# Principles of Light Microscopy

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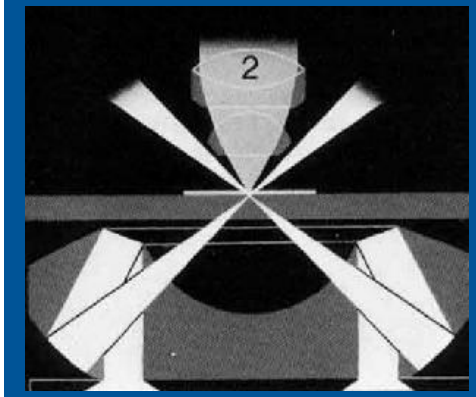
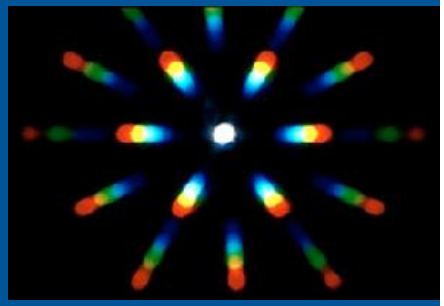
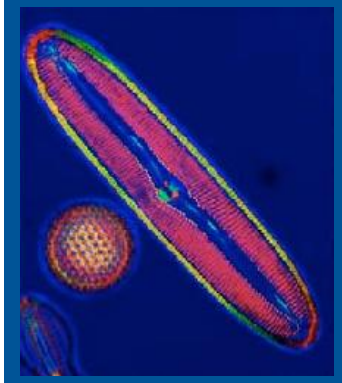
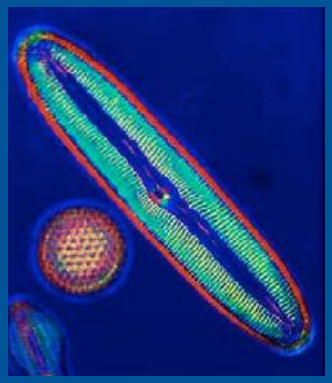
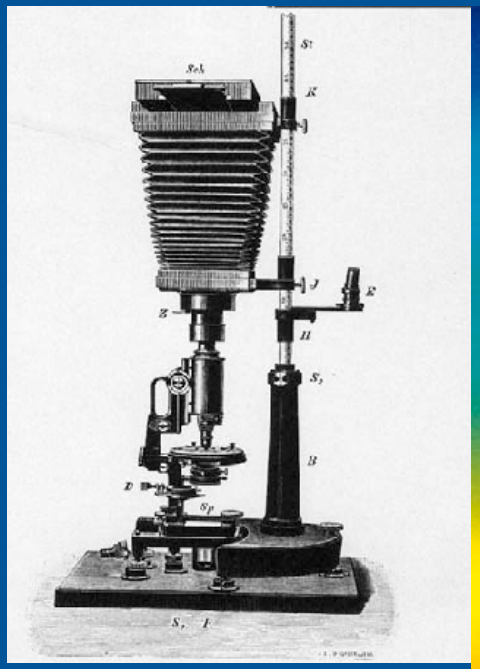
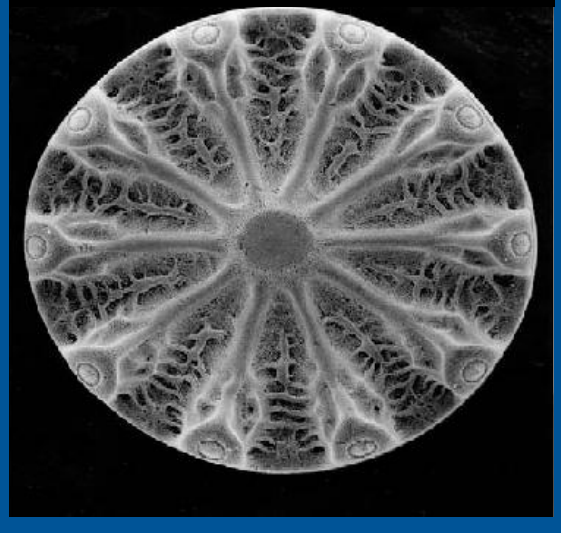


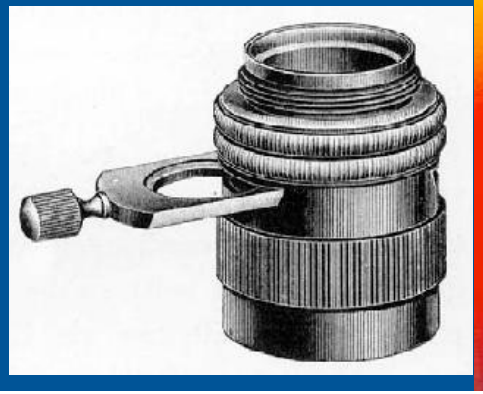











# True or False ?

- Light microscopy is out of date now that we have electron microscopes.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.

# MICROGRAPHIA:

OR SOME

*Physiological Descriptions*

OF

## MINUTE BODIES

MADE BY

MAGNIFYING GLASSES.

WITH

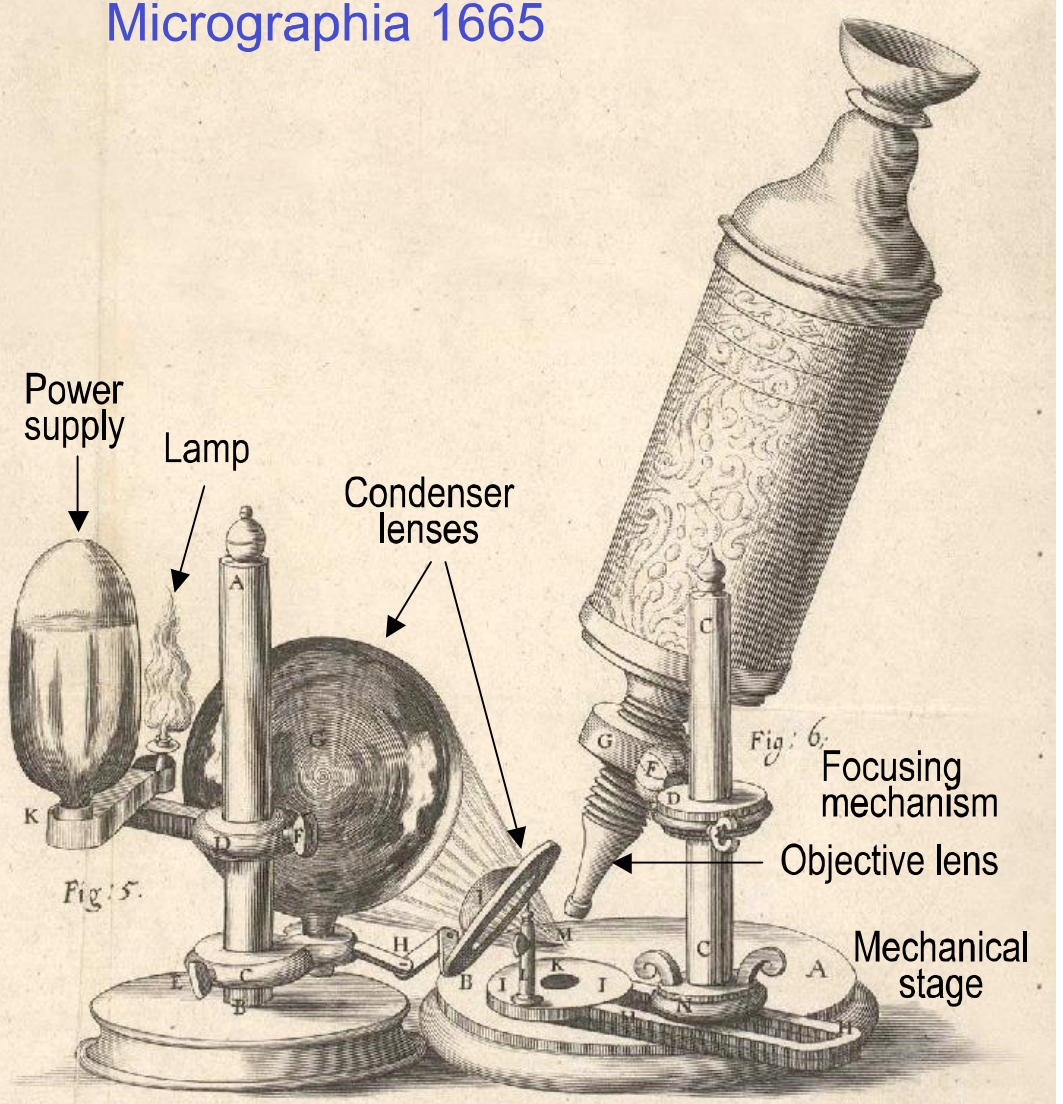
OBSERVATIONS and INQUIRIES thereupon.

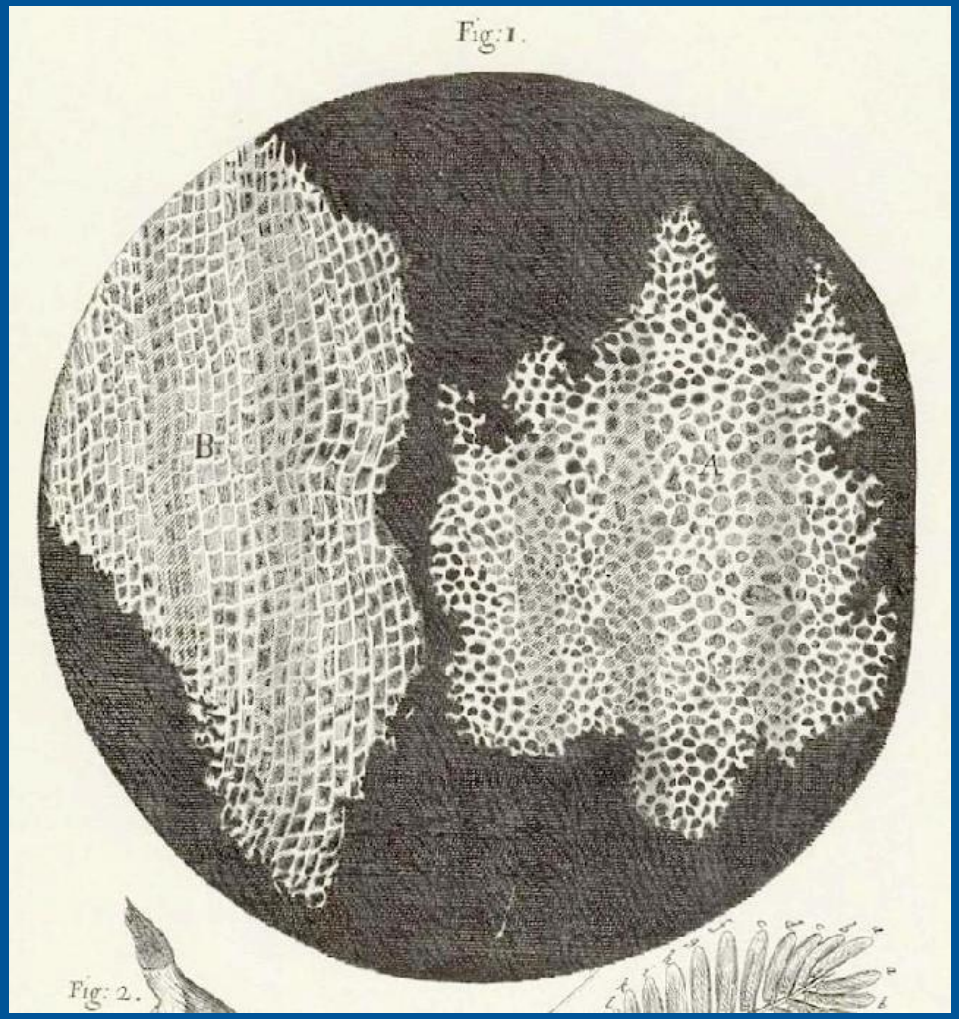
By *R. HOOKE*, Fellow of the ROYAL SOCIETY.

*Non possis oculo quantum contendere Lynceus,*
*Non tamen idcirco contemnas Lippus inungi.* Horat. Ep. lib. 1.


*LONDON*, Printed by *Jo. Martyn*, and *Ja. Allestry*, Printers to the ROYAL SOCIETY, and are to be sold at their Shop at the *Bell* in S. *Paul's* Church-yard. MDCLXV.

## Robert Hooke 1635 - 1703
## Micrographia 1665






Thin slice of Cork

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

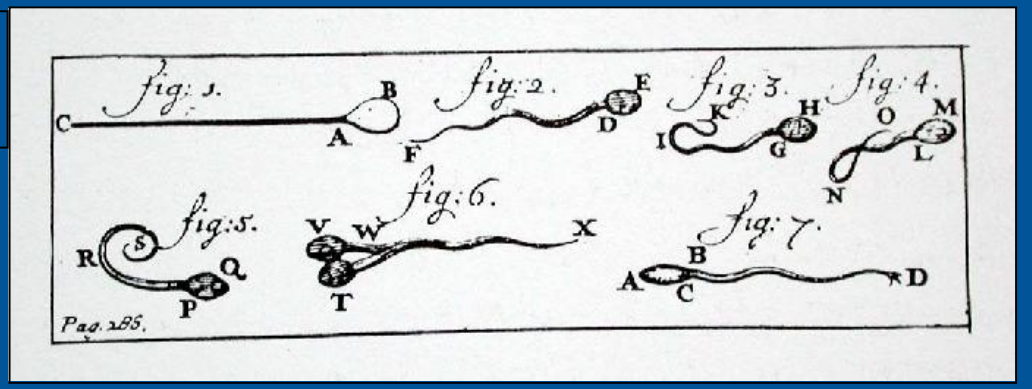
“ … ‘tis possible with a single Microscope to make discoveries much better than with a double one, because the colours which do much disturb the clear Vision in double Microscopes is clearly avoided and prevented in the single.”

1665

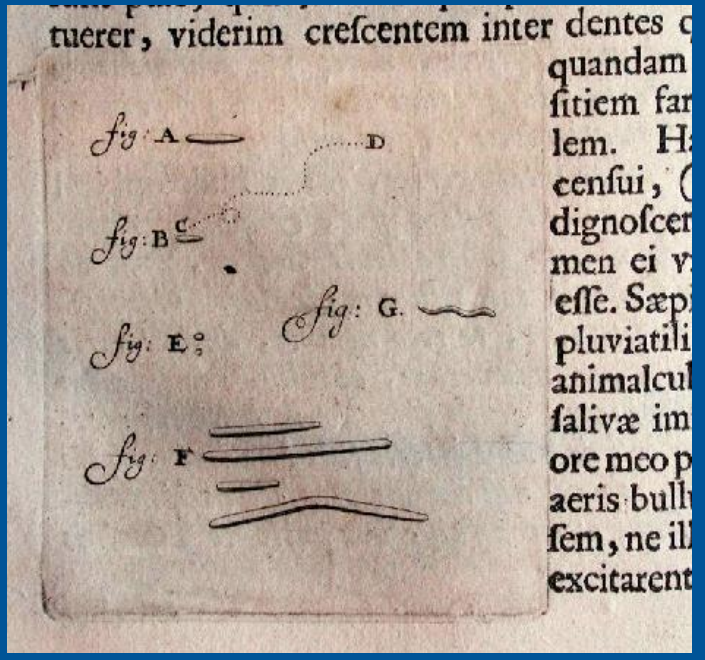
# A different approach – using a single lens

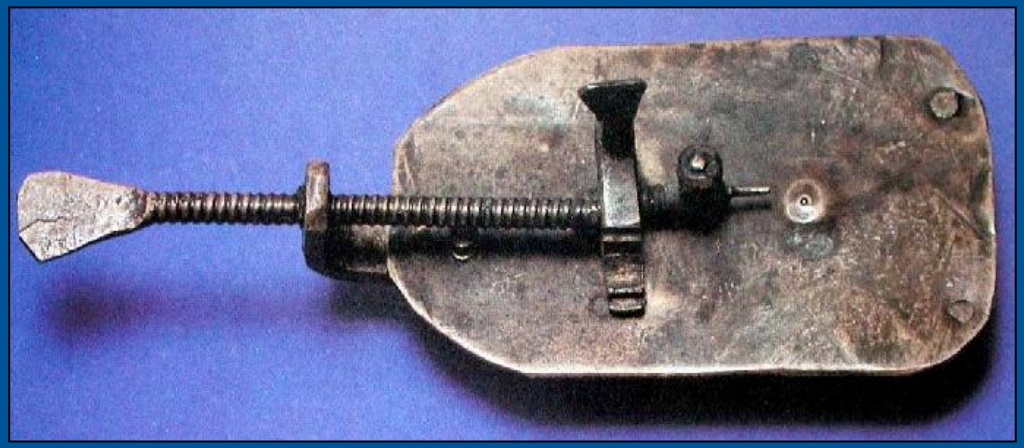
Antony van Leeuwenhoek,1632 - 1723

Human sperm

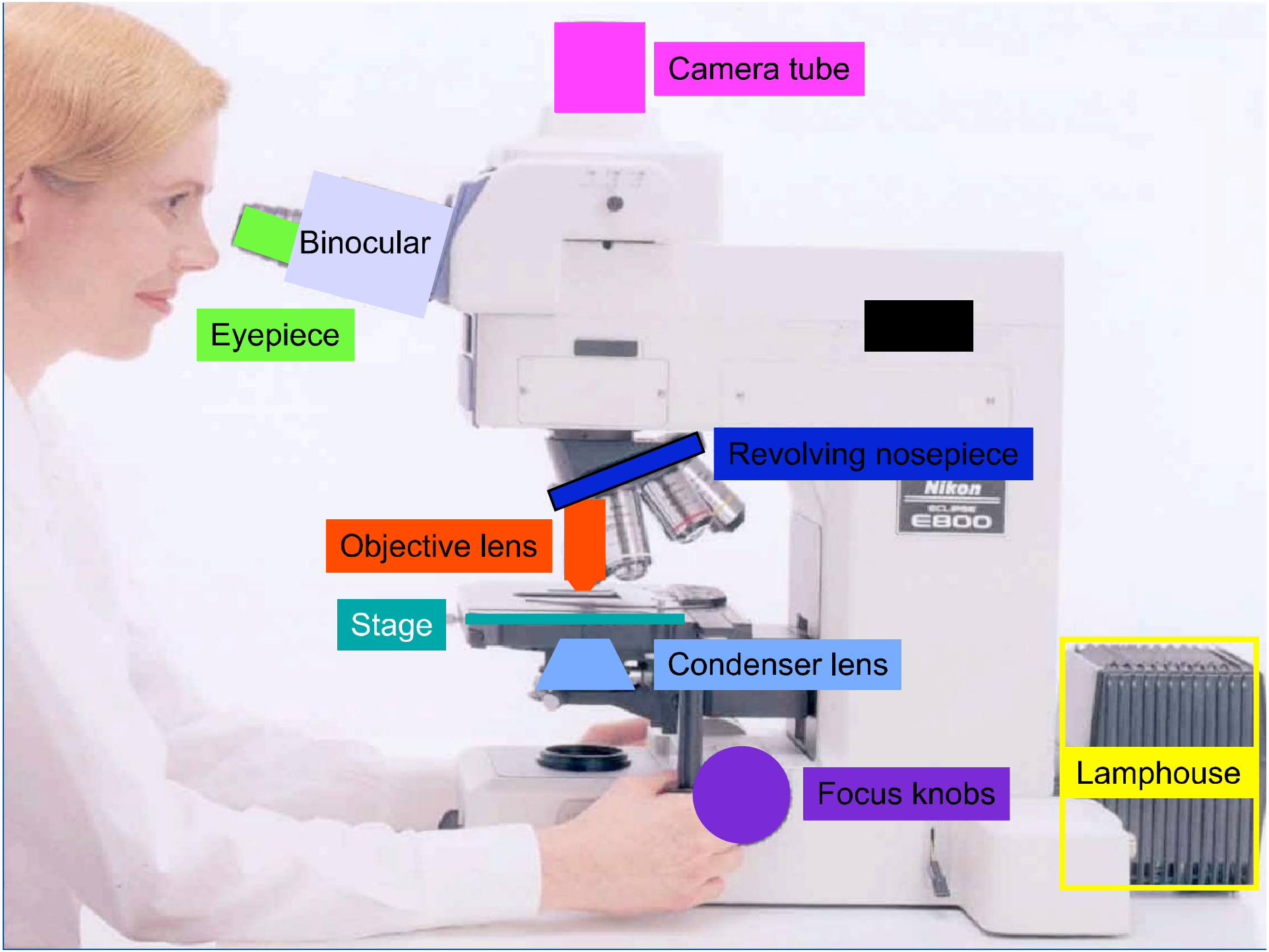



Bacteria



50mm


Camera tube
Binocular
Eyepiece
Revolving nosepiece
Objective lens
Stage
Condenser lens
Focus knobs
Lamphouse
Nikon
ECLIPSE
E800

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’.

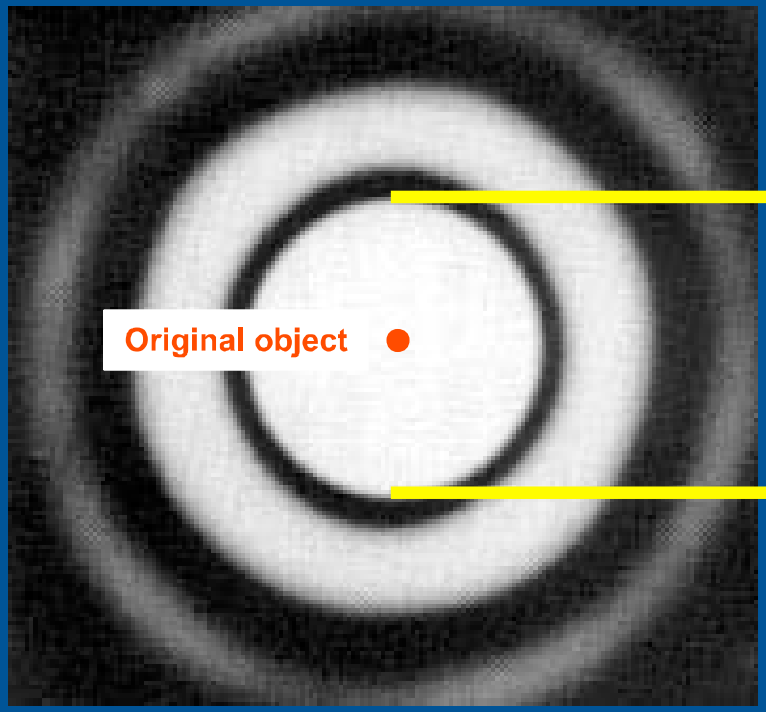
The smaller they are and the more you have, the better.

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 limit to resolution




Airy disc

Image of a point source:
The *Airy Pattern*

*This is caused by* ***Diffraction***

The size of the Airy disc depends on:

The ***aperture*** of the lens

The ***wavelength*** of the imaging radiation

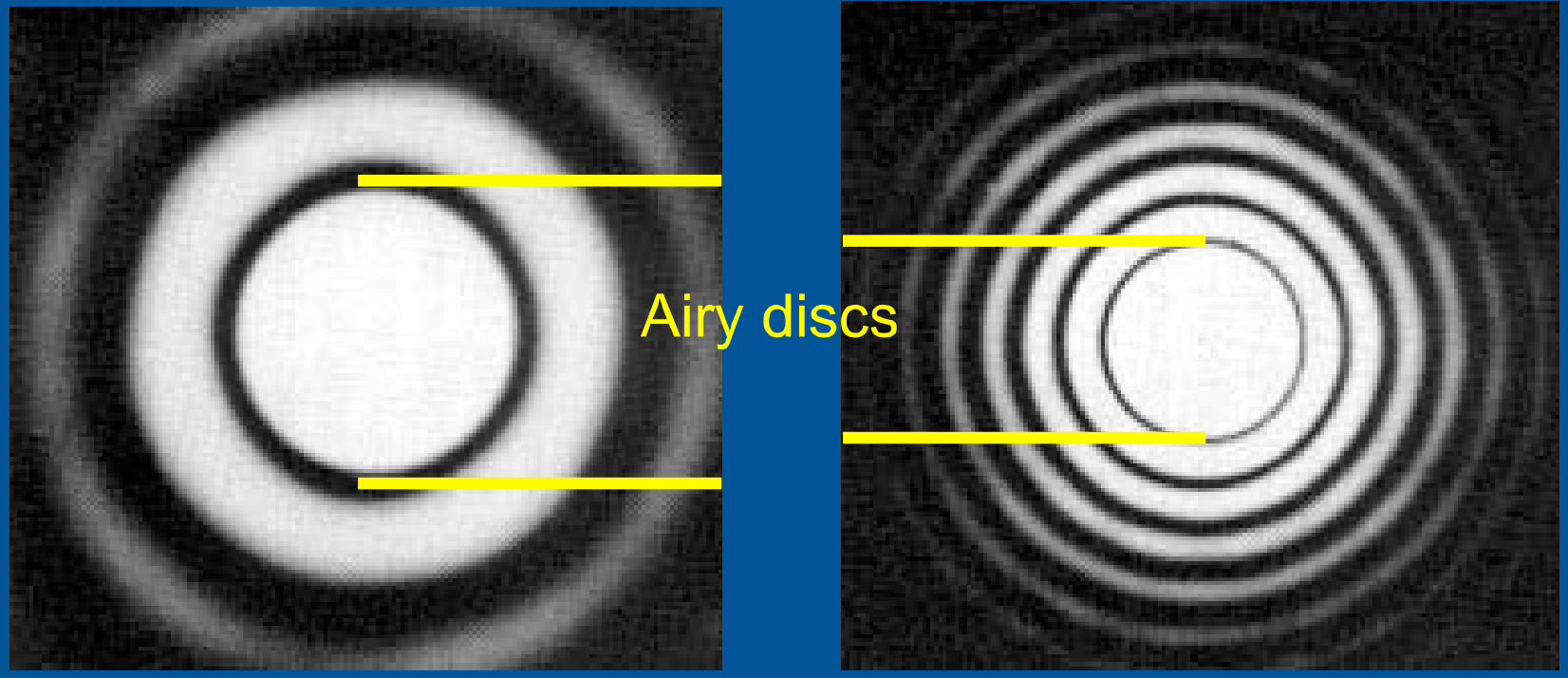
Larger aperture and shorter wavelength

→ smaller disc → better resolution

# Image of a point source:
# the *Airy Pattern*
## with most of its brightness in the central *Airy disc*

Small aperture

Larger aperture



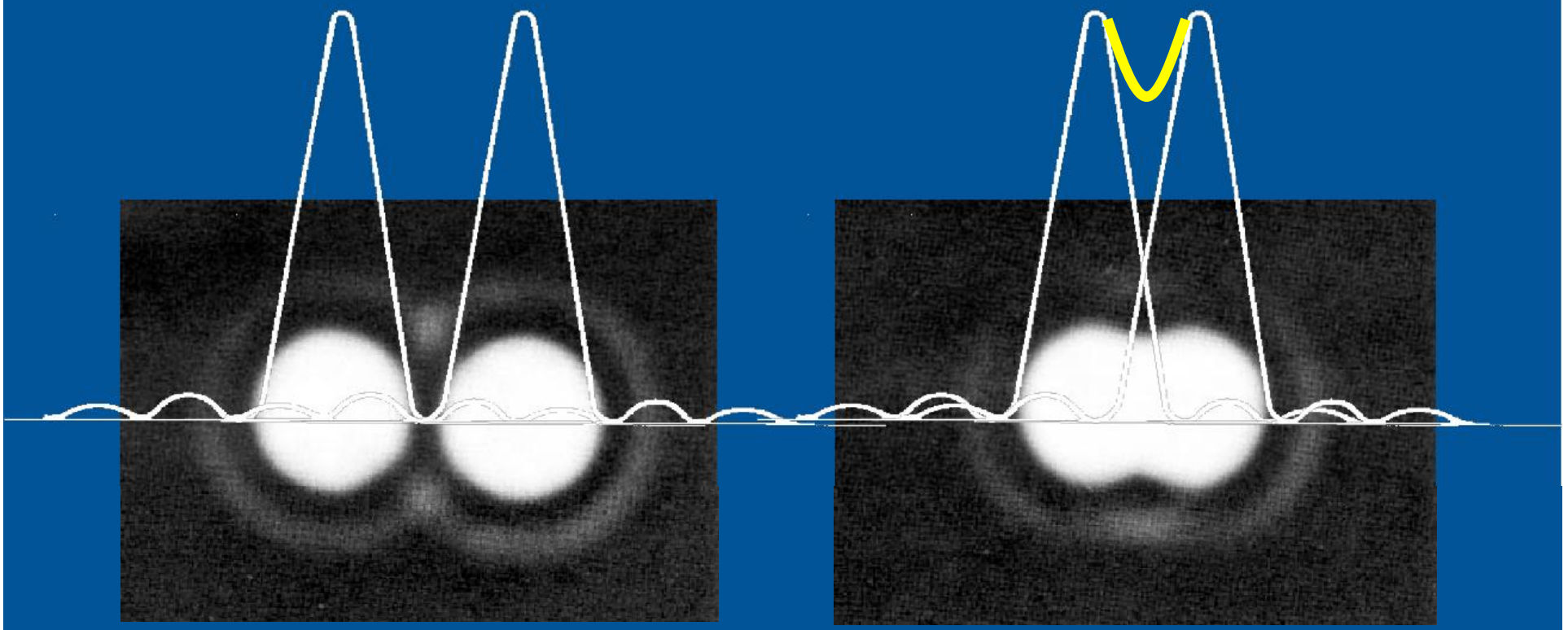
# Diffraction in the microscope

- 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.

# Two Airy patterns: brightness plots

Points resolved

Not resolved

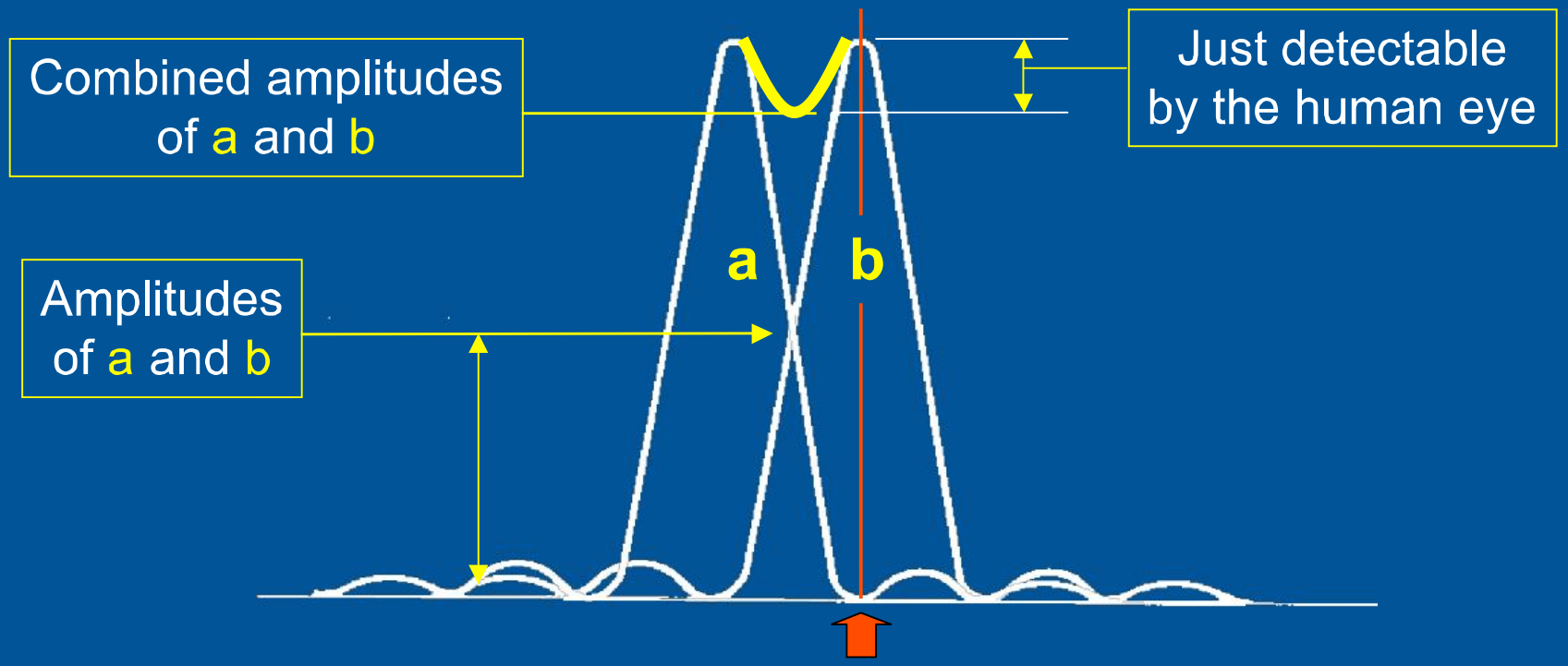


The *Rayleigh Criterion* states that two objects can be resolved in an image when they are separated by

distance **$0.61\lambda$ / n sin $\alpha$**

# The Rayleigh Criterion for Resolution

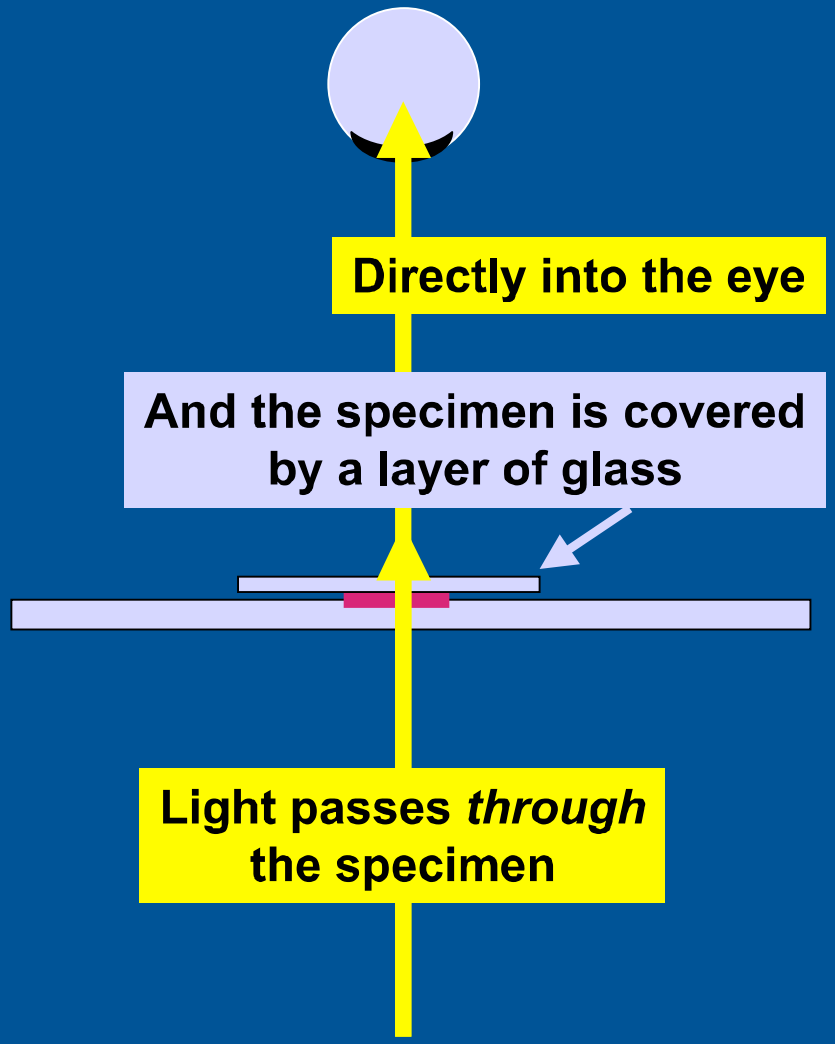
## calculated from aperture of objective lens



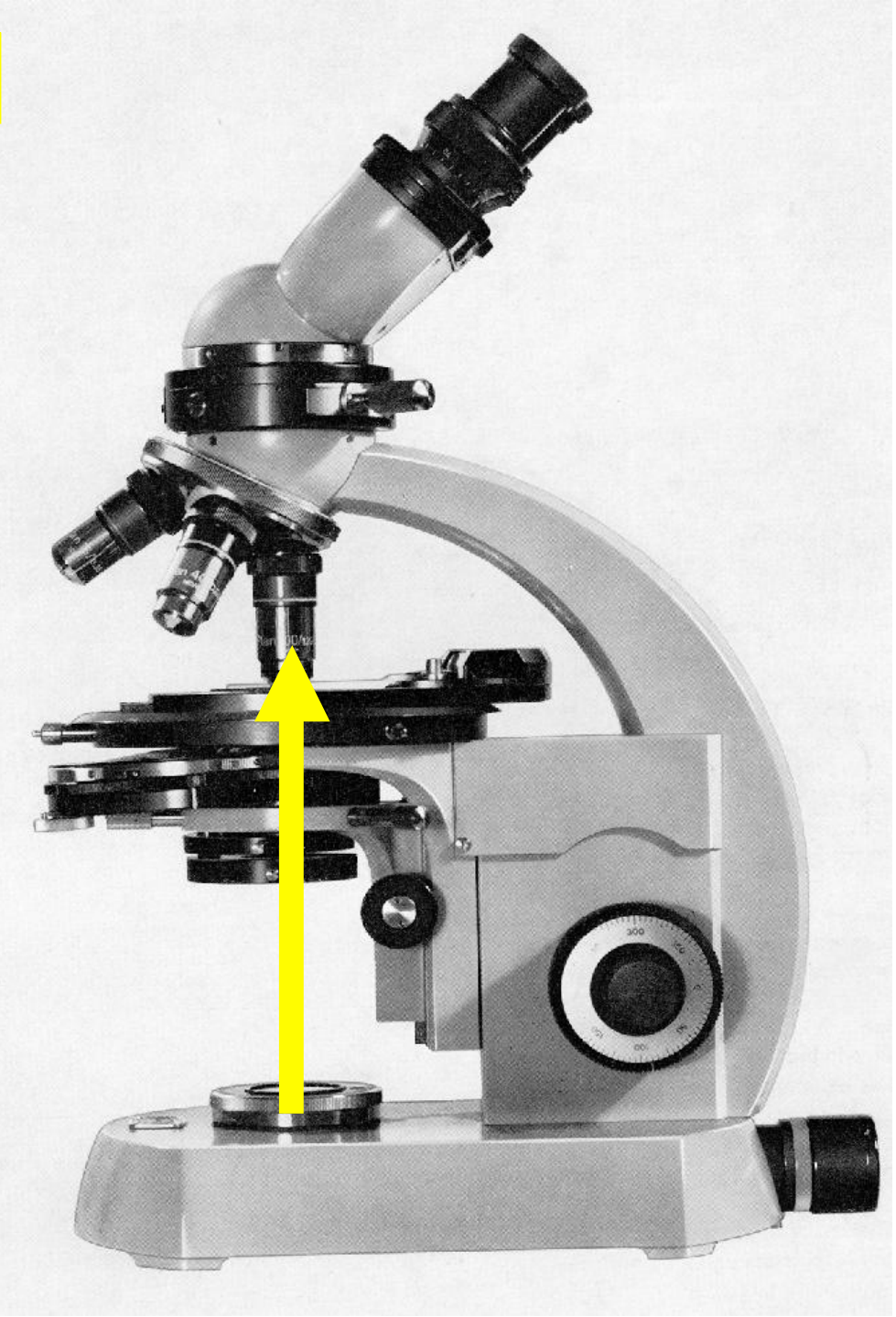

The peak of one curve falls (approximately) over the centre of the first dark ring of the other

**radius = λ / 2 n sin α**

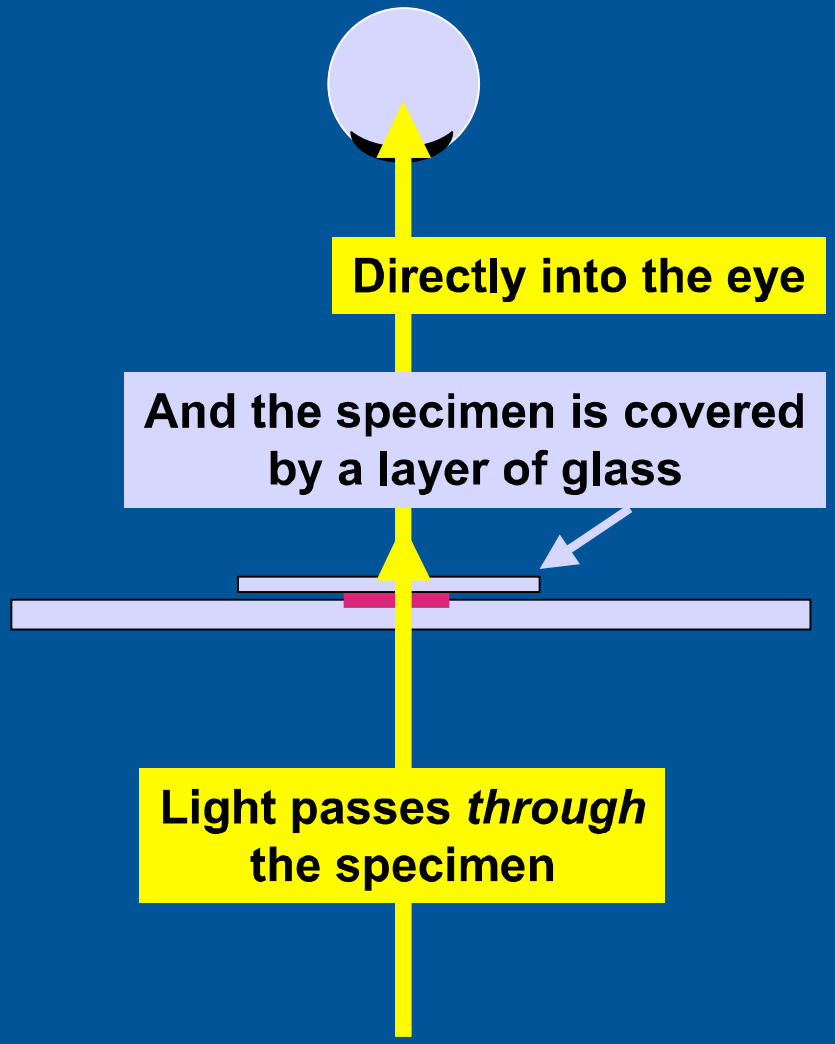
# Transmitted-light, Bright-field




**A most unusual way of looking at things!**



# Transmitted-light, Bright-field



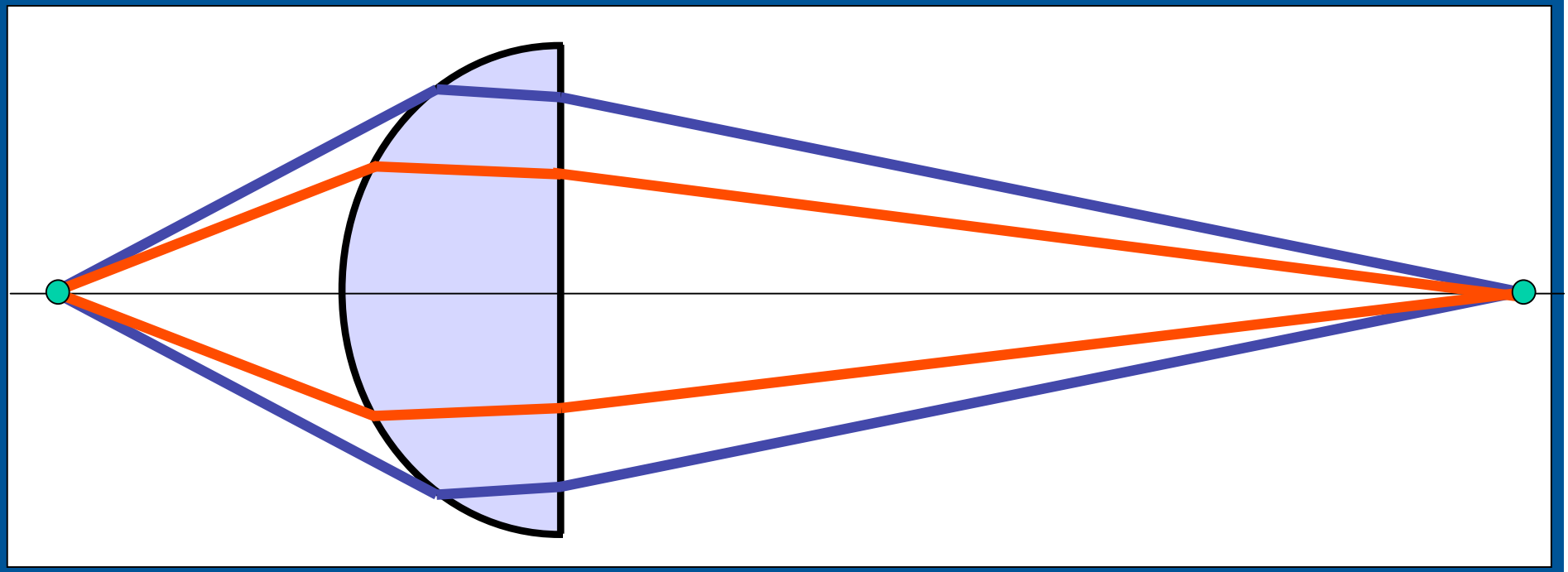

**A most unusual way of looking at things!**

And the microscope uses light for a job that 'it wasn't designed for'

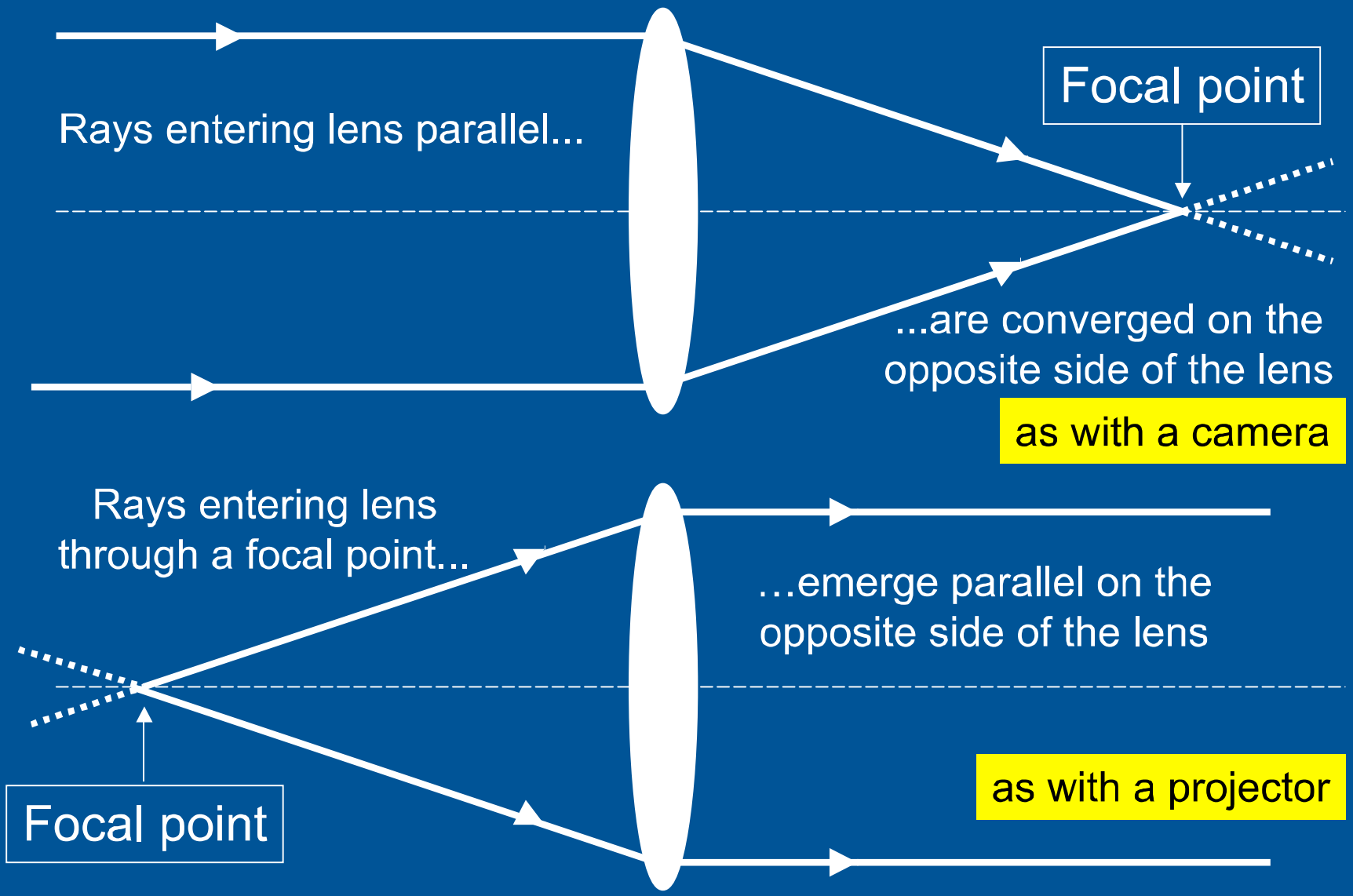
- looking at things that are around the same dimensions as the wavelength of light

# The job of an ideal lens

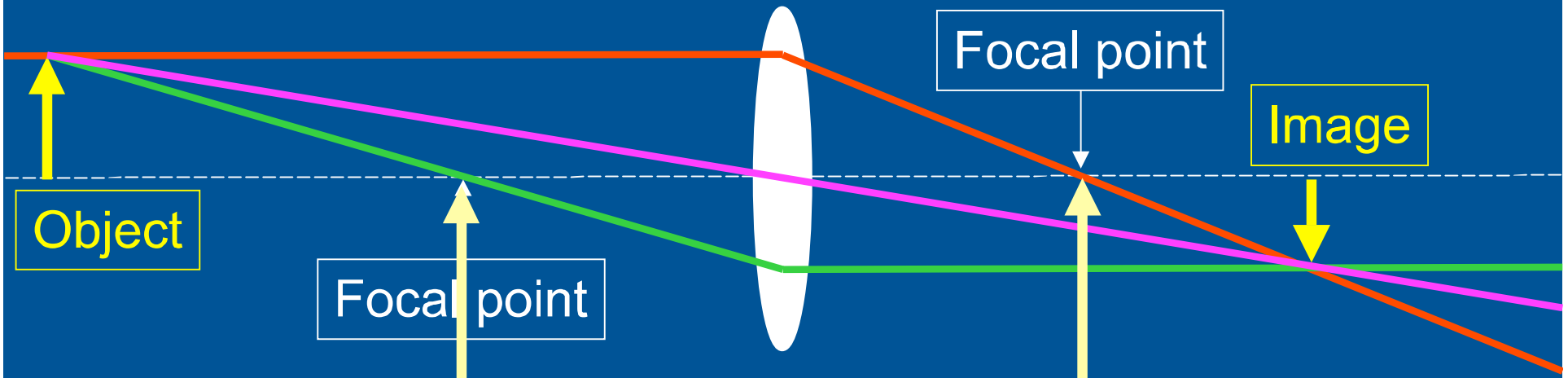
- To accept as many rays as possible from each point in an object
- To reassemble all the rays from each point at corresponding points in the image…
- In such a way that the distance travelled by all the rays from each object point to its corresponding image point is the same
  - so that they all arrive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



# A lens has two focal planes



# 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 undeviated

# 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

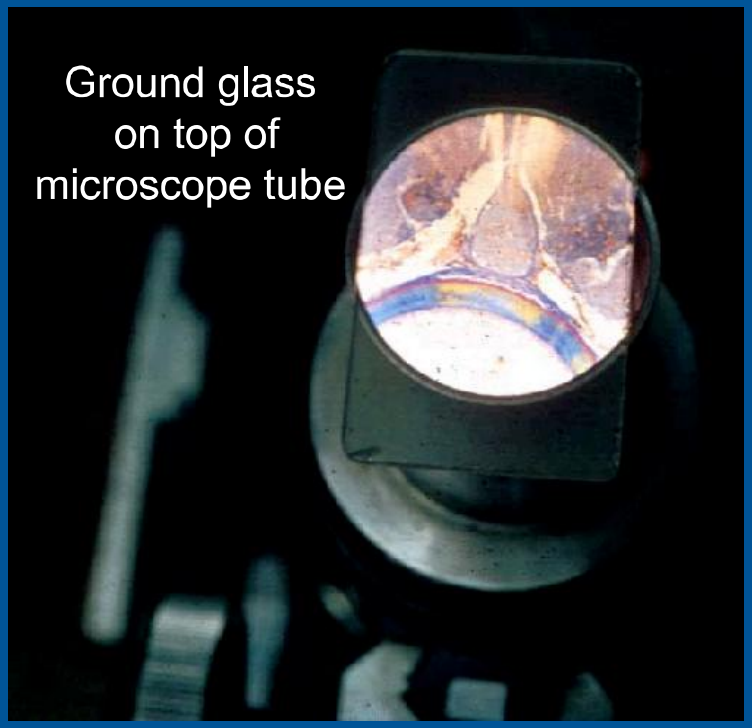
## Magnifying glass

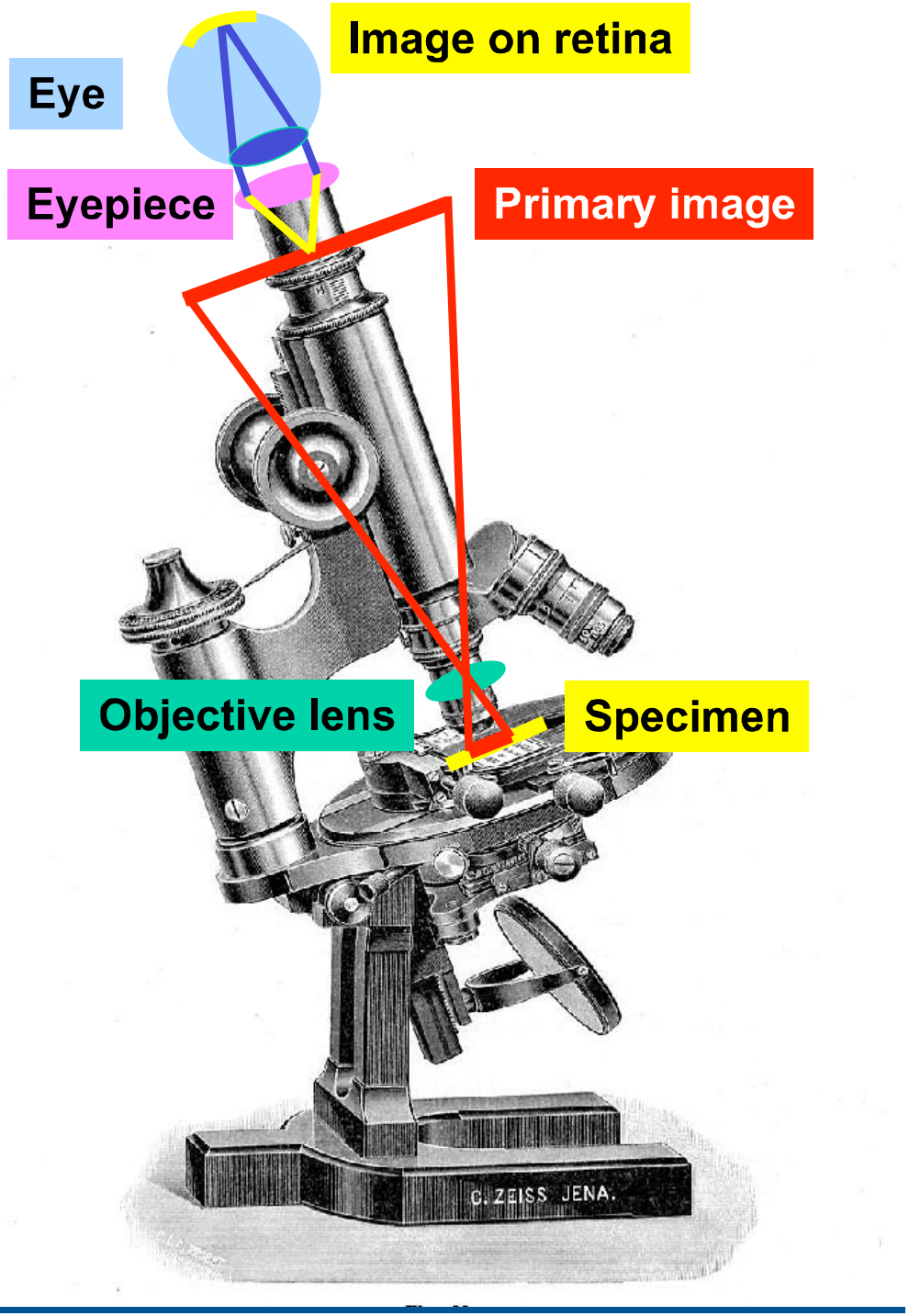
- not forming a real image; parallel rays to infinity


Focal length of lens
Object at infinity
Image in focal plane
Camera - landscape
f
f'
Image smaller than object
Camera - portrait
Image same size as object
Camera - macro lens
2f
2f'
Projector
Image larger than object
Magnifying glass
Object in focal plane
Image on retina
Image at infinity

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



# Magnification

Depends on:

Image distance

longer → higher mag

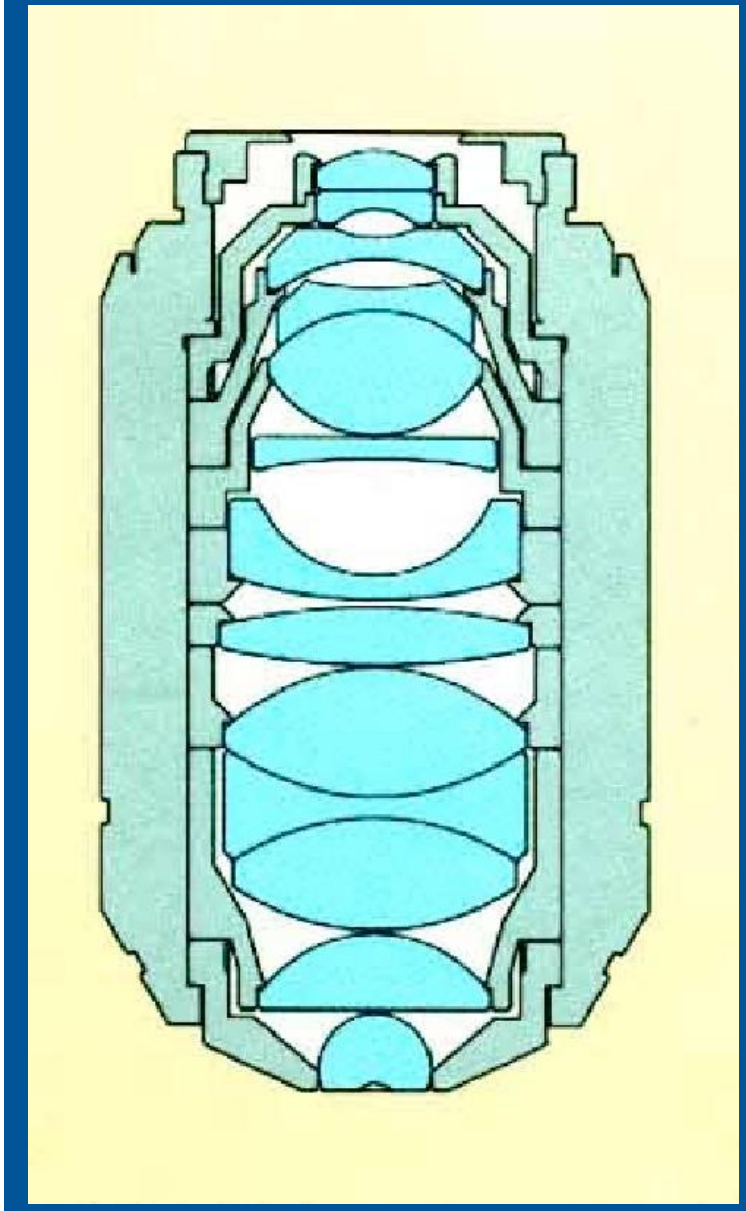
Objective focal length

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

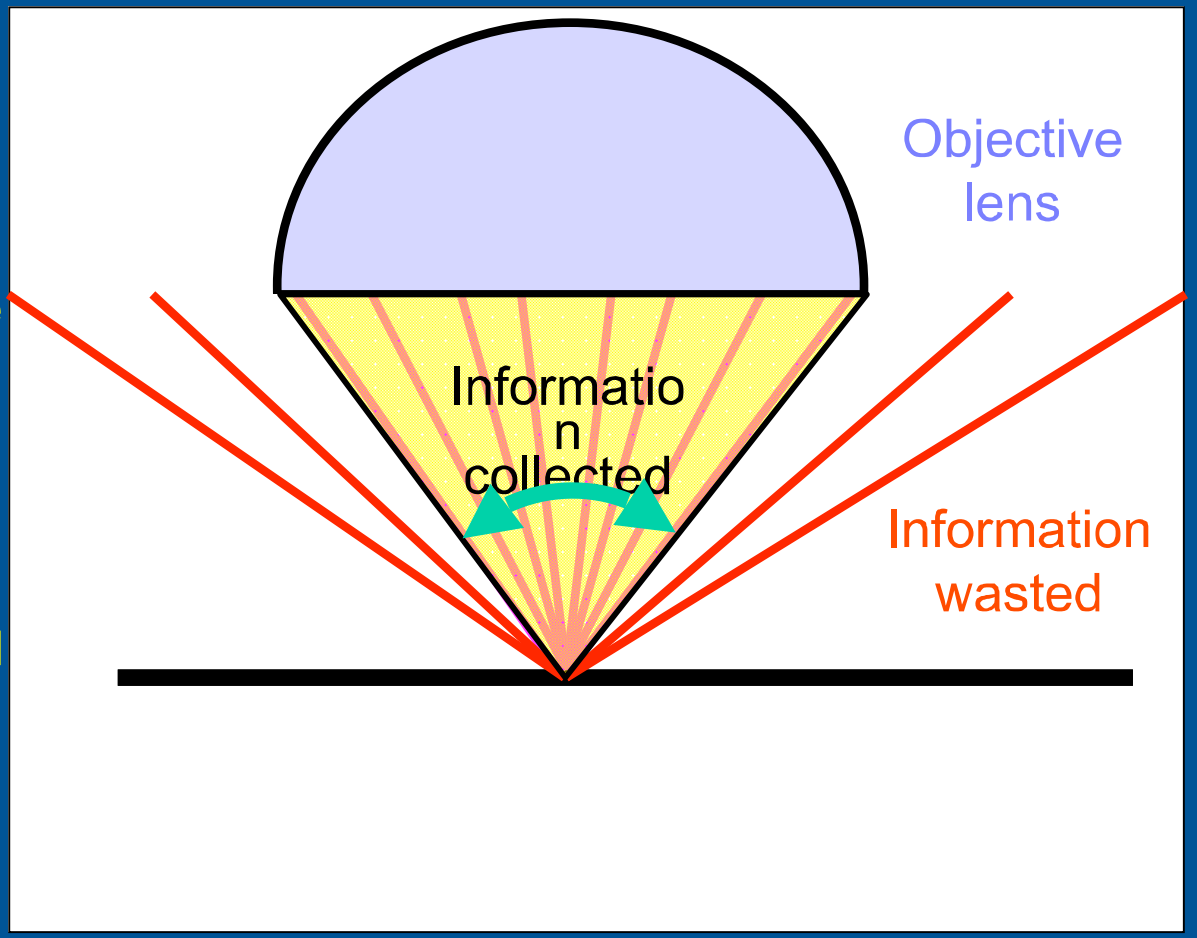
– and some information –

will *NOT* be collected,

and will be wasted

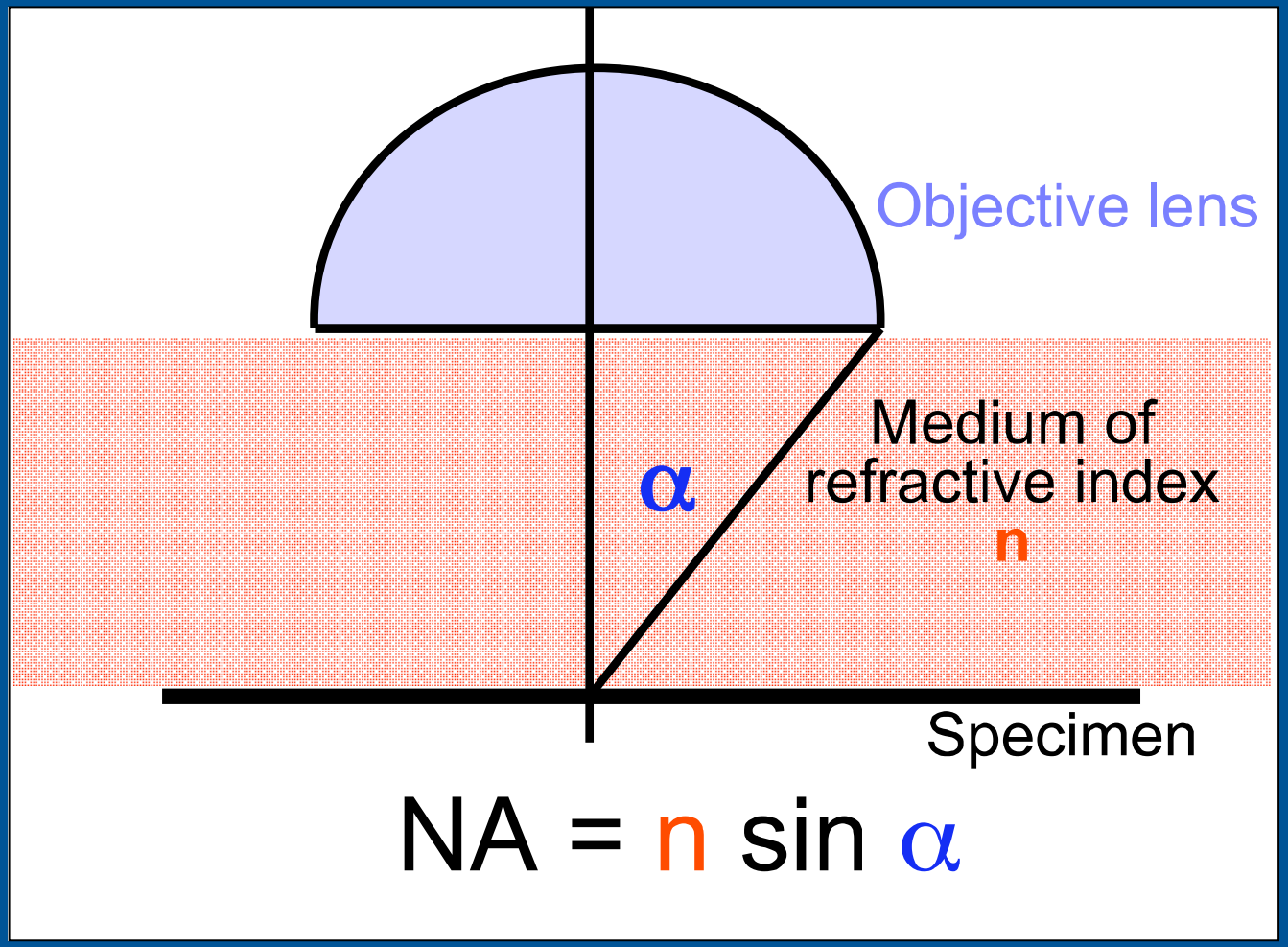
Resolution will therefore depend on the angular *aperture* of the objective -

the larger the imaging aperture the higher the resolution



# Numerical Aperture

NA




$$NA = n \sin \alpha$$

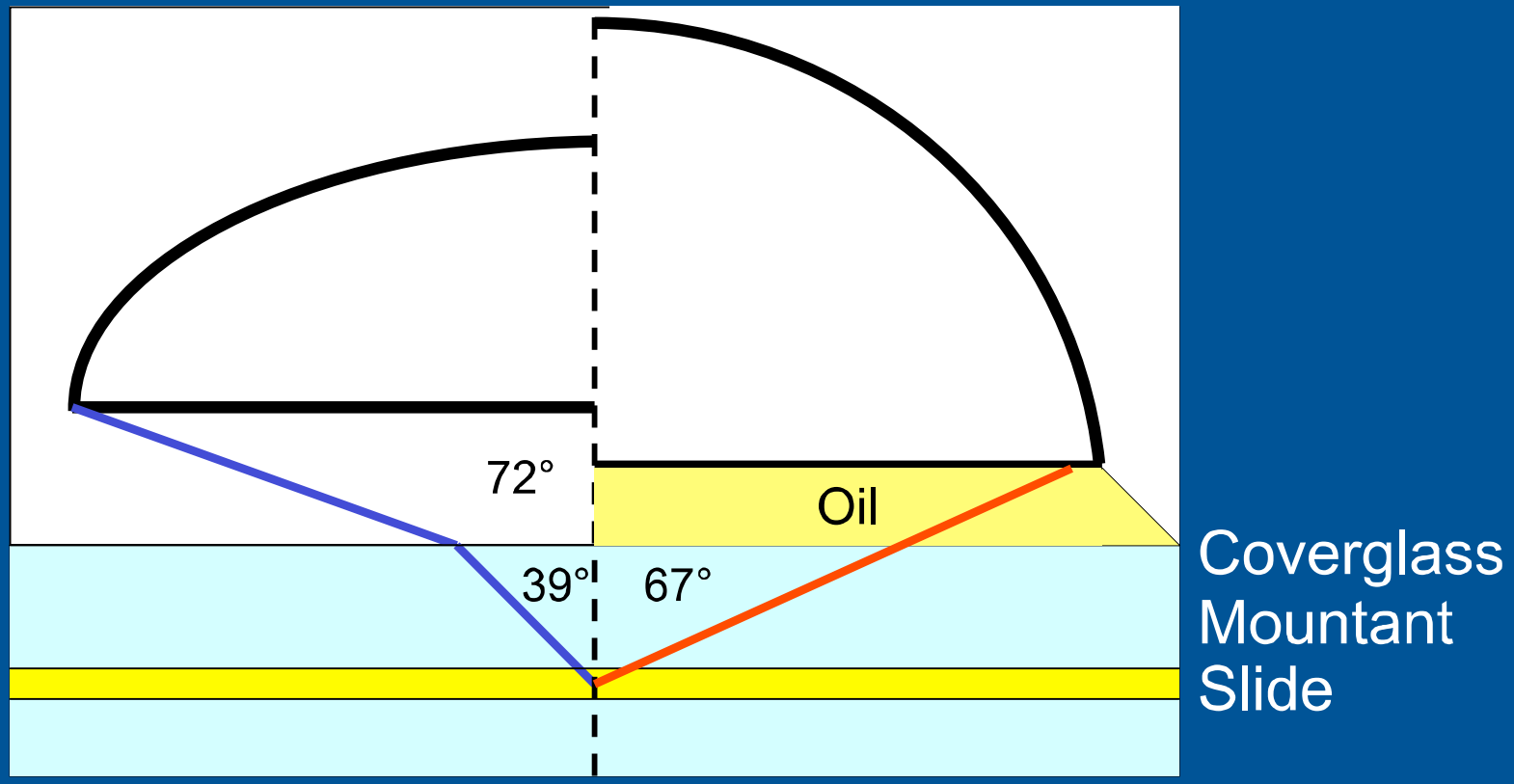
# Numerical Aperture

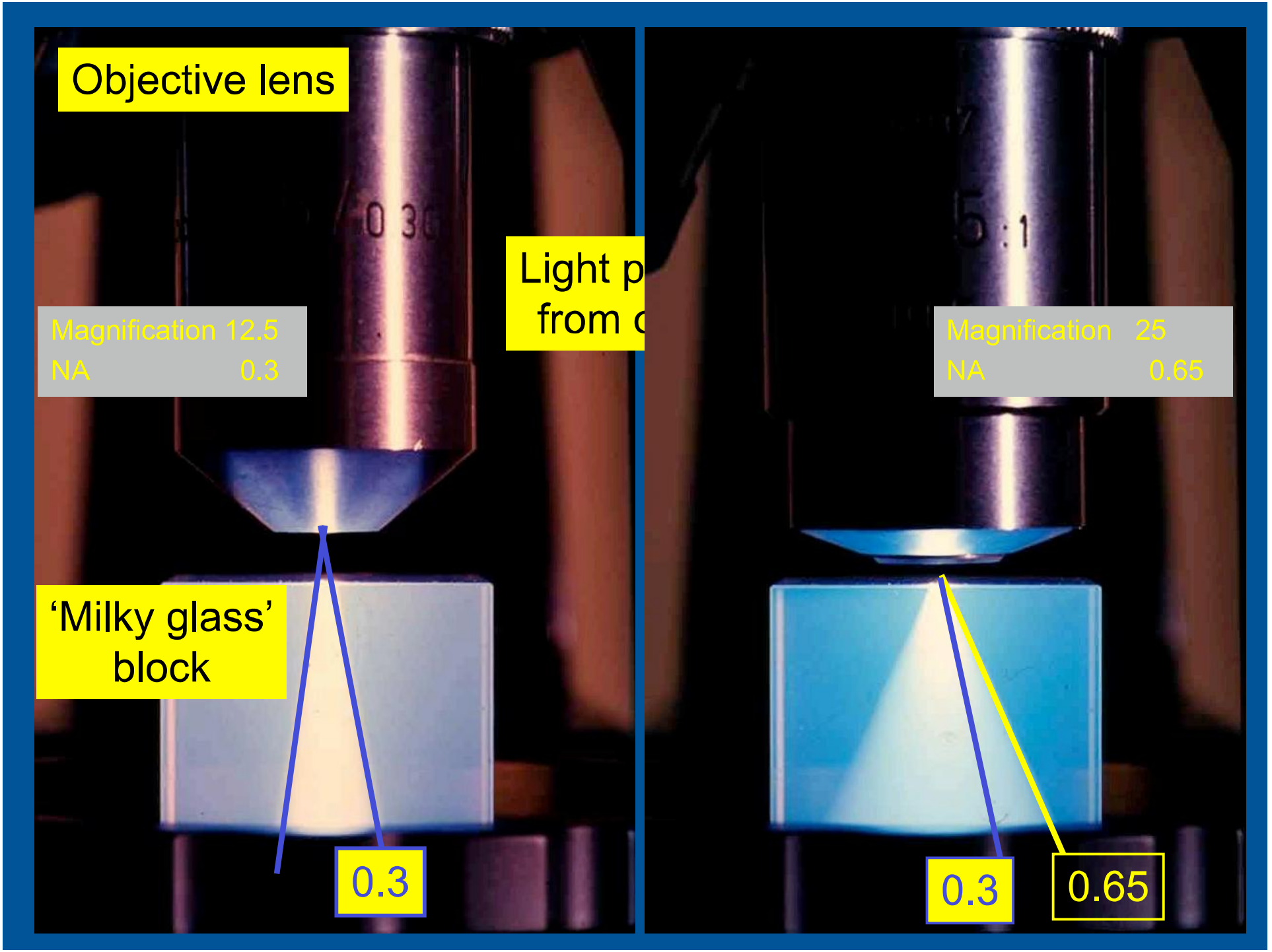
**Dry Objective**

NA = 1 x sin 72°
= 1 x 0.95
= 0.95

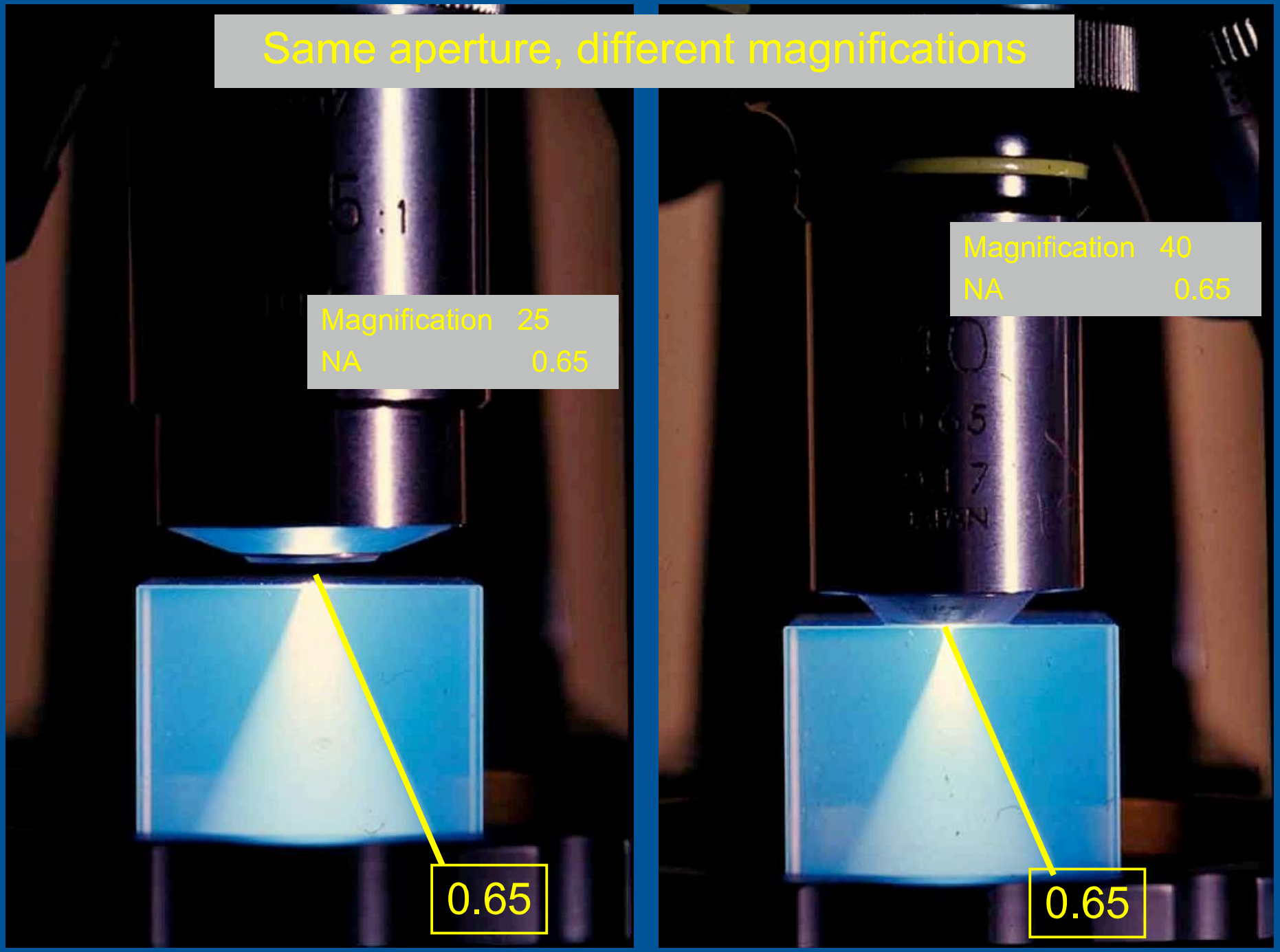
**Immersion Objective**

NA = 1.515 x sin 67°
= 1.515 x 0.92
= 1.4



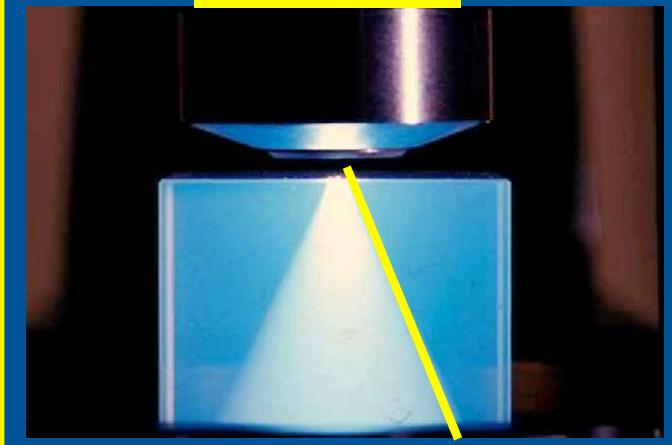

Objective lens
Light p
from c
Magnification 12.5
NA 0.3
'Milky glass' block
0.3
Magnification 25
NA 0.65
0.3
0.65

# Same aperture, different magnifications



Same aperture
different magnifications

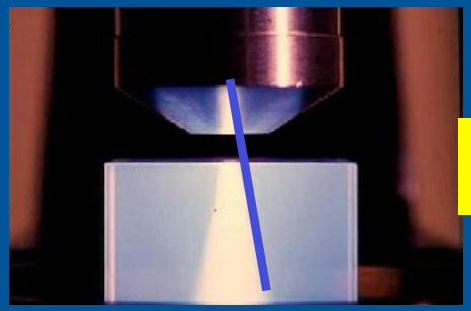
25 / 0.65



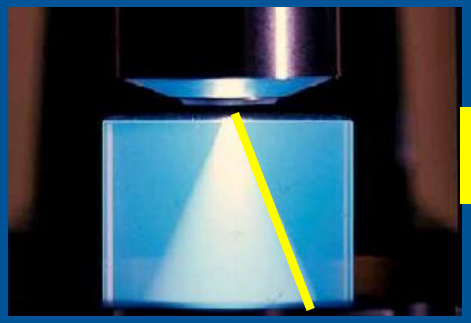
40 / 0.65

40 / 0.95

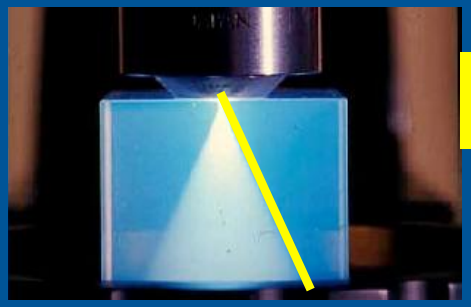

Same magnification
different apertures



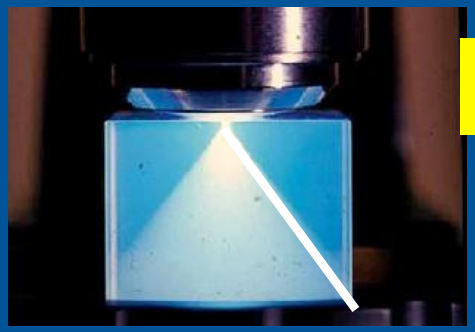
12.5 / 0.3



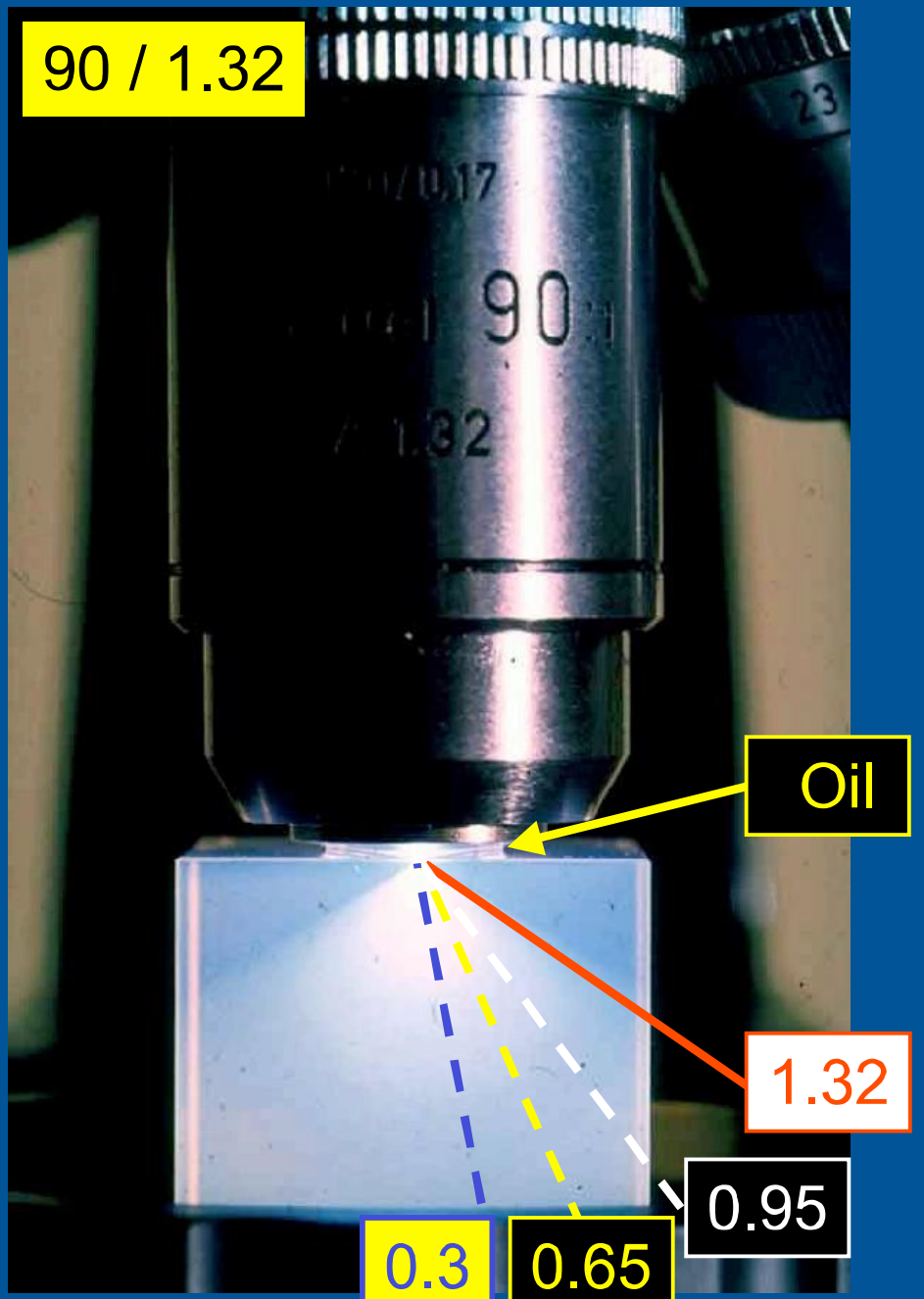
25 / 0.65



40 / 0.65

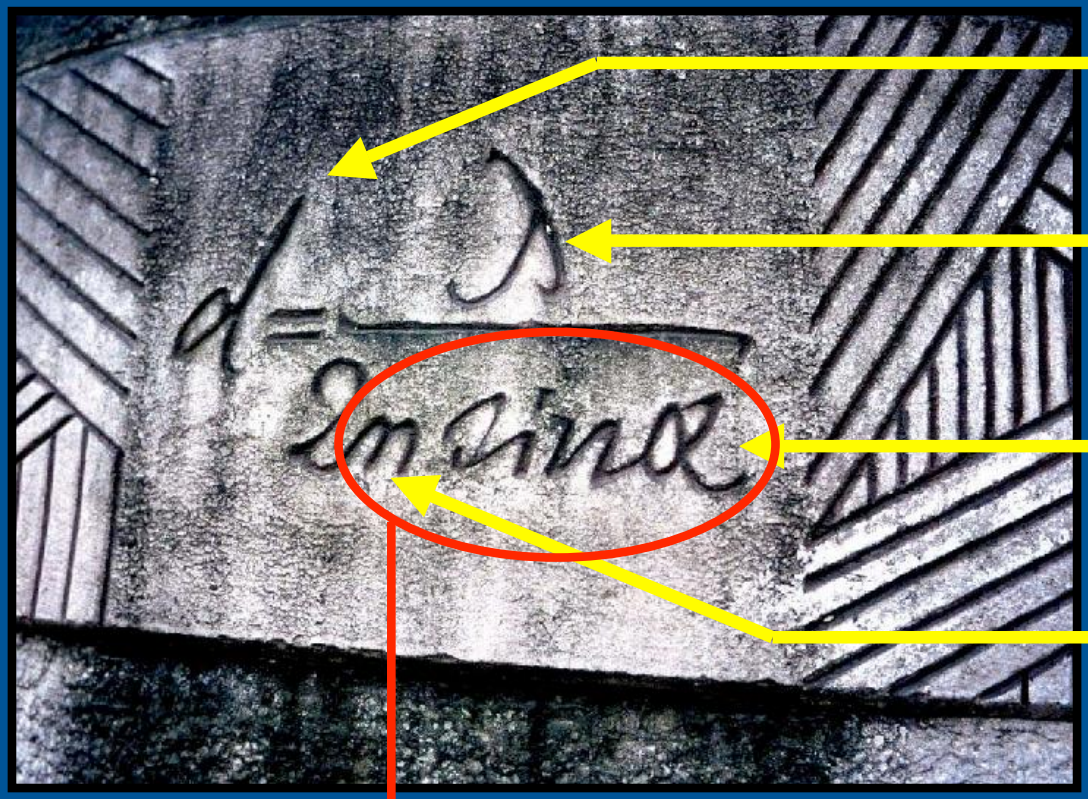


40 / 0.95



# Why is Numerical Aperture Important?

*Inscription on Ernst Abbe's memorial*




d — Minimum resolved distance

λ — Wavelength of imaging radiation

α — Half-aperture angle

n — Refractive index of medium

Numerical Aperture

Minimum resolved distance is now commonly expressed as

$d = 0.61 \lambda / NA$

# 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 $NA^2$