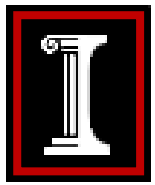


Optical Imaging
Chapter 4 – Microscopy

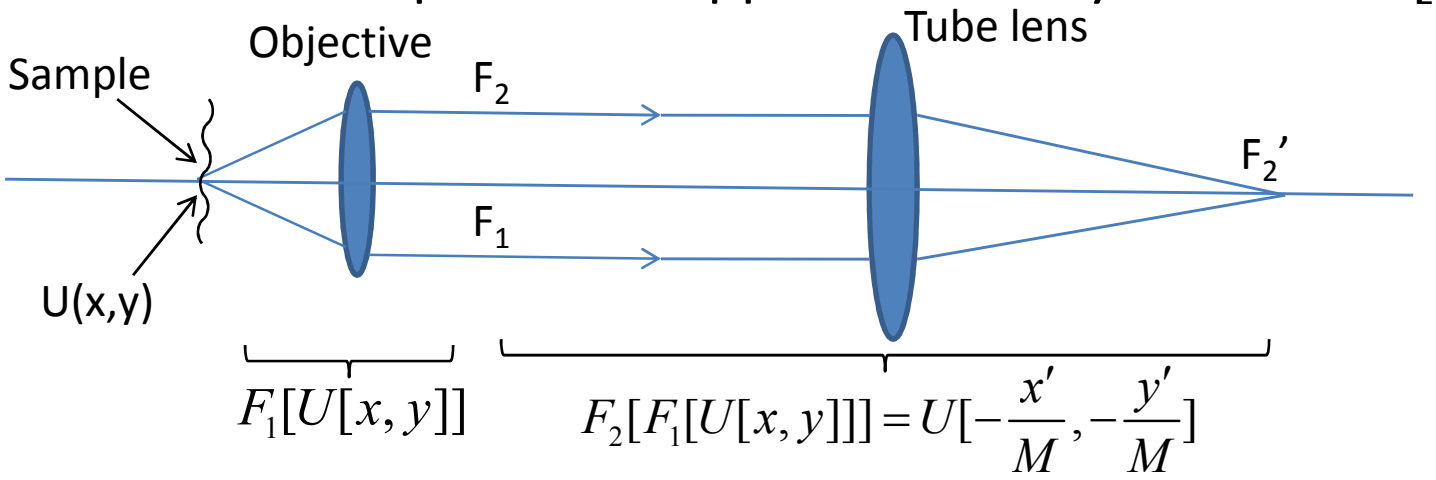
Gabriel Popescu

University of Illinois at Urbana-Champaign
Beckman Institute

Quantitative Light Imaging Laboratory
http://light.ece.uiuc.edu

4.1 Resolution of Optical Microscopes

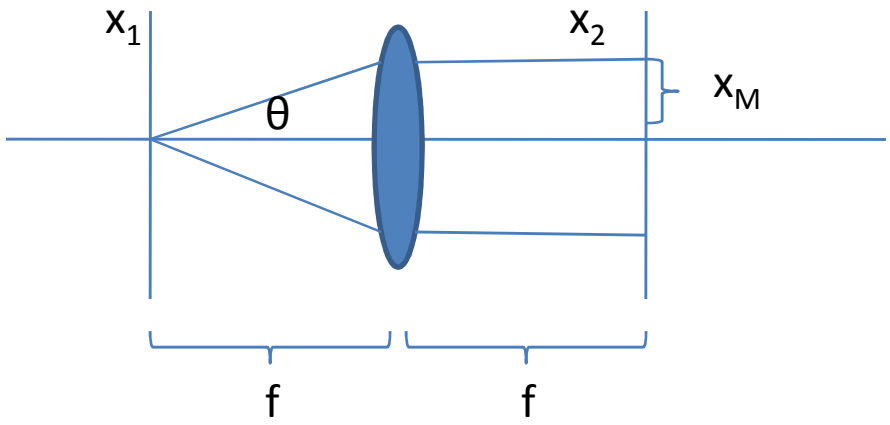
- The Microscope can be approximated by 2 lenses $F[F[U[x,y]]]$



- "-" sign means inverted
- The objective is the most important part of the microscope
- Usually the third lens (occular) images F_2' at ∞, such that we can visualize it with the relaxed eye.

4.1 Resolution of Optical Microscopes

- The objective lens dictates the resolution or size of the smallest object that the microscope can resolve.
- Contrast is generated by absorption, scattering, etc.
- Microscopes can be categorized by the methods that they use to produce contrast.
- Let's consider an infinitely small object (point):



How small can we see?

4.1 Resolution of Optical Microscopes

- Fourier properties of the lens; the reconstructed field is:

$$U[x,y] = \int_{-\infty}^{\infty}\int_{-\infty}^{\infty} U[x,y]e^{2i\pi(x_1\xi + y_1\eta)}d\xi d\eta \qquad (4.1)$$

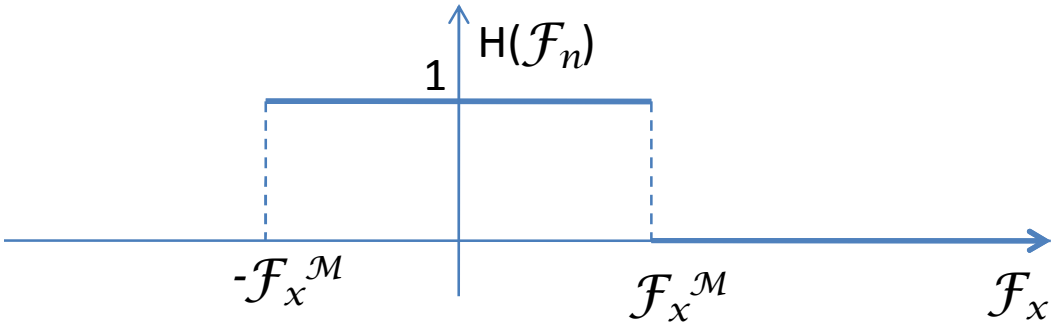
- We know that $\xi < \infty$ and $\eta < \infty$ because $\xi^M < \frac{x_M}{\lambda f}$ and $\eta^M < \frac{y_M}{\lambda f}$
- We can access only a finite frequency range and therefore we can only achieve finite resolution.
- We would need an infinite spectrum to reconstruct a δ-function (in this case a point)

4.1 Resolution of Optical Microscopes

- Given the finite frequency support we can write:

$$U[\xi', \eta'] = U[\xi, \eta] \cdot H[\xi, \eta] \tag{4.2}$$

- Where $H = \begin{cases} 1 \text{ if } \xi < \xi^M, \ \eta < \eta^M \\ 0 \text{ otherwise} \end{cases}$



- So, eq. 4.1 becomes

$$\bar{U}[x, y] = F[U[\xi, \eta] \cdot H[\xi, \eta]] \tag{4.3}$$

4.1 Resolution of Optical Microscopes

- Use the Convolution Theorem once more (which states that convolution in one domain is multiplication in another) to get:

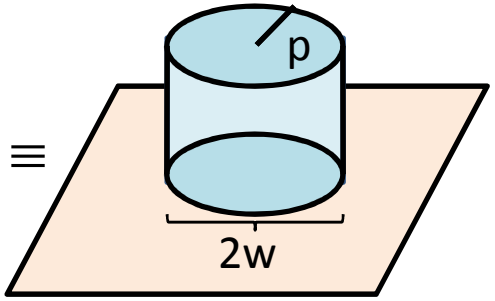
$$\bar{U}[x,y] = U[x,y] \circledv h[x,y] \tag{4.4}$$

- Where $\bar{U}[x,y]$ is the ideal image
  $U[x,y]$ is the real image
  $h[x,y]$ is the impulse response

$$e^{i(x\cdot\xi + y\cdot\eta)}$$

$$h[x,y] = F[H[\xi,\eta]] \tag{4.5}$$

- $H[f_x, f_y] = \left\{ \begin{matrix} 1 \text{ if } f_x{}^2 + f_y{}^2 \le w^2 \\ 0 \text{ otherwise} \end{matrix} \right\} \equiv circ[\frac{L}{w}] \equiv$ (4.6)



4.1 Resolution of Optical Microscopes

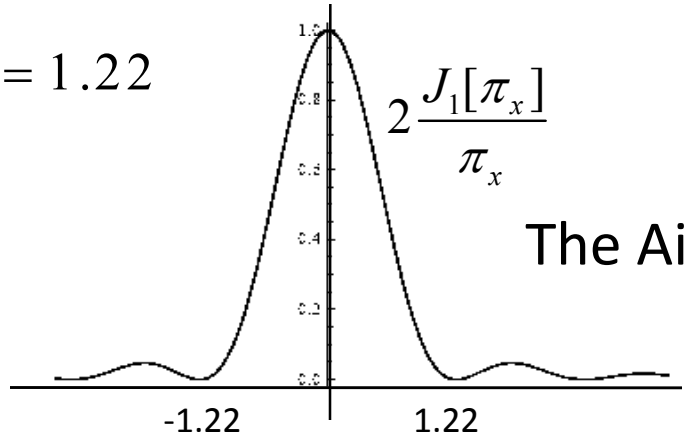
- So, $F[H] = A \cdot \frac{J_1[2\pi W \rho]}{2\pi W \rho} = h$, where J_1 is a Bessel function of the 1st kind and order
- So the image of a "point" becomes:

$$|h[x, y]|^2 = A^2 \cdot \left| \frac{J_1[2\pi W \rho]}{2\pi W \rho} \right|^2 \qquad (4.7)$$

- Since $W_M = \frac{x_M}{\lambda f}$ and $2W_M \rho = 1.22$

$$2\frac{x_M}{\lambda f}\rho = 1.22$$

- so, $\boxed{\rho = .61\frac{\lambda f}{x_M}}$ (eq. 4.8)

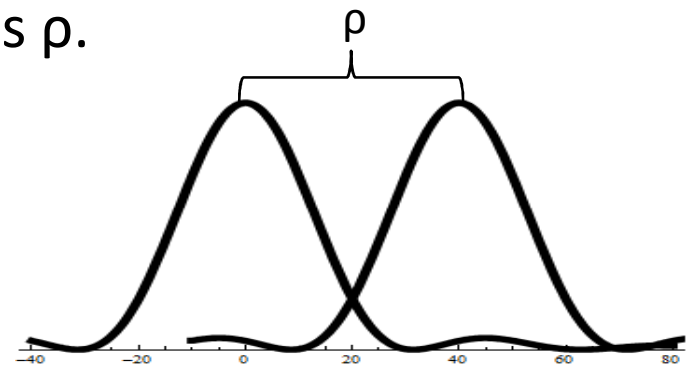


The Airy Function

- A point will be imaged as a smeared spot of diameter $\rho = .61\frac{\lambda f}{x_M}$

4.1 Resolution of Optical Microscopes

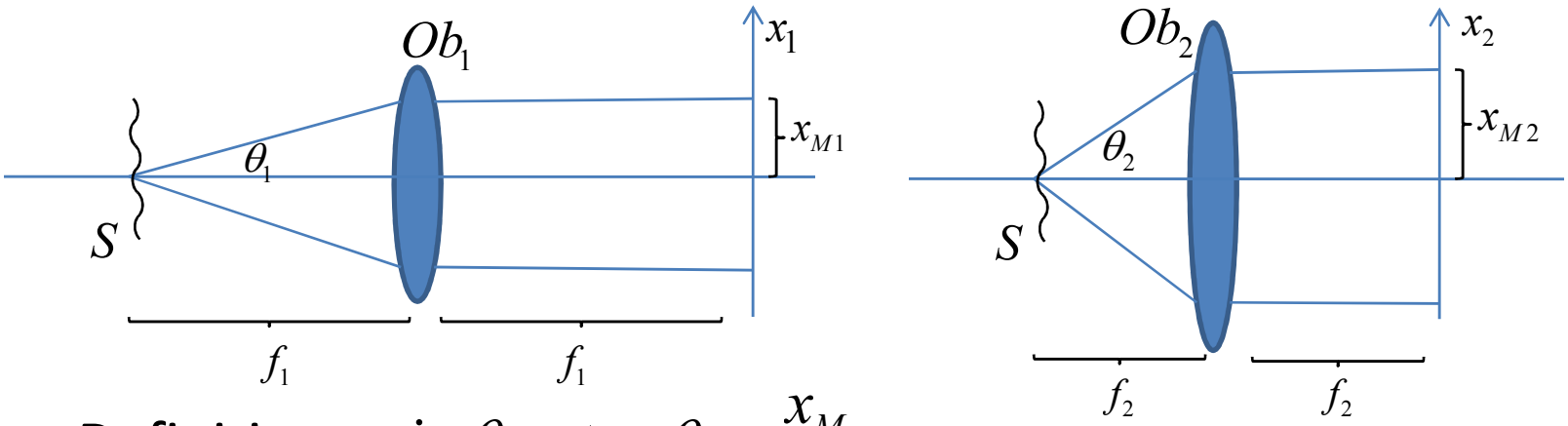
- Imagine that we have two such points. Then the resolution is the minimum distance between the points that are separated, which is ρ.



$$\rho = \text{resolution}$$

- An objective lens that allows higher spatial frequencies (or angles) provides a higher resolution.

4.1 Resolution of Optical Microscopes



- Definition: $\sin\theta_1 = \tan\theta_1 = \dfrac{x_M}{f}$

$$\boxed{\sin\theta_1 = NA} = \text{Numerical Aperture} \qquad (4.10)$$

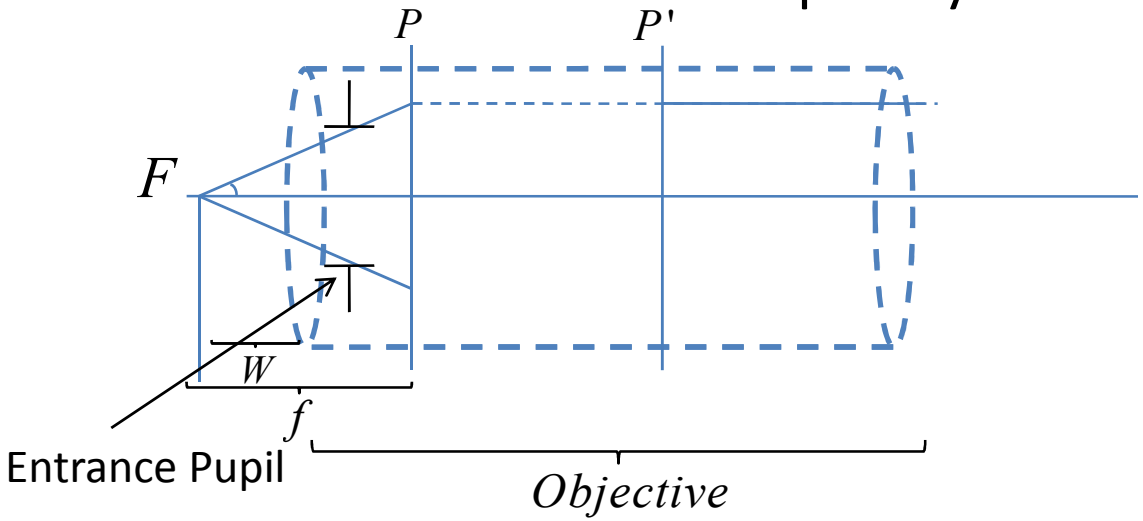
- The resolution becomes $\rho = .61\dfrac{\lambda}{NA}$ but $\rho = \dfrac{\lambda}{2NA}$ is good enough
- Compare Ob_1 and Ob_2 above:

$$\theta_1 < \theta_2, \quad x_{M1} < x_{M2} \Rightarrow \boxed{NA_1 < NA_2} \Rightarrow \boxed{\rho_1 > \rho_2} \qquad (4.11)$$

- So Ob_2 provides a better resolution.

4.1 Resolution of Optical Microscopes

- This discussion applies for all lenses where the physical aperture overlaps with the lens. In general, objectives are made out of several lenses => complex systems



f = focal distance measured from principal plane

W = working distance = distance from F to physical surface of lens

Entrance Pupil = image of physical aperture

$\Rightarrow$ f and entrance pupil determine numerical aperture i.e. resolution

4.1 Resolution of Optical Microscopes

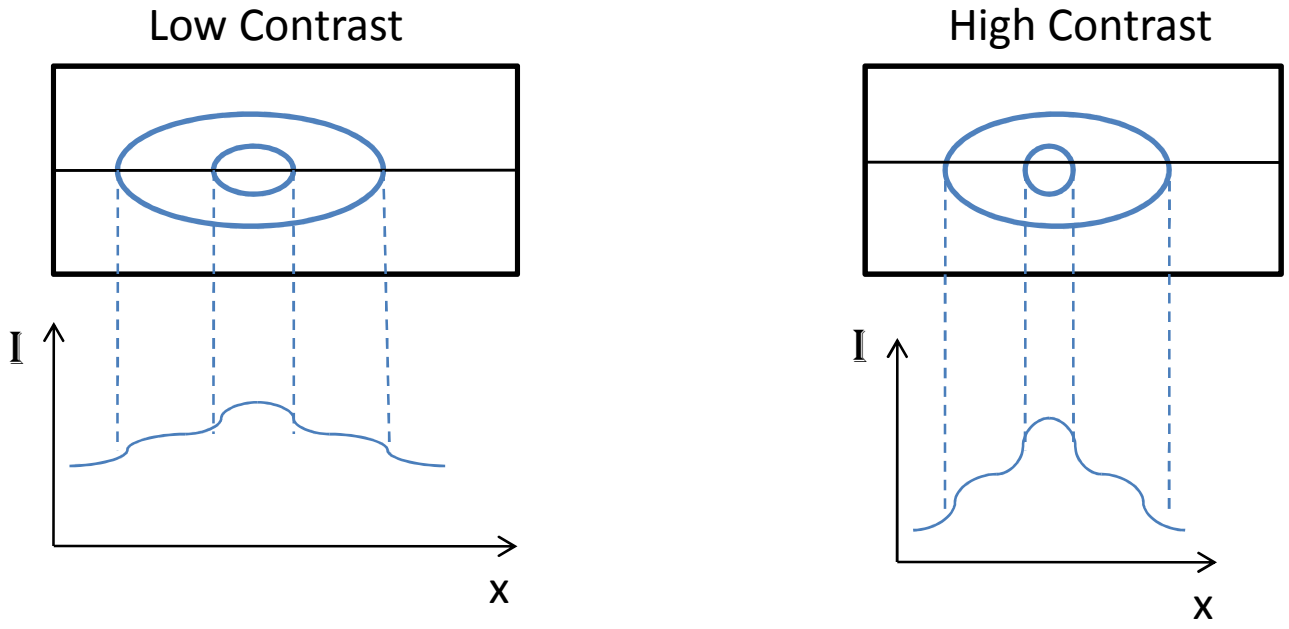
- Note: if the objective lens is immersed in a medium for which n≠1, then

$$f' = \frac{f}{n} \quad \Rightarrow \quad NA' = n \cdot NA \quad \Rightarrow \quad \rho' = \frac{\rho}{n} \qquad (4.12)$$

- This means that it is possible for immersed objective lenses to have a better resolution.

4.2 Contrast

- The final image consists of a distribution $I[x, y]$ which is the result of absorption, scattering/diffraction, etc.
- Contrast = a measure of the intensity fluctuations across the image. In general, the more contrast the better.



4.2 Contrast

- Analogy from contrast of fringes in interferometery

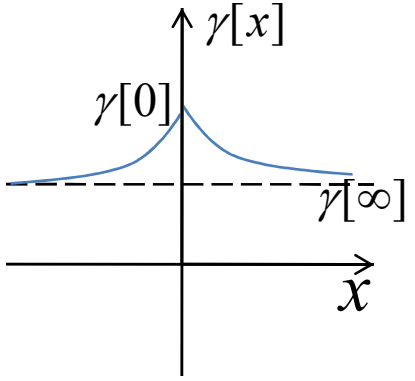
$$\gamma = \frac{I_{\max} - I_{\min}}{I_{\max} + I_{\min}}, \in [0:1] \qquad (4.13)$$

- One could see a statistically meaningful definition :

$$\Gamma[\bar{\rho}] = \langle I[\bar{r}] \cdot I[\bar{r} + \bar{\rho}] \rangle = \int I[\bar{r}] \cdot I[\bar{r} + \bar{\rho}] \, d^2 r$$

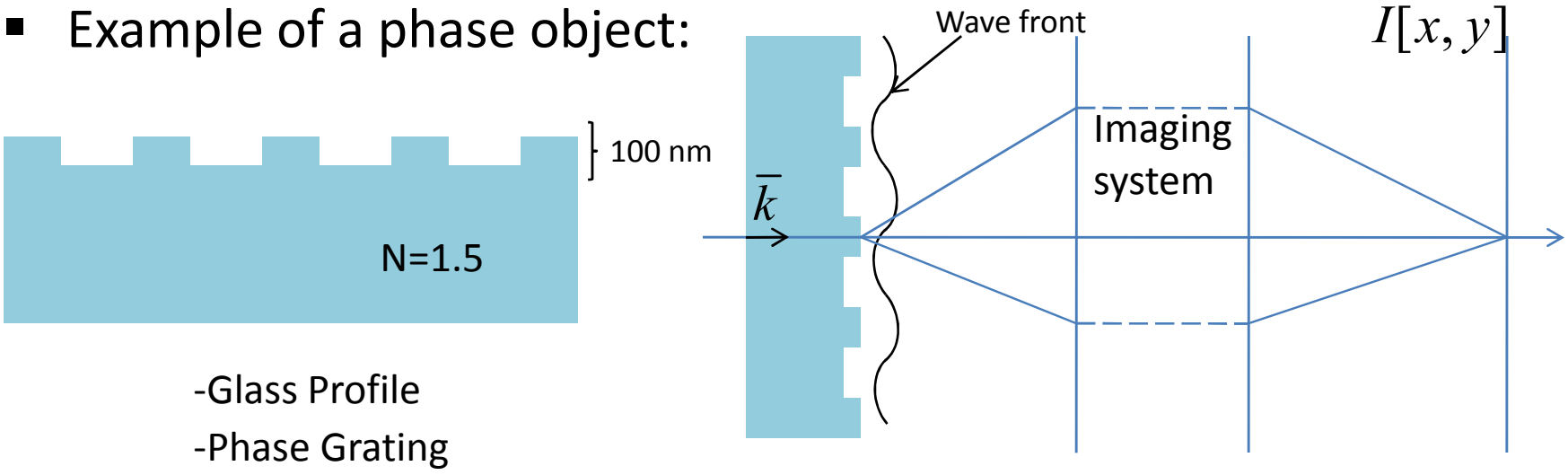
$$= \text{intensity spatial autocorrelation}$$

$$\Rightarrow \gamma[\rho] = \frac{\Gamma[\rho]}{\Gamma[0]} \Rightarrow \text{contrast} = \frac{\gamma[0]}{\gamma[\infty]} - 1$$



4.2 Contrast

- While resolution is given by the instrument, the contrast is given by the instrument/sample combination.
- Most biological structures (i.e. cells) are very transparent so
- $I[x, y]$ is flat, which means there is low contrast
- They can be assumed "phase objects"
- Example of a phase object:



-Glass Profile
-Phase Grating

4.2 Contrast

- No absorption so $I[x,y] = \text{constant} \quad \Rightarrow \quad \text{contrast} = 0$
- <u>BUT:</u> the wave front carries information about the sample

$$E[x,y] = E_0 \cdot e^{i\phi[x,y]} \tag{4.14}$$

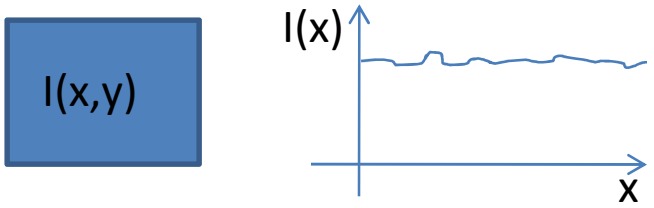
- This is the expression for the field in the vicinity of a phase object.
- <u>Bright Field</u> microscopy produces low contrast images of phase objects

4.2 Contrast

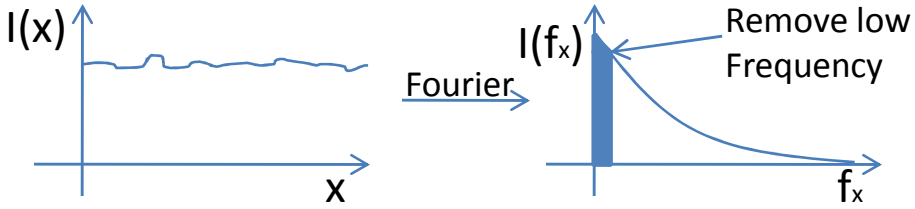
- There are several ways to enhance contrast:
  - Endogeneous Contrast
    - Dark field
    - Silierien
    - Phase contrast
    - Schlerein
    - Qualitative phase microscopy
    - Confocal
    - Endogeneous florescence
  - Exogeneous Contrast Agents
    - Staining
    - Florescent tagging — Full field / Confocal
    - More recently
      - Beads (dielectric and metallic)
      - Nano
      - Quantum Dots

4.3 Dark Field Microscopy

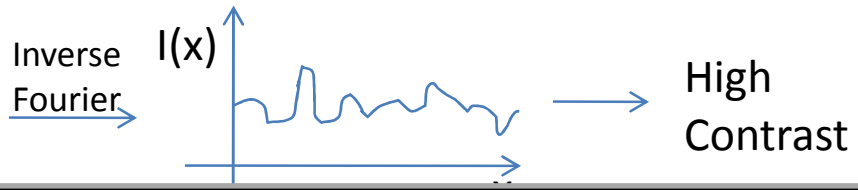
- Consider the low contrast image



- Typical low pass filtering = remove ΔC

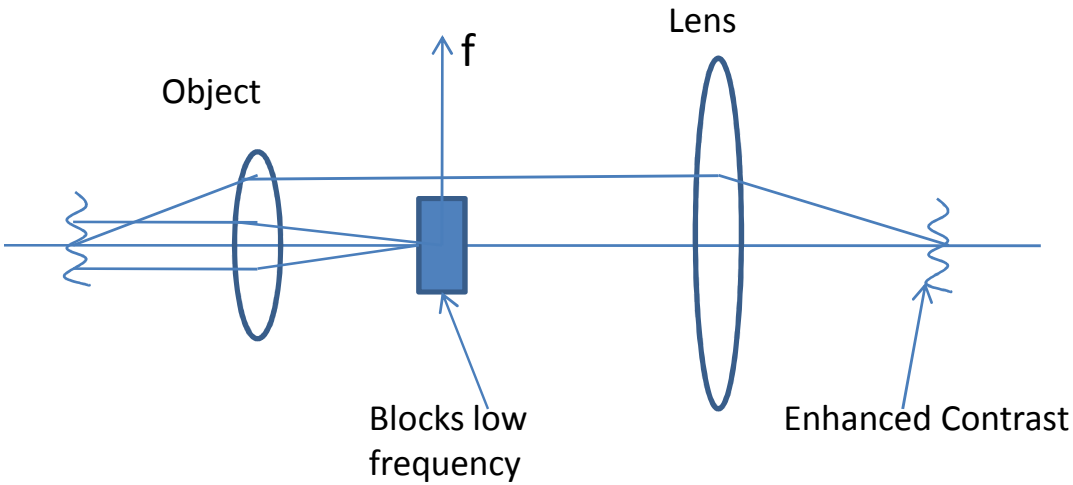


- Then take the inverse Fourier Transformation



4.3 Dark Field Microscopy

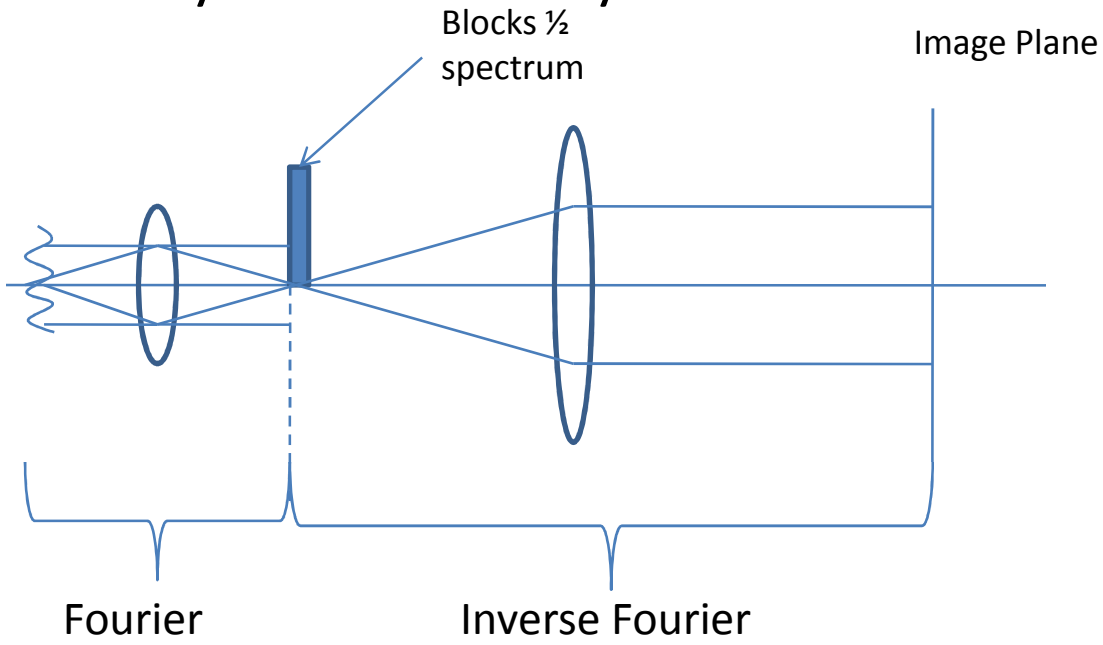
- Actual Microscope



- High frequency components are enhanced (eg. edges)
- Without the sample ⟶ Dark Field

4.4 Schlieren Method

- Not used very often nowadays



- Enhances Contrast
- Phase objects can be rendered visible
- Edges are enhanced
- Relates to Hilbert Transform.

4.4 Schlieren Method

- Exercise: Show the following for a real signal f(x)

$$f(x) \xrightarrow{\text{Fourier}} F(g) \xrightarrow[\text{spectrum}]{\text{Cut ½}} F_t(g) \xrightarrow[\text{Fourier}]{\text{Inverse}} \tilde{f}(x)$$

$$\tilde{f}(x) \in \mathbb{C} \text{ and}$$

$$\tilde{f}(x) = \frac{1}{2} f(x) + i \frac{P}{2\pi} \int \frac{f(x')}{x - x'} dx' \longrightarrow \text{Hilbert}$$

To the left: David Hilbert a German Mathematician, recognized as one of the most influential and universal mathematicians of the 19th and early 20th centuries.

4.5 Phase Contrast Microscopy

- Developed by Frits Zernike (1935) yielding noble prize in 1953(Physics)
- Very powerful, commonly used today.
- Consider a phase object:

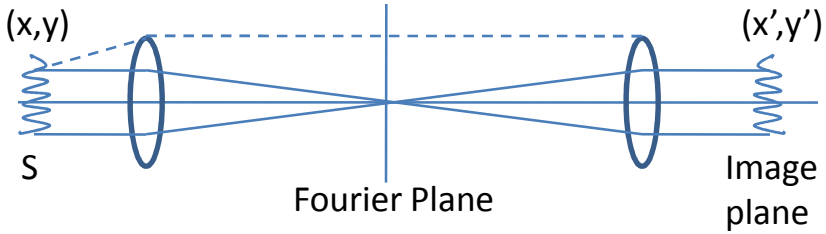
$$U(x,y) = e^{i\phi(x,y)} \qquad (4.15)$$

- Intensity distribution:

$$I(x,y) = |U|^2$$

$$= 1 \Rightarrow \text{ No Contrast}$$

- Assume: The microscope has a magnification M=1

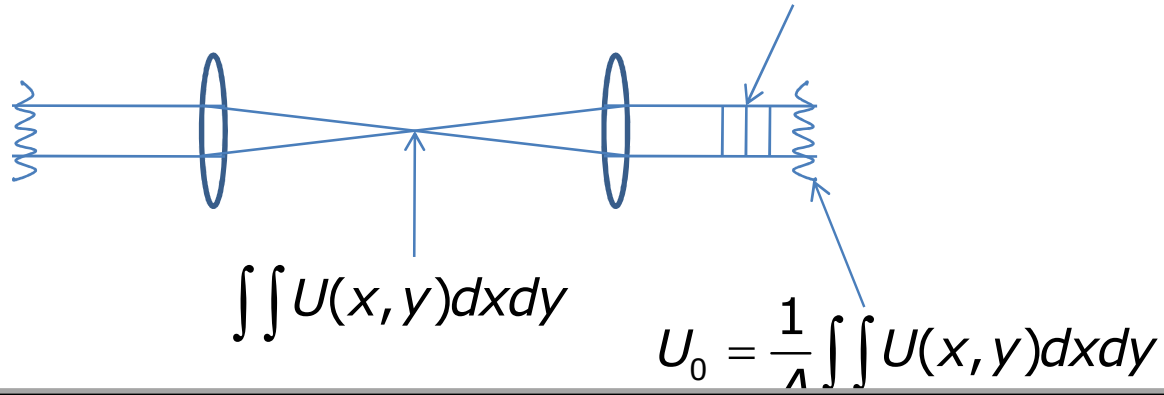


4.5 Phase Contrast Microscopy

$$\tilde{U}(f_x, f_y) = \iint_{-\infty} U(x,y) e^{-i2\pi(\xi f_x + \eta f_y)} dxdy \quad (4.16)$$

$$f_x = \frac{x}{\lambda f}; f_x = \frac{y}{\lambda f}$$

- Note: $\tilde{U}(0,0) = \iint U(x,y)dxdy$ (4.17)
- Central Ordinate Theorem
- Zero Frequency component corresponds to a plane wave in the image plane(constant of (x,y))



4.5 Phase Contrast Microscopy

- Note:
- U_0 has no information about the structure of the sample.

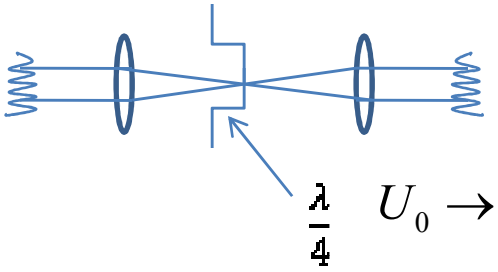
$$U_0 = \frac{1}{A}\int\int U(x,y)dxdy \quad = \text{Average field}$$

Image formation is an interference between the average field and high frequency components.

$$U(x,y) = U_0 + \underbrace{[U(x,y) - U_0]}_{\text{High Frequency Component}} \rightarrow U_1(x,y) \qquad (4.18)$$

4.5 Phase Contrast Microscopy

- Phase contrast relies on shifting the phase of U_0 by



- Assume $|U_0| = 1$ $U_0 \to U_0 a e^{i\alpha}$ becomes: $U(x,y) = a e^{i\alpha} + [U(x,y) - 1]$
- The intensity distribution in the image plane becomes: (4.19)

$$I(x,y) = |U(x,y)|^2 =$$

$$= \left| a e^{i\alpha} + e^{i\phi(x,y)} - 1 \right|^2 =$$

$$= a^2 + 1 + 1 + \mathrm{Re}[2a e^{i(\alpha+\phi)} - 2a e^{i\alpha} - 2e^{i\phi}] =$$

$$= a^2 + 2[1 - a\cos\alpha - \cos\phi + a\cos(\alpha+\phi)] \qquad (4.20)$$

4.5 Phase Contrast Microscopy

- Note: For a = 0 recover Dark Field Microscopy
- Assume "small" phase shift

$$\cos\phi \simeq 1; \alpha = \pm\frac{\pi}{2}$$

$$I(x,y) \simeq a^2 + 2a \cdot \sin\alpha \cdot \sin\phi$$

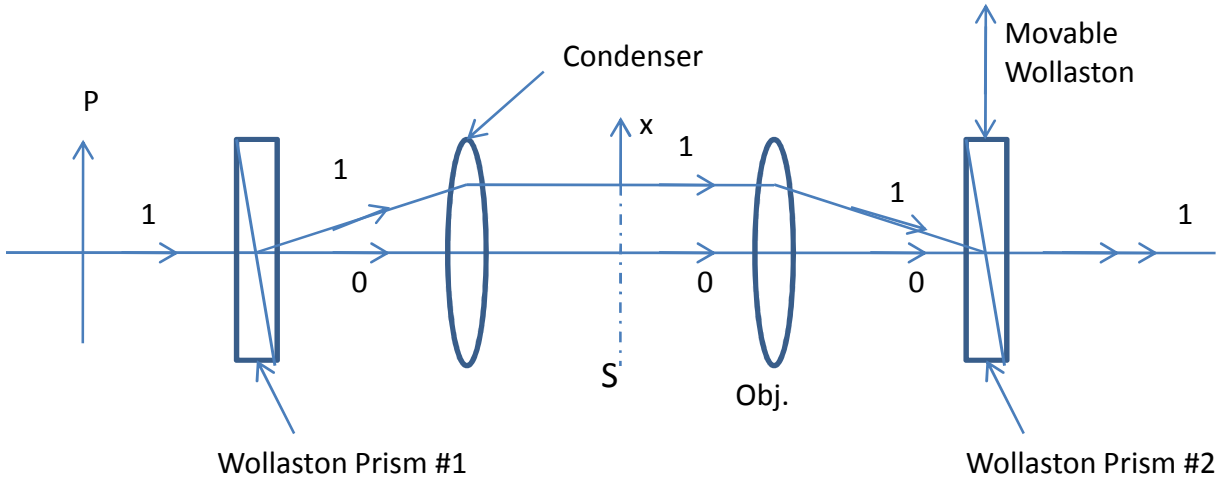
$$= a^2 + 2a \cdot \phi(x,y) \cdot \sin\alpha$$

$$I(x,y) = a^2 \pm 2a \cdot \phi(x,y) \qquad (4.21)$$

- P.C.M couples ϕ into intensity
- a<1 enhances contrast (best modulation for $|U_0| \simeq |U_1|$)

4.6 Nomarski/Differential Interference Contrast Microscopy

- DIC= Differential Interference Contrast



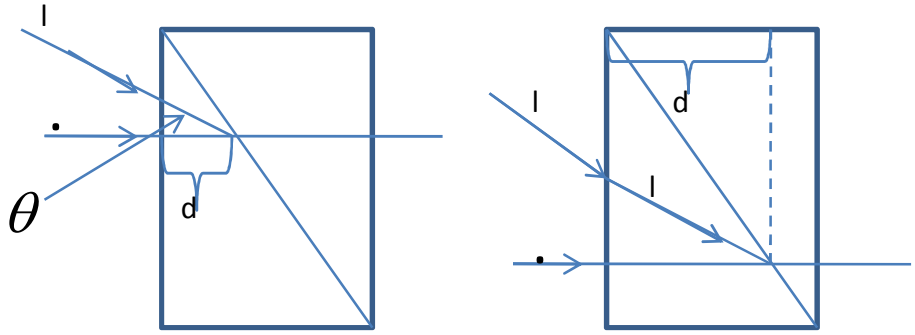
- Use polarization discrimination to create 2 interfering beams
- Illuminate sample(s) with 2 drifted beams (orthogonal pal.)

4.6 Nomarski/Differential Interference Contrast Microscopy

- "Dust" amount ≃Airy disk $\simeq \frac{\lambda}{2NA} \simeq 1\mu m$

- Wollaston prism #2 brings the 2 beams together through interference.

$$E_{Total} = E_1 + E_0 = A_1 \cdot e^{i\phi_1} + A_0 \cdot e^{i\phi_0} \qquad (4.22)$$



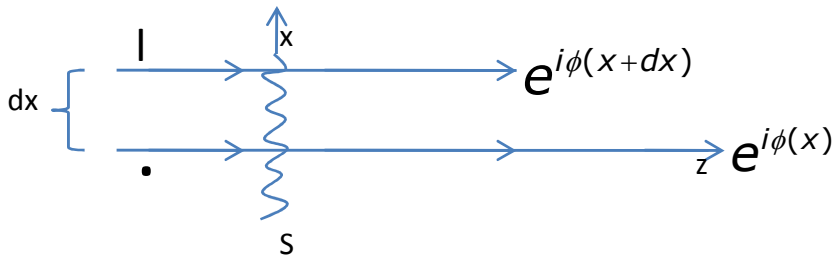
$$\delta = \phi_1 - \phi_0 = n_1 \frac{d}{\cos\theta} k - n_0 \cdot d \cdot k$$

4.6 Nomarski/Differential Interference Contrast Microscopy

- By varying the position of Wollaston prism

one can adjust $\delta = \phi_1 - \phi_0$

- Phase Shift through the sample:



- becomes:

$$E_{Total} = A_n e^{i(\phi_n + \delta)} + A_0 \cdot e^{i\phi_0} =$$

$$\simeq A_0 e^{i\phi_0}[1 + e^{i(\phi_{11} - \phi_0 + \delta)}] \qquad (4.23)$$

4.6 Nomarski/Differential Interference Contrast Microscopy

- The Intensity in the image plane (as a function of displacement x).

$$I(x) = 2I_0(1+\cos[\phi(x+dx)-\phi(x)+\delta]) \qquad (4.24)$$

- Note: For small ϕ , best results obtained for $\delta = \frac{\pi}{2}$

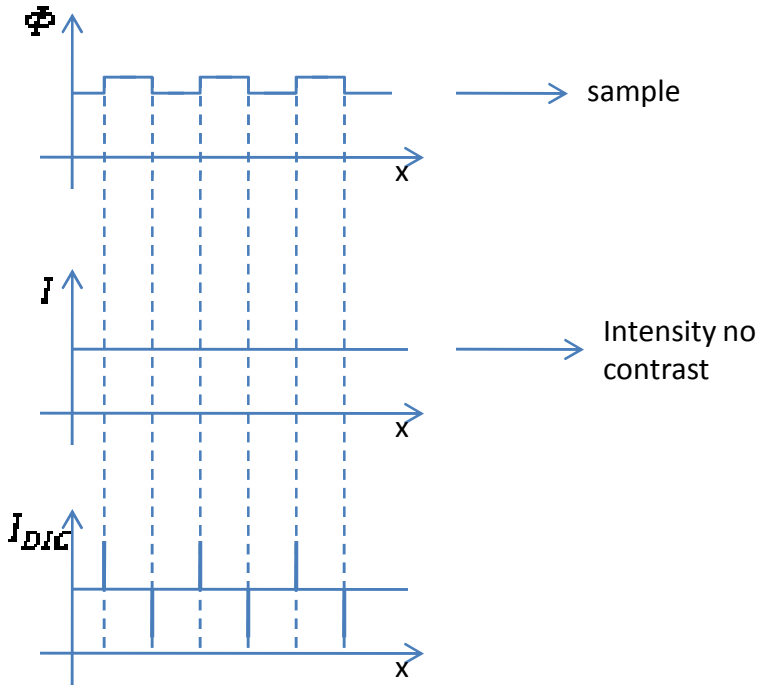
$$I(x) = 2I_0(1+\sin[\phi(x+dx)-\phi(x)])$$

$$\simeq 2I_0[1+dx\cdot\frac{\phi(x+dx)-\phi(x)}{dx}] \qquad (4.25)$$

- So the final intensity distribution is related to the gradient of the phase: $\frac{\delta\phi(x)}{\delta x}$

4.6 Nomarski/Differential Interference Contrast Microscopy

- DIC is a very sensitive to edges, even though the actual phase shifts are “small”.
- Example:



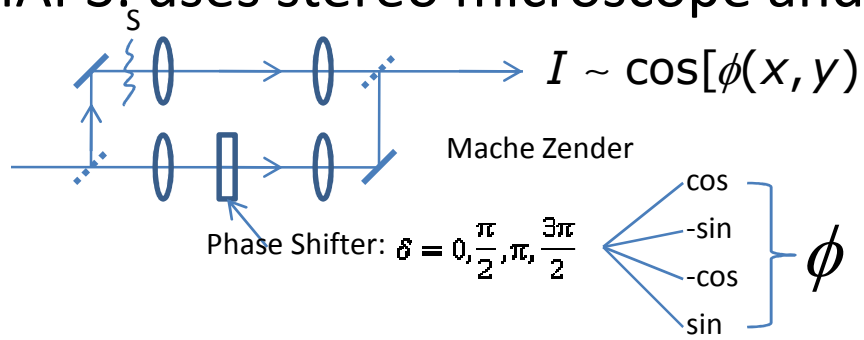
- Phase contrast and DIC heavily used today, especially for investigating live biological structures (cells) noninvasively.

4.7 Quantitative Phase Microscopy

- PC &DIC are great, but qualitative in terms of
- Knowing $\phi(x, y)$ quantitatively offers some advantages, i.e. gives a map of structure density; for homogeneous structures, gives molecular information.
- QPM is a “rather new” domain; several methods so far.
- Main obstacle is noise

4.7 Quantitative Phase Microscopy

a) DRIMAPS: uses stereo microscope and phase shifting



b) Defocusing method: collect 3 images one in focus and 2 defocused

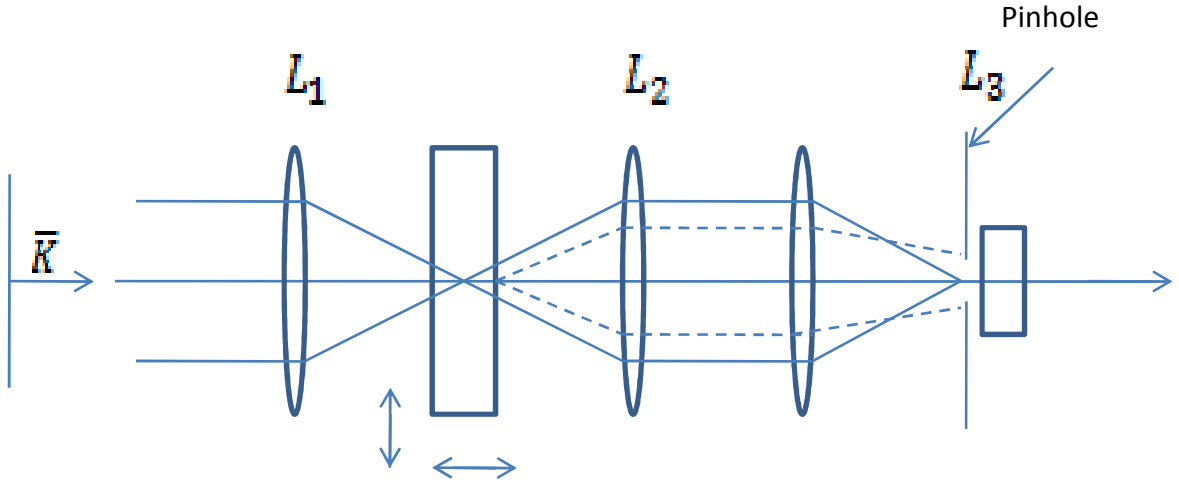
- Uses the irradiance transport equation
- It's called non-Interferometric, but uses the imaging as an interference phenomenon.

c) Spec lab at MIT(2000-2004)

- Various QPM techniques: differential, actively stabilized, PC + phase-shifting

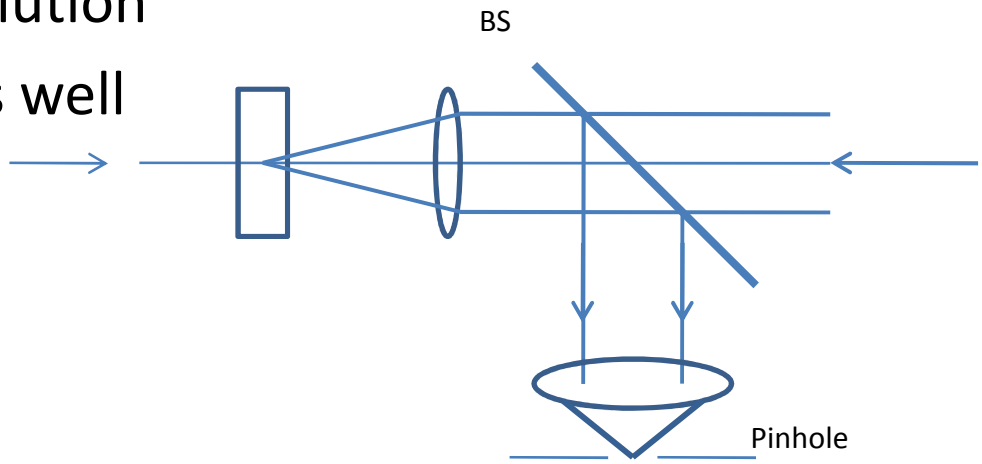
4.8 Confocal Microscopy

- So far, we discussed full- field imaging, i.e obtaining the entire image at once(great feature: imaging as a parallel process).
- The image can be recorded point by point also(like TV), sometimes with some advantages.
- Confocal = same focal point for illumination and collection



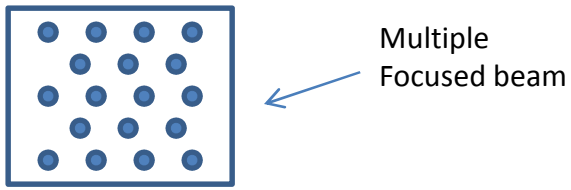
4.8 Confocal Microscopy

- Due to pinhole, light out of focus is rejected, which can create stacks of slices, hence 3D rendering
- Scanning: either by scanning the sample or the beam
- Note:
  - 3D Info
  - large field of view (limited by aperture)
  - up to $\sqrt{2}$ better resolution
- ! It works in reflection as well



4.8 Confocal Microscopy

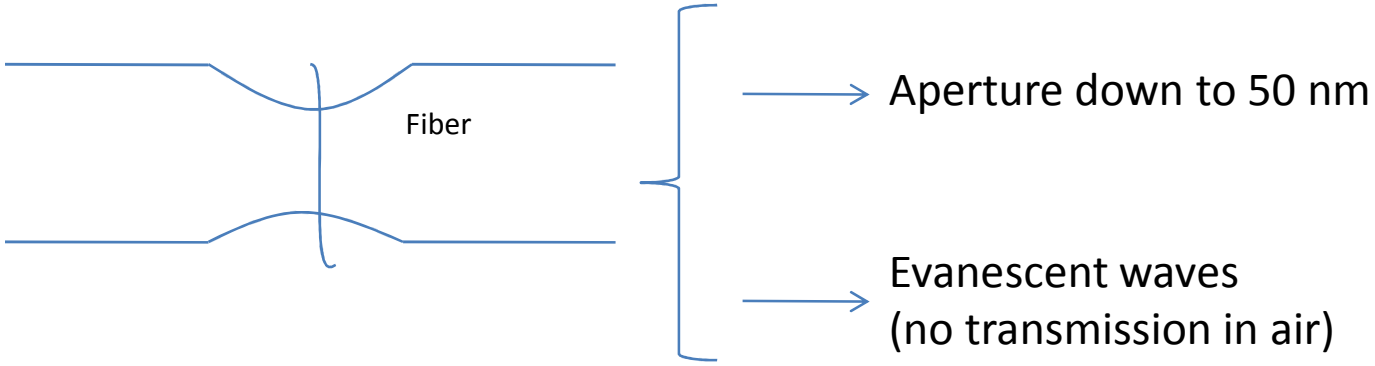
- Recent development: Multi Foci
  - Improves acquisition time
  - Need more power → Trade-off



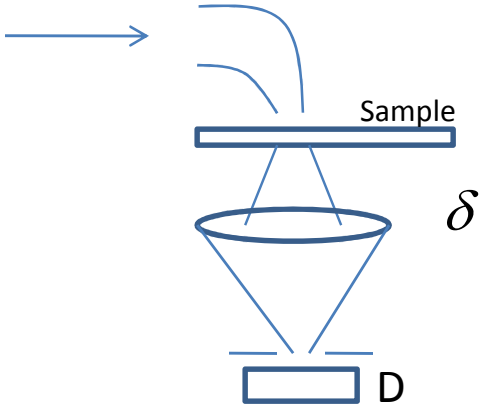
- Confocal can provide many frames/seconds(video rate)
- Leading to 4D imaging(x,y,z,t)

4.8 Confocal Microscopy

- **Near Field Scanning Optical Microscope(NSOM)**
  - Continuation of confocal & AFM
  - Tappered fiber as cantilever :



4.8 Confocal Microscopy



$$\delta = 50 - 100nm$$

→ Evanescent waves couple into sample

→ Became propagating

→ Not limited by diffraction

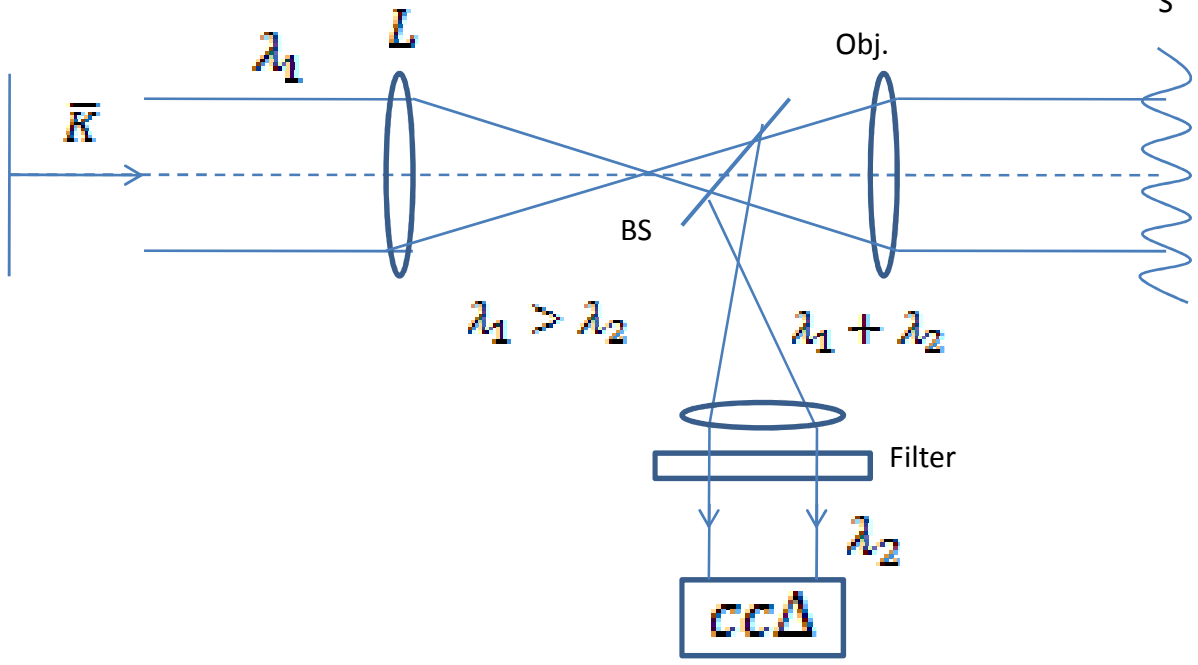
- Drowback: scanning time; difficult in liquids

4.9 Fluorescence Microscopy

- Illumination and emission have different wavelengths
- Endogenous “Fluorophos” eg. NA ΔH
- Most commonly exogenous
- Recently:
  - GFP technology (given fluorescent protein)
  - genetically encoded, fused with NA
  - GFP live cell imaging
  - allows for multiple “fluorophores”
  - dynamic monitoring of processes(cell signaling)

4.9 Fluorescence Microscopy

- Fluorescence adds specificity to the measurement. (organelle dynamics, process specific)
- Typically- epi-fluorescence(reflection geometry)



- Filter blocks the excitation light

4.9 Fluorescence Microscopy

- Full-Field is limited to thin samples
- Combine fluorescence &confocal leads to deeper penetration
- Issues when imaging live cells:
  - acquisition time, sensitivity, damage.
- Photo-bleaching can produce cell damage:
  - limit duration of illumination→need efficiency sensitivity
  - use intensified CCD
- Acquisition speed: improve with multi-foci &Nipkow disk scanning

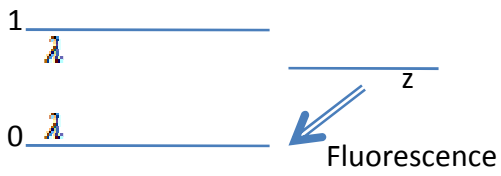
4.9 Fluorescence Microscopy

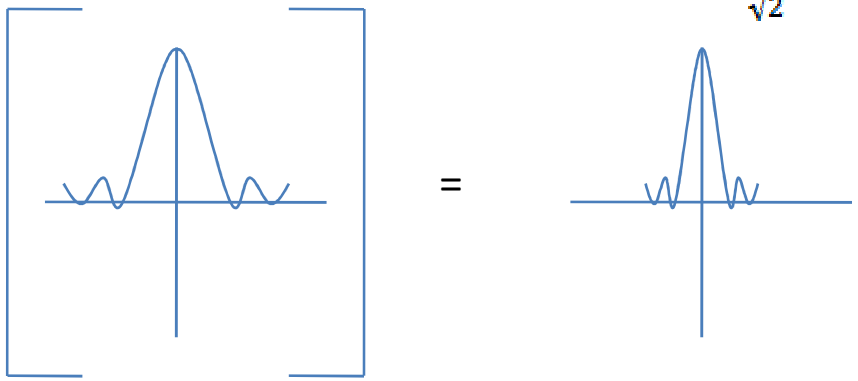
- Other Fluorescence Techniques:
  - Total internal reflection
  - FCS-Fluorescence correlation spectroscopy
  - FRAP-Fluorescence recovery after photoblocking
  - FRET-Fluorescence resonance energy transfer.
  - FLIM-fluorescence lifetime imaging.
  - STED-Stimulated emission depletion → 100nm spot
  - STED+ 4Pi confocal microscopy 33nm diffraction spot single molecule imaging.

4.10 Multiphoton Imaging

- 2- Photon laser scanning microscopy
- Nonlinear process
- Deep Penetration
- Requires high power distribution

$$I_f \sim I^2_x \longrightarrow$$ Improves Resolution





Illumination spot is reduce by $\cong \frac{1}{\sqrt{2}}$

4.10 Multiphoton Imaging

- 2nd harmonic Imaging-recent:
- Endogenous SNG molecules(e.g collagen)
- $P = \chi^{(L)} \cdot E^2$ coherent process (phase matching)
- Same advantage of smaller illumination spot