Tips and Pitfalls in Microscopy

Alexia Ferrand
Imaging Core Facility
Biozentrum
Basel
Basic optics...

Images are not identical to the object

Image = Object x Point spread function
Basic optics...

**The Airy Disk**

Caused by diffraction on objective aperture  
*Infinitely small self luminous points*  
Negative impact on resolution  
*(approx 200nm limit)*

\[
\text{resolution} = 0.61 \times \frac{\lambda}{n \sin \alpha}
\]

*Where:*  
\[n \sin \alpha = \text{NA} \]
\[n = \text{RI of medium} \]
\[\alpha = 1/2 \text{ objective collection angle} \]
\[\lambda = \text{wavelength of light} \]

*after GB Airy, British astronomer, 1834*

Adapted from Bob Fleming, Bio-Imaging, The Roslin Institute Edinburgh
Basic optics…

The Airy Disk

Rings caused by interference at optical wavefronts

Adapted from Bob Fleming, Bio-Imaging, The Roslin Institute Edinburgh
Basic optics...

**The Airy Disk**

*Classic Point Spread Function (PSF) of object (bead) in Z projection should be “hourglass” shape*
Basic optics…

The Airy Disk

*If PSF is “wineglass”…*

To improve your image:

There are key steps to be aware of

Adapted from Bob Fleming, Bio-Imaging, The Roslin Institute Edinburgh
Outline

- Image Acquisition for fixed samples
- Image Acquisition for live imaging
- Image Processing
Image Acquisition for fixed samples

The brighter, the better!*  

*Except when it saturates…

Image taken on our confocal SP5-Matrix room 187 (AF)
Fixation of your samples

- «Standard» Fixation: PFA
- Alternative: 100% MeOH
- Methanol works nicely for microtubules and centrosomes
- For fluorescent proteins, DON’T USE MeOH!
- Also poor results with phalloidin when using MeOH

- Store in the dark at 4°C or -20°C depending on your mounting media.
Preparation of your samples

- The sample is part of the optics
- The last 500 um are important

1 – coverglass
Preparation of your samples

- The sample is part of the optics
- The last 500 um are important
  1 – coverglass (spherical aberrations)

<table>
<thead>
<tr>
<th>Numerical Aperture</th>
<th>0.01 mm Deviation</th>
<th>0.02 mm Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.30</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>0.45</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>0.70</td>
<td>2 percent</td>
<td>8 percent</td>
</tr>
<tr>
<td>0.85</td>
<td>19 percent</td>
<td>57 percent</td>
</tr>
<tr>
<td>0.95</td>
<td>55 percent</td>
<td>71 percent</td>
</tr>
</tbody>
</table>
Preparation of your samples

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2 – mounting media (avoid bubbles)

<table>
<thead>
<tr>
<th>Medium</th>
<th>Refractive Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permount (Homaica)</td>
<td>1.54</td>
</tr>
<tr>
<td>glas</td>
<td>1.52</td>
</tr>
<tr>
<td>Immersion oil</td>
<td>1.52</td>
</tr>
<tr>
<td>Canacia balsam</td>
<td>1.52</td>
</tr>
<tr>
<td>DPX</td>
<td>1.52</td>
</tr>
<tr>
<td>Fluorescence Mounting Medium (DAAB)</td>
<td>1.47 – 1.50</td>
</tr>
<tr>
<td>Mowiol</td>
<td>1.49</td>
</tr>
<tr>
<td>Prolong Gold (Prokala)</td>
<td>1.47 – 1.41</td>
</tr>
<tr>
<td>Kaisers glycerol jelly</td>
<td>1.47</td>
</tr>
<tr>
<td>Vectashield</td>
<td>1.46</td>
</tr>
<tr>
<td>Vectashield / glycerol + 2% DAAB</td>
<td>1.46</td>
</tr>
<tr>
<td>glycerol / water in different ratios</td>
<td>1.47 – 1.33</td>
</tr>
<tr>
<td>glycerol (100%)</td>
<td>1.47</td>
</tr>
<tr>
<td>glycerol 50% (w/w)</td>
<td>1.46</td>
</tr>
<tr>
<td>glycerol 60% (w/w)</td>
<td>1.45</td>
</tr>
<tr>
<td>Aquatex</td>
<td>1.40 – 1.41</td>
</tr>
<tr>
<td>albumin/water</td>
<td>1.42 – 1.33</td>
</tr>
<tr>
<td>Fluoromount-G (Southern Biotech Assoc.)</td>
<td>1.39</td>
</tr>
<tr>
<td>Gelmount (Homaica)</td>
<td>1.36</td>
</tr>
<tr>
<td>water</td>
<td>1.33</td>
</tr>
<tr>
<td>air</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Always check immersion medium for compatibility with your dyes!

Source: Leica

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Bleaching of various fluorophores in $\rho$-Phenylenediamine

Mounting medium: $\rho$-Phenylenediamine

Alexa Fluor 594 and Alexa Fluor 488 are the fluorophores of choice
Preparation of your samples

- The sample is part of the optics
- The last 500 um are important
  3 – matching the refractive indexes (spherical aberrations)

U Plan-Apo 100X / 1.35 NA objective
Microtubules, centrosomes and DNA are stained. Seeing is believing? A beginners’ guide to practical pitfalls in image acquisition. AJ North, JCB, 2006
Preparation of your samples

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Preparation of your samples

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  4 – Schmutz effect
Choice of the lens

- What information can you get from the lens?
Choice of the lens

- What influences the resolution?

Wing imaginal disk taken on our confocal SP5-MP room 235 (F.Hamaratoglu)
Choice of the lens

- What influences the resolution?
  - NA of the objective
    * Light gathering ability
  - The higher the NA, the better the details,
Choice of the lens

- What influences the resolution?
  - NA of the objective
    * Light gathering ability
  - The higher the NA, the better the details,
  - and the higher the intensity

MDCK epithelial cells imaged with a confocal using identical settings. A uses a 10X / 0.25 NA. B uses a 10X / 0.45 NA. Seeing is believing? A beginners’ guide to practical pitfalls in image acquisition. AJ North, JCB, 2006
What influences the resolution?
- NA of the objective
  * Light gathering ability
  • The higher the NA, the better the details,
  • and the higher the intensity
  • A high NA reduces the working distance
Choice of the lens

- What influences the resolution?
  - NA of the objective
  - Good refractive index match

A refractive index mismatch gives rise to geometrical aberrations.
Choice of the lens

- What influences the resolution?
  - NA of the objective
  - Good refractive index match
  - Pixel size
Question

- What objective will have the best resolution?
  - 60X, 1.42 NA, zoom 1.6
  - 60X, 1.4 NA, zoom 1.6
  - 100X, 1.4 NA, zoom 1

Drosophila brain with Neurotactin in red and GFP-tubulin clone in green. Sample N. Riebl, Image W. Büttner taken on our LSM 700 inverted confocal, room 186.

Tips and Pitfalls/ A. Ferrand
Optimizing images

Increase signal

• Choose bright dyes
• Image through a clean N° 1.5 coverslip
• Mount specimen as close to the coverslip as possible
• Use high NA clean objective with the lowest magnification
• Use glycerol-based medium containing anti-bleaching
• Remove DIC Wollaston prism and analyzer from light path

Decrease background

• Clean coverslip and optics
• Perfect fluorophore labeling protocol to minimize nonspecific labeling
• Turn off the room lights

Adapted from Accuracy and precision in quantitative fluorescence microscopy. JC Waters, JCB, 2009
Transmitted light

- Köhler illumination
- Bright and even illumination with good contrast and resolution
- Also important for epifluorescence

The Image and Illumination planes are aligned in the microscope
A clear example...

Before and after images of HeLa cells taken on a DeltaVision room 229 (AF).
Fluorescence

- The brighter the better, **BUT NO SATURATION**
- Otherwise, no quantification will be possible
- Check your histogram

Histogram Basic Tutorial

8 bit (0-255 gsl)

The brighter the better, **BUT NO SATURATION**

Otherwise, no quantification will be possible

Use the visual help from the acquisition software

**Detector saturation**

If PMT gains, laser powers, lamp intensities or camera exposure times are set too high the detector can saturate causing a loss of structural information within bright structures. It is best to have no saturated (red) pixels.
Multi-labeling

When > 1 color, cross-talk and bleedthrough can happen
- Excitation and Emission spectra are not well separated.

Do proper controls
- Single labelled controls with each fluorochrome to check all channels

Check different settings on the confocal
- Use sequential scan instead of exciting the sample with multiple laser lines at once
- Check the filter sets before choosing your fluorophores
# Multi-labeling

## SIMULTANEOUS
- Faster

## SEQUENTIAL
- Less bleedthrough
  - Blue then Green then Red
Image Acquisition for live imaging

Think viability and photostability more than nice picture!
HeLa GFP-CENP-A cell line
Overnight movie using a widefield DeltaVision Core
12 bit camera (0-4095 gsl)

Neutral Density 32%
Exposure time 25 ms

Normal mitosis timing under those conditions.
Min (noise) 70
Max (signal) 320
Signal/noise = 4.5
Min (noise) 70
Max (signal) 320

Signal/noise = 4.5

LIVE IMAGING:
TARGET 1:3-1:5 ratio
Keep your cells alive

- And also, keep your cells in focus: do not hesitate to contact us to show you the autofocus options
Optimizing live imaging

**Increase signal**
- Choose bright dyes
- Image through a clean N° 1.5 glass bottom dish
- Mount specimen as close to the coverslip as possible
- Use high NA clean objective with the lowest magnification
- Remove DIC Wollaston prism and analyzer from light path
- Minimize exposure of specimen to fluorescence excitation light prior to image acquisition
- It is often necessary to sacrifice SNR to maintain cells alive
- Consider binning to increase SNR

**Decrease background**
- Clean coverslip and optics
- Use media **without phenol red** for imaging
- Turn off the room lights

Adapted from Accuracy and precision in quantitative fluorescence microscopy. JC Waters, JCB, 2009
Image Processing
1) Always keep the original data.
2) When converting image data from a proprietary format, save your image data as TIFF (tagged image file format) files.
3) Simple adjustments to the entire image are usually acceptable.
4) Acquire your images under identical conditions, and any post-acquisition image processing should also be identical.
Tips for your analysis

1) Always keep the original data.
2) When converting image data from a proprietary format, save your image data as TIFF (tagged image file format) files.
3) Simple adjustments to the entire image are usually acceptable.
4) Acquire your images under identical conditions, and any post-acquisition image processing should also be identical.
5) Be careful when you change your image size, especially in Photoshop.

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What to add in your M&Ms

- Manufacturer and type of system/microscope (eg Zeiss LSM700 confocal mounted on an Axio Observer microscope stand)
- Objective used (eg 63x 1.4 NA oil Plan-Apochromat)
- Illumination light source, wavelengths of excitation and emission (eg 488 nm line of Argon laser with a longpass 500nm filter)
- Camera manufacturer and model (eg CoolSNAP HQ2, Photometrics)
- Software for acquisition (eg LASAF 2.6) and image acquisition settings (eg ND, exposure time, binning, interval in t and z)
- Details of any image processing or analysis routine– raw images may be required also.

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Take-home messages

- The sample is part of the optics – Avoid sample-induced aberrations
- Try to increase signal and decrease background
- Do not overexpose your images
- Adjust exposure time, laser power (confocal), and neutral density (widefield/spinning disk) to keep your cell happier (alive and functional) for longer

...  

Always keep your raw data and work on copies
Be ethical with your images!
Thank you!

Alexia Ferrand
Imaging Core Facility
Biozentrum
Uni Basel
alexia.ferrand@unibas.ch
www.biozentrum.unibas.ch/imcf

Where to find us?
=> Kragenbau: G1054/1055