ZEISS Celldiscoverer 7

High quality LSM imaging with autocorrection objectives using plastic labware.

Seeing beyond

Authors: Sandra Orthaus-Müller, Volker Döring, Frank Vogler, Harald Schadwinkel
Carl Zeiss Microscopy GmbH, Germany
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ZEISS Celldiscoverer 7 – an automated live cell imaging system designed to make complex microscopy simple – harbors many automation functions such as automatic sample recognition, measurement of bottom material and bottom thickness, an auto-calibration routine for micro-well-plates, an auto-immersion water objective and a variety of autofocus options to name but a few. All these features were assembled in this machine to enable easy execution of sophisticated experiments for various types of samples and sample carriers.

Introduction

The optical concept of this boxed microscope consists of two elements: Up to four objectives are situated in a 4-position objective turret underneath the sample and a 3-position magnification changer enables fast and easy switching of the total magnification of each objective. In this way, 12 different combinations are available allowing a range from 2.5× up to 100× magnification with high NA (see figure 1). In addition, all objectives in the Celldiscoverer 7 feature apochromatic correction to enable artefact-free multicolor imaging.

To ensure optimal environmental conditions for live cell experiments, the front lenses of the objectives as well as the bottom and lid of the sample chamber are heated to the desired temperature.

The three objectives with the highest numerical apertures include a motorized autocorrection ring. In combination with the information regarding the sample carrier bottom material (including its refractive index and thickness – both are determined automatically upon sample loading), optimal image acquisition is guaranteed by adjusting the objective to the optimal autocorrection ring position (see figure 2). The automatic bottom material detection corrects for any refractive index mismatch, while the automatic bottom thickness measurement is used to avoid spherical and chromatic aberrations. For an in-depth review of the influence of objective and sample features as well as the advantage of autocorrection optics please refer to the info box.

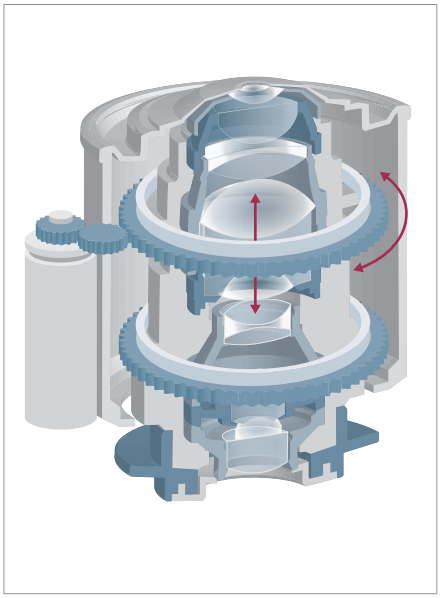


Figure 2 *Autocorrection objectives. ZEISS Celldiscoverer 7 objectives include an autocorrection ring to automatically adopt to bottom material and thickness. This way an optimal image quality is ensured for all sample carriers in every acquisition.*

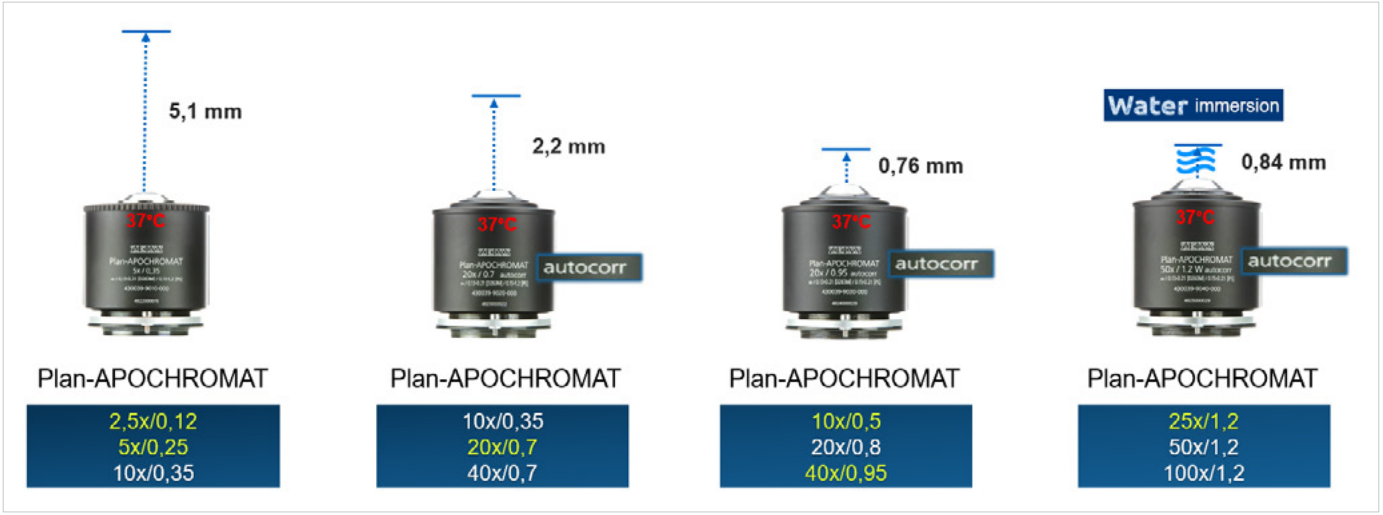
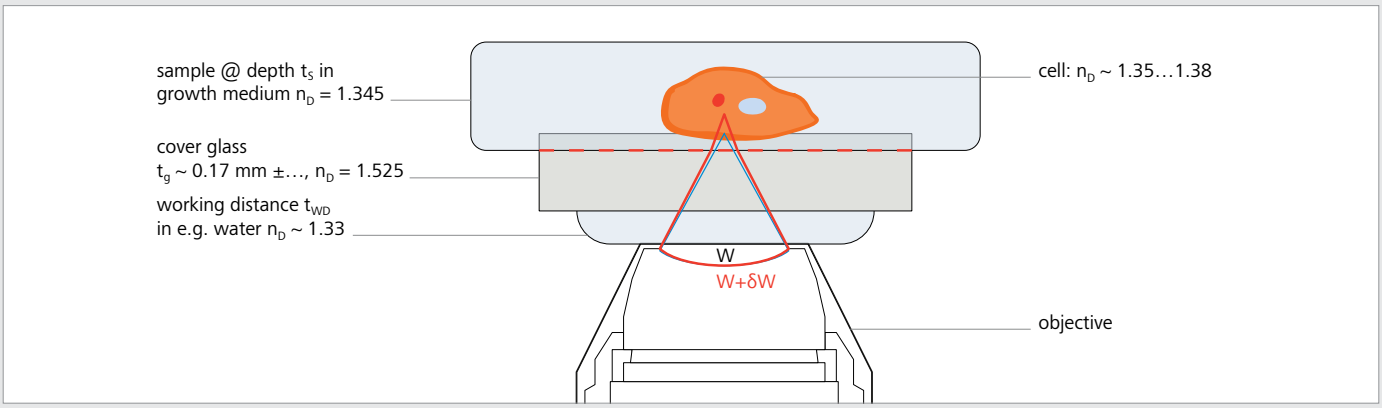


Figure 1 *Overview of ZEISS Celldiscoverer 7 objectives. In combination with the 3-position magnification changer, a total of 12 magnifications with individual numerical apertures can be used.*

Autocorrection Objectives – a powerful tool to optimize image quality

Microscope objectives, especially those with high *numerical apertures* (NA) are masterpieces in optics and precision mechanics. Superb image quality and – for confocal systems – highest signal levels ensure that optical aberrations are only magnitudes of much less than the wavelength of light. These requirements translate to the tightest tolerances for the elements INSIDE the objectives. Each of the individual lenses have their surfaces polished to within nanometers in terms of accuracy, they are mounted with micrometer precision, and the optical glass that is used has a tolerance down to ±0.0001 for the refractive indices.

In addition to the objective, there are several "optical elements" that have an equal contribution to the image quality, but the thicknesses (t_x) and refractive indices (n_x) of sample, cover glass, immersion medium, and sample embedding medium are usually not so compliant in terms of tolerance.



Deviations in the thicknesses t_x and refractive indices n_x from the nominal values result in aberrations, and these in turn lead to deterioration in image quality and reduced signal levels. In addition, the values of t_x and n_x are influenced by changes in wavelength and temperature but the impact of this effect is negated in ZEISS Celldiscoverer 7 since there is robust temperature control preventing any temperature fluctuations. The Strehl ratio S gives the aberrated intensity of a point-like object at the image point divided to the intensity without any aberrations present.

1) $$S = \frac{1}{\pi^2}\left|\iint e^{i2\pi\Delta W(\rho,\theta)}\rho d\rho d\theta\right|^2$$

Here the wavefront or optical path difference ΔW is integrated over the complete objective opening or pupil (with radial and angular coordinates ρ and θ) and contains the aforementioned aberrations in all layers. This includes especially *spherical aberration* which is the most critical aberration with high NA objectives:

2) $$\Delta W = \sum_{all\ t_x\ and\ n_x} t_x \cdot \left(\sqrt{n_x^2 - NA^2\rho^2} - n_x\right)$$

The general expression can become quite lengthy, with 6 terms for t_x and n_x, and due to their non-linear nature, they unfortunately cancel each other out in very special constellations only. Nevertheless, we can simplify matters and give a few rules of thumb. Obviously, the bigger the deviations of t_x and n_x from their nominal values the bigger the aberrations, but there are other factors that affect the magnitude of the aberrations and the sensitivity of an objective to such deviations:

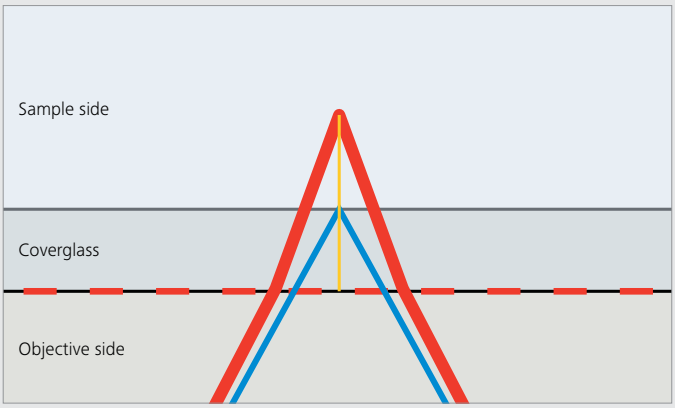
a) If immersion and embedding medium have (nearly) the same refractive indices (like water immersion and a sample in aqueous medium), the focusing depth $t_{embedding}$ inside the sample space is quite uncritical, as a longer path in the embedding medium is simply compensated by a shorter path in the immersion medium by refocusing. Consequently, imaging deep into aqueous medium with an oil immersion objective is much more critical. This is the main reason for choosing water immersion for live cell imaging with ZEISS Celldiscoverer 7.

b) The larger the refractive index step between the individual layers the larger the spherical aberration. For oil immersion, a variation in the thickness of the cover glasses is not usually critical since oil and glass have very similar refractive indices n~1.518. For air objectives, the large index step makes it more critical if both objectives have the same numerical aperture. The Plan-Apochromat 20×/0.95 in ZEISS Celldiscoverer 7 is a very sensitive objective in this respect as it has both a high index step and a high numerical aperture.

c) The numerical aperture is the most critical parameter, and this is easily seen in the formula for ΔW and in the enlarged view of the figure. The yellow ray on axis (represented by ρ=0 in formula (2)) travels a shorter distance in the medium than the edge rays (ρ=1, blue for the nominal situation and red for the aberrated). And these distances differ much more if the numerical aperture is increased, making spherical aberration extremely dependent on NA (ΔW~ NA^4).

Even for the nominal situation the yellow and blue rays differ in length, but this is almost perfectly compensated for by clever engineering inside the objective. If now the thicknesses t_x and

refractive indices n_x differ from the nominal values, this compensation is no longer perfect and spherical aberration reduces Strehl ratio, and hence image quality and signal strength.



Beside spherical aberration also chromatic aberration is influencing the image quality.

Longitudinal chromatic aberration is easy to understand when considering that the refractive index depends on the wavelength. Thus, not all colors come to the same image location along the optical path. With apochromatic objectives as in the Celldiscoverer 7, these focus differences are minimized over a broad range of wavelength and shifts from the ideal image location are kept well below the depth of focus. Making objectives APOCHROMATIC is one of the major efforts in optical engineering, because the refractive indices of all lens glasses inside the objective and all media outside the objective (immersion, embedding, cover or bottom material) vary with the wavelength in a non-linear fashion. The craft and art of the optical engineer is in part to carefully select and combine lens materials, shapes and thicknesses INSIDE the objective in order to compensate for the media OUTSIDE the objective. This works under two conditions

a) The media OUTSIDE the objective comply with the assumptions in the engineering process and do not vary. This is obviously not the case, if different bottom materials and thicknesses come into play with the same objective.
b) If the media OUTSIDE the objective (like bottom materials and thicknesses) change in the application also the objective INSIDE needs to adapt to this in order to stay apochromatic.

The spherical as well as chromatic aberration can be compensated for in correction objectives by very specific precision movement of one or more lens groups inside the objective. The underlying concept is to compensate the aberration term ΔW generated by imperfections in sample space by a compensation term $-\Delta W$ inside the objective. Generally, such a single correction measure for up to six parameters t_x and n_x would not suffice. But here comes the strength of the full automation of the ZEISS Celldiscoverer 7 which makes the motorized correction objectives highly adaptive: The refractive index of the immersion is known with high precision, the thickness and refractive index of the cover glass are measured using the built in Definite Focus and the distances traveled (in immersion and embedding medium) are measured with high precision by the motorized focus, making ΔW a known quantity.

Hence for essentially all variations of thicknesses t_x and refractive indices n_x in sample space, ZEISS Celldiscoverer 7 not only stays in focus, but also maximizes Strehl ratio, and hence image quality and signal strength in a highly automated and adaptive fashion.

The introduction of the LSM 900 including the Airyscan 2 transforms ZEISS Celldiscoverer 7 into the first fully automated confocal imaging system on the market. The High Sensitivity (HS) mode of the Airyscan 2 detector enables very gentle imaging because the amount of excitation light necessary for image acquisition is greatly reduced. At the same time, the signal-to-noise ratio and hence the resolution is clearly improved when compared to standard confocal imaging. Applying the Airyscan multiplex 2Y mode using two lines for imaging in one sweep, the acquisition speed is also increased. The three classic features of confocal imaging: speed, resolution and signal-to-noise ratio, must be carefully balanced with respect to one another. Focusing on one aspect automatically means a reduction in the two other parameters. With Airyscan multiplex 2Y mode, all three aspects can now be improved at the same time. This turns ZEISS Celldiscoverer 7 into an extremely sensitive high-resolution point scanner that is ideally suited for long term live-cell imaging experiments especially in combination with 25× water autoimmersion objective with an outstanding numerical aperture of 1.2.

Very often, experimental conditions or cost prohibit the use of optical grade glass bottom material with a typical thickness of 170 µm. As an alternative, in many cases rather thick (~1 mm) optically plastic material is used to accommodate demanding cell cultures. In a classic fluorescence microscope this kind of setup would lead to sub-standard imaging results with many aberrations as well as loss of excitation and emission light.

Since ZEISS Celldiscoverer 7 automatically adjusts to the vessel bottom (material as well as thickness), the highest image quality is always maintained ensuring that the maximum available information can be gathered from the sample. This is especially true when imaging in LSM mode through optically low-quality material like plastic vessels with thicknesses of 1mm or more. In this particular case, the focused laser beam that is scanned into the sample, as well as the collected emitted light from the sample that needs to pass the pinhole at the conjugated plane, is heavily distorted if no proper correction is performed.

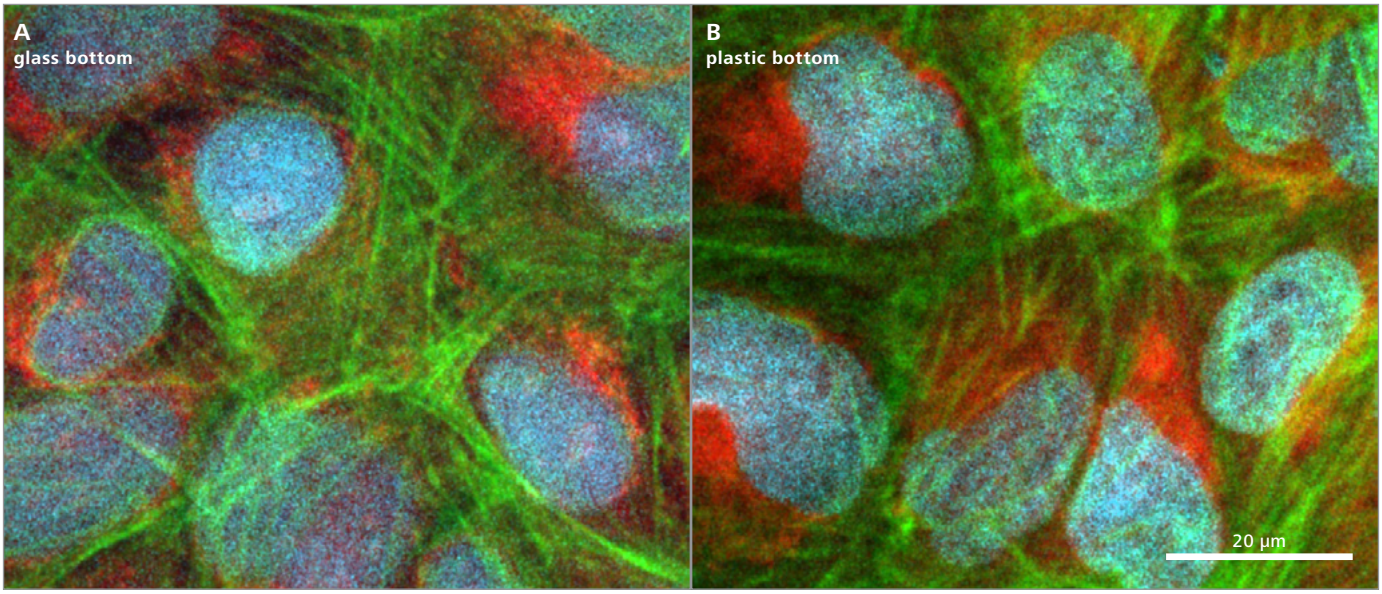


Figure 3 *Comparison of image quality of A) thin glass bottom (170 µm) and B) thick plastic bottom when the optimal correction is applied. U2OS Cells stained with Hoechst (blue), MitoTracker™ Red (red), Alexa Fluor™ 488 Phalloidin (green)*

In this article the difference in confocal image quality, signal-to-noise ratio (SNR) and excitation power is compared between optimal and incorrect autocorrection ring settings for cells grown on thick plastic (>1000 µm). The findings demonstrate the power of the autocorrection objectives for high-quality confocal imaging using plastic bottom sample carriers.

Results

As described above, the image quality – or more precisely resolution and SNR – are being influenced by the illumination and detection objective as well as sample features like bottom material and thickness of the sample carrier, refractive index of the medium and imaging depth. If this information is known, the image quality can be improved by reducing spherical and chromatic aberrations with the help of correction optics. ZEISS Celldiscoverer 7 automatically detects bottom material and thickness upon sample loading. This information is then used to automatically set the autocorrection rings at the objectives to the optimal position to correct for any negative influence on the image quality.

The following examples showcase adherent cells grown on different imaging vessels that have either thin glass (~170 µm) or thick plastic (~1000 µm) bottom material – the confocal imaging was performed using the Airyscan 2 detector deploying an automatically corrected 20× objective with a numerical aperture of 0.7.

Figure 3 shows the strength of the adaptive optics of ZEISS Celldiscoverer 7. Cells that are either grown on optically well-suited glass of a thickness of 170 µm compared to cells on a thick 1000 µm plastic bottom – a challenging setup for standard confocal fluorescence microscopy. While there is a higher clarity of the cells grown on glass, the plastic harbored cells still showcase an astounding amount of detail due to the optimal adaptation to bottom material and thickness that was automatically applied to the objective.

When comparing the image quality between the automatically calculated setting for a vessel bottom thickness of 1020 µm versus an incorrect setting manually overwritten to a thickness of 210 µm, it is apparent that due to the missing optimization the image quality of the latter is severely impacted when compared to the corrected version (see figure 4). As the light is far blurrier due to spherical aberrations and refractive index mis-

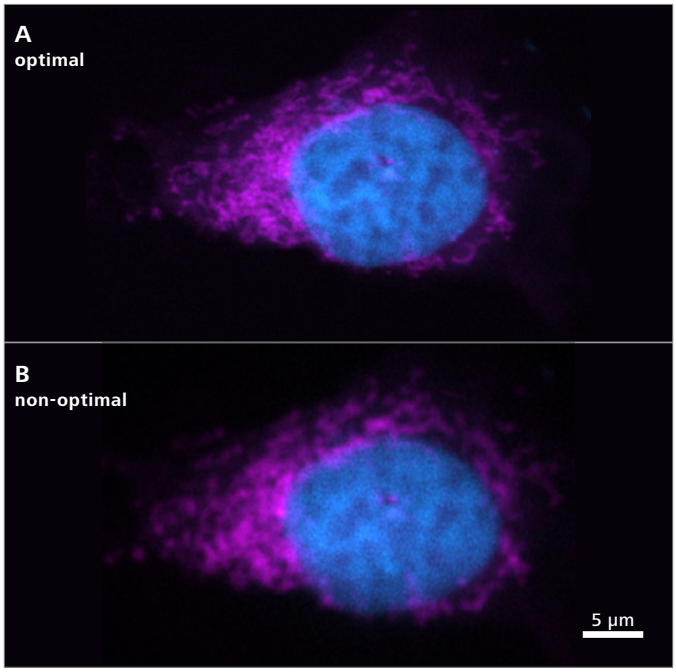


Figure 4 *Comparison of image quality and different laser powers under A: optimal autocorrection ring settings (1,020 µm). Laser power: DAPI (blue) 2.5 % 405 nm, Mitotracker (magenta) 1.5 % 561 nm; B: non-optimal autocorrection ring settings (210 µm). Laser power: DAPI (blue) 25 % 405 nm, MitoTracker™ Red (magenta) 80 % 561 nm.*

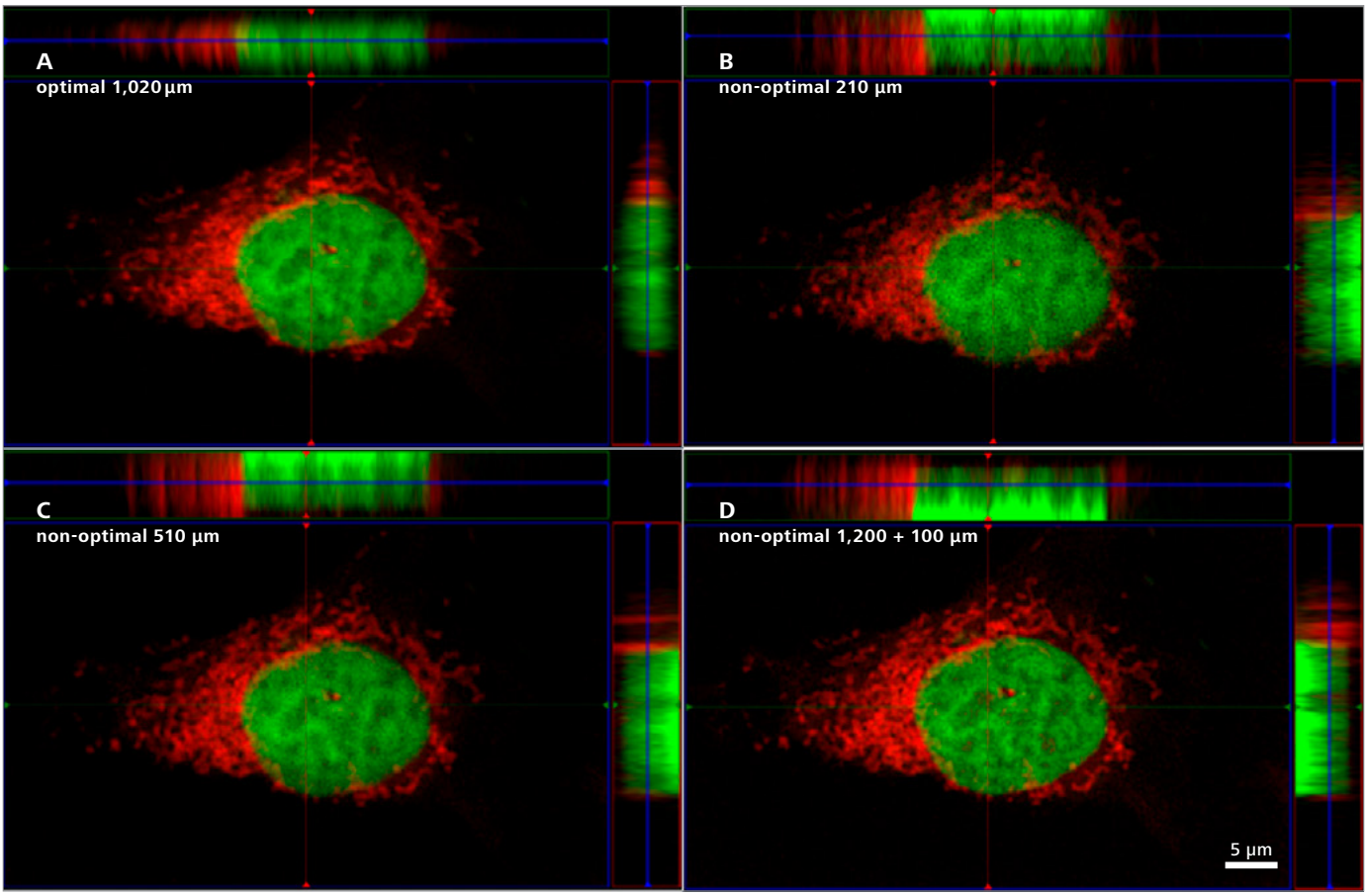


Figure 5 *The influence of autocorrection objectives on chromatic and spherical aberrations as well as laser intensities and overall image quality. ZEISS Celldiscoverer 7 automatically measures bottom thickness and adjusts the autocorrection rings to correct for these artefacts. Comparison of 3-dimensional datasets from a DAPI (green) and MitoTracker™ Red (red) stained U2OS Cell. A: optimal autocorrection ring settings (@1,020 µm bottom thickness; 405 nm = 3.8%; 561 nm = 2.8%). B: non-optimal autocorrection ring settings (@210 µm bottom thickness; 405 nm = 80%; 561 nm = 25%) C: non-optimal autocorrection ring settings (@510 µm bottom thickness; 405 nm = 80%; 561 nm = 50%) D: non-optimal autocorrection ring settings (1,200 µm bottom thickness + 100 µm imaging depth; 405 nm = 20%; 561 nm = 20%). In the autocorrection ring settings, the refractive index of the bottom material is also considered.*

match, the image appears fuzzy and fine details are no longer visible resulting in a loss of resolution. In addition, aberrations not only manifest themselves when light is detected from the sample but also appear when light is being focused (or in this case rather not properly focused) into to specimen to stimulate fluorescence. This combination of loss of optical quality on the detection-side and reduced excitation efficiency means that excessive amounts of excitation light (10-50-fold increase in this example) are needed to get an image with comparable brightness values. An alternative to increasing the laser power could be an increase in pixel dwell time, however neither option is ideal for live cell imaging experiments as the former very likely induces phototoxic effects while the latter prevents the observation of fast processes as the maximum acquisition speed is significantly reduced.

When looking at 3-dimensional data (figure 5), it is apparent that the z-resolution is drastically impacted if the autocorrection ring is not set properly. When the correct auto-detected and -adjusted setting of 1020 µm thickness is used, the cell appears thin in its z-expansion and both colors align in the same z-plane (figure 5A). One can clearly distinguish the flat lower side of the cell that resides on the plastic surface from the more rounded top side that extends into the medium. Manually changing the autocorrection ring setting to a different (= wrong) value will clearly decreases the image quality – the cell appears to be elongated in the z-direction and the two colors no longer line up in the same z-plane (figure 5B-D).

This demonstrates the appearance of spherical and chromatic aberrations if the optics are not properly corrected for bottom thickness. In addition to these aberrations, the laser power needs to be significantly increased to compensate for the improper correction ring settings.

Summary

Modern long term Live-Cell-Imaging applications that last up to several days have a high demand for instrument performance like environmental control (temperature and CO_2), a stable focus and gentle illumination parameters to mimic the *in vivo* conditions of the investigated sample as closely as possible. All these features are included in ZEISS Celldiscoverer 7. In addition, autocorrection objectives supplied with the instrument allow for the use of different types of sample carriers consisting of different bottom material and thickness. The machine will automatically detect and adjust these parameters whenever a new sample is being loaded. This way even optically non-ideal vessels such as those with thick plastic bottoms (> 1 mm) can be used to generate high quality imaging data. The information gathering capability is maintained. This even works in confocal mode in combination with the Airyscan 2 detector of the integrated LSM 900 as shown in this article. Distortions like chromatic and spherical aberrations are corrected for ensuring that image quality parameters like high resolution and signal-to-noise ratio are optimized. In addition, correctly adjusted optics allow for high-speed image acquisition and guarantee low phototoxicity for long-term experiments, as the necessary light dosage for fluorescence imaging is greatly reduced. This, in combination with the other features, turn ZEISS Celldiscoverer 7 into an ideal live cell imaging system that is fully automated and enables the execution of very sophisticated applications.

Cover image: Sample courtesy of J. Hartmann and D. Gilmour, EMBL, Heidelberg, Germany

Carl Zeiss Microscopy GmbH
07745 Jena, Germany
microscopy@zeiss.com
www.zeiss.com/celldiscoverer