Super-resolution Optical Microscopy

Chau-Hwang Lee（李超煌）

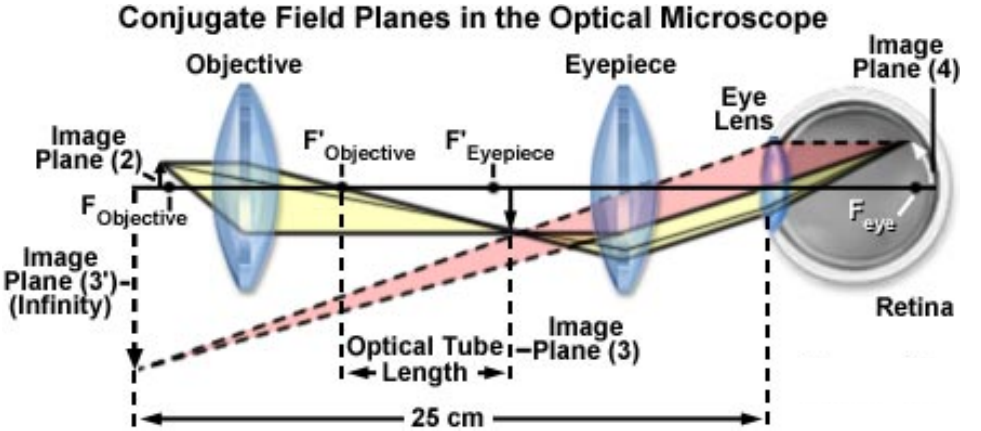
Associate Research Fellow

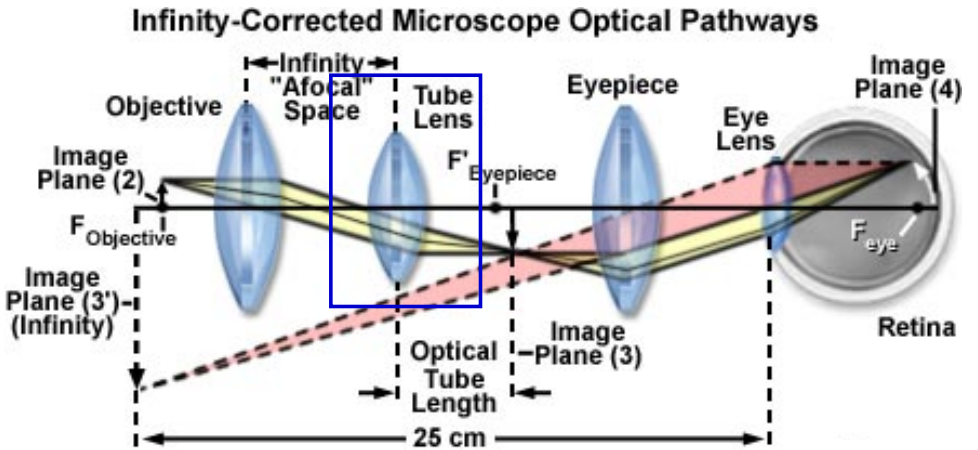
Research Center for Applied Sciences, Academia Sinica
128 Sec. 2, Academia Rd., Nankang, Taipei 11529, Taiwan

E-mail: clee@gate.sinica.edu.tw

Principles of optical imaging

Image formation in an optical microscope





Images are from http://micro.magnet.fsu.edu/

Abbe's image formation theory

Gratings represent the Fourier components of an object.

The diffraction patterns of every Fourier component interfere at the image plane to form the image.

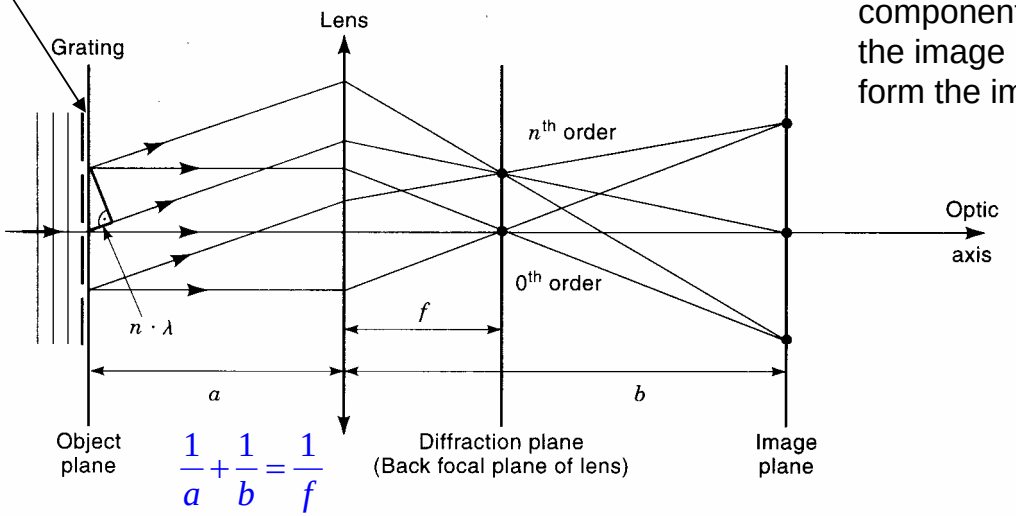


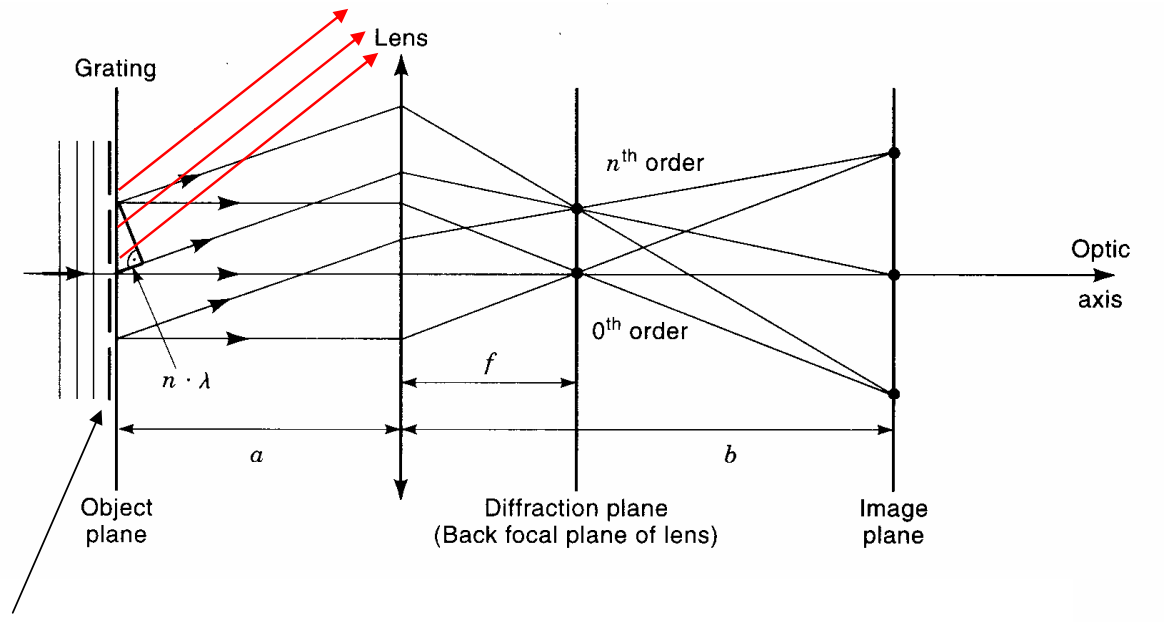
Figure 5-14

Abbe's theory for image formation in a light microscope. An objective lens focused on a grating ($2f > a > f$) in the object plane produces a magnified real image of the grating in the image plane. The diffraction plane is located at $1f$ in the back aperture of the lens. An incident planar wavefront is shown. Diffracted nth-order and nondiffracted 0th-order rays are separated in the diffraction plane, but are combined in the image plane.

Ref: D. B. Murphy, *Fundamentals of Light Microscopy and Electronic Imaging* (Wiley-Liss, New York, 2001).

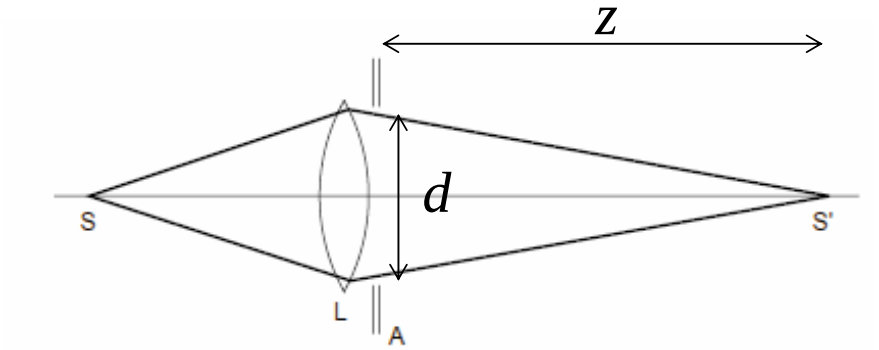
Why does optical resolution have a limit?

Higher spatial frequency components lead to larger diffraction angles. Resolution limit is inverse of the lowest spatial-frequency that cannot pass the lens aperture.



spatial frequency: black/white periods per unit length

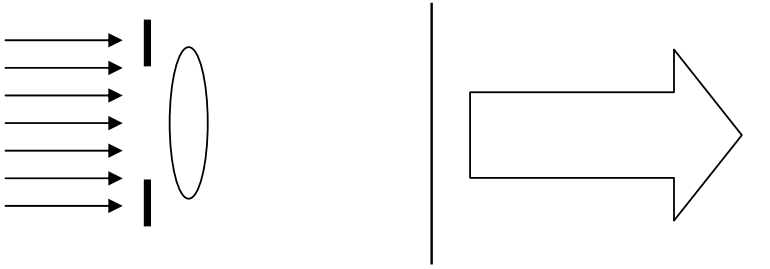
Fraunhofer diffraction of a lens

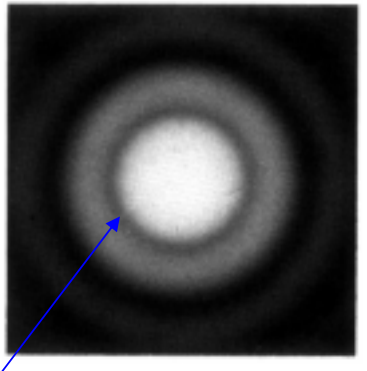


If $z >> d^2/\lambda$, we can describe diffraction of a lens by the Fraunhofer diffraction theory.

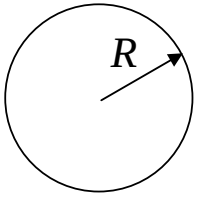
$$\psi(x,y,z) = \left(-\frac{i}{\lambda z}\right)\exp(ikz)\iint_A \psi(x_o,y_o,0)\exp\left[-\frac{ik}{z}(xx_o+yy_o)\right]dx_o dy_o$$

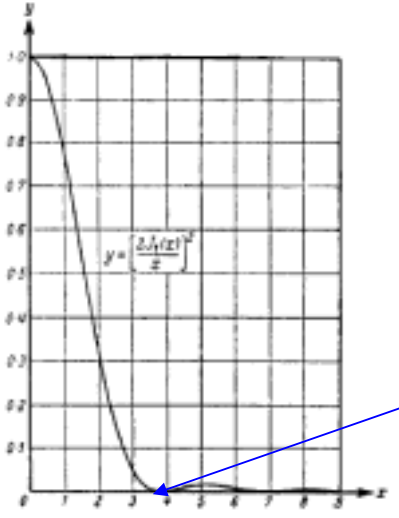
Fraunhofer diffraction: a circular aperture





From R. Guenther, *Modern Optics* (Wiley, 1990).





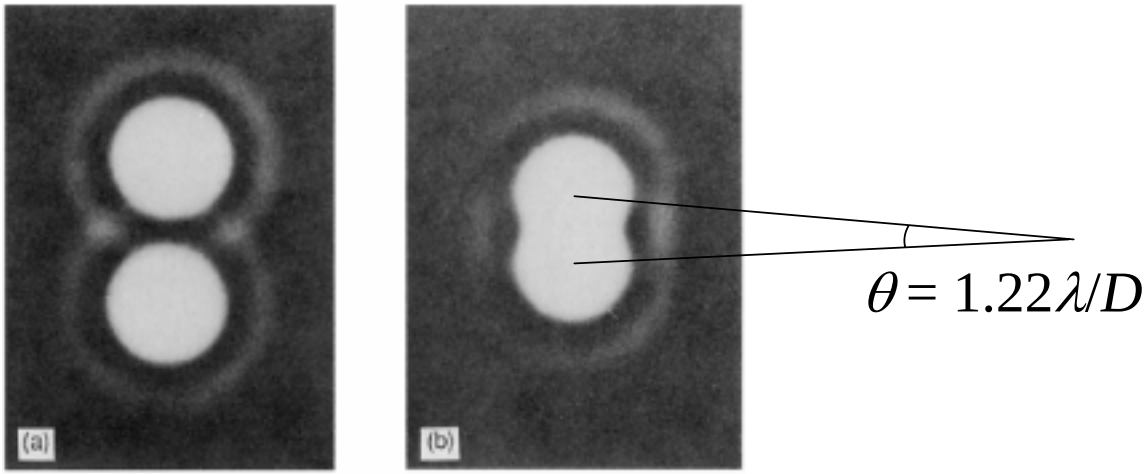
$$I = I_0\left[\frac{2J_1(\rho)}{\rho}\right]^2$$

$$\rho = kR\sin\theta$$

$\rho = 3.832$; or $\sin\theta = 1.22\lambda/2R$

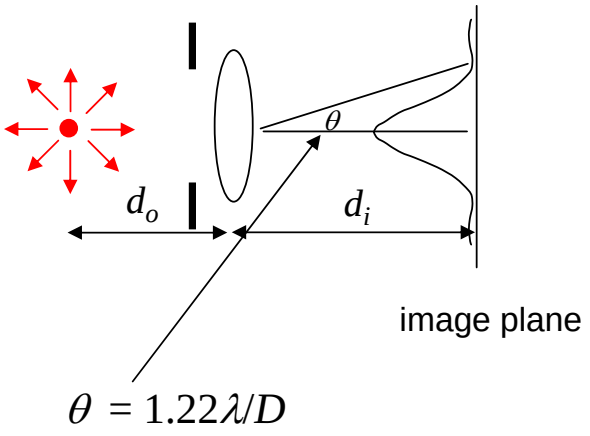
This pattern is called Airy disc.

Angular resolution of an aperture

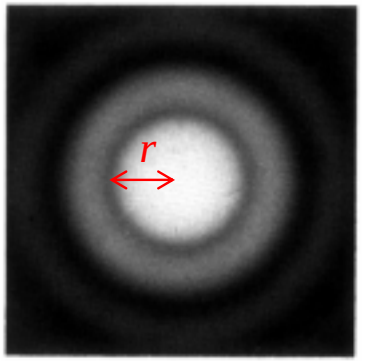


Consider two stars (point sources) in the space imaged by a telescope. The angular radius of the image is $1.22\lambda/D$ ($D = 2R$). This is called the angular resolution of the telescope, also known as the Rayleigh criterion.

Radius of the Airy disc



$\theta = 1.22\lambda/D$

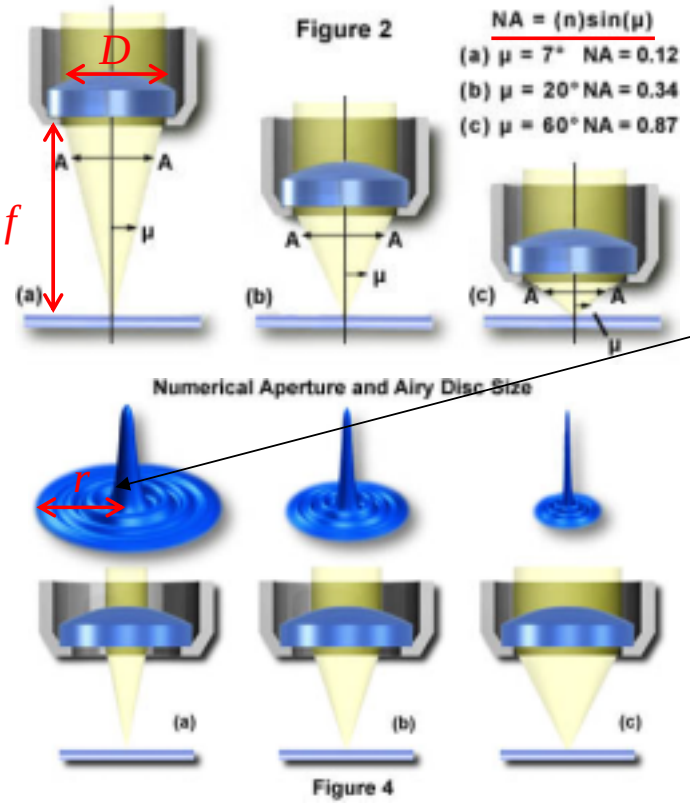


$$I = I_0\left[\frac{2J_1(\rho)}{\rho}\right]^2$$

On the image plane, $r_i = d_i\theta = 1.22\lambda(d_i/D)$. The observer will see the disc as the image of a point. And the radius of the disc on the object plane is thus $r = r_i d_o/d_i = 1.22\lambda(d_o/D)$.

The Airy disc on the object plane is called the point-spread function (PSF) of the lens.

Radius of the Airy disc formed by an objective



For an objective, $d_o \approx f$. So $r = 1.22\lambda(f/D)$. From the left figure, we see NA ~ $D/2f$. Therefore $f/D = 1/2\text{NA}$.

We then have $r = 1.22\lambda/2\text{NA} = 0.61\lambda/\text{NA}$.

To have an image through an objective, one may consider that an image is formed by summation of many "discs." Each disc has a finite radius of r.

$$g(y) = \int h(y-x) f(x)\, dx$$

image, object, Airy disc

Images are from http://micro.magnet.fsu.edu/

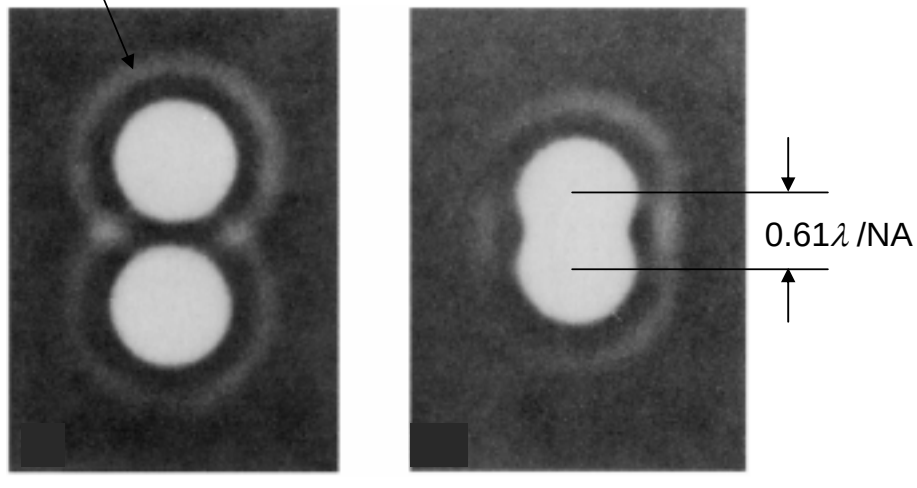
Numerical aperture and resolution

This is called “point-spread” function (PSF).

Rayleigh criterion:

resolution ~ 0.61λ /NA

For dry samples, NA < 1.0



clearly resolved

resolution limit

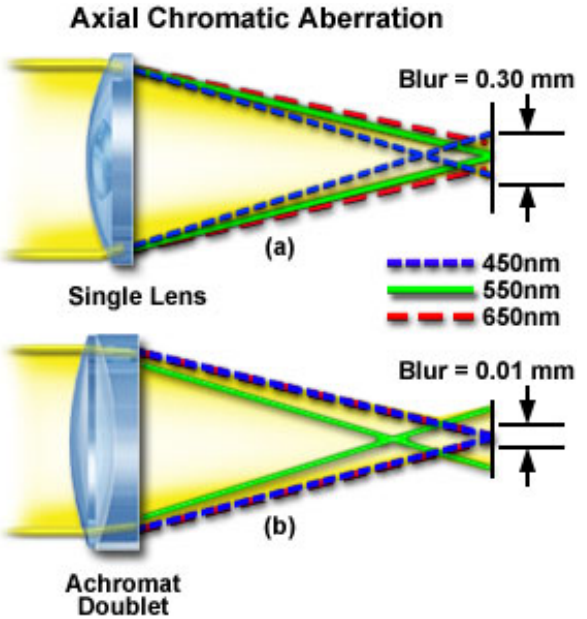
Ref: M. Born and E.Wolf, *Principles of Optics*, 6th ed. (Pergamon, Oxford, 1980), Chap. 8.

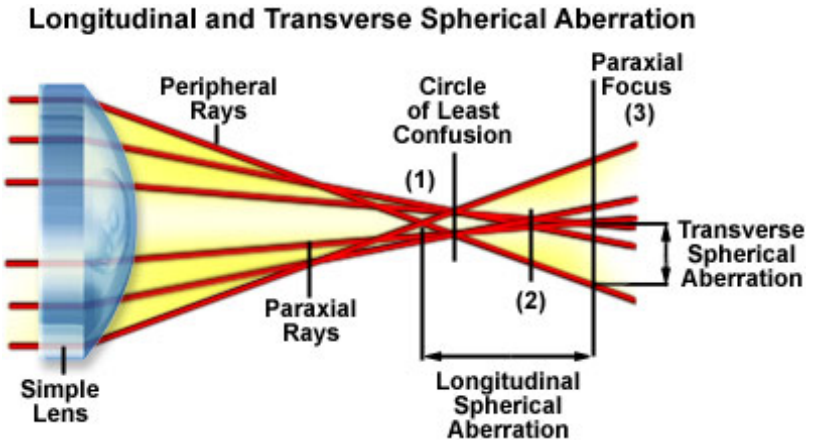
Specifications of an objective

Figure 1

Images are from http://micro.magnet.fsu.edu/

Aberrations





Images are from http://micro.magnet.fsu.edu/

Compensation of objectives

Objective Type	Spherical Aberration	Chromatic Aberration	Field Curvature
Achromat	1 Color	2 Colors	No
Plan Achromat	1 Color	2 Colors	Yes
Fluorite	2-3 Colors	2-3 Colors	No
Plan Fluorite	3-4 Colors	2-4 Colors	Yes
Plan Apochromat	3-4 Colors	4-5 Colors	Yes

Images are from http://micro.magnet.fsu.edu/

Confocal Microscopy

Confocal microscopy

Improved depth resolution

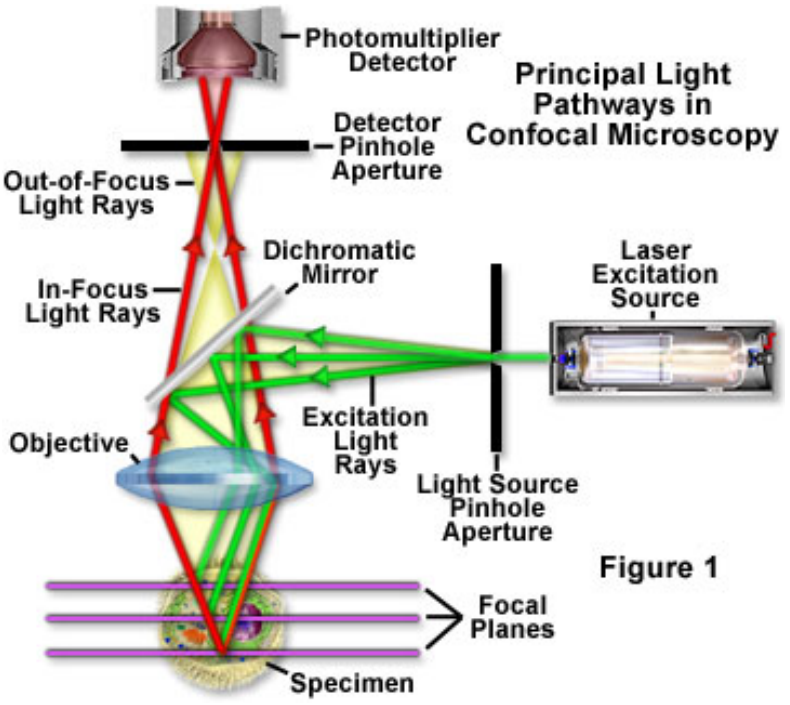
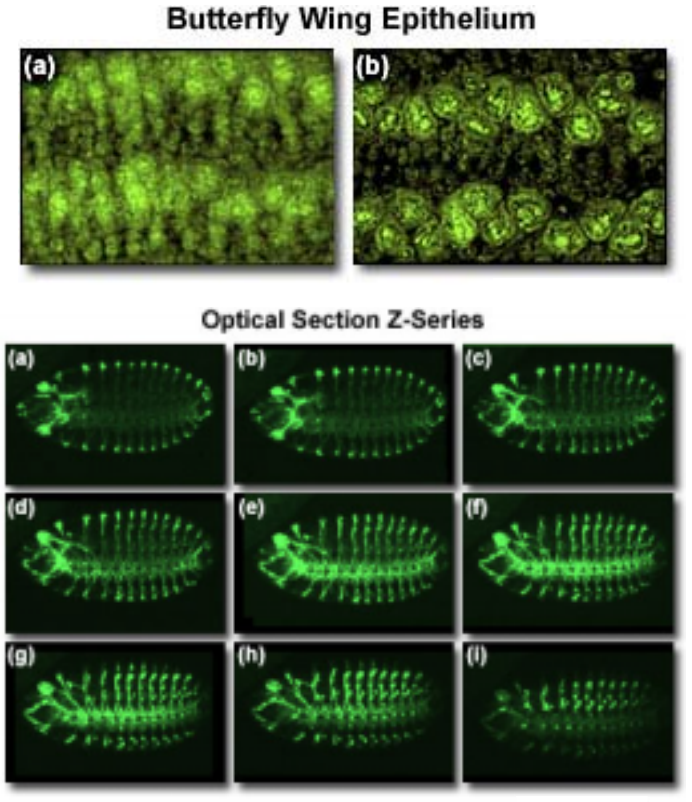
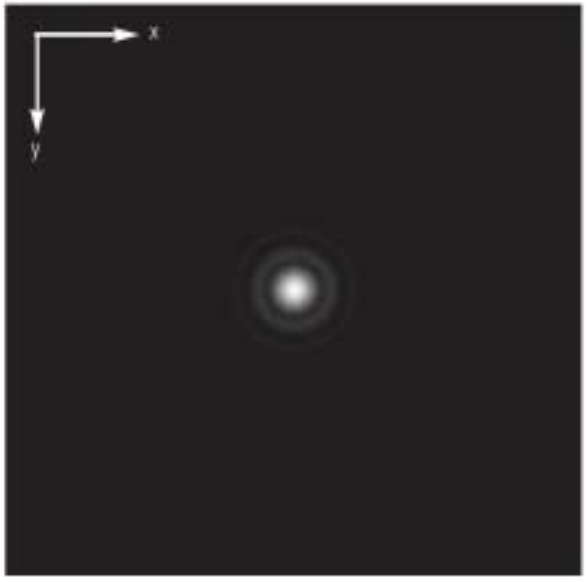


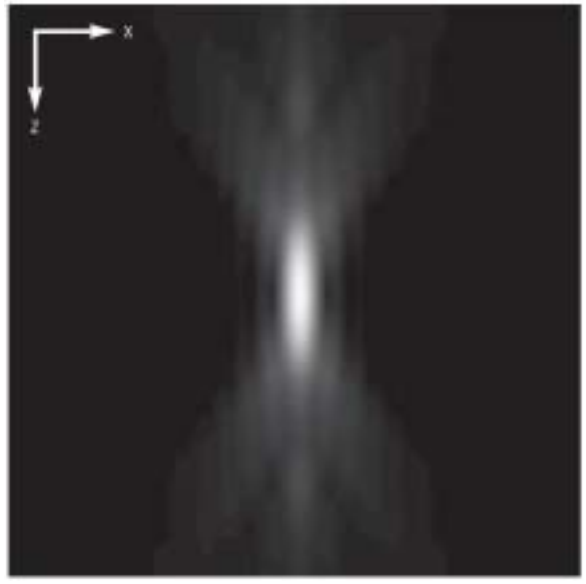
Figure 1



Images are from http://micro.magnet.fsu.edu/

Three-dimensional point-spread function





Lateral:

$$FWHM_{III,lateral} = 0.51 \frac{\lambda_{exc}}{NA}$$

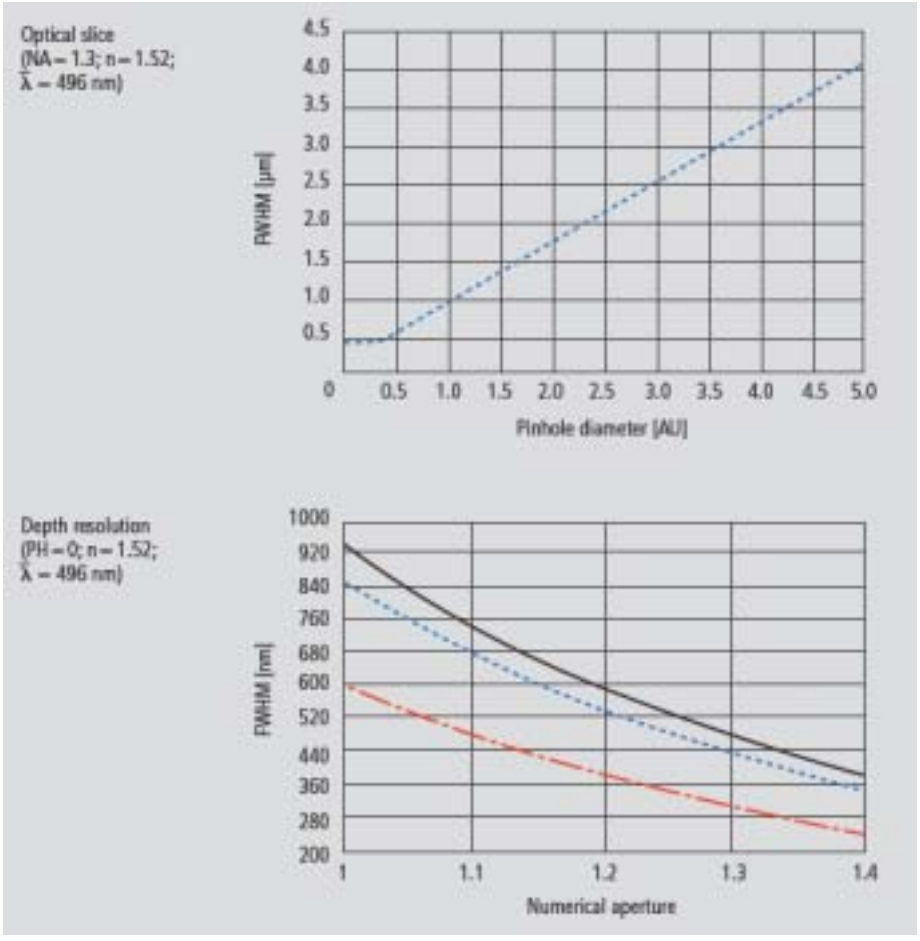
Axial:

$$FWHM_{III,axial} = \frac{0.88 \cdot \lambda_{exc}}{(n - \sqrt{n^2 - NA^2})}$$

n = *refractive index of immersion liquid,*
NA = *numerical aperture of the microscope objective,*
λ_{exc} = *wavelength of the excitation light*

Ref: Carl Zeiss, *Confocal Laser Scanning Microscopy*

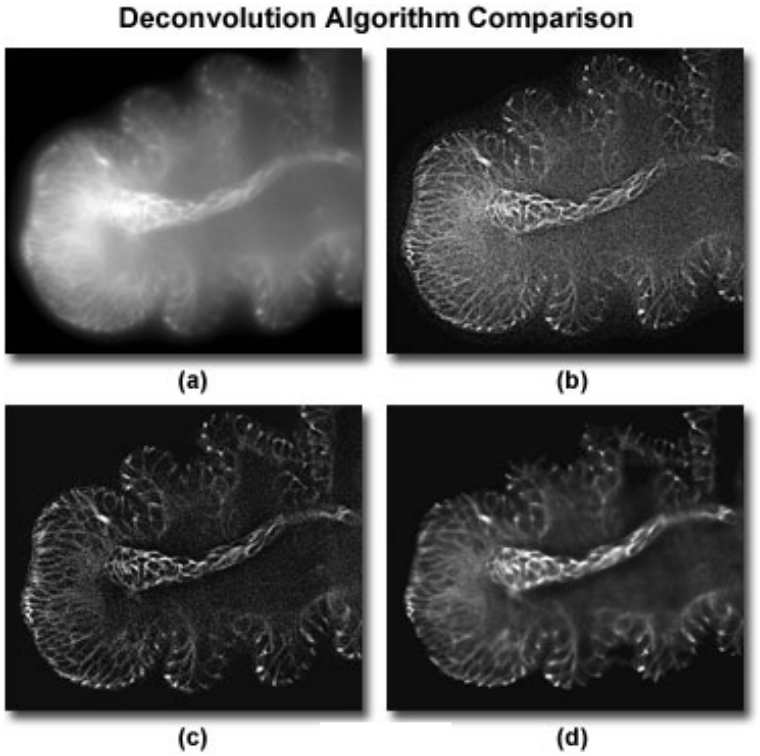
Effect of the pinhole diameter and NA



Ref: Carl Zeiss, *Confocal Laser Scanning Microscopy*

Resolution Enhancement

Deconvolution in optical microscopy



(a) original (raw) image

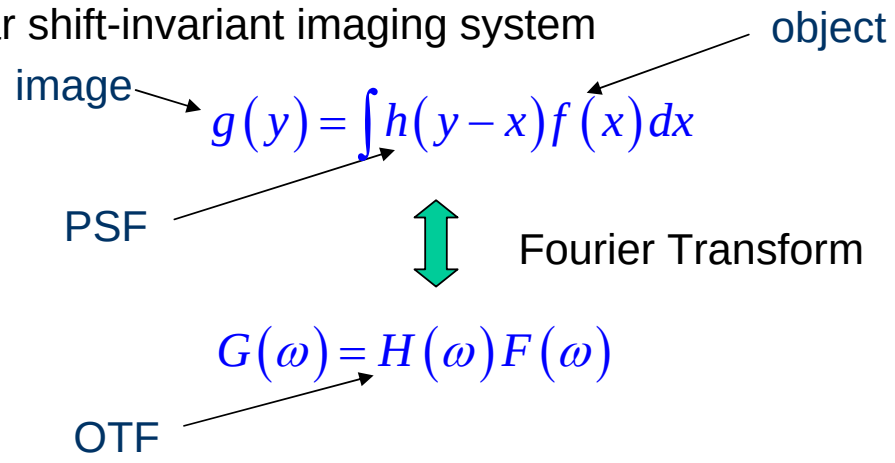
(b) deblurring by a nearest neighbor algorithm

(c) deconvolution by an inverse (Wiener) filter

(d) by iterative blind deconvolution incorporating an adaptive point spread function

Image-formation of an optical system

- Ideal linear shift-invariant imaging system



- Practical linear shift-invariant imaging system

$$g(y) = \int h(y-x) f(x) dx + n(y)$$

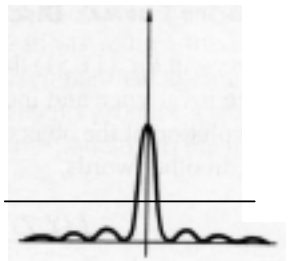
Fourier Transform

$$G(\omega) = H(\omega) F(\omega) + N(\omega)$$

Why direct deconvolution fails

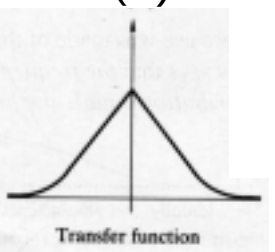
$$F(\omega) = \frac{G(\omega) - N(\omega)}{H(\omega)}$$

h(y-x)



Fourier Transform

H(ω)



- Band-limited character of the optical transfer function (OTF) $H(\omega)$

  ➔ results in divide-by-zero for high spatial frequencies;

  ➔ enhances high-frequency noise.

  ➔ If $H(\omega)$ also has noise, deconvolution does not work at all.

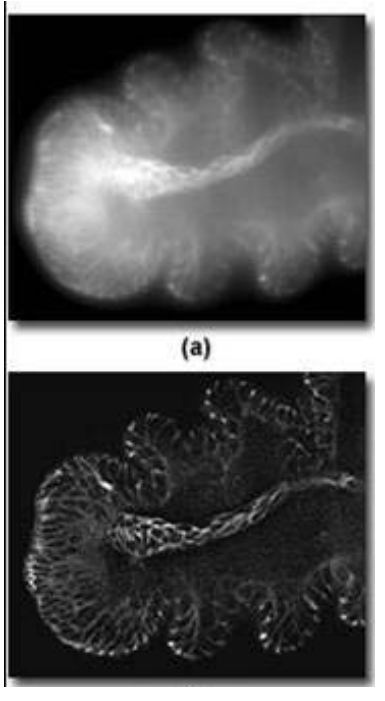
Weiner filtering

By adding a non-zero value *K* in this form, the problem of small values of $H(\omega)$ can be avoided:

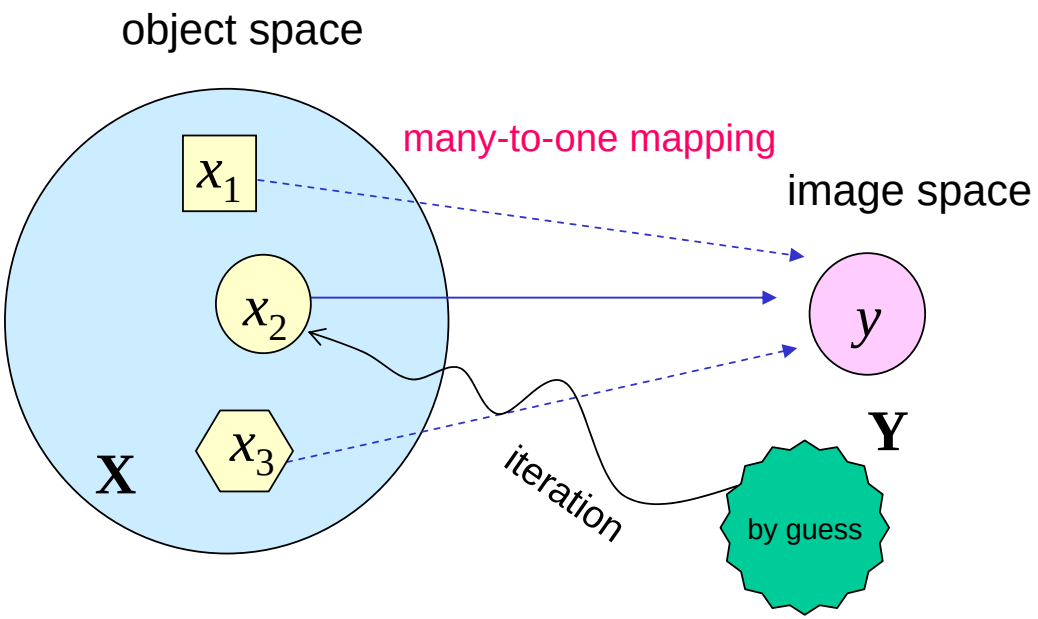
$$F(\omega) = \frac{1}{H(\omega)} \left[\frac{|H(\omega)|^2}{|H(\omega)|^2 + K} \right] \left[G(\omega) - N(\omega) \right]$$

Usually, the value of *K* is determined according to the width of $H(\omega)$ and the magnitude of $N(\omega)$. This is called Weiner filtering.

Weiner filtering is capable of enhancing the edges. However, it reduces the intensity in the flat area.

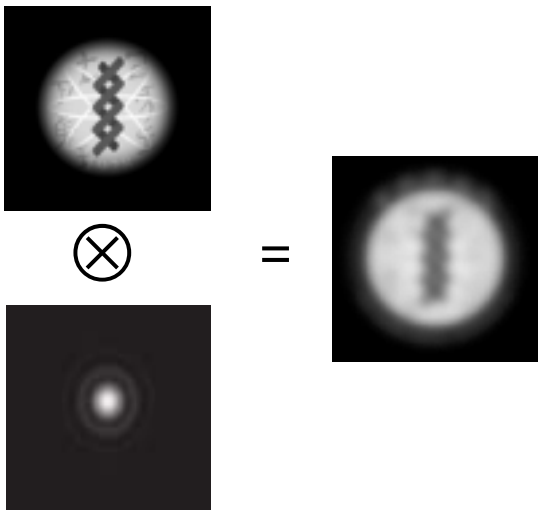


Maximum-likelihood estimation

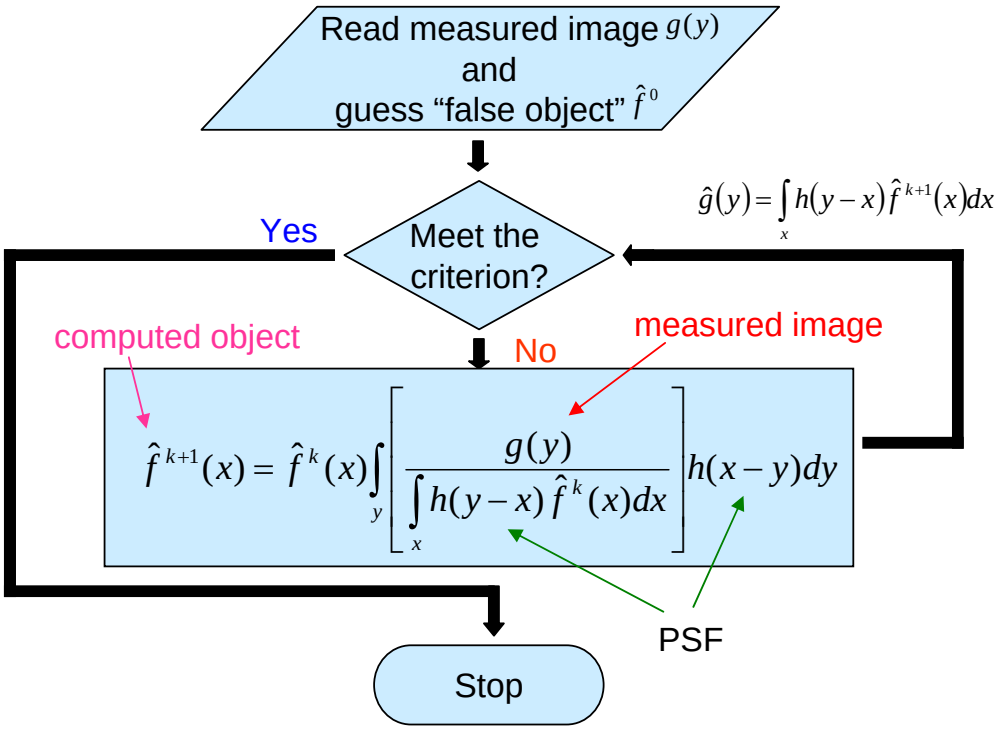


Maximum-likelihood estimation can be used to find a solution for such a “many-to-one” mapping problem.

Iterations of maximum-likelihood estimation

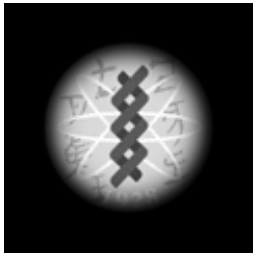


Maximum-likelihood estimation

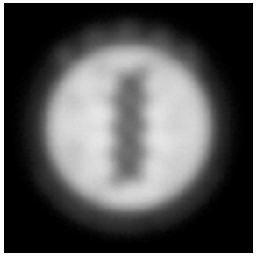


Ref: G. M. P. van Kempen *et al*., *IEEE Eng. Med. Biol*. **15,** 76 (1996).

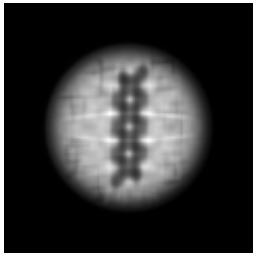
Resolution improvement: high-frequency components



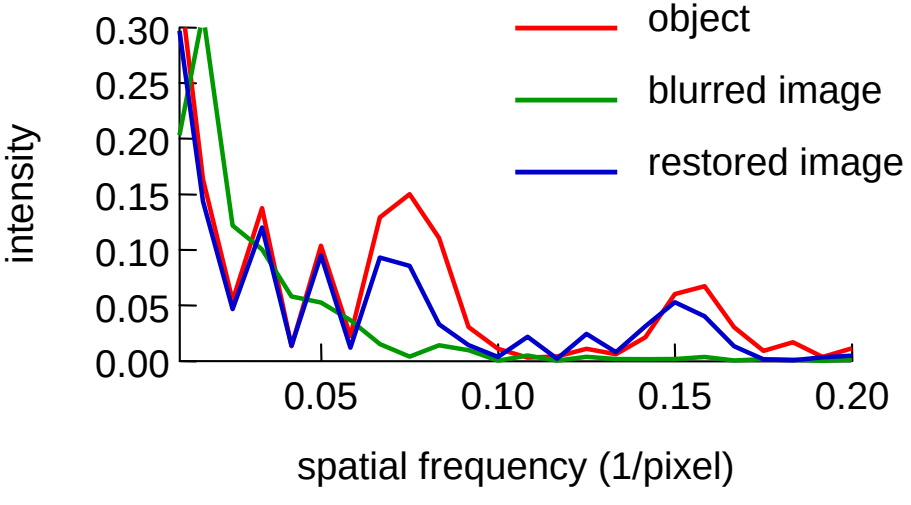
object



blurred image



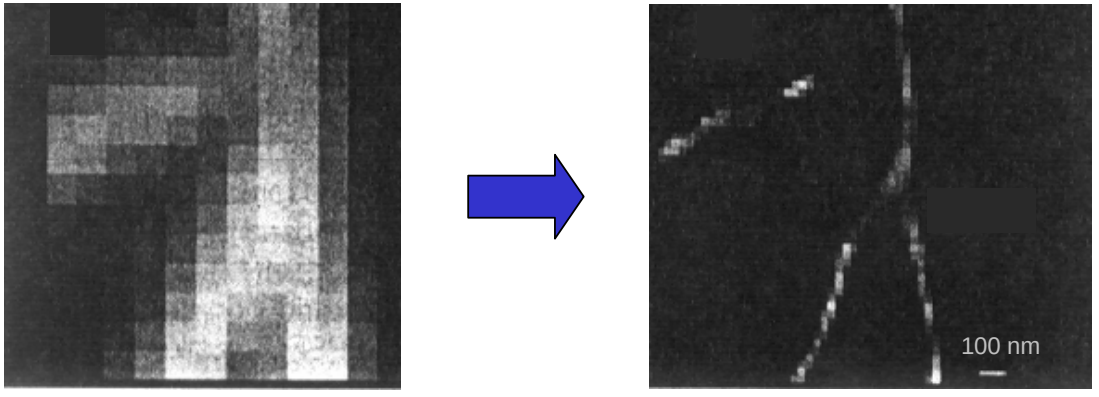
restored image
(1000 iterations)



Super resolution by restoring high-contrast image

To approach super resolution we need high contrast, such as that provided by fluorescent or scattering labelling.

Restored by an iterative algorithm

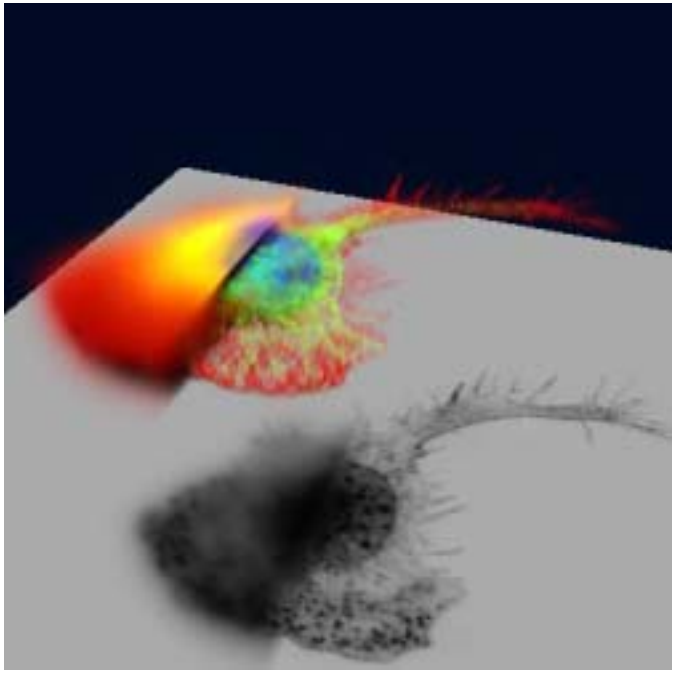


Sample: microtubules in a rat kidney cell

After 2000 iterations, ~ 50-nm lateral resolution is achieved.

Ref: W. A. Carrington *et al*., *Science* **268,** 1483 (1995).

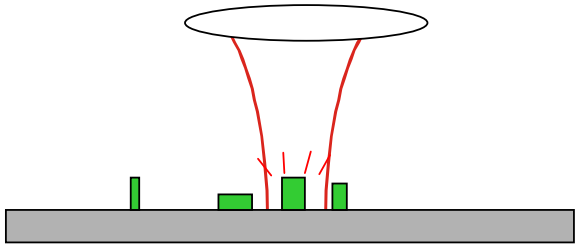
Commercial software products are available



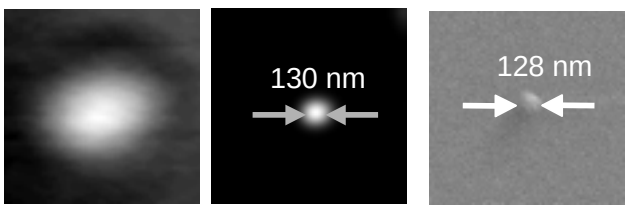
This image is from Scientific Volume Imaging BV. http://www.svi.nl/

Deconvolution based on topographic contrast

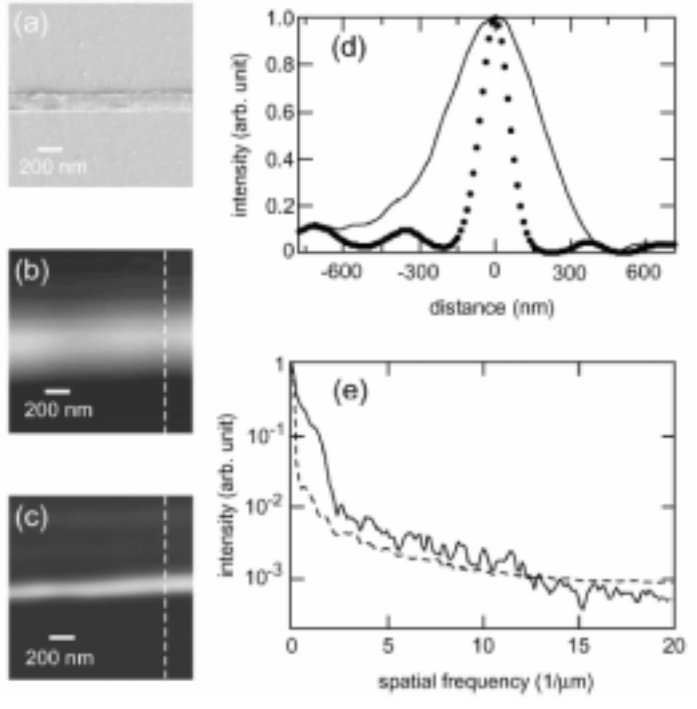
Topographic contrast is also useful for resolution enhancement



Objects smaller than the resolution limit can have high contrast because we can detect the topographic features.



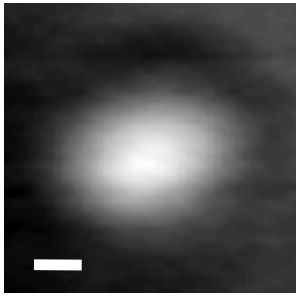
DCM image restored image SEM image



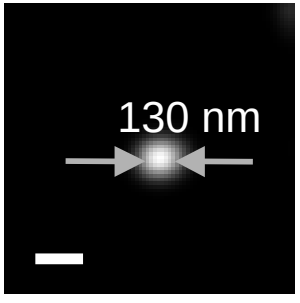
A 140-nm-wide line observed by differential confocal microscopy.

C.-H. Lee, H.-Y. Chiang, and H.-Y. Mong, *Optics Letters* **28,** 1772 (2003).

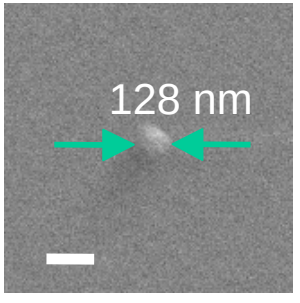
Resolution limit



DCM image
(pixel size : 15 nm
pixel : 80 ⅹ 80)



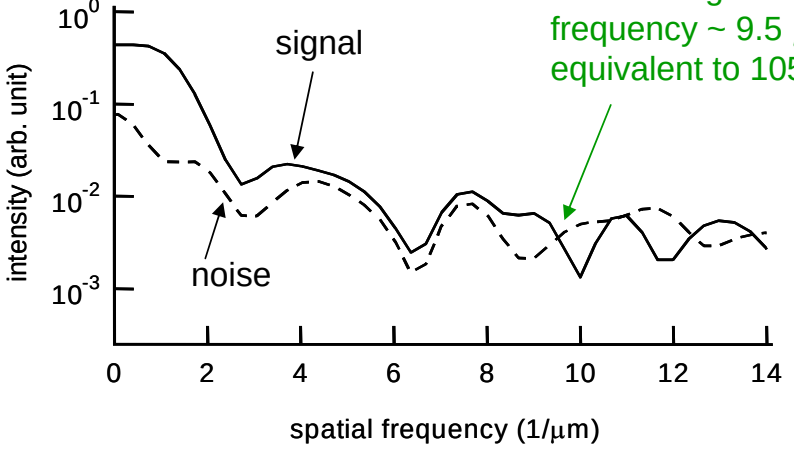
restored image



SEM image

sample : bead
(coated 15-nm gold)

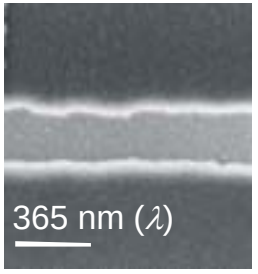
scale bar = 200 nm



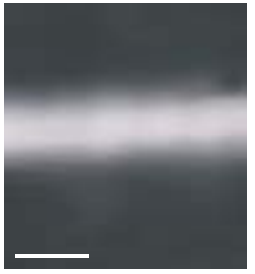
"…frequency components above the crossover frequency cannot be recovered…"
D. L. Snyder *et al.*, *IEEE Trans. Medical Imaging* **6,** 228–238 (1987).

Resolution enhancement based on widefield optical profilometry

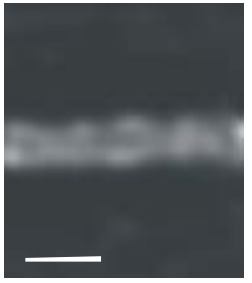
200-nm Cr line



SEM image

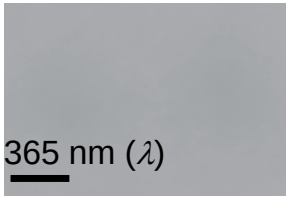


Topographic image

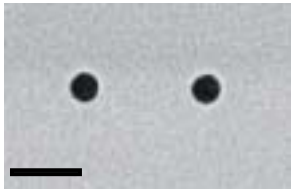


Restored topographic image

100-nm holes

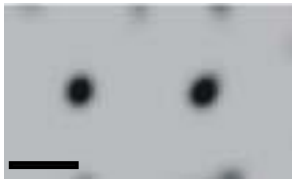


Optical image



SEM image

Topographic image

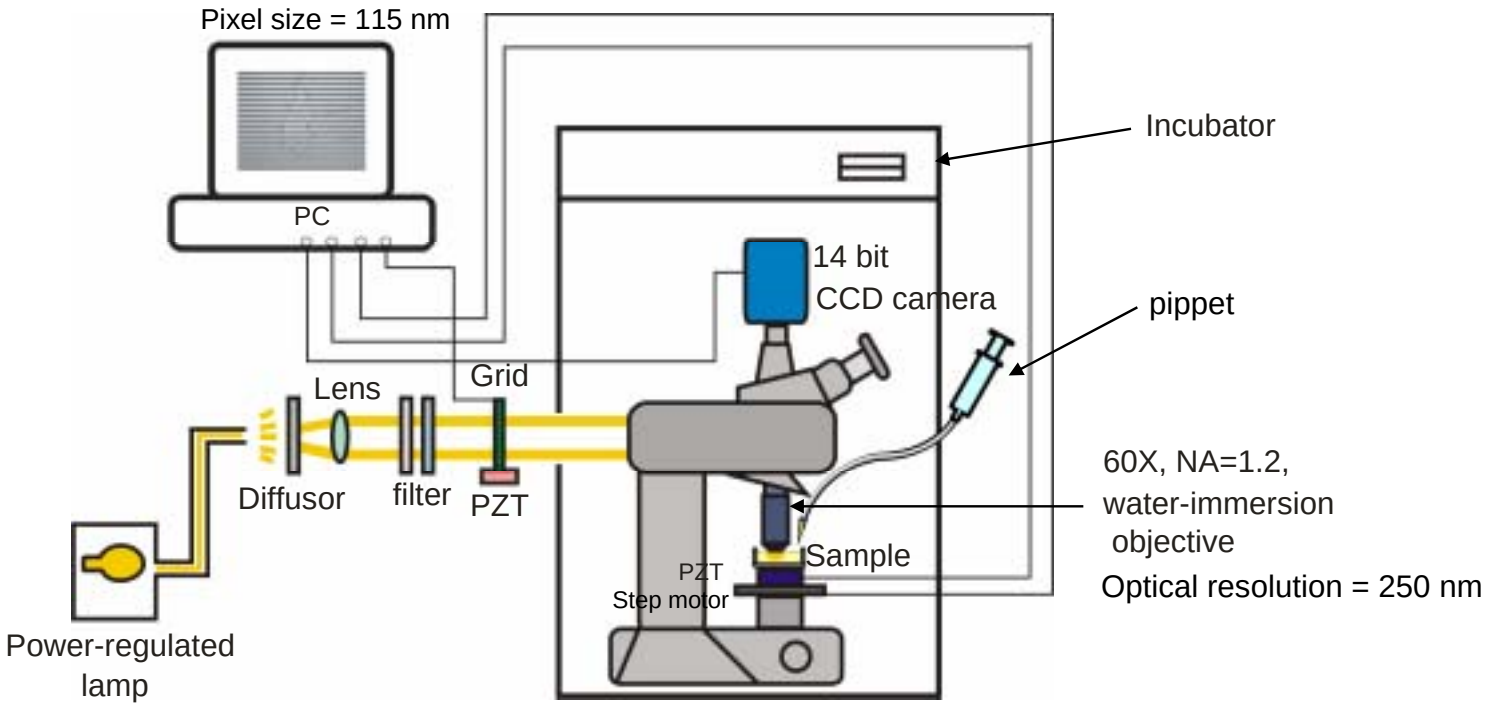


Restored topographic image

S.-W. Huang, H.-Y. Mong, and C.-H. Lee, *Microsc. Res. Tech.* **65,** 180 (2004).

Applications on cell dynamics

The NIWOP system

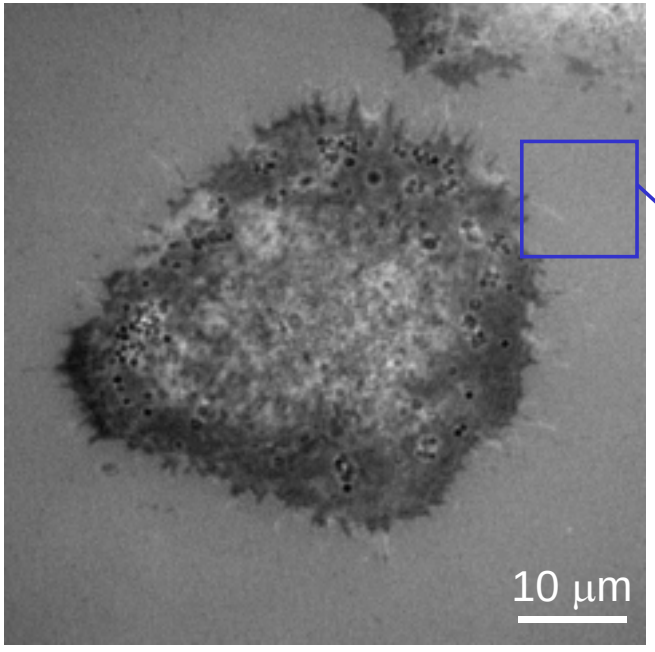


A conventional bench-top microscope is used to construct a NIWOP system.

NIWOP in an incubator

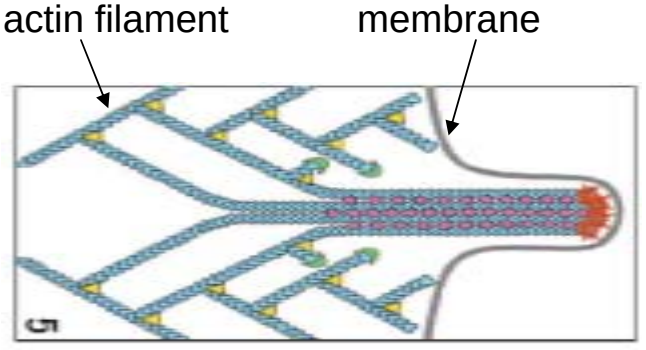
The filopodium

Lung cancer cell CL1-0



Filopodium (絲狀偽足)

1. Bundles of actin filaments
2. Related to cell migration
3. Related to cancer metastasis
4. Diameters about 100–300 nm

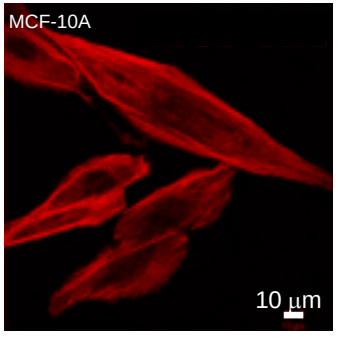


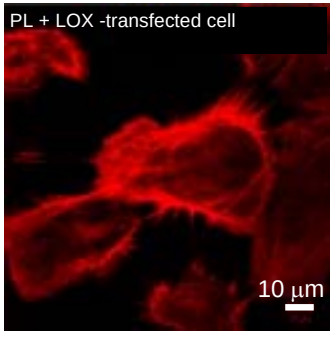
Ref: T. M. Svitkina, et al., *J. Cell Biol.* **160,** 409 (2003).

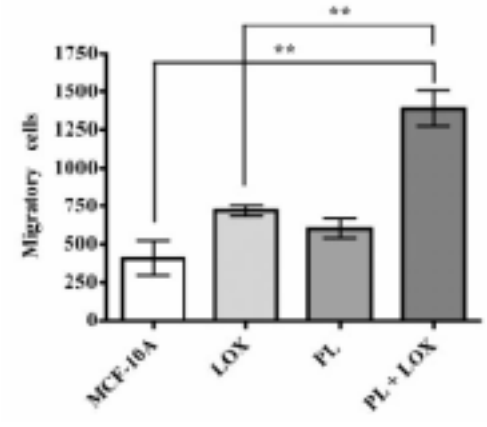
Filopodia are related to cell migration

Breast epithelial cell

MCF-10A : Breast epithelial cell
PL + LOX: placental lactogen + Lysyl oxidase



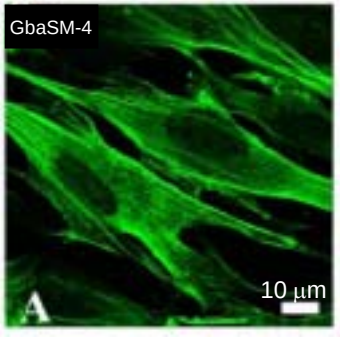


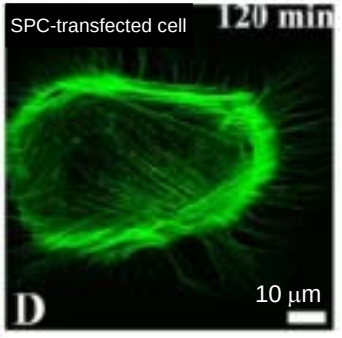


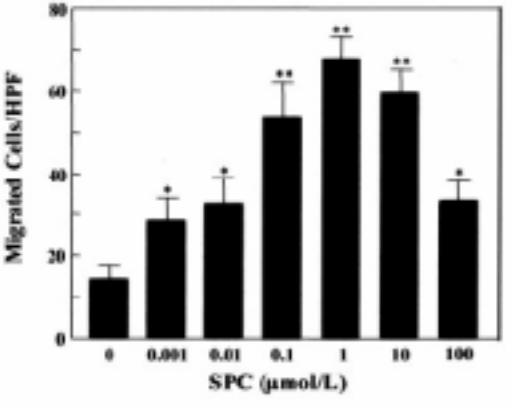
Ref: N. Polgar et al., *J. Biol. Chem.* **282,** 3262 (2007).

vascular smooth muscle cell

SPC: sphingosylphosphorylcholine
GbaSM-4: vascular smooth muscle cells

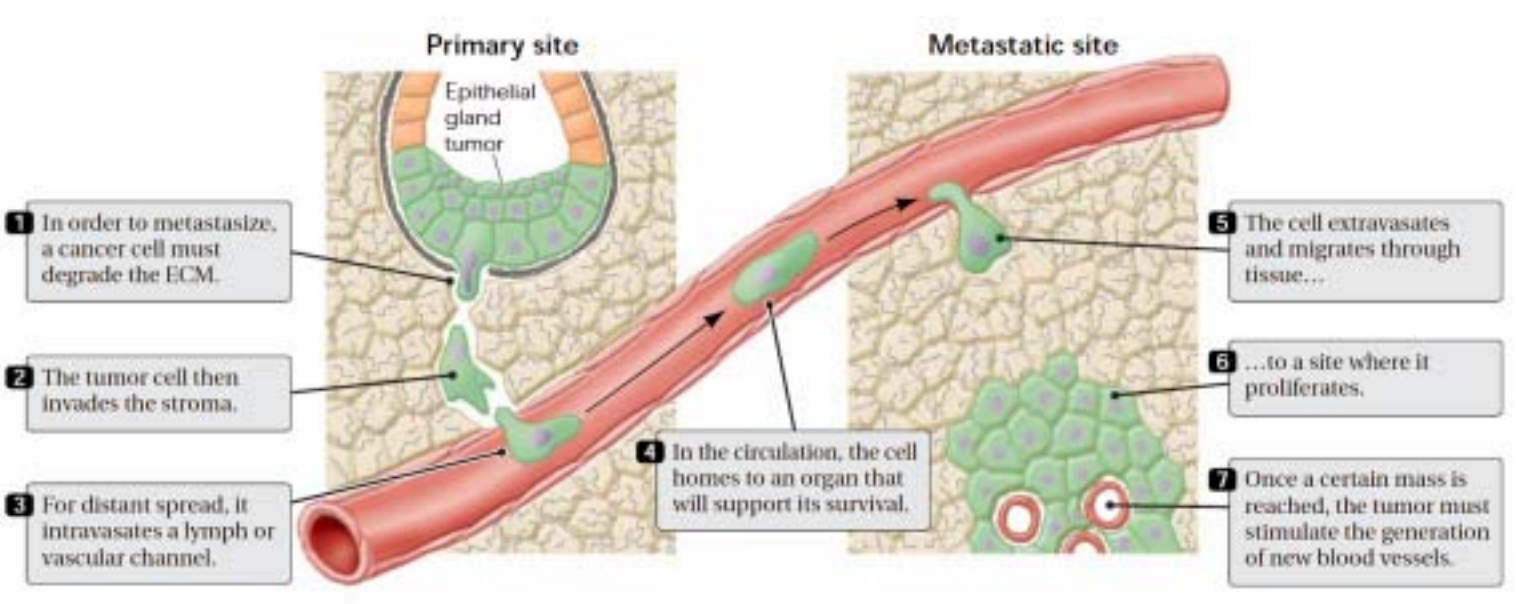






Ref: S. Li, et al., *Am. J. Physiol Heart Circ. Physiol.* **291,** 1262 (2006).

Migration is related to cancer cell metastasis

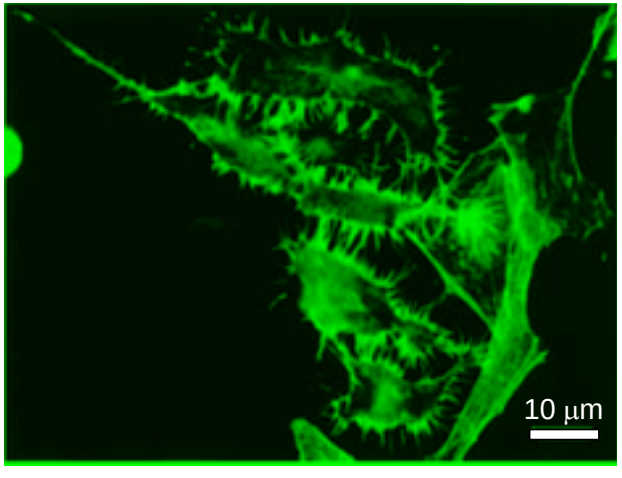


Ref: D.-H. Geho et al., *Physiology* **20,** 194 (2005).

Filopodia are related to cancer metastasis

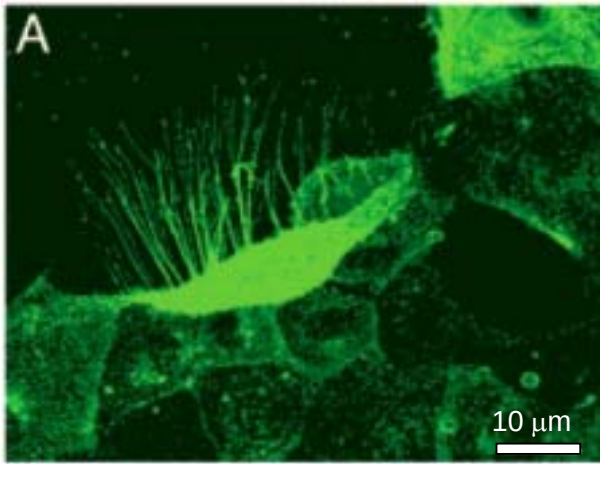
Filopodia are thought to be positively related to cancer cell motility.

Lung cancer cell



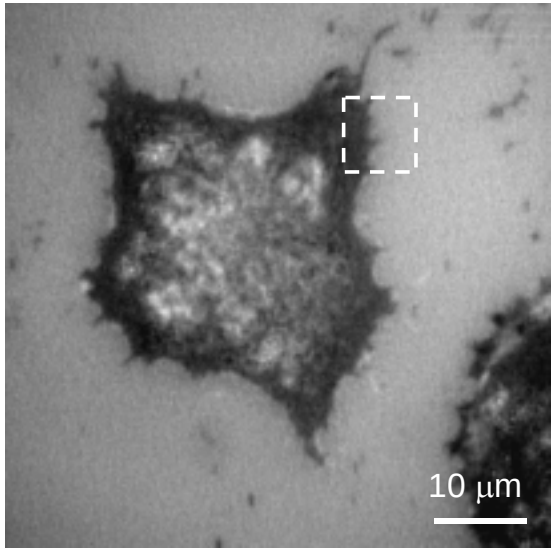
Ref: J.-Y. Shih et al., *J. Natl. Cancer Inst.* **93,** 1392 (2001).

Colon cancer cell



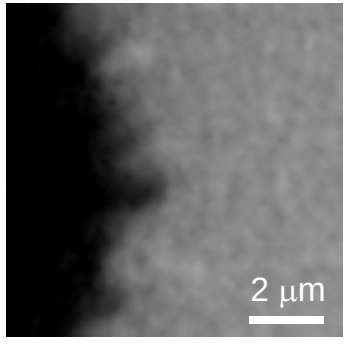
Ref: O. Kovbasnjuk et al., *Proc. Natl. Acad. Sci. USA* **102,** 19087 (2005).

Seeing the filopodia without fluorescence labeling

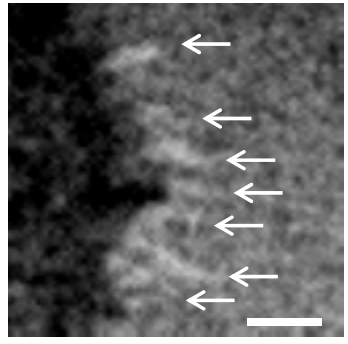


Lung cancer cell CL1-0

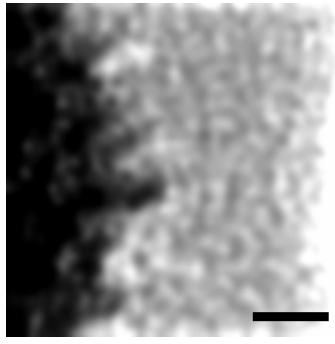
Bright field



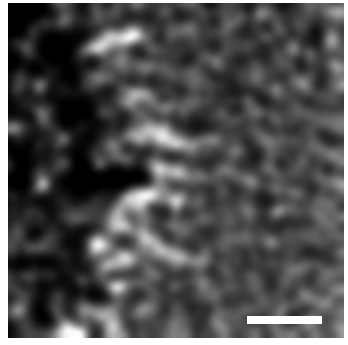
NIWOP



Bright field + Restoration



NIWOP + Restoration

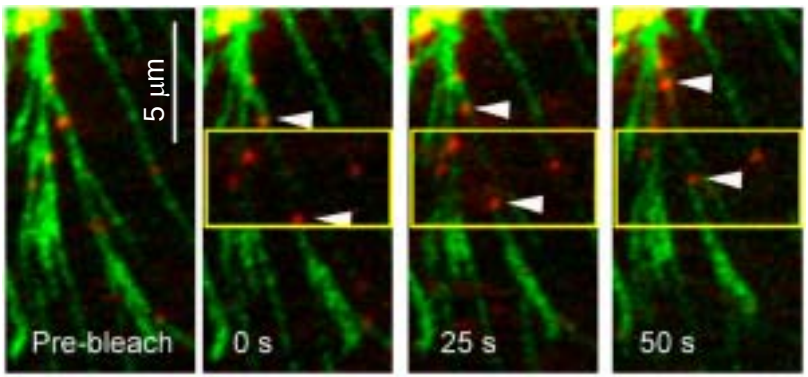


T.-H. Hsu et al., *Optics Express* **15,** 76 (2007).

Epidermal growth factor (EGF)

Ref: H. Ogiso et al., *Cell* **110**, 775 (2002).

- EGF regulates cell proliferation and differentiation by binding to the extracellular region of the EGF receptor (EGFR).
- EGFR is abundant in cancer cell.
- Filopodia play a major role for retrograde signal transduction related to EGF.

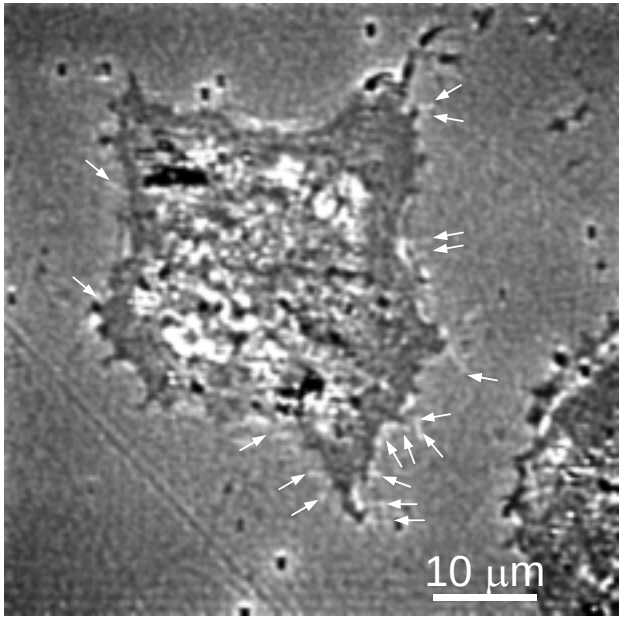


Ref: D. S. Lidke et al., *J. Cell Biol*. **170**, 619 (2005).

Number of filopodia affected by EGF

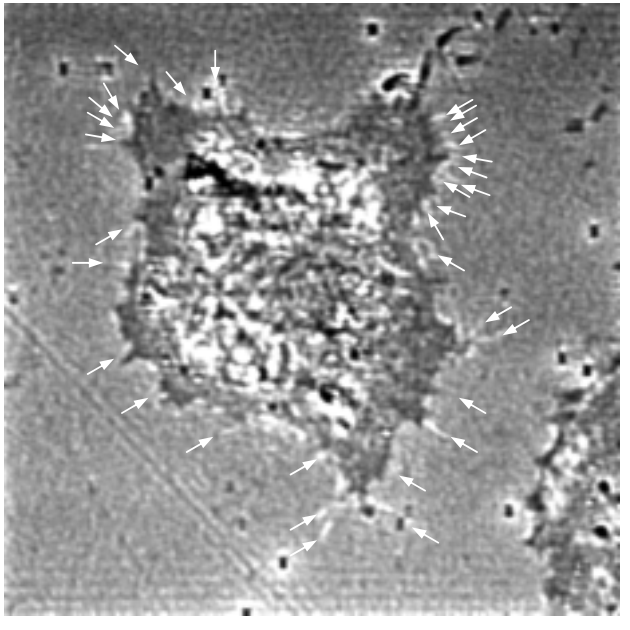
Filopodia threshold: contrast > 20% and length > 1 μm

(a) Before the treatment of EGF



Number of filopodia: 17

(b) 10 minutes after the treatment of 50 ng/ml EGF

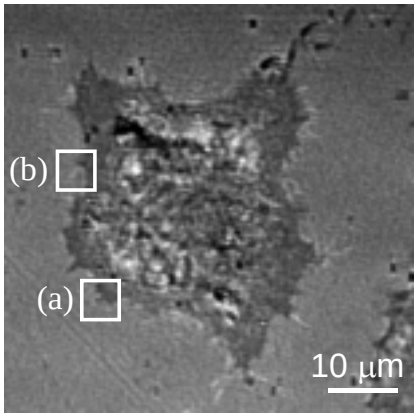


Number of filopodia: 32

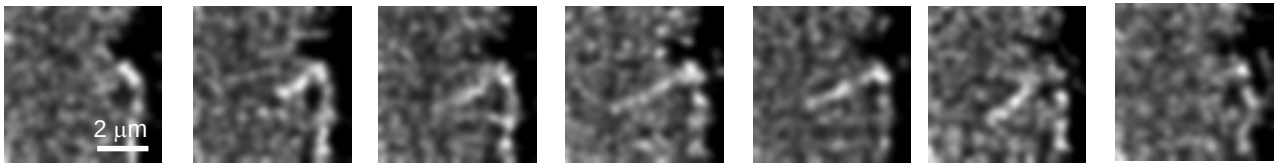
T.-H. Hsu et al., *Optics Express* **15,** 76 (2007).

Dynamics of single filopodia

(a) Before treatment of EGF



(b) After treatment of EGF

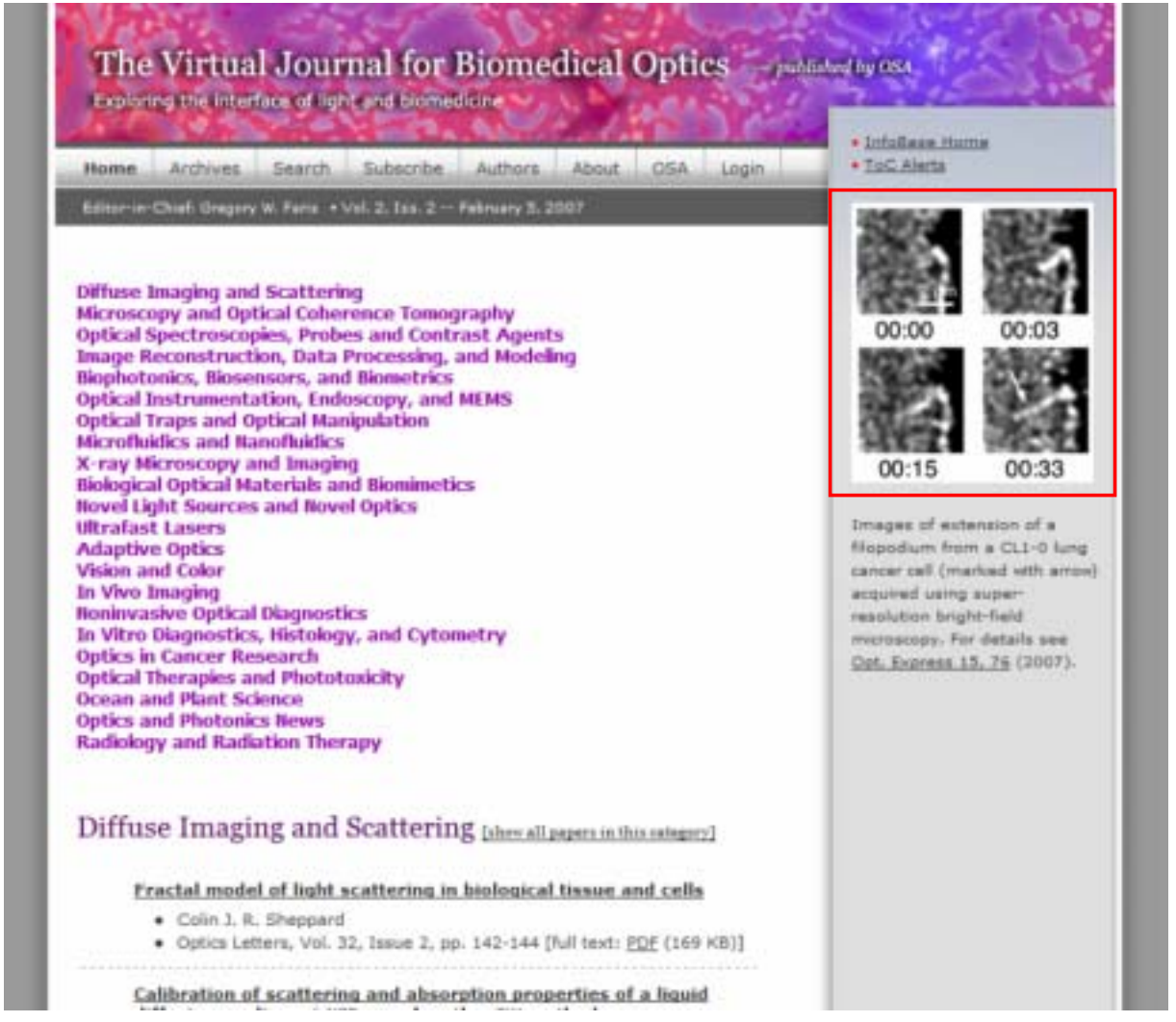


Nine filopodia of this cell are measured:

	Elongation rate (nm/sec)	Shrinkage rate (nm/sec)
Before EGF treatment	90 ± 11	75 ± 6
After EGF treatment	110 ± 12	100 ± 15

T.-H. Hsu et al., *Optics Express* **15,** 76 (2007).

Highlighted in *Virtual Journal for Biomedical Optics* (February 2007)



Conclusions

1. Optical resolution (point-spread function) is determined by the wavelength and the numerical aperture of the objective.

2. With images of high signal-to-noise ratios, deconvolution techniques can greatly improve the resolution.

3. With nanometer topographic contrast, bright-field images can also be improved to "super-resolution."

4. Without strong illumination required by fluorescence microscopy, super-resolution bright-field microscopy is more suitable for long-term observation of cell dynamics.