MICROSCOPY DAY 2011:
Basics of Microscopy
Martin Spitale

- Properties of light:
  - Magnification and defraction limit
  - Contrasting techniques in transmitted light microscopy
- Fluorescence microscopy techniques:
  - Epifluorescence technique
  - Widefield microscopes
  - Confocal microscopes
- Light as a tool:
  - photo-bleaching, activation and switching
  - phototoxins
  - laser tweezers
- Light detectors:
  - noise
  - resolution and sampling rate
Microscopy tools in FILM

- **Conventional (widefield) microscopes (WF1, WF2)**
  - low-light live imaging
  - automated multi-position XYZT acquisition
  - ratiometric imaging
  - high-speed acquisition

- **Confocal microscopes (CF1, CF2, CF5)**
  - fixed and live 3D imaging
  - high-speed imaging
  - photobleaching, photoswitching, laser tweezers
  - spectral imaging
  - automated multi-position XYZT acquisition

- **Multiphoton / FliM microscopes (CF3, CF4)**
  - in vivo imaging
  - fluorescence lifetime, FRET

- **Superresolution microscope (WF3)**
  - TIRF
  - PALM
  - STORM

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Transmitted light microscopy: basic design

**Magnification**

\[ M = \frac{d_i}{d_o} \]

- **Lens**
- **Object plane**
- **Focal plane**
- **Image plane**

Louis Pasteur’s microscope (ca. 1850)

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Transmitted light microscopy: basic design

Light

Reflection

Absorption

Image

Lamp

focal plane:
confoc. pinhole

obj. plane:
sample

image plane:
eye / detector

obj. plane:
sample

lens:
objective

d_i

d_o

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Transmitted light microscopy: basic design

- Light
- Collector
- Condensor
- Objective
- Image
- Eye / detector
- (Infinity corrected)
- Objective plane: sample
- Confoc. pinhole
- Focal plane: sample
- Tube lens / eyepiece
Transmitted light microscopy: basic design

chromatic aberration
Transmitted light microscopy: basic design

chromatic correction
(Achromat, Apochromat, ...)

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Transmitted light microscopy

Zebrafish (Brachydanio rerio)

Mariya Moosaje: Zebra fish embryo development

long time courses (4 days)
Transmitted light microscopy: diffraction limit

$\text{d}_{xy} = 0.61 \cdot \frac{\lambda}{n \cdot \sin(\mu)}$

Abbe’s Law

$\lambda = \text{wavelength}$

$n \cdot \sin(\mu) = \text{numerical aperture (NA)}$
Transmitted light microscopy: diffraction limit

\[ d_{xy} = 0.61 \ast \frac{\lambda}{n \ast \sin(\mu)} \]

Abbe’s Law

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Transmitted light microscopy: diffraction limit

Refractive Index $n = \text{speed of light in vacuum} / \text{speed of light in medium}$

$$d_{xy} = 0.61* \frac{\lambda}{n* \sin(\mu)}$$

Abbe’s Law

Immersion:
- Air ($n=1$)
- Water ($n=1.33$)
- Glycerin ($n=1.47$)
- Oil ($n=1.51$)
Transmitted light microscopy: increasing contrast

- Reflection
- Absorption
- Scattering
- Phase shift
- Diffraction
- Interference

Light wave

Particle (photon)

Brightness change

Water / oil immersion
Transmitted light microscopy: increasing contrast

Darkfield illumination
Transmitted light microscopy: increasing contrast

Darkfield illumination

Brightfield

Darkfield
Transmitted light microscopy: increasing contrast

Phase contrast

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Transmitted light microscopy: increasing contrast

Phase contrast
Transmitted light microscopy: Increasing contrast

Phase contrast

Georgina Cornish: Migrating T cells
Transmitted light microscopy: increasing contrast

Differential Interference Contrast (DIC)

from ‘microscopy primer’ (http://micro.magnet.fsu.edu)

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Transmitted light microscopy: Increasing contrast

Cell growth, division and death
Transmitted light microscopy: Increasing contrast

Sample staining
Fluorescence microscopy

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Fluorescence microscopy: Basics

Exciting light → Fluorophor → emitted light

Jablonsky diagram

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Imperial College London
Fluorescence microscopy: Epifluorescence

Transmitted light path

Epifluorescence light path

(Invented 1965 by Johan Ploem)
Fluorescence microscopy: Epifluorescence

Transmitted light path

Obj. plane: sample

Image plane: eye
(infinity corrected)

Epifluorescence light path

(Invented 1965 by Johan Ploem)

Obj. plane: sample

Excitation filter

Dichroic mirror

Emission filter

Image plane: eye
(infinity corrected)

Lamp

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Fluorescence microscopy: Epifluorescence

- Single channel
- Channel overlay (including transmitted light)
Fluorescence microscopy: Epifluorescence

Transmitted light path

Epifluorescence light path

(Invented 1965 by Johan Ploem)
Fluorescence microscopy: Epifluorescence

Transmitted light path

Epifluorescence light path

(Invented 1965 by Johan Ploem)
Fluorescence microscopy: Deconvolution

Stack of images along z axis

Point spread function (PSF)

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Fluorescence microscopy: Deconvolution

Potential artefacts

Stretching

Edge artefacts

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Fluorescence microscopy: Confocal

Standard (widefield) microscope

sample

focal plane

camera

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Fluorescence microscopy: Confocal

Confocal microscope

optical slice

pinhole

Photo Multiplier Tube

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Fluorescence microscopy: Confocal

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Fluorescence microscopy: Confocal

Widefield

Confocal
Other uses of lasers in microscopes:
• pulsed lasers (2P, SHG, Flim, Flim-Fret) → Mark Scott: Special techniques I
• switching / uncaging → Steve Rothery: Special techniques II
• bleaching (FRAP, FLIP)
• phototoxins
• laser tweezers
Photobleaching techniques:
FRAP (Fluorescence Recovery After Photobleaching) / FLIP (Fluorescence Loss In Photobleaching)

Principle:
• An Region Of Interest (ROI) is bleached
• Movement (diffusion, transport) of the visible fluorescence into the ROI (FRAP) or loss of fluorescence outside the ROI is measured over time

Problems:
• incomplete bleaching
• slow (sample movement)
• high phototoxicity

\[
\frac{F(t)}{F_p} = \alpha \sum_{n=0}^{\infty} \left[ \left( \frac{-K}{n!} \right) \left( \frac{1}{1 + \frac{1}{n(1 + 2t/\tau_D)}} \right) \right] + (1 - \alpha) \frac{F_o}{F_p}
\]
Light as a tool

Photobleaching techniques:
FRAP (Fluorescence Recovery After Photobleaching) / FLIP (Fluorescence Loss In Photobleaching)

Single-molecule analysis with photobleaching

source: Kevin Teng, Univ. Illinois, USA
**Light as a tool**

**Light-switches: Phototoxins, uncaging**

*Light pulses are used to:*
- activate phototoxins
- uncage drugs
- activate photo-sensitive ion channels (opsins)
- Optogenetics

Ioanna Stamati (Mahendra Deonarain lab):
**Apoptosis (cell death) induced by photoactivation of a phototoxin**
Laser tweezers / laser dissection

Intensive infrared laser light is used to cut (laser dissection) or move objects (e.g. whole cells)

Stefane Oddos (French / Davis labs):
Signalling clusters in the immunological synapse
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Light detectors

Types of detectors in light microscopes:
• cameras:
  • CCD
  • EM-CCD
  • back-illuminated EM-CCD
  • CMOS
• photomultiplier tubes (PMT)

Essential considerations:
• sensitivity (signal-to-noise ratio)
• dynamic range
• linearity
• sampling rate ('pixel size')

Light detectors: noise

Signal-To-Noise ratio (SNR): 30 15 5

SNR: commonly measured as

- intensity (objects)
- STDEV (background)
Light detectors: noise

Types of noise:

1) Photon noise:
   • inherent statistical variation in the arrival rate of photons (Poisson statistical distribution)
   • equivalent to the square-root of the signal.

2) Dark noise:
   • electrons thermally generated within the silicon structure of the CCD
   • independent of photon-induced signal
   • cooling the CCD reduces the dark current dramatically

3) Read-out noise:
   • inherent to the process of converting CCD charge carriers into a voltage and the subsequent processing and analog-to-digital conversion
   • Usually added uniformly to every image pixel (except CMOS)

Reducing noise:

• brighter labelling
• higher-sensitivity detector
• longer integration time / lower scan speed
• averaging multiple exposures
• image processing: median / Gauss filter
Abbe’s Law

\[ d_{xy} = 0.61 \frac{\lambda}{n \sin(\mu)} \]

Nyquist theorem

To capture the full information, the sampling frequency needs to be at least twice the highest sample frequency.

Microscopy:
sampling distance
\[ \leq 0.5 \text{ smallest structure or diffraction limit} \]
Light detectors: sampling rate

\[ d_{xy} = 0.61 \frac{\lambda}{n \sin(\mu)} \]

**Abbe’s Law**

To capture the full information, the sampling frequency needs to be at least twice the highest sample frequency

**Nyquist theorem**

**Microscopy:**
- Sampling distance
- \( \leq 0.5 \) smallest structure or diffraction limit
Light detectors: sampling rate

\[ d_{xy} = 0.61 \times \frac{\lambda}{n \times \sin(\mu)} \]

**Abbe’s Law**

To capture the full information, the sampling frequency needs to be at least twice the highest sample frequency

**Nyquist theorem**

**Microscopy:**
sampling distance
\[ \approx 0.5 \text{ smallest structure or diffraction limit} \]
Light detectors: sampling rate

\[ d_{xy} = 0.61 \times \frac{\lambda}{n \cdot \sin(\mu)} \]

Abbe’s Law

To capture the full information, the sampling frequency needs to be at least twice the highest sample frequency

Nyquist theorem

Microscopy:
sampling distance \( \leq 0.5 \) smallest structure or diffraction limit

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Light detectors: sampling rate

\[ d_{xy} = 0.61 \times \frac{\lambda}{n \sin(\mu)} \]

**Abbe’s Law**

To capture the full information, the sampling frequency needs to be at least twice the highest sample frequency

**Nyquist theorem**

**How to achieve the ‘Nyquist Rate’:**

- **Widefield:**
  - combination objective / pixel size
  - distance of Z-slices
- **Confocal:**
  - zoom
  - distance of Z-slices

**Microscopy:**

- sampling distance
- \( \leq 0.5 \) smallest structure or diffraction limit