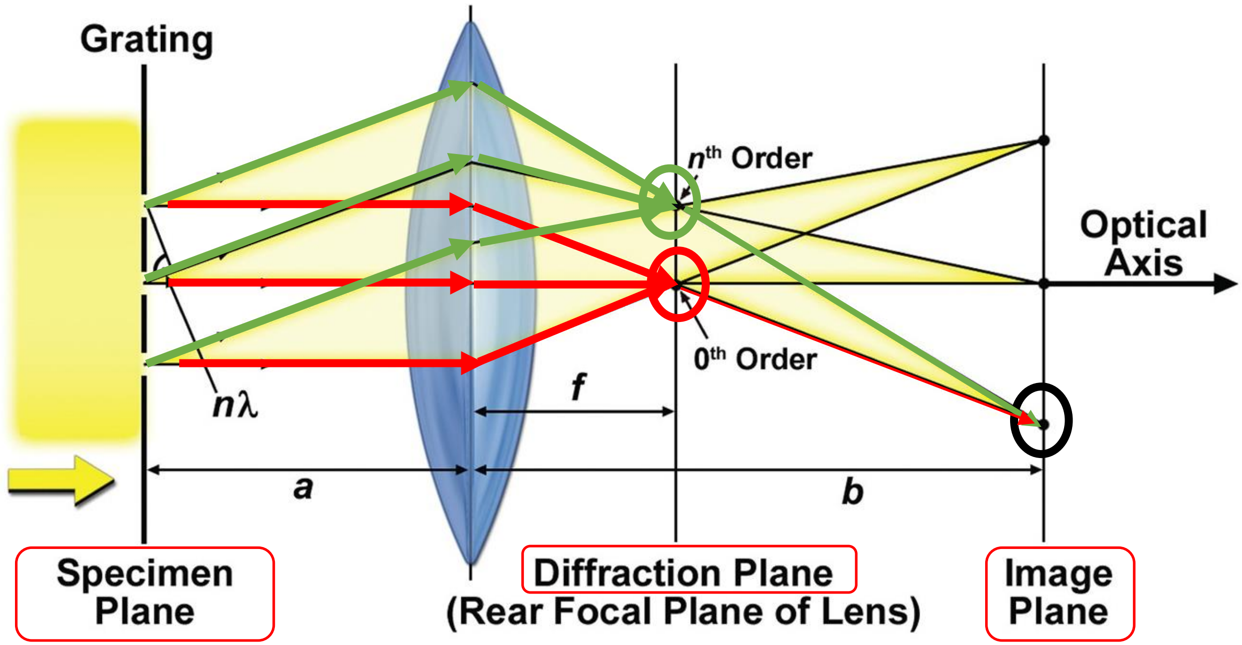
MICRO-561

Biomicroscopy I

Syllabus (tentative)

Lecture 1	Introduction & Ray Optics-1
Lecture 2	Ray Optics-2 & Matrix Optics-1
Lecture 3	Matrix Optics-2
Lecture 4	Matrix Optics-3 & Microscopy Design-1
Lecture 5	Microscopy Design-2
Lecture 6	Microscopy Design-3 & Resolution -1
Lecture 7	Resolution-2
Lecture 8	Resolution-3
Lecture 9	Resolution-4, Contrast-1
Lecture 10	Contrast-2, Fluorescence-1
Lecture 11	Fluorescence-2
Lecture 12	Sources, Filters
Lecture 13	Detectors
Lecture 14	Bio-application Examples

Reminder: Fourier (a.k.a Diffraction) Plane



- Let's assume that our object is a grating, and it is placed on specimen plane, which is located between f & 2f (2f>a>f).

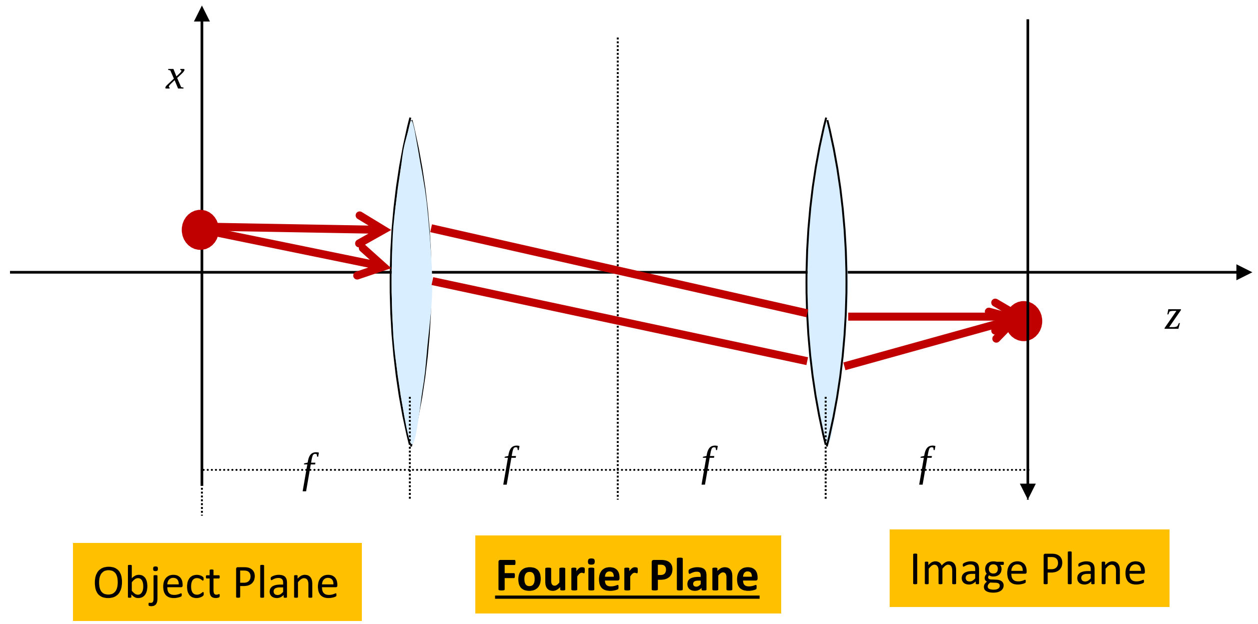
- The objective lens produces a magnified real image of the grating (i.e. object) in the image plane.

- **The Fourier (or diffraction) plane is located at the back focal plane of the lens.**

- The rays of different orders are separated at the *diffraction plane*

- But, the rays of different orders are merged (interfere) at the *image plane*

“4-f” Imaging System



- Unity magnification (inverted)

  - Two cascaded Fourier transforming sub-systems (2-lenses with 4f setting) :
    1st lens performs F.T. & 2nd lens performs I.F.T.

Object plane Fourier plane

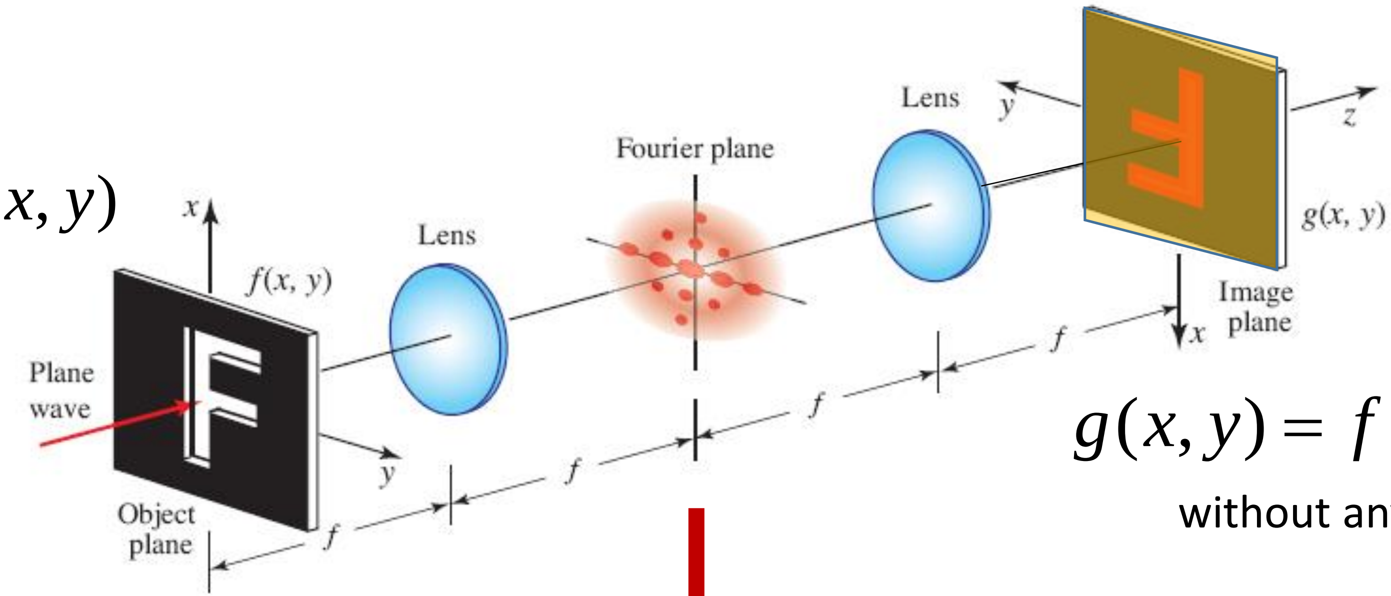
$$(x, y) \rightarrow (\upsilon_x, \upsilon_y)$$

Fourier plane Image plane

$$(\upsilon_x, \upsilon_y) \rightarrow (-x, -y)$$

4-f Imaging System

$f(x,y)$



$$g(x,y) = f(-x,-y)$$

without any filter

All of the "spatial frequencies" of the object are separated here (so called "Fourier plane")

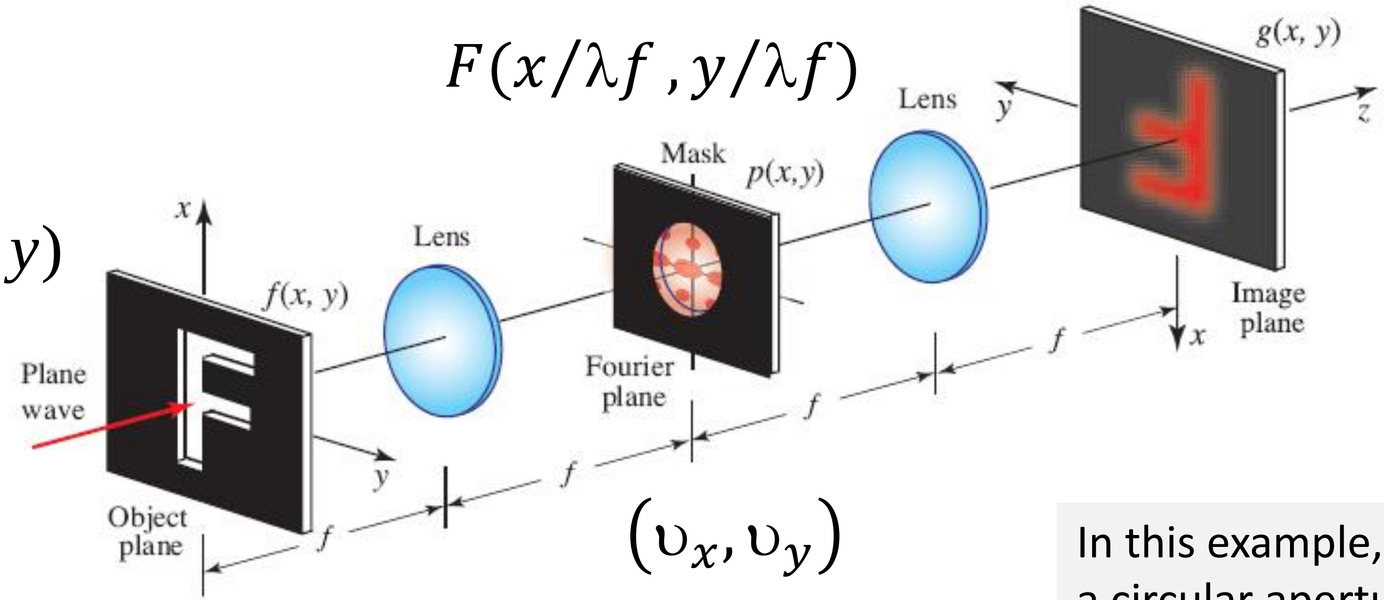
Let's insert a spatial filter in the F.P:

This will lead to generation of a new image **g(x,y)**, which is the **filtered version of f(x,y)**

Spatial Filtering

$F(x/\lambda f\,, y/\lambda f)$

$f\,(x, y)$



(υ_x, υ_y)

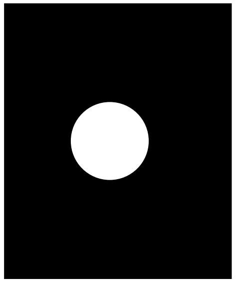
(x, y)

In this example, the filter is a circular aperture

υ_x is the spatial frequency ➔ $\upsilon_x = x/(\lambda f)$ Its unit is [1/length]

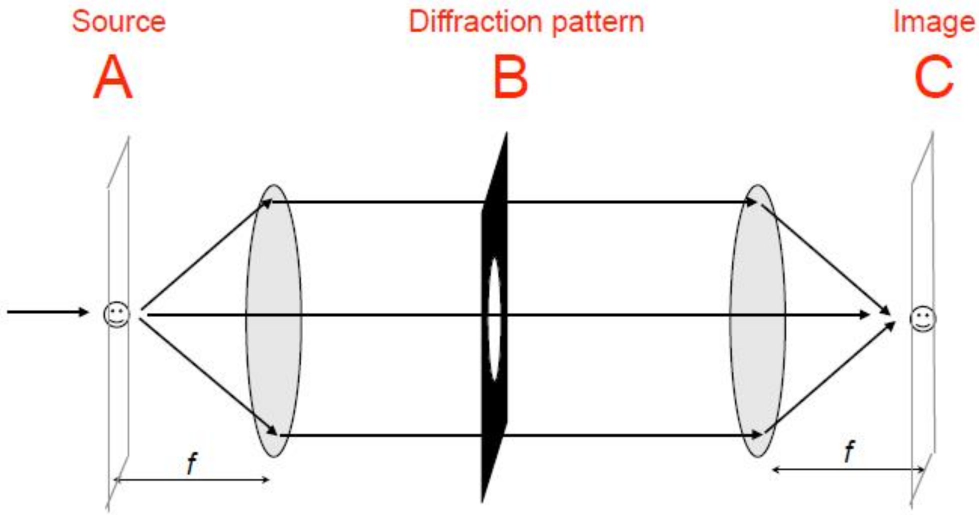
Low-pass filter

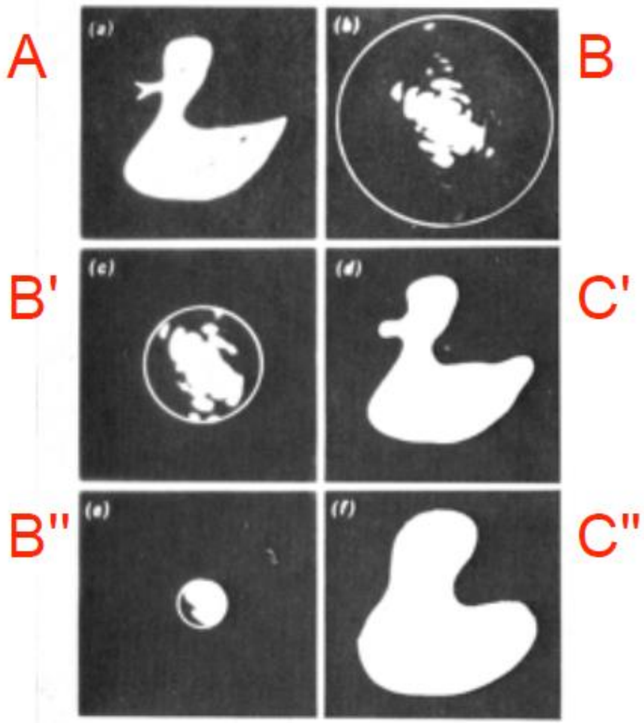
Max Planck



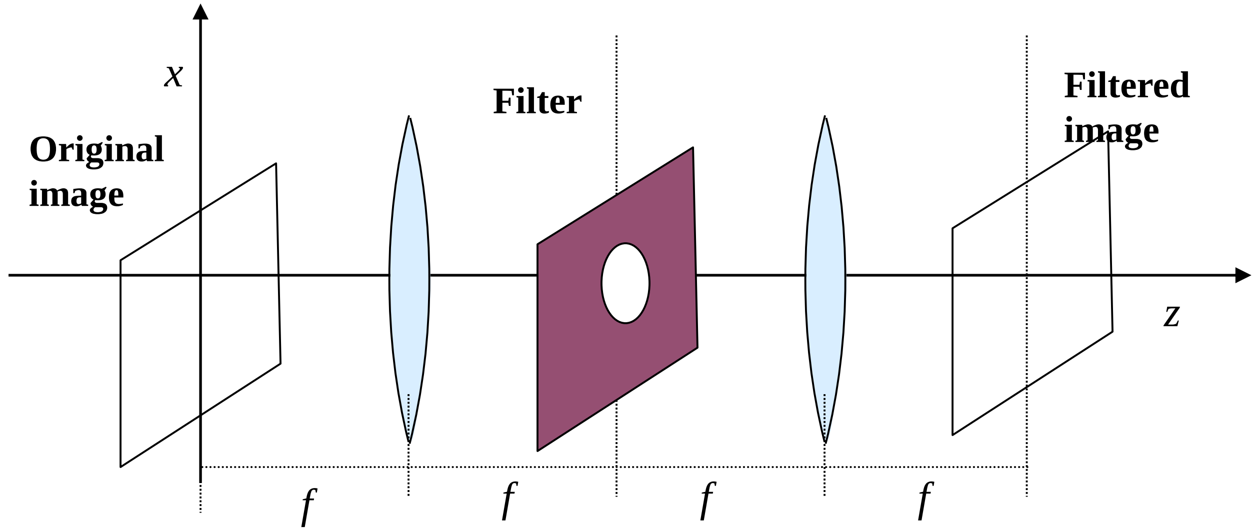
Removing higher diffracted orders in the Fourier Plane reduces the resolution of the final image

A mask is inserted at the Fourier (diffraction) plane to allow only the 0^{th} order and lower order light to pass



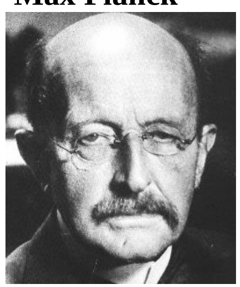
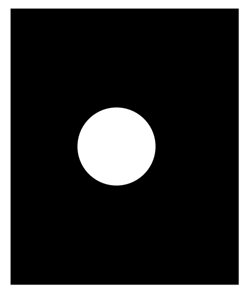


Examples of Spatial Filters

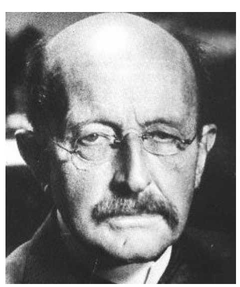
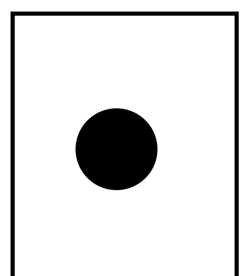
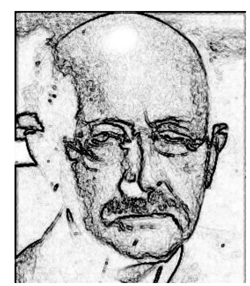


Max Planck

Low-pass filter


High-pass filter

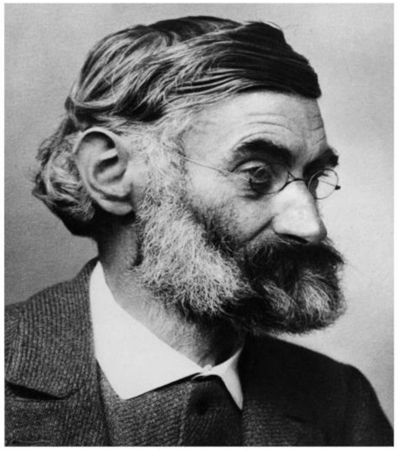
Determining **microscopy resolution** by 2 methods

1. **Abbe's theory of image formation**

→ Collecting more **diffraction orders** for higher resolution

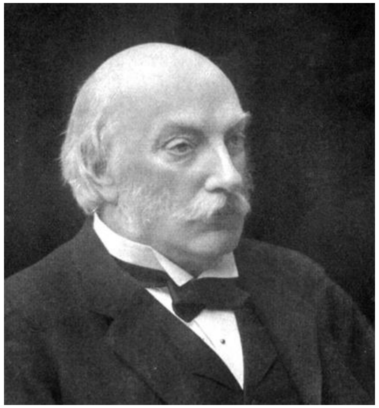
2. **Rayleigh criteria**

→ Diffraction of a circular aperture & Airy disks



Ernst Abbe [1840-1905]

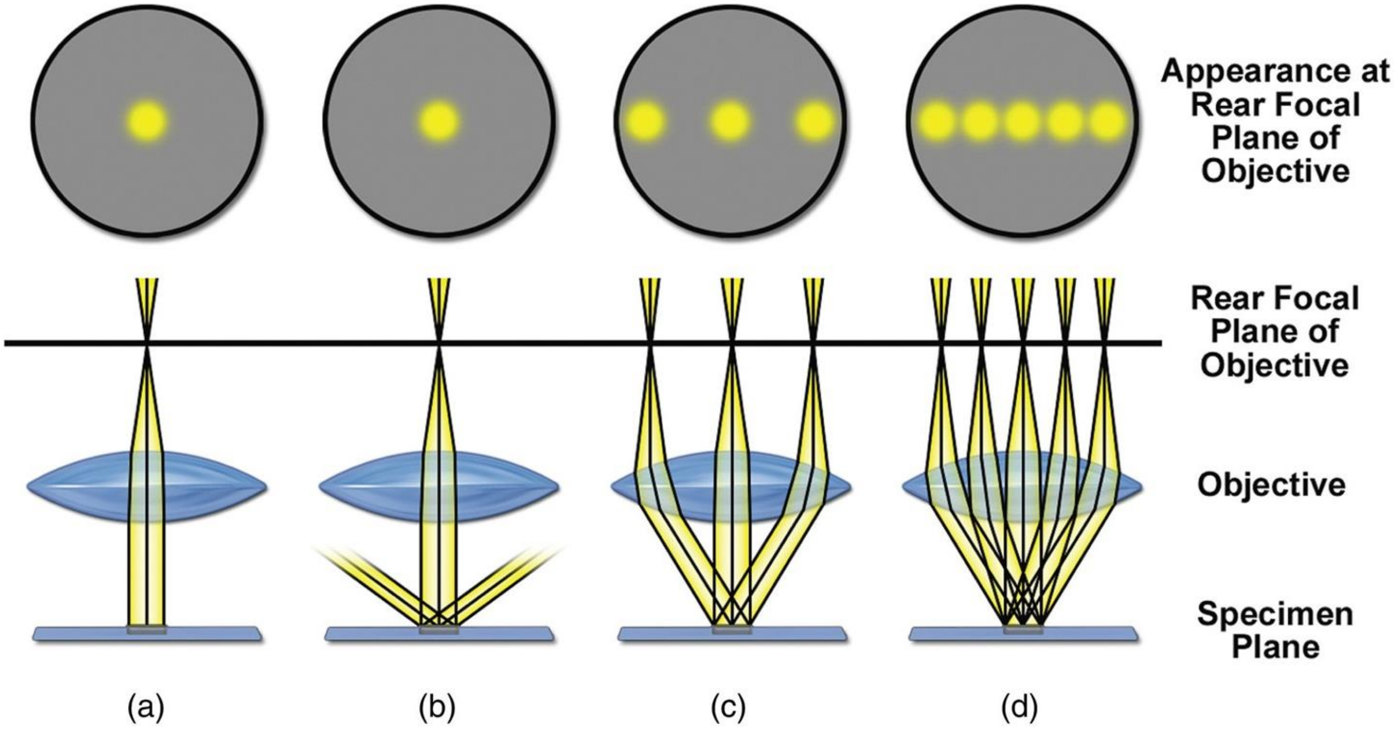
Collaborated with Carl Zeiss (owner of a microscopy company) & Otto Schott (owner of a glass company) in Jena, Germany.



The Lord Rayleigh (**John William Strutt**) [1842-1919]

Discovered Rayleigh scattering (which is used to explain why the sky is blue!). Discovered Argon (with Ramsay), got Nobel Prize for Physics in 1904

Abbe's theory



(a) No diffracting object in the specimen plane: no image (only background)

(b) Grating as specimen. Collect no diffraction other than 0^{th} order ➔ no new content for image

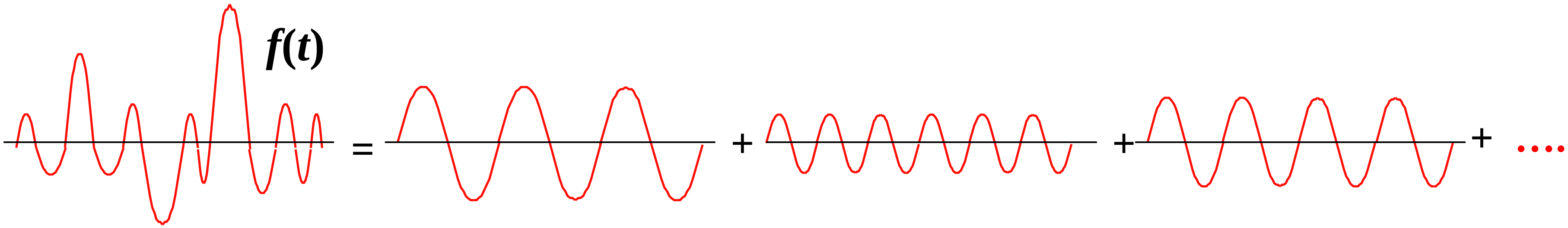
(c) Grating as specimen. Collect 0^{th} & 1^{st} orders diffraction ➔ Image with minimum resolution

(d) Grating as specimen. Collect multiple higher orders ➔ Image with higher resolution

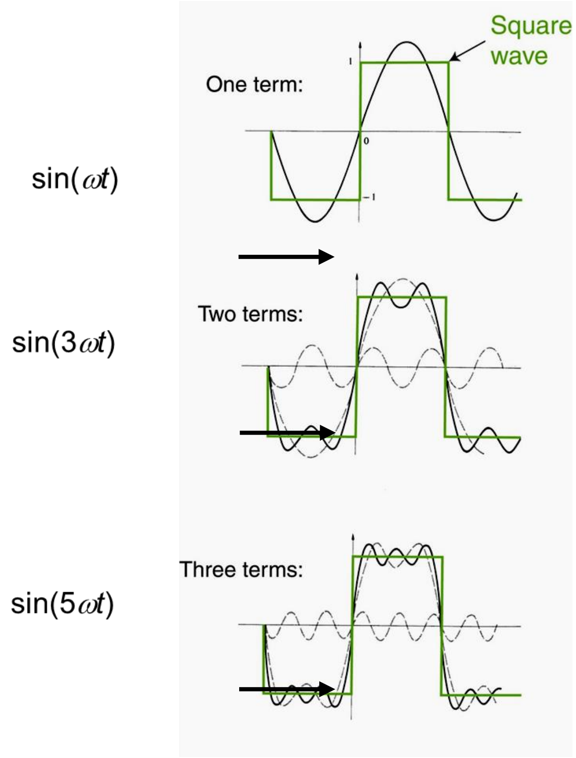
Abbe showed that we need at least TWO adjacent orders
Example: 0^{th} & 1^{st}

Adding More Fourier Terms (~Diffraction Orders)

Expansion of a function $f(t)$ as a sum of harmonic functions (Fourier terms)

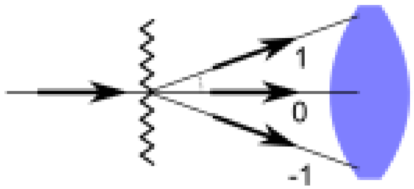


Analogy in microscopy: image is formed by the summation of diffraction orders



Accuracy (i.e. resemblance between the green & solid black curves) increases as we include more Fourier terms (i.e. higher orders terms)

Abbe's theory & resolution



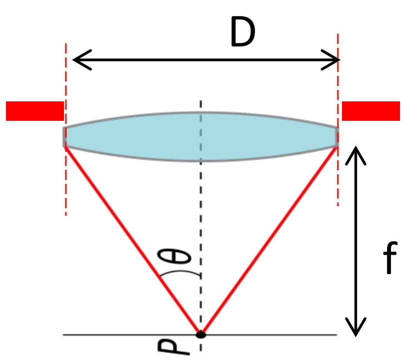
direct illumination

- A periodic specimen with a period ***d*** gives rise to the grading orders.
- For a direct illumination, the grating equation is:

$$\boldsymbol{d\ sin\theta_m = m\ \lambda_0\ \ (m = 1, 2, \dots)}$$

- In order to resolve the object, the NA of the lens should be large enough to collect at least the first order:

$$NA = nSin(\boldsymbol{\theta}) > \frac{1x\lambda_o}{d}$$



- **Numerical aperture:** A dimensionless number that defines the range of angles over which the lens can accept light.

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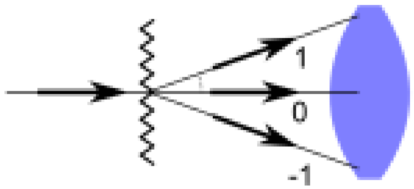
- n is the refractive index of the medium surrounding the lens

Abbe's theory & resolution



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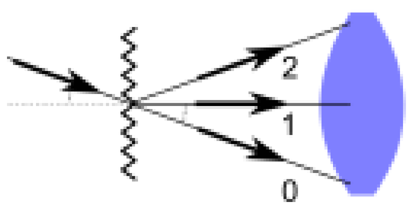
$$d > \frac{\lambda_o}{NA} = \frac{\lambda_0}{nSin(\boldsymbol{\theta})}$$



oblique illumination

- For oblique illumination, Abbe's criteria becomes:

$$d > \frac{\lambda_0}{2NA} = \frac{\lambda_0}{2nSin(\boldsymbol{\theta})}$$

References:
https://arxiv.org/pdf/1412.3255.pdf (by Weisenburger & Sandoghdar)
Experimental – physic 2 (Elektrizitat und Optik) by Wolfgang Demtroder

Determining **microscopy resolution** by 2 methods

1. **Abbe's theory for image formation**

→Collecting more diffraction orders for higher resolution

$$d_{min} \propto \frac{\lambda_o}{2nsin(\theta)}$$

University of Jena (Germany)
Abbe School of Optics

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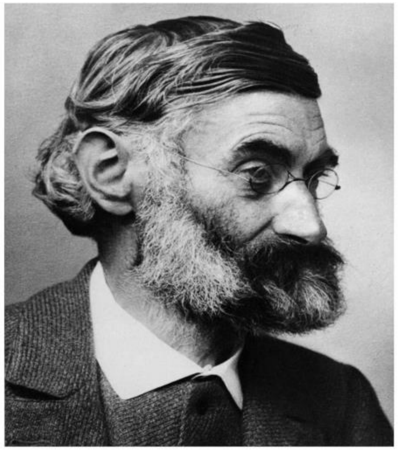
→ Diffraction of a circular aperture & Airy disks



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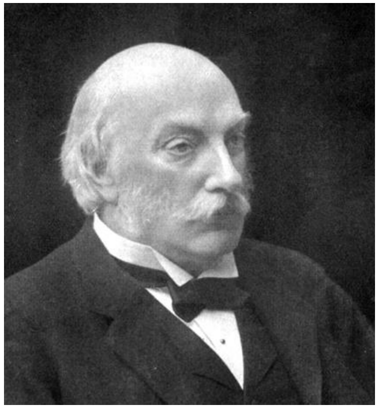

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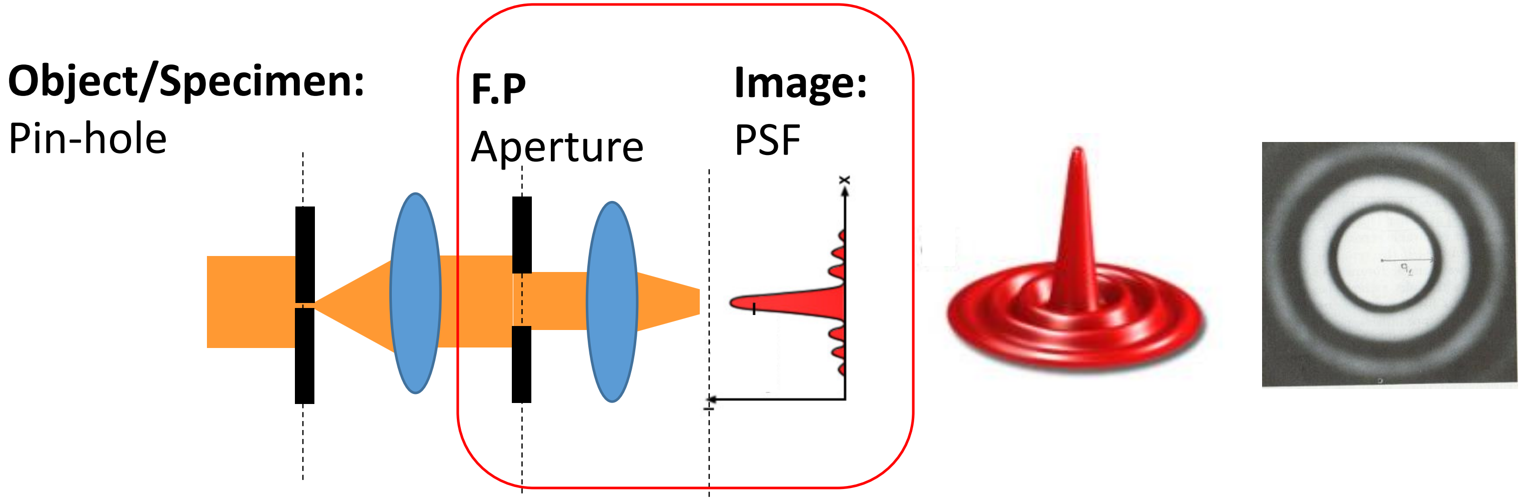
Rayleigh Criteria - **P**oint **S**pread **F**unction



Consider a 2-lens imaging system in “4f” configuration.

At the object plane: As an input, let’s put an ideal point source (~zero diameter) → $\delta(x)$

At the Fourier plane: Let’s INSERT a circular aperture.

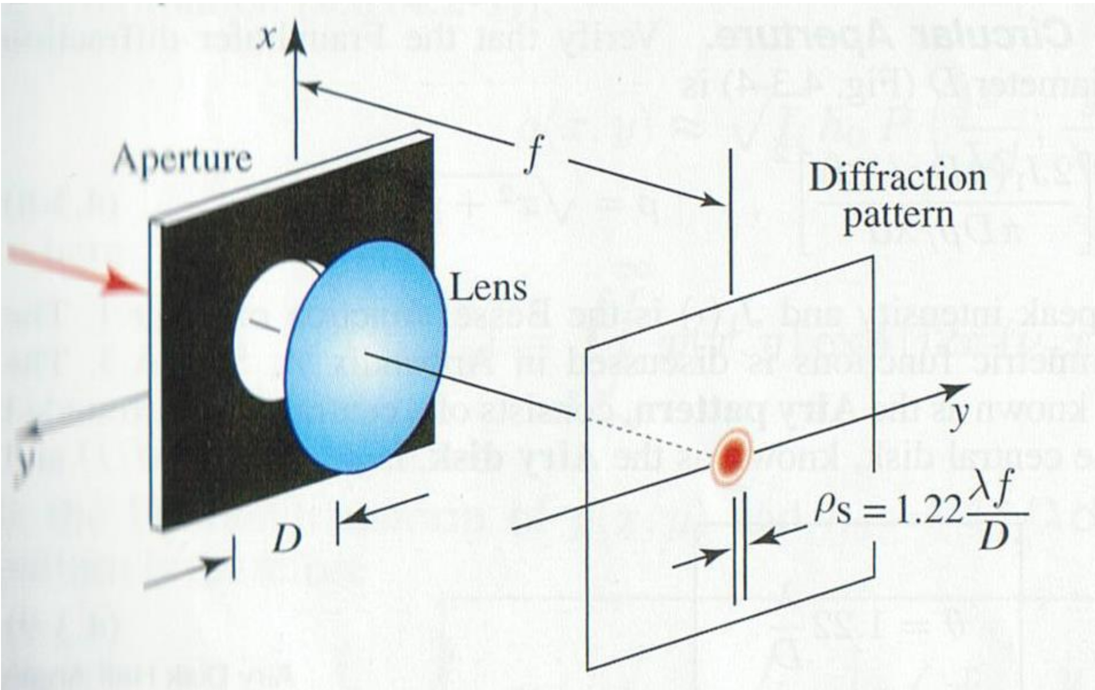
→the aperture filters (such that removes) the higher frequencies!

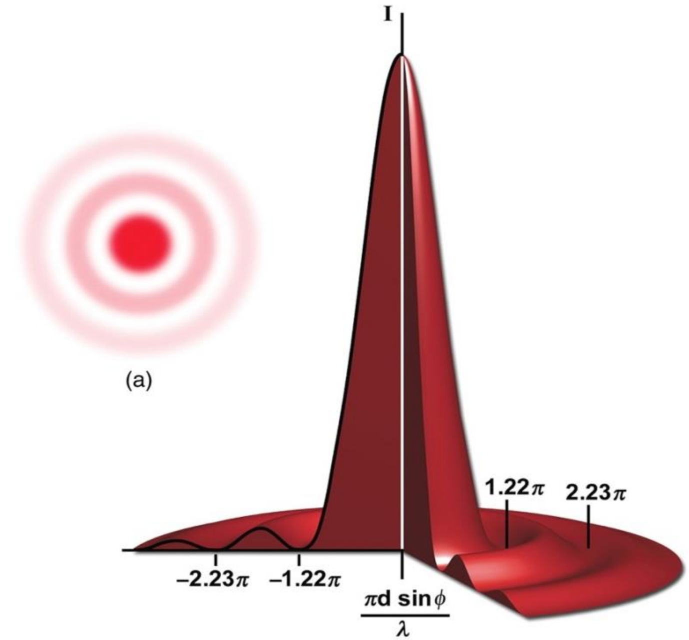
(this aperture can represent the finite/limited size of a lens, aperture stop, etc)

At the image plane: We get the F.T. of the circular aperture → diffraction pattern (**Airy Disks**)

The system response to a point source (a.k.a. impulse function) is called **P**oint **S**pread **F**unction (**PSF**)

Diffraction Limited Spot Size According to Rayleigh Criteria



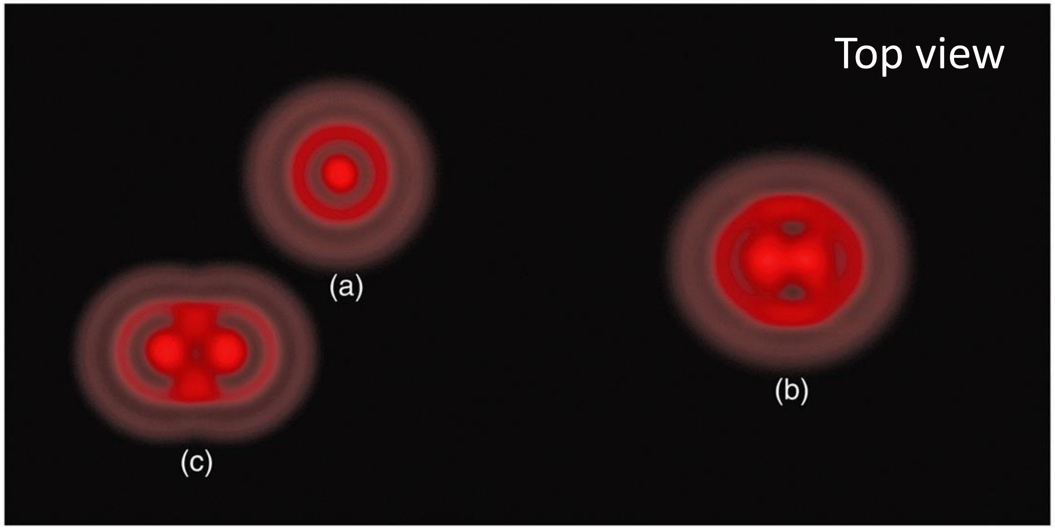


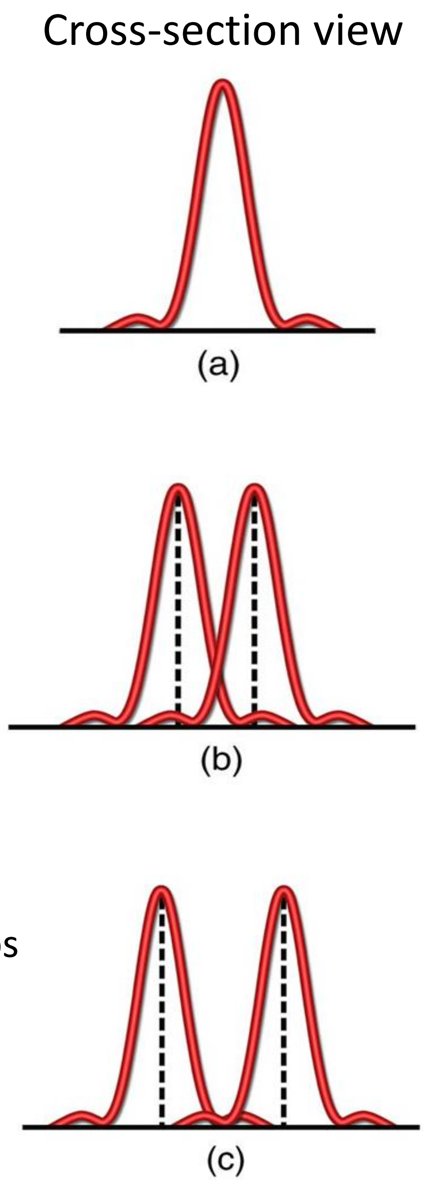
- At the image plane, a collimated light beam with a finite size (D) will form a finite spot with a "diffraction-limited spot size".
- This spot corresponds to the *central* **Airy disk** of the diffraction pattern and its radius is given by:

$$\rho_s = 1.22\,\lambda f / D$$

- The **central disk** contains 84% of power

Rayleigh Criterion for Resolution





The resolution of a microscope is the shortest distance between two points that can be separated and still observed as 2 points.

At the image plane:

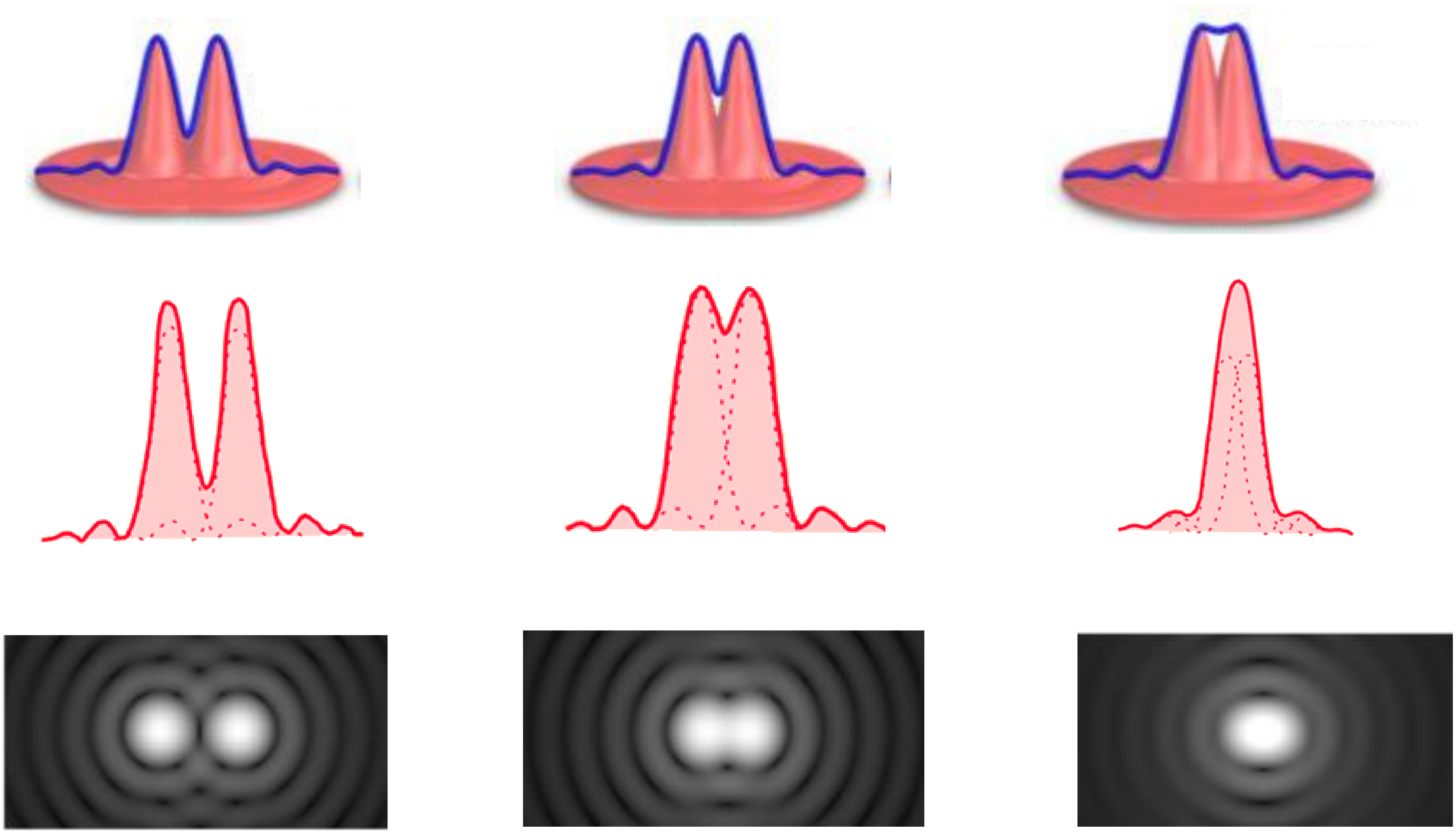
a) Profile of a single diffraction pattern.

b) Profile of two disks separated at the Rayleigh limit: maximum of one disk overlaps with the 1st minimum of the other disk. These two points are barely resolvable.
➔ **Minimum resolvable feature size is ρ_s**

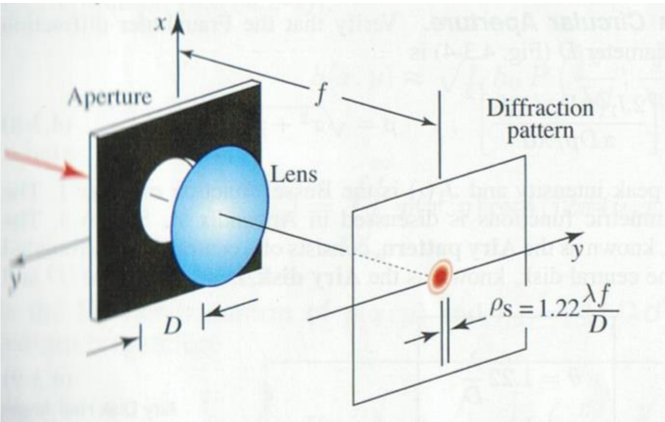
c) Profile of two disks at a separation such that the maximum of each disk overlaps the 2nd minimum of the other disk. These two points are clearly resolvable.

Airy Disk Separation and the Rayleigh Criterion

Resolved | **Rayleigh Limit** | **Not Resolved**

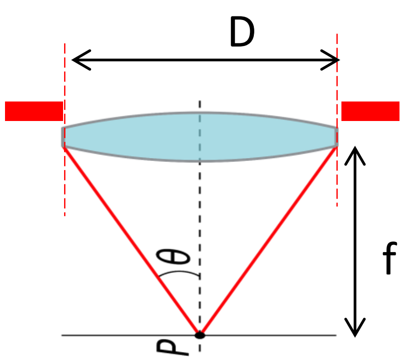


Rayleigh Criterion & Resolution



- Diffraction-limited spot corresponds to the central **Airy disk** of the diffraction pattern and its radius is given by:

$$\rho_s = 1.22\,\lambda f / D$$



- **Numerical aperture:** A dimensionless number that defines the range of angles over which a lens can accept light.

$$\mathrm{NA} = \mathrm{n}\sin(\theta)$$

- θ is the half angle of the light cone accepted by the lens
- n is the refractive index of the medium surrounding the lens

$$\sin(\theta) \approx \frac{D}{2f} \Rightarrow \boxed{\rho_s \approx \frac{1.22\,\lambda_o}{2NA} \approx 0.61\frac{\lambda_0}{n\sin(\theta)}}$$

Summary: Microscopy resolution determined by 2 methods

1. **Abbe's theory for image formation/resolution**

→Collect at least two diffraction orders to form an image (collecting more orders will provide better resolution)

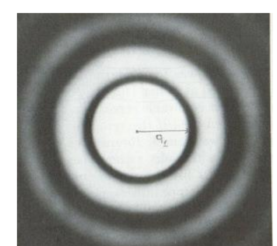
$$d_{min} \approx 0.5\frac{\lambda_0}{NA} \approx 0.5\frac{\lambda_0}{nsin(\theta)}$$

2. **Rayleigh criterion for image resolution**

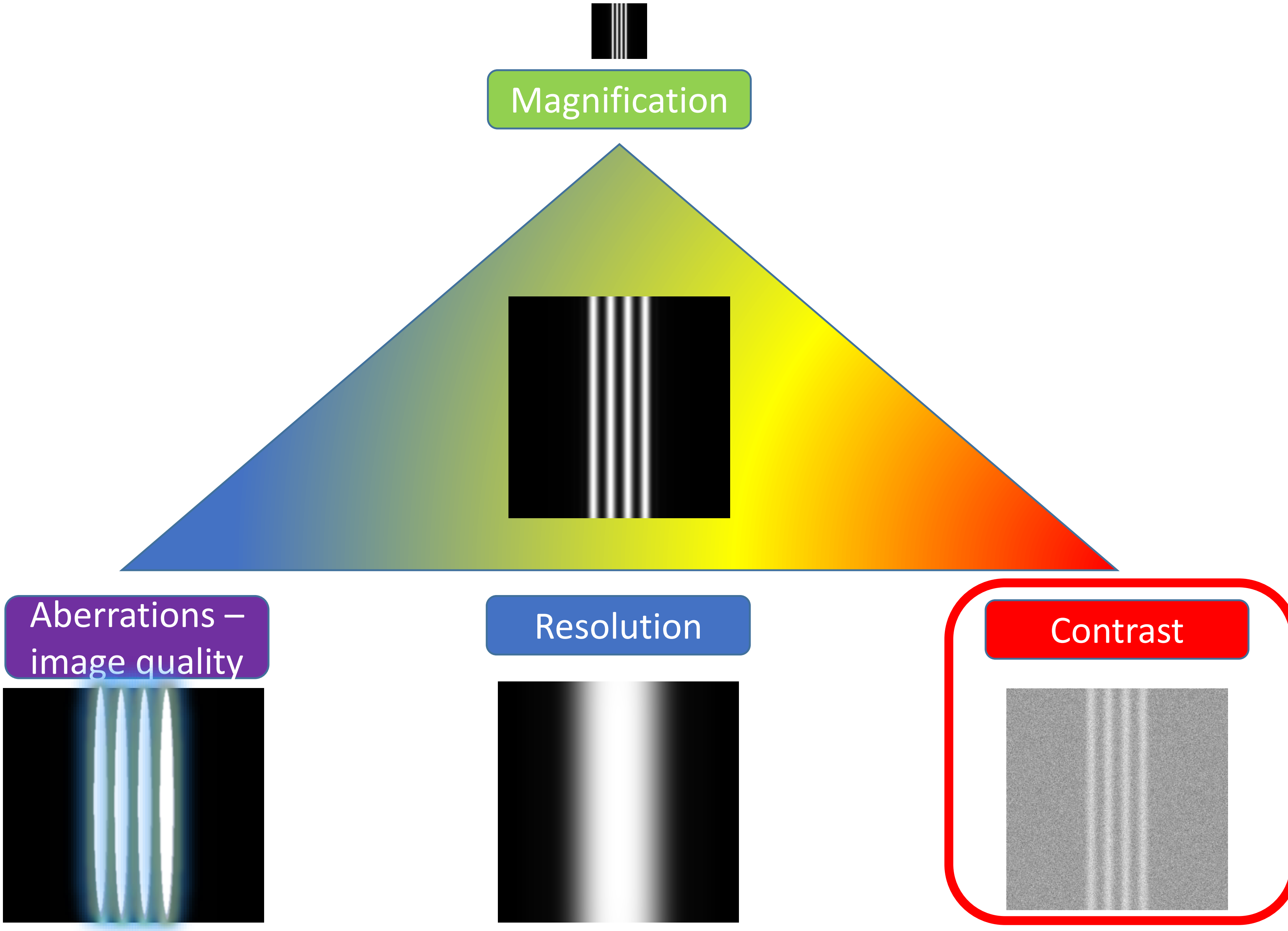
→ Diffraction of a circular aperture & Airy disk

$$\rho_s \approx 0.61\frac{\lambda_0}{NA} \approx 0.61\frac{\lambda_0}{nsin(\theta)}$$

P.S.F



Important aspects in microscopy



Important aspects in microscopy:

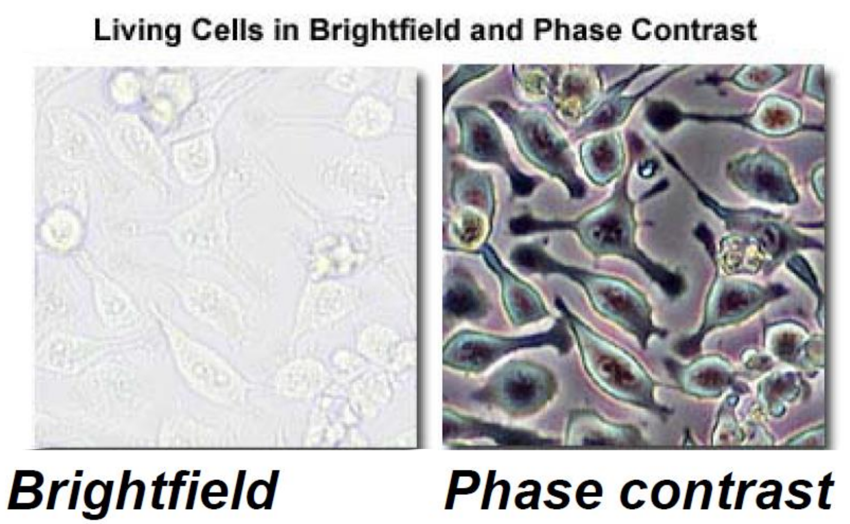
- Magnification
- Image quality – aberrations, alignment, illumination condition etc
- Resolution
- **Contrast**

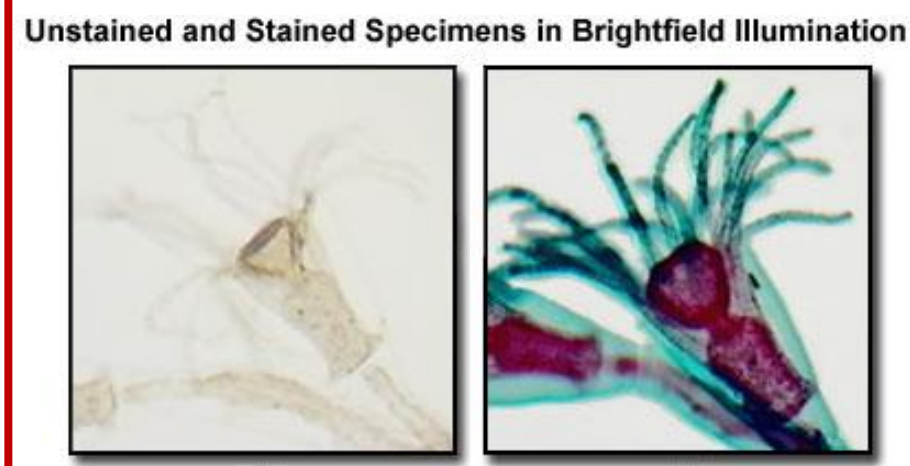
- **Contrast** is necessary to detect/differentiate details from *background*.
- Contrast can be achieved when the captured light from an object has different in **intensity** or color (= wavelength) from the background light.

Contrast in Bright-Field Microscope

Why is the contrast low?

Most of the biological samples (e.g. cell, tissue etc..) are optically thin and transparent:
➔ They do not absorb, scatter etc.. → **we get low contrast w.r.t. background**
➔ They are hard to see!!





Contrast depends on the difference between the sample brightness and background brightness

Background signal and contrast

$$\frac{Brightness\ of\ Specimen}{Brightness\ of\ Specimen + Brightness\ of\ Background} \times 100\%$$

Background: 100 Units
Specimen: 50 Units

50/(100+50) X 100% = 33%

Background: 50 Units
Specimen: 50 Units

50/(50+50) X 100% = 50%

Background: 0 Units
Specimen: 50 Units

50/(50+0) x 100% = 100%