Digital Micrography
Talk outline:

• Types of hardware we have available
• Using your digital camera
• Microscope techniques to improve your images
Hardware for digital micrography

- SLR-type camera with macro lens
  - up to around 5x mag
  - good depth of field
  - benefits from a copy stand

- Consumer compact camera with adapter lens
  - inexpensive, versatile
  - can be difficult to focus
  - can be some image vignetting
Hardware for digital microscopy (contd)

- Specialised microscopy cameras:
  1. “Ordinary”
     - tethered or untethered
     - uncooled $\rightarrow$ not for fluorescence
     - e.g. Olympus DP12
  2. “Scientific”
     - tethered, low noise, high sensitivity, high resolution…
     - expensive ($25K+)$
     - e.g. Zeiss Axiocam
     - Leica DC500
The Imaging CCD
- An array of pixels – photon wells

Requirements:

Sensitivity
• Need high signal to noise ratio
  → use cooling (-20º C) – allows long exposure (up to 10 min)

Rapidity
• Need high frame rates
  → use advanced circuitry – allows smooth focusing and framing in previews

Accuracy
• Need colour images
  → use RGB filters

Detail
• Need high resolution
  → use large CCD

-the last two of these create problems
The Colour Problem

Pixels are masked with tiny filters allowing only red, green or blue light through

On any pixel, only light of wavelengths transmitted by the filter is recorded

The lost component must be made good by data interpolation
The Resolution Problem:

• Sampling theory: for the full resolution of a lens to be utilised, the smallest resolvable distance for that lens must be projected onto at least two pixels

• For a typical 10x objective, this distance projects to about 7µm

• →Ideally we need pixels around 3µm square

• For a 2/3” CCD this equates to 12 Mpixel
A solution: Microscanning of the CCD array

- 4 steps of 1 pixel each to cover all colours
- 4 or 9 sub-pixel steps to increase resolution
- a combination of these up to 36-shots
Pixel shifting – ”Microscanning”

- Zeiss Axiocam, Leica DC500

• Pros:
  - True colour
  - High resolution
  - Flexibility

• Cons:
  - Not suitable for moving specimen → use 1-shot
  - Increases exposure time → risks from vibration etc.
  - True colour required for best fluorescence
Using the digital camera
Preparation

• Appropriate accessories
  - fast PC, CD burner, selection of lenses…

• Parfocality:
  - of eyepieces and camera

• Bulb alignment:
  - halogen and UV lamps

• Flat field correction:
  - white and black-corrected

(this is done in the camera software; repeat it if your images all have a graded background of colour or shading)
Basic method for digital micrography

(not necessarily in this order!)

• Preview

• Focus

• If necessary…
  - adjust exposure
  - adjust white balance
  - make colour correction
  - select frame (ROI)

• Acquire

• Check the result in Photoshop at 100%
Some jargon…

• Binning

→ faster exposure
→ smaller files
→ less resolution
Some jargon…

- **Binning**
  - Increase this to boost the value assigned to each photon reaching the CCD
    - → brighter image
    - → faster exposure
    - → more noise
Some jargon…

- Binning
- Gain
- Bit depth

<table>
<thead>
<tr>
<th>14 bit camera</th>
<th>16,000 levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 bit: 8 R + 8 G + 8 B</td>
<td></td>
</tr>
<tr>
<td>48 bit: 16 R + 16 G + 16 B</td>
<td></td>
</tr>
</tbody>
</table>

24 bit is fine for most things. Use 48 bit for quantitation and for processing difficult images—it takes up more space, and doesn’t suit all image processing software.
Some jargon…

- Binning
- Gain
- Bit depth
- Histogram
Some jargon...

- Binning
- Gain
- Bit depth
- Histogram
- Gamma

\[ \gamma = 0.6 \]

\[ \gamma = 1 \]

\[ \gamma = 2 \]
Sharpening the image

• Sharpening before Acquiring, or…

• Photoshop Unsharp Mask
  
  - gives more precise control
  - in the Filters → Sharpen menu
  - try 50-100%, about 2 pixels radius, threshold 0-2
Glitches with the Leica DC500…

• Failure to acquire (alter histogram setting)

• Flickering preview (alter brightness setting or binning)

• White balance out of range (alter exposure setting)
Some general points:

• Set file size to suit the end need
  - basic setting is good for 11 x 8.5 cm print (1300x1030 pixels @ 300 ppi)

• Can montage images very well using software

• Use the microscope to magnify the image, rather than relying on enlargement of high resolution images
  - but bear in mind depth of field falls with higher magnifications
3 best tips:

• Ensure your microscope is set up properly
• Take the time to make good slides
  - use no. 1 1/2 cover slips if possible
• Use the microscope correctly

Rubbish in → rubbish out
Using the compound microscope
## Microscopy techniques

<table>
<thead>
<tr>
<th>Technique</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bright field</td>
<td>Stained/ pigmented material</td>
</tr>
<tr>
<td>Phase contrast</td>
<td>Unstained preps, e.g cultured cell monolayers</td>
</tr>
<tr>
<td>Polarised light</td>
<td>Birefringent molecules, e.g amylin, cellulose</td>
</tr>
<tr>
<td>Differential Interference Contrast (DIC) (=Nomarski)</td>
<td>Variety of stained and unstained specimens</td>
</tr>
<tr>
<td>Dark field</td>
<td>Fine filaments, microorganisms</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>Autofluorescence, labelled probes</td>
</tr>
</tbody>
</table>
Bright field
Setting up your microscope

- Set the eyepieces
Spectacles icon appears on most modern eyepieces—it means the lens is designed to be viewed from a few mm back—the distance of the eyepiece caps, or of a pair of glasses.
If you don’t wear glasses, viewing is more comfortable if the eyepiece caps are in place.

If you use glasses, unclip the caps and keep your glasses on – it makes viewing the PC monitor easier, while improving your field of view down the eyepieces.
The band indicates the right position for parfocality with the camera, if you have normal vision.
Setting up your microscope

- Set the eyepieces
- Set condenser top lens to suit magnification
Condenser top lens:

In $\geq 10x$

- if the top lens is left out your image will look terrible at higher magnifications

Out $< 10x$

- otherwise you can’t illuminate the full field of view
Setting up your microscope

- Set the eyepieces
- Set condenser top lens to suit magnification
- Use ND filters to adjust brightness
Most microscopes have a marker or pushbutton setting for optimal bulb brightness (in this case a dot on the voltage wheel). Changing the voltage from this setting will alter the colour of your lighting.

By using neutral density filters, brightness can be reduced without colour change. ND filters are usually engaged using pushbuttons labelled with N or ND + a number.
Setting up your microscope

- Set the eyepieces
- Set condenser top lens to suit magnification
- Use ND filters to adjust brightness
- Ensure objective lenses are clean
Dirt on the objective lens can degrade your image quality greatly.

The condition of a lens can be inspected in situ using a Bertrand lens or phase telescope.

Lens oil is a weak solvent. It should always be cleaned off objectives at the end of a session using a good quality lens tissue.

For more thorough cleaning use ethanol or diethyl ether.
Setting up your microscope

- Set the eyepieces
- Set condenser top lens to suit magnification
- Use ND filters to adjust brightness
- Ensure objective lenses are clean
- Set Köhler illumination
Köhler illumination

This should be done every time you set up your microscope. All modern microscopes are designed to work in this condition.

1. Focus specimen
2. Close field diaphragm
3. Focus condenser
4. Open field diaphragm
5. Adjust aperture
Numerical aperture: generally, bigger is better

The smallest distance a lens can resolve, $d$, reduces as NA increases: $d = \frac{0.61\lambda}{NA}$

Definition: $NA = n \sin \theta$, where $\theta$ is the angle of light that can enter the lens, and $n$ is refractive index. For air $n=1$, for oil $n=1.515$, so oil lenses are better.

There is a price: depth of field is inversely proportional to NA.
Consequently...

Lower magnification = more depth of field
   -since lower magnification lenses tend to have lower NA

Closed condenser aperture = more depth of field
   -since this reduces $\theta$ and hence NA

= poorer resolution
   -also due to lower NA

= more contrast
   -as less light floods the specimen

Typically, set condenser aperture to just less than objective NA
   -but go by what looks best!
Phase contrast
Phase contrast

• Phase rings must be aligned
  -check with Bertrand lens or phase telescope

• Open fully the aperture diaphragm
  -as phase rings cut out much incoming light
Bertrand lens

Phase telescope
PH position of condenser aperture is fully open for phase contrast viewing.
Polarised light
Polarised light

• Need properly crossed polars, giving a dark field

• Rotate stage to observe birefringence
DIC (Nomarski)
DIC – Differential Interference Contrast

- Need crossed polars
- Requires correct prism/ objective pairs
- Adjust prism angle for best look
Upper and lower DIC prisms

Prism adjuster
DIC – Differential Interference Contrast

- alternative is Hoffman modulation contrast
  
  - found on some inverted microscopes
  
  - good for looking at plastic culture dishes, as birefringence makes these unsuitable for DIC
Dark field
Dark field

- Can use HP phase rings for LP objectives
- Specialised dark field rings in the condenser are used up to c.40x
- Specialised condenser and low NA objective (with iris diaphragm) is required for 100x
- Avoid dust! – it interferes greatly with dark field images
Fluorescence
Specimen fluoresces by absorbing light of certain wavelengths and emitting it at longer wavelengths:

Use filters to select particular wavelengths
1 first barrier filter: lets through only green light with a wavelength between 515 and 560 nm

2 beam splitting mirror: reflects light below 580 nm but transmits light above 580 nm

3 second barrier filter: cuts out unwanted fluorescent signals, passing the specific red rhodamine emission above 590 nm
Fluorescence microscopy

- Use an antifade
- Use long pass or band pass filters as appropriate
- UV lamp - first on, last off  
  - ignition causes electromagnetic pulse
  - 20 minute rule  
    - allow warm-up and cool-down time
- fill in the log book
- Optimise fluorescence with the field and aperture diaphragms
Field diaphragm allows sensitive specimens to be protected by restricting illuminated area.

Aperture diaphragm controls overall brightness of fluorescence.
Fluorescence microscopy

• Need fluor lenses for UV illumination (e.g. DAPI)
  - standard glass does not transmit UV wavelengths

• Brightness $\propto NA^4/M^2$

  $\rightarrow$ 63x lens a good choice

  - giving both sufficient brightness and enough resolution to see subcellular detail; 100x lenses aren’t as bright.
The End