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Corresponding Author:	Mikhail Skliar, Ph.D. University of Utah Hospital Salt Lake City, UT UNITED STATES
Corresponding Author's Institution:	University of Utah Hospital
Corresponding Author E-Mail:	mikhail.skliar@utah.edu
Order of Authors:	Mikhail Skliar, Ph.D.
	Vasiliy S. Chernyshev
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TITLE:

Imaging of Extracellular Vesicles by Atomic Force Microscopy

AUTHORS AND AFFILIATIONS:

Mikhail Skliar[1,2], Vasiliy S. Chernyshev[3,4]

[1]Department of Chemical Engineering, University of Utah, Salt Lake City, UT, USA
[2]The Nano Institute of Utah, University of Utah, Salt Lake City, UT, USA
[3]Center for Photonics and Quantum Materials, Skolkovo Institute of Science and Technology, Skolkovo Innovation Center, Moscow, Russia
[4]Biopharmaceutical Cluster 'Northern', Moscow Institute of Physics and Technology, Dolgoprudny, Russia

Corresponding Author:
Mikhail Skliar (mikhail.skliar@utah.edu)

Email Address of Co-author:
Vasiliy Chernyshev (v.chernyshev@skoltech.ru)

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SUMMARY:

A step-by-step procedure is described for label-free immobilization of exosomes and extracellular vesicles from liquid samples and their imaging by atomic force microscopy (AFM). The AFM images are used to estimate the size of the vesicles in the solution and characterize other biophysical properties.

ABSTRACT:

Exosomes and other extracellular vesicles (EVs) are molecular complexes consisting of a lipid membrane vesicle, its surface decoration by membrane proteins and other molecules, and diverse luminal content inherited from a parent cell, which includes RNAs, proteins, and DNAs. The characterization of the hydrodynamic sizes of EVs, which depends on the size of the vesicle and its coronal layer formed by surface decorations, has become routine. For exosomes, the smallest of EVs, the relative difference between the hydrodynamic and vesicles sizes is significant. The characterization of vesicles sizes by the cryogenic transmission electron microscopy (cryo-TEM) imaging, a gold standard technique, remains a challenge due to the cost of the instrument, the expertise required to perform the sample preparation, imaging and data analysis, and a small number of particles often observed in images. A widely available and accessible alternative is the atomic force microscopy (AFM), which can produce versatile data on three-dimensional geometry, size, and other biophysical properties of extracellular vesicles. The developed protocol guides the users in utilizing this analytical tool and outlines the workflow for the analysis of EVs by the AFM, which includes the sample preparation for imaging EVs in hydrated or desiccated form, the electrostatic immobilization of vesicles on a substrate, data acquisition, its analysis, and

interpretation. The representative results demonstrate that the fixation of EVs on the modified mica surface is predictable, customizable, and allows the user to obtain sizing results for a large number of vesicles. The vesicle sizing based on the AFM data was found to be consistent with the cryo-TEM imaging.

INTRODUCTION:

Extracellular vesicles (EVs) are present in all body fluids, including blood, urine, saliva, milk, and the amniotic fluid. Exosomes form a district class of EVs differentiated from other EVs by endosomal biogenesis, the markers of the endosomal pathway, and the smallest size among all EVs. The size of exosomes is often reported with substantial variability between studies. The sizing results were found to be method dependent, reflecting the difference in physical principles employed by different analytical techniques to estimate EV sizes[1,2]. For example, the nanoparticle tracking analysis (NTA) — the most widely used size characterization technique — estimates the size of EVs as their hydrodynamic diameters, which characterize the resistance to the Brownian mobility of EVs in the solution. A larger hydrodynamic diameter of a vesicle implies its lower mobility in liquid. The coronal layer around vesicles, consisting of surface proteins and other molecules anchored or adsorbed to the membrane surface, substantially impedes the mobility and increases the hydrodynamic size of EVs. In relative terms, this increase is particularly large for the exosomes[3], as illustrated in **Figure 1**.

The cryogenic transmission electron microscopy (cryo-TEM) imaging is a definitive technique in characterizing vesicle sizes and morphology in their hydrated states. However, the high cost of the instrumentation and the specialized expertise needed to use it correctly motivate the exploration of alternative techniques that can image hydrated EVs. A relatively small number of EVs observed or characterized in the acquired cryo-TEM images is another notable disadvantage of this technique.

Atomic force microscopy (AFM) visualizes the three-dimensional topography of hydrated or desiccated EVs[4–6] by scanning a probe across the substrate to raster the image of the particles on the surface. The essential steps of the protocol to characterize EVs by AFM are outlined in this study. Before imaging the vesicles in liquid, they must be immobilized on a substrate by either tethering to a functionalized surface, trapping in a filter, or by electrostatic attraction[7]. The electrostatic fixation on a positively charged substrate is a particularly convenient option for immobilization of exosomes known to have a negative zeta potential. However, the same electrostatic forces that immobilize the extracellular vesicles on the surface also distort their shape, which makes post-imaging data analysis essential. We elaborate this point by describing the algorithm that estimates the size of the globular vesicles in the solution based on the AFM data on the distorted shape of the exosomes immobilized on the surface.

In the developed protocol, the procedure for the robust electrostatic immobilization of vesicles is presented and followed by the steps needed to perform atomic force imaging in the hydrated or desiccated states. The factors that influence the surface concentration of the immobilized vesicles are identified. The guidance is given on how to perform the electrostatic immobilization for samples with different concentrations of EVs in the solution. The selection of experimental conditions permitting the estimation of empirical probability

distributions of different biophysical properties based on a sufficiently large number of immobilized vesicles is discussed. Examples of post-imaging analysis of the AFM data are given. Specifically, an algorithm is described for determining the size of vesicles in the solution based on the AFM characterization of immobilized EVs. The representative results show the consistency of the vesicle sizing by AFM with the results of cryo-TEM imaging.

PROTOCOL:

1. Isolation of EVs from a biofluid

1.1. Isolate EVs by one of the established methods, such as the differential ultracentrifugation[8], precipitation, or size-exclusion chromatography[9].

1.2. Confirm the presence of expected surface and luminal biomarkers and the absence of biomarkers indicating cross-contamination of the preparation. Confirm the lipid bilayer morphology of the isolated particles by electron microscopy.

NOTE: When isolating the exosomes, the hydrodynamic size distribution measured by nanoparticle tracking analysis (NTA) or dynamic light scattering should be in the expected range. The details of EV and exosome isolation are beyond the scope of this protocol. The selected method will depend on experimental questions and the goal of the study[10]. The following steps provide a concrete illustration of the procedure to enrich the exosomes by precipitation from the growth medium of MCF-7 breast cancer cells using a commercially available precipitation kit (**Table of Materials**).

1.3. Before cell culture expansion, store MCF-7 breast cancer cells in liquid nitrogen. Thaw cells to subculture.

1.4. Following aseptic practices, perform cell plating on 150 mm plates. Use the growth medium composed of the Eagle's minimum essential medium, 0.01 mg/mL human recombinant insulin, and 10% exosome-free fetal bovine serum.

1.5. Aerate the culture by 95% air and 5% CO_2 and incubate at 37 °C.

1.6. After the cells are settled (approximately 24 h after plating), change the media. Split the plate at 1:10 ratio and culture ten plates, each containing 20 mL of media.

1.7. Harvest and pool media from 9 of these plates (180 mL) at ~70−80% confluence when cells are still in the growth phase.

1.8. Divide the media into 60 mL and 120 mL, further split into 30 mL/tube, and centrifuge at 3,000 x *g* for 15 min.

1.9. Transfer the supernatant from each tube to a new sterile 50 mL tube and perform the exosome isolation.

1.10. Isolate exosomes by precipitation according to published protocols (see, for example, reference[11]) or follow the manufacturer's instructions if a commercial isolation kit (**Table of Materials**) is used. As a first step in the latter case, centrifuge cell medium at 3,000 x *g* for 15 min. Withdraw supernatant and discard cells and cell debris.

1.11. Add the precipitation solution (1:5 volume ratio) to the supernatant, mix, and refrigerate overnight.

1.12. Centrifuge at 1,500 x *g* for 30 min at room temperature. Discard the supernatant after centrifugation.

1.13. Spin the remaining exosome pellet for another 5 min at 1,500 x *g*. Without disturbing the pellet, remove the remaining precipitation solution by aspiration.

1.14. Resuspend the pellet in 100−500 µL of 1x phosphate-buffered saline (PBS) buffer and divide into multiple aliquots as needed for the downstream analysis.

1.15. Immediately proceed to the surface immobilization of the isolated exosomes for AFM imaging. If necessary, freeze the aliquots at -80 °C for later use while taking precautions to avoid damage to the sample during the freeze-thaw cycle.

2. Surface fixation of extracellular vesicles

2.1. Use strong double-sided tape, epoxy, or an alternative adhesive to firmly attach a mica disk to an AFM/scanning tunneling microscope (STM) magnetic stainless-steel specimen disk.

2.2. Cleave mica disc by using a sharp razor or utility knife, or by attaching an adhesive tape to the top surface and then pealing it off to remove a layer of material.

NOTE: Either method should reveal a virgin surface by removing a thin layer of mica previously exposed to the environment. After the procedure, the attachment of mica to the AFM/STM metal specimen disk must remain firm.

2.3. At room temperature, treat the top surface of mica for 10 s with 100 µL of 10 mM $NiCl_2$ solution, which modifies the surface charge from negative to positive.

2.4. Blot $NiCl_2$ solution with a lint-free wipe or blotting paper. Wash the mica surface 3x with deionized (DI) water and dry it with a stream of dry nitrogen.

NOTE: It is a good practice to scan the modified surface with AFM to confirm it is free from contaminants.

2.5. Place the AFM specimen disk with the attached surface-modified mica in a petri dish.

2.6. Dilute the exosome sample from step 1.14 with 1x PBS to obtain a concentration between 4.0 x 10^9 and 4.0 x 10^{10} particles per mL of solution. Validate the diluted particle concentration using NTA.

2.7. Form a sessile drop on the surface of mica by emptying 100 µL of the diluted exosome solution from a pipette.

2.8. Place lid on the petri dish and seal it with a paraffin film to reduce sample evaporation. Incubate the sample for 12−18 h at 4 °C.

NOTE: The surface density of the immobilized exosomes will increase with the incubation time and the concentration of EVs in the liquid. Longer incubation time may be necessary if exosomes are present in the sample at lower concentrations.

2.9. After incubation, aspirate 80−90% of the sample without disturbing the surface. At this point, the exosomes will be electrostatically immobilized on the mica substrate.

2.10. Before imaging hydrated EVs, rinse the surface with 1x PBS. Repeat 3x. Take care to keep the sample hydrated throughout the rinsing process.

2.11. After washing the mica surface with 1x PBS, remove 80%−90% of liquid, and pipette ~40 µL of fresh 1x PBS to cover the sample.

2.12. When imaging the desiccated EVs, rinse the substrate with DI water. Repeat 3x.

NOTE: Rinsing with DI water will prevent the formation of salt crystals and the deposition of solutes on the surface as the substrate dries.

2.13. Before imaging desiccated EVs, aspirate as much liquid as possible without touching the surface and dry the rest with a stream of dry nitrogen.

3. AFM imaging

3.1. To image the desiccated EVs, select a cantilever designed for scanning in the air in tapping and non-contact imaging modes and mount it onto the probe holder.

NOTE: The characteristics of an example cantilever listed in **Table of Materials** (123 µm length, 40 µm width, 7 nm tip radius, and 37 N/m spring constant) may be used as a guide when selecting a probe compatible with the available AFM instrumentation.

3.1.1. Place the preparation from step 2.13 on the AFM stage. The magnetic stainless-steel specimen disk will immobilize the sample on the stage. Allow time for the preparation and the stage to equilibrate thermally.

3.1.2. Use the tapping mode to scan a sufficiently large area of the mica's surface. For example, choose an area of 5 x 5 µm, rastered in 512 lines at a scan rate of ~1 Hz. Acquire

both the height and phase images as they provide complementary information on the topography and the surface properties of the sample.

NOTE: The scan time will increase with the imaged area and the number of lines selected to form the image but decrease with the scan rate defined as the number of lines scanned per second. Fast scan rates may impact the image quality. Therefore, the speed of rastering should judicially balance the tradeoff between the acquisition time and the image quality.

3.2. To image hydrated vesicles, select a cantilever appropriate for scanning soft, hydrated samples and mount the cantilever onto a probe holder designed for scanning in liquids.

NOTE: When selecting a probe compatible with the available AFM instrumentation, the specifications of the probe listed in **Table of Materials** (triangular cantilever with 175 µm nominal length, 22 µm width, 20 nm tip radius, 0.07 N/m spring constant, and optimized for imaging with the drive frequency of ~7 kHz) may be used as a guide.

3.2.1. Wet the tip of the cantilever with 1x PBS to reduce the likelihood of introducing air bubbles into the liquid during scanning.

3.2.2. Place the preparation from step 2.11 on the AFM stage. The magnetic stainless-steel specimen disk will immobilize the attached mica containing immobilized EVs on its surface.

3.2.3. Allow time for the preparation and the AFM stage to equilibrate thermally.

3.2.4. Image the hydrated mica surface in the tapping mode. Acquire both the height and phase images.

NOTE: The imaging quality is influenced by the instrumentation, selected probe, and scan parameters. When optimizing the scanning conditions, the following choices may be used as a starting point: 5 x 5 µm area scanned in 512 lines with ~0.8 Hz scan rate and ~7 kHz drive frequency.

4. Image analysis

NOTE: The following data processing and analysis steps are applied to the acquired height images. A similar procedure may be adapted to analyze the phase data. The description below is specific to Gwyddion[12], a free and open source software available under GNU General Public License. Similar capabilities are available in alternative software tools.

4.1. Go to **Data Process**, **SPM modes**, **Tip** and choose **Model Tip** (**Figure 2**). Select the geometry and the dimensions of the tip used to scan the sample and click **OK**.

4.2. Correct the tip erosion artifacts by performing the surface reconstruction. Open the image. From the menu, select **Data Process**, **SPM modes**, **Tip**, then choose **Surface Reconstruction** and click **OK** (**Figure 3**).

4.3. Align the imaging plane to match the laboratory XY plane by removing the tilt in the substrate from the scan data. To accomplish this task, select **Data Process**, **Level** and choose **Plane Level** (**Figure 4**).

4.4. Align rows of the image by selecting **Data Process**, **Correct Data** and then choose **Align Rows**. Several alignment options are available (**Figure 5**). For example, **Median** is an algorithm that finds an average height of each scan line and subtracts it from the data.

4.5. Go to **Data Process**, **Correct Data** and choose **Remove Scars** (**Figure 6**), which removes common scanning errors known as scars.

4.6. Align the mica surface at the zero height, Z = 0, by selecting **Flatten Base** in **Level** drop-down menu accessible from **Data Process** (**Figure 7**).

4.7. Identify EVs on the scanned surface by using **Mark by Threshold** in **Grains** drop-down menu (**Figure 8A**). This algorithm identifies surface-immobilized exosomes as particles protruding from the zero-surface substrate by the height above the user-selected threshold. Select a threshold in the range between 1 and 2 nm, which will eliminate most of the background interference.

NOTE: The threshold in **Figure 8A** is 1.767 nm. The outcome of the MCF-7 exosome identification with this thresholding is shown in **Figure 8B**. Gwyddion offers several alternatives to thresholding as the algorithm to automatically identify vesicles in the image, including automated thresholding (Otsu's method), edge detection, and the watershed algorithm.

4.8. Perform geometric and dimensional characterization of the identified EVs using the available **Distributions** algorithms accessible from **Grains** menu.

NOTE: Gwyddion provides tools to assess the distribution of scalar-valued, areal, volumetric, and other properties of immobilized EVs in a hydrated or dedicated state. An example of a scalar-values property is shown in **Figure 9,** which gives the distribution of maximum heights within the footprint of each identified exosome.

4.9. Export the AFM data from Gwyddion for specialized analysis by other computational tools and custom computer programs.

REPRESENTATIVE RESULTS:

Surface fixation of EVs is a critical step in the imaging sequence. Electrostatic surface immobilization of exosomes, known to have a negative zeta potential, will robustly occur after the mica's substrate is modified to have a positive surface charge. Without the treatment with $NiCl_2$ to impart positive surface changes, the immobilization of EVs on the substrate was found to be ineffective. The height image in **Figure 10A**, acquired in the air after the MCF-7 exosome sample containing 2.59 x 10^{10} vesicles per mL of PBS was incubated for 12 h on *unmodified* surface of freshly cleaved mica, shows very few vesicles remaining on the surface after it was cleaned with DI water. The vesicles visible in **Figure**

10A are, most likely, the result of incomplete aspiration of DI water, which resuspended vesicles not fixed to the surface and then deposed them on the substrate as it evaporated.

After modifying the surface charge with nickel chloride, it is advisable to confirm that the surface remains free of contaminants after the treatment. The height image in **Figure 10B** (obtained in the air) gives an example of a clean surface after it was treated with $NiCl_2$ and then washed three times with DI water. The roughness of cation-derivatized surface was below 0.3 nm, which is consistent with the previous report[13].

The dramatic positive impact of the surface charge modification on the efficiency of the fixation of MCF-7 exosomes is illustrated by **Figure 10C,D**. These two panels show the height scans acquired in the air after the sample, previously imaged in **Figure 10A**, was incubated for 24 h and 12 h, respectively, on the surface treated with nickel chloride.

The time a given sample is incubated on the treated surface determines the surface concentration (vesicles per area) of the immobilized EVs. The height image in **Figure 10C** illustrates the case of excessively dense surface coverage by the immobilize vesicles obtained after the described MCF-7 exosome sample was incubated for 24 h. A number of algorithms rely on having sufficient unoccupied substrate between the grains to perform image correction and data analysis. For example, leveling and shifting the substrate to the zero plane, line correction, and estimation of the grains' volume need the intervening flat surface to perform accurate calculations. When the concentration of the immobilized vesicles is as high as in **Figure 10C**, these algorithms will not function reliably. An example of an adequate surface concentration of vesicles immobilized from the same MCF-7 sample is shown in the height image in **Figure 10D**, which was obtained after shorter (12 h) incubation.

The post-processing of the acquired raw AFM data is needed to correct for common scanning errors. The following description is specific to Gwyddion. Similar functionality is available in other AFM/SPM data analysis tools.

Within Gwyddion, **Plane Level** function is used to correct for a tilt in the substrate. Such background correction is accomplished by first finding the plane of the substrate using all data points in the image and then subtracting it from the raw data. The correction along the scan lines is accomplished by **Align Rows** function. For example, one of the implemented algorithms performs the alignment by computing the median height of each scan line and the then subtracting the result from the corresponding row of image data. The contribution of the local faults in the feedback loop can be removed by applying **Remove Scars** function, which fills the gaps in the aligned data and eliminates the scars by comparing the data in the adjacent scan lines. The shift of the substrate to the elevation Z = 0 can be accomplished by a combination of a facet and polynomial leveling of the surface after masking grains and other features. Gwyddion's **Flatten Base** tool performs this task autonomously or with a user-specified mask. After the described background and line corrections, the electrostatically fixated vesicles can be identified on the substrate by executing **Mark Grains** function.

Figure 11A and **Figure 11B** show height and phase images of hydrated MCF-7 exosomes immobilized on a mica surface and acquired in PBS using the tapping mode. A total of 561 hydrated vesicles were identified in the scanned area using **Threshold** algorithm of **Mark Grains** function with the threshold value set to ~20%. The phase lag of the probe's response at the drive frequency is sensitive to localized stiffness variations in soft samples. The consistency between the height and phase images, seen in **Figure 11A,B**, is, therefore, an important confirmation that the imaged grains are, indeed, soft vesicles immobilized on the substrate.

Figure 11C shows the cross-section of the height image through exosomes located on the white line in **Figure 11A**. While the exosomes in a biofluid have a globular geometry[1,14–16], their shape on the substrate is severely distorted by the electrostatic attraction to the positively charged surface. The oblate pancake-like geometry of electrostatically immobilized vesicles is further illustrated in **Figure 11D** by the close-up height image (and its cross section) of an exosome boxed in **Figure 11A**. The corresponding phase image is shown in **Figure 11E**. The empirical probability density function (pdf) of peak heights above the surface for all 561 hydrated vesicles identified in the AFM scan is shown in **Figure 12A**. The mean value for this distribution is 7.9 nm, which is approximately equal to twice the thickness of a phospholipid bilayer[17] in the absence of deforming forces.

The area on the substrate occupied by an immobilized exosome was approximated as a circle with the diameter equal to the mean distance from the vesicle's "center of mass" to its boundary on the mica's surface. The distribution of these projection diameters is shown in **Figure 12A** and has the mean equal to 69.6 nm. The obtained height and the diameter distributions further quantify the significant impact of the electrostatic surface immobilization on the distorted shape of immobilized exosomes.

The robustness and reparability of the protocol procedures were confirmed by reanalyzing the same MCF-7 sample three times, from sample preparation to imaging, with each repeat producing results statistically similar to those shown in **Figure 12**.

The deformation of immobilized vesicles caused by electrostatic forces may be compensated or interpreted to provide an insight into the properties of the imaged EVs. For example, the AFM data may be used to estimate the globular size of the vesicles in the solution. As a starting point, we can calculate the volume encapsulated by the membrane envelopes of immobilized vesicles. The volume is found by integrating the difference between the surface level of the identified vesicles and the substrate elevation underneath them. The substrate level under the vesicles is not directly accessible but can be estimated by the Laplace or alternative interpolation of data points for unoccupied substrate surrounding the vesicles. Within Gwyddion, such volume calculation is performed using **Distribution of Various Grain Characteristics** function. The result exported from Gwyddion can then be mapped into the diameters of volume-equivalent spheres.

The application of the described algorithm to the AFM data for 561 analyzed hydrated MCF-7 vesicles produced the distribution of the diameters of volume-equivalent spheres shown in **Figure 12B**. This distribution estimates the size of membrane vesicles in their innate globular form in a biofluid before their electrostatic fixation on the mica surface. The vesicle

sizing obtained from the analysis of the AFM data was compared with the results of cryo-TEM imaging of the same sample and was found to be in close agreement[3] (**Figure 12B**). The comparison of the hydrodynamic diameters measured by the NTA with the obtained vesicle sizes (**Figure 1**) indicates that the mobility of exosomes is much smaller than would be expected from the size of their vesicles determined from the AFM and cryo-TEM measurements. The difference between the hydrodynamic and vesicle sizes characterizes the thickness of the coronal layer surrounding exosomal vesicles.

FIGURE LEGENDS:

Figure 1: Comparison of hydrodynamic and geometric diameters of EVs. The geometric size of the exosomal vesicle is substantially smaller than its hydrodynamic size determined from its diffusion in a liquid. The difference is the coronal layer formed by membrane-conjugated and adsorbed molecules that impede the mobility of EVs. This figure is modified from reference[3] and reprinted with permission.

Figure 2: Properties of the AFM probe. The geometry and the dimensions of the AFM probe can be specified using **Model Tip** function.

Figure 3: Correction of imaging artifact caused by tip-sample convolution. By performing **Surface Reconstruction**, the acquired AFM data can be corrected for tip artifacts.

Figure 4: Correction for a tilt in the substrate. Plane Level determines the plane of the substrate and subtracts it from the AFM data.

Figure 5: Correction of misalignments in scan rows. A conventional algorithm to align the scan data is to find an average height along each scan line and subtracts the result from the corresponding row of data points in the image.

Figure 6: Correction for the gaps in the aligned data. Common scanning errors, known as scars, can be removed from the AFM data by applying **Remove Scars** function.

Figure 7: Alignment of a substrate at zero elevation. Flatten Base option in Level menu allows the user to place the substrate surface at the base level corresponding to the zero height.

Figure 8: Identification of immobilized vesicles on the scanned surface. (**A**) The surface-immobilized exosomes are identified as grains protruding above the substrate by a user-selected height threshold specified in **Mark by Threshold**. (**B**) The outcome of the identification.

Figure 9: Analysis of the AFM data. The distribution of maximum heights above the substrate within the area occupied by the identified exosomes is shown as compiled by **Grain Distributions** tool.

Figure 10: Impact of surface modification and EV concentration of the surface density of immobilized vesicles. (**A**) The AFM height image of freshly cleaved mica substrate after 12 h

incubation with MCF-7 exosome sample followed by cleaning with DI water and drying. The immobilization of EVs from the liquid to the substrate is inefficient without imparting a positive charge to mica's surface. Few particles seen in the scan are likely the result of incomplete removal of the MCF-7 sample before the substrate was dried. (**B**) The height scan of mica's surface in the air after the treatment with nickel chloride shows the substrate free of contaminations. Panels (**C**) and (**D**) show AFM height scans obtained after the modification of the surface charge and the incubation with the same MCF-7 sample as in panel (**A**) for 24 h and 12 h, respectively. The surface concentration of immobilized vesicles is excessively dense after 24 h incubation. The 12 h incubation leads to fewer exosomes immobilized on the surface and the scan data that are easier to analyze accurately.

Figure 11: AFM images of hydrated MCF-7 exosomes electrostatically immobilized on the modified mica surface. (**A**) The height image. (**B**) The corresponding AFM phase image confirms that the grains in the height image are soft nanoparticles, as should be expected for membrane vesicles. (**C**) The height data for the three vesicles crossed by the line shown in panel (**A**) illustrate a flattened shape caused by the electrostatic attraction of exosomes to the positively charged surface of the modified mica. (**D**) The shape distortion is apparent in an enlarged view the immobilized vesicle boxed in panel (**A**) and its cross section. The phase image of the same vesicle is shown in (**E**). This figure is modified from reference[3] and reprinted with permission.

Figure 12: Dimensional characterization of hydrated vesicles immobilized on the surface and the estimation of their globular size in the solution. (**A**) The distribution of peak heights above the surface (red curve) has the mean equal to 7.9 nm. The area occupied by immobilized exosomes has 69.6 nm average diameter (blue curve). (**B**) AFM height image for one of the immobilized exosomes illustrates its highly oblate shape caused by electrostatic forces. The globular size of exosomal vesicles in the solution can be estimated by matching volumes enclosed by surface-immobilized and spherical membrane envelopes. (**C**) The size distribution of globular vesicles in the solution (red curve) was determined from the AFM data of 561 immobilized vesicles. The vesicle sizes in cryo-TEM images (blue curve) are consistent with the AFM results. This figure is modified from reference[3] and reprinted with permission.

Figure 13: Surface concentration and size segregation artifacts during passive deposition of EVs from evaporating liquid. (**A**) The scanning electron microscopy (SEM) image shows that the surface concentration of exosomes passively deposited from a drying liquid is spatially variable when surface immobilization from a suspending biofluid is not performed. (**B**) Passive deposition of EVs from a drying sample causes vesicles size segregation. The substantial size variability is quantified by the probability density functions (pdf) for the vesicles in different regions in the image (**A**) defined by white diagonal lines. This figure is modified from reference[1] and reprinted with permission.

Figure 14: Cup-shaped geometry of desiccated vesicles passively deposited on the surface during the liquid evaporation. The surface desiccation of vesicles which were not immobilized by electrostatic forces is known to result in a cup-shaped appearance often observed in SEM images of EVs. This figure is modified from reference[1] and reprinted with permission.

DISCUSSION:

The immobilization of EVs from a biological fluid, surface scanning, and image analysis are the essential steps of the developed protocol for the AFM characterization of EVs in liquid. The number of vesicles amenable to AFM imaging scales with the imaged surface area and the surface concentration of the vesicles immobilized on the substrate. Given a negative zeta potential of EVs and exosomes[18], we advocate electrostatic fixation of EVs from liquid samples to the AFM substrate. The immobilization is effective when the surface is positively charged. Prior to EV immobilization, the positive surface charge may need to be imparted to the substrate, as in the case of mica — a layered silicate mineral with general formula $KAl_2(AlSi_3O_{10})(OH)_2$. Freshly cleaved mica's surface is close to perfectly flat, which is ideal for imaging nanoparticles by the AFM, but its surface charge is negative and, thus, must be modified. The protocol describes the procedure to impart a positive surface change to the AFM substrate. The representative results show marked improvement in EV fixation from a biofluid to the modified mica substrate.

When imaging hydrated vesicles, it is important to minimize sample evaporation which causes the surface deposition artifacts and convective flows and increases the liquid concentration of the vesicles with time, leading to higher surface concentration of immobilized EVs than expected, especially during prolonged incubations. Probe holders explicitly designed for liquid samples eliminate or slow evaporation and should be used to image hydrated EVs. Nonspecific bindings to the scanning probe are reduced in the presence of ionic species. Therefore, when imaging hydrated EVs, it is preferable to cover the substrate with a buffered medium, such as PBS, instead of DI water.

Importance of surface immobilization

The consistent and predictable immobilization of EVs on the modified substrate removes the primary source of variability in the AFM results. All downstream steps, from scanning to data analysis, are more easily controlled by the selection of instrumentation, probes, scanning parameters, and the data analysis sequence and algorithms. The user should be aware of upstream variability in biological samples and EV isolation protocols, which are important issues beyond the scope of this work.

We recommend performing surface immobilization of EV on the modified mica's surface from liquid samples even when the goal is to characterize desiccated vesicles in the air — the need is less evident since vesicles will unavoidably deposit on any substrate as the liquid evaporates. In fact, the AFM results for desiccated EV obtained without modifying mica's surface charges, which is a prerequisite step for electrostatic immobilization of EVs from a liquid, have been reported in the past[19–21]. When EVs are not fixed to the surface from the liquid sample, however, their passive deposition by evaporation will produce artifacts collectively known as a coffee ring effect[22]. Two such artifacts, occurring as a drying liquid recedes, are illustrated in the SEM image (**Figure 13A**) of serum exosomes deposited by evaporation on a negatively charged glass surface. Significant variations in the surface concentration of precipitated vesicles are immediately apparent. The second artifact, quantified in **Figure 13B**, is the considerable variability in vesicle sizes in different areas within the perimeter of the dried sample. Given these artifacts, the AFM characterization of

passively deposited vesicles from a drying liquid may produce biased or inconsistent results unless the entire surface area initially occupied by a now-dried liquid sample is scanned.

Two additional issues should be considered when imaging the desiccated samples obtained without firm immobilization of vesicles on the substrate. Recall that our protocol instructs users to thoroughly wash the surface with DI water after the vesicles are immobilized from a liquid sample. This step intends to prevent ionic and other non-vesicular solutes from forming surface deposits during the evaporation of complex biofluids with considerable osmolarity. If EVs are not fixed, thorough washing will detach a large number of vesicles from the surface, potentially biasing the results and leaving too few particles for analysis. Another common difficulty, reduced by immobilizing EVs on the modified mica surface before the AFM imaging, is the adhesion of particles to the probe[23] and the misleading artifacts caused by this phenomenon.

Control of surface density of immobilized EVs

The two easily controllable factors identified in the protocol allow the user to customize the surface concentration of the immobilized EVs on the modified mica substrate: the concentration of the vesicles in the liquid sample and the time the sample is incubated on the substrate. A high density of immobilized vesicles, achieved with longer incubation times and higher concentration of EVs in the liquid, increases the number of vesicles analyzed during the scanning and the statistical power of conclusions arrived by the analysis of the AFM data. At the same time, an excessively dense surface concentration, as in the case shown in **Figure 10C** where particles tightly cover the entire surface with no intervening areas of the substrate, complicates the image analysis and the interpretation of results and may lead to scanning artifacts caused by the interaction between closely spaced particles.

Influence of electrostatic screening and hydrodynamic mobility of EVs

The transparent control over the surface concentration of immobilized EVs as a function of factors influencing it allows a user to customize experimental conditions to meet the specific needs of a study. When performing customization, it is important to recognize that the electrostatic surface immobilization is a transport-limited process influenced by the ionic strength of the biofluid.

The concentration of ionic and positively charged species inversely impacts the Debye length over which the surface and vesicle charges are screened. Beyond this length, the electrostatic forces are negligible. The boundary layer of the substrate's electrostatic attraction will be much smaller in ionically-rich PBS than in DI water. This difference implies that, after a short incubation corresponding to the time needed to deplete the layer of liquid where the electrostatic attractions are felt, a surface density of EVs immobilized from the suspension in DI water will be higher than from PBS suspension, assuming the concentration of EVs is the same in both liquids. Put differently, more vesicles must be immobilized to deplete a thicker attraction layer in DI water than in PBS under otherwise identical conditions.

After the vesicles are depleted from the boundary layer, the immobilization becomes an entirely transport-limited process. In this regime, the rate of the deposition will not depend on the suspending medium (e.g., DI water or PBS) as long as the viscosity is the same and

the transport is entirely diffusive. However, the transport of vesicles into the attraction boundary layer may not be entirely diffusive. For instance, if the sample in a sessile drop on the AFM substrate partially evaporates during the incubation, the fluid inside the drop will be subjected to the evaporation-driven flow, and the transport of the vesicles towards the substrate will have, both, diffusive and convective contributions. When the evaporation is not adequately controlled, the contribution of a convective transport will be considerable, and the rate of immobilization will be higher than expected. The impact of the convective transport will change with the thickness of the attraction layer, which itself depends on the ionic content of the liquid. Furthermore, the evaporation will enhance the vesicle immobilization on the substrate by concentrating EVs in the solution. At higher EV concentrations, the concentration gradient between the attraction layer and the adjacent liquid will increase, creating a larger thermodynamic driving force to the migration of vesicles towards the substrate.

Immobilized vesicles may represent a liquid sample with a bias. For the case when the rate of immobilization is limited by diffusion, vesicles with smaller hydrodynamic sizes, determined by the combination of the vesicle size and the thickness of the coronal layer surrounding it (**Figure 1**), are more likely to enter the attraction layer because of their higher mobility. Consequently, after the initial depletion period, the hydrodynamically small EVs will be overrepresented on the substrate compared to their contribution to the EV population in the liquid sample. Note that a smaller hydrodynamic size does not automatically point at EVs with smaller vesicle sizes because of the heterogeneity in the thickness of the coronal layer[3]. The biased representation is avoided with long incubations that deplete the entire population of EVs in the liquid by its immobilization on the substrate. When a user aims at immobilizing all EVs from the biofluid, to avoid excessively dense coverage of the surface with the immobilized vesicles, it may be necessary to reduce the EV concentration in the liquid below the range suggested in the protocol.

Deformation of EVs on the substrate

Extracellular vesicles in their native hydrated state and after desiccation can be characterized by the AFM, as described in the protocol. The electrostatic forces[24] that immobilize EVs on the mica surface also distort their shape from globular geometry in which they exist in the solution. The impact of the desiccation on the size and morphology of immobilized EVs may be analyzed by rescanning the same surface area before and after the sample is allowed to dry.

It is instructive to examine the impact of the sample preparation on the shape of the desiccated EVs. The electrostatically immobilized EVs maintain the highly oblate geometry after drying but are further flattened by the desiccation. The height above the surface of the desiccated vesicles becomes smaller than in **Figure 12A**, while their footprint area increases (data not shown). On the other hand, when vesicles are deposited passively during the liquid evaporation and without prior immobilization on the surface, they tend to attain a cup-shape geometry upon desiccation, as has long been observed in the SEM images and, more recently, in AFM scans. This cupped shape is now recognized as a sample preparation artifact[25] caused by non-uniformity in capillary forces during surface desiccation, as mechanistically explained in **Figure 14**[1].

Image analysis and interpretation of AFM data

The responses to electrostatic and capillary forces acting to distort the shape of EVs provide valuable information on structural and compositional properties of EVs. For example, a multidimensional set of biophysical characteristics, such as the deformed size and shape extracted from the AFM data, were recently used to demonstrate the feasibility to differentiate between exosomes secreted by different host cells[5]. The distortions can also be taken into account and compensated. For example, we showed how to use the AFM data to characterize the globular size of vesicles in the solution by estimating the diameters of spheres that encapsulate the same volume as immobilized exososmes[3].

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DISCLOSURES:

The authors have nothing to disclose.

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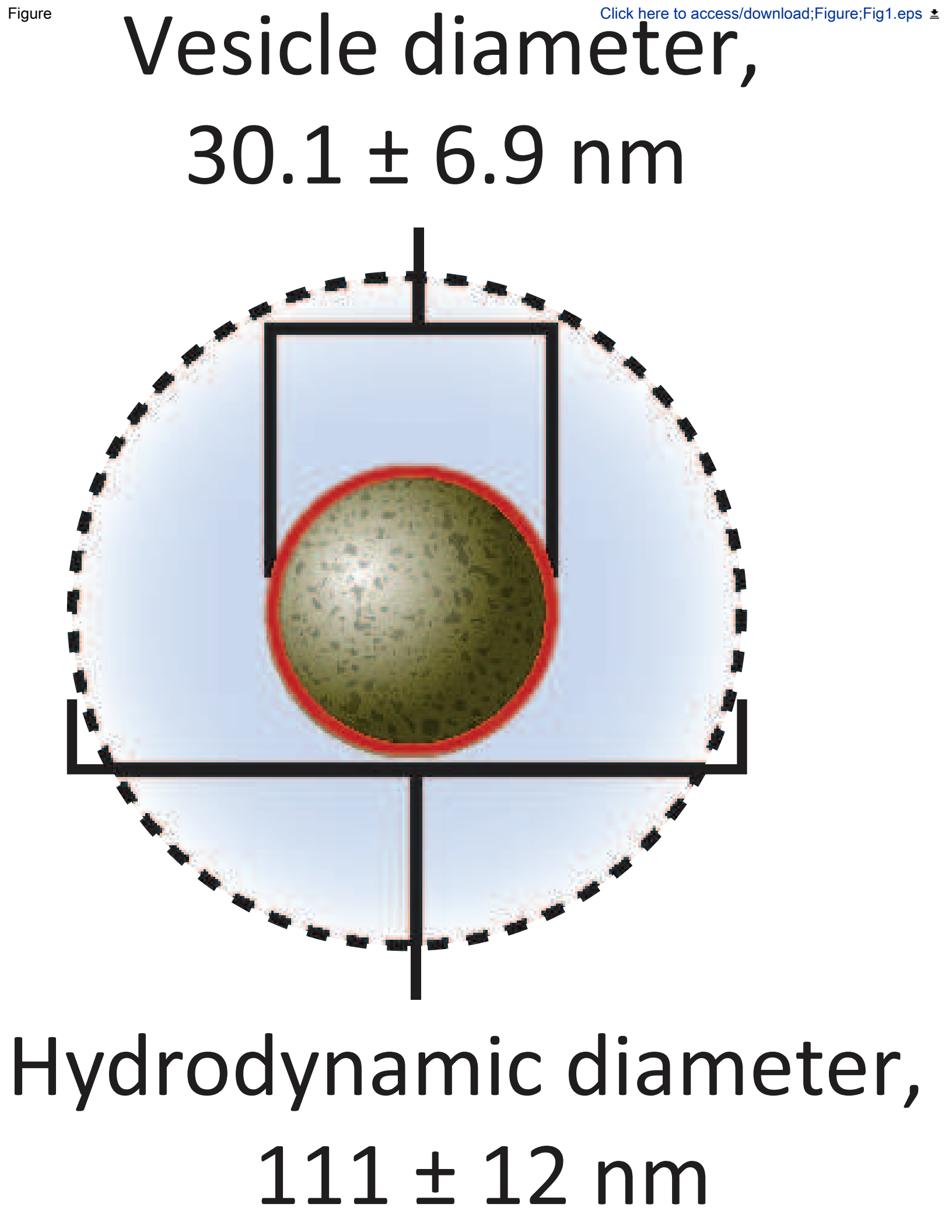
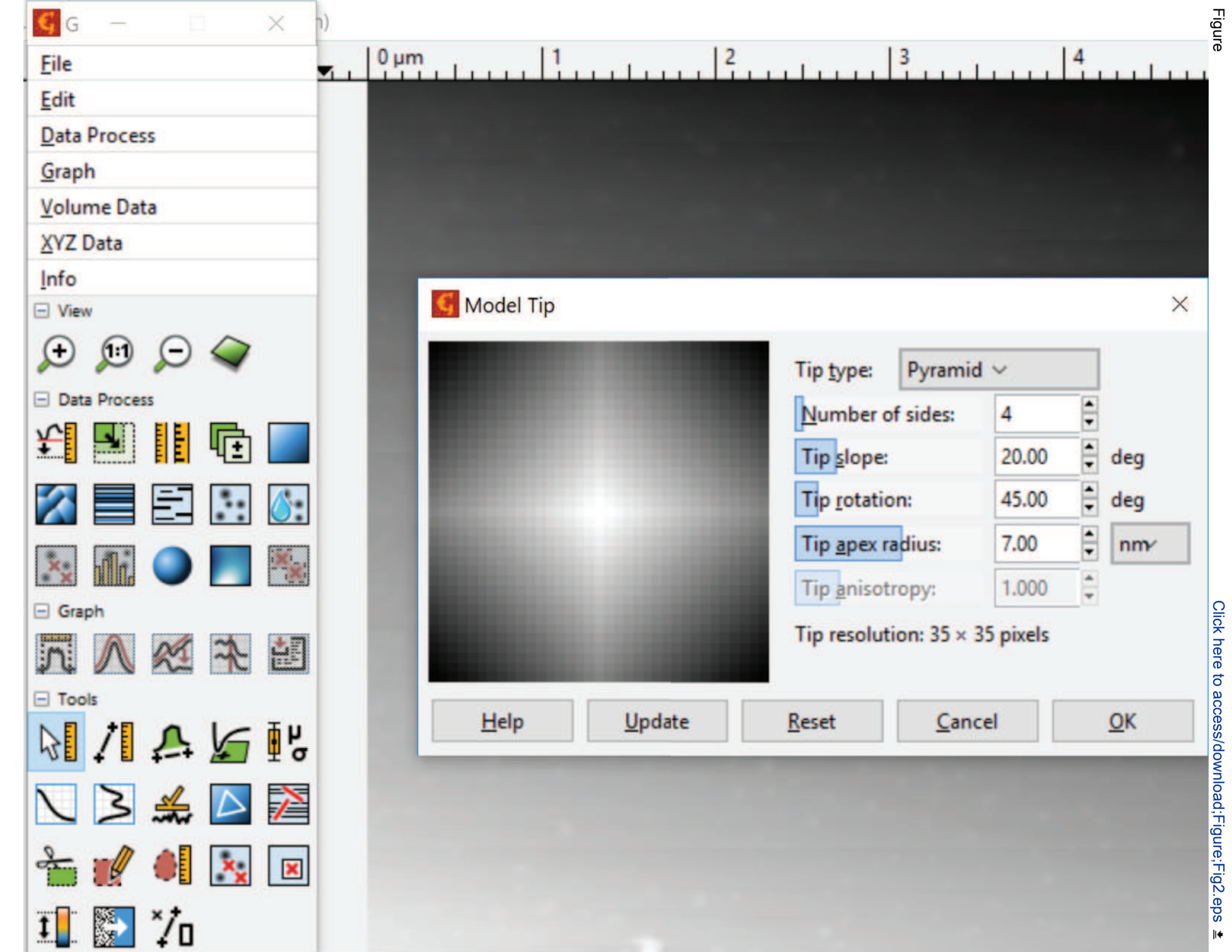
Figure





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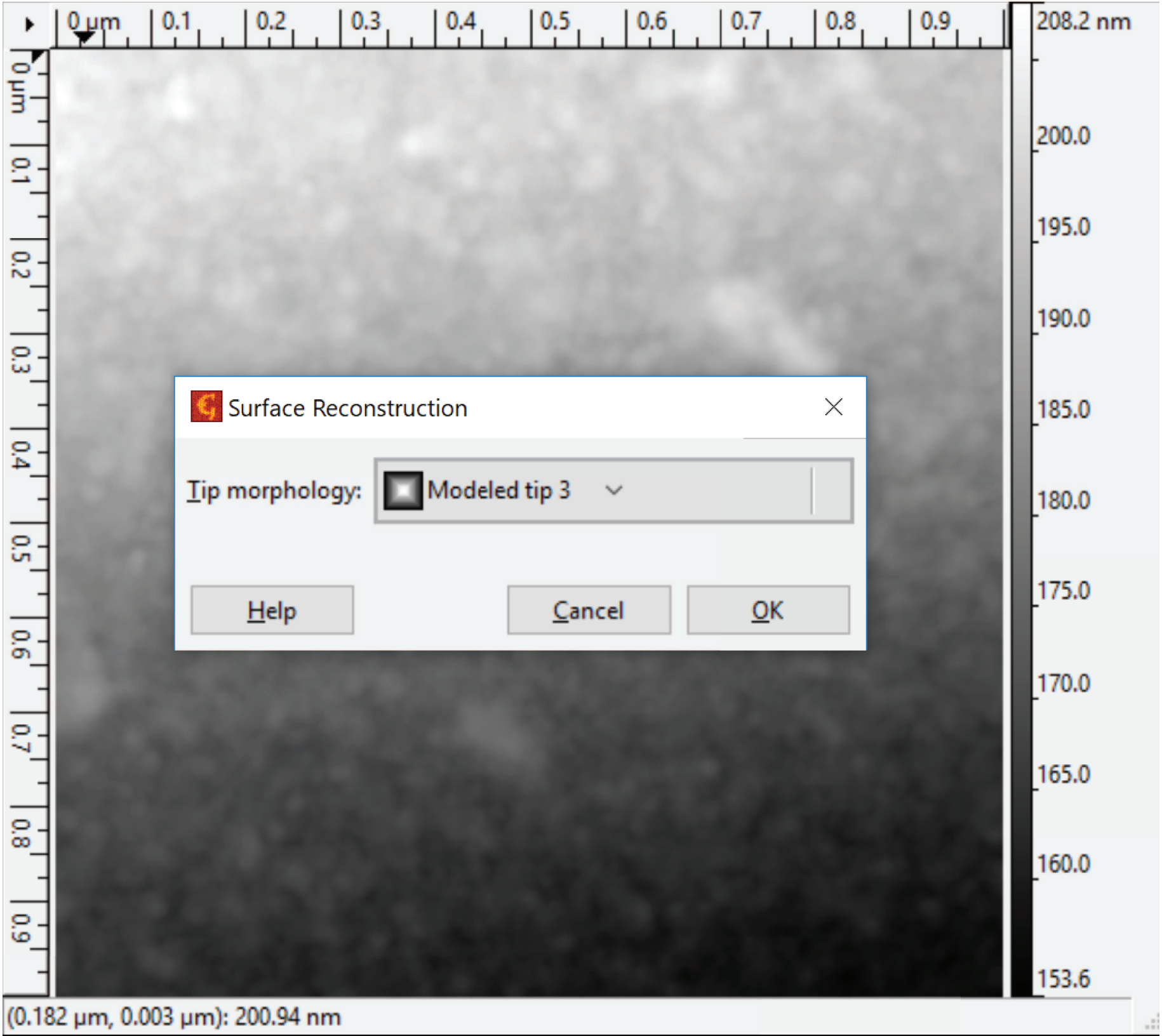

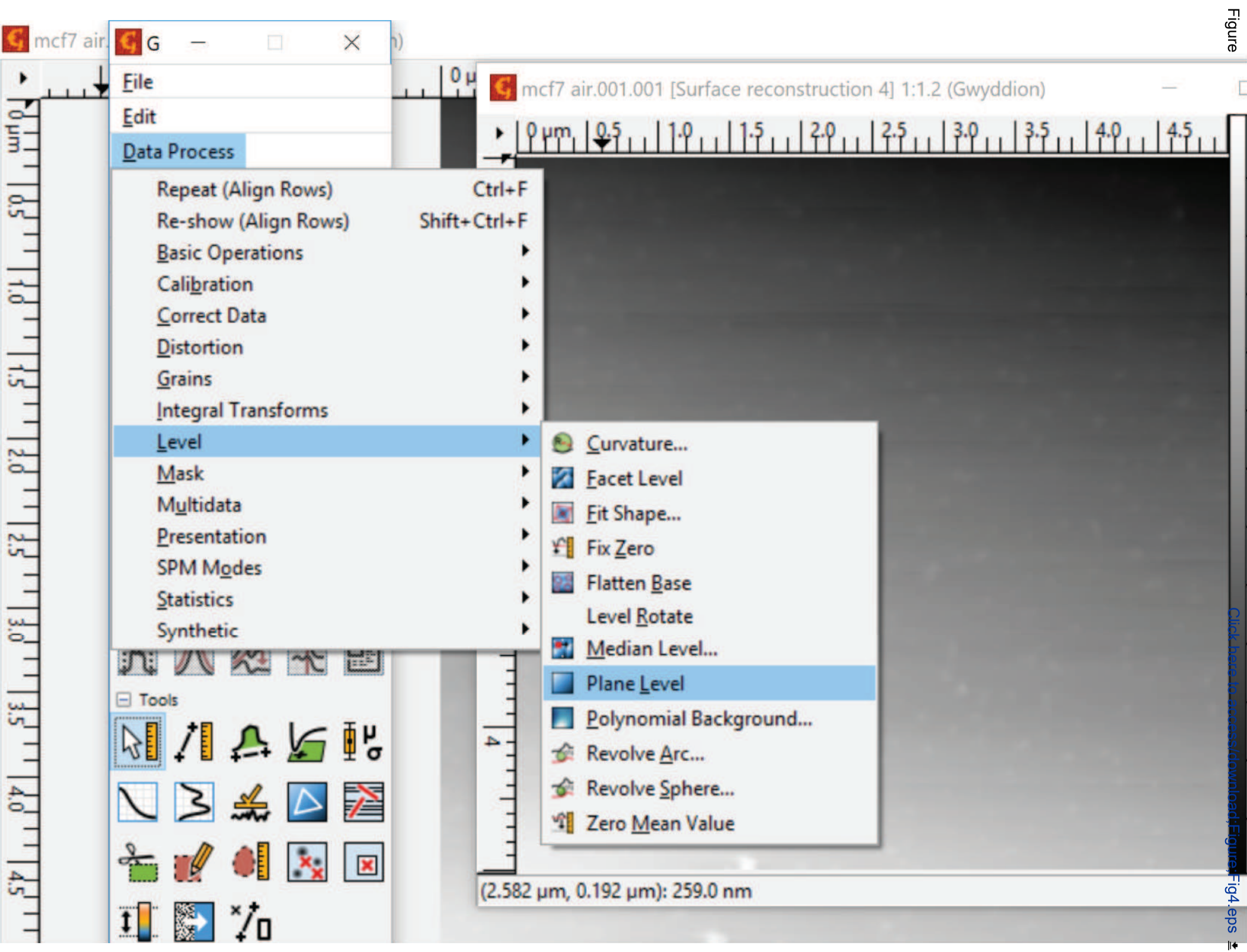

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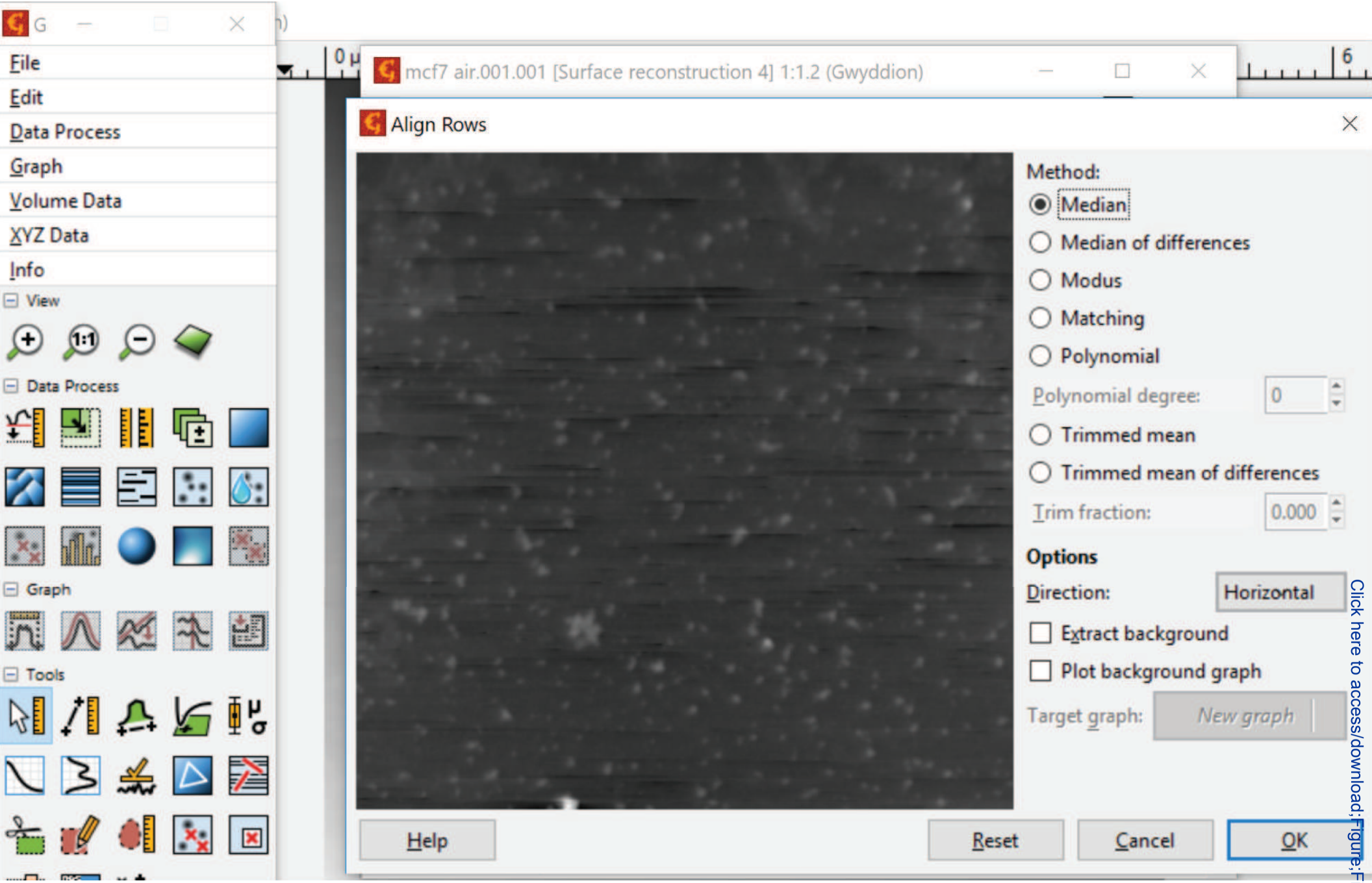

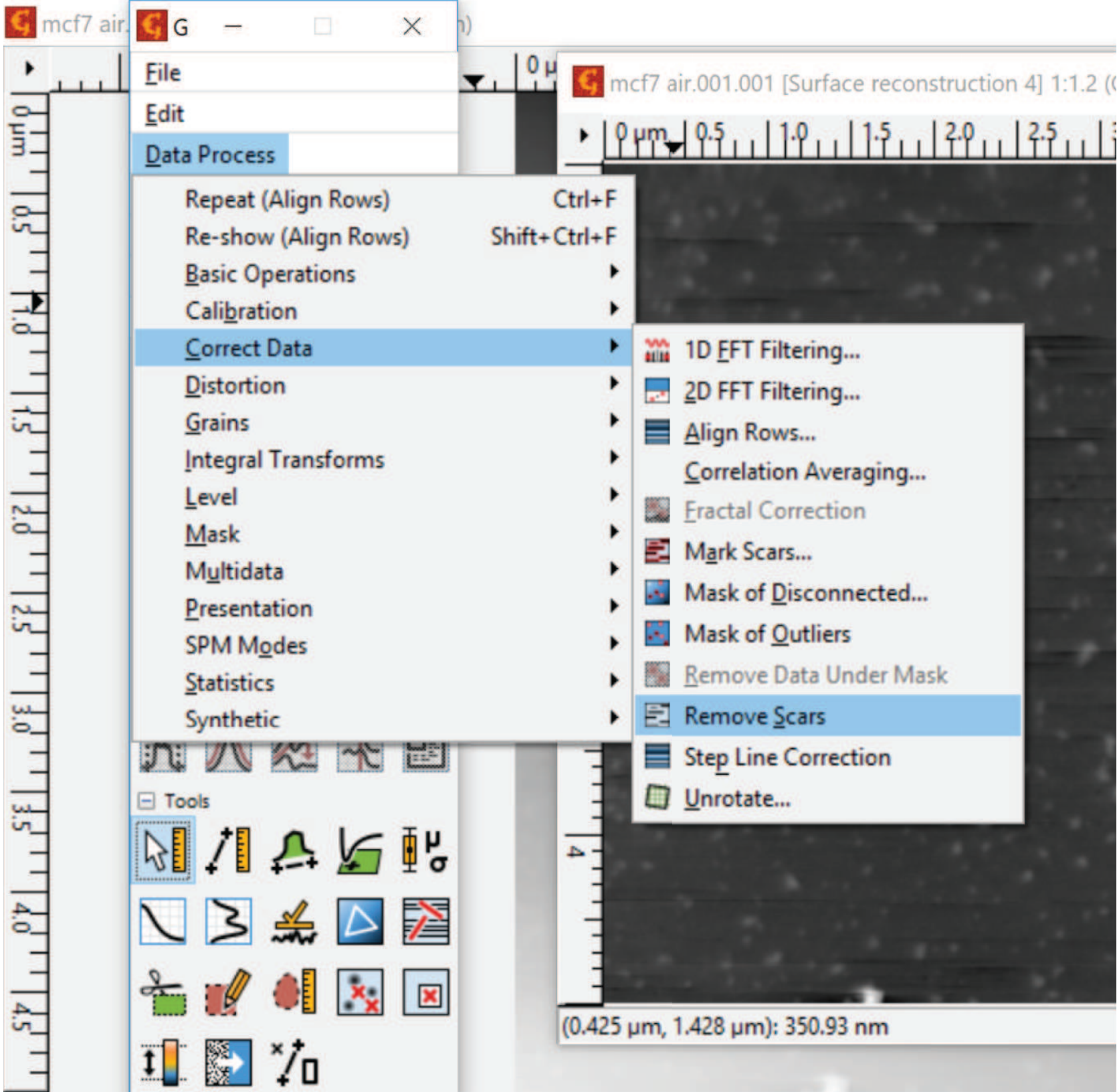

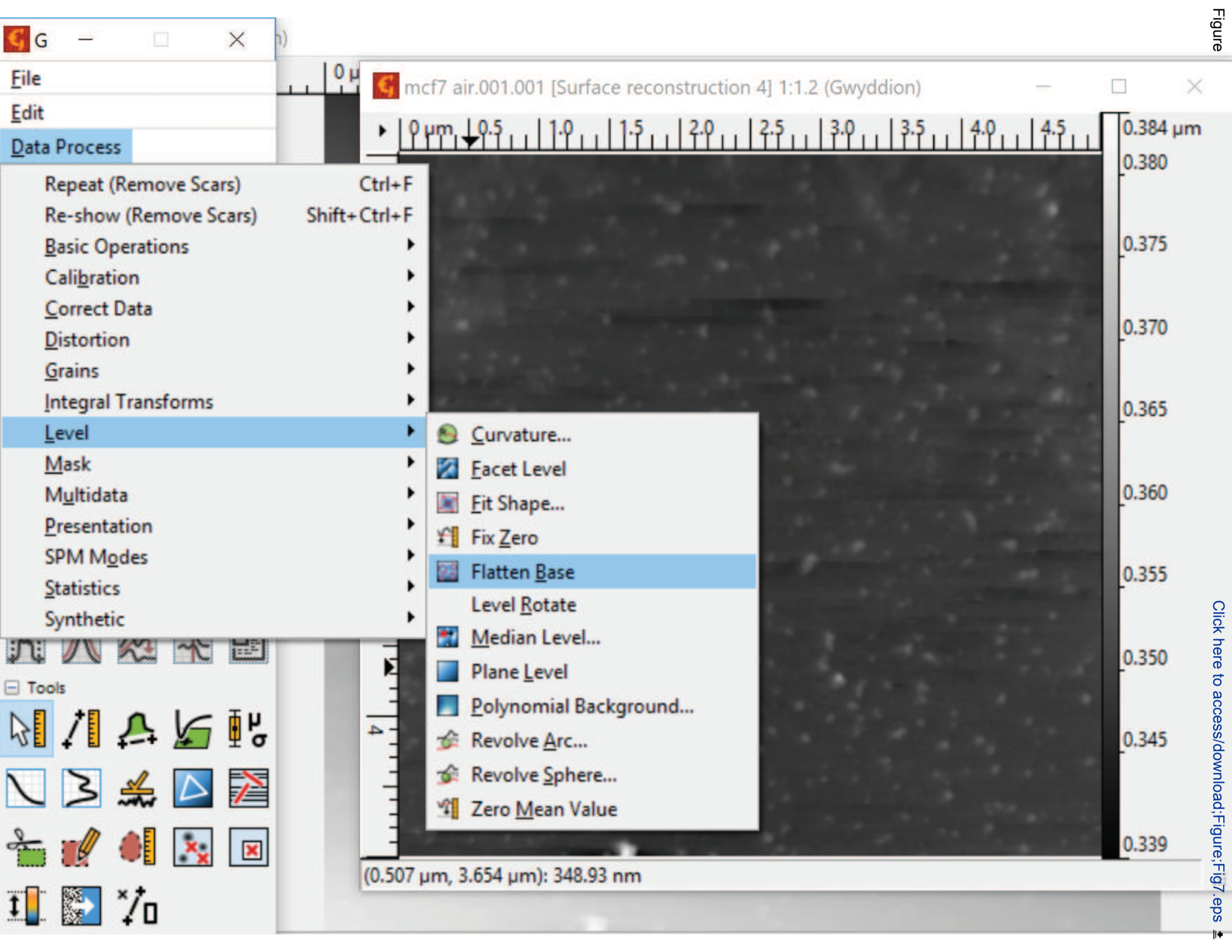

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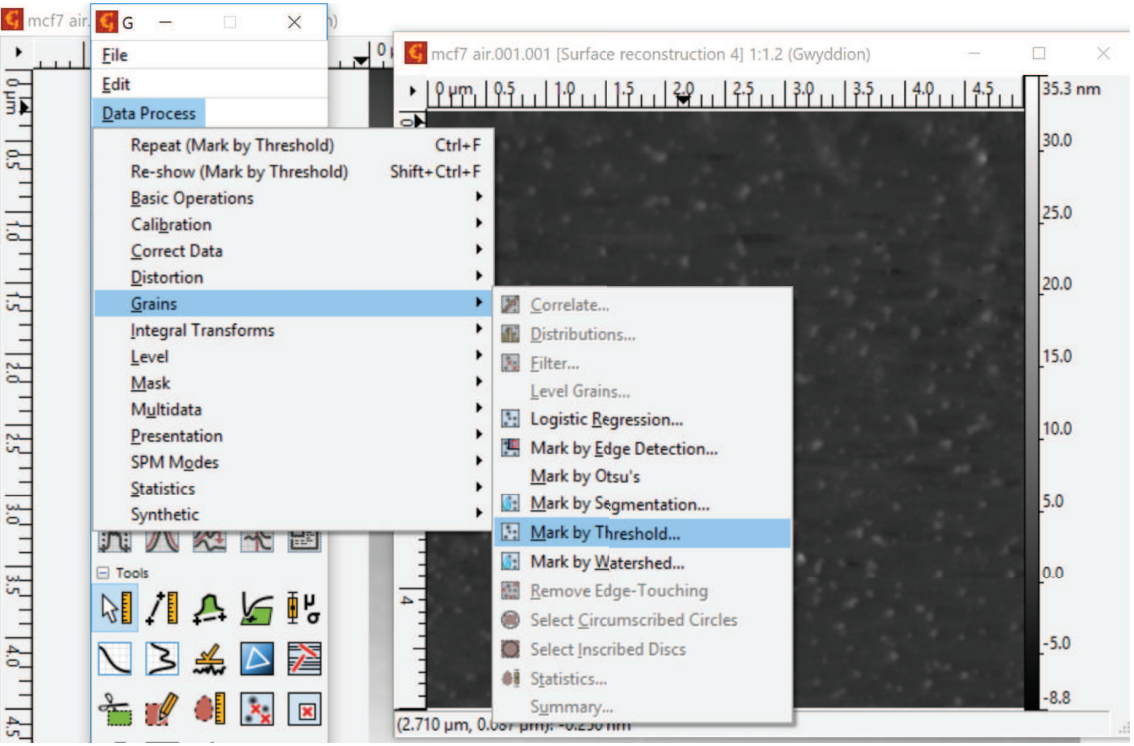
A



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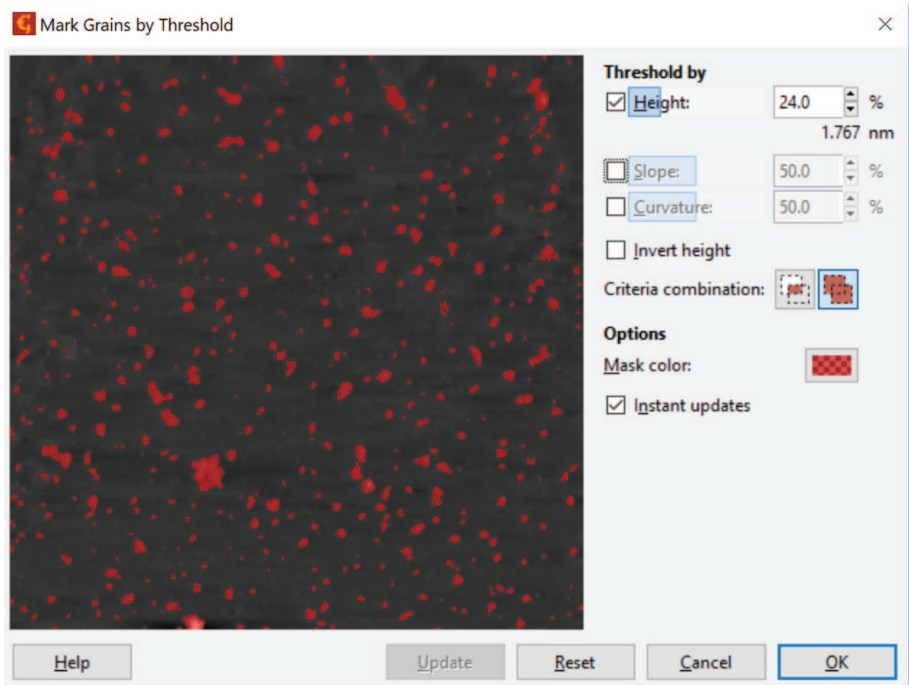

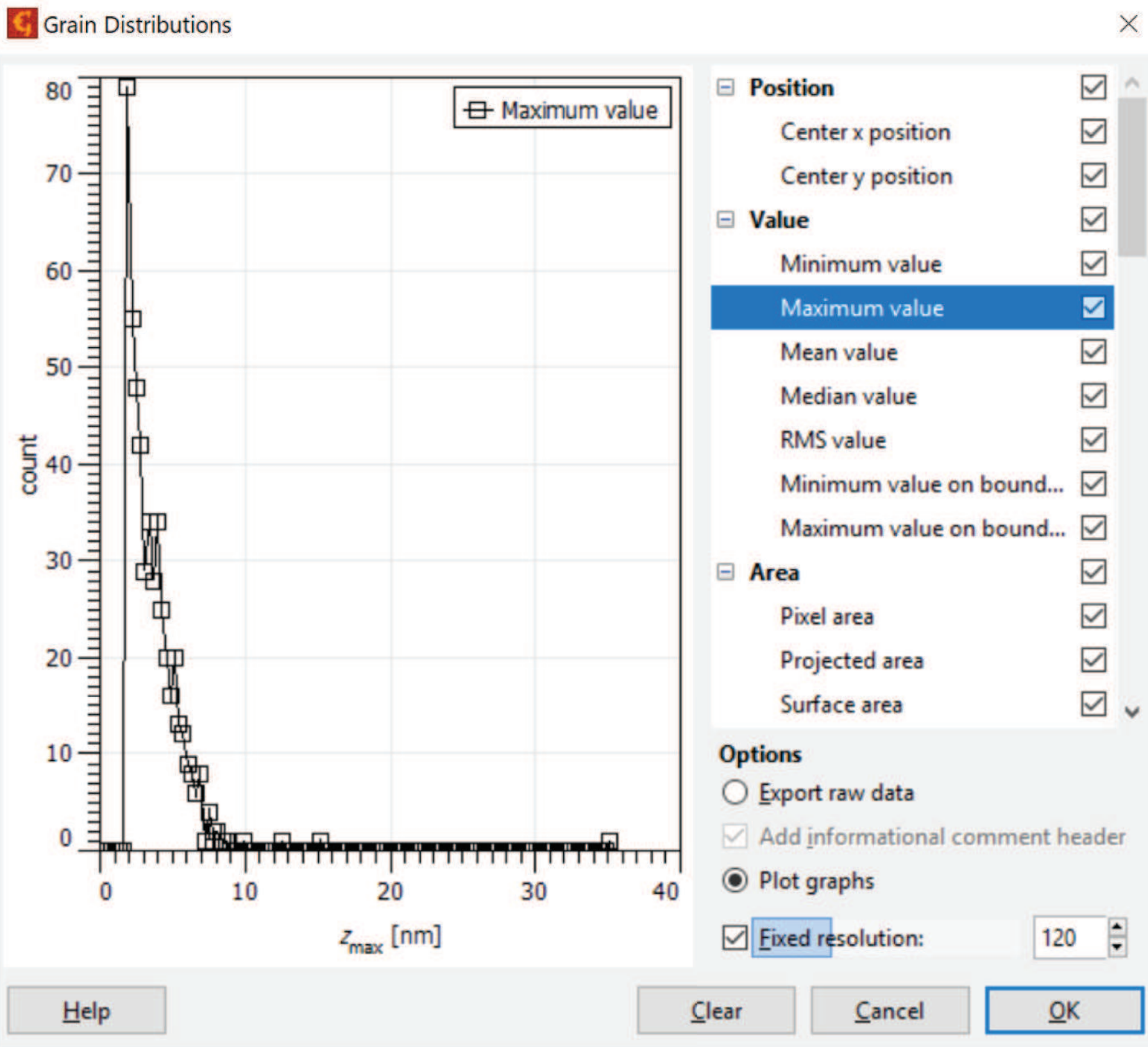

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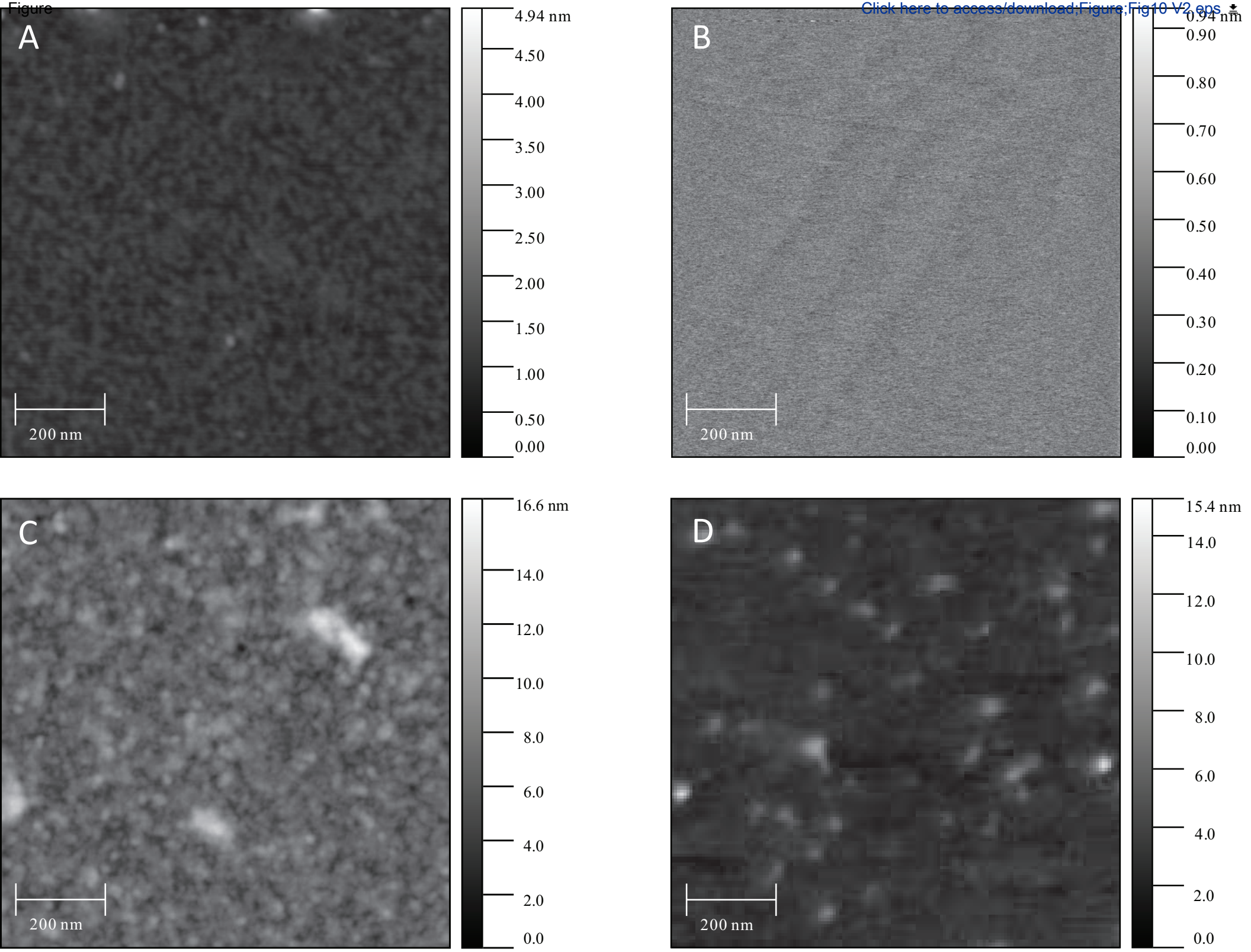

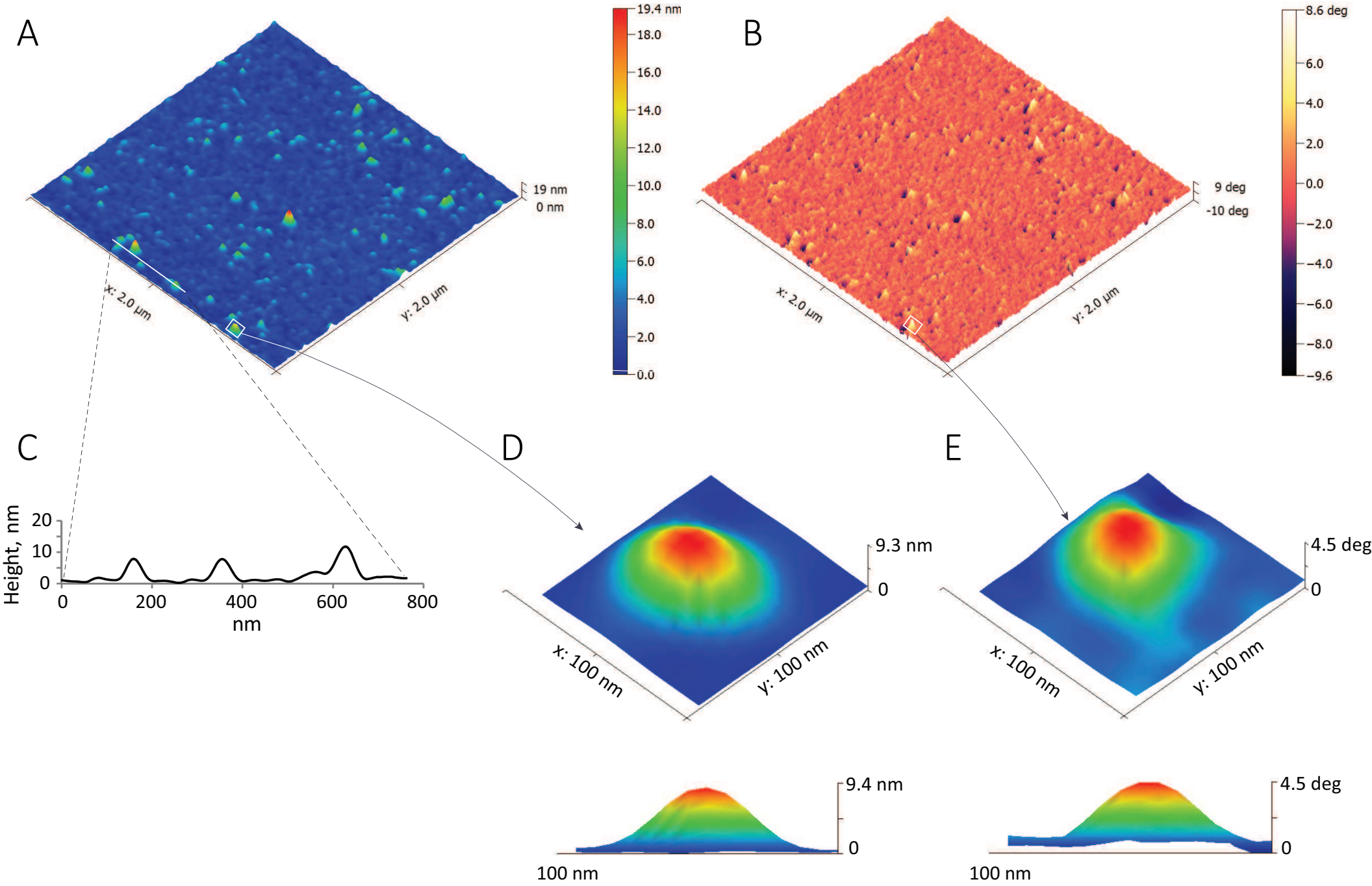

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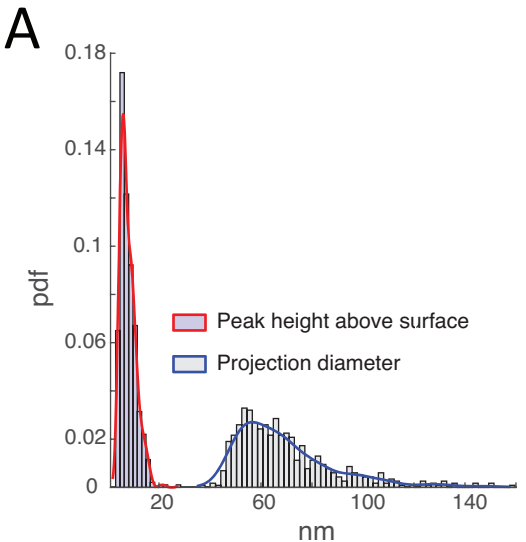

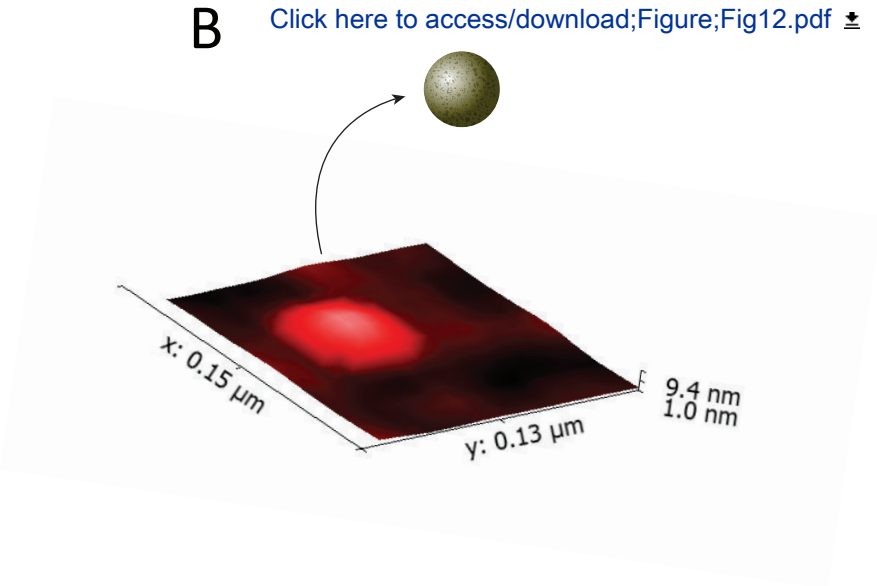

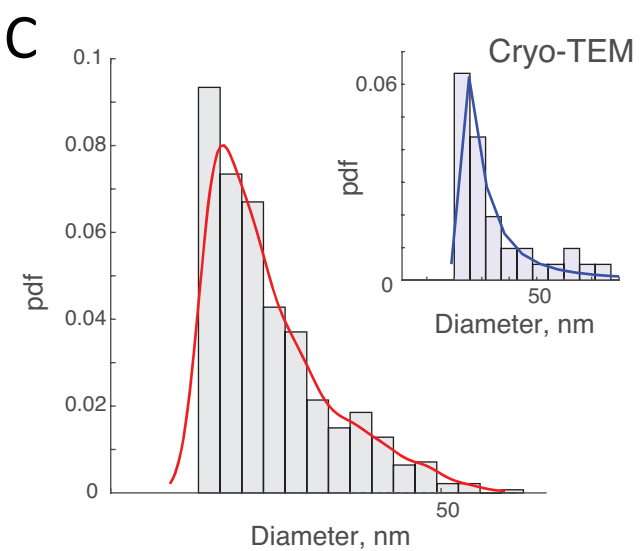

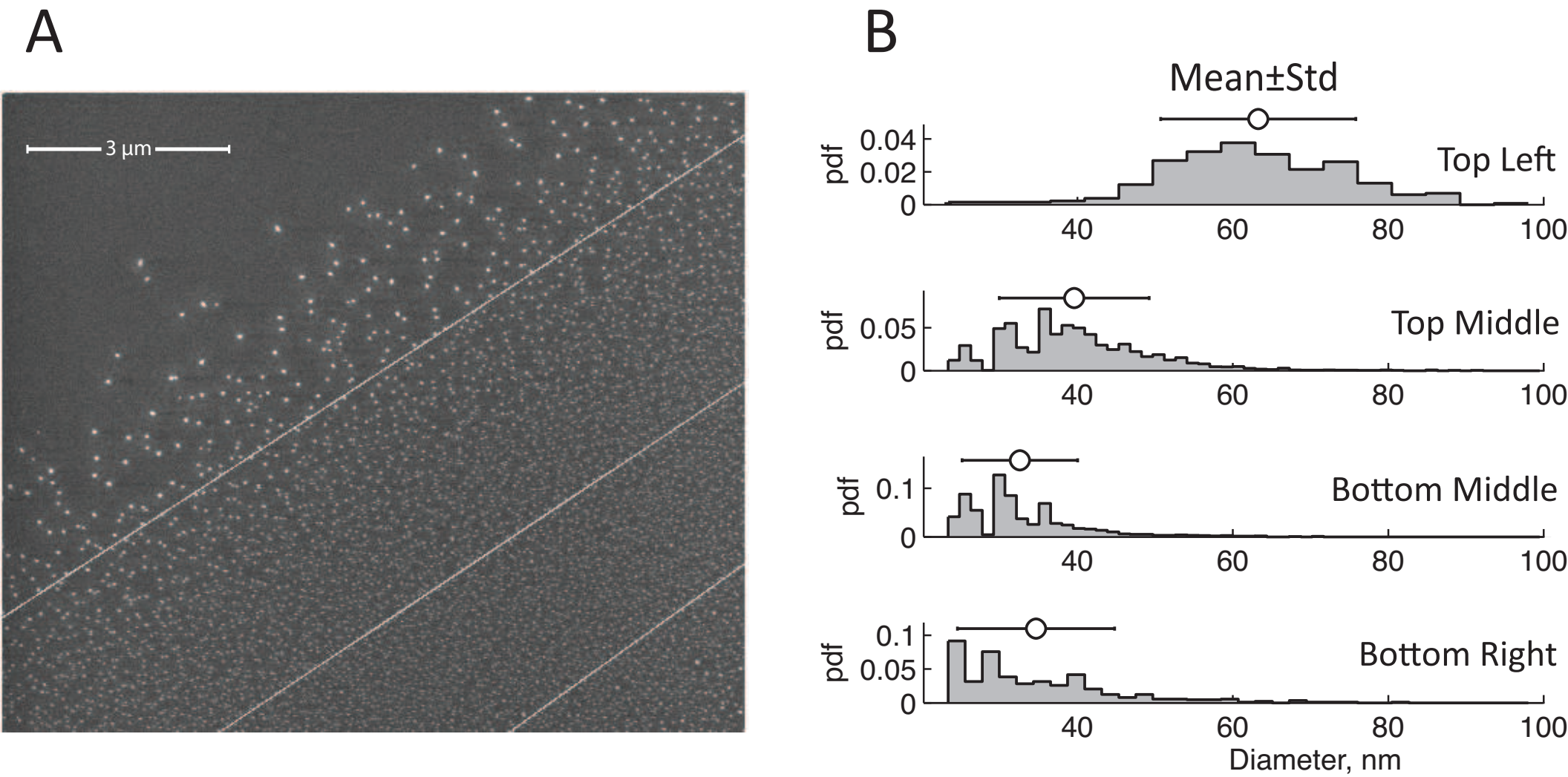

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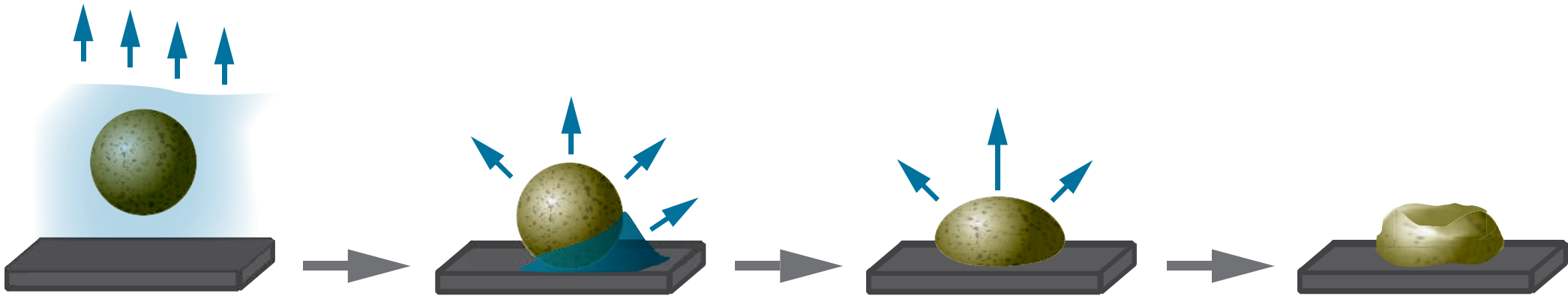

Table of Materials

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Name of Material/ Equipment	Company	Catalog Numbe	Comments/Description
AFM/STM Controller	Bruker	Multimode Na	This AFM controller supports imaging
AFM/STM metal specimen discs (1(	TedPella	16207	Metal specimen disc on which a mica (
AFM/STM Mica discs (10 mm)	TedPella	50	Highest quality grade V1 mica, 0.21mr
AFM probe for imaging in the air	Bruker	TESP-V2	High quality etched silicon probes for
AFM probe for soft sample imaging	Bruker	MLCT	Soft silicon nitride cantilevers with sili
Double sided tape	Spectrum	360-77705	Used to fix the mica disk on the metal
ExoQuick-TC	System Biosci	EXOTC50A-1	ExoQuick-TC is a proprietary polymer-
Glass probe AFM holder for imagin	Bruker	MTFML-V2	This glass probe holder is designed for
Gwyddion	Czech Metrol	Version 2.52	Open Source software for visualizatior
Lint-free blotting paper	GE Healthcare	Grade GB003	Use this blotting paper to remove NiCl
Lint-free cleanroom wipes	Texwipe	AlphaWipe TX	Use these polyester wipes for surface
Nickel(II) chloride ($NiCl_2$)	Sigma-Aldrich	339350	Powder used to make 10 mM $NiCl_2$ in
Phosphate Buffered Saline (1x)	Gibco	10010023	PBS, pH 7.4

of biological samples in liquid and air.
disk is attached by a double-sided tape or other means.
n (0.0085”) thick. Interleaved, in packages of 10. Can be mounted on AFM/STM discs. Available
tapping mode and non-contact mode for scanning in the air.
con nitride tips, which are well-suited for liquid operation. The range in force constants enable
specimen disc.
based kit designed for exosome isolation from tissue culture media.
scanning in fluid with the MultiMode AFM. The holder can be used in peak force tapping mod
n and analysis of data fields obtained by scanning probe microscopy techniques.
I_2 after the modification of the mica's substrate.
cleaning.
DI water

in four diameters

s users to image extremely soft samples in contact mode as well as high load vs distance spectr

e, contact mode, tapping mode, and force modulation. The probe is acoustically driven by a se

oscopy.

parate piezo oscillator for larger amplitude modulation. The holder is supplied with two ports,

required fittings, and accessories kit for adding and removing fluids.

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CORRESPONDING AUTHOR:

Name:	Mikhail Skliar
Department:	Chemical Engineering
Institution:	University of Utah
Article Title:	Characterization of extracellular vesicles by atomic force microscopy
Signature:	
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Dear Dr. Cao:

We have completed the revision of our manuscript (JoVE submission JoVE59254) which, following the recommendation of the 2nd reviewer, is not titled *Imaging of Extracellular Vesicles by Atomic Force Microscopy*. The permissions to re-print certain figures or their modifications have been obtained and submitted with the revision.

The detailed description of changes and the response to the comments follow. We thank you and the reviewers for the constrictive comments that helped improve the manuscript.

Sincerely,

MS and VSC

Response to editorial comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

- We have thoroughly rewritten the manuscript for clarity and completeness. We did our best to proofread it carefully, and to catch spelling errors and grammar issues.

2. Please revise lines 268-270, 272-274, 278-281, 287-297, 341-346 to avoid previously published text.

- Done.

3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

- Done.

4. Please remove the embedded figure(s) and Table of Materials from the manuscript. All figures/tables should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

- Done.

5. Figure 1: Please include a space before and after the “±” symbol.

- Done.

6. Figure 3: Please use the uppercase letters A, B, C and D for panel labels.

- Done.

7. Keywords: Please provide at least 6 keywords or phrases.

- Done.

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- Done.

9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

- Done.

10. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

- Done.

11. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

- Done.

12. Lines 94-109, 214-220: The Protocol should contain only action items that direct the reader to do something. Please either write the text in the imperative tense as if telling

someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.), or move them into the Introduction/Discussion section.

- Done.

13. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

- Done.

14. 4.2.2: Please provide the composition of precipitation solution. If it is purchased, please cite the Table of Materials.

- Cited in the ToM. An alternative protocol that does not use proprietary commercial product is now cited.

15. 4.2.4: What volume of PBS is used to re-suspend the pellet?

- Answered. See step 1.14.

16. Line 215: Please verify whether the reference numbers are correct.

- We removed these referenced from the Protocol section. They now appear in Representative Results.

17. Lines 222-252: Software must have a GUI (graphical user interface) and software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps.

- We added multiple GUI screen captures illustrating all the essential steps in data analysis.

18. Table of Materials: Please sort the items in alphabetical order according to the Name of Material/Equipment.

- Done.

Response to reviewers' comments

Reviewer #1:

Manuscript Summary:
The protocol reported by Skliar and Chernishev is dealing with the characterisation of Extracellular Vesicles by means of Atomic Force Microscopy. The paper is definitely targeting a challenging issue in the exciting field of Extracellular Vesicles characterisation. The protocol is sound and the reference to previous protocols on AFM on EVs is correctly addressed.

I've only few concerns: the authors claim that without modification of Mica surface with NiCl2 they cannot observe vesicles on the surface while measuring in air, while in several reports the unmodified mica has been used for imaging in air (es. Sharma et al ACS Nano 2010, Radeghieri et al, Biophys Biochem Res. Comms 2017, Woo et al. J Circulation Res 2016).

- We added an extensive discussion of the advantages and disadvantages of these two approaches to imaging EVs in the air. Please see Importance of Surface Immobilization in the Discussion section. We added all the references you suggested. We clarified that images A, C, and D in Figure 10 (Figure 2a, c, and d in the original manuscript) were obtained with the same sample. We also made it clear that panels A and D were acquired after the same incubation time but with and without surface modification (D and A, respectively), which illustrates that, without imparting positive surface charge, the immobilization of EVs is not effective.

Moreover looking at the image in Figure 1a we can see in the top part some bright spots that in principle could be ascribed to the sparse presence of vesicles.

- We cleaned the surface with DI water which was then removed from the surface by aspiration. However, not all liquid could be pipetted out. Those vesicles that are visible, most likely, are the result of incomplete aspiration of DI water that resuspended vesicles not fixed to the surface and then deposed them on the substrate during the evaporation. The number of vesicles is very small in absolute terms and relative to the results when mica surface was modified. Although not included in the paper a similar result was obtained when working with negatively charged liposomes. A significantly higher number of liposomes remained on modified mica after washing.

Then even though the paper is not related to novelty it is puzzling me that they are using as Figure 2 the exact figure reported in the supporting information of their paper (Skliar et al Biochem Biophys Res. Comms 501 (2018) 1055) without a proper referencing.

- Additional citations added. The expanded Discussion now clarifies the importance of many factors (charge screening, the impact of vesicle mobility on immobilization, desiccation artifacts, to mention a few) on the AFM characterization, which should be instructive to most readers.

Reviewer #2:

Manuscript Summary:
AFM is undoubtedly a powerful characterization tool for EVs. Overall, the manuscript is timely and well described.

Major Concerns:

**Abstract: "The fixation and imaging protocols described by us are simple, repeatable, and produce data for a large number of vesicles. The versatility of the AFM data provides rich information about biophysical properties for a large number of exosomes, including the size distribution and the morphology of vesicles".

Rigor and reproducibility of EV analysis is a prime concern in the biomedical field in general. The authors present no data to suggest the repeatability of the results.

- We responded to your comment by completely re-writing the paper. In the revision, we emphasize that the reproducibility of AFM imaging results primarily depends on the reproducibility of EV fixation on the substrate. We summarize this point in the abstract by stating " The representative results demonstrate that the fixation of EVs on the modified mica surface is predictable, customizable, and allows the user to obtain sizing results for a large number of vesicles." The variability in biological samples and EV isolation protocols are important issues beyond the scope of the paper, as we now clearly annunciate.

- The immobilization of the EVs on the surface is the primary source of variability in AFM data since all downstream steps (scanning and data analysis) are easily controlled by the selection of instrumentation, probes, scanning parameters, and the data analysis sequence and algorithms. The revised manuscript substantially expands the discussion of factors that influence the immobilization of EVs including the charge screening, the influence of the liquid medium, EV transport from the liquid to the substrate, and the evaporation of the sample. We added new subsections in the Discussion where we analyze potential variability in the immobilization results caused by drying artifacts (and included a new Figure 13 as the illustration), the difference in EVs' hydrodynamic mobility, and surface deformation of the vesicles produced by electrostatic attraction to the substrate and non-uniform capillary forces during desiccation (Figure 14).

- The explicitly identified causes of potential variations and the suggestions on how to reduce or account for their influence are the most important changes in the revised

manuscript. These changes are meant to help the users to obtain reproducible AFM characterization results for any given sample and provide the guidance in understanding the likely sources when variations do occur.

- We now point in the Representative Results that the same MCF-7 sample was reanalyzed three times, from sample preparation to imaging. Each repeat produced statistically similar results to those shown in Figure 12.

Also, can they clarify approximate numbers of EVs amenable to AFM imaging. Both EM and AFM are typically used as qualitative methods to probe EV structures, so this would be helpful for the readers to know.

- In the Discussion section, we now explicitly state that " The number of vesicles amenable to AFM imaging scales with the imaged surface area and the surface concentration of the vesicles immobilized on the substrate." We now include an expanded discussion of factors controlling the density of the immobilized EVs on the modified mica's surface. A dedicated subsection on Control of Surface Density of Immobilized EVs is now included in the Discussion. We now explicitly state that "A high density of immobilized vesicles... increases the number of vesicles analyzed during the scanning and the statistical power of conclusions arrived by the analysis of the AFM data," caution the user that "an excessively dense surface concentration, as in the case shown in Fig. 10C where particles tightly cover the entire surface with no intervening areas of the substrate, complicates the image analysis and the interpretation of results and may lead to scanning artifacts caused by the interaction between closely spaced particles." We explicitly describe the reason why it is not desirable to have concertation of EVs as dense as in Figure 10C; from the Representative Results section: "A number of algorithms rely on having sufficient unoccupied substrate between the grains to perform image correction and data analysis. For example, the leveling and shifting the substrate to the zero plane, the line correction, and the estimation of the grains' volume need the intervening flat surface to perform accurate calculations." We provide the suggested liquid concentration of EVs and sample incubation times to obtain adequate surface concentration of immobilized EVs. We also discuss factors that allow the user to customize the surface concentration of the immobilized EVs on the modified mica substrate to meet the goals of a study.

**Use a double-sided tape to attach a mica disk (10 mm diameter, Ted Pella Inc., Redding, CA) to the AFM/STM metal specimen stage.
Double sided tape will drift and create issues when imaging, particularly so for fluid imaging?

- We had no adverse issues with using a particular laboratory-grade double-sided tape (listed in Table of Materials). It held well with no drift in the results. The tape was placed on the sample holder, and mica (not yet cut) was firmly pushed down to get

good bonding. The sessile drop of PBS on mica's surface was small enough and never touched the tape.

- Nevertheless, to address your concern and to caution a user, we now state in the Protocol: " Use strong double-sided tape, epoxy, or an alternative adhesive to firmly attach a mica disk to the AFM/STM magnetic stainless steel specimen disk."

**Mica is a layered silicate mineral with general formula K(Al2)Si3Al)O10(OH)2 that naturally has a negatively charged surface and a "near perfectly flat surface" ideal for imaging nanoparticles by the AFM.
Can the authors provide quantitative assessment of the flat surface, eg. surface roughness, and why it matters for imaging EVs.

- The following Figure 1 shows the roughness of our sample was comparable with the previously published results (Figure 2 taken from DOI: 10.1021/am100697z). In Representative Results, we added the following quantification of flatness of the modified surface: " The roughness of cation-derivatized surface was below 0.3 nm, which is consistent with the previous report[13]." However, because mica is such a common and widely used substrate in AFM imaging of biological samples, we did not add further details in the paper but will do so if advised.

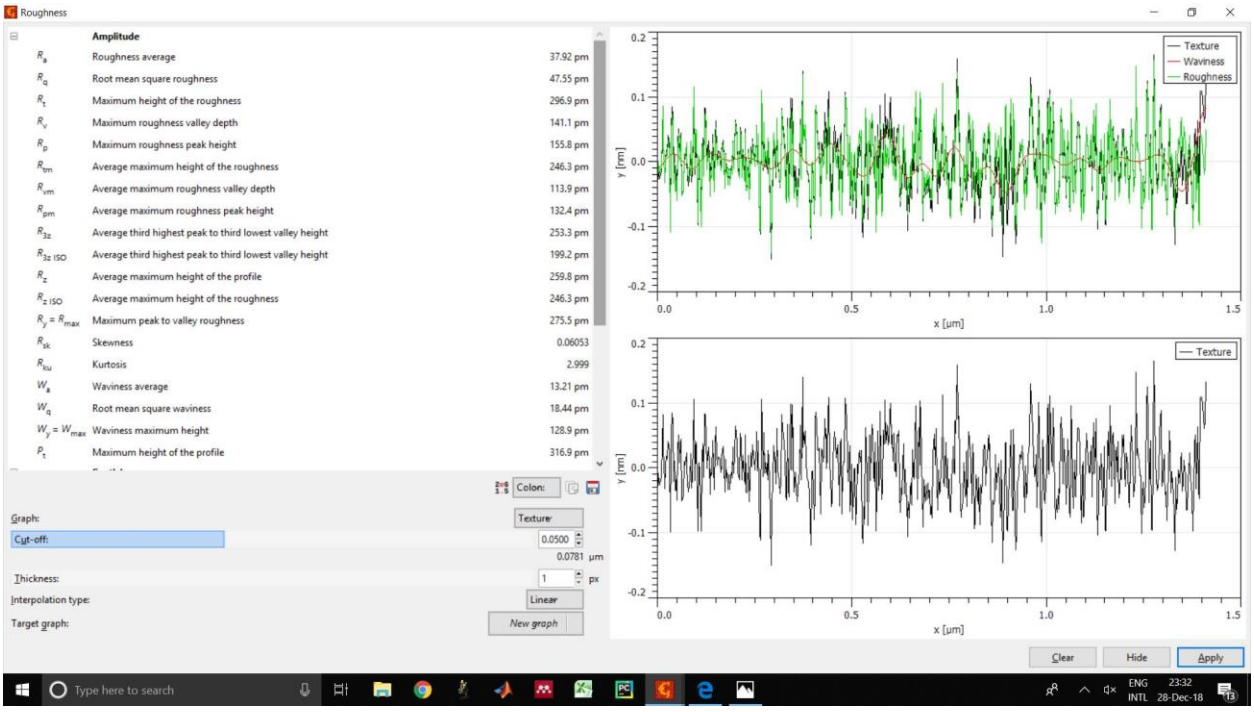

Figure 1: Surface roughness of the modified mica used in our study.

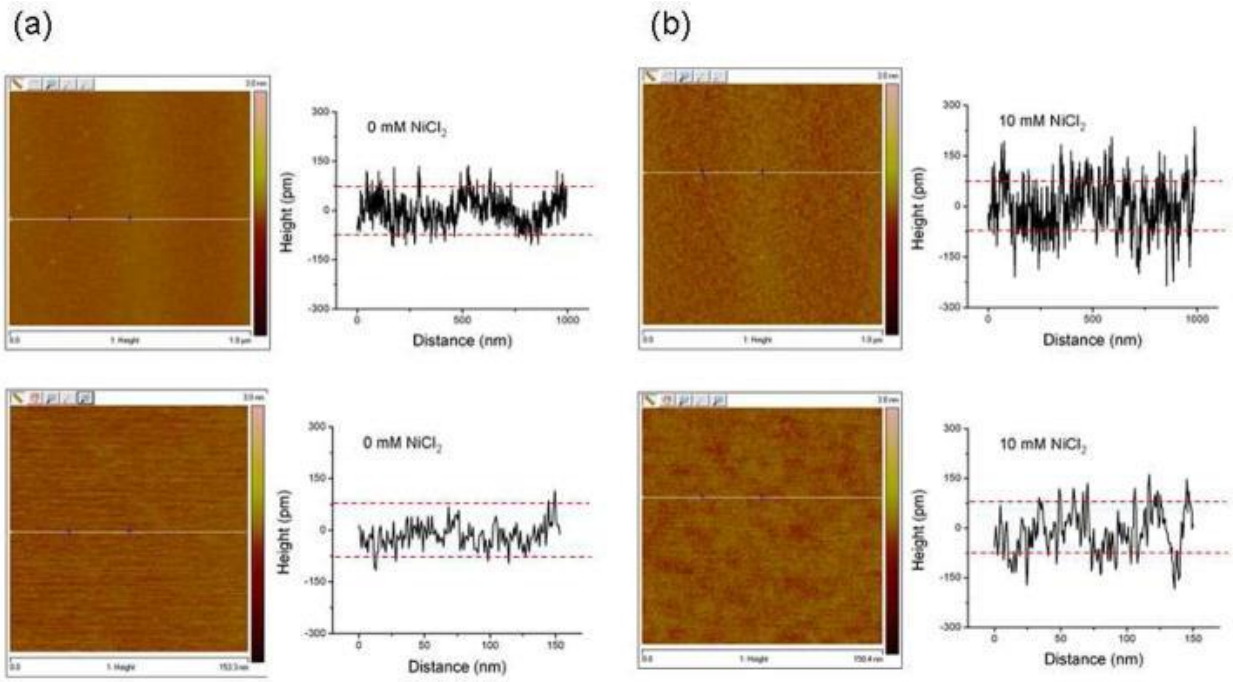

Figure 2: Surface roughness reported in DOI: 10.1021/am100697z

**2.Cleave mica disc by using a sharp razor or utility knife. An initial cut on the side of the mica disc will cause a thin layer of the material to easily detach. Remove the cleaved layer. The remaining thin layer must still be firmly attached to the AFM/STM metal specimen disc.
Peeling with double-sided tape should suffice?

- We added this option to the Protocol.

** for AFM imaging, the scan rate and approximate time it takes to acquire a 5X5 micron image of certain resolution must be included.

- We include the following Note in the Protocol which will allow users to estimate the time it would take to image the selected surface area (# of lines/rate). We also give suggestions on the selection of default values.

Note: The scan time will increase will the imaged area (5×5 µm being a common choice), the number of lines selected to form the image (512 lines are often acquired), and the scan rate defined as the number of lines scanned per second. The scan rate is often set to ~1 Hz. Fast scan rates may impact the image quality. Therefore, the speed of rastering should judicially balance the tradeoff between the acquisition time and the image quality.

** 24 hours of EV incubation the surface seems longer than most protocols?

- Figure 10C, obtained after 24 h incubation, shows a surface that is covered with an excessively large number of exosomes. So, this was an example of what not to do.

- More generally, we do think that longer incubations are important to avoid the fixation bias. This issue is addressed in new subsection in the Discussion titled Immobilized Vesicles May Represent a Liquid Sample with a Bias, which states: "For the case when the rate of immobilization is limited by diffusion, vesicles with smaller hydrodynamic sizes, determined by the combination of the vesicle size and the thickness of the coronal layer surrounding it (Figure 1), are more likely to enter the attraction layer because of their higher mobility. Consequently, after the initial depletion period, the hydrodynamically small EVs will be overrepresented on the substrate compared to their contribution to the EV population in the liquid sample. Note that a smaller hydrodynamic size does not automatically point at EVs with smaller vesicle sizes because of the heterogeneity in the thickness of the coronal layer[3]. The biased representation is avoided with long incubations that deplete the entire population of EVs in the liquid by its immobilization on the substrate."

** Can authors suggest a dilution expt to optimize the time and concentration for EV imaging, for greater efficiency of imaging.

- We added a subsection in the Discussion on Control of Surface Density of Immobilized EVs. We also added the recommendation to use the NTA to confirm that the desired liquid concentration of EVs was attained after the dilution.

**Fig 2. It would be helpful to show baseline zero height for all panels for easy comparison. Is Fg 2B a height or amplitude image? Fig 2(C) The AFM scan of the surface after an excessively long 24-hours exposure to an exosome sample shows a surface concentration of immobilized vesicles that is too high for an accurate analysis. The features are all aligned ~45degree angle in 2(C). Is that draft or directional effect of washing the surface?

- We clarified in the text of the manuscript that all images in Figure 10 (old Figure 2) are height images. We agree that there is a drift in the old Fig. 2C. We have replaced the old image with a scan of the same sample without a drift. The height images now show Z=0 as a baseline.

**Fig 3D. Can the authors include a phase image of the same particle for comparison, to show the complementarity of height and phase images of EVs?

- Done. See updated Figure 11.

The authors should also discuss the limitations of AFM imaging and comparisons to EM. For instance, time, surface scanning features, loss of particles that fail to adhere to

the surface.

- We now discuss in more details the issues related to surface deformation of EVs due to electrostatic immobilization -- an issue not present in cryo-TEM results. We also added a discussion of desiccation artifacts (illustrated in new Figures 13 and 14) that do not affect cryo-TEM (they can affect SEM results, though!) or when hydrated samples are imaged by AFM. We now include the direct comparison of the exosome sizing results based on cryo-TEM images and AFM data (Figure 12C) to show their consistency. The discussion related to the potential difference between the population of immobilized EVs and EVs in the solution was also included during the revision.

**A useful resource on AFM and EM imaging of vesicles from researchers in the imaging and characterization of EVs, is covered in the MOOC "Basics of Extracellular vesicles" and should be available as a reference to interested readers.

- The field of Extracellular Vesicles has become so active that it is difficult to recommend a single review of the subject to a beginner. Fortunately, our audience are EV researchers interested in the adoption of AFM in their projects, which is why we avoided citing review papers, textbooks, and course material.

Minor Concerns:

**Given the scope of the characterization of EVs discussed in the current manuscript, it would be rather appropriate to use change the title to Imaging of EVs by AFM.

- Thank you for an excellent suggestion which has now been adopted.

**Typos and grammar
After the incubation, remove 80-90% of sample from the surface by aspiration. If the imaging of the hydrated exosomes is desired, was rinse the surface 4 times with 1x PBS, making sure that the sample remains hydrated throughout the process.

- This part of the protocol was rewritten.

Reviewer #3:

Comments:

Line 150: Double sided tape is inherently a bad idea for any AFM samples - often times the tape thermally expands/contracts leading to drift that can be devastating for experiments characterizing exosomes on the scale of tens of nanometers. Epoxy or some other adhesive is much better.

- We added a comment to the protocol that notes the need to thermally equilibrate the specimen after the incubation and before scanning. We also added the suggested

alternatives to a double-sided tape when attaching mica disk to the AFM/STM magnetic specimen disk.

Line 155: Why wouldn't you use tape to cleave the mica? Much simpler and less room for error.

- We added this option to the Protocol.

Line 162: you should specify blot the mica puck with some lint-free wipes (kimtech is actually awful in my experience, I would suggest a knit cloth like alpha wipes, or anything suitable for a class 100 clean room - even this can be risky for introducing particulates to your substrate).

- We added the need to use lint-free blotting paper when blotting NiCl2 from mica's surface. We added options for lint-free wipes and lint-free blotting paper to Table of Materials.

Line 164: please clarify/rewrite "Place the AFM specimen stage with the surface-modified mica in a petri dish". Are we putting the Mica puck into a petri dish, and then putting that into the AFM stage? If so, later is step 3 you say incubate at 4C, which almost no AFMs have the capability to control their temperature that low.

- It should have said "specimen disk." Corrected. We now refer to it as "magnetic specimen disk" to clarify the mechanism of its attachment to the AFM stage.

Line 176: I assume the 1X PBS is with Mg & Ca ions?

- We use PBS (listed in ToM) manufactured without Calcium and Magnesium.

Line 187: Even fresh DDW on freshly cleaved mica can leave reside/particulates when air drying in lieu of quicker nitrogen drying - why do you suggest this?

- We do use and recommend drying in a stream of dry nitrogen. The protocol is now corrected.

Line 301: please list the detailed results of the AFM size vs cryo-TEM size analysis including mean, standard deviation, and number of exosomes measured for each data set in a table.

- We added the comparison of AFM and cryo-TEM sizing results as Figure 12C. Additional details are given in the cited reference (DOI: 10.1016/j.bbrc.2018.05.107) and its Supplemental Information.

Figure 2:
* Please clarify - is this done in air (i.e. DI wash then dry) or liquid? It seems like there may be some exosomes on the very top - please confirm that this control was done in

the exact same manner as the NaCl2 runs (i.e. same density of exosomes incubated with the same conditions/time/rinse steps).

- All scans shown in Figure 10 (old Figure 2) were performed in the air, as we now clarify in the manuscript. The same sample was used in scans A, C, and D.

- As mentioned in our response to the 1st reviewer, we cleaned the surface with DI water which was then removed from the surface by aspiration. However, not all water could be pipetted out after washing. Those visible vesicles are, most likely, the result of incomplete aspiration of DI water that resuspended vesicles not fixed to the surface and then deposited them on the substrate during the evaporated.

* 2A seems to be very rough compared to 2B, given that the Z scales of the two are nearly an order of magnitude different. Is it due to contaminants/precipitates left over from the exosome incubation (see comment below about SBI products), or other debris?

- The difference in roughness is too small to be seen by eye in the images. But, since you asked, (A) is, indeed, rougher than (B) (400-700 pm roughness vs. ~350 pm for mica modified with NiCl2). The likely cause is a more complex composition of a bio-sample, which contain proteins, peptides, and amino acids found in growth medium (positively charged residues would be attracted to the negatively charged surface of mica). Some PEG (the active ingredient in ExoQuick, as far as I can tell) may contribute to an increased roughness, but likely less than proteins because of smaller MW.

* Please make the z-scales in each 2A, 2C, and 2D the same range for a better comparison of the three cases (I think the lower scale on 2B makes sense given how smooth the surface is).

- The z-scale was changed.

In general:
While I understand you may not have the capability at your facilities/lab, PeakForce Tapping AFM by Bruker is truly the best suited AFM modality for delicate samples in fluid. This could easily be addressed in the discussion section.

- Thank you. Noted.

ExoQuick TC by SBI is known to contain polymeric precipitates from their proprietary process/product, which is rather frustrating. How does this method give confidence in the measured topography is due to solely that of exosomes vs. polymeric precipitates? It seems as if they could be present in Figures 2 & 3. This should also be addressed in the discussion section.

- The discussion of the EV isolation is the topic beyond the scope of this paper. This hotly debated subject has plenty of opinionated opponents and proponents of every EV enrichment option (usually with solid justifications!). A small increase in surface roughness in the scan shown in Figure 10A (old 2A), incubated with the biological sample containing leftover PEG, proteins and other molecular mêlée from the growth medium, points to a minimal contribution of precipitants to the AFM results even when a desiccated sample is imaged.

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