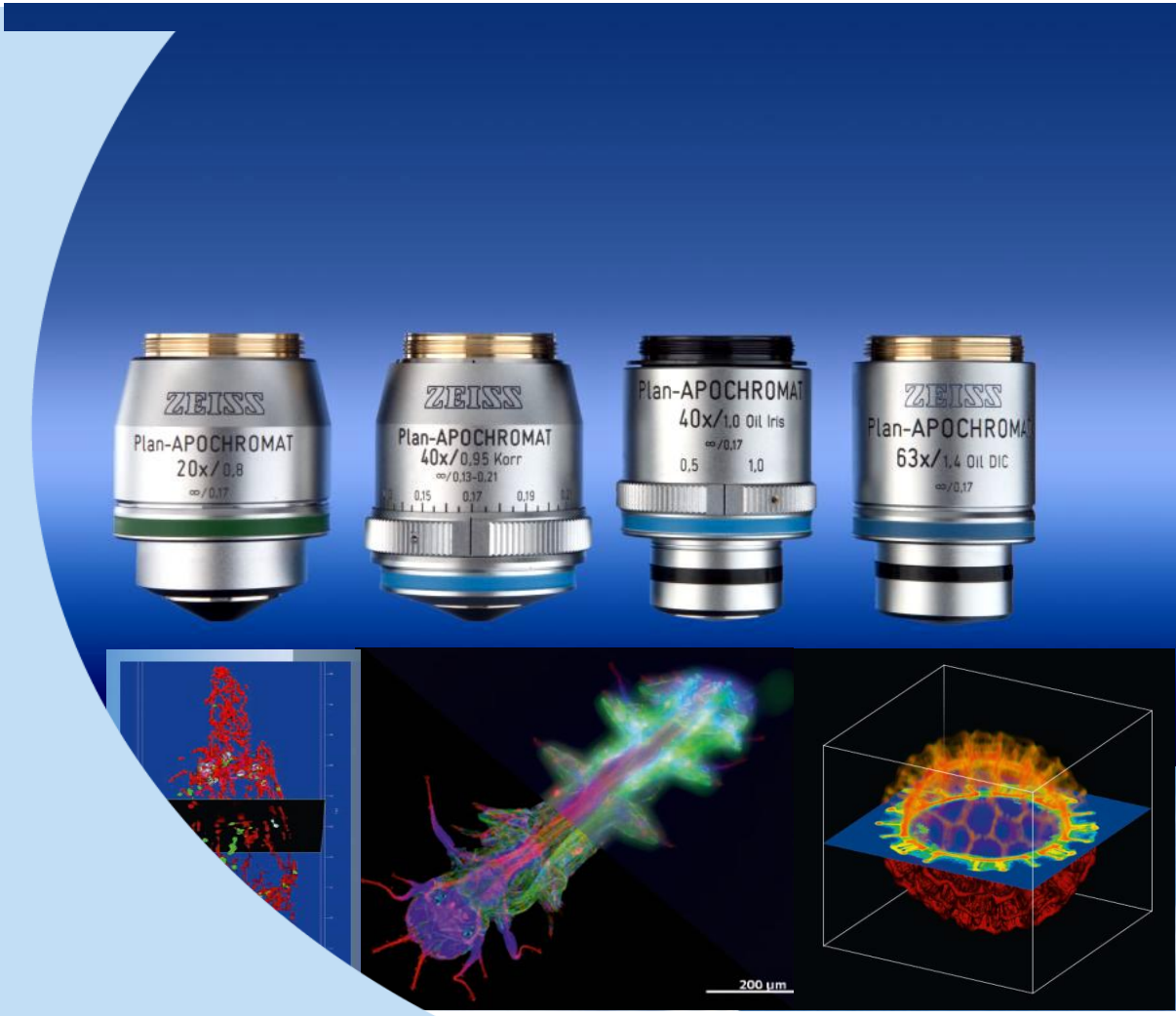
McGill University Life Sciences Complex
Imaging Facility

Systems Biology
Microscopy Workshop

The Microscope Objective

Carl Zeiss Canada

8 December 2010

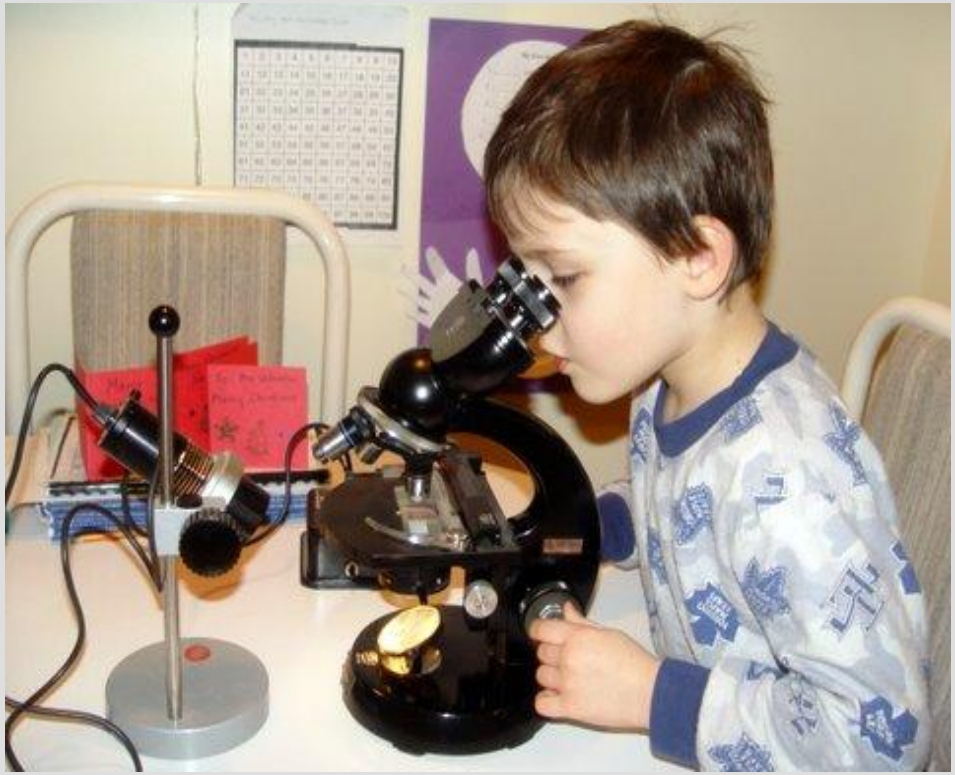


Fundamentals in Microscopy

Why study the objective?

Single most critical component of the microscope.

Gain some practical insight into the optimization of an optical microscope.

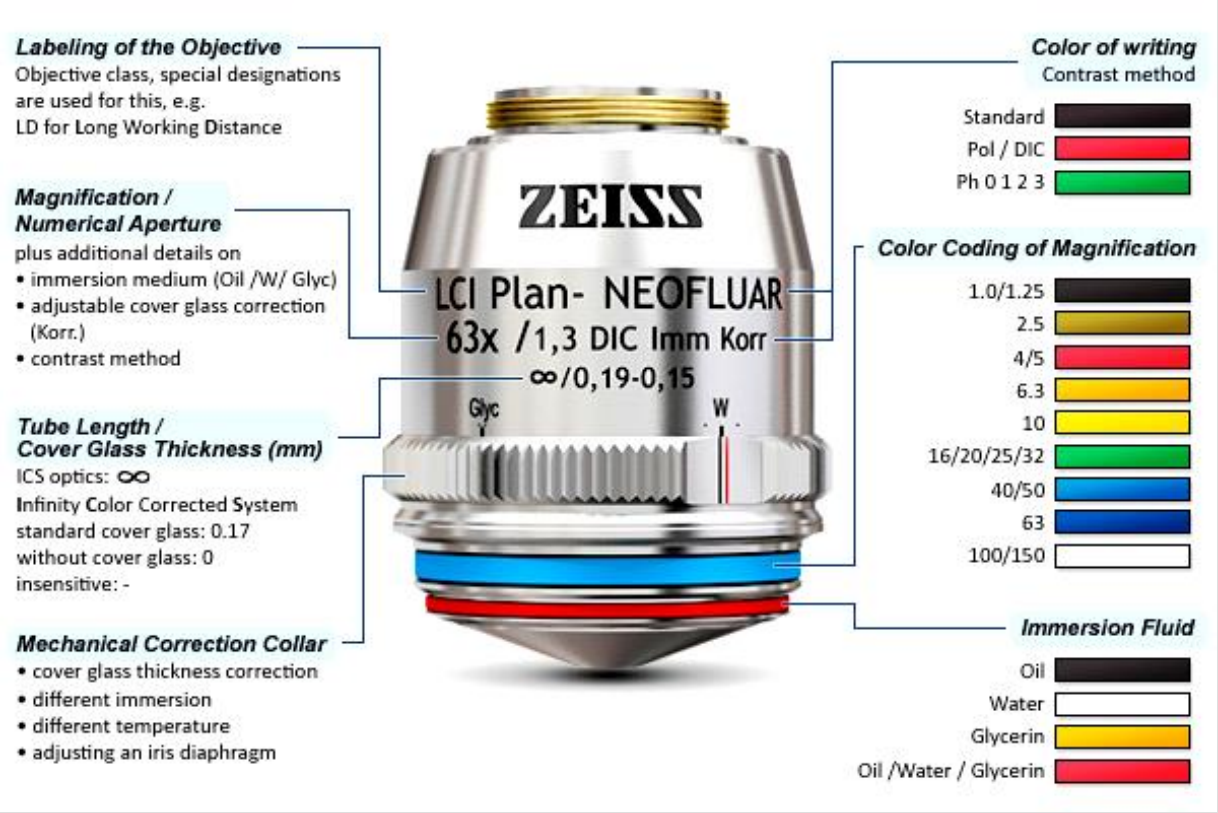


Resident Microscopist

The Objective Road Map

Today, for ease of use, all microscope objectives follow a colour code that allows immediate recognition of important objective parameters
(e.g. Magnification, imm fluid

The standard colour code of objectives was introduced to microscopy in 1953 by Dr. Kurt Michel at CARL ZEISS



The Objective Lens

The Perfect Microscopical Image

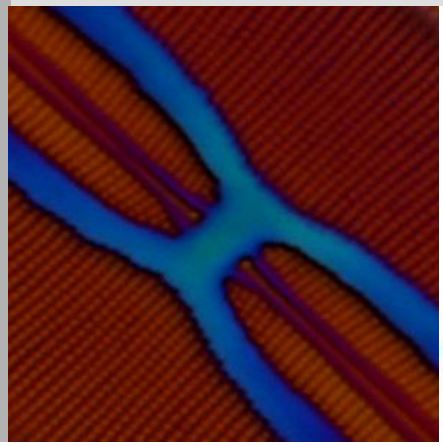
The Microscope Objective is Responsible for the Formation of a Perfect Image

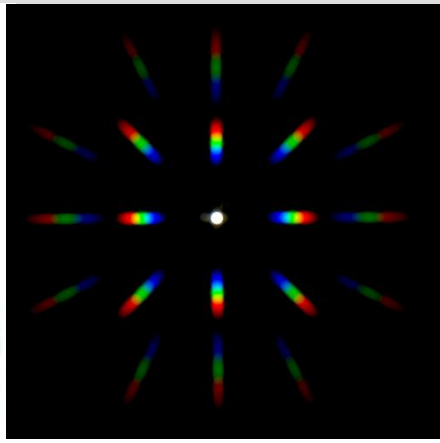
The perfect microscopical image

has a magnification that matches with a given structure size

is of maximum possible detail rendition in x,y and z

has the highest possible contrast





The Objective Properties

The Microscope Objective is the most important Optical Component for Imaging

The microscope objective properties influence:

The resolution of the imaged structure (n. A.)

The diffraction behavior of the captured sample light (Strehl value)

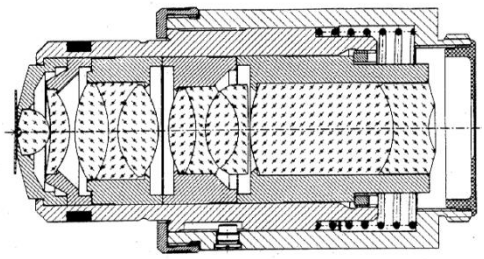
Amount of residual spherical aberration

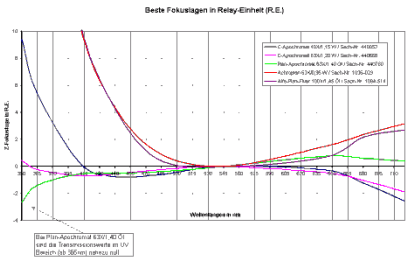
The presence / absence of colour fringes (chromatic aberrations)

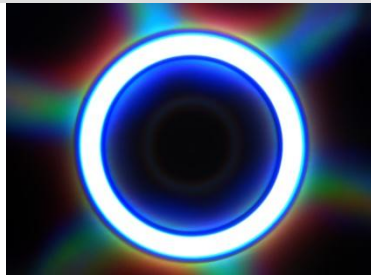
The transmission for different light colours (glass transmission range)

The flatness of field (plan correction)

The minimum possible distance between structure and objective (FWD)





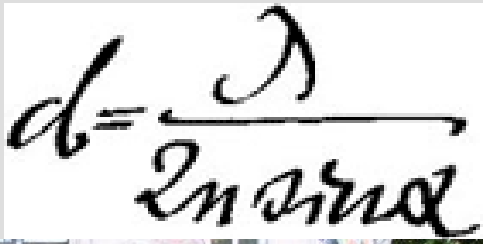


Objective correction: Resolution and Numerical Aperture (n. A.)

The resolution of a microscopical image depends on the actual numerical aperture (n. A.) of the given objective and the wavelength of light used

$$d_0 = \frac{\lambda}{2 \text{ n. A.}}$$

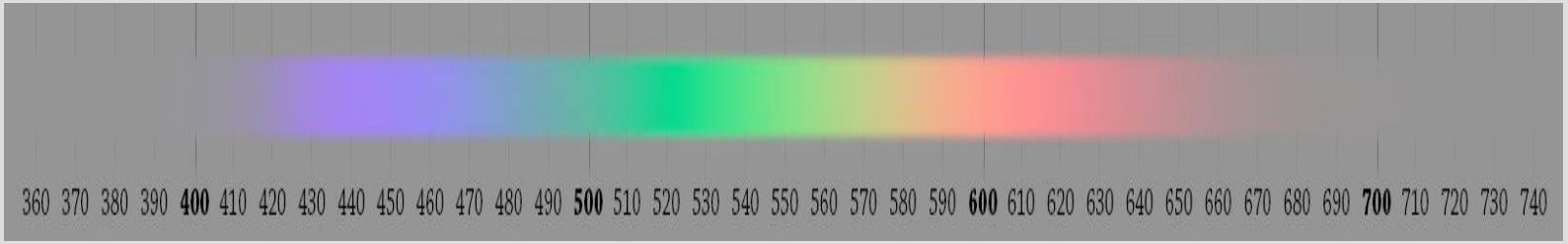
The resolution formula of the microscope was developed in 1872 by Prof. Ernst Abbe at CARL ZEISS



Resolution

One Formula

$$d_{\min} = \frac{\lambda}{NA_{Objective} + NA_{Condenser}}$$



Objective correction: Numerical Aperture (n. A.) Increase by Immersion

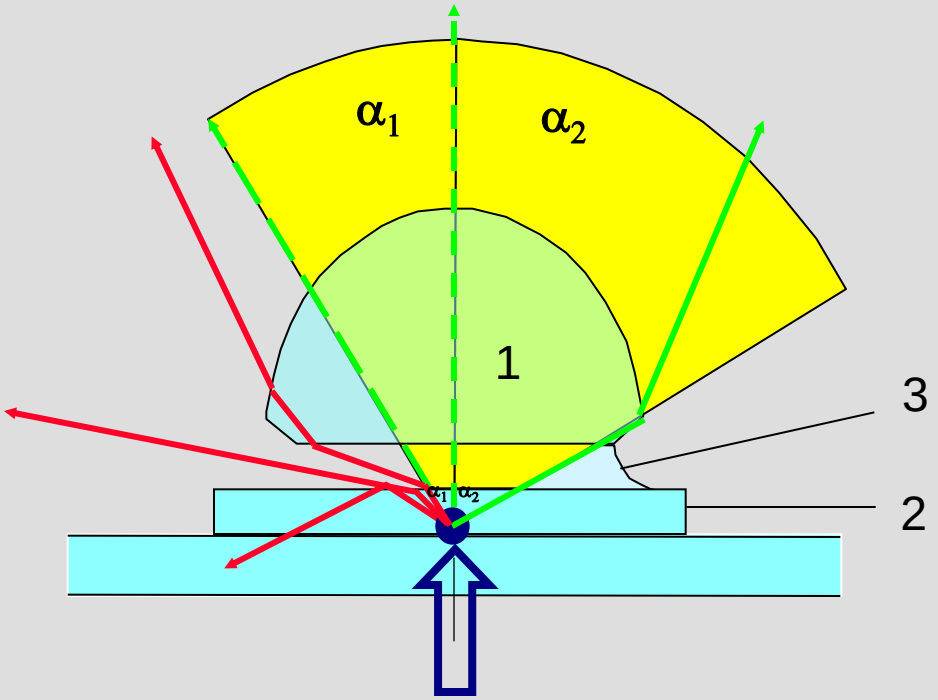
The higher n. A. the higher is the resolving power of the objective

- Theoretical max.
  n. A. in air = 1:
  a = 90°, sin a = 1,
  $n_{air} = 1$
- In practice:
  n. A. $max_{air} = 0.95$
  opening angle ca. 72°

Improvement of resolution:

Increase of working n. A. through increase of n Medium (Immersion)

- Immersion oil (3)
  $n_{Oil} = 1.518$ between coverglass (2) and objective (1)
  - no total reflection
  - full use of objective aperture



n. A. max Air = 0,95
n. A. max Immersol ~ 1,46
(n. A. max Monobromnaphtalene ~ 1,68 Toxic!)

Objective correction: Immersion works against Spherical Aberration

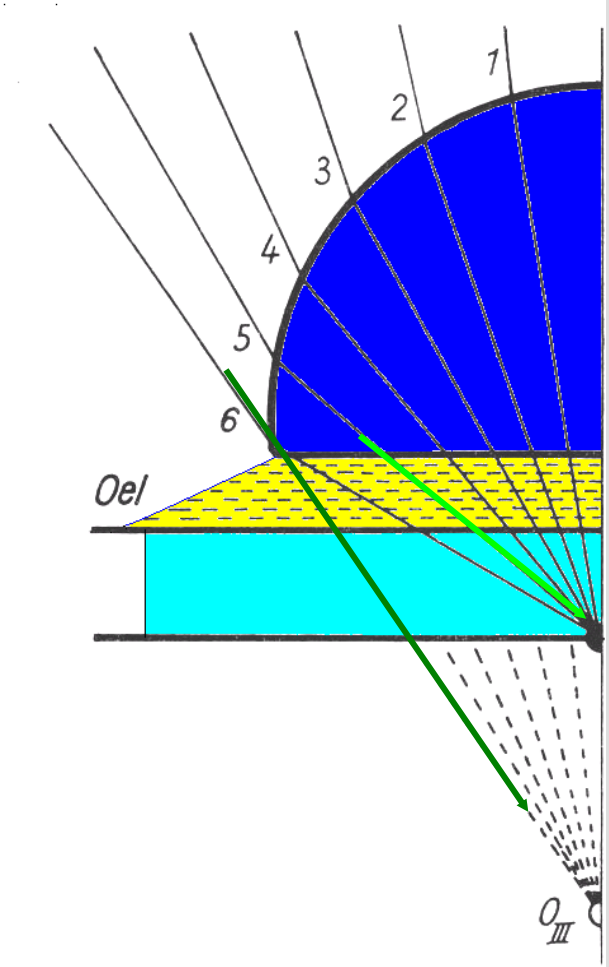
If cover glasses vary slightly from 0,17 mm, spherical conditions are also optimal for those structures that are close to the cover glass, if an immersion objective is used

Water embedded specimens work best with water immersion objectives (n. A. $_{max}$ = 1,2)

Oil objectives can reach higher n. A. values (~ 1,46)

Among many other milestones, Ernst Abbe optimized the principle of „homogeneous immersion“ where the refractive behavior of front lens, immersion medium and cover glass is practically identical.

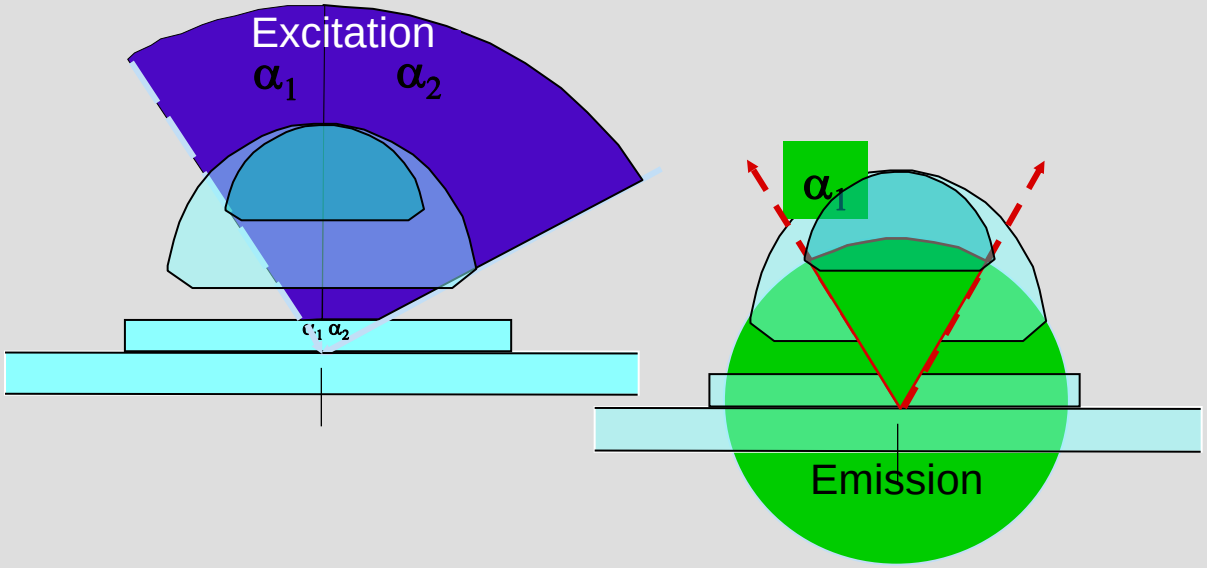
The first oil- immersion objective was introduced in 1879 by CARL ZEISS



Objective correction: Numerical Aperture (n. A.) and Brightness in Fluorescence (n. A.)^4

The larger n. A. the higher is the fluorescence brightness:

A high n. A. objective illuminates the sample with a larger cone of excitation light and can also capture a larger cone of emission light



The fluorescence brightness *theoretically* growths with the (n. A.)^4

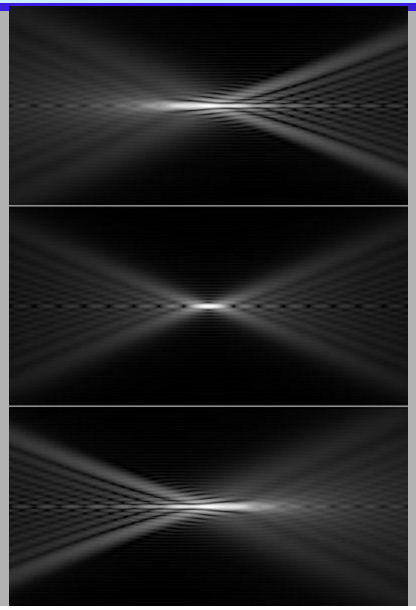
Example: **N Achroplan 40/ 0,65 Dry compared with EC Plan- NEOFLUAR 40/ 1,30 Oil**

The EC Plan- NEOFLUAR with the double n. A. value is 2^4= 16times brighter!

Objective correction: Aberrations

The major aberrations are:

Spherical
Coma
Astigmatism
Flatness
Distortion
Chromatic



Spherical Aberration

Lenses with spherical surfaces focus axial rays differently from peripheral rays.

You have a significant degree of control over the amount of spherical aberration in your image!

Objective correction: Spherical Aberration

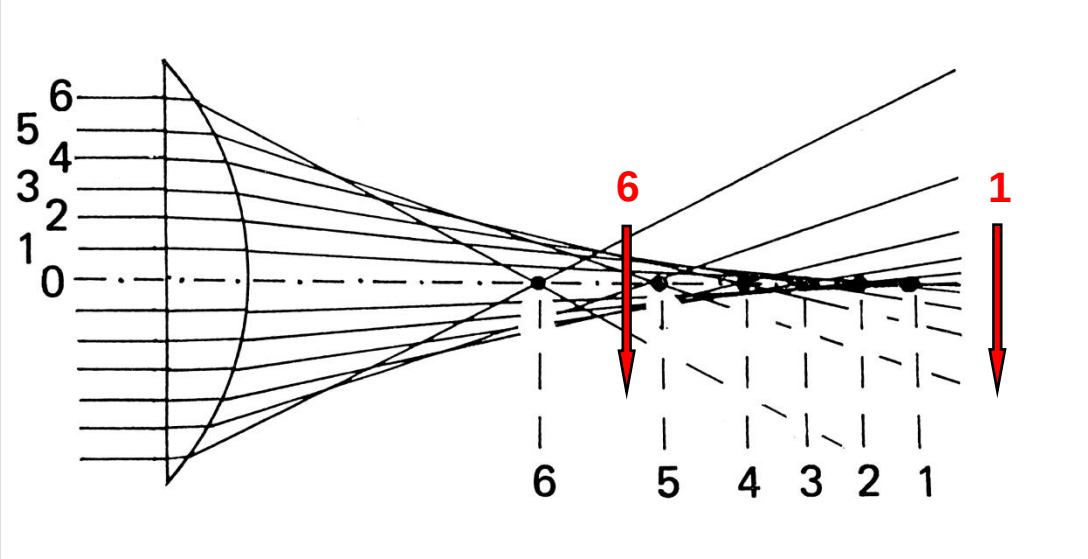
A simple spherical lens has a different focal length for light rays passing the outer zones (4-6) compared to the central rays (1-3)

This phenomenon is called “Spherical aberration“ or “Opening error“

Spherical aberration introduces a haze into the image

It is prominent with all (dry) objective magnifications 40:1 and higher

Spherical aberration can be compensated by the objective lens design



Objective correction: Correction Collar compensates Spherical Aberration

Biological objectives of apertures > ~ 0,4 are calculated for a specific cover glass thickness:

0 (direct water immersion) or 0,17 mm (**all other**)

Spherical aberration is mainly introduced by:

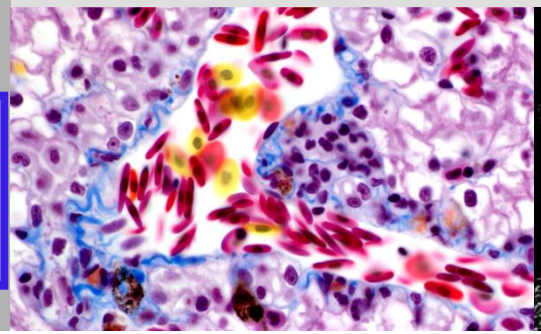
- Too thick sample
- A cover glass thickness deviating from 0,17 mm
- Structure remote from cover glass

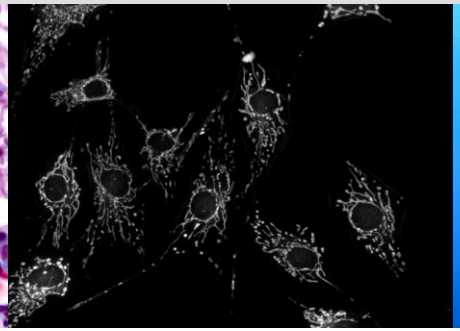
The correction collar moves a compensating lens group inside the objective

Correction (= Korr, German) collar dry and immersion systems often can compensate for (much) larger amounts of spherical aberration, e.g. compared to oil immersion „no- Korr“ objectives

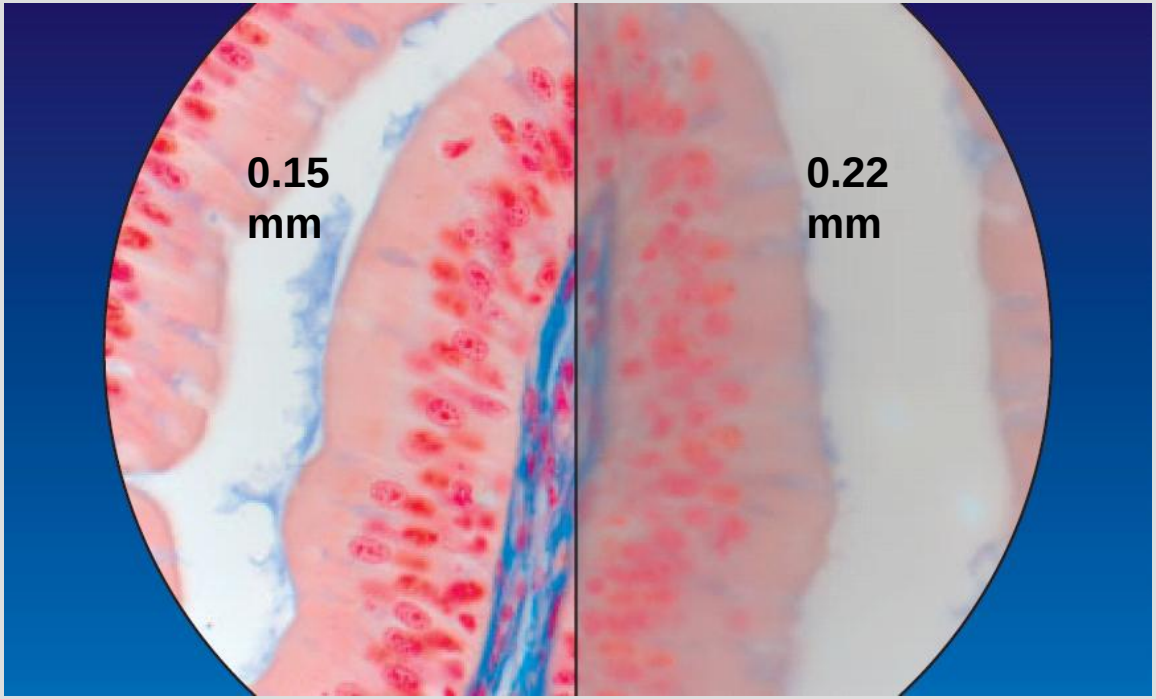
The correction collar is turned until the contrast / signal to background noise ratio is best

In fluorescence applications, beads are often used to become familiar with the spherical behavior of high- n. A. Korr objectives





If the microscopical image does not appear as crisp as expected, spherical aberration is often likely to be the reason.



The duodenum of a frog was perfectly sectioned to approx. 5 µm and Azan-stained. A Plan-APOCHROMAT 40/0.95 Korr. but different cover glass settings (0.15 and 0.22) were used.

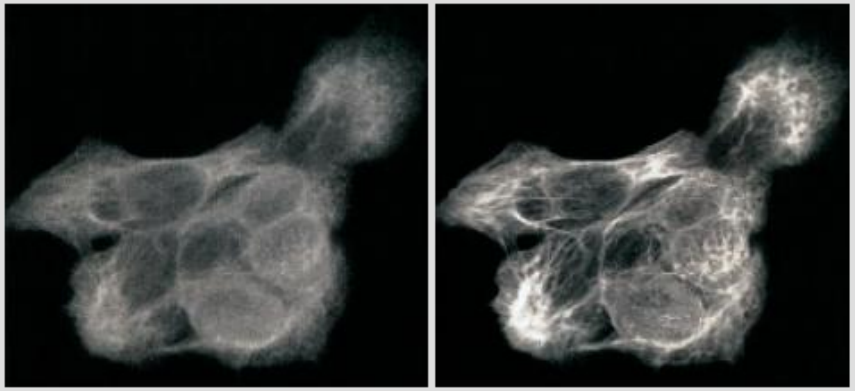
Resolution

Choice of Immersion Media

To avoid refractive index mismatch and the resulting spherical aberration choose the right type of immersion objective

•Example:

- Imaging the same sample with immersion medium that has a mismatched refractive index to the sample i.e. oil into water (blurry) and a matched refractive index i.e. water into water (sharp).



oil immersion objective

NA=1.4

water immersion objective

NA=1.2

Objective correction: Multi- Immersion Korr LCI- objectives

Multi- Immersion objectives can be used when working with different immersion media (oil, glycerol, water)

Today, our multi-immersion objectives are called **L**(ive)**C**(ell)**I**(maging)- objectives

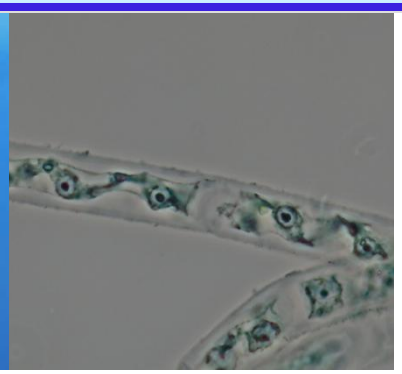
Multi- Immersion objectives have been invented in 1979 by Dr. Rudolf Conradi at CARL ZEISS

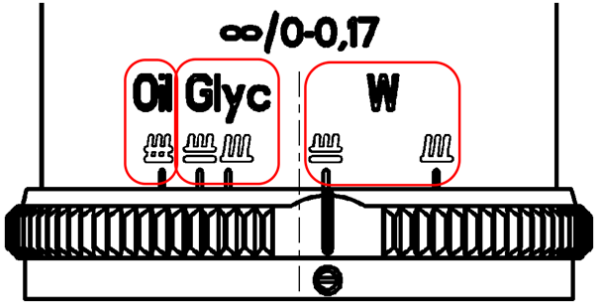
Usually they have an exceptionally **strong visual contrast** and fine working distances, as well as outstanding transmission values and a high n. A.

They are also strongly recommended, if the spherical aberration caused by poor sample conditions (e.g. too thick a cover glass, refractive index to low) has to be compensated in water embedded cell preparations

We offer a full magnification range of multimmersion objectives from 16:1 – 63:1

As a rule of thumb, if spherical aberration cannot be compensated by a C- APOCHROMAT and high apertures are required, go for the LCI- objective





Objective correction: Aberrations

The major aberrations are:

Spherical
Coma
Astigmatism
Flatness
Distortion
Chromatic

Coma

Asymmetrical aberration for peripheral image points, resulting from the failure of a lens to focus rays from peripheral object points which pass through the center of the lens and its periphery to the same image point.

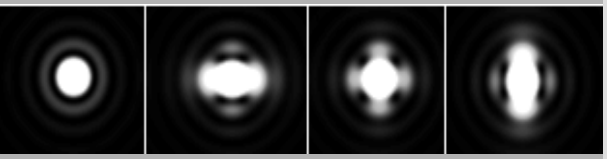
When these aberrations occur, the image of a point is focused at sequentially differing heights producing a series of asymmetrical spot shapes of increasing size that result in a comet-like

Coma is well corrected for in properly constructed modern microscope objectives.

Objective correction: Aberrations

The major aberrations are:

Spherical
Coma
Astigmatism
Flatness
Distortion
Chromatic



Astigmatism

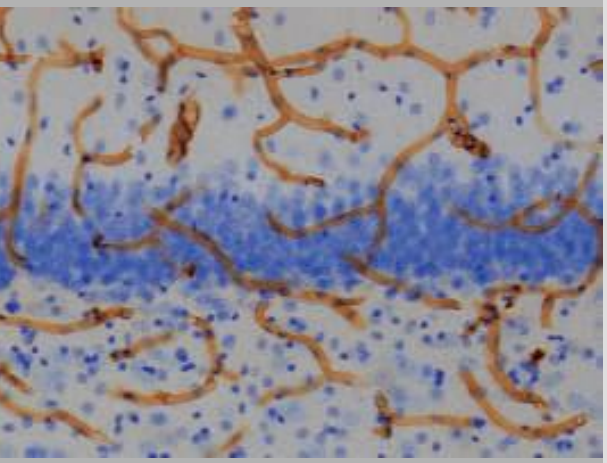
Off axis image points are either tangentially or radially distorted, resulting in loss of peripheral sharpness. The larger the field diameter, the more difficult it is to correct for the aberration.

Astigmatism is well corrected for in properly constructed modern microscope objectives.

Objective correction: Aberrations

The major aberrations are:

Spherical
Coma
Astigmatism
Flatness
Distortion
Chromatic



Flatness

With an uncorrected lens system a flat specimen does not appear in sharp focus over the entire field. Either the periphery or the center of the field of view is out of focus. A flatfield lens eliminated this aberration and renders images sharp from the center to the edge.

Objectives with the prefix “PLAN” in their name are corrected for flatness.

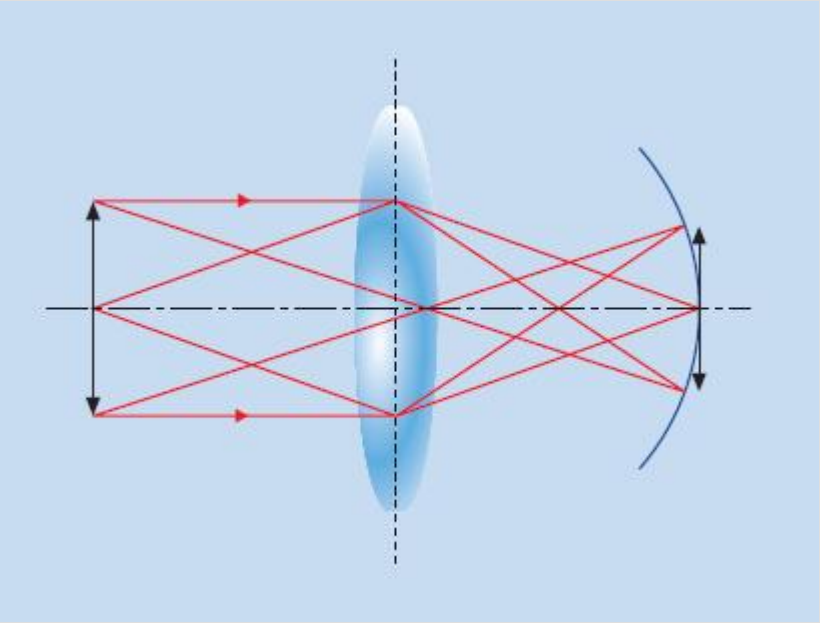
Objective correction: Flatness of Field (“Plan- “)

Objectives have to be forced to provide images that are sharp over a large field of view

Objectives providing a flattened image are called “Plan”-corrected

“Plan-” correction was invented by Dr. Hans Boegehold in 1938 at CARL ZEISS

The flatness of field is achieved by introducing steeply curved lens surfaces into the rear lens elements as well as using a concave meniscus within the front lens



Objective correction: Aberrations

The major aberrations are:

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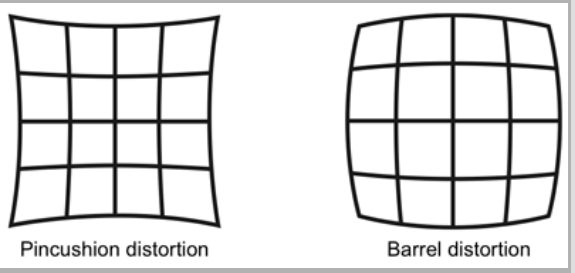


Distortion

Distortion is an aberration resulting from a change in magnification between axial image points and peripheral image points. Straight geometric lines, which do not intersect the optical axis, are either curved in or out resulting in pin cushion or barrel distortion.

Distortion is well corrected for with well designed objectives. It is more noticeable at low magnification and with stereo microscopes.

Objective correction: Aberrations

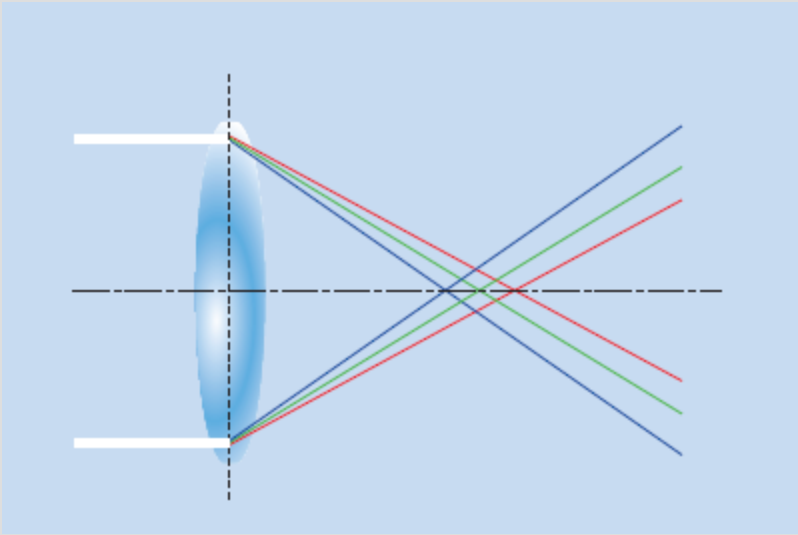
The major aberrations are:

Spherical
Coma
Astigmatism
Flatness
Distortion
Chromatic

Chromatic

Axial Chromatic Aberration

Rays of different spectral colours are focused in different planes producing severe colour fringing around image structures.



Objective correction: Aberrations

The major aberrations are:

Spherical
Coma
Astigmatism
Flatness
Distortion
Chromatic

Chromatic

Lateral Chromatic Aberration

Different wavelengths are focused at different positions in the **focal plane** (because the magnification of the lens also varies with wavelength).

The effect is also known as Chromatic Magnification Difference.

The correction of chromatic aberration is determined through the choice of the type of objective used and can scarcely be influenced in practice.

Objective correction: Chromatic

Depending on the degree of correction, a distinction is made between, in order of increasing color error elimination:

Achromats

Fluorites

Apochromats

“**Achromat**”

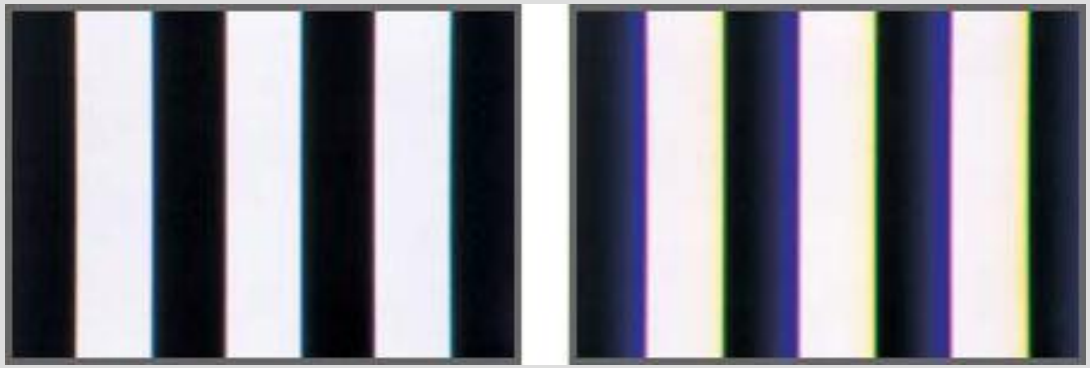
Two colours: Usually Red and Blue

“Neofluar” or **“Fluorite”** (Semi Apochromat)

Two colours and near correction of the third

“Apochromat” (free from colour)

Commonly three colours: Red Blue Green



Objective correction: Chromatic

Objectives have colour artifacts (e.g. colour fringes) in x/y and z (= Chromatic Aberration)

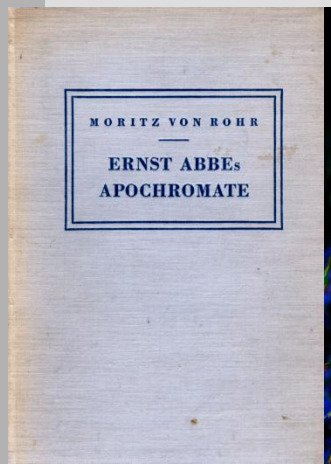
Optics delivering images free from traces of colour fringes and having a focus match for at least 3 spectral lines are called “apochromatic”

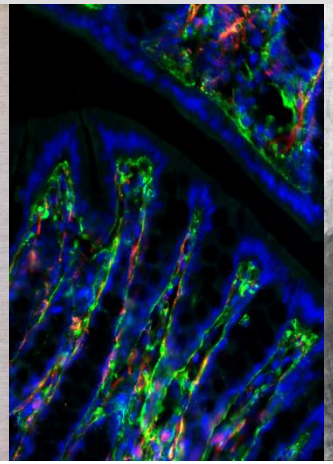
CARL ZEISS APOCHROMATs are fully colour corrected no longer for only 3 – 4 spectral lines, but for a full spectral range, i.e. 7 wavelengths from UV through to IR

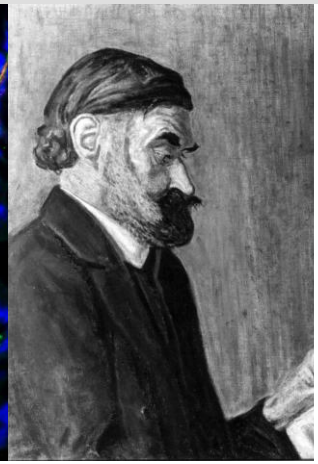
e.g. ***C- APOCHROMAT 40/ 1.2 W Korr UV- VIS- IR*** is fully colour corrected from ~ 365 to ~ 900 nm

The apochromatic correction was invented by Prof. Ernst Abbe in 1886 at CARL ZEISS

Apo correction is achieved by a smart combination of unique glasses with different colour refractive behavior (dispersion)

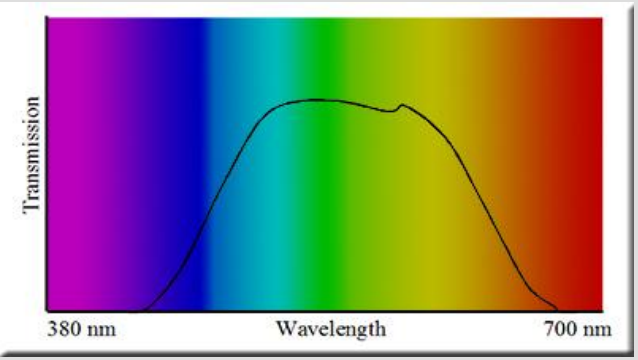


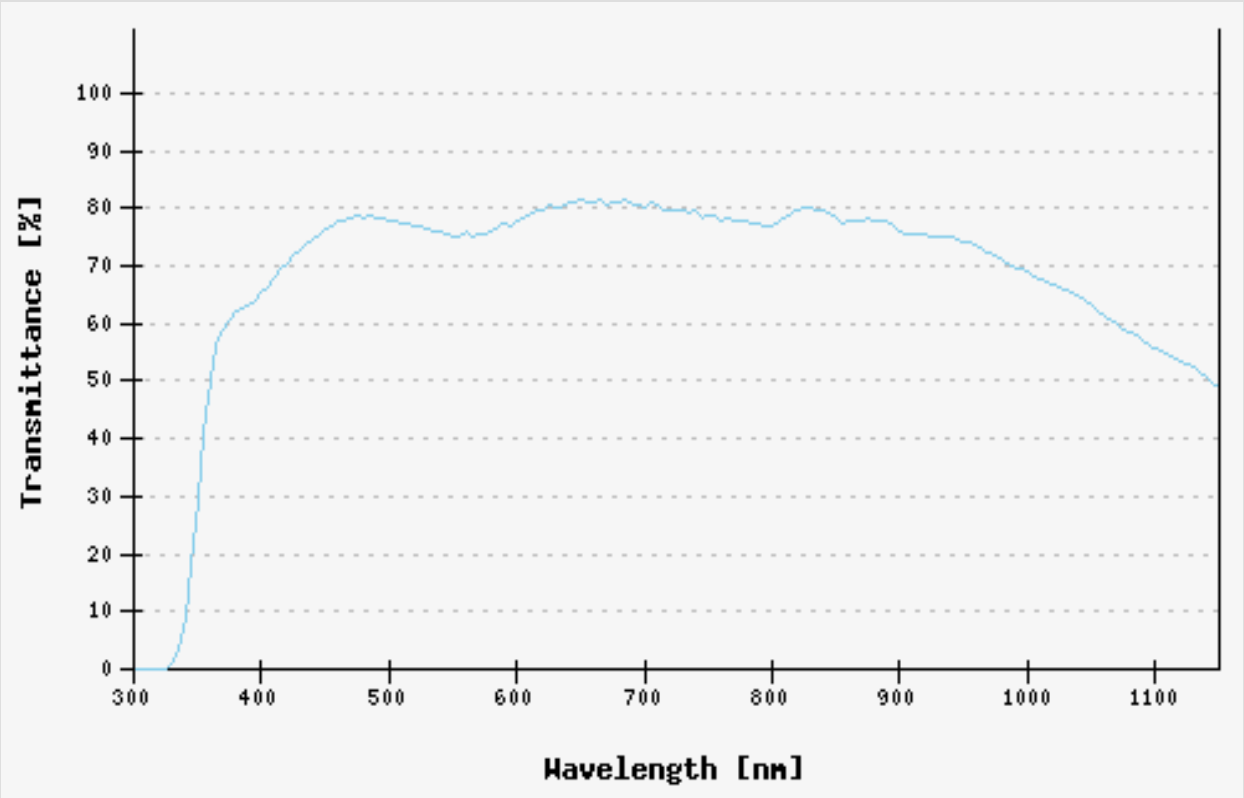




Objective correction: Glass Transmission

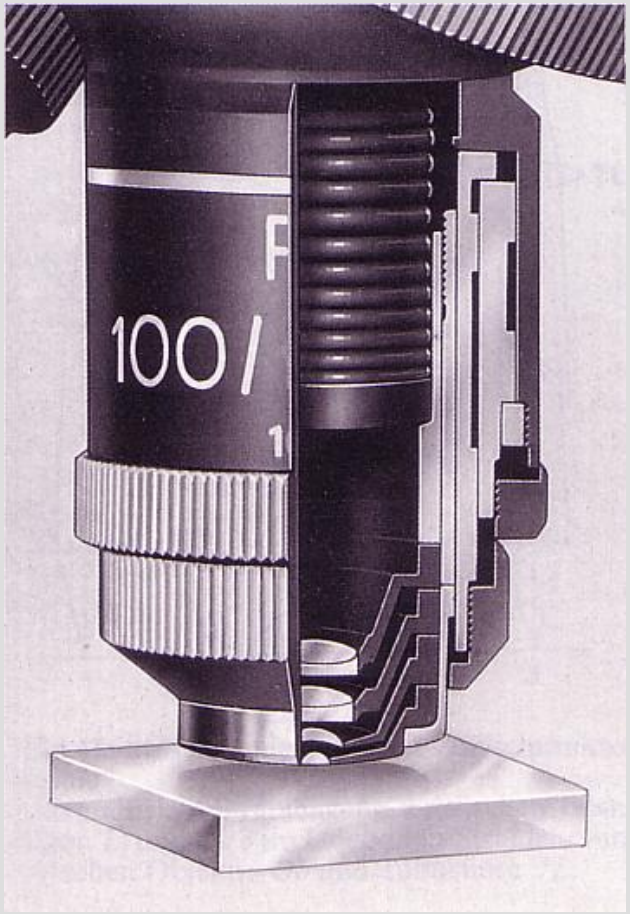
The degree to which an objective can transmit a given wavelength range of light describes its transmission profile.





C- APOCHROMAT 40/ 1.2 W Korr UV- VIS- IR

Working Distance



Many microscopists think that the free working distance of an objective tells them exactly about how deep the objective can focus into the sample without producing a spherically bad image.

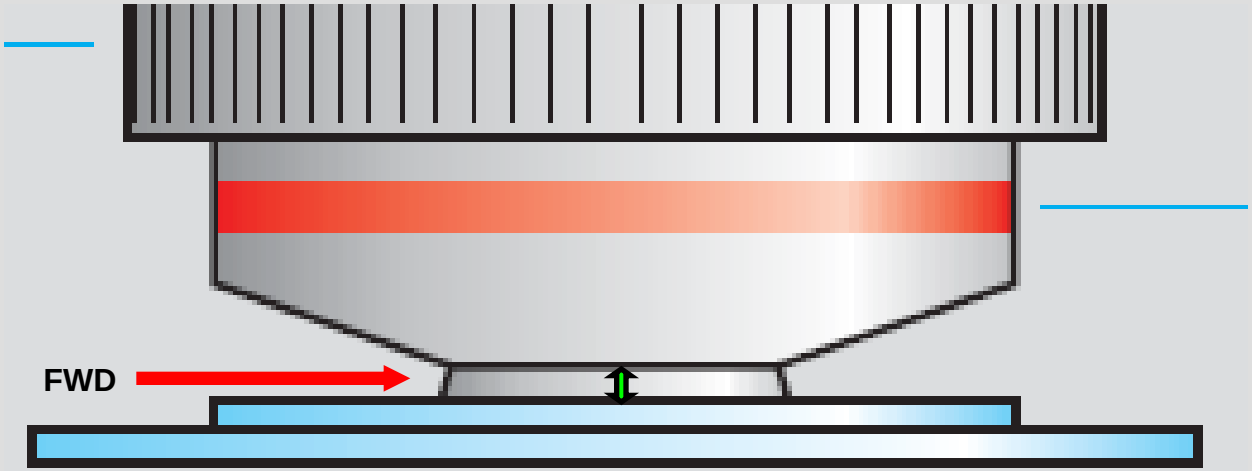
In most cases, this is wrong.

Working Distance

To clarify things, how is working distance exactly defined?

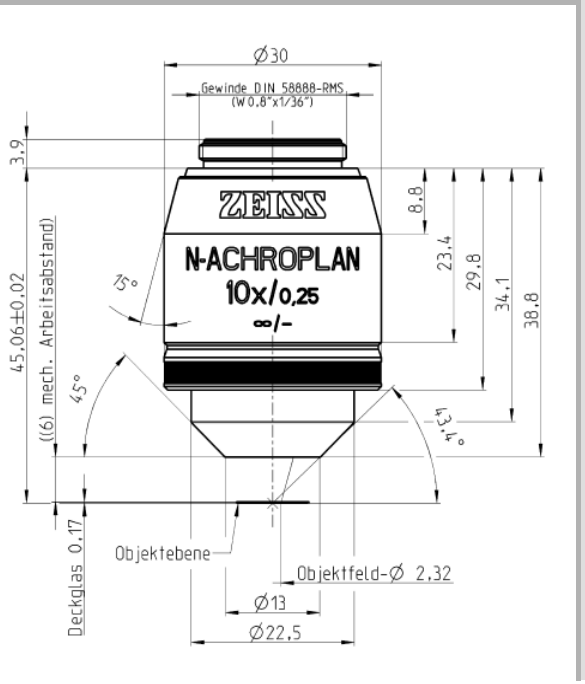
The free working distance (FWD, a) is the distance between the objective front lens metal mounting surface and the upper side of the cover glass surface when the objective is in focus.

It is also assumed that the cover glass thickness is exactly the requested one (usually D= 0.17 mm) and that the focussed structure is attached to the cover glass underside.



Working Distance

This is the distance between the objective front lens and the top of the cover glass when the specimen is in focus.



Working distance tends to decrease with increasing n.A.

C-APOCHROMAT 40X/1.20 W CORR WD=0.28mm

LD C-APOCHROMAT 40X/1.1 W CORR WD = 0.62mm

EC PLAN-NEOFLUAR 40X/0.75 WD=0.71mm

Working distance tends to decrease with increasing magnification:

EC PLAN-NEOFLUAR 10X/0.30 WD=5.2mm

EC PLAN-NEOFLUAR 40X/0.75 WD=0.71mm

EC PLAN-NEOFLUAR 100X/1.3 OIL WD=0.2mm

Any space between the cover slip and the sample adds to the working distance since the value for the working distance of a lens presumes that the sample is just under the cover slip

The Objective Types

The lines of distinction between objective classification are becoming blurred.

The application should determine choice.

Many specialty objectives exist:

E.g. TIRF, Deep UV, LD, 2P

- Achromats
- Plan Achromats
  - LD Plan Achromats
  - LD Dipping D=0
- Fluar
  - α Plan Fluar
  - Ultrafluar
- Plan Neofluar
  - LD Plan Neofluar
  - LCI Immersion (Water/Glyc/Oil)
- Plan Apochromat
  - C-Apochromats
  - LD C-Apochromats
  - LD Dipping D=0
  - α Plan Apochromat

The Sample Properties

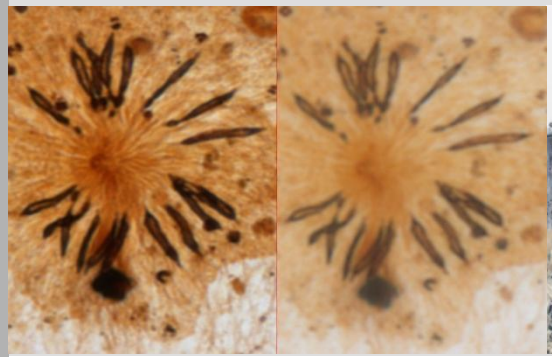
The Sample Properties

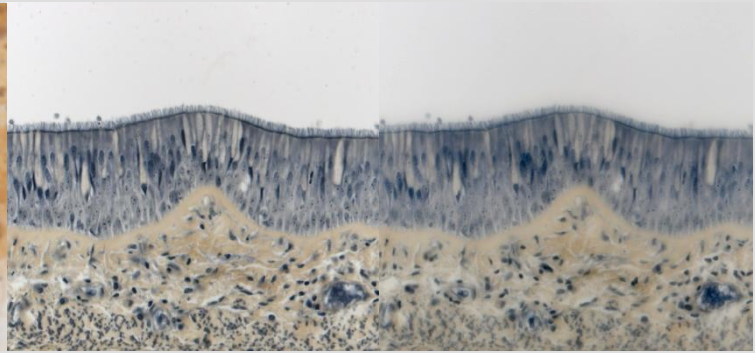
The sample properties

influence the presence or absence of image blurr (spherical aberration)

impair the contrast (signal- to- backround- noise ratio)

determine whether -due to the sample topography- the objective is able to produce an image at all or not (free working distance)





Sample Influence:

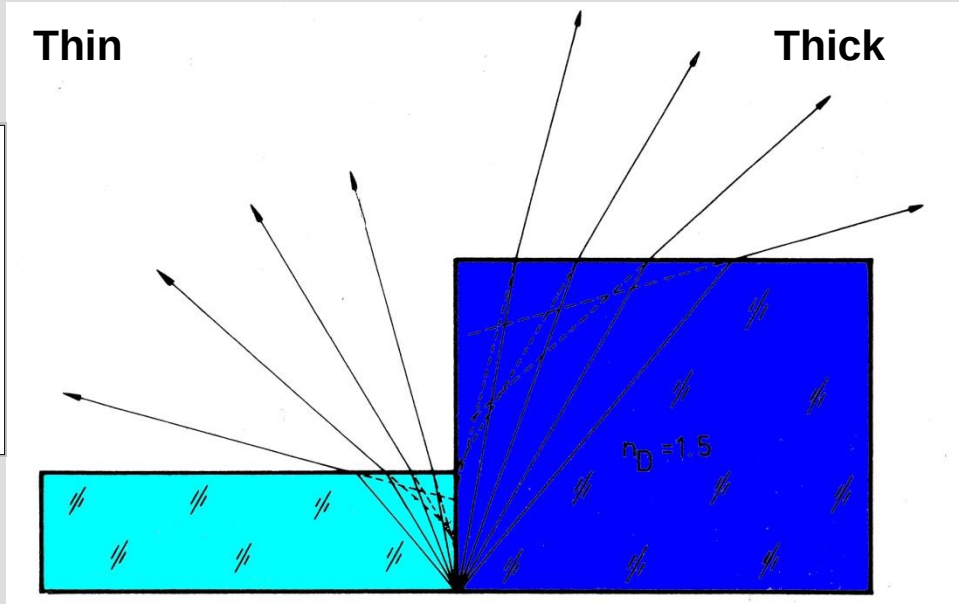
Spherical aberration
Cover Glass Influence

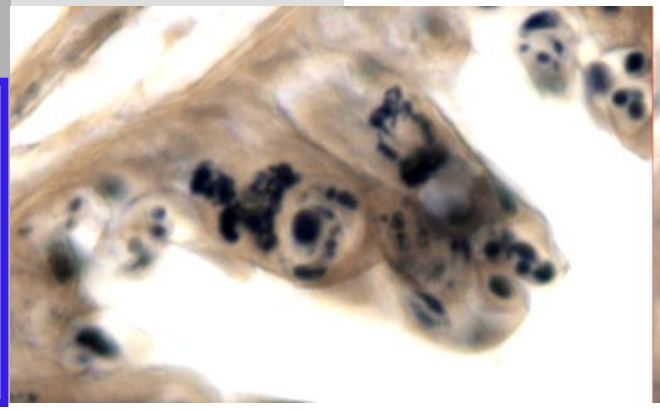
The cover glass refracts the sample light irradiating from one point with angles. The angles depend on cover glass thickness

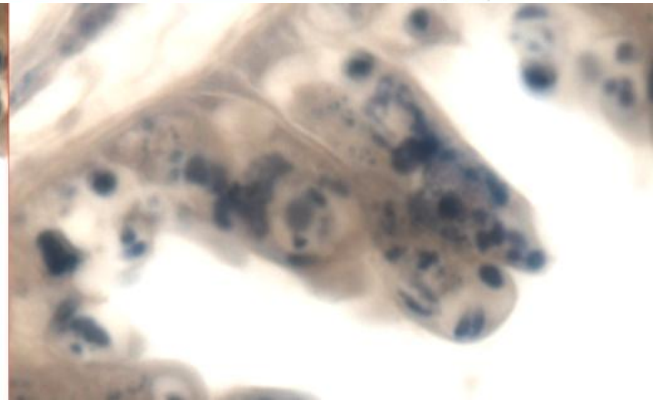
These rays are offered to the objective

The higher the numerical aperture of a given objective, the more sensitive it looks onto different light angles, the more visible is this image blurr

All objectives with an n. A. > ~ 0,35 are already sensitive here and especially dry objectives with n. A. values > 0,6 are most sensitive against spherical aberration





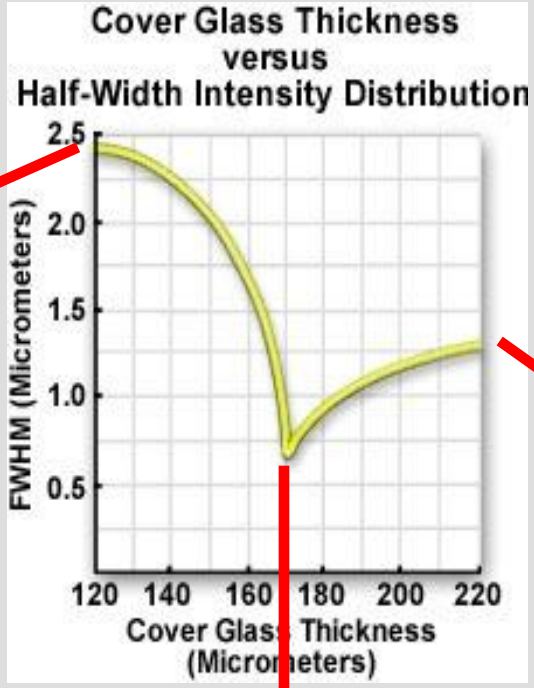


Sample Influence:

Spherical aberration
Cover Glass too thin

Image quality **decreases faster when** choosing **too thin** cover glasses

This is true for the normal cover glass thickness range of unsorted batches



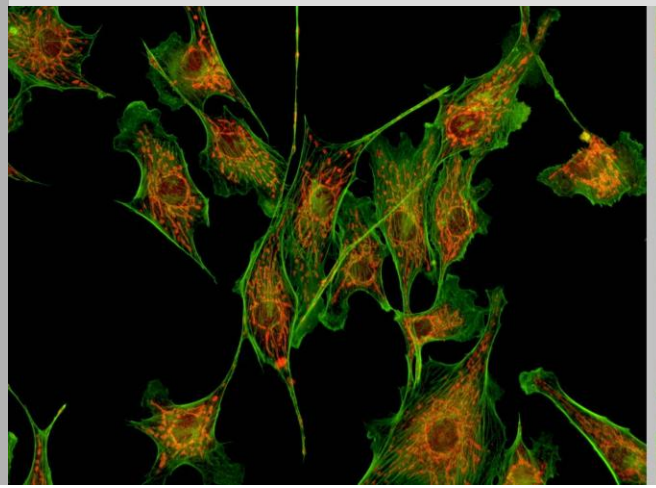
D= 0.17 mm

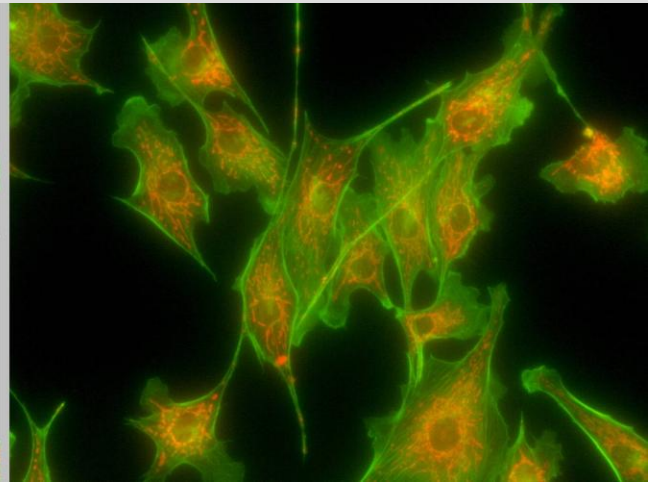
Sample Influence:

Contrast (Signal- to background noise ratio)

Image blurr caused by non-compensated spherical aberration is likely to be confused with other preparation artifacts, e.g. improper washing of specimen

The **Plan- APOCHROMAT 40/ 0,95 Korr** has a strong compensation power for such optical contrast problems, for lower magnifications we recommend the LCI- objectives to solve this problem





Sample Influence: Wrong Refractive Index

A wrong immersion medium with a deviant refractive index and/ or dispersion will introduce spherical and chromatical aberration to the image

Examples:

•Using immersion oil with a water immersion type objective

•Applying low viscosity immersion media instead of immersion oil (e.g. IMMERSOL ™)

•Employing embedding media with a refractive behavior strongly deviant from immersion oil will add to an inferior signal to background noise ratio in fluorescence

The use of proper immersion media is a prerequisite in live cell imaging

For optimum results:

Oil immersion systems with IMMERSOL ™ 518 **F**

Water immersion objectives with distilled water or IMMERSOL **W** (artificial non- evaporating, low- viscosity „water“). A must for long-time experiments

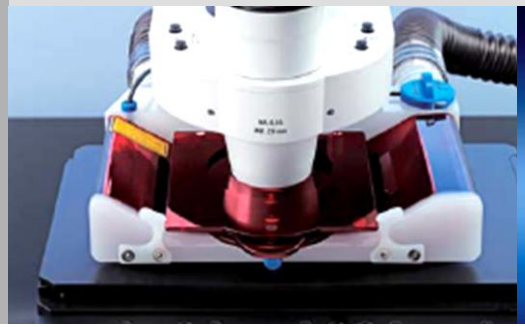
ALWAYS REMOVE OLD RESIDUES OF IMMERSION MEDIUM FROM THE FRONT LENS. DO NOT MIX BATCHES

Sample Influence: Heating the Specimen/ Objective

Sometimes, samples have to be heated. To overcome a temperature gradient between immersion objectives and the connected sample, objectives can also be heated

To stabilize the heat efflux from a “warm” sample to the “cold” microscope, CARL ZEISS invented calorimetrically insulated objectives, called
“i- objectives” for live cell imaging and incubation applications

All objective temperatures different from 20°C will slightly introduce spherical aberration. This is critical if structures are very minute with lots of background signal



We make it visible.

How to choose

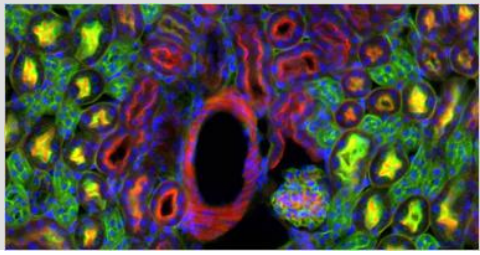
The Optimal Objective Choice: Magnification and n. A.

The Optimal Objective Choice is dictated by the Sample and Application

The optimal objective choice in microscopy of living specimens follows a canon of simple questions:

What is the specimen size?

Objective magnification as such is defined (e.g. 10x or 150x)



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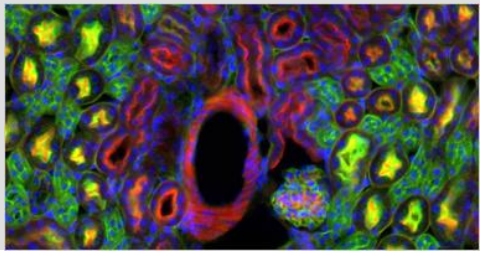
What is the size of minute details inside the specimen?

Objective resolution (n. A.) is defined (e.g. 1,46)



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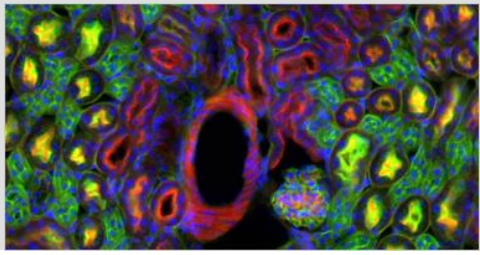
What is the imaged field of view and required resolution?

Objective magnification in respect of objective n. A. is defined (e.g. Plan- APO 20/ 0,8 for MosaiX)



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What is the specimen size?

Objective magnification as such is defined (e.g. 10x, 100x or 150x)

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Objective resolution (n. A.) is defined (e.g. 1,46)

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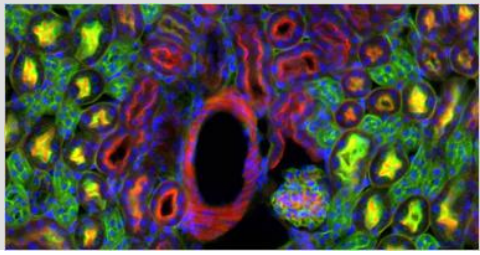

The Optimal Objective Choice: Brightness and Fluorescence

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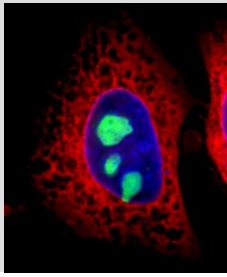
The optimal objective choice in microscopy of living specimens follows a canon of simple questions:

How bright is the fluorescence signal?

Objectives with a high n. A. are employed for weak signals (e.g. Plan- APO 20/ 0,8, 40/ 1,30; for UV use C- APO or FLUAR)



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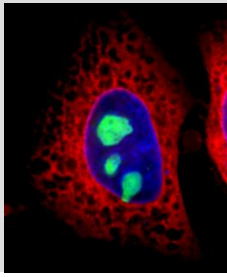
The optimal objective choice in microscopy of living specimens follows a canon of simple questions:

Is more than 1 fluorescence colour channel used?

Apochromatic objectives have best colour match



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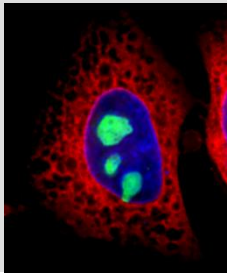
The optimal objective choice in microscopy of living specimens follows a canon of simple questions:

Is *extremely* colour- critical multichannel work done?

Use **C**- APOCHROMAT objectives ("**C**"= **C**onfocal)



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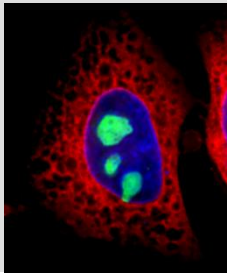

The Optimal Objective Choice: Water Immersion

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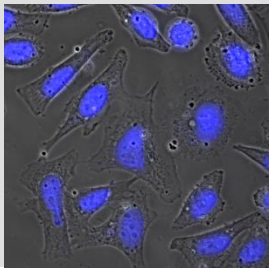
The optimal objective choice in microscopy of living specimens follows a canon of simple questions:

Is the sample immersed within an aqueous medium?

Water immersion objectives are recommended



The Optimal Objective Choice: Water Immersion

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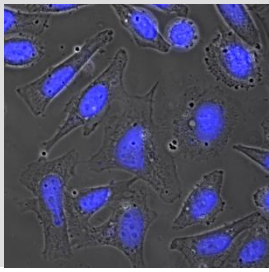
The optimal objective choice in microscopy of living specimens follows a canon of simple questions:

Are those structures very thick?

Water immersion with LD- water immersion objectives



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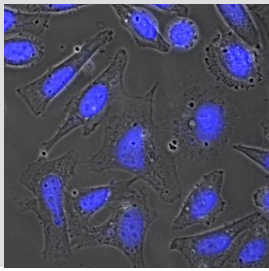
The optimal objective choice in microscopy of living specimens follows a canon of simple questions:

Are the structures uncovered (no cover glass possible)?

Water immersion objectives for direct front lens immersion



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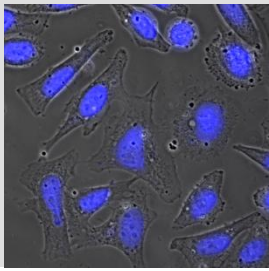


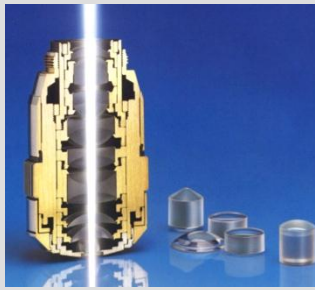
The Optimal Objective Choice: Contrasting Techniques

The Optimal Objective Choice is dictated by the Sample and Application

The optimal objective choice in microscopy of living specimens follows a canon of simple questions:

Is the sample birefringent (e.g. Microtubuli aggregates)?

Special strainfree POL- contrast objectives



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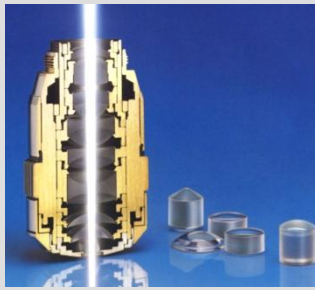
Are the structures very thick (> ~ 100 - 200 µm)?

→ Optical sectioning with DIC (use DIC-objectives, if possible)



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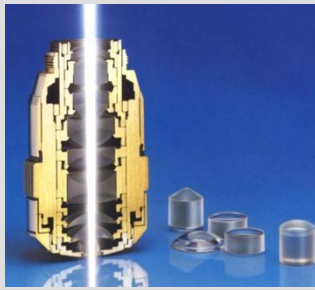

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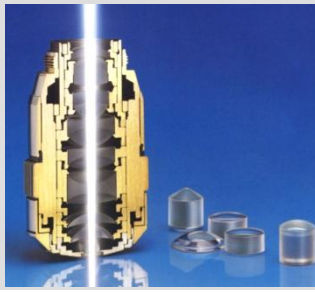
Is the specimen very thin (< ~ 10 µm)?

Strong contrast with Phase Contrast



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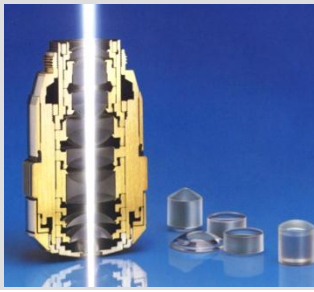
Is the specimen extremely thin (< ~ 2 – 0,02 µm)?

Best contrast with Ultra Darkfield



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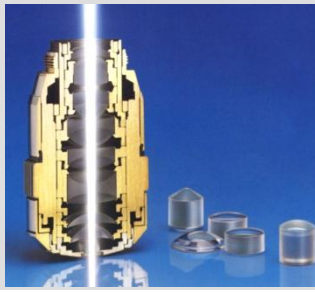


Optical Conflicts:

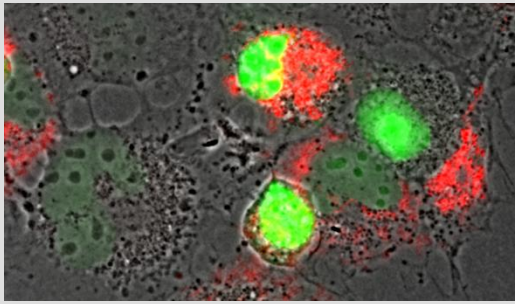
Ph-/ LD- Objectives
Older Objectives

The Optimal Objective Choice is restricted by conflicts

However, the optimal objective choice can be disturbed by some optical conflicts caused by the objective and the sample properties

Phase Contrast Objectives Ph

Not recommended for best brightfield, darkfield and DIC images with all magnifications 40x and higher.
Also the fluorescence brightness is slightly reduced here (light absorption of phase ring= ~ 88%)



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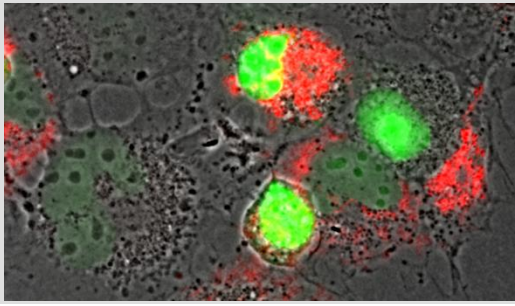
Commonly, only recent objectives with a known diffraction behavior are suited for DCV and ApoTome- work (good objectives are C- APO, Plan- APO, EC- objectives of higher classes)



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LD objectives

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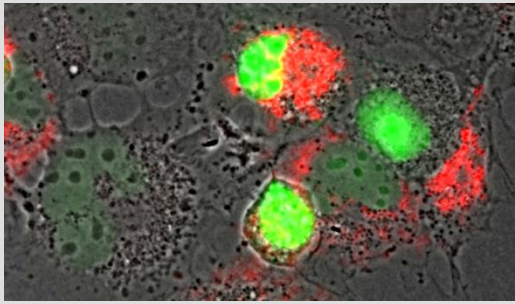

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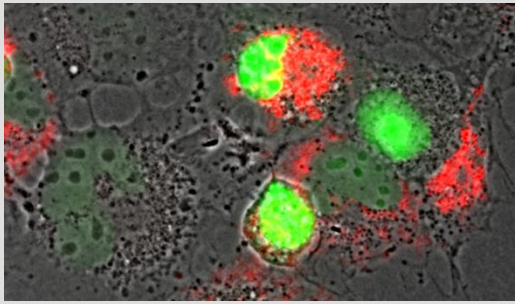

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