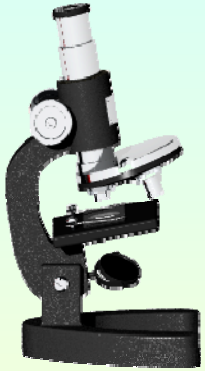
2006 Oct 30, Nov 6, 13, 20 & **27**

Quantification & Image Processing - A very brief introduction

$E = h\nu$



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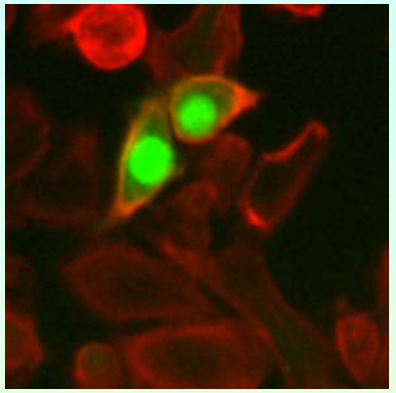
- Quantification
  - Image Photometry
  - Morphometry
  - Pixel intensity depends on 5 general factors
- Image Processing
  - Linearity
  - Overload/underload, noise margin
  - Enhancements (very briefly)
    - Linear: contrast, noise reduction, edge enhancement,
    - Non linear: selection filters median, deconvolution 2D & 3D
- Bleed through
- Colocalization – notes after the end
- Live Cell Imaging - Wendy

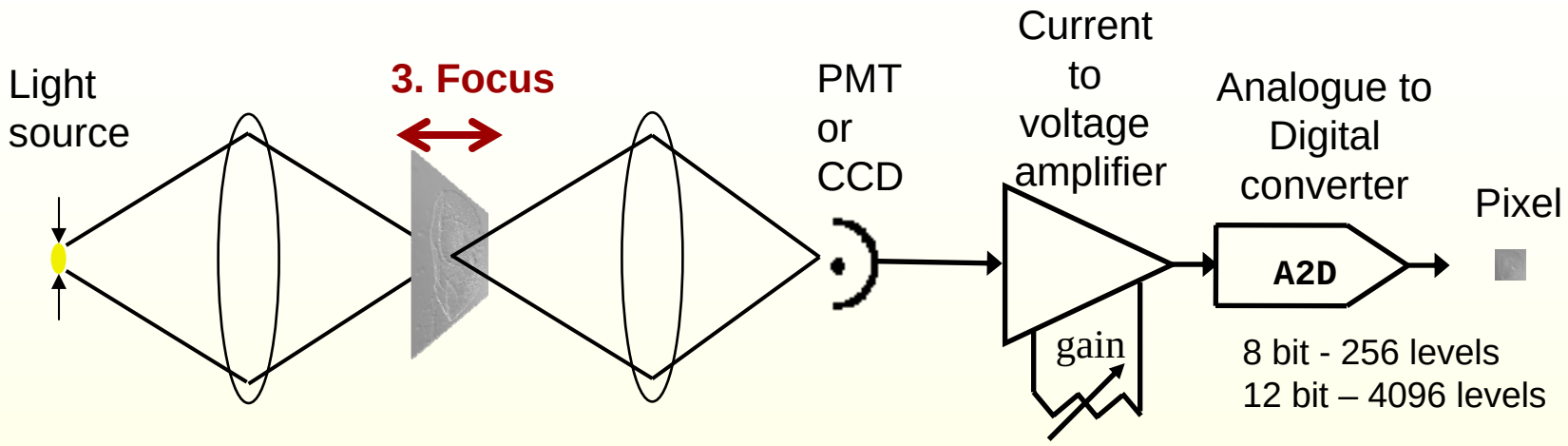
Quantification

– Image Photometry: intensity, “weight” fluorescent material in a unit region.



– Morphometry: shape, analysis of structure

Quantification



1. Illumination **2. [dye]** **4. Optical collection** **5. Detector**

i.e. objective, lenses, filters, apertures, mirrors, Pin hole size, etc.

Pixel value depends on:

1. Illumination intensity
2. Dye concentration
3. Focus
4. Optical collection
5. Detector gain

Quantification

Pixel value depends on:
1. Illumination intensity
2. Dye concentration
3. Focus
4. Optical collection
5. Detector gain

Really a multitude of detailed parameters.

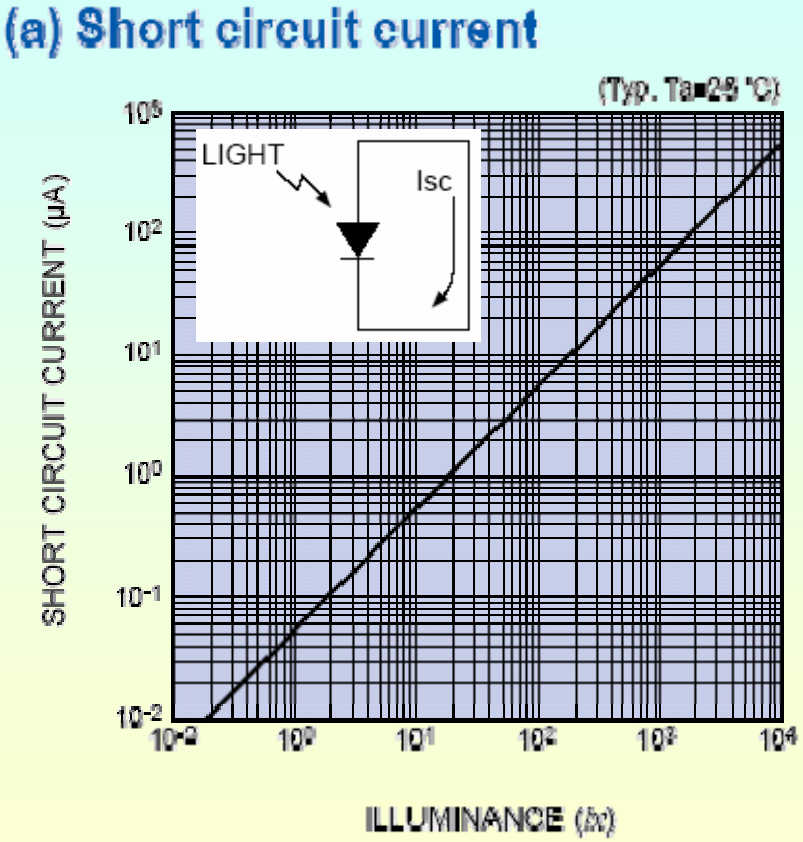
1. Illumination: arc lamp light flicker, laser oscillations, stable control of lamp voltage, long term drift, age of lamp, laser, good Kohler setup, aperture size, coupling lens efficiency, etc, etc, etc.
2. Dye concentration: light absorbance by other material, fluorescent dye not light saturated, photobleaching, etc, etc, etc.
3. Focus: stage does not drift, live cell does not move away, thickness of sample, etc, etc, etc.
4. Optical collection: objective NA, objective glass, objective aperture open, confocal pin hole size, etc, etc, etc.
5. Detector gain: exposure time, detector gain, PMT voltage, electrical gain, in linear range of detector, not overloaded A2D converter (saturation), not underloaded A2D converter (black clipping), intensifier gain, etc, etc, etc.

James Pawley published 39 steps: now has even more steps.

Work hard to keep them constant. E.g. parallel processing of samples. E.G. Time lapse can be good control.

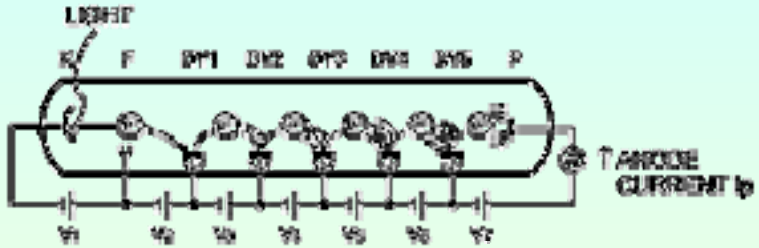
Quantification

CCD in photoconductive mode



Current out is proportional to photons/s in.

PMT at fixed anode cathode voltage

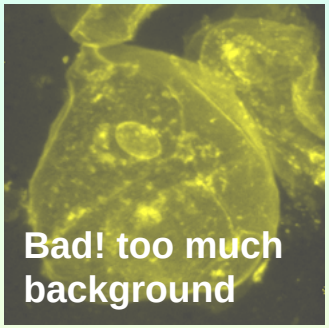


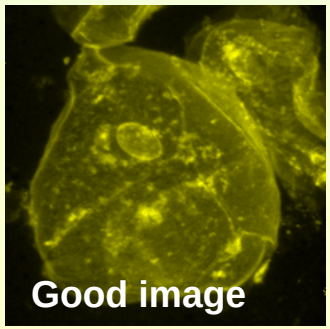
Current out is proportional to photons/s in.

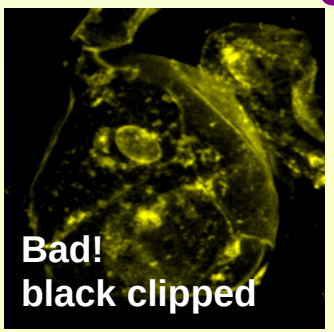
Quantification

In a well designed system
the A2D converter sets the minimum
and maximum value which can be
digitized.

Minimum is 0
Maximum set by number of levels







Forbidden
pixel values

255

Pixel value

Slope = gain = contrast

Photon flux

Offset = black level = brightness

0

Offset

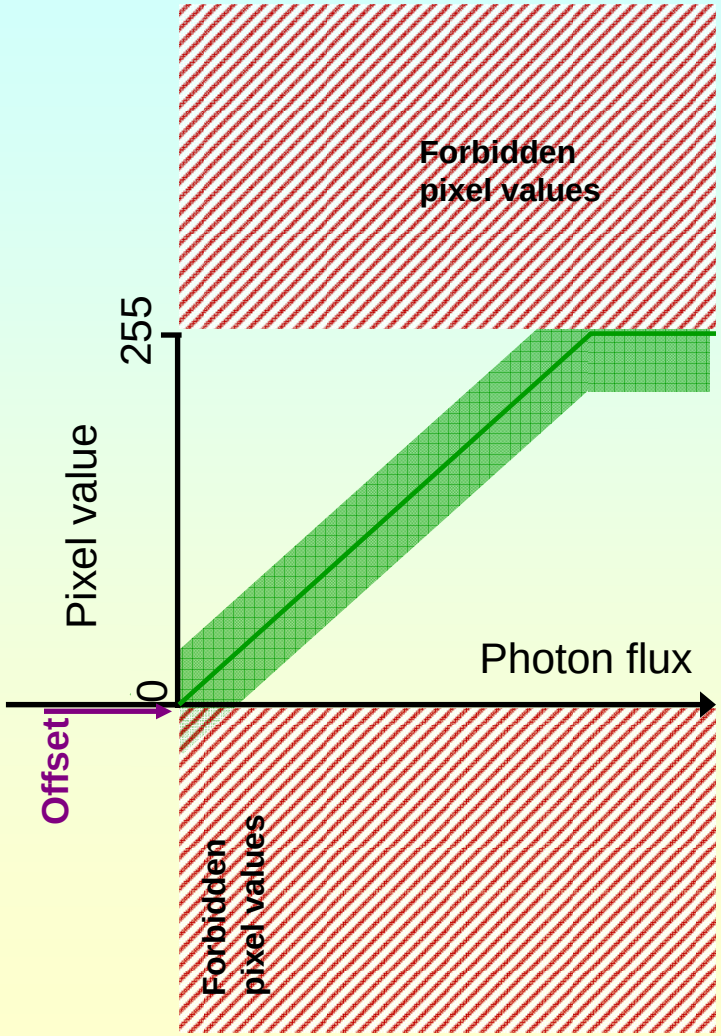
Forbidden
pixel values

-ve

Quantification

Noise adds linearly to photon signal
Noise will average to zero if sampled without clipping

Reduced range – restore contrast after averaging



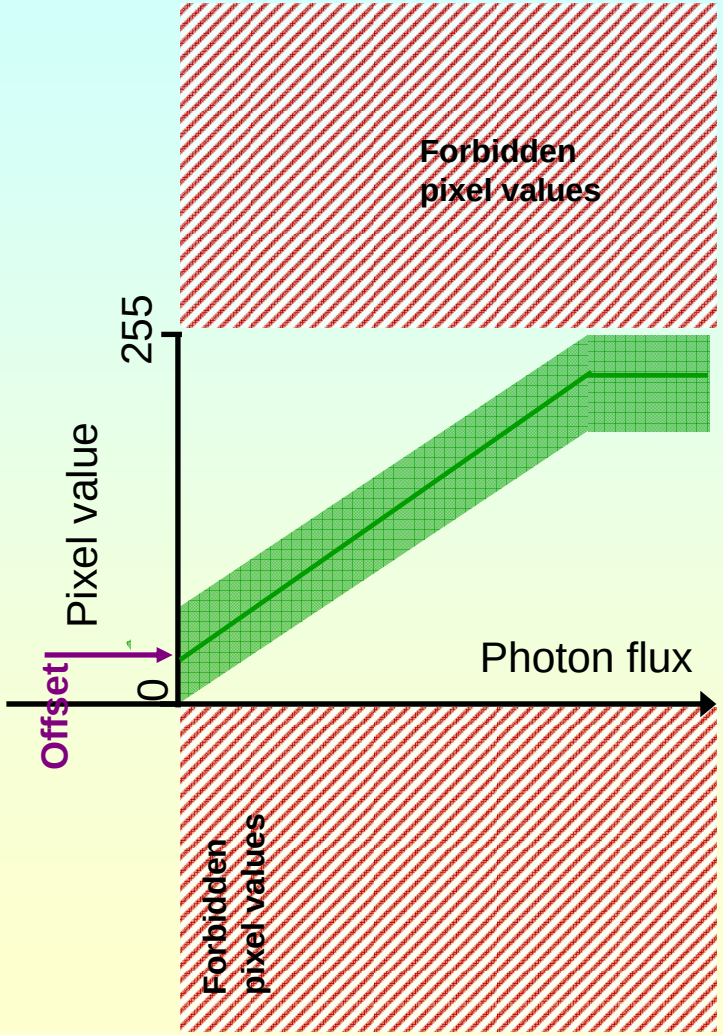
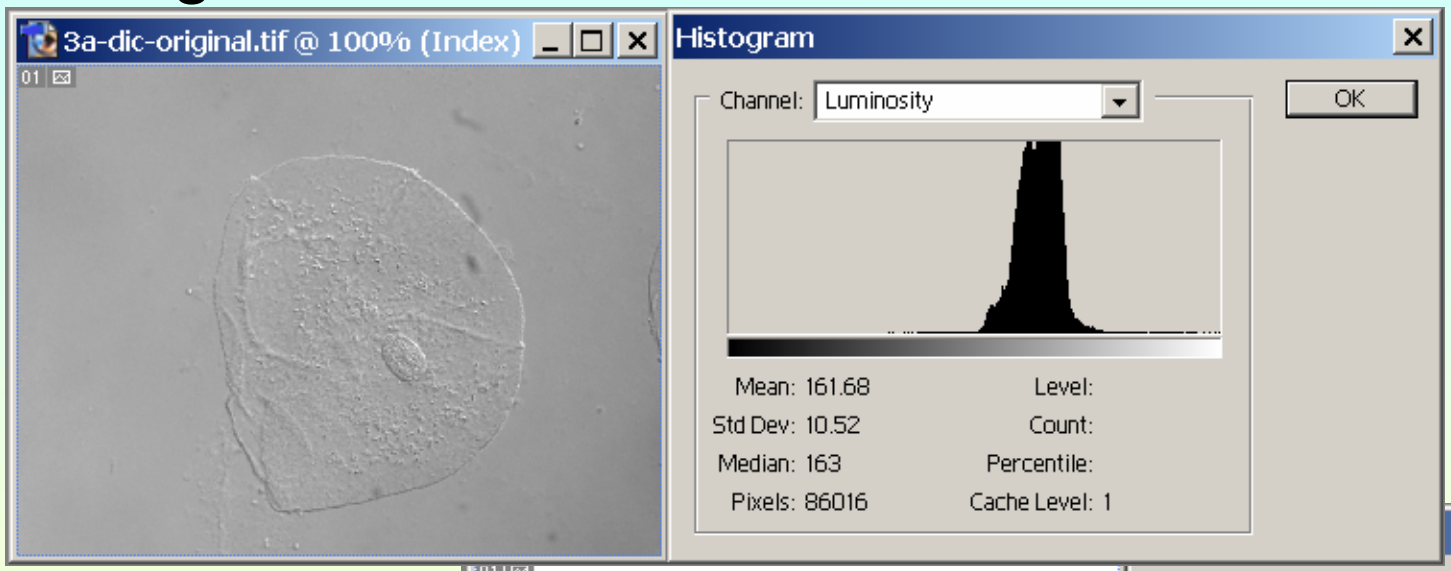
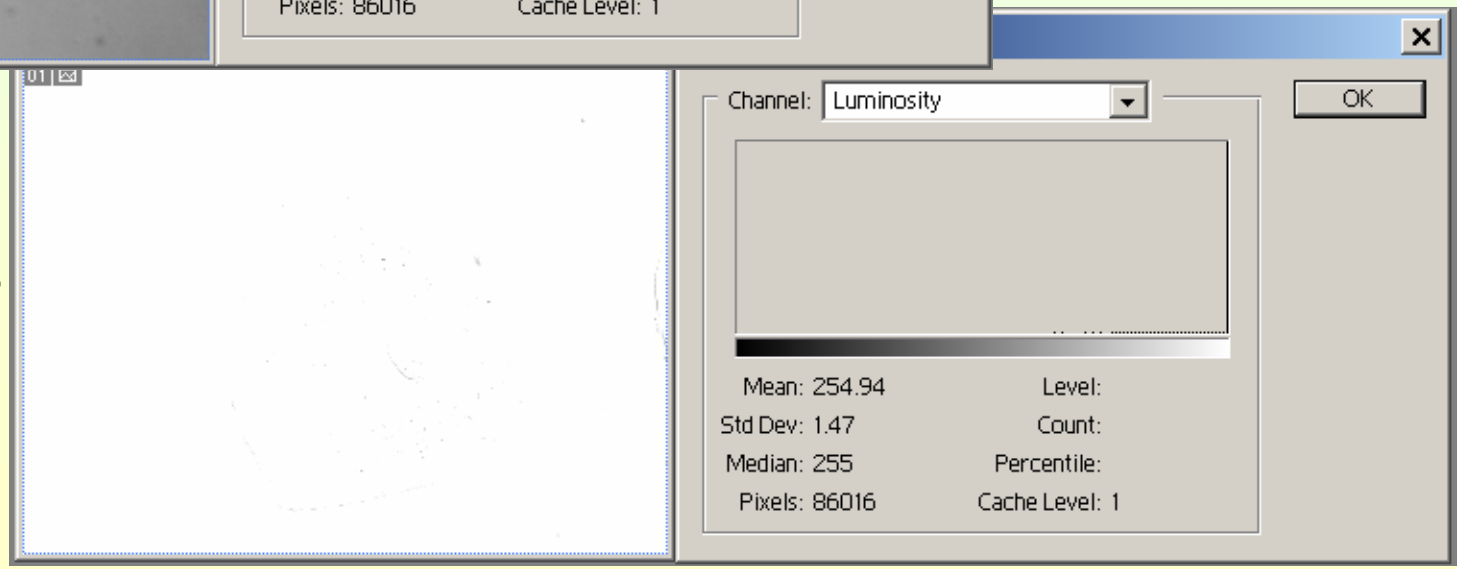


Image Processing

- Gain is same as contrast
- Brightness is the same as black level



Original image



Original times 2 (Clipped)

Image Processing

- Auto levels in Photoshop is not good.
- Black and white clipping produced

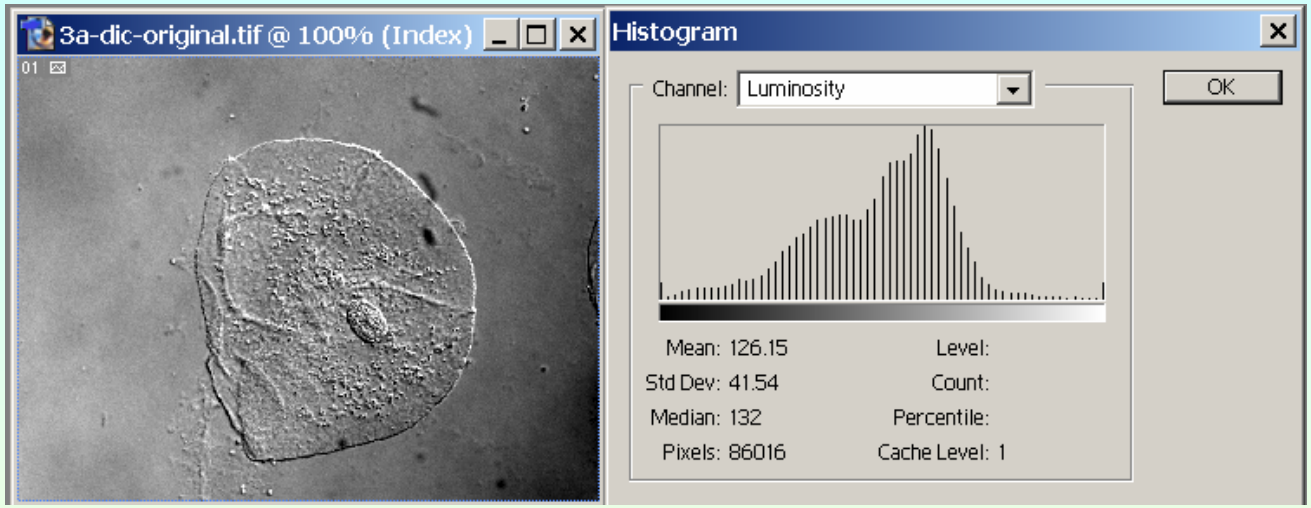


Image Processing

Manual levels. Original high resolution images lack contrast – use image processing to improve contrast

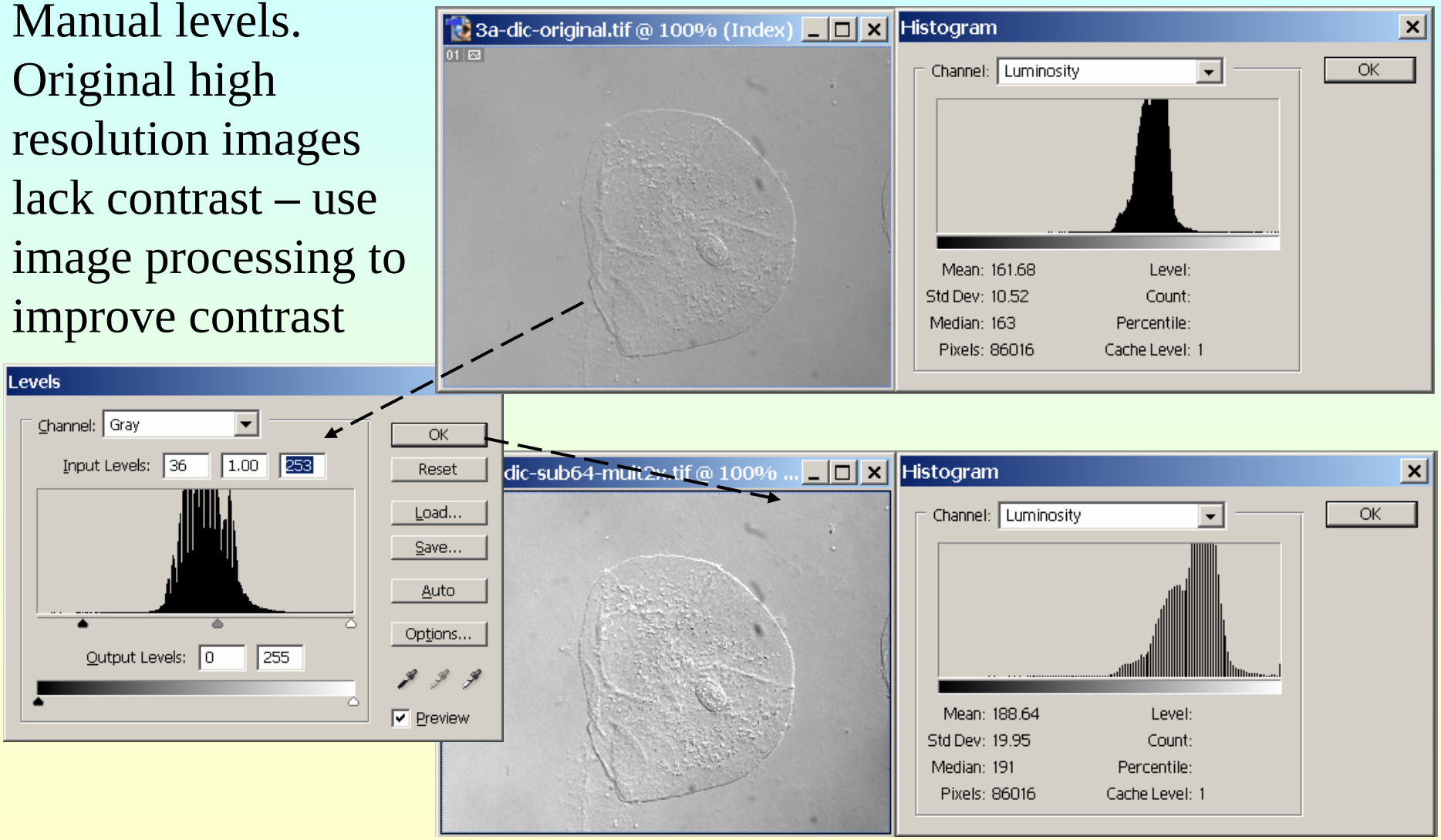


Image Processing

- 2D Filtering – kernel – enhance local contrast

e.g. to sharpen edges

-1 –1 –1
-1 9 –1
-1 –1 –1

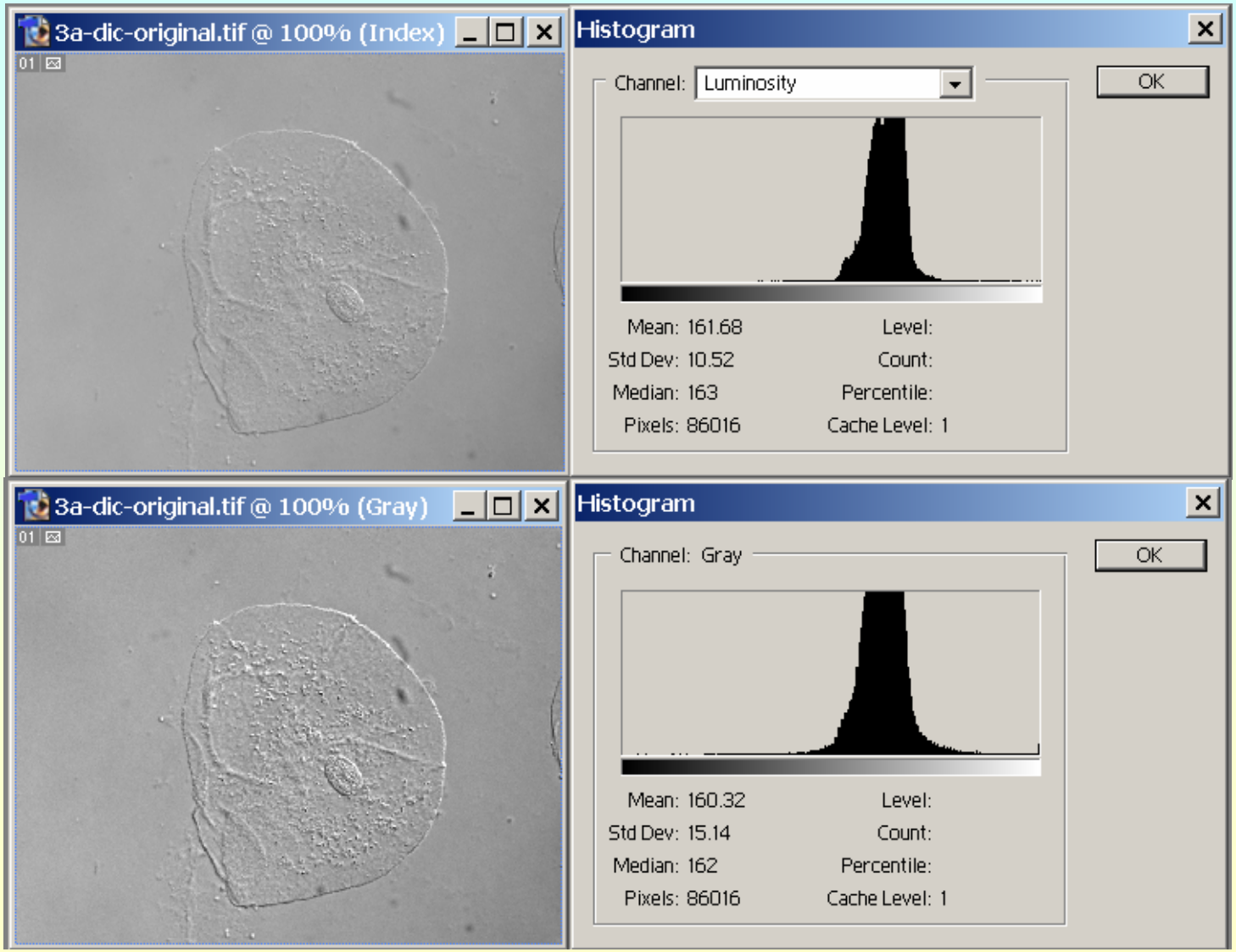


Image Processing

- 2D Filtering – kernel – median filter

Reduces noise

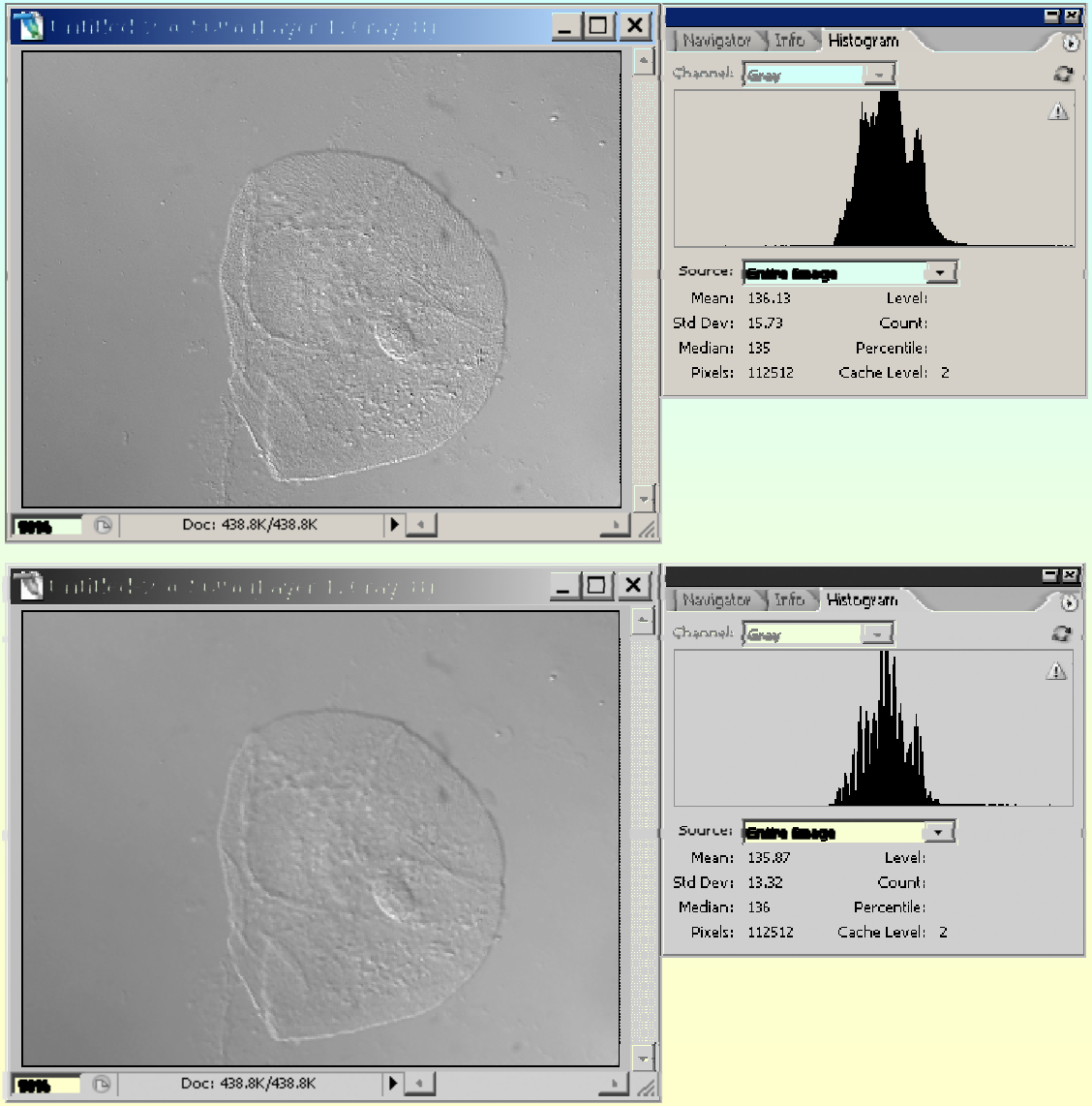
10	40	40
30	30	40
40	120	30

↓

central pixel becomes median value

↓

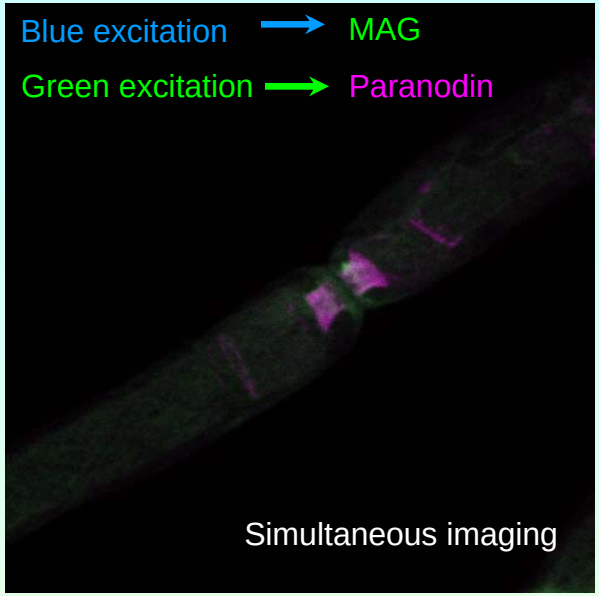
40



Barely scratched the surface of digital imaging. Some references:

- The Image Processing Handbook, 4th ed., Russ, J., CRC Press
- Video Microscopy: the Fundamentals, Inoue, S., Spring, K., 2nd ed., Plenum Press
- http://www.olympusmicro.com/
- http://www.microscopyu.com/
  (Fun and interesting. Some of the principals especially depicted in some figures are confusing or not entirely correct.)
- ImageJ http://rsb.info.nih.gov/ij/ (Free, kind of. Your taxes!)

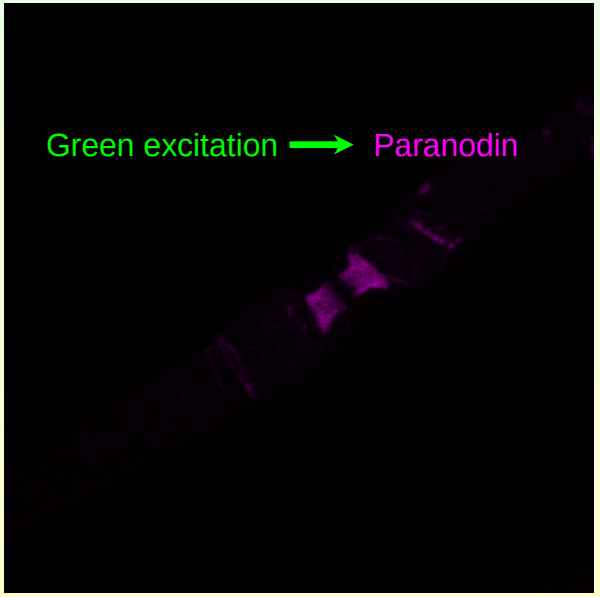
Emission Bleed Through Test



→ Turn off green excitation →

Bad!

Turn off blue excitation →



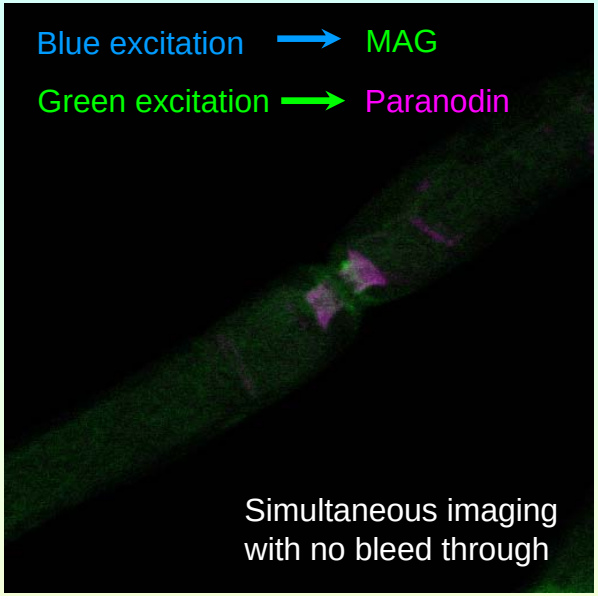
Expected

(MAG = Myelin Associated Glycoprotein)

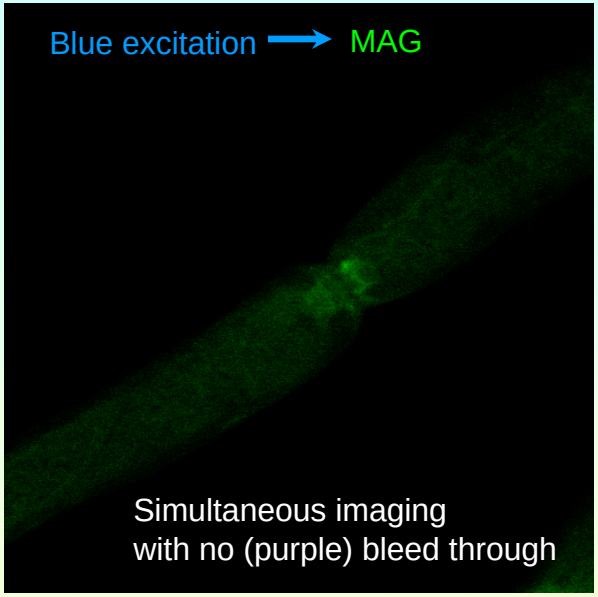
Paranodin = RED

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

Summary

Kohler illumination

Condenser aperture

- Open: best resolution, even illumination
- Minimum: high contrast, larger depth of focus

$d = \lambda / 2\ NA$, d=resolution, λ=wavelength, NA=numerical aperture

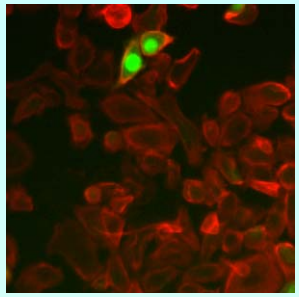
Confocal

- Removes out of focus information
- Improves x+y-axis resolution, z-axis resolution substantially

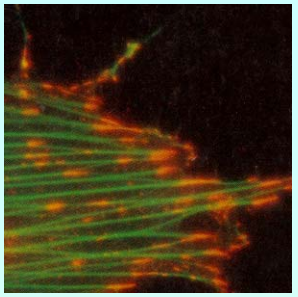
Acquire carefully

- Avoid overload and underload

http://microscopy.unc.edu/notices2



Colocalization in Optical Microscopy



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

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

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

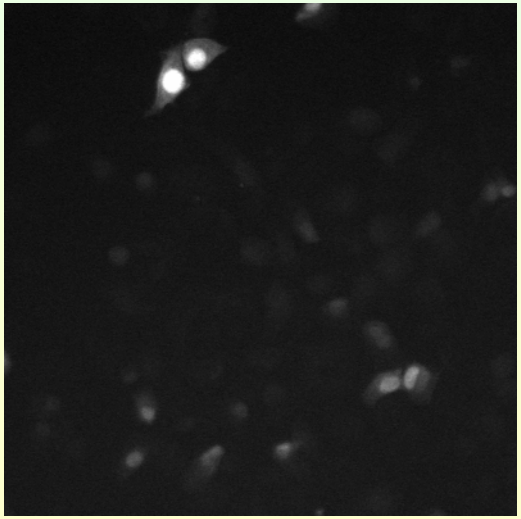
Co-location at the cell level

mixed cells +

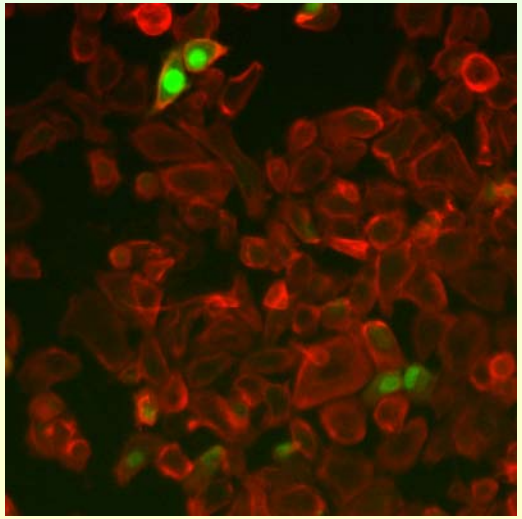
GFP vector +

dsRed vector =

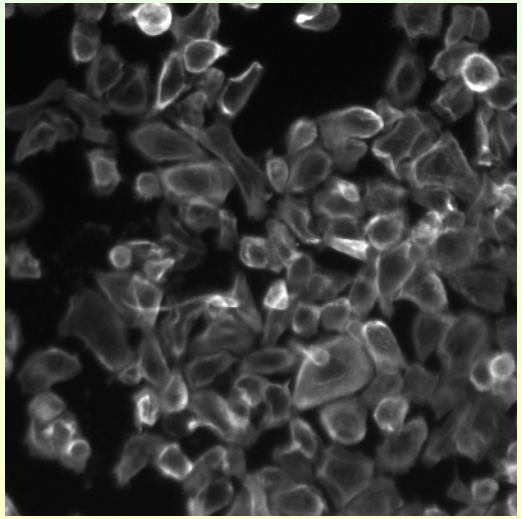
GFP



Overlay

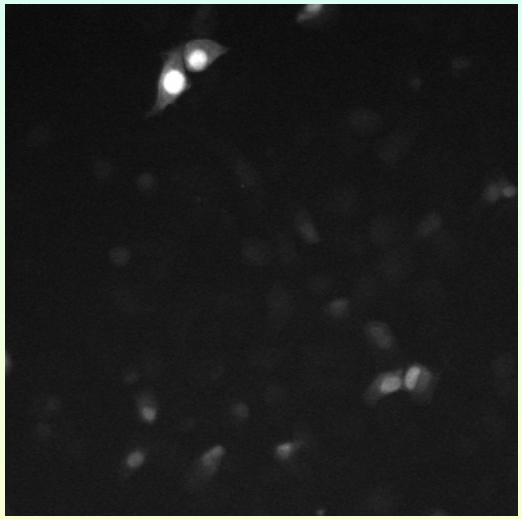
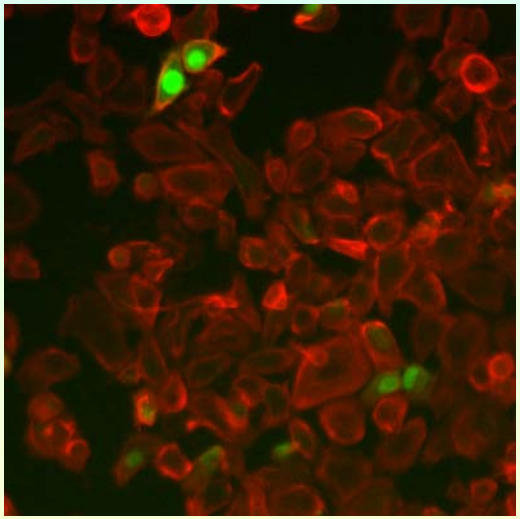
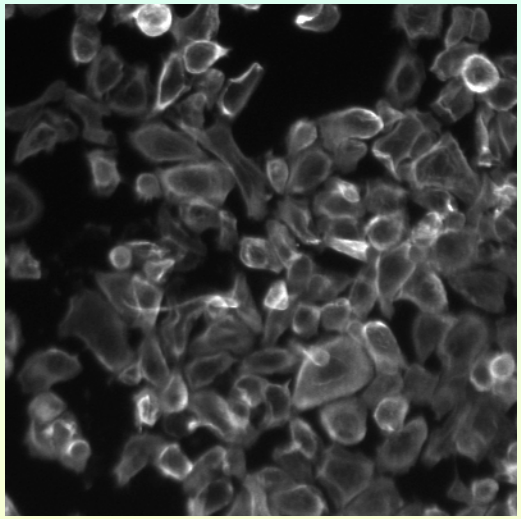


dsRed

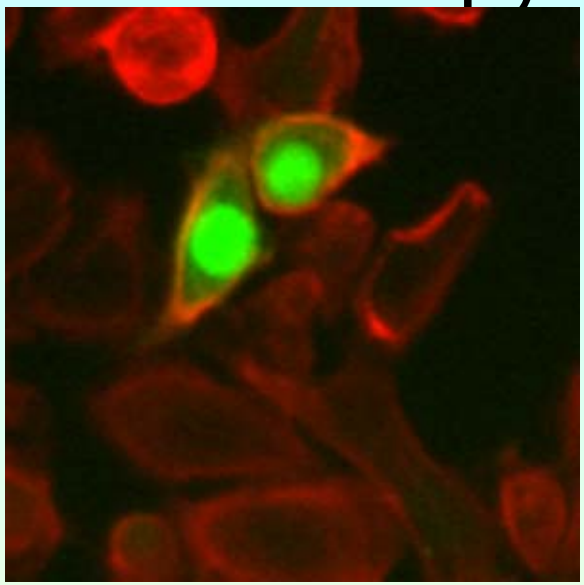


Co-location at the cell level

Count cell types based on expression in cell using a microscope (e.g. Flowcytometry)

Microscopy



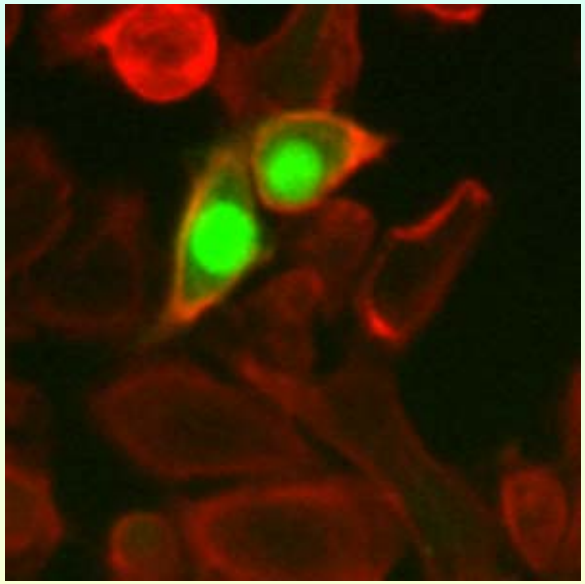
Spatial information – see structures inside the cell

With Fluorescent labels can also see sub resolution structures

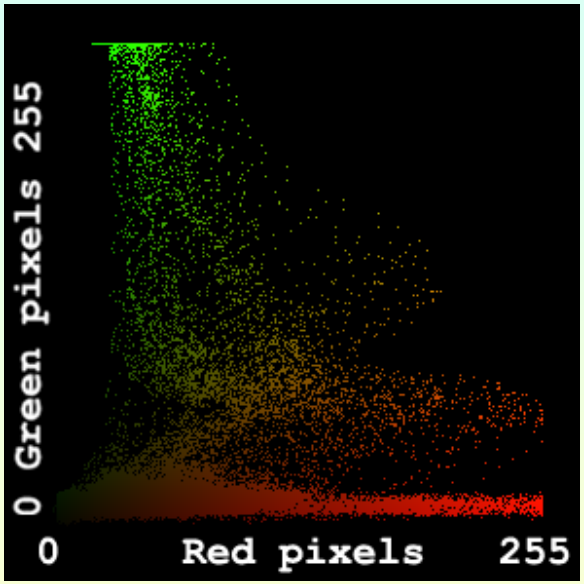
- See emitted light without necessarily resolving them

Fluorograms

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

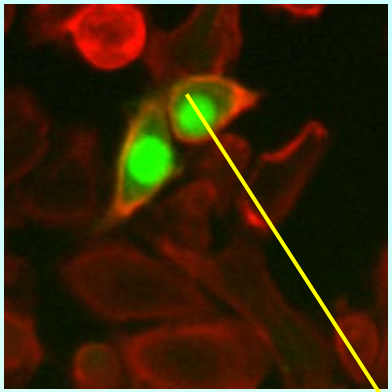


correlation 0.147

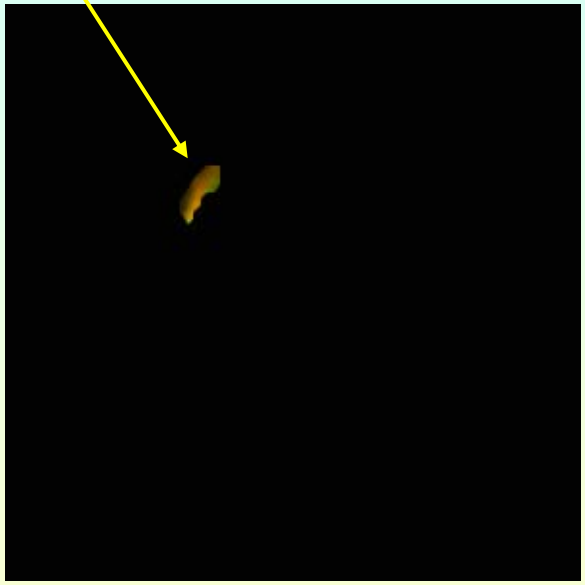


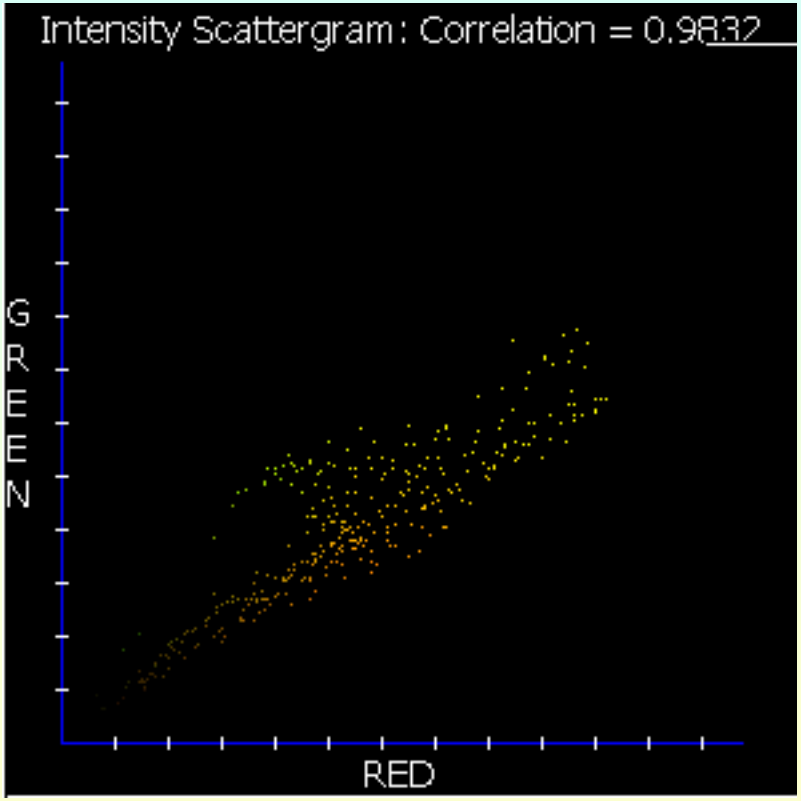
Green vertical and red horizontal scattered points indicate that red & green labels do not associate

Fluorograms



Subregion from a patch of cell membrane





Diagonal scatter show that red & green
Labels colocalize in the membrane