Refractive index and axial distance measurements in 3-D microscopy

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Refractive index and axial distance measurements in 3-D microscopy. We use a simple geometrical model to show that when a converging wave is focused into a dielectric object, the axial movement of the object stage is not followed by the focus. This has important consequences for depth measurements in 3-dimensional microscopy. It was found for objects immersed in a watery solution that the apparent axial dimension can be up to three times larger than the actual size.

Brechungsindex und axiale Abstandsmessung in der 3-D Mikroskopie. Wir benutzen ein einfaches geometrisches Modell um zu zeigen, daß bei Fokussierung einer konvergierenden Welle in ein dielektrisches Medium die axiale Bewegung des Objekthalters nicht bei dem Fokus gefolgt wird. Das hat wichtige Konsequenzen für die Tiefenmessung in der 3-D Mikroskopie. Es wurde für in Wasser eingebettete Objekte gefunden, daß die auftretende axiale Dimension bis zu drei mal größer sein kann als die wirkliche.

1. Theory

In 3-D microscopy the axial dimension or depth of an object is measured by first focusing on the "top" and then on the "bottom" of said object. Next it is determined how much the object stage has been moved in this process. This distance is then taken as the object's depth. We shall argue that this method is incorrect since the focal position (i.e. the spot that is imaged) does not simply follow the axial movement of the object stage but, in the case of objects immersed in an aqueous solution, is moved over a much smaller distance, depending on refractive index conditions. This "focal anomaly" thus gives rise to an apparent depth which can be up to three times larger than the actual depth.

Consider a converging light cone travelling in a medium with refractive index n_1 which is focused by a lens into a second medium with refractive index n_2 (see fig. 1). The marginal rays of the light cone make an angle θ_1 with the normal to the interface. The light is then refracted according to Snell's Law:

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

Let the half-width of the light cone at the interface be x. The depth of focus z_1 is then given by

$$z_1 = \frac{x}{\tan\theta_2} . \tag{2}$$

Suppose now that the object stage is moved a distance Δ_s along the z-axis towards the lens (i.e. upwards in fig. 1). The half-width of the light cone is then increased by an amount δx, for which we have

$$\delta x = \Delta_s \tan\theta_1 . \tag{3}$$

The new depth of focus z_2 is now according to (2) equal to

$$z_2 = \frac{x + \delta x}{\tan\theta_2} . \tag{4}$$

Substitution of (3) yields for the axial focal shift, which we call Δ_f,

$$z_2 - z_1 \equiv \Delta_f = \frac{\tan\theta_1}{\tan\theta_2}\Delta_s . \tag{5}$$

This means that an axial movement Δ_s of the object stage, results in an amplified or decreased shift of the focus, when n_1 is smaller or greater than n_2, respectively. Alternatively, this can be expressed in terms of NA, the numerical aperture of the objective, for which we have

$$NA = n_1 \sin\theta_1 . \tag{6}$$

Substituting this together with eq. (1) gives

$$\Delta_f = \frac{\tan(\sin^{-1}(NA/n_1))}{\tan(\sin^{-1}(NA/n_2))}\Delta_s . \tag{7}$$

In 3-D microscopy one typically uses oil immersion objectives with $NA = 1.30$ and $\theta_1 = 60°$. When this is used to image a (biological) watery object, according to eq. (7) the apparent depth of the object will be more than two and a half times the actual depth. Note that the effect will be smaller for low numerical aperture lenses. The model predicts this anomaly only in the axial direction.

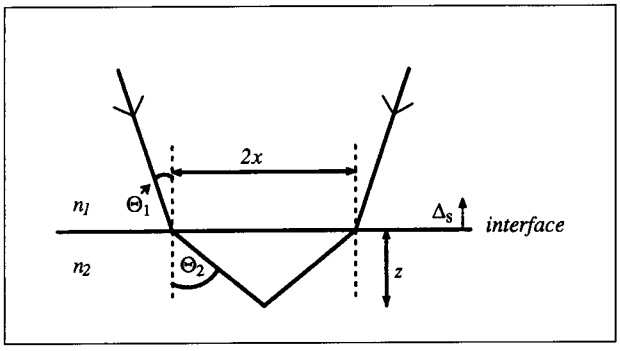

Fig. 1. Geometry of the system. A convergent light wave which travels through a medium with refractive index n_1 is focused in a second medium with refractive index n_2. Indicated is the normal to the interface (dashed). The marginal rays make an angle θ_1 with the normal before refraction, and an angle θ_2 after refraction. In this example $n_1 > n_2$.

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The lateral length of an object remains unaffected. When $n_2 > n_1$, e.g. when an oil-immersion lens is used to study an object embedded in a medium with refractive index greater than 1.51, the model predicts an apparent depth which is smaller than the actual one.

It should be emphasized that this focal anomaly has great consequences to the fields of image restoration, image reconstruction and of course, image analysis. To illustrate this point we refer, out of many possible choices, to the recent study of Geiger et al. [1]. They used an oil immersion objective to make 3-D images of lymphocyte nuclei embedded in glycerol gelatine ($n = 1.47$), and found that these had an ellipsoid shape. The authors are puzzled by the fact that the longer axis of the ellipsoids is consistently pointing in the z-axis. Our model gives an explanation of this phenomenon. A second example is the paper by Rigaut et al. [2] in which confocal data sets are used to estimate cell volumes. As they do not take the focal anomaly into account, the volumes they report should be larger as compared to volumes obtained by means of microtomy.

Our model only uses marginal rays to determine the focal position. This is justified since most of the rays originate from the marginal region of the objective. The interface, however, gives rise to spherical aberration. That means that the paraxial rays do not focus at precisely the same position as the marginal rays. Especially when one is focusing deep into the second medium this may become a factor of importance, causing the diffraction pattern to become wider. As a result of this small objects appear to be larger. However, spherical aberration alone can by no means account for the factor of about 2.5 that is predicted for some cases.

2. Experiment

In order to test the above predictions, we have measured the dimensions of small fluorescent spheres which were immersed in liquids with varying refractive indices. Watery solutions were made with sucrose ($n = 1.359$, 1.415 and 1.449). Furthermore aqua dest. ($n = 1.334$), ethyleneglycol ($n = 1.428$) and immersion oil ($n = 1.518$) were used. Three-dimensional images of the spheres (5.6 µm "fluorospheres", Coulter Corp., Hialeah, Florida, U.S.A.) were made with a confocal microscope (Biorad 600M), equipped with an $NA = 1.30$ oil objective and operating at $\lambda = 488$ nm. The axial distance between the spheres and the interface was 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 (see fig. 2). The more the refractive index of the embedding liquid is closer to that of oil ($n_{oil} = 1.518$), the more the apparent axial diameter approximates the lateral diameter. For spheres immersed in oil, the artefact did indeed not occur. We found, inter alia, that objects immersed in oil were imaged much sharper than those immersed in water. This is an indication of the presence of spherical aberration when watery biological objects are studied. The experiment was therefore repeated with the spheres now 170 µm below the interface. It was found that the ratio between the axial and lateral dimension of the spheres was somewhat increased compared to the other series of measurements (fig. 2). This may be due to a loss of axial resolution caused by the now greater amount of spherical aberration.

Until now, we have not able to test the model for liquids with $n > 1.518$, because in all such liquids we have examined so far, the spheres dissolved.

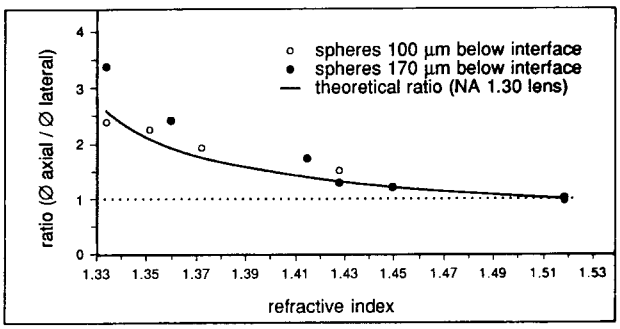

Fig. 2. Ratio of the axial and the lateral diameter of fluorescent microspheres as a function of the refractive index of the embedding medium. Notice that for spheres in a watery medium the apparent axial diameter is around three times the real diameter.

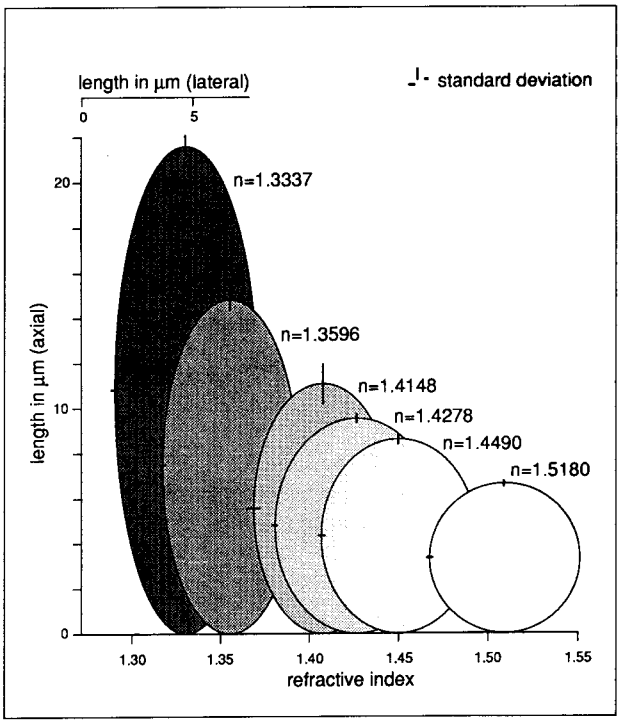

Fig. 3. View in the zx-plane of 6 µm fluorescent microspheres in media with varying refractive index. In this case the spheres were all 170 µm below the interface. Only the spheres immersed in oil appear to be truly spherical.

3. Conclusion

We have presented a geometrical model of the three-dimensional imaging process predicting that the apparent axial dimension of an object depends on immersion conditions and can be up to three times its actual size. This effect is caused by the fact that the focal position does not simply follow the vertical movement of the object stage, but (in general) moves over a smaller distance. This causes an overestimation of depths and volumes. It was experimentally verified that this effect indeed occurs, as is nicely illustrated in fig. 3. Reasonable agreement with the theory was found. The precise value of the anomaly depends on the index of refraction of the medium in which the object is embedded and on the distance between the object and the interface.

This result has major implications for all forms of quantitative 3-D microscopy. One way of determining the focal anomaly in a practical situation may be to insert a fluorescent sphere in the sample which is studied and measure the ratio of the sphere's lateral and axial diameter. This value can then be used to calibrate distances. Finally, it should be noted that the use of water immersion objects to study biological objects may lessen the anomaly but does not prevent it from occurring. This is because the index of refraction of such objects typically varies from 1.33 to 1.39, so there still remains some mismatch, causing the effect.

Acknowledgements

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References

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NOTE ADDED IN PROOF:

After this paper was submitted an article appeared by K. Carlsson [J. Microscopy, **163**, (1991) 167–178] in which, in passing, the discussed effect is mentioned.