



Particle Size analysis of nanomaterials using ImageJ/Fiji

Standard Protocol for Image processing.

V1.1

This document outlines step-by-step instructions for semi-automated particle size analysis of nanoparticles and other materials from digital electron micrographs. It is important to ensure the highest image quality in terms of particle resolution and focus. For improved accuracy in determining particle size, select a magnification/image resolution combination that provides a minimum of 10 pixels per particle. At least 500 particles should be analyzed to ensure adequate statistical confidence.

In terms of image content, the particles to be measured should ideally be isolated to minimize errors introduced by aggregates or agglomerates. For more information on how to improve sample dispersion and minimize sample preparation artifacts, please visit www.dunesciences.com to learn about our **SMART Grids™** product line for enhanced specimen preparation.

Images should be saved in high resolution TIFF format. Alternately, plug-ins are available for IMAGEJ or Fiji that can accept digital micrograph (.DM3) files or TIA (.SER) files.

Note: **ImageJ** is a public domain, image processing program developed at the National Institutes of Health. **Fiji** is also a public domain image processing software that can be described as a distribution of ImageJ together with Java, Java 3D, and numerous plug-ins. The following protocol will work with either ImageJ or Fiji.

PROTOCOL:

1. Download and install **ImageJ** <http://rsbweb.nih.gov/ij/download.html> or **Fiji** <http://pacific.mpi-cbg.de/wiki/index.php/Downloads> following instructions (if needed). There is no charge.
2. **Open ImageJ:**
From the menu bar, select: **File> Open**
browse for filename. Select sample image file.
3. **Set measurement scale:** Use either the scale bar from TEM or other measurement of pixel size (e.g. direct from TEM). If setting the scale using scale bar, use the 'Magnifying glass' tool button below the menu bar to zoom in on the scale bar. Select the 'Straight line' tool button located below the menu bar and draw a line the length of the scale bar. This line length is reported in pixels. In some cases the file format will transfer the dimensions with image.

Note: If the scale bar begins and ends with vertical tick marks, begin and end at the same point in the x- and y-direction at each endpoint (e.g., right edge of beginning tick mark to right edge of ending tick mark).



From the menu bar, select: **Analyze> Set Scale...**:

The field 'Distance in pixels' will be filled automatically by the software

Enter the value of the scale bar in the field 'Known distance'

Enter the unit of the scale bar in the field 'Unit of Length' (e.g. nm)

Click ok

From the menu bar, select: **Image> Zoom> Original scale** to return zoom back out.

- 4. Crop image:** Select the 'Rectangle tool' button from below the menu bar. Outline desired area for sizing particles with rectangle thereby cropping away scale bars or other image artifacts that might affect contrast or particle analysis.

From the menu bar, select: **Image> Crop**.

From the menu bar, select: **Image> Zoom> Original scale** to return zoom back out

Re-save file using a different filename (e.g. RM8011_f1.tiff).

- 5. Adjust image brightness.**

From the menu bar, select: **Image> Adjust> Brightness/Contrast...**

Press the 'Auto' button

Press the 'Apply' button

Note: brightness and contrast can be manually selected.

SAVE processed image as a TIFF file.

- 6. Threshold particles:** Thresholding removes unwanted background information, leaving behind only the particle themselves.

From the menu bar, select: **Image> Adjust> Threshold...** to open the dialog box (Fig. 1A).

Use the 'Threshold' function to automatically or interactively set lower and upper threshold values, segmenting the image (Fig. 1B) into features of interest (particles) and background. Depending on the particle size and magnification, it can be helpful to use the zoom tool to set threshold more precisely to the particle edges.

While adjusting, the thresholded features are displayed in red and background is displayed in grayscale (Fig. 1C).

When you select "set" or "apply", the background is removed (becomes white) leaving only the black images of the particles (Fig. 1D).

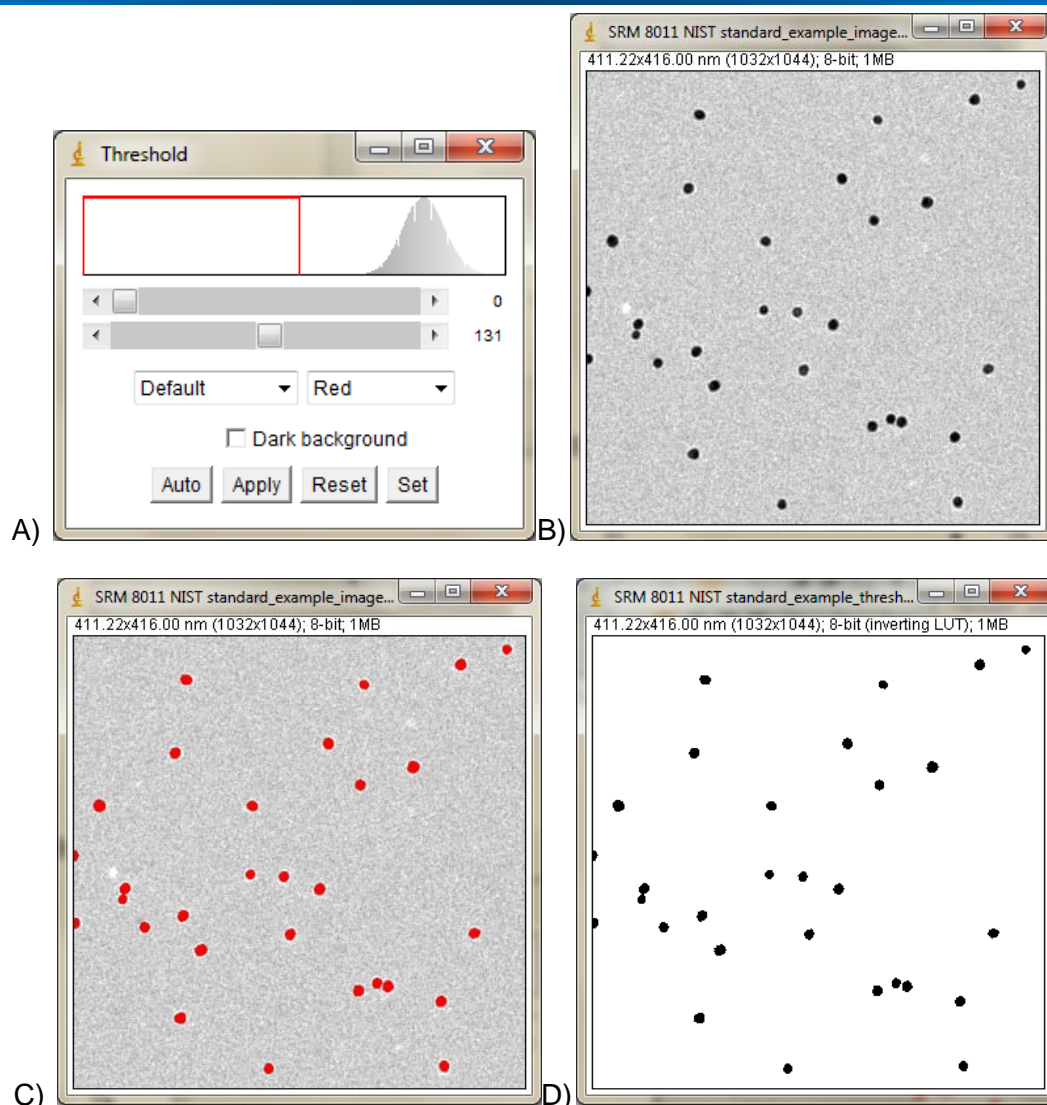


Fig. 1. ImageJ 'Thresholding' function that segments the background from the features of interest. A) Threshold dialog box. B) Image of gold nanoparticles prior to thresholding. C) Threshold adjustment to match particles. D) Final binary threshold image showing black particles on white background.

Note: In some cases, it may be difficult to completely separate the background from the particles of interest. In this case, you can get it close to the correct threshold value and then despeckle or erode followed by dilate the image. These functions will remove any single pixel particles that are not of interest.

Despeckle: From the menu bar, select: **Process>Noise>Despeckle**



Erode/Dilate: From the menu bar, select: **Process> Binary> Erode**, followed by **Process> Binary> Dilate**

Either of these methods work, but one may work better than the other depending on the image quality. Be sure to *overlay* the final thresholded image with the original image to ensure the accurate representation.

*To OVERLAY images: Save thresholded image with different filename. Open processed image. Zoom in to see edges of particles. From the menu bar, select: **Image>Overlay>Add image**. Select thresholded image file with 15% opacity. Ensure “create image selection” is not checked. Click “OK”. To compare images, toggle back and forth between **hide overlay** and **show overlay** from the **Image>Overlay** menu.*

Note: Depending on image quality, optional processing steps may be required in order for appropriate thresholding of particles in image. The inset below highlights some common processing tools that should be used prior to thresholding if necessary. In general, the fewest processing steps are required.

1. To clean up edges of particles:

Select: **Process> Filters> Gaussian Blur: Radius (2-3) pixels> OK**

>Select: **Process> Filters> Unsharp mask: GaussianRadius (2-3), Mask weight (0.6)> OK**. Save processed image (does this remove thresholding?)

2. To correct for uneven background resulting from non-uniform electron beam illumination:

Select **FFT> Bandpass filter: filter structures down to 100 pixels, up to 3 pixels, Unselect “Saturate image when autoscaling” > OK**. Save

7. Set Measurements:

From the menu bar, select: **Analyze> Set Measurements**.

Tick the boxes that correspond to the measurements that you want included (Fig 2). At a minimum, select ‘Area’, ‘Fit ellipse’, ‘Shape descriptors’, and ‘Ferret’s diameter’ then select ‘Limit to Threshold’. Click ‘ok’.

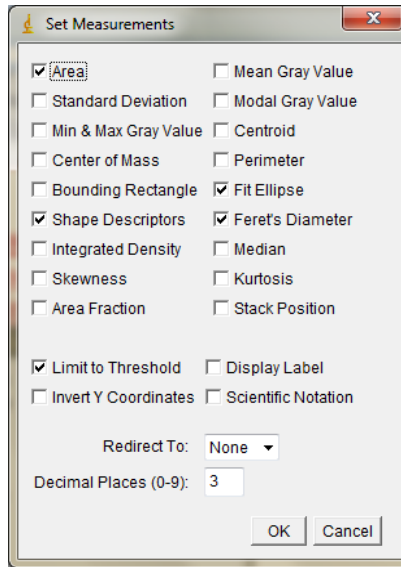


Fig 2. Image J 'Set measurements' function illustrating available measurement options.

- Analyze Particle Size:** Ensure that thresholded image is selected prior to analyzing particle size.

From the menu bar, select: **Analyze > Analyze Particles** to measure features individually. *This action will open a dialog box. Select 'Show outlines' from the drop down menu and tick the boxes 'Display results', 'Include Holes', and 'Exclude on edges' as shown in Fig. 3. Click OK.*

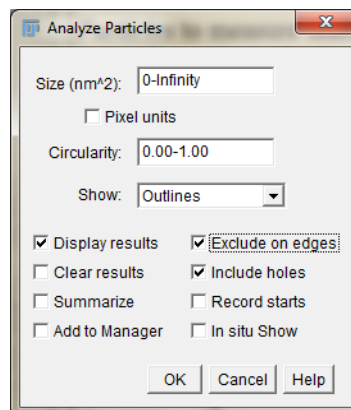


Fig. 3. Image J 'Analyze particles' dialog box. Select 'Show outlines' from the drop down menu and 'Display results' by ticking the box.

Particle Size Analysis SOP



Two new windows will pop up after completing the analyze particles (*Fig. 4*). One window is an image file that shows the outlines and numbers of the particles included in the measurements. The second window is a spreadsheet entitled 'Results' that has the individual measurement values. These files must be saved separately. See step 8 for instructions on file naming.

From the menu bar, select: **File> Save As > .tiff** (or other format such as .jpeg for image file)

From the menu bar, select: **File> Save As... Measurements > filename.xls** for tabulated results.

*Note: Alternatively, the tabulated results data can be saved from the Results window menu bar. Select: **File> Save As... filename.xls***

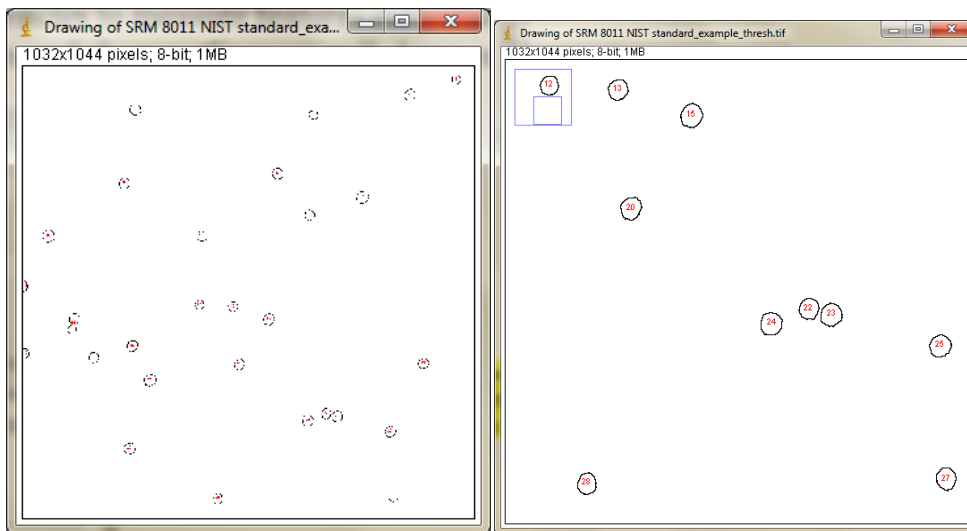


Fig. 4. Drawing of particles counted as part of analysis. Individual particles are numbered as shown in the blow-up to allow for exact measurement tracking of individual particles.

	Area	Major	Minor	Angle	Circ.	Feret	FeretX	FeretY	FeretAngle	MinFeret	AR	Round	Solidity
1	81.611	10.579	9.822	73.798	0.919	11.021	582.957	22.713	40.601	9.962	1.077	0.928	0.946
2	68.750	9.839	8.897	153.122	0.861	10.459	261.394	22.713	130.365	9.165	1.106	0.904	0.928
3	59.700	8.769	8.668	94.286	0.861	9.505	672.213	27.494	123.024	8.697	1.012	0.988	0.918
4	48.903	8.031	7.753	10.524	0.847	8.847	72.919	35.464	35.838	7.969	1.036	0.965	0.917
5	61.605	9.462	8.290	27.972	0.888	9.898	73.318	46.222	49.899	8.453	1.141	0.876	0.936
6	74.942	10.145	9.406	38.086	0.917	10.766	79.295	53.793	51.009	9.563	1.079	0.927	0.946
7	66.686	9.812	8.693	119.410	0.898	10.182	213.578	56.981	149.421	9.065	1.134	0.882	0.940
8	68.750	9.787	8.944	53.678	0.866	10.527	652.290	90.053	60.524	9.447	1.094	0.914	0.930
9	64.780	9.245	8.922	139.679	0.905	9.858	293.271	92.843	165.964	9.165	1.036	0.965	0.934
10	70.655	9.561	9.409	94.486	0.872	10.337	578.175	113.961	27.553	9.563	1.016	0.984	0.931

Fig. 5. Data table generated by ImageJ. Data can be save as .xls or .dat and imported into spreadsheet software such as Excel.



9. Determination of particle size and size distribution.

Once the data has been saved, it can be imported to data analysis software such as MSEXCEL for statistical analysis. Particle size can be determined from the area number alone if one assumes the particles are roughly spherical. In this case, the area should be converted to an effective radius using excel or other spreadsheet program according to:

$$r = \sqrt{\frac{A}{\pi}}$$

The diameter ($D=2r$) can then be averaged for all of the particles being analyzed.

For elongated particles, it is possible to determine the average for each of the major and minor axes from the Major and Minor data columns. Similarly, one can use the Feret's Diameter which is the longest distance between any two points along the particle boundary, also known as maximum caliper. The angle (0-180 degrees) of the Feret's diameter is displayed as FeretAngle, as well as the minimum caliper diameter (MinFeret).

Other measurements such as shape factors (circularity) allow the data to be sorted to eliminate aggregated particles (assuming spheroidal particles). Sorting based on circularity and including only those with circularity values > 0.8 will ensure any aggregates are not included in the measurement.



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