Tips and Pitfalls in Microscopy

ALEXIA FERRAND
IMAGING CORE FACILITY
BIOZENTRUM
BASEL
Why use a microscope?

- **Super-resolution**
- **Light microscopy**
- **Electron microscopy**

- **Naked eye**

- small molecule: 1 nm
- protein: 10 nm
- virus: 100 nm
- mitochondrion: 1 μm
- bacterium: 10 μm
- mammalian cell: 100 μm
- hair: 1 mm
- ant: 1 mm
Why use a microscope?

Magnification

- Retina
- Simple magnifying lens
- Subject
- Virtual image

- Retina
- Lens of eye
- Eyepiece
- Image formed by objective
- Tube lens
- Objective
- Specimen
- Condenser
- Virtual image
Why use a microscope?

Resolution
Why use a microscope?

Resolution: Smallest distance between 2 point sources such that their diffraction patterns show a detectable drop in intensity between them.
Why use a microscope?

Magnification versus Resolution:

Magnification can be meaningless if the necessary resolution is lacking.
Basic optics...

Images are not identical to the object

Image = Object x Point Spread Function
Basic optics...

- **Point Spread Function:** Describes the response of an imaging system to a point source or point object.
Basic optics...

- The Airy Disk*: Caused by diffraction on objective aperture
- Negative impact on resolution

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

Where: \( n \sin \alpha = NA \)
\( n = \text{RI of medium} \)
\( \alpha = 1/2 \text{objective collection angle} \)
\( \lambda = \text{wavelength of light (Emission)} \)

*after GB Airy, British astronomer, 1834
Basic optics...

- **Point Spread Function**: A good PSF of an object (bead) should have an “hourglass” shape.
Basic optics...

- Point Spread Function: A good PSF of an object (bead) should have an “hourglass” shape.

To improve your images

There are tips to follow!
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...
SAMPLE Fixation

- «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
Immuno-Staining

• Blocking step with BSA or serum
• Primary Ab
  • 30 min-1h (cells)
  • 1h – days (tissues sections/organs)
• Secondary Ab
  • 30 min-1h
• Washing steps
  • Many short ones better than couple long ones

• Store in the dark at 4°C or -20°C depending on your mounting media.
Careful experiment planning

- Do proper controls
  - Single labelled controls with each fluorochrome to check all channels (Pos/Neg)

- When > 1 color, cross-talk and bleedthrough can happen
  - Make sure that Excitation and Emission spectra are well separated.
  - Use sequential scan if needed
  - Check the filter sets before choosing your fluorophores
Careful experiment planning

https://www.biozentrum.unibas.ch/imcf/links-downloads
Careful experiment planning

Antibodies, fluorescence dyes, fluorescent proteins, filters:

- **BenchSci** - A.I. Driven Antibody Search
- Fluorescence spectra viewers:
  - FPbase - a database of fluorescent proteins.
  - SearchLight (Semrock)
  - Fluorescence SpectraViewer - Plot and compare spectra and check the spectral compatibility for many fluorophores.
  - Table of Fluorochromes @ Iowa State University - check for excitation and emission maxima of most of the current fluorochromes.
- **Fluorescent Biosensor Database** - Repository of genetically encodable fluorescent biosensors.

Biology resources:

- **The Human Protein Atlas** - Database of proteins’ distribution in cells, tissues and organs.
- **Allen Brain Map** - Brain atlases and advanced tools for neurobiologists.
- **Image Data Resource (IDR)** - Public repository of image datasets from published scientific studies.
- **BioNumbers** - Search tool for molecular biology associated numbers
Multi-labeling

SIMULTANEOUS
Faster

SEQUENTIAL
Less bleedthrough

Blue then Green then Red
Sample Preparation

The sample is part of the optics
The last 500 um are important
1 – coverglass

MOST COMMON SIZE
0.17 mm = N°1.5
Sample Preparation

The sample is part of the optics

The last 500 um are important

1 – coverglass (spherical aberrations)

http://www.olympusmicro.com
Sample Preparation

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

Grow cells directly on the coverglass
//
Mount your specimen on the coverglass
Sample Preparation

The sample is part of the optics
The last 500 um are important
2 – mounting media (avoid bubbles)

<table>
<thead>
<tr>
<th>Mounting Media</th>
<th>Refractive Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permount (Biomeda)</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>Canada balsam</td>
<td>1.52</td>
</tr>
<tr>
<td>DPX (Fluka)</td>
<td>1.52</td>
</tr>
<tr>
<td>Fluorescence Mounting Medium (DAKO)</td>
<td>1.47 – 1.50</td>
</tr>
<tr>
<td>Mowiol (Calbiochem)</td>
<td>1.49</td>
</tr>
<tr>
<td>ProLong Gold (Molecular Probes)</td>
<td>1.47 – 1.41 (increases over time)</td>
</tr>
<tr>
<td>Kaisers glycerol jelly</td>
<td>1.47</td>
</tr>
<tr>
<td>Vectashield (Vector Labs)</td>
<td>1.46</td>
</tr>
<tr>
<td>Vectashield / glycerol + 2% DABCO</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 90% (w/w)</td>
<td>1.46</td>
</tr>
<tr>
<td>glycerol 80% (w/w)</td>
<td>1.45</td>
</tr>
<tr>
<td>Aquatex (Merck)</td>
<td>1.40</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 (Biomeda)</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>

Source: Leica

THERE IS NO «one fits all» MOUNTING MEDIA
Preserve 3D information

Some mounting media can flatten the sample

- 50% PBS-Glycerol
- Prolong Diamond
- Euparal
- Vectashield
- Prolong Gold
- Fluoromount G

- DAPI
- aTubulin
- Phalloidin
Which mounting media?
Sample Preparation

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
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4 – Schmutz effect
What information can you get from the objective lens?

- Mounting threads
- Manufacturer
- Aberration corrections
- Immersion medium
- Numerical aperture
- Working distance
- Color code
- Finger grip
- Front lens
- Flat field correction
- Magnification
- Special properties
- Tube lens
- Cover glass thickness
Choice of the lens

What influences the resolution?

*C. elegans larvae* taken on a Zeiss Elyra 7 Lattice SIM (P. Ray and A. Ferrand)
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**
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
  - A high NA reduces the working distance
A refractiveindex mismatch gives rise to geometrical aberrations.

Choice of the lens

What influences the resolution?

- Good refractive index match
Choice of the lens

What influences the resolution?
- 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. Riebli, Image W. Büttner taken on our LSM 700 inverted confocal.
Fluorescence

The brighter the better, BUT NO SATURATION

• Otherwise, no quantification will be possible
• Check your histogram

Histogram Basic Tutorial

http://www.digitaldarrell.com/article-understandingyourdigitalcamerashistogram.asp
Fluorescence

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.
Optimizing images

- 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

Increase signal

Adapted from Accuracy and precision in quantitative fluorescence microscopy. JC Waters, JCB, 2009
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Decrease background
- Clean coverslip and optics
- Perfect fluorophore labeling protocol to minimize nonspecific labeling
- Turn off the room lights

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Image Acquisition for live imaging

Think viability and photostability more than nice picture!
Careful experiment planning

- The previous rules apply
  - Controls
  - Fluorescent proteins matching the microscope configuration
  - No crosstalk/Bleedthrough
  - Sample preparation in appropriate dishes (#1.5 thickness)
  - Choice of the objective

- Extra Challenges
  - Bleaching
  - Phototoxicity
  - Autofluorescence (biological material, material and/or media) influencing the SBR
  - Controlled environment (Temperature, Humidity, pH, CO2)
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
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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
Prospects & Overviews

Phototoxicity in live fluorescence microscopy, and how to avoid it

Jaroslav Icha (1)†, Michael Weber (2)*,†, Jennifer C. Waters (2) and Caren Norden (1)*

Bioassays 2017
Optimizing live imaging

- Choose bright fluorophores
- Image through a clean N° 1.5 glass bottom dish/ibiTreat
- Mount specimen as close to the coverslip as possible
- Use high NA objective with the lowest magnification
- Remove DIC 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

Clean coverslip and optics
Use media without phenol red for imaging (i.e. DMEM GFP, Evrogen)

Turn off the room lights

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Optimizing live imaging

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Image Analysis and Processing

In focus  Simple Gaussian blur
Tips for your analysis

- Always keep the original data.
- When converting image data from a proprietary format, save your image data as TIFF (tagged image file format) or ome.tiff (lossless compression).
- Simple adjustments to the entire image or cropping are usually acceptable.
- Acquire your images under identical conditions, and any post-acquisition image processing should also be identical.

For more details, please read: Digital Images Are Data: And Should Be Treated as Such, by Douglas W. Cromey
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- Acquire your images under identical conditions, and any post-acquisition image processing should also be identical.
- Be careful when you change your image size, especially in Photoshop.
- Use of software filters to improve image quality is usually not recommended for biological images.
- Cloning or copying objects into a digital image, from other parts of the same image or from a different image, is very questionable.

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What to add in your M&Ms

- Manufacturer and type of microscope (eg. Zeiss LSM880 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. Prime 95B, 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 also be required
Quantitative imaging

- Avoid Bias – the difference needs to be statistically relevant
- Illumination of the field of view needs to be homogenous
- Avoid focus drift in a timelapse
- Be careful of laser power/illumination fluctuation
- Avoid Photobleaching
- Be careful with mounting your samples
- In a full set of experiments, make sure that there are no software updates
- Stats: Nature has a collection of short articles called ‘Statistics for Biologists’ (https://www.nature.com/collections/qghhqm)
Tutorial: guidance for quantitative confocal microscopy

James Jonkman, Claire M. Brown, Graham D. Wright, Kurt I. Anderson and Alison J. North
Take-home message

- The sample is part of the optics – avoid sample-induced aberrations
- Try to increase signal and decrease background
- Do not overexpose your images
- For live 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
University of Basel
alexia.ferrand@unibas.ch
www.biozentrum.unibas.ch/imcf

This lecture found some inspiration from the work of others:
Dr C. Janz (Ibidi), Dr L. Gelman (FMI Basel)
B. Fleming (Roslin Institute Edinburgh)
Jonkman et al., Nature Protocols 2020