Aaron Ponti

Microscopy Network Basel

Image processing course

Deconvolution basics

v1.2

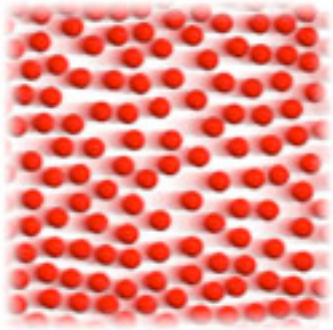
http://micro.magnet.fsu.edu/primer/lightandcolor/particleorwave.html

What is light?

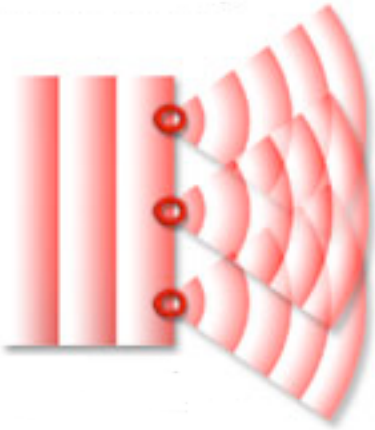
Particle or Wave

Pythagoras
(~580-500 BC)

"steady stream of particles, like droplets of water sprayed from a hose nozzle "



"ripples spreading across the surface of a pond disturbed by a stone"



Aristotle
(384-322 BC)

vs.

Sir Isaac Newton
(1642-1727)

Christiaan Huygens
(1629 – 1695)

James Clerk Maxwell
(1831-1879)
Philipp Lenard
(1862-1947)

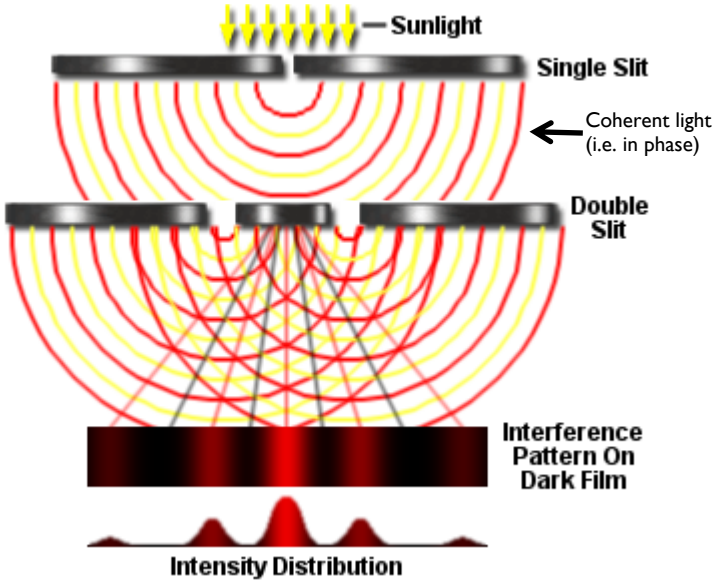
Both?

Albert Einstein
(1879-1955)
Arthur H. Compton
(1892-1962)
Louis-Victor de Broglie
(1892-1987)

Augustin-Jean Fresnel
(1788-1827)
Thomas Young
(1773-1829)

Thomas Young's experiment (1803)

- Because he believed that light was composed of waves, Young reasoned that some type of interaction would occur when two light waves met.
- He used a screen containing a single, narrow slit to produce a coherent light beam (containing waves that propagate in phase) from ordinary sunlight.
- When the sun's rays encounter the slit, they spread out or **diffract** to produce a single wavefront. When this front hits a second screen with two closely spaced slits, two additional sources of coherent light, perfectly in step with each other, are produced.
- When the light waves from the second set of slits are diffracted, they meet each other and overlap – either fully in step (**constructive interference**), or fully out of step **(destructive interference)**, or somewhere in between.



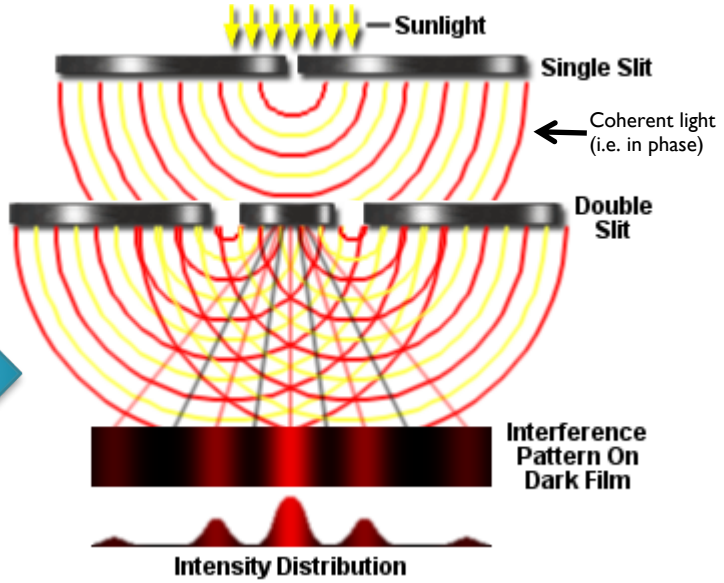
Thomas Young's experiment that seemed to prove once and for all that light is a wave.

- This gives rive to an **interference pattern** (**fringes**) on the screen that could **not** be explained by the particle theory.

Interference and diffraction effects are not restricted to light: waves produced on the surface of a pool or pond will spread in all directions and undergo an identical behavior; a person in a room hears the noise coming from another room as if it were originating from the doorway.

Thomas Young's experiment (1803)

If we want to see an **image** of the two slits on the screen instead of a diffraction pattern, we need to refocus the wavefront: we need a **lens**!

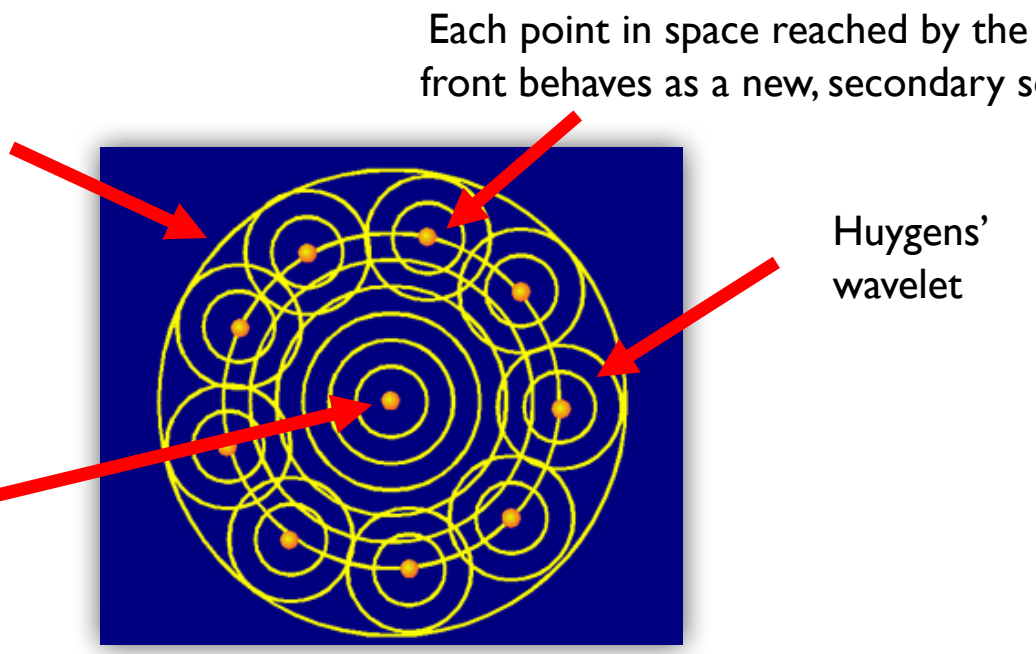


Thomas Young's experiment that seemed to prove once and for all that light is a wave.

The Huygens – Fresnel principle

- Simplified model of image formation to describe the behavior of **waves** in space that explains phenomena like refraction, diffraction and interference: in particular, it applies to image formation in light microscopy.

Huygens…



… Fresnel

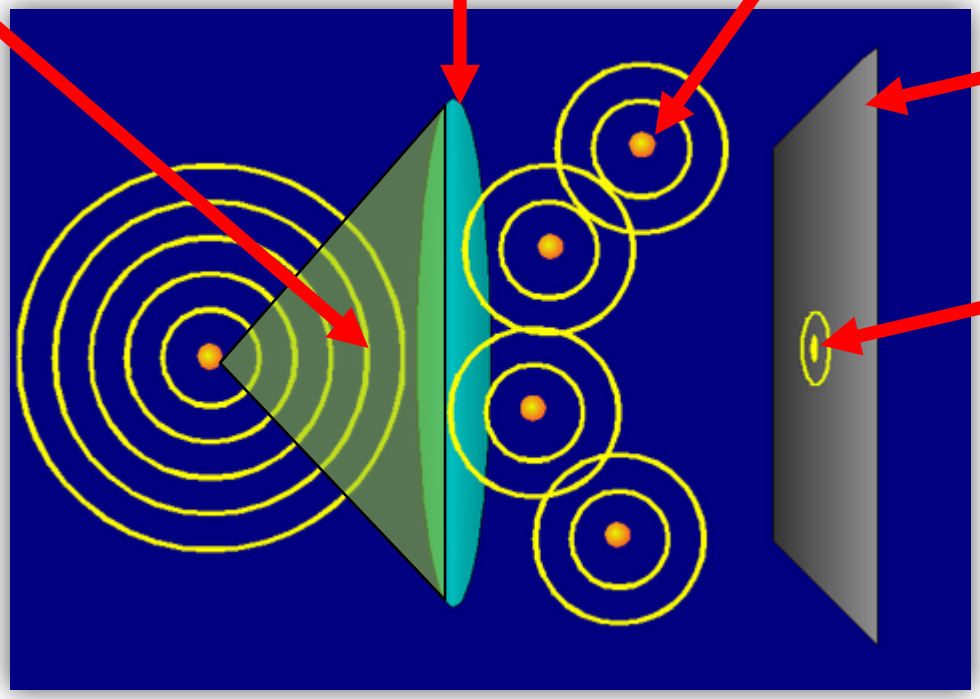
The amplitude of the wave at any given point in space is given by the superposition (interference) of the amplitudes of all secondary wavelets at that point.

The role of the objective

Cone of captured light: a large fraction of the wave front is not captured by the lens and the light source cannot be refocused to a point

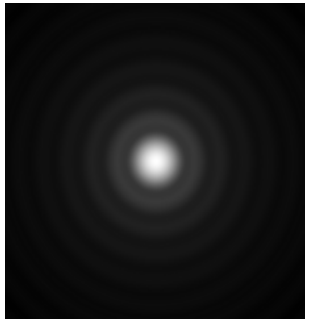
Focusing, convergent lens

Huygens' wavelet



Screen or detector, where all wavelets interfere

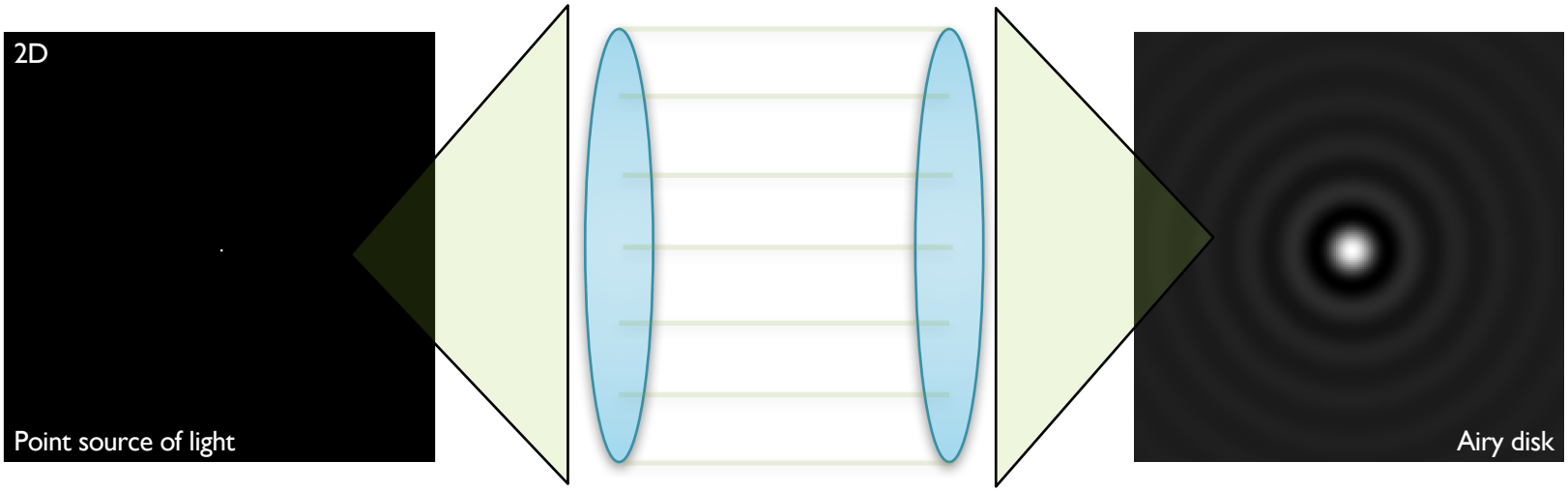
Airy disk, **NOT** a point!

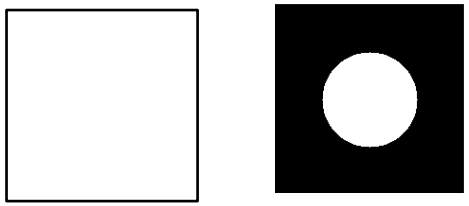


Interference pattern

The lens delays the light more in the center and less toward the periphery and "inverts" ("mirrors") the shape of the spherical wave front

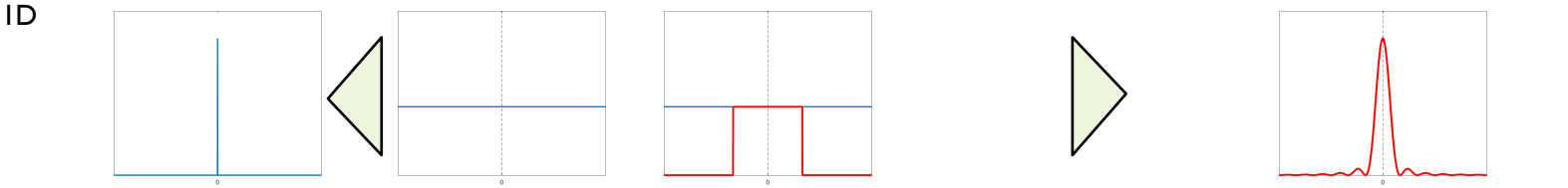
The role of the objective





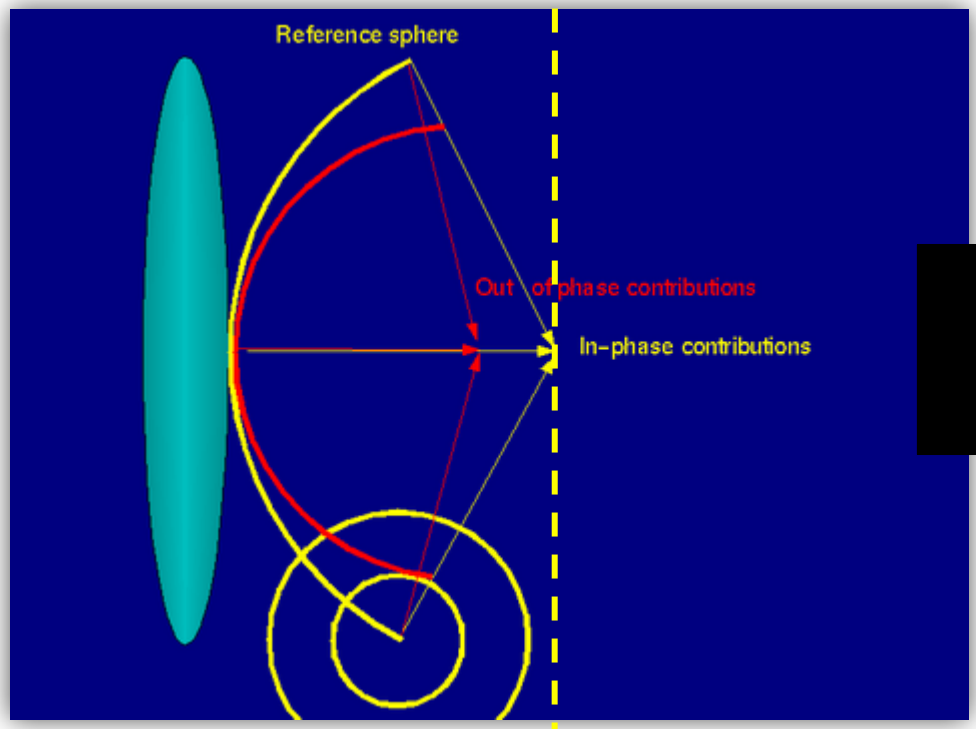
The lens drops all frequencies above a given cutoff.

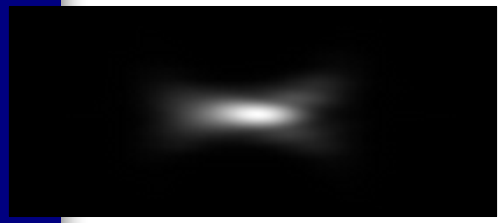
Frequency domain



The Point-Spread Function

The interference behavior in the image plane (Airy disk) also extends along the optical axis and gives rise to the (3D) Point Spread Function (PSF).

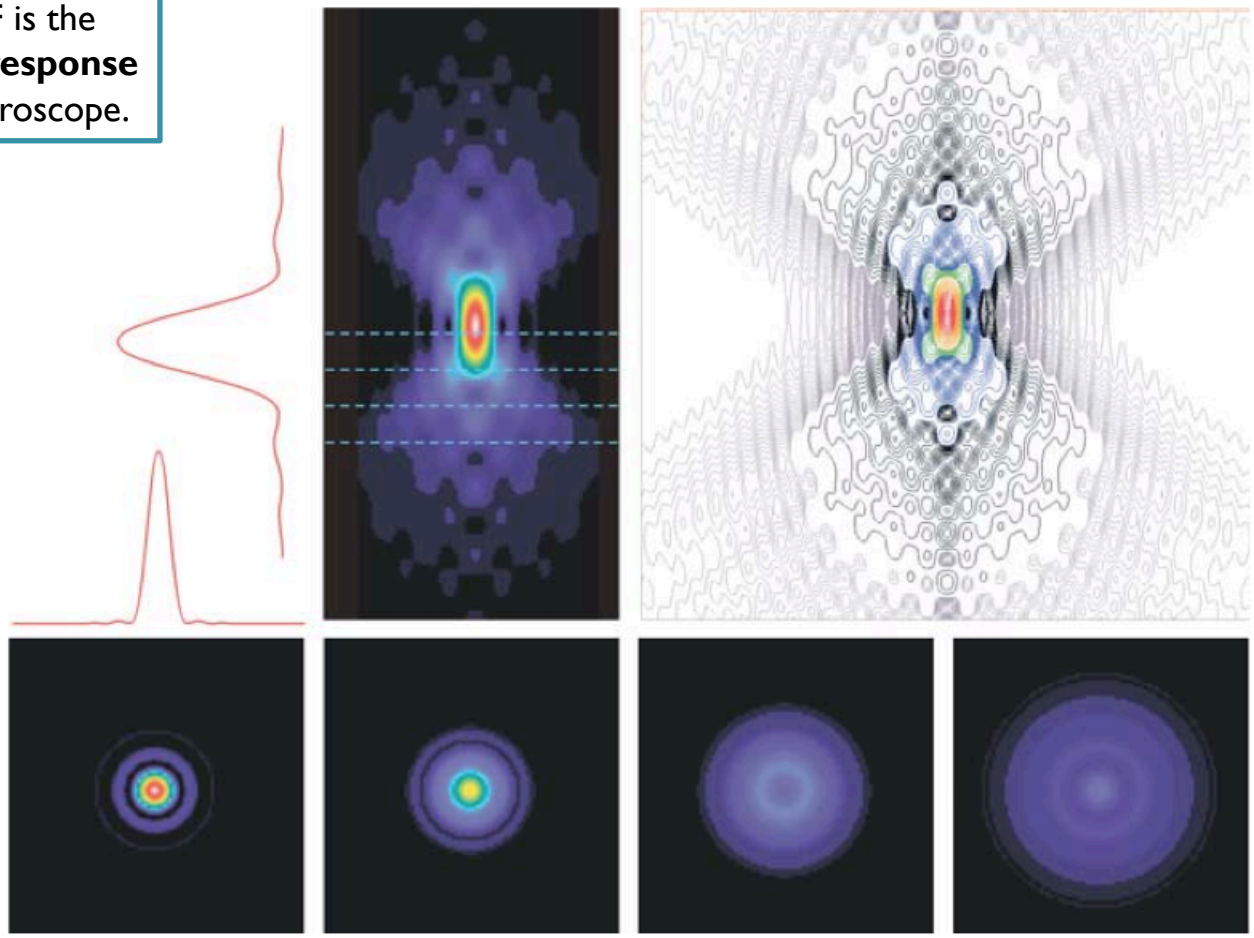




Lateral (XZ) view

The Point-Spread Function

The PSF is the **impulse response** of the microscope.



Diffraction pattern of an **ideal**, **diffraction-free** microscope in one, two, and three dimensions.

Resolution

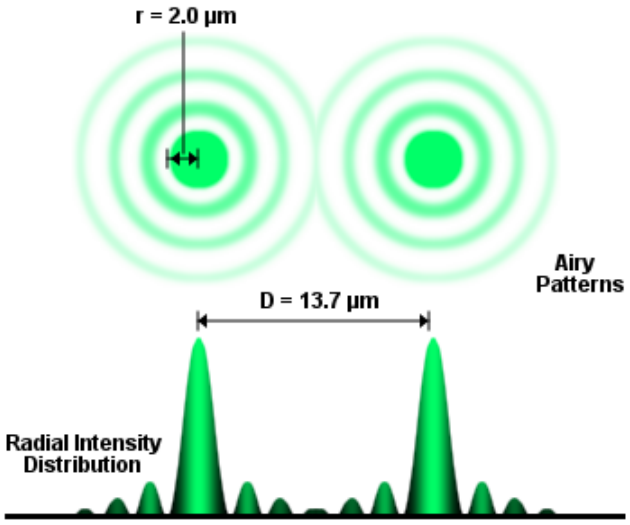
- The amount of light that the lens can collect is measured by the Numerical Aperture (NA).
- NA and wavelength λ define the size of the Airy disk (PSF)
- The radius $r_{lateral}$ of the Airy disk (the distance between the central maximum and the first minimum) at the focal plane is:

$$r_{lateral} = \frac{0.61\lambda}{n\sin\alpha} = \frac{0.61\lambda}{NA}$$

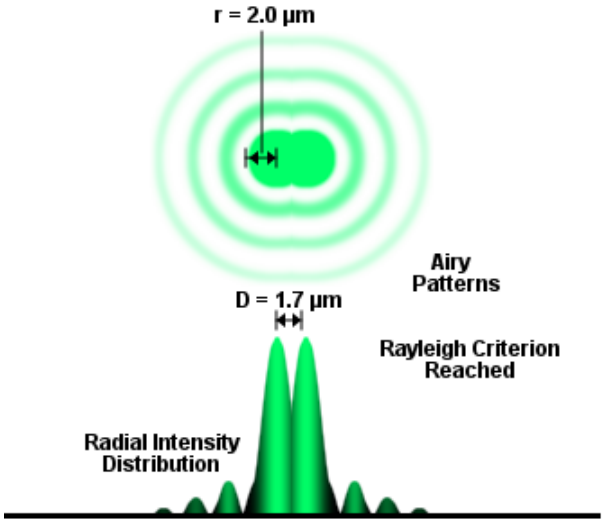
larger NA, higher resolution

- The **Rayleigh criterion** establishes a standard to characterize the spatial resolution of an optical device: the minimum resolvable detail, or how much can two points be close to each other before they become indistinguishable.
  - This is one of the possible definitions of resolution, others being **Abbe's diffraction limit** and the **Sparrow limit**.
- In the axial direction, the resolution limit is given by: $r_{axial} = \frac{2\lambda n}{NA^2}$

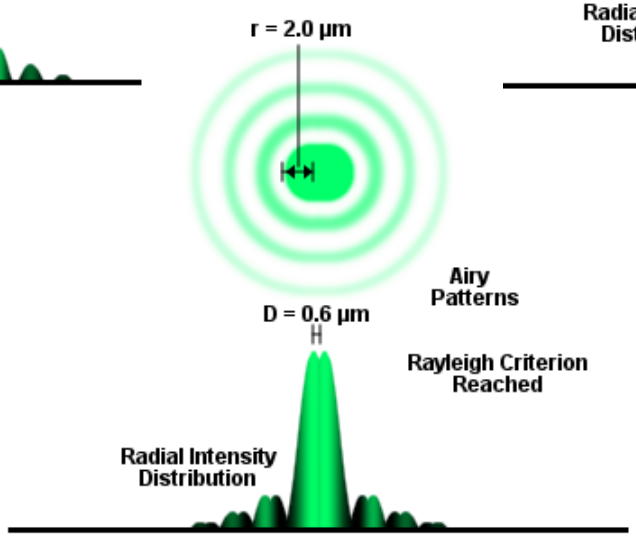
Resolution



Fully resolved



Rayleigh



Unresolved

Image formation model

- Fluorescent microscopes are incoherent[1] imaging systems → the image formation process is linear and described by linear system theory:

"Linear means that two different light signals (or of any other nature, in general) coming from two different points of the object do not interfere with each other. The resulting image of two emitting points is equal to the addition of the images that would arise by measuring the two points separately."

- An ideal (fluorescence) light microscope is therefore:
  - aberration free
  - completely described by a **shift-invariant** 3D PSF
- This is mathematically represented by a Convolution equation.

$$g = f \otimes h = \iiint_{-\infty}^{\infty} f(x)\, h(x' - x)\, d^3x$$

[1] For a specimen illuminated by a large-angle cone of light, or for self-luminous objects, the light rays forming adjacent Airy patterns are incoherent and do not interfere with each other.

Image formation model

- Image formation in the fluorescent microscope is completely described by its PSF, but two additional elements are involved in the process of bringing the acquired image *g* to disk: **noise** and **sampling**.

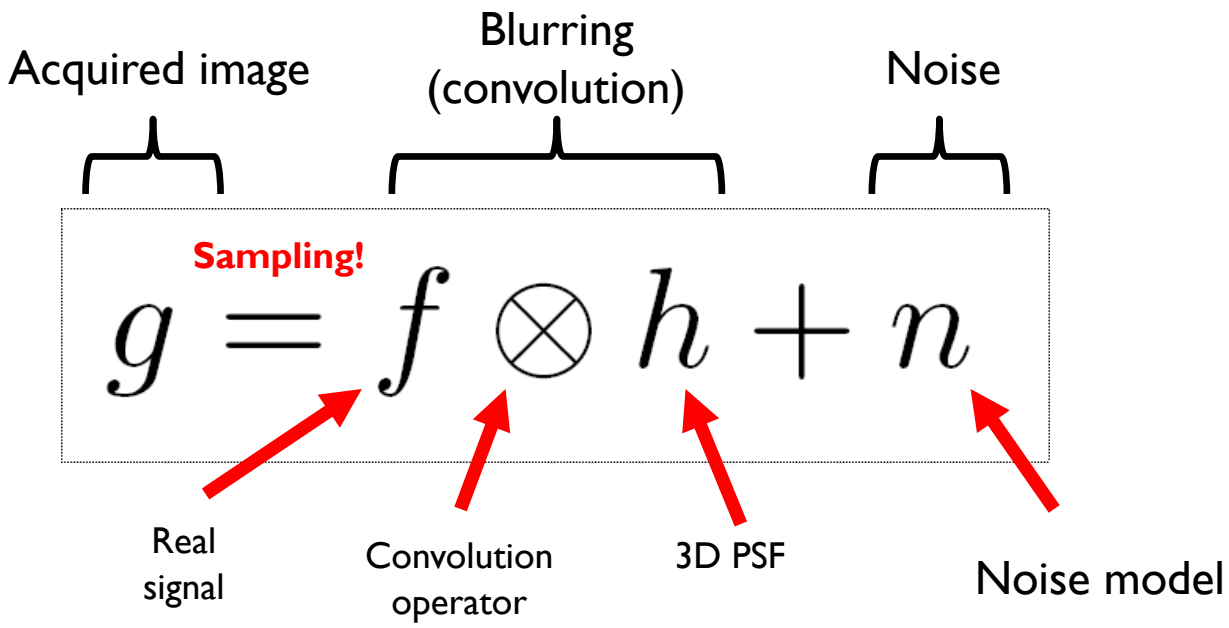
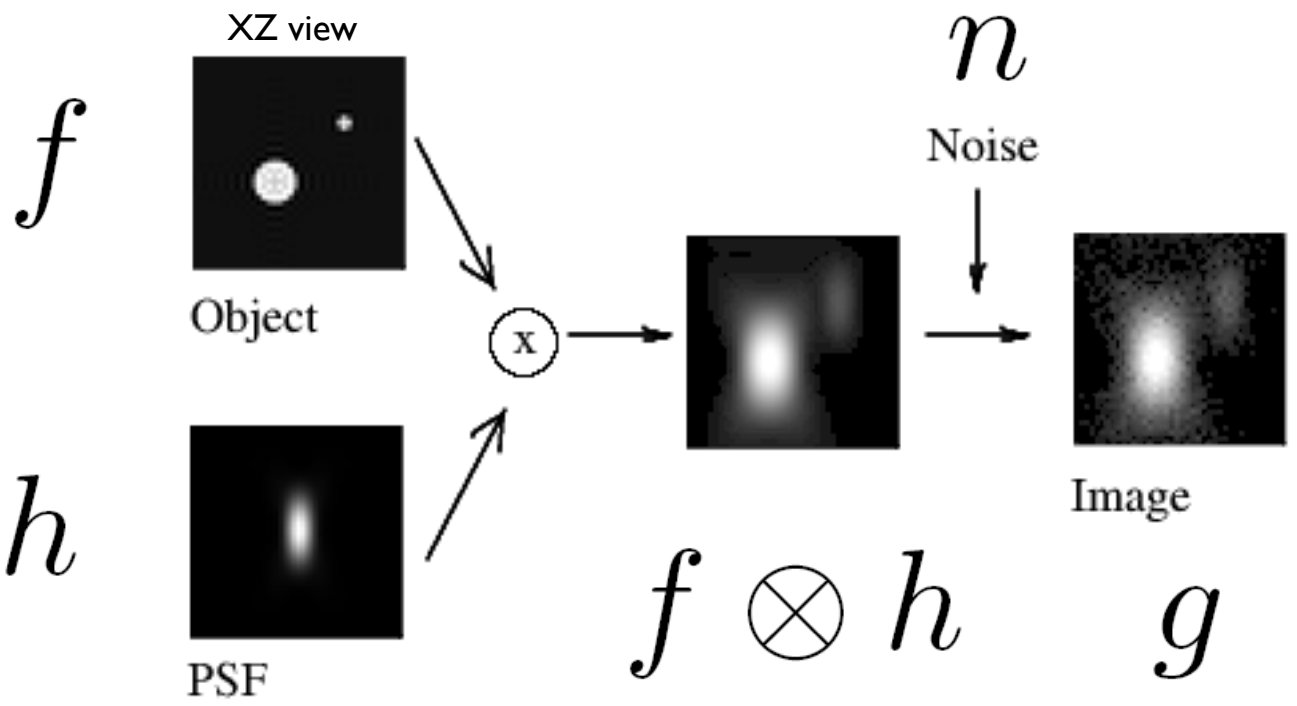
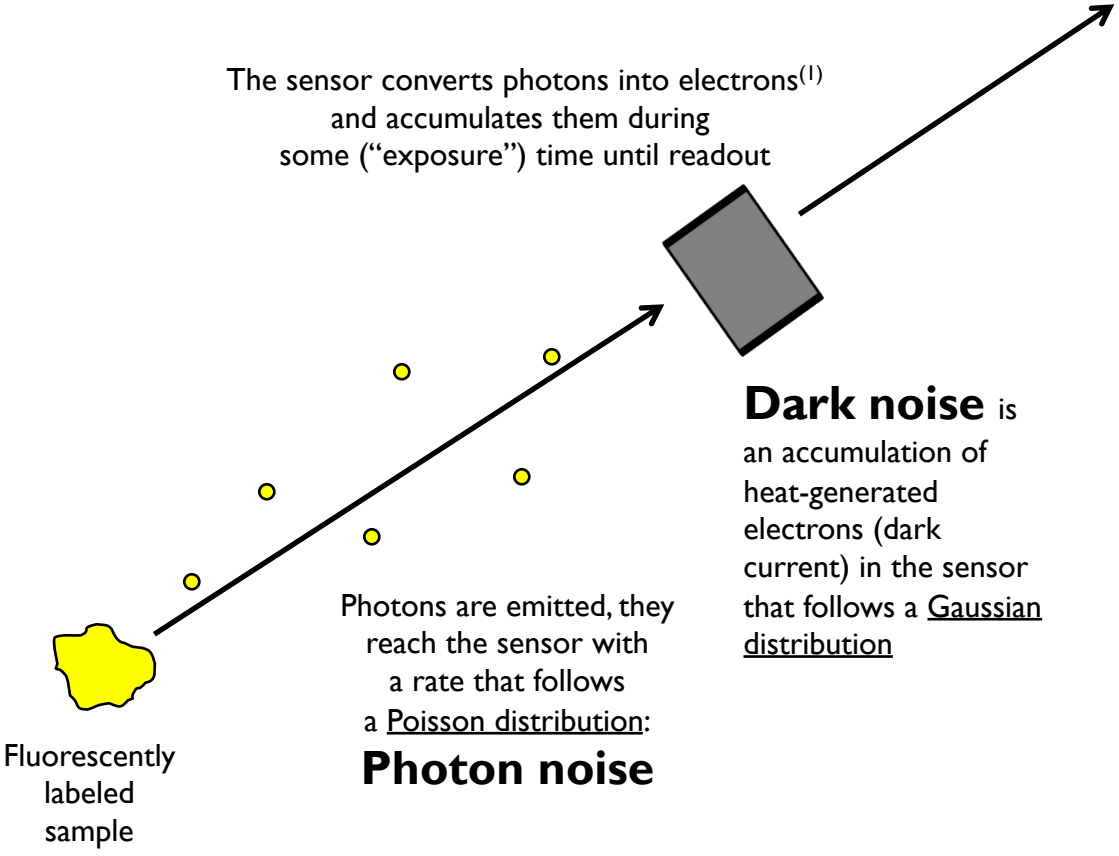


Image formation model



Noise

A simplified sensor (e.g. a CCD pixel)

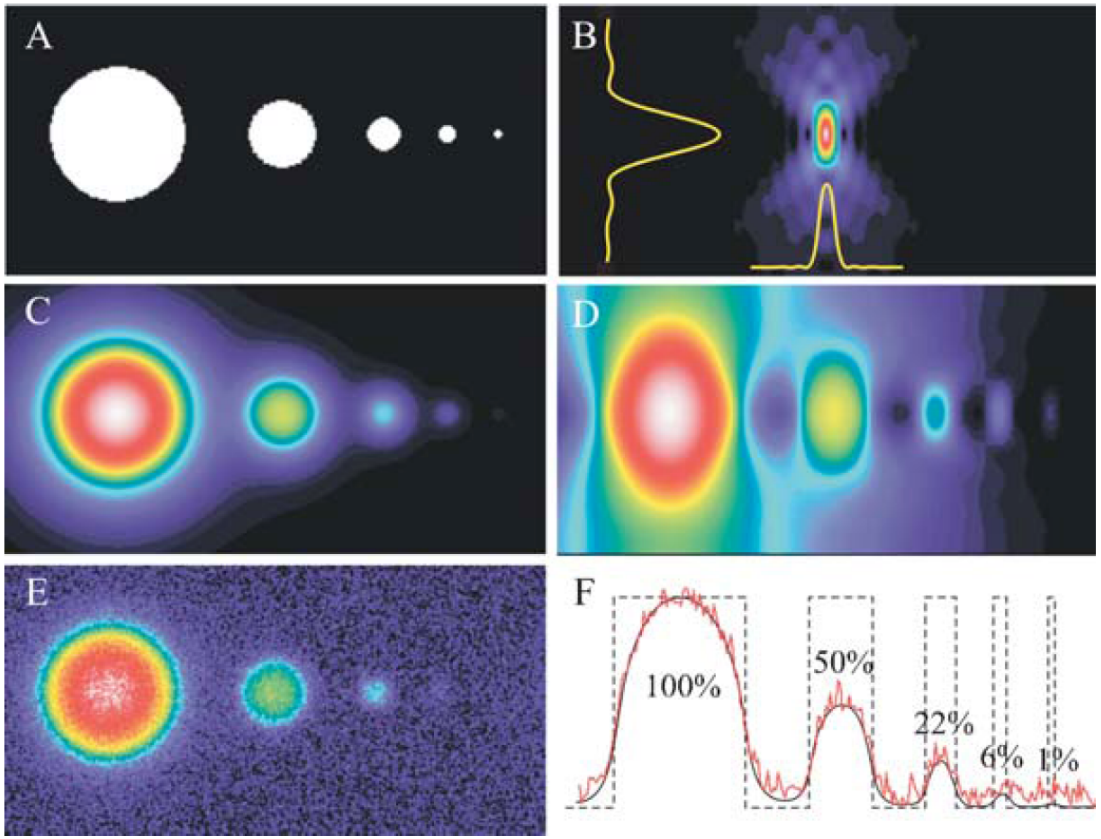


Once the exposure is over, the charge at each sensor is measured, and the measurement is converted into a digital value. This measurement process is called readout.

Readout noise: since the accumulated signal has to be amplified to be read, and there is no such thing as a perfect amplifier, the amplifier adds a bit of noise, similar to static in a radio signal, to the charge it is amplifying. The readout noise follows a Gaussian distribution.

(1) With given Quantum Efficiency

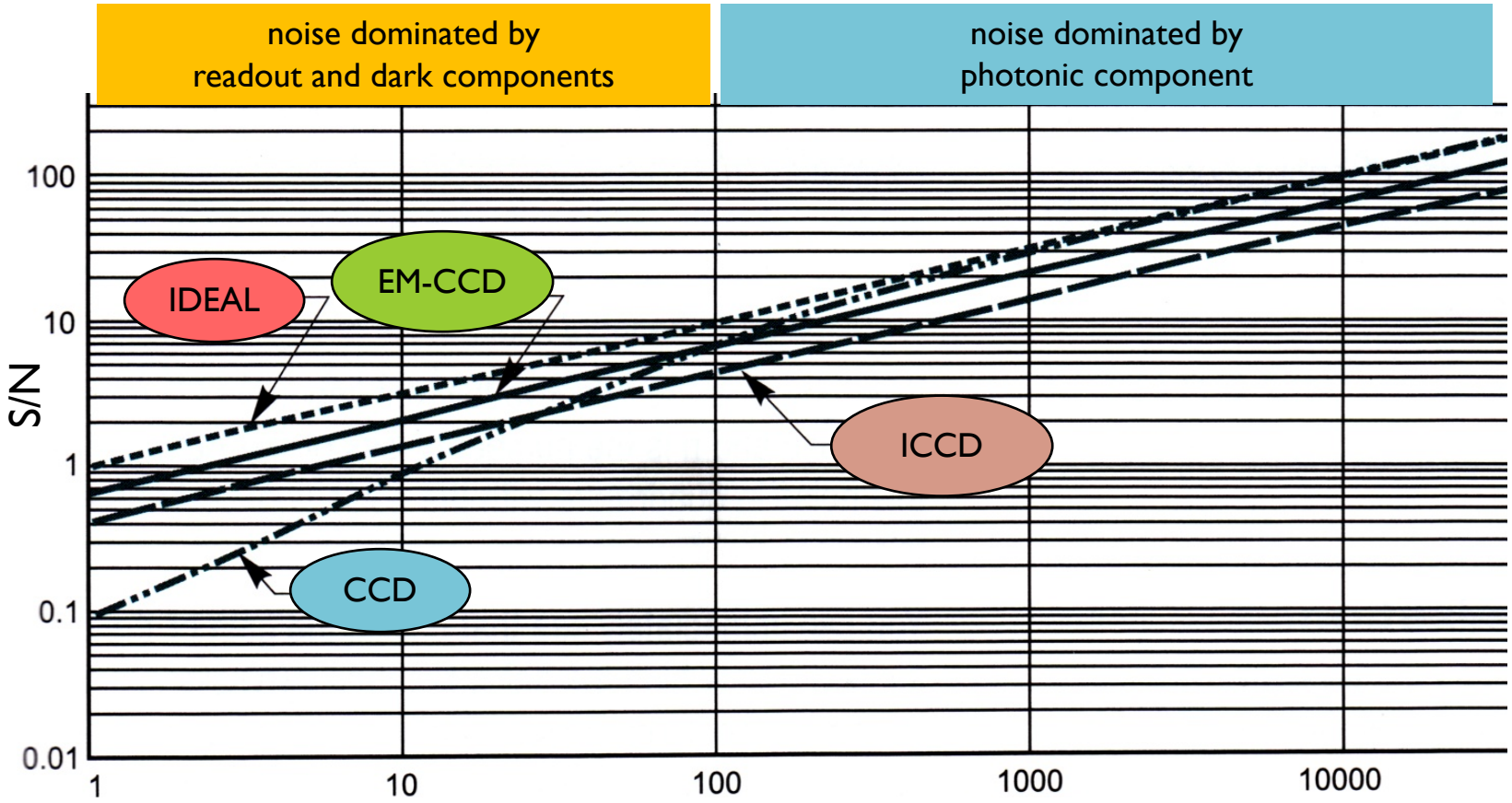
Noise



Three-dimensional simulation of an object observed through a microscope, in the presence of optical blur and noise. The object consists of five spheres with different diameters but the same fluorescence density. **A** initial object; **B** point spread function (PSF) of the microscope; **C, D** lateral and axial cross sections of the object after convolution with the microscope's PSF; **E** lateral cross section of the object after blurring and the addition of noise; **F** intensity profiles and percentage of the object's maximum intensity of original (dashed), blurred (black) and blurred + noisy (red) data. Following blurring, the smaller the object, the weaker its maximum intensity is likely to be. Noise reduces the likelihood of detecting small and highly attenuated objects.

Noise

The signal to noise ratio $$SNR = \frac{signal}{\sqrt{dark^2 + readout^2 + photon^2}}$$



Sampling

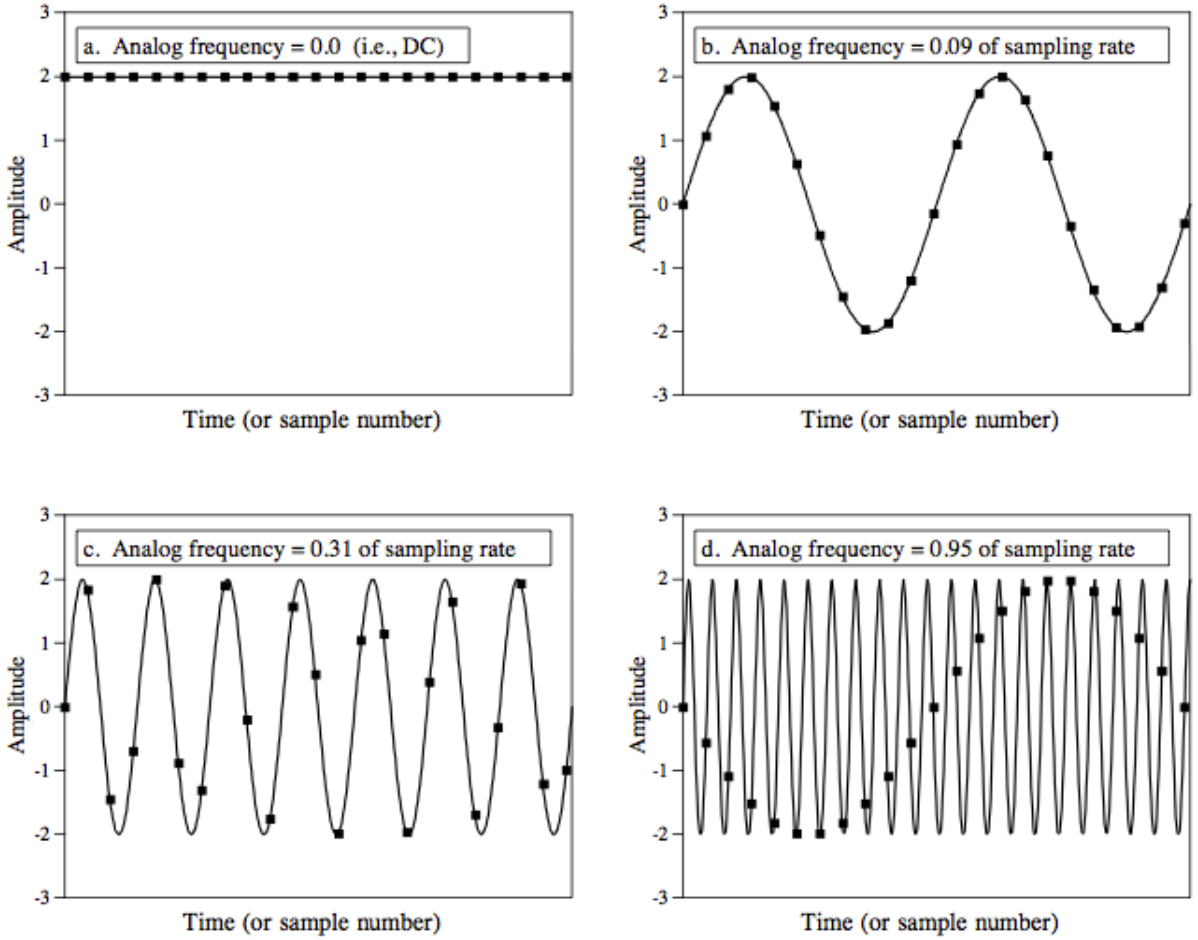
- Sampling is the process of converting a signal (e.g. a function of continuous time or space) into a numeric sequence (a function of discrete time or space).
- The **Nyquist-Shannon sampling theorem** establishes that when sampling a signal (converting from an analog signal to a digital signal), the sampling frequency must be greater than **twice** the bandwidth B of the input signal (Nyquist rate) in order to be able to reconstruct the original perfectly from the sampled version.

$$\Delta_{lateral} = \frac{r_{lateral}}{2} \qquad \Delta_{axial} = \frac{r_{axial}}{2}$$

- Given the low SNR of light microscopy images, in practice a sampling frequency of about **3x** is recommended.
- Such oversampling is not always practically possible: phototoxicity, photobleaching, …

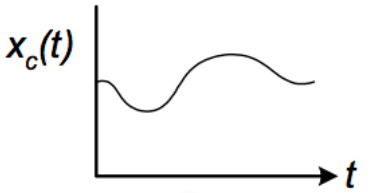
Sampling

- Sampling must generate an **univocal representation** of the original (analog) signal.



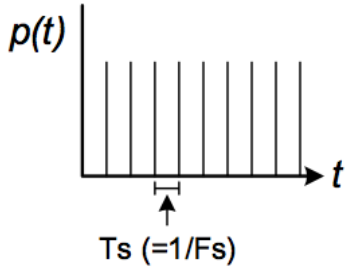
Frequency considerations of **sampling**

Band-limited, continuous signal

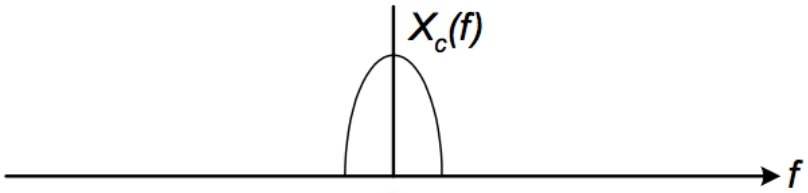


Multiplication

Sampling function (periodic impulse function)

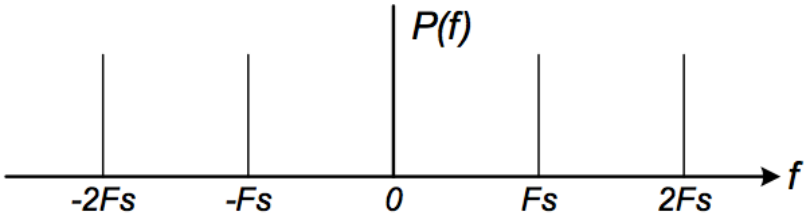


Fourier transform



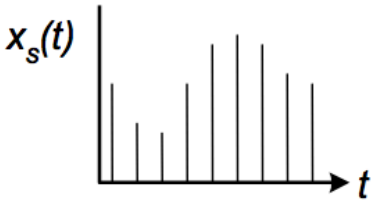
Convolution

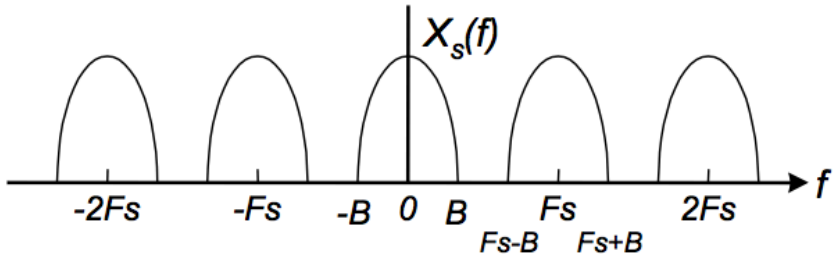
Fourier transform



Fs: sampling frequency

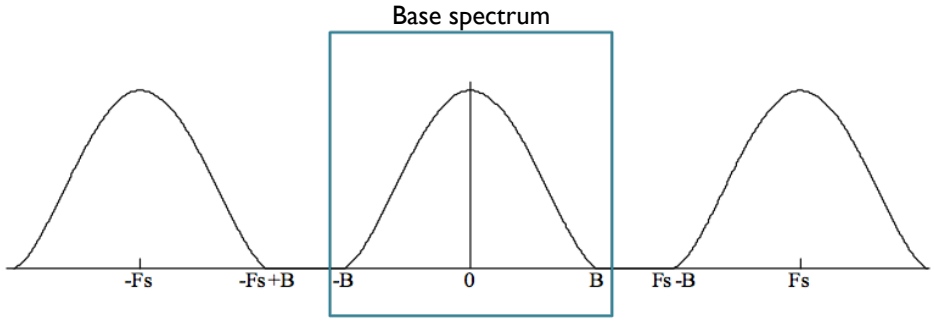
The frequency-domain representation of a sampled signal is the **convolution** of the Fourier transforms of the signal and of the sampling function:



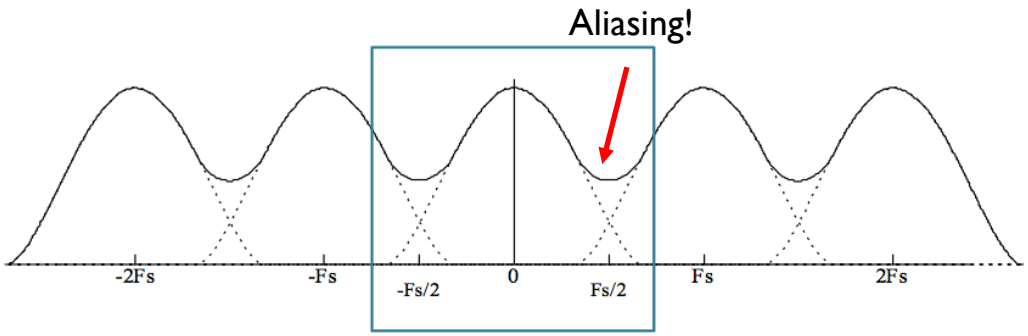


Frequency considerations of **sampling**

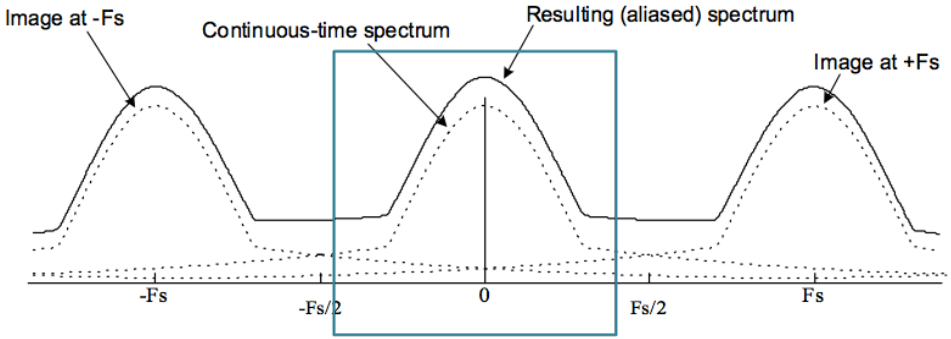
Signal is band-limited and *Fs* > 2B



Signal is band-limited and *Fs* < 2B



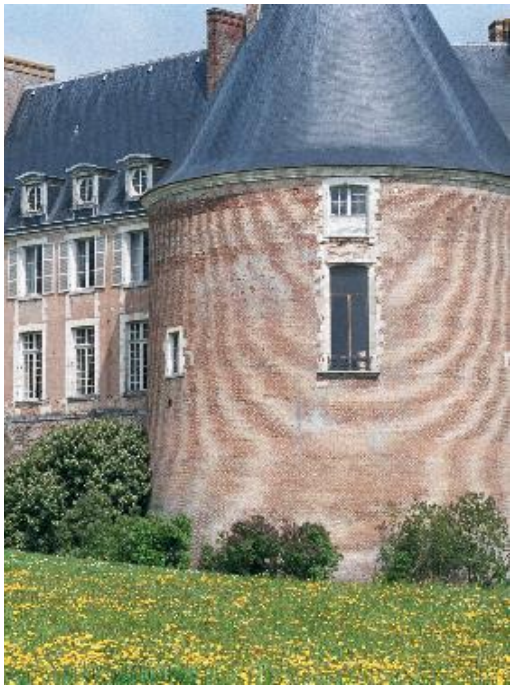
Signal is **not** band-limited



Sampling artifacts

- If the Nyquist criterion is not met, all frequencies above Nyquist will **not** be properly represented.
- More disturbingly, these high frequencies will not simply be missing in the sampled signal, they will *contaminate* the lower frequencies, giving rise to **aliasing** artifacts.

Properly sampled.



Undersampled (and aliased).

Sampling in the microscope

- In a widefield microscope, **lateral sampling** is achieved by the CCD chip and is a function of the CCD pixel size P_s, camera binning b, and objective magnification M:

$$\Delta_{lateral} = \frac{P_s b}{M}$$

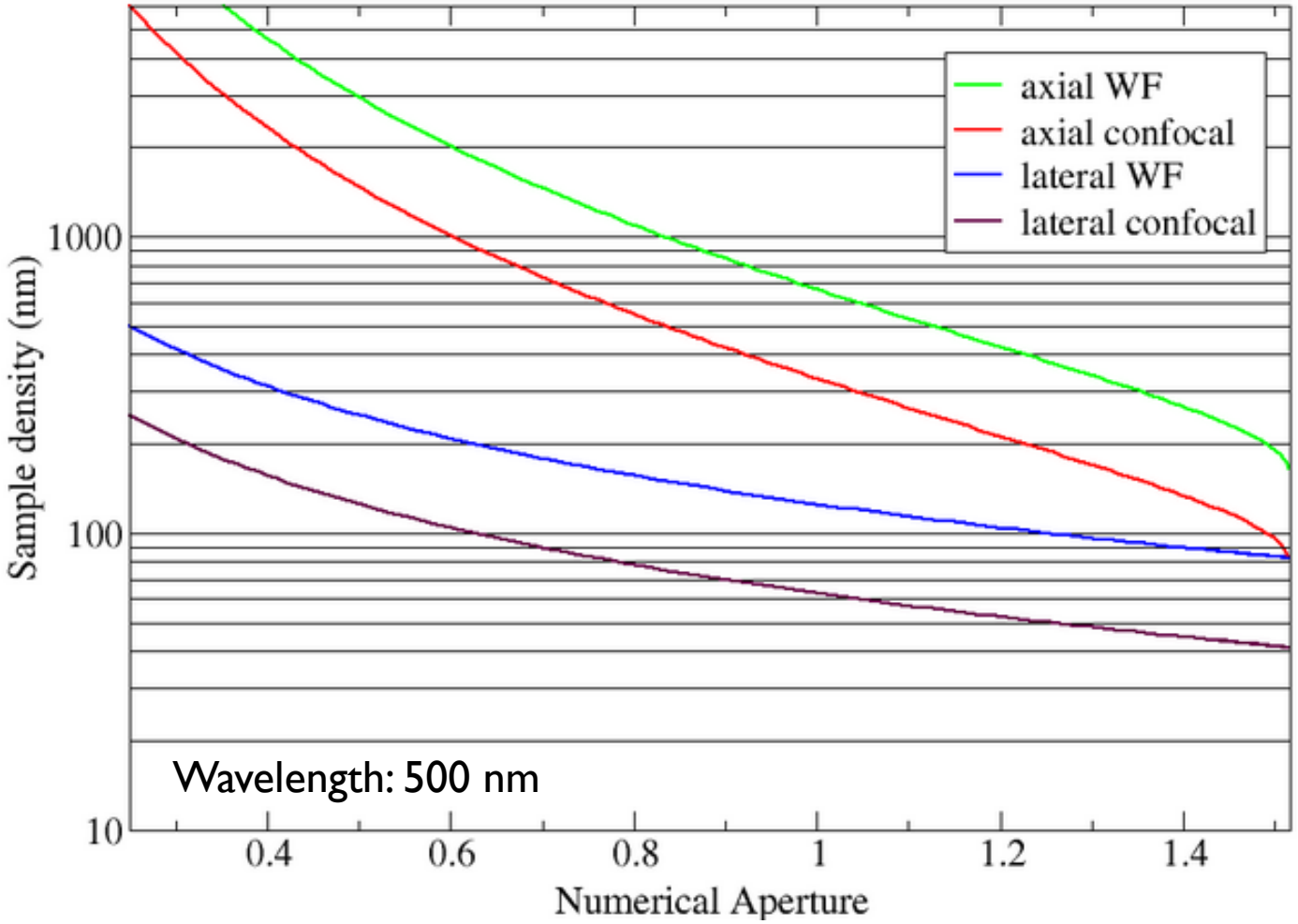
- By incorporating the lateral resolution equation, the lateral sampling criterion can be expressed as:

$$\frac{P_s}{M} \leq \frac{0.61\lambda}{2NAb}$$

- This allows checking the sampling against the Nyquist theorem and ensuring compatibility with deconvolution.
- For a scanning confocal microscope, the **lateral sampling** can be adjusted with the zoom command to satisfy the Nyquist theorem.
- For both widefield and confocal microscope, **axial sampling** must be adjusted by selecting an appropriate plane spacing distance.

Sampling in the microscope

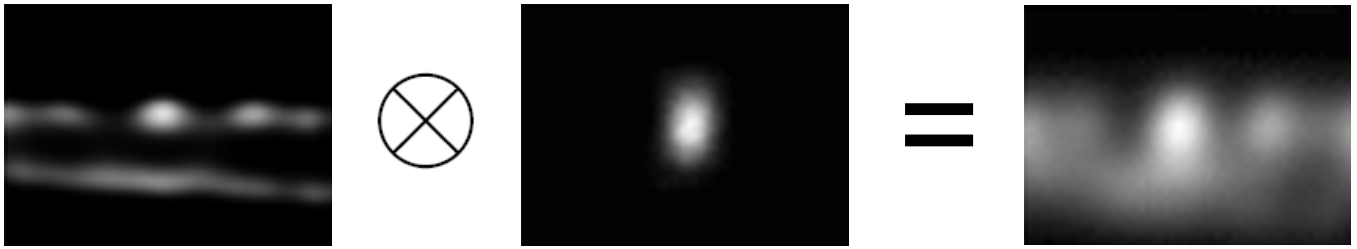
- The optimal sample density is a function of the PSF extension, which is defined by wavelength and Numerical Aperture:



Deconvolution: Intuition

Convolution by the microscope lens

$$g = f \otimes h$$



Object f PSF h Image g

Can we reverse it?

a.k.a. *Can we read a newspaper on Saturn from Earth?*

Convolution theorem

$$\iiint_{-\infty}^{\infty} f(x)\, h(x' - x)\, d^3x$$

$$g = f \otimes h$$

Fourier transform

$$G = F \cdot H$$

Convolution in direct space = multiplication in frequency (Fourier) space

(and vice versa!)

The **Fourier transform**, named after **Joseph Fourier**, is a mathematical transform that decomposes any function into a sum of (complex) sinusoidal basis functions of different frequencies.

The Fourier transform is also a reversible operation: thus, given the frequency representation *F*, one can determine the original function, *f*.

The convolution theorem states that a convolution of two functions in real space is the same as a the inverse transform of the multiplication of their Fourier transforms.

Inverse filtering

$$\iiint_{-\infty}^{\infty} f(x)\, h(x' - x)\, d^3x$$

$$g = f \otimes h$$

Fourier transform

$$G = F \cdot H$$

Convolution in direct space = multiplication in frequency (Fourier) space

(and vice versa!)

"Deconvolution"

$$f \longleftarrow F = G/H$$

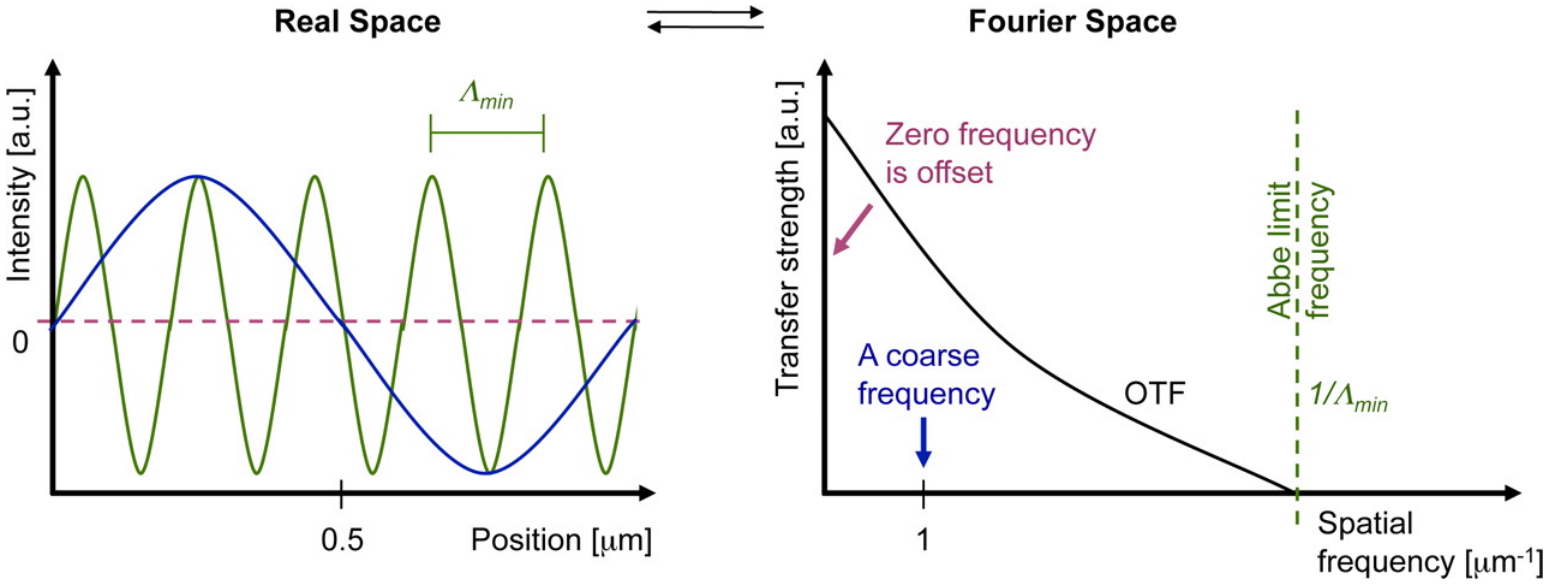
Inverse filtering

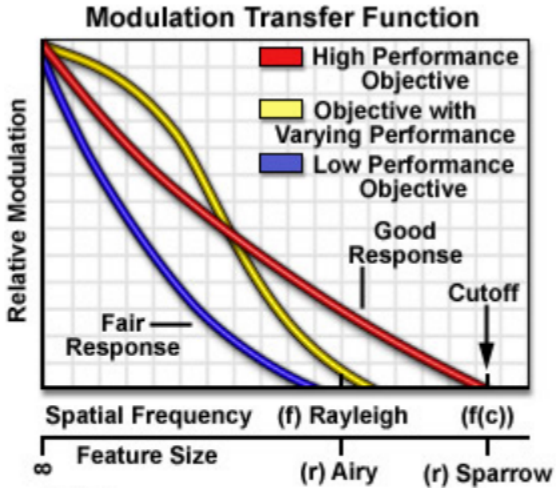
Inverse Fourier transform

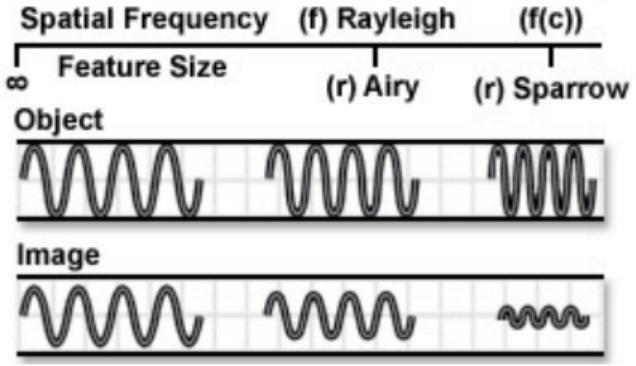
Can we do this?

Modulation Transfer Function

$$\Lambda_{min} = \frac{\lambda}{2n \sin(\alpha)} = \frac{\lambda}{2NA}$$





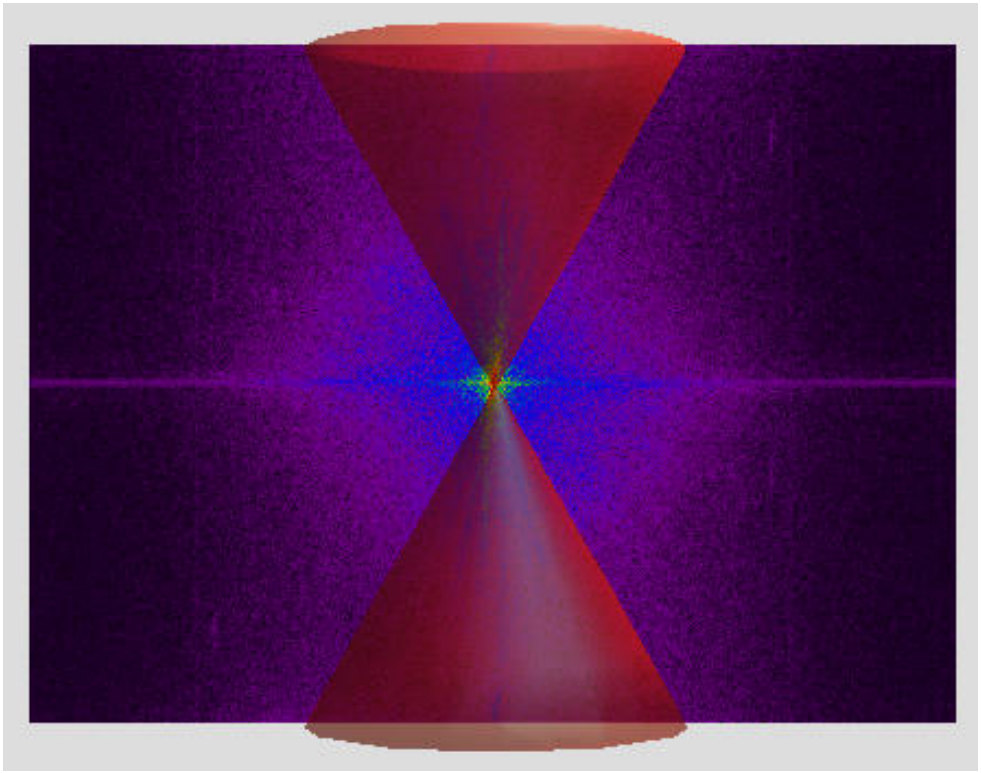


Cookie cutter

- All frequencies above the Abbe limit are cut off. In a widefield microscope, this is particularly obvious in the axial direction.

Cone of missing frequencies

XZ



The Fourier transform of the PSF of a widefield microscope has cone of missing frequencies along the z axis.

Problems of inverse filtering

$$g = f \otimes h + n$$

$$F = \frac{G - N}{H}$$

Artifacts!

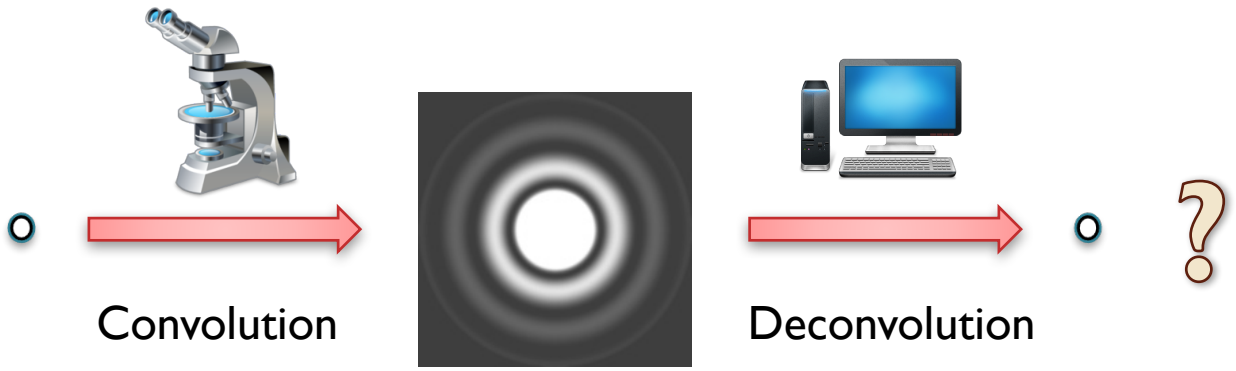
Extreme noise amplification!

H = 0 (or very small) at many places!

- Inverse filtering will never allow us to recover the true object function *f*.

Deconvolution

- Deconvolution is an **algorithmic** inversion method for **restoring** an image distorted during the image formation process, based on **prior knowledge of the degradation phenomenon**.



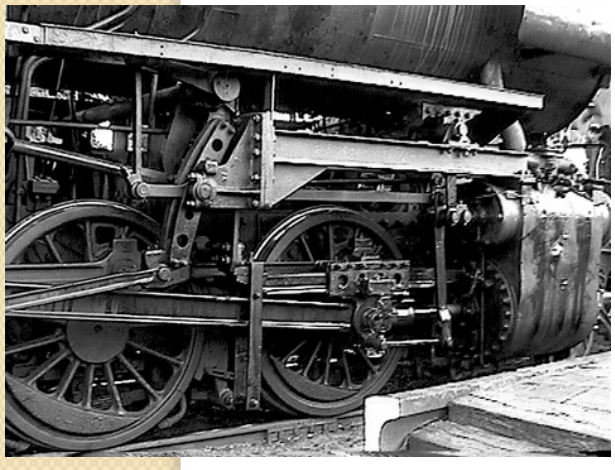
- In microscopy, the goal is to **reassign the optical blur to its original position and to reduce (statistical) noise**.
- Deconvolution belongs to the *ill-posed* problem category and does not generate a single solution: the result is an *estimate* of the object that is closer to the observed object than the acquired image.

Deconvolution algorithms

- Since inverse filtering is not applicable, several extensions and alternative approaches have been suggested (incomplete list):
  - **Nearest neighbors**
    - subtracts from each plane a weighted average of the nearest planes – not really a deconvolution...
  - **No neighbors**
    - subtracts from each plane a blurred version of itself – not really a deconvolution...
  - **Wiener filter**
    - an inverse filter that tries to minimize the impact of deconvolved noise at frequencies which have a poor SNR
  - **Constrained iterative algorithms**
    - iteratively refines an estimate of the object by convolving it with the known PSF and comparing it with the acquired image; the weighted (by a relaxation factor) difference is added to the estimate as guess for the next round; it is an iterative inverse filter.
      Examples: **Jansson Van-Cittert Algorithm**, **Gold Algorithm**
  - **Blind deconvolution**
    - an iterative constrained algorithm that tries to estimate both the original object and the PSF simultaneously from the degraded image
  - **Maximum likelihood estimation algorithms (statistical algorithms)**
    - iteratively optimizes the likelihood of an estimate of the object given the measured image and the PSF; the noise should be Poisson-distributed
    - some versions use a regularization function to avoid convergence problems and increase speed
  - **Wavelet-based deconvolution**

http://www2.ujf-grenoble.fr/medecine/iab/clientzone/plforme9/fichiers/DeconvolutionMicroscopy_Sibarita_Springer.pdf

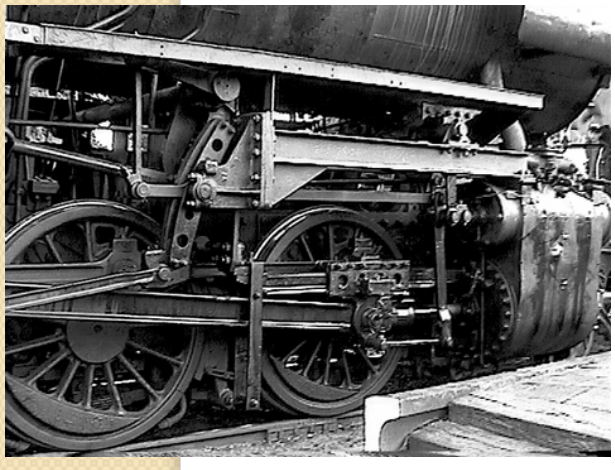
Deconvolving trains



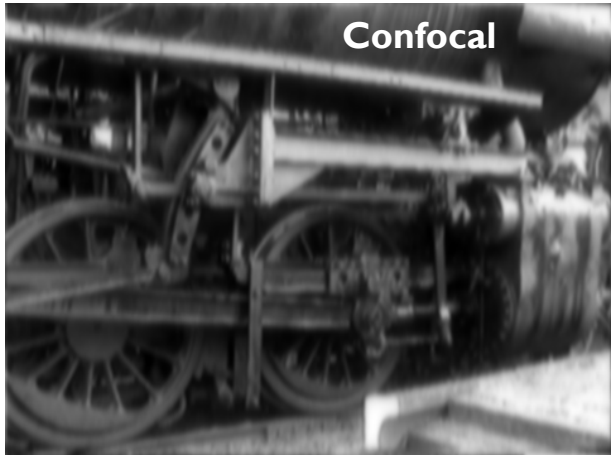
"Sub-resolution train"

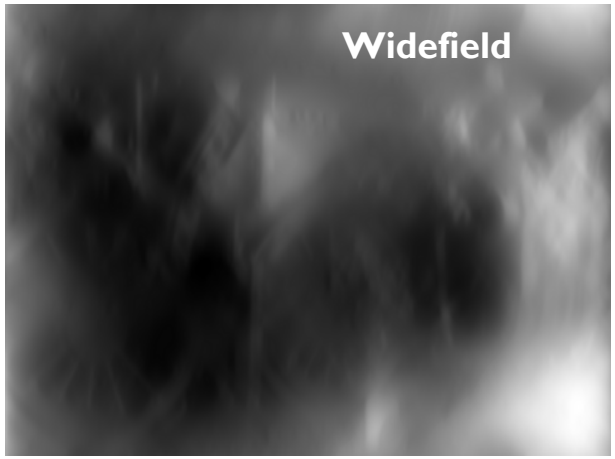
Noise-free convolution and deconvolution

Deconvolving trains

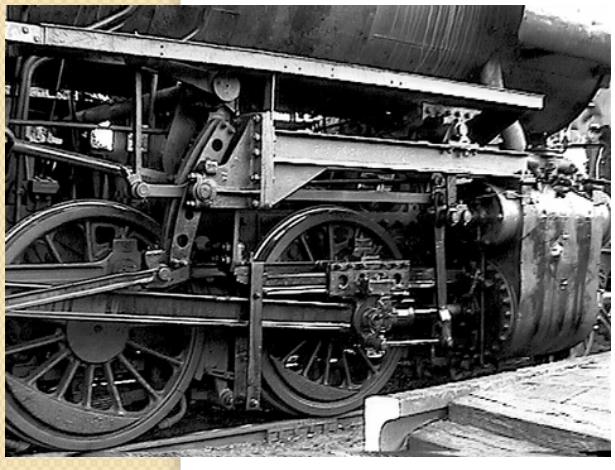


"Sub-resolution train"
Noise-free convolution and deconvolution

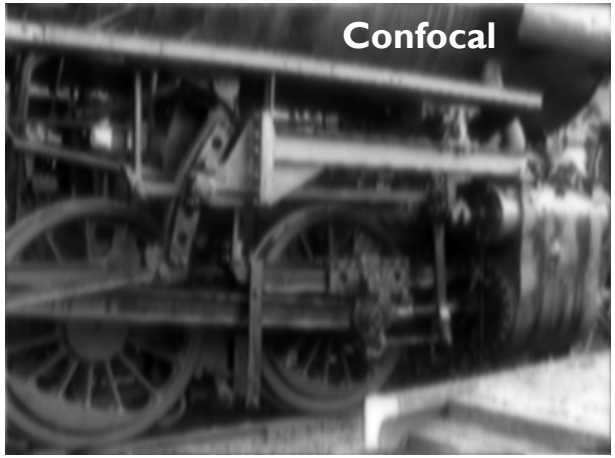


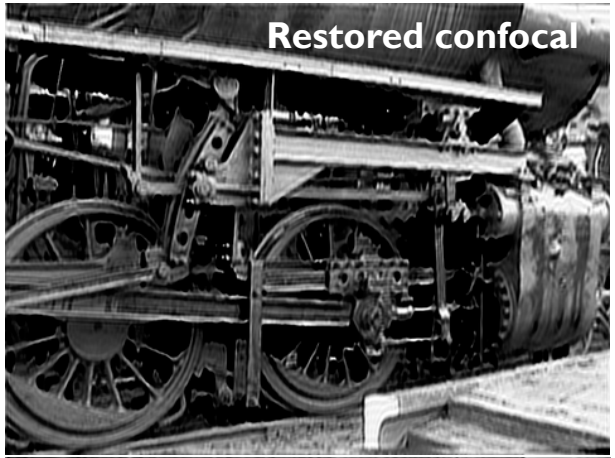


Deconvolving trains



"Sub-resolution train"
Noise-free convolution and deconvolution





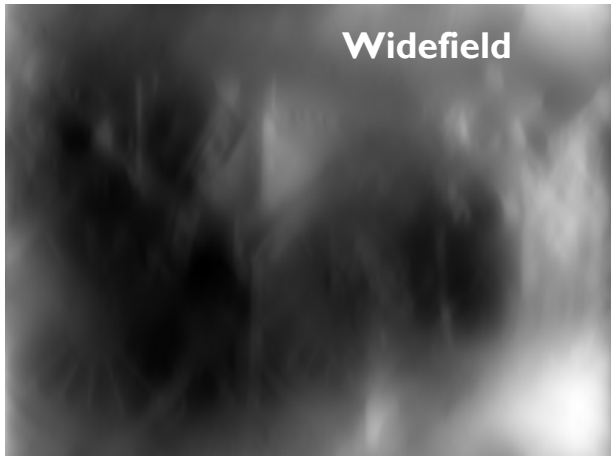


Image formation in a real objective with aberrations

- The ideal model assumes that image formation is governed by a **linear**, **shift-invariant** system:
  - this implies that a **single** 3D PSF is enough to describe completely image formation throughout the 3D object space
  - however, most frequently, shift invariance does **not** apply in axial direction
- Axial shift variance results from **refractive index mismatches**
  - between the sample medium and the objective-embedding medium (can be modeled)
  - due to the emitted light passing through biological matter that has locally different refractive index (**scattering**, cannot be modeled)
  - these mismatches give rise to **spherical aberrations**
- In general, only the immediate proximity to the coverslip will give an aberration-free image (up to 10-15 μm, depending on the sample)
- Therefore, the real PSF will most likely be different from a theoretical one as one moves away from the transition between the two media, and this divergence will increase the more one moves the focus into the specimen.

Image formation in a real objective with aberrations

Example: confocal microscopy, 1.3 oil objective (n = 1.518) with watery medium (n = 1.33), 520-nm wavelength

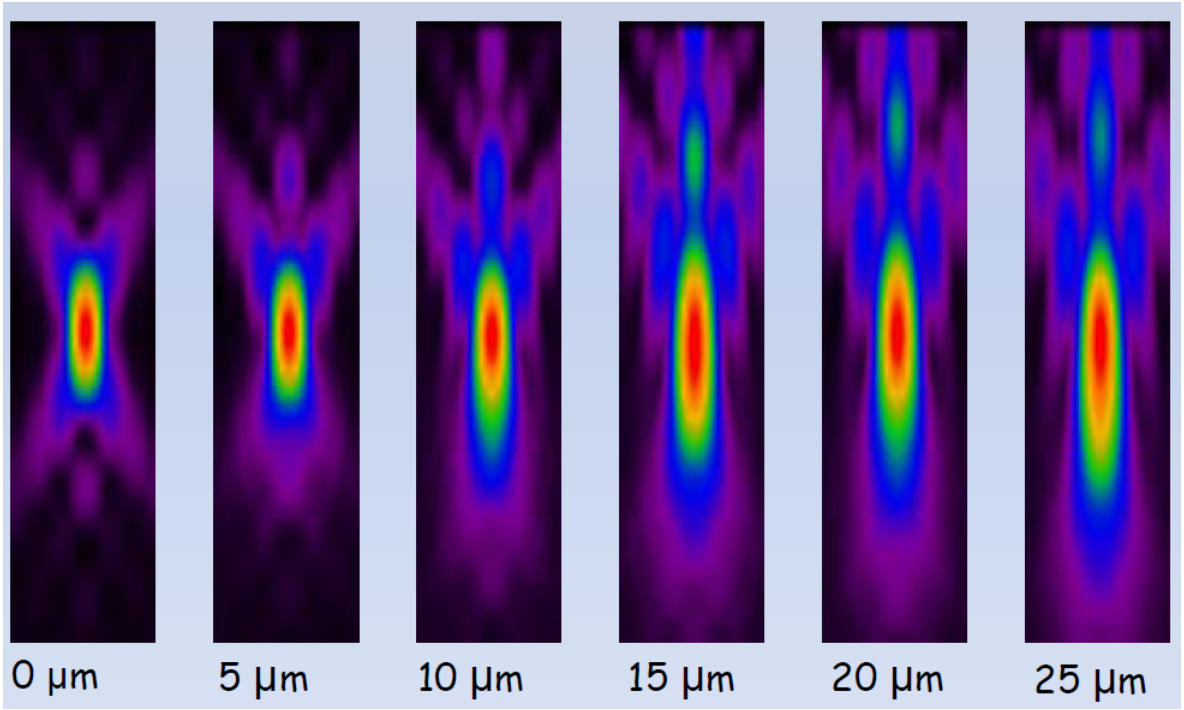
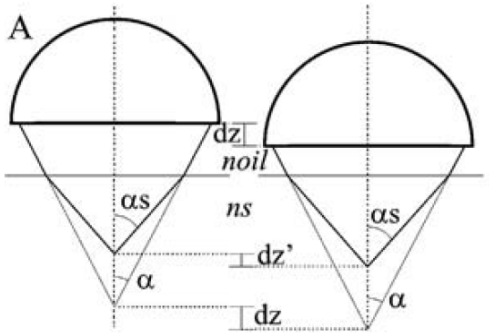
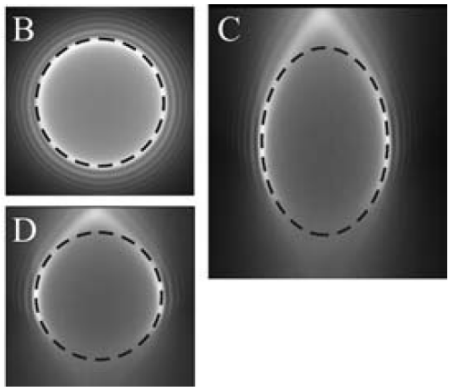


Image formation in a real objective with aberrations

- Refractive index mismatch also gives rise to **geometrical aberration**:

"Fish tank effect"





A Axial distance mismatch due to the presence of layers with different refractive indices along the optical path. **B** Axial section of the bead. **C, D** Observations of a 6μm fluorescent calibration bead without (C) and with (D) distance calibration.

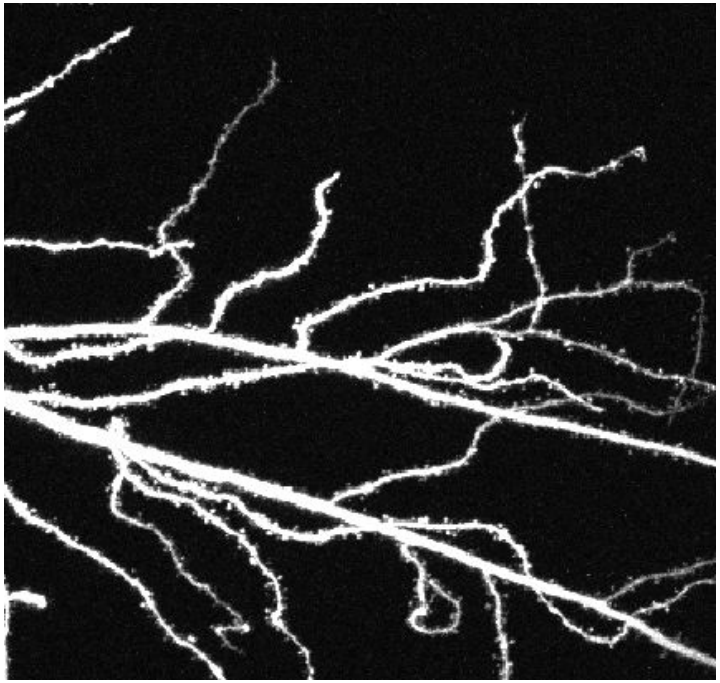
- If we ignore the difference between the refractive indices of the immersion oil and the sample, the displacement error can be expressed as:

$$dz' = \frac{n_{smp}}{n_{obj}} dz$$

dz	physical movement of the objective or stage
n_{smp}	refractive index of the sample medium
n_{obj}	refractive index of the objective-embedding medium
dz'	movement of the focal plane

Clipping or saturation

Clipping during acquisition occurs when the dynamic range of the input signal exceeds that of the analog-to-digital converter (ADC), i.e. when you are *saturating* your detector.



This is an example of a clipped image, where all the inner regions in the objects of interest are saturated to a constant maximum value, and the internal structure can no longer be observed.

Apical dendritic tree of a CA1 pyramidal neuron in an acute hippocampal slice. Image courtesy of Dr. T.M. Hoogland, Dept. of Neuroscience, Baylor College of Medicine, Houston.

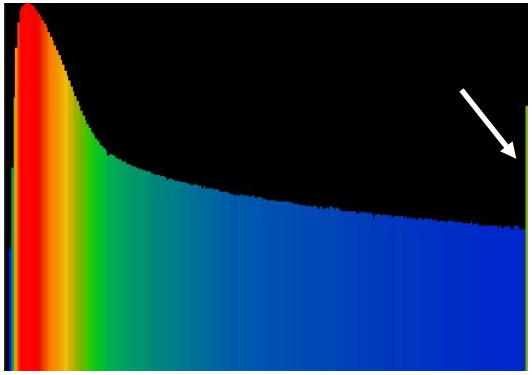
?

It is a bit like trying to recognize this person from this picture…

Yes, it is Indiana Jones.

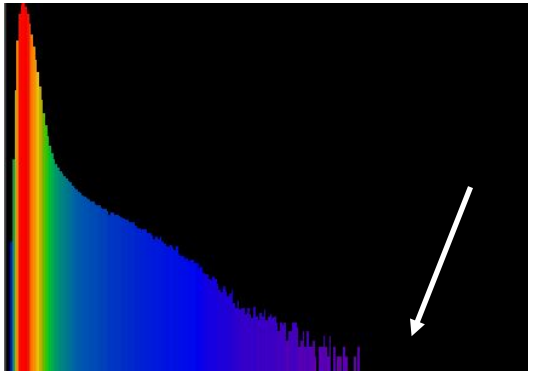
Clipping or saturation

The image histogram is a good indicator of whether the signal is likely to be saturated or not.



From the shape of the histogram it looks likely than many pixels would have had an intensity higher than allowed by the detector.

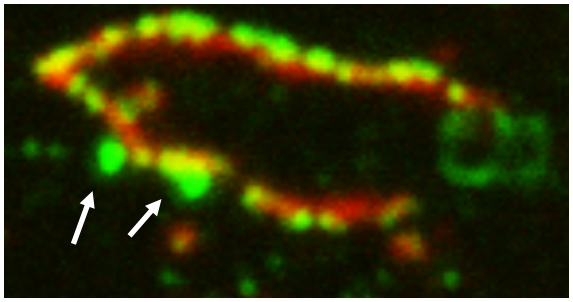
vs.

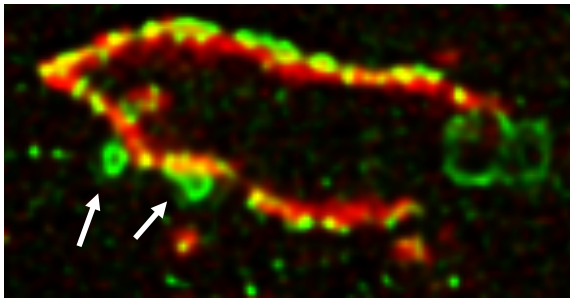


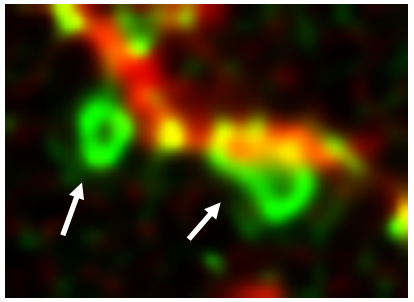
This image is clearly not saturated.

Example: artifacts of deconvolution of saturated signal

The (2D) restoration of the saturated regions of the green channel (arrows) results in hollow regions being created. These are very probably just an artifact resulting from deconvolving a clipped region.







(Detail) Golgi apparatus of a goblet cell (the mucus secreting epithelial cells in intestine). Images courtesy of Dr. J.A. Valentijn, Molecular Cell Biology Dept., Leiden University Medical Center.

Determination of the PSF

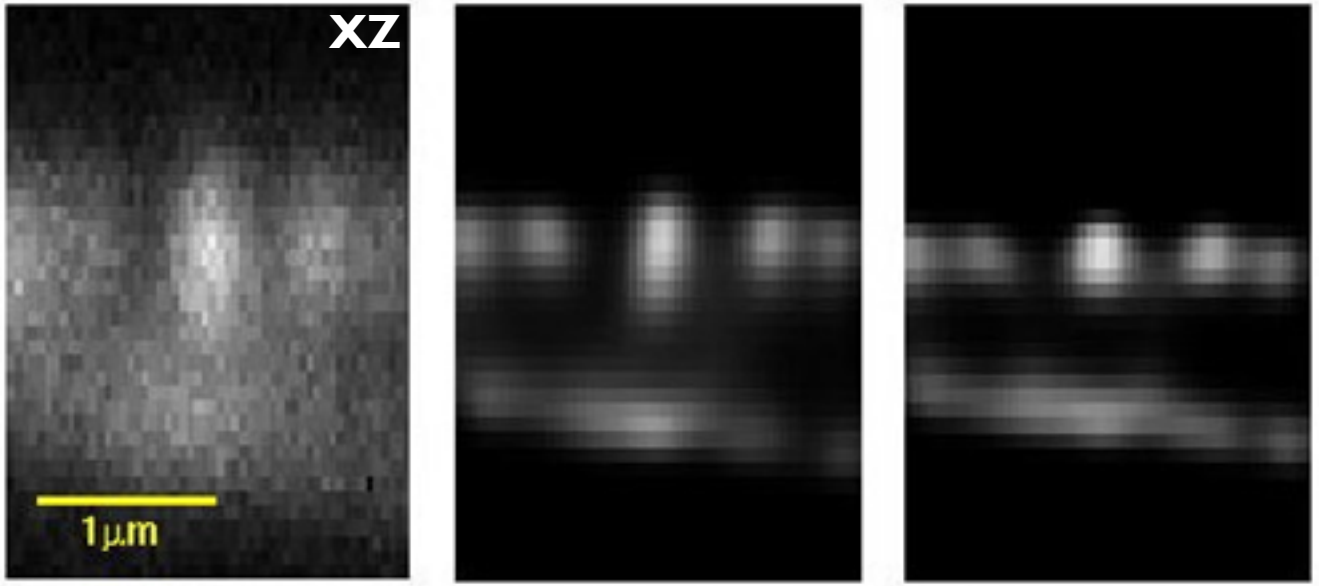
- The accuracy and quality of the PSF are essential to ensure the correct performance of any deconvolution algorithm.
  - Noise, incorrect estimates of aberrations and incorrect scaling of the PSF may cause major artifacts in the restored image.
- The PSF may be **calculated theoretically** or **measured empirically**.

- Theoretical PSFs:
  - are easy to calculate, simplify the deconvolution process by avoiding the need for time-consuming PSF extraction, are noise free;
  - they apply only to perfect lenses and well defined and calibrated optical paths (not found in practice), but *can* model simple aberrations (like spherical/geometrical aberration at different z levels due to refractive index mismatch).

- Empirical PSFs:
  - are a more faithful representation of the real image formation model and can be measured in a simple manner, involving the **acquisition** and **averaging** of **images of sub-resolution fluorescent beads**. From these bead images, the PSF of the system can be **extracted**;
  - *could* be obtained at different z levels to quantify spherical aberration due to refractive index mismatch, since it is more difficult to model the way they get deformed by axial distance.

Determination of the PSF

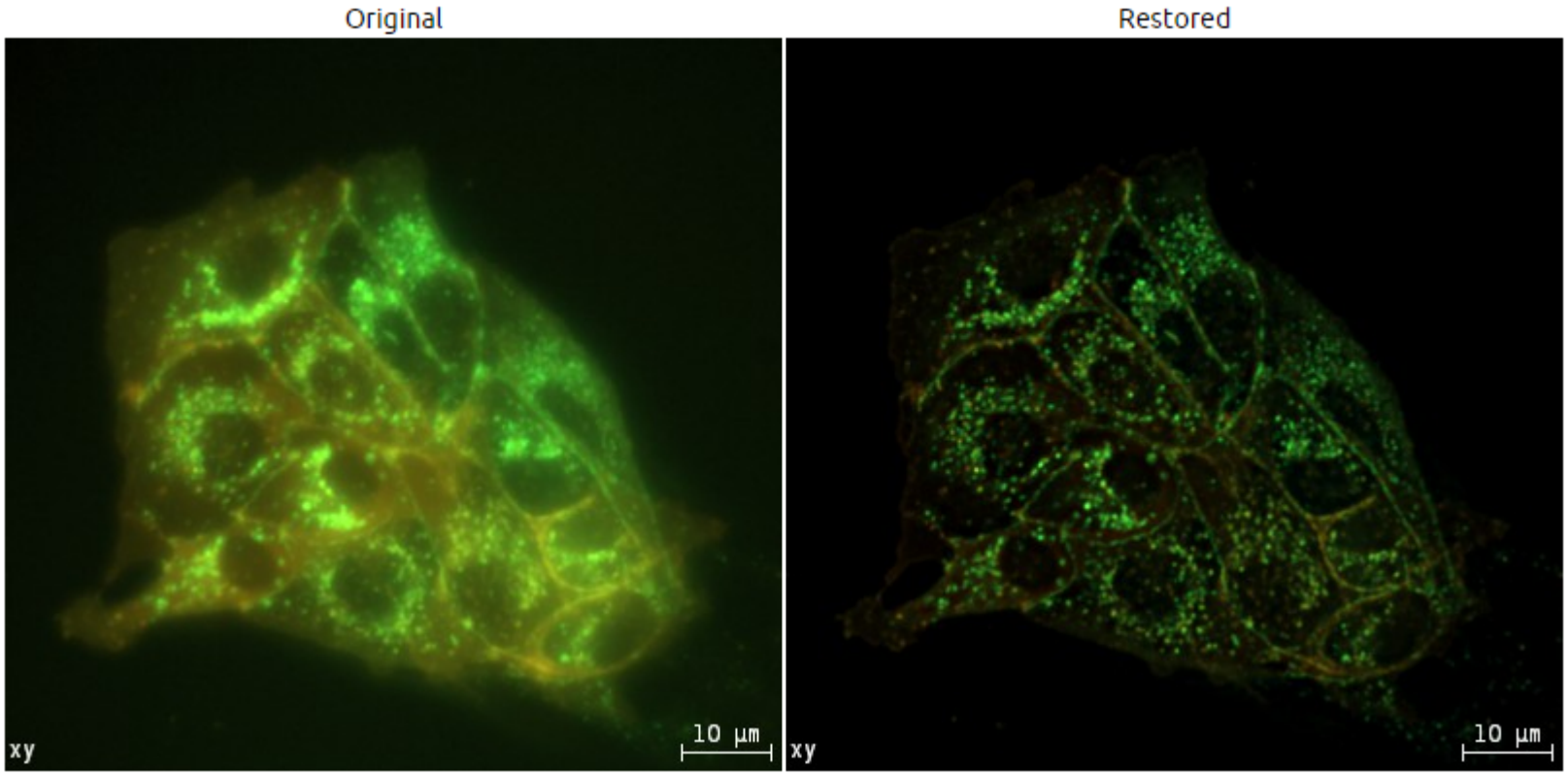
Example: Restoration of actin filaments acquired on a 2-photon microscope, with theoretical and empirical PSF.



Measured data | Restored, theor. PSF | Restored, meas. PSF

- The Huygens software:
  - Increases resolution (especially in the axial direction)
  - Removes noise
  - Subtracts the backgound
  - Increases contrast
  - Corrects for spherical and geometrical aberrations by adapting the PSF as a function of the axial distance from the coverslip and the refractive indices used
  - Corrects for bleaching and illumination instability
- It uses one of the following algorithms:
  - Classic maximum likelihood estimation (good for almost any type of microscopy image, and well-suited for low-signal images and to restore point-, line-, and plane-like features)
  - Quick maximum likelihood estimation (much faster than classic and with *almost* the same quality – optimal for time series)
  - Iterative constrained Tikhonov-Miller (fast and particularly good for low-noise wide field images)
  - Quick Tikhonov Miller (is an inverse filtering method that can give noise amplification; used in very specific circumstances only)
- Uses either theoretical or measured PSFs
- Offers a tool to extract (*distill*) PSFs from sub-resolution bead images.

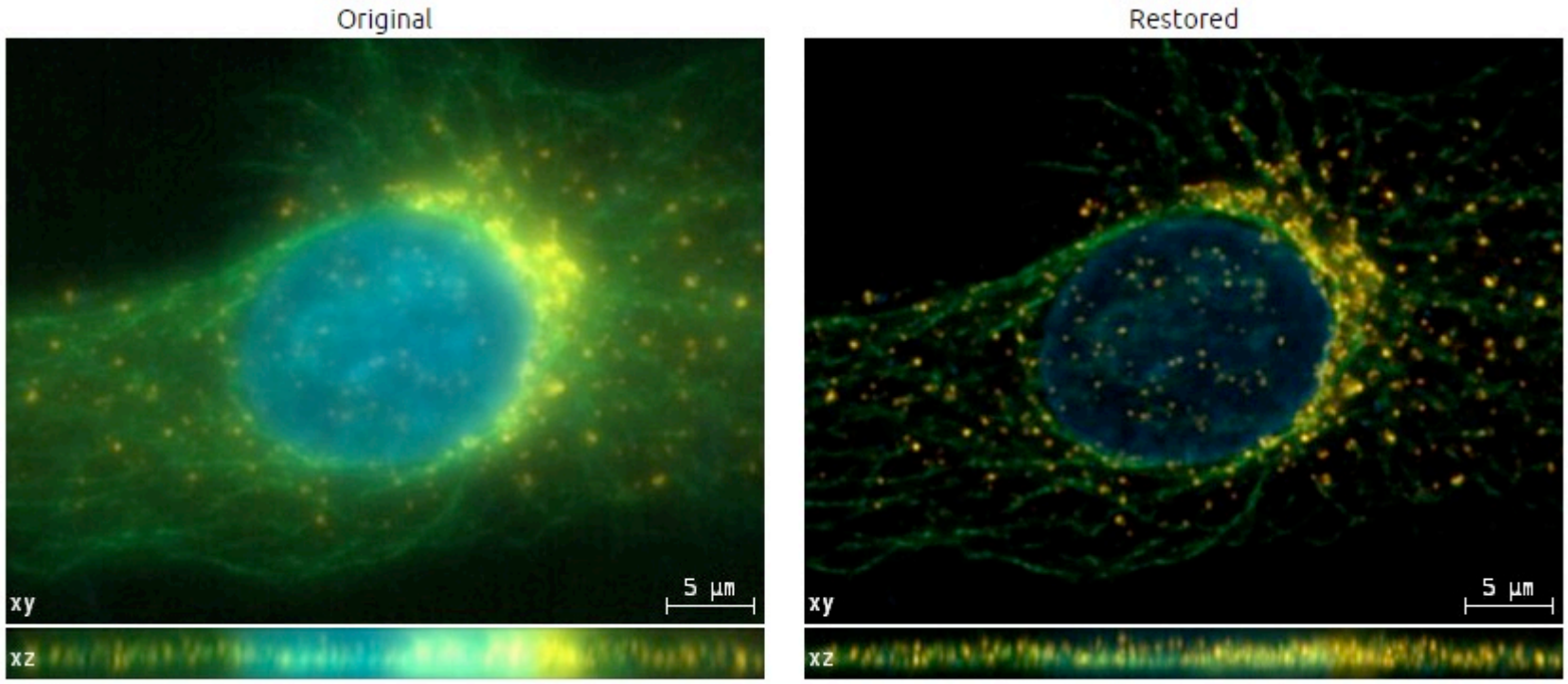
Example



Deconvolution of cell-cell junctions of MDCK cells. MDCK (Madin-Darby Canine Kidney) cells cultured for 3 days, were stained for p120-catenin (mCherry - red) and Claudin3 (EGFP-green), and imaged with a Nikon Ti widefield microscope (Objective 40x; 1.3 NA oil lens). Shown are a single slice of the original z-stack either background-corrected (left) or deconvolved with Huygens (right).

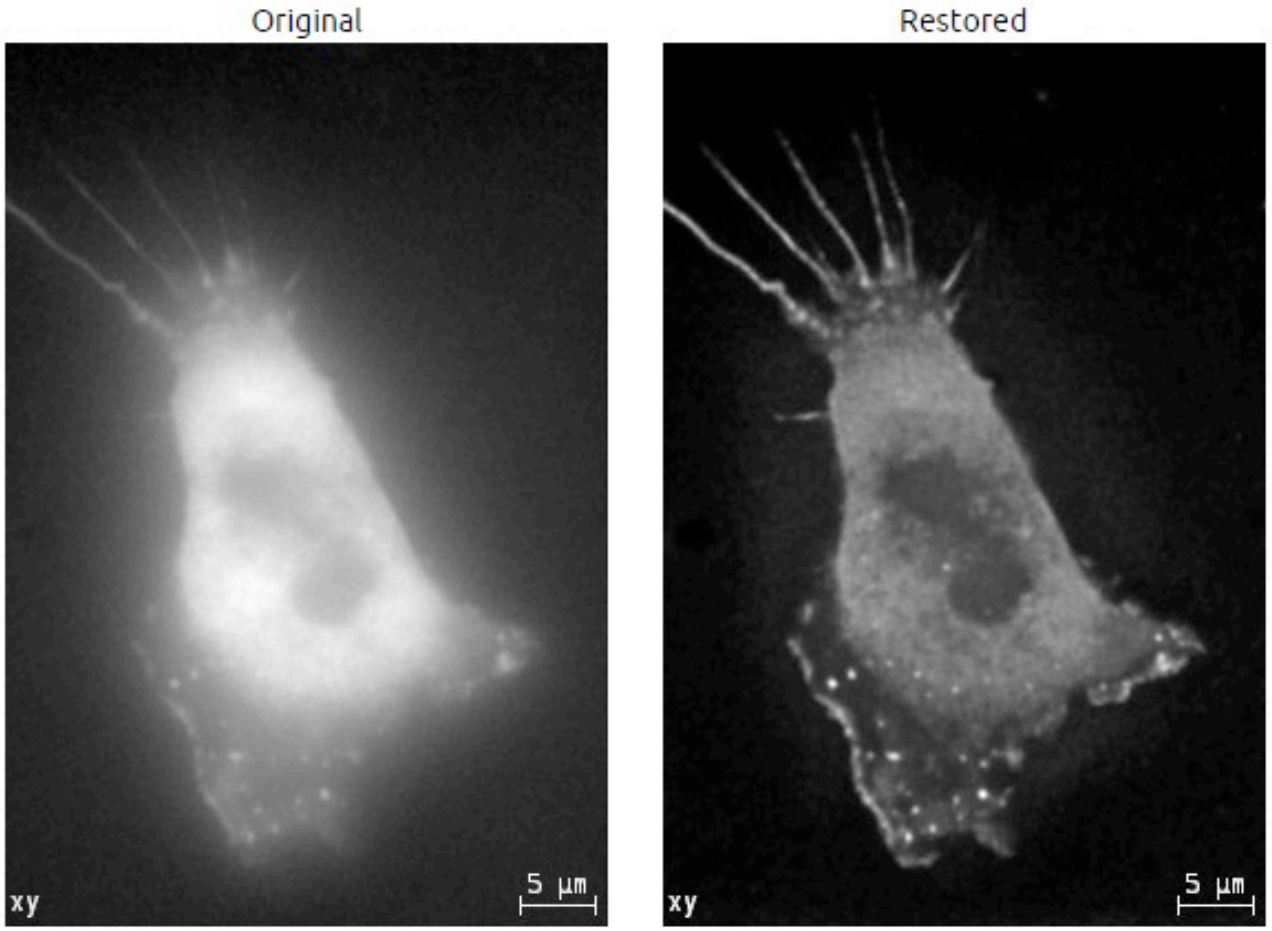
Image acquired by Dr. Johan de Rooij, Hubrecht Institute, Utrecht, The Netherlands (source: http://www.svi.nl)

Example



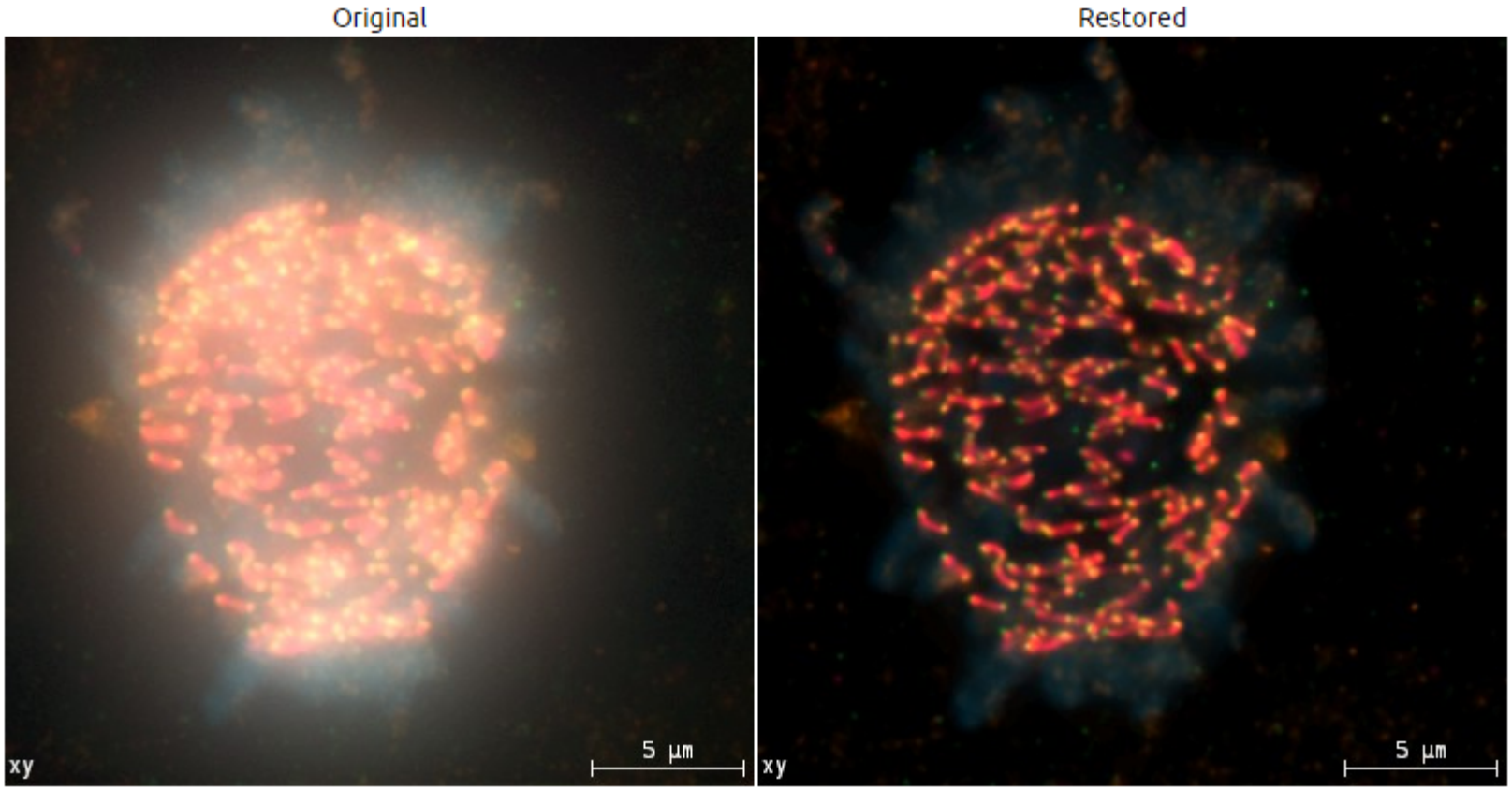
Deconvolution of an HeLa cell acquired on a widefield microscope.
Image courtesy Dr. Yury Belyaev. EMBL, Heidelberg, Germany.

Example



Widefield image of migrating cell.
Source: https://svi.nl/DemoImages

Example



Deconvolution of U2OS human osteosarcoma cell line.
Image from Dr. Livio Kleij (facility) and Dr. Martijn Vromans, Medical Oncology, UMC Utrecht, The Netherlands.
Microscope: Deltavision microscope (widefield). Channels: Ch0: DAPI (DNA); Ch1: Mis12_GFP (kinetochore complex component); Ch2: pDsn1-Alexa 568 (kinetochore complex component); Ch3: CREST-Alexa647.

http://huygens-rm.org