Biomedical Imaging & Analysis

Lecture 6, Part 1. Fall 2014

Basic Image Processing / Filtering (I)

*[**Text**: Ch. 1 and Ch. 2 (until 2.4, Linear Filtering) of Insight into Images edited by Terry Yoo, et al.]*

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Outline

- Review: Lecture 5 (Linear Model for Microscope)
- Basic concept: spatial filtering
- Basic concept: image gradient calculation
- The Gaussian filter
- Overview of image feature detection
- Point feature detection

- Review: Lecture 5
  - Linear Systems Model of a Microscope
- Basic concept: spatial filtering
- Basic concept: image gradient calculation
- The Gaussian filter
- Overview of image feature detection
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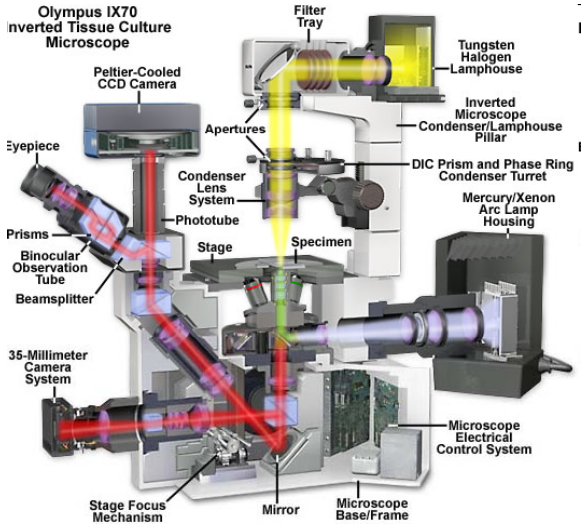
A Microscope can be modeled as a Linear System



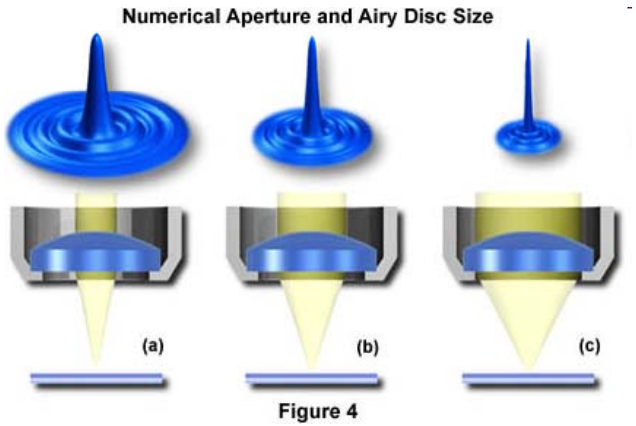
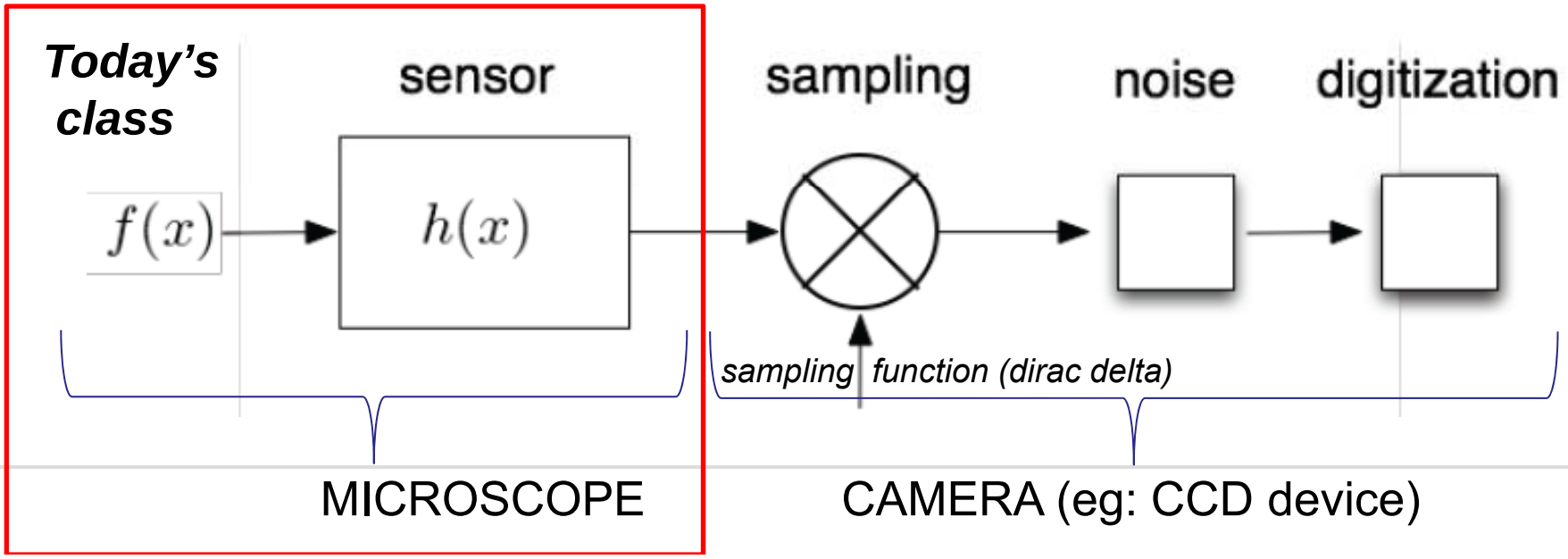
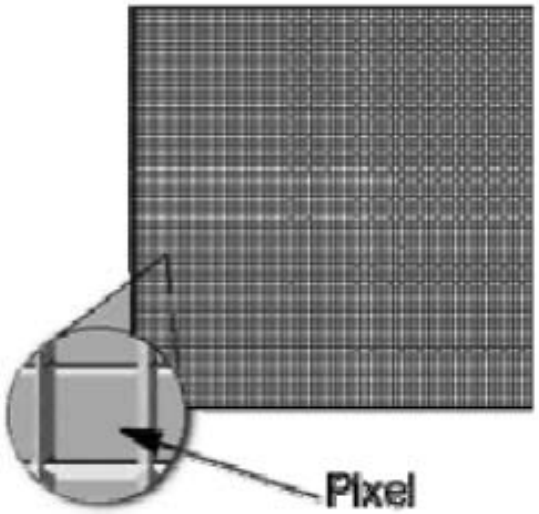
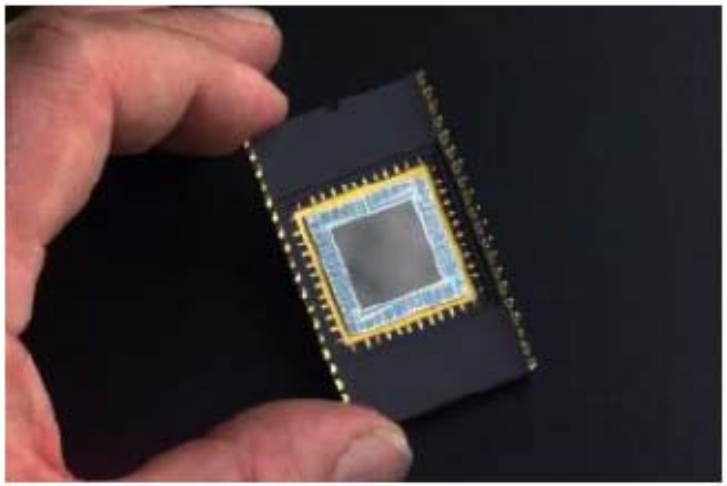


Figure 4



Review: Basic Concepts (I)

- An image records spatiotemporal information of a biological process.
- An image can be considered as both a matrix and a surface.
- 



Camera Noise Model

- Signal $S = I \cdot QE \cdot T$

  T: exposure/integration time
  QE: quantum efficiency

- Signal shot noise

$$N_{shot} = \sqrt{S}$$

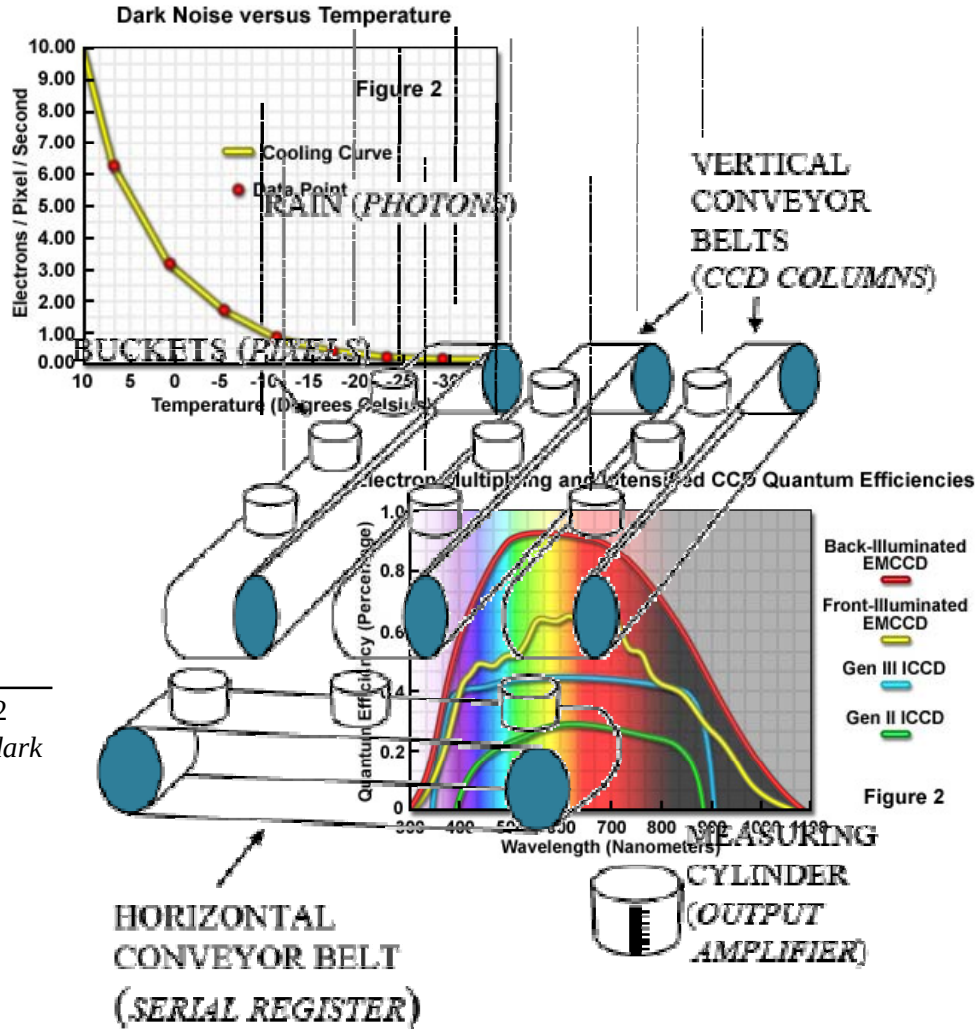
- Camera noise

$$N_{dark} = \sqrt{D \cdot T} \qquad N_{camera} = \sqrt{N_{read}^2 + N_{dark}^2}$$

  D: dark current

- Total noise

$$N_{total} = \sqrt{N_{shot}^2 + N_{read}^2 + N_{dark}^2}$$



http://micro.magnet.fsu.edu/primer/java/digitalimaging/russ/shotnoisereduction/index.html

Thermal Noise

- Gaussian distribution:

  Given a Gaussian process, the probability of receiving *x* photons is given by

$$f(x, \mu, \sigma) = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(x-\mu)^2}{2\sigma^2}}$$

- Thermal energy in the silicon lattice causes Gaussian distributed noise.

Poisson Image Noise (Shot Noise)

- Poisson distribution:

  Given a Poisson process, the probability of receiving n photons is given by

$$P_v(n) = \frac{\lambda^n e^{-\lambda}}{n!} \quad \approx N(\mu=\lambda, \sigma=\sqrt{\lambda})$$

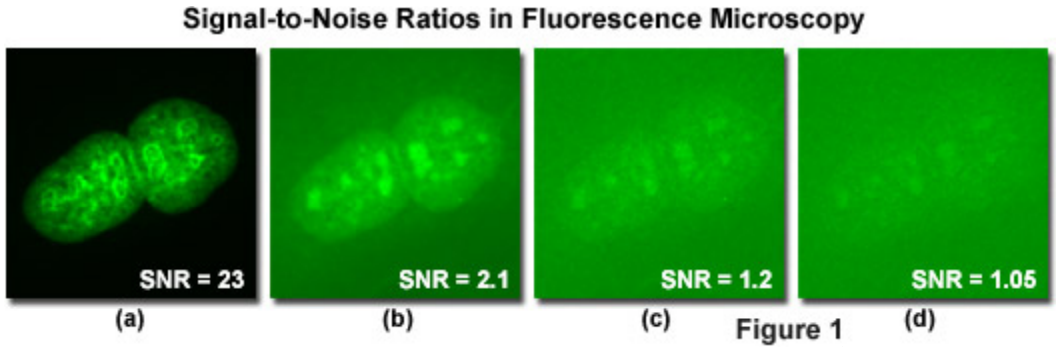
$$\mu = \sigma^2 = \lambda \leftarrow \text{Average number of photons}$$

Poisson to Normal Distribution Approximation:
http://www.stat.ucla.edu/~dinov/courses_students.dir/Applets.dir/NormalApprox2PoissonApplet.html

- The process of photon counting in a CCD is statistically described by a Poisson distribution…

Signal-to-Noise Ratio

- Signal-to-Noise ratio defines image quality.



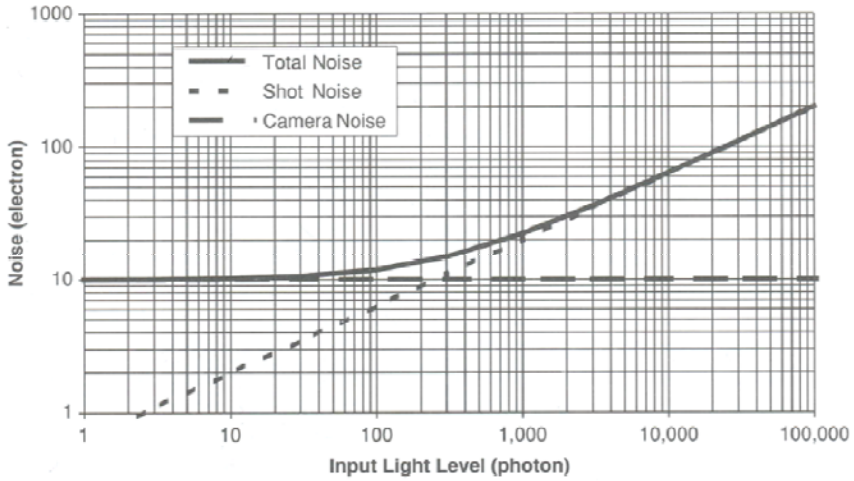


Fig. 4 Noise vs. light level (camera noise = 10 electron, QE = 0.4).

$$SNR = \frac{P_{signal}}{P_{noise}} = \left(\frac{A_{signal}}{A_{noise}} \right)^2$$

$$SNR = \frac{\sigma^2_{signal}}{\sigma^2_{noise}}$$

$$SNR = \frac{A_{signal}}{\sigma_{noise}}$$

High Resolution Microscopy

- Size of cellular features are typically on the scale of a micron or smaller.

- To resolve such features require

  - Shorter wavelength (electron microscopy)
  - High numerical aperture (resolution)
  - High magnification (spatial sampling)

$$D = \frac{0.61\lambda}{NA}$$

- Resolution (D) improves (gets smaller) if λ ↓ or n↑ or α↑ (parameters affecting NA)

The Bane of Imaging: **Diffraction Limit**

Abbe's diffraction limit:

"broadening" of a point caused by diffraction is known as the "point spread function" (Δ)

$$\Delta_{x\text{-}y} = (0.61\ \lambda)/(\eta\ \sin(\alpha))$$

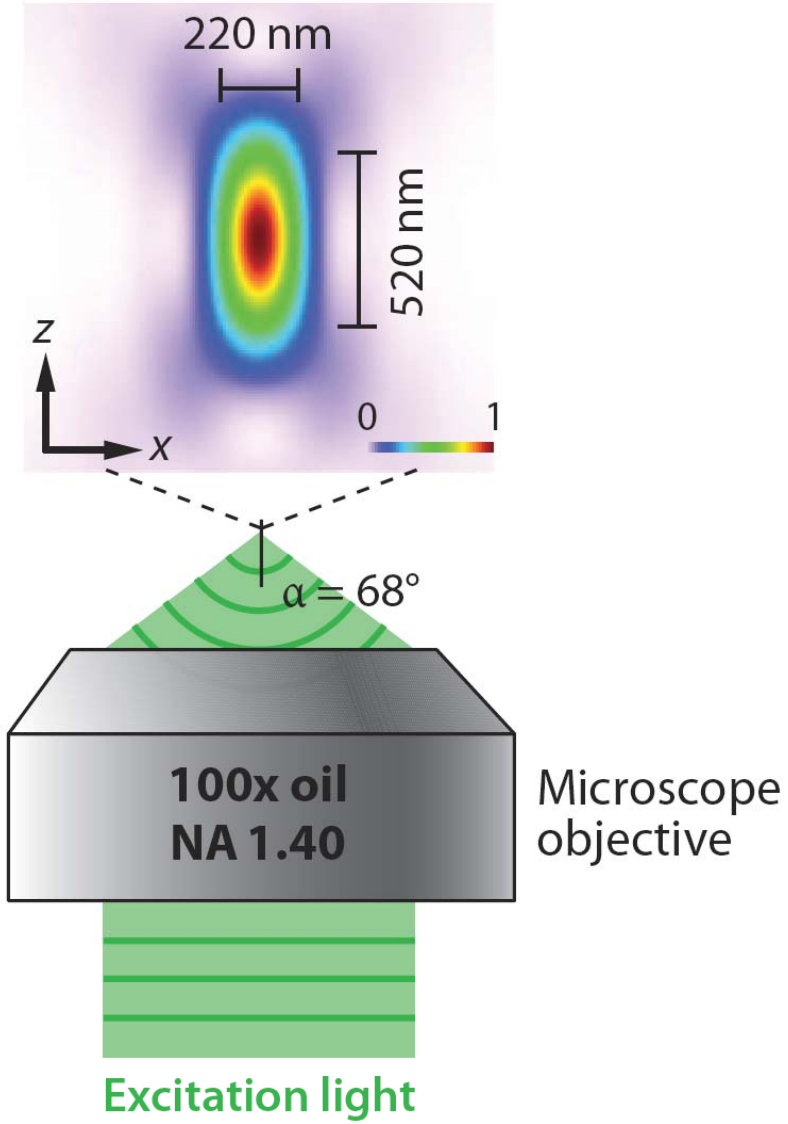
η = refractive index medium

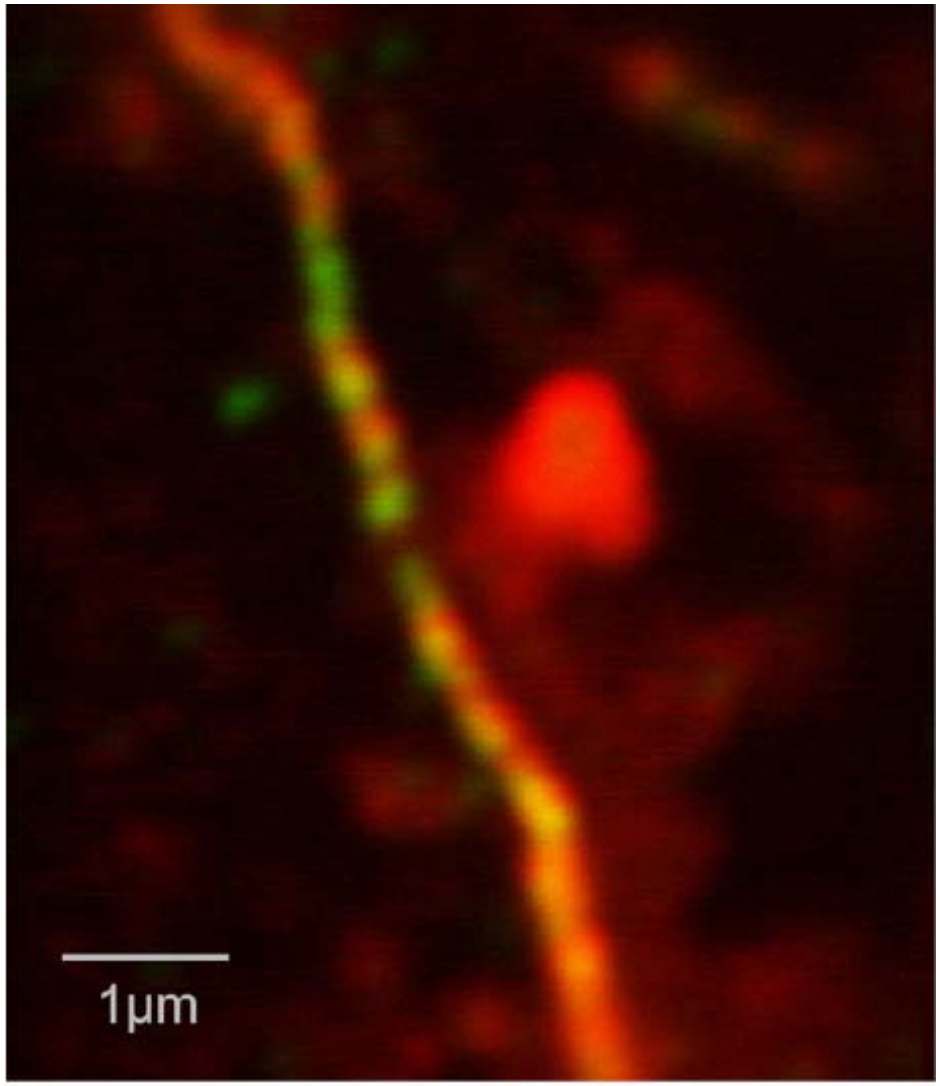
α = half-cone angle of focused light

- Practical limit obtained when imaging very small objects by magnification.

- Diffraction causes blurring of objects when imaging smaller than ~200-500 nm (diffraction limit).

Examples of Diffraction Limit





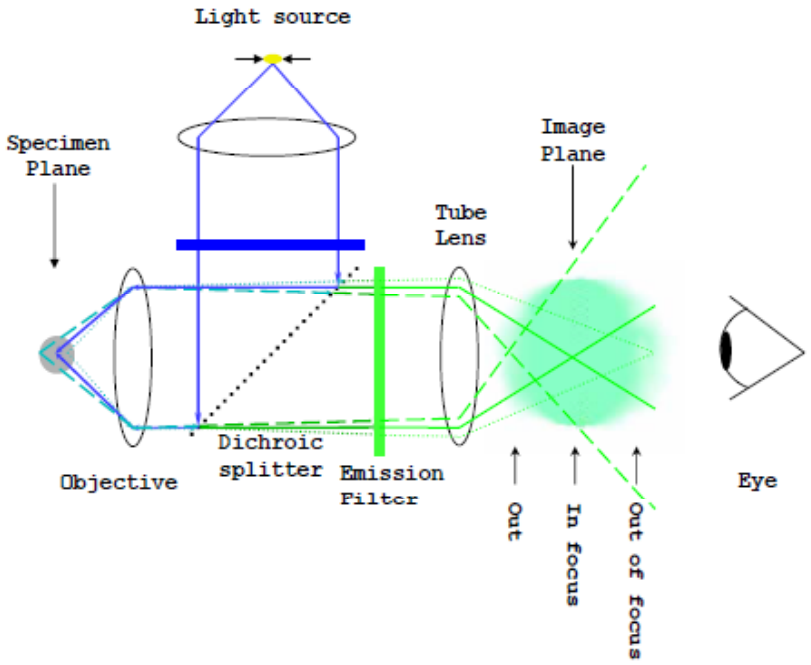
Ways to Circumvent Limit

- Near Field Microscopy: Place microscope distance less than 1 wavelength from sample. 20-50 nm resolution
- Far Field Microscopy
  - Confocal, 4pi and I^5M, Structured-Illumination Microscopy (SIM)
- Super-Resolution
  - Spatially Patterned Excitation
    - STED
    - RESOLFT
    - SSIM
  - Localization Methods
    - total internal reflection fluorescence (TIRF)
    - STORM
    - PALM, FPALM

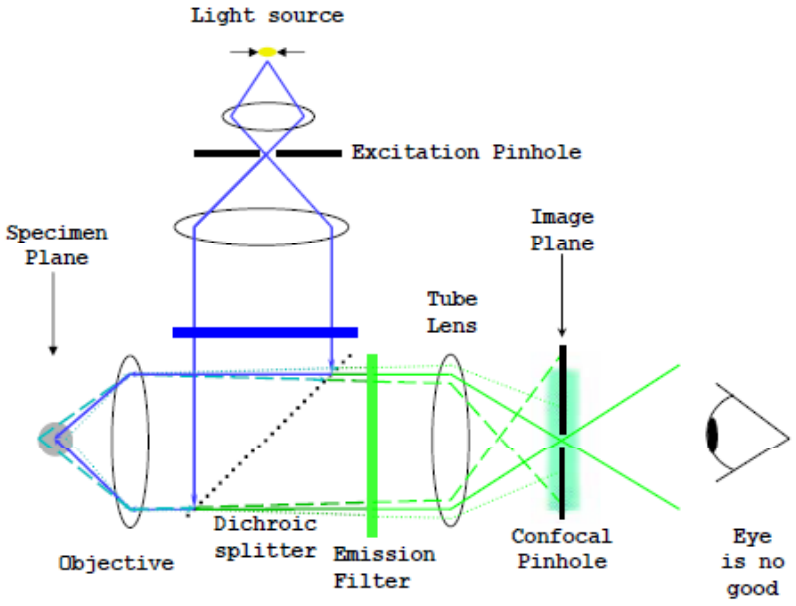
Far Field: Confocal Microscopy

- Non-linear 2-photon excitation and pinhole detection decrease PSF size beyond classical limits for a given wavelength of incident light.
- $2^{1/2}$ improvement in resolution.
- Problem: 2-photon excitation uses high wavelengths which increase diffraction : $\Delta_{x\text{-}y} = (0.61\ \lambda)/(\eta\ \sin(\alpha))$

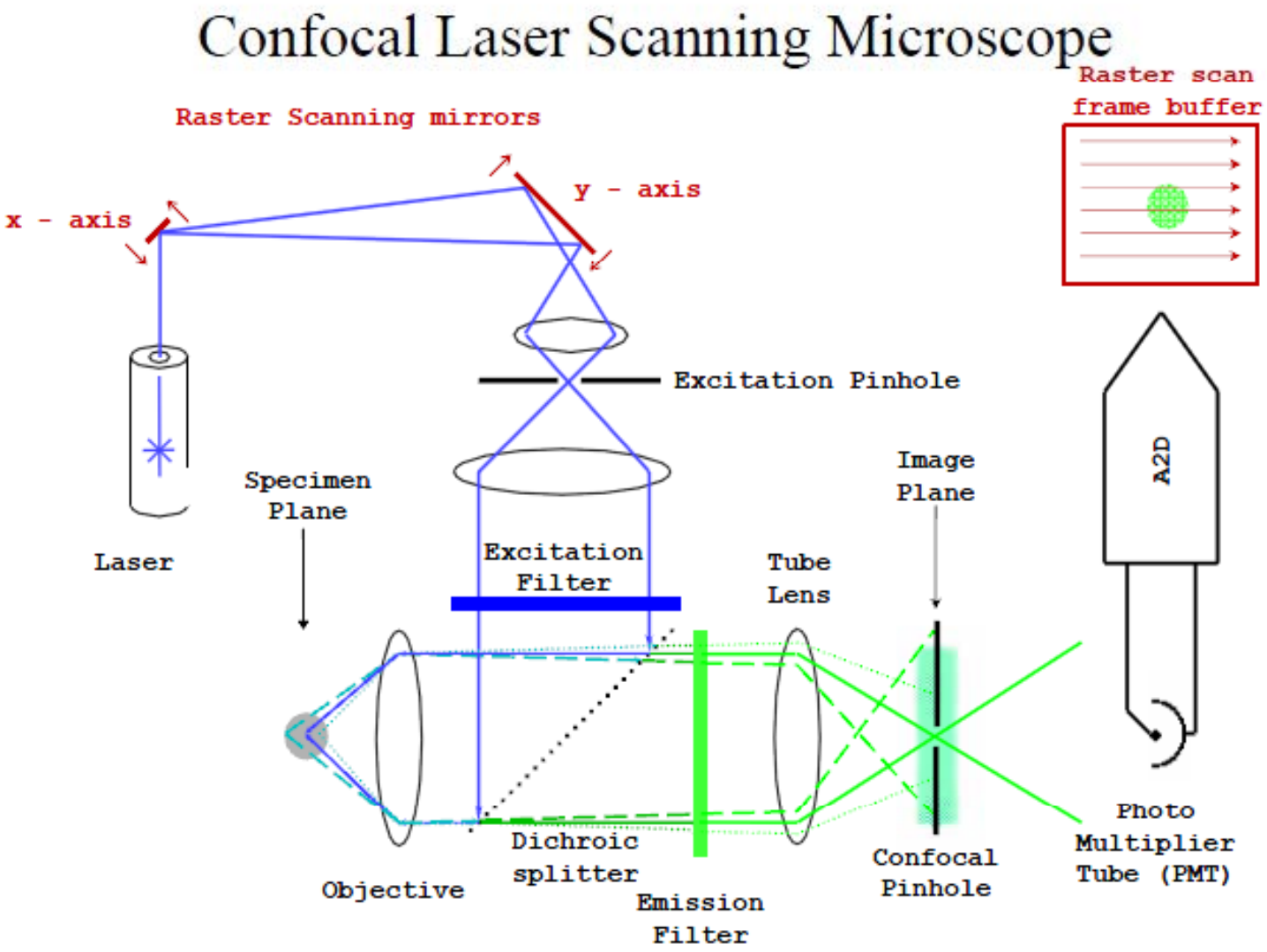
Limitation of wide field microscopy



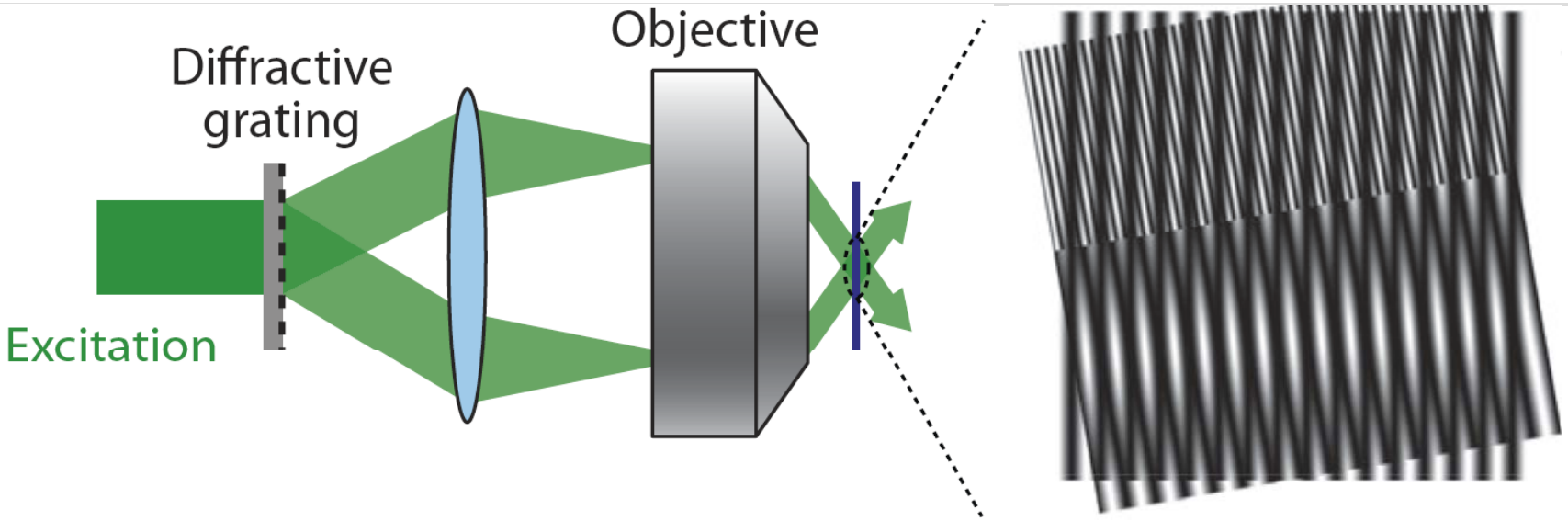
Confocal Principal – confocal pinhole



Far Field: Confocal Microscopy (I)

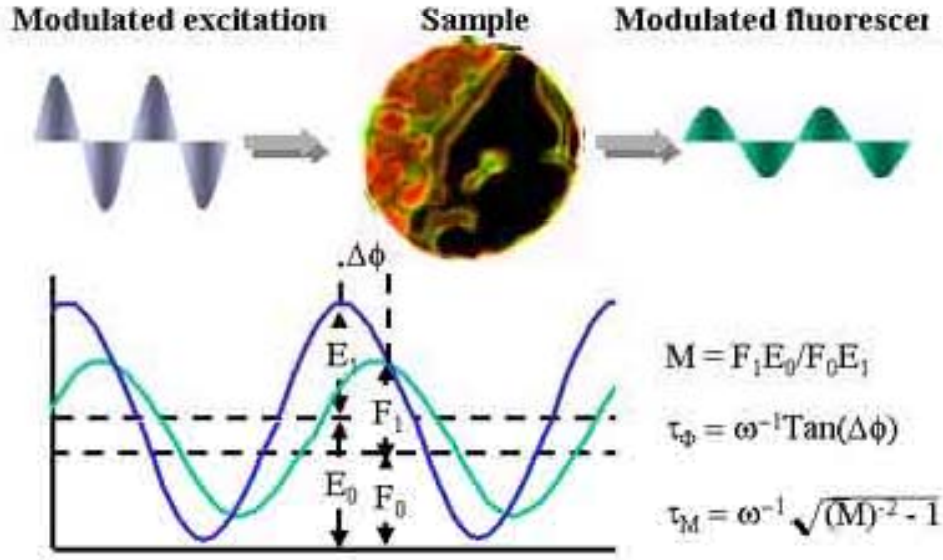


Structured-Illumination Microscopy (SIM)



100 nm resolution possible

Fluorescence lifetime imaging microscopy/ Fluorescence resonance energy



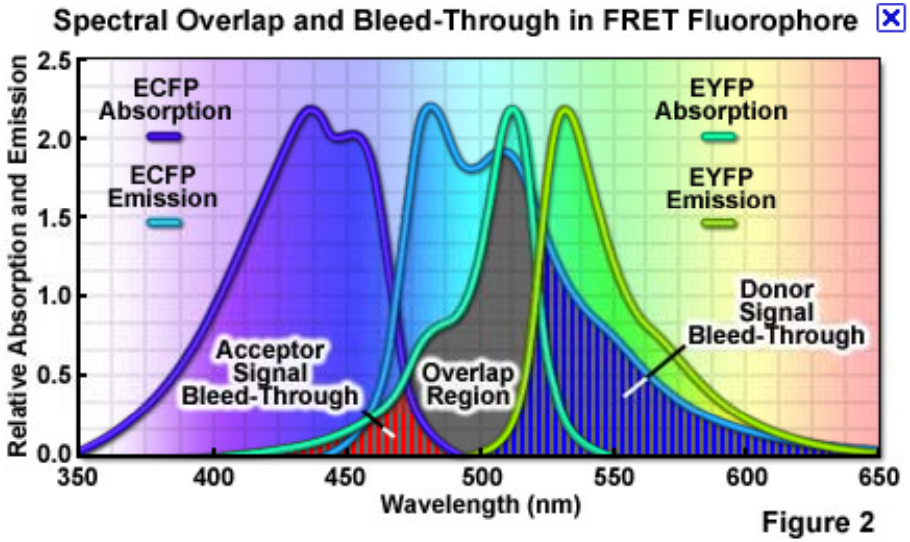


Figure 2

Far Field: Confocal Microscopy (II)

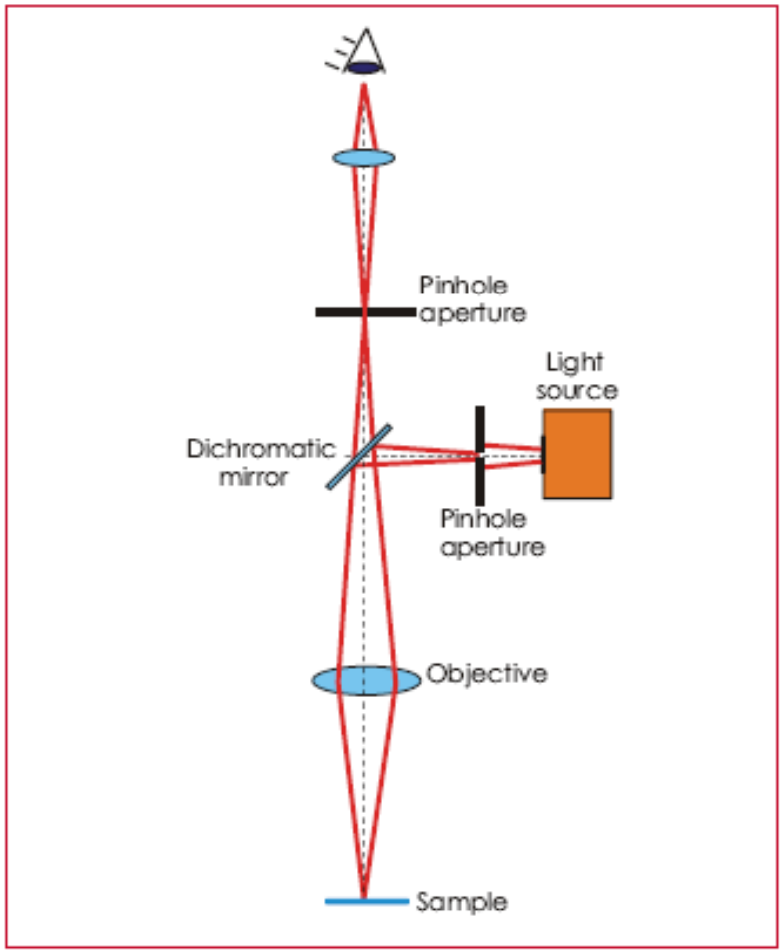


Figure 4. Simplified scheme of the reflective confocal microscope with two pinhole apertures.

Beyond the Diffraction Limit

- **methods use common dyes (good)**
- **confocal is easiest, most widely used**

- **best resolution obtainable only 100 nm (SIM)**
- **single molecule is problematic**

Super-Resolution Microscopy

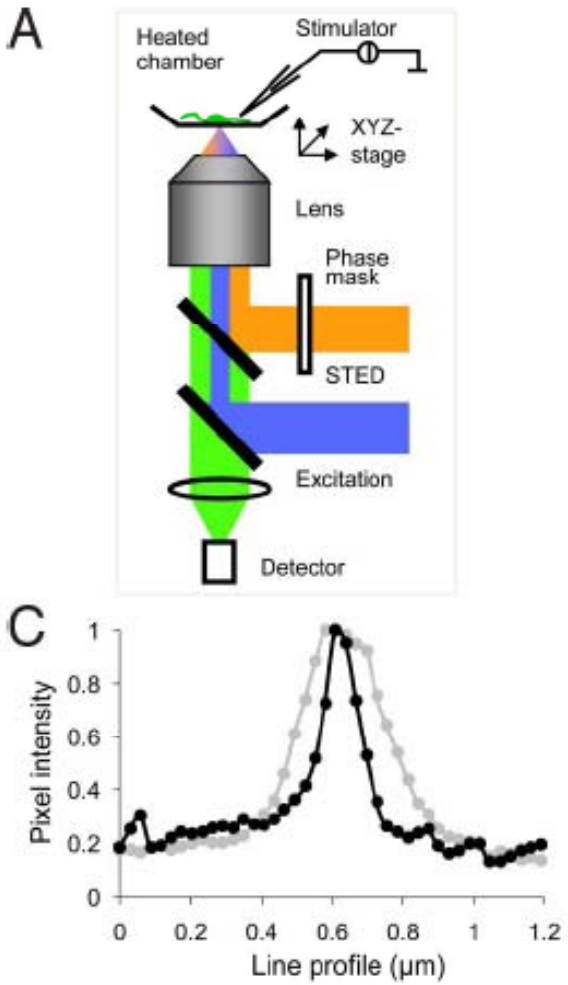
- Goal: obtain sub-100 nm resolution.
- Pioneered by Stefan Hell in mid-1990s
- Max Plank Institute (Germany). Chemistry Nobel Laureate (2014).

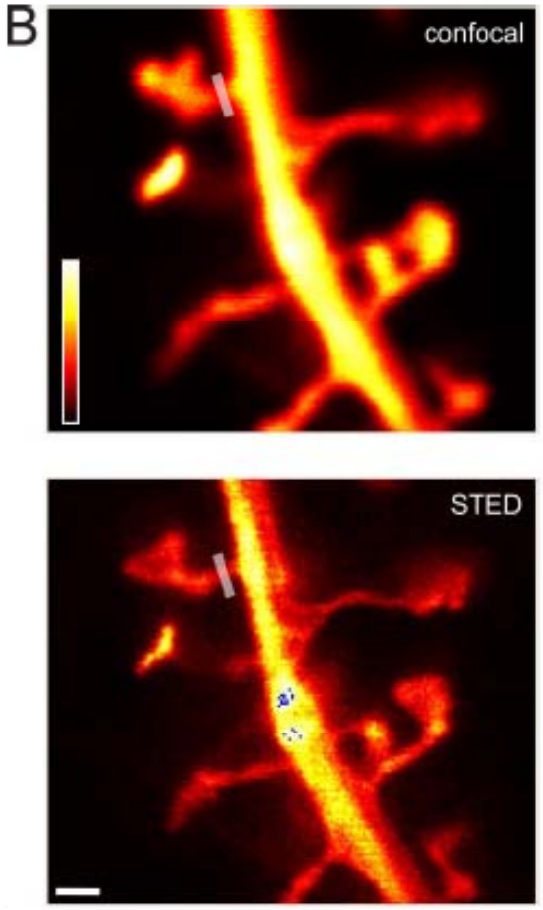
- **Two methods:**

  (i) Spatially Patterned Excitation:
  STED, RESOLFT, SSIM

  (ii) Localization Methods:
  TIRFM, STORM, PALM, FPALM

Stimulated emission depletion microscopy (STED)





STED microscopy operates by using two laser beams to illuminate the specimen. An excitation laser pulse (generally created by a multiphoton laser) is closely followed by a doughnut-shaped red-shifted pulse that is termed the **STED beam**.

Excited fluorophores exposed to the STED beam are instantaneously returned to the ground state by means of stimulated emission. The non-linear depletion of the fluorescent state by the STED beam is the basis for superresolution.

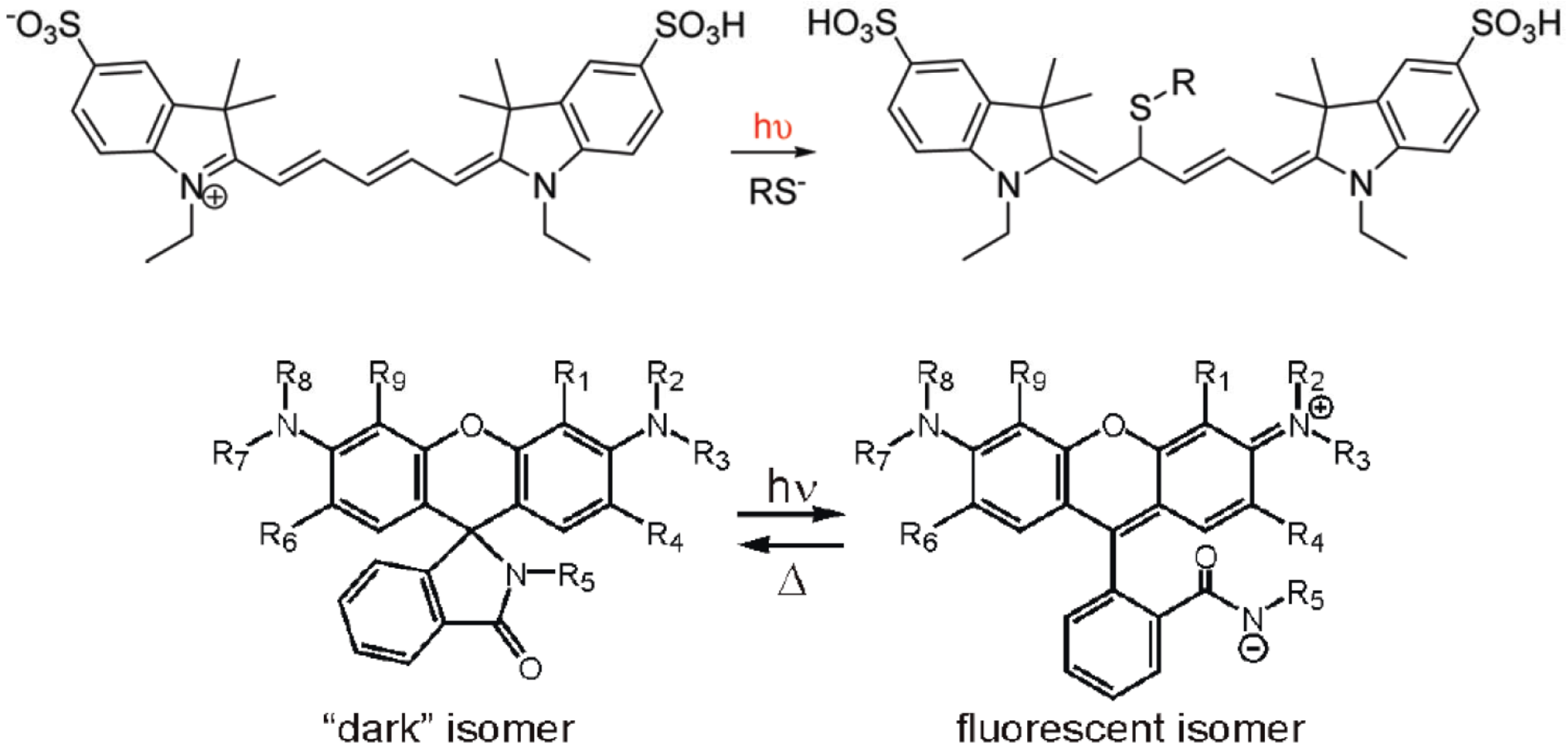
When the two laser pulses are superimposed, only molecules that reside in the center of the STED beam are able to emit fluorescence, thus significantly restricting emission. This action effectively narrows the point spread function and ultimately increases resolution beyond the diffraction limit.

To generate a complete image, the central zero is raster-scanned across the specimen in a manner similar to single-photon confocal microscopy, as illustrated in the tutorial. STED microscopy is capable of 20 nanometer (or better) lateral resolution and 40 to 50 nanometer axial resolution.

Dyes for **Localization** Microscopy

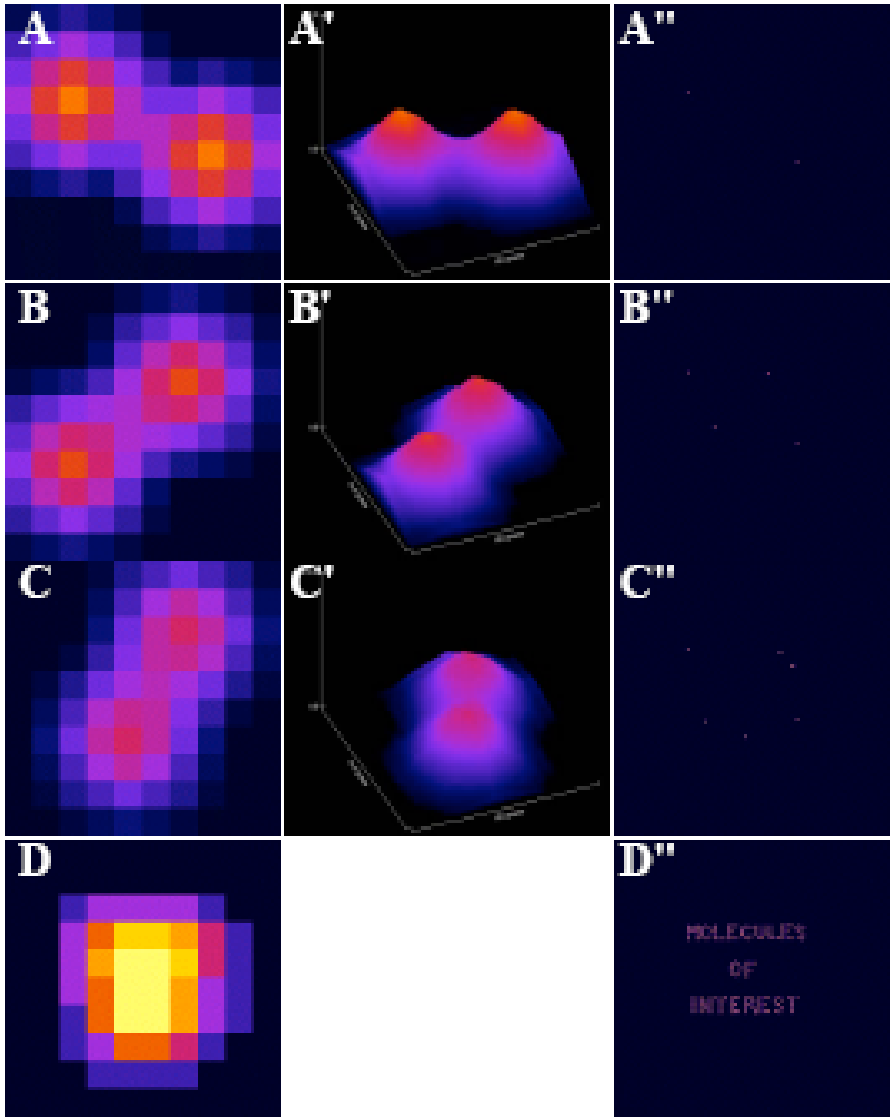
- have on and off state
- easily able to switch from on/off state
- on/off can be non-fluorescent or have a change in *either* excitation or emission wavelengths
- best if reversible but not necessary

Examples of Photoswitchable Dyes



Fluorophore		Activation wavelength (nm)	Before activation		After activation		Reversible	References
			Ex[a] (nm)	Em (nm)	Ex (nm)	Em (nm)		
Cyan/dark-to-green FP	PA-GFP	405	400	517	504	517	No	(88)
	PS-CFP2		400	468	490	511		(89)[b]
Green-to-red FP	Kaede	405	508	518	572	582	No	(90)
	EosFP	405	505	516	569	581		(91)
	Dendra2	405–488	490	507	553	573		(92)[b]
Dark-to-red FP	PAmCherry	405	NF		564	595	No	(62)
Reversible FP	Dronpa	405	NF		503	518	Yes	(93)
	Dronpa2				486	513		(94)
	Dronpa3				487	514		(94)
	rsFastLime				496	518		(95)
	bsDronpa				460	504		(61)
	EYFP	405	NF		513	527		(66)
Caged dyes	Caged fluorescein	<405	NF		497	516	No	[c]
	Caged Q-rhodamine[d]				545	575		
Cyanine dyes	Cy5 & Alexa 647	350–570[e]	NF		647	665	Yes	(46, 58)
	Cy5.5				674	692		
	Cy7				746	773		
Photochromic rhodamine	SRA545	375	NF		Green	545	Yes[f]	(59, 96)
	SRA552					552		
	SRA577					577		
	SRA617					617		

Photoactivated localization microscopy (PALM)



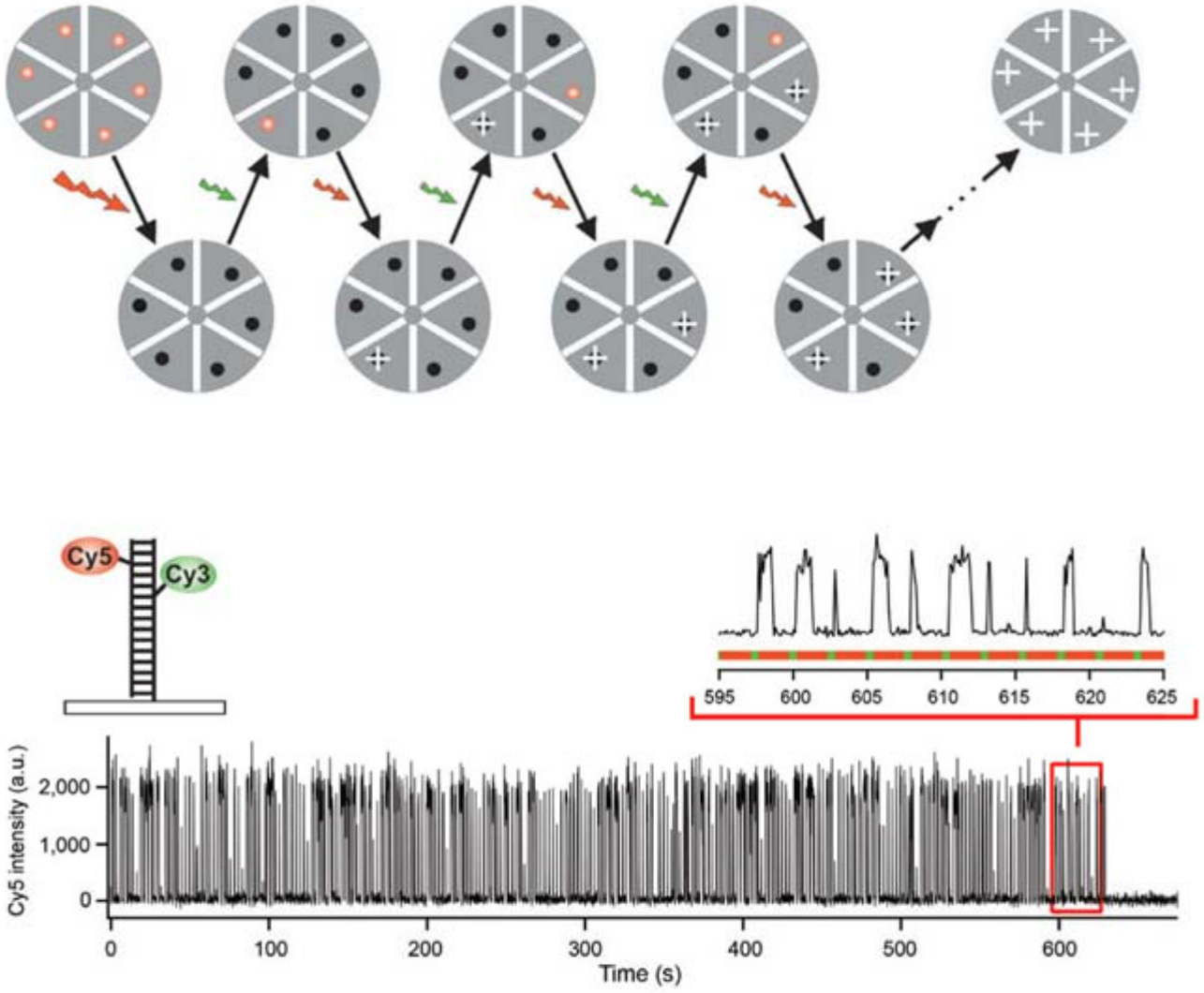
A single fluorescent molecule forms a diffraction-limited image having lateral and axial dimensions defined by the excitation wavelength, refractive index of the imaging medium, and the angular aperture of the microscope objective:

$$\textbf{Resolution}_{x,y} = \lambda / 2[\eta \cdot \sin(\alpha)]$$
$$\textbf{Resolution}_{z} = 2\lambda / [\eta \cdot \sin(\alpha)]^2$$

In PALM and other single-molecule localization methods, images can be rendered with the molecules localized to the highest precisions (a nanometer or two) by selectively excluding data points with poor localization.

This comes at the expense of the molecular density (and thus, resolution) in the final image.

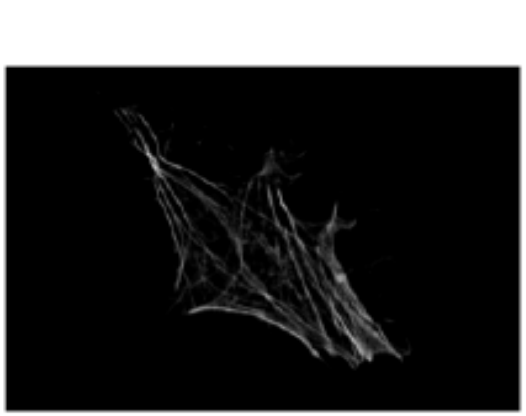
Stochastic optical reconstruction microscopy (STORM)



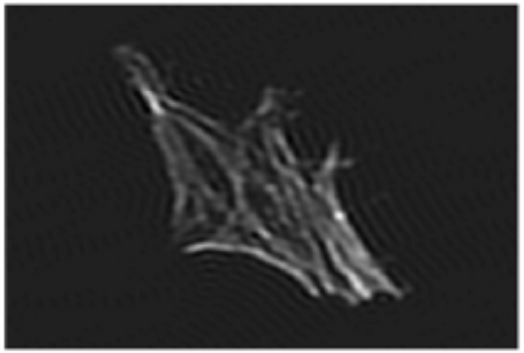
Single-molecule localization is demonstrated through the analysis of the stochastic blinking of quantum dots.

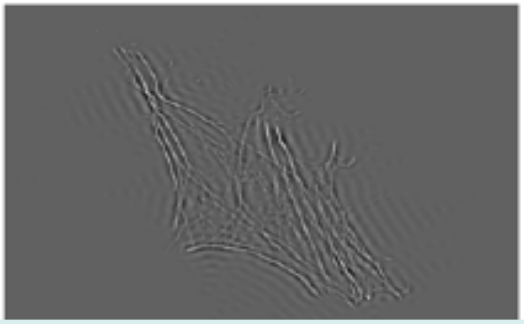
Review: Basic Concepts (II)

- Image noise usually has high-frequency .

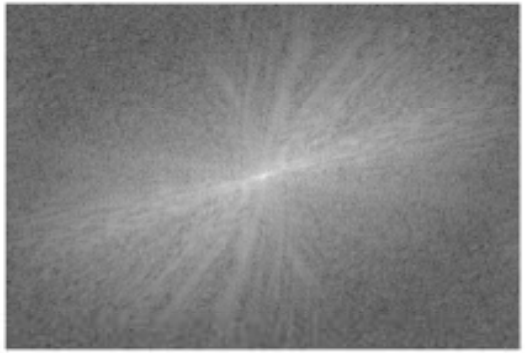


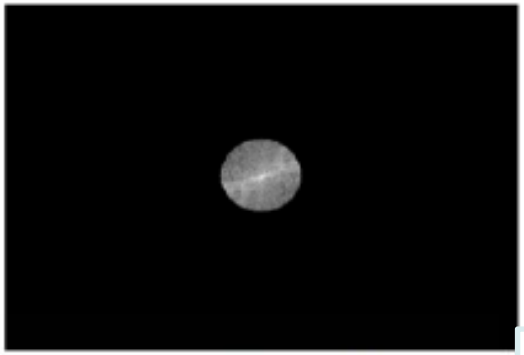
Image



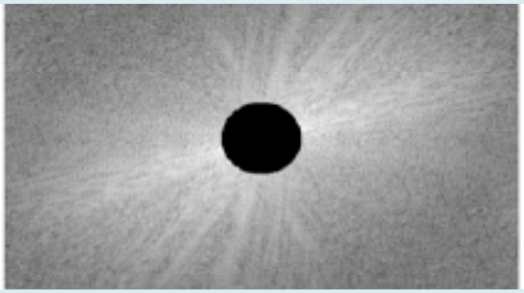


But the High Frequencies of the Image also has Edge Information!





Fourier Transform



*De-noising often comes at the cost of "**BLURRING**".*

- Review: Lecture 5
- **Basic concept: spatial filtering**
- Basic concept: image gradient calculation
- The Gaussian filter
- Overview of image feature detection
- Point feature detection

Basic Concept: Spatial Filtering (I)

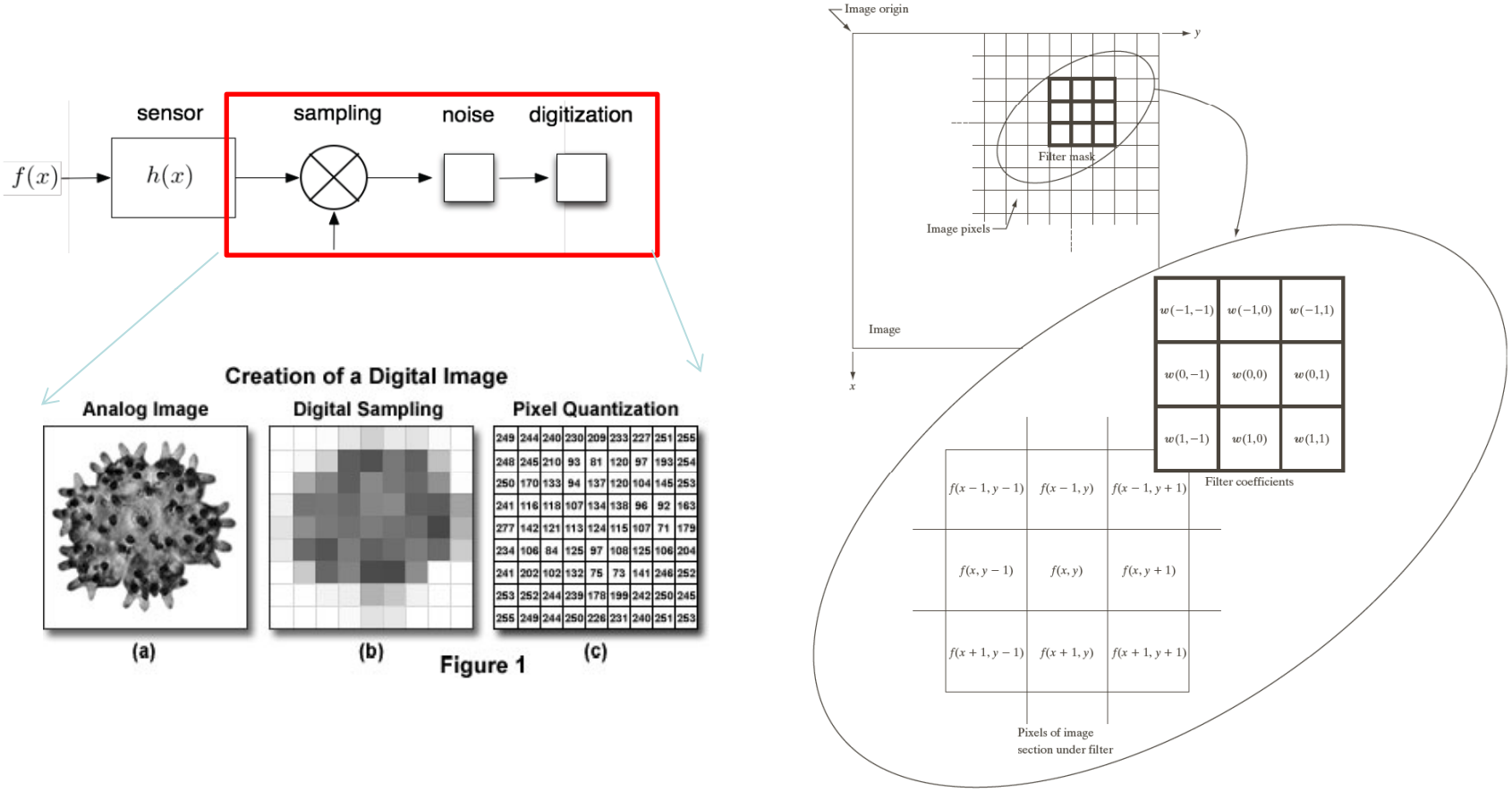
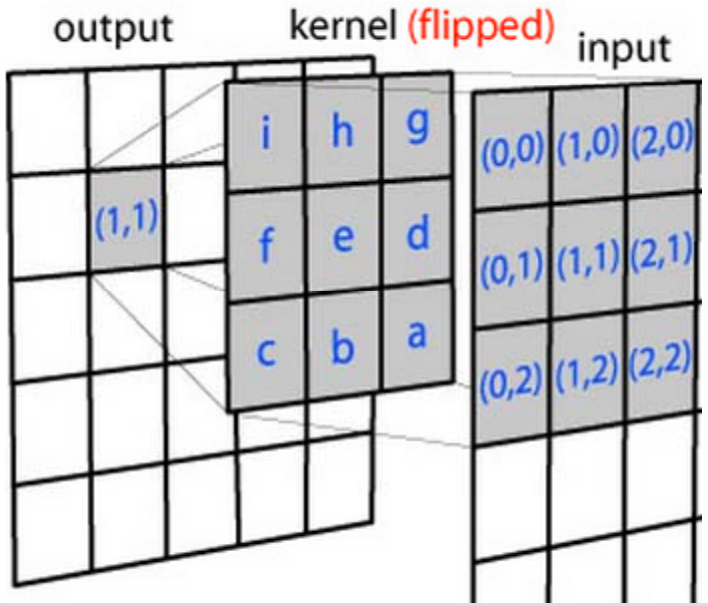


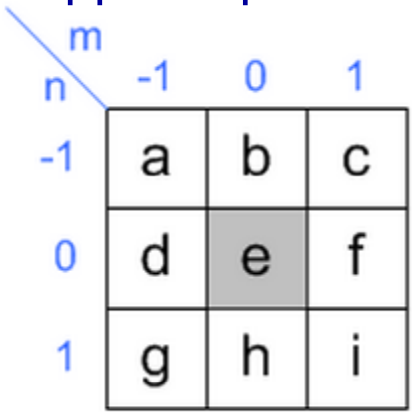
FIGURE 3.28 The mechanics of linear spatial filtering using a 3×3 filter mask. The form chosen to denote the coordinates of the filter mask coefficients simplifies writing expressions for linear filtering.

Gonzalez & Woods, DIP 3/e

The Convolution Operator – what happens to an input 1D image function, f(x,y) as it passes through an aperture ..?

- Simple example of convolution of input image (matrix, S) and impulse response (kernel) in 2D spatial. *Y[n] = (S * h) [n].*
- Notice that the kernel matrix is flipped both horizontal and vertical direction before multiplying the overlapped input data.



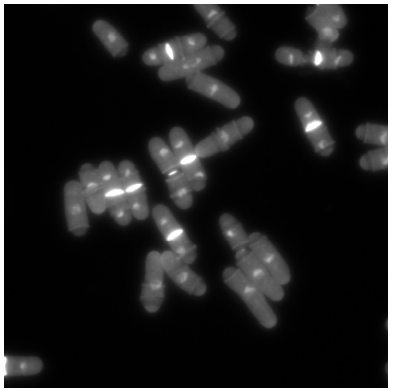


$$y[1,1] = \sum_{j=-\infty}^{\infty} \sum_{i=-\infty}^{\infty} x[i,j] \cdot h[1-i, 1-j]$$

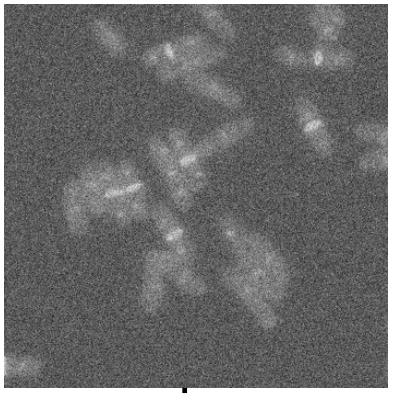
$$\begin{aligned} = \; & x[0,0] \cdot h[1,1] + x[1,0] \cdot h[0,1] + x[2,0] \cdot h[-1,1] \\ & + x[0,1] \cdot h[1,0] + x[1,1] \cdot h[0,0] + x[2,1] \cdot h[-1,0] \\ & + x[0,2] \cdot h[1,-1] + x[1,2] \cdot h[0,-1] + x[2,2] \cdot h[-1,-1] \end{aligned}$$

Basic Concept of Image Filtering (I)

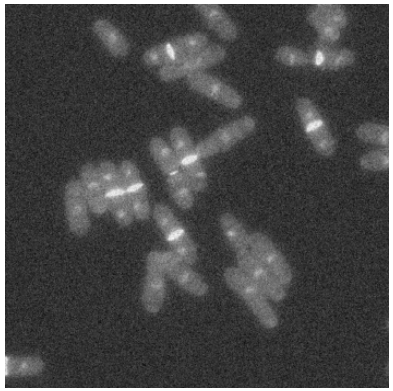
- Application I: noise suppression



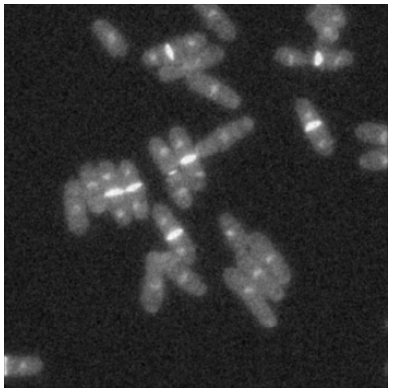
original



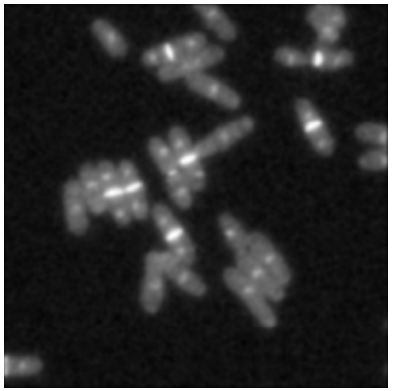
noise added



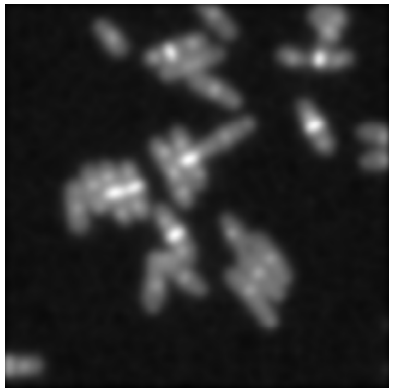
σ=1



σ=2



σ=5



σ=10

Basic Concept of Image Filtering (I)

- Application II: image conditioning

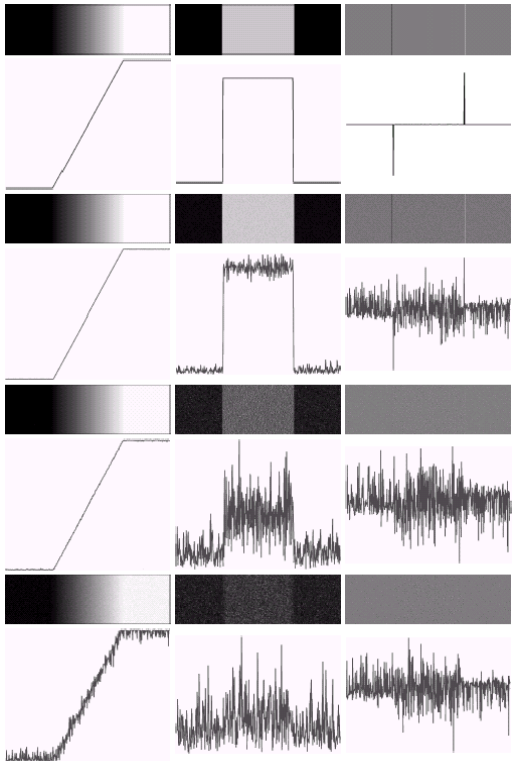


FIGURE 10.7 First column: images and gray-level profiles of a ramp edge corrupted by random Gaussian noise of mean 0 and $\sigma = 0.0, 0.1, 1.0,$ and 10.0, respectively. Second column: first-derivative images and gray-level profiles. Third column: second-derivative images and gray-level profiles.

a
b
c
d

Gonzalez & Woods, DIP 2/e

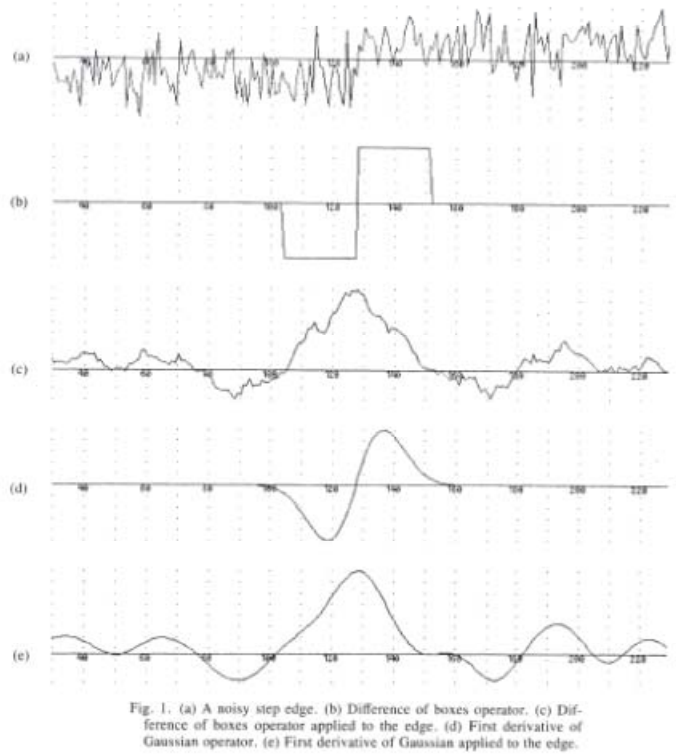


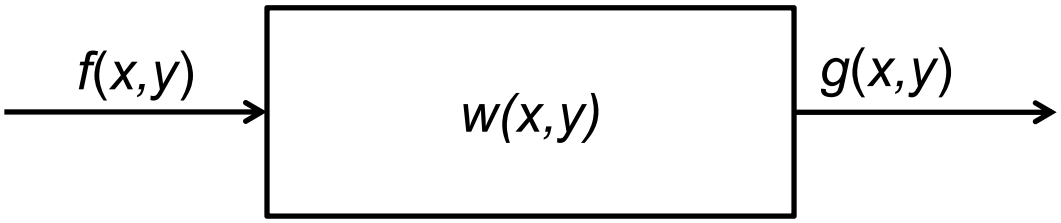
Fig. 1. (a) A noisy step edge. (b) Difference of boxes operator. (c) Difference of boxes operator applied to the edge. (d) First derivative of Gaussian operator. (e) First derivative of Gaussian applied to the edge.

Canny, J., *A Computational Approach To Edge Detection*, IEEE Trans. Pattern Analysis and Machine Intelligence, 8(6):679–698, 1986.

Basic Concept: Spatial Filtering (I)

- A spatial filter is often referred to as a mask, a kernel, a template, or a window.

$$\sum_{s=-a}^{a}\sum_{t=-b}^{b} w(s,t)\, f(x+s,y+t) = \sum_{s=-a}^{a}\sum_{t=-b}^{b} w(-s,-t)\, f(x+s,y+t) = w(x,y) \otimes f(x,y)$$



$$g(x,y) = w(x,y) \otimes f(x,y)$$

$$G(u,v) = W(u,v) \cdot F(u,v)$$

http://www.imageprocessingplace.com/

Oppenheim et al, Signals & Systems, 1997

Basic Concept: Spatial Filtering (I)

- Image filtering in the spatial domain

$$g(x,y) = \sum_{s=-a}^{a}\sum_{t=-b}^{b} w(s,t)\, f(x+s, y+t)$$

$$= \sum_{s=-a}^{a}\sum_{t=-b}^{b} w(-s,-t)\, f(x+s, y+t) \leftarrow \text{if } w(\cdot) \text{ is symmetric w.r.t. the origin}$$

$$= \sum_{s=-a}^{a}\sum_{t=-b}^{b} w(s,t)\, f(x-s, y-t)$$

$$= w(x,y) \otimes f(x,y)$$

What happens when we have a Sequence of processing operations?

- Chain matrix multiplication
  - Matrix multiplication ORDER matters.

Basic Concept: Spatial Filtering (I)

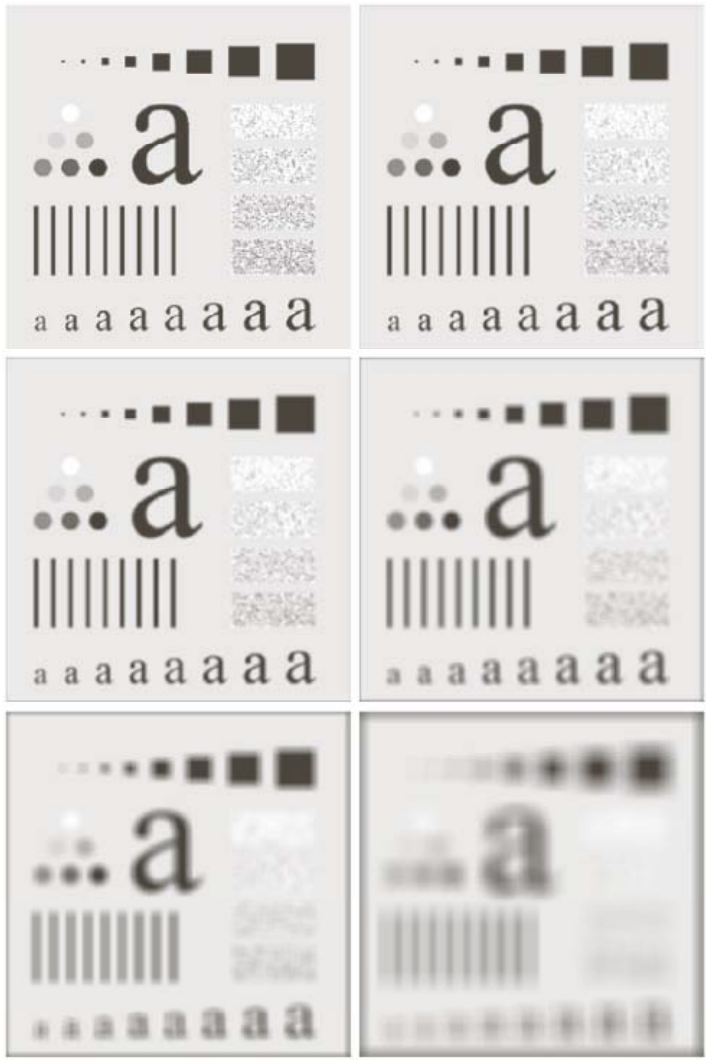


FIGURE 3.33 (a) Original image, of size 500 × 500 pixels. (b)–(f) Results of smoothing with square averaging filter masks of sizes $m = 3, 5, 9, 15$, and 35, respectively. The black squares at the top are of sizes 3, 5, 9, 15, 25, 35, 45, and 55 pixels, respectively; their borders are 25 pixels apart. The letters at the bottom range in size from 10 to 24 points, in increments of 2 points; the large letter at the top is 60 points. The vertical bars are 5 pixels wide and 100 pixels high; their separation is 20 pixels. The diameter of the circles is 25 pixels, and their borders are 15 pixels apart; their intensity levels range from 0% to 100% black in increments of 20%. The background of the image is 10% black. The noisy rectangles are of size 50 × 120 pixels.

a b
c d
e f

Basic Concept: Spatial Filtering (II)

- Frequency response of the averaging filter

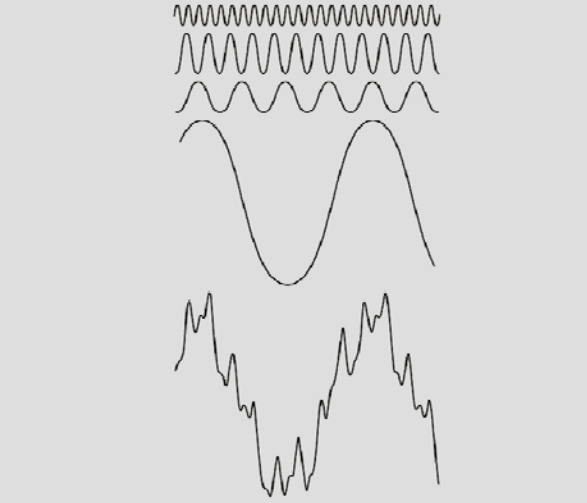
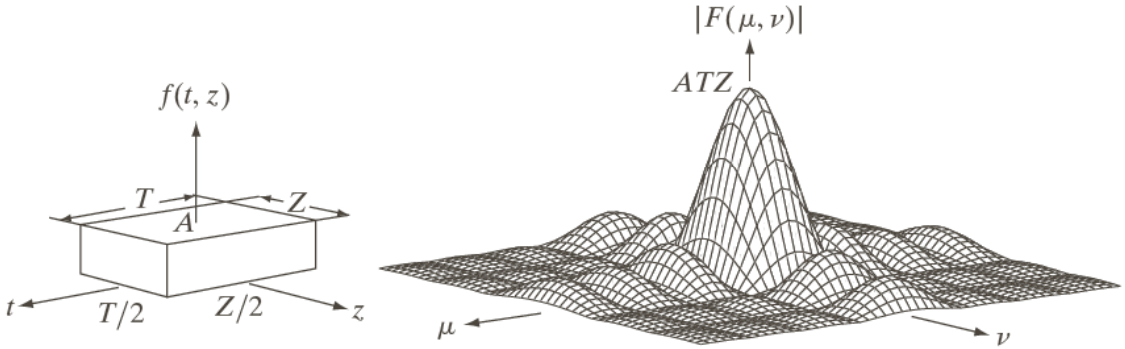


FIGURE 4.1 The function at the bottom is the sum of the four functions above it. Fourier's idea in 1807 that periodic functions could be represented as a weighted sum of sines and cosines was met with skepticism.

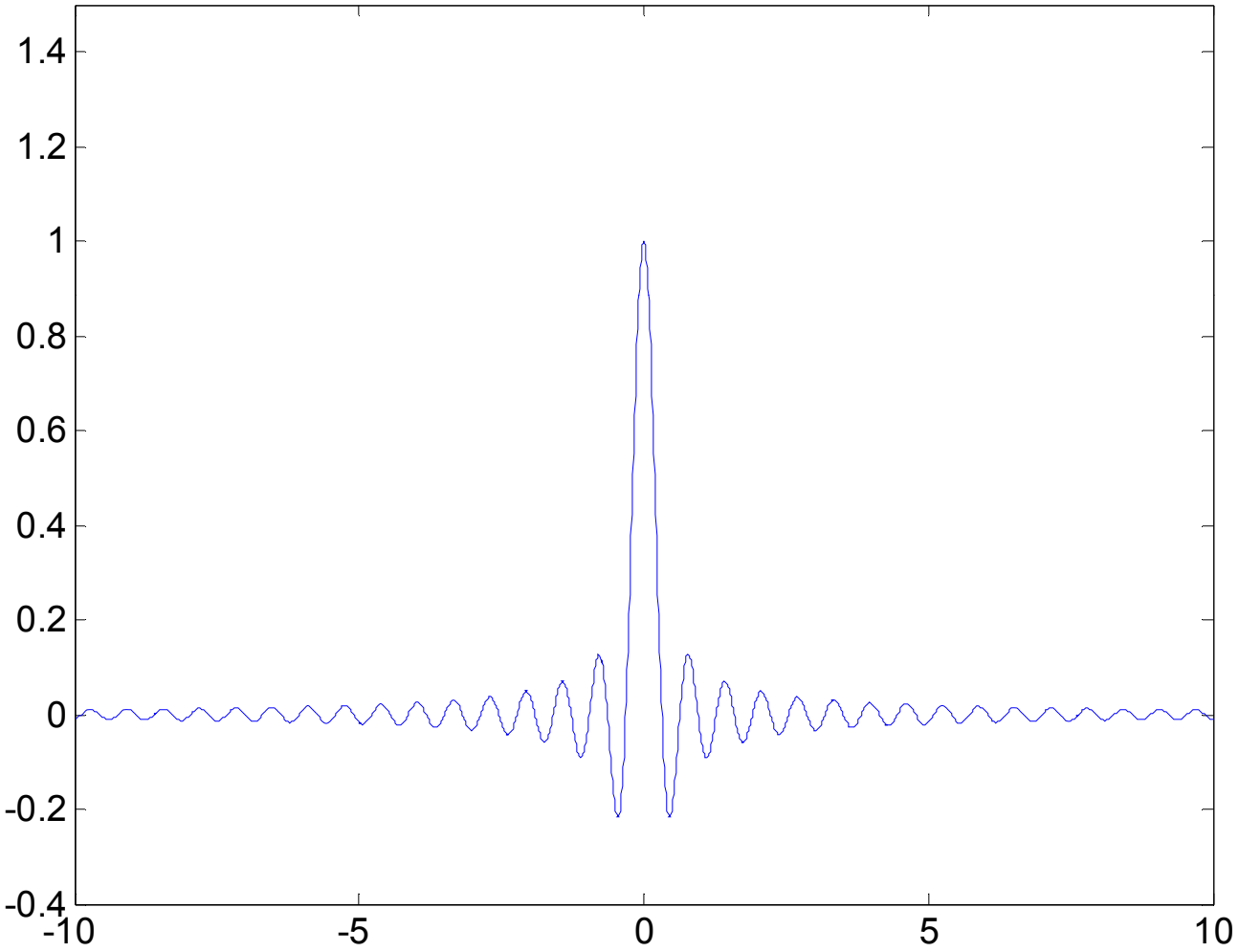


a b

FIGURE 4.13 (a) A 2-D function, and (b) a section of its spectrum (not to scale). The block is longer along the t-axis, so the spectrum is more "contracted" along the μ-axis. Compare with Fig. 4.4.

$$\left|F(\mu,\nu)\right| = A \cdot T \cdot Z \left|\frac{\sin(\pi\mu T)}{\pi\mu T}\right| \left|\frac{\sin(\pi\nu Z)}{\pi\nu Z}\right|$$

Basic Concept: Spatial Filtering (II)



- Review: Lecture 5
- Basic concept: spatial filtering
- Basic concept: image gradient calculation
- The Gaussian filter
- Overview of image feature detection
- Point feature detection

Basic concept: Gradient Estimation Kernel (I)

- Implementation

$$I_x(i,j) = \frac{I(i+1,j) - I(i-1,j)}{2}$$

$$I_y(i,j) = \frac{I(i,j+1) - I(i,j-1)}{2}$$

- Notation:

  J: raw image;

  I: filtered image after convolution with Gaussian kernel G.

- A basic property of convolution

$$\frac{\partial(G \otimes J)}{\partial x} = \frac{\partial I}{\partial x} = I_x = \frac{\partial G}{\partial x} \otimes J \qquad \frac{\partial(G \otimes J)}{\partial y} = \frac{\partial I}{\partial y} = I_y = \frac{\partial G}{\partial y} \otimes J$$

Basic concept: Image Gradient Estimation (II)

- First order derivative

$$I_x(i,j) = \frac{I(i+1,j) - I(i-1,j)}{2h} + O(h^2)$$

$$I_y(i,j) = \frac{I(i,j+1) - I(i,j-1)}{2h} + O(h^2)$$

- Second order derivative

$$I_{xx}(i,j) = \frac{I(i+1,j) - 2I(i,j) + I(i-1,j)}{h^2} + O(h)$$

$$I_{yy}(i,j) = \frac{I(i,j+1) - 2I(i,j) + I(i,j-1)}{h^2} + O(h)$$

The Gaussian Filter (III)

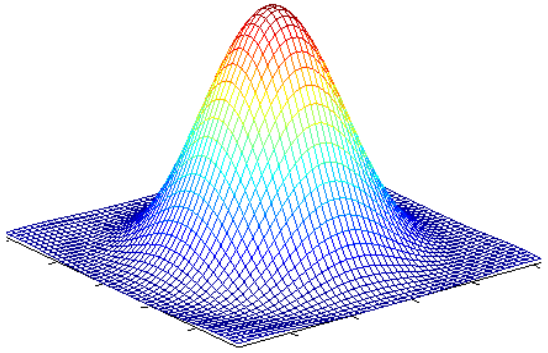
- Gaussian kernel in 1D & 2D

$$G(x;\sigma) = \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{x^2}{2\sigma^2}}$$

$$G(x,y;\sigma_x,\sigma_y) = \frac{1}{2\pi\sigma_x\sigma_y} e^{-\left(\frac{x^2}{2\sigma_x{}^2}+\frac{y^2}{2\sigma_y{}^2}\right)}$$

- First order derivative

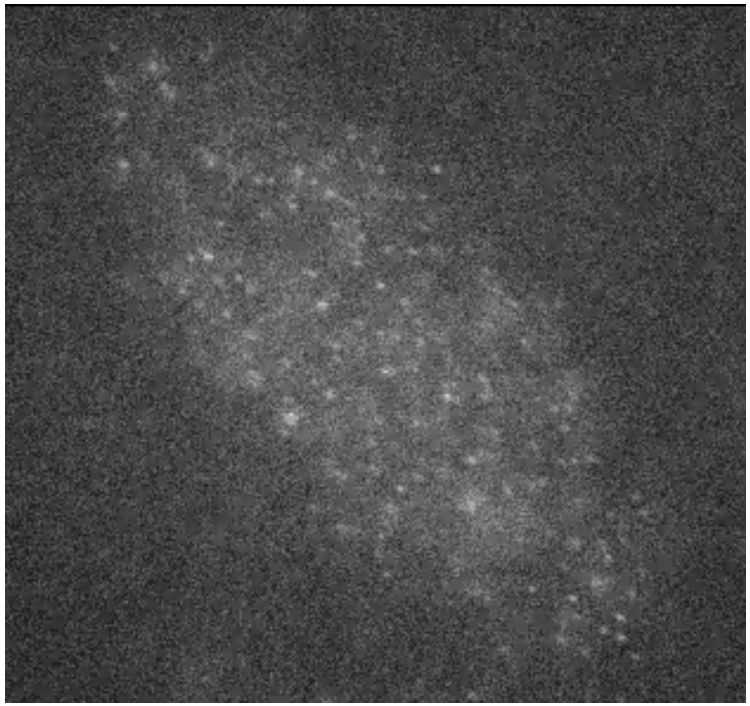
$$G'(x;\sigma) = \frac{-x}{\sqrt{2\pi}\sigma^3} e^{-\frac{x^2}{2\sigma^2}}$$



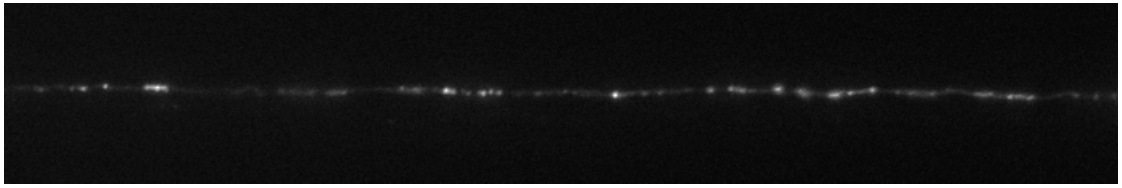
- Second order derivative

$$G''(x;\sigma) = \frac{-x}{\sqrt{2\pi}\sigma^3} e^{-\frac{x^2}{2\sigma^2}} \left[1-\frac{x^2}{\sigma^2}\right]$$

Feature Detection: Points/Particles

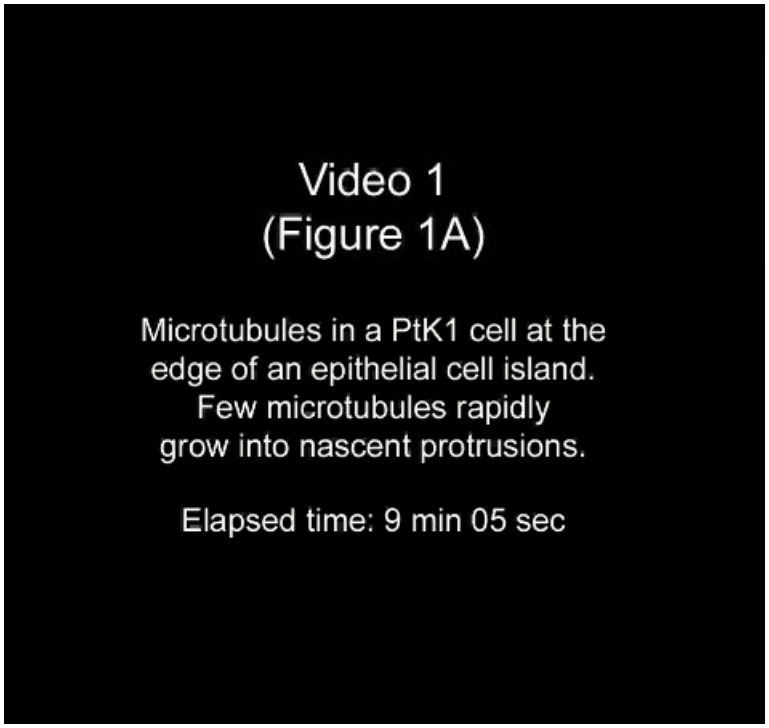


Fluorescent speckles in a Xenopus extract spindle



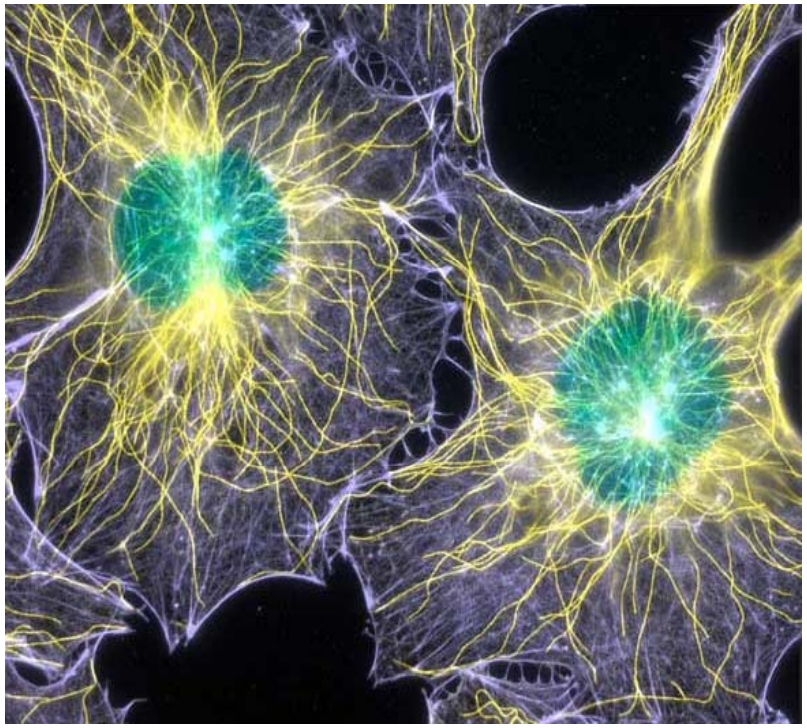
Vesicles transported in a Drosophila motor neuron

Feature Detection: Lines/Curves



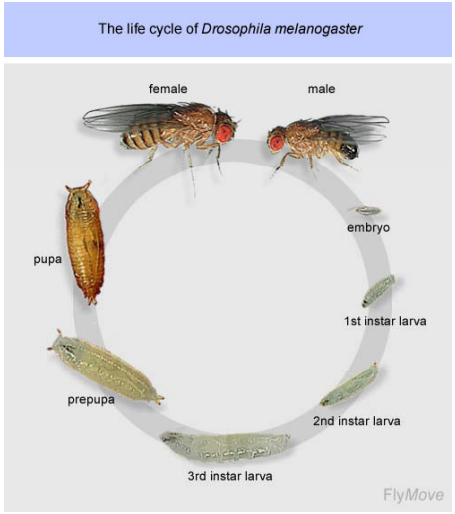
T. Wittmann et al, *J. Cell Biol.*, 161:845, 2003.

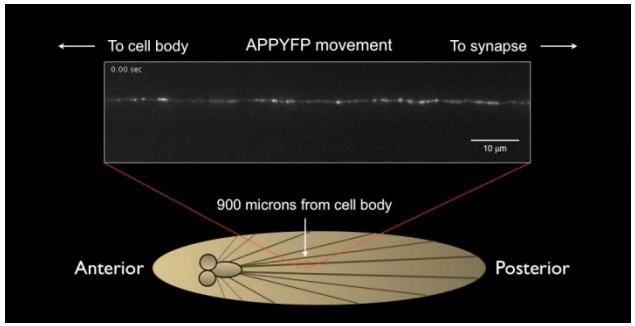
http://www.cell.com/cell_picture_show

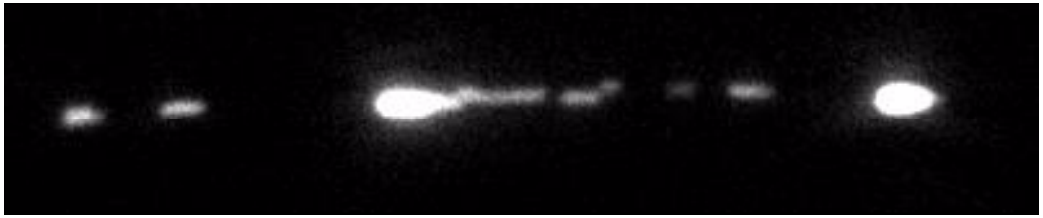


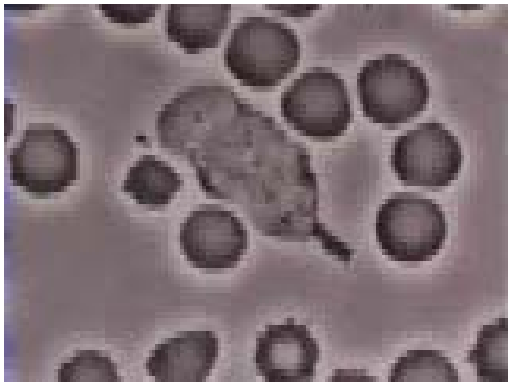
Nikon Small World, 2003
Torsten Wittmann, UCSF
Filamentous actin and microtubules (structural proteins) in mouse fibroblasts (cells) (1000x)

Feature Detection: Points vs Clusters / Regions









A neutrophil chasing a bacterium.
Devreotes Lab, Johns Hopkins U.

Image Gradient Calculation Under Gaussian Filtering

- A basic property of convolution

$$\frac{\partial (G \otimes J)}{\partial x} = \frac{\partial I}{\partial x} = I_x = \frac{\partial G}{\partial x} \otimes J \qquad \frac{\partial (G \otimes J)}{\partial y} = \frac{\partial I}{\partial y} = I_y = \frac{\partial G}{\partial y} \otimes J$$

- This is the basis of "Canny Edge Detection".

Algorithm **Canny Edge Detection**

1. *Smooth the image with a Gaussian filter.*
2. *Compute the gradient magnitude and orientation using finite-difference approximations for the partial derivatives.*
3. *Apply nonmaxima suppression to the gradient magnitude.*
4. *Use the double thresholding algorithm to detect and link edges.*