PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Procedure for Examining the PEG Modification of the Surface of Gold Nanoparticles Using Time-of-Flight Secondary Ion Mass Spectroscopy

This is a SOP recommended for external use by PATROLS

Adapted from the NanoImpactNet SOP, Clift *et al* (Deliverable 5.4 under the European Commission's 7th Framework Programme, Grant Agreement 218539).

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Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	10/01/2021	Initial Document	Hyun Kyong Shon
2.0	15/04/2021	Additional details added	Jin Gyeong Son

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1 Introduction:

DOMAIN: Material characterization

Over the past decade, the nanomaterial sector has been developing remarkably. In particular, gold nanoparticles are widely used for the delivery of medicines, food additives, and bio applications, which have a major interest in the amount of contents. To make and utilize effective and safe nanomaterials, we need to know the surface characteristics of nanoparticles. Reliable analysis methods are needed to refine the surface of gold nanoparticles and evaluate their condition. In particular, as the use of nanomaterials increases, concerns are also growing over safety issues of nanomaterials. In this SOP, we present a method by time-of-flight secondary ion mass spectrometry (ToF-SIMS). ToF-SIMS analysis to evaluate the characteristics of nanoparticles in the form of surface modified with polyethylene glycol (PEG) on the most commonly utilized gold nanoparticles.

1.1 Scope and limits of the protocol

SCOPE:

This SOP was established with the intention to be used by participants of the RR study within the project PATROLS. This SOP provides instructions on how to measure the surface modified ligands on gold nanoparticles (AuNPs) using ToF-SIMS.

Specifically, this procedure guide describes the procedure for confirming the PEG modification of the surface of AuNPs. PEG is a biocompatible substance that inhibit the non-specific adsorption. To examine the PEG modification of the surface of AuNPs, free PEG ligands unadsorbed on the AuNPs surface were removed from the solution by centrifuging it for four times. Sampling was carried out by spotting 10 nL of the PEG-modified AuNPs solution on a silicon (Si) wafer. This procedure guide presents the ToF-SIMS measurement and data processing methods for examining the PEG surface modification of AuNPs.

Limitations:

This method can be used to determine whether gold nanoparticles are modified only when PEG is modified, and cannot be used to determine whether or not other nanoparticles or the same nanoparticles are modified when other ligands are bound.

1.2 Validation state of protocol

Level of advancement towards standardization	Level reached (please mark only one with "X")
Stage 1: Internal laboratory method under development	
Stage 2: Validated internal laboratory method	X
Stage 3: Interlaboratory tested method	
Stage 4: Method validated by Round Robin testing	
Standardisation plans	
Is the method considered for standardisation (OECD SPSF or similar)?	N
Has the method been submitted for standardisation (to OECD, CEN, ISO,…) in its own right or as part of another standardisation project?	N
Is the method included in an existing standard (or ongoing standardisation work)	N

2 Terms and Definitions:

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: 2016, definition 2.4]

Engineered nanomaterial
Nanomaterial designed for specific purpose or function

[SOURCE: ISO/TS 80004-1: 2016, definition 2.8]

Manufactured nanomaterial
Nanomaterial intentionally produced to have selected properties or composition.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.9]

Particle
Minute piece of matter with defined physical boundaries.

Note 1 to entry: A physical boundary can also be described as an interface.

Note 2 to entry: A particle can move as a unit.

Note 3 to entry: This general particle definition applies to *nano-objects*.

[SOURCE: ISO 26824:2013, 1.1]

Substance
Single chemical element or compound, or a complex structure of compounds.

[SOURCE: ISO 10993-9:2009, definition 3.6]

R statistics program
is a statistics program used for deriving the Pearson product Moment correlation coefficient of a relationship.

Scatter plot

It is a plot where the X and Y values of two variables meet on the Cartesian coordinate system to indicate their relationship.

Pearson product moment correlation coefficient

It is a plot It is a coefficient that indicates the degree of correlation between two variables X and Y.

3 Abbreviations:

- ToF-SIMS ; time-of-flight secondary ion mass spectrometry
- PEG ; poly ethylene glycol, structural formula: H-(O-CH2-CH2)n-OH
- AuNPs ; gold nanoparticles

4 Principle of the Method:

4.1 Measured item

Pearson correlation coefficient obtained from the image showing specific peaks of PEG, which was used to modify the surface of AuNPs.

4.2 Intensity of peaks

The intensity of the detected peaks was calculated according to Equation (1).

$$Y=Y_s \bullet P\,(A \rightarrow A_i) \bullet T \bullet D \quad (1)$$

Y_s : Sputtering efficiency

$P\ (A \rightarrow A_i\ )$: Secondary ionisation rate

T: Efficiency of the generation of secondary ions on the surface to reach the detector

D: Detector efficiency

Assuming that the same sample was being measured, the experiment was carried out under the same conditions. Thus, $P\ (A \rightarrow A_i\ ),\ T,\ D$ are same. However, the intensity of the peaks varies depending on the amount of substance on the surface. The mass peaks from the spectrum obtained in a two-dimensional plane represent the mass images.

4.3 Image processing method

(1) Smoothing

The image data obtained (256 pixels × 256 pixels) are smoothed using the following method. When using SurfaceLab software from IONTOF, ‘average 1’ is selected from the Image - Filter to automatically process the image data.

3 x 3 pixel array

1	2	3
4	5	6
7	8	9

Average 1 calculates the new intensity of pixel 5= I(5) :

$$I(5) = \frac{1}{9} * \sum_{i=1}^{9} I(i)$$

Figure 1. Image smoothing method.

(2) Binning

In the smoothed image (256 pixels × 256 pixels), four pixels are combined to form a single pixel. When SurfaceLab software from IONTOF is used, ‘4’ is selected from Image - Binning to automatically process the image data.

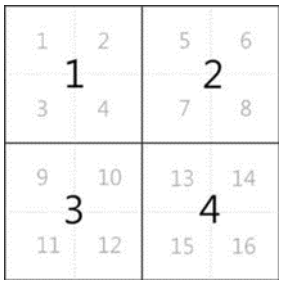


Figure 2. Image binning method.

4.4 Pearson correlation coefficient

(1) Scatter plot

A scatter plot is used for illustrating the relationship between two variables using an orthogonal coordinate system. The image of the AuNPs (Au^-) is used as one of the variables and the image showing the PEG ligands used for surface modification ($C_3H_3O_2^-$) is used as the other variable. Each image is converted to an ASCII file and the resulting files are combined to create a single ASCII file (Figure 3).

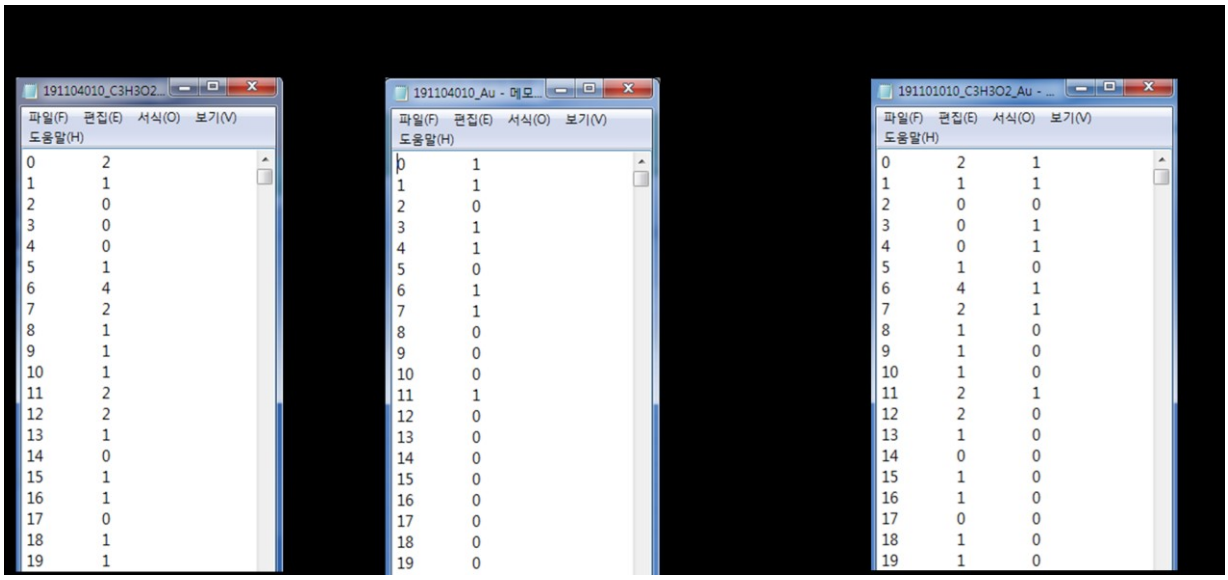


Figure 3. Method of creating the ASCII file by drawing the scatter plot.

While various programs are available for drawing scatter plots, we used the free open source R statistic program. In the R statistics program, the following command draws the scatter graph and the Pearson correlation coefficients can be calculated.

```
Data1 <- read.table("file name.txt")
Data2 <- subset(Data1, V2>0)
Data3 <- subset(Data2, V3>0)
smoothScatter(Data3[,2:3])
```

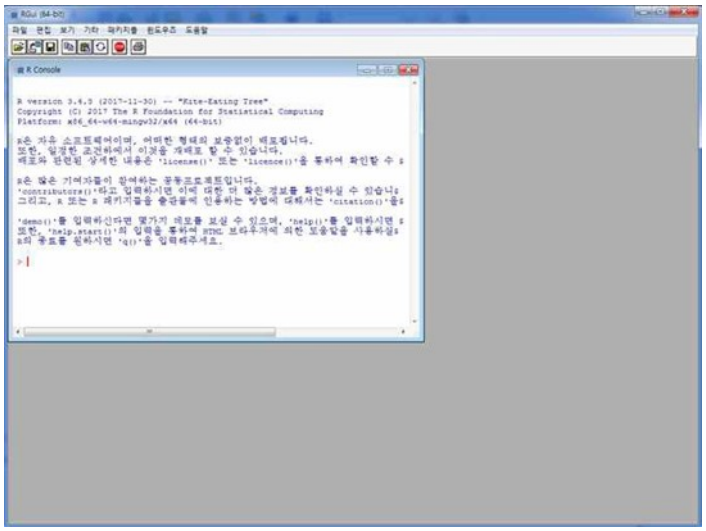


Figure 4. Open source R statistics program.

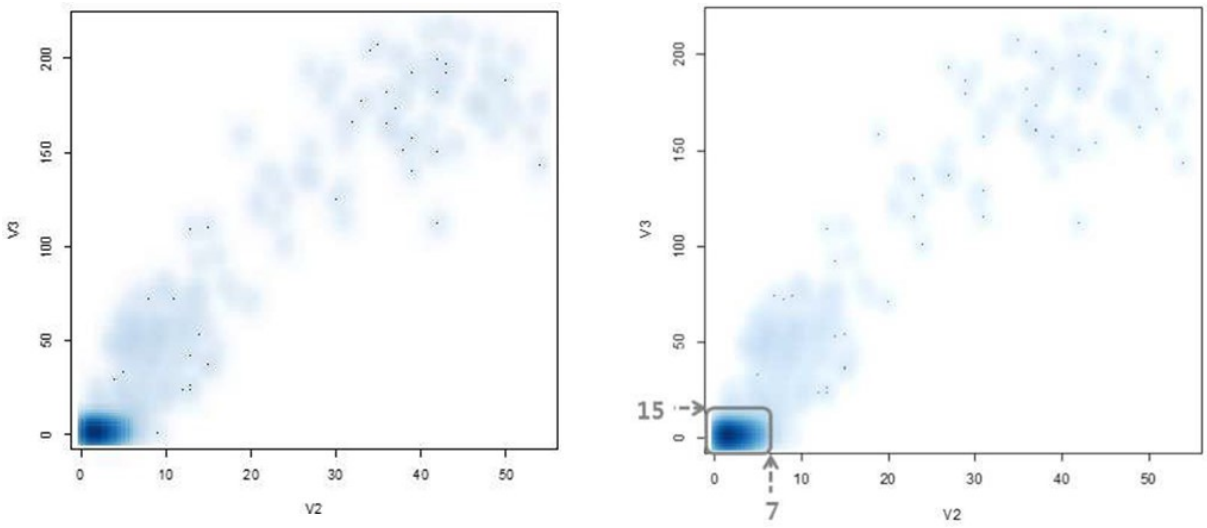
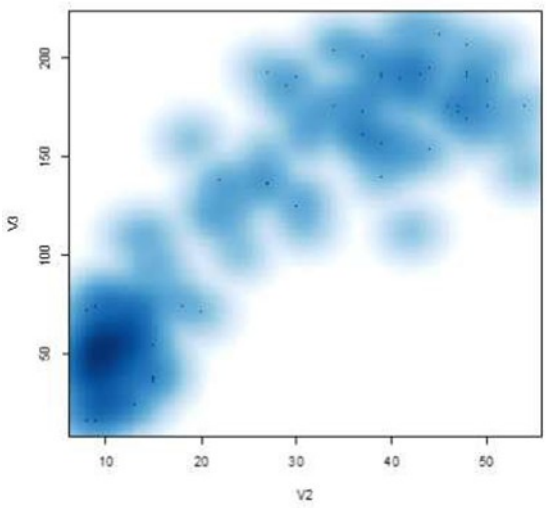


Figure 5. Scatter plot obtained without removing the background signal.

The bottom left corner of the scatter plot with low X and Y axes values represents the background with a group of low-intensity signals (Figure 5). If this area is not removed, erroneous Pearson correlation coefficients can be derived. To remove the background, it is necessary to set objective values for the boundary of the background. V2 in the X axis and V3 in the Y axis represent the background boundary values. By giving the following command, the scatter plot and Pearson correlation coefficient (with the background removed) can be obtained (Figure 6).

```
Data1 <- read.table("file name.txt")
```

```
Data2 <- subset(Data1, V2>7)
Data3 <- subset(Data2, V3>15)
cor(Data3)
smoothScatter(Data3[,2:3])
```



	V1	V2	V3
V1	1.0000	-0.0789	-0.0979
V2		1.0000	**0.9501**
V3			1.0000

Figure 6. Scatter plot and Pearson correlation coefficients of the single ASCII file with the background removed.

5 Description of the Method:

5.1 Test system used

- ToF-SIMS ToF-SIMS

ToF-SIMS (IONOF, Germany) generally uses Au^+, Bi_3^+, and Ar_n^+ as the primary ions, and is used to analyse the elements present on the surface of a sample by detecting the secondary ions generated when a beam of primary ions is irradiated on the surface. In the experiment reported in this procedure guide, a Bi_3^+ primary ion beam was irradiated on a PEG-AuNPs sample to detect the peaks corresponding to Au^- and PEG charateristic peak $C_3H_3O_2^-$ ions (Figure 7).

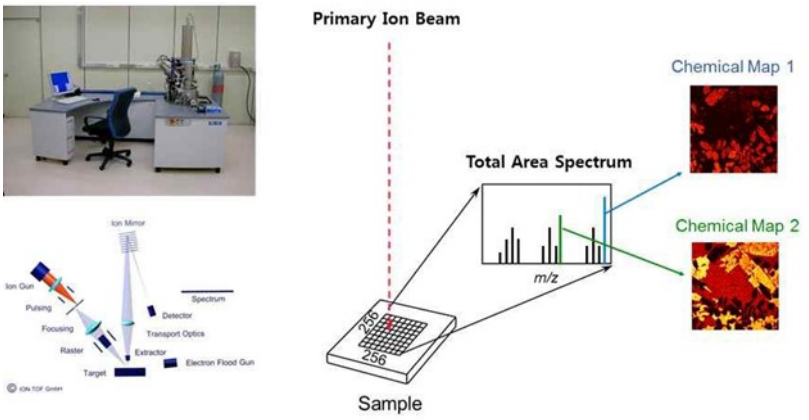


Figure 7. Instrument and schematic of ToF-SIMS V (IONTOF, Germany)

[ToF-SIMS measurement conditions]

▷ Primary

Primary ion: Bi_3^+ primary

Energy: 25 kV

Primary ion current: 1.5 pA @ 150 μs

Primary ion Dose density: 4.0 E+11 ions/cm^2

Analysis area: 500 μm × 500 μm (256 pixels × 256 pixels)

▷ Analyser

Extractor energy: 2 kV

Reflector voltage: 20 eV

▷ Detector

Post-acceleration voltage: 20 kV

MCP: 900 V

Photomultiplier voltage: 1500 V

- Sampler

- Spotter: It is a system for sampling the AuNPs solution on a Si wafer as an array, which can be used to spot the solution at the pL ~ nL level (sciFLEXARRARYER 3 (Scienion, Germany); Figure 8 a). In the experiment reported in this procedure guide, 10 nL of the AuNPs solution was spotted.

- Si wafer: The substrate for spotting the AuNPs solution (Figure 8 b)

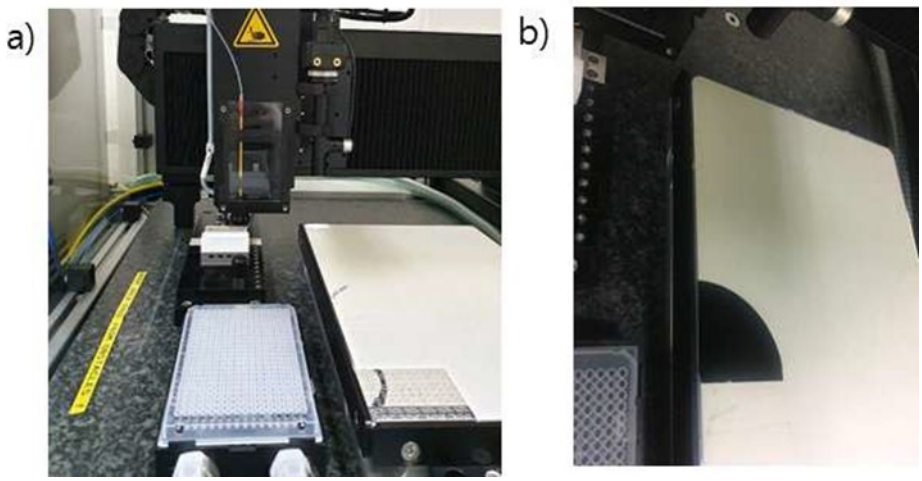


Figure 8 a) sciFLEXARRARYER 3 system, b) Si wafer used as the substrate

5.2 Chemicals and reagents used:

Chemicals, Nanomaterials and reagents are divided in three classes:

1) Recommendation of the supplier, alternative supplier is feasible, information about supplier and batch should be carefully documented in the raw data

2) Ordering from the defined supplier is mandatory, batch is flexible; information about the batch should be carefully documented in the raw data

3) Ordering from the defined supplier and the defines batch is mandatory. If the batch is not available, material can be exchanged between partners, if possible.

- PEG (m.w.2000, Sigma) – 2
- Ultrapure water – 1
- AuNPs (30 nm, BBI solution) – 2

5.3 Apparatus and equipment used:

- Centrifuge (Hanil) – 1
- 1.5 mL and 2 mL Centrifuge Tubes – 1
- Microbalance (Mettler-Toledo) – 1
- P20, P200 and P1000 micropipettes (e.g. Ependorf) – 1
- Sterile 20 µl, 200 µl and 1000 µl Pipette tips – 1

5.4 Health and safety precautions:

Standard health and safety precautions associated with working within a laboratory environment and handling nanomaterials, as described by the Korean Industrial

Safety and Health Act (https://www.kosha.or.kr/kosha/index.do), should be adopted when conducting this SOP. The experiment should take into account the factors that cause health problems by invading the human body due to the form of the substance (liquid, dust) where chemical factors should be considered. All health and safety precautions outlined in the MSDS data sheets associated with the specific chemicals required must also be followed.

5.5 Applicability:

The application of this SOP is a method that can be used only to determine if a PEG ligand is modified in gold nanoparticles. The validity of the identified verification method has not been confirmed in cases where the composition of nanoparticles is different or if the type of ligand is changed.

5.6 Reagent preparation:

- Prepare PEG-modified AuNPs.

1. The optical density of citrated AuNPs is 1 OD. Dissolve PEG ligands in distilled water to achieve a concentration of 50 μM.

2. After adding 100 μL of the 50-μM PEG 2000 ligand to 900 μL of the AuNPs solution with an optical density of 1 OD, stir the mixture for at least 2 h to modify the surface of the AuNPs with PEG.

3. The 1 mL of the PEG-modified AuNPs solution in a tube and centrifuge (8000 rpm) for 15 min. Discard 19/20 of the supernatant. Replace the discarded supernatant with equal volume of distilled water and vortex to re-disperse. Label the sample obtained by repeating this procedure four times as AC4 (‘after centrifuging four times’).

- Sample storage

- Store the PEG-modified AuNPs that has not been centrifuged in a refrigerator (4 ℃).

- The centrifuged PEG-modified AuNPs solution should be discarded immediately after use because PEG dissociates from AuNPs over time.

5.7 Procedure:

5.7.1 Testing for nanomaterial interference

When analyzing ligands of nanoparticles, there are many free ligands without attachment that can act as interfecce. In order to prevent excessive free ligands from acting as a inhibitor in the analysis of adsorption ligands, this SOP established free ligands washing conditions. The free ligand removal process involves using a

centrifuge to induce spin-down of nanoparticles and remove the upper layer of the nanoparticles after adsorption. The process is specified in 5.6 reagent registration.

5.7.2 Test procedure

1. Use a spotter to spot the PEG-modified AuNPs solution (AC4) on a Si wafer for sampling.

1.1 Use filtered and degassed deionised water to wash the piezo dispensing capillary (PDC) nozzle.

1.2 Adjust the voltage and pulse of the PDC nozzle to the recommended level and optimise so that deionised water droplets are formed on the tip of the nozzle.

1.3 Dispense AC4 on the 384-wells set-up inside the spotter device.

1.4 After loading 30 µL of AC4 on the PDC nozzle, spot 10 nL on a Si wafer as array type.

1.5 When forming the array for AC4, spot with a 350-µm space between the droplets to ensure that they do not overlap.

2. Preparation for ToF-SIMS analysis

2.1 Check the vacuum state: Ensure the vacuum state of the main chamber where the equipment can be used (≤ 1.0E-9 mbar).

2.2 Number of primary ions: Check whether the incident amount of Bi_3^+ primary ions is sufficient and measure the secondary negative ions.

2.3 Measurement conditions

① Prior to the experiment, the equipment for 60 min to stabilise the actuated signal of the mass spectrometer.

② Place the Si wafer with AC4 sampled as an array on the sample holder and load the sample holder into the preparation chamber.

③ After loading the sample on the sample holder, set the analysis range, primary ion dose density (PIDD), and the number of secondary ions. Ensure that the peaks for PEG- ($C_3H_3O_2^-$) and Au ions are detected in the secondary negative ion spectrum.

④ Save the optimised conditions in the computer for analysing the sample.

* The general conditions for this experiment are mentioned in Section 5.1

2.4 Mass conversion (MS calibration)– The measured values obtained by ToF-SIMS represent the ToF of ions to pass through the analysis tube and the intensity of peaks. Therefore, the ToF of ions to pass through the analysis tube must be converted to mass. Peaks observed in the spectrum are used, and in the case of the negative ion mass spectrum, CH^-, C_2H^-, C_3H^-, C_4H^-, and C_5H^- peaks are used for the conversion.

3. Detecting AuNPs and PEG ligand peaks using ToF-SIMS

3.1 Load the prepared sample to the ToF-SIMS sample holder and place inside the preparation chamber. When the degree of vacuum reaches approximately ≤ 1.0 E –5 mbar, transfer the sample holder to the main chamber.

3.2 Check whether the mass resolution of the ^{29}Si peak is 7000 under the analytical conditions described in Section 5.1.

3.3 Check whether the Au^- and PEG($C_3H_3O_2^-$) peaks have been accurately measured under the analytical conditions described in Section 5.1.

3.4 Analyse five points for each sample.

4. Image processing using SurfaceLab, an analytical program for ToF-SIMS

4.1 Obtain Au^- and $C_3H_3O_2^-$ images from the measured data.

4.2 For image smoothing, select each image in SurfaceLab and right-click to open a pop-up menu. Select ‘average 1’ from the filter option to automatically run image smoothing (Figure 9).

4.3 For image binning, select each image in the SurfaceLab program and choose ‘4 pixel’ from the binning option to automatically run image binning (Figure 10).

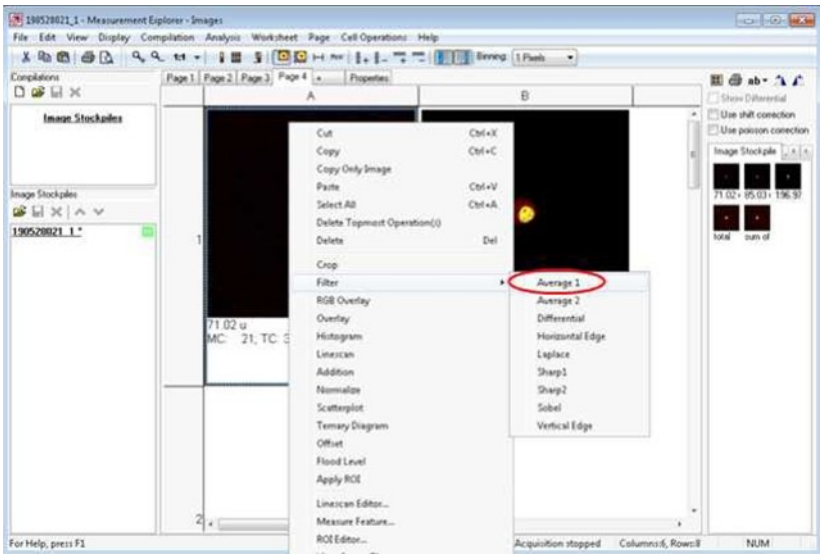


Figure 9. Image smoothing in SurfaceLab image program.

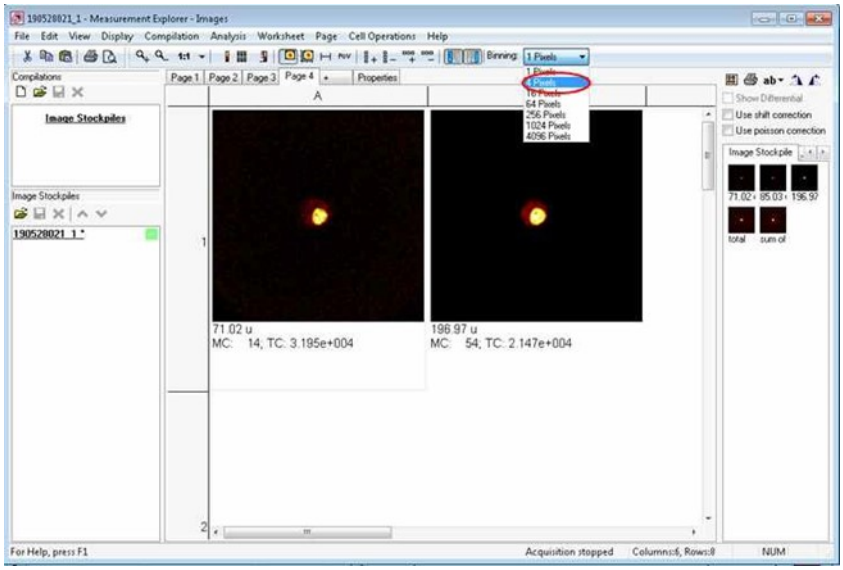


Figure 10. Image binning in SurfaceLab image program

5. Scatter plot and Pearson correlation coefficient

Use R statistic program, which is a free open resource.

5.1 Obtain the ASCII file from each image obtained in Section 4. Right-click on each image and choose the 'Export' option from the pop-up menu to obtain the ASCII files.

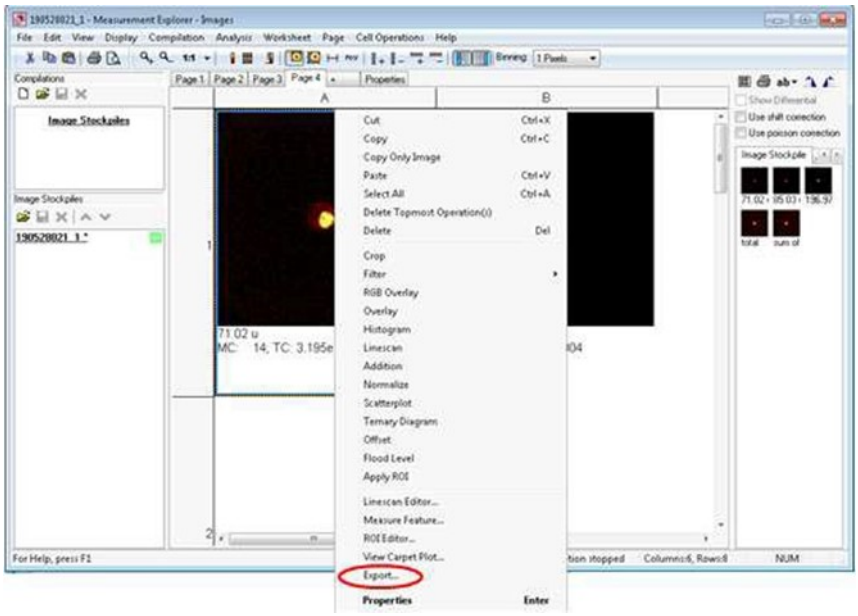


Figure 11. Obtaining an ASCII file in SurfaceLab image program

5.2 To draw the scatter plot, combine the ASCII files obtained from the two images into a single ASCII file (Figure3).

5.3 Use the R statistics program to draw the scatter plot and calculate the Pearson correlation coefficients.

6 Data Analysis and Reporting of Data:

To confirm whether the surface of the AuNPs has been modified by PEG, Type A and B uncertainties are calculated as discussed below.

- Type A uncertainty

In experiments using ToF-SIMS, the analysis must be carried out at Primary ion dose density (PIDD) ≤ 1.0 E12 ions/cm^2. In the experiment reported in this procedure guide, the intensity of the Au^- and $C_3H_3O_2^-$ peaks was low. Therefore, the analysis of a single sample was carried out at 4.0 E11 ions/cm^2, which made it difficult to obtain multiple values from the sample, thereby making the calculation of Type A uncertainty difficult.

- Type B uncertainty

1. Uncertainty in equipment performance

An uncertainty should be calculated based on the data obtained from multiple measurements of the same sample. However, we encountered problems with respect to the absence of samples with certified homogeneity and inability to perform repeated measurements on the same sample. Accordingly, the mass accuracy certified by IONTOF was used as a substitute. The mass accuracy certified by

IONTOF is ≤ 10 ppm, and because this value is negligible as compared to other uncertainties, it did not affect the Pearson correlation.

2. Mean and standard deviation (SD) for determining the background boundary values

It would be ideal to have a program that can automatically determine the background boundary values from the scatter plot. However, there is no such program. Therefore, the background boundary values must be set manually. For setting the background boundary values manually, SD is calculated and used as u(x1).

Number of measurement	1	2	3	4	5	6	7	8	9	10	ave	std
AC4	0.9577	0.9582	0.9553	0.9518	0.9573	0.9553	0.9462	0.9518	0.9555	0.9553	0.9544	0.0036

3. Uncertainty in sample homogeneity and finite number of measurement samples

The uncertainty generated by the finite number of samples and sample homogeneity used to confirm whether the surface of AuNPs has been modified by PEG cannot be assessed separately. Hence, this uncertainty is calculated using the SD of five repeated measurements. When calculating the value, if the difference between the limits aB_- (lower limit) and aB_+ (upper limit) is 2aB (= $aB_+ - aB_-$), then ′a′ can be divided by √3 to calculate the uncertainty as follows.

$$u(x_2) = \frac{aB}{\sqrt{3}}$$

$$a = \frac{(aB_+ - aB_-)}{2}$$

aB_+ :*upper limit* , aB_- : *lower limit*

Here, aB_+ is 1.0000 and aB_- is 0.8500, and thus, a = 0.575. Accordingly, u(x_2) = 0.3320.

・ Expanded uncertainty (U)

To determine the coverage factor *k* for estimating the interval with the desired confidence level, v_{eff}, the effective degree of freedom of u_c, is calculated.

$$v_{eff} = \frac{u_c^4}{\sum \frac{u_i^4}{\nu i}}$$
$$= \frac{(0.0036)^4}{\frac{(0.0036)^4}{9} + \frac{(0.3320)^4}{\infty}}$$
$$\approx 9$$

The expanded uncertainty is expressed as U = ku_c. In Type B uncertainty, v_{eff} = 9. Thus, the confidence interval is estimated to be approximately 95% using k = 2 from the t-distribution. In this procedure guide, U = 0.664.

Table 1. Measurement of uncertainties

Standard uncertainty components	**Cause of uncertainty**	**Standard uncertainty value**	**Sensitivity coefficient**	**Contribution to the combined standard uncertainty**	**Degree of freedom**
Uncertainty in the process of determining the background boundary, $u(x_1)$	Uncertainty in the process of determining the background in the scatter plot	0.0036	1	0.0036	9
Uncertainty in sample homogeneity and finite number of measurement samples, $u(x_2)$	Uncertainty in sample homogeneity and finite number of measurement samples	0.3320	1	0.3320	∞

$$u_c^2 = \sum u_i^2 = 0.11023696$$
$$u_c = 0.3320$$
$$v_{eff} = 9$$

7 Publications:

[1] Hyun Kyong Shon, Jin Gyeong Son, Sunho Joh, Jeong Hee Moon, Tae Geol Lee, 'Numerical evaluation of polyethylene glycol ligand conjugation to gold nanoparticle surface using ToF-SIMS and statistical analysis', *Biointerphases*, 15(3), 031008

8 References

[1] ISO/IEC 17025, General requirements for the competence of testing and calibration laboratories.

[2] ISO/IEC 98-3:2008, Uncertainty of measurement — Part 3: Guide to the expression of uncertainty in measurement (GUM:1995)