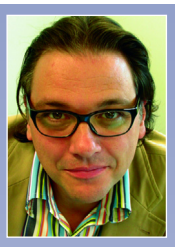
Properties and Selection of Objective Lenses for Light Microscopy Applications

Peter Drent, Nikon Instruments Europe, Amsterdam, The Netherlands

BIOGRAPHY



Peter Drent obtained his degree in biology at Utrecht University in the Netherlands. He is now working as a manager for microscopy products for Nikon Instruments Europe BV.

ABSTRACT

Microscopes have been traditionally designed to use the human eye to observe the image. But nowadays more and more digital cameras are used as the imaging device in both standard and confocal microscopes. These new digital detectors are more sensitive to intensity and flatness differences than the human eye and require new strategies for objective lens design.
This article describes the optical performance of modern objective lenses and discusses criteria for the selection of objectives for a variety of microscope applications including fluorescence microscopy, confocal microscopy, and live cell imaging.

KEYWORDS

light microscopy, confocal microscopy, digital imaging, objective lens, numerical aperture, oil immersion, water immersion

AUTHOR DETAILS

Peter Drent,
Product Manager Microscopy,
Nikon Instruments Europe BV,
Schipholweg 321,
1171PL Badhoevedorp,
The Netherlands
Tel: +31 20 4496258
Email: drent@nikonbv.nl

Microscopy and Analysis 19(6):5-7 (UK), 2005

INTRODUCTION

Prior to reviewing modern objective lens selection, it is worthwhile reflecting on some key drivers in microscope development. Ever since the days of Hooke and Leeuwenhoek, microscope lenses and optical systems have traditionally been designed to provide optimum performance when samples are viewed by the human eye. It is only relatively recently that the differing needs of the digital camera have had to be taken into account. A key advance in sample observation was the Koehler set-up (developed by August Koehler in1893), which ensured even illumination of the specimen plane. However, it was not until the 1950s that microscopes were equipped with a trinocular eyepiece tube that used a photoeyepiece to enable connection of photographic equipment. At that time, bright field, dark field and phase contrast were the most common illumination techniques; fluorescence was not yet in use. In the 1960s film cameras began to be connected to the microscope, again via the photoeyepiece.

Nowadays, a multitude of imaging systems are regularly added to the modern microscope and fluorescence has become one of the most popular contrasting techniques. The latest additions for improved specimen observation, documentation and analysis include digital cameras, laser scanning confocal systems, and advanced systems equipped with fluorescent live-cell imaging capabilities and spectral detector systems. In essence, the modern microscope has grown from being a mere observation station into a complete imaging analysis workstation.

Although the traditional microscope set-up, with correct Koehler illumination, ensures even illumination of the specimen plane, modern sensors, such as those in digital cameras, are far more sensitive to differences in the evenness of illumination than the human eye. This has driven the development of a new illumination strategy in modern microscopes, in effect, a digital Koehler illumination equivalent.

OBJECTIVE LENS DEVELOPMENTS

Along with the progression of the microscope from an observation station into an analysis workstation, the microscope objective lens has also evolved considerably. A key step in this evolution was the transition from the achromat objective lens, which was adequate in the early days of microscopy when the eye was the only method of observation, to the planachromat objective lens, needed when photographic systems arrived and image flatness became an issue. Plan correction of the microscope objective lens ensured flat image reproduction, resulting in uniformly focused photographs. Later, when fluorescence became popular as a contrasting technique, plan fluor objective lenses were introduced and, for special applications that needed ultraviolet excitation, super fluor objective lenses were developed.

Plan fluor objective lenses are essentially planachromat-style lenses with respect to correction but because of different glass selection they show higher transmission rates in the infrared (IR) and blue violet (BV) parts of the spectrum. In addition, planapochromat objective lenses are regarded as being superior in performance with respect to resolution and

Figure 1:
Modern violet-corrected objective lenses for light microscopy. From left to right: Plan Apo VC 60x water immersion, numerical aperture 1.2, working distance 0.27 mm, coverglass thickness 0.15-0.18 mm; Plan Apo VC 100x oil immersion, NA 1.4, WD 0.13 mm, CG 0.17 mm; Plan Apo VC 60x oil immersion, NA 1.4, WD 0.13 mm, CG 0.17 mm.

correction and are regularly selected for higher-level photographic applications.

Typically, achromat objective lenses are designed to focus blue (about 486 nm, also called the f line) and red (656 nm, c line) in the same plane, providing axial chromatic aberration correction. Furthermore, plan fluor objective lenses are also designed to correct for the green (588 nm, also called the d line). In addition to the above, planapochromat objective lenses are corrected for the g line (436 nm). Just recently, a new type of objective lens has been introduced — the planapochromat violet-corrected (VC) series (Figure 1), that covers the h line (405 nm) as well, totally correcting axial chromatic aberration for five lines, ranging from 405-656 nm (Figure 2).

Live cell imaging

In recent years, live cell imaging has become extremely popular. However, since live cells are often kept in climatically controlled chambers, these impose special demands on the microscope objective lens, such as a long working distance. (Extra long working distance may be needed for observing specimens that are contained in thick walled vessels.) Long working distance correction typically covers around 2 mm, enough for Petri dishes or multiwell plates. If the cells are kept in more elaborate climate control chambers, or cell culture bottles, a longer correction for working distance of 4-6 mm is needed, which can normally be found with extra long working distance objective lenses. Super long working distance lenses are also available.

Water immersion

The demands of high-resolution microscopy on live cells also resulted in the introduction of two other types of objective lenses: water immersion and water dipping. Water-immersion objective lenses use water instead of oil as the immersion medium. The maximum numerical aperture (NA) of these water-immersion lenses (e.g. planapochromat 60× with NA 1.2) is lower than oil-immersion types (e.g. planapochromat 60× with NA 1.45) and this has an important effect on resolution. This is because the resolution of an objective lens depends directly on its NA, and can be described as the wavelength divided by twice the NA. When live cells or tissues need to be observed deeper inside the specimen, water-immersion lenses show superior performance with respect to resolution and aberration correction. These lenses offer additional benefits since most cells live in a watery environment. By using water as the immersion medium, the optical system becomes symmetric. If we follow the optical axis from specimen to objective lens, light passes through the following materials: water (cell environment), glass (coverglass), water (immersion medium) and glass (objective lens). As a rule of thumb, when observing up to 25 μm into the specimen, high-NA water-immersion lenses should be selected as these will give the brightest and best resolved images.

Water-dipping lenses are designed to work without a cover glass, and can be submerged directly into the Petri dish or a climate-controlled chamber. Typically, their NA is slightly lower than water-immersion lenses (e.g. plan fluor 100× NA 1.1 compared to plan fluor 100× NA 1.3) but the working distance is much longer, possibly up to 2.5 mm (e.g. plan fluor 100× NA 1.1) compared to 0.2 mm (e.g. plan fluor 100× NA 1.30). This combination of water-dipping and long working distance enables micromanipulation to be undertaken at extremely high magnifications while the tissue being observed is kept in an optimal microenvironment. Because the NA of the water-dipping lenses is still relatively high, this high-resolution micromanipulation can also be combined with digital imaging or even with laser scanning confocal microscopy.

The relative intensity of the image in an optical system is given by the expression:

$$NA^4 / TM^2$$

where NA is numerical aperture of the objective lens and TM is total magnification. Using this formula, it can be shown that the planapochromat 60× NA 1.45 is the highest resolution lens available, while the plan fluor 40× NA 1.3 is the brightest.

The most flexible microscope objectives lenses currently available are the multi-immersion type (plan 20× MI). These can be used dry or with water, glycerin or oil, offering greater flexibility, and are often used as a lower magnification companion lens for a high-NA, high-resolution immersion lens, especially in confocal microscopy.

Correction collars

There are several types of correction rings that allow the performance of the microscope objective lens to be offset against certain parameters, such as cover glass thickness, immersion medium refractive index, temperature correction, or correction for depth penetration into tissues (refractive index). By correcting for these parameters, optimum performance can be readily achieved.

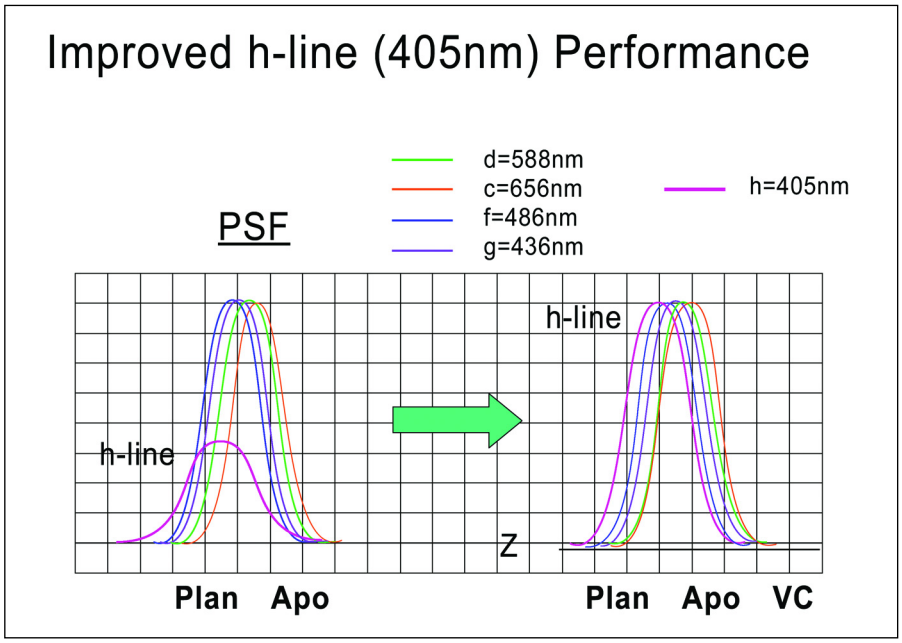


Figure 2:
Chromatic aberration correction of objective lenses. In the CFI60 planapochromat VC series the h line (405 nm) is focused in the same focal plane as the g, f, d and c lines (right), whereas with traditional Plan Apochromat lenses the h line is focused in a different plane (left). CFI60 planapochromats are fully corrected from 405 nm up to 656 nm.

Physical dimensions

Along with the increase in microscope objective lens performance, the physical properties of the objective have also changed. The parfocal distance has grown from 33 mm to RMS 45 mm and, with Nikon, to 60 mm. A similar evolution has occurred for the objective lens diameter, which has grown from 20.3 mm to 25 mm — a move which has given microscope designers more latitude in their optical designs.

Zoom systems

However, it is not only the microscope optics that have evolved significantly, the entire optical system has had to adapt too. When digital sensors became the prime detector, the

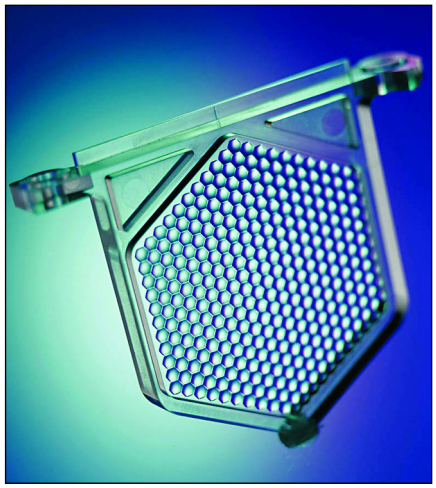


Figure 3:
Fly-eye lens for uniform specimen illumination.

microscope needed to be equipped with some form of magnification changer. We already know (see above) that a microscope objective lens has a resolving power that is determined by its NA. This optical resolution therefore needs to be matched to the digital resolution of the camera. To balance the output resolution with the resolution of the digital sensor the microscope needs to be equipped with a zooming system. According to Nyquist, the sampling frequency needs to be 2-3 times higher than the object that is being sampled. As a result, the modern microscope is equipped with a system that allows zooming. When this is motorised it allows automatic setting of the correct zooming position for each individual microscope objective lens.

Uniformity of illumination

As has been described, the modern microscope objective lens has been specifically designed to cover a broad range of corrections. However, as these lenses are almost always used as part of a high-level imaging system, the evenness of the image they create has also had to be redesigned. The traditional Koehler illumination has had to go 'digital'.

Uniformity of illumination is particularly important for digital images as vignetting, which is sometimes not detected by film photography or through the eyepieces, may have a deleterious influence on image quality.

Another factor that can compromise the uniformity of the image is uneven illumination, which can be eliminated by introduction of a 'fly-eye' lens arrangement in the microscope illuminator (Figure 3). This is the digital equivalent to the traditional Koehler illumination.

In traditional Koehler illumination the lamp filament is spread out by a diffuser and then projected onto the specimen. To the human eye, and even for film photography, this appears to be even, but digital cameras, with their high sensitivity, can easily detect any unevenness. The fly-eye lens element multiplies and projects the single illuminating filament as more than 300 filaments, evenly distributed across the view field. This approach is ideal for the most sensitive digital cameras.

One choice for digital photomicrography, the planapochromat VC lens, not only shows a correction for up to five lines, but is also designed to be perfectly vignetting free. This means that the resolution of the objective lens is even across the entire view field, as shown in Figure 4.

OBJECTIVE SELECTION

In summary, selecting appropriate objective lenses can be the most critical decision when setting up a digital imaging system, or when building up a laser scanning confocal microscope. When using bright field applications in a digital microscope system, light intensity is almost never a limiting factor. Evenness of illumination (fly-eye illumination) correction and vignetting-free objective lenses (planapochromat VC series) are desirable specifications. Zoom optics are very important to balance the digital and optical resolution.

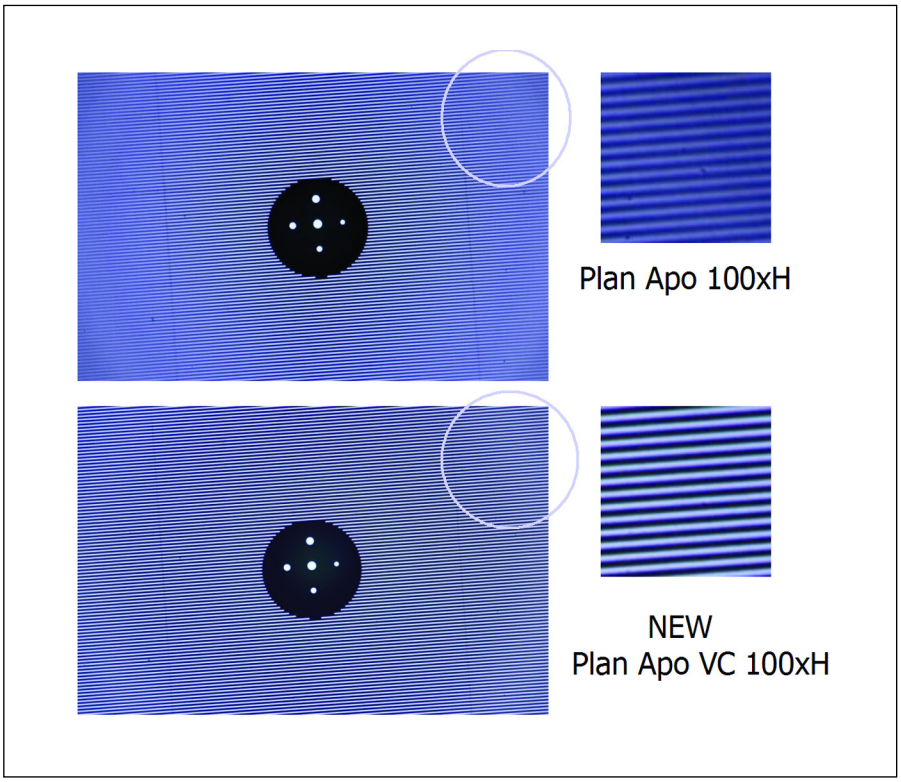


Figure 4:
Comparison of resolving power of normal (top) and violet-corrected (bottom) planapochromat oil-immersion (H) objectives.

Fluorescence microscopy

When using fluorescence applications in a digital microscope system, a choice needs to be made between maximum signal collection (plan fluor 40× NA 1.3), in case of photon limiting applications, or super fluor lenses when using dyes that work at UV or IR wavelengths. When the signal is not limited, correction, as offered by planapochromat lenses, is the preferred option.

Confocal microscopy

For laser scanning confocal microscope systems, objective lens selection is arguably the most critical selection choice in producing superior results. A laser scanning confocal microscope scans the view field point by point and collects the signal. By adjusting the focus position of the specimen, the next plane within the specimen can be scanned in a similar fashion, resulting in a digital 3D image of the signal distribution within the specimen.

Most laser scanning confocal microscopes work with blue (488 nm), green (543 nm) or red (633 nm) excitation. At these wavelengths correction and transmission of the objective lens is at its optimum (for plan fluor and plan apochromat series microscope objective lenses). When using infrared (IR) light to excite the specimen, as in two-photon laser scanning microscopy, the IR transmission of a lens is critical. Planapochromat VC lenses not only work well in the violet part of the spectrum, but also perform extremely well in the IR. One of the benefits of multiphoton microscopy is the depth penetration into the specimen. The long working distance of the fluor water-dipping lenses (up to 2.5 mm) even permit imaging into larger tissues like mouse brain.

In summary, good lenses for laser scanning confocal microscopy are:

1. For most general, highest resolution, vignetting-free operation: planapochromat 60× VC, oil immersion, NA 1.4.
2. For living cell imaging deeper than 25 μm, vignetting free: planapochromat 60× VC, water immersion, NA 1.2.
3. For brightest imaging in cases of photon limitation: plan fluor 40×, oil immersion, NA 1.3.
4. For living cell imaging deeper than 250 μm, vignetting free: fluor 60×, water dipping, NA 1.0.

CONCLUSIONS

With sophisticated optical design, the latest in illumination techniques and advanced detectors — all working together in harmony — modern microscopes are now capable of delivering superior performance for anything from observing sections of tumours, single molecules or cells growing in culture dishes, to reconstructing sets of chromosomes in the nucleus.

However, even the performance of the most flexible of microscopes is still dependent on the design and correct selection of the part closest to the specimen itself. Fortunately, objective lens have responded to the challenge and now offer the high performance needed to meet the demands posed by even the very latest illumination and detection techniques.