

MorphCol supplement#18 - Vertical correction for the Auto-focus in AMOR 3.5

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Introduction

In the Automeasurement Mode of AMOR 3.5 the autofocus function does not operate ideally during Autotilt, when the magnification 3.2x or higher are reached. The cause of the difficulty is, that the geometry of the stage is not truly eucentric, i.e. the optical axis of the microscope is located slightly excentric with respect to the Pitch- and Roll-axis: If the stage is in horizontal position, the optical axis intersects the plane of the sample slide about 5 mm to the right side of the roll-axis and about 4 mm in front of the pitch-axis (see Figure 1). In addition, the Pitch-and Roll axes do not perfectly intersect each other but also may have a slight distance in between (see Figure 2).

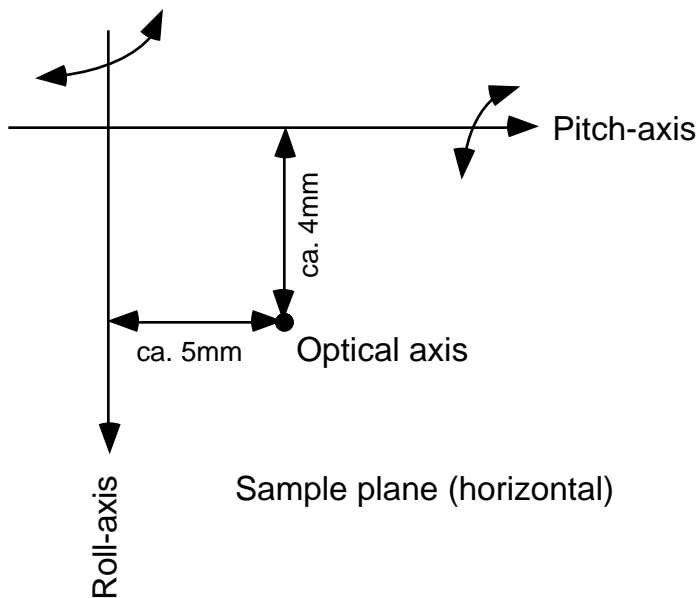


Figure 1. View on the stage from above (optical-axis is vertical). The Roll-axis is directed towards the viewer.

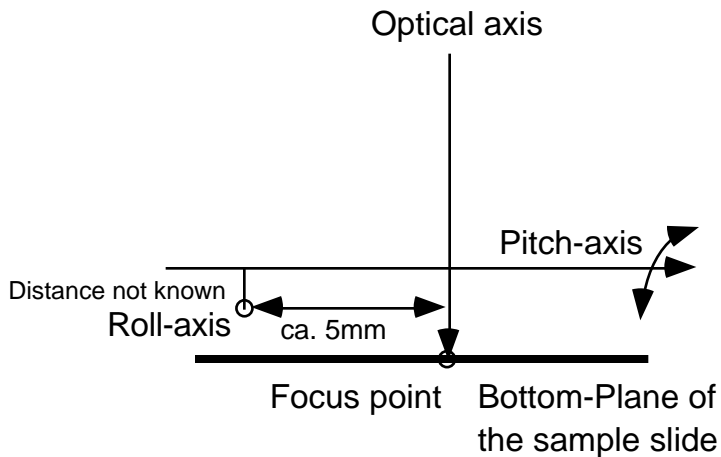


Figure 2. Orientation of the stage axes seen in front view. The roll-axis is now directed towards the reader. The pitch axis and the roll-axis have eventually no intersection and the distance in between them is not known (though small). The focus point is the intersection between the optical axis and the bottom plane of the sample slide.

The bottom of the sample-slide is located slightly underneath the pitch- or roll-axis. Consequently, at high magnification, a specimen will move out of focus (under- or overfocus depending on the direction and extent of tilting) when the stage is tilted about the Pitch- or Roll axis. The defocussed situation becomes evident at high magnification, where the depth of focus of the lense is only a very narrow band (see Figure 3). At lower magnifications (until about 2x to 2.5x) the vertical displacement remains relatively small in comparison to the depth of focus if the inclinations of the stage is not too strong.

The defocussing-effect is thus most disturbing in the case of very small specimens where strong magnifications are required. The specimen becomes underfocused (located below the focus point of the lense) if tilt about the pitch-axis is directed towards the observer (=negative Pitch inclination). The specimen is underfocused too, if tilt about the roll-axis is inclined to the right side (=negative Roll inclination). In such situations the vertical range of the autofocus is often not sufficient to reach in-focus condition, and the microscope motor needs an extra amount (DZ) of vertical correction (in the above case to a lower level).

Such a vertical correction for Autotilt is not implemented in AMOR because all test-objects during stage programming were comparably large. Thus, the stage was only tested at comparably low magnifications, where the focussing difficulty is not apparent because of large depth of focus.

One solution to overcome this difficulty would be to implement a mathematical model describing the three-dimensional geometry of the three axes (Pitch, Roll, optical axis) in space. In practice however, it became clear, that the measurement of the three axes cannot be realized with sufficient precision (sub-millimeter precision required).

Therefore, the necessary vertical correction to reach focus conditions were determined empirically as a function of the inclinations about the Pitch- and Roll-axes at the highest possible magnification of 4x (AMOR reading at 3.93x).

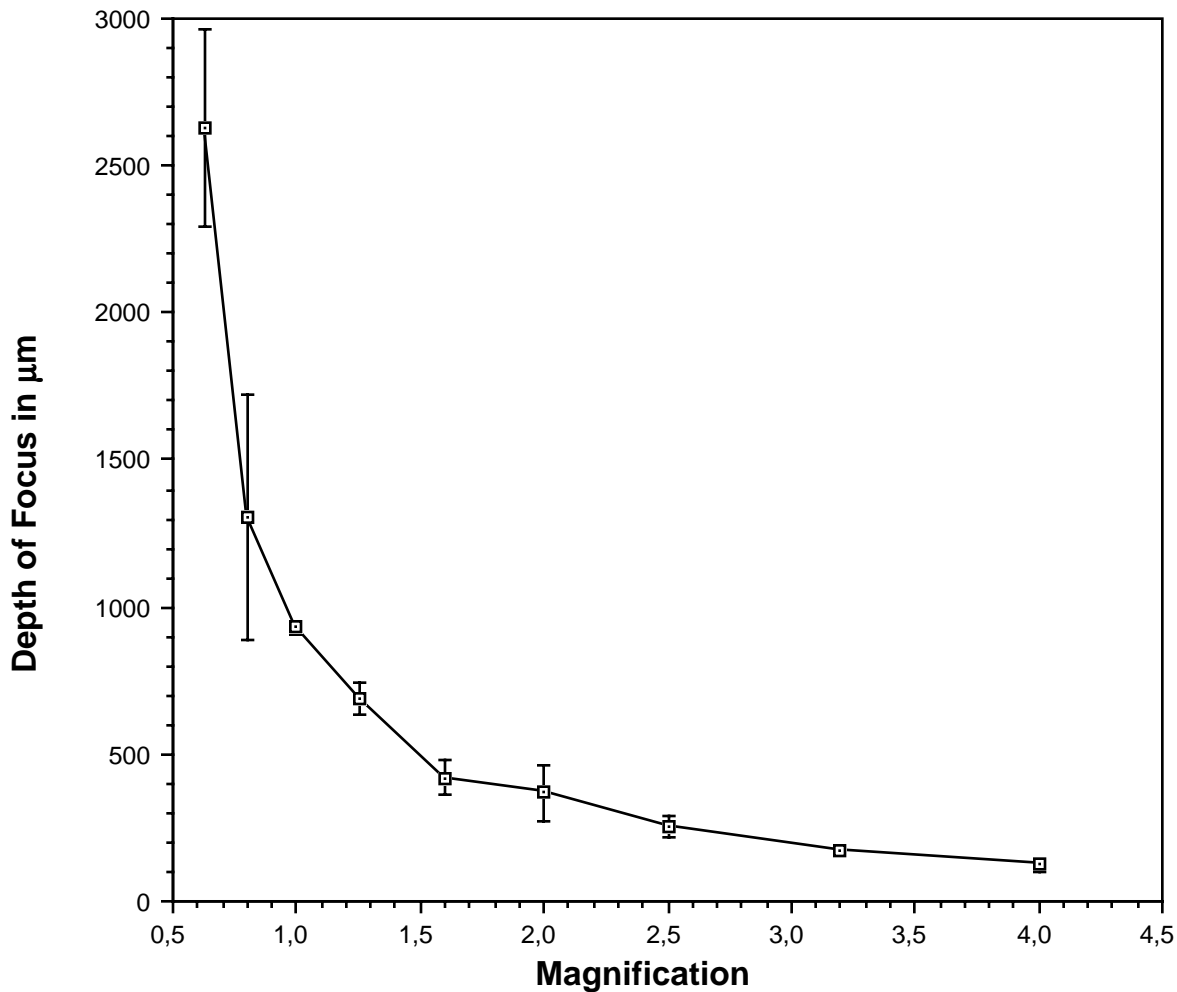


Figure 3. Empirically estimate of the depth of focus (in μm) of the objective Achromat 1x for the Leica MZ6 binocular microscope as a function of magnification. Vertical bars indicate 95% confidence intervals about the means. For the data see Table 1.

Magnification	DOF, μm	$\pm 95\%$ CI
0,63	2625	335,6
0,80	1308	416,2
1,00	931	24,8
1,25	690	53,2
1,60	419	59,4
2,00	368	95,2
2,50	258	36,1
3,20	170	8,1
4,00	123	25,5

Table 1. Data for Figure 3. The depth of focus (DOF) is in micrometers. Column 3 indicates $\pm 95\%$ confidence interval about the mean. (Data from Knappertsbusch, 2003).

Empirical determination of the vertical displacement from focus condition in function of the inclination about the Pitch- or Roll-axis

For this experiment sample 18304B, Surf, 0-1cm, 150-250 μ m, 1/8 split, slide a was used which has very small specimens.

Testing corner-fields and center of the slide:

All experiments were made in the single-specimen mode of AMOR 3.5. It was tested whether the slide can geometrically be mapped as a plane, when driving individual fields into focus positions at no tilt. Corner-fields 1, 12, 60, and 49 and the central field No 31 were checked by the following procedure:

```
1   Initialize stage
2   Apply calibrated slide
3   Select single-specimen mode
4   Move to field 31 --> stage is in horizontal position (pitch=0, roll=0)
5   Increase Magnification to 4x and AutoCenter object
6   Focus to bottom of slide --> -14 mouse clicks needed for focus motor (sensitivity=fine)
7   Reset           ! = Motorfocus in start position, stage horizontal

C   First loop begins (stage horizontal)
8   Move to field X (X=1,12,49, and 60, if 60 continue with 13)
9   Increase Magnification to 4x and AutoCenter object
10  Focus to bottom of slide --> -4 mouse clicks needed for
    focus motor (sensitivity=fine) ! = Start positions
11  Reset
12  Goto 8
C
C
13  Reset           ! = Motorfocus in start position, stage horizontal
C
C   Second loop begins (tilt about pitch axis, tilting forward)
14  Tilt about pitch axis (-16 clicks at sensitivity=normal)
15  Move to Field X (X=31,1,12,49, and 60, if 60 continue with 20)
16  Increase Magnification to 4x and AutoCenter object
17  Focus to bottom of slide --> Record number of mouse clicks (sensitivity=fine)
18  Reset
19  Goto 14
C
C
20  Reset           ! = Motorfocus in start position, stage horizontal
C
C
C   Third loop begins (tilt about pitch axis, tilting backward)
21  Tilt about pitch axis (+21 clicks at sensitivity=normal)
22  Move to Field X (X=31,1,12,49, and 60, if 60 continue with 27)
23  Increase Magnification to 4x and AutoCenter object
24  Focus to bottom of slide --> Record number of mouse clicks (sensitivity=fine)
25  Reset
26  Goto 21
C
C
```

```
27  Reset          ! = Motorfocus in start position, stage horizontal
C
C
C  Fourth loop begins (tilt about roll axis, tilting right)
28  Tilt about pitch axis (-21 clicks at sensibility=normal)
29  Move to Field X (X=31,1,12,49, and 60, if 60 continue with 34)
30  Increase Magnification to 4x and AutoCenter object
31  Focus to bottom of slide --> Record number of mouse clicks (sensibility=fine)
32  Reset
33  Goto 28
C
C
34  Reset          ! = Motorfocus in start position, stage horizontal
C
C
35  Sixth loop begins (tilt about roll axis, tilting left)
36  Tilt about pitch axis (+21 clicks at sensibility=normal)
37  Move to Field X (X=31,1,12,49, and 60, if 60 continue with 42)
38  Increase Magnification to 4x and AutoCenter object
39  Focus to bottom of slide --> Record number of mouse clicks (sensibility=fine)
40  Reset
41  Goto 36
C
C
42  Reset          ! = Motorfocus in start position, stage horizontal
C
43  End program
```

The interspersed Reset operations are necessary in order to place the microscope to identical start positions before each field is checked and between loops. The first start position of the motor-focus after initialization of AMOR attains a value of -14 clicks. After a first Reset is done, the motor focus always starts at -3 to -4 mouse clicks, which corresponds to an absolute vertical focus position at about -131mm.

For the focus motor one mouse-click at sensibility=fine results in a vertical displacement of the microscope by 100 μ m. A negative mouse click indicates a lowering, a positive mouse click indicates a movement in top direction.

For the Pitch- and Roll movements one mouse click results in a change of the tilting angle by 1.5°. For pitch, a negative mouse click means a tilting of the stage towards the observer, a positive mouse click is a tilting in backward direction. For roll, a negative mouse click means an inclination of the slide plane to the right side, a positive mouse click is an inclination of the sample plane to the left side.

The result (see Table 2) of this experiment was an average lowering for all fields by about -30 clicks (= -300 μ m) at a Pitch about -16 clicks (= -24°) and an average rise of +26 vertical clicks (= +260 μ m) at a Pitch of +21 clicks (= +31.5°).

About the roll-axis a lowering of the microscope by about 310 μ m (= -31 clicks fine) was reached when the stage was tilted by 31.5° to the right (= -21 clicks normal), and a rise of the microscope by about 170 μ m (= +17 clicks fine) was attained if the stage was tilted by 31.5° to the left (= +21 clicks normal). The conclusion from this test is, that the vertical position of the focus point (DZ) is only a function of the tilting amounts but is *not* dependent on the field number.

Testing central field 31 at various inclinations about Pitch- and Roll-axes.

In order to determine the relationship between vertical correction (DZ) to attain focus condition as a function of the tilt angle, the above experiment was repeated for the central field No. 31 but at varying inclinations about the Pitch- and Roll-axes. The procedure modifies then to

```
1 Initialize stage
2 Apply calibrated slide
3 Select single-specimen mode
4 Move to field 31 --> stage is in horizontal position (pitch=0, roll=0)
5 Increase Magnification to 4x and AutoCenter object
6 Focus to bottom of slide --> -14 mouse clicks needed for focus motor (sensitivity=fine)
7 Reset ! = Motorfocus in start position, stage horizontal
C
C
C First loop begins (tilt forward about pitch axis)
8 Tilt about pitch axis (increments of -2 clicks, sensitivity=normal)
9 If (Tilt=-16 mouse clicks normal) Goto 13
10 Increase Magnification to 4x and AutoCenter object
11 Focus to bottom of slide --> Record number of mouse clicks (sensitivity=fine)
12 Goto 8
C
C
13 Reset ! = Motorfocus in start position, stage horizontal
C
C
C Second loop begins (tilt backward about pitch axis)
14 Tilt about pitch axis (increments of +2 clicks, sensitivity=normal)
15 If (Tilt > +21 mouse clicks normal) Goto 19
16 Increase Magnification to 4x and AutoCenter object
17 Focus to bottom of slide --> Record number of mouse clicks (sensitivity=fine)
18 Goto 14
C
C
19 Reset ! = Motorfocus in start position, stage horizontal
C
C
C Third loop begins (tilt right about roll axis)
20 Tilt about roll axis (increments of -2 clicks, sensitivity=normal)
21 If (Tilt < -21 mouse clicks normal ) Goto 25
22 Increase Magnification to 4x and AutoCenter object
23 Focus to bottom of slide --> Record number of mouse clicks (sensitivity=fine)
24 Goto 20
C
C
25 Reset ! = Motorfocus in start position, stage horizontal
C
C
C Fourth loop begins (tilt left about roll axis)
26 Tilt about roll axis (increments of +2 clicks, sensitivity=normal)
27 If (Tilt=+21 mouse clicks normal) Goto 31
```

```
28 Increase Magnification to 4x and AutoCenter object
29 Focus to bottom of slide --> Record number of mouse clicks (sensibility=fine)
30 Goto 26
C
C
31 Reset          ! = Motorfocus in start position, stage horizontal
C
32 End program
```

The results of this experiment are shown in Table 3a (Pitch) and 3b (Roll) and in Figure 4.

Pitch	DZ(pitch) Run1	DZ(pitch) Run2	DZ(pitch) Run3	DZ(pitch) Run4
-16	-26	-26	-27	-26
-14	-22	-23	-23	-22
-12	-18	-19	-19	-18
-10	-15	-15	-15	-15
-8	-12	-12	-12	-12
-6	-8	-9	-9	-9
-4	-6	-6	-6	-6
-2	-2	-3	-3	-3
0	0	0	0	0
2	3	3	3	3
4	6	6	6	6
6	9	9	9	8
8	12	12	12	11
10	14	15	14	14
12	17	17	17	16
14	20	20	20	19
16	23	23	23	22
18	25	25	26	25
20	28	29	29	28
21	30	30	30	30

Table 3a. Vertical displacements DZ in order to meet the focus point at the bottom of the slide at a magnification 4x and as a function of the tilt angle about the pitch axis. Pitch is given in number of mouse clicks (sensibility =normal). DZ is given in mouse clicks to move the focus motor up or down at sensibility=fine. The test was repeated four times (=runs 1 through 4). Negative Pitch means tilting towards the observer. Negative DZ means downward movement.

Roll	DZ(roll) Run1	DZ(roll) Run2	DZ(roll) Run3	DZ(roll) Run4
-21	-29	-27	-28	-28
-19	-25	-24	-24	-24
-17	-21	-21	-21	-20
-15	-18	-17	-17	-17
-13	-15	-14	-14	-15
-11	-12	-11	-12	-12
-9	-10	-9	-9	-9
-7	-8	-7	-7	-7
-5	-6	-5	-5	-5
-3	-4	-3	-3	-3
-1	-1	-1	-1	-1
0	0	0	0	0
1	1	1	1	1
3	3	3	3	3
5	5	5	5	5
7	7	7	7	7
9	9	9	9	8
11	11	11	10	10
13	12	12	12	12
15	14	14	14	14
17	16	16	16	16
19	18	18	18	18
21	20	20	20	20

Table 3b. Vertical displacements DZ in order to meet the focus point at the bottom of the slide at a magnification of 4x and as a function of the tilt angle about the roll axis. Roll is given in number of mouse clicks (sensibility=normal). DZ is given in mouse clicks to move the focus motor up or down at sensibility=fine. The test was repeated four times (=runs 1 through 4). Negative Roll means stage is inclined to the right side. Negative DZ means downward movement.

DZ follows a curved line (probably a tangent function) for tilting about the Pitch and Roll-axes. The vertical corrections can be parametrized as third order polynomials by the following two equations

$$\begin{aligned}
 \text{DZ (pitch)} &= 0.0011453 + 1.3956x - 0.0073118x^2 + 0.00039349x^3 \\
 \text{DZ (roll)} &= 0.17778 + 0.94687x - 0.0087772x^2 + 0.00043296x^3
 \end{aligned}$$

The vertical movement DZ is given in mouse clicks (sensibility=fine), the tilt angle x is indicated in clicks (sensibility=normal) for pitch or roll movements. For DZ 1 motorstep is equal to 1µm, 1 mouseclick (sensibility=fine) equals 100 motorsteps or 100 µm.

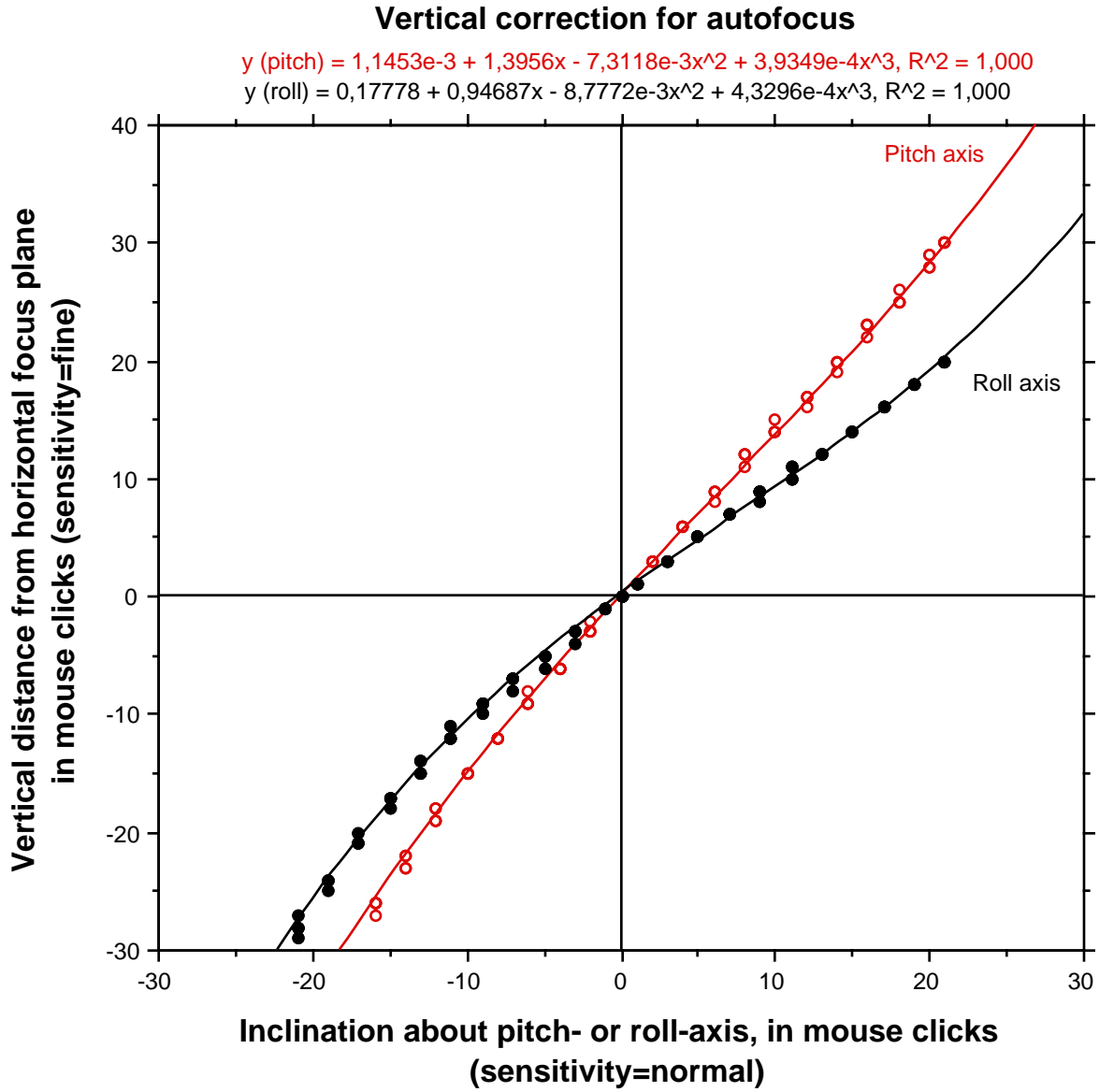


Figure 4. DZ as function of inclination about the Pitch- and Roll-axis. The lines are third order polynomial approximations.

Conclusions

Calculation of the total vertical correction for the focus point as a function of roll- and tilt

1.) With the above tests the total amount for focus correction DZ can be calculated as the sum of all individual vertical displacements (in vertical mouseclicks for the focus motor at sensibility=file):

$$DZ = K_0 + DZ(\text{pitch}) + DZ(\text{roll})$$

where K_0 is

$K_0 = -14$ if no Reset was done after stage initialization (starting at $Z = -112'000$ steps)

$K_0 = -4$ if a Reset has been done (starting at $Z = -113'000$ steps)

$$DZ(\text{pitch}) = 0.0011453 + 1.3956x - 0.0073118x^2 + 0.00039349x^3$$

$$DZ(\text{roll}) = 0.17778 + 0.94687x - 0.0087772x^2 + 0.00043296x^3$$

2.) The value of K_0 (in mouseclicks at sensibility=fine) indicates the vertical distance from the vertical default position (Z , in motorsteps) of the microscope to the position of the microscope, where the bottom of the slide appears in focus if the stage is horizontal (Pitch and Roll are then both 0).

3.) K_0 is independent of the magnification used. However, focus condition is best recognized at maximum magnification (i.e. at 4x).

4.) K_0 can vary from slide to slide due to variation in thickness of the bottom-plate of the slide caused during manufacturing the slides. Through determination of K_0 prior to routine measurements with AMOR, variation in slide quality can be compensated for.

5.) In Z -direction, 1 motorstep is equal to $1\mu\text{m}$, 1 mouseclick at sensibility=fine equals 100 motorsteps or $100\mu\text{m}$. Negative values of DZ correspond to a downward movement of the microscope.

References

Knappertsbusch, M. (2003). Estimation of the Depth of Focus for the 1x Achromat objective of the Leica MZ 6 binocular at various magnifications. MorphCol Supplement#17. Unpublished report, 9 April 2003.