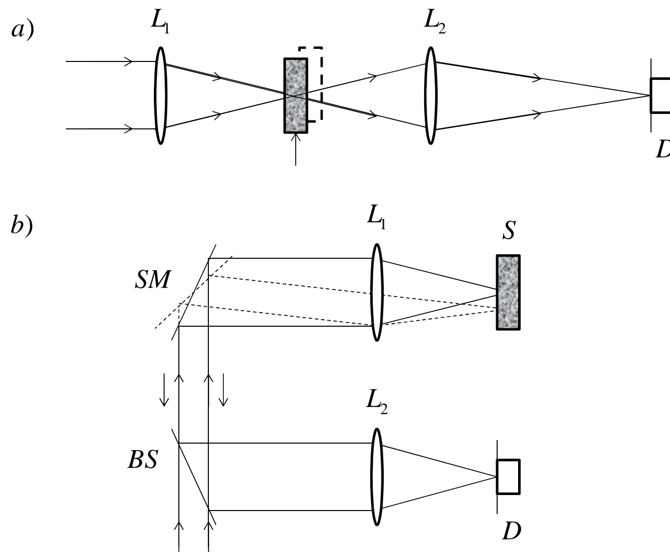
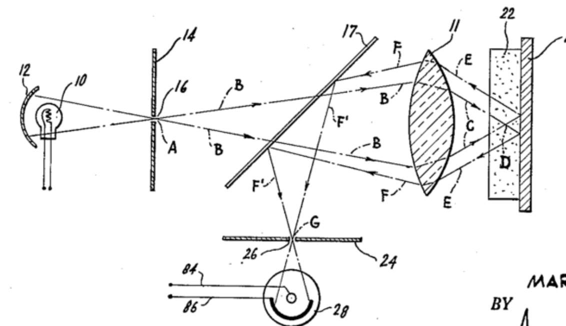
12. CONFOCAL MICROSCOPY

- Confocal microscopy can render depth-resolved slices through a 3D object by rejecting much of the out of focus light via a pinhole.
- The image is reconstructed serially, i.e. point by point, using a single photodetector, rather than in parallel (in bright field, phase contrast, etc.), where a 2D image is recorded via a detector array, such as a camera.
- The illumination light is focused down to a small spot at the sample plane, while the detector records the light originating from the same spot, hence the name *confocal*.
- Works with intrinsic/scattering contrast and fluorescence (much more common)
- Wilson, T. and C. Sheppard (1984). *"Theory and practice of scanning optical microscopy"*, London, Academic Press.

12.1. Principle.

- The principle of confocal microscopy was described prior to the invention of lasers [Minsky, Patent 1957].
- Today most confocal systems use lasers for illumination ("Laser scanning confocal microscope"). Can operate either in transmission or reflection, as shown in Fig. 1.

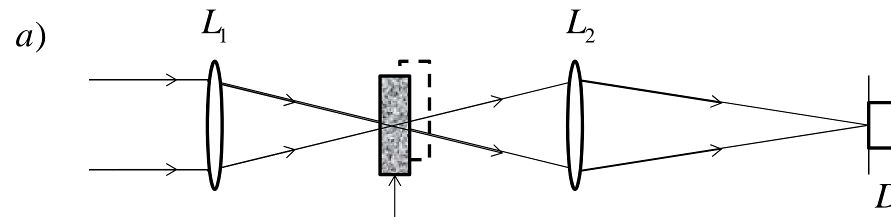




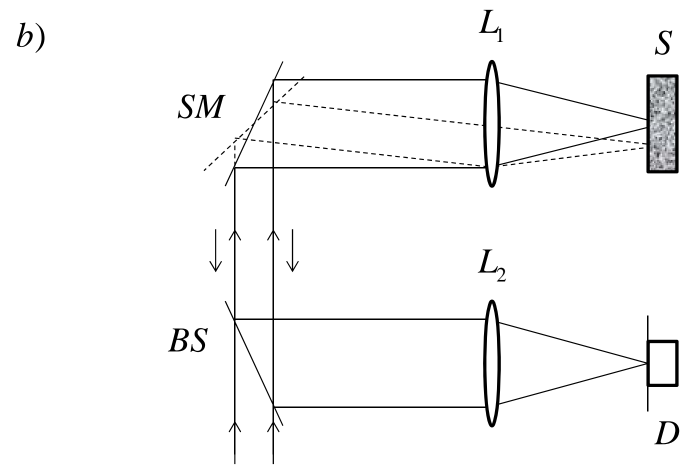
(wikipedia)

Figure 1. a) Confocal imaging in transmission: L1,2 lenses, specimen, D detector; arrow indicates the plane of focus. b) Confocal imaging in reflection (epi-illumination): L1,2 lenses, S specimen, BS beam splitter, SM scanning mirror.

- Out of focus light is rejected by the <u>pinhole</u> in front of the detector, which is placed at a plane conjugate to the illumination plane
- The image reconstruction is performed either by scanning the sample or the beam.
- Of course, scanning the beam can be made much faster by using galvo-mirrors; translating the specimen is limited by inertia.
- Note that the transmission geometry (Fig. 1a) requires that the specimen is translated. WHY?



- Scanning the illumination beam requires that the pinhole translates synchronously, which is impractical.
- in reflection, the light is automatically "descanned."



- For imaging the specimen in the second transverse direction, a second scanning mirror is necessary.

- Clearly, the “epi” geometry (reflection, Fig. 1b) is the main choice for *fluorescence* confocal microscopy, because the excitation light can be filtered out more efficiently than in transmission.

12.2. Resolution.

- Since confocal microscopy renders 3D images, we must define both transverse and longitudinal resolutions (Fig. 2),

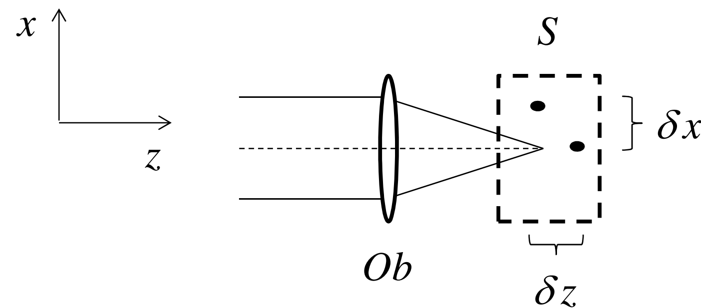
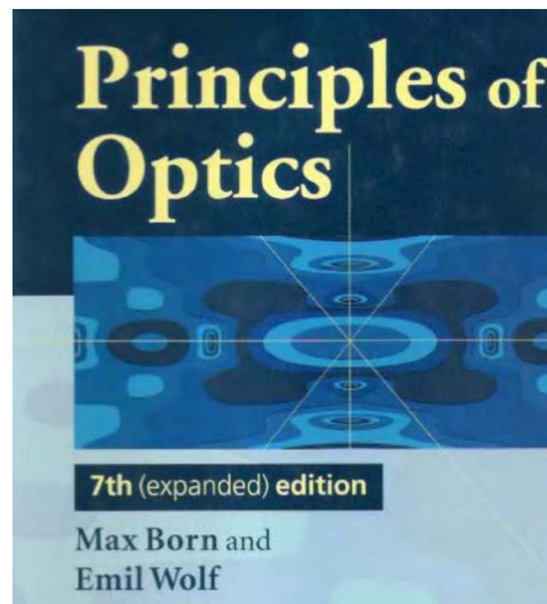


Figure 2. Transverse (dx) and longitudinal (dz) resolution in confocal microscopy: Ob objective, S specimen.

- The closest two points that can be "resolved" perpendicular to and along the optical axis, respectively. What we consider *resolved* is subject to convention.
- Still, no matter the convention, the Green's function for the confocal system (impulse response) is given by the 3D distribution of field in the vicinity of focus.

Side note: in 1956, Linfoot and Wolf provided the field near focus for a plane wave incident.

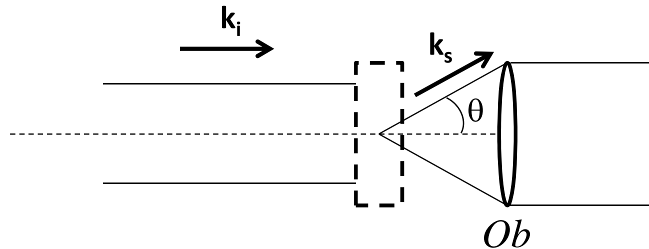


Let's look at the scattering problem: Recall the Born approximation

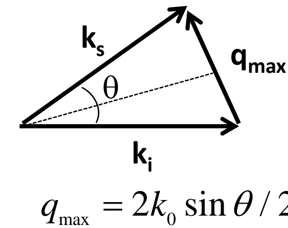
$$F(\mathbf{r}) \leftrightarrow U(\mathbf{q}); \quad \mathbf{q} = \mathbf{k_s} - \mathbf{k_i}$$

$$\delta x \propto \frac{1}{\Delta q_x}; \quad \delta z \propto \frac{1}{\Delta q_z}$$

Transmission- plane wave incident

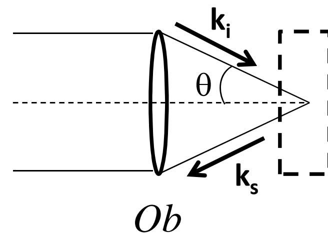


Max. momentum transfer

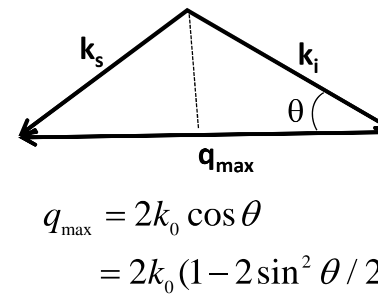


$$q_{\max} = 2k_0 \sin\theta/2$$

Reflection- Gaussian beam



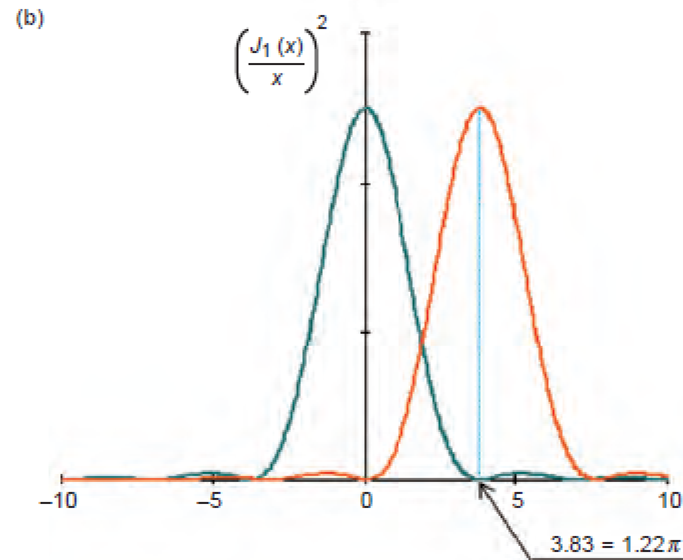
Max. momentum transfer



$$q_{\max} = 2k_0 \cos\theta$$
$$= 2k_0(1 - 2\sin^2\theta/2)$$

Better z-sectioning!

- Abbe's criterion:



$$\delta x = 1.22\frac{\lambda}{\sin\theta_{Condenser} + \sin\theta_{Objective}}$$

$$= 0.61\frac{\lambda}{\sin\theta_{Objective}} \qquad (12.1)$$

$$\delta z = \frac{2\lambda}{\left(\sin\theta_{Objective}\right)^2}$$

- where λ is the wavelength in vacuum ; axial resolution has a stronger dependence on NA.

    - Recall Gaussian beams

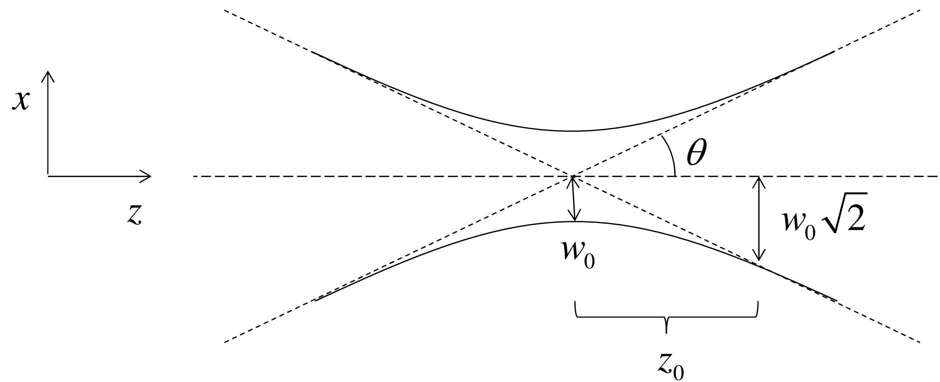


Figure 4. Diffraction of a Gaussian beam: w0 minimum waist, z0 Rayleigh range , theta diffraction angle.

- $$z_0 = \frac{\pi w_0^{\;2}}{\lambda}; \qquad z_0 = \frac{\lambda}{\pi\theta^2}$$
  $$w^2 = w_0^{\;2}\left(1 + \frac{z}{z_0}\right); \quad w_0 = \frac{\lambda}{\pi\theta} \qquad (12.2)$$

- Aberrations, both chromatic and geometrical, lower the resolving power of the confocal microscope. Aberration correction, adaptive optics, is a critical issue especially when operating at very high NA (current research)
- However, even with an instrument that is capable in principle of producing high resolution images, the amount of information in the final image depends also on how different the various regions of instrument appear, i.e. on *contrast*.

12.3. Contrast.

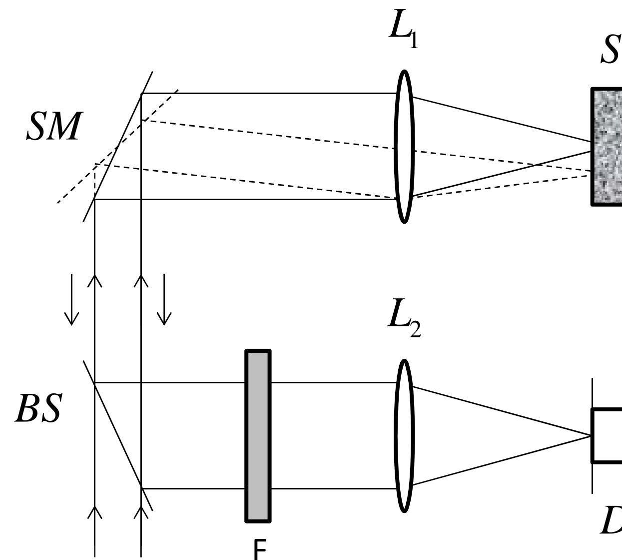
- Recall:

  -Contrast $C_{AB} = |S_A - S_B|$

  -Contrast to noise
  $$\mathrm{CNR}_{AB} = \frac{C_{AB}}{\sigma_N} = \frac{|S_A - S_B|}{\sigma_N}$$

- Note that confocal microscopy is intrinsically *intensity-based* method; therefore, we anticipate low contrast when operating in *reflection mode* (non-fluorescence), as the refractive index variation within tissues is low.
- Out of focus light blurs the image, lowering both the contrast and resolution
- Small pinhole, better contrast, less light➔ compromise

- Most commonly, confocal microscopy is used with fluorescence➔ higher contrast
- Theoretically, the contrast in a fluorescence image is infinite, i.e. untagged structures (background) give zero signal. However, practical issues related to dark signals in the detector, limited dynamic range, saturation, and out of focus light , lower the contrast.



12.4. Further Developments.

- Past and current research in confocal microscopy deals mainly with achieving higher acquisition rates and deeper penetration. The <u>“spinning disk”</u> confocal microscope was built upon an idea due to Nipkow (Nipkow, 1884). It was a rotating disk with perforated pinholes arranged in a spiral, such that the entire field of view is scanned upon one rotation of the disk, which allows fast acquisition rates

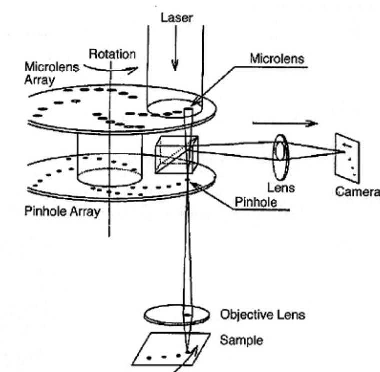


FIGURE 2-71. Yokogawa high-speed confocal system. Microlenses on a second Nipkow disk increase disk transmission to 40–60% instead of a fraction of a percent as in conventional, single-Nipkow-disk systems. The microlens and pinhole arrays are patterned to give a homogeneous field with no sign of scan lines. (From Ichihara *et al.*, 1996.)

"Video Microscopy", Inoue and Spring, 1997

- The penetration depth has been improved significantly once confocal microscopy was combined with nonlinear optics, in particular <u>two-photon fluorescence</u> [Masters, B. R. and P. T. C. So (2008)];

12.5. Limitations

- Serial
- Low intrinsic contrast
- Fluorescence: photobleaching, photoxicity
- High illumination irradiance
- Still, fluorescence confocal microscopy offers a great tool for biological studies, especially cell biology.
- **But- deconvolution microscopy➔ better images, slow…**
- **Pawley, J. B. (2006). Handbook of biological confocal microscopy. New York, Springer.**

EXAMPLES (Price, R. L. (2011). Basic confocal microscopy. New York, Springer.)

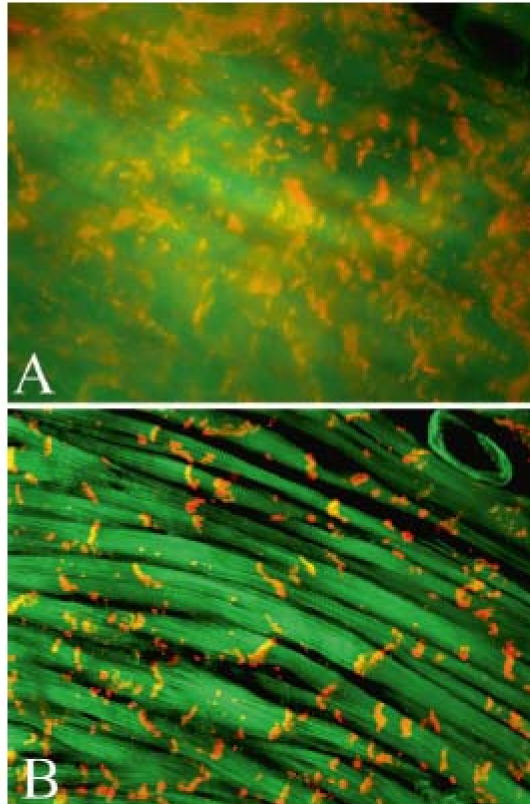
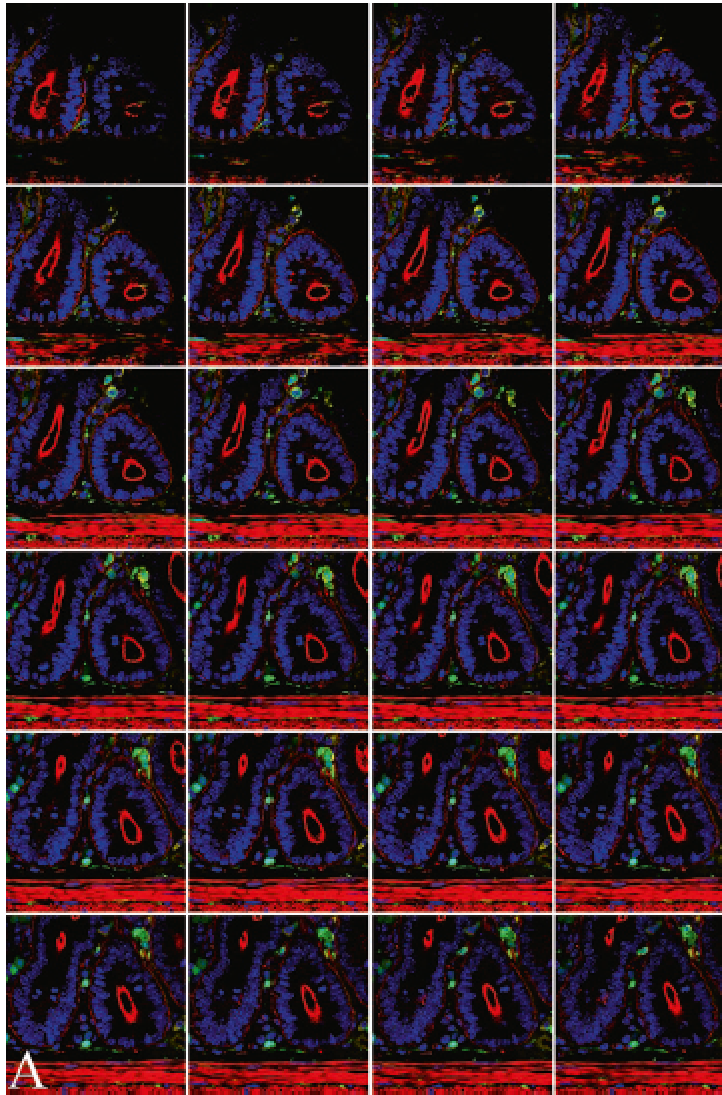


Fig. 1.3 Widefield fluorescent (*top*) and single photon confocal scanning laser microscope (CSLM) (*bottom*) images taken from a 100-μm thick vibratome section of mouse heart that has been stained for f-actin (*green*) and connexin 43 (*red*). In the widefield image out-of-focus light that contributes to the formation of the image significantly decreases the resolution and contrast of the image. Use of the pinhole in the confocal image to remove the out-of-focus light results in an image of much higher contrast and resolution as shown by the striated pattern of the myocyte sarcomeres and distinct cell: cell junctions labeled by the connexin 43 antibody



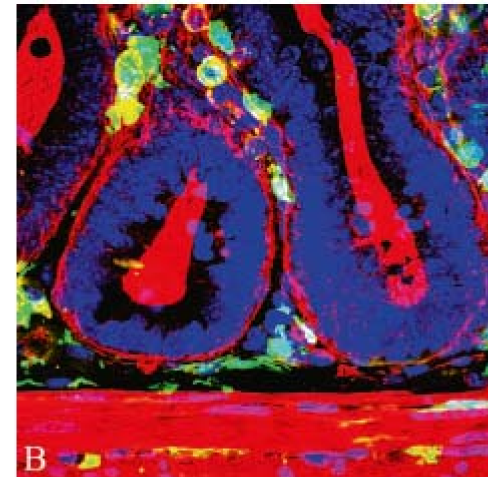


Fig. 1.4 Confocal optical sections (Z-series) through a section of intestine stained with multiple fluorescent dyes. Images were collected at 1 μm intervals through a 50-μm thick section of tissue and every other section (2 μm intervals) are shown in (**a**). All sections were then projected into a single composite image as shown in (**b**). The procedures for collection and projection of data sets are discussed in later chapters. *Blue* – DAPI stain for nuclei, *red* – f-actin stain, *green* – green fluorescent protein, *yellow* – mRNA stabilizing protein

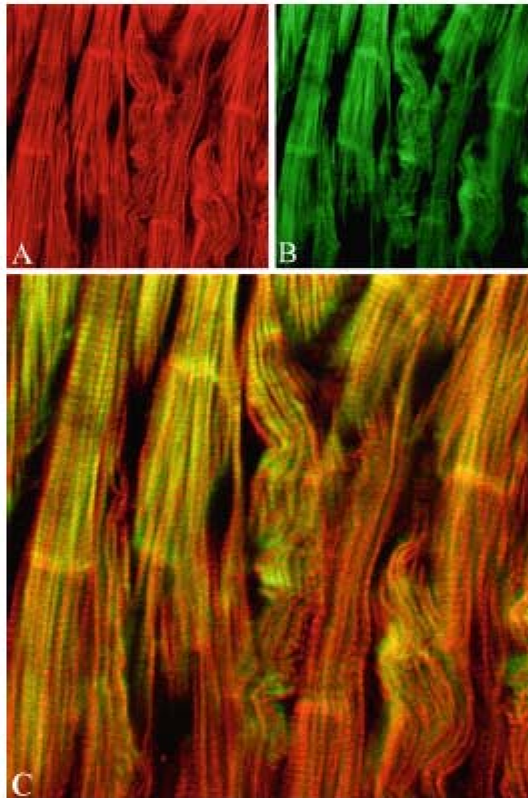


Fig. 3.7 A section of adult mouse heart labeled with 633 phalloidin (**a**) and 405 phalloidin (**b**). Both are labeling f-actin in the cardiac myocytes but due to chromatic aberrations in the system, the *red* and *green* signals do not show 100% overlap as shown in the merged image (**c**)