



## **NIST - NCL Joint Assay Protocol, PCC-6**

### **Size Measurement of Nanoparticles Using Atomic Force Microscopy**

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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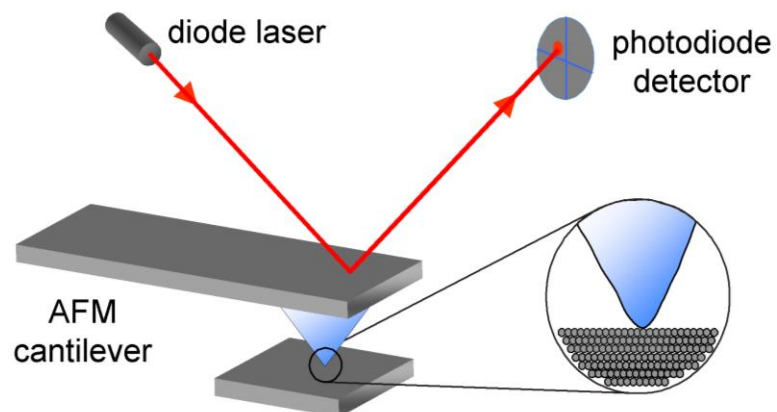
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## 1. Introduction

This assay protocol outlines the procedures for sample preparation of gold and the determination of nanoparticle size using atomic force microscopy (AFM). An AFM utilizes a cantilever with a sharp probe to scan a specimen surface. The cantilever beam is attached at one end to a piezoelectric displacement actuator controlled by the AFM. At the other end of the cantilever is the probe tip that interacts with the surface. At close proximity to the surface, the probe experiences a force (attractive or repulsive) due to surface interactions, which imposes a bending moment on the cantilever. In response to this moment, the cantilever deflects, and this deflection is measured using a laser beam that is reflected from a mirrored surface on the back side of the cantilever onto a split photodiode. A schematic diagram of the system is shown in Figure 1. The cantilever deflection is measured by the differential output (difference in responses of the upper and lower sections) of the split photodiode. The deflections are very small relative to the cantilever thickness and length. Thus, the probe displacement is linearly related to the deflection. The cantilever is typically silicon or silicon nitride with a tip radius of curvature on the order of nanometers.

Based on the nature of the probe-surface interaction (attractive or repulsive), an AFM can be selected to operate in various modes, namely contact mode, intermittent contact mode, or non-contact mode. In contact mode, the interaction between the tip and surface is repulsive, and the tip literally contacts the surface. At the opposite extreme, the tip interacts with the surface via long-range surface force interactions; this is called non-contact mode. In intermittent contact mode, the cantilever is oscillated close to its resonance frequency perpendicular to the specimen surface, at separations closer to the sample than in non-contact mode. As the oscillating probe is brought into proximity with the surface, the probe-surface interactions vary from long range attraction to weak repulsion and, as a consequence, the amplitude (and phase) of the cantilever oscillation varies. During a typical imposed 100 nm amplitude oscillation, for a short duration of time, the tip extends into the repulsive region close to the surface, intermittently touching the surface and thereby reducing the amplitude. Intermittent contact mode has the advantage of being able to image soft surfaces or particles weakly adhered to a surface and is hence preferred for nanoparticle size measurements.



**Figure 1.** Schematic diagram of an AFM. A laser beam is focused on the back of a cantilever that has a tip that interacts with a surface; the beam reflects into a four-quadrant photodetector. Normal forces between the tip and surface deflect the cantilever up or down. Lateral forces twist the cantilever left and right. These deflections are measured by monitoring the deflection of the reflected laser.

A microscope feedback mechanism can be employed to maintain a user defined AFM set point amplitude, in the case of intermittent contact mode. When such feedback is operational, constant vibration amplitude can be maintained by displacing the built-in end of the cantilever up and down by means of the piezo-actuator. (Operation of an AFM with feedback off enables the interactions to be measured and this is known as force spectroscopy.) This displacement directly corresponds to the height of the sample. A topographic image of the surface can be generated by rastering the probe over the specimen surface and recording the displacement of the piezo-actuator as a function of position.

Unlike electron microscopes, which provide a two-dimensional projection or a two-dimensional image of a sample, AFM provides a three-dimensional surface profile. Although the lateral dimensions are influenced by the shape of the probe, the height measurements can provide the height of nanoparticles with a high degree of accuracy and precision. If the particles are assumed to be spherical, the height measurement corresponds to the diameter or size of the particle. In this assay protocol, procedures for dispersing gold nanoparticles on various surfaces such that they are suitable for imaging and height measurement via intermittent contact mode AFM are first described. Generic procedures for AFM calibration and operation to make such

measurements are then discussed. Finally, the procedures for data analysis and reporting are provided. The nanoparticles used to exemplify these procedures are National Institute of Standards and Technology (NIST) Au nanoparticle Reference Materials, RM 8011 (nominally 10 nm), RM 8012 (nominally 30 nm), and RM 8013 (nominally 60 nm), all of which contain citrate-stabilized negatively charged Au nanoparticles in an aqueous solution.

## 2. Sample Preparation and Inspection

*Always wear appropriate personal protective gear (e.g., gloves, lab coat, goggles, and respirator) and take appropriate precautions during sample preparation and measurement.*

The sample preparation (deposition) procedures outlined below were developed specifically for application to NIST Reference Materials RM8011, RM8012 and RM8013, which consist of negatively charged citrate-stabilized gold nanoparticles in aqueous solution at a mass concentration nominally 50  $\mu\text{g/g}$ . The procedures should work with other nanoparticles that carry a negative surface charge or zeta potential. As suggested below, these procedures can also be applied to positively charged or neutral nanoparticles with some modification. Each procedure may require some optimization by the user in order to obtain satisfactory deposition density and to minimize artifacts such as aggregate formation on the substrate or build up of organic films resulting from additives that might be present in the solution phase.

### 2.1 Nanoparticle Deposition Procedures

Nanoparticle samples need to be dispersed on flat surfaces for AFM measurements. The roughness of the surface should be much less than the nominal size of the nanoparticles in order to provide a consistent baseline for height measurements. High-quality mica, atomically flat gold (111) (deposited on mica), or single crystal silicon can all be used as substrates to minimize the effect of surface roughness on nanoparticle measurements. In this section, we will describe three different procedures for depositing nanoparticles on these various substrates, using the NIST gold RMs as model test materials.

### 2.1.1 Mica Substrate

Mica is a layered mineral that can be readily cleaved along alkali-rich basal planes to form clean, atomically flat surfaces extending over large areas. To prepare the substrate, a mica disc must be cleaved to produce a clean surface. Place the disc on a clean, lint-free cloth or directly on an AFM puck. Press a piece of adhesive tape against the surface of the disc and then smoothly remove the tape from the mica. The top layer of the mica should appear on the tape. Continue to cleave the mica until a full layer is removed and the exposed surface is smooth. Typically, this step needs to be repeated several times.

After cleaving, the mica disc is ready to be activated so as to promote adhesion between the substrate and the Au nanoparticles. The NIST Au nanoparticle RMs are dispersed in solution and stabilized by adsorbed citrate ions that give the particles a negative charge. The mica substrate can be activated to have a positive charge that readily binds negatively charged particles to the surface. The substrate is activated using dilute 0.1 % poly-l-lysine (PLL) solution to provide a positively charged surface. To create the solution, dilute 0.1 % PLL solution 1:10 with filtered deionized (DI) water (*e.g.*, add 0.5 mL PLL to 4.5 mL DI water). Use clean glassware for dilution and coating. Keep the diluted PLL solution stored at 2 °C to 8 °C until needed. Fully immerse the mica disc in the diluted PLL solution for 30 min at room temperature. To minimize evaporation, cover the solution with a glass dish. After the time has elapsed, remove the mica from the solution and blow dry with a compressed nitrogen stream.

After drying, apply  $\approx 25 \mu\text{L}$  of undiluted gold nanoparticle solution onto the PLL-modified mica substrate using a micropipette. The gold solution should spread evenly across the surface. Incubate at room temperature using the following schedule:

- 60 nm particles: 10 min
- 30 nm particles: 5 min
- 10 nm particles: 30 sec

The incubation time is appropriate for 50 ppm Au suspensions, but can be varied to modify the particle density on the surface as required. Rinse the

substrate with filtered DI water and gently dry with a nitrogen gas stream. The sample is now ready to image.

### 2.1.2 Silicon Substrate

The electrostatic deposition procedure described above for negatively charged Au nanoparticles on mica can also be conducted using silicon as the substrate material. Cleave a small sample (5 mm × 5 mm) from a silicon wafer. Clean the sample using the following procedure: 5 min in a plasma cleaner, 10 min in a glass container with ethanol placed in an ultrasonic cleaner, 5 min in a plasma cleaner. At this time, the untreated wafer supports a thin, native oxide layer. The substrate can then be treated to produce a positive surface using an amino-silane coupling agent, such as 3-aminopropyldimethylethoxysilane (APDMES). Place a drop of APDMES on the Si surface. Allow the APDMES to react with the underlying substrate for 2 h inside a closed vial. Remove the excess APDMES by rinsing with ethanol followed by DI water.

After drying, apply  $\approx 25 \mu\text{L}$  of undiluted gold nanoparticle solution onto the APDMES-modified silicon substrate using a micropipette. The gold solution should spread evenly across the surface. Incubate at room temperature using the following schedule:

- 60 nm particles: 60 min
- 30 nm particles: 30 min
- 10 nm particles: 15 min

To prevent evaporation, the substrate with Au solution droplet should be closed inside a humidified chamber (closed container with DI water present). Following incubation, rinse the sample first with ethanol, followed by DI water, and dry with nitrogen prior to analysis.

### 2.1.3 Gold Substrate

An atomically flat gold (111) surface (deposited on mica) can also be used as the substrate. If necessary, clean the gold surface using ethanol and dry with

nitrogen. It is not recommended that an ultrasonic cleaner be used, as this may delaminate the gold layer from the underlying mica.

The gold substrate can be functionalized in a manner similar to that described for mica and silicon above, but using thiolated compounds that react chemically with the gold surface. For instance, an amino-thiol compound could be used to impart a positive surface charge to deposit negatively charged nanoparticles. In this case, one should follow the procedure described for APDMES above, but instead use an appropriately selected thiol compound.

The nanoparticles can also be deposited in the absence of a functionalized surface using the drop-cast method. However, to minimize agglomeration, gold solutions are typically stabilized with a surfactant or other agent, some of which may also be dissolved in the solution phase. As a result, AFM imaging may show a residual organic layer on the substrate and nanoparticle surface, which can potentially influence the accuracy of the particle measurements. If this is the case, the user may wish to adopt the following alternative preparation procedure, which utilizes a centrifuge to remove the additional stabilizing agent (*e.g.*, citrate ions) from the solution.

Place an approximately 1 mL aliquot of Au suspension from into a 1.5 mL microtube and centrifuge at the rotation speed and time listed below. Remove and discard a portion of the supernatant from the microtube (according to the dilution ratio given below), then replace with DI water to obtain the proper dilution of the native suspension. No change in the stability of the suspension should be observed during this process. The following guidelines are appropriate for the NIST Au reference materials; it may be necessary to vary these parameters in order to optimize deposition and minimize artifacts.

- 60 nm particles: dilution ratio 1:3; speed 5,000 rpm; time 5 min; volume of the suspension between 0.8 mL and 1 mL
- 30 nm particles: dilution ratio 1:5; speed 8,000 rpm; time 6 min; volume of the suspension between 0.8 mL and 1 mL
- 10 nm particles: dilution ratio 1:8; speed 14,000 rpm; time 20 min; volume of the suspension between 0.8 mL and 1 mL



After the dilution and centrifuge process, a droplet ( $\approx 0.05$  mL) of the suspension can be placed on the substrate using a micropipette and dried in air at 70 °C. The sample is now ready to image.

## 2.2 Optical Microscope Inspection

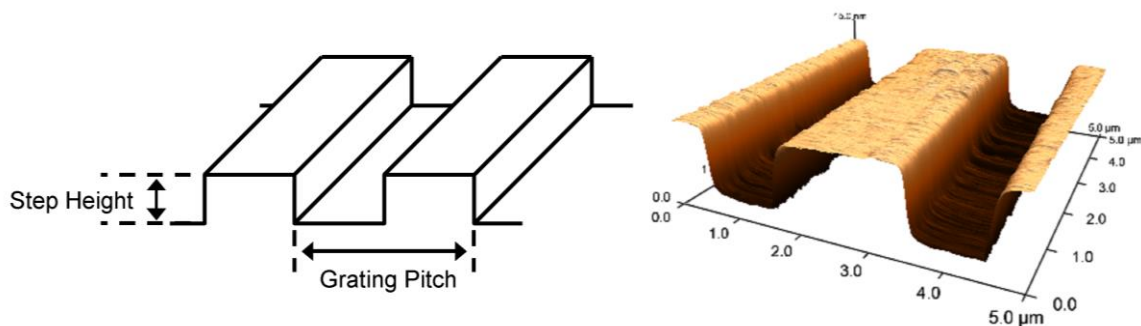
Inspect each sample using an optical microscope before AFM imaging to find possible areas where one can expect a reasonably good dispersion of the particles. In most cases, the exterior of the dried droplet includes excess stabilizing agents (*e.g.*, citrate), while the interior is free of these agents with suitable particle distributions.

## 3. AFM Imaging and Size Measurement Procedure

### 3.1 Accuracy (Height Calibration)

In order to obtain accurate measurements, the axial (*z*)-displacement of the piezoelectric stage needs to be calibrated using available traceable standards. In Figure 2, we show a schematic diagram and AFM image of a calibration grating, which consists of a one-dimensional array of rectangular SiO<sub>2</sub> steps on a Si wafer. For this particular grating, the step height was certified to be 19.5 nm  $\pm$  0.8 nm. The step height is measured with an AFM calibrated using step height reference standards. The reference standards are three calibration gratings that were measured and certified by NIST (NIST Reports 821/261141-99 and 821/265166-01).

After choosing a suitable grating (the step height of the grating should be similar to the characteristic height of the nanoparticles), measure the calibration grating in several locations using a sharp AFM tip and compare the average measured value to the certified step height. If the values are markedly different, consult the AFM manufacturer on how to re-calibrate the *z*-displacement of the piezoelectric stage.



**Figure 2.** Schematic diagram and AFM image of a calibration grating consisting of an array of rectangular SiO<sub>2</sub> steps on a Si wafer. For this particular grating, the step height was certified to be 19.5 nm ± 0.8 nm.

### 3.2 Imaging Mode

Nanoparticles are fixed to the substrate via weak physical forces (*e.g.*, electrostatic and van der Waals forces). As a result, intermittent contact mode is a suitable imaging mode in which the cantilever is driven to oscillate up and down near its resonance frequency by a small piezoelectric element mounted in the AFM tip holder. The amplitude of this oscillation is greater than 10 nm, typically 100 nm to 200 nm.

### 3.3 Cantilevers

Probes consist of a cantilever integrated with a sharp tip on the end. The properties and dimensions of the cantilever and sharp tip play an important role in determining the sensitivity and resolution of the AFM. Several of the key features that should be considered when choosing an AFM cantilever are listed and discussed below:

- Tip radius and geometry: A topographic AFM image is actually a convolution of the tip and sample geometry. While this does not affect height measurements, it does affect the overall representation of surface features. To minimize the convolution, it is best to use tips with radii < 10 nm.

- Cantilever stiffness: Stable cantilever oscillations are required to successfully image a surface in intermittent contact mode, and are only possible when the cantilever has enough energy to overcome adhesive forces (*e.g.*, those arising from capillary menisci, van der Waals, and electrostatic forces) between the tip and sample. To overcome these forces, cantilevers with stiffness  $\approx 40 \text{ N m}^{-1}$  are recommended.
- Cantilever tilt: In most AFM instruments, the cantilever itself is tilted by  $\approx 10^\circ$  to  $20^\circ$  relative to the surface. This is to ensure that the tip makes contact with the sample before any other component, such as the nearby sides of the cantilever chip. While this does not affect height measurements, it does result in an asymmetric representation of the features. In cases where this may be a problem, some cantilever manufacturers offer "on scan angle" symmetric tips, which compensate for the cantilever tilt via the tip geometry.

### 3.4 Scan Size

AFM images have a lateral ( $x$ ,  $y$ ) resolution and a vertical ( $z$ ) resolution. The radius of curvature of the end of the tip will determine the highest lateral resolution obtainable with a specific tip. However, another factor that needs to be considered during image analysis is the number of data points, or pixels, present in an image in the  $x$  and  $y$  scan-direction. For example, in acquiring a  $10 \mu\text{m} \times 10 \mu\text{m}$  image with 512 pixels, the pixel size is  $\approx 19.5 \text{ nm}$  ( $10 \mu\text{m}/512$  pixels). In this case, it is not possible to resolve features smaller than 19.5 nm at a  $10 \mu\text{m}$  scan size. Thus, it is important to consider the particle size when choosing the scan size. The scan parameters shown below can be used as starting points.

- 60 nm particles: scan size  $2.0 \mu\text{m} \times 2.0 \mu\text{m}$ ; scan rate 1 Hz (per scan line)
- 30 nm particles: scan size  $1.0 \mu\text{m} \times 1.0 \mu\text{m}$ ; scan rate 1 Hz
- 10 nm particles: scan size  $0.5 \mu\text{m} \times 0.5 \mu\text{m}$ ; scan rate 1 Hz

### 3.5 Acquiring Images

After completing the general setup for the AFM (*e.g.*, calibrating the z-displacement of the piezoelectric stage, choosing and mounting the appropriate cantilever, tuning the cantilever, etc.), the instrument is now ready to begin the nanoparticle measurement process. At first, use a large scan size to identify a region with a homogeneous nanoparticle distribution. Once a suitable region has been identified, start collecting the nanoparticle images, using the scan parameters above as a starting point. Adjust the oscillation amplitude feedback gains (proportional and integral) to ensure that the forward and backward line scans (profiles) look identical. Store the images on the computer with incremental filenames for post-imaging analysis.

## 4. Image Analysis

Once images are captured during real-time operation, they can be viewed, modified, and analyzed offline using the software supplied by the AFM manufacturer. Some of the more useful data visualization and processing features for nanoparticle measurements will be discussed here.

### 4.1 Flatten Images

Usually, the first step in AFM image processing is a line-wise flattening to remove artifacts of the image acquisition process. For instance, samples are not always mounted perfectly perpendicular to the AFM tip, resulting in some tilt that is not actually present on the sample surface. Other sources of artifacts include thermal drift and non-linearity in the scanner. The flattening technique will correct these non-idealities by fitting each scan line with a polynomial and subtracting it from the data. A first order (linear) correction is normally enough to remove any artifacts.

In the presence of nanoparticles, the flattening procedure becomes a bit more difficult. The software attempts to fit the polynomial to both the substrate and the nanoparticles, instead of just fitting to the substrate. To “eliminate” certain features during the flattening process, most AFM software packages include an

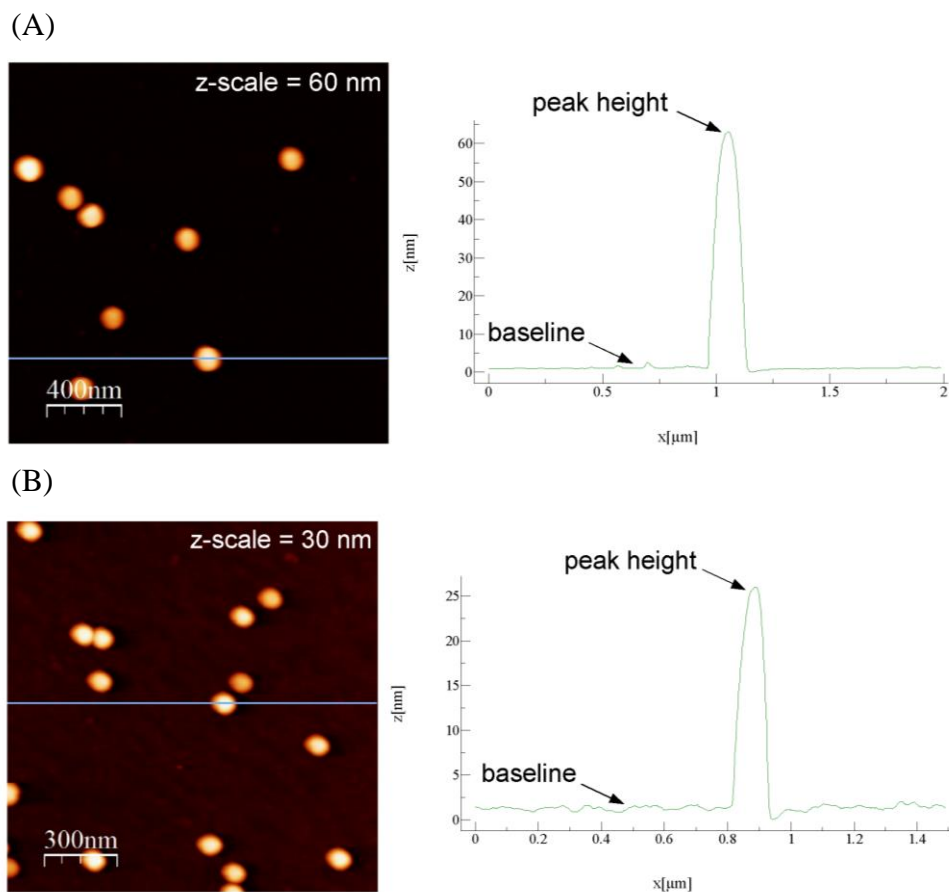
“exclude points” function. Basically, this function will exclude all selected points during the flattening process, effectively ignoring the nanoparticles while flattening the underlying substrate.

#### 4.2 Cross-sectional Line Profiles

Another common feature included in most AFM software packages is the cross-section tool. A cross-sectional line can be drawn across any part of the image, and the vertical profile along that line is displayed. The cursors can be moved to make horizontal, vertical, and angular measurements. By making several cross-sectional line profiles through a nanoparticle, it is not only possible to calculate the particle height, but also to determine if the particle is isolated and sitting on a flat region (*e.g.*, not on a step edge).

#### 4.3 Height Measurement Procedure

Draw a fixed, moving, or averaged cross-section through each particle as shown in Figure 3. Use the cursors to find both the average value for the baseline (on both sides of the nanoparticle) and the peak height. If the flattening procedure was done properly (*i.e.*, the nanoparticles were excluded from the flattening process), the baseline should be relatively flat over the line scan. Subtract the average baseline height from the peak height to find the nanoparticle height. Repeat this procedure for at least 100 nanoparticles for statistical analysis. (To ensure representative and unbiased sampling during particle analysis, please see the theory in references 7 and 8 for discussion on the proper number of particles to count. This theory offers a general guidance for estimating the number of particles to be counted at a certain confidence level.) Large agglomerates and obvious extraneous particles should be excluded from averaging.



**Figure 3.** AFM images and cross-sections for 60 nm (A) and 30 nm (B) nanoparticles (nominal). The difference between the peak height and the average baseline (from both sides of the nanoparticle) is the particle height.

#### 4.4 Automated Batch-Mode Particle Analysis

Most AFM software packages offer an automated particle analysis function. The software can measure the height of particles based on the height of pixel data by using the threshold method and plot a histogram distribution. Prior to performing batch mode measurements, the above mentioned flattening procedure must be applied to ensure a flat substrate. By adjusting the height threshold, the particles above this threshold can be included for analysis, while the particles below this threshold will be excluded. After selecting a group of particles, the

distance between the maximum height of pixel data from individual particles and substrate can be automatically measured, and the height mean value and standard deviation of particles can be calculated. This batch-mode height analysis allows researchers to carry out the analysis of large numbers of particles during a short period of time.

## 5. Reporting Particle Size Data

Calculate the mean and the expanded uncertainty for the particle height distributions according to the following procedure (for a more detailed description of the analysis, see the references below). The average, or arithmetic mean, particle height  $\bar{X}$  is given by

$$\bar{X} = \frac{1}{n} \sum_{i=1}^{i=n} X_i, \quad (1)$$

where  $n$  is the number of measurements and  $X_i$  is the height of each particle. The most common method for describing the variation about the mean value is the standard deviation, or, more simply, the square root of the variance:

$$u_X = \sqrt{\frac{1}{n-1} \sum_{i=1}^{i=n} (X_i - \bar{X})^2}. \quad (2)$$

However, the uncertainty associated with  $\bar{X}$  is not defined by the standard deviation  $u_X$ , but by the standard deviation of the mean, or the standard error,  $u_{\bar{X}}$ . The standard error is related to the standard deviation via  $u_{\bar{X}} = u_X / \sqrt{n}$ , which yields

$$u_{\bar{X}} = \sqrt{\frac{1}{n(n-1)} \sum_{i=1}^{i=n} (X_i - \bar{X})^2}. \quad (3)$$

It is important to consider not just the uncertainty associated with the mean particle height but other sources of experimental uncertainty. In particular, it is necessary to

include the uncertainty associated with the step height grating used to calibrate the AFM. In the case here, the calibration grating had a mean height ( $G$ ) of 19.5 nm, with a standard uncertainty ( $u_G$ ) of 0.35 nm (not to be confused with the *expanded* uncertainty of 0.8 nm). The combined standard uncertainty ( $u_c$ ) is obtained by combining the individual standard uncertainties,  $u_{\bar{x}}$  and  $u_G$ , using the following expression

$$u_c = \sqrt{u_{\bar{x}}^2 + u_G^2} . \quad (4)$$

The corresponding effective degrees of freedom  $\nu_{eff}$  is obtained from the Welch – Satterthwaite equation

$$\frac{u_c^4}{\nu_{eff}} = \frac{u_{\bar{x}}^4}{\nu_{\bar{x}}} + \frac{u_G^4}{\nu_G} , \quad (5)$$

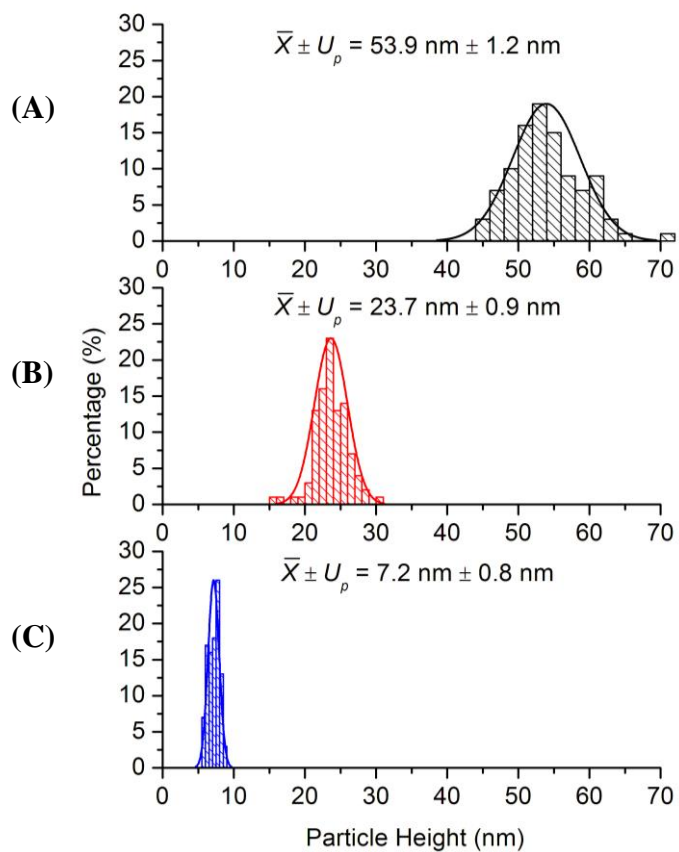
where  $\nu_{\bar{x}}$  and  $\nu_G$  are the degrees of freedom for the height measurements and the calibration grating measurements, respectively. The *expanded* uncertainty  $U_p$ , or the uncertainty that defines an interval having a level of confidence  $p$ , is then given by

$$U_p = k_p u_c \quad (6)$$

where  $k_p$  is the coverage factor. The coverage factor is selected to achieve a desired level of confidence  $p$  using  $t$ -distribution tables (assuming  $\nu_{eff}$  degrees of freedom). This yields a mean and expanded uncertainty for each data set that can be described by  $\bar{X} \pm U_p$ .

The height distributions for 60 nm, 30 nm, and 10 nm nanoparticles (deposited using procedure 2.1.1 on a mica substrate) are shown in Figure 4. For each data set, the mean and expanded uncertainty were calculated with a 95 % confidence level. Note that the uncertainty in the mean is a lot less than the characteristic width of the distribution.





**Figure 4.** Histograms for 60 nm (A), 30 nm (B), and 10 nm (C) nanoparticles (nominal).

## 6. References

### 6.1 Atomic Force Microscopy

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## 7. Abbreviations

AFM	atomic force microscopy
APDMES	3-aminopropyldimethylethoxysilane
Au	gold
cm	centimeter
DI	deionized
<i>G</i>	mean height of calibration grating
Hz	Hertz
<i>k<sub>p</sub></i>	coverage factor
m	meter
min	minutes
mL	milliliter
mm	millimeter
μL	microliter
μm	micrometer
NIST	National Institute of Standards and Technology
n	number of measurements
N	Newtons
nm	nanometer
<i>p</i>	confidence level
PLL	poly-L-lysine
RM	reference material
rpm	revolutions per minute
sec	seconds
Si	silicon
SiO <sub>2</sub>	silicon dioxide

$u_c$	combined standard uncertainty
$u_G$	standard uncertainty
$U_p$	expanded uncertainty
$u_X$	standard deviation
$u_{\bar{X}}$	standard deviation of the mean
$\nu_{eff}$	effective degrees of freedom
$\nu_G$	degrees of freedom for calibration grating measurements
$\nu_{\bar{X}}$	degrees of freedom for height measurements
$\bar{X}$	average particle height
$X_i$	particle height