The widefield microscope
Illuminations

The goal is to illuminate your sample uniformly in a field of view simultaneously

- Trans illumination/ Brightfield:
  - white light (LED or incandescent) or can add filters if needed (e.g. to prevent optogenetic stimulation).
  - Used to image stains such as Golgi, silver, H&E. Usually limited to two colors.
  - Used in contrast methods such as differential interference contrast microscopy (DIC) or phase contrast microscopy

- Fluorescence light source:
  - Arc lamp (older systems) or LED-based to use specific wavelengths.
  - Used in epifluorescence microscopy with dyes or proteins with wavelength specific properties.
  - Used in functional imaging, (calcium/voltage), optogenetics, FRAP...
Brightfield

**CRITICAL ILLUMINATION**

sample plane

**aperture diaphragm**

PROBLEM OF CRITICAL ILLUMINATION:

an image of the light source is formed at the sample plane
KÖHLER ILLUMINATION

- **condenser lens** - resolution
- **aperture diaphragm** - contrast
- **field lens** - depth of field
- **field diaphragm** - determine the filling of the condenser aperture
- **field diaphragm** - contrast (preventing glare)
- **collector lens** - collimate light

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Arc lamp or why we ask you not to turn it on and off rapidly.

Turning on the lamp creates a current between anode and cathode of the light bulb. The electric arc needs to strike and to stabilize on one spot of the cathode. This usually takes around 10 minutes. When turning it off, the lamphouse needs to cool and that usually takes another 20 minutes. Every time the lamp is turned on, roughly 10 hours of its lifetime is used (lifetime ~200-300 hours). So when using a core microscope, don’t turn off the lamphouse if someone is using the microscope after you, but do switch it off if you’re the last person to use at night or weekends!
Light Emitting Diode (LED)
Epifluorescence light path
Fluorescence selection in a microscope

A filter set is composed of:

- Excitation filter: to limit the wavelengths reaching your sample.
- Emission filter: to only collect the emitted wavelengths you’re interested in.
- Dichroic mirror that reflects certain wavelengths and let others pass through.
Fpbase and filter choices

Created and maintained by Talley Lambert here at HMS: www.fpbase.org

To help you choose filters for your fluorophores: www.semrock.com

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Beyond the tube lens: detection
Trans illumination with a camera

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Widefield detectors

• Chip sensors such as CCD, cMOS.
• Takes a snapshot of a plane that holds spatial information
• Usually monochromatic (except for histology)
• Has a certain number of pixels and a specific pixel size (spatial resolution constraint) and refresh rate (kHz or less)
Image Construction on a camera based system
Analog to Digital Conversion

Source: https://people.cs.clemson.edu/~dhouse/courses/405/notes/pixmaps-rgb.pdf
Display Range and the Histogram
Common terms

• **Background** – This is a temporally stable source of photons, meaning something that is constant in one field of view.

• **Noise** – Not temporally stable, random seeming. The sources of this are often the system itself like heat generated by current running through the camera/detector

• **Digital Pixel** – The building block of our digital image.

• **Point Source (physical pixel)** – The point of light we are projecting onto a sample and then collecting.
Chip sensor types

- CCD (Charged coupled device)
- emCCD (electron multiplying CCD)
- CMOS (Complementary Metal Oxide Semiconductor)
Things to consider before capturing an image on wide field..

• Sample prep:
  1. Proper mounting media (no histo mounting media when doing fluo).
  2. Sectioned at an appropriate thickness, minimizes background.
  3. Good antibodies and fresh markers e.g. DAPI.
  4. Marker controls so I know I don’t have crosstalk or nonspecific binding.

• What is the size of my object I am quantifying? What am I quantifying?
  • You should always know (roughly) the size of the object you are trying to resolve. Be sure that your objective of choice will achieve a pixel size < half the size of your object. This is known as “Nyquist sampling”.

• What are the ways I can enhance my signal while minimizing background?
  • Exposure time.
  • Gain.

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### 4.12. Specifications

<table>
<thead>
<tr>
<th>Specification</th>
<th>Details</th>
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<tbody>
<tr>
<td><strong>Effective number of pixels</strong></td>
<td>1344 (H) × 1024 (V)</td>
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<tr>
<td><strong>Cell size</strong></td>
<td>6.45 μm (H) × 6.45 μm (V)</td>
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<td><strong>Effective area</strong></td>
<td>8.67 mm (H) × 6.60 mm (V)</td>
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<td><strong>Dual scan mode</strong></td>
<td>Normal scan / Fast scan</td>
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<tr>
<td><strong>Pixel clock rate</strong></td>
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<td></td>
<td>Fast scan: 28.00 MHz/pixel</td>
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<td><strong>Readout noise (r.m.s.) typ.</strong></td>
<td>Normal scan: 6 electrons</td>
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<tr>
<td></td>
<td>Fast scan: 10 electrons</td>
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<tr>
<td><strong>Full well capacity typ.</strong></td>
<td>High dynamic range mode: OFF</td>
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<tr>
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<td>High dynamic range mode: ON</td>
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<tr>
<td><strong>Dynamic range typ.</strong></td>
<td>3 000 : 1 (at Normal scan / 1×1)</td>
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<tr>
<td><strong>Cooling method / temperature</strong></td>
<td>Forced-air cooled: -35°C</td>
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<tr>
<td></td>
<td>Water cooled: -40°C (Water temperature: +20°C)</td>
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<tr>
<td><strong>Dark current</strong></td>
<td>0.0005 electrons/pixel/s (at - 40°C)</td>
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<td><strong>Dual A/D converter</strong></td>
<td>12 bit or 16 bit</td>
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<td><strong>Exposure time</strong></td>
<td>10 μs to 4200 s</td>
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<td><strong>Binning</strong></td>
<td>2 × 2, 4 × 4, 8 × 8</td>
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<tr>
<td><strong>Sub-array</strong></td>
<td>Yes</td>
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<td><strong>Dual light mode</strong></td>
<td>Low light mode / High light mode</td>
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<tr>
<td><strong>High dynamic range mode</strong></td>
<td>Yes</td>
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</table>

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Exposure

1 ms  10 ms  50 ms  100 ms
Gain

- Rely on gain when you think that your exposure time is going to be too long to reasonably acquire your image.
- Longer exposure times on cooled cameras typically have a lowered impact on noise. This means better signal to noise ratio.
- Gain is simply light amplification. Even the light (background or noise) you don’t want!
- The camera manufacturer may have some specific guidelines for default gain values.
Binning

- Create super-pixels
- This is an almost noiseless efficient way of increasing the brightness of your image but at the cost of resolution.

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<th>Speed</th>
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<th>Resolution</th>
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<td>sCMOS</td>
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A way to think about sampling rate.... Nyquist

![Graph showing Pixel Size Effect on Contrast with n values of 2, 12, 24, and 36.](image)
Nyquist in Action

Plot of 64 example

53.748x100.89 (61x355): 8-bit; 213K

Plot of 64 example

10.41x70.30 (61x355): 8-bit; 213K

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Find the core online for more info on equipment and services: https://nif.hms.harvard.edu/

Follow us on Twitter for events and classes: https://twitter.com/hmsneuro

HMS wide microscopy cores and services, register for emailing list: https://microscopy.hms.harvard.edu/

Resources:
• microscopyU
• Olympus microscopy resource center
• Leica Science lab