Introduction to Light Microscopy and Confocal Microscopy

Nick Barry
Light Microscopy at the LMB

- We have a range of microscopes.
- They each have strengths and weaknesses.
- We can help you to choose the best microscope for your sample/scientific question.
FIXED AND STAINED CELLS

LIVE DROSOPHILA LARVA

Yara Sanchez-Corrales
Katja Roeper
Quantitative Imaging
WHEN WAS THE LIGHT MICROSCOPE INVENTED?

- Lenses
- Z Jansen 1609
- Galileo 1609
- Robert Hooke Micrographia 1665
- Antony van Leeuwenhoek (1632–1723)
- Abbe Imaging Theory 1873

Nick Barry
Robert Hooke
Micrographia 1665

First described the cell

Robert Hooke
Antony van Leeuwenhoek (1632 – 1723)

Observations published in
Philosophical Transactions of the Royal Society c 1673

BIOLOGICAL MICROSCOPY HAD ARRIVED
Principle of the Microscope

\[ d > f \]

Magnification = 100X

What is special about microscopes?

THEY REVEAL THE FINE DETAILS

Magnification
Resolution

With a microscope we are often trying to observe structures smaller than the wavelength of light

(Telescopes are optimised to see very distant objects)

This has some profound implications in relation to how they are made and used.
LIGHT MICROSCOPY

**Objects are smaller than the wavelength of light**

Ever increasing scatter and absorption

**light microscopy**

Improving the resolving power of the microscope

1) Diffraction
The wave-like nature of light and resolution

2) Refraction
The bending of rays of light by spherical surfaces and optical aberrations

3) Dispersion
Wavelength dependence of optical properties

Nick Barry
Improving the resolving power of the microscope

1) Diffraction

What happens when the size of the object that you want to look at is about the same size as the light that you are shining on it?

2) Refraction

3) Dispersion

Nick Barry
Young’s Slits - Interference and diffraction

Snells Law (1621)
Huygens, Treatise on Light (1690)
Newton, Opticks (1704)
Thomas Young (1801)

1846 Zeiss starts building microscopes
Zeiss asks Abbe for help in 1866

Carl Zeiss
Ernst Abbe

Nick Barry
Abbe’s Experimental Apparatus

Eyepiece for regular image or Bertrand lens to look at objective back focal plane

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Diffraction

Object

Diffraction pattern in the back focal plane of the objective

A finer pattern leads to larger diffraction angles

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Higher NA also means more light is collected giving a brighter image.

Immersion gives higher NA.

\[ \text{NA} = n \sin \alpha \]

**Importance of Objective Numerical Aperture:**

- **NA is King**

**RESOLUTION**

\[ R = \frac{1.22 \lambda}{2\text{NA}} \]
Importance of Objective Numerical Aperture :- NA is King

1. Object on the microscope stage
2. Fine structure leads to diffraction
3. Lens only captures a limited number of the diffraction orders
4. ALL Detail not reproduced in the image

Diffraction Limited Imaging

diffraction orders - some miss the lens

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Improving the resolving power of the microscope

1) Diffraction
   - Lenses bend and focus rays of light. However, ..........
2) Refraction
   - In real microscopes, not all rays end up in a perfect (diffraction limited) focal spot resulting in an aberrated or distorted and blurred image.
3) Dispersion

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Sample induced aberrations (i.e. what we do wrong)

Consider this simple case

With the high quality Plan Apochromatic objectives available to us, if we used them EXACTLY as the lens designer intended the images would be almost free of aberrations.

Three rays of light coming to a common point (focal spot)

Sample induced aberrations (i.e. what we do wrong)

Consider this simple case

Slab of higher refractive index

Because of refraction, described by Snell’s law, rays move (out of their ideal position) around real optical elements in an Angle, Thickness and Wavelength dependent manner.
Sample induced aberrations (i.e. what we do wrong)

\[
\begin{align*}
n_1 &\quad \text{IMMERSION MEDIUM} \\
n_2 &\quad \text{COVERGLASS WRONG THICKNESS} \\
n_3 &\quad \text{SAMPLE REFRACTIVE INDEX NOT MATCHED} \\
n_4 &\quad \text{TOO MUCH MOUNTING MEDIUM} \\
n_3 &= n_2 = n_1 \text{ for oil immersion}
\end{align*}
\]
Improving the resolving power of the microscope

1) Diffraction

2) Refraction

3) Dispersion

Spreading out the spectrum of light with a prism

Nick Barry

1550 1600 1650 1700 1750 1800 1850

Hooke  Leeuwenhoek  Chester Moore Hall  Adams  Tulley

Achromatic doublet invented (?) by Chester Moore Hall around 1730

Around 1830, in collaboration with Joseph Jackson Lister, William Tulley made one of the first microscopes that corrected for both chromatic and spherical aberration. Nick Barry
The Modern Microscope Format

Older style

"Finite Tube Length"

Newer research grade

"Infinity Corrected"

Object plane

Primary image plane

Conjugate planes

Objective back aperture

Objective Markings

Nick Barry

OLYMPUS manufacturer

plan apochromat
numerical aperture
magnification
infinity correction

40x /1.0 oil iris at 0.17

corrected for coverglass
thickness of 0.17 mm

1.0 > < 0.5

color-coded band
special feature: dark-field iris

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Objective Types

5X 10X 20X

Low NA Air

AIR GAP

40X 60X 100X

Refractive index matching \( n(\text{oil}) = n(\text{coverslip}) = n(\text{sample}) \)

Oil immersion
NA 1.0 – 1.45

Higher NA = Higher resolution

Not good for thick watery samples

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Objective Types

5X 10X 20X

Low NA Air

20X 40X

Very small air gap

High NA Air (NA < 1)

Coverslip thickness must be correct

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Objective Types

Good for live biological samples

Water immersion
NA 1 - 1.2
Correction collar must be set to the correct coverslip thickness

Water on both sides of the coverslip

Good for electrophysiology, intravital and lightsheet imaging

Water dipping
Upright microscopes only, NA ~ 1

The Eyepiece

Microscope primary image plane

Too close for comfort

Remember to zero the diopter setting

http://micro.magnet.fsu.edu/primer/anatomy/oculars.html
The Eyepiece

Image is now a comfortable distance away and has additional magnification

Remember to zero the diopter setting

The Condenser, Conjugate planes and Kohler Illumination

GETTING THE BEST IMAGE OUT OF THE MICROSCOPE

http://micro.magnet.fsu.edu/primer/anatomy/oculars.html

http://www.microscopyu.com/print/articles/formulas/formulasconjugate-print.html
Why it is important to focus the condenser?

FOR ALL SHARED MICROSCOPES IN THE LMB THE DEFAULT CONDITION IS THAT THE CONDENSER IS OUT OF FOCUS AND OUT OF ALIGNMENT

Uniform illumination of the sample
Most efficient use of light
Highest resolution
Minimises background glare and scatter
Sets the microscope up for contrast enhancement such as Phase or DIC
You get the best images

Steps to Kohler illumination
1) Identify the condenser focusing and centering knobs
2) Identify the Field Aperture and Condenser Aperture
3) Put a sample on the microscope and focus it
4) Close down the Field Aperture and focus the Condenser
5) Center the Condenser
6) Open the Field Aperture until it is about matched to the field of view
7) Check that the Condenser Aperture is fully open

Bringing Contrast to Transparent Samples

A typical cell culture monolayer is almost invisible in white light illumination

Interference methods can be used to bring contrast by exploiting the phase retardation of light by the slightly increased refractive index of the cell compared to the surrounding medium.

Phase Contrast, Frits Zernike 1934

Differential Interference Contrast F.H. Smith 1955, Normarski
Phase Contrast

The Phase Plate is located at the back aperture of the objective. Diffracted and undiffracted light lie in different positions. Phase plate sets up conditions for interference at the image.

Phase Contrast Alignment

Adjust the microscope for Kohler illumination
The objective indicates which condenser mask to use
Identify and select the correct illumination mask in the condenser
Using a Bertrand Lens align the bright and dark rings
You are putting optical elements into conjugate planes

http://www.olympusmicro.com/primer/anatomy/specialobjectives.html

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Differential Interference Contrast

Shearing interferometer with very small shear (a few hundred nanometers)

Figure 1

Relative phase retardation

Optical Sectioning in Differential Interference Contrast Microscopy

http://micro.magnet.fsu.edu/primer/techniques/dic/dicintro.html

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INCREASING THE USEFULNESS OF LIGHT MICROSCOPY

ADD CONTRAST TO A SPECIFIC MOLECULAR SPECIES IN THE SAMPLE

FLUORESCENT MOLECULE LINKED TO A MOLECULAR TARGETING METHOD

e.g. Antibodies
     Toxins
     Binding partners
     GFP
     ...

FLUORESCENCE MICROSCOPY

ADVANTAGES OF FLUORESCENCE MICROSCOPY

- Good resolution
- High sensitivity
- High specificity
- Compatible with living things

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A TYPICAL FLUORESCENT LABEL

Nick Barry

FLUORESCENCE SPECTROSCOPY

Jablonski diagram

Molecule energy states

Photon in

Photon out

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EXCITATION AND EMISSION FILTERS

MULTIPLEXED FLUORESCENT LABELS
WIDE FIELD FLUORESCENCE MICROSCOPE

'CUBES' HAVE 3 FILTERS

**Emission filter**
Fluorescence emission

**Dichroic filter**
Separates excitation and emission

**Excitation filter**
Fluorescence excitation

The excitation light and the emitted fluorescence both pass through the same objective. The whole field of view is illuminated at once.

Nick Barry
In the fifties, Minsky wanted to look at the detailed structure of brain tissue. He reasoned:
1) Why not illuminate the sample point by point to reduce deleterious effect of scatter?
2) Why not also use a pinhole to get rid of scatter out of plane?

THE CONFOCAL MICROSCOPE HAD BEEN INVENTED
CONFOCAL IMAGING Marvin Minsky 1957

image

Conjugate
AKA
Confocal
planes

scanning mechanism

focal plane

detector
pinhole
3D mask

light source

objective

object point

one point illuminated
one point detected

MINSKY’S APPARATUS AND PATENT

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COMBINED EFFECT OF POINT SCANNING AND PINHOLE

Widefield Fluorescence  Confocal Fluorescence

CONFOCAL IMAGING: Depth resolved images in a 3D object

1) Illumination intensity falls off as $Z^2$
2) Detection efficiency falls off as $Z^2$

For an out of plane point:
- Confocal image intensity $\propto 1/Z^4$
- Confocal sectioning

Nick Barry
CONFOCAL MICROSCOPY AT THE LMB

John White, Brad Amos, Michael Fordham, Richard Durbin

How the Confocal Laser Scanning Microscope entered Biological Research

Fig. 1. The prototype confocal laser scanning microscope in 1980. The optical axis of the microscope is horizontal, with a telescope linking the scanning eyepiece to a 25-sector polygon mirror on the right. This prototype was capable of scanning speeds in excess of 4000 lines per second (unsuccessful).
CONFOCAL MICROSCOPY AT THE LMB

MRC 600 Confocal Microscope
Biorad

CONFOCAL FLUORESCENCE IMAGING

Multiple dyes
3D Rendering

Ilaria Chiaradia
(during training)

Optical resolution "diffraction limited"
No Out Of Focus Fluorescence

Nick Barry
CONFOCAL FLUORESCENCE IMAGING

Multiple dyes
3D Rendering

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Optical resolution “diffraction limited”
No Out Of Focus Fluorescence

Nick Barry

CONFOCAL FLUORESCENCE IMAGING

The confocal image is a depth resolved slice out of a three dimensional sample.

By acquiring image stacks it is possible to reconstruct the 3D architecture of a sample

Nick Barry
IMAGE OF A POINT OBJECT AND THE DIFFRACTION LIMIT

Point source
Lens
Image of a point source

AIRY PATTERN
POINT SPREAD FUNCTION PSF

CONFOCAL IMAGING :- Setting the pinhole

Laser spot diameter in the sample = $1.22\lambda/\text{NA}$
(diameter of the first minimum)

Fluorescence spot size at pinhole = $1.22\lambda/\text{NA} \times \text{Objective Magnification} \times \text{Confocal Magnification}$

50 to 100 microns

DEFINING THE PINHOLE DIAMETER IN AIRY UNITS

If the pinhole matches the diameter of the first minimum then it is said to be set at 1 AIRY

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CONFOCAL IMAGING :- Setting the pinhole

In reality the excitation spot is imaged onto the pinhole in the confocal scan head

Default setting = 1 Airy

Bigger pinholes let through out of focus fluorescence
HOW TO SET THE PIXEL ARRAY SIZE

To see the finest sub-cellular detail you will need a high NA objective. Oil immersion or water immersion for thick watery samples.

Using the scan mirror controls you can zoom into the image. Typically the confocal samples the image in an array of 512 by 512 pixels. With Zoom the effective pixel size changes. However since the NA of the objective is fixed, the resolution stays the same.

Confocal Imaging:

- The PSF is set by the objective NA
  - Spot diameter = \(1.22\frac{\lambda}{NA}\)
    - \(480\text{nm} \at \lambda = 550\text{nm}\)
  - Resolution = \(1.22\frac{\lambda}{2NA}\)
    - \(240\text{nm}\)

Nyquist Sampling

Information theory says that you need two pixels to sample the minimal resolvable object in an image.
HOW TO SET THE PIXEL ARRAY SIZE

Calculate the lateral and axial resolution and set the pixel spacing to be about half of these values.

Laser beam scanning over the sample

Pixelated image

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HOW TO SET THE PIXEL ARRAY SIZE

OVER SAMPLING: PIXELS ARE TOO SMALL

Scanner moves a small fraction of the PSF

Do not get any extra resolution
Image takes longer to acquire

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HOW TO SET THE PIXEL ARRAY SIZE

UNDER SAMPLING

Gaps between image points

Still get 4 adjacent pixels in the image

Information is lost
Not necessarily obvious in the image

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HOW TO SET UP THE DETECTOR

Sample

Excitation in Fluorescence out

User variable

User variable

User variable

fixed

The signal arriving at the digitiser must match the dynamic range of the digitiser

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HOW TO SET UP THE DETECTOR

Most confocal microscopes use a photomultiplier to detect the emitted light.

**Photomultiplier**

**Gain**

**Amplifier**

**Gain = 1**

**A to D converter**

**255**

**Digitiser Units**

**0**

**User adjustments**

**Gain (Volts)**

**Offset**

**Set for correct use of the A to D dynamic range**

**Photobleaching ?**
- Use less laser
- Anti fade agents
- Different label

**Noisy Image ?**
- Use more laser
- Averaging

**Image of the brightest part of the sample**

**“Range Indicator” view**

**Red = saturated pixels**

**Blue = zeros**

**FLUORESCENCE INPUT**

**A to D converter**

**255**

**Digitised OUTPUT**

**Clipped**

**Saturation**

Nick Barry
NON LINEAR OPTICAL MICROSCOPY

Two-Photon Laser Scanning Fluorescence Microscopy

Winfried Denk,* James H. Strickler, Watt W. Webb

Molecular excitation by the simultaneous absorption of two photons provides intrinsic three-dimensional resolution in laser scanning fluorescence microscopy. The excitation of fluorophores having single-photon absorption in the ultraviolet with a stream of strongly focused subpicosecond pulses of red laser light has made possible fluorescence images of living cells and other microscopic objects. The fluorescence emission increased quadratically with the excitation intensity so that fluorescence and photobleaching were confined to the vicinity of the focal plane as expected for cooperative two-photon excitation. This technique also provides unprecedented capabilities for three-dimensional, spatially resolved photochemistry, particularly photolytic release of caged effector molecules.

SCIENCE, VOL. 248
6 APRIL 1990

Brad Amos M.R.C.

PRINCIPLE OF TWO-PHOTON FLUORESCENCE MICROSCOPY

Excitation
2 Low Energy Photons
This type of event has a very very low probability

High Peak Power Low Average power laser
Mode locked Ti:Sapphire Laser
100 femtosecond pulse 80 million pulses per second
Peak power 100,000 watts + focusing ⇒ 2PE

Excitation only at focus
IMAGING IN INTACT TISSUE

single molecules  subcellular organelles  single cells  fixed tissue slices  intact tissue  animal models

USE NEAR INFRARED TO BEAT

scatter and absorption

THE GOAL IS TO OBSERVE PROCESSES IN PHYSIOLOGICALLY INTACT TISSUE EVENTUALLY TO WORK IN LIVING ANIMALS (INTRAVITAL MICROSCOPY)

IMAGING: ADVANTAGES OF TWO-PHOTON EXCITATION

• Sectioning effect
• No pinhole, No de-scanning in detection path
• Separation of excitation and emission
• No out-of-plane photobleaching
• Single excitation wavelength for many dyes
• NIR has deeper penetration into tissue (a few hundred microns)
NIR IMAGING WINDOW

2 PHOTON EXCITATION AND EMISSION

Excitation is well separated from Emission facilitating spectroscopic analysis of multiple species over a broad wavelength span.
DETECTION OF ATHEROSCLEROTIC LESIONS

Plaque quantitation by traditional enface imaging

RAT AORTA: AUTOFLUORESCENCE IMAGING
SHG AND TWO PHOTON AUTOFLUORESCENCE IMAGING

XZ section in thoracic aorta

XYZ section of Right Carotid

25X water immersion 0.8 NA
360 x 360 x 128 microns

BLUE SHG generation in Collagen
GREEN Elastin Autofluorescence

AUTOMATED STACK ACQUISITION AND IMAGE STITCHING

NILE RED STAINING TO HIGHLIGHT LIPID DEPOSITS

Coverslip plane
X Z SECTIONS

Lumen
Exterior

Internal Lesion  External SHG  External Lipid

INTRAVITAL MICROSCOPY

SKIN  LUNG  KIDNEY
EYES  BRAIN  CAROTID ARTERY  ABDOMEN  LIVER  INTESTINES  LYMPH NODES

N Barry
Two-Photon Imaging of Lymphocyte Motility and Antigen Response in Intact Lymph Node

Mark J. Miller, Sindy H. Wei, Jan Parker

Michael D. Cahalan

SCIENCE VOL 296 7 JUNE 2002

B AND T CELL MIGRATION WITHIN A LYMPH NODE (CAHALAN GROUP)
Measurement of microhemodynamics in the ventilated rabbit lung by intravital fluorescence microscopy

GERHARD E. H. KUHNL, FRANZ H. LEIPFINGER, AND ALWIN E. GOETZ
Institutes for Surgical Research and of Anesthesiology, Ludwig-Maximilians University,
D-8000 Munich 70, Germany
0161-75967/93 $2.00 Copyright © 1993 the American Physiological Society

INTRAVITAL MICROSCOPY OF THE PULMONARY MICROCIRCULATION

FIG. 1. Thoracic window.

Two-Photon Imaging of Dendritic Spine Development in the Mouse Cortex

Feng Pan, Wen-Biao Gan
Molecular Neurobiology Program, Skirball Institute, Department of Physiology and Neuroscience,
New York University School of Medicine, New York, New York 10016

Transgenic GFP-expressing mouse
Functional studies of the kidney of living animals using multicolor two-photon microscopy
Kenneth W. Dunn, Ruben M. Sandoval, Katherine J. Kelly, Pierre C. Dagher, George A. Tanner, Simon J. Atkinson, Robert L. Bacallao and Bruce A. Molitoris

TWO PHOTON EXCITATION FLUORESCENCE INTRAVITAL MICROSCOPY
RUSSO et al
KI 2007

S3 The infusion of green albumin and red dextran
Movie was generated at 1 frame/s with the time lapse increased ×10
COME AND SPEAK TO US TO USE THE MICROSCOPES

THANK YOU FOR YOUR ATTENTION

Nick Barry

Further Information

General microscopy websites
- Molecular Expressions  http://micro.magnet.fsu.edu/
- Zeiss Campus  http://zeiss-campus.magnet.fsu.edu/
- Nikon MicroscopyU  https://www.microscopyu.com/
- iBiology Microscopy Short Course  http://www.ibiology.org/online-biology-courses/short-microscopy-series/

Fluorescence spectra viewers
- BD Spectrum Viewer  http://www.bdbiosciences.com/eu/s/spectrumviewer
- Life Technologies SpectraViewer  https://www.thermofisher.com/order/spectra-viewer
- Chroma Spectra Viewer  https://www.chroma.com/spectra-viewer