Principles of Light Microscopy
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True or False?

- Light microscopy is out of date now that we have electron microscopes.
- All graduates are taught how to use the light microscope.
- Because their fittings are standardised, most objectives and eyepieces can be interchanged and used on any microscope.
- Oil immersion is necessary only for high magnifications.
- It is best to use thick coverglasses because they are stronger.
- It is best to use very thin coverglasses for top-quality work.
- Microscopes are fitted with diaphragms designed to control the intensity of illumination.
- A good microscope provides a higher magnification than a poorer one.
- An image of the lamp filament should not occur anywhere in the microscope.
- Light microscopy is so much simpler than electron microscopy, that it is unnecessary to attend a course on it.
Robert Hooke 1635 - 1703
Micrographia 1665
I could exceedingly plainly perceive it to be all perforated and porous, much like a Honey-comb…

…these pores, or **cells**...
Robert Hooke on Simple and Compound microscopes

“… ‘tis possible with a single Microscope to make discoveries much better than with a double one, because the colours which do much disturb the clear Vision in double Microscopes is clearly avoided and prevented in the single.”

1665
A different approach – using a single lens

Antony van Leeuwenhoek, 1632 - 1723

Human sperm

Bacteria
With a microscope you don’t look at the specimen:

you look at an *image* of the specimen.

Like paintings, photographs or sculptures in an art gallery, the microscope image is a *representation* or a *likeness*, an *artefact*, and it will be different from the original object.

In order properly to understand microscope images, we need to understand these artefacts, and how they are produced.
The *input* requirements of the eye determine the *output* requirements of the microscope:

- The image must be presented at infinity, so that the image-forming rays are parallel on entering the eye
- Exit pupils must be 3-5mm diameter, to match the pupils of the eyes...
- and separated by about 65mm to match the interocular distance.
Microscopy

Microscopes must provide:

• **Resolution**
  ability to carry information about fine detail in the specimen to the image

• **Contrast**
  differences in the image between features and their surroundings

• **Magnification**
  to make the image large enough for the eye to appreciate the resolved detail
The limit to resolution

All optical systems

- cameras, telescopes, eyeballs, microscopes

have a limit to the smallness of each image point – like ‘optical pixels’.

The smaller they are and the more you have, the better.
Because of Diffraction at the image-forming lens:

All optical systems - the eye, cameras, projectors, telescopes, microscopes - image a point of light as a disc of light surrounded by bright and dark rings.

And the diameter of these rings is related to the aperture of the lens.
The size of the Airy disc depends on:

- The *aperture* of the lens
- The *wavelength* of the imaging radiation

Larger aperture and shorter wavelength → smaller disc → better resolution
Image of a point source: the *Airy Pattern*
with most of its brightness in the central *Airy disc*
Diffraction in the microscope

• Diffraction occurs whenever light or other wave motion encounters any kind of obstacle.
• The Airy pattern is the result of diffraction at the objective lens.
• Diffraction also occurs at the specimen.
• Whether you consider resolution to be limited by diffraction at the objective lens or the specimen, the result is the same.
The Rayleigh Criterion states that two objects can be resolved in an image when they are separated by

\[
\text{distance} \quad 0.61\lambda / n \sin \alpha
\]
The Rayleigh Criterion for Resolution
calculated from aperture of objective lens

The peak of one curve falls (approximately) over the centre of the first dark ring of the other

Combined amplitudes of $a$ and $b$
Amplitudes of $a$ and $b$

Just detectable by the human eye

radius = $\lambda / 2 n \sin \alpha$
Transmitted-light, Bright-field

Directly into the eye

And the specimen is covered by a layer of glass

Light passes *through* the specimen

A most unusual way of looking at things!
Light passes through the specimen directly into the eye. And the microscope uses light for a job that 'it wasn’t designed for' - looking at things that are around the same dimensions as the wavelength of light. A most unusual way of looking at things!
The job of an ideal lens

- To accept as many rays as possible from each point in an object
- To reassemble all the rays from each point at corresponding points in the image…
- In such a way that the distance travelled by all the rays from each object point to its corresponding image point is the same - so that they all arrive ‘in phase’.
A lens has two focal planes

Rays entering lens parallel... are converged on the opposite side of the lens as with a camera

Rays entering lens through a focal point... emerge parallel on the opposite side of the lens as with a projector
Ray diagrams - three simple rules

1. Rays entering lens parallel to axis... … cross the axis at the focal point on the opposite side of the lens

2. Rays entering lens through focal point... leave the lens parallel to axis

3. Rays passing through centre of lens are undelected
What can lenses do??

Lenses can act in a way similar to those of three familiar optical devices:

Camera
- forming a reduced-size, real image, close to the lens

Projector
- forming an enlarged, real image, distant from the lens

Magnifying glass
- not forming a real image; parallel rays to infinity
Object at infinity

Image in focal plane

Focal length of lens

Image smaller than object

Camera - portrait

Image same size as object

Camera - macro lens

Image larger than object

Camera - landscape

Object in focal plane

Image on retina

Image at infinity

Magnifying glass

Projector
The **objective lens** works like a *projector lens* and forms the **Primary Image** 10mm below the top of the viewing tube.

and the **eyepiece** acts as a *magnifying glass* and examines the centre of this image.

Ground glass on top of microscope tube
Magnification

Depends on:

Image distance
   longer → higher mag

Objective focal length
   shorter → higher mag

Eyepiece focal length
   shorter → higher mag

...and in recent microscopes on the focal length of the tube lens (more later)
The objective lens is the microscope. The other parts support its function and adapt the image to the receiving device.
The importance of Aperture in the Microscope

Consider that every ray leaving the object carries some information about fine detail in the object. Some of these rays – and some information – will be collected by the objective and some rays – and some information – will NOT be collected, and will be wasted. Resolution will therefore depend on the angular aperture of the objective - the larger the imaging aperture the higher the resolution.
Numerical Aperture (NA) is calculated as $NA = n \sin \alpha$.

- **Objective lens** is the lens that magnifies the specimen when viewed through the microscope.
- **Specimen** is the object to be observed under the microscope.
- **Medium of refractive index $n$** refers to the media through which light travels between the objective lens and the specimen.
Numerical Aperture

\[
\text{Dry Objective: } \quad \text{NA} = 1 \times \sin 72^\circ \\
\quad = 1 \times 0.95 \\
\quad = 0.95
\]

\[
\text{Immersion Objective: } \quad \text{NA} = 1.515 \times \sin 67^\circ \\
\quad = 1.515 \times 0.92 \\
\quad = 1.4
\]

Refraction makes angle $\alpha$ in air represent less-oblique rays at the specimen - which is where it really matters!
Objective lens

Light passing from objective into glass block

‘Milky glass’ block

Magnification 12.5
NA 0.3

Magnification 25
NA 0.65
Same aperture, different magnifications
Same aperture
different magnifications

25 / 0.65

40 / 0.65

40 / 0.95

Same magnification
different apertures

0.65
0.95
Minimum resolved distance is now commonly expressed as
\[ d = 0.61 \frac{\lambda}{NA} \]

**Why is Numerical Aperture Important?**

- **Minimum resolved distance**
- **Wavelength of imaging radiation**
- **Half-aperture angle**
- **Refractive index of medium**

Inscription on Ernst Abbe's memorial

\[ d = \frac{\alpha}{n} \]
Why is Numerical Aperture Important?

- Resolution depends on NA
- Light transmission of objective depends on $NA^2$
- Depth of field of objective is (approximately) inversely proportional to $NA^2$
Once you have bought the objective lenses, there is little you can be done to improve resolution...

...but it can easily be made worse by poor illumination of the specimen.
What are we trying to do when illuminating a microscopical specimen??

- Light up the specimen uniformly
  - over a controllable area
- Illuminate the objective aperture uniformly
  - over a controllable angle
Two basic methods of illumination:

Source-focused (or ‘Critical’) Illumination:
Light-source imaged on to specimen

Köhler Illumination:
Light-source imaged in the aperture of the condenser
Source-focused Illumination

Bench lamp imaged on ground glass on stage by condenser lens

Ground glass

Image of bench lamp
Light sources suitable for source-focused illumination:

- Uniformly-illuminated sky *
- Flame of oil-lamp
- Surface of opal light bulb *
- Uniformly-illuminated white paper or ground glass *

*note that these are really ‘secondary sources’

Condenser lens acts like a camera lens
- throws an image of source on to underside of slide
But looking for a region of uniformly illuminated sky in Leeds…

gave an image of the stink-pipe on the Chemistry Building

...when the microscope was set up correctly
Source-focused Illumination

Using a normal electric lamp gives an image of the writing on the end of the bulb.

Köhler Illumination solves this problem.

...and a modern halogen lamp is even worse.
Conjugate planes

An image of the object forms the primary image and this is transferred to the retina.

These are three conjugate planes - successive images of one another ... and there are more.
August Köhler  
1866 - 1948

published

A new system of illumination for photomicrographic purposes

(in German) in 1893.
The back focal plane of the objective

Image of objects at ‘infinity’ in back focal plane of objective
How do we light up the specimen *uniformly*?

Imagine a light source in the first focal plane of the condenser. Light will pass parallel through the object and be brought into focus in the back focal plane of the objective. Into the objective.
In Köhler Illumination an extra lens, the Lamp collector lens throws an *image* of the filament into the first focal plane of the condenser. This image of the filament falls also on the aperture diaphragm of the condenser, the *Illuminating aperture diaphragm*. The *Illuminated field diaphragm* fitted just after the lamp collector is imaged on to the object by the condenser lens. In this situation the lamp collector lens appears to be uniformly filled with light.

**How do we light up the specimen uniformly?**
How do we light up the specimen *uniformly*?

Note that

- **Each** point in the **object** receives light from **many** points on the **filament**

  and that

- **Each** point of the **filament** provides light to **many** points on the **object**
Why is it necessary to…

Light up the specimen uniformly over a *controllable area*?

It is unnecessary, and often detrimental, to illuminate parts of the specimen outside the field of view:

- some specimens are light-sensitive, and could be damaged
- light can be scattered into field of view from outside this area
- illuminating a large area of specimen produces a large primary image, and light can reflect from internal walls of microscope, reducing contrast in the image
Why control the area illuminated?

Large area of object illuminated provides large disc of light at primary image causing reflections from walls of microscope and reduction in contrast.
How do we control the area illuminated?

1. An adjustable diaphragm here

2. Is imaged on to the specimen, so that the area illuminated is restricted

3. And the disc of light at the primary image is kept off the walls of the microscope
**Why is it necessary to...**

Illuminate the *objective aperture uniformly* over a *controllable angle*?

Consider that every ray leaving the object carries some information about fine detail in the object. *Some* of these rays – and *some* of the information – will be collected by the objective.

And some rays – and some information – will *NOT* be collected, and will be wasted.

Resolution will thus depend on the *angular aperture* of the objective - the larger the aperture the higher the resolution.
Why is it necessary to…

Illuminate the *objective aperture uniformly* over a *controllable angle*?

‘Common sense’ suggests that if we expect to receive light over a large angle, it is important for good resolution that *most* of the objective aperture should be illuminated.

But why just *most*? Why not *all*? Why not a *very wide* cone of light?
Why is it necessary to... Illuminate the objective aperture uniformly over a controllable angle?

If the illuminating aperture is too large, light will be scattered from the edges of the objective lens, thus reducing contrast.
Why is it necessary to illuminate the *objective aperture uniformly* over a *controllable angle*?

**Worse**

If the illuminating aperture is too *small*, resolution will be reduced and image quality will be impaired though contrast will be increased.
Why is it necessary to... Illuminate the *objective aperture uniformly* over a *controllable angle*?

So for best resolution the *illuminating aperture* should approach the *imaging (objective) aperture*

60 to 75% is often recommended
How do we control the angle of illumination?

1. Closing the condenser diaphragm
2. Narrows the angle of rays passing though the object

View down tube (objective back focal plane)
How do we control the angle of illumination?

The aperture diaphragm of the condenser thus acts as the Illuminating Aperture Diaphragm – so called because it is the diaphragm which regulates the Illuminating Aperture.
What are the diaphragms for?

IF Diaphragm also imaged in primary image plane - prevents light from reflecting from inside of tube

Image of IF Diaphragm on specimen

Condenser lens

Illuminated Field Diaphragm
What are the diaphragms for?

Illuminating aperture diaphragm imaged in back focal plane of objective...

...and also in Exit Pupil of eyepiece

Illuminating aperture diaphragm
What are the diaphragms for?

1. Closing the condenser diaphragm

2. Narrows the angle of rays passing through the object

View down tube (objective back focal plane)

Condenser lens

Illuminating aperture diaphragm closed
What are the diaphragms for?

The diaphragms are NOT intended for adjusting the brightness of the image.

The **Illuminating Aperture Diaphragm** sets the angle of the cone of light illuminating the specimen, and is adjusted according to **objective NA**.

The **Illuminated Field Diaphragm** sets the area of specimen illuminated, and is adjusted according to **magnification**.
Illuminating system completely out of adjustment
Illuminated Field Diaphragm closed; its image is not centred on field of view.

Image of Illuminated Field Diaphragm not sharply focused on specimen.

Centre of image of diaphragm.

Fuzzy edge.
Condenser focus adjusted

Image of Illuminated Field
Diaphragm now sharply focused on specimen

Sharp edge
Image of Illuminated Field Diaphragm now centred on field of view
Image of Illuminated Field
Diaphragm centred on field of view

How?
• moving the condenser lens in x-y
• moving the Illuminated Field Diaphragm in x-y
• waggling the microscope mirror
• moving the entire lamp about on the bench
• centring the objective lens
Illuminated Field Diaphragm opened to illuminate full field of view
Back Focal Plane of objective

Lamp not centred with collector lens
Back Focal Plane of objective

Lamp centred with collector lens
Diffuser inserted between lamp and collector lens
Illuminating Aperture Diaphragm closed to c75% of objective aperture
Illuminating Aperture too large

Image hazy and ‘washed out’
Illuminating Aperture correct

Image contrast optimal
Illuminating Aperture too small

Image contrast too high; artefacts present
Illuminating Aperture

Correct

Too small

75%
Illuminating Aperture too small
Illuminating aperture too small

Note this object

This is what it ‘should’ look like!

Illuminating aperture correct
Illuminating aperture wide open: much larger than objective aperture

Gradually close illuminating aperture diaphragm. Illuminating aperture now equal to objective aperture

Removing stray light

Optimum setting: Stray light removed but still have adequate illuminating aperture

Reducing illuminating aperture

Poor image!

Image brightness

Setting the Illuminating aperture diaphragm – a simple way
Illuminating aperture too large
Illuminating aperture correct
Illuminating aperture too small
Illuminating aperture much too small – an extreme example
Köhler Illumination provides

Control of Area illuminated by the Illuminated Field Diaphragm, which is adjusted according to magnification.

Control of Angle of illumination by the Illuminating Aperture Diaphragm (the condenser diaphragm), which is adjusted according to objective aperture.
Imagine illuminating system is ‘folded over’ about the object plane, so that ...
Illuminated Field Diaphragm inserted in plane corresponding to Primary Image Plane

Illuminating Aperture Diaphragm inserted here. Filament must now be repositioned

Cannot insert Illuminating Aperture Diaphragm here - would also restrict imaging aperture

Objective lens acting as its own Condenser

Reflector inserted at 45°
Illuminated Field Diaphragm

Primary image

Illuminating Aperture Diaphragm

Filament

Can not insert Illuminating Aperture Diaphragm here - would also restrict *imaging* aperture

Objective lens acting also as Condenser