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Nanotechnologies — Measurements of particle size and shape distributions by scanning electron microscopy

Nanotechnologies — Détermination de la distribution de taille et de forme des particules par microscopie électronique à balayage

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/ TC 229, *Nanotechnologies*.

Any feedback or questions on this document should be directed to the user’s national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

This document provides guidance for measuring and reporting the size and shape distributions of nanometer-scale particles using images acquired by the scanning electron microscope (SEM). This document applies to the SEM-based measurement of larger particles also. Nanoparticles are three-dimensional (3D) objects, but the SEM image is only a two-dimensional (2D) representation of the 3D shape from a certain viewing angle. The SEM image carries valuable information about the size and shape of particles. While the SEM image does contain a certain amount of 3D information, for sake of simplicity, this document does not deal with reconstructing 3D information. Rigorous three-dimensional characterization of nanoparticles would include size, shape, surface structure (e.g. texture), surface and internal material composition, and their locations in the investigated 3D volume. This document deals with two attributes of morphology, size and shape, for discrete and aggregated nano-objects (materials with at least one dimension in the nanometer-scale, i.e. within 1 nm to 100 nm). Suitable sample preparation is essential to obtaining high-quality electron microscope images and preferred techniques often vary with the sample material. It is equally important to make sure that the SEM itself is suitable to carry out the measurements with the required uncertainty. Typical guidance suggests that a large number, several hundreds or thousands of particles need to be measured for statistically sound size and shape distribution results. The actual number of nano-objects needed to be measured depends on the sample, the required uncertainty and on the performance of the SEM. Statistical evaluation of the data and the evaluation of uncertainty of the measurands are included as part of the measurement and reporting procedures.

This document contains measurement procedures, particle and data analysis and reporting clauses. In the Annexes, there are specific examples for measurements and guidance for the qualification of the SEM for reliable quantitative measurements. Automation of the image acquisition and data analysis can reduce cost and improve the quality of the results. Measurements of samples of discrete nanoparticles are generally easier to carry out with automated image acquisition and particle analysis systems. Measurements of complex discrete nanoparticles, and aggregates or agglomerates of nanoparticles may require operator-assisted image acquisition and analysis. Evaluation of particle shape is facilitated by many pertinent analysis software solutions that allow for automatic selection of various shape attributes as well.

Nanotechnologies — Measurements of particle size and shape distributions by scanning electron microscopy

1 Scope

This document specifies methods of determining nanoparticle size and shape distributions by acquiring and evaluating scanning electron microscope images and by obtaining and reporting accurate results.

NOTE 1 This document applies to particles with a lower size limit that depends on the required uncertainty and on the suitable performance of the SEM, which is to be proven first -according to the requirements described in this document.

NOTE 2 This document applies also to SEM-based size and shape measurements of larger than nanoscale particles.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO/IEC Guide 99, *International vocabulary of metrology — Basic and general concepts and associated terms (VIM)*

ISO 9276-1, *Representation of results of particle size analysis — Part 1: Graphical representation*

ISO 9276-2, *Representation of results of particle size analysis — Part 2: Calculation of average particle sizes/diameters and moments from particle size distributions*

ISO 9276-3, *Representation of results of particle size analysis — Part 3: Adjustment of an experimental curve to a reference model*

ISO 9276-5, *Representation of results of particle size analysis — Part 5: Methods of calculation relating to particle size analyses using logarithmic normal probability distribution*

ISO 9276-6, *Representation of results of particle size analysis — Part 6: Descriptive and quantitative representation of particle shape and morphology*

ISO 13322-1, *Particle size analysis — Image analysis methods — Part 1: Static image analysis methods*

ISO 16700, *Microbeam analysis — Scanning electron microscopy — Guidelines for calibrating image magnification*

ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories*

ISO/TS 24597:2011, *Microbeam analysis — Scanning electron microscopy — Methods of evaluating image sharpness*

ISO 26824, *Particle characterization of particulate systems — Vocabulary*

ISO/TS 80004-1, *Nanotechnologies — Vocabulary — Part 1: Core terms*

ISO/TS 80004-2, *Nanotechnologies — Vocabulary — Part 2: Nano-objects*

ISO/TS 80004-3, *Nanotechnologies — Vocabulary — Part 3: Carbon nano-objects*

ISO/TS 80004-4, *Nanotechnologies — Vocabulary — Part 4: Nanostructured materials*

ISO/TS 80004-6, *Nanotechnologies — Vocabulary — Part 6: Nano-object characterization*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/IEC Guide 99, ISO 9276-6, ISO 26824, ISO/TS 80004-1, ISO/TS 80004-2, ISO/TS 80004-3, ISO/TS 80004-4, ISO/TS 80004-6, and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at https://www.iso.org/obp
- IEC Electropedia: available at http://www.electropedia.org/

3.1 General terms

3.1.1
nanoscale
length range from approximately 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from larger sizes are predominantly exhibited in this length range.

[SOURCE: ISO/TS 80004-1:2015, 2.1]

3.1.2
nano-object
discrete piece of material with one, two or three external dimensions in the *nanoscale* (3.1.1)

[SOURCE: ISO/TS 80004-1:2015, 2.5, modified — Note 1 to entry and the source have been deleted.]

3.1.3
particle
minute piece of matter with defined physical boundaries

[SOURCE: ISO/TR 16197:2014, 3.10, modified — Notes 1, 2 and 3 to entry and the source have been deleted.]

3.1.4
primary particle
original source *particle* (3.1.3) of *agglomerates* (3.1.5) or *aggregates* (3.1.6) or mixtures of the two

[SOURCE: ISO 26824:2013, 1.4, modified — Notes 1, 2 and 3 to entry have been deleted.]

3.1.5
agglomerate
collection of weakly or medium strongly bound *particles* (3.1.3) where the resulting external surface area is similar to the sum of the surface areas of the individual components

Note 1 to entry: Agglomerate originates from the Latin "agglomerare" meaning "to form into a ball".

Note 2 to entry: The forces holding an agglomerate together are weak forces, for example van der Waals forces or simple physical entanglement.

Note 3 to entry: Agglomerates are also termed secondary particles and the original source particles are termed *primary particles* (3.1.4).

[SOURCE: ISO 26824:2013, 1.2, modified — Note 1 to entry has been added.]

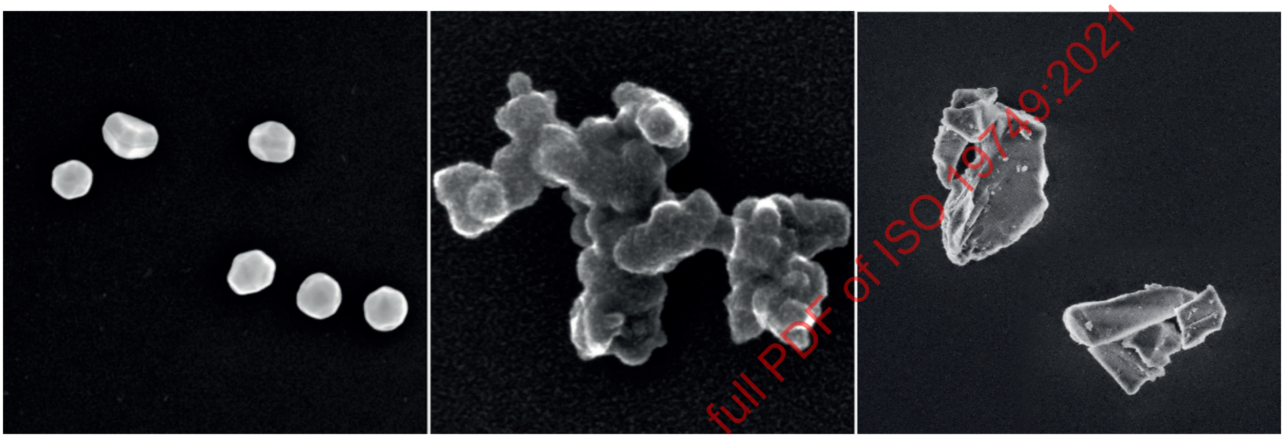
3.1.6
aggregate

particle (3.1.3) comprising strongly bonded or fused particles where the resulting external surface area is significantly smaller than the sum of surface areas of the individual components

Note 1 to entry: The forces holding an aggregate together are strong forces, for example covalent bonds, or those resulting from sintering or complex physical entanglement, or otherwise combined former *primary particles* (3.1.4).

Note 2 to entry: Aggregate comes from the Latin “aggregat” meaning “herded together”.

Note 3 to entry: Figure 1 shows examples of individual, aggregate and *agglomerate* (3.1.5) particles.



NOTE The images are projected views from certain angles of the 3D objects. Depending on the viewing angle, the observable size of particles can vary substantially.

Figure 1 — SEM images of individual gold (left) and carbon black aggregate (middle) and corundum agglomerate (right) particles

[SOURCE: ISO 26824:2013, 1.3, modified — Notes 2 and 3 to entry have been added.]

3.1.7
nanoparticle

nano-object (3.1.2) with all external dimensions in the *nanoscale* (3.1.1) where the lengths of the longest and shortest axes of the nano-object do not differ significantly

[SOURCE: ISO/TS 80004-2:2015, 4.4, modified — Note 1 to entry has been deleted.]

3.1.8
particle size

x

dimension of a *particle* (3.1.3) determined by a specified measurement method and under specified measurement conditions

Note 1 to entry: Different methods of analysis are based on the measurement of different physical properties. Independent of the particle property actually measured, the particle size can be reported as a linear dimension, an area, or a volume.

Note 2 to entry: The symbol x is used denote linear *particle* (3.1.3) size. However, it is recognized that the symbol d is also widely used. Therefore, the symbol x may be replaced by d.

3.1.9
particle size distribution

distribution of the quantity of *particles* (3.1.3) as a function of *particle size* (3.1.8)

[SOURCE: ISO/TS 80004-6:2021, 4.1.2, modified — Notes 1 and 2 to entry have been deleted.]

3.1.10

particle shape

external geometric form of a *particle* (3.1.3)

Note 1 to entry: Shape description requires two scalar descriptors, i.e. length and breadth.

[SOURCE: ISO/TS 80004-6:2021, 4.1.3, modified — Note 1 to entry has been added.]

3.1.11

analytical sample

portion of material, resulting from the original sample or composite sample by means of an appropriate method of sample pretreatment and having the size (volume/mass) necessary for the desired testing or analysis

Note 1 to entry: The sample in analytical chemistry is a portion of material selected from a larger quantity of material. The term needs to be qualified, for example, bulk sample, representative sample, primary sample, bulked sample, test sample. The term 'sample' implies the existence of a sampling error, i.e. the results obtained on the portions taken are only estimates of the concentration of a constituent or the quantity of a property present in the parent material. If there is no or negligible sampling error, the portion removed is a test portion, aliquot or specimen. The term 'specimen' is used to denote a portion taken under conditions such that the sampling variability cannot be assessed (usually because the population is changing), and is assumed, for convenience, to be zero. The manner of selection of the sample should be prescribed in a sampling plan.

[SOURCE: ISO 11074:2015, 4.1.3, modified — Note 1 to entry has been added.]

3.2 Core terms: image analysis

3.2.1

binary image

digitized image consisting of an array of *pixels* (3.2.2), each of which has a value of 0 or 1, whose values are normally represented by dark and bright regions on the display screen or by the use of two distinct colors

[SOURCE: ISO 13322-1:2014, 3.1.2]

3.2.2

pixel

smallest element of an image that can be uniquely processed, and is defined by its spatial coordinates and encoded with colour values

[SOURCE: ISO 12640-2:2004, 3.6, modified — Note 1 to entry has been deleted.]

3.2.3

pixel resolution

number of imaging *pixels* (3.2.2) per unit distance of the detector

Note 1 to entry: The typical unit is sometimes expressed as dots per inch (dpi).

[SOURCE: ISO 29301:2017, 3.24, modified — the hyphen has been deleted in this term.]

3.3 Core terms: statistical symbols and definitions

3.3.1

arithmetic mean

sum of values divided by the number of values

Note 1 to entry: See ISO 9276-1:1998 for other quantity measures and types of distributions.

3.3.2
standard deviation

measure of the dispersion of a series of results around their mean, equal to the positive square root of the variance and estimated by the positive square root of the mean square

[SOURCE: ISO 4259-1:2017, 3.21]

3.3.3
coefficient of variation

ratio of the *standard deviation* (3.3.2) to the *arithmetic mean* (3.3.1)

[SOURCE: ISO 27448:2009, 3.11]

3.3.4
relative standard error

standard error (SE_x) divided by the mean ($\bar{x}$) and expressed as a percentage

3.3.5
analysis of variance
ANOVA

technique which subdivides the total variation of a response variable into components associated with defined sources of variation

3.3.6
p-value

probability of observing the observed test statistic value or any other value at least as unfavorable to the null hypothesis

Note 1 to entry: 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.

Note 2 to entry: 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 favour of the research hypothesis.

[SOURCE: ISO/TR 14468:2010, 3.13, modified — Note 1 to entry has been modified and Note 2 to entry has been added.]

3.3.7
residual deviation

difference between the observed value of the response variable and the estimated value of the response variable

3.3.8
residual standard deviation

scatter of the information values about the calculated regression line

Note 1 to entry: It is a figure of merit, describing the *precision* (3.5.3) of the calibration.

Note 2 to entry: For this document, the *standard deviation* (3.3.2) of the method means the standard of deviation of the calibration procedure.

[SOURCE: ISO 8466-1:1990, 2.5, modified — the symbol has been deleted and the entire entry has been editorially revised.]

3.3.9
quantile plot
graphical method of comparing two distributions where the quantiles of the empirical (data) distribution are plotted on the y-axis while the quantiles of the theoretical (reference) distribution with the same mean and variance as the empirical distribution are plotted on the x-axis

Note 1 to entry: The quantile-quantile (q-q) plot is a probability plot, a graphical technique for determining if two data sets come from populations with a common distribution. A q-q plot is a plot of the quantiles of the first data set against the quantiles of the second data set. See ISO/TS 80004-6.

3.4 Core terms: measurands and descriptors

3.4.1
measurand
quantity intended to be measured

[SOURCE: ISO/IEC Guide 98-4:2012, 3.2.4]

3.4.2
Feret diameter
distance between two parallel lines which are tangent to the *perimeter* (3.4.5) of a *particle* (3.1.3)

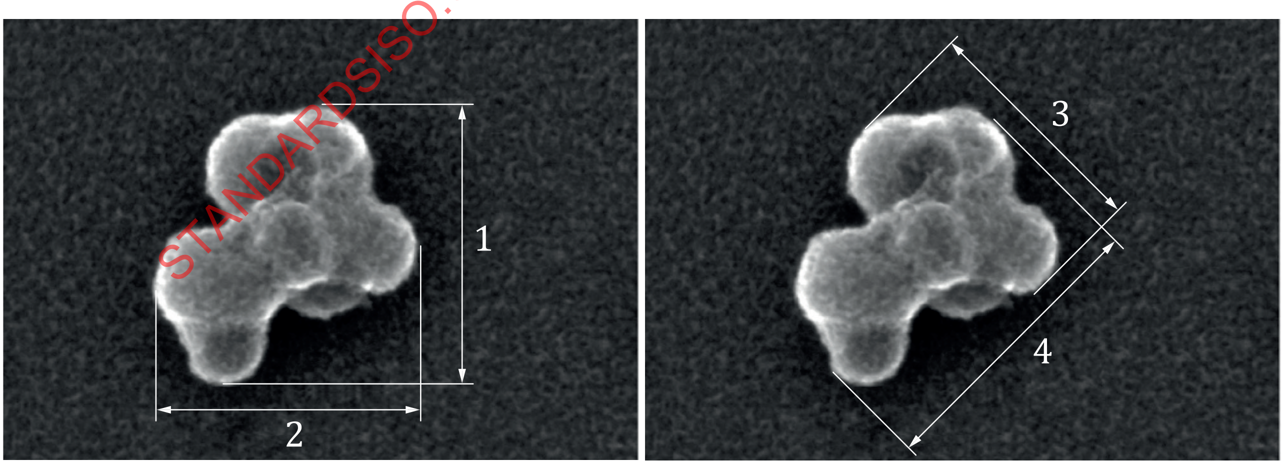
[SOURCE: ISO 10788:2014, 2.1.4, modified — Note 1 to entry has been deleted.]

3.4.3
maximum Feret diameter
maximum length of an object whatever its orientation

[SOURCE: ISO/TR 945-2:2011, 2.1, modified — the word "Féret" in the term has been changed to "Feret" and Note 1 to entry has been deleted.]

3.4.4
minimum Feret diameter
minimum length of an object whatever its orientation

Note 1 to entry: The *Feret diameter* (3.4.2) or Feret's diameter is a measure of an object size along a specified direction; it is applied to projections of a three-dimensional object on a two-dimensional plane, see Figure 2. It is also called the caliper diameter.



Key

1	vertical Feret diameter
2	horizontal Feret diameter
3	breadth
4	length

Figure 2 — Horizontal Feret diameter (88 nm) and vertical Feret diameter (93 nm), and length (99 nm) and breadth (79 nm) of a carbon black particle

Note 2 to entry: The maximum Feret diameter x_{Fmax} is the “length” of the *particle* (3.1.3). The *minimum Feret diameter* (3.4.4) x_{Fmin} is the ”breadth” of the particle.

Note 3 to entry: The Feret diameter depends on the orientation of the particle with respect to tangents, so a single measurement cannot always be representative. If all possible orientations are considered, for a convex particle with the particle *perimeter* (3.4.5) P: $P = \pi\, x_{\mathrm{Fmean}}$. (Cauchy theorem). There is no such relation between P and x_{Fmean} for a concave object.

3.4.5
perimeter
total length of the object contour

[SOURCE: ISO/TR 945-2:2011, 2.3, modified — the symbol "P" has been deleted.]

3.4.6
convex hull
smallest convex set containing a given geometric object

[SOURCE: ISO 19123:2005, 4.1.2]

3.4.7
aspect ratio
ratio of the *minimum Feret diameter* (3.4.4) to the *maximum Feret diameter* (3.4.3)

3.4.8
ellipse ratio
ratio of the lengths of the axes of the Legendre ellipse of inertia

[SOURCE: ISO 26824:2013, 4.4, modified — Note 1 to entry has been deleted.]

3.4.9
extent
ratio of *particle* (3.1.3) area to the product of the *maximum Feret* (3.4.3) and the *minimum Feret* (3.4.4) diameters

3.4.10
compactness
degree to which the projection area A of the *particle* (3.1.4) is similar to a circle, considering the overall form of the particle with the *maximum Feret diameter* (3.4.3)

[SOURCE: ISO 26824:2013, 4.9, modified — the formula and Note 1 to entry have been deleted.]

3.4.11
convexity
ratio of the *perimeter* (3.4.5) of the *convex hull* (3.4.7) envelope bounding the *particle* (3.1.3) to its perimeter

3.4.12
circularity
form factor
degree to which the projected area of the *particle* (3.1.3) is similar to a circle, based on its *perimeter* (3.4.5)

3.4.13
roundness
square of the *circularity* (3.4.12)

3.4.14
solidity
ratio of the projected area A to the area of the *convex hull* (3.4.7) A_{C} (envelope)

3.5 Core terms: metrology

3.5.1

repeatability condition of measurement

condition of measurement, out of a set of conditions that includes the same measurement procedure, same operators, same measuring system, same operating conditions and same location, and replicate measurements on the same or similar objects over a short period of time

[SOURCE: ISO/IEC Guide 99:2007, 2.20, modified — Notes 1 and 2 to entry have been deleted.]

3.5.2

measurement accuracy

closeness of agreement between a measured quantity value and a true quantity value of a *measurand* (3.4.1)

Note 1 to entry: The concept of measurement accuracy is not a quantity and is not given a numerical quantity value. A measurement is said to be more accurate when it offers a smaller *measurement uncertainty* (3.5.4).

Note 2 to entry: The term “measurement accuracy” should not be used for measurement trueness and the term measurement *precision* (3.5.3) should not be used for ‘measurement accuracy’, which, however, is related to both these concepts.

Note 3 to entry: Measurement accuracy is sometimes understood as closeness of agreement between measured quantity values that are being attributed to the measurand.

[SOURCE: ISO/IEC Guide 99:2007, 2.13, modified — the second and third terms have been deleted.]

3.5.3

precision

closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions

Note 1 to entry: Measurement precision is usually expressed numerically by measures of imprecision, such as *standard deviation* (3.3.2), variance, or *coefficient of variation* (3.3.3) under the specified conditions of measurement.

Note 2 to entry: The specified conditions can be, for example, repeatability conditions of measurement, intermediate precision conditions of measurement, or reproducibility conditions of measurement (see ISO 5725-1:1994).

Note 3 to entry: Measurement precision is used to define measurement repeatability, intermediate measurement precision, and measurement reproducibility.

Note 4 to entry: Sometimes “measurement precision” is erroneously used to mean measurement accuracy.

[SOURCE: ISO/IEC Guide 99:2007, 2.15, modified — the first term has been deleted.]

3.5.4

measurement uncertainty

non-negative parameter characterizing the dispersion of the quantity values being attributed to a *measurand* (3.4.1), based on the information used

Note 1 to entry: Measurement uncertainty includes components arising from systematic effects, such as components associated with corrections and the assigned quantity values of measurement standards, as well as the definitional uncertainty. Sometimes estimated systematic effects are not corrected for but, instead, associated measurement uncertainty components are incorporated.

Note 2 to entry: The parameter may be, for example, a *standard deviation* (3.3.2) called standard measurement uncertainty (or a specified multiple of it), or the half-width of an interval, having a stated coverage probability.

Note 3 to entry: Measurement uncertainty comprises, in general, many components. Some of these may be evaluated by *Type A evaluation of measurement uncertainty* (3.5.7) from the statistical distribution of the quantity values from series of measurements and can be characterized by standard deviations. The other components, which may be evaluated by *Type B evaluation of measurement uncertainty* (3.5.8), can also be characterized by standard deviations, evaluated from probability density functions based on experience or other information.

Note 4 to entry: In general, for a given set of information, it is understood that the measurement uncertainty is associated with a stated quantity value attributed to the *measurand* (3.4.1). A modification of this value results in a modification of the associated uncertainty.

[SOURCE: JCGM 200:2012, 2.26]

3.5.5
combined standard measurement uncertainty

standard *measurement uncertainty* (3.5.4), a non-negative parameter characterizing the dispersion of the quantity values being attributed to a *measurand* (3.4.1), based on the information use, is obtained using the individual standard measurement uncertainties associated with the input quantities in a measurement model

Note 1 to entry: In case of correlations of input quantities in a measurement model, it is essential to take covariances into account when calculating the combined standard measurement uncertainty; see also ISO/IEC Guide 98-3:2008, 2.3.4.

Note 2 to entry: Measurement uncertainty includes components arising from systematic effects, such as components associated with corrections and the assigned quantity values of measurement standards, as well as the definitional uncertainty. Sometimes estimated systematic effects are not corrected for but, instead, associated measurement uncertainty components are incorporated.

Note 3 to entry: The parameter maybe, for example, a *standard deviation* (3.3.2) called standard measurement uncertainty (or a specified multiple of it), or the half-width of an interval, having a stated coverage probability.

Note 4 to entry: Measurement uncertainty comprises, in general, many components. Some of these maybe evaluated by *Type A evaluation of measurement uncertainty* (3.5.7) from the statistical distribution of the quantity values from series of measurements and can be characterized by standard deviations. The other components, which maybe evaluated by *Type B evaluation of measurement uncertainty* (3.5.8), can also be characterized by standard deviations, evaluated from probability density functions based on experience or other information.

Note 5 to entry: In general, for a given set of information, it is understood that the measurement uncertainty is associated with a stated quantity value attributed to the measurand. A modification of this value results in a modification of the associated uncertainty.

[SOURCE: JCGM 200:2012, 2.31]

3.5.6
expanded measurement uncertainty

U

product of a *combined standard measurement uncertainty* (3.5.5) and a factor larger than the number one

Note 1 to entry: The factor depends upon the type of probability distribution of the output quantity in a measurement model and on the selected coverage probability.

Note 2 to entry: The term "factor" in this definition refers to a coverage factor.

Note 3 to entry: Expanded measurement uncertainty is termed overall uncertainty in paragraph 5 of Recommendation INC-1 (1980) (see the GUM) and simply "uncertainty" in IEC documents.

[SOURCE: ISO/IEC Guide 99:2007, 2.35, modified — the second term "expanded uncertainty" has been changed to "*U*".]

3.5.7
Type A evaluation of measurement uncertainty

evaluation of a component of *measurement uncertainty* (3.5.4) by a statistical analysis of measured quantity values obtained under defined measurement conditions

[SOURCE: ISO/IEC Guide 99:2007, 2.28, modified — the second term and the Notes 1, 2 and 3 to entry have been deleted.]

3.5.8
Type B evaluation of measurement uncertainty

evaluation of a component of *measurement uncertainty* (3.5.4) determined by means other than a *Type A evaluation of measurement uncertainty* (3.5.7)

[SOURCE: ISO/IEC Guide 99:2007, 2.29, modified — the second term, the example and the Note 1 to entry have been deleted.]

3.5.9
reference material
RM

material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process

3.5.10
certified reference material
CRM

reference material (3.5.9) characterized by a metrologically valid procedure for one or more specified properties, accompanied by a reference material certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability

[SOURCE: ISO Guide 30:2015, 2.1.2, modified — the Notes 1, 2, 3 and 4 to entry have been deleted.]

3.6 Core terms: scanning electron microscopy

3.6.1
scanning electron microscope
SEM

instrument that produces magnified images of a specimen by scanning its surface with a well-focused electron beam

Note 1 to entry: A conventional SEM utilizes an electron source filament either made from W or LaB_6 materials that are heated to produce a source of electrons by thermionic emission. The electron beam probe sizes are in the range of 3 nm to 4 nm, which is not sufficient to resolve the smallest nanometer-scale details. The range of useful analysis is generally under 100 000 times magnification. Conventional SEMs typically operate at high *accelerating voltages* (3.6.2) (5 kV to 30 kV) and often require non-conducting samples to be coated with a thin layer of conductive metal (gold, gold-palladium, chromium, osmium) for imaging or carbon for analytical purposes (only).

Note 2 to entry: A field emission scanning electron microscope (FESEM) has an extremely sharp cathode tip that generates a smaller diameter probe size compared to a conventional SEM, even at low accelerating voltages (0,5 kV to 5 kV). In FESEMs, electron beam probe sizes can be 0,5 nm or less, expanding the useful magnification range by an order of magnitude higher. Non-conducting materials can be imaged without applying a conductive coating using low or very low accelerating voltages. A FESEM is sometimes referred to as a high-resolution SEM (HRSEM).

Note 3 to entry: Variable pressure SEM (VPSEM) or controlled pressure SEM which can operate with a pressure in the specimen chamber from 1 Pa up to 5,000 Pa so that direct secondary emission is no longer detectable and images are obtained with the detection of electrons by gas amplification, environmental SE detector, ions, photoemission or using other signals such as backscattered (BSE) or absorbed current. This type of SEM can be also used with a normal vacuum in the chamber.

3.6.2
accelerating voltage
potential difference applied between the filament and the anode to accelerate the electrons emitted from the source

[SOURCE: ISO 23833:2013, 5.1]

3.6.3
secondary electron
SE
electron emitted from a specimen as a result of bombardment by the primary electrons

Note 1 to entry: By convention, an electron with energy less than 50 eV is considered as a secondary electron.

[SOURCE: ISO 22493:2014, 3.4]

3.6.4
secondary electron image
SEI
scanning electron beam image in which the signal is derived from a detector that selectively measures *secondary electrons* (3.6.3) (electrons having less than 50 eV) and is not directly sensitive to backscattered electrons

[SOURCE: ISO 23833:2013, 4.4.11]

3.6.5
scanning transmission electron microscopy
STEM
method that produces magnified images or diffraction patterns of the sample by a finely focused electron beam, scanned over the surface and which passes through the sample and interacts with it

Note 1 to entry: In SEMs the energy of the primary electron beam is significantly lower than in traditional transmitted electron microscopes (TEMs), but still useful for STEM imaging and measurements of *nanoparticles* (3.1.7).

[SOURCE: ISO/TS 80004-6:2021, 4.5.7, modified — Notes 1 to entry has been modified and Note 2 to entry has been deleted.]

4 General principles

4.1 SEM imaging

Particles are three-dimensional. The SEM provides a projection, a view from a certain angle of the three-dimensional particles. The digital SEM image is a two-dimensional matrix of locations identified by horizontal and vertical parameters and the intensity of a signal at those locations. This matrix can be viewed as an image on the computer screen.

SEM imaging and analysis is especially useful for nanoparticle visualization and measurements of various particle size and shape distribution measurands. Imaging the nanoparticles is the first step to visualize their presence, but without suitably good repeatability in imaging, the SEM cannot provide useful information for particle size measurements. Depending on the resolving power of the SEM, it is possible to image well sub-10 nm particles. Figure 3 shows an example for various size nanoparticles with the approximate sizes for two of them. The horizontal field width is 300 nm.

There are 50 atoms in 1 nm^3 Si; a 1-nm diameter Si sphere particle contains about 26 atoms, a 2-nm diameter Si sphere particle contains about 210 atoms, so small nanoparticles cannot generate large signals. To achieve sufficiently noise-free images, proper setting of the SEM and the imaging parameters is indispensable.

Many modern SEMs have scanning transmission imaging capability. The images of nanoparticles obtained in the SEM in this scanning transmission electron microscopy working mode are very similar to images taken in the transmission electron microscope (TEM) and in scanning transmission electron microscope (STEM), which generally work at much higher accelerating voltages. STEM images taken in the SEM can be particularly useful for particle size and shape measurements. Obviously for this working mode, the same sample preparation requirements apply as for traditional TEM or STEM investigations. A similar ISO document is available on the size and shape measurements of particles with these techniques, which has information and examples for sample preparation and imaging useful for SEM-based measurements.

4.2 SEM image-based particle size measurements

Size measurements determine a one-dimensional (1D) parameter, a number related to the size of a three-dimensional (3D) particle, e.g. the circular equivalent diameter in a projected view image. SEM images can contain some volume-related, additional information also. Depending on the instrument settings and the sample material, more signal electrons might be generated by a spherical particle than by a flat one, even if they have the same size and shape projected image.

For the determination of particle size, relevant sections of ISO 13322-1, ISO 9276-1, ISO 9276-2, ISO 9276-3 and ISO 9276-5 shall be used.

Depending on the resolving power of the SEM, it is possible to measure the size and size distribution of sub-10 nm particles. The smallest measureable nanoparticles start at 1-nm size for the highest resolution SEMs that have a resolution of 0,4 nm or better. Other instruments, especially those that are equipped with conventional tungsten electron source, have a spatial resolution of a few nanometers, cannot suitably image and measure the smallest nanoparticles. The actual minimum value of the measurable nanoparticle size depends on the instrument, its environment, and the sample as well. When reliable sizing of the nanoparticles — due to lack of sufficiently high resolution — is not possible, particle counting still might be feasible. For this, it might be sufficient to detect the presence of nanoparticles. Signal intensity might also be used to determine size-related information with reduced accuracy. For example, in Figure 3, the approximately 2-nm size gold particle gives less intense signal than the 10-nm size. The detection limit of the presence of very small size nanoparticles shall be determined with comparisons of the results of higher-resolution and more sensitive SEM or TEM or other measurements, using suitable samples.

Additionally, the measurement error also depends on the resolving power of the SEM. Generally, sharper, more focused electron beam will result in images that show more details of particles that appear smaller, while lack of focus or resolving power makes them appear larger. This does not necessarily mean that good-quality size and shape distribution measurements cannot be carried out. Proper calibration using relevant reference samples with known particle sizes could help to significantly reduce measurement uncertainty.

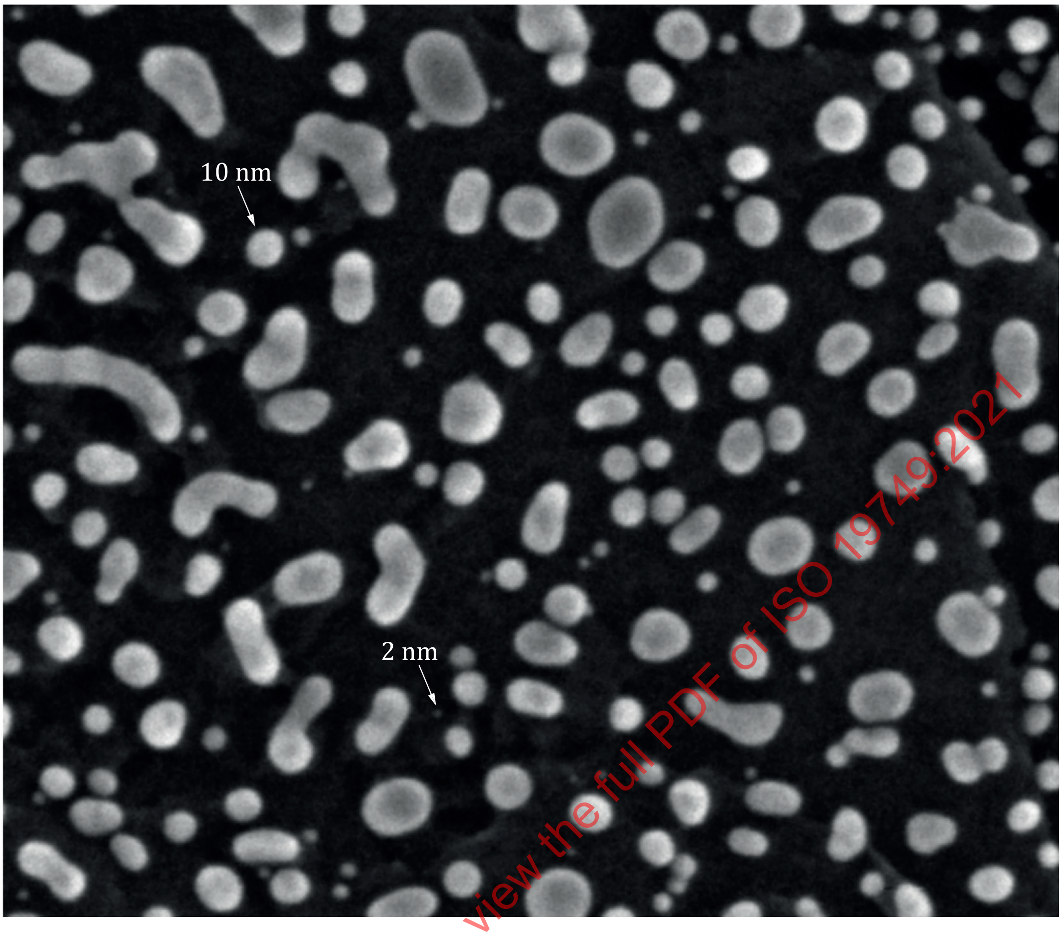


Figure 3 — SEM image of gold nanoparticles on carbon substrate showing large variation of size and shape (300 nm HFW)

4.3 SEM image-based particle shape measurements

Shape measurements determine a two-dimensional function related to projection, a view from a certain angle of the three-dimensional particle. ISO 9276-6 shall be used in SEM image-based particle shape measurements.

Accurate determination of the shape nanoparticles requires higher resolving power images than simpler size measurements. ISO 9276-6:2008 states that: “The optimum resolution shall not and cannot be stated absolutely but shall rather be related to the size of the element features to be determined (e.g. agglomerate branches, roughness scale). Analysis of image parameters is generally based on a digitized image. The process of digitizing an image can result in information loss because of the transformation of the continuous features innate to the particle into discrete elements of finite size — the finite resolution. For pixel errors, smaller than 5 % for a circle, the necessary pixel numbers per particle range from 100 to 200; for robust parameters, like projection area and ellipse ratio, up to 5,000 for parameters using the perimeter.”

5 Sample preparation

5.1 Sample preparation fundamental information

The quality of SEM nanoparticle size and shape measurement results strongly depends on sample preparation. The relevance of the result obtained on prepared samples to the whole amount of the raw nanoparticle material can be ensured by proper sampling and with careful preparation. There is no single best method for preparation of all nanoparticles, rather methods determined by the characteristics of nanoparticles in the raw nanoparticle material.

The measurand, quantity or property which is intended to be measured is also fundamental in sample preparation. Depending on exactly what information with what certainty shall be determined, the best sample preparation method might be different. It is up to the user to decide the most appropriate one.

The information on the health effects of various nanoparticles is not yet complete. It is possible that some nanoparticles are hazardous to health while others are not or do not cause adverse health effects following exposure. For this reason, it is recommended to always wear appropriate personal protective equipment, including disposable gloves, safety glasses, laboratory coats, filter respirators and take appropriate precautions when handling nanomaterial. Sample preparation should be carried out in a vented fume hood equipped with suitable air filters.

Tools used for preparation and handling nanoparticles shall 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 shall be taken to remove all traces of the cleaning detergent as this can impact the nanomaterial properties. If available, store tools under high-efficiency particulate air (HEPA) filtered air (e.g. in a clean enclosure/bench).

5.2 General recommendations

Nanoparticle samples are typically powders or suspensions. Powder samples are often dispersed in liquids prior to their deposition on a substrate for electron microscopy evaluation. Suspensions often need to be diluted to image discrete particles. Typically, out of a large volume of raw sample material analytical samples are taken that contain a smaller number of nanoparticles. Out of these samples a number of particles are separated and deposited on a substrate surface. Key sample preparation objectives are: use a representative sample of the raw powder or liquid, minimize agglomeration in the dispersion used for mounting the sample, minimize particle-particle associations on the support, distribute the sample uniformly across the substrate and select a substrate that enhances the contrast between particle and background.

The prepared samples shall be relevant, i.e. the size and shape distribution of nanoparticles of the small amount needed to prepare samples for measurements, the original raw sample material shall be divided in a manner that ensures that the portion taken will be representative of the whole original sample material. This is the so-called sampling. The method used to divide the sample shall be determined by the sample preparation method and shall be decided by the laboratory performing the measurements.

The dispersion of the nanoparticles over the surface so that particles do not touch each other, i.e. the particles are clearly visible in the images with their boundaries easily discernible. There should be as few particles as possible touching each other or piling up. Measuring touching particles as one particle without proper separation will introduce a measurement error. It is also desired that the particles to be not far from each other, so many nanoparticles can be observed across the acquired images.

5.3 Ensuring good sampling of powder or dispersion-in-liquid raw materials

5.3.1 Powders

It is essential to sample powder samples carefully, particularly if they are aggregated as material can redistribute under the vibrations and forces of transportation. ISO 14887:2000, ISO 14703:2008, and ISO 14488:2007 provide guidance on how to take and handle powder samples. Most powders and nanoparticles will be negatively charged in the gas phase, so loading dry samples on substrate surfaces, which are also usually negatively charged, often results in large agglomerates that are difficult to image and measure. Powder samples are prepared for SEM and STEM imaging by dispersed in a liquid phase, which is then deposited on the measurement substrate or grid.

5.3.2 Nanoparticle dispersions in liquids

Nanoparticles can be dispersed in colloidal liquids or liquid sols. Stable colloidal dispersions in the liquid phase usually require either electrostatic or steric stabilization methods to prevent agglomeration. Concentrated nanoparticle dispersions shall be diluted to reduce touching nanoparticles on the measurement substrate or grid. The stability of colloidal dispersions affects whether they remain as discrete particles or agglomerate, aggregate, or flocculate. Colloid stability depends on the particle surface chemistry, coatings or adducts on the particles, solvent chemistry, and inorganic and organic ligands in the fluid phase.

For all raw samples, it is essential to minimize particle agglomeration in the sample dispersion, and sample preparation.

5.4 Ensuring representative dispersion

Forces that affect the stability of colloidal systems include: excluded volume repulsion, electrostatic interaction, van der Waals forces, entropic forces and steric interactions. Mutual repulsion of like electrical charges on nanoparticle colloids leads to electrostatic stabilization. Electrical double layers can form at the nanoparticle/liquid interface. Due to their small size, nanoparticles can have very large surface areas compared to their volume (mass), so repulsive surface forces can overcome buoyancy forces that might lead to particulate settling. Steric stabilization of particles occurs when coatings, such as surfactants, polymers or oligomers, are attached to the nanoparticle surfaces. These coatings prevent agglomeration of nanoparticles by keeping them apart enough that attractive forces would not become effective.

There are commercial nanoparticle preparation products that have surface coatings or functionalized surfaces, which are designed to control particle dispersion in specific media. Destabilization of nanoparticle colloids in liquid dispersions can occur via a variety of mechanisms. The common methods for controlling the interactive forces between particles are selection of the dispersing liquid and using surfactants (see ISO 26824). Selection of the dispersing liquid is usually preferred, as surfactants could cover the surface of nanoparticles with thin layer of material. Depending on the size of the nanoparticles, this surfactant coating can affect the results of the characterization and measurements of the nanoparticles. Also, surfactants are susceptible to desorption from the nanoparticles or can also induce nanoparticle agglomeration with changes in temperature and liquid phase composition; it is essential to choose them with proper care.

Sample dispersion with ultrasonic treatment (sonication) is frequently required to minimize aggregation and agglomeration.

Particles can appear very different in size and shape, depending on the viewpoint. In the determination of their size and shape, it is indispensable to assess and use the information about how the 3D particles were deposited on the 2D sample surface. It is similarly important to assess and use the information related to the angle from which the images used for analysis were taken.

5.5 Nanoparticle deposition on a substrate

5.5.1 General

An important objective of sample preparation is to generate a uniform distribution of particles across the entire measurement substrate. Several common methods for depositing nanoparticles on measurement substrates are described, for example, in ISO/TS 24597, ISO 26824 and Reference [17]. The knowledge about and the number of methods of particle deposition are growing; it is advisable to explore and use the methods in pertinent literature.

Several common methods for depositing nanoparticles on measurement substrates are available (see ISO/TS 80004-3).

A preferred support has most of the following characteristics: relatively flat of the frame size selected, uniform and low background intensity across the frame size, and good contrast between particles and background. Many nanoparticles will need to be imaged and analysed to obtain a high-quality data on the particle size distribution. Particle analysis time can be reduced and data quality can improve if image analysis software is used. When preparation methods are developed for unknown samples, it can be useful to go through the entire process to determine which factors are critical for the sample at hand.

Commercial or open-source image analysis software is used to analyse the acquired images. The images shall be of sufficient quality such that individual particles can be resolved and their dimensions measured. An important objective of sample preparation is to generate a uniform distribution of particles across the entire measurement substrate.

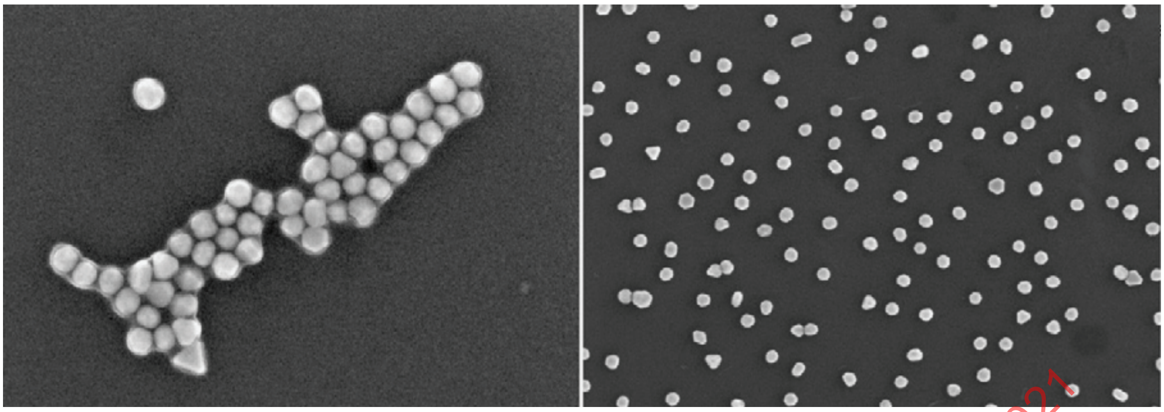
5.5.2 Nanoparticle deposition on wafers and chips of silicon or other materials

This is one of the easiest and often the most economical way of preparing sample for SEM measurements. Blank silicon wafers and chips have good electrical conductivity and are sufficiently flat and allow for high-quality secondary and backscattered electron-based imaging and measurements. The chips shall 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 water (H_2O) and ammonium hydroxide (NH_4OH). The beaker is then exposed for 5 min with low intensity ultrasonic cleaning with a non-heated clean water bath. Then the chips are rinsed with clean water and an ethanol (C_2H_6O) jet and dried with a blast jet of pressurized oil-free nitrogen gas. 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 shall be completely free from any visible particles or discoloration. Another procedure is described in Reference [17].

- Etch and clean the surface using a strong cleaning agent, such as the so-called acidic piranha solution (mixture of 3:1 ratio of 30 % hydrogen peroxide (HOOH) solution added to concentrated sulphuric acid (H_2SO_4 vitriol)) [35r] or a mixture of ethanol (420 ml), sodium hydroxide, NaOH (70 g) and water (280 ml).
- The surface can be positively charged by using poly(l-lysine) or silane coupling agents. A variety of silanes can be used. For example, immerse the surface in a solution of aminopropyltrimethoxysilane (APTES, 1 % w/v) dissolved in 95 % ethanol and 5 % water mixture, followed by four ethanol rinses. The negatively charged nanoparticles adhere to the positively charged surface so that most of them are well separated. The substrates are dried and heated to 100 °C to remove surface water.

It is not unusual that a positively or negatively charged capturing layer of molecules (e.g. poly-L-lysine) can be used well for grabbing and adhering the nanoparticles to the surface of the chip. Within several minutes of room air exposure, an approximately 1,3 nm thin, so called “native oxide” forms on the surface of the Si chips, which can be sufficient for the preparation of the capturing layer. However, it has been shown that a thicker oxide layer is preferable. A one-minute wet oxygen plasma treatment of the Si chips forms thicker layer of silicon dioxide. The chips with their shiny surface facing up are to be treated in a plasma etcher with 40 W of power at 2×10^{-1} millibar (20 Pa) oxygen or air pressure.

The oxygen plasma oxidizes and removes organic contaminants from the chip surface and increases 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 surface loses its ability to conduct away charge generated by the primary electron beam of the microscope. Figure 4, a) shows sample preparation examples for simple dilution and droplet deposition and air drying type of sample preparation of nanoparticles on the left and the same raw material prepared with the procedure described above,[17] yields much more desirable samples to measure on the right. The sample on the left has the particles too close to each other, surrounded by some residual colloidal material that is also on the surface of the Si substrate producing a noisy uneven background. The use of capturing layer allows for thorough rinsing away the gooey colloidal material, which results in clean nanoparticle surfaces, and better, more even contrast between the particles and the substrate.



a) Secondary electron images of 30 nm colloidal gold nanoparticles deposited on Si chip surfaces after simple dilution and drying only

b) Secondary electron images of 30 nm colloidal gold nanoparticles deposited on Si chip surfaces prepared using a layer of electrostatic capturing molecules

NOTE Secondary electron images of 30 nm colloidal gold nanoparticles deposited on Si chip surfaces after simple dilution and drying only (left), resulted in undesired sample. The same nanoparticle raw sample material prepared using a layer of electrostatic capturing molecules (right) yielded a much more favourable sample. The horizontal field width is 640 nm (left) and 1,2 µm (right).

Figure 4 — Secondary electron images of 30 nm colloidal gold nanoparticles deposited on Si chip surfaces

Nanoparticle liquid suspensions shall be completely dried before the sample can be evacuated for SEM observation. During drying uneven distribution and aggregation of nanoparticles can occur. To prevent aggregation and to achieve representative distribution of the nanoparticles across the deposited area, the liquid shall be removed carefully. Simple methods use a piece of filter paper placed gently to the top or to the edge of the nanoparticle suspension bead deposited over the substrate. Once most of the liquid is removed with the filter paper, the sample on the substrate is dried under ambient conditions, i.e. room temperature air or in an inert (N_2) gas. Another possibility is to spread the nanoparticle liquid suspension very thinly over the substrate, so aggregation during drying can be avoided. It is indispensable to carry out these steps so that representative distribution of the nanoparticles across the deposited area is ensured.

Capturing nanoparticles on a substrate by electrical charge depend on the size of the particles, so for example, the capturing efficacy of 10 nm and 100 nm diameter particles are different. If particle size distributions or bi- or multimodal, complex particle samples are to be measured, sample preparation by this method should be carried out such that samples are as representative as possible. Comparison with the results of other particle size measurement methods is recommended.

5.5.3 Nanoparticle deposition on TEM grids

Transmission electron microscope (TEM) grids can be used well for nanoparticle imaging and measurements in SEMs also. While the sample preparation might be simpler with the use of Si chips, the lack of bulk material under the nanoparticles can be advantageous, partly because the particle-related portion of the generated contrast can be higher. For very small particles this could be the opposite, the signal generated from the particles alone is weaker and longer acquisition times are necessary. Additionally, TEM grids allow for the use of secondary, backscattered and transmitted electron-based imaging and measurements in many SEM simultaneously. TEMs work at high electron landing energies, typically 60 keV to 300 keV. The 5 keV to 30 keV landing energy operation of the SEM in STEM image acquisition mode can be very advantageous for nanoparticle imaging and measurements, as these low energies might be still sufficient to generate useful transmitted signals. The simplest TEM grids are copper mesh with thin support films. Carbon and silicon monoxide coatings are common and have greater mechanical strength and stability compared to plastic films. There is growing variety of TEM support grids and films available from various vendors; in many cases speciality, more expensive substrates prove to be the best choice.

- Carbon coated grid: Disperse the nanoparticles in a non-aqueous or low salt concentration media. Pipette a small amount (~5 µl) of suspension directly on the coated grid and allow drying. The nanoparticles can agglomerate during the drying process. Should this occur, different solvent media can be selected, usually by considering the Hamaker constant for the solid and liquid pair.
- The surface can be positively charged by using silane treatments. Incubation of the nanoparticle suspension on the coated grid should allow the negatively charged nanoparticles to adhere on the positively charged coated grid. Several methods are available (see ISO 9276-6 and ISO/TS 24597).

Place the TEM grid with the sustaining membrane up on a filter paper and spread the nanoparticle liquid suspension over it. The extra liquid can be absorbed by the filter paper, and the suspension on the grid is dried naturally. It is indispensable to carry out these steps so that representative distribution of the nanoparticles across the deposited area is ensured. Comparison with the results of other particle size measurement methods is recommended.

5.6 Number of samples to be prepared

The number of samples needed to obtain statistically valid measurement results on nanoparticle size and shape distribution depends on the sample material to be investigated, the measurands, the sample preparation and the required measurement uncertainty. A major factor affecting measurement uncertainty of particle size distributions is the number of particles that need to be imaged and measured to achieve the required degree of confidence in coefficients modelling the sample distribution, i.e. the sample mean and standard deviation. Typically, from couple of hundred to a couple of thousands of particles need to be imaged, analysed and reported. For the measurements of the shape of nanoparticles much higher resolution images are needed (see 5.3), therefore the number of images to be acquired can be very large.

5.7 Number of particles to be measured for particle size determination

The number of particles measured shall be determined based on the particle-size distribution and the desired confidence limits. Assuming the particles are log-normally distributed, the required number (N) of particles with a given error (δ) and a given confidence limit is estimated in accordance with Formula (1):

$$\log N = -2 \log \delta + K \tag{1}$$

where K is numerically determined by the confidence limit, particle distribution and other parameters.

Statistical error caused by the number of sample particles has been studied using computer simulation and compared with the theoretical equation developed by Masuda and Iinoya (see ISO/IEC Guide 99 and Reference [6]). The theory assumes a log-normal size distribution and gives general analytical solutions for the distribution of sample-mean diameter and the number of particles required. A computer simulation study has confirmed the theory. Therefore, the error caused by an inadequate number of sample particles or number of particles required in a measurement can be analytically estimated.

An example application of the theoretical equation concluded that about 61,000 particles are required to get the mass median diameter within 5 % error with 95 % probability for the powder particle sample with the standard deviation of 1,6. However, if the evaluation based on the mean volume diameter is applied, the required number drops to only 6,100 particles. The number of particles required can be reduced somewhat both by changing the evaluation basis and by increasing the assigned admissible error. Even if the geometric standard deviation of powder remains at 1,6, an evaluation based on the mean-volume diameter and a permissible error of 20 % requires only 381 particles. However, size measurement results are usually transformed into a mass basis, and therefore, the mass median diameter should be adopted as the evaluation basis.

NOTE See ISO 13322-1.

5.8 Number of particles to be measured for particle shape determination

The physical and chemical behaviour of nanoparticles strongly depend on their shape. In most cases of production processes and product performance, the shape of nanoparticles is even more important than their size. Shape-based image processing can be a very powerful way of assessing various properties of nanoparticles and nano-engineered materials. The measurement of particle shape is essentially determining a two-dimensional descriptor of a three-dimensional object. There are various definitions and standardized shape factors available that can provide additional descriptors to a particle population of interest. The number of particles measured for shape shall be determined based on the particle-shape descriptor, the particle-shape distribution and the desired confidence limits.

6 Qualification of the SEM for nanoparticle measurements

To find out whether a given SEM can acquire the images sufficient for the size measurements of nanoparticles, the qualification of the SEM as a measuring system shall be carried out first to identify the limits of performance of the given SEM, and to determine the uncertainty components of measurement results caused by various imperfections of the SEM and its environment.

Many SEMs are in use for taking images, others also for obtaining repeatable and accurate quantitative information about a wide variety of sample properties, including the size of nanoparticles. Suitable quality SEM images can be excellent inputs for the analysis, the quantitative assessment of the size and shape distribution of nanoparticles. Almost all SEMs can carry out the necessary imaging, but their limits are certainly set by:

- spatial resolution (the primary electron beam focusing ability, image sharpness);
- drifts (of the sample stage and of the electron optical column);
- cleanliness (absence of disturbing beam-induced contamination);
- scale and linearity (in both horizontal and vertical directions);
- noise;
- primary electron beam current;
- few more instrument setting-related parameters.

The required measurement uncertainty and the sample itself are also determining factors. Depending on these, the lower limit can be at about 10 nm, a few nm or even smaller. Annex A shall be followed for proving suitable SEM performance.

7 Image acquisition

7.1 General

The nanoparticles can be so small, that some SEMs cannot generate images with sufficiently high magnification and high resolution to accurately reveal their size and shape. This is especially true for 10 nm or smaller size particles. To obtain good results, it is essential to collect images with suitable quality and use appropriate procedures to extract the information sought. In the SEM, just like with any other high-quality imaging systems, it is essential to choose a number of parameters properly to obtain adequate quality data and images.

High-performance SEMs can have spatial resolution of 1 nm or better at optimum electron optical system and electron beam parameter settings. For the best results the accelerating voltage, the beam current and the pixel dwell time shall be set to an optimum, along with the sample working distance. These parameters depend on the SEM and shall be used throughout the study to ensure good image-to-image consistency. The contrast and brightness of the images shall set so that a good balance of the particle detail and distinction from the background can be achieved.

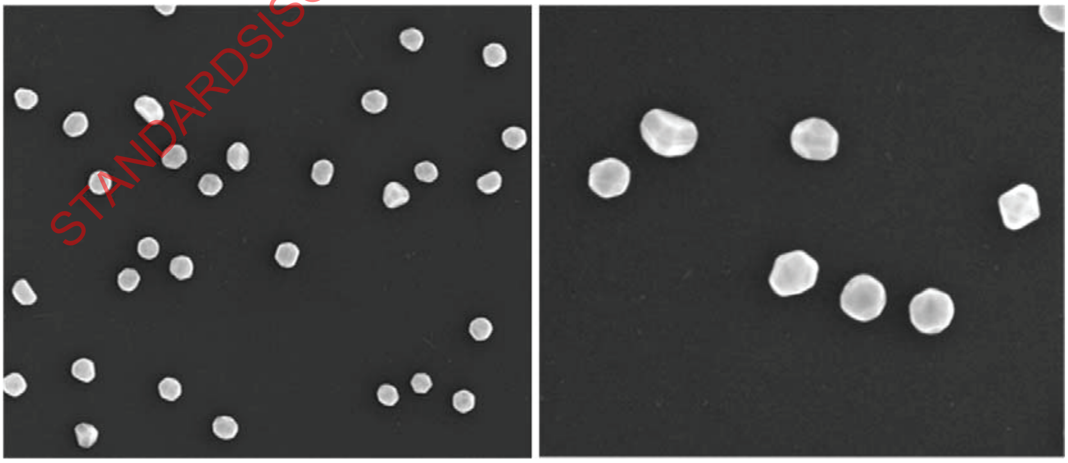
Generally, secondary or transmitted electron mode operation is suitable for obtaining the best quality, highest resolution images that are appropriate for measurements of nanoparticle size and shape distribution.

Before the whole measurement process takes place, it is advantageous to check the quality all samples for aggregation and the particle size distribution should be roughly estimated. The whole process might involve the measurement of several thousands of particles, so it can be time consuming. This is the phase in the measurement process when the optimal observation and image acquisition conditions, and settings of SEM should be determined. The settings of the SEM shall be so that all particles (including small particles) have sufficient contrast for particle size measurement. The particles in the image frames should be sufficiently isolated, i.e. far away from each other.

There is one more important matter in image acquisition: just before starting the acquisition of the images used for the measurements of nanoparticle size and shape distribution, align the SEM column according to the manufacturer's procedures to minimize stigmatism and maximize image sharpness, i.e. achieve the best resolution. The best focus settings shall be regularly checked throughout the acquisition of all images used for nanoparticle size and shape determination.

The examples given in 7.1 and in Figures 5 and 6 illustrate the image quality and general appearance needed for reliable measurement results. The SEM images of the NIST 60 nm, 30 nm and 10 nm nominal size colloidal gold particle reference materials (RMs) were used. Most of these samples were imaged at 250,000 times magnification, which provides a good balance between high spatial details and the number of particles within an image frame [Figure 5, a) and b)]. Figure 5 right reveals the facets of the nominally 60 nm size particles but shows only 7 particles. It is possible to take more images at higher magnifications, but other considerations such as rate of contamination, acquisition times, drifts, might lead to somewhat lower optimized magnification images. Out of the nominally 30 nm size particles a lot more fit into the same 250,000 times magnification image, see Figure 6, b). Too low magnification images might not have sufficient pixel resolution, so the size measurement results will be less accurate, and shape measurements might not have sufficient number of pixels.

For the nominally 10 nm size gold nanoparticles, it was necessary to use 500,000 times magnification to ensure sufficient pixel resolution [Figure 5, a)]. To meet the demand of measurement precision at these small fields of view, a digital capture resolution of 2,048 square pixels by 1,886 square pixels was used for all images. At 250,000 times magnification, such image resolution provides an area approximately 100 pixels by 100 pixels for a nominally 60 nm particle, and an area of 50 pixels by 50 pixels for a nominally 30 nm particle. Similarly, at 500,000 times magnification the same image resolution yields an area of about 35 pixels by 35 pixels for a nominally 10 nm particle.



a) SEM image of NIST RM 8013 colloidal gold particles at 250,000 times magnification

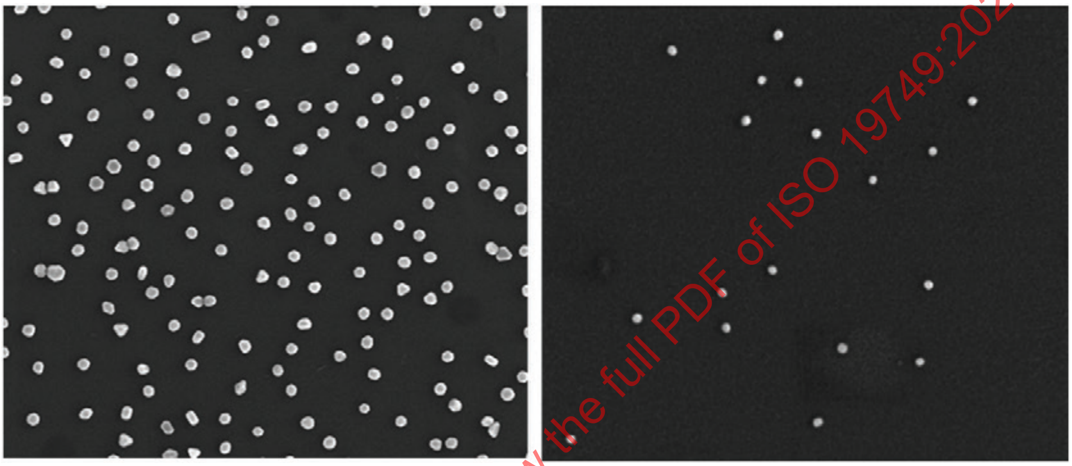
b) SEM image of NIST RM 8013 colloidal gold particles at 500,000 times magnification

NOTE The horizontal field width is 600 nm (right). The horizontal field width is 1,2 µm (left). Note the facets of the particles.

Figure 5 — SEM images of NIST RM 8013 (nominally 60 nm) colloidal gold particles

For particle shape measurements, higher pixel resolution images are necessary than for particle size measurements. Figure 5 a) and Figure 9 show that modern SEM with sub-nanometer focusing ability can acquire images that allow for the excellent quality measurements of shape of nanoparticles.

The optimal image acquisition settings, the number of pixels recorded depend on the task at hand, it is up to the user to prove that these were chosen properly.



a) SEM image of NIST RM 8012 colloidal gold particles at 250,000 times magnification

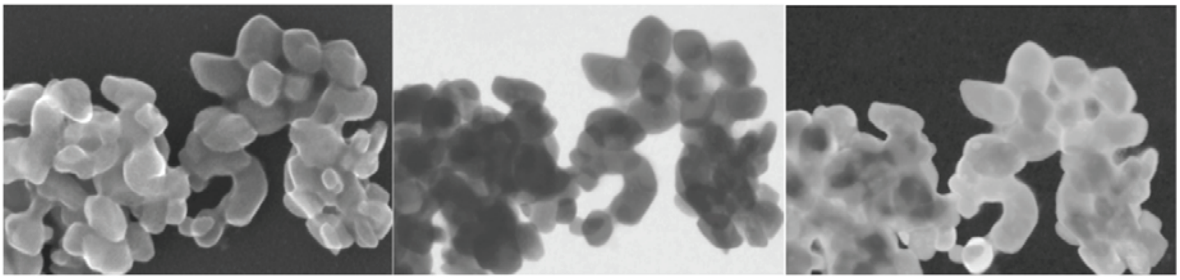
b) SEM image of NIST RM 8011 colloidal gold particles at 500,000 times magnification

NOTE The horizontal field width is 1,2 µm (left). The horizontal field width is 600 nm (right).

Figure 6 — Typical SEM image of NIST RM 8013 (nominally 30 nm) colloidal gold particles

There is no single best way of acquiring SEM images, because the optimum settings are dependent on the instrument and the sample, and the required uncertainty. It is up to the SEM operator to set the image acquisition parameters correctly. Since piled up particles and the particles that are at the borders of the image frames generally cannot be used for automated analysis, the number of individual image frames to capture also varies. There is no scientifically valid, universal number of particles that it is necessary to acquire and analyse. The actual, optimized, case by case number depends on a set of parameters, and it is essential not to measure too few particles or too many, because in the first case wrong results will arise, and in the second the time spent with carrying out needless measurements will result in unnecessary expenses.

The signal used for imaging in the SEM can also have significant influence on the images and potentially on the measurement results. Figure 7 shows images of titania (titanium oxide, TiO_2) particles taken with an SEM that is equipped with a transmitted electron detector. The images on the middle and on the right, are akin to images taken of TEMs with much more energetic electrons. The bright-field [Figure 7, b)] and dark-field [Figure 7, c)] STEM images are possible at 15 kV accelerating voltage because the particles are small.



a) Secondary electron electron image of a titanium oxide agglomerate

b) Bright-field transmitted electron image of a titanium oxide agglomerate

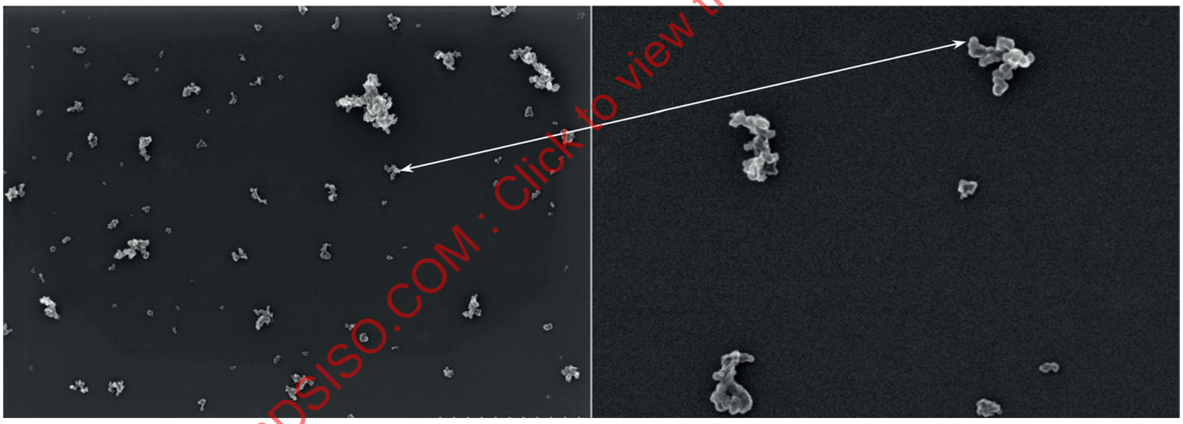
c) Dark-field transmitted electron image of a titanium oxide agglomerate

NOTE 424 nm HFW images.

Figure 7 — Electron images of a titanium oxide agglomerate

These images show different contrast than the surface-related SE images, and reveal additional information that are advantageous in differentiating and segregating *primary particles* (3.1.4) in the agglomerate.

Figure 8 shows a high pixel resolution image and a portion of it in pixel-to-pixel digital magnification. The high pixel resolution image of carbon black agglomerate sample has many particles across it, and suitable for measuring many particles per frame.

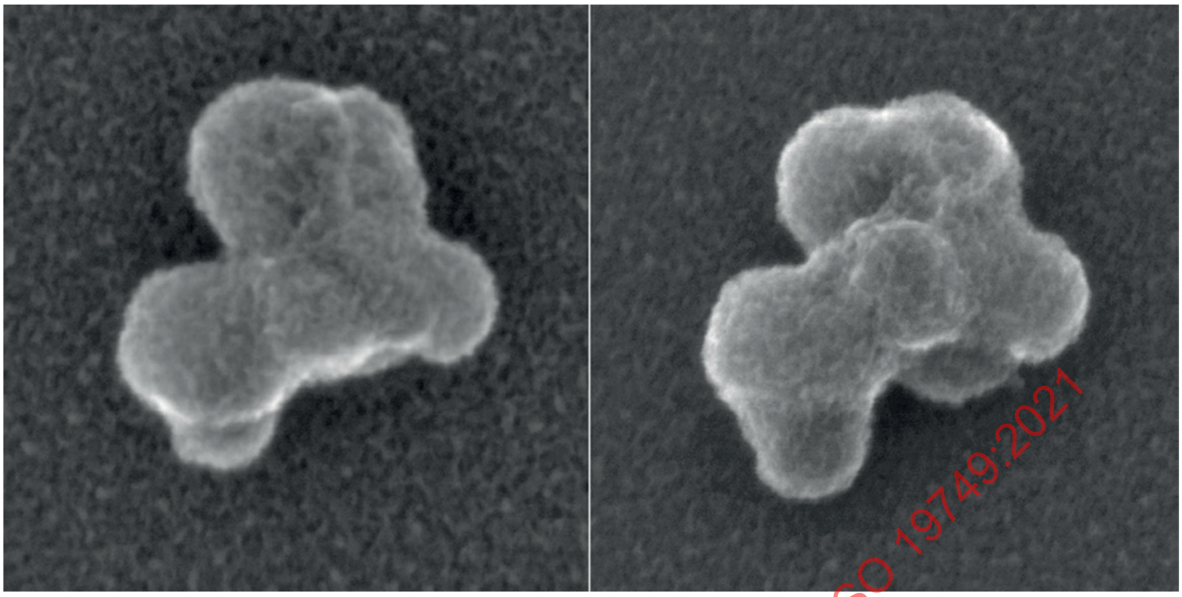


a) High (5,120 by 3,840) pixel resolution image of a carbon black agglomerate sample

b) Portion as pixel-to-pixel digitally magnified image of a carbon black agglomerate sample

Figure 8 — Carbon black agglomerate sample

The images above are examples, the actual images in practice can differ in appearance.



a) High-magnification SEM image of the same carbon black particle taken at −14° angle

b) High-magnification SEM image of the same carbon black particle taken at 15° angle

NOTE The horizontal field width is 133 nm.

Figure 9 — High-magnification SEM images of the same carbon black particle

The two images of Figure 9 illustrate the difficulty of complete three-dimensional measurements. Ultimately one would need many images taken from many directions, and a sophisticated solution to merge those results into one 3D rendered representation of the particle. The shapes and sizes measured based on these two images are significantly different, but if the nanoparticles land on the substrate in random directions, the average of a sufficiently large number of particles will approach a shared average value, which is easier to measure and likely more relevant to industrial or commercial applications.

7.2 Setting suitable image magnification and pixel resolution

The SEM magnification shall be set by considering the range of particle sizes to be analysed and the image capture software to be used for analysis. The field of view should be large enough to include the largest particles while having sufficient resolution to distinguish the smallest particles from noise. Consider a square-shaped particle has a length dimension of 10 nm and an area of 100 nm^2 for which the resolution is 0,5 nm/pixel. A 1-pixel error in measuring the length dimension of the particle would give a 5 % error in a diameter. A 4-pixel error in measuring the area would give a one percent error in the particle area. The ASTM standard for carbon black provides a starting point for setting the operating magnification. Table 1 shows the error in diameter measurements, as a percent, for a 1-pixel error in reporting a particle length. Note that length measurement errors can occur during image capture or particle analysis. Operators can determine the operating magnification to achieve the needed measurement uncertainty for a descriptor by using the steps provided in this protocol.

Table 1 — Suggested starting points for setting instrument magnification for different sized nanoparticles

Particle size range nm		Resolution nm/pixel		Uncertainty in diameter measurement for a 1-pixel difference in image %	
low	**high**	**low**	**high**	**low**	**high**
14	21	1,5	2,0	11 %	10 %
22	26	2,0	2,5	9 %	10 %
27	37	2,5	3,0	9 %	8 %
38	49	3,0	4,0	8 %	8 %
50	62	4,0	5,0	8 %	8 %
63	100	5,0	6,0	8 %	6 %
101	199	6,0	12,0	6 %	6 %
200	400	12,0	20,0	6 %	5 %

For pixel errors smaller than 5 % for a circle, the necessary pixel numbers per particle range from 100 to 200 (see ISO 9276-6). At a specific magnification, the number of pixels per nanometer can be estimated, which allows estimates of the number of pixels per square nanometer. Thus, it is often possible to determine, during image capture, whether the error on smaller particles is sufficient, and adjust the magnification if needed. If the particle size distribution is broad, it can be necessary to adjust the resolution to provide accuracy for small particles. In some cases, it can be necessary to take images at two different magnifications to generate data with good size accuracies for large and small particles.

The magnification and pixel resolution shall be set by considering acquisition speed, i.e. pixel dwell time, pixel coverage of the smallest particles, and the number of particles within the acquired images. The pixel dwell time shall be set so the sufficiently noise-free images can be acquired. The number of pixels will set the frame time, which shall be minimized, while acquiring sufficiently noise-free images. All these are also influenced by the required measurement uncertainty, larger acceptable uncertainty measurements can be carried out on quickly acquired, noisier images than those that need to be performed with smaller uncertainty.

Commonly used distributions require at least two parameters, one that describes the size and the other that describes the shape of the particles. For each application, the relative standard uncertainty needed for the mean and standard deviation of the distribution will set the number of nanoparticle images required. The methods for curve-fitting distributions to particle size data provide relative standard uncertainty for each of the two descriptors (size and shape). If the fitted distribution provides a good description of the data, one can determine the effect of the number of particles analysed on the relative standard uncertainty for the samples and adjust the number of particles counted to produce data sets with the needed accuracy.

8 Particle analysis

8.1 Particle analysis fundamental information

The overall objective for measuring particle size and shape distributions is to transform digital micrographs from grey-scale images into a binary image consisting of discrete particles and background and using various criteria generate the needed information with an uncertainty statement.

The analysis should be representative of the sample. In general, the sample should be split so that measurements can be made on three or more parts. Statistical analysis of the data should demonstrate whether the samples are truly representative of the whole raw sample (see ISO/TS 80004-2). A more complete discussion is provided by ISO 13322-1:2014.

The SEM generates projected images of particles deposited onto an electron-transparent substrate, in the case of TEM type samples or not, as with Si chips. Both internal structure and surface morphology can contribute to the image acquired. See, for example Figure 5 b), where contrast – due to crystal stacking – is also shown beyond the facets of the triangular particle.

Since many nanoparticles are needed to be measured for high-quality particle size distribution measurements, the work shall be carried out with image analysis software. Both commercial and open source software are available. The effects of sample size on the measurement uncertainty of mean particle diameter has been well-studied, with larger numbers of particles resulting in lower measurement uncertainties. Measurement uncertainty for the distribution parameters, sample mean and standard deviation should also be lower for larger number of particles.

No image analysis method or software is perfect. Generally, it is much easier to analyse completely isolated particles than aggregated ones where the separation of the individual particles in many cases is difficult. With aggregated particles, some additional measurement errors can arise:

a) two or more aggregated particles are counted as one particle,

b) a part of aggregated particles is perceived as a particle,

c) an area enclosed by particles is taken as a particle, and

d) shot noise is mistaken for a particle.

The item a) given above leads to an increase in particle size (e.g. maximum Feret's diameter), the items b), c) and d) increase the number of non-existent small particles.

8.2 Individual particle analysis

ISO 13322-1:2014, sublauses 5.2 through 5.3 and Clause 6 provide guidance on counting procedures, particle edges, particles cut by the edge of the measurement frame, touching particles, measurements, calibration and traceability, and distortion for individual (manual) particle analysis. The measurements of the size and shape of many individual particles are more demanding than measuring the average of many of them. Annexes B, C and D show examples for the results of nanoparticle measurements.

8.3 Automated particle analysis

There are many commercial software for automated and manual particle analysis, these might cost several hundreds or thousands of US dollars. ImageJ, Fiji[1] is in the public domain, therefore it is free.

The overall objective for measuring particle size distributions is to transform the digital micrograph from a grey-scale image into a binary image consisting of discrete particles and background. Each pixel value shall be classified by thresholding the image and tabulating the number pixels for each nanoparticle. Imaging threshold operations are subject to user bias and automation is preferred. When the background values are not uniform across the image (caused by the presence of, for example, dust particles, residues from improper cleaning), automated methods can fail. The user would need to manually select threshold values for different regions of the image (see ISO/TS 80004-2). Most of the data reported in the case studies have been analysed using a public domain software, called ImageJ.

After thresholding is completed, nanoparticles shall be identified. A nanoparticle will consist of many contiguous pixels that meet the thresholding criteria. There possibly are some background pixels with binary values that exceed the thresholding criteria. These will usually be singular or small numbers of pixels, which should be eliminated from analysis. In ImageJ, there is a 'despeckle', an erode, and a dilate function. Applying this combination of steps usually removes artefacts created by the software or noise. A visual comparison of the original image and the processed image should confirm that appropriate artefacts were removed. Similarly, some pixels within the nanoparticle can have values below the threshold limit. These holes shall be 'filled' by the software to permit analysis. Some specific

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1) ImageJ and Fiji are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

examples on automated analysis are provided by PCC-15 (see ISO/TS 80004-3), NIOSH/DUNE, and a good practice guide (no. 119, see Reference [22] pp. 36-40). There are many free plug-in programs that can be used in ImageJ or Fiji to detect, count and measure shape and number of particles, one such example is Graylevel Watershed that could speed up the time to results.

8.4 Automated particle analysis procedure example

This protocol assumes that all images were taken in digital format. The procedure steps for ImageJ software are:

- Create working copies of all images/frames (preserve the original unmodified images).
- Open ImageJ and open the image 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 artefacts that might affect contrast or particle analysis.
- Check and correct brightness and contrast to ensure that all images have histograms centred and wide enough to cover at least 80 % of the possible gray levels.
- The thresholding operation can result in frame files with single pixel artefacts 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 artefacts 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.
- Touching particles should not be addressed by using automated separation algorithms (watershed, in the case of ImageJ). Rather, all particle analyses should be recorded, and touching particles should be removed by sorting from the spreadsheet of the results.
- Set the list of measurands desired (such as area, shape descriptors, Feret diameter, fit ellipse, and limit to threshold).

  NOTE 1 Several size and shape descriptors will help identify imaging and measurement issues as well as assist with the characterization of the particle sample under study.

- Analyse 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).

  NOTE 2 Additional example with guidance and short explanation is provided in Annex E as a macro for ImageJ.

Imaging threshold operations are subject to user bias, so automation is preferred. When the background values are not uniform across the image (caused by, for example, the presence of dust particles, residues from improper cleaning), automated methods can fail. The user would need to manually select threshold values for different regions of the image (see ISO 9276-6).

9 Data analysis

9.1 General

There are several applications for statistical analysis of particle size data: assessment of data robustness within one measurement and among several measurements, fitting reference models to the size distributions, and assessment of grand statistics for several measurements. Table 2 shows typical examples used in many cases. Measurement uncertainties are computed from coefficients of variation determined from the fitted parameters of each data set in the measurement's data set.

Table 2 — Typical statistical analysis methods

Statistical method	Reported statistics
	***p*-values**
ANOVA	There is no difference between the mean value of the descriptor for a specific data set and the grand mean value of the descriptor for all data sets.
Pair-wise ANOVA	There is no difference between the mean values of the descriptor for the data set pair.
Bivariate analysis	Pair-wise comparison of descriptor cumulative distributions: there is no difference between the descriptor distributions of the data set pair. Size-shape distributions: there is no difference between the descriptor distributions of the data set pair.
	Fitted scale and width parameter estimates plus their standard errors
Nonlinear regression; maximum likelihood	C_{v} of fitted parameters (scale and width); measurement uncertainties computed from several C_{v}s

9.2 Raw data screening: detecting touching particles, artefacts and contaminants

It can be useful to perform data screening prior to full analysis of data sets. For example, touching particles should be removed from the data set, artefacts should be identified and removed, and contaminants should be identified and analysed separately. Raw data screening workflow can be developed at the inception of a particle size and shape distribution measurements. This can ensure that the proper measurand is measured indeed with optimal use of resources and time.

9.3 Fitting models to data

Three reference models commonly fitted to cumulative particle size distribution data are: lognormal, Rosin-Rammler-Bennett, and Weibull. These and the normal distribution can be compared to the cumulative frequency data. ISO 9276-3 provides guidance on fitting data to reference models (see ISO 9276-1).

9.4 Assessment of measurement uncertainty

9.4.1 General

NOTE Standards organizations require statements on measurement uncertainty. There are differences between Comité Européen de Normalisation (CEN), International Standards Organization (ISO), and American Society for Testing and Materials (ASTM) approaches, although all of these report pooled uncertainties. CEN uses the ISO 5725 series[6] and ISO Guide to the expression of uncertainty in measurement (GUM methods, which generally qualify the test results. ASTM uses precision and bias estimates (ASTM E456[42]), which generally qualifies the test method. Versailles Project on Advanced Materials and Standards (VAMAS) generally follows CEN guidelines. The CEN/ISO approach is: define the measurands, identify all sources of uncertainties in the test, quantify each source of uncertainty with a probability distribution, calculate the combined (pooled) standard uncertainty, and estimate the expanded uncertainty at the 95 % confidence interval. Some references provide great detail on measurement uncertainty for measurement of particle size alone.

Type A uncertainty components include those that are evaluated by statistical methods, such as precision. Type B uncertainty components are evaluated by other means, such as calibration errors or effects of temperature variations. The Type A components are: the degree of closeness of measurement to the reference value, and the precision (the degree to which different measurements show the same result). Precision is usually separated into two components, repeatability, the variance observed for the same operator and same instrument but different conditions (sample preparation or day of testing, for example) and reproducibility, the variance observed due to the same process being performed by different operators on different instruments (as in this case study). When a reference material is being investigated for its certified descriptor, the trueness for each laboratory can be computed [the difference between the certified value and the average descriptor value for each laboratory (bias)].

9.4.2 Example: Measurement uncertainty for particle size measurements

For a size descriptor, the pooled measurement uncertainty, $u_c(x)$, is based on reproducibility $u(ir)$, the trueness, $u(t)$, and the instrument calibration error, $u(c)$. $U(ir)$ and $u(t)$ are Type A uncertainty components while $u(c)$ is a Type B uncertainty component. The pooled measurement uncertainty is the square of the sum of the squares of its individual components:

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

In most SEM-based particle measurement cases, there are more components of measurement uncertainty, as it is shown in Table 3. It shows a spreadsheet of a 60 nm size nanoparticle measurement, with typical uncertainty components. Depending on the particular case, there might be more components. In this case, all components were assumed to be uncorrelated and their values' squares were added together in the uncertainty calculation formula (Cell 20).

Table 3 — SEM-based particle size measurement uncertainty components

SEM particle measurement uncertainty calculator

Component	**Source/reason**	nm	%	**Measured (M) or Estimated (E)**	**Type A or Type B**
Mean particle size		60		M	A
Uncertainty due to					
X, Y calibration of the SEM	Standard deviation of nanolattice	0,1		E	B
X, Y calibration nanolattice	Uncertainty of C-AFM measurement of nanolattice	0,03		M	A
Drift of stage and beam	Drift maps and fast scan and slow scan comparison	0,3		E	B
Focus and astigmatism	Beam geometry	0,5		E	B
Global thresholding	Background unevenness	0,3		M	A
Repeatability	Standard deviation of repeated measurements	0,5		M	A
Threshold correction	Dependant on particle material composition	1,3		E	B
Pixel resolution	The particle is represented by a pixellated image	0,8	1,3	E	B
Greyscale resolution	Greyscale image binarised by thresholding	0,6	1	E	B
Incertitude		**1,83**	**3,05**		
Incertitude étendue k **= 2**		**3,66**	**6,10**		
Type in the values					

Some of these uncertainty components might be correlated, if so, a more complex way of calculation of the expanded measurement uncertainty calculation shall be carried out.

9.4.3 Bivariate analysis

Bivariate analysis can be used to compare two-dimensional data sets independently from an assumed model. Considering both types of descriptors will be important to characterization of such materials. This method is a nonparametric test for equal distributions in high dimension, testing the composite hypothesis of equal distributions when the distributions are unspecified. Several types of two variable comparisons can be analysed by this method: empirical distributions (descriptor – cumulative distribution data) and descriptor-descriptor data (size-size, size-shape, or shape-shape). The bivariate method is very useful for statistical comparison of data sets but does not provide metrics that can be used to compute the measurement uncertainty of descriptors.

10 Reporting the results

The report of the measurements derived from the application of this document shall comply with the corresponding requirements of ISO/IEC 17025. The SEM-based particle measurement report shall contain information about:

- case or study identification, organization, laboratory, address, author(s), date;
- sample preparation, materials, substrate, placement and drying method, sample identification, personnel;
- SEM instrument, type, identification, calibration, settings, operator;
- image acquisition, analysis, dates, software/method, manual or automated acquisition;
- image acquisition conditions, magnification, nm/pixel, image pixel size, signal-to-noise ratio;
- itemize all measurands analysed, number of particles reported, number of image frames;
- particle analysis, thresholding conditions, minimum particle area (>200 pixels), number of image frames, particles;
- data analysis, raw data screening, software/method, detection of touching particles, descriptors, ranges for detection and differentiation;
- detection of artefacts, descriptors, ranges for detection and differentiation;
- retained particles, descriptors, ranges for detection and differentiation;
- average yield, the percentage of particles retained;
- repeatability and/or reproducibility;
- software/method, Software/ANOVA; bivariate analysis; other;
- repeatability (or reproducibility), p-value for grand mean analysis; percentage similar p-values for pairwise analysis;
- descriptor selection, 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, estimates and standard errors, C_v %;

- measurement uncertainty;
- descriptor residual standard error, descriptor parameter residual standard error if computed;
- inter-laboratory measurement uncertainty, if computed;
- residual deviations; correlations, software/method, residual standard deviation, if computed;
- plots: residual deviations, quantile, residual deviation plot, quantile plot, showing range over which, the model fits the data;
- correlations found among descriptors.

Depending on the purpose of the study, some of these can be omitted.

See Annex G example for reporting the results of a case study on well-dispersed bimodal (18 nm and 82 nm size) SiO_2 nanoparticles.

Annex A
(normative)

Qualification of the SEM for nanoparticle measurements

A.1 Background

SEMs are very versatile, widely used instruments. While they are easy to operate and provide results quickly, there could be notorious problems, that hinder operating them at their best performance, which is necessary for nanometer-scale measurements to achieve excellent repeatability and accuracy. The most bothersome are unintended motions of the sample stage and the primary electron beam, geometry distortions, wrong scale, image blur (lack of sharp focus), noise and electron beam-induced contamination. Quantification of these essential performance parameters is necessary to ensure that all SEMs perform at or better than their manufacturers specifications, and reliable nanoparticle measurements can be carried out. Quantified knowledge helps in the calculation of measurement uncertainty, and in necessary repairs.

A.2 General

Many SEMs are in use for taking images, others also for obtaining repeatable and accurate quantitative information about a wide variety of sample properties, including the size of nanoparticles. Suitable quality SEM images can be excellent inputs for the analysis, the quantitative assessment of the size and shape distribution of nanoparticles. Almost all SEMs can carry out the necessary imaging, but their limits are certainly set by:

- spatial resolution (the primary electron beam focusing ability, image sharpness);
- drifts (of the sample stage and of the electron optical column);
- cleanliness (absence of disturbing beam-induced contamination);
- scale and linearity (in both horizontal and vertical directions);
- noise;
- primary electron beam current;
- few more instrument setting-related parameters.

The required measurement uncertainty and the sample itself are also determining factors. Depending on these, the lower limit can be at about 10 nm, a few nm or even smaller.

A.3 Measurement of spatial resolution

The spatial resolution of the SEM, which is related to its ability of focusing the primary electron beam, is one of the most often reported performance parameters. It is related to image sharpness, which is easier to measure objectively. Without proving that the SEM is meeting or exceeding its specified resolution performance, it is not possible to carry out nanoparticle measurements with the smallest uncertainty. For the purposes of this document, both the SEM manufacturer's resolution measurement procedure and the pertinent ISO procedure shall be followed, which is given in ISO/TS 24597:2011. Carry out at least seven measurements using images acquired at the landing energies specified by the SEM vendor for the performance of the SEM, determine the average, the standard deviation and the correlation among the results. Include one typical SEM image for each specified landing energy.

Annex F and the ISO 19749 Qualification of the SEM for nanoparticle measurements excel spreadsheet has useful examples.

NOTE The ISO 19749 Qualification of the SEM for nanoparticle measurements excel spreadsheet is provided in a machine-readable file at: https://standards.iso.org/iso/19749/ed-1/en.

The results of the measurement of the spatial resolution of the SEM shall be included in the report of nanoparticle measurements. Determine the Type A or Type B uncertainty related to spatial resolution of the SEM and use it in the calculation of the accuracy of the measurement results.

A.4 Measurement of drifts

Drift of the sample stage and of the electron optical column can seriously distort the SEM image of nanoparticles, especially in the traditional slow-scan image acquisition mode. In many instances, drifts cause the single most detrimental problem to high-quality SEM-based nanoparticle measurements. Fast imaging and two-dimension Fourier transform-based drift compensation could solve this problem. The ISO 19749 Qualification of the SEM for nanoparticle measurements excel spreadsheet presents examples for the results of drifts, as well as solutions for measuring and minimizing them.

NOTE The ISO 19749 Qualification of the SEM for nanoparticle measurements excel spreadsheet is provided in a machine-readable file at: https://standards.iso.org/iso/19749/ed-1/en.

To measure the drift performance, first set the focus to its best, magnification, landing energy (accelerating voltage), beam current, to the values specified by the instrument manufacturer for proving the best resolution performance. Then without intentionally changing the sample location, perform at least the 1-min and 10-min measurements. If it is pertinent to the task at hand, perform further, longer ones, as are relevant to automated measurement systems that measure samples over a long time without operator assistance.

One-minute measurement: take 6 consecutive images at every 10 s, play them in sequence to visualize the nature and the extent of the drift, find the largest displacement in x and y directions among them, report it as 1-min drift performance value. The point is to accomplish the image sequence acquisition within 1 min. If these fast images prove to be too noisy, use somewhat higher beam current.

Ten-minute measurement: take 10 images, one at start and then at every minute, play them in sequence to visualize the nature and the extent of the drift, find the largest displacement in x and y directions among them, report it as 10-min drift performance value.

One-hour measurement: take 20 images, one at start and then every 3 min, play them in sequence to visualize the nature and the extent of the drift, find the largest displacement in x and y directions among them, report it as 1-h drift performance value.

Long-term measurement: take images at every 3 min, play them in sequence to visualize the nature and the extent of the drift, find the largest displacement in x and y directions among them, report it as long-term drift performance value.

Automatic, software-based measurements can also be performed.

The results of the measurement of the drifts of the SEM shall be included in the report of nanoparticle measurements. Determine the Type A or Type B uncertainty related to drifts and use it in the calculation of the accuracy of the measurement results.

A.5 Measurement of electron beam-induced contamination

Electron beam-induced contamination, caused by desorption of highly mobile molecules on surfaces where the electron beam interacts with the sample, is possibly significant problem. In worst cases, it can completely preclude nanoparticle measurements. The carbonaceous layer that forms under electron irradiation could easily obscure the smallest nanoparticles and significantly change the size of larger ones or change the generated signal, even during the time when a single image is taken. See Annex A and Annex F for more information on this topic.

For the measurement of cleanliness follow this procedure:

- On amorphous silicon oxide patterns of the NIST Contamination Research Grade Material (RGM 10100) or a similar Si chip using the best resolution imaging parameters take one image at the magnification specified by the manufacturer used for proving that the SEM meets its specifications. Save the image.
- Go up to twice as high magnification and continuously image for 10 min.
- Go back to the original magnification and take another image.
- If there is any visible darkening or carbonaceous structure in the middle of the second image, the instrument or the sample or both fail to meet cleanliness requirement.
- If the specification was not met, first clean the sample in the mixture of 3:1 ratio of 30 % hydrogen peroxide solution added to concentrated sulphuric acid (acid piranha solution).

CAUTION — When preparing the piranha solution keep in mind that the reaction is exothermic. The solution will become hot. It is essential to add the peroxide to the acid, not the other way around, and then let the mixture cool to room temperature.

This ferocious oxidizer will clean all hydrocarbon residues from the sample in less than 30 min.

If the instrument – with the clean sample – fails the test again, it needs to get cleaned with low-energy plasma or other cleaning process, so this measurement procedure will result in a contamination-free image.

Repeat this procedure at all primary electron-beam landing energies (accelerating voltage) the SEM has resolution specification for, and for other values used by measurements.

Upon successful accomplishment of this measurement, the results shall be included in the report of nanoparticle measurements with the final image pairs (before and after the 10 min continuous imaging at the specified magnification) that prove contamination-free operation, record beam current as well.

Fortunately, there are reliable methods that can essentially eliminate this problem. The measurements shall prove that the SEM itself is free from electron beam-induced contamination. If, with a clean SEM, there is no negligible contamination, then the sample preparation shall be reviewed and changed to produce clean nanoparticle samples.

If it is unavoidable to acquire images without observable contamination, determine the Type A or Type B uncertainty related to contamination and use it in the calculation of the accuracy of the measurement results.

A.6 Measurement of scale and linearity

The SEM shall be calibrated to properly convert pixels into SI length units (e.g. nanometer). The calibration procedure shall include verification of the uniformity of the field of view. To ensure accuracy and traceability, this shall be done by calibrating the SEM with a certified standard reference material (CRM), a sample with suitable patterns at distances accurately known with proper, traceable uncertainty.

The scale and linearity in both horizontal and vertical directions are important to ensure that the size and shape distribution information derived from SEM images are correct. The scale or magnification of the SEM image is generally set by the manufacturer and can be recalibrated by service personnel. To realize scale or magnification calibration within ±10 % accuracy is generally simple, but to carry out measurements with better than ±5 % accuracy is not simple, in most cases can only be done with a calibrated reference material placed next to the sample under evaluation at the same level to ensure identical distance from the objective lens (working distance). Annex A presents solutions for measuring scale errors.

To set the scale of the SEM, follow the procedure described in ISO 16700. Other methods using traceable, calibrated artefacts can also be used. The uncertainty of scale calibration in both horizontal and vertical directions shall be calculated and used in the determination of the uncertainty of particle size and shape.

Linearity in both horizontal and vertical directions is another important requirement with SEM-based nanoparticle measurements. Lack of linearity or nonlinearity causes nanoparticles appear different in size and shape depending on where they are located in the measurement frame. This error could reach ±10 % at the sides of the image frame, especially in SEMs with coil-based electron beam deflection (scanning) systems in fast imaging mode. In some instances, using only the centre 2/3 of the image frames could lessen the problem satisfactorily.

The results of the measurements of scale and linearity in both horizontal and vertical directions of the SEM shall be included in the report of nanoparticle measurements. Determine the Type A or Type B uncertainty related to scale and linearity and use it in the calculation of the accuracy of the measurement results. See the ISO 19749 Qualification of the SEM for nanoparticle measurements excel spreadsheet for examples of scale calibration results.

NOTE The ISO 19749 Qualification of the SEM for nanoparticle measurements excel spreadsheet is provided in a machine-readable file at: https://standards.iso.org/iso/19749/ed-1/en.

A.7 Measurement of noise

SEM images are not noise-free. The signals, especially those that arrive at the detectors from structures that are comprised by only a few dozens of atoms are inherently weak. Additionally, SEMs in their signal chain can pick up various types of noise. Noise causes errors in nanoparticle size and shape distribution measurements, so it is indispensable to ensure that images used for particle size and shape measurements have sufficiently high signal-to-noise ratio (R_{SN}).

To measure the noise of the images used for nanoparticle size and shape distribution measurements set the instrument to acquire suitable images on one of the samples prepared for these measurements. Set all pertinent instrument parameters, contrast and brightness, magnification (field of view), image pixel dwell or frame time, beam current to their optimized settings and levels, and take one representative image of the nanoparticles.

Calculate the R_{SN} with this formula:

$$R_{SN} = \frac{A_{sig}}{\sigma_{sig}}$$

where

A_{sig} is the average value of the brightest or darkest portions of the image that show the nanoparticles;

σ_{sig} is the standard deviation of the signal (generally, it is recommended to achieve *SNR* 5 to *SNR* 7 or higher).

The results of the measurements of the noise of the SEM shall be included in the report of nanoparticle measurements. Determine the Type A or Type B uncertainty related to noise and use it in the calculation of the accuracy of the measurement results, see the ISO 19749 Qualification of the SEM for nanoparticle measurements excel spreadsheet.

NOTE The ISO 19749 Qualification of the SEM for nanoparticle measurements excel spreadsheet is provided in a machine-readable file at: https://standards.iso.org/iso/19749/ed-1/en.

A.8 Measurements of primary electron beam current

The primary electron beam current value and stability influence the achievable particle size and shape measurement repeatability. Excessive primary electron beam current can alter the sample, too low beam current can result in images with too much noise, and the instability of the current can produce images that make particle differentiation and thresholding difficult, especially for automated measuring setups. Generally, 1 min or shorter acquisition times are adequate for most high-resolution, high-magnification imaging of nanoparticles, so a 10-min measurement is sufficient for these measurements. Longer acquisition times and long-term automated imaging and measurement require primary electron beam current measurements over longer time, commensurate with the imaging times.

Using a Faraday cap and a suitable picoampere meter, measure the dark current 11 times at start and then at every minute, calculate the mean and standard deviation values, and turn on the primary electron beam aiming it into the centre of the Faraday cap, so that all electrons fly in and none could come out.

Ten-minute assessment: take 11 readings at start and then at every minute, calculate the mean and the standard deviation values, report them as 10-min beam current performance metric, report all readings as well.

Long-term assessment: take readings at start and then at every minute, calculate the mean and the standard deviation values, report them as long-term beam current performance metric, indicate the beginning and the end of the measurements, report all readings as well. The time of this assessment depends on the task at hand and shall be at least as long as the measurement procedure itself.

The results of the measurements of the primary electron beam current of the SEM shall be included in the report of nanoparticle measurements. Determine the Type A or Type B uncertainty related to beam current and use it in the calculation of the accuracy of the measurement results. For beam current measurement result examples, see the ISO 19749 Qualification of the SEM for nanoparticle measurements excel spreadsheet.

NOTE The ISO 19749 Qualification of the SEM for nanoparticle measurements excel spreadsheet is provided in a machine-readable file at: https://standards.iso.org/iso/19749/ed-1/en.

Annex B
(informative)

Cross-sectional titanium dioxide samples preparation

B.1 Background and objectives

Preparation of cross-sectional titanium dioxide (TiO_2) samples has proved to be useful for SEM-based size and shape characterization of industrial particles, which can have irregular shape, broad size distribution and a strong propensity of cohesion and agglomeration. The preparation process uses macroscopic amounts of raw particle sample that is mixed with a hot-mounting resin. The separation of particles is ensured by a vibration mill and dispersion beads (that are removed right before the cross-sectional sample is made). This way all sizes are well represented within the prepared sample, as flushing or sedimentation effects can be ruled out. Typical cross-sectional sample sizes of 25 mm or 30 mm give sufficient area for the acquisition of many images. With these samples the particle-resin contrast is good and overlapping particles are rare, so automated image acquisition and analysis procedures can be used with confidence.

B.2 Cross-sectional sample preparation

a) Representative sampling: from a macroscopic amount of raw sample, a representative amount is separated by using a sample riffler, e.g. a rotary or spinning riffler available from various suppliers. In the case of pigment-size TiO_2, a rotary micro-riffler was used to separate 2 g of pigment.

b) Choosing the resin: in order to avoid sedimentation, an appropriate embedding medium is a hot mounting resin that maintains high viscosity during the embedding process. For optimal results, the filler material of the resin is removed by sieving. The selected amount of raw sample is dispersed within the resin. For the dispersion of titanium dioxide particles (either pigmentary or nanometer size), the use of a vibration mill in combination with dispersion beads was shown to be a good choice. 2 g of TiO_2 is dispersed in 4 g of hot-mounting resin using 22,5 g of (0,4 µm to 0,6 µm size) ZrO_2 dispersion beads for a dispersion time of 10 min at 20 Hz, after dispersion the beads are removed by sieving.

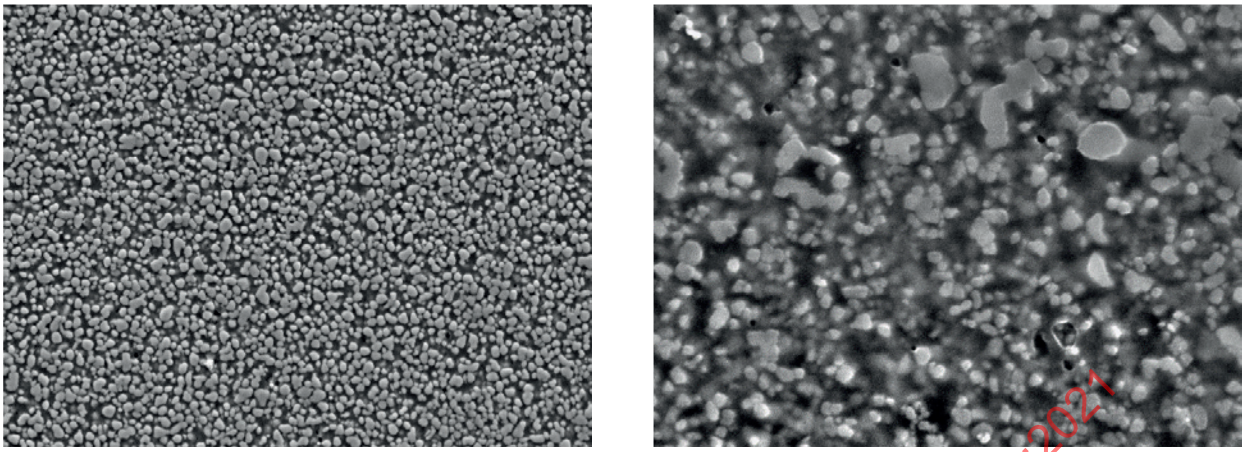
c) The dispersion is then hot-mounted (compressed) at 180 °C temperature and 4 bar pressure for 8 min to 12 min.

d) Preparation of a polished surface for SEM investigation:

    1) A five-step polishing process, with active oxide polishing as the final step, leads to smooth, scratch-free cross sections of the resin-embedded particles.

    2) Before SEM investigation, to avoid sample charging, a sufficiently conductive, 1-nm thin or thinner layer of carbon or Os is deposited using a commercially available sputter coater.

Figure B.1 shows typical results for pigment size TiO_2 particles in Figure B.1, a), and TiO_2 nanoparticles on the Figure B.1, b).



a) SE image of pigment-sized TiO_2 particles, taken with side-mounted Everhart-Thornley detector, 5 kV acceleration voltage, 7 mm working distance, 17,78 µm HFW

b) SE Image of TiO2 nanoparticles, taken with in-lens detector at 5 kV acceleration voltage, 2,5 mm working distance, 1,2 µm HFW

Figure B.1 — SE images of pigment-sized TiO_2 particles

Annex C
(informative)

Case study on well-dispersed 60 nm size silicon dioxide nanoparticles

C.1 Background and objectives

Silicon dioxide (SiO_2) nanoparticle is one of the most important industrial nanoparticle, and widely used as part of raw materials in commercial products. For the quality control of products, there are increasing needs on the evaluation of size distribution of SiO_2 nanoparticles by means of quick observation via SEM. This inter-laboratory comparison (ILC) aimed to establish a protocol for the size distribution measurement by SEM based on the best practices, which are extracted through the processes of sample preparation, SEM observation and image analysis in two-step test.

C.2 ILC

This ILC has been carried out in two phases under the leadership of the Nanotechnology Business Creation Initiative (NBCI) and National Institute of Advanced Industrial Science and Technology (AIST) in Japan. Phase 1 was conducted to assess the size distribution measurement methods and their results of each participant and their practices in sample preparation. In Phase 1, thus, the protocol had large degree of freedom, while in Phase 2, after identifying the best performing sample preparation and measurement methods, participants were asked to prepare and measure samples according to the best performing ideas. The results of Phase 2 have shown significant improvement over the results of Phase 1.

C.3 ILC participants

The participants in this ILC were an industrial producer and two users of nanoparticles, three analytical instrument makers, two analytical service suppliers and a Metrology Institute in Japan, totaling nine contributors (participants *a* to *i* for Phase 1 and participants *A* to *I* for Phase 2).

C.4 Summary of the protocol in the ILC Phase 1

The mandatory conditions in this case study were listed in Table C.1. Other conditions regarding the size distribution measurement were left to each participant but reported as supporting information.

Table C.1 — Summary of the protocol in Phase 1

Sample	Distributed as aqueous suspension of SiO_2 particles (mass fraction of 0,1 %) with dispersant
Sample preparation	Up to the participants, but concentration of suspension is kept as supplied. Procedures were reported.
Image acquisition	Digital image taken with scanning electron microscope
Maximum pixel size	1,5 nm/pixel
Data analysis	Edge of particles are extracted manually or automatically to measure the particle size
Number of particles	More than 500 particles to be measured
Report	Maximum and minimum Feret’s diameter, area, equivalent circular diameter (ECD)

C.5 Details of sample preparation and observing conditions in ILC Phase 1

Most of the participants employed the drop-cast method, except organization a that had a TEM grid contacted to the surface of the suspension. The participant c added trace amount of hydroxyethyl cellulose (HEC) and NH_3 to the suspension. The substrate and drying procedures are summarized in Table C.2.

In image acquisition, all participants chose secondary electron signal. The details of the observing conditions are listed in Table C.3.

Table C.2 — Summary of sample preparation

		Drying			
		Room temp.	**Vacuum**	**Heating**	**Remove excess suspension**
Substrate	Si chip	d, i, e		f	g
	TEM grid		a		c, h
	Al foil				
	Others	b			

Table C.3 — Summary of image acquisition conditions

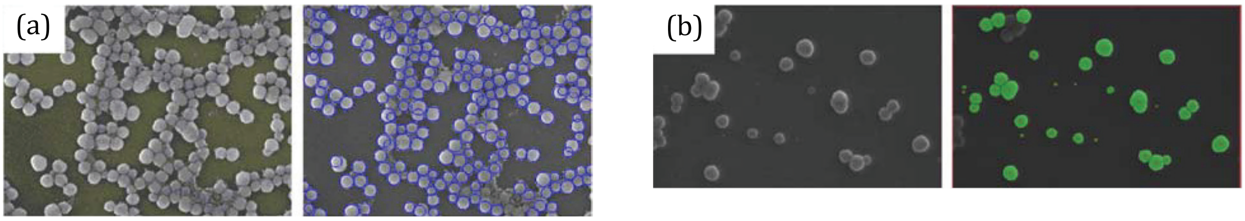
Participant	**SEM**	**Maker**	**SE detector**	V_{acc} kV	**Magnification**	**Pixel size** nm/px	**Calibration**
a	JSM-7800F	JEOL	UED	3	70,000×	1,33	As installed
b	JSM-7610F	JEOL	SE	5	80,000×	1,18	microscale (house standard)
c	S-4300	HHT	ET	15	80,000×	1,25	25,14 µm grid
d	Merlin	CZ	In-lens	3	25,000×	1,1	microscale HJ-1000
e	Gemini SEM 500	CZ	In-lens	0,5	60,630×	1,06	1 µm grid
f	S5500	HHT	ET	3	50,000×	1,01	Hitachi Standard Microscale Type 595−4706
g	SU-8230	HHT	Upper	2	30,000×	0,83	MRS-3
h	SU9000	HHT	Upper	30	100,000×	0,99	Microscale
i	JSM-7100F	JEOL	LED	5	50,000×	0,93	MRS-6

V_{acc} is the accelerating voltage of the SEM.

C.6 Size distribution measurement results in ILC Phase 1

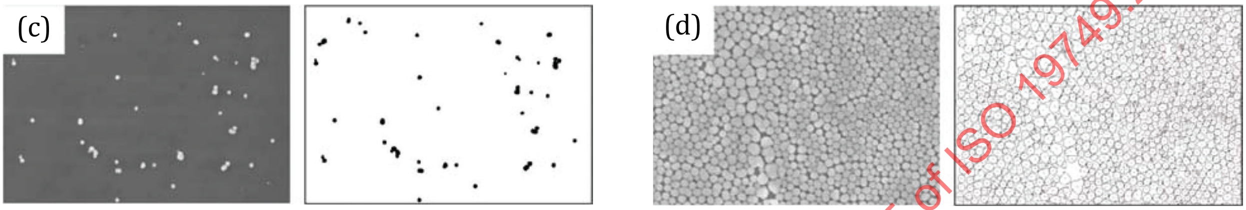
Figure C.1 shows pairs of typical SE image and analysed image, in which the extracted particles are indicated with various colours. The appearance of reported images depends on the participants, i.e. sample preparation. Some of participants observed agglomerated SiO_2 particles on the substrate, on the hand, the particles are well-dispersed by some participants. The conditions for image analysis are summarized in Table C.4. Most of the participant chose automatic analysis.

The reported data on individual SiO_2 particles were organized into histograms shown in Figure C.2, a). The median values for 9 participants are plotted in Figure C.2, b). The average value of medians of equivalent circular diameter is 64,7 nm ± 5,1 nm (1 σ). Although this standard deviation for median values, 7,9 %, is not so large, the shape of histograms differs between participants. One of possible reasons for this divergence is difficulty in the analysis of images with agglomerated particles. Since the edge line of particles is complicated in such images as shown in Figures C.1, a) and d), trace of the edge of the particles can include more error comparing to images with well-dispersed particle shown in Figures C.1, b) and c). In addition, particles tend to get agglomerated with similar size particles.



a) Typical SE images (left pane) and analysed images (right pane) in Phase 1, reported by participant b

b) Typical SE images (left pane) and analysed images (right pane) in Phase 1, reported by participant c



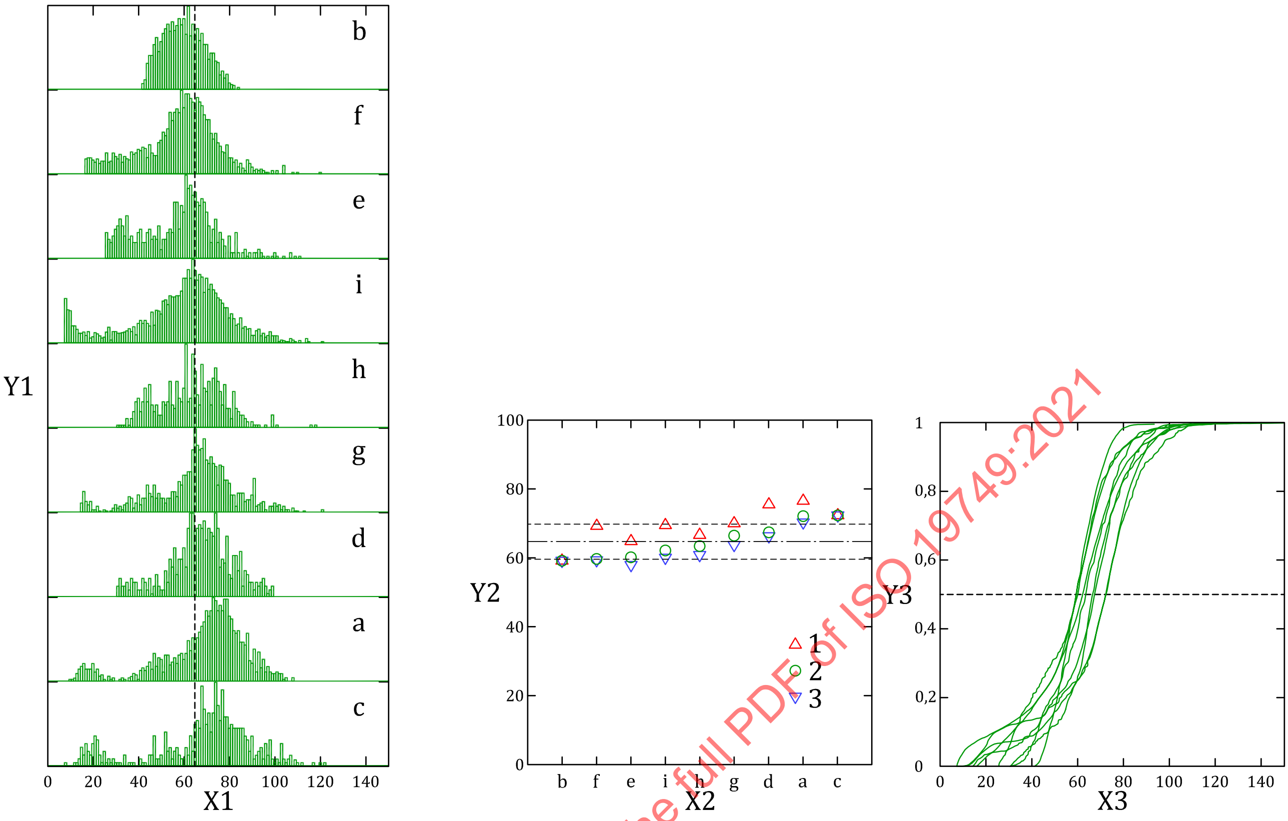
c) Typical SE images (left pane) and analysed images (right pane) in Phase 1, reported by participant d

d) Typical SE images (left pane) and analysed images (right pane) in Phase 1, reported by participant f

Figure C.1 — Typical SE images (left pane) and analysed images (right pane) in Phase 1

Table C.4 — Image analysis conditions

Participant	Edge detection	No. particles	No. images
a	auto (partially manual)	1725	120
b	auto (circular Hough transform)	2289	11
c	auto (partially manual)	521	14
d	auto	565	13
e	auto	593	2
f	auto	2140	2
g	auto (partially manual)	1149	24
h	auto	501	79
i	auto	4020	9



a) Reported size distributions

b) Plot of median values for maximum Feret's diameter, minimum Feret's diameter and equivalent circular diameter

c) Plots of cumulative relative frequency against equivalent circular diameter

Key

X1	equivalent circular diameter (nm)
Y1	normalized frequency (a.u.)
	bin width: 1 nm
X2	participants
Y2	median values (nm)
1	maximum Feret
2	ECD
3	minimum Feret
X3	equivalent circular diameter
Y3	cumulative relative frequency CRF (a.u.)

NOTE The chain line and the dotted lines in (b) shows the average values of ECD and that ±1 σ, respectively.

Figure C.2 — Reported size distributions (a), plot of median values for maximum Feret's diameter, minimum Feret's diameter and equivalent circular diameter (b), and plots of cumulative relative frequency, against equivalent circular diameter (c)

C.7 Summary of the protocol in ILC Phase 2

Out of several sample preparation methods used in the ILC Phase 1, one, by participant c, proved to be the best to prepare samples suitable for size distribution measurements. In Phase 2 all samples were prepared using this method. In the part of image observation and analysis, several directions were added based on the findings in Phase 1 to improve the accuracy and reliability of measurements. Table C.5 shows the summary of the protocols for the ILC Phase 2.

Table C.5 — Summary of the protocol in Phase 2

Sample	Water suspension of SiO_2 particle (mass fraction of 0,1 %) with dispersant (not identical to Phase 1 specimen)
Sample preparation	HEC addition method
Image acquisition	Digital secondary electron image
Maximum pixel size	1,5 nm/pixel
Survey	Before taking images for size measurement, perform survey to make sure that no remarkable size gradient is present in the samples. Adjust contrast and brightness to improve suitable contrast for small particles.
Data analysis	Edge of particles are extracted manually or automatically to measure the particle size. In case of automatic image analysis, check if there are no undetected particles due to size filter or thresholding.
Number of particle	More than 500 particles to be measured
Report	Maximum and minimum Feret's diameter, area, equivalent circular diameter

C.8 Details of sample preparation and image acquisition conditions in ILC Phase 2

In the sample preparation protocol for Phase 2, participants were asked to prepare their samples according to the procedures below (used by participant c in Phase 1).

a) Prepare 1 % HEC aqueous suspension and add 2 µl to 5 µl to 1 ml of the SiO_2 aqueous suspension.

b) Drop a couple of microlitres of SiO_2 aqueous suspension onto TEM grid with film support placed over a filter paper. Adsorb excess SiO_2 aqueous suspension by the filter paper. Clean Si chip is also acceptable substrate instead of TEM grid, for this, remove the excess SiO_2 aqueous suspension on the Si chip by filter paper before the droplet gets dry.

c) Dry the TEM grid or Si chip for more than 30 min at room temperature.

d) Add conductive coating, if excess ample charging makes it necessary.

e) Acquire SEM images.

Three participants chose TEM grid as substrate while the other six used Si chip. Participant A and I used the TEM grid with formvar film and elastic carbon film, respectively. The substrate and drying procedures are summarized in Table C.6.

In image acquisition, all participants chose secondary electron signal except participant I, who used transmitting electron detector. The range of accelerating voltage was from 2 kV to 5 kV for the most of participants. The details of the observing conditions are listed in Table C.7.

Table C.6 — Summary of sample preparation

		Drying			
		Room temp.	Adsorb by filter paper	Vacuum	Air blower
Substrate	TEM grid	B, I(Formvar)		A(elastic carbon)	
	Si Chip		C, D, F, G, H		E[a]

Hydrophilization of the substrate or conductive coating of the surface after particle deposition.

[a] Preparation procedure out of the protocol.

Table C.7 — Summary of image acquisition conditions

Participant	SEM	Maker	Electron detector	V_{acc} kV	Magnification	Pixel size nm/px	Scale calibration
A	JSM-7800F	JEOL	UED	3	70,000×	1,33	As installed
B	S-4300	HHT	SE	15	80,000×	1,25	25,14 µm grid
C	Merlin	CZ	In-lens SE	3	25,000×	1,12	microscale HJ-1000
D	SU-8230	HHT	Upper SE	2	30,000×	0,82	Cristal dimension
E	SU-8200	HHT	Upper SE	5	35,000×	1,42	Microscale
F	JSM-7610F	JEOL	SEI	5	80,000×	1,18	Microscale (in-house standard)
G	S5500	HHT	ET	3	50,000×	1,01	Hitachi standard Microscale (595–4706)
H	Gemini SEM 500	CZ	In-lens SE	3	50,000×	1,10	Zeiss standard method
I	JSM-7100F	JEOL	TE	30	50,000×	1,03	MRS-5

C.9 ILC Phase 2 size distribution measurement results

Figure C.3 shows typical SE image and analysed image pairs, in which the extracted particles are indicated by various colours. The conditions for image analysis are summarized in Table C.8. Most of the participants chose automatic analysis.

Table C.8 — Conditions on image analysis

Participant	Edge detection	Software	No. particles	No. images
A	auto (partially manual)	Photoshop (ver. 7), Particle analysis (ver. 3.5)	1262	98
B	auto	LUZEX AP	572	9
C	auto	Fiji (ImageJ 1,49 m)	1042	16
D	auto	Image-Pro Premier Ver.9.2	767	23
E	auto	Image-Pro Premier 3D (9.2.1)	750	10
F	auto	homemade code	2452	12
G	auto	ImageJ 1,50i	508	11
H	auto	SPIP version 6.6.3	847	31
I	auto	ImageJ (1,50 g)	3470	86

The reported data on SiO_2 particles were reduced into histograms as shown in Figure C.4, a). The median values for 10 data sets are plotted in Figure C.4, b). The average value of medians of equivalent circular diameter is 62,0 nm ± 3,1 nm (1 σ). By comparing the curves of cumulative relative fraction in Phase 1 Figure C.2, c) and that in Phase 2 Figure C.4, c), both the scattering of median value and the convergence in the reported size distributions have improved.

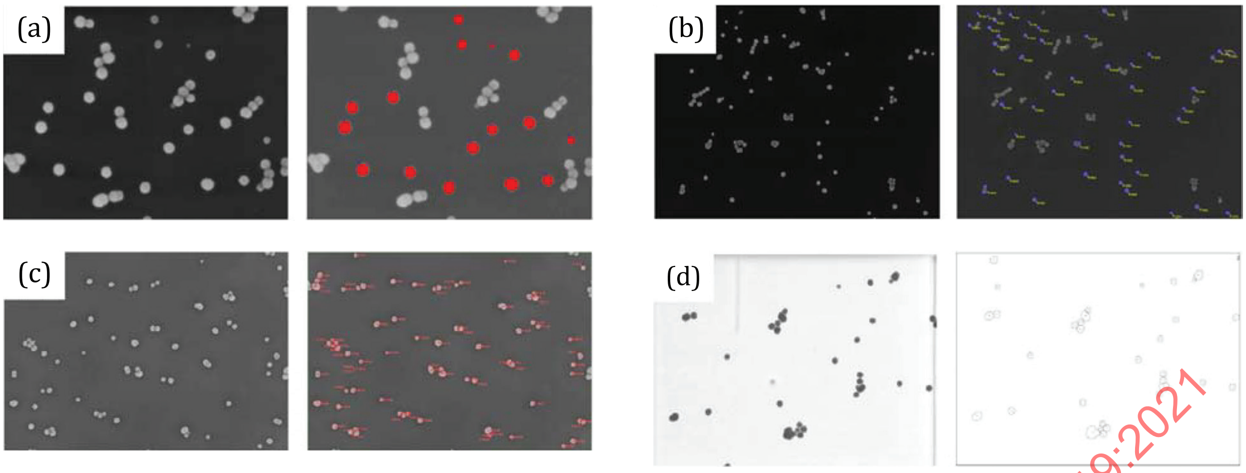
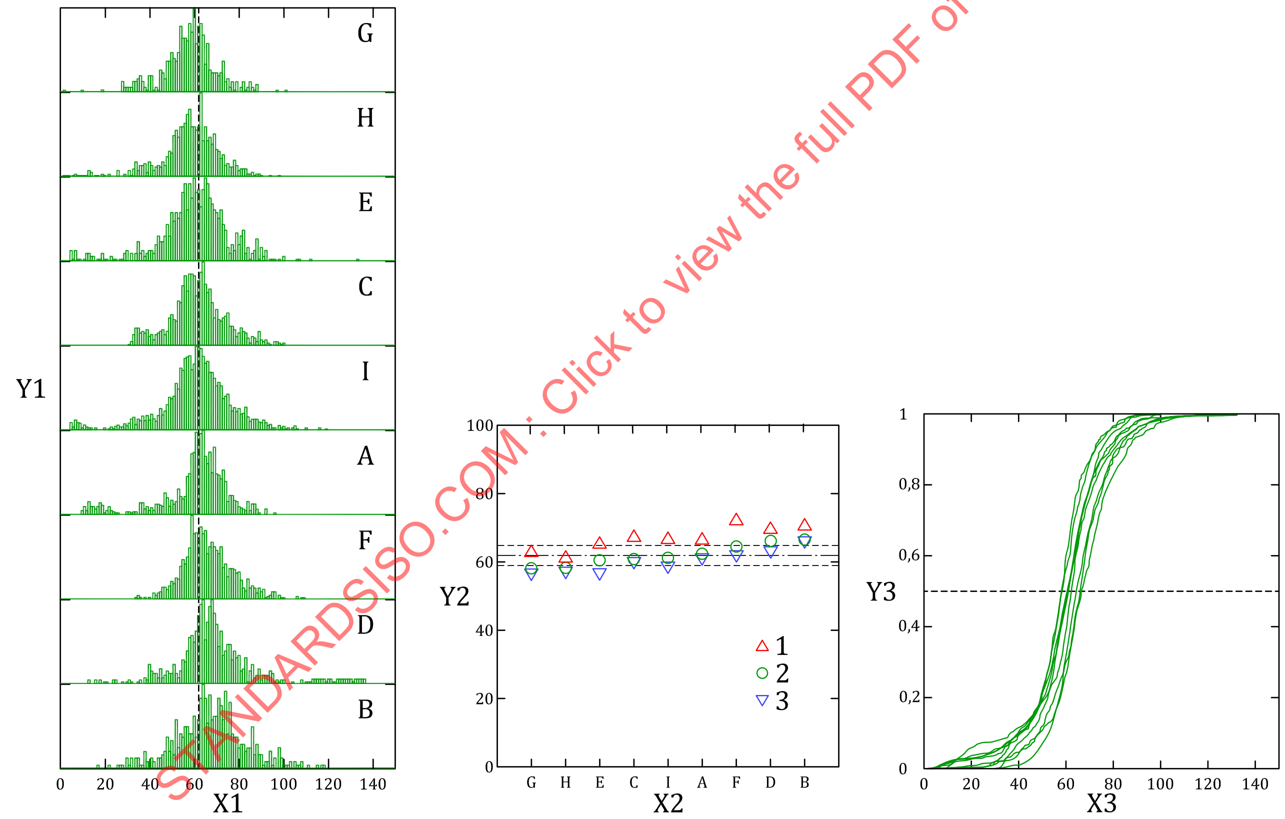


Figure C.3 — Typical SE images (left pane) and analysed images (right pane) in Phase 2, reported by participant A (a), D (b), E (c) and I (d)



a) Reported size distributions

b) Plot of median values for maximum Feret’s diameter, minimum Feret’s diameter and equivalent circular diameter

c) Plots of cumulative relative frequency against equivalent circular diameter

Key

X1	equivalent circular diameter (nm)	X2	participants	X3	equivalent circular diameter
		Y2	median values (nm)		
Y1	normalized frequency (a.u.)	1	maximum Feret	Y3	CRF (a.u.)
		2	ECD		
	bin width: 1 nm	3	minimum Feret		

NOTE The chain line and the dotted lines in Figure C.4, b) shows the average values of ECD and that ±1 σ, respectively.

Figure C.4 — Reported size distributions (a), plot of median values for maximum Feret's diameter, minimum Feret's diameter and equivalent circular diameter (b), and plots of cumulative relative frequency against equivalent circular diameter (c)

C.10 Remarks

With the contribution from 9 nanomaterial relating organizations in Japan, this two-phase ILC revealed the current situation of SiO_2 nano particle size distribution measurement by SEM in industry. The result in Phase 1 shows that even in the case of well-dispersed nano particles, sample preparation can cause agglomeration of the particles leading the difficulty in SEM observation and following image analysis. In the first phase of this test, the "best practices" were found in sample preparation out of various methods. By adopting these methods into the protocol in Phase 2, a large improvement was recognized in the reported images and convergence in the reported size distribution.

As the nature of an imaging method, specimen preparation is a very important process in the size distribution measurement by SEM. Although specimen preparation is material dependent issue, the practices reported in this annex might be applicable not only SiO_2 nano particles, but also other well-dispersed particles. By accumulating the knowledge on specimen preparation, material by material, robust size and shape distribution measurement by SEM should be realized.

Annex D
(informative)

Case study on 40 nm size titanium dioxide nanoparticles

D.1 Background and objectives

Scanning electron microscopy is regarded as one of most realistic solution for size distribution measurement of nanoparticles in the industry due to its capability to quickly and easily observe the particles. In SEM, as an imaging method, specimen preparation is an important factor to achieve reliable measurement. Since how to prepare specimen is generally material dependent, it is important to accumulate the knowledge for the sample preparation for representative nanoparticle in industry. In this ILC, titanium dioxide (TiO_2) was chosen as an example of nanoparticle that can easily get agglomerated in the specimen preparation. We aimed to identify best practices and establish a protocol for the size distribution measurement by SEM for such nanoparticles through this ILC.

D.2 ILC

This ILC was held under the chairmanship of NBCI and National Institute of AIST in Japan. The ILC has two steps, Phase 1 and Phase 2. Phase 1 aimed to compare the skill on size distribution measurement of each participants and to identify good practices on sample preparation for SEM observation. In Phase 1, thus, the protocol has larger degree of freedom than that in Phase 2, asking the participants to prepare sample according their idea. In Phase 2, the protocols were modified based on the best practice on the sample preparation identified in Phase 1 to check the convergence of measurement result.

D.3 ILC participants

The participants in Phase 1 in this ILC were two nanoparticle producers, three nanoparticle users, three analytical instrument makers and a metrology institute in Japan, totaling 9 organizations (participants n to v). Phase 2 has 10 organizations: two nanoparticle producers, three nanoparticle users, three analytical instrument makers, an analytical service supplier and a metrology institute in Japan (participants N to W.)

D.4 Protocols in the ILC Phase 1

The mandatory conditions in this case study were listed in Table D.1. Other conditions regarding the size distribution measurement were left to each participant but reported as supporting information.

Table D.1 — Summary of the protocol in Phase 1

Sample	Distributed as aqueous (water) suspension of TiO_2 particle (0,1 weight %) with dispersant
Sample preparation	Up to participants, but concentration of suspension is kept as supplied procedures were reported
Image acquisition	by scanning electron microscope
Maximum pixel size	1,5 nm/pixel
Data analysis	Edge of particles are extracted manually or automatically to measure the particle size
Number of particles	More than 500 particles to be measured
Report	Maximum and minimum Feret's diameter, area, equivalent circular diameter

D.5 Details of sample preparation and image acquisition conditions in ILC Phase 1

Most of the participants used drop-cast method. The substrate and drying procedures are summarized in Table D.2. In image observation, all participants chose secondary electron as image signal. The details of the observing conditions are listed in Table D.3.

Table D.2 — Summary of sample preparation

		Drying			
		Room temp.	Vacuum	Heating	Remove excess suspension
Substrate	Si chip	q, v		r	s
	TEM grid	t		u	
	Al foil	n, p			
	Other	o			
Hydrophilization of the substrate.					

Table D.3 — Summary of image acquisition conditions

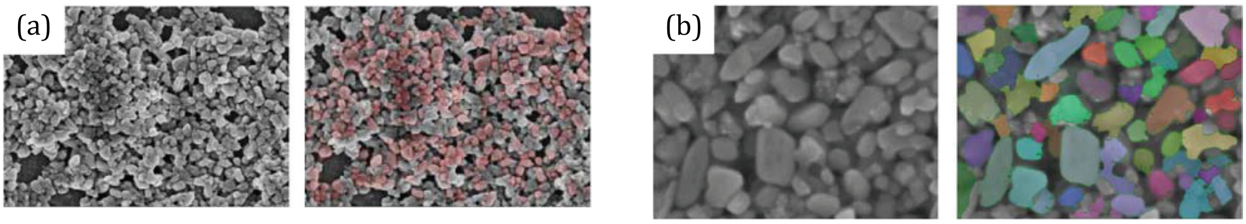
Participant	**SEM**	**Maker**	**SE detector**	V_{acc} kV	**Magnification**	**Pixel size** nm/px	**Calibration**
n	S-5500	HHT	SE	2	100,000×	1	Microscale (240 nm pitch)
o	JSM-7610F	JEOL	SEI / GB_LOW	3	300,000×	0,31	Microscale (house standard)
p	S-4800	HHT	ET	5	100,000×	0,5	Grating
q	GeminiSEM 500	CZ	In-lens SE	0,5	100,000×	1,09	1μm grid
r	S5500	HHT	ET	3	50,000×	1,01	Hitachi Standard Microscale (595–4706)
s	SU-8230	HHT	Upper	2	30,000×	0,83	MRS-3
t	SU9000	HHT	Upper	30	100,000×	0,99	Microscale
u	S-4800	HTT	Upper (SE)	3	100,000×	0,99	25,4 μm grid
v	JSM-7100F	JEOL	LED	5	50,000×	0,93	MRS-6

D.6 Size distribution measurement results in ILC Phase 1

Figure D.1 shows pairs of typical SE image and analysed image, in which the isolated particles are indicated with various colours. While most of the participants reported SE images with agglomerated TiO_2 nanoparticles, participant s reported SE images of well-dispersed nanoparticles.

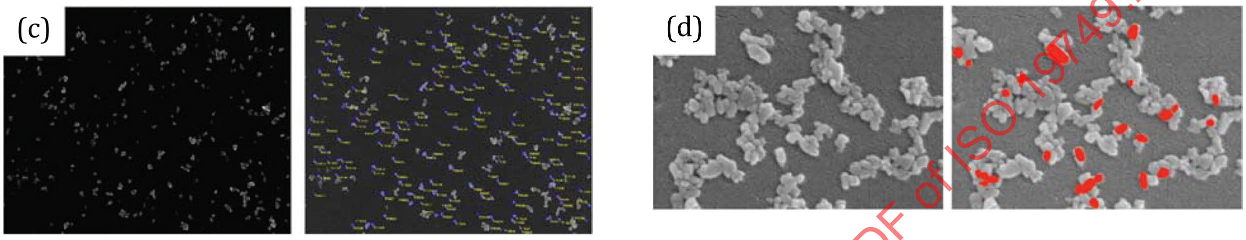
The conditions for image analysis are summarized in Table D.4. Five participants analysed images manually and four used automatic image analysis. The reported data on individual TiO_2 particles were reduced into histograms as shown in Figure D.2, a). The median values for 9 participants are plotted in Figure D.2, b). The average value of medians of equivalent circular diameter is 36,1 nm ± 10,6 nm (1 σ).

As shown in Figures D.2, a) and c), the shape of histograms and cumulative relative frequency curves widely varies with the participants. This large scattering can be attributed to the error in the edge detection. Since TiO_2 nanoparticles tend to easily get agglomerated in the drop-cast specimen preparation process, the SE images from many participants show stacked TiO_2 particles with complicated edge line, which is difficult to trace even manually Figures D.1, a), b) and d). On the other hand, participant s, as shown in Figure D.1, c), reported SE images of well-dispersed TiO_2 nanoparticles.



a) Typical SE images (left pane) and analysed images (right pane) in Phase 1, reported by participant n

b) Typical SE images (left pane) and analysed images (right pane) in Phase 1, reported by participant o



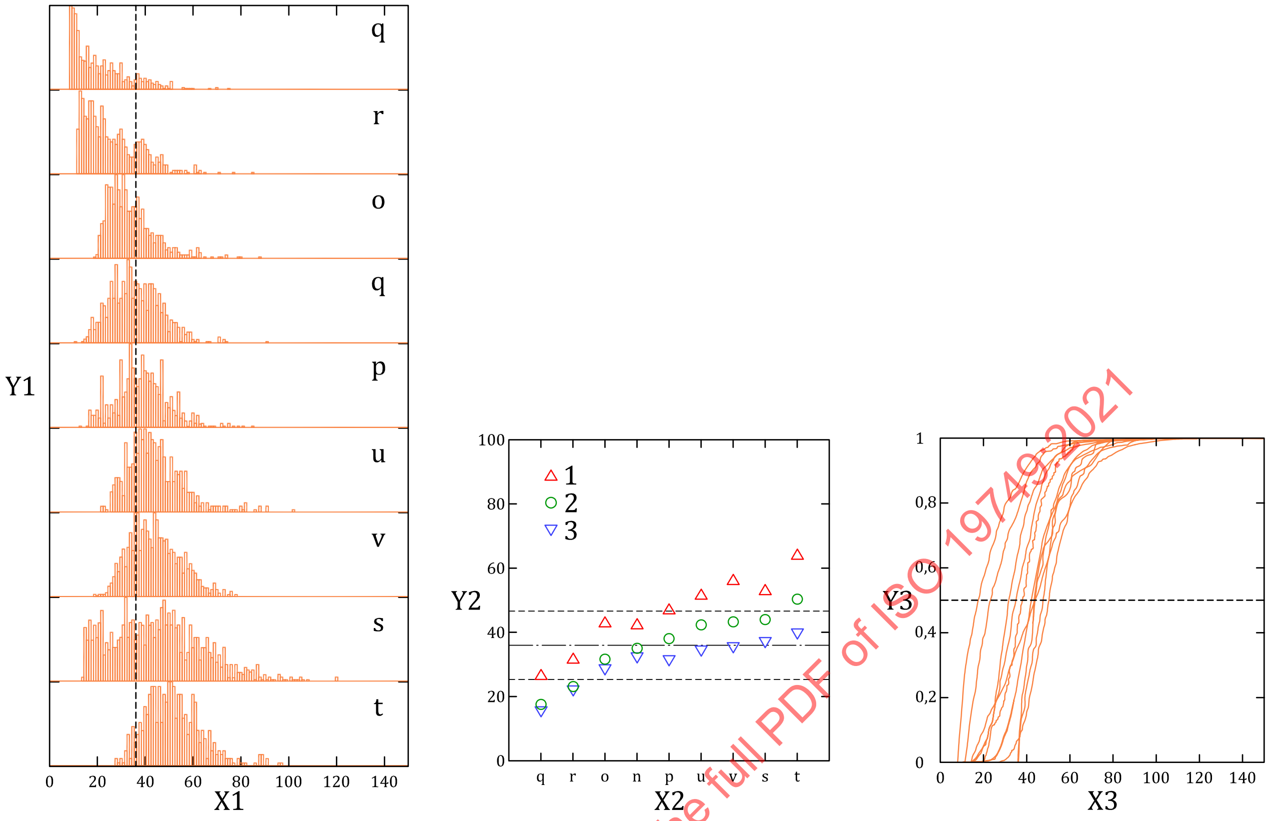
c) Typical SE images (left pane) and analysed images (right pane) in Phase 1, reported by participant s

d) Typical SE images (left pane) and analysed images (right pane) in Phase 1, reported by participant u

Figure D.1 — Typical SE images (left pane) and analysed images (right pane) in Phase 1

Table D.4 — Image analysis conditions

Participant	Edge detection	Software	No. particles	No. images
n	manual	Mac-View (Ver.3)	717	3
o	auto	Homemade code	603	12
p	manual	LUZEX AP	562	15
q	auto	SPIP (Ver. 6.6.1)	629	2
r	auto	ImageJ (1,50i)	581	1
s	auto	Image-Pro Premier (Ver.9.2)	1229	7
t	manual	Image-Pro Premier 3D (Ver. 9.2.1)	503	37
u	manual	Mac-View (Ver.4)	533	31
v	manual	ImageJ (1,50g)	1190	4



a) Reported size distributions

b) Plot of median values for the maximum Feret diameter, minimum Feret diameter and for equivalent circular diameter

c) Plots of cumulative relative frequency against equivalent circular diameter

Key

X1 equivalent circular diameter (nm)

Y1 normalized frequency (a.u.)

bin width: 1 nm

X2 participants

Y2 median values (nm)

1 max feret

2 ECD

3 min feret

X3 equivalent circular diameter

Y3 CRF (a.u.)

NOTE The chain line and the dotted lines in Figure D.2, b) shows the average values of ECD and that ±1 σ, respectively.

Figure D.2 — Reported size distributions (a), plot of median values for the maximum Feret diameter, minimum Feret diameter and for equivalent circular diameter (b), and plots of cumulative relative frequency against equivalent circular diameter (c)

D.7 Protocols in the ILC Phase 2

Out of several sample preparation methods used in the ILC Phase 1, participant s, proved to be the best to prepare samples suitable for size distribution measurements suppressing the agglomeration of particles. In Phase 2 all samples were prepared using this method. In the part of image observation and analysis, several directions were added based on the findings in Phase 1 to improve the accuracy and reliability of measurements. Table D.5 shows the summary of the protocols for the ILC Phase 2.

Table D.5 — Summary of the protocol in Phase 2

Sample	Water suspension of TiO_2 particle (0,1 weight %) with dispersant (not identical to Phase 1 specimen)
Sample preparation	Drop casting onto hydrophilic Si chip (see C.2.8)
Image acquisition	Digital secondary electron image
Maximum pixel size	1,5 nm/pixel
Survey	Before taking images for size measurement, perform survey to make sure that no remarkable size gradient is present in the samples. Adjust contrast and brightness to improve suitable contrast for small particles.
Data analysis	Edge of particles are extracted manually or automatically to measure the particle size. In case of automatic image analysis, check if there are no undetected particles due to size filter or thresholding.
Number of particle	More than 500 particles to be measured
Report	Maximum and minimum Feret's diameter, area, equivalent circular diameter

D.8 Details of sample preparation and image acquisition conditions in ILC Phase 2

In the sample preparation protocol for Phase 2, participants were asked to prepare their samples according to the procedures below (used by participant s in Phase 1).

a) Hydrophilize a Si chip for substrate.

b) Drop about one microlitres of TiO_2 aqueous suspension onto the Si chip. After waiting for 10 s, adsorb excess TiO_2 aqueous suspension by a filter paper.

c) Dry the Si chip for more than 30 min at room temperature.

d) Add conductive coating, if excess sample charging makes it necessary.

e) Acquire SEM images.

For hydrophilization of Si chip, several methods were used. The participants N, Q, S, U, W and V made plasma treatment on Si chip. The participant O washed Si chip and soaked it in ethanol for 24 h, while the participant R used ethanol only for washing Si chip. The participant P chose chemical etching to get hydrophilic surface. The participant T employed UV ozone cleaner for hydrophilization.

In image acquisition, all participants chose secondary electron signal. The range of accelerating voltage was from 2 to 7 kV. Table D.6 gives a summary of sample preparation methods in this annex, while details of the observing conditions are listed in Table D.7.

Table D.6 — Summary of sample preparation

TiO_2 aqueous suspension		**Drying**	
Substrate: Si wafer		**Filter paper**	**Air blower**
Substrate hydrophilization method	Plasma	N, Q, S, U, W	V[a]
	Ethanol	O (24H), R(rinse)	
	$H_2SO_4+H_2O_2$	P	
	UV + O_3	T	
Conductive coating of the surface after particle deposition.			

[a] Preparation procedure out of the protocol.

Table D.7 — Summary of image acquisition conditions

Participant	SEM	Maker	SE detector	V_{acc} kV	Magnification	Pixel size nm/px	Calibration
N	S-4800	HTT	Upper (SE)	3	100,000×	0,99	25.4 µm mesh Cu grid
O	S-5500	HHT	SE	2	100,000×	1,00	25 µm mesh Cu grid
P	JSM-6320F	JEOL	SE	5	150,000×	0,83	S2009ST
Q	SU-8230	HHT	Upper (In-lens SE)	2	30,000×	0,82	Cristal dimension
R	S-4800	HHT	SE(U)	2.5	100,000×	0,50	Grating
S	JSM-7610F	JEOL	ET SE	5	300,000×	0,31	Microscale (house standard)
T	S-5500	HHT	SE	3	50,000×	1,01	Hitachi Standard Microscale Type 595–4706
U	Gemini SEM 500	CZ	In-lens SE	2,5	50,000×	1,10	ZEISS standard method
V	SU8200	HHT	Upper (SE)	5	35,000×	1,42	Microscale
W	JSM-7100F	JEOL	ET SE	7	70,000×	0,67	MRS-6

ET SE: Everhart-Thornley secondary electron detector.

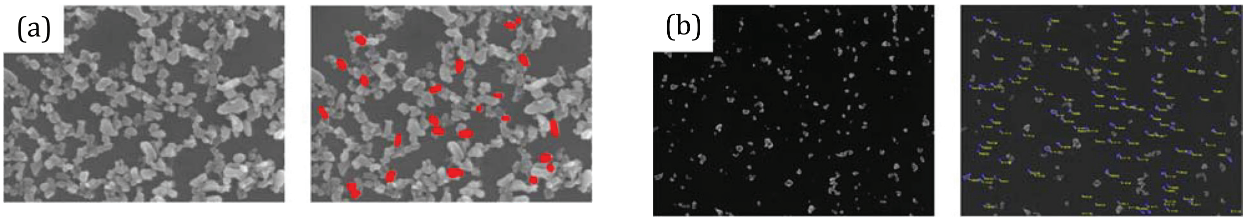
D.9 ILC Phase 2 size distribution measurement results

Figure D.3 shows typical SE image and analysed image pairs, in which the extracted particles are indicated by various colours. Compared to those in Phase 1, reported SE images shows that agglomeration of TiO_2 particles was remarkably suppressed. Several participants succeeded in placing the particles independently on the substrate. Thus, by introducing the best practice of sample preparation method in the first phase into the protocol in Phase 2, sample quality has been drastically improved.

The conditions for image analysis are summarized in Table D.8. Similar to Phase 1, five participants analysed images manually and four used automatic image analysis. The reported data on individual TiO_2 particles were reduced into histograms as shown in Figure D.4, a). The median values for 10 data sets are plotted in Figure D.4, b). The average value of medians of equivalent circular diameter is 37,7 nm ± 6,5 nm (1 σ).

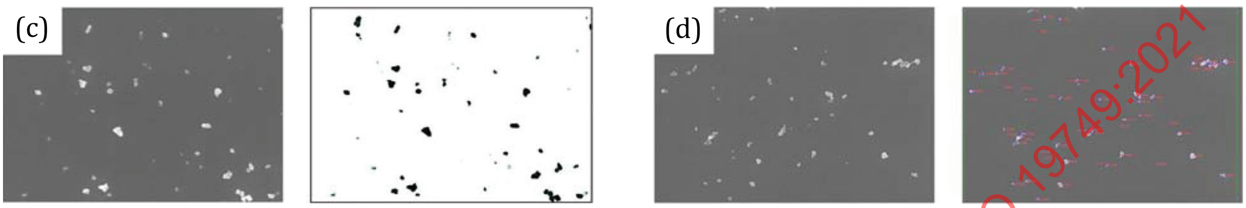
As shown in Figure D.4, a), the size distribution for seven participants (N, O, P, Q, R, V and W) shows similar shape, which have a peak around 40 nm in equivalent circular diameter. By comparing the histograms in Phase 1 Figure D.2, a) and those in Phase 2 Figure D.4, a), both the scattering of median value and the convergence in the reported size distributions have improved.

The histograms for participants T and U have entries at particle size of several nanometers, which is an artefact caused by noise. Since these two participants used automatic edge detection, there could have been an inappropriate setting for size filter. Even though the shape of distribution was distorted by this noise, another peak is recognizable at around 40 nm in diameter indicating that nanoparticles were also detected. By choosing appropriate filter condition to remove noise, these results could be improved. To obtain reliable measurement result, not only good specimen preparation, but also careful consideration for image analysis condition is important.



a) Typical SE images (left pane) and analysed images (right pane) in Phase 2, reported by participant N

b) Typical SE images (left pane) and analysed images (right pane) in Phase 2, reported by participant Q



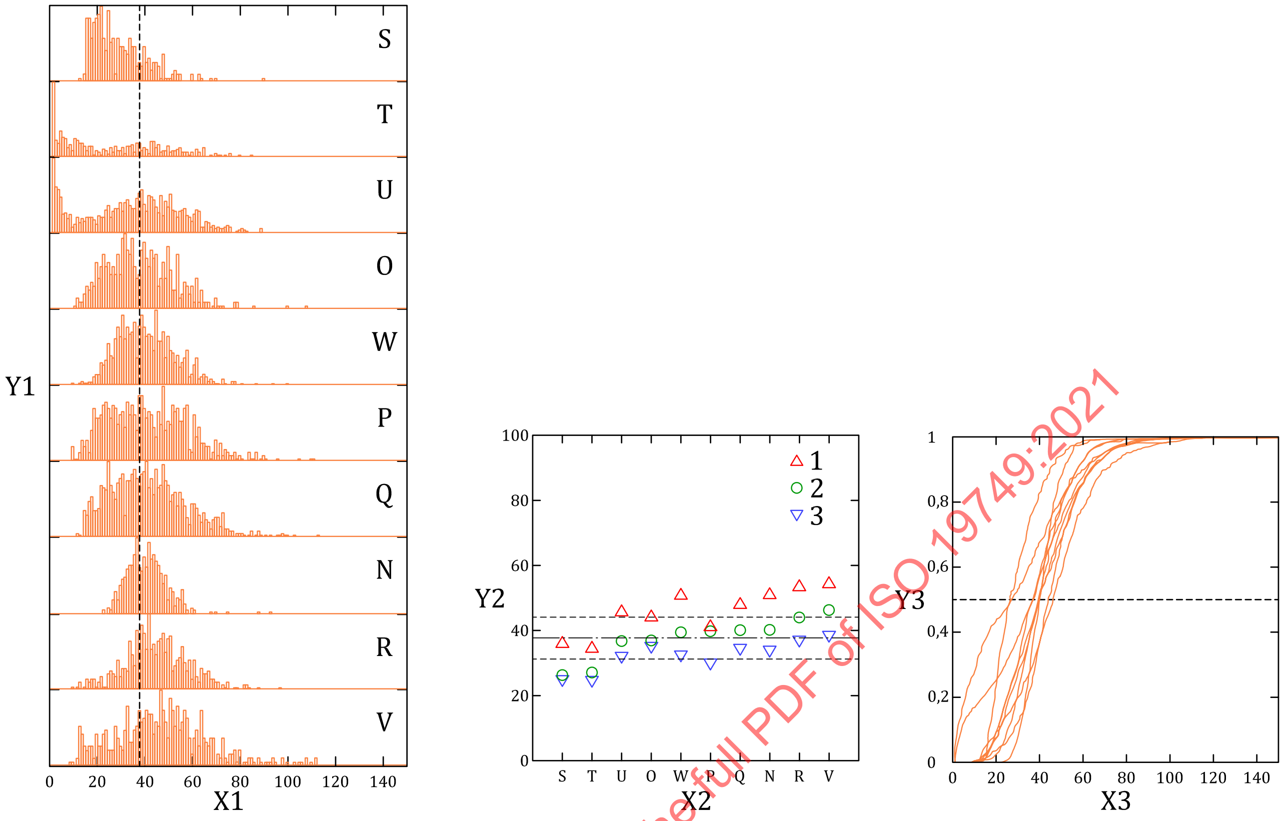
c) Typical SE images (left pane) and analysed images (right pane) in Phase 2, reported by participant T

d) Typical SE images (left pane) and analysed images (right pane) in Phase 2, reported by participant V

Figure D.3 — Typical SE images (left pane) and analysed images (right pane) in Phase 2

Table D.8 — Image analysis conditions

Participant	Edge detection	Software	No. particles	No. images
N	manual	Mac-View (Ver.4)	586	25
O	manual	Mac-View (Ver.3)	517	10
P	manual	Photoshop, ImageJ (1,50i)	777	15
Q	auto	Image-Pro Premier (Ver.9.2)	1121	12
R	manual	LUZEX AP	587	30
S	auto	Homemade code	324	12
T	auto	ImageJ (1,50i)	503	4
U	auto	SPIP (Ver. 6.6.3)	1199	30
V	auto	Image-Pro Premier 3D (Ver. 9.2.1)	564	11
W	manual	ImageJ (1,50g)	772	34



a) Reported size distributions

b) Plot of median values for maximum Feret's diameter, minimum Feret's diameter and for equivalent circular diameter

c) Plots of cumulative relative frequency against equivalent circular diameter

Key

X1	equivalent circular diameter (nm)	X2	participants	X3	equivalent circular diameter
		Y2	median values (nm)		
Y1	normalized frequency (a.u.)	1	maximum Feret	Y3	CRF (a.u.)
		2	ECD		
	bin width: 1 nm	3	minimum Feret		

NOTE The chain line and the dotted lines in b) shows the average values of ECD and that ±1 σ, respectively.

Figure D.4 — Reported size distributions a), plot of median values for maximum Feret's diameter, minimum Feret's diameter and for equivalent circular diameter b), and plots of cumulative relative frequency against equivalent circular diameter c)

D.10 Remarks

In this two-phase ILC, a protocol for the size distribution measurement of TiO_2 by SEM has been developed. Phase 1 identified a good practice in the sample preparation to suppress the agglomeration of TiO_2 particles. By introducing this technique into the protocol, the reported results have shown convergence in Phase 2.

As the nature of an imaging method, specimen preparation is a very important process in the size distribution measurement by SEM. The practices reported here might be helpful in the preparation for not only TiO_2 nano particles, but also other nanomaterials. To achieve robust size and shape distribution measurement by SEM, accumulating “best practices” through case studies, material by material, is important.

Annex E
(informative)

Example for extracting particle size results of SEM-based nanoparticle measurements using ImageJ

This example macro for Image J or Fiji is an example with step-by-step details of the various functions.

```
//**************This is a macro designed to process images of spherical particles
on heterogeneous surfaces and subsequently analyze and measure particle areas using
predefined crop, BG subtract, and circularity ********
//*********  Code was written by Samuel J. Underwood with editing and optimization by
Justin M. Gorham of NIST.
//*********  Version 1.0 draft 08/04/2016 current revision 10/28/2016
//*********  National Institute of Standards and Technology


//1. Titling macro and identification of the hotkey, a, to launch macro
macro"Analyze and process [a]"

//2. The following script calculates the size of a pixel in nanometers by user input of
horizontal field width (HFW) and extraction of pixel width
{
Dialog.create("Set Scale");
Dialog.addNumber("Horizontal Field Width(nm)", 3200);
Dialog.show();

//this allows the input values to be incorporated into the Set Scale Program
d=Dialog.getNumber();//sets the HFW as d
e=getWidth();//gets the Width in pixels and sets it as e
run("Set Scale...","distance=e known=d pixel=1 unit=nm");

//3. The following script makes a rectangle and crops to a predefined size to remove
pixels with metadata
makeRectangle(0,0,2048,1765);
run("Crop");

//4. The following line of code subtracts the background using a 40 pixel wide radius ball

run("Subtract Background...","rolling=40 black");

//5. Despeckle salt and pepper noise in background and smooth an arbitrary number of times
run("Despeckle");
run("Smooth");
run("Smooth");
run("Smooth");
run("Smooth");
run("Smooth");

//6. Setting Brightness and Contrast with a pause built in for user inspection and
adjustment
run("Brightness/Contrast...");//brings up brightness window
title="Contrast Adjustment"//Title for pausing
msg="Complete?";//Message on pause dialog
run("Enhance Contrast", "saturated=0.35");//automatically adjusts contrast
waitForUser(title,msg);//pause command
run("Apply LUT");//sets and applies new brightness
run("Close")//close brightness/contrast window.

//NOTE:  the built-in Pause was modeled after the script taken from https:
//imagej.nih.gov/ij/macros/WaitForUserDemo.txt

//7. Setting the threshold and saving it with a pause built in for user inspection and
adjustment
```

```
run("Threshold...");//brings up threshold window
title="Threshold Adjustment"//Title for pausing
msg="Complete?";//Message on pause dialog
waitForUser(title,msg);//pause command
getThreshold(lower,upper);//get the set threshold window
run("Close")//close threshold window

//8. Setting up structure for size range analyzed by defining minimum pixel value used
Dialog.create("Minimum radius analyzed");//Created dialog box with title
Dialog.addNumber("Lower size limit-radius (pixels)", 5);//Sets up line for input and a
description to the left.  5 pixels default minimum
Dialog.show();//displays dialog box until value is added and 'OK' is selected

//calculating minimum area for running particle analysis
h=Dialog.getNumber();//sets the radius minimum as h
g=h*d/e;//converts minimum from pixels to nm
f=3.14*g*g;//area in nm^2 of a circle with an h pixel radius

//9. Particle analysis and summary of values
run("Analyze Particles...",
     "size=f-Infinity circularity=0.75-1.00 show=Outlines display exclude clear
summarize");
}
```