Nanomaterial FAte and Speciation in the Environment (NanoFASE)

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NanoFASE Scientific Protocol

Quantification of Nanoparticle Localisation in Tissue by TEM

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1. **Name(s) of scientific protocol:**

   Quantification of Nanoparticle Localisation in Tissue by TEM

2. **Scope and Domain:**

   - This document contains a protocol for the assessment of nanoparticle localisation in cells or tissues (organisms).
   - The method involves embedding of the tissue in epoxy resin and cutting into thin slices for imaging.
   - TEM is the gold standard for ultraresolution of cellular structures, and provides a semi-quantitative method for particle counting.

3. **Principle of the scientific protocol:**

   TEM provides ultrastructural resolution of cellular and tissue structure

4. **Description of scientific protocol:**

   Cells or tissue are fixed with 2.5 % gluteraldehyde / 2 % paraformaldehyde (EMS, Hatfield, Pennsylvania) in 0.1 M Cacodylate Buffer (CB) (1h – overnight) then washed with 3 x 0.1 M CB buffer. Cells were then stained with 2 % osmium tetroxide (OsO_4) (EMS, Hatfield, Pennsylvania) (1 hour) followed with 3 x dH_2O washes. Cells were then fixed with uranyl acetate (1 hour) followed by washing with 3 x dH_2O. Cells were then dehydrated with a series of ethanol washes x 2 (50 %, 60%, 70 %, 80 %, 90% and 100 %) before infiltration with 50:50 absolute alcohol:EmBed 812 (1 hour). EmBed 812 was made up as per the standard protocol for hard resin (EMS, Hatfield, Pennsylvania). Two subsequent infiltrations were performed (45 min) with

EmBed 812 alone, before inverting and mounting on resin-filled embedding BEEM® capsules (EMS, Hatfield, Pennsylvania) with care taken to remove all bubbles from within the capsule. Samples were then baked overnight at 60°C in an oven (Thelco Laboratory Apparatus by Precision Scientific Co., India). Coverslips were separated from BEEM capsules by plunging into liquid nitrogen, and samples were then allowed to re-equilibrate with room temperature. The area of interest (0.5 mm^2) was visualised, and trimmed and isolated with a sharp single edge razor blade (EMS, Hatfield, Pennsylvania) under a light microscope (Leica UltraCut UCT, Leica Microsystems Inc., IL, USA). Following trimming, 70 nm or 150 nm serial sections were cut using a Diatome diamond knife (EMS, Hatfield, Pennsylvania). Sections were then collected onto 200 mesh copper (Cu) grids or slotted grids (EMS, Hatfield, Pennsylvania) that had been pre-treated with alcohol. Samples were then stored for staining.

Grids containing sections were stained by inverting on top of small blobs of 2% uranyl acetate (10 minutes) (EMS, Hatfield, Pennsylvania) inside petri dishes; grids were then washed with dH_2O and air dried before repeating this step with Reynolds lead citrate ($CaCO_3$ crystals used to remove air from within the chamber). Following this, grids were washed with dH_2O and left to dry before TEM imaging.

The approach can also be applied to organisms as follows :

TEM cross sections of daphnids follwoing exposure to Nanopartciles were prepared as follows : whole Daphnia were euthanised and fixed immediately

in a 2.5% glutaraldehyde in a 0.1M phosphate buffer suspension. Daphnids were dehdydrated in ethanol and embedded in epoxy resin before sectioning using a ultramicrotome to cut 0.1 µm sections with a diamond knife. Images were visualized using JEOL 1200EX 80kV and JEOL 1400EX 80kV microscopes.

5. Environment:

Specialised TEM facility.

6. Biological and/or environmental models used:

Has been applied to all cell types, but also to whole organisms including daphnia, earthworms, and others.

7. Chemicals and reagents used:

2.5 % gluteraldehyde

2 % paraformaldehyde

0.1 M Cacodylate Buffer (CB)

absolute alcohol

EmBed 812

2% uranyl acetate

Reynolds lead citrate ($CaCO_3$ crystals)

8. Nanoparticles used:

All types of particles are suitable for TEM. Metal, metal oxide, carbon based and even polymeric such as Polystyrene. Applied to cerium dioxide, Iron oxide, silver and gold in cells, and gold, silver, titania and polystyrene in Daphnia within NanoFASE.

9. Apparatus and equipment used:

JEOL 1200EX 80kV

JEOL 1400EX 80kV microscopes

Ultramicrotome and diamond knife

General plastics and glassware

10. Health and Safety Precautions:

Samples: The protocol should follow Control Of Substances Hazardous to Health (COSHH) standards, and general health and safety precautions apply.

Instrument: View instrument manual for health and safety precautions regarding general, electrical warnings and manual handling precautions. Users should be fully trained before utilising TEM instrument.

11. Data analysis and Reporting the Data:

- Analysis of the images is performed to identify the sub-cellular / tissue localisation of the nanoparticles. Features of interest are identified.
- Automated software was developed to analyse the images and identify the nanoparticles and organelles.

- Stereological approaches can be utilised to get some semi-quantitative information on the distribution of the nanoparticles in the tissue.[1]

Abbreviations:

CB - Cacodylate Buffer

COSHH - Control Of Substance Hazardous to Health

TEM - Transmission Electron Microscopy

12. Limitations:

- Measurements by NTA are limited by size (detection of dim scattering particles / particle movement below detectable limit) and concentration boundaries. These limits will be sample dependent.
- This protocol is applicable across a range of powder dispersions but will be limited by particle concentration range provided by dilution steps.

13. Publications:

Guggenheim et al, Confocal and Superresolution Imaging of Nanoparticle Uptake into Cancer Cells. PLoS One. 2016.

Nasser F, Lynch I. Updating traditional regulatory tests for use with novel materials: Nanomaterial toxicity testing with Daphnia magna. Safety Science, 2019, 118, 497-504

14. References:

(1) Elsaesser A, Barnes CA, McKerr G, Salvati A, Lynch I, Dawson KA, Howard CV. Quantification of nanoparticle uptake by cells using an unbiased sampling method and electron microscopy. Nanomedicine, 2011, 6. https://doi.org/10.2217/nnm.11.70