Principles of Image Processing (mostly for microscopy)

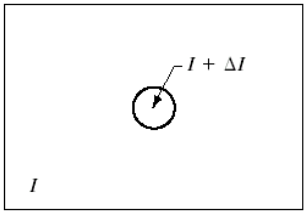
Costas Pitris, MD PhD
KIOS Research and Innovation Center of Excellence
Department of Electrical and Computer Engineering
University of Cyprus

Image Formation and Digitization

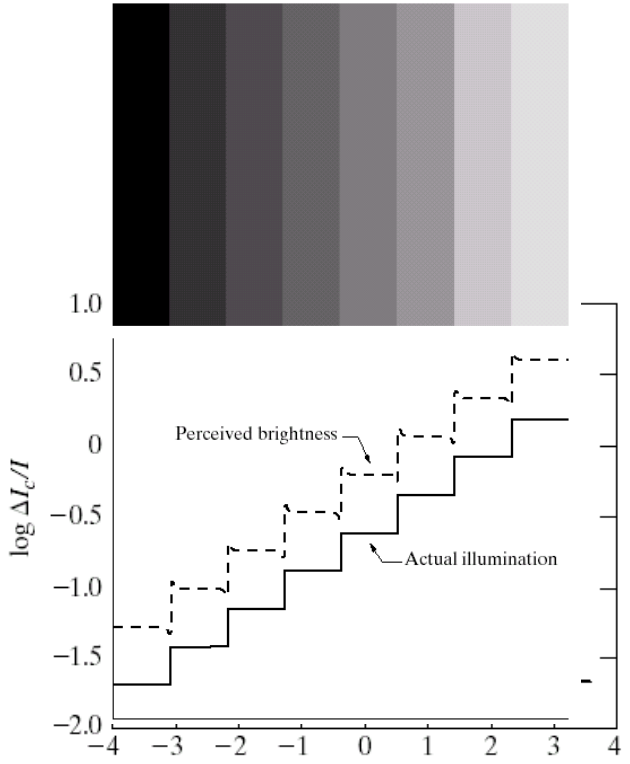
Real World → Optics → Sensor → Digital Image

Visual Perception

- **Perception ≠ Sensing**
- **Webber ratio**
  - ΔIc/I where 50% can not perceive change
  - Lower at lower illumination levels

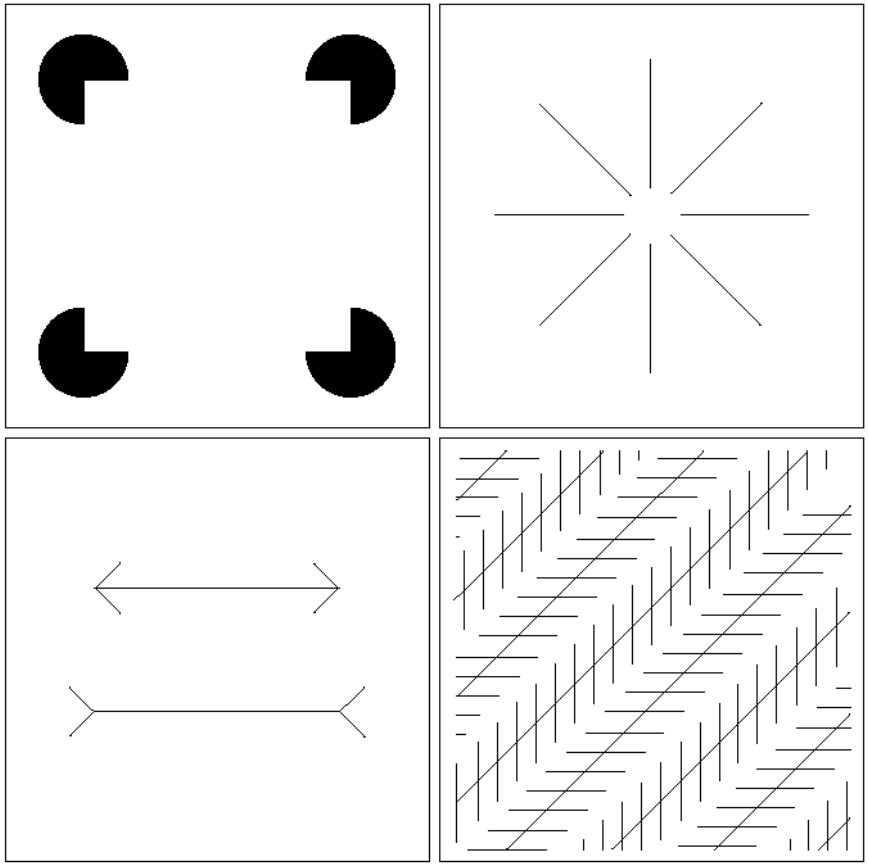


- **Perception of Brightness**
  - Not just a function of brightness
  - Depends on the environment
  - Has to be considered when designing projector systems



Visual Perception

- **Visual illusions**
  - The brain completes missing details
  - The brain can be fooled by additional details



Lenses

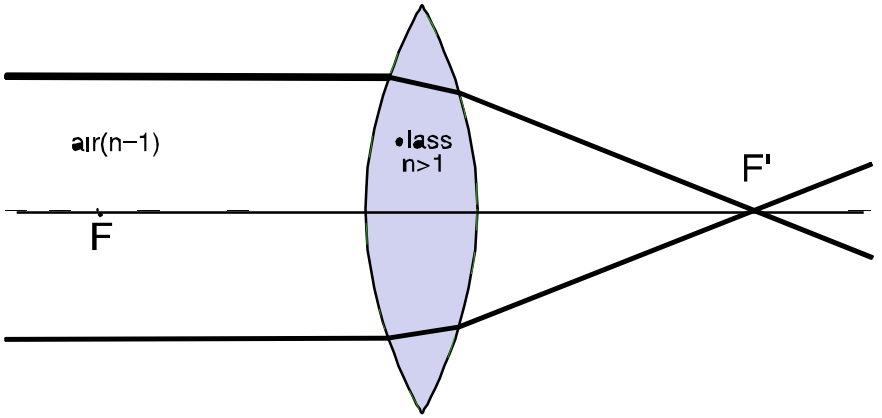
- **Imaging with thin lenses**
  - Ideal lens!
- **Real lenses**
  - Thick
  - Two curvatures
  - Lensmaker's equation

$$\frac{1}{s_o}+\frac{1}{s_i}=(n_2-1)\left(\frac{1}{R_1}-\frac{1}{R_2}\right)$$

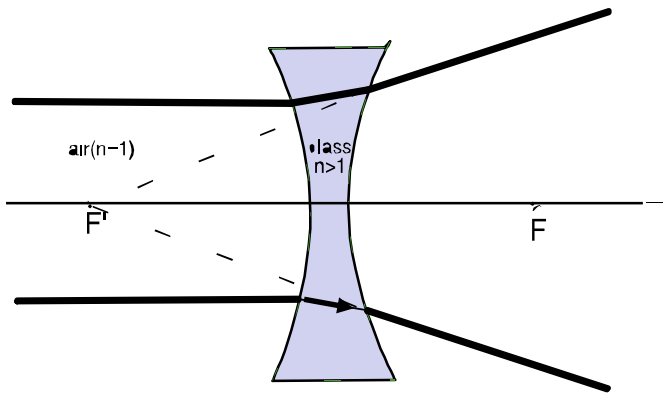
  - Focal length

$$\frac{1}{f}=(n_2-1)\left(\frac{1}{R_1}-\frac{1}{R_2}\right)$$

Converging (focusing) Lens



Diverging (defocusing) Lens



(The focal length is negative)

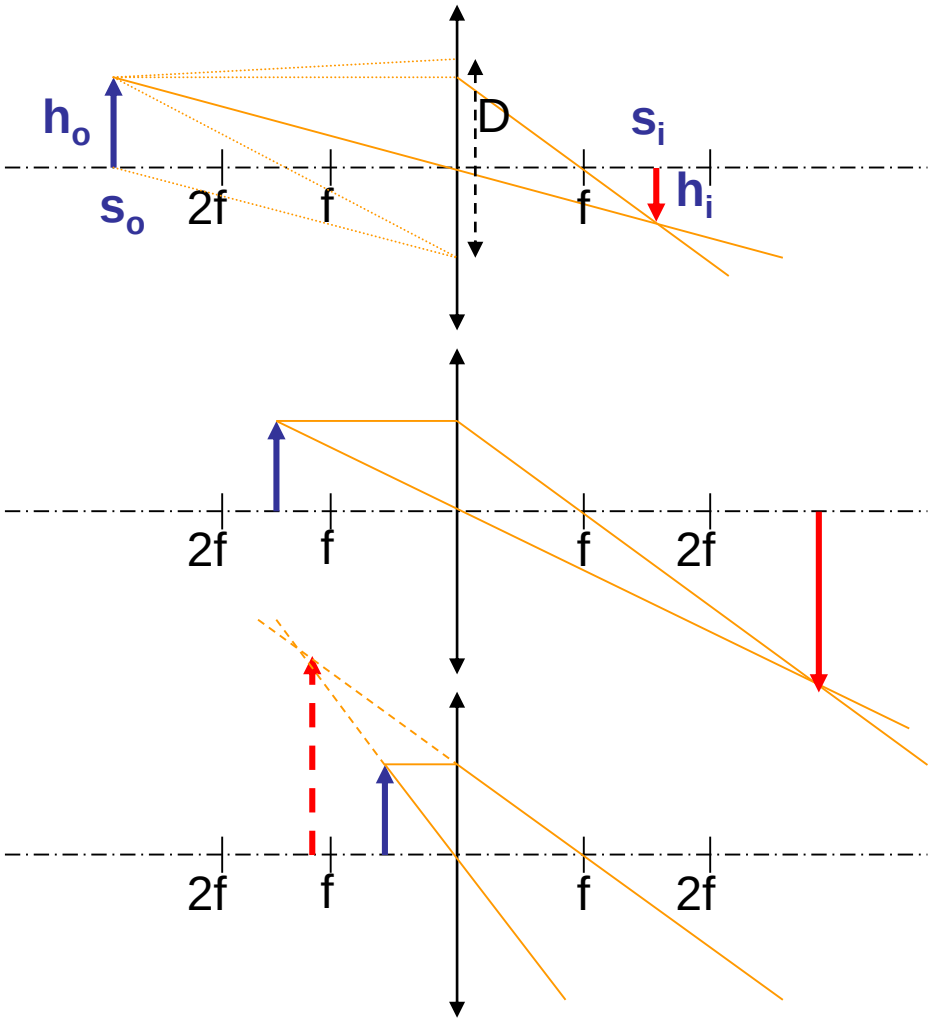
Lenses for Imaging

- **Paraxial Approximation**
  - Geometrical Optics → Ray Tracing
- **Image formation by a lens**
- **Ideal thin lens**

$$\frac{1}{f} = \frac{1}{s_o} + \frac{1}{s_i}$$

  - f = focal length
  - s_i = image distance
  - s_o = object distance
- **Magnification**

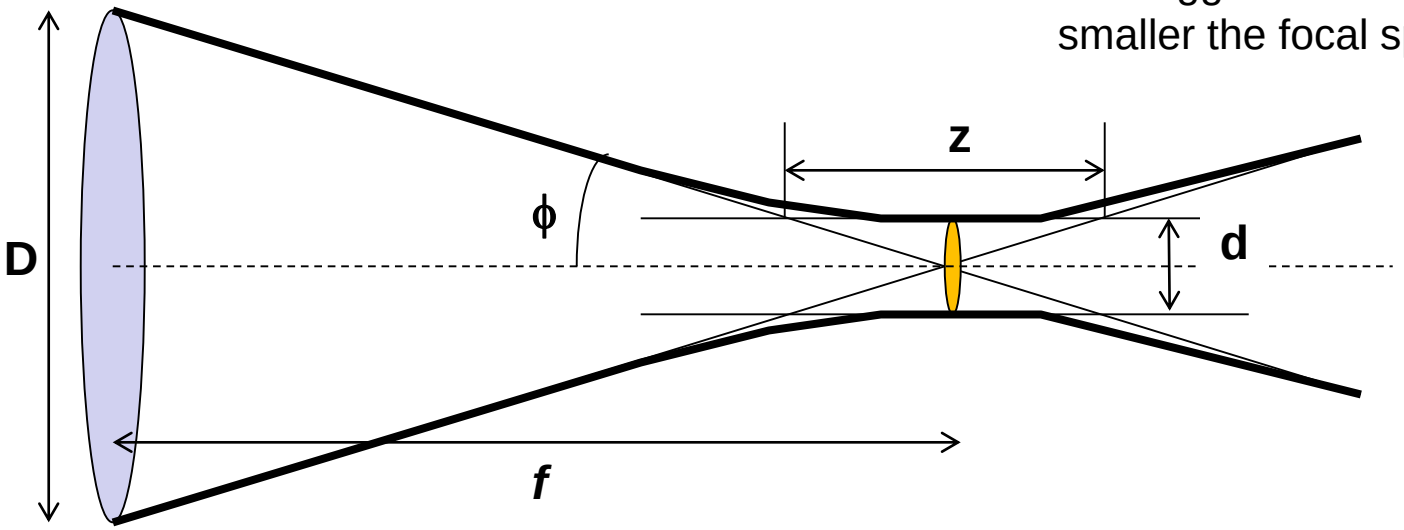
$$M = -\frac{s_i}{s_o} = -\frac{h_i}{h_o}$$

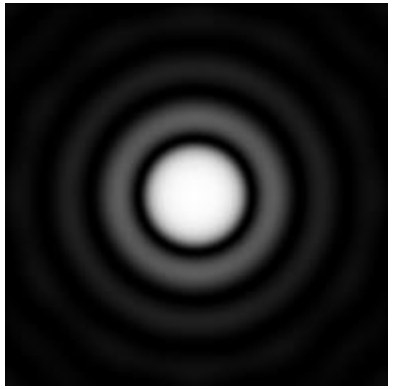


Lenses and Wave Optics

	Lens with Uniform Beam	Lens with Gaussian Beam
Size of the diaphragm	D	2w
Size of the focal point	$d = \frac{2.44 f \lambda}{D}$	$d = 2w_0 = \frac{2.54 f \lambda}{2w}$
Depth of focus	$z = 1.22\lambda \left(\frac{2f}{D} \right)^2$	$z = 1.27\lambda \left(\frac{2f}{2w} \right)^2$

The bigger the diameter (D) the smaller the focal spot (d)





Lens Characteristics

- **Lens characteristics**
  - Numerical Aperture (NA)
  - F Number (f/#)
- **Gromit with f/22 (left) and f/4 (right).**

$$NA \approx \frac{D}{2f} \qquad f/\# \approx \frac{f}{D}$$

Small diameter, D
Long depth of focus

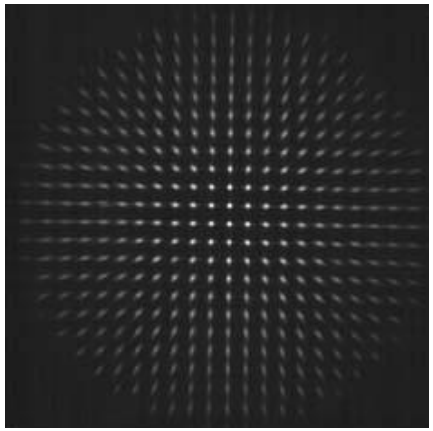
DOF ↑, Resolution ↓

Large diameter, D
Short depth of focus

DOF ↓, Resolution ↑

Optical Aberrations

- **Aberrations**
  - Chromatic
  - Spherical
  - Curvature of Field
  - Astigmatism
  - Coma
  - Distortion
  - Vignetting

- Reduced with better (and more complicated / expensive) optical system

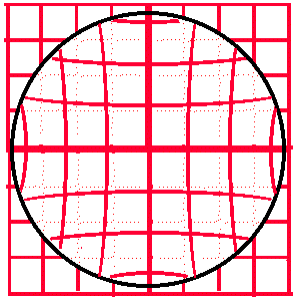
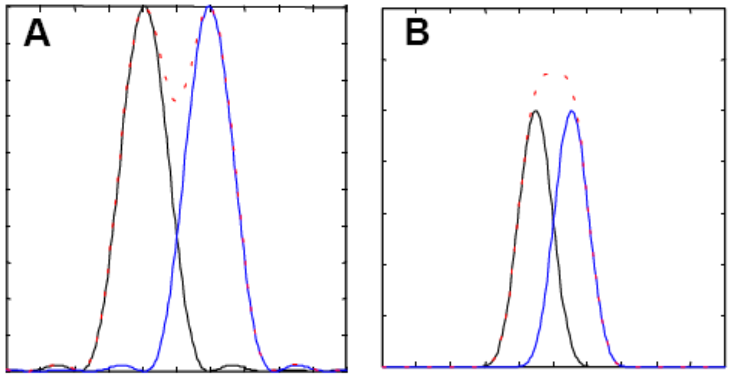
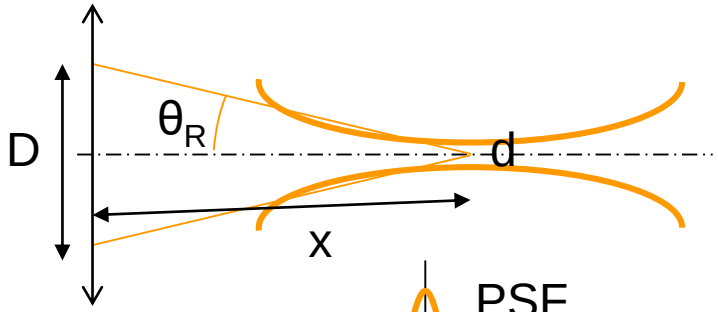




Image formation

- **"Resolution" of an image**
  - The smallest distinguishable detail = optical resolution
  - It is also equivalent to the highest spatial frequency
- **Optical Resolution**
  - Rayleigh criterion
    - 3 dB fall between peacks
  - Sparrow criterion
    - Diameter of Point Spread Function (PSF)
  - Aberrations further degrade the resolution



$$d_{3dB} = \frac{1.02\lambda}{NA} \qquad d = \frac{1.22\lambda}{NA}$$



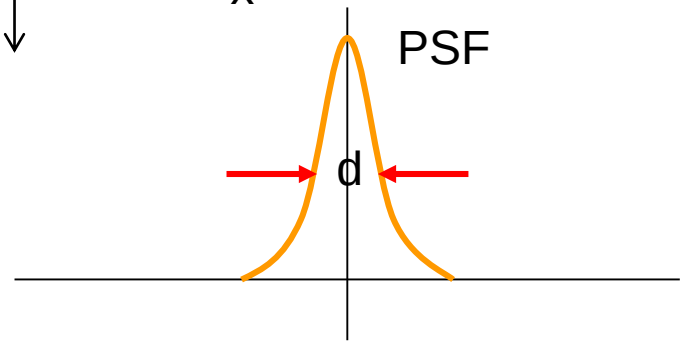


Image Digitization

- **Two stages in the digitization process:**
  - **Spatial sampling**: Create the pixels
    - When a continuous scene is imaged on the sensor, the continuous image is divided into discrete elements - picture elements (pixels)
  - **Quantization**: Create the gray levels or colors
    - Choose number of gray levels (according to number of image bits)
    - Divide continuous range of intensity values

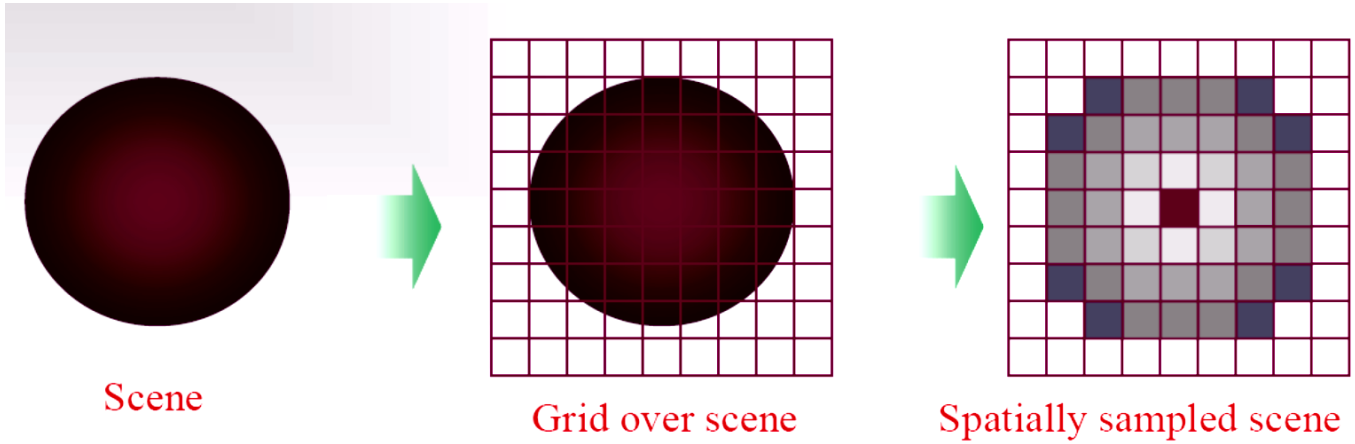


Image Digitization

- **Spatial sampling**
  - Camera Resolution
    - Number of pixels (e.g. 1024x1024)
    - Must be enough to correctly digitize the optical resolution
      - Otherwise the image resolution will be degraded because of the camera
    - Κριτήριο Nyquist
      - $d_{camera} = d_{optical} / 2$
      - N_{pixel} = Field of View / d_{camera}
  - Under sampling issues

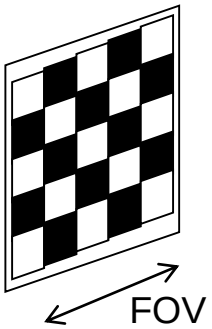
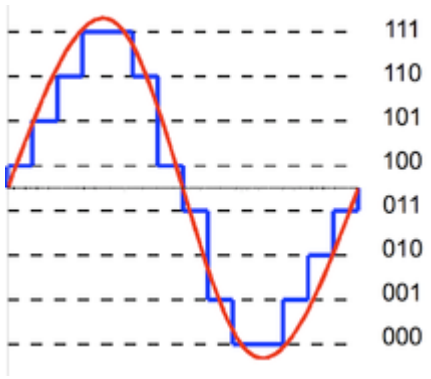


Image Digitization

- **Quantization**
  - Enough levels so as not to loose gray/color detail
  - Match the gray levels to the entire bit range
    - Change the “gain” and “sensitivity”
  - Low frequency (uniform) areas are more sensitive to quantization
  - Large dynamic range requires more bits



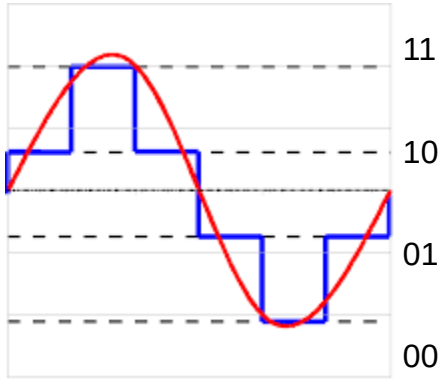


Image Digitization

- **Quantization**

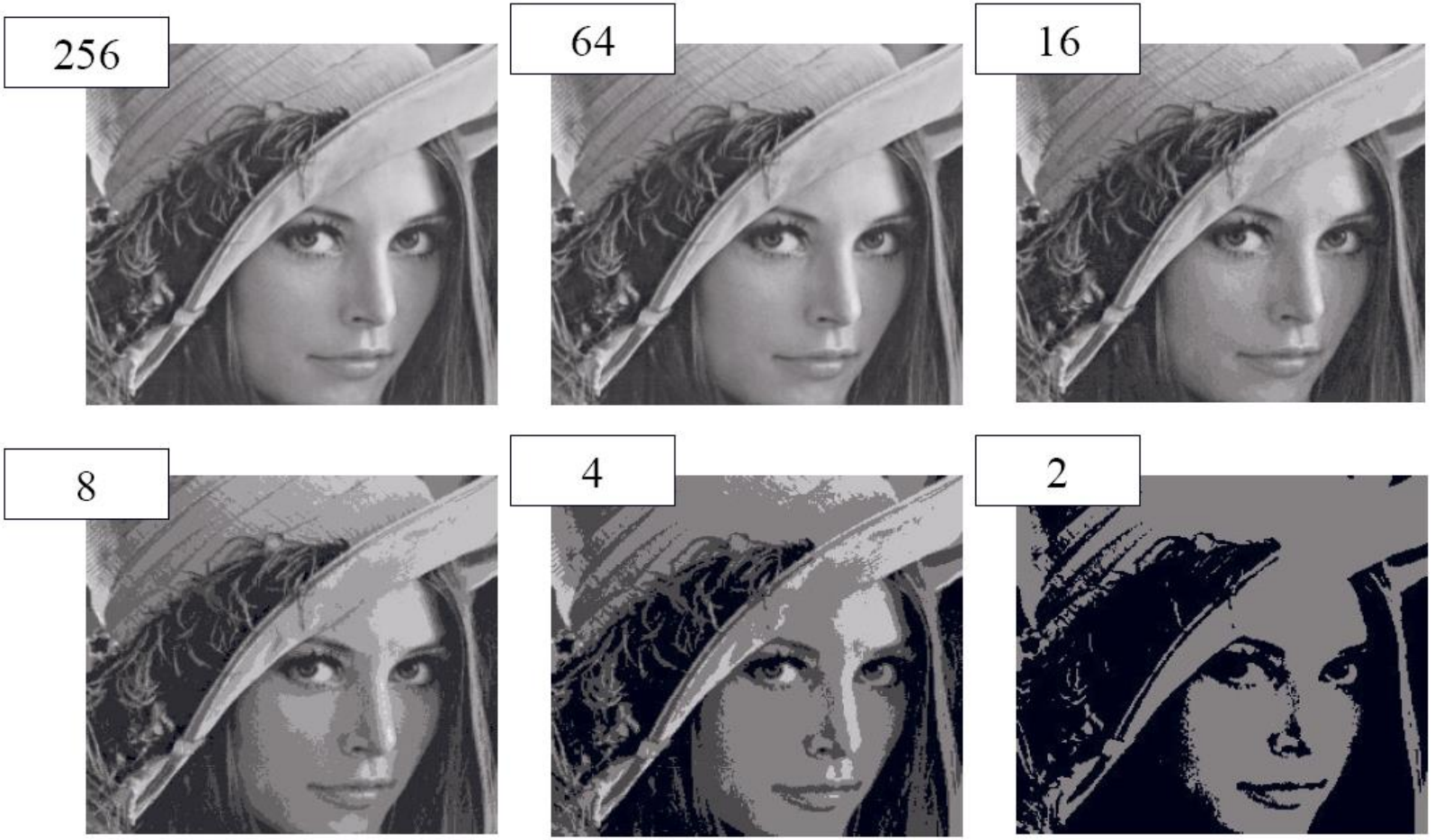
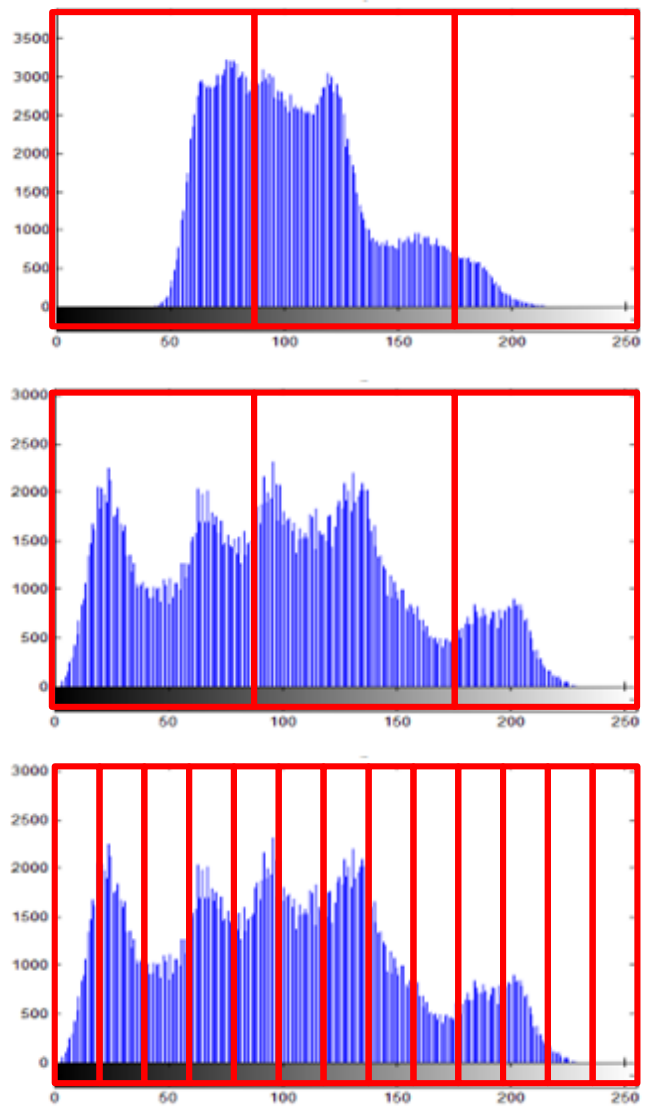


Image Digitization

- **Quantization**
  - Match the histogram to get the most of the bits!
    - Watch out for noise when adjusting gain/sensitivity!
  - Use enough bits for your range and details!



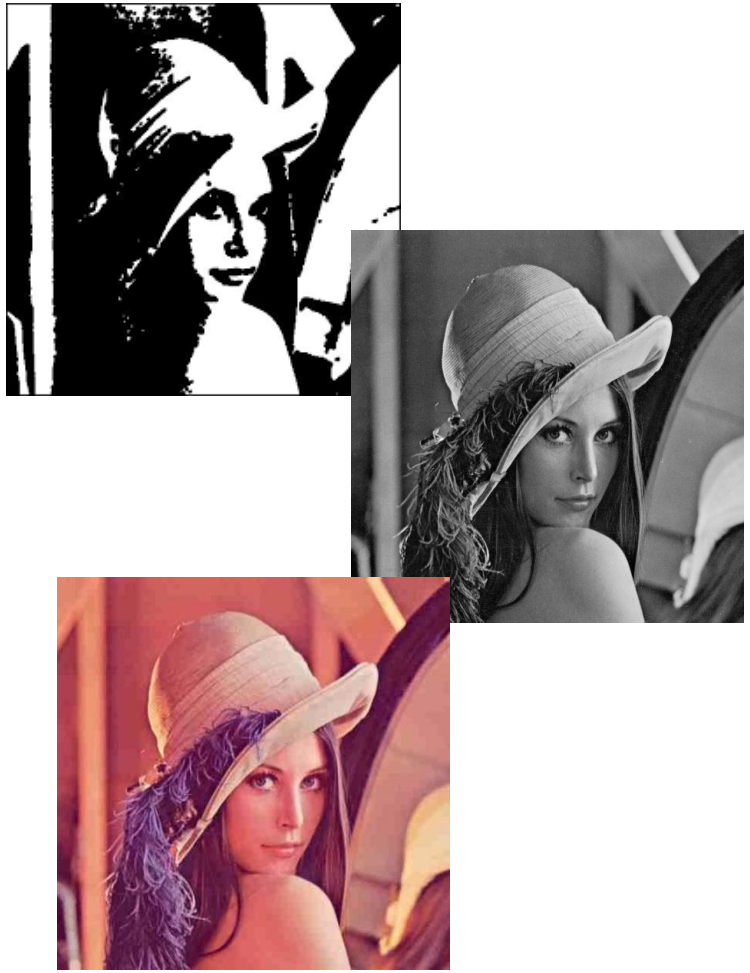
Noise in Images

- **An important limitation in low intensity level imaging**
  - Not background, autofluorescence, etc
  - Good image has SNR > 4
  - If SNR is low, uses a more sensitive or cooled camera
  - Increasing the gain and/or sensitivity can only go so far
    - Noise increases as well after a point
- **Signal-To-Noise-Ratio**

$$SNR = \frac{Signal}{Variation} = \frac{Mean}{Std}$$

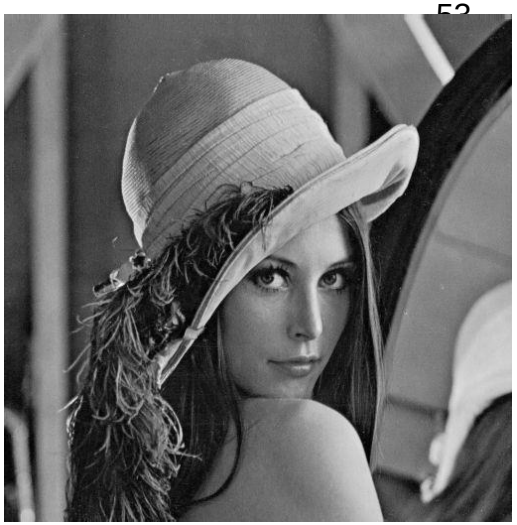
Image Types

- **Three types of images:**
  - Binary images
    - g(x,y) $\in$ {0 , 1}
  - Gray-scale images
    - g(x,y) $\in$ typically {0,…,255}
  - Color Images
    - Three channels:
    - R(x,y) G(x,y) B(x,y)
    - Each typically {0,…,255}



Gray Scale Image

x =	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72
y =															
41	210	209	204	202	197	247	143	71	64	80	84	54	54	57	58
42	206	196	203	197	195	210	207	56	63	58	53	53	61	62	51
43	201	207	192	201	198	213	156	69	65	57	55	52	53	60	50
44	216	206	211	193	202	207	208	57	69	60	55	77	49	62	61
45	221	206	211	194	196	197	220	56	63	60	55	46	97	58	106
46	209	214	224	199	194	193	204	173	64	60	59	51	62	56	48
47	204	212	213	208	191	190	191	214	60	62	66	76	51	49	55
48	214	215	215	207	208	180	172	188	69	72	55	49	56	52	56
49	209	205	214	205	204	196	187	196	86	62	66	87	57	60	48
50	208	209	205	203	202	186	174	185	149	71	63	55	55	45	56
51	207	210	211	199	217	194	183	177	209	90	62	64	52	93	52
52	208	205	209	209	197	194	183	187	187	239	58	68	61	51	56
53	204	206	203	209	195	203	188	185	183	221	75	61	58	60	60
	200	203	199	236	188	197	183	190	183	196	122	63	58	64	66
	205	210	202	203	199	197	196	181	173	186	105	62	57	64	63



Color Image

Color Quantization

2 colors

8 colors

256 colors

4 colors

16 colors

Original
($2^{8x3} = 2^{24}$ colors)

Image Filtering

- **Median Filtering**
  - Replace the pixel value with the median of a neighborhood around the pixel
  - Removes noise but also smooths the image
    - Matlab: medfilt2
  - In quantitative microscopy except for thresholding purposes (see next)

Image Filtering

- **Frequency domain filtering**
  - Low pass filtering
    - Let the low f through and remove the high f
    - Equivalent to removing the details → blurring
    - E.g. Gaussian blur
  - High pass filtering
    - Let the high f through and remove the low f
    - Equivalent to removing the uniform areas and enhancing the sharp areas
    - E.g. Prewitt or Sobel edge detection
  - Matlab: imfilter

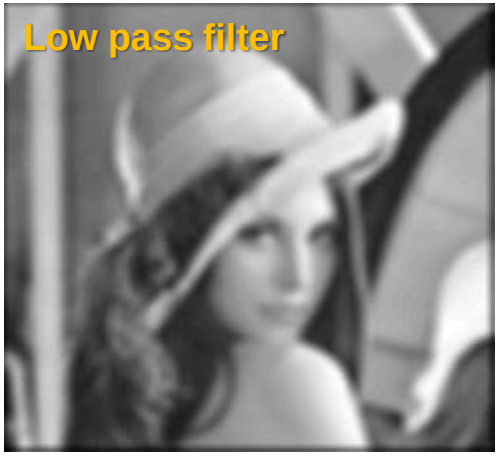


Image Filtering

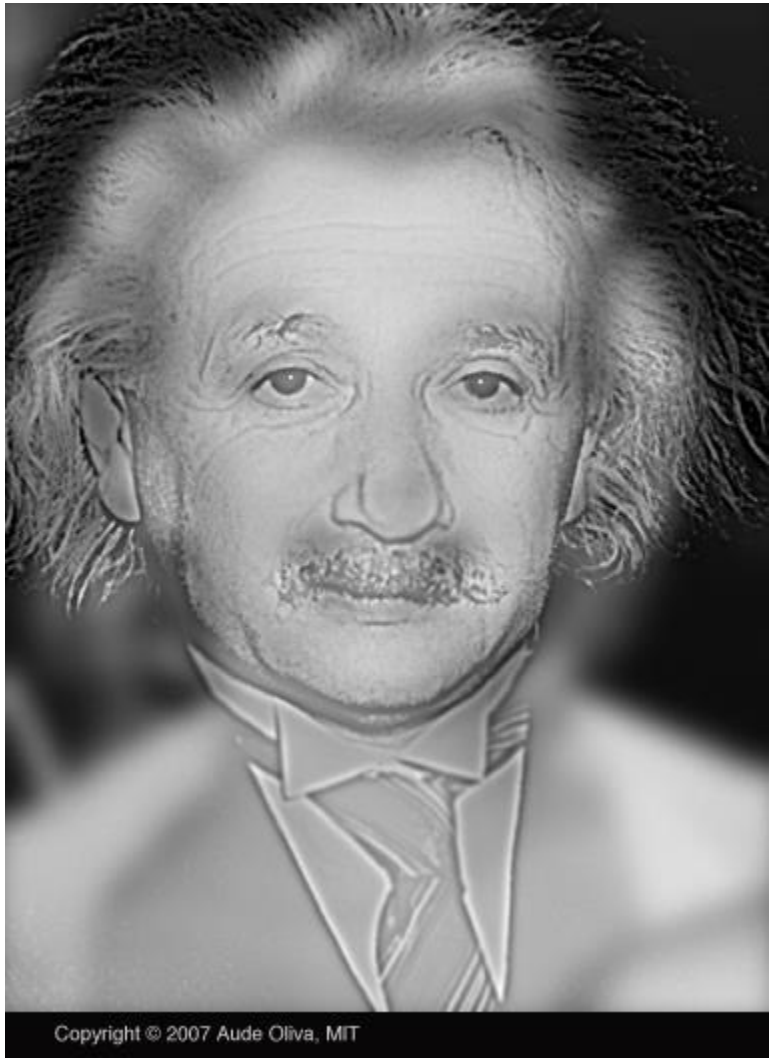
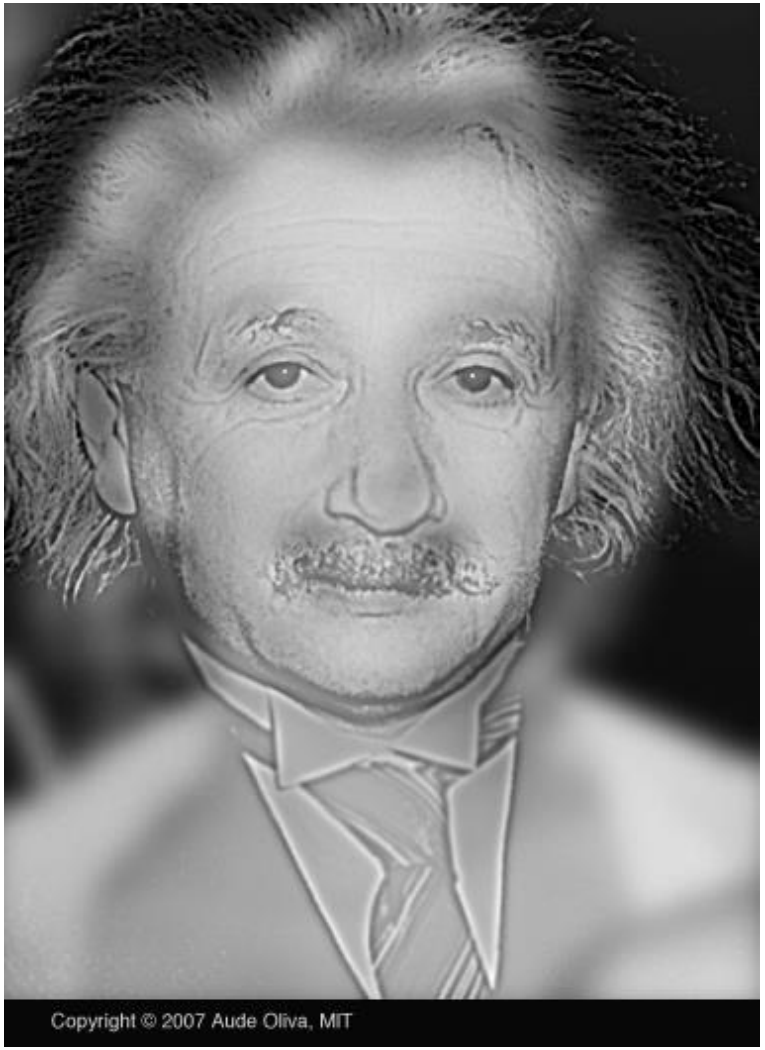


Image Filtering



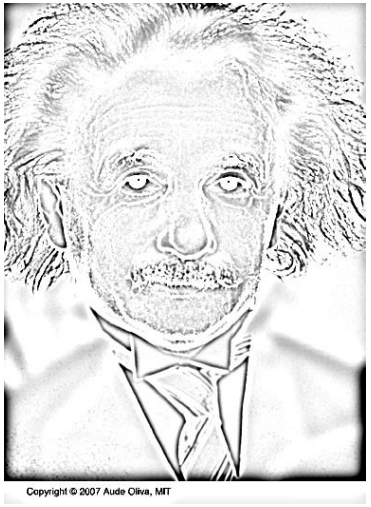
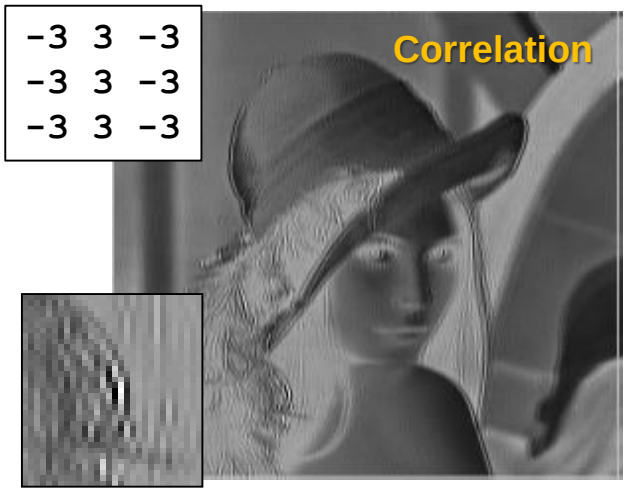


Image Filtering

- **Filtering with convolution and correlation**
  - Correlation is equivalent to enhancing a certain pattern in the image
    - Matlab: xcorr2
  - Convolution is equivalent to blurring each pixel by a certain pattern
    - matlab: xconv2
- **Deconvolution**
  - Deconvolution can be performed to reverse the effect of convolution
  - The results are not perfect
    - Matlab: deconvblind



Quantitative Fluorescence Microscopy

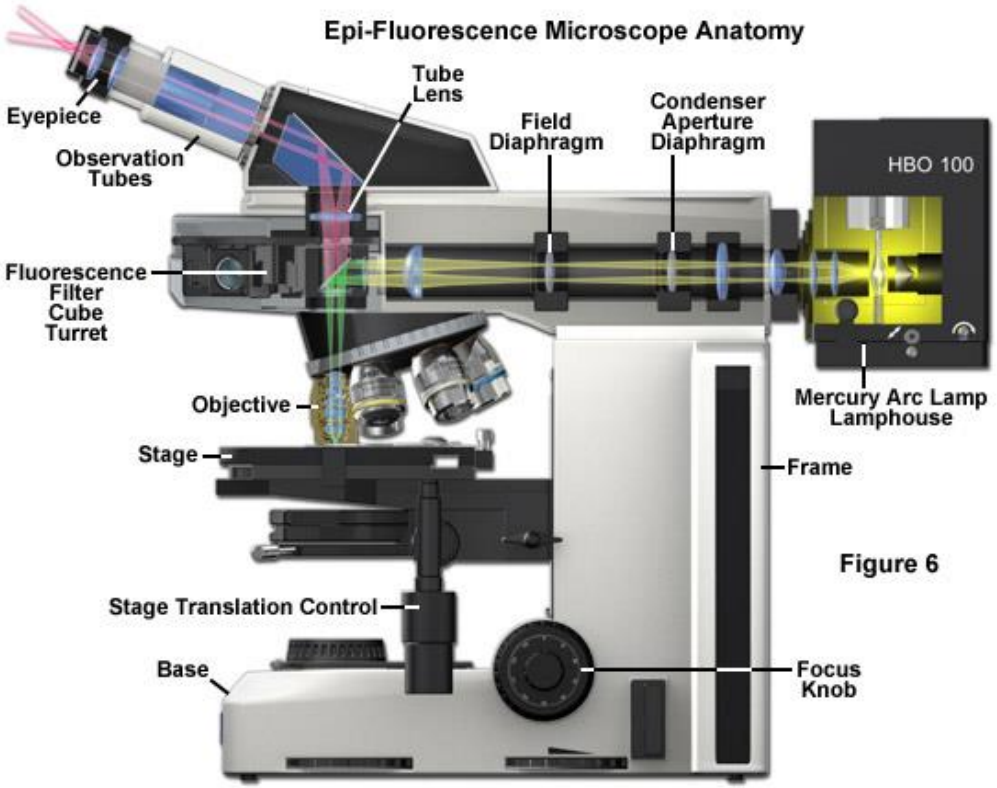


Figure 6

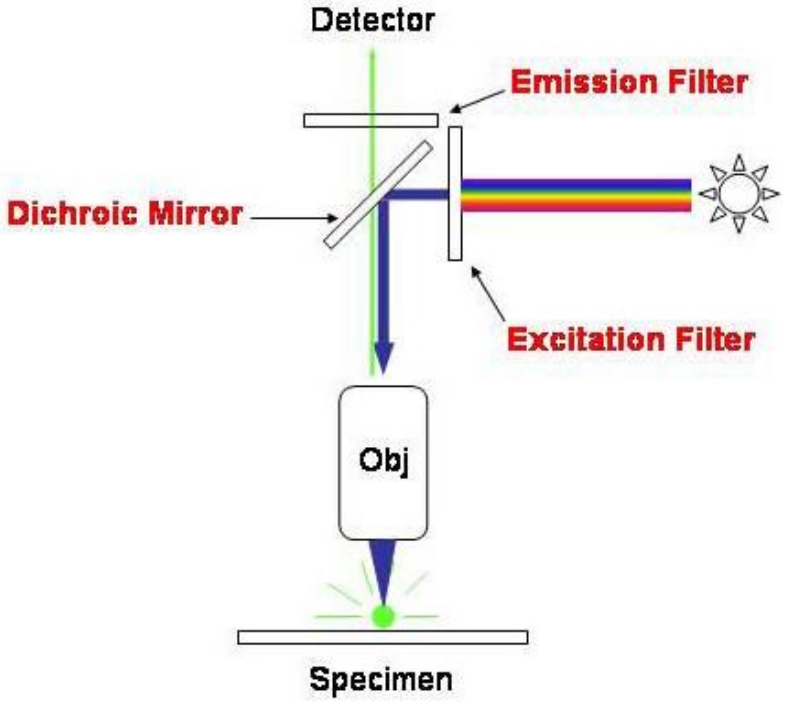
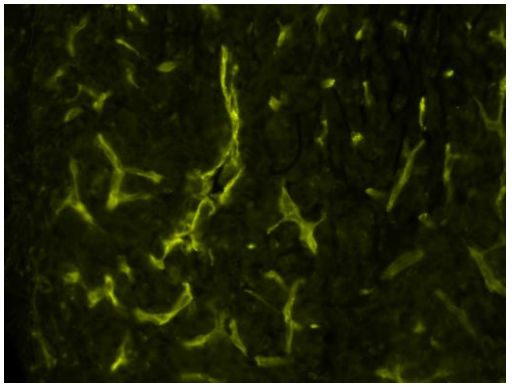
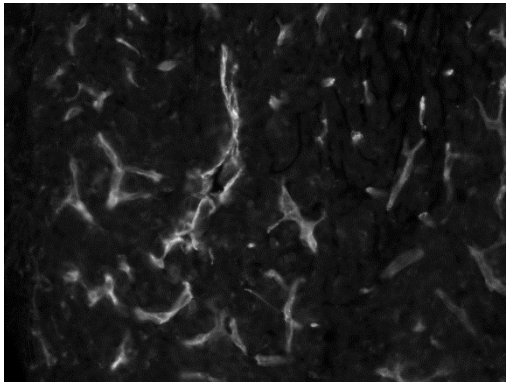


Image Type Conversion: Color→Gray

- **Color Image**
  - In fluorescence microscopy, usually contains light through one filter
  - No need to be color
  - Better sensitivity and higher SNR when taken with a grayscale camera or in grayscale mode
- **Gray Scale Image**
  - Gray = (R+G+B)/3
  - Matlab: rgb2gray





Histogram

- **Histogram**
  - Distribution of intensity values
  - Matlab: imhist
- **Normalization**
  - Make the minimum value 0 and the maximum value 1
- **Histogram equalization**
  - Spreads the values to enhance contrast
  - Low intensity features become more visible
  - Similar results by taking log of the image
  - Matlab: histeq
- **Notes**
  - Useful when trying to create algorithms that work on images with dissimilar intensities
  - Not for making quantitative measurements

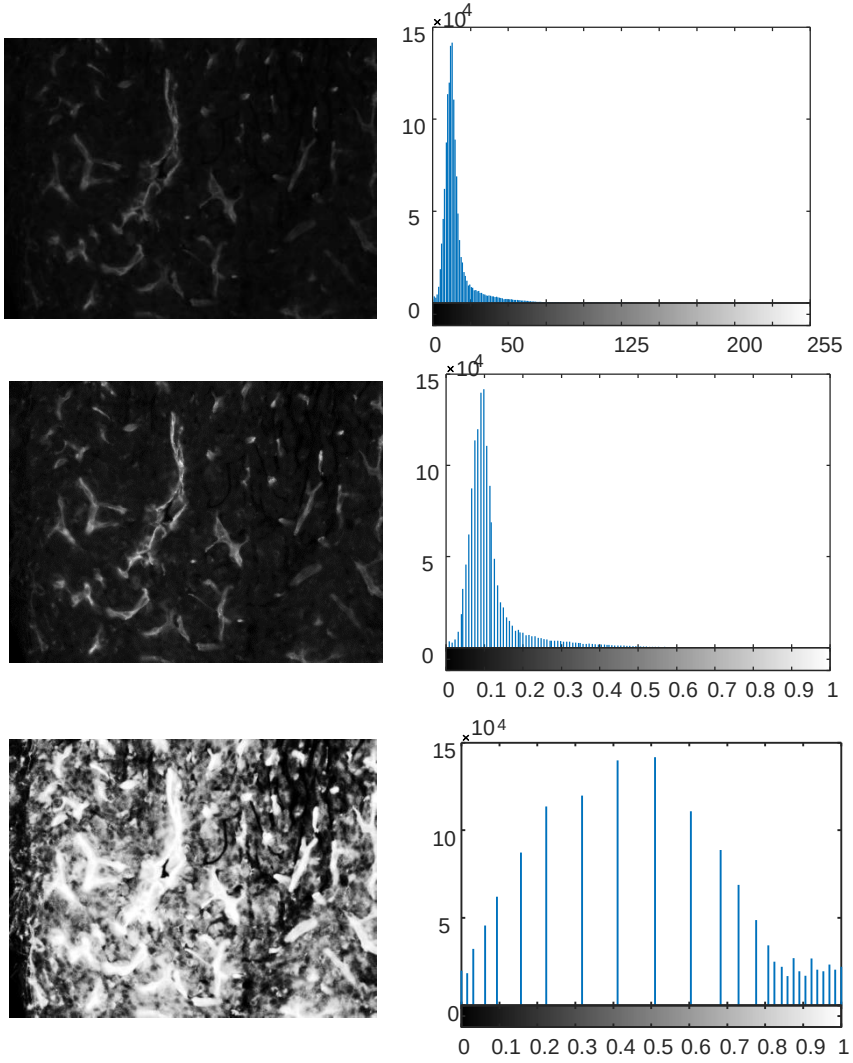
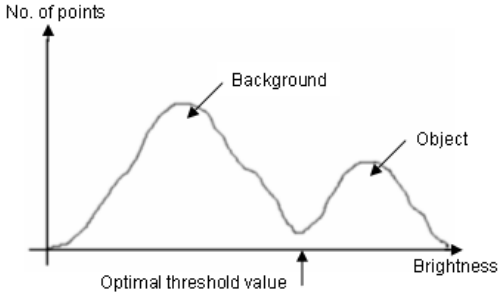
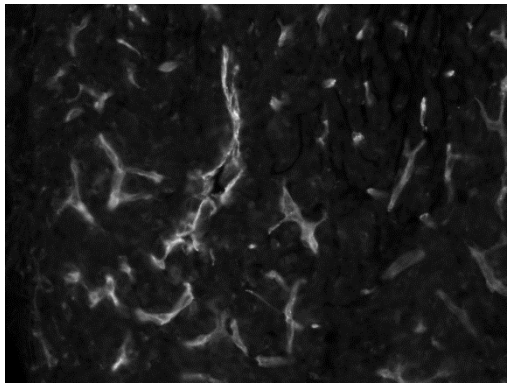


Image Type Conversion: Gray→BW

- **Thresholding**
  - Convert a grayscale image to binary
  - Usually to identify and segment particular areas of interest
    - “Mask”
  - Everything above a threshold set to 1 and everything below set to 0
    - Matlab: im2bw
  - Threshold can be set manually or automatically (Otsu’s method)
    - Matlab: graythresh





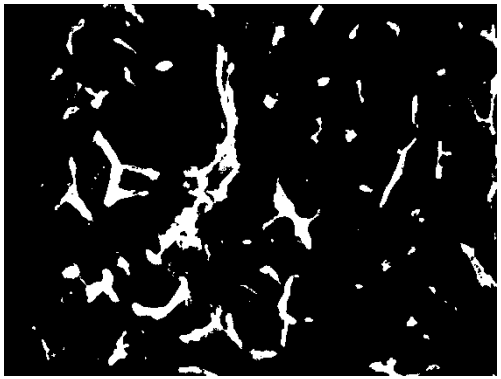
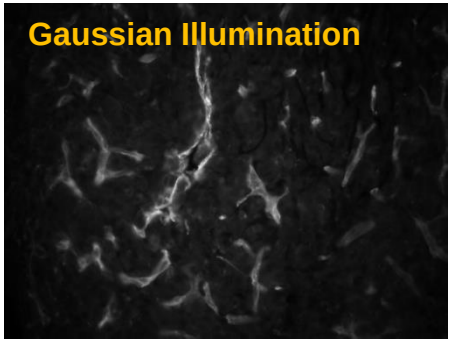
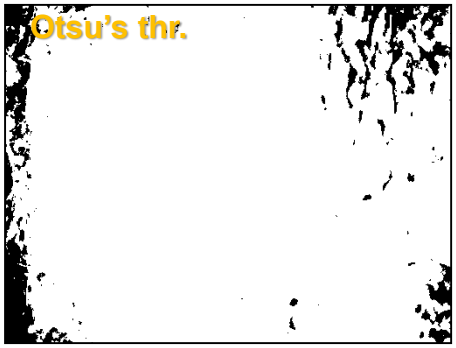
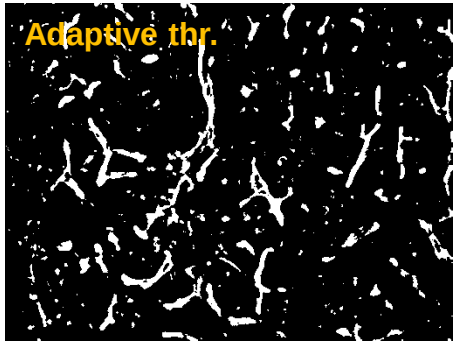


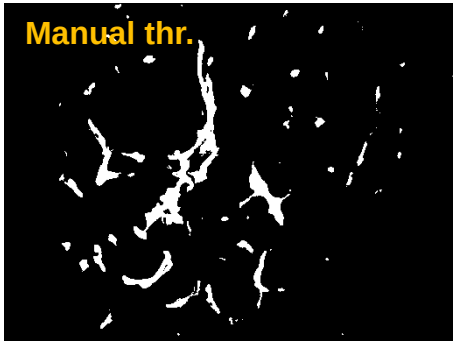
Image Type Conversion: Gray→BW

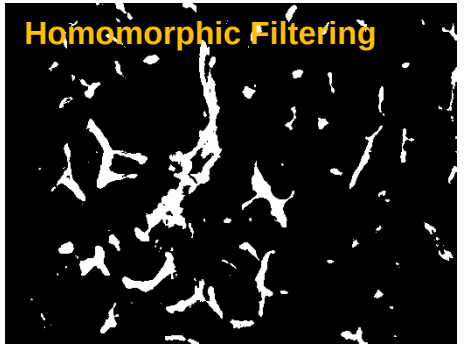
- **Thresholding**
  - Uneven illumination can be a problem
  - Solutions:
  - Adaptive thresholding
    - Matlab: imbinarize(I, 'adaptive');
  - Homomorphic filtering
    - Normalizes the brightness across an image and increases contrast
    - The high-frequency components are increased and low-frequency components are decreased
    - Closest compared to the previous slide
- **Remember**
  - Even the best image processing can not correct for everything!
  - Take better images!
  - You have to make compromises for the algorithm to work on ALL images
  - Be consistent for quantitative results





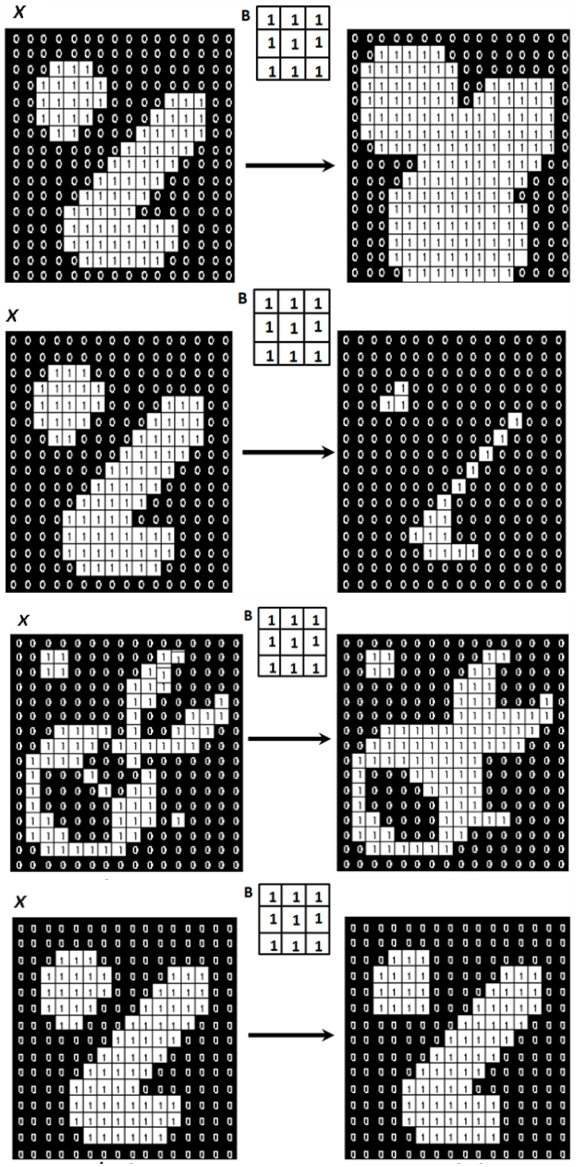






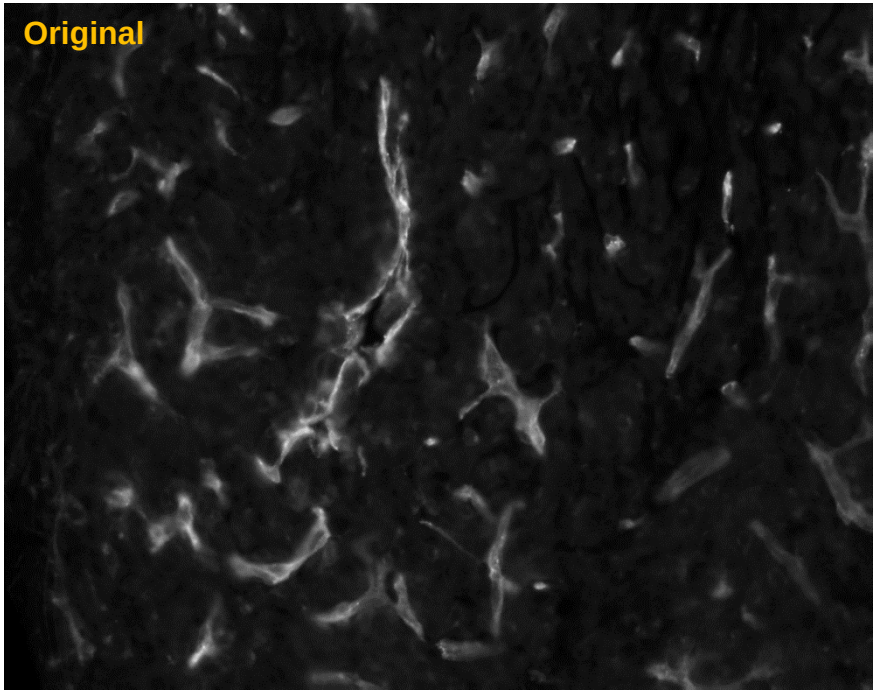
Morphological Operations

- **Dilate**
  - Add pixels around the periphery
  - “Convolution” with the structuring element
  - Makes shapes thicker
  - Matlab: imdilate
- **Erode**
  - The opposite of dilate
  - Makes shapes thinner
  - Matlab: imerode
- **Close**
  - Dilate followed by erode
  - Closes gaps smaller than the structuring element and connects adjacent objects
  - Matlab: imclose
- **Open**
  - Erode followed by dilate
  - Disconnects adjacent objects and removes objects smaller than the structuring element



Morphological Operations

- **Example - Vessels**
  - Threshold
    - Matlab: im2bw
    - Threshold=0.9 x graythresh
  - Close
    - Matlab: imclose
    - Structuring Element: Disk, 9 pixels
  - Open
    - Matlab: imopen
    - Structuring Element: Disk, 5 pixels



Morphological Operations

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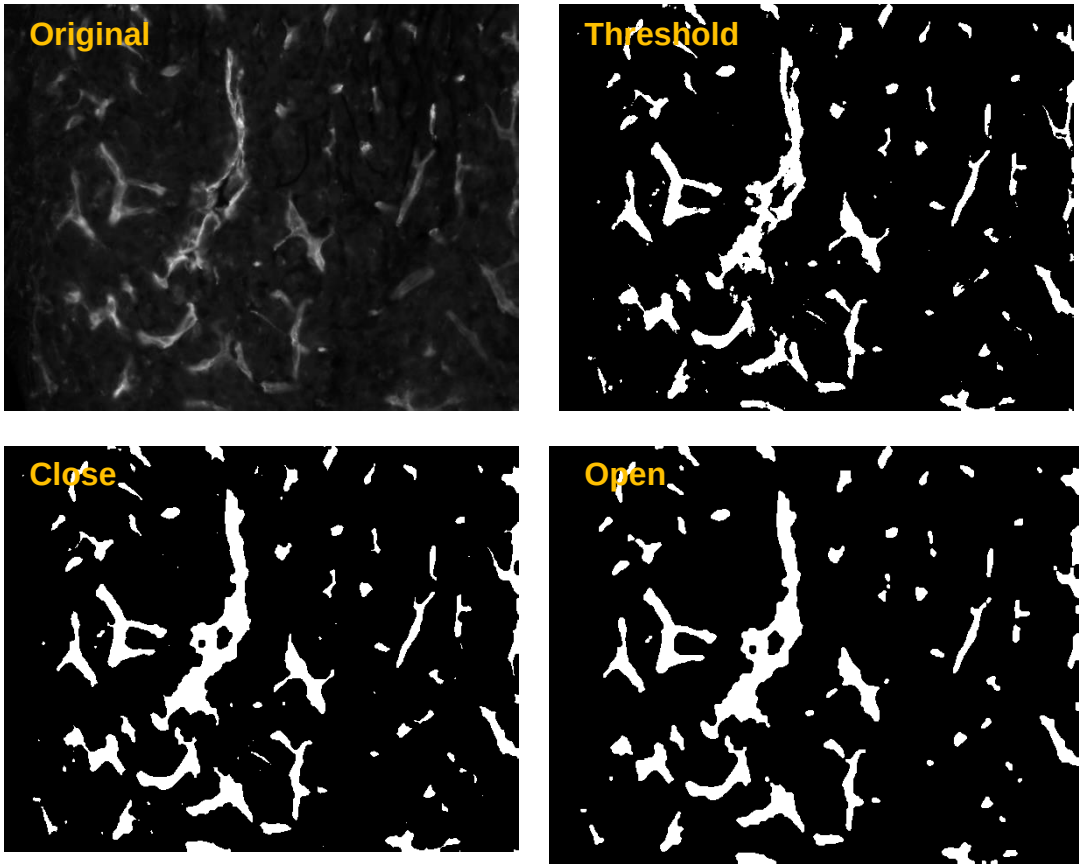
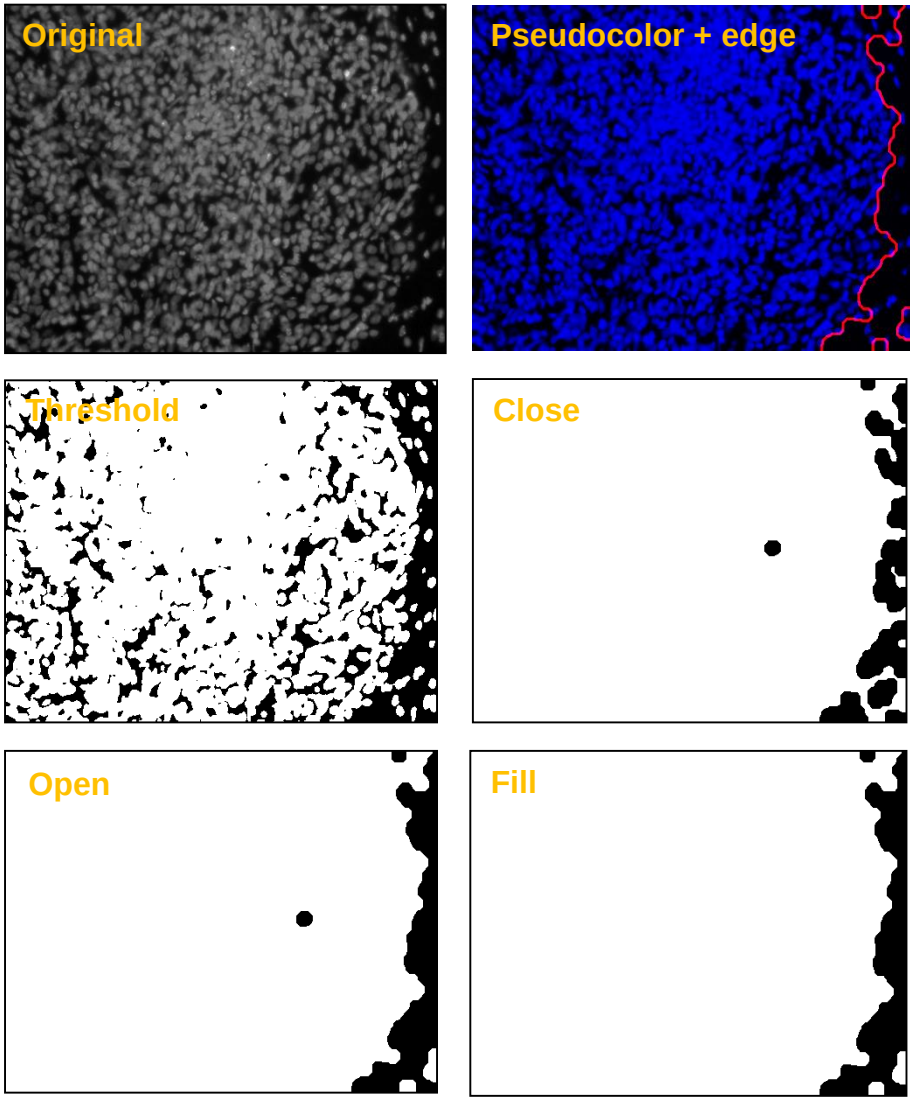
    - Structuring Element: Disk, 5 pixels



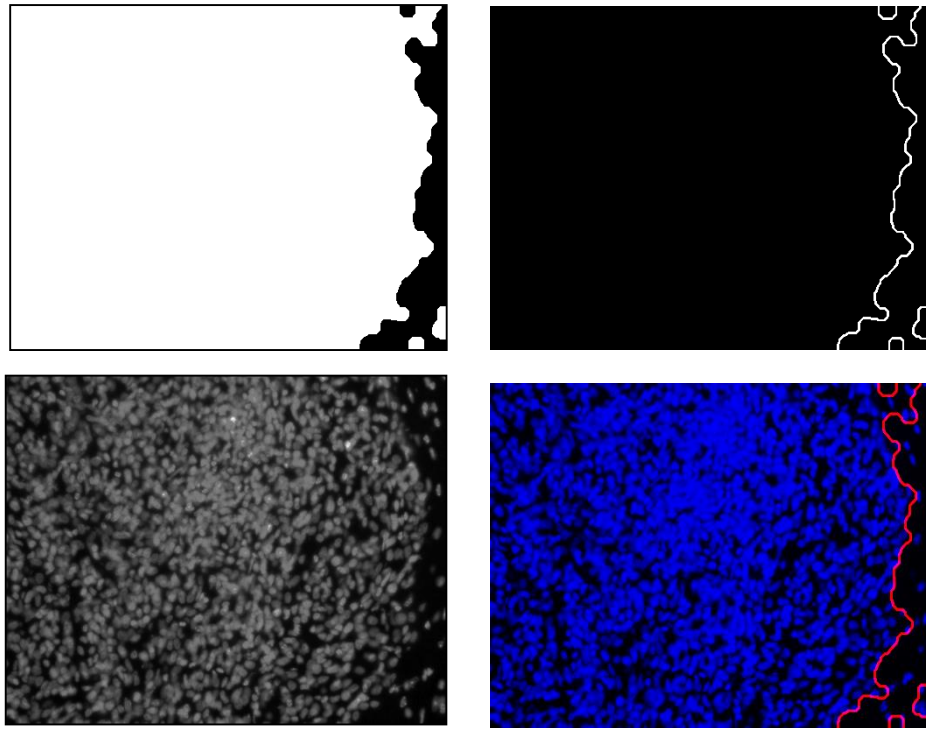
Morphological Operations

- **Example - Tissue**
  - Threshold
    - Matlab: im2bw
    - Threshold=0.45 x graythresh
  - Close
    - Matlab: imclose
    - Structuring Element: Disk, 21 pixels
  - Open
    - Matlab: imopen
    - Structuring Element: Disk, 21 pixels
  - Fill the holes and remove small regions
    - Matlab: imfill



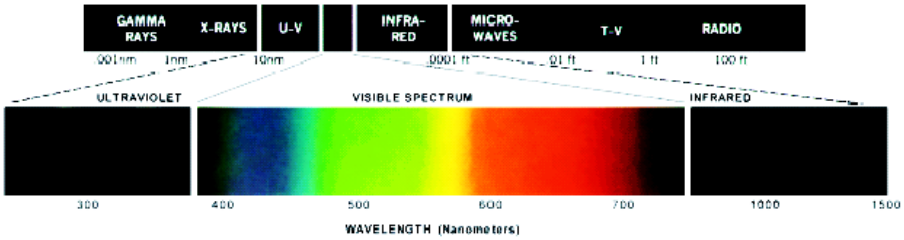
Morphological Operations

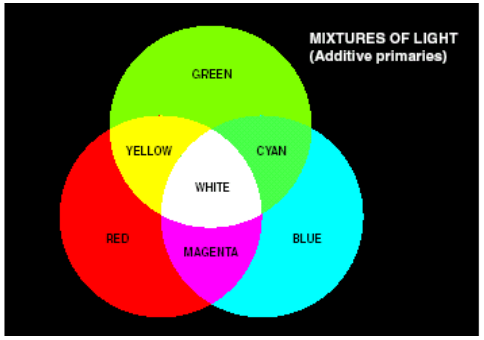
- **How did we get the edge?**
  - Erode
    - Matlab: imerode
    - Structuring Element: Disk, 8 pixels
  - Subtract
    - Original – Eroded
- **How did we get the color overlay?**
  - Let's talk more about color!

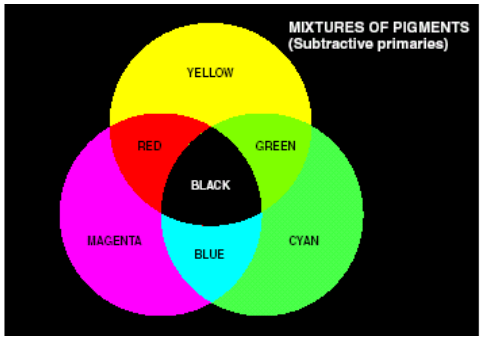


Color

- **Color Images**
  - We can differentiate thousands of colors vs. ~ 24 gray levels
  - Real color images
  - Pseudo-color images
- **Color light**
  - Radiance: Total power (Watts - W)
  - Luminance: observed power (lumens – lm)
  - Brightness: Intensity (energy). Hard to measure
- **Primary Colors – CIE Standard**
  - Red = 700 nm
  - Green = 546.1 nm
  - Blue = 435.8 nm
- **Secondary colors**
  - Magenta = R+B
  - Cyan = G+B
  - Yellow = R+G
- **Paints (and printers)**
  - Primary: MCY
    - Absorb one color and let the other two pass

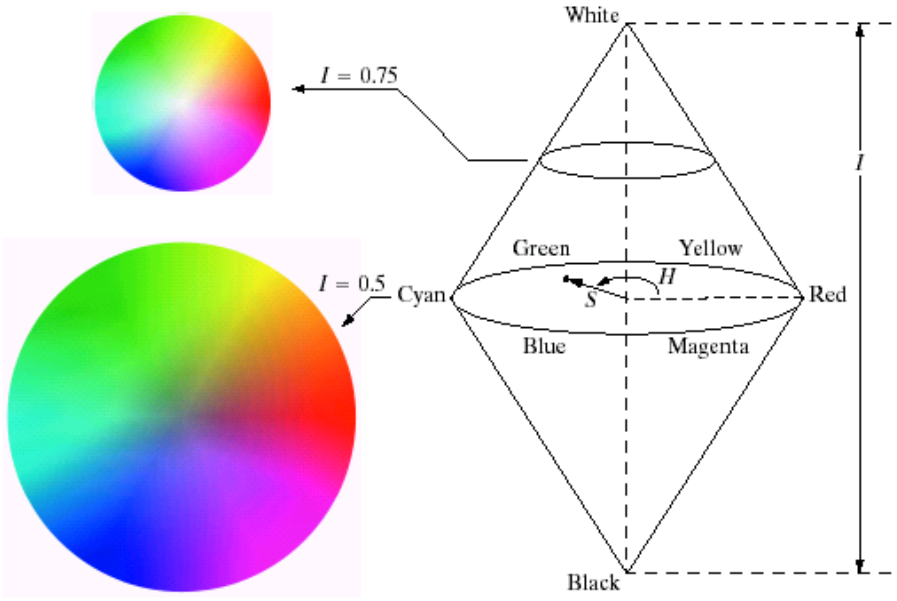






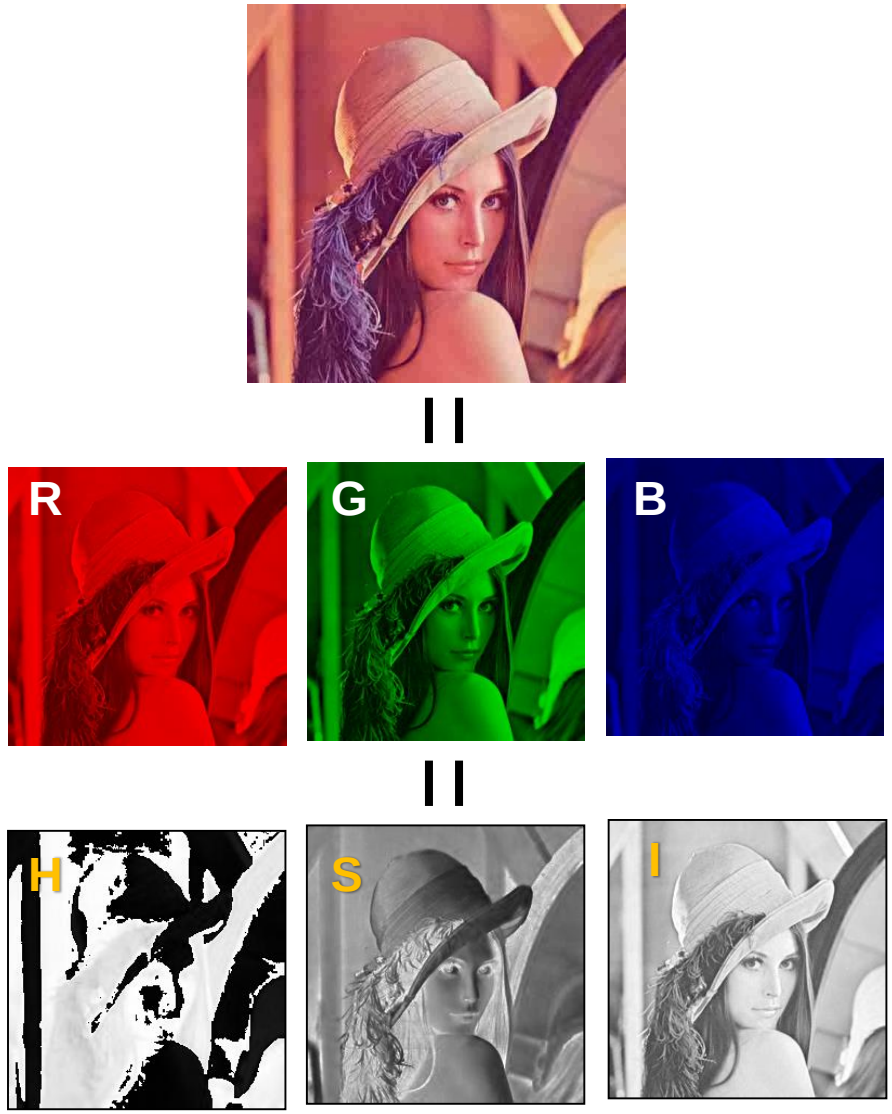
HSI Color Model

- **Closer to human perception**
- **Color characteristics**
  - **Hue**: the major color
    - Value of angle (0 to 1 or 0 to 360^o)
    - Primary Colors: every 120^o
    - Secondary Colors: between the primary
  - **Saturation**: Amount of major color
    - E.g. Pink = Red + White → less saturated
    - Value of axis r (0 to 1)
  - **Intensity**: like achromatic light
    - Value of axis z



Color Images

- **RGB Color Image**
  - Three images each representing the R, G and B value of each pixel.
- **HSI Color Image**
  - Three images each representing the H, S and I value of each pixel.

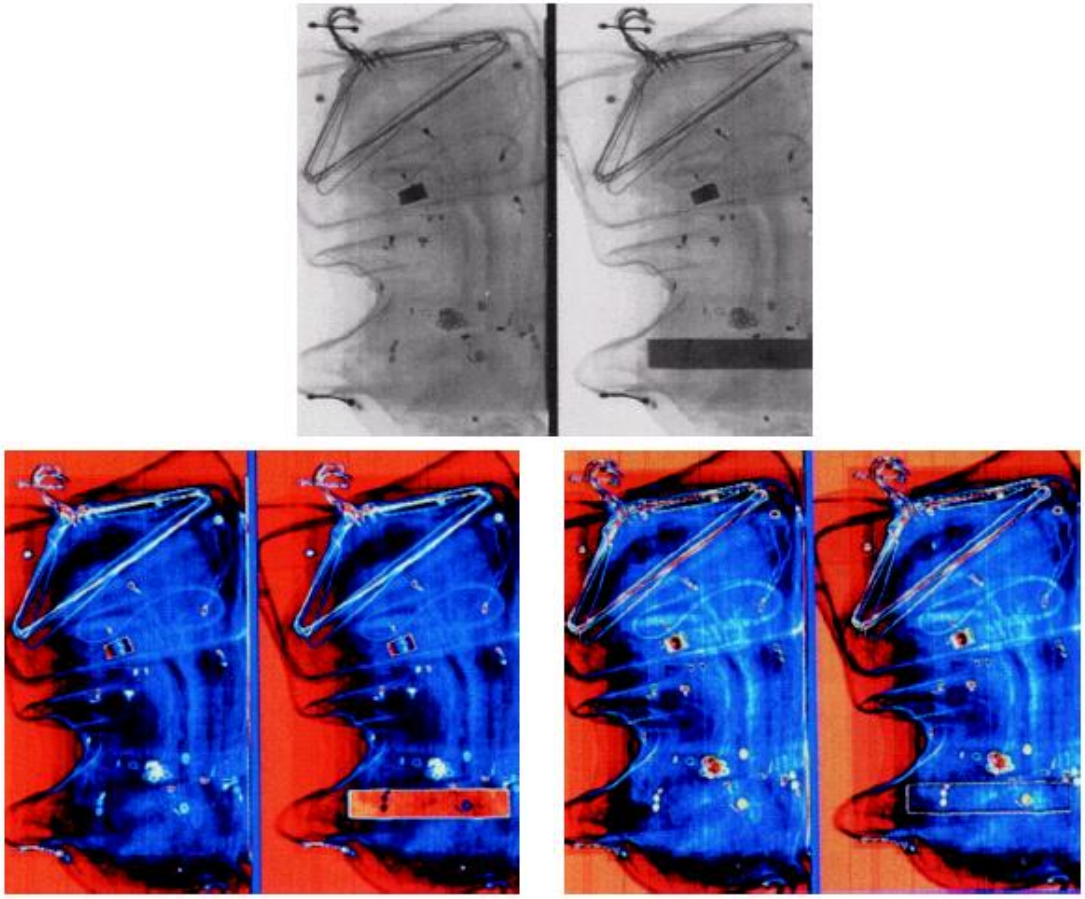


Pseudocolor Display

- **Why pseudocolor?**
  - Enhance visual contrast
  - Display more information
- **Pseudocolor using colormaps**
  - Enhance contrast
  - Assign different colors to different gray levels → subtle differences are easier to see
  - Can choose any color scheme and make it uniform (linear) or not
  - Matlab: imshow(im, map)
    - jet, lines, pink, prism, spring, summer, white, winter, autumn, bone, cool, copper, flag, gray, hot, hsv, parula

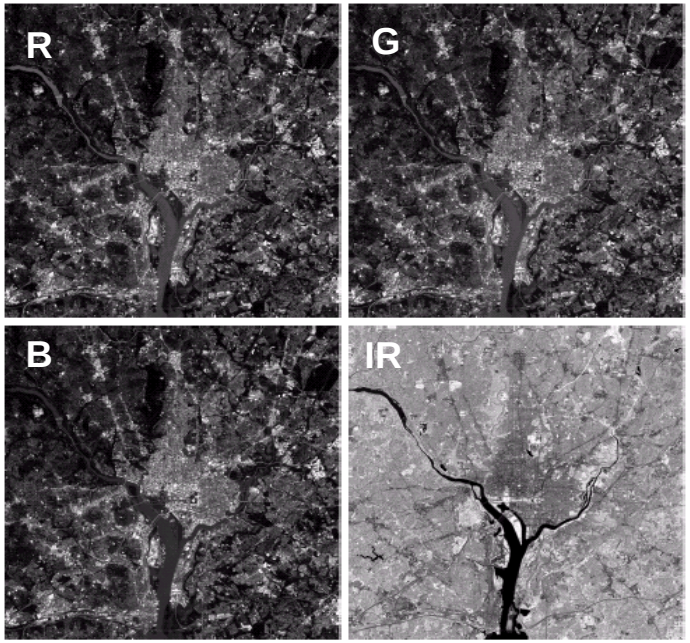
Pseudocolor Display

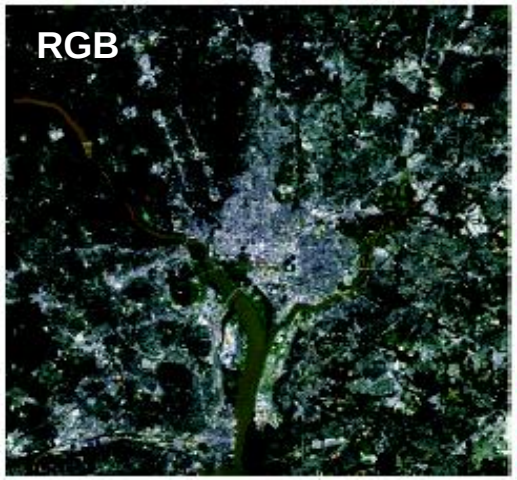
- **Pseudocolor using colormaps**
  - Practical applications: X-ray pseudocolors for airport security

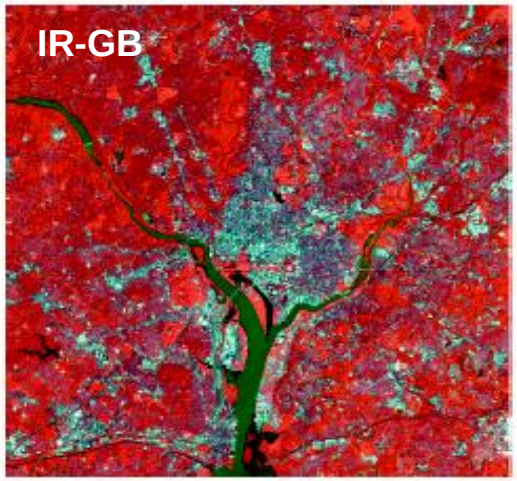


Pseudocolor Display

- **RGB Pseudocolor**
  - Display more than one channels of information
  - Do not have to correspond to actual RGB values
    - Different modalities
    - Different wavelengths
  - Can be mixed or left separated







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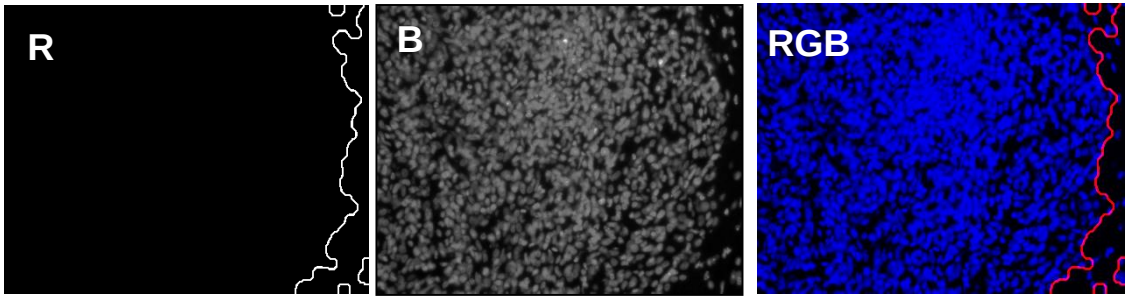

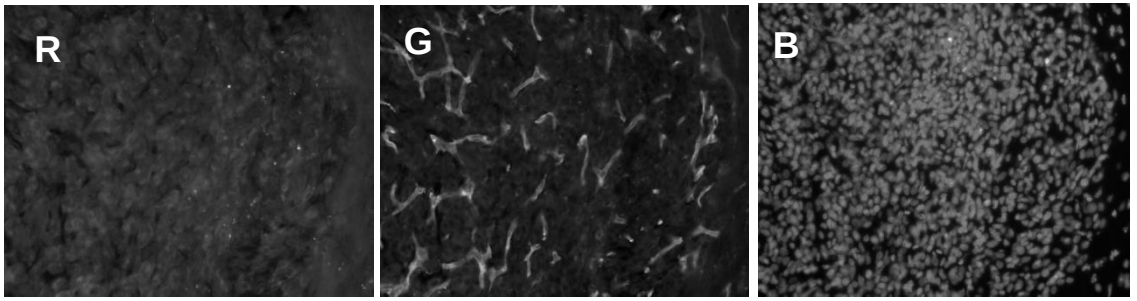

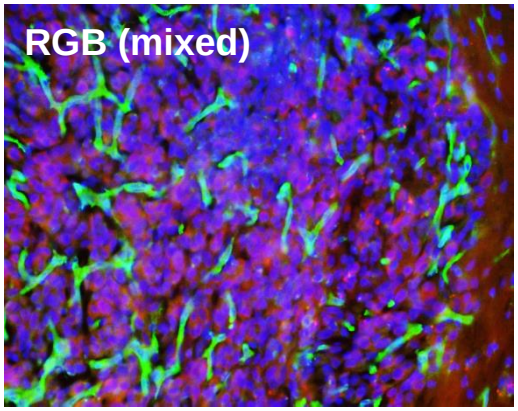

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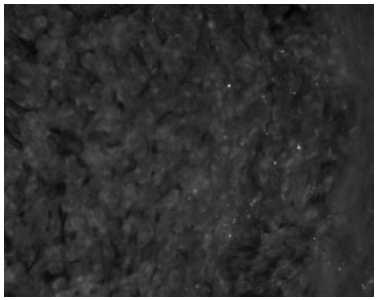
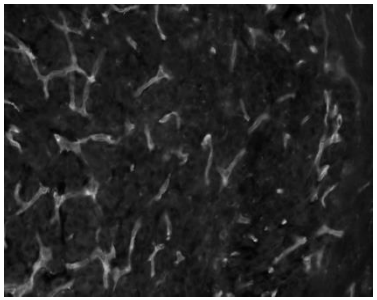
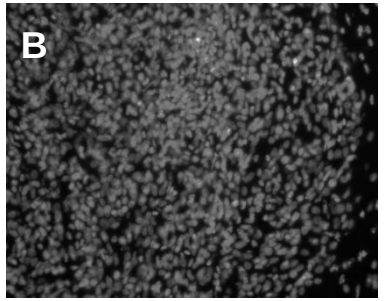
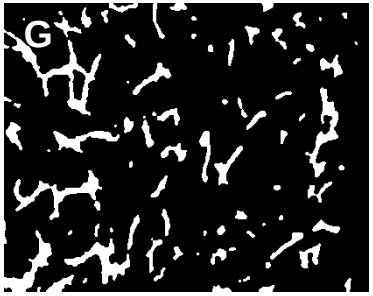
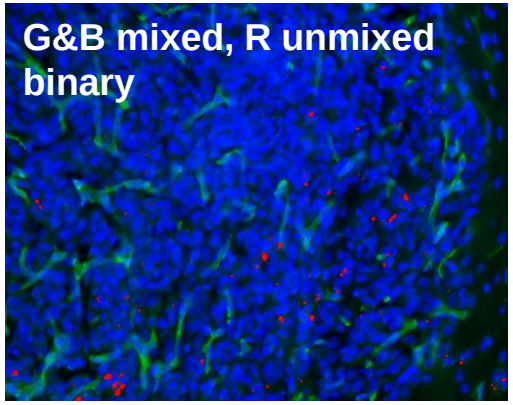
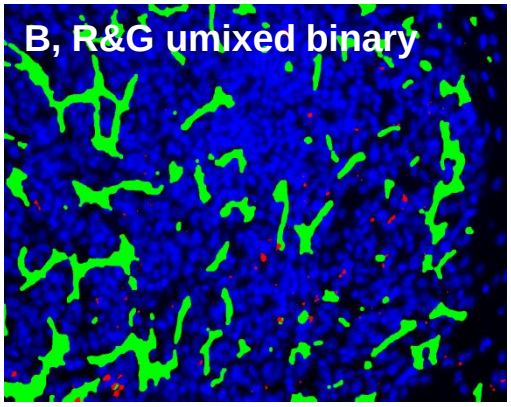






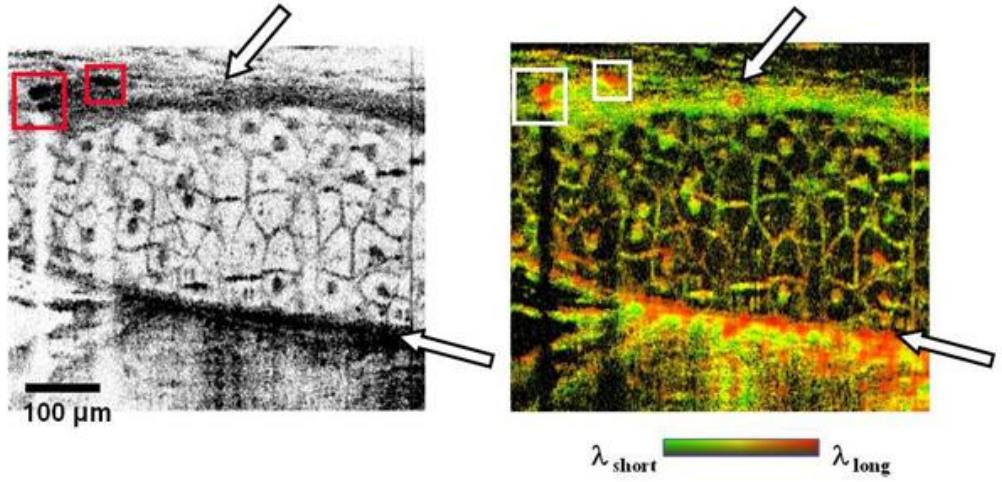


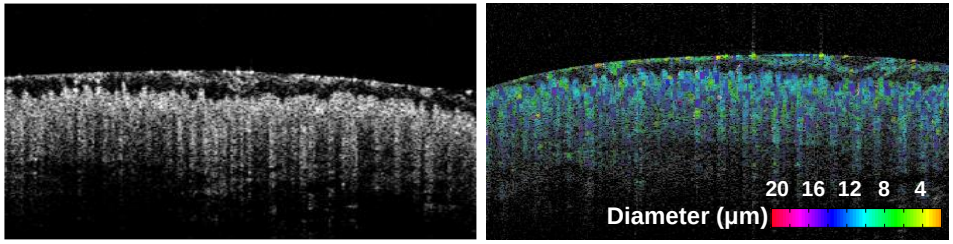




Pseudocolor Display

- **HSI Pseudocolor**
  - Useful for overlaying structure (intensity) and another characteristic (color)
- **Examples:**
  - Intensity: OCT Images (structure)
  - Hue (color):
    - Centroid of the spectrum
    - Size of the scatterer
    - Amount of dispersion
  - Saturation:
    - Set to 1





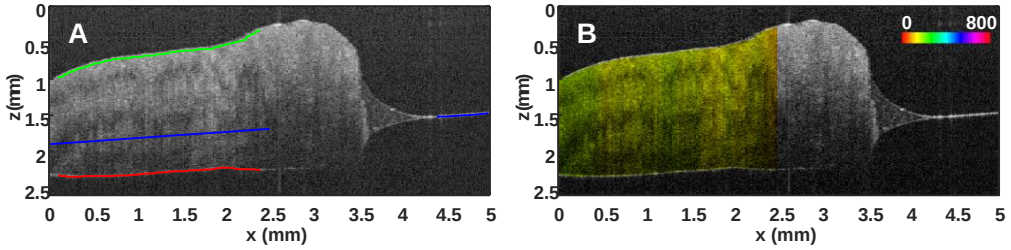


Image Segmentation

- **Thresholding**
  - Simplest method of segmentation
  - Works well when the regions have different intensity than the background and are not touching.

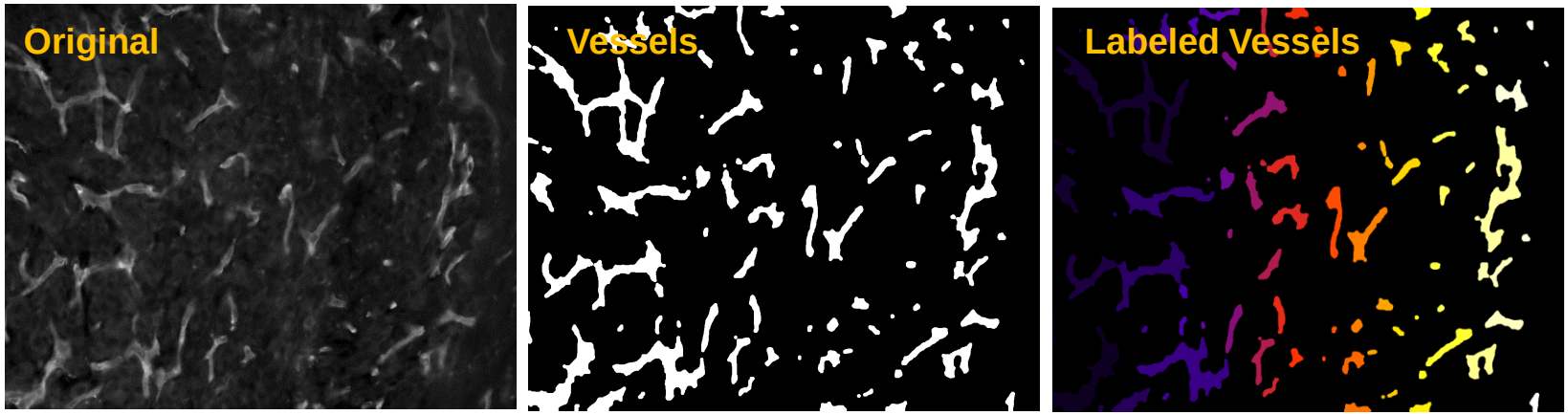
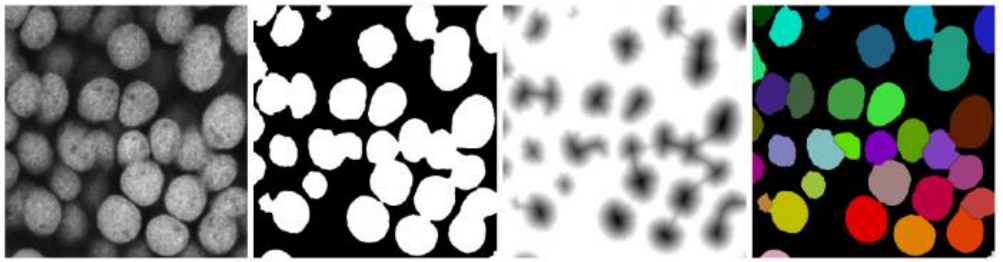


Image Segmentation

- **Watershed transformation**
  - Place a water source at each regional minimum
  - Flood the entire image
  - Build barriers when different water sources meet
  - The resulting set of barriers constitutes a watershed segmentation by flooding
  - Matlab: watershed
- **To avoid over-segmentation:**
  - Suppress shallow minima
  - Matlab: imhmin



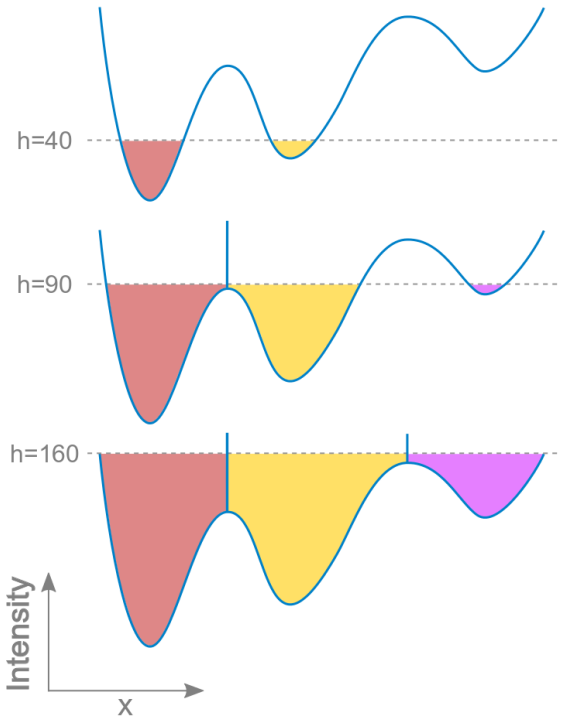
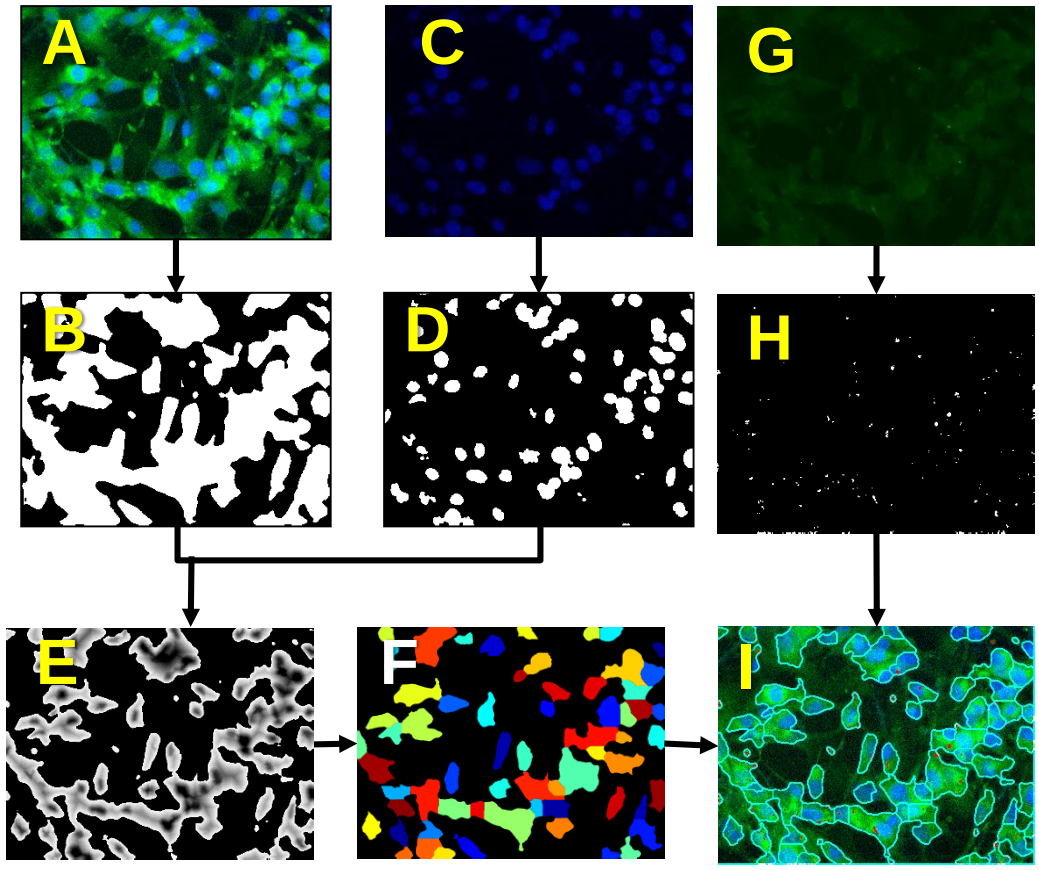


Image Segmentation

- **Watershed Example**
  - u87mg gfp cells quantification



A: Contrast enhanced composite image with DAPI (blue) and Cy3 (green)

B: Segmented image A, showing the area covered by cells in white

C: The DAPI image

D: Segmented image C, showing the area covered by the nuclei in white

E: Image of the negative of the binary distance of C plus the negative of the binary distance of D

F: The results of the watershed segmentation (using image E) showing each cell in a different colour

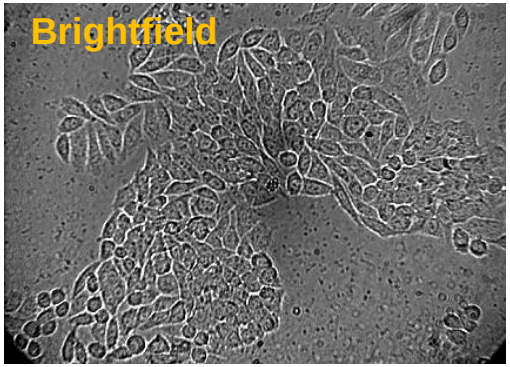
G: The Cy3 image

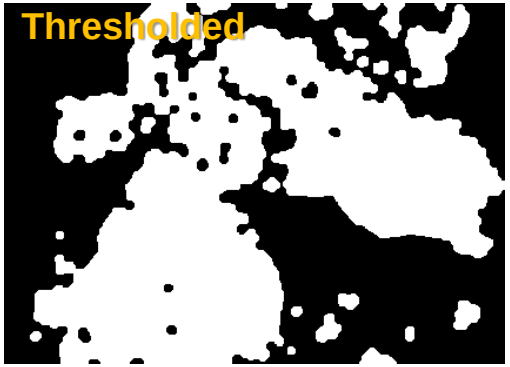
H: Segmented image G where the cell fluorescence was removed and the nanoparticles are shown in white

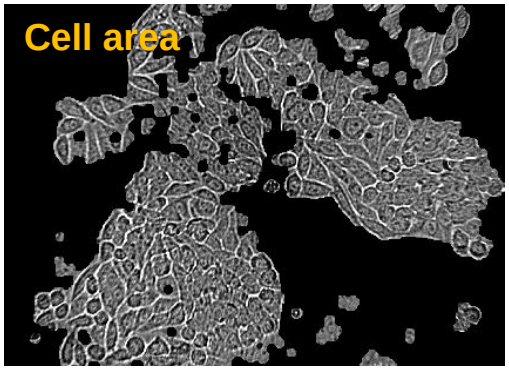
I: Contrast enhanced composite image with DAPI (blue), Cy3 (green), cell boarders (cyan), and nanoparticles (red)

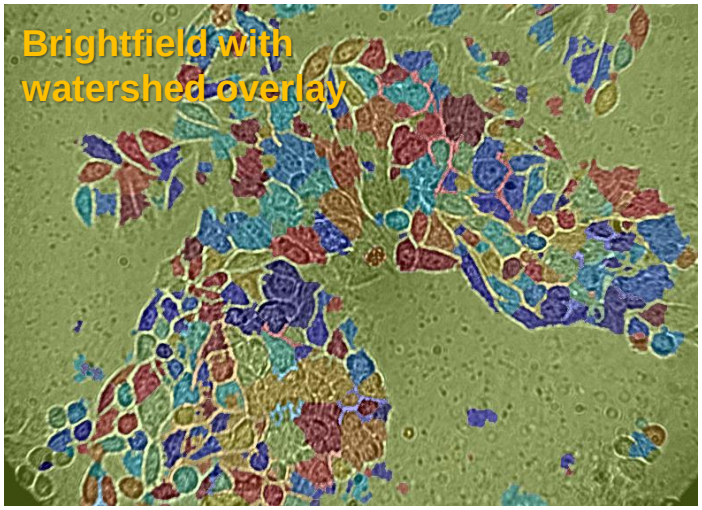
Image Segmentation

- **Watershed Segmentation**
  - Finding stem cells in SW620 culture (bright field only)









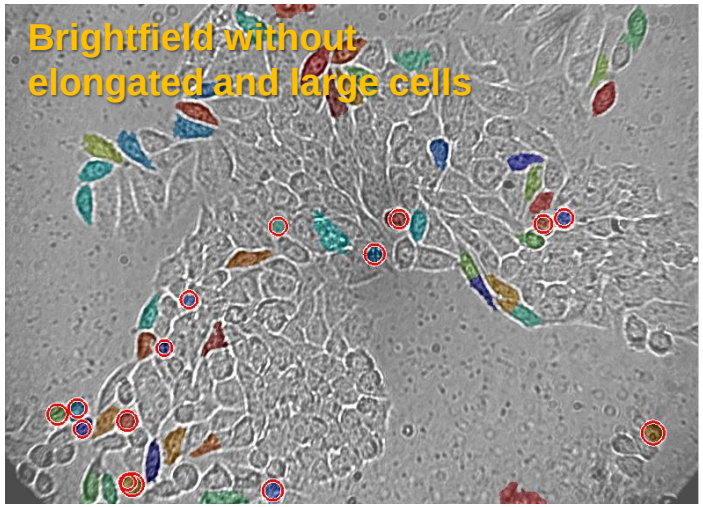


Image Segmentation

- **Extract information regarding the regions**
  - Number, size, shape, etc.
- **Shape and Value Measurements (Matlab: regionprops)**

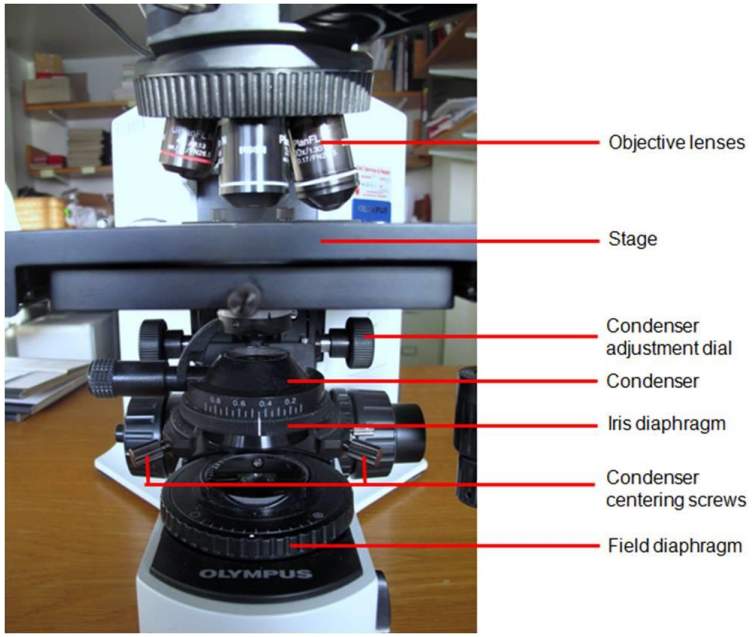
• Area	• EulerNumber	• Orientation	• MaxIntensity
• BoundingBox	• Extent	• Perimeter	• MeanIntensity
• Centroid	• Extrema	• PixelIdxList	• MinIntensity
• ConvexArea	• FilledArea	• PixelList	• PixelValues
• ConvexHull	• FilledImage	• Solidity	• WeightedCentroid
• ConvexImage	• Image	• SubarrayIdx	
• Eccentricity	• MajorAxisLength		
• EquivDiameter	• MinorAxisLength		

Quantitative Microscopy

- **Remember what makes a good image**
  - Good image data has a high S:N ratio (count more photons)
  - Correctly sampled to reproduce the optical resolution
    - pixel = optical resolution/2
  - Avoid aberrations (sample prep / choice of objective / technique)
    - Spherical aberration (SA)
    - Motion blur
    - Bad system alignment
  - Correctly annotated (Metadata retained)
  - **Image Processing is NOT a substitute for a good image**

Quantitative Microscopy

- **Before you begin**
  - Clean the microscope of dust and other debris
    - **Warning**: Lens paper and pure methanol ONLY!
  - Align the lamp and microscope
    - Instructions
  - **Once you optimized, the microscope should not be modified again during the entire study**
  - **If you are unsure, DON'T DO IT!!!!**



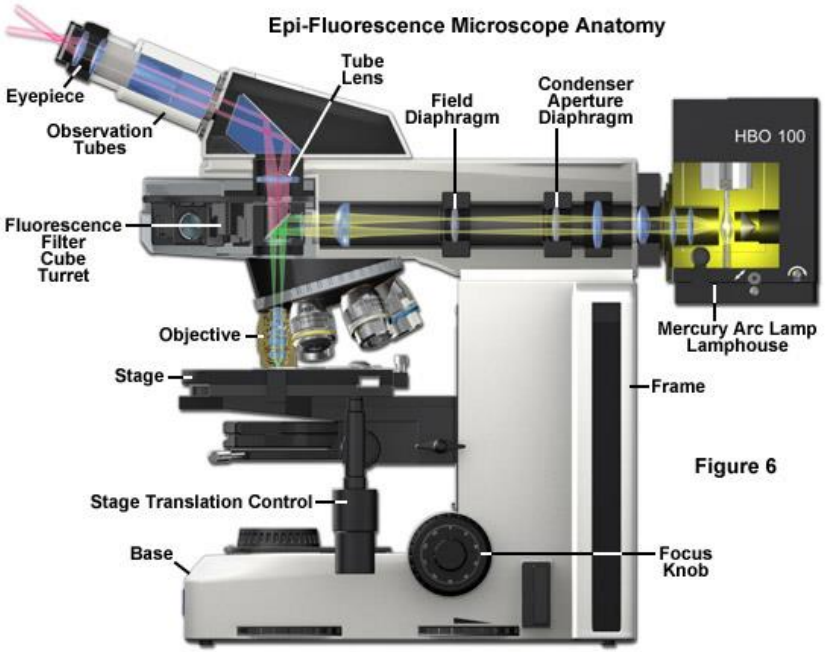
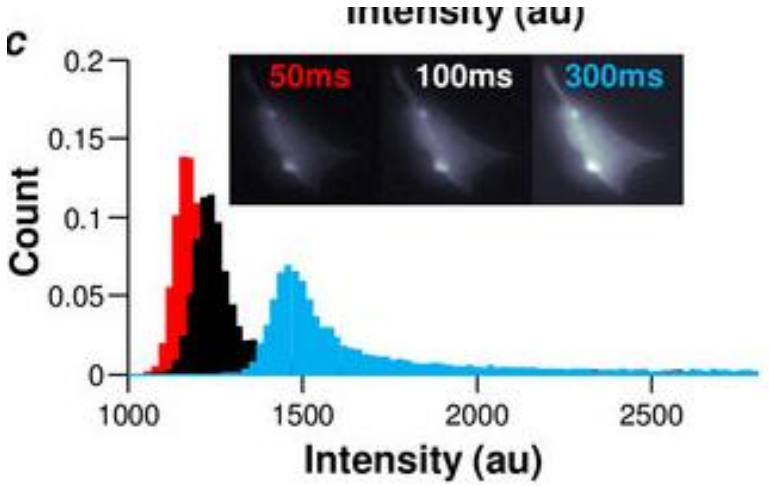
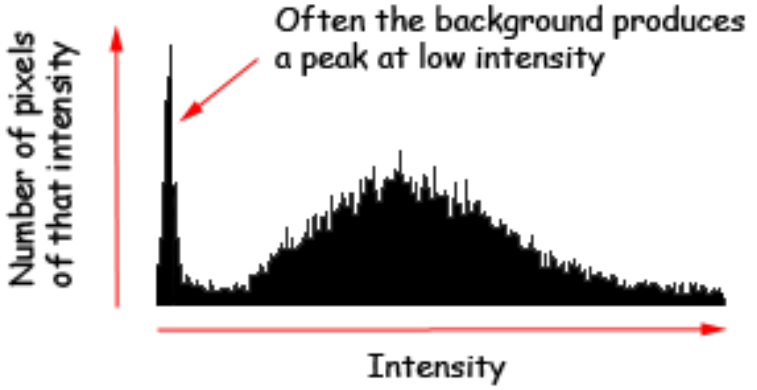


Figure 6

Quantitative Microscopy

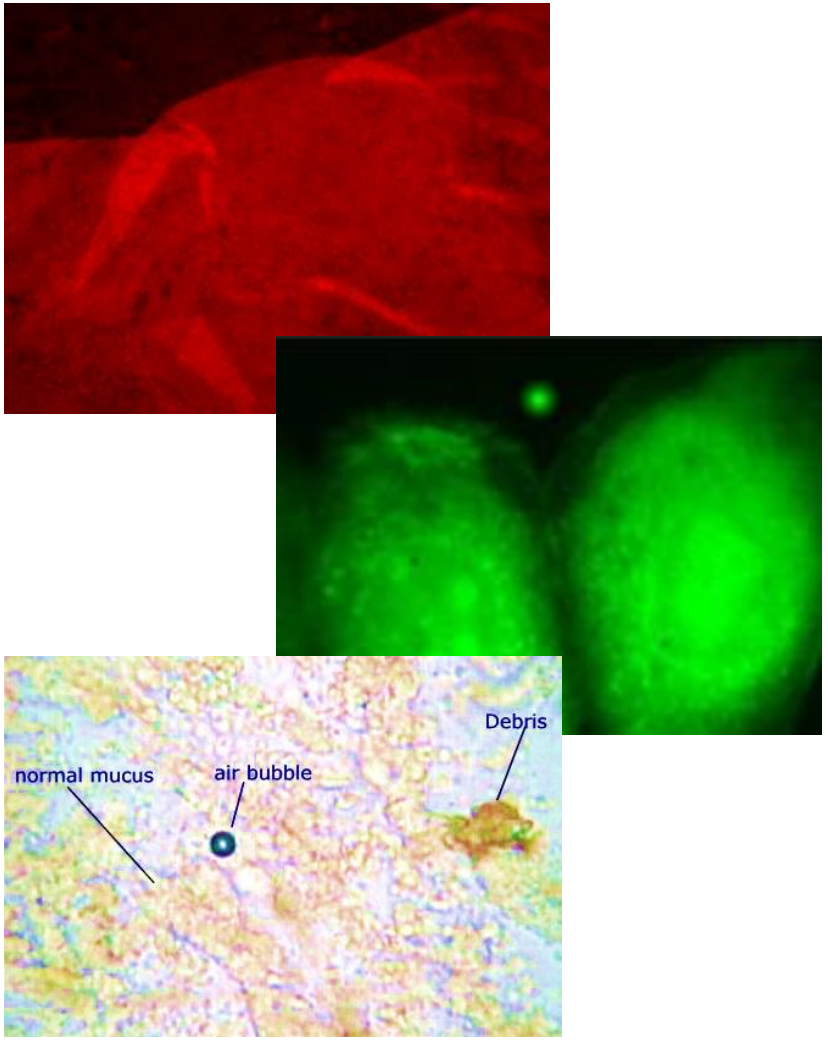
- **Adjust the settings for your sample**
  - Choose appropriate excitation/emission filters
  - Set the exposure, sensitivity and/or gain
    - Maximum setting (histogram filling the range
    - No saturation
    - Avoid too high sensitivity/gain since the noise may increase
  - Test your most fluorescent sample → Images with saturation **CANNOT** be used for quantitative measurements
  - Note that settings
    - **Must be kept the same throughout the experiment**
    - **Can be different for different fluorophores as long as you are consistent**





Quantitative Microscopy

- **Avoid areas with processing artifacts or debris**
  - They are very hard to remove by post-processing
  - Introduce significant outliers
- **Choice of areas**
  - Random or using the same pattern for all samples
  - **DO NOT choose areas based on what you think better fits your hypothesis!**



Quantitative Microscopy

- **Do not corrupt the integrity of the original data**
  - Retain your original data in its original file format and original metadata associations
  - Ideally use Uncompressed TIF (tagged image file format) for processed data
  - AVOID compressed file formats when processing: JPEG, PSD, PDF, compressed TIF…This will cause data corruption and loss
  - Most data is collected as single channel grey scale images at 8 or 16 bit depth
  - Avoid saving primary image data in color formats (RGB)
  - Avoid repeated inter-conversions of file formats

Original Lena Image (12KB size)

Lena Image, Compressed (85% less information, 1.8KB)

Lena Image, Highly Compressed (96% less information, 0.56KB)

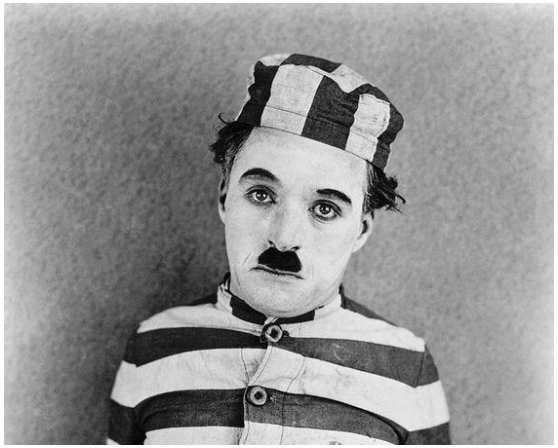
Quantitative Microscopy

- **What is OK?**
  - Denoising, background subtraction
  - If finding objects, normalization and thresholding. If measuring intensity, raw data
  - **Whatever you do, apply to all images the same way!**
- **Image manipulation (alteration) - Bad practice!**
  - **BAD**: manipulated but does not alter interpretation
  - **VERY BAD**: Changes interpretation with intention to defraud
  - Adjustments necessary to reveal a feature ALREADY PRESENT in the original data are acceptable if they can be justified

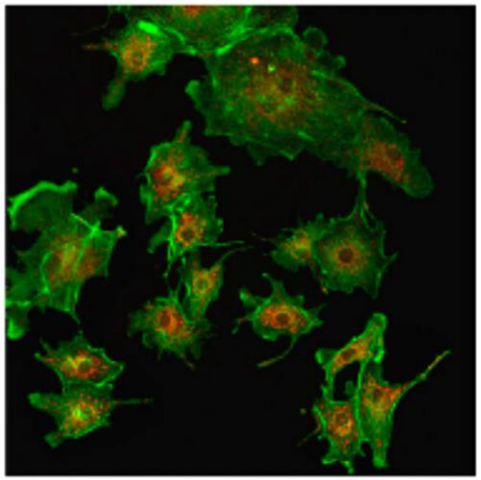
Lena Söderberg (born 31 March 1951) , centerfold of the November 1972 issue of Playboy

Quantitative Microscopy

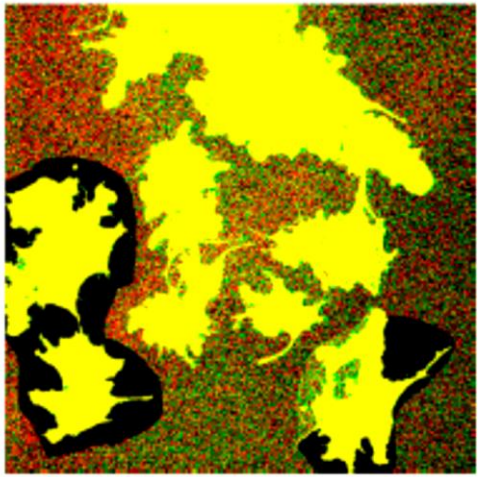
- **THEY HAVE WAYS OF FINDING OUT WHAT YOU DID!**



Manipulated image



Manipulation revealed by contrast adjustment



Misrepresentation of image data.
Cells from various fields have been juxtaposed in a single image, giving the impression that they were present in the same microscope field. A manipulated panel is shown at the top. The same panel, with the contrast adjusted by us to reveal the manipulation, is shown at the bottom.

Rossner & Yamada (2004). What's in a picture? The temptation of image manipulation. J. Cell Biology 166: 11–15.

Thank you!

"I want you to make me the fairest of them all."