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Improved Array Method for Size Calibration of Monodisperse Spherical Particles by Optical Microscope*

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Key Words:

- Polystyrene
- Standard Reference Material
- Uncertainty
- National Bureau of Standards (NBS)
- National Institute of Standards and Technology (NIST)
- Community Bureau of Reference (BCR)
- NIST Traceable
- Optical Microscope

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ABSTRACT

Monodisperse or highly uniform spheres, when placed on a flat surface in a liquid medium, align themselves into systematic hexagonal arrays characterized by straight rows of particles. Using an optical microscope, the length of a row can be measured and divided by the number of spheres in the row to calculate the average diameter of the spheres. Limitations of the traditional array methods have been avoided by improved sample preparation methods and careful selection of measurement rows. Using the improved method, a series of monodisperse spherical particles from 0.5 to 40 micrometers (µm) was calibrated and certified with a stage micrometer calibrated by the National Institute of Standards and Technology (NIST).

1. INTRODUCTION

When placed on a flat surface in a liquid medium, monodisperse or highly uniform spheres align themselves into systematic hexagonal arrays characterized by straight rows of particles. Using a calibrated optical microscope, the length of a row can be measured, then divided by the number of spheres in the row to calculate the average diameter of the spheres.

Array methods for determining the mean diameter of spherical particles have been in use at the authors' laboratory since 1977. The methods were developed because of the difficulty of determining the edge of spherical particle images with high precision as shown in *Figure 1*. When the spheres are in contact in a straight line on a flat surface, the uncertainty of defining the outside edge of the first and last particle in an array is the same as for both edges of a single particle. When the uncertainty is divided by the number of spheres in the row, the edge uncertainty per sphere becomes very low, greatly improving the accuracy of the mean size determination. *Figure 2* shows a typical array

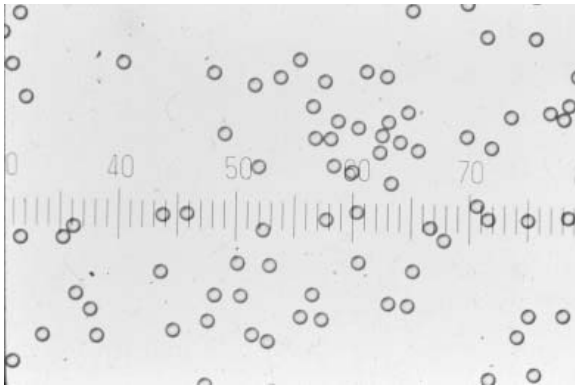

Figure 1. Typical edge images for 9.87 µm spheres, 8 µm per division.

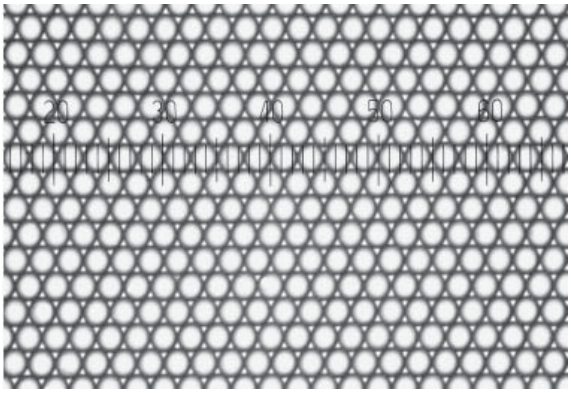

Figure 2. 9.87 µm spheres in arrays, 4 µm per division.

Other laboratories have also used array methods successfully[1]. Kubitschek[2] and Hartman[3,4] have described errors in previous methods which can be overcome with the techniques we have developed. When the mean diameter of monodisperse particles is of primary importance, rather than the size distribution, the array method is a convenient and practical method. This report describes our method and gives the results of the measurement of selected reference standards from 0.46 to 40 µm.

2. EQUIPMENT AND METHODS

2.1. Microscope

The microscope used in this work is an Olympus BHA. It has 15x eyepieces equipped with an eyepiece reticle, and objectives of 10, 20, 40, 60 and 100x magnification.

2.2. Stage Micrometer - Primary Standard

The primary calibration standard is a stage micrometer calibrated for 31 intervals by laser interferometry by the National Institute of Standards and Technology (NIST)[5]. The uncertainty of the micrometer calibration, from NIST Report #5524, is less than 0.00004 mm (0.04 µm) for lengths less than 0.2 mm, the longest length used to calibrate the eyepiece reticle. The micrometer was calibrated at 20°C and has a thermal coefficient of expansion of 8.5 parts per million per °C. The maximum error due to thermal expansion is 0.004%. The micrometer is 2 mm in length divided in 200 divisions, with line widths of 2 µm, and sharp line edges as shown in *Figure 3*.

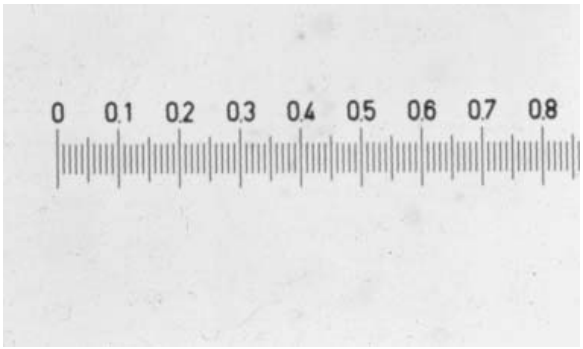

Figure 3. The NIST-calibrated stage micrometer, 10 µm per division.

2.3. Verification Standards

Our own in-house size standards and several certified particle size standards from NIST and from the Community Bureau of Reference (BCR)[6] were used as verification standards. They were measured for spherical diameter using the improved array method. The three BCR Standard Reference Materials analyzed are BCR #165A (2.223 µm), BCR #166A (4.821 µm), and BCR #167A (9.475 µm), calibrated by the optical array method. The three NIST Reference Materials are SRM #1690 (0.895 µm), SRM #1960 (9.89 µm) and SRM #1961 (29.64 µm)[7].

The eight Nanosphere size standards were calibrated by transmission electron microscopy (TEM) using the internal standard method[8,9] with SRM #1690 (0.895 µm) as the reference standard.

2.4. Calibration

The microscope eyepiece reticle was calibrated by measuring intervals on the NIST-calibrated stage micrometer with the eyepiece reticle (*Figure 4*). It is critical that the eyepiece reticle be well focused for the microscope operator, and that the eyepieces of binocular microscopes be carefully focused to the operator's eyes.

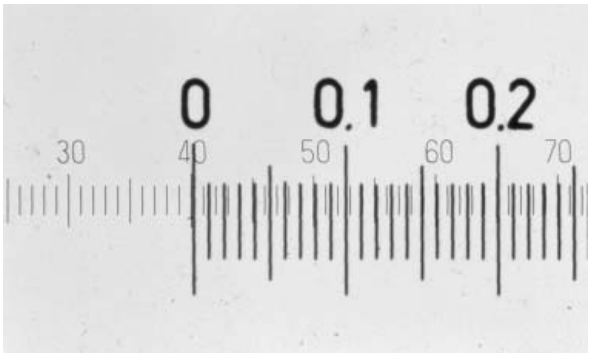

Figure 4. The stage micrometer through the eyepiece reticle, 8 µm per reticle division.

To minimize the effect of spherical aberration, only the central 20% of the eyepiece reticle, which has no apparent distortion, was calibrated. No lengths were measured at more than 25 divisions (<20% of the field), as beyond this point, the field is not optically flat. In general, the reticle should be calibrated as close as possible to the length of the array being measured.

2.5 Array Preparation

There are several methods of preparing measurable arrays, but the general method involves inducing the spheres to array in monolayers by drawing the microsphere suspension between microscope slide and cover-glass by capillary action. Anything that disturbs this smooth flow will interrupt array formation. If the array formation is too slow, the microspheres will array loosely, making them appear larger than they actually are. This can be detected by measuring the array in two different directions; if there is any variation, the section of arrays in question should not be used. If the microspheres array too fast, they will pack too tightly, and not all of them will be in contact with the slide. This produces arrays that appear striated when slightly defocused, and will show an average diameter smaller than the actual diameter.

The greatest problems in producing measurable arrays are flocculation, or the presence of clumped particles or large spheres. These can cause the microspheres to array in multilayers. Near-size large and small particles can make holes or gaps in the arrays, causing the rows to crack or bend. Preparation of good, measurable arrays requires microsphere suspensions that contain a minimum of large or small outliers or clumps of particles, and have proper dispersing agents to prevent flocculation during array formation.

2.6. Row Selection

Rows were selected that were in flat monolayer arrays, without large or small particles, cracks or gaps. They were perfectly straight when compared to the eyepiece

reticle, and were at least 10 divisions long whenever possible. Row lengths were measured on the eyepiece reticle by placing a reticle line exactly between two beads and counting the number of beads until the edge of a bead corresponds closely with another line. The length (to the nearest tenth of a division) and the number of spheres in the row were recorded. At least 9 rows were measured for each sample. The row lengths can be measured directly by the microscope operator or photographed for later analysis.

These values were entered in a double precision computer program created specifically for the array method which automatically adjusts for the scale calibrations. The mean is calculated as the sum of the row lengths in micrometers divided by the total number of spheres measured. *Figure 5* shows a typical row with the eyepiece reticle in place.

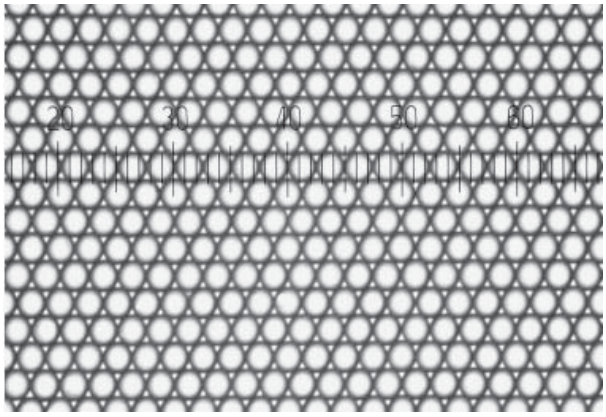

Figure 5. 9.87 µm arrayed spheres through the eyepiece reticle, 4 µm per division.

2.7. Analysis of Uncertainty[10]

The total uncertainty is the sum of the random measurement error and the calibration uncertainty (Table 1). The calibration uncertainty was calculated as the sum of the stage micrometer calibration uncertainty (from NIST report #5524) and the estimated uncertainty of determining the edge of the stage micrometer lines by the microscope operator.

To determine the random error of the measurements, the mean diameter of each row measurement was considered as one determination. The precision of the measurements is the standard deviation of the mean diameters for each individual row. Errors in locating the edges of the spheres are included in the row-length variation.

Error Source	Uncertainty Amount
A. Calibration Uncertainty	
1. Uncertainty in the Stage Micrometer	0.05 µm per measurement
2. Location of Micrometer Line Edges	0.17 µm per measurement
(5% of 1.7 µm width x 2)	
Total Calibration Uncertainty	0.22 µm per measurement
3. Average spheres per measurement	14 spheres
4. Calibration error per sphere	0.016 µm per sphere
(0.22 µm/14 spheres)	
B. Random Measurement Uncertainty	
5. Standard Deviation of measurements	0.040 µm per sphere
C. Total Uncertainty of the Mean Diameter	
6. Sum of A-4 and B:	0.056 µm per sphere

Table 1. Sources of Uncertainty for 10 µm Spheres

3. RESULTS

The expected values and the values observed by array method for the certified reference standards are summarized in *Table 1*. There was no bias observed, meaning that the systematic error was not significant. The average percent differences between the observed and expected values, 0.11%, can be considered random or measurement error. The measured value was within the uncertainty of the certified value for the standards in all cases. *Figure 6* is a graph of the expected vs. observed values.

Reference Material	Certified Diameter (µm)	Array Value	Difference	
			(nm)	(%)
3450A (TEM)	0.460 ± 0.004	0.458 ± 0.019	-0.002	-0.43
3500A (TEM)	0.496 ± 0.004	0.497 ± 0.023	0.001	0.20
3600A (TEM)	0.597 ± 0.005	0.597 ± 0.017	0	0
3700A (TEM)	0.705 ± 0.006	0.706 ± 0.021	0.001	0.14
3800A (TEM)	0.798 ± 0.007	0.799 ± 0.017	0.001	0.13
NIST SRM 1690	0.895 ± 0.008	0.895 ± 0.008	0	0
4009A (TEM)	0.993 ± 0.021	0.992 ± 0.017	-0.001	-0.10
BCR 165A #3	2.223 ± 0.013	2.224 ± 0.032	0.001	0.04
BCR 166A #4	4.821 ± 0.019	4.821 ± 0.036	0	0
BCR 167A #1	9.475 ± 0.018	9.471 ± 0.062	-0.004	-0.04
NIST SRM 1690	9.89 ± 0.04	9.896 ± 0.066	0.006	0.06
NIST SRM 1690	29.64 ± 0.06	29.58 ± 0.06	-0.06	-0.20

Table 2: Comparison of the Array Method with Certified Diameters of Reference Standards

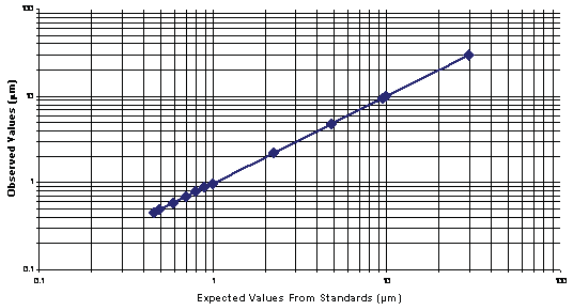

Figure 6. Array Method: Observed vs. Expected Values for Standards.

4. CONCLUSIONS

Although limited primarily to the measurement of monodisperse microspheres, the array method offers improved mean size analysis compared to most one-by-one particle sizing methods, provided the arrays are measured by the recommended procedures. It correlates extremely well with more sophisticated and complicated methods for calibrating particle size standards, can be NIST traceable, and is relatively easy to perform. Using the improved array method, a new series of particle size standards from 1.0 to 100 µm has been calibrated and certified by the authors' laboratories.

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10. The original publication of this paper pre-dates NIST Technical Note #1297, therefore the uncertainty calculation follows the guidelines in place at the time instead of the TN1297 / ISO GUM methods.

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