Light and fluorescence microscopy

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CHEE 6327

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Reference: www.olympusmicro.com for online tutorial

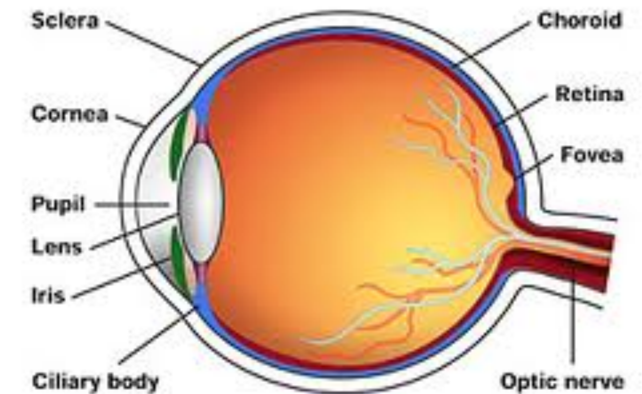
Outline for lecture

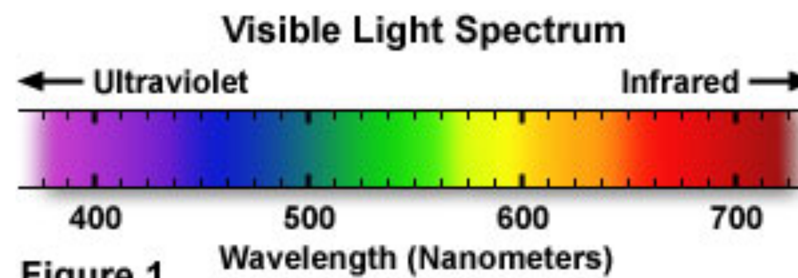
- Introduction to optics and brightfield microscopy
- Contrast-enhancing techniques
  - Darkfield, phase contrast, DIC, polarized
- Fluorescence and confocal microscopy
- Digital imaging
- Image processing
  - Histograms
  - Convolution
  - Fourier transforms and Fourier shift theorem
  - Particle tracking

Introduction

- Microscopes are designed to produce **magnified images** of small objects
  - Visual images: to eye
  - Photographic images: to camera
- Tasks in a typical microscopy experiment:
  - Produce a magnified image of the specimen
  - Separate out the details in the image
  - Render details visible to human eye or camera

Microscopy techniques





Scanning electron microscope (SEM)
Transmission electron microscope (TEM)
Scanning tunneling microscope (STM)
Atomic force microscopy (AFM)

Light microscopy

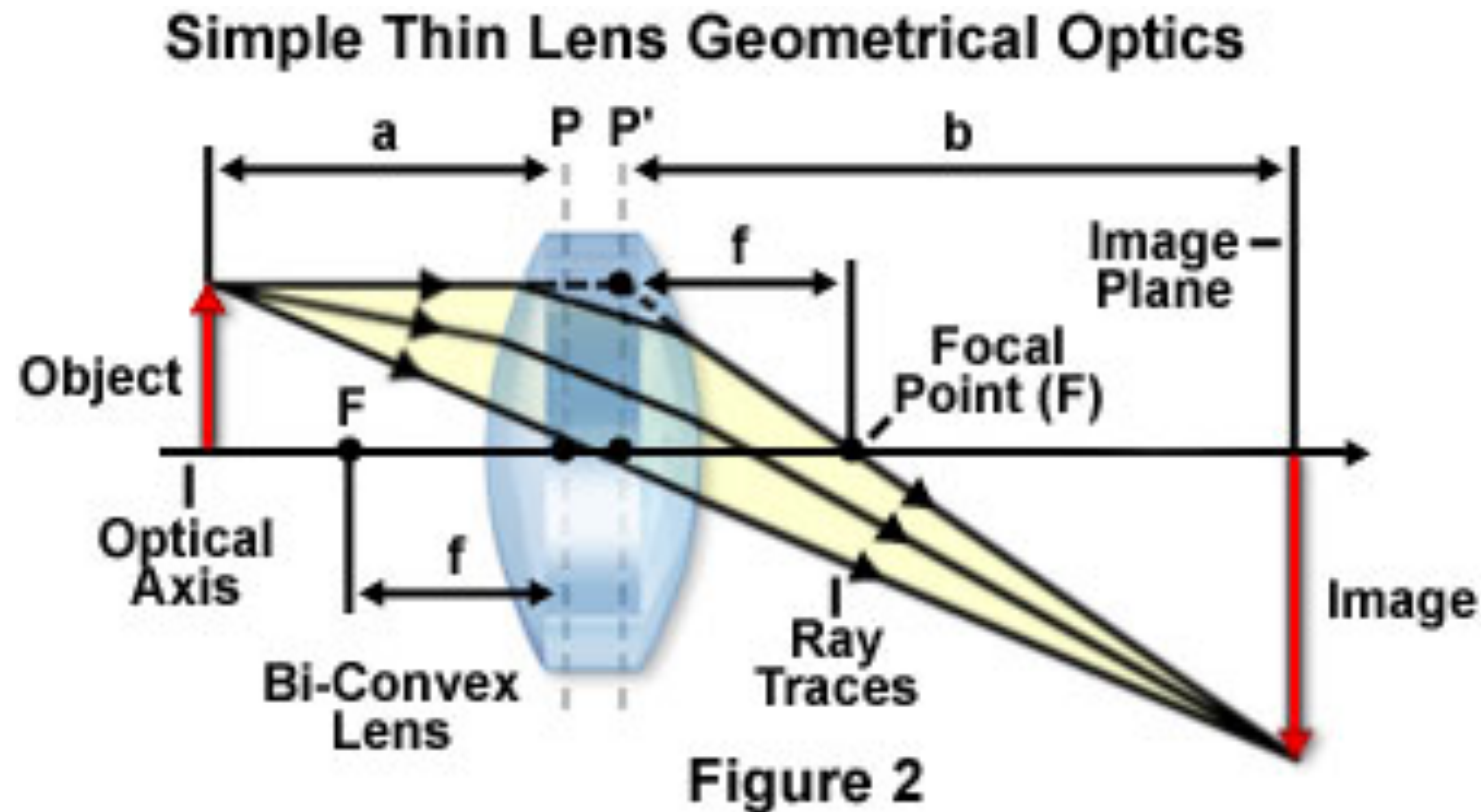
Magnifying glass

200 nm

500 μm

object size

Geometric optics: thin lens equation



Thin lens equation:

$$\frac{1}{a} + \frac{1}{b} = \frac{1}{f}$$

Sign conventions:

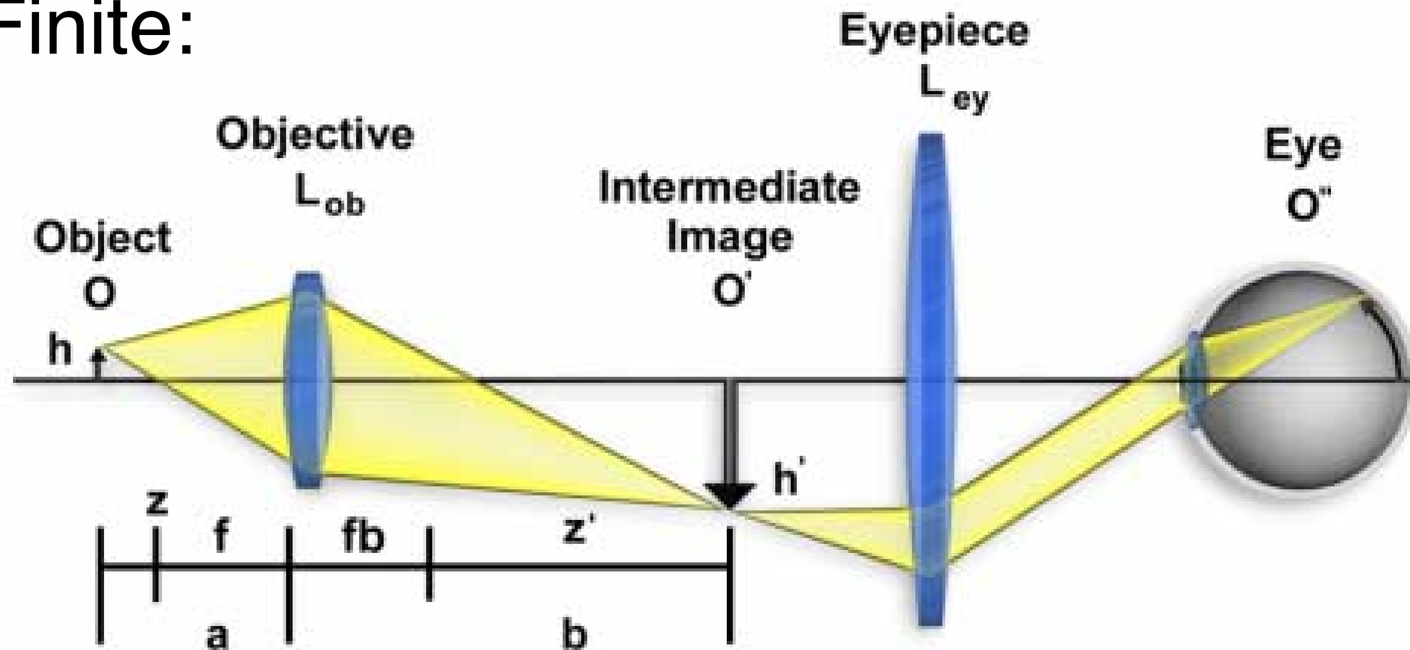
- a : object distance (from object to lens vertex)
- b: image distance (from image to lens vertex)

Lateral magnification:

$$M = \frac{h_{\mathrm{im}}}{h_{\mathrm{obj}}} = \frac{b}{a}$$

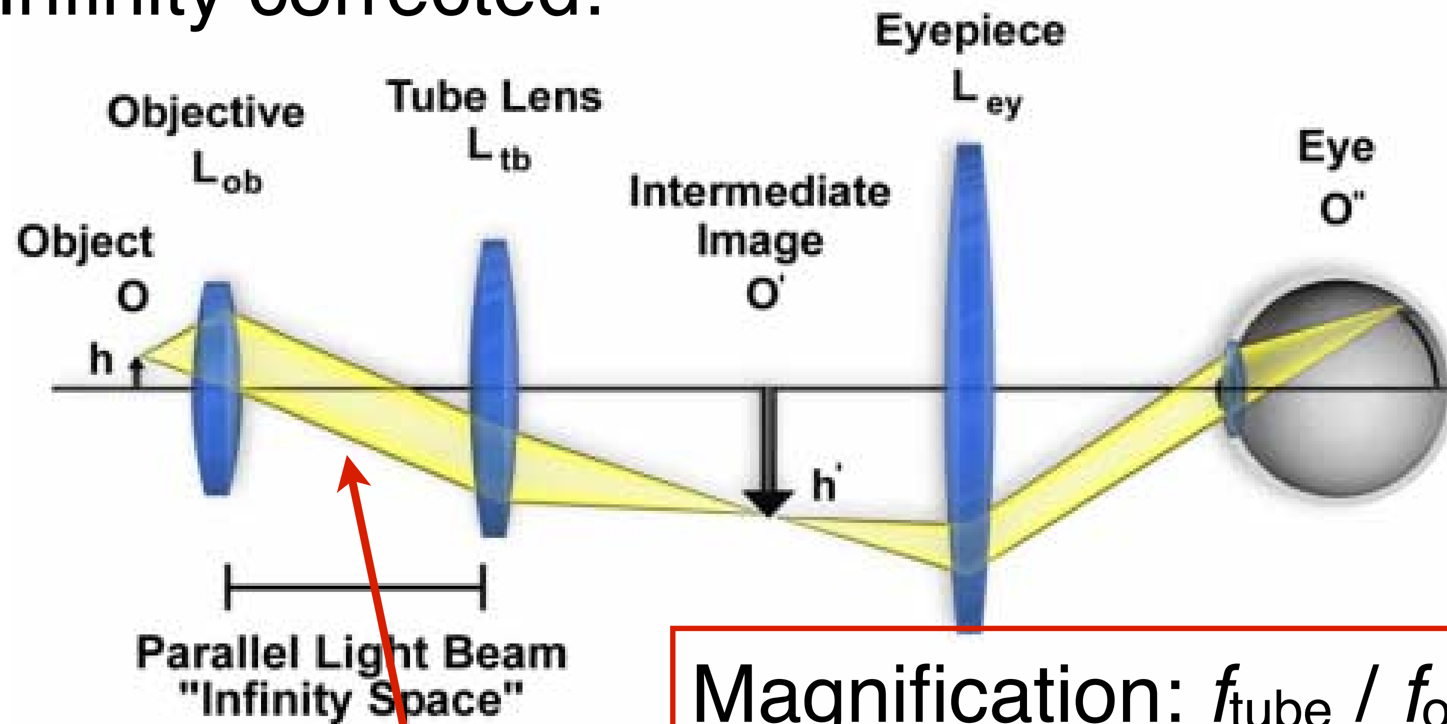
Optical microscopy basics

Finite:



Magnification: b/a

Infinity corrected:



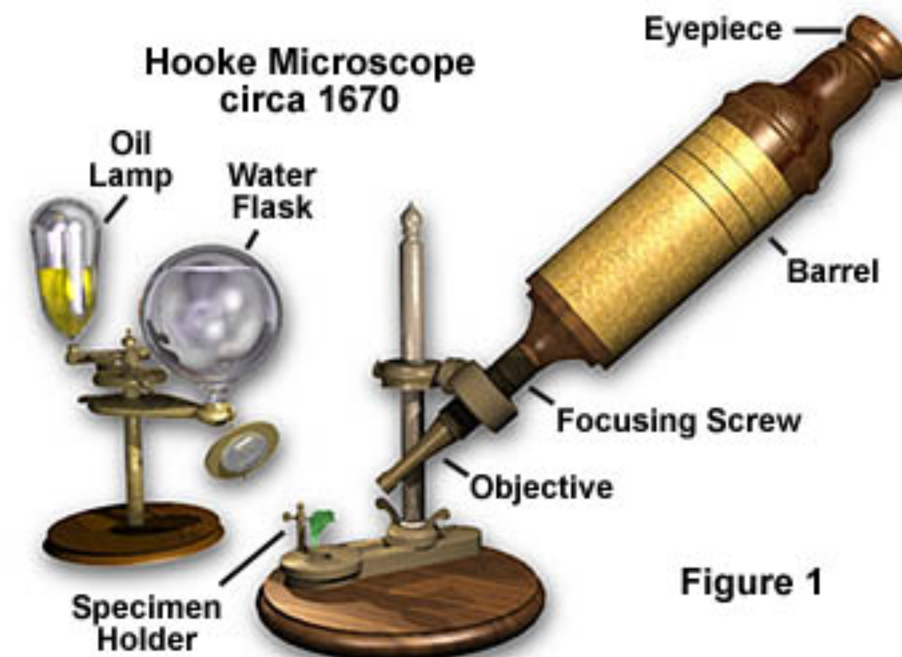
Magnification: f_{tube} / f_{obj}

additional accessories go here (minimal distortion)

- Compound microscopes typically have at least two lenses:
  - Objective
  - Ocular / eyepiece
- Goal: form a magnified image that is spread out on the eye or camera
  - This enables small features to be resolved resolved

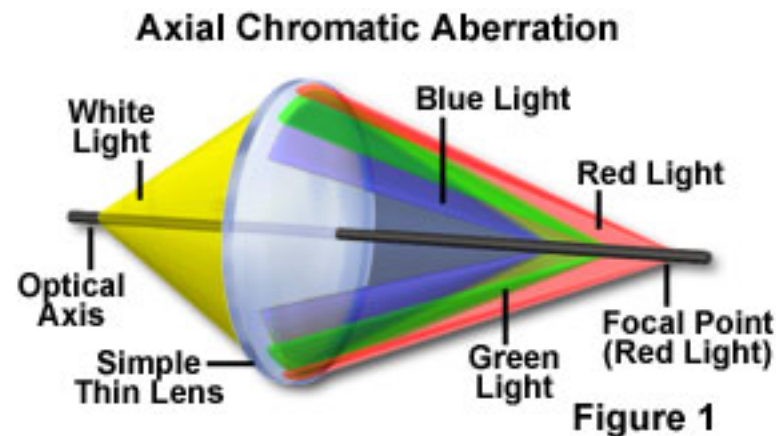
History of microscopy

- **Magnifying glass**:
  - First used: by Romans in 1st century AD as "burning glasses" to focus light
  - Ability to magnify (rather than focus) first used in 13th century
- **Microscopes**:
  - First compound microscopes: Janssen, Hooke 1660s

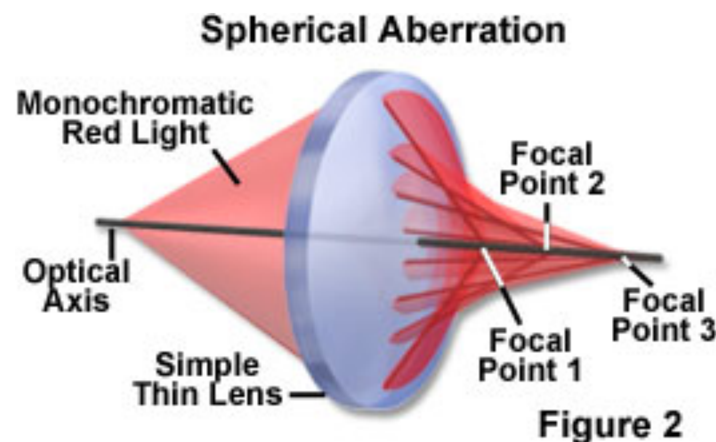


Problems in early compound microscopes

- **Chromatic aberration**: different colors are focused at different locations
  - Due to variations in refractive index *n* across visible light frequencies



- **Spherical aberration**: light entering the lens at different positions is focused at different locations
  - Due to variations in thickness along convex lenses



Optics in a modern microscope

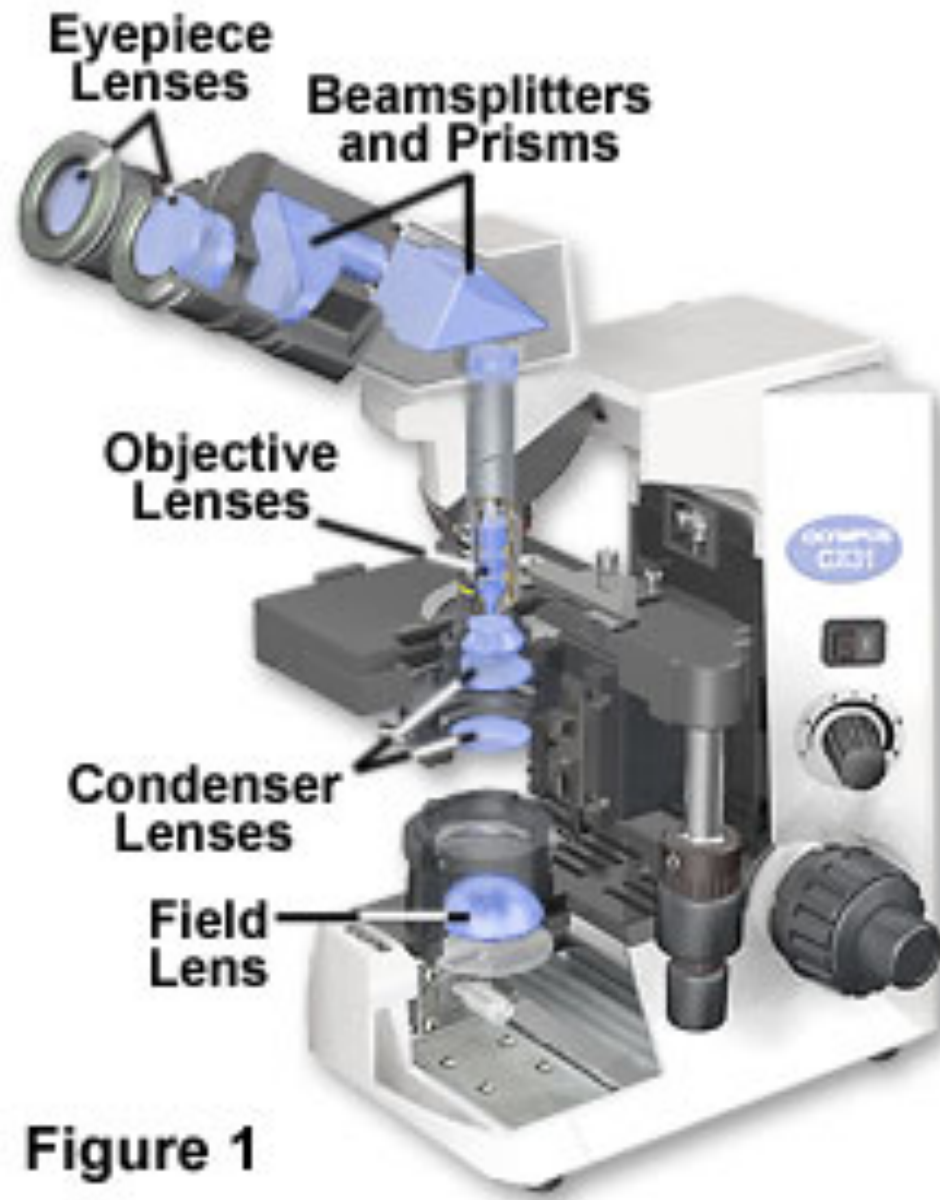


Figure 1

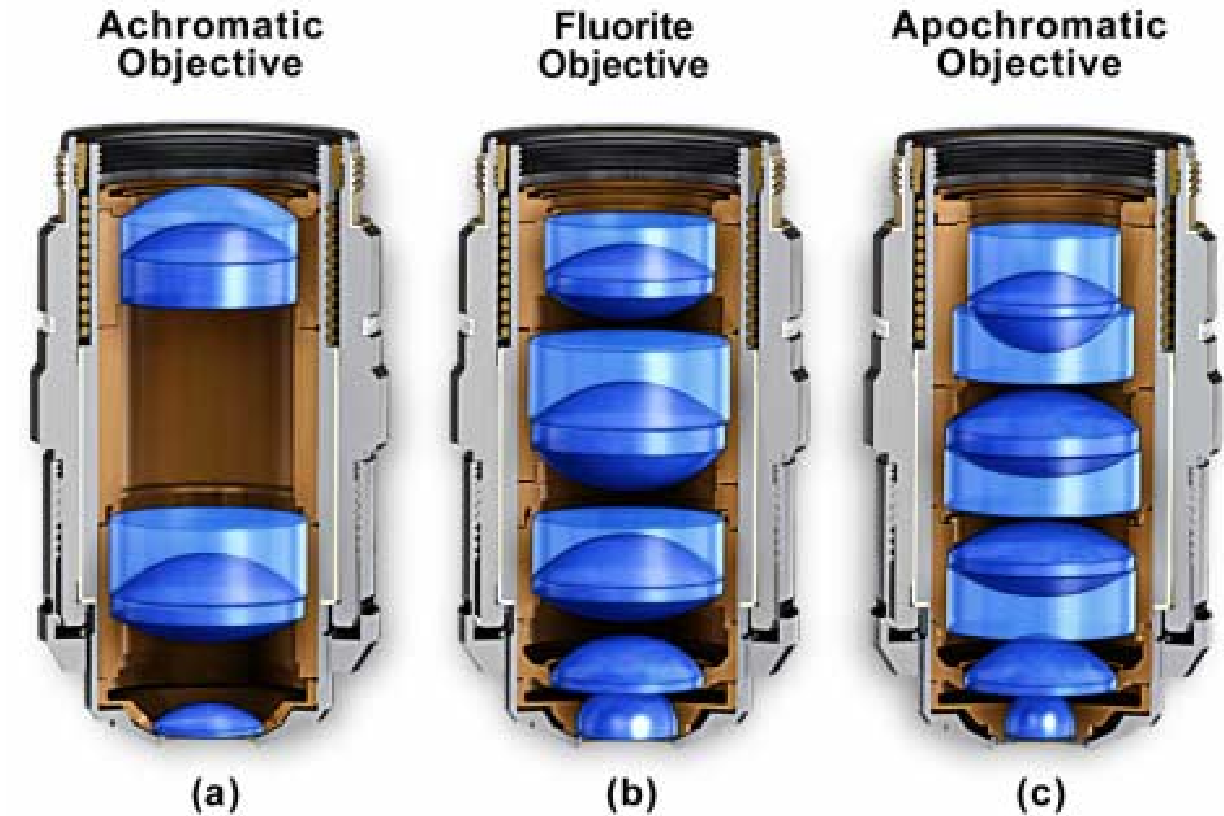
- **Objective:**
  - Gather light coming from all parts of specimen
  - Reconstitute light into corresponding points in image
  - Produce magnified, real image up into body tube
- **Condenser**:
  - Gathers light from microscope and concentrates into cone that uniformly illuminates specimen
- **Eyepiece**:
  - Further magnifies real image

Objective lenses

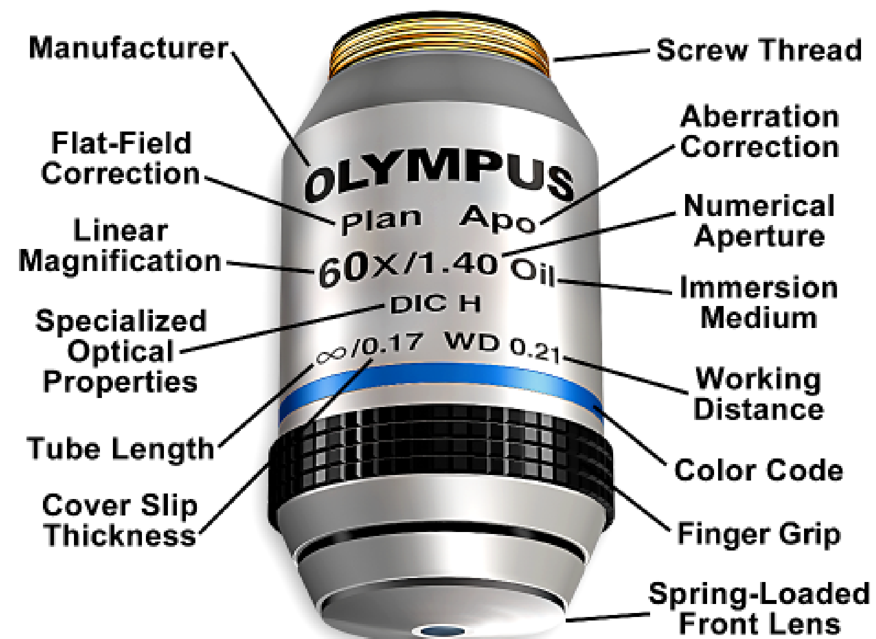
Corrections for Aberrations

Type	Spherical	Chromatic	Flatness Correction
Achromat	* [b]	2 [c]	No
Plan Achromat	* [b]	2 [c]	Yes
Fluorite	3 [d]	< 3 [d]	No
Plan Fluorite	3 [d]	< 3 [d]	Yes
Plan Apochromat	4 [e]	> 4 [e]	Yes

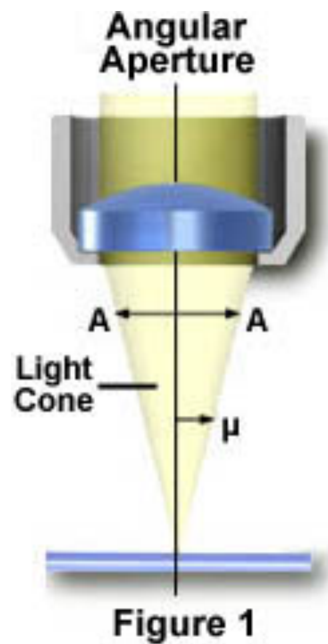
[a] Source: Nikon Instrument Group
[b] Corrected for two wavelengths at two specific aperture angles.
[c] Corrected for blue and red - broad range of the visible spectrum.
[d] Corrected for blue, green and red - full range of the visible spectrum.
[e] Corrected for dark blue, blue, green and red.



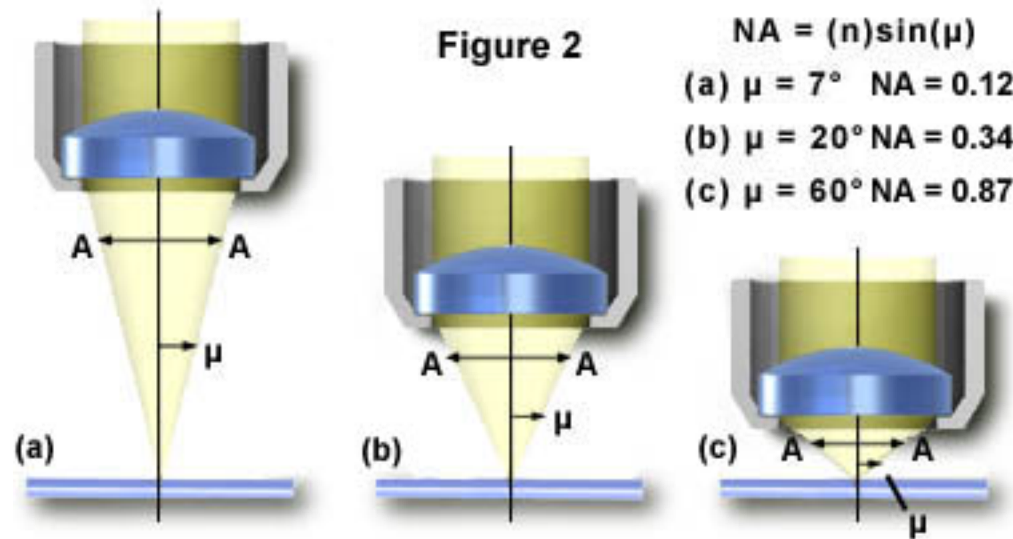
Objective Specifications



Resolution: numerical aperture



Numerical aperture (NA): $\mathrm{NA} = n \sin \mu$



Resolution: $$R = \frac{\lambda}{2\mathrm{NA}}$$

Resolution and Numerical Aperture by Objective Type

	OBJECTIVE TYPE					
	Plan Achromat		Plan Fluorite		Plan Apochromat	
Magnification	N.A	Resolution (μm)	N.A	Resolution (μm)	N.A	Resolution (μm)
4x	0.10	2.75	0.13	2.12	0.20	1.375
10x	0.25	1.10	0.30	0.92	0.45	0.61
20x	0.40	0.69	0.50	0.55	0.75	0.37
40x	0.65	0.42	0.75	0.37	0.95	0.29
60x	0.75	0.37	0.85	0.32	0.95	0.29
100x	1.25	0.22	1.30	0.21	1.40	0.20
N.A. = Numerical Aperture						

Resolution versus Wavelength

Wavelength (nanometers)	Resolution (micrometers)
360	.19
400	.21
450	.24
500	.26
550	.29
600	.32
650	.34
700	.37

Improving resolution with immersion lenses

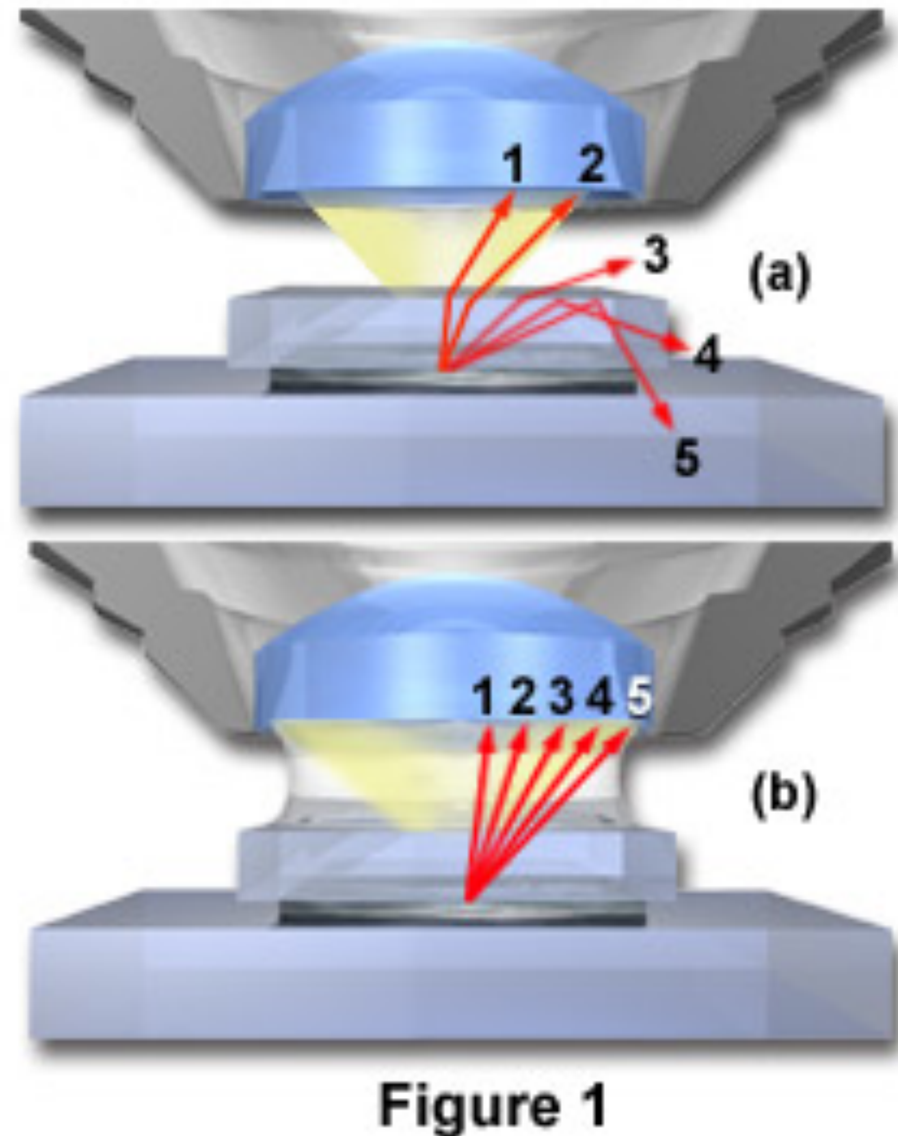


Figure 1

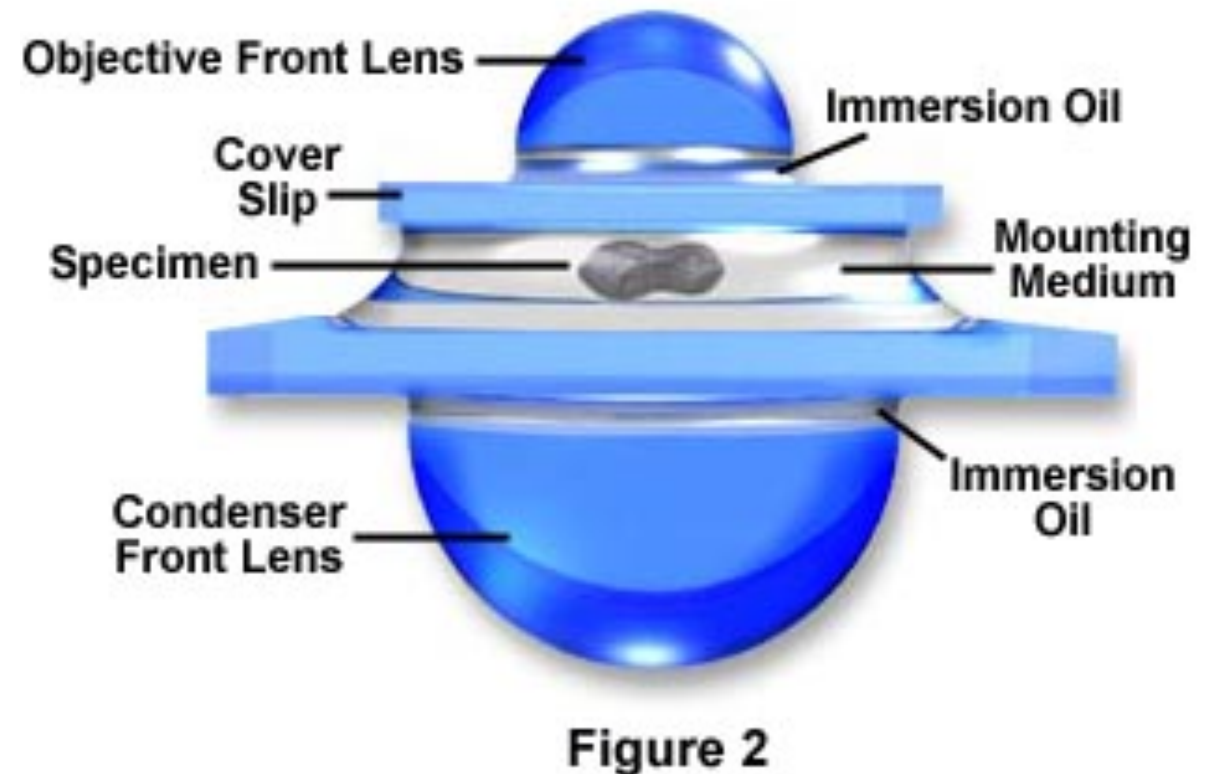


Figure 2

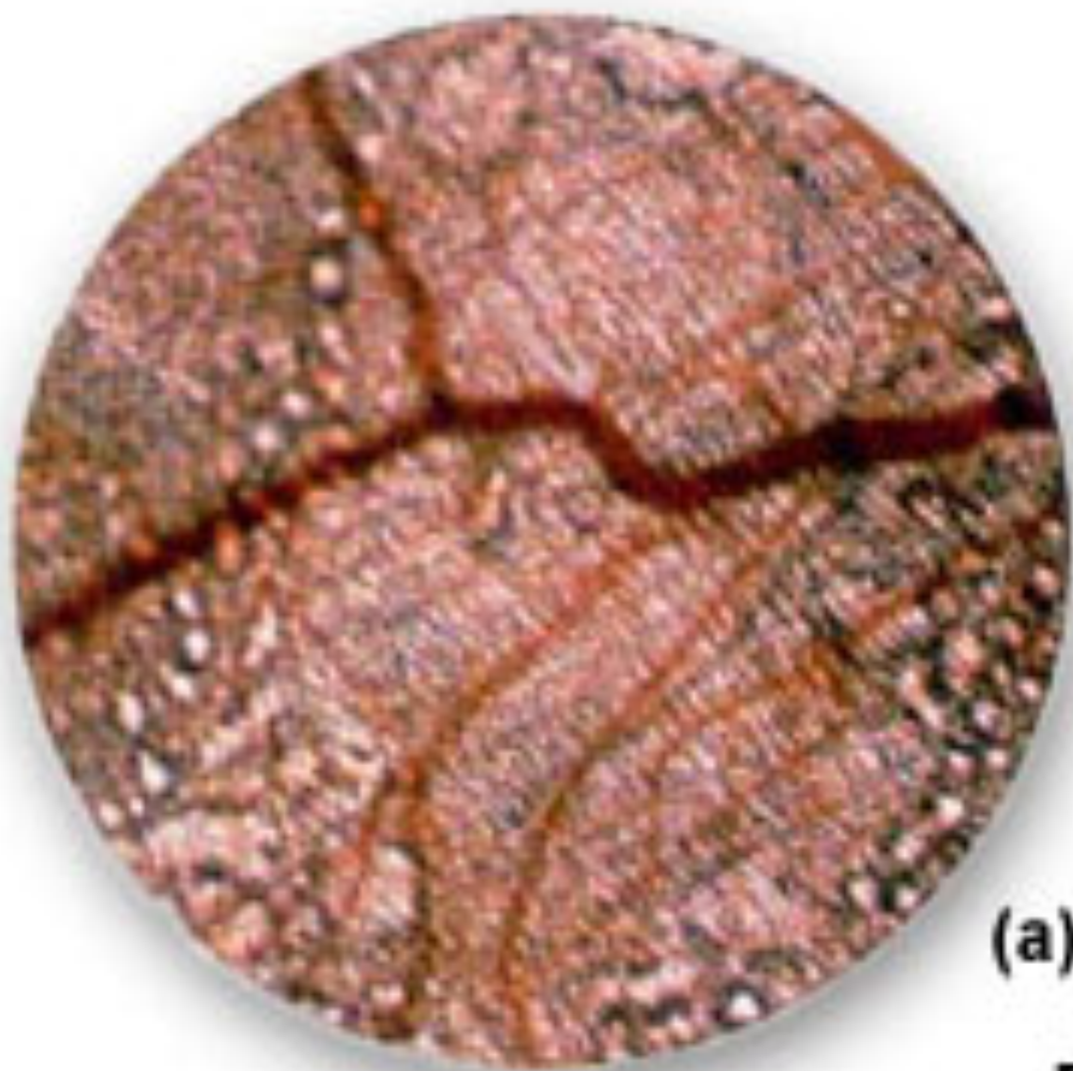
Basic idea: adding a higher-*n* medium between coverslip and lens reduces light deviation due to refraction

Two major types of immersion lenses:

- **oil:** best for imaging samples with index of refraction close to glass
- **water:** best for imaging biological samples in aqueous media

Dry versus immersion lens

Rat Cremaster Muscle in Physiological Saline



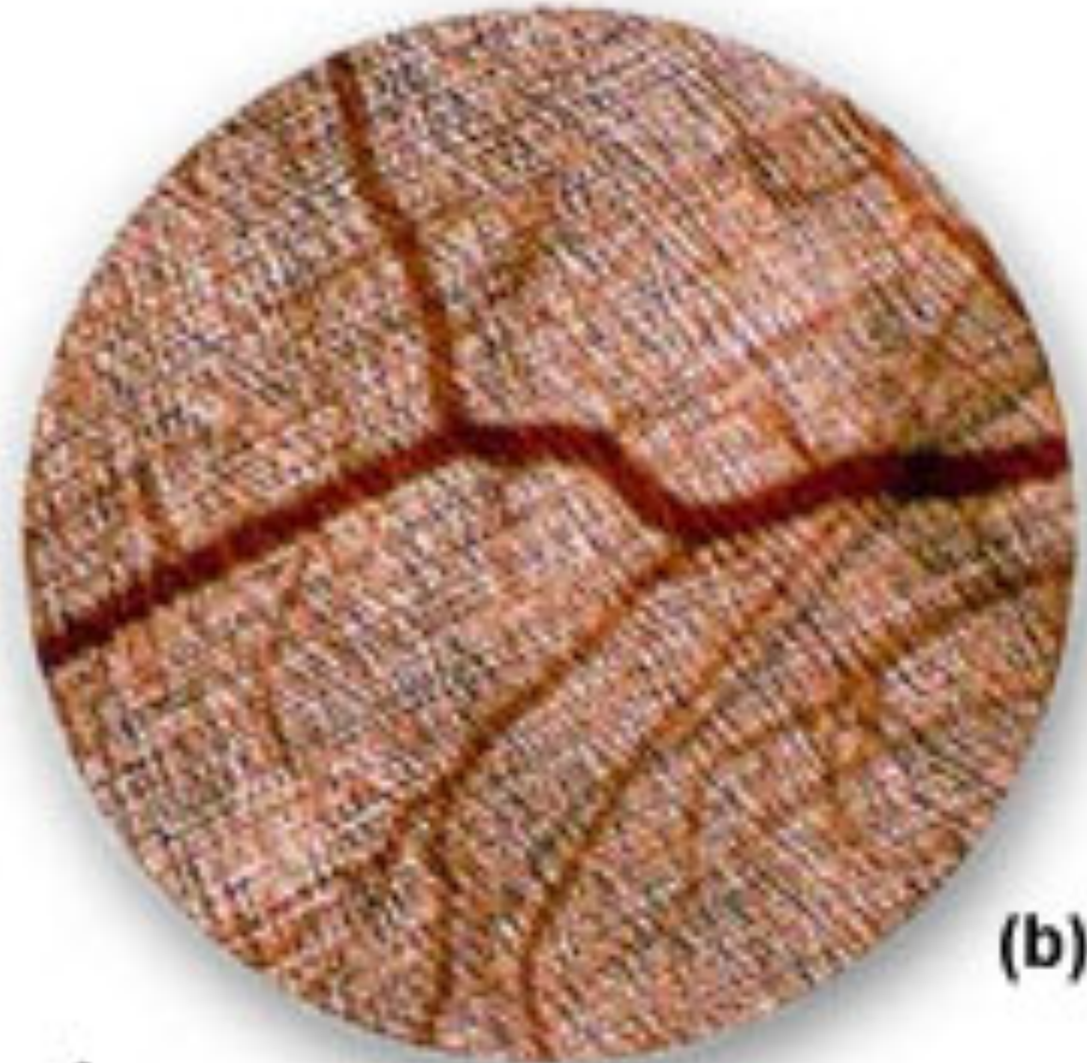


Figure 4

dry 4x lens

water immersion 4x lens

Microscope illumination system

Microscope Illumination System

Substage Condenser
Stage
Aperture Diaphragm
Field Diaphragm
Condenser Vertical Position Control
Coarse and Fine Focus
Lamphouse
Mirror
Field Lens
Collector Lens
Tungsten Halogen Lamp

Figure 2

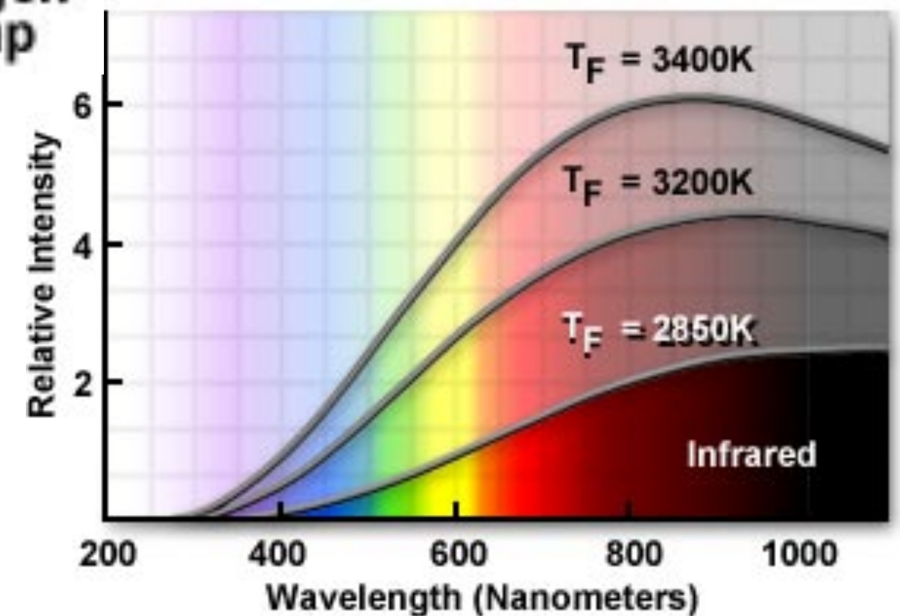
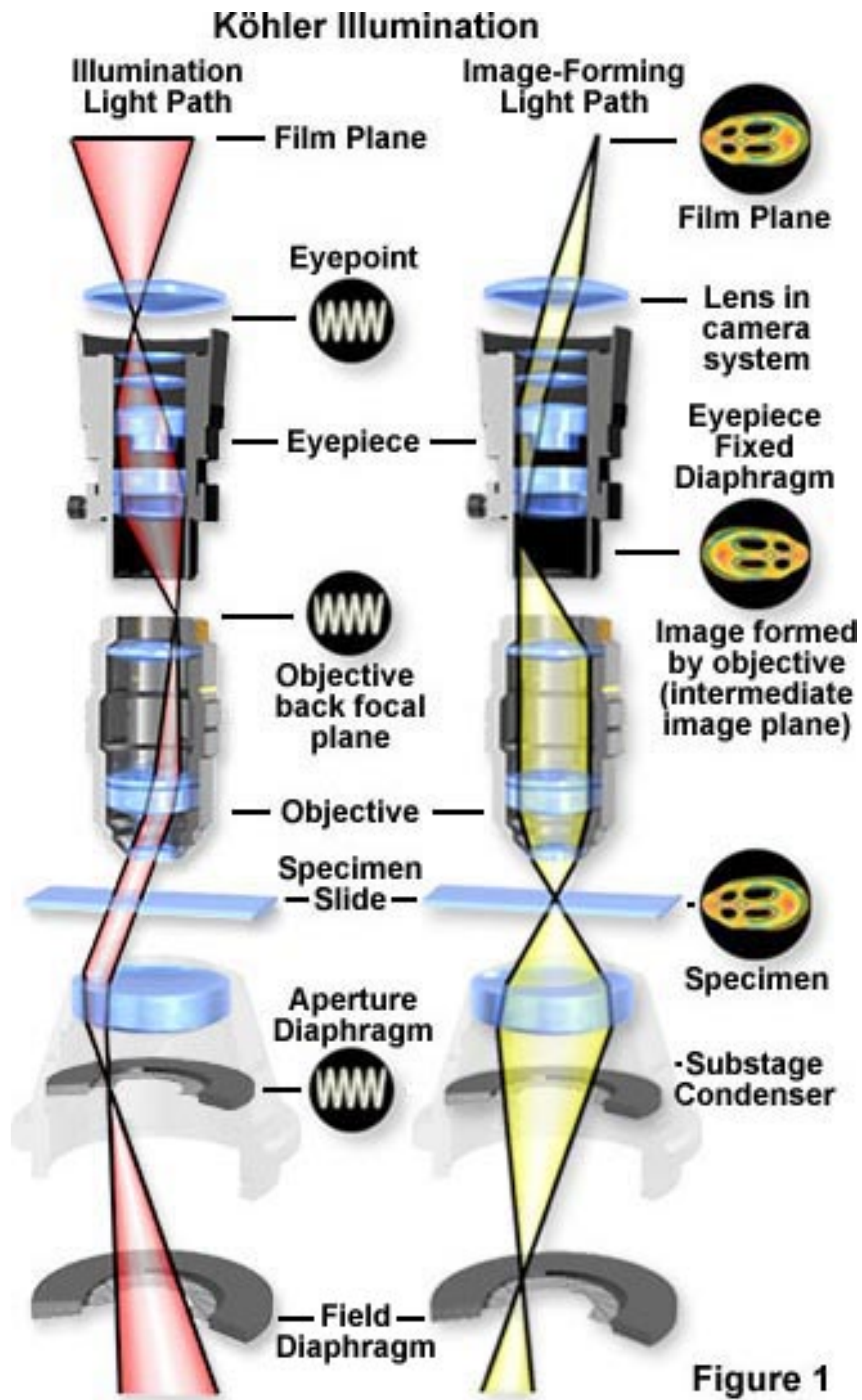


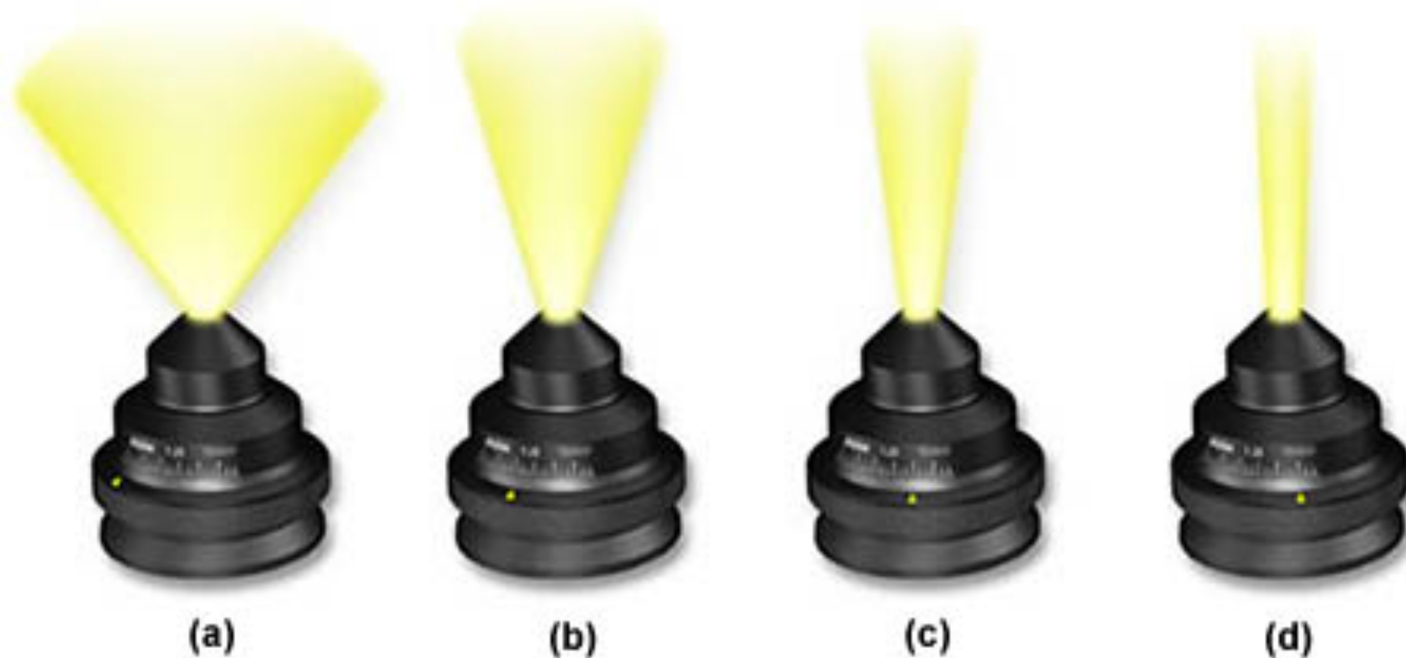
Figure 2

Köhler illumination



- Technique for producing a uniformly bright field of view
- Image of light source is focused at condenser aperture diaphragm
  - Produces parallel, unfocused light
- Magnified image of light source below condensed (at aperture diaphragm)
  - Produces wide cone of illumination required for optimum resolution
- **Advantages**
  - Size of condenser aperture diaphragm used to control NA
  - Reduce unwanted stray light, glare
  - Minimize effects of dust, imperfections on surface of condenser in image

Effect of condenser on NA



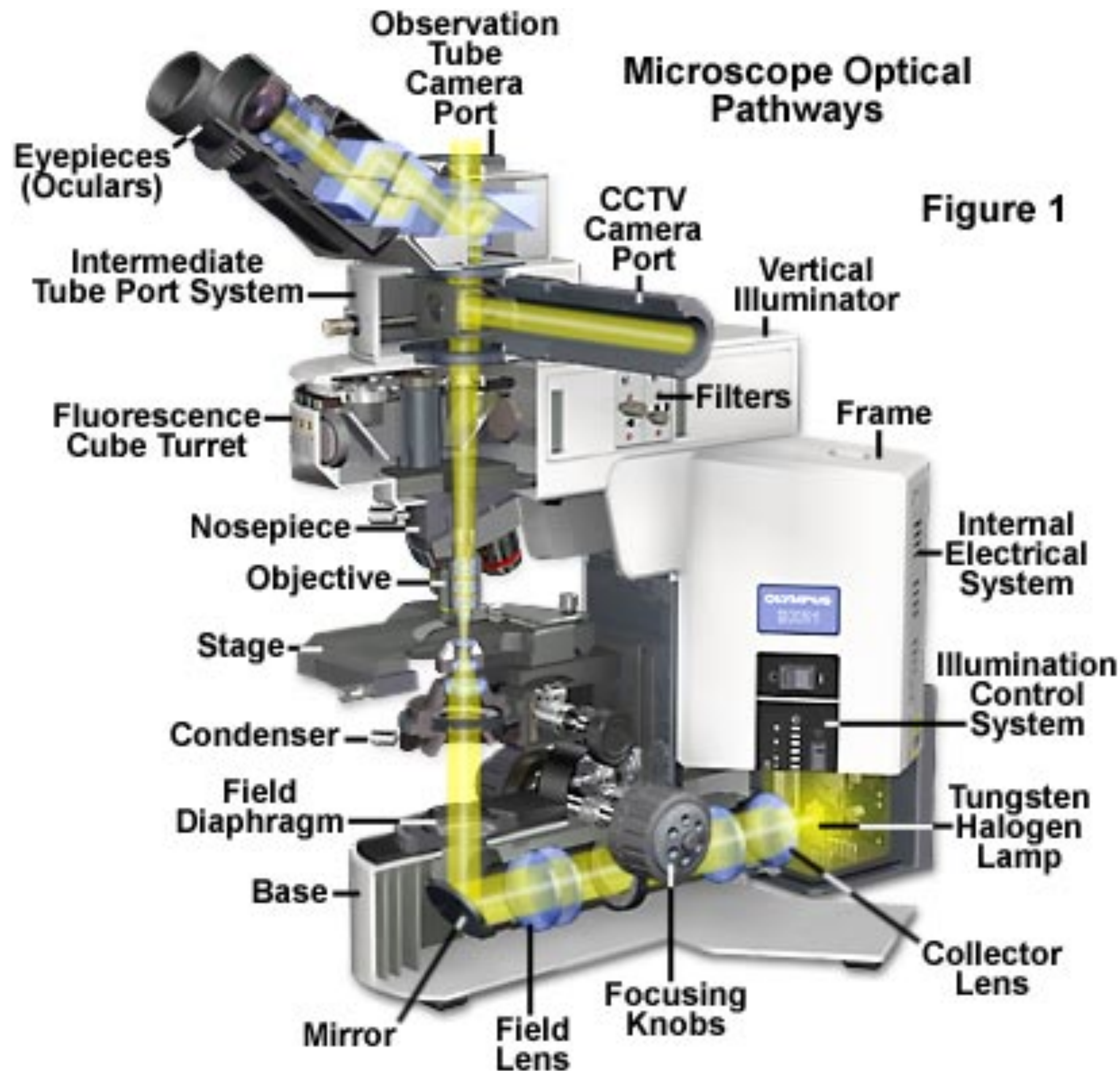
- The condenser opening angle determines its NA
- Larger cone leads to larger NA
- Condenser NA can limit overall NA of imaging system even if lens NA is higher!

Modified expression for resolution:

$$R = \frac{1.22\lambda}{\mathrm{NA}_{\mathrm{obj}} + \mathrm{NA}_{\mathrm{cond}}}$$

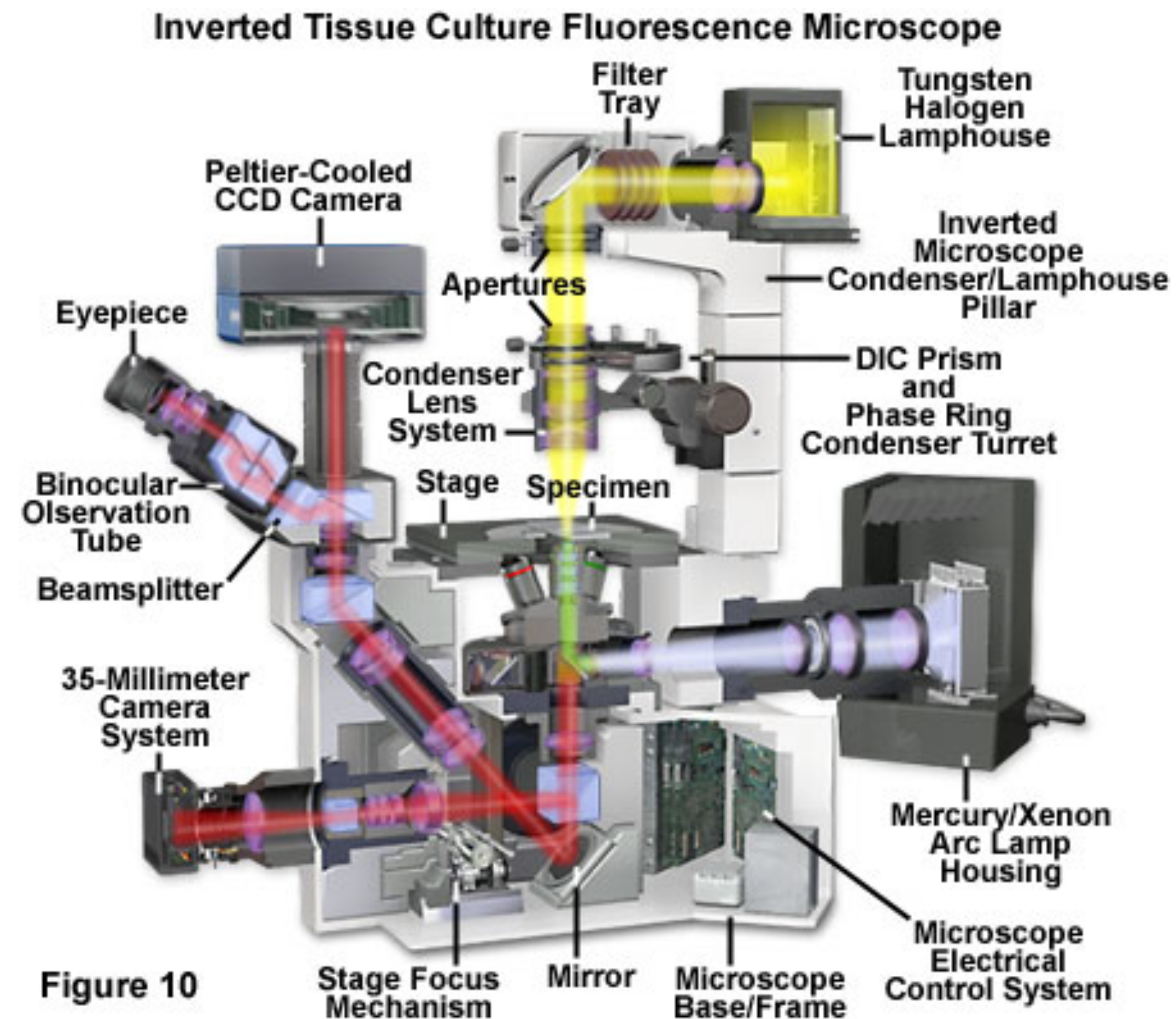
Choice of microscope geometry

Upright



Most commonly found

Inverted



Especially useful for:
- metallurgy
- tissue and cell culture
- microfluidics / PDMS
- large samples

Choice of light: transmitted vs. reflected

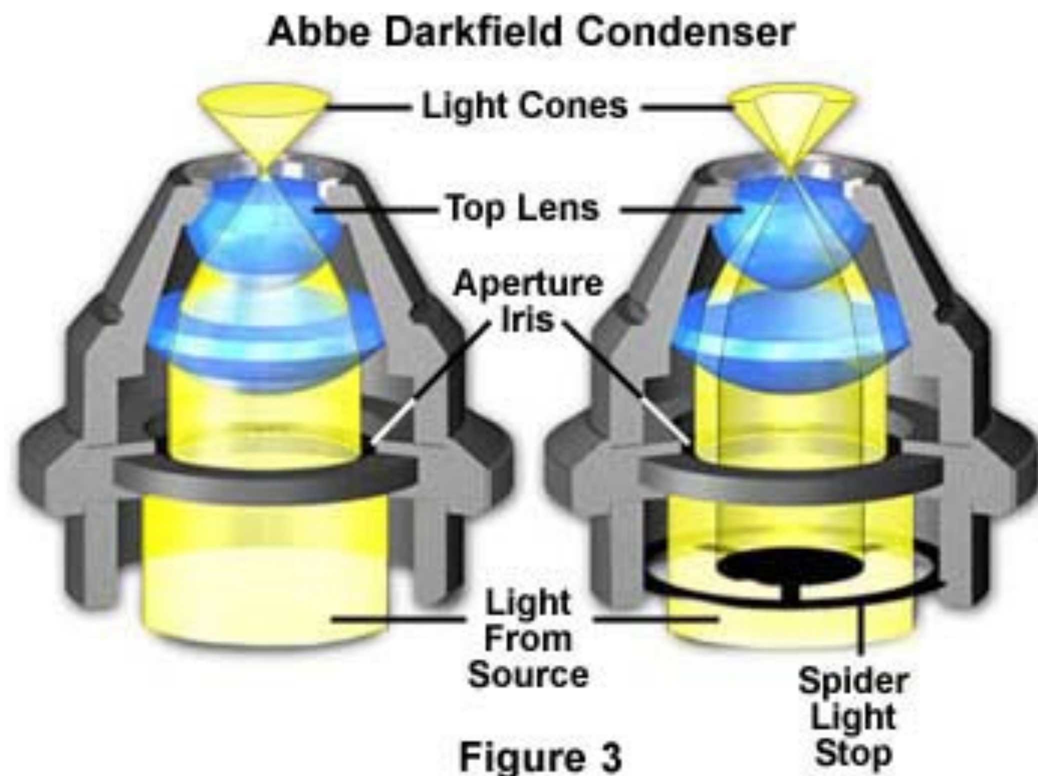
- **Transmitted** (diascopic):
  - Light passes through the sample
  - Used for thin, almost transparent samples (biology, porous media)
- **Reflected** (episcopic):
  - Light is reflected from the sample
  - Used for opaque objects like integrated circuits (materials science) and metals

Techniques for improving contrast

Percent contrast: $$\frac{(B_I - S_I)}{B_I} \times 100$$

- **Darkfield microscopy**
- **Phase contrast microscopy**
- **Differential interference contrast (DIC) microscopy**
- **Polarized light microscopy**
- **Fluorescence microscopy**
- **Confocal microscopy**
- Hoffman modulation contrast microscopy
- Rheinberg illumination

Darkfield microscopy



- **Basic idea:** contrast enhanced by blocking out the central light that normally illuminates the specimen, allowing only *oblique* rays from every angle to strike it
- **Advantages:**
  - Good for imaging edges, *n* gradients, boundaries, outlines
  - Simple, can be improvised
  - Can be combined with fluorescence
  - Can be used with either transmitted or reflected light
- **Disadvantages:**
  - Requires thin, clean specimens on slides of thickness 1.0 mm
  - High-intensity illumination needed
  - Not good at revealing internal details

Sample darkfield images

Transmitted light

Figure 2

Reflected light

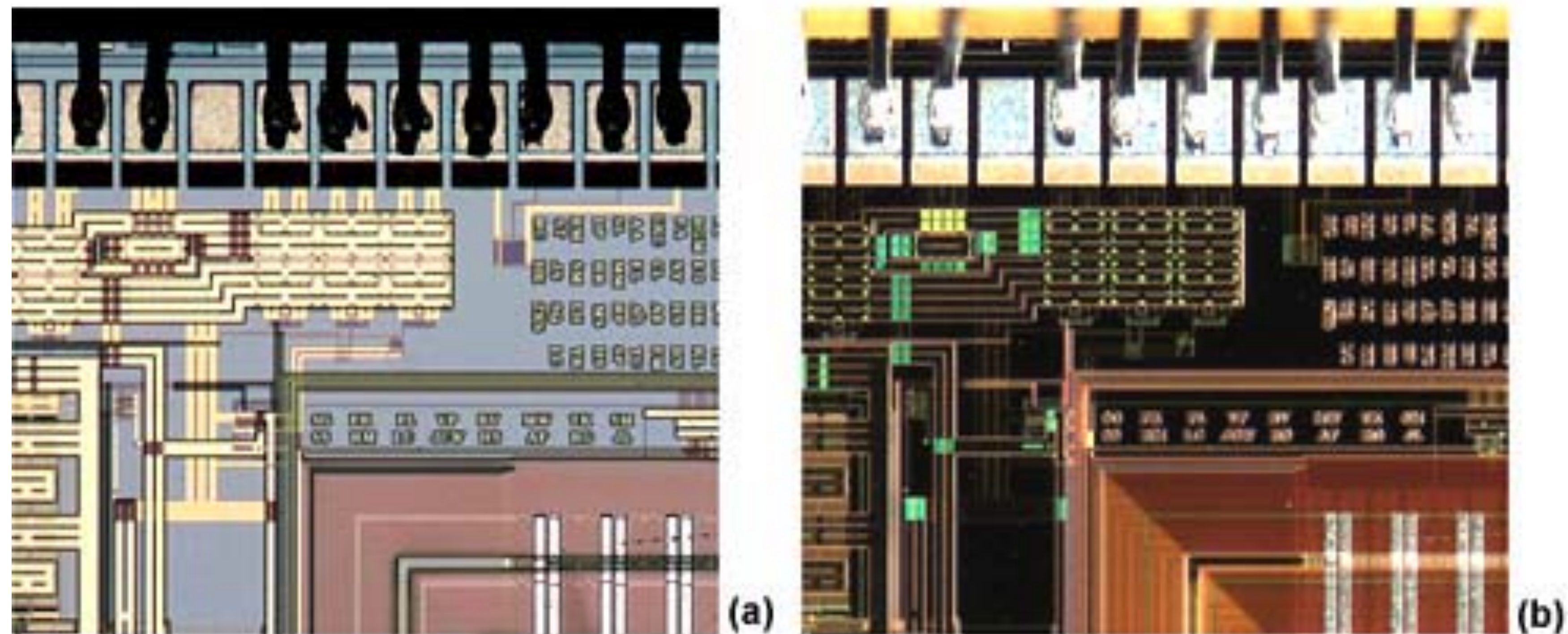
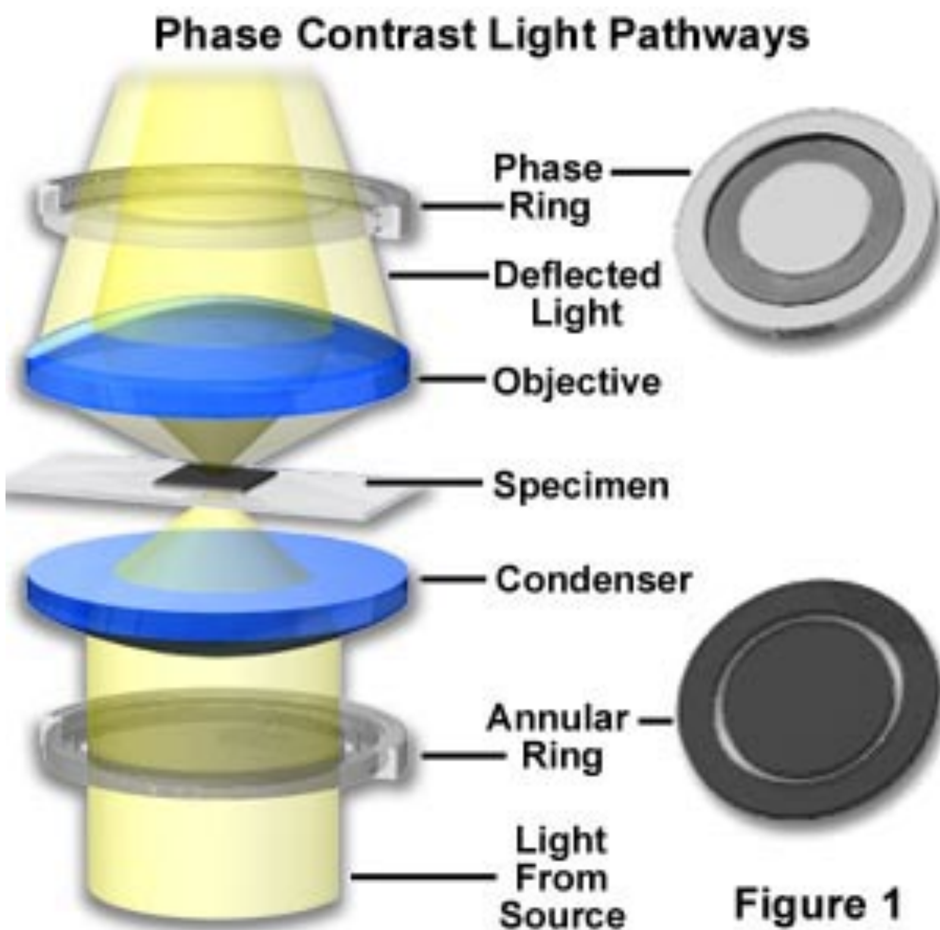


Figure 4

Phase contrast microscopy

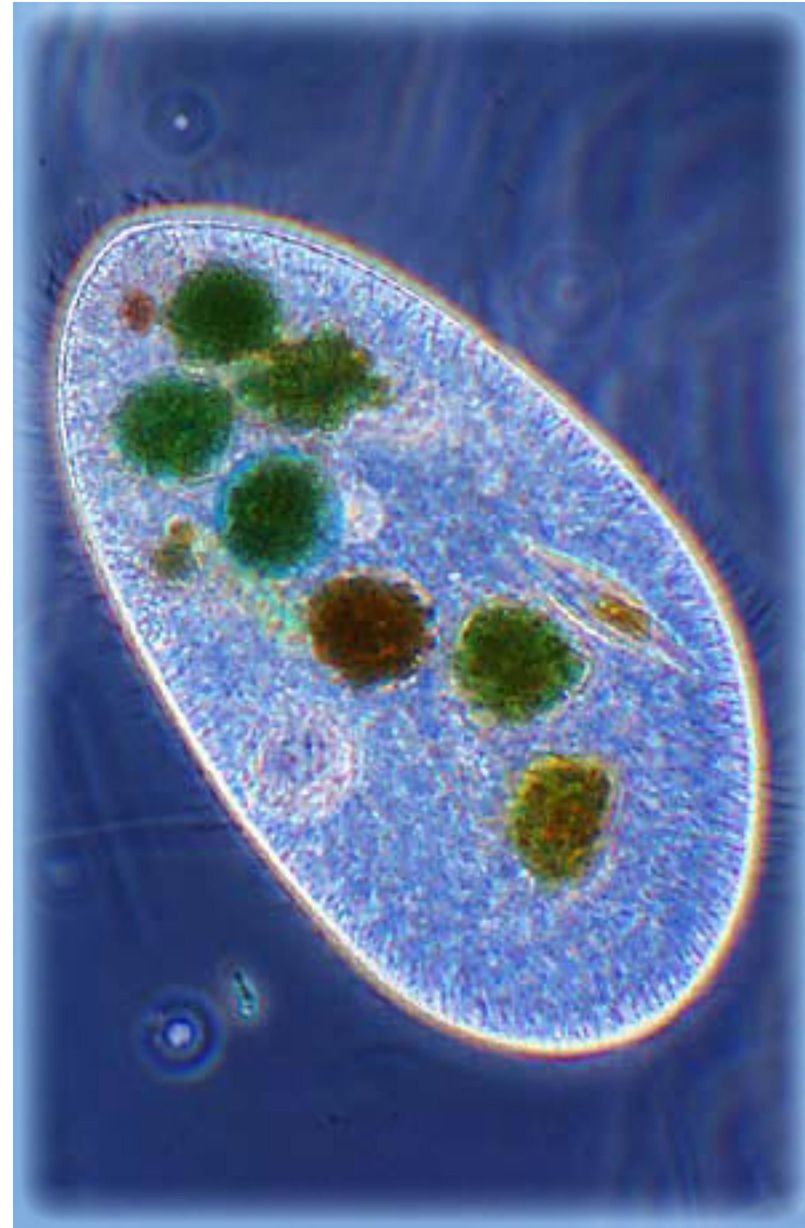


- **Basic idea:** speed up direct light by $\lambda/4$ to create destructive interference, thereby converting phase information into amplitude information (via **magnitude**)
- **Advantages:**
  - No stains required
  - Can be combined with reflected light fluorescence
  - Particularly useful for thin specimens scattered in field of view
- **Disadvantages:**
  - Images usually surrounded by halos (optical artifacts)
  - Phase annuli limits working NA and reduce resolution
  - Does not work well with thick specimens

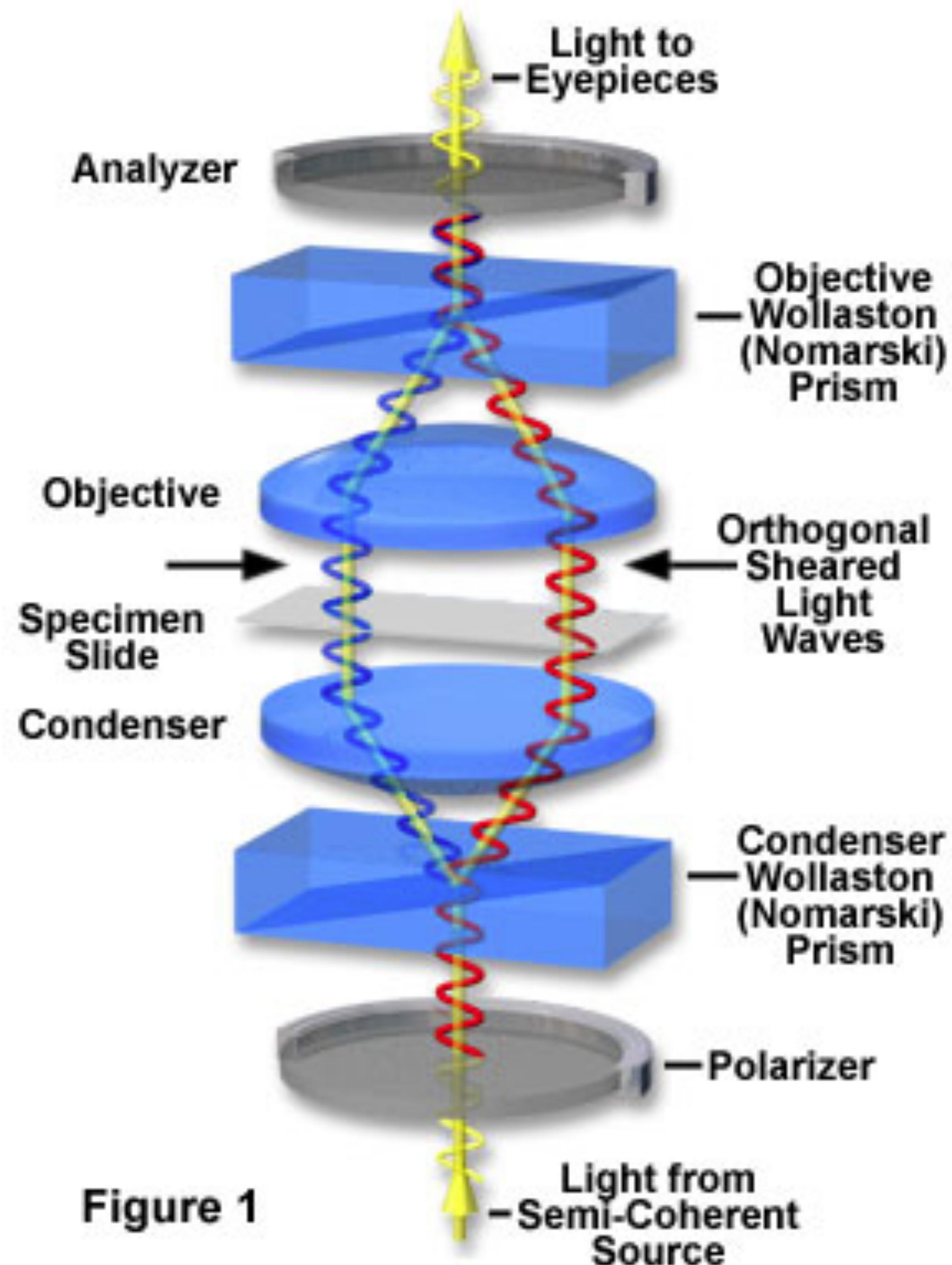
Sample phase images

Radiolarians

Paramecium



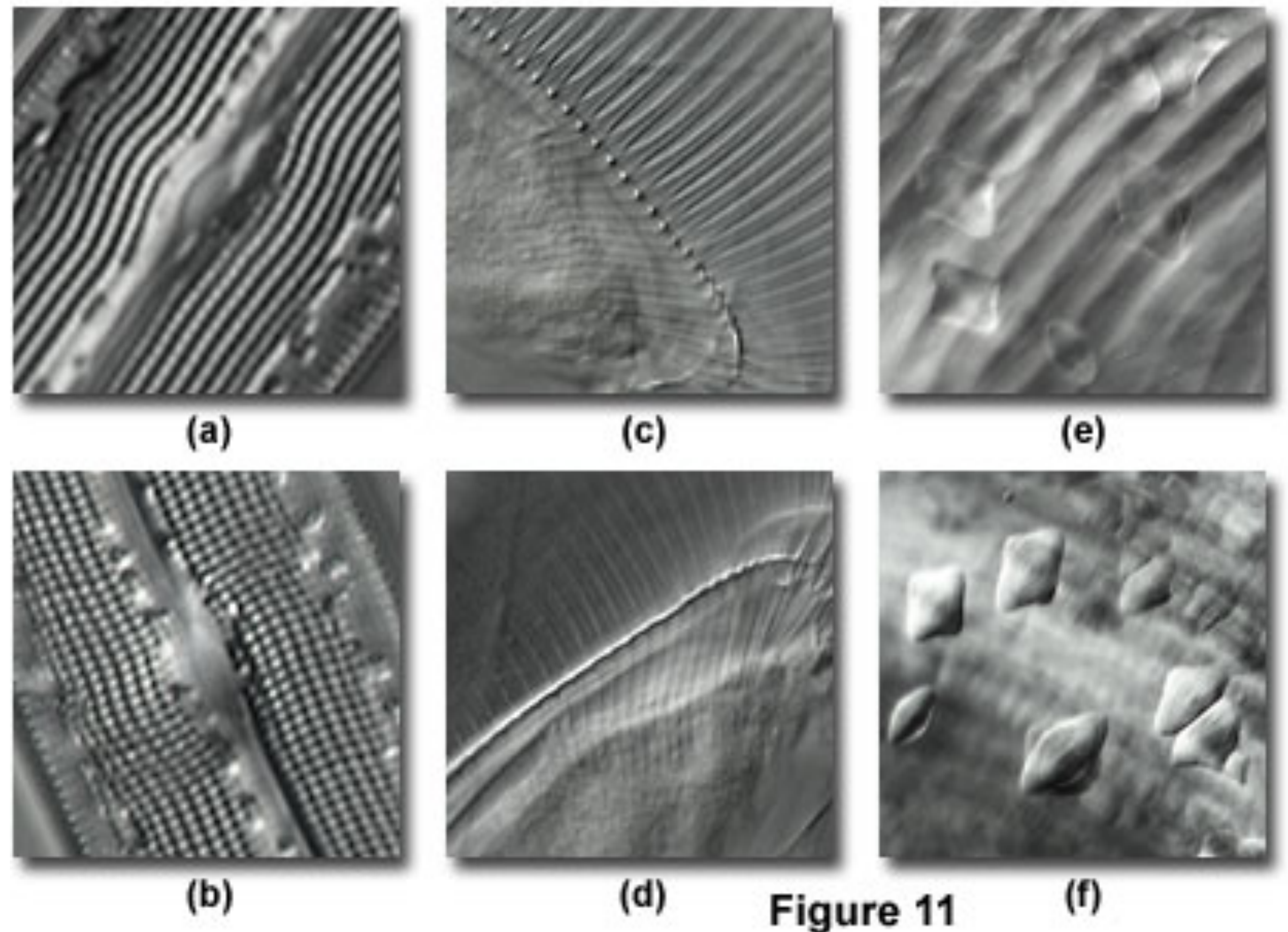
Differential interference contrast (DIC)



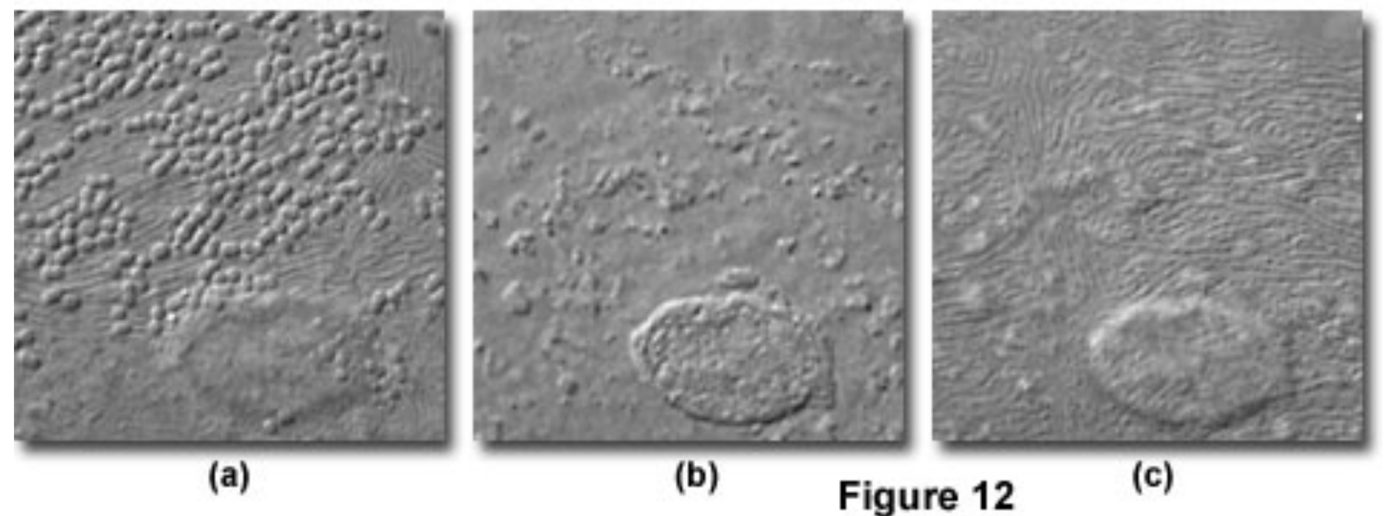
- **Basic idea:** "beam-shearing interferometer": convert **gradients** in specimen optical path length into amplitude differences
- **Advantages:**
  - Optical components do not mask condenser/objectives, and hence do no loss of resolution
    - Especially along optical axis
  - Elimination of halos (phase) -- do not obscure smaller features
  - Can be used on relatively thick specimens
- **Disadvantages:**
  - Qualitative, not quantitative
  - Cannot be used with plastic
  - Expensive optics

DIC sample images

Compare (a)-(b), (c)-(d), (e)-(f): Sample orientation influences level of detail that can be resolved



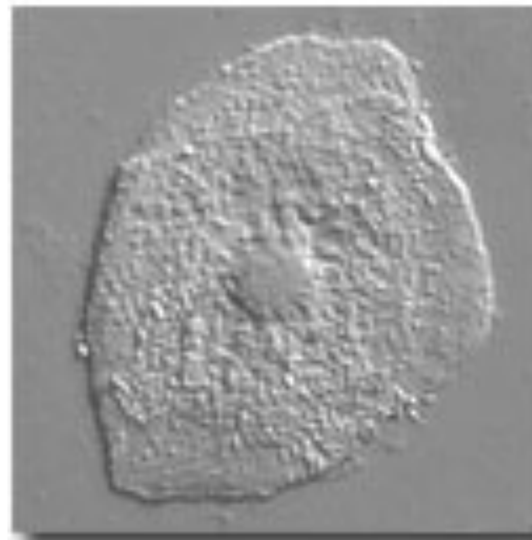
Optical sectioning possible, but quantitative interpretation requires comparison with computer models



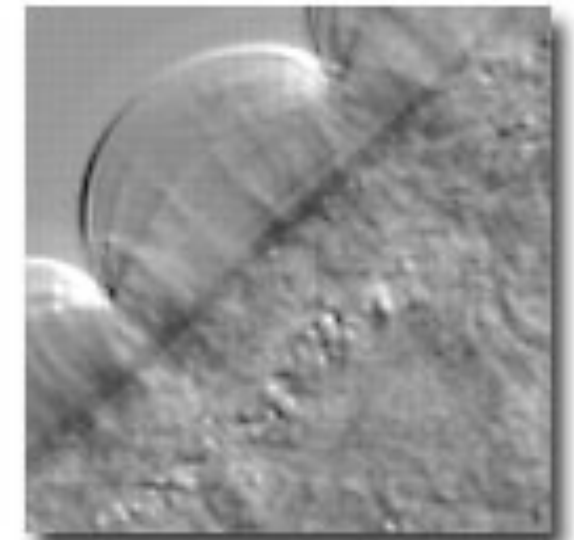
Phase versus DIC I

Transparent Specimens in Phase Contrast and DIC

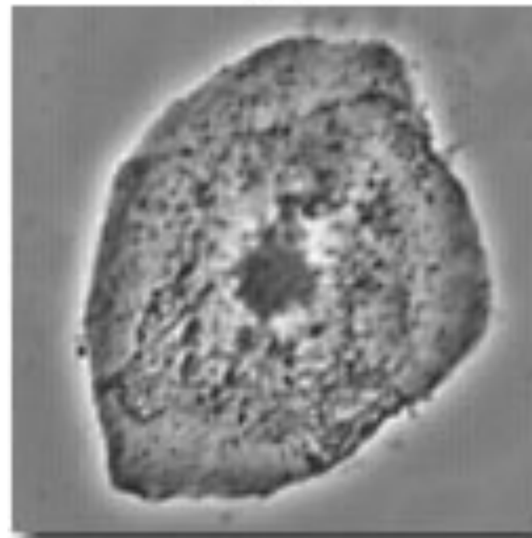
DIC
(optical path length **gradients**)

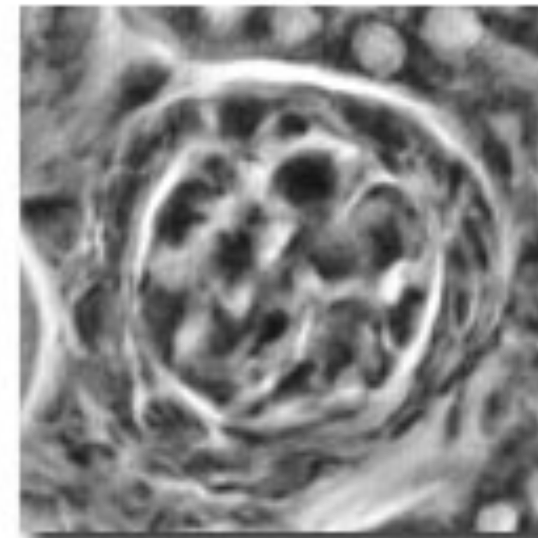

(a)

(c)


(e)

Phase
(optical path length **magnitude**)


(b)


(d)

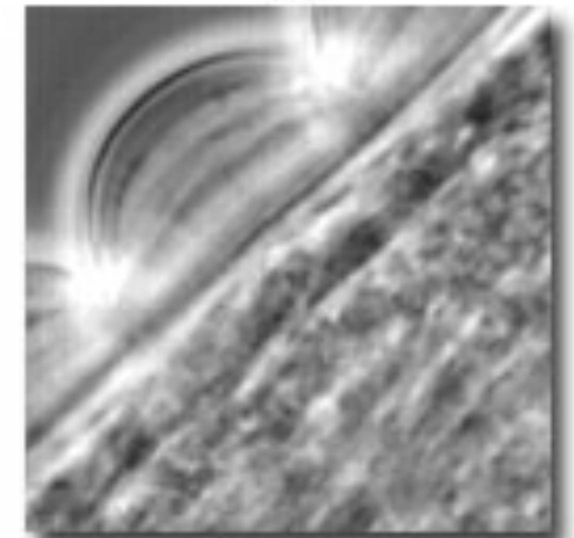
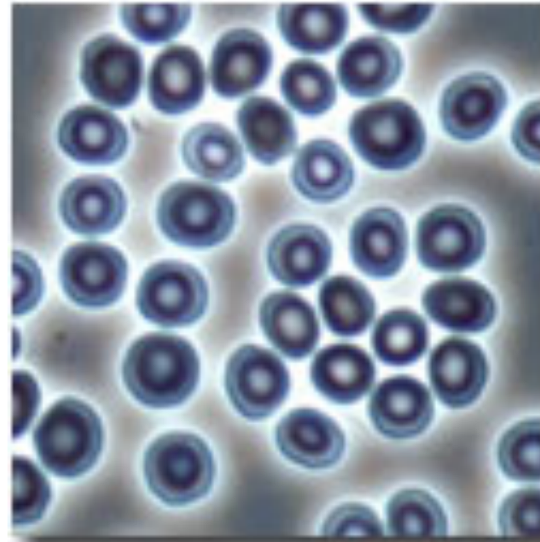

(f)

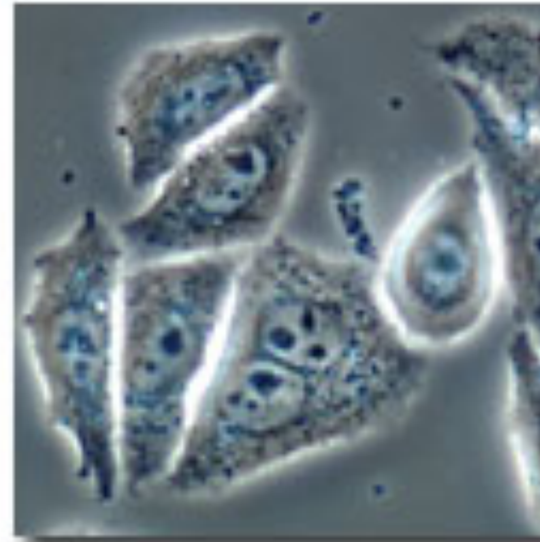
Figure 1

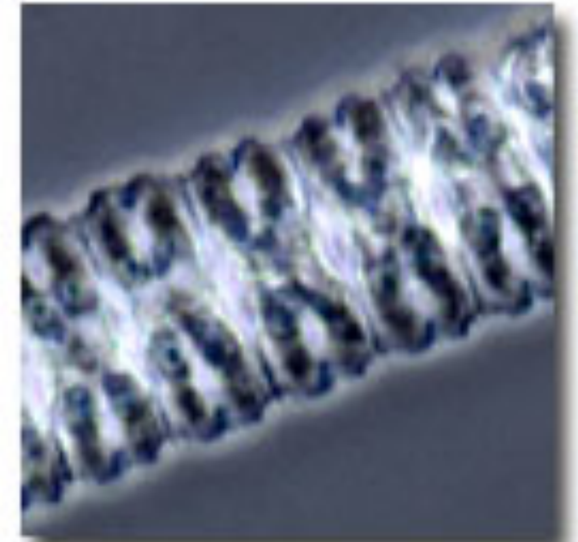
Phase versus DIC II

Halos in Phase Contrast and DIC Microscopy

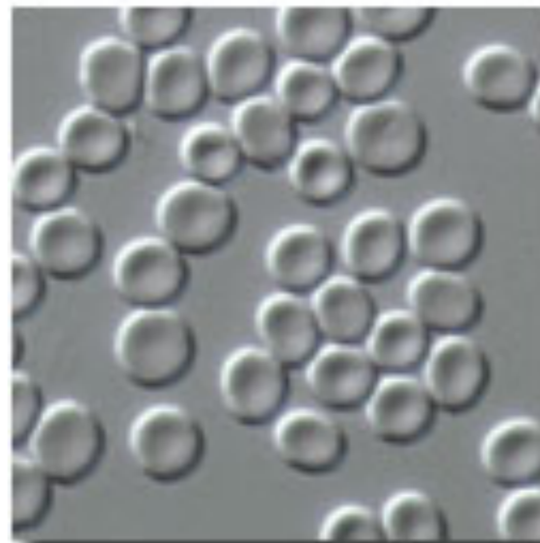
Phase
(optical path length
magnitude)

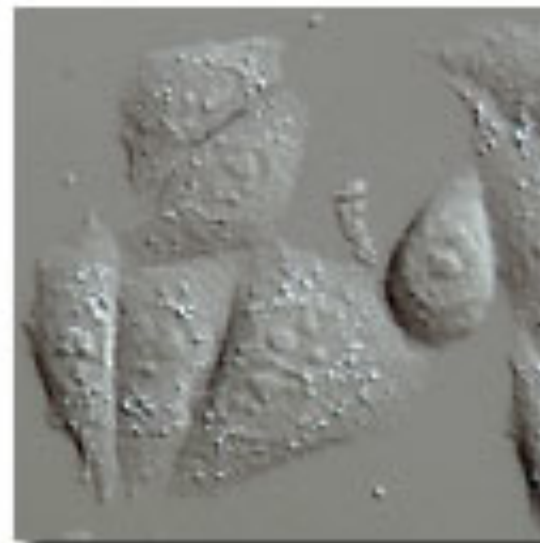

(a)


(c)


(e)

DIC
(optical path length
gradients)


(b)


(d)

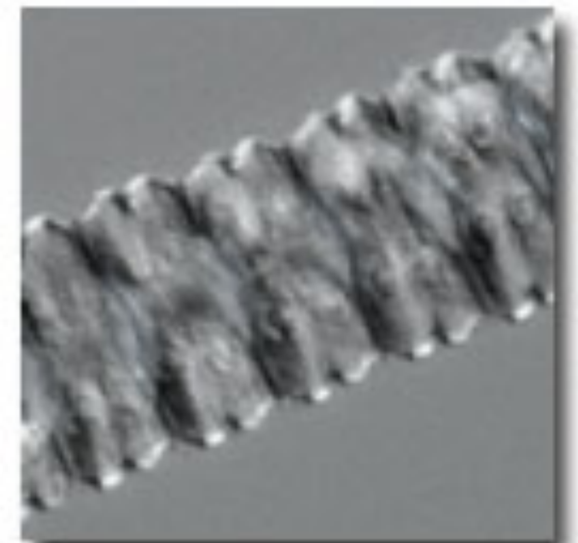
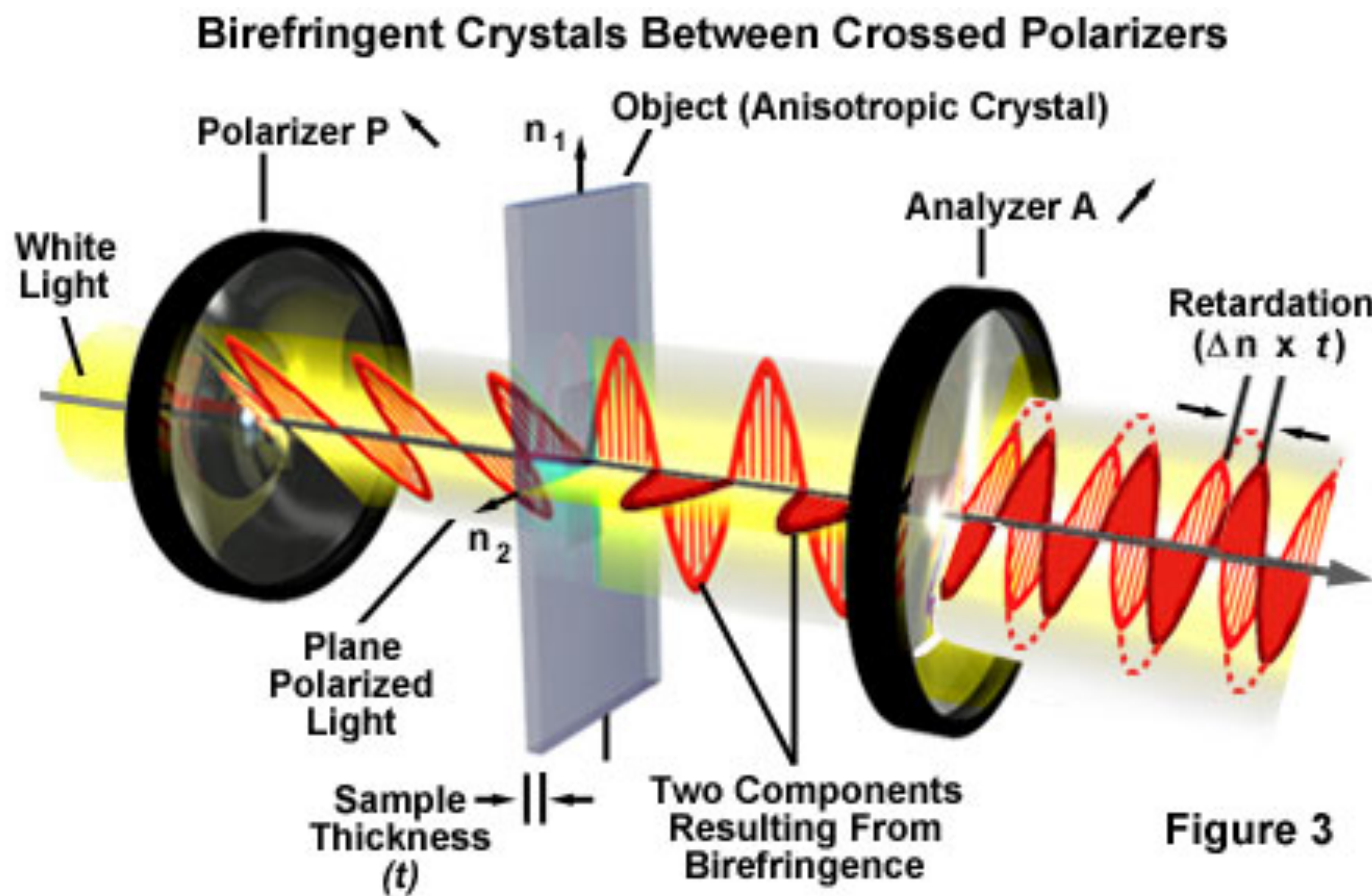

(f)

Figure 4

Polarized light microscopy



Birefringence:

$$B = |n_{\max} - n_{\min}|$$

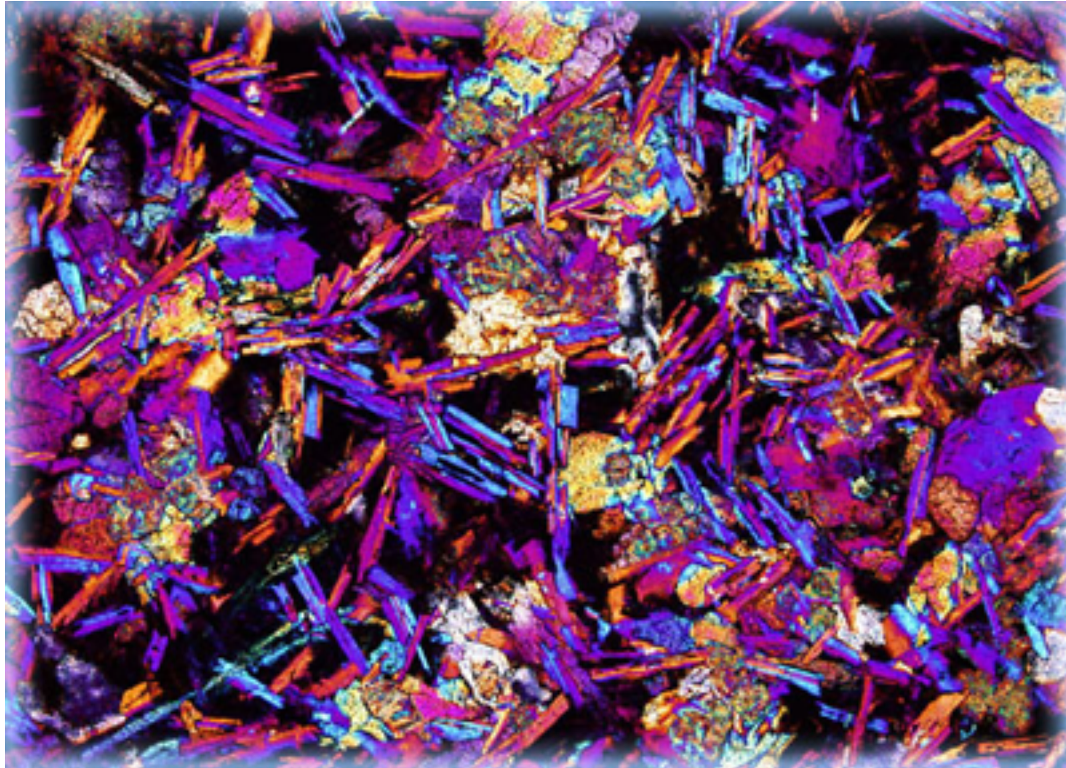
Retardation:

$$\Gamma = tB = t|n_{\max} - n_{\min}|$$

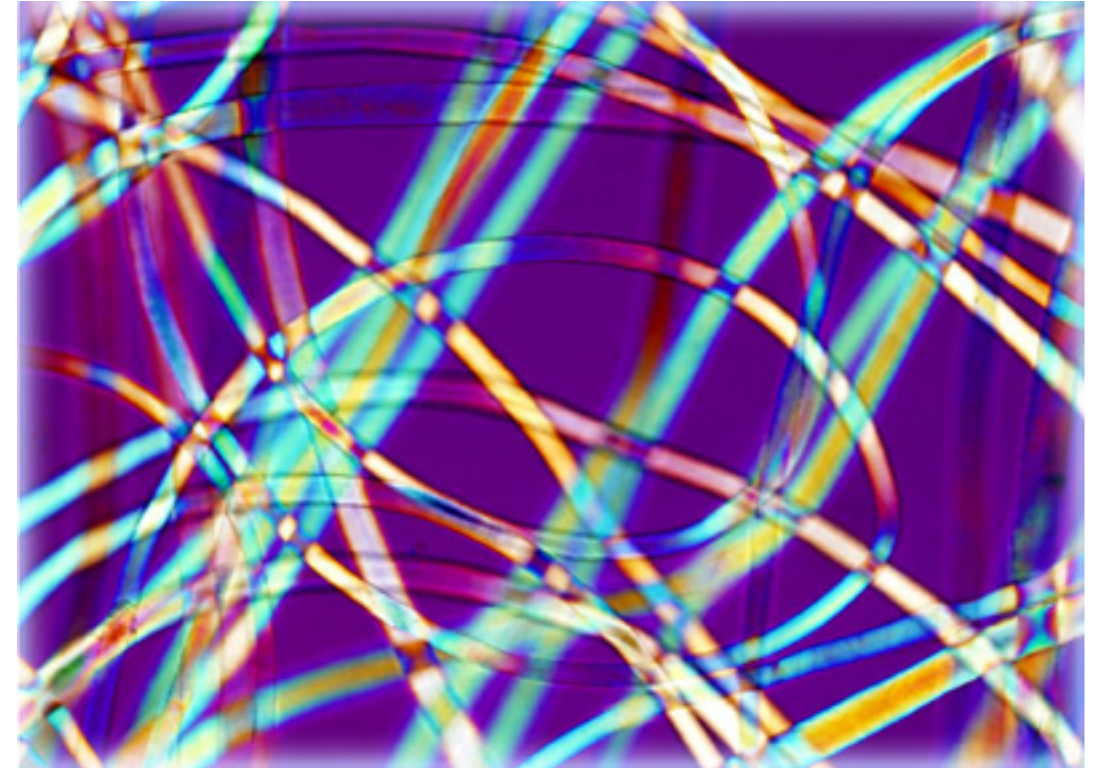
- **Basic idea:** image contrast is created by the interaction of plane-polarized light with a birefringent specimen
- **Advantages:**
  - Colors yield quantitative information on path differences
- **Disadvantages:**
  - Only works for birefringent samples
  - Requires strain-free objectives
- **Useful for:** liquid crystals, crystals, oriented polymers

Polarized microscopy sample images

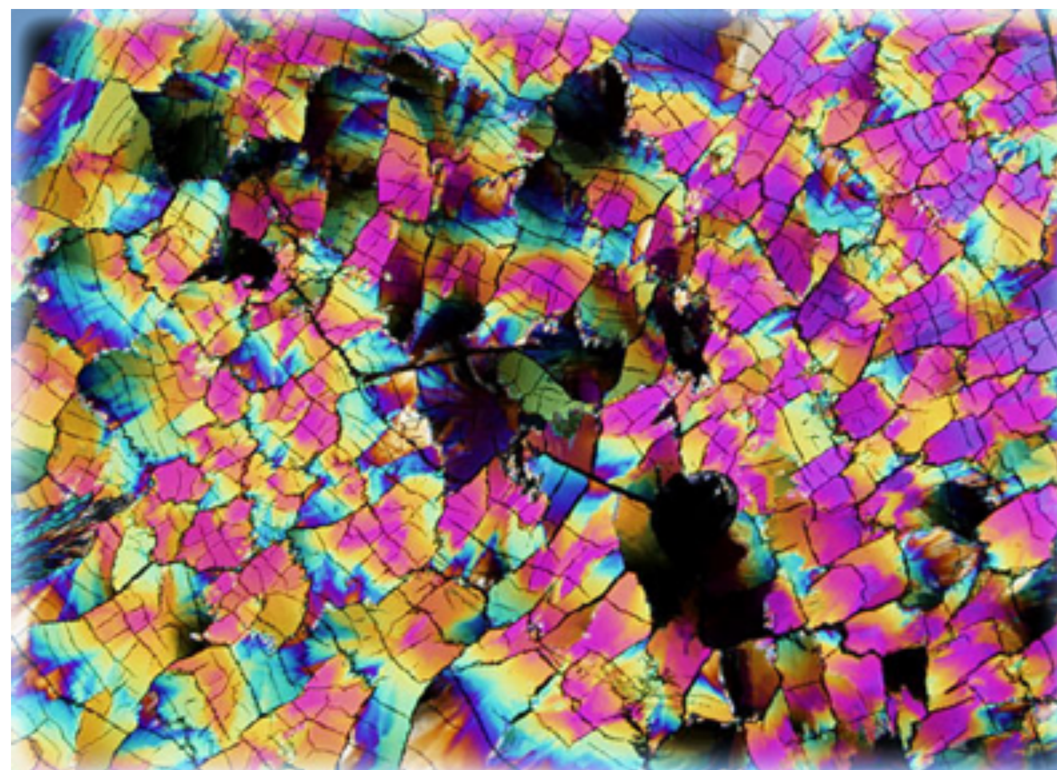
Basalt (solidified volcanic rock)



Wool



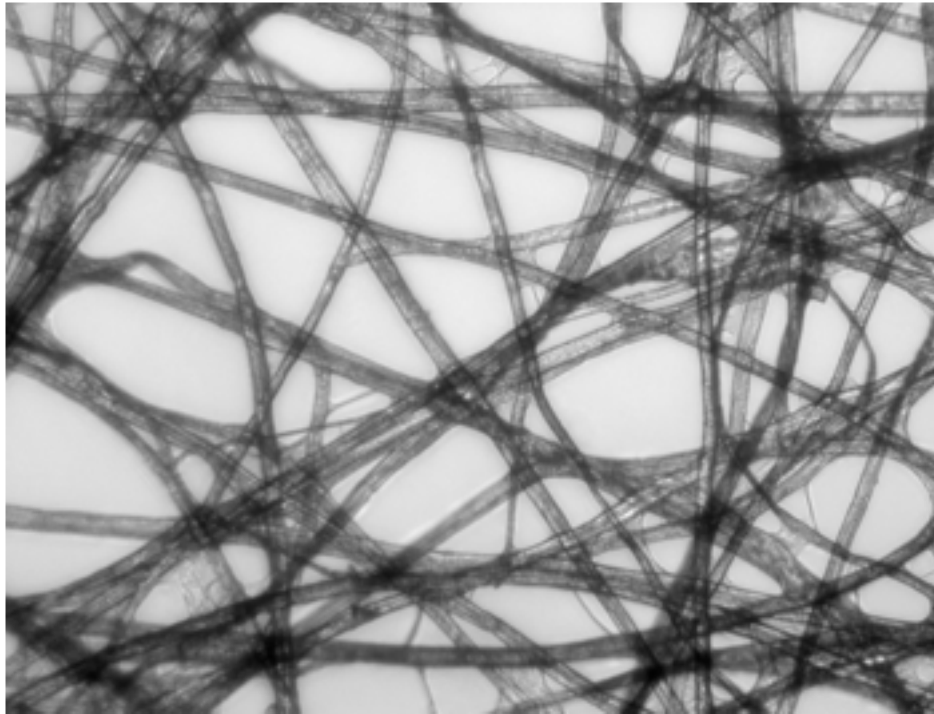
Cholesterol



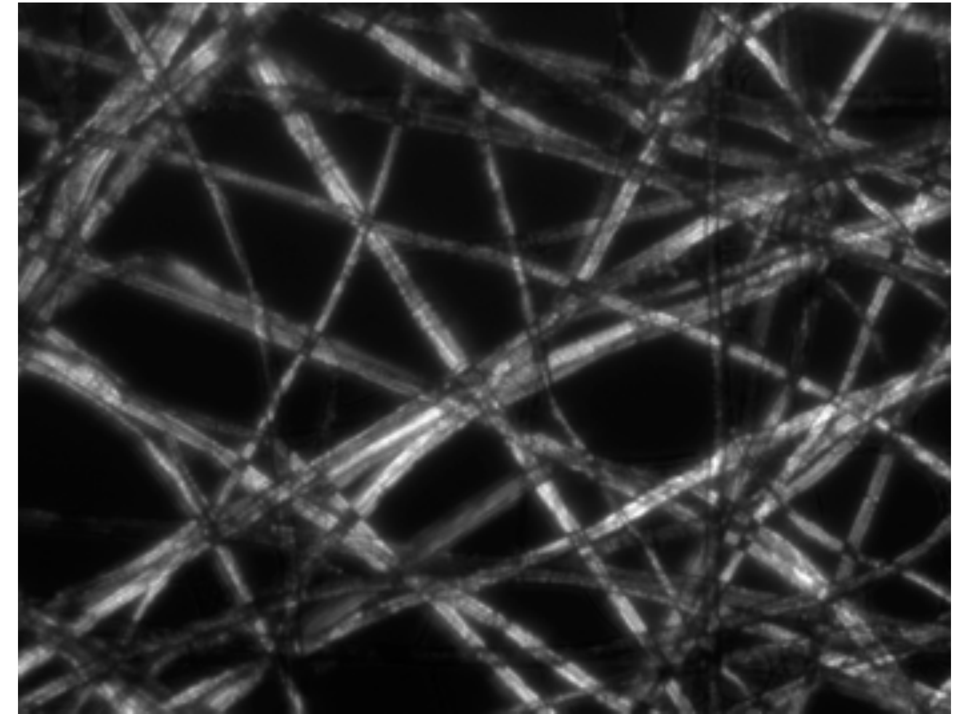
Comparison of darkfield, phase, polarized

Paper fibers

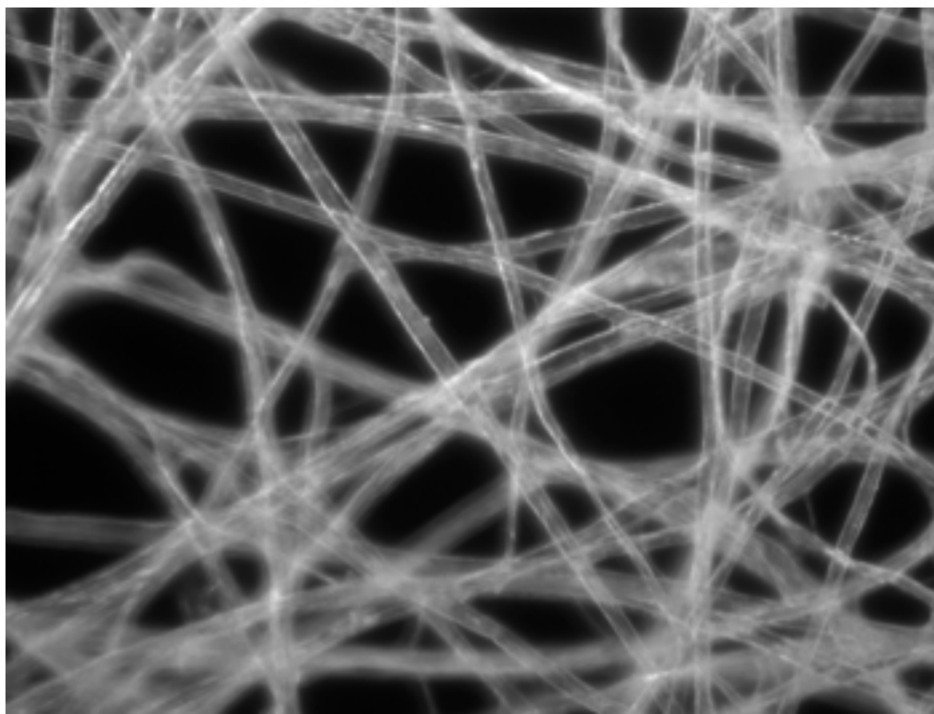
Brightfield



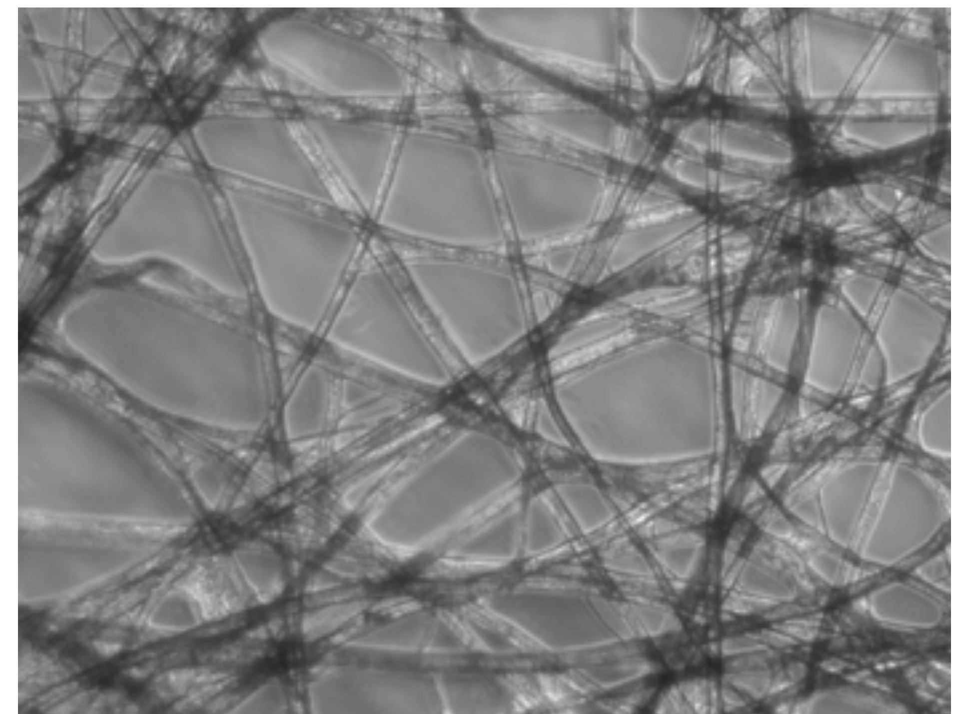
Polarized



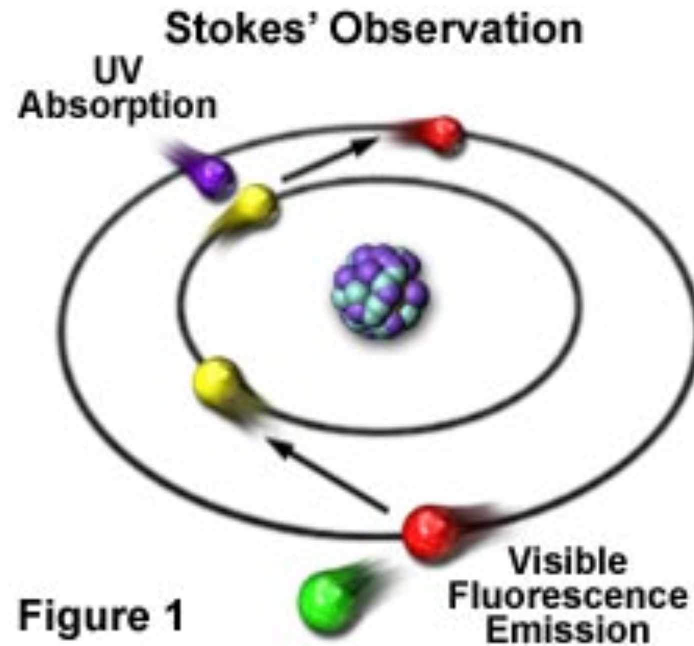
Darkfield

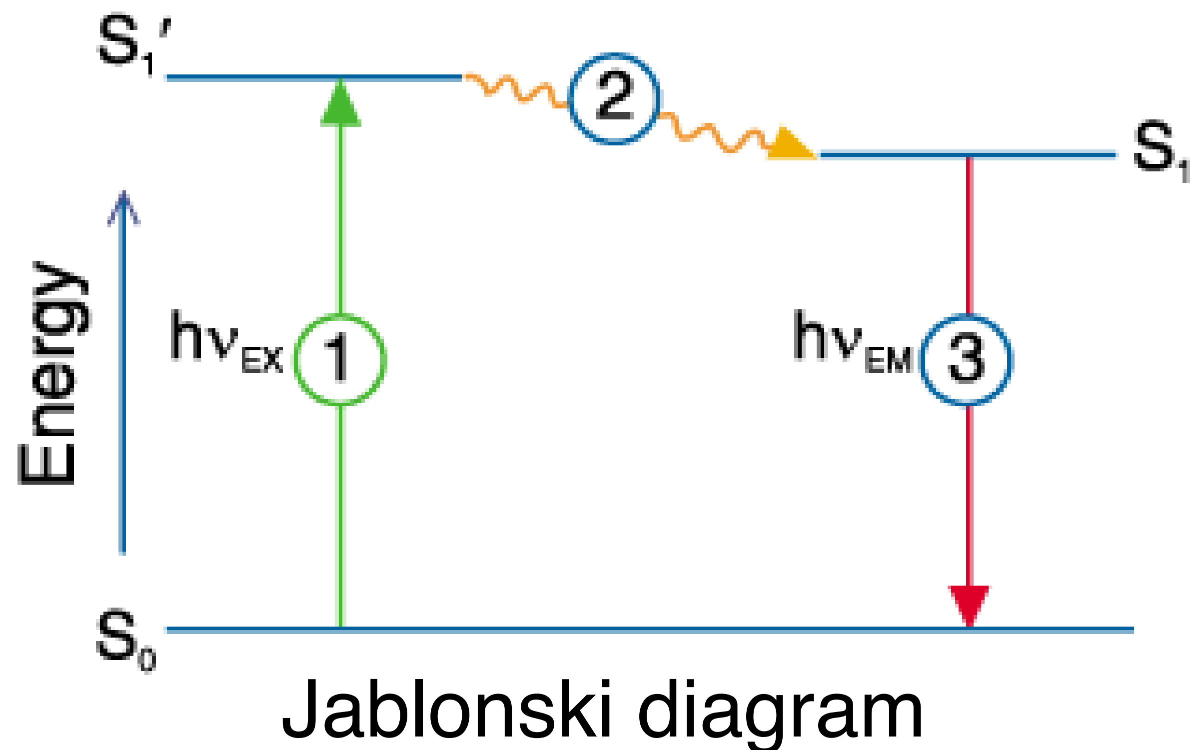


Phase



Fluorescence

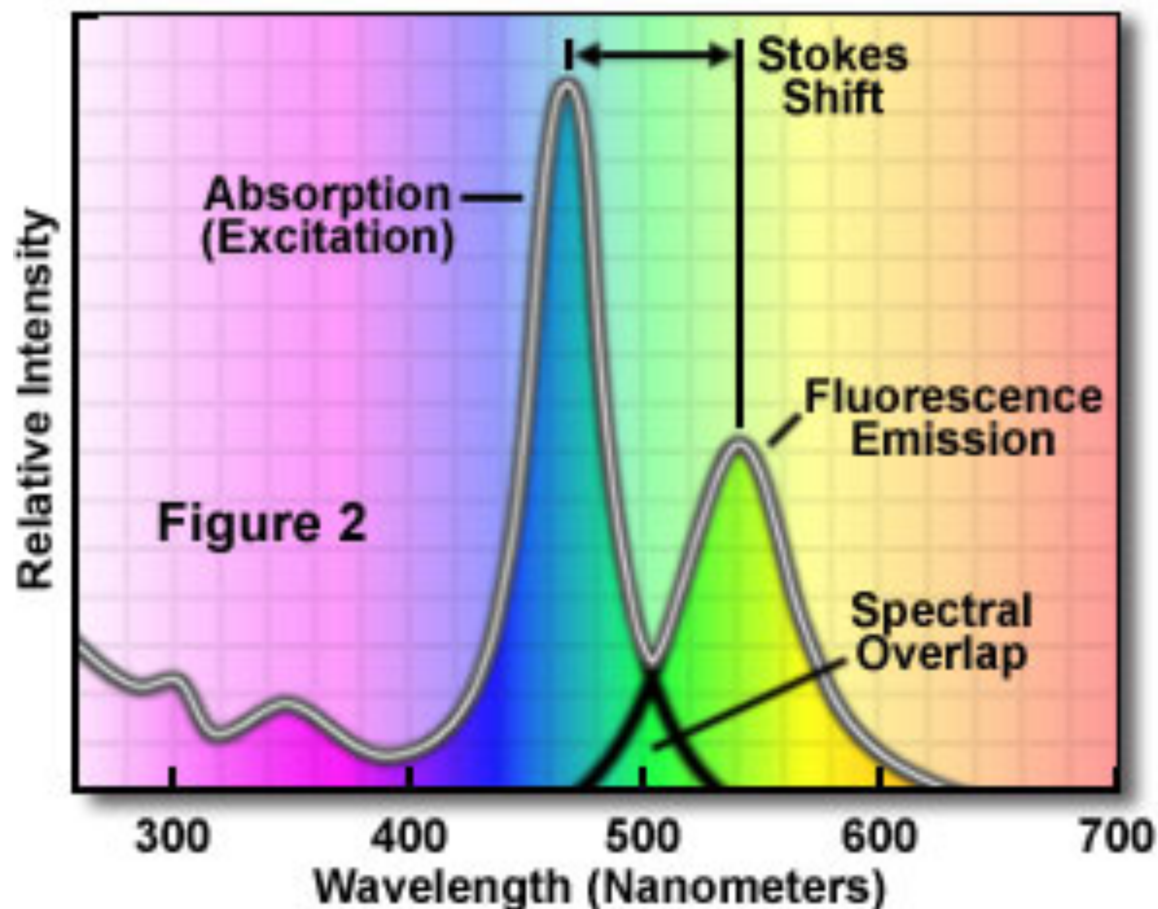




Jablonski diagram

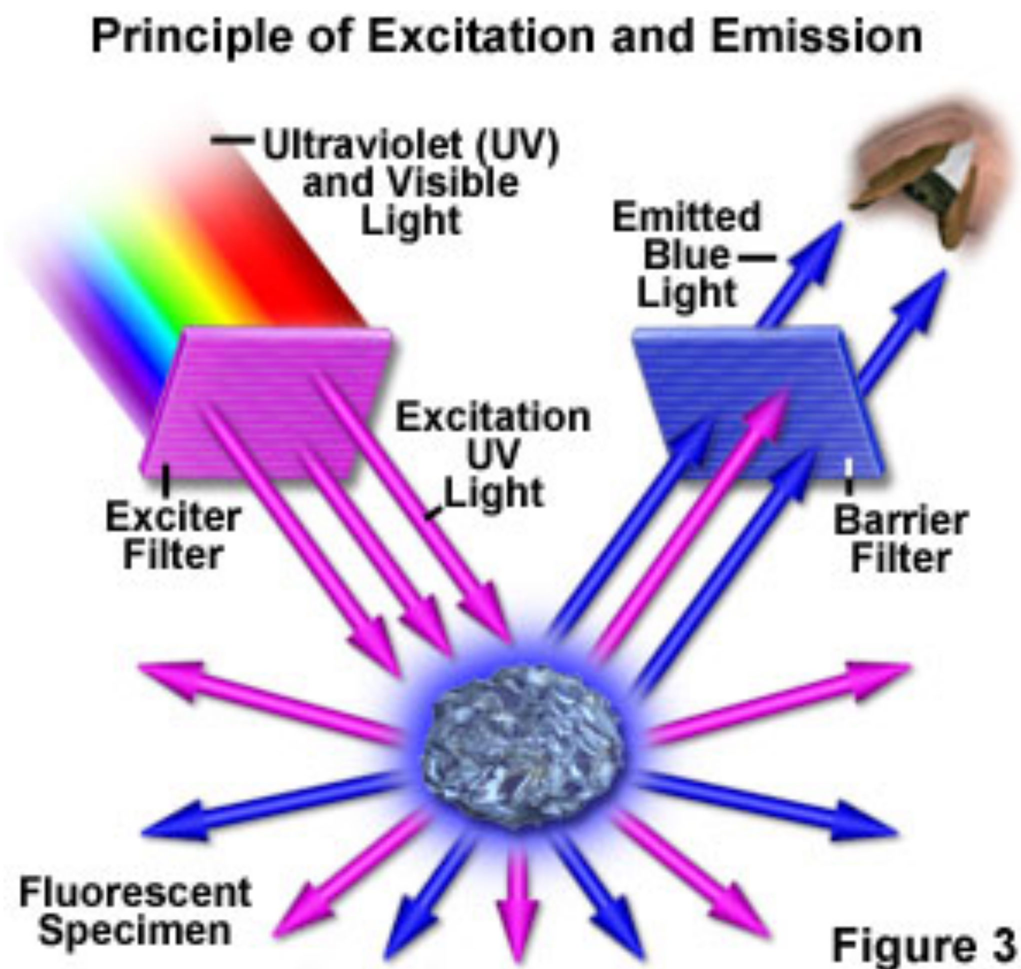
- **Basic idea:** molecules excited at a particular wavelength will emit light at a longer wavelength
- **Sequence of events:**
  1. Excitation ($h\nu_{ex}$)
  2. Excited state lifetime
  3. Fluorescence emission ($h\nu_{em}$)
- **Stokes shift:** $h\nu_{ex}$ - $h\nu_{em}$
- **Quantum yield:** photons emitted / photons absorbed (range: 0.1-0.9)

Quantitative properties of fluorescence



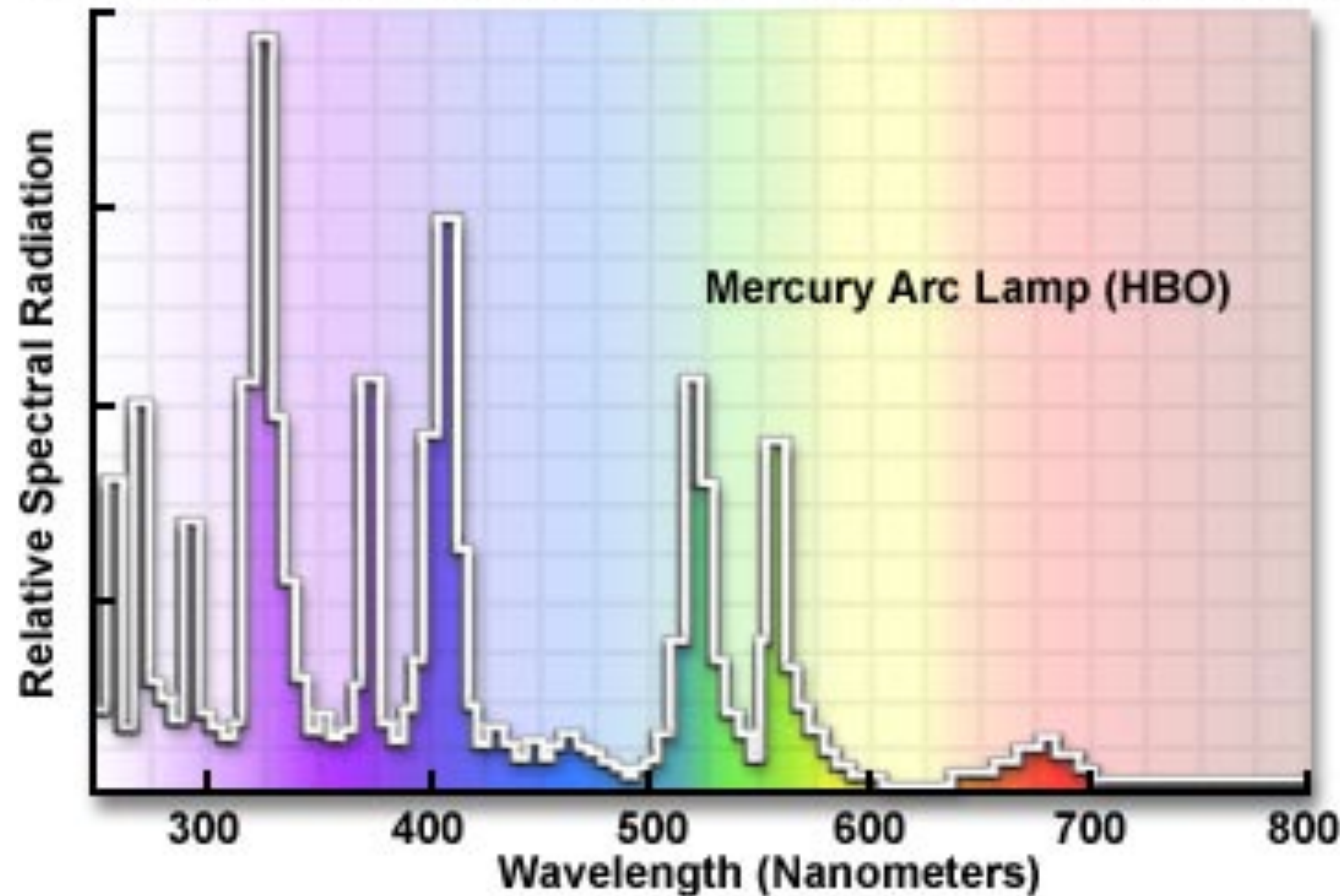
- For molecules in solution, spectra are broad
  - Excitation spectrum = absorption spectrum
- Emission spectrum independent of excitation wavelength
- Emission intensity proportional to excitation intensity
- Process is cyclical unless dye molecules get destroyed in excited state (“**photobleaching**”)

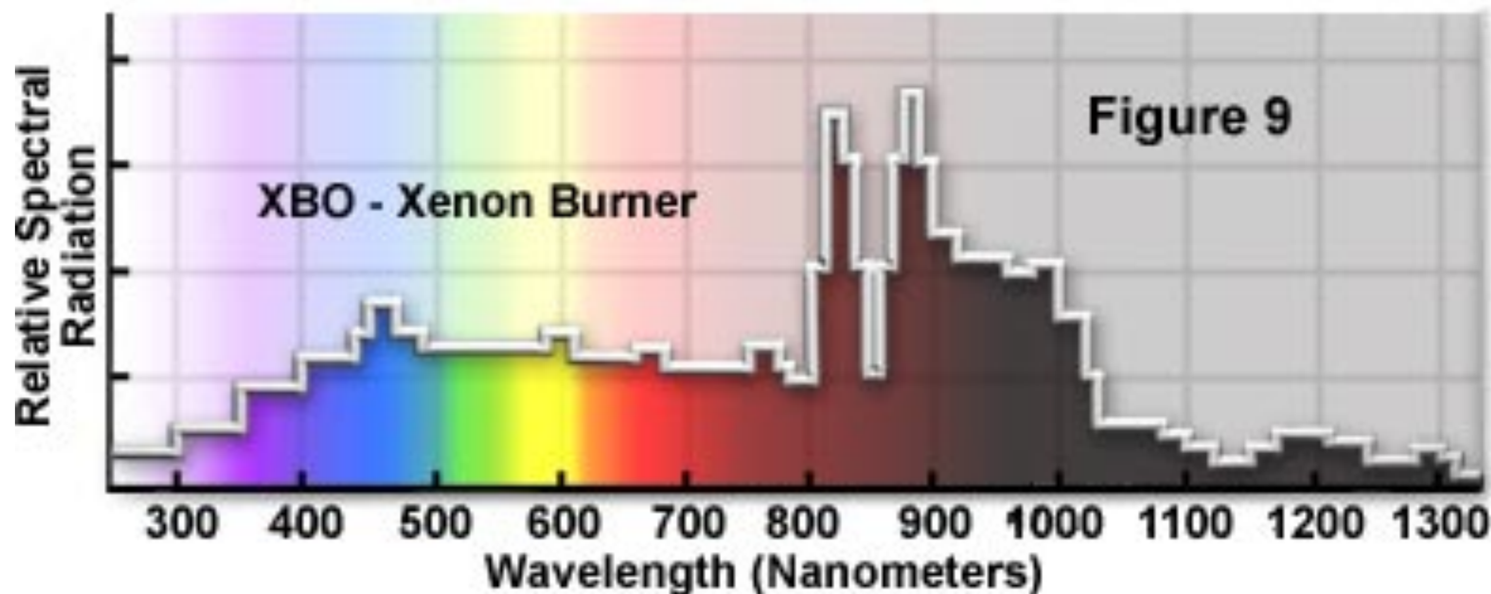
Fluorescence microscopy



- **Basic idea:** illuminate sample with light and detect the emitted fluorescence light ("epifluorescence")
- **Advantages:**
  - Image single molecule distribution
  - Multiple labels possible
  - High specificity: target labels to specific regions of interest
  - Local probe of conditions (pH, viscosity, ion concentration, etc.)
- **Disadvantages:**
  - Requires dye-specific filters and dichroics that are matched to the emission, excitation wavelengths

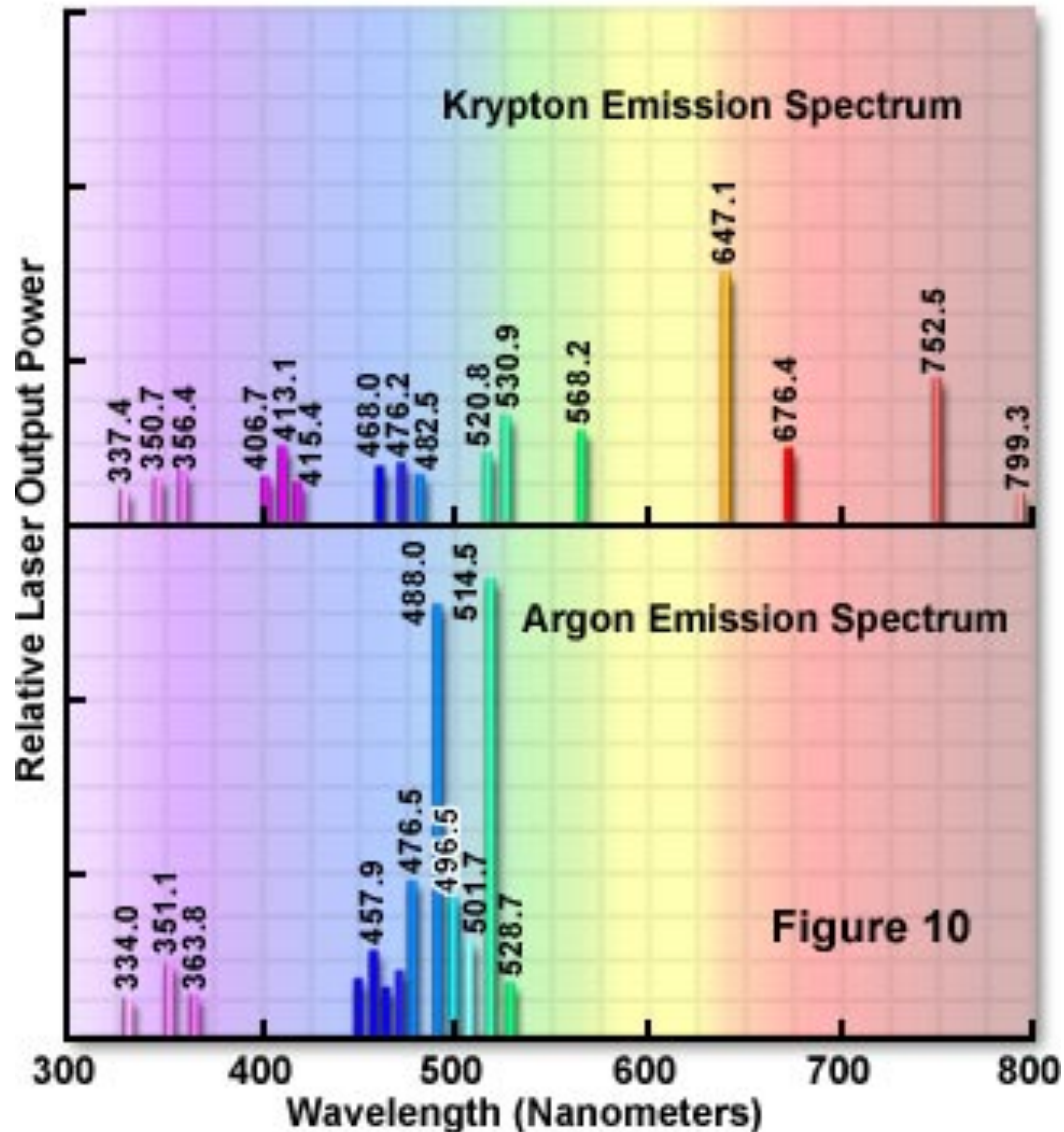
Arc lamp emission spectra



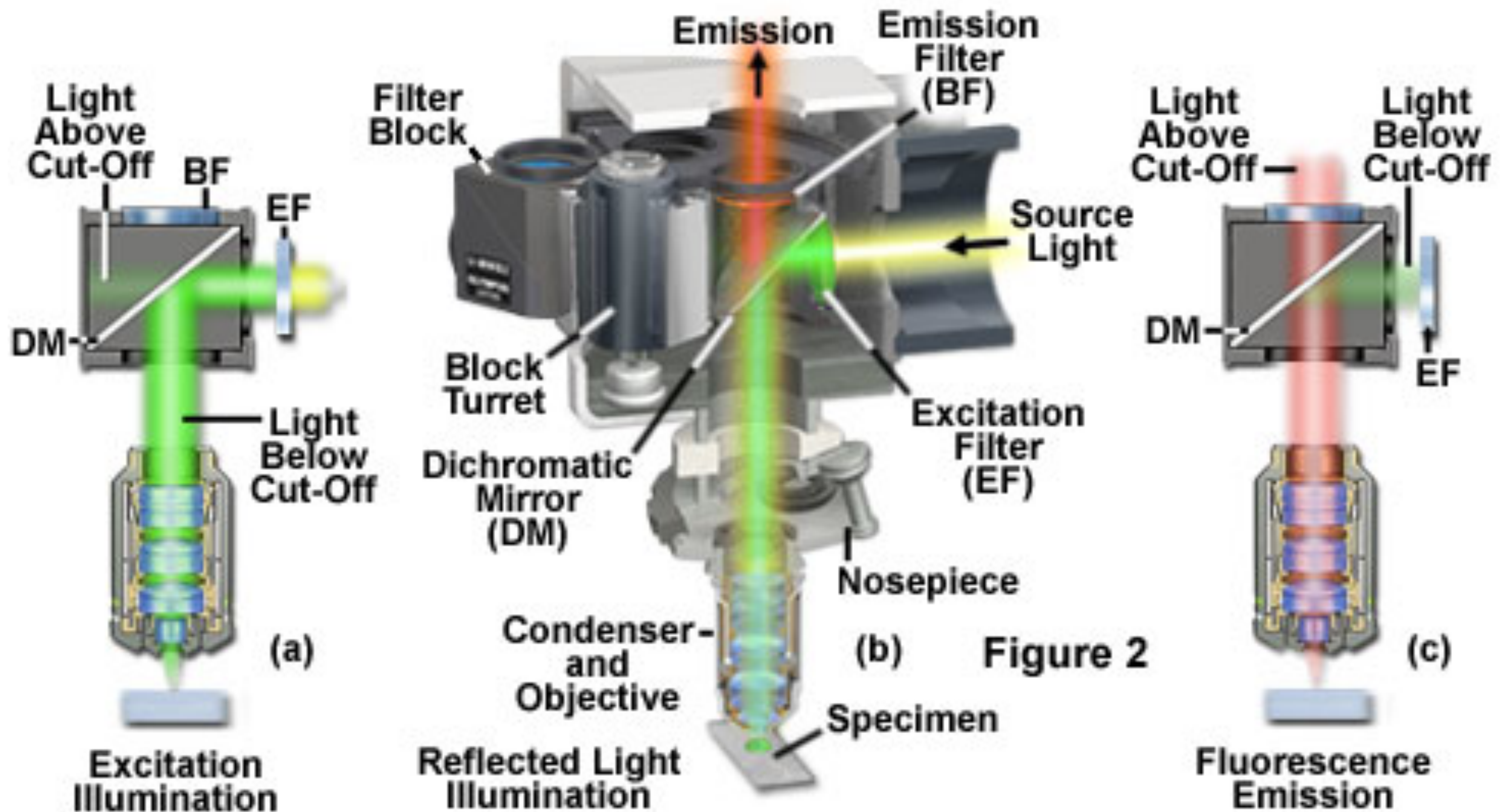


- **Mercury spectrum**:
  - Strongly peaked in visible range (especially in 500-600nm range)
  - Peaks can be used for monochromatic light for fluorescence
  - Should not be used for quantitative brightfield, darkfield, DIC, or polarized color
- **Xenon spectrum**:
  - More uniform across visible wavelengths
  - Deficient in UV

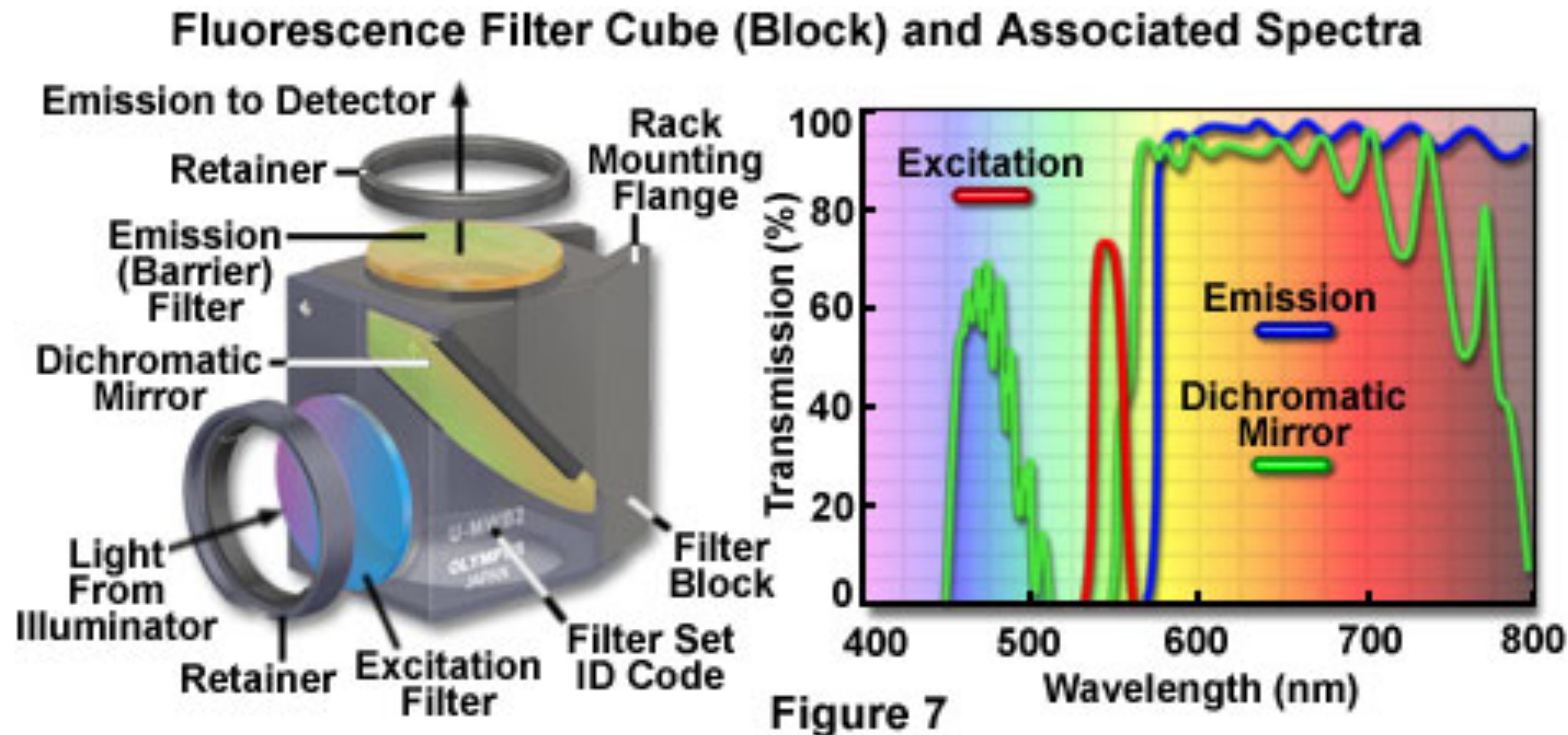
Common laser illumination sources



Filters for epifluorescence



Use of filter cubes to separate ex, em



- **Excitation filter**:
  - Typically **bandpass filter**: lets through wavelengths of a specific range ($\lambda_b \pm d\lambda$).
- Dichromatic mirror or **dichroic** (λ_d):
  - Designed to reflect light with $\lambda < \lambda_d$ and transmit light with $\lambda > \lambda_d$
- **Emission filter**:
  - Typically either **bandpass** ($\lambda_b \pm d\lambda$) or **longpass** (attenuates light with $\lambda < \lambda_l$ and transmits light with $\lambda > \lambda_l$)

Common fluorophores I

Dye	Absorbance Wavelength	Emission Wavelength	Visible color
Hydroxycoumarin	325	386	blue
methoxycoumarin	360	410	blue
Alexa fluor	345	442	blue
aminocoumarin	350	445	blue
Cy2	490	510	green (dark)
FAM	495	516	green (dark)
Alexa fluor 488	494	517	green (light)
Fluorescein FITC	495	518	green (light)
Alexa fluor 430	430	545	green (light)
Alexa fluor 532	530	555	green (light)
HEX	535	556	green (light)
Cy3	550	570	yellow
TRITC	547	572	yellow
Alexa fluor 546	556	573	yellow
Alexa fluor 555	556	573	yellow
R-phycoerythrin (PE)	480;565	578	yellow
Rhodamine Red-X	560	580	orange
Tamara	565	580	red
Cy3.5 581	581	596	red
Rox	575	602	red
Alexa fluor 568	578	603	red
Red 613	480;565	613	red
Texas Red	615	615	red
Alexa fluor 594	590	617	red
Alexa fluor 633	621	639	red
Allophycocyanin	650	660	red
Alexa fluor 633	650	668	red
Cy5	650	670	red
Alexa fluor 660	663	690	red
Cy5.5	675	694	red
TruRed	490;675	695	red
Alexa fluor 680	679	702	red
Cy7	743	770	red

Common fluorophores II

Nucleic acid probes (DNA/RNA):

Dye	Absorbance Wavelength	Emission Wavelength	Visible color
DAPI	345	455	blue
Hoechst 33258	345	478	blue
SYTOX blue	431	480	blue
Hoechst 33342	343	483	blue
YOYO-1	509	509	green
SYTOX green	504	533	green
TOTO 1, TO-PRO-1	509	533	green
SYTOX orange	547	570	yellow
Chromomycin A3	445	575	yellow
Mithramycin	445	575	yellow
Propidium iodide	536	617	red
Ethidium bromide	493	620	red

Quantum dots as fluorophores

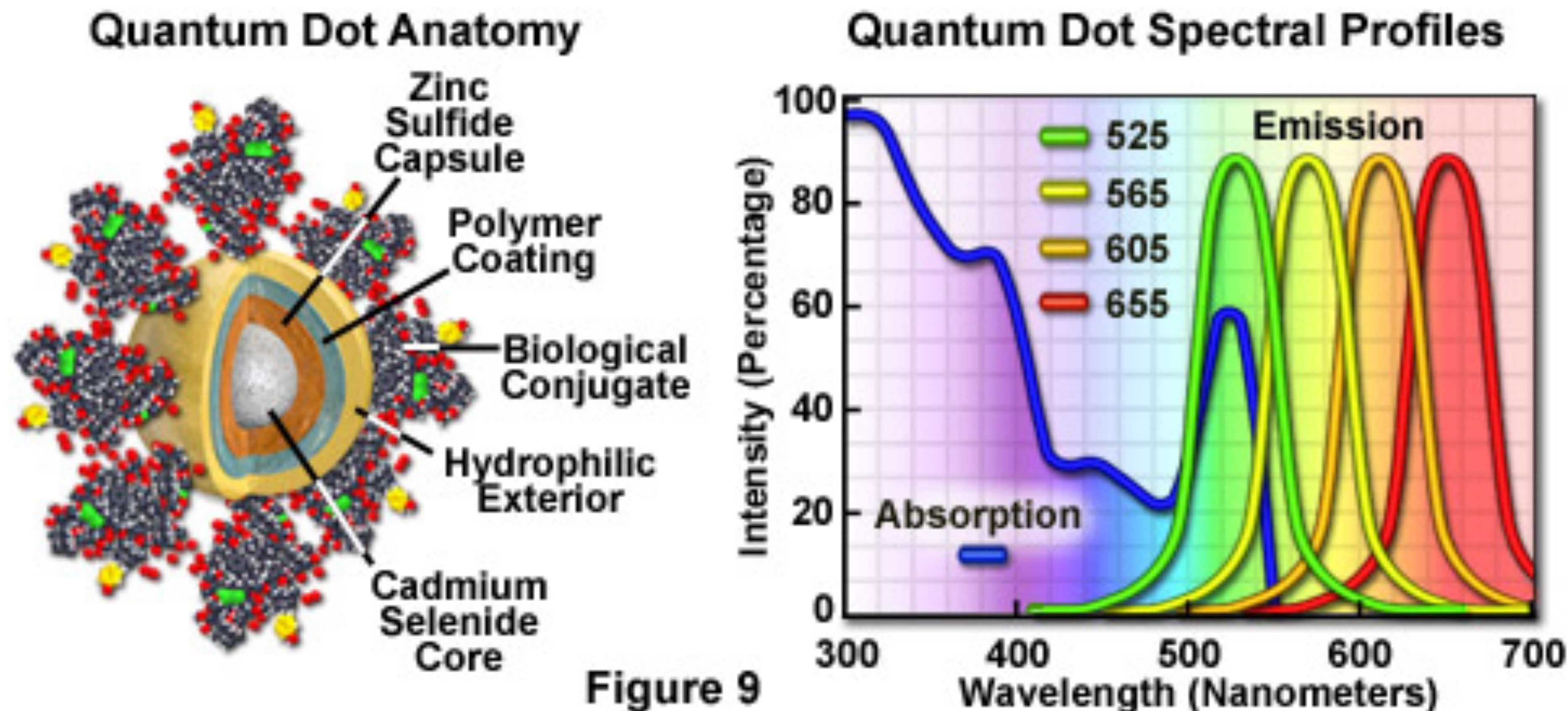
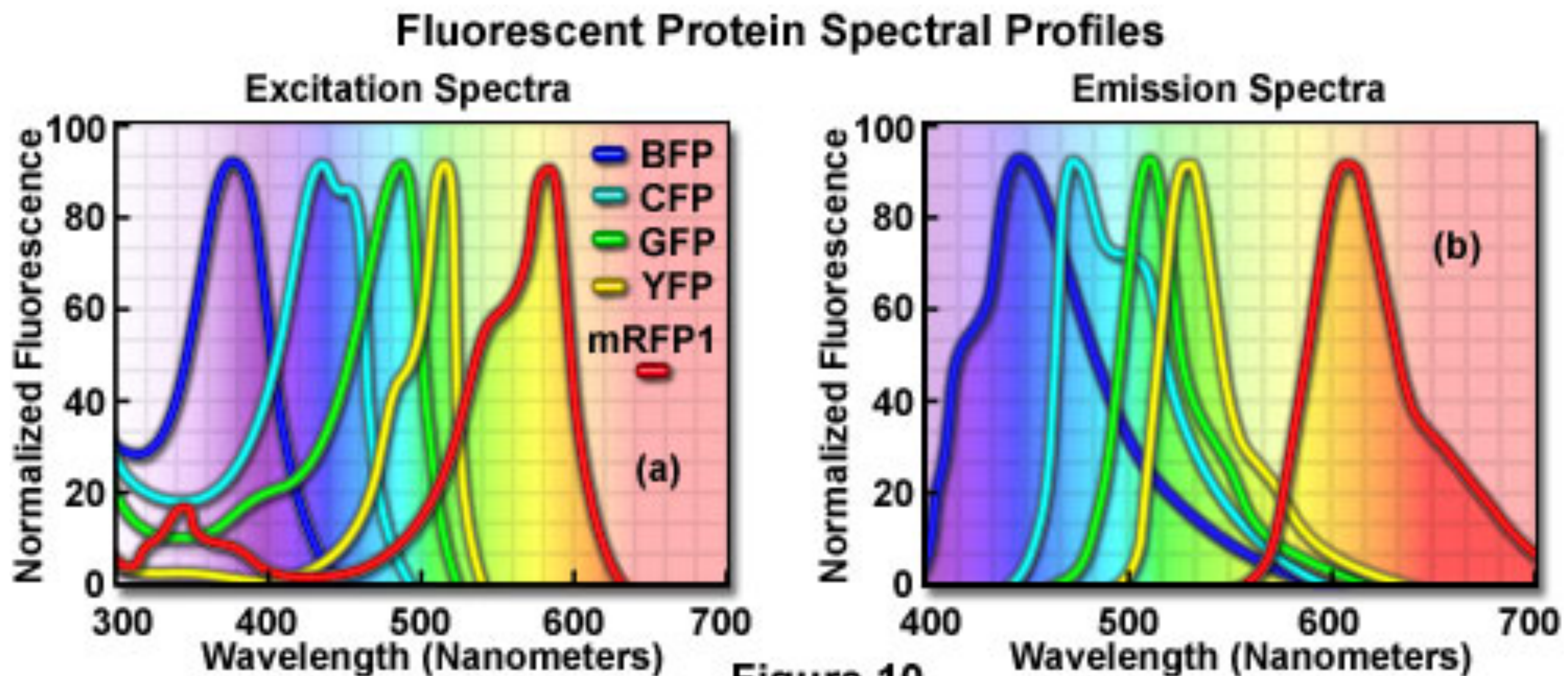


Figure 9

- **Quantum dot**: semiconductor with excitons confined in all three spatial dimensions
  - Properties are intermediate between bulk semiconductors and discrete molecules
- Emission wavelength directly related to qdot size, independent of excitation wavelength
- **Advantage**: very photostable (resists photobleaching)
- Used (in nanoparticle form) for single particle tracking of proteins, organelles, etc.

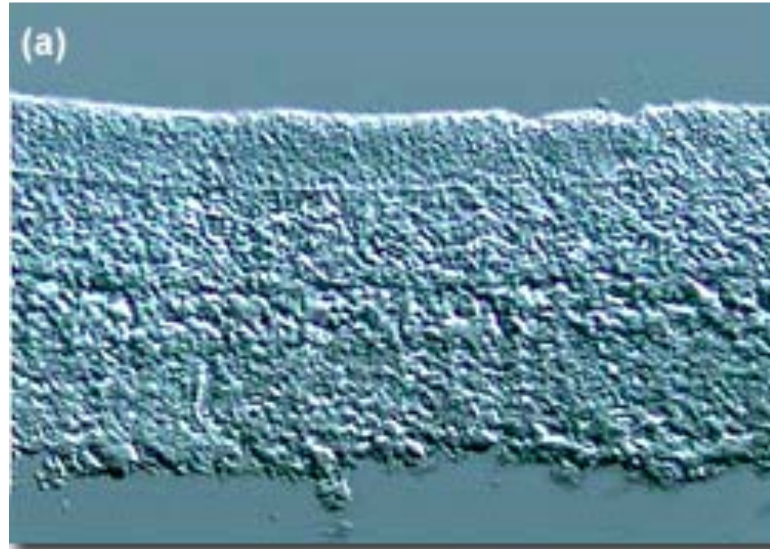
GFP and biologically-derived fluorophores

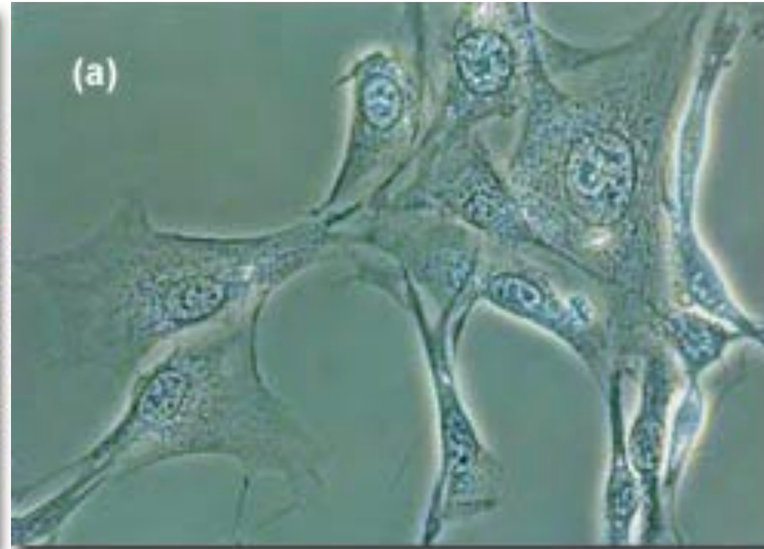
- **Green fluorescent protein** (GFP) isolated from north Atlantic jellyfish
  - Can be conjugated into mammalian and bacterial cells via transfected plasmids
- Replacement for fluorescein/FITC dyes
- **Advantage**: less toxic to cells



Phase or DIC + fluorescence

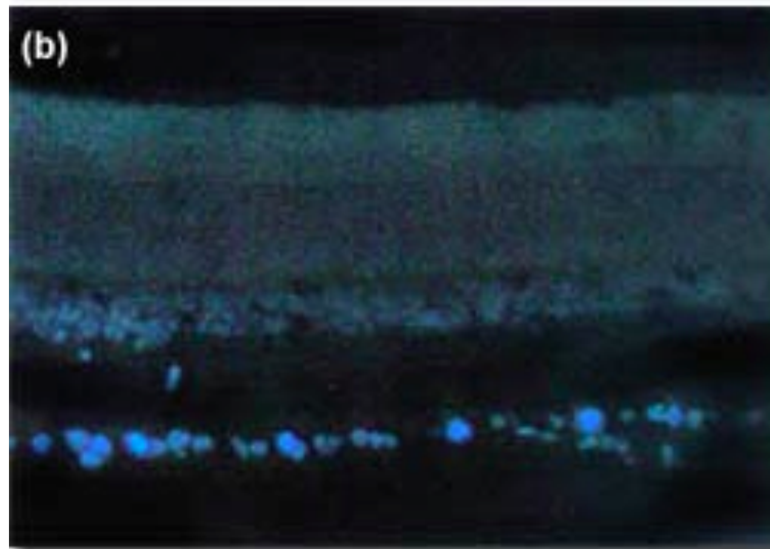
DIC

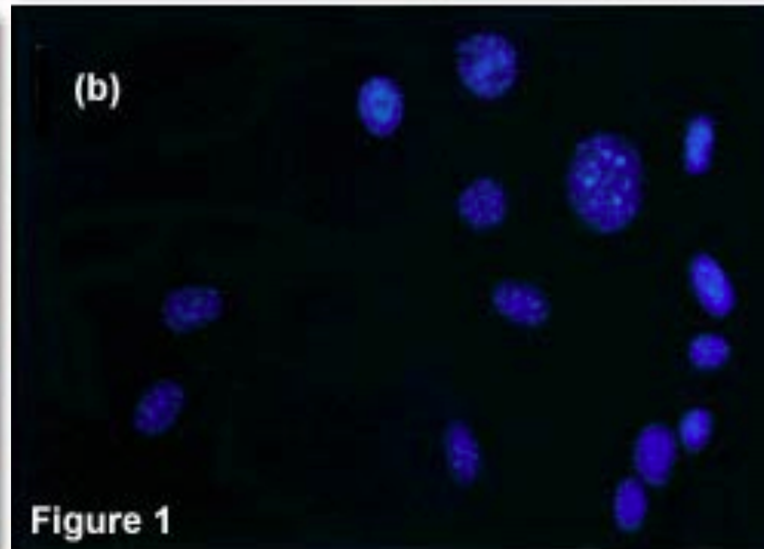




Phase

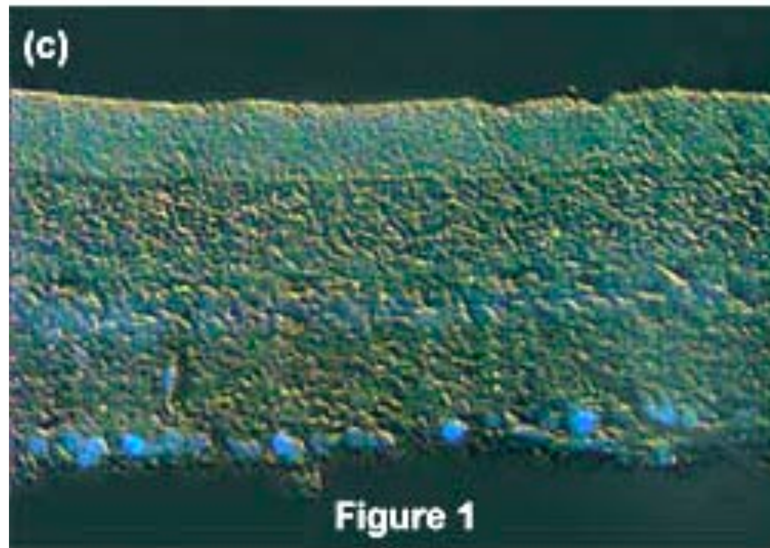
Fluorescence

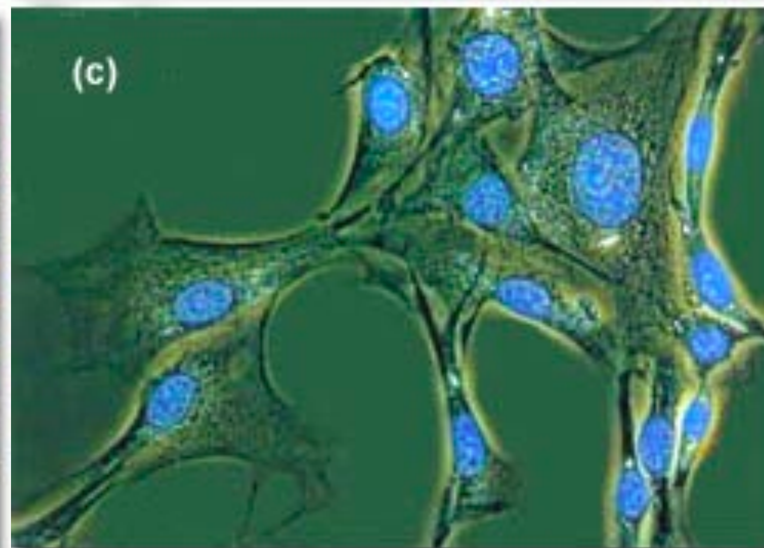




Fluorescence

Combined

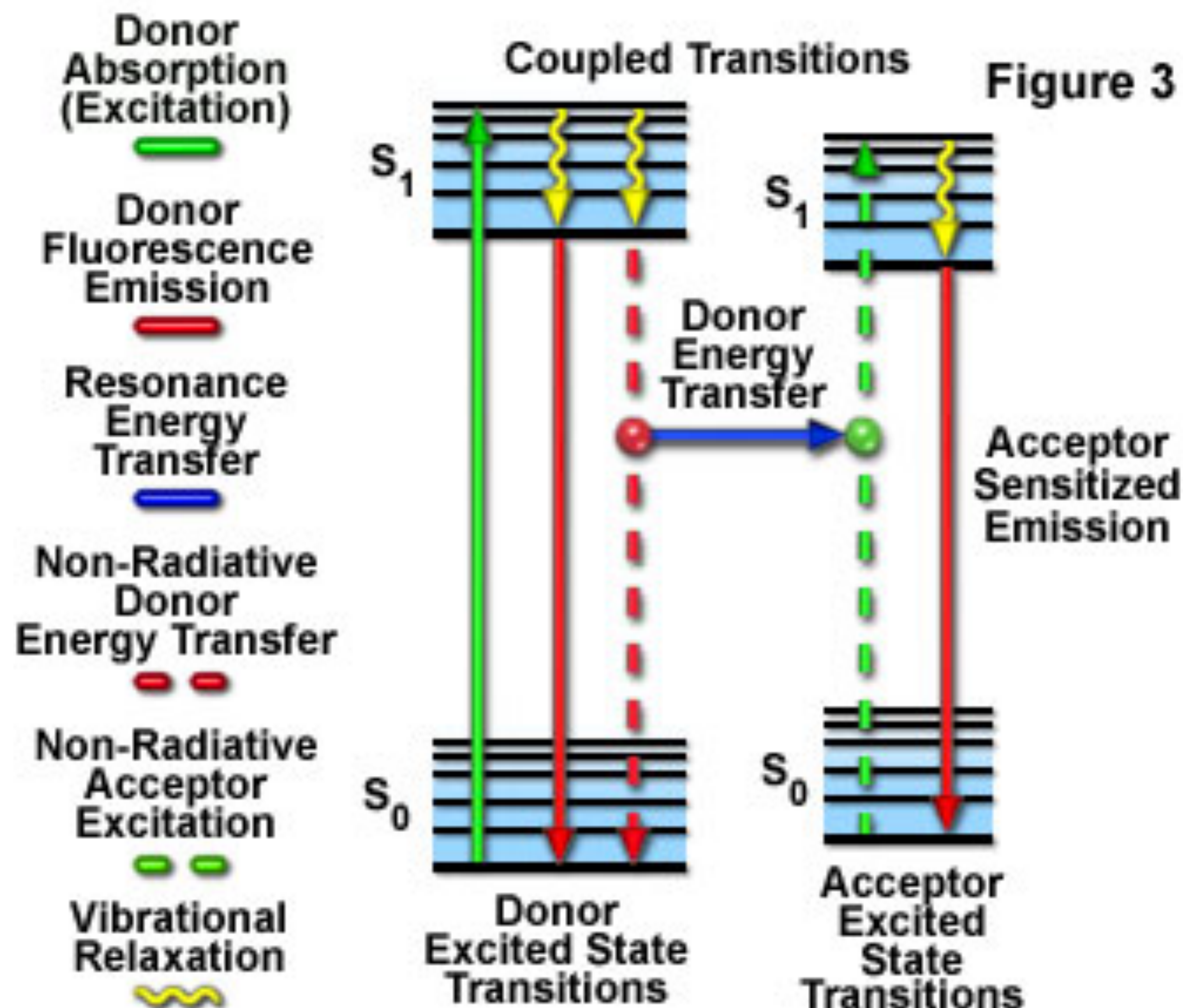


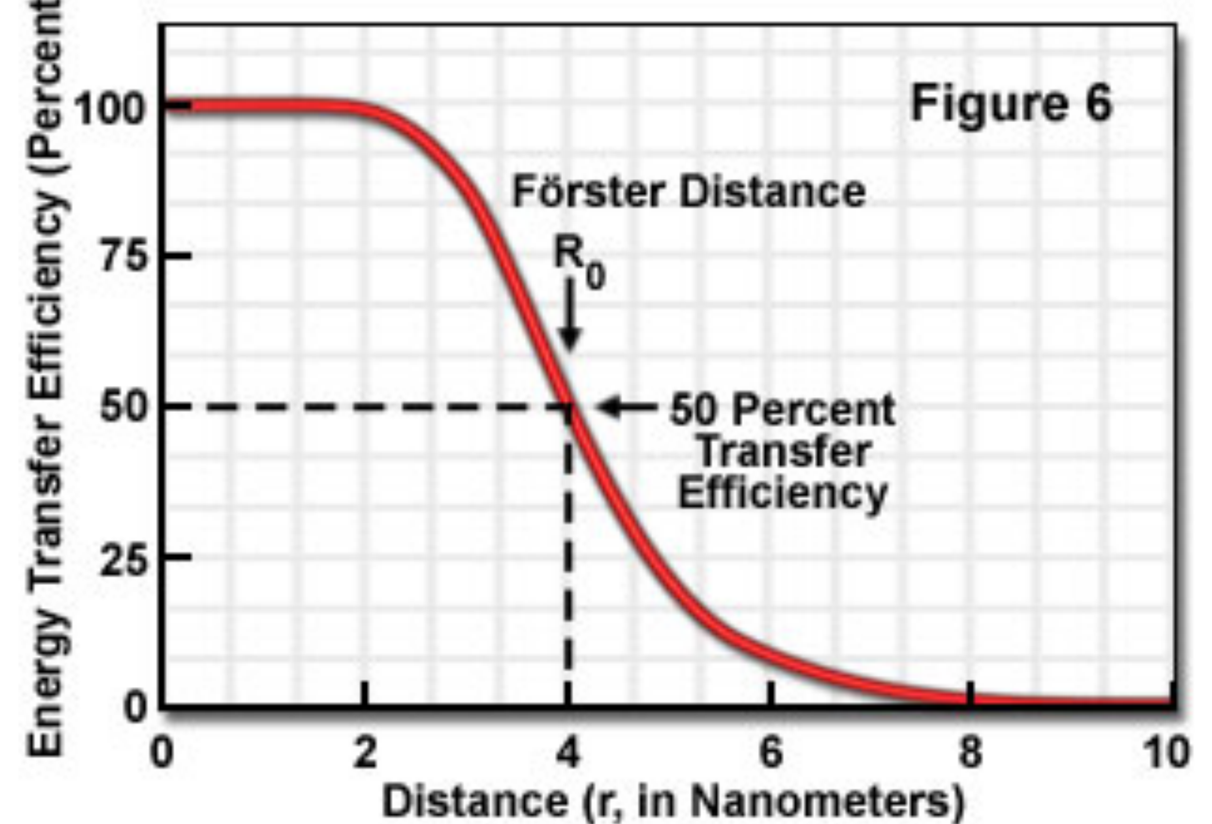


Combined

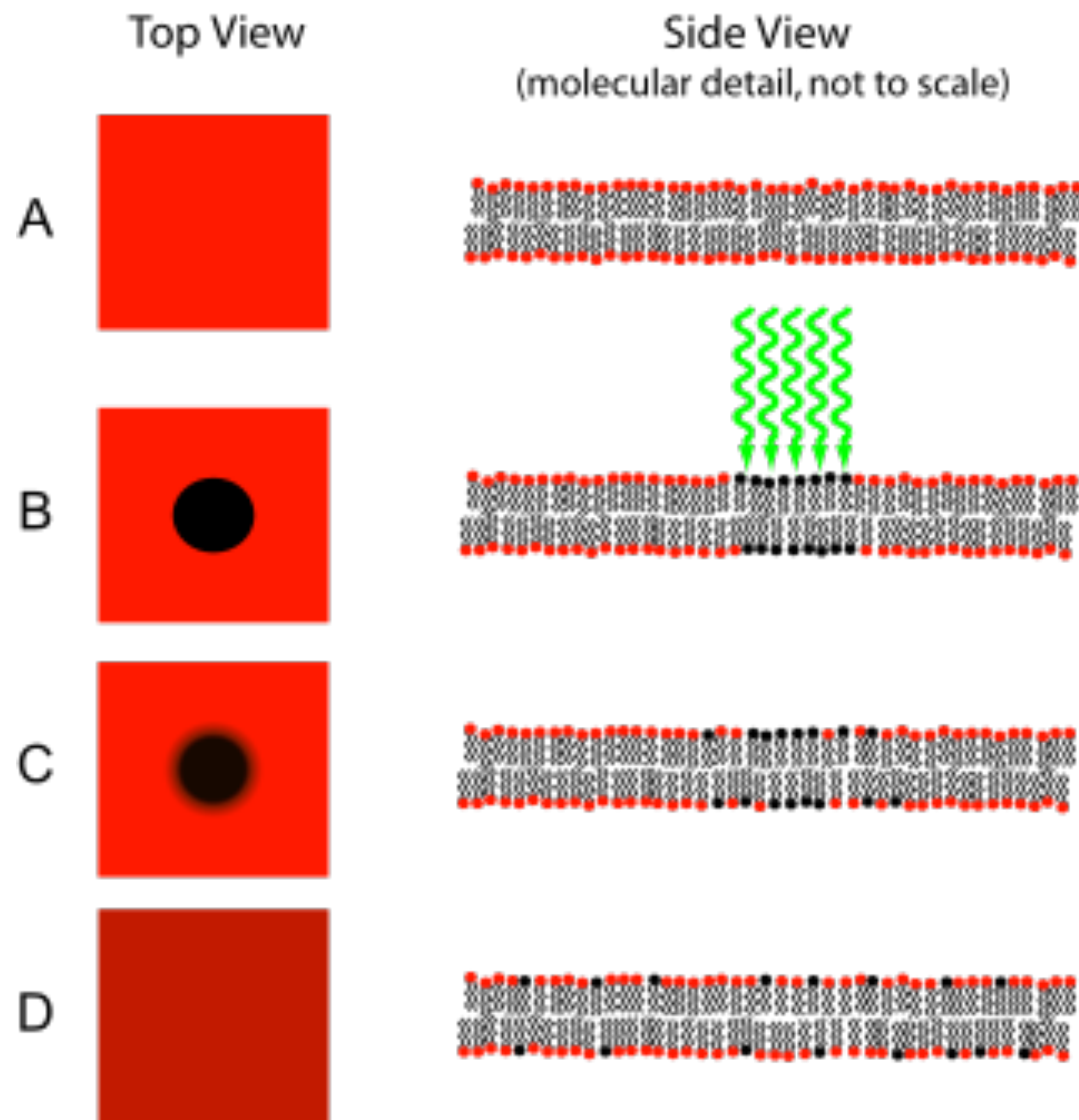
Variants of fluorescence microscopy I

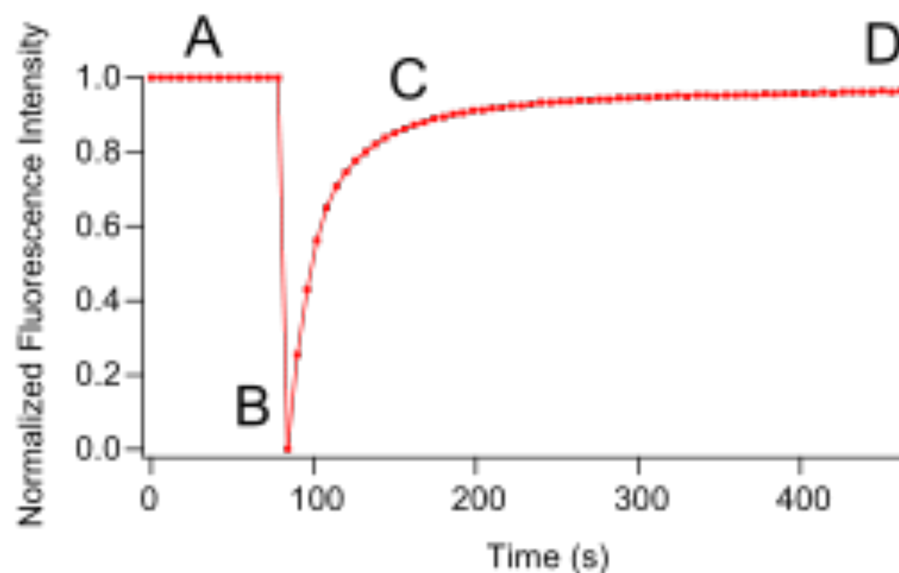
- **Fluorescence resonance energy transfer** (FRET)
  - **Basic idea:** Use fluorescence emission from one fluorophore to excite another
  - Measure fluorescence intensity ratio to sensitively measure nm-scale distances
  - Used to measure conformational changes, reaction rates, ...





Variants of fluorescence microscopy II





- **Fluorescence recovery after photobleaching** (FRAP)
  - **Basic idea:** a region of sample is bleached with a short burst of light, and the recovery of fluorescence (via diffusion of fluorophores back into the region) is monitored
  - Useful for measuring kinetics, diffusion coefficients in membranes

$$D = \frac{w^2}{4t_{1/2}}$$

Variants of fluorescence microscopy III

- **Calcium ratio imaging**
  - **Basic idea:** spectral properties of dyes change upon binding calcium
  - Typical dyes based on BAPTA (EGTA homologue)
  - Measure e.g. intensity of emission
  - Useful for measuring concentrations of calcium (and other ions or pH using similar fluorophores)

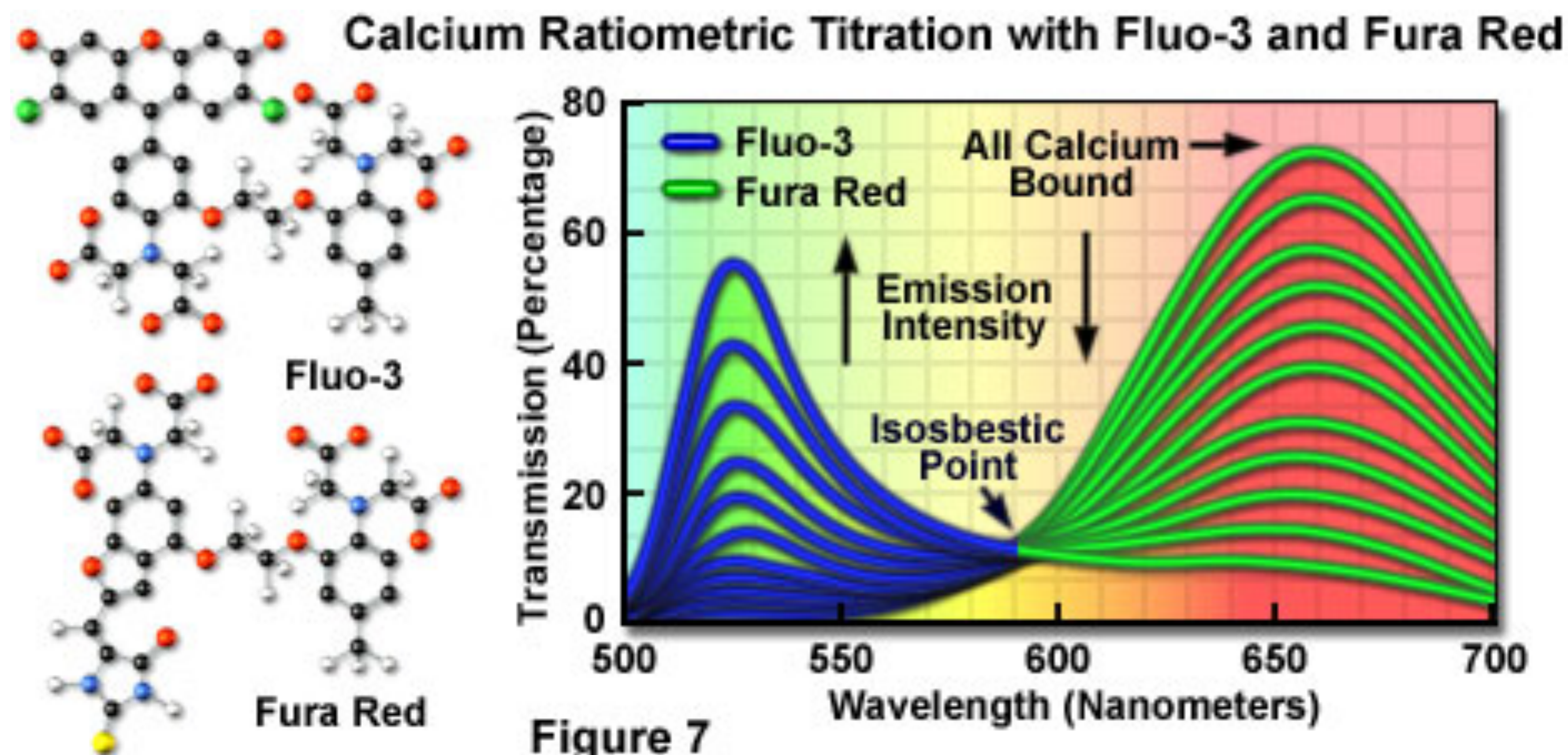
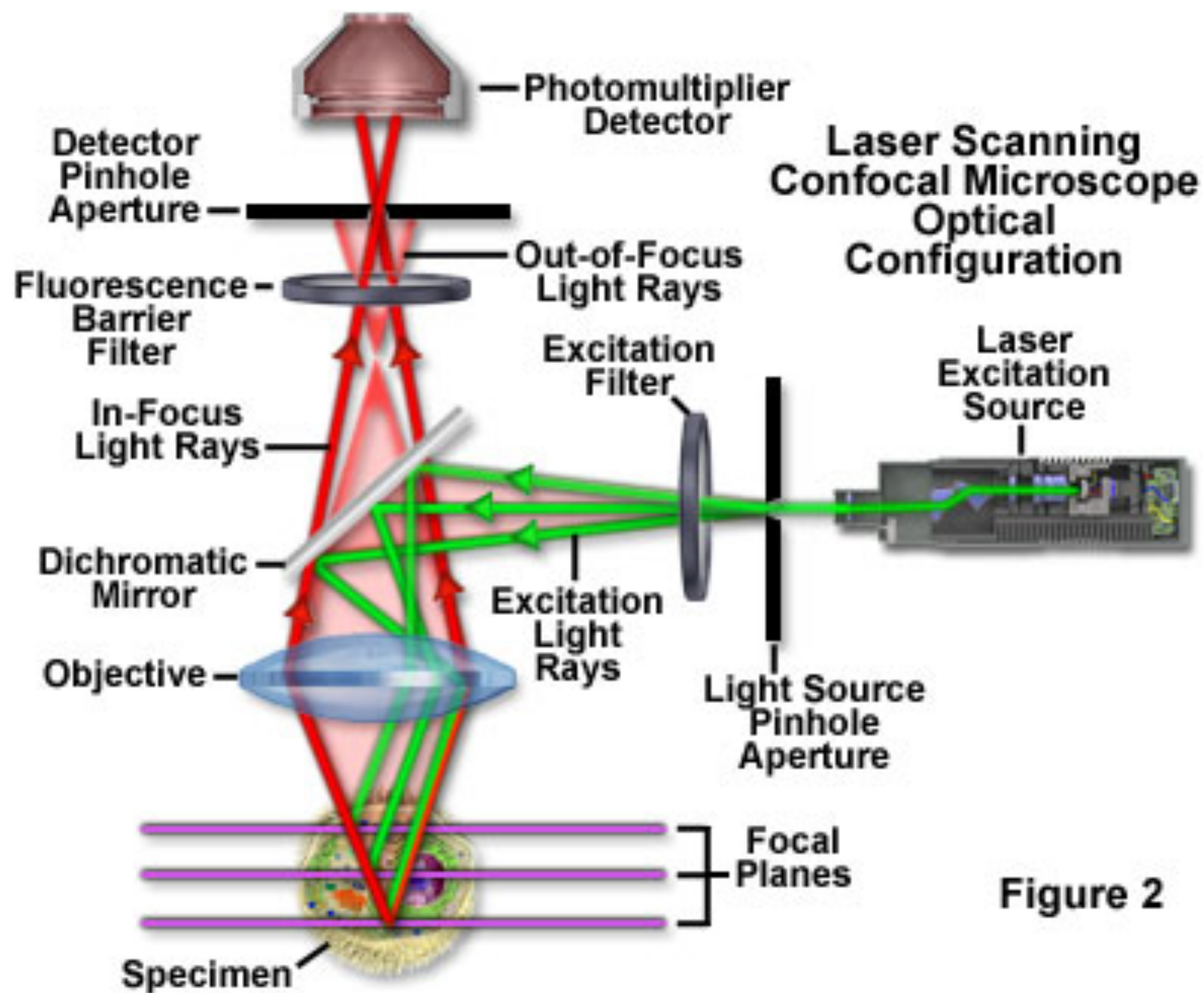


Figure 7

Variants of fluorescence microscopy IV

- **Spectrofluorometry and microplate readers**
  - **Basic idea:** Measure average properties of bulk samples (micro- to milliliter) over continuous range of wavelengths
- **Fluorescence scanners and microarray readers**
  - **Basic idea:** Use 2-D fluorescence to characterize macroscopic objects such as electrophoresis gels, blots, chromatograms, microfluidic devices, DNA sequences
- **Flow cytometry**
  - **Basic idea**: Quantify subpopulations within a large sample by measuring fluorescence per cell in a flowing stream

Confocal microscopy



Lateral resolution:

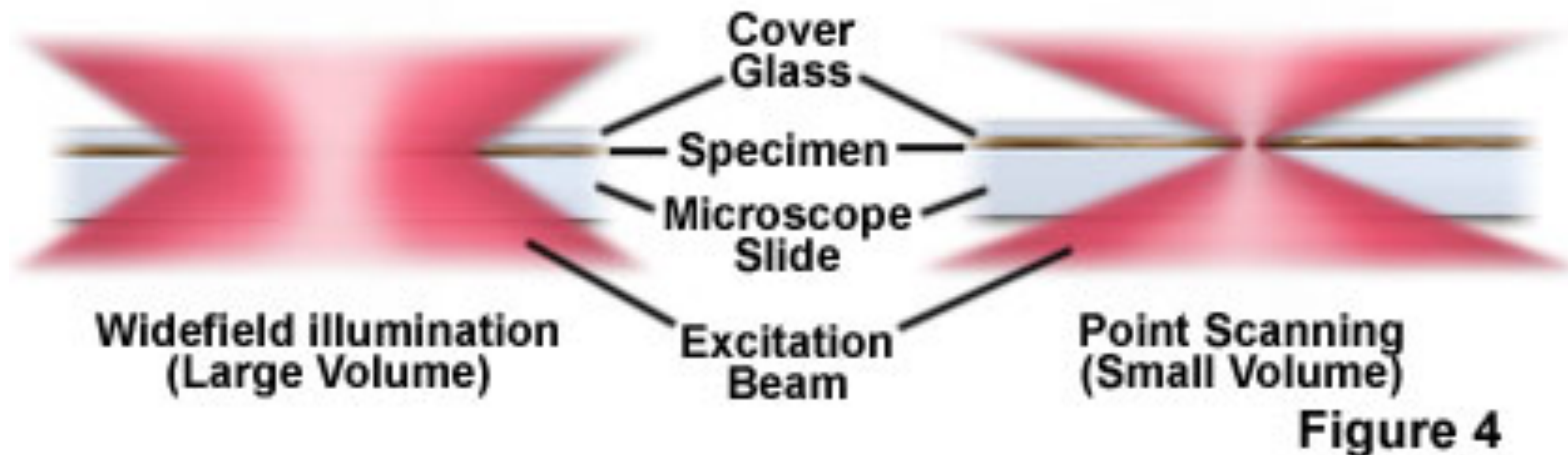
$$R_{\mathrm{lateral}} = \frac{0.4\lambda}{\mathrm{NA}}$$

Axial resolution:

$$R_{\mathrm{axial}} = \frac{1.4\lambda n}{\mathrm{NA}^2}$$

- **Basic idea**: thin optical slices are attained by blocking out-of-focus light with pinholes at light source, detector
- **Advantages:**
  - Non-invasive 3-d imaging via serial 2-d thin slice images
  - Improved contrast, definition compared to fluorescence
  - Reduction in background noise
- **Disadvantages:**
  - High intensity lasers can damage biological samples
  - Samples must be nearly index-matched
  - Expensive!
  - Limited number of lasers

Widefield versus confocal illumination

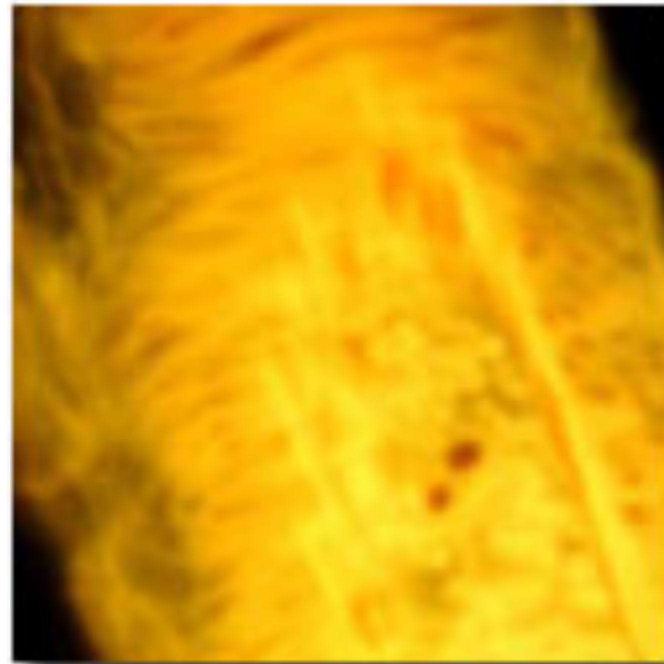


- Size of illumination point in confocal microscopy: 0.2-0.8 μm in diameter, 0.5-1.5 μm in height
- Focused beam is moved in a raster to scan across a 2-d image using two mirrors (or a mirror and a galvanometer)
- Confocal image never exists as a real image in eyepieces
  - Reconstructed through software
- Multiple-beam scanning confocal microscopes use spinning Nipkow disks containing an array of pinholes and microlenses
  - Use arc discharge lamps rather than lasers

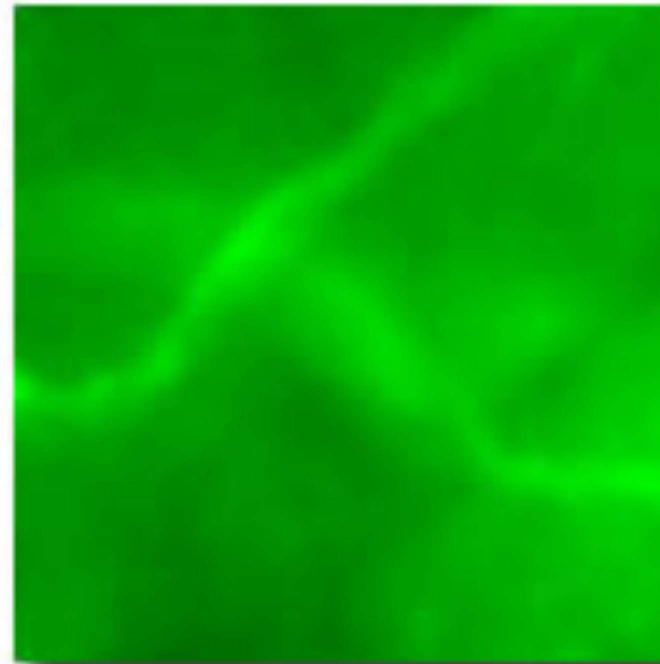
Comparison of confocal and widefield

Confocal and Widefield Fluorescence Microscopy

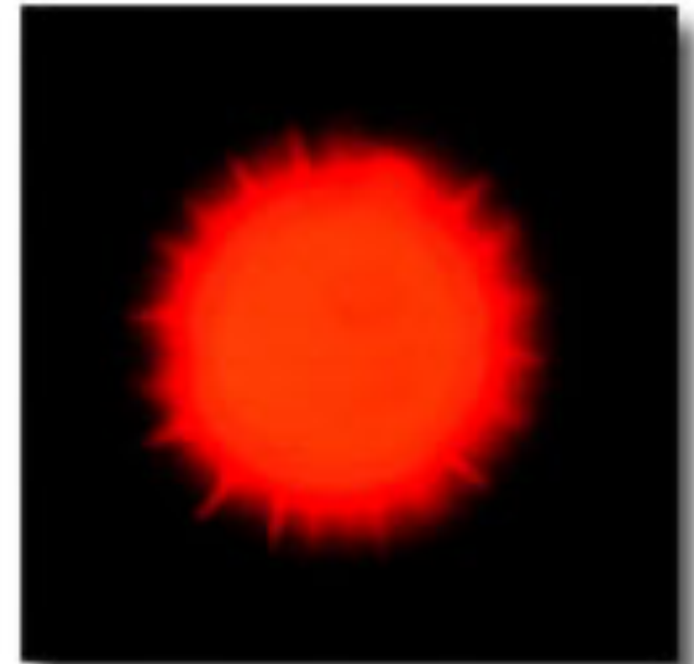
widefield



(a)

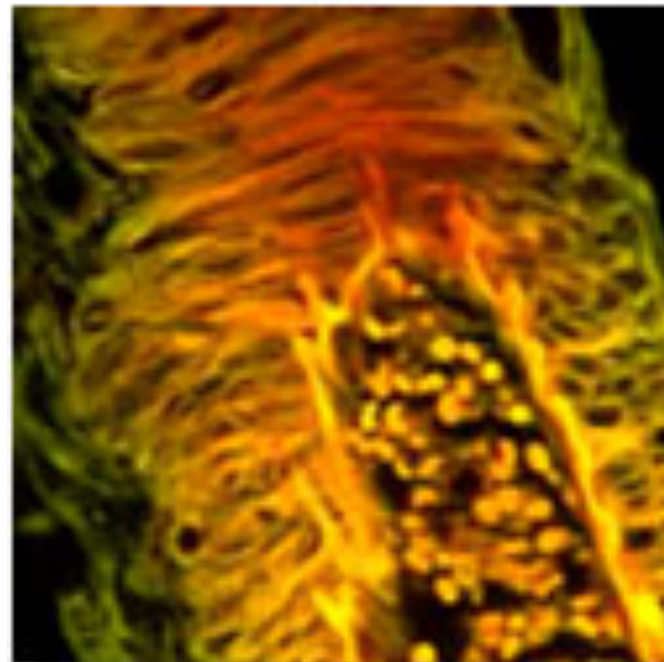


(b)

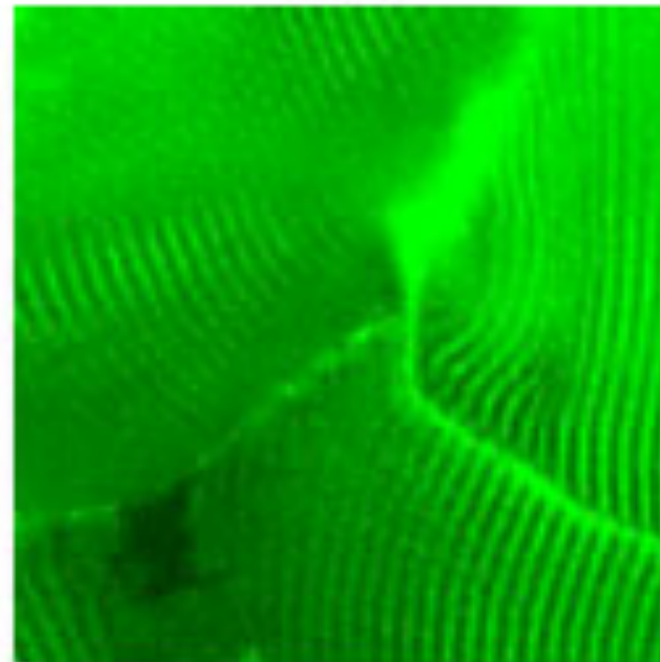


(c)

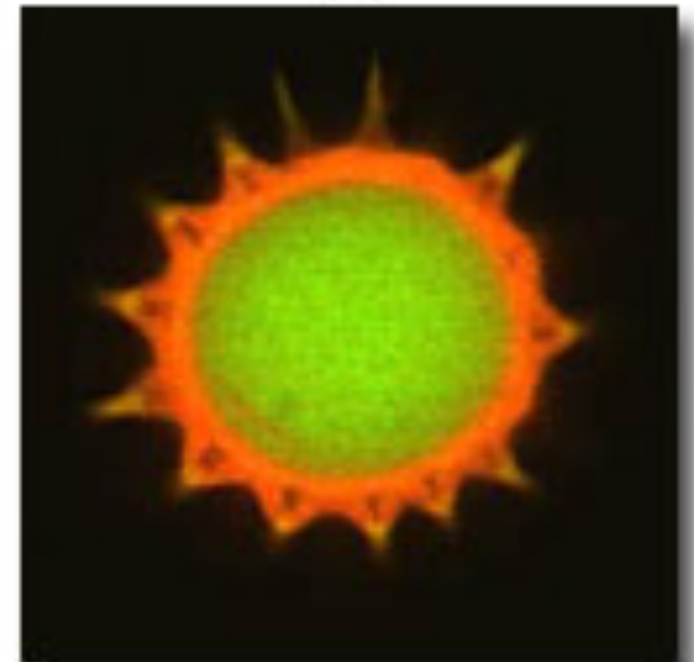
confocal



(d)



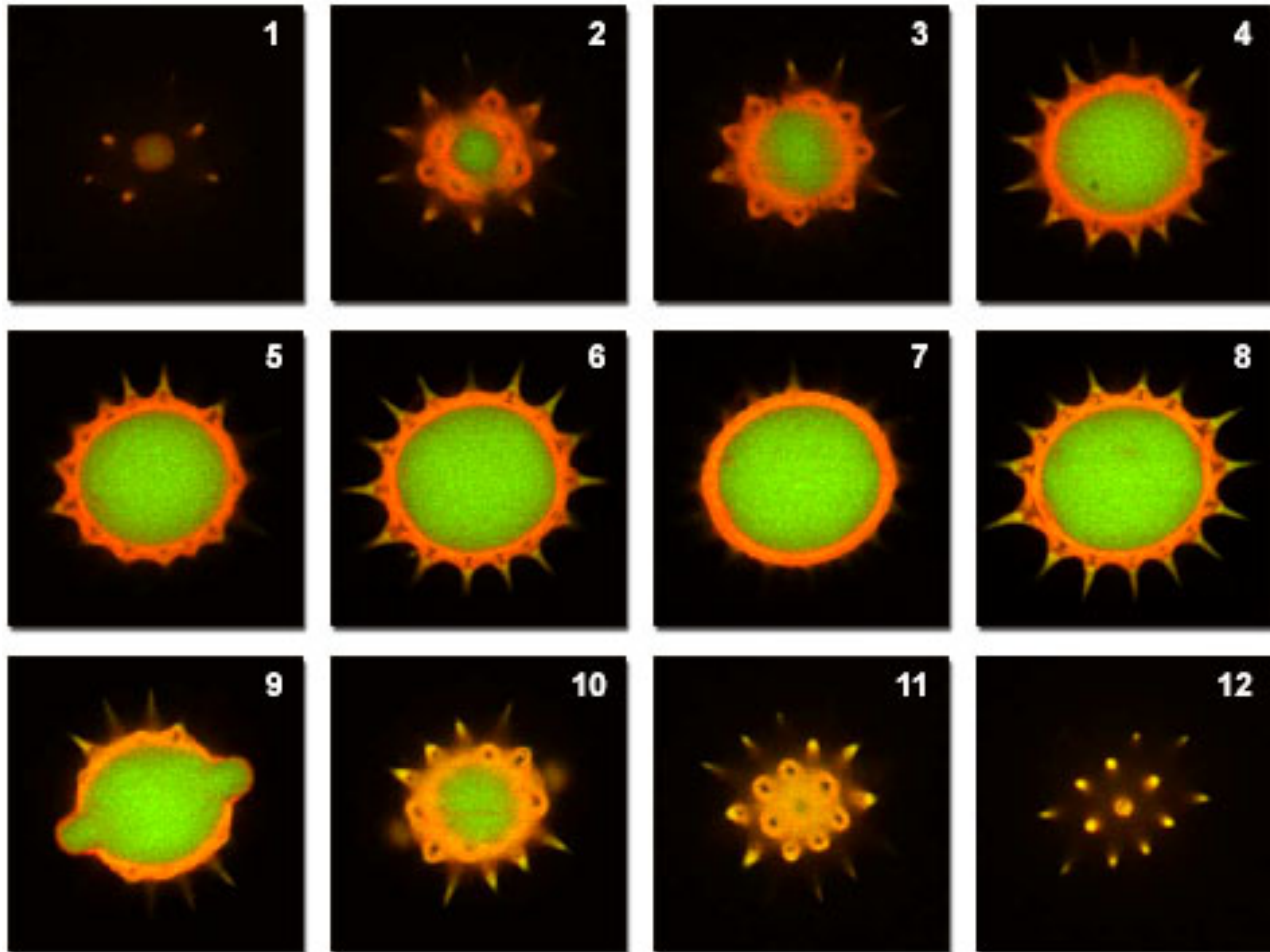
(e)



(f)

Figure 1

Optical sectioning via confocal microscopy



Digital imaging: CCDs

- **Charge-coupled device** (CCD)
  - Convert light energy into electronic charge
  - Electrons generated by interaction of photons with silicon
  - Transferred across chip through registers to amplifier

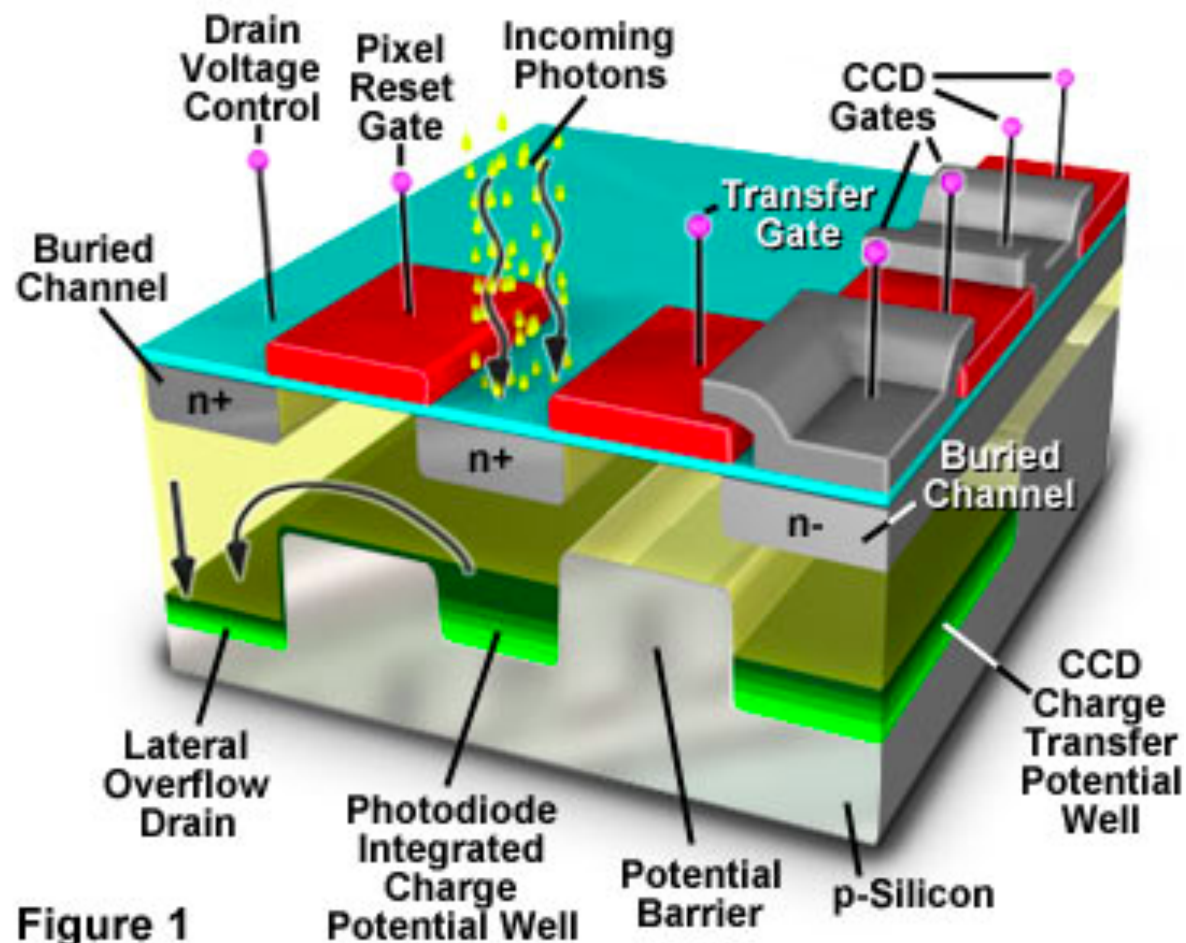


Figure 1

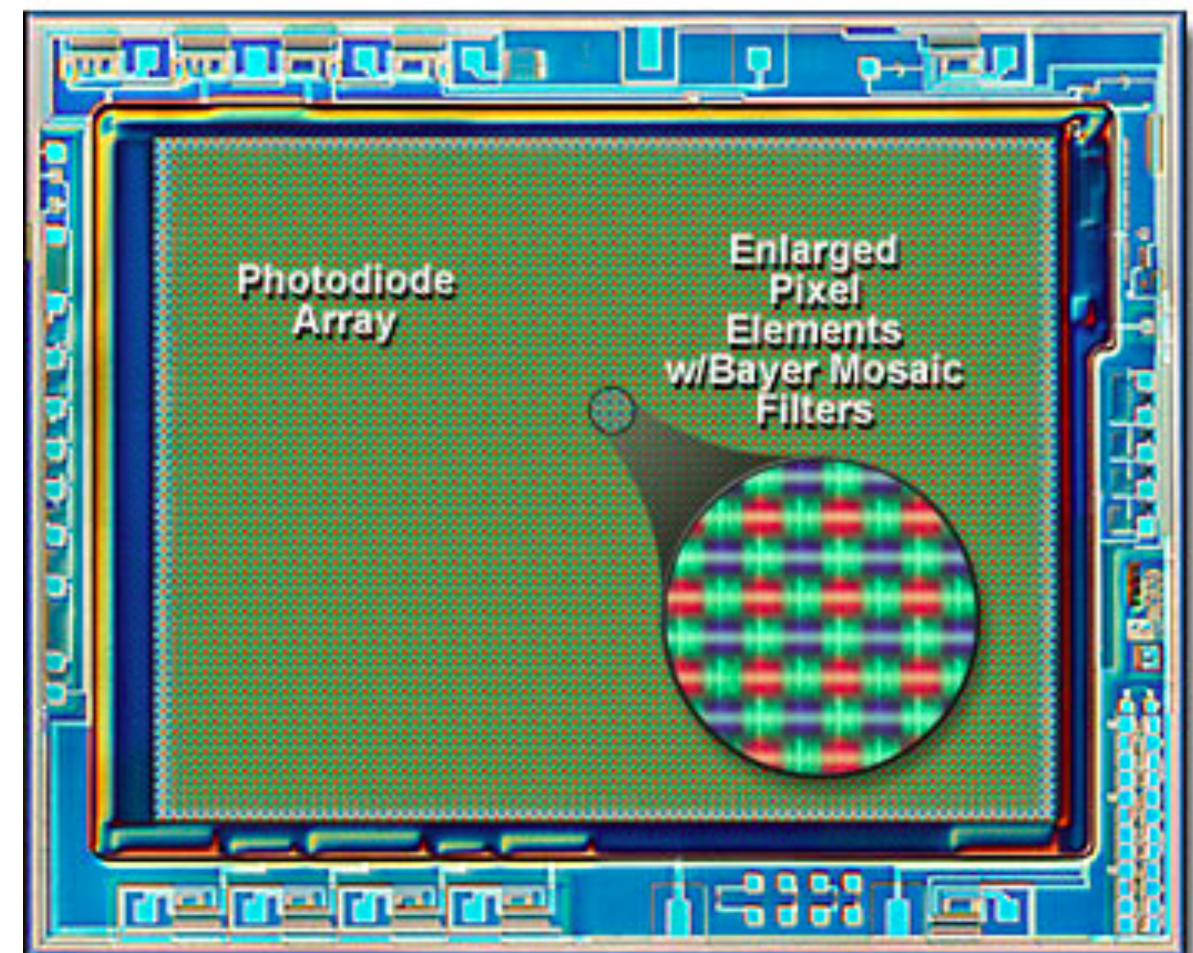


Figure 2

Digital imaging: camera types

- Camera
  - **Monochrome** cameras
    - Sensitive, fast, for quantitative imaging
    - Requires less light
    - Pixel value represents intensity (8-bit, 12-bit, 16-bit ranges)
  - **Color** cameras
    - Qualitative, slow, non-sensitive
    - Requires more light

Digital image creation

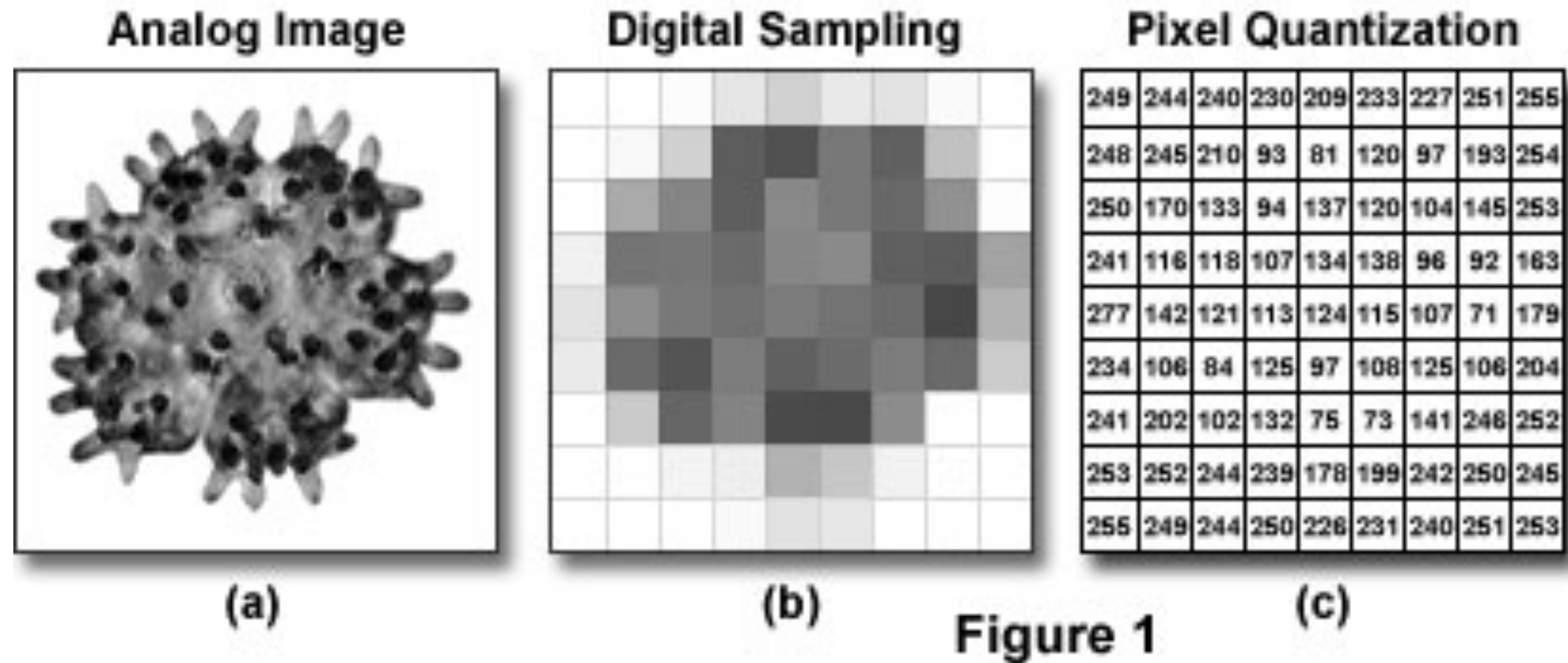


Figure 1

Spatial resolution and pixelation

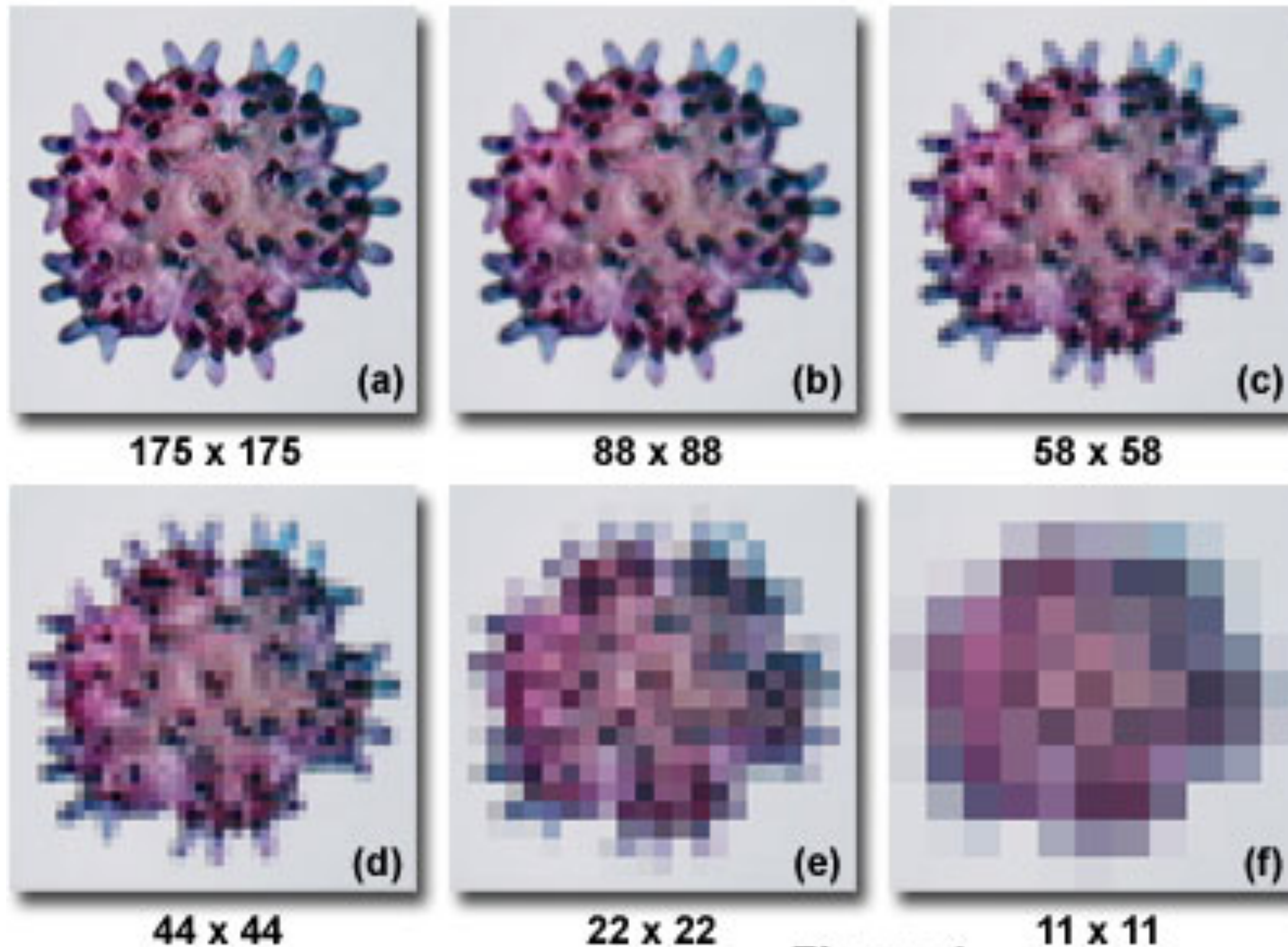


Figure 4

Recommended: 2.5-3 samples per smallest resolution figure

Grayscale pixel values and resolution

Increased bit depth leads to improved resolution in image

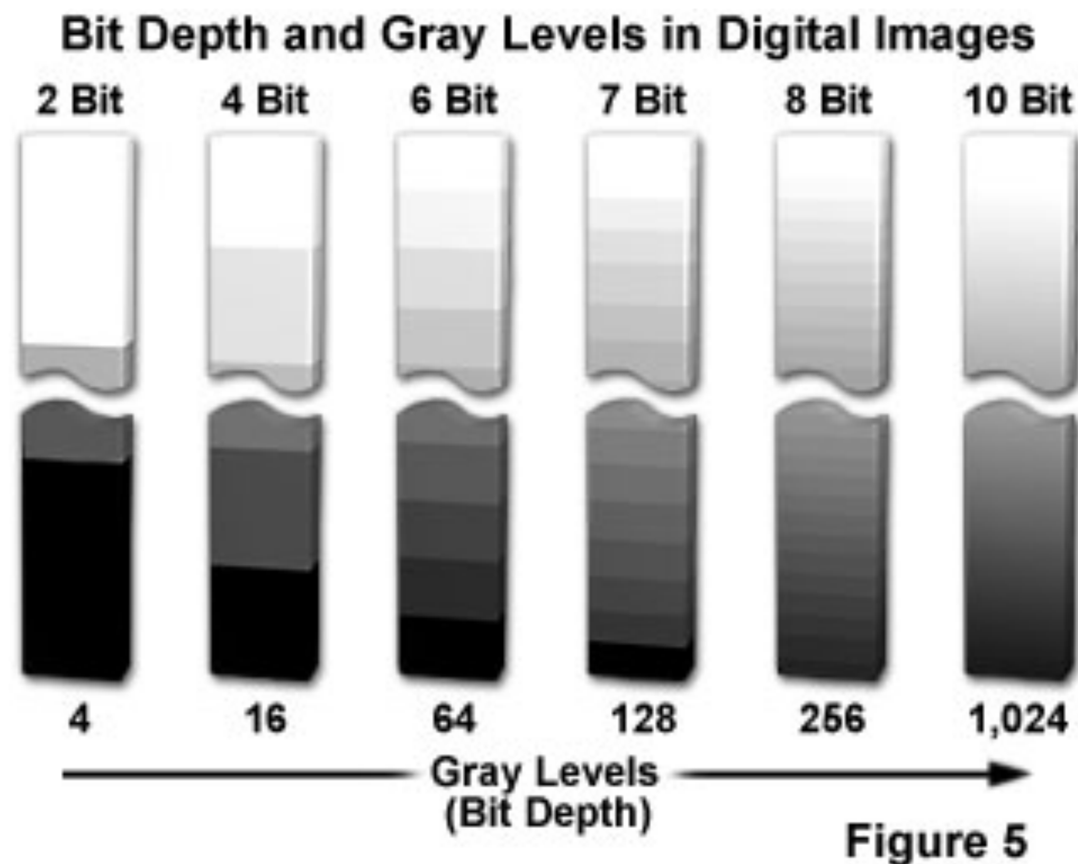


Figure 5

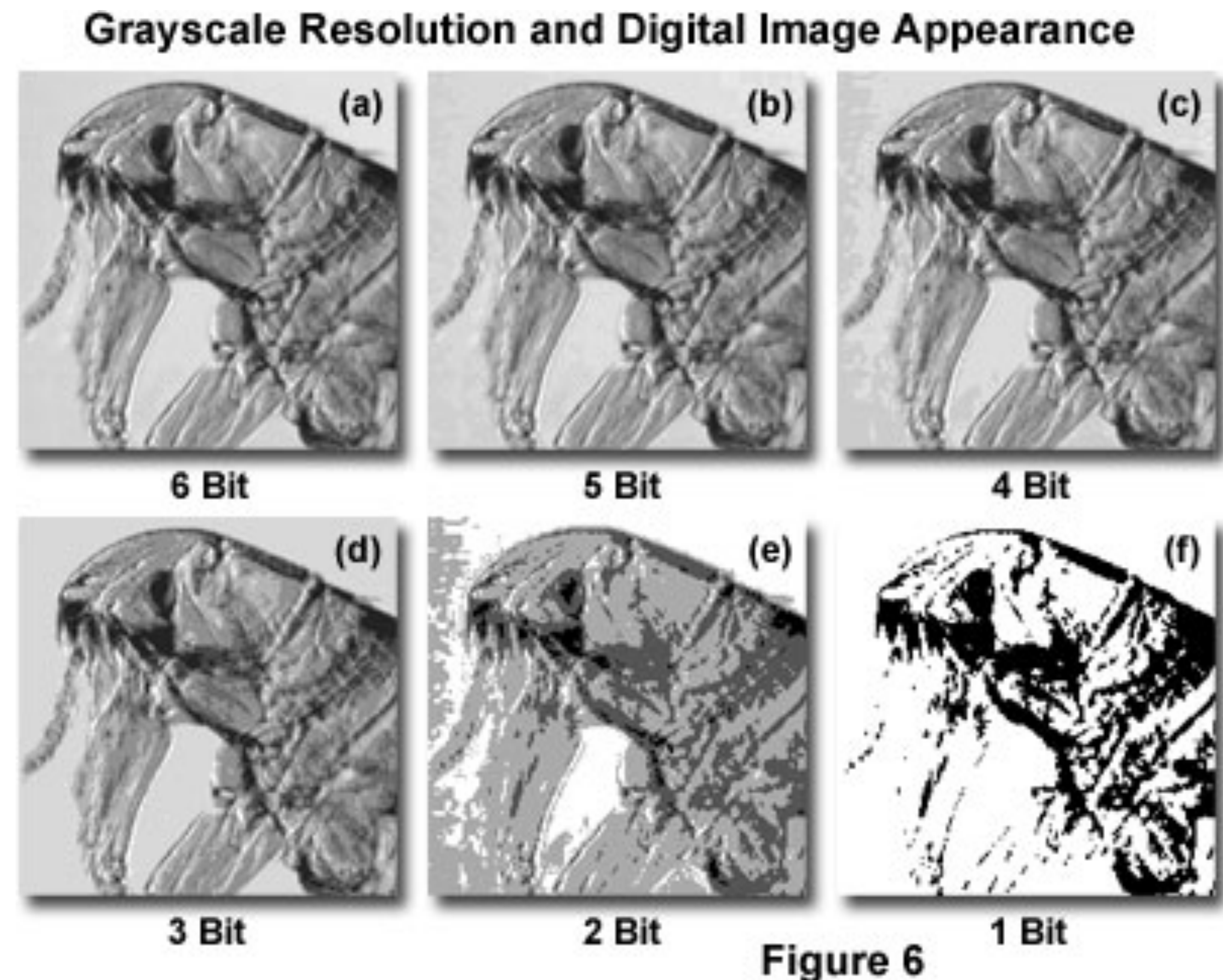


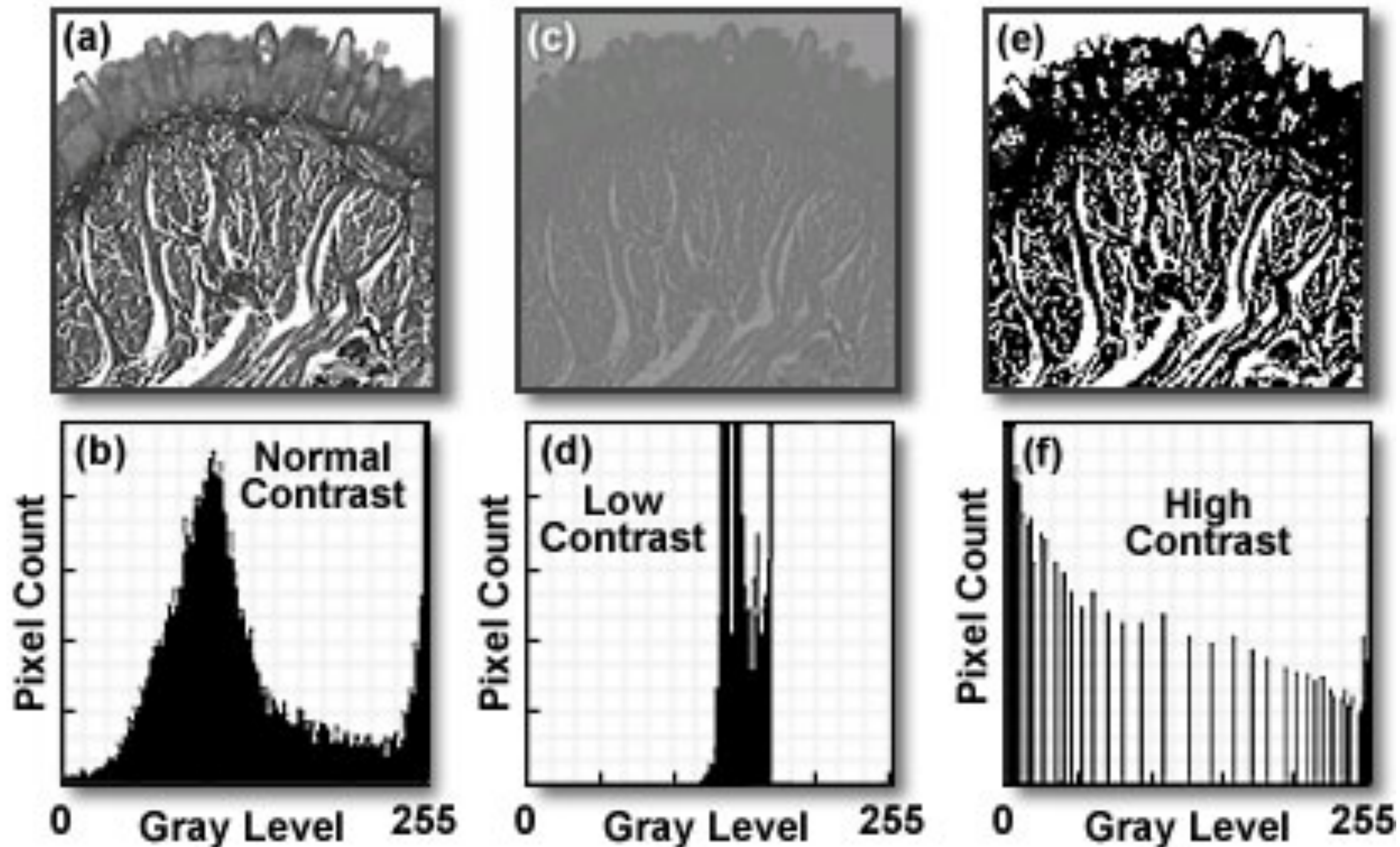
Figure 6

Word of caution: make sure that your analysis package can read images of the bit depth that you select!

Software packages for image processing

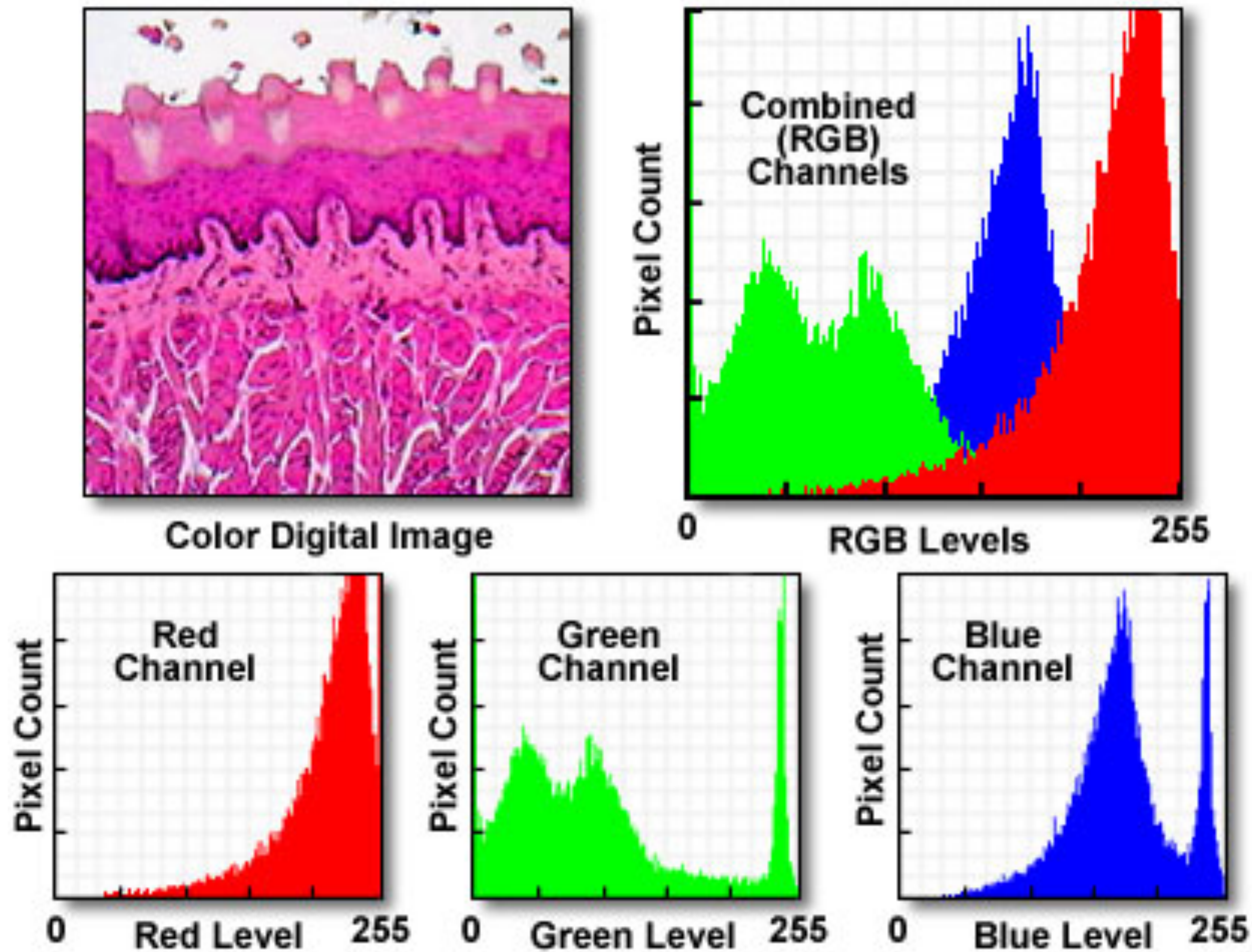
- Microscope acquisition packages (Leica, Zeiss, Olympus, Nikon)
- ImageJ
  - (http://rsbweb.nih.gov/ij/)
  - Free, lots of plug-ins available
- Matlab
  - Image processing toolbox, avialable through CCoE
- Adobe Photoshop
- Image Pro Plus
- NI Vision
- Many others
- **NOTE: for publications all parts of an image must be processed equally!**

Image contrast: histogram



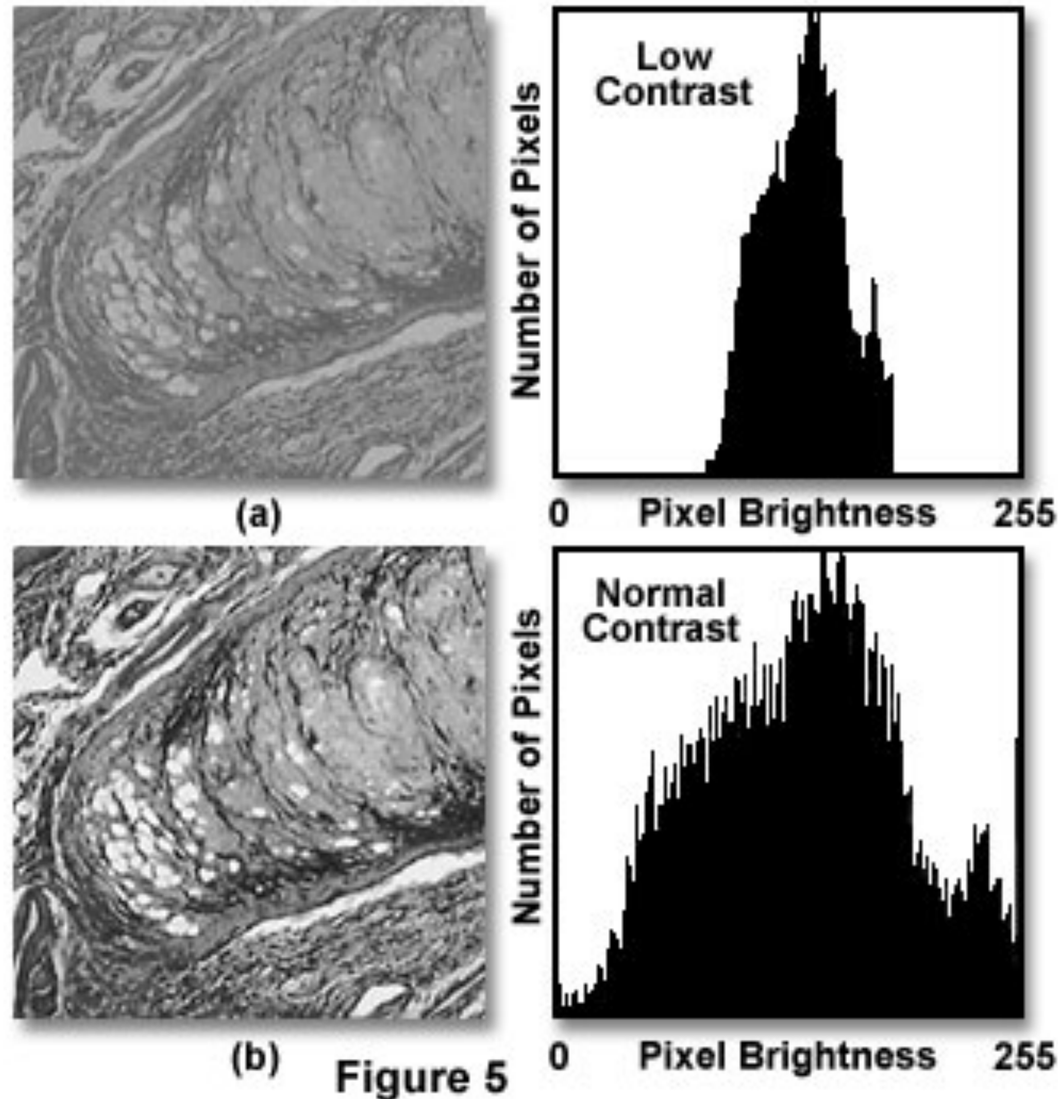
- Resolution is maximized by using the full dynamic range of the camera
- Saturating at either low or high levels reduces resolution

Image contrast: color histograms



Intensities in each color channel reflect distributions of light

Histogram stretching



$$O(x, y) = \frac{I(x, y) - B}{W - B}$$

- Enhance contrast by stretching the histogram to cover the full dynamic range
- However: better to optimize the image contrast during acquisition!

Flat field correction

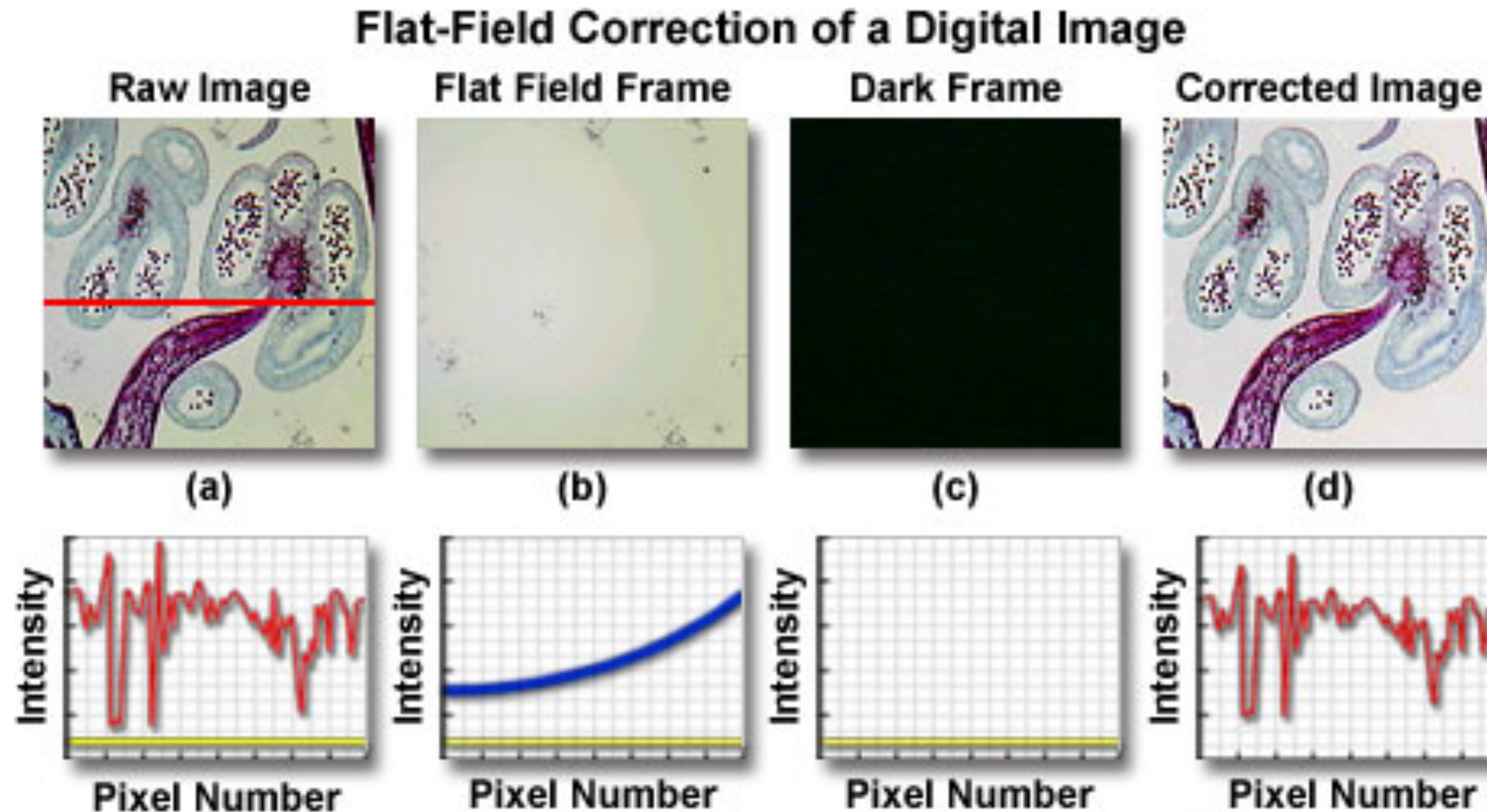
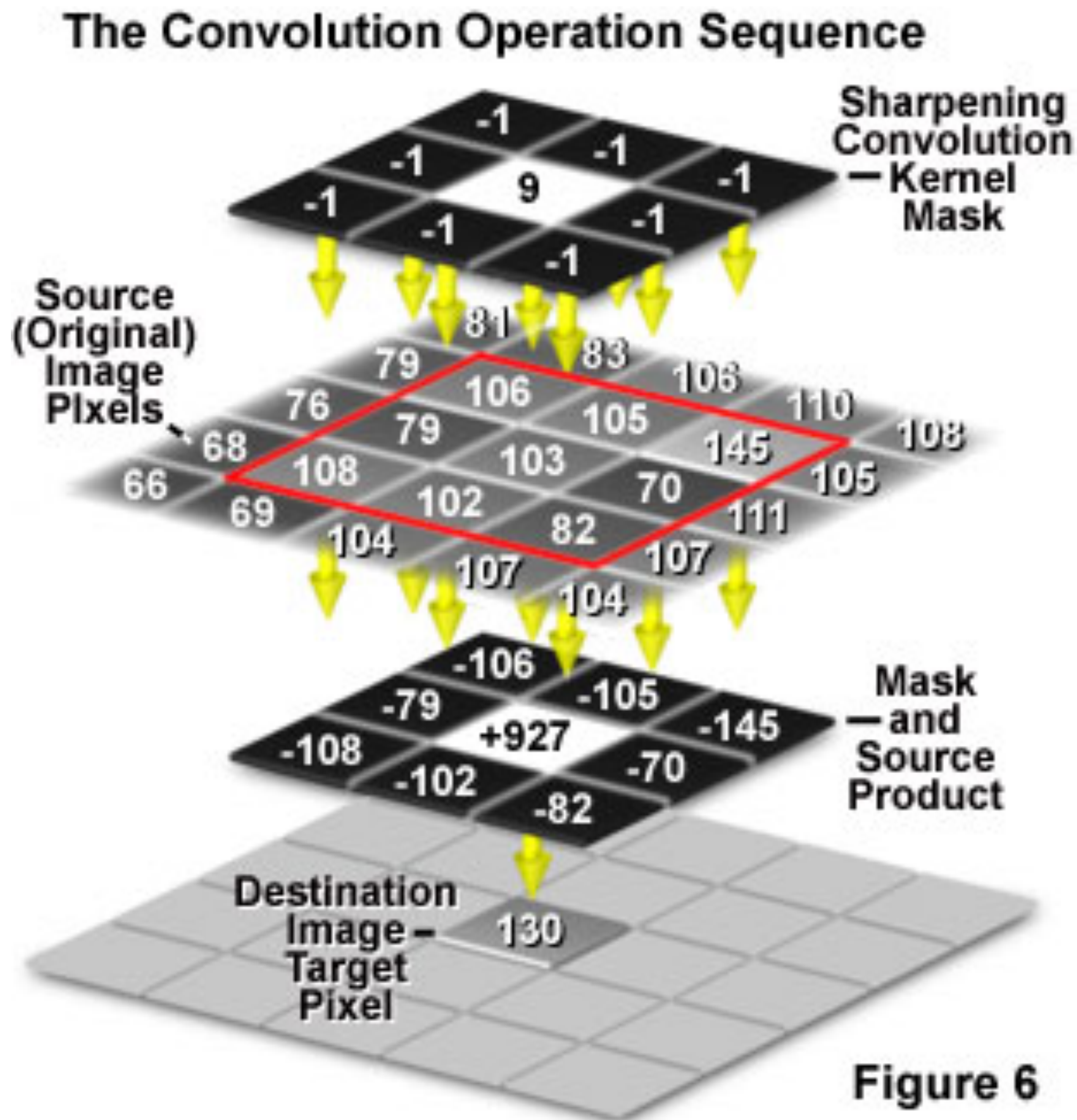


Figure 3

- Requires two additional images:
  - **Flat field frame:** image of the field of view without the sample (to capture dust, etc, in field of view)
  - **Dark reference frame:** image of a flat black background (to capture variations in the camera sensitivity)
  - Many modern microscopes do this correction for you
    - Good idea to take these images just in case

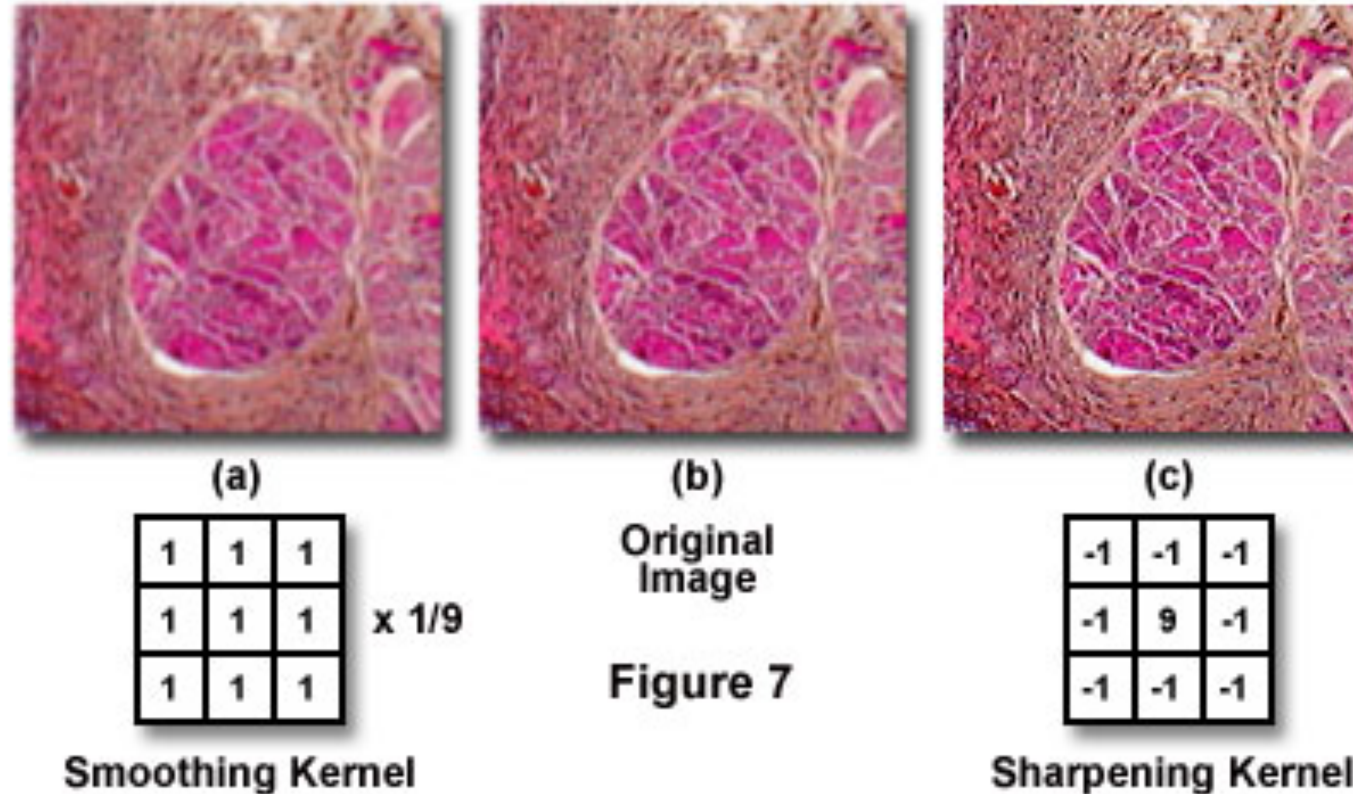
Convolution: feature finding



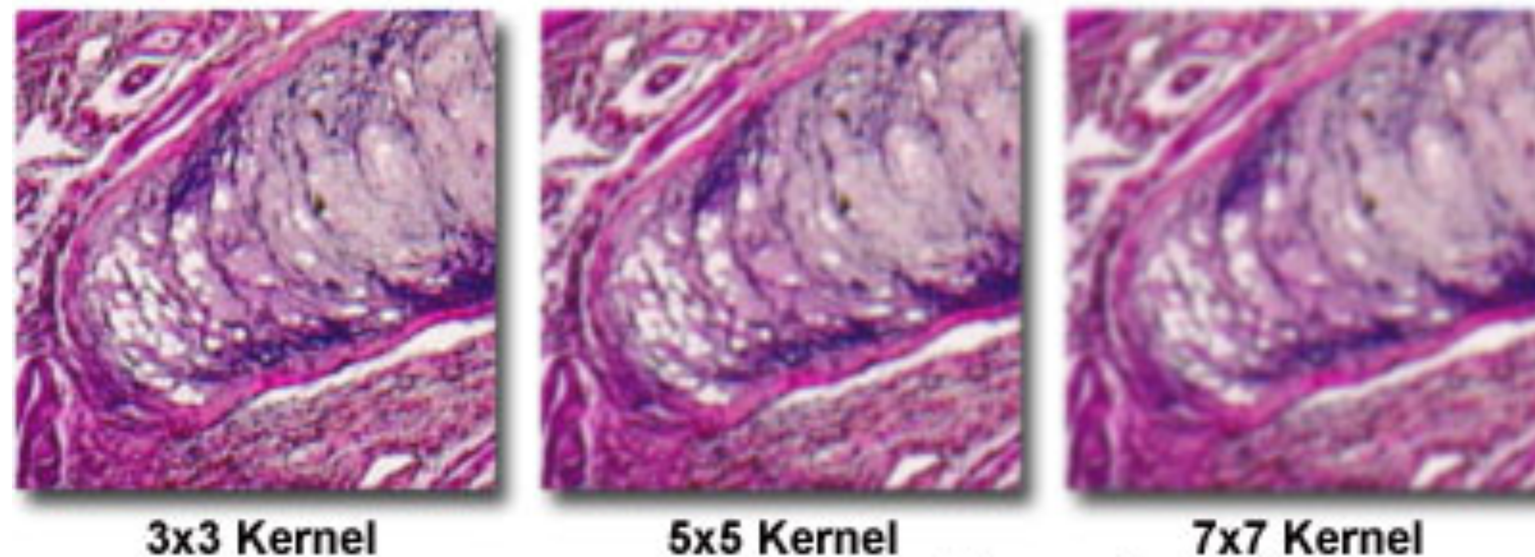
- **Basic idea:** integer value of each output pixel is altered by contributions from adjoining input pixel values
- **Box convolution:**
    - Source pixel values inside mask multiplied by corresponding mask values
    - Destination target pixel is sum of products
- Many built-in options available in software packages for convolution
    - Can also write your own in Matlab, etc.

Types of convolution filters I

- Smoothing and sharpening kernels



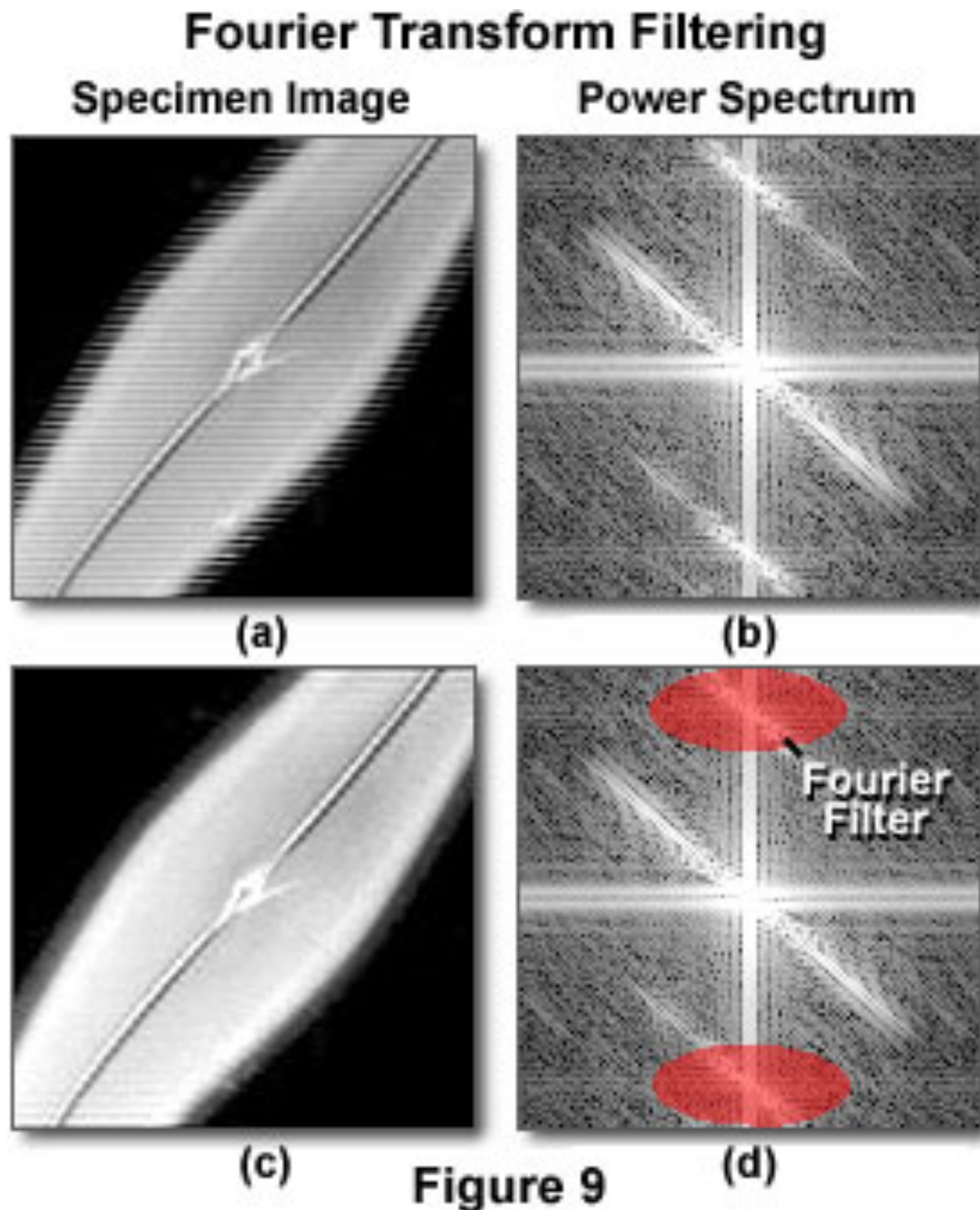
- Kernel size effects



Types of convolution filters II

- **Median convolution filters**
  - Effective at eliminating “faulty” pixels (unusually high or low brightness) and image noise
  - Source pixel value replaced by median of pixel values in the convolution kernel
  - Good for images with high contrast (preserves edges)
- **Derivative filters**
  - e.g. **Sobel filter**: produces a derivative in any of eight direction depending on matrix choice
  - Used for edge enhancement
- **Laplacian filters** (operators)
  - Used to calculate second derivative of intensity as a function of position
  - Generates sharp peaks at the edges
  - Enhances brightness slopes
- **Unsharp masks**
  - Subtraction of blurred image from original image, followed by adjustment of gray values
  - Preserves high-frequency detail while allowing shading correction, background suppression
  - User-adjustable but also increases noise -- **use with caution**

Fourier transforms



- **Basic idea:** Any harmonic function can be decomposed into sums of sines and cosines
- Fourier transform converts function varying in **space** to function varying with **frequency**
- Fast Fourier transform (FFT) routines implemented in many software packages
- In image processing:
  - Used to manipulate images through deletion of high- or low-spatial-frequency info
  - Used to stitch multiple images together into a large image

Math note: convolutions and FTs

Convolution theorem: the Fourier transform of the convolution of two functions is the product of their Fourier transforms.

Mathematically:

Definition of Fourier transform: $\hat{f}(\xi) \equiv \int_{-\infty}^{\infty} f(x) e^{-2\pi i x \xi} dx$

Convolution theorem:

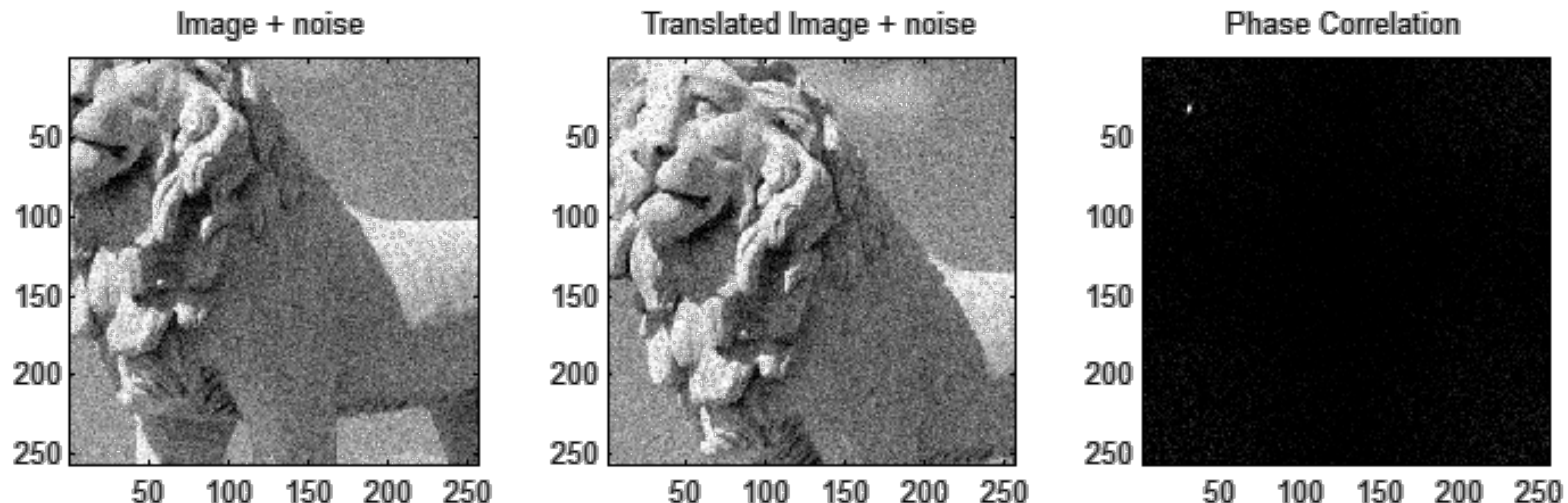
$$h(x) = (f * g)(x) = \int_{-\infty}^{\infty} f(y) g(x-y) dy$$

$$\hat{h}(\xi) = \hat{f}(\xi) \cdot \hat{g}(\xi)$$

Mathematical efficiency: FTs require more memory/computational power but are generally faster than convolutions

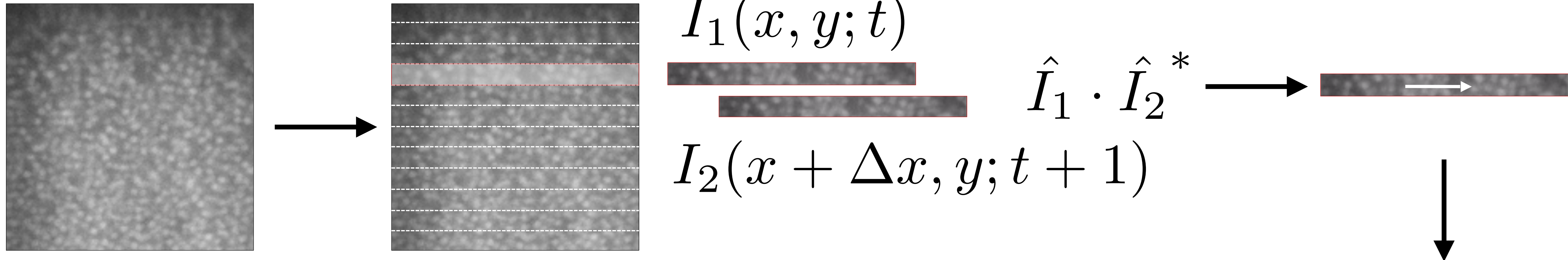
Image stitching with Fourier transforms

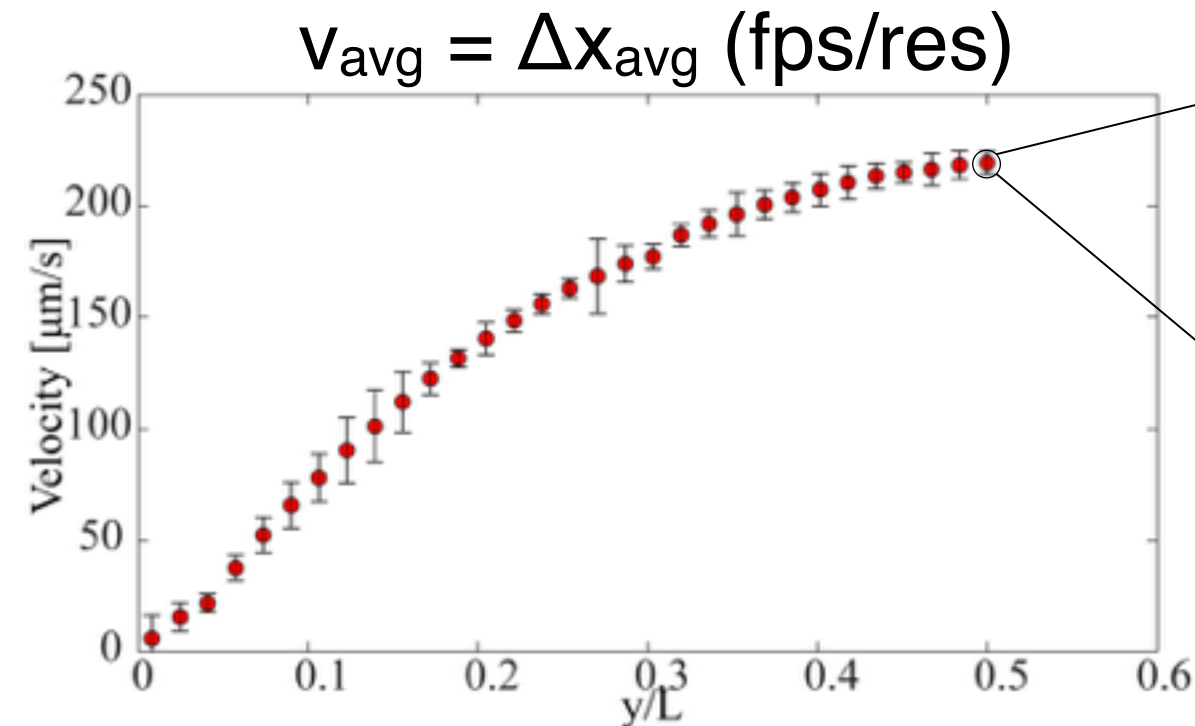
- **Basic idea:** Use Fourier transforms to calculate the maximum overlap between two images *f* and *g* ("Fourier shift theorem")
- Typical procedure:
  - Calculate discrete FT of both images $\hat{f}, \hat{g}$
  - Calculate the cross-power spectrum $\hat{r} = \dfrac{\hat{f}\hat{g}^*}{|\hat{f}\hat{g}^*|}$
  - Obtain normalized cross-correlation via inverse Fourier transform
  - Determine location of peak in *r*



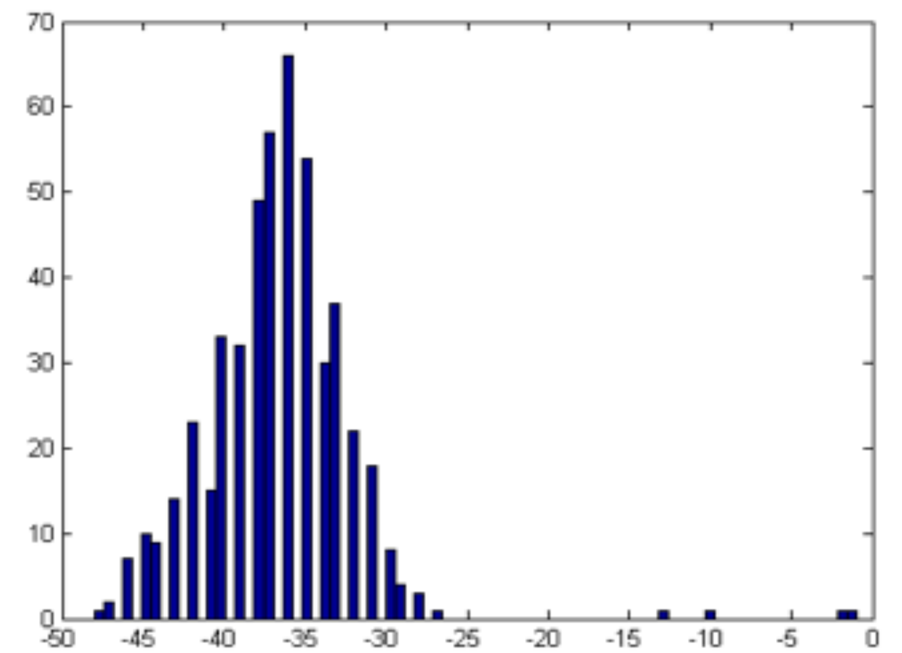
Application: velocimetry

Basic idea: apply image correlation techniques to measure velocities in flowing suspensions





number of particles

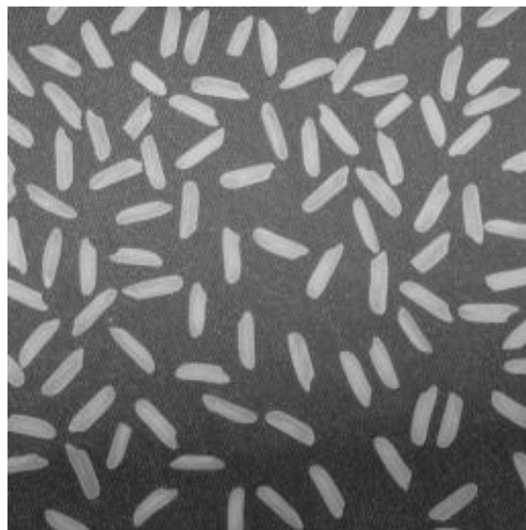


particle displacement (pixels)

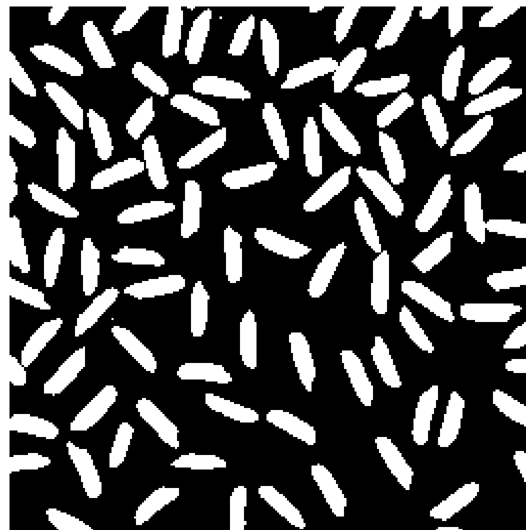
Graduate student: Rahul Pandey

Image segmentation

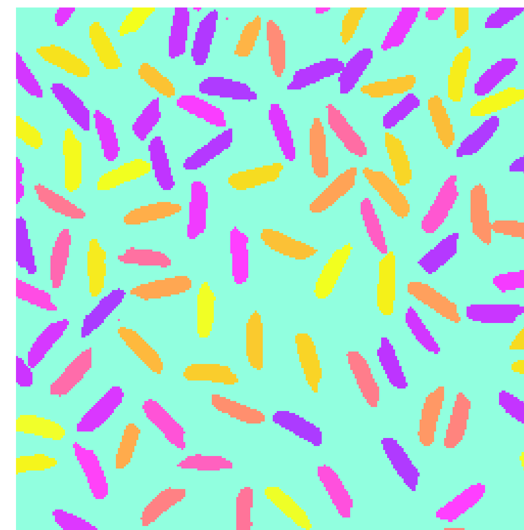
- **Basic idea:** locate and quantitatively characterize distinct features in an image
- Typical process:
  - Segment image via thresholding
  - Label objects
  - Calculate statistics
- Can be simply done in Matlab via regionprops



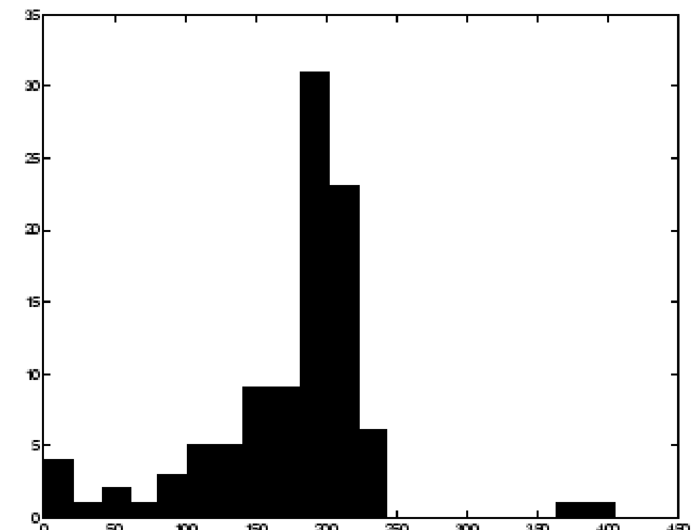
original (grayscale)



segmented (b/w)

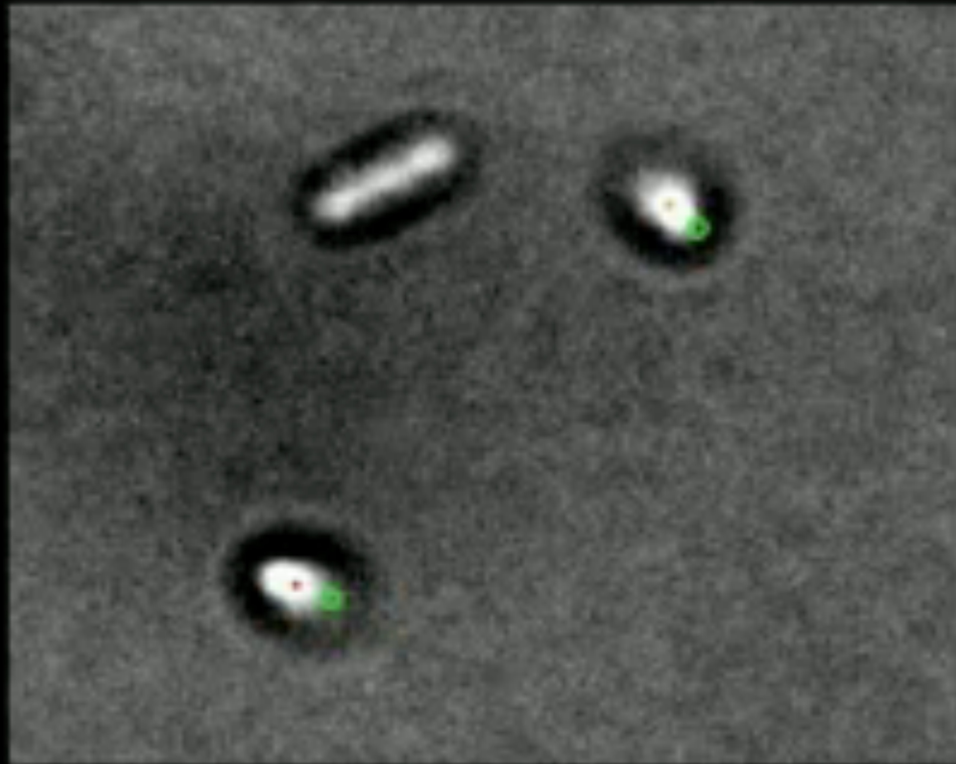


labeled



area histogram

Particle tracking routines



F. Jin, J. C. Conrad, G. C. L. Wong *et al.*, PNAS (2011)
Bacteria moving on glass substrates

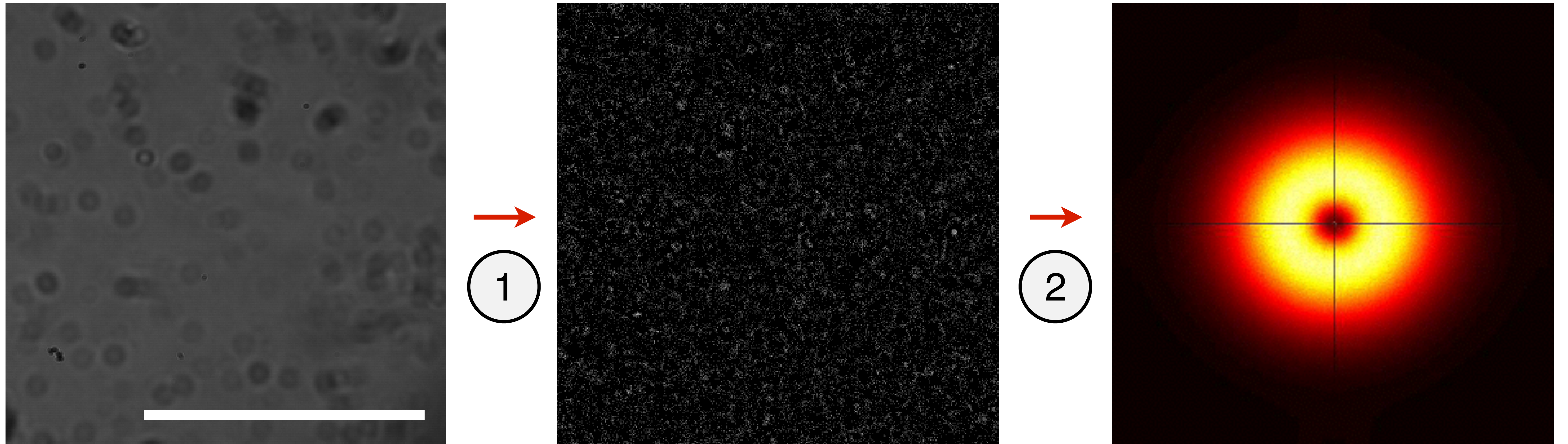
- **Basic idea:** particle tracking routines allow features to be followed over long times by minimizing the total displacement of features between frames
- Typical process:
  - Spatial filtering (convolution)
  - Feature identification (segmentation)
  - Linking of positions into trajectories

Particle-tracking code repository (Matlab):
http://www.physics.georgetown.edu/matlab/

Image difference (frames separated by fixed lag time Δt subtracted): fluctuations (= dynamics) readily visualized!

Safari, Vorontsova, Poling-Skutvik, Vekilov, and JCC, *Phys. Rev. E* (2015)

Differential dynamic microscopy



1. Subtract images separated by fixed lag time:

$$\delta f(x, y; \Delta t) = f(x, y; t + \Delta t) - f(x, y; t)$$

2. Fourier transform image differences:

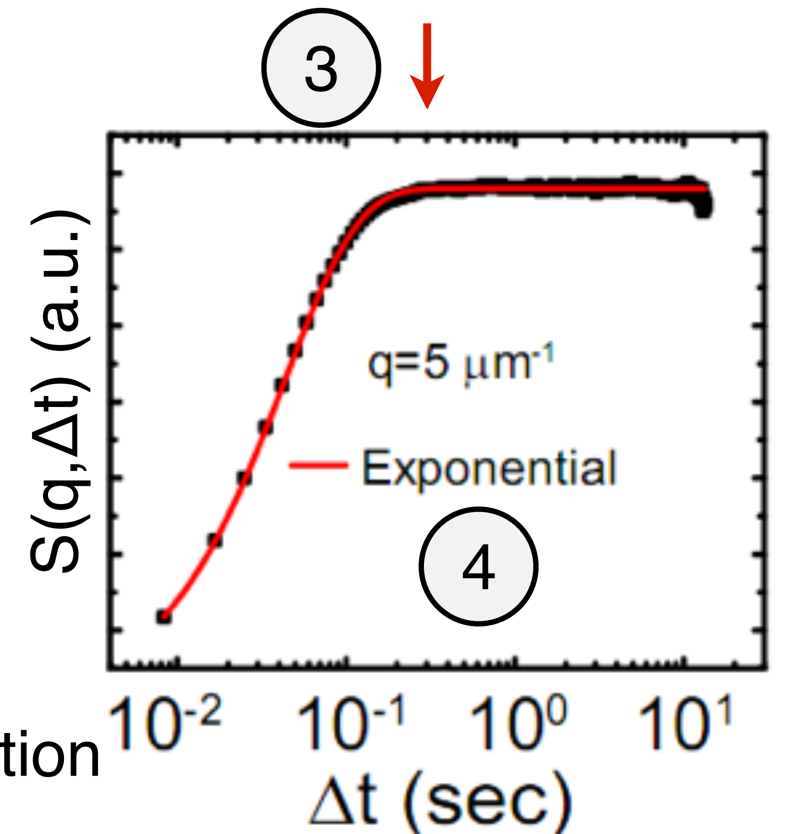
$$S(u_x, u_y; \Delta t) = \left\langle \left|\delta I(u_x, u_y; \Delta t)\right|^2 \right\rangle$$

3. Azimuthally average to obtain image structure function:

$$S(u_x, u_y; \Delta t) \rightarrow S(q, \Delta t)$$

4. Fit structure function to obtain intermediate scattering function

$$S(q, \Delta t) = A(q)\left[1 - f(q, \Delta t)\right] + B(q)$$



Framework also works for other linear space-invariant imaging methods (fluorescence DDM)

Summary for microscopy lecture

- Introduction to optics and brightfield microscopy
- Contrast-enhancing techniques
  - Darkfield, phase contrast, DIC, polarized
- Fluorescence and confocal microscopy
- Digital imaging
- Image processing
  - Histograms
  - Convolution
  - Fourier transforms and Fourier shift theorem
  - Particle tracking
  - Fluctuation analysis (differential dynamic microscopy)