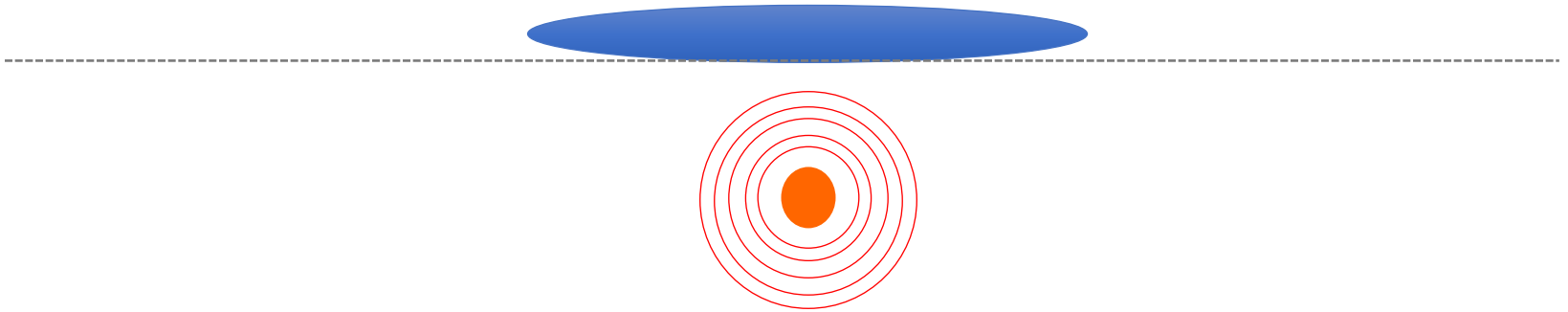
Microscopy, Optics & Fluorescence

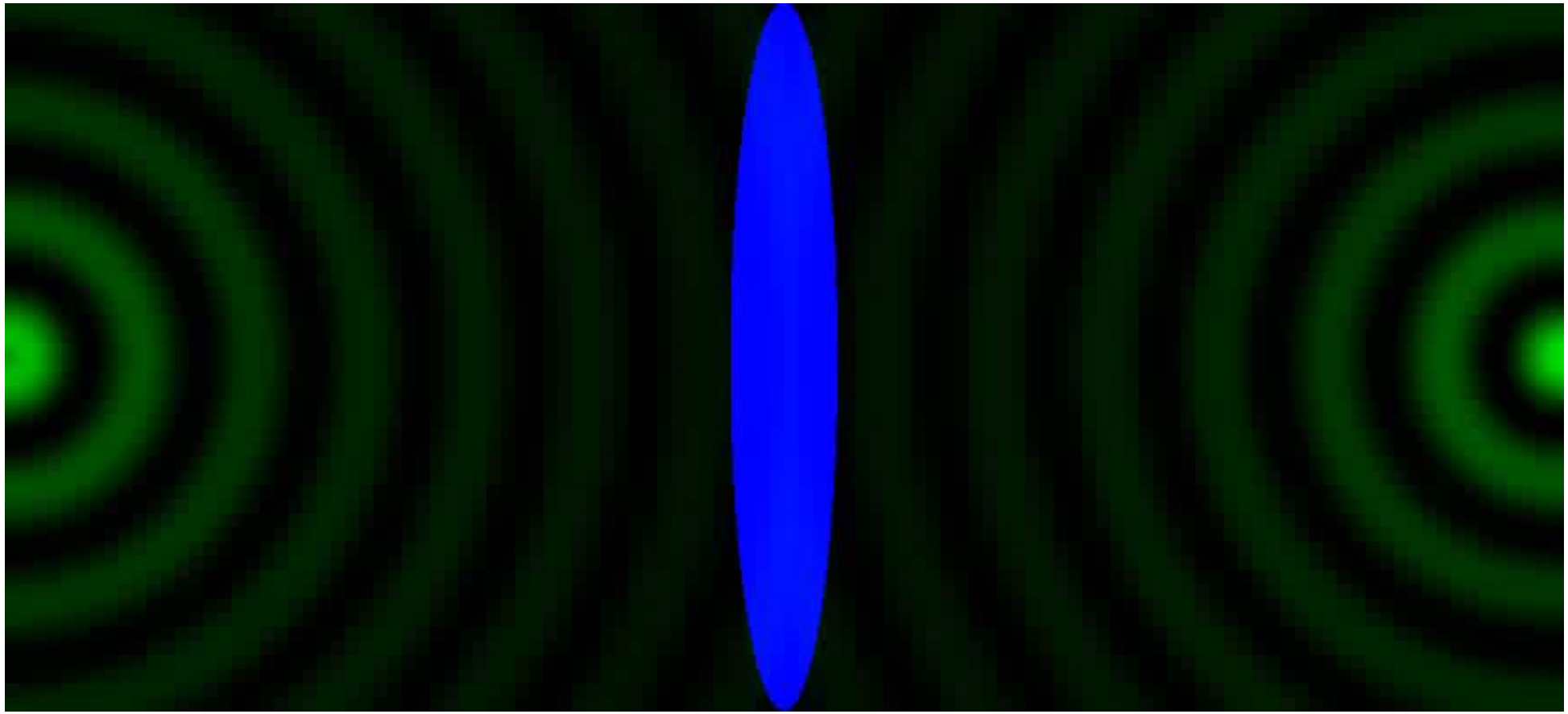
Felix & Edna

aperture and resolution



a sub diffraction object scatters and emits in a –for all real word purposes - perfect spherical wave front

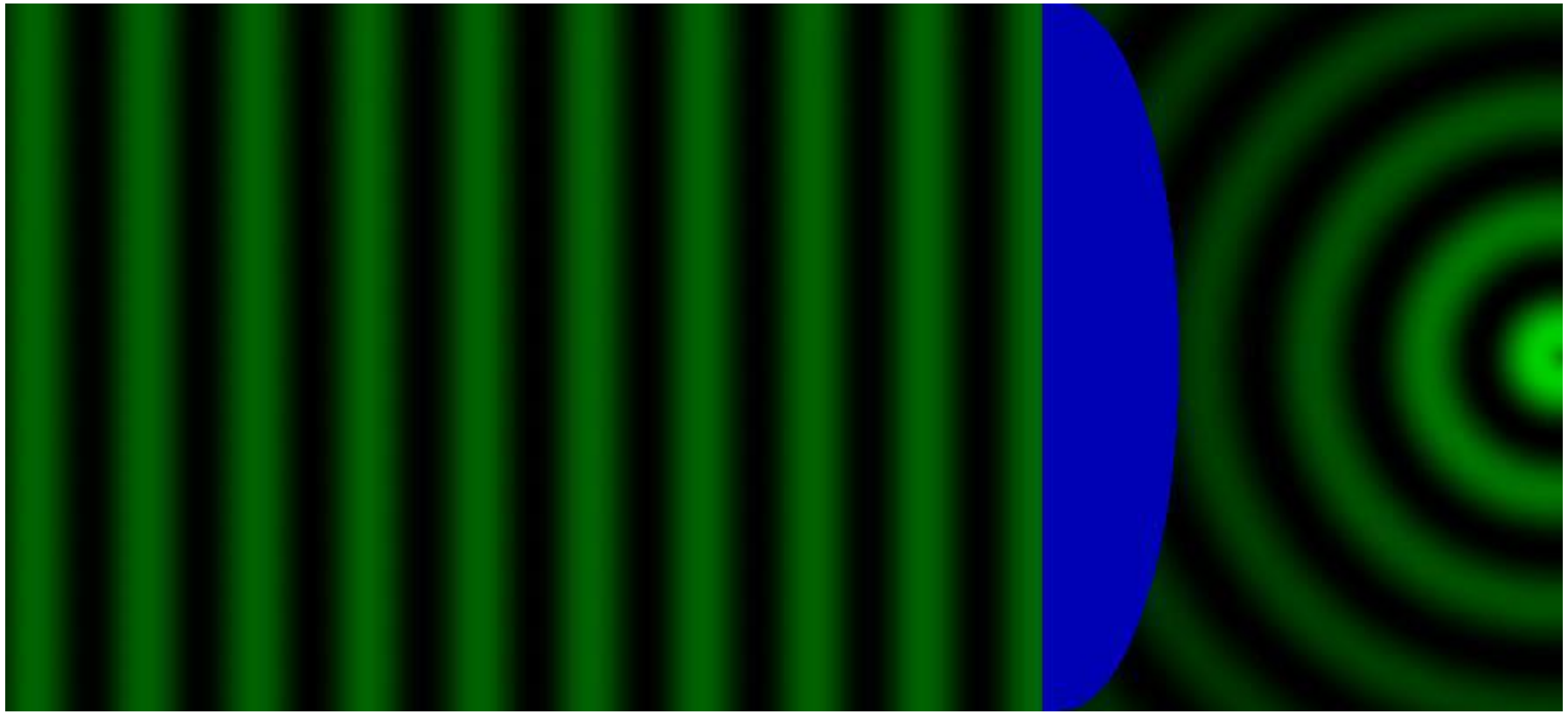
the lens recollects the light



.. by retarding the central waves more than the peripheral ones

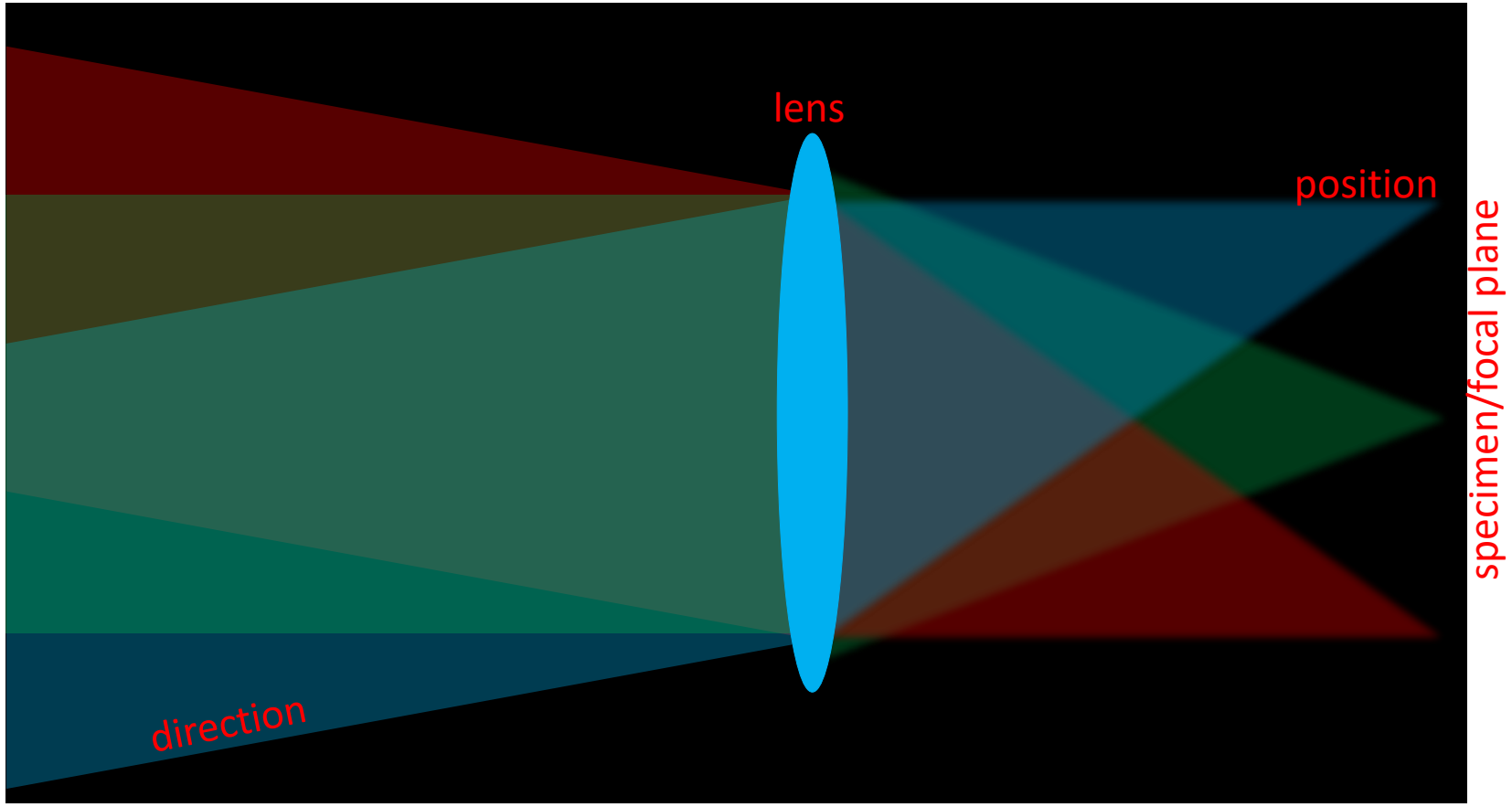
the definition of a lens

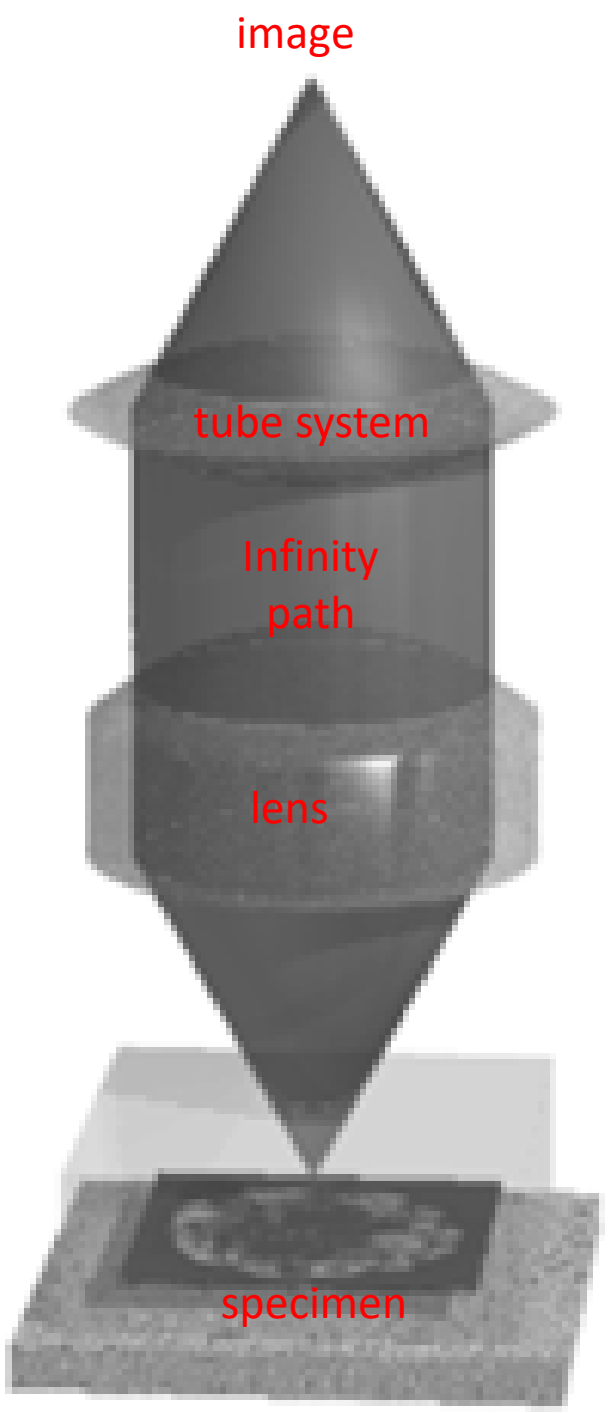
FELIX



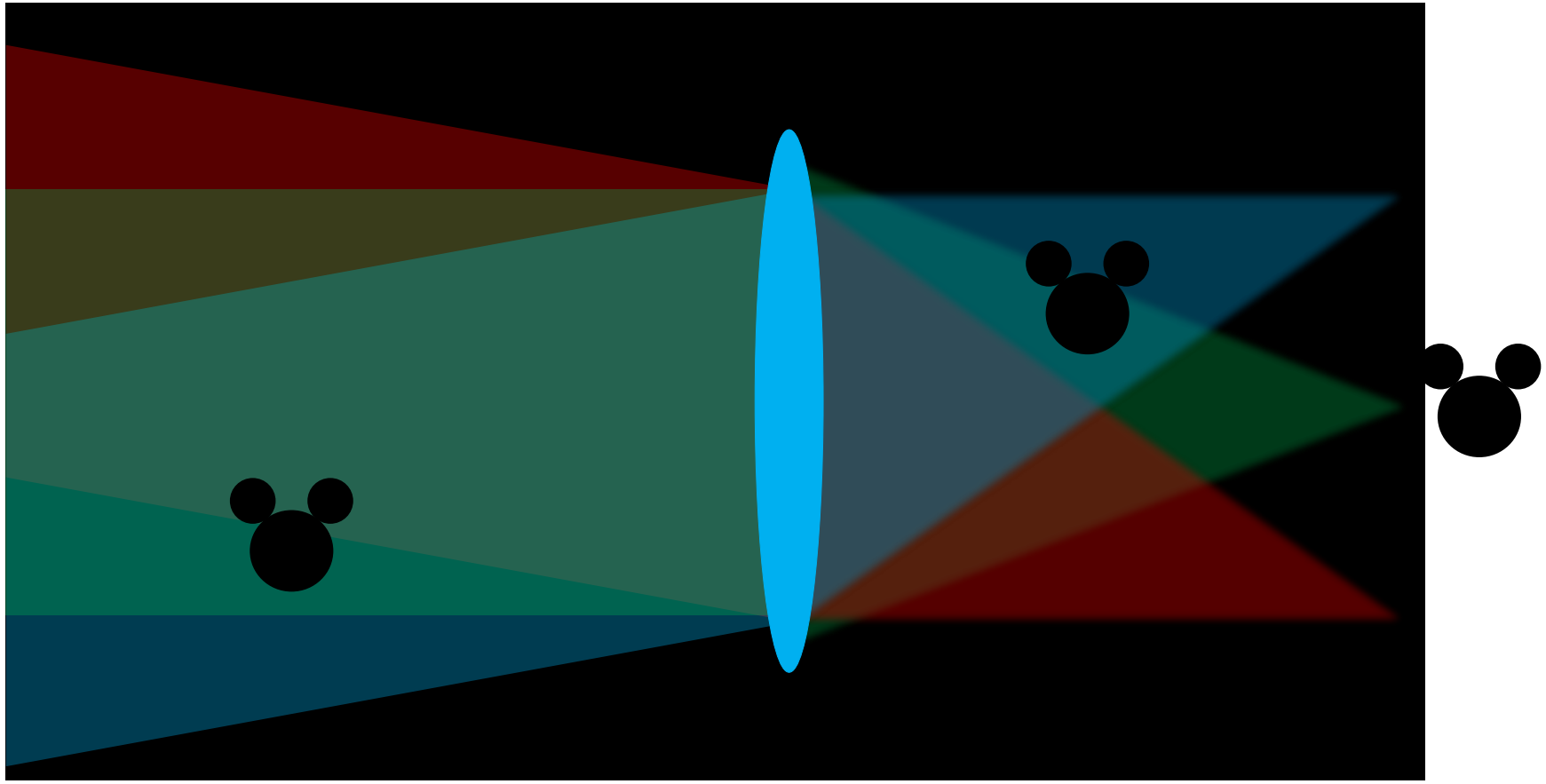
a laser emits a single wave which then be focused onto a single point

the wave direction is translated into position

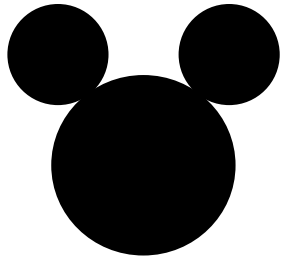




the wave direction is translated into position

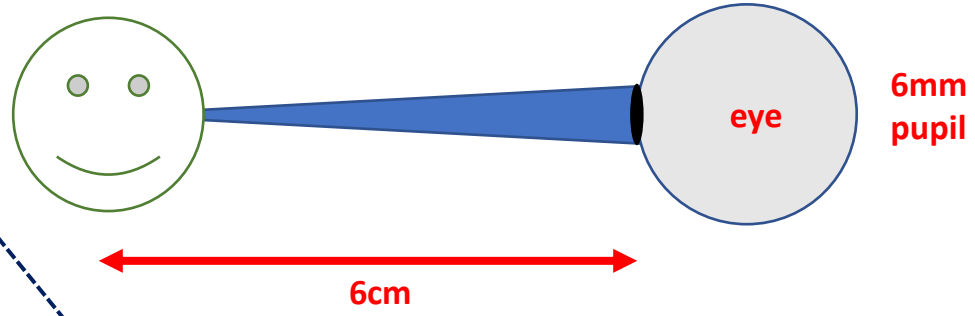


dimming shadow blocking

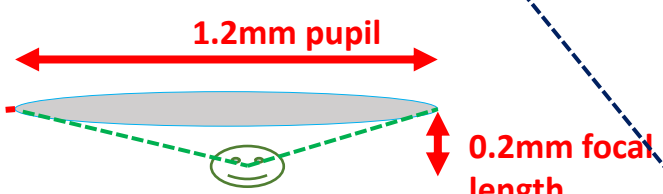


What happens, when we put an absorbing objects at different positions in the light path?

Eye vs Confocal Lens

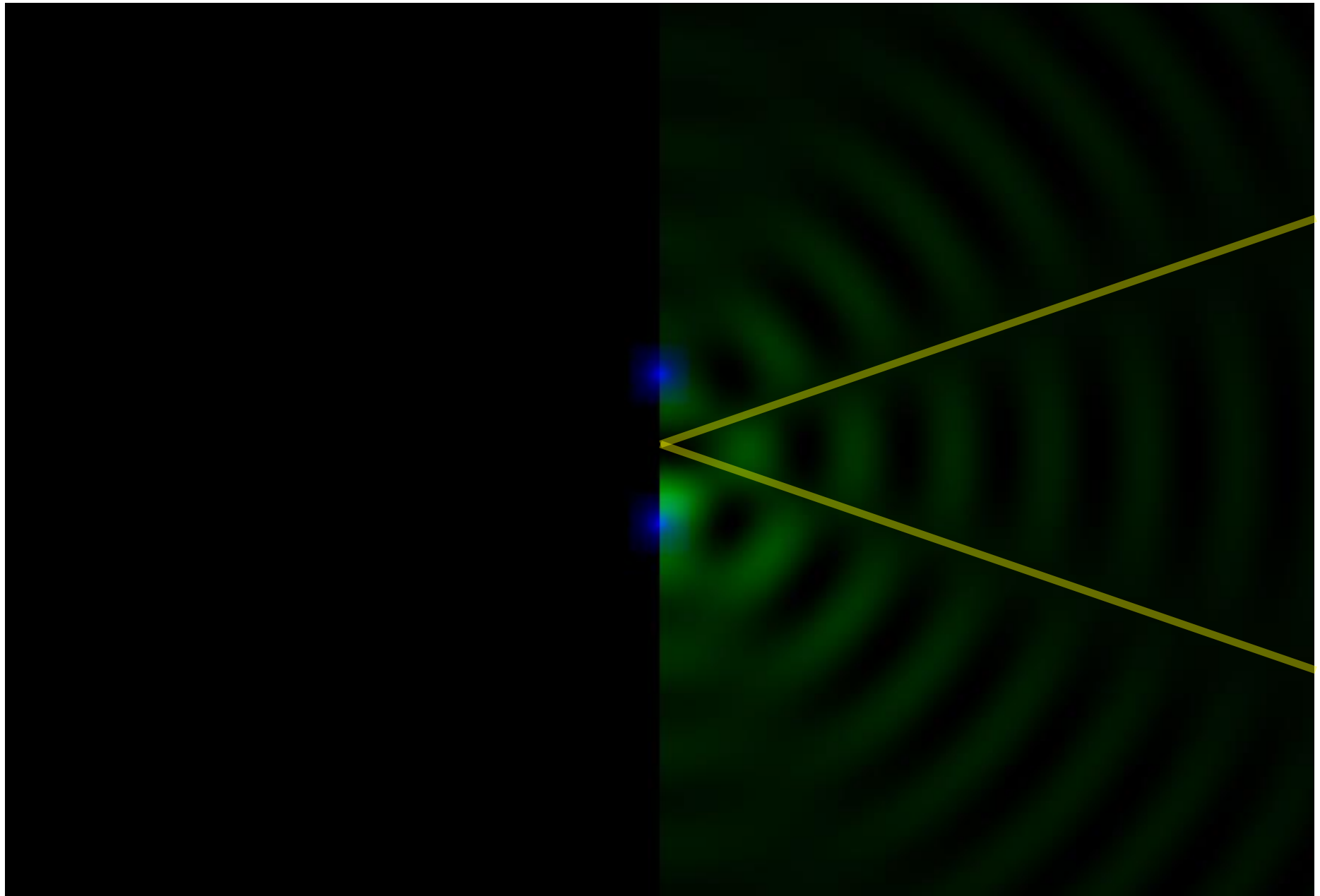


f-number
f/10



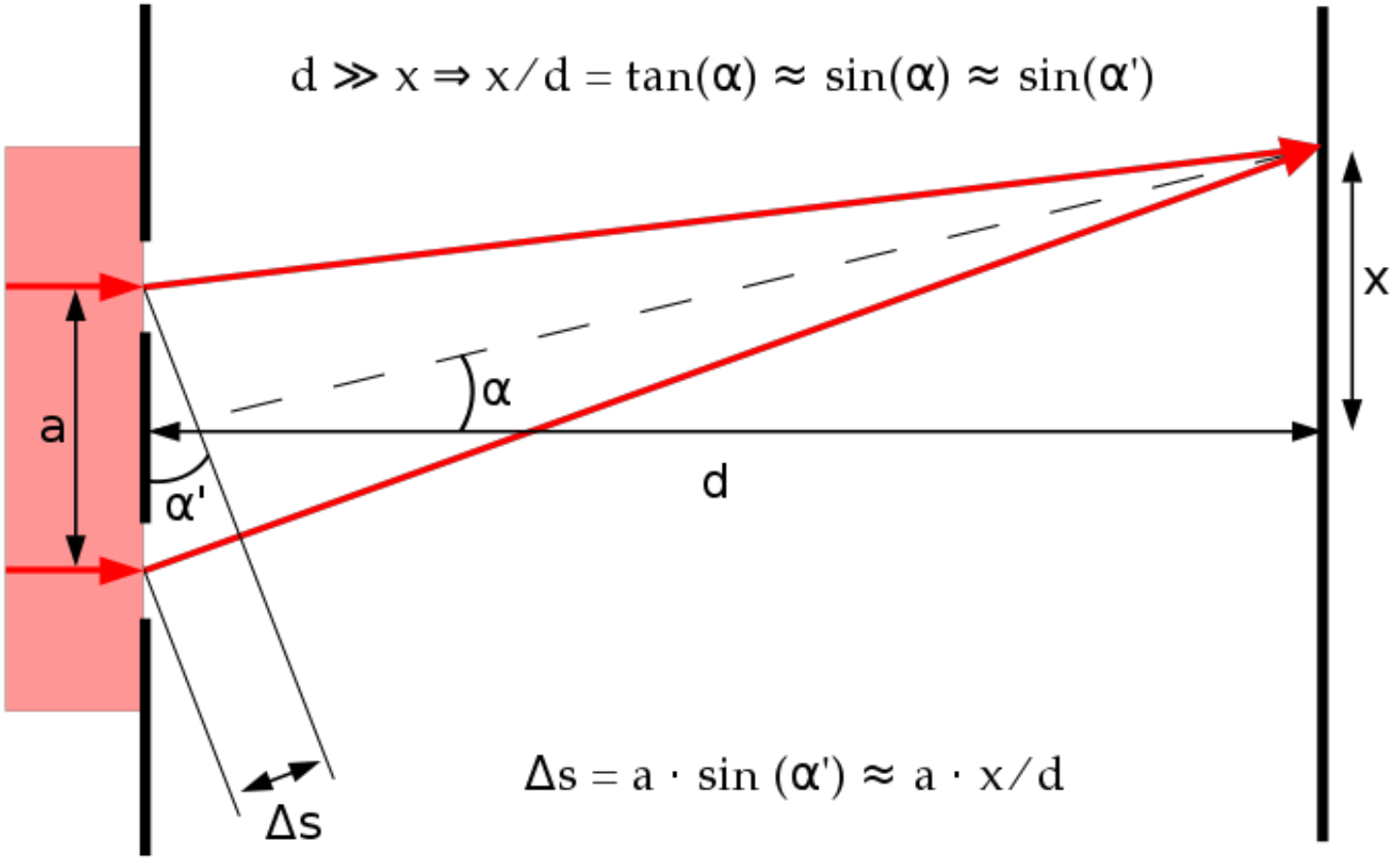
f-number
f/0.12

the lens’ resolution

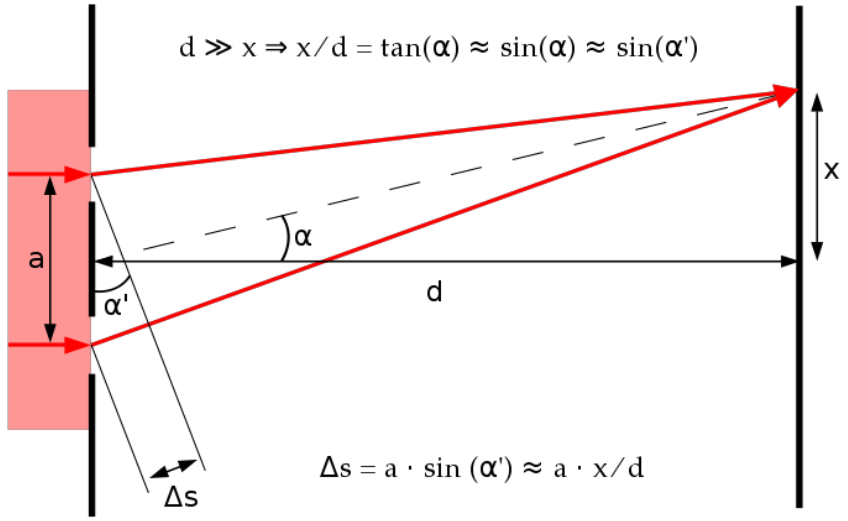


aperture and resolution

interference is positive if the path difference between the scatteres and the lens pupil is a multiple of the wavelength



aperture and resolution



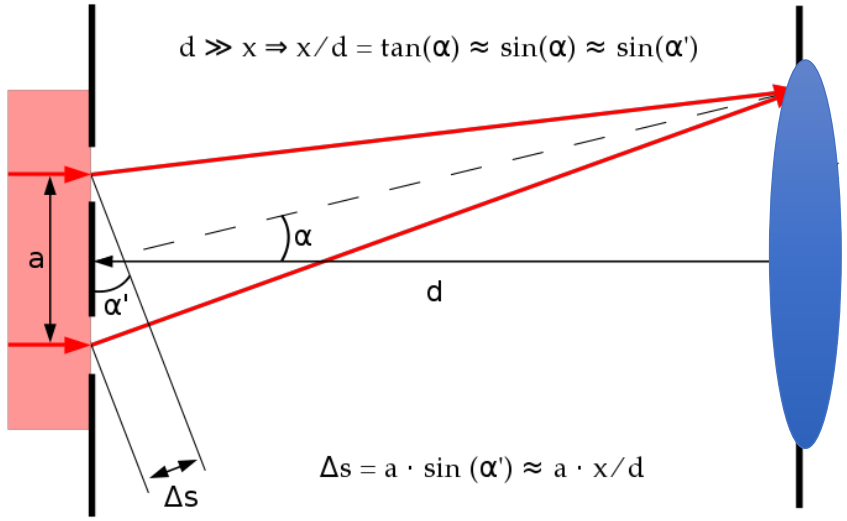
$\Delta s = m\lambda$ m=0,1,2,3….

$\lambda/a = \sin(\alpha)$

the primary maximum is m=0 for both emitters

if m=1 (or higher) is observed, an image of the emitters will be formed!

aperture and resolution

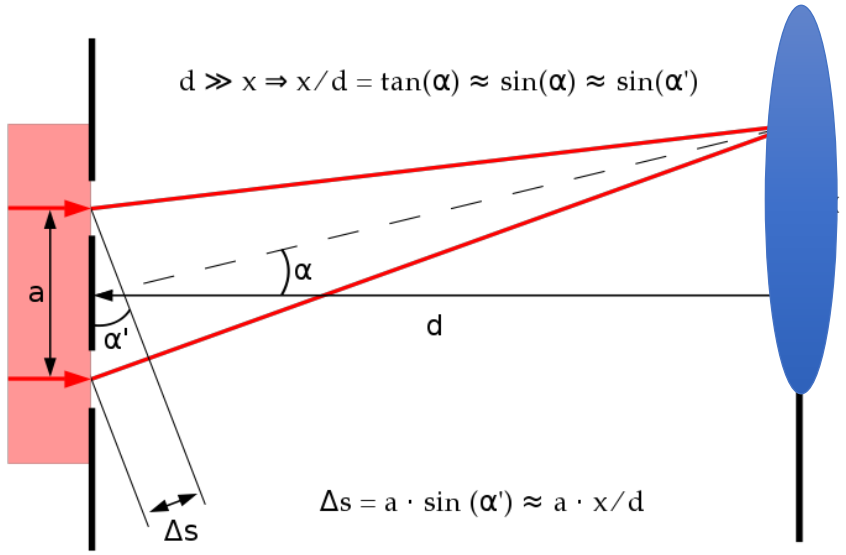


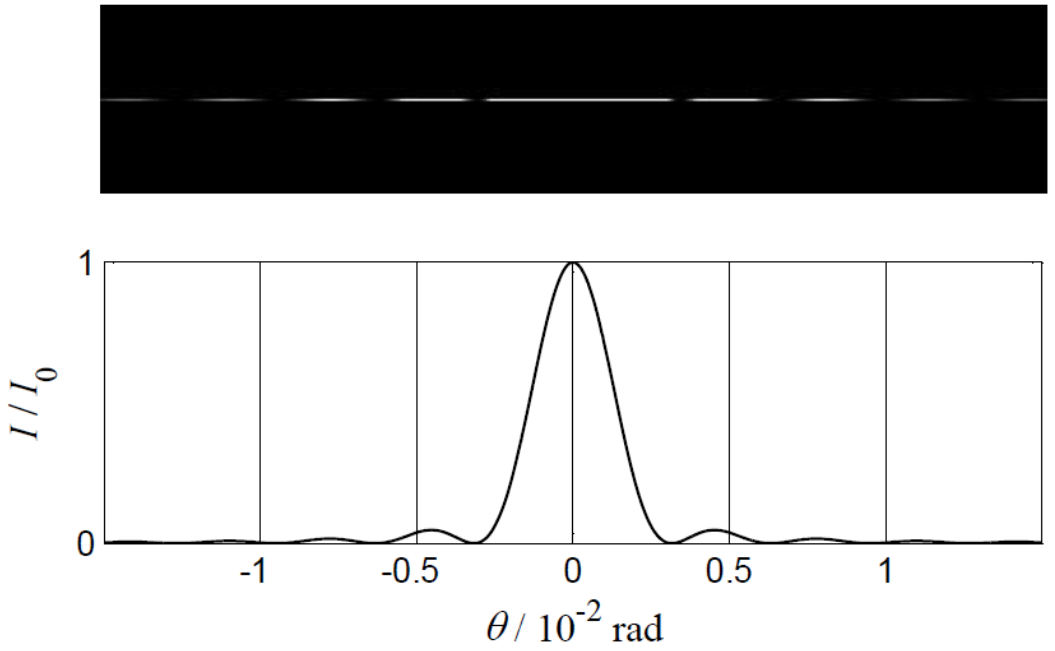
$\lambda/a=\sin(\alpha)$

$\alpha$is also the half aperture of the lens!

$\alpha$and hence the resolution for a planar illumination is $a= \lambda /\sin(\alpha)$

diffraction profile

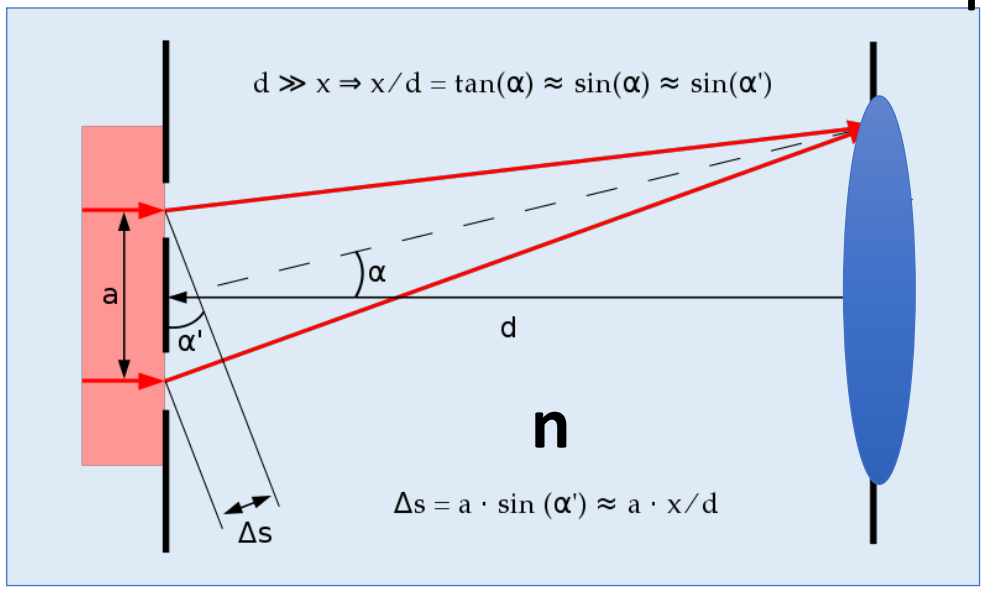




lines are not perfectly sharp

but there is an absolute and hard boundary which separation can still be visualized with a microscope!

immersion and numerical aperture

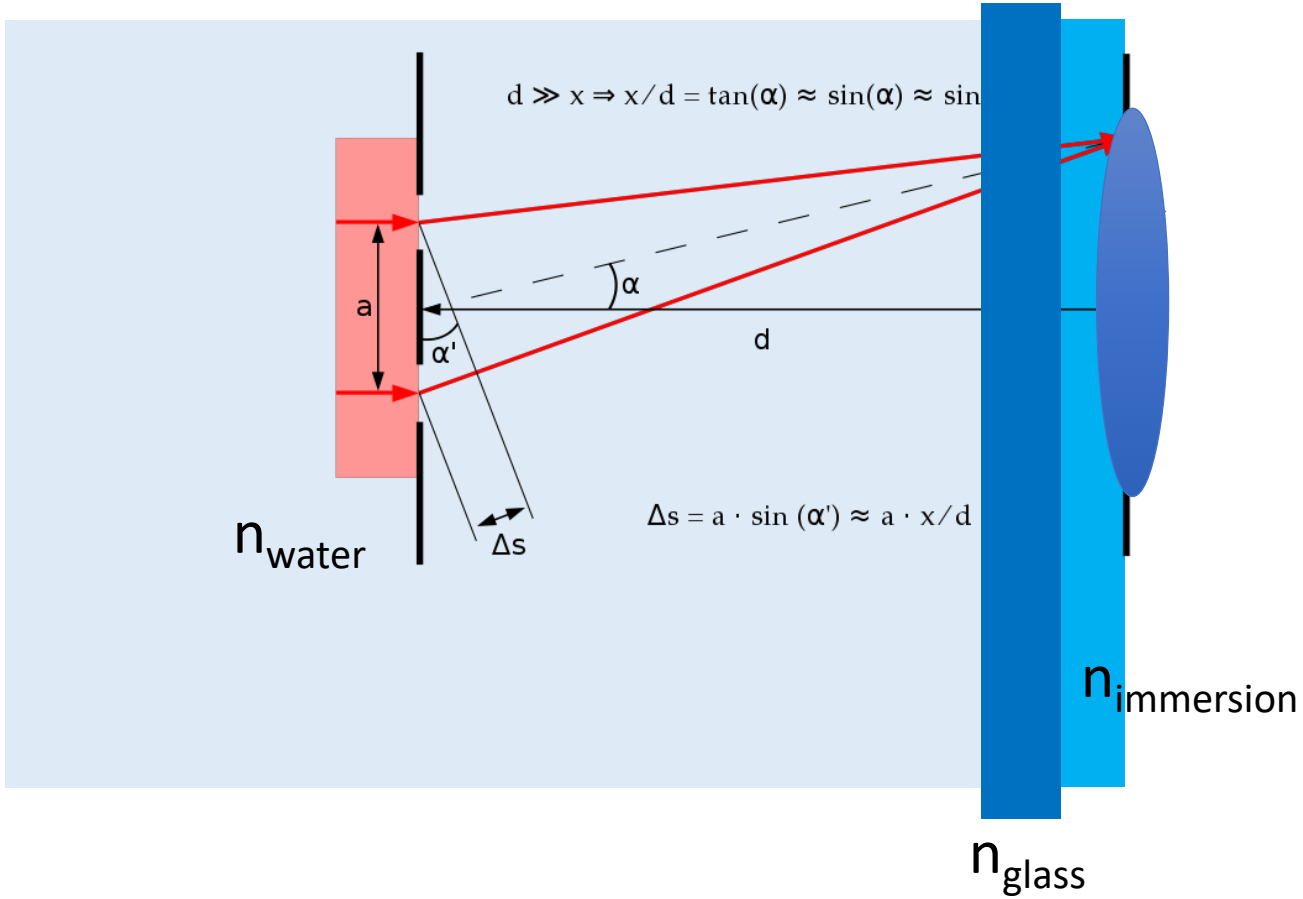


$\lambda=\lambda_0/n$ $\lambda/a=\sin(\alpha)$

$a= \lambda_0 /n \sin(\alpha)$ $NA:=n \sin(\alpha)$

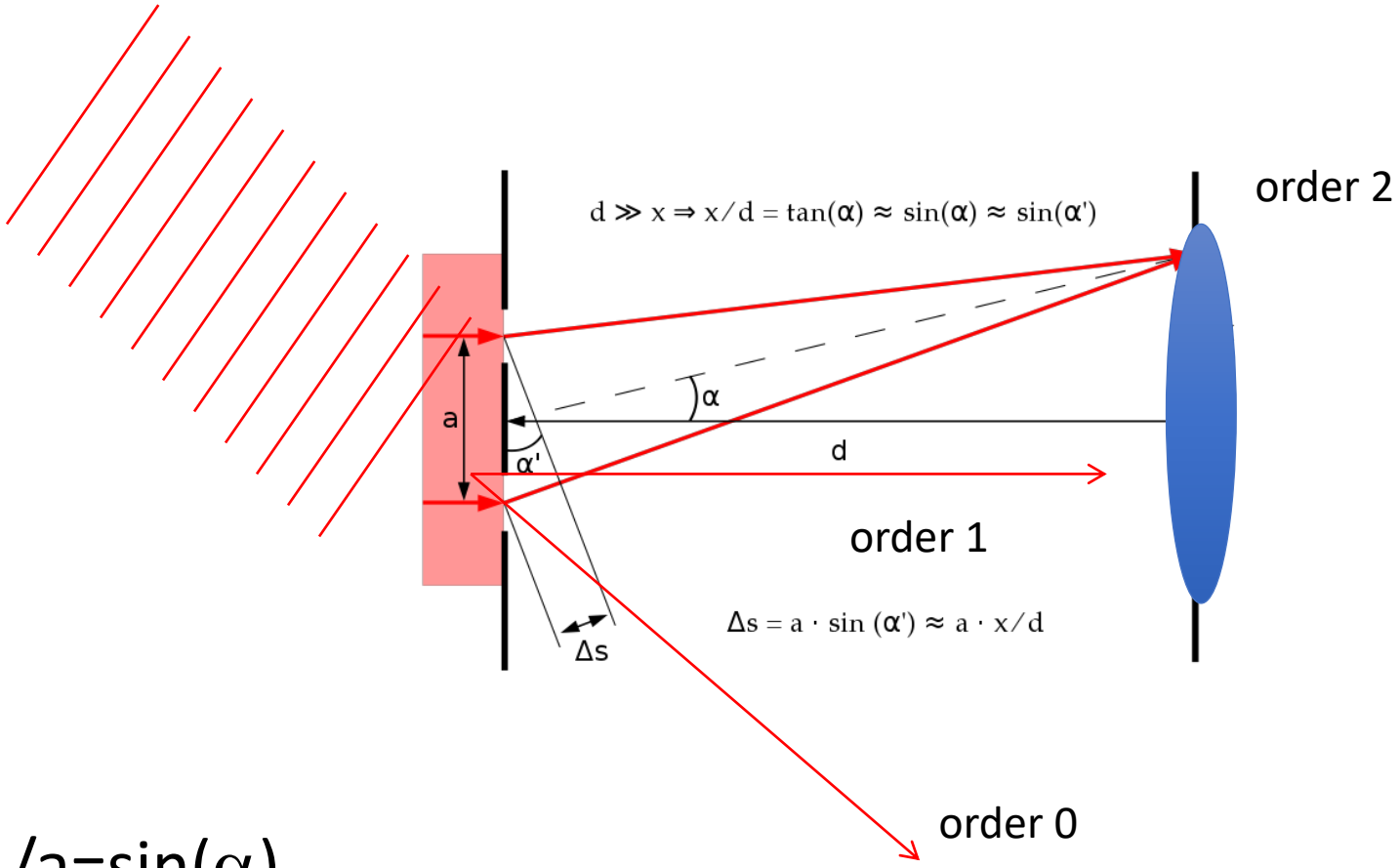
immersion captures higher diffraction orders at the same aperture angle by changing the ratio between resolution and local wavelength

immersion medium is only a part of the entire immersion system!



immersion captures higher diffraction orders at the same aperture

oblique light

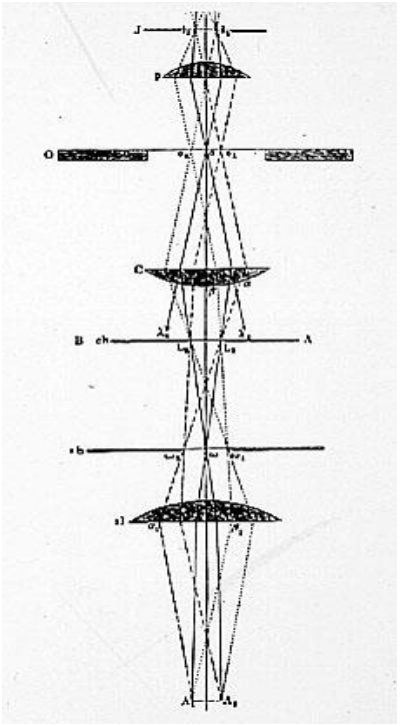


$2\lambda/a=\sin(\alpha)$

$a=\lambda/2\sin(\alpha)$

Best known oblique light source Koehler Illumination

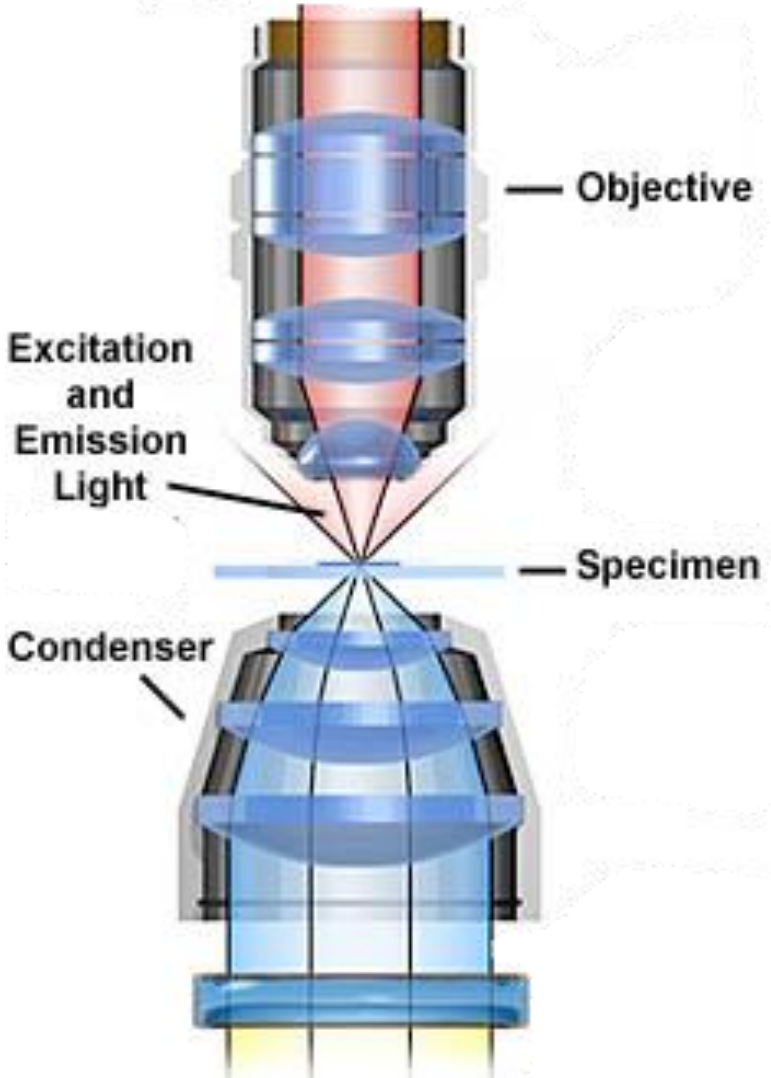
Prof. August Köhler
(1866 - 1948)



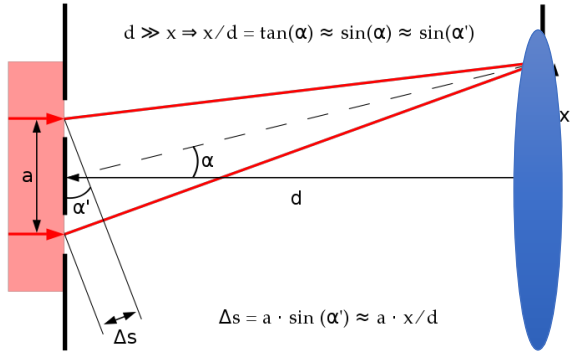
also provides very homogenous illumination from an uneven filament by backfocal plane illumination

lenses

unlike telescopes, objectives aim at collecting a large fraction of the light



quantum mechanic resolution



uncertainty principle:

$$\Delta p \Delta x \geq h$$

$$\Delta x = a$$

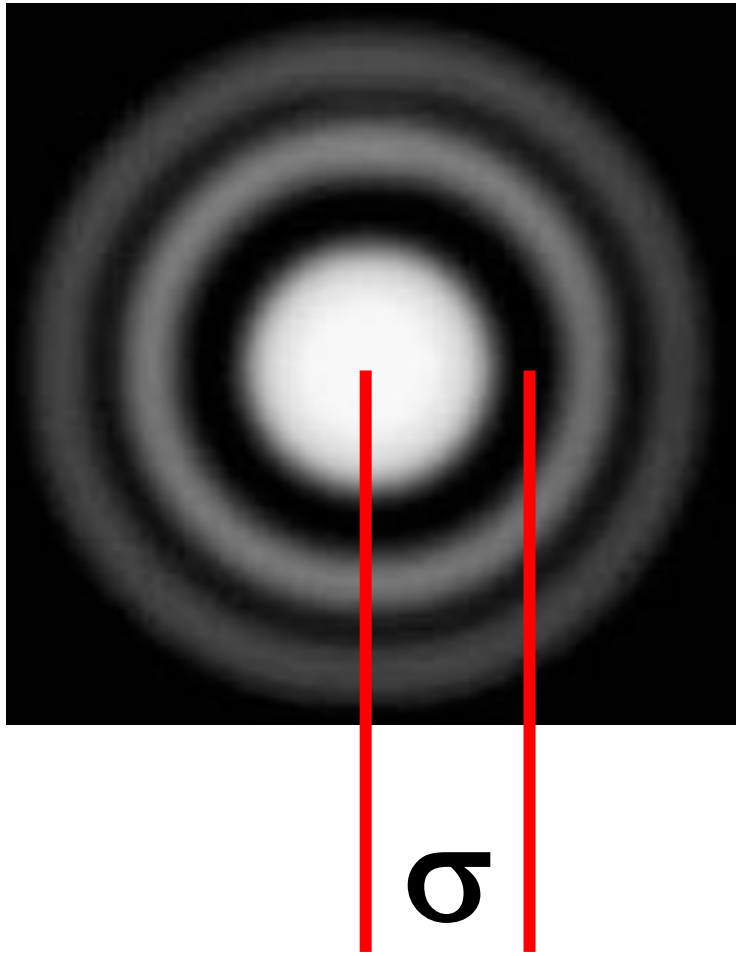
$$\Delta p = 2p \cdot sin(\alpha)$$

$$p = \hbar k = h\frac{n}{\lambda}$$

$$\cancel{h}\frac{2n}{\lambda} sin(\alpha) a = \cancel{h}$$

$$a = \frac{\lambda}{2NA}$$

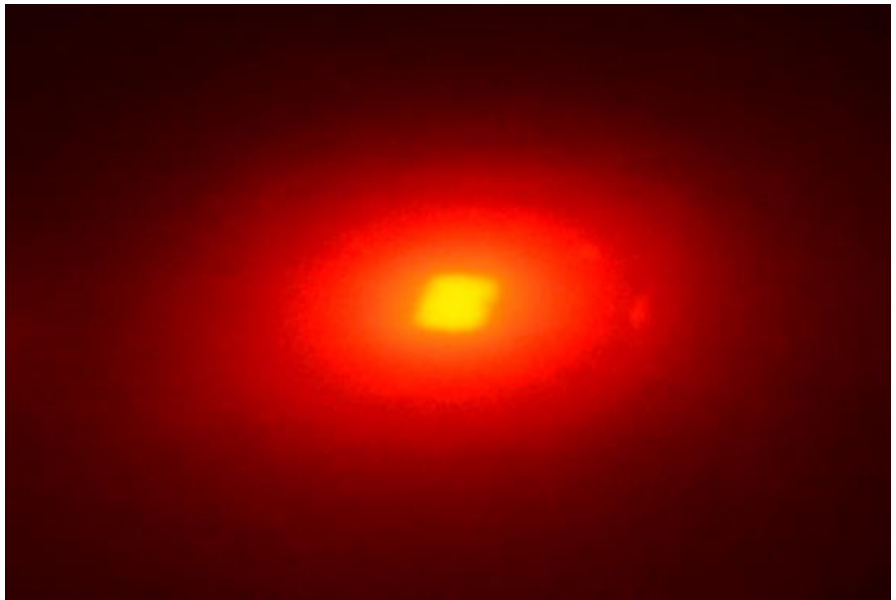
imaging expends excitation cycles



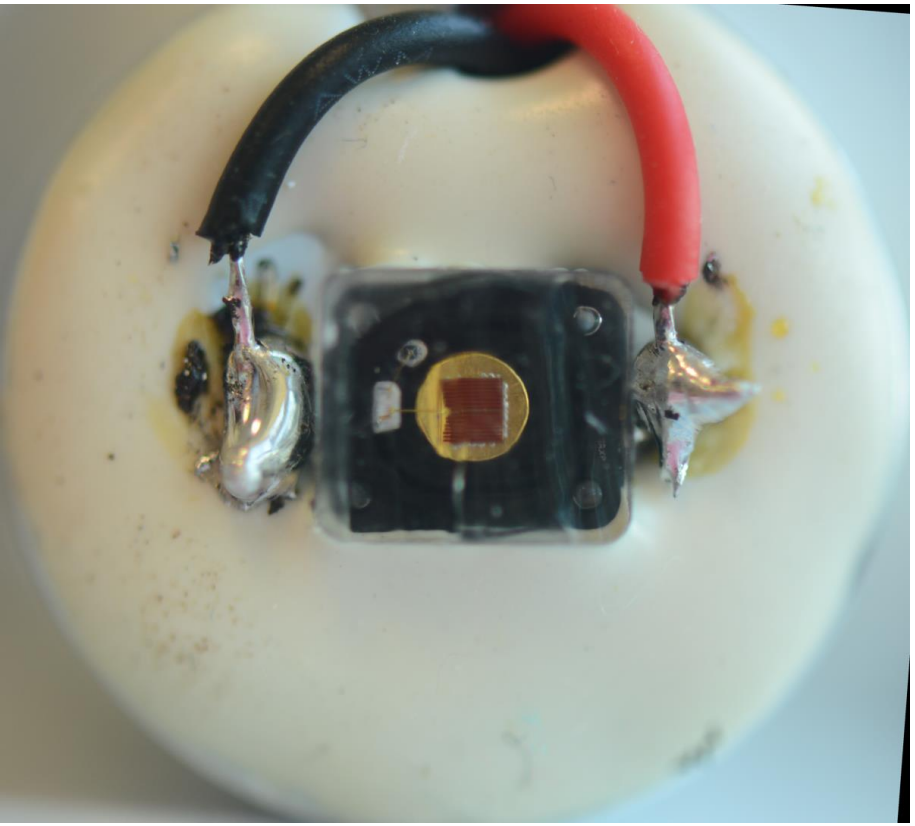
$$\sigma = 0.61 \frac{\lambda}{NA}$$

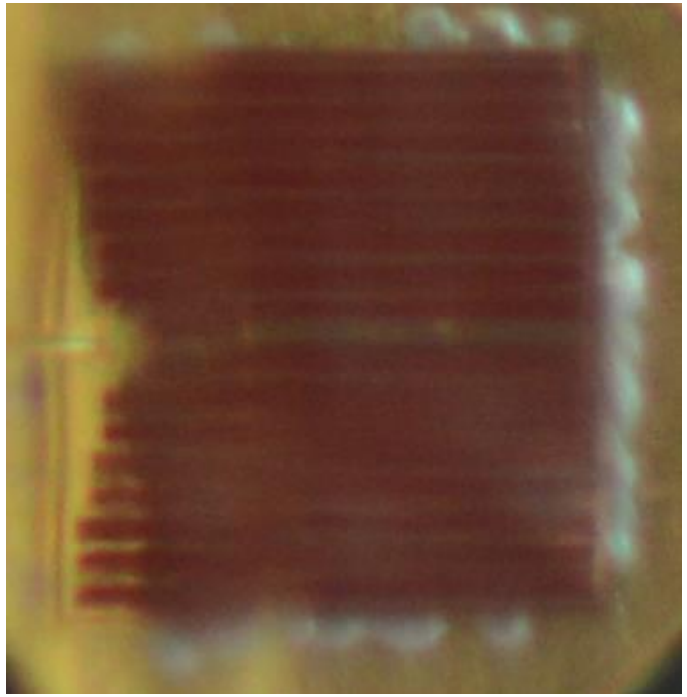
homogenous illumination - condenser

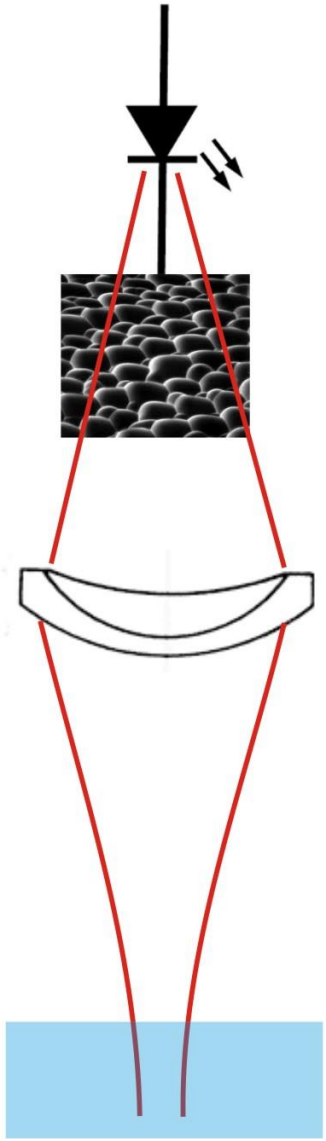
images the source



homogenous illumination – modern area LED

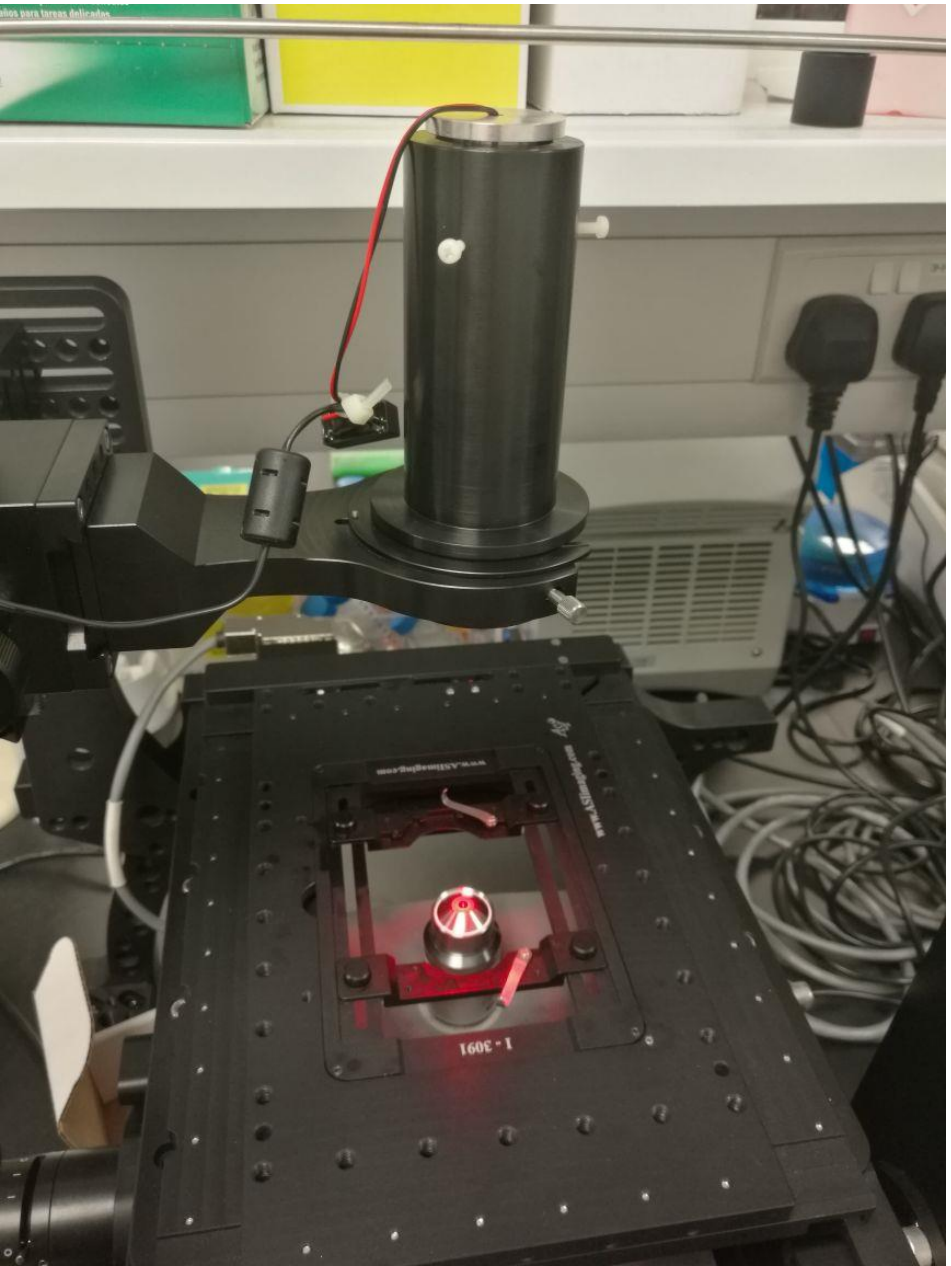




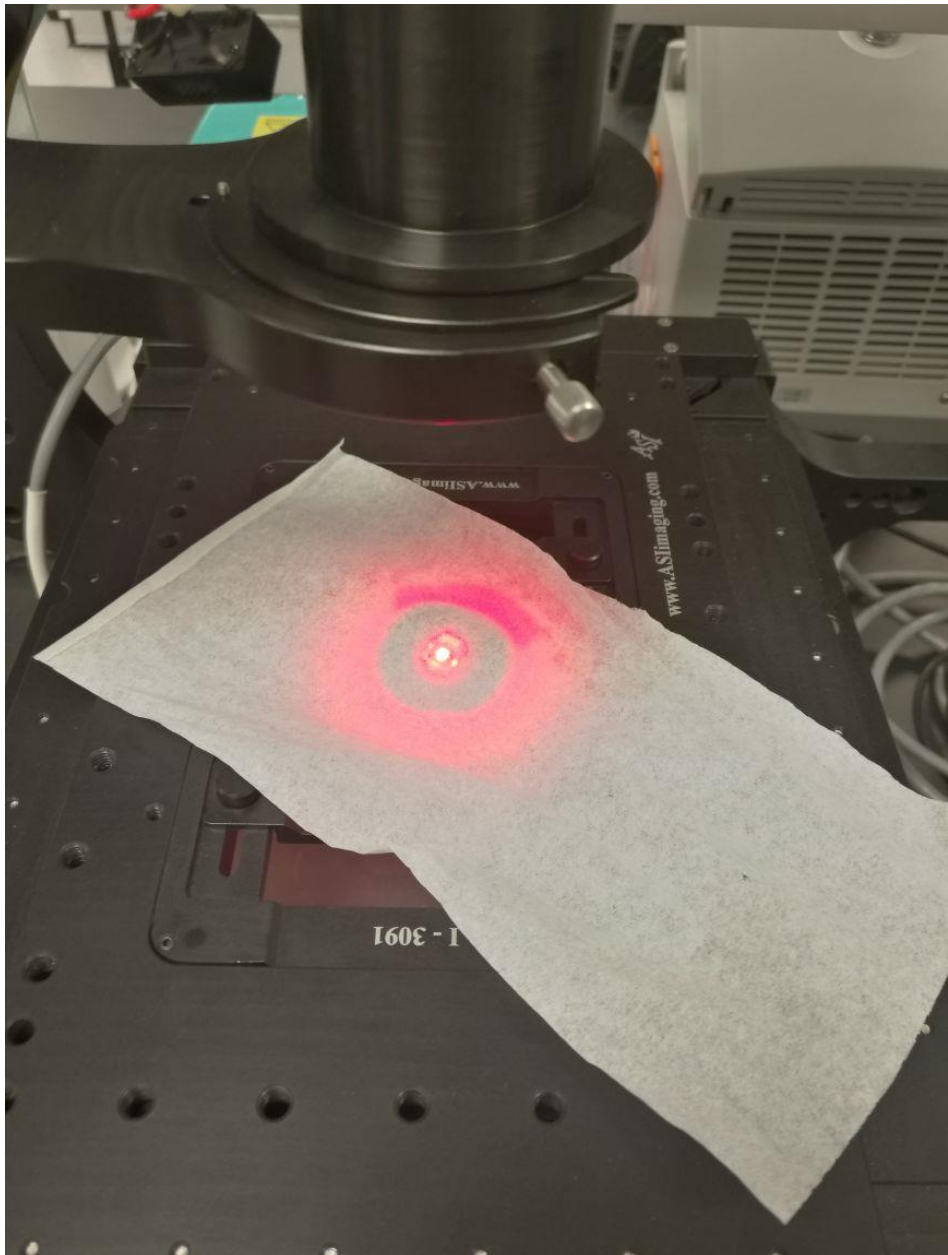


refocus the source

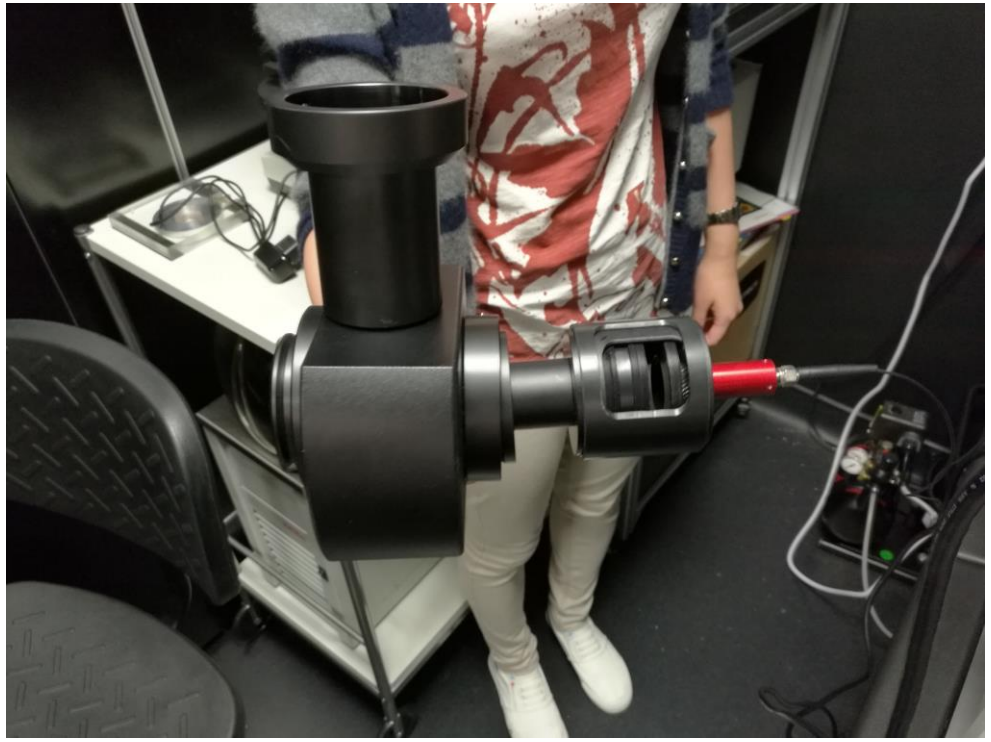
homogenous illumination – modern area LED

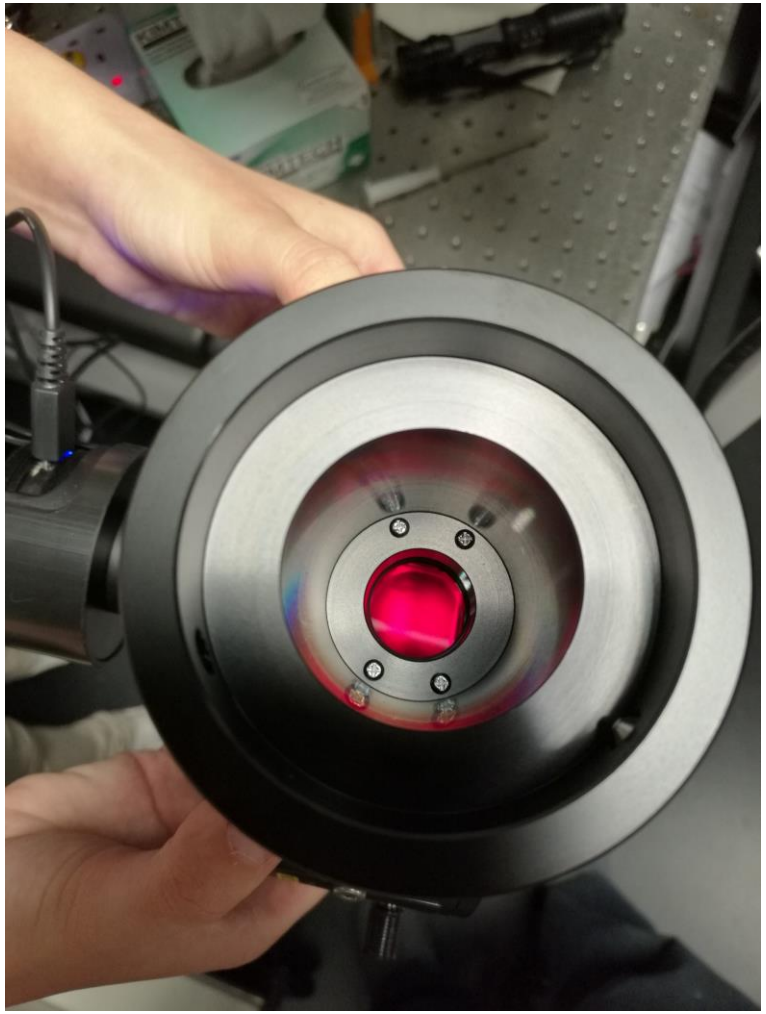


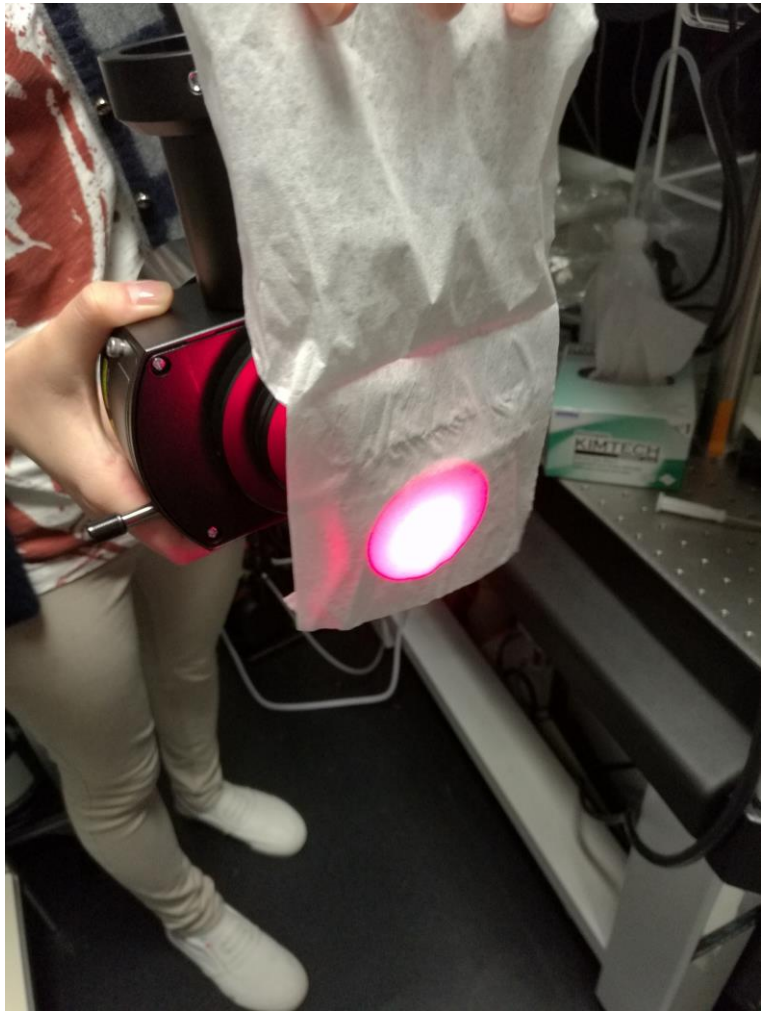
useful flat area
is very small



laser homogenizer
70% loss

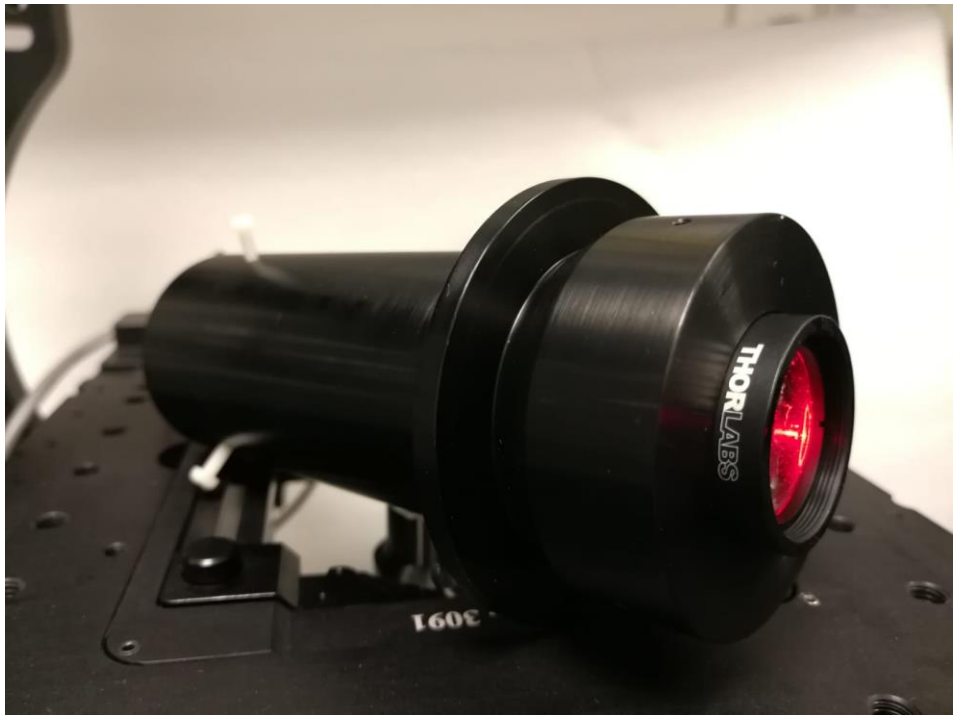


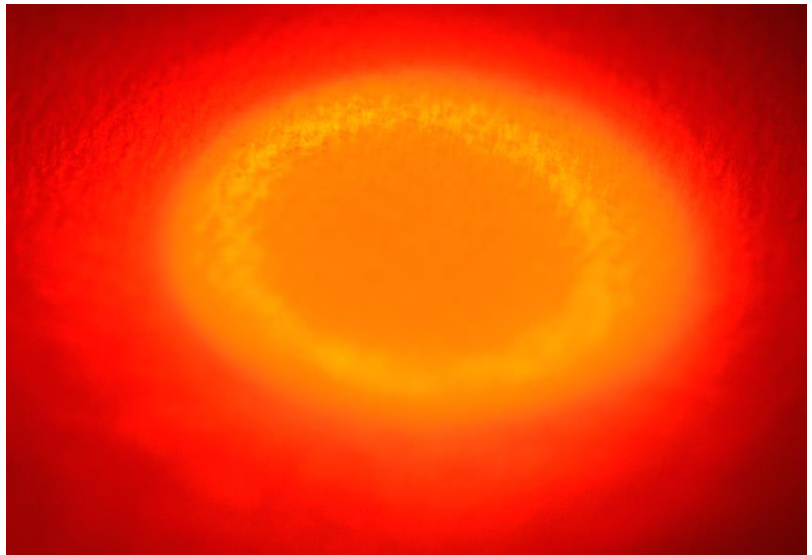


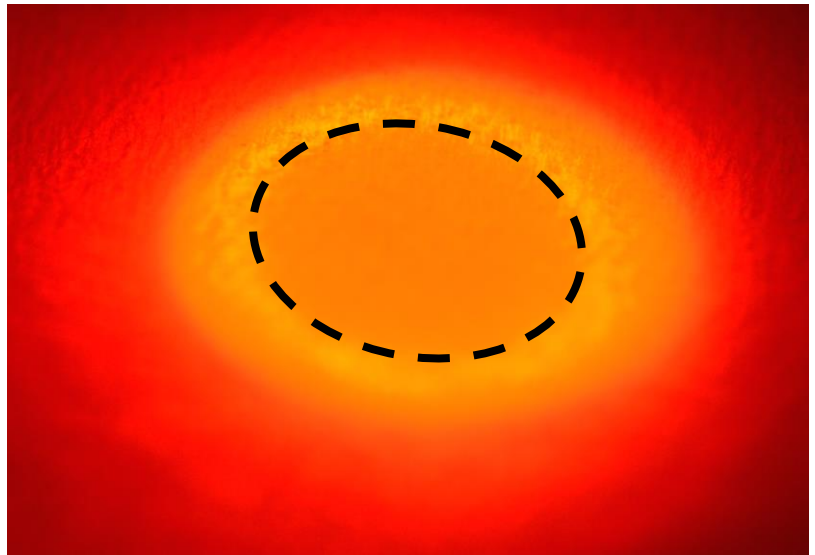


safety issue when using laser radiation

homogenous illumination – LED emitters







homogenous illumination
LED emitters

emitters are hard to feed – they need
a fairly high voltage and drift

charge pump mechanism
much more stable than lasers

Light Microscope Types

Felix & Edna

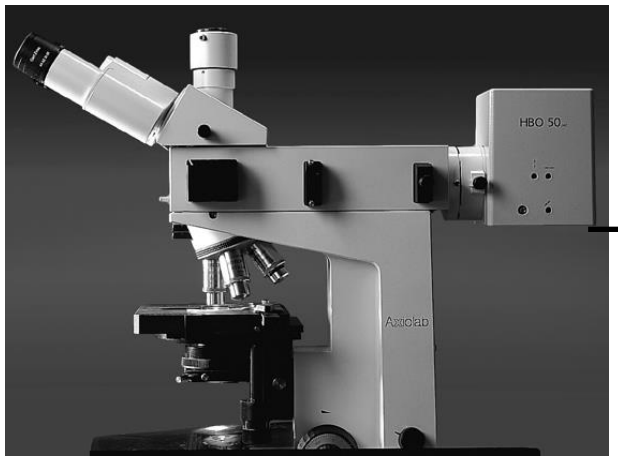
images from

Modern Microscope Bodies

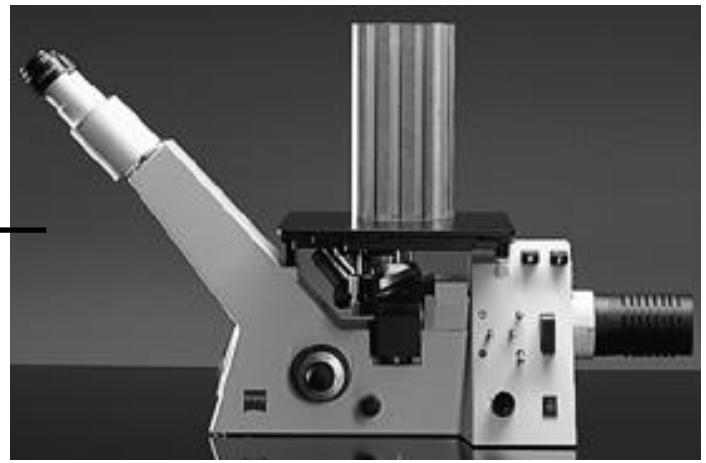
Transmitted Light (transmission)

Upright Microscopes

Inverted Microscopes

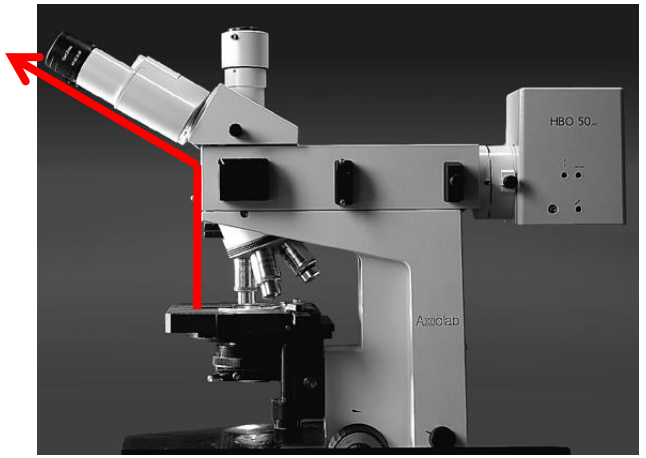


Incident Light (epi illumination)



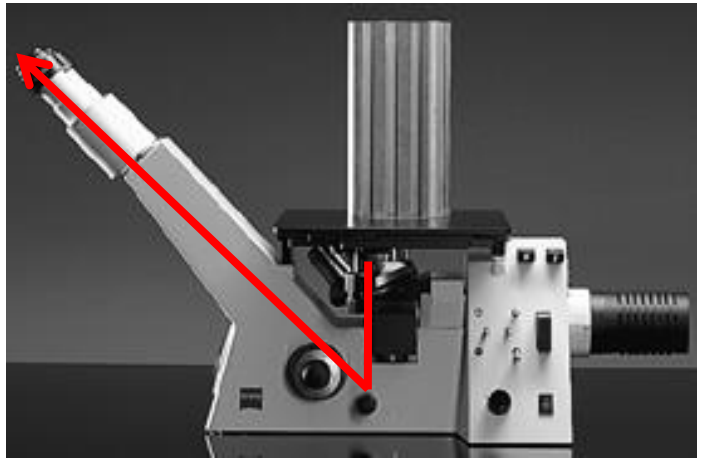
Modern Microscope Bodies

Upright Microscopes



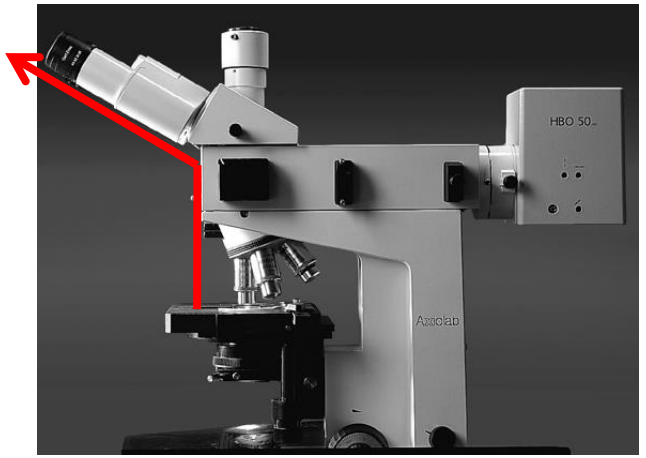
stage is one block – solid design
optics is short and direct
specimen lies on slide
much cheaper to build

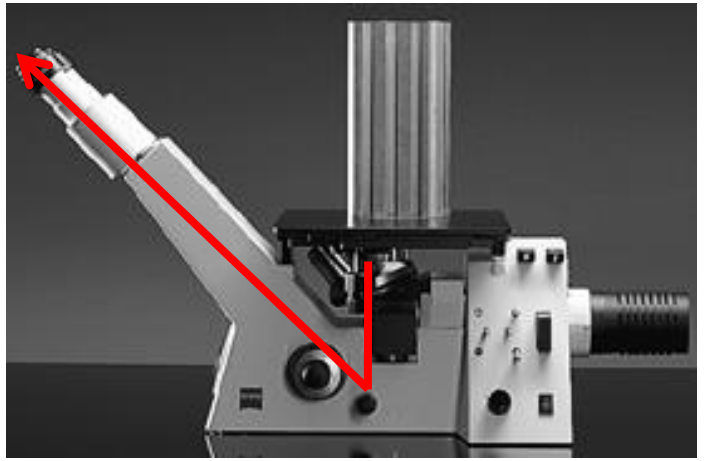
Inverted Microscopes

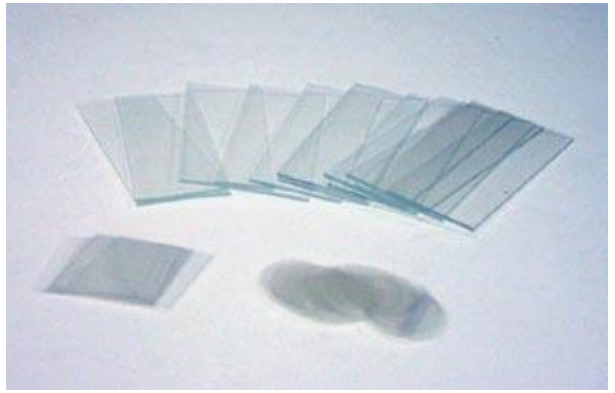


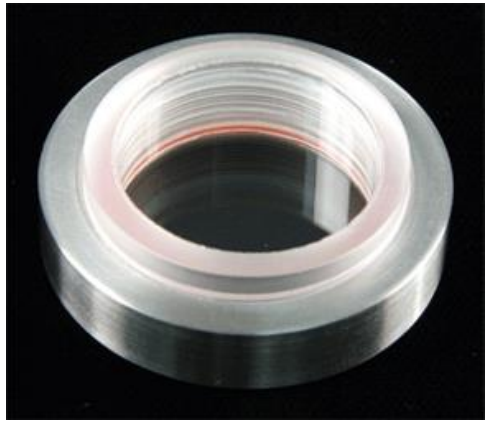
optics goes through stage – complex
optics has a turn in it
specimen lies on cover slip
very expensive to build

Cover Slips and Holders







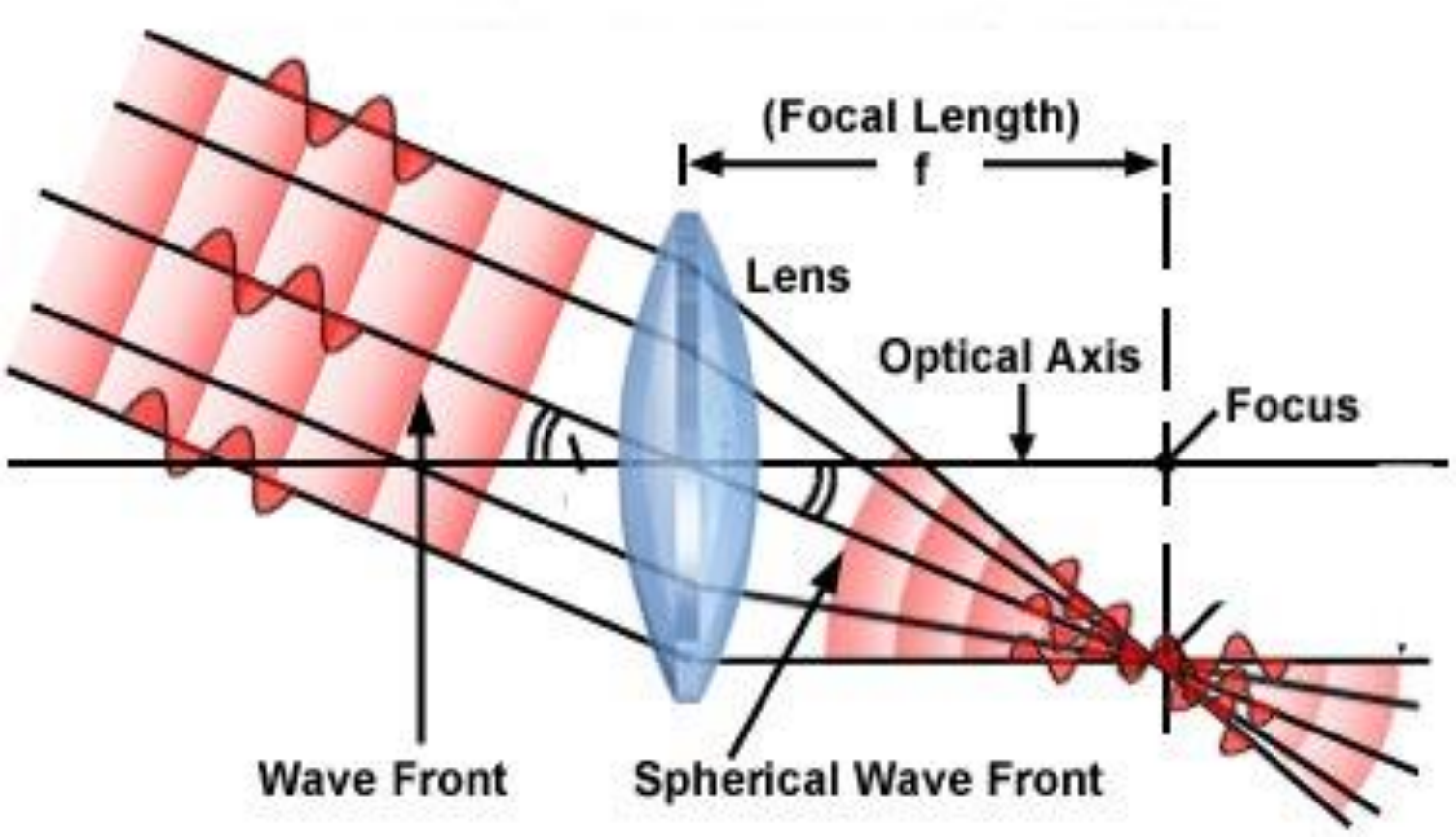


bodies

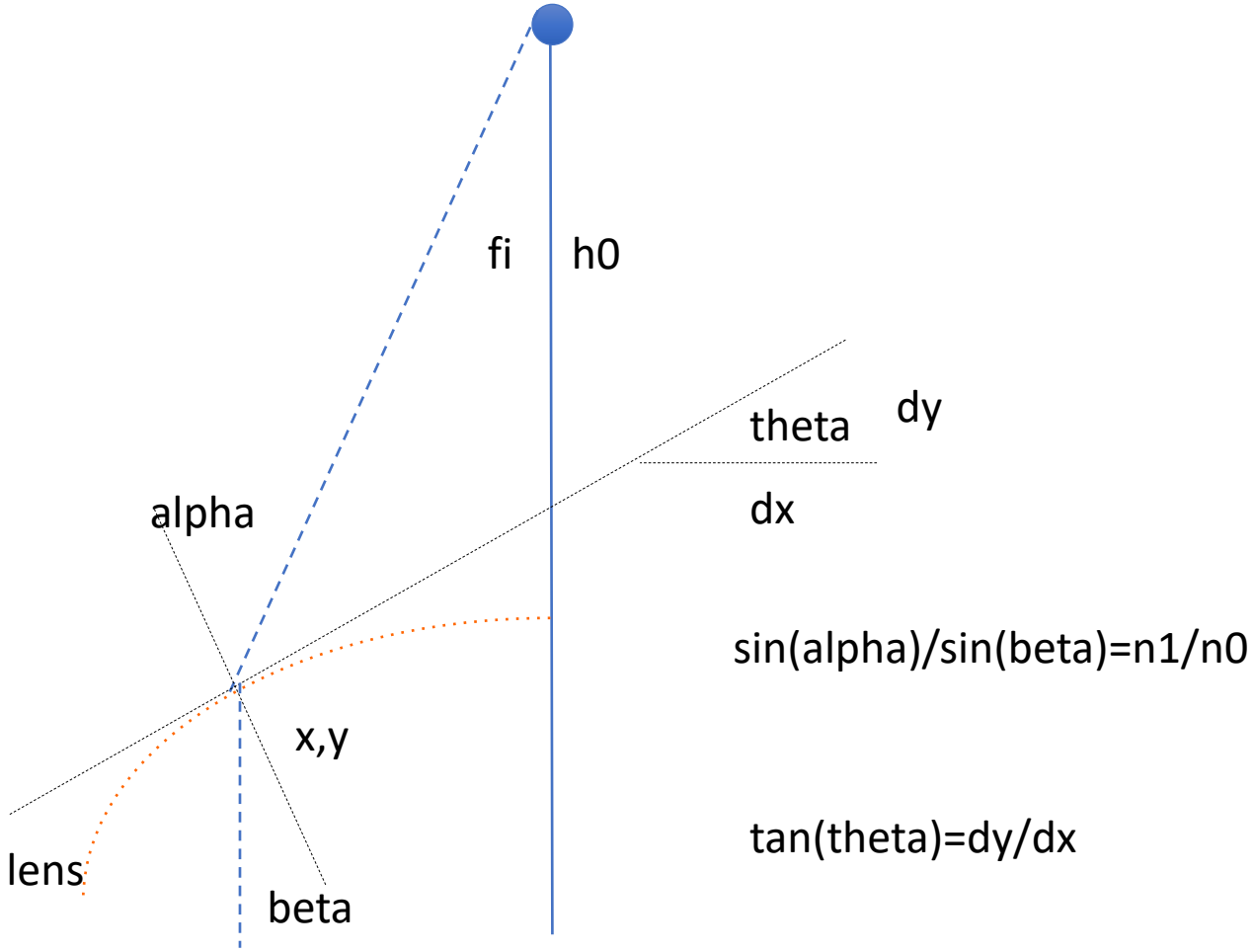
immersion systems are some 140 years old … so the problem is not new …

objectives again

the focusing element
the front element re-directs all light waves coming from a certain point at a certain distance onto a single point in the object plane and vice versa



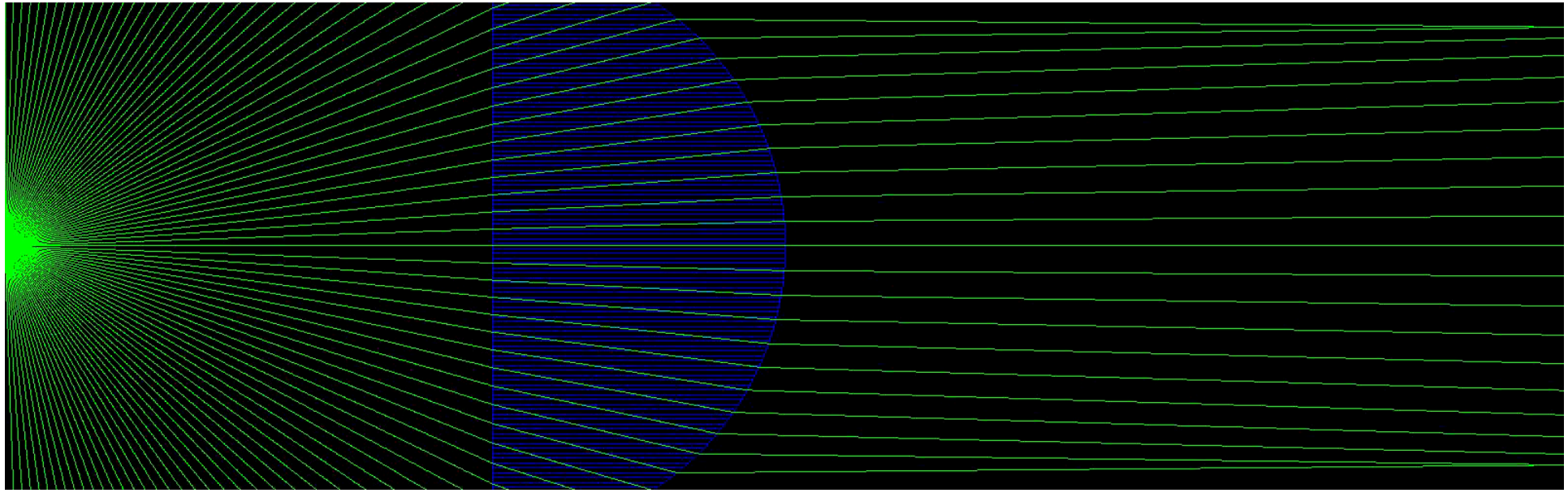
use snell's law



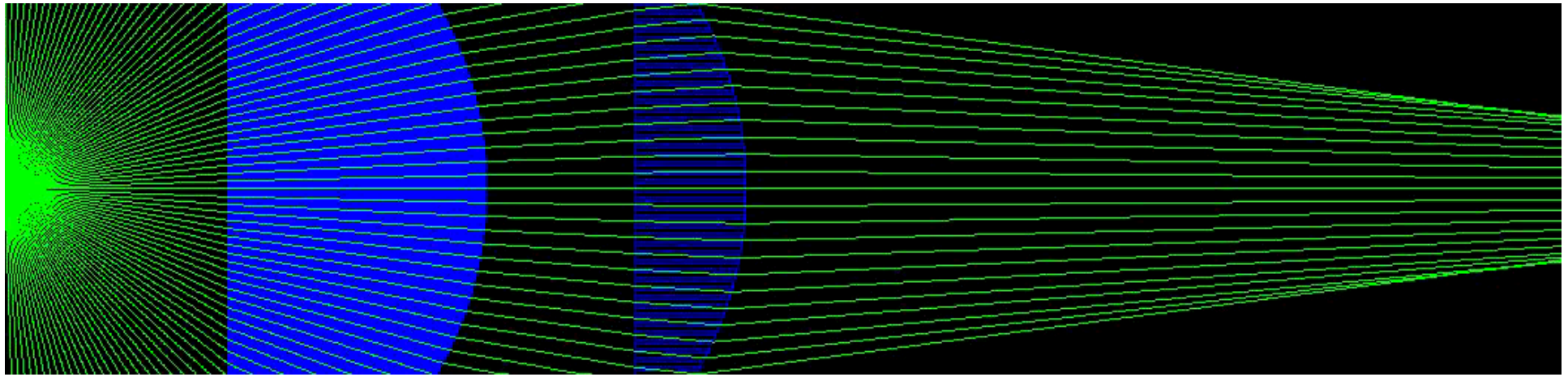
$$\sin(\alpha)/\sin(\beta)=n1/n0$$

$$\tan(\theta)=dy/dx$$

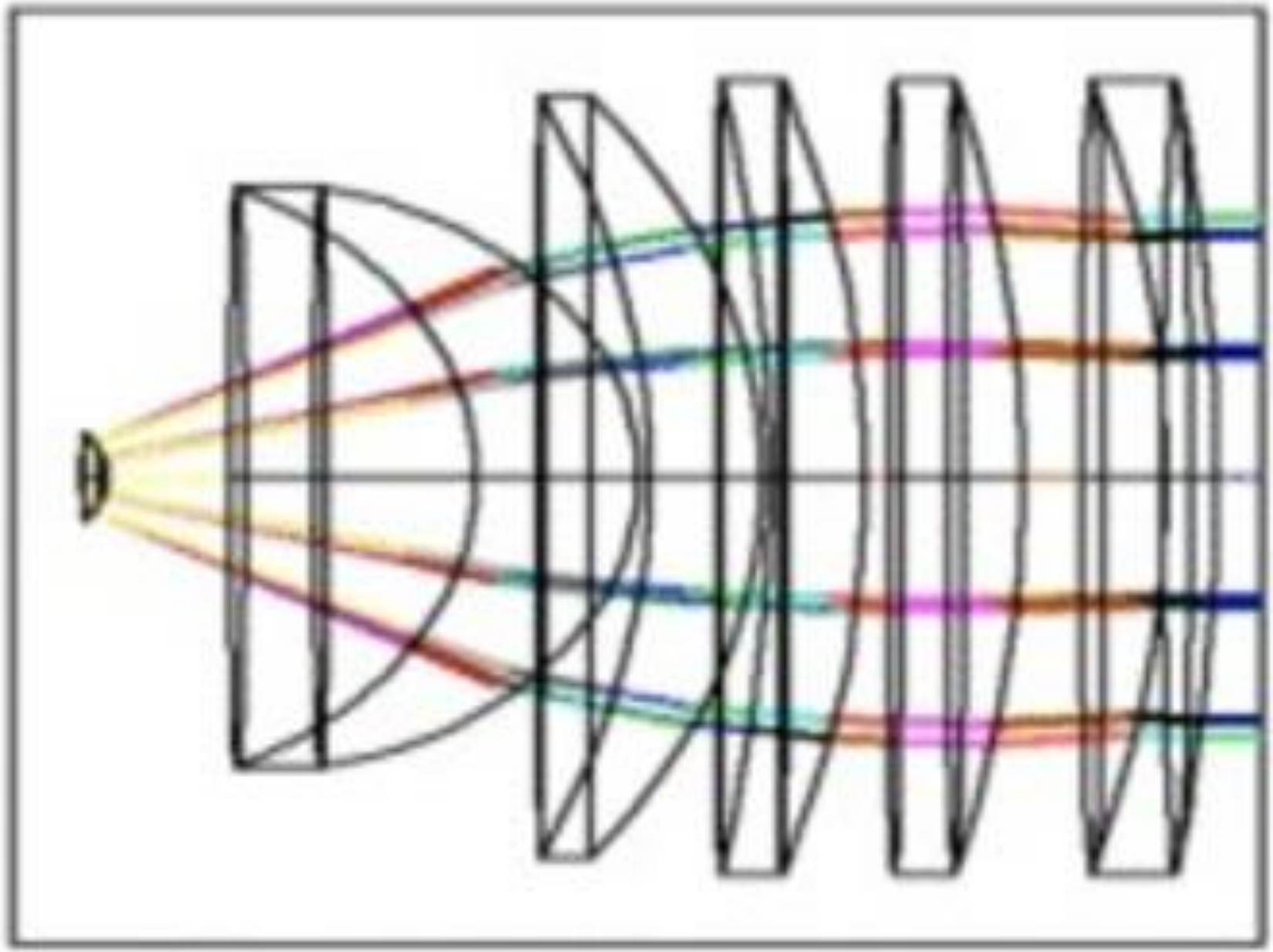
lens limitations - N



lens limitations- 1st radius

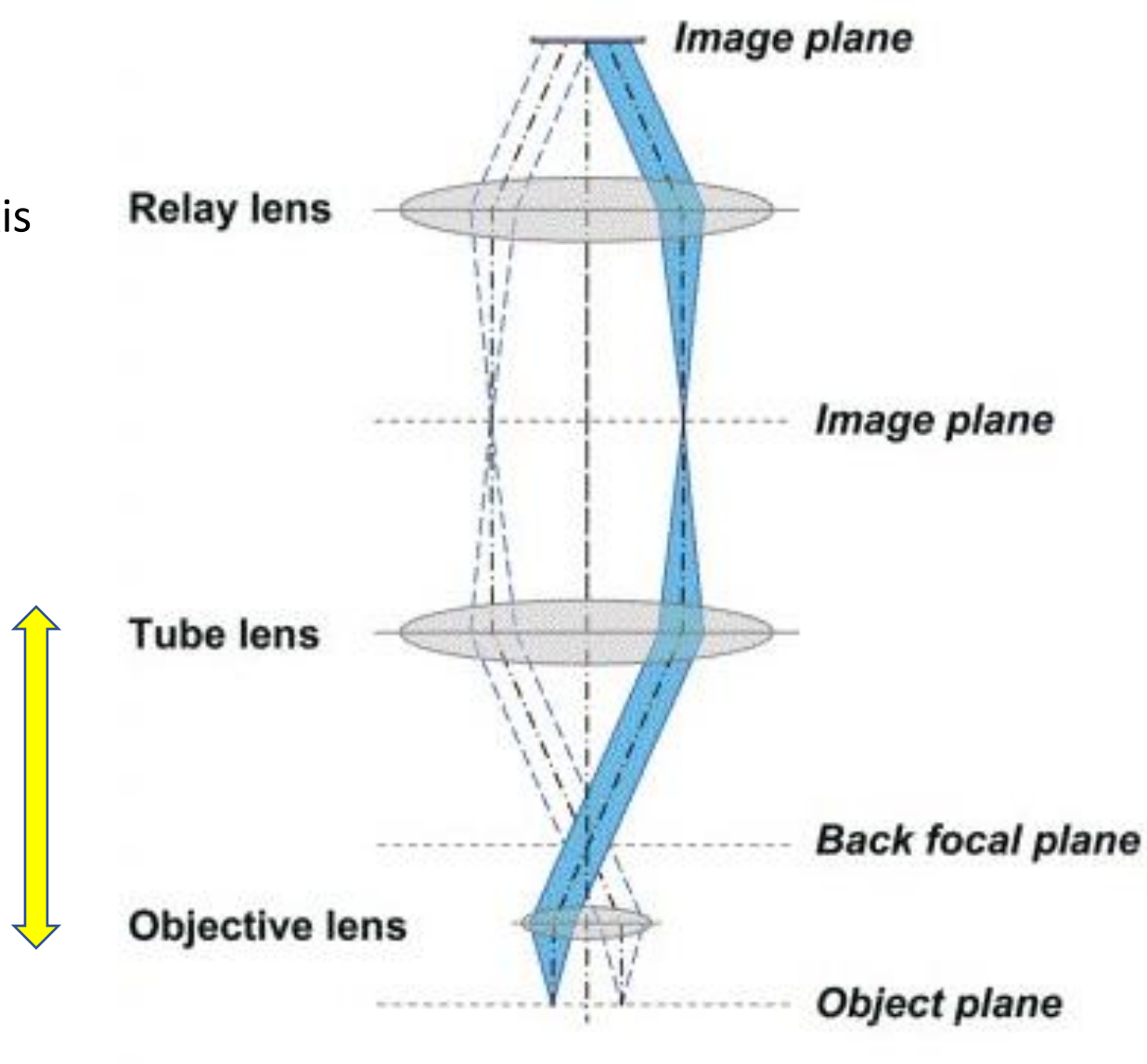


=> lots of elements



optical elements

the microscope
high NA
rays far from axis



parallel light?

Rayleigh diffraction at backfocal plane $\varphi=\lambda/D\sim4.6e\text{-}5$ for a 12mm field stop

parallel light?

yes – for each position in the object plane!

angle encoding in the infinity path

$\varphi=\lambda/D\sim4.6e\text{-}5$ (rad)

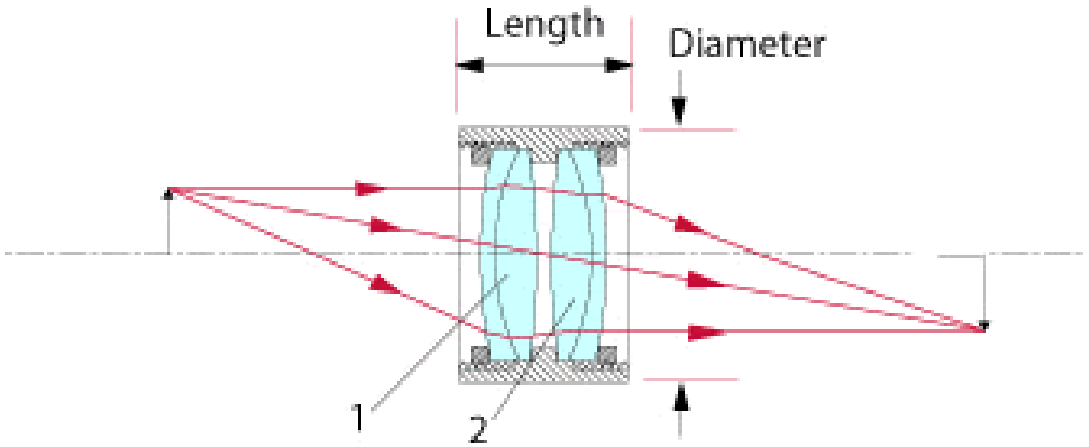
if we need 500 lines: $\Delta\varphi=500x4.6e\text{-}5=0.023$

or 1.31 degrees!

over a 110mm tube, the beams drifts up to 2.5mm off axis in the very best case!

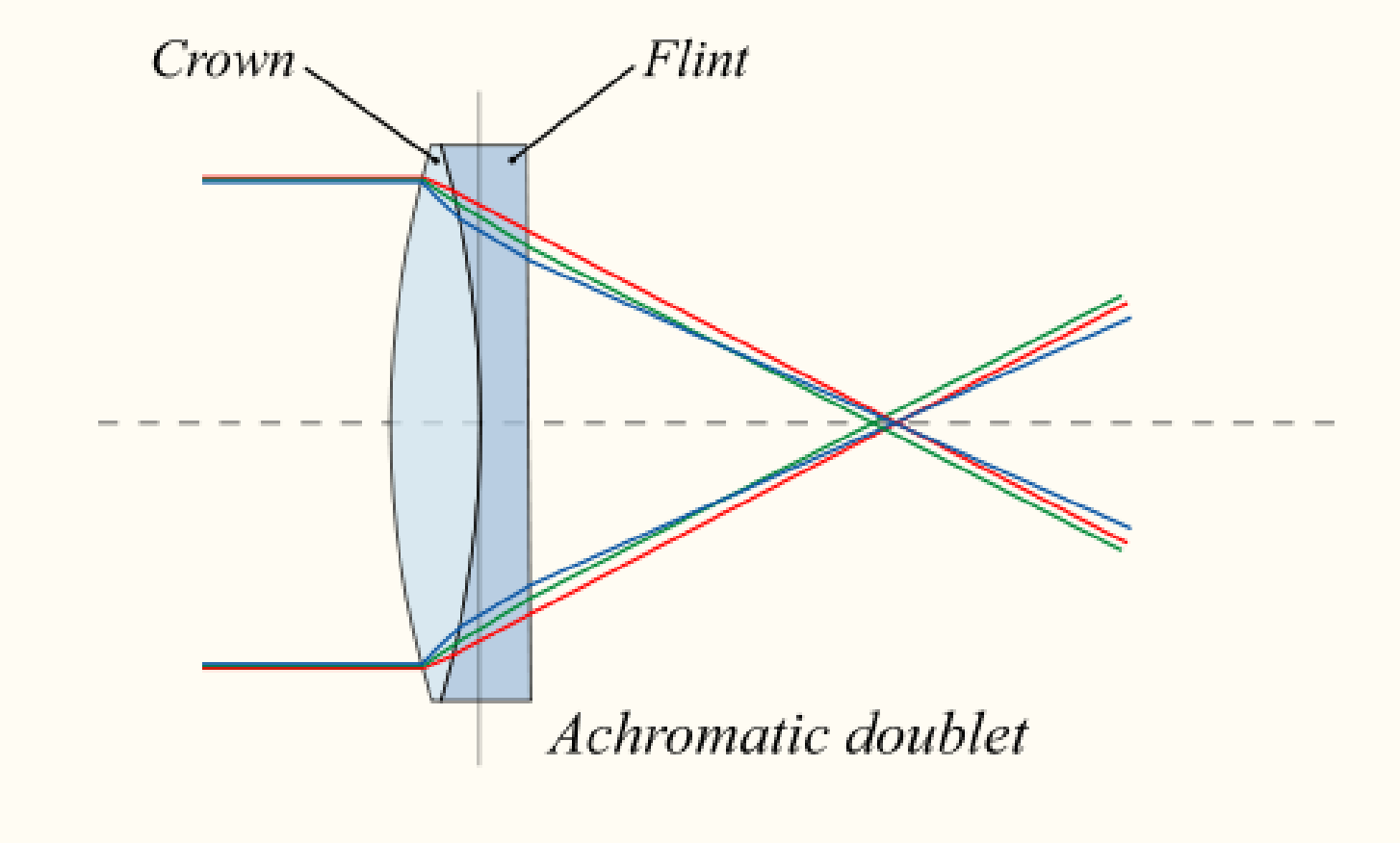
optical elements

the relay lens



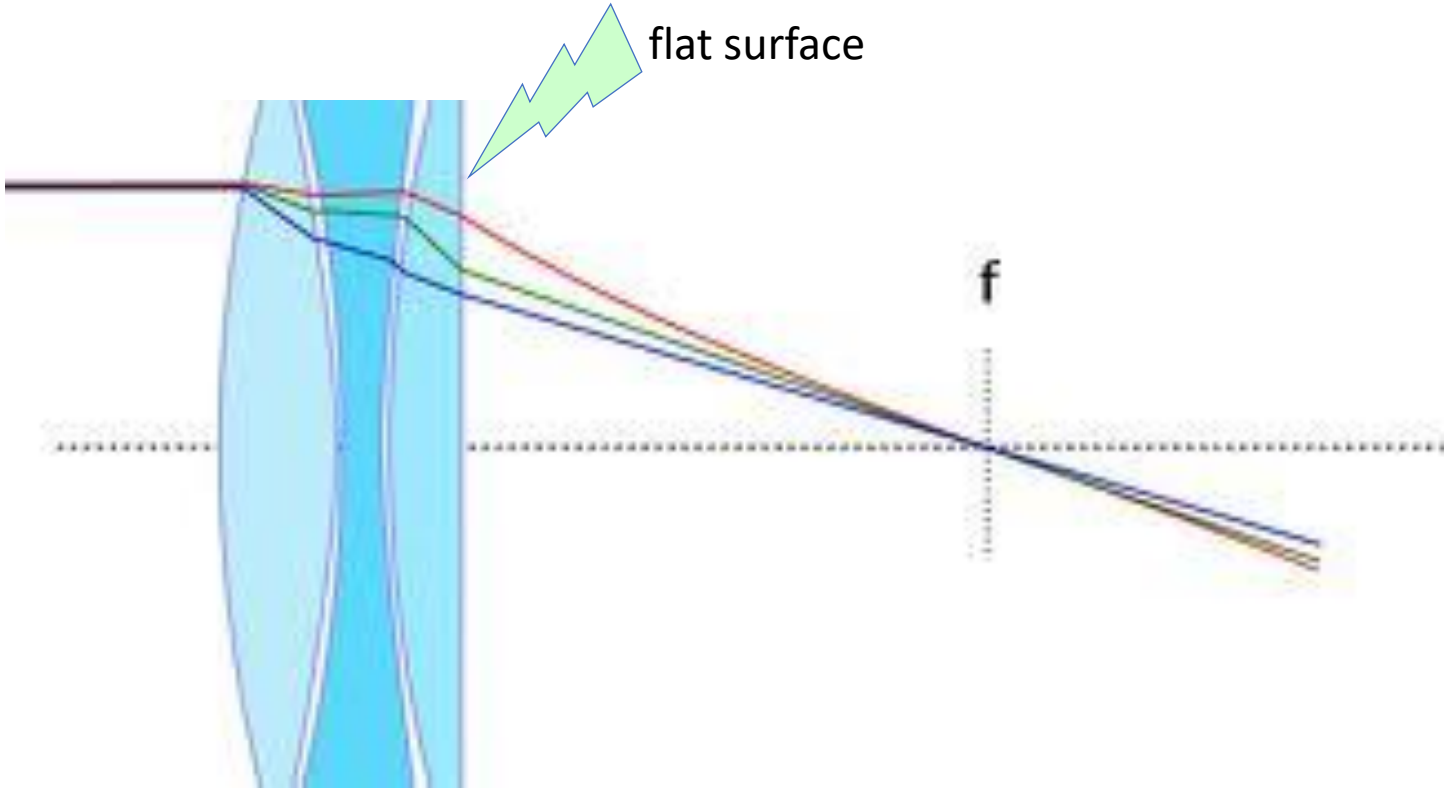
objectives

this needs to work with different wavelengths (dispersion)



objectives

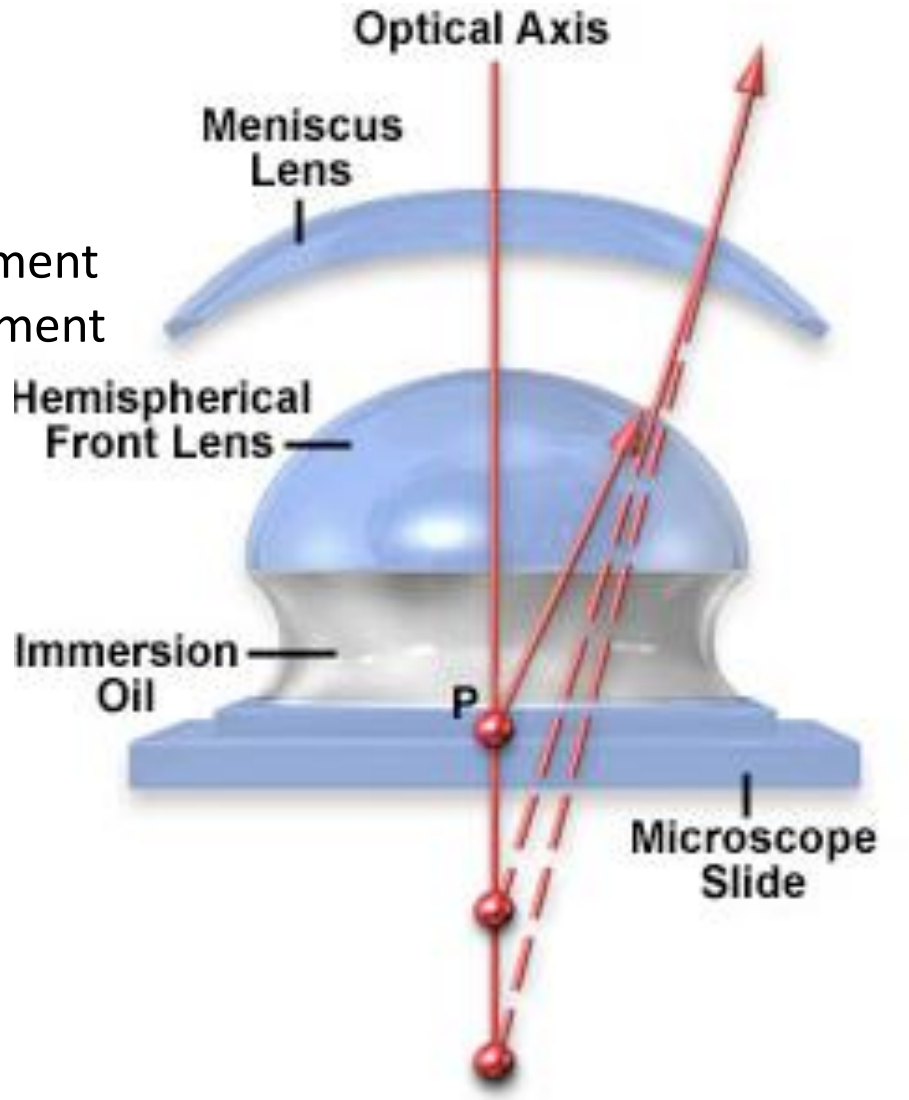
low NA apochromatic system



objectives

the oil immersion

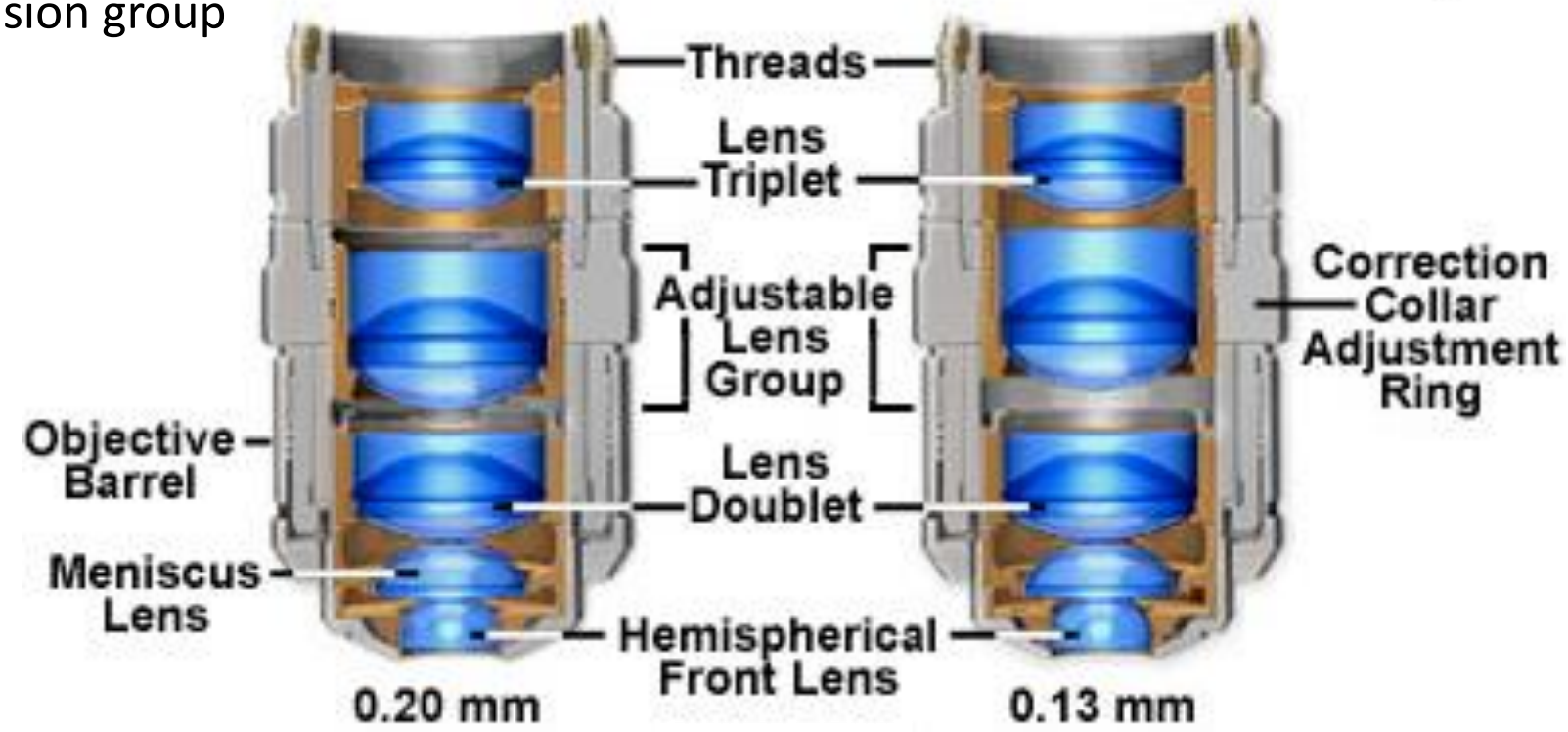
very high NA
no dispersion at front element
scratch resistant front element



objectives

nightmare water immersion

dispersion at front element
scratch sensitive front element
high precision group

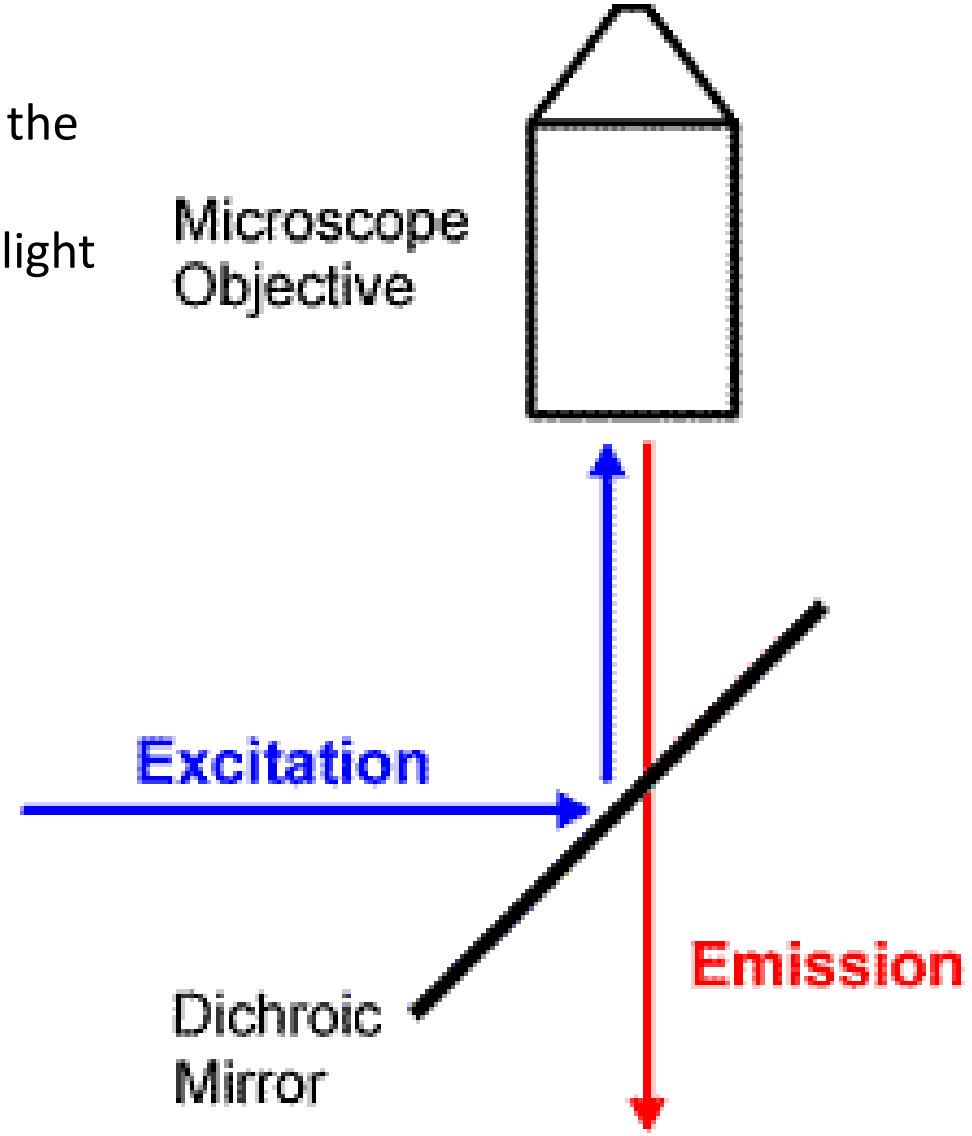


Basic Course Light Microscopy
Objectives - Transmitted Light

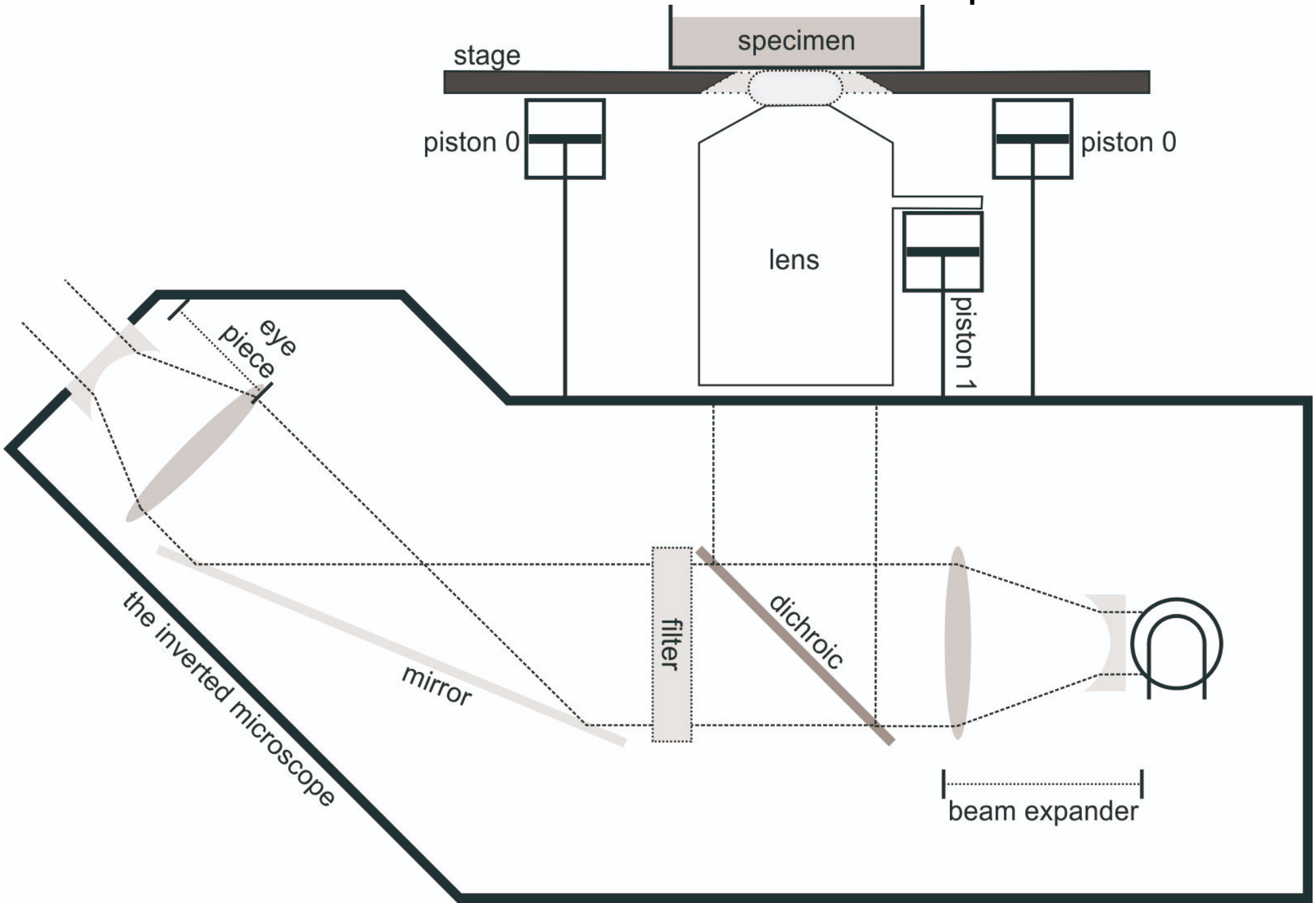
Name	Transmission (nm)	N.A. (all / 100x (Oil))
• CP Achromat	405 - 1000	low / 1,25
• Achrostigmat	405 - 1000	medium / 1,25
• A-Plan	405 - 1000	medium / 1,25
LD A-Plan	405 - 1000	low
• Achroplan	405 - 1000	medium / 1,25
• EC Plan-Neofluar	340 - 700	high / 1,3
LD EC Plan Neofluar	340 - 700	medium
• Fluar	340 - 700	very high / 1,3
• Plan-Apochromat	390 - 700	very high / 1,4
• C-Apochromat	340 - 700	very high / 1,2 (63xW)

EPI & dichroics

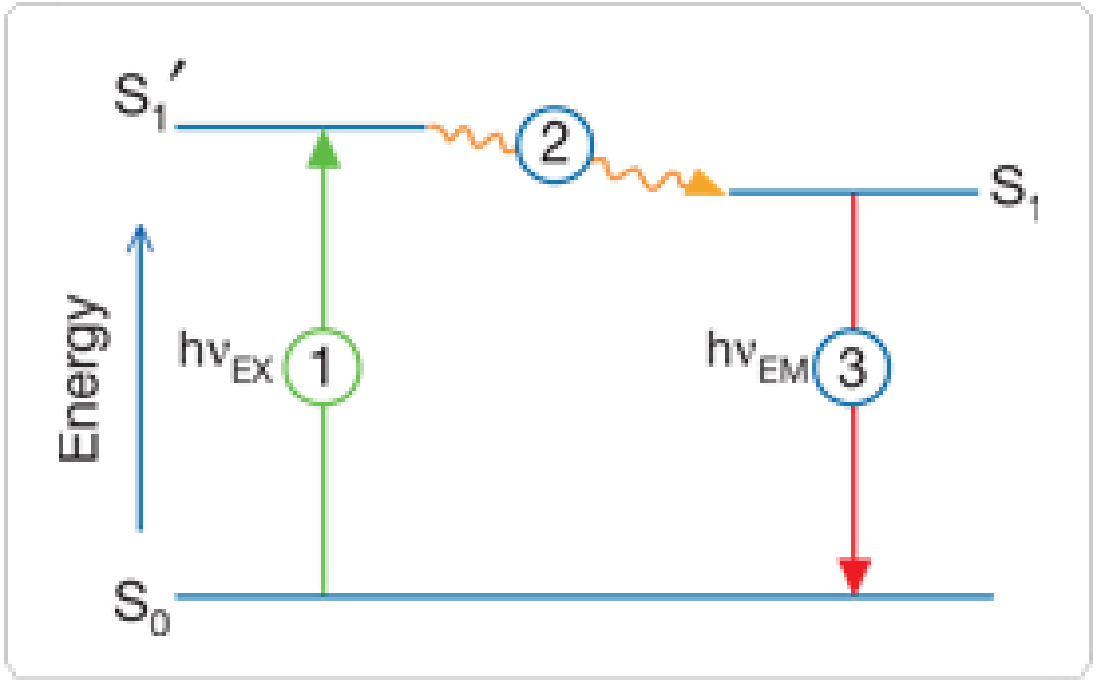
the dichroic directs the excitation light and removes excitation light from the emission



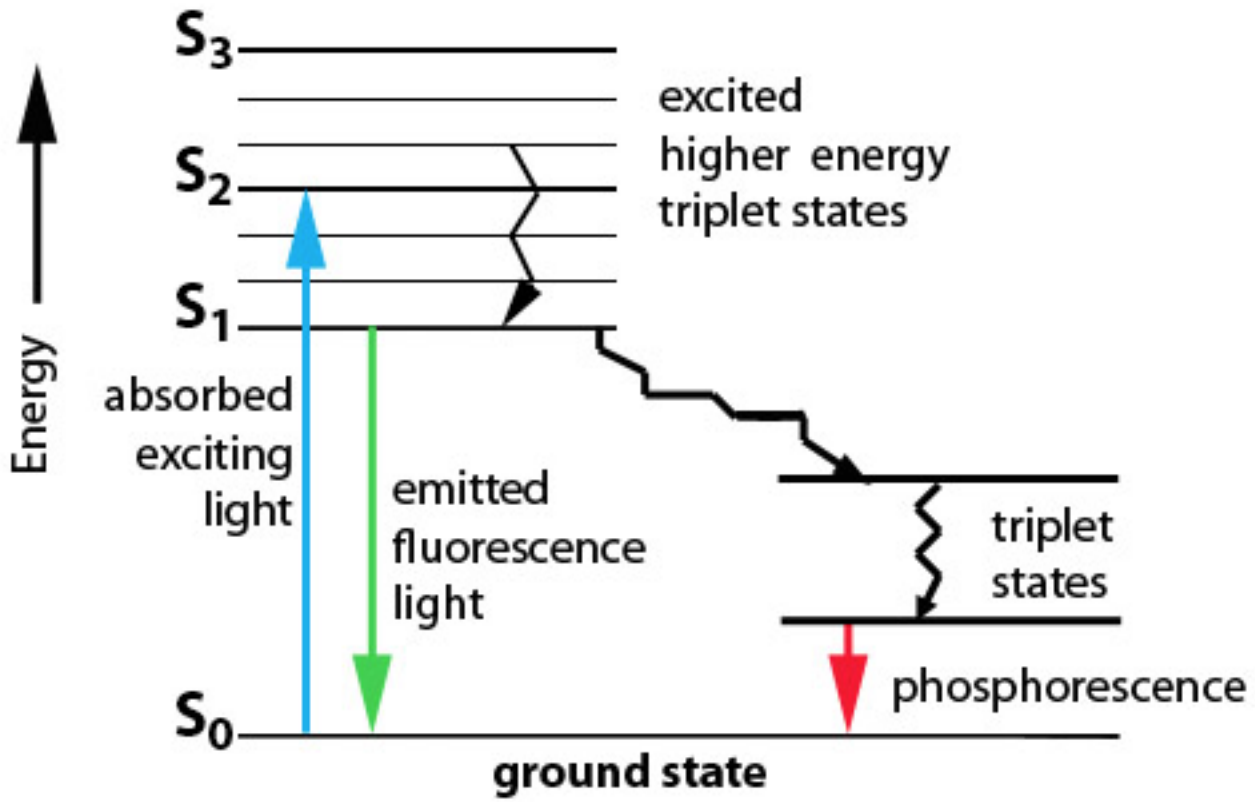
how it looks inside a microscope:



Fluorescence: absorption scheme



absorption scheme



molar extinction

$A = l \cdot \epsilon \cdot c$	Absorbance - Beer Lambert Law
$I = l_0 \cdot e^{-A}$	absorption
ϵ	extinction [m^2/Mol] [L / mol cm]

molecule cross section

$A = l \cdot \epsilon \cdot c$ Absorbance - Beer Lambert Law

$I = l_0 \cdot e^{-A}$ absorption

ϵ extinction [m^2/Mol] [L / mol cm]

$\sigma := 3.8 \cdot 10^{-21} \epsilon$ cross section

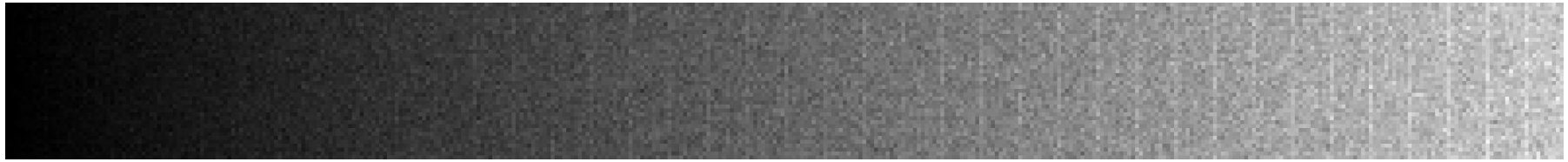
Poisson Noise

$$P_N(k) = \frac{N^k}{k!} e^{-N}$$

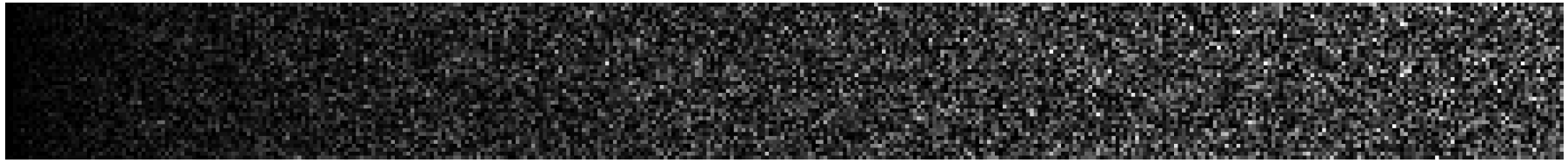
If the average deviation from N actually is $\sqrt{N}$ which is exactly the noise level.

Poisson noise (0p to 200 counts)

signal – 10% steps



noise level – 10% steps



unique characteristic of Poison noise!

The probability that a measurement is brighter than a threshold T is:

$$P_N(k > T) = \sum_{i=T+1}^{\infty} P_N(i) = \sum_{i=T+1}^{\infty} \frac{N^i}{i!} e^{-N}$$

tedious to calculate…

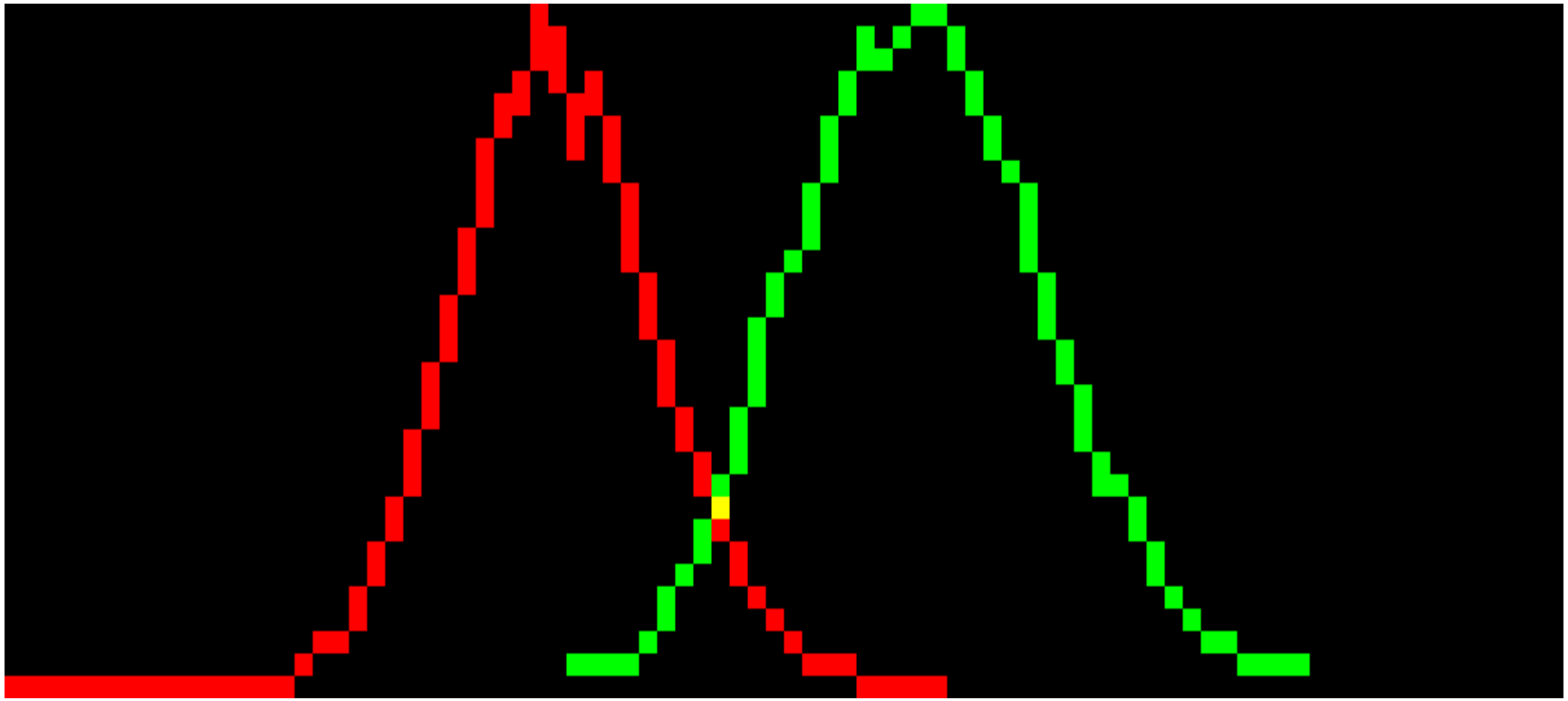
Sum of all probabilities that a value of k or greater is measured:

$$P_N(k \geq 0) = \sum_{i=0}^{\infty} P_N(i) = 1$$

If we simplify this for k greater than threshold T:

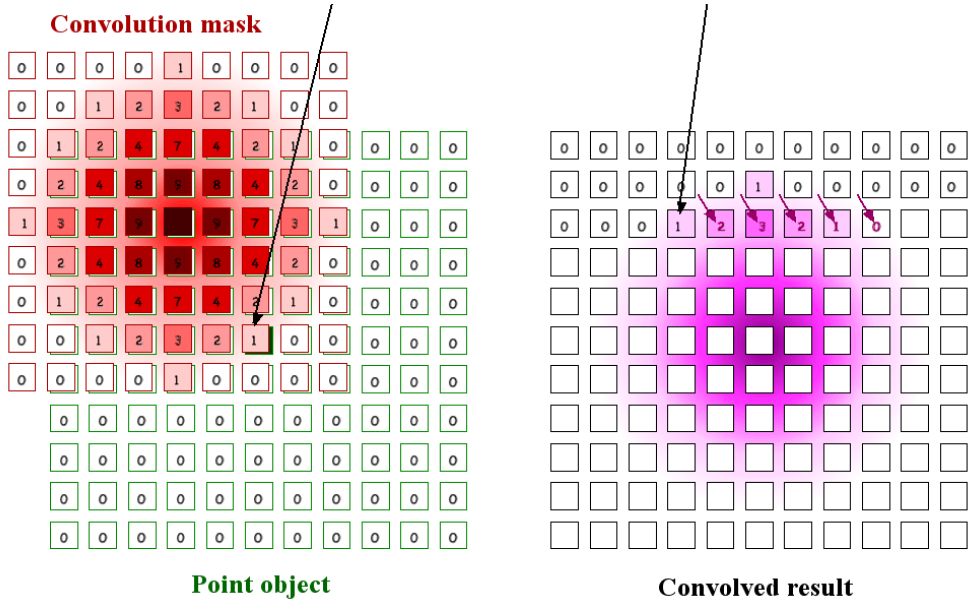
$$P_N(k > T) = \sum_{i=T+1}^{\infty} P_N(i) = 1 - \sum_{i=0}^{T} P_N(i)$$

overlapping intensity distributions



DECONVOLUTION

the process of convolution mimics the blurring cause by imaging,

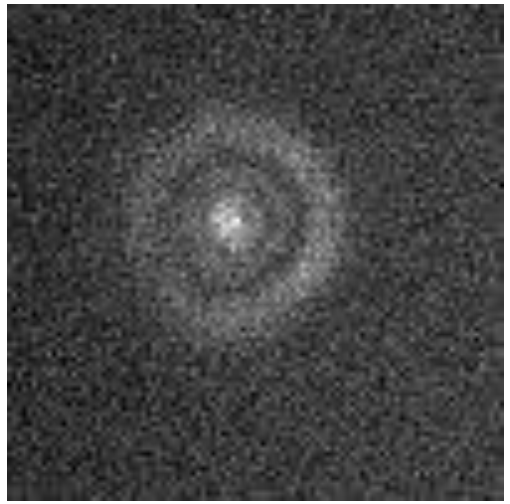


one can partially reverse this blurring if one knows the PSF.

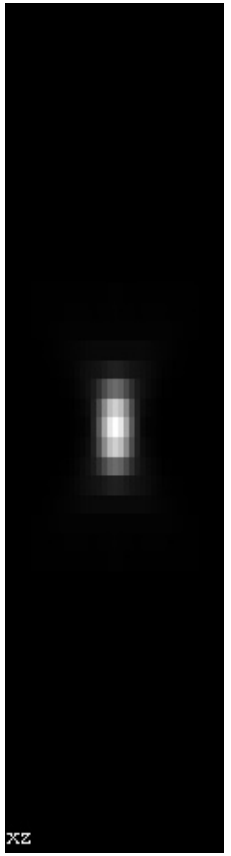
The PSF is measured by recording a series of images of a point-like object in adjacent focus planes.

As point-like objects are small, they contain little dye.

Therefore their images are regraded by Poisson Noise.

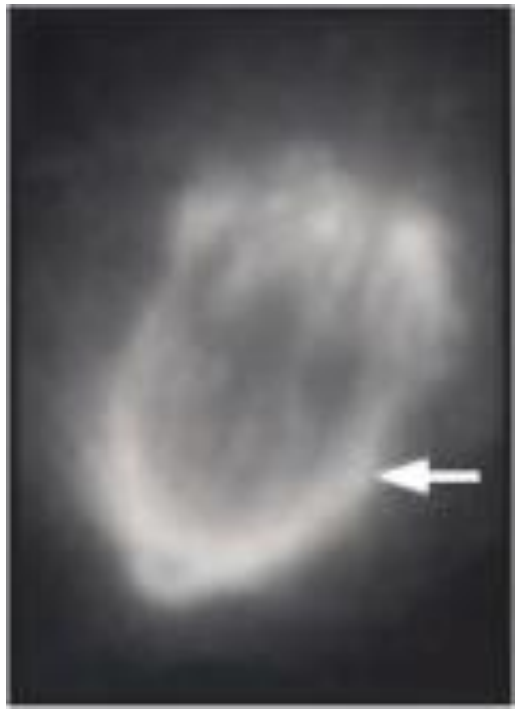


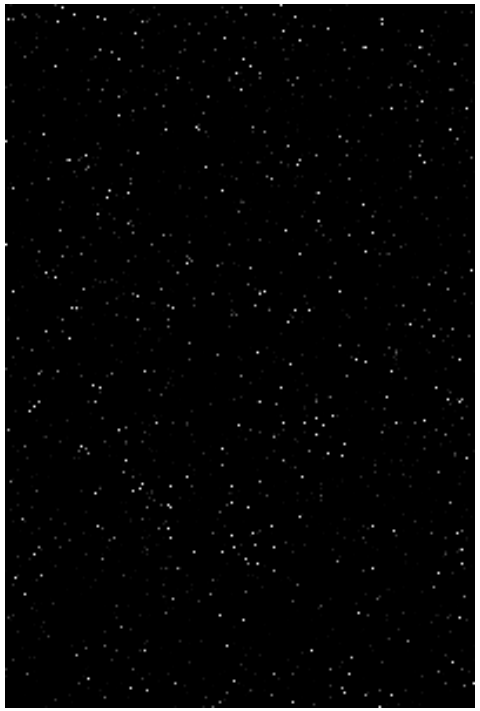
This noise can be reduced by summing or averaging the images of a large number of beads.

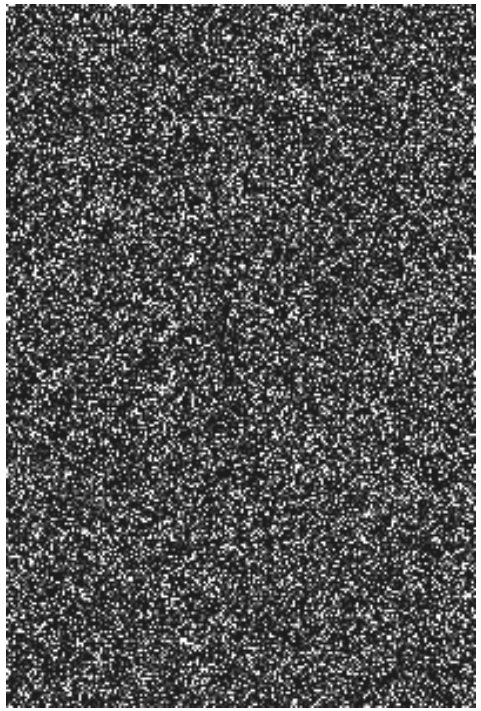


inverse filtering

Can’t we just invert the convolution process?
Yes, but it doesn’t work.
The signal to noise goes to zero and only noise is left





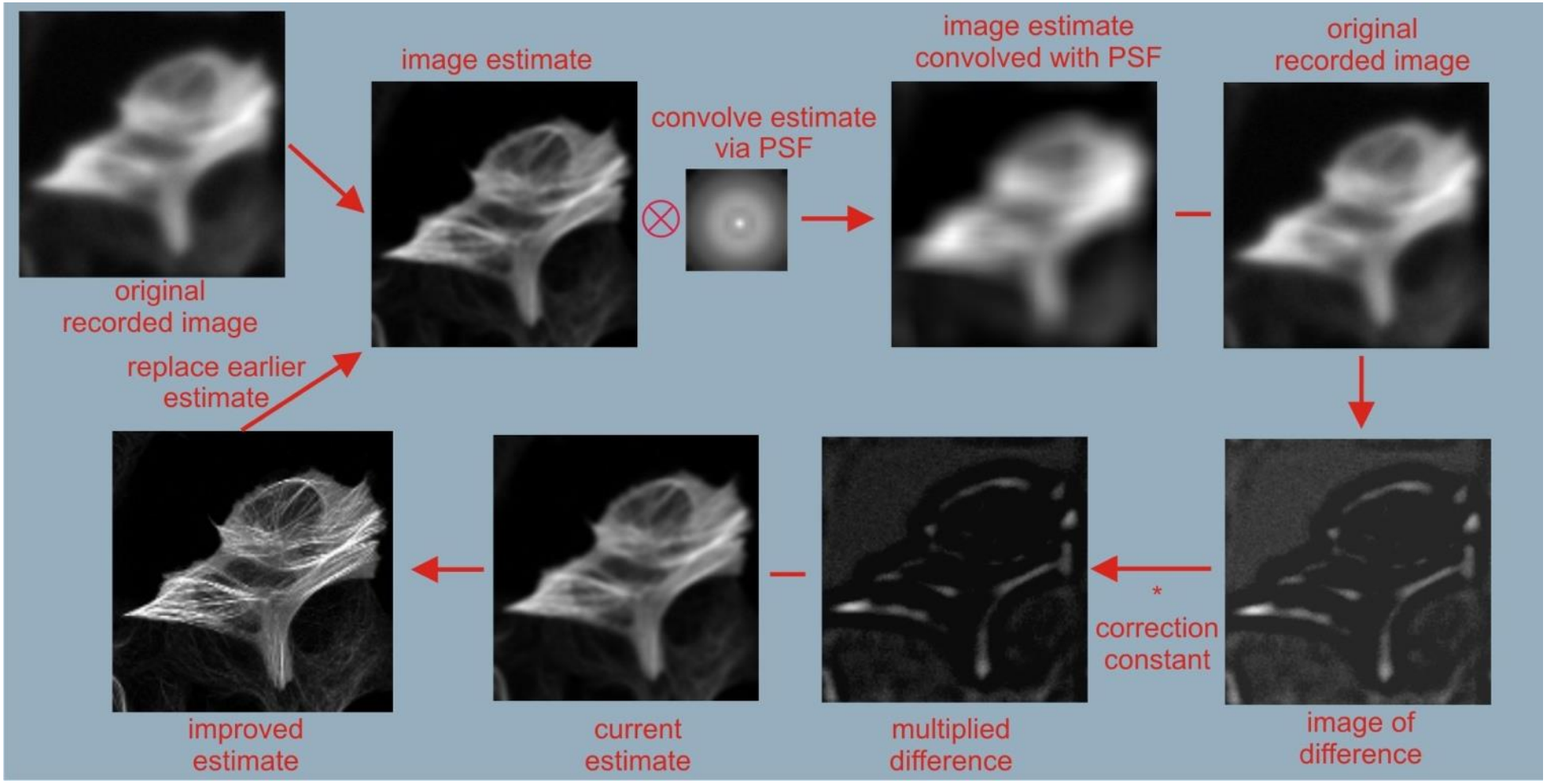


Classic deconvolution

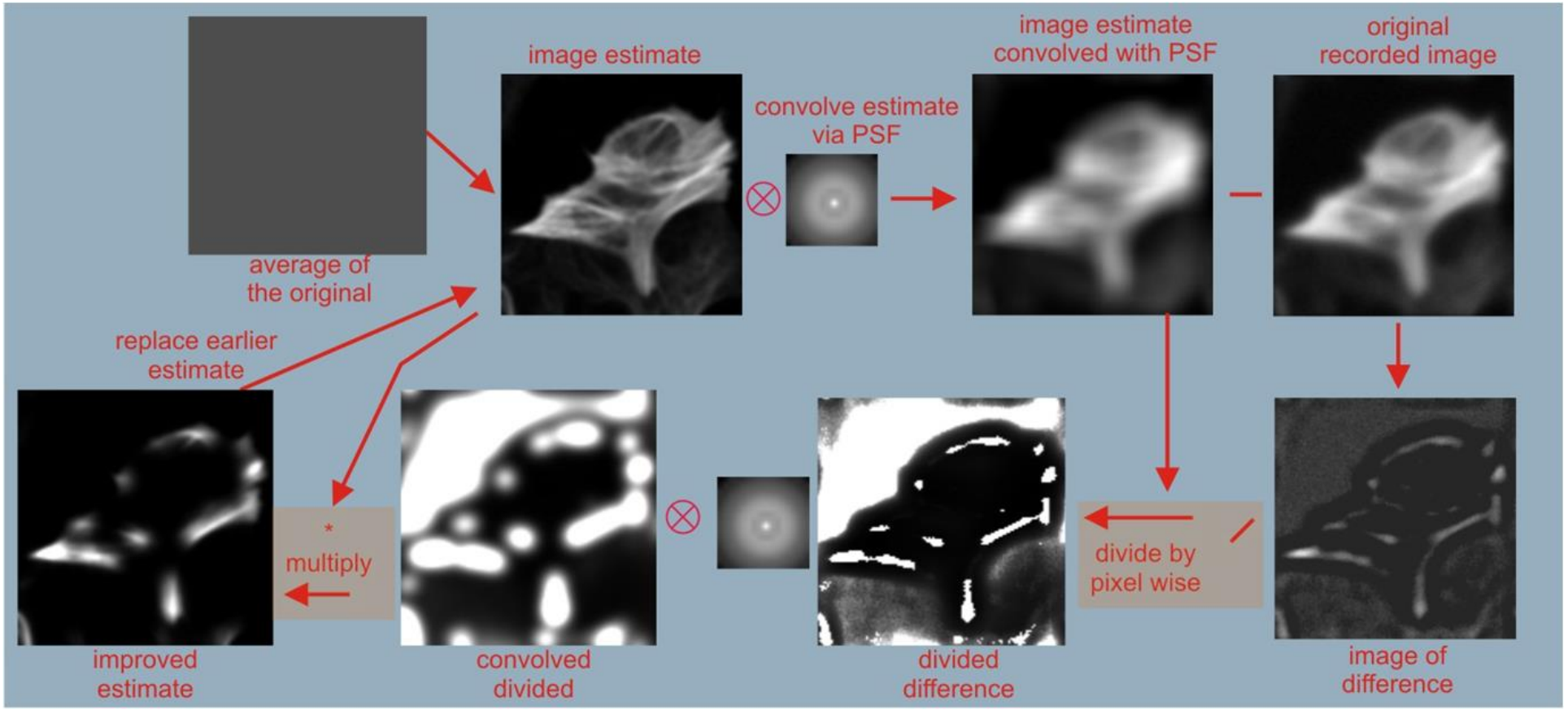
There are 3 major approaches:

- van Cittert
- Richardson Lucy
- ML Iterative-Constrained, Steepest gradient

VanCittert deconvolution

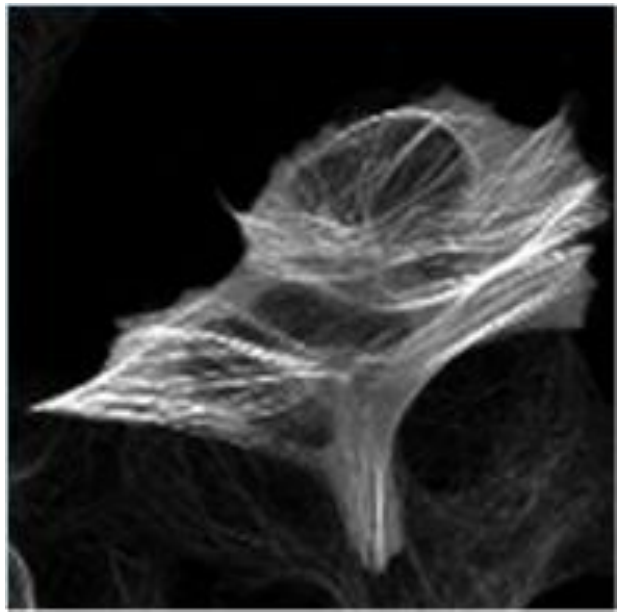


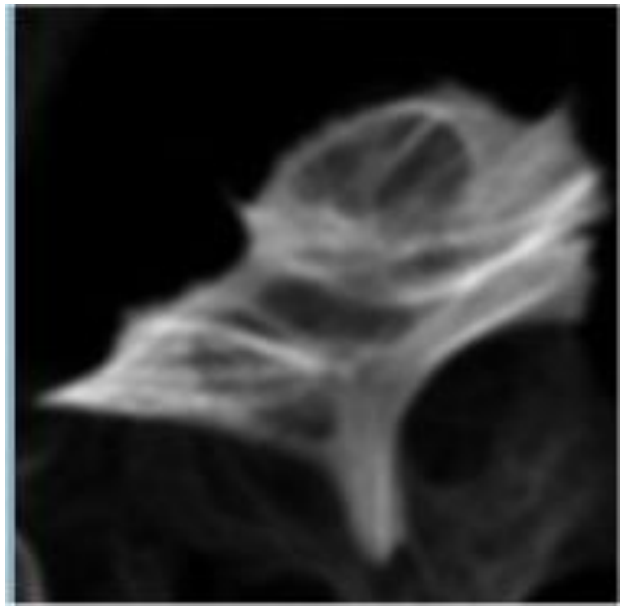
Richardson-Lucy Decon



Richardson Lucy uses the same PSF in the convolve step as is used in the correction step.

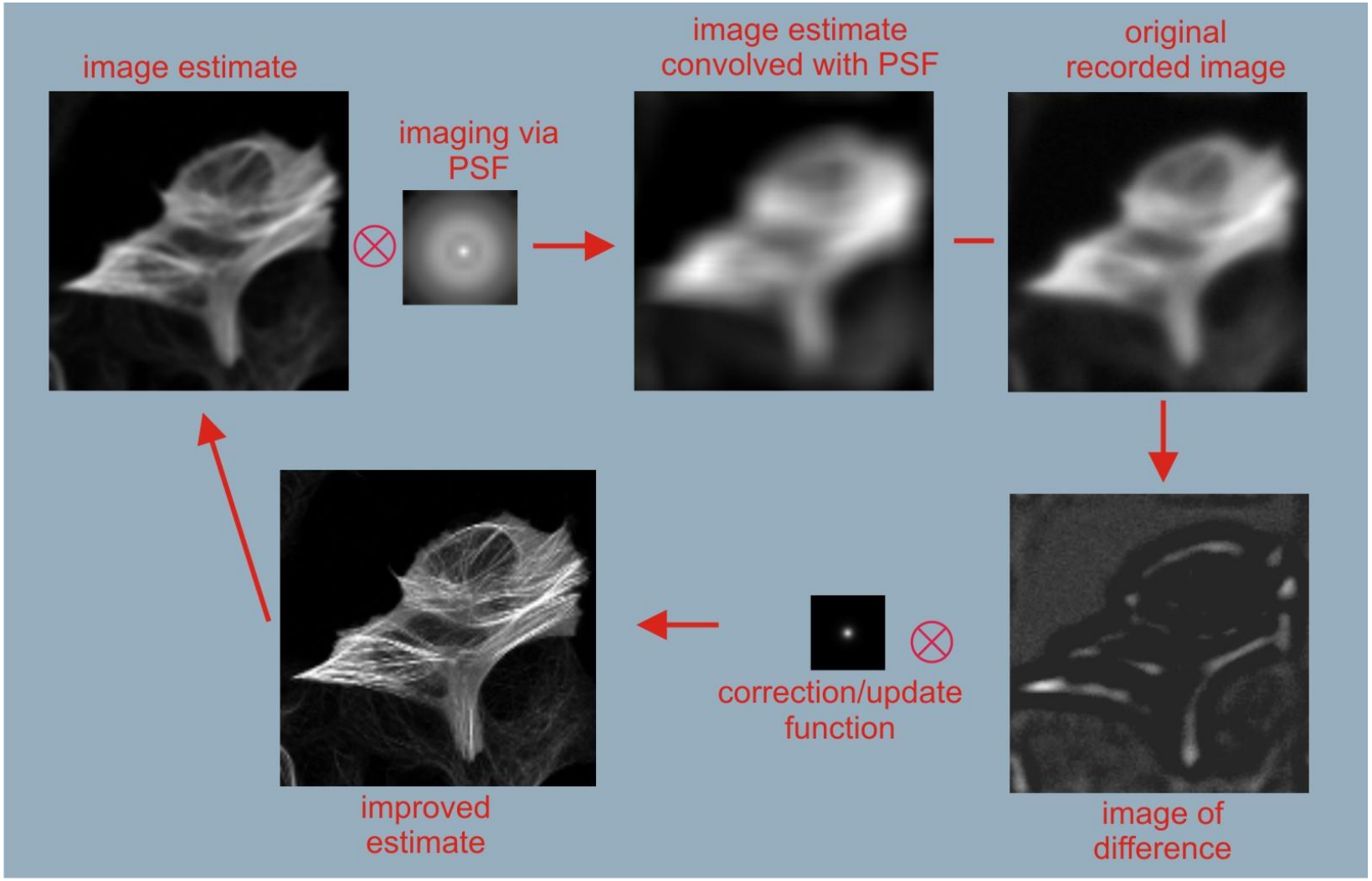
The estimator produces consecutively higher and higher resolution images.



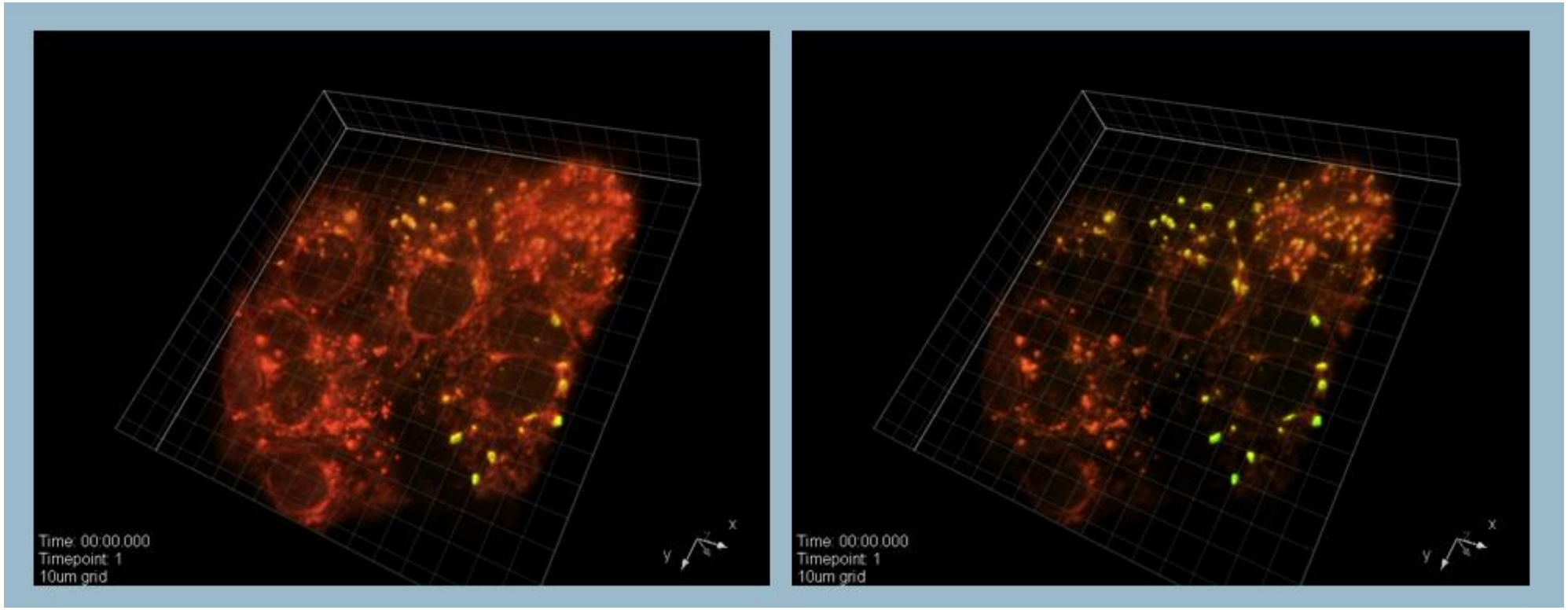


Maximum Likelihood Gradient Methods..

..use a common iteration scheme but the initial estimate and the correction function differ substantially and often the results are stored in frequency space.

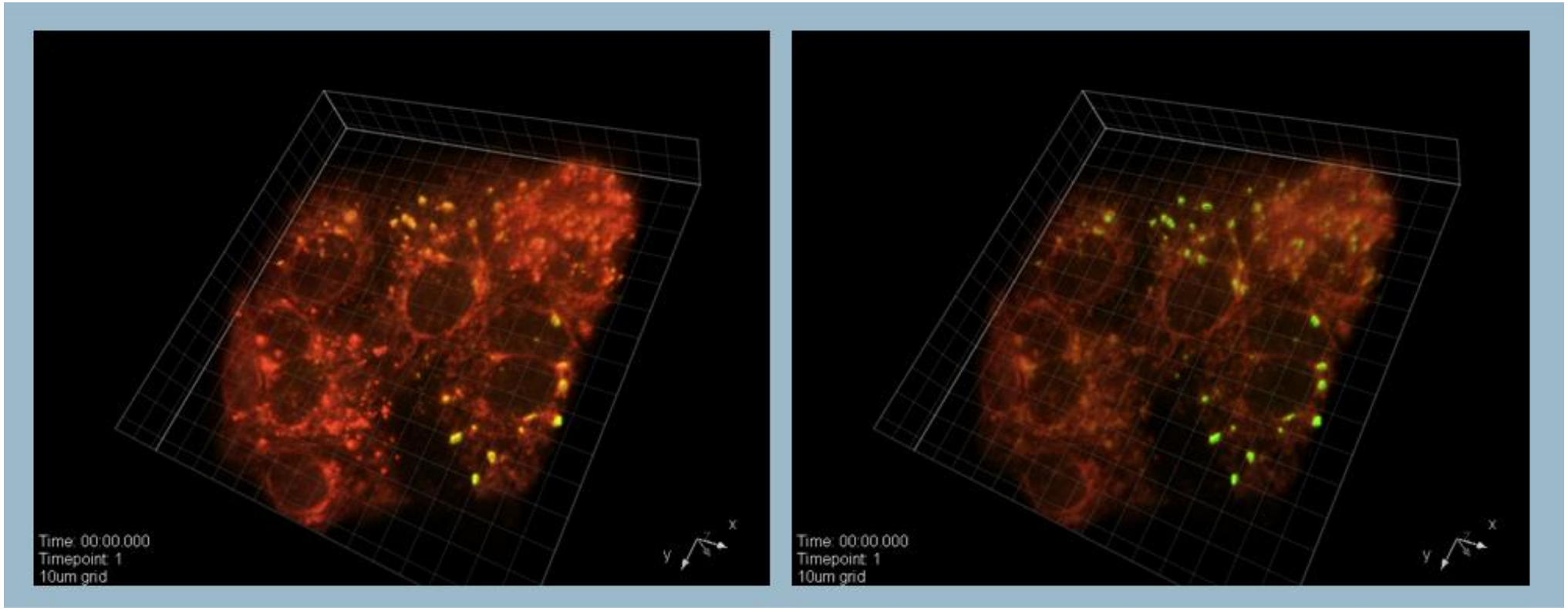


Original vs. MLE deconvolution (Slidebook)
note the reduced background



Gradient rendering

Decon vs. Gaussian filter. (Slidebook)

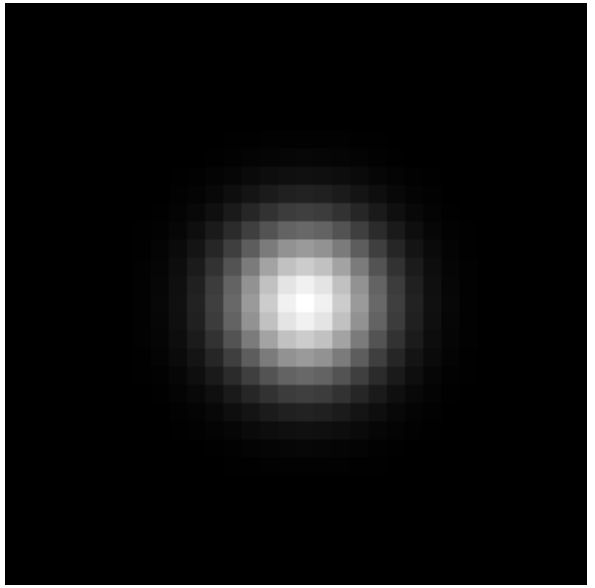


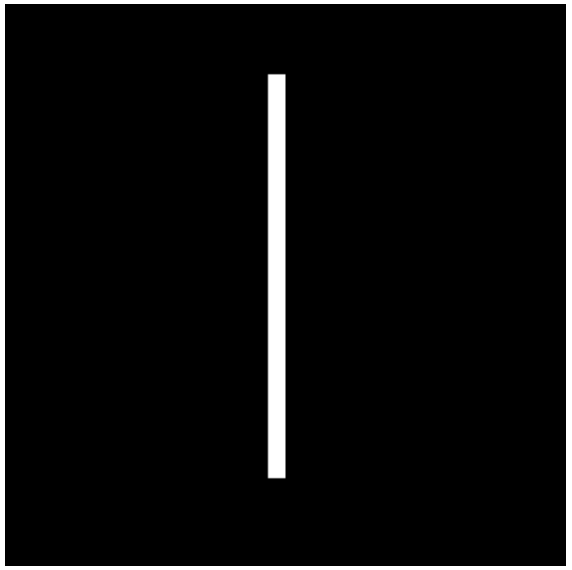
Gradient rendering

Gradient rendering

theoretical performance of decon algorithms

PSF





Object

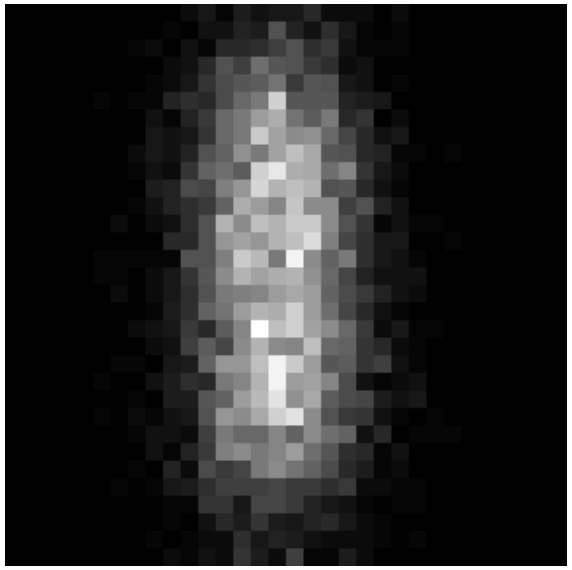
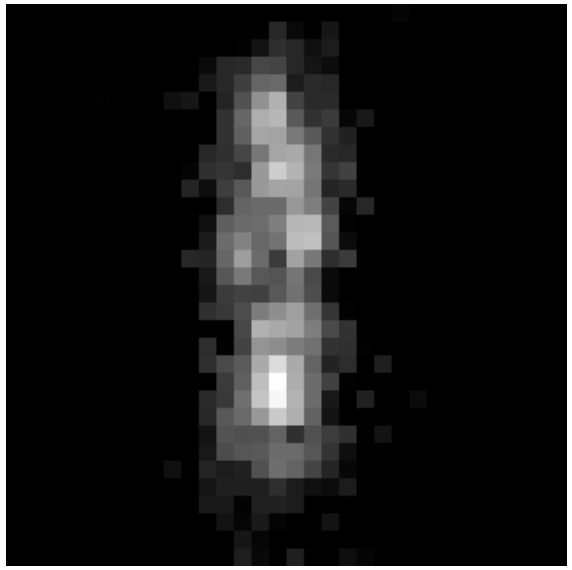


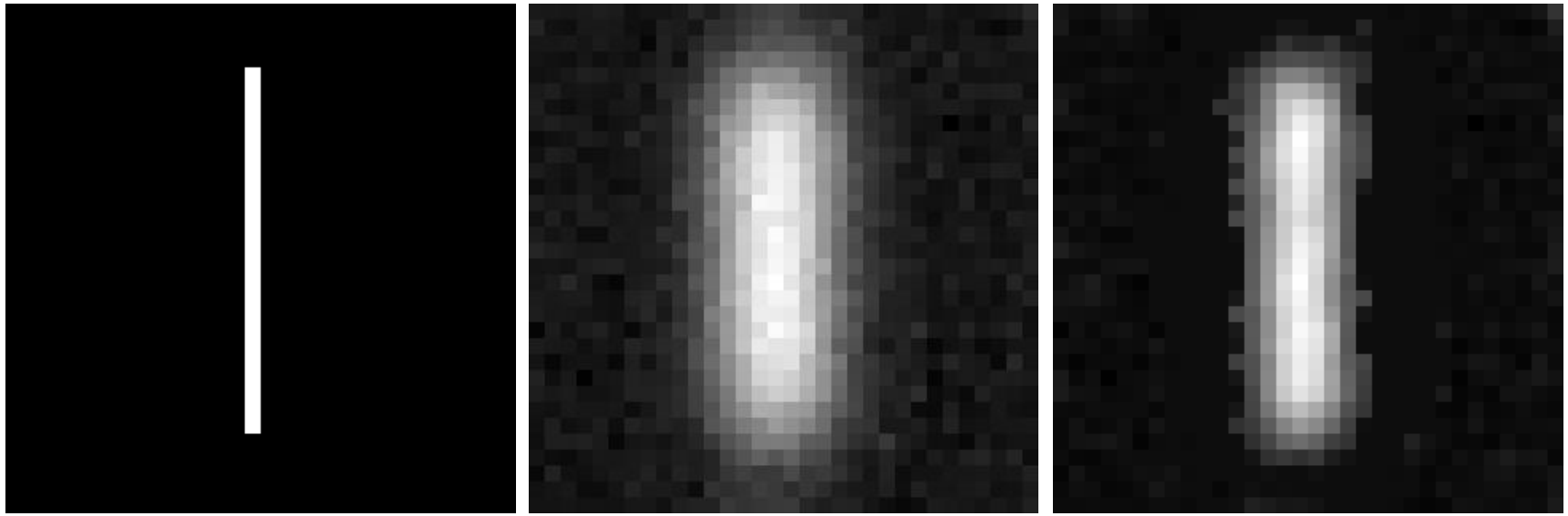
Image w/Poisson (photon) noise



R-L decon result

(Image-J “auto-restraint algorithm”)

theoretical performance of decon algorithms Gaussian (electronic) noise*



Object

Image w/Poisson (photon) noise

R-L decon result

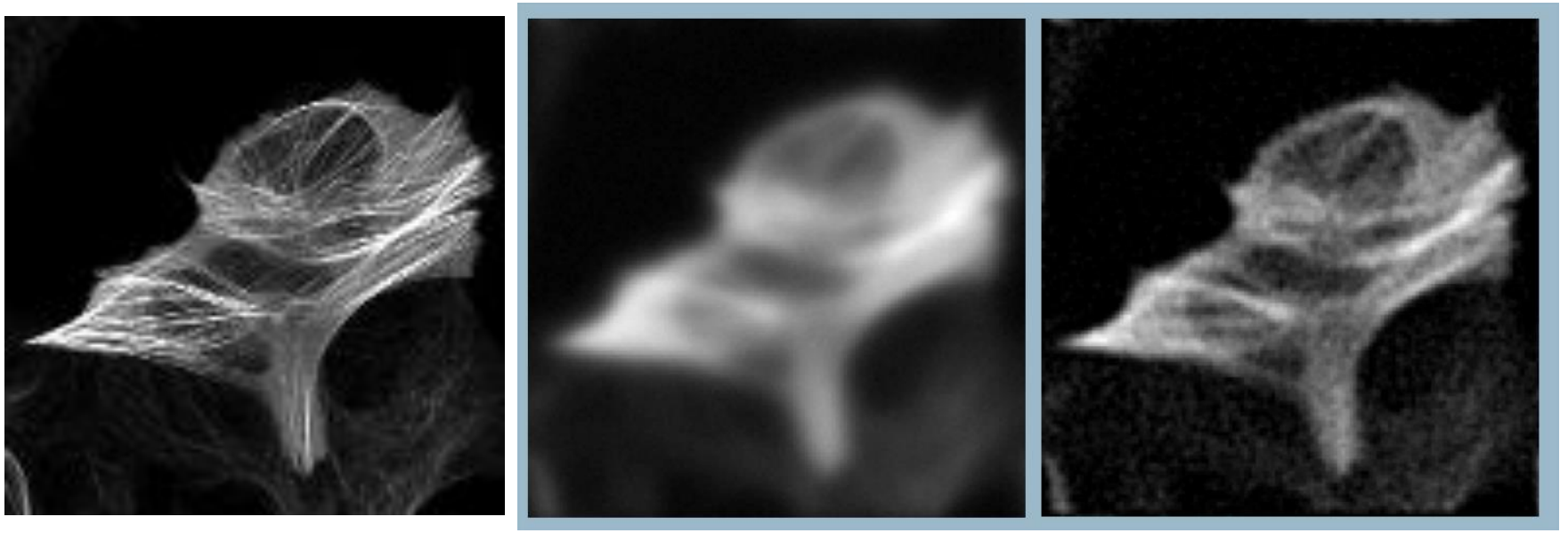
(Image-J “auto-restraint algorithm”)

* Poisson noise is proportional the sqrt of the singal.

Gaussian noise is a fixed uncertainty unrelated to the size of the signal intensity. It is usually observable only at low level signals from the CCD.

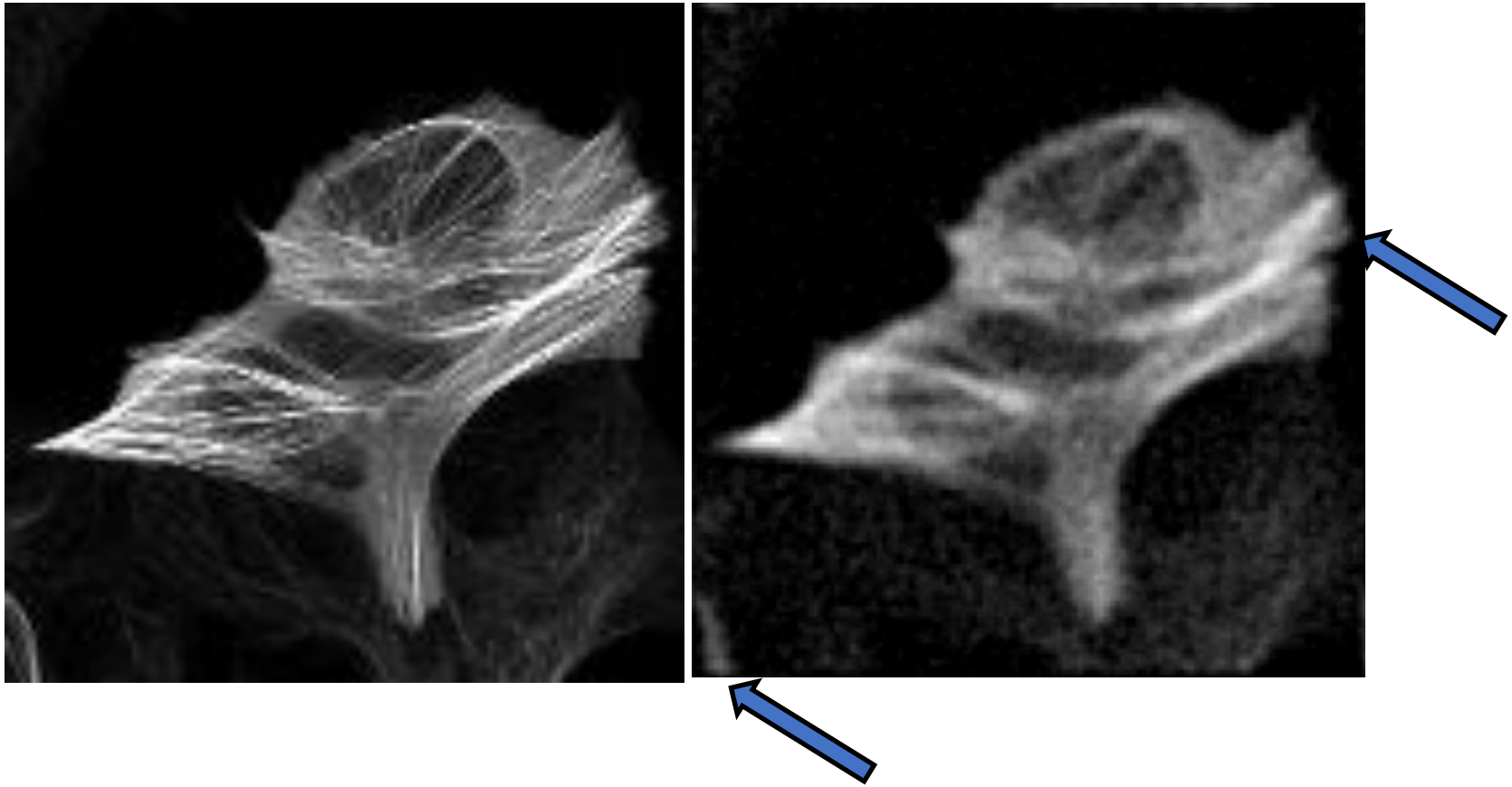
theoretical performance of decon algorithms

RL *converges faster if parts or the boundaries of the volume have little signal.*

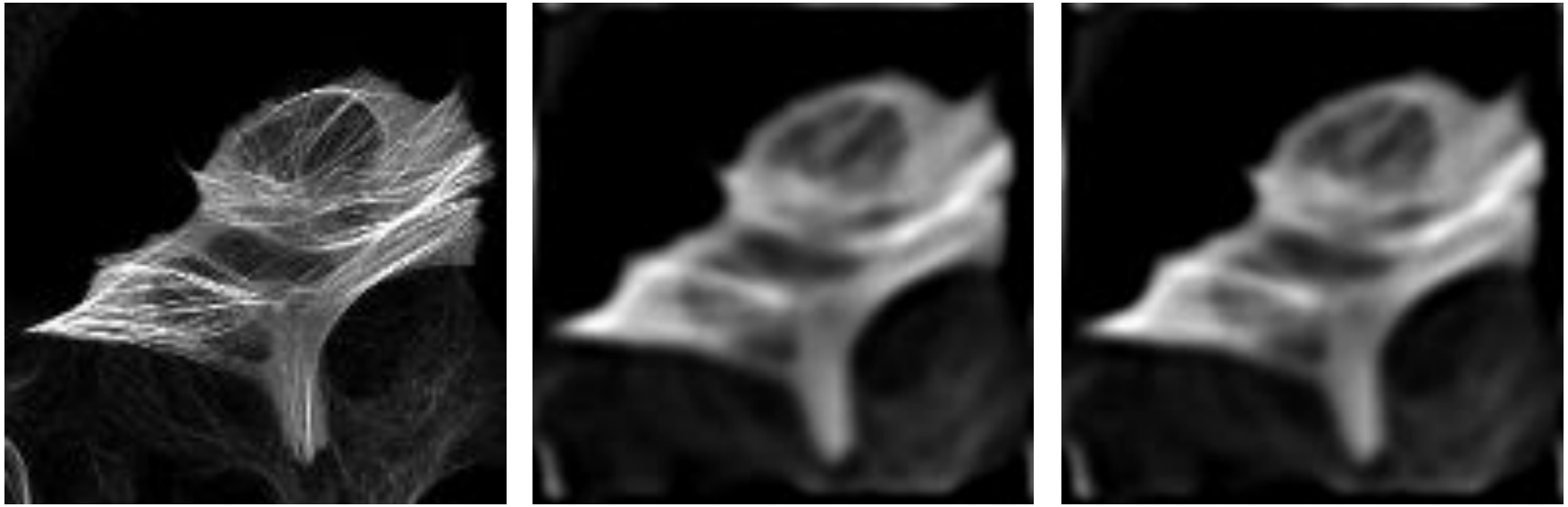


All iterative deconvolution algorithms degrade near the volume boundaries

For the simple reason that the image information in that area is incomplete

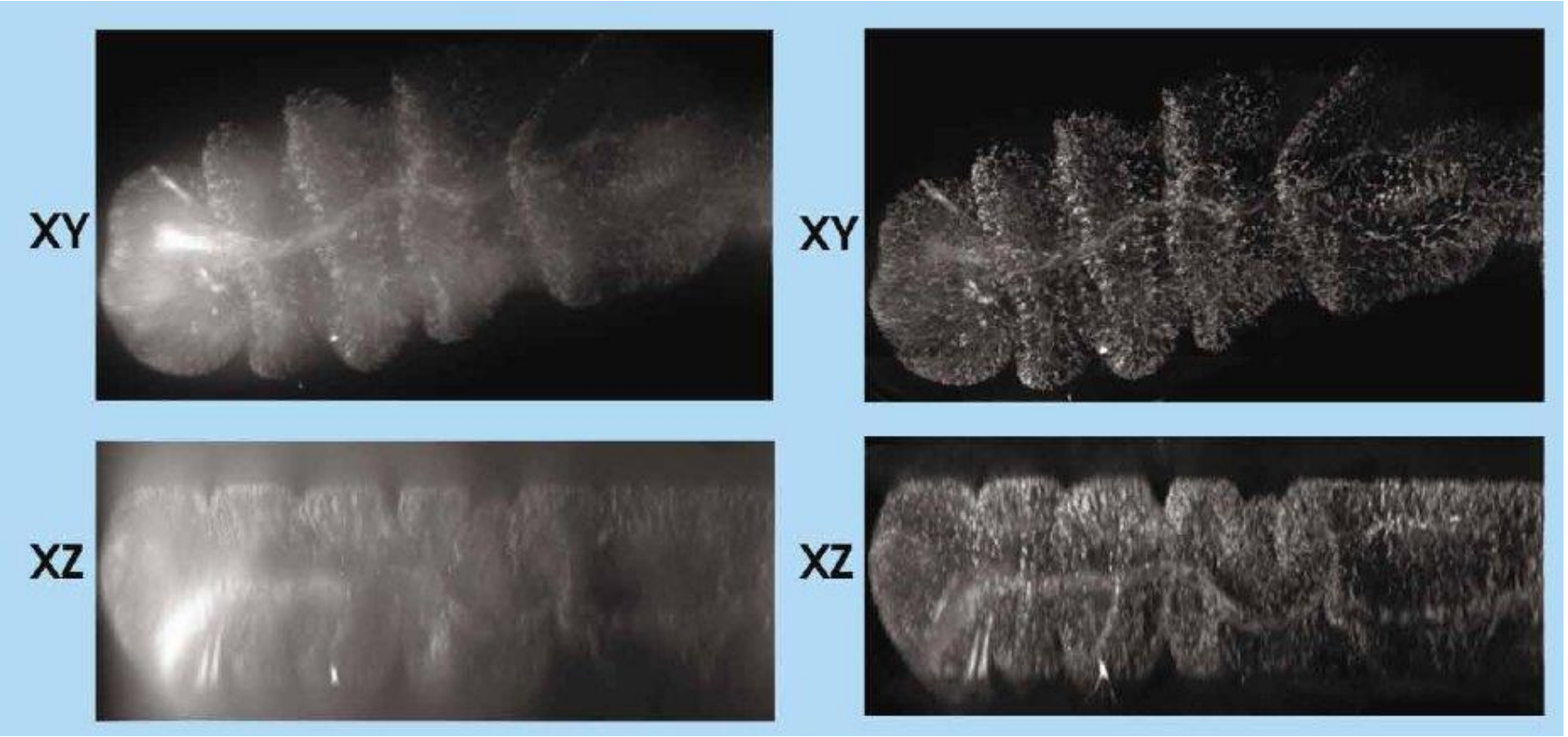


PSF inaccuracies



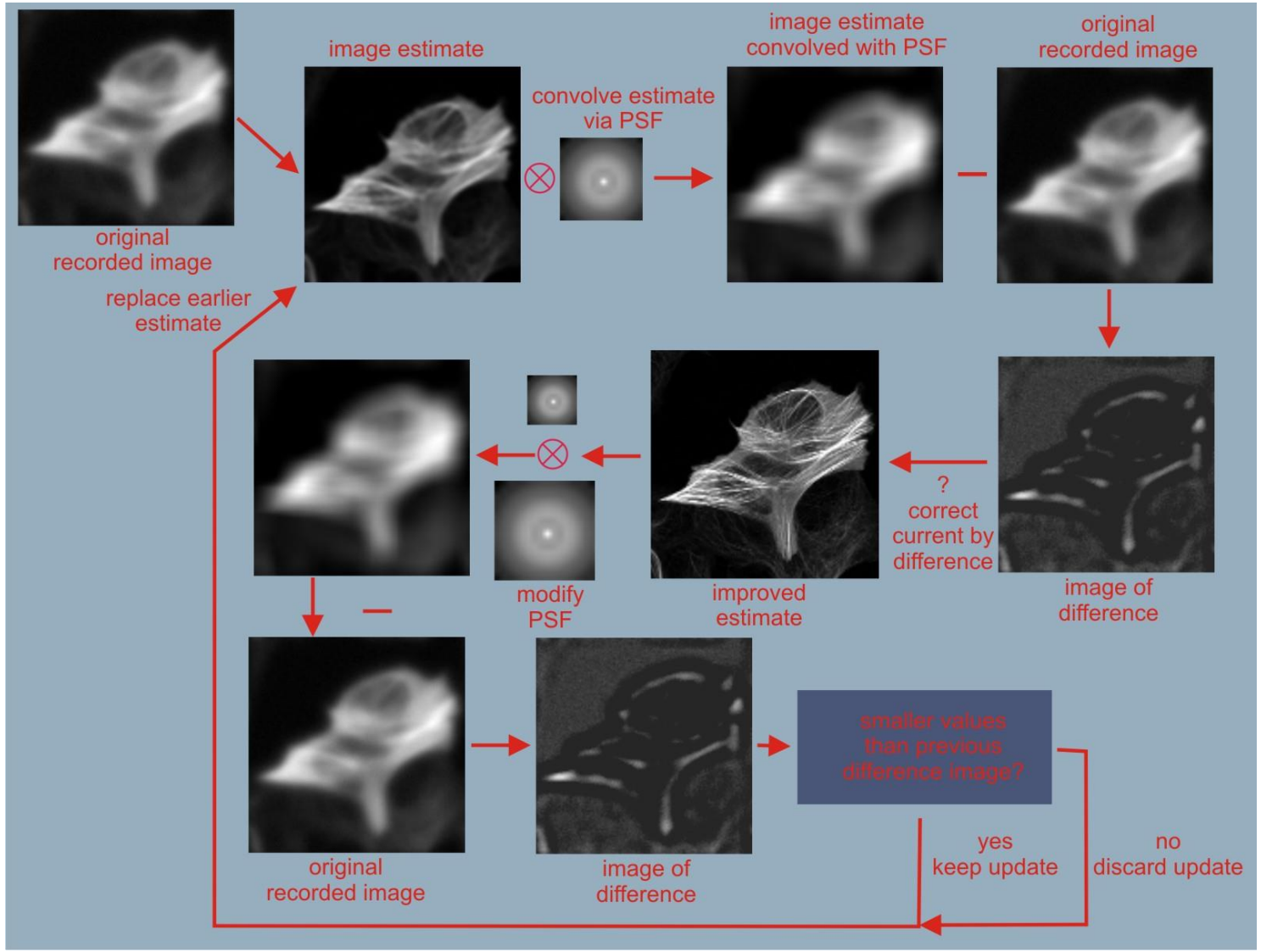
original – measured PSF – theoretical PSF
often good enough

“Blind” Decon

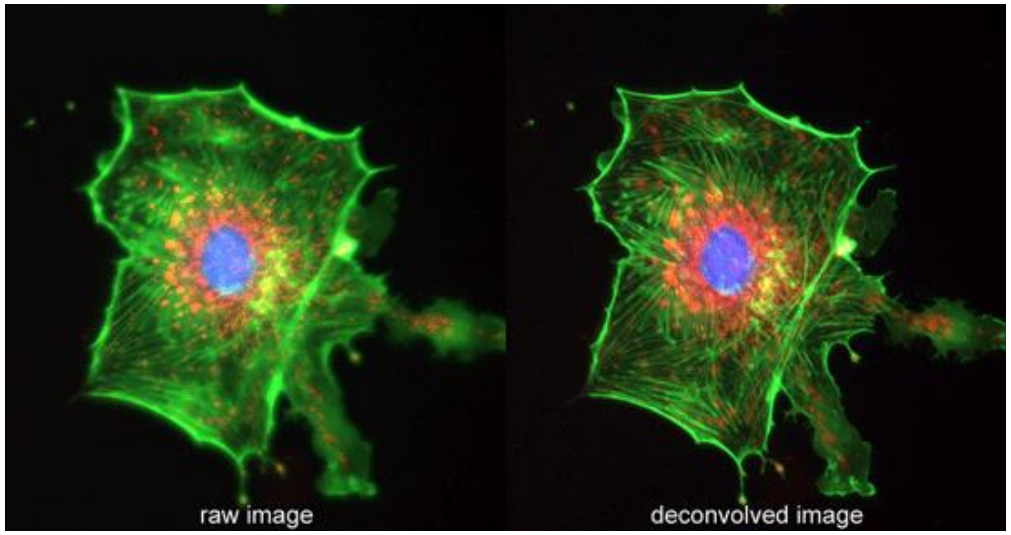


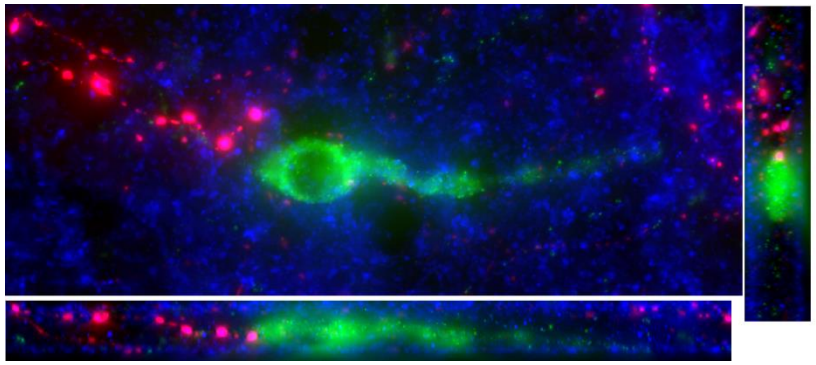
from "Clearing Up Deconvolution" D.S.C. Biggs, AutoQuant Imaging Inc.

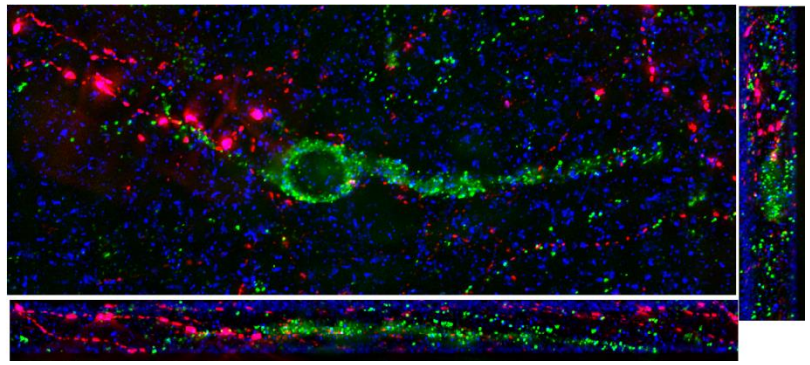
“Blind” Decon


original
recorded image
image estimate
convolve estimate
via PSF
image estimate
convolved with PSF
original
recorded image
replace earlier
estimate
?
correct
current by
difference
image of
difference
modify
PSF
improved
estimate
original
recorded image
image of
difference
smaller values
than previous
difference image?
yes
keep update
no
discard update

Maximum Likelihood (ML)

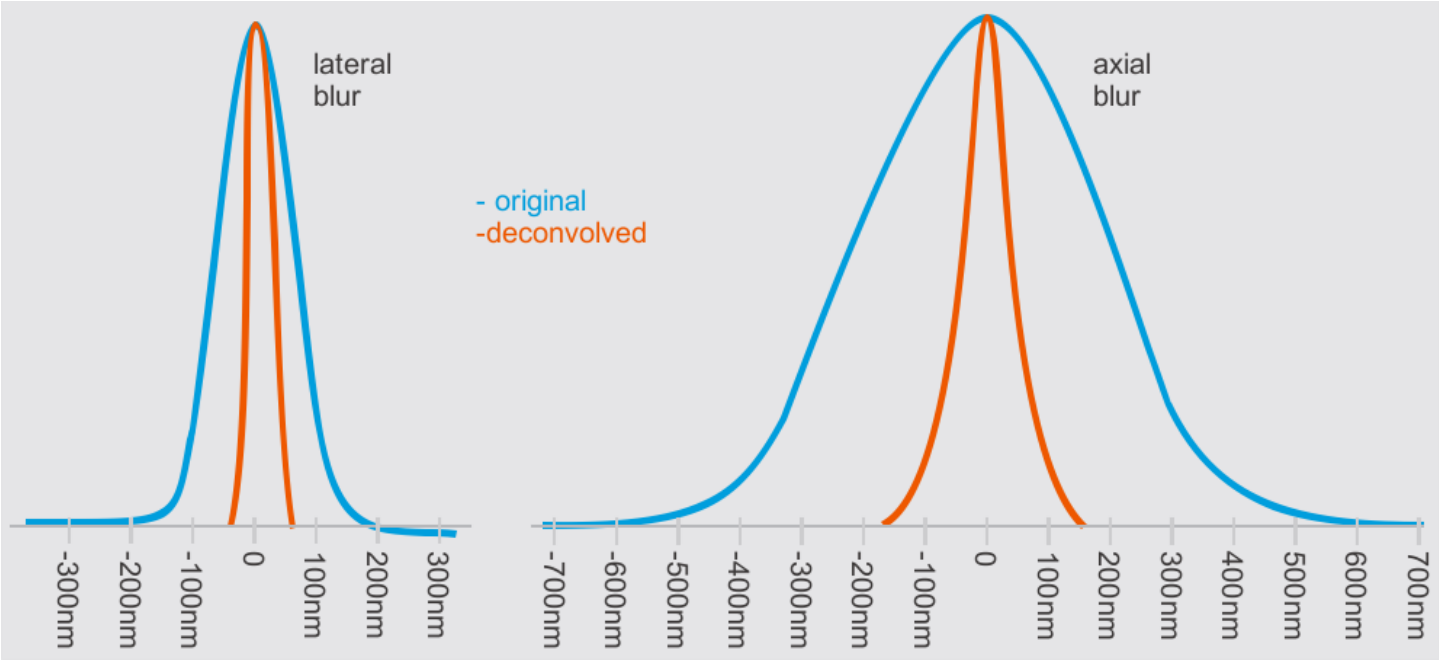






source: Anda Cornea NCRR: Shared Instrumentation Grant, presented @ UBC

As a result, deconvolution can result in stronger improvement of the Z axis!



50nm vs. about 100nm - only 2x left (ML <10% noise energy)