The benefits of automated water immersion lenses for high-content screening.

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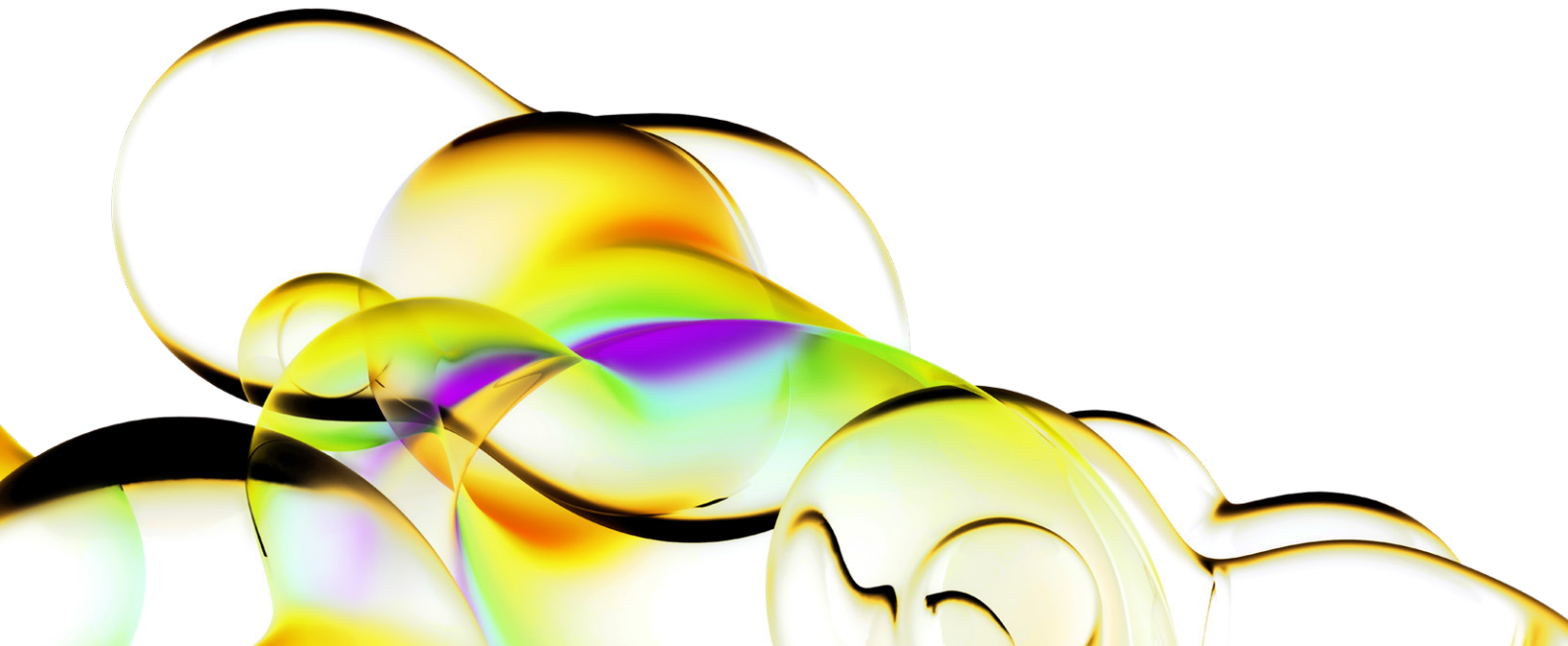
Introduction

High-content screening (HCS) combines automated microscopy and multiparametric analysis of cellular events. When setting up an HCS assay, there are a number of factors that should be considered. Key factors such as the resolution and sensitivity required for capturing the desired level of sub-cellular detail and the number of cells needed for robust statistics must be weighed against the need for speed or throughput. Maintaining sample integrity is another important factor to be considered especially for live cell microscopy, where repeated imaging can cause not only photobleaching but even phototoxicity.

Figure 1: Top right: Operetta CLS™. Lower left: Opera Phenix® Plus.



The key parameters in HCS imaging - speed, sensitivity or intensity and resolution - are often visualized in the form of a triangle (Fig 2). This illustrates that they cannot be optimized independently as altering one of them influences the others. For example, increasing the resolution by choosing a higher magnification requires imaging of more fields to maintain the same cell number – at the expense of speed; or increasing the fluorescence intensity can be achieved by increasing the exposure time – again at the expense of speed. For spinning disc confocal microscopes, increasing the pinhole size would be another way to increase the fluorescence signal. However, this would compromise the resolution. Nevertheless, there is a way to overcome some of these obstacles and here we explain why the choice of the objective lens is critical.

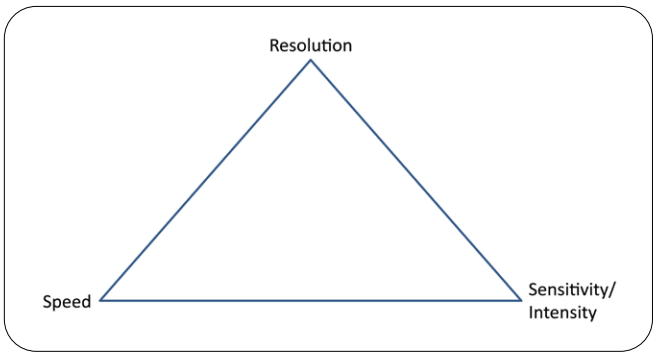


Figure 2: Triangle of "compromises". Increasing one of the parameters will result in a decrease in at least one of the others.

Water immersion objectives improve light collection

Most HCS assays are based on fluorescence detection. Fluorescence microscopy requires that excitation light is absorbed by a molecule which then emits red-shifted light. The objective lens is one of the key microscope components as it focuses excitation light onto the specimen, collects the emitted light, and then focuses it onto a detector, typically a camera chip, where the image is formed.

The most important parameter describing the objective performance is its Numerical Aperture (NA). The NA is defined as the sinus of half of the opening angle of the objective's front lens multiplied by the refractive index of the immersion medium (Fig 3)[1]. As air has a refractive index of 1, the limit of the NA is close to 1 for air objectives. Water has a higher refractive index (n = 1.33), therefore water immersion objectives can achieve higher NAs.

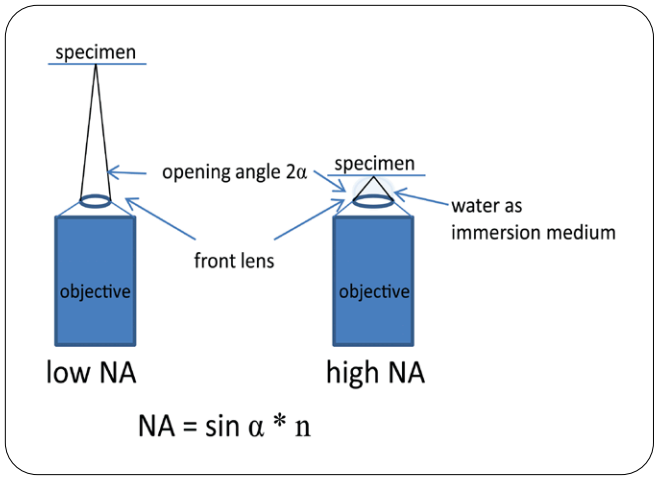


Figure 3: Schematic of a low (left) and a high (right) NA objective. Mathematically, the NA is defined as the sinus of half of the opening angle α of the objective's front lens multiplied by the refractive index of the immersion medium (n).

This is important as the brightness of an objective with a magnification M in an epi-fluorescence setting ($F_{(epi)}$) is defined as[2]:

$$(F_{(epi)}) = 10000 * NA^4 / M^2$$

Consequently, even small positive changes in NA have a large impact on image brightness because more light is delivered into the specimen and more light is collected. In addition, the NA is directly proportional to the resolution of an objective, so using a high NA water immersion lens will not only increase fluorescence intensity but also the resolution.

A real-life example is shown in Figure 4. Cells were imaged using either a 40x water immersion objective NA 1.1 (W), 40x air objective NA 0.75 (hNA) or 40x air objective NA 0.6 (WD). Increasing the NA from 0.6 to 0.75 and further to 1.1 using a water immersion lens increased the measured intensities by a factor of 2.3 (Fig 4, yellow column) and a factor of 7.2 (Fig 4, blue column) respectively. This means that the exposure times can be reduced by the same factors, significantly speeding up the assay. This is a significant advantage, particularly for live-cell imaging as it reduces both photobleaching and phototoxicity.

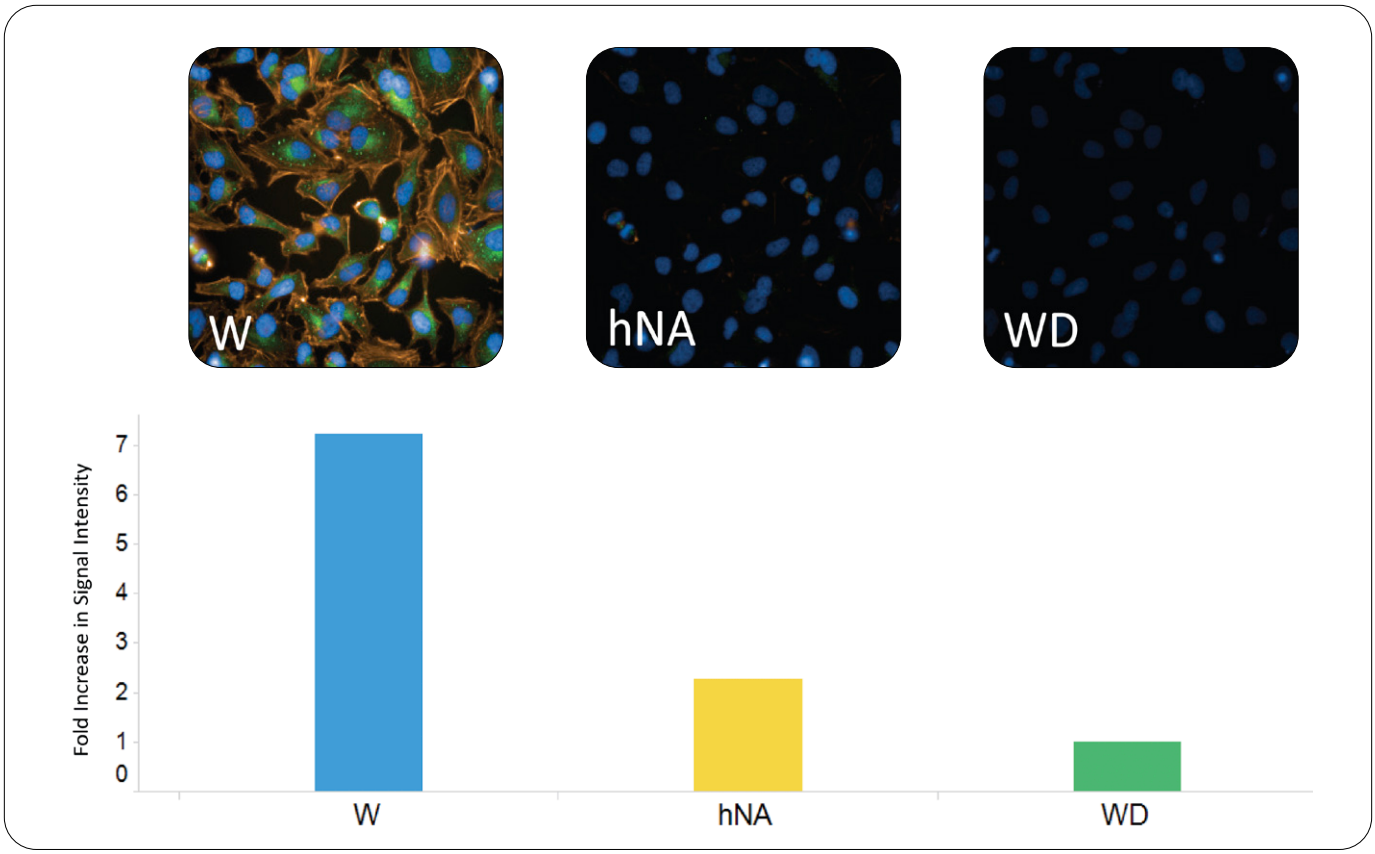


Figure 4: Water immersion objectives allow much brighter images compared to air objectives. The images shown were acquired on the Operetta CLS high-content analysis system with the same settings for exposure time and excitation power using either a 40x water immersion NA 1.1 (W), a 40x air NA 0.75 (hNA) or a 40x air NA 0.6 (WD) objective. Images are displayed with exactly the same contrast and brightness settings. Cells were stained with phalloidin (actin cytoskeleton, orange), anti alpha-tubulin antibody (microtubules, green) and Hoechst (nuclei, blue). The relative intensity shown in the bar graph was calculated by averaging the blue, green and orange channel intensities obtained with the different objectives and subsequently normalizing them to the average intensity of the 40x WD objective. Increasing the NA from 0.6 (WD) to 0.75 (hNA) and further to 1.1 (W) using a water immersion lens increased the measured intensities by a factor of 2.3 (yellow column) and a factor of 7.2 (blue column) respectively.

Water immersion objectives reduce bleaching

To demonstrate the reduction in bleaching when using a water immersion objective, a z-stack with 60 planes using either the 40x low NA, 40x high NA or 40x water immersion objective was recorded. The excitation power was kept constant, but exposure times were adjusted to obtain similar intensities with all objectives. Two images of the same plane were taken before and after the z-stack acquisition to calculate the percentage of bleaching (Table 1).

The reduction in bleaching is primarily important for live cell assays, where repeated imaging of the same cells is necessary to understand the biological effect. An example would be the study of NFκB signaling dynamics on a single cell level[3].

Water immersion objectives improve 3D imaging

As a result of more researchers using complex 3D cell models such as spheroids for their screening assays, 3D image quality is becoming more important. In high-content screening, most samples are kept in aqueous buffers or cell culture media, with refractive indices close to water. When using water immersion objectives, the refractive indices of the immersion medium and the medium within the well are closely matched. This reduces optical aberrations and greatly improves resolution in Z, as the fluorescent light from the sample is less distorted. To illustrate this effect, a multi-layered HepG2 culture was labeled with the nuclear dye Hoechst. Subsequently stacks were acquired

(as described above) and displayed in the XYZ view using the Harmony® software (Fig 5). When comparing the nuclei in the YZ (left) and XZ (top) viewing panes, it becomes clear that nuclei imaged with the water immersion objective are less distorted in z-direction than when imaged with the two air objectives[4]. This can make a substantial difference for quantification of, for example, cell invasion assays where it is important to accurately count nuclei that have invaded a 3D matrix.

Large 3D structures like spheroids pose an even greater challenge for 3D image quality and image analysis.

If subcellular details are to be analyzed, it is important to reduce background signal from other planes as much as possible to obtain sharp images. Water immersion objectives have a smaller focal depth and therefore an improved background rejection compared to air objectives. It is therefore important to note that the best image quality from a 3D object can be achieved in the confocal mode with a water immersion lens (Fig 6). This allows the analysis of even fine subcellular structures such as spots or membranes, or structures such as bile canaliculi in liver microtissues as previously shown on the Opera Phenix Plus high-content screening system[5].

Table 1: Loss of fluorescence signal due to bleaching. A z-stack of 60 planes was acquired in confocal mode using either a 40x low NA air objective (40x WD), 40x high NA air objective (40x hNA) or 40x water immersion objective (40x W). The exposure time was varied to obtain similar mean intensities with all objectives. Compared to the 40x WD, the water immersion objective only required 10% of the excitation energy (Relative Exposure Energy: LED Power x Exposure time / Exposure time of 40x WD). This significantly reduces bleaching of fluorophores as quantified by the percentage intensity loss.

Objective lens	NA	LED power	Exposure time in ms	Relative exposure energy	Time per z-stack in s	Mean intensity before stack acquisition	Mean intensity after stack acquisition	Percent intensity loss (bleaching)
40x WD	0.6	100%	500	100%	34.4	3987	2815	29%
40x hNA	0.75	100%	160	32%	14	4018	3434	14.50%
40x W	1.1	80%	60	10%	8	4078	3758	8%

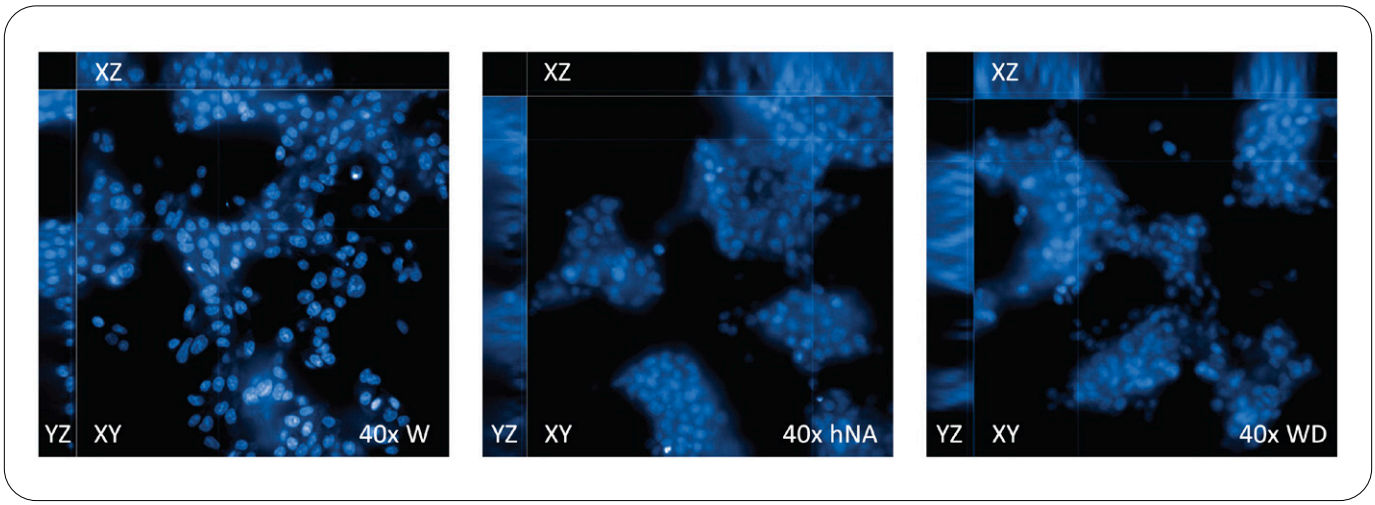


Figure 5: Harmony XYZ view of a multi-layered HepG2 culture imaged with a 40x water immersion objective NA 1.1 (left), 40x air objective NA 0.75 (middle) and 40x air objective NA 0.6 (right). The XYZ view shows a single plane in the XY direction and the whole z-stack in the YZ (left) and XZ (top) direction. Images were acquired in confocal mode on the Operetta CLS system.

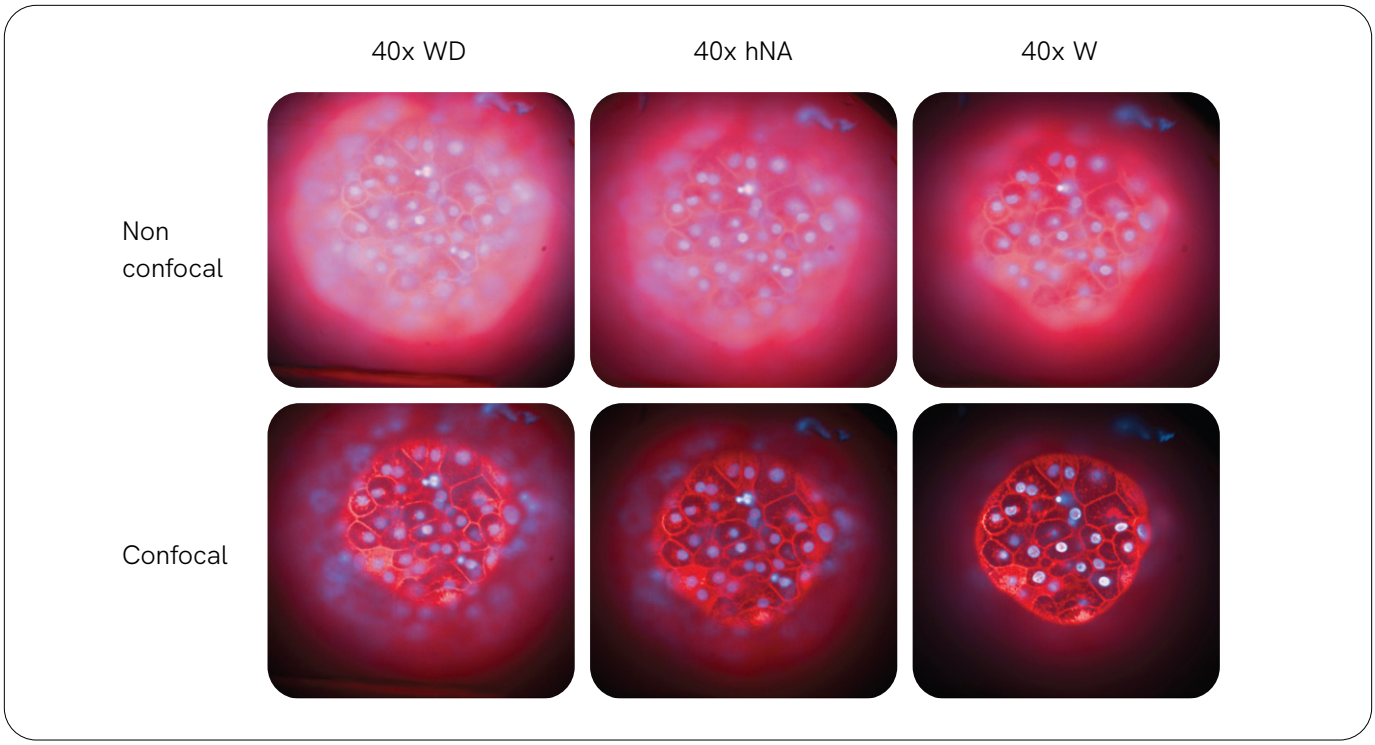


Figure 6: Human liver microtissue imaged in confocal or non-confocal mode with air and water immersion objectives. InSphero 3D InSight™ human liver microtissues were labeled with Hoechst (nuclei, blue) and CellMask™ Deep Red (membranes, red). A single plane (z= 20 µm) was imaged on the Operetta CLS system either in confocal or non-confocal mode with three different 40x objectives. The higher the NA, the better the background rejection. The sharpest image is achieved with the 40x water immersion NA 1.1 objective in confocal mode.

Summary

Water immersion objectives can provide significantly brighter images from typical HCS samples compared to air objectives. This allows a tenfold decrease in excitation energies, and consequently much faster and gentler imaging. In addition, water immersion objectives increase the resolution and decrease optical aberrations, providing higher quality images from both 2D and 3D samples. Revvity's proprietary water collar system enables the use of water immersion lenses for screening by fully automating the process of delivering immersion water to the lens and removing excess liquid. This proven technology is now available both on the Opera Phenix Plus high-content screening system and on the Operetta CLS high-content analysis system where it has been combined for the first time with powerful LED illumination.

References

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