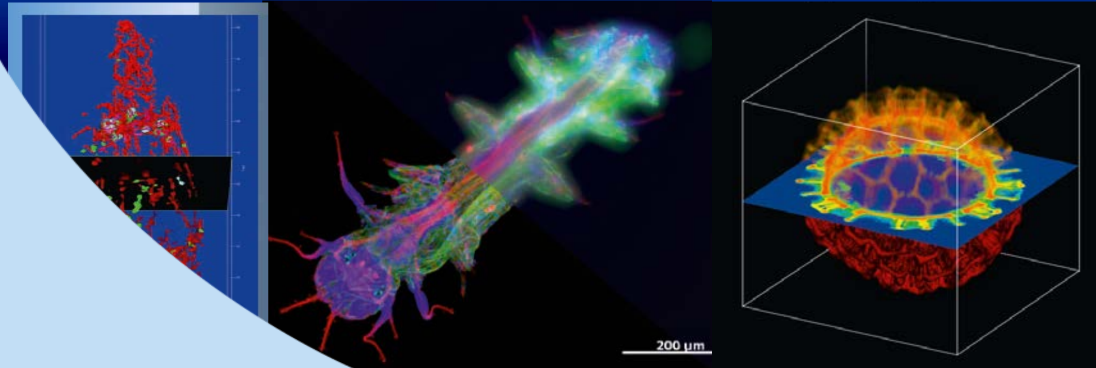
How to choose the optimal objective?



Dr. Sebastian Gliem
CARL ZEISS Microscopy
Embedded Specialist, HCBI

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AGENDA

I. Importance
II. Types/Tasks
III. Properties
IV. How to choose
V. Choices

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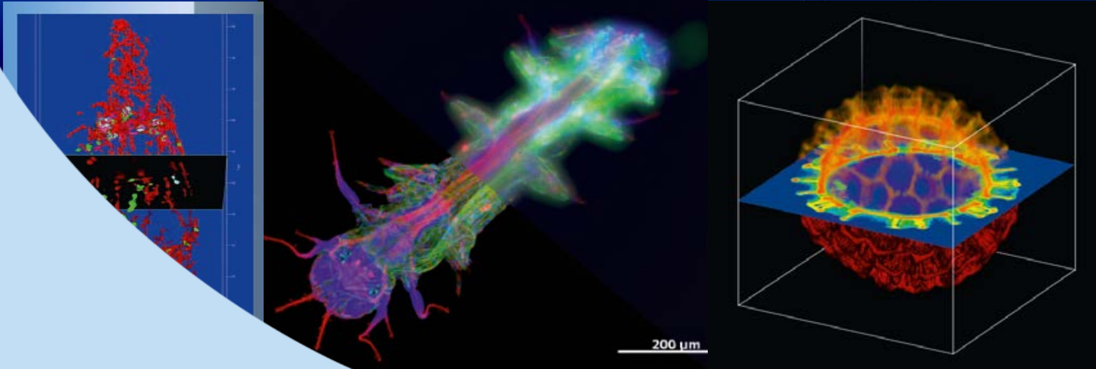
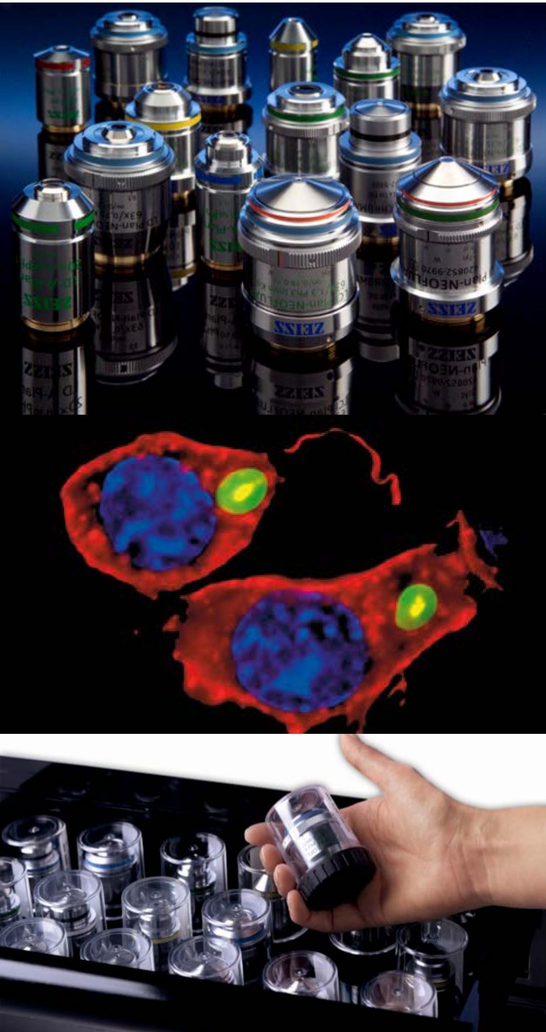
Why to think about objective selection?



Why to think about the right objective?



- Challenging applications in Life Cell Microscopy
- Variety of methods in Life Cell Microscopy

Challenging Applications

Demanding applications in light microscopy of living cells are

- high- resolution microscopy of very thick samples
- high- resolution microscopy of structures remote from the cover glass
- high- resolution microscopy of fast moving structures

Usually these applications are linked to fluorescence microscopical methods!

Method Approaches in Living Cell Microscopy

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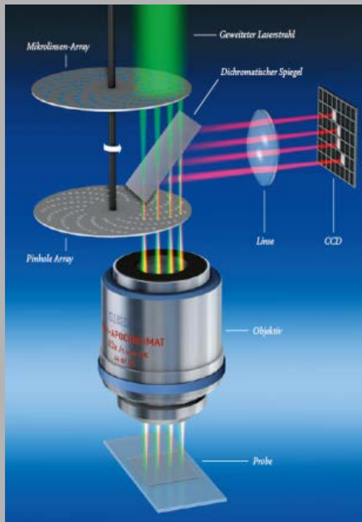
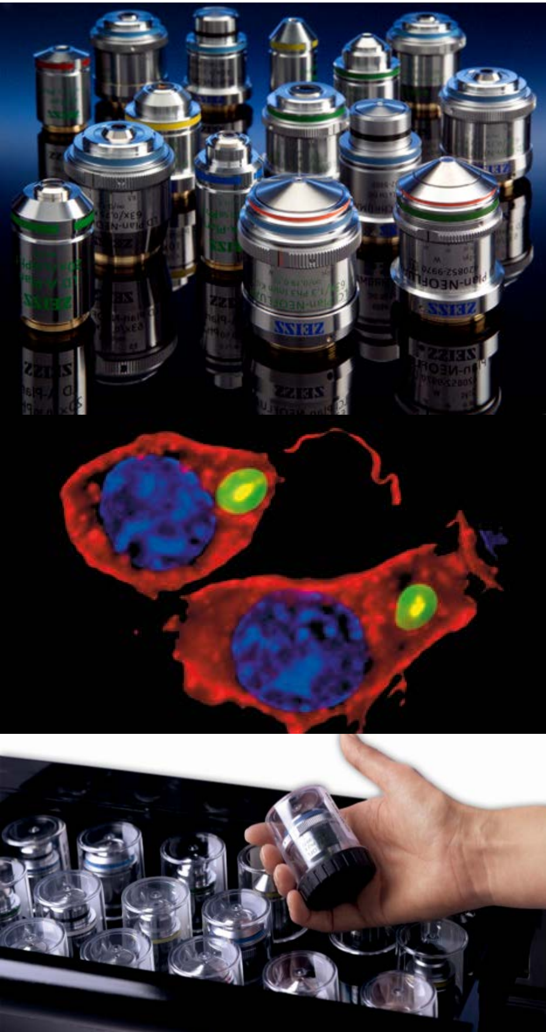
Optical Sectioning results in more Information



Methods applied to achieve perfect images in demanding fluorescence applications are

- Optical sectioning with structured illumination (e.g. ApoTome)
- Confocal pinhole techniques
  (e.g. Confocal Laser systems, Spinning Disc systems)
- Evanescent fields (TIRF)
- Single plane illumination (LSFM)
- Mathematical approaches (e.g. 3D/ 2D Deconvolution)

Why to think about the right objective?

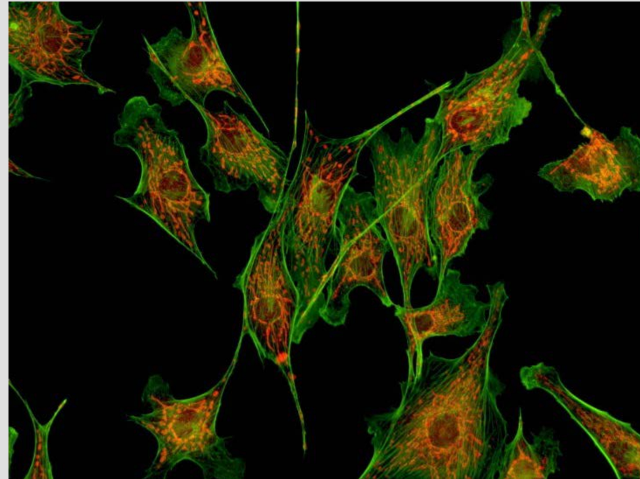


- Challenging applications in Life Cell Microscopy
- Method approaches in Life Cell Microscopy
- Sample preparation, e.g. immersion
- Sample properties, e.g. sample size

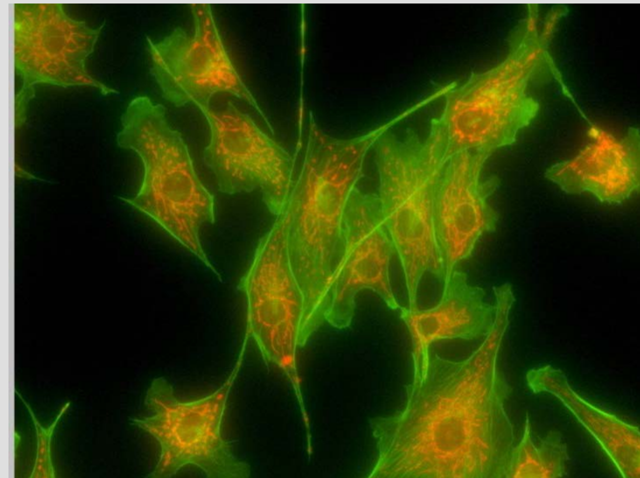
Influences of the sample preparation

Immersion

- Wrong immersion
- Always use a clean objective!



- Acquired with clean lens



- Acquired with old oil remnants

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 (e.g. anisol) 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 our proprietary CARL ZEISS immersion media is a prerequisite in live cell imaging

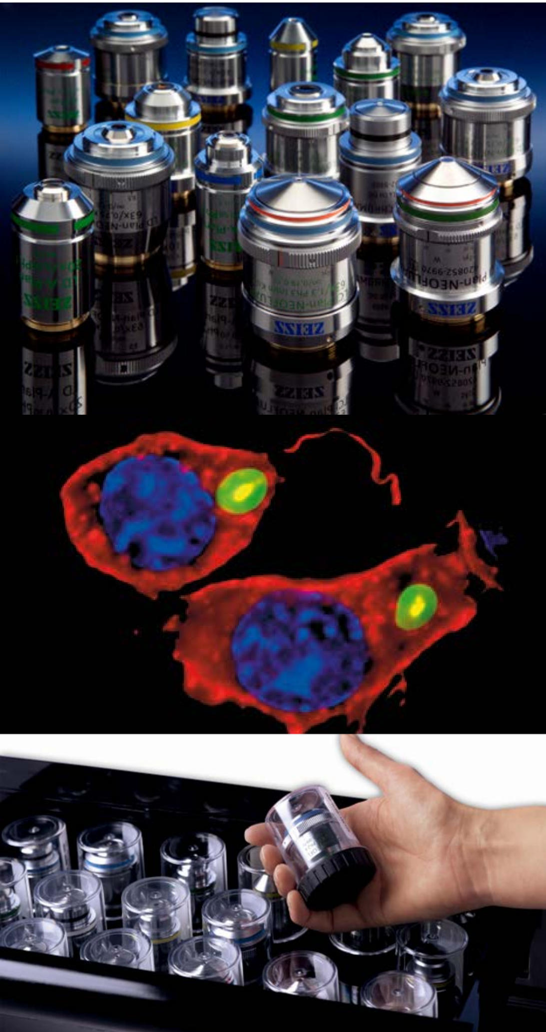
For optimum results:

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

Why to think about the right objective?



- Challenging applications in Life Cell Microscopy
- Method approaches in Life Cell Microscopy
- Sample preparation, e.g. background staining
- Sample properties, e.g. sample size

- Different types of objectives
- Different objective properties
- Different objective corrections

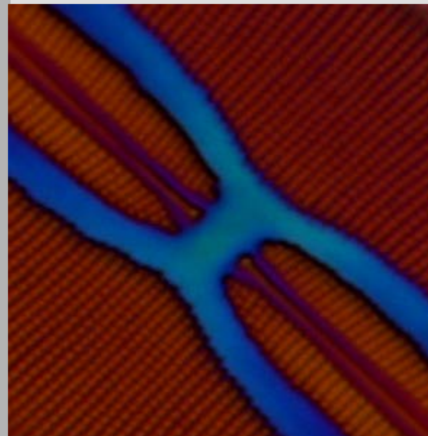
➢ The Perfect Microscopical Image

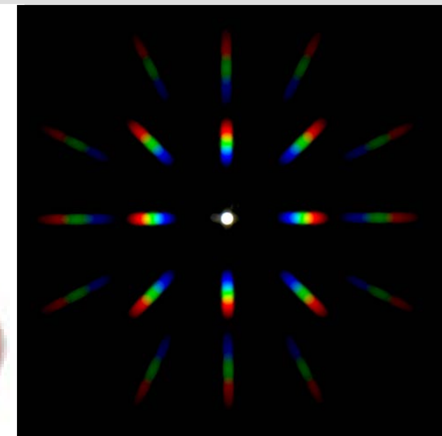
The Perfect Microscopical Image

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





Types of objectives

Types of objectives
(selection)

Ultrafluar

A-Plan

Fluar

Achroplan

C-Apochromat

Plan-Neofluar

LD Plan-Neofluar

Apochromat

EC Plan-Neofluar

LD LCI Plan-Apochromat

Plan-Apochromat

Types of objectives
(selection)

Main differences

Glass material

Amount of lenses

Shape of the glass

Glass coatings

Consequences

- Light transmission efficiency (λ)
- Light ray representation in your image (= degree of correction)

The Objective Colour Code

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Today, for ease of use, all microscope objectives follow a colour code that allows immediate recognition of important objective parameters

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

Labeling of the Objective
Objective class, special designations are used for this, e.g.
LD for Long Working Distance

Magnification / Numerical Aperture
plus additional details on
- immersion medium (Oil /W/ Glyc)
- adjustable cover glass correction (Korr.)
- contrast method

Tube Length / Cover Glass Thickness (mm)
ICS optics: ∞
Infinity Color Corrected System
standard cover glass: 0.17
without cover glass: 0
insensitive: -

Mechanical Correction Collar
- cover glass thickness correction
- different immersion
- different temperature
- adjusting an iris diaphragm

ZEISS
LCI Plan- NEOFLUAR
63x /1,3 DIC Imm Korr
∞/0,19-0,15
Glyc
W

Color of writing
Contrast method
Standard
Pol / DIC
Ph 0 1 2 3

Color Coding of Magnification
1.0/1.25
2.5
4/5
6.3
10
16/20/25/32
40/50
63
100/150

Immersion Fluid
Oil
Water
Glycerin
Oil /Water / Glycerin

ZEISS

Tasks of objectives

Tasks of objectives

The microscope objective is the most important optical component for imaging

The tasks of microscope objectives

- magnify (M) the image structure
- resolve (n.A.) the image structure
- offer inter-sample-objective correction capabilities (e.g. immersion, corr-ring)
- provide a necessary working distance
- avoid blurred edges (spherical aberration)
- image different colors in one point (chromatic aberration)
- high light transmission for required λ
- applicable for wanted contrast techniques (BF, DF, Ph, DIC, Pol, Fl)

Properties of objectives

Objective properties:

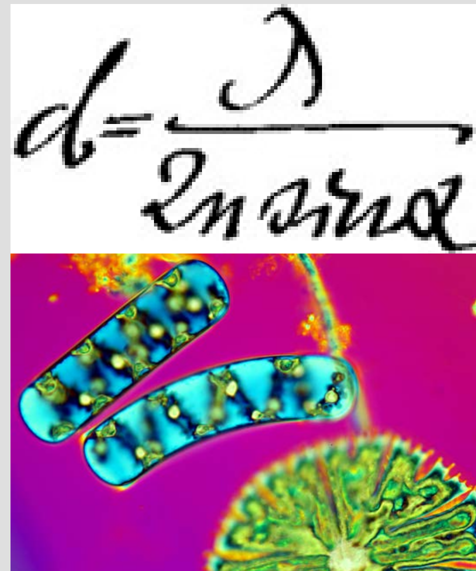
Resolution and numerical Aperture

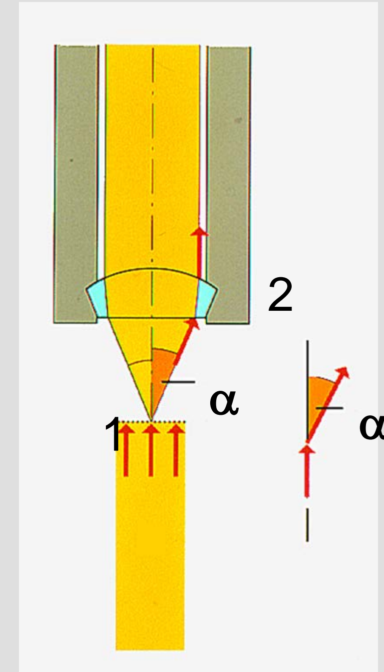
The resolution of a microscopical image depends on the actual numerical aperture (n. A.) of the given objective/ and the wavelength of light used

$$d_0 = \frac{\lambda}{2\ n.\ A.} = \frac{\lambda}{2n * \sin\alpha}$$

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

In 1905 Dr. Moritz von Rohr at CARL ZEISS invented the first objective with an n. A. = 1,68 (toxic immersion medium)





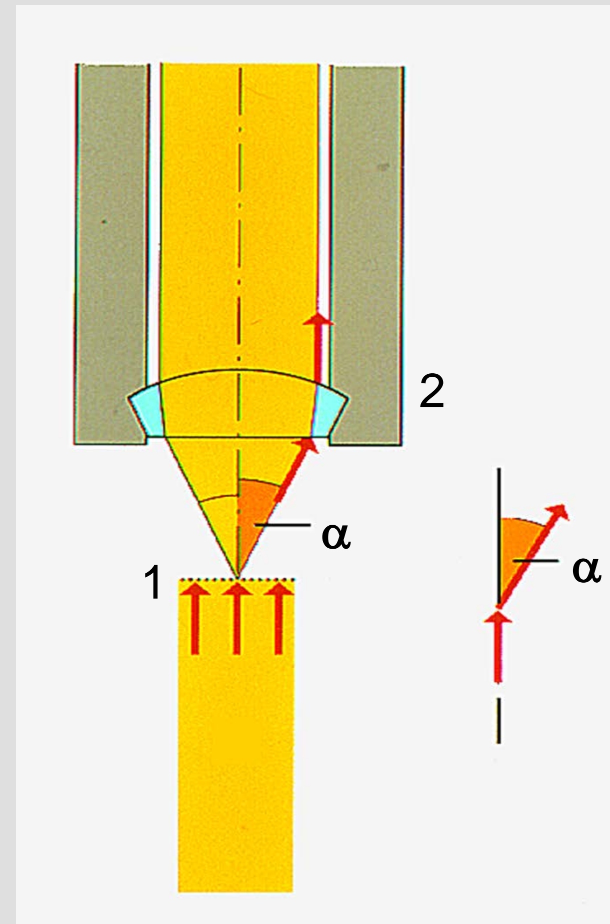
n. A. max $_{\text{Immersol}}$ ~ 1,46
(n. A. max $_{\text{Monobromnaphtalene}}$ ~ 1,68 Toxic!)

Objective properties:

Resolution and numerical Aperture

The wider the opening angle of the objective (2)

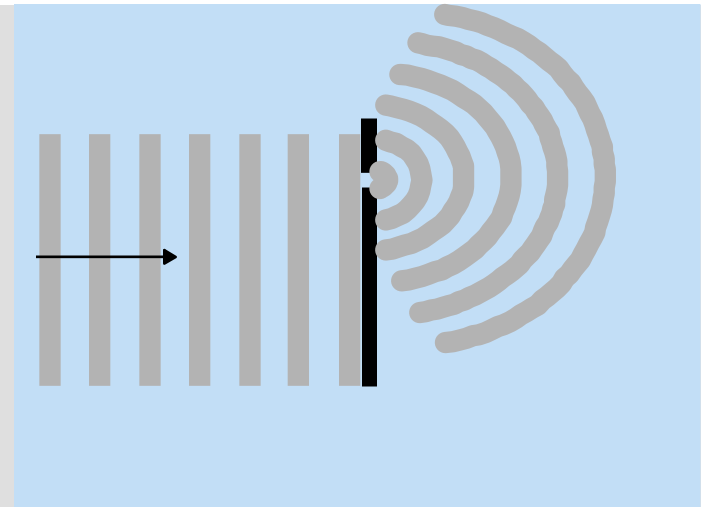
- **the more of the diffracted light can be captured**
- **the smaller details (1) can be resolved**



- n = refractive index of the medium between object and objective
  $n_{air} = 1$, $n_{glass} = \sim 1.52$
- a = half the opening angle of the objective

Differentiation between two points

What is the requirement that two points can be differentiated from each other?

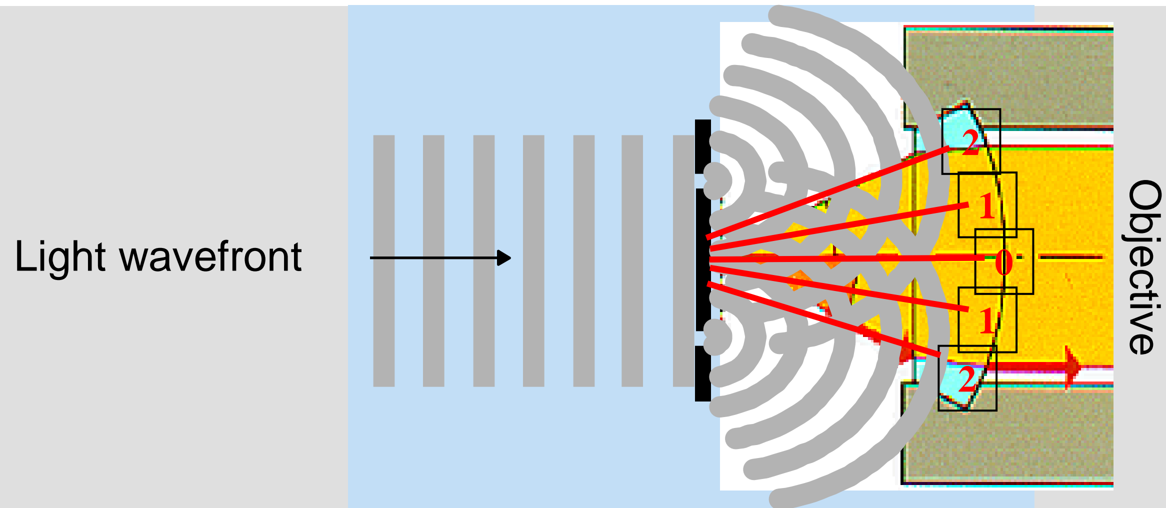


When light originates from single points, it generates so-called diffraction patterns, that contain structural information

Objective properties:

Differentiation between two points

When two diffraction patterns of neighbouring points interfere with each other they generate interference maxima that are captured by the objective (intermediate image)



Objective properties:

Differentiation between two points

- To perceive two points as separate points, the objective needs to collect at least the 1st order maximum of the diffraction interference pattern

Light wavefront

Fine structure

Objective

2

1

0

1

2

1

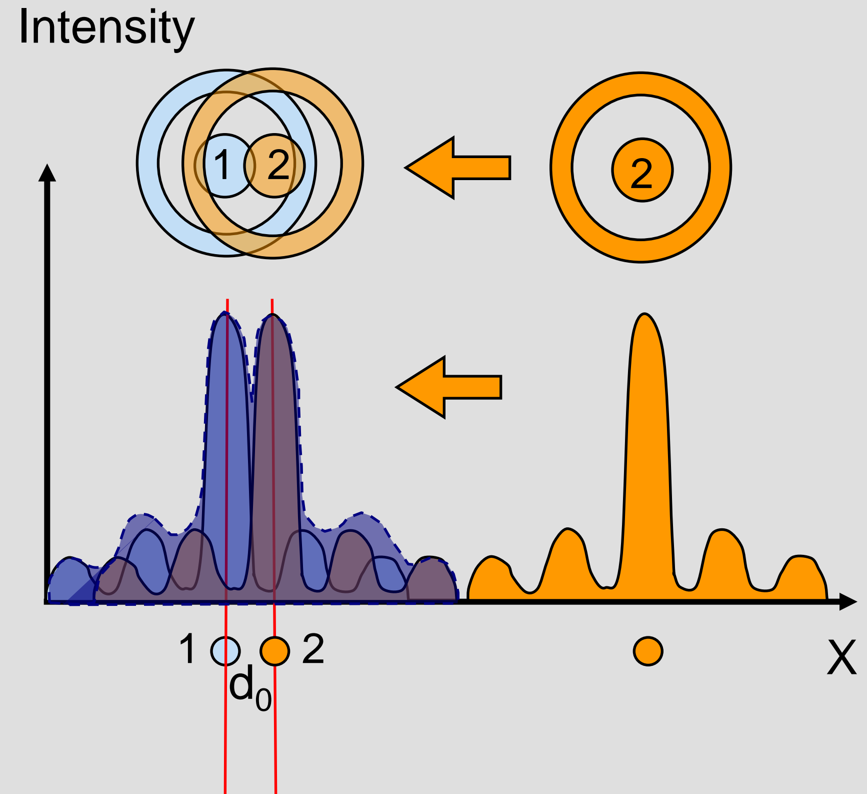
0

1

Differentiation between two points

Principal maximum of object 1 (centre of Airy Disc) coincides with first minimum of object 2:

- Minimum distance d_0 is reached (limiting resolution)
- Rayleigh-criterion to achieve sufficient contrast:
  Intensity of maxima 20% higher than intensity of minimum



Objective properties:

Differentiation between two points

Theoretical maximal resolution d_0

$$d_0 = \frac{\lambda}{n.A._{Objective} + n.A._{Condenser}}$$

Simplified formula (wo condensor) for resolution d_0

$$d_0 = \frac{\lambda}{2\, n.A._{Objective}}$$

Maximal resolution d_0 in reality

$$d_0 = \frac{1.22 \times \lambda}{2\, n.A._{Objective}}$$

Example

Green light λ = 550 nm, n. A. = 1.4 (Oil immersion)

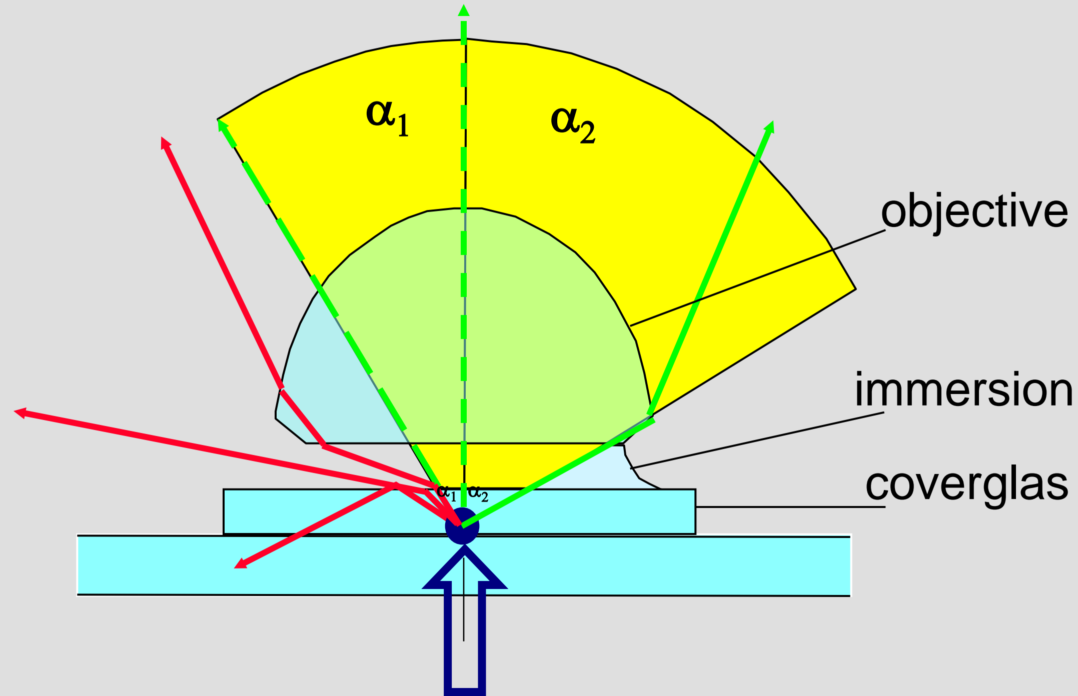
d_0 = 671 nm / (2 x 1.4) = 239 nm = 0.239 µm

Objective properties:

Immersion enables higher n.A.

Immersion prevents light rays from being deflected between two materials, e.g. between objective front lens and coverglas

- More order maxima of the diffracted light pattern can be collected
- Higher resolution!



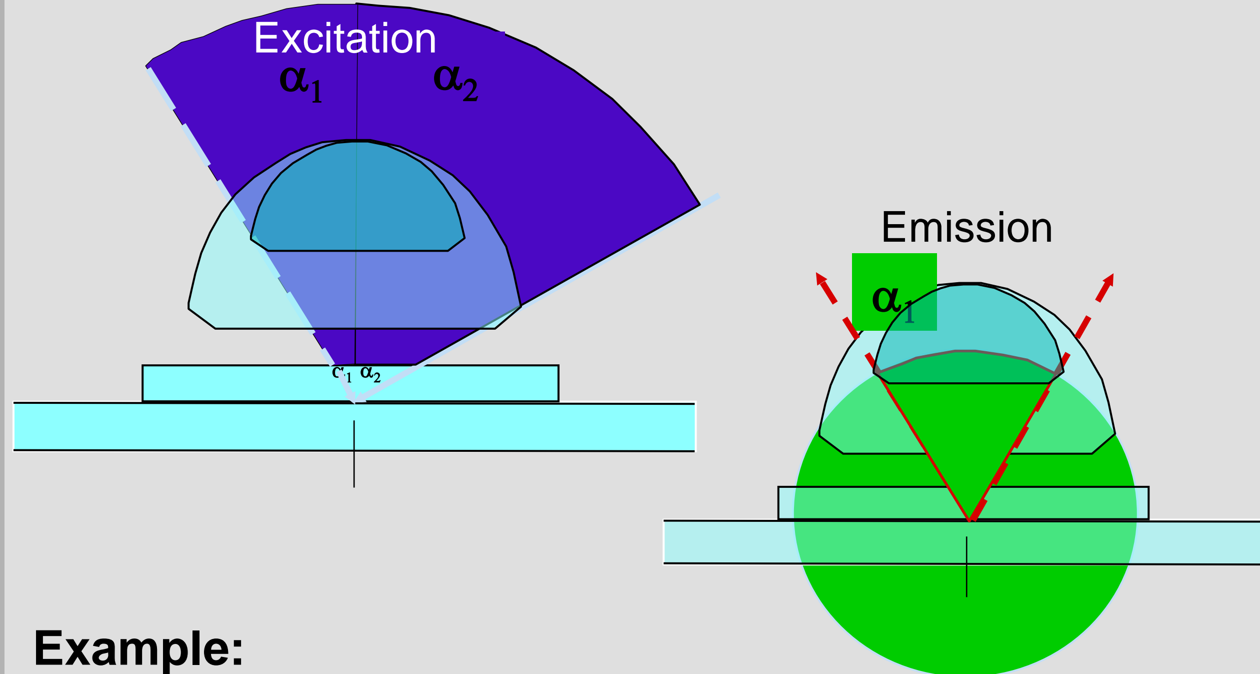
n. A. max $_{\text{Air}}$ = 0,95
n. A. max $_{\text{Immersol/ Immersol HI}}$ ~ 1,46 - ~ 1,57
(n. A. max $_{\text{Monobromnaphtalene}}$ ~ 1,68 Toxic!)

Objective properties:

Higher n.A. = More 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

- Fluorescence brightness theoretically growths with $(n.A.)^4$



Example:

N Achroplan 40x/ 0,65 Dry
vs
EC Plan- NEOFLUAR 40x/ 1,30 Oil

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

Objective properties:

Higher n.A. = More Brightness

CAVE

Next to n.A……

Additionally, glass properties and applied wavelength determine light transmission efficiency

Higher n.A. = Smaller depth of field

The depth of field is the z-thickness inside the object field that is imaged sharp together

The depth of field is increasing with the decrease of the n.A.

- $(n.A.)^2 \sim 1/$ Depth of Field

Example:

Depth of field α Plan-Apochromat 100x/1,46 = 0,23 µm
Depth of field Plan-Apochromat 20x/0,8 = 1,32 µm

Lens aberration

Types of lens aberrations

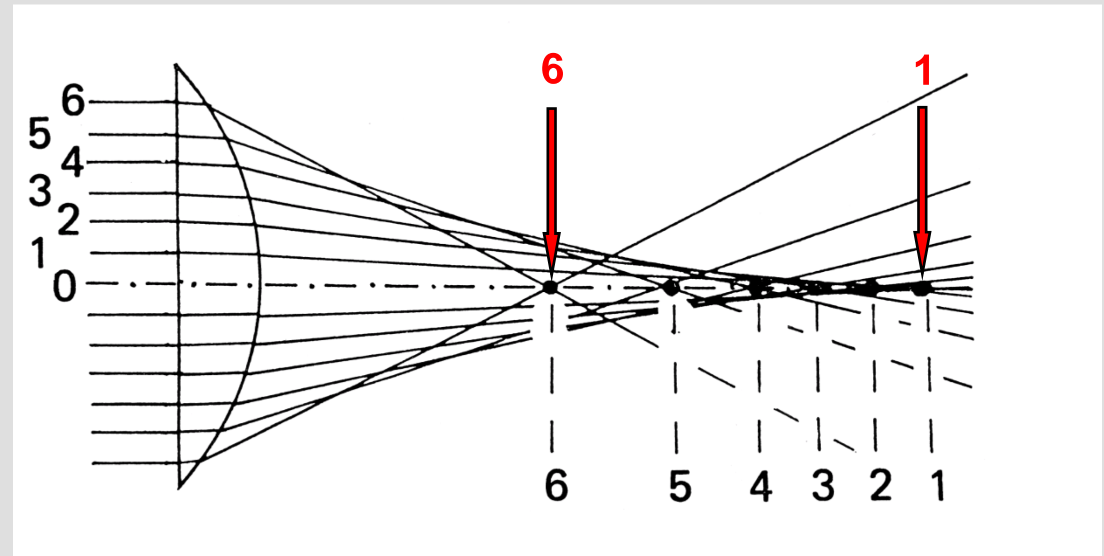
- Spherical aberration
- Chromatic aberration
- Curvature of the field
- Astigmatism
- Distortion
- Coma

Objective properties:

Spherical aberration

Difference of the focal point for rays traveling at different distances to the optic axis

- Blurred image
- Compensated by aspherical lenses

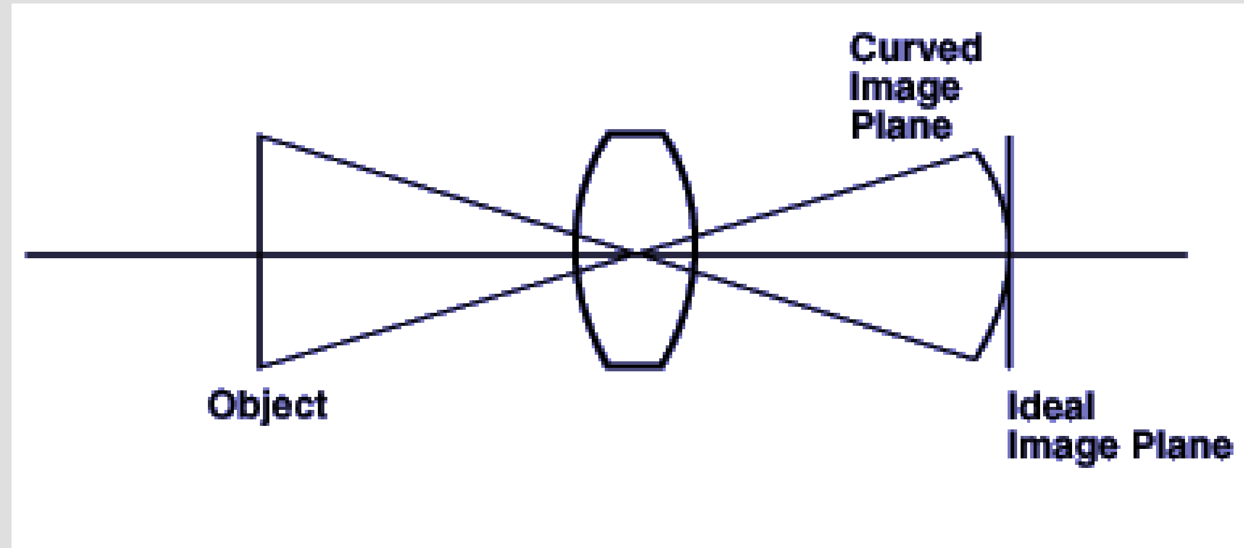


Objective properties:

Curvature of the field

Imaging through a curved lens surface causes a curved image plane

- ➢ Image with unsharp edges

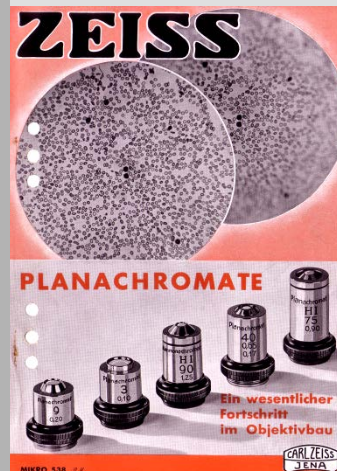


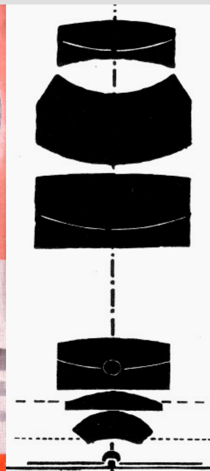
Objective properties:

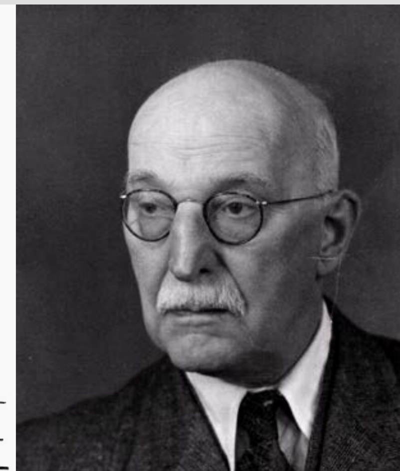
Flatness of field ("Plan-Objectives")

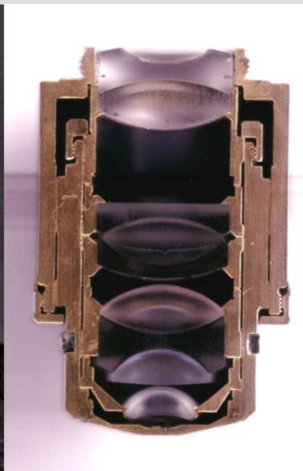
- Compensated by steeply curved lens surfaces at the back of the objectives and by a concave meniscus within the front lens
- "Plan-" correction invented by CARL ZEISS in 1938

APOCHROMAT	Flat Field	>> 25!
EC- Plan- NEOFLUAR	Flat Field	> 25!
W- N ACHROPLAN	Flat Field	~ 20
F- FLUAR	Flat Field	~ 17
FLUAR (non flattened!)	Flat Field	~ 14



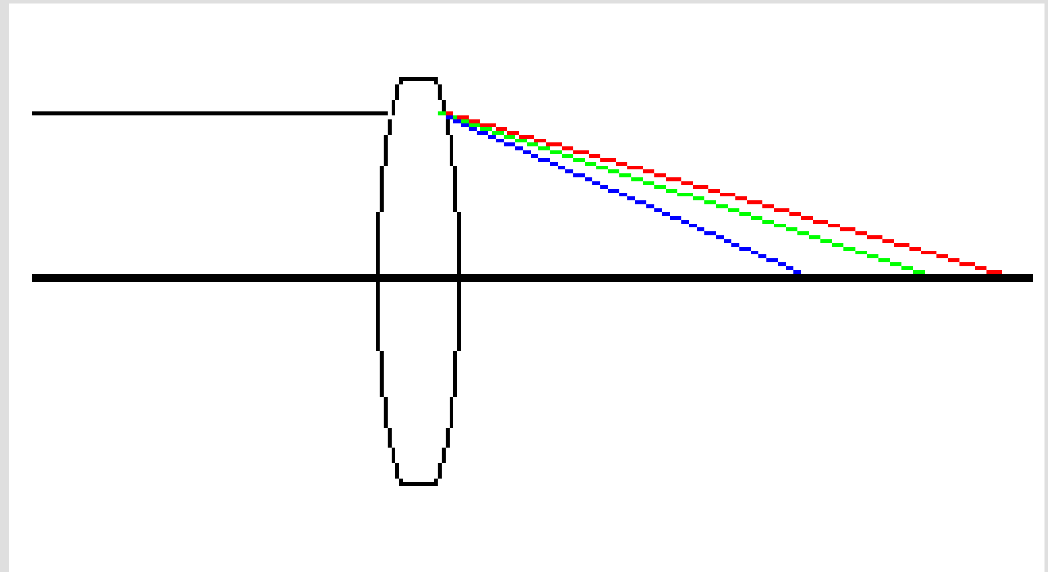






Chromatic aberration

Objectives have color artefacts (e.g. color fringes) in x/y and z

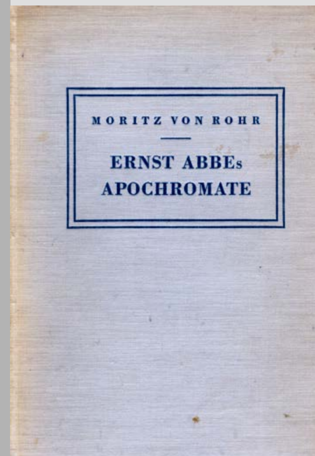


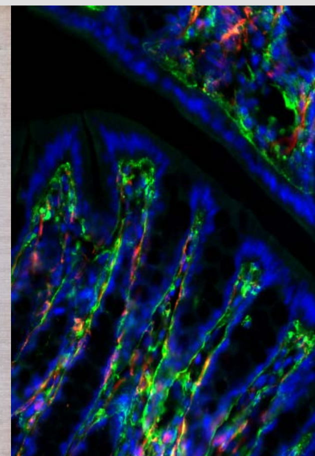
Objective properties:

Chromatic aberration

- Compensated by a combination of unique glasses with different color refractive properties
- "Apochromatic-" correction invented by CARL ZEISS in 1886

APOCHROMATs are fully colour corrected
for 3 – 4 spectral lines





Objective properties:

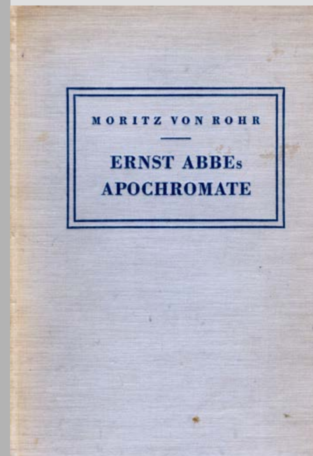
Chromatic aberration

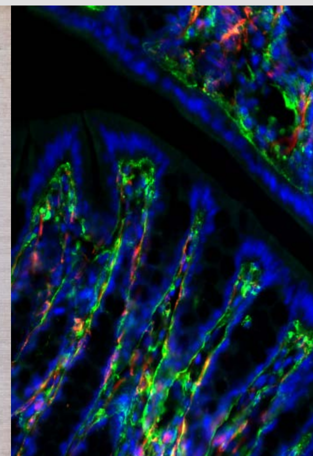
- Compensated by a combination of unique glasses with different color refractive properties

- “Apochromatic-” correction invented by CARL ZEISS in 1886

CARL ZEISS APOCHROMATs are fully colour corrected no longer for only 3 – 4 spectral lines, but **for a full spectral range** (this corresponds to a correction of up to **14 (!)** spectral lines on the „old scale“)

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





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How to choose the right objective

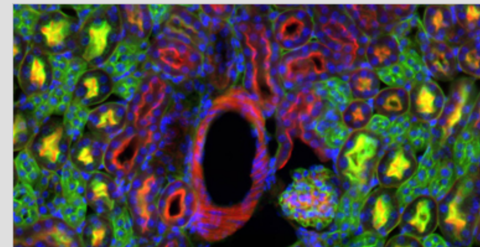
Magnification and Resolution

The optimal objective choice is dictated by the sample and the application

The optimal objective choice in microscopy follows a canon of simple questions….

What is the specimen size?

- Objective magnification varies (e.g. 10x to 150x)



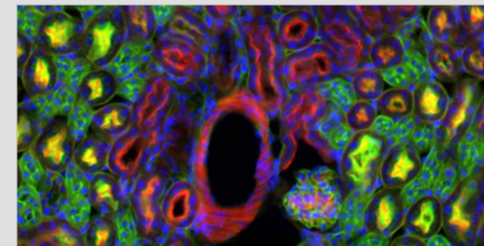
Magnification and Resolution

The optimal objective choice is dictated by the sample and the application

The optimal objective choice in microscopy follows a canon of simple questions….

What is the size of minute details inside the specimen?

- Determination of the required n.A.



Objective choice:

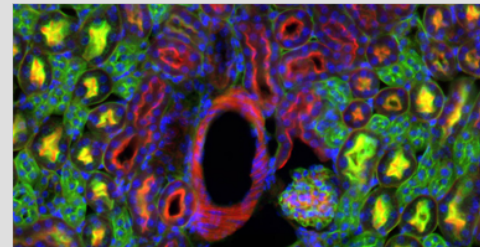
Magnification and Resolution

The optimal objective choice is dictated by the sample and the application

The optimal objective choice in microscopy follows a canon of simple questions….

What is the imaged field of view and required resolution?

- Definition of magnification in respect of n.A.
- Best compromise for Tiles imaging



Objective choice:

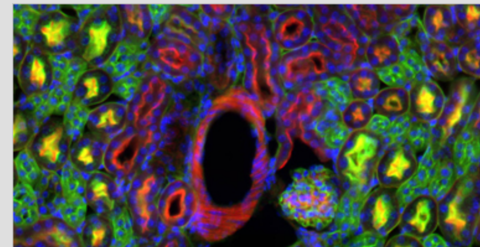
Brightness and Fluorescence

The optimal objective choice is dictated by the sample and the application

The optimal objective choice in microscopy follows a canon of simple questions….

How bright is my fluorescence signal?

- Objectives with a high n.A. are employed for weak signals
- E.g. Plan-Apo for 400-700 nm
- E.g. C-Apo or Fluar for UV



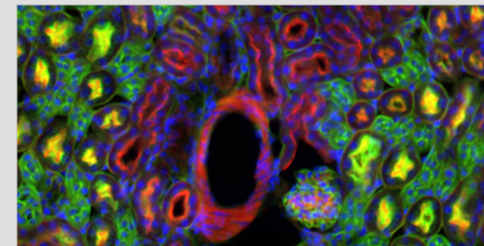
Brightness and Fluorescence

The optimal objective choice is dictated by the sample and the application

The optimal objective choice in microscopy follows a canon of simple questions….

How many fluorescent signals do you expect?

- Apochromatic objectives have best color match



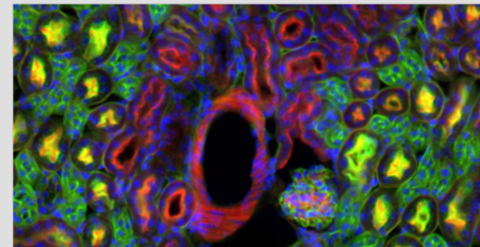
Brightness and Fluorescence

The optimal objective choice is dictated by the sample and the application

The optimal objective choice in microscopy follows a canon of simple questions….

Do you want to investigate colocalization with different dyes?

- Use C-Apochromatic ("C" = confocal) for best quantitative results on confocal microscope systems



Objective choice:

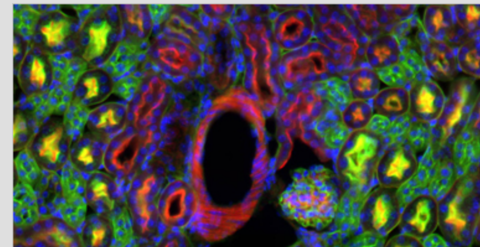
Water immersion

The optimal objective choice is dictated by the sample and the application

The optimal objective choice in microscopy follows a canon of simple questions….

Is the sample immersed within an aqueous medium?

- Water immersion objectives are recommended



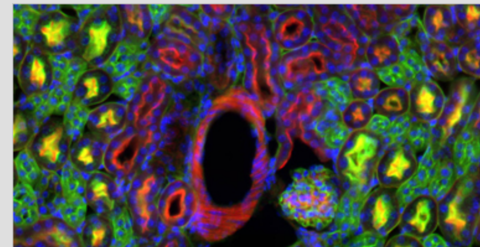
Water immersion

The optimal objective choice is dictated by the sample and the application

The optimal objective choice in microscopy follows a canon of simple questions….

Are the structures of interest very thick?

- Use water immersion objectives with Long-Distance (LD-) characteristics



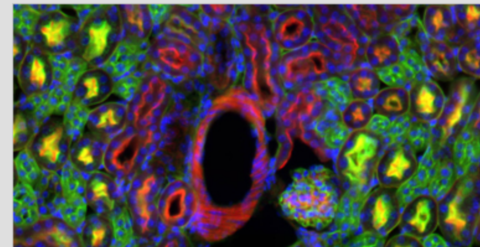
Water immersion

The optimal objective choice is dictated by the sample and the application

The optimal objective choice in microscopy follows a canon of simple questions….

Are the structures of interest uncovered (no cover glass possible)?

- Use water immersion objectives for direct front lens immersion



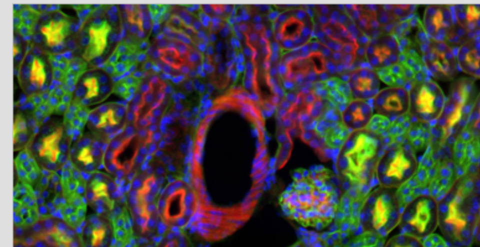
Brightfield contrasting techniques

The optimal objective choice is dictated by the sample and the application

The optimal objective choice in microscopy follows a canon of simple questions….

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

- Use strainfree POL-contrast objectives



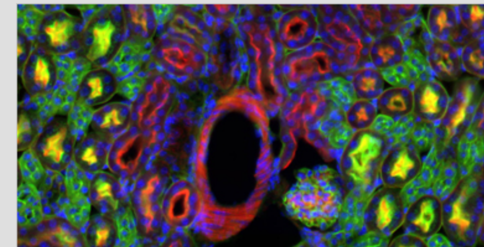
Brightfield contrasting techniques

The optimal objective choice is dictated by the sample and the application

The optimal objective choice in microscopy follows a canon of simple questions….

Are structures very thick in BF (100-200 µm)?

- Use DIC objectives



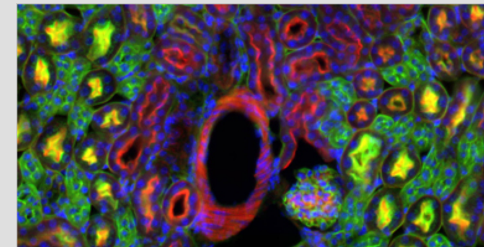
Brightfield contrasting techniques

The optimal objective choice is dictated by the sample and the application

The optimal objective choice in microscopy follows a canon of simple questions….

Are structures very thin in BF (<10 µm)?

- Strong contrast with Phase Contrast objectives



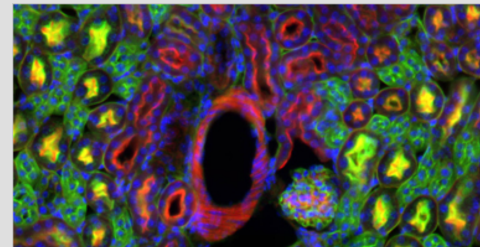
Brightfield contrasting techniques

The optimal objective choice is dictated by the sample and the application

The optimal objective choice in microscopy follows a canon of simple questions….

Are structures extremely thin in BF (<2 µm)?

- Best contrast with Darkfield



The Choice

(examplary)

confocal Laser Scanning Microscopes (e.g. LSM 880)

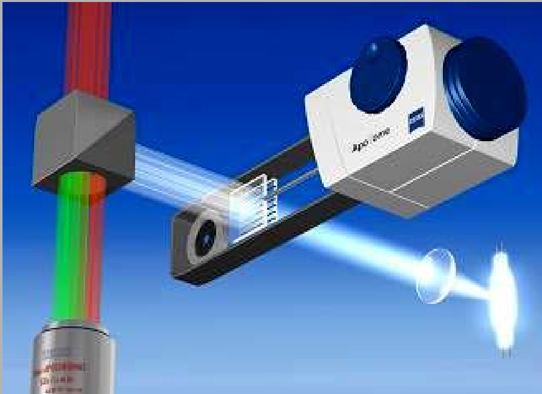
Objectives suitable for confocal work must allow to produce images with minimum spherical aberration, maximum signal strength and good contrast

- **For best results use C-Apochromats and LD C-Apochromats**
- **Alternative: Plan-Apochromats produce very good results too**

Objective choice:

ApoTome

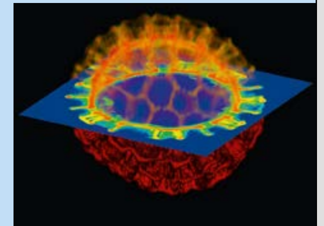
The ApoTome section thickness depends on the grid stripe thickness, grid frequency, objective n. A. and magnification



- **Use Plan-Apochromats as general workhorses**
- **For critical colocalization use C- APOCHROMAT 40x/ 1,2 W Korr**
- **When working distance is critical use LD C- APOCHROMAT 40x/ 1,1 W Korr UV-VIS-IR**

Deconvolution algorithms employs the Point Spread Function to calculate an optical section image

- In principal, all objectives work
- PSF needs to be known or measured
- Images with minimal aberrations are required
- To use a large field of view, it is recommended to employ Plan-Apochromat or C-Apochromat objectives



Objective choice:

Samples with strong impact of spherical aberration

- Cover glass thickness deviates from 0,17 mm (thinner is worse)
- Embedding medium with a refractive index little lower than 1,518

- **Typical sample: Multicolor and embedded**
- **Embedded in anti-bleaching media that has poor optical performance**

➢ **Workhorse:**
Plan-Apochromat 40x/0,95 Korr

Objective choice:
Samples with extreme impact of spherical aberration

- Cover glass thickness > 300 µm
- Petridish bottom, 1,2 mm
- Chamber glasses, > 3 mm

➢ **Only conventional widefield microscopy recommended with…**

LD EC Plan-Neofluar Korr objectives

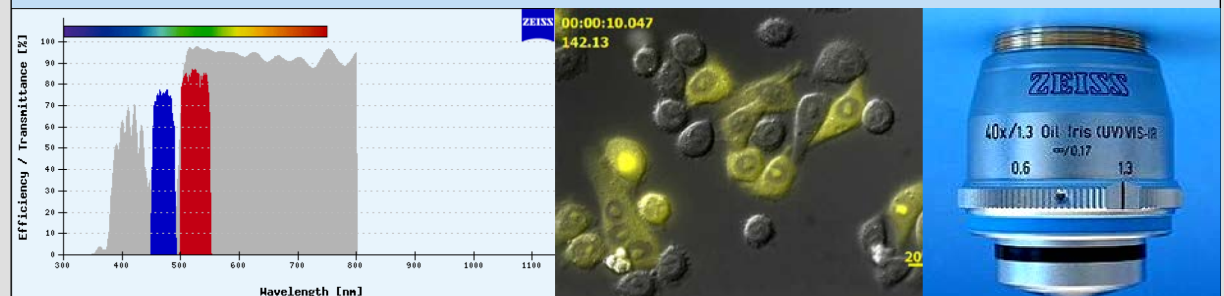
➢ **These samples do not allow high-resolution imaging work!**

Objective choice:

High Speed and High Resolution

For fluorescence imaging application, use HE- filter sets, light attenuators, maximum n.A. objectives and sensitive cameras

- UV/Ca^{2+}-Imaging: Fluar objectives
- Water-embedded: C-Apochromat
- Fixed sample: Plan-Apochromat
- Minute structures: α Plan-Aprochromat



CARL ZEISS Internet Objective Data Base

A dedicated objective data base is available on

https://www.micro-shop.zeiss.com

Objective Assistant

→ Deutsch

Print

Objective Assistant

pdf Brochure: Objectives from Carl Zeiss (5 MB)

→ Objectives Text Search

Objective Class	Magnific.	Contrast Method/Application	Options ◉ AND ○ OR
CP-Achromat	1,0x	B BrightField	Without Immersion
A-Plan	1,25x	BD BrightField/DarkField	Water
LD A-Plan	2,5x	DIC Differential Interference Contrast	SC
Achroplan/N-Achroplan	5x	RL DIC Reflected Light DIC	Clearing
W Achroplan/W N-Achroplan	10x	HC DIC High Contrast DIC	Silicone oil

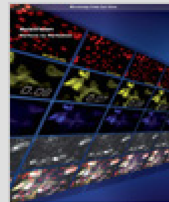
For multiple selection: hold [PC: 'Ctrl'-key] [Mac: 'Command'-key] down.

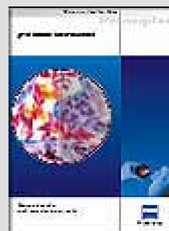
Search

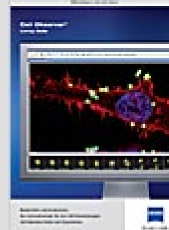
→ Description of Classes of Objectives

Objective Selection by Contrast Method/Application:

→ Phase Contrast
→ PlasDIC Contrast
→ DIC Contrast
→ Fluorescence
→ DarkField
→ Polarization Contrast
→ C-DIC Contrast
→ TIC Contrast
→ TIRF
→ ApoTome
→ Microdissection
→ Confocal Microscopy







We make it visible.