Advanced Optical Microscopy
lecture

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Today:

**Optical transfer functions (OTF)** and point spread functions (PSF) in incoherent imaging.

1. Quick revision: the incoherent wide-field OTF (and the missing cone)
2. Filling the missing cone: the confocal microscope
3. Imaging through two objectives: the 4Pi microscope
4. Superresolution imaging: Structured illumination microscopy (SIM)
Quick revision: the incoherent wide-field OTF (and the missing cone)
Optical Transfer Function (OTF):

For incoherent microscopy techniques, e.g. fluorescence microscopy
The missing cone and optical sectioning:

- Lateral support
- Axial support
2. Filling the missing cone: the confocal microscope
The confocal microscope

- Pinhole
- PMT detector
- Tube lens
- Emission filter
- Dichromatic mirror
- Objective
- Laser
The confocal PSF:

Point spread function, PSF:

- **Wide-field imaging:** The image generated by a point-source.

Or:

- **Scanning:** The amount of signal detected from a point-source in dependence on the source’s position.
The confocal PSF:

Sample scan \rightarrow \text{scan position: } s

Let’s first look at the detection only (i.e. constant illumination everywhere):

- The sample is a point source, shifted to the scan position $s$.
- The point source emits light, which forms an image (\(\rightarrow\) PSF around the position $s$)
  - in the image plane: $h_{\text{emission}}(r - s)$
- Light not falling onto the (centered) pinhole $p(r)$ is blocked. Right behind the pinhole the light distribution is:
  \[ h_{\text{emission}}(r - s)p(r) = h'_{\text{emission}}(s - r)p(r) \text{ with } h'_{\text{em}}(r) := h_{\text{em}}(-r) \]
- The resulting light distribution is integrated on the PMT detector, yielding the final confocal PSF:
  \[ h_{\text{detection}}(s) = \int h'_{\text{emission}}(s - r)p(r)dr \]
    \[ = [h'_{\text{emission}} \otimes p](s) \]
- The detection PSF is a convolution of the (mirrored) emission PSF with the pinhole.
The confocal PSF:

Sample scan $\rightarrow$ scan position: $s$

Let's combine this with **point scanning illumination**:

- The illumination is centered, i.e. fixed at position 0. The shape of the illumination is given by the illumination PSF: $h_{\text{illumination}}(r)$

- The point source at position $s$ is illuminated with light of brightness: $h_{\text{illumination}}(s)$

- The detection signal $h_{\text{detection}}(s)$ has to be scaled with this brightness: $h_{\text{illumination}}(s) h_{\text{detection}}(s)$

- This combined signal is the confocal PSF:

$$h_{\text{confocal}}(s) = h_{\text{illumination}}(s) h_{\text{detection}}(s)$$

$$h_{\text{confocal}}(s) = h_{\text{illumination}}(s) [h'_{\text{emission}} \otimes p](s)$$
Confocal fluorescence microscopy

Reduction of out of focus light

Resolution in confocal microscopy

Comparison of axial (x-z) point spread functions for widefield (left) and confocal (right) microscopy
The confocal OTF:

$$\text{PSF}(r) = \text{PSF}_{\text{Excitation}}(r) \times \text{PSF}_{\text{Detection}}(r)$$

$$\text{OTF}(k) = \text{OTF}_{\text{Excitation}}(k) \otimes \text{OTF}_{\text{Detection}}(k)$$

Increasing the aperture angle ($\alpha$) enhances resolution!!

Missing cone has been filled!!

Axial support has been increased.

Lateral support has been increased.
Top view

Missing cone
We have circumvented the Abbe-limit, BUT:
Comparison of widefield (upper row) and laser scanning confocal fluorescence microscopy images (lower row).

(a) and (b) Mouse brain hippocampus thick section treated with primary antibodies to glial fibrillary acidic protein (GFAP; red), neurofilaments H (green), and counterstained with Hoechst 33342 (blue) to highlight nuclei.

(c) and (d) Thick section of rat smooth muscle stained with phalloidin conjugated to Alexa Fluor 568 (targeting actin; red), wheat germ agglutinin conjugated to Oregon Green 488 (glycoproteins; green), and counterstained with DRAQ5 (nuclei; blue).

(e) and (f) Sunflower pollen grain tetrad autofluorescence.
3.

Imaging through two objectives: the 4Pi microscope

Filling (part of) the missing cone by enlarging the NA.
Aperture increase: 

4 Pi Microscope (Type C)

Sample between Coverslips

Illumination Emission

Detector Pinhole

Dichromatic Beamsplitter

Laser

Fluorescence Intensity

High Sidelobes

2 Photon Effect

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ATF

OTF

widefield

4Pi
4Pi PSFs

widefield, $\lambda=500\text{nm}$

4Pi, $\lambda=500\text{nm}$
Leica 4Pi

Image: http://www.leica-microsystems.com

Optical Principle
Phase- and wavefront-corrected imaging system

1. Special high NA objectives
2. Sampleholder
3. UHQ surface mirrors
4. UHQ non-polarizing beamsplitter
5. 4Pi point spread function
6. Two photon point spread function
4Pi images

Deviding *Escherichia Coli*

4. Superresolution imaging: Structured illumination microscopy (SIM)
Limited resolution in conventional, wide-field imaging

Real space
“Sample” for simulation

Fourier space
Fourier transform of “Sample”

Sample will be “repainted” with a blurry brush rather than a point-like brush.
Moiré effect

high frequency detail

high frequency grid

low frequency moiré patterns
Moiré effect

Structured Illumination

Illumination with periodic light pattern down-modulated high-frequency sample information and makes it accessible for detection.
Structured Illumination Microscopy

Sample  Sample with structured illumination  Illumination

Multiplication of sample and illumination
Structured Illumination Microscopy

Real space

\[\rightarrow\]

Fourier space

Convolution of sample and illumination

Multiplication of sample and illumination
Structured Illumination Microscopy

Sample

Illumination
Structured Illumination Microscopy

Sample
Structured Illumination

Sample

Sample & Illumination
Imaging leads to loss of high frequencies (OTF)
Separating the components...
Separating the components...
Shifting the components...
Separating the components...
Shifting the components...
Recombining the components...

Sample
Separating the components...
Shifting the components...
Recombining the components... using the correct weights.
sample wide-field SIM (x only)
1 focus in back focal plane

Full-field illumination

Missing cone – no optical sectioning
2-foci in back focal plane

2-beam structured illumination

Missing cone – no optical sectioning
Missing cone filled – optical sectioning

3 foci in back focal plane

2-beam structured illumination

better z-resolution
Fourier space

1 μm

Liisa Hirvonen, Kai Wicker, Ondrej Mandula, Rainer Heintzmann
99 beads averaged

WF: 252 nm
SIM: 105 nm
Axon Actin (Growth Cone)

2 µm

excite 488nm, detect > 510 nm
24 lp/mm = 88% of frequency limit
Plan-Apochromat 100x/1.4 oil iris

Samples Prof. Bastmeyer, Universität Karlsruhe (TH)
Axon Actin (Growth Cone)

excite 488nm, detect > 510 nm
24 lp/mm = 88% of frequency limit
Plan-Apochromat 100x/1.4 oil iris

Samples Prof. Bastmeyer, Universität Karlsruhe (TH)
Doublets in Myofibrils

Isolated myofibrils from rat skeletal muscle
Titin T12 – Oregon green

L. Hirvonen, E. Ehler, K. Wicker, O. Mandula, R. Heintzmann, unpublished results
3d live cell SIM

cytosol (green), actin (red)

Images by Reto Fiolka,

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