**What’s the numerical aperture (NA)?**

The NA is the product of the refractive index (n) of the immersion medium and the sine of the half opening angle of the light path:

\[ \text{NA} = \sin \alpha \times n \]

As a rule of thumb, the larger the NA, the more light is collected and the better is the resolution. Of course, there are also other things that determine the quality of the objective. Furthermore, the higher the NA, the smaller is the working distance at a given magnification. Therefore, you simply cannot use a 100x NA=1.4 objective to look at a preparation that is a couple of hundred microns thick.

It also should be noted that “large NA” is a relative term and means different values for different immersion media. The refractive indices for immersion media are:

- Air: \( n = 1 \)
- Water: \( n = 1.333 \)
- Oil: \( n = 1.518 \) (matches the n of the cover slip glass)

Therefore, an NA of 0.6 is about as large as it gets for air objectives, while 1.0 for oil objectives is still pretty low (a 100x oil objective with an NA of 1.4 is about as good as it gets).
Determining axial scaling and shift with geometric optics

**note:** this is not completely accurate because only the peripheral rays are taken into account. Therefore, these calculations should only be used for objectives with a lower NA (up to 0.4 air or 0.85 oil)!

In an optical situation with different optical media and mismatching refractive indices, the Actual Focal Position (AFP) is different from the Nominal Focal Position (NFP), the latter being the focal position in an optical situation without refractive index mismatch. Due to this phenomenon, moving the microscopic stage over a defined distance in the z-axis causes a movement of the focus over a distance different from this. Thus, images are scaled in the axial dimension.

For objectives with a relatively low Numerical Aperture (NA), it is possible to calculate the correction factor that is needed to rescale the image by just using geometric optics, trigonometry and linear equations. Higher NA objectives require the use of wave optics simulation because with greater opening angles limiting the calculation to peripheral rays results in significant errors.

The aim is to calculate the Actual Focal Position for two different given Nominal Focal Positions. Since AFP and NFP are linearly related, a simple linear equation can be used to derive scale and shift. The figure shows which dimensions are known (green), and which have to be calculated (red).

**notes:**

- **NFP:** nominal focal position (as defined by the opening angle of the objective: \( \alpha \))
- **VFP:** virtual focal position (as defined by the angle changed at the first refractive plane: \( \beta \))
- **AFP:** actual focal position (as defined by the angle changed at two refractive planes: \( \gamma \))
- **NA:** numerical aperture
- **n1:** refractive index of the immersion medium. 1.518 (oil), 1.0 (air), 1.33 (water).
- **n2:** refractive index of the cover slip glass. This should be 1.518, removing one refractive plane when using oil objectives.
- **n3:** refractive index of the mounting medium. Examples: 1.5409 (methyl salicylate), 1.443 (Vectashield).
- **c:** thickness of the cover slip. Should be measured! Objective correction for spherical aberrations is valid for 170mm. Both thicker and thinner cover slips will decrease the signal amplitude significantly. Unfortunately, most commercially available cover slips are only 120-150mm thick.
- **a:** leg opposite to \( \alpha \) and \( \beta \).
- **b:** leg opposite to \( \beta \) and \( \gamma \).
All calculations are based on the following equations:

1) \[
\frac{n_1}{n_3} \frac{\sin \beta}{\sin \alpha} \quad \text{and} \quad \frac{n_2}{n_3} = \frac{\sin \gamma}{\sin \beta} \quad \text{(Snellius' law)}.
\]

2) \[NA = \sin \alpha \times n_1 \quad \text{(numerical aperture of the objective)}\]

3) \[\tan \alpha = \frac{a}{\left( NFP + c \right)}\]

4) \[\tan \beta = \frac{a}{\left( VFP + c \right)} = \frac{b}{VFP}\]

5) \[\tan \gamma = \frac{b}{AFP}\]

The aim is to calculate the AFP at a given NFP:

<table>
<thead>
<tr>
<th>known</th>
<th>unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>(\alpha)</td>
</tr>
<tr>
<td>(n_1)</td>
<td>(\beta)</td>
</tr>
<tr>
<td>(n_2)</td>
<td>(\gamma)</td>
</tr>
<tr>
<td>(n_3)</td>
<td>(a)</td>
</tr>
<tr>
<td>(c)</td>
<td>(VFP)</td>
</tr>
<tr>
<td>(\text{NFP})</td>
<td>(b)</td>
</tr>
<tr>
<td>(\text{AFP})</td>
<td></td>
</tr>
</tbody>
</table>
How to rescale the z-axis for accurate three-dimensional measurements (from wave optics simulations)

These values have been obtained using wave optics calculations (rather tedious). For low NA objectives (<0.5 air, <1 oil), you can calculate the scaling factor using geometric optics (see above) if your mounting medium is neither vectashield (/glycerol) nor methyl salicylate. Glycerol (n=1.455) is slightly different from vectashield.

Correction factors:

<table>
<thead>
<tr>
<th></th>
<th>0.4 air</th>
<th>0.6 air*</th>
<th>0.85 oil</th>
<th>1.0 oil</th>
<th>1.4 oil*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vectashield</td>
<td>1.4813</td>
<td>1.4800</td>
<td>0.9400</td>
<td>0.9350</td>
<td>0.9413</td>
</tr>
<tr>
<td>(n=1.443)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl salicylate</td>
<td>1.5813</td>
<td>1.5963</td>
<td>1.0175</td>
<td>1.0200</td>
<td>1.0213</td>
</tr>
<tr>
<td>(n=1.5409)</td>
<td></td>
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</tbody>
</table>

*for high NA objectives, these values may be a little smaller for 633nm light.