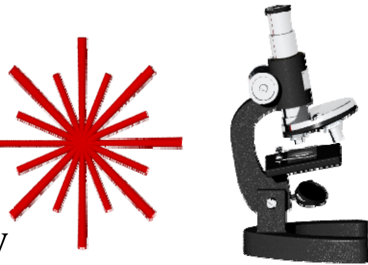
Introductory Guide to Light Microscopy - Biomedical Confocal Microscopy

~~14 May~~ 11 June 2007

E = hν



Michael Hooker Microscopy Facility

1. Live Cell Imaging
2. Bleed Through
3. Co-localization
4. Deconvolution
5. Software

MHMF acknowledges the support of the SOM Road Map Initiative

Michael Chua
microscopy@unc.edu
843-3268
6007 Thurston Bowles

Wendy Salmon
wendy_salmon@med.unc.edu
966-7051
6129 Thurston Bowles

1. Live Cell Imaging

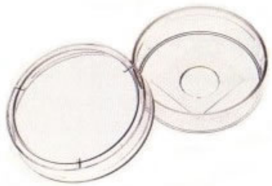
- 1. Live Cell Imaging
  - Morphology (natural structure)
  - Photometric analysis (intensity, e.g. [Ca],)
  - Dynamics (changes in shape &/or intensity with time)

1. Live Cell Imaging

- Conditions
  - Oxygen
  - Temperature
  - CO_2 / pH
  - Physiological ions
  - Osmolarity - humidity – drying - condensation
  - Flow – drug addition, O_2, metabolites
  - Working distance

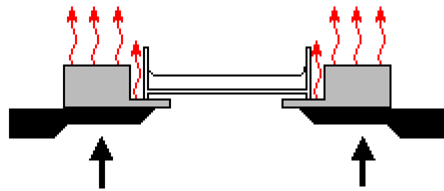
1. Live Cell Imaging

- Control - simple to complex



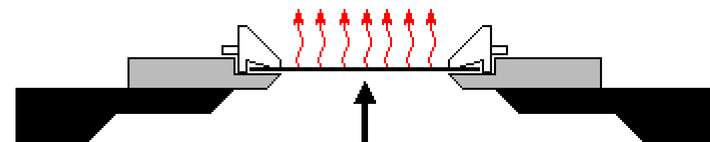
#1.5 cover slip glued to hole in petri dish

- Ambient temperature
- Ambient O_2 good!
- No CO_2 (poorly controlled $[pH]_i$)
- Drying – OK short term
- Simple
- Commercially available



Electrically heated metal block

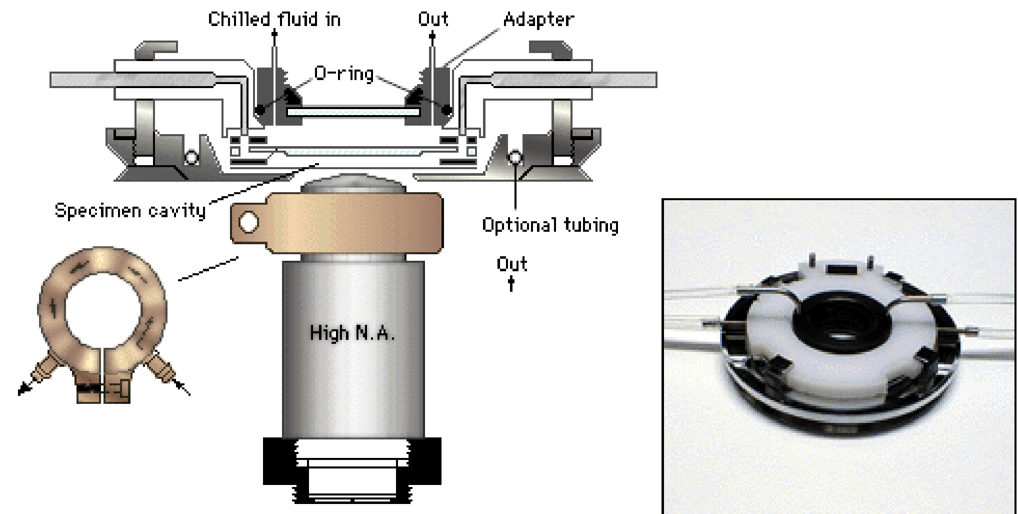
Better temperature control



Electrically heated Indium Tin Oxide transparent coating on petri dish

1. Live Cell Imaging

- Control – complex



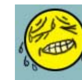
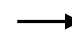
- Well controlled temperature (to +40C)
- Controlled flow though solution with:
  - CO_2 (intracellular pH controlled)
  - Drugs
  - O_2
  - No drying
- Good long term, e.g. 24 hr
- Heat loss through oil immersion to objective negated by heated collar

1. Live Cell Imaging

- CO_2 / pH
- 15 mM HCO_3 + 5% CO_2 → intra & extra cellular pH control → long term viability
- Hepes, etc, does not cross membrane → extra cellular pH control only
- Cell types have different resiliency to non ideal conditions

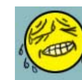
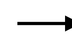
External pH indicated by Phenol Red

pH = 6.5 5% CO_2

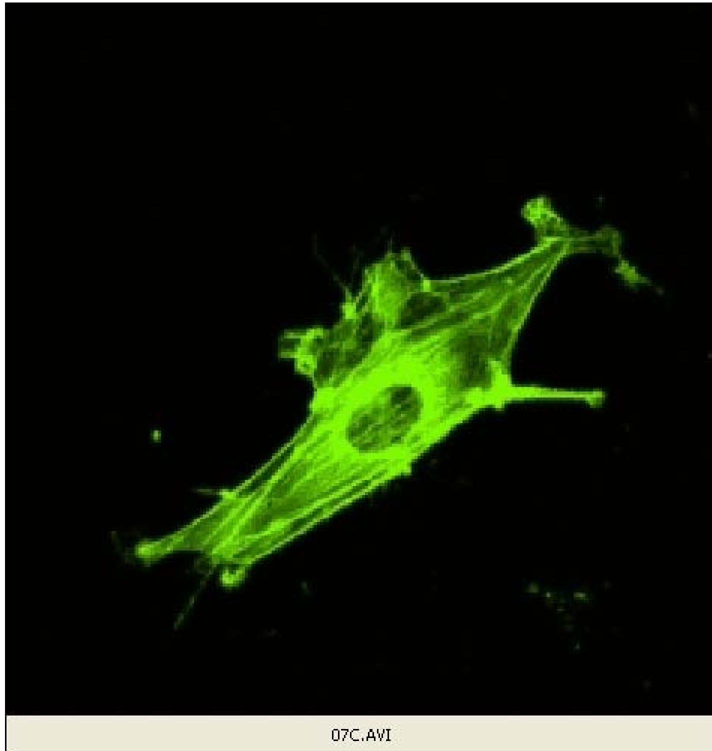



pH = 7.4 5% CO_2 + HCO_3

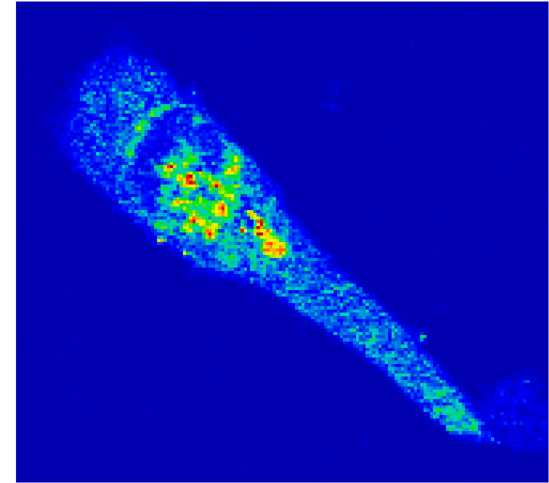
pH = 8.0 air only + HCO_3

1. Live Cell Imaging



Cultured neuron transfected with GFP - migration

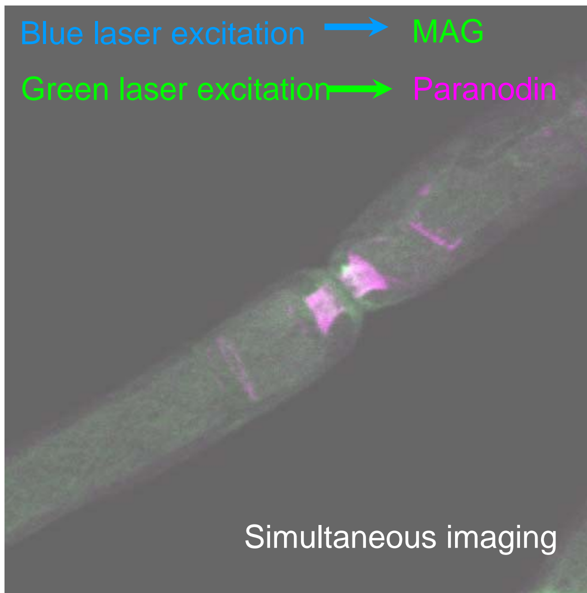


Cardiac myocyte labeled with Fluo-3 – $[Ca]_I$ showing spontaneous activiety

2. Bleed Through

- 1. Live Cell Imaging
- 2. Bleed Through
  - Confocal simultaneous scanning
  - Sequential scanning
- 3. Co-localization
- 4. Deconvolution
- 5. Software

Emission Bleed Through Test



→ Turn off green excitation →

Bad!

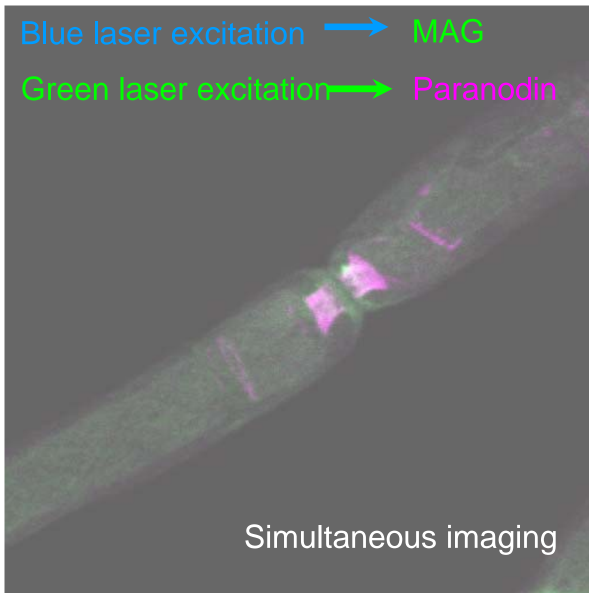
→ Turn off blue excitation →

Expected

(MAG = Myelin Associated Glycoprotein)

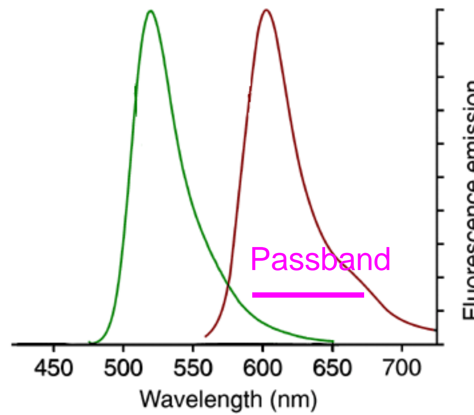
Paranodin = RED

Emission Bleed Through Test



→ Turn off green excitation →

Bad!



→ Turn off blue excitation →

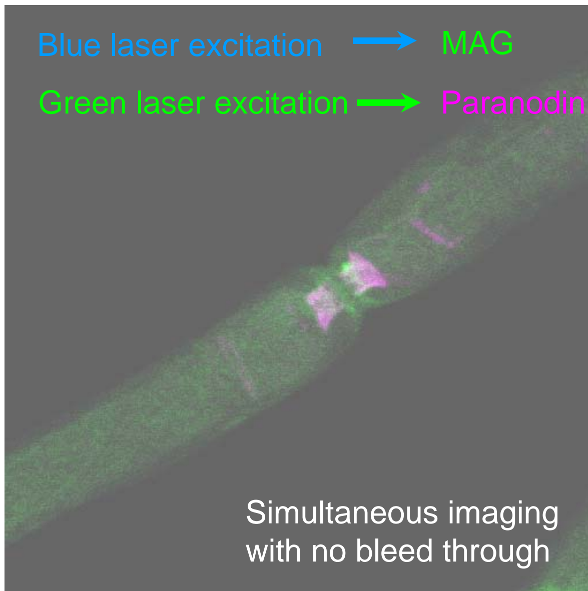
Expected

(MAG = Myelin Associated Glycoprotein)

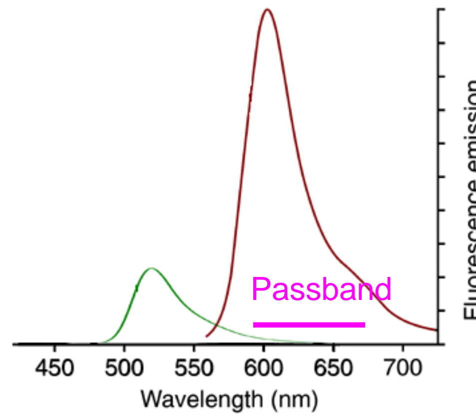
Paranodin = RED

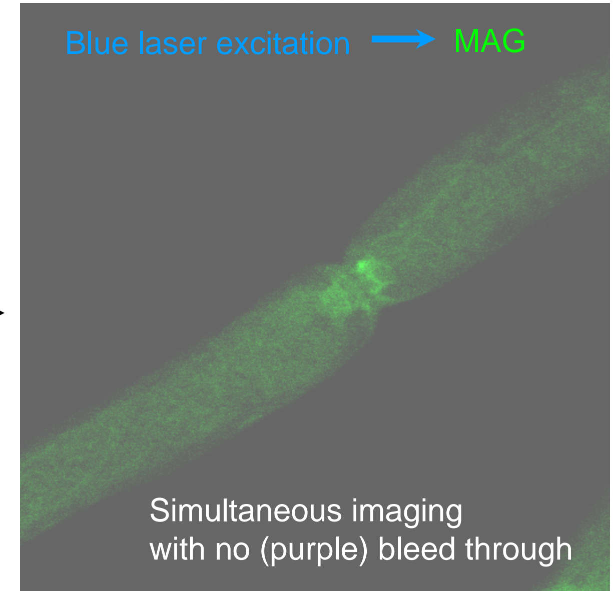
Emission Bleed Through Corrected

Blue excitation reduced to 25%
Green detection sensitivity increased 4 fold



→ Turn off green excitation →



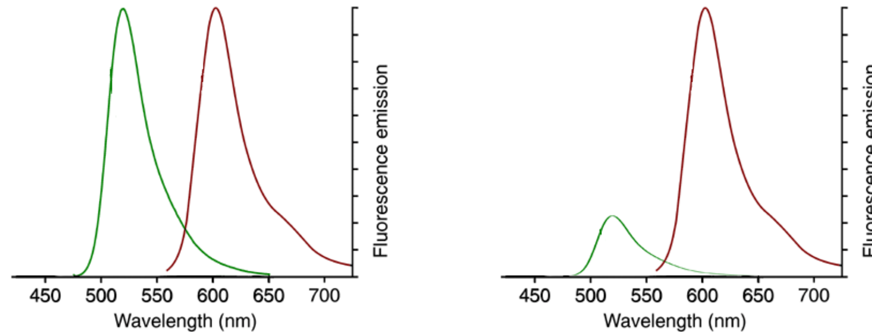


Good!

Note: short wavelength fluorophores bleed into longer wavelength detection channels

Simultaneous/Sequential Scanning

Simultaneous – scan multiple dyes at once
- No filter switching, faster
- Can educe cross talk by lowering excitation power of shorter wavelength excitation

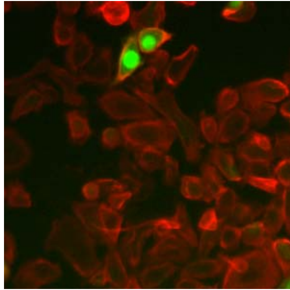


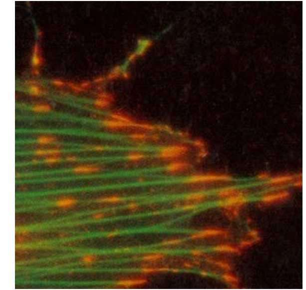
Sequential
- Excite and detect one dye at a time
- Emission cross talk eliminated
- Be careful of cross excitation
- Can speed up filter switching with fully electronic filters e.g. Leica AOBS confocal systems- AOTFs instead of dichroics

3. Co-localization

- 1. Live Cell Imaging
- 2. Bleed Through
- 3. Co-localization
- 4. Deconvolution
- 5. Software

Determining Co-localization with Microscopy





Determining whether fluorescent tags co-localize is not necessarily a trivial issue.

Simple to complex considerations and techniques for addressing this question will be presented.

Such considerations are important to all who view photomicrographs in journals, during seminars, as well as practicing microscopists.

Introduction

- Co-localization at the whole cell level

- Co-localization at the subcellular level

- Resolution, resolution, resolution, i.e. confocal microscopy

- Red / Green pseudo color overlay – the pitfalls

- Fluorograms

- Is there interaction?

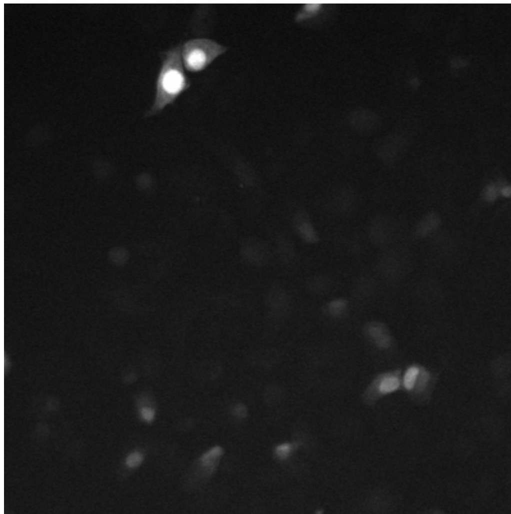
Co-location at the cell level

mixed cells +

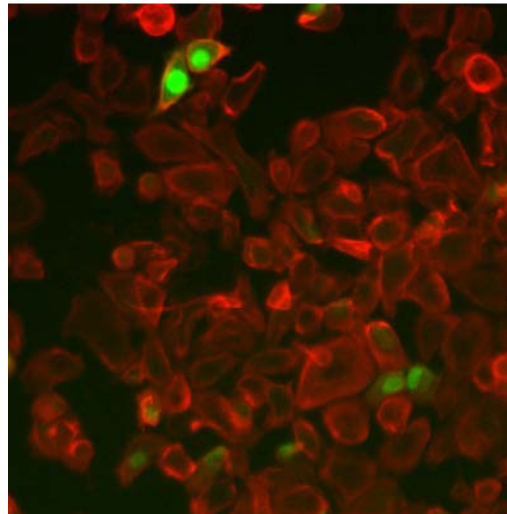
GFP vector +

dsRed vector =

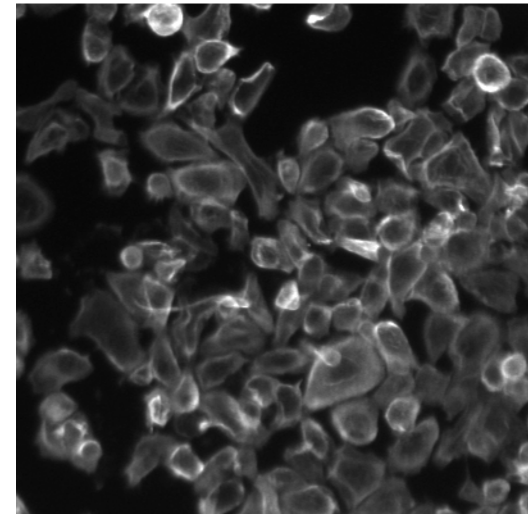
GFP



Overlay

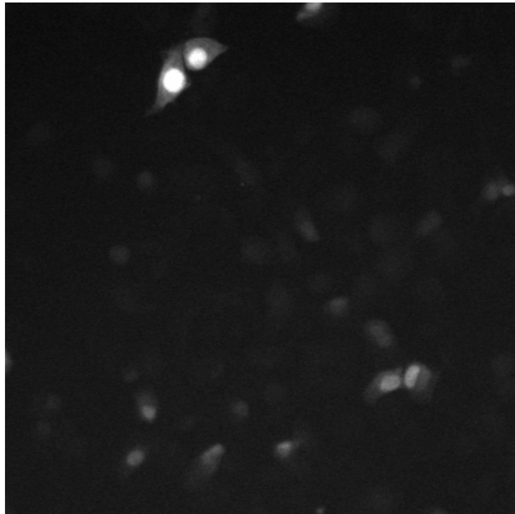


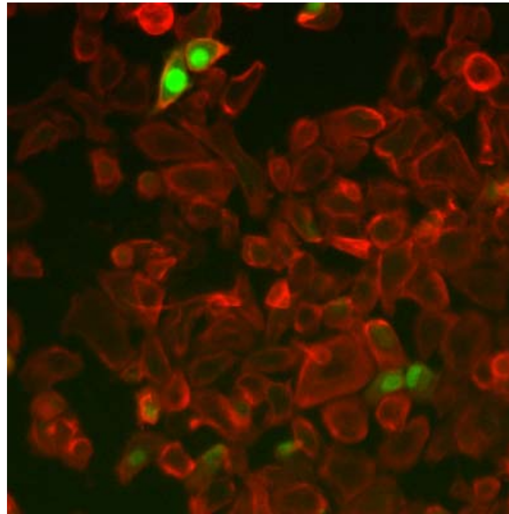
dsRed

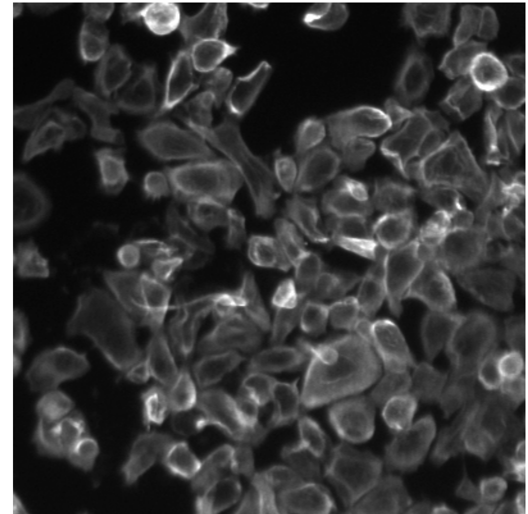


Co-location at the cell level

Count cell types based on expression in cell using a microscope

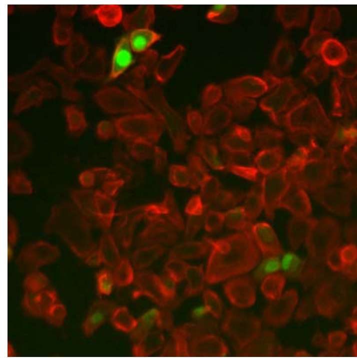




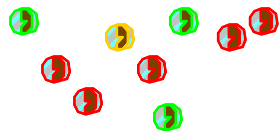


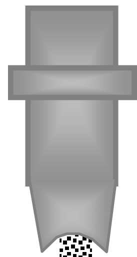
Co-location at the cell level

Flow Cytometer



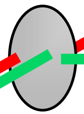
Dissociate cells

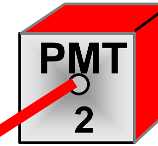


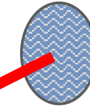


Flow cell

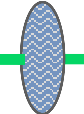
Dichroic Filter

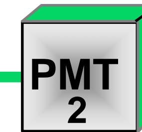




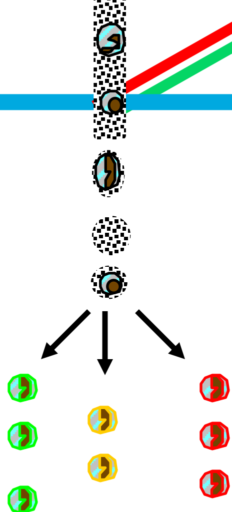


Red Bandpass Filter





Green Bandpass Filter

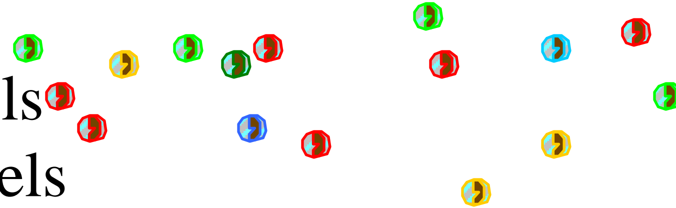


Sort large numbers of cells based on GFP & dsRed expression

Flow Cytometry Data

Advantages

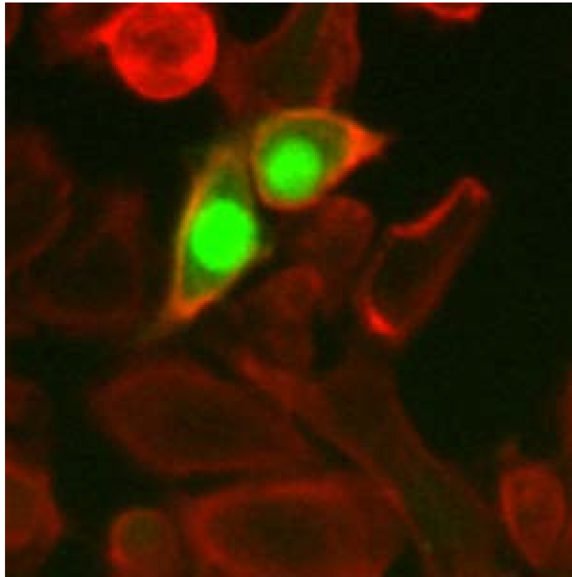
- Count large number of cells
- Sorting using multiple labels
- Fast sorting



Minuses

- Cells can be damaged by dissociation
- Mechanical stress to cells through flow plumbing
- Spatial resolution is low.
  - Effectively size of cell.
  - No information of location of label in cell.

Microscopy

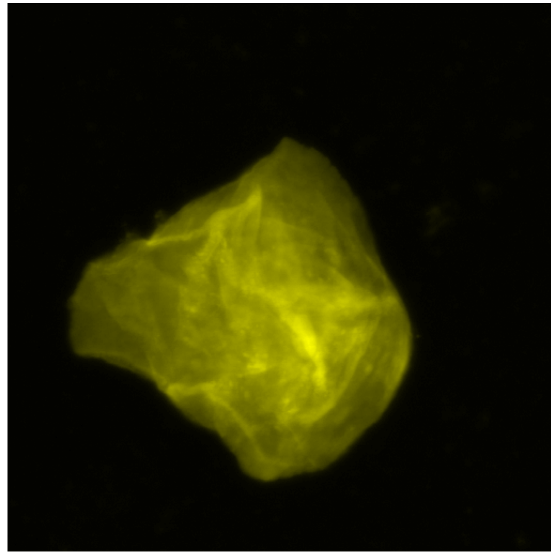


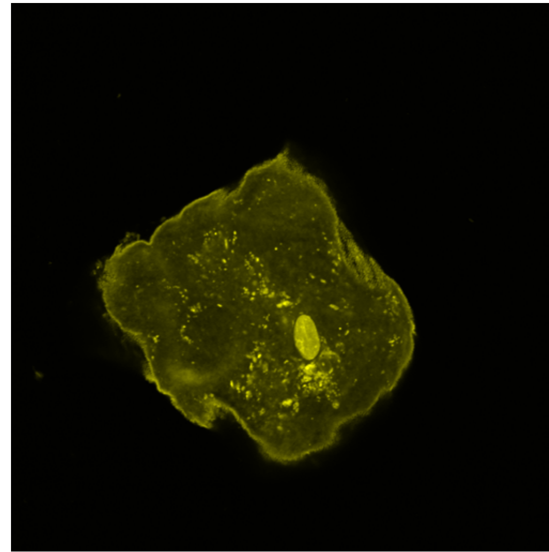
Spatial information – see structures inside the cell

With Fluorescent labels can also see sub resolution structures
- See emitted light without necessarily resolving them

Wide Field versus Confocal

Wide field | 63X NA 1.4 FM 1-43 | Confocal





r_{airy} = 0.61 λ / NA
= ~0.22 µm
~8 µm thick (measured)

Structures in same x-y position may not be in the same plane

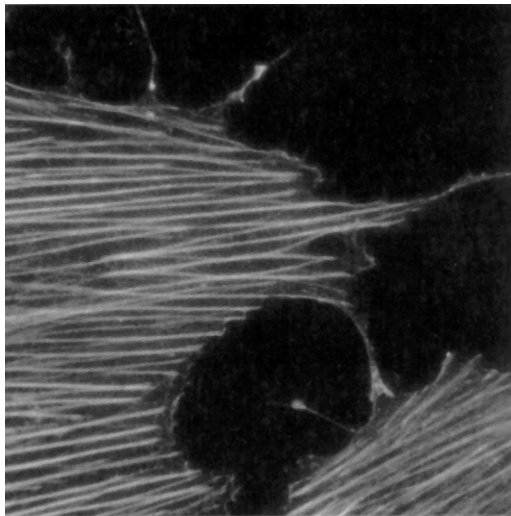
r_{airy} = 0.61 λ / NA / √2
= ~0.15 µm

r_{axial} = 1.77 λ / NA^2
= ~0.45 µm

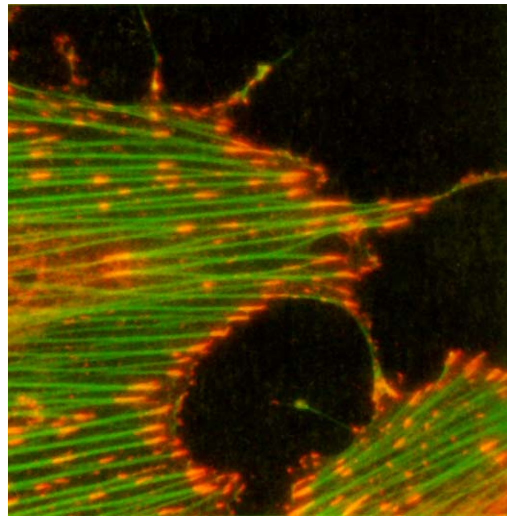
Optical sectioning separates structures

Therefore need to use confocal scanning in order to separate structures in the z axis.

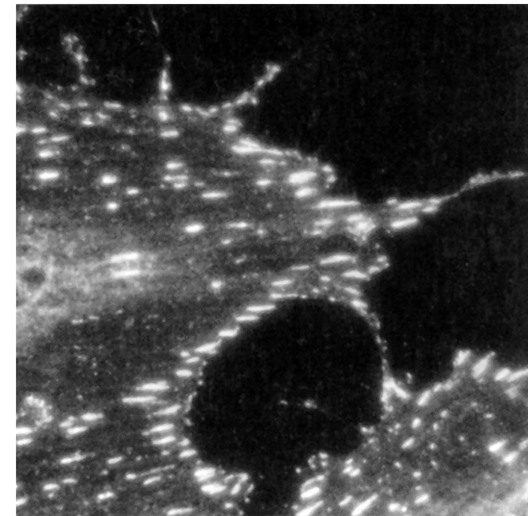
Microscopy – Red Green Overlay



Green
(Actin)

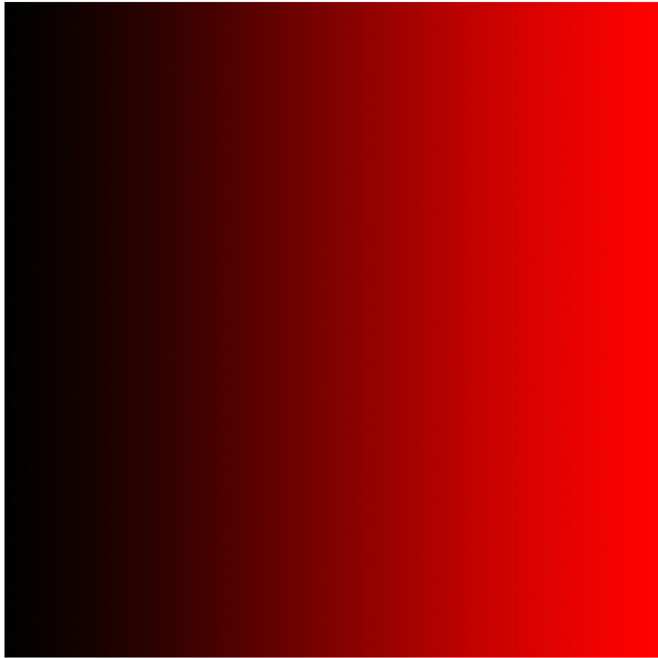


Does anyone
see yellow at the
focal adhesions?

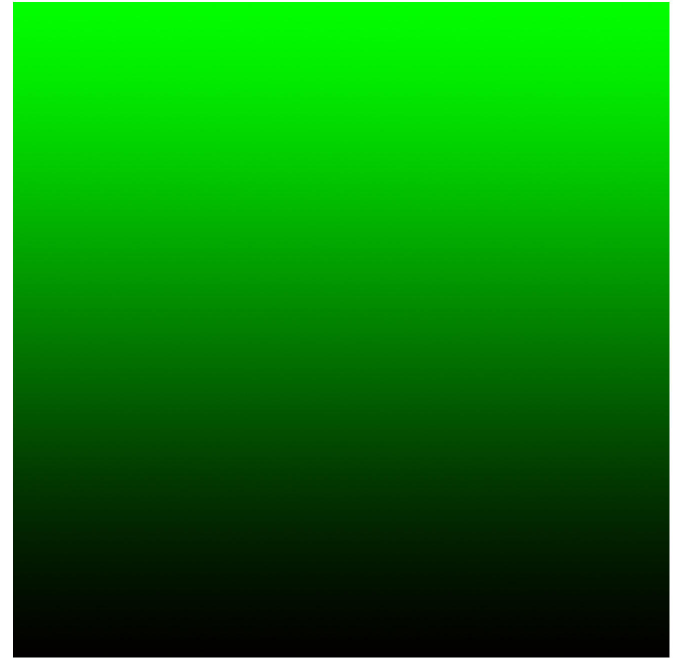


Red
(Phosphotyrosine)

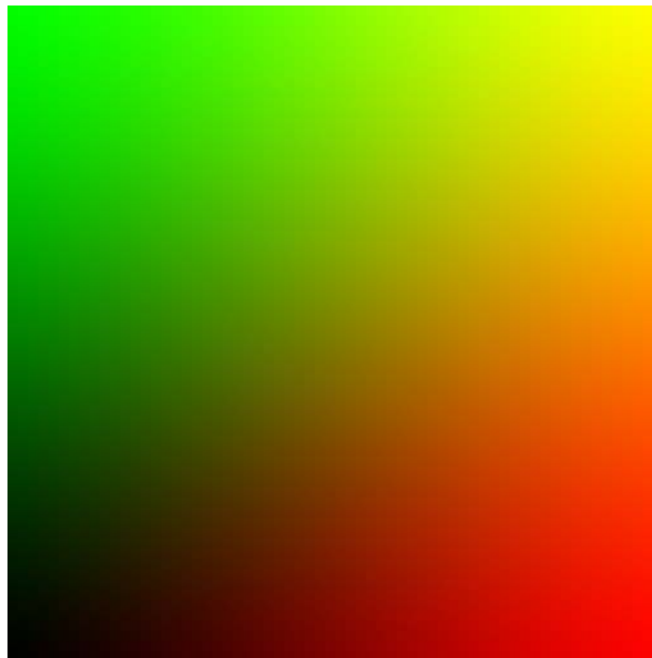
Red + Green



Red 0% to 100%

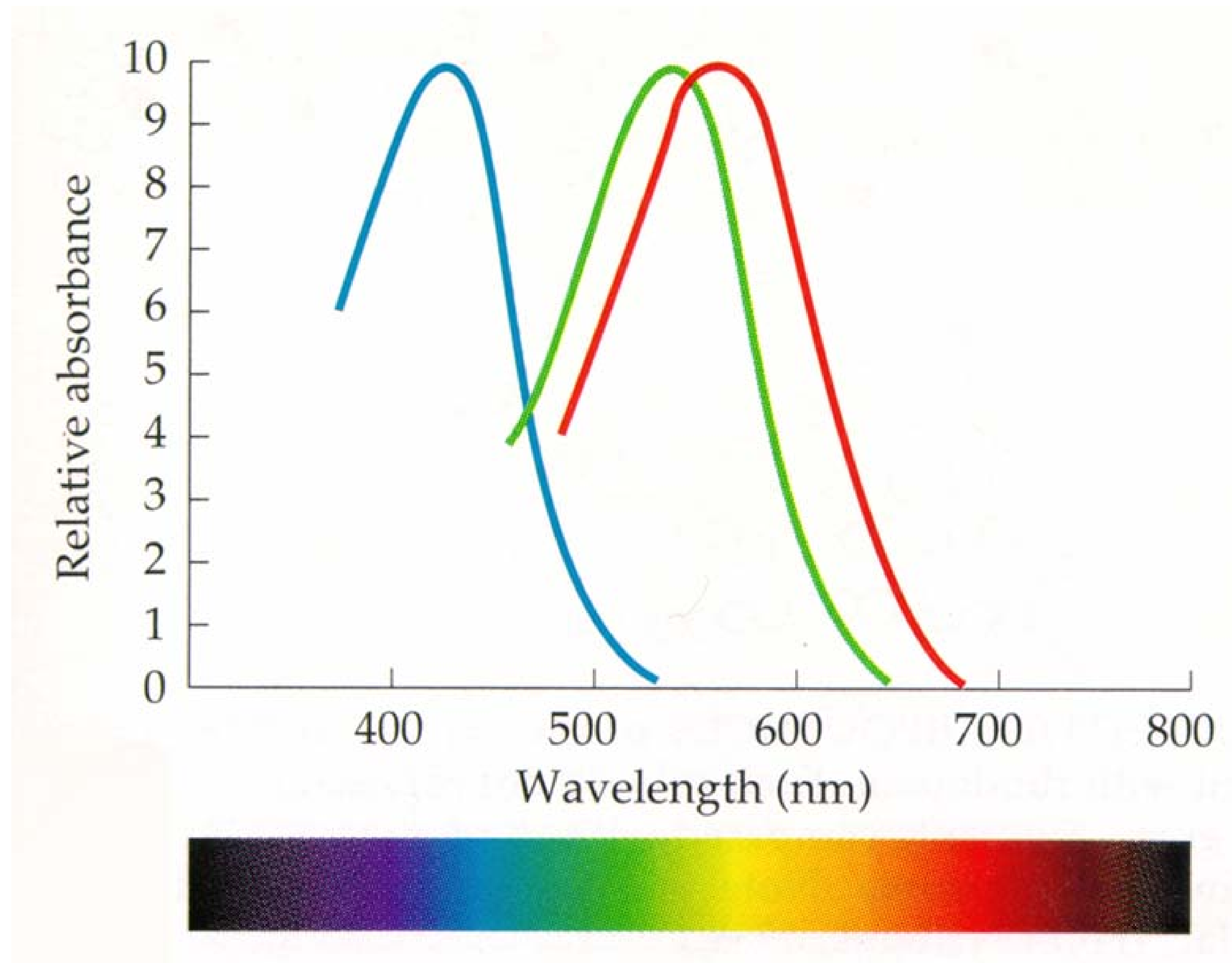


Green 0% to 100%

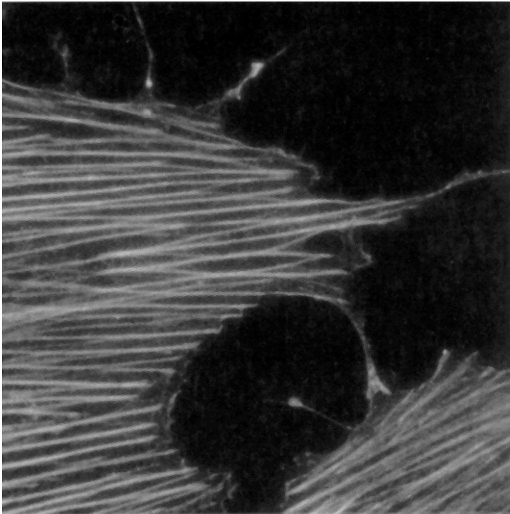


Sum of Red & Green gradients

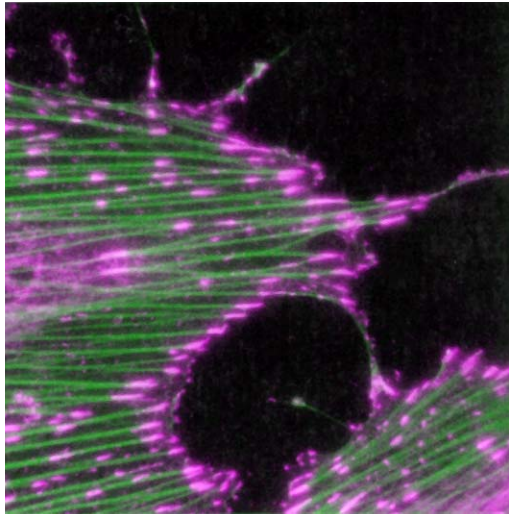
Cones – Trichromat (normal color vision)



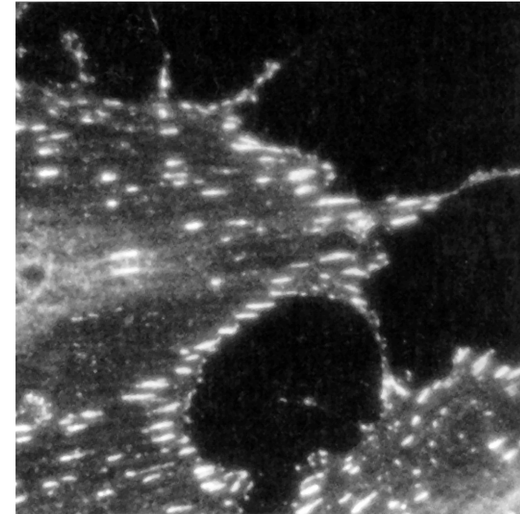
Microscopy –Green Magenta Overlay



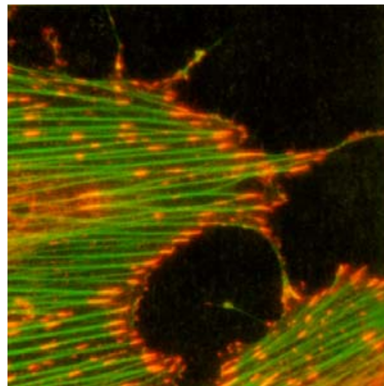
Green
(Actin)



Dichromats
are happy

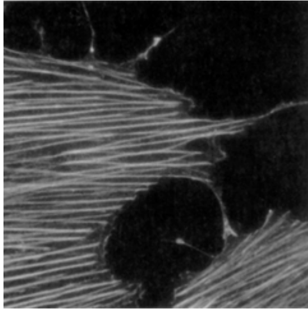


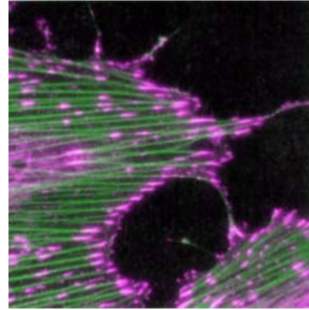
Magenta
(Phosphotyrosine)

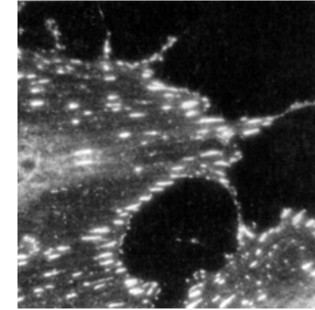


Dichromats
are unhappy

Overlay Summary







Display channels separately in gray scale

Include color overlay for pizzazz factor

Green and magenta good for colorblind viewers

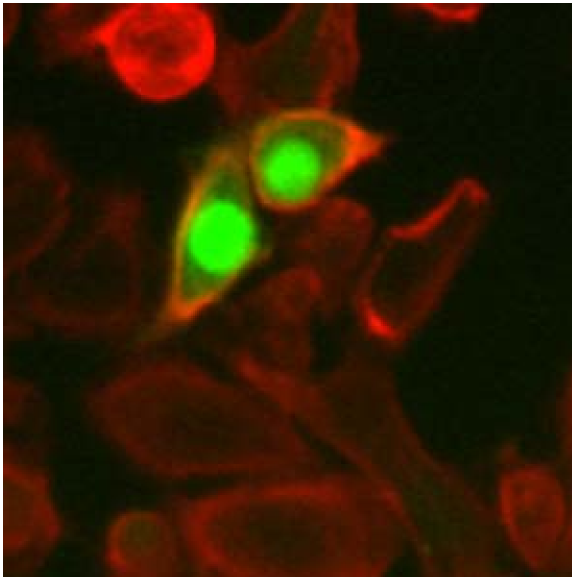
Still can not see different color where green and magenta structures overlay.

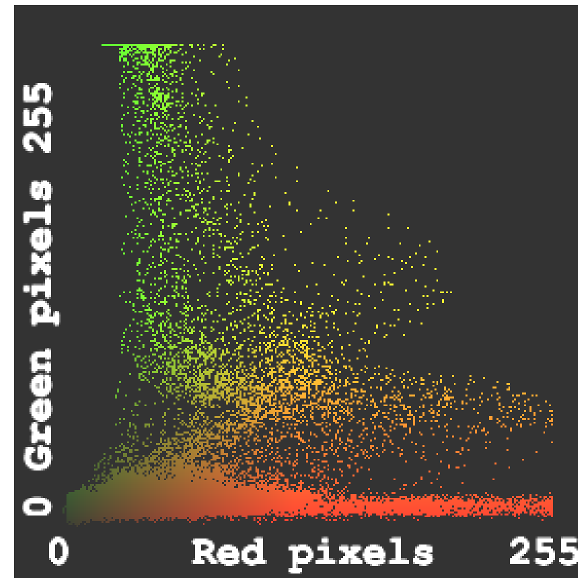
Quantitative solution to measuring overlay - fluorogram

Fluorograms

Use confocal images in order to get good spatial resolution in the x-y & z-axes

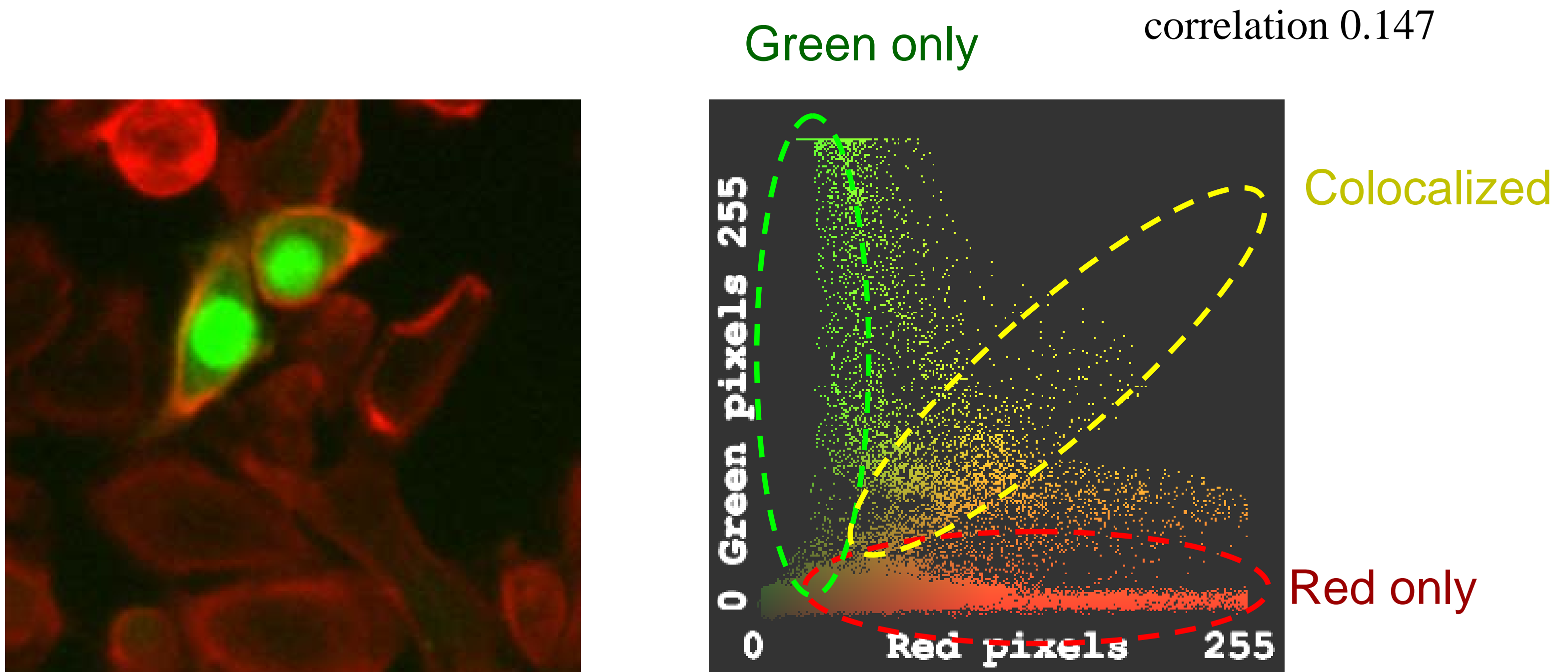
correlation 0.147





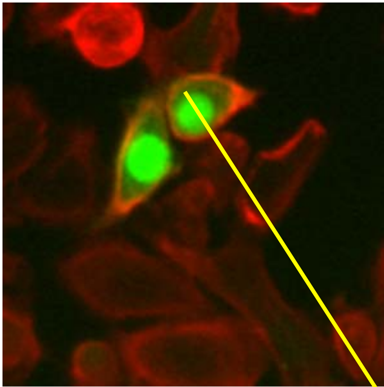
Fluorograms

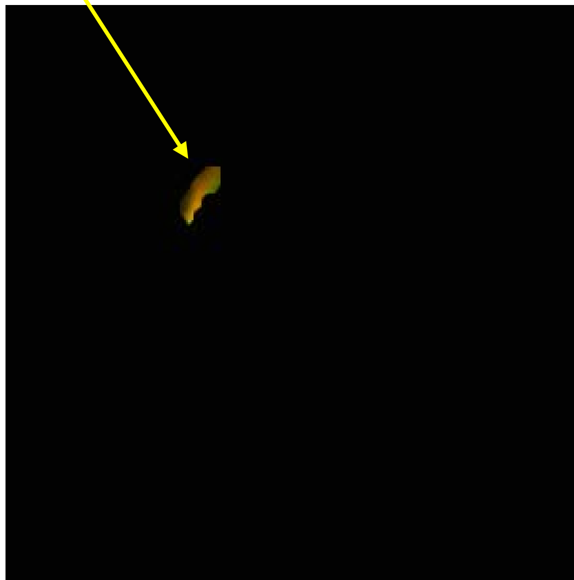
Use confocal images in order to get good spatial resolution in the x-y & z-axes

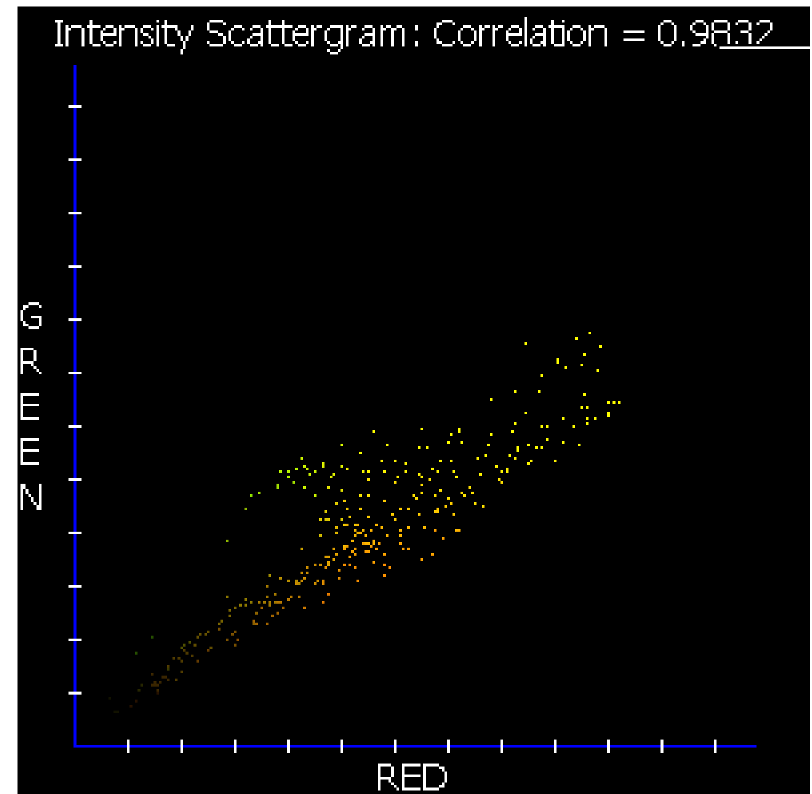


Fluorograms

Subregion from a patch of cell membrane







Fluorogram summary

- Fluorograms are a objective measure of co-localization

- Images must be of thin sections

- Region to analyze must be carefully chosen

- Dyes, filters & excitation must be carefully chosen to avoid cross talk

- Software for generating fluorograms is available
  e.g. C-Imaging, Zeiss and Leica software, ImageJ plugin

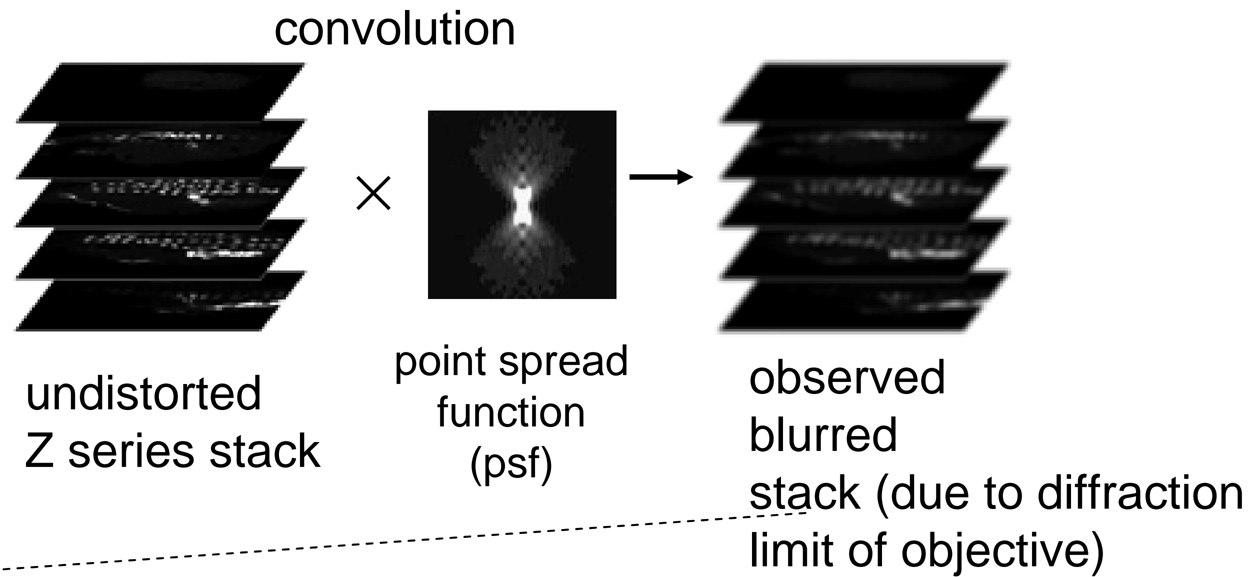
- Co-localization does not necessary imply interaction

- Interaction can be measured with FRET, FCS and other techniques

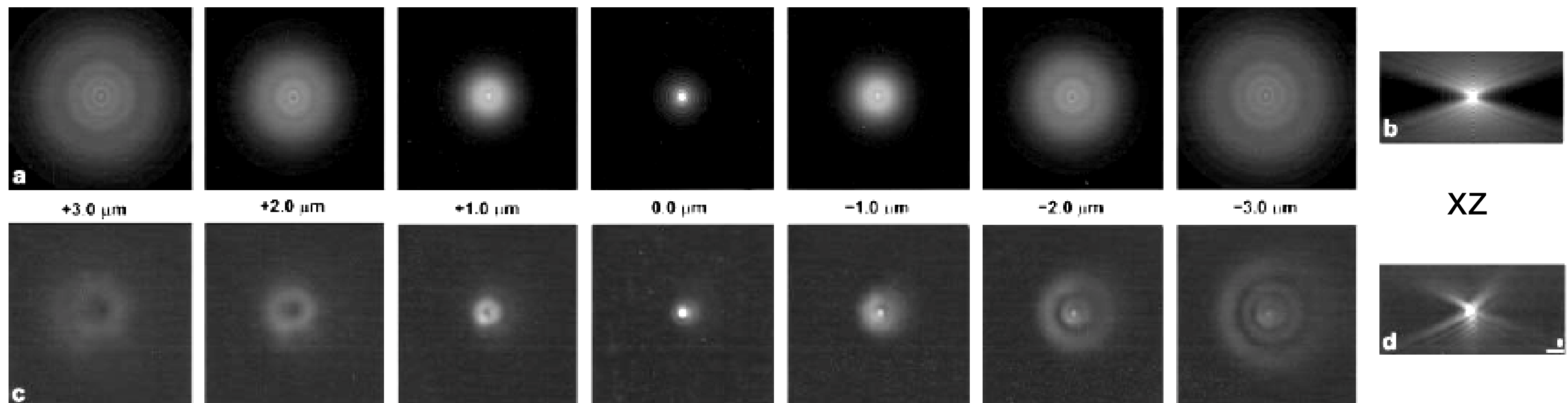
4. Deconvolution

- 1. Live Cell Imaging
- 2. Bleed Through
- 3. Co-localization
- 4. Deconvolution
    - Principals
    - Examples
- 5. Software

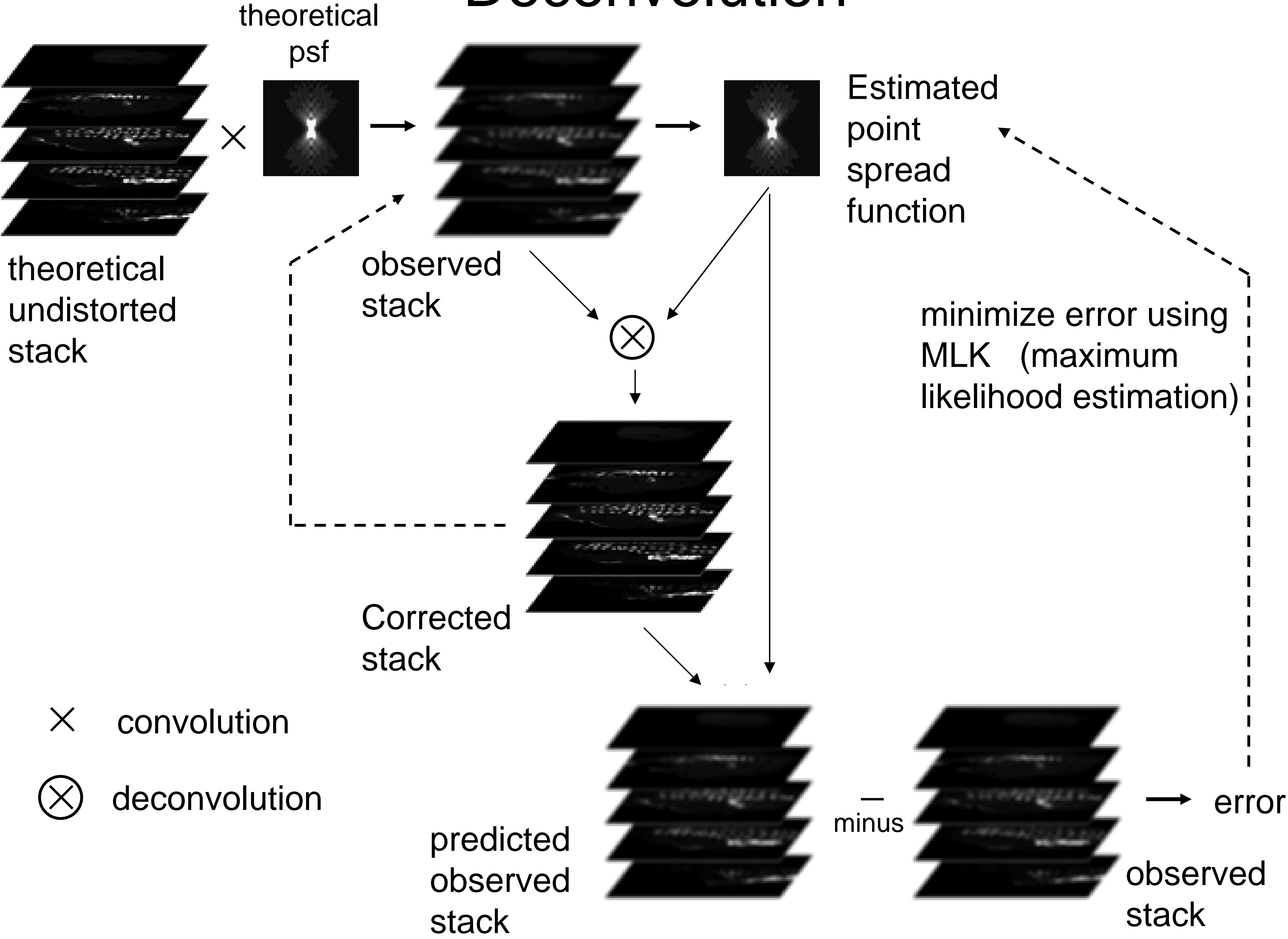
Deconvolution



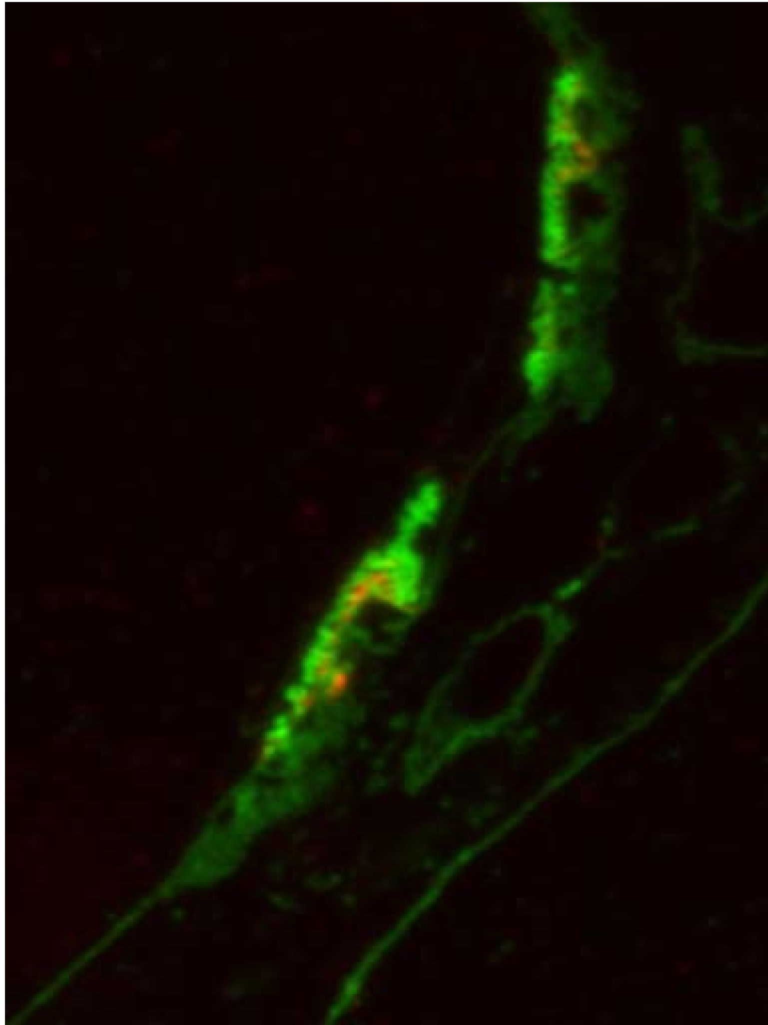
Calculated psf

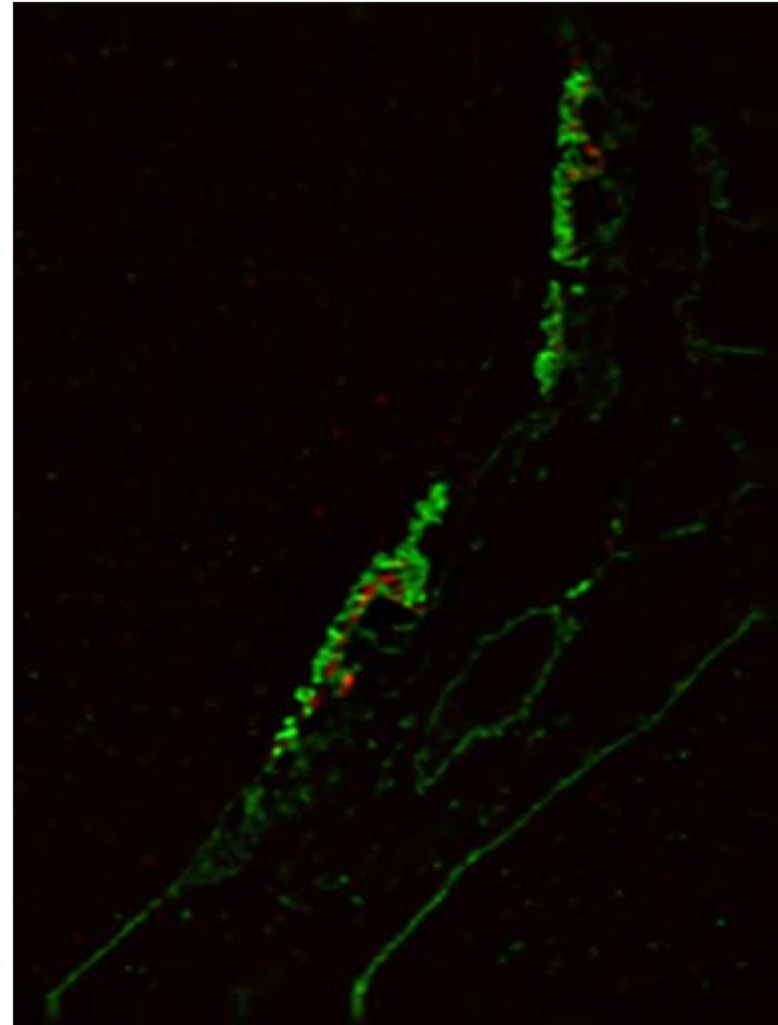


Measured psf (using a < 0.2 um bead)

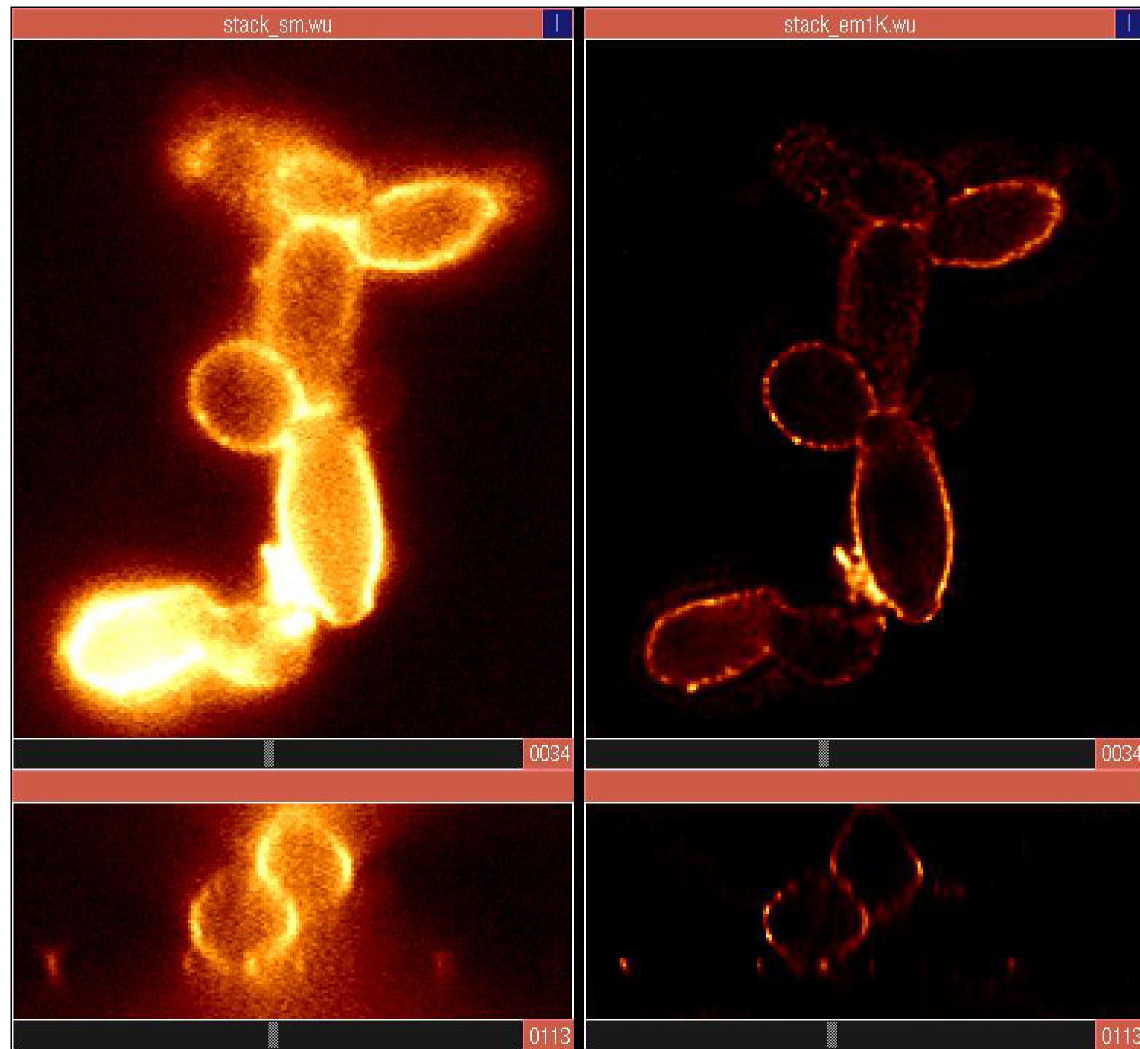

Deconvolution
theoretical
psf
Estimated
point
spread
function
theoretical
undistorted
stack
observed
stack
minimize error using
MLK (maximum
likelihood estimation)
Corrected
stack
convolution
deconvolution
predicted
observed
stack
minus
error
observed
stack

Neuro Muscular Junction – thin section





Deconvolution



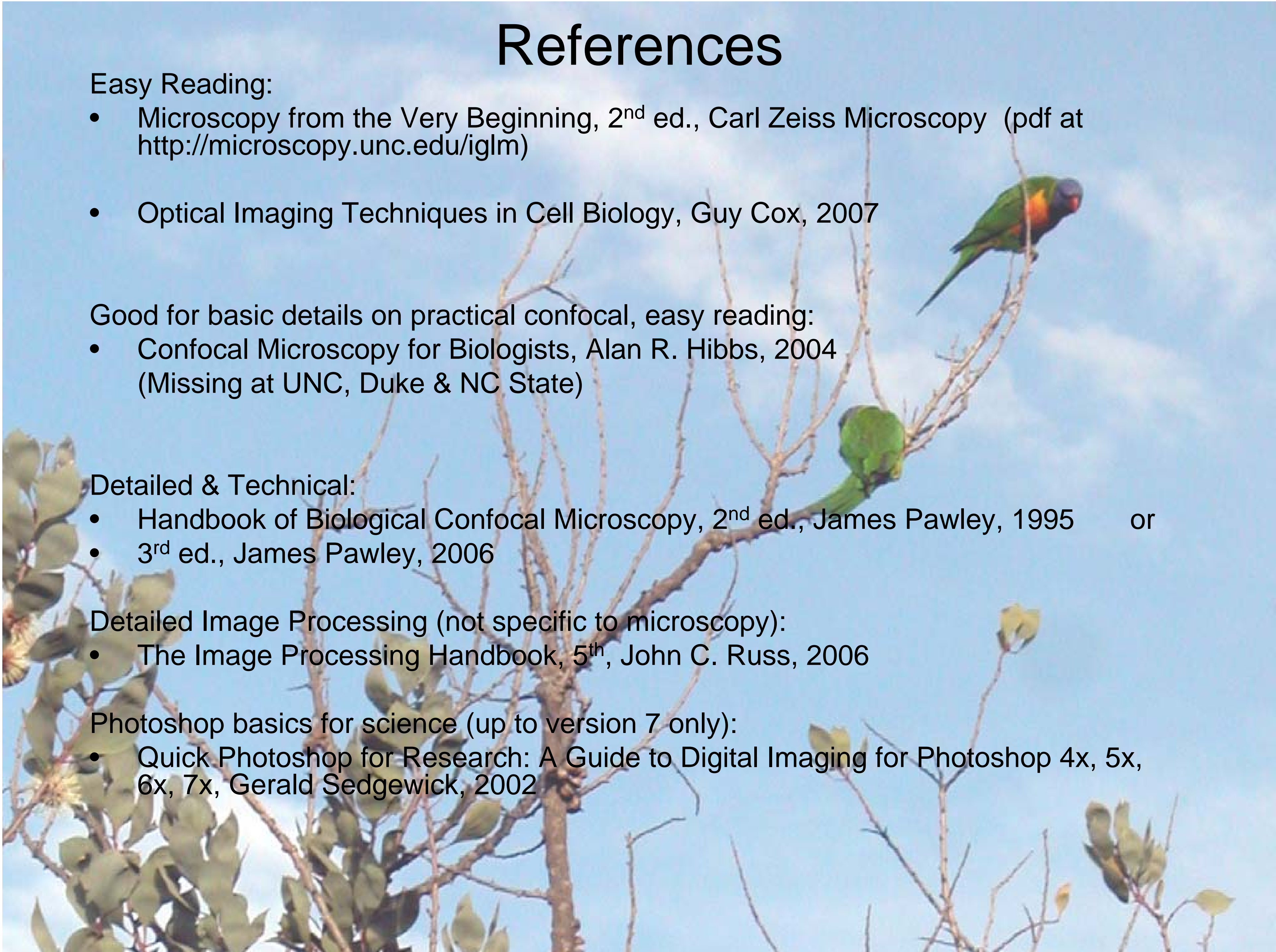
Deconvolution

- Images are blurred due to:
  - Out of focus light
  - Diffraction limiting of imaging
  - Spherical aberration
- These effects are linear and can be undone
- Use point spread function of optical system to characterize diffraction and other optical aberrations. Obtain PSF:
  - By measuring with a subresolution bead (<100 nm): "measured deconvolution"
  - By calculating from image stack through the sample by assuming point spread is same for any location in the sample volume. "blind deconvolution"
- Can apply to widefield stacks or confocal stacks
- Several algorithms. Different results depending on structure
- Work well when features are distinct and thin. e.g. chromosomes. Not so good when features are indistinct, i.e. volumes
- Computationally intensive
- Transmitted light deconvolution is being developed

- Software available for deconvolution:
    - C-Imaging (one workstation at MHMF)
    - ImageJ plugin (slow, but free see plugins at http://rsb.info.nih.gov/ij/)
    - Volocity (not at MHMF, deconvolution module $12K)
    - Several other packages, e.g. Imaris ($20K), Autoquant/ImagePro ($15K)

5. Software at MHMF

	Photo shop	ImageJ	C-Imaging	Metamorph	Volocity	Matlab	Leica Zeiss
Image processing	+++	++	+	++	+	++	+
Quantification	+	++	++	++	+	++	++
Results to file		+	+	+	+	+	+
Batch processing	+		+	+		+	
Plugins	limited	+++				+	
3D		++	+	+	+++	++	+
Motion tracking		+	+	+	++	+	
Co-localization		+	+	+	++	+	++
FRAP/FRET		+					+
Deconv		+	+	?	+	?	
Cost	$ @ UNC	free	$$$	$$$$$	$$$$$	Free at UNC	
Comments	Must have		Can be difficult		Difficult to learn	Difficult to learn	



References

Easy Reading:

- Microscopy from the Very Beginning, 2nd ed., Carl Zeiss Microscopy (pdf at http://microscopy.unc.edu/iglm)
- Optical Imaging Techniques in Cell Biology, Guy Cox, 2007

Good for basic details on practical confocal, easy reading:

- Confocal Microscopy for Biologists, Alan R. Hibbs, 2004
  (Missing at UNC, Duke & NC State)

Detailed & Technical:

- Handbook of Biological Confocal Microscopy, 2nd ed., James Pawley, 1995 or
- 3rd ed., James Pawley, 2006

Detailed Image Processing (not specific to microscopy):

- The Image Processing Handbook, 5th, John C. Russ, 2006

Photoshop basics for science (up to version 7 only):

- Quick Photoshop for Research: A Guide to Digital Imaging for Photoshop 4x, 5x, 6x, 7x, Gerald Sedgewick, 2002