Measuring *Z*-Distances using a Confocal Microscope

A confocal microscope can be used to characterize colloidal structures. However, care should be taken when measuring distances within a colloidal structure along the *z*-direction, which means along the optical axis of the microscope objective. In order to determine distances along that axis, the confocal microscope measures the displacement of the stage on which the sample is mounted. However, the desired distance is the difference in height above the dispersion-slide interface. The two distances are not equal if the laser beam crosses one or several refractive index boundaries after leaving the objective. Therefore, it might be necessary to perform a correction on distances measured using a confocal microscope. The appropriate correction factor can be calculated quite easily using geometric optics [1].

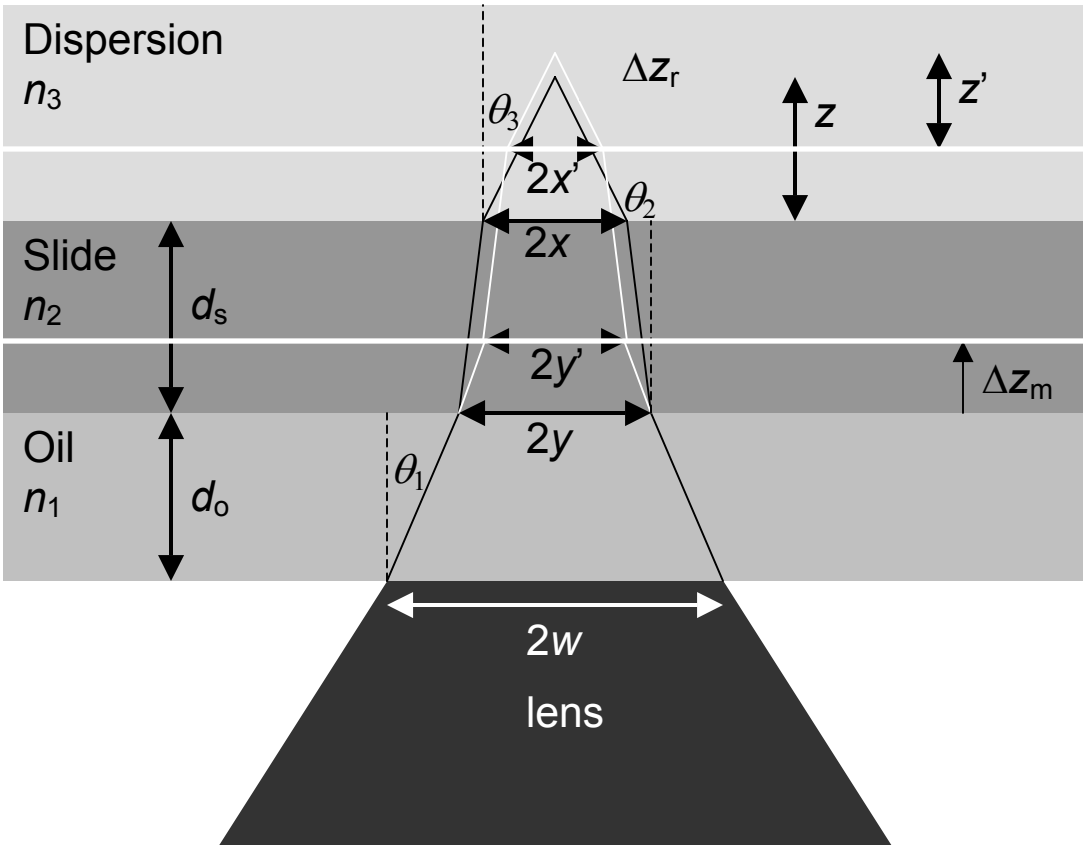

Figure 1: Schematic picture of a common geometry for *z*-measurements using confocal microscopy. The laser probe beam enters from above; the emitted light is collected by an objective lens below. Since the confocal lens has a fixed acceptance angle, which is expressed by its numerical aperture, only light that is emitted by the focal point within a certain angular range is detected. Before entering the lens, the light goes through the dispersion, the cover slide and the immersion oil with refractive indices n_1, n_2 and n_3 respectively.

Figure 1 shows a schematic representation of a common geometry for z-measurements using confocal microscopy[1]. The displacement of the stage is denoted by Δz_m, whereas the height difference relative to the dispersion-slide interface is Δz_r. Before entering the lens, the light goes through the dispersion, the cover slide and the immersion oil with refractive indices n_1, n_2 and n_3 respectively (in Figure 1 $n_2 > n_3 > n_1$). In order to keep matters simple, it is assumed that the colloidal particles within the dispersion are (nearly) matched to the fluid surrounding them at the wavelength at which the (fluorescent) colloids emit. Furthermore, it is assumed that $0° \leq \theta_1 < 90°$, such that its sine, cosine and tangent are all positive real numbers. This is no unphysical assumption: if it is not met, the laser beam will not converge and it will will not be focussed into a single point, which will make imaging impossible.

Before moving the stage, the position of the focal point z relative to the slide-dispersion interface can be expressed as,

Equation 1: Position of focal point relative to the slide-dispersion interface before moving the stage.

$$z = \frac{x}{\tan(\theta_3)},$$

in which x is the half-width of the light cone at the border between the cover slide and the dispersion, and θ_3 is the angle of incidence of the light coming from the focal point onto the cover slide (see Figure 1). After moving the stage, the position of the focal point relative to the slide-dispersion interface is z'. Since the refractive indices of the system have not changed and the acceptance angle of the microscope has not changed, the angles are the same before and after moving the stage. As the displacement of the cover slide equals the displacement of the stage (the cover slide is assumed to be a rigid object that is rigidly connected to the stage) the displacement of the focal point is equal to $|z - z'|$. In short,

Equation 2: Heigth difference of focal point relative to slide-dispersion interface.

$$\Delta z_r = |z - z'| = \left| \frac{x}{\tan(\theta_3)} - \frac{x'}{\tan(\theta_3)} \right| = \frac{|x - x'|}{\tan(\theta_3)},$$

in which x' is the half-width of the light cone at the border between the cover slide and the dispersion after moving the stage.

The difference $|x - x'|$ can be written as a function of θ_1. From Figure 1,

Equation 3: The difference $|x - x'|$ as a function of θ_1.

$$x' = w - d_o \tan(\theta_1) - d_s \tan(\theta_2) - \Delta z_m \tan(\theta_1)$$

[1] The situation before moving the stage is indicated by black lines, while the situation after moving the stage is indicated by white lines.

$$x = w - d_o \tan(\theta_1) - d_s \tan(\theta_2)$$
$$|x - x'| = w - d_0 \tan(\theta_1) - d_s \tan(\theta_2) - w + d_o \tan(\theta_1) + d_s \tan(\theta_2) + \Delta z_m \tan(\theta_1) = \Delta z_m \tan(\theta_1),$$

in which θ_1 is half the acceptance angle of the microscope objective, d_o is the thickness of the immersion oil layer before moving the stage, d_s is the thickness of the cover slide and Δz_m is the distance over which the stage has been moved. Thus, the height difference relative to the slide-dispersion interface can be expressed as in Equation 4.

Equation 4: Height difference relative to the slide-dispersion interface as a function of θ_1 ans θ_3.

$$\Delta z_r = \frac{\tan(\theta_1)}{\tan(\theta_3)} \Delta z_m$$

Using Snell's law,

Equation 5: Snell's law.

$$n_i \sin(\theta_i) = n_j \sin(\theta_j),$$

in which i and j are adjacent media, it is even possible to rewrite Equation 4 in terms of the numerical aperture NA of the microscope objective, which is defines as $n_1 \sin(\theta_1)$.

Equation 6: Height difference relative to slide-dispersion interface as a function of *NA*.

$$\Delta z_r = \frac{\tan(\theta_1)}{\tan(\theta_3)} \Delta z_m = \frac{\frac{\sin(\theta_1)}{\cos(\theta_1)}}{\frac{\sin(\theta_3)}{\cos(\theta_3)}} \Delta z_m = \frac{\frac{n_1 \sin(\theta_1)}{n_1 \sqrt{1 - \sin^2(\theta_1)}}}{\frac{n_3 \sin(\theta_3)}{n_3 \sqrt{1 - \sin^2(\theta_3)}}} \Delta z_m$$
$$= \frac{\frac{NA}{\sqrt{n_1^2 - (NA)^2}}}{\frac{n_1 \sin(\theta_1)}{\sqrt{n_3^2 - n_3^2 \sin^2(\theta_3)}}} \Delta z_m = \frac{\frac{NA}{\sqrt{n_1^2 - (NA)^2}}}{\frac{NA}{\sqrt{n_3^2 - (NA)^2}}} \Delta z_m = \sqrt{\frac{n_3^2 - (NA)^2}{n_1^2 - (NA)^2}} \Delta z_m$$

Finally, the confocal correction factor c_c is defined as the ration of Δz_r and Δz_m. Thus,

Equation 7: Confocal correction factor.

$$c_c = \frac{\Delta z_r}{\Delta z_m} = \sqrt{\frac{n_3^2 - (NA)^2}{n_1^2 - (NA)^2}}.$$

1. Visser, T.D.;Oud, J.L.and Bakenhoff, G.J., *Refractive index and axial distances measurements in 3-D microscopy.* Optik, 1992. **90**(1): p. 17-19.