SCANNING Vol. 16, 198–200 (1994)

Received November 17, 1993

Volume Measurements in Three-Dimensional Microscopy

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Summary: A refractive index mismatch between the oil immersion and the microscopic object can lead to a severe overestimation of the object's size. The cause of this effect is explained and a simple calibration method to compensate for its occurrence is presented. A practical example is discussed. The analysis applies to both conventional three-dimensional, and confocal microscopy.

Key words: three-dimensional microscopy, confocal microscopy, volume measurement, focal anomaly

Introduction

When an object is imaged by a high NA system (e.g., a confocal fluorescence microscope), there often will be a refractive index mismatch between the immersion medium and the cover glass ($n = 1.51$) on the one hand, and the object and its embedding on the other. For biological objects, the index will be comparable to water, that is, $n \sim 1.33$. The effects of this mismatch can hardly be overstated, for when the object stage is moved vertically (i.e., in the z-direction), the object point or plane that is imaged is *not* moved over the same distance as the stage. The size of this "focal anomaly", as will be shown in the next sections, is appreciable. It can lead to an overestimation of the object size by a factor of two or more. The effect occurs only in the vertical direction and affects both conventional and confocal microscopy.

A possible remedy is to insert microscopic perfect spheres into the embedding medium. Then the (apparent) axial and lateral diameters of the spheres are determined. The focal anomaly will cause the former to be larger. The ratio of the two diameters yields the factor by which all vertical distances must be divided to recover the "true" size.

A geometric explanation of the elongation effect is given in the following section. Under "Results" two experiments are reported: one which demonstrates the occurrence of the focal anomaly and another in which the proposed gauging method is applied to study cell volumes of primary root cells of *Zea mays*.

Geometric Theory of the Focal Anomaly

Figure 1 depicts the illumination part of the microscope. A converging light cone (not indicated) emanates from the lens, travels through the immersion oil and the cover glass (both with refractive index n_1), and is then brought to focus in the object and its embedding (refractive index n_2). Consider a ray which in the first medium makes an angle θ_1 with the normal to the cover glass-object interface. The ray is refracted according to Snell's Law as

$$n_1 \sin\theta_1 = n_2 \sin\theta_2. \tag{1}$$

Let the radius of the light cone at the interface be r_1. The distance below the interface where this ray crosses the vertical or z axis is called z_1. Then

$$r_1 = z_1 \tan\theta_2. \tag{2}$$

Let the focal length of the lens be f and the combined thickness of the immersion oil and the cover glass d_1. This yields

$$r_1 = (f - d_1)\tan\theta_1. \tag{3}$$

Equating the two relations gives

$$z_1 = (f - d_1)\frac{\tan\theta_1}{\tan\theta_2} = (f - d_1)\frac{n_2 \cos\theta_2}{n_1 \cos\theta_1}. \tag{4}$$

Suppose now that the object stage is moved a distance Δ_{stage} along the vertical axis. The thickness of oil immersion plus cover glass is now d_2 for which

$$d_2 - d_1 = \Delta_{stage}. \tag{5}$$

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Thus, after the stage has moved, the new point of focus for the ray we are considering has shifted to a point z_2, for which

$$\Delta_{\text{focus}} = z_1 - z_2 = \Delta_{\text{stage}} \frac{n_2 \cos\theta_2}{n_1 \cos\theta_1}. \quad (6)$$

Thus we find that an axial displacement Δ_{stage} of the object stage results in a shift of the focus Δ_{focus} which is a factor $n_2 \cos\theta_2 / n_1 \cos\theta_1$ less. For the marginal rays with, say, $\theta_1 = 60°$, $n_1 = 1.51$, and $n_2 = 1.33$ (corresponding to an $NA = 1.30$ objective), this factor is about 0.37. For paraxial rays Eq. (6) reduces to

$$\Delta_{\text{focus}} = \Delta_{\text{stage}} \frac{n_2}{n_1}, \quad (7)$$

which in our example yields a factor of $n_2/n_1 = 0.88$. The interface causes all rays to focus at different positions, so there is no longer a single focal spot, but rather a region along the z axis that is illuminated. The apparent depth of an object that is measured will be somewhere between $(0.88)^{-1}$ and $(0.37)^{-1}$ times the actual depth. In other words, the overestimation of the object's vertical size will be between 1.14 and 2.73.

Notice that the model predicts the anomaly only in the axial direction. The lateral size of an object remains unaffected. It also follows that when $n_2 > n_1$, for example, when a water-immersion lens is used to study an object embedded in a medium with refractive index > 1.33, the apparent depth will be *smaller* than the actual one.

It should be emphasized that the effect will occur not only in confocal, but also in conventional three-dimensional (3-D) microscopy.

In the next sections we discuss several experiments which corroborate our findings.

Materials and Methods

For the first experiment, microspheres embedded in media with various indices of refraction, the embeddings were obtained by dissolving sucrose in water ($n = 1.359$, 1.415, and 1.449). In addition aqua dest. ($n = 1.334$), ethyleneglycol ($n = 1.428$), and immersion oil ($n = 1.518$) were used. Three-dimensional images were made with a confocal microscope (Biorad 600M), equipped with a 100× NA 1.30 oil objective, and operating at $\lambda = 488$ nm. The axial distance between the spheres (5.6 µm "fluorospheres", Coulter Corp., Hialeah, Fla., USA) and the interface were kept constant at 100 µm. Both the lateral and the axial diameter were measured. Reasonable agreement with the theoretical predictions was found: the spheres embedded in water indeed appeared to have an axial diameter equal to 2.4 times their lateral diameter, (Fig. 2). The closer the refractive index of the embedding liquid is to that of oil ($n_{\text{oil}} = 1.518$), the more the apparent axial diameter approximates the lateral diameter. For spheres immersed in oil, the artefact did indeed not occur.

For the second experiment, the determination of cell sizes, primary roots of corn (*Zea mays*) were fixed overnight in 3:1 absolute methanol: glacial acetic acid (v/v), thereafter washed in acetate buffer (pH 4.6), and macerated in a mixture of 1% cellulase and 1% pectinase in the same buffer (v/v) for 2 h at 37°C. After washing, first in acetate buffer, then in PBS buffer (pH 7.0), the root tips were stained with the DNA/RNA specific fluorochrome propidium iodide [0.1 µg/ml solution in PBS (v/v) for 1 h at room temperature]. This procedure resulted in a suspension of root tip cells, which was then mixed with a diluted

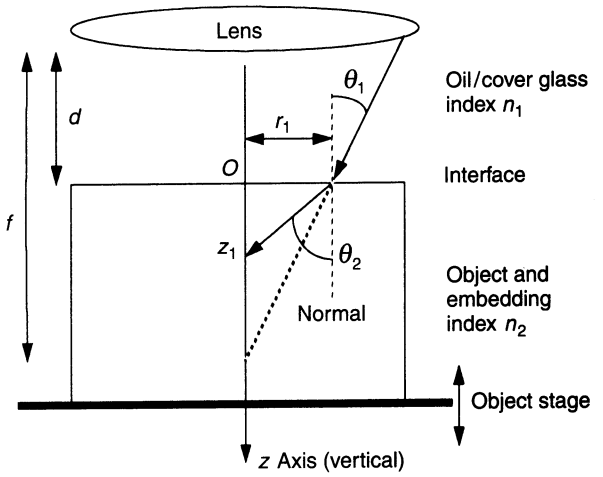


FIG. 1 Geometry of the system. A ray of light leaves the lens and travels through the immersion oil and the cover glass, both with refractive index n_1. In the embedding medium (with index n_2), the ray makes an angle θ_2 with the normal (dashed line) to the interface. In this example $n_1 > n_2$. The origin O of the z axis is at the interface.

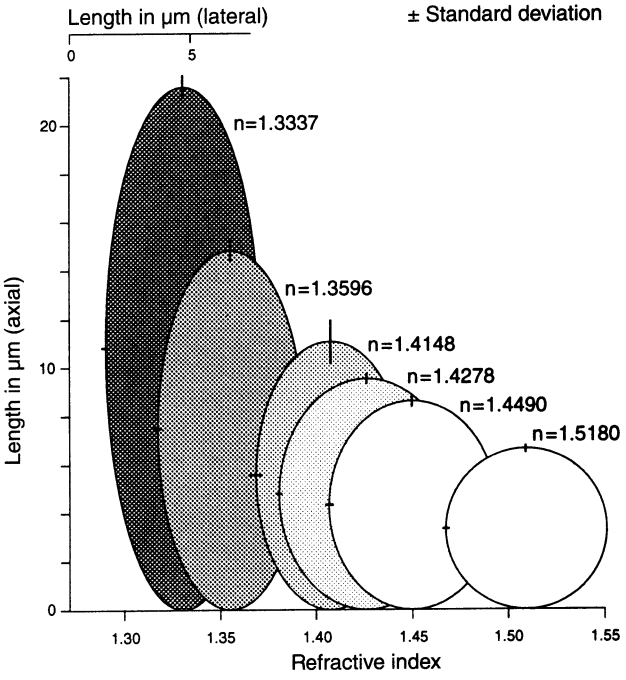


FIG. 2 Side view (zx plane) of the ~ 6 µm fluorescent microspheres embedded in media with varying refractive index. Only the spheres immersed in oil appear to be truly spherical. However, the elongated spheres on the left are more typical for biomedical objects.

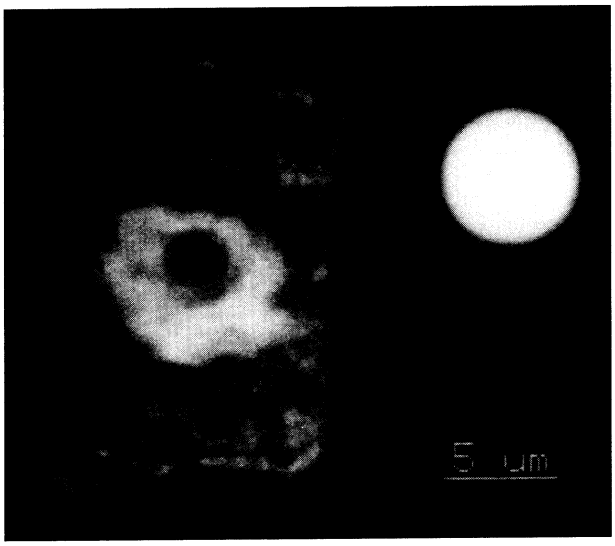
suspension of FITC coated (~ 6 μm) latex spheres ("Fluoresbrite", PolySciences Inc.). The mixture of cells and spheres was embedded in 2% low-melting-point agar in distilled water (v/v). The preparations were analysed with a Leica confocal microscope equipped with a 100× (NA 1.32) oil objective, using both the 488 and the 514 nm lines of the Argon laser for fluorescence excitation of FITC and propidium iodide, respectively. The refractive index of the embedding medium and the immersion oil was 1.344 and 1.518, respectively. We measured the dimensions in the x-, y-, and z-direction of 10 cells which had a sphere in their close proximity at the same depth in the preparation. The lateral and axial diameters of each sphere were measured.

Results

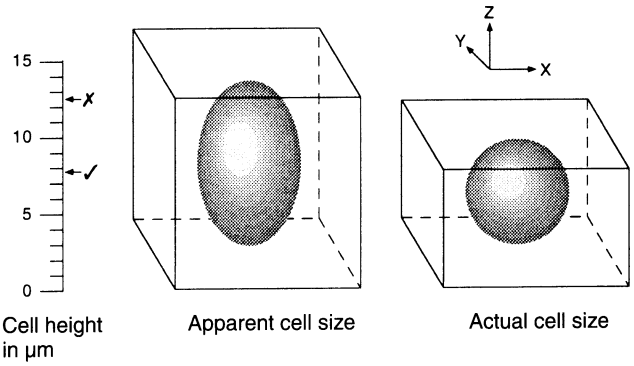
Two different experiments were carried out: one to show that the predicted effect indeed exists, and one which uses a simple gauging method to compensate for the effect.

The model was first tested on fluorescent microspheres which were immersed in watery solutions with different refractive indices. The spheres were imaged with an oil immersion objective. The predicted elongation was indeed observed (Fig. 2). Especially for watery objects ($n = 1.33$), which are typically dealt with in biomedical practice, the effect is quite huge. But also for objects embedded in, for example, glycerol ($n = 1.475$), which has an index much closer to that of oil and glass, it follows from interpolation that the effect will still be appreciable (~ 17%). Only spheres embedded in oil appear to be truly spherical.

In order to gauge the vertical axis, microspheres can be mixed with the object that is to be measured (Fig. 3a). By determining the ratio of the lateral and the axial sizes of the spheres, the precise elongation factor can be found. By dividing all vertical dimensions by this factor, one has taken the effect into account. This method was applied to the measurement of cell sizes of corn (see Fig. 3b). The overestimation factor was found to range from 1.60–2.02, with an average of 1.74. Notice that after correction for the elongation effect the cell nucleus appears much more spherical in shape.



(a)



(b)

FIG. 3 Illustration of the method to determine the overestimation of size along the z axis, due to a refractive index mismatch. (a) One optical section of a series of 16 confocal images, which shows a corn root cell (stained with propidium iodide) and the adjacent FITC stained microsphere; (b) schematic representation of the same root cell before (left) and after (right) correction of the axial size, based on the ratio of measured lateral and axial diameter of the microsphere. On average, the overestimation of the axial size was found to be 1.74×. Note that (a) is a top view of the cell, whereas the schematic cell (b) is depicted in side view.

Discussion

The systematic overestimation of sizes in 3-D microscopy, due to the different refractive indices of immersion oil and the object's embedding that our model predicts, is indeed found in experiments. Under typical conditions the apparent elongation may be as large as 2.5. It is clear that this "focal anomaly" has great consequences for all sorts of quantitative microscopy, such as volume measurement, image restoration, image reconstruction and, of course, image analysis. When the refractive indices are known, the size of the effect can be estimated, as rule of thumb, as the average of the effect on paraxial and marginal rays [see Eqs. (6), (7)], A more accurate vectorial analysis of the effect is now in preparation (Wiersma and Visser).

Calibration of the distance between the different image layers by the insertion of a fluorescent microsphere is shown to be a relatively simple and reliable solution, at least when one is studying cell suspensions.

It should be realized that our remarks are equally valid for both conventional and confocal 3-D microscopy.

Acknowledgments

The authors gratefully acknowledge the technical assistance of Marlies Buys. They also thank Sjors Wiersma for carefully reading the manuscript.

Reference

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