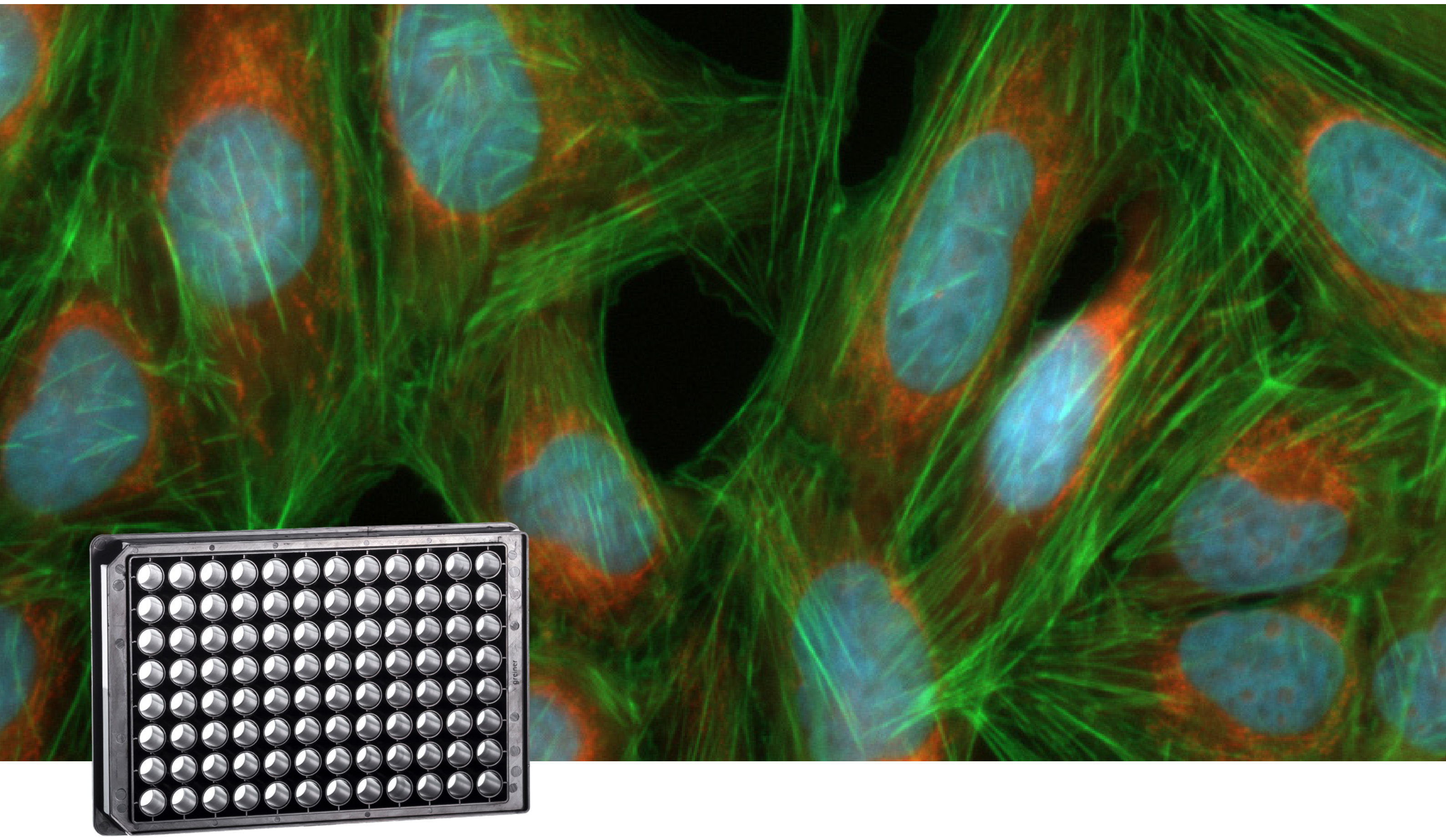
Technology Note

Plastic Labware for Optimal Results in Modern Life Science Microscopy Using ZEISS Axio Observer and ZEISS Celldiscoverer 7



Seeing beyond

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Within the field of life science research, light microscopy is a fundamental and constantly evolving tool. In the early years samples could only be viewed by eye. The quality of the resulting hand-drawn images depended strongly on the artistic skills of the observer. Combining the light microscope with cameras – first analog and later digital – allowed for a steady increase in scientific grade representations of minute details of life. Advances in incubation systems enabled the environmental control of temperature, pH value and O_2 levels facilitating the execution of more and more complex long-term live imaging experiments with living cell cultures, organs, or even small model organisms.

A very important property in the context of biological light microscopy is the way in which the sample is prepared for microscopic observation. For fixed biological specimens the default was and still is an object sandwiched between a glass slide and a thin cover glass. However, for modern live cell imaging experiments more advanced vessels like dishes and multiwell plates are necessary to mimic the natural environment of the specimen but at the same time allow for an uninhibited view for the microscope objective. Inverted microscope setups, where the objective is positioned below the specimen and imaging takes place through the material the sample is mounted on are ideal for this purpose and are now the standard for cell culture imaging applications. The optical properties of the bottom materials of these vessels are critical, as they have a significant impact on the quality of the microscopic image. These parameters can influence the image quality and thereby the scientific significance of a microscope assay (Figure 1).

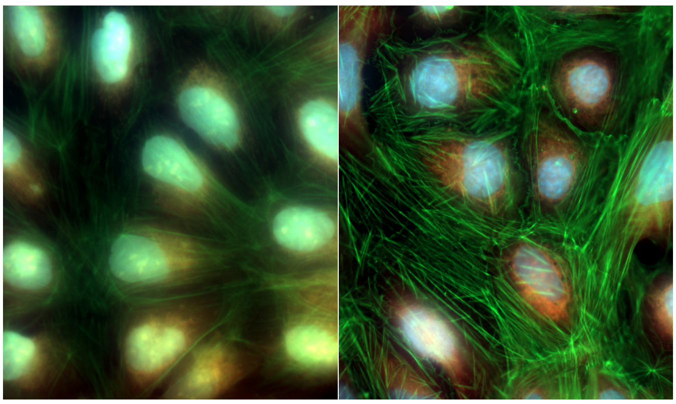

Figure 1 *Suboptimal (left) and optimal (right) combinations of microscope objectives and sample bottom materials have a drastic impact on image quality.*

In this article we provide an overview of the performance of two inverted microscopes – ZEISS Axio Observer (a classic stand) and ZEISS Celldiscoverer 7 (a boxed microscope system) – when working with different plastic labware products (SCREENSTAR, µClear and CELLview) from Greiner Bio-One.

Microplates for Advanced Microscopy and High Content Screening

Need for specialized products in microscopy

Depending on the complexity of live cell imaging experiments and the requirements of the corresponding microscope, Greiner Bio-One provides tailored product solutions for high content imaging and phenotypic screening of live cells.

For low throughput imaging, Greiner Bio-One offers glass bottom disposables of the CELLview product line. This includes the CELLview slide, a transparent slide with an embedded cover glass and a black detachable compartmentalization and the CELLview dish, a standard cell culture dish with embedded cover glass bottom (Figure 2). Recent advances in imaging and multiparametric image analysis techniques have led to an increasing importance of high throughput imaging for all aspects of drug discovery and system biology. The ease of automation, combined with rapid data analysis and interpretation, enables a high turnover of tested compounds. Therefore, Greiner Bio-One provides several high content screening microplates ranging from 96 to 1536 well.

Figure 2 *CELLview product family – CELLview slide (bottom), CELLview dish (top right) and the 96 well CELLview microplate (top left)*

Image courtesy: Greiner Bio-One GmbH, Frickenhausen, Germany

Choosing the right substrate for each application

Glass:

The gold standard for biological samples in microscopy is glass. Glass possesses excellent optical properties as it is highly transparent and less susceptible to scratching and dust contamination. Imaging products with glass bottoms feature excellent intra- and inter-well flatness due to the rigidity of the material. They offer optimal thermal conductivity that grants ideal conditions for live cell imaging. Since microscope objectives are corrected to the bottom thickness and refractive index of cover glass (170 µm and n about 1.52, respectively), generally the best imaging results with the highest resolution can be achieved using glass substrates.

Cyclic olefin:

Cyclic olefins have become increasingly popular for use as a base material for microplates. The material possesses excellent optical features with a high spectral transmission and a low background in the lower UV range. With a refractive index which is very close to that of glass, cyclic olefin bottom plates combine excellent optical properties with improved cell growth and cell attachment due to their organo-polymeric nature. Although cyclic olefin bottom plates typically do not achieve the planarity of glass substrates, they show a very good inter- and intra-well flatness.

Polystyrene:

Polystyrene is the most widely used base material for microplates. Polystyrene foil bottom plates are an optimal solution for routine high throughput and imaging applications. Although polystyrene substrates are well suited for many cell-based assays, they are not optimized for high magnifications i. e, use of high numerical aperture (NA) objectives with short working distances. This does not only refer to the standard plate geometry but also to the optical properties of the polystyrene polymer. Major advantages are the high variety of cell culture surfaces that facilitate optimal cell culture condition for various applications and the attractive entry price level of the plates.

To be used for high resolution microscopy the microplates must fulfill specific requirements, which are reflected in both the choice of raw materials and the plate design (grey box).

Requirements to Microscopy Microplate

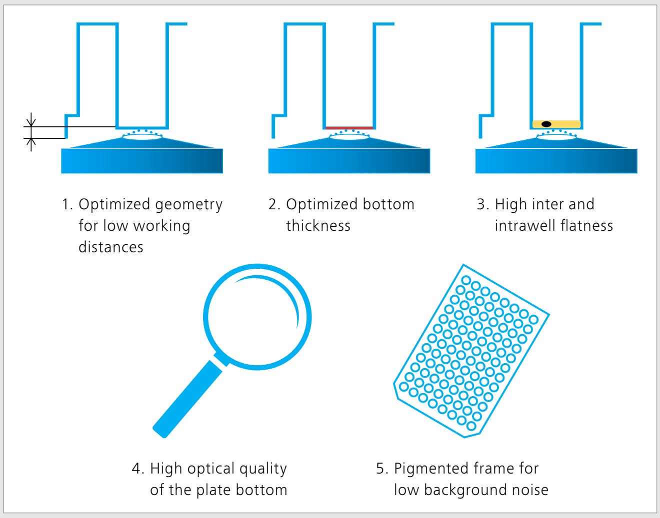

Figure 3 *General requirements for microplates used for microscopy*

1. A larger distance between the plate bottom and the outer rim, as often seen for classical screening microplates, can be a limiting factor for the choice of the objective. For highest image quality only microplates with a recessed well bottom enable a complete periphery access for objectives with a high NA or immersion applications which typically have a short working distance.
2. Microscopic objectives are typically calibrated to a 170 µm bottom thickness, the standard thickness of coverslip glass. The typical bottom thickness of microplates for high content screening is in the range of 170 to 190 µm. This enables the use of high NA objectives with short working distances for the best imaging results.
3. The planarity of the microplate is a decisive criterion and strongly depends on the bottom material. Glass bottom microplates feature a maximum level of planarity due to its rigidity, but also cyclic olefin plates show good planarity at various plate densities. Those microplates can significantly reduce the autofocus times and enhance the overall screening throughput.
4. To ensure best imaging results, only materials with a high optical quality should be used for the well bottom material. As discussed, glass followed by cyclic olefin foils achieve the best imaging results. However, for many applications, polystyrene is an adequate solution with an excellent price-performance ratio.
5. The majority of imaging applications rely on fluorescence-based readouts, consequently a black pigmented cyclic olefin frame is the optimal solution for minimum background noise.

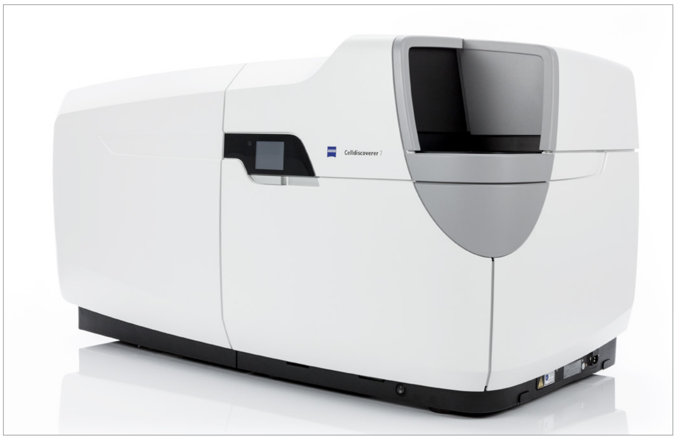

Figure 4 *ZEISS Celldiscoverer 7 – fully automated boxed microscope system*

The microplate portfolio for imaging applications comprises the three product families CELLview, SCREENSTAR and µClear to meet the specific needs of the various microscopic applications. The microplates are optimized with regard to imaging-relevant features such as bottom material, bottom flatness, well bottom elevation and cell culture surface. CELLview products combine the convenience of a plastic disposable with the high optical quality of a 170 µm thin cover glass bottom, providing superior high-resolution microscopic images of in-vitro cultures. The 190 µm thin cyclic olefin film bottom of the SCREENSTAR microplates features outstanding glass-like optical properties while providing an excellent surface for adherent cell culture. µClear plates possess a 190 µm thin polystyrene film bottom and are well suited for standard microscopic application with low to medium magnification.

ZEISS Celldiscoverer 7 – an automated live cell imaging system designed to make complex microscopy simple – harbors many automation functions like automatic sample recognition, an auto-immersion water objective, a variety of autofocus options and many more. Well integrated incubation options (temperature, gas, humidity control) allow for long-term live cell observations.

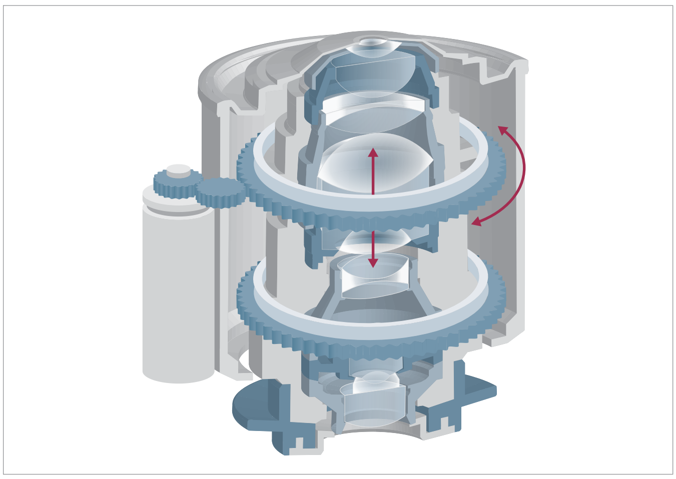

Figure 5 *ZEISS Celldiscoverer 7 – fully automated boxed microscope system*

Figure 6 *ZEISS Axio Observer 7 – flexible inverted microscope platform for multimodal imaging*

The optical concept of this boxed microscope consists of two elements: Four objectives that are situated in an objective turret and a 3-position magnification changer enable 12 different combinations allowing a range from 2.5× up to 100× magnification. All objectives in ZEISS Celldiscoverer 7 feature apochromatic correction to enable artifact-free multicolor imaging.

The three objectives with the highest numerical apertures have an 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 (Figure 5). 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.

ZEISS Axio Observer is the flexible inverse platform for demanding multimodal imaging of living and fixed specimens. It can be combined with a wealth of technologies and accessories to support even complex experiments precisely. Because of its flexible character, additional features and equipment it can be upgraded later, which means that the ZEISS Axio Observer remains functional and future-proof.

ZEISS Axio Observer offers a broad portfolio of objectives. Depending on the application, the ideal objective can be selected for each application. Objectives with the highest numerical aperture deliver the best resolution, while objectives with long working distances are designed for deep tissue imaging. More specialized versions are optimized, e.g., for live cell imaging or for different immersion media. Many objectives have a correction collar enabling correction for different cover glass thicknesses. By using a motorized version this correction can also be automated.

	A1			A6			A12	
left	top	center	left	top	center	left	top	center
172	171	170	167	167	167	168	167	168
	D1			**D6**			**D12**	
left	top	center	left	top	center	left	top	center
172	171	172	167	166	166	168	168	168
	H1			**H6**			**H12**	
left	top	center	left	top	center	left	top	center
173	172	172	168	168	168	169	168	169

Table 1 *Bottom thickness measurement at 3 positions in selected wells of 96 well CELLview plate with glass bottom*

Even though ZEISS Axio Observer is the flexible platform for life science research, it is equipped with several automation functions addressing the need for increasingly large statistics in modern science. Featuring the AI Sample Finder for optimal user guidance and efficient operation, ZEISS Axio Observer makes sample placement easier than ever and significantly reduces the time to conduct experiments.

Results

Geometric and optical properties of microplate formats with different bottom materials

Many features influence the optical quality of microscopic images. A very important – and sometimes neglected – contribution comes from the bottom material used when working with an inverted microscope setup, especially in fluorescence microscopy where the excitation light required to generate fluorescence within the sample as well as the emitted light will have to pass through the bottom material. Here, not only the thickness plays a crucial role but also the substrate, as any non-ideal conditions will influence the resulting image quality in a negative way. Shown here are a couple of features of different 96 well plates that were measured using some of the automation functions of ZEISS Celldiscoverer 7.

Thickness

To optimize optical quality, high end objectives at inverted microscope setups are corrected for a defined bottom material – typically glass with a refractive index of around 1.5 – with a specific thickness of 170 µm. Some objectives have a correction collar that allows for adaption to a range of different thickness values. If a specific thickness is known, the objective can be adjusted accordingly to optimize image quality.

ZEISS Celldiscoverer 7 was used to measure the bottom thickness at three different positions within 9 wells across a 96 well plate. The three different types of plates show a very good consistency of thickness values for their respective bottom material types. The analyzed CELLview glass bottom showed an average thickness of 169 ± 4µm (Table 1). The SCREENSTAR plate with a cyclic olefin bottom was measured with a mean bottom thickness of 184 ± 3µm and the µClear polystyrene bottom plate showed a mean thickness of 197 ± 4µm. All these values lie within the specifications by the manufacturer and present optimal conditions for image acquisition – given that the correction ring of the used objective is correctly set, and the objective is suited to work with the bottom material type.

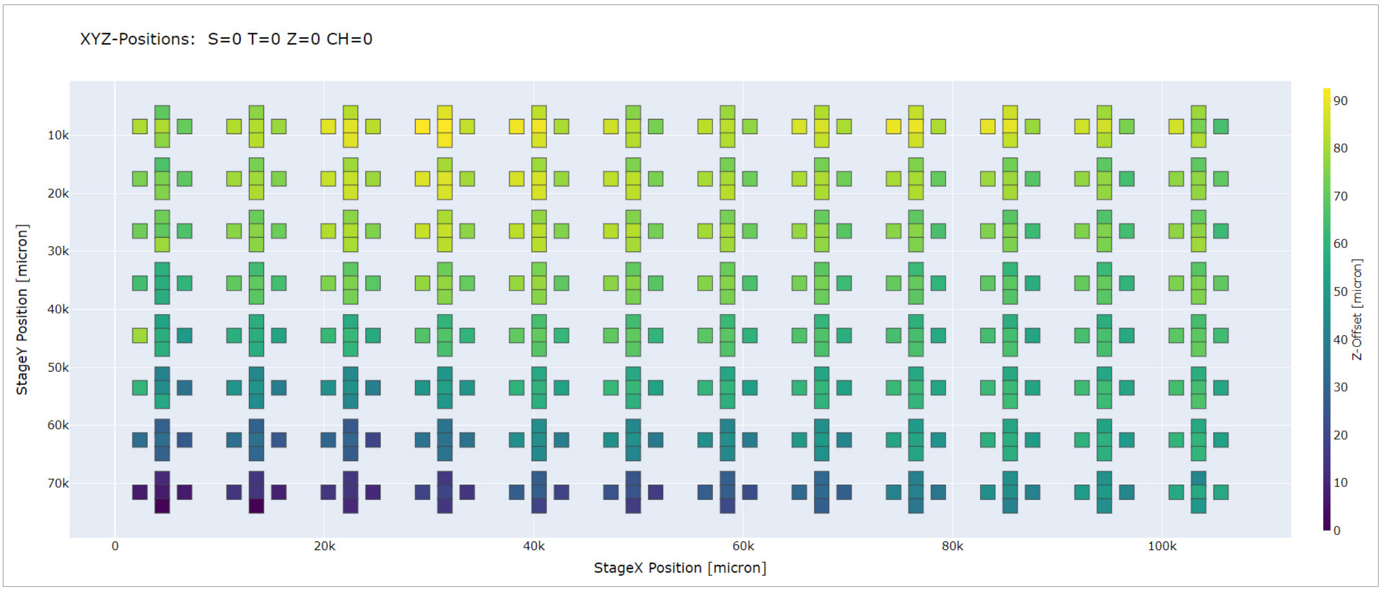

Figure 7 *Flatness measurement at 5 positions in each well of a 96 well CELLview plate with glass bottom*

Flatness

For optimal imaging conditions a flat bottom surface is very important when scanning larger areas of a sample. While modern microscopes like ZEISS Celldiscoverer 7 and ZEISS Axio Observer have sophisticated auto focusing strategy options that automatically find the optimal focus plane, they can only correct for a certain amount of change over a given area. If bottom surfaces are bent or warped too much, unsharp areas within images will be the result. To test the flatness of the three types of plates ZEISS Celldiscoverer 7 was used again. The z-position for 5 positions (XY distance between individual positions: ~1 mm) in each well of the 96 well plates were determined and analyzed. Figure 7 shows a graphical representation of the flatness measurement for a CELLview plate.

The best values for inter- and intra-well flatness showed the CELLview (glass bottom) with around 100µm and 10µm respectively. The two plates with foil bottom showed slightly higher variations as the foil is more prone to warping: SCREENSTAR (cyclic olefin) Inter-well: ~120 µm; Intra-well: ~40 µm vs. µClear (polystyrene) Inter-well: ~140; Intra-well: ~50 µm.

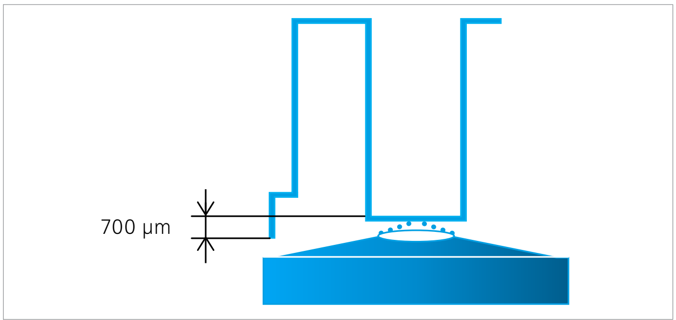

Figure 8 *Well bottom elevation – distance between outer rim and internal well bottom.*

Well Bottom Elevation (Skirt Height)

The height difference between the rim that surrounds a microplate and the optical bottom is called well bottom elevation (Figure 8). It mainly determines the maximum achievable imaging area of any given objective. A high well bottom elevation requires the objective to be raised higher above the plate bottom to bring the sample into focus at its working distance. A short well bottom elevation therefore is always preferable as it allows for larger coverage of the sample area in the X and Y dimension without the risk of colliding with the rim of the plate. In particular, high NA objectives (which usually have a rather short working distance) benefit from low well bottom elevation plates. CELLview plates (glass bottom) and SCREENSTAR plates (cyclic olefine bottom) both have a low well bottom elevation of around 700 µm and therefore are ideally suited for applications requiring high NA objectives. µClear plates (polystyrene bottom) have a well bottom elevation of 3700 µm making them better suited for screening applications that only require low magnification and NA – so objectives with a high working distance can be used to optimize the travel range in X and Y.

Image comparisons

Low SCREENSTAR vs high µClear

ZEISS Axio Observer offers a large portfolio of objectives. For this review a Plan-Apochromat" 20×/0.8 and a Plan-Apochromat 63×/1.4 Oil which are corrected for glass with a thickness of 170 µm were used. These objectives are used representatively for imaging of large areas with a decent magnification (20×/0.8) and for acquiring finest details with maximum resolution (63×/1.4). As the objectives are correct for glass, the image quality degrades if non-glass substrates not having a thickness of 170 µm are used.

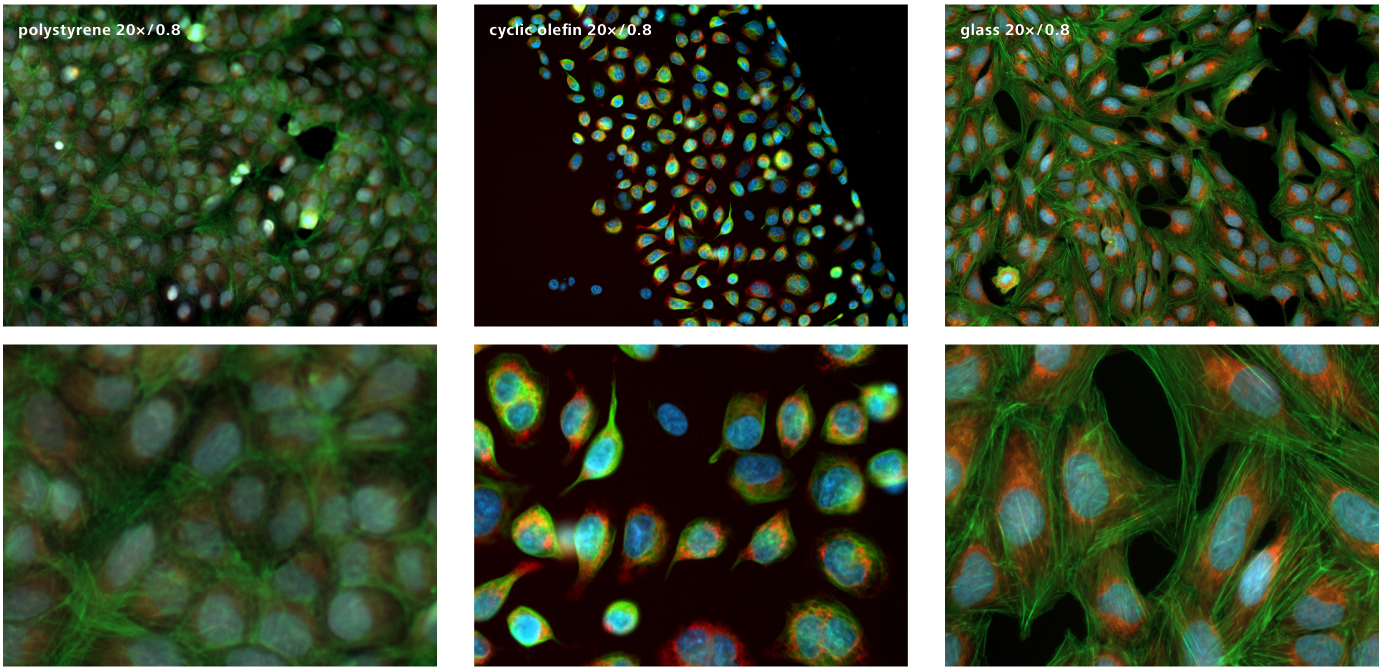

Figure 9 *Image comparison of U2OS grown on polystyrene (left), cyclic olefin (middle) and glass (right) bottom material. Imaged with a 20×/0.8 objective. Blue – nuclei, red – mitochondria, green – actin fibers. Image size top row: 625 µm, bottom row: 200 µm.*

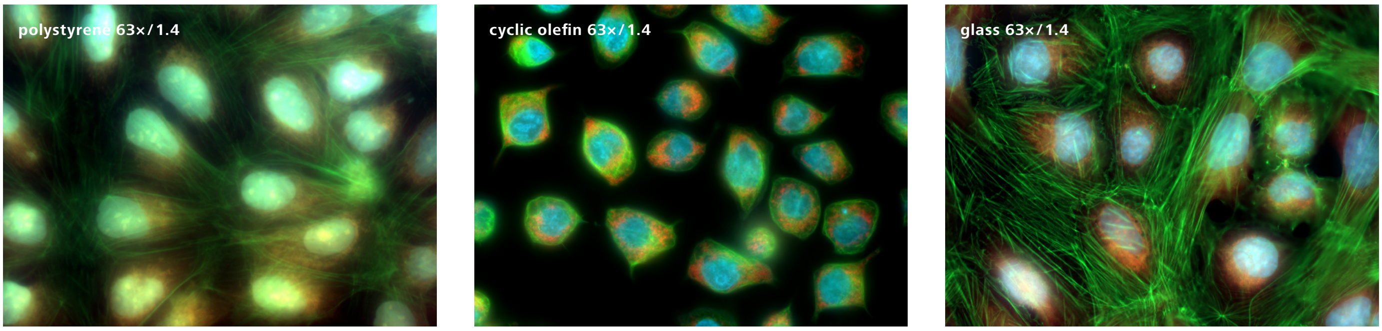

Figure 10 *Image comparison of U2OS grown on polystyrene (left), cyclic olefin (middle) and glass (right) bottom material. Imaged with a 63×/1.4 oil immersion objective. Blue – nuclei, red – mitochondria, green – actin fibers. Image size: 200 µm.*

Comparing the image quality of the different bottom materials of the microplates clearly shows that glass yields the sharpest images with highest contrast and resolution (Figure 9). The actin fibers of the cells can be well identified, and mitochondria structures are visible. Comparing the image quality with the cyclic olefin and polystyrene substrates at high magnifications shows that polystyrene leads to reduced sharpness due to the optical properties of the substrate not matching the objective specifications. Cyclic olefine ranges between both image qualities.

With the Plan-Apochromat 63×/1.4 Oil, finest structures can be resolved when using the glass substrate (Figure 10). However, objectives with such a high NA like the 63×/1.4 Oil objective require substrates matching their specification. This becomes evident when switching to polystyrene. As magnification increases, the image loses contrast and structures appear blurred due to optical aberrations.

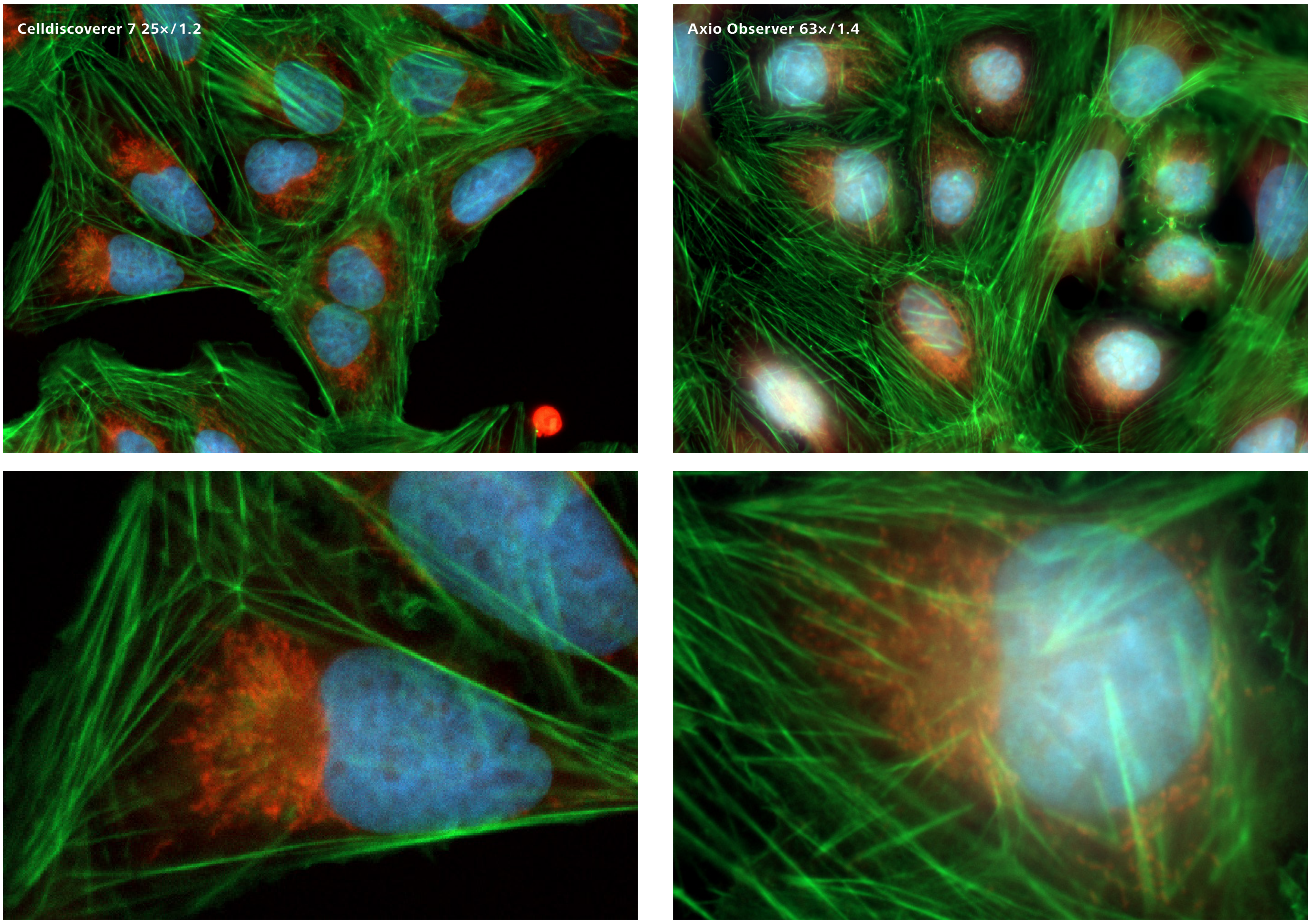

Figure 11 *Image comparison of U2OS grown on glass bottom material. Imaged with ZEISS Celldiscoverer 7 using a 25×/1.2 water immersion objective (left) and with ZEISS Axio Observer using a 63×/1.4 oil immersion objective (right). Blue – nuclei, red – mitochondria, green – actin fibres. Image size top row: 200 µm, bottom row: 50 µm.*

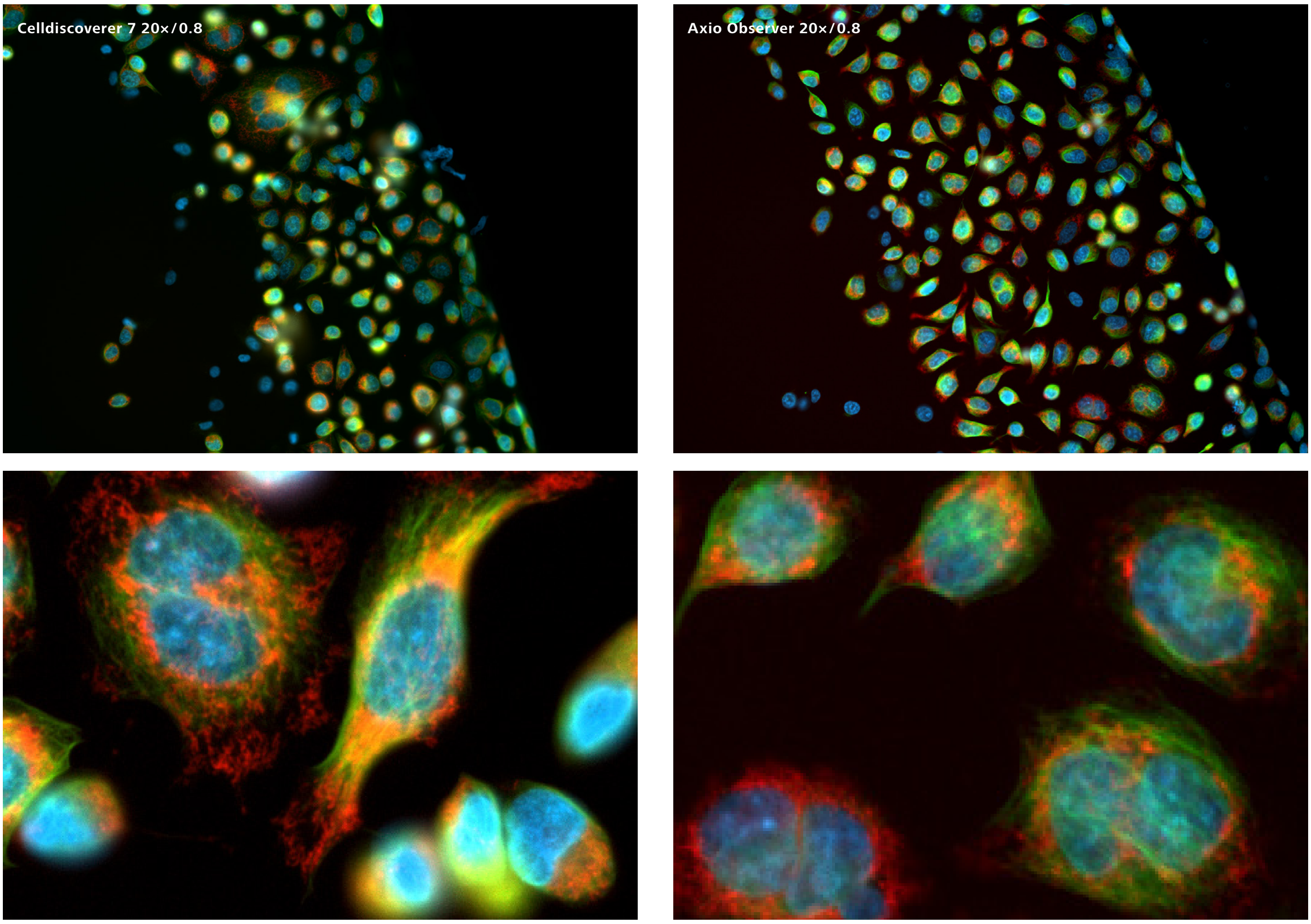

Figure 12 *Image comparison of U2OS grown on cyclic olefin bottom material. Imaged with ZEISS Celldiscoverer 7 using a 20×/0.8 objective (adjusted, left) and with ZEISS Axio Observer using a 20×/0.8 objective (not adjusted, right). Blue – nuclei, red – mitochondria, green – microtubules. Image size top row: 625 µm, bottom row: 100 µm.*

Images acquired with cyclic olefin substrates also show a loss of sharpness when compared to glass; however, the image quality is still suitable for many imaging applications.

Comparison of ZEISS Celldiscoverer 7 and ZEISS Axio Observer

Both ZEISS Celldiscoverer 7 and ZEISS Axio Observer can acquire high-resolution images at high magnifications. However, the focus of both microscopes is different. While ZEISS Celldiscoverer 7 has many automation features allowing it adapt to the optical properties of the plastic flexibly and automatically, ZEISS Axio Observer is a flexible platform with many ports and accessories but requires more manual settings and corrections. However, due to the flexible character of the ZEISS Axio Observer platform, it supports more objectives reaching higher magnifications and numerical apertures. For this reason, the achievable resolution and sensitivity of ZEISS Axio Observer are higher if the optical properties (e.g., thickness, refractive index) of the sample carrier match the requirements of the objectives. This means that highest resolution and sensitivity can be achieved by using substrate with a glass bottom and a thickness of 170 µm (Figure 11).

If the optical properties of the sample carrier differ from the optimal values, some corrections can be applied e.g., by adjusting a correction collar to the thickness of the cover glass. However, ZEISS Axio Observer requires these corrections to be made manually or by using motorized objectives. In contrast, ZEISS Celldiscoverer 7 comes with many automation functions allowing automatic correction for these deviations in thickness. Consequently, this allows the acquisition of relatively high-quality images in an automated fashion even for substrates having non-ideal optical properties (Figure 12).

Summary

Glass, cyclic olefin, and polystyrene film bottom plates deliver a significantly improved image quality, as compared to microplates not optimized for microscopy. Within this group of plates, the properties of the different materials differ with respect to inter- and intra-well flatness, bottom thickness, and achievable image quality.

Glass bottom microplates have a bottom with a thickness close to 170 µm for which most objectives are corrected. They show excellent uniformity for both inter- and intra-well flatness over the entire size of the plate. This makes glass bottom microplates the perfect choice for demanding applications requiring the highest optical quality. However, from the investigated materials, glass is the most expensive due to its complex production process. Many applications like high throughput screening where overall costs play a significant role, are well addressed by polystyrene microplates. As the polymer material does not have the same rigidity as glass, the inter- and intra-well flatness is reduced as compared to glass bottom plates. Moreover, due to the bottom thickness of 190 µm, µClear polystyrene film bottom plates require objectives with correction collars to avoid aberrations for optimal image quality. In combination with a standard plate geometry, µClear plates are therefore well suited for standard imaging applications at low to medium magnification with larger working distances. SCREENSTAR microplates with a cyclic olefin bottom are an ideal compromise for many applications as they combine high optical features with high quality surfaces for cell culture. The bottom thickness of ~190 µm yields a high image quality at medium magnifications, and differences to glass become visible only at high magnifications and numerical apertures. This makes the SCREENSTAR plate a very popular HCS plate optimized for high throughput imaging.

For optimal results, not only the microplate but also the imaging system is of utmost importance. Depending on the application, different criteria like achievable highest resolution and sensitivity, possibility for automation of workflows and motorization of components are of varying importance. Celldiscoverer 7 allows automatic adjustments of the correction collar to the bottom thickness, thus delivering optimal image quality even for optically non-ideal bottom materials like polystyrene. Workflows can be saved and executed via an external control panel, making it a great solution for higher throughputs. ZEISS Axio Observer 7 is a flexible platform with a large portfolio of accessories allowing it to customize the microscope for almost every application. The large objective portfolio covers a wide range including high-end objectives with large numerical apertures for highest resolution and sensitivity. Corrections for different cover glass thicknesses can be done manually or by using motorized objectives with a correction collar.

The interplay of microscope and microplate is important for achieving best results as the applicative requirements may differ significantly. ZEISS microscopes have been validated with Greiner Bio-One microplates ensuring full compatibility and highest optical performance with respect to the application requirements. Combining the correct microscope with the optimal microplate allows the configuration of a package fulfilling the application requirements best at minimal cost.

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