Introduction to light microscopy

(concepts in imaging and overview)
Microscopy with light
Components of a light microscope

1. Light source
2. Objective
3. Sample or specimen holder
4. Focusing mechanism
5. Lens for focusing light on specimen
6. Eyepiece / Camera
Interaction of light with matter

Samples

mouse
tissue
cells
Fundamentals of light microscopy

Compound microscope:

Microscope composed of an objective and an additional lens (eyepiece, occular, tube lens)

Magnification:

What is the maximal magnification?

Is there a limit in useful magnification?

\[ M_{\text{final}} = M_{\text{objective}} \times M_{\text{ocular}} \]

\[ \Rightarrow \text{Why is there a limit in resolution?} \]
Diffraction at an aperture or substrate

Disturbance of the electric field of a planar wavefront by diffraction upon passage through an aperture

A mixture of particles diffracts an incident planar wavefront inversely proportional to the size of particles
Plane wave

Grating

Lens

Object plane

Back focal plane

Image plane
Image formation in the microscope

Diffracted light from a periodic specimen produces a diffraction pattern of the object in the back focal plane.

Not interacting incident light is transmitted undeviated and produce the evenly illuminated image plane.

Diffraction spots in the back focal plane correspond to constructive interference of waves differing in 1, 2, … wavelengths.

Image formation in the image plane is by interference of undeviated and deviated waves.
Diffraction image of a point source of light

The image of a self-luminous point in a microscope is a pattern created by interference in the image plane.

The pattern is a central bright spot surrounded by a series of rings.

The central spot contains ≈ 84% of light.

The image is called: Airy disk (after Sir George Airy (1801 – 1892))
Resolution and aperture angle

The objective aperture must capture light from a wide angle for maximum resolution (diffracted or emitted light)

$$NA = n \sin \alpha$$

$\alpha$: half angle of the cone of specimen light accepted by the objective

$n$: refractive index of medium between lens and specimen
Resolution limits

\[ d_{xy} = \frac{0.61 \times \lambda}{NA} \]

\[ d_z = \frac{n \times \lambda}{NA^2} \]

These formulas are used for the calculation of resolution in widefield microscopy.

In other techniques like confocal laser scanning, multiphoton microscopy, etc, other formulas are used.
Fluorescence in microscopy

Advantages:

- Very high contrast resulting in high sensitivity
- Tagging of specific entities possible
- Excitation / emission allows for various variants of microscopy techniques

Jablonski scheme
Confocal laser scanning microscopy: true 3D microscopy

Regular widefield fluorescence

Problem: out of focus light

Reduced contrast from out of focus light

http://smokingdesigners.com/34-stunning-depth-field-photographs/
### Excitation of fluorescence in sample:

Sample is excited by a laser focused to a point.

### Notes:

Sample is excited by a laser focused to a point.
**Emission of fluorescence from sample:**

Sample is excited by a laser focused to a point. Emitted fluorescent from focus is focused to a point and then reaches a detector measuring the incoming fluorescent light.

**Notes:**

A computer records the amount of emitted light and computes an image point by point over time.
Excluding out of focus light:

Sample is excited by a laser focused to a Point

Emitted fluorescent from focus is focused to a point and then reaches a detector measuring the incoming fluorescent light.

A computer records the amount of emitted light and computes an image point by point over time.

Emitted fluorescent from out-of-focus is also out-of-focus at pinhole and largely excluded from detector by the presence of the pinhole.
Excluding out of focus light:

Sample is excited by a laser focused to a point. Emitted fluorescent from focus is focused to a point and then reaches a detector measuring the incoming fluorescent light.

A computer records the amount of emitted light and computes an image point by point over time.

Emitted fluorescent from out-of-focus is also out-of-focus at pinhole and largely excluded from detector by the presence of the pinhole.
Comparison of widefield and confocal microscopy

Confocal microscopy has a very high signal to noise ratio (prominent in thick samples)

Confocal microscopy allows well resolved 3D imaging (without any image processing)

\[ d_z = \frac{n \times \lambda}{NA^2} \]

Image acquired with a widefield microscope

\[ d_z = \sqrt{\frac{0.88 \cdot \lambda_{em}}{n - \sqrt{n^2 - NA^2}}}^2 + \left(\frac{n \cdot \sqrt{2 \cdot PH}}{NA}\right)^2 \]

Image acquired with a confocal microscope
Temporal resolution – Nipkow disk (spinning disk – tandem) scanning microscopy

http://zeiss-campus.magnet.fsu.edu/tutorials
Multiphoton microscopy

Imaging deep into tissue
Multiphoton microscopy

Imaging in scattering tissue

All fluorescent photons provide useful signals.

Helmchen and Denk, Nature Methods 2005
Multiphoton microscopy

Deep tissue two-photon microscopy

Helmchen and Denk, Nature Methods 2005
Light sheet microscopy

Confocal microscopy

A

Objective lens

Evenly illuminated

Plane of interest

B

Scan

Pinhole rejects out-of-focus light and confines the detection plane

Light-sheet microscopy

C

Laser

Light shear microscopy is characterized by the use of a laser to illuminate a plane of interest, which is then imaged using a detector. This method provides a high degree of contrast and resolution.

D

Widefield detection with a CCD camera

This diagram illustrates the key differences between confocal and light-sheet microscopy.
Super-resolution Microscopy at a Glance

Catherine G. Galbraith and James A. Galbraith

**Diffraction**
The size that a point source appears in a conventional microscope.
- Lateral resolution: 200–250 nm
- Axial resolution: 500–700 nm

**Defining super resolution**
Super resolution is defined as any method that improves resolution by a factor of two over diffraction. Two basic approaches have been used to achieve this goal: ensemble (SIM, STED) and single-molecule (PALM, fPALM, STORM, dSTORM) techniques.

**Super-resolution techniques**

<table>
<thead>
<tr>
<th>Technique</th>
<th>Resolution improvement relative to diffraction</th>
<th>Method of illumination</th>
<th>Method of image generation</th>
<th>Probes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ensemble</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIM</td>
<td>Two fold</td>
<td>Patterned widefield</td>
<td>Multiple images combined</td>
<td>Conventional fluorescent proteins and dyes</td>
</tr>
<tr>
<td>STED, GSD</td>
<td>Several fold</td>
<td>Hardware-shaped excitation beam</td>
<td>Scanned excitation beam</td>
<td>STED, GSD photobleachable probes</td>
</tr>
<tr>
<td>Single molecule</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PALM, fPALM</td>
<td>Order of magnitude</td>
<td>Stochastic fluorescence activation</td>
<td>Summed single-molecule frames</td>
<td>Photobleachable fluorescent probes</td>
</tr>
<tr>
<td>STORM, dSTORM</td>
<td>Order of magnitude</td>
<td>Stochastic fluorescence activation</td>
<td>Summed single-molecule frames</td>
<td>Inorganic, photobleachable dyes</td>
</tr>
</tbody>
</table>

**PALM, fPALM, STORM, dSTORM**
Single-molecule techniques turn on and localize individual molecules.
- Lateral resolution: 10–60 nm
- Axial resolution: 500–700 nm wide field; 100 nm TIRF; 70 nm astigmatic lens; 10 nm interferometry

**STED, GSD**
A doughnut-shaped deexcitation beam decreases the effective size of the scanned excitation beam.
- Lateral resolution: 30–60 nm
- Axial resolution: 500–700 nm wide field; 30 nm localization

Electron microscopy measures the diameter of microtubules at ~25 nm. Light microscopy measures the diameter of microtubules from 25–250 nm, depending on the imaging technique used.
Super resolution microscopy

Beyond the diffraction limit

The common feature: switching fluorophores on and off sequentially in time and space

so that the signals can be recorded consecutively below the diffraction limit
Literatur

**Fundamentals of light microscopy and electronic imaging**, Douglas B. Murphy; Wiley-Liss, 2001
ISBN 0-471-25391-X (Sehr verständliches Buch mit allem nötigen Grundlagenwissen zu Lichtmikroskopie)

**Light Microscopy in Biology – A practical approach**, A. J. Lacey; Oxford University Press, 2004 (Einfache Beschreibung der Lichtmikroskopie mit praktischen Übungen und Anleitungen)


http://microscopy.fsu.edu/primer/index.html (Ausführliche und vorzügliche Beschreibung der Lichtmikroskopie mit Demonstrationen, sehr empfehlenswert)