Digital Imaging Systems
Light Microscopy

conventional optical microscopes, contrast techniques, fluorescence

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Introduction

- Microscopy:
  - Optical Microscopy (visible light)
  - Micro-CT (X-ray)
  - Electron Microscopy (electron beam)
  - Atomic Force Microscopy (no radiation)

- Uses:
  - Biology
  - Crystallography
  - Material Sciences
  - Engineering
Contents

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The Bright Field Microscope

- Simplest, oldest form of microscopy
- All other microscopy techniques we will see today (and most we'll see tomorrow) are implemented by adding optical elements into the bright field microscope's optical path
- Uses wide-field illumination (vs spot illumination)
- Contrast obtained by absorption
  - Less transparent regions seen darker
The Bright Field Microscope

- Light
- Sample
- Objective
- Intermediate image plane
- Image plane
- Ocular
- A.K.A. eyepiece
- Eye

Lenses typically are compound lenses, not a single lens.
The Bright Field Microscope

- Light
- Sample
- Objective
- Intermediate Image Plane
- Projection Lens
- Image Plane
- CCD
Köhler Illumination

Illuminating your samples since 1893

Note how each point of the light source illuminates the whole sample. This makes for a very uniform illumination. Improved uniformity often accomplished with frosted glass over the lamp.
Putting It All Together

- Collector lens
- Lamp
- Field diaphragm
- Condenser diaphragm
- Sample
- Objective lens
- Tube lens
- Afocal space
- Intermediate image plane
- Projection lens
- Image plane
- CCD
Old, but Still Current!

Nikon's First Microscope (circa early 1900s)

Nikon Eclipse E200 Student Microscope (circa 1999)
Rayleigh's inequality for resolving power: $r \geq 1.22 \frac{\lambda}{2NA}$

For a diffraction-limited microscope:

$$psf(r) = \left(2 \frac{J_1(\pi 2NA/\lambda r)}{\pi 2NA/\lambda r}\right)^2$$
Spherical Aberration

- When the lens curvature deviates from the ideal, light going through different parts of the lens focuses at different distances from the lens.
- There is no perfect focus point: loss of resolution.
- Correction always takes into account the cover slip.

Different cover slip thickness will cause spherical aberration!
Chromatic Aberration

- A normal lens focuses each wavelength at a different spot
  - Compare to a prism
- Chromatic corrections: achromat, fluorite, apochromat
  - Using different materials: crown glass, flint glass, fluorspar
Field of View Flatness

- Field curvature corrected lenses are able to keep the whole field of view in focus.
- Very important for quantitative microscopy – even more important for 3D microscopy!
Immersion Media

- air ($n = 1.00$)
- cover slip ($n = 1.50 - 1.54$)

Also used:
- water ($n = 1.33$)
- immersion oil ($n = 1.51$)
- cover slip ($n = 1.50 - 1.54$)
Objectives

minimum CCD sampling density:

\[ \text{pixel size} < \frac{1}{2} \cdot M \cdot 1.22 \cdot \lambda / 2 \cdot NA \]
Objective
Dark Field Microscopy

- Tiny modification to the bright field setup
- Contrast obtained by diffraction
Dark Field Microscopy

Light scatters at sample

Objective collects only scattered light

Light stop at condenser diaphragm stops all light that would go directly into the objective
Phase Contrast Microscopy

- Yet another small modification to bright field setup
- Contrast obtained by change in phase
Phase Contrast Microscopy

Phase ring lets only a “ring of light” through to illuminate the sample. Direct light gets delayed by $\frac{1}{4} \lambda$ (90 degree phase change).

Light scattered at sample is not delayed. Light interferes with the object and phase object to create an interference pattern. Constructive and destructive interference create the image.
Phase Contrast Microscopy

Delay or advance phase with $\frac{1}{4} \lambda$?
DIC: Differential Interference Contrast Microscopy

- A slightly more difficult modification
- Contrast obtained by difference in phase change
  - That is: optical gradients converted to intensity differences
- Produces fake 3D effect
- Orientation-dependent
On Polarization and Wollaston Prisms

Birefringent crystal (quartz or calcite)
DIC: Differential Interference Contrast Microscopy

the two perpendicular components are delayed in phase differently

lamp

field diaphragm

unpolarized

linearly polarized 45 deg.

linearly polarized

0 deg. and 90 deg. polarized light separated spatially

elliptically polarized

linearly polarized

CCD

image plane
DIC: Differential Interference Contrast Microscopy
Structured Illumination
Structured Illumination

Image in frequency domain

Uniform illumination corresponds to impulse in FD

Sinusoidal illumination corresponds to shifted impulse in FD
Structured Illumination

normal area of FD imaged in microscope

multiplication in image domain is convolution in frequency domain

normal “field of view” of frequencies is shifted

resolution can be increased to about double the system's
Structured Illumination

Fluorescence Microscopy
Fluorescence Microscopy

• The use of fluorescence in microscopy is the most important innovation to quantitative biology since the invention of the microscopy itself

• Images show only location of fluorescent markers, all non-stained tissue remains perfectly transparent
  – Perfect when measuring the extent of stained tissues or organelles

• Amount of fluorescence can be measured accurately
  – Direct relation between amount of fluorescence and number of proteins
Fluorescence emission is always a longer wavelength (less energy) than the absorption (or excitation).

The difference is the Stokes shift.
Fluorophores

Most common fluorophores:
1. Small molecules (this slide)
2. Fluorescent proteins
3. Quantum dots

![Graphs of Fluorophores](image)

- **Fluorescein derivatives**: (e.g. FITC)
- **Cyanine derivatives**: (e.g. Cy3, Cy5)
- **Rhodamine derivatives**: (e.g. Rhodamine green, Rhodamine phalloidin)
Photo-Bleaching and Photo-Toxicity

- Fluorescent molecules can lose their fluorescence ability
  - An excitation/emission cycle has a fixed probability of causing the loss of fluorescence
  - Molecule reacts with something in its environment (usually oxygen), and converts into a non-fluorescent molecule
  - Limit light exposure to avoid photobleaching

- Fluorescent molecules can kill the cells stained with it
  - Fluorescence excitation can result in free oxygen radicals
  - Free radicals kill the cell because they are chemically highly reactive

- Excitation light can also destroy the specimen through heat transfer
Green Fluorescent Protein

M. Chalfie, O. Shimomura, R.Y. Tsien
2008 Nobel Prize in Chemistry

man-made GFP derivatives:
EGFP (enhanced GFP)
BFP (Blue)
CFP (Cyan)
YFP (Yellow)
mCherry
mRaspberry
mPlum
mBanana
mStrawberry
dTomato
Quantum Dots

Zinc Sulfide Capsule
Polymer Coating
Biological Conjugate
Hydrophilic Exterior
Cadmium Selenide Core

~20 atoms diameter

2.3 Size (nanometers) → 5.5

QDot 525
QDot 565
QDot 605
QDot 655
QDot 705

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The Epifluorescence Microscope

- Objective works also as condenser
- Objective
- Dichroic
- Tube lens
- Excitation filter
- Emission filter
- Projection lens
- CCD
- Sample
- Condenser diaphragm
- Collector lens
- Field diaphragm
- Lamp
- Image plane
The Filter Cube

- Dichroic:
  - Shorter wavelengths are reflected
  - There also exist mirrors that reflect only a small band of wavelengths

- Filter:
  - Selected wavelengths are transmitted
  - Long pass: \( \lambda \) and longer are transmitted
  - Band pass: \( \lambda \pm d \) are transmitted

- Excitation light
- Emission
- Fluorescence emission light out
- White light in
The Epifluorescence Microscope
Autofluorescence

A single optical section from a thick section of human skin
green: anti-basal lamina protein
red: neuronal processes
collagen and elastin autofluoresce in blue
(source: zeiss.com)

pollen is strongly autofluorescent

autofluorescence in fruit fly
Cross-Talk
Spectral Unmixing

By imaging many channels ("hyperspectral image"), it is possible to distinguish overlapping emission spectra.

stained with 3 different red dyes...

705 nm QDot (shown green)
food autofluorescence (red)
skin autofluorescence (white)

source: www.cri-inc.com
Further Reading

• Microscopy U (Nikon)
  - http://www.microscopyu.com/

• Microscopy Primer (Molecular Expressions)
  - http://microscopy.fsu.edu/

• Olympus Microscopy Resource Center
  - http://www.olympusmicro.com/

• The Handbook (on fluorescent probes)
  - http://probes.invitrogen.com/handbook/

• Optical Microscopy

Images in these slides that I didn't draw myself, came mostly from Microscopy U, some from Wikipedia, and some from other, quoted sources.