An Introduction to the Theory and Practice of Light Microscopy

Michael Chua
Cell & Molecular Physiology
microscopy@unc.edu
http://microscopy.unc.edu
6007 Thurston Bowles
843-3268

Michael Hooker
Microscopy Facility

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The Five Talk Plan

- Oct 30. A brief history of microscopy, theory of operation, key parts of a typical microscope for transmitted light, Kohler illumination, the condenser, objectives, Nomarski, phase contrast, resolution

- Nov 06. Fluorescence: Why use it, fluorescence principals, contrast, resolution, filters, dichroic filter cubes, immuno staining, fluorescent proteins, dyes.


- Nov 27. Advanced Confocal: Live cell imaging, co-localization, blead through/cross talk, FRAP, fluorescence recovery after photobleaching, deconvolution
History and Evolution

- 4 Centuries of light microscopy!
- Imaging transmitted, DIC, phase contrast, fluorescence. Live cell techniques – dyes targeting organelles, time lapse, spectral detection, FRAP, FRET
- Resolution improving techniques

Zeiss compound microscope with confocal scan head (2001)

~400 years
Zacharias Janssen was a Dutch spectacle-maker credited with inventing the first compound microscope in ~1590. Magnifications ~3x to ~9x.
Huygens

Christiaan Huygens (1629–1695) was a Dutch mathematician, astronomer and physicist developed an improved two lens eye piece. Optical errors in two half curved lenses tend to cancel out. Can get more magnification.

Hooke

Robert Hooke improved the design of the new compound microscope, including a light source ~1655. Developed micrometer. Resolution ~5
Anton van Leeuwenhoek simple microscope (~1675) used a single lens which yielded high magnifications (~70x to ~300x) and excellent resolution (~1 um). He reported seeing many kinds of microorganisms including bacteria!
Chester Moore Hall & John Dollond

- Achromatic refracting lens was invented in 1733 by an English barrister named Chester Moore Hall
- Patented by John Dollond.

Joseph Jackson Lister (1786-1869) design and construct superior complex lenses by combining lenses of crown and flint glasses of different dispersion, but separated in order to both correct chromatic aberration and minimize spherical aberration.
Abbe

Ernst Abbe (1840-1905) applied mathematical principles to the design of lenses, which dramatically facilitated the manufacturing high quality optical instruments by the Carl Zeiss corporation. Abbe's realization that the performance of a microscope was limited by the diffraction of light was not well accepted for decades.

The Abbe limit formula

\[ d = \frac{\lambda}{2 \cos \alpha} \]
Köhler

In 1893 August Köhler (1866-1948) invented a method of providing optimum illumination of a microscope specimen while working at the Zeiss Corporation. Improved resolution and evenness of light illumination made photomicrography possible.

Georges Nomarski (1919-1997) developed the differential interference contrast (DIC) microscopy technique, which bares his name.
Johas Ploem invented the epi-illumination cube used in fluorescence microscopy.
Inoue & D. Allen & N. Allen

Video enhanced microscopy – use electronic camera and computer generated contrast enhancement.

Shinya Inoue  Nina Allen
Marvin Minsky

1957 Patented the Confocal Scanning Microscope: U.S.Patent 3013467

Practical Confocal microscope systems became available in the late 1980s. Yields improved contrast, resolution and optical sectioning.
1980s Digital deconvolution microscopy removes haze.

Mathematical Transformation + time

+ a powerful computer with extensive storage
History Summary - Recent Evolution

Dyes – fixed, vital, indicators
Immunostaining /Antibodies
Molecular Biology
Illumination
Lasers
Electronics
Cameras – CCDs, Intensifiers, high speed
Optics – ATOF, ATOM, fibers
Computers
Algorithms & software
Techniques – Time lapse, FRAP, FRET, FLIM
Control systems – focus, x-y movement, shutters
Live cell environmental control

Better resolution
More sensitivity
Lower noise
Faster detection
Greater specificity
Easier analysis
Bigger storage
New capabilities

Increased complexity
Increased cost
More raw data
Key Parts of a typical microscope

Note that the lamp is missing.
Simplified Optical Path

Illumination and observation light paths

Problem: See image of lamp filament
Kohler Illumination

Condenser has double apertures

Condenser aperture changes the cone angle of light at the specimen

Field diaphragm changes diameter of light at the specimen
The Condenser

- **Is your friend**
  - When trying to find your sample
    - Minimum aperture gives:
      - Thick depth of focus (easy to find sample since greater chance of being in focus)
      - High contrast (can see edges of colorless cells, and also see dust & scratches)
      - Less light (but have more than enough anyway)
      - Poorer resolution (who cares when just locating sample)
  - When taking images
    - Maximum aperture provides:
      - Thinner depth of focus (less overlying material seen)
      - Lower contrast (more even background, no dust and scratches)
      - More light (good for dark samples)
      - Best resolution (camera will notice the difference but eye will not)
Parts of a typical microscope

MICROSCOPE COMPONENTS

Camera
Camera Adapter
Binocular
Eyepiece
Beam Switch
Magnification Changer
Filter Cube Changer
Slot for Analyzer Body Tube
Slot for DIC Prism Objective Nosepiece Objective Stage
Condenser: Diaphragm&Turret Centering Focus
Slot for Polarizer Field Diaphragm
Upright Microscope Stand

Coarse/Fine Specimen Focus Filters and Diffuser Lamp: Focus, Centering

Focus, Centering

Epi-Lamp Housing

Shutter
Filters

Epi-Field Diaphragm & Centering

Epi-Condenser Diaphragm

Focus, Centering

Trans-Lamp Housing

Mirror: Focus and Centering

From E. Salmon
Parts of a typical microscope

- Incandescent lamp (transmitted)
- Field diaphragm (2)
- Condenser focus (5)
- Neutral density & polarizer filters (3)
- Condenser ring (5)
- Condenser aperture (4)
- Mercury arc lamp (fluorescence)
- Slide holder (6)
- Wallston prism, obscured (7)
- Mercury lamp block off rod (14)
- Dichroic filter wheel (15)
- DIC wave plate (analyser), slider (8)
- Fine/course focus (6)
- (9) Camera port sliders (10)
- Incandescent light (transmitted) power wheel (12)
- Incandescent light power (1)
Objectives

- Magnification
- Numerical Aperture
- Immersion – oil – glycerol – water – air
- Tube length system (infinity)
- Cover slip (0.17 mm or 0 or variable)
- WD = working distance
- Corrections – cover slip – iris
- Field and aberration
- Plan Apo – Fluor – Acromat
Magnification

• Note more magnification (M) gives less light intensity at detector. (due to inverse square law & also often more absorbing lens elements)

• Brightness proportional to $1 / M^2$

![Diagram showing the relationship between magnification (M) and light intensity.](image)
Numerical Aperture (NA)

Numerical aperture is loosely related to resolving power. High NA leads to smaller working distance.

\[ u = \text{half angle of cone of illumination} \]
\[ n = \text{refractive index of medium} \]

- Brightness proportional to \( NA^2 \)
Immersion

Refractive Indexes:
- Dry (air) 1.00
- Water 1.33
- Glycerol 1.47
- Immersion oil, glass 1.52

Snell’s law of refraction
\[ n_1 \sin(o_1) = n_2 \sin(o_2) \]

\( (n = \text{refractive index}) \)
Numerical Aperture (NA) - resolution

NA = n.sin (a),  a = half cone angle
    n = refractive index of medium
    λ = wavelength

Wide Field  d  = λ / 2 NA
              = 0.22 μm
Tube length

- Modern scopes are “infinity” corrected
- Older scopes may be 160 mm
Cover Slips

- For non oil immersion objectives coverslip is really a lens element.
- Use (number) #1.5
- With non immersion lenses with a cover slip correction collar it is very important the adjust it appropriately.

This objective’s correction collar lists cover slip thickness in mm. Set to 0.17 for numbers #1.5 cover slips, or use maximum contrast method if your sample has structures with good contrast.
### Typical Objectives

<table>
<thead>
<tr>
<th>Mag.</th>
<th>NA</th>
<th>type</th>
<th>WD</th>
<th>corrections</th>
<th>cover slip</th>
<th>Immersion</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x</td>
<td>0.3</td>
<td>PL Fluotar</td>
<td>3.6 mm</td>
<td></td>
<td>#1.5</td>
<td>air</td>
</tr>
<tr>
<td>16x</td>
<td>0.5</td>
<td>PL Apo</td>
<td>150 um</td>
<td></td>
<td>#1.5</td>
<td>oil/glycerol/water</td>
</tr>
<tr>
<td>20x</td>
<td>0.7</td>
<td>PL Apo</td>
<td>590 um</td>
<td></td>
<td>#1.5</td>
<td>air</td>
</tr>
<tr>
<td>40x</td>
<td>0.85</td>
<td>PL Apo</td>
<td>240 um</td>
<td>corr</td>
<td>0.14-0.18</td>
<td>water</td>
</tr>
<tr>
<td>40x</td>
<td>1.25 to 0.75</td>
<td>Apochromat</td>
<td>240 um</td>
<td>aperture</td>
<td>#1.5</td>
<td>oil</td>
</tr>
<tr>
<td>63x</td>
<td>1.4 to 0.6</td>
<td>PlanApo</td>
<td>90 um</td>
<td>aperture</td>
<td>#1.5</td>
<td>oil</td>
</tr>
<tr>
<td>63x</td>
<td>1.2</td>
<td>Apo</td>
<td>220 um</td>
<td>corr</td>
<td>0.14-0.18</td>
<td>water</td>
</tr>
<tr>
<td>L40x</td>
<td>0.8</td>
<td>HCX Apo</td>
<td>3 mm</td>
<td>U-V-I</td>
<td>none</td>
<td>water **</td>
</tr>
<tr>
<td>L63x</td>
<td>0.9</td>
<td>HCX Apo</td>
<td>2 mm</td>
<td>U-V-I</td>
<td>none</td>
<td>water **</td>
</tr>
</tbody>
</table>
Care of Objectives

Gentle, gentle, gentle, gentle!
When rotating objective turret. Slowly!
When changing slides. Focus away. Remove slide slowly!
When unscrewing objectives. Two hands! Don’t let drop. Store immediately in objective holder tube.

Cleaning:
Should be infrequent with dry lenses.
Lens tissue or surgical cotton only! Never Kim Wipes, Kleenex, Q-Tips, etc.
With immersion lenses, blot front lens gently. Do not wipe or rub.
Dry or immersion lenses of residue or oil use coated lens cleaner (pH ~6.0 to 7.0, non-ionic detergent, short chain alcohol)
Common Modes of Imaging

- Transmitted
  - Wide field (standard transmitted)
  - Phase contrast
  - Nomarski (DIC)
  - Polarization (material science)
- Epi-illumination (including confocal)
  - Fluorescence
  - Reflection (material science)
Transmitted light – (wide field)

Live Buccal Epithelial cell (unstained)
Wave Nature of Image Formation

Ernst Abbe (1872) Image formation:
Collection of diffracted rays around sample by objective
Interference of these rays in the image plane

$$D = \frac{\lambda}{2n \sin(\alpha)}$$

$$n = \text{refractive index of medium}$$
$$\lambda = \text{wavelength of light}$$
Phase contrast

Easy to set up.
Works through plastic
Loose resolution
Normarski (Differential Interference Contrast, DIC)

Setup, setup, setup!
1. Kohler illumination
2. Cross polarizers
3. Push in Wollaston prisms
4. Adjust shear (Wollaston II)
   No birefringent material in light path, e.g. plastic, collagen

But easy to do once practiced.
Nomarski enhances local gradients of refractive differences

Wide field

Nomarski (DIC)
Standard White Light Microscope

Transmitted light microscopy
  Sample needs to be mostly transparent
  Dyes give contrast/color

Reflected light microscopy
  Sample needs to be reflective
  See surface

Exciting light adds background – reduces contrast
Fluorescence – Live Buccal Epithelial cells
More on November 6!

Transmitted

Fluorescence
FM 1-43 membrane dye
Limitation of wide field

A solution is confocal imaging: November 20!
Barely scratched the surface of light microscopy.

References

• Fundamentals of Light Microscopy and Electronic Imaging, D. Murphy


• Microscopy from the Very Beginning, 2nd ed., Carl Zeiss Microscopy
Hydroethidrine Mosquito larva from a pond in Durham
Michael Hooker Microscopy Facility

http://microscopy.unc.edu

Michael Chua
microscopy@unc.edu
843-3268
6007 Thurston Bowles

Wendy Salmon
wendy_salmon@med.unc.edu
966-7051
6129 Thurston Bowles