Microscopy Showdown
The microscope
Illuminations
Light Emitting Diode (LED)
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Depending on material and band gap thickness, different colors emitted. Highly divergent.
Pros and cons of LED

• Efficient compared to light bulbs/incandescent light sources.

• Colors

• Warmup time and cycling: Don’t have to wait 15 minutes like for an arc lamp, can turn on and off rapidly. Can be used for train of optogenetic stimulation that way or duty cycling to reduce the amount of power delivered.

• Lifetime: 10 000+ hours compared to hundreds to a few thousand hours for lamps.

• Temperature dependence: can overheat due to ambient temperature resulting in shutdown or even damage.

• Area light source: Single LEDs do not approximate a point source of light giving a spherical light distribution, but rather a lambertian distribution. LEDs cannot provide divergence below a few degrees (need collimation optics for microscopes).
Lasers
Lasers

A chain reaction of photons begins and the laser begins to "lase."

Full operation.
Lasers

- The medium or laser material can be a gas or it can be a solid like a crystal doped with specific ions to obtain different properties (wavelength, pulse width etc...).
- The laser beam comes out coherent: propagating in one direction, phase locked, same waveform and same frequency. Can be focused in a diffraction limited spot.
- The intensity profile follows a gaussian distribution.
Detectors
Beyond the tube lens: detection
Cameras aka chip based sensors
CD sensors - Charged Coupled Device: From the serial register charges are passed one by one to the read out electronics where the signal is converted into a voltage, amplified, quantified, and digitized. So all the data within a CCD sensor is usually read out through a single output node.

EMCCD sensors - Electron Multiplying CCD: EMCCD cameras employ back thinned sensor technologies typical offering peak QE >90%. These types of cameras are used for extreme low light applications and can be single photon sensitive. The price of these cameras is typically significantly higher than for regular CCD-based cameras.

CMOS - Complementary Metal Oxide Semiconductor: Originally used in cell phones and low end cameras. The major difference compared to CCDs is the intra pixel electronics and time saving sensor read out principle with thousands of read out nodes compared to the single read out node.

sCMOS - scientific CMOS: Introduced a couple of years ago this type of sensor overcomes common drawbacks of CMOS sensors like high noise level. This type of sensor is used for high-end fluorescence imaging which benefits from the fast frame rates, high dynamic range and low noise.
Noise (temporally unstable)

- **Dark noise** - also known as **dark current** - is a fundamental noise present in the sensor. Dark noise is caused by thermal energy in the silicon randomly generating electrons in pixels. Dark noise builds up in pixels with exposure time. It is less of a concern for fast applications with short exposure times. When it comes to long exposure times e.g. one second or more for weak fluorescent signals, this noise type can become a major issue. Dark noise is reduced by *cooling* the sensor.

- **Read noise** originates from the electrical readout circuitry of the sensor involved in quantifying the signal. As a rule of thumb the read noise can be reduced by reducing the pixel readout rate. This **pixel readout rate** defines how fast charge can be read out from the sensor (MHz). As this determines the frame rate of the camera read noise has to be taken into account for fast experiments like high-speed time-lapse of living cells.

- **Photon shot noise** is based on the uncertainty in counting the incoming photons. In other words it arises from the stochastic nature of photon impacts on the sensor but is not introduced by the sensor itself. It is best explained by imagining you are trying to catch rain drops in buckets. Even if every bucket is of identical size and shape, not every bucket will catch exactly the same numbers of drops hence detection of photons on the chip can be visualized as a Poisson distribution.

- Under low light conditions such as fluorescence imaging when the signal intensity is low, the different noise sources can have a major impact on the quality of the image as they impact the signal-to-noise ratio.
Background (temporally stable)

Common sources of background:
- Thick samples scattering light
- Unspecific binding of antibodies
- Bad mounting medium
Photodiodes

Avalanche photodiode

- high gain (4000:1 photons)
- low dark noise
- very small dynamic range

Photomultiplier tube (PMT)

- low gain (limited by dynodes)
- high dark noise
- wide dynamic range
HyD (best of both worlds)
Microscopes
What microscopes are available to you?
Scanning microscopy
Point scanning
Confocal microscopy

Single photon excitation illuminates the entire hourglass. Confocal microscopes were designed to reduce background fluorescence generated above and below the focal plane. Addition of the pinhole aperture blocking photons not coming from the focal plane.
Out of focus fluorescence
Multiphoton microscopy

\[ P_{2P} = \frac{\langle I \rangle^2}{\tau R} \]

- \( P_{2P} \): probability of two-photon effect
- \( I \): light intensity (# photons per area per time)
- \( \tau \): pulse duration
- \( R \): pulse repetition rate
2-photon excitation

Limited out of focus fluorescence because the excitation volume is already restricted.
Uses longer wavelengths, lower energy photons
Increases the penetration depth of light
Super resolution
STORM (Stochastic Optical Reconstruction Microscopy)
STED (Stimulated Emission Depletion)
Super resolution

**STORM**
- Have to use specific dyes and buffers to increase signal
- Often TIRF based and can be done in 3D (a few microns)
- Full field usually
- Resolution >15 nm

**STED**
- Can be used with most fluorophores
- Scanning method and prone to bleaching
- Can be used in tissue and even in vivo.
- Resolution >30 nm
Lightsheet
Lightsheet (Volume and more volume)

At least for the one in NIF. Lattice light sheet microscopes got a lot of publicity recently. One version in the Nikon Imaging Center but only for small thin samples (20x20 microns FOV). For the fly reconstruction 160 x160 um fields tiled.
Recap

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<tr>
<td>Resolution</td>
<td>240 nm</td>
<td>240 nm</td>
<td>260 nm</td>
<td>20 nm</td>
<td>30 nm</td>
<td>60 nm to 5 um</td>
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<tr>
<td>Live sample</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sample type</td>
<td>Anything under 100 um</td>
<td>Anything under 100 um</td>
<td>In vitro, in vivo</td>
<td>Cells and very thin sections (&lt;20 um)</td>
<td>Anything under 100 um</td>
<td>Whole organs (realistically with WD, samples up to 4 mm)</td>
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<tr>
<td>Main benefit</td>
<td>Background rejection</td>
<td>Confocal + speed</td>
<td>Tissue penetration</td>
<td>Single molecule</td>
<td>Super res for all</td>
<td>Cleared tissue and volume</td>
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• Interested in more in depth discussion about optics/microscopy?
  • Email me aurelien_begue@hms.Harvard.edu

• Need scientific help on a project involving microscopy or other core services like clearing/in situ?

• Feedback appreciated (anonymous solution tbd)