Height Measurements under the Compound Microscope

Gregor Overney, San Jose, California, USA

The compound microscope is a precision tool for measuring the height (or thickness) of small objects. Unfortunately, it is not common knowledge what equations have to be used to ensure the most accurate measurements. With this paper, I give a short introduction to improving the accuracy of vertical measurements under the compound microscope. The study is based on G. W. White's paper published in 1970 [**3**].

First, we will look at a simple equation used to measure the height or thickness of a sample when observed with a microscope objective that has a small numerical aperture ($NA \sim 0.25$ or less). Second, we will look at a more accurate equation that enables us to conduct height measurements with a microscope objective with a high numerical aperture ($NA \sim 0.65$ or more). Third, we proof that the simple equation indeed satisfies the more accurate equation for very small numerical apertures ($NA \ll 1$).

Part A: Height measurements with "low power" objectives (small numerical aperture)

We will follow the notations used in the Figure 1.

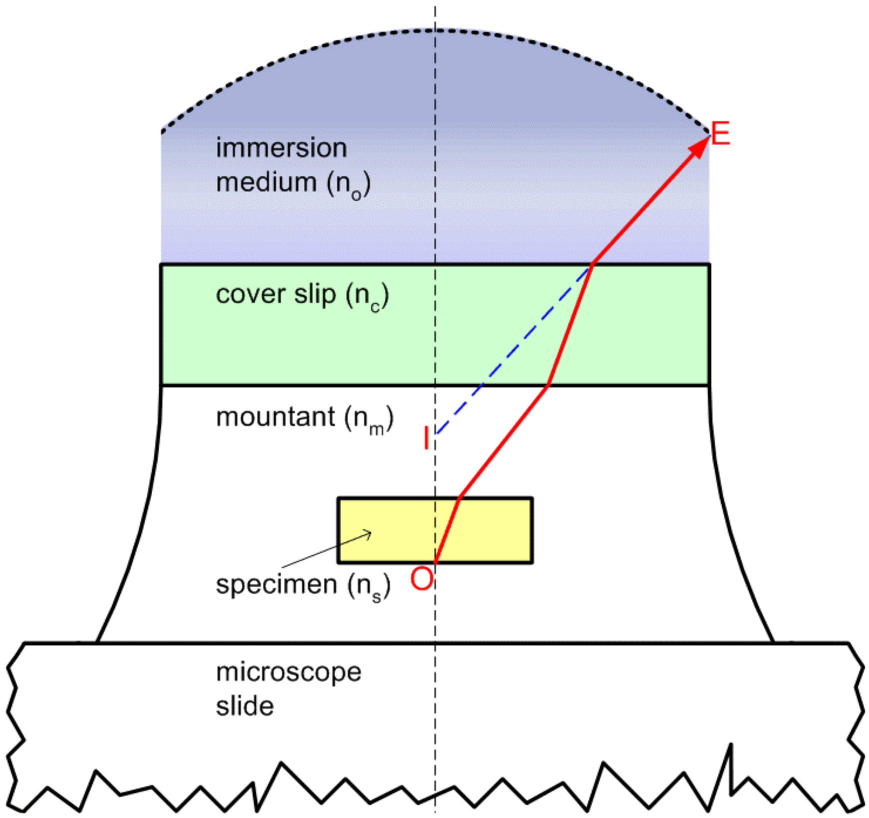

Figure 1: Illustrates the various interfaces along the *z*-direction (direction perpendicular to the cover slip). The optical ray path is indicated with a red polygon. The blue dashed line indicates the location of the apparent beam. The ray of light, traveling from point 'O' to 'E', will undergo refraction at various interfaces and will appear to come from point 'I'.

Let the *z*-direction be the direction along which we want to measure the height (or thickness) of a sample. If the numerical aperture of the microscope objective is small enough, the height Δz is given by

$$\Delta z = \frac{n_s}{n_o} \times \left(h_2 - h_1 \right), \quad (1)$$

with n_s = refractive index of the specimen, n_o = refractive index of the medium between the objective's front lens and the cover glass (which is very close to 1.0 for air or 1.515 for immersion oil), h_2 = the larger value read from the fine focus knob, and h_1 = smaller value read from the fine focus knob. Unfortunately, in some literature, n_s is defined as the refractive index of the mountant, which is not necessarily identical to the refractive index of the specimen. Remember, equation (1) is independent of the refractive index of the cover slip and the refractive index of the mountant. But equation (1) depends on the refractive index of the specimen and the refractive index of the medium between cover slip and objective lens (immersion medium).

To clarify equation (1), let us perform a simple experiment used to measure the thickness of a cover glass [**1**]. Following Paul James' recommendation, a cover glass with some dust and/or markings on both sides is placed in top of a clean microscope slide. A "low power" objective ($NA \sim 0.25$ or less) is then focused on the top and then on the bottom surface of the cover glass to produce the two readings h_1 and h_2 for bottom and top surface, respectively. For this little experiment, n_s is around 1.52 (glass) and n_o is close to 1.0 (air). From equation (1), we can easily determine the thickness of the cover glass, as

$$\Delta z = 1.52 \times \left(h_2 - h_1\right). \tag{2}$$

This is the simple equation used in [1] that relates the real thickness (Δz) to the apparent thickness ($h_2 - h_1$). But we have to be careful when trying to use this equation with a "high power" objective ($NA \sim 0.65$ or more), which is explained in the Part B of this paper.

Fine focusing mechanisms for various brands of microscopes are different. On most scopes, the minimum fine focus adjustment gradation corresponds to a change in height of 1 µm and the total motion of the stage is just 0.1 mm per full rotation. Other scopes are less precise and move the stage by 0.2 mm or even 0.3 mm per full rotation.

The following table lists a few selected compound microscopes without motorized focus movement.

Compound microscope	**Minimum fine focus adjustment gradation**	**Stage motion per full rotation**
Leica DM E	3 µm	0.3 mm
Leitz Ortholux	1 µm	0.1 mm
Nikon Eclipse E200	2 µm	0.2 mm
Nikon Labophot-2, Optiphot-2, Microphot-SA and FXA, E400, E600, E800, 50i, 55i, 80i	1 µm	0.1 mm
Olympus CX21	2.5 µm	?
Olympus BX2 series, BX40, BX50, BX60, AX70	1 µm	0.1 mm

Some of the above microscopes, such as the Leitz Ortholux, have a fine focusing mechanism that is more precise than its minimum fine focus adjustment gradation.

The precision of the focusing mechanism should be calibrated. One simple way to calibrate a microscope stage is to use a cover glass of known thickness (see experiment explained above).

Up to this point, everything was rather straightforward. Unfortunately, a fine adjustment mechanism of a compound microscope is frequently not linear with rotation over the range of travel [**2**]. To measure this non-linearity, we would have to repeat the above measurement for different positions of the <u>coarse</u> focus and plot the measured thickness as a function of the turns of the <u>fine</u> focus from the lowest zero. In this case, focusing should always be attempted from the same direction to avoid backlash errors. We also must be aware that we tend to make a reading once the object is in precise focus. Therefore, the depth of field (in the object space) determines the error in one reading. For a thickness reading, we perform two independent measurements. Since the variance of the difference of two independent variables is equal to the sum of their variances, and since the standard error of the difference is the square root of the variance of the difference, we obtain an error *E* for our determination of the apparent height that is given by $E = \pm dl\sqrt{2}$, where *dl* is the depth of field. (The depth of field of a microscope lens is given by $dl = n\lambda / NA^2$, with λ = wavelength of light in air and *n* = refractive index of the medium between the lens and the object.)

Part B: Height measurements with "high power" objectives (high numerical aperture)

What is the equation when the numerical aperture of the microscope objective is no longer small but comparable to the refractive indices? (For instance *NA* = 0.75 or higher.) For this case, we must look at the *weighted mean apparent depth* for all the rays that can be captured by the objective, which depends on the numerical aperture (*NA*) of the objective. In 1970, G. W. White published his equation that takes a weighted mean apparent depth into account [**3**]. It states for parallel-sided specimens

$$\Delta z = \frac{n_o - \sqrt{n_o^2 - NA^2}}{n_s - \sqrt{n_s^2 - NA^2}} \times \left(h_2 - h_1 \right). \tag{3}$$

(We omit the mathematical proof of this equation. The interested reader can read more about this in [**3**].) Using immersion oil between objective and cover glass and a medium that has exactly the same refractive index ($n_s = n_o$), we obtain the simple relation that the apparent height is equal to the real height ($\Delta z = \Delta h \equiv h_2 - h_1$). On the other hand, with a small numerical aperture and air between objective lens and cover glass ($NA \ll 1$ and $n_o = 1$), we get our known equation (1), $\Delta z = n_s \times \Delta h$, which we used in our experiment to measure the thickness of a cover glass (see above). We will proof this limiting case in Part C of this paper.

Next, we shall look at some quantitative results that equation (3) offers.

The following two figures show the difference between equation (1) and equation (3) for various refractive indices. In both figures, the value obtained with equation (1) is 100%.

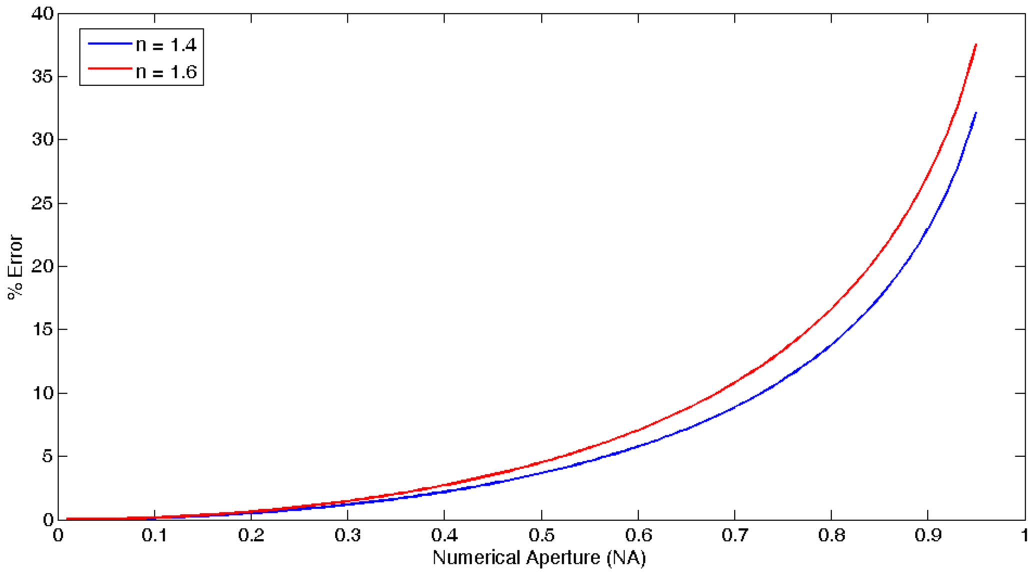

Figure 2: Percent error between equation (1) and equation (2) plotted as a function of the numerical aperture (NA) of a microscope lens (equation (1) is 100%). The refractive index of the immersion medium is 1.0 (air). The refractive index of the specimen is 1.4 and 1.6.

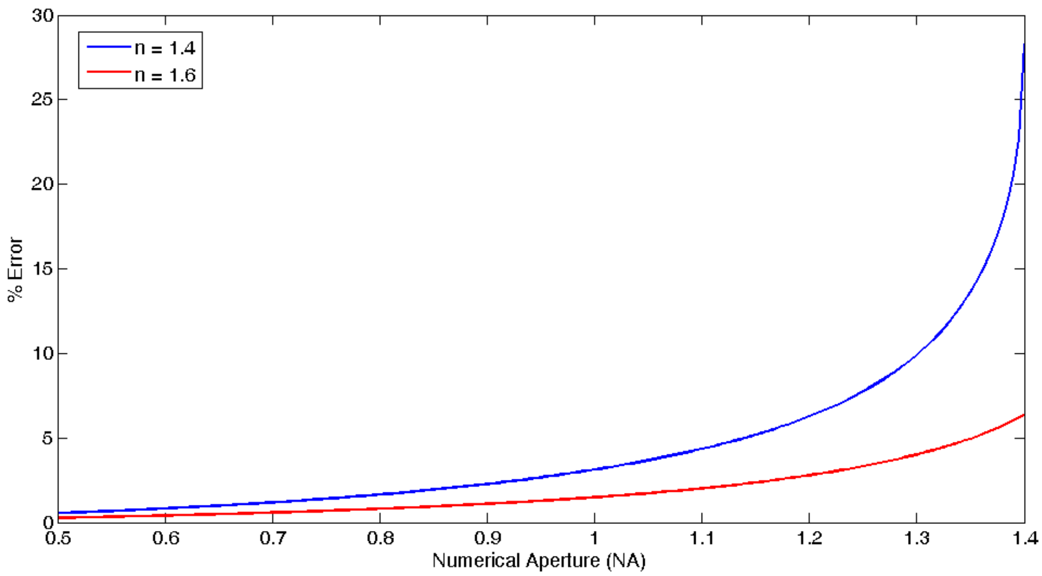

Figure 3: Percent error between equation (1) and equation (2) plotted as a function of the numerical aperture (NA) of a microscope lens (equation (1) is 100%). The refractive index of the immersion medium is 1.524 (oil). The refractive index of the specimen is 1.4 and 1.6.

It is easily ascertained that equation (3) is not valid for $NA > n_s$ or $NA > n_o$. Due to refraction, light rays, which are barely caught by the objective's frontlens, do no longer originate inside the zone with a refractive index smaller than the numerical aperture of the lens. We know this phenomenon as total internal reflection.

Part C: The limiting case of $NA \ll 1$ and $n_o = 1$

The remaining part of this paper focuses on one limiting case of equation (3). We study the case of $NA \ll 1$ and $n_o = 1$. A closer look at equation (3) immediately suggests expanding the terms under the root signs using the binomial theorem, which states

$$(1+q)^{m/n} = 1 + \sum_{i=1}^{\infty} \frac{\prod_{j=0}^{i-1}\left(\frac{m}{n} - j\right)}{\prod_{k=1}^{i} k} q^i \ . \qquad (4)$$

Equation (4) looks more complicated than it is. For small values of q only the first few terms of the sum must be considered. To apply this equation to our case, we must rewrite equation (3) such as

$$\Delta z = \frac{1-\sqrt{1-NA^2}}{n_s - n_s\sqrt{1-\frac{NA^2}{n_s^2}}} \times \left(h_2 - h_1\right). \qquad (5)$$

Now we expand the terms under the root sign $\sqrt{1-NA^2}$ using equation (4) so that

$$\sqrt{1-NA^2} \equiv (1-NA^2)^{1/2} = 1 - \frac{1}{2}NA^2 - \frac{1}{8}NA^4 - \cdots . \qquad (6)$$

Since we assume $NA \ll 1$ (only axial rays considered), equation (6) simplifies to

$$\sqrt{1-NA^2} = 1 - \frac{1}{2}NA^2 . \qquad (9)$$

After repeating this for the second root sign in equation (3), we obtain

$$\Delta z = \frac{1}{n_s} \frac{1-1+\frac{1}{2}NA^2}{1-1+\frac{1}{2}\frac{NA^2}{n_s^2}} \times \left(h_2 - h_1\right) = \frac{1}{n_s} n_s^2 \times \left(h_2 - h_1\right) = n_s \times \left(h_2 - h_1\right), \qquad (10)$$

which is identical to $\Delta z = n_s \times \Delta h$. – Additionally, using equation (4), we can show that equation (3) becomes equation (1) for $NA \ll 1$.

References

[**1**] Paul James, *A case for thin coverslips*, *Micscape Magazine*, **114**, April 2005. (The URL is http://www.microscopy-uk.org.uk/mag/artapr05/pjcoverslip.html).
[**2**] Maksymilian Pluta, Chapter 13 in Measuring Techniques, Advanced Light Microscopy, Volume 3, Elsevier, Amsterdam, 1993.
[**3**] G. W. White, Improving the accuracy of vertical measurements under the microscope, *The Microscope*, **18** (1970), 51-59.

