Ultrahigh-NA oil immersion optics with spherical aberration correction capability

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In the pursuit of scientific discovery, research applications often drive the development of new technology. Conversely, new technology also extends the boundaries of scientific research. This symbiotic relationship is a force that facilitates scientific progress. This article addresses novel optical technology designed for the application of total internal reflection fluorescence (TIRF), but will increase optical performance for a great number of research applications.

TIRF optics

Although TIRF has been utilized for more than two decades to provide an extremely high signal-to-noise ratio,[1–3] initiating scientific investigation at the single-molecule level, it was not until recently that application-specific optics were developed for this technique. Historically, TIRF was accomplished by introducing a focused laser to the glass specimen interface via a prism in an open optical system (*Figure 1a*). This type of configuration had many limitations,[4] most notably limited access to cells for manipulation, and the necessity to image through the tissue, which scatters light, degrading image quality. Ideally, the best approach for TIRF with respect to performance, ease of use, and flexibility in experimental design is to introduce a focused collimated laser through the objective lens at extremely large angles (Figure 1*b*). These angles are necessary for TIRF so that incident light can strike the coverglass specimen interface at an angle greater than or equal to the critical angle for total internal reflection.

To put into perspective the breadth of the cone of illumination in a TIRF objective, a standard oil immersion lens with a numerical aperture (NA) of 1.45 can collect or transmit a cone of light at 73.16º, with respect to the optical axis, therefore spanning greater than 146º.

The call for ultrahigh-NA optics capable of large angles of illumination (*Figure 2*) was answered in the late 1990s. Currently, three of the four major microscope manufacturers produce optics with high enough NA for TIRF.

A TIRF oil immersion lens was recently manufactured that incorporates a correction collar. Furthermore, the correction collar of the 60× Plan Apo 1.45-NA TIRF objective lens (**Nikon Instruments Inc.,** Melville, NY) was calibrated for a temperature range from 23 ºC (room temperature) to 37 ºC (physiological temperature). This makes it possible to correct for spherical aberration that induces blurriness in the image. Spherical aberration can be a serious concern at higher NA, especially when imaging extremely small structures such as single molecules.

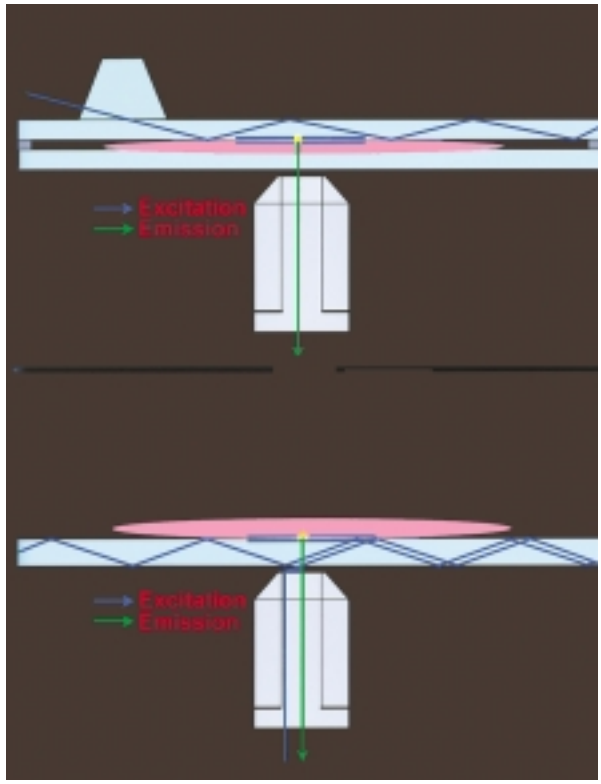

Figure 1 *Two major instrumental approaches to TIRF. a) Configuration for prism-based instrumentation. Incident laser excitation (blue) enters the coverslip via a prism mounted to the surface. Emission is shown in green. b) objective lens based TIRF configuration. Incident laser illumination (blue) enters the coverslip via an objective lens with sufficiently high numerical aperture. Emission is shown in green. (Figure adapted from Ref. 4.)*

Spherical aberration

An ideal simple convex lens has a focal point at a fixed distance called the focal length. In a theoretically perfect lens, rays of light passing through the lens concentrate at this focal point whether they pass through the center (axis) of the lens or through the periphery of the lens. In reality, it is not possible to manufacture an ideal simple lens. High-performance objective lenses for microscopy often contain greater than a dozen optical elements in multiple optical groups.[5] These complex lenses are designed to correct aberration so that light focuses at the same focal point whether passing

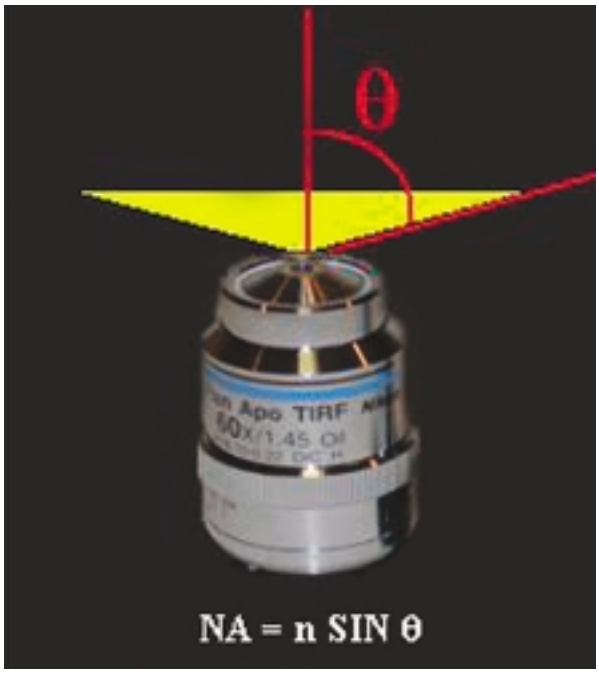

Figure 2 *The angles of light exiting an objective lens are a function of the NA.*

through the axis or the periphery of the lens. In addition, they are corrected to focus a broad spectrum of colors in the same location and provide a wide field of view without curvature. To design such complex correction into optics, it is necessary to account for the different refractive indices of every component in the optical path, from the optical elements themselves, through the immersion media, cover glass, and specimen. The optical pathlength from the objective to the focal point consists of the sum of each element length multiplied by its refractive index (*n*) (Eq. [1]). The complex optical prescription of an objective lens must be set for a specific optical pathlength. However, the optical pathlength is variable, since coverslip thickness is not consistent, the refractive index of immersion media is temperature and wavelength dependent, and specimens are rarely of homogeneous optical density. Consequently, even with highly corrected optics, variable conditions can smear the focal point in the *Z* direction, inducing blur in the image. Therefore, it is important to correct for this variable optical pathlength to eliminate spherical aberration in the image (*Figure 3*).

$$OPL = ((L_1 \times n_1) + (L_2 \times n_2) \ldots + (L_n \times n_n)) \quad (1)$$

where OPL is the optical pathlength, *L* is the length, and *n* is the refractive index; (*n*) oil = 1.515, (*n*) glycerin = 1.47, (*n*) water = 1.33, and (*n*) air = 1.0.

Many high-performance air, water immersion, and multi-immersion objective lenses are equipped with a correction collar that can adjust for a range of optical pathlengths. This type of correction was originally developed to correct for coverglass thickness, since the glass had a different refractive index than the immersion media. This was not adapted to high-NA oil immersion optics, since the refractive index of immersion oil matches that of glass. However, many research applications require imaging at a range of temperatures and utilize multiple wavelengths of light. These experimental variables can induce spherical aberration, decreasing image quality while using oil immersion optics. When using high-NA oil immersion optics with the capability to correct for spherical aberration, it is possible to obtain the sharpest images and extract the most meaningful data.

The advantages of this optical technology for several applications are shown in this paper. It is by no means a complete list, but merely a few representative examples.

Epifluorescence

For biological and biophysical investigation, fluorescence imaging has long been a very powerful technique. The force of this technique gained momentum in 1997, when it was shown that the genetically encoded fluorophor green fluorescent protein (GFP), which has its origins in the jellyfish *Aequoria Victoria*, could be expressed across species in both eukaryotes and prokaryotes.[6] Since those pioneering experiments, GFP mutants have been produced in a variety of colors, and their stability and brightness have been enhanced. The genes for GFPs have been stably introduced into many specific genes of interest in a variety of species used as experimental models, making it possible to study a particular protein in a noninvasive manner.

For epifluorescence imaging, new TIRF optics offer three distinct benefits: 1) The higher NA (1.45 vs 1.40 for the standard Plan Apo 60×) can collect more light due the broad cone of illumination; 2) resolution, or two-point discrimination, is directly dependent on NA, making these optics capable of a higher resolution compared to other Plan apochromatic lenses; 3) spherical aberration correction capability eliminates blur in the *Z* direction, which would result in an overall decline in the S/N, decreasing the quality of data that can be extracted from the images. An example of the effect of spherical aberration in an epifluorescence image is shown in *Figure 4*.

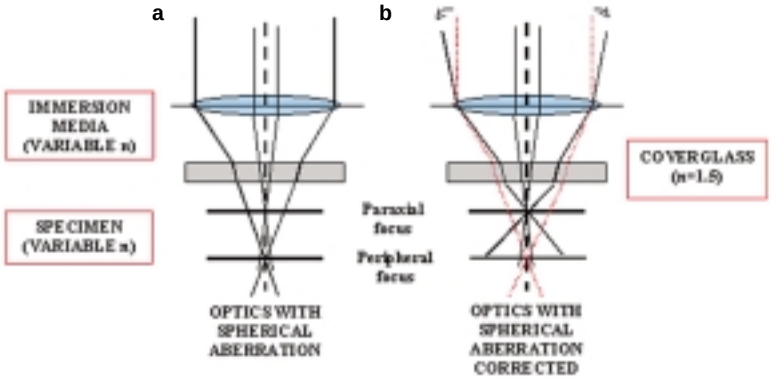

Figure 3 *Correction of spherical aberration by parfocal adjustment. a) Ray diagram illustrating misfocused light from the peripheral regions of a spherical lens (spherical aberration). b) Ray diagram illustrating correction of the spherical aberration. Uncorrected light shown as dotted lines, correction shown with arrows.*

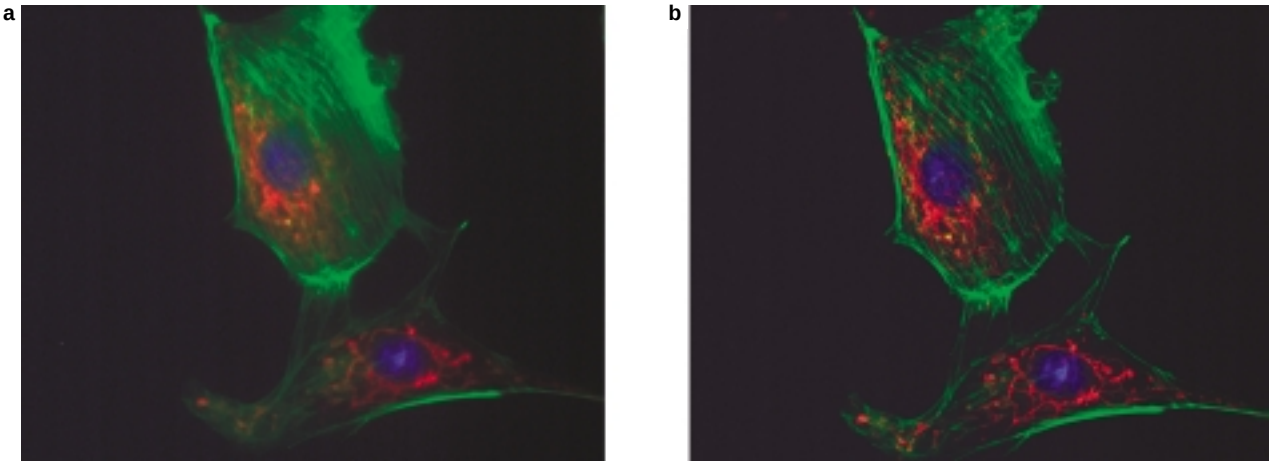

Figure 4 *Spherical aberration decreases S/N in epifluorescence images. Triple labeled cells (**Molecular Probes,** Eugene, OR): mitochondria (red), tubulin (green), DNA (blue). a) Image shows significant spherical aberration. Note that the image is in perfect focus, yet still shows excessive blur. b) The same specimen imaged with spherical aberration corrected. Imaged with the Plan Apo 1.45-NA TIRF objective lens.*

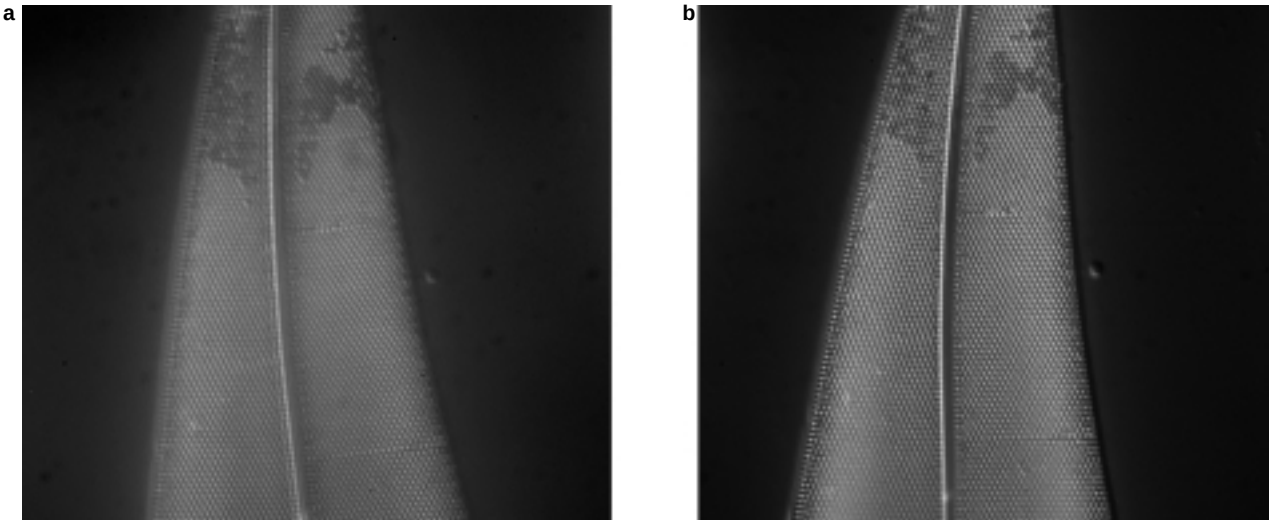

Figure 5 *Spherical aberration induces blur in DIC images. Surface structure of a diatom imaged in high-NA DIC. a) Image shows significant spherical aberration. Note that the image is in perfect focus, yet still shows excessive blur. b) The same specimen imaged with spherical aberration corrected. Imaged with the Plan Apo 1.45-NA TIRF objective lens, and high-NA oil immersion condenser.*

Differential interference contrast

Differential interference contrast (DIC) is an important technique that can be utilized to provide contrast when imaging a specimen that is unstained, and when high resolution is essential. With DIC, the microscopist can image structures in cells that are below the optical resolution limit and would be invisible using standard transmitted illumination. One such example is the ability to image in vitro microtubules in solution that have a diameter of 25 nm or approx. ⅒ of the resolution limit.[5] Because live cells are aqueous, and many of the organelles have different optical densities, spherical aberration can become a serious problem when imaging deep structures. Additionally, refractive index mismatch can cause blur when looking at fine surface structures.

TIRF objective lenses offer a significant improvement and enhance image quality in DIC. First, the NA of 1.45 makes it possible to achieve resolution in DIC images that was not previously possible. Second, the ability to correct for spherical aberration adds sharpness to the images, irrespective of variability in the optical pathlength. Dr. Shinya Inoué (Marine Biological Laboratories, Woods Hole, MA), considered by many to be the world's leading authority on video-enhanced live-cell DIC imaging, had the opportunity to use the Plan Apo 1.45-NA TIRF objective with a spherical aberration correction ring for high-NA DIC imaging, and responded: "The image in DIC is spectacular. I have never in my life seen such sharp, detailed, and clear images of the surface ridges on oral epithelial cells. They showed exquisitely resolved fine detail, and remarkably shallow depth of field (i.e., outstanding *Z*-axis resolution)." *Figure 5* illustrates the effect of spherical aberration in a DIC image.

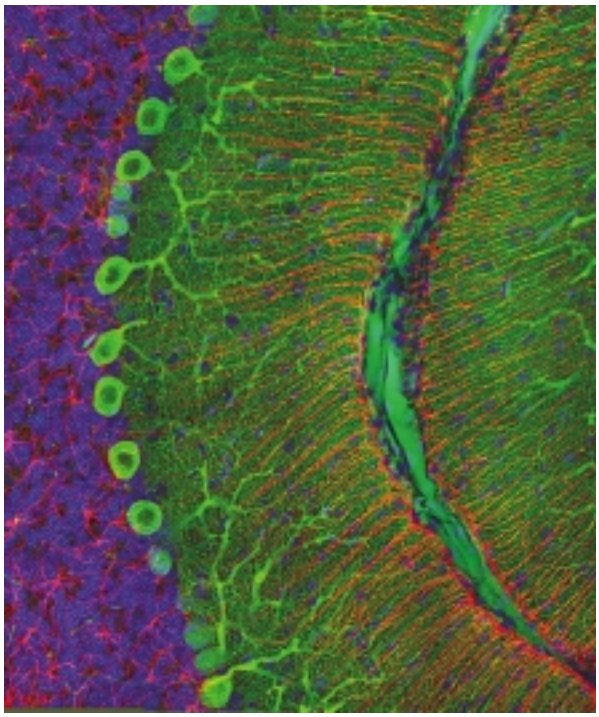

Figure 6 *TIRF optics can be utilized to acquire extremely high quality confocal images. This image shows a sagittal section of mouse cerebellum, imaged in fluorescence using the 60× Plan Apo TIRF lens with spherical aberration correction on the* ***Nikon*** *RCM (real time confocal microscope). (Image courtesy of Hiroyuki Hakozaki, Thomas J. Deerinck, and Sunny Chow, in the laboratory of Mark H. Ellisman, UCSD, La Jolla, CA.)*

Confocal microscopy

Confocal microscopes effectively remove out-of-focus light, decreasing the noise in fluorescent images. A typical laser scanning confocal microscope accomplishes this by raster scanning a diffraction-limited laser spot across the specimen point by point. Then, the emission passes through a pinhole in the conjugate (or confocal) primary image plane, physically blocking out-of-focus light. By removing this light, it is feasible to image relatively far into specimens with fluorescent structures labeled at multiple *Z* positions that would cause a serious problem resolving signals above the noise floor with standard epifluorescence. The most significant improvement in the image comes from cleaning up out-of-focus light laterally for each fluorescent spot in the image. Performance is hindered further in the axial direction, and the quality of 3-D images produced when reconstructing multiple *Z* sections into an image is limited by the amount of spherical aberration produced by the optics. Therefore, the higher resolution provided by the ultrahigh NA of TIRF optics and the ability to correct for spherical aberration elevate the value of the data acquired. The quality of the confocal images produced using TIRF optics is illustrated in *Figure 6*.

Conclusion

The applications discussed above are only an example of the versatility and power of this optical technology. Additionally, ultrahigh NAs and the ability to eliminate spherical aberration can provide significant benefits for many other techniques. Further, because this new generation of optics has extremely high transmission in the infrared (1064 nm), they may enhance the data quality in multiphoton and laser-trapping systems as well.

In the future, it is likely that these optics, originally designed for TIRF, will be used in place of standard high-NA oil immersion optics for a large number of research applications.

References

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