A common European approach to the regulatory testing of nanomaterials

Standard Operating Procedure

Title	***Confocal Raman Microspectroscopy (CRM)***
Subtitle	
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Objective

Confocal Raman Microspectroscopy (CRM) as a label-free and non-invasive imaging technique provides unique information about translocation and fate of nanoparticles at subcellular level. CRM allows the simultaneous visualization of NMs and their biological environment. CRM has a strong potential as a label-free, nondestructive technique for time-course imaging of individual cells and tracking of cell metabolism.

Principle of the method

The combination of confocal microscopy with Raman spectroscopy provides very high content of molecular-spectroscopic information in every pixel of image at a singular cell level. CRM offers a label-free way to complement histopathology, diagnose and characterize culture cells and tissue sections. CRM provides 3D chemical composition images with a resolution of about 250 nm. CRM reveals not only the 3D NM distribution but also their co-localization within cell compartments.

Equipment & chemicals

1. Confocal Raman Microspectrocopy (Alpha300 R, WITec GmbH, Germany)
2. 532, 630, and 785 nm laser source
3. 63x water immersion objective (W Plan-Apochromat 63x/1, Zeiss, Germany)
4. Heating stage (WITec GmbH, Germany)
5. CaF_2 substrate: 25mm x 25mm (CRYTAL GmbH, Germany), quartz slices: 25mm diameter (ZELL QUARZGLAS und Technische Keramik Technologie GmbH, Germany), silica wafers
6. Superfibronectin from human plasma (S5171, Sigma, St. Luis, MO)
7. Dulbecco`s Phosphate Buffered Saline (PBS w/o Ca/Mg) (Biowest, France)
8. Formaldehyde
9. Xylol

Settings for CRM measurements

- Laser: 532nm
- Grating: 600 g mm^{-1}
- Laser power: NM212, NM103, NM104, NM110 & lung tissue: 34mW
  NM300-K: 16mW
- Integration time: NM212, NM103, NM104, NM110 & lung tissue: 0.07s
  NM300-K: 0.14s

Sample preparation

1. Culture cells
   - Cell cultivation on CaF_2, quartz or silica wafer (NP dependent)
   - Coating of substrate with superfibronectin (5µg/ml in PBS): 2h at 37°C
   - Wash 3-times with PBS, cultivate cells in culture medium for 24h (cell density: 75000 cells/ml)
   - NP exposure:
     1. *during the exposure*: live cell imaging of NPs
     2. *after the exposure*: remove cell medium with NPs, wash 3-times with PBS, fix cells 30min at room temperature with 3.7% Formaldehyde, wash 3-times with PBS, store cells at 4°C and measure in PBS

2. Lung slides
   - Paraffin-embedded tissues on quartz slides: removal of paraffin by means of xylol (3-times, 10min)
   - Measure in Millipore-water

Data analysis

Acquired spectra were processed using the Project FOUR PLUS software (WITec GmbH, Germany).