Evolution of the microscope
Lenses are *old* – 1600s

A single lens is really just a ‘magnifying glass’, but high *magnifications* are possible (300x).
Optical theory understood by 1860s

Used multiple lenses for high resolution and a separate lens to provide illumination.

Ernst Abbe

\[ d = \frac{\lambda}{2NA} \]
‘Modern’ light microscope by 1890s

Multiple lens scheme to provide uniform illumination when using non-uniform light sources (e.g. electric filament bulbs)
Same optical principles used to build electron microscope by 1930s
Microscopy 2018: So what’s new?

- Advanced Lasers
- Advanced optoelectronics
- Microscope
- New dyes and probes
How a transmitted light microscope works
Bright-field microscopy is the most basic form of transmitted-light microscopy. It relies on absorbance of light and so the sample can be conceptualized as a collection of small apertures that diffract ~much of the incident light. **All other** kinds of microscopy are variations on this basic design!
The illuminating optics provide uniform illumination

The sample is placed in the condensers BFP, where the light is most concentrated and defocused. Diaphragms control illumination ‘quality’.

Köhler Illumination System

Light Source

Collector Lens

Field diaphragm

Aperture diaphragm

Condenser Lens

Objective Lens

Sample

No filament structure seen at sample
QUIZ

Which rays are coming from O2?

How does the ray path change when the field diaphragm is closed?
The NA of the condenser lens impacts depth of field

The more the rays through a given point in the sample are ‘bundled’ (small NA) the more they tend to all experience the same portions of the sample.

Large condenser NA = small depth of field, low contrast

Small condenser NA = large depth of field, high contrast
The NA of the condenser lens impacts resolution

Illuminating with plane waves at larger angles allows more higher diffraction orders generated by sample to be collected by the objective.

Large condenser NA = Higher resolution

Small condenser NA = lower resolution
The NA of the condenser impacts resolution

Larger condenser NA = Higher resolution.

\[ \frac{K \lambda}{NA_{\text{obj}}} \]

Resolution with a Condenser

Imaging optics provide magnification

The detector sits a fixed distance from the optics, at the focal point of the tube lens. Thus, when the sample is closer than this distance magnification occurs.

\[ M = \frac{I_d}{O_d} \]

(Recall resolution is determined by the objective’s NA)
The focal length of most tube lenses is about 200 mm. If the magnification of the system is 100x, how far is the sample from the objective?
Visualization optics are need to look by eye

When there is no camera, a additional lens (‘eye piece’) is added after the intermediate image to make the rays again parallel for the eye.

For Human Convenience
Often adds 10x magnification
An complete microscope uses many (sets of) lenses to produce good illumination, provide engineering convenience, and provide light that is comfortable for the human eye.
Transmitted electron microscopy
Why Use Electrons?

Recall that diffraction due to the wave nature of light is what limits resolution in a light microscope. Electrons have a much shorter wavelength and so they diffract less (for a given aperture), allowing many more diffraction orders to be collected and thus far higher resolutions.

\[ \lambda = \frac{h}{mv} \]

Comparing a photon and electron of the energy, the electron’s wavelength is about 1000x shorter. Thus, an EM can have ~1000x higher resolution!
Transmission LM’s and EM’s are Largely Similar in Concept and Design
EM’s use Electric or (Electro)Magnetic Fields to Focus Electrons

Electrostatic Lens

Electromagnetic Lens

Electrons can only travel in a vacuum!
## Transmission LM versus EM

<table>
<thead>
<tr>
<th></th>
<th>LM</th>
<th>EM</th>
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<tbody>
<tr>
<td><strong>Wave Source:</strong></td>
<td>- Visible light (1 eV)</td>
<td>- Electrons (120 keV)</td>
</tr>
<tr>
<td><strong>Lensing:</strong></td>
<td>- Curved glass</td>
<td>- Electromagnetic field</td>
</tr>
<tr>
<td></td>
<td>- Curvature fixed</td>
<td>- Curvature variable</td>
</tr>
<tr>
<td><strong>Sample Environment:</strong></td>
<td>- Air, water, oil etc</td>
<td>- High vacuum</td>
</tr>
<tr>
<td></td>
<td>- Live imaging possible</td>
<td>- Dehydrated only!</td>
</tr>
<tr>
<td><strong>Staining:</strong></td>
<td>- Fluorescent or colored dyes; often specific</td>
<td>- Heavy metals</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Non-specific</td>
</tr>
<tr>
<td><strong>Resolution:</strong></td>
<td>- 200 nm</td>
<td>- &lt;1 nm</td>
</tr>
</tbody>
</table>
Variations on bright field microscopy
Review of Bright-Field Microscopy

Recall that bright-field microscopy relies on absorbance of light and so the sample can be conceptualized as a collection of small apertures that diffract almost all of the incident light.
Limitation of Bright-Field Microscopy

Unstained samples (e.g. living cells) absorb/diffract very little light. Most of the light imaged passed straight through the sample without interacting...
Variations of the bright-field light path

The bright-field light path can be modified to specifically select out or accentuate light that did interact with the sample.

Extra optical elements are added in these locations.
Phase Contrast Microscopy
The Goal of Phase Contrast Microscopy

Phase contrast microscopy is designed to emphasize the little bit of light that is diffracted from the large majority of light that is not diffracted.

Most light passes straight through.
The Goal of Phase Contrast Microscopy

Bright field images ALL light that passes through the sample.

In Phase Contrast, the direct light is strongly attenuated...

With direct light gone, regions that caused diffraction are clearly seen.
How Phase Contrast Microscopy Works

An annulus (open ring) is placed in the FFP of the SCL, so that the sample is illuminated with only oblique light.

All illumination is oblique

Diffracted light covers the entire BFP, but un-diffracted light is focused to a specific ring location.
A ‘phase plate’ is placed in the BFP of the objective where the oblique, direct light is focused. This plate attenuates (and phase shifts) the direct light. *The diameter of the phase plate must match the image of the phase annulus.*
Bright-field vs. Phase Contrast Microscopy

Bright-field

Phase-Contrast

Bright ‘fringes’ due to scattered direct light
Differential Interference Contrast Microscopy (DIC)
The Goal of DIC Microscopy

Light’s phase shifts as it passes through sub-cellular materials of different refractive index. Differential interference contrast (DIC) microscopy forms an image whose contrast is related to the amount of phase shift at nearby locations. No diffraction is required!
Refraction causes a phase delay

When light’s EM field travels through a material with bonded electrons, it causes them to oscillate, just like little antennas. The oscillating electrons produce their own little light waves, with a short delay. The total light field in the material is the sum of the EM fields produced by the electrons and the impinging wave. Thus, the total field experiences a phase delay.
How DIC Works

1. A birefringent crystal is used to simultaneously send two columns of light through the sample, each slightly offset and with a different polarity.
2. The refraction in the sample shifts the phase of the light.
3. A second birefringent crystal ‘undoes’ the initial offset. Thus, light from adjacent sample locations interferes. The amount of interference is proportional to the phase difference between adjacent regions.

Using first polarity

Using second polarity

Phase map 1

Phase map 2

Slightly offset the phase maps

Give each map the same polarity so the light can interfere

Result is a phase difference map

Same polarity:
The light from nearby locations is brought together using a birefringent material.

Asymmetric crystals have two different refractive indices. They can split light into two paths, each with its own polarity.

Unpolarized Light

Refractive Index 1
R.I.1 = 1.49

Refractive Index 2
R.I.1 = 1.66

Polarity 1

Polarity 2

http://igs.indiana.edu/images/rocksandminerals/CalciteArticle_Figure2.jpg
The DIC Light Path

- Lamp Filament
- Lamp Condenser Lens
- Wollaston Prism
- Polarizer (Rotatable)
- ‘Sub-stage’ Condenser Lens
- Objective Lenses

**Interference happens here**

No diffraction required

**Object**

Undoes the offset

**FFP of LCL**

Creates a light field with two orthogonal polarities and a small offset
Caution! DIC images contain NO 3D information

DIC images look ‘3D’: One side of an object looks bright and one side and dark, similar to how oblique sunlight is cast on a mountain. However, the bright/dark shading is due to the direction of the phase derivative (offset). **THERE IS NO 3D INFORMATION IN THE INTENSITY.**

The intensities encode the amount of phase difference – the dark areas are not a shadows!
Bright-field vs. DIC Microscopy

Bright-field

Differential Interference Contrast