How to Choose the Optimal CARL ZEISS Objective

CARL ZEISS MicroImaging GmbH
Training & Application Centre, TASC
Objective Choice: 

Agenda

• Challenging Applications in Living Cell Microscopy
• Method Approaches in Living Cell Microscopy
• The Perfect Microscopical Image
• The Objective Properties
• The Objective Correction
• The Sample Properties
• The Sample Influence
• The Optimal Objective Choice
• Recommended Outfits
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

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)
- Optical sectioning with confocal pinhole techniques (e.g. Confocal Laser systems, Spinning Disc systems)
- Optical sectioning with evanescent fields (TIRF, SIRF)
- Mathematical approaches (e.g. 3D/2D Deconvolution)
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
General Aspects:
Objective design and function
The microscope objective properties are influencing:

- the resolution of the imaged structure (n. A.)
- the diffraction behavior of the captured sample light (Strehl value)
- amount of residual minimum spherical aberration
- the presence/absence of colour fringes (chromatical 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)
The diffraction pattern contains information about the diffracting structure.

The intermediate image is formed through interference of diffracted and undiffracted light (at least its 1st order maximum).

Capturing of diffracted light by the objective is necessary for image formation and resolution of details.

Objectives with a wide opening angle "observe" a wide angle in space and are able to capture more of the diffracted light than objectives with a small opening angle.

The image formation theory was founded by Prof. Ernst Abbe in 1872 at CARL ZEISS.

Objective correction: Numerical Aperture (n. A.)

Coarse structure

Fine structure
Objective correction: Numerical Aperture (n. A.)

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

Measure of the opening aperture of the objective:

Numerical Aperture = n. A. = n x sin a

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

\[ d_0 = \frac{\lambda}{2 \cdot \text{n. A.}} \]

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 Immersol ~ 1.46
(n. A. max Monobromnaphtalene ~ 1.68 Toxic!)
Objective correction:

Resolution and Numerical Aperture (n. A.)

Theoretical maximal resolution $d_0$

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

Simplified formula for resolution $d_0$

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

Maximal resolution $d_0$ in reality

$$d_0 = \frac{1.22 \times \lambda}{n. \text{A. Objective} + n. \text{A. Condenser}}$$

Example:

Green light $\lambda = 550$ nm, n. A. = 1.4 (Oil immersion)

$$d_0 = \frac{550 \text{ nm}}{(2 \times 1.4)} = 200 \text{ nm} = 0.2 \mu\text{m}$$
The wavelength has a strong influence on the resolution.

Blue light (shorter wavelengths) provide images with higher resolution compared to red light (longer wavelengths) illumination.

The wavelength determines distance between the maxima of the diffracted light:

Blue light is diffracted to a lesser extent than red light.

Objective correction: Wavelength depending image resolution

- Weak Diffraction
- Strong Diffraction
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:
  \( a = 90^\circ, \sin a = 1, \ n_{\text{air}} = 1 \)

- In practice:
  n. A. max_{\text{air}} = 0.95
  opening angle ca. 72°

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

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

n. A. max_{\text{Air}} = 0.95
n. A. max_{\text{Immersol}} \sim 1.46
(n. A. max_{\text{Monobromnaphtalene}} \sim 1.68 Toxic!)
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.)

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 \textit{theoretically} grows 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 \(2^4 = 16\) times brighter!
Objective correction: 

Resolution and 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.

\[
\text{Depth Field (µm)} = \frac{1000}{7 \times \text{n. A. Objective} \times \text{Magn. Total}} + \frac{\lambda (µm)}{2 \times \text{n. A. Objective}^2}
\]

\( (\text{n. A.})^2 \sim 1/ \text{Depth of Field} \)

The sharpness depth of the image on the sensor side is called “Depth of Focus” and is reciprocal with “Depth of Field”.

High n. A. objectives have a narrow depth of field.

Depth of field alpha Plan- APOCHROMAT 100/ 1,46 = 0,23 µm

Depth of field Plan- APOCHROMAT 20/ 0,8 = 1,32 µm!

\( \lambda = 0,55 \mu m, M_{\text{Eye piece}} = 10x \)
A point shaped structure is imaged by the microscope objective as a slightly blurred disc (limited by diffraction).

The diffraction behavior of an objective is described by looking at the intensity distribution inside the diffraction pattern (Airy disc).

The opening of objective (2) diffracts light.

The image of an object point (1) is a small disk surrounded by diffraction rings forming the Airy Disc (3).

The imaging of a point as a disc reduces resolution of the microscope by the factor “1,22”.

**Objective correction:**

**Diffraction limited image formation**
Objective correction: Resolution limits due to diffraction limited image formation

Airy Discs of 2 points wide apart:
2 object points clearly imaged as 2 separate 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)

Superimposing of the intensity profiles at limiting resolution meets Rayleigh-criterium:
Intensity of maxima 20% higher than intensity of minimum between maxima
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: Spherical Aberration and Diffraction Behavior

Spherical aberration is usually explained by geometrical optics.

Today, it is also described by non-geometrical parameters using the wave nature of light analyzing the diffraction behavior (Airy discs) and the corelated image brightness (Strehl value).

Deviations from the theoretically expected „best pattern shape“ are caused by spherical aberration and are therefore a very good measure for image quality.

Optimal

Non-Optimal
Objective correction: Airy Disc FWHM

Best Airy disc shapes have a narrow „full-width at half maximum“ (= FWHM), compared to a non-optimal pattern.
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

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

The correction collar moves a compensating lens group inside the objective
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.

Objective correction: **Multi- Immersion Korr LCI- objectives**

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 too low) has to be compensated in water embedded cell preparations.

We offer a full magnification range of multimersion 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 requested, go for the LCI-objective.
Objective correction: Long working distance
LD- objectives

Objectives specially designed for a cover glass thickness range between 0 – ~2 mm have a large free working distance. They are called LD- objectives.

LD- objectives usually have (strongly) reduced apertures for a given magnification.

LD EC Plan- NEOFLUARs are not recommended for optical sectioning (ApoTome, SD) or DCV work.

They can be employed to give a superimposed topography image in DIC (Nomarski) or phase contrast.

LD- objectives for high resolution work are our LD APOCHROMAT- type objectives.
Objective correction: Apochromasie

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

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

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

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

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 40/ 1.2 W Korr UV- VIS- IR is fully colour corrected from ~ 365 to ~ 900 nm
Objective correction: Focus match for the different colours

The focus varies with different wavelengths

The focus match for different colours is given in Raleigh Units (R.U.)

1 R.U. = λ / n. A^2

Focus deviations of +/- 2 R.U. are practically tolerable

The higher the apochromasie the better the colour match over a wider spectral range

Example, curves valid up to ~2005
Objective correction: Glass Transmission and Matched Coating

Objective types differ also in their transmission range

In a CARL ZEISS objective, anti-reflex coatings must still work for the full specified transmission range

The anti-reflex coating technology was invented by Prof. Adolf Smakula in 1936 at CARL ZEISS

<table>
<thead>
<tr>
<th>UV(A)</th>
<th>VIS</th>
<th>(N)IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC Plan- NEOFLUAR</td>
<td>-</td>
<td>436</td>
</tr>
<tr>
<td>Plan- APOCHROMAT</td>
<td>365</td>
<td>-</td>
</tr>
<tr>
<td>Plan- APOCHROMAT W</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C- APOCHROMAT</td>
<td>365</td>
<td>405</td>
</tr>
<tr>
<td>C- APOCHROMAT UV-VIS-IR</td>
<td>365</td>
<td>-</td>
</tr>
<tr>
<td>(ULTRAFLUAR</td>
<td>320</td>
<td>365</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>CARL ZEISS APOCHROMAT types</th>
<th>Flat Field</th>
<th>&gt;&gt; 25!</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC- Plan- NEOFLUAR</td>
<td>Flat Field</td>
<td>&gt; 25!</td>
</tr>
<tr>
<td>W- N ACHROPLAN</td>
<td>Flat Field</td>
<td>~ 20</td>
</tr>
<tr>
<td>F- FLUAR</td>
<td>Flat Field</td>
<td>~ 17</td>
</tr>
<tr>
<td>FLUAR (non flattened!)</td>
<td>Flat Field</td>
<td>~ 14</td>
</tr>
</tbody>
</table>
Objective correction: Free Working Distance

The free working distance (FWD) 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.

Precondition:
Cover glass thickness
D = 0.17 mm and focused structure is directly attached to the cover glass underside.

All dry and immersion objectives D = 0.17 mm demand a closely attached specimen regardless of FWD number due to spherical aberration correction for D = 0.17.

With immersion objectives D = 0 the FWD exactly tells you how deep you can penetrate into the sample.
The Objective Colour Code

Today, for ease of use, all microscope objectives follow a colour code that allows immediate recognition of important objective parameters (e.g. magnification, n. A.)

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

The sample properties

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

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

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

can have an impact on additional and artificial amplification of colour fringes (additional CVD due to dispersion problems)
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 blur.

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.

**Diagram:**
- **Cover Glass Thickness versus Half-Width Intensity Distribution**
- D = 0.17 mm
Sample Influence:  

Contrast (Signal-to-background noise ratio)

Image blur 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.
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.
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.

Heat has a negative impact on the lifetime of complex objectives (delamination of optical cement)!

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.
External Influence: Focus Drift

Long-time experiments with living cells are difficult to carry out also due to focus drift (x, y, z) problems.

A focus drift mainly is the result of thermal influence on the geometry of objective, nosepiece, stand and stage (open windows, air condition varies temperature, too short waiting time after switching on all components).

By creating a thermal equilibrium the focus will practically be stable.

The first long-time imaging system was introduced to microscopy by Dr. Kurt Michel in 1952 at CARL ZEISS.

To overcome focus shift over time problems (drift), the environment must have a constant temperature for at least 4-8 hrs before starting a long-time experiment.

A stabilized thermal environment can be achieved with our incubator XL and a constant room temperature (air condition must be stable as well).

Additionally, advanced focus re-adjusting devices, e.g. Definite Focus™ are recommended sometimes (no z-stacks!)
How to choose the correct objective
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)
The optimal objective choice in microscopy of living specimens follows a canon of simple questions:

What is the size of minute details inside the specimen?

Objective resolution (n. A.) is defined (e.g. 1,46)
The optimal objective choice in microscopy of living specimens follows a canon of simple questions:

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

1. **What is the specimen size?**
   - Objective magnification as such is defined (e.g. 10x, 100x or 150x)

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3. **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)
The Optimal Objective Choice: Brightness and Fluorescence

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, 40/1.3; for UV use C-APO or FLUAR)
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
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" = Confocal)
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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 in microscopy of living specimens follows a canon of simple questions:

- Are those structures very thick?

Water immersion with LD-water immersion objectives
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
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

- **Are those structures very thick?**
  - Water immersion with LD- water immersion objectives

- **Are the structures uncovered (no cover glass possible)?**
  - Water immersion objectives for direct front lens immersion
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
The optimal objective choice in microscopy of living specimens follows a canon of simple questions:

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

Optical sectioning with DIC (use DIC-objectives, if possible)
The optimal objective choice in microscopy of living specimens follows a canon of simple questions:

Are the structures extremely thick (> ~ 200 µm)?

Contrasting with Oblique Illumination (e.g. Dodt contrast)
The optimal objective choice in microscopy of living specimens follows a canon of simple questions:

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

Strong contrast with Phase Contrast
The optimal objective choice in microscopy of living specimens follows a canon of simple questions:

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

Best contrast with Ultra Darkfield
The Optimal Objective Choice: Contrasting Techniques

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

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  - Special strainfree POL- contrast objectives

- **Are the structures very thick (> ~ 100 - 200 µm)?**
  - Optical sectioning with DIC (use DIC-objectives, if possible)

- **Are the structures extremely thick (> ~ 200 µm)?**
  - Contrasting with Oblique Illumination (e.g. Dodt contrast)

- **Is the specimen very thin (< ~ 10 µm)?**
  - Strong contrast with Phase Contrast

- **Is the specimen extremely thin (< ~ 2 – 0,02 µm)?**
  - Best contrast with Ultra Darkfield
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%)
Optical Conflicts: Ph-/ LD- Objectives

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Older objectives

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)
Optical Conflicts: Ph-/ LD- Objectives

Older Objectives

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LD objectives

Not suitable for high-resolution tasks, due to lower n. A.
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LD objectives
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The optimum microscope choice
The ApoTome section thickness depends on the grid stripe thickness, grid frequency, objective n. A. and magnification.

The section thickness is around 1 R. U.

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Outfit: ApoTome

No LD- and/or Phase contrast objectives recommended
No older ICS objectives recommended
Use FLUAR only for smaller central fields of view

100x objectives only if required by structure dimensions; for best UV transmission use alpha Plan- FLUAR 100/1.45 or alpha Plan- APOCHROMAT 100/1.46 (e.g. high resolution work on organelle level)

Plan- APOCHROMAT 20/0.8 (overview topography, e.g. developmental biology)
Plan- APOCHROMAT 40/1.3 (total cell morphology, Colibri illumination, e.g. slow moving cells)
Plan- APOCHROMAT 63/1.4 (general high resolution work)

For critical colocalization use C- APOCHROMAT 40/1.2 W Korr or, if working distance is critical, LD C- APOCHROMAT 40/1.1 W Korr UV-VIS-IR

If working conditions are very special, e.g. water embedded thick samples, you use the outfit recommended there.
Outfit: Yokogawa Spinning Disc

The Yokogawa Spinning Disc (SD) system works with a synchronized multiple helical lens/pinhole arrangement. The multiple pinhole diameter is fix.

The section thickness is around 1 R. U. only for objective magnification 100x.

Due to the number of pinholes the SD can acquire up to ~100 images/sec (EM-CCD camera).

Low bleaching impact is beneficial.

FLUAR only for UV-applications, central field of view is useable only.

Due to the fixed pinhole size and the speed of the rotating disc, a 100x objective is the paramount here due to n. A. and magnification.

For best results, the structure should be close to the cover glass and not be thicker ~ 30 – 40 µm. Single cell layers are perfect samples.

For best confocality use alpha Plan- APOCHROMAT 100/1,46 (UV) VIS-IR, also Plan- APOCHROMAT 40/1,30 (UV) VIS-IR and 63/1,4 work well here.
In Confocal Microscopy no special objective parameters are required.

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

For multichannel applications the focus match for the used different colours should be perfect.

Nevertheless, if confocal work with UV/ blue excitation (e.g. DAPI- labeled samples) is done, the objective must allow a good transmission and *apochromatic* focus correction for the *laser line* 405 nm. This is difficult to achieve.

The 40:1 magnifications of *C- APOCHROMAT* and *LD C- APOCHROMAT* have the highest apochromatic correction (= narrowest focus match for a given correction range), also including 405 nm (405nm laser diode). That is why they are marked „UV“ and not „(UV)“.

„UV“= **full apochromatic correction (focus match)** including the engraved spectral range

„(UV)“= sufficient to very good **transmission** in that engraved spectral range

Usually, normal Plan- APOCHROMATs have a perfect apochromasie from at least 436 – 644 nm. For monochromatic imaging, a lot of them work from 405 – 950 nm. If monochromatic work at 405 nm is done, a lot more objectives will deliver a perfect image (in a different focal position though).
Outfit: Deconvolution

The Deconvolution algorithms employ the exact theoretical diffraction parameters (PSF= point spread function) of our objectives.

The diffraction behavior can also be measured with small fluorescent beads, resulting in a calibrated PSF.

Only objectives with a known diffraction behavior (PSF) will work here.

Additionally for highest accuracy, piezo-focussing devices for a single objective or a stage piezo focus have to be used.

For DCV all objectives must be set to conditions that produce images with minimum spherical aberration. For DCV work, Korr objectives can minimize the spherical aberration strongest.

In DCV work, objective choice depends mainly on structure size.

Deconvolution is mainly used on small object fields (calculation time is field size depending). Because of that, our Plan- APOCHROMAT 40/0.95 Korr, 40/1.30 and 63/1.4 are standard objectives for this application.

For DCV work with water embedded specimens our LD LCI Plan- APOCHROMAT 20/0.8 Korr UV-VIS-IR and the LD C- APOCHROMAT 40/1.1 W Korr UV-VIS-IR are best.
Outfit:

In TIRF, the illumination light is totally reflected by the cover glass. The electromagnetic field of the reflected light can still excite a fluorescence within a very thin layer of ~ 50 - 300 nm (evanescent field). The evanescent field thickness equals the optical section thickness.

To produce total reflection, the illumination angle has to be very steep (> 72°).

Only objectives of maximum n. A. (> 1,4) provide large enough TIRF-illumination angles.

TIRF

As TIRF needs high objective apertures for ease of angle inclination adjustment, highest n. A. values are essential here. The recommended TIRF aperture is 1,46 for ease of instrument use.

For 1-channel work in UV, our alpha Plan-FLUAR 100/1,45 is a good value.

For best colour match in multicolour TIRF use the alpha Plan-APOCHROMAT 100/1,46 (UV) VIS-IR. This is the universal TIRF application objective. As many TIRF samples are very thin, the phase contrast version of this objective is useful.
Outfit:

Samples with strong impact of Spherical Aberration

Cover glass thickness deviating from 0.17 mm (thinner is worse)

Glass thickness still between ~ 0.10 - ~ 0.25 mm

Embedding medium with low refractive index compared to n= 1.518

Typical samples are fixed, embedded multi-colour labelled fluorescence specimens

A lot of the anti-bleaching embedding media used here have a poor optical performance. As long as the cells are close to the cover glass the objective will deliver good structure rendition and perfect signal to background noise ratio.

The workhorse objective is our unique (no focus shift during adjustment!)

Plan- APOCHROMAT 40/ 0.95 Korr
Outfit:

Samples with extremely strong impact of Spherical Aberration

Cover glass thickness
300 µm and thicker

Petri dish bottoms (~1,2 mm) etc.

Chamber glasses (> ~3 mm)

These working conditions are related to very thick cover glasses

In conventional widefield microscopy we recommend our LD EC Plan- NEOFLUAR Korr- objectives

Usually these sample conditions do not allow any high-resolution imaging work!

Heating chamber glasses are of 0,17 mm thickness! LD- objectives are used here due to influence of heat
Outfit: Thicker Samples (Water) with Cover Glass

When imaging living cells, results are perfect if the structures are attached to the underside of a 0,17 +/- 0,01 mm cover glass.

Unfortunately living cells are often remote from there, or cell clusters are looked at, as in developmental biology.

UV/ Ca2+- Imaging: FLUAR 10/0,5 (no flat image, strong z- colour shift between fluorescence channels)

Water embedded specimens: C- APOCHROMAT- type objective

Fixed samples of medium thickness: Plan- APOCHROMAT 40/1,30 (UV) VIS-IR
Outfit:

Extremely Thick Samples (Water) with Cover Glass

If water embedded samples are covered with a standard cover glass and specimen is in a thickness range of ~ 400 µm use our LD C- APO

We strongly recommend to use our LD C- APOCHROMAT 40/ 1,1 UV-VIS-IR if the full transmission range and best colour match in z is required
Outfit:

Extremely Thick Samples (Water) and no Cover Glass possible

In neurobiology, cellular regions of interest are often hidden inside a thick layer of tissue (e.g. thick brain slice)

For maximum free working distance: **N W ACHROPLAN** used with Axio Examiner tube lens

Highest image definition (also in DIC): **Plan- APOCHROMAT 20/ 1,0 W**

With this objective, additional magnification should be achieved by using our **magnification changer 4x**

Recommended contrasting method is a special, rotatable oblique illumination, the **Dodt contrast (Axio Examiner)**
In live cell imaging the topography of thicker structures has to be corellated to the superimposed fluorescence image. Sometimes the samples are not single cell layers, as in developmental biology.

High- Resolution of Thicker Samples with DIC (Nomarski)

Usually, DIC (Nomarski) is recommended if samples have to be „optically sectioned“. A typical application is developmental biology

A perfect image can be obtained using the following specially suitable objectives:

- **Plan- APOCHROMAT 20/ 0,8**
- **Plan- APOCHROMAT 40/ 1,30 DIC (UV) VIS-IR**
- **Plan- APOCHROMAT 63/ 1,4 DIC** (use oil- immersion DIC- condensor n. A. 1,4 for minimum optical section size)

**Senarmont- DIC** should be set to its bluish background colour

**Plan- APOCHROMAT 63/ 1,4 DIC**: With slightly thicker samples (mostly having a poorer contrast due to spherical aberration) use HR- slider, with thin structures (also having a low DIC- contrast) employ HC- slider

Differential interference contrast (DIC) allows good contrast with fully opened aperture: Optical sectioning is possible
Outfit:

High Resolution of Thin Samples with Phase Contrast

Structures < ~ 10 µm are too thin to provide a good DIC-contrast

Phase contrast works better here

Phase contrast is very sensitive against spherical aberration and dirt

Phase contrast was developed in 1941 by CARL ZEISS

Phase contrast is superior to all other contrasting techniques when it comes to look at structures with a thickness of ~ 5 – ~ 10 µm

Sample must be attached to the underside of the cover glass

In phase contrast the allowed chromatical aberration residues from the objective are more visible compared to other optical contrasting methods (use interference green filter). That is why Plan-APOCHROMAT-type objective deliver the sharpest image

Usually objective magnifications from 40x – 100x are needed in phase contrast microscopy, because suitable samples are small

For high-resolution work we recommend:

Plan- APOCHROMAT 63/1,4 Ph 3
Alpha- Plan APOCHROMAT 100/1,46 Ph 3

For maximum visual contrast use: EC Plan- NEOFLUAR 63/1,25 Ph3
Outfit:

Extremely Thin Samples/ Small Structures in Ultra Darkfield

If structures are isolated, extremely minute in size (< ~ 0,5 µm), e.g. single forming microtubular structures, virus particles, colloids, etc. ultra-darkfield is needed.

Ultra-darkfield is done with an oil-immersion cardioid „ultra-condensor“ n. A. 1,2/1,4. Objective aperture is between ~ 0,9 and ~ 0,7 (Iris).

Ultra-darkfield was invented by Prof. Henry Siedentopf in 1905 at CARL ZEISS.

You need an apochromatic objective with the possibility to reduce the n. A. by means of an in-built iris.

Only apochromats provide a darkfield with best signal to background noise ratio. Also structures below the resolution limit of the microscope will have maximum contrast.

Typically the Plan-APOCHROMAT 40/1,0 Oil Iris is used here. If maximum magnification is needed, we recommend our new projection optics (housed inside the magnification changer 4x 60N-60N).

In ultra darkfield, the thickness of the slide must not exceed 1,2 mm, all optical parts have to be meticulously clean.
Outfit:

Small Birefringent Structures in Polarized Light

If cellular structures are orientated, they often show a weak birefringence, e.g. microtubules within the spindle apparatus.

Linear polarized light and strain-free immersion objectives are used for detection.

Sometimes, cellular structures are birefringent. Usually it is caused by a high orientation grade/parallelility of the macromolecules composing these structures.

The most prominent example is the microtubular spindle apparatus responsible for the proper distribution of the chromosomes during cell division.

Also, orientated molecules will have a different vibration plane of the emitted fluorescence light which sometimes has to be detected as well.

Our Plan-APOCHROMAT 150/1.35 DIC Glyc Korr VIS-IR is also recommended for such extreme detection applications of weak birefringence.

It is recommended to use fully rotatable analyzer and polarizer as well as a stage of at least rotation capability. The immersion principle and the small exit pupil diameter $d = 2.96 \text{ mm} \ (d = 2f \times \text{N.A.}; \ f = 164.5/M)$ of this objective makes it especially suitable for such work.
Outfit:

Automatized image acquisition of extremely large object fields with high resolution is done with multiple single shot images (high aperture) stitched together. This is called MosaiX.

For MosaiX a motorized stage and a low-power high n. A.- objective is needed.

High- Resolution Imaging of Large Object Fields (MosaiX)

- Use an objective with an as low magnification as possible (object field size/acquisition time) but highest aperture. Do not use immersion objectives, as the immersion film will rip off during acquisition!

- Perfect MosaiX objectives are our Plan- APOCHROMAT 10/0.45 and especially the Plan- APOCHROMAT 20/0.8
Outfit: High-Speed and High-Resolution

If high speed and high resolution have to be merged within a fluorescence imaging application, HE-filter sets, light attenuators, maximum n. A. objectives and sensitive cameras are paramount.

Spherical aberration should be removed for best contrast/signal to background noise ratio.

- UV/ Ca$^{2+}$- Imaging: FLUAR-type objectives (no flat image, strong z-colour shift between fluorescence channels)

- Water embedded specimens: C-APOCHROMAT-type objective

- Fixed samples of medium thickness: Plan-APOCHROMAT 40/1.30 (UV) VIS-IR

- Minute structures: alpha Plan-APOCHROMAT 100/1.46 UV-VIS-IR
Outfit:

Maximum objective magnification is required, if structures are at the border of the resolution of the light microscope. Such structures can only clearly be detected if they are quite isolated and no spherical aberration is present.

We have designed a special high-magnification n. A. 1,35 glycerol-immersion objective:

**Plan-APOCHROMAT 150/1,35 DIC Glyc Korr VIS-IR.** In monochromatic work this objective is also perfect for 405 nm and 850 nm (diffraction limited).

Results are good, only if spherical aberration is low, no background structures overlaying and objects are directly attached to a 0,17 +/- 0,01 mm cover glass!

Additional magnification with superb contrast retaining comes with our new magnification changer outfittable with a maximum magnification projection module 5,0x.
We offer a vast amount of unique support through your CARL ZEISS sales force, a powerful in-house application department and via the Internet

A dedicated objective data base is available on
https://www.micro-shop.zeiss.com
We make it visible.