Idiosynchratic primer on principles of light microscopy for neuroscience
Basic features of a microscope for transmission and fluorescence
The lens maker’s formula (thin lens, paraxial beam, …):
A good start to understand imaging

\[(d_1 - f)(d_2 - f) = f^2\]
Infinity corrected microscopes: Two pathways in brightfield imaging

(1) Imaging path
(2) Illumination path
Imaging path: Three essential lenses to magnify object

- An objective with focal length \( f_O \)
- A tube lens with focal length \( f_T \) (typically \( f_T = 160 \text{ mm} \)).
- An eyepiece with focal length \( f_E \).

Magnification at intermediate plane, set by objective and tube lens, is

\[
\frac{r_3}{r_1} = -\frac{f_T}{f_O}
\]

Magnification at eye, set by eyepiece, is

\[
\frac{r_5}{r_3} = -\frac{d_5}{d_4}
\]

Total magnification of object is

\[
\frac{r_5}{r_1} = \frac{f_T}{f_O} \frac{d_5}{d_4}
\]
Extended source illumination: Two essential lenses for illumination

Range of angles at the sample depends on the height of the source, with slope

\[ r_6' = \frac{r_1}{d_6 - \frac{f_{\text{Con}}}{f_{\text{Col}}}} \]
Infinity corrected microscopes: Two pathways in brighfield imaging

(1) Imaging path

(2) Illumination path

- Aperture diaphragm controls the angular spread of the light and thus depth-of-field.
- Field diaphragm controls the size of the illumination spot
Modes of imaging

Transmission: Contrast based on absorption and scattering
Transmission: Contrast based on refraction (change in optical path length)

CW from 9 o’clock: bright field; enhanced bright field; differential interference contrast (DIC); phase
Example: DIC microscopy to image $\nabla_{\text{optical path}}$
Fluorescence: Contrast based on absorption and incoherent emission
Fluorescein – Large-scale labeling in biology and the Windy City
\[ \varepsilon = 9 \times 10^4 \text{ M}^{-1}\text{cm}^{-1} \text{ at } \lambda = 500 \text{ nm} \]

Rule of thumb: \( \varepsilon_{\text{maximum}} \sim 20,000 \times \) (number of rings)

Example: \( \varepsilon = 90,000 \text{ M}^{-1}\text{cm}^{-1} \text{ at } \lambda = 500 \text{ nm} \) for fluorescein
Spatial resolution: Set by the wavelength and geometry

What does a point object look like? Expect to visualize it as a dot of order one wavelength.

First zero of pattern, \( J(r)/r \), at \( r = \lambda \times (0.6 / NA) \).

What is numerical aperture (NA)?

\[
NA = (n)\sin(\mu)
\]

(a) \( \mu = 7^\circ \) \( NA = 0.12 \)
(b) \( 20^\circ \) \( 0.34 \)
(c) \( 60^\circ \) \( 0.87 \)
At the very best, NA = 1 and $r = 0.3 \, \mu m$ for green light.

Better resolution for samples embedded in high index media.

Sampling theorem requires $\geq 2$ samples per resolution unit, so we need to sample at $0.15 \, \mu m$.

For the example CCD with 7200 pixels on edge, the maximum field is $7200 \times 0.15 \, \mu m = 1.1 \, mm$.

In practice, most lenses do not have such a large fields e.g., Zeiss 40X 1.2 NA water objective has 500 $\mu m$ field and $r = 0.25 \, \mu m$.

This implies a CCD resolution of $(500/0.25/2)^2 = (4000)^2 \sim 16 \, Mpixels$; quite reasonable.
Detection versus discrimination

Detection has arbitrary accuracy.
Resolution implies a minimum distance between identical objects.
Palm Imaging of overlapping, identical fluorophores that switched on at low density

Betzig, Patterson, Sougrat, Lindwasser, Olenych, Bonifacino, Davidson, Lippincott-Schwartz & Hess (Science 2006)  
Rust, Bates & Zhuang (Nature Methods 2006)
Scanning microscopy for optical sectioning

Pixel-by-pixel excitation for confocal, multiphoton, harmonic, STED, ... microscopy

Change in slope of the mirror, $r_1'$, is turned into a change in position in the focal plane, $r_4$, with 

$$r_4 = -\frac{1}{f_3} \frac{f_1}{f_2} r_1'$$

0
Confocal scanning microscopy (Minsky, Amos, ...., ça 1960)

Each image has a depth of field that is given by $\Delta z \sim 2 \lambda / (\text{NA})^2$

Images must be reassembled to form a 3-D view
Two-photon laser scanning microscopy (Denk et al. Science 1990)

Integrated Absorption Probability
Area A is scanned in a time t with power P

One photon
\(N_{\text{absorption}} \sim P t\)
\[\frac{P_0 A}{A} = P_0\]

Two photon
\(N_{\text{absorption}} \sim P^2 t\)
\[\left(\frac{P_0}{A}\right)^2 = \frac{P^2}{A}\]

Out of focus

In focus

\(\sim \text{Constant}\)

Out of focus

\(\frac{1}{A}\)
In vitro and in vivo two-photon microscopy for structural studies

Currently, one typically images 500 µm deep and can image to 1000 µm with special equipment.
Two-photon microscopy with free-ranging animals for cell function

Sawinski, Wallace, Greenberg, Grossmann, Denk & Kerr (2009 PNAS)
Super-resolution scanning microscopy through stimulated emission: STED (Hell & coworkers 1994)
The goal is science, not (only) pretty pictures: Vectorization of data