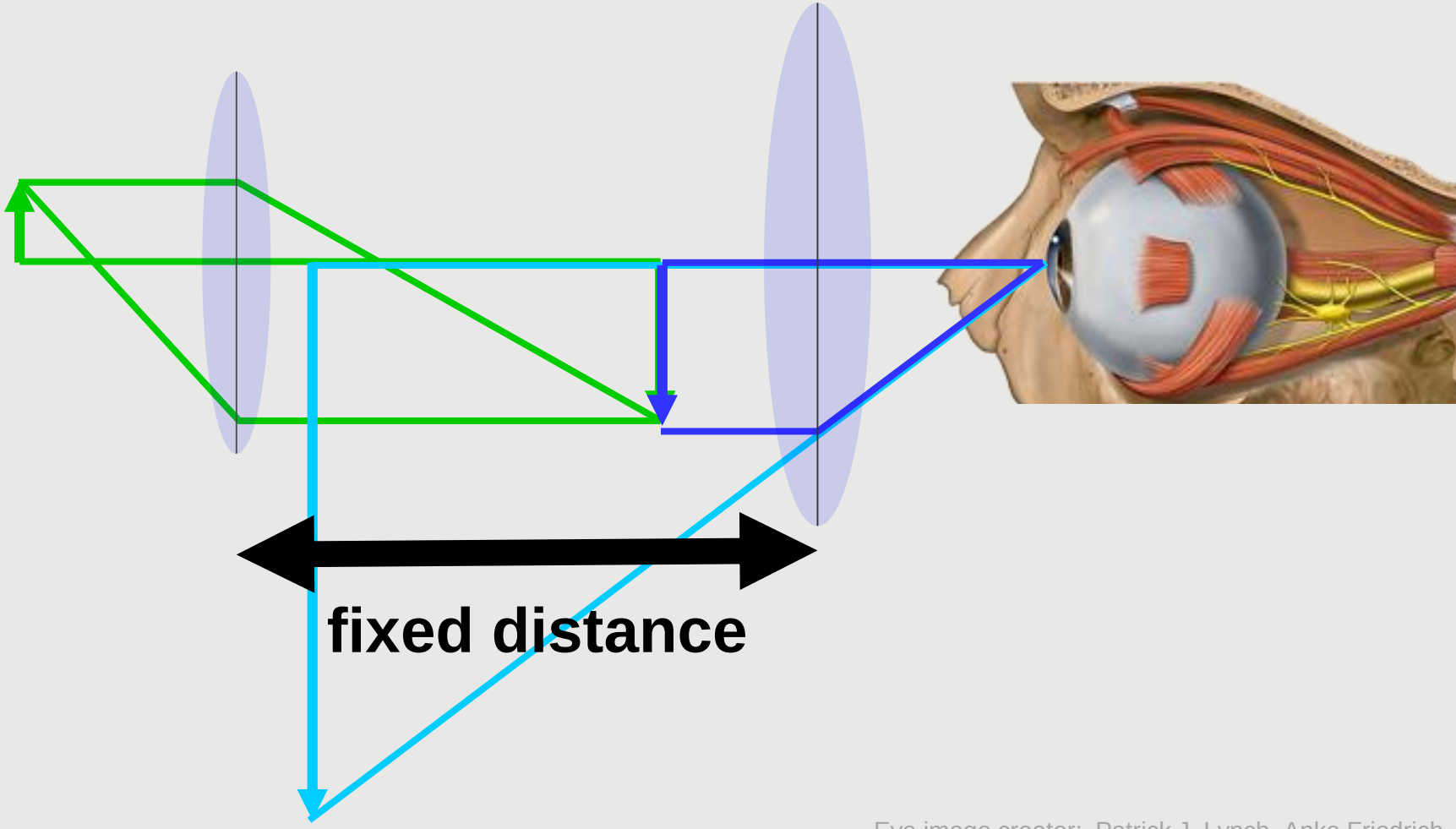
The compound microscope



Eye image creator: Patrick J. Lynch, Anka Friedrich
https://commons.wikimedia.org/wiki/File:Lateral_orbit_nerves_chngd.jpg

Beam path in the compound microscope

- With this ‚finite' optics, objective and eye piece must have a constant distance. Else, the intermediate image will not be at the only correct position.

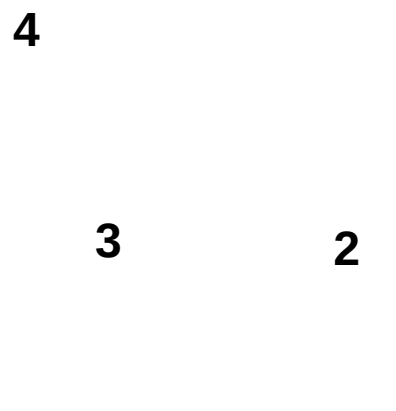
- Consequence: To focus, the specimen must be moved along the optical axis (z-direction)

Compound microscope with finite optics and 160 mm tube length

http://www.mikroskopie.de/kurse/objektive.htm

Objectives for finite optics (160 mm)

Where is the intermediate image?

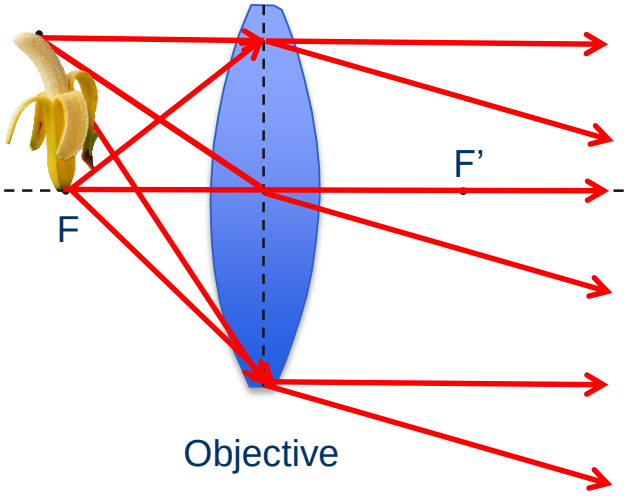


http://www.mikroskopie.de/kurse/objektive.htm

Objectives for finite optics (160 mm)

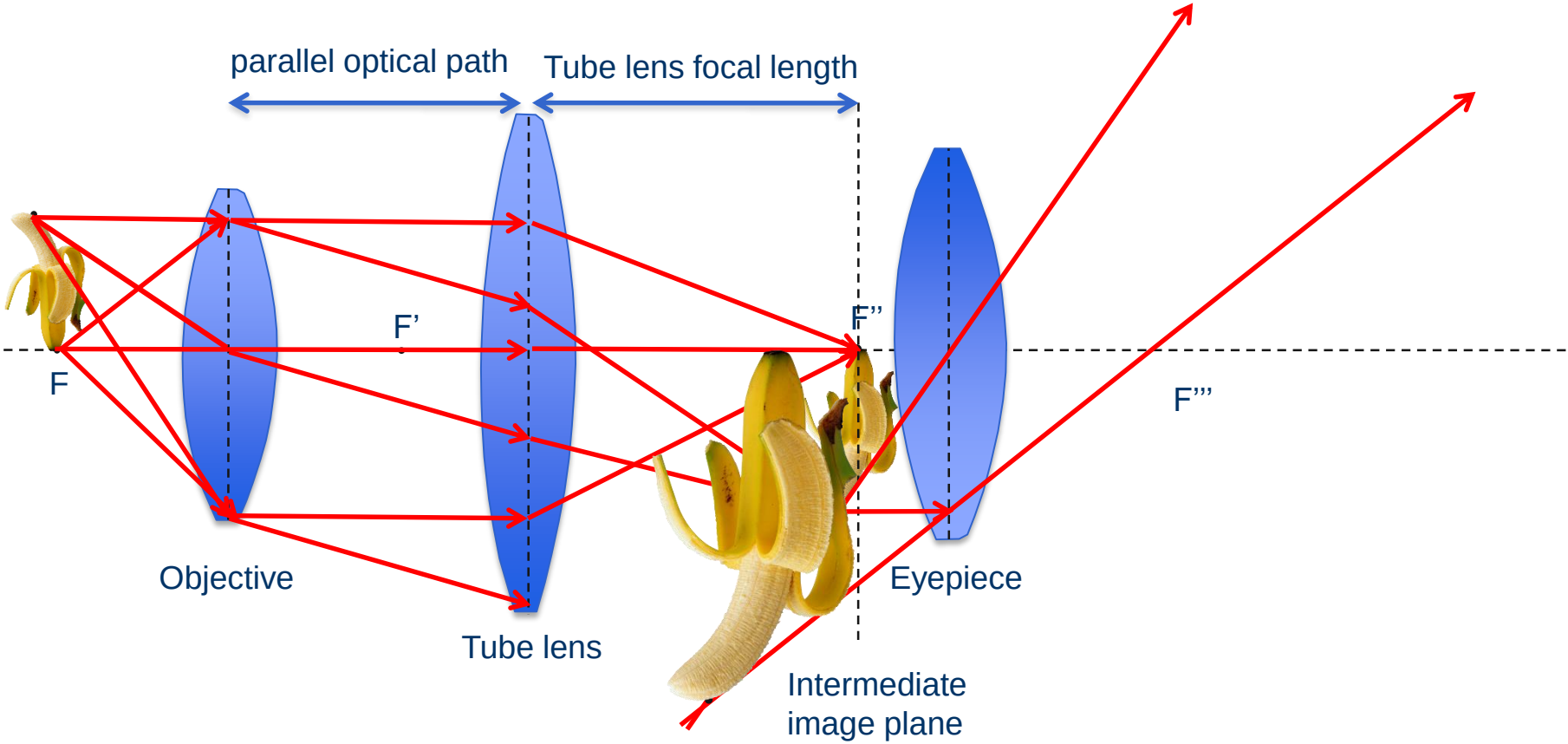
The Compound Microscope

Infinite optical Systems



The Compound Microscope

Infinite optical Systems

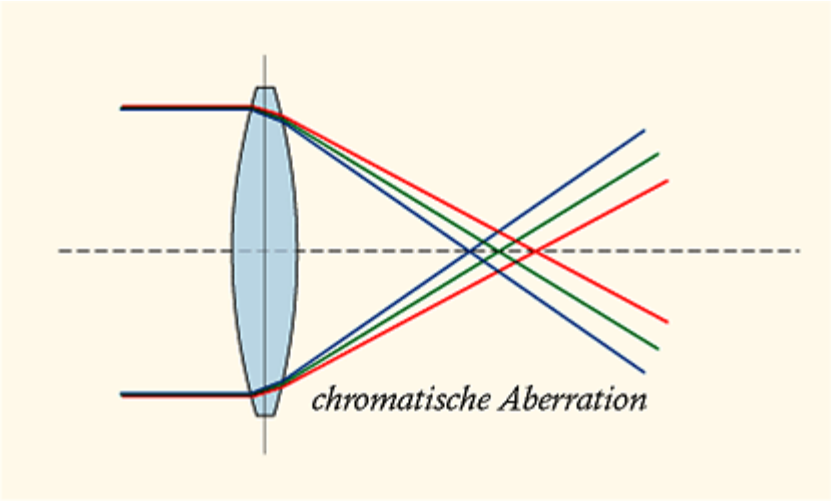


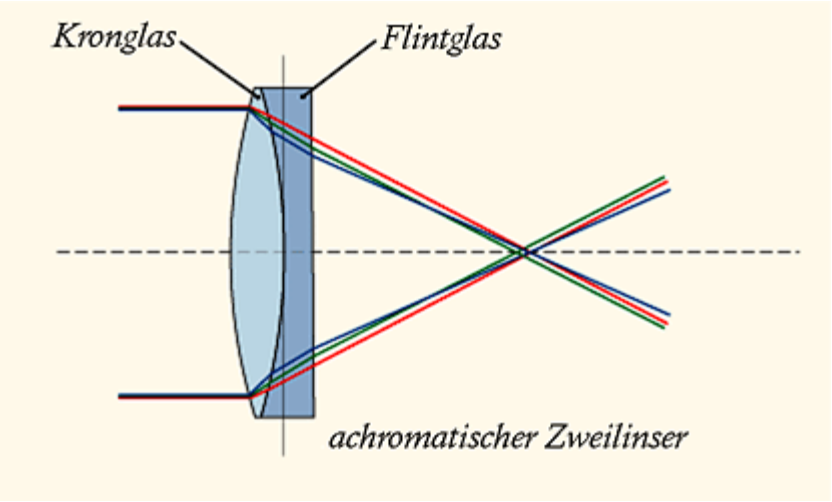
Aberrations

Chromatic aberration

Chromatic Aberration

- Due to the use of two lens systems (objective + eye piece), chromatic aberrations are multiplied. This was solved only in the 19th century





http://de.wikipedia.org/wiki/Abbildungsfehler

Dispersion

Chromatic aberration

- Achromatic objectives = corrected for green and red

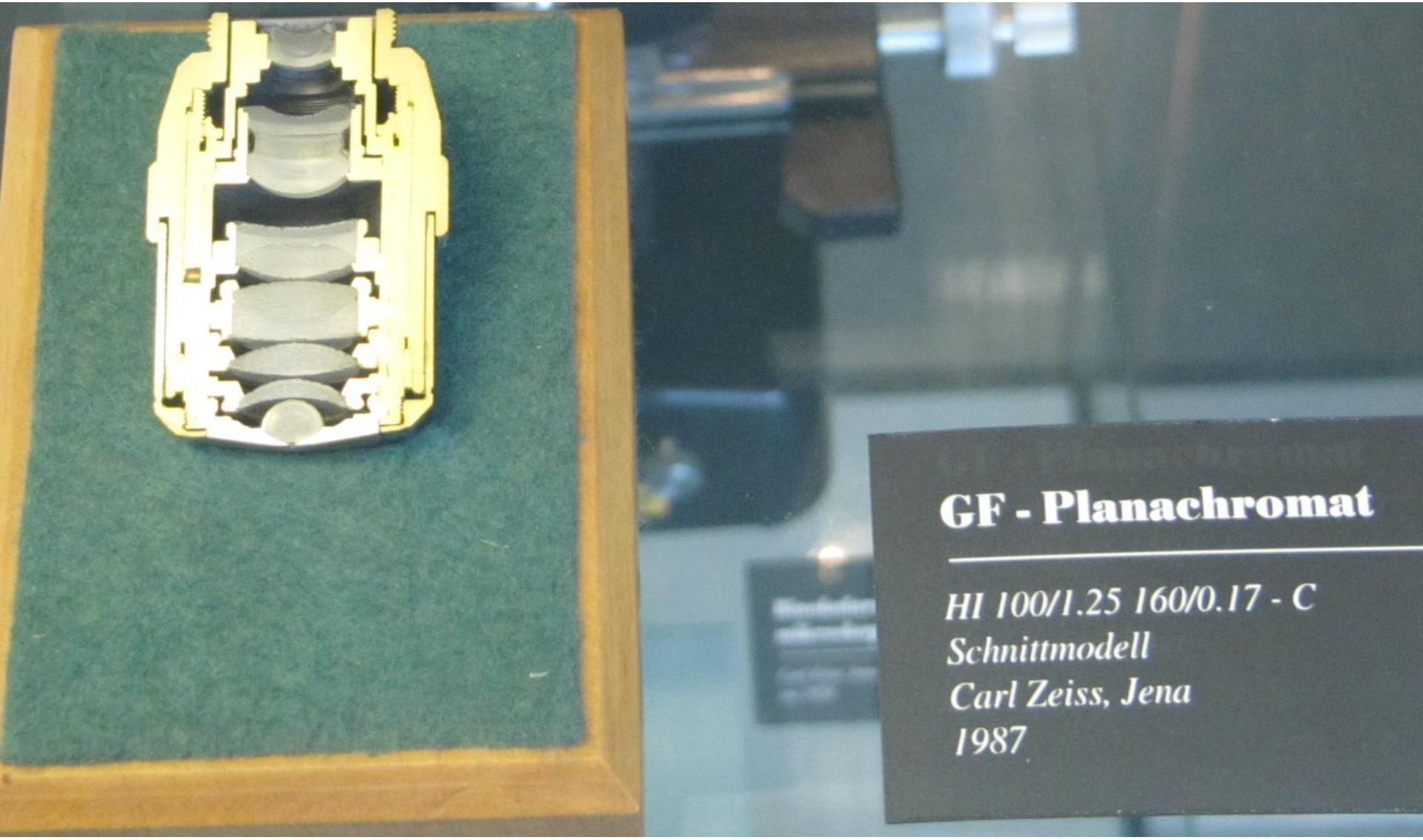
- Apochromatic objectives = corrected for blue, green, and red

- Fluorite objectives are similar to Achromats but make use of material with lower dispersion than normal glass, such as fluorite.

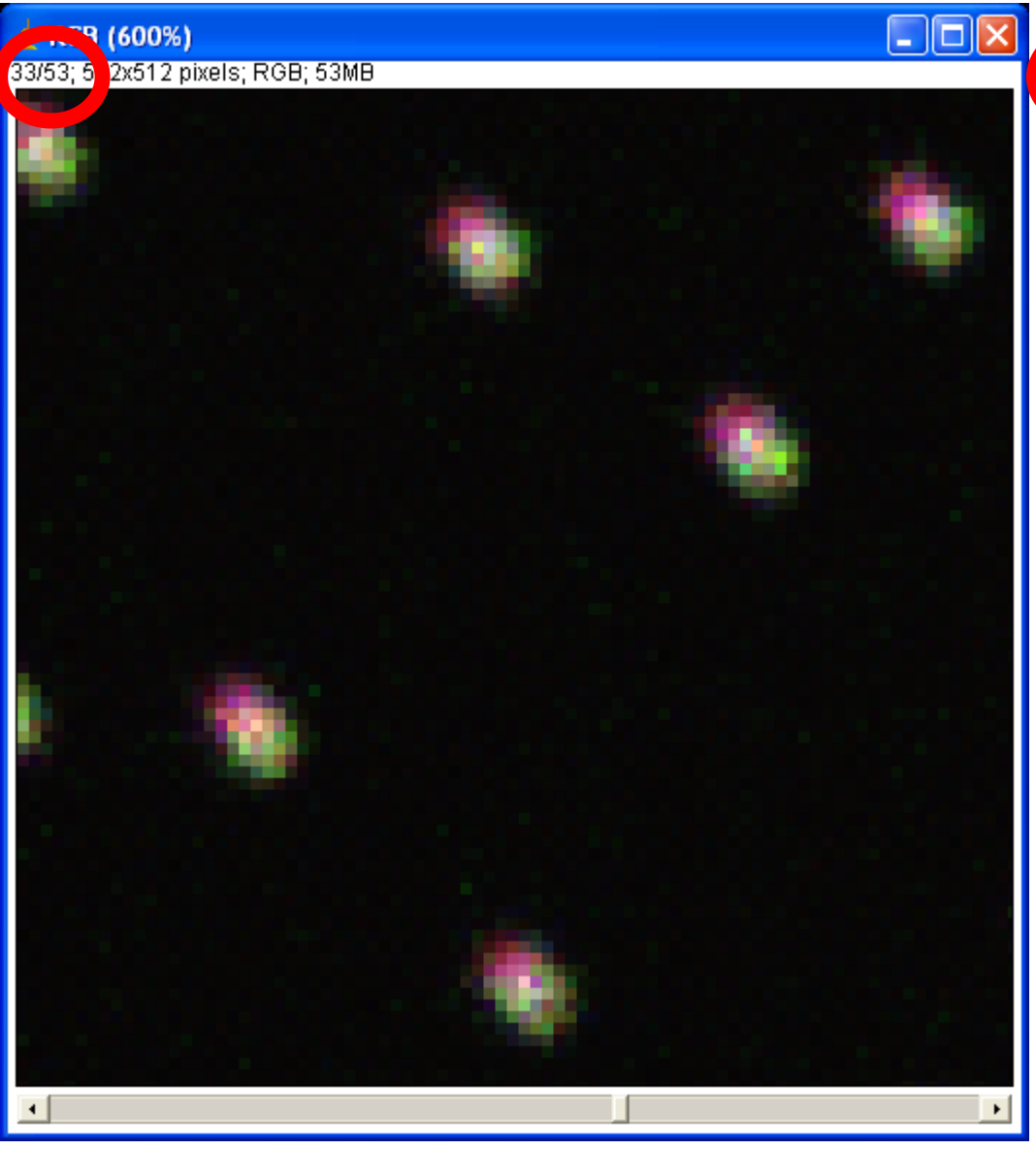
∞/0
HCX APO
L 20x/1.00 W
∞/0.17/D
PL APO
100x/1.40-0.7 OIL
∞/-/C
PL FLUOTAR
40x/1.00-0.50 OIL
∞/0.17/D
HCX PL FLUOTAR
20x/0.50
160/0.17
PL FLUOTAR
40/0.70
PHACO 2
160/-
PL FLUOTAR
10/0.30
PHACO 1

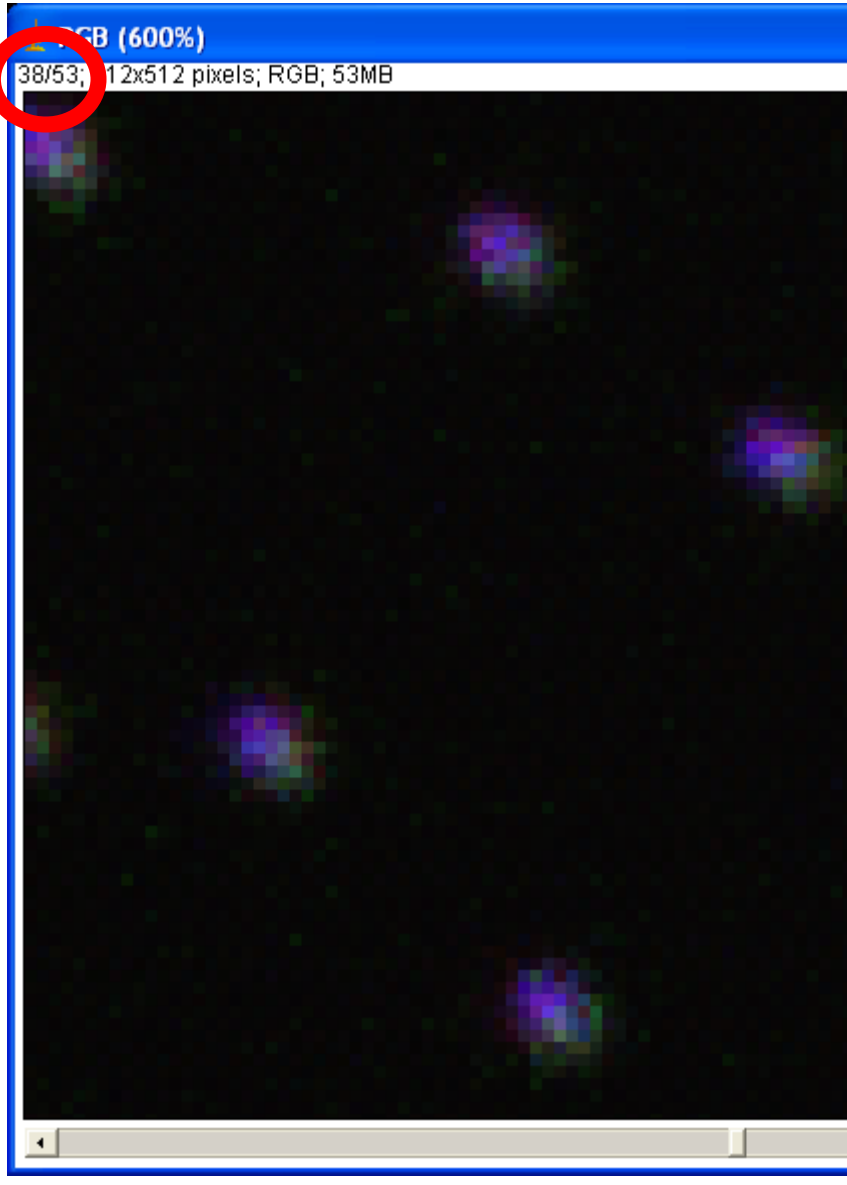
- A modern microscope contains lenses made from various glass varieties.

- The more lenses are used to achieve better correction, the more expensive it gets.

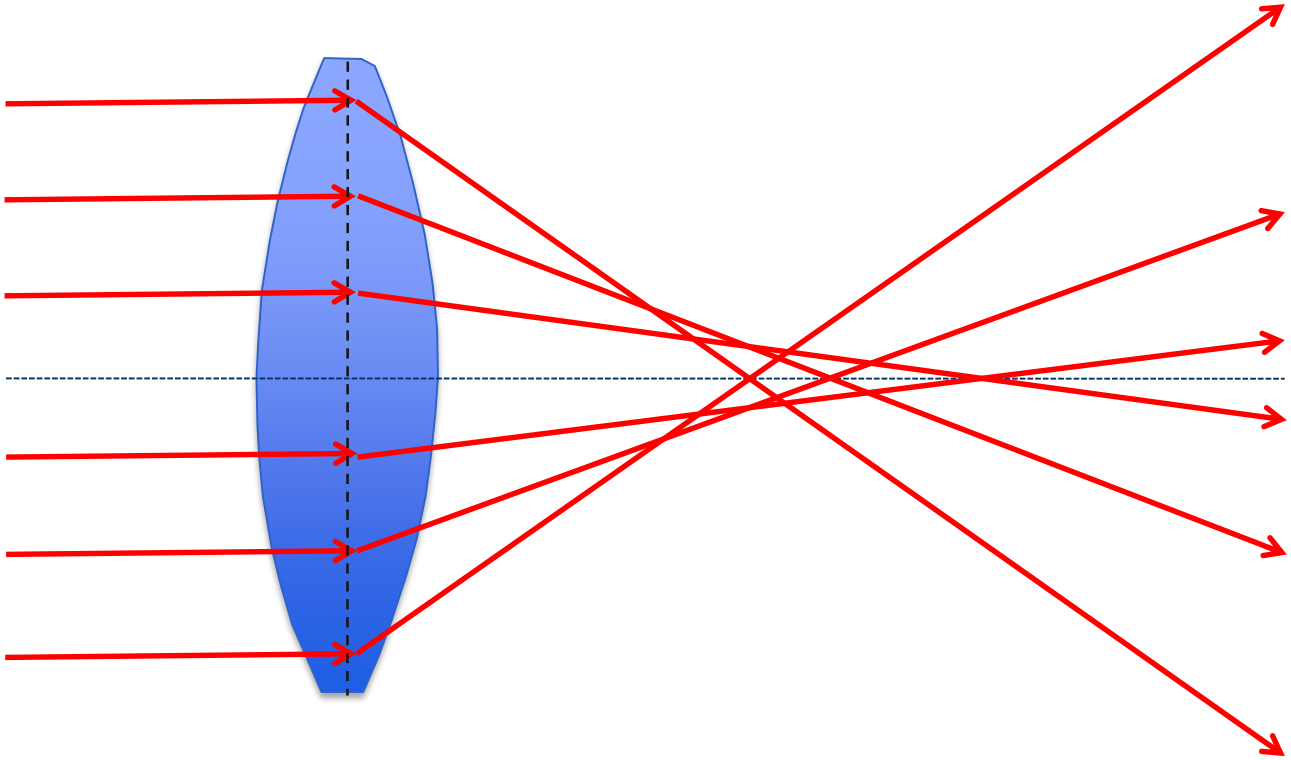

GF - Planachromat
HI 100/1.25 160/0.17 - C
Schnittmodell
Carl Zeiss, Jena
1987

Determination of chromatic aberration





Spherical Aberration



Further aberrations

- Field curvature
- Pincushion or barrel distortion
- …

Magnification vs. Resolution

Useful and empty magnification vs. resolution

Usefull and empty magnification

- Obviously, magnification says nothing about image quality, i.e. resolution.

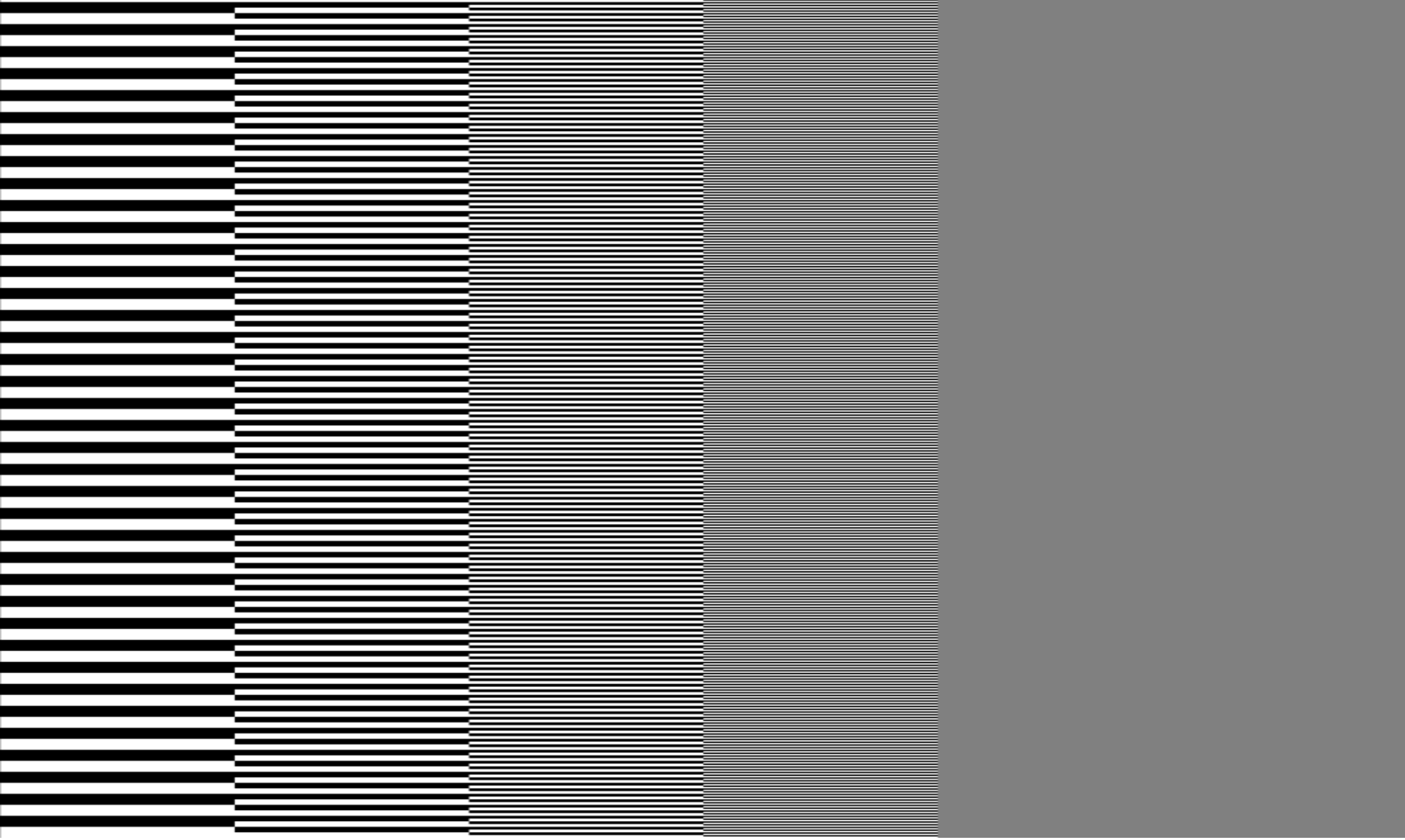
Resolution in light microscopy

- In microscopy, resolution is defined as the **distance** that two structures must have so that they can be resolved as two structures

- Resolution is typically limited by diffraction

Diffraction (dt: Beugung)

Light can be regarded as a wave…



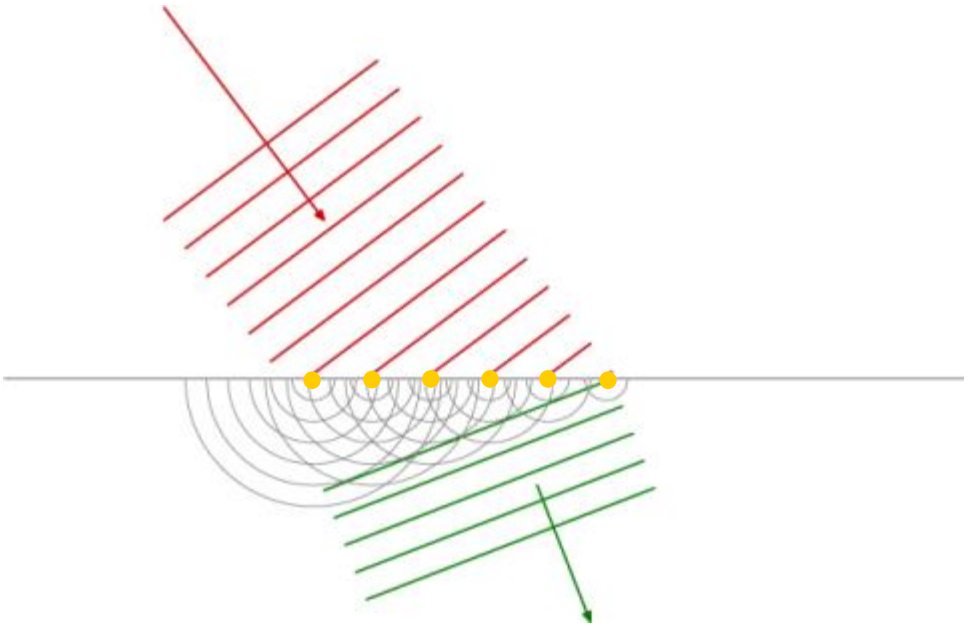
Huygens Principle

- Every point of a wave front can be regarded as the origin of a new wave

http://gigant.kgw.tu-berlin.de/~baalman/concept/intro.html

Huygens Principle

- Reason for *refraction*: the velocity of the light wave (speed of light) is less in optically dense media.

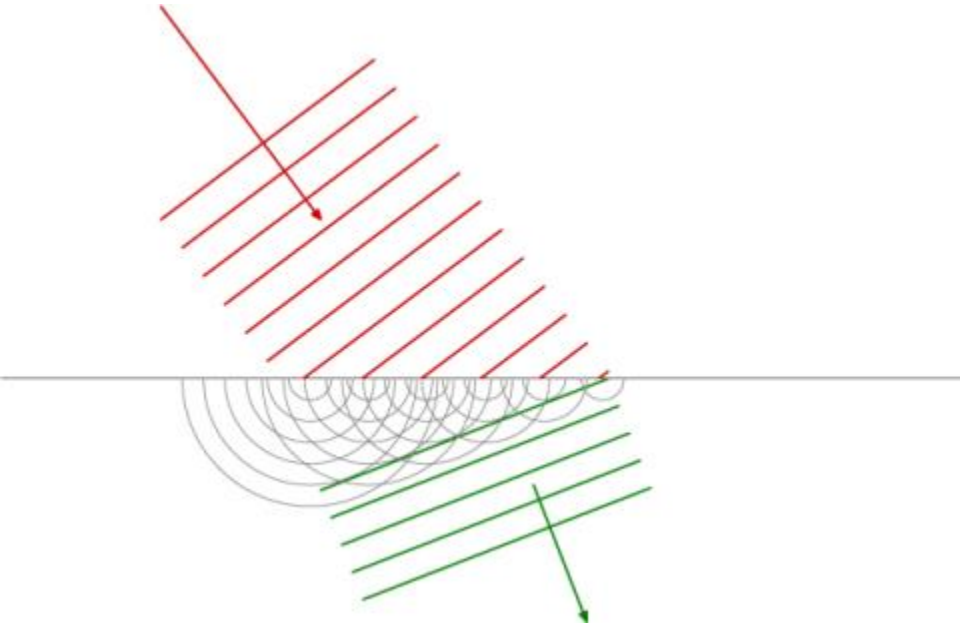


- Thus, the frequency is constant, but the wavelength changes

Refraction index Ri or n

$$n = \frac{c_0}{c}$$

- n = Refraction index
- c_0 = speed of light in vacuum
- c = speed of light in medium

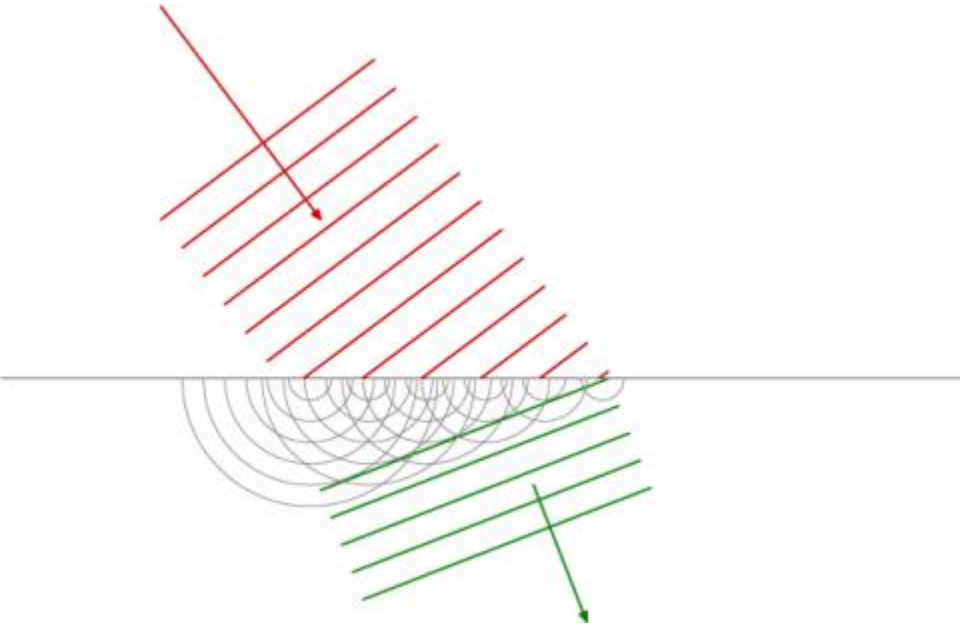


http://de.wikipedia.org/wiki/Brechzahl

Refraction index Ri or n

$$n = \frac{c_0}{c}$$

- Example: for water n = 1.33
- Speed of light in water:
  c= (300 000 km/s) / 1.33
  = 226 000 km/s



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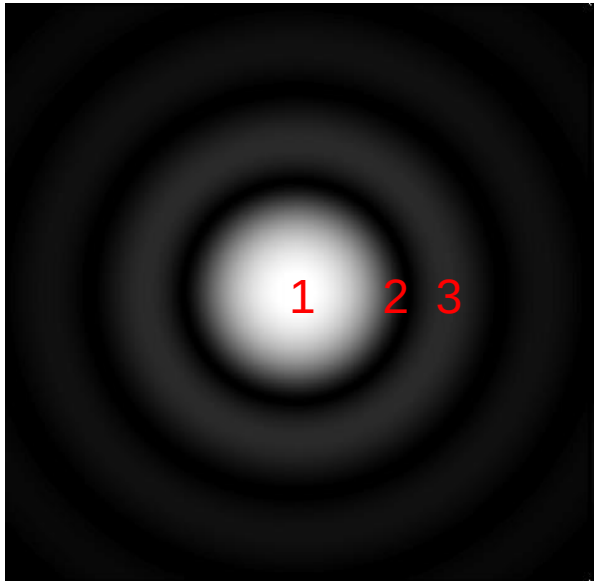
Diffraction and Fluorescence

- A microscopic fluorescence image is created by the light emitted by many fluorochromes.
- Each fluorochrome is a point source of light.

Diffraction and Fluorescence

- A microscopic fluorescence image is created by the light emitted by many fluorochromes.
- Each fluorochrome is a point source of light.
- Due to diffraction, the image of a point light source made with the microscope is not a point, but an Airy disk.

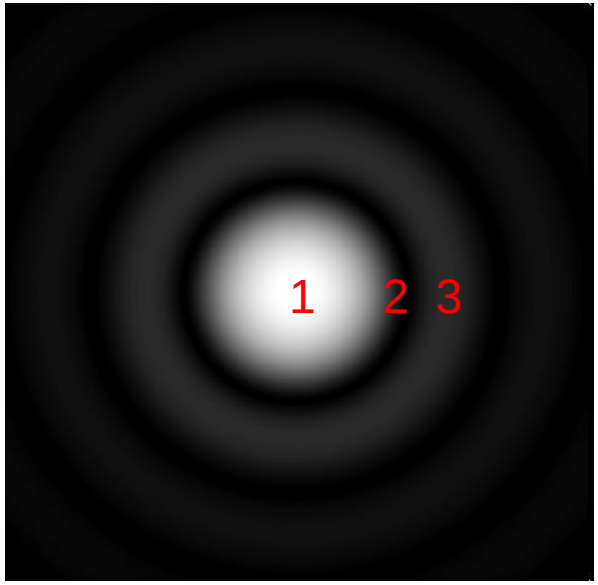


Airy disk with central maximum (1), first minimum (2) and first ring maximum

Image source:
http://commons.wikimedia.org/wiki/Image:Diffraction_disc_calculated.png

Diffraction and Fluorescence

- A microscopic fluorescence image is created by the light emitted by many fluorochromes.
- Each fluorochrome is a point source of light.
- Due to diffraction, the image of a point light source made with the microscope is not a point, but an Airy disk.
- The fluorescence microscopic image is thus a superposition of many Airy patterns.

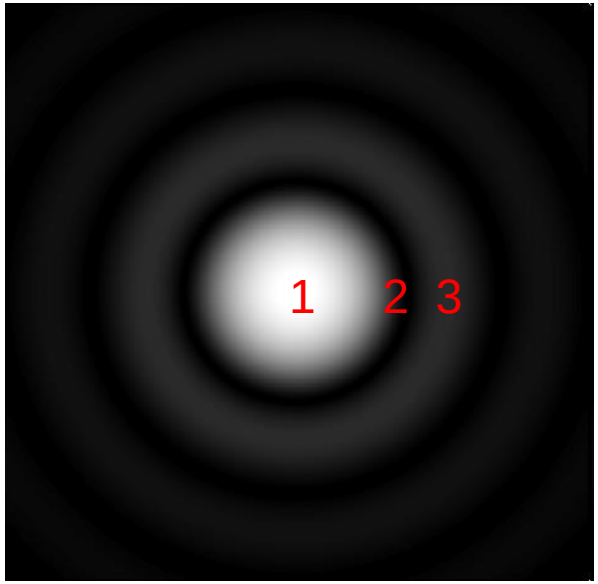


Airy disk with central maximum (1), first minimum (2) and first ring maximum

Image source:
http://commons.wikimedia.org/wiki/Image:Diffraction_disc_calculated.png

Diffraction and Fluorescence

- The size of the Airy disk depends on the resolving power (NA) of the objective
- The 3D-distribution of the light is called the Airy pattern or point spread function (PSF).



Airy disk with central maximum (1), first minimum (2) and first ring maximum

Image source:
http://commons.wikimedia.org/wiki/Image:Diffraction_disc_calculated.png

Resolution in light microscopy

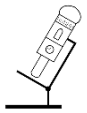
- In microscopy, resolution is defined as the **distance** that two structures must have so that they can be resolved as two structures

- What does that mean?

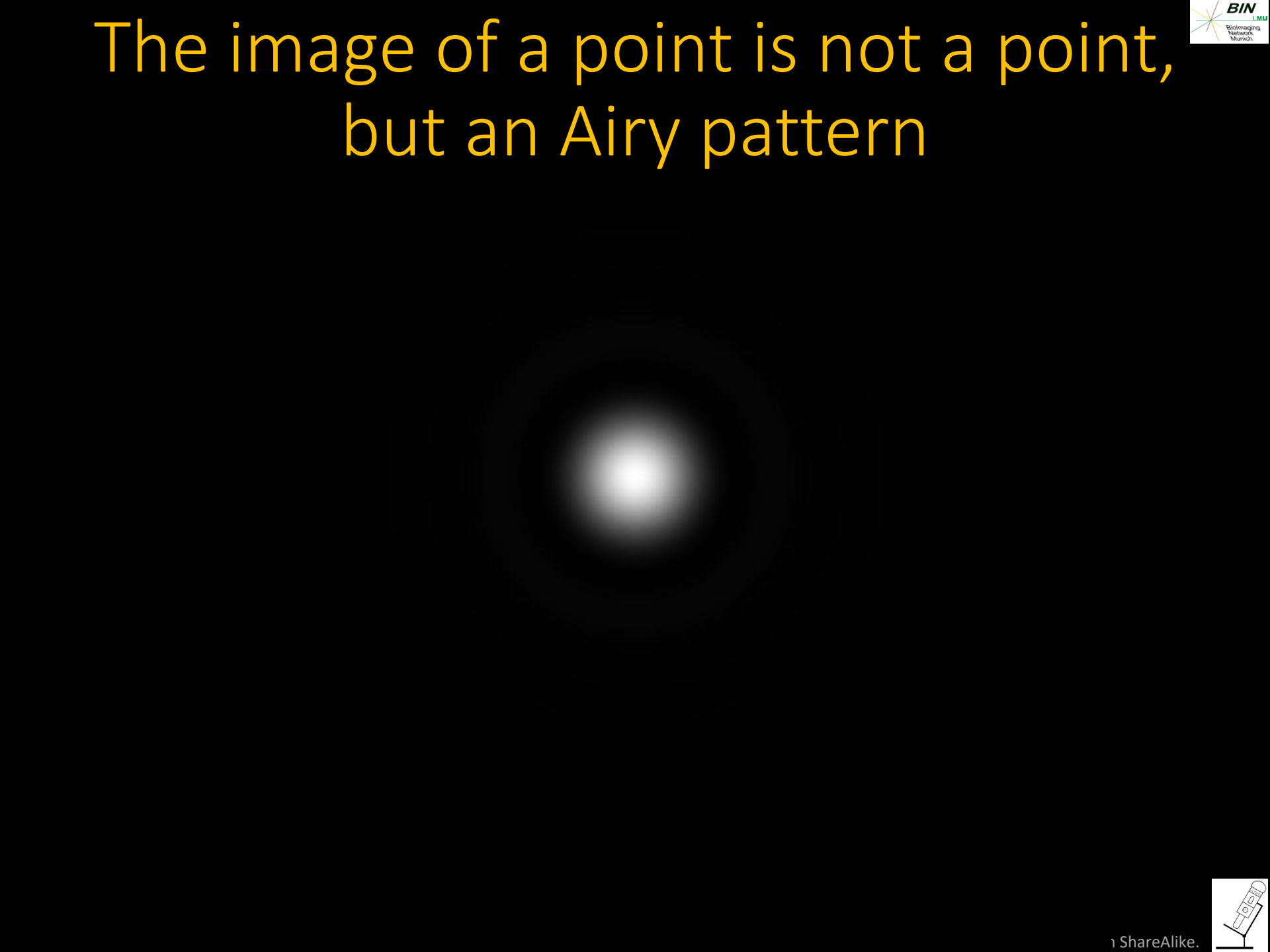
- Note: The following explains the situation for fluorescence microscopy

The idealized situation

- Unlimited number of photons
- Two point-like sources emit the same number of photons (identical intensity)
- No other sources in the neighborhood
- No spherical aberration, Ri mismatch
- No noise

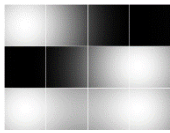


The image of a point is not a point, but an Airy pattern



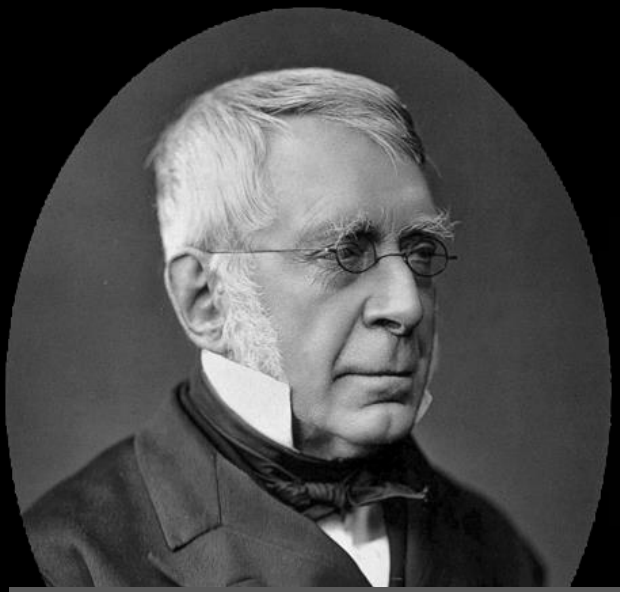
How the Airy patterns and intensity plots in this presentation were created

- COSM PSF (http://cirl.memphis.edu/cosmos.php) was used to produce a PSF image (.wu file), see settings in screenshot left. Reassuringly, the first minimum turned out to be at 217 nm away from the Airy pattern maximum, as it should be according to d=0.61*λ/NA.
- The .wu image was opened in Fiji (http://fiji.sc/Fiji) using the Import-Raw function with the following parameters: Type 32-real, Offset 1024, Gap 0, Little-endian and the width, height and number of images as defined in COSM PSF.
- In Fiji, the image's type was changed to 16 bit and it was then saved as Text-Image to be reopened in Excel 2013. In the 2001x2001 pixel image, the position of the maximum gray value of 65535 was labeled in Fiji position 1001;1001 and in Excel in Line 1002, Column ALN.
- The numbers of line 1002 were then used to draw the "wide-field" diagrams (blue) in GraphPad Prism. For the confocal-with-closed-pinhole diagram (green), the numbers of this line were squared.
- For import in Powerpoint, images of Airy patterns in Fiji were first adjusted to the desired grayscale range and then converted to 8-bit,
- For images with the 'added pixel intensities' (see right), 80x80 pixel (=nm) sized boxes were defined in Fiji (y-value for all: 961. x-values: 961, 1041, 1121, 1201, 938, 1018, 1098, 1178). Two images were added using the "Calculator Plus" (k1=0.5; k2=0). All 12 'pixel-'images were combined in a stack to create the montage shown here. The average intensity was determined using the 'measure' command. Values were normalized in Excel.

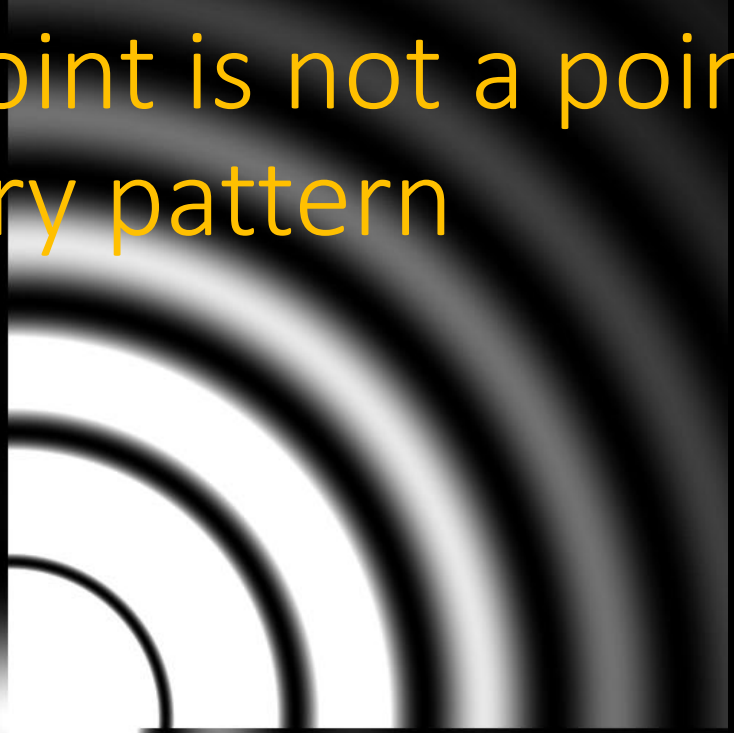


- One exception applies: The image with two neighboring Airy patterns was created by Katharina Nekolla with Matlab code kindly provided by Peng Xi. This code is published in his book "Optical Nanoscopy and Novel Microscopy Techniques" published by CRC.
- I would like to thank the contributors to the 'confocal list' (http://lists.umn.edu/cgi-bin/wa?A0=CONFOCALMICROSCOPY). Without the tips of Arne Seitz, Sergey Tauger, David Baddeley, Peng Xi and others, I would have not been able to generate those images.

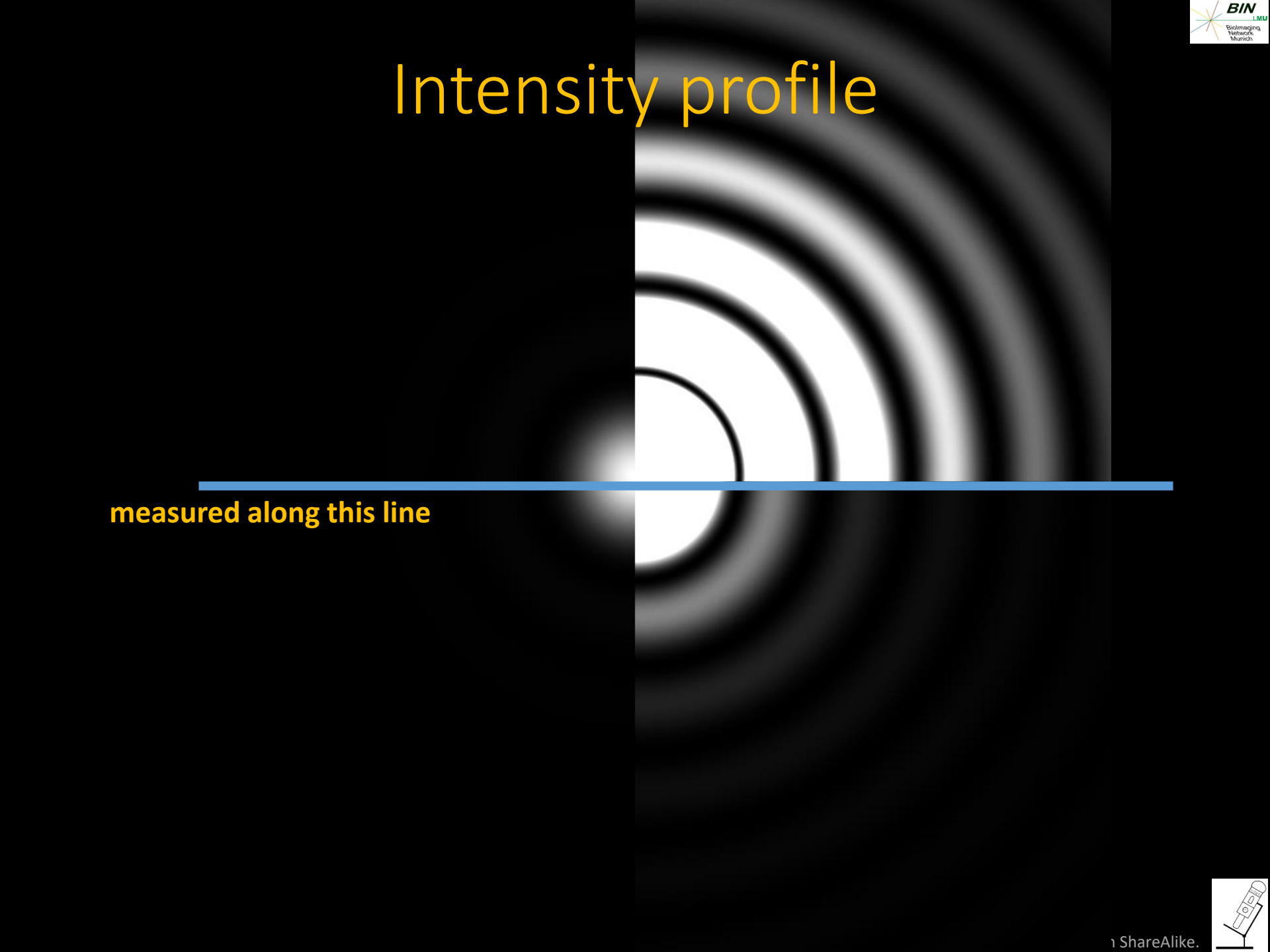
The image of a point is not a point, but an Airy pattern



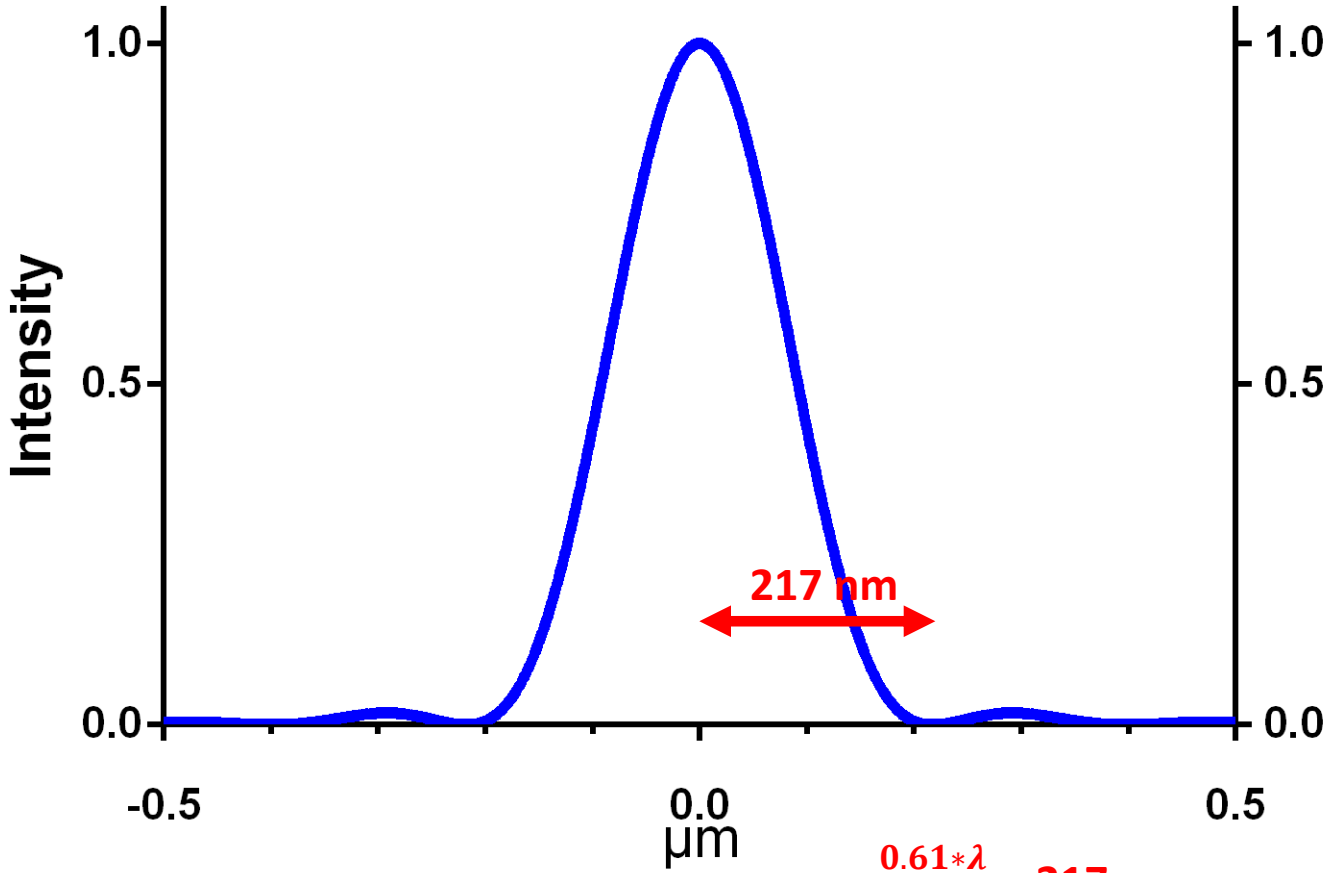
Sir George Biddell Airy,
27.07.1801–02.01.1892
Astronomer Royal 1835-1881



Intensity profile

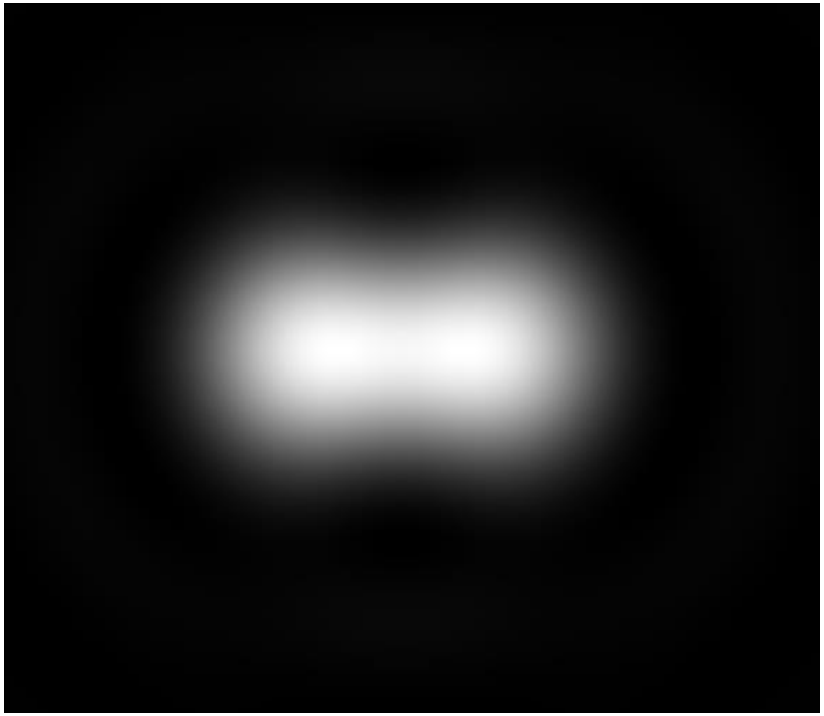


Example: Intensity distribution in the Airy pattern of a point with an NA 1.4 objective with λ=500 nm



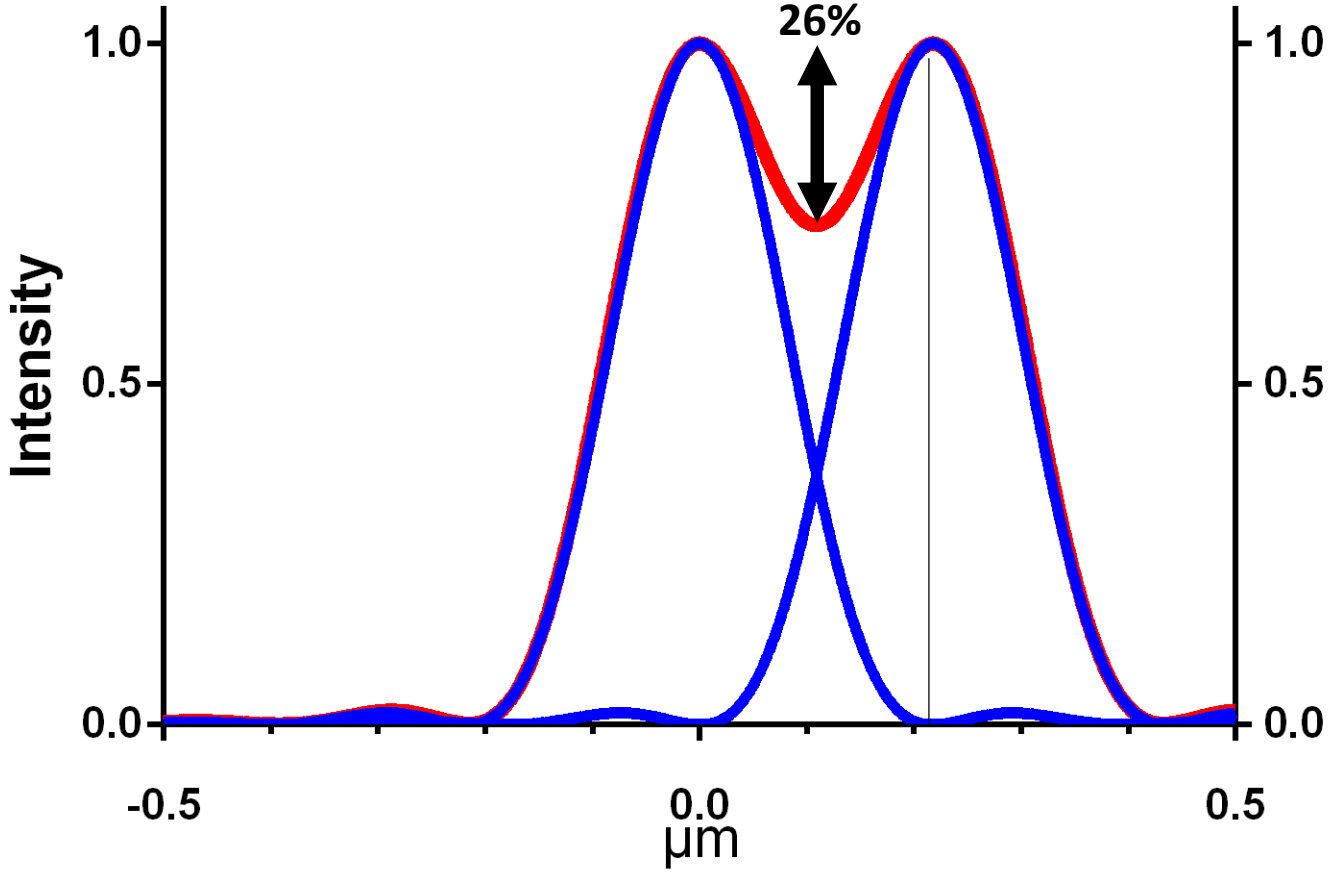
$$r = \frac{0.61 * \lambda}{NA} = 217 \text{ nm}$$

How close can two Airy patterns be together and still be ***resolved*** = recognized as two?



Lord Rayleigh
(John William Strutt)
12.11.1842-30.07.1919

Example: Intensity distribution in the Airy pattern of a point with an NA 1.4 objective with λ=500 nm



Rayleigh criterion

“..This rule is convenient on account of its simplicity; and it is sufficiently accurate in view of the necessary uncertainty as to what exactly is meant by resolution.”

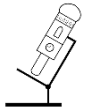
Rayleigh (1879) ‘XXXI. Investigations in optics, with special reference to the spectroscope, Phil. Mag. Series 5,8: 49, p 267

Lord Rayleigh won the Nobel Prize in Physics 1904 "for his investigations of the densities of the most important gases and for his discovery of argon in connection with these studies"

Lord Rayleigh
(John William Strutt)
12.11.1842-30.07.1919

But how do we measure that?

- It is very difficult to exactly determine the location of the minimum experimentally
- Therefore, for measurements a different criterion is needed:
- The Full Width Half Maximum, FWHM



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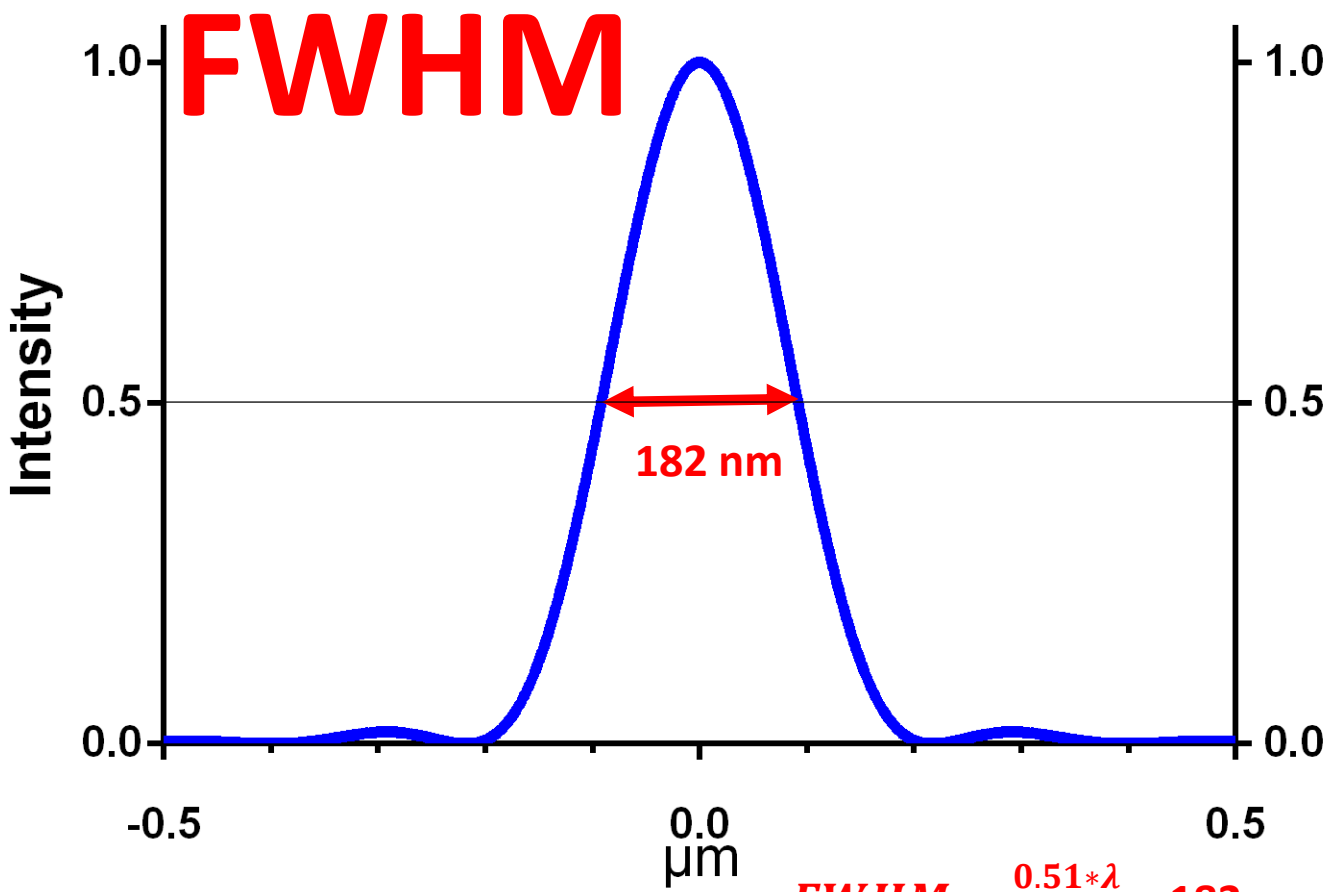
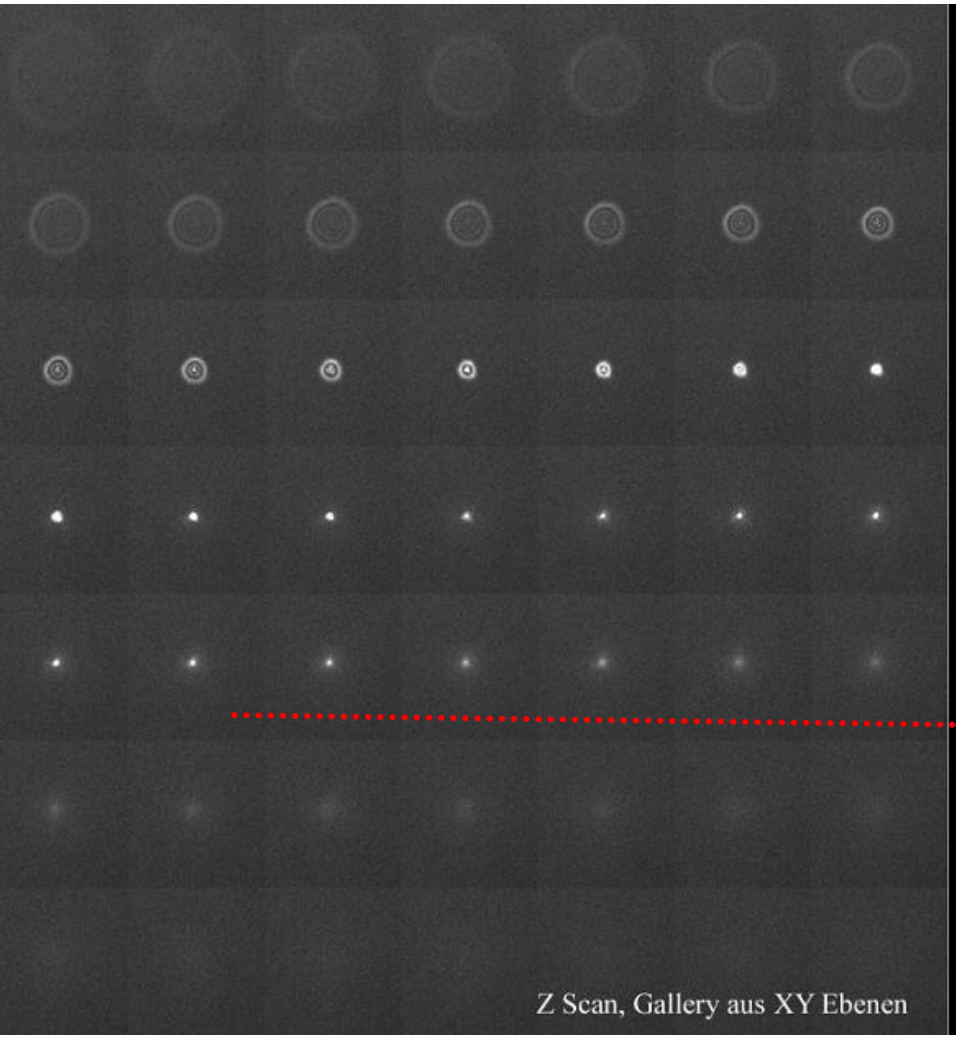
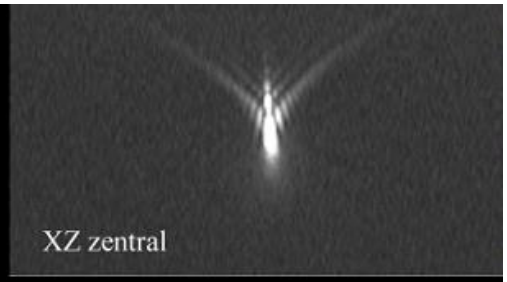


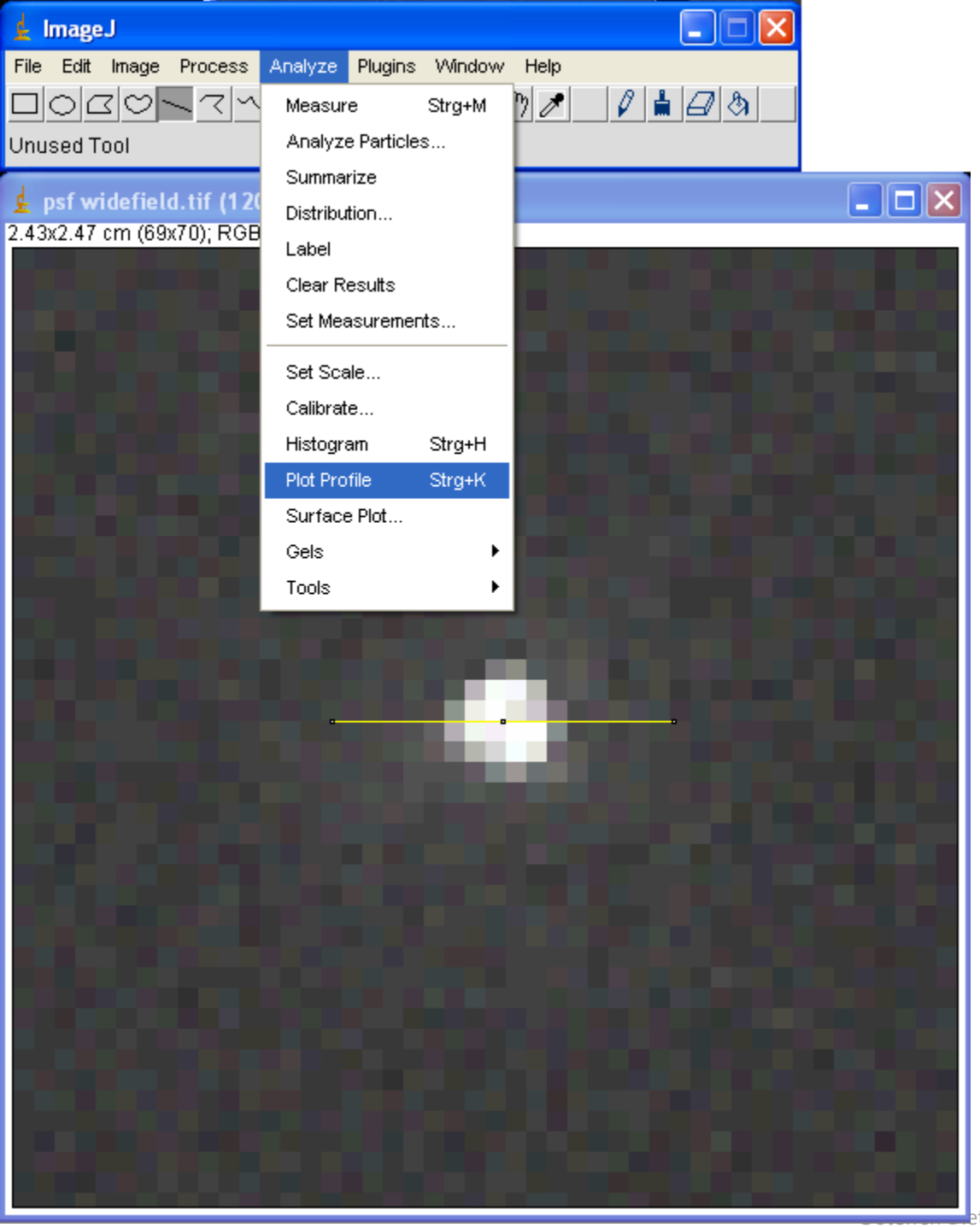
How to measure the FWHM of a Point-spread-function

- Z-sections through a 175 nm bead



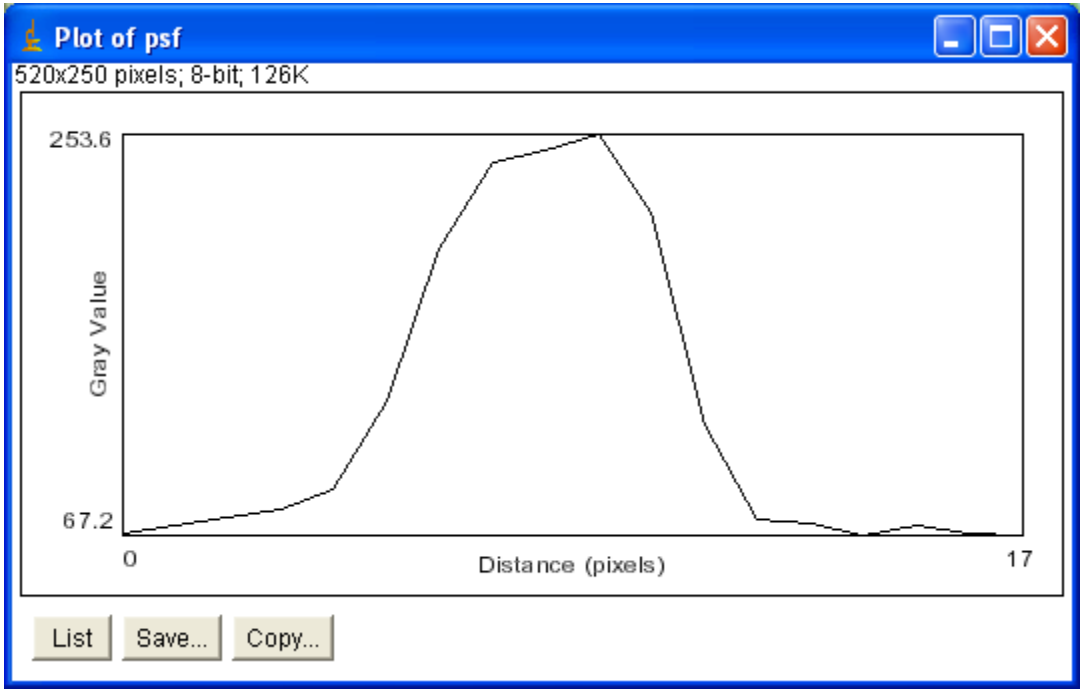


PSF widefield microscope, 175 nm bead Cy3

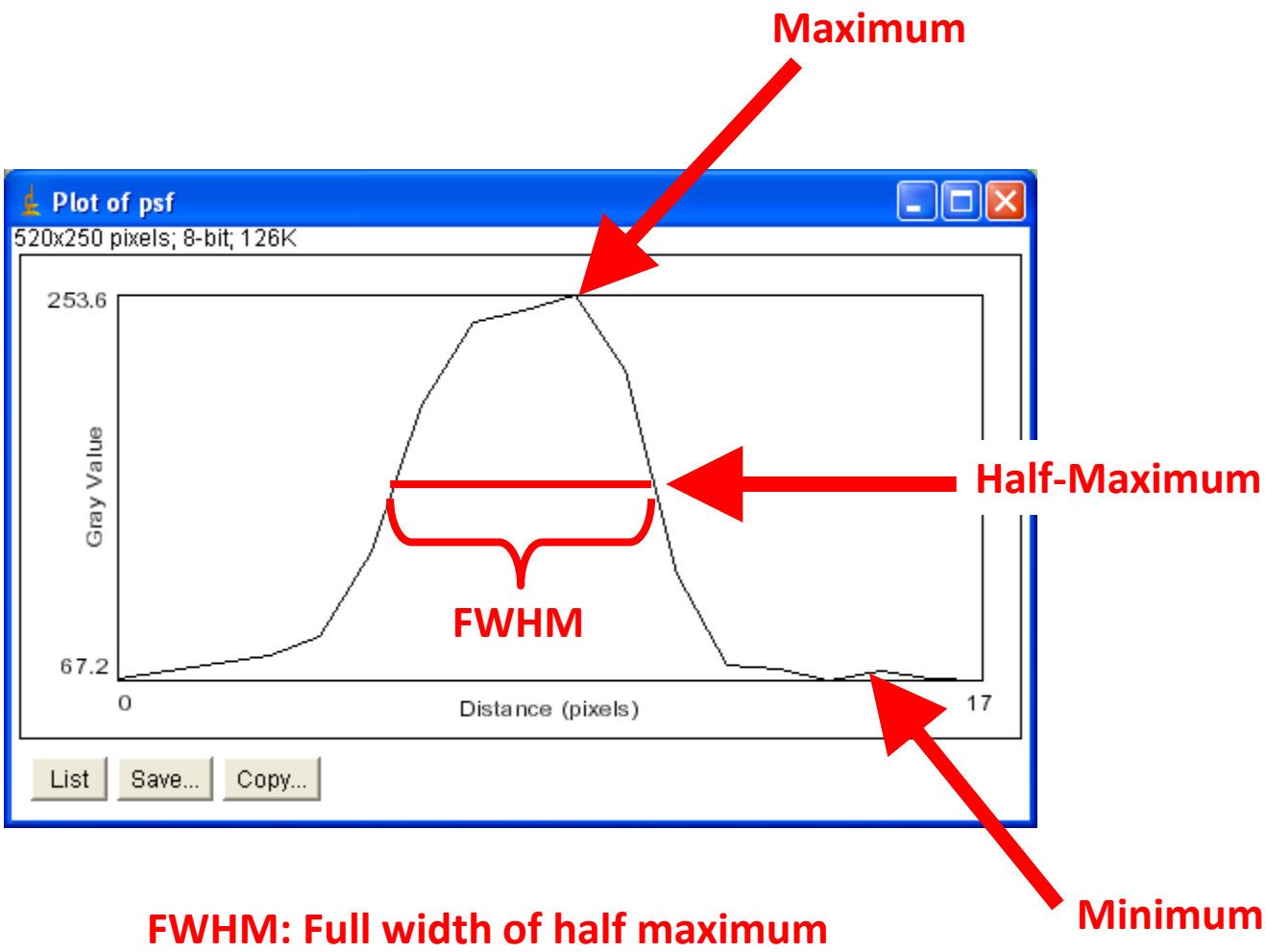


With Fiji / ImageJ

Intensity distribution along the line selection



X-axis: Pixels
Y-axis: Intensity (gray level)

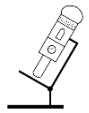


FWHM: Full width of half maximum

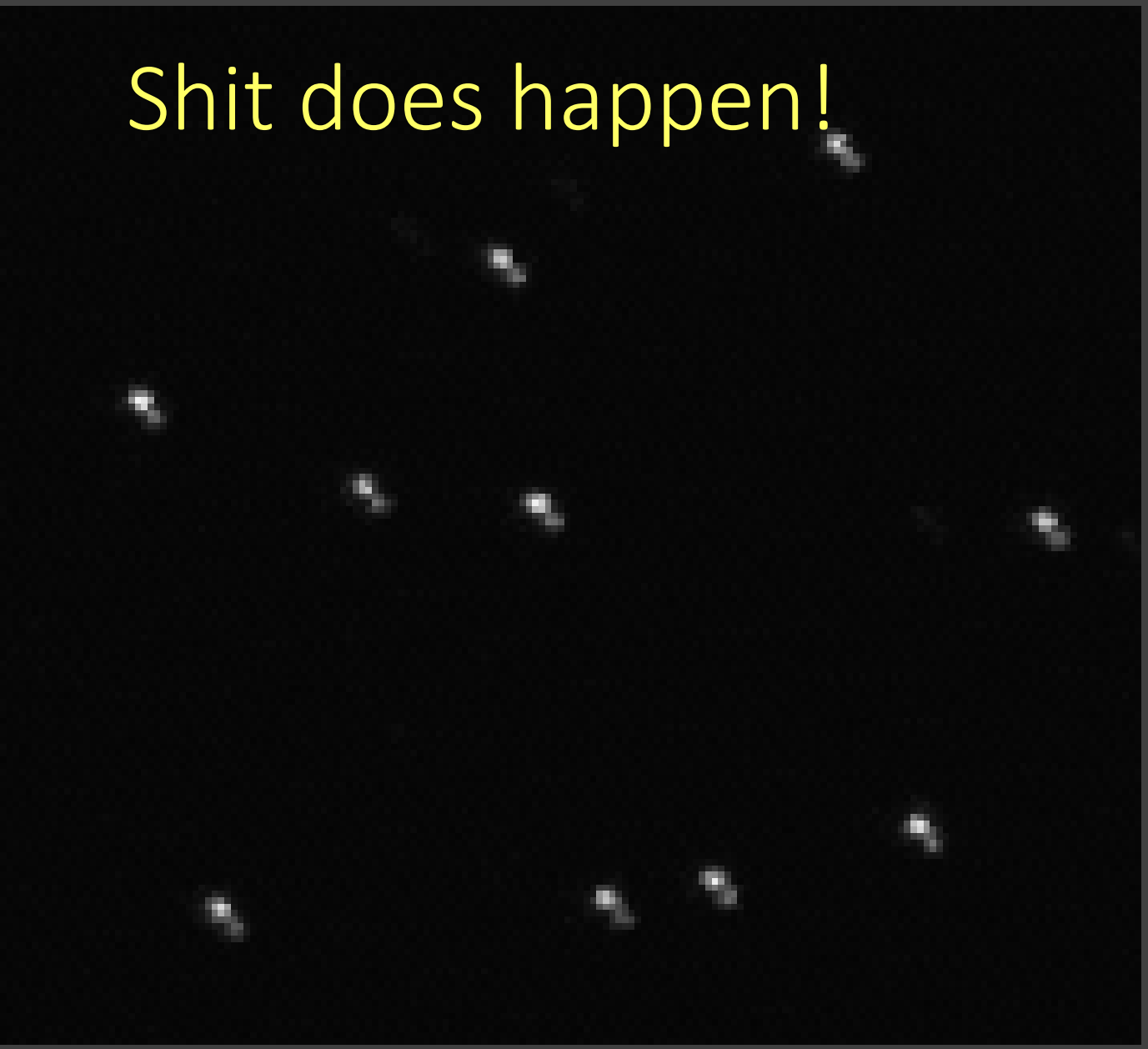
Test your microscope!

- Record point spread function (PSF) for every color channel you plan to use.
- For immersion objectives, use 175 nm or smaller beads (e.g. from Molecular Probes) or individual quantum dots or gold beads.

- Measure the Full width half maximum (FWHM) of the PSF (e.g. with Fiji/ImageJ).
- Do this measurement a couple of times and average the result.
- Some objectives are good only in the center, so test in center and near the edge of field of view
- Theory: $FWHM_{x,y}=0.51*\lambda/NA$

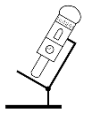


Shit does happen!



Leica SP1, 63x,
with DIC Prism in the beam path

All this was about imaging in the focal plane – what about z?

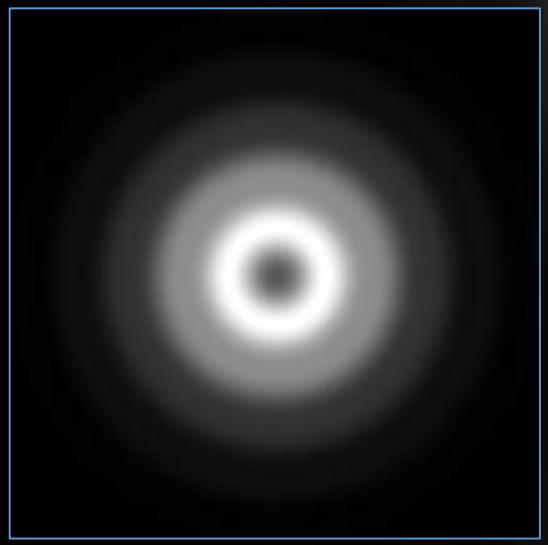


The Airy pattern in z-direction

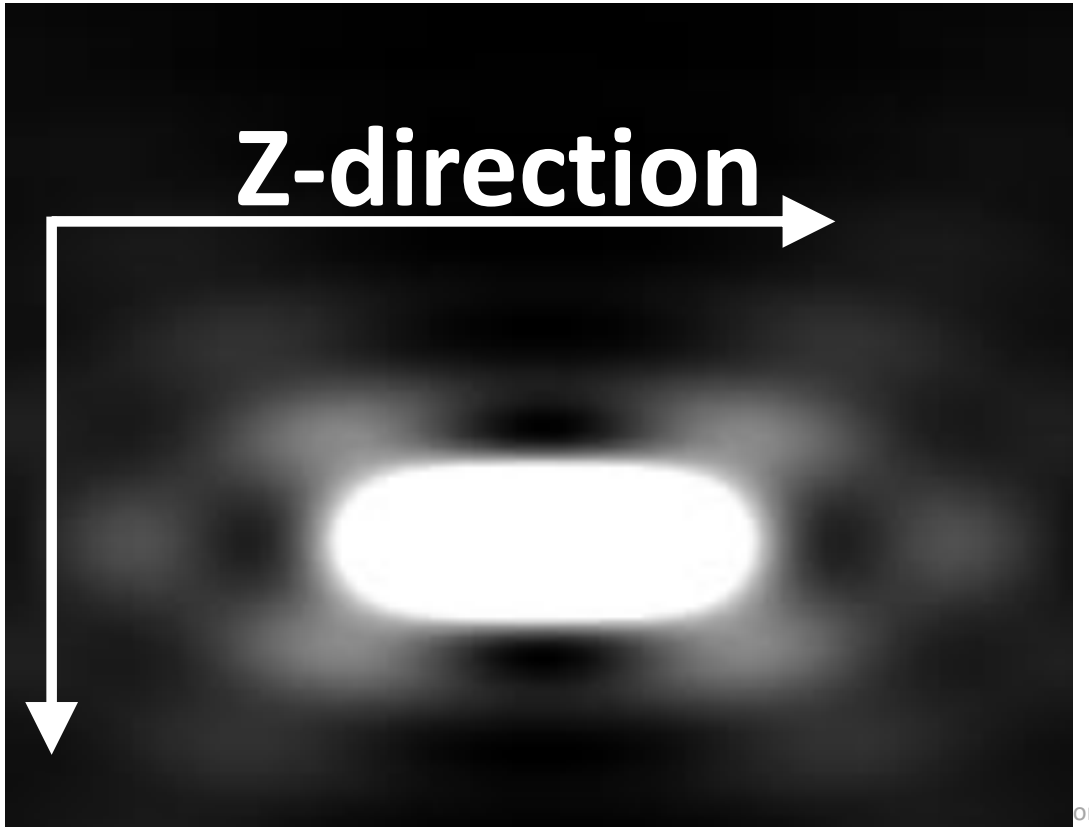
The Airy pattern in z-direction

The Airy pattern in z-direction

Cut here



Example: Intensity distribution in the Airy pattern of a point with an NA 1.4 objective with λ=500 nm



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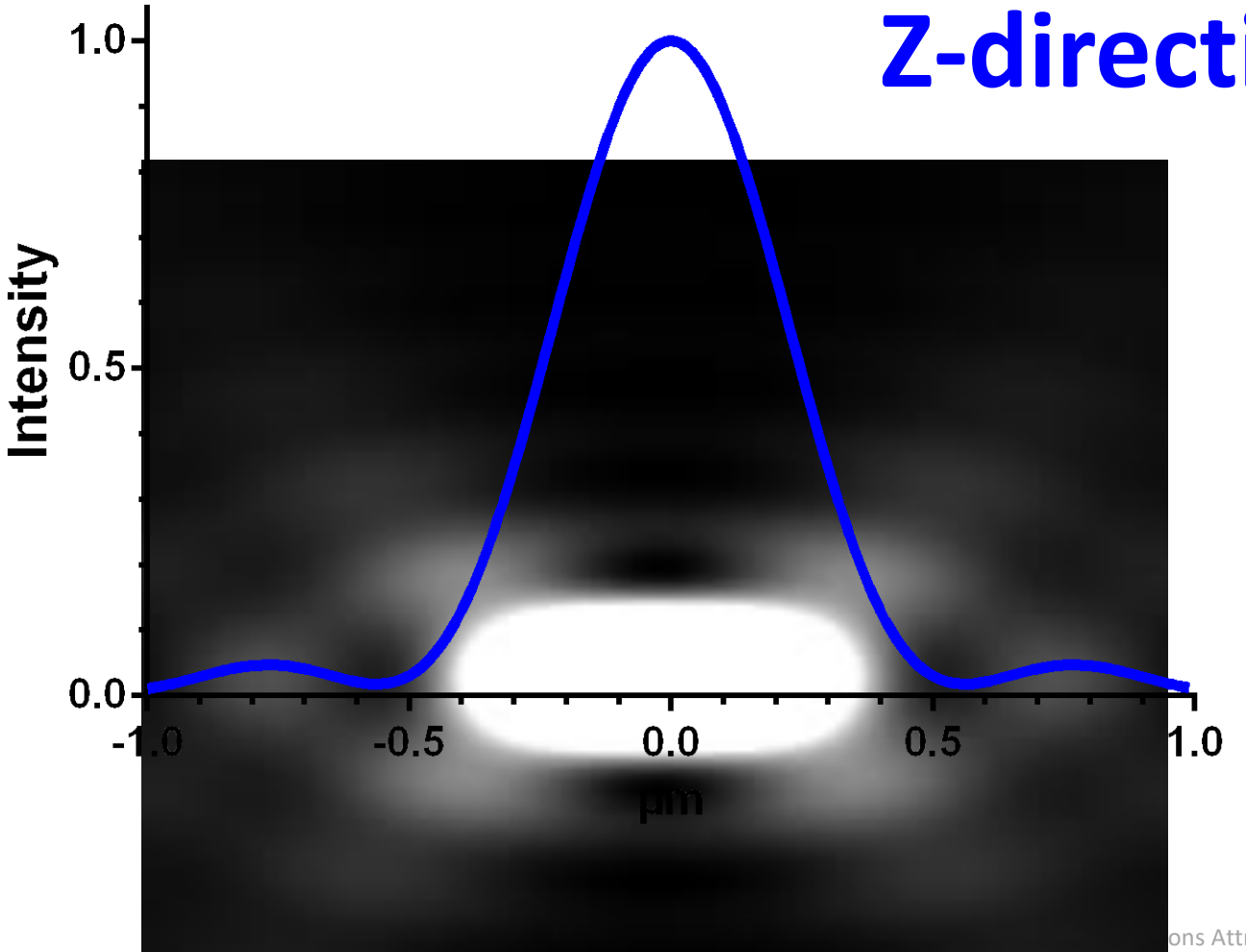

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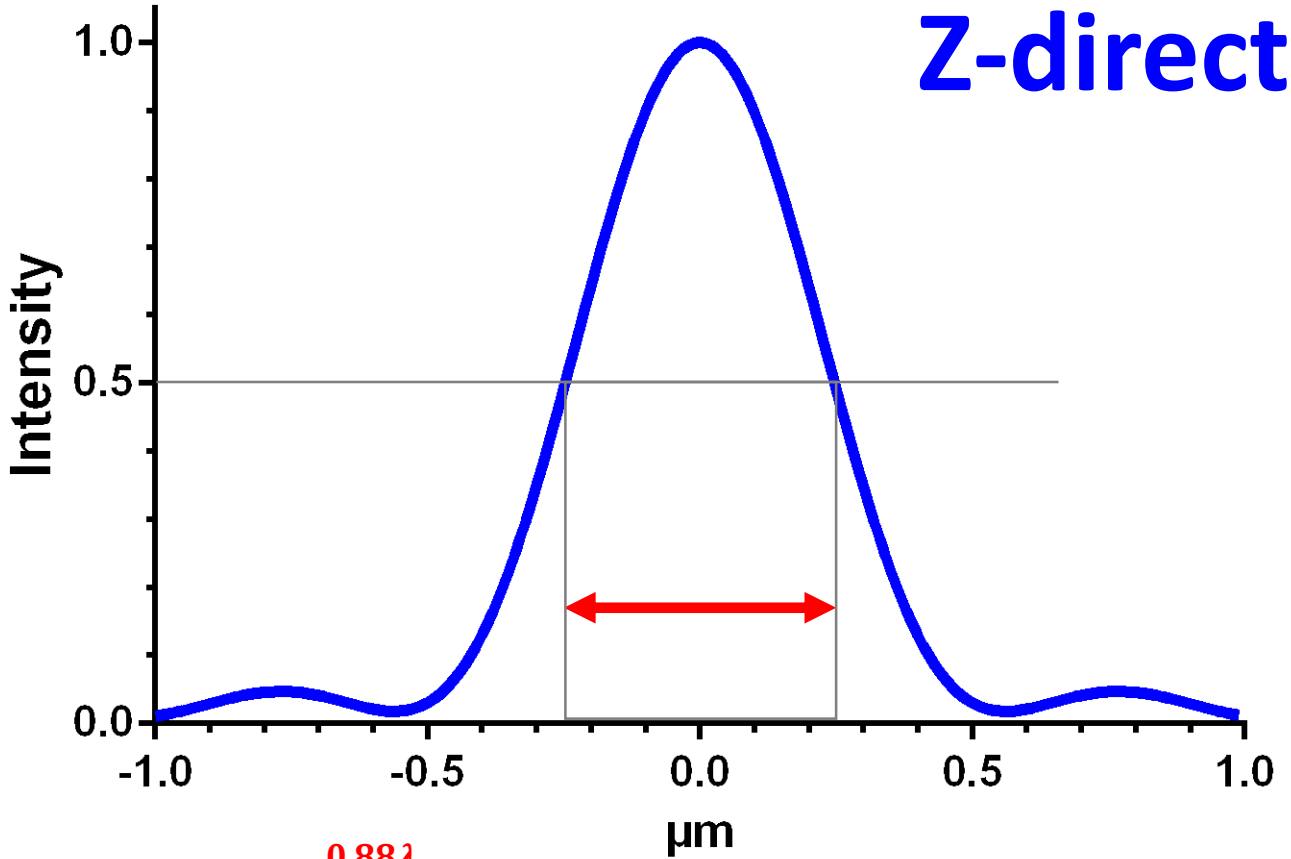
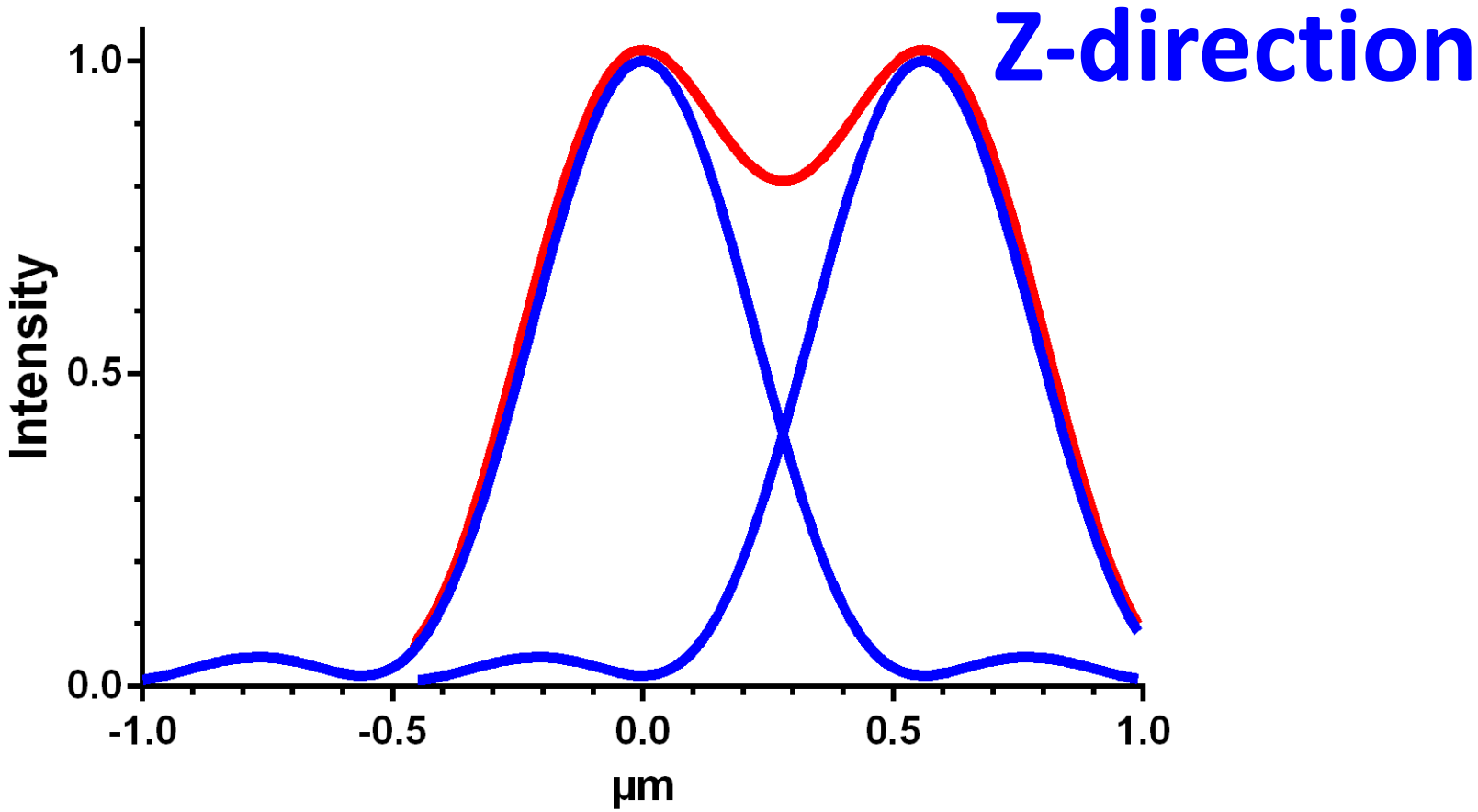


$$\text{FWHM}_{(z)} = \frac{0.88\lambda}{n - \sqrt{n^2 - NA^2}} = 472 \text{ nm}$$

Example: Intensity distribution in the Airy pattern of a point with an NA 1.4 objective with λ=500 nm



Rayleigh criterion

- The Rayleigh criterion is a good criterion for self-luminous objects such as in fluorescence microscopy.
- For brightfield (‘non-fluorescence’) microscopy the Abbe-limit is more appropriate (but we don’t go into that)
- In the focal plane, $\Delta R = 0.61 \cdot \lambda / NA$

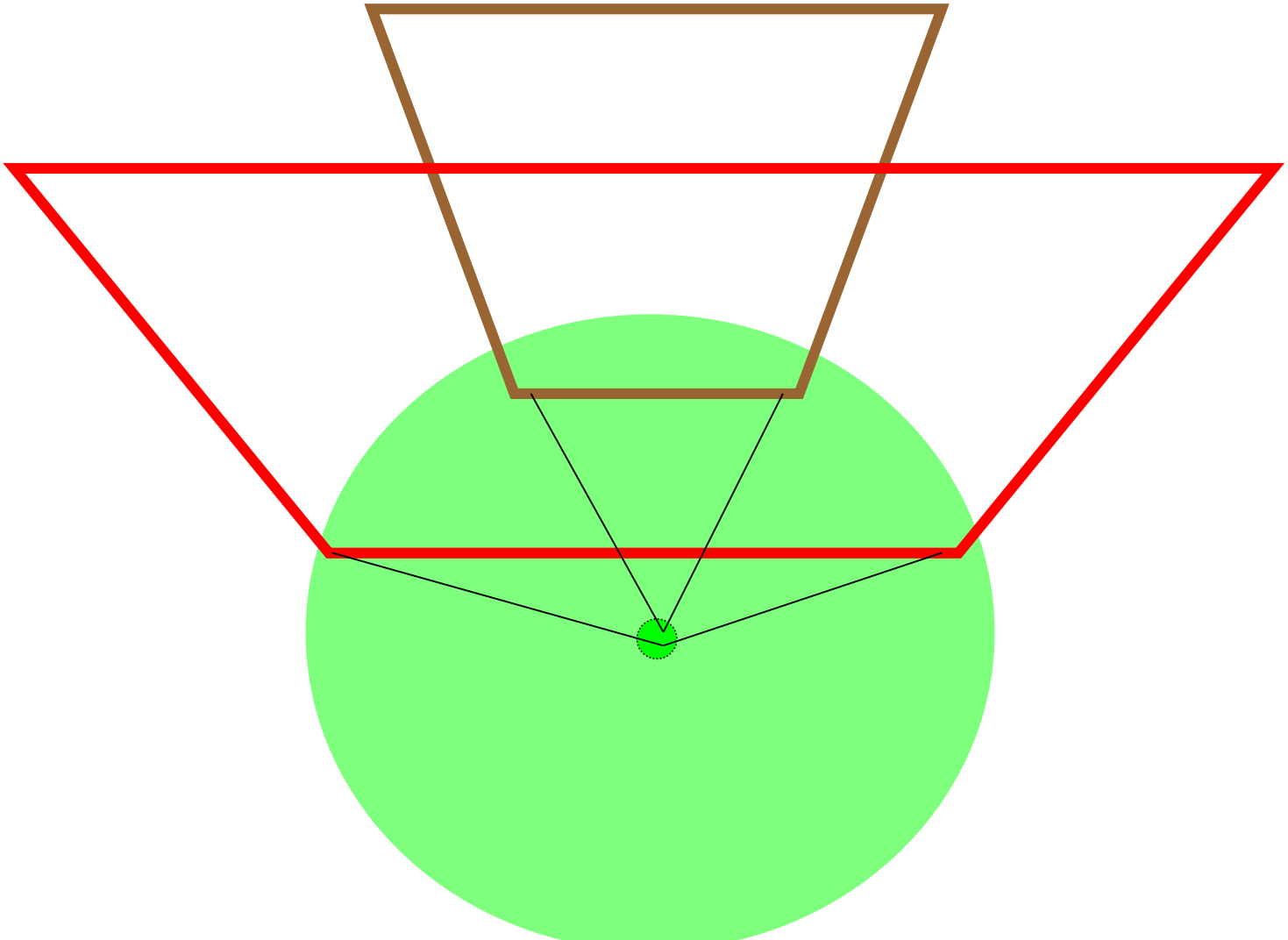
What is NA?

- Numerical Apertur
- Given on every professional objective
- Range: about 0.04 – 1.45

$\Delta R = 0.61 \cdot \lambda / NA$

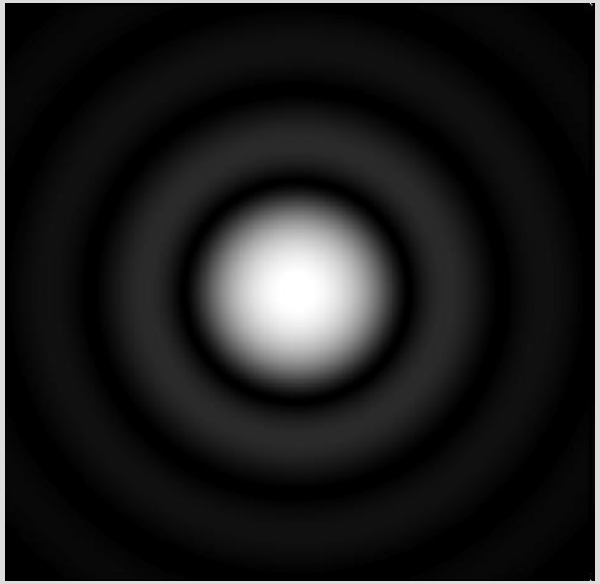
The aperture angle or acceptance angle

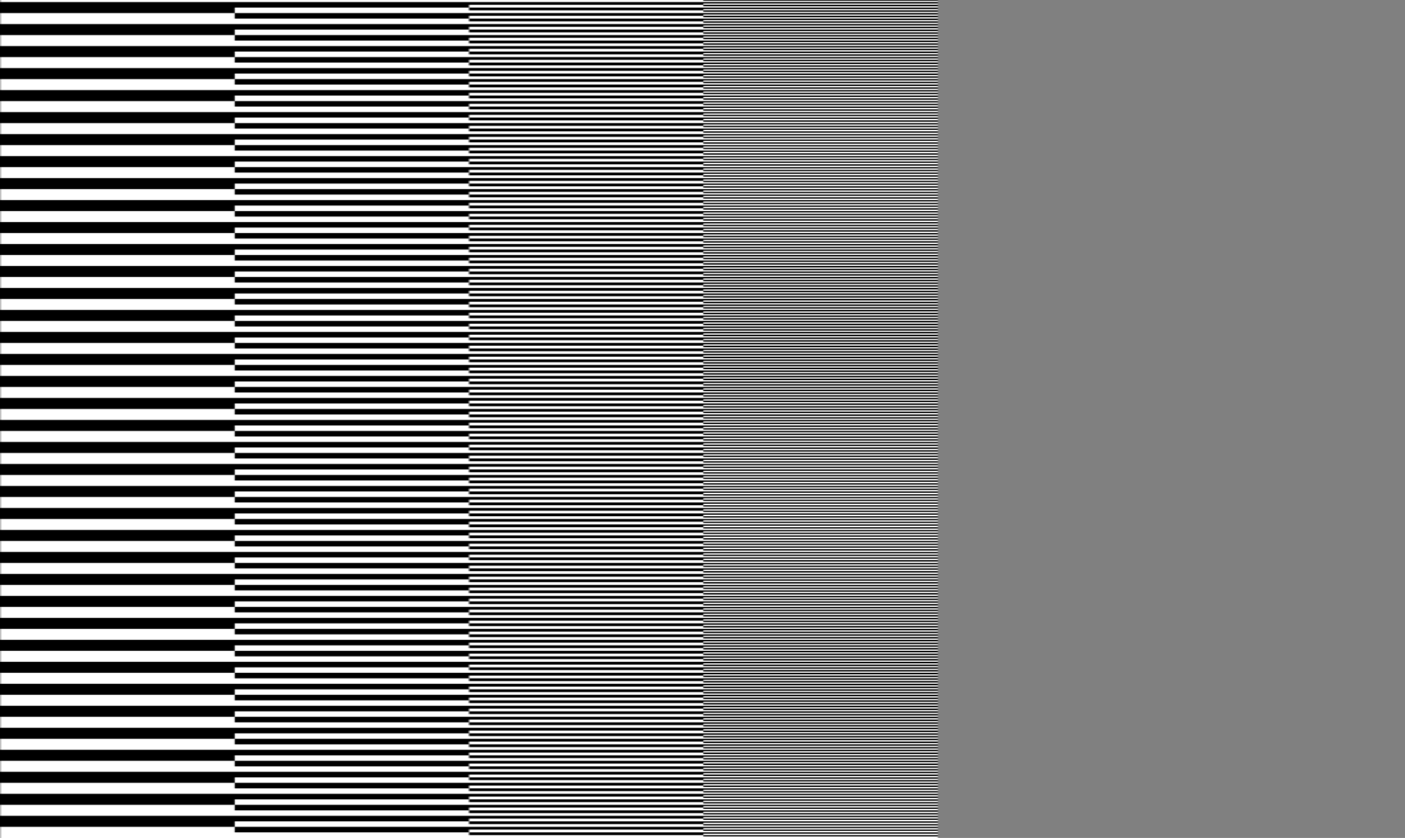
(deutsch: Öffnungswinkel)



The aperture angle of the objective

- With a larger acceptance angle of the front lens, additional diffraction rings will contribute to the image
- In particular, for some strongly diffracting (i.e. small) objects, the first maximum is now collected, which is otherwise missed
- => Better resolution is achieved.





Ernst Abbe, 1873:

- „durch kein Mikroskop können Teile getrennt (oder die Merkmale einer real vorhandenen Struktur wahrgenommen) werden, wenn dieselben einander so nahe stehen, dass auch der erste durch Beugung erzeugte Lichtbüschel nicht mehr gleichzeitig mit dem ungebeugten Lichtkegel in das Objektiv eintreten kann“.

- No microscope can separate parts (or properties of an existing structure), if they are so close to each other, that even the first ring maximum generated by diffraction will not enter the objective together with the undiffracted light.

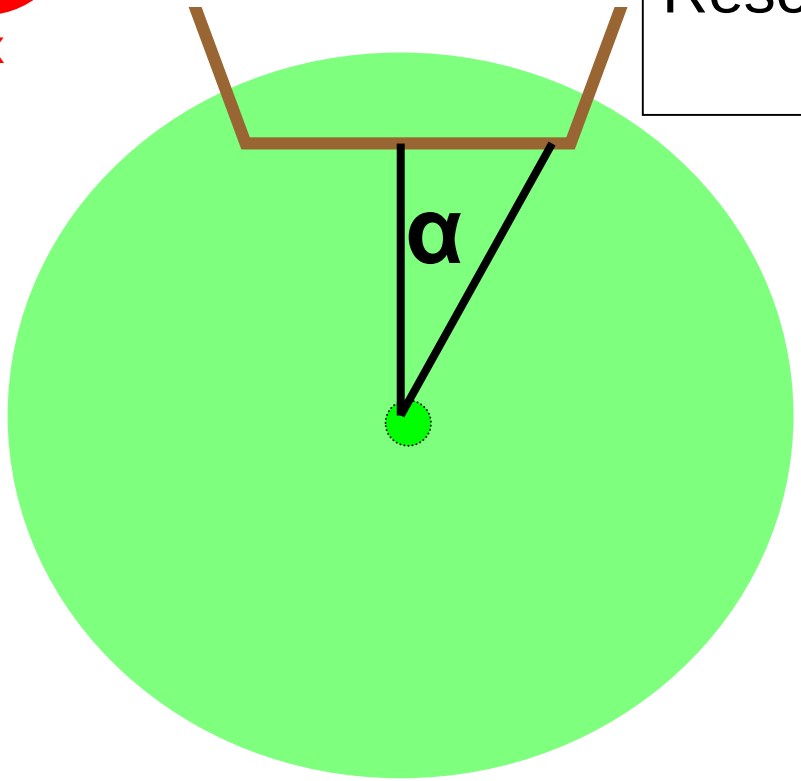
http://www.zeiss.de/C1257173002D0F60/0/06AF63BB52D85C74C1257185003F8553/$File/Innovation_15_18.pdf

Aperture Angle and NA

- The aperture angle is 2α
- Definition: **Numerical Aperture**
  NA = n • sinα

Refractive Index

Resolution = 0.61• λ / NA
= 0.61• λ /(n• sinα)



Why is the refractive index important for resolution?

But let‘s have some examples first

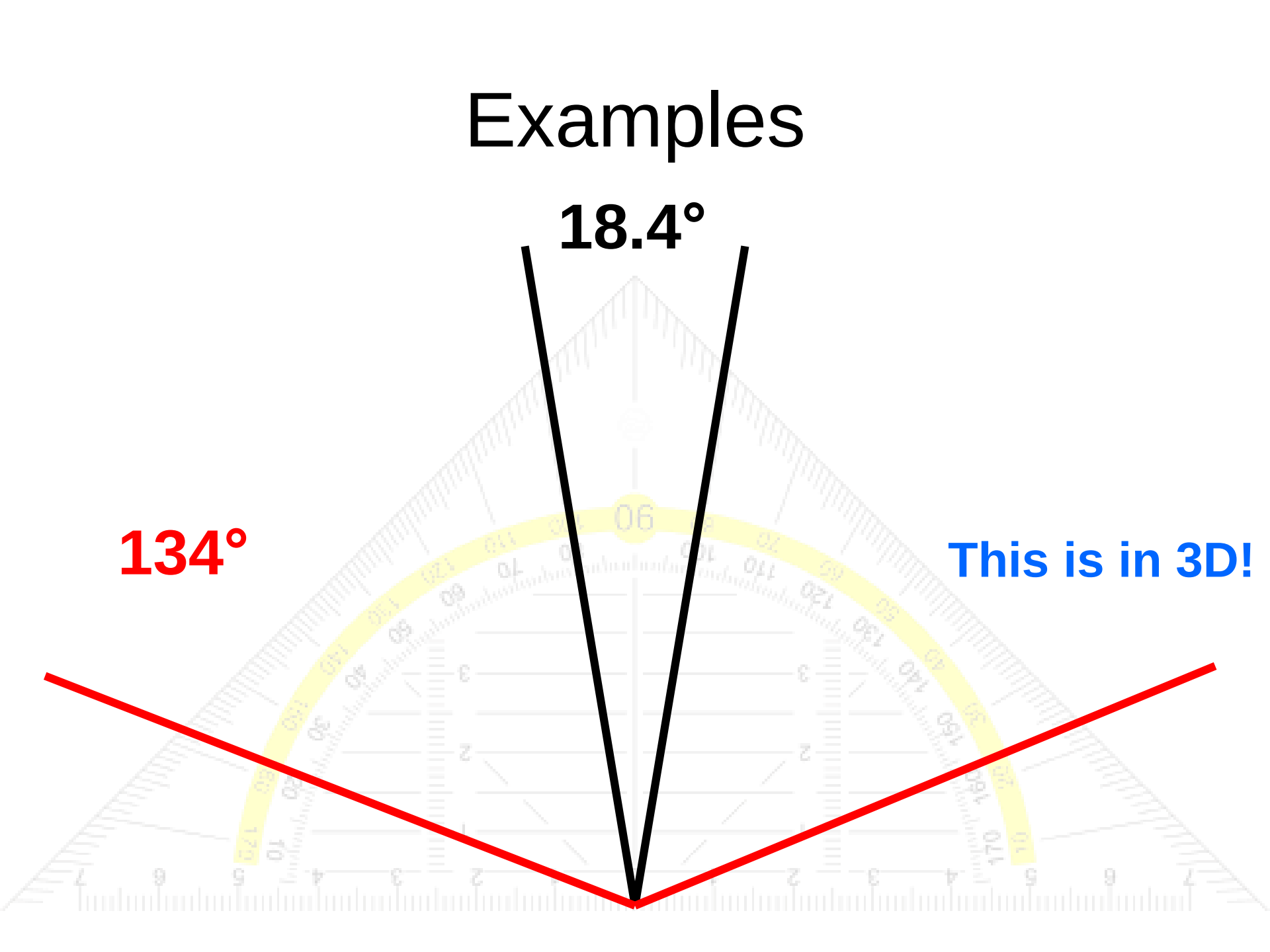
Examples

- NA = n • sinα

- 4x air objective NA = 0.16
  0.16 = 1 • sinα, α = 9.2°
  aperture angle 2α = 18,4°

- 100x oil objective NA = 1.4
  1.4 = 1.518 • sinα
  sinα = 0.92 , α = 67°
  aperture angle 2α = 134°

Examples



Take-home-message:

Higher NA means

- better resolution

and

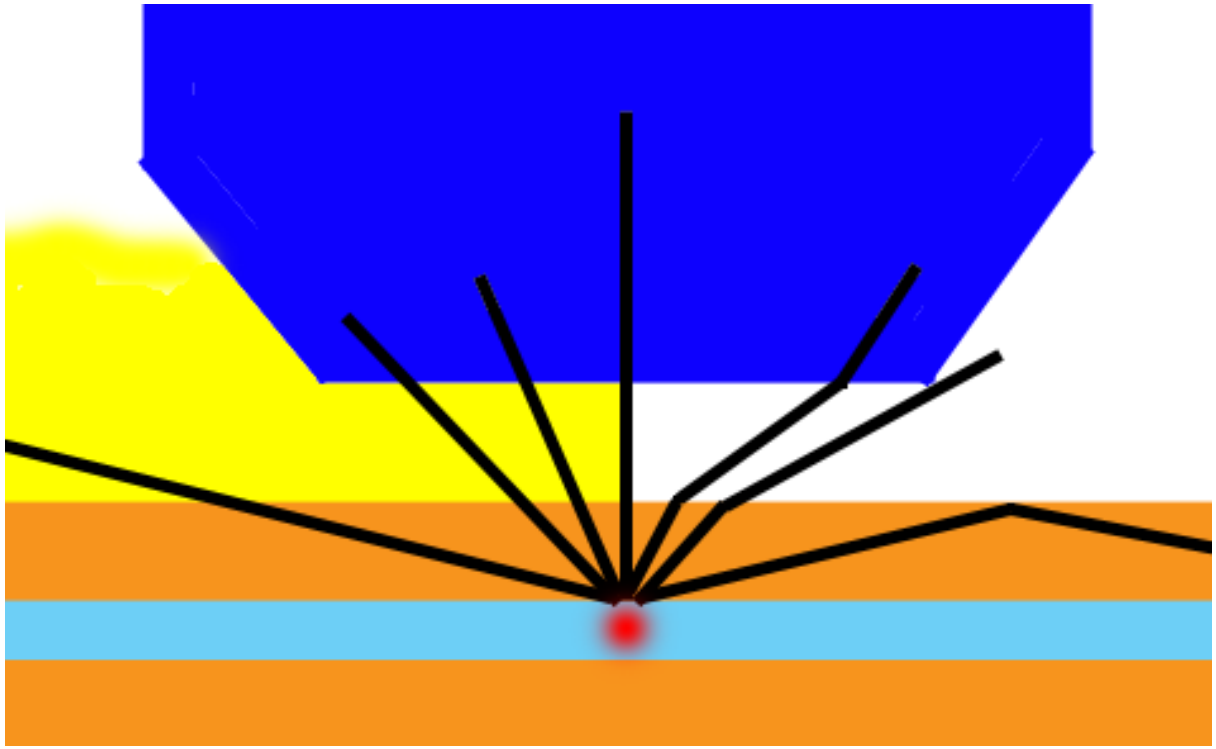
- more light

Why is the refractive index (Ri) important for resolution?

The Ri of the medium between coverslip and objective, that is.

Oil immersion

has better resolution because light from a larger angle (=more information) is collected



Optical density (refractive index = Ri) of oil is similar but not identical to glas

Maximal NA

- The theoretical maximum for the opening angle 2α is 180° (if the diameter of the front lens is infinite and the working distance 0.) For NA = n • sinα then applies:

- Dry objectives:
  NA = 1 • sin (90°) = 1
  Actual values are at most at 0.95, reflecting an opening angle of 72°.

- Oil objectives:
  NA = 1.518 • sin (90°) = 1.518
  Actual values are at most at 1.45.

NA

- Some objectives have an adjustment ring for the NA. This is <u>not a correction collar</u>!
- For fluorescence, make sure this is set to maximum!

$\Delta R = 0.61 \bullet \lambda / NA$

Maximal resolution with todays normal fluorescence microscopes (Rayleigh criterion)

Resolution = 0.61• λ / NA

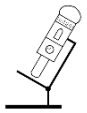
for a NA=1.4 Oil immersion objective:

Maximal theoretical resolution in xy

for λ = 500 nm:
d = 0.61 x 500 nm / 1.4 = 217 nm

For λ = 450 nm: d = 196 nm
For λ = 700 nm: d = 305 nm

Embedding medium and coverslips
–
Why you shouldn‘t just take what you find in the drawer



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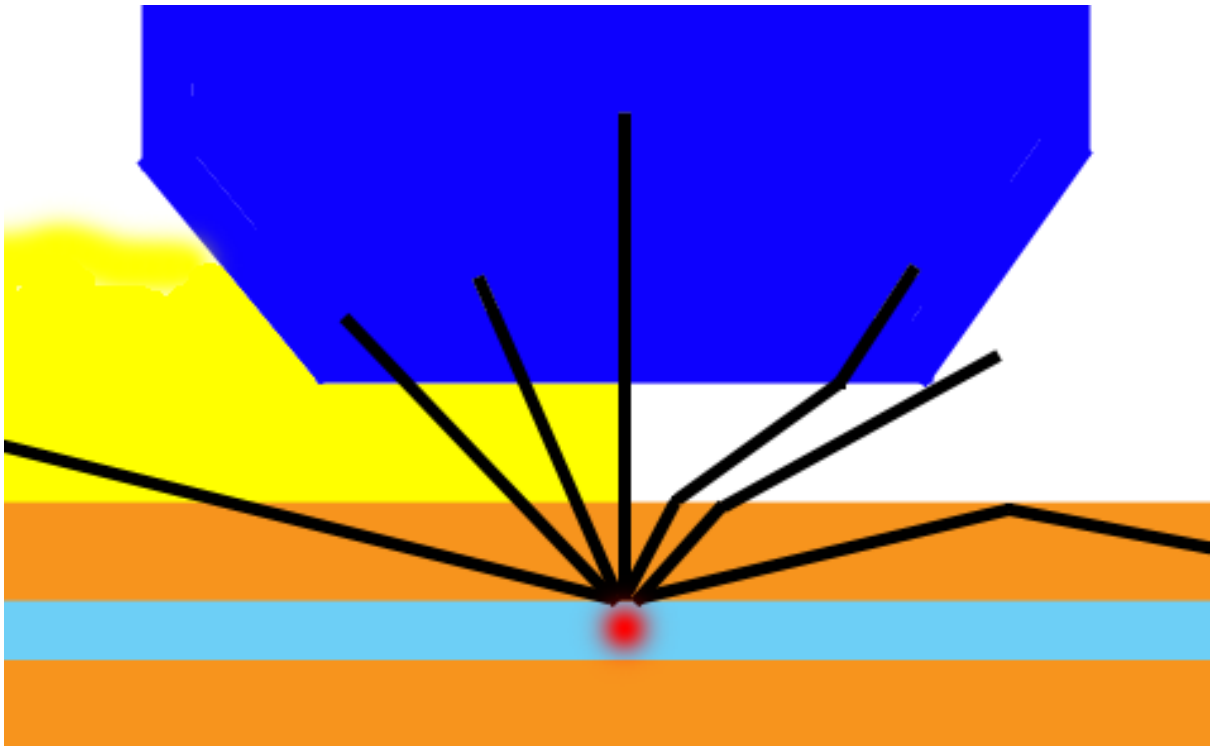
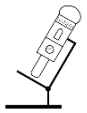
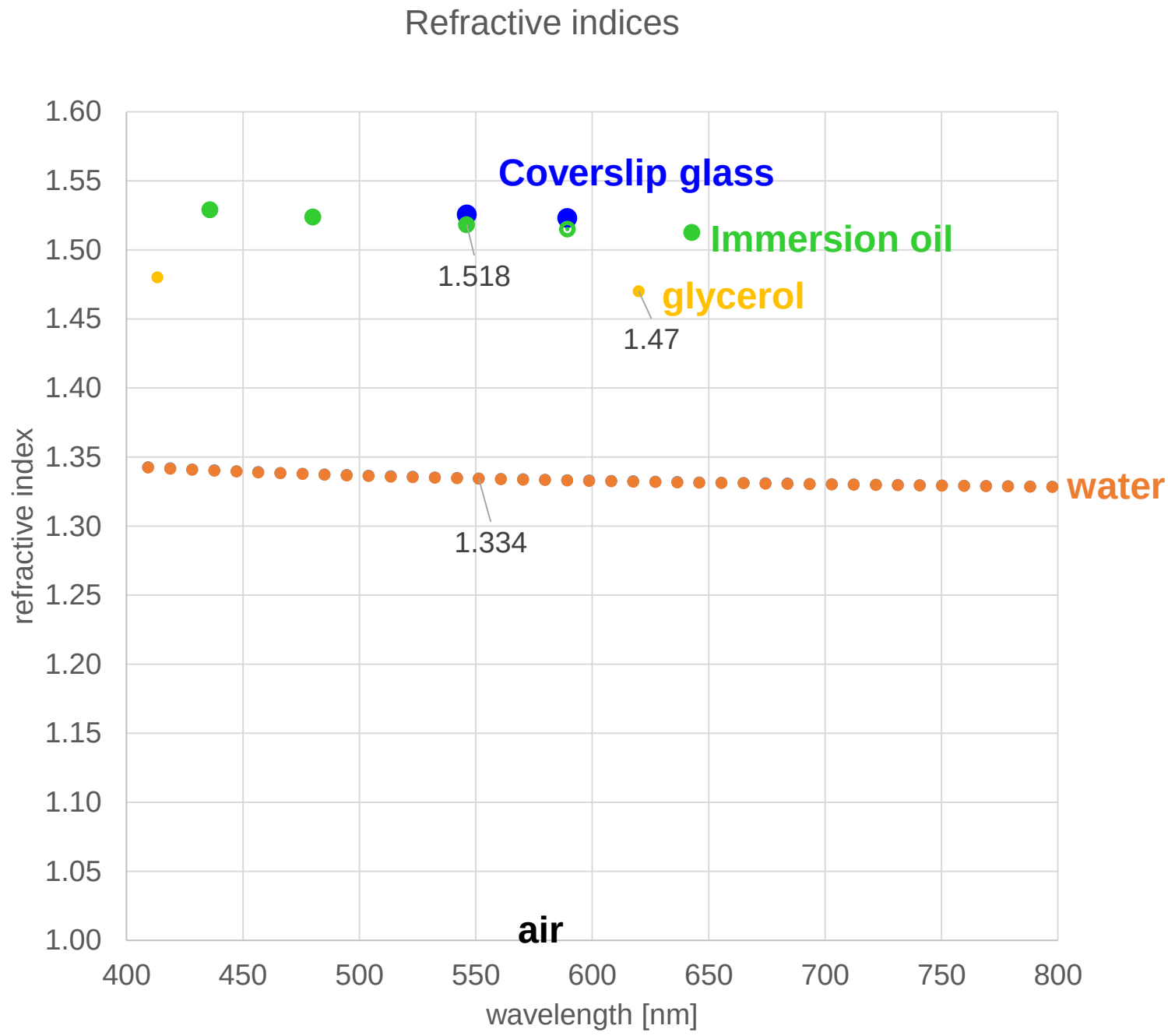


Ri mismatch

- Oil immersion objectives are calculated to be used with oil on **both** sides of the coverslip.
- Scientists don‘t usually embed their preparations in oil.
- So, we get an Ri mismatch
- Similar if you use a coverslip with a dipping water objective.

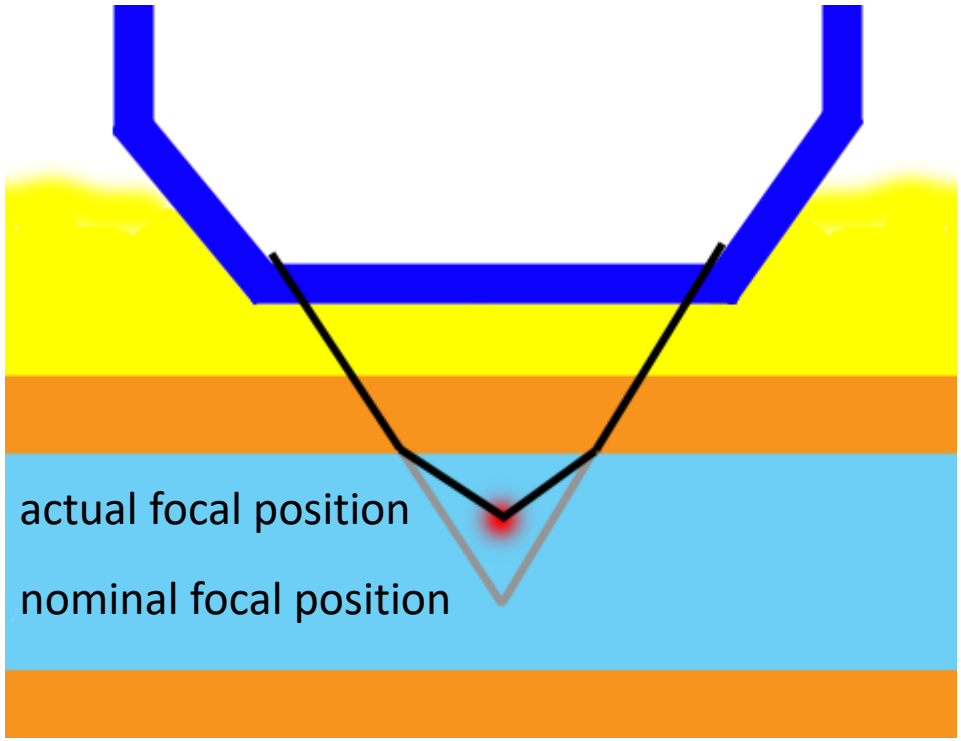




Data from http://refractiveindex.info

Ri mismatch

- **Consequence 1:** The focus you are looking at is not where the microscope (software) tells you it is.
- Example: Oil objective but cells are in water: You are only 8.2 µm away from the coverslip instead of 10.



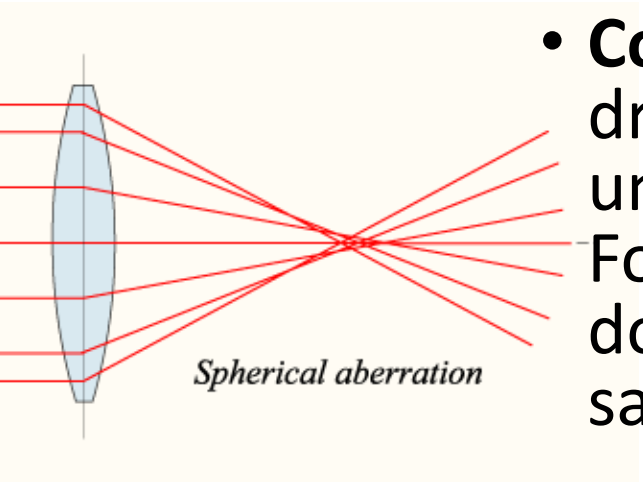
focal shift

oil
coverslip
water

Ri mismatch

Hell SW, Stelzer EHK: Lens aberations in confocal fluorescence microscopy. In: *Handbook of biological confocal microscopy.* 2nd edition. Edited by Pawley JB. New York: Plenum Press; 1995: 347-354.

Ri mismatch



- **Consequence 2**: The resolution goes down dramatically with depth, due to uncorrected spherical aberration.
  For distances from the coverslip > 20 µm don‘t use an oil immersion objective with a sample in water (or glycerol, respectively).

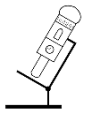
- **Which is why cultured cells should always be on the coverslip, not on the slide.**

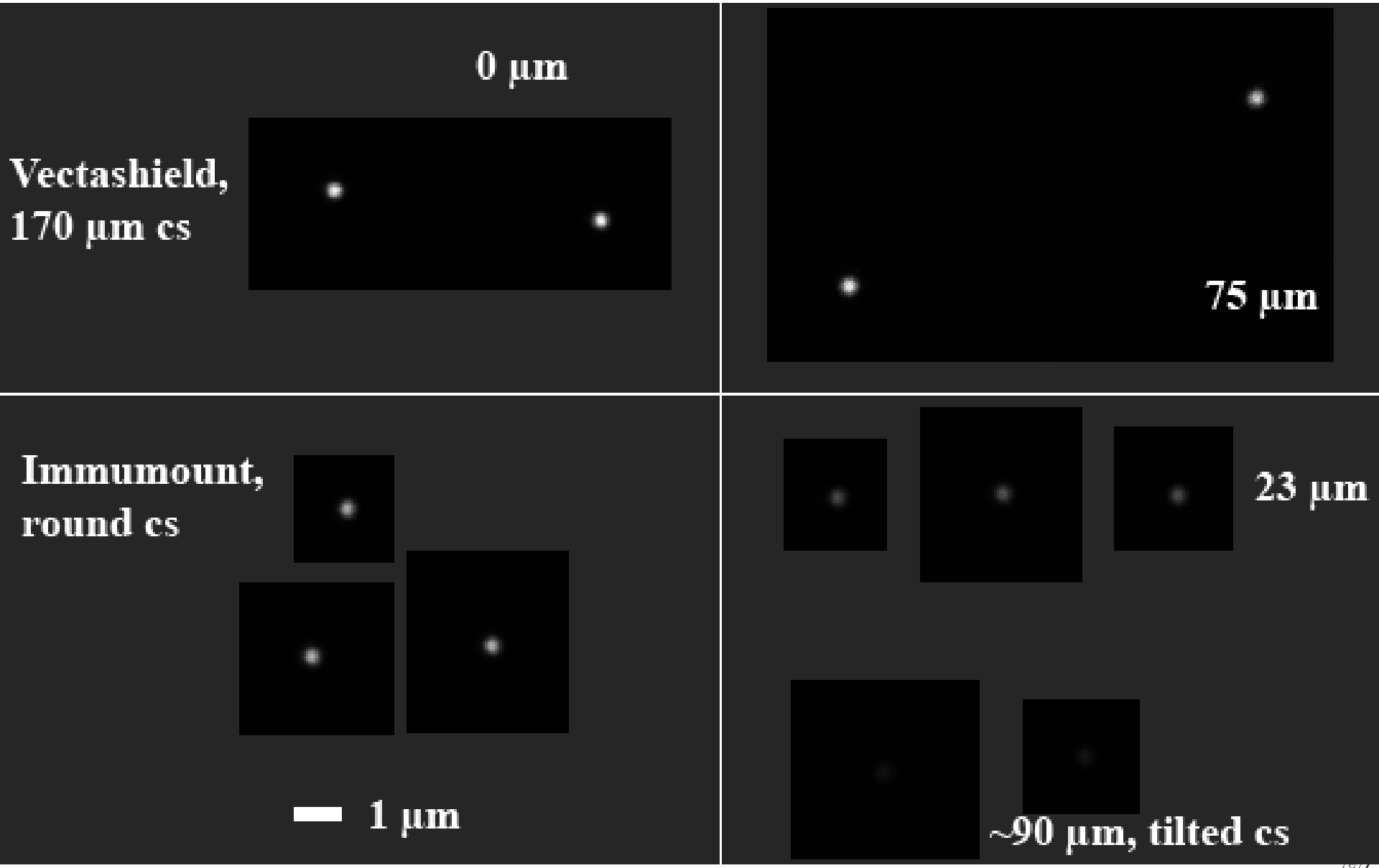
Image source:
https://commons.wikimedia.org/wiki/File:Lens-sphericalaberration.png
Created by DrBob and published under CC BY-SA 3.0

Ri mismatch

- **Consequence 3**: The brightness goes down dramatically with depth, due to uncorrected spherical aberration.

- **Which is why you should care even if you don‘t need a good resolution.**

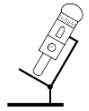




Are you in trouble with your preparation?

Find out with self-made test slides:

- Coverslips with dried-on fluorescent beads (or Qdots or gold beads)
- Object slides with dried on beads.
- Make several test preparations with variable amounts of that old mounting medium you found in some fridge. This leads to a variable distance beteween coverslip and slide.
- Measure and compare FWHM and brightness of beads on coverslip with those on slides in variable depths.

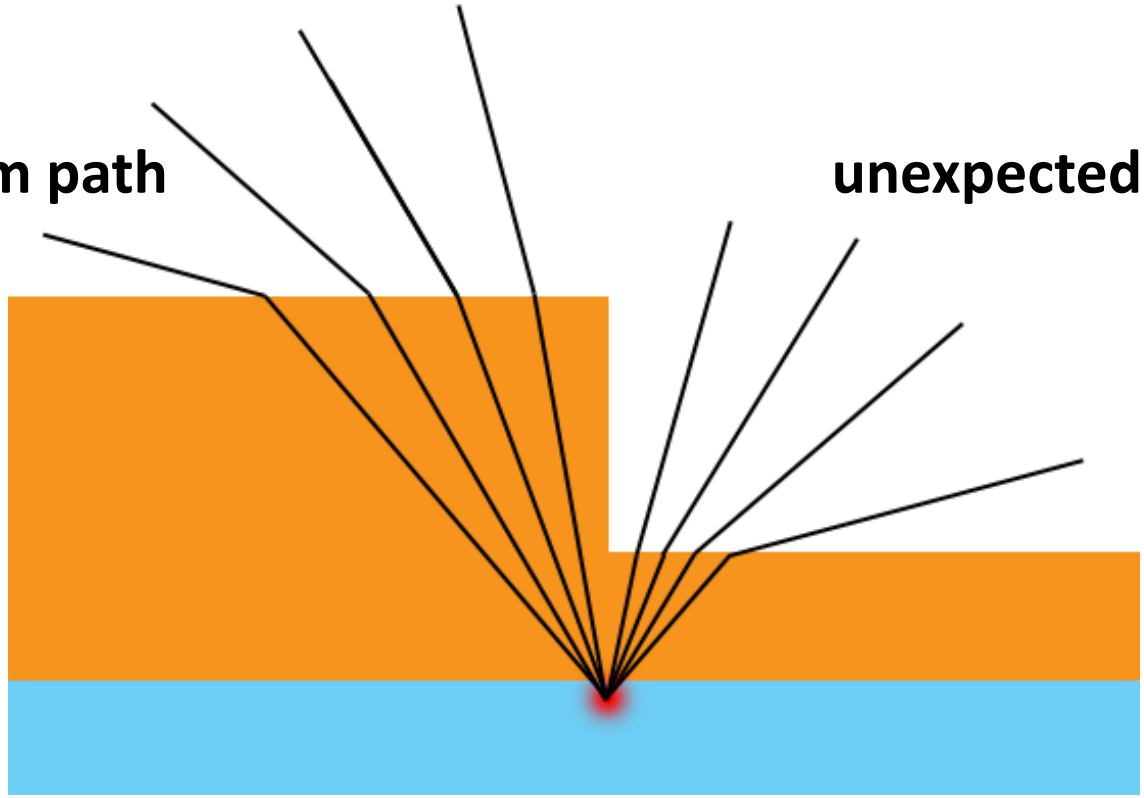


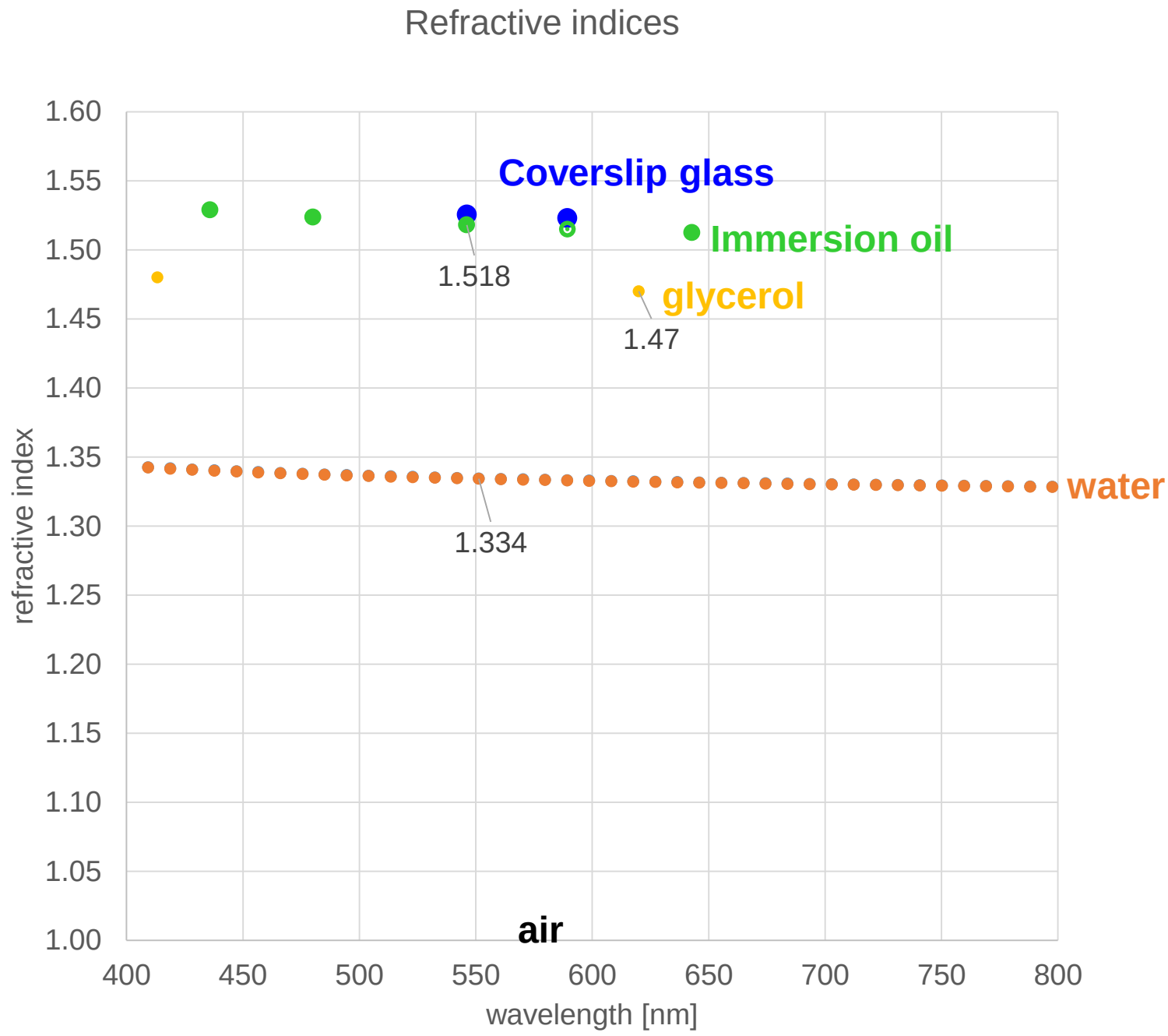
Coverslip thickness

- To avoid uncorrected spherical aberration, the coverslip must have the right thickness!

expected beam path

unexpected beam path





Data from http://refractiveindex.info

Coverslip thickness

- The right thickness is 0.17 mm = 170 µm. This is what microscopy companies have their objectives designed for.

- Unfortunately, this is not what you usually get.

Coverslip thickness

- Problems are even worse with water immersion or dry objectives with high N.A.

- If you want to do high resolution microscopy, you should get 170 µm coverslips, (“Thickness 1½ “ or better).

- We use the ones from www.hecht-assistent.de

Coverslips

- Some objectives („Corr“) have correction collars with which they can be adapted to varying coverslip thicknesses.
- Some can handle 0-2 mm.

Coming Up:

Principles of Fluorescence and Fluorescence Microscopy

Image processing and image presentation

Some Aspects of Digital Imaging

Walter Brendel Zentrum München

BIN LMU BioImaging Network Munich

Steffen Dietzel

Walter-Brendel-Zentrum für Experimentelle Medizin

Core Facility Bioimaging at the Biomedical Center

Principles of Fluorescence and Fluorescence Microscopy

XXX. *On the Change of Refrangibility of Light.*

By G. G. Stokes, *M.A., F.R.S., Fellow of Pembroke College, and Lucasian Professor of Mathematics in the University of Cambridge.*

Received May 11,—Read May 27, 1852.

black cloth or velvet behind, or by some similar contrivance. It has been usual to speak of the colour so exhibited as displayed by reflexion. As however the cause now appears to be so very different from ordinary reflexion, it seems objectionable to continue to use that term without qualification, and I shall accordingly speak of the phenomenon as *dispersive reflexion**. Thus dispersive reflexion is nothing more than internal dispersion considered as viewed in a particular way.

* I confess I do not like this term. I am almost inclined to coin a word, and call the appearance *fluorescence*, from fluor-spar, as the analogous term *opalescence* is derived from the name of a mineral.

Fluorescence

Loss of thermal energy

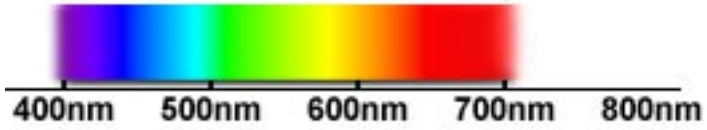
Emission

- Fluorescence is the property of some substances to get excited by light of certain wavelengths and to emit light of other wavelengths.
- In contrast to phosphorescence, there is no „after-glow“

Image sources: http://www.olympusmicro.com/primer/lightandcolor/fluorointroduction.html

Fluorescence

- Usually, the emitted photons have less energy than the absorbed ones. Thus, fluorescence has a longer wavelength than excitation light.



- Every fluorochrome has a characteristic excitation and emission spectrum

- The distance between the excitation and emission maxima is called Stokes-Shift

Fluorochromes

- Apart from the spectra, three properties are of paramount importance:
- Extinction Coefficient: The larger, the more photons are absorbed.
- Quantum Yield: The ratio of absorbed and emitted photons. The theoretical maximum is 1.
- Extinction Coefficient x Quantum Yield = Brightness.

Brightness = ε Φ

Marcel P. Bruchez, „State of the Art and Beyond:
Fluorescent Probes for Living Cells„

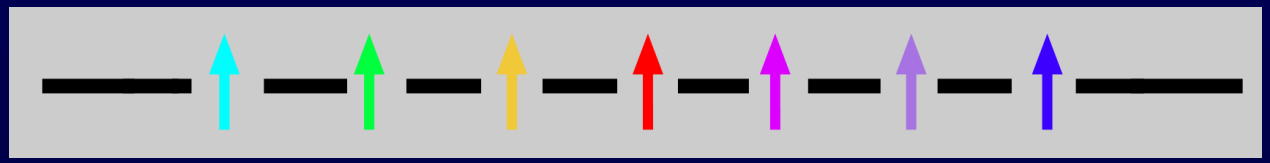
Maxima of popular fluorochromes

	Excitation Max.	Emission Max.	
Nucleic Acid binding fluorochromes			
Hoechst (both versions)	350	461	
Dapi	**358**	**461**	
Chromomycin A3	445	575	
TOTO-1	514	533	
Ethidium Bromide	518	605	
Propidium Iodide	535	617	
LDS-751	543	712	
To-Pro3	642	661	
General fluorochromes			
AMCA	349	448	
DEAC	426	480	
Fluorescin (FITC)	**494**	**518**	
Alexa488	495	519	
Cy2	489	506	
OregonGreen488	496	524	
Alexa532	532	554	
Tamra	552	575	
Cy3	**553**	**575**	
Tetramethylrhodamine (TRITC)	**555**	**580**	
Alexa564	556	573	
Alexa568	578	603	
Cy3.5	581	596	
Alexa594	590	617	
Texas Red	595	615	
Alexa633	632	647	
Cy5	**649**	**670**	
Alexa660	663	690	
Cy5.5	675	694	
Alexa680	679	702	
Cy7	743	767	
Fluorescent Proteins			
EBFP	380	440	
ECFP	434	477	
GFP (S65T, EGFP)	489	508	
EYFP (10C)	514	527	
dsRed	558	583	

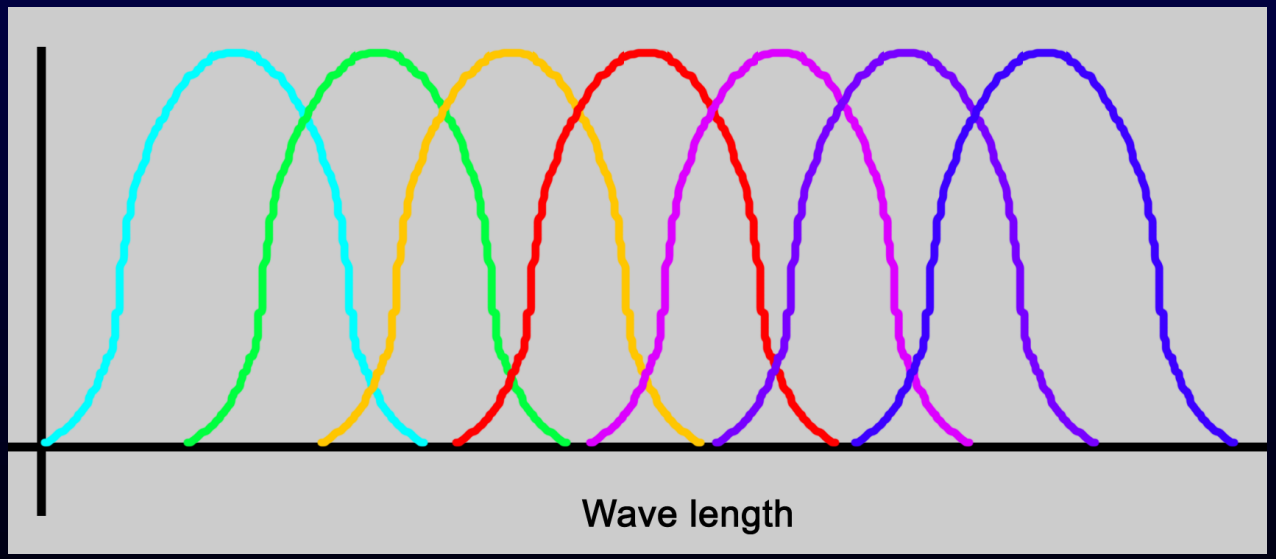
Web-sites:
http://www.probes.com/servlets/spectra
http://www.apbiotech.com/product/publication/lsn/17/p2.1.html

How many fluorochromes can be used in microscopy simultaneusely?

Cooled charged-couple device (CCD) camera



DEAC SG Cy3 Cy3.5 Cy5 Cy5.5 Cy7



I.Solovei 2003

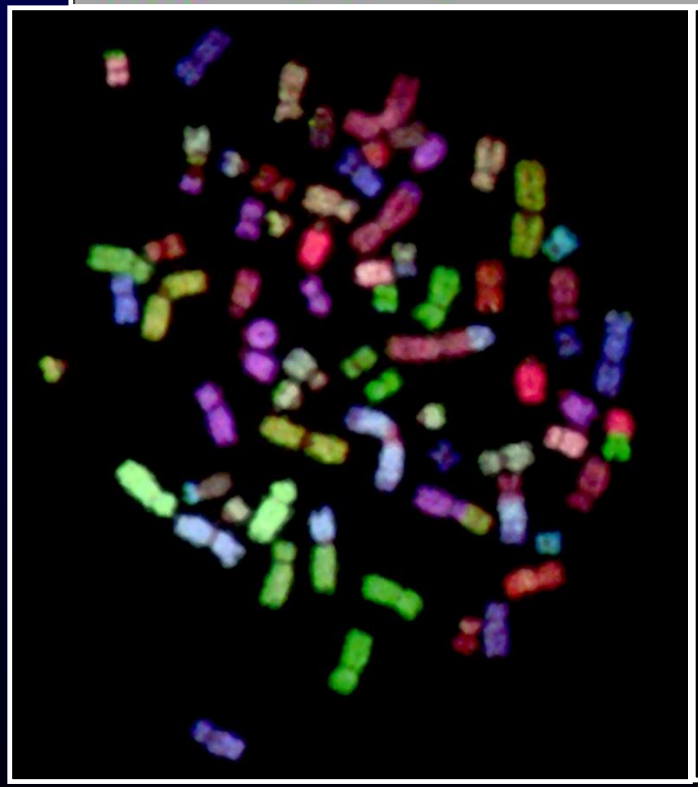
Classical scheme with 5 fluors for M-FISH on 2D chromosomes:

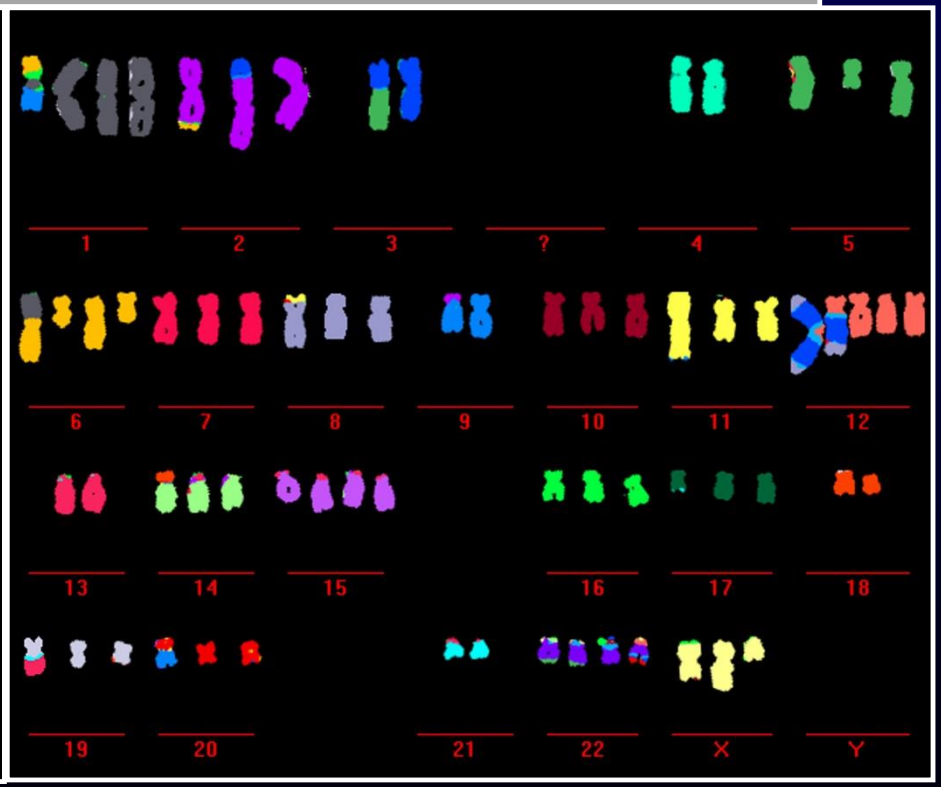
Display Combinations and Colours

Number of combinations: 25

Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	ps
Colour																									
FITC	X					X				X			X			X							X		X
Cy3	X			X	X		X											X	X		X				
Cy3.5			X			X		X			X				X			X		X					
Cy5			X	X					X	X							X					X		X	X
Cy5.5		X										X	X		X		X				X				

Cy 7





I.Solovei 2003

Fluorescence Microscopy

Barrier Filter

Beam Splitter

Excitation Filter

Beam path in the classic fluorescence microscope

Fluorescence microscope requirements

- A lot of excitation light is needed. Therefore very effective emission filters must completely suppress reflected excitation.
- All glass lenses must be very transparent to minimize loss of the (comparatively) week fluorescence light.
- For excitation of DAPI, glass must be UV transparent.

Fluorescence microscopy

- A new light source is created in the specimen!
- The direction of the excitation is therefore not important (allowing light sheet microscopy).
- Emitted photons are distributed equally in all directions.
- Doubling the NA increases amount of light four fold.

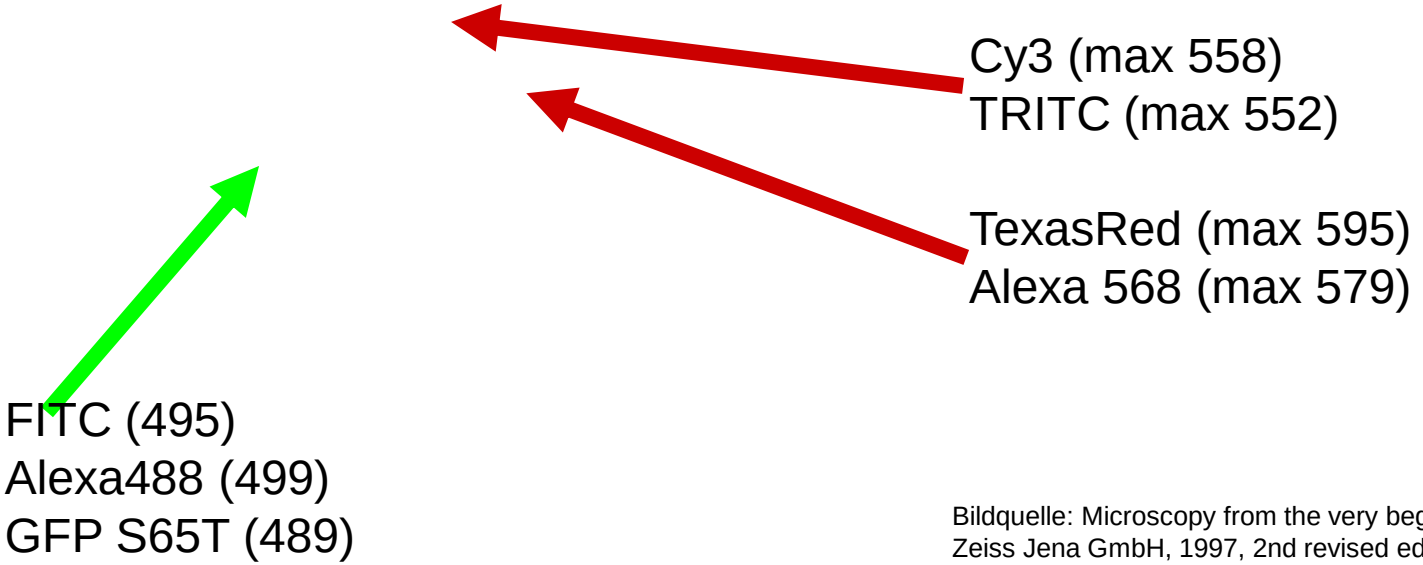
Fluorescence microscopy

- Only a small fraction of the excitation light causes fluorescence. Therefore a powerful light source is needed.

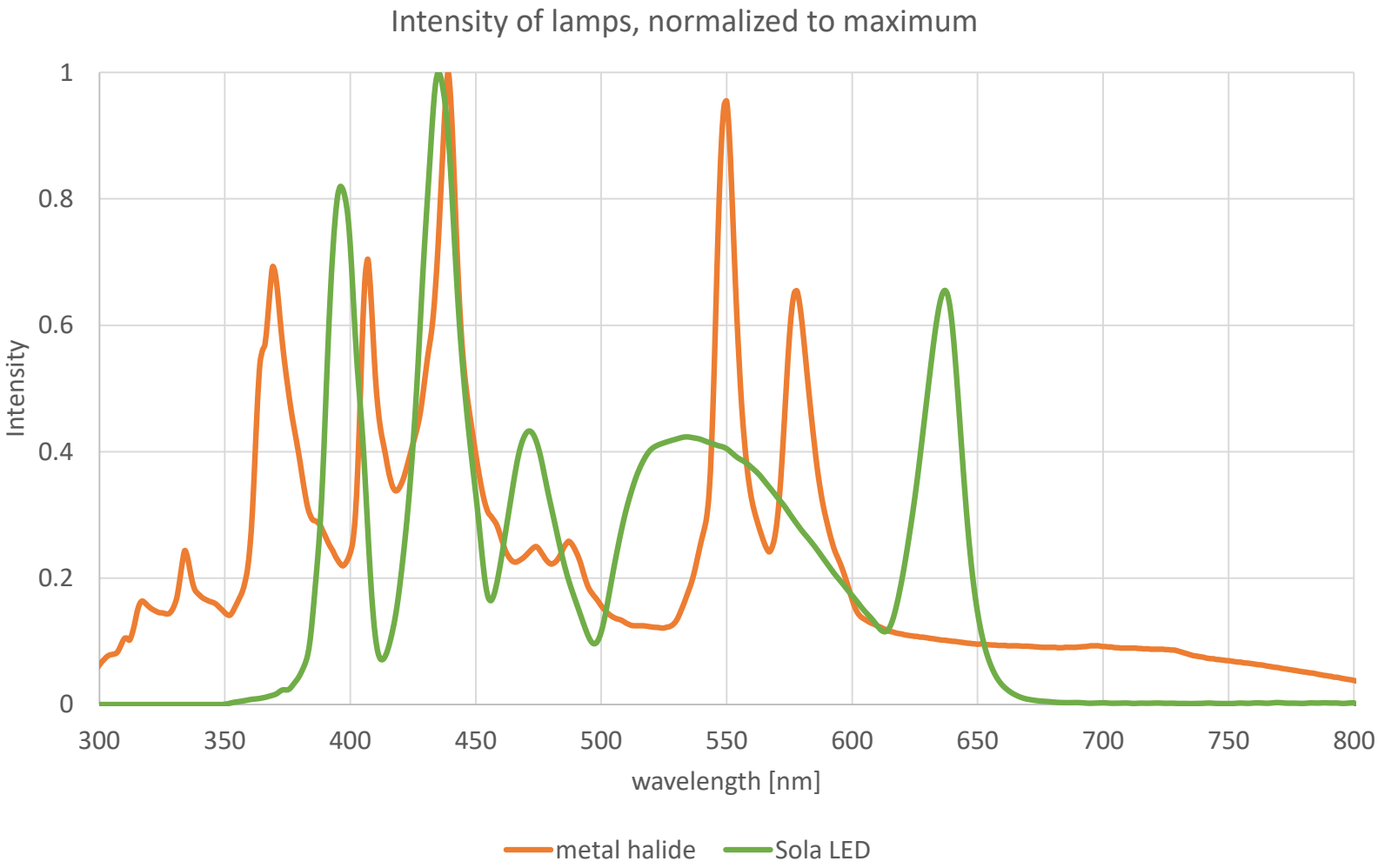
- Caution: Very strong excitation may cause saturation.

Mercury lamp

- Emission spectrum



Bildquelle: Microscopy from the very beginning Carl Zeiss Jena GmbH, 1997, 2nd revised edition



Data kindly provided by Iain Johnson, Lumencor, in 2015.

Bleaching and Anti-Fade

„In CLSM, fluorochromes under incident light are excited and emit fluorescence. A certain proportion ... of the excited fluorochromes are oxidized and will no longer emit fluorescence ... This is the phenomenon of photobleaching“

M. Ono et al, Journal of Histochem. 49 / 2001

Anti-Fading Reagents

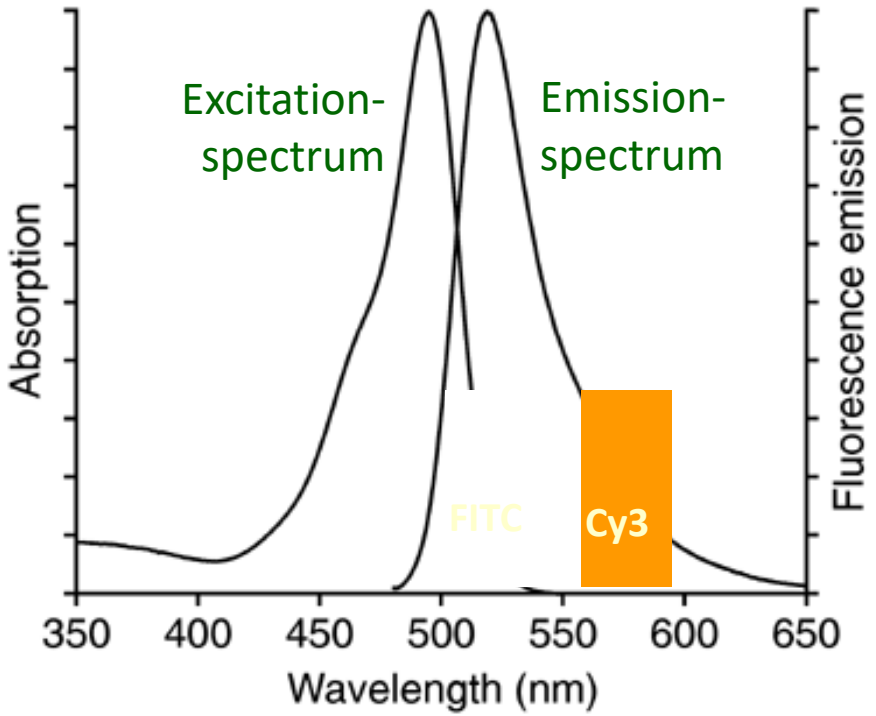
M. Ono et al, Journal of Histochem.
49 / 2001

FITC–
phalloidin-stained NIH 3T3 cells

Choose fluorochromes and filters carefully

Bleed through = cross talk

- Check each fluorochrome for bleed through to other filters.
- Sequential recording (instead of simultaneous) helps



FITC goat anti–mouse IgG antibody/pH 8.0
http://probes.invitrogen.com/servlets/spectra?fileid=143iggp8

How to select fluorochromes

The following parameters should be considered:

- Spectral properties of the light source
- Spectral properties of available filters
- Spectral properties of the detector. Example: The eye is most sensitive with green, followed by orange, but not sensitive in the far red (>650 nm)
- Spectral properties of the fluors
- (Theoretical) brightness (Absorption coefficient x Quantum yield)
- Potential bleed through in neighboring color channels (Example: FITC, Cy3/Cy3.5)

Fluorescence microscopy

- Structures smaller than the resolution limit can be detected
- Fluorochromes can bleach and thus distort results.
- To reduce bleaching, always block excitation when it is not used!!!!!!!

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

BIOMEDICAL CENTER
CORE FACILITY BIOIMAGING

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Image processing and image presentation

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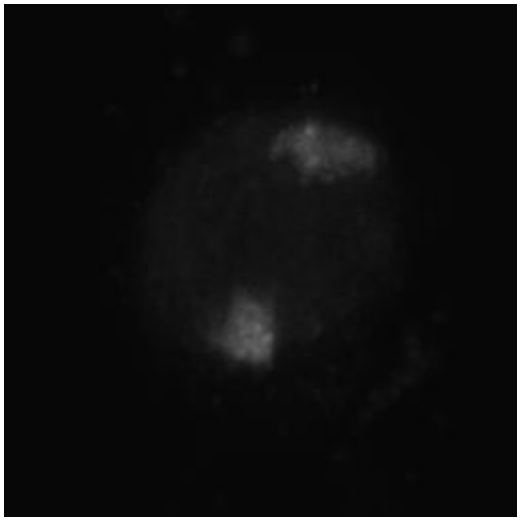
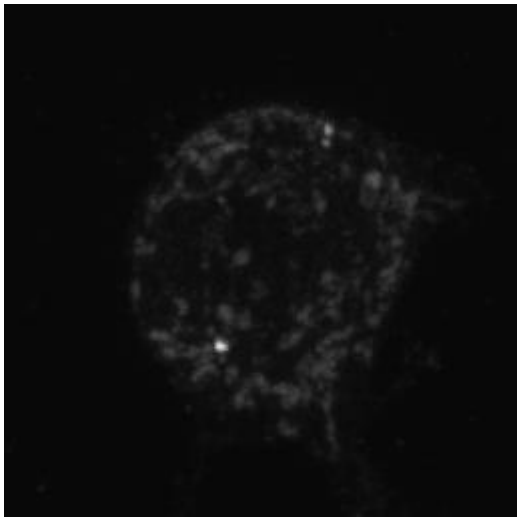
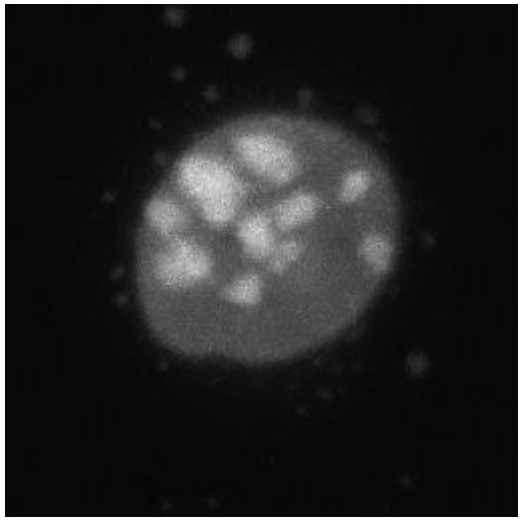
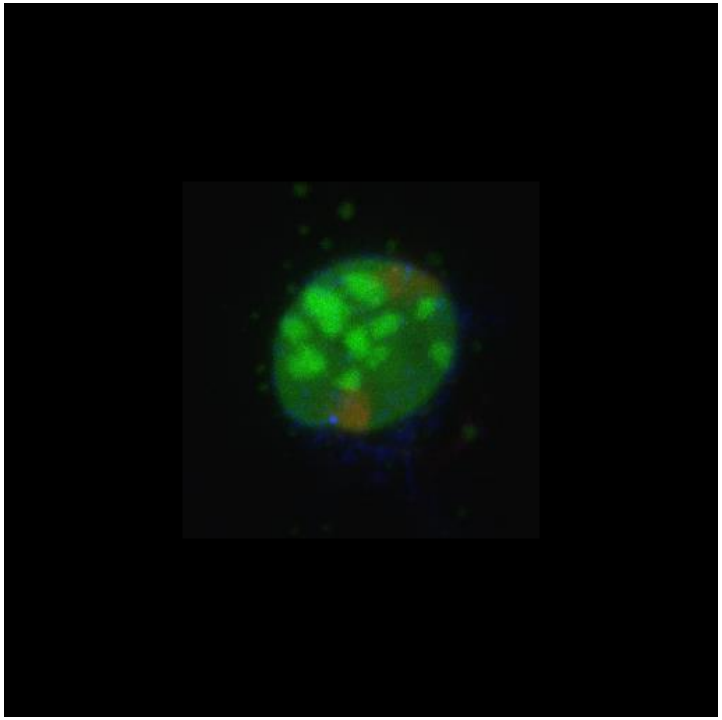
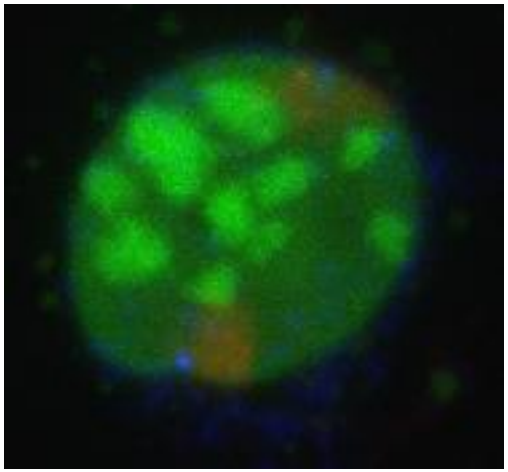
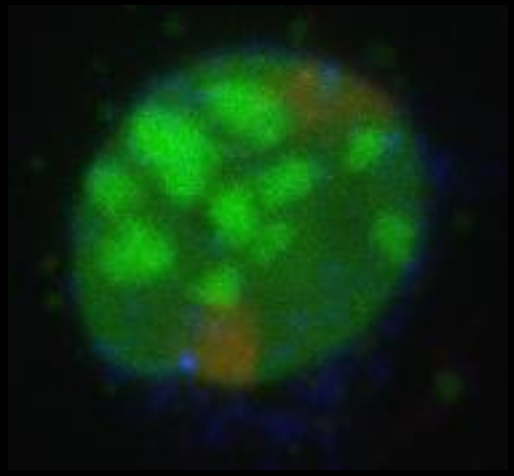






Who cares about the black space around the nucleus?

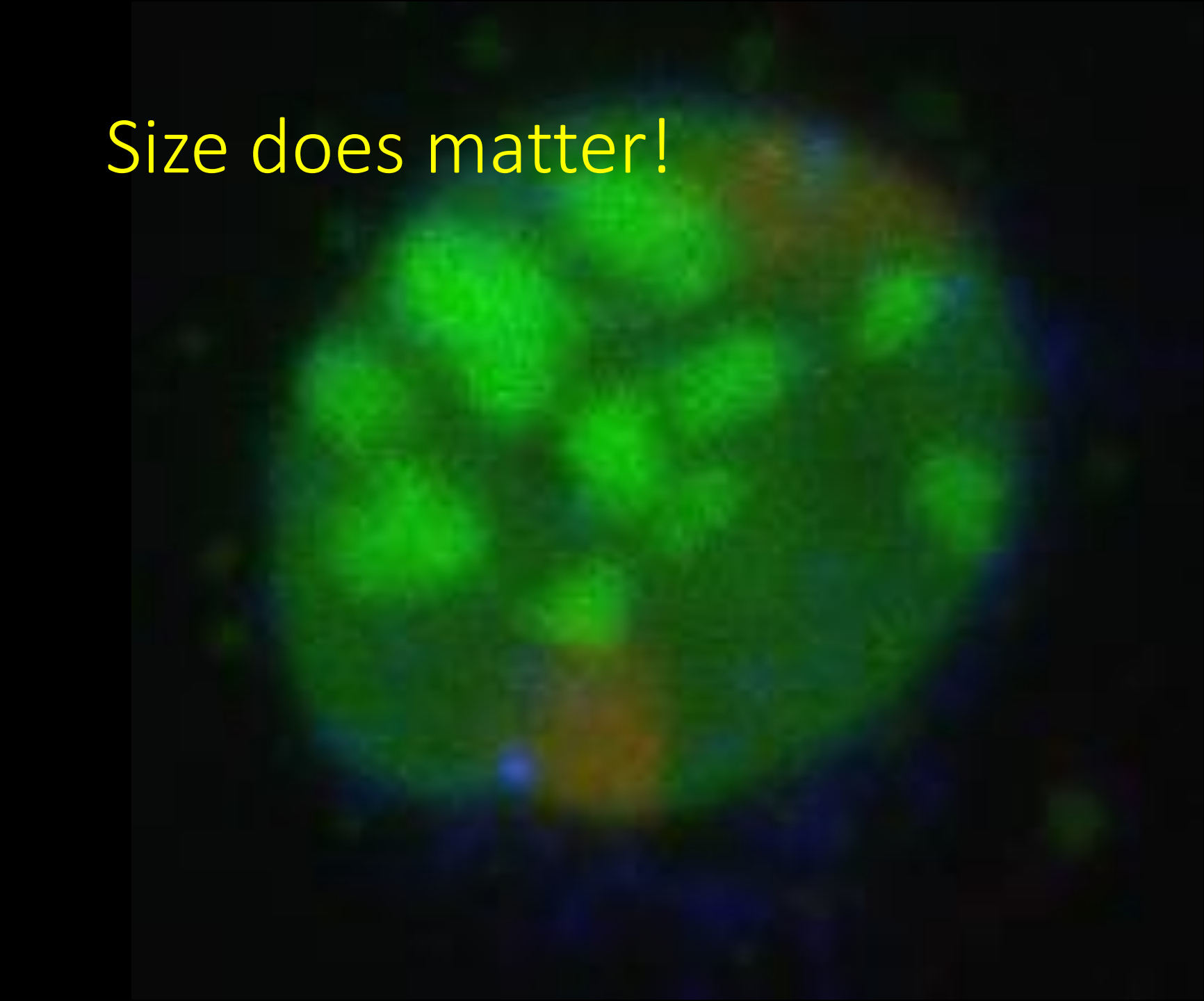


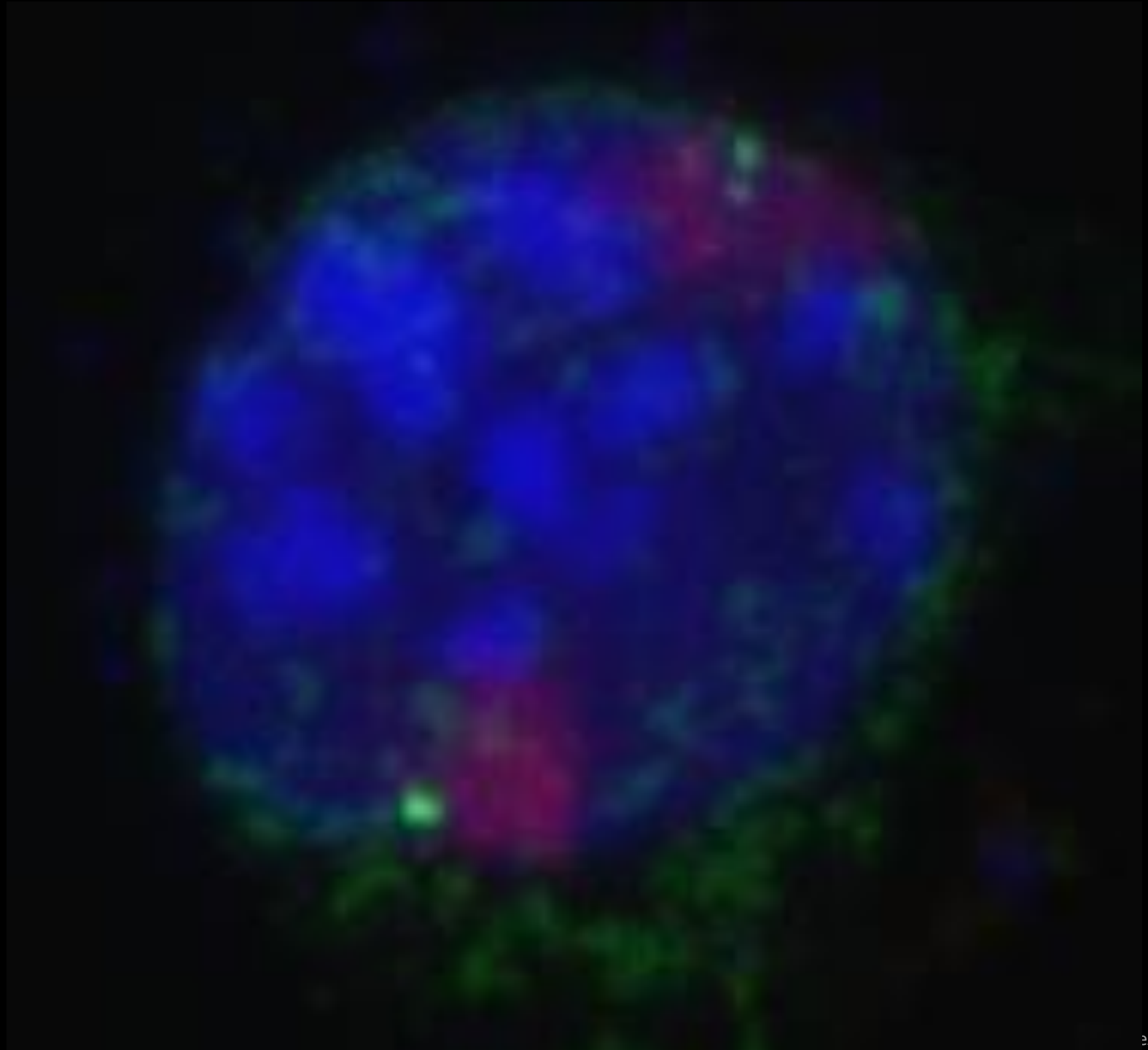
Don‘t use huge bright background with fluorescence images

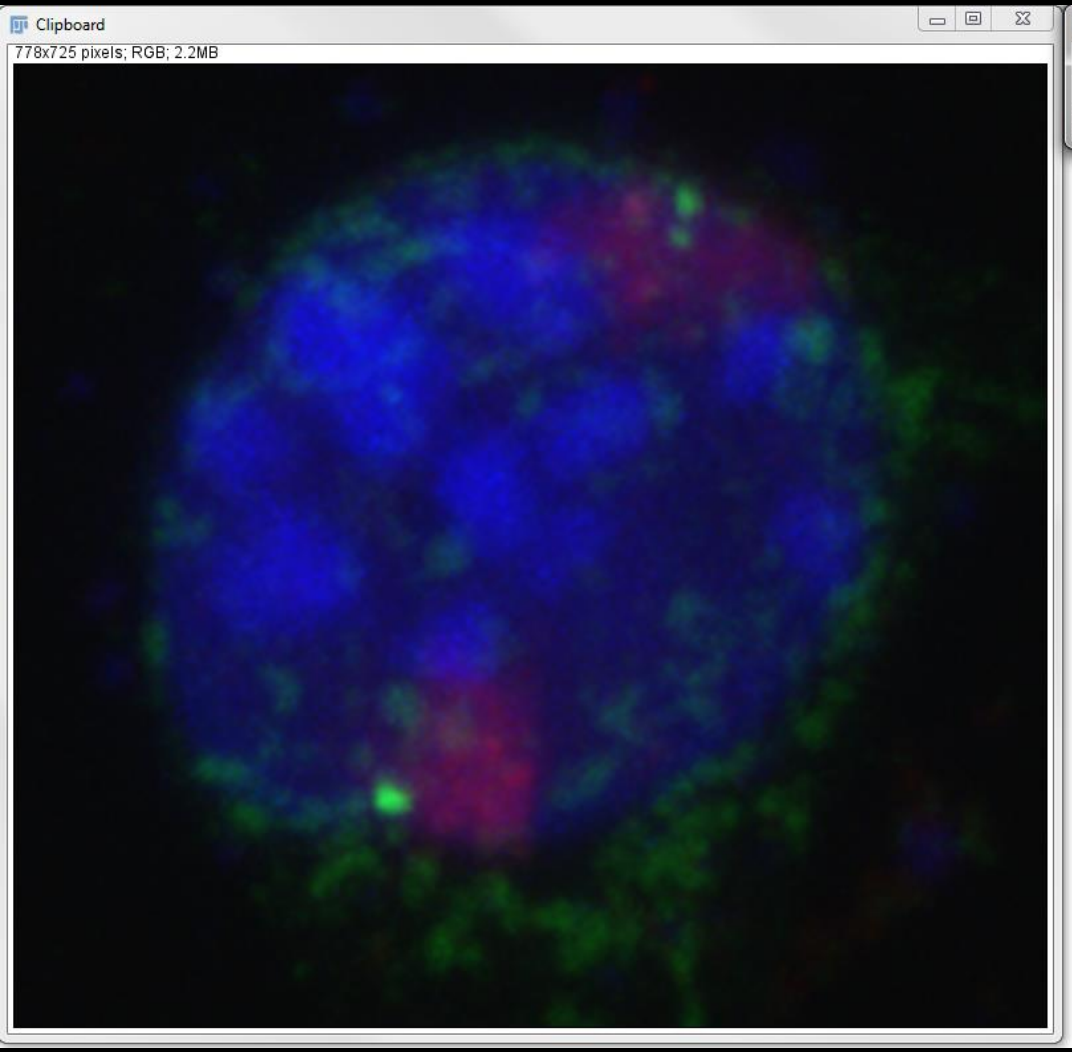


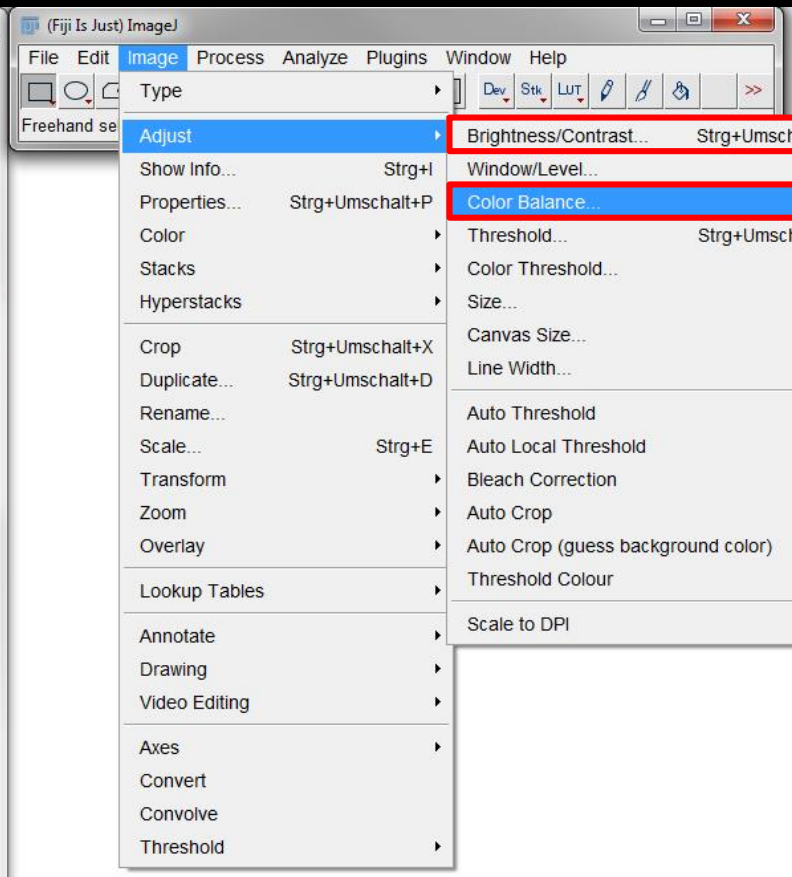
Don‘t use huge bright background with fluorescence images

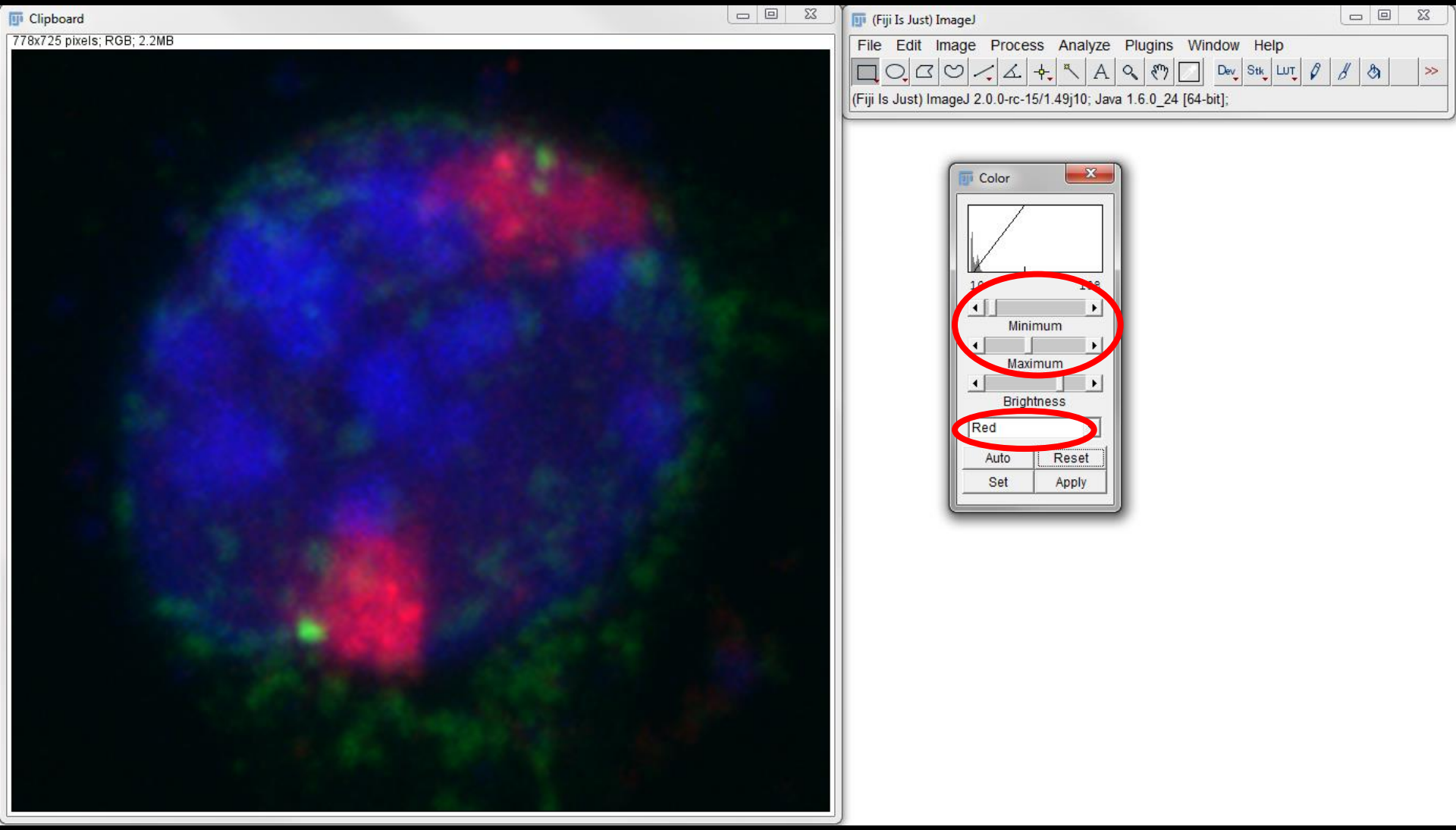
Size does matter!

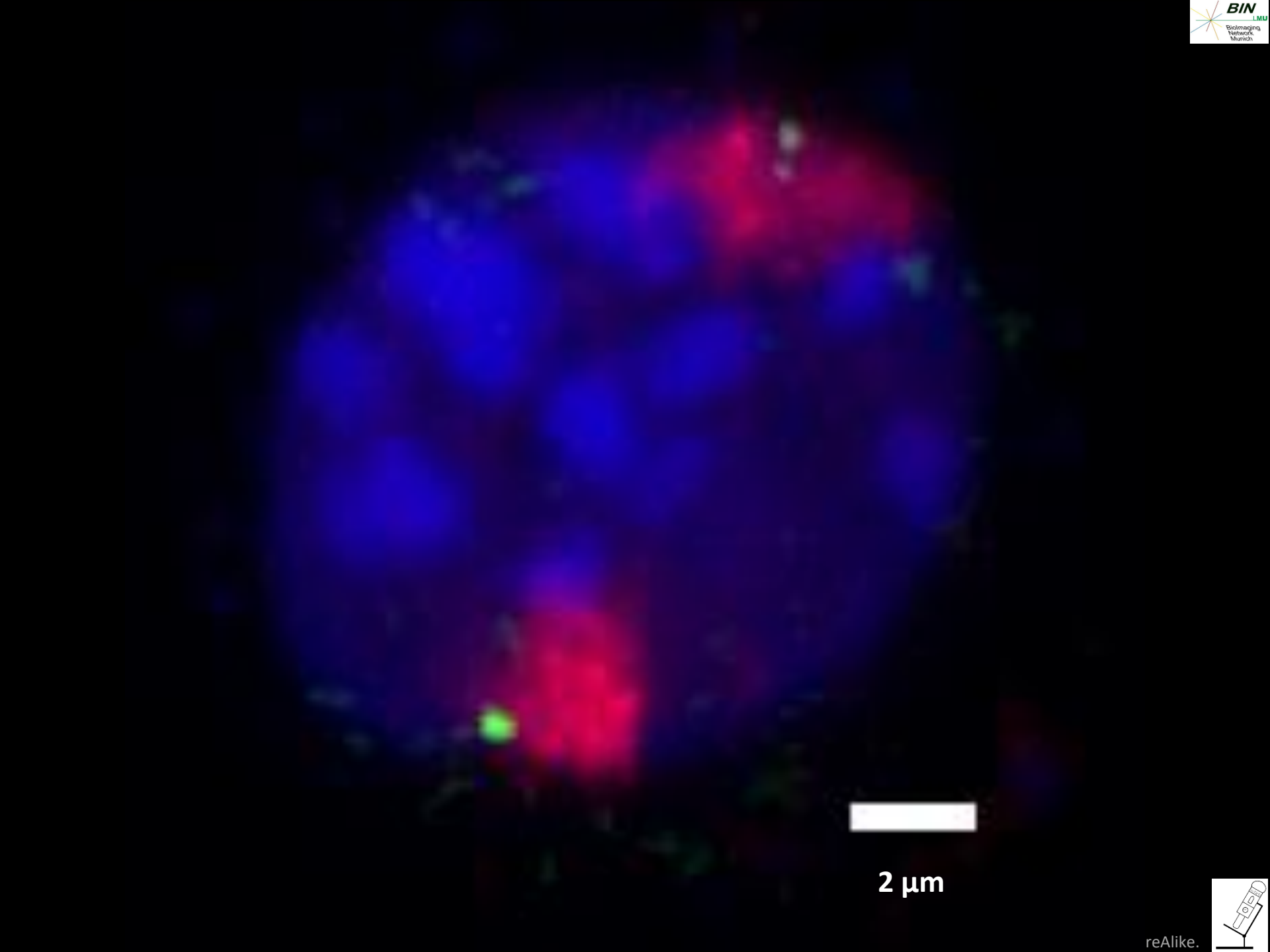












JCB

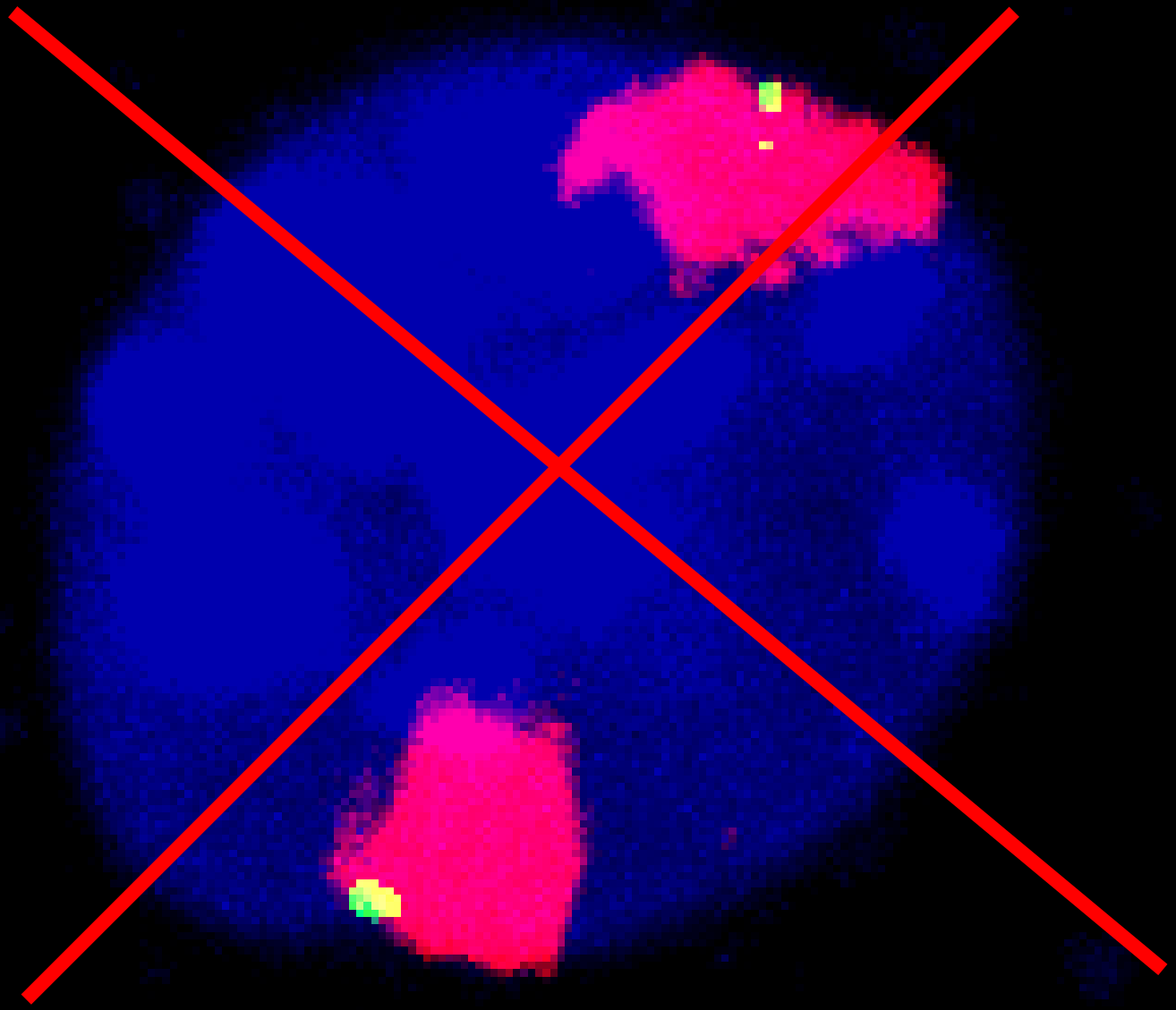
Instructions to authors,
Version November 2014

Image manipulation:

All digital images in manuscripts accepted for publication will be scrutinized by our production department for any indication of manipulation that is inconsistent with the following guidelines. Manipulation that violates these guidelines may result in production delays or revocation of acceptance.

- No specific feature within an image may be enhanced, obscured, moved, removed, or introduced.
- The grouping of images from different parts of the same gel, or from different gels, fields, or exposures, must be made explicit by the arrangement of the figure (i.e., using dividing lines) and in the text of the figure legend.
- Adjustments of brightness, contrast, or color balance are acceptable if they are applied to every pixel in the image and as long as they do not obscure, eliminate, or misrepresent any information present in the original, including the background. Non-linear adjustments (e.g., changes to gamma settings) must be disclosed in the figure legend.

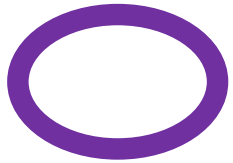
Questions raised by the production department will be referred to the Editors, who will request the original data from the authors for comparison to the prepared figures. If the original data cannot be produced, the acceptance of the manuscript may be revoked. Any case in which the manipulation affects the interpretation of the data will result in revocation of acceptance. Cases of suspected misconduct will be reported to an author's home institution or funding agency.

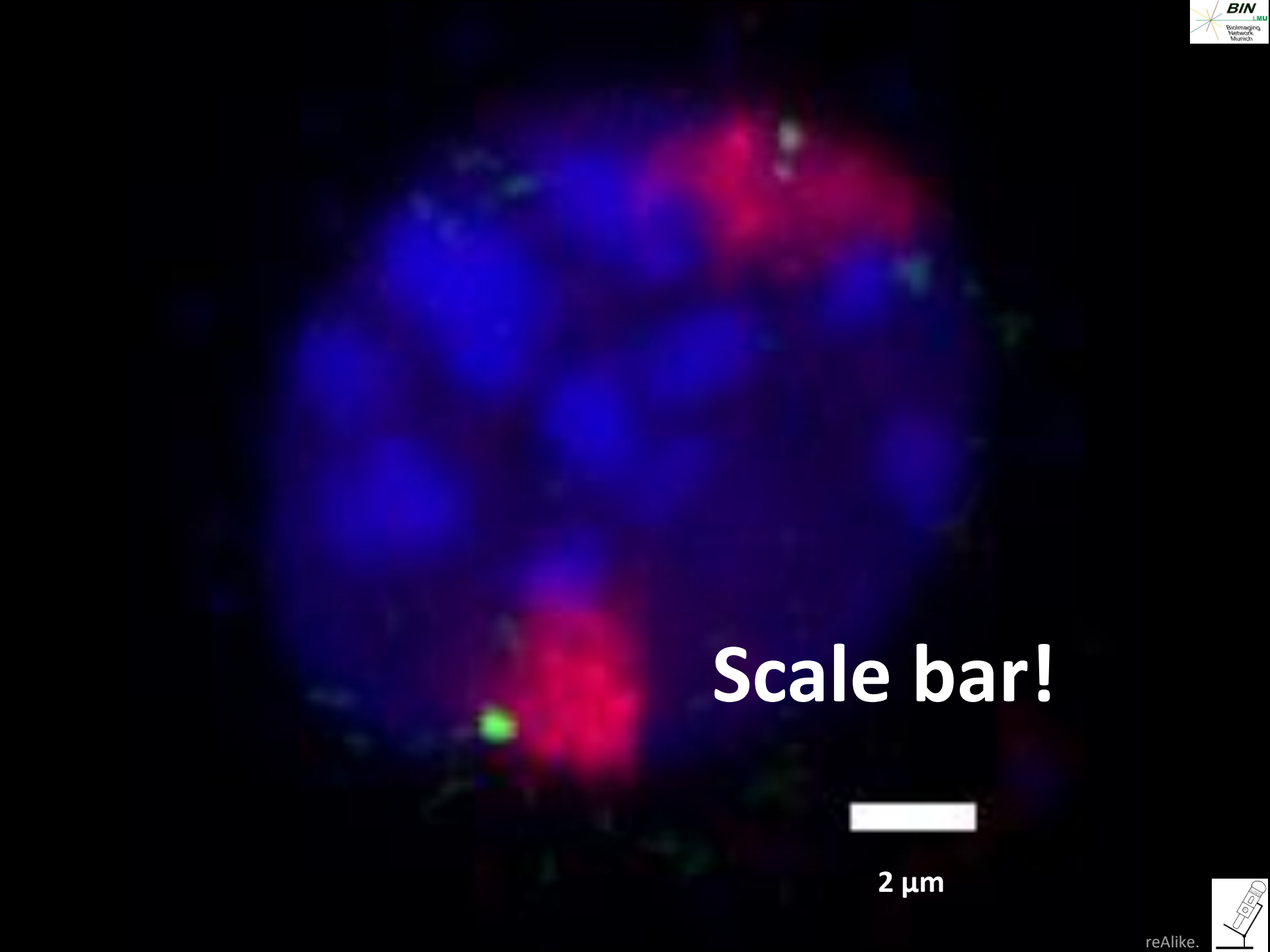


Don‘t overdo it!

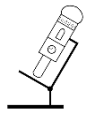
Usefull and empty magnification

Bullshit





Red and green



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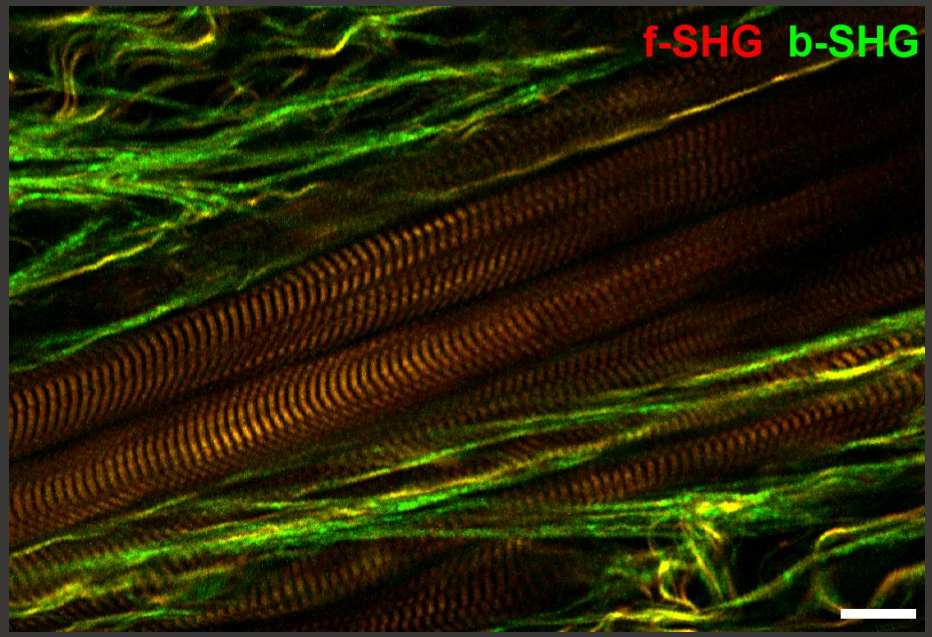
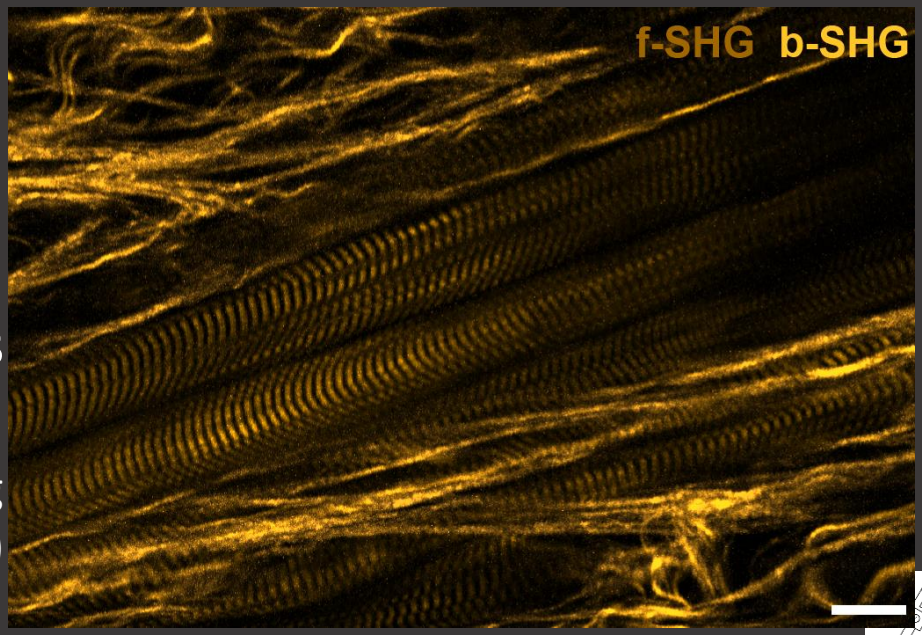


8% of all men will see this

(made with the Visicheck plugin, setting ‚deuteranop')



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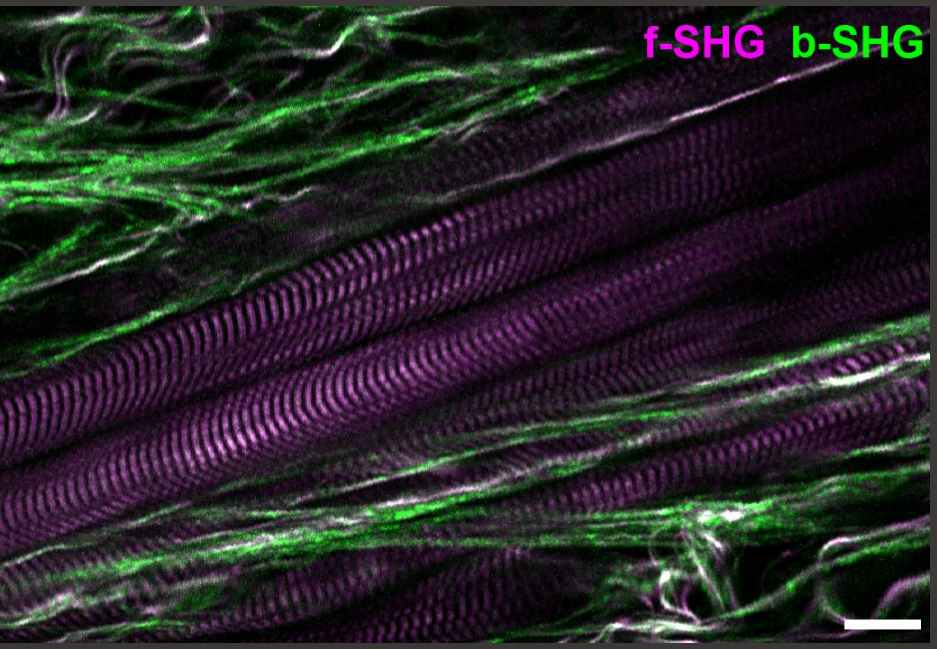

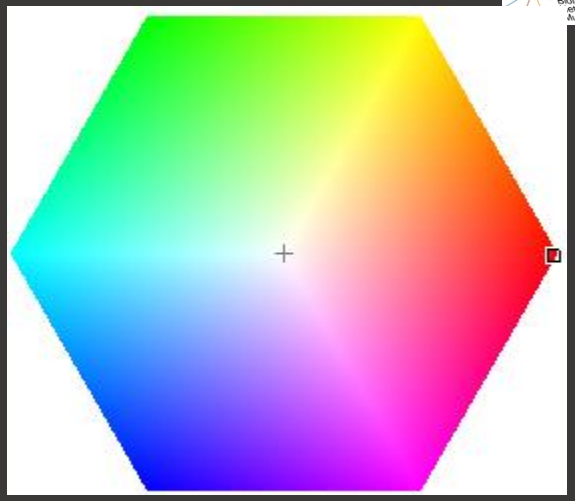

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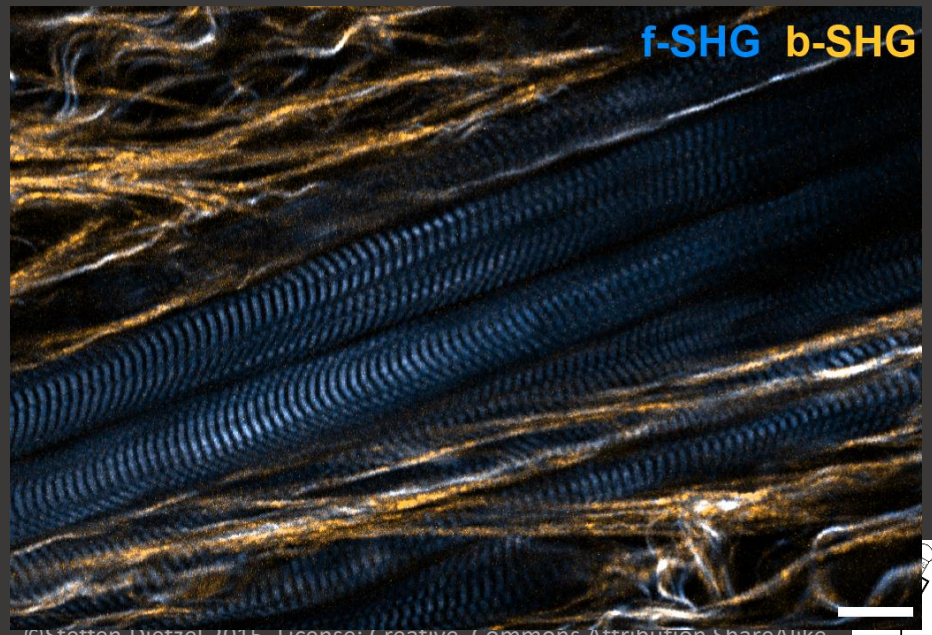

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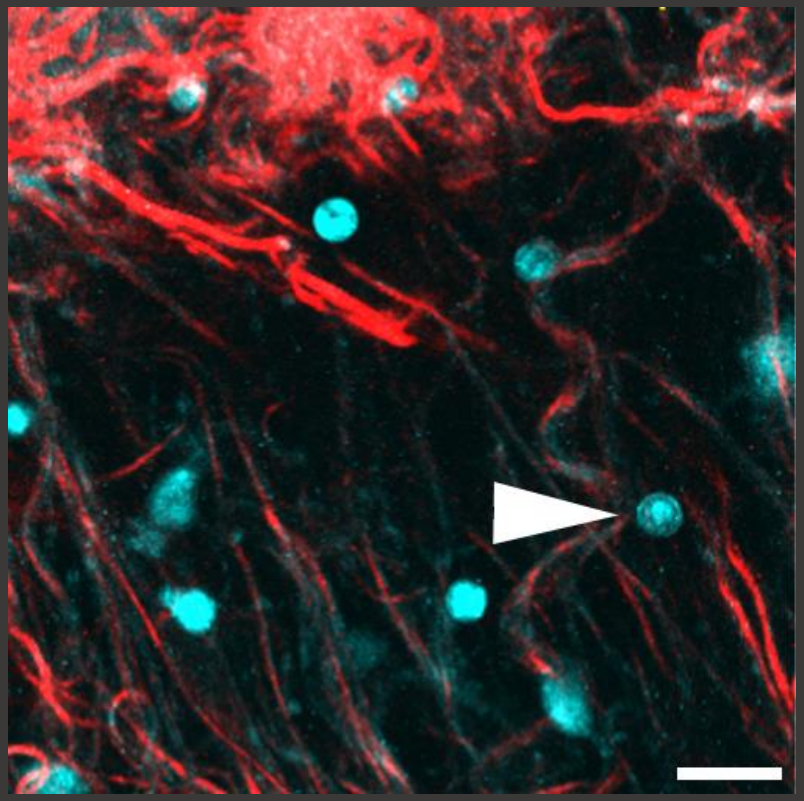
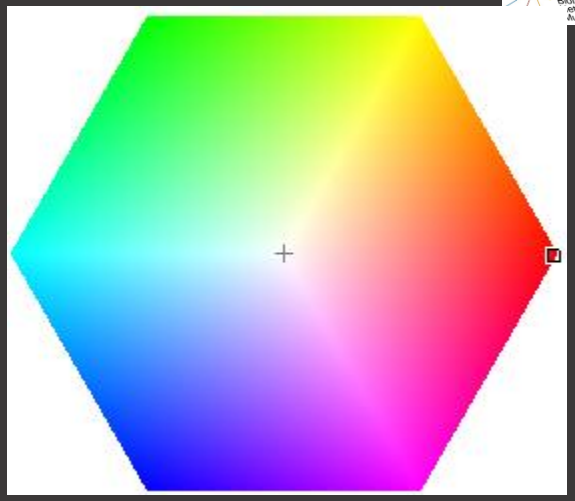
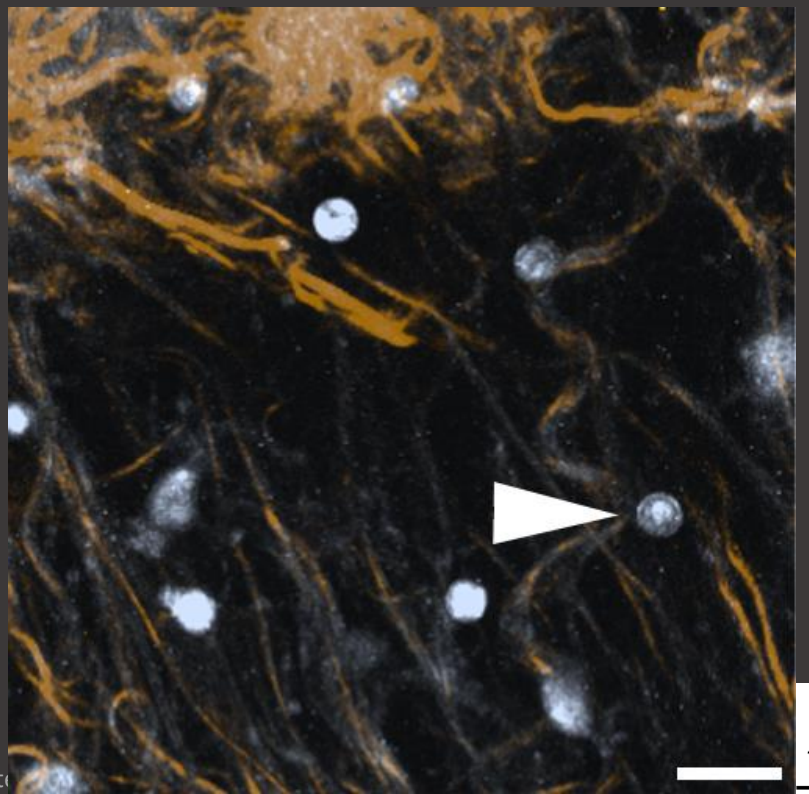


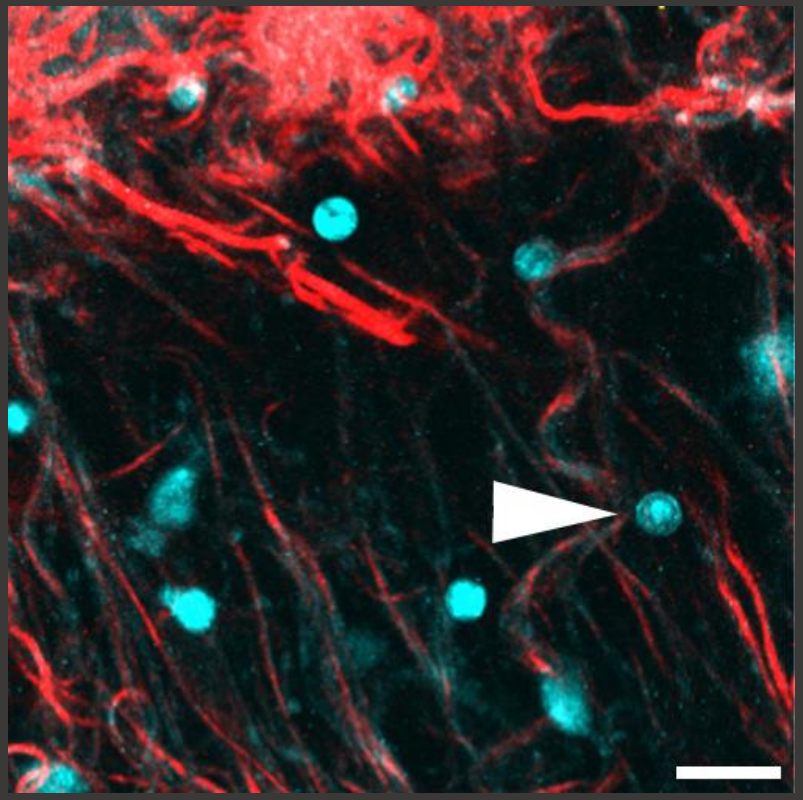




8% of all men will see this

(made with the Visicheck plugin, setting ‚deuteranop')

Conclusion



- If your image contains only two colors, do not use red and green!

Steffen Dietzel

Walter-Brendel-Zentrum für Experimentelle Medizin

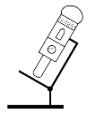
Core Facility Bioimaging at the Biomedical Center

Some Aspects of Digital Imaging

Pixels and Voxels

A pixel (=picture element) is the element of a digital 2D image

A voxel (= volume pixel) is the element of a digital 3D image

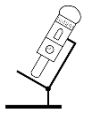


A Pixel Is *Not* A Little Square,
A Pixel Is *Not* A Little Square,
A Pixel Is *Not* A Little Square!
(And a Voxel is *Not* a Little Cube)

Microsoft Technical Memo 6
Alvy Ray Smith, July 17, 1995. (available online)

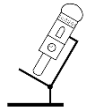
*"*A pixel is a *point* sample.*"*

*"*There are cases where the *contributions* to a pixel can be modeled … by a little square, but not ever the pixel itself.*"*



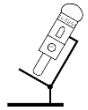
“But the pixels on my CCD camera chip are little squares…”

- The recording elements on the chip may be square shaped. But the light intensity that is recorded from all the square‘s surface is represented in just one value.
- Thus, although the ‚contributions to the image may be modelled by a square‘, the pixels that build up the image are still point samples.



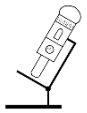
„Pixel size“

- Since a pixel is a point sample, it really has no size.
- Whenever we talk about pixel size in microscopy, what we really mean is „pixel distance“.
- It may help to imagine this as the distance from the center of one pixel to the center of the next.
  (Although strictly speaking a point has no center…)
- Despite of the above, „pixel size“ is the established term for the pixel distance and broadly used.



Resolution and “pixel size” in digital images

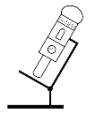
(Nyquist)



We talked about resolution. But how to capture the resolution on the digital image?

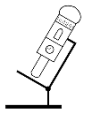
- If pixels of an image are spaced 2 µm, obviously the image does not resolve structures with a 200 nm resolution.

- Which pixel distance (“pixel size”) do we need to digitally capture the resolution (Rayleigh Criterion) that the microscope provides?

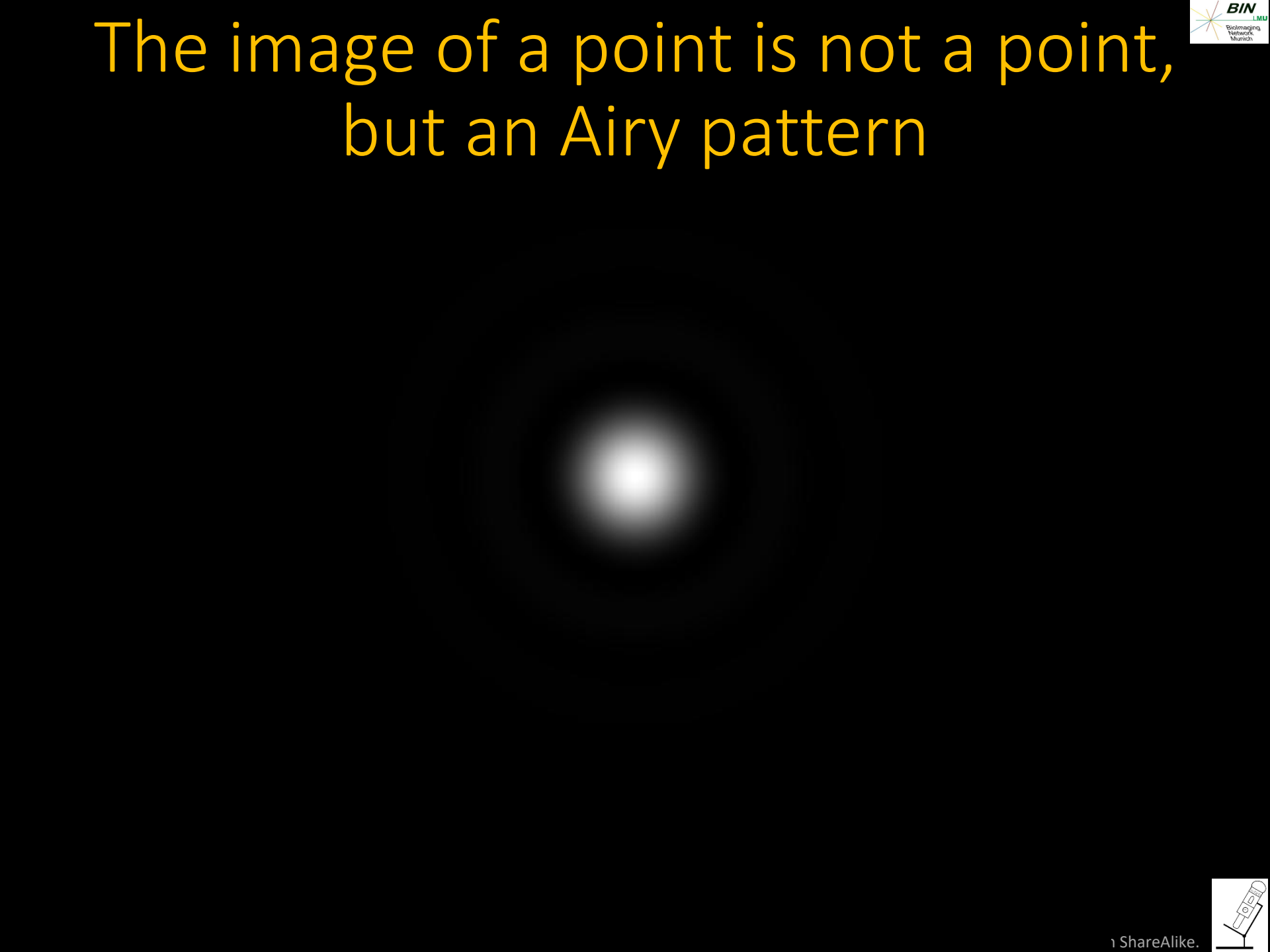


The idealized situation

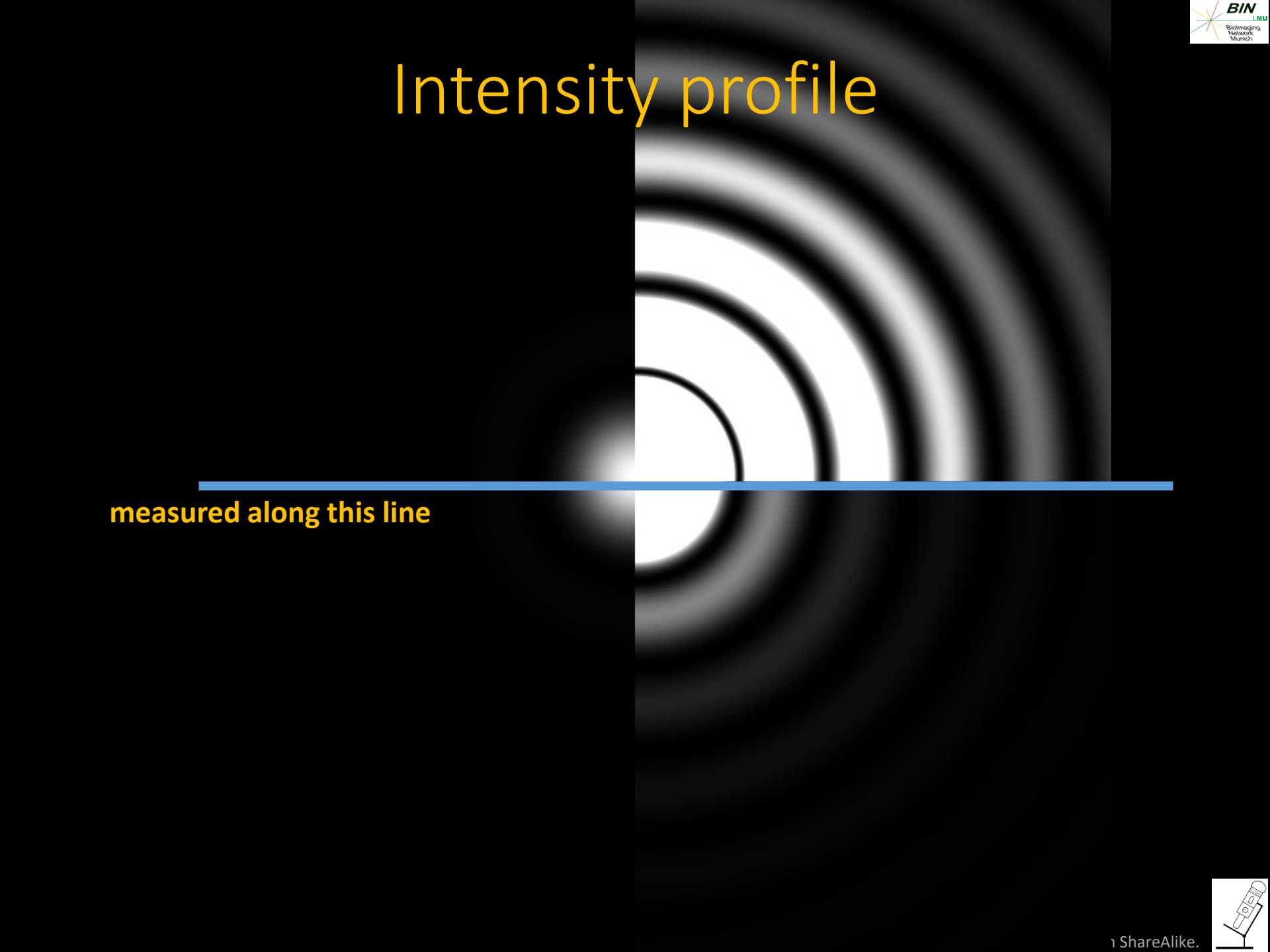
- Unlimited number of photons
- Two point-like sources emit the same number of photons (identical intensity)
- No other sources in the neighborhood
- No spherical aberration, Ri mismatch
- No noise



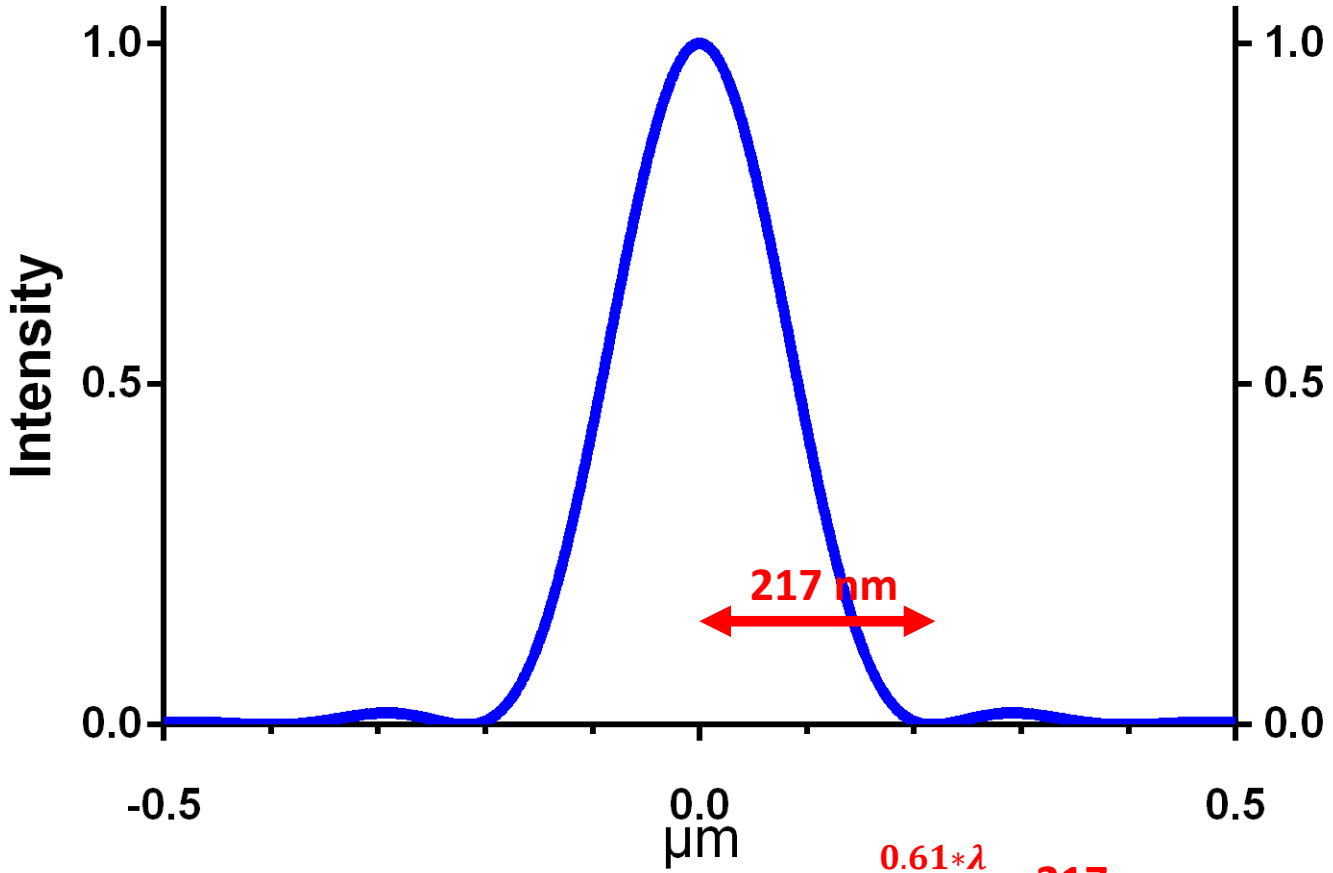
The image of a point is not a point, but an Airy pattern



Intensity profile

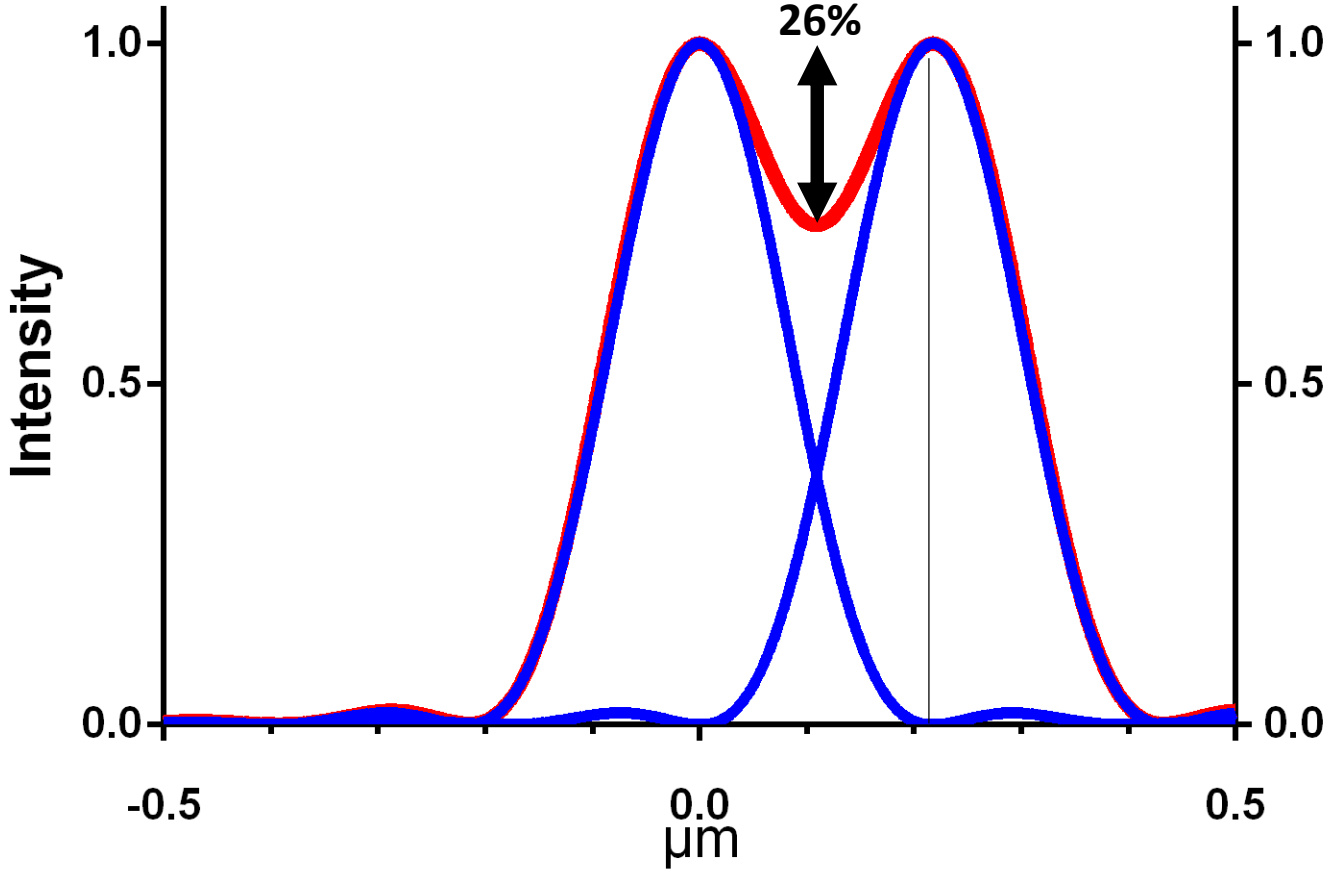


Example: Intensity distribution in the Airy pattern of a point with an NA 1.4 objective with λ=500 nm



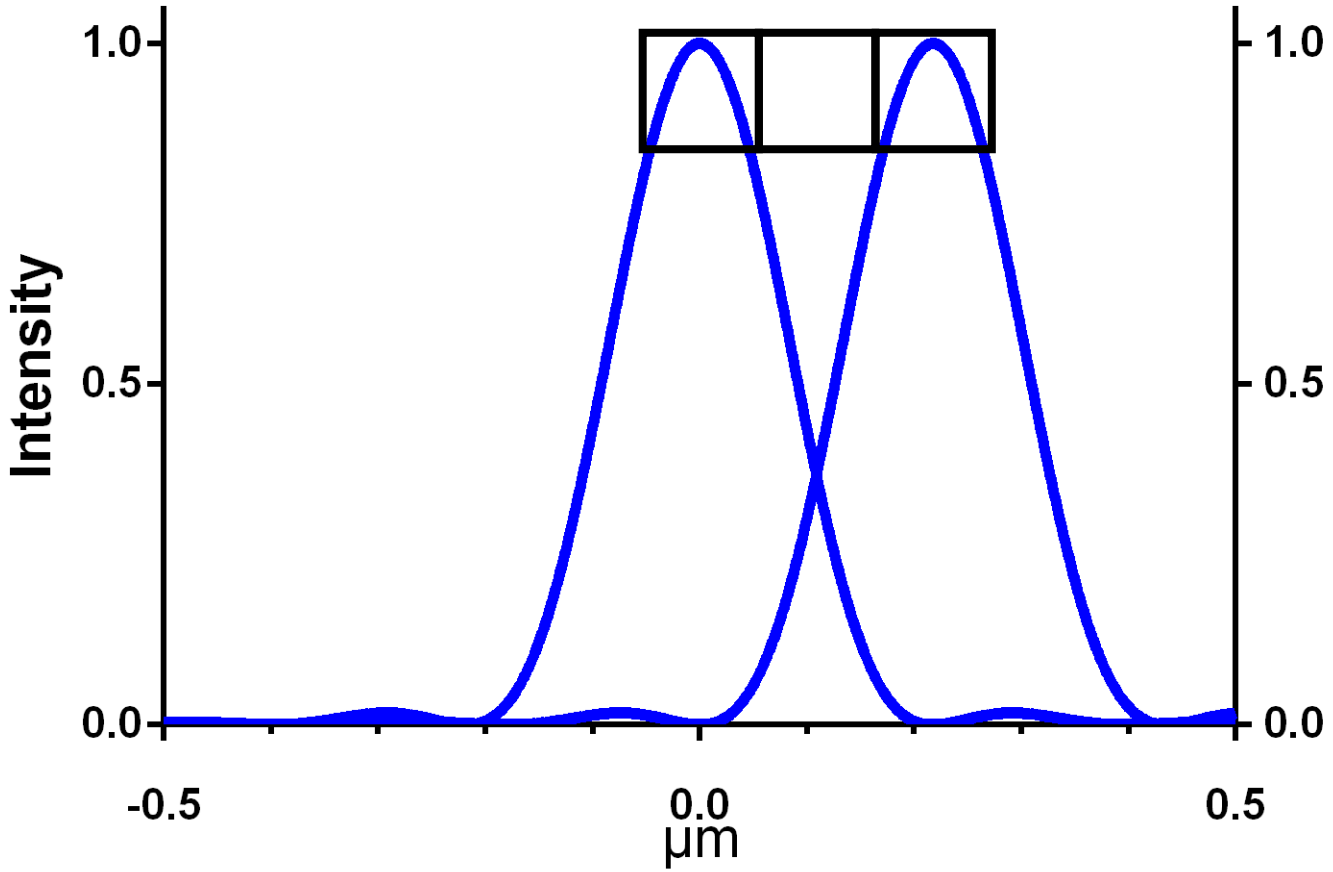
$$r = \frac{0.61 * \lambda}{NA} = 217 \text{ nm}$$

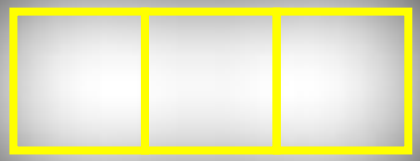
Example: Intensity distribution in the Airy pattern of a point with an NA 1.4 objective with λ=500 nm



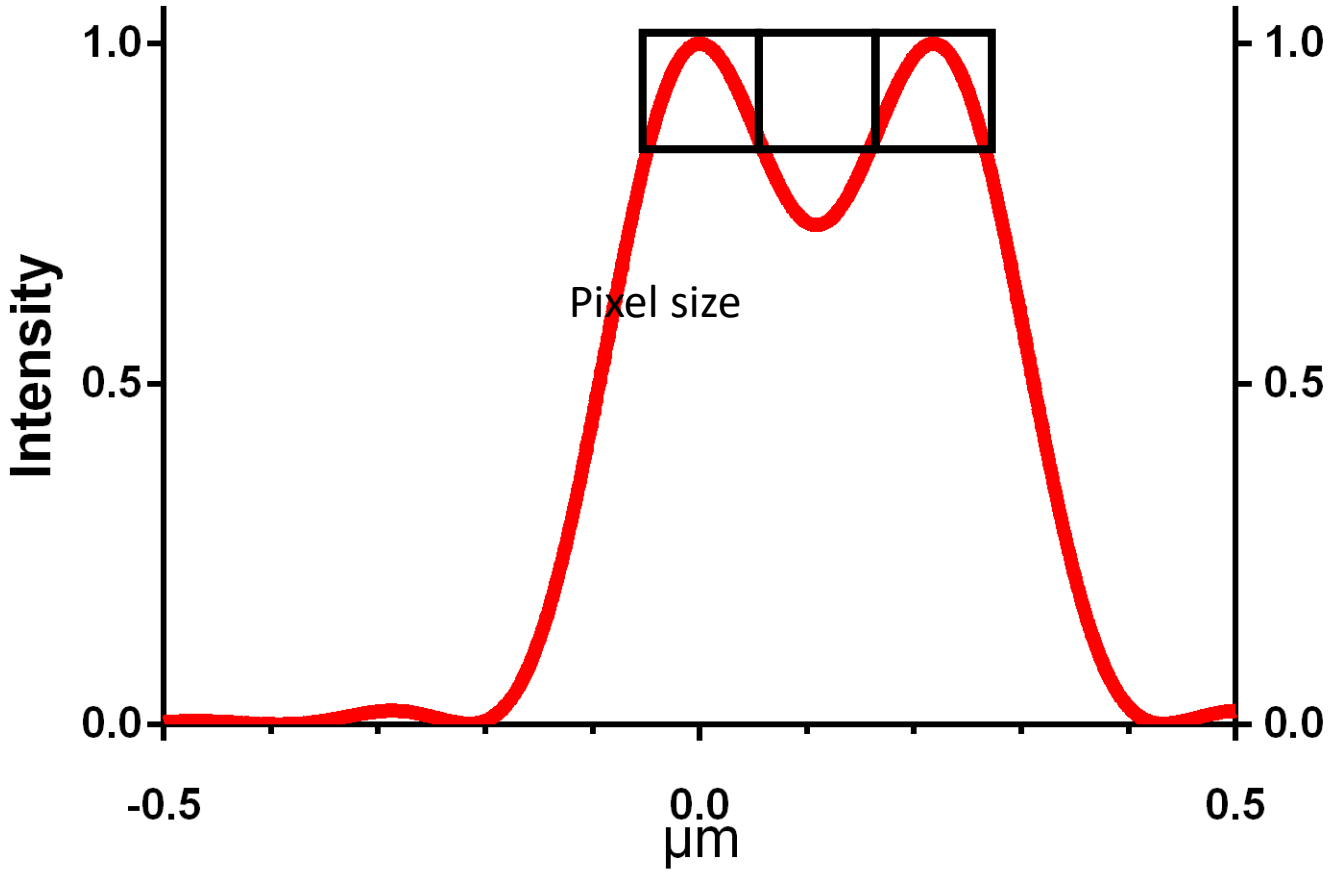
Rayleigh criterion

How many pixels do we need?

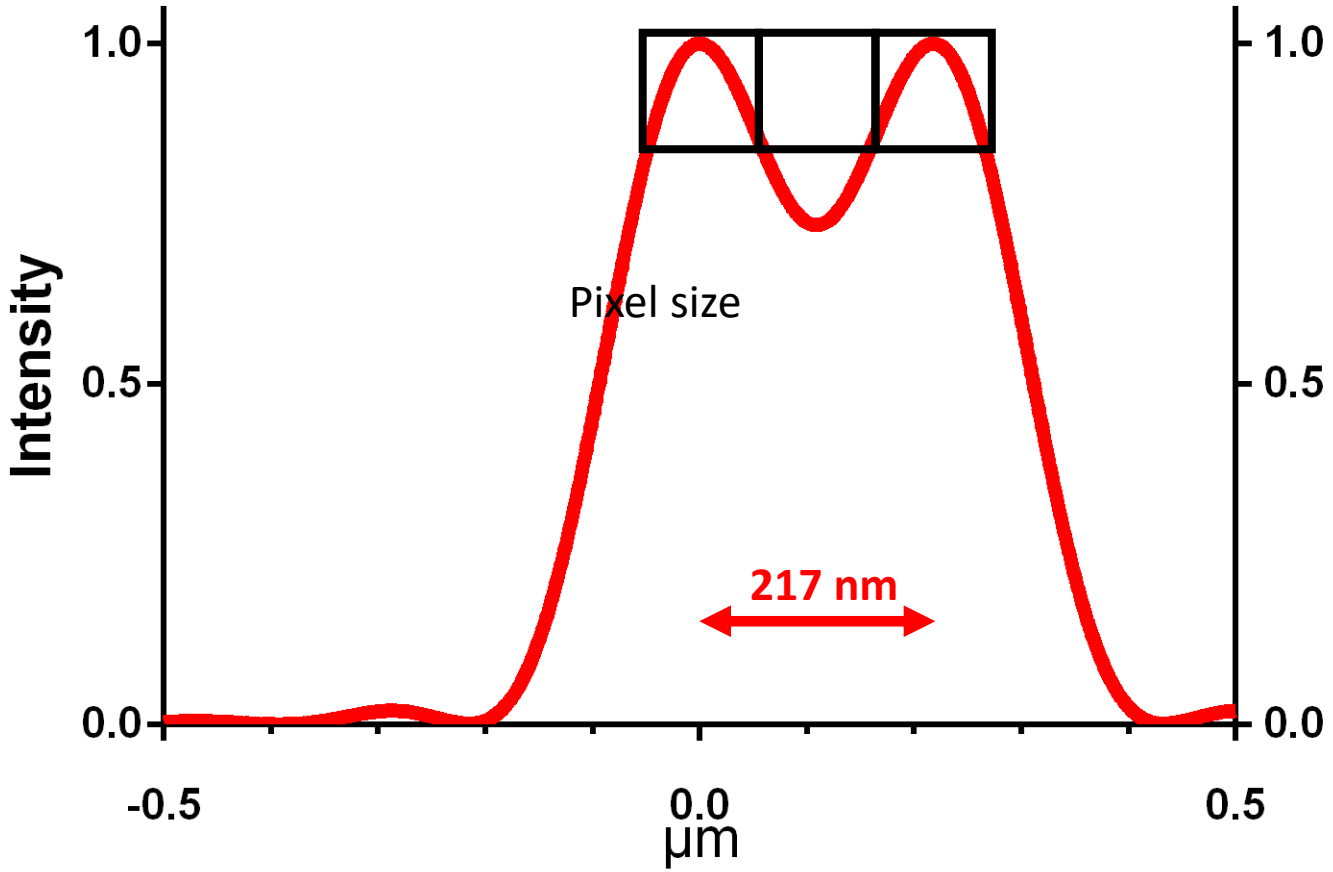




How many pixels do we need?



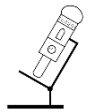
Which spacing do the pixels have to have?



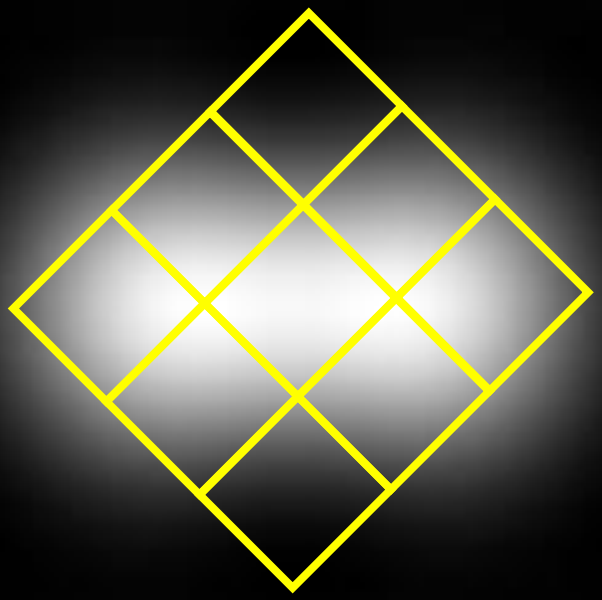
Which spacing do the pixels have to have?

- To realize the physical resolution, pixel size must be < 2x smaller
  than the resolution
  (Nyquist criterion, after Harry Nyquist, 07.02.1889 - 04.04.1976.)

- „< 2x smaller“ is often interpreted as 2.3x smaller.
  In the given example that would be 94 nm.



What if the structure is diagonal to the pixel pattern?

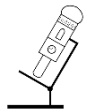


Which spacing do the pixels have to have?

- Pixel distance at the 45°-angle is $\sqrt{2} = 1.41$ times larger than along the axis.

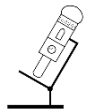
- To cover diagonal point sources, pixel size must be < 2.82x smaller
  than the physical resolution

- "< 2.82x smaller" can be interpreted as
  1/3 of the resolution.

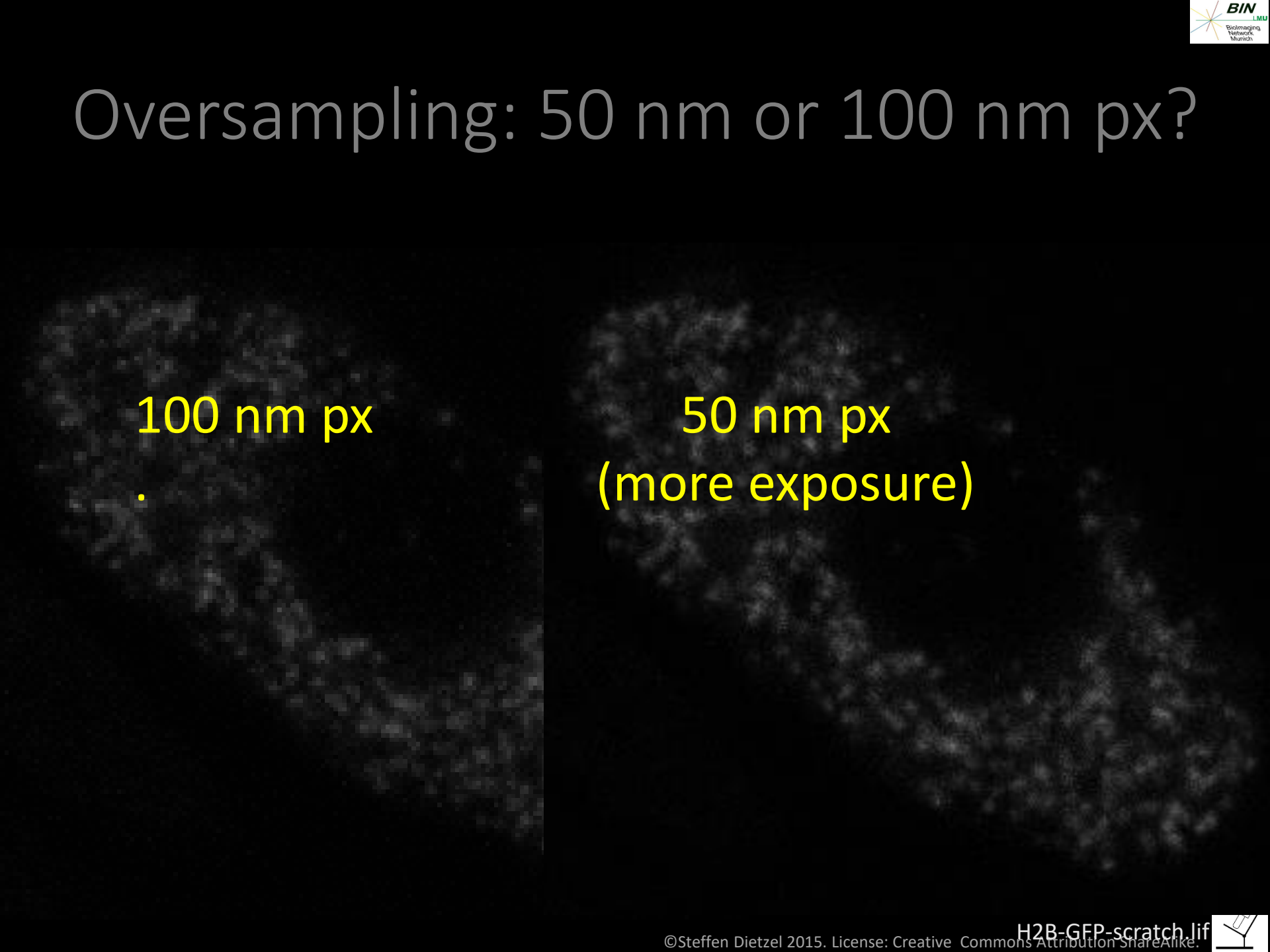


Which spacing do the pixels have to have?

- On the one hand, in real life we don‘t usually reach the theoretical resolution (spherical aberration, etc.), so we could use somewhat larger pixels.
- On the other hand, a little oversampling may smoothen the image. Also, we may want to allow for potential confocal resolution improvement, arguing for smaller pixels.
- For an NA 1.4 objective, 70 - 80 nm pixel size can be recommended for many cases.
- Much smaller pixels do not usually improve image quality but do increase scanning time, bleaching and phototoxicity.



Oversampling: 50 nm or 100 nm px?



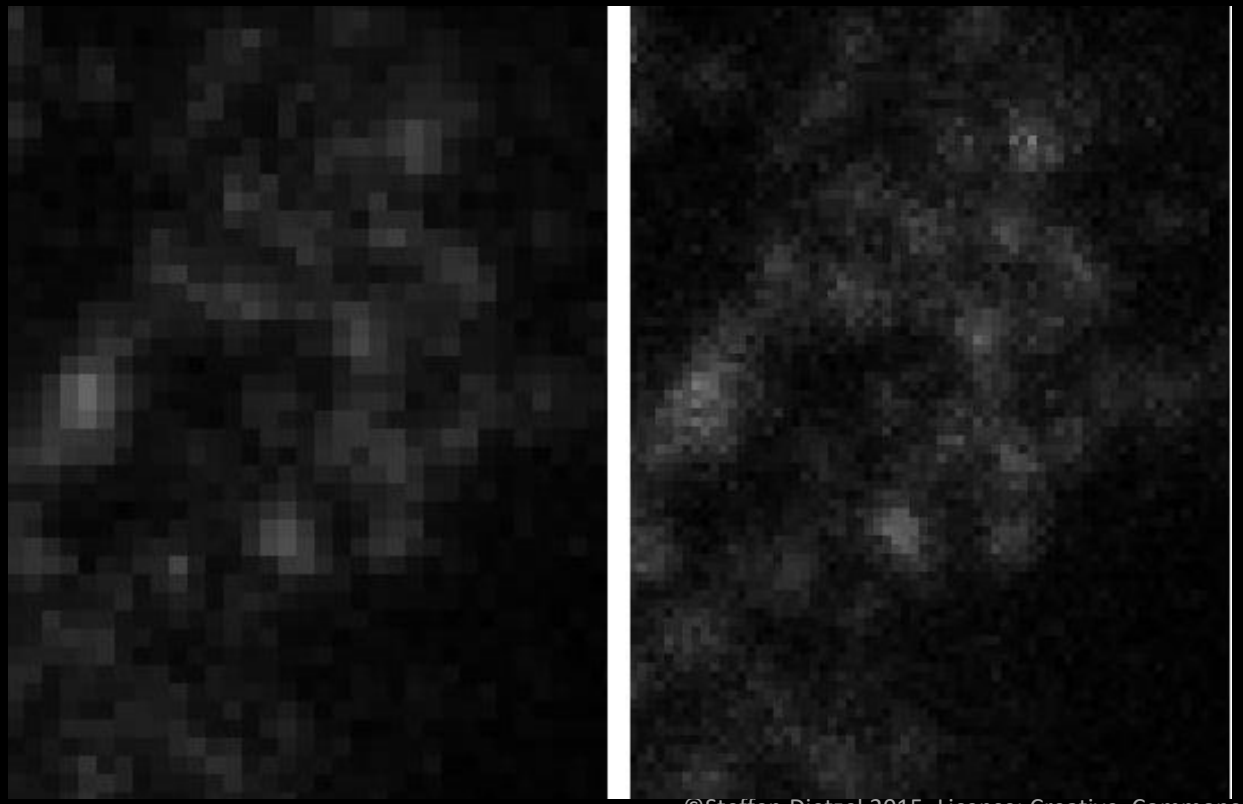
H2B-GFP-scratch.lif

Oversampling: 50 nm or 100 nm px?

100 nm px

.

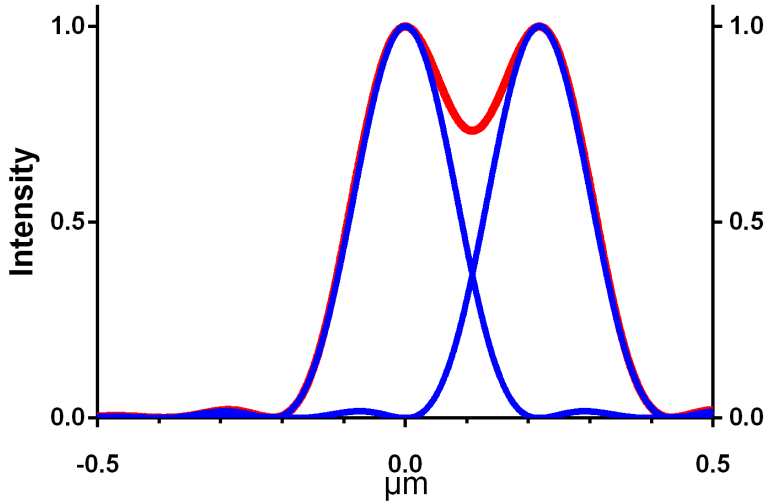
50 nm px
(more exposure)



H2B-GFP-scratch.

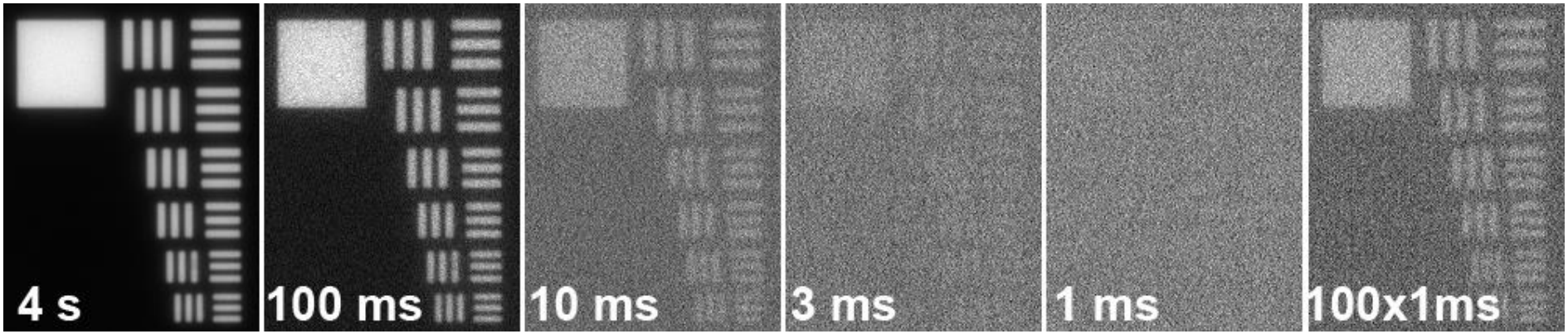
Resolution is not everything,

You also need contrast!



Signal (=number of collected photons) must be above the noise.

Poisson noise, contrast and resolution, demonstrated with a CCD camera



Low light conditions and a USAF 1956 target, brightfield transmission microscopy with a CCD camera. The intensity of the images was adjusted post exposure.

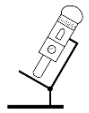
With 3 ms exposure the remaining contrast is just barely sufficient to visualize the larger bars, but not the smaller ones. No bars can be recognized with 1 ms exposure, due to the statistical character of photon detection.

When hundred 1-ms-exposures are averaged, the statistics is good enough to increases the signal-to-noise ratio above the detection limit.

Statistical noise = Poisson noise

- **<u>Poisson noise is a hard physical limit</u>**, just as diffraction!

  Only counting more photons helps!



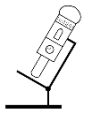
Photon statistics

- Only if a large enough number of photons is collected to detect differences in brightness, structures can be resolved.

- This fundamentally limits the minimal exposure time and the number of exciting photons.

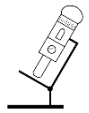
- This is true for all kinds of microscopic image generation. In practice, it plays a role only for fluorescence, since only here too short exposure times are relevant.

- Every photon lost in the microscope decreases signal-to-noise ratio, leading to longer exposure times and more damage to the sample.



Shot noise = Poisson noise = statistical noise

- In a perfect fluorescence microscopy setup, Poisson noise will be the major problem. It can be attenuated by increasing exposure time (camera) or by averaging sequentially recorded images (confocal).



Insufficient image quality can be caused by

Noise

- Poisson noise
- Electronic detector noise: dark noise and read noise

Blur

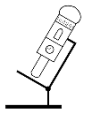
- Optical aberrations like Ri mismatch, scattering in deep samples, dirty lenses or slides

Unwanted signal (photons)

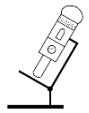
- Unspecific dye binding, autofluorescence in sample or immersion oil, room lights

System or sample variations

- Uneven staining, uneven illumination, uneven blur.



Contrast and Resolution – Reality Check

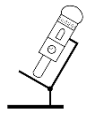


Which intensity difference can we expect for 80 nm pixels in the idealized situation?

Assuming the parameters from the example above:

NA 1.4 objective with λ=500 nm

and thus Rayleigh criterion $r = \frac{0.61 * \lambda}{NA}$ =217 nm



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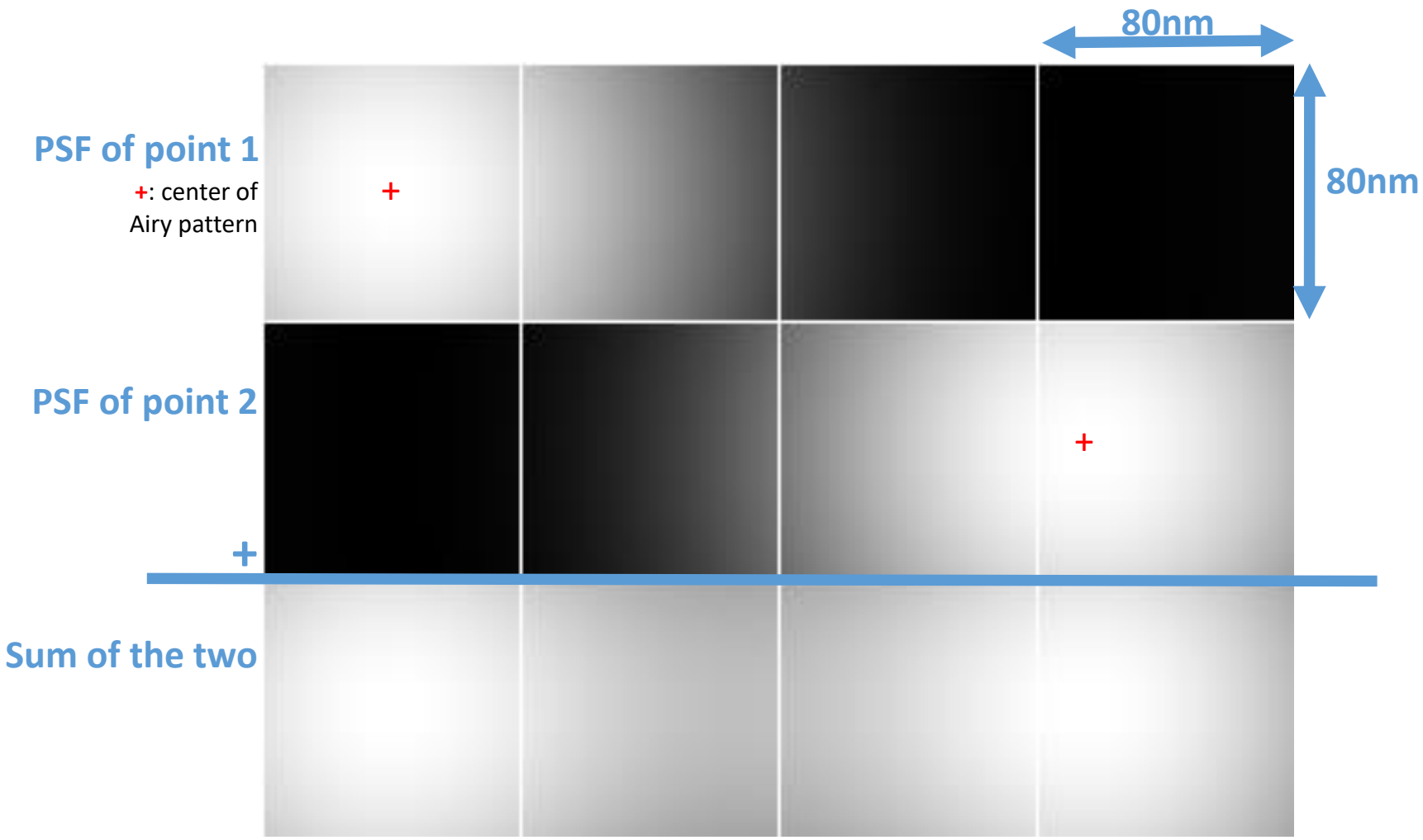

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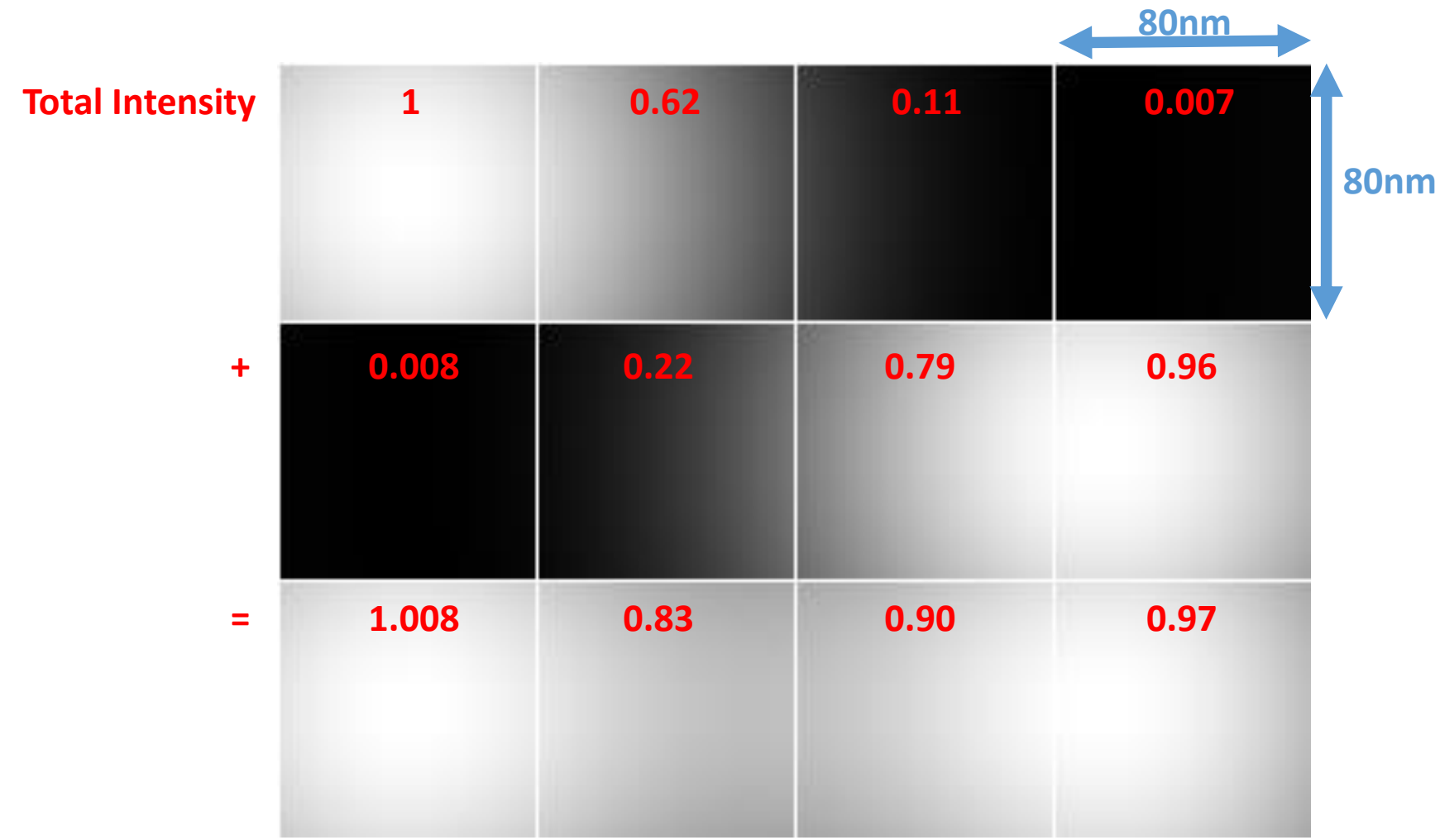

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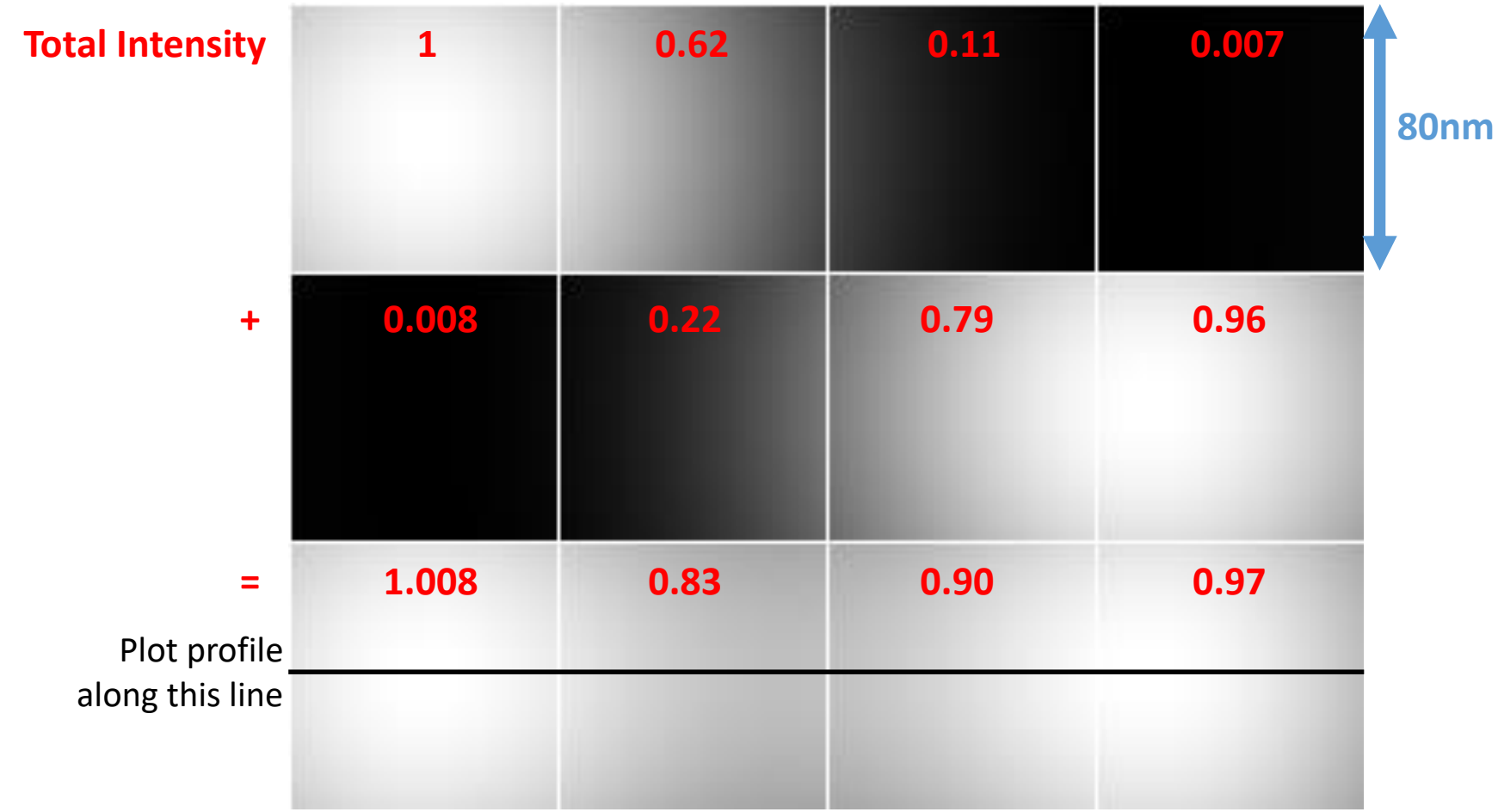

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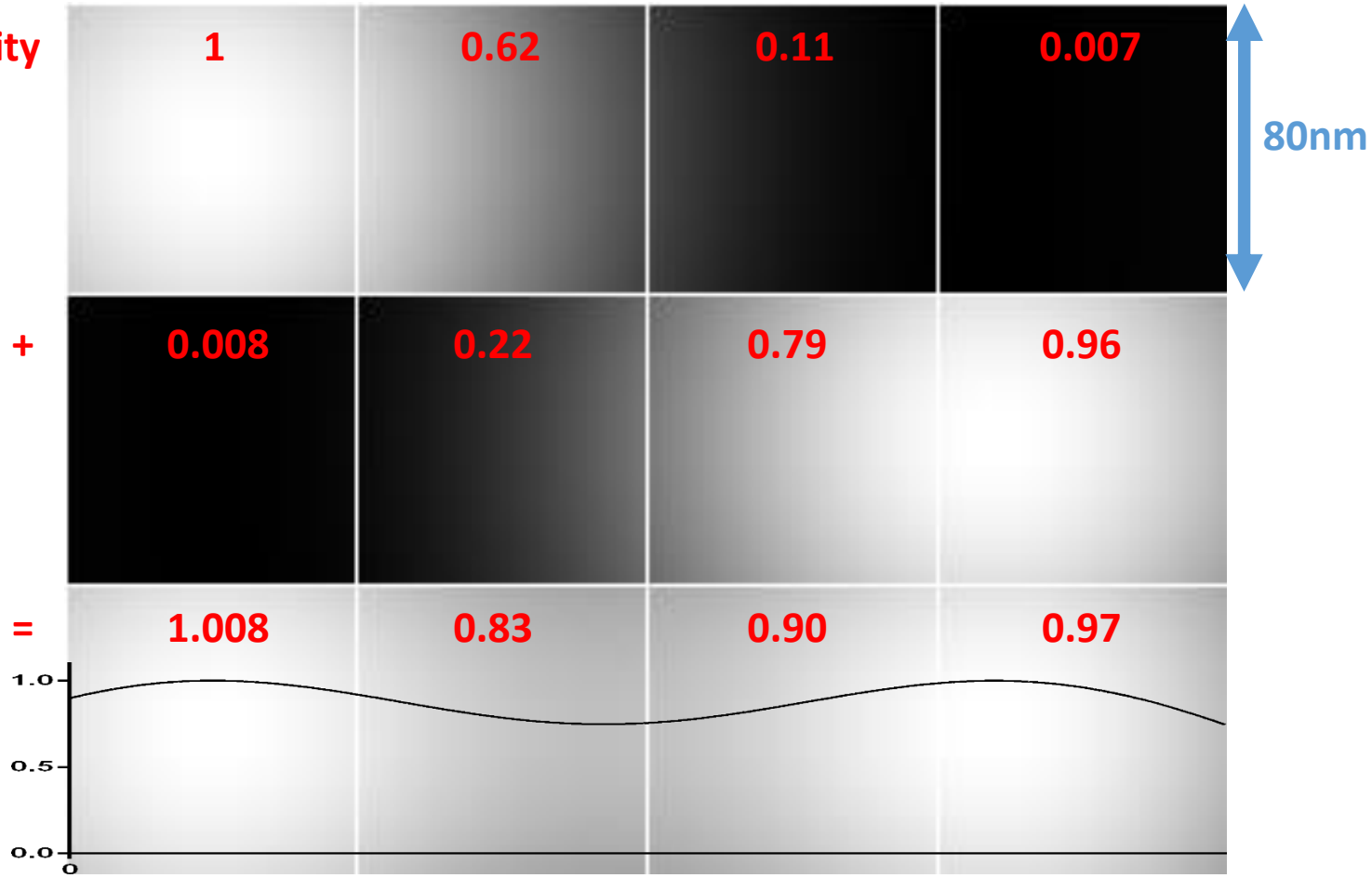
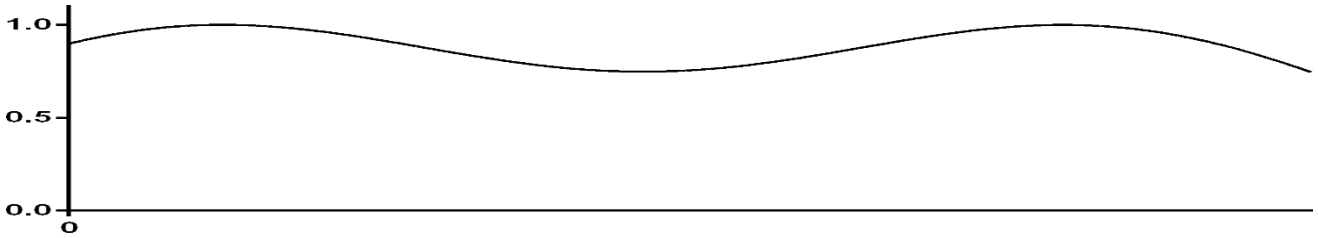
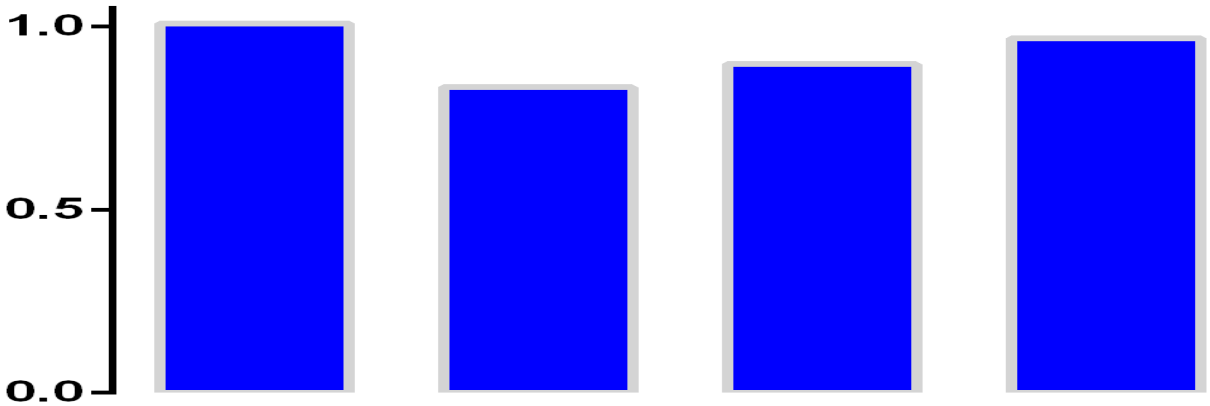
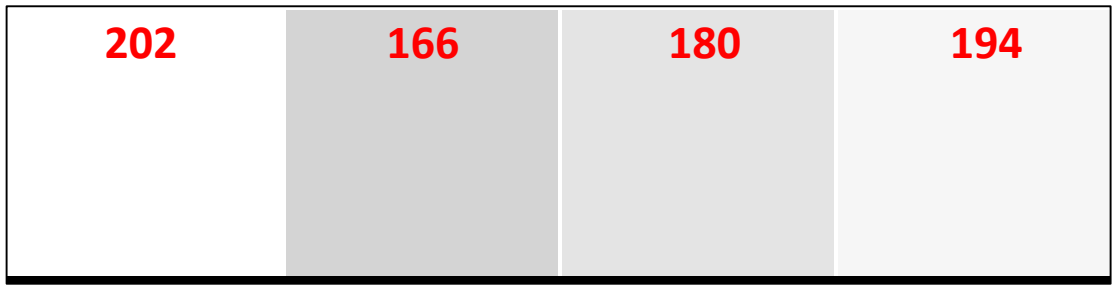


Which intensity difference can we expect for 80 nm pixels in the idealized situation?



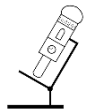


for example



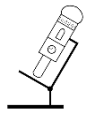
Reminder:
This is for the idealized case!

- Unlimited number of photons
- Two point-like sources emit the same number of photons (identical intensity)
- No other sources in the neighborhood
- No spherical aberration, Ri mismatch
- No noise

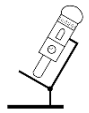


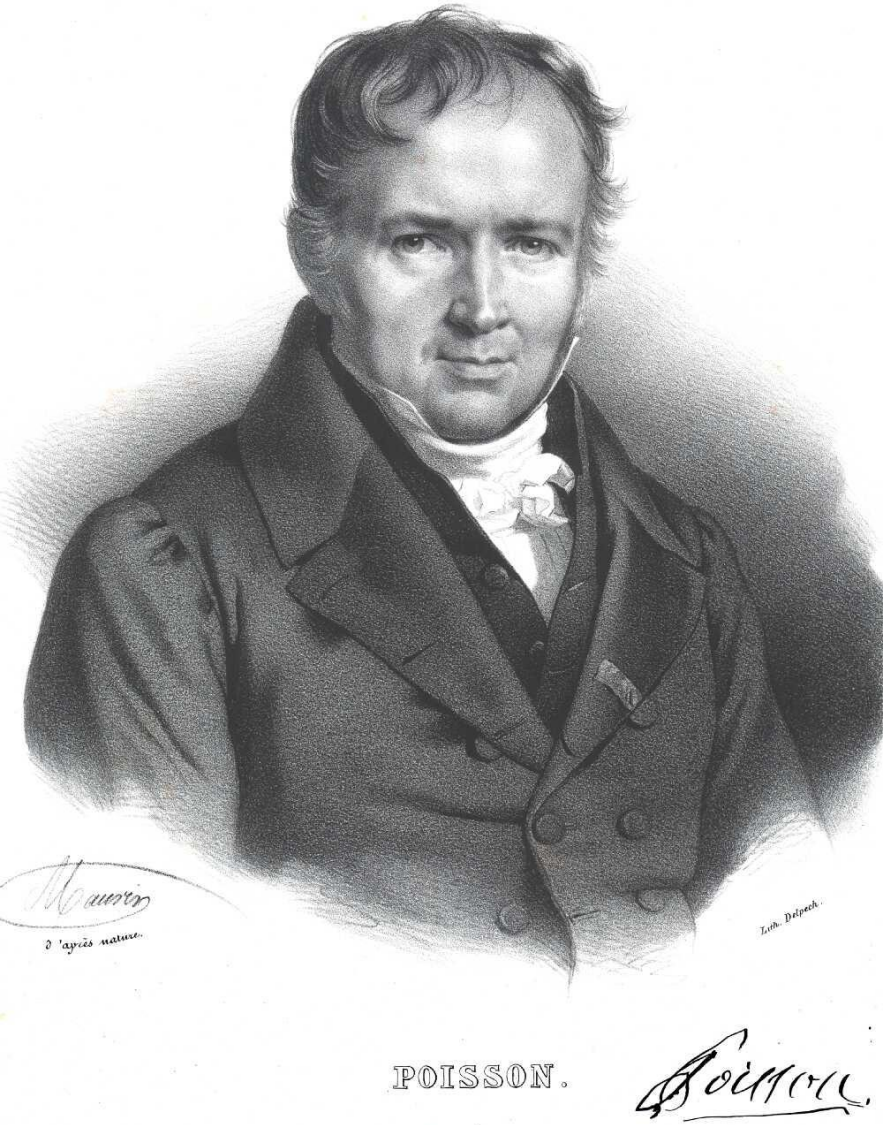
Contrast and Resolution – Reality Check

And then there is noise….



Statistical noise
= Poisson noise
= shot noise





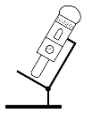
Siméon Denis Poisson

21 June 1781 – 25 April 1840
French mathematician and physicist.

Statistical noise = Poisson noise

- Whether any particular fluorescent molecule emits a photon or not is a random, statistical process: Good fluorochromes have a higher probability than bad ones.

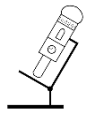
- Poisson noise is independent of the microscope hardware!
  (But hardware may cause other noise).



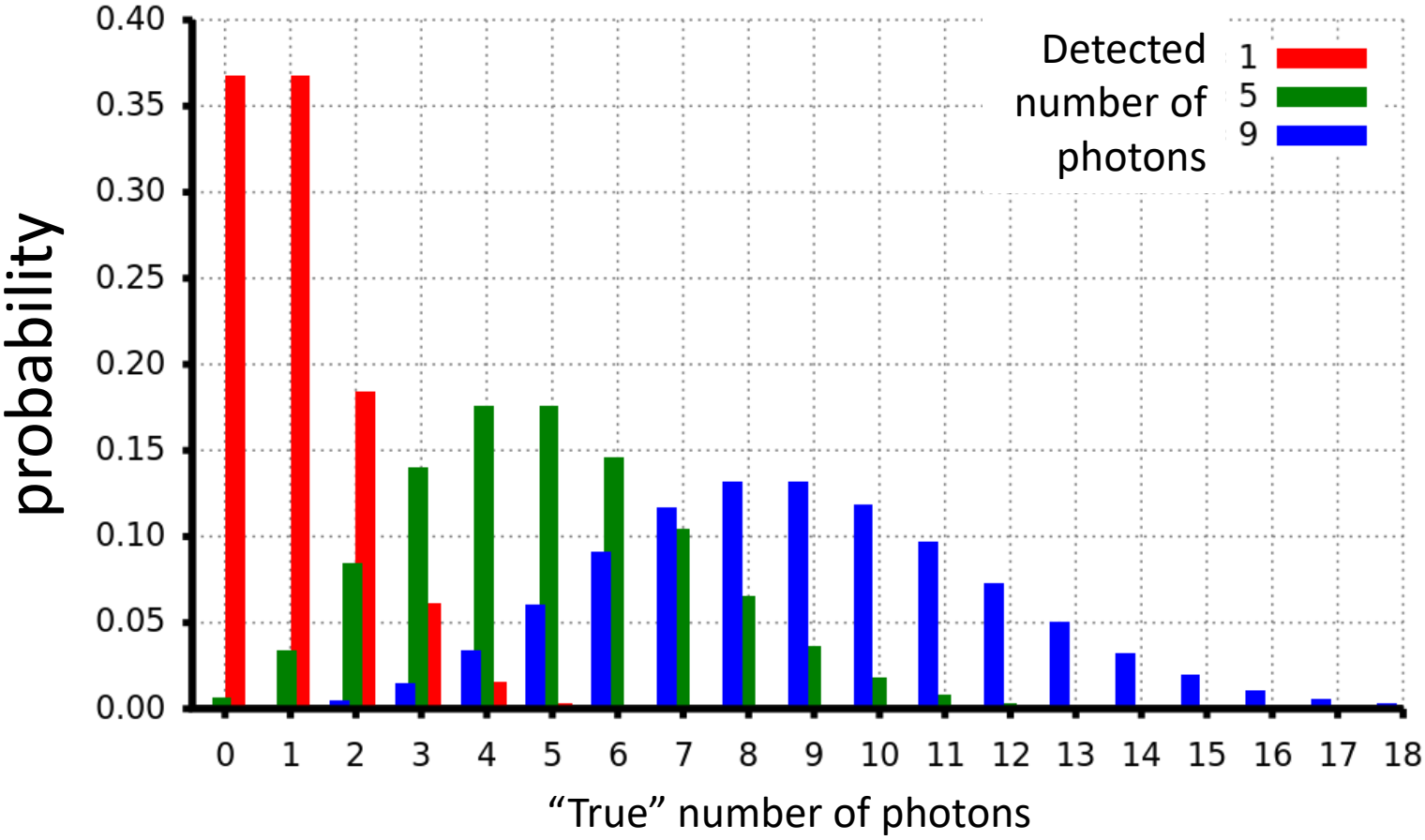
Statistical noise = Poisson noise

- …is the square root of the number of photons detected, meaning 2/3 of theoretical photon numbers are within this range.

- Example: 16 photons are read (Poisson noise √16=4). 2/3 of such measurements “should have been” between 12 and 20 photons (if there were no noise). 1/3 deviate even stronger.



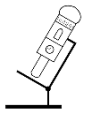
Poisson distribution with 1, 5 and 9 detected photons



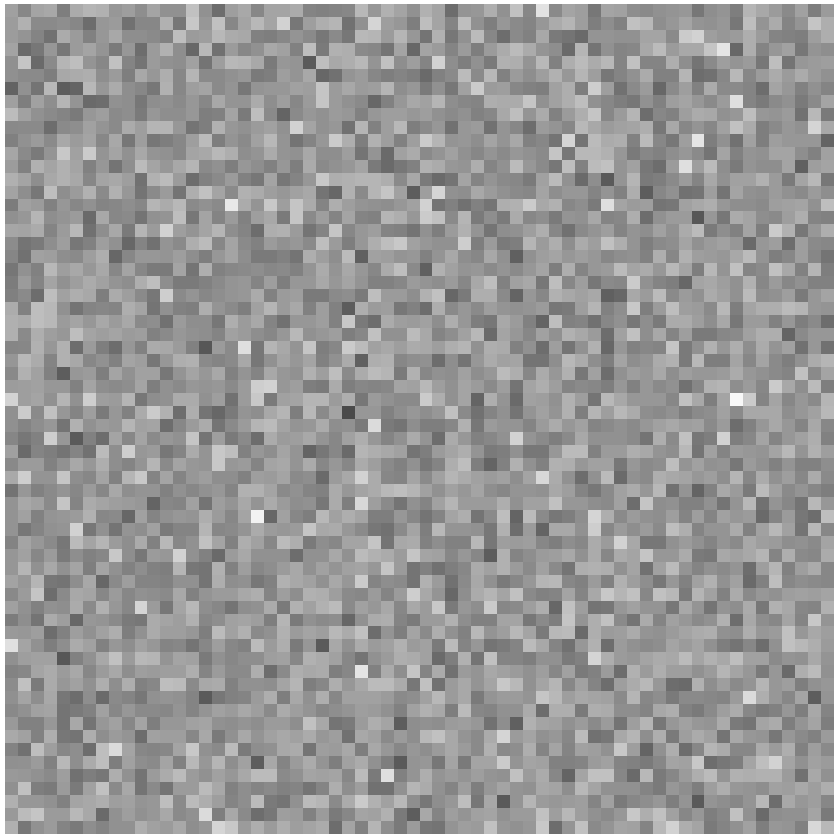
Signal to noise ratio, SNR

Examples

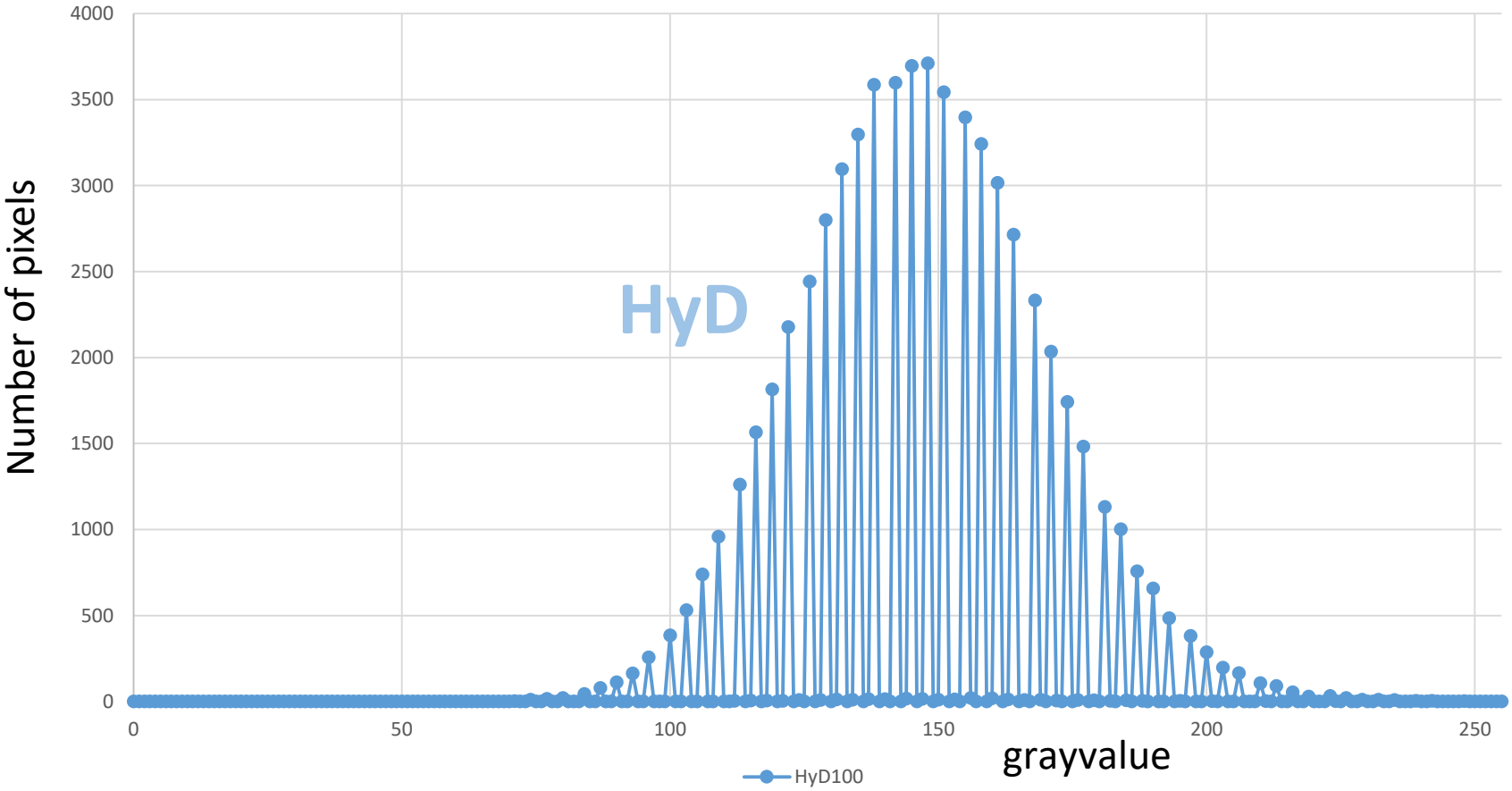
- 16 photons are read.
  Poisson noise √16=4
  SNR = 16/4 = 4
- 64 photons are read.
  Poisson noise √64=8
  SNR = 64/8 = 8
- 4 photons are read.
  Poisson noise √4=2
  SNR = 4/2 = 2



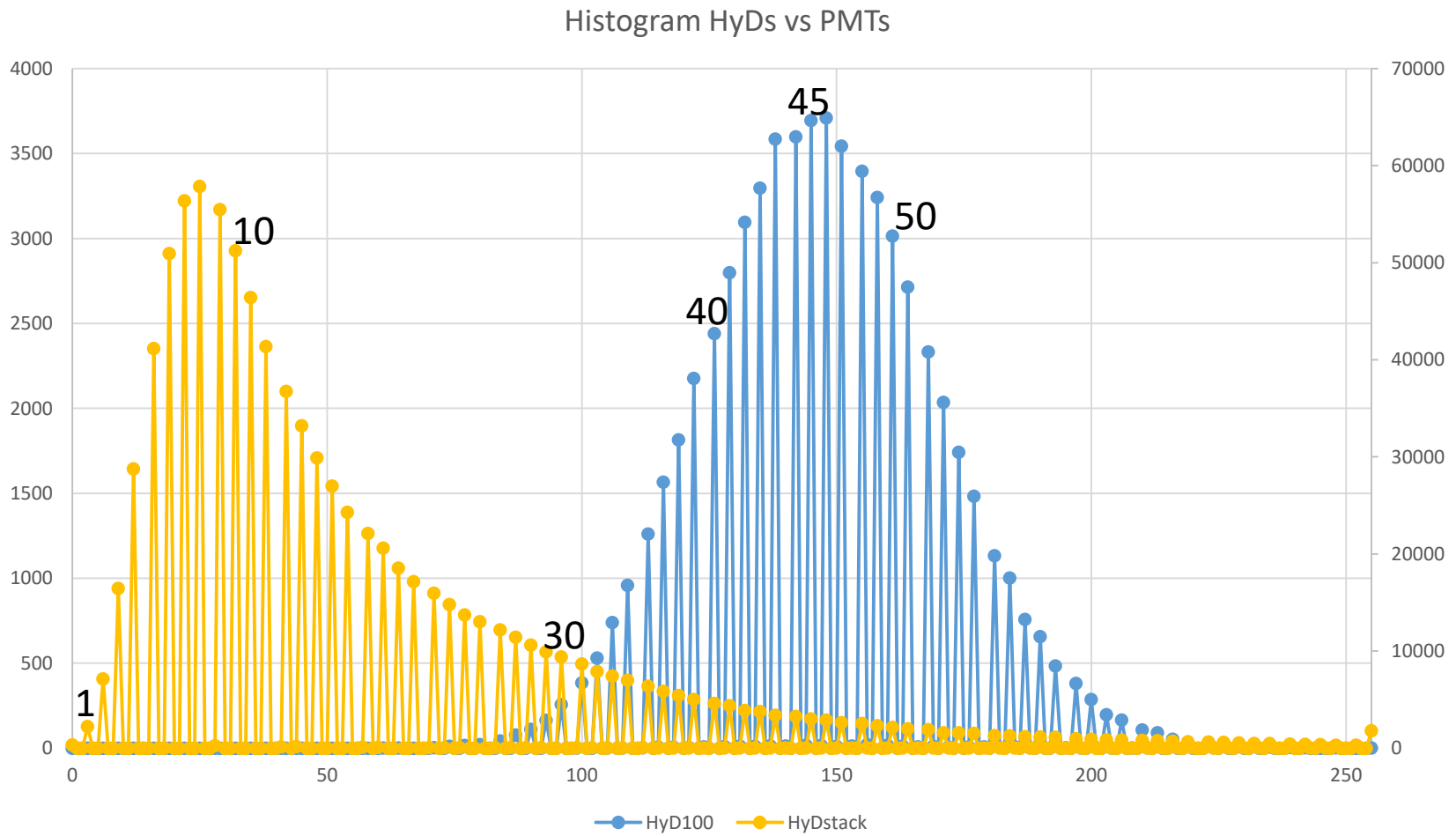
Single scan of a homogeneously fluorescent slide with a hybrid detector



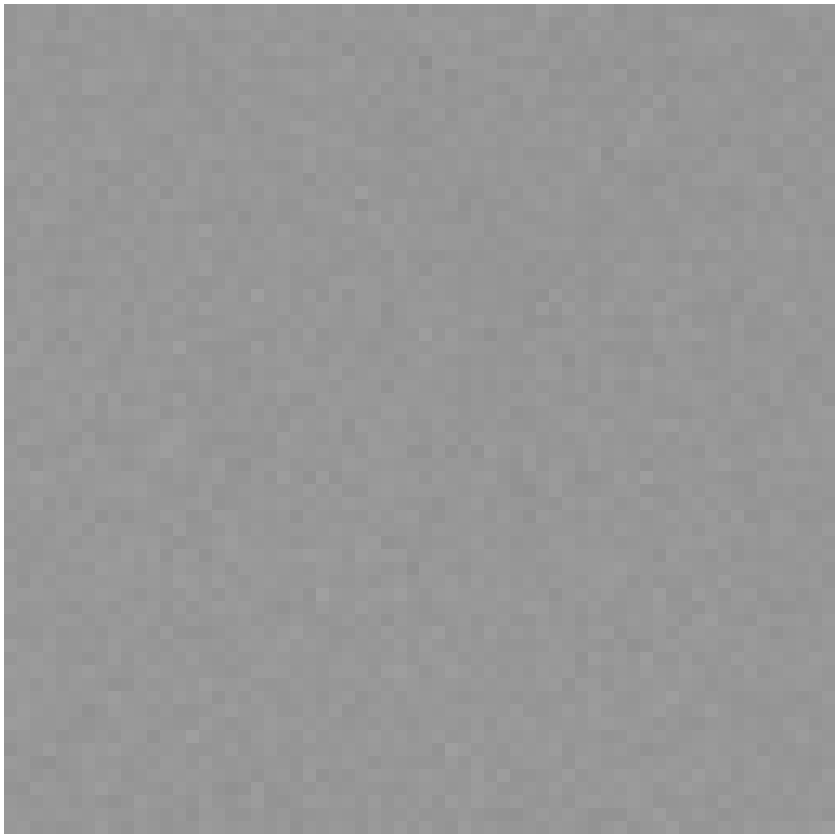
Histogram of a single 256x256 scan of a fluorescent slide



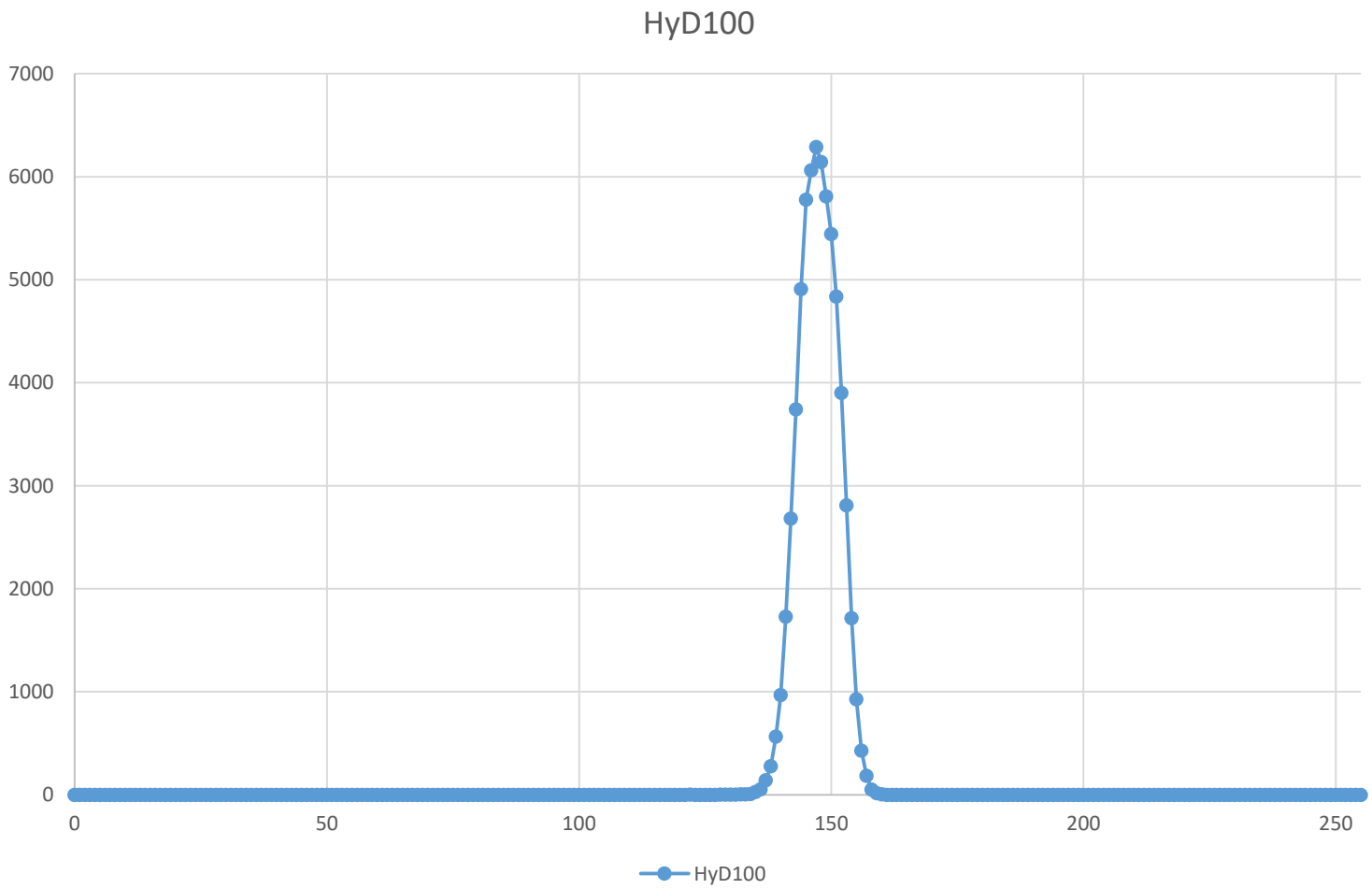
Hybrid detectors allow counting of photon (photoelectron) numbers



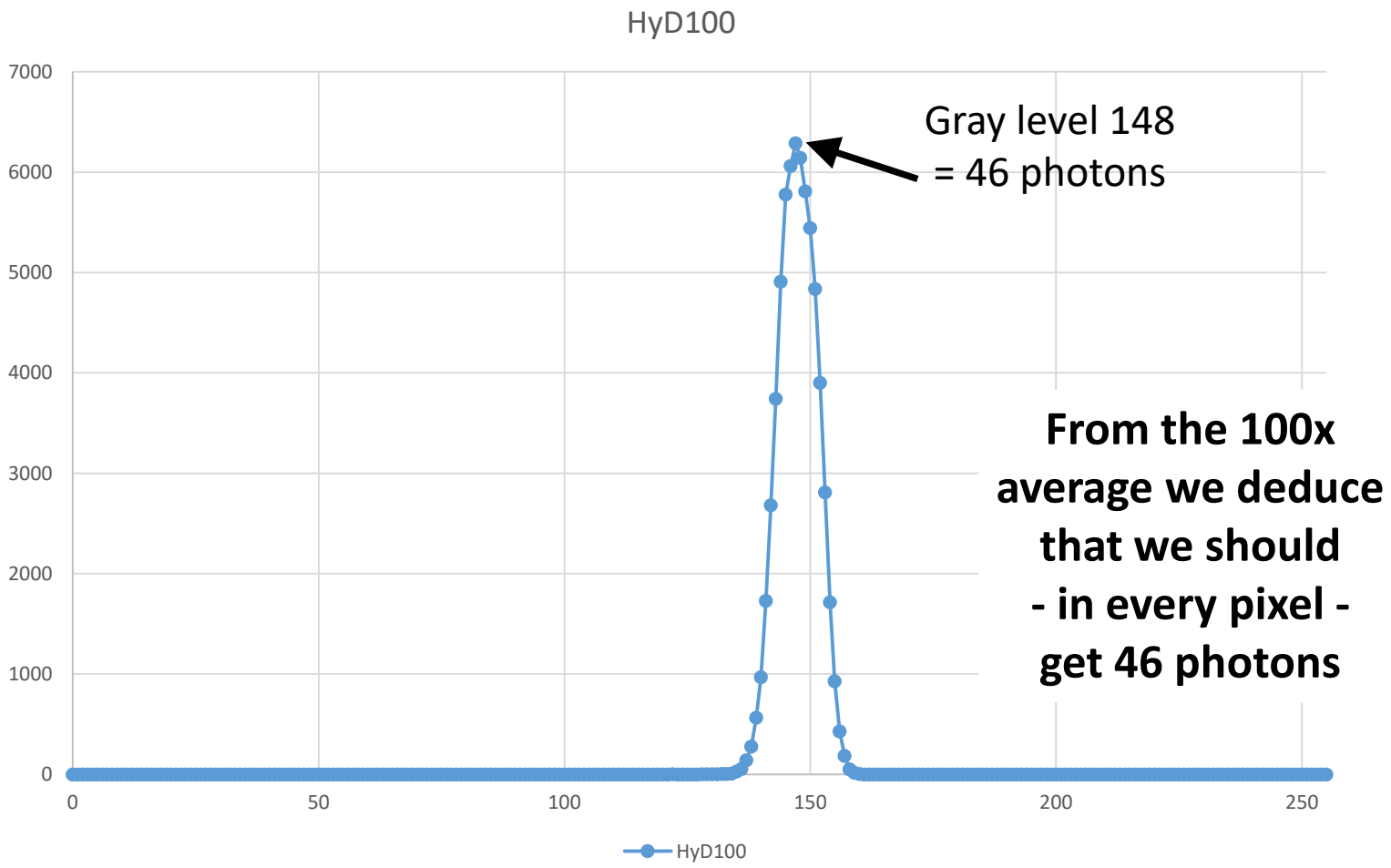
Average Projection of 100 Scans (frame average) with a hybrid detector



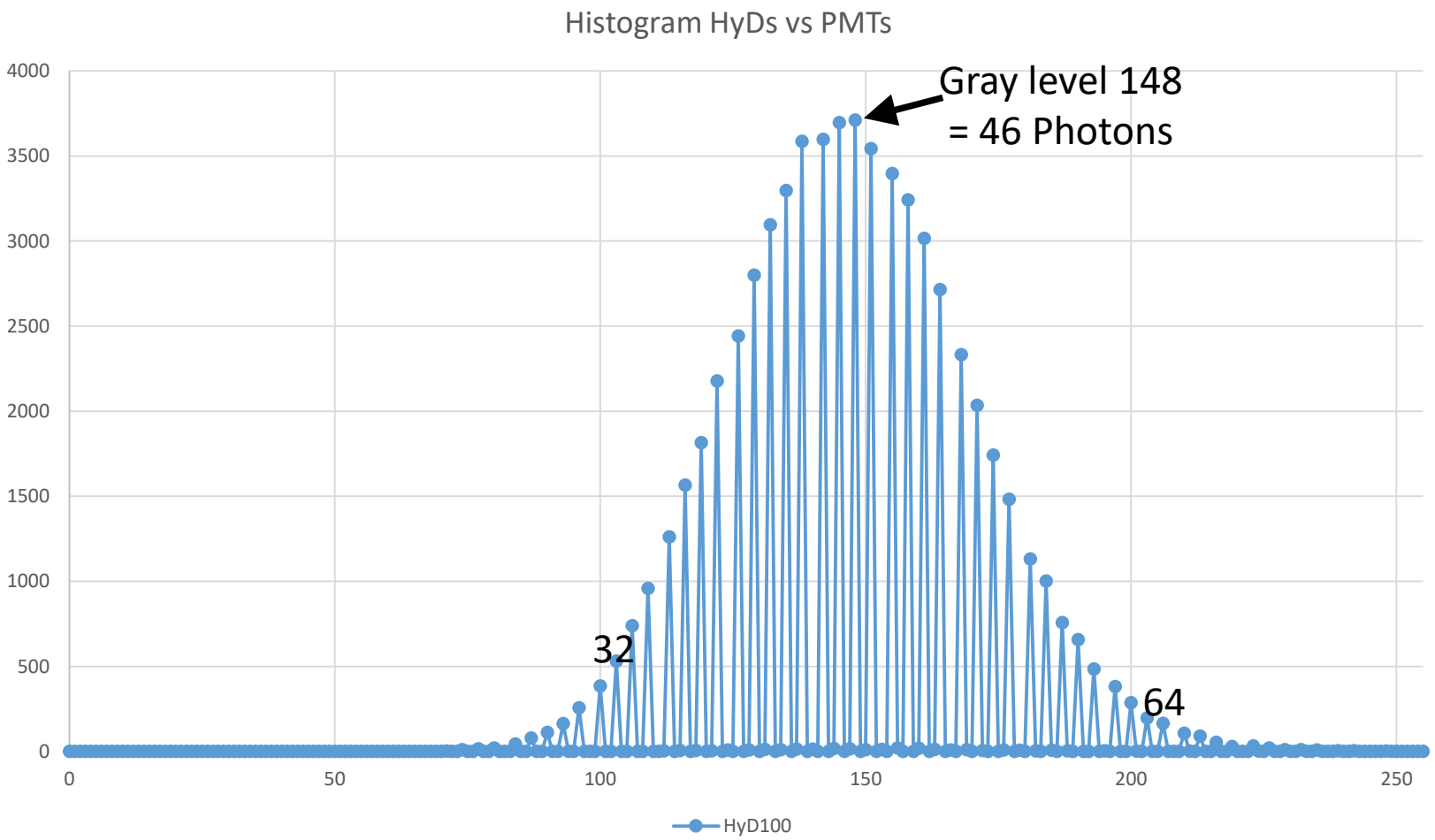
Histogram of a 100x average 256x256 scan of a fluorescent slide



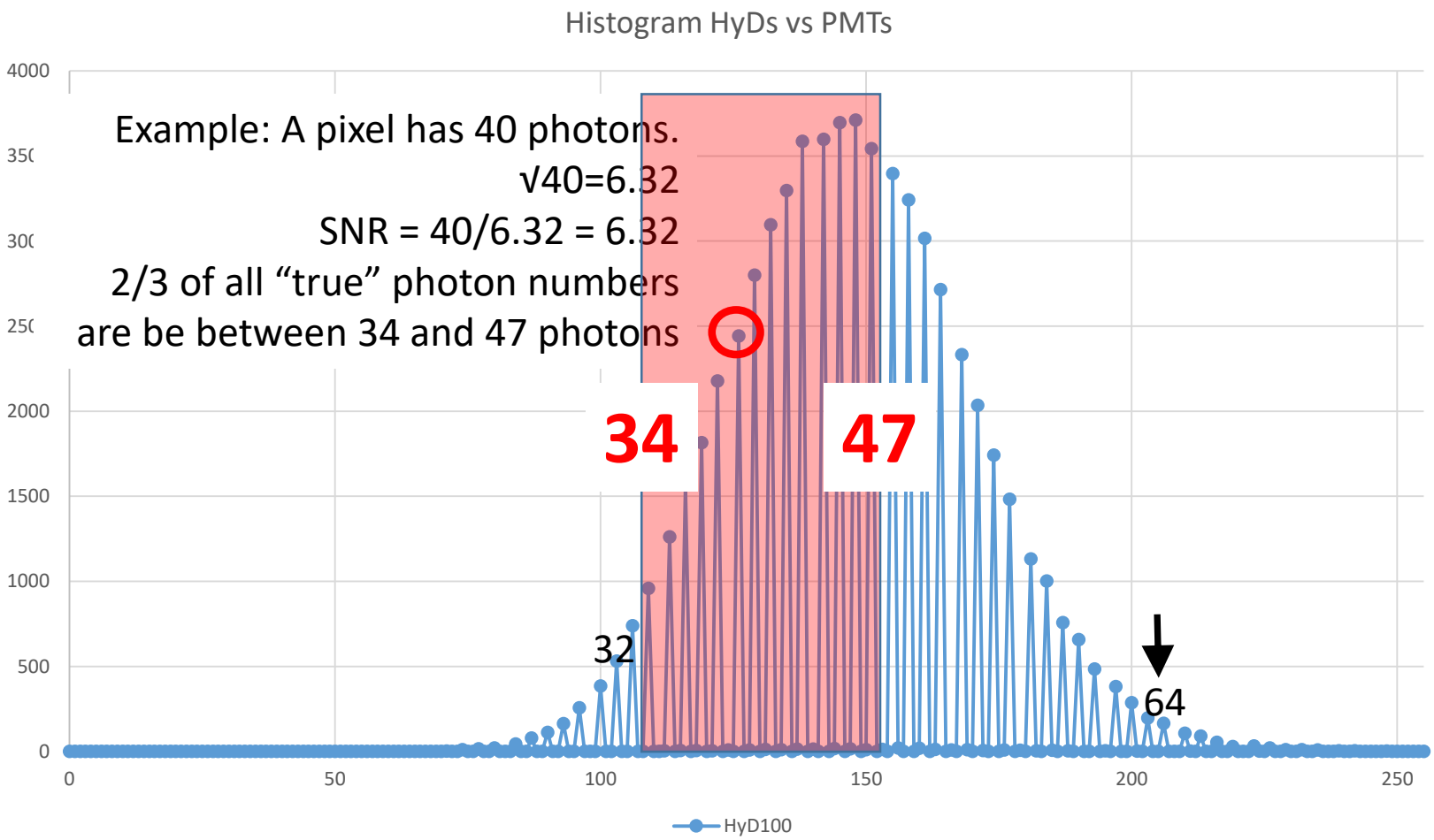
Histogram of a 100x average 256x256 scan of a fluorescent slide



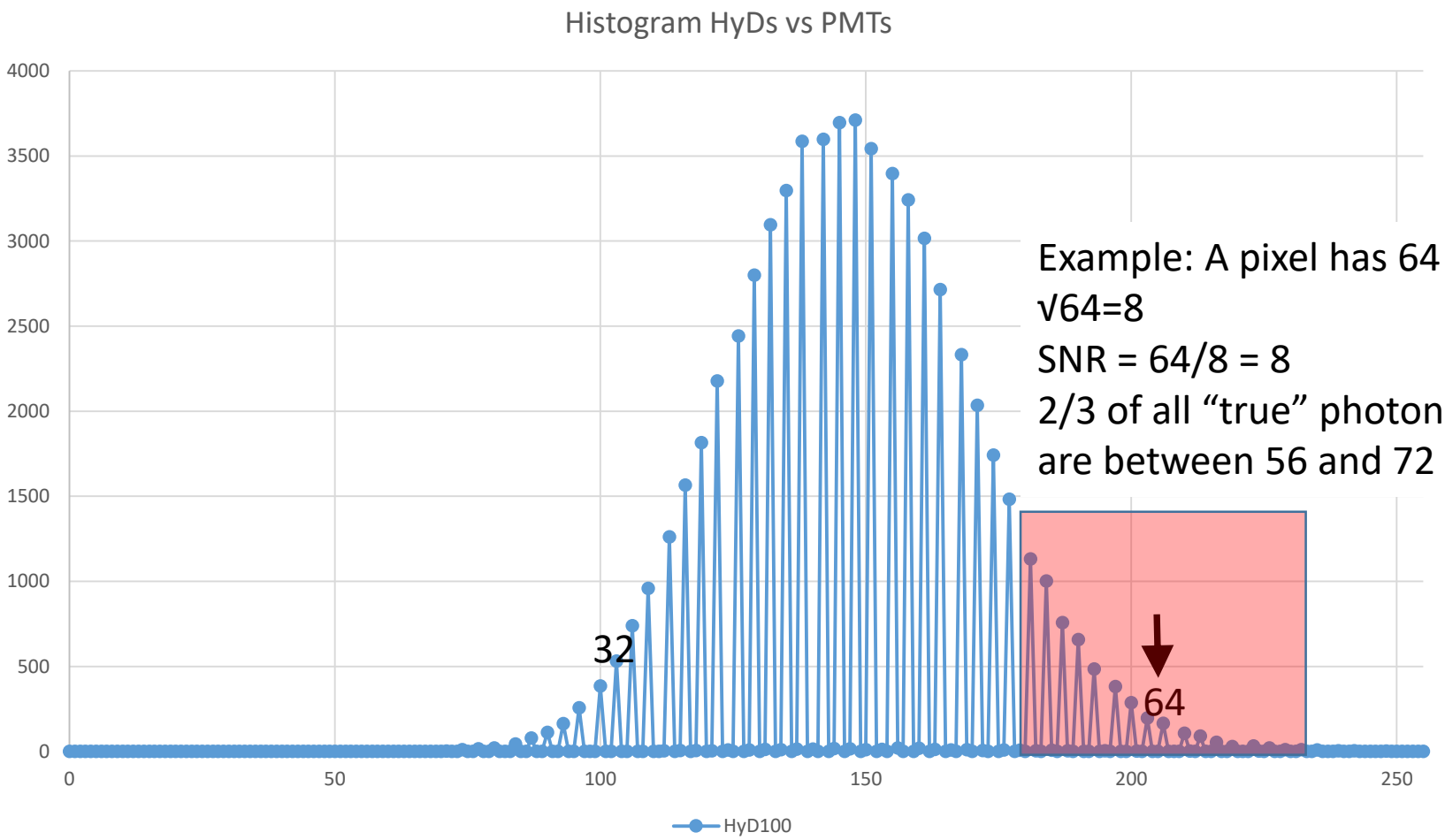
Histogram of a single 256x256 scan of a fluorescent slide



Histogram of a single 256x256 scan of a fluorescent slide



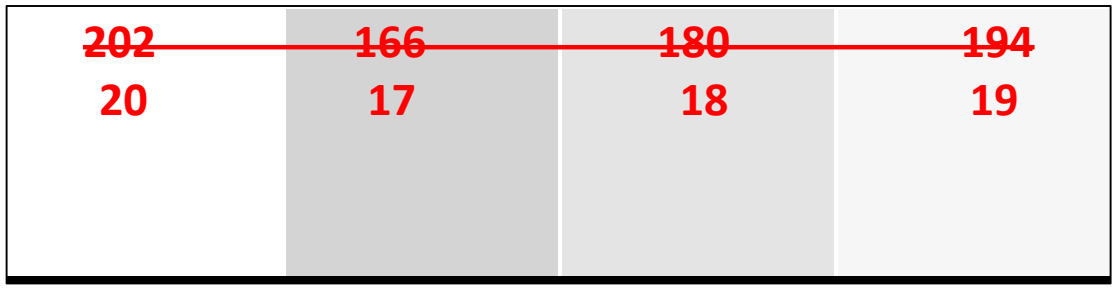
Histogram of a single 256x256 scan of a fluorescent slide



Example: A pixel has 64 photons.
√64=8
SNR = 64/8 = 8
2/3 of all “true” photon numbers are between 56 and 72 photons

In real life, we are happy if a confocal collects 20 photons for bright pixels

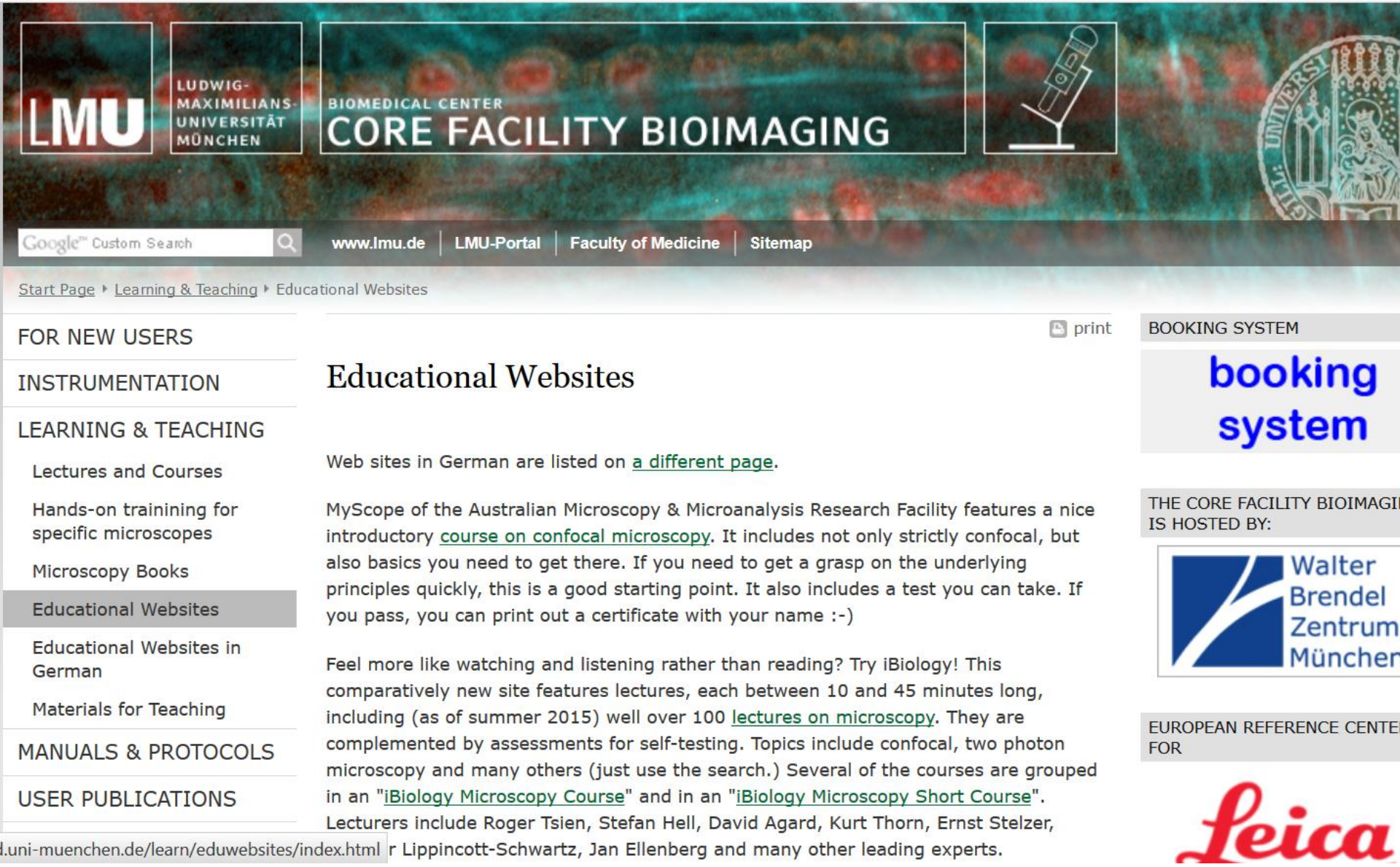
for example



- However, the above is ***not*** a prediction how such an image will look like.
- Rather, it is a ***probability map***. The leftmost pixel simply has a slightly (1.17x) higher probability of receiving a photon than the one right to it.
- We might just as well collect the following

  18 **21** **20** **18**

- In typical settings, we may not be able to collect sufficient photons to be certain of such a small difference.



http://www.bioimaging.bmc.med.lmu.de/learn/eduwebsites/index.html

http://www.ammrf.org.au/myscope/confocal/introduction/

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  - Tailor this module
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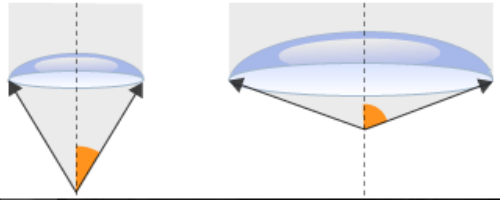
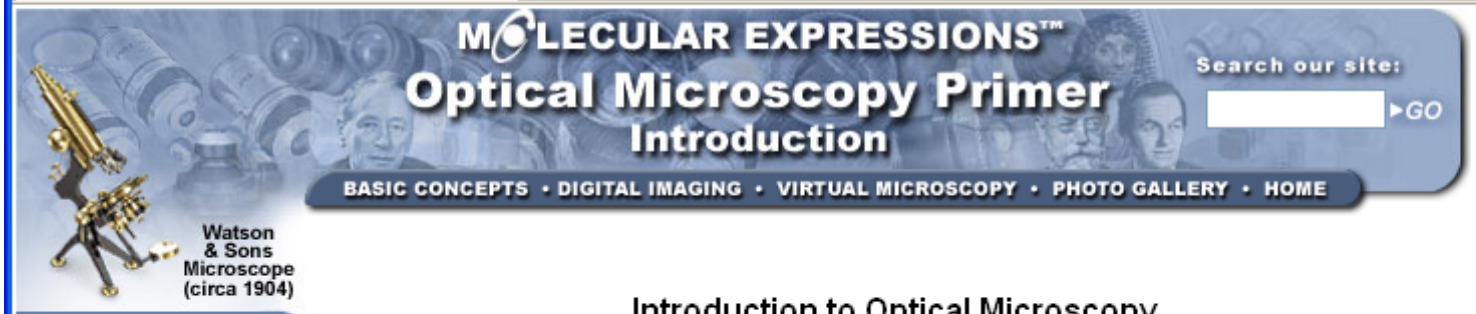
Introduction

Optical microscopy uses visible light, and its performance is inherently limited by the wavelength of light. This ranges from 400nm (violet) to 650nm (red). There are two different ways of calculating resolution, according to whether the specimen is illuminated externally (Abbe calculation) or is effectively self luminous as in fluorescence microscopy (Rayleigh calculation). Both give similar results, the difference lies in how to set up the microscope for best performance. With an ideal lens, diffraction limits the resolution to about half the wavelength of light, and our best objectives come within 95% of this.

What makes an objective good?

The most important quality of a lens is not its magnification, essential though that is, but its numerical aperture. This is defined as the sine of the maximum angle (from the vertical) at which light can enter. It is 'numerical' because it is a ratio – the actual size of the lens makes no difference. It could be – and usually is – a small lens very close to the slide, but where a large working distance is required it can be larger and further away (and probably more expensive).





Microscopy Primer
Light and Color
Microscope Basics
Special Techniques
Digital Imaging
Confocal Microscopy
Live-Cell Imaging
Photomicrography
Microscopy Museum
Virtual Microscopy
Fluorescence
Web Resources

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Introduction to Optical Microscopy, Digital Imaging, and Photomicrography

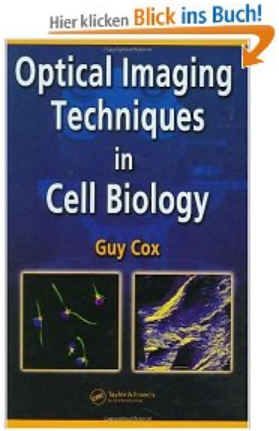
This treatise on optical microscopy is divided into several sections that are available through the links displayed immediately to the left (in the darker navigational boxes) and below. In order to print the entire microscopy primer as a paper document, you must download each link independently, send the file to your printer, and put the results together.

In the Bibliography, we have included links to other works on optical microscopy and our section on Web Resources contains links to other microscopy sites on the Internet. This material is targeted for educational purposes only, and is not available to be posted on remote websites (either commercial or educational) or distributed in any electronic format.

Frequently Asked Questions - Mortimer Abramowitz, senior microscopist at Olympus America Inc., answers the 50 most commonly asked questions about microscopy and photomicrography.

Physics of Light and Color - Visible light represents only a small portion of the entire electromagnetic spectrum of radiation that extends from high-frequency gamma rays through X-rays, ultraviolet light, infrared radiation and microwaves to very low frequency long-wavelength radio waves. The complex phenomenon of visible light is classically discussed in terms of rays and wavefronts. Starting with the nature of electromagnetic radiation, a wide variety of topics are covered in this section, including refraction, reflection, diffraction, interference, birefringence, polarization, primary colors, human

Recommendable textbooks on (confocal) microscopy



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Guy Cox (Autor)

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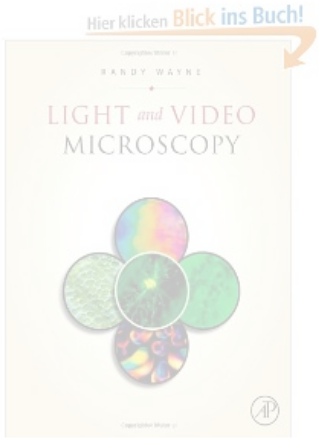

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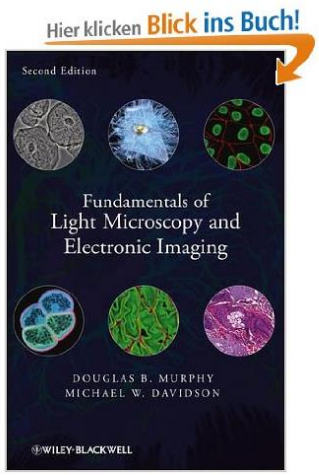
More elaborate, good for general background but only 4 confocal pages (+digital imaging, fluorescence etc.)



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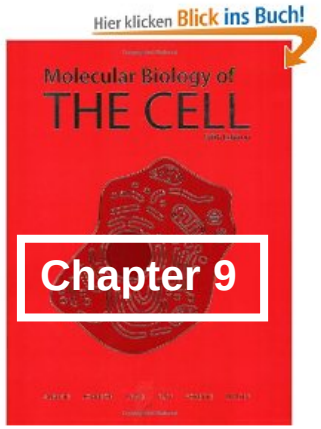
2nd, much improved edition 2013. 42 confocal pages (plus mulit-photon, digital imaging, fluorescence etc.)



Molecular Biology of the Cell [Taschenbuch]

Bruce Alberts (Autor), Alexander Johnson (Autor), Peter Walter (Autor), Julian Lewis (Autor), Martin Raff (Autor), Keith Roberts (Autor), Nigel Orme (Fotograf)

(6 Kundenrezensionen) Gefällt mir (3)

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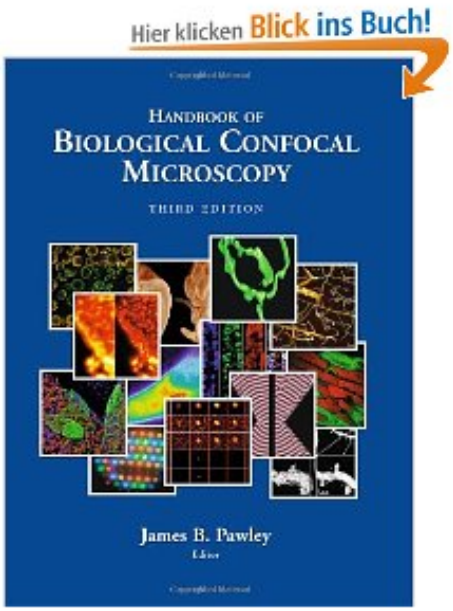
Only because you might already have it

the reference book on confocal microscopy, 988 pages



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James Pawley (Herausgeber)

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Recommended reading:

- Chapter 35: Tutorial on practical confocal microscopy.....
- Chapter 36: Practical confocal microscopy