A common European approach to the regulatory testing of nanomaterials

Standard Operating Procedure

Title	***Electron microscopic image analysis of primary particles in aggregated nanomaterials***
Subtitle	
NANoREG Work package/task:	WP2 Synthesis, supplying and characterization
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Title: Electron microscopic image analysis of primary particles in aggregated nanomaterials

Goal:

This procedure aims to analyse the 2D properties of the primary particles on EM micrographs. To be suitable for quantitative characterisation, the images should have a homogeneous background and the particles should be clearly distinguishable from the background.

Field of application:

This method allows characterising NM on EM-micrographs using image analysis software.

- The NM can be metallic consisting for example of Ag or Au, an oxide including SiO_2 ,TiO_2 ,Fe_2O_3, Fe_3O_4 ,and other.
- Imaging can be performed as described in SOP/NANoREG/D2.10/TEMIma, or by other imaging methods
- The NM can be monodisperse or polydisperse.
- Freeware and/or commercial image analysis softwares (iTEM, ImageJ, Fiji, Visilog, Digital micrograph,…) can be used.

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1 Goal

This procedure aims to analyse the 2D properties of the primary particles on EM micrographs. To be suitable for quantitative characterisation, the images should have a homogeneous background and the particles should be clearly distinguishable from the background.

2 Domain of application

This method allows characterising NM on EM-micrographs using image analysis software.

- The NM can be metallic consisting for example of Ag or Au, an oxide including SiO_2 ,TiO_2 ,Fe_2O_3, Fe_3O_4 ,and other.
- The NM can be monodisperse or polydisperse.
- Freeware and/or commercial image analysis softwares can be applied.

3 Definition, abbreviations, references and norms

- **Pixel size**: the size of one pixel in an EM image (in nm).
- Size: Any dimensional size measure (ECD, Feret, Diameter, …)
- Minimal size in one dimension: The minimal size in one dimension of a particle, generally the Feret min, width (filamentous particles) or Maximal inscribed circular diameter is used.
- Particle: minute piece of matter with defined physical boundaries [ISO 14644-6:2007, definition 2.102]
- Aggregate: particle comprising strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components [ISO/TS 27687:2008]
- Agglomerate: collection of weakly bound particles or aggregates or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components [ISO/TS 27687:2008]
- TEM: Transmission electron microscopy
- NM: Nanomaterial

4 Principle of the method

The image analysis program detects particles on an EM micrograph based on their grey value. All detected particles can be semi-automatically measured. Multiple measurands are measured simultaneously on individual particles.
A typical particle analysis consists of following steps:

- Image preparation
- Setting and adjusting the threshold value
- Defining the detection area
- Setting the detection parameters
- Detection of the primary particles in aggregated NM
- Selection of the primary particle parameters
- Defining the classification schemes
- Classification of the particles according the selected parameters
- Exporting of results in excel spreadsheets and storage of the (annotated) images in the NM database

5 Equipment

- Image analysis software: following freeware and/or commercial image analysis softwares iTEM, ImageJ, Fiji, Visilog, Digital micrograph can be applied.
- Database software to store the images together with the image analysis characteristics.

6 Instruction

6.1 Image Storage

- The original image is stored in a database with sample preparation and imaging information.

6.2 Image preparation

- (Optional) Preparing the image for grey value thresholding (Comment 1).
  - Verify whether the image background (e.g. due to heterogeneous image illumination during image acquisition) is homogenous. The mean intensity profiles in the X- and Y-axes should have no specific tendency.
  - Modify the gray values to optimize the contrast between particles and background.
  - Apply a N*N filter to reduce border effects when thresholding.
- Manually set the grey scale threshold on the image separating the particles from the background (Comment 2).
- Binarize the image
- Add the binarized image to the NM database, attached it to the original micrograph for later reference.

6.3 Particles cut by the edge of the measurement frame

If all the objects that appear in the image frame are accepted for measurement, the accuracy of the final distribution will be impaired because some of the objects will be cut by the edge of the image frame. To overcome this, a measurement frame is defined within the image frame. The measurement frame can be used in the following two ways.

- Particles are excluded based on a measurement frame:
  - The frame is set at 10% from top and left size of the micrograph
  - Particles in this 10% band that touch the top and left border of the frame are included in the analysis.
  - Particles touching the right and bottom border of the frame are excluded from analysis
- Particles are excluded based on their X-Y coordinates.
  - The X-Y coordinates of each particle in the image are recorded.
  - Particles with an X-Y coordinate that is closer than 5% of the micrograph size to any of the borders of the micrograph are excluded from the dataset.

6.4 Select the particle measurements

- (Optional) All possible parameters that the image analysis software allows to measure are selected.
- Four types of characteristic measurands are essential to be selected (size, shape and surface topology measurements).

- Two size measurands are essential: ECD, to compare results with certified reference materials and the minimal size in one dimension (e.g. Feret min and MICD)
- The Gray value Max measurement (measured on the Euclidean distance map, used for an estimator of the minimal external dimension of the primary particles)
- Shape measurements (e.g. aspect ratio, roundness) describing the shape of the particle
- Surface topology (e.g. solidity, shape factor, convexity)

- The X-Y coordinates of the centre of the particle.

6.5 Image processing

- A threshold is set selecting the particles of interest.
- Separate and detect the primary particles in the aggregates.
  - Binarise the image
  - Duplicate the binarized image
  - Apply separator filter on the First Image (Watershed)
  - Apply Euclidean distance map on the Second image
  - Merge the two images using an Image calculator with operation Min (Background = Black) or Max (Background = white)
  - Detect the primary particles
- The minimal size of the particles is set to at least at 100 pixels
- Particles cut by the edge of the measurement frame are excluded from analysis as described in 6.3
- Detected particles are indicated in the image with a number that relates to the number in the spreadsheet
- Holes in the particles are excluded from analysis (Comment 3)
- Particle measurements are recorded in a spread sheet

6.6 Data storage

Essential data is stored and includes:

- The original micrograph with sample preparation and imaging information
- Annotated micrographs, including image analysis information
  - Image preparation, filters used and settings
  - Method to handle particles that are cut by the measurement frame
  - Summary of the measurands measured
  - Image processing steps followed
- Spreadsheeds with the characteristics of the particles in the sample includes
  - Particle measurements

- Image name
- Particle number
- X-Y coordinates of the particles

7 Specific safety measures

8 Comments

Comment 1

- Heterogeneous image illumination resulting in a gradient of the grey values in background of the TEM micrographs should be avoided by saturation and perfect alignment of the TEM filament and by sufficient broadening of the beam.
- Particles with a high contrast between particle and background generally do not need adjustment of the grey values.
- When a rough boundary is observed while thresholding in low contrast materials, an N*N filter might be appropriate.

Comment 2

This generally does not work for grids that do not have a uniform background (like holey grids). For these special cases "Magic wand" or local thresholding can be applied.

Comment 3

For non-overlapping particles the fill holes option can be used. However for nanostructured materials and overlapping particles real holes can occur and the fill holes option should not be selected.