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Supplementary material

Size and shape distributions of primary crystallites in titania aggregates

Table of contents

Note: corresponding authors are identified for each element of Supplementary Material.

S1 Reporting template

Eric A. Grulke
University of Kentucky, Lexington, KY, USA
Corresponding author: eric.grulke@uky.edu

Table 1 shows a detailed reporting template that can be used to summarize metadata for intra- or interlaboratory studies. The sections are: report, sample preparation, instrument factors, image capture and particle analysis, and data analysis. The template is intended to be flexible; not all entries are appropriate for all samples, and entries can be added if desired.

Table S1 Example reporting template
Inserts in light gray are example responses

Report

Project element	Response
Study title	
Study objective	
Laboratory	
Author	
Date submitted	

Sample preparation

Item	Response
Sample ID	
Date received	
Substrate	
Sample type	Powder mounted on a lacey carbon grid
Sample condition	
Sample treatment	
Sample division/splitting	
Grid pretreatment	
Particle deposition	
Incubation	
Washing	
Staining	
Drying	
Substrate	Type of substrate, substrate pretreatment,…
Placement method	
Drying method	

Instrument factors

Item	Response
Organization	
Operator	
Analysis Dates	
TEM instrument manufacturer	
Instrument model	
Operating voltage	
Beam current settings	
Objective lens excitation	
Diffraction aperture	
Calibration standards	
Calibration procedure	
Most recent calibration date	

Image capture and particle analysis

Item	Response
Image capture	
Software/method	Software/manual or automated acquisition
Measurement conditions	Magnification, nm/pixel, frame size, signal-to-noise ratio
Descriptors retained	Itemize all measurands analyzed
Particle analysis	
Thresholding	Auto thresholding (ImageJ)
Minimum particle area (> 200 pixels)	
Number of frames	37
Total number of particles	621

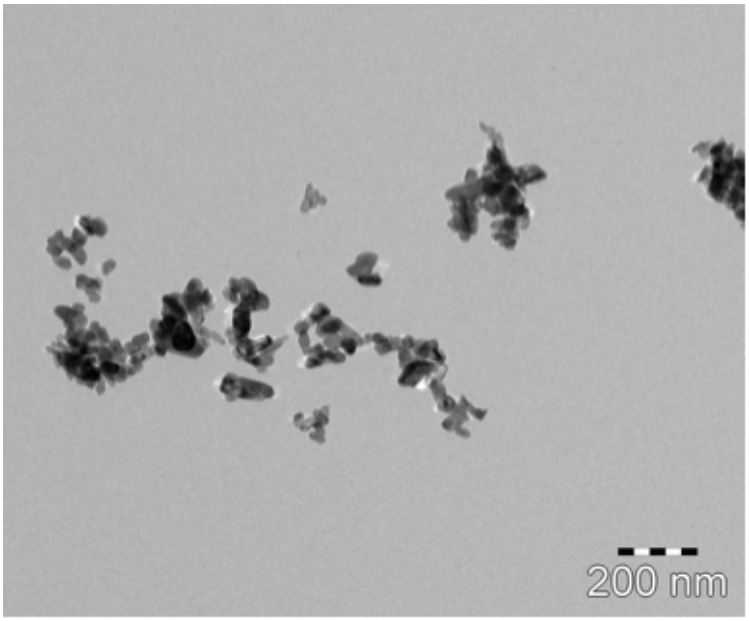
Data analysis

Item	Response
Raw data triage	
Software/method	
Detection of touching particles	Report descriptors, ranges for detection and differentiation
Detection of artifacts	Report descriptors, ranges for detection and differentiation
Retained particles	Report descriptors, ranges for detection and differentiation
Average yield, %	Report the % of particles retained
Other triage steps	None
Repeatability and/or reproducibility	
Software/method	Software/ANOVA; bivariate analysis; other
Repeatability (or reproducibility if ILC)	Report p-value for grand mean analysis; % similar p-values for pairwise analysis
Descriptor selection	Report method for description selection, if applicable
Fitting distributions to data	
Software/method	Software/nonlinear regression; maximum likelihood; other
Preferred reference model	Report normal, lognormal, Weibull, or other distribution
Parameter values	Report estimates and standard errors, C_v %
Parameter residual standard error	Report descriptor parameter residual standard error if computed
Measurement uncertainty	
U_{ILC}	Report measurement uncertainty
Residual deviations; correlations	
Software/method	
Residual standard deviation	Report if computed
Plots: residual deviations, quantile, other	Report residual deviation plot, quantile plot showing range over which the model fits the data
Correlations	Report correlations developed between descriptors

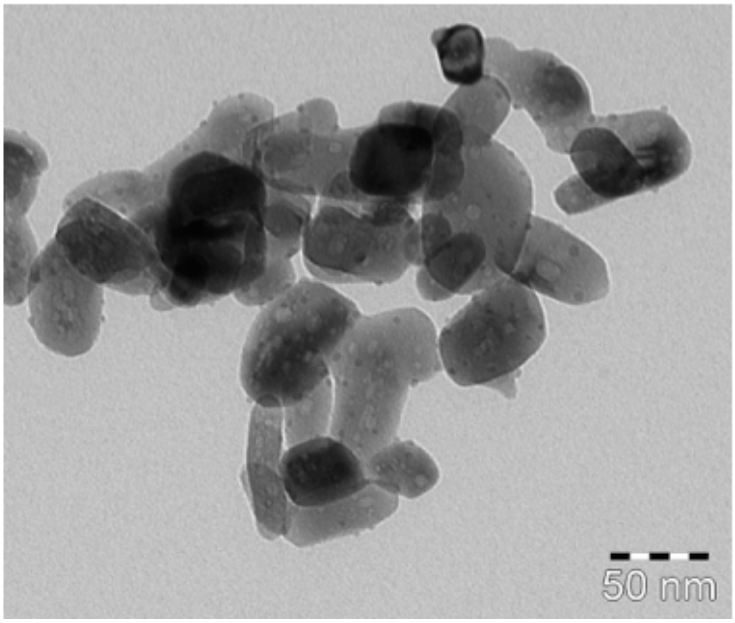
S2 Sample preparation

Kazuhiro Yamamoto[a]
[a]National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan,
Corresponding author: k-yamamoto@aist.go.jp

TiO_2 dispersions



TiO_2 particles (25 mg/ml) were dispersed in an aqueous solution of sodium hexametaphosphate, a mixture of polymeric metaphosphates that is commonly used as a sequestrant and a deflocculant for clay particles ($(NaPO_3)_6$; 1 mg/ml). The solution was ultrasonicated for one hour to fracture titania aggregates to submicron particles, resulting in stable TiO_2 dispersions (Figure S1a). A coating of the sodium hexametaphosphate is not visible at this magnification. However, small spheroidal crystals of the sodium hexametaphosphate, about 3-5 nm in radius, can be observed on the surface of the primary crystallites. These would be ignored during manual tracing. As this is a commercial sample, it also contains some small seed crystallites of titania, which appear as square or rectangular shapes on the surfaces of the primary crystallites.



Large aggregates with sizes greater than 900 nm (as detected by laser diffraction) are reduced to aggregates with sizes between 100 – 300 nm (Figure S1b). Smaller aggregates distributed over a relatively flat support have similar heights, making it easier to get clear in-focus images of primary crystallites.

Phosphate salts appear to aid the dispersion of titania aggregates in water dispersions. However, phosphate salts are also known to dissolve titanium dioxide and, therefore, can complicate image analysis. When phosphate salts are used, it is important to mount the sample immediately after sonication in order to reduce possible effects of salts on the particle morphology.
crystallites.

Figure S1a TEM of titania aggregates dispersed from an aqueous sodium metahexaphosphate solution

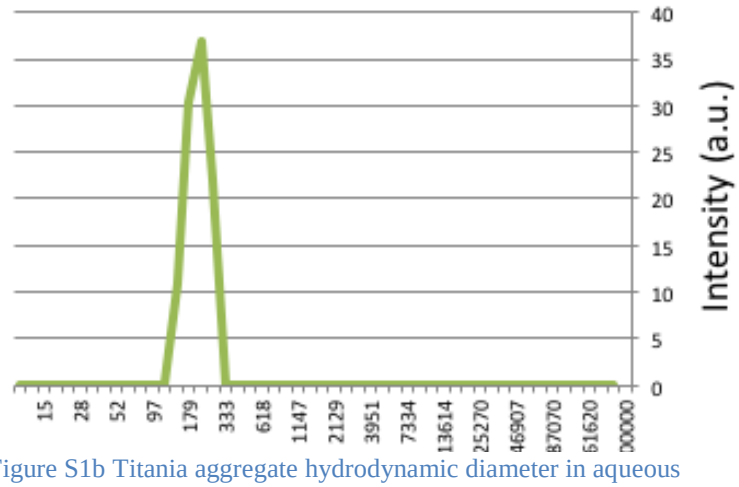


Figure S1b Titania aggregate hydrodynamic diameter in aqueous sodium metahexaphosphate solution

Other choices of phosphate salts are possible. Figure S2a shows titania aggregates from aqueous solutions containing sodium dihydrogen phosphate (NaH_2PO_4). In this case, the aggregates distribution is bimodal when analyzed by dynamic light scattering and the aggregate size ranges from ~ 100 to 1,500 nm (Figure S2b). The larger aggregates have greater heights above the flat support and make it more difficult to image the primary crystallites.

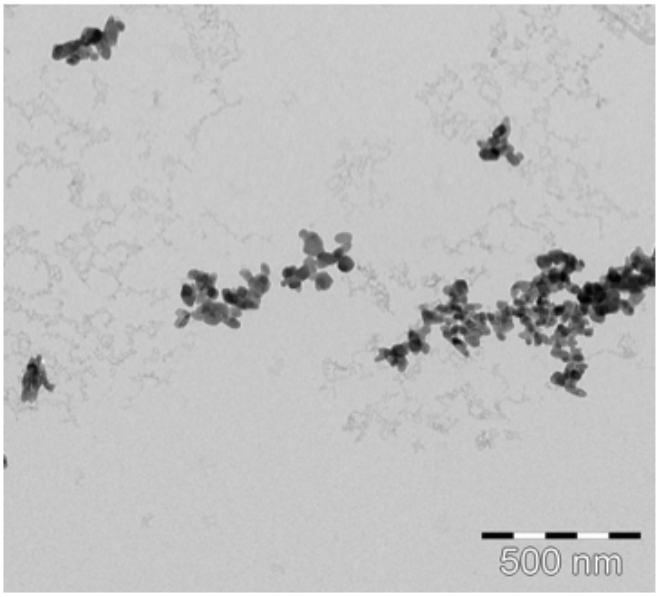


Figure S2a TEM of titania aggregates in aqueous sodium dihydrogen phosphate solution

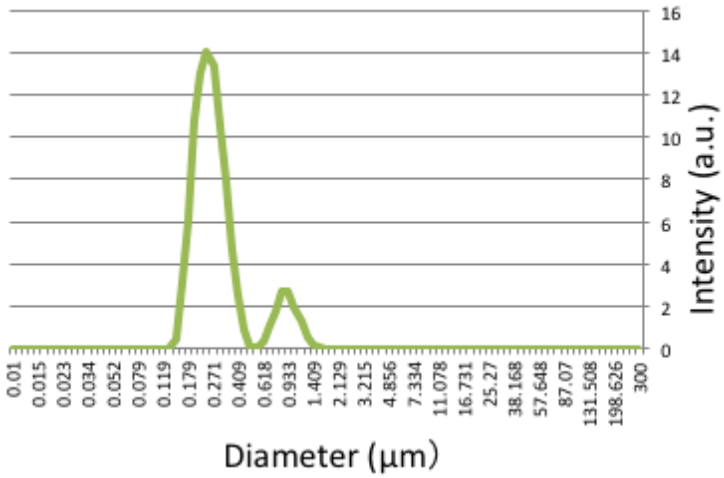


Figure S2b Titania aggregate size in aqueous sodium dihydrogen phosphate solution

TEM grid preparation

Copper metal TEM grid with amorphous carbon support membrane was used. The support membrane surface of a TEM grid is made hydrophilic using a glow discharge cleaning system, similar to a plasma cleaner. Such systems are commercially available. Filter paper is placed on a hot plate that has been warmed at 100C, and the hydrophilized support membrane is placed on the top of this. 15μL of the TiO_2 dispersion liquid is collected using a micropipette, and dripped onto the support membrane on the hot plate. The TEM grid with support membrane is dried on a hot plate.

S3 Instruments and protocol factors

Kazuhiro Yamamoto[a]
[a]National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan,
Corresponding author: k-yamamoto@aist.go.jp

Operating conditions

Tables S2 and S3 list the instruments used in this study and the protocol factors. ISO 13322-1:2004 [1] provides guidance on the operating conditions for electron microscopes used for particle size imaging. Specific steps include: adjust the desired peak signal level using the image contrast mode, set the accelerating voltage according to the material to be imaged, use the sample working distance specified by the electron microscope manufacturer for high resolution imaging, mount the sample flat on the specimen holder with the stage tilt set to zero, switch off dynamic focus and tilt correction, select the operating magnification using the reference in Annex B, ISO 13322-1, include an internal reference length in all images, and align the instrument according to the manufacturer's procedures. It is important to obtain images at the eucentric height and at magnifications used explicitly for the instrument's calibration.

Table S2 Instruments used in this ILC

Laboratory	Instrument	Model	Accelerating voltage
AIST	TEM	EM922 (Carl Zeiss)	200kV
AIST	STEM	JSM-7100F (JEOL)	30kV
BAM	TEM	JEM-2200FS (JEOL)	200kV
BAM	STEM	Supra40 (Carl Zeiss)	10kV
DuPont	TEM	JEM-2000FX (JEOL)	200kV
KRISS	TEM	Tecnai F30 (FEI)	300kV
NMIAU	TEM	JEM-2100 (JEOL)	200kV
FDA	TEM	JEM-1400 (JEOL)	80kV
Ishihara	TEM	H-7000 (HITACHI)	100kV

NIOSH	TEM	JEM-1220 (JEOL)	80kV
TAYCA	TEM	JEM-1230 (JEOL)	80kV

Calibration protocols

Kazuhiro Yamamoto[a]
[a]National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan,
Corresponding author: k-yamamoto@aist.go.jp

This section describes calibration standards, a general calibration procedure, and a protocol for setting TEM operating conditions for calibration. It includes example images of primary crystallites that are appropriate to trace manually as well as images of primary crystallites that do not have clearly distinguishable boundaries and which should not be analyzed.

Calibration standards

Since TEMs have a wide range of magnification and many operating modes, the actual magnification at any set of instrument settings may differ from the indicated magnification by up to 10%. Calibration of the instrument to a known length scale at optical conditions similar to those used for analysis is preferred. Standards should be run periodically to provide verification of correct instrument operation within manufacturer specifications and to validate measurement procedures. Typical examples are shown in Table S3. It is important not to use a standard sample after its expiration date.

Table S3 Calibration materials

Standard size, nm	Material	Source
2160 lines/mm	Gratings; linear or cross-grating	
20 nm	Polystyrene spheres (NIST-traceable)	NIST
2 – 20 nm	Colloidal gold	Various
< 2 nm	Crystals of catalase enzyme	

General calibration procedure

Figure S3 shows examples of the image quality based on aperture settings. The aperture should be chosen to provide good resolution of primary crystallite edges for tracing.

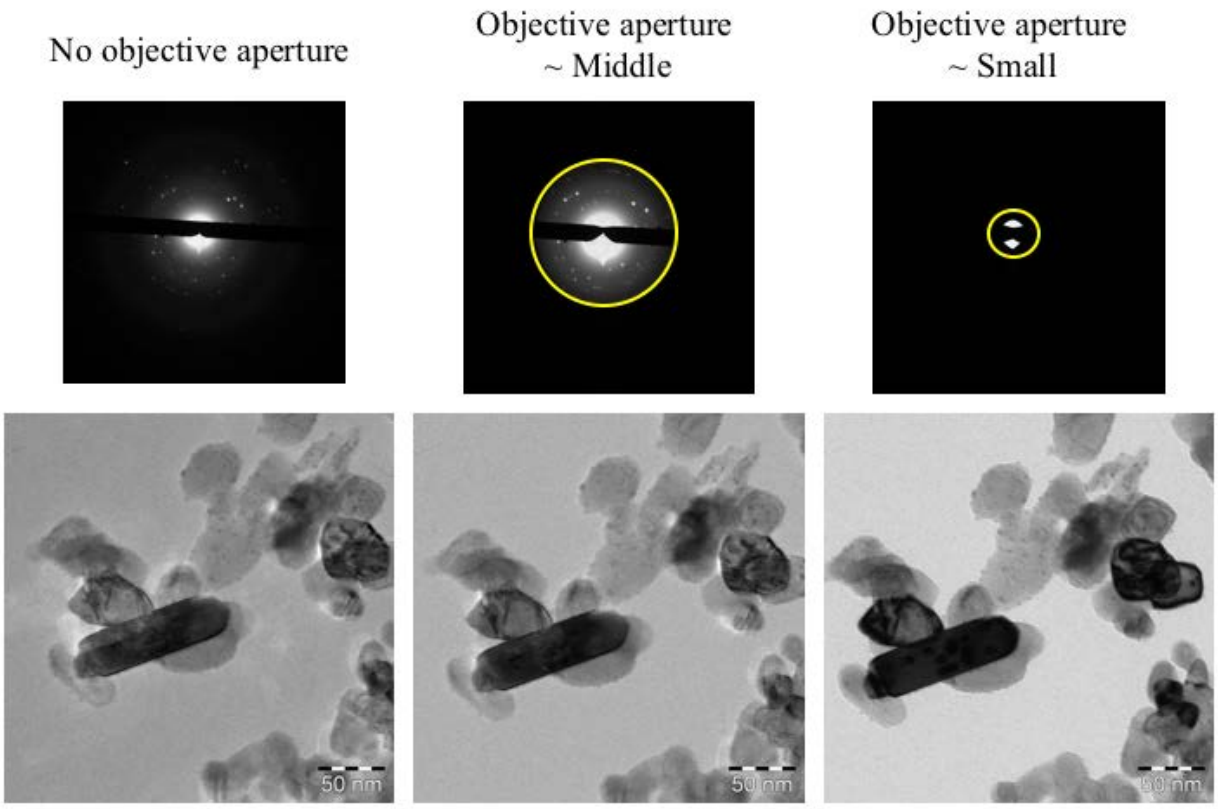


Figure S3 Effects of objective aperture selection in images

As the calibration is strongly depend on the electron optics condition, it must be done under optimum lens conditions using suitable reference materials (RMs). Then, the particle size measurement should be done under the same conditions of the calibration with RM. (e.g., objective lens current and the other lens parameters, and specimen height). The objective lens should calibrate to the eucentric axis using the "focus reset" or "focus calibrate" button. The specimen must be set to this position by adjusting the Z-axis. The "wobbler" functions can be used as the focus aid. When the sample is positioned on the eucentric axis, the image at the first image plane will have the minimum error in its length (see Figure S4). While the sample is positioned on the eucentric axis, tilting the sample around this axis is the same as tilting on first image plane, so the sample image doesn't move on the fluorescent screen. If the sample position is not at the eucentric axis, there will be errors in its measured dimensions. Both reference material calibrations and sample measurements must be done with the specimens positioned on the eucentric axis.

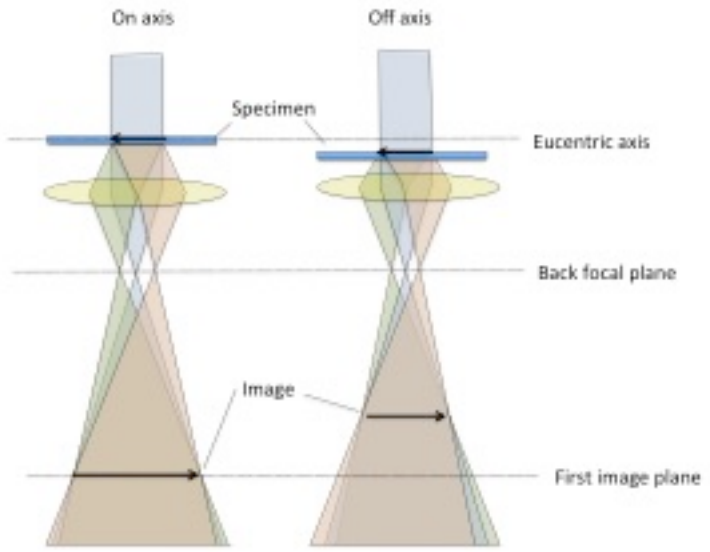


Figure S4 Example of scale size error due to the specimen plane not coinciding with the eucentric axis. Left hand side: proper alignment. Right hand side: sample displaced from the eucentric axis

If there is a wide range of primary crystallite sizes, TEM projection lenses will need to be switched to make accurate measurements across the entire size range of a particle distribution. The lens ranges will depend on the TEM model, for example, low magnification < 100k and high magnification > 100k. Within each range, linearity of scaling will be achieved. However, there are discontinuities between calibration lines for different lenses: scale calibration needs to be done for each projection lens used to acquire images. Figure S5 demonstrates such differences in scaling calibration lines. Details of scale calibration are provided in ISO 29301:2010 Microbeam analysis -- Analytical transmission electron microscopy -- Methods for calibrating image magnification by using reference materials having periodic structures [2]. Calibration equations change between each lens system, resulting in discontinuities (offsets) between the lines for different lenses.

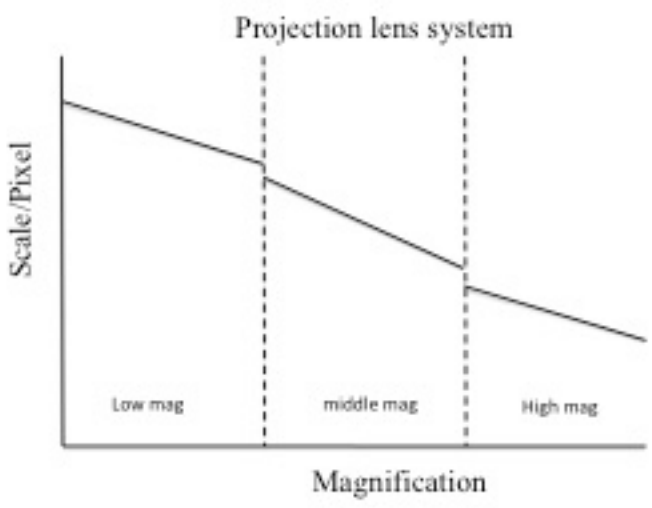


Figure S5 Linear scaling of magnification and scale for different projection lens systems

Setting TEM operating conditions for calibration

[Note: please see the procedure of ISO 29301:2010 [2]]

Set the operating condition of the TEM according to the following procedures to ensure, as far as possible, use of the same conditions.

a) Check that the degree of vacuum in the TEM column is lower than 10^{-4} Pa and stable.

b) The high voltage shall be applied and an appropriate time be allowed for it to stabilize.

NOTE Oil-filled 100 kV tanks take about 2,5 h; gas-filled tanks take about 45 min. Higher voltage instruments are normally operated with the high voltage continually applied, therefore a stabilization period is not usually required.

c) Use an anti-contamination device, if needed.

d) Select a specimen region of interest (ROI) for the calibration that is clean and free from damage, verify the eucentric height of the ROI, and adjust the height of the ROI, if necessary.

e) In order to minimize the effect of the magnetic hysteresis of the lenses, set the magnification of the TEM to the target value for calibration according to the same sequence, for example, adjust higher magnification than the target magnification at first, then set the target magnification after that.

f) Set the excitation of the objective lens to the desired reproducible value; the standard condition is recommended.

g) Adjust the specimen height to focus the magnified image projected on the fluorescent screen, the TV monitor or the personal computer (PC) screen.

NOTE If the TEM in question is not equipped with a specimen-height control function, this procedure can be omitted.

h) Correct astigmatism at a slightly higher magnification than the target value and adjust the accelerating voltage center. For example, if the target calibration is at a magnification of **x** 100k, set the magnification in the range **x** 150k ~ **x** 200k for alignment.

i) Switch the observation mode of the TEM to the selected-area electron-diffraction (SAED) mode or the convergent-beam electron-diffraction (CBED) mode from the image mode. Also, make sure that the objective aperture is removed.

NOTE For the SAED mode, it is necessary to insert a selected-area aperture over the area of interest of the specimen in order to project a selected-area electron-diffraction pattern on the viewing device (fluorescent screen/TV monitor/PC screen).

j) Adjust the condenser lens system to provide nearly parallel illumination conditions.

k) Align a low-index zone axis of the crystal parallel to the optical axis (i.e. zone-axis illumination), if the specimen is a single crystal, see Figure 2.

l) Insert the objective aperture, centering it about the electron optical axis. Also, switch the observation mode of the TEM back to the image mode.

m) Return the magnification to the target value of calibration, and set the excitation current of the objective lens to the standard exciting condition again.

n) Apply a relaxation function to relax the magnetic hysteresis of the objective lens, if the TEM has it.

o) Adjust the specimen height to focus the magnified image roughly.

NOTE If the TEM in question is not equipped with a specimen-height control function, this procedure can be omitted.

p) Adjust the fine focus by varying the exciting current of the objective lens.

NOTE If necessary, it is possible to use the Image Wobbler function for focusing the image.

q) Turn off the auto-focus correction function to the optimum under-focus condition linked with the Image Wobbler function, if the TEM is equipped with this function.

r) Adjust the illumination condition of the condenser lens system (spot size and brightness) with reference to the dynamic range of each detector to obtain image contrast in the whole dynamic range.

The condenser lens system should be operated under conditions that approach parallel illumination. Alternatively, they should be done under a condition where it is documented that the beam convergence no longer affects the image focus. Recording multiple images under varying degrees of beam convergence can help make this determination.

Instrument operation

Focusing conditions and specimen heights are very important to making size and shape measurements by TEM. An internal reference length is calibrated using calibration standards (Table S2). All particle size measurements must be done with the same defocus and specimen height conditions of calibration. Specimen height should be at the eucentric position. The Scherzer defocus condition is generally used. The zero defocus is defined using the Fresnel

fringe first, followed by using the Scherzer defocus. It is important to obtain images at the eucentric height and the same defocus condition at magnifications used explicitly for the instrument's calibration.

The microscope should be tuned for good contrast between background and particles. To achieve this, bright field imaging is usually preferred but dark field or STEM imaging modes were also acceptable. The Online Resource has images demonstrating the effects of objective aperture on image quality (Figure S3), alignment (Figure S4), linear scaling (Figure S5), examples of particles to capture (Figure S6) and not capture (Figure S7).

Each laboratory participating in this case study had a different electron microscopy (Table S1). There were differences between datasets with respect to the instrument type used, the number of particles for which data was reported, the number of frames containing these particles, the calibration method, the instrument resolution, and the software used for image analysis (Table S2).

S4 Image capture and particle analysis

Kazuhiro Yamamoto[a]
[a]National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan,
Corresponding author: k-yamamoto@aist.go.jp

Image acquisition.

It is important to acquire images with good contrast between the background and the aggregated particles. The guidelines for this ILC were: select operating conditions to minimize drift, collect images at an appropriate accelerating voltage, set the ratio of the image resolution to the average particle diameter to control measurement uncertainty, and ensure reproducible calibration settings by performing lens normalization (intended to minimize hysteresis) at the selected magnification for imaging. For a nominal particle diameter of 30 nm, a resolution of 0.5 nm/pixel gives an uncertainty of 1.6%. At this resolution, smaller particles would have larger measurement uncertainties. A well-aligned, stable TEM should be operated at a magnification that allows many particles to be visible in the field of view and ensures that each particle is recorded with a large number of pixels. For example, nanoparticles with D_{ave} = 50 nm, imaged a magnification of ~ 50,000 × and recorded on a CCD camera with pixel dimensions (square matrix) of 14 μm, would have a diameter of about 180 pixels. A CCD camera with 2048 x 2048 pixels would contain about 120 particles within the field of view of a single micrograph [3]. Enough micrographs should be recorded to image a minimum of 500 nanoparticles per sample from a minimum of 2 widely separated regions of the grid. Image recording times should be sufficiently long so that the average grey-scale ratio of particle to background is at least 5:1. Recording times also should be kept short (1 – 2 s) to minimize contributions from stage drift [3]. The number of particles measured will have a direct effect on the quality of the coefficients of the lognormal model fitted to the data. Therefore, the statistical requirements for the reported results will help determine the number of nanoparticles counted for each application.

Manual particle tracing

The primary crystallites of this commercial titania sample cannot be separated physically into individual nanoparticles. Manual particle tracing was used with the traced images being saved for each frame. Only those particles that have clear and distinguishable edges or boundaries were reported. This protocol assumes that all images were taken in digital format. Procedure steps were provided for the open source software, ImageJ (*http://rsbweb.nih.gov/ij/download.html).*
Figure S6 shows some possible selections of particles for capture. Yellow rectangles indicate three areas of an image. The top right rectangle shows a particle that has easily distinguishable edges, which is outlined in yellow in the next frame. The lower left rectangles shows additional examples of particles that should be captured; these are outlined in yellow in the following frame. Figure S7 enlarges the middle center rectangle; it shows an example of a particle that does not have clearly distinguishable edges and should not be captured. There is likely to be a line of fusion between the two segments of the blackened particle, but the line of demarcation is not obvious.

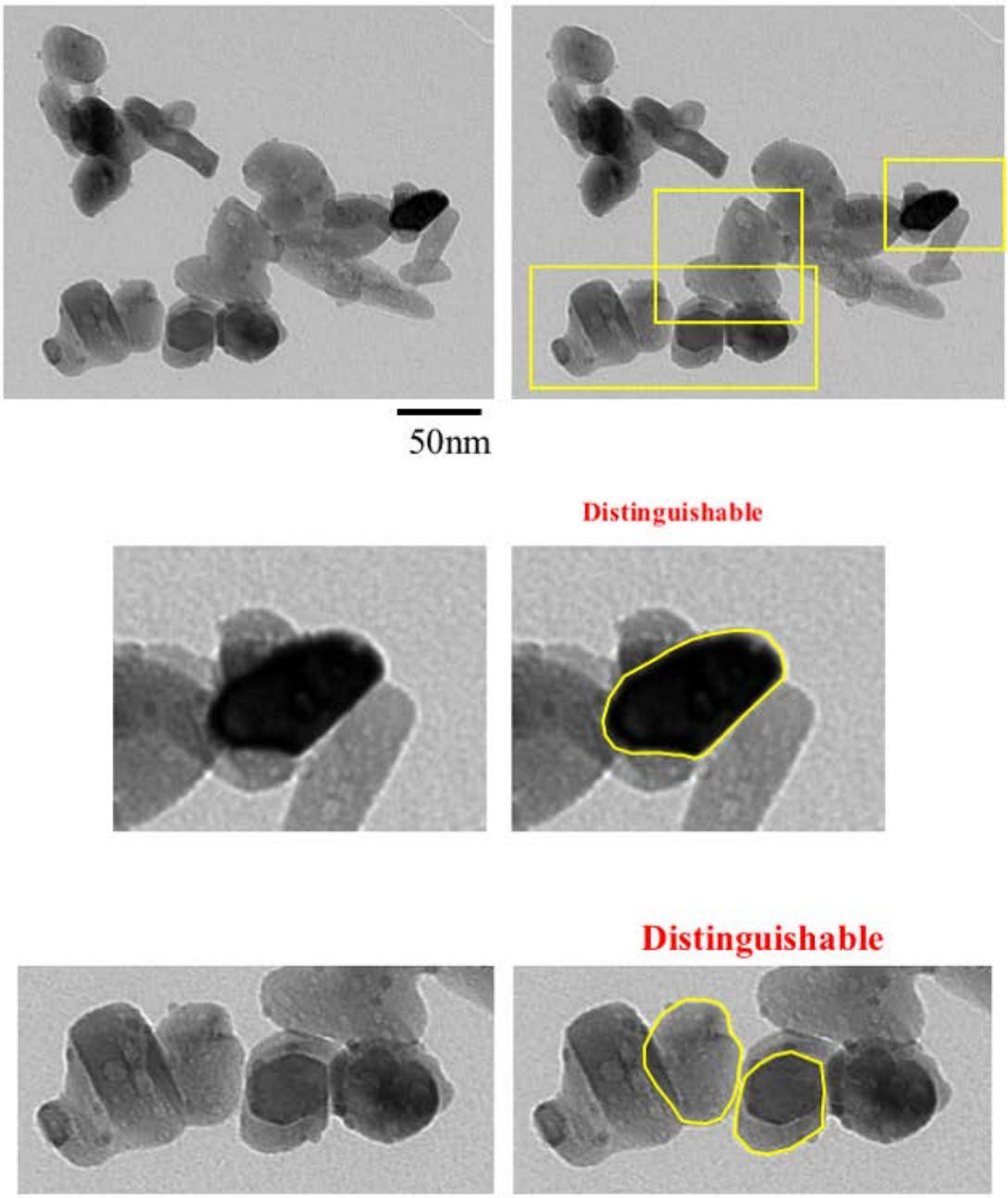


Figure S6 Examples of distinguishable primary crystallites

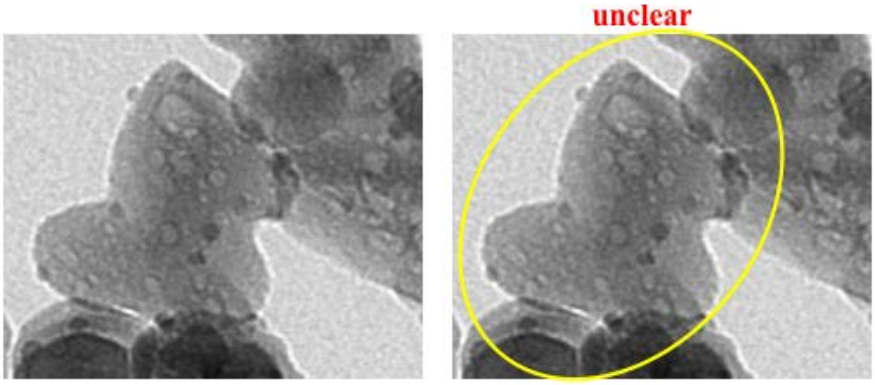


Figure S7 Example of a primary particle that should not be captured

Image analysis procedure using ImageJ

Aleksandr Stefaniak
U.S. National Institute of Occupational Safety and Health, Morgantown, WV
Corresponding author: boq9@cdc.gov

This section provides a generic image analysis procedure for ImageJ [4], freeware that is available from NIH (https://**imagej.nih**.gov/ij/). Follow the following steps to analyze particle images:

- Create working copies of all images/frames (preserve the original unmodified images)
- Open ImageJ and open the frame file
- Set the measurement scale using the scale bar or another measurement of pixel size, returning to the original scale prior to continuing
- Crop the image to remove scale bars and other image artifacts that might affect contrast or particle analysis
- Check and correct brightness and contrast to ensure that all images have histograms centered and wide enough to cover at least 80 % of the possible gray levels
- The thresholding operation may result in frame files with single pixel artifacts or poor image quality, e.g. rough particles or uneven background due to non-uniform electron beam illumination. In the case of the former, apply the despeckle and erode/dilate processes to remove these artifacts and save the changes. In the case of poor image quality, the operator could clean up the edges of particles or correct for uneven background by applying special filters. Assess the image transformation and save changes.
- Identify and manually outline primary crystallites with clearly defined edges, as described previously.

- Set the measurements desired, such as size and shape descriptors. For new or unknown samples, it is preferable to include as many descriptors as possible, since the most critical descriptors may not be identified at the inception of the process. In this case, additional descriptors would be perimeter and fit ellipse. These would be relevant for optical applications in which surface plasmon resonance is expected to affect application performance.
- Analyze the particles (ImageJ specific settings should include: show outlines, display results, include holes, and exclude on edges)
- Save each image file that shows particle outlines and their number sequence (filename.tif) and the spreadsheet (Results.xls, with all measurand values for all particles plus particle number and frame number).

S5 Descriptor distributions and definitions

Eric A. Grulke
University of Kentucky, Lexington, KY, USA
Corresponding author: eric.grulke@uky.edu

Descriptor distributions

Descriptor distributions generated from two-dimensional TEM images will be count-based or number-based. The error associated with the mean value of a particle size distribution has been shown to vary with the square root of 1/n, where n is the number of particles measured [5]. Therefore, imaging a large number of particles should reduce the error of the mean value of the distribution and the error associated with the distribution of the sample population. This protocol used 500 particles as the minimum number of particles to be reported. ISO 13322-1:2004 provides a measurement uncertainty analysis specifically for lognormal distributions, which would be a common model for size descriptors of nanoparticle systems. Other standards and guidelines [6] also address statistical uncertainty.

For a specific application, the target standard errors for the mean and standard deviation parameters of the distribution will determine the number of nanoparticle images required. The methods implemented for curve-fitting model distributions to particle size data in this protocol provide relative standard errors (RSEs) for each of the two descriptors (scale and width). Assuming that the fitted distribution provides a good description of the data, investigators can determine the effect of the number of particles analyzed on the RSEs for their samples, and adjust the number of particles counted to develop data sets with the needed accuracy or measurement uncertainty.

Descriptor definitions

These terms are referenced from: ISO 9276-6 Representation of results of particle size analysis – Part 6: Descriptive and quantitative representation of particle shape and morphology [7].

Size or scale descriptors

Area basis

A — projection area

Length basis

ECD — equivalent circular diameter based on area

$$ECD = \sqrt{\frac{4 \cdot A}{\pi}}$$

Feret — maximum Feret diameter; distance between parallel tangents; corresponds to the “length” of the particle

minFeret — minimum Feret diameter; distance between parallel tangents; corresponds to the “breadth” of the particle

Note 1: volume-equivalent diameter, x_V, and surface-equivalent diameter, x_S, are not included as they are three-dimensional descriptors

Proportion descriptors

Descriptors with values ranging from 0 to 1 are preferred. The aspect ratio provides macroshape information.

Aspect ratio ratio of the minimum Feret diameter to the maximum Feret diameter

$$aspect\ ratio\ = \frac{x_{Fmin}}{x_{Fmax}}$$

Mesoshape descriptors

Compactness degree to which the diameter of the particle is similar to that of a circle with the same area; ratio of the area-equivalent diameter to the Feret diameter

$$compactness = \frac{\sqrt{\frac{4 \cdot A}{\pi}}}{X_{Fmax}}$$

S6 Data analysis

Woodrow W. Burchett[j], Arnold J. Stromberg[j], and Eric A. Grulke[k]
[j]Applied Statistics Laboratory, University of Kentucky, Lexington, KY, USA
[k]Chemical & Materials Engineering, University of Kentucky, Lexington, KY, USA
Corresponding author: eric.grulke@uky.edu, +1-859-257-6097

ANOVA decision tree

Figure S8 shows a decision tree for analysis of variance (ANOVA) of ensemble or pairwise data. Ensemble analysis can be done to determine intralaboratory repeatability or interlaboratory reproducible. For example, ANOVA of one descriptor over all frames can be used to demonstrate intralaboratory repeatability; if $p > 0.05$, then all frames have mean values similar to that of the grand mean for the lab. ANOVA of one descriptor over all labs (for example, A_1 through A_n) can be used to demonstrate interlaboratory reproducibility; if $p < 0.05$, then at least one lab has a descriptor mean different from its ensemble grand mean. ANOVA results can be used to determine which descriptor measurements appear similar across all labs. If $p < 0.05$ for a specific descriptor in the ensemble test, pairwise ANOVA of all dataset pairs, such as dataset A_1 compared to dataset A_2, can be used to determine whether there are any pairs for which $p > 0.05$ for that descriptor. This approach provides additional information about descriptor reproducibility and the datasets themselves.

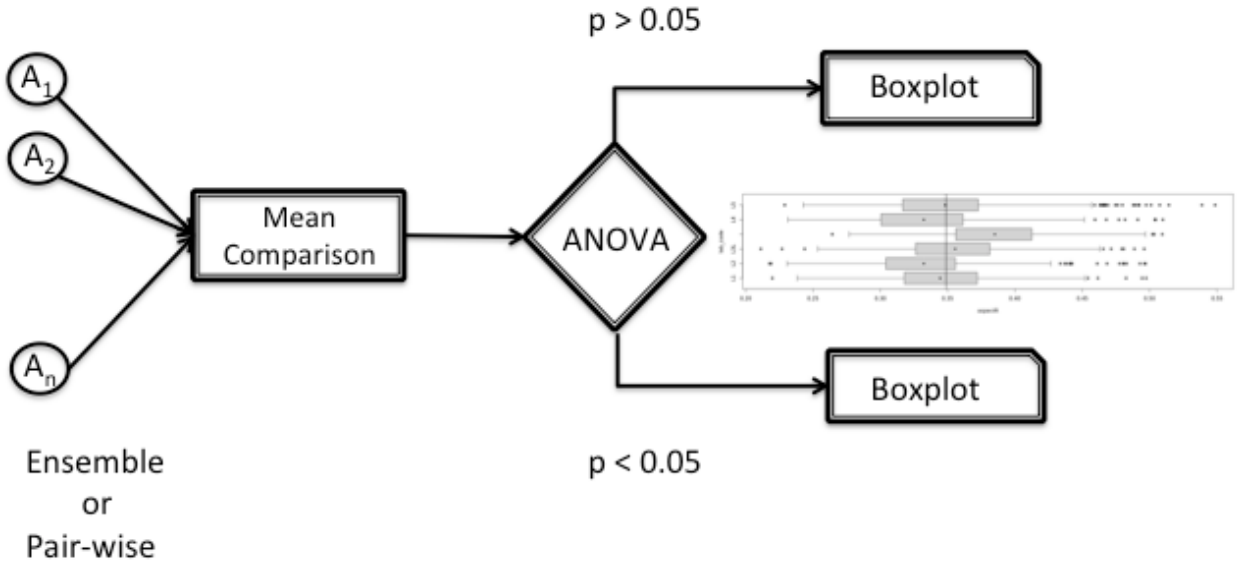


Figure S8 Decision tree for ANOVA analysis

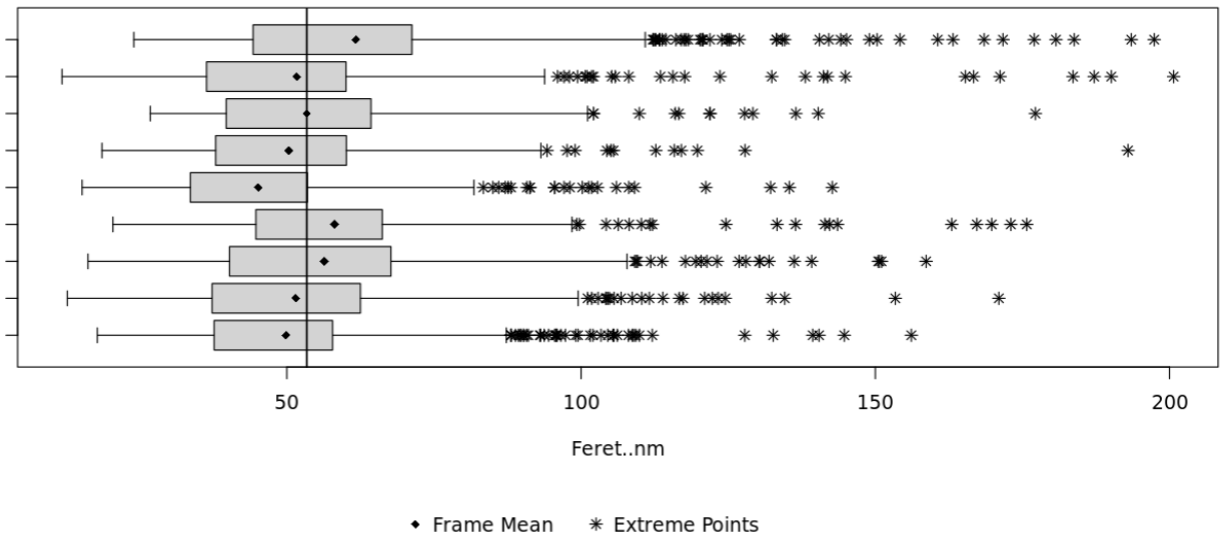


Figure S9 ANOVA boxplots showing the grand mean (vertical line) and individual lab means (black diamonds) for the primary crystallite Feret diameters

Figure S9 shows ANOVA Feret diameter boxplots for the ILC. The grand mean of the complete dataset is shown as the vertical line. The equivalent circular diameter (ECD) data is shown with a gray box plot for each laboratory. The box area represents ±1.5 σ about the mean, which is indicated by a point. In this case, eight of the nine subsets have means different from the grand mean. Only one lab has a Feret diameter mean value similar to the grand mean. "Extreme" points are shows as asterisks, but were not removed from the datasets. Table S6 shows the results of ANOVA analyses of the six descriptors using calibration method and software as the group variable.

Bivariate analysis decision tree

The decision tree for bivariate analysis [8] is shown in Figure S9. The bivariate analysis approach is a nonparametric test for equal distributions in high dimension. It tests the composite hypothesis of equal distributions when the distributions are unspecified and is implemented by conditioning on the pooled sample to obtain an approximate permutation test that is distribution free. Several types of two variable comparisons were considered in this work: empirical distributions (descriptor – cumulative distribution data) and descriptor-descriptor datasets (size-size, shape-shape, or size-shape). The Shiny App®, https://shiny.as.uky.edu/bivariate-fitting-app/, provides three choices of plots (heat map, contour plot, or scatter plot) plus reports the energy and p-value statistics. The bivariate analysis Shiny App is very useful for statistical comparisons of datasets, but does not provide metrics that can be used to compute measurement uncertainty of descriptors.

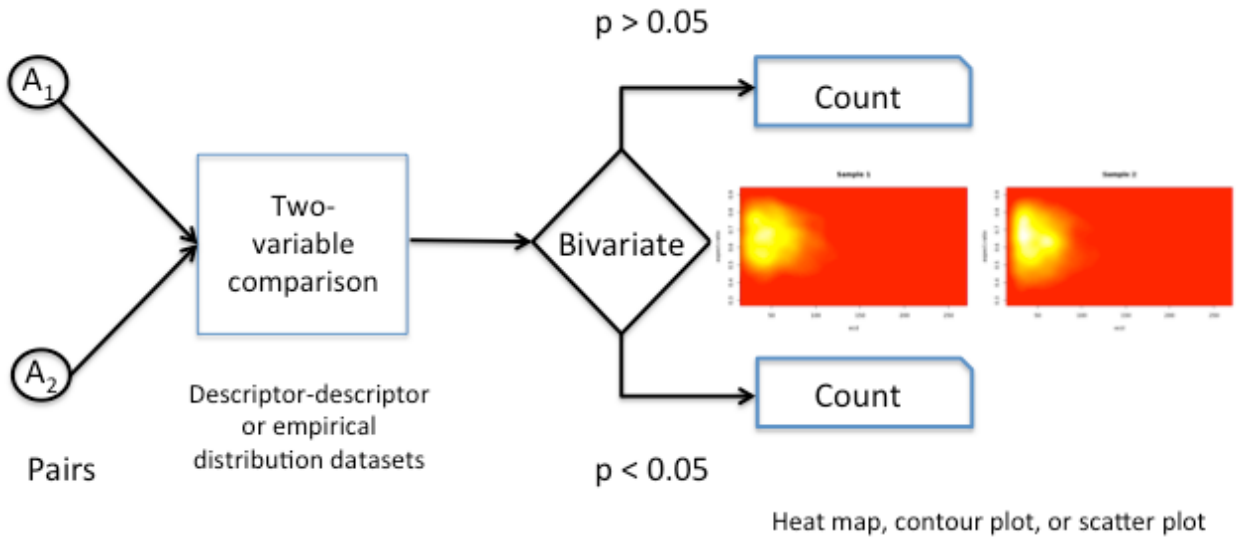
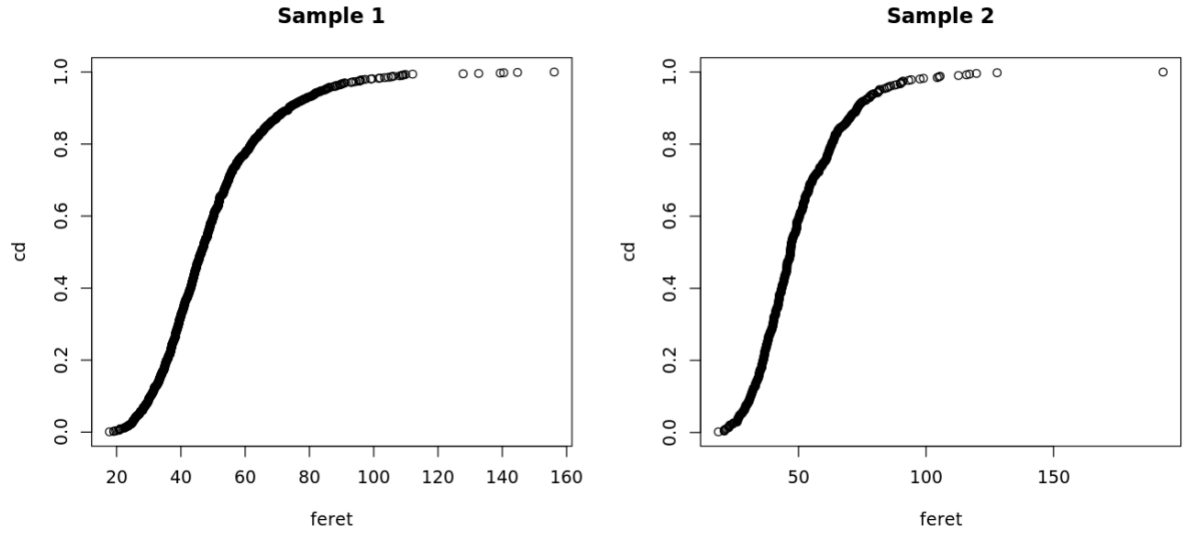


Figure S10 Decision tree for bivariate analysis

Figure S11 shows the bivariate analysis comparison of the empirical Feret cumulative distribution for Labs L1 and L3. This dataset pair has similar lognormal scale and width parameters and an ANOVA p-value > 0.05. As shown in the gray frame, the E-value for the pair is low (7.4) and the p-value (0.871) is greater than 0.05 for the bivariate evaluation.



E-test of Equal Distributions from Szekely & Rizzo (2004):

```
        Multivariate 2-sample E-test of equal distributions

data:  sample sizes 1033 519, replicates 1000
E-statistic = 7.3888, p-value = 0.8731
```

Figure S10 Bivariate analysis of Feret cumulative distribution for Labs L1 and L3

Fitting distributions to data decision tree

The model fitting decision tree is shown in Figure S11. Three different reference models can be selected: normal, lognormal, and Weibull. Commonly used model distributions require at least two parameters, one that describes scale and one that characterizes the width (or spread, or breadth) of the data. For clarity in this study, we define

"size" as a characteristic dimension of the particle and "shape" as the shape of the particle. The characteristic dimension or mean of a descriptor distribution is defined as its scale, while its standard deviation is defined as the width of the distribution. Scale and width are applied specifically to parameters estimated using distribution data. For example, the scale of a normal distribution would be its arithmetic mean while its width would be the standard deviation. Two fitting techniques, maximum likelihood estimates and nonlinear regression estimates, were used for each distribution. The Shiny App, https://shiny.as.uky.edu/curve-fitting-app/, provides several plots: the empirical histogram (the bin size can be varied), the empirical cumulative distribution, fitted model plots compared to data for the differential or cumulative distributions, and a residual deviation plot. The parameter estimates for scale and width are reported along with their standard errors. These can be converted to coefficients of variation and then to measurement uncertainties. Selection of preferred reference models was guided by considering three elements, the computed measurement uncertainty, the fit visualizations, and the residual deviations plots.

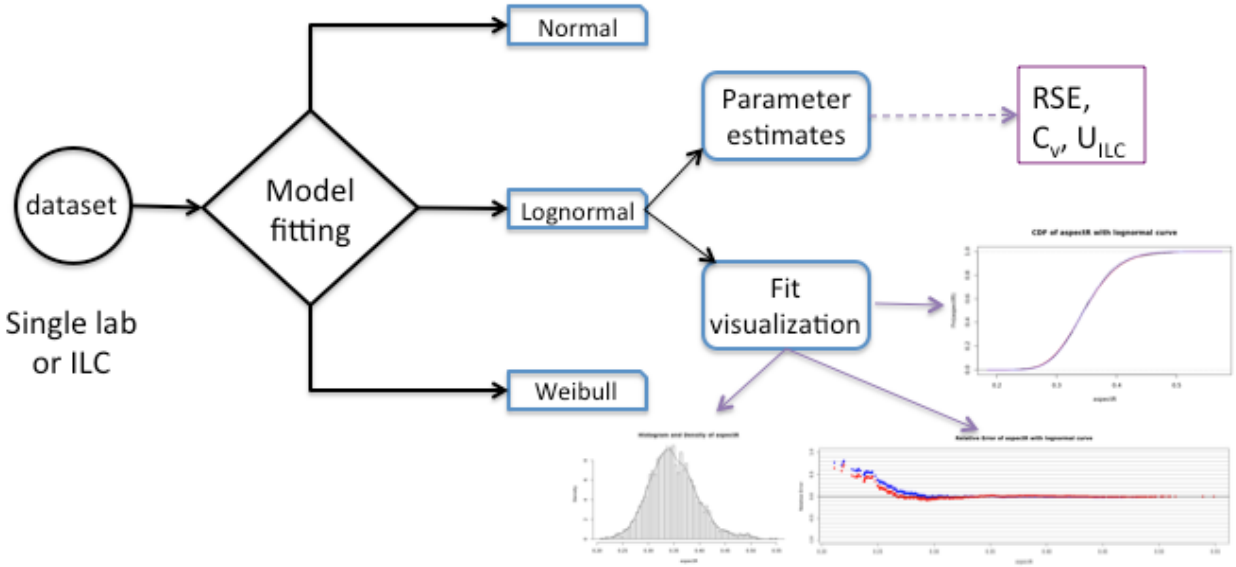


Figure S11 Decision tree for fittings distributions to data

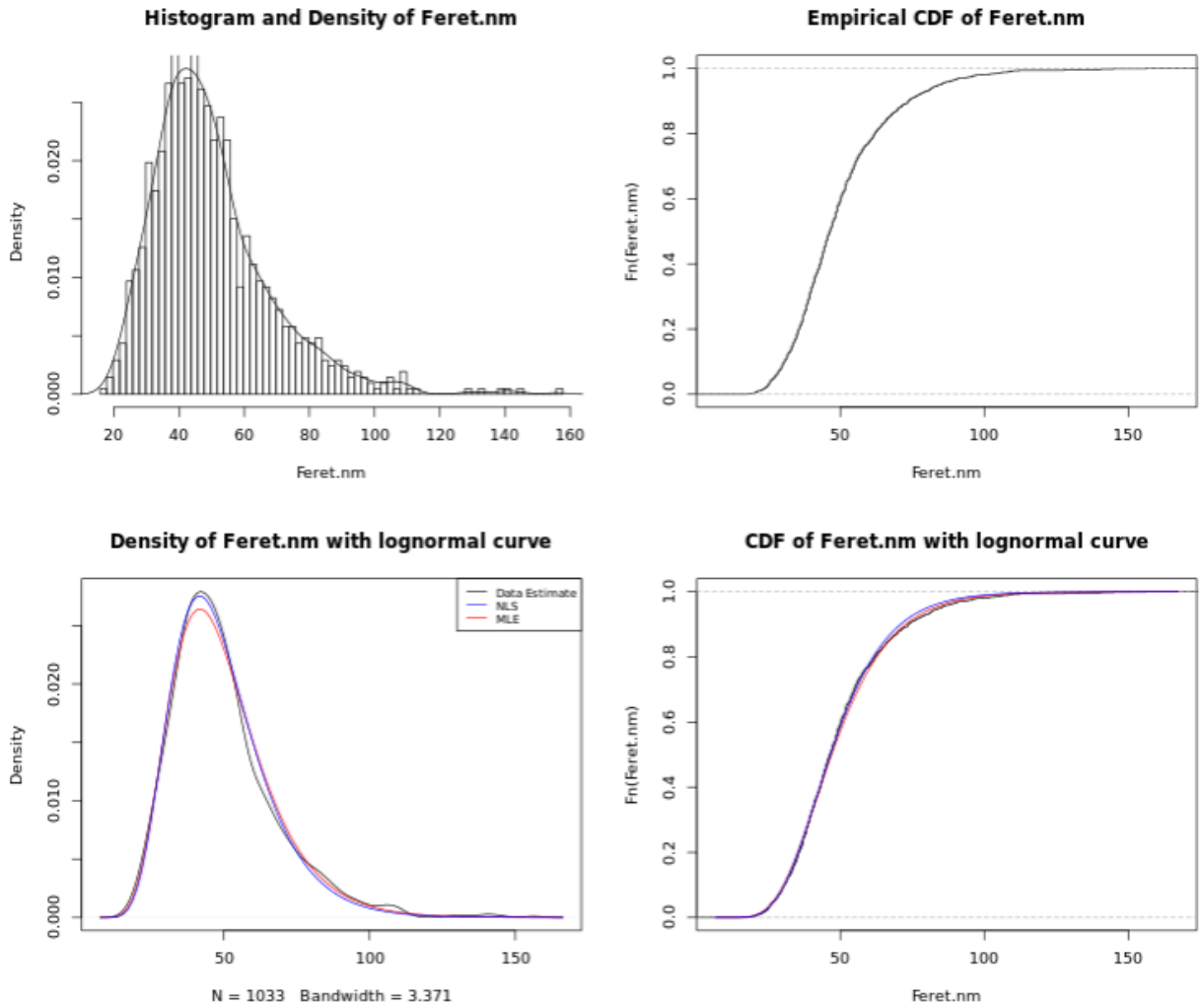
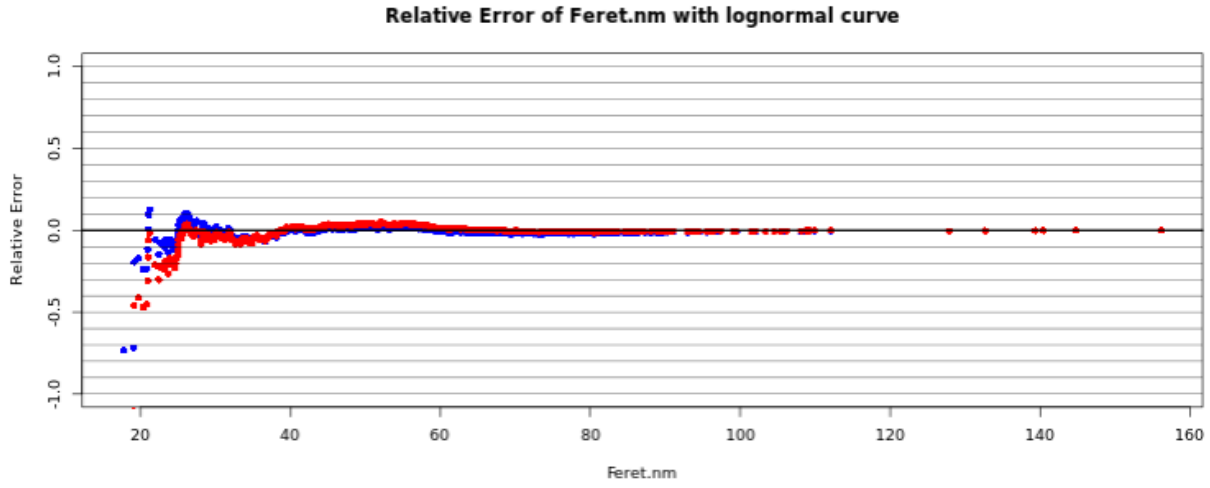


Figure S12 Lognormal model fitted to Feret descriptor data, Lab L1. Empirical histogram, top left. Empirical cumulative distribution, top right. Lognormal density distribution models, bottom left. Lognormal cumulative distribution models, bottom right.



Parameter Values:

	meanlog	SE	sdlog	SE
MLE	3.8500	0.0106	0.3401	0.0075
NLS	3.8406	0.0003	0.3281	0.0005

Figure S13 Residual deviations of the models to the data, Feret descriptor data, Lab L1. Maximum likelihood estimates (MLE), red circles. Nonlinear regression analysis (NLS), blue circles.

Measurement Uncertainties

Woodrow W. Burchett[j], Arnold J. Stromberg[j], and Eric A. Grulke[k]
[j]Applied Statistics Laboratory, University of Kentucky, Lexington, KY, USA
[k]Chemical & Materials Engineering, University of Kentucky, Lexington, KY, USA
Corresponding author: eric.grulke@uky.edu, +1-859-257-6097

Procedure A

In this procedure, the size and shape data were fitted to the normal distribution; this distribution has much conventional use, but did not describe datasets as well as other reference models. Notice that determining the parameter values using the maximum likelihood or nonlinear regression methods gives different values than computing the average and standard deviation of the data. The coefficients of variation and measurement uncertainties for the complete set of size and shape descriptors are shown in Table S8 and S9. For the full ILC dataset, the coefficients of variation are in the range of 10% for descriptor length scales and in the range of 15% or less for descriptor widths. For the subset of laboratories with similar descriptor means, the coefficients of variation is less than 2% for descriptor scales and less than 8% for descriptor widths.

Table S8 Coefficients of variation and measurement uncertainties for size descriptors of normal distributions

Statistic	Normal distribution parameter estimates							
	Area		D_{ecd}		Feret		minFeret	
	scale	width	scale	width	scale	width	scale	width
$\bar{x}$	1391	991	39.8	12.9	52.9	20.9	31.8	10.3
S	293	300	4.12	1.88	4.99	2.57	3.09	1.28
c_v, %	21.1%	30.3%	10.3%	14.6%	9.43%	12.3%	9.72%	12.4%

Table S9 Coefficients of variation and measurement uncertainties for shape descriptors of normal distributions

Stastistic	Normal distribution parameter estimates			
	Aspect ratio		Compactness	
	scale	width	scale	width
$\bar{x}$	0.632	0.148	0.773	0.0929
S	0.0188	0.00559	0.0196	0.00523
c_v, %	2.97%	3.78%	2.54%	5.64%
U_{ILC}	6.27%	8.0%	5.35%	11.9%

Procedure B

For this procedure, the coefficients of variation and measurement uncertainties are reported for the preferred reference models, as determined by ANOVA and bivariate analysis. For size descriptors (Table S9), lognormal distributions were used. For shape descriptors (Table S10), Weibull distributions were used. The size descriptor scales had coefficients of variation that differed by less than 5%, and the size descriptor widths had coefficients of variation that differed by less than ~10%. These coefficients of variation are much lower than those computed for the normal distribution, illustrating the value of using reference models that better describe the data.

Table S10 Coefficients of variation and measurement uncertainties for lognormal parameters of size distributions

Statistic	Lognormal distribution fitted parameters							
	Area		D_{ecd}		Feret		minFeret	
	scale	width	scale	width	scale	width	scale	width
$\bar{x}$	6.91	0.637	3.58	0.318	3.84	0.360	3.36	0.326
S	0.322	0.066	0.161	0.033	0.149	0.040	0.156	0.034
c_v, %	4.65%	10.4%	4.49%	10.5%	3.87%	11.1%	4.63%	10.5%
U_{ILC}	9.81%	21.9%	9.47%	22.0%	8.17%	23.4%	9.77%	22.0%

Table S11 Coefficients of variation and measurement uncertainties for Weibull parameters of shape distributions

Statistic	Weibull fitted shape parameters			
	Aspect ratio		Compactness	
	scale	width	scale	width
$\bar{x}$	0.693	4.94	0.815	10.0
s	0.023	0.609	0.019	0.856
c_v, %	3.32%	12.3%	2.28%	8.55%
U_{ILC}	7.00%	26.0%	4.81%	18.0%

The coefficients of variation for the size descriptors of the full ILC are significantly larger than those reported by Rice for a gold nanoparticle certified reference material [9]. Since a key objective of this certified reference material is to provide a known standard for size, its coefficient of variation for the size descriptor should be relatively small. Reference distributions often fail to represent the data well for very low and very high values of the descriptor (data ranges outside of ± 2σ from the mean), so the full data should show higher uncertainty values than the model. Of course, the modeled distributions will be easier to use over their ranges of application.

S7 Statistical and metrological definitions

Woodrow W. Burchett[j], Joshua W. Lambert[j], Arnold J. Stromberg[j], and Eric A. Grulke[k]
[j]Applied Statistics Laboratory, University of Kentucky, Lexington, KY, USA
[k]Chemical & Materials Engineering, University of Kentucky, Lexington, KY, USA
Corresponding author: eric.grulke@uky.edu, +1-859-257-6097

Statistical definitions

The definitions provided here generally follow the guidance of ISO 5725 [10].

Mean

The **mean** (or **arithmetic mean**) is the sum of all the values in a group (x_i) divided by the number of values in that group (n).

$$\overline{x} = \frac{\sum_{i=1}^{n} x_i}{n}$$

Estimates for the fitted mean begin with the standard definition and then are iteratively updated to minimize the sum of differences between the reference model and the data, either by the maximum likelihood estimate or the nonlinear regression estimate methods.

Standard deviation

The **standard deviation**, denoted by ***s*** or ***SD***, represents the average amount of variability in a set of sample measurements. That is, it is the average distance of each sample measurement (x_i) from the mean$(\bar{x})$.

$$s = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}$$

Estimates for the fitted mean begin with the standard definition and then are iteratively updated to minimize the sum of differences between the reference model and the data, either by the maximum likelihood estimate or the nonlinear regression estimate methods.

Coefficient of variation

The **coefficient of variation** is also known as the unitized risk, variation coefficient, or relative standard deviation. The coefficient of variation is used in this protocol to evaluate each descriptor reported for the interlaboratory assessment. As an example, the coefficient of variation for a descriptor mean is:

$$\hat{c}_v = \frac{s}{\bar{x}}$$

where $\bar{x}$ is the descriptor's mean and s is the descriptors's standard deviation for the interlaboratory comparison. These 'grand statistics' are used to evaluate reported data and descriptors for the ILC.

Standard error

Example: standard error of the mean. The standard error is the standard deviation of the sampling distribution of a statistic. Standard error of the mean is an estimate of how close the sample mean is to the population mean. This value decreases as the sample size increases.

$$SE_{\bar{x}} = \frac{s}{\sqrt{n}} \ ;$$

For fitted parameters, the standard error is computed using Wald confidence intervals.

Relative standard error

The relative standard error (RSE) is the standard error divided by its statistic and expressed as a percentage.
Example: relative standard error of the mean

$$RSE_{\bar{x}} = \frac{SE_x}{\bar{x}}$$

P-value

If the null hypothesis were true and if the experiment were repeated many times, a **p-value** is the probability that a value at least as extreme as the computed test statistic would be observed. In hypothesis testing, a statement claiming that the null parameter is the true parameter is called the null hypothesis. The purpose of a hypothesis test is to determine whether the data provide evidence against the null hypothesis. When a statistic is obtained that is very different from the null parameter, the null hypothesis can be rejected. An alternative, or research hypothesis, is a hypothesis that states that the true parameter is not (or is less than or is greater than) the null parameter; it is the hypothesis that corresponds to the research question. The goal of a hypothesis test is to reject the null hypothesis in favor of the research hypothesis.

Variance

The variance, Var(x), between a model and data can be defined as:

$Var(x) = \sum_1^n \left(x_{i,model} - x_{\mathrm{i},data}\right)^2$

Residual deviation

The **residual deviation** of an observed value is the difference between the observed value of the response variable and the estimated value of the response variable.

Quantile-quantile plot

The quantile-quantile (q-q) plot can be used to determine whether two data sets come from populations with a common distribution (described in ISO 9276-3:2008(en) [11]). The q-q plot compares the quantiles of the first data set with those of the second data set. Q-q plots work naturally with cumulative distributions. Q-q plots allow several distributional elements to be tested simultaneously, such as shifts in location, shifts in scale, changes in symmetry, and the presence of outliers.

Metrological definitions

Measurement uncertainty sample calculation

For the area-equivalent diameter, factors of the pooled measurement uncertainty ($u_c(x)$) could include the inter-laboratory reproducibility ($u(ir)$), the trueness ($u(t)$), and the image resolution error ($u(c)$). The image resolution error is set by the image scale and the particle size. For example, for a distribution of 30 nm particles (nominal size), the image scale might be set to 0.5 nm/pixel. The range of particle sizes imaged might range between 15 and 50 nm, based on the breadth of the distribution. The image resolution error depends on particle size, ranging from 3.3 % to 1.7 % to 1 % for particle sizes of 15, 30, and 50 nm.

$$u_c(x) = \sqrt{u(ir)^2 + u(t)^2 + u(c)^2}$$

Expanded measurement uncertainty sample calculation

The Report of Investigation for RM8012 [12] gives the expanded measurement uncertainty for 30 nm gold nanoparticles, based on the combined standard uncertainty[13] for different ampoules of the reference material (a Type A evaluation). The expanded measurement uncertainty, U = 2.1, was computed using:

$$U = k \cdot s_{ampoule\ means} \cdot \sqrt{1 + \frac{1}{N}}$$

where k is the coverage factor for 95% expanded uncertainty intervals (=2), $\square_{\text{ampoule means}}$ is the standard deviation of the means of the area-equivalent diameter computed for different ampoules (s = 0.94 nm), and the radical term adjusts for the number of ampoules studied (N=4).

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