Principles of Light Microscopy

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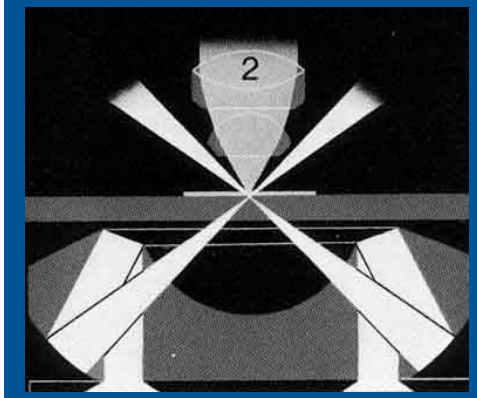
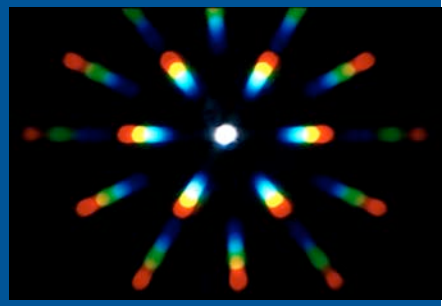
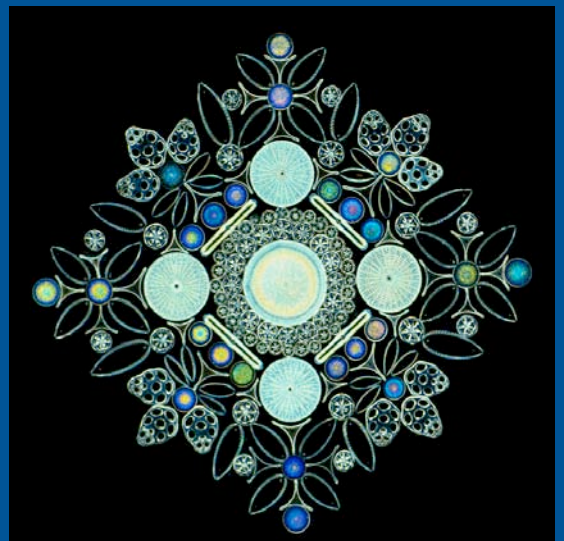
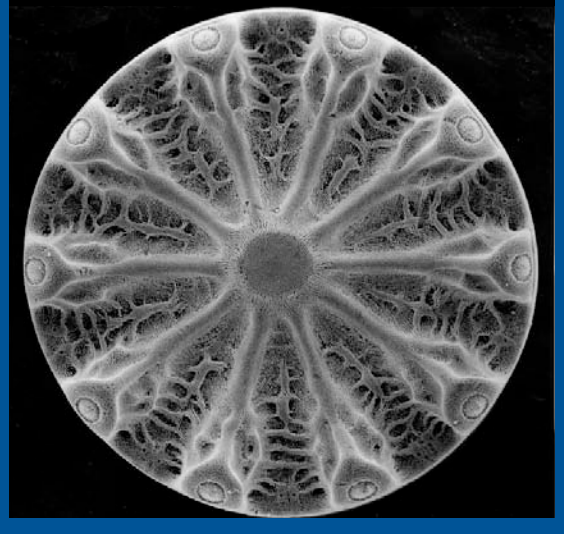
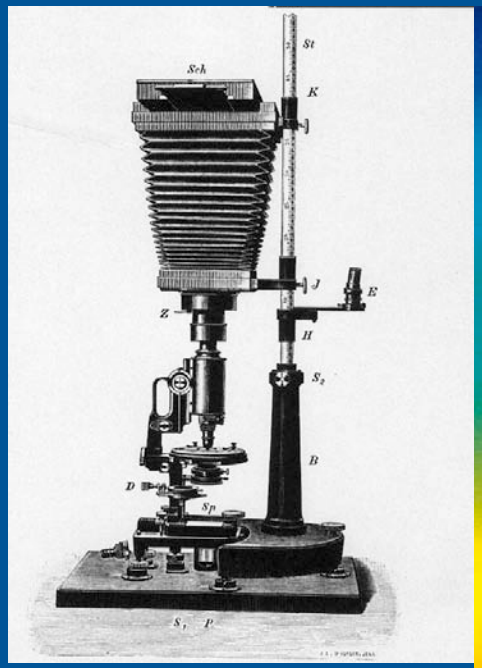
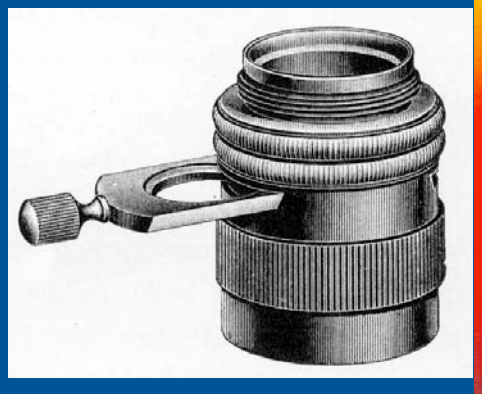












True or False ?

- Light microscopy is out of date now that we have electron microscopes.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

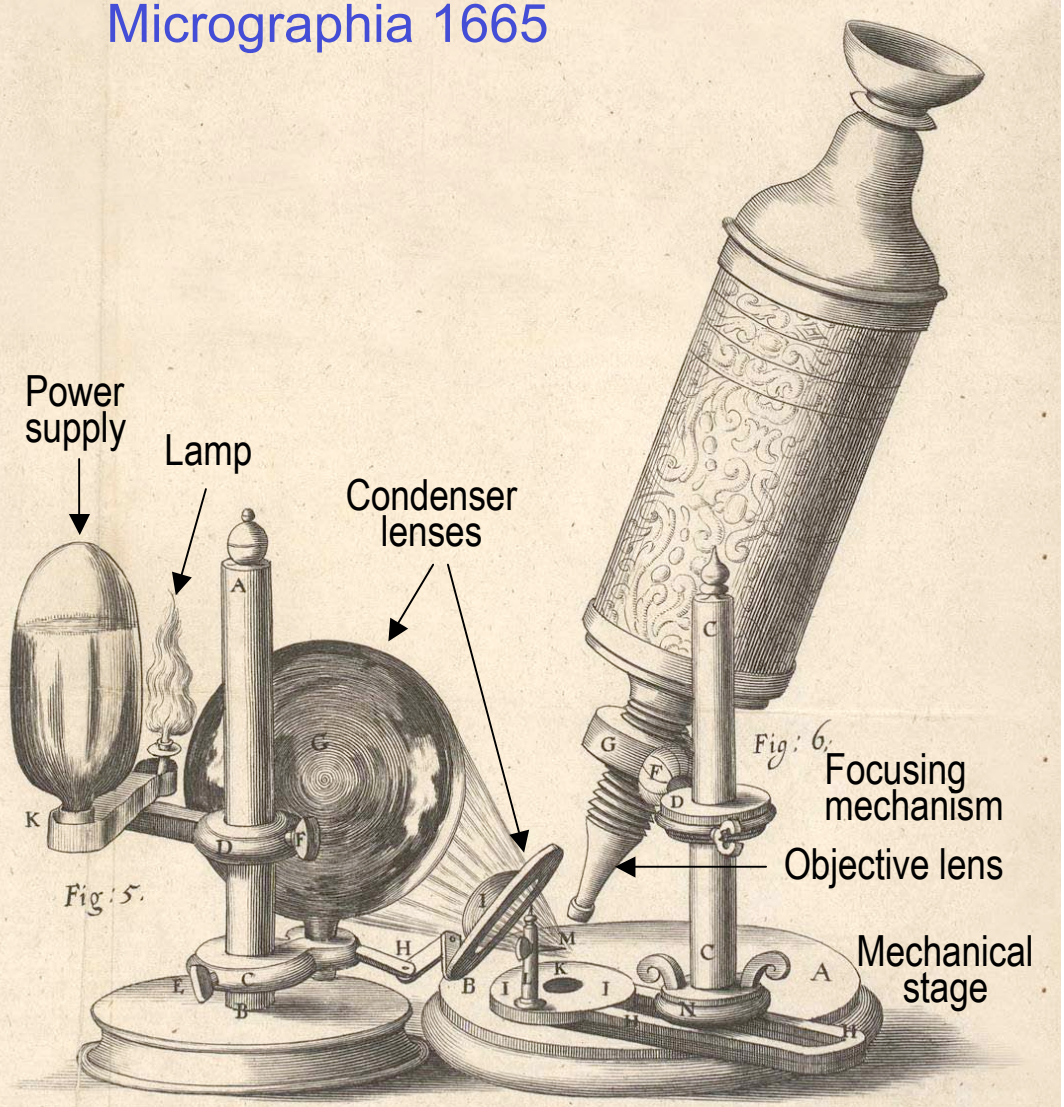
OBSERVATIONS and INQUIRIES thereupon.

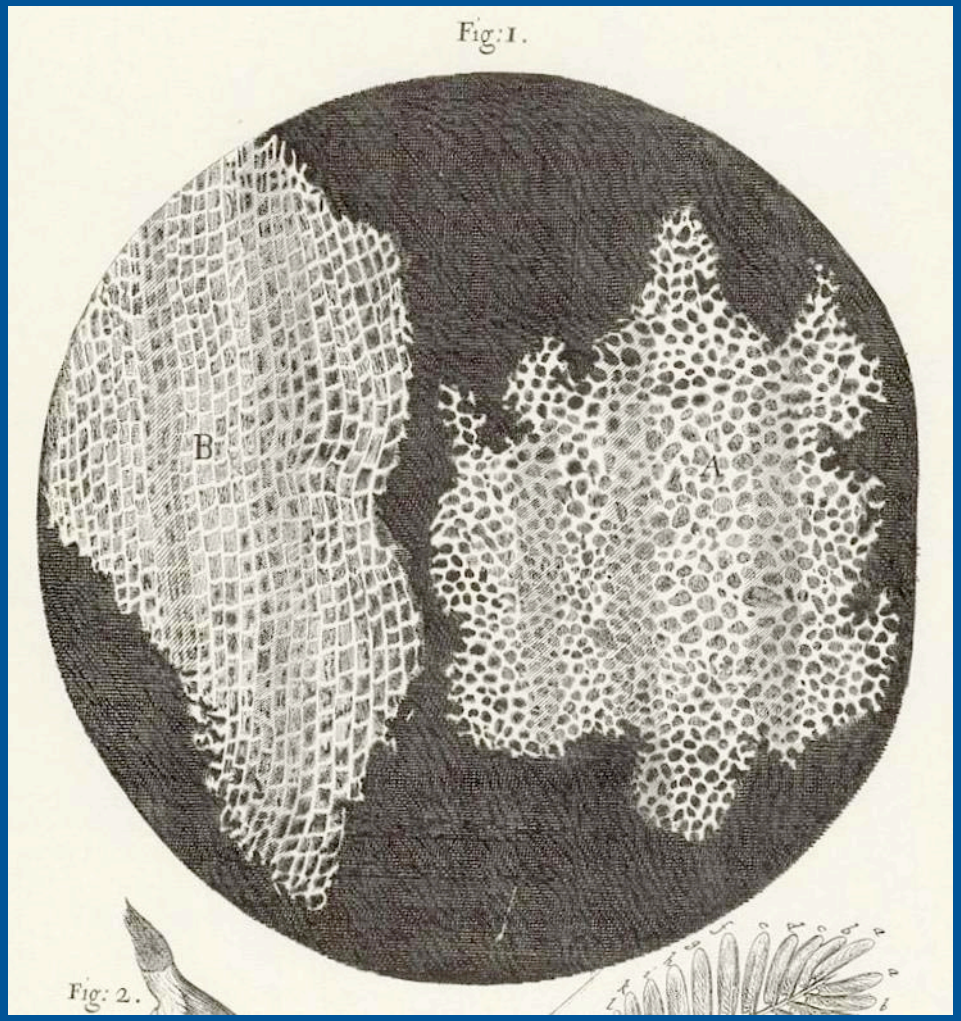
By *R. HOOKE*, Fellow of the ROYAL SOCIETY.

Non possis oculo quantum contendere Linceus,
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. M DC LXV.

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

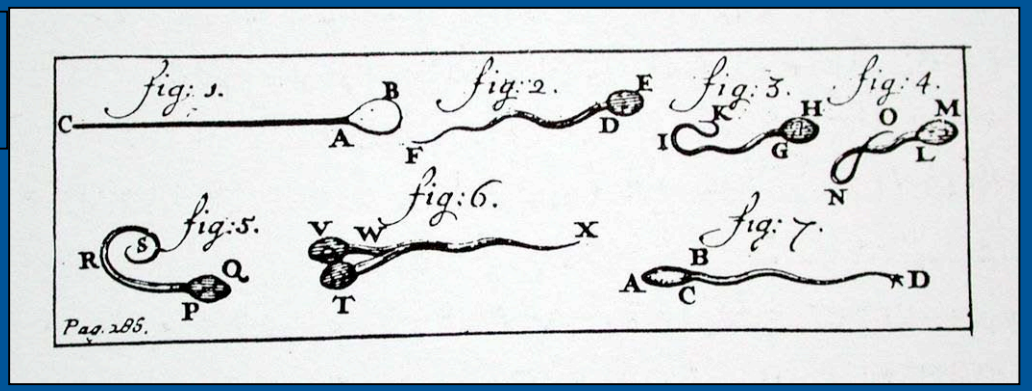
" … 'tis possible with a single Microscope to make discoveries much better than with a double one, because the colours which do much disturb the clear Vision in double Microscopes is clearly avoided and prevented in the single."

1665

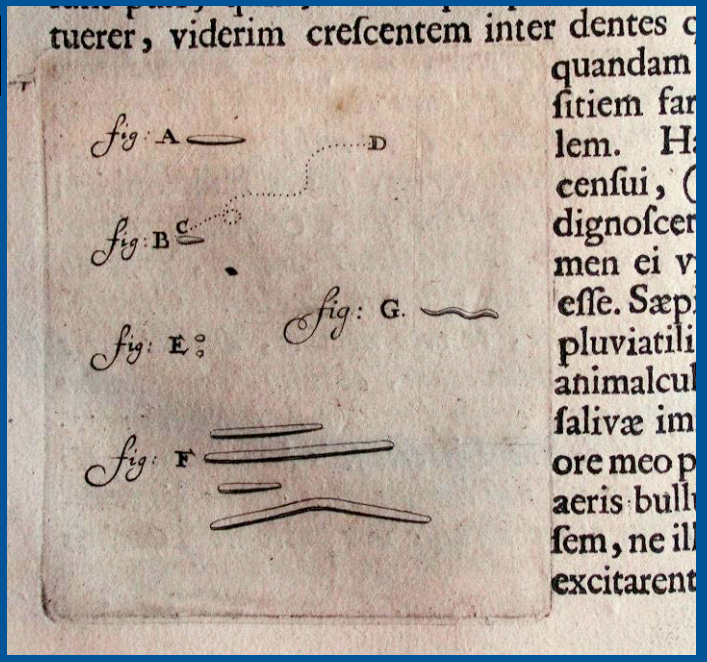
A different approach – using a single lens

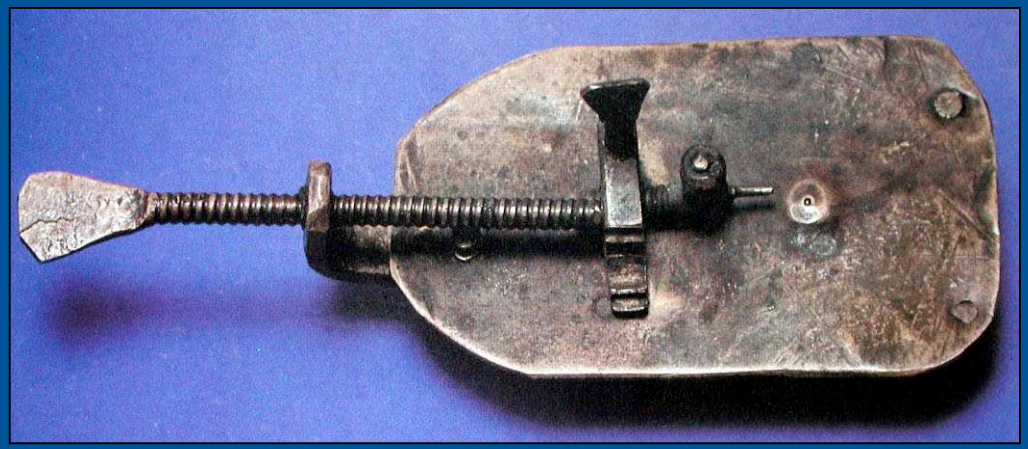
Antony van Leeuwenhoek,1632 - 1723

Human sperm

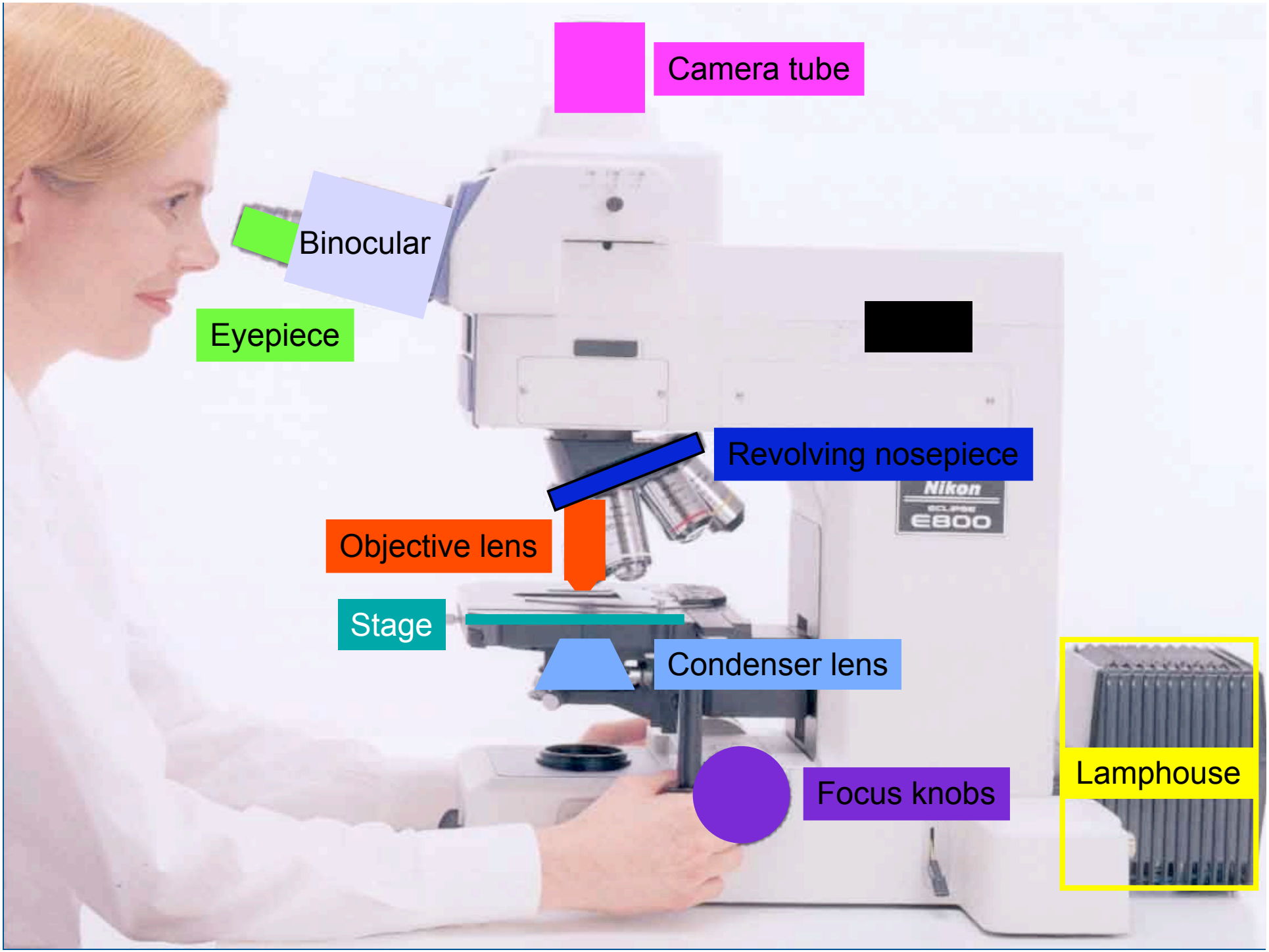


Bacteria





50mm


Camera tube
Binocular
Eyepiece
Revolving nosepiece
Objective lens
Stage
Condenser lens
Focus knobs
Lamphouse
Nikon
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’.

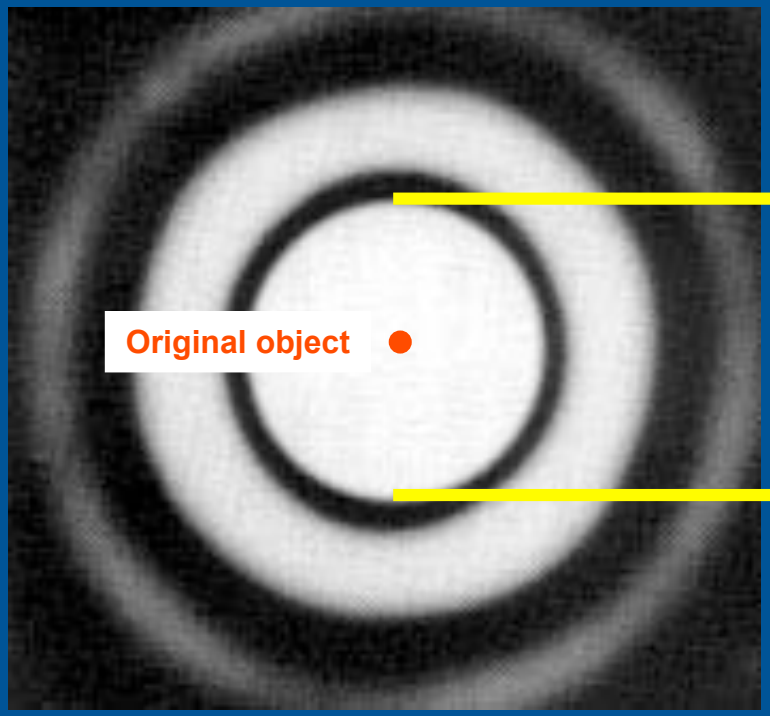
The smaller they are and the more you have, the better.

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

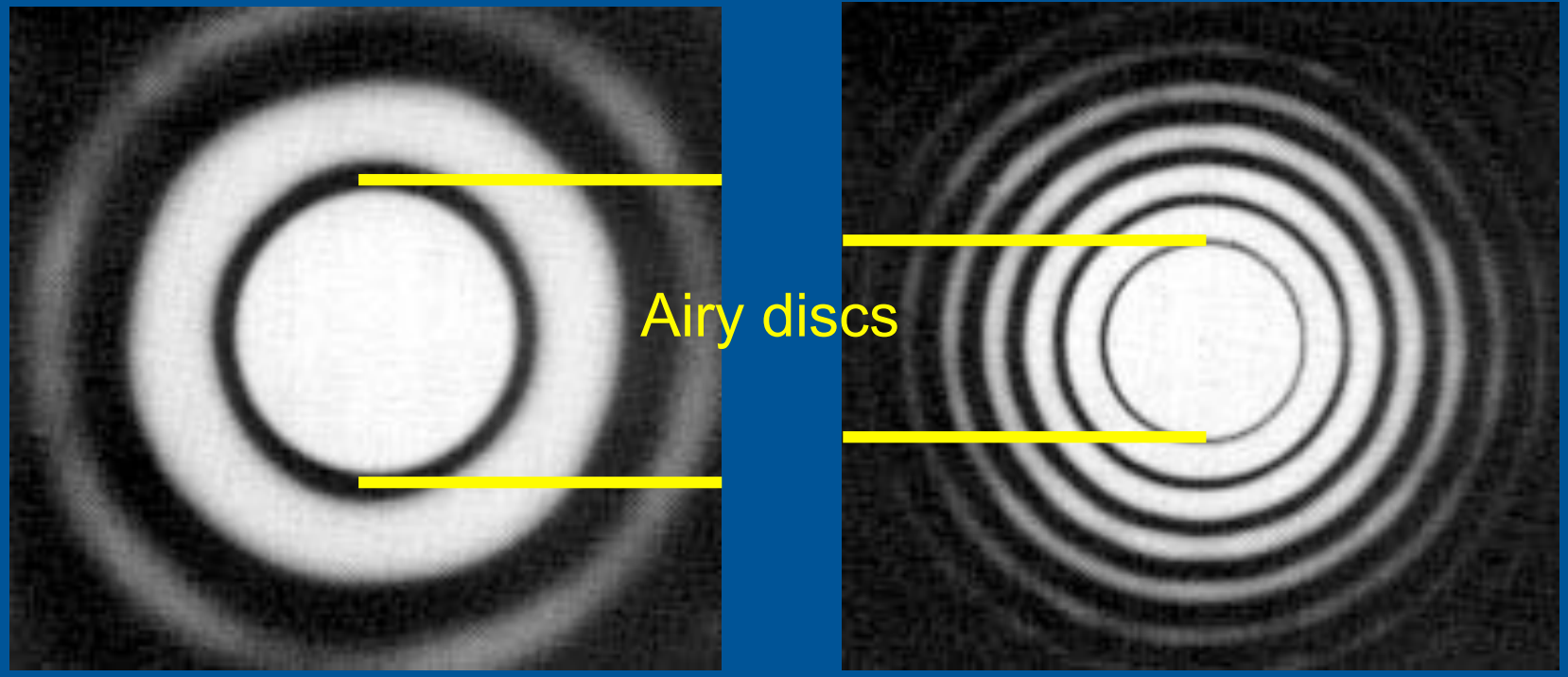
→ smaller disc → better resolution

Image of a point source: the *Airy Pattern*

with most of its brightness in the central *Airy disc*

Small aperture

Larger aperture



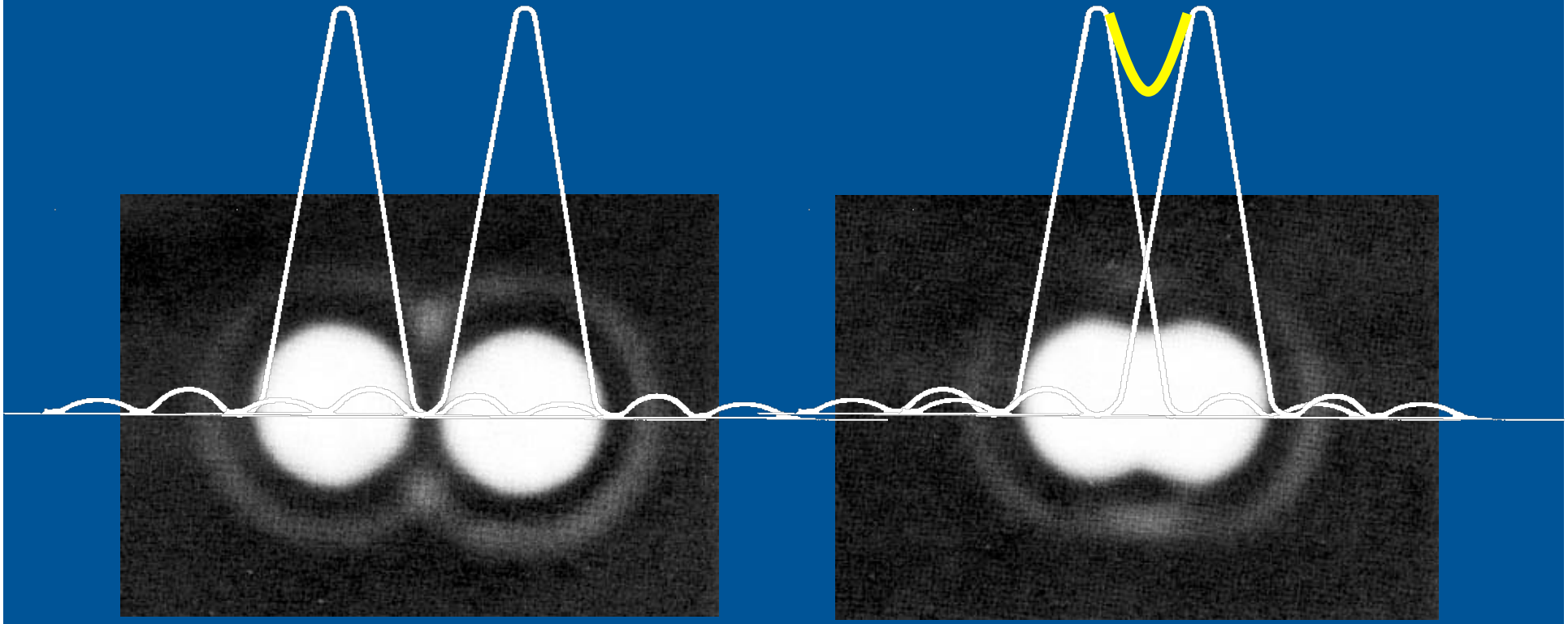
Diffraction in the microscope

- 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

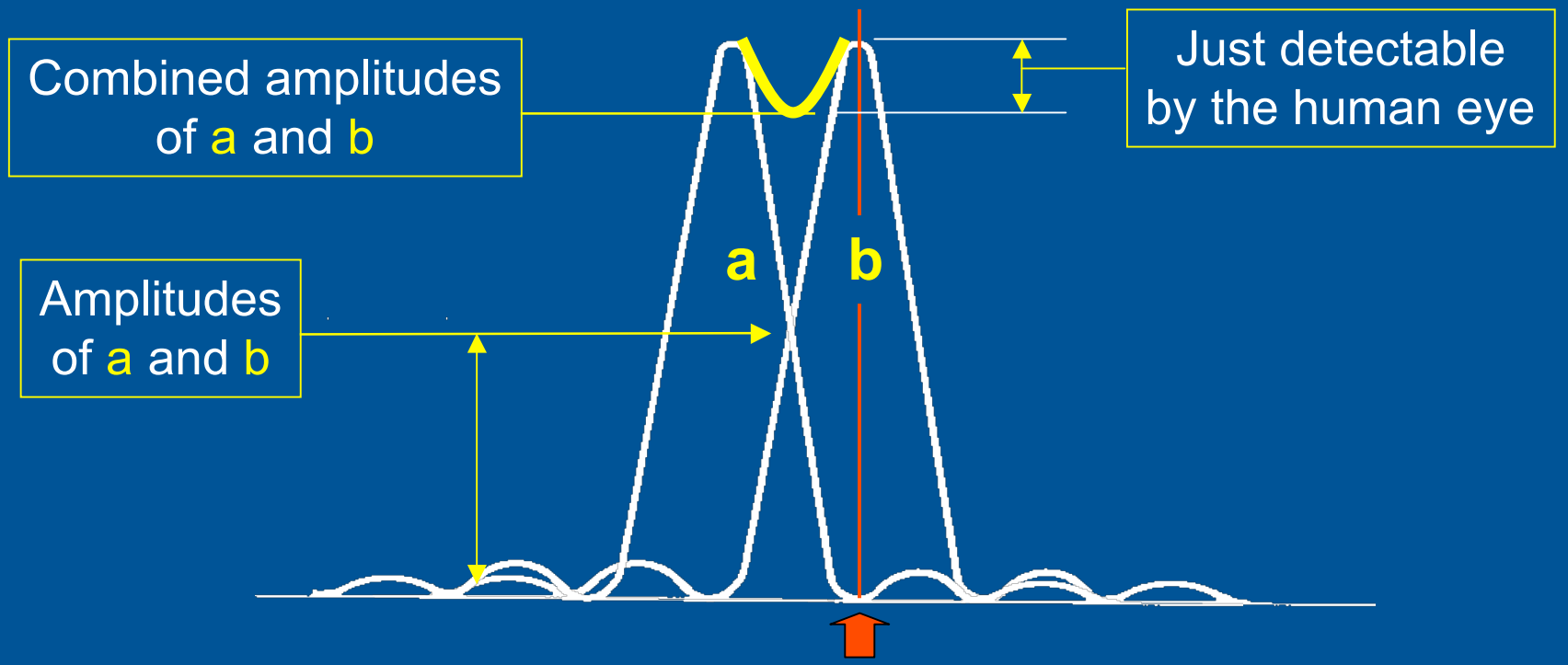


The *Rayleigh Criterion* states that two objects can be resolved in an image when they are separated by

distance **0.61λ / n sin α**

The Rayleigh Criterion for Resolution

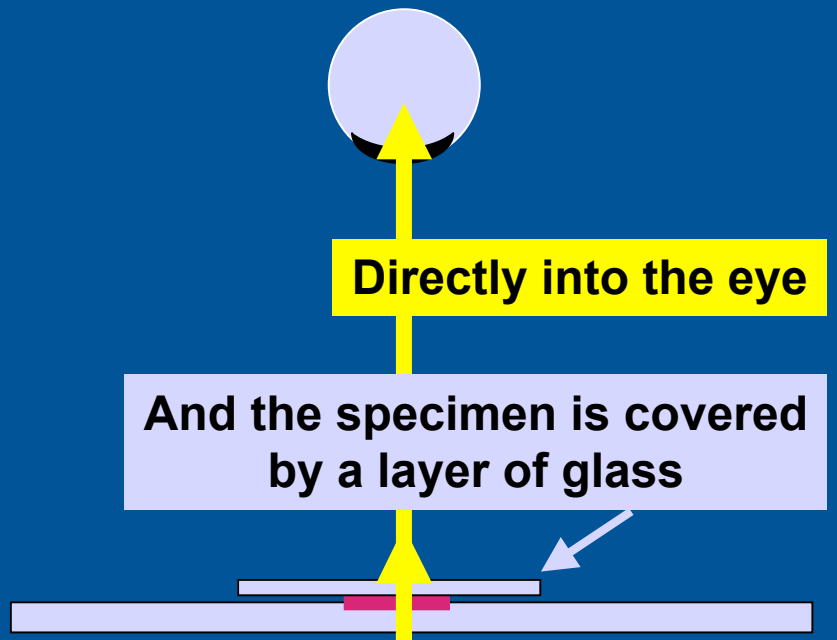
calculated from aperture of objective lens



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

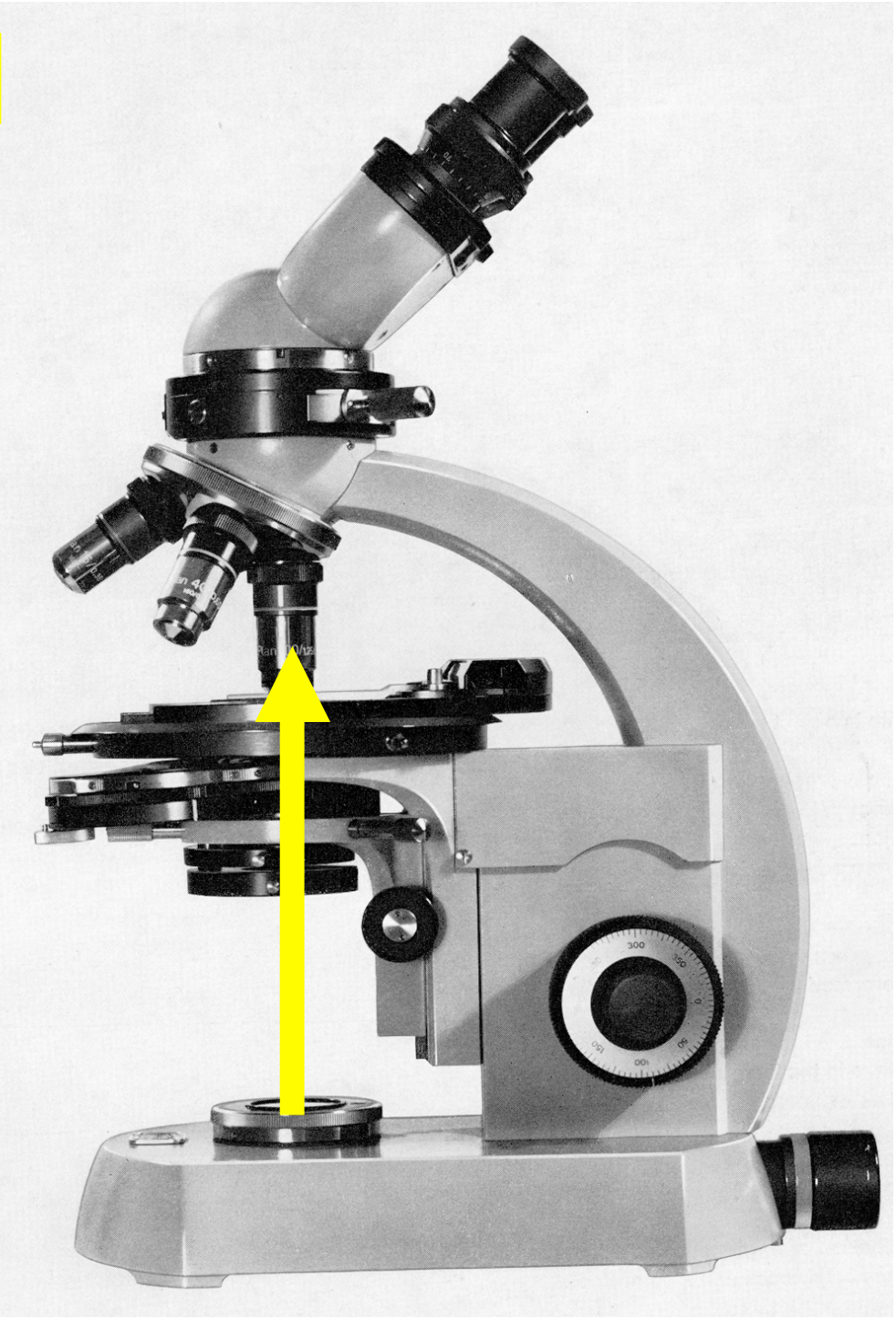


Directly into the eye

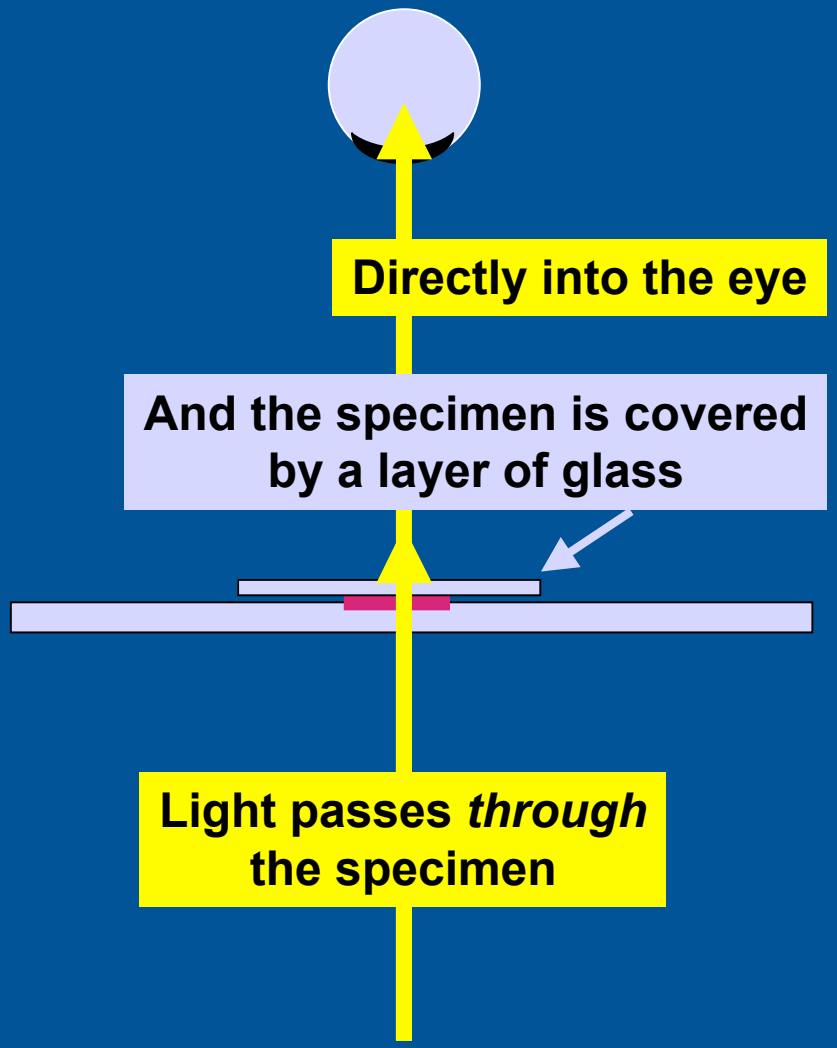
And the specimen is covered by a layer of glass

Light passes *through* the specimen

A most unusual way of looking at things!



Transmitted-light, Bright-field



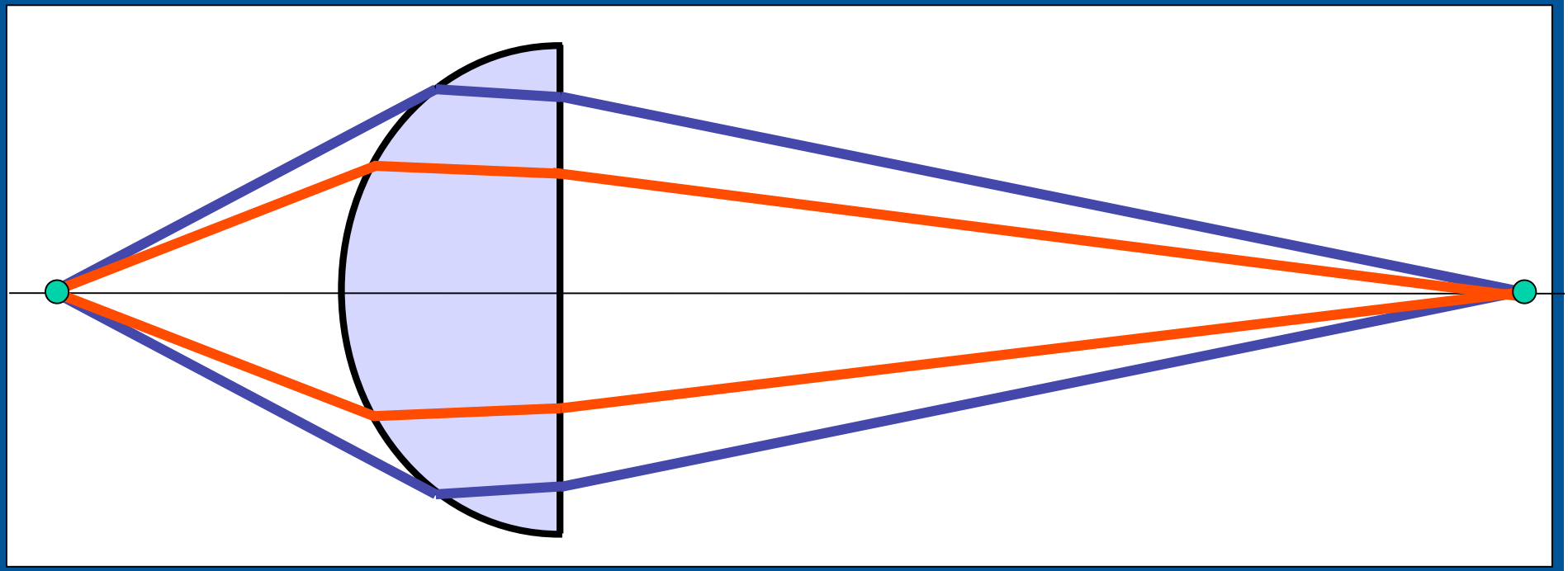
A most unusual way of looking at things!

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

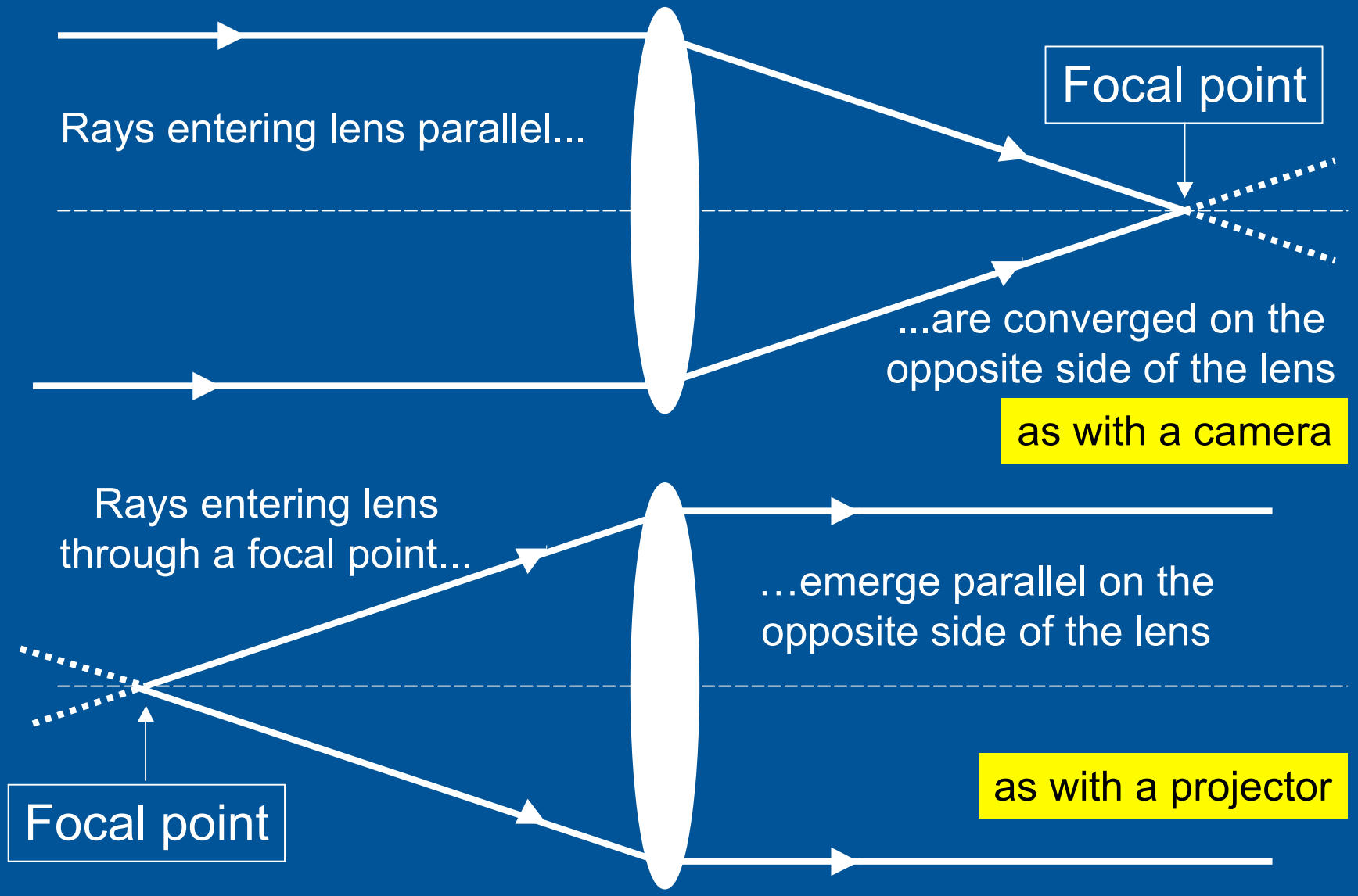
- looking at things that are around the same dimensions as the wavelength of light

The job of an ideal lens

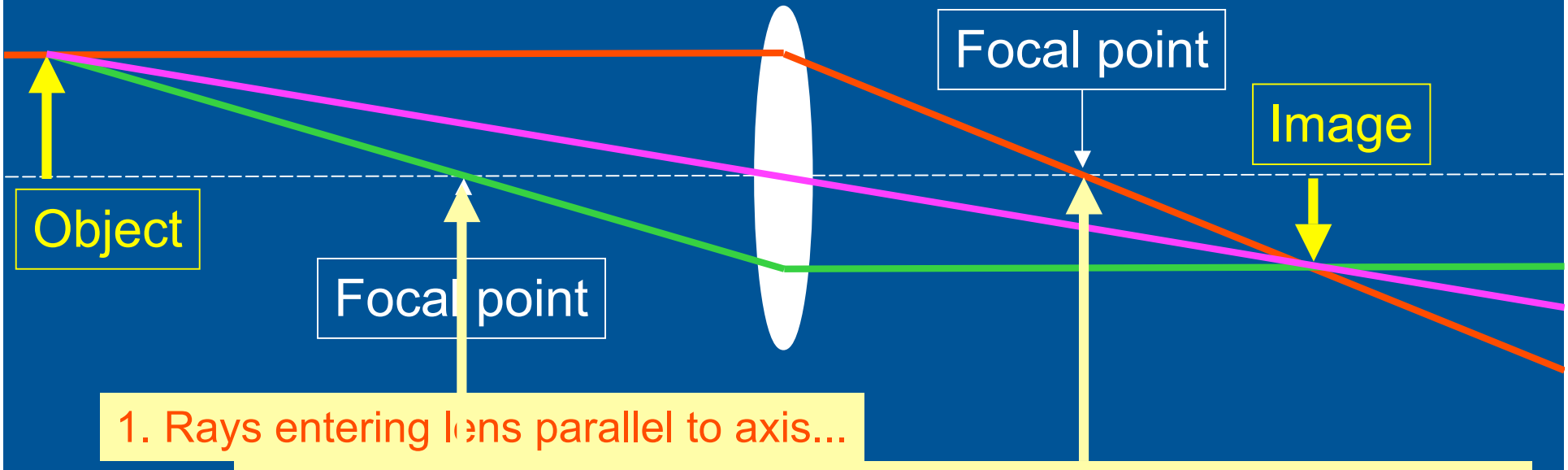
- To accept as many rays as possible from each point in an object
- To reassemble all the rays from each point at corresponding points in the image…
- In such a way that the distance travelled by all the rays from each object point to its corresponding image point is the same
  - so that they all arrive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

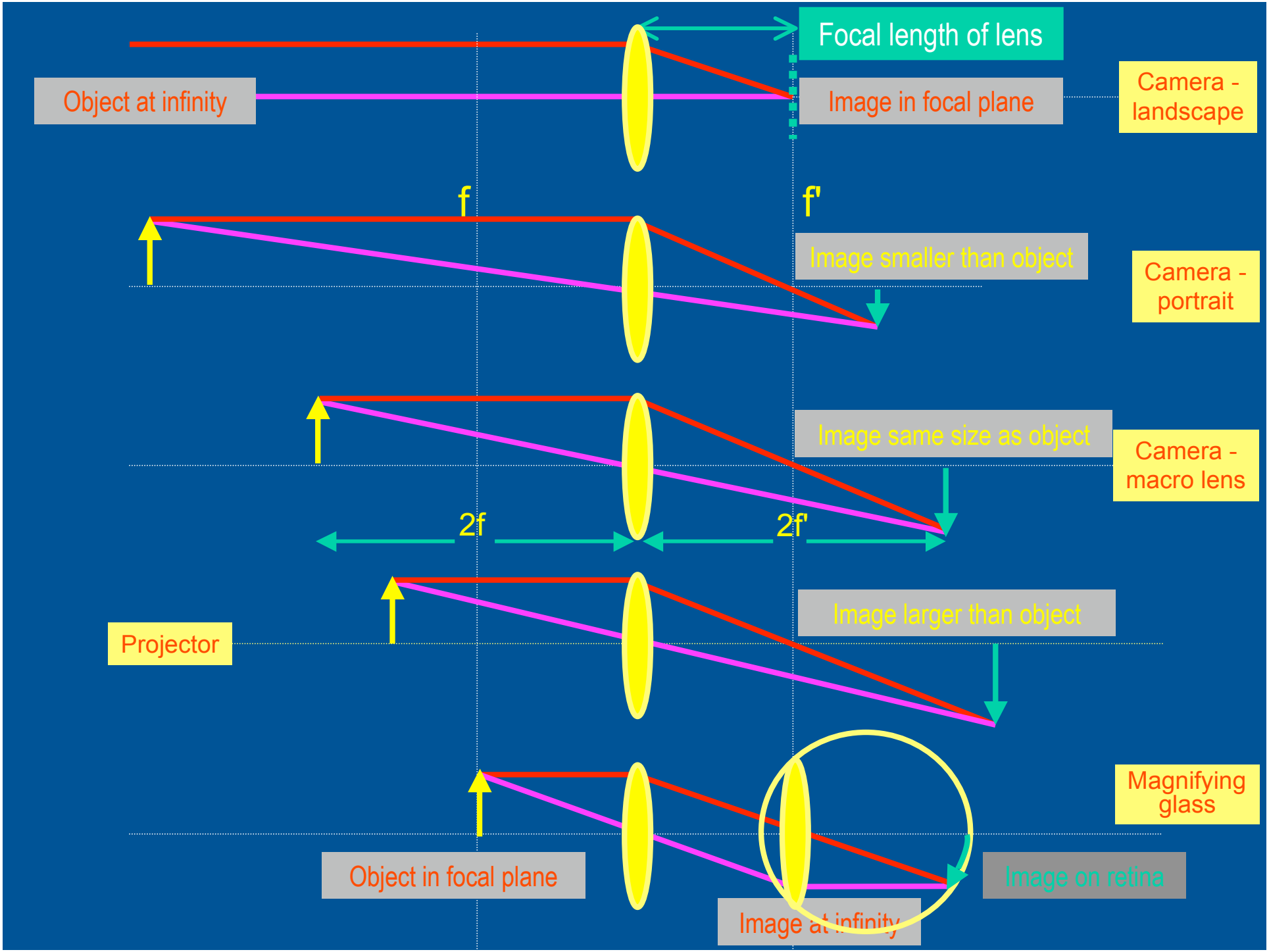
- forming a reduced-size, real image, close to the lens

Projector

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

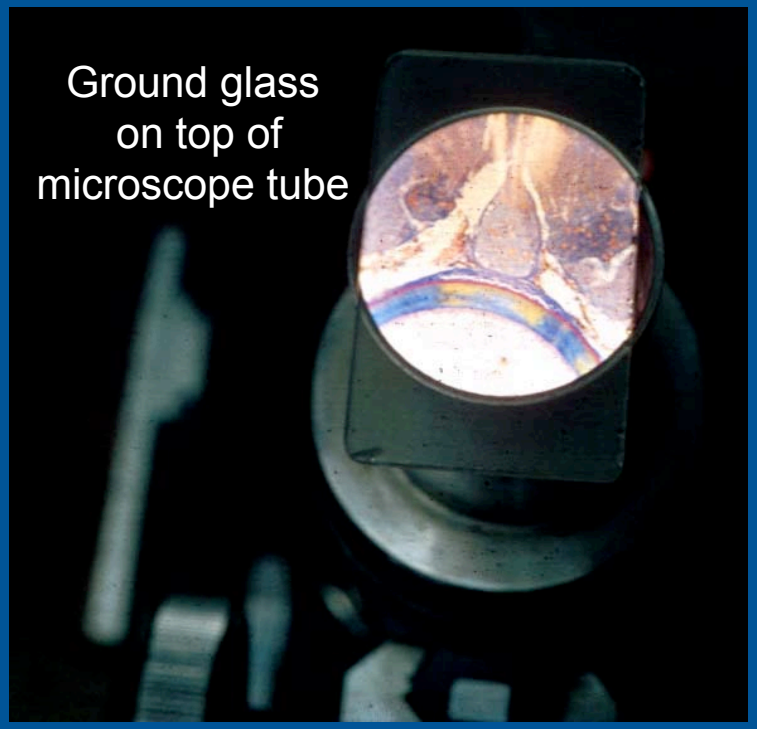
Magnifying glass

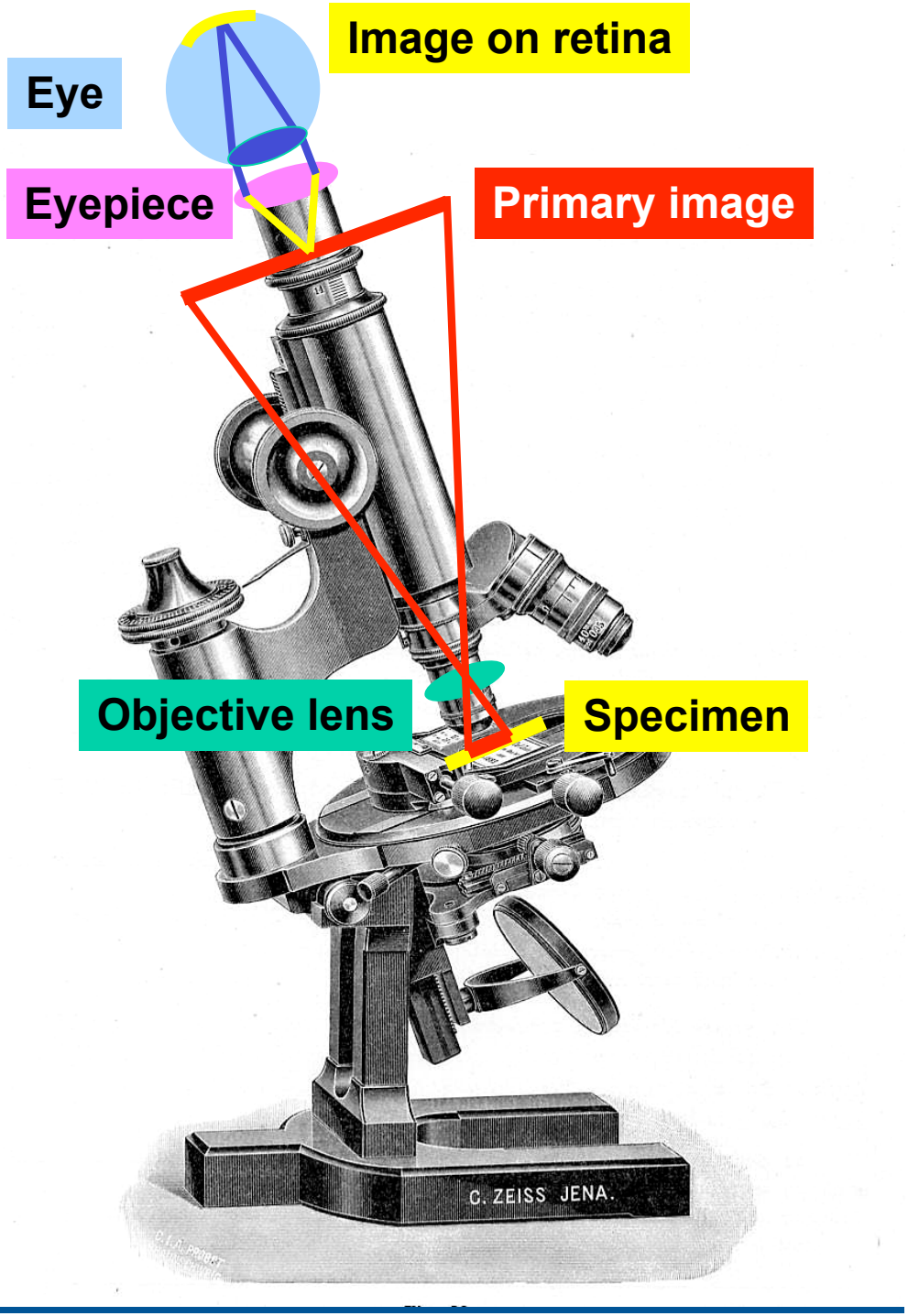
- not forming a real image; parallel rays to infinity


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

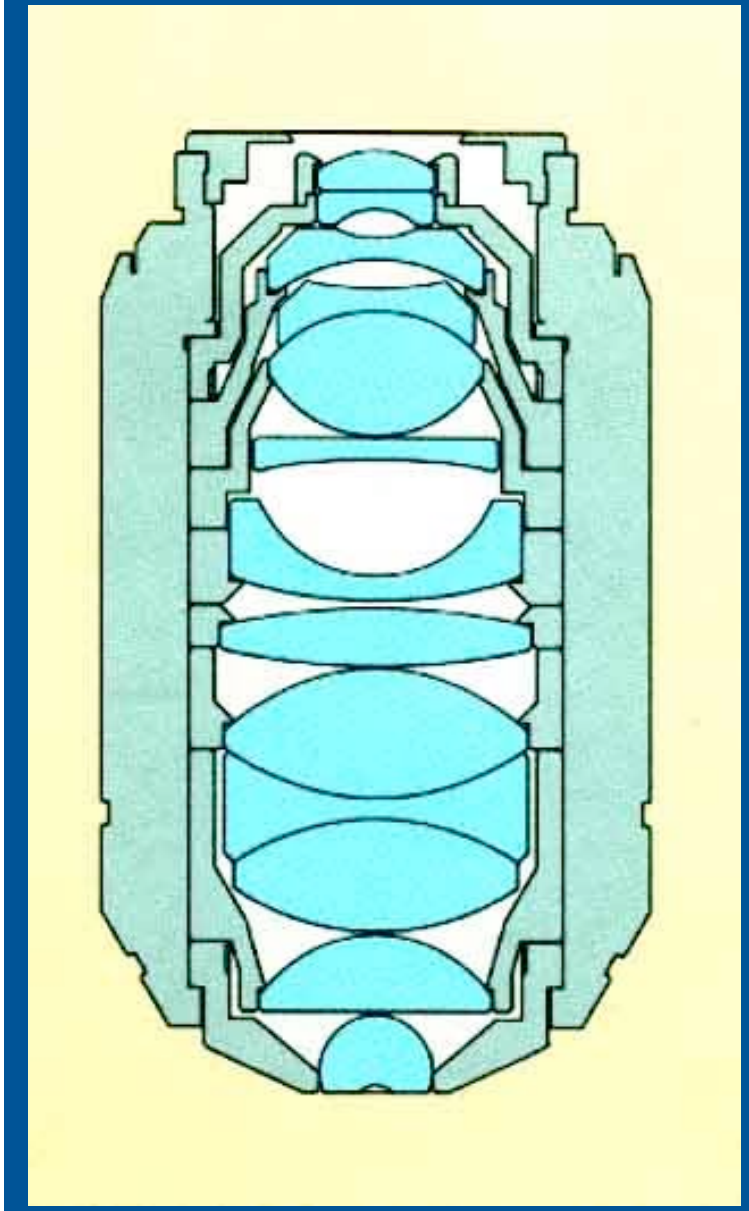
Objective focal length

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

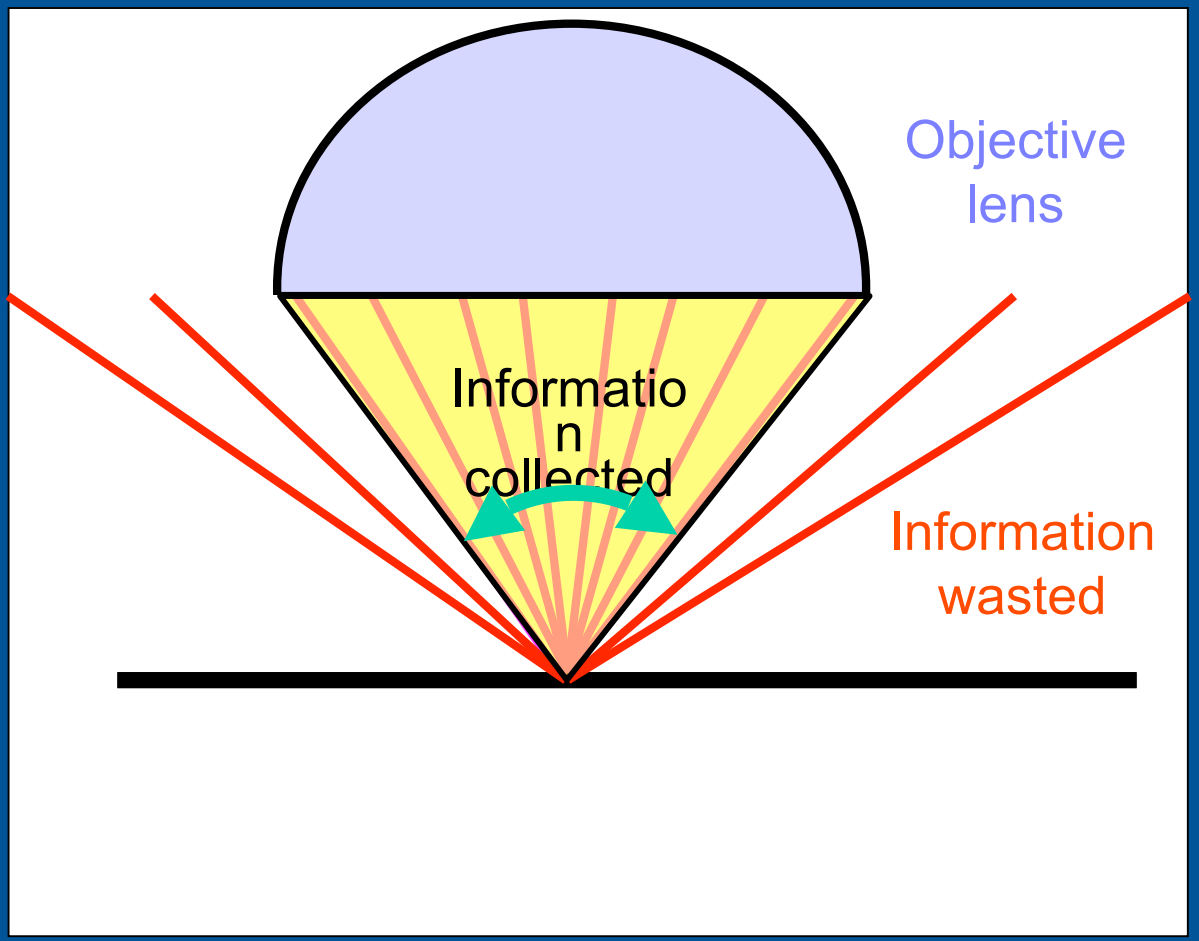
– and some information –

will *NOT* be collected,

and will be wasted

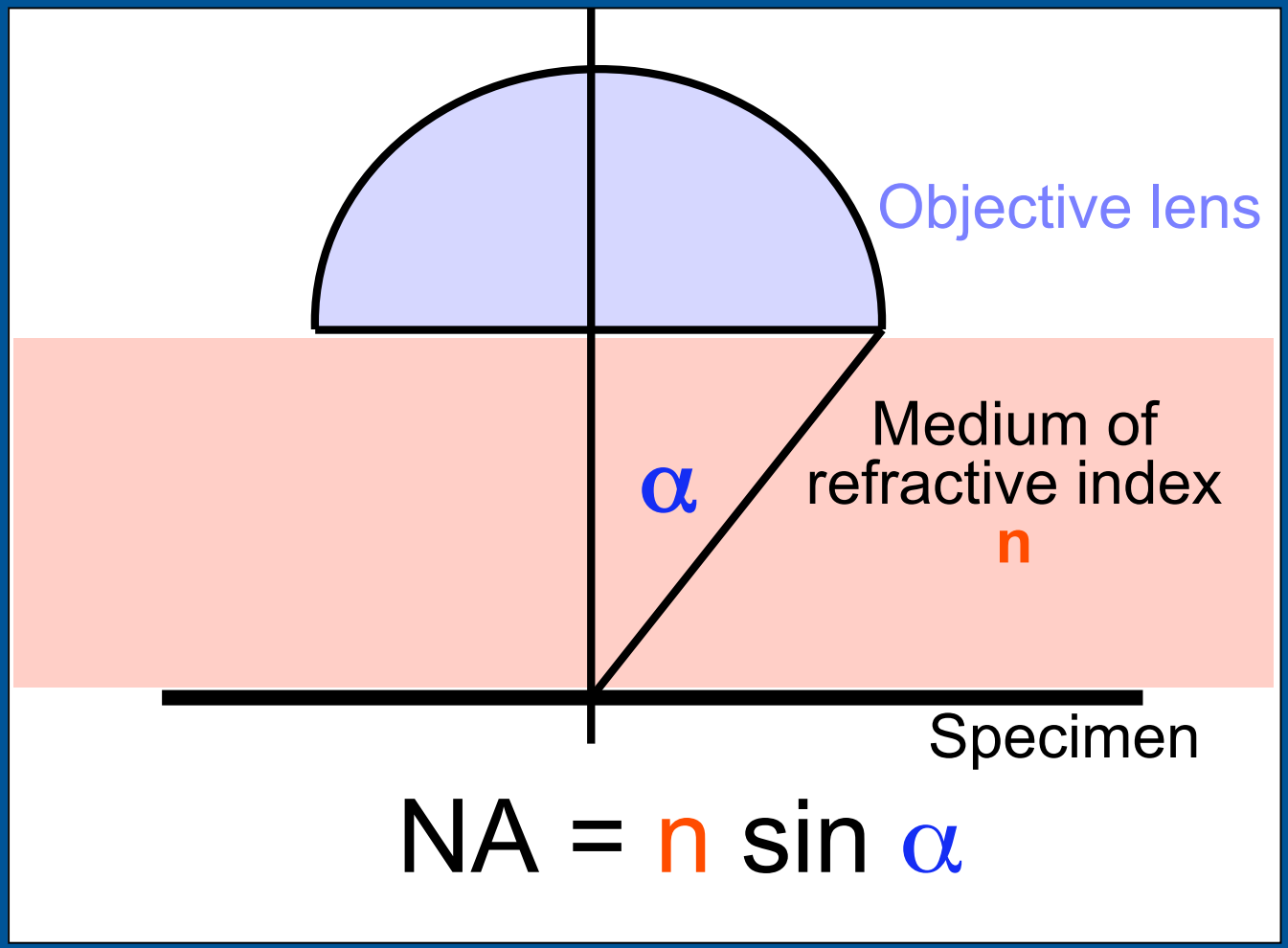
Resolution will therefore depend on the angular *aperture* of the objective -

the larger the imaging aperture the higher the resolution



Numerical Aperture

NA



$$NA = n \sin \alpha$$

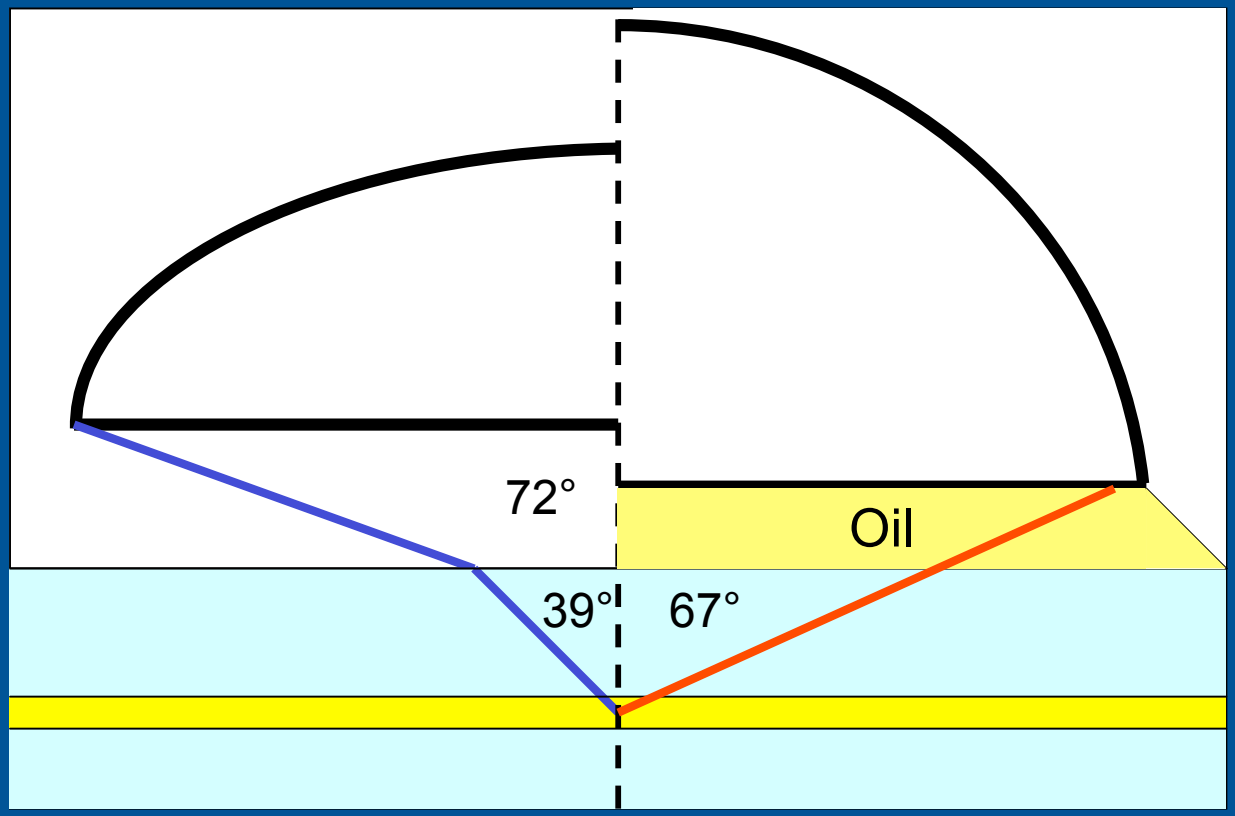
Numerical Aperture

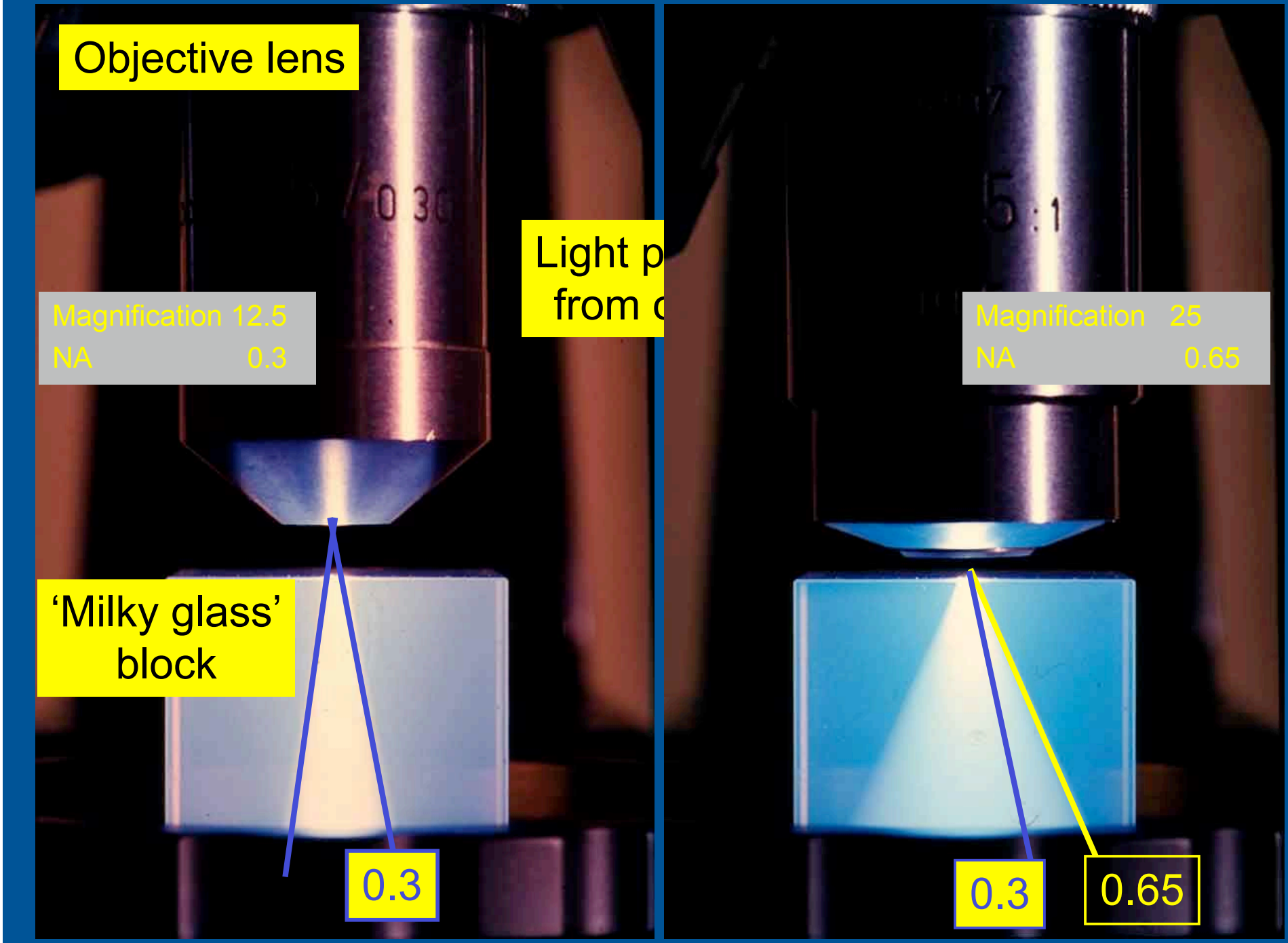
Dry Objective

NA = 1 x sin 72°
= 1 x 0.95
= 0.95

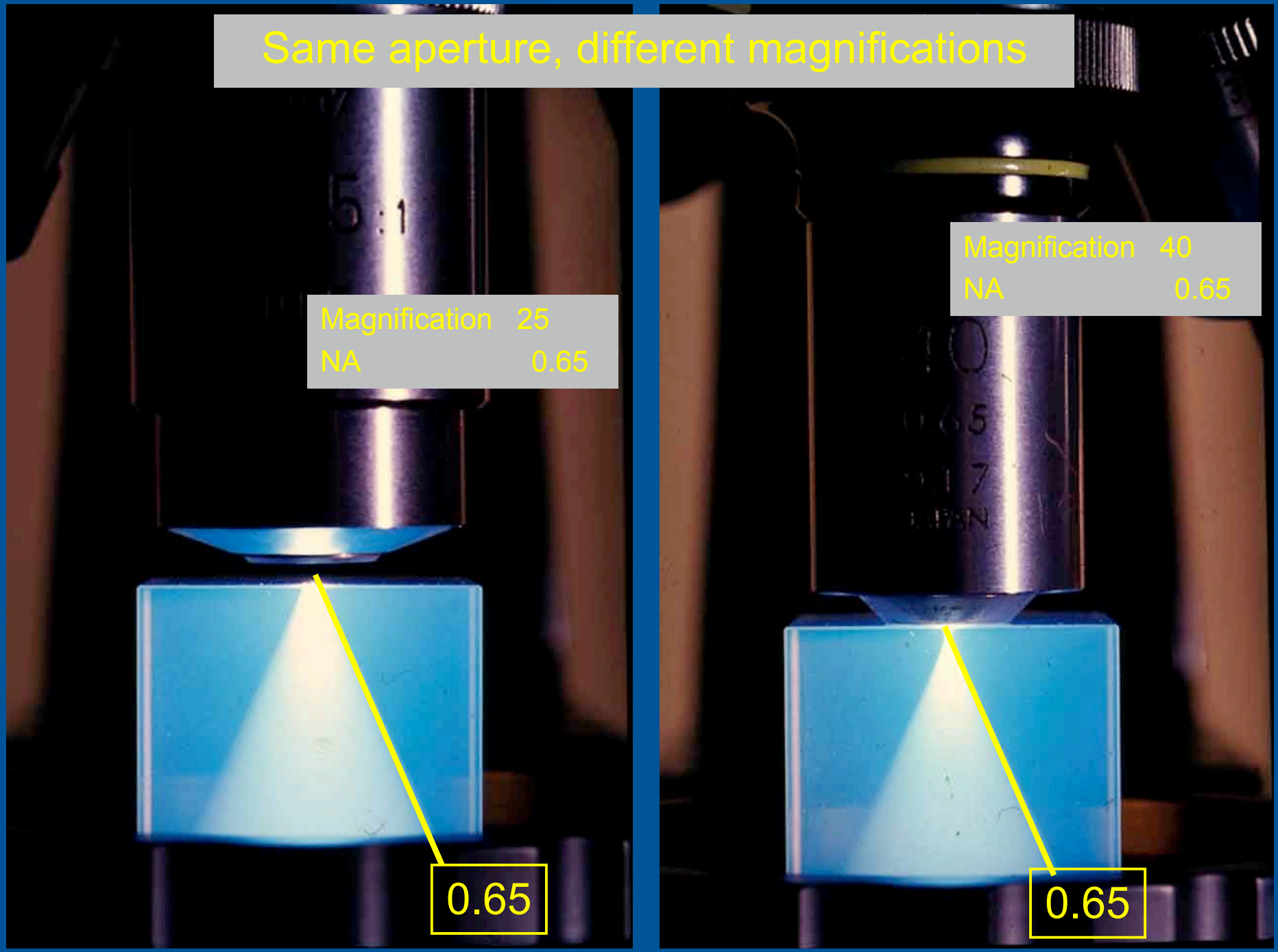
Immersion Objective

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



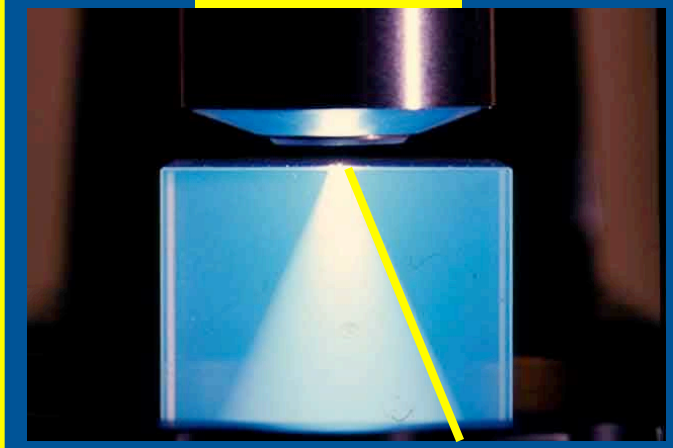

Objective lens
Light p
from c
Magnification 12.5
NA 0.3
‘Milky glass’ block
0.3
Magnification 25
NA 0.65
0.3
0.65

Same aperture, different magnifications

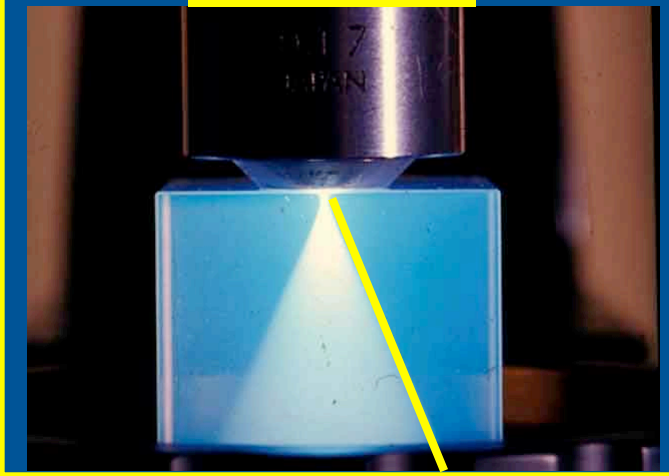


Same aperture
different magnifications

25 / 0.65

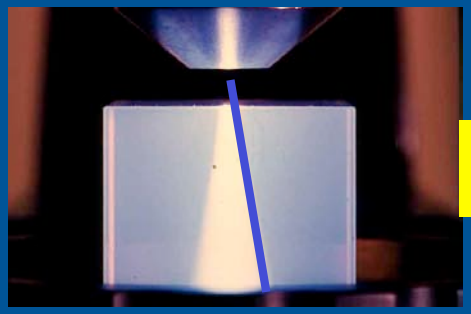


40 / 0.65

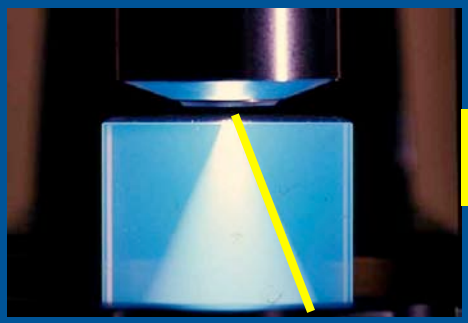


40 / 0.95

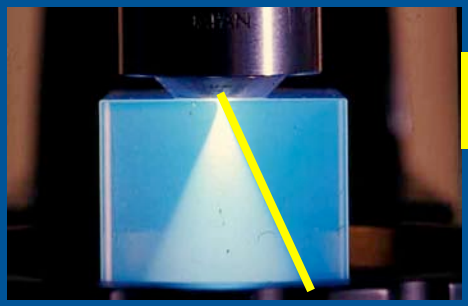
Same magnification
different apertures



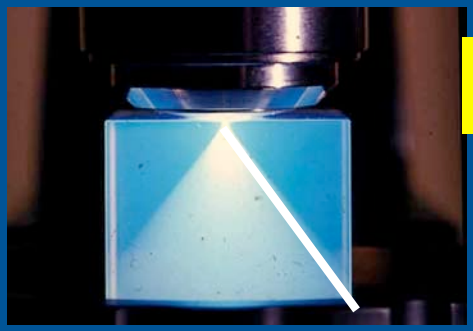
12.5 / 0.3



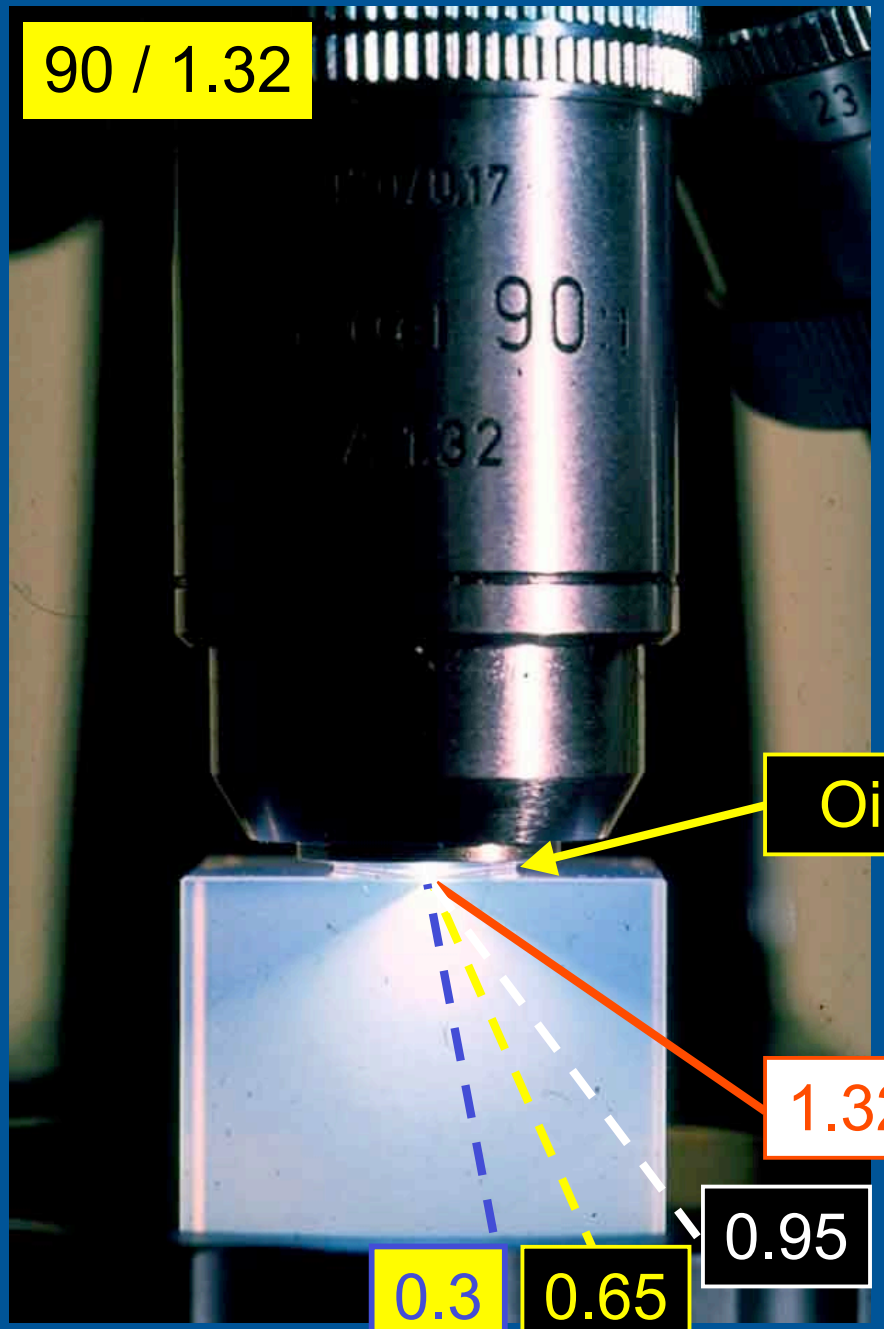
25 / 0.65



40 / 0.65

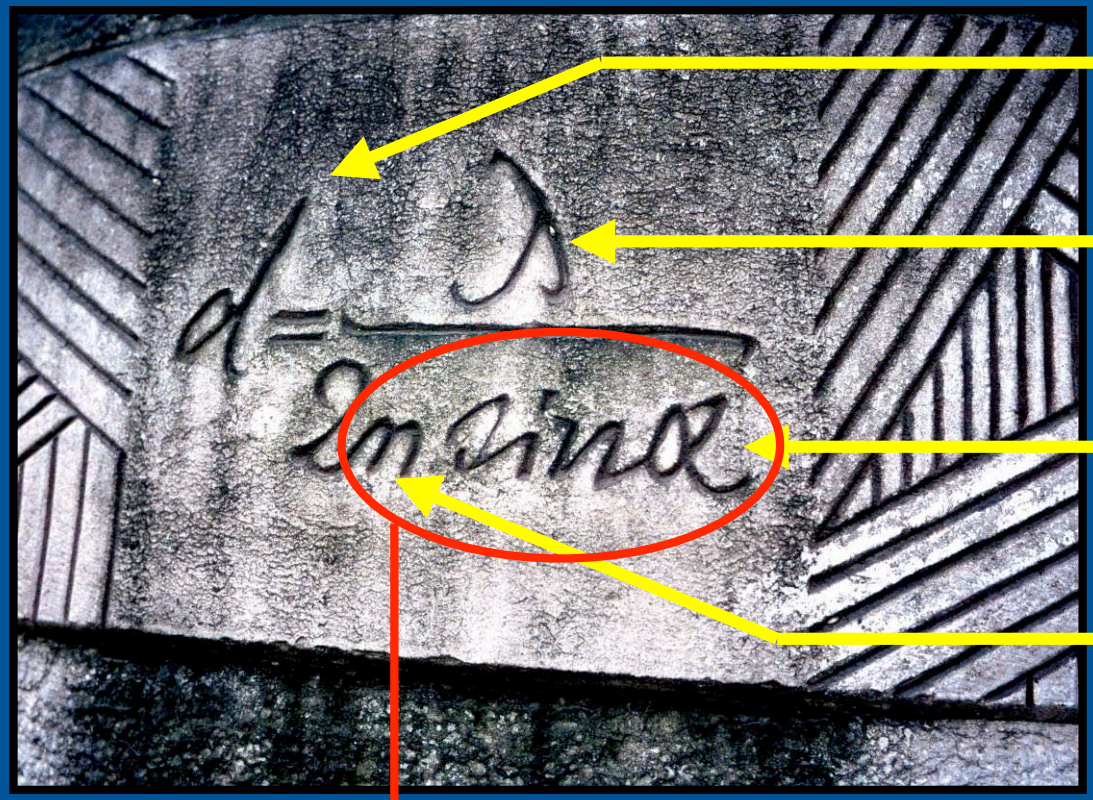


40 / 0.95



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

Microscope Illumination

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***

Microscope Illumination

Two basic methods of illumination:

Source-focused (or 'Critical') Illumination:

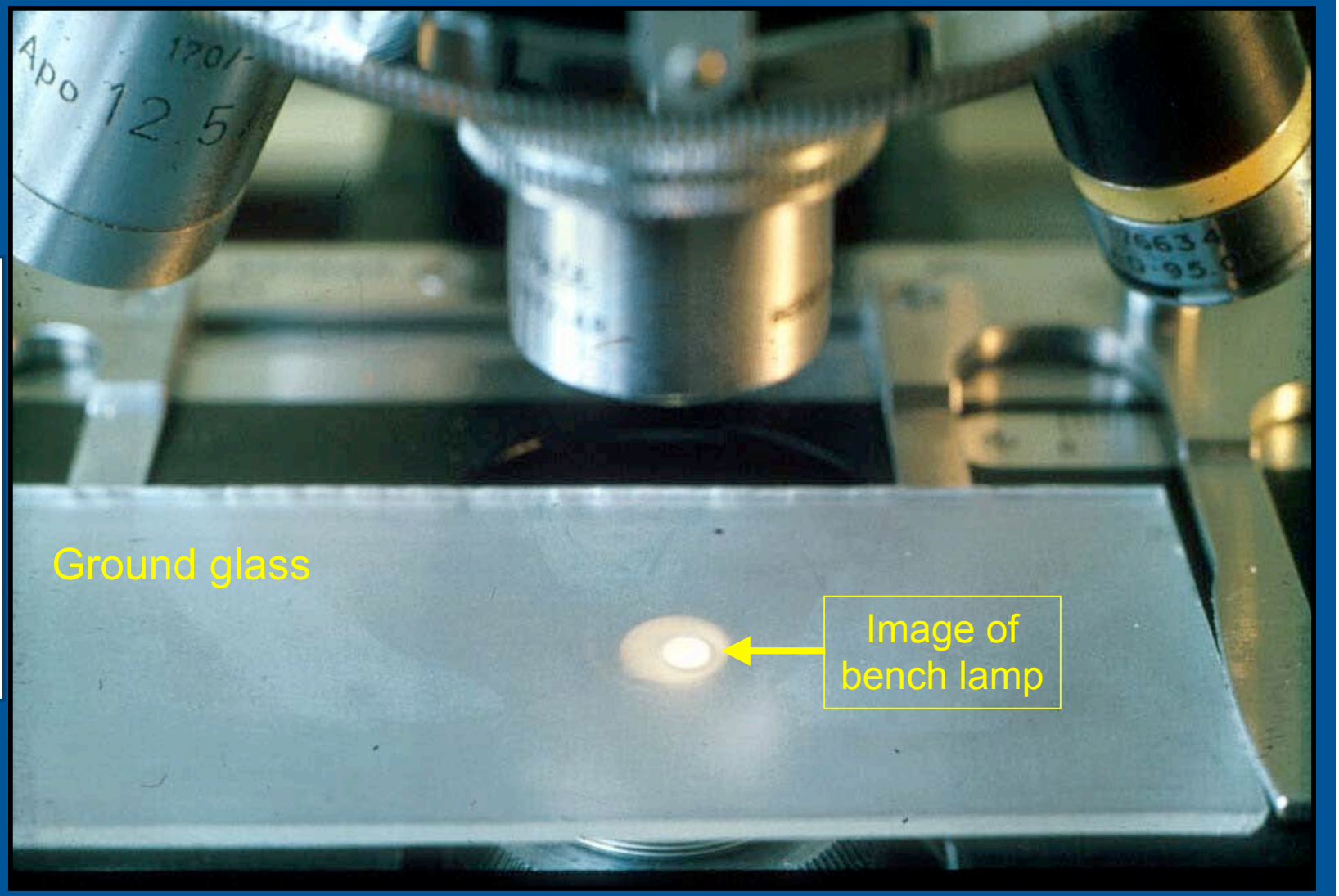
Light-source imaged on to specimen

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



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

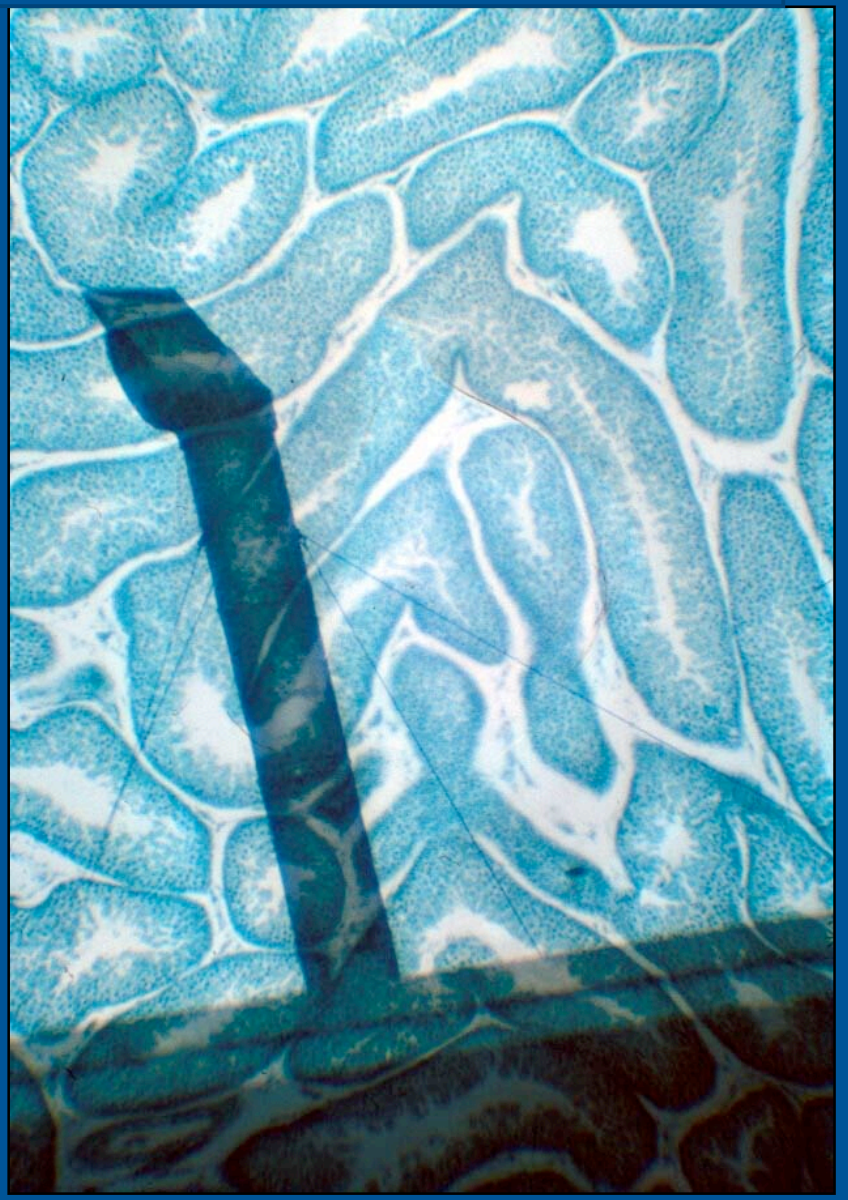
- throws an image of source on to underside of slide

Source-focused Illumination

But looking for a region of uniformly illuminated sky in Leeds…

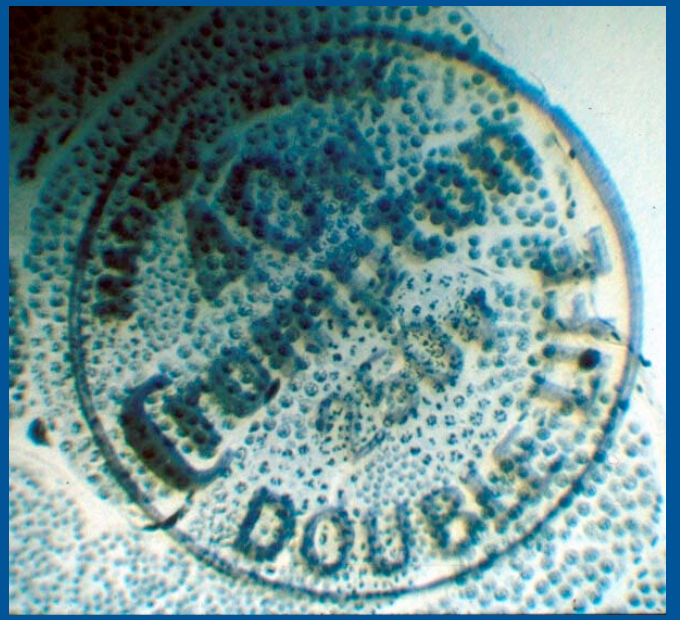
gave an image of the stink-pipe on the Chemistry Building

…when the microscope was set up *correctly*



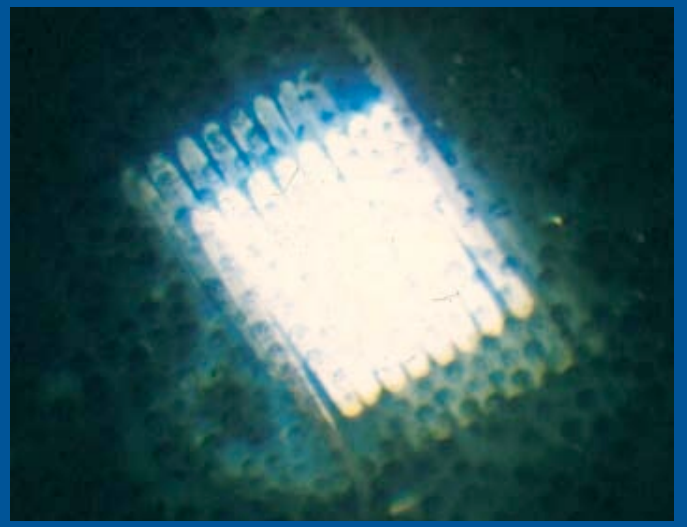
Source-focused Illumination

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

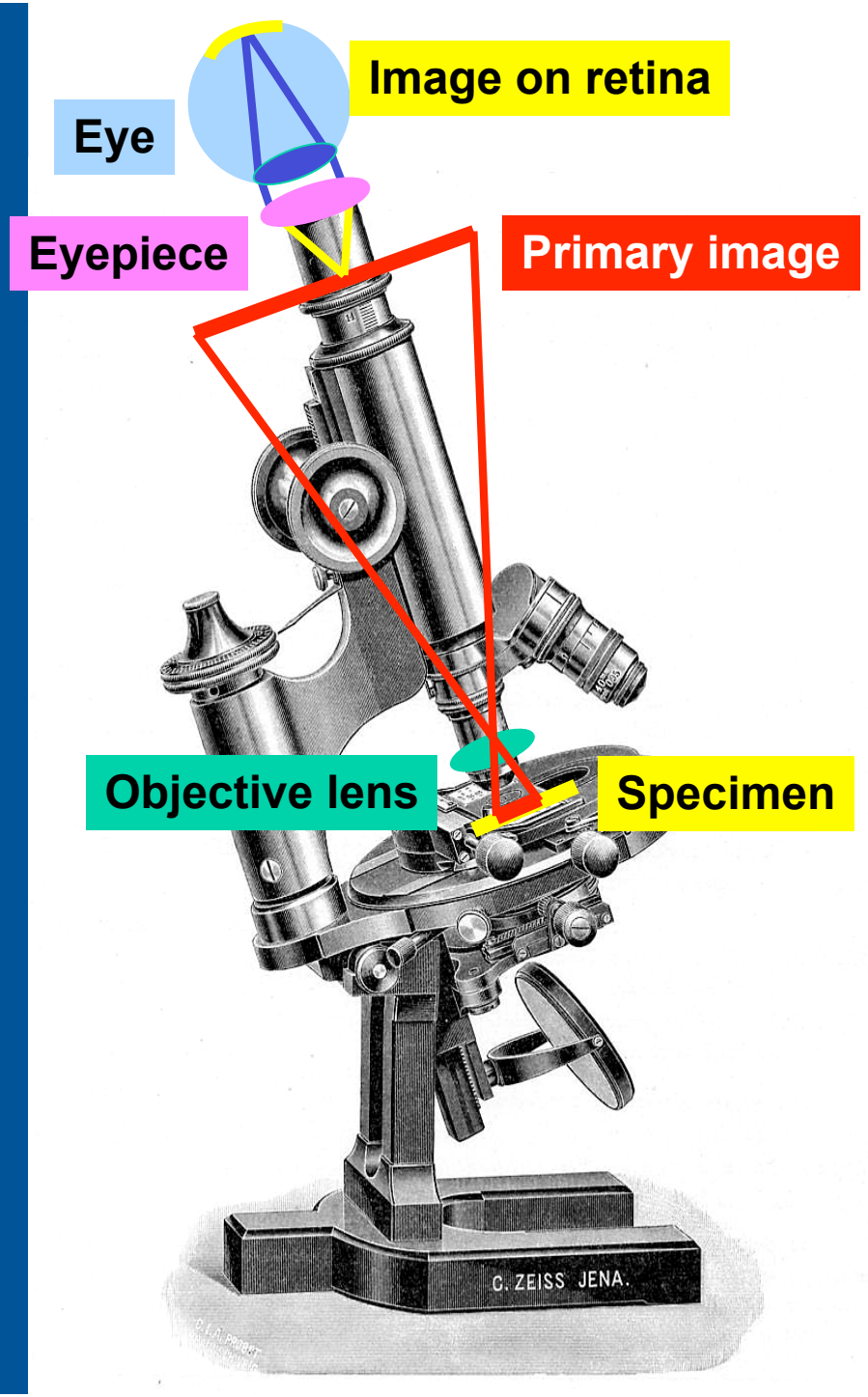
to the retina

These are three

conjugate planes

- successive images of one another

… and there are more.



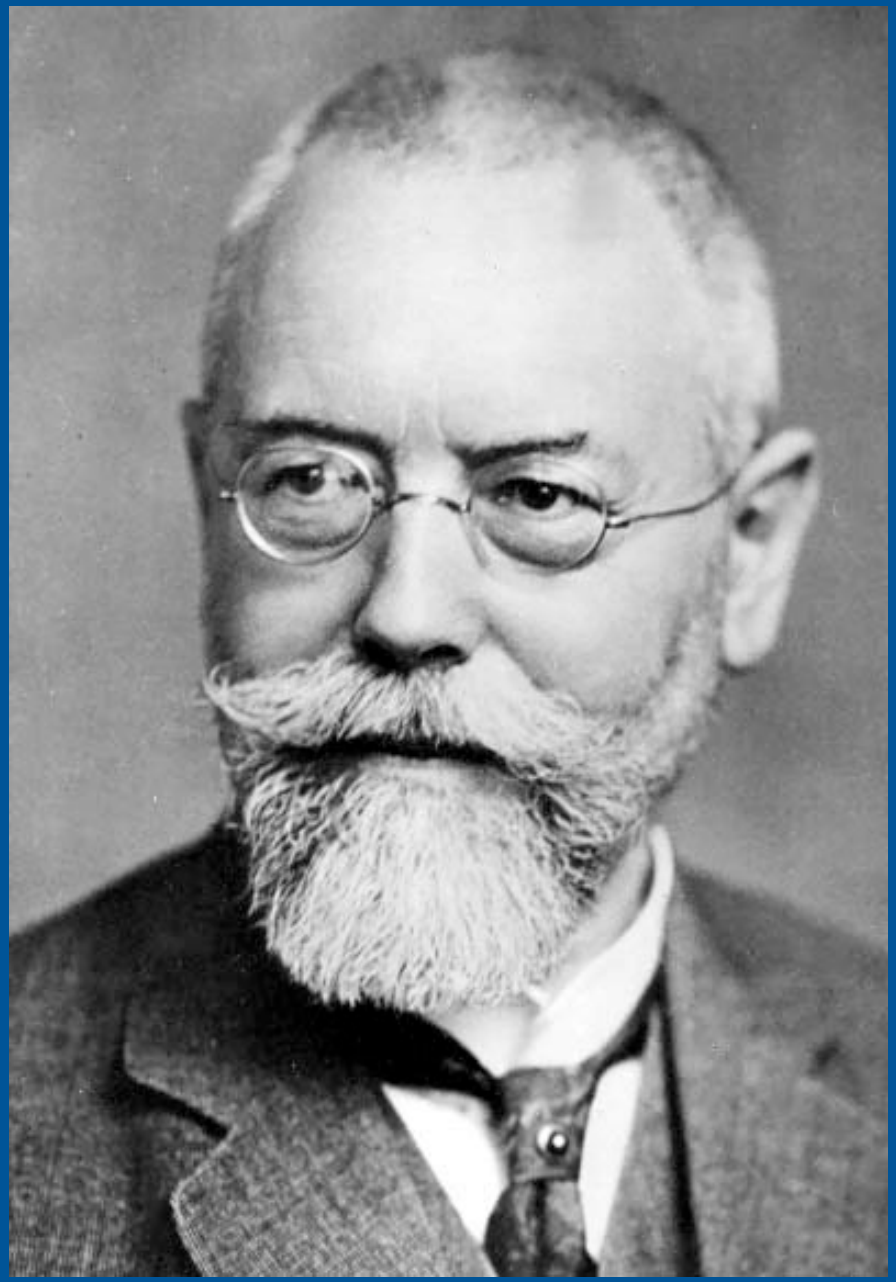
August Köhler

1866 - 1948

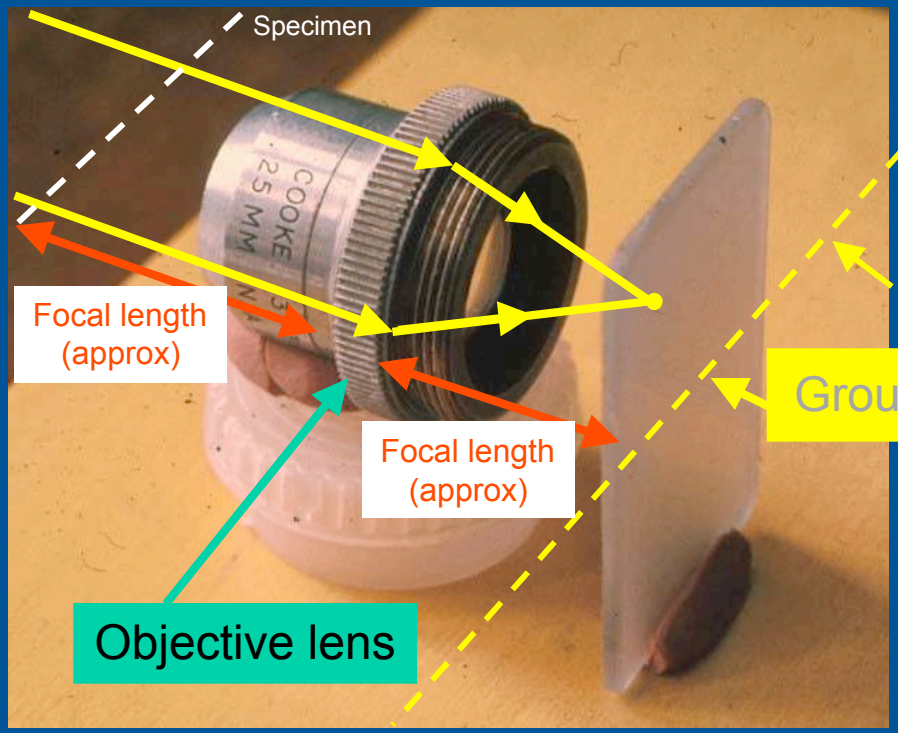
published

A new system of illumination for photomicrographic purposes

(in German) in 1893.



The back focal plane of the objective



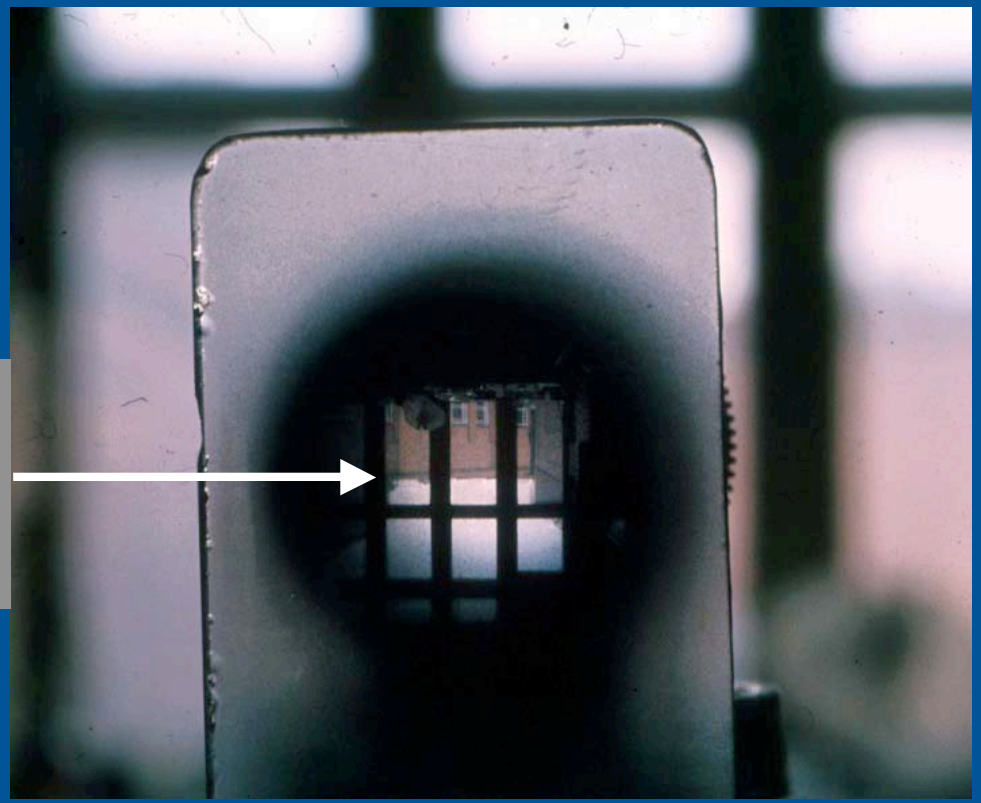


Image of objects at ‘infinity’ in back focal plane of objective

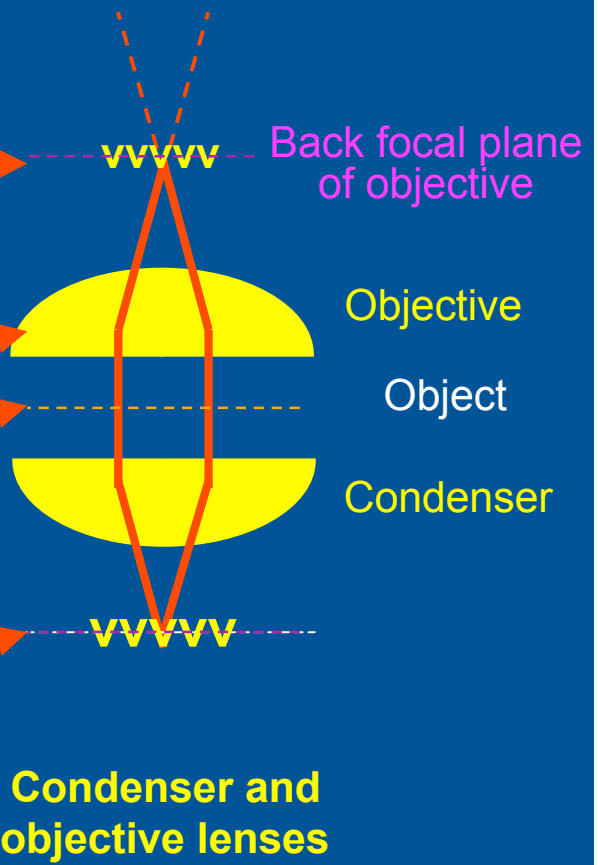
How do we light up the specimen ***uniformly***?

And be brought into focus in the back focal plane of the objective

Into the objective

Light will pass *parallel* through the object

Imagine a light source in the first focal plane of the condenser



Condenser and objective lenses

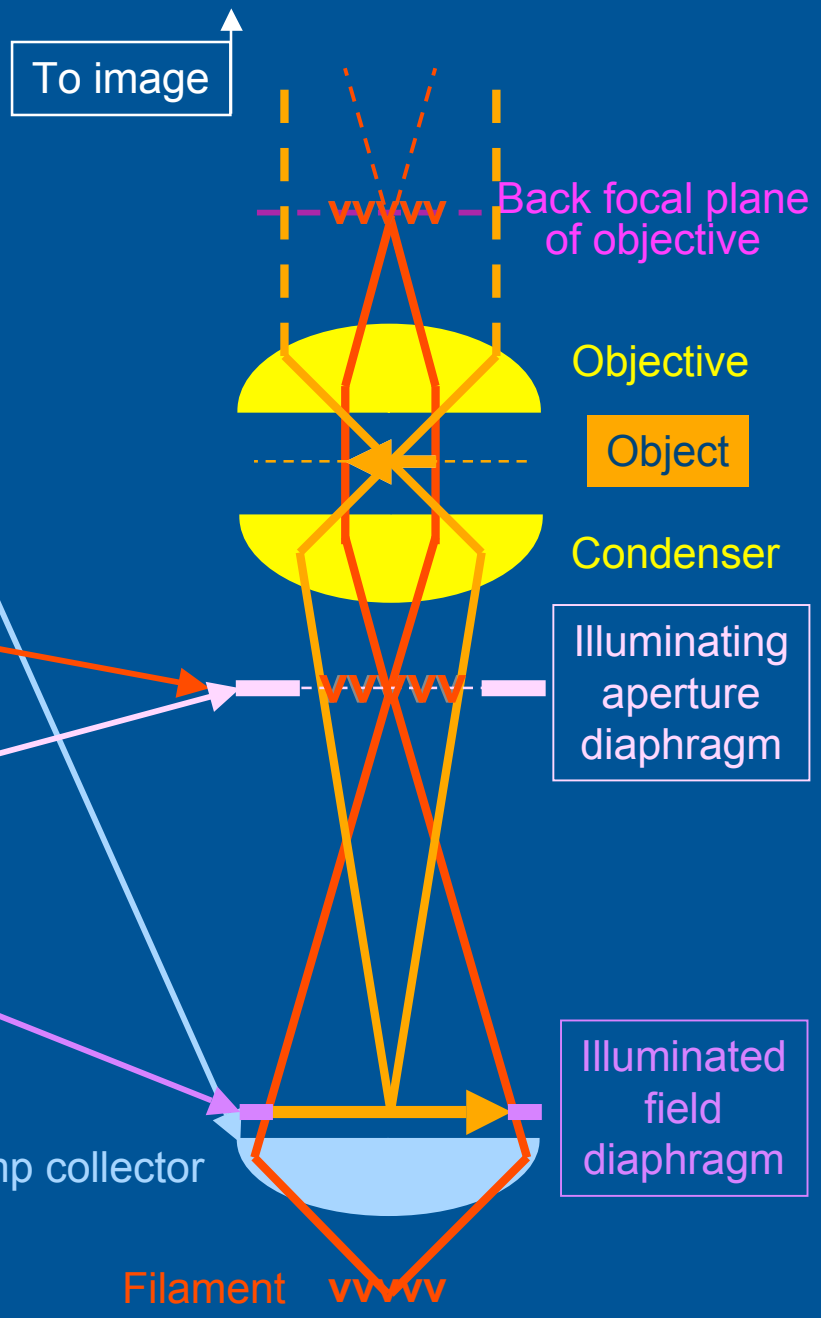
How do we light up the specimen ***uniformly***?

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



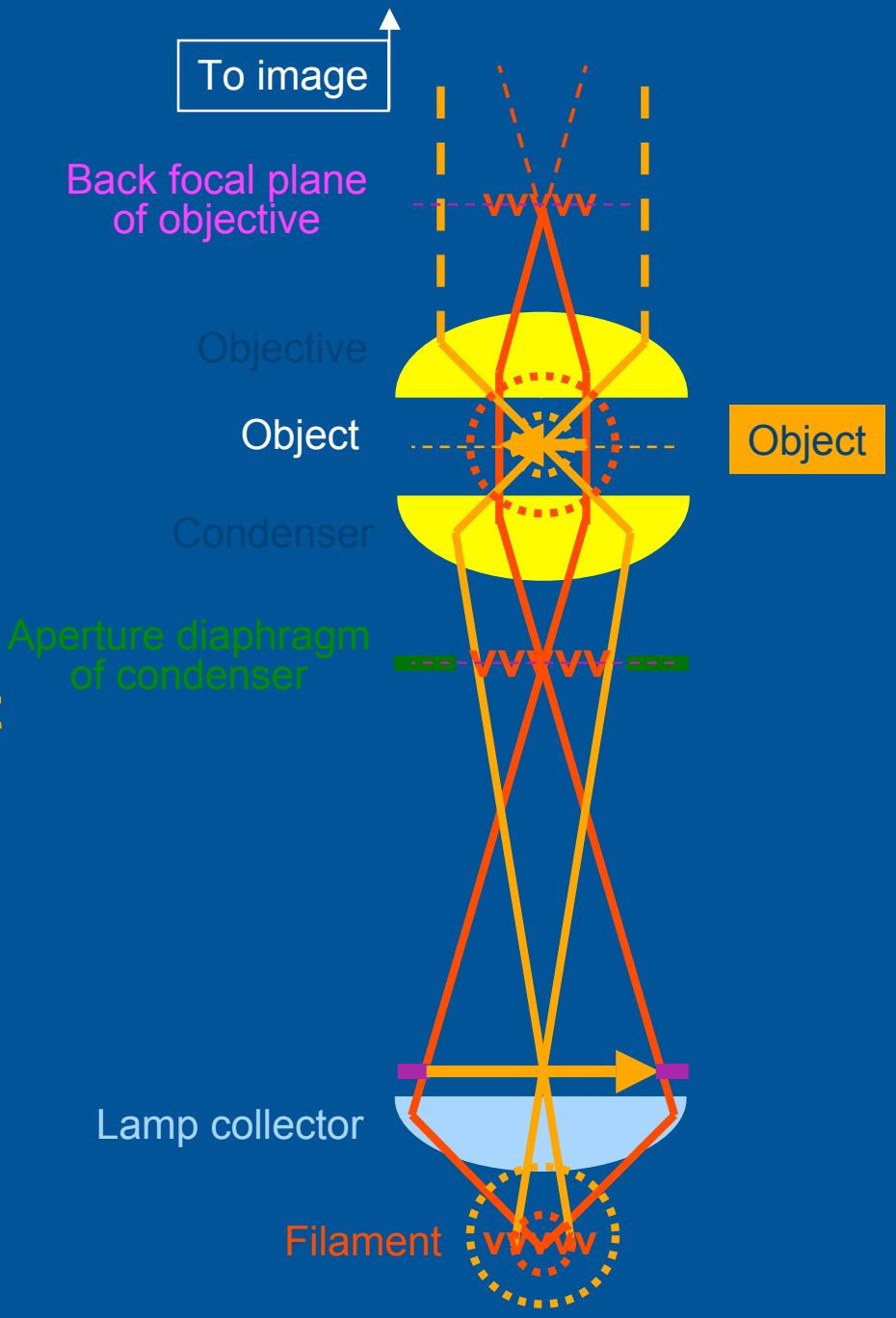
How do we light up the specimen ***uniformly***?

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***?

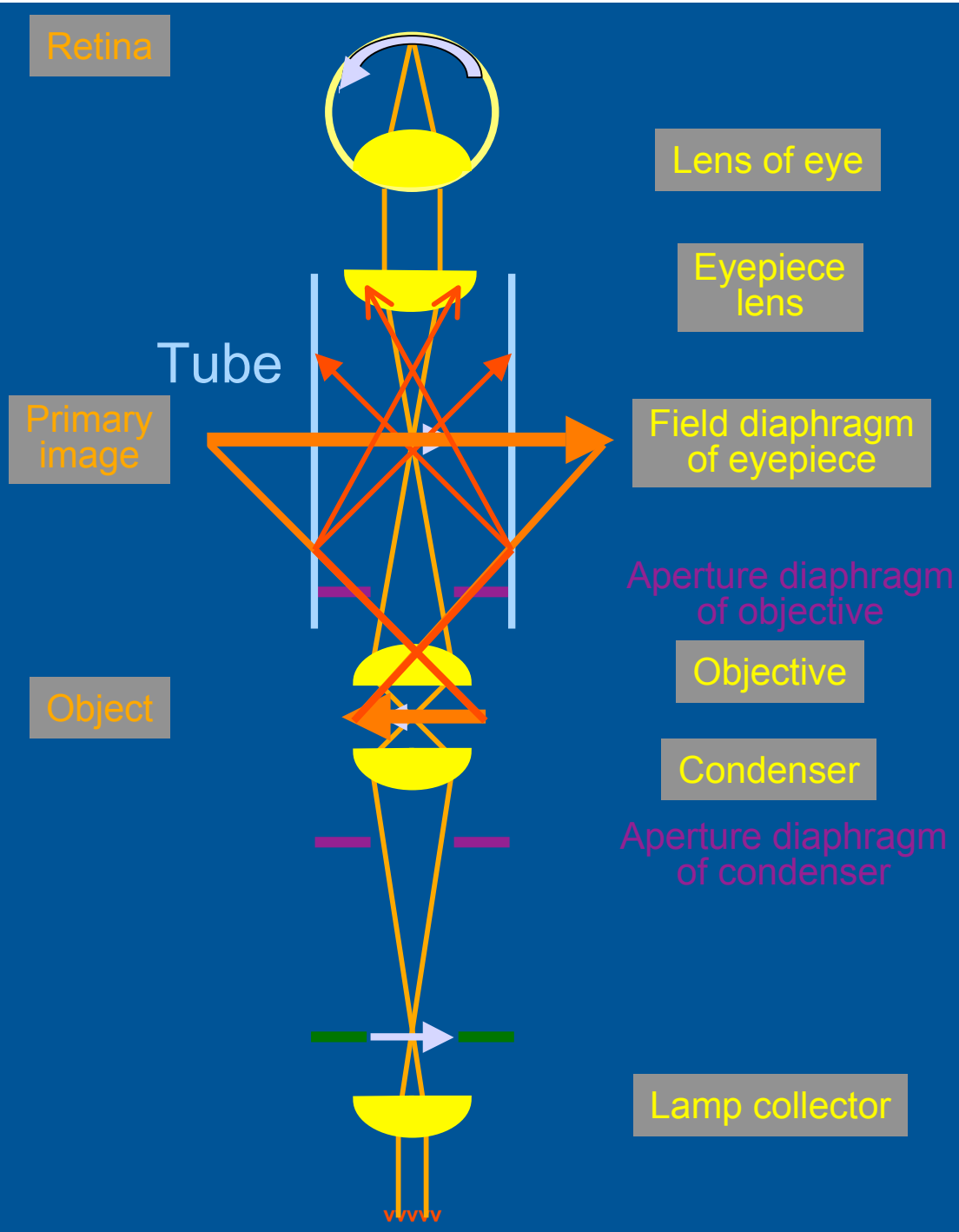
It is unnecessary, and often detrimental, to illuminate parts of the specimen outside the field of view

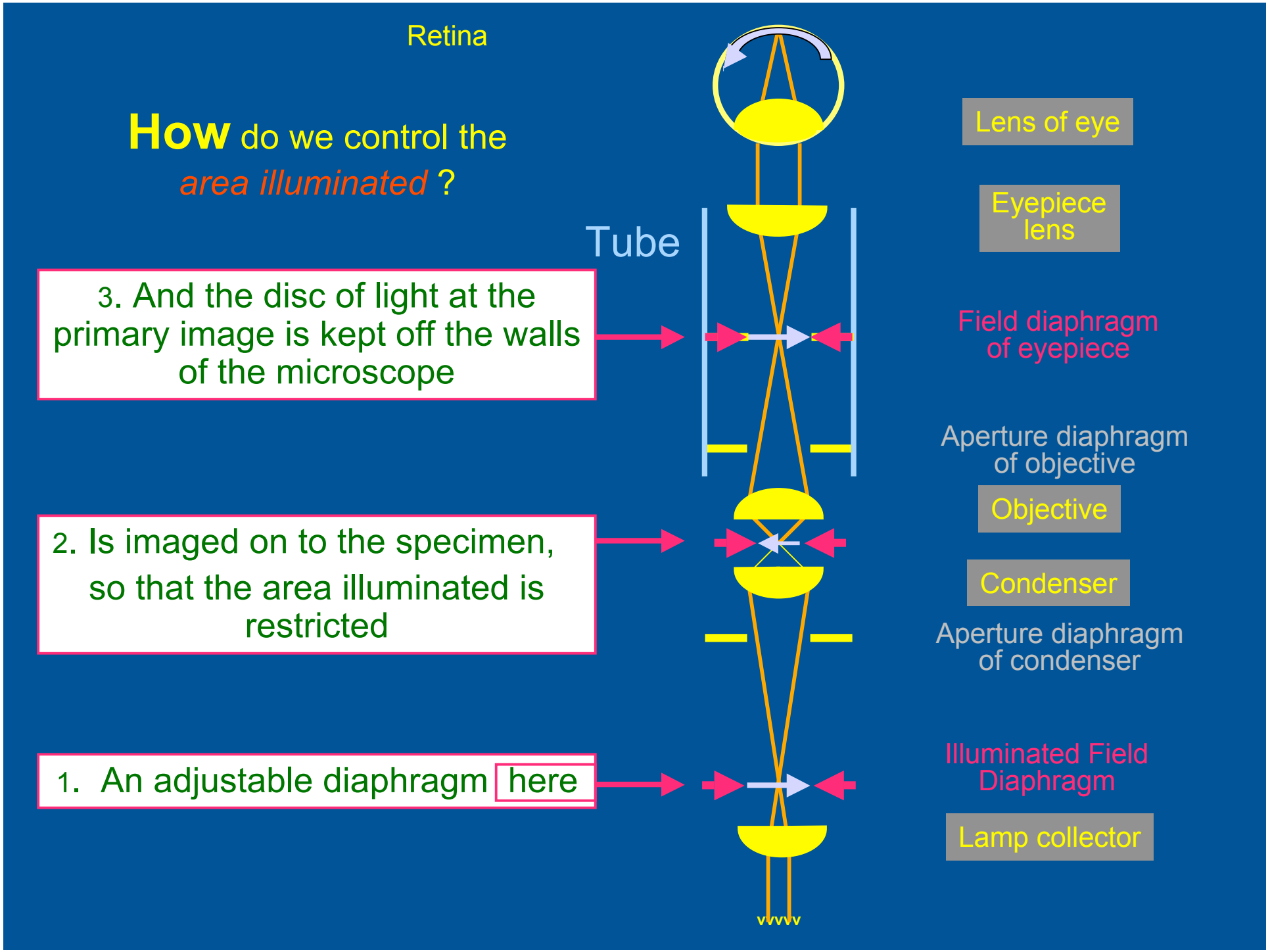
- some specimens are light-sensitive, and could be damaged
- light can be scattered into field of view from outside this area
- illuminating a large area of specimen produces a large primary image, and light can reflect from internal walls of microscope, reducing contrast in the image

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




Retina
How do we control the area illuminated ?
Tube
3. And the disc of light at the primary image is kept off the walls of the microscope
2. Is imaged on to the specimen, so that the area illuminated is restricted
1. An adjustable diaphragm here
Lens of eye
Eyepiece lens
Field diaphragm of eyepiece
Aperture diaphragm of objective
Objective
Condenser
Aperture diaphragm of condenser
Illuminated Field Diaphragm
Lamp collector

Why is it necessary to…

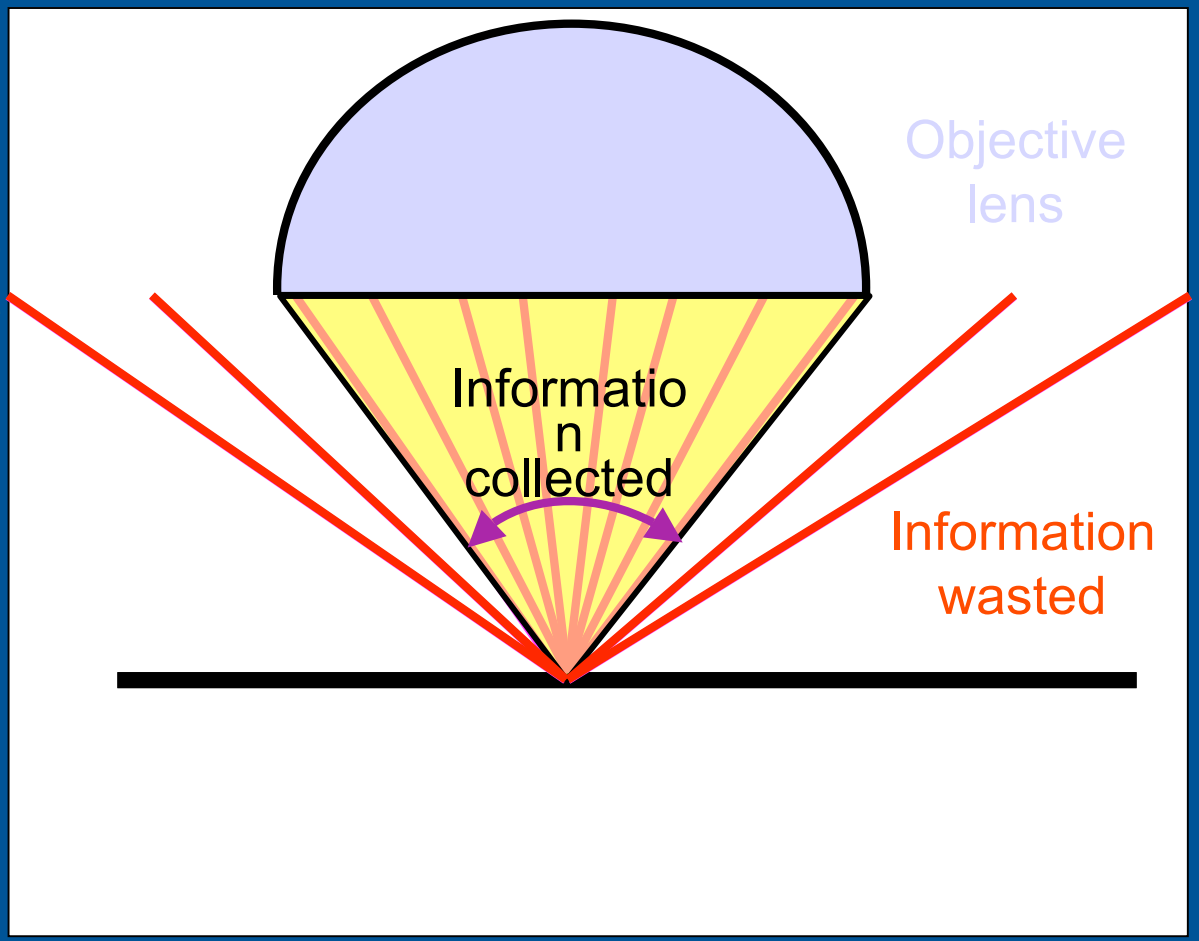
Illuminate the *objective aperture uniformly* over a *controllable angle*?

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…

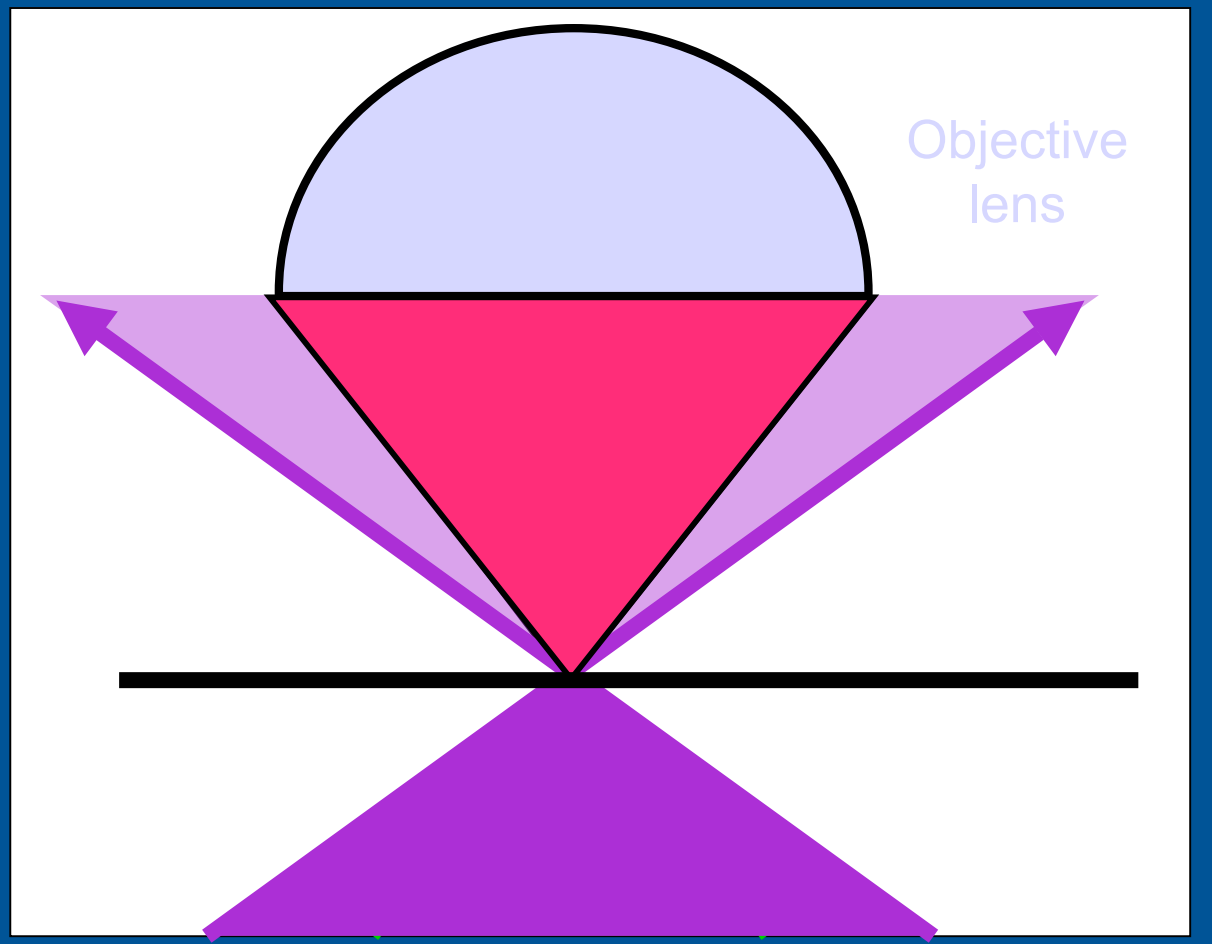
Illuminate the *objective aperture uniformly* over a *controllable angle*?

'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* ?

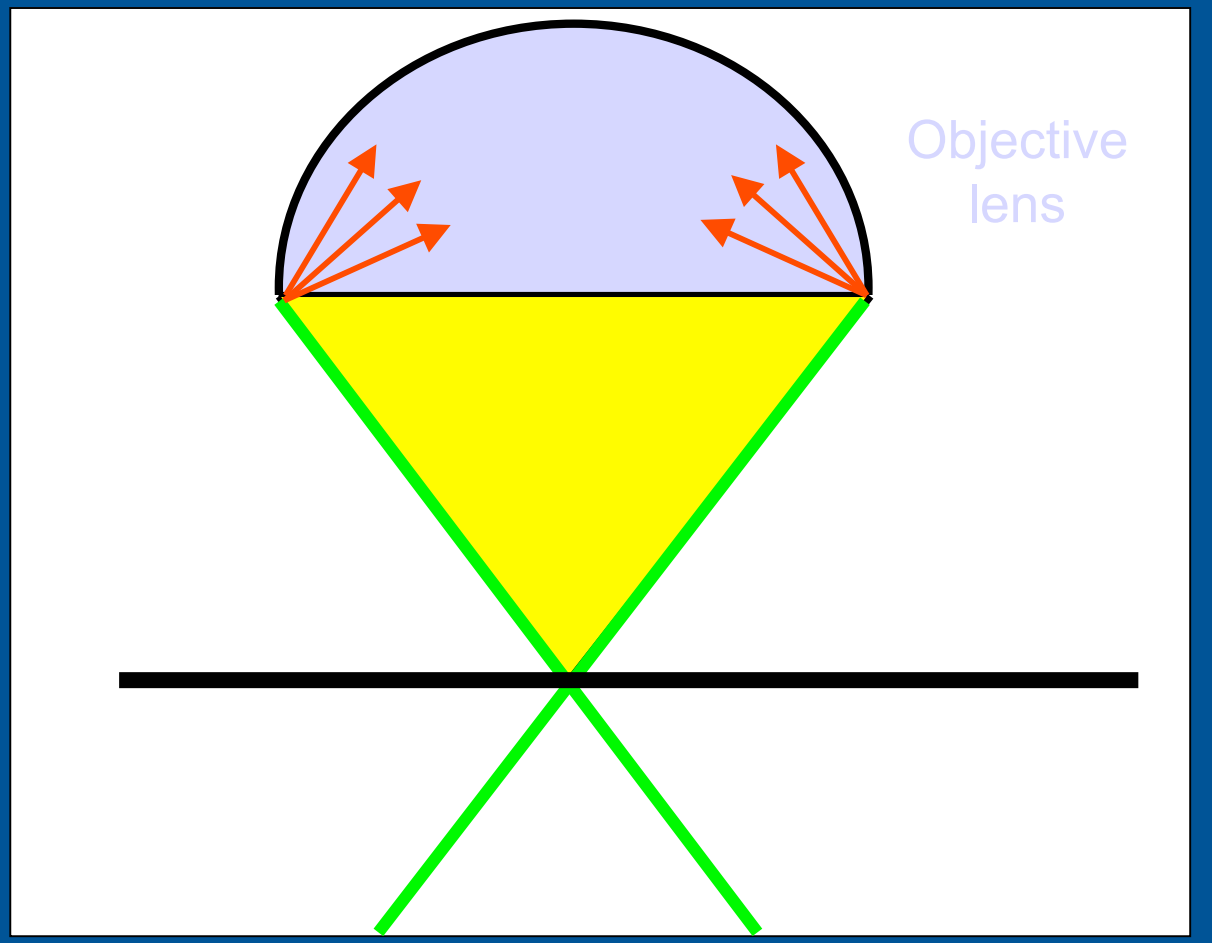
Why not a *very wide* cone of light ?



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

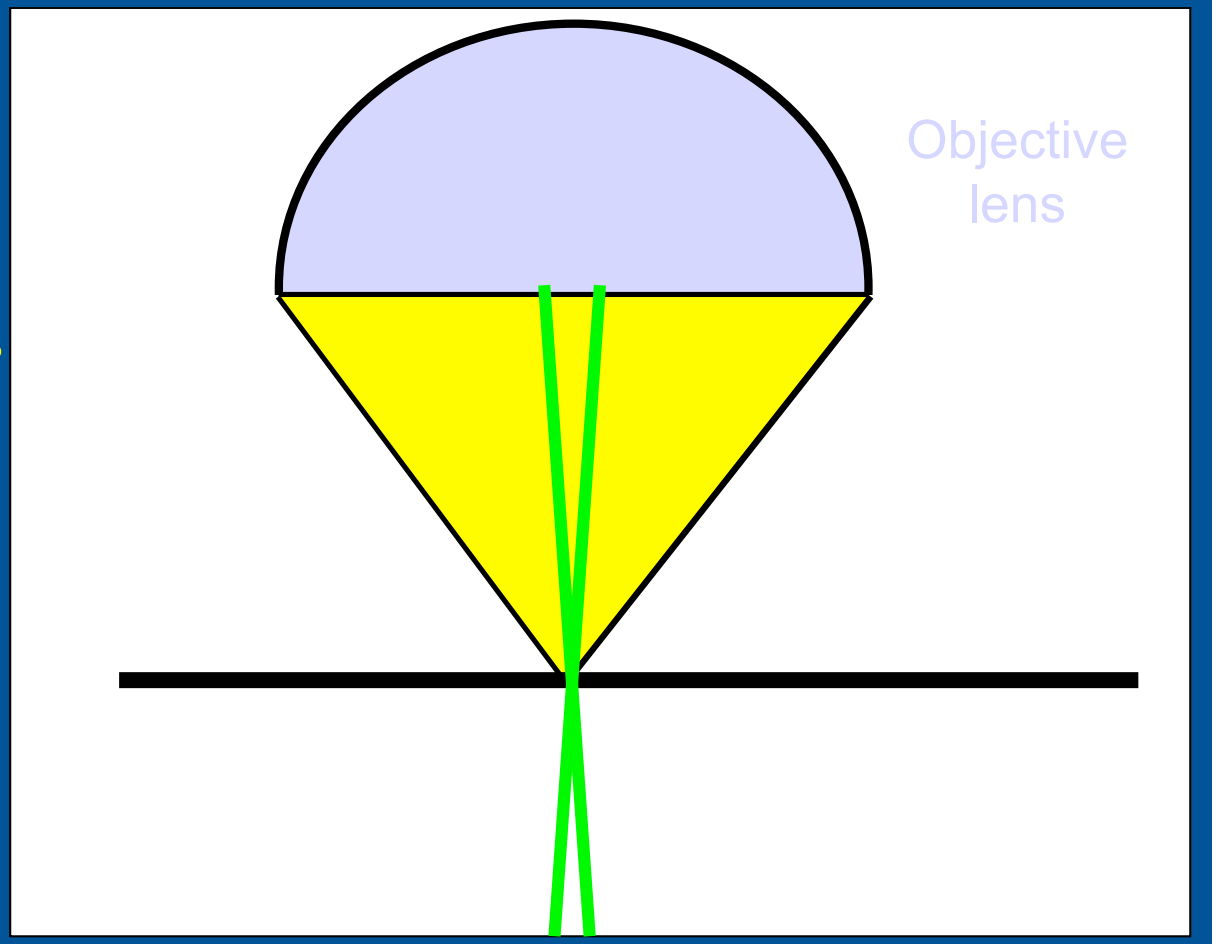
If the illuminating aperture is too ***small***,

resolution will be reduced

and

image quality will be impaired

though contrast will be increased.

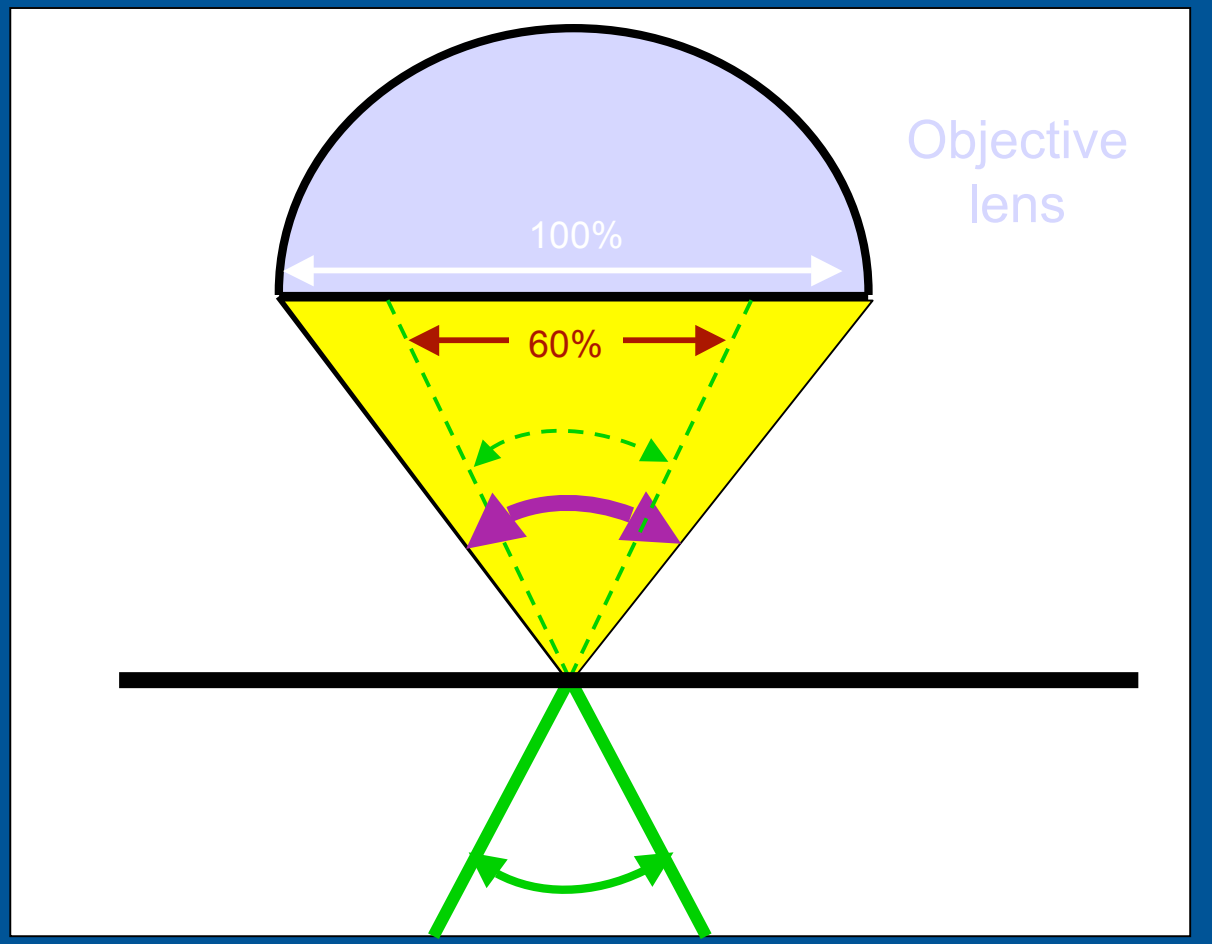


Why is it necessary to…

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

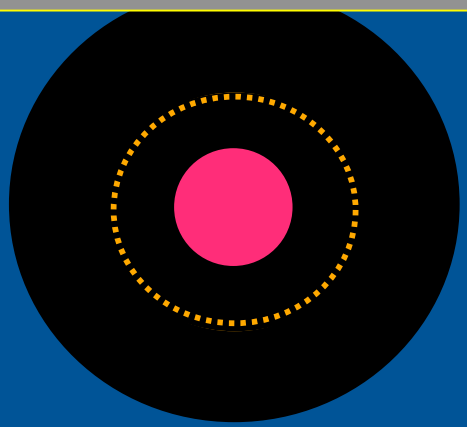
So for best resolution the illuminating aperture should approach the imaging (objective) aperture

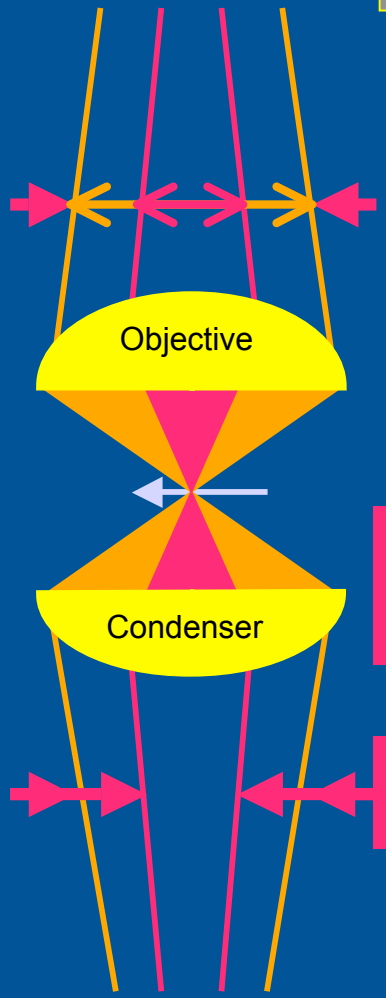
60 to 75% is often recommended



How do we control the *angle of illumination* ?

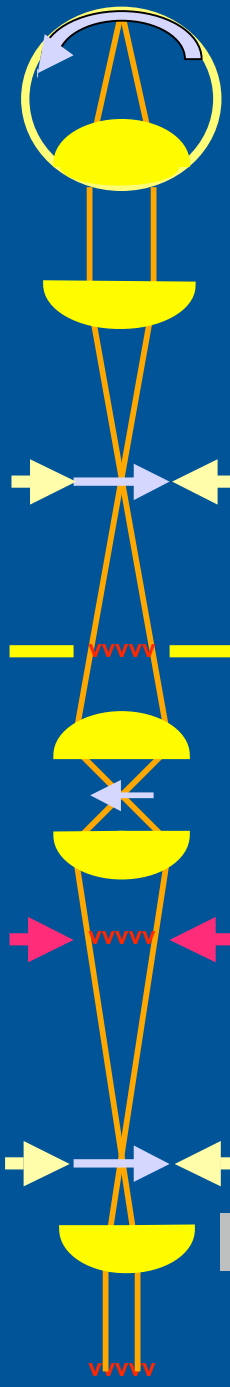
View down tube
(objective back focal plane)





2. Narrows the angle of rays passing though the object

1. Closing the condenser diaphragm



Retina

Lens of eye

Eyepiece

Field diaphragm of eyepiece

Aperture diaphragm of objective

Objective

Object

Aperture diaphragm of condenser

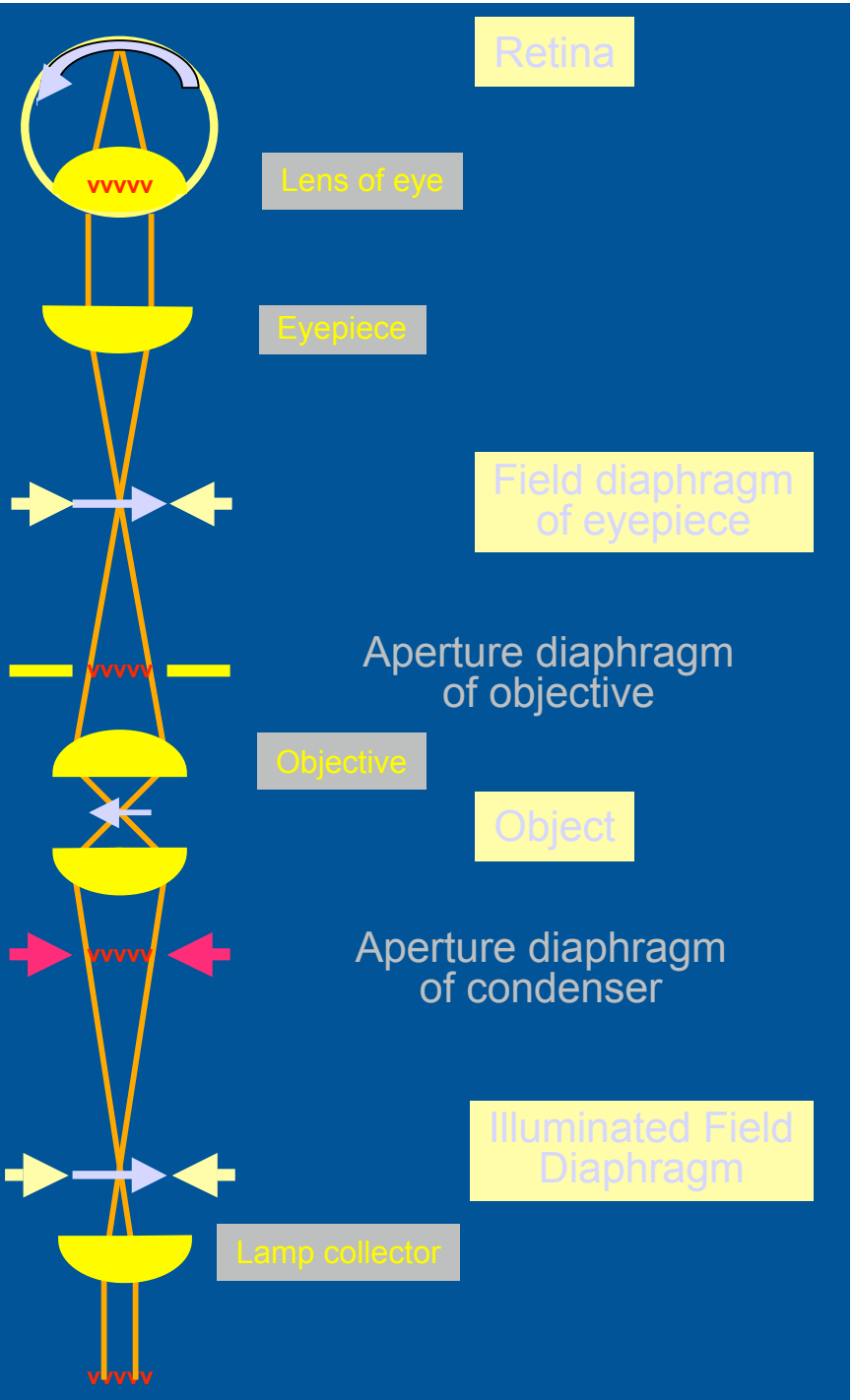
Illuminated Field Diaphragm

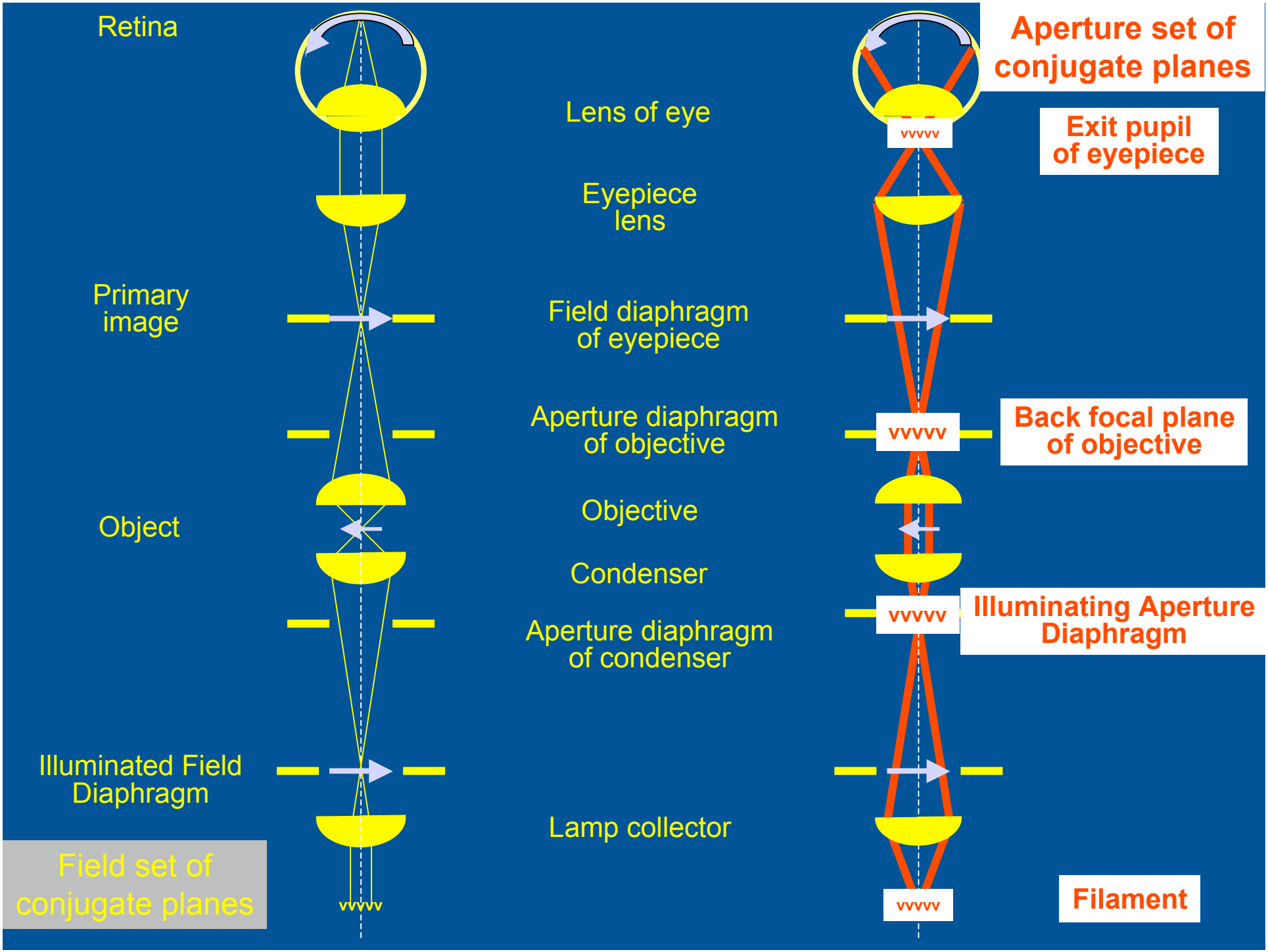
Lamp collector

How do we control the *angle of illumination* ?

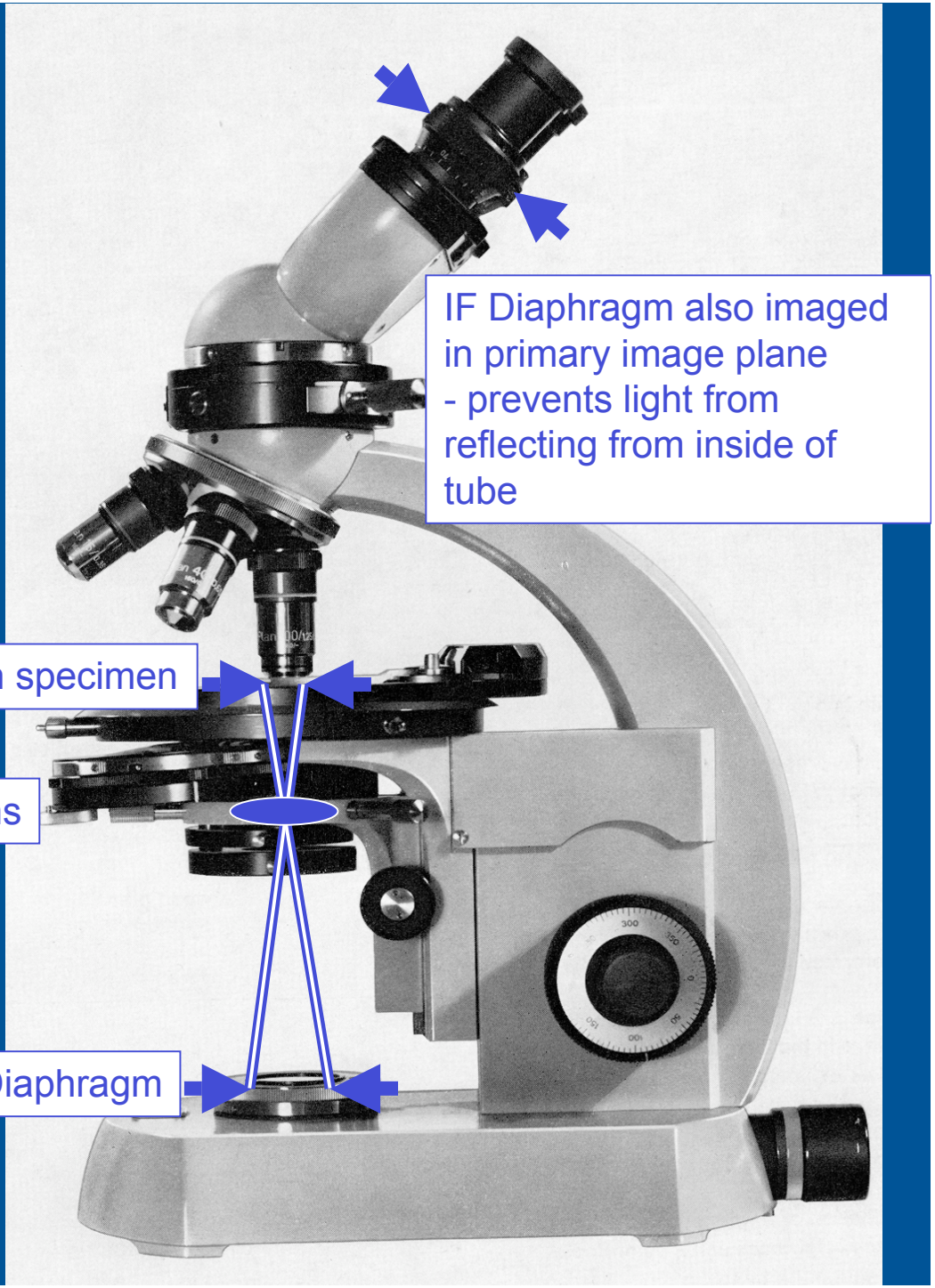
The aperture diaphragm of the condenser thus acts as the *Illuminating Aperture Diaphragm*

– so called because it is the diaphragm which regulates the Illuminating Aperture

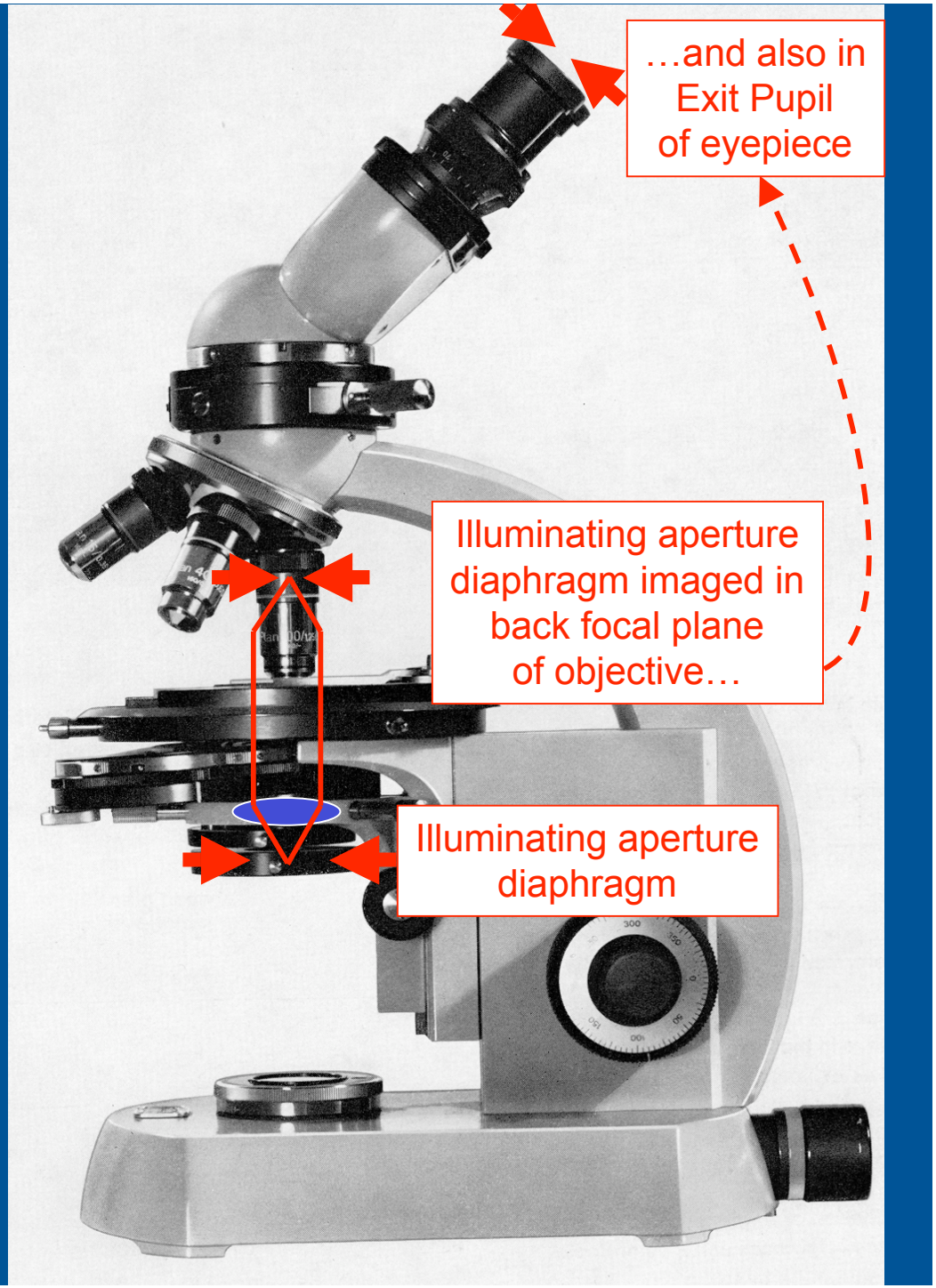



Retina
Lens of eye
Eyepiece lens
Primary image
Field diaphragm of eyepiece
Aperture diaphragm of objective
Object
Objective
Condenser
Aperture diaphragm of condenser
Illuminated Field Diaphragm
Lamp collector
Field set of conjugate planes
vvvvv
Aperture set of conjugate planes
Exit pupil of eyepiece
Back focal plane of objective
Illuminating Aperture Diaphragm
Filament

What are the diaphragms for?

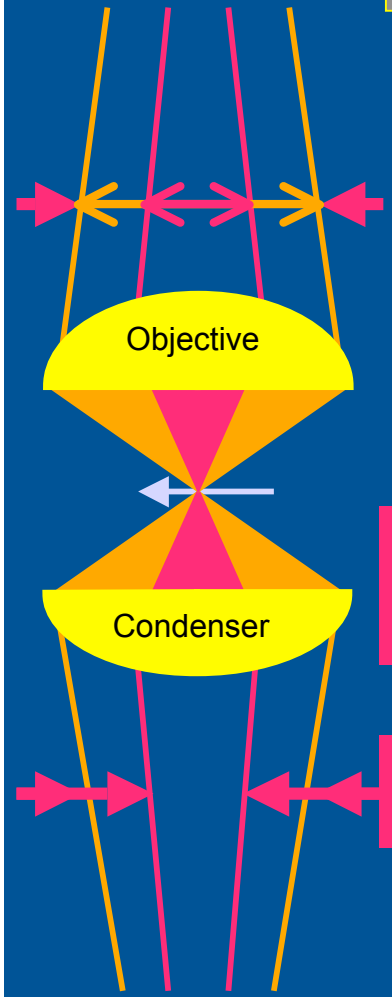


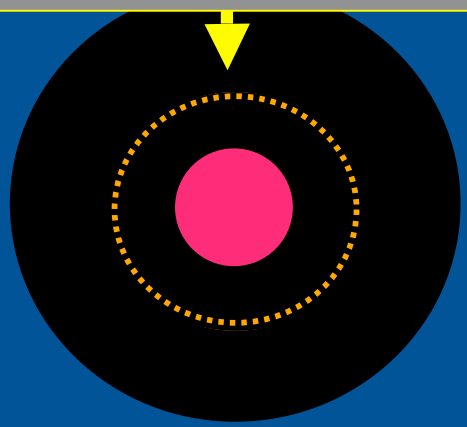
What are the diaphragms for?



What are the diaphragms for?

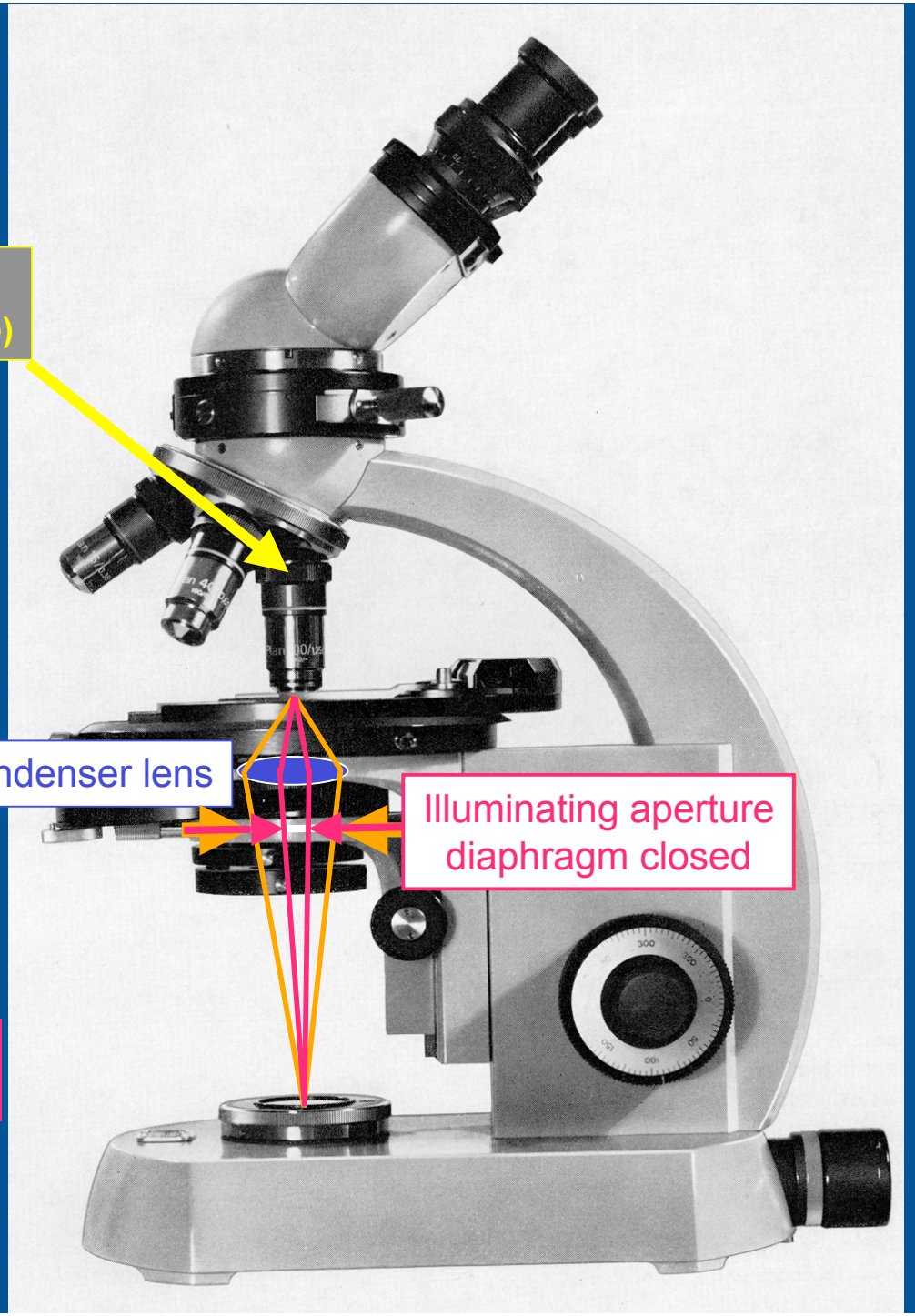
View down tube
(objective back focal plane)





2. Narrows the angle of rays passing though the object

1. Closing the condenser diaphragm



What are the diaphragms for?

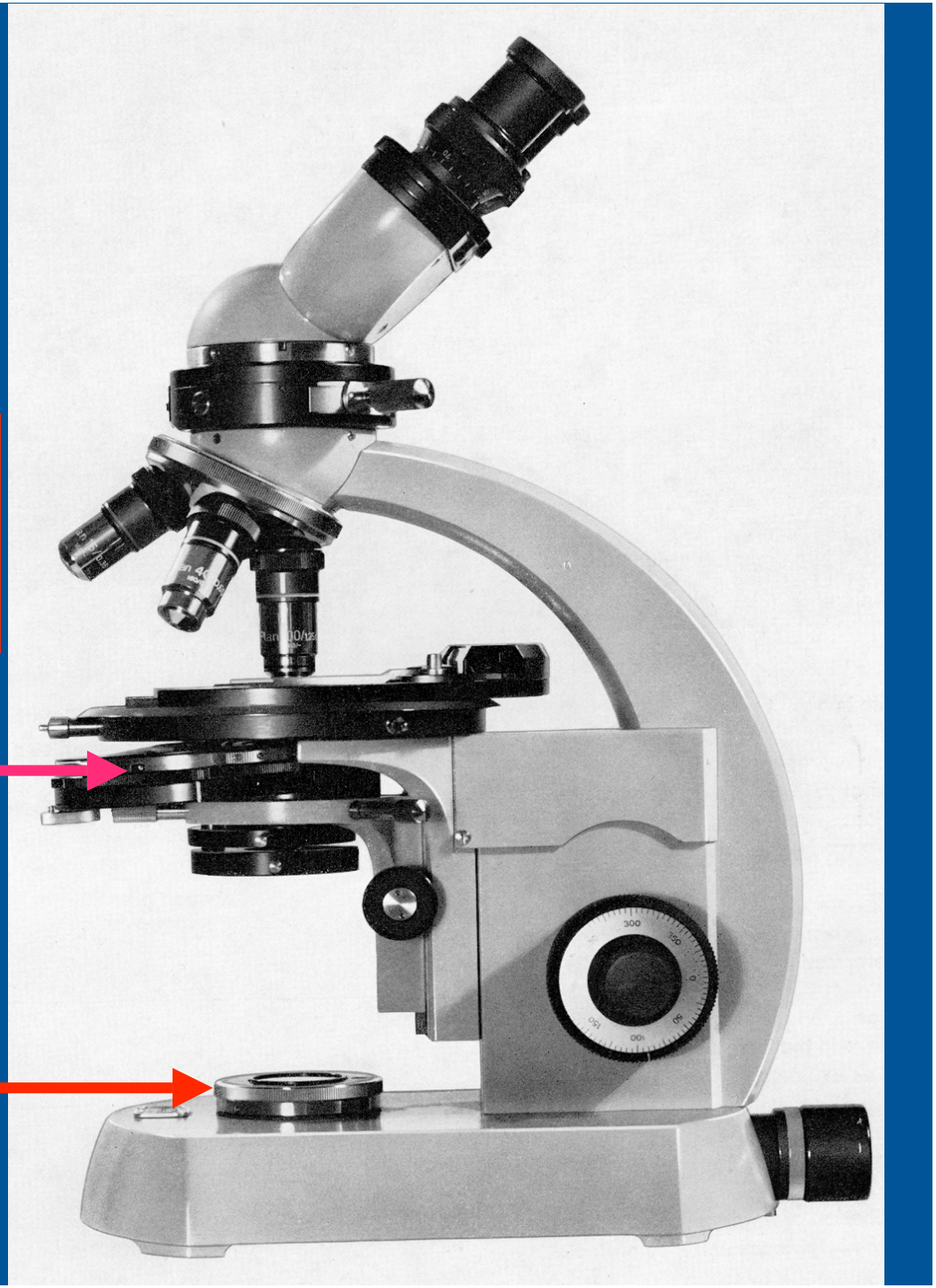
The diaphragms are NOT intended for adjusting the brightness of the image

The

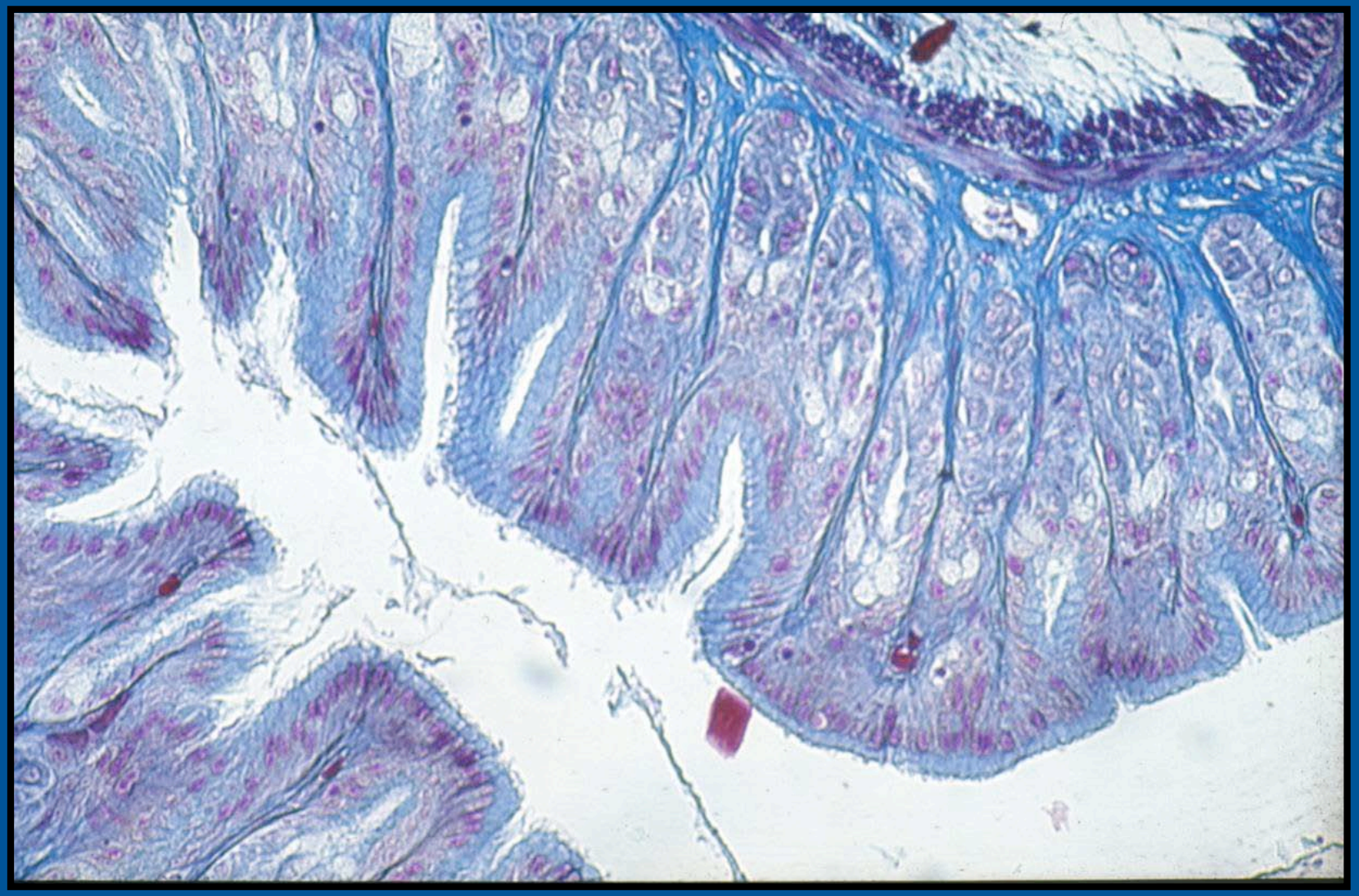
Illuminating Aperture Diaphragm

sets the ***angle*** of the cone of light illuminating the specimen, and is adjusted according to ***objective NA***

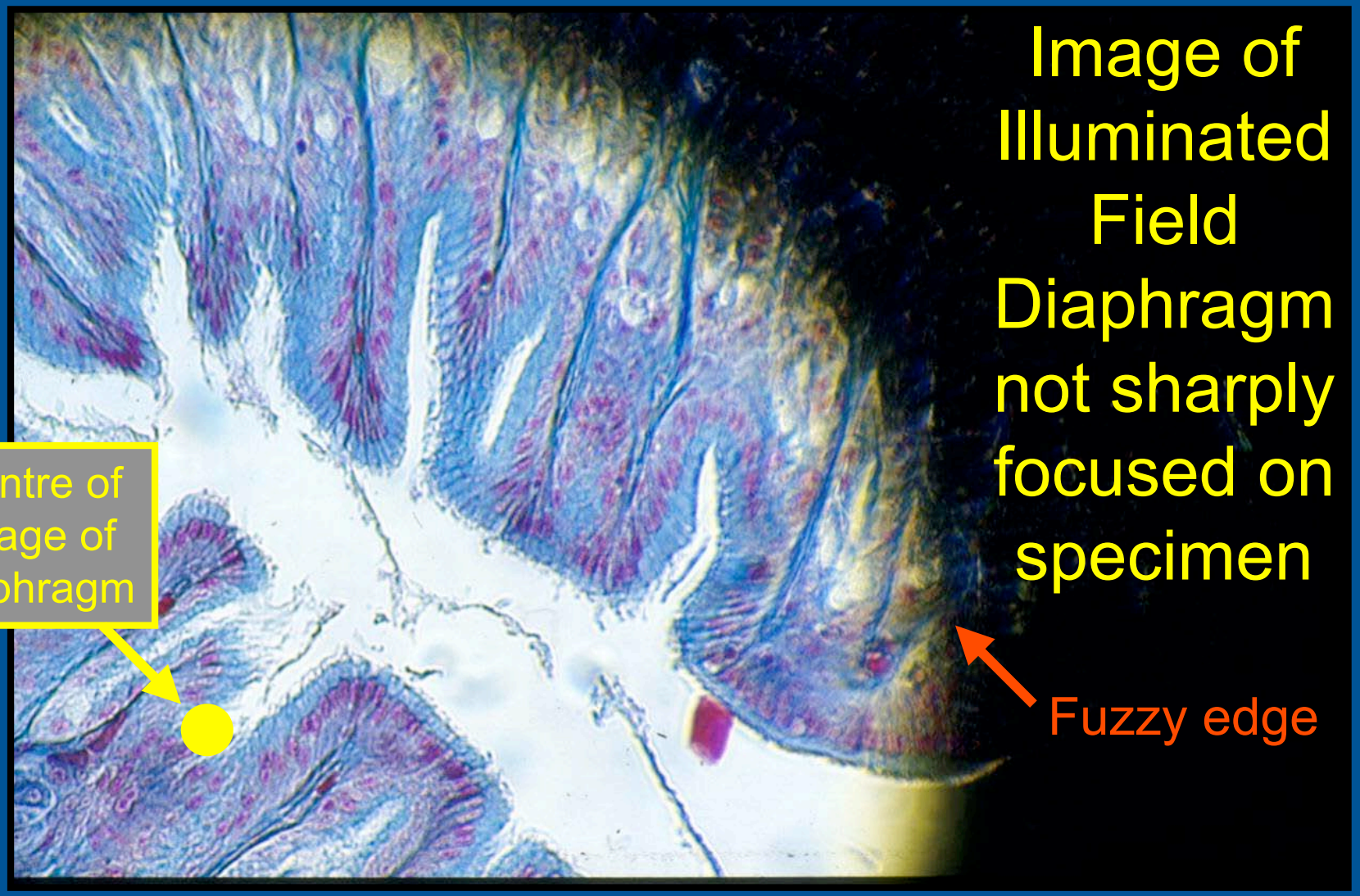
The *Illuminated Field Diaphragm* sets the ***area*** of specimen illuminated, and is adjusted according to ***magnification***



Illuminating system completely out of adjustment



Illuminated Field Diaphragm closed; its image is not centred on field of view



Condenser focus adjusted

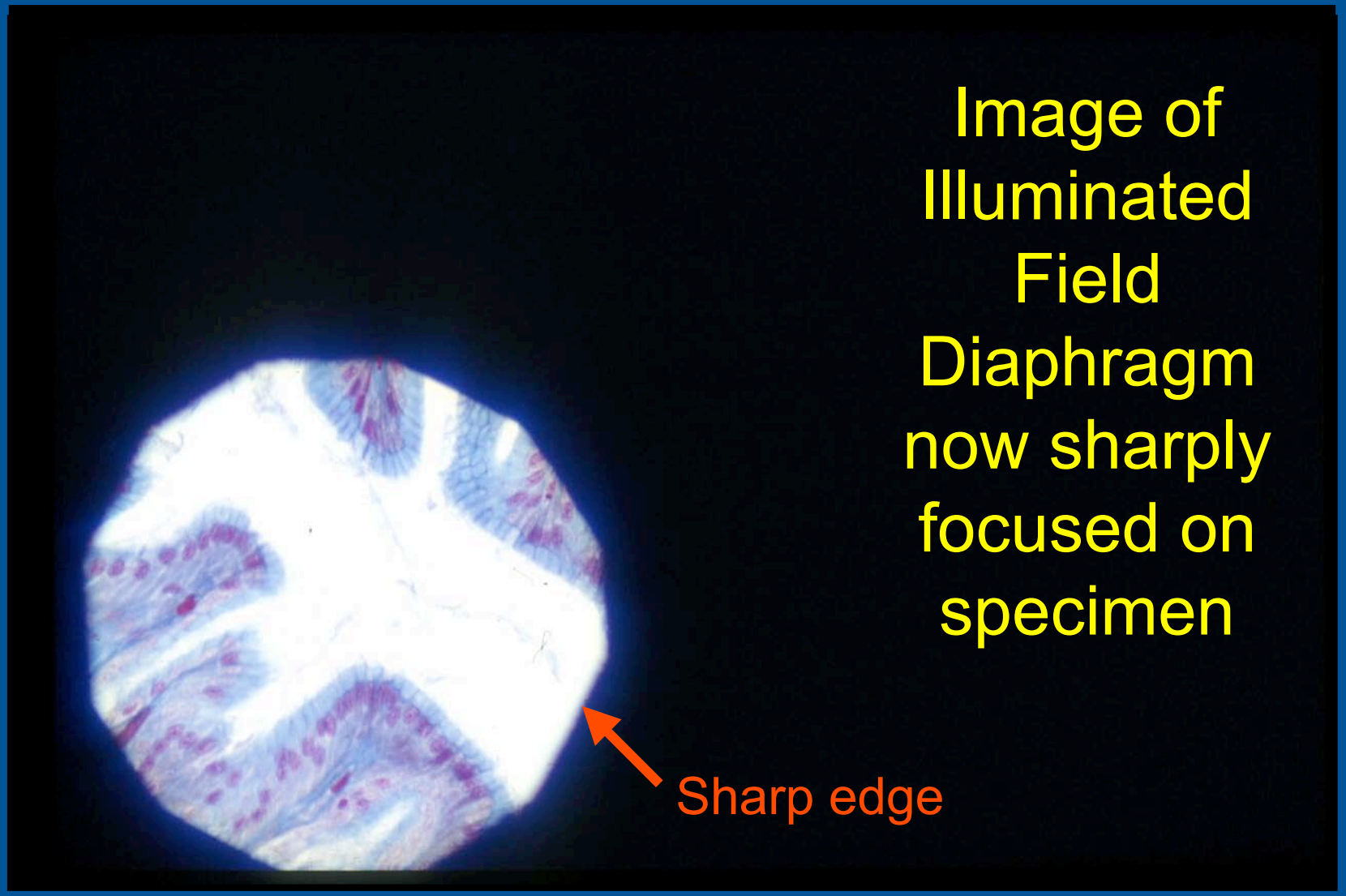


Image of Illuminated Field Diaphragm now centred on field of view

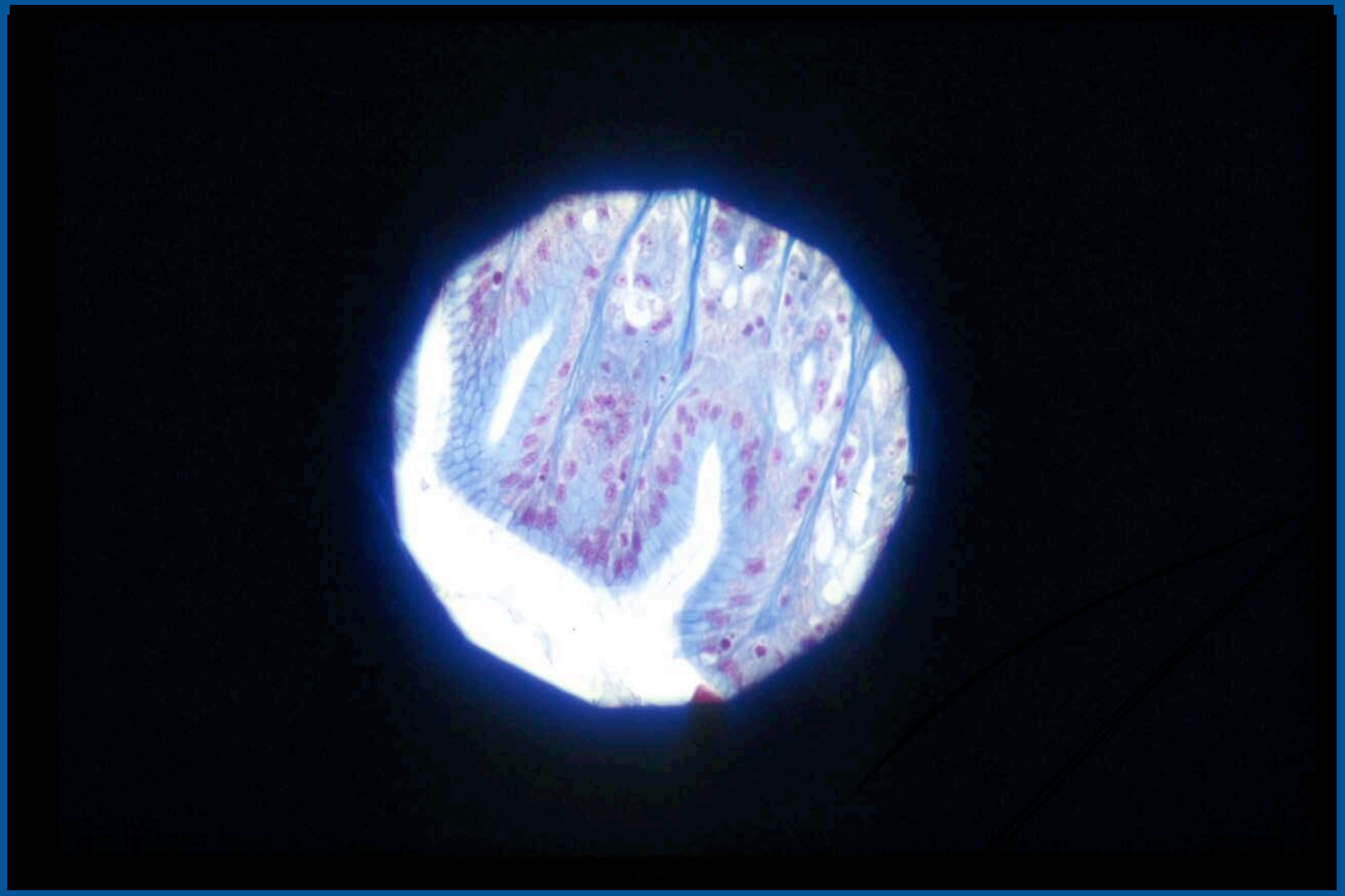


Image of Illuminated Field
Diaphragm centred on field of view

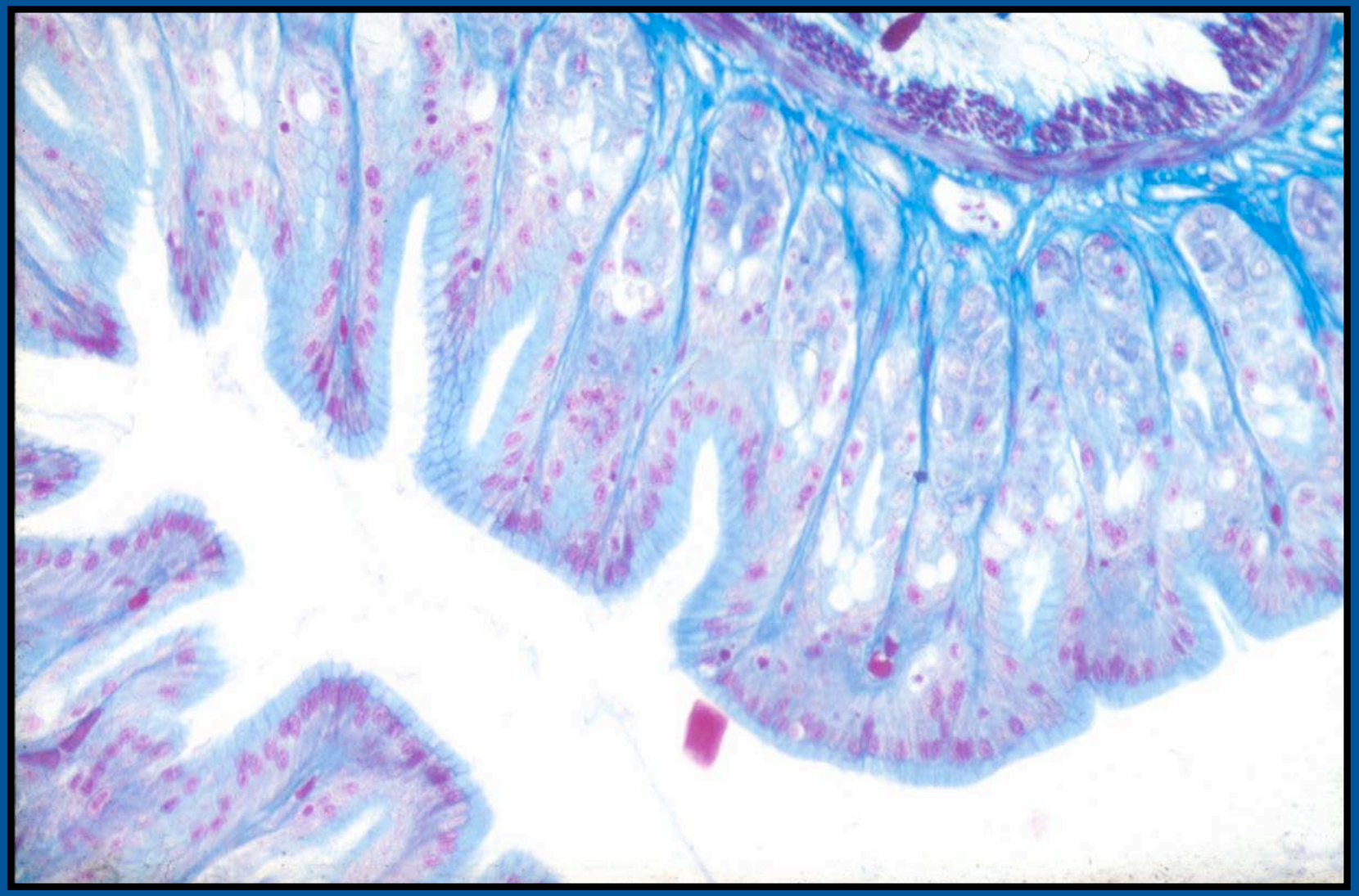
How?

- moving the condenser lens in x-y

Most common system

- 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



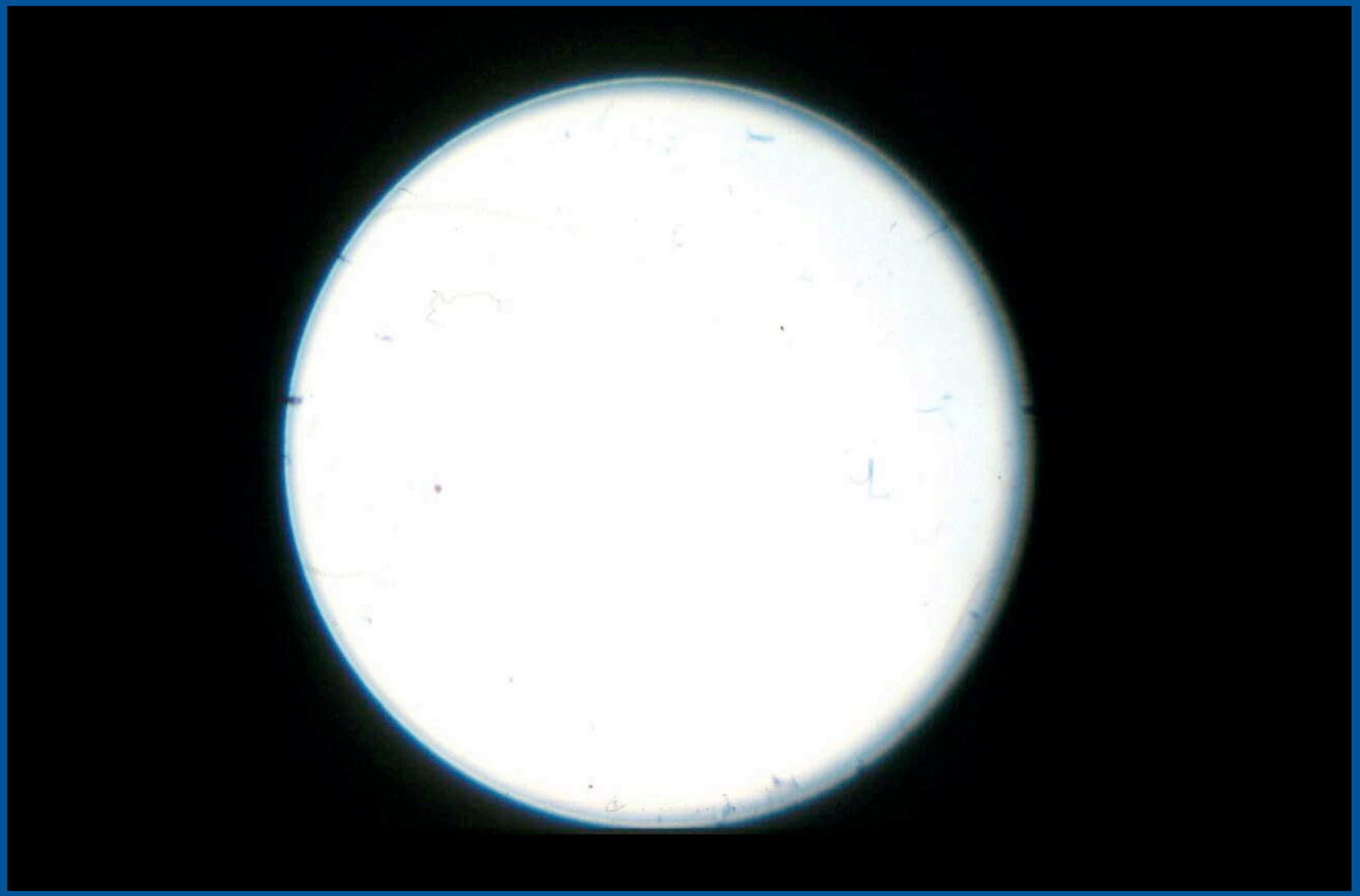
Back Focal Plane of objective

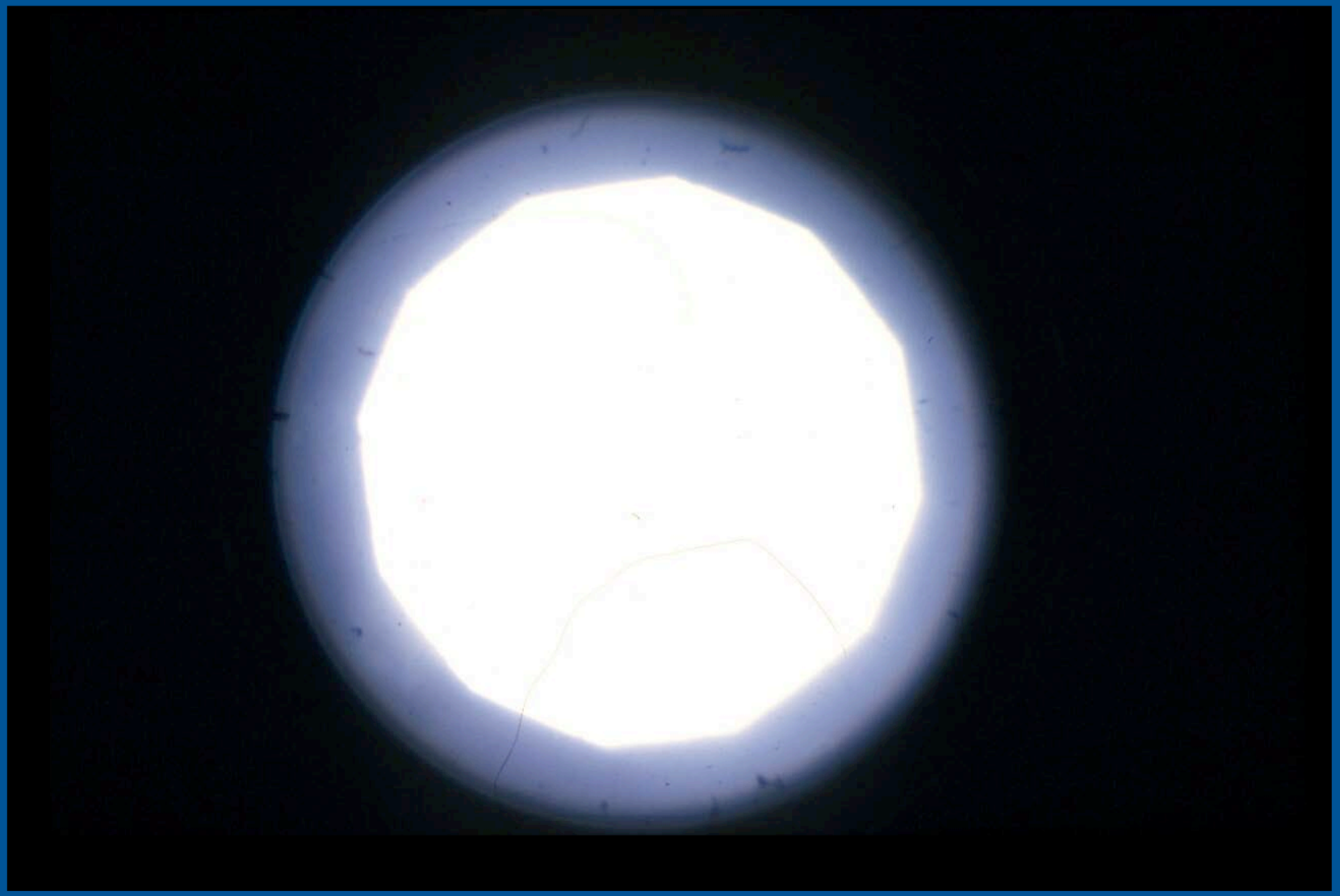
Lamp not centred with collector lens

Back Focal Plane of objective

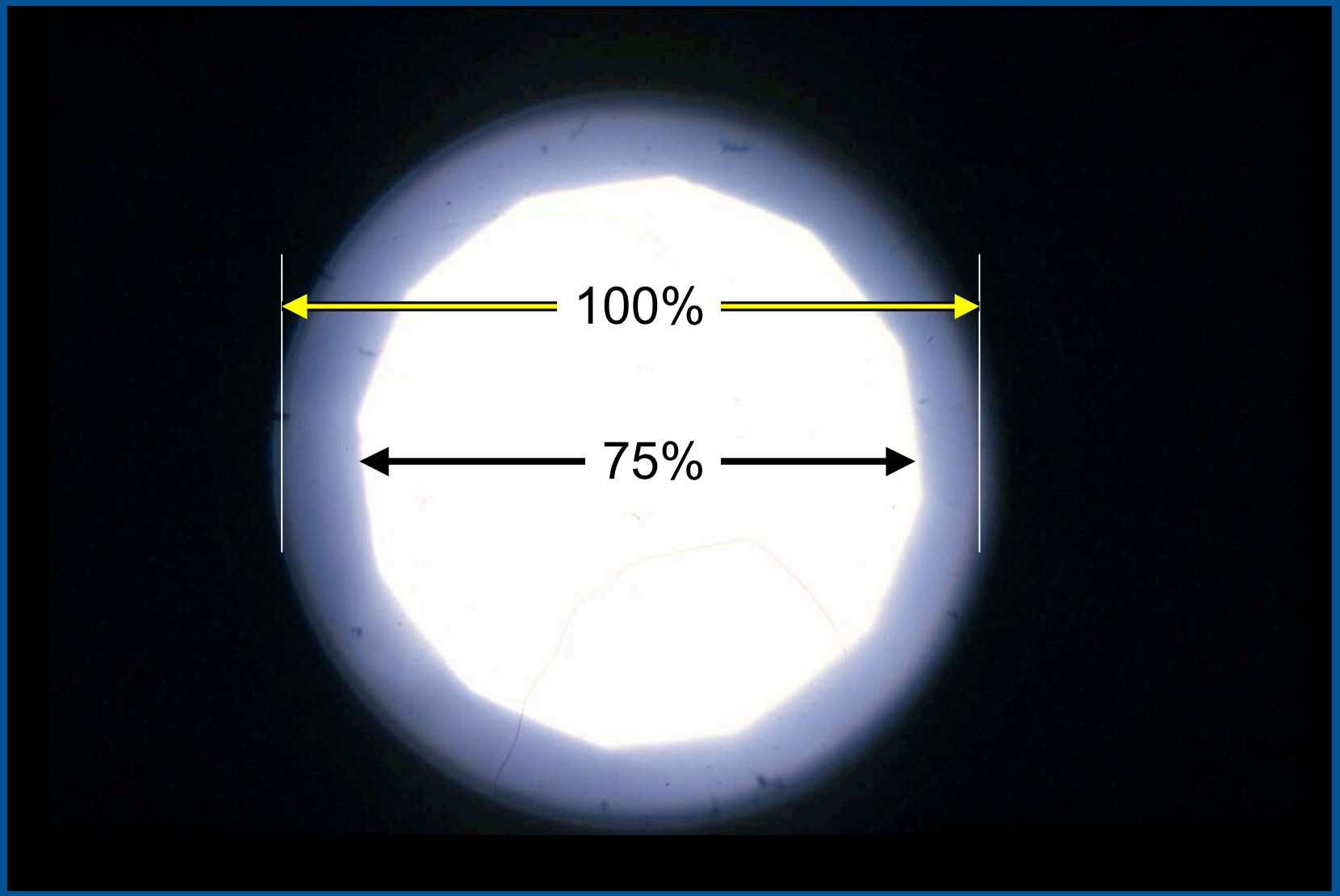
Lamp centred with collector lens

Diffuser inserted between lamp and collector lens





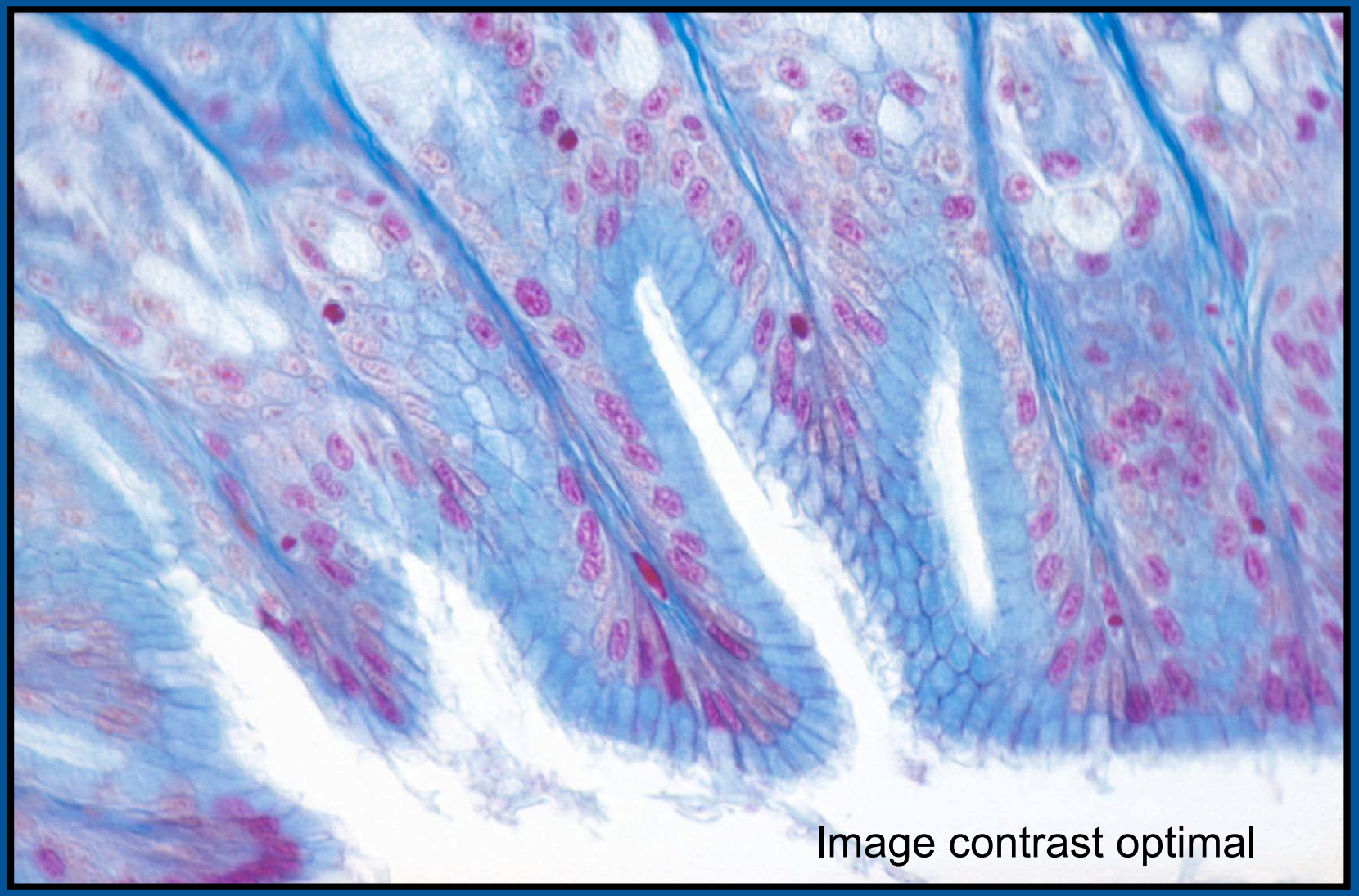
Illuminating Aperture Diaphragm closed to c75% of objective aperture



Illuminating Aperture too large

Image hazy and ‘washed out’

Illuminating Aperture correct



Illuminating Aperture too small

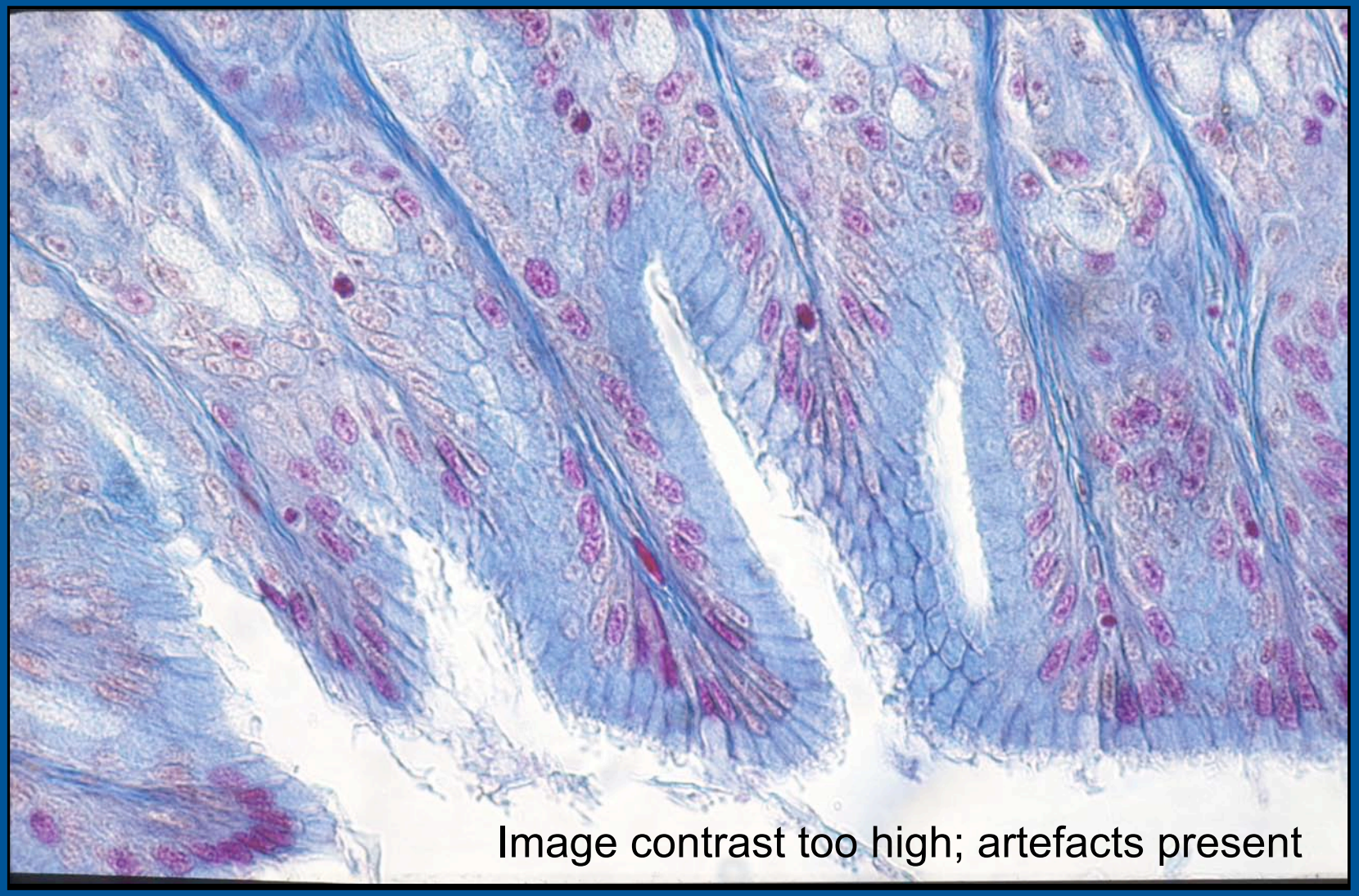
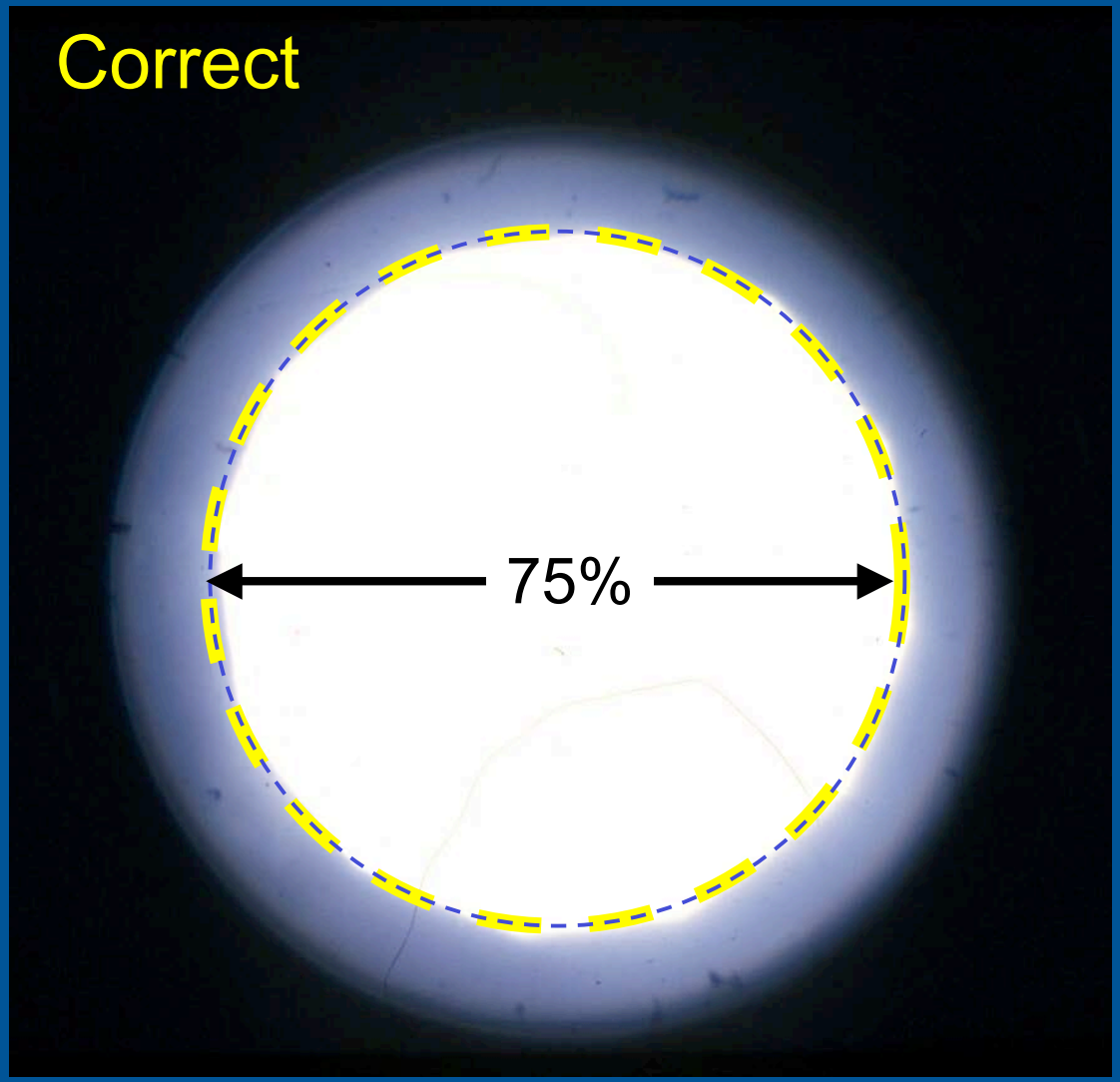
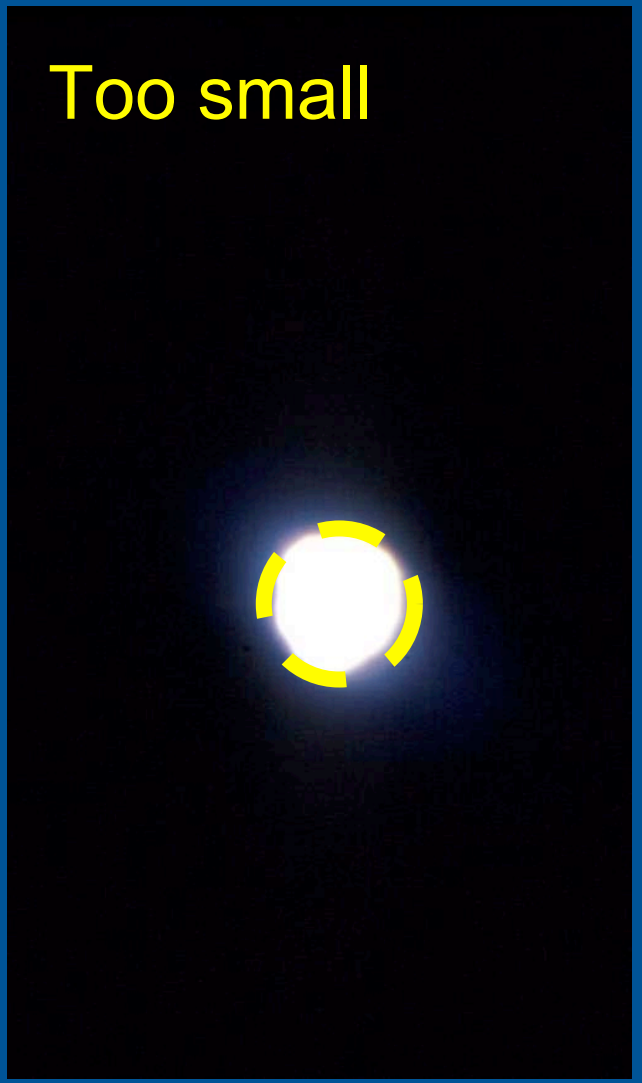


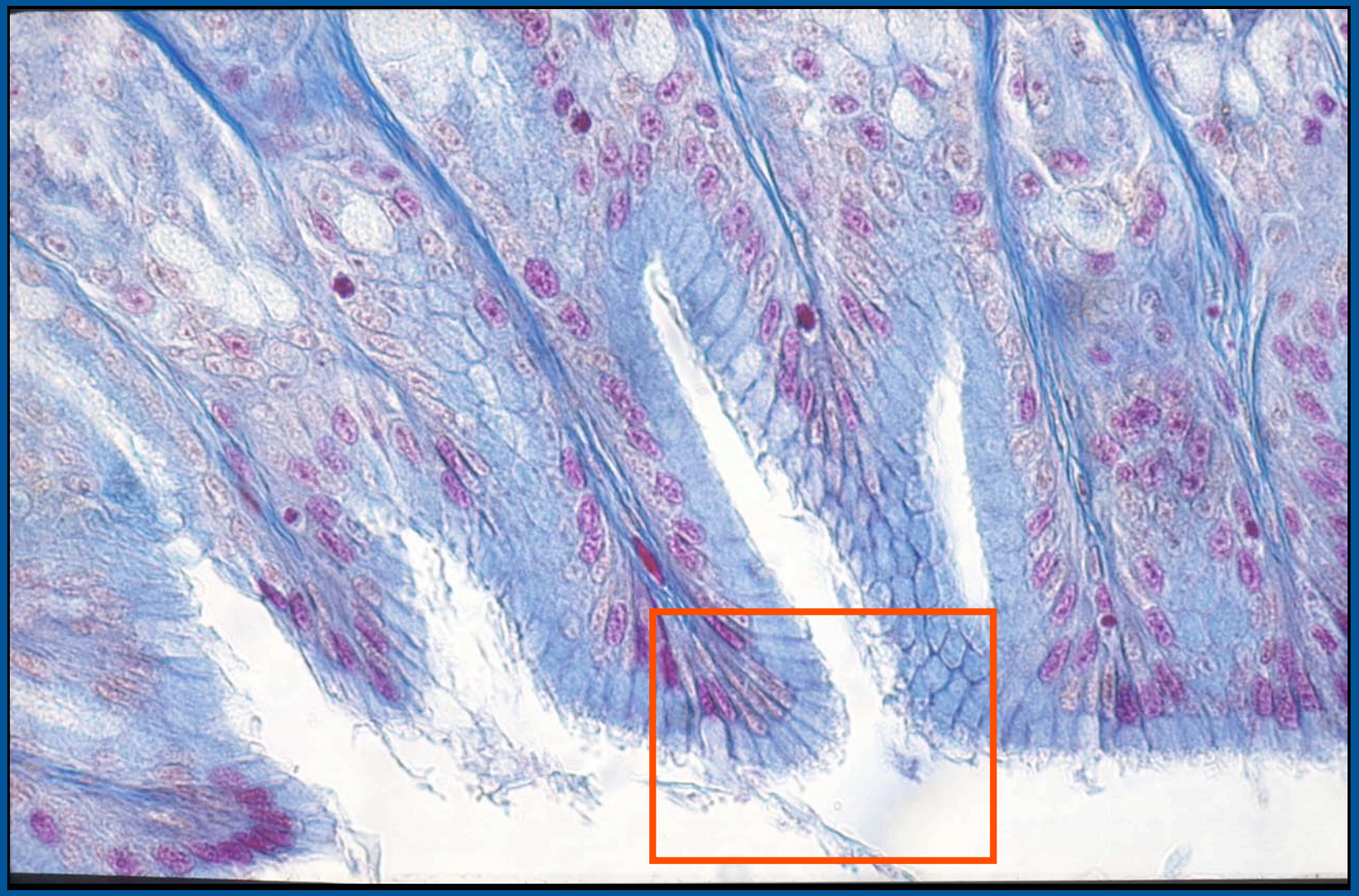
Image contrast too high; artefacts present

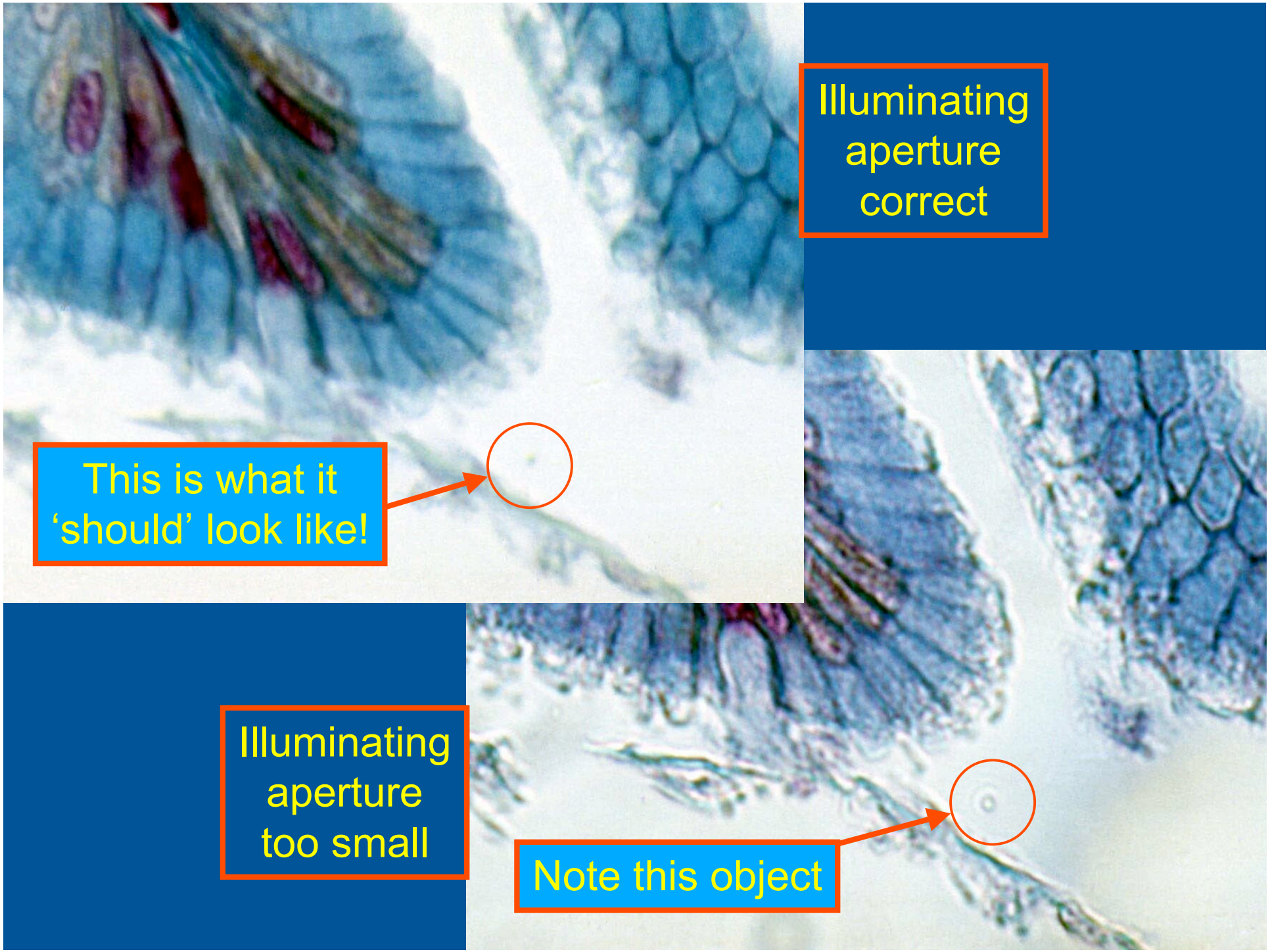
Illuminating Aperture

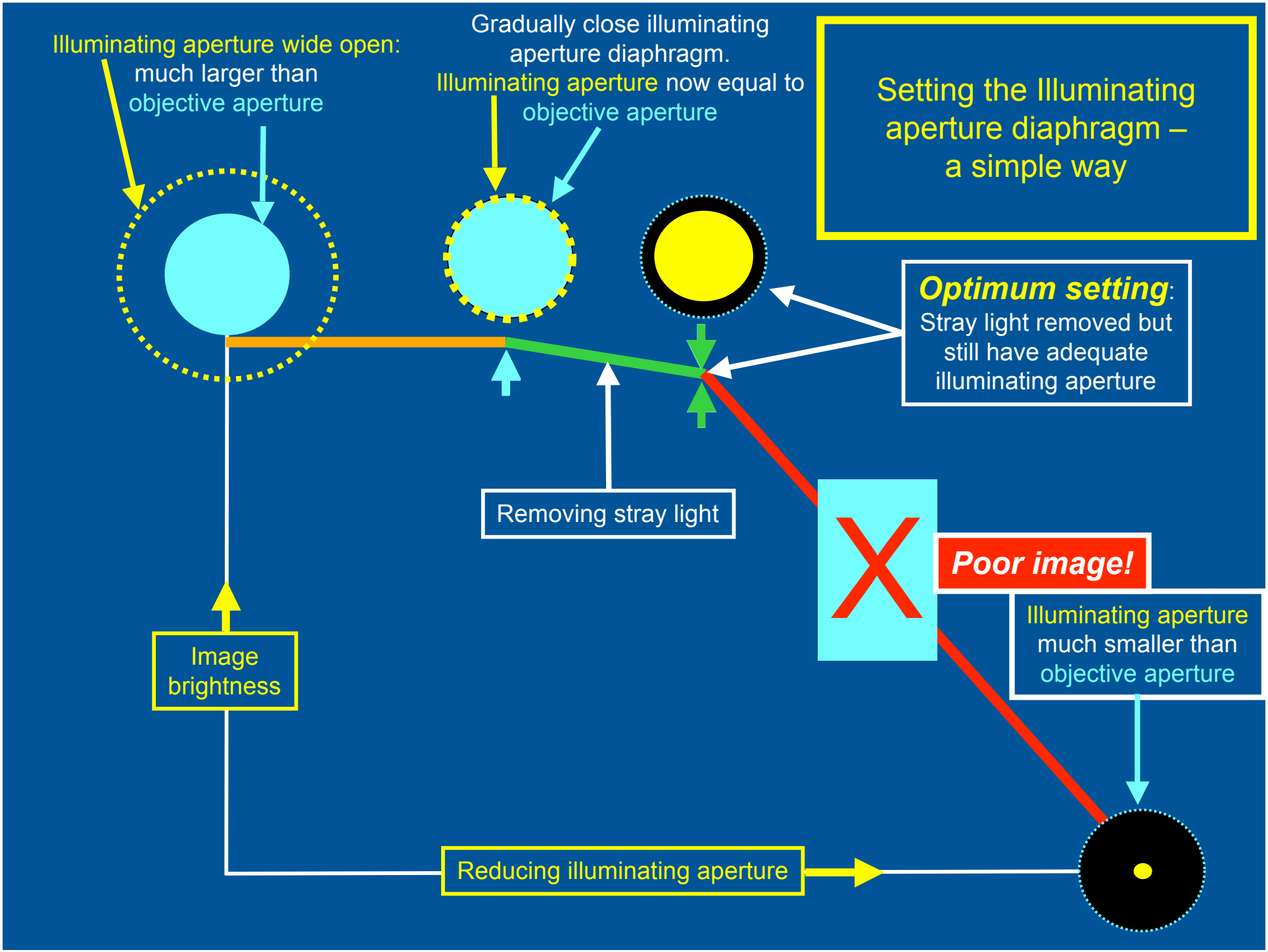


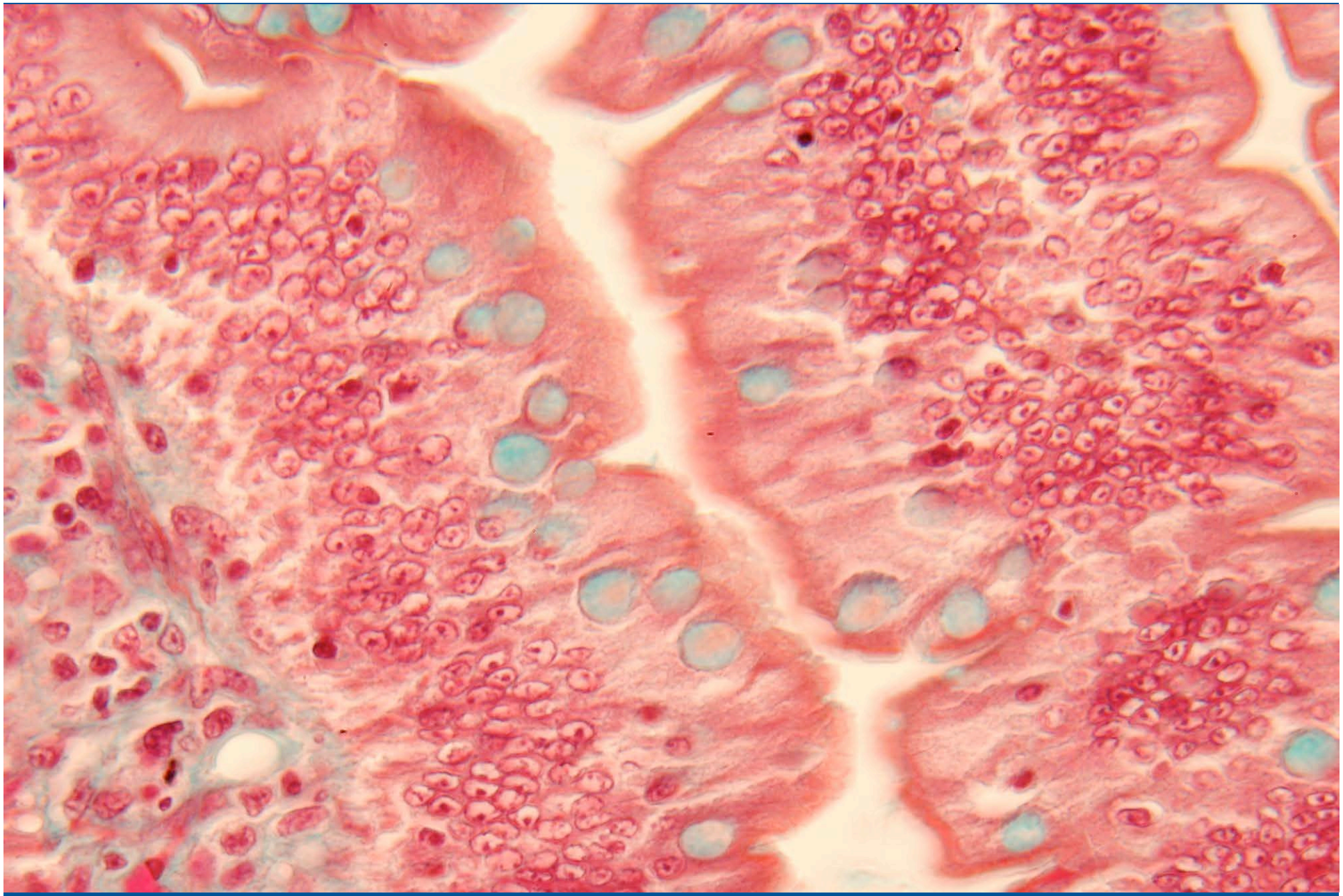


Illuminating Aperture too small

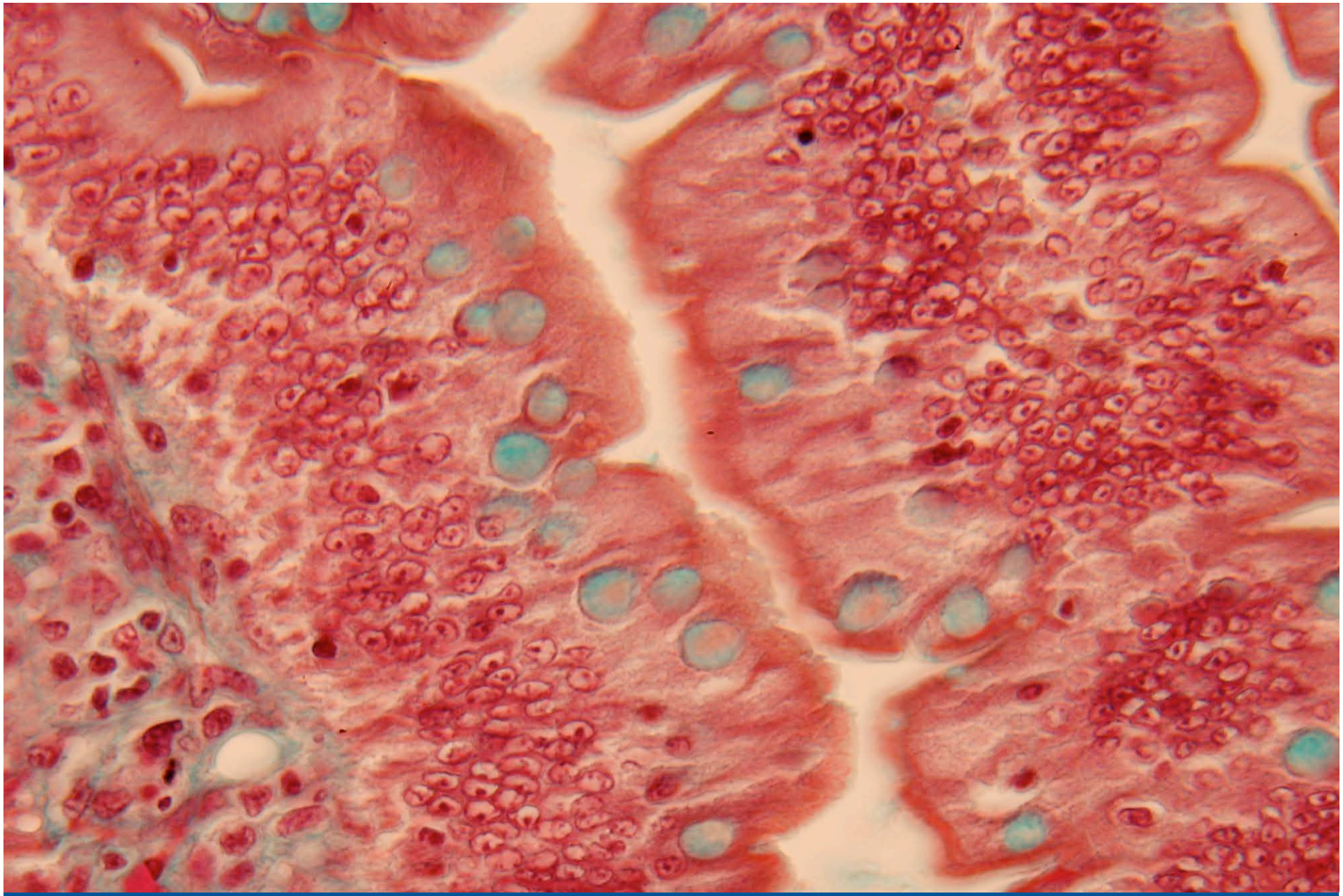



Illuminating aperture correct
This is what it ‘should’ look like!
Illuminating aperture too small
Note this object

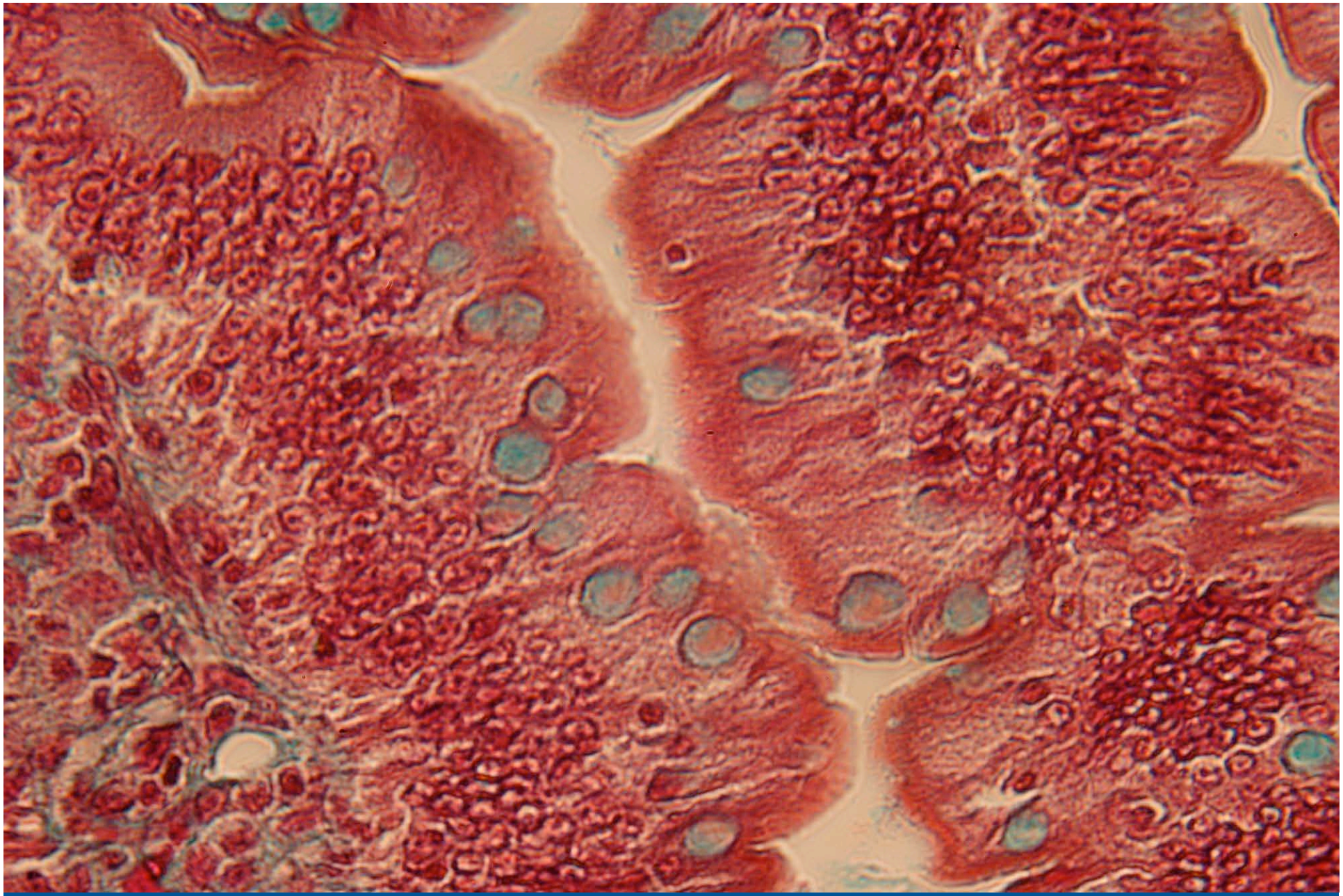

Illuminating aperture wide open:
much larger than
objective aperture
Gradually close illuminating
aperture diaphragm.
Illuminating aperture now equal to
objective aperture
Setting the Illuminating
aperture diaphragm –
a simple way
Optimum setting:
Stray light removed but
still have adequate
illuminating aperture
Removing stray light
Poor image!
Illuminating aperture
much smaller than
objective aperture
Image
brightness
Reducing illuminating aperture



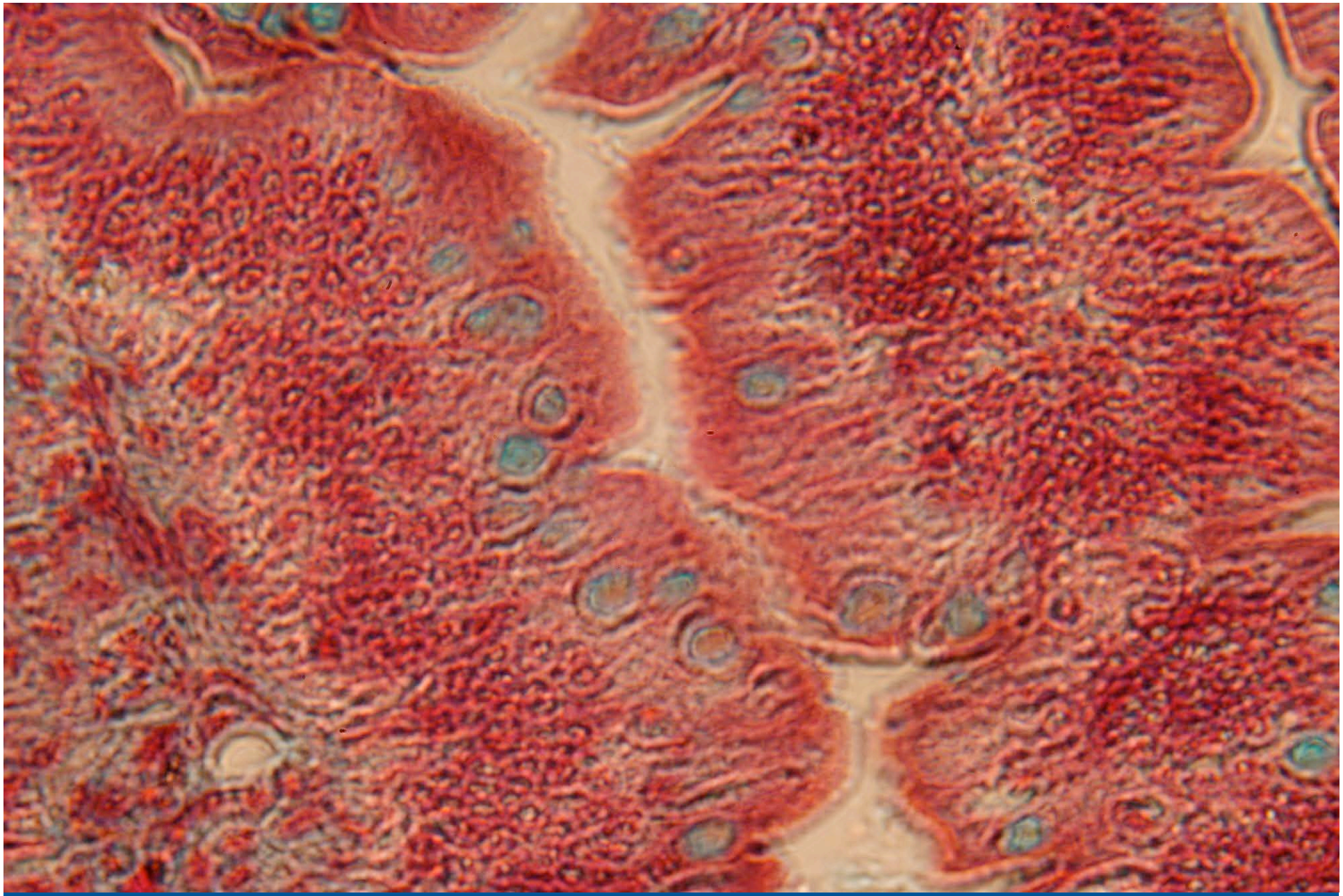
Illuminating aperture too large



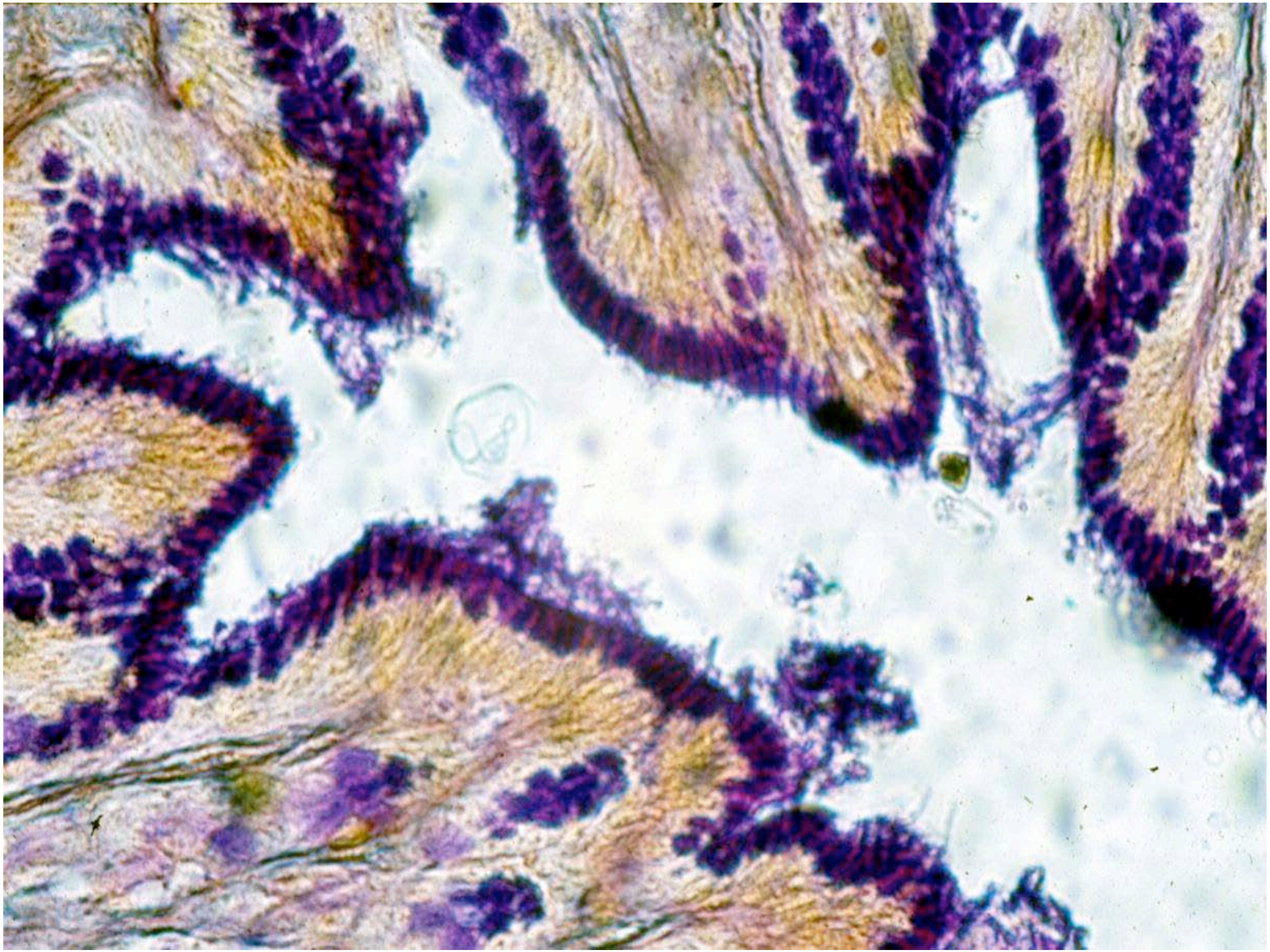
Illuminating aperture correct

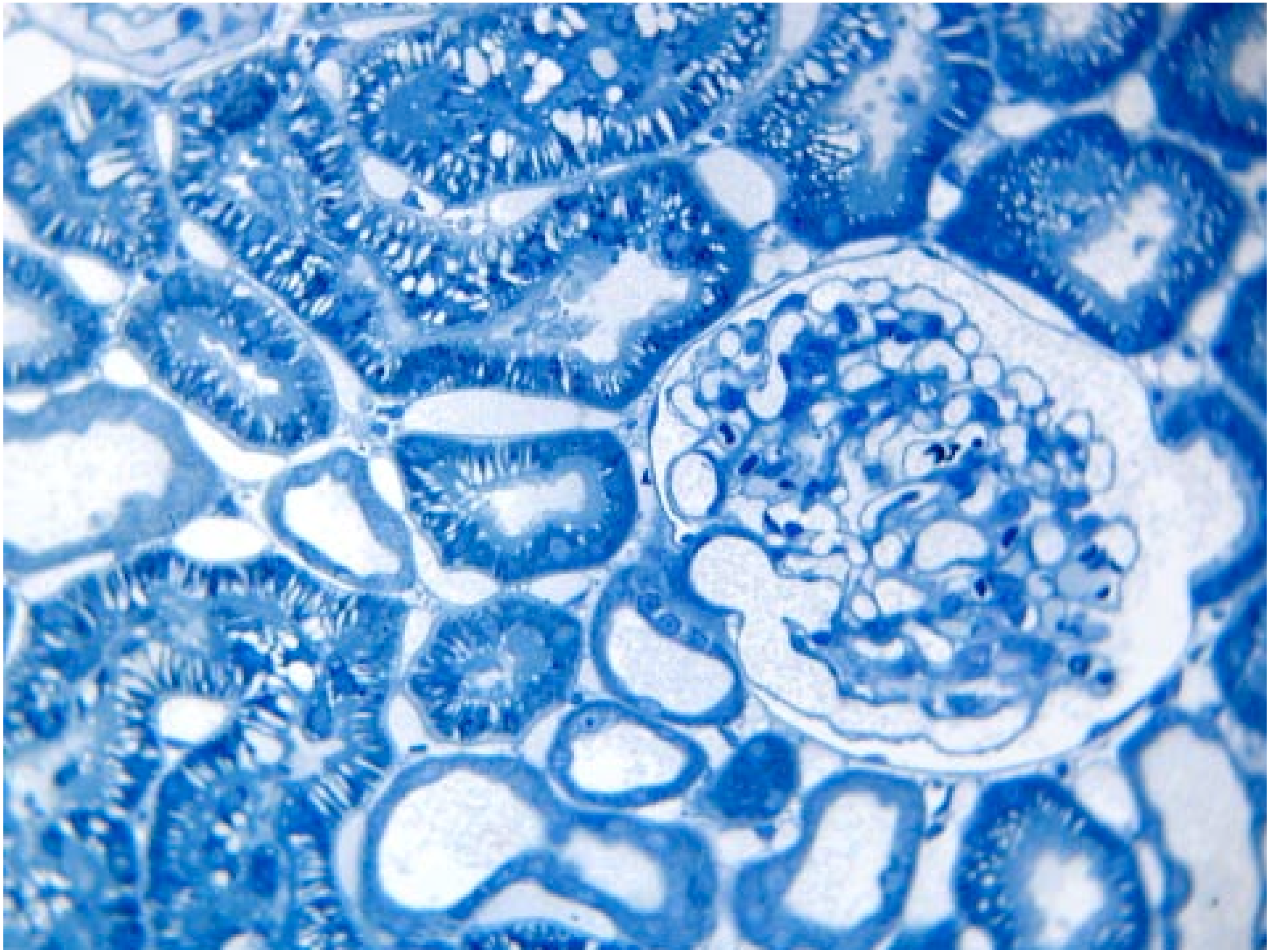


Illuminating aperture too small



Illuminating aperture much too small – an extreme example

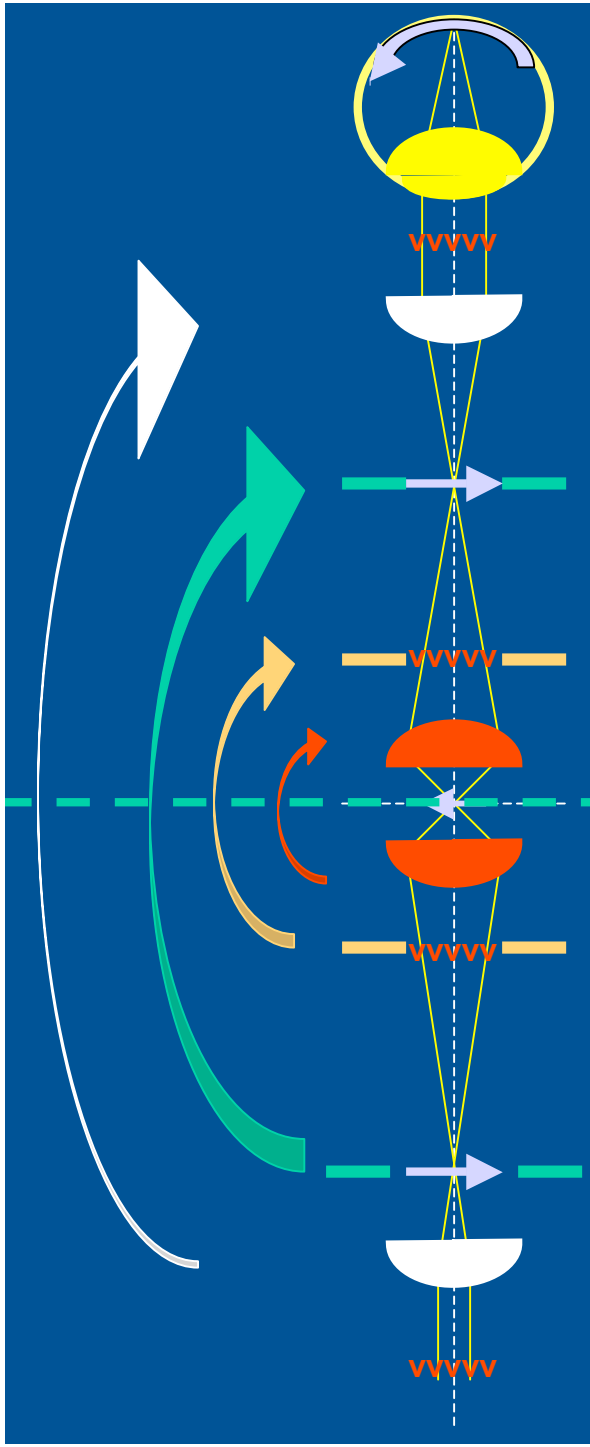




Köhler Illumination provides

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 ...

Eyepiece lens

Lamp collector falls on Eyepiece lens

Primary image

Illuminated Field Diaphragm falls in Primary Image plane

Illuminating Aperture Diaphragm falls in Back Focal Plane of Objective

Objective

Condenser

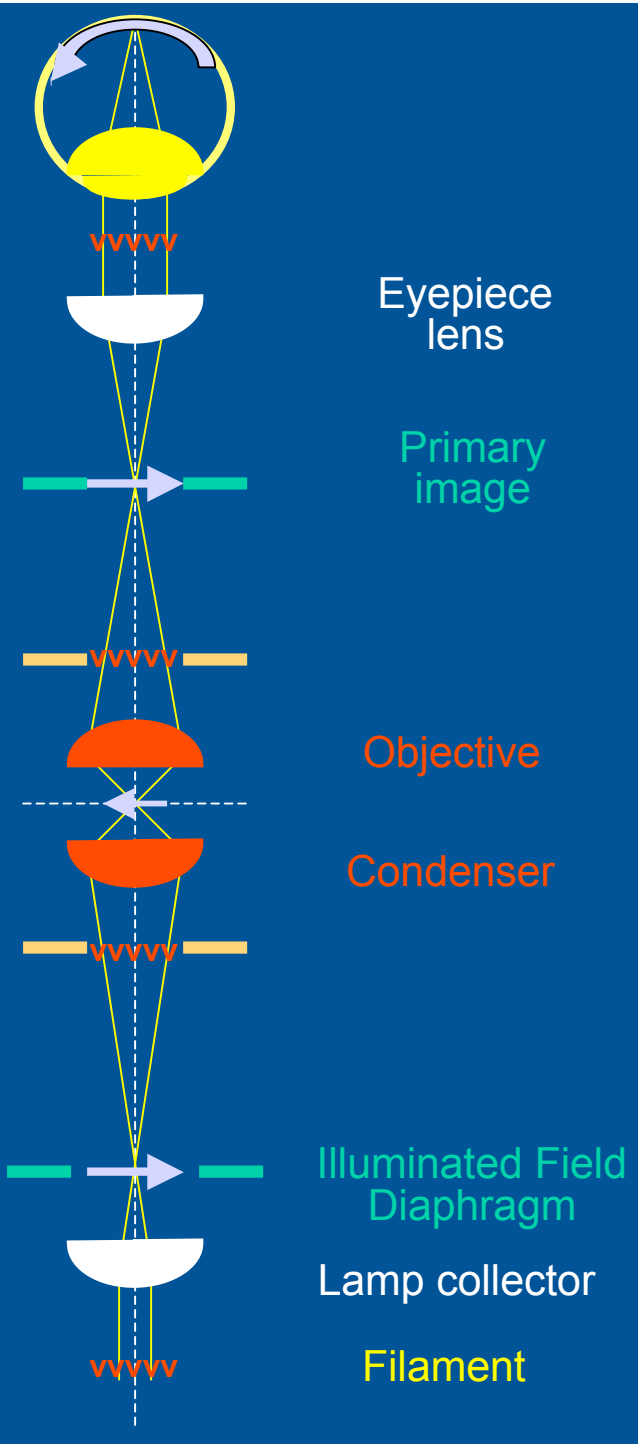
Condenser falls on Objective

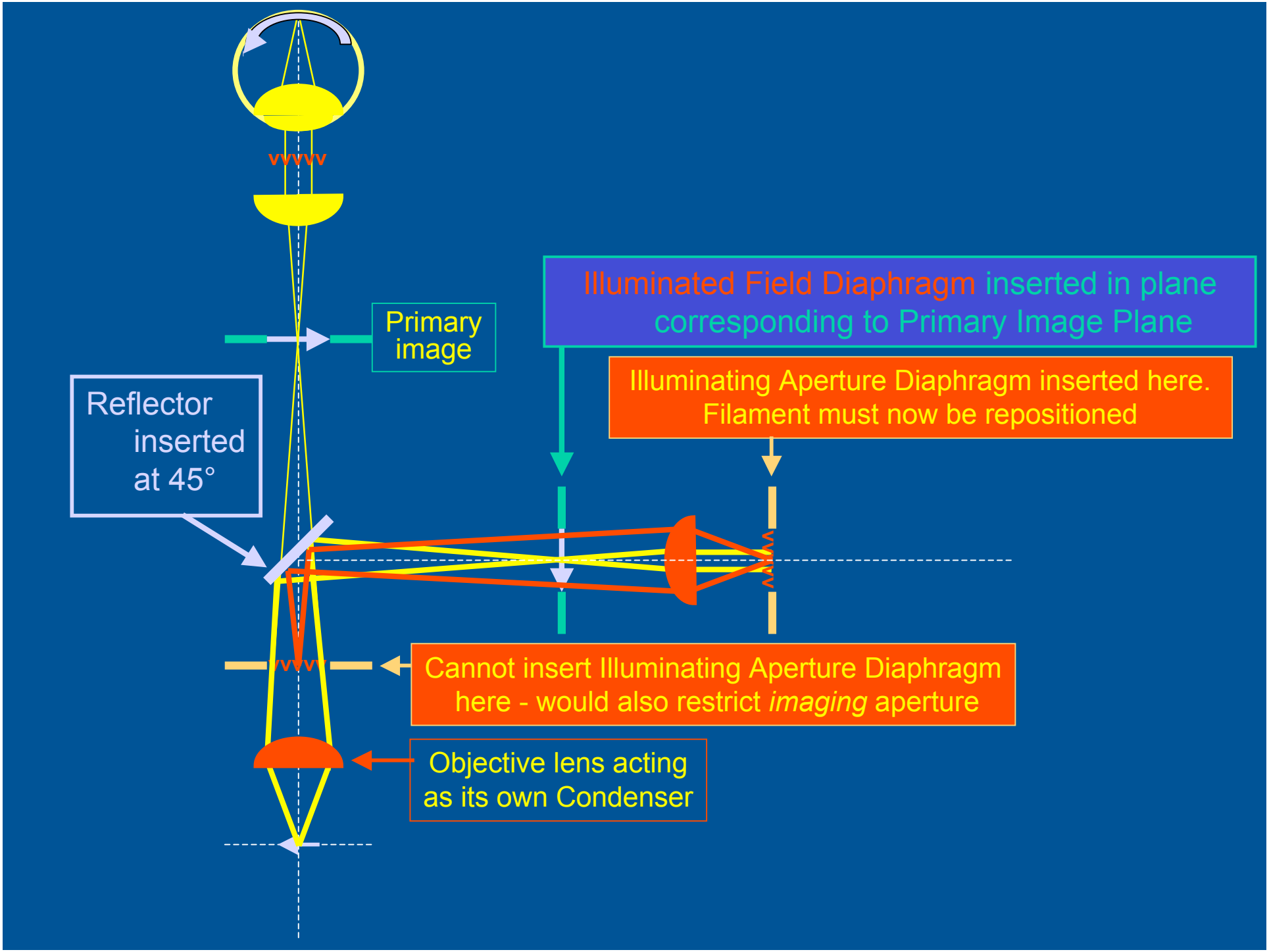
Epi-illumination

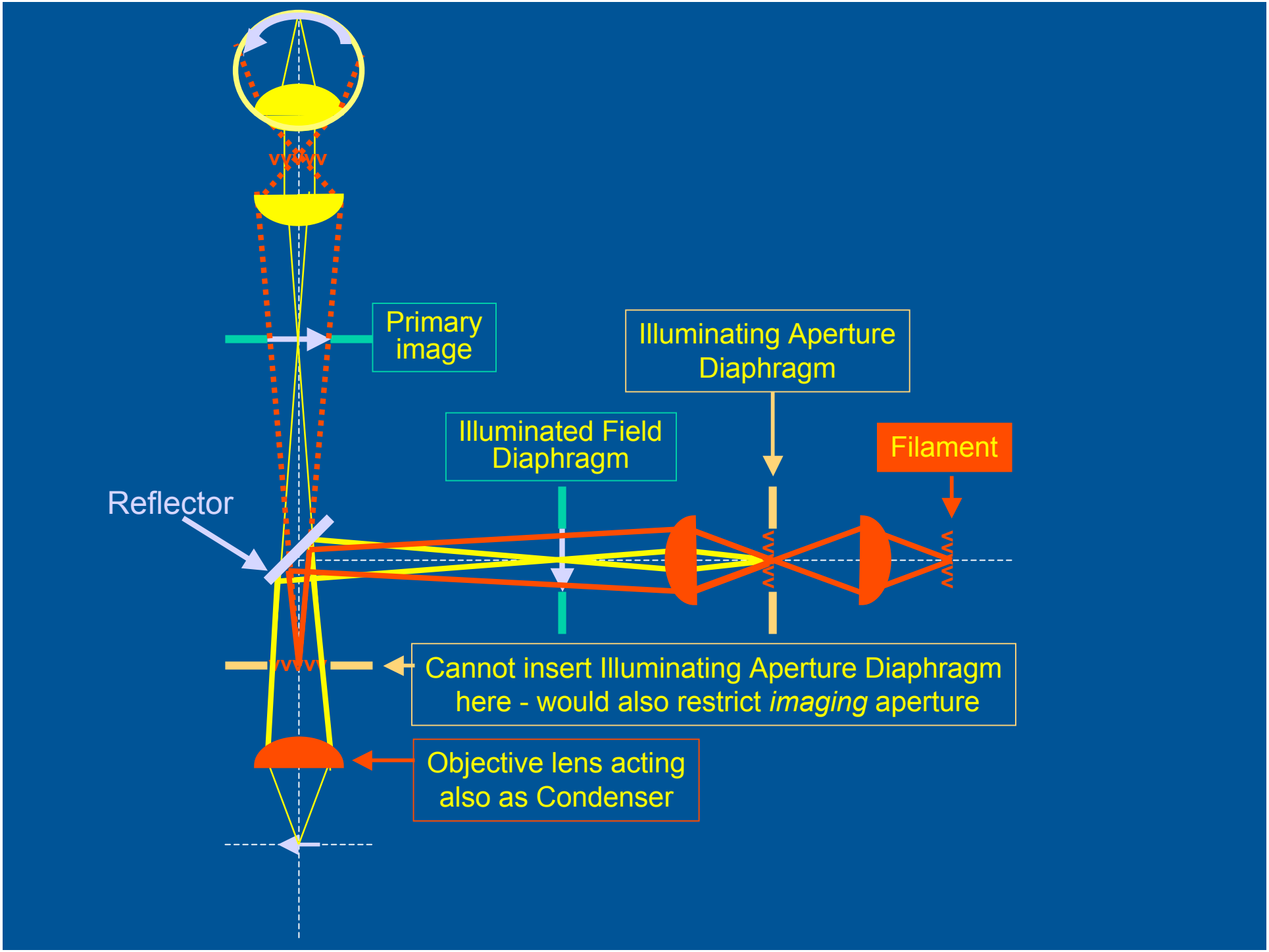
Illuminated Field Diaphragm

Lamp collector

Filament


Eyepiece lens
Primary image
Objective
Condenser
Illuminated Field Diaphragm
Lamp collector
Filament


Primary image
Illuminated Field Diaphragm inserted in plane corresponding to Primary Image Plane
Reflector inserted at 45°
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


Primary image
Illuminating Aperture Diaphragm
Illuminated Field Diaphragm
Filament
Reflector
Cannot insert Illuminating Aperture Diaphragm here - would also restrict *imaging* aperture
Objective lens acting also as Condenser