Quantification & Image Processing
- A very brief introduction

E = hν

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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]
3. Focus
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

PMT at fixed anode cathode voltage

(a) Short circuit current

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.

Slope = gain = contrast
Offset = black level = brightness

Forbidden pixel values

Good image
Bad! too much background
Bad! black clipped

Photon flux
Pixel value

-ve
255
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
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

  \[
  \begin{matrix}
  -1 & -1 & -1 \\
  -1 & 9  & -1 \\
  -1 & -1 & -1 \\
  \end{matrix}
  \]
Image Processing

- 2D Filtering – kernel – median filter

Reduces noise

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central pixel becomes median value

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Barely scratched the surface of digital imaging. Some references:


- 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**

Blue excitation → MAG
Green excitation → Paranodin

Simultaneous imaging

→ Turn off green excitation →

Blue excitation → MAG

Should just see green MAG! But just see bleed through swamping green detection channel

Bad!

Green excitation → Paranodin

→ Turn off blue excitation →

(MAG = Myelin Associated Glycoprotein) Paranodin = RED

Expected
Emission Bleed Through Corrected

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

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

\[ d = \frac{\lambda}{2 \, \text{NA}}, \quad d = \text{resolution}, \quad \lambda = \text{wavelength}, \quad \text{NA} = \text{numerical aperture} \]

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