TECHNICAL SPECIFICATION

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# Nanotechnologies — 3D image reconstruction of rod-supported nano-objects using transmission electron microscopy

*Nanotechnologies — Reconstruction d'images 3D de nano-objets soutenus par des tiges à l'aide de la microscopie électronique à transmission*



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# Foreword

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This document was prepared jointly by Technical Committee ISO/TC 229, *Nanotechnologies*, and Technical Committee IEC/TC 113, *Nanotechnology for electrotechnical products and systems.*

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

Electron tomography, in transmission electron microscope (TEM), has impact on nanotechnology and nanomaterial metrology like that of computer tomography in medicine. For example, industries using nanotechnologies have requirements to verify materials, processes and products. Quantitative measurement at the nanoscale, including three-dimensional (3D) image reconstruction of nano-objects using TEM, responds to this need.

TEM, a two-dimensional (2D) imaging instrument, can provide 2D projection images of materials at the nanoscale, in the length range from below 1 nm to above 100 nm. From multiple 2D TEM images collected at suitable tilt increments, the 3D shape, size and volume parameters can be determined. This document describes sample preparation, instrumentation setup, data acquisition and processing for 3D image reconstruction of nano-objects using TEM, from which dimensional parameter values can be determined and interpreted. Variation in methodology for use with scanning transmission electron microscopy (STEM) is included in an informative annex.

The method described herein is limited to samples dispersed on or within an electron-transparent rod-shaped support. This method is particularly useful when the detailed shape of a limited number of objects, such as nanoparticles, is sought. For example, when 2D measurements yield a non-uniform distribution of objects, 3D image reconstruction can be used applied to study a small number of the objects in more detail. A variant of sample preparation is described that allows 3D reconstruction to be used in conjunction with 2D TEM analysis of a sample area of interest, such as an area containing outliers.

Potential applications for 3D image reconstruction of nano-objects using TEM are broad and might include validation of metrological artefacts, such as polystyrene latex nanoparticles, and site-specific analysis of interfaces buried within devices, and measurement of individual objects such as nanoparticles. The method might also be utilized to obtain detailed shape of non-symmetric nano-objects such as nanorods and nanocrystals.

Other applications include calibration for a variety of nanoscale characterization tools, particularly nanoscale characterization instruments and artefacts, to ensure that they are applied in a consistent way.

Case studies are provided in informative annexes, including variations of sample preparation, data acquisition, alignment and reconstruction methods. It is noted that placing of alternative data acquisition, alignment and reconstruction methods in annexes does not imply that a method is inferior to the one described in the main body of the document. Conversely, such might be the subject of future revisions of this document. However, the process, from sample preparation on a rod-shaped support to extraction of measurands, has been tested in accordance with the steps described in this document and tested on samples described in the annexes.

Figure 1 summarizes the procedure steps in this document. Normative aspects are highlighted in red. Informative aspects are highlighted in blue and appear in annexes. Additional annexes not listed in this figure are also included.

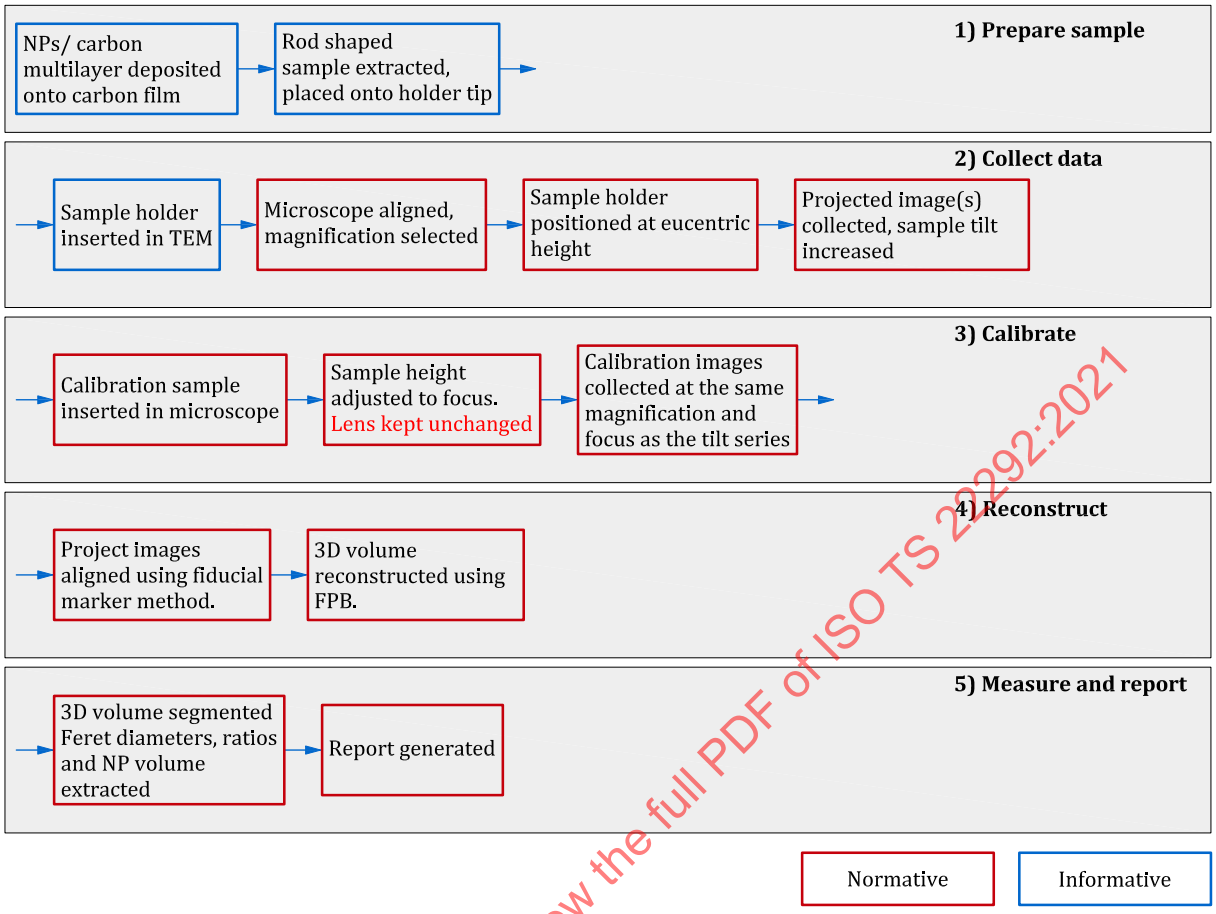



**Figure 1 — Procedure steps**

# Nanotechnologies — 3D image reconstruction of rod-supported nano-objects using transmission electron microscopy

## 1 Scope

This document provides guidance for sample preparation, data acquisition by transmission electron microscopy, data processing, and three-dimensional image reconstruction to measure size and shape parameters of nano-objects on rod-shaped supports. The method is applicable to samples dispersed on or within an electron-transparent rod-shaped support.

## 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 99:2007, *International vocabulary of metrology — Basic and general concepts and associated terms (VIM)*

ISO/TR 945-2:2011, *Microstructure of cast irons — Part 2: Graphite classification by image analysis*

ISO/TS 10797:2012, *Nanotechnologies — Characterization of single-wall carbon nanotubes using transmission electron microscopy*

ISO 21363, *Nanotechnologies — Measurements of particle size and shape distributions by transmission electron microscopy*

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

ISO 26824:2013, *Particle characterization of particulate systems — Vocabulary*

ISO/TS 80004-1:2015, *Nanotechnologies — Vocabulary — Part 1: Core terms*

ISO/TS 80004-2:2015, *Nanotechnologies — Vocabulary — Part 2: Nano-objects*

ISO/TS 80004-6:2021, *Nanotechnologies — Vocabulary — Part 6: Nano-object characterization*

ISO/TS 80004-8:2020, *Nanotechnologies — Vocabulary — Part 8: Nanomanufacturing processes*

## 3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/IEC Guide 99:2007, ISO/TR 945-2:2011, ISO/TS 10797:2012, ISO/TS 24597:2011, ISO 26824:2013, ISO/TS 80004-1:2015, ISO/TS 80004-2:2015, ISO/TS 80004-6:2021, ISO/TS 80004-8:2020 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 Nanotechnology-related terms

**3.1.1**
**nanoscale**
length range approximately from 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**
**nanomaterial**
material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale

Note 1 to entry: This generic term is inclusive of nano-object and nanostructured material.

[SOURCE: ISO/TS 80004-1:2015, 2.4]

**3.1.3**
**nano-object**
discrete piece of material with one, two or three external dimensions in the nanoscale

Note 1 to entry: The second and third external dimensions are orthogonal to the first dimension and to each other.

[SOURCE: ISO/TS 80004-1:2015, 2.5]

**3.1.4**
**nanoparticle**
nano-object with all external dimensions in the nanoscale where the lengths of the longest and the shortest axes of the nano-object do not differ significantly

Note 1 to entry: If the dimensions differ significantly (typically by more than three times), terms such as nanofibre or nanoplate may be preferred to the term nanoparticle.

[SOURCE: ISO/TS 80004-2:2015, 4.4, modified — the abbreviation "NP" has been deleted".]

## 3.2 Instrument-related terms

**3.2.1**
**scanning electron microscopy**
**SEM**
method that examines and analyses the physical information (such as secondary electron, backscattered electron, absorbed electron and X-ray radiation) obtained by generating electron beams and scanning the surface of the sample in order to determine the structure, composition and topography of the sample

[SOURCE: ISO/TS 80004-6:2021, 4.5.5]

**3.2.2**
**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: Typically uses an electron beam with a diameter of less than 1 nm.

Note 2 to entry: Provides high-resolution imaging of the inner microstructure and the surface of a thin sample (or small particles), as well as the possibility of chemical and structural characterization of micrometre and sub-micrometre domains through evaluation of the X-ray spectra and the electron diffraction pattern.

[SOURCE: ISO/TS 80004-6:2013, 4.5.7]

**3.2.3**
**transmission electron microscope**
**TEM**
method that produces magnified images or diffraction patterns of the sample by an electron beam which passes through the sample and interacts with it

[SOURCE: ISO/TS 80004-6:2021, 4.5.6]

**3.2.4**
**focused ion beam instrument**
**FIBI**
instrument and method that allows to fabricate objects at nanoscale using a focused ion beam (FIB), typically Gallium, and observe the fabricated area using an SEM column located in the same instrument chamber

Note 1 to entry: For FIB lithography, refer to ISO/TS 80004-8:2020, 7.1.9.

Note 2 to entry: For FIB focused ion-beam deposition refer to ISO/TS 80004-8:2020, 7.2.12.

**3.2.5**
**dual beam instrument**
**DBI**
instrument combining the instruments used in the *SEM* (3.2.1) and *FIB* (3.2.4) methods

## 3.3 Measurement-related terms

**3.3.1**
**Feret diameter**
distance between two parallel tangents on opposite sides of the image of a particle

[SOURCE: ISO 26824:2013, 8.6]

**3.3.2**
**maximum Feret diameter**
maximum value of Feret diameter of an object, whatever its orientation

[SOURCE: ISO/TR 945-2:2011, 2.1]

**3.3.3**
**minimum Feret diameter**
minimum value of Feret diameter of an object whatever its orientation

[SOURCE: ISO 21363:2020, 3.4.5]

**3.3.4**
**pixel**
smallest non-divisible image-forming unit on a digitized TEM image

[SOURCE: ISO/TS 24597:2011, 3.1, modified — the abbreviation "TEM" has been changed to "SEM".]

**3.3.5**
**measurand**
quantity intended to be measured

[SOURCE: ISO/IEC Guide 99: 2007, 2.3]

# 4 Sample considerations

## 4.1 General

Clause 4 discusses physical properties of the sample rod. For methods that can be applied to prepare the sample rod, see Annexes A, E and F.

## 4.2 Choice of sample rod diameter

Sample rod diameter considerations apply to both TEM and scanning TEM (see Annex B) as follows:

a) The sample shall be rod-shaped with cross section shape no more that 50 % different from circular cross section (1:1,5 ratio of axis length for elliptical sample rod cross section);

NOTE 1 Rectangular cross-section that does not exceed the 1:1,5 aspect ratio is acceptable.

b) The sample rod shall be made of low atomic number material such as carbon;

c) The sample rod diameter shall be less than one inelastic mean free path for the incident electron energy in the TEM chosen. For example, at 300 keV incident electron energy a carbon rod with less than 250 nm diameter shall be utilized;

d) Sample rod diameter that exceeds twice the inelastic mean free path shall be avoided to reduce the effect of plural electron scattering in the sample rod and the associated loss of spatial resolution[5][6][7];

e) The effect of geometrical broadening of the electron beam shall be kept at a small fraction of desired resolution of the final 3D reconstructed volume. The geometrical broadening can be estimated from instrument convergence semi-angle and collection semi-angle [1];

f) To ensure adequate image resolution, the sample rod diameter shall not exceed two times the depth of focus [15].

NOTE 2 Typical imaging conditions in conventional TEM mode at 300 keV electron energy allow for about 250 nm sample rod diameter.

NOTE 3 The choice of imaging parameters for TEM and STEM tomography, depth focus, and rod diameter is described in detail in Reference [15].

# 5 Instrument factors

## 5.1 Microscope set up

### 5.1.1 General

This clause provides guidance on conventional parallel beam transmission electron microscope (TEM) instrumentation set up for data acquisition. For scanning TEM (STEM) instrumentation set up, see Annex B.

The critical set up parameters for TEM data acquisition are:

a) acceleration voltage (see 5.1.2);

b) convergence semi-angle (see 5.1.3);

c) collection angle (see 5.1.4);

d) microscope magnification (see 5.1.5);

e) number of pixels of the detector (see 5.1.6);



f) image acquisition time (see 5.1.7).

### 5.1.2 Acceleration voltage

The acceleration voltage shall be selected as described in A.2.4 d) on sample thickness. Typically, 300 kV or 200 kV should be used.

Using the maximum voltage available on a particular TEM is preferred. Using the maximum available voltage means maximum allowable sample rod diameter and maximum depth of focus. For example, using 300 kV rather than 200 kV allows for 250 nm diameter carbon rod rather than 200 nm diameter rod at 200 kV.

### 5.1.3 Convergence semi-angle

The convergence semi-angle shall be selected so that the illumination at the sample plane is uniform over the imaged area of the sample. Furthermore, the illumination uniformity has to be such that the apparent sample focus does not visibly change over the observed area.

NOTE The illumination uniformity can be verified by performing an intensity profile across the image (e.g. diagonally from corner to corner of the image). The uniformity of the defocus value is typically not a concern and it can be verified by performing a fast Fourier transform (FFT) of sub-areas of the image. For example, a 2048 × 2048 image can be divided into 256 × 256 pixels regions of interest. The image composed of absolute value of FFT can be compared among the various regions of interest to ensure that the FFT does not vary too much from region to region. An FFT that has same numbers of circular rings over an arbitrary sub-field of view is an indication that illumination is sufficiently uniform.

### 5.1.4 Collection angle

In conventional TEM mode the collection angle is selected by the objective aperture size. The collection angle is determined as the square root of the sum of squares of the convergence angle and acceptance angle of the objective aperture. In practise the convergence angle in TEM mode is much smaller than the acceptance angle of the objective aperture. Therefore, the collection angle is determined by the objective aperture acceptance angle alone.

The main criteria for collection angle is the avoidance of diffraction contrast while maximizing the contrast in the image. The contrast increases with decreasing collection angle,[9][10] but at the same time, the diffraction contrast increases with decreasing collection angle. The presence of diffraction contrast could make the data unsuitable for 3D reconstruction.[9][10] The number of counts collected by the detector decreases with decreasing collection angle leading to increase in acquisition time.

### 5.1.5 Microscope magnification

Microscope magnification and detector pixel size are critical parameters to ensure correct sampling of the object for 3D reconstruction. The higher the desired number sampling, the lower the effect of the detector point spread function. At the same time high sampling, i.e. high microscope magnification and small pixel size, decreases the field of view that can be acquired without region stitching by subsequent offset acquisitions. High magnification also decreases the number of counts per pixel at a given beam current density and acquisition time. Typically, the magnification is chosen so that the desired projected image resolution is sampled by 5 pixels or more[11].

EXAMPLE If a desired projected image resolution is 1 nm, the pixel size is chosen to be about 0,2 nm. A 2 048 pixel × 2 048 pixel camera can then cover a 410 nm × 410 nm field of view that is adequate for most practical purposes. For example, a camera with physical 5 µm × 5 µm pixel size and 0,2 nm pixel size at the sample plane requires microscope magnification 25,000×. Typically, a somewhat higher magnification, for example, 30,000×, can be chosen to slightly oversample the object.

### 5.1.6 Number of pixels of the detector

The highest number of pixels on the camera should be used to achieve optimum image resolution and field of view. For example, it is advisable to use binning 1 for a 2 048 pixel × 2 048 pixel camera so that

there are 2 048 pixel × 2 048 pixel in the images. Number of pixels, their size, the field of view and the image resolution are related. See 5.1.5 for an example of magnification estimate.

If the point spread function of the camera is poor, it can be necessary to combine camera pixels (bin the pixels). In such case, the necessary magnification should be estimated for the combined pixel size. Combining (binning) the camera pixels results in a corresponding decrease of the field of view as compared to binning 1 images. For example, a 1 000 $nm^2$ × 1 000 $nm^2$ field of view with 0,5 nm per pixel obtainable with 1 000 pixel × 1 000 $pixel^2$ camera would be reduced to 500 nm × 500 nm if the camera is binned by 2 while maintaining sampling 0,5 nm per pixel.

### 5.1.7 Image acquisition time

Image acquisition time shall be chosen such that enough signal to noise ratio is obtained in the projected images to allow for projected image alignment and for reconstruction. The acquisition time needs to be chosen such that the sample drift is less than the pixel size at the sample plane. If the number of counts per pixel is too low at the drift limited acquisition time, either the microscope beam current can be increased or multiple images at each tilt can be acquired. Typically, about 100 electrons per pixel of the detector are sufficient for alignment and reconstruction[12].

## 5.2 Microscope calibration

To ensure correct microscope calibration, the microscope shall be calibrated under the same imaging conditions as used for the tomography data acquisition. The calibration shall be performed either immediately before or immediately after the data acquisition. Identical conditions in this case refers to using the same lens currents, the objective lens and the intermediate and projector lens system currents, as used for the tomography data acquisition. The calibration of the TEM instrument shall be performed as described in ISO 21363.

NOTE Calibrating before or after collection of projected images is equivalent as long as the lens settings are not changed between calibration and collection of projected images.

For precise size measurement by using TEM, the same condition of TEM lens and specimen height between a measurement specimen and a calibration specimen is important. An internal reference length for TEM instruments must be calibrated using calibration standards. All size measurements should be done with the same lens and specimen height conditions of calibration. Focusing condition of the lens can affect the size measurement. A Scherzer defocus condition is generally used. The zero defocus is defined using Fresnel fringe at first, then focusing goes to Scherzer defocus. Specimen height should be at the eucentric position. It is important to obtain images at eucentric height and the same defocus condition at magnifications as used explicitly for the instrument's calibration.

For most microscopes the selection of identical conditions is achieved by selecting the same nominal magnification and same focus value of the objective lens current as used for the tilt series acquisition. The sample focusing shall be achieved by utilizing the mechanical Z height of the stage. Focusing by changing the imaging lens in a TEM or probe forming lens in STEM should be limited to a range no more than ±1 µm to prevent magnification change or image rotation. The lens currents must be same for microscope calibration as for data collection. Calibration should be performed at the eucentric height and at the limits of the range of lens current focusing. For calibration a suitable calibration sample with known dimensions shall be used. An example of such sample is available from several suppliers[13].

The pixel size obtained using the above calibration procedure shall be used to calibrate the reconstructed 3D volume.

See Annex D for recommended microscope and data collection parameters to record. See Annex H for an example of uncertainty budget.



# 6 Image capture (data acquisition)

## 6.1 General

This clause covers image capture to obtain projected images in 2D.

NOTE ISO 21363 provides extensive information on collection of projected images, that is also applicable to collection of projected images for electron tomography reconstruction.

## 6.2 Procedure

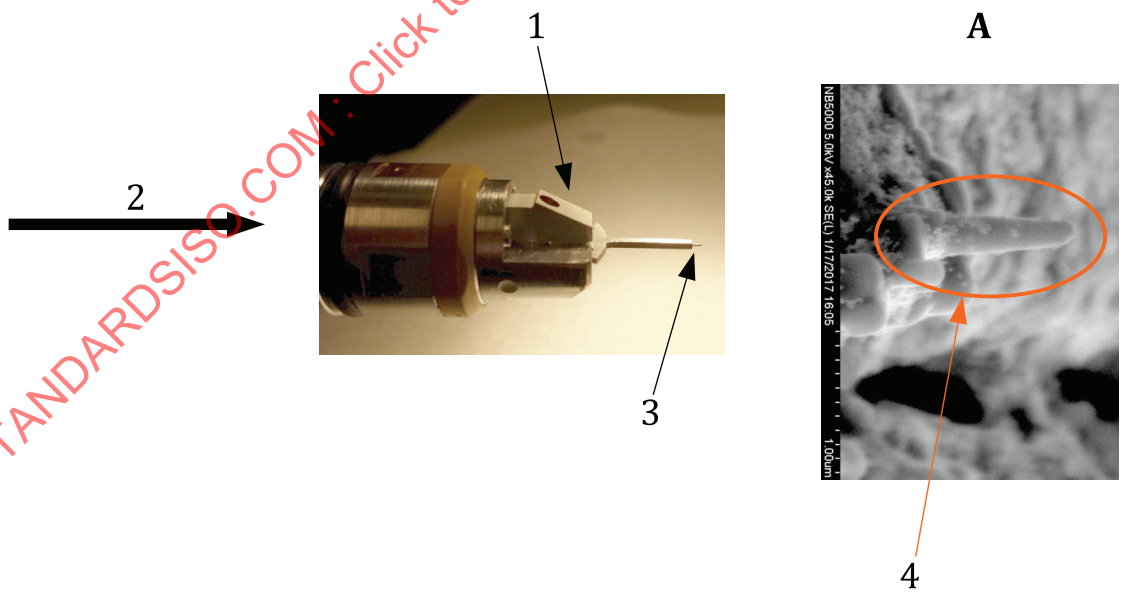
The procedure is as follows.

a) The data shall be collected in a transmission electron microscope (TEM) in conventional TEM mode. Alternatively, a scanning TEM (STEM) mode can be used, see Annex B. The TEM shall be operated at incident energy between 100 kV and 400 kV. The microscope can have thermal electron source ($LaB_6$), field assisted thermal electron source (Schottky electron source) or a field emission electron source. The point resolution of the microscope shall be 0,3 nm or less. The information limit of the microscope shall be 0,2 nm or less.

b) The microscope shall allow sample to be tilted over −90° to +90° around at least one axis perpendicular to the incident beam. The microscope vacuum pressure near the sample chamber shall be $1 \times 10^{-7}$ torr or less.

c) Data acquisition shall be bright field TEM. Other methods such as scanning TEM (STEM) bright field (BF-STEM), annular dark field (ADF-STEM), and high angle annular dark field HAADF-STEM are acceptable if they provide contrast that is predominantly monotonic with sample thickness regardless of tilt angle. See Annexes B and C for examples.

d) The sample shall be mounted as depicted in Figure 2 with the sample long axis perpendicular to the electron beam and parallel with the tilt axis of the sample stage.




**Key**

1 sample stage clamp
2 tilt axis = sample holder axis
3 sample tip with carbon rod
4 carbon rod with nanoparticles
A SEM image of sample tip with sample rod

**Figure 2 — Sample rod at the tip of a TEM holder**

e) The electron beam convergence angle and collection angle, as determined by objective aperture shall be selected such that the contribution of diffraction contrast is small. Typically, ~100 mrad collection angle is sufficiently large at 300 kV to avoid artefacts arising from diffraction contrast. Typically, the smallest effect of diffraction contrast can be achieved by using no objective aperture. The image contrast of nanoparticles shall be maximized while keeping the diffraction contrast to a minimum.

f) Eucentric height shall be adjusted by minimizing the sample lateral movement when the sample is tilted over the entire −90° to +90° tilt. Sample focusing using objective lens excitation shall be limited to ±1 µm range to prevent change in magnification and sample rotation. Sample focusing exceeding the ±1 µm range shall be achieved by adjusting mechanical Z height of the sample stage.

g) The lateral (X, Y) positioning of the sample on the camera within ±1 µm range shall be done using deflector coils. Outside the ±1 µm range mechanical movement of the sample stage shall be used.

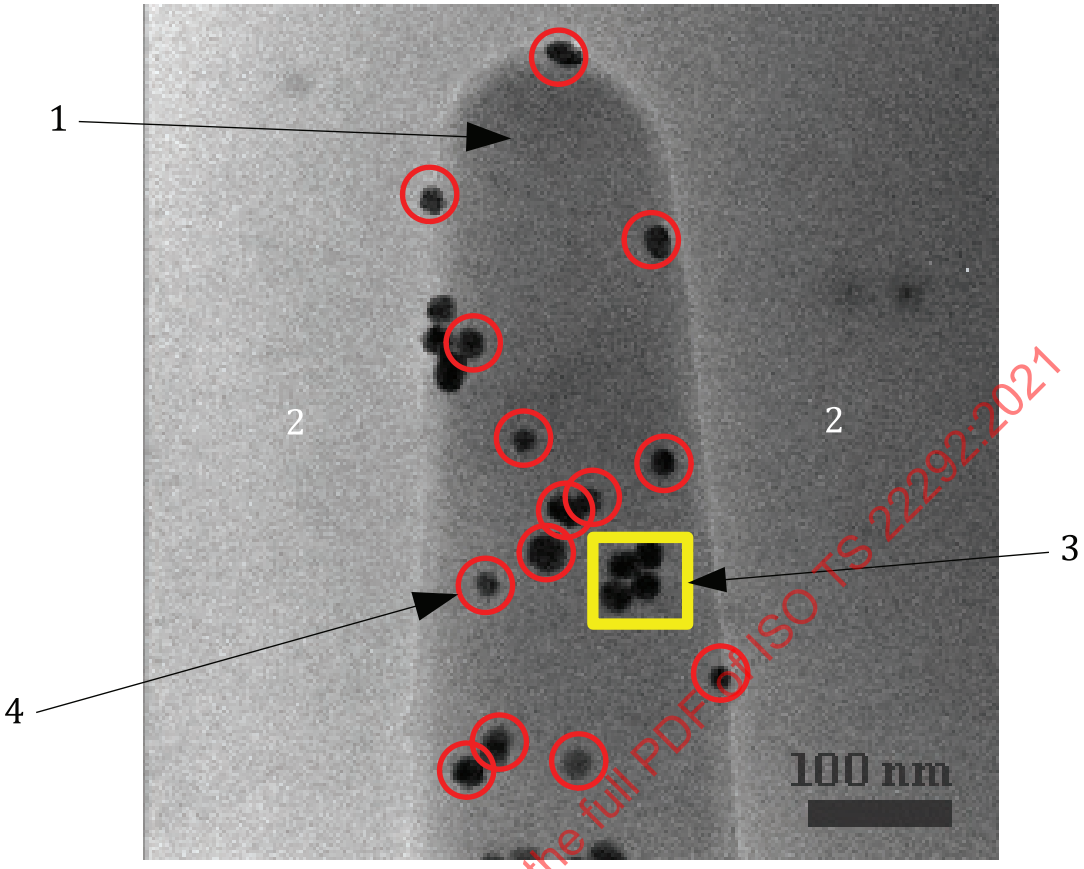
h) The microscope magnification shall be set up to acquire about 5 pixels per desired resolution in the reconstructed volume. For example (0,2 × 0,2) $nm^2$ image pixel size shall be used for 1 nm intended resolution of the 3D reconstructed volume.

i) The data can be collected manually, or an automated data acquisition package can be used.

j) The bit depth of the saved images shall be 16 bit or more. The bit depth should be chosen such that the digitization of the data does not lead to loss of dynamic range or insufficient sampling of image greyscale levels. The camera output can provide more than 16-bit dynamic range. The data should be saved with at least 16-bit dynamic range.

k) The field of view should include sufficient number of nanoparticles, as shown in Figure 3 to ensure a maximum number of well-separated particles. The number achievable will vary from sample to sample and also will vary for deposited vs embedded particles. To obtain sufficient total number of nanoparticles, multiple fields of view along the rod axis can be collected. For example, two fields of view, about 500 nm each, can be obtained with a 1000 nm long sample rod.

NOTE 2 ISO 21363 provides guidance on the number of nanoparticles needed for statistical analysis.




**Key**

1 carbon rod
2 vacuum
3 clustered nanoparticles not suitable for analysis
4 well separated nanoparticles suitable for analysis

**Figure 3 — Experimental projected TEM image showing 20 nanoparticles visible within the field of view**

l) Projected images shall be acquired in 3° or less increments from −90° to +90° stage tilt. Here 0° corresponds to tilt value where the sample rod is not tilted and corresponds to sample exchange position in most microscopes.

Suggestion for file naming: file names should indicate the nominal tilt at which they were acquired. For example, the positive tilt angles can be marked by underscore "XXXXXXXX_zzz.tiff" while the negative tilt angles can be marked by a hyphen "-" such as "XXXXXXXX_-zzz.tiff". Here "XXXXXXXX" is arbitrary name up to 8 characters long, "zzz" is image number. The projected data can be saved as uncompressed tiff or in Gatan Digital Micrograph format. When Gatan Digital Micrograph file format is used, the file name extension can reflect that by using a ".dm3" or ".dm4" filename extension.

NOTE 3 Stack data formats, such as MRC and various binary formats can be used.

Use of data formats with publicly available documentation of the format are recommended.

m) The images acquisition time shall be selected such that about 100 electrons per pixel are collected in each image of the tilt series.

n) The procedure for individual image acquisition described in ISO 21363 shall be used.

# 7 Data alignment and volume reconstruction

## 7.1 General

This clause covers steps to obtain a 3D reconstruction from collected 2D projected images. One needs to use a 3D reconstruction algorithm implemented in software. Filtered back projection (FPB) is described here.

NOTE Alternative reconstruction algorithms, such as simultaneous iterative reconstruction technique (SIRT), are described in Annex G.

## 7.2 Procedure

The procedure is as follows:

a) A suitable tomography reconstruction software package shall be utilized.

NOTE 1 See Annex C.

b) The images within the tilt series shall be aligned to eliminate lateral RMS displacement among images in the tilt series to less than 1 pixel over the entire tilt range −90° to +90°. The alignment can be performed manually or using an automated procedure. The nanoparticles themselves can be utilized as fiducial markers for image alignment.

c) Using the aligned image tilt series, the 3D volume shall be reconstructed using standard filtered back projection (FBP) algorithm. A linear ramp filter shall be used for the back-projection step. The reconstruction shall be performed for example using a suitable package as listed in Annex C.

d) The reconstructed volume shall be saved as a cube of raw data with X, Y, Z axis and reconstructed intensity in all three dimensions. For example, a reconstructed volume shall be saved as a volume of 500 volume pixels × 400 volume pixels × 300 volume pixels, 16 bit per pixel.

NOTE 2 16 bit = 2 byte / pixel results in 0,25 GB uncompressed file for a 500 volume × 500 volume × 500 volume.

NOTE 3 The use of bit depth higher than 16 bit is acceptable.

# 8 Reconstructed volume evaluation and data analysis

## 8.1 General

Clause 8 describes how to extract data from the reconstructed 3D data cube.

The particles selected for analysis shall not be clustered and they shall be visibly separated from adjacent particles.

Reliable separation of particles implies particle centre separation of about 2,5× their radius in case of spherical particles[14]. When the particles are non-spherical, the distance between particles larger than the 2,5× radius of the sphere fully enclosing the particles is recommended.

## 8.2 Identification of nanoparticles and 3D volume

The steps for identification of nanoparticles in the reconstructed 3D volume are as follows.

a) The first step toward extracting particle characteristics is the identification of the particles and their boundaries. This is achieved by suitable software.[15] All processing shall be performed on the 3D volume. This document does not make use of 2D projections of the 3D volume.

NOTE 1 Figure 4 shows the workflow diagram for the steps outlined in 8.2. An open source TomoMi software can be used to perform the nanoparticle extraction[15].



b) The reconstructed 3D data volume shall be loaded in a suitable software. A 3D median filter with 3 pixel × 3 pixel × 3 pixel is applied to the reconstructed volume.

c) A small cube-shaped box of the volume is selected around each nanoparticle selected for analysis. The nanoparticle selection is performed manually. The box around the nanoparticle should be about 40 % larger than the nanoparticle itself. Multiple nanoparticles are selected for a reconstructed 3D data set. Each box with nanoparticle is assigned an identifier number (ID#).

A cube shaped volume about 50 pixel × 50 pixel × 50 pixel should be selected around a spherical nanoparticle with a 30-pixel diameter.

d) The software shall perform local thresholding limited to the box with each selected nanoparticle. The thresholding is defined in 8.3. The nanoparticle boundary is thus identified by its boundary.

e) The software shall identify the minimum and maximum Feret diameter. The minimum Feret diameter $F_{min}$ is obtained by finding the minimum distance connecting the nanoparticle internal boundary in 3D. The maximum Feret diameter $F_{max}$ is obtained by finding the maximum internal distance connecting the nanoparticle boundary in 3D.

NOTE 2 The $F_{min}$ and $F_{max}$ obtained in 3D in general do not agree with $F_{min}$ and $F_{max}$ obtained from 2D projections, as described in ISO 21363.

f) The software shall calculate the Feret diameter ratio as $F_{rat} = F_{max} / F_{min}$;

g) The software shall extract the nanoparticle volume V by counting the voxels enclosed inside nanoparticle internal boundary;

h) The software shall export a CSV file with the following columns:

ID# $F_{min}$ $F_{max}$ $F_{rat}$ Volume[15].

## 8.3 Thresholding for measurand extraction

The method for local thresholding for detection of nanoparticle boundaries is as follows:

a) A volume containing an individual nanoparticle shall be selected, see 8.2 c).

b) A three-dimensional median filter with 3 pixel diameter shall be applied to the volume containing an individual nanoparticle. All voxels, including those of the nanoparticle itself, shall be included in the threshold estimate.

c) The 3D volume obtained in b) shall be binarized (grayscale pixel values converted to 0 and 1 values.) by applying threshold $T$ obtained as:

$$T = (B_{min,NP} - B_{back}) \times t + B_{back}$$

where

$t$ is the parameter that optimizes variations among reconstruction methods such as FBP and SIRT; $t$ = 0,5 shall be used for volume reconstructed by FBP, see Reference [16] and 8.3, Note 1;

$B_{min,NP}$ is the minimum brightness within the nanoparticle;

$B_{back}$ is the brightness of the sample background outside the nanoparticle.

For nanoparticles that appear dark relative to the 3D volume background, as applicable to bright field TEM data.

$$T = (\mathrm{B}_{\mathrm{max,NP}} - B_{\mathrm{back}}) \times t + B_{\mathrm{back}}$$

where $B_{\mathrm{min,NP}}$ is the maximum brightness within the nanoparticle.

For nanoparticles that appear bright relative to the 3D volume background, as applicable to annular dark field scanning TEM images.

Voxels with values below the threshold value $T$ shall be assigned 0 (black) and voxels above the threshold value $T$ shall be assigned 1 (white).

The brightness of background shall be taken as the value of the highest number of background voxels in a histogram of the entire volume including the single nanoparticle, see 8.2 c) and a) above. Therefore, the brightness of background is obtained from all pixels in the volume including pixels of the nanoparticle itself.

NOTE 1 Interlaboratory comparison (ILC) data discussed in the annexes were processed using value $t$ = 0,5. See Annexes E and F.

NOTE 2 See Figures 4 and 5.

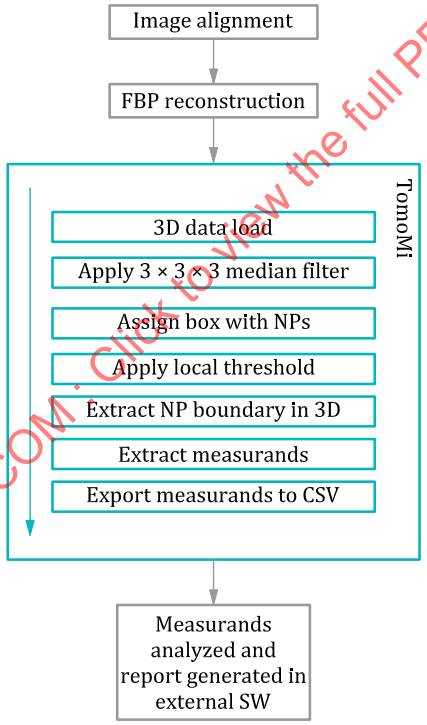
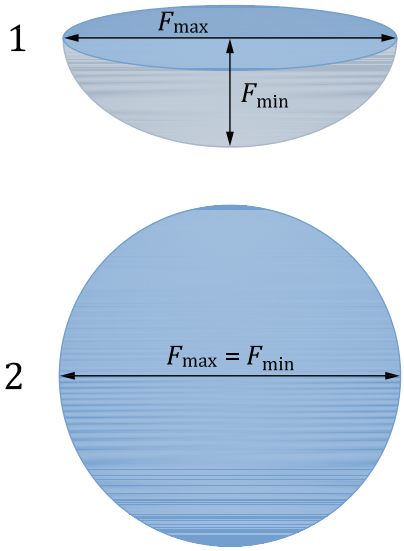



**Figure 4 — Flow chart diagram for volume segmentation and extraction of measurands**




**Key**

1 $F_{min}$ and $F_{max}$ obtained in 3D

2 $F_{min}$ and $F_{max}$ obtained from 2D projection

NOTE The concept can be also applied to objects that have non-circular 2D projection.

**Figure 5 — Concept of 3D segmentation and the difference as compared to 2D segmentation**



# 9 Expression of results

## 9.1 Extracting parameters for each well-separated nano-object

Primary size and shape parameters may include the following:

a) Minimum Feret diameter $F_{min}$.

NOTE 1 In this document, parameter evaluation uses the binary 3D volume rather than a 2D image discussed in ISO 21363.

b) Maximum Feret diameter $F_{max}$.

NOTE 2 In this document, parameter evaluation uses the binary 3D volume rather than a 2D image discussed in ISO 21363.

c) Feret ratio $F_{rat} = F_{max}/ F_{min}$.

NOTE 3 Figure 5 illustrates the difference of $F_{max}$ and $F_{min}$ obtained between 2D and 3D. In 2D, the depicted object would have $F_{rat} = 1$. In 3D, the same object can have $F_{rat} \neq 1$.

d) Nano-object volume.

Nano-object volume is the volume of an individual nano-object in $nm^3$ and is obtained by summing the pixels with digital 0 that belong to a particular particle for all binary image slices in which the particle is detected. The number of the pixels where a particle is detected is then multiplied by the pixel area in square nanometers and by the slice thickness in nanometers. The process is repeated for each binary image slice in which a particular particle is detected to obtain particle volume in each slice where it is detected. The volume values for each slice are then summed to obtain the entire particle volume. The process is repeated for all well separated particles present in the reconstructed volume.

NOTE 4 The method described in this document makes no assumption on object symmetry. The measurands described in 9.1 can be equally applied to nearly spherical nanoparticles as they can be applied to highly non-symmetrical objects such as rods, prisms and platelets.

## 9.2 Measurement uncertainty

The relevant documents on measurement uncertainty include but are not limited to ISO/IEC 17025:2005, Clause 5, ISO/IEC Guide 98-3 (GUM:1995) and ISO/IEC Guide 99 (VIM). Also, ISO 19749:2021, 10.3.1 as applicable to assessment of measurement uncertainty, e.g. formulae provided and information on statistical error and number of samples for size measurements in ISO 19749:2021, 6.6.

NOTE 1 ISO 21363 provides detailed information on measurement uncertainty and sources of error in 2D TEM analysis. The information in ISO 21363:2020, 9.4, is relevant to reconstruction imaging in 3D.

NOTE 2 The errors specific to 3D reconstruction and measurement of minimum and maximum Feret diameter and nanoparticle volume are discussed in Reference [15]. Annexes E, F and H in this document describe particular application examples and provide insight on the sources of errors and their magnitude for a given application.

## 9.3 Sources of errors

The sources of error of the measurand arise from each step of the process:

a) sample that is not representative of the object of interest (9.3.1);

b) acquisition of 2D projected image acquisition (9.3.2);

c) instrument calibration (9.3.3);

d) alignment of the projected images (9.3.4);

e) reconstruction of the 3D volume (9.3.5);

f) discrete representation of the objects (nanoparticles) in 3D (9.3.6);

g) interpretation of the obtained measurands, see 9.3.7;

h) limited number of observed objects (nanoparticles) (9.3.8).

### 9.3.1 Error arising from sample that is not representative of the object of interest

The method described in this document requires a subset of the studied objects (nanoparticles) to be selected for detailed analysis in 3D. A systematic error can arise if the selected nanoparticles are not representative of the large volume population. A sample preparation method shown in Annex A can reduce the likelihood that a subset that is not representative of the large volume is selected. The sample nanoparticles are repeatedly deposited onto a flat carbon film the same way as used for 2D TEM analysis, see ISO 21363. The sample deposited onto thin carbon film can be examined by 2D TEM analysis (see ISO 21363) prior to extracting the desired area of the sample for 3D analysis.

NOTE 1 The 3D analysis can be applied for example to outliers identified by 2D TEM analysis of large number of nanoparticles. An area of the sample containing large concentration of outliers can be selected based on a 2D TEM analysis (see ISO 21363) of the sample.

ISO 21363 shall be consulted to ensure use of representative sample for the 2D sample preparation.

NOTE 2 Preparation of sample that is representative of the investigated nanomaterial is discussed in ISO 21363:2020, 5.2.

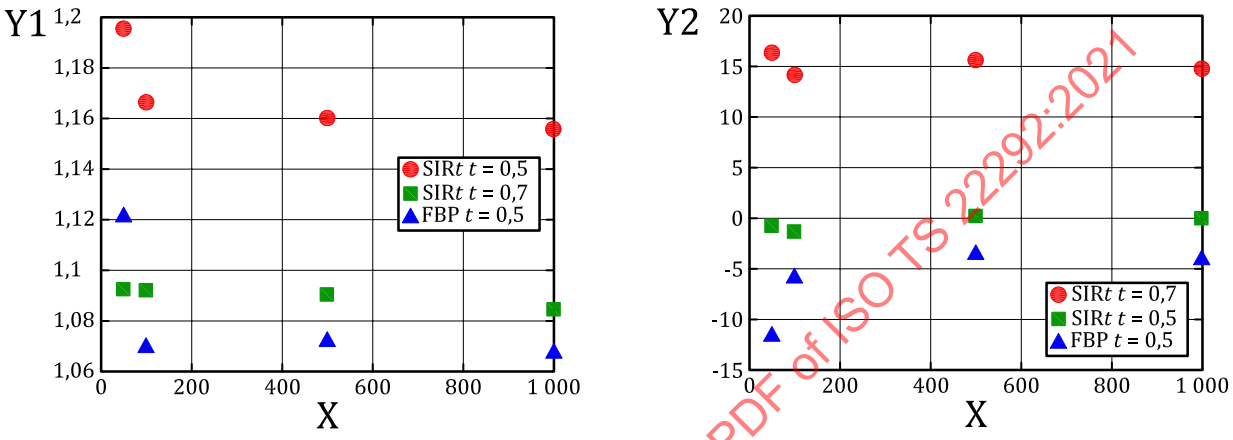
### 9.3.2 Acquisition of 2D projected images

The same concepts apply as described in ISO 21363:2020, Clause 7. Furthermore, the image acquisition dose should be kept low to avoid radiation damage of the sample during image acquisition. About 100 electrons per pixel per each projected image provides sufficient signal to noise ratio for image



alignment and 3D volume reconstruction.[15] See Figure 6 illustrating the error in measured and anticipated volume of a spherical particle. A model of a spherical particle was generated in a computer. Sixty projected images in 3-degree tilt increment were generated and Gaussian shot noise added to each of the projected images correspond to number of electrons per pixel indicated on the x abscissa. The difference between the computer-generated sphere and the reconstructed sphere aspect ratio and volume is plotted on the y abscissa.[15] The plot suggests that FBP reconstruction of projected images with about 100 electrons per pixel provides reasonable estimate of Feret diameter ratio and volume of the particle. The SIRT reconstruction method may tolerate lower signal to noise ratio, but systematically overestimates aspect ratio, see Annex G.




**a) Effect of reconstruction method and threshold on measured aspect ratio**

**b) Error in measured of volume as function of irradiation dose**

**Key**

X count per pixel

Y1 aspect ratio

Y2 error of volume (%)

NOTE The x-abscissa indicates the number of electrons i.e. number of counts per pixel in each of the 60 projected images collected over 0-degree to 180-degree tilt range. Threshold value calculated using $t$ = 0,5, see 8.3, is plotted for both FBP and SIRT reconstruction methods. Additionally, threshold value calculated using $t$ = 0,7 in 8.3, is also investigated for SIRT. a) Measured aspect ratio and b) error in measured volume as function of irradiation dose. Dose exceeding about 100 electrons per pixel does not improve precision of the measurement but can result in additional radiation damage.

**Figure 6 — Error arising from limited signal to noise ratio**

Beam-induced nanoparticle movement can lead to images that are impossible to align. Beam current density at the sample that does not lead to beam induced movement of the nanoparticles shall be used[15].

Sample rod azimuthal tilt within typical values encountered in TEM tomography has limited effect on the quality of reconstructed volume[11].

NOTE 2 The sampling of objects in projected images intended for electron tomography is lower than that used for 2D TEM analysis. See 9.3.6.

NOTE 3 Beam-induced nanoparticle movement can be detected by comparing the images acquired at 0-degree sample tilt (start of acquisition) and 180-degree sample tilt (end of acquisition). The beam-induced nanoparticle movement can be also detected by inability to obtain correct image alignment.

### 9.3.3 Instrument calibration

The instrument calibration process for 3D TEM is same as in ISO 21363:2020, 6.2 and 6.3. Therefore, the same error estimate as in ISO 21363 applies.

### 9.3.4 Alignment of the projected images

The alignment of the projected images prior to 3D volume reconstruction can be a major source error. However, using fiducial markers[11] or the nanoparticles themselves in-lieu of fiducial markers can reduce the alignment error to below 1-pixel value.

### 9.3.5 Reconstruction of the 3D volume

The error arising from 3D volume reconstruction strongly depends on the method used for reconstruction. Here we described limitations applicable to filtered back projection. Alternative methods are discussed in the annexes. See Figure 6 for an illustration of the effect of reconstruction method, signal to noise ratio and threshold for nanoparticle segmentation on measured volume and ratio of Feret diameters[16].

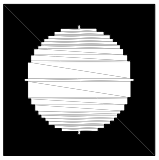
### 9.3.6 Discrete representation of the objects (nanoparticles) in 3D

A nanoparticle is an object that has continuous boundaries down to atomic scale. However, in a computer a nanoparticle is represented by discrete pixels in three dimensions. Figure 7 and Table 1 illustrate the error arising from discrete representation of a nanoparticle. The values in Table 1 were obtained by comparing a sphere (aspect ratio $F_{max}/ F_{min}$ = 1 with known volume obtained from its diameter in pixels) to discretized version of the sphere. The $F_{min}$ and $F_{max}$ were obtained as indicated in Figure 4. The volume was obtained by counting voxels that are inside the discretized sphere.[15] The effect of discretization does not include the above effect of reconstruction and thresholding. Figure 7 and Table 1 indicate that a nanoparticle should be sampled by about 30 pixels or more to reduce effect of discretization on measured Feret diameter ratio.

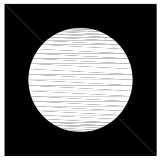
The desired resolution of the reconstructed 3D volume should be about 1/30 of the size of the smallest investigated nanoparticles.

NOTE 1 Routine resolution of 3D reconstruction in a TEM is approximately $(1\ nm)^3$, implying that the precision of shape evaluation of nanoparticles less than ~30 nm in diameter could be limited by the intrinsic resolution of the method.

NOTE 2 Detection of nanoparticles sampled by fewer than 5 pixels can be possible. However, it is not always possible to measure shape of such nanoparticles.



a) Discretized shape



b) Original shape

**Figure 7 — Effect of discretization of a continuous nanoparticle**

**Table 1 — Effect of discretization of a continuous nanoparticle**

| Sphere original diameter pixel | Maximum Feret diameter | Minimum Feret diameter | Aspect ratio | Error of volume % |
|---|---|---|---|---|
| 8 | 8 | 7,0 | 1,15 | 4,1 |
| 16 | 16 | 15,2 | 1,05 | 1,7 |
| 32 | 32 | 31,1 | 1,03 | 0,5 |
| 64 | 64 | 63,6 | 1,01 | 0,1 |

### 9.3.7 Interpretation of the obtained measurands

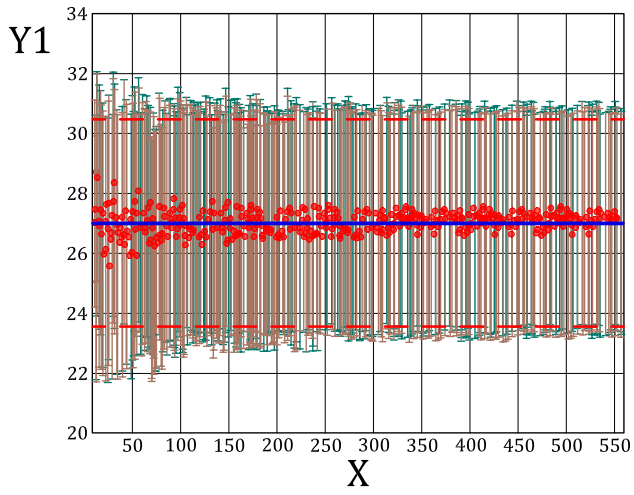
The summary measurands describing a nanoparticle, such as Feret diameter ratio and volume, may allow for statistical evaluation and sample comparison even for a small number of nanoparticles typically evaluated by electron tomography, see 9.3.8. However, the description of the nanoparticle by the measurands may not fully convey the nanoparticle shape and the shape relevancy to its properties. Visual inspection of the reconstructed 3D shape of the nanoparticles is recommended.

NOTE In some applications, such as catalysis, a small fraction of nanoparticles can have a disproportional impact for that application. The 3D reconstruction of outliers identified by 2D TEM (see ISO 21363) enables detailed study of the outliers.

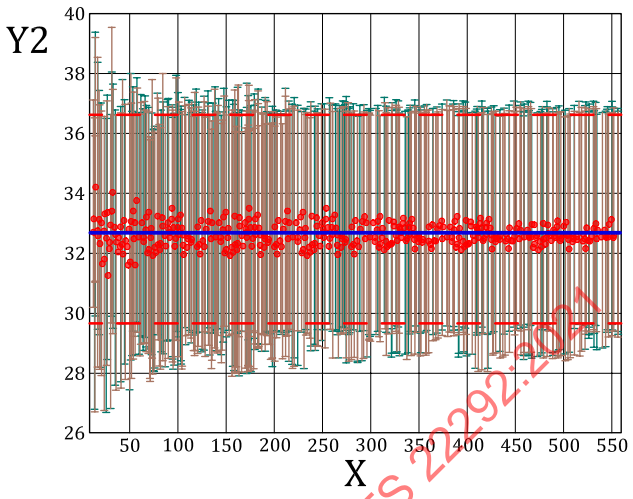
### 9.3.8 Limited number of observed objects (nanoparticles)

The finite (limited) number of particles analysed results in error arising from limited counting statistics. Figure 8 illustrates the effect of limited number of analysed particles on minimum and maximum Feret diameter, see a) and b), as well as on Feret ratio, see c). A set of 507 nanoparticles was analysed. Consequently, a subset of the 507 nanoparticles with sample size from 10 nanoparticles to 507 nanoparticles was processed. The size of the subset is indicated on x abscissa of Figure 8 a), b) and c). The measured value of the mean is indicated by red circle and the standard deviation of the distribution is indicated by the size of the error bar for each subset size that is number of nanoparticles randomly selected from the 507-nanoparticle data set.

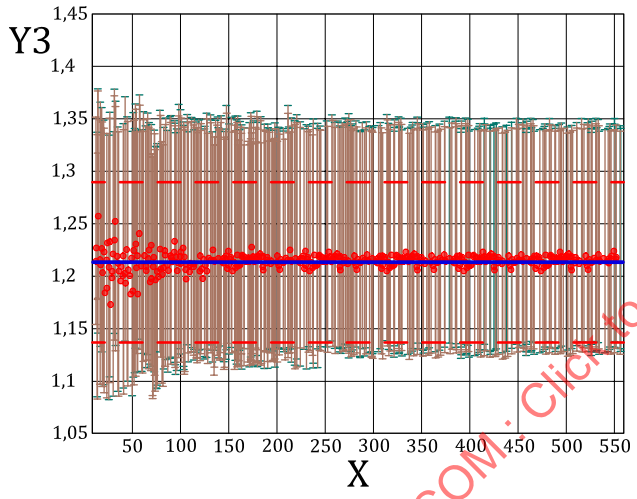
Log-normal distribution is assumed for the symbols (mean) and error bars (standard deviation). For comparison, mean and standard deviation of the entire set of 507 nanoparticles is indicated in blue solid (mean) and dashed red (standard deviation) under the assumption that the sample size and Feret ratio obey normal distribution.



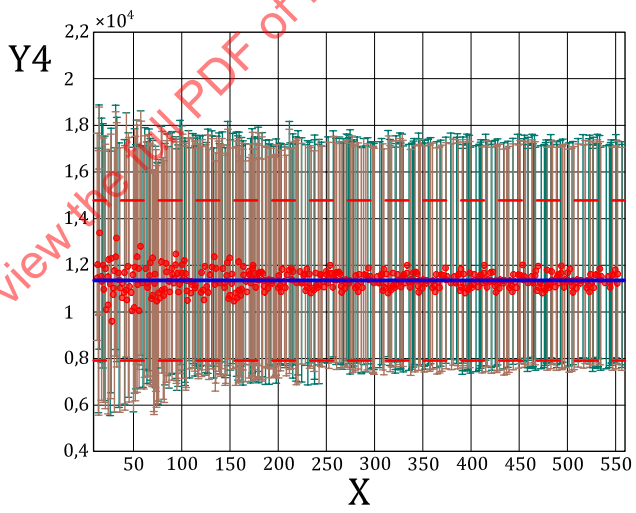

**a) Effect of limited number of nanoparticles on minimum Feret diameter**




**b) Effect of limited number of nanoparticles on maximum Feret diameter**




**c) Effect of limited number of nanoparticles on Feret ratio**




**d) Effect of limited number of nanoparticles on volume**

**Key**

| | | | |
|---|---|---|---|
| X | # of particles included in subset | | log-normal distribution |
| Y1 | subset of NPs: min. Feret diameter $\mu$ and $\sigma$ (nm) | | normal distribution |
| Y2 | subset of NPs: max. Feret diameter $\mu$ and $\sigma$ (nm) | | all NPs mean, normal distribution |
| Y3 | subset of NPs: Feret ratio $\mu$ and $\sigma$ | | all NPs std. dev., normal distribution |
| Y4 | subset of NPs: volume $\mu$ | | |

NOTE 1 The x abscissa indicates the number of randomly selected nanoparticles in a subset. The red symbols indicate sample mean for the corresponding subset size. The error bar indicates the standard deviation for the corresponding subset size. Log-normal nanoparticle size. The solid blue line indicates the mean of the entire 410 nanoparticle set assuming that the nanoparticle size obeys normal distribution. The dashed red lines indicate the standard deviation of the entire set of 410 samples assuming normal distribution of nanoparticle sizes.

NOTE 2 The figure shows the results under the assumption that the particle size obeys lognormal distribution (in blue) or normal distribution (in red).

a) The effect of limited number of analysed nanoparticles on minimum Feret diameter, $F_{min}$;

b) The effect of limited number of analysed nanoparticles on measured maximum Feret diameter, $F_{max}$;

c) The effect of limited number of analysed nanoparticles on measured Feret ratio, $F_{rat}$;

d) The effect of limited number of analysed nanoparticles on measured particle volume, $V$.

**Figure 8 — Effect of limited number of analysed nanoparticles on measured minimum and maximum Feret diameter, Feret diameter ratio and volume**

## 10 Test report

The test report shall include:

a) Lab identification (location);

b) Analyst name and contact information;

c) Identification of the nanomaterial analysed;

d) Date the data was collected and date the data was processed;

e) Sample preparation;

f) Data acquisition parameters (instrument parameters, see Table D.1);

g) Data analysis method/software;

h) Sample property evaluation (example provided in Table 2);

i) Reconstructed volume raw data (e.g. 500 volume pixel × 400 volume pixel × 300 volume pixel, signed 16 bit per pixel little endian encoding is recommended;

NOTE For example, TomoMi software is designed to load signed 16-bit little-endian 3D data sets.

j) The particle minimum and maximum Feret diameters evaluated in all three dimensions along all possible directions.

The evaluation of Feret diameter only along X, Y and Z axis or in slice images along only three planes can lead to incorrect measurement of Feret diameter. The Feret diameter shall be evaluated along all possible directions in 3D.

k) Non-sphericity of the particle taken as the ratio of the minimum and maximum Feret diameter evaluated in all three dimensions.

l) Nanoparticle volume in cubic nm.

m) Location of original projected images, 16-bit uncompressed TIFF or Digital Micrograph format (DM3). When raw data are used, 16-bit signed little endian is recommended. Three-dimensional data stack formats such as MRC and various binary formats can be used.

n) Note any differences from the described protocol.

**Table 2 — Nanoparticle measurement results**

| | **Date data collected** | **YYYY-MM-DD** | | **Date data processed** | **YYYY-MM-DD** | |
|---|---|---|---|---|---|---|
| Particle # | Minimum Feret diameter $F_{max}$ nm | Maximum Feret diameter $F_{min}$ nm | Aspect ratio $R = F_{max} / F_{min}$ | Volume $nm^3$ | Surface area $nm^2$ | |
| 1 | | | | | | |
| 2 | | | | | | |
| … [*add rows as needed*] | | | | | | |

# Annex A
(informative)

# Sample preparation

## A.1 General

This annex describes the steps that can be taken to process a sample from bulk to a form that can be examined by electron tomography. In all methods described here, the resulting sample is rod-shaped.

NOTE Rod composition has little effect on the measured results. It is important for the contrast difference between studied material and sample rod to be high. Additionally, it is important that the composition of the support rod is uniform to avoid spurious contrast for the rod affecting the 3D results[15].

Rod-shaped sample preparation steps are described for:

a) nanoparticles suspended in liquid;

b) nanocomposites and nanoparticles embedded in a matrix.

## A.2 Nanoparticles suspended in liquid

### A.2.1 General

This subclause provides information on how to prepare samples from nano-objects (e.g. nanoparticles, nanocubes, nanorods) suspended in a liquid (e.g. water or alcohol) by deposition onto a carboneous sample rod. It is applicable to nano-object suspensions for which the nano-objects readily adhere to carboneous sample rod.

### A.2.2 Carbon rod fabrication in a FIB

A carbon rod 100 nm to 250 nm in diameter can be prepared by FIB induced deposition from carboneous precursor.

NOTE 1 The carbon rods can be mass produced by lithography methods.

NOTE 2 The rod diameter is determined by the incident electron energy, intended electron energy and the nature of the sample, such as particle size and atomic number. See 4.2 and Reference [15].

The carbon rod for nanoparticle deposition can be fabricated under the example conditions described here. The rod is fabricated using a Gallium ion beam. Focused electron beam in a scanning electron microscope (SEM) or other types of focused ion beam may be also used, but the deposition rate may be low.

The carbon rod shall be fabricated as follows:

a) A square carbon area about 3 µm × 3 µm is deposited onto the sample by scanning the Ga ion beam on the substrate in the presence of a suitable carbon precursor, such as phenanthrene or naphthalene. The acceleration energy of the Ga ions is between 10 kV and 40 kV. A beam with sub 10 nm diameter should be used.

b) After a suitable thickness of carbon is deposited the flow of carbon precursor is terminated and beam is blanked. The carbon film thickness shall be same or larger than the desired length of the carbon rod.

c) The deposited carbon 3 µm × 3 µm area is then fabricated by milling probe into a rod of desired diameter. See 4.2 discussing the choice of rod diameter.

NOTE 3 An example set of parameters: deposited area 3 µm × 3 µm, Ga ion probe current 0,07 nA and 40 kV ion beam acceleration used in a dual beam instrument.

### A.2.3 Suspension deposited onto prefabricated rod

The procedure is as follows:

a) The nano-object suspension concentration can be adjusted by evaporating the liquid component of the suspension or diluting the liquid component to obtain a sufficient number of nano-objects deposited onto a rod. The number of nanoparticles to be deposited depends on the rod diameter, the size of nanoparticles and the desired statistic of the measured parameters.

NOTE 1 For example, the native volume of NIST reference material 8012[12] containing 30 nm diameter Au nanoparticles was decreased by evaporating 90 % of the solvent by heating vial with the suspension to 50 °C on a hot plate in laboratory air. The suspension with increased concentration was then used in subsequent steps of sample preparation described below. The concentration should be adjusted based on an evaluation of number of nanoparticles present on a rod using SEM imaging[12].

b) A 5 uL drop of the concentrated suspension shall be deposited on the carboneous rod using a micropipette. Excess suspension shall be removed by using the same micropipette after 1 s to 3 s.

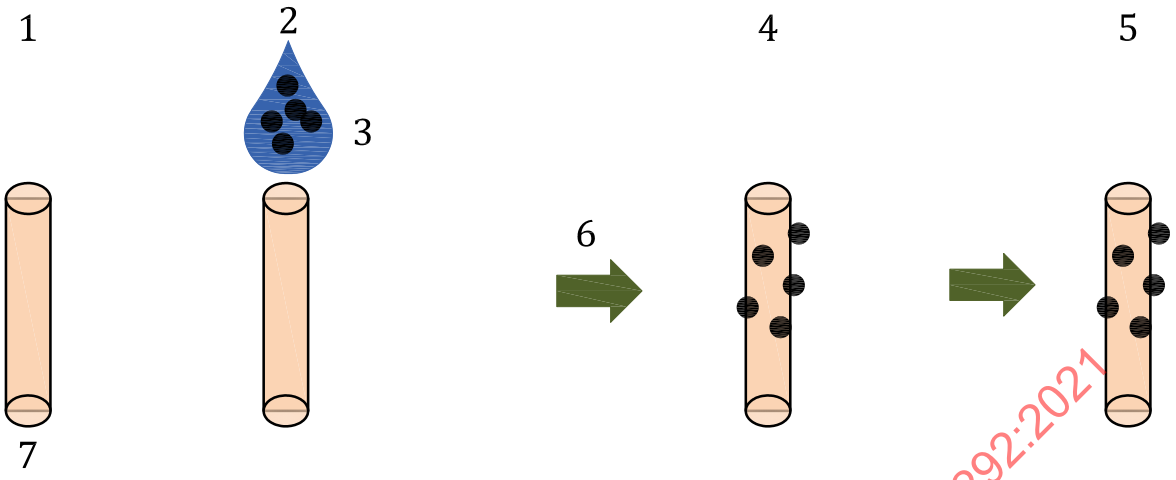
c) The sample should be allowed to dry overnight in laboratory air. The drying time has to be adjusted depending on parameters such as the nature of the solvent, the laboratory humidity and temperature. Alternatively, a desiccator or a vacuum oven can be also utilized to decrease the drying time if the nanoparticles are not damaged by an elevated temperature or fast drying.

NOTE 2 The objective of this step is to ensure that the solvent is removed, and nanoparticles are well attached to the carbon rod. Inspection in an SEM or in an optical microscope may be used to assess the presence of a solvent.

d) After drying, the sample rod with deposited nano-objects can be examined in an SEM. The deposition of suspension followed by SEM or TEM inspection can be repeated on the same rod until enough nano-objects are present on the carboneous rod. The number of nanoparticles attached to the rod depends on many parameters, such as their surface treatment and the nature of the solvent. Therefore, the number of deposition steps and the concentration of nanoparticles in the solvent must be determined by observation of the sample in a TEM or SEM.

e) The deposition and drying cycle can be repeated 3 times to 10 times. The number of cycles shall be decided iteratively after examining the number of nanoparticles already present on the rod. To estimate the number of nanoparticles already present on the rod the sample shall be examined by 2D TEM or SEM imaging.

The sample preparation is shown in Figure A.1.




**Key**

1 carbon rod prepared in FIB
2 nanoparticles deposited
3 liquid suspension of liquid nanoparticles
4 samples prepared for TEM
5 TEM observation
6 dry
7 carbon rod

NOTE 1 The above described method was published in Reference [17];

NOTE 2 Alternative sample preparation methods are described in References [1] and [9].

**Figure A.1 — Sample preparation**

### A.2.4 Suspension deposited onto silicon wafer

This section provides information on how to prepare samples of nano-objects (e.g. nanoparticles, nanocubes, nanorods and nanocrystals) suspended in a liquid (e.g. water or alcohol) by deposition onto a silicon wafer. A rod-shaped sample is then fabricated by a FIB.

The rod can be fabricated as follows.

a) A suspension of the nano-object sample in a suitable liquid can be deposited using a pipette onto a silicon wafer. Care must be taken to ensure the concentration of nano-objects in the suspension is such that the deposit on the wafer has sufficient number of nano-objects within ~100 nm diameter. At the same time, the concentration of the nano-objects in the suspensions should be such that the nano-objects are well separated. The number of nano-objects can be estimated using scanning electron microscope images. The number of nano-objects per area, can be inspected by SEM to estimate the number and separation of the nano-objects on the substrate and the suitability of the deposited sample for sample rod fabrication. If the number of objects per area is too low, a subsequent deposition can be applied or a new suspension with higher concentration of nanoparticles can be prepared and deposited. If the number of objects per unit area is too high, the concentration of nanoparticles in the suspension can be decreased and suspension should be applied to a new silicon wafer.

b) The wafer with the sample drop is allowed to dry overnight. The drying time should be adjusted depending on parameters such as the nature of the solvent, the laboratory humidity and temperature. Alternatively, a desiccator or a vacuum oven can be also utilized to decrease the drying time if the nanoparticles are not damaged by an elevated temperature or fast drying. The sample can be prepared in multiple steps, for example by depositing a drop of the nano-objects suspension, then depositing a separation layer to ensure nano-objects are well separated, and then

depositing another drop of nano-object suspension. The liquid component of the suspension can be such that it embeds the nano-objects upon drying. For example, nanoparticles in epoxy resin can be prepared in this manner.

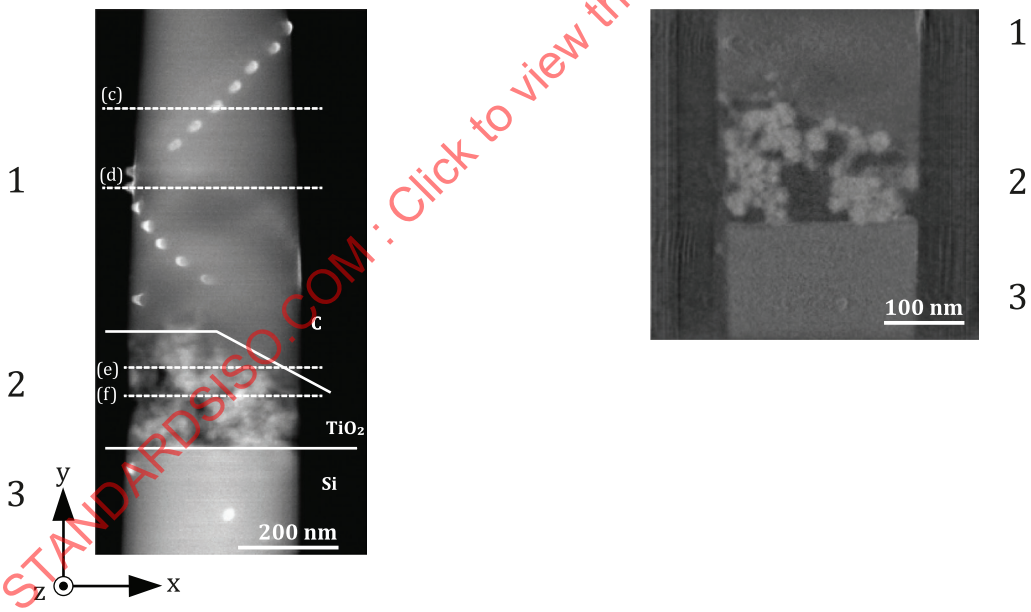
c) After a sufficient amount of nano-object suspension is deposited onto a silicon wafer and has dried, a protective layer should be deposited to cover the nano-objects on the silicon wafer. For example, an electron beam or a FIB-beam induced deposition of carbon can be utilized to create such protective layer. Alternatively, a carbon layer deposited by sputtering in vacuum or electron beam or thermal evaporation can be used as a protective layer.

When the FIB-induced carbon deposition is used the Ga ion beam current should be as low as possible. The electron beam induced carbon deposition can be utilized for an initial carbon layer deposition.

d) Following the protective layer deposition, a focused ion beam can be used to fabricate a rod-shaped sample containing the deposited nano-objects. The diameter of the rod-shaped sample shall be no more than 1 inelastic mean free path for the electron energy used for imaging in the TEM. For example, for low atomic number objects the maximum rod diameter is about 200 nm at 200 kV incident electron energy in the TEM. The rod diameter can be increased with increasing incident energy and can be decreased with decreasing incident electron energy in the TEM for nano-objects and embedding materials that contain high atomic number elements.

e) The fabricated sample rod can be transferred to the TEM sample holder using standard pluck out FIB sample preparation methods.

A set of fiducial markers can be fabricated on the protective layer section of the sample rod. See Figure A.2 that shows nanoparticles deposited on a silicon wafer[13].




**a) Projected TEM image of TiOx nanoparticles within a sample rod for electron tomography**

**b) Slice image extracted from the reconstructed volume of the same sample**

**Key**

1 carbon protective layer with fiducial markers

2 TiOx nanoparticles

3 silicon substrate

NOTE 1 The sample was prepared by depositing suspension onto a Si wafer followed by carbon deposition in Figure A.1, a).

NOTE 2 The nanoparticles overlap in a) but can be identified in b).

**Figure A.2 — FIB sample preparation from nanoparticles dropped in Si, including fabrication of fiducial markers**

## A.3 Nanocomposites and nanoparticles embedded in matrix

### A.3.1 General

This subclause is applicable to preparation of samples of nano-objects embedded in a matrix. The matrix and the embedded nano-objects must be sufficiently stable under electron and focused ion beam irradiation.

The methods described are:

a) multilayer samples of nano-objects deposited onto a carbon coated TEM sample support grid, as an alternative to samples described in A.2 (A.3.1);

b) an example for catalyst nanoparticles embedded in a support material such as carbon black (A.3.2);

c) nanoparticles embedded in a polymer matrix (A.3.2).

### A.3.2 Multilayer sample deposited onto carbon coated TEM sample support grid

The method is described as:

a) A suspension of nano-objects, such as nanoparticles, can be deposited onto a carbon-coated TEM grid. The suspension should be dropped onto the carbon grid using a micropipette.

b) A thin carbon film can be vacuum evaporated or sputtered in a vacuum deposition system onto the TEM sample grid with carbon film and with the deposited nano-objects.

c) The areal concentration of nano-objects that are present on the carbon film can be inspected using an SEM. SEM images can be acquired in either secondary electron or transmitted electron modes.

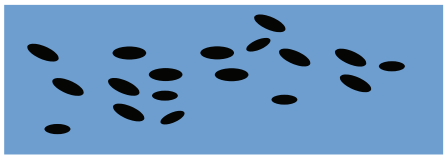
d) Steps a) to c) can be repeated until desired areal density of nano-objects is achieved. The overall thickness of the multilayer nano-object / carbon film sample should not exceed 300 nm. For example, 10 layers of 30 nm diameter gold nanoparticles separated by 5 nm thick carbon film deposited onto a TEM sample support grid with a 10 nm carbon film may result in a sample that contains 20 nanoparticles to 50 nanoparticles within an area 1,000 nm long, 300 nm wide and 300 nm thick. Note that the number of nano-objects per unit area may not be uniform across the sample. Only limited number of areas with desired areal density of nano-objects may exist.

e) Area with desired areal density of nano-objects is identified using electron beam in a dual beam (FIB/SEM) instrument or using ion beam in a FIB instrument.

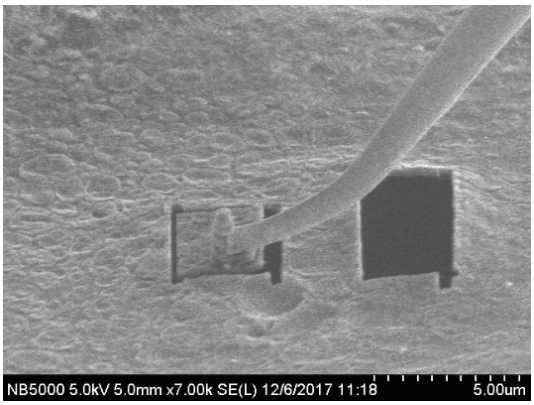
f) The area that is deemed to contain desired areal number of nano-objects is cut by FIB on three sides leaving only one side connected to the rest of the sample. The sample is oriented such that the remaining (fourth) side can be cut off without interference with the FIB microsampling needle, see Figure A.3.

g) The FIB microsampling probe should be placed onto the area of interest and the microsampling probe should be bonded to the carbon composite film with nano-objects. The remaining side of the sampled area should be cut off and the sample can be transferred onto a TEM support stub.

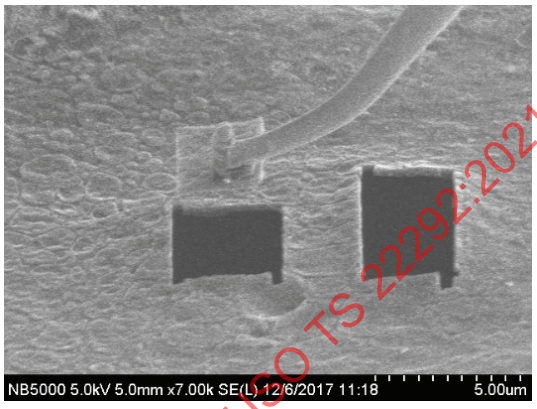
h) The sample can be cut and polished by FIB to dimensions that should not exceed rod diameter described in 4.2 in the direction perpendicular to the tilt axis.



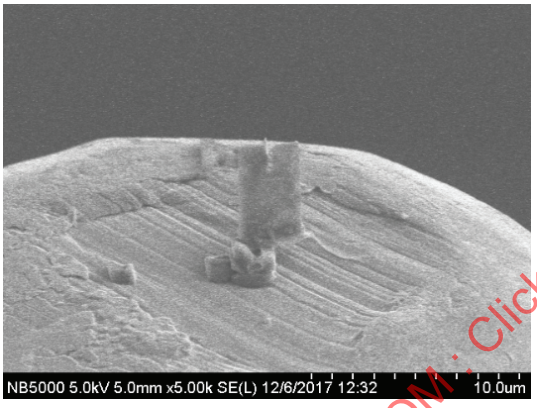
**a) Carbon multilayer embedded Au NPs on a TEM grid**



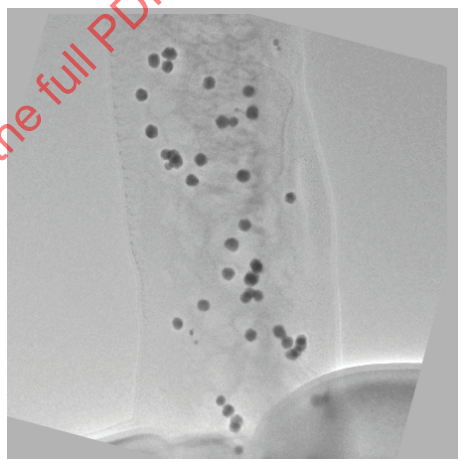

**b) FIB cut, probe attached**




**c) FIB pluck out**




**d) Placement onto sample needle**



**e) Example TEM image**

NOTE 1 Figure A.3, a) shows a TEM grid square with carbon film-supported multilayer of 30 nm Au nanoparticles embedded in sputtered carbon layers. Ten layers Au and carbon each were deposited in the example shown. The micromaniplator needle is attached to the desired area of the carbon film. The sample is cut by FIB Ga beam so that the sample can be extracted.

NOTE 2 In Figures A.3, b), c) and d), the sample area attached to micromanipulator in a) is placed onto a TEM support pin for transfer into a TEM.

NOTE 3 Figure A.3, e) shows an example image extracted from TEM tilt series showing Au nanoparticles (dark) embedded in carbon film (brighter) sample rod with rectangular cross section. The sample is surrounded by vacuum (bright areas around the sample)[21].

**Figure A.3 — Microsampling images from a sample of nanoparticle-carbon multilayer deposited on a TEM grid**

### A.3.3 Catalyst nanoparticles embedded in a support material and nano-objects embedded in a polymer matrix

Sample can be prepared by established microsampling method. A bulk sample can be placed in a focused ion beam and a desired area of the sample can be cut out and placed onto microsampling probe. The sample can be transferred from the microsampling probe onto a TEM holder by established method of choice. The sample can be polished by ion beam in FIB so that it does not exceed the rod diameter in the direction perpendicular to the tilt axis as described below.

NOTE 1 See Reference [10] for example.

NOTE 2 Ion beam in FIB can result in organic material shrinkage. Extremely low beam current can reduce the beam induced shrinkage.

# Annex B
(informative)

# STEM set up

## B.1 General

This subclause provides guidance on STEM instrumentation set up for data acquisition. While the parameters that are adjusted are often the same as for TEM, preferred parameter values might differ between TEM and STEM modes.

The critical set up parameters for STEM data acquisition are:

a) acceleration voltage (B.2);

b) convergence semi-angle of the incident probe (B.3);

c) collection semi-angle of the detectors (B.4);

d) pixel dwell time (B.5);

e) number of pixels and pixel size (B.6).

## B.2 Acceleration voltage

The acceleration voltage should be selected as described in 4.2 on sample thickness. Typically, 300 kV or 200 kV should be used.

## B.3 Convergence semi-angle of the incident probe

The convergence semi-angle alpha of the probe should be chosen so that the geometrical probe broadening within the sample thickness is less than the desired resolution.[15] For example, to collect data from a diameter rod $D$ = 100 nm at a desired resolution $p$ = 1 nm the probe convergence semi-angle must not exceed alpha = arcsin ($D$ / $p$) = arcsin (1 nm / 100 nm) = 10 milli radians. To reduce the effect of limited accuracy of focusing of the sample, it is typically desirable to reduce the probe convergence angle further.[15] An aperture placed above the probe forming objective lens can be used to limit the convergence semi-angle. Alternatively, the optics of the condenser lens system can be adjusted to obtain small convergence semi-angle at the sample plane.

The probe should be positioned on an amorphous region of the sample at the same sample height along the beam path as the sample (such as at the tip of the sample rod). The probe stigmatism and focus is then adjusted for example using the Ronchigram method.[6] An additional consideration for probe convergence angle is that the convergence angle can be selected such that the effect of the probe forming objective lens aberrations is minimized. The selected angle limiting aperture size should be such that only flat region of the Ronchigram is included [6].

NOTE 1 For most modern instruments for the convergence angles of interest for electron tomography the lens aberrations are excluded by the requirement for limited geometrical probe broadening.

NOTE 2 Alignment methods other than Ronchigram are acceptable providing that adequate image quality is obtained.

## B.4 Collection semi-angle of the detectors

The collection semi-angle theta should be selected such that the effect of diffraction contrast is minimized while collection efficiency is maximized. In practise, the collection angle theta is adjusted by changing the excitation of the lens directly above the signal collecting detector.

## B.5 Pixel dwell time and pixel size

The data should be collected with pixel dwell time selected such that the effect of sample drift over the entire image acquisition is less than the desired resolution. If the signal-to-noise ratio is lower than desired for data reconstruction, multiple images may be collected at each tilt.[12] Collecting multiple images at each tilt may be beneficial in 3D imaging of objects where the measurand estimate depends on signal to noise ratio of the collected data. An extrapolation to noise free data set can be then obtained. [12] An alternative to adjusting the pixel dwell time is the adjustment of the microscope beam current.

## B.6 Number of pixels

The requirements on pixel size and image size in number of pixels are the same as for conventional TEM. See 5.1.6.

# Annex C
(normative)

# Tomography reconstruction and visualization software packages

## C.1 Validation of reconstruction software packages and algorithms

### C.1.1 General

There are many software packages for reconstruction of the 3D volume from the projected 2D images. Some of the packages include provision for 2D image alignment, while others assume that already aligned 2D projected images are provided as an input for the reconstruction process. Various reconstruction methods, filters and other parameters may be implemented.

This annex provides information on how to evaluate differences arising from the use of a particular software package as compared to FBP with linear ramp filter as implemented in the normative aspect of the document.

NOTE In-house software by Dr. Hayashida, TEMography.com and IMOD have been applied in this document. For other methods, see C.2.

### C.1.2 Validation of a reconstruction software package

The validation of a software package should be done at least once before it is put to use for 3D reconstruction of nano-objects. The following steps should be taken.

a) A 3D volume with an ideal sphere should be generated in a computer. Sphere diameters listed in Table 1 should be used.

b) A set of 60 projected 2D images with 3-degree tilt step over the 0 to 180 tilt range should be generated. Noise should not be added to the images.

c) Alignment of the 2D projected images should be verified, but lateral displacement of computer generated 2D projected images is expected to be negligible.

d) The tested software package should be used to reconstruct the 3D volume.

e) The reconstructed volume should be compared to the original computer-generated volume.

f) The errors should be compared to those in Table 1.

g) The errors should be noted in the measurement protocol and suitability of the software package determined.

NOTE 1 As listed in C.2, the TomoMi software does not provide alignment or reconstruction capabilities. It is solely intended to extract measurands from the reconstructed 3D volume.

NOTE 2 It is often convenient to save 3D data cube after reconstruction as 16-bit signed little-endian encoding. TomoMi, as listed in C.2, is tested to read signed 16-bit little endian data.

To generate 3D reconstructed volume suitable for measurand extraction by TomoMi, software packages for tomography reconstruction and visualization include, but are not limited to, those listed in C.2.

## C.2 List of reconstruction software packages

### C.2.1 Data acquisition packages

Data acquisition packages include, but are not limited to the following:

a) TEMography.com: http://temography.com/;

b) Hitachi EMIP;

c) SerialEM: http://bio3d.colorado.edu/SerialEM/;

d) Amira-Avizo: https://www.fei.com/software/avizo/?LangType=2052.

NOTE Package a) has been extensively used to reconstruct data for this document.

### C.2.2 Alignment, reconstruction and interpretation packages

Alignment, reconstruction and interpretation packages include, but are not limited to the following:

a) IMOD: https://bio3d.colorado.edu/imod/;

b) TomoJ: https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-8-288;

c) ImageJ: https://imagej.nih.gov/ij/ ... general image processing tool;

d) TEMography.com: http://temography.com/;

e) Inspect3D: https://www.thermofisher.com/us/en/home/electron-microscopy/products/software-em-3d-vis/inspect-3d-software.html

f) Matlab TIGRE: https://www.mathworks.com/matlabcentral/fileexchange/58042-tigre-tomographic-iterative-gpu-based-reconstruction-toolbox;

g) Matlab tomobox: https://www.mathworks.com/matlabcentral/fileexchange/28496-tomobox;

h) Hitachi EMIP;

i) TOMViz: https://tomviz.org/.

NOTE Field experience with packages a) and d) have been positive.

# Annex D
(informative)

# Microscope data collection parameters

The microscope and data collection parameters in Table D.1 should be recorded in same or similar format and should be included in the test report.

**Table D.1 — Microscope and data collection parameters to be recorded**

| | **Date data collected** | YYYY-MM-DD |
|---|---|---|
| | **Date data processed** | YYYY-MM-DD |
| | **Sample description** | |
| **Item #** | **Description** | **Value** |
| 1 | Microscope manufacturer and model | |
| 2 | Acceleration voltage used [kV] | |
| 3 | Objective polepiece type (e.g. high res, cryo) and point resolution of the microscope (e.g. 0,2 nm) | |
| 4 | Collection angle or objective aperture used (e.g. 100 mrad or objective aperture #1) | |
| 5 | Illumination probe used (e.g. spot size and alpha – nominal information is sufficient) | |
| 6 | Camera pixel size [nm/pix] | |
| 7 | Camera size in pixels (such as 2 048 pixel × 2 048 pixel) | |
| 8 | Camera sensitivity in counts per incident electron [counts/$e^-$] | |
| 9 | Camera manufacturer and model (e.g. Gatan, Ultrascan 1000, model 892) | |
| 10 | Microscope nominal magnification in [kx] (e.g. 30 kx) | |
| 11 | Exposure time for individual images in seconds (e.g. 1s per image) | |
| 12 | Tilt angle increments in [degrees] (e.g. 3°) | |
| 13 | Total number of images (e.g. 62, 60 images at 3° are sufficient, additional images allow to inspect damage by comparison of 1st and last images) | |
| 14 | Tilt range in degrees (e.g. −90° to +90°) | |
| 15 | Microscope calibration method | |
| 16 | Image alignment and software (e.g. manual alignment, IMOD 4.9) | |
| 17 | Volume reconstruction method and software<br>(e.g. filtered back projection, linear ramp filter, IMOD 4.9) | |
| 18 | Max. and min. Feret diameter evaluation method<br>(e.g. manual in visualized volume using IMOD 4.9 for volume visualization) | |
| 19 | Nanoparticle volume evaluation method<br>(e.g. thresholding and volume pixel counting in IMOD 4.9 or slice area sum multiplied by slice thickness) | |
| 20 | Number of particles evaluated (e.g. 25) | |
| 21 | Differences between the protocol and the actual experiment | |

# Annex E
(informative)

# Case study: Metal nanoparticle, ILC results

## E.1 Sample preparation and use

The purpose of ILC is to evaluate repeatability and reproducibility of the normative steps described herein, see Figure 1. Six laboratories were involved in the ILC. Laboratory A prepared the samples, analysed each sample before shipping to participating laboratories B to F. Six instrument models from three instrument manufacturers were used. Three instruments were operated at 300 kV and three instruments were operated at 200 kV. The raw data and reconstructed volume were then sent to lab A to compile and perform statistical analysis. See ISO 21363:2020, 9.2, and Reference [19] for information on the statistical analysis of the data.

The investigated samples were gold nanoparticles with 30 nm nominal diameter suspended in a liquid (Sigma Aldrich 741,973). A carbon rod 200 nm to 350 nm cross section was prepared using the multilayer composite method, A.3.1. The cross section of the rod had a ratio of long to short axis less than 1,5.

A total of 13 different samples or sample regions were prepared, each nanoparticle examined at least twice, resulting in 29 data sets containing 410 measurements. Each data set has between 10 and 30 nanoparticles. The samples were examined at 200 kV in three laboratories and at 300 kV in two laboratories. Three instrument manufacturers tools were used, each manufacturer instrument used at two different laboratories A to F. A total of six different instrument models were used. All data were collected in TEM mode. Results from one laboratory had 15° missing wedge data collected at 200 kV.

## E.2 ILC example results

Figure E.1 shows result of one-way analysis of variance for minimum Feret diameter $F_{min}$, maximum Feret diameter $F_{max}$, Feret ratio $F_{rat}$ and volume $V$. The x abscissa identifies the data set (1 to 35) while the y abscissa describes the properties of the data set. The bottom and top of the blue box for each of the 35 data sets represents the 25th and 75th percentile, the red line in the centre of the box indicates the median value, the dashed whiskers indicated the extreme values and the plus sign indicates outliers in each data set.