

Depth-of-Focus in Microscopy

I.T. Young, R. Zagers, L.J. van Vliet, J. Mullikin, F. Boddeke, H. Netten
Pattern Recognition Group of the Faculty of Applied Physics
Delft University of Technology
Lorentzweg 1, NL-2628 CJ Delft, The Netherlands
e-mail: young@ph.tn.tudelft.nl

Abstract

Digital image processing and analysis provide a powerful tool for describing optical systems. In this paper we show that conventional formulas for the depth-of-focus (Δz) of a high numerical aperture, diffraction-limited optical system are incorrect. Using standard diffraction wave theory we have (re-)discovered a formula for Δz and then tested that formula under controlled experimental conditions using digital image processing and analysis.

Our expression for Δz is given by:

$$\Delta z = \frac{\lambda}{4 \cdot n \cdot \left(1 - \sqrt{1 - \left(\frac{NA}{n} \right)^2} \right)}$$

where λ is the (near monochromatic) wavelength of illumination, NA is the numerical aperture of the optical system, and n is the index of refraction. This formula is in agreement with empirical measurements of Δz to within the experimental error associated with our measurement system.

1 Introduction

A major project within our group is the automation of certain tasks in molecular cytogenetics. Molecular cytogenetics deals with the use of specific molecules to label various parts of DNA in the biological cell. Modern developments in *in situ* hybridization coupled with fluorescent markers make it possible to detect and count the presence of any particular chromosome (1-22,X,Y) or the presence of disease specific (sub-chromosomal) genetic markers in non-dividing nuclei. In research as well as clinical practice this means there is an urgent need for instrumentation to count hundreds to thousands of cells to assess, for example, aneuploidy, trisomies, and minimum residual disease (MRD).

To develop the appropriate instrumentation we have had to examine a number of problems in depth. One of these concerns an accurate description of the depth-of-focus for the type of high numerical aperture lenses that are typically used in microscopy and, in particular, fluorescence microscopy. (High numerical apertures are especially needed in epi-illumination fluorescence where the light gathering efficiency is proportional to NA^4 .)

2 Theory

The depth-of-focus (Δz) of a microscope system has been described by a number of authors. Unfortunately, these descriptions do not agree. In general, the authors propose that Δz should be a function of the wavelength of light, λ , the numerical aperture of the objective lens NA , the index of refraction, n , and the total magnification of the optical system M . The numerical aperture is defined as $NA = n \cdot \sin \alpha$ where n is the index of refraction of the medium between the lens and the specimen being studied and α is the angle illustrated in Figure 1. Two of the most well-known formulas (from Piller [1] and Born & Wolf [2]) are:

$$\Delta z_P = \frac{1000}{7 \cdot NA \cdot M} + \frac{\lambda}{2 \cdot NA^2} \quad \Delta z_{BW} = \frac{\lambda}{2 \cdot NA^2} \quad (1)$$

The fact that neither of these two formulas seems to give the values of Δz that are observed in practice has led us to examine this problem from the theoretical as well as the experimental side. Using wave theory we have developed a “new” formula which agrees with objective measurements made using a variety of lenses and wavelengths:

$$\Delta z = \frac{\lambda}{4 \cdot n \cdot \left(1 - \sqrt{1 - \left(\frac{NA}{n}\right)^2}\right)} \quad (2)$$

That is, for a given (λ , NA , n , and M) we predict Δz using the above formula.

The basis of this formula is as follows. Consider two light emitting points along the axis of the optical system: one in-focus at position R and one out-of-focus at position $R + \Delta z$. Each point produces (according to the Huygens’ model) a spherical wavefront of light. Given a pupil that subtends an angle α with the in-focus object, the maximal difference, W , between these two wavefronts will occur at the angle α . This is illustrated in Figure 1.

Using the law of cosines we have the following:

$$(R + \Delta z)^2 = (R + W)^2 + (\Delta z)^2 + 2(\Delta z)(R + W)\cos(\alpha) \quad (3)$$

This equation is quadratic in W and can be solved for W which, for $\Delta z \ll R$, reduces to:

$$W \cong \Delta z(1 - \cos \alpha) = 2\Delta z \sin^2\left(\frac{\alpha}{2}\right) \quad (4)$$

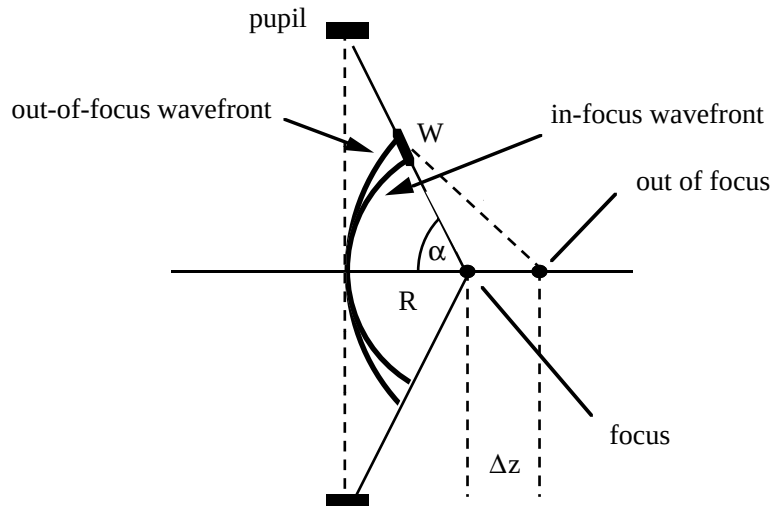


Figure 1: Two points: one is in-focus and one out-of-focus. Each produces a spherical wavefront. The wavefront approaches the entrance pupil of an optical system that subtends an angle α as shown. The maximum distance between the wavefronts, W , occurs at angle α .

A widely used criterion for characterizing the distortion due to defocusing is the Rayleigh limit which in this context states that, if we wish to maintain a diffraction limited system, the maximal path difference may never exceed a quarter wave length, that is, $W \leq \lambda/4$ [3]. Application of this condition to equation 4 results in equation 2.

On purely numerical grounds, our value of Δz (equation 2) can differ by as much as a factor of two for high NA lenses from that given by Δz_{BW} . This is illustrated in Figure 2. Note that for low values of the NA ($NA < 0.5$), an asymptotic expansion of equation 2 gives Δz_{BW} .

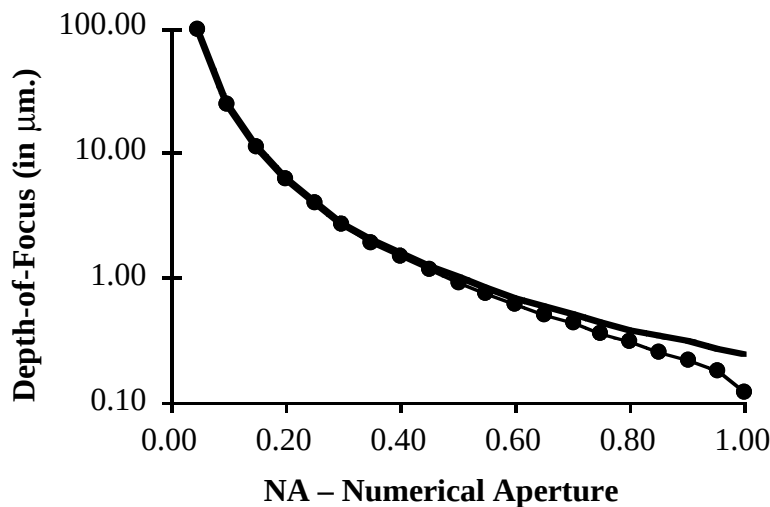


Figure 2: The depth-of-focus according to Born and Wolf (–) and the depth-of-focus according to equation 2 (–•–).

We can measure Δz using a technique based upon the Struve ratio [4]. The Struve ratio $\rho(z)$ measures the degradation in the optical transfer function (OTF) response to a line source as a function of the position z of the microscope table. As can be seen from equation (5), the Struve ratio measures the “volume” under the OTF for the line source. If the “volume” decreases, then the Struve ratio decreases as well.

$$\rho(z) = \frac{\tau(x=0, y=0, z)}{\tau(x=0, y=0, z=0)} = \frac{\int OTF(\omega, z) d\omega}{\int OTF(\omega, z=0) d\omega} \quad (5)$$

This change is illustrated in Figure 3 in data that we have measured. We see that, as z increases away from the position of optimum focus, the OTF decreases. This effect is particularly strong in the middle frequency range as the minimum frequency $\omega=0$ and the maximum frequency $\omega=4\pi NA/\lambda$ are always “pinned” to the values of 1.0 and 0.0, respectively [5].

3 Materials and methods

The key, of course, is to measure the line response of the optical system. We have implemented this by using a test slide produced by e-beam lithography at the Delft Institute for Micro-Electronics (DIMES). A bar grating on the test slide is used and the height of each bar is 10 nm. of chromium deposited through the lithography process. The images are acquired with an inverted Nikon microscope. The condenser is a Nikon Achr Apl 1.35, especially corrected for chromatic aberration and aplanatism. Its numerical aperture is variable within the range [0.1,1.35]. Via a microscope objective the test slide is imaged onto a CCD camera (Photometrics Model 250 with the KAF 1400 CCD chip). The z -position of the microscope objectives can be changed via a DC-motor controlled by a computer. The smallest step in the axial (z) direction is 24.7 nm. $\pm$ 1.3% [6].

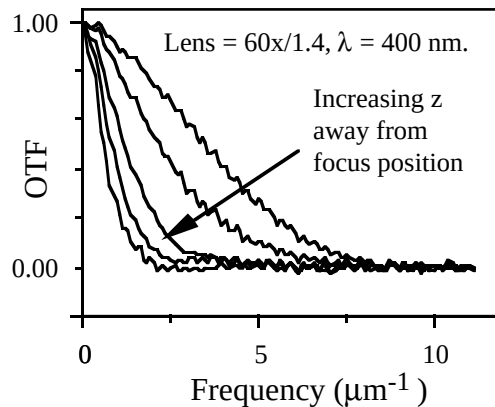


Figure 3: As we move away from optimum focus, as Δz increases, the $OTF(\omega_r/2\pi)$ “sags”. This effect is particularly pronounced in the middle frequencies. These *measured* data describe a 60× lens with an NA of 1.4 (oil-immersion), and a wavelength of $\lambda = 400$ nm. (blue).

The sampling density in the specimen plane is determined by the magnification of the objective lens, further magnification by various relay lenses, and the center-to-center pixel spacing of the CCD camera. For example for a 40× lens, a 2× relay magnification and 6.8 μm. CCD pixel size, the sampling density is $(6.8 \mu\text{m.})/(2 \times 40) = 0.085 \mu\text{m.}$ per pixel for a sampling frequency of 11.75 pixels per μm. Given that the 40× lens has an NA = 0.85, this means that at a wavelength of .510 μm. the sampling frequency should exceed the Nyquist frequency given by $4NA/\lambda$ ($= 4 \times 0.85/.510 = 6.67$ pixels per μm.) to ensure that aliasing is avoided [5]. This sampling frequency of 11.75 clearly exceeds the Nyquist frequency of 6.67 indicating that for this lens–camera combination aliasing is not a problem.

4 Image processing

After correction for background shading using standard techniques, the edge-response images are differentiated to produce line-response images. The line response is equivalent to the point spread function (PSF) of the microscope lens if the lens is circularly symmetric [7]. Next the (radial) OTF of the lens is computed from the Fourier transform of the PSF. The Struve ratio can then be computed using equation 5.

A collection of Struve ratios as a function of z is shown in Figure 4. From these curves we can measure the value of Δz associated with a particular lens. For a lens to remain in the “diffraction limited state” the amount of aberration (W) associated with defocusing must remain less than $\lambda/4$. This can be shown to translate into a Struve ratio of 0.86. In other words to be “diffraction limited” the Struve ratio $\rho(z) \geq 0.86$. The maximum value of z for which this is true gives Δz .

5 Results and discussion

For a collection of eight different lenses and numerical apertures the theoretical depth-of-focus was predicted from equation (2) and the measured depth-of-focus was extracted from Figure 4. This has led to the results in Figure 5. If our model is correct then not only should there be a linear relationship between the values of the form $y = ax+b$ but the values of the coefficients should (ideally) be $a = 1$ and $b = 0$.

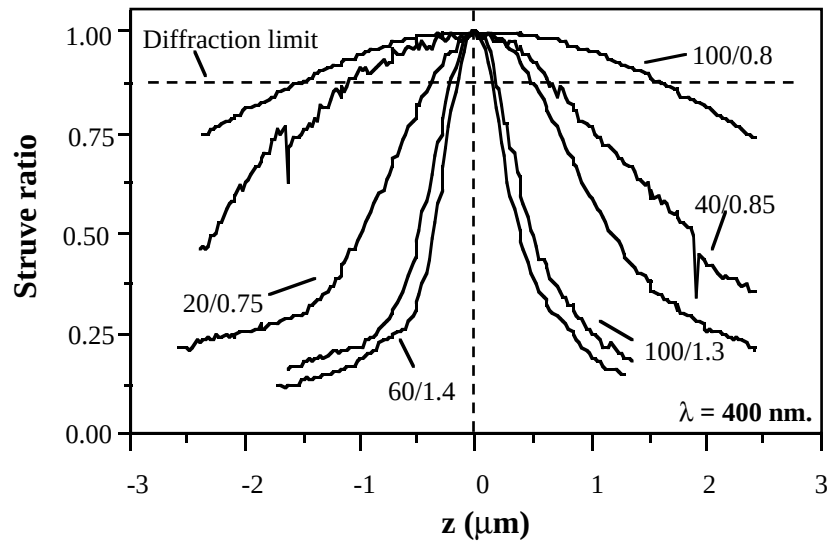


Figure 4: The Struve ratio $\rho(z)$ is measured for a variety of microscope lenses of varying magnifications and NAs. Given that the aberration W must be less than $\lambda/4$ to maintain a diffraction-limited optical system (see text), the depth-of-focus for each lens can be defined as that value of $z=\Delta z$ for which $\rho(z=\Delta z) = 0.86$.

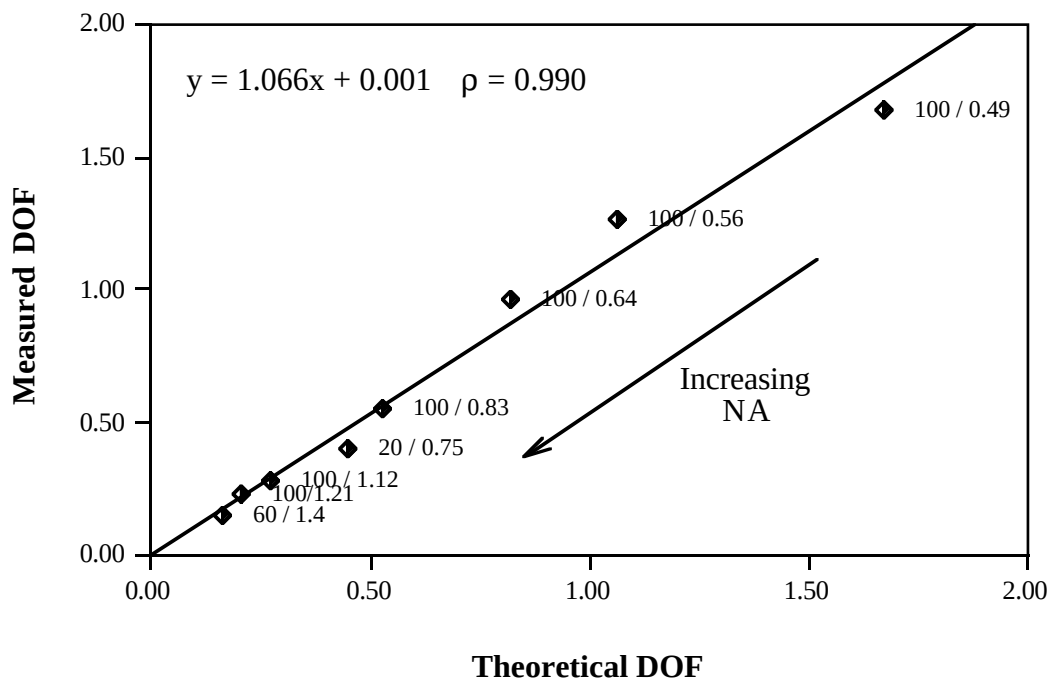


Figure 5: The depth-of-focus ($\text{DOF}=\Delta z$) measured at 400 nm versus the theoretical prediction based upon equation 2. The correlation coefficient, and regression line are shown in the figure and discussed in the text.

An examination of Figure 5 shows that the linear fit is excellent with the correlation coefficient $\rho = 0.99$. The slope of the regression line ($a = 1.066$) as well as the intercept ($b = 0.001$) show that the agreement between theory and experiment is not just a straight line relationship but an excellent *prediction* of the measured values.

One of the most interesting results is the direct demonstration that the magnification M plays no role in Δz . This can be seen easily in the results of an experiment where NA , λ and n were held constant and only M was varied by using a zoom relay lens.

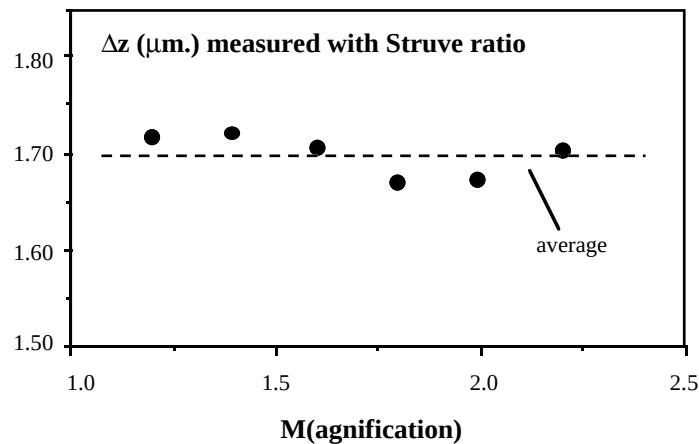


Figure 6: The depth-of-focus (Δz) measured for a constant λ , NA , and n but varying M .

It is clear from Figure 6 that within the experimental error the depth-of-focus is not a function of magnification, M , and thus the formula given in Piller is *not* appropriate for quantitative microscopy. The formula that matches the experimental data – particularly at high numerical aperture – is equation (2) above.

Acknowledgments

This work was partially supported by The Netherlands' Foundation for Scientific Research, NWO Grant 900-538-016 and by the SPIN research program, Three-Dimensional Image Analysis.

References

- [1] Piller H. Microscope Photometry. Berlin: Springer-Verlag, 1977
- [2] Born M, Wolf E. Principles of Optics. (Sixth ed.) Oxford: Pergamon Press, 1980
- [3] Reynolds GO, DeVelis JB, Parrent GBJ, Thompson BJ. Physical optics notebook: Tutorials in Fourier optics. Bellingham, Washington: SPIE Optical Engineering Press, 1989
- [4] Barakat R. Application of the sampling theorem to optical diffraction theory. J. Optical Society America 1964;54(7):920-930.
- [5] Young IT. Image Fidelity: Characterizing the Imaging Transfer Function. In: Taylor DL, Wang YL, ed. Fluorescence Microscopy of Living Cells in Culture: Quantitative Fluorescence Microscopy – Imaging and Spectroscopy. San Diego: Academic Press, 1989: 1–45. (Wilson L, ed. Method in Cell Biology; vol 30:B).
- [6] Van Vliet LJ, Boddeke FR, De Jong P, Netten H, Young IT. Autofocusing in Fluorescence Microscopy. In: Clausen OP, Laerum OD, Lindmo T, Steen HB, ed. XV Congress of the International Society for Analytical Cytology. Bergen, Norway: Wiley-Liss, 1991: 70.
- [7] Papoulis A. Systems and Transforms with Applications in Optics. New York: McGraw-Hill, 1968