# NIST - NCL Joint Assay Protocol, PCC-15

# Measuring the Size of Colloidal Gold Nano-particles using High-Resolution Scanning Electron 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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## 1. Introduction

This assay protocol outlines procedures for sample preparation and determination of mean size (effective diameter) of citrate stabilized colloidal gold nano-particles by high-resolution scanning electron microscope (SEM). The gold particles are three-dimensional structures and while most of them may be properly described as irregular, some possess certain distinct shapes. These may have more or less triangular, hexagonal and rectangular facets or spherical shapes. Some particles even have complex shapes such as diamonds and cylinders, and are clearly multi-faceted. SEM images are essentially two-dimensional projections of the non-spherical gold particles sitting on the Si substrate, which make the average size information somewhat ambiguous. However, by counting and measuring sufficiently large number of particles placed at random on the substrate, it is possible to arrive at the effective particle diameters.

## 2. Sample Preparation

2.1. Information on the health effects of colloidal gold nano-particles is not yet complete, and while there are no known serious problems, it is recommended to always wear appropriate personal protective gear (e.g., gloves, lab coat, goggles, respirator, etc.) and take appropriate precautions when handling nano-materials.

2.2. Tools used for preparation and handling nano-particles should be cleaned with filtered de-mineralized water and stored dry. The use of commercial cleaning agents formulated specifically for optical components is acceptable to remove residues, but care must be taken to remove all traces of the cleaning detergent as this may impact the nano-material properties. If available, store tools under high efficiency particulate air (HEPA) filtered air (e.g., in a clean bench).

2.3. Suspending, diluting or rinsing media (i.e., solvent, dispersant, solution) should be filtered prior to sample preparation using a 0.1 μm or smaller pore size membrane, if possible.

2.4. For scanning electron microscope measurements the colloidal gold nano-particles can be deposited on many surfaces, either in their original concentration, or after

dilution. Nevertheless, this may lead to samples that are less than ideal. The gold nano-particles might pile up and or stick to each other as shown on Figure 1a, and the colloid material can act as a source of electron beam-induced contamination. If the samples are used for scanning probe measurements, the colloid material might stick to the probe. All these issues might adversely affect or prevent high-resolution imaging and measurements. The sample preparation procedure described here was found to be useful to avoid these problems and facilitate high-resolution SEM and atomic force microscopy (AFM) imaging and measurements.

The goal is to capture the gold nano-particles from suspension onto chemically derivatized silicon chips that are suitable for SEM and AFM investigations. This is reached by forming a covalently bound amino silane monolayer on the thin silicon dioxide layer that covers the Si chip. The positively charged amino group captures and holds the citrate stabilized negatively charged individual gold nano-particles from the colloidal suspension. The strength of the forces that hold the particles is strong enough to withstand the necessary rinsing, so at the end the nano-particles strongly adhere and deposit cleanly on Si chip surface (Figure 1b). The Si and gold give very good contrast in the SEM and disturbing sample charging does not occur if the silicon dioxide layer is thin enough.

**A.** **B.**

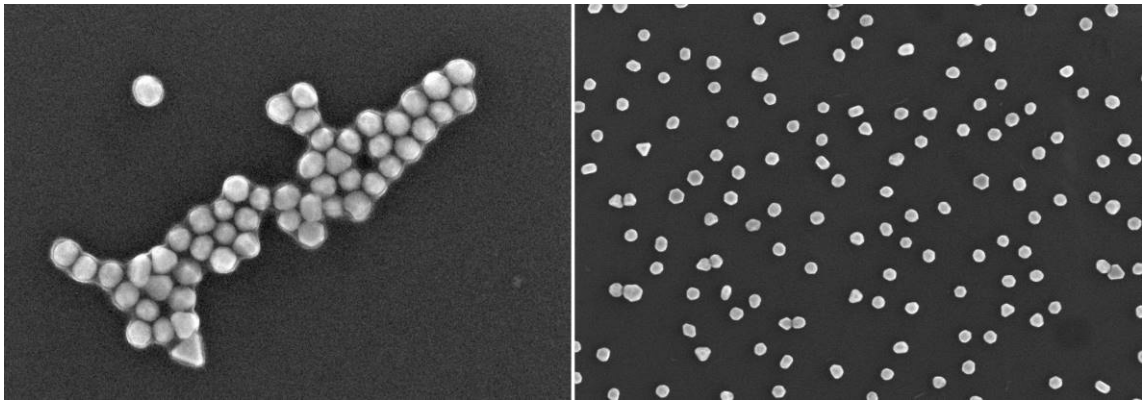

**Figure 1.** High-resolution 15 keV landing energy secondary electron images of 30 nm colloidal gold nano-particles deposited on Si chip surfaces after dilution and drying only (a) and after

using the positively charged surface with procedure described in this document (b). The field-of-view is 640 nm (a) and 1.2 μm (b).

2.5. Preparation of sample holders on which the colloidal nano-particles will be deposited starts with cutting silicon wafers into 5 mm by 5 mm chips. Any diameter Si wafer will suffice; in the NIST measurements 100 mm (4 inch) conductive (doped) wafers were used for their low price and availability. A wafer-dicing machine that holds the wafer with a vacuum chuck cut and sawed grooves into it according to a computer controlled sawing scheme. The grooved wafers were then broken into individual chips using forceps (Figure 2).

Alternatively the Si wafer can be cleaved into small chips, after using a diamond scribe to delineate the cleaving location. The cleaving lines will follow the crystal orientation of the silicon. With some practice one can make excellent chips with <100> Si wafers. Cleaving rectangular chip samples out of <111> wafers can be difficult, as cleaving tends to happen at a 60° angle.

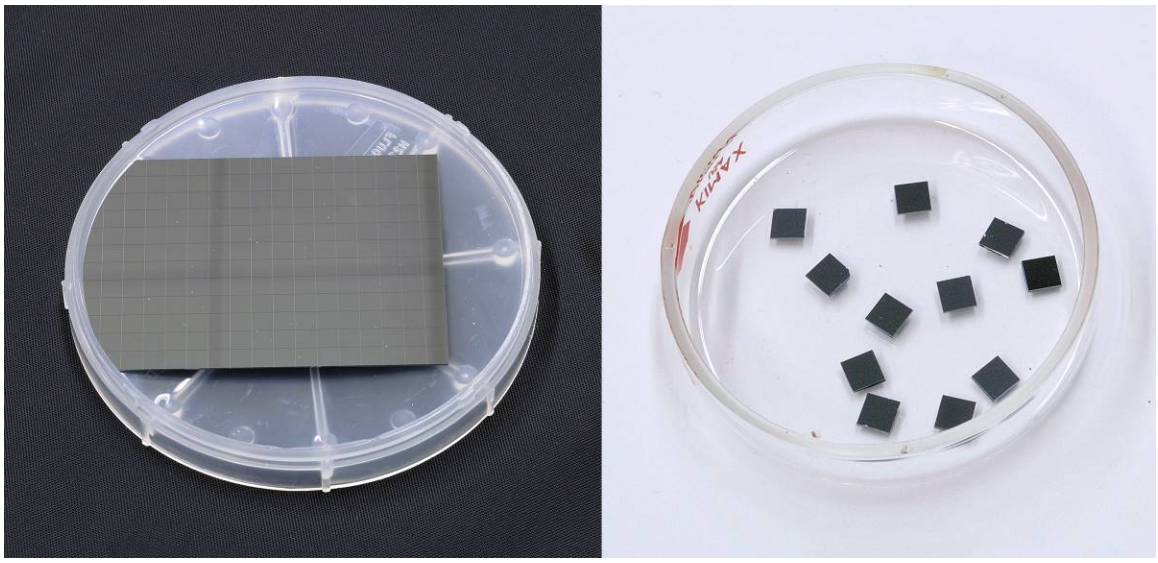

**Figure 2.** A portion of a Si wafer with groves already cut (left) and several 5 mm by 5 mm chips (right).

2.6. The chips have to be cleaned to remove dust and silicon fragments from their surfaces. The chips are placed into a small beaker filled with a 9:1 solution of $H_2O$ and $NH_4OH$. The beaker is then exposed for 5 minutes to a low intensity ultrasonic cleaning with a non-heated water bath. The chips were rinsed with clean water and an ethanol jet and dried with a blast jet of pressurized oil-free nitrogen. Alternatively, one could use an anion-active or non-ionic surfactant and clean water, but this will require more rinsing. The shiny surface of the chips at the end of this step must be completely free from any visible particles or discoloration.

2.7. The approximately 1.3 nm thickness of the native oxide on the surface of the Si chips is not ideal for the preparation of the capturing layer. The chips with the native oxide might work sufficiently, but a somewhat thicker oxide layer was found to work better. To form the thicker layer of silicon dioxide, a one-minute wet oxygen plasma treatment of the chips is performed. With their shiny surface facing up, the chips were treated in a pressure plasma etcher with 40 watts of power at $2x10^{-1}$ millibar (20 Pa) pressure.

The oxygen plasma oxidizes and removes organic contaminants from the chip surface and may slightly increase the thickness of the silicon dioxide layer. This layer is needed for the following chemical derivatization. However the silicon dioxide, which as a bulk material is an insulator, should not be so thick (thicker than 4 nm to 5 nm) that the chip looses its ability to conduct charge away from the region where the microscope's electron beam scans over the sample.

Water vapor is added to the oxygen in the plasma chamber to optimize the formation of silanol groups on the silicon dioxide layer. The silanol groups react with the silane applied in the next step. After the plasma treatment, the chips are very hydrophilic. This can be demonstrated by applying a drop of water, which forms a small wetting angle. It is important proceed with the next step within a few minutes.

2.8. In this step prepare an approx. 10:1:1 (volume) mixture of 95 % (190 proof) ethanol (ethyl alcohol)/ pure water/ aminopropyldimethylethoxysilane (APDMES). The chemical composition of APDMES is $NH_2CH_2CH_2CH_2Si(CH_3)_2OCH_2CH_3$. Roughly, 10 mL should be sufficient. Let the above mixture hydrolyze for about 5 minutes.

2.9. Place the chips onto a Teflon surface (sheet). Apply one large drop of the mixture onto the chips. Keeping the drop size within a very small range is not necessary, but it is recommended to dispense a large enough drop to cover a large part of the chip (as shown in Figure 3). Wetting is expected to be poor, which is evidenced by the fact that the drop of the silane mixture clearly recedes from the edges. It was found that a special, well-type chip holder worked well, because it conveniently and safely holds the chips and helps the drop of silane mixture stay on the top of the chips. One can get similarly good results without the use the well-type chip holder. Figure 3 shows the Teflon chip holder used at NIST. The holder facilitates the process by holding more liquid on the top of the chips, and by decreasing evaporation that may otherwise cut the derivatization time short.

Chemical derivatization of the Si chips takes approximately one-hour. Depending on sample cleanliness and silicon dioxide layer quality and thickness, somewhat longer or shorter times may work; some experimentation may be needed. After the waiting period is over, wash the chips thoroughly with low-pressure ethanol-jet and dry them with a low-pressure gas jet. Finally, place the chips into a heated oven set to 110°C for 10 minutes. Once this procedure is completed, use the derivatized chips within a few minutes, as their surface will loose activity with time.

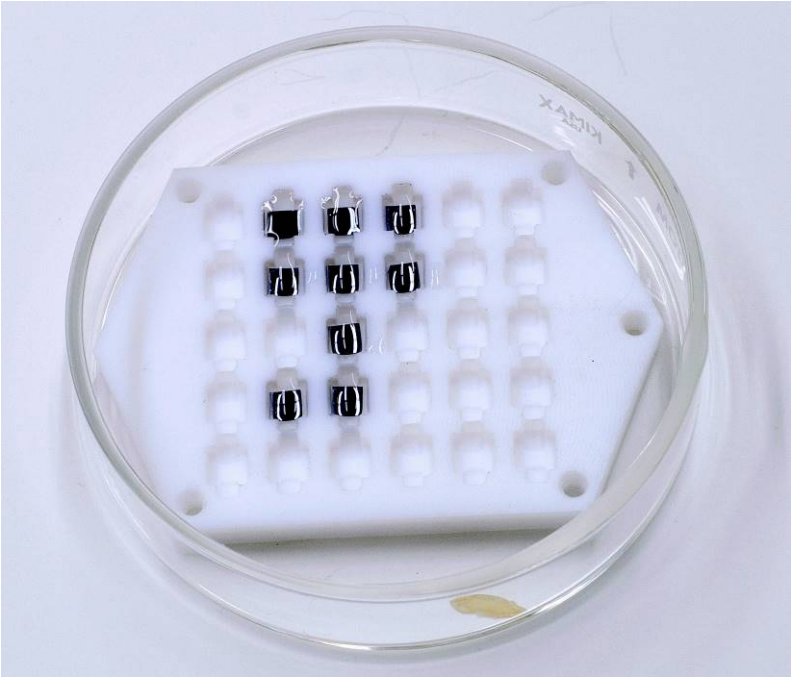

**Figure 3.** The Teflon chip holder used for conveniently holding and separating many Si chips at once.

2.10. The next step applies the colloidal gold nano-particle solution to the shiny top surface of the derivatized Si chips. The required time depends on the size and concentration of gold nano-particles, so some experimentation may be needed. It was found that one to two hours worked well for samples at their original concentration. With shorter times, it is possible that the particles will be far away from each other, which, especially for the 10 nm size particles is not desired. It is useful to support the chip by a Teflon surface (sheet) and the Teflon chip holder (shown on Figure 3). A large drop of colloidal material on the chips over time will allow for the deposition of the gold nano-particles. Keeping the drop size within a very small range is not necessary, but it is recommended to dispense a large enough drop to cover a large part of the chip. Wetting will be poor, and the drop of colloidal gold can recede from edges.

Finally, the chip is gently but thoroughly rinsed with isopropanol (isopropyl alcohol) and clean de-ionized water, and dried by gently blowing clean nitrogen gas over the substrate held at a slant angle.

Samples prepared this way have particles randomly placed on the substrate at suitably high density, and without significant particle touching or pile-up. It is important to point out that these procedures do not introduce undesirable size or shape bias, i.e. the original size and shape population distributions of the gold nano-particles were left unchanged. Once produced, the chips with the deposited gold nano-particles on them must be placed into clean storage. It is best to measure them as soon as possible.

## 3. Measurement Procedure

The particles of the colloidal gold samples are very small, so small, that many SEMs will not be able to generate images with sufficiently high magnification and high resolution to accurately reveal their size and shape information. This is especially true for the nominally 10 nm size samples. To obtain good results, one must collect good quality data and images and use appropriate procedures to extract the information sought. In the SEM, a number of parameters must be chosen properly to obtain adequate quality data and images. The SEM parameters described here are guides to obtaining good results, but not necessarily the best settings for all SEMs capable of carrying out the required measurements. The measurement procedures described here do not lead to accurate and traceable measurement results. In the calculations to obtain the mean values of the effective particle diameters not all measurement errors were accounted for, and therefore accuracy of the results cannot be stated. Accuracy is described as "the closeness of agreement between a measured value and a true quantity value of a measurand", and traceability as "the property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons, all having stated uncertainties". The true value is generally unknown, so accuracy is not typically assigned a numerical value. Measurements with lower uncertainty are said to be more accurate. The measurements carried

out at NIST lead to Reference Materials (RMs) and not Standard Reference Materials (SRMs) [1,2].

### 3.1. SEM Imaging and Data Collection

The samples were imaged by a FEI Helios Dual-Beam SEM-based measuring system, which is equipped with a high-performance electron beam column and sample stage. The best spatial resolution of the system is 1 nm at optimum settings at 15 kV accelerating voltage. For this study, the best results were obtained at that accelerating voltage, with 86 pA beam current and 30 µs beam dwell-time for each image pixel, and with the sample at 3.5 mm working distance. These parameters were used throughout the study to obtain image-to-image consistency. The contrast and brightness of the images were set so that a good balance of the particle detail and distinction from the background was achieved. It is necessary to set the electron-optical column and imaging conditions properly to acquire images with very good spatial resolution, and with no evidence of astigmatism, excessive noise, or other disturbances which would lead to erroneous measurement results. Pertinent information is available in the literature. [3,4]

Most samples were imaged at 250 000 times magnification (at 1.2 µm field-of-view), which provides a good balance between high spatial details and particle density (Figure 4 and Figure 6). Figure 5 reveals the facets of the nominally 60 nm size particles, but shows fewer of them. One, of course can opt for taking more images at higher magnifications, but other considerations such as rate of contamination, etc. might lead to somewhat lower magnification images. Too low magnification images might not have sufficient pixel resolution, so the measurement results will be less reliable. For the nominally 10 nm size gold nano-particles, it was necessary to use 500 000 times magnification (600 nm field-of-view) to ensure sufficient resolution of the smaller particles (Figure 7). To meet the demand of measurement precision at such small scale lengths, a digital capture resolution of 2048 by 1886 square pixels was used for all images. At 250000 times magnification such an image resolution provides an approximately

100 by 100 pixel area for a nominally 60 nm particle, and a 50 by 50 pixel area for a nominally 30 nm particle. Similarly, at 500 000 times magnification, the same image resolution yields an area of roughly 35 by 35 pixels for a nominally 10 nm particle.

With any SEM, it is important to keep the electron beam-induced contamination at negligible levels. This is especially important in the case of the smallest particles that could easily get obscured by a layer of contamination. It was found that some samples were slightly more prone to contamination than others. This was likely due to the difference in sample preparation, especially in the two rinsing steps. Samples made by simple direct deposition of the colloidal gold nano-particles on Si substrates sometimes showed prohibitively strong contamination. It is known that the insufficiently clean vacuum and sample stage in the SEM also contribute to contamination. In this study the sample and instrument cleanliness were kept at levels that allowed for taking several images of the samples without inducing significant sample contamination.

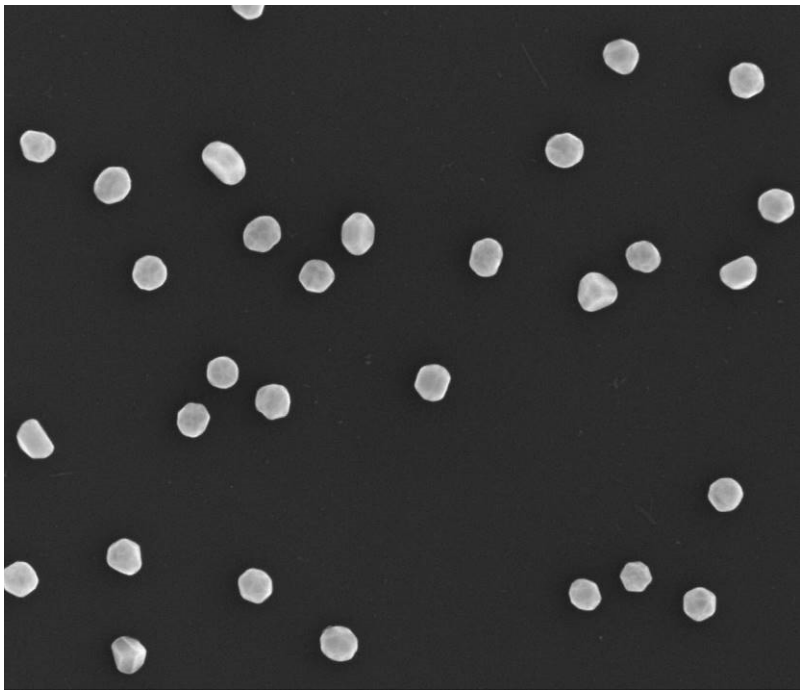

**Figure 4.** Typical SEM image of RM 8013 (nominally 60 nm) colloidal gold particles at 250 000 times magnification. The field-of-view is 1.2 µm.

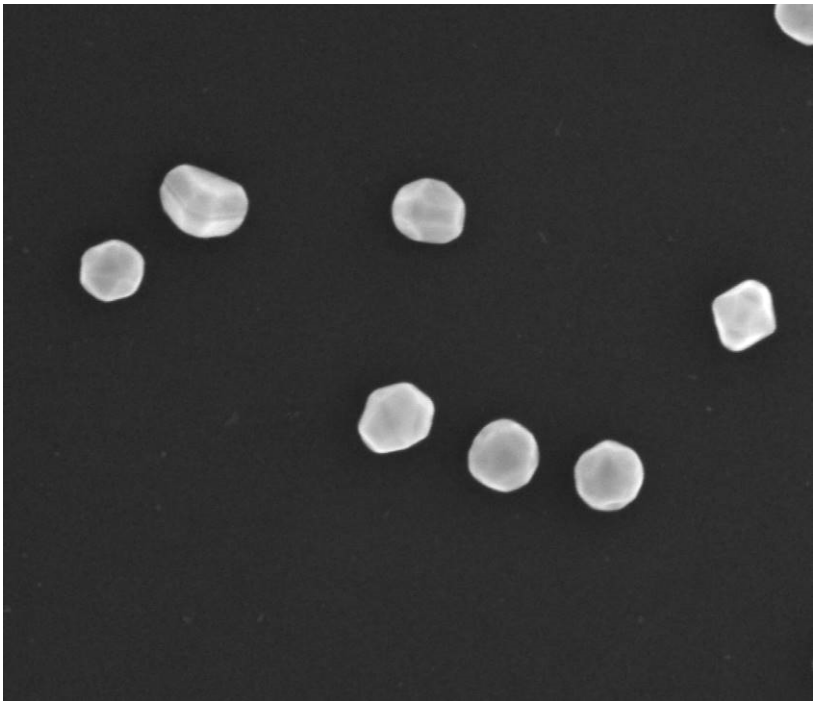

**Figure 5.** Typical SEM image of RM 8013 (nominally 60 nm) colloidal gold particles at 500 000 times magnification. The field-of-view is 600 nm. Note the facets of the particles.

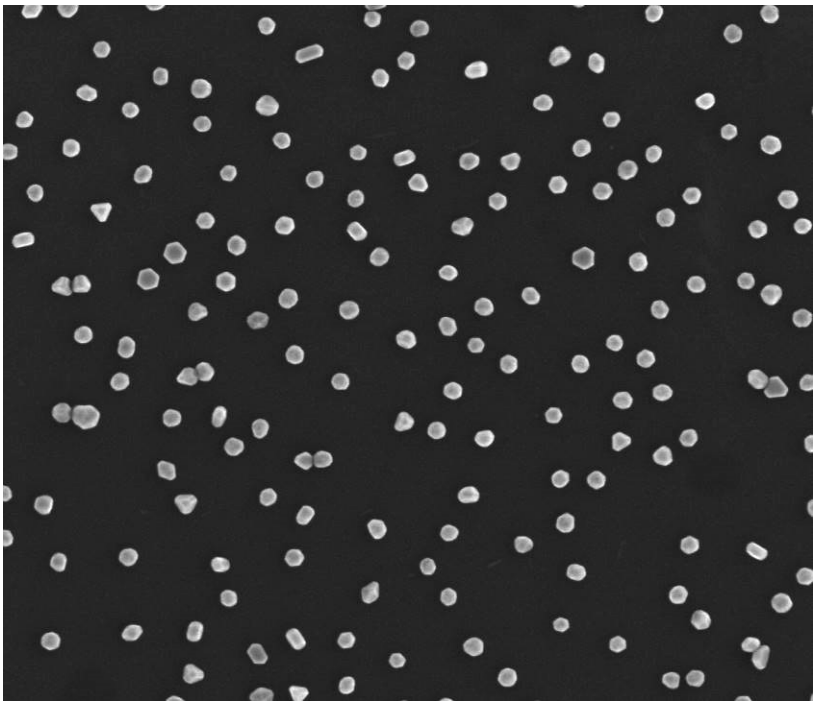

**Figure 6.** Typical SEM image of RM 8012 (nominally 30 nm) colloidal gold particles at 250 000 times magnification. The field-of-view is 1.2 µm.

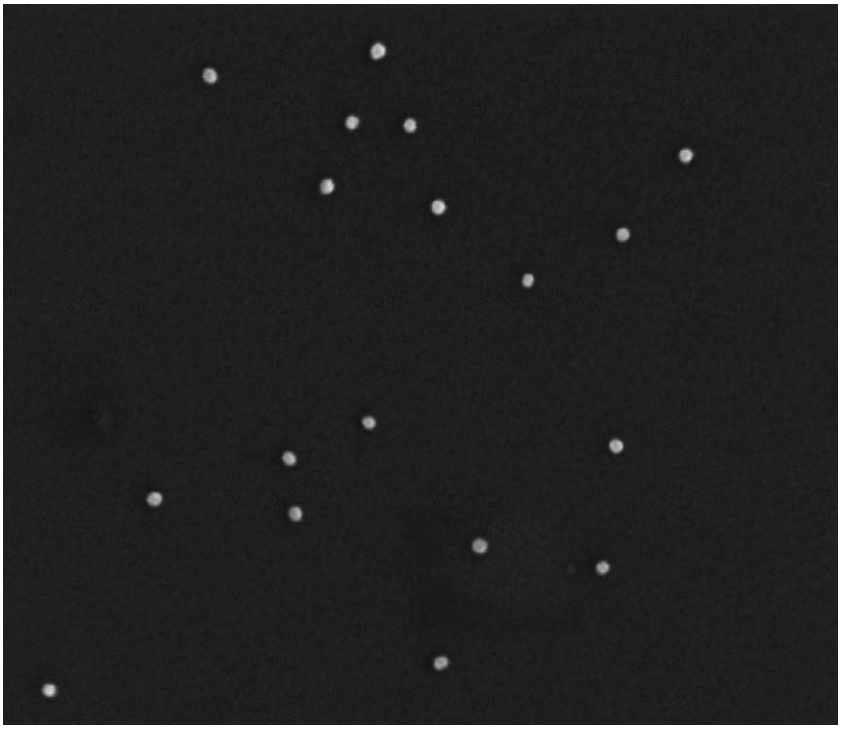

**Figure 7.** Typical SEM image of RM 8011 (nominally 10 nm) colloidal gold particles at 500 000 times magnification. The field-of-view is 600 nm.

## 4. Image Processing and Analysis

For scale calibration a VLSI Standards Nano-Lattice sample was used for both X and Y directions. This artifact was calibrated on NIST's Calibrated Atomic Force Microscope by Ronald Dixson of MMD, PML, who has found that the pitch value is 99.936 nm with an uncertainty of 0.062 nm (coverage factor k=2). The pitch measurement of this calibration sample by FEI Helios SEM was performed with NIST SEM Pitch Metrology software, which is freely available from the Nanometer-Scale Metrology Group [5]. The scale calibration or fiducial mark provided with the SEM images and set at the factory was found to be within 3% of the traceable scale calibration as determined by the pitch measurement described above. This is a reasonable agreement, so no adjustment to the image collection parameters of the SEM was needed.

Version V1.37 of NIH ImageJ (freely available sophisticated image processing software from the National Institute of Health [6]) was used for image processing, analysis and measurements. The key step of particle segmentation was performed with different segmentation algorithms (entropy threshold, Otsu threshold, K-means clustering, mixture modeling, etc.) available within the ImageJ program, and the Otsu threshold algorithm was found to provide the most accurate and consistent data. This process results in a binary version of the images, in which the particles are white and the background is black. After segmentation the "Analyze Particles" command of ImageJ was used to count and measure particles in the binary images. The outlines of the particles as traced by ImageJ were used to check the quality of the particle separation from their background. ImageJ generated the measurement results for each numbered particle in the entire image and the results were exported to a spreadsheet program for further analysis.

The area data for each particle as obtained from ImageJ in the spreadsheet were converted to an effective diameter value in pixel units, regardless of the particular particle shape. Using the calibration results from the prior pitch measurements, this value was subsequently converted into nanometers. From many particles in one image and many images obtained from multiple samples, statistical results such as standard deviation, the average effective particle diameter values were obtained (Table 1- 3 and Figures 8-10).

## 5. Observations of SEM images

The sample images shown here illustrate that SEM imaging can capture the size and shape information of all gold nano-particles. The particles are randomly placed on the Si substrate with suitable densities and are clearly distinguishable from the background, which is required for accurate particle measurements. Furthermore, the images provide more than just size information about each particle. The particles are manifestly three-dimensional structures and while most of them may be described as irregular, some possess various distinct shapes. Some have triangular, hexagonal and rectangular facets or spherical shapes. Other particles even have multi-faceted, symmetrical, diamond or cylinder shapes.

## 6. Statistical analysis of particle size measurement results

The scope of the work was limited to the study and determination of the average size of gold nano-particles. SEM images are essentially two-dimensional projections of the gold particles on the Si substrate. The shapes of the particles are not completely regular spheres or polyhedrons, which makes the average size information somewhat ambiguous. However, by counting and measuring a sufficiently large number of such particles placed at random on the substrate, it was possible to arrive at the results of effective particle diameters. The information given in other NIST - NCL Joint Assay Protocols give guidance on obtaining consistent and meaningful statistical results.

The table below shows the measured effective particle diameter distributions for the three RMs. To evaluate the uncertainty of the results, the re-sampling technique known as Bootstrap can be used. This is especially applicable in cases like this, where the sampling distribution is not well known. The 0.025 and 0.975 quantiles from the bootstrap distribution are used to obtain the uncertainty. (Notice that there is no need to multiply by a coverage factor since a probabilistic interval is directly obtained from the bootstrap results).

**Table 1.** Statistical results obtained by the Bootstrap evaluation method.

| RM | Particle size (nm) |
|---|---|
| 8011 | 9.9 ± 0.1 |
| 8012 | 26.9 ± 0.1 |
| 8013 | 54.9 ± 0.4 |

Another approach: because there is a sufficiently large number of measurements, a more traditional uncertainty analysis could also be used. The summary values are the means of the SEM measurement results. The expanded uncertainty displayed is the confidence limit associated with the sample average calculated as $U=ku_c$. The combined uncertainty, which in this case, the estimated standard deviation of the mean, is given by $u_c$ calculated according to the methods in NIST and ISO Guides [7,8]. The coverage factor, $k$, is the expansion factor of 2 associated with a confidence level of 95 %.

**Table 2.** Statistical results obtained by the traditional evaluation method.

| RM | Particle size (nm) | k |
|---|---|---|
| 8011 | 9.9 ± 0.1 | 2 |
| 8012 | 26.9 ± 0.1 | 2 |
| 8013 | 54.9 ± 0.4 | 2 |

Since both approaches yield identical values for mean particle sizes and uncertainty ranges for all three RMs, the measurement results can be summarized as follows:

**Table 3.** The results of SEM measurements of RM 8011, RM 8012 and RM 8013 with the number of measured particles and the details of the measured results.

| | Number of particles measured | Mean value of particle area ($nm^2$) | Mean value of effective particle diameter (nm) | Minimum effective diameter measured (nm) | Maximum effective diameter measured (nm) |
|---|---|---|---|---|---|
| **RM 8013** (nominally 60 nm) | **425** | **2368.9** | **54.9 ± 0.4** | **45.7** | **72.9** |
| **RM 8012** (nominally 30 nm) | **1185** | **566.2** | **26.9 ± 0.1** | **20.6** | **37.2** |
| **RM 8011** (nominally 10 nm) | **140** | **77.0** | **9.9 ± 0.1** | **8.0** | **12.4** |

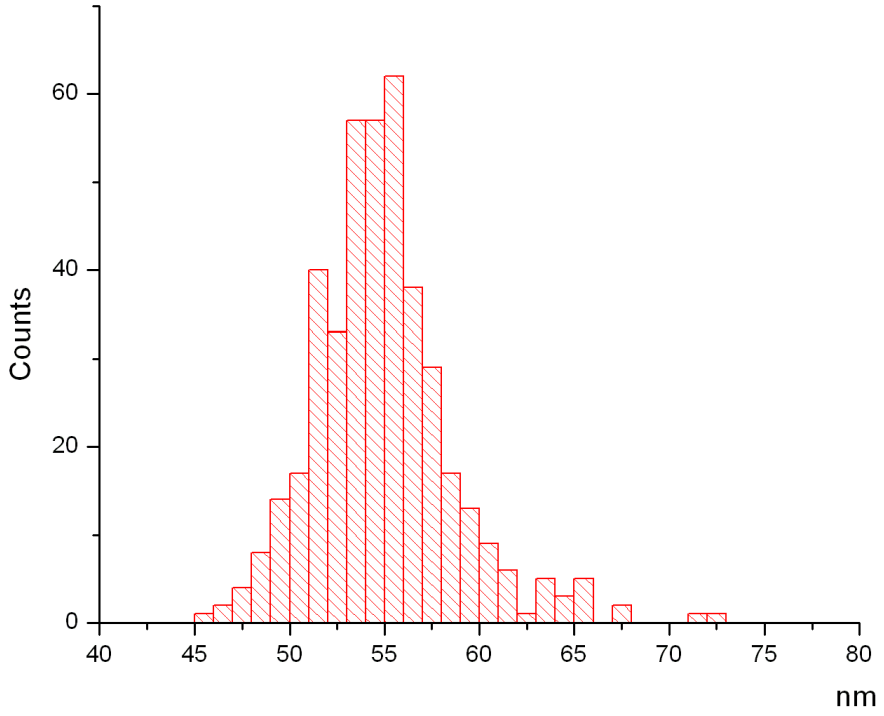


**Figure 8.** Measured effective particle diameter distribution for the RM 8013 (nominally 60 nm)

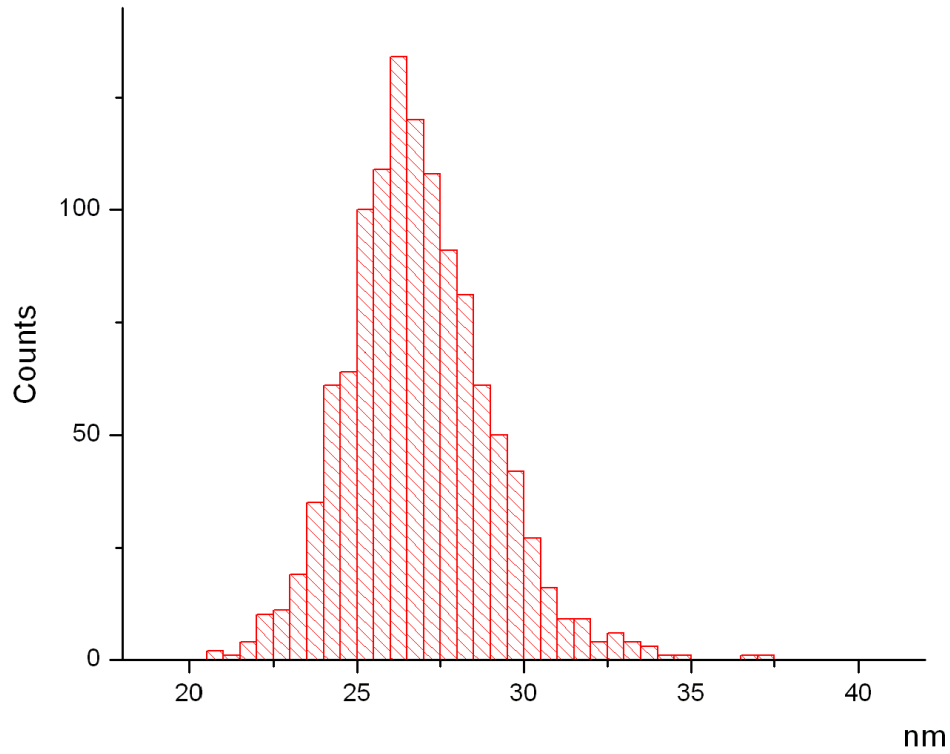


**Figure 9.** Measured effective particle diameter distribution for the RM 8012 (nominally 30 nm)

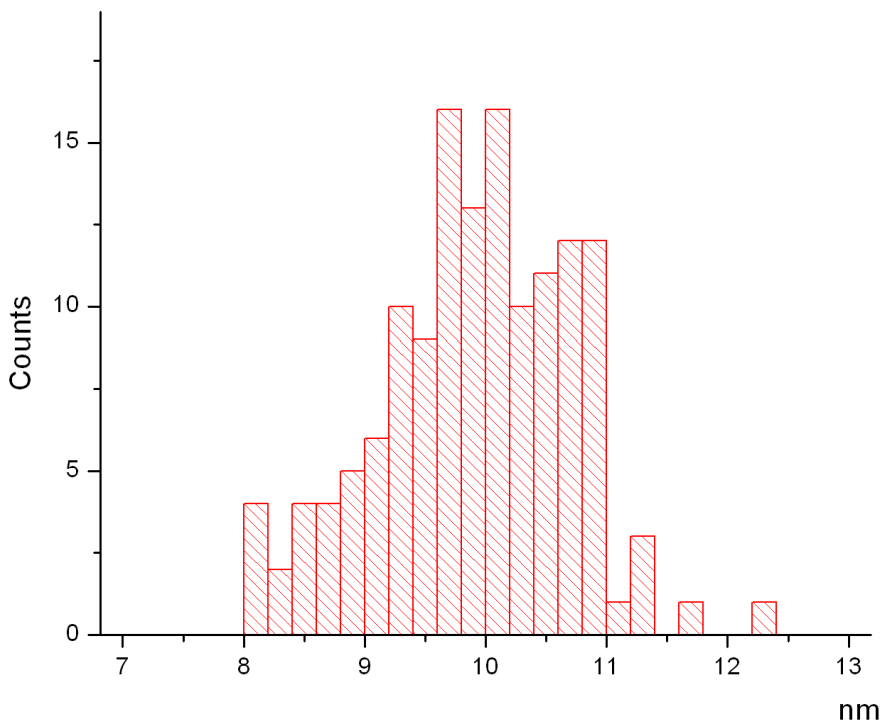


**Figure 10.** Measured effective particle diameter distribution for the RM 8011 (nominally 10 nm)

## 7. References


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5. NIST SEM Pitch Metrology freely available software is available from the Nanometer-Scale Metrology Group (andras@nist.gov)
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8. ISO 13322-1:2004. Particle size analysis - Image analysis methods - Part 1 http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=38664

## 8. Abbreviations

| | |
|---|---|
| AFM | atomic force microscopy |
| APDMES | aminopropyldimethylethoxysilane |
| C | Celsius |
| $H_2O$ | water |
| HEPA | high efficiency particulate air |
| keV | kiloelectron volts |
| kV | kilovolts |
| mL | milliliter |
| mm | millimeter |
| ms | millisecond |
| µm | micrometer |
| µs | microsecond |
| NCL | Nanotechnology Characterization Laboratory |
| $NH_4OH$ | ammonium hydroxide |
| NIST | National Institute of Standards and Technology |
| nm | nanometer |
| Pa | Pascal |
| RM | Reference Material |
| SEM | scanning electron microscope |
| Si | silicon |
| SRM® | Standard Reference Material® |