SHORT TECHNICAL NOTE

Calibration of the automated z-axis of a microscope using focus functions

F. R. BODDEKE, L. J. VAN VLIET & I. T. YOUNG
Pattern Recognition Group of the Faculty of Applied Physics, Delft University of Technology,
Lorentweg 1, 2628 CJ Delft, The Netherlands

Summary

Applications in automated microscopy and three-dimensional microscopy require careful calibration of the microscope system. This paper presents methods for calibration of the motorized z-axis (focus or optical axis) of an automated microscope. Apart from the automated microscope the procedures require a CCD camera and a test slide containing a simple bar pattern. The calibration embraces the following characteristics of the z-axis: (a) measuring the motor step size in nanometres; (b) measuring the mechanical backlash in the focus mechanism of the microscope and (c) measuring the reproducibility and the stability of the focus position over time. The measurements employ focus functions to determine the z-position of the microscope stage.

Introduction

An automated microscope system is equipped with an image sensor, a computer-controlled stage, focus drive, filter wheels and shutters. Quantitative analysis requires calibration of the image sensor and the automated axes: xy stage and focus (z-axis). The image sensor converts a light intensity pattern into a digital image. Important characteristics are sampling density, linearity of photometric response, signal-to-noise ratio, sensitivity and spatial resolution (Mullikin et al., 1994). The automated axes are driven by stepping motors or DC motors with position encoders. Both systems use ‘steps’ to encode position. Calibration of these axes yields the physical distance of each step, e.g. 25 nm per step. Calibration of the xy-axes (object plane perpendicular to the optical axis) can be done using a camera for which the sampling density is known (Mullikin et al., 1994). The difference in xy-position of an object before and after stage movement yields a physical distance that corresponds to the number of steps.

Calibration of the z-axis cannot be done using a similar scheme. Three-dimensional imaging of a fluorescently labelled microsphere is not the simple solution it seems to be. The measured height of the sphere in z-steps cannot be related to the calibrated diameter in the xy image plane owing to a mismatch of refractive index and the unknown depth of the bead in the surrounding medium (Visser et al., 1992; Hell et al., 1993; Jacobsen & Hell, 1995). To overcome this problem we offer an alternative solution based on focus functions. When a 2D flat object is moved along the z-direction it moves in and out of focus. The focus position is measured using a technique described by Boddeke et al. (1994). Here the focus criterion is the energy after filtering with the \{1, -1\}-filter, i.e. the average (over an image or line) of the squared difference of the values of adjacent pixels in one direction.

In the following three sections the measurement of the z-axis motor step size, the measurement of the backlash in the focus mechanism (z-axis) and the measurement of the stability of the z-axis will be discussed. All images were acquired using a Nikon Diaphot inverted microscope in brightfield mode using a Nikon 20/0.75 Plan Apo objective and a Photometrics CC200 CCD camera.

Measurement of the z-axis motor step size

The test object is a microscope slide with a flat bar pattern (or knife edge) along the length of the slide. The slide is tilted with respect to the object plane (cf. Fig. 1). Only one line perpendicular to the ramp (i.e. a line of equal height) will be in focus. Moving the tilted slide along the z-axis will cause a change in the lateral position of this in-focus line. This change is proportional to the tangent of the tilt angle.
The z-axis motor step size is derived by determining the change in the in-focus z-position in the object plane as a result of a step in the z-direction. Using the known tilt angle the horizontal displacement can be converted into a vertical distance. The set-up used for this measurement is depicted in Fig. 1 and the measured parameters are listed in Table 1.

The geometry (cf. Fig. 1) of this configuration leads to

$$\tan(\alpha) = \frac{\Delta h}{\Delta x} = \frac{\rho_{cylinder}(1 + \cos(\alpha)) - D_{slide} \sin(\alpha)}{L_{slideholder} - \rho_{cylinder}(1 + \sin(\alpha))}$$

(1)

with $\Delta h$ the height of the tilted slide at the contact point with the cylinder, and $\Delta x$ the distance between the support points of the slide in the object plane.

A focus criterion is used to determine the in-focus position. Therefore a 1D focus criterion is applied to each line in the image, resulting in a focus function along the tilted slide. This 1D focus criterion is the energy after filtering this line with a simple difference filter $\{-1, 1\}$. The bar pattern on the slide must be aligned so that the bars are perpendicular to the in-focus line. Figure 2 depicts such an image of a part of the slide (about 0.7 mm in length) with the focus function as an overlay. The in-focus line is the line in the image with the highest focus value. The z-axis motor step size ($s$) can now be determined by the angle of tilt ($\alpha$), the measured difference in x-position of the in-focus line ($\Delta x_{\text{pixel}}$ measured in pixels) that correspond to the number of z-axis motor steps moved ($\Delta z_{\text{step}}$) and the sampling density ($d$) of the camera.

$$s = \frac{\Delta x_{\text{pixel}} \tan(\alpha)}{\Delta z_{\text{step}}}$$

(2)

Table 1. Parameters and their values as used to calculate the z-axis motor step size.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radius of the cylinder (mm)</td>
<td>$\rho_{cylinder}$</td>
</tr>
<tr>
<td>Thickness of the slide (mm)</td>
<td>$D_{slide}$</td>
</tr>
<tr>
<td>Length of the slide holder (mm)</td>
<td>$L_{slideholder}$</td>
</tr>
<tr>
<td>Tilt angle (rad)</td>
<td>$\alpha$</td>
</tr>
<tr>
<td>Sampling density in x-direction (pixels $\mu$m$^{-1}$)</td>
<td>$d$</td>
</tr>
</tbody>
</table>

To achieve more accuracy in the estimation of the z-axis motor step size we acquire a series of images as a function of z-axis motor step size. For all these images the focus values along the image are calculated (as in Fig. 2) and stacked. This stack of focus functions yields a new image (top of Fig. 3). We determine the in-focus z-position for each x-position (the maximal value along all vertical lines). These in-focus z-positions are plotted against the x-position as depicted in the bottom part of Fig. 3. A linear least-squares fit through these data points yields the estimate for the slope of the line $dx_{\text{pixel}}/dz_{\text{step}} = 0.894 \pm 0.005$ pixels/step.

Before applying the above method to measure the z-axis step size, it is confirmed with that same method applied to a nontilted slide that the imaged part of the slide is perpendicular to the z-axis. The linear fit through these data points showed in good approximation a horizontal line (a slope smaller than the measurement error). All points of the imaged part of the slide are in focus at the same time.

The z-axis motor s step size is given by

$$s = \frac{dx_{\text{pixel}} \tan(\alpha)}{dz_{\text{step}} \cdot d} = \frac{c \tan(\alpha)}{d}$$

(3)

and the relative error ($\delta s/s$) in the measured step size is

$$\left(\frac{\delta s}{s}\right)^2 = \left(\frac{\delta c}{c}\right)^2 + \left(\frac{\delta \alpha}{\alpha}\right)^2 + \left(\frac{\delta d}{d}\right)^2 + \left(\frac{\delta \alpha}{\cos^2(\alpha) \sin^2(\alpha)}\right)^2.$$  

(4)
For our z-axis the measured motor step size is (calculated with the values in Table 1) \( s = 24.88 \pm 0.20 \) nm.

**Measurement of the backlash in the z-direction**

Mechanical backlash is present in all microscope stages. It is important to avoid backlash problems when reproducible positions are desired. To do so, a motorized stage must always approach a position from the same side, preferably from below. Coming from above the stage must first pass the desired position by at least the size of the backlash before actually going back up to the desired position. The backlash in the z-axis is measured by comparing two focus functions. The first focus function is calculated from successive images while moving the stage up. The second focus function is acquired while moving the stage in the opposite direction. The two focus functions are shown in Fig. 4. The shift in z-position between the two focus functions is the mechanical backlash we were looking for. The mechanical backlash in the focus mechanism of our microscope is \( 0.60 \pm 0.08 \) μm.

**Fig. 2.** Image of the tilted slide. The focus values of single lines as a function of the x-position in the image are superimposed on the image.

**Fig. 3.** (top) Image constructed of vertically stacked focus functions (black indicates low value). (bottom) The in-focus x-position as a function of the z-position retrieved from the top image. A straight line was fit through the data points using a least-squares fit.
Measurement of stability of the z-position

Small temperature variations due to thermostat-controlled heaters and/or air conditioning may cause the object under study to drift away from focus over time (see also Mason & Green, 1975). Air disturbances (e.g. from air conditioning) and mechanical vibrations may cause slight vibrations of the microscope slide. Monitoring the focus criterion as a function of time allows us to study the stability of the z-axis (focus position) of the microscope.

A simple test object with constant illumination (light source with stabilized power supply) should produce the same focus value. We measure the focus value of a stable scene as a function of time. Using the corresponding focus function, all these values can be mapped onto positions along the z-axis. In this way the drift in the z-direction of an object with a known focus function can be followed. Figure 5 shows the focus function of an object and the focus values of this object as they change in time without moving the z-axis. These measurements show that the focus position of our microscope could drift about 0.2 μm in less than 10 min. This causes unnecessary blurring, especially when using high-NA objectives (Young et al., 1993). This same procedure can also be used to examine the reproducibility of the z-axis.

Conclusions

We have presented three methods based on focus functions to calibrate the z-axis of an automated microscope. The change of focus position between images of a tilted slide as a function of z-axis motor step size permits us to measure this step size very accurately. The backlash in the focus mechanism, i.e. the difference in physical position between approaching a motor position from below or from above, is estimated by the shift between two focus functions. The first focus function is calculated from successive images while moving the stage up. The second focus function is acquired while going in the opposite direction. This backlash cannot be neglected. The stability measurements show that although the z-axis is not moving, the slide can move up or down in a range of 0.2 μm in less than 10 min, owing to temperature variations in the laboratory (air conditioner).

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References


