Microscopy 1
Classification of Microscopy Techniques

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Microscopy Methods

• Light microscope
• Principles of image formation
• Main requirements for image formation
• Resolution (Airy-disk, Abbe principle, diffraction limit)
• Numerical aperture
• Immersion medium
• Phase contrast microscopy
• Atomic Force Microscopy
• Electron microscopy
Closing the Resolution Gap -

Increasing Biological Complexity

Fluorescence-microscopy

X-ray-microscopy

Cryo-electron tomography

Transcriptomics

Proteomics

MDS

NMR

Single particle cryo-EM

X-ray crystallography

Increasing Resolution

µm

nm

Å
## Classification of Microscopic Methods

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Design Principles in Light Microscopy

Parts of the Compound Light Microscope

- Body Tube
- Revolving Nosepiece
- Objectives
- Stage Clips
- Diaphragm
- Light Source
- Base
- Ocular Lens (Eyepiece)
- Arm
- Stage
- Coarse Adjustment Knob
- Fine Adjustment Knob
- Stage Plate

Parts of the Stereomicroscope

- Eyepiece
- Diopter
- Stereo Head
- Rotating Objectives
- Top/Bottom Lighting
- Focus Knob
- Lighting Controls
- Stage Clips

Image Courtesy: img.docstoccdn.com/thumb/orig/20473017.png
The Light Microscope

The beam path in the microscope comprises two essential parts: The object itself is clearly magnified by the objective (up to 100 times). This creates a so-called real intermediate image. The magnification is generated by several converging lenses arranged one behind the other.

The real intermediate image is magnified 10 to 20 fold by means of the eyepiece. This creates a so-called virtual intermediate image that can be seen with the eye or captured with a camera. The following figure shows the beam path in the microscope:
Glossar

• **Diffraction** is the deflection of waves at an obstacle.

• **Interference** describes the change in amplitude when two or more waves are superimposed.

• In contrast to diffraction, **scattering** involves the deflection of light by interaction with particles.

• **Reflection** is the term used for rectified, coherent scattering.

• In the case of **refraction**, the deflection of radiation is based on the change in propagation speed as it occurs when passing through a phase boundary.
Thought-experiment on the Transition from Geometric to Wave Optics
Numerical aperture

The numerical aperture of an optical system is a dimensionless number that indicates the resolving power of an objective.

The numerical aperture of an objective lens is defined by $\text{NA} = n \sin \theta$, where $n$ is the index of refraction of the medium in which the lens is working (1.00 for air, 1.33 for pure water, and typically 1.52 for immersion oil).

Increasing the magnification and the numerical aperture of the objective reduces the working distance, i.e. the distance between the front lens and the specimen.
Immersion Media

Immersion avoids total reflection at the cover glass => larger observation angle.
The use of an immersion medium allows a higher numerical aperture (N.A.) and thus an improvement of the resolving power. With water, an angle of incidence of 55° can be achieved at the interface between lens and water. When using air, the practical limit is already about 35°.

\[
N.A. = n \cdot \sin \theta
\]

Due to the refractive index of air, dry lenses, i.e. those without immersion, achieve a maximum theoretical N.A. of 1 (at \( \sin \theta = 1 \), corresponding to an aperture angle \( 2\theta \) of 180°) and practically a N.A. of 0.95 (aperture angle 144°).
For an oil immersion objective with a numerical aperture N.A. = 1.4 for immersion oil with a refractive index of 1.518, the following applies: \( 1.4 = 1.518 - \sin \theta \). From this follows the aperture angle \( 2\theta \) with 134°.
N.A. and Resolving Power - Abbe limit

Objective and condenser with the same N.A.
In this case, we obtain Abbe's formula in its most familia

\[ d = \frac{\lambda}{2n \cdot \sin \theta}; \text{ where } \lambda \text{ is the wavelength of light.} \]

or, due to \( n \cdot \sin \theta = \text{N.A.} \)

\[ d = \frac{\lambda}{2\text{N.A.}}. \]

Condensor lens with smaller N.A. than the objective

\[ d = \frac{\lambda}{\text{N.A. (Objective)}} + \text{N.A. (Condensor)} \]
Resolving power (Airy-disc, diffraction limit)

The Airy disc describes the optimally focused point of light that a perfect lens with a circular aperture can produce due to the limitations caused by light diffraction.
The size of the Airy disc decreases with increasing numerical aperture and light cone angle.
Phase Contrast Microscopy

- In phase contrast microscopy, differences in the density of structures are displayed. Objects with higher density and thus higher refractive index (e.g. cell nucleus and mitochondria) are normally imaged darker than structures with lower density and thus lower refractive index (e.g. cytoplasm, ER, Golgi).
- Only thin, transparent or weakly stained objects are suitable for phase contrast microscopy.
- The phase contrast microscope transforms phase shifts through the object into amplitude changes and thus differences in brightness.

Phase Contrast Movie of a Dividing Normal Rat Kidney Cell
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Phase contrast microscopy of Rat1 cells.
https://ibidi.com/content/213-phase-contrast
Differential Interference Contrast Microscopy (DIC)

- DIC works by separating a polarized light source into two orthogonally polarized mutually coherent parts which are spatially displaced (sheared) at the sample plane, and recombined before observation.
- As in polarization, interference contrast also works with polarized light. In addition, however, 2 Wollaston prisms are introduced into the beam path.
- In contrast to phase contrast, DIC provides infinitely variable contrast..
- Unlike phase contrast, DIC allows optical sections to be made. DIC can therefore be used for thicker preparations.
- A relief contrast is created at interfaces. This 3D effect is created because the phase of the two partial beams is particularly strongly shifted here with the interfaces appearing darker or lighter.

DIC focus series through a mixed culture of human buccal epithelial cells and bacteria.
http://olympus.magnet.fsu.edu/primer/techniques/dic/dicintro.html
ATOMIC FORCE MICROSCOPY – AFM

Cantilever

AFM modalities: (a) Contact-, (b) Contact-free and (c) Intermittent- or tapping mode

- In a raster process, the surface to be examined is scanned line by line.
- Piezo crystals in the measuring stage, which expand or contract depending on the applied voltage, move the measuring probe over the sample.
- As the tip of the probe is only a few atoms thick, the resolution reaches down to the atomic scale.
- The deflection of the probe is often amplified and detected by a reflected laser beam.
With the help of AFM topographic images of surfaces can be measured with a spatial resolution in the nanometer range.

AFM can be performed under physiological conditions (pH, temperature, ionic strength of media) and is therefore very well suited for the investigation of biological systems.

In addition to surface imaging, AFM can be used to study the mechanical properties of cells and biopolymers.
AFM-Applications

AFS - Single molecule ligand-receptor unbinding

AFS - Unfolding of proteins

Cell Elasticity

Cell Height

http://biomechanicalregulation-lab.org/afm/
Single Molecule Force Spectroscopy

Single molecule force spectroscopy is used to measure binding forces in single molecules such as proteins.

The biomolecule under investigation is covalently bound to a sample carrier and the measuring tip and then stretched by retracting the measuring tip.

As protein folding is predominantly the result of hydrogen bonds and van der Waals forces, stretching causes the molecule to unfold step by step before, after complete unfolding, one of the covalent bonds in the molecule or on the surface is torn.

(A) Schematic diagram of the experimental set-up. (B) The force-distance curve shows the complete unfolding of (titin I91)x8 and xmod dockerin covalently anchored to the sample surface and cohesin III-CBM covalently anchored to the cantilever tip. The signature curve shows the unfolding of a single CBM domain, followed by the unfolding of 8 titin I91 domains and finally the dissociation of the dockerin-cohesin III complex.

In the associated force-distance curve, the unfolding of structural domains can be identified by a sawtooth-like structure of the force curve. To understand the measurement results one needs at least knowledge of the protein sequence.
Electron Microscopy

• Since fast electrons have a much shorter wavelength than visible light, resolutions down to about 0.1 nm can be achieved in the electron microscope.
• The theoretically possible resolution for an EM with 100 keV electron energy is about 0.0037 nm. In practice, resolutions down to 0.05 nm can be achieved.
• For biomedical applications transmission electron microscopes (TEM) and scanning electron microscopes (SEM/SEM) are used.
• The cross section of electrons is considerably larger than that of photons, which limits the maximum sample thickness to 100 nm.
• Electrons lose energy on contact with the sample through a variety of mechanisms.
• The formation of low-energy secondary electrons goes along with the release of energy in the form of heat, light emission (cathodoluminescence) or X-ray emission.
• All signals provide information about the properties of the sample surface, e.g. its topography and element composition.
The main components of an electron microscope are:

- The **electron gun**, which generates the free electrons in an electron source and accelerates them towards an anode located in a ring around the beam axis.

- The **HIGH-voltage** between cathode and anode determines the energy and thus the wavelength of the electrons.

- **Electron lenses**, which can deflect the trajectories of the electrons, have the same function as glass lenses in a light microscope. While the focal length of glass lenses is fixed, the focal length of electron lenses is adjustable.

- In addition to lenses, as in the light microscope, **apertures** and **energy filters** are also used.

- A **high-vacuum system** ensures that the electron source works efficiently and that the electrons are not hindered in their path by collisions with gas molecules.

- **Sample holder** for stabilization and controlled displacement, rotation, tilting, heating, cooling of the sample.

- **Detectors** that register the electrons themselves or secondary signals.

- The **microscope column** forms the frame for all electron-optical components and shields the interior from external influences such as disturbing magnetic fields, vibrations, temperature fluctuations.
Cryo-EM

- Cryoelectron microscopy (cryo-EM) is a form of TEM in which biological samples are examined at cryogenic temperatures ($\lesssim -150 \, ^\circ C$) in amorphous ice.
- The radiation dose should be below 10 electrons per Å2.
- Under these conditions, cryo-EM allows imaging of the examined object close to its native state.
- Contrast enhancement is achieved using phase plates and energy filters.
- The main variants are cryo-EM single particle analysis, cryo-EM tomography and cryo-EM microED.
- The resolution of cryoelectron microscopically determined 3-D structures can currently reach 0.2 nanometers (2 Å).
- Cryo-EM is particularly suitable for resolving the structure of large MDa complexes.
Thank you for your attention