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SHORT COMMUNICATION

Simple optimization procedure for objective lens correction collar setting

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Summary

We present a new method for setting a coverglass correction collar on an objective lens. Axial scans across the interface between the specimen volume and the slide are used together with a quantitative function of merit to determine the optimum setting of the correction collar. The method, which simplifies the adjustment for the user and reduces photobleaching, was implemented within the software environment of a scanning microscope system.

Introduction

The presence of spherical aberration reduces signal intensity and axial resolution in confocal or multiphoton microscopy. This can often be introduced by an incorrect coverglass thickness or a mismatch in refractive index between the embedding medium and the lens immersion medium. Modern microscope objective lenses of high numerical aperture (NA) are often equipped with a correction collar that may be used to remove the spherical aberration introduced by different coverglass thicknesses. However, the setting of the correction collar is often difficult due to the lack of a quantitative quality criterion and problems caused by the focal shift introduced during the adjustment. Even more importantly, inefficient adjustment procedures may bleach the fluorescent sample, which is an unwanted side-effect. Here we present a simple optimization method for the correction collar adjustment that is based on a quantitative criterion and minimizes photobleaching. We implemented an automated and user-friendly optimization approach within the software environment of a scanning microscope system.

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Limitations of previous optimization procedures

Objective lenses are typically designed to provide diffraction-limited performance when focusing through a particular coverglass thickness into a particular refractive index. It is well known that a deviation from the nominal coverglass thickness or a refractive index mismatch introduces spherical aberration (Hell *et al.*, 1993; Török *et al.*, 1995; Booth *et al.*, 1998; Keller, 1995), especially when high-NA lenses are used. In the case of a refractive index mismatch the additional aberration depends linearly on the focusing depth. The axial extension of the point spread function of the lens is especially sensitive to spherical aberration, which leads to reduced axial resolution of the instrument and a decrease in the intensity signal level. The manufacturers of microscope lenses have recognized this problem and offer lenses that are equipped with a coverglass correction collar that may be used to compensate for the spherical aberration. However, setting the correction collar to the nominal coverglass thickness is not necessarily sufficient because the thickness of the coverglass is only specified within a particular tolerance and is often different from the nominal thickness. Furthermore, the exact refractive indices of the sample, the embedding medium and the focusing depth are also rarely known in practice but are required to estimate the spherical aberration components. It would therefore be desirable to have a simple, user-friendly optimization procedure for the correction collar adjustment.

It is common practice to repeatedly scan the sample while adjusting the correction collar in an attempt to maximize the intensity and resolution in the image. This is clearly challenging and subjective. Furthermore, the adjustment of the correction collar also introduces defocus and changes the location observed within the specimen (Pawley, 2002). This requires tracking the object feature of interest in the axial direction while scanning the specimen, adjusting the correction collar and judging the intensity, which is clearly impractical. The wide-field fluorescence mode of the microscope delivers a real-time image but is

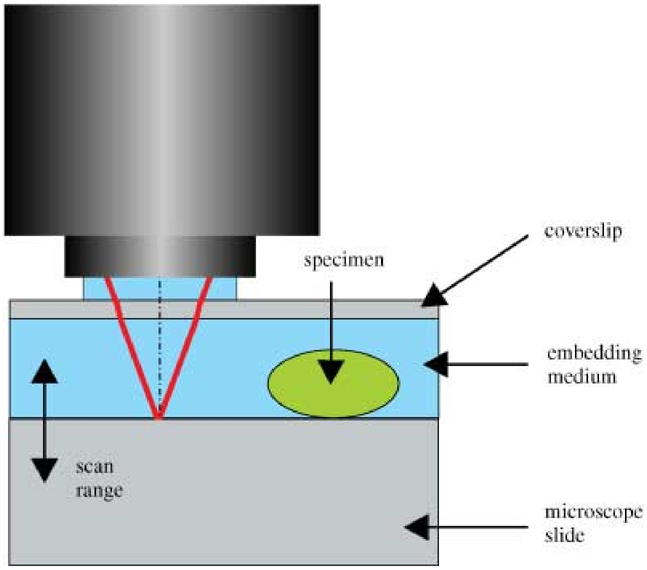

Fig. 1. The focusing configuration for the acquisition of axial scans.

not appropriate for the adjustment. If, for example, an extended region of constant fluorophore concentration is observed, the overall intensity stays the same even when different amounts of spherical aberration are present. When the adjustment is performed in the confocal mode, the separation of the effects of aberration adjustment and photobleaching on the intensity can be a problem.

New approach for the optimization procedure

An optimization procedure should be based on a quantitative criterion, work with a variety of specimens and should be simple and easy to implement. We introduce a criterion based upon the reflection from the interface between the specimen volume and the microscope slide to obtain the optimum correction collar setting. A fast axial scan through a region that includes the interface makes tracking of the reflection during the adjustment unnecessary (Fig. 1). In order to reduce or prevent photobleaching, the axial scan can be performed next to the region to be imaged. Alternatively, one could use a different wavelength that does not excite and bleach the fluorophore.

Axial scans for different settings of the correction collar for a 1.2 NA water-immersion lens are shown at the top of Fig. 2. In each image the same axial range was scanned. A shift in the focal position of about 20 µm over the correction collar range from 140 to 170 µm is visible. Corresponding intensity profiles are displayed at the bottom of Fig. 2. The axial width of the reflection signal is reduced and the maximum intensity increases when the setting is changed towards the optimum. Theoretical modelling for the presence of spherical aberration confirms this behaviour of the axial response from a reflection at an interface (Wilson & Sheppard, 1984).

We can choose different criteria that evaluate the correction collar setting from an axial scan of a reflection such as those in Fig. 2. For instance, one can use the peak intensity of an axial scan. Another useful criterion is the value of the squared intensity, integrated along the axis:

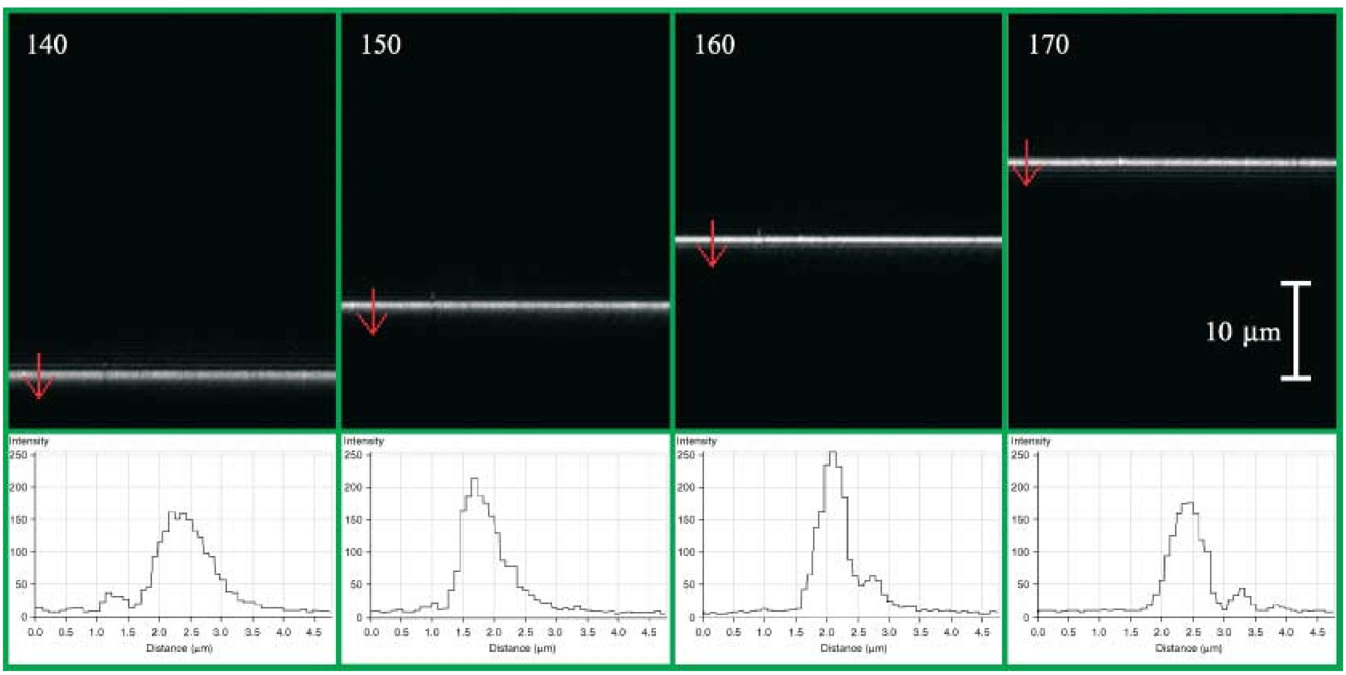

Fig. 2. Axial scans of the reflection from the interface between the microscope slide and the embedding medium. The numbers within the panels indicate the correction collar setting in micrometres of coverglass thickness; the red arrows indicate the location where the intensity profiles were extracted.

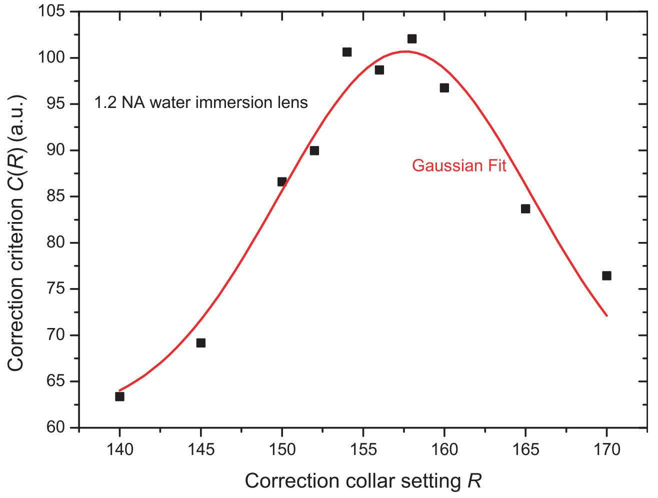

Fig. 3. Experimental values for $C(R)$, measured for a 1.2 NA water-immersion lens.

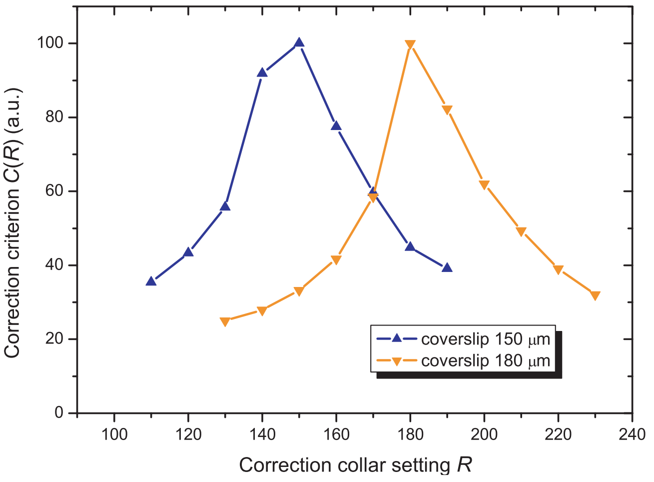

Fig. 4. Spherical aberration correction criterion, $C(R)$, plotted against the correction collar setting for two different coverglasses using a 0.95 NA dry lens.

$$C(R) = \int_{-\infty}^{\infty} I^2(R,z)dz,$$

where $I(R,z)$ denotes the intensity signal and R is the correction collar setting. The value of $C(R)$ can be easily calculated. Other criteria such as the integral of higher powers of the intensity in the axial direction may also be used. In practice it is useful to average over several line scans in order to make the algorithm less susceptible to noise. Experimental values for $C(R)$ using a 1.2 NA water-immersion lens are shown in Fig. 3. The specimen was embedded in a mixture of glycerol and water. The constant scan range in the axial direction was set such that the reflection from the interface between the specimen volume and the microscope slide was included despite the focal shift that was introduced by the adjustment of the correction collar. The peak of the correction criterion is distinct and fitted a Gaussian shape. The goal of the optimization procedure was to locate the setting R of the correction collar where the function $C(R)$ has a maximum. As mentioned before, the two main sources of spherical aberration are inappropriate coverglass thickness and refractive index mismatch. In Fig. 3 the refractive index mismatch of the embedding water/glycerol mixture caused a deviation of the optimum setting from the setting of the nominal coverglass thickness. In this case, the criterion is particularly useful because even an accurate measurement of the coverglass thickness would not allow correct estimation of the optimum setting. The measured thickness of the coverglass was 145 µm but the optimum setting was found to be 158 µm because of the refractive index mismatch.

In a second experiment, a lens with a larger range of correction collar adjustment was used (0.95 NA dry lens) and the correction criterion $C(R)$ was recorded for two different nominal coverglass thicknesses of 150 and 180 µm. As shown in Fig. 4, the resulting functions are similar in shape but the position of the peak depends on the aberration conditions. The peak of the correction criterion coincides with the optimum setting. Assuming that $C(R)$ is known for a particular lens, one may fit this function to a few measured values of the correction criterion and extract the peak position. We have implemented a software macro that records axial scans of the reflection, calculates $C(R)$ and predicts the position of the optimum setting from three single measurements assuming a Gaussian shape of the correction function. The graphical interface of the software macro is shown in Fig. 5. There are two ways to operate the module: the first mode monitors the correction quality close to real-time and the adjustment can be performed manually.

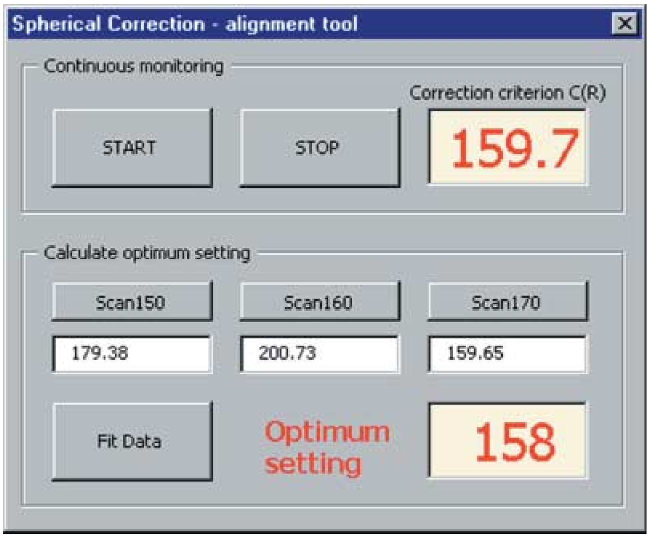

Fig. 5. User interface for the software macro. The top section displays the value of the correction criterion $C(R)$. The bottom section predicts the optimum setting of the correction collar from three axial scans.

In the second mode one can set the correction collar to three different positions, record three values and obtain a prediction for the optimum value. The prediction procedure requires calibration for each individual lens. Optimum settings were found by this method and verified by manual adjustment to confirm that this was indeed the optimum position. In principle, full automation of the optimization would be feasible if the correction collar of the lens could be controlled via the PC. It should be pointed out that in both modes of the software there is no need to refocus the specimen while adjusting the correction collar as long as the axial scan contains the reflection from the interface.

The same optimization can be performed for the interface between specimen and coverglass. Optimum settings for intermediate planes within the specimen volume can then be inferred by linear interpolation between the settings for the bottom and top of the specimen.

Discussion

Spherical aberration has a strong influence on the imaging quality of confocal and multiphoton microscopes and can be caused by inappropriate coverglass thickness and refractive index mismatch. Lenses with adjustment facilities for spherical aberration are available but the common adjustment procedures have practical problems such as unwanted photobleaching, the need for object tracking during the adjustment and the lack of a quantitative function of merit that can be optimized.

We proposed using the reflection from the interface between the specimen volume and the microscope slide to obtain information on the aberration correction setting. A criterion based on the integral of the squared intensity in axial direction is used to monitor correction quality. Importantly, this method does not rely on fluorescence intensity and minimizes bleaching of the sample. In addition, there is no need for object tracking during the adjustment. The position of the optimum setting can be predicted from three simple measurements.

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