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Event: SPIE/COS Photonics Asia, 2018, Beijing, China

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ABSTRACT

Two kinds of 3D label free Bio-Transfer-Standards (BTS) have been further developed at the University of Helsinki (UH). The first one, NanoRuler, is a staircase BTS featuring eight fatty acid bilayers which allows vertical calibration in the range of 5 to 40 nm. The second one, NanoStar, is a V-shaped BTS featuring two 5 nm tall bilayers that overlap at 10° angle. This standard enables the determination of the Instrument Transfer Function (ITF). A stability test was conducted on the BTSs, during which the standards were stored in laboratory conditions, and were profiled each week. Profiling was done using a custom-built Scanning White Light Interferometer (SWLI). The stability of NanoStar was ± 0.3 nm, and of NanoRuler ± 0.5 nm to ± 2.5 nm. The BTSs maintained their specified properties for at least six months and therefore allow vertical calibration and ITF determination. In addition, changes in surface morphology of one NanoRuler subjected to water immersion are presented. This paper reports intermediate findings during an ongoing stability test that will run for 24 months.

Keywords: Nanometer scale metrology, Bio transfer standard, Optical profiler, Scanning White Light Interferometry, Instrument Transfer Function

1. INTRODUCTION

The global impact of bio-imaging is growing quickly in the academic and commercial setting. 3D quantitative imaging, label-free and fresh sample imaging, as well as super-resolution and large throughput imaging are main trends. For quantitative bio-imaging, there is a demand for a soft transfer standard. Traceability is required to compare measurements done at different times, with different instruments, in different laboratories. Modern instruments, with high repeatability and resolution, can give a delusion of high accuracy. Therefore, to achieve trustworthy and accurate measurements, persistent calibration and high resolution are required.

There are two distinct approaches in profilometry: contacting and non-contacting. Most non-contacting techniques give, or have the potential to produce 3D information. However, these instruments are calibrated using methods from an ISO standard specified for contacting instruments. Hence the 3D area information is neglected, e.g. when measuring a step height specimen, where results are analysed only in one dimension. New methods are suggested where the 3D areal information is taken into account, which permits classification of 3D measurement instruments [1–5]. The ISO 25178-700 framework considers this matter [6].

Usually, for an instrument's amplification coefficient (i.e. the effective measurement accuracy) the calibration is done using a standard featuring a single traceable height difference. That instrument then has traceability in future measurements done in the proximate range of the height calibration standard. Heights measured outside the calibration range have neither direct traceability nor good accuracy. Therefore, to ensure linearity the amplification coefficient should be calibrated using a height calibration standard with several steps inside the desired measurement range.

In ISO 25178 part 700 the Instrument Transfer Function (ITF) is considered to describe the spatial resolving power of optical instruments. As the optical transfer function (OTF) for a traditional microscope specifies the spatial frequency response of the instrument, similarly ITF describes the spatial frequency response of 3D measurement instruments. Hard materials (metal, silicon, and glass) are used as a base material in commercial vertical calibration devices [7–9]. In a few

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Optical Metrology and Inspection for Industrial Applications V, edited by Sen Han,
Toru Yoshizawa, Song Zhang, Proc. of SPIE Vol. 10819, 108190D · © 2018 SPIE
CCC code: 0277-786X/18/$18 · doi: 10.1117/12.2501258

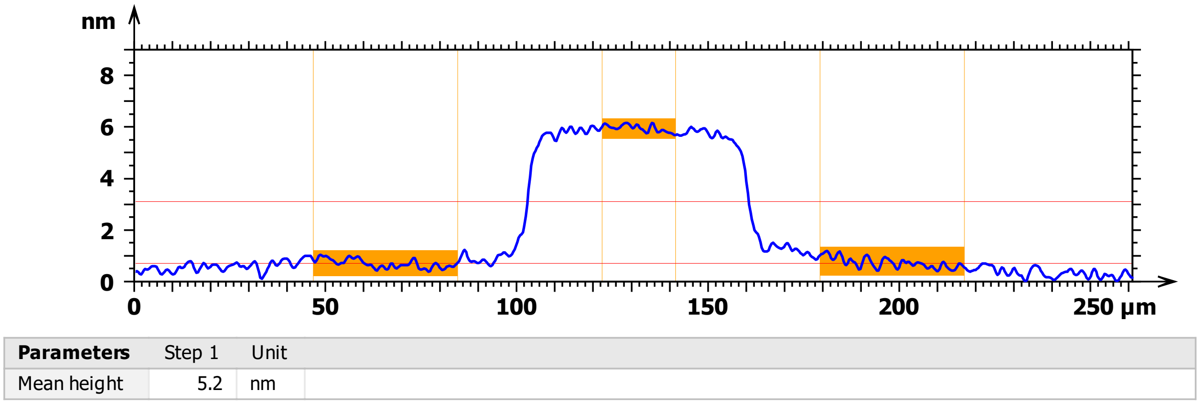
commercial devices, the step height is less than 50 nm and generally, these devices have a step height exceeding 1 µm. There have been efforts to produce a step height calibration standard with a step height of less than 10 nm, using methods including DNA origami self-assembled structures or crystalline surfaces [10,11]. There are devices that can be used for 2D lateral calibration, e.g. the Siemens-Star. Nevertheless, for metrological purposes 3D calibration devices are needed [12].

Here, we study the height stability of three NanoRuler and one NanoStar BTS developed and manufactured at the University of Helsinki (UH). The BTSs were profiled with a custom built SWLI [13], also developed at UH. The data is presented for measurements over a time period of 6 months. In addition, we studied the resistance to water of the BTS structures as a function of immersion time.

2. METHODS

2.1. ISO 5436-1

The ISO 5436-1 describes the calibration analysis procedure for a step height measurement. The procedure is meant for groove step height standards. The device should have a well defined step with two reference locations on its sides. In ISO 5436-1, it is specified that the step width (W) should be measured. The measurement width should be W/3 and should be centred on the step (the distance from the step edges is W/3) while the two references are 2W/3 wide and away from the step centre [14]. The ISO 5436-1 analysis method is implemented in some commercially available analysis software (e.g. MountainsMap [15], see Fig. 1.)



Parameters	Step 1	Unit
Mean height	5.2	nm

Figure 1. Example of extracted profile of NanoStar using step height ISO 5436-1 analysis method in MountainsMap

2.2. Error budget

The step height measurement is modelled as:

$$H_s = \mu_a - \mu_b = \frac{1}{N_a}\sum_{i=1}^{N_a} a_i - \frac{1}{N_b}\sum_{i=1}^{N_b} b_i \quad , \tag{1}$$

where a_i is the region of interest (N_a samples), b_i is the selected reference region (N_b samples), μ_a and μ_b are the respective means of these regions. The uncertainty of eq. (1) propagates and is

$$u_c = \sqrt{u_a^2 + u_b^2} \, , \tag{2}$$

where u_a and u_b are the standard uncertainties of the upper and lower levels, when taking into account the maximum deviation from the mean level in the corresponding regions. Table 1 shows the error budget calculated using Eqs. (1) and (2).

Table 1. Error budget for the height measurement in Fig. 1.

Uncertainty component	Unit	Value	Standard uncertainty $u(x_i)$ (nm)	Sensitivity coefficient $c_i=\frac{\partial f}{\partial x_i}$	Contribution $u_i(y)=\|c_i\|u(x_i)$ (nm)
Region of interest, µa	nm	5.0	0.2	1	0.2
Reference region, µb	nm	-0.2	0.4	-1	0.4
Calculated quantity	Function	Unit	Value	Standard uncertainty $u_c(y)=\sqrt{\sum_i u_i^2(y)}$ (nm)	Expanded uncertainty $U=2u_c(y)$ (nm)
Step height, H_s	$H_s=\mu_a-\mu_b$	nm	5.2	0.5	1.0

2.3. Bio transfer standards

Both BTSs; NanoRuler and NanoStar, were produced using a Langmuir-Blodgett (KSV Mini, KSV Instruments, Finland). A fatty acid monolayer was spread across a liquid subphase, and was compressed to surface pressure of 30 mN/m, which was maintained over the whole procedure. A glass coverslip attached to the instrument's programmable dipper acted as a substrate, onto which bilayers of fatty acid were transferred. The number of steps on either BTS is determined by the number of sequential transfers, i.e. how many times the coverslip is dipped through the compressed film. A single step is at the edge of a folded monolayer that is transferred onto the substrate during both the downstroke and the upstroke. The resulting bilayers were uniform and of acceptable quality. Due to the monolayer-by-monolayer construction, the thickness could be compared to literature data with good agreement [16,17].

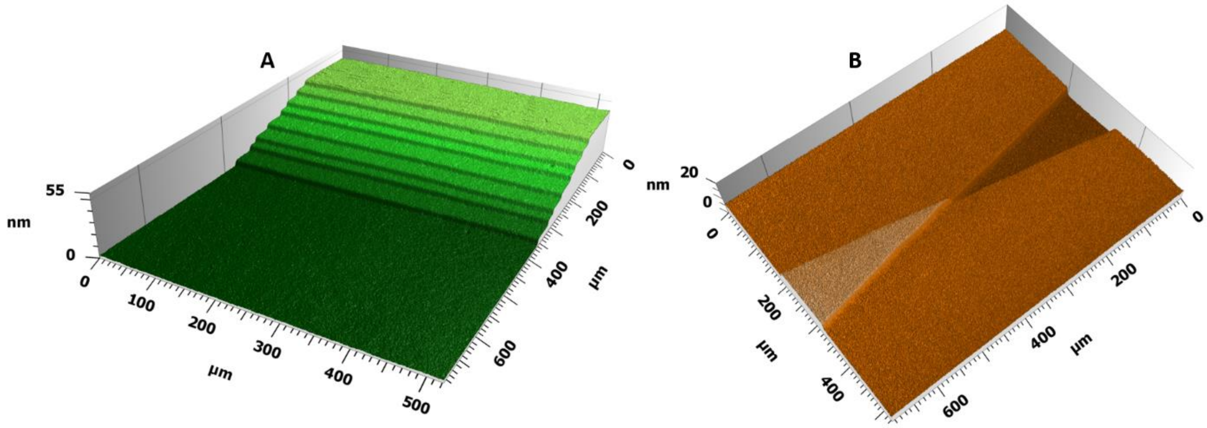


Figure 2. 3D image of Langmuir-Blodgett films based A) NanoRuler and B) NanoStar BTS.

2.4. Optical measurement (Scanning white light interferometry - SWLI)

The SWLI instrument was used in the height measurements of the NanoStar and NanoRuler BTS. The instrument is constructed in a side-illuminated microscope frame (Nikon, Japan, type L-UEPI), using a broadband halogen light source (Philips, Netherlands, type 7724). In all measurements, a 10x Mirau type interferometric objective (Nikon, Japan type CF IC EPI Plan DI) was used together with 1x tube lens (Nikon, Japan, type MXA20696). Finally, the image was acquired using a CMOS camera (Hamamatsu, Japan, type Orca Flash 2.8, (C11440-10C)). The Mirau objective contains a beam splitter that splits the incident light into two beams, one for the reference mirror and one for the device under study. Both light beams reflect (from the specimen and from the mirror) and interfere to produce an interferometric image, which is focused onto the camera cell. Changing the optical path difference between the reference mirror and specimen, e.g. by means of a piezo translator, generates a stack of interferograms that are recorded. In this work, five images in each measurement were processed to create the topological map of the sample.

2.5. Measurement / Analysis protocol

A measurement protocol for NanoRuler BTS was followed to make measurements done on different dates comparable. All three NanoRuler BTS were marked beforehand, to show the vertical position of the steps. From the mark, a lateral offset of 300 µm was made normal to the rising staircase using a motorized microstage (Standa, Lithuania 8MTF-102LS05). This position was measured sequentially three times using the SWLI instrument. The same protocol was repeated for the remaining NanoRuler BTS. This protocol ensures that the spatial position is the same across different dates for the same BTS. In the NanoStar BTS, we identified the spatial location of the V-shape on the device and did three consecutive SWLI measurements. Since the NanoStar BTS featured only one cross the spatial location is the same in each measurement done on different dates. The temperature and relative humidity of the environment was recorded for each measurement run. On average, the temperature was 21.6 ºC ± 0.6 ºC whereas the relative humidity was 18.1 % ± 4.6 %.

An analysis protocol for the BTSs was developed. A commercial software, MountainsMap version 7.4.8114 (Digital Surf, Paris, France) [15], was used to analyse the data and to produce 3D images. For the NanoRuler BTS, three z-profiles, perpendicular to the stairs, were determined at different relative x-coordinate positions; 25 %, 50 %, and 75 % (see Fig. 3A). From these profiles, the steps were identified and their heights were acquired. Accordingly, two z-profiles were obtained from the NanoStar BTS, so that the relative x-coordinate from the crossing position was -25 % (negative step profile) and 25 % (positive step profile), respectively (see Fig. 3B). For the NanoStar BTS an ISO 5436-1 method was used. Before the profile extraction, the software's median filter was applied (3x3 kernel) and a 2nd, order polynomial form was removed from the surface as described in [18].

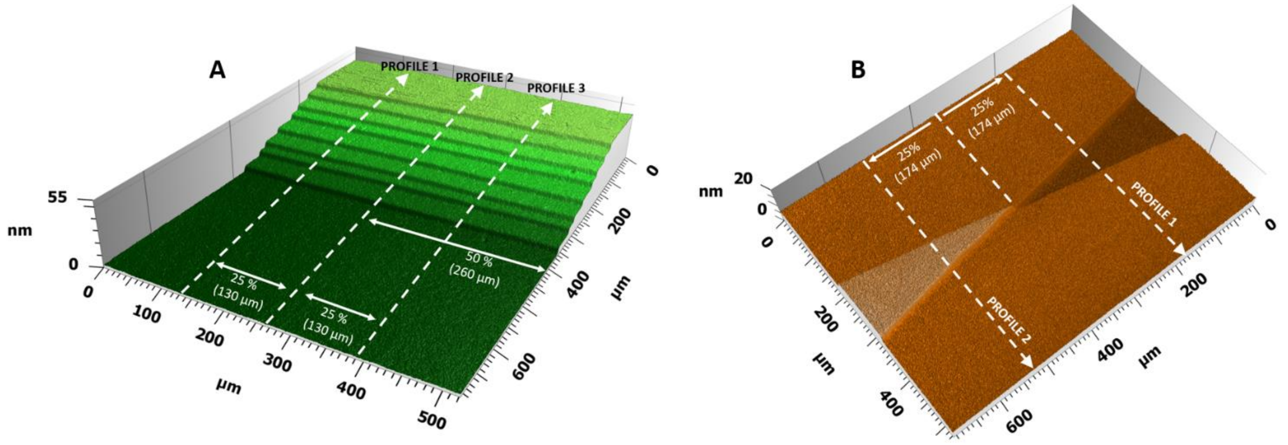


Figure 3. Graphical representation of the measurement protocols for A) NanoRuler and B) NanoStar BTS, where "PROFILE 1" indicates the negative step and "PROFILE 2" the positive step. The arrows indicate measurement direction.

2.6. Water immersion protocol

In the immersion test, one NanoRuler BTS was immersed into ion-exchanged water for 10 seconds, one minute, 15 minutes, 30 minutes, one hour, and 12 hours, starting from the shortest. After each immersion, the BTS was left to dry in open air for minimum 15 minutes, after which the BTS was measured using the same protocol as before. After each measurement, the BTS immersion test was resumed with the next longer period. The MountainsMap software was used to determine the roughness of the BTS. The lowest plane was identified and extracted from each measurement. A median filter was applied (3x3 kernel) and 2nd order polynomial form was removed from the extracted plane. Finally, the remaining data was thresholded, so that the highest and lowest 0.1% heights were omitted. The surface roughness was evaluated by taking a root-mean-square height (Sq) according to the ISO 25178.

3. RESULTS

Figure 4 shows the individual mean heights for the three NanoRuler BTS as a function of measurement date. The same information is in Figure 5 for the NanoStar BTS. The means of the mean heights and their standard deviations (SD) for the steps in both types of BTS over the full time interval are shown in table 2. These are also shown as solid and dashed lines in Figures 4 and 5.

There is no trend in the step heights over the measurement time period (Fig. 4 & 5), and the same single step height is obtained for both NanoRuler and NanoStar BTSs (Table 2). In NanoRuler BTS all step heights are integer multiples of the single step height, 5.1 nm, within their error bars. The SD value is higher for each consecutive step in the NanoRuler BTS, whereas in the NanoStar BTS, the SD value is the same for the negative and positive steps.

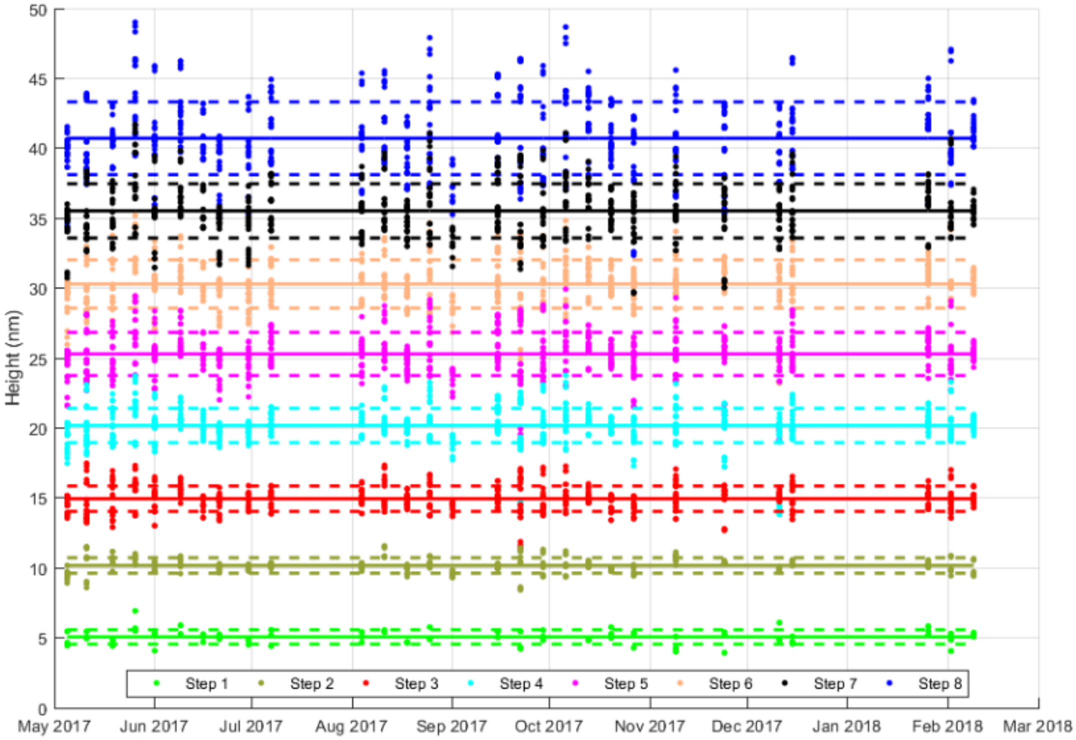


Figure 4. Mean height values of three NanoRuler BTS vs measurement date (solid points), calculated mean value (solid line, from Table 2) of each step together with SD of the sample population (dashed line).

Table 2. Mean heights and SD of each step in NanoRuler and NanoStar BTS.

Staircase Height BTS									V-shaped BTS		
Step	1	2	3	4	5	6	7	8	Step	Positive	Negative
Mean Height (nm)	5.1	10.2	15.0	20.2	25.3	30.3	35.5	40.7	Mean Height (nm)	5.1	-5.1
SD (nm)	0.5	0.6	0.9	1.2	1.5	1.7	1.9	2.5	SD (nm)	0.3	0.3

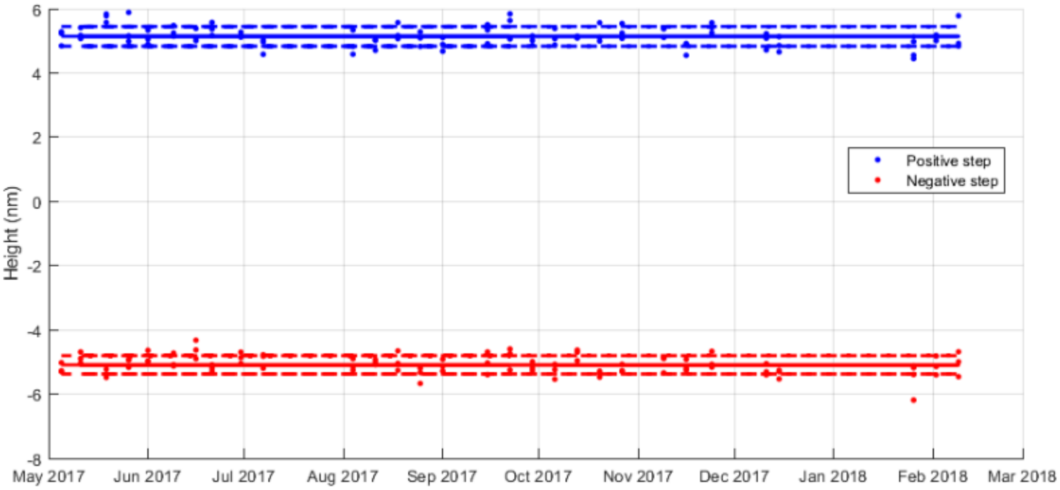


Figure 5. Measured mean step heights of NanoStar as a function of measurement date (solid points), calculated mean value (solid line, from Table 2) together with the SD of the sample population (dashed line).

Figure 6 shows the 3D image of the measured NanoRuler as a function of immersion time. The change in Sq value relative to the value before immersion (ΔSq) versus total immersion time are shown (Fig. 7). Figure 6 shows that there is little change after the shortest immersion of 10 seconds, but after one minute, changes are evident. Furthermore, the analysed ΔSq in Figure 7 shows that the surface gets rougher with increasing immersion time.

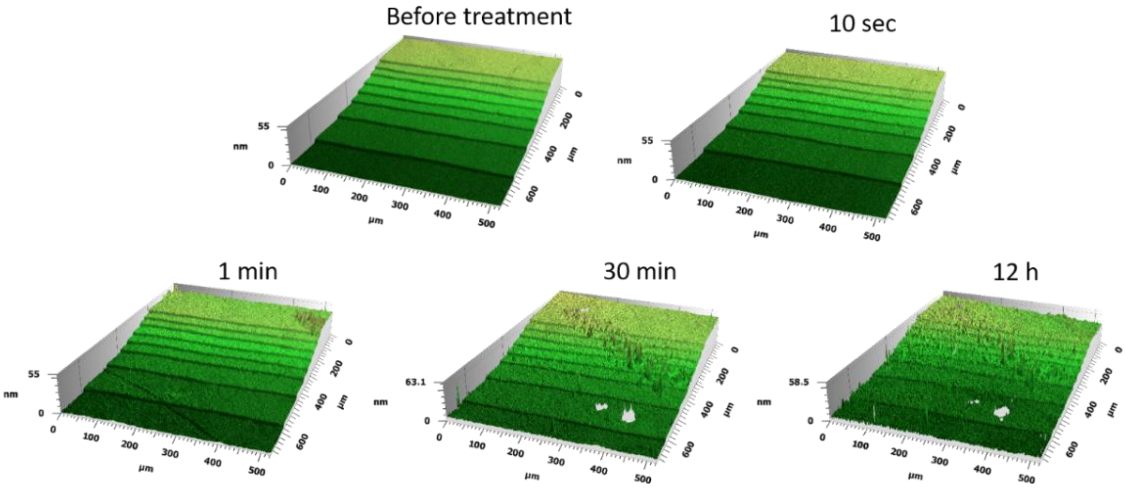


Figure 6. 3D reconstruction of SWLI data from the measured NanoRuler after different immersion times. The images obtained after 15 minutes and 1 hour are not shown for the sake of clarity.

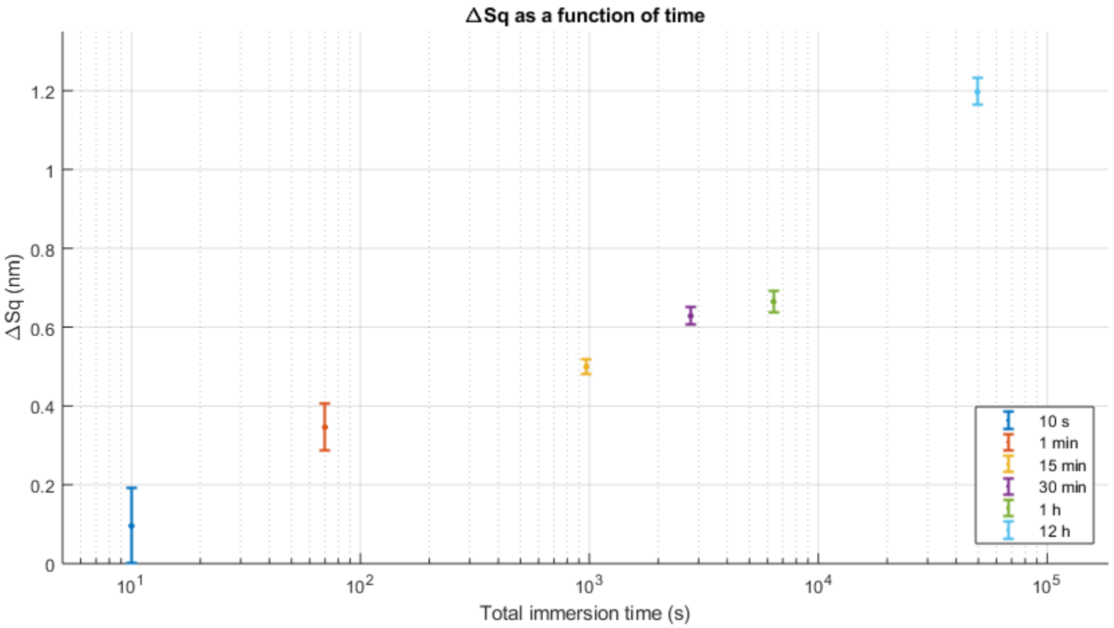


Figure 7. ΔSq as a function of cumulative immersion time. Error bars represent 95 % confidence limits in each measurement.

4. DISCUSSION

The SD value increases for each consecutive step with the NanoRuler BTS, but for NanoStar it is the same for both steps. The reason might be in the form removal and geometric differences between the two BTSs. In NanoStar there are only three height planes, the reference plane, the negative plane, and the positive plane. When the 2nd order polynomial form is fitted to the reference plane and removed from the data there might variations between measurements, but for two equal steps the effect is small. The NanoRuler BTS has eight steps, each at an increasing lateral distance from the reference plane that is used for form removal. Hence, uncertainty in the form removal propagate into bigger SD at higher steps. Another contribution to the magnitude of the SD value is the rather low number of repetitions. The employed protocols do not take advantage of the 3D areal information (i.e. we use profiles), which would be better suited for the instrument that measures heights. Alas, there is no standard (ISO) way of doing these measurements; therefore, there is ambiguity as to what measurement protocol should be used. This makes it difficult to compare results obtained at different laboratories. We aim to address this deficiency.

The absence of linear or second order trends in the stability data tells us that there are no changes in the BTS heights during the prolonged measurement period. The water immersion tests show that the surface gets rougher as a function of immersion time. Since we immersed one NanoRuler cumulatively for different times into the water solution, the amount of drying cycles for the sample increases in each measurement. Hence, there is ambiguity as to which process (immersion or drying) contributes to the surface roughening. To address this question, in future work we will do a multi sample experiment where we study the ΔSq as a function of drying time. However, even after 12 hours of immersion, the staircase structure of the NanoRuler BTS remains intact.

5. CONCLUSIONS

We presented results from a 6 month stability test of four BTSs (3 NanoRulers and 1 NanoStar). The mean height of both steps in NanoStar across the 6 month period was 5.1 nm, and the sample SD was 0.3 nm. For the Nanoruler BTSs the mean heights for the 8 steps were 5.1 nm, 10.2 nm, 15.0 nm, 20.2 nm, 25.3 nm, 30.3 nm, 35.5 nm and 40.7 nm. The respective sample SDs were 0.5 nm, 0.6 nm, 0.9 nm, 1.2 nm, 1.5 nm, 1.7 nm, 1.9 nm and 2.5 nm. No linear nor 2nd order trends were seen in the stability data. This shows that the developed BTSs (NanoRuler and NanoStar) perform well as a calibration device. The water immersion test indicates the possibility of using LB based techniques to produce BTS for immersion microscopy.

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