Particles in Cell - User Manual

Particles in Cell is a FIJI (ImageJ) routine for a fast analysis method to quantify micro and nanoparticle uptake from images produced from dual-color confocal fluorescence microscopy. During the image analysis routine, cells are reconstructed and split into two areas, intracellular and membrane region. Next, particles are localized, and color coded accordingly. The mean intensity of particles, measured in calibration experiments, is used to determine the absolute number of particles. Extra parameters, namely, the ratio of fluorescence intensity and a calibration factor, can be used for a more precise quantification.

The routine needs the dual-color confocal fluorescence microscopy image to be separated by channels. As the image for the cells and the particles are to be given separately.

FIJI can be downloaded from: https://imagej.net/Fiji

The routine builds upon a previously developed code made possible by Torrano et al. and fully described in:

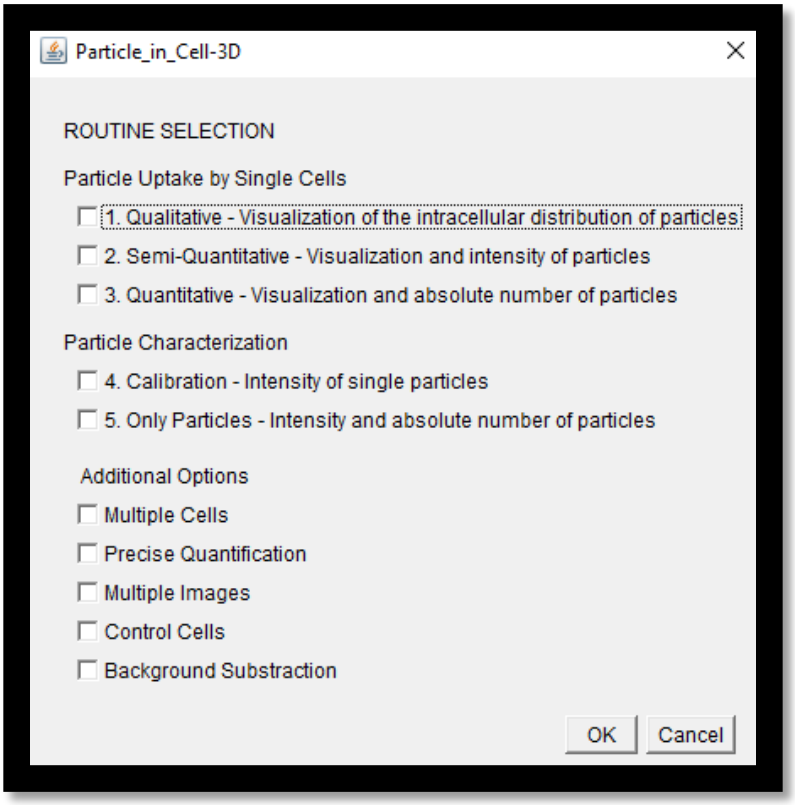
Torrano, A. A., Blechinger, J., Osseforth, C., Argyo, C., Reller, A., Bein, T., ... & Bräuchle, C. (2013). A fast analysis method to quantify nanoparticle uptake on a single cell level. *Nanomedicine*, *8*(11), 1815-1828.

The code described here takes the original ParticlesInCell3D routine and improves it to allow for single image, multi-cellular particle uptake quantification. Additionally, the new code adds new options to enhance the quantification process.

Both codes (the stack and single image versions) can be found and downloaded from: https://github.com/Broude97/Particle-In-Cell

1. **Load Particles in Cell**
   To start with the analysis the routine must first be loaded into FIJI. From the FIJI menu select:
   - Plugins >> Macros >> Run… >> Particles in Cell
2. **Select routine.**

Particles in Cell is separated into five routines. The first three are devoted to the visualization and quantification of particles in cell uptake experiments. They permit quantification with increasing levels of accuracy.



Check the box on the dialog above for the desired routine. Check only one routine. Choose the additional options to be included in the analysis.

- *Qualitative.* Visualization of the intracellular distribution of particles. Final visualization will be found on the results directory under _Uptake- *"Cell's image name".*
- *Semi – Quantitative.* Measure and compare the amount of particles in different cells or regions based on particles' fluorescence intensity. Final visualization will be found on the results directory under _Uptake- *"Cell's image name".*
- *Quantitative.* Count the absolute number of particles internalized by cells. Final visualization will be found on the results directory under _Uptake- *"Cell's image name".*

The last two routines are aimed at the characterization of nanoparticles, micro particles, and agglomerates.

- *Calibration.* Used to determine the mean particle intensity to be used for uptake quantification experiments.
- *Only Particles.* Count the absolute number of particles in cell free regions.

Routine 1. Qualitative.

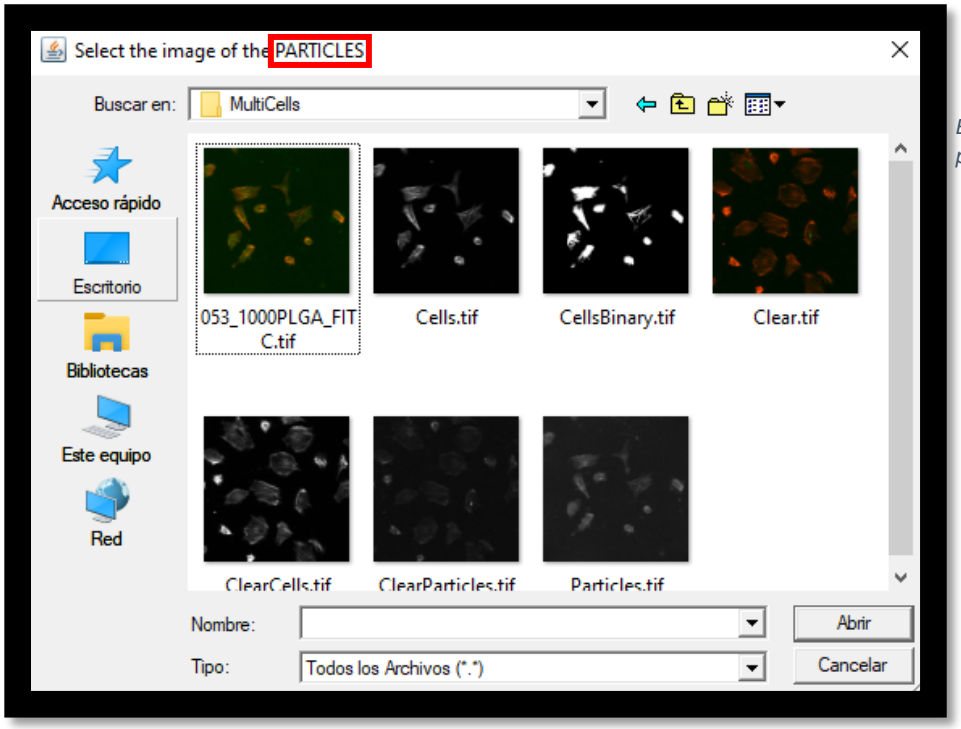
Visualization of the intracellular distribution of particles. Final visualization will be found on the results directory under _Uptake- *"Cell's image name".*

1. **Select additional options.**

Additional options for the fast analysis of multiple cells and images are available. As optional additional parameters for a more precise quantification.

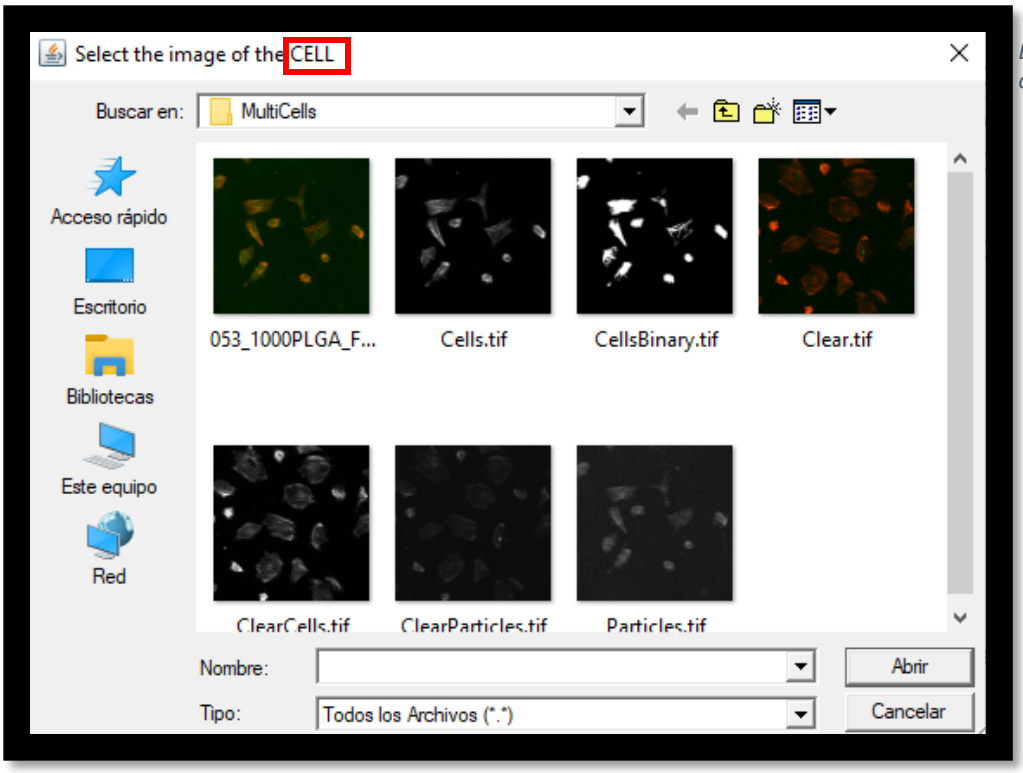
- *Multiple Cells.* Allows for selection of more than one cell in the same image for analysis.
- *Precise Quantification.* Allows for fluorescence ratio and calibration factor as additional parameter for more accurate results.
- *Multiple Images.* Allows analysis of more than one image.
- *Control Cells.* Allows for the selection of control cell to use as background subtraction for the particles.
- *Background Subtraction.* Allows for personalized values of background to be subtracted from the particles.

2. **Select particles image.**
   A file browser opens where the particles channel image to be analyzed must be selected.



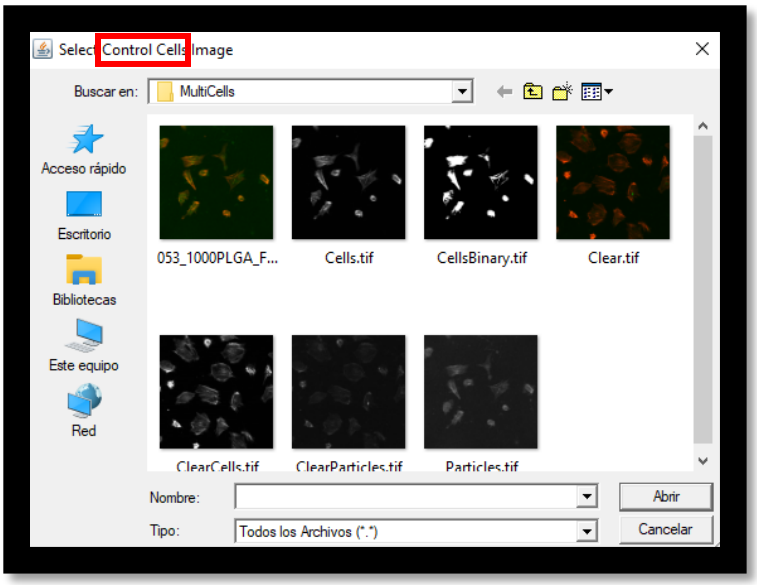
Browse to the desired particles image.

3. **Select cells image.**
   A file browser opens where the cells channel image to be analyzed must be selected.



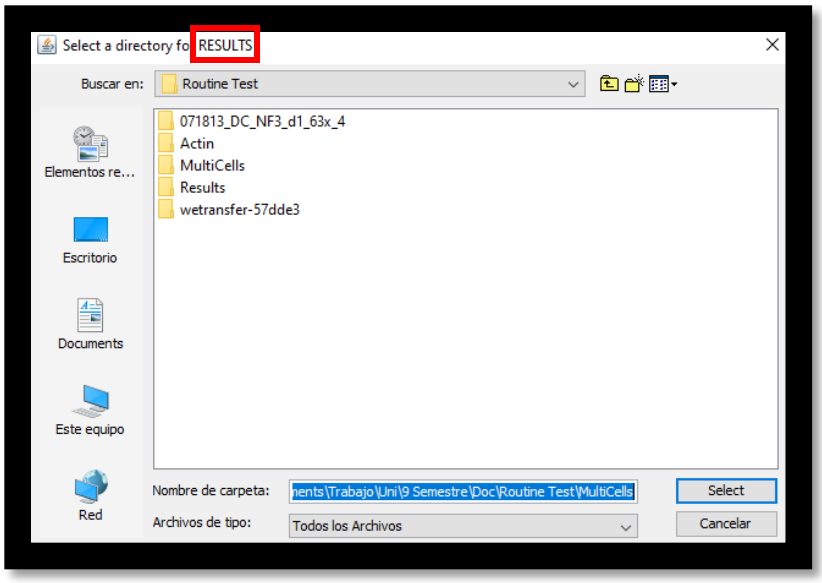
Browse to the desired cell image.

- *If Control Cells was selected. Select an image to use as control cells as well.*



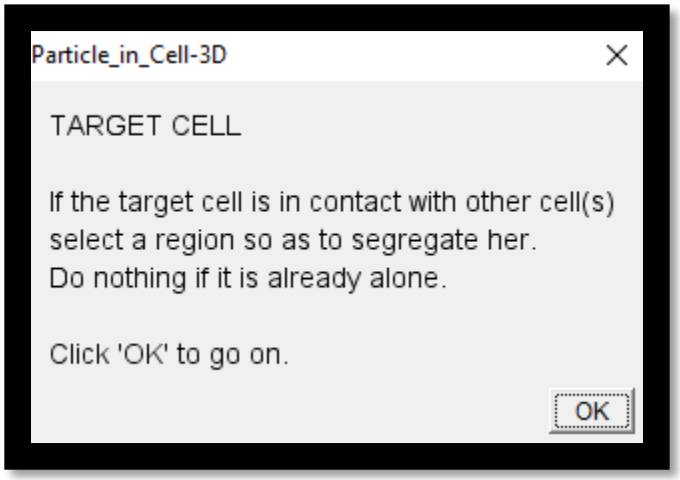
Browse to the desired control cell image.

4. **Select Results directory**
   A file browser opens where the desired results directory must be selected.



Browse to the desired results directory.

5. **Select target area.**
   Select the region of interest within the image selected. If the region of interest is the whole image do not select anything.

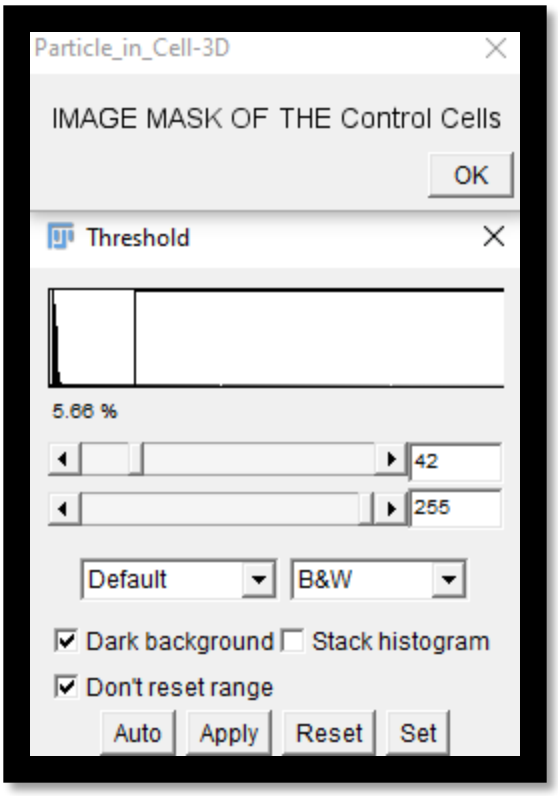


Before clicking "OK", make sure the target area is selected. If the target area is the whole image, then just click "OK".

If Control Cells was selected

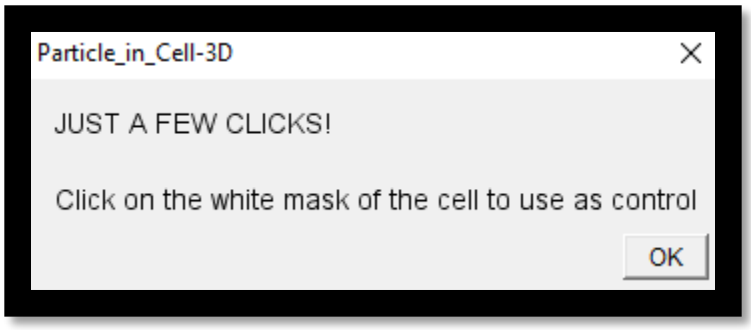
When using the Control Cells feature:

- Threshold control cells image.

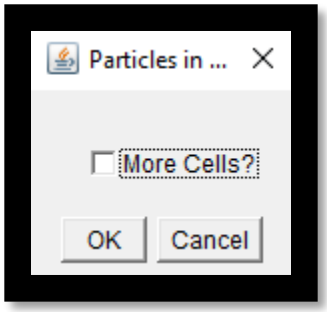


Threshold the control cells image before clicking "OK". Once a satisfactory threshold has been selected click "OK".

- Select cells to use as control.



Before clicking "OK" select a cell to use as control by clicking on it.



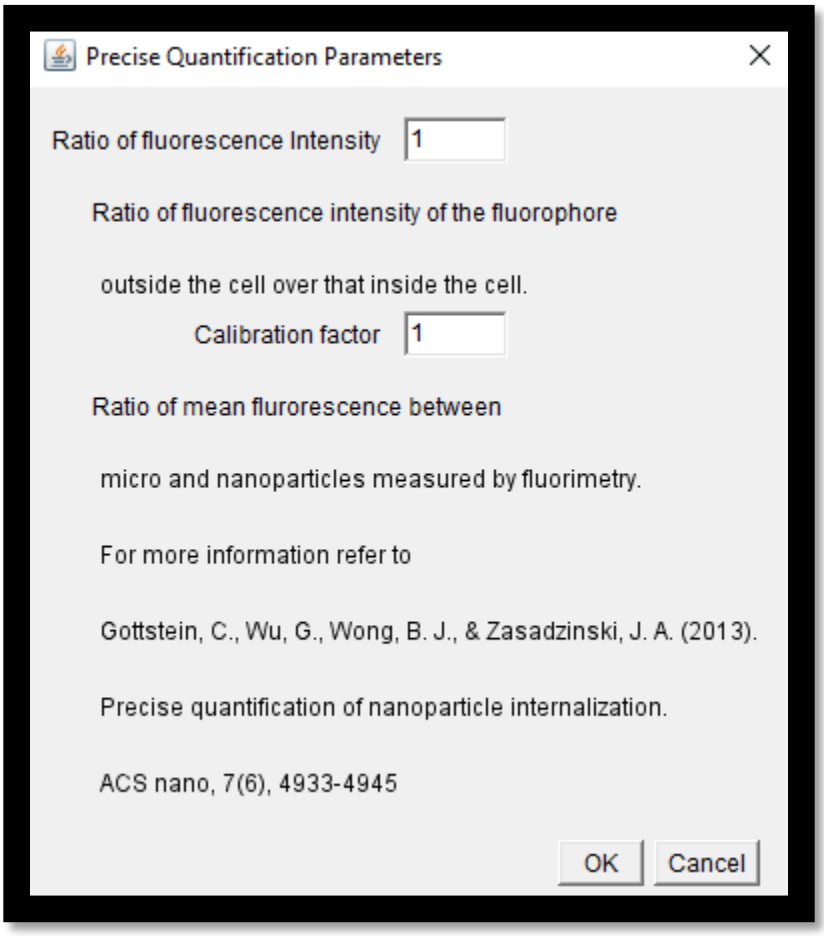
If more than once cell is to be used as control, check the "More Cells?" box before clicking "OK". This will allow for a new cell to be selected. Repeat for as many cells as desired. When done simply leave the "More Cells?" box unchecked and click "OK".

If Precise Quantification was selected

The routine allows for a more precise quantification by means of two parameters. A ratio of fluorescence intensity and a calibration factor. The ratio is the change of fluorescence intensity some dyes experience when going from outside the cell to inside the cell. The calibration factor is used mainly when the mean intensity is obtained from microparticles by flow cytometry. The calibration factor relates the intensity of the microparticles to that that would be obtained by nanoparticles. This calibration factor is the ratio of the mean fluorescence of micro and nanoparticles as measured by fluorimetry at the same conditions as the ones used at flow cytometry.

When using the Precise Quantification feature:

- Choose ratio of fluorescence and calibration factor parameters.

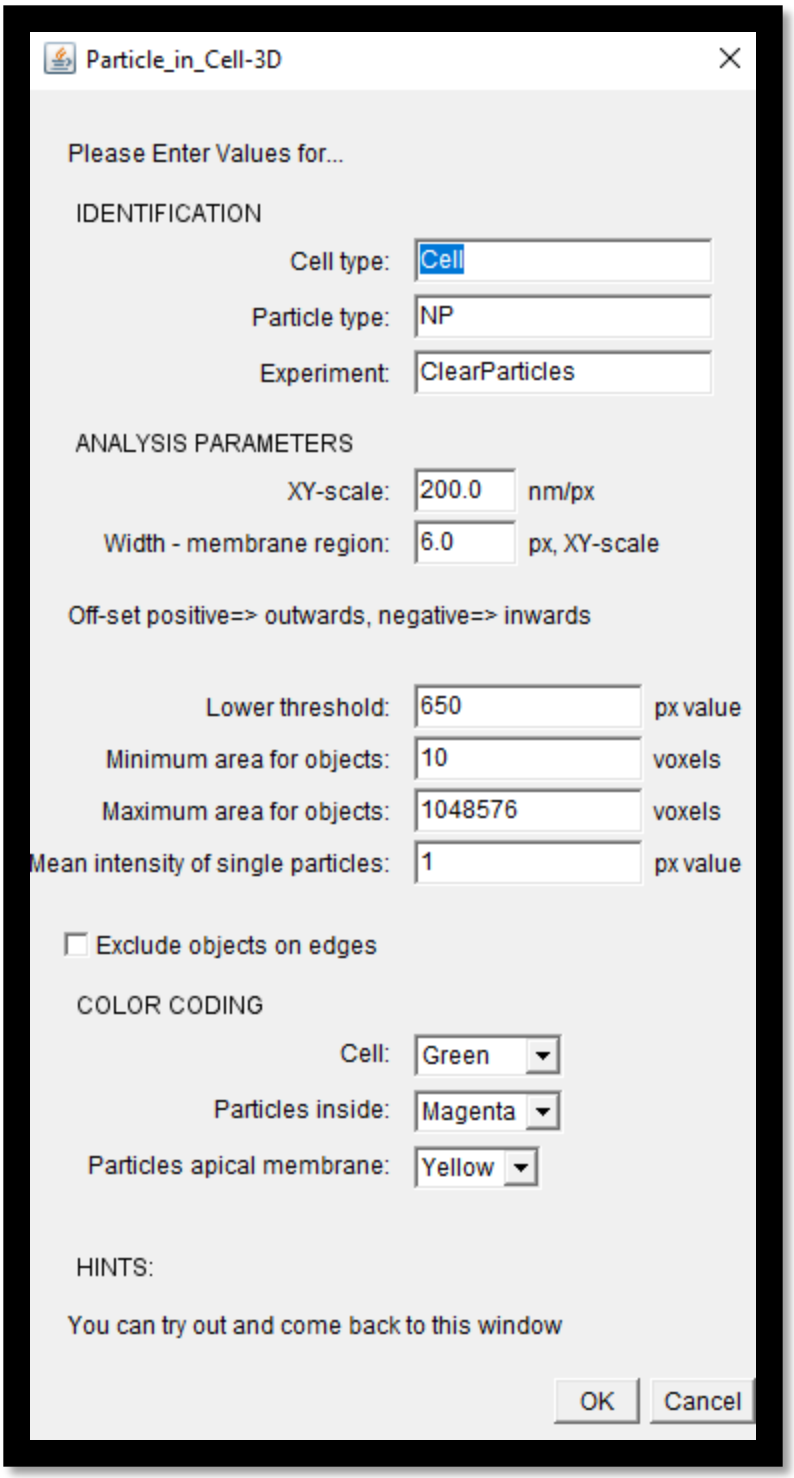


Choose the values to be used for the ratio of fluorescence intensity and the calibration factor.

These parameters are usually obtained through flow cytometry. For more information review:

Gottstein, C., Wu, G., Wong, B. J., & Zasadzinski, J. A. (2013). Precise quantification of nanoparticle internalization. ACS nano, 7(6), 4933-4945

6. Select parameters.



Choose the parameters to be used for the analysis.

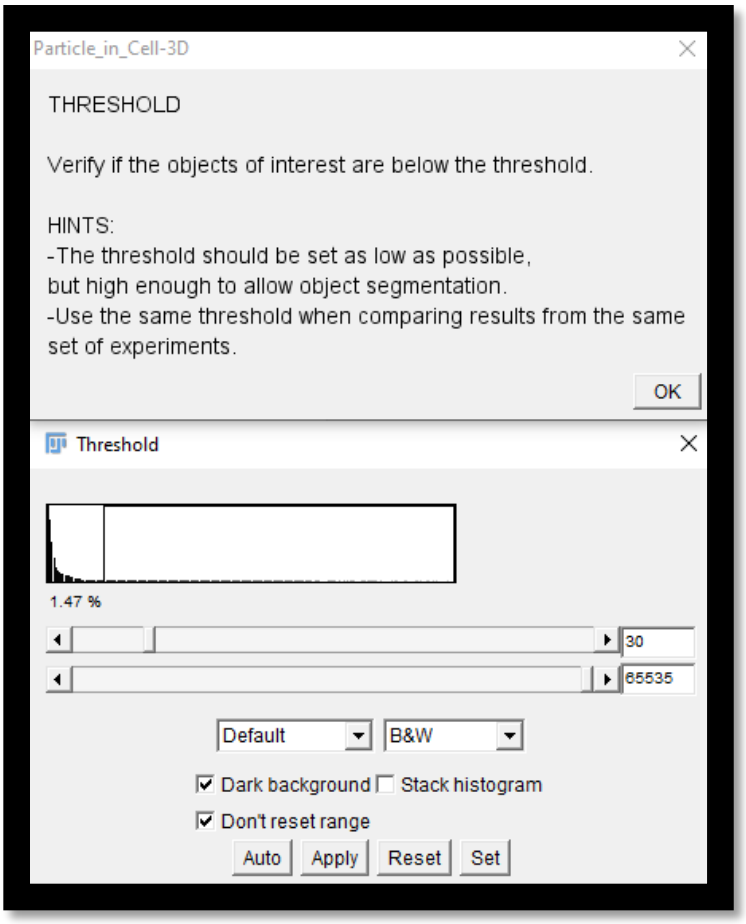
- *Cell Type.* Name to save intermediate steps cells images.
- *Particle Type.* Name to save intermediate steps particles images.
- *Experiment.* Name to identify the current experiment.
- *XY Scale.* Image XY scale. Used for conversion between pixels and nanometers.
- *Membrane region width.* Width to use as the membrane region.

- *Lower threshold.* Value to use to threshold the particles. Can be previewed and changed later.
- *Minimum object area.* Minimum area of objects to consider.
- *Maximum object area.* Maximum area of objects to consider.
- *Mean particle intensity.* Mean particle intensity. Obtained from calibration experiments. Used for the absolute quantification of internalized particles.
- *Exclude objects on edges.* Whether to include in the quantification objects on the edge of the regions of interest.
- *Cell color.* Choose the color by which to identify the cell.
- *Particles inside color.* Choose the color by which to code intracellular particles.
- *Particles Apical Membrane color.* Choose the color by which to code membrane particles.

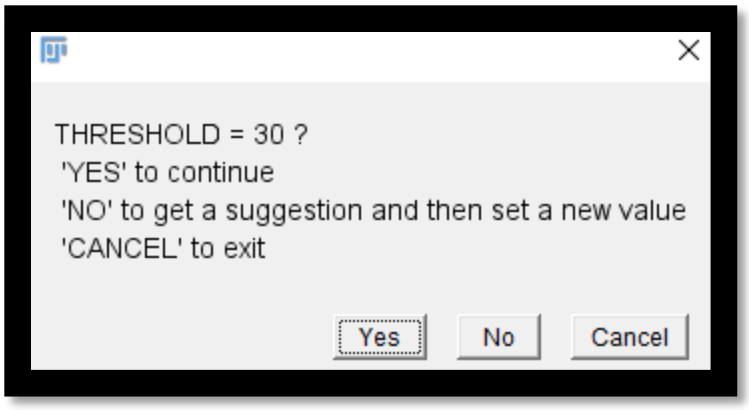
7. **Threshold particles.**

Threshold the particles. The value entered in the precious step is used by default. A preview is visualized where the user can manually change the threshold value. When a satisfactory value is not found a new value will be suggested and the user will return to the previous step where the suggestions is set as default, but it can be changed by the user. This process is repeated until a satisfactory value is found.

The aim is to set the threshold as low as possible while still allowing for object segmentation



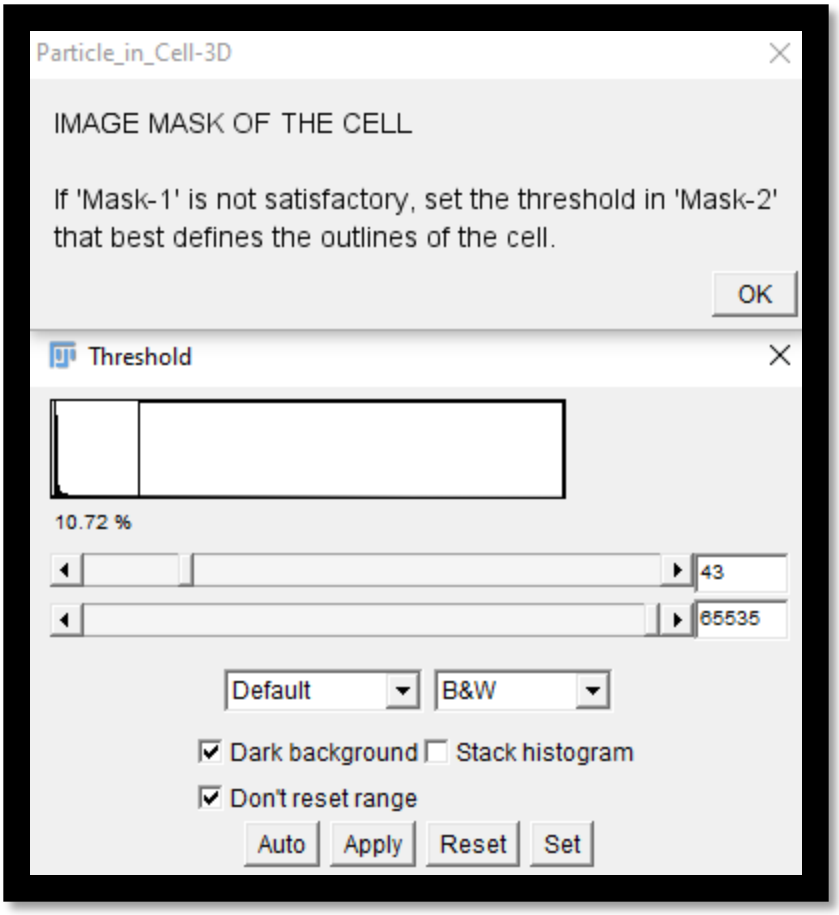
Before clicking "OK", make sure that the thresholding is satisfactory. If not, it can be manually changed before clicking "OK".



Click "YES" if satisfied with the current thresholding value. "No" to get a new suggestion and set a new value.

8. **Threshold cells.**
   An automatic mask of the cells is generated. However, a second image is opened where the user can set a threshold value manually.

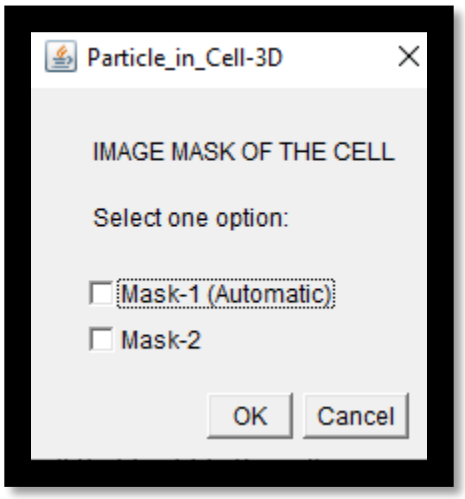
   The objective is to achieve a mask that accurately represents the cell.



If the automatic thresholding (Mask-1) is unsatisfactory. A manual thresholding (Mask-2) can be done before clicking "OK".

9. **Select best cell mask.**
   Selection of the mask of the cell that best fits the cells.
   - *Mask 1.* Automatic.

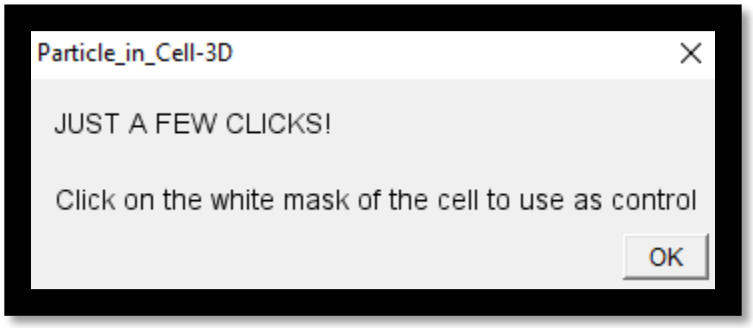
- *Mask 2.* User defined.



Select best representation of the cell.

10. Select cells to analyze.

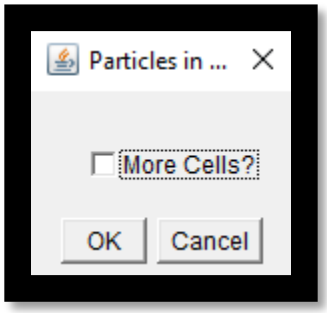
Selection of one or more cells to be analyzed.



Before clicking "OK" select a cell to be analyzed by clicking on it.

If Multiple Cells was selected.

After selecting one cell a prompt will appear asking the user if he wants to select more cells for analysis

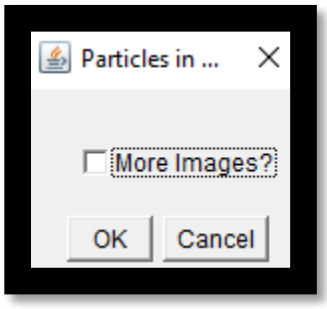


If more than once cell is to be analyzed, check the "More Cells?" box before clicking "OK". This will allow for a new cell to be selected. Repeat for as many cells as desired. When done simply leave the "More Cells?" box unchecked and click "OK".

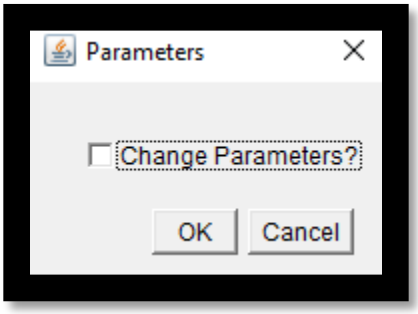
If Multiple Images was selected.

Select next images to be analyzed and new results directory to save the results from the new images.

When choosing more images there is the option to either keep the same parameters as for the previous analysis or to choose new ones.



If another image is to be analyzed, check the "More Images?" box before clicking "OK". This will allow for a new image to be selected. Repeat for as many images as desired. When done simply leave the "More Images?" box unchecked and click "OK".



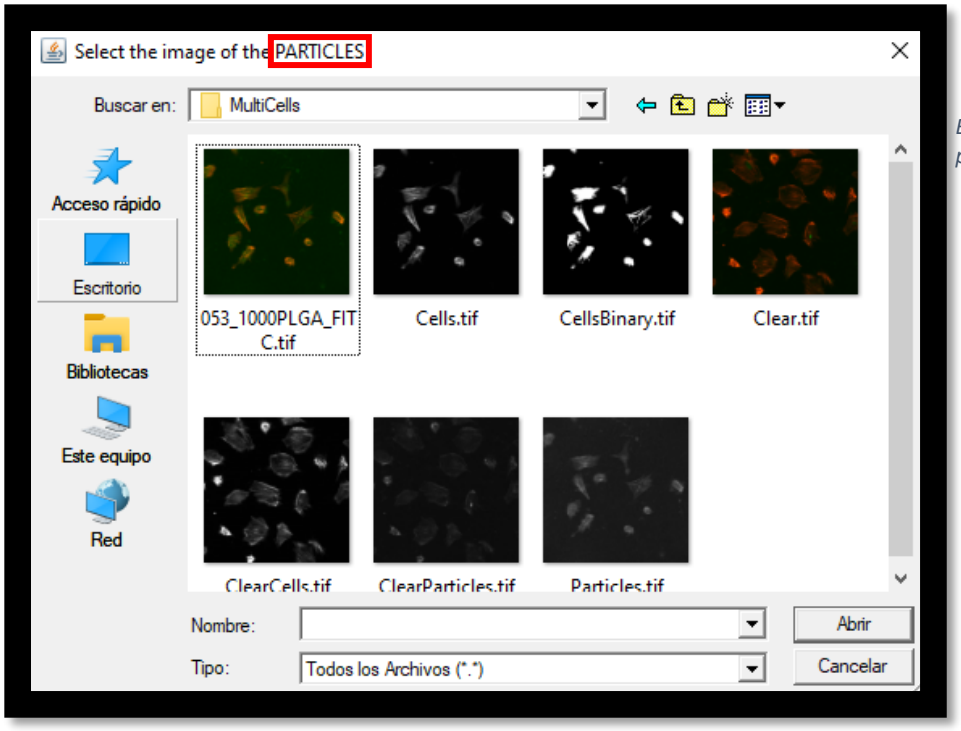
If the same parameters as the previous analysis are desired leave the box unchecked. If new parameters are desired check the box before clicking "OK".

Routine 2. Semi-Quantitative.

Measure and compare the amount of particles in different cells or regions based on particles' fluorescence intensity. Final visualization will be found on the results directory under _Uptake- *"Cell's image name".*

1. **Select additional options.**
   Additional options for the fast analysis of multiple cells and images are available. As optional additional parameters for a more precise quantification.
   - *Multiple Cells.* Allows for selection of more than one cell in the same image for analysis.
   - *Precise Quantification.* Allows for fluorescence ratio and calibration factor as additional parameter for more accurate results.
   - *Multiple Images.* Allows analysis of more than one image.
   - *Control Cells.* Allows for the selection of control cell to use as background subtraction for the particles.
   - *Background Subtraction.* Allows for personalized values of background to be subtracted from the particles.
2. **Select particles image.**

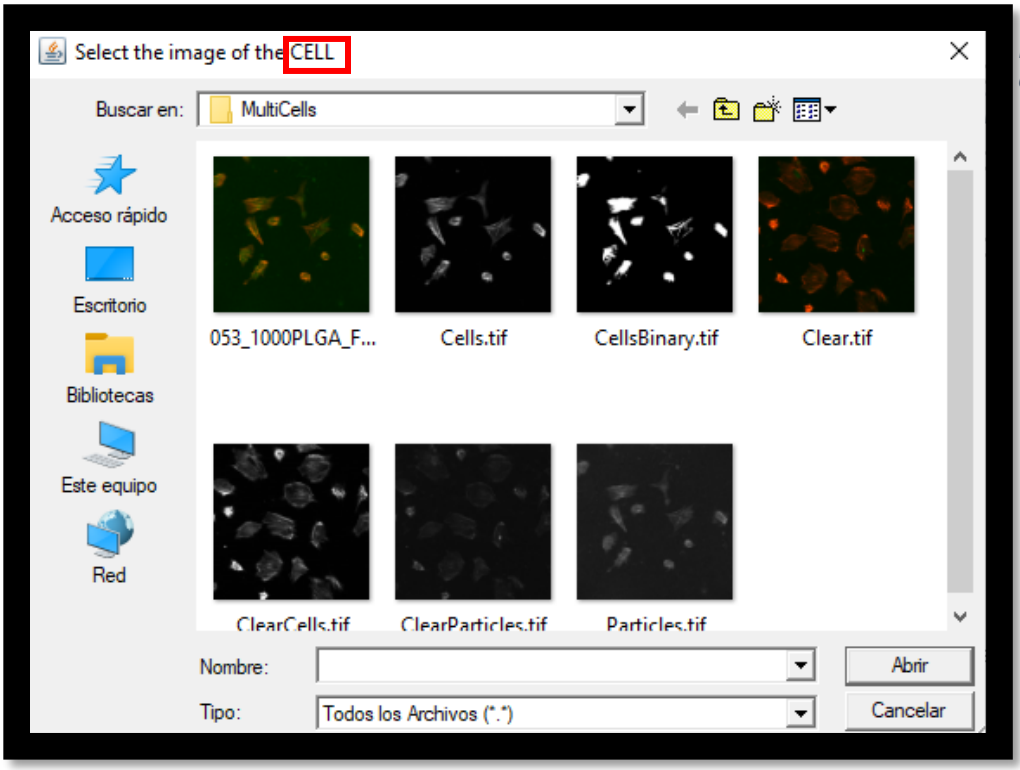
A file browser opens where the particles channel image to be analyzed must be selected.



Browse to the desired particles image.

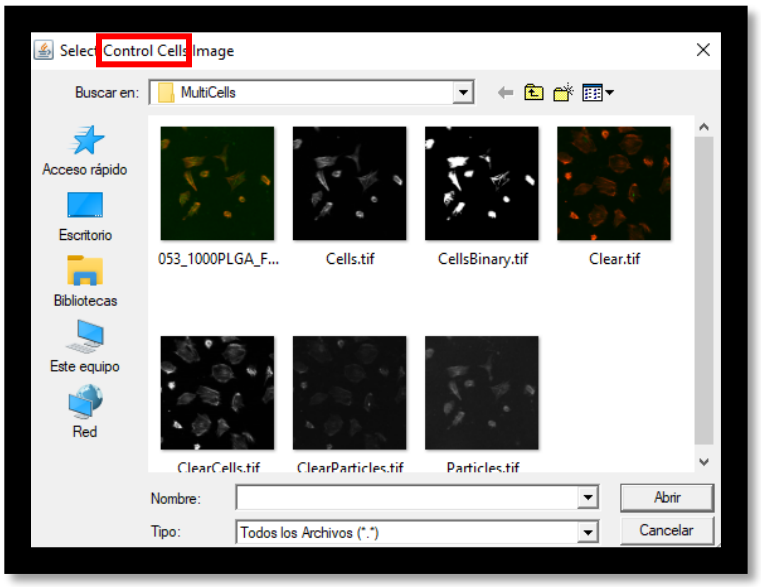
3. **Select cells image.**

A file browser opens where the cells channel image to be analyzed must be selected.



Browse to the desired cell image.

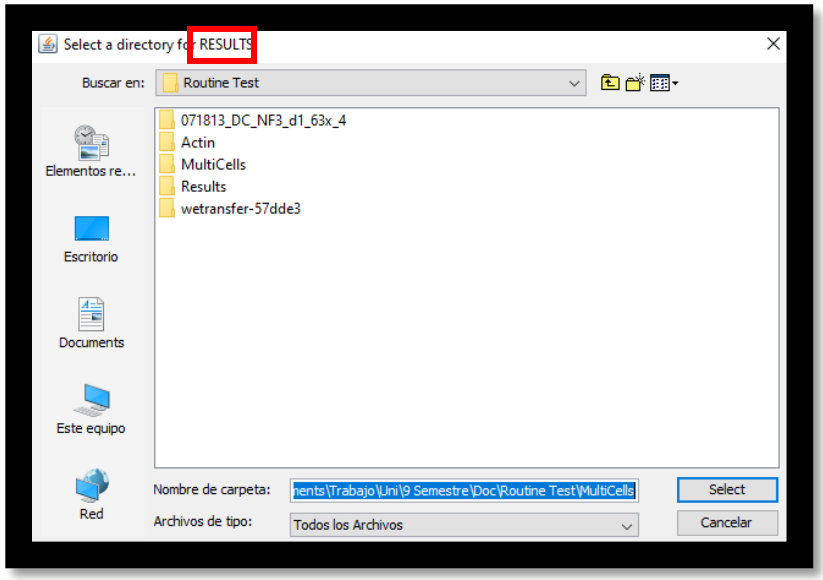
- *If Control Cells was selected. Select an image to use as control cells as well.*



Browse to the desired control cell image.

4. Select Results directory

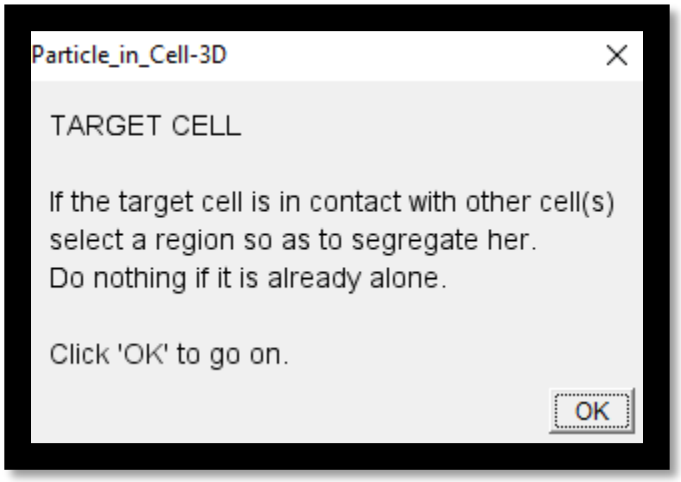
A file browser opens where the desired results directory must be selected.



Browse to the desired results directory.

5. Select target area.

Select the region of interest within the image selected. If the region of interest is the whole image do not select anything.

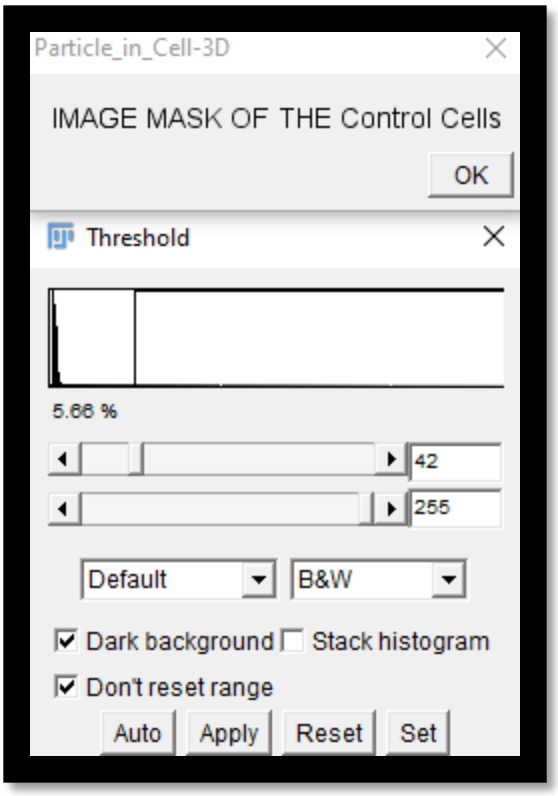


Before clicking "OK", make sure the target area is selected. If the target area is the whole image, then just click "OK".

If Control Cells was selected

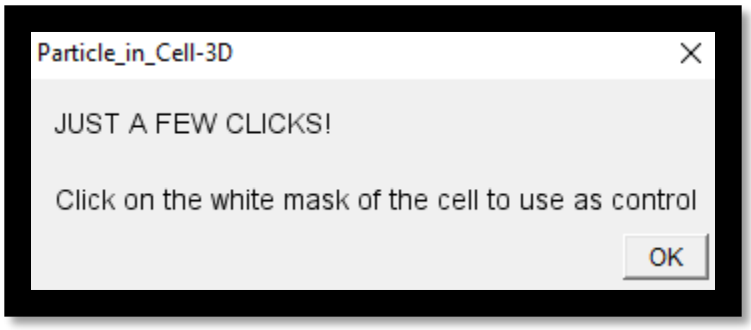
When using the Control Cells feature:

- Threshold control cells image.

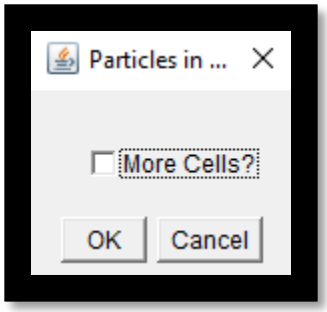


Threshold the control cells image before clicking "OK". Once a satisfactory threshold has been selected click "OK".

- Select cells to use as control.



Before clicking "OK" select a cell to use as control by clicking on it.



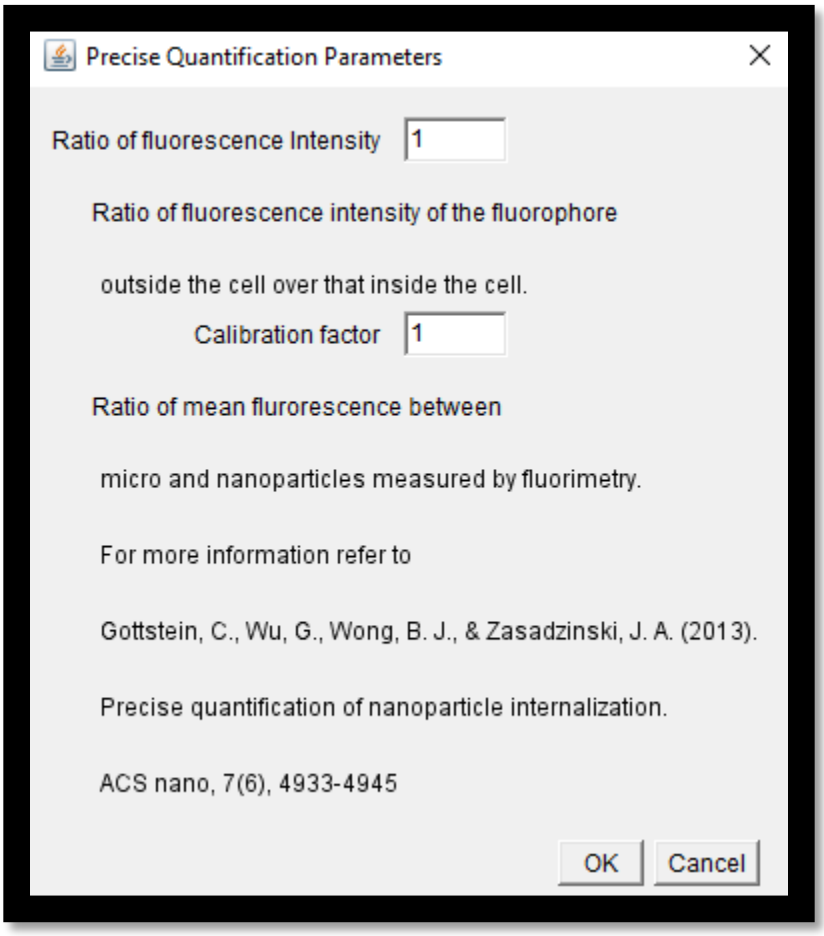
If more than once cell is to be used as control, check the "More Cells?" box before clicking "OK". This will allow for a new cell to be selected. Repeat for as many cells as desired. When done simply leave the "More Cells?" box unchecked and click "OK".

If Precise Quantification was selected

The routine allows for a more precise quantification by means of two parameters. A ratio of fluorescence intensity and a calibration factor. The ratio is the change of fluorescence intensity some dyes experience when going from outside the cell to inside the cell. The calibration factor is used mainly when the mean intensity is obtained from microparticles by flow cytometry. The calibration factor relates the intensity of the microparticles to that that would be obtained by nanoparticles. This calibration factor is the ratio of the mean fluorescence of micro and nanoparticles as measured by fluorimetry at the same conditions as the ones used at flow cytometry.

When using the Precise Quantification feature:

- Choose ratio of fluorescence and calibration factor parameters.

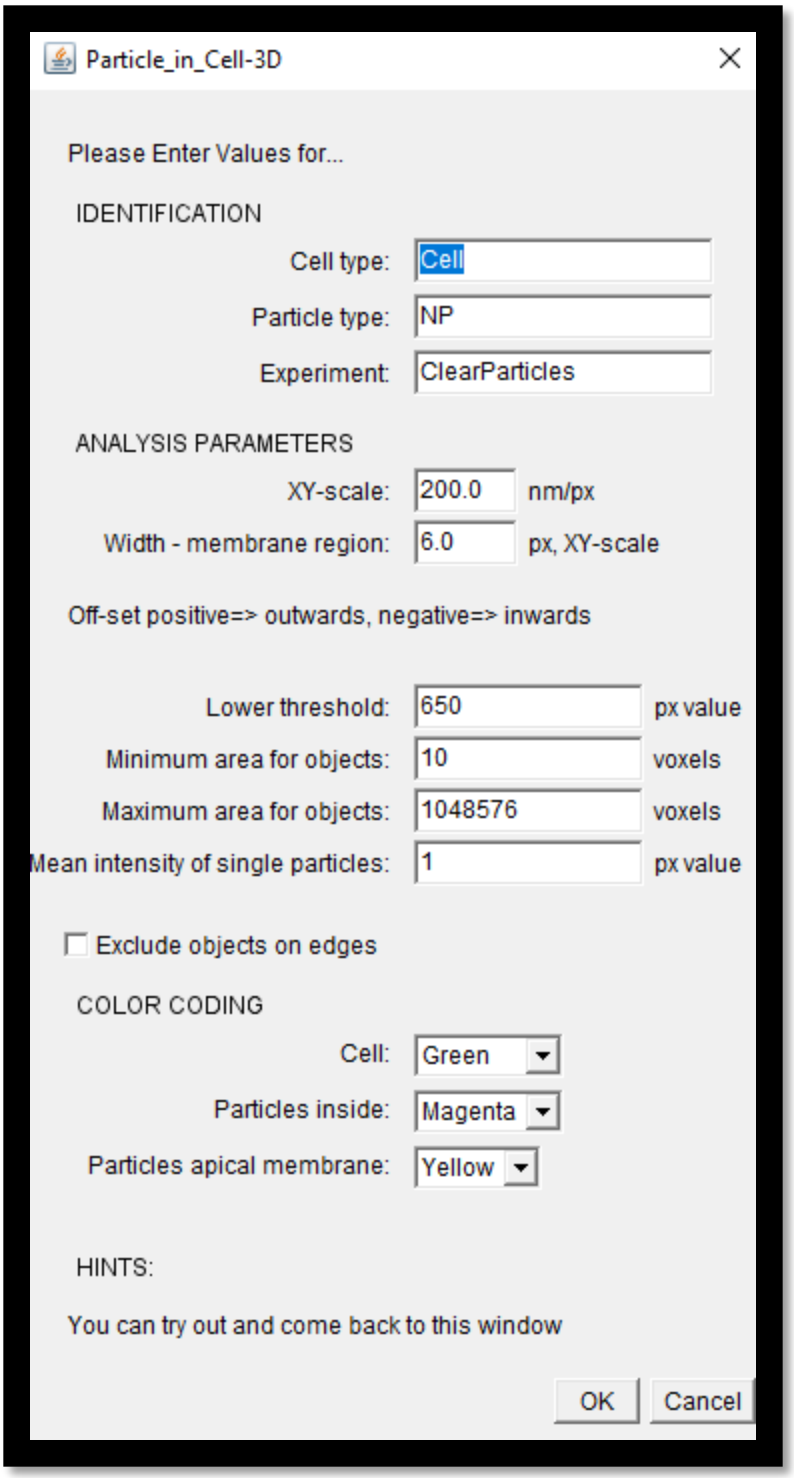


Choose the values to be used for the ratio of fluorescence intensity and the calibration factor.

These parameters are usually obtained through flow cytometry. For more information review:

Gottstein, C., Wu, G., Wong, B. J., & Zasadzinski, J. A. (2013). Precise quantification of nanoparticle internalization. ACS nano, 7(6), 4933-4945

6. Select parameters.



Choose the parameters to be used for the analysis.

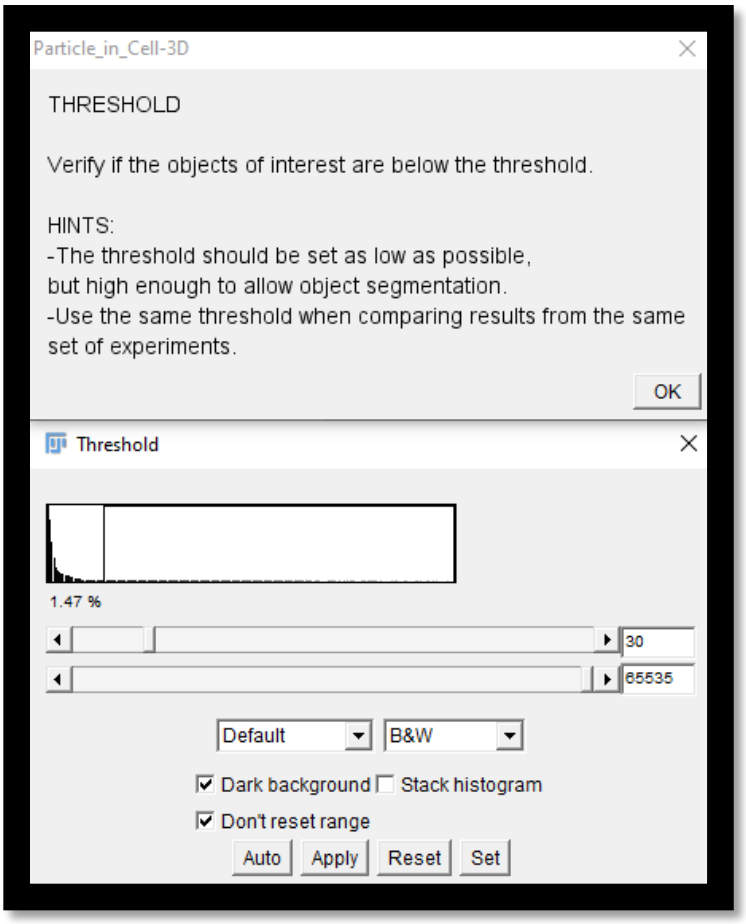
- *Cell Type.* Name to save intermediate steps cells images.
- *Particle Type.* Name to save intermediate steps particles images.
- *Experiment.* Name to identify the current experiment.
- *XY Scale.* Image XY scale. Used for conversion between pixels and nanometers.
- *Membrane region width.* Width to use as the membrane region.

- *Lower threshold.* Value to use to threshold the particles. Can be previewed and changed later.
- *Minimum object area.* Minimum area of objects to consider.
- *Maximum object area.* Maximum area of objects to consider.
- *Mean particle intensity.* Mean particle intensity. Obtained from calibration experiments. Used for the absolute quantification of internalized particles.
- *Exclude objects on edges.* Whether to include in the quantification objects on the edge of the regions of interest.
- *Cell color.* Choose the color by which to identify the cell.
- *Particles inside color.* Choose the color by which to code intracellular particles.
- *Particles Apical Membrane color.* Choose the color by which to code membrane particles.

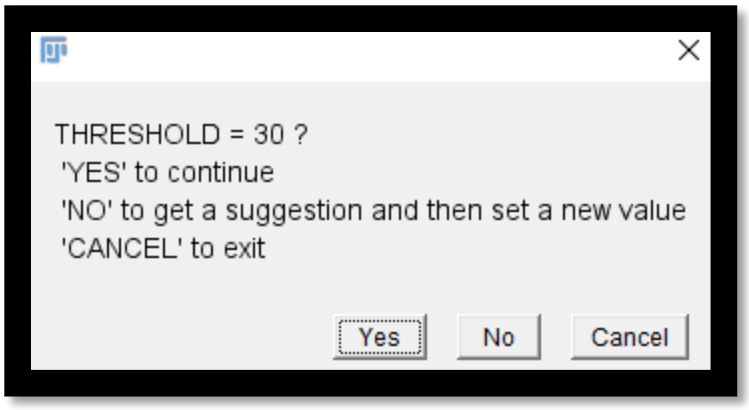
7. **Threshold particles.**

Threshold the particles. The value entered in the precious step is used by default. A preview is visualized where the user can manually change the threshold value. When a satisfactory value is not found a new value will be suggested and the user will return to the previous step where the suggestions is set as default, but it can be changed by the user. This process is repeated until a satisfactory value is found.

The aim is to set the threshold as low as possible while still allowing for object segmentation



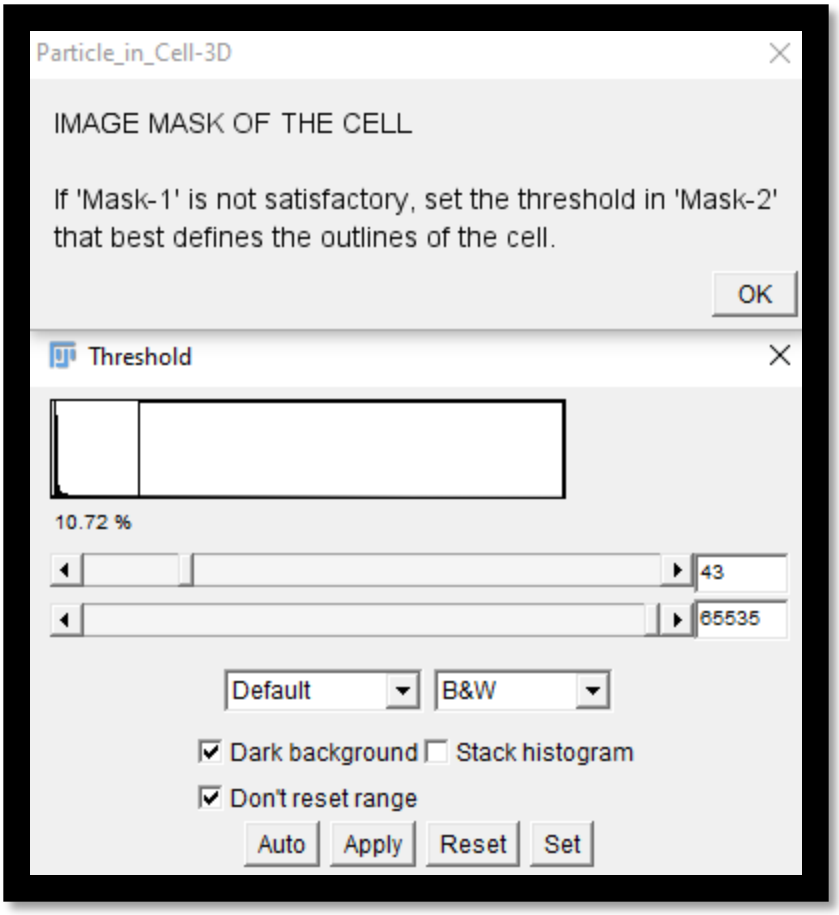
Before clicking "OK", make sure that the thresholding is satisfactory. If not, it can be manually changed before clicking "OK".



Click "YES" if satisfied with the current thresholding value. "No" to get a new suggestion and set a new value.

8. **Threshold cells.**
   An automatic mask of the cells is generated. However, a second image is opened where the user can set a threshold value manually.

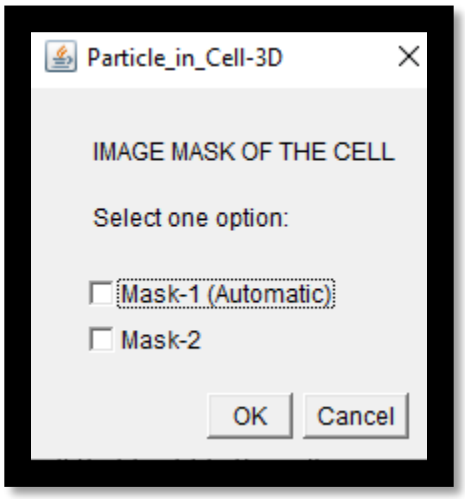
   The objective is to achieve a mask that accurately represents the cell.



If the automatic thresholding (Mask-1) is unsatisfactory. A manual thresholding (Mask-2) can be done before clicking "OK".

9. **Select best cell mask.**
   Selection of the mask of the cell that best fits the cells.
   - *Mask 1.* Automatic.

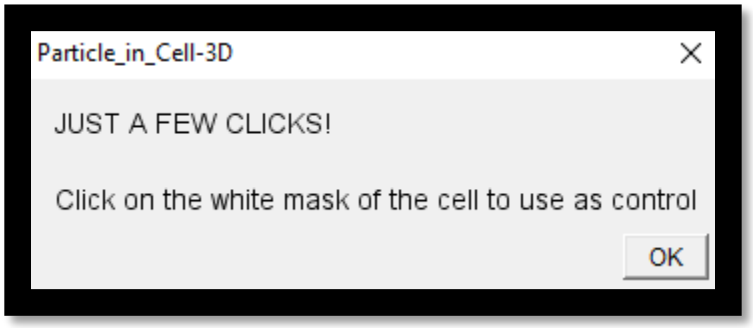
- *Mask 2.* User defined.



Select best representation of the cell.

10. Select cells to analyze.

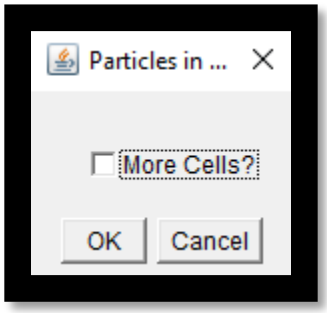
Selection of one or more cells to be analyzed.



Before clicking "OK" select a cell to be analyzed by clicking on it.

If Multiple Cells was selected.

After selecting one cell a prompt will appear asking the user if he wants to select more cells for analysis



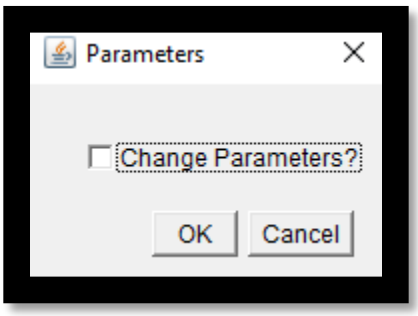
If more than once cell is to be analyzed, check the "More Cells?" box before clicking "OK". This will allow for a new cell to be selected. Repeat for as many cells as desired. When done simply leave the "More Cells?" box unchecked and click "OK".

If Multiple Images was selected.

Select next images to be analyzed and new results directory to save the results from the new images.

When choosing more images there is the option to either keep the same parameters as for the previous analysis or to choose new ones.

If another image is to be analyzed, check the "More Images?" box before clicking "OK". This will allow for a new image to be selected. Repeat for as many images as desired. When done simply leave the "More Images?" box unchecked and click "OK".

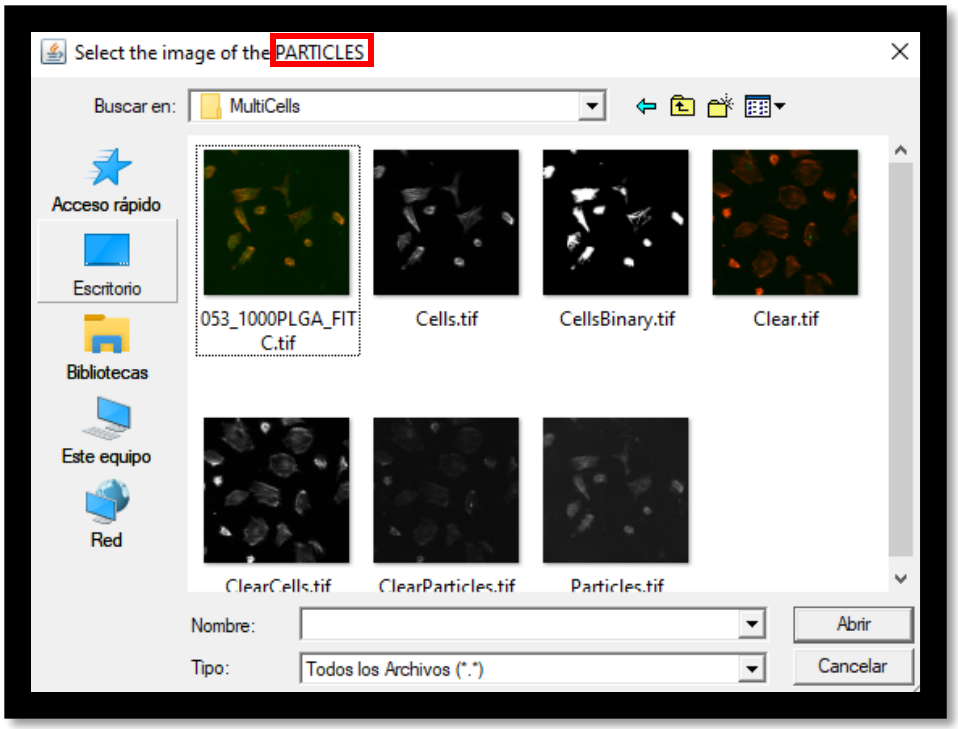


If the same parameters as the previous analysis are desired leave the box unchecked. If new parameters are desired check the box before clicking "OK".

Routine 3. Quantitative.

Count the absolute number of particles internalized by cells. Final visualization will be found on the results directory under _Uptake- *"Cell's image name".*

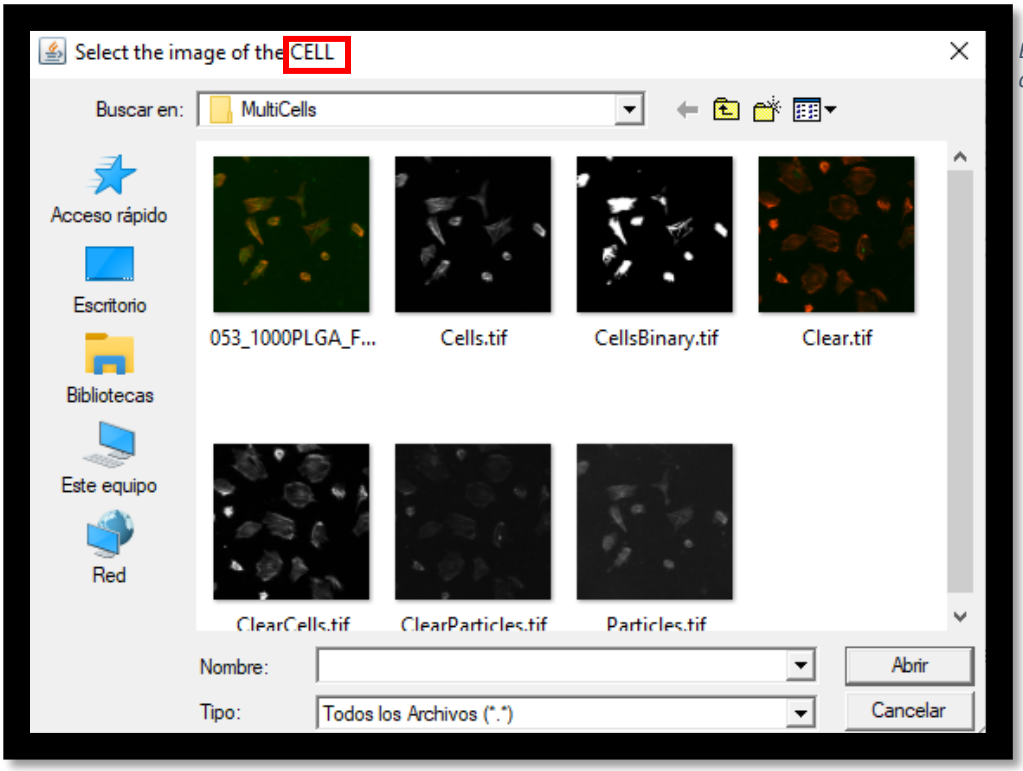
1. **Select additional options.**
   Additional options for the fast analysis of multiple cells and images are available. As optional additional parameters for a more precise quantification.
   - *Multiple Cells.* Allows for selection of more than one cell in the same image for analysis.
   - *Precise Quantification.* Allows for fluorescence ratio and calibration factor as additional parameter for more accurate results.
   - *Multiple Images.* Allows analysis of more than one image.
   - *Control Cells.* Allows for the selection of control cell to use as background subtraction for the particles.
   - *Background Subtraction.* Allows for personalized values of background to be subtracted from the particles.
2. **Select particles image.**
   A file browser opens where the particles channel image to be analyzed must be selected.



Browse to the desired particles image.

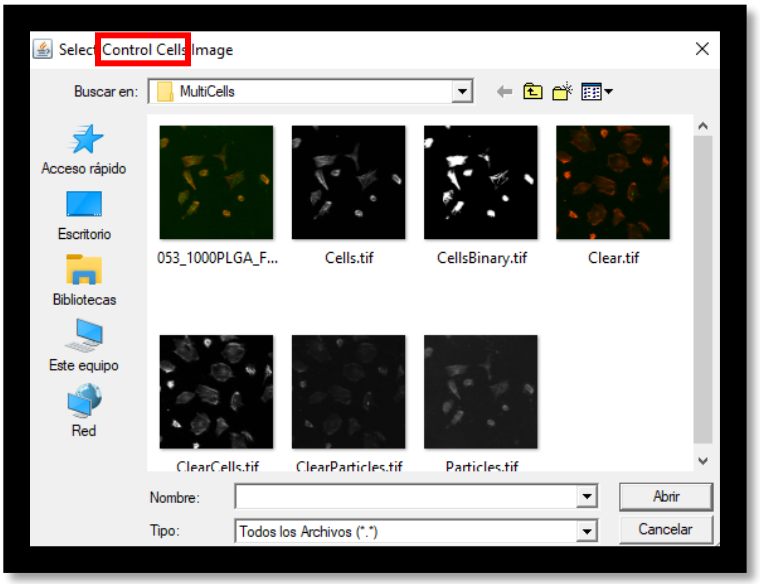
3. **Select cells image.**

A file browser opens where the cells channel image to be analyzed must be selected.

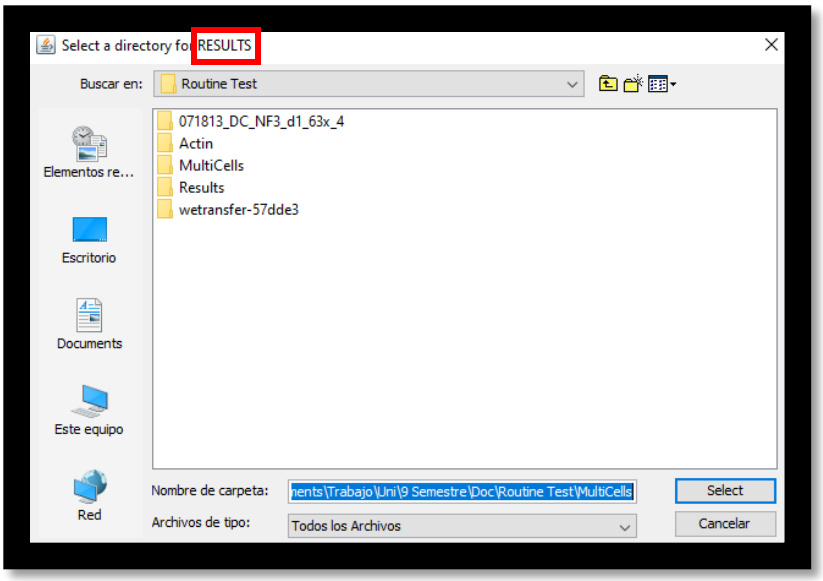


Browse to the desired cell image.

- *If Control Cells was selected. Select an image to use as control cells as well.*



Browse to the desired control cell image.

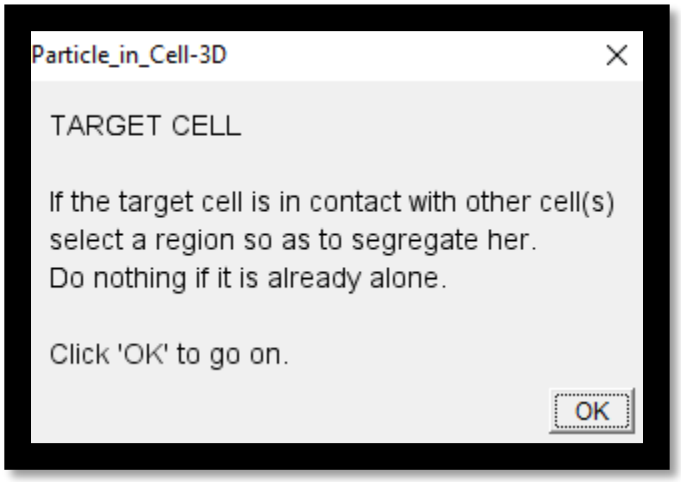


Browse to the desired results directory.

4. **Select Results directory**
   A file browser opens where the desired results directory must be selected.

5. **Select target area.**

Select the region of interest within the image selected. If the region of interest is the whole image do not select anything.

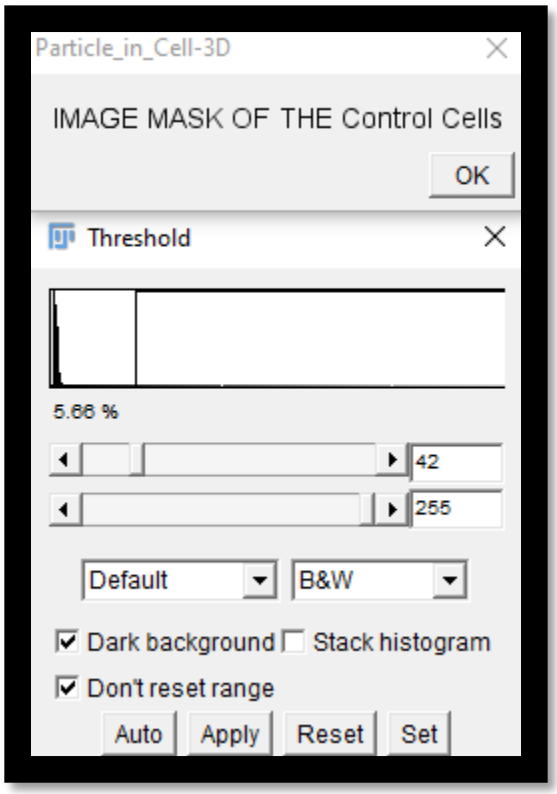


Before clicking "OK", make sure the target area is selected. If the target area is the whole image, then just click "OK".

If Control Cells was selected

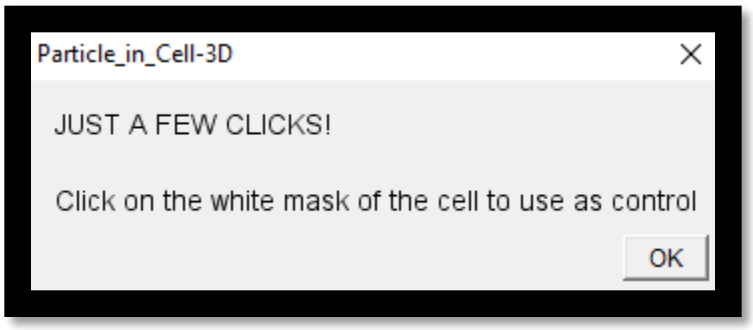
When using the Control Cells feature:

- Threshold control cells image.

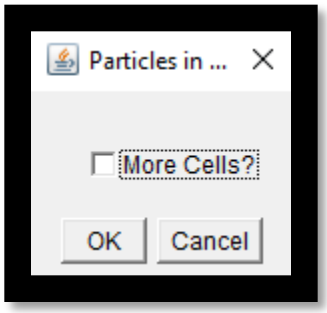


Threshold the control cells image before clicking "OK". Once a satisfactory threshold has been selected click "OK".

- Select cells to use as control.



Before clicking "OK" select a cell to use as control by clicking on it.



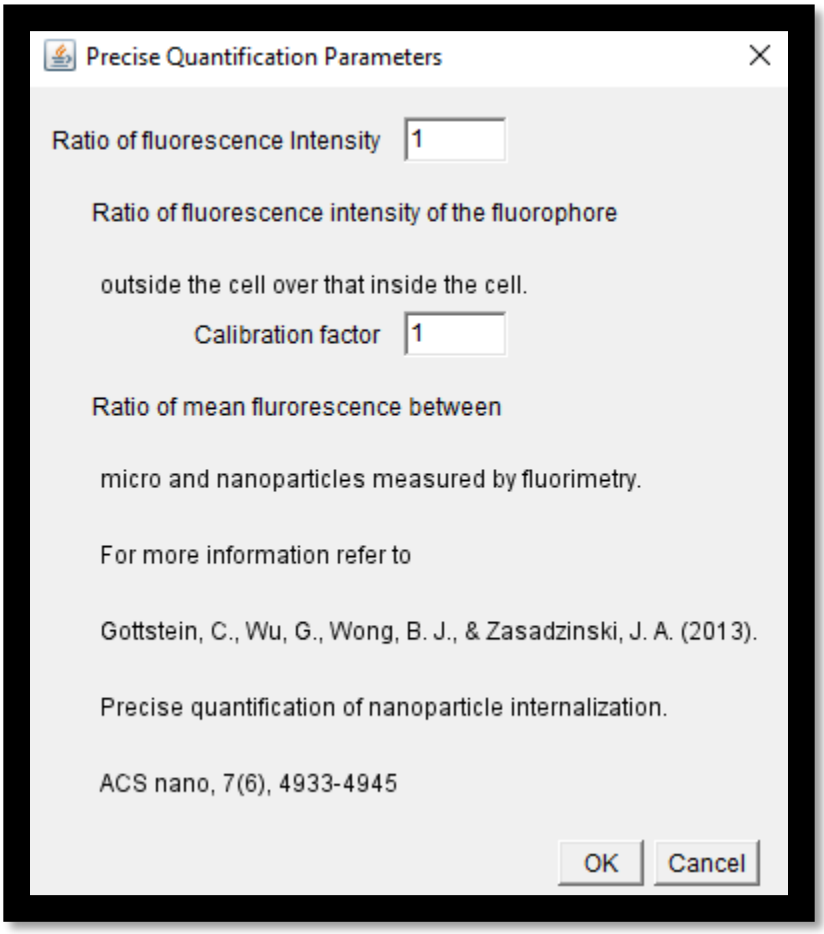
If more than once cell is to be used as control, check the "More Cells?" box before clicking "OK". This will allow for a new cell to be selected. Repeat for as many cells as desired. When done simply leave the "More Cells?" box unchecked and click "OK".

If Precise Quantification was selected

The routine allows for a more precise quantification by means of two parameters. A ratio of fluorescence intensity and a calibration factor. The ratio is the change of fluorescence intensity some dyes experience when going from outside the cell to inside the cell. The calibration factor is used mainly when the mean intensity is obtained from microparticles by flow cytometry. The calibration factor relates the intensity of the microparticles to that that would be obtained by nanoparticles. This calibration factor is the ratio of the mean fluorescence of micro and nanoparticles as measured by fluorimetry at the same conditions as the ones used at flow cytometry.

When using the Precise Quantification feature:

- Choose ratio of fluorescence and calibration factor parameters.

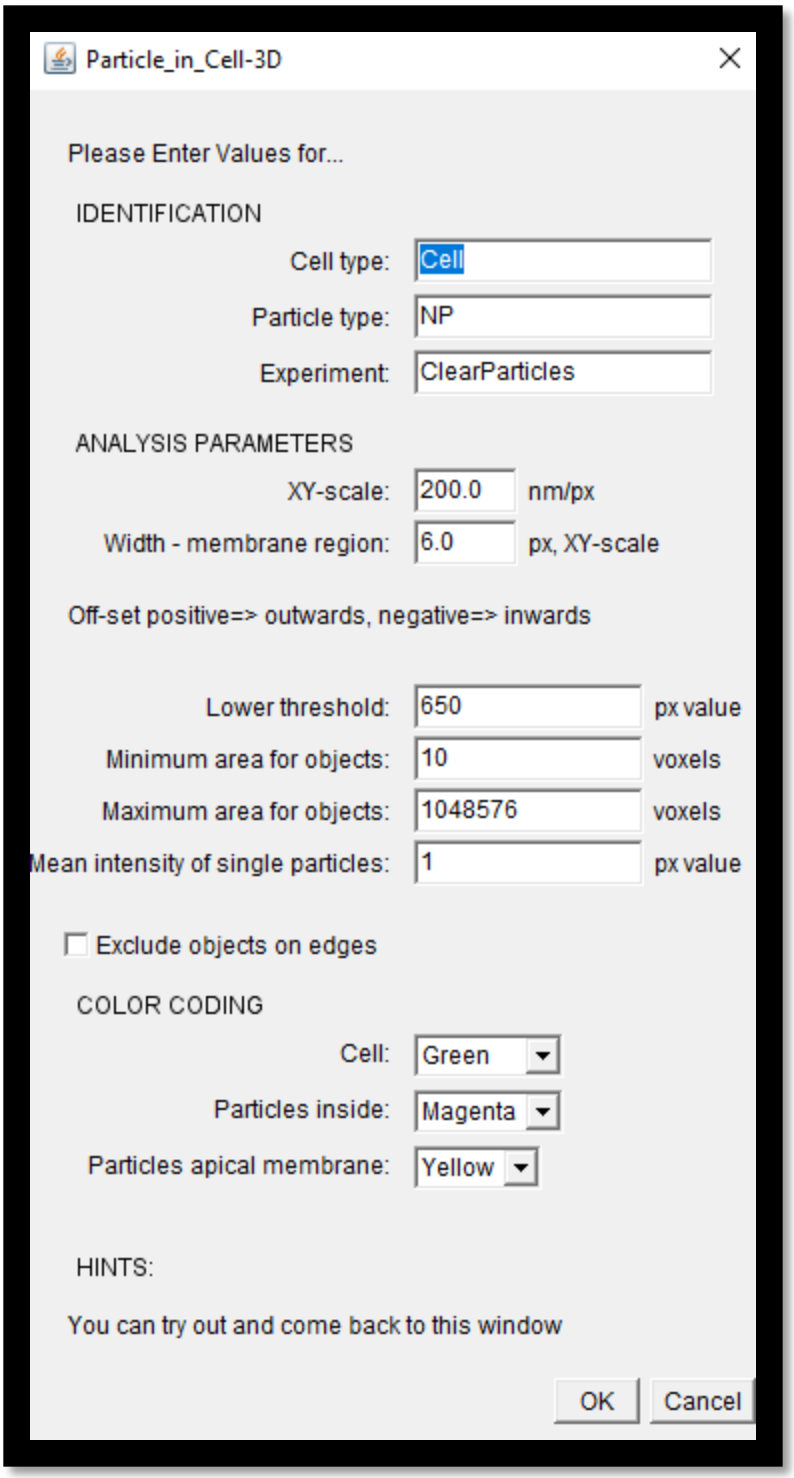


Choose the values to be used for the ratio of fluorescence intensity and the calibration factor.

These parameters are usually obtained through flow cytometry. For more information review:

Gottstein, C., Wu, G., Wong, B. J., & Zasadzinski, J. A. (2013). Precise quantification of nanoparticle internalization. ACS nano, 7(6), 4933-4945

6. Select parameters.



Choose the parameters to be used for the analysis.

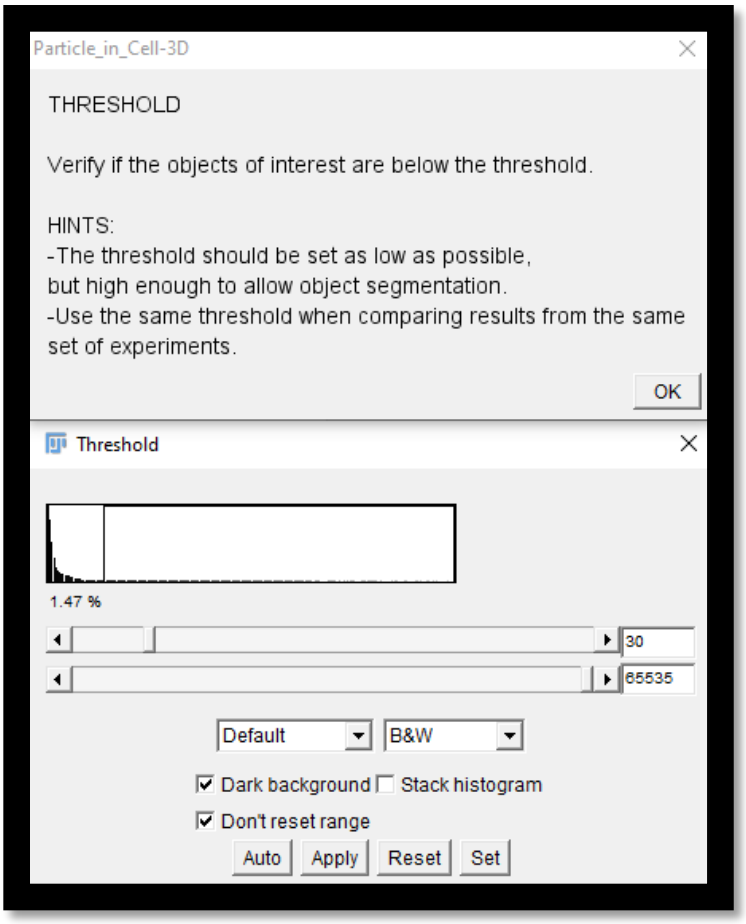
- *Cell Type.* Name to save intermediate steps cells images.
- *Particle Type.* Name to save intermediate steps particles images.
- *Experiment.* Name to identify the current experiment.
- *XY Scale.* Image XY scale. Used for conversion between pixels and nanometers.
- *Membrane region width.* Width to use as the membrane region.

- *Lower threshold.* Value to use to threshold the particles. Can be previewed and changed later.
- *Minimum object area.* Minimum area of objects to consider.
- *Maximum object area.* Maximum area of objects to consider.
- *Mean particle intensity.* Mean particle intensity. Obtained from calibration experiments. Used for the absolute quantification of internalized particles.
- *Exclude objects on edges.* Whether to include in the quantification objects on the edge of the regions of interest.
- *Cell color.* Choose the color by which to identify the cell.
- *Particles inside color.* Choose the color by which to code intracellular particles.
- *Particles Apical Membrane color.* Choose the color by which to code membrane particles.

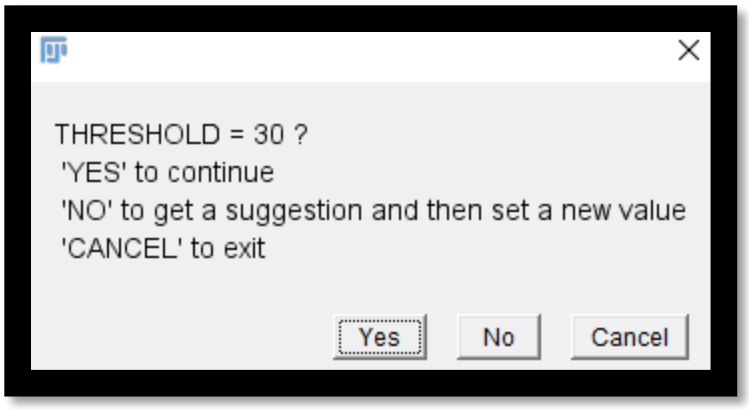
7. **Threshold particles.**

Threshold the particles. The value entered in the precious step is used by default. A preview is visualized where the user can manually change the threshold value. When a satisfactory value is not found a new value will be suggested and the user will return to the previous step where the suggestions is set as default, but it can be changed by the user. This process is repeated until a satisfactory value is found.

The aim is to set the threshold as low as possible while still allowing for object segmentation



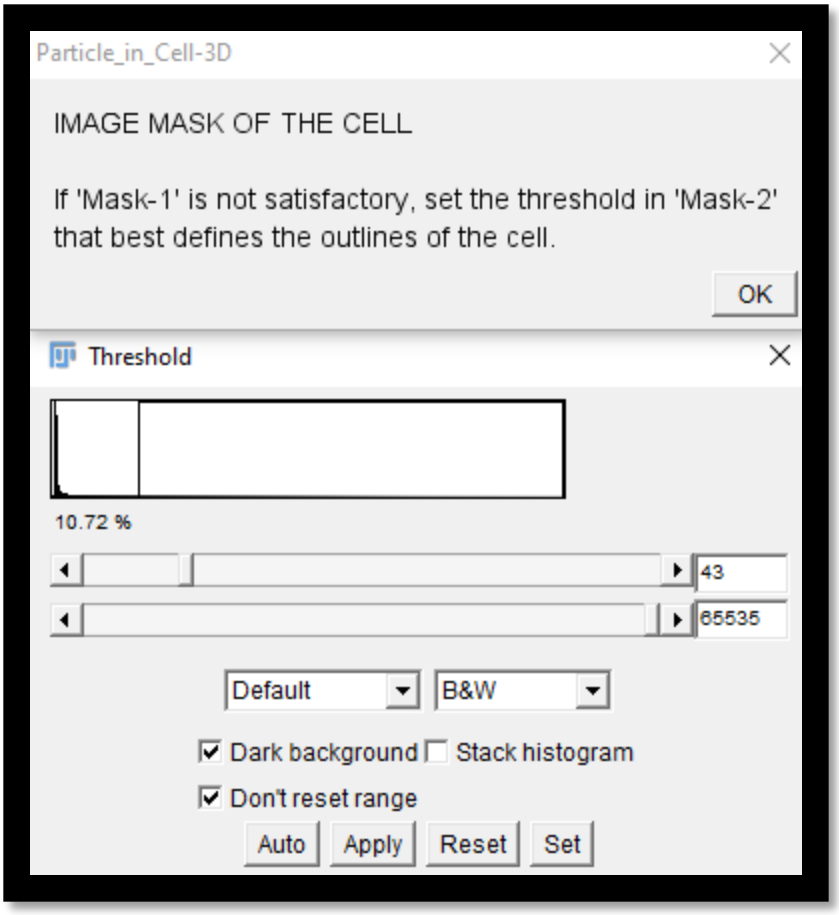
Before clicking "OK", make sure that the thresholding is satisfactory. If not, it can be manually changed before clicking "OK".



Click "YES" if satisfied with the current thresholding value. "No" to get a new suggestion and set a new value.

8. **Threshold cells.**
   An automatic mask of the cells is generated. However, a second image is opened where the user can set a threshold value manually.

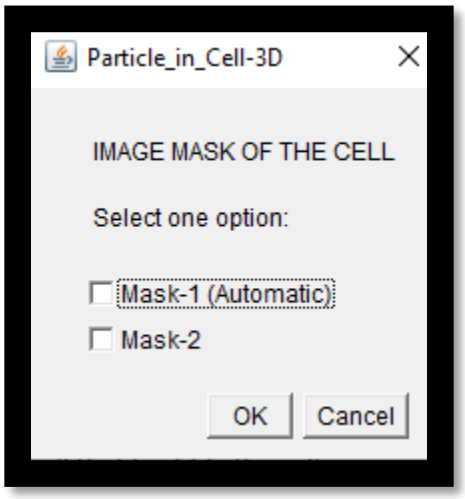
   The objective is to achieve a mask that accurately represents the cell.



If the automatic thresholding (Mask-1) is unsatisfactory. A manual thresholding (Mask-2) can be done before clicking "OK".

9. **Select best cell mask.**
   Selection of the mask of the cell that best fits the cells.
   - *Mask 1.* Automatic.

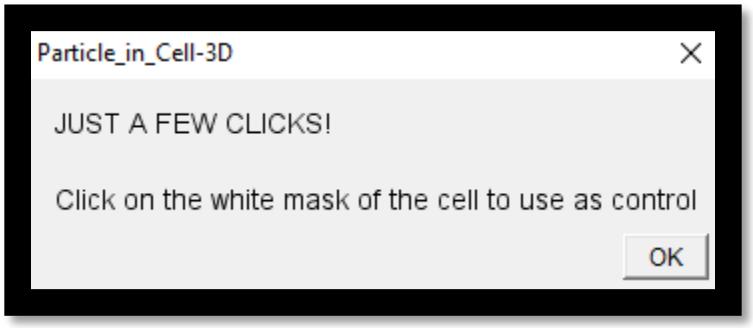
- *Mask 2.* User defined.



Select best representation of the cell.

10. Select cells to analyze.

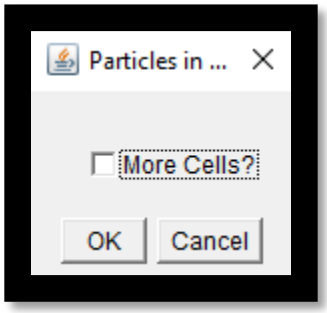
Selection of one or more cells to be analyzed.



Before clicking "OK" select a cell to be analyzed by clicking on it.

If Multiple Cells was selected.

After selecting one cell a prompt will appear asking the user if he wants to select more cells for analysis



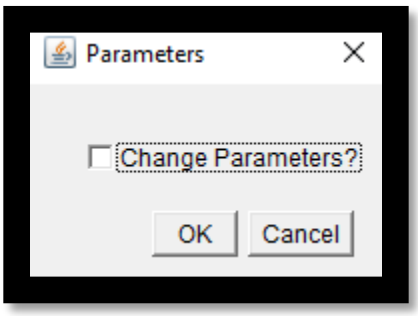
If more than once cell is to be analyzed, check the "More Cells?" box before clicking "OK". This will allow for a new cell to be selected. Repeat for as many cells as desired. When done simply leave the "More Cells?" box unchecked and click "OK".

If Multiple Images was selected.

Select next images to be analyzed and new results directory to save the results from the new images.

When choosing more images there is the option to either keep the same parameters as for the previous analysis or to choose new ones.

If another image is to be analyzed, check the "More Images?" box before clicking "OK". This will allow for a new image to be selected. Repeat for as many images as desired. When done simply leave the "More Images?" box unchecked and click "OK".



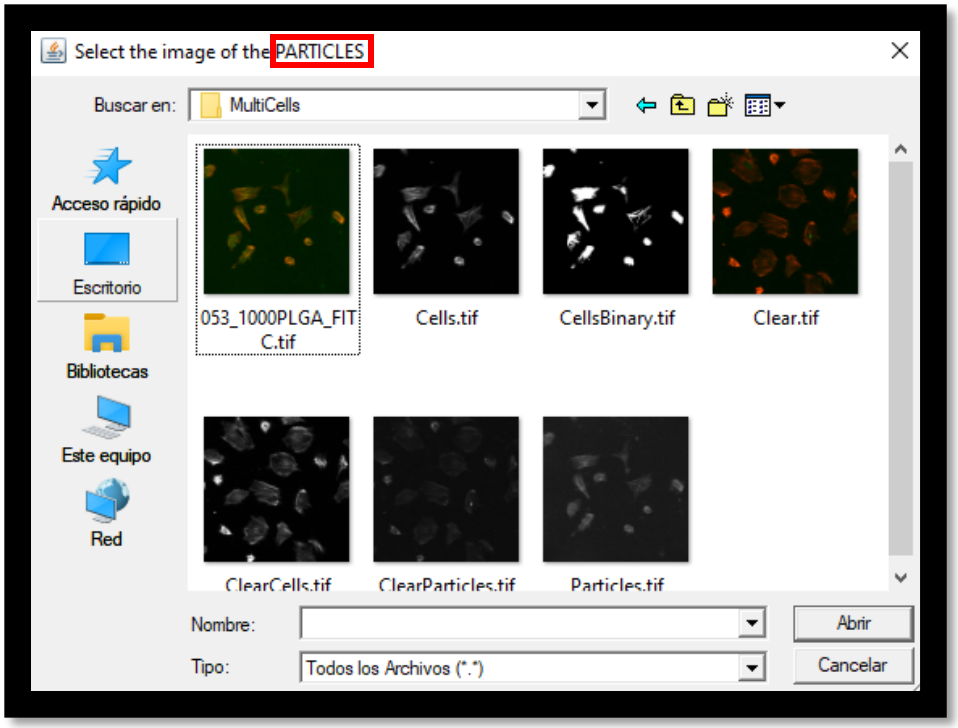
If the same parameters as the previous analysis are desired leave the box unchecked. If new parameters are desired check the box before clicking "OK".

Routine 4. Calibration.

Used to determine the mean particle intensity to be used for uptake quantification experiments.

1. **Select additional options.**
   Additional options for the fast analysis of multiple cells and images are available. As optional additional parameters for a more precise quantification.
   - *Multiple Cells.* Allows for selection of more than one cell in the same image for analysis.
   - *Precise Quantification.* Allows for fluorescence ratio and calibration factor as additional parameter for more accurate results.
   - *Multiple Images.* Allows analysis of more than one image.
   - *Control Cells.* Allows for the selection of control cell to use as background subtraction for the particles.
   - *Background Subtraction.* Allows for personalized values of background to be subtracted from the particles.
2. **Select particles image.**

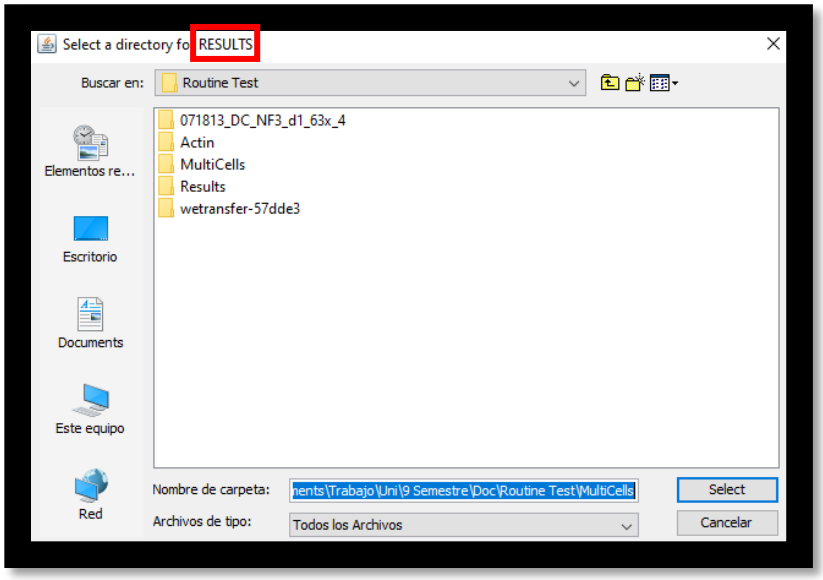
A file browser opens where the particles channel image to be analyzed must be selected.



Browse to the desired particles image.

3. Select Results directory

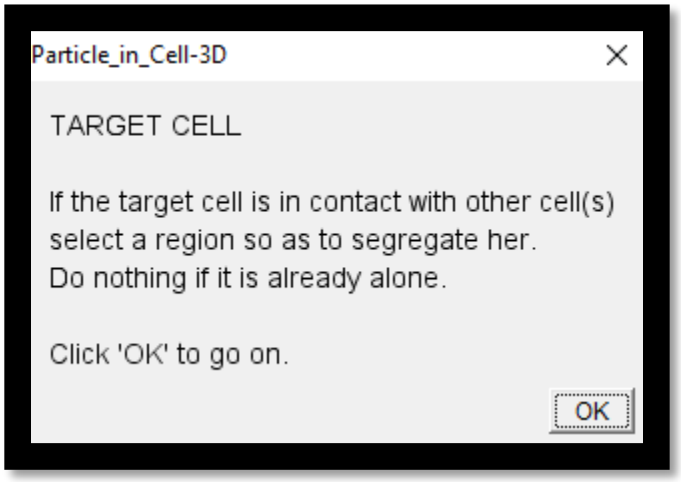
A file browser opens where the desired results directory must be selected.



Browse to the desired results directory.

4. Select target area.

Select the region of interest within the image selected. If the region of interest is the whole image do not select anything.

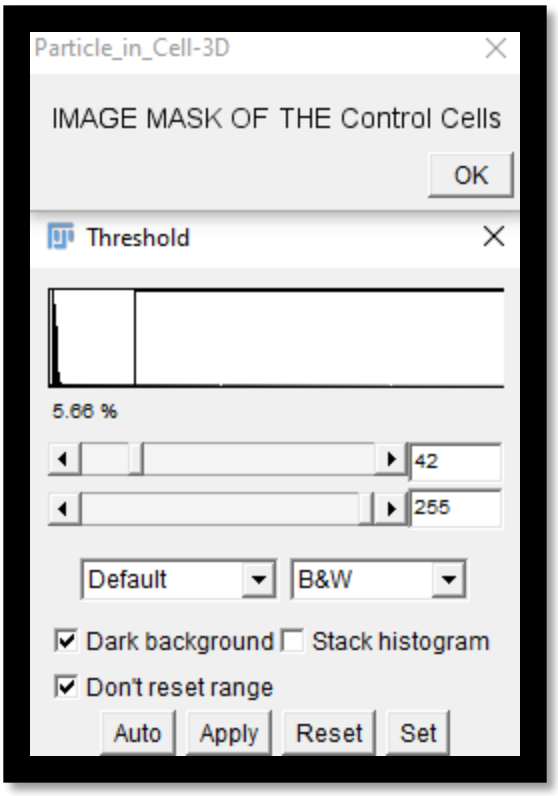


Before clicking "OK", make sure the target area is selected. If the target area is the whole image, then just click "OK".

If Control Cells was selected

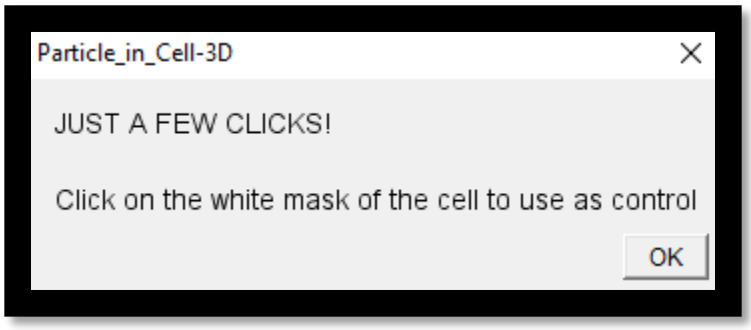
When using the Control Cells feature:

- Threshold control cells image.

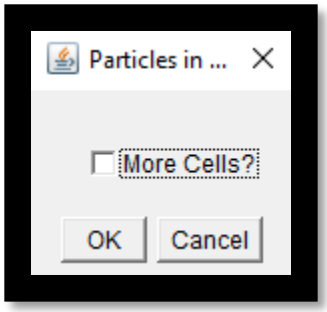


Threshold the control cells image before clicking "OK". Once a satisfactory threshold has been selected click "OK".

- Select cells to use as control.



Before clicking "OK" select a cell to use as control by clicking on it.



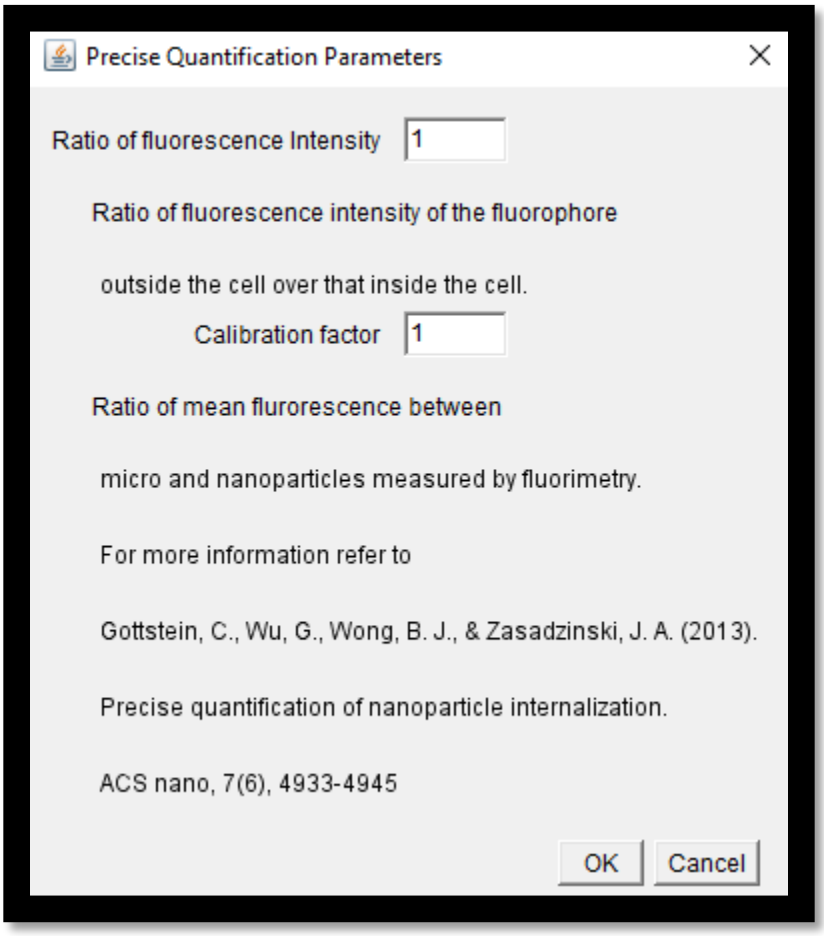
If more than once cell is to be used as control, check the "More Cells?" box before clicking "OK". This will allow for a new cell to be selected. Repeat for as many cells as desired. When done simply leave the "More Cells?" box unchecked and click "OK".

If Precise Quantification was selected

The routine allows for a more precise quantification by means of two parameters. A ratio of fluorescence intensity and a calibration factor. The ratio is the change of fluorescence intensity some dyes experience when going from outside the cell to inside the cell. The calibration factor is used mainly when the mean intensity is obtained from microparticles by flow cytometry. The calibration factor relates the intensity of the microparticles to that that would be obtained by nanoparticles. This calibration factor is the ratio of the mean fluorescence of micro and nanoparticles as measured by fluorimetry at the same conditions as the ones used at flow cytometry.

When using the Precise Quantification feature:

- Choose ratio of fluorescence and calibration factor parameters.

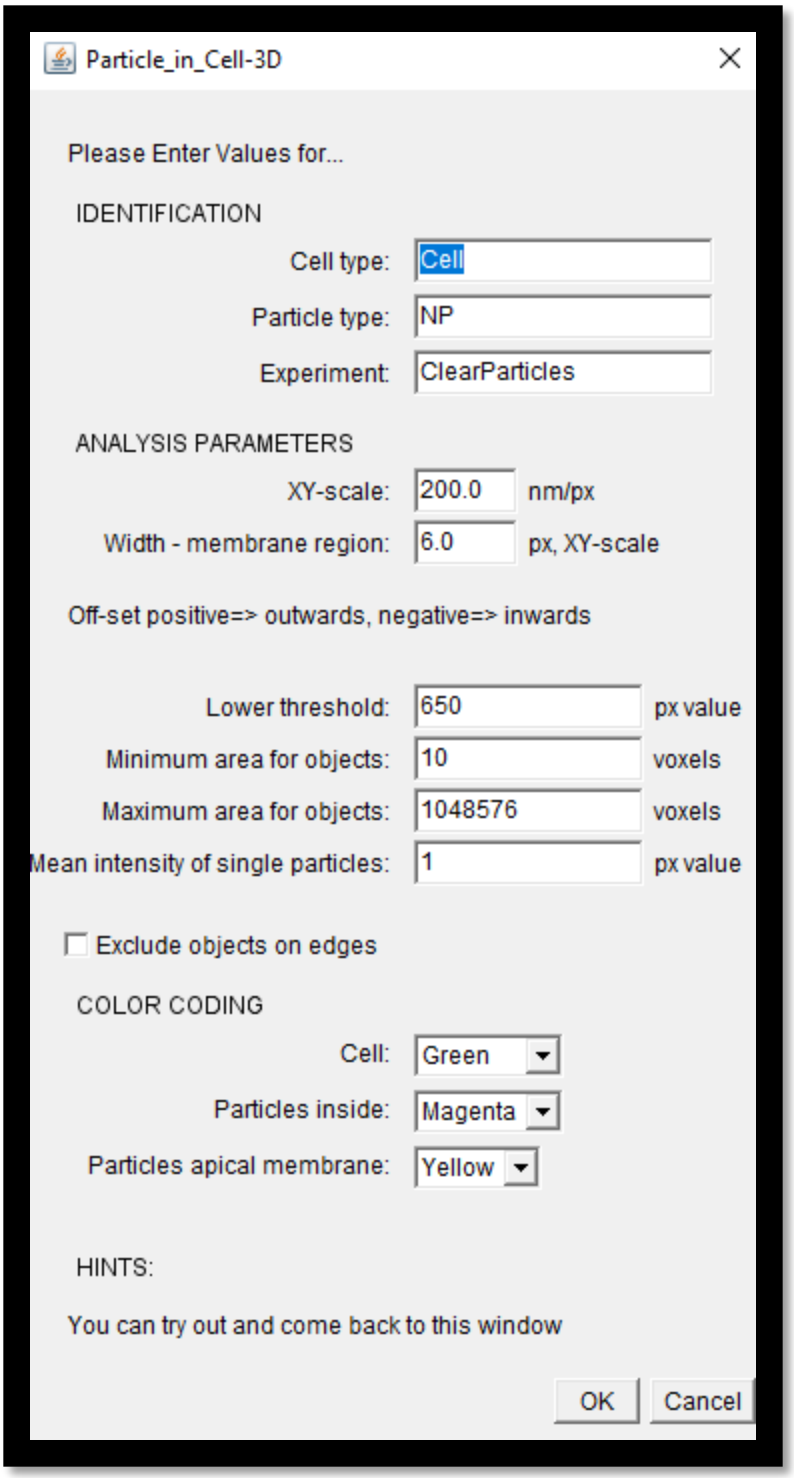


Choose the values to be used for the ratio of fluorescence intensity and the calibration factor.

These parameters are usually obtained through flow cytometry. For more information review:

Gottstein, C., Wu, G., Wong, B. J., & Zasadzinski, J. A. (2013). Precise quantification of nanoparticle internalization. ACS nano, 7(6), 4933-4945

5. Select parameters.



Choose the parameters to be used for the analysis.

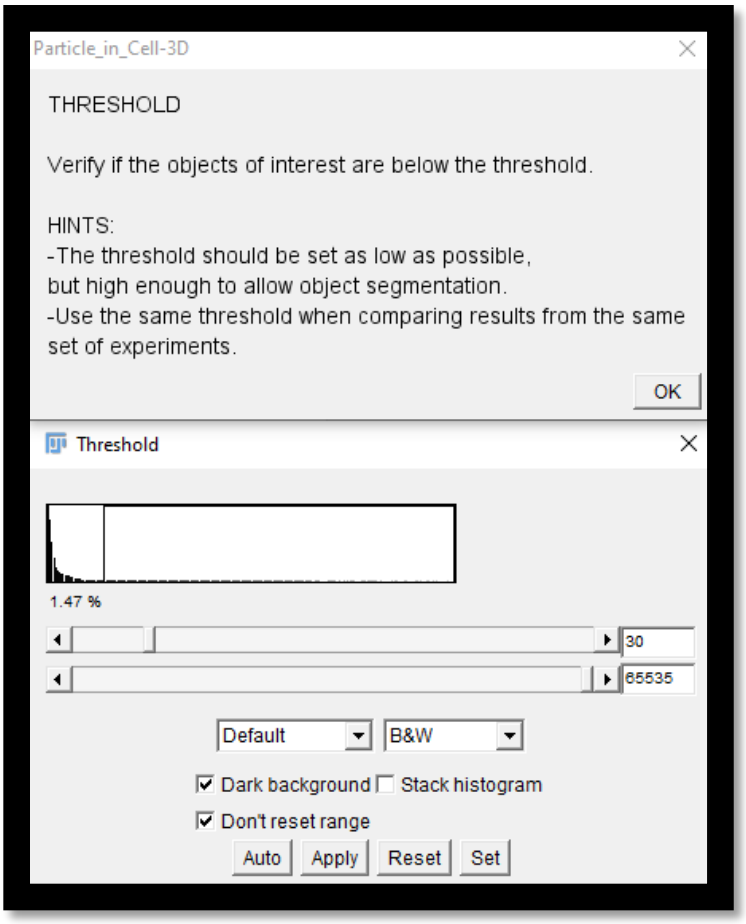
- *Cell Type.* Name to save intermediate steps cells images.
- *Particle Type.* Name to save intermediate steps particles images.
- *Experiment.* Name to identify the current experiment.
- *XY Scale.* Image XY scale. Used for conversion between pixels and nanometers.
- *Membrane region width.* Width to use as the membrane region.

- *Lower threshold.* Value to use to threshold the particles. Can be previewed and changed later.
- *Minimum object area.* Minimum area of objects to consider.
- *Maximum object area.* Maximum area of objects to consider.
- *Mean particle intensity.* Mean particle intensity. Obtained from calibration experiments. Used for the absolute quantification of internalized particles.
- *Exclude objects on edges.* Whether to include in the quantification objects on the edge of the regions of interest.
- *Cell color.* Choose the color by which to identify the cell.
- *Particles inside color.* Choose the color by which to code intracellular particles.
- *Particles Apical Membrane color.* Choose the color by which to code membrane particles.

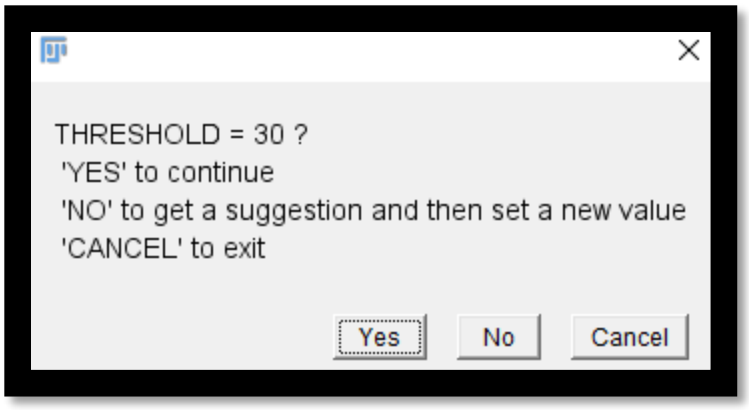
6. **Threshold particles.**

Threshold the particles. The value entered in the precious step is used by default. A preview is visualized where the user can manually change the threshold value. When a satisfactory value is not found a new value will be suggested and the user will return to the previous step where the suggestions is set as default, but it can be changed by the user. This process is repeated until a satisfactory value is found.

The aim is to set the threshold as low as possible while still allowing for object segmentation



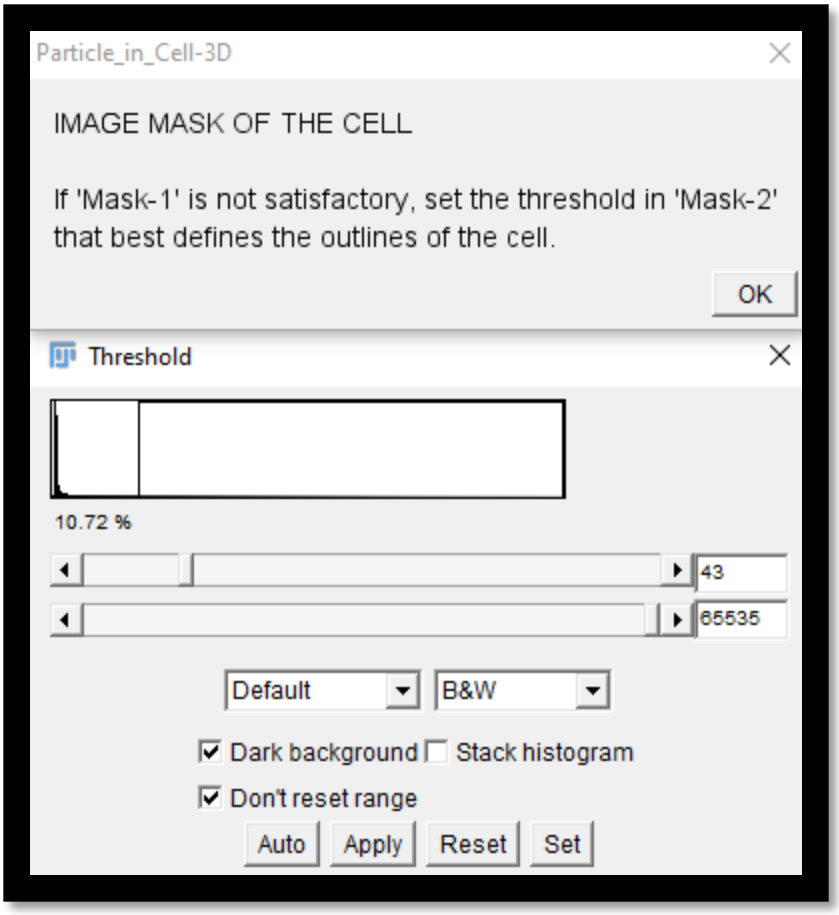
Before clicking "OK", make sure that the thresholding is satisfactory. If not, it can be manually changed before clicking "OK".



Click "YES" if satisfied with the current thresholding value. "No" to get a new suggestion and set a new value.

7. **Threshold cells.**
   An automatic mask of the cells is generated. However, a second image is opened where the user can set a threshold value manually.

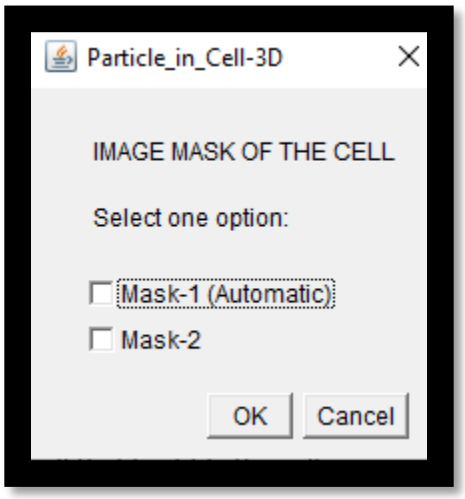
   The objective is to achieve a mask that accurately represents the cell.



If the automatic thresholding (Mask-1) is unsatisfactory. A manual thresholding (Mask-2) can be done before clicking "OK".

8. **Select best cell mask.**
   Selection of the mask of the cell that best fits the cells.
   - *Mask 1.* Automatic.

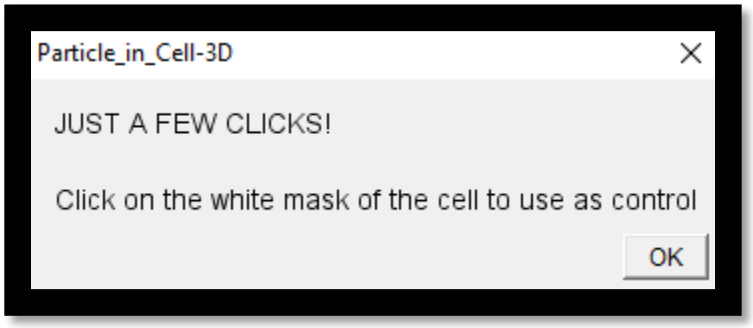
- *Mask 2.* User defined.



Select best representation of the cell.

9. Select cells to analyze.

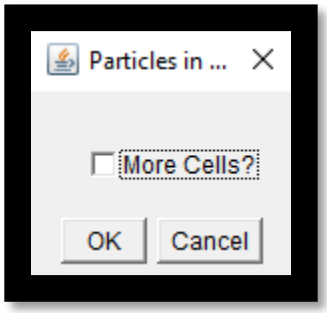
Selection of one or more cells to be analyzed.



Before clicking "OK" select a cell to be analyzed by clicking on it.

If Multiple Cells was selected.

After selecting one cell a prompt will appear asking the user if he wants to select more cells for analysis



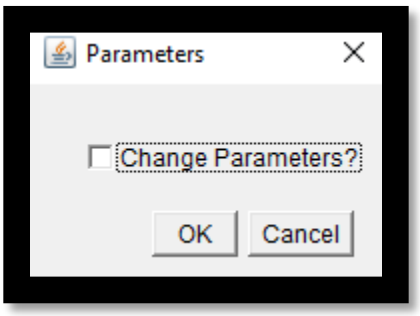
If more than once cell is to be analyzed, check the "More Cells?" box before clicking "OK". This will allow for a new cell to be selected. Repeat for as many cells as desired. When done simply leave the "More Cells?" box unchecked and click "OK".

If Multiple Images was selected.

Select next images to be analyzed and new results directory to save the results from the new images.

When choosing more images there is the option to either keep the same parameters as for the previous analysis or to choose new ones.

If another image is to be analyzed, check the "More Images?" box before clicking "OK". This will allow for a new image to be selected. Repeat for as many images as desired. When done simply leave the "More Images?" box unchecked and click "OK".



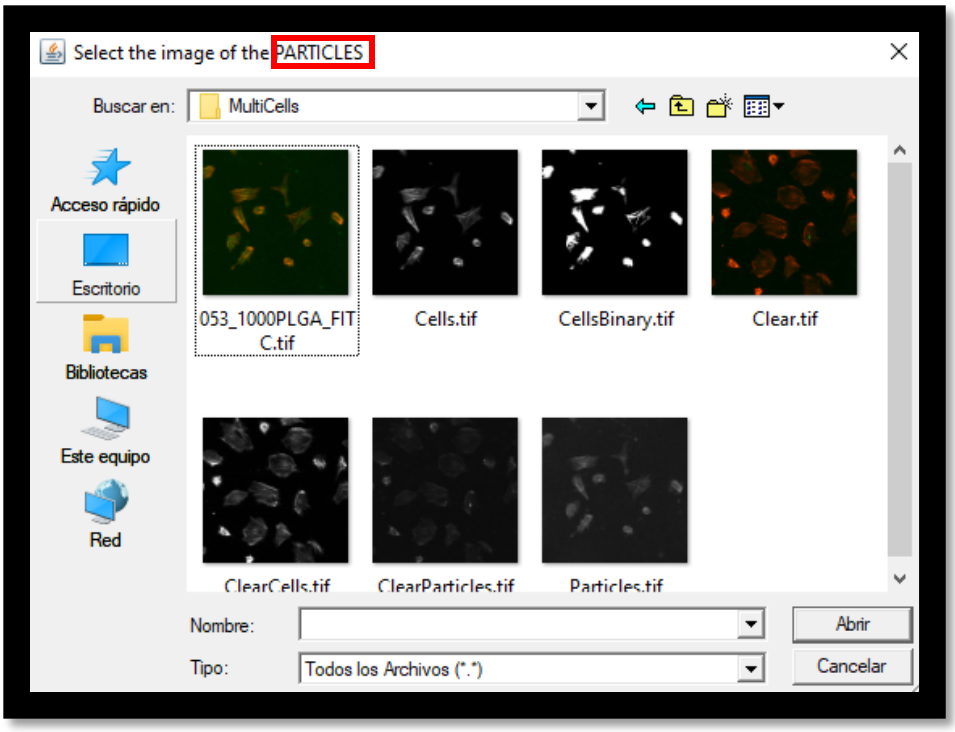
If the same parameters as the previous analysis are desired leave the box unchecked. If new parameters are desired check the box before clicking "OK".

Routine 5. Only Particles

Count the absolute number of particles in cell free regions.

1. **Select additional options.**
   Additional options for the fast analysis of multiple cells and images are available. As optional additional parameters for a more precise quantification.
   - *Multiple Cells.* Allows for selection of more than one cell in the same image for analysis.
   - *Precise Quantification.* Allows for fluorescence ratio and calibration factor as additional parameter for more accurate results.
   - *Multiple Images.* Allows analysis of more than one image.
   - *Control Cells.* Allows for the selection of control cell to use as background subtraction for the particles.
   - *Background Subtraction.* Allows for personalized values of background to be subtracted from the particles.
2. **Select particles image.**

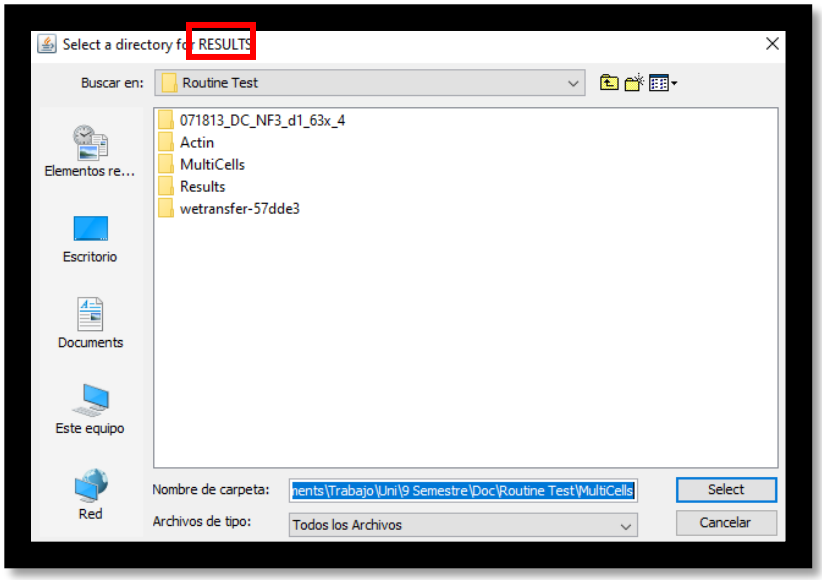
A file browser opens where the particles channel image to be analyzed must be selected.



Browse to the desired particles image.

3. **Select Results directory**

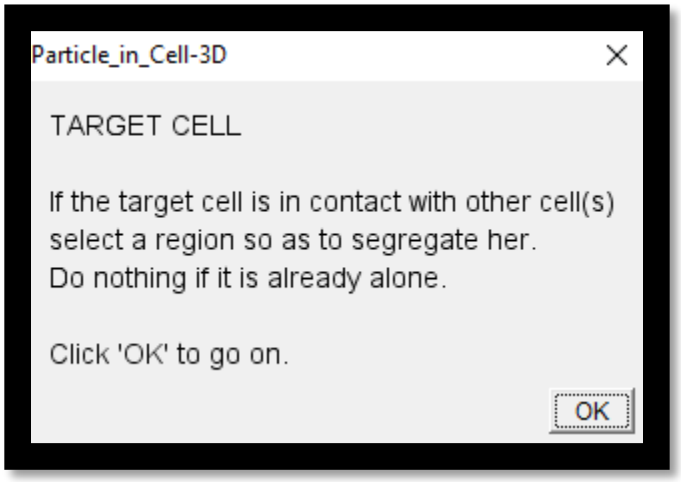
A file browser opens where the desired results directory must be selected.



Browse to the desired results directory.

4. **Select target area.**

Select the region of interest within the image selected. If the region of interest is the whole image do not select anything.

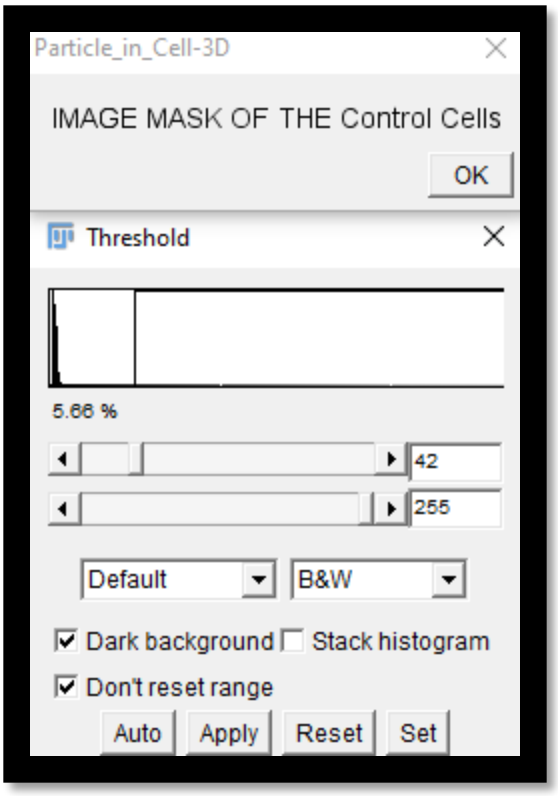


Before clicking "OK", make sure the target area is selected. If the target area is the whole image, then just click "OK".

If Control Cells was selected

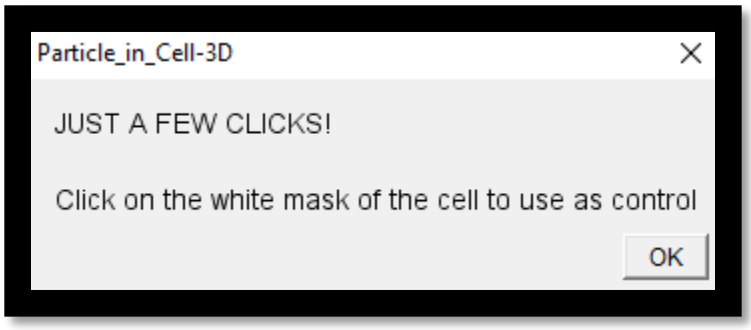
When using the Control Cells feature:

- Threshold control cells image.

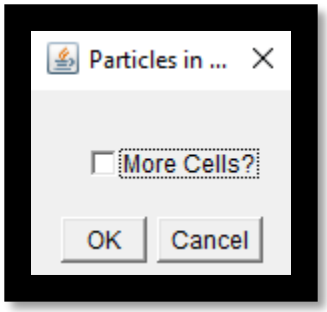


Threshold the control cells image before clicking "OK". Once a satisfactory threshold has been selected click "OK".

- Select cells to use as control.



Before clicking "OK" select a cell to use as control by clicking on it.



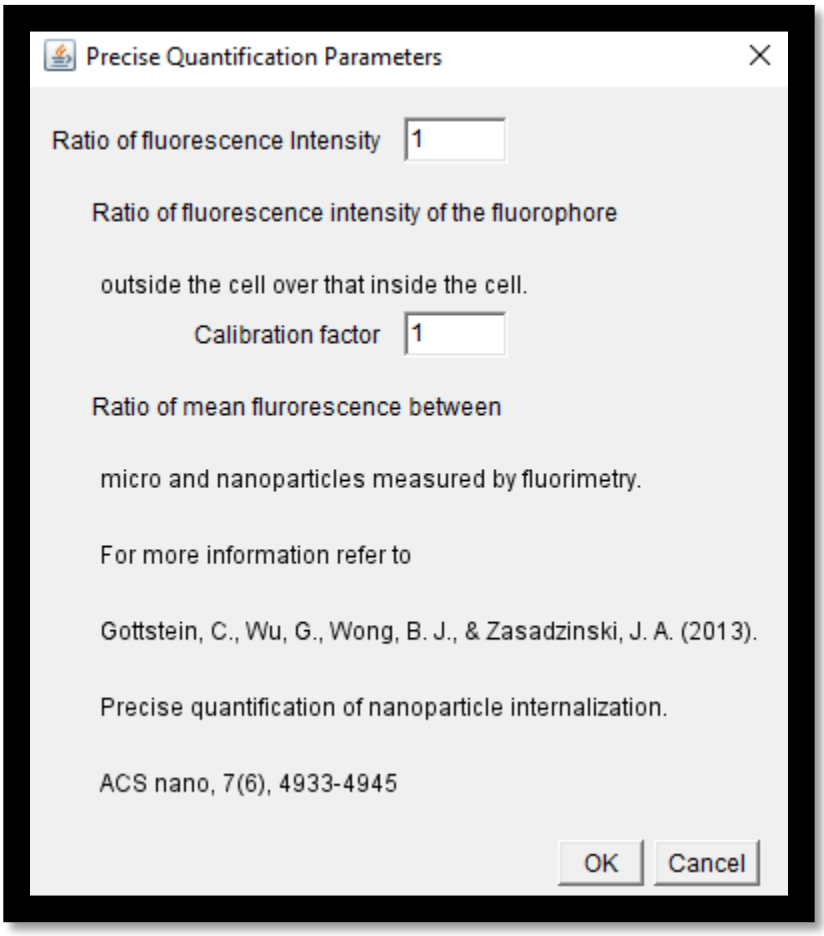
If more than once cell is to be used as control, check the "More Cells?" box before clicking "OK". This will allow for a new cell to be selected. Repeat for as many cells as desired. When done simply leave the "More Cells?" box unchecked and click "OK".

If Precise Quantification was selected

The routine allows for a more precise quantification by means of two parameters. A ratio of fluorescence intensity and a calibration factor. The ratio is the change of fluorescence intensity some dyes experience when going from outside the cell to inside the cell. The calibration factor is used mainly when the mean intensity is obtained from microparticles by flow cytometry. The calibration factor relates the intensity of the microparticles to that that would be obtained by nanoparticles. This calibration factor is the ratio of the mean fluorescence of micro and nanoparticles as measured by fluorimetry at the same conditions as the ones used at flow cytometry.

When using the Precise Quantification feature:

- Choose ratio of fluorescence and calibration factor parameters.

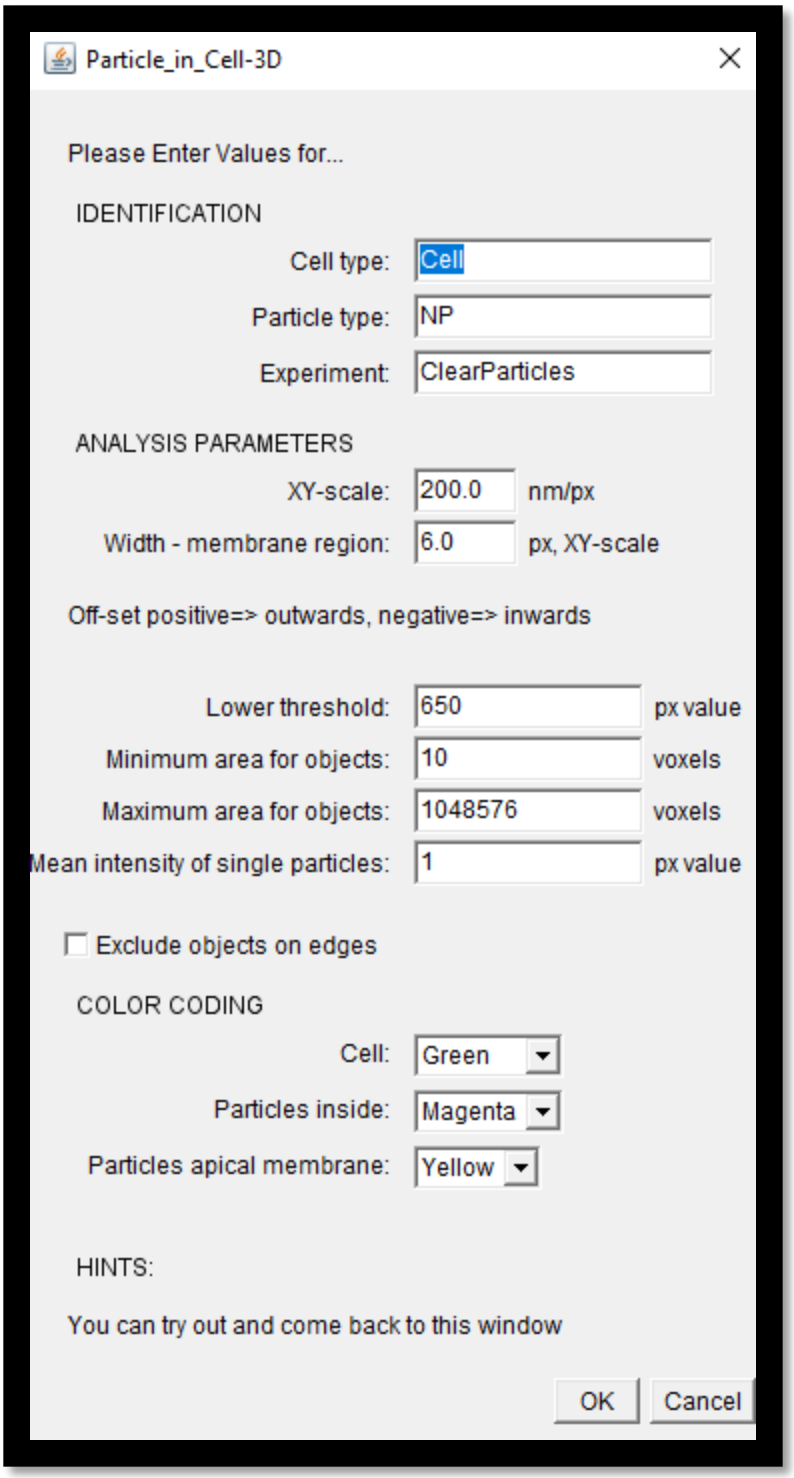


Choose the values to be used for the ratio of fluorescence intensity and the calibration factor.

These parameters are usually obtained through flow cytometry. For more information review:

Gottstein, C., Wu, G., Wong, B. J., & Zasadzinski, J. A. (2013). Precise quantification of nanoparticle internalization. ACS nano, 7(6), 4933-4945

5. Select parameters.



Choose the parameters to be used for the analysis.

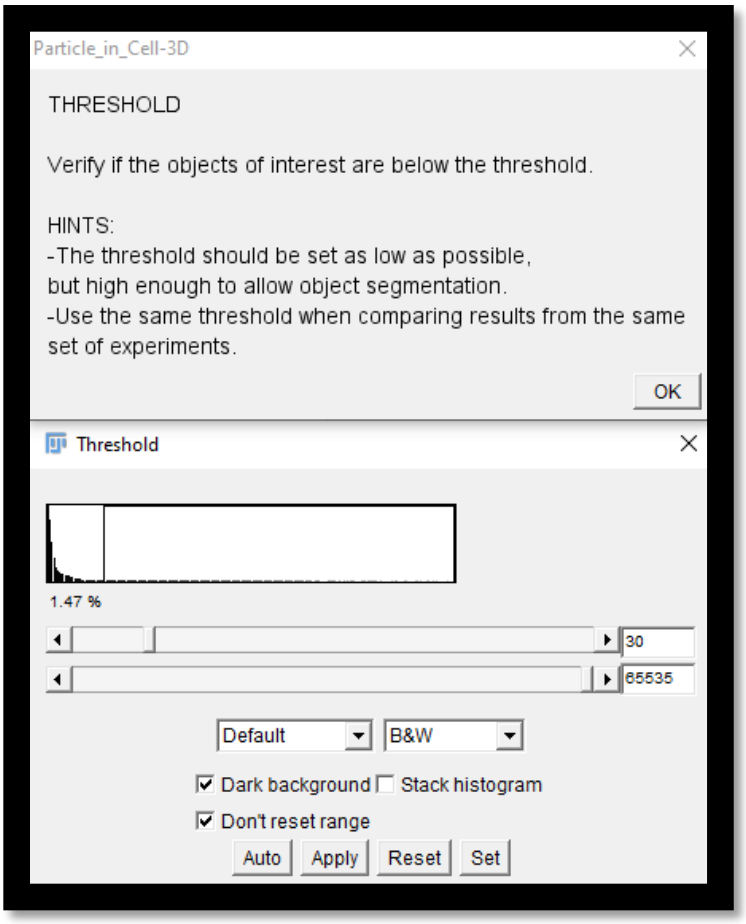
- *Cell Type.* Name to save intermediate steps cells images.
- *Particle Type.* Name to save intermediate steps particles images.
- *Experiment.* Name to identify the current experiment.
- *XY Scale.* Image XY scale. Used for conversion between pixels and nanometers.
- *Membrane region width.* Width to use as the membrane region.

- *Lower threshold.* Value to use to threshold the particles. Can be previewed and changed later.
- *Minimum object area.* Minimum area of objects to consider.
- *Maximum object area.* Maximum area of objects to consider.
- *Mean particle intensity.* Mean particle intensity. Obtained from calibration experiments. Used for the absolute quantification of internalized particles.
- *Exclude objects on edges.* Whether to include in the quantification objects on the edge of the regions of interest.
- *Cell color.* Choose the color by which to identify the cell.
- *Particles inside color.* Choose the color by which to code intracellular particles.
- *Particles Apical Membrane color.* Choose the color by which to code membrane particles.

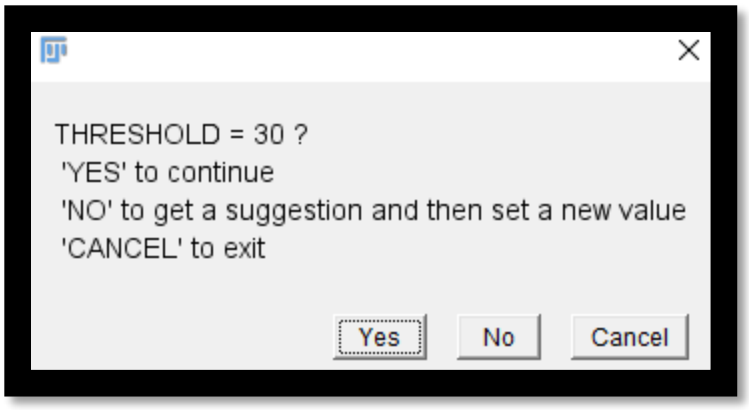
6. **Threshold particles.**

Threshold the particles. The value entered in the precious step is used by default. A preview is visualized where the user can manually change the threshold value. When a satisfactory value is not found a new value will be suggested and the user will return to the previous step where the suggestions is set as default, but it can be changed by the user. This process is repeated until a satisfactory value is found.

The aim is to set the threshold as low as possible while still allowing for object segmentation



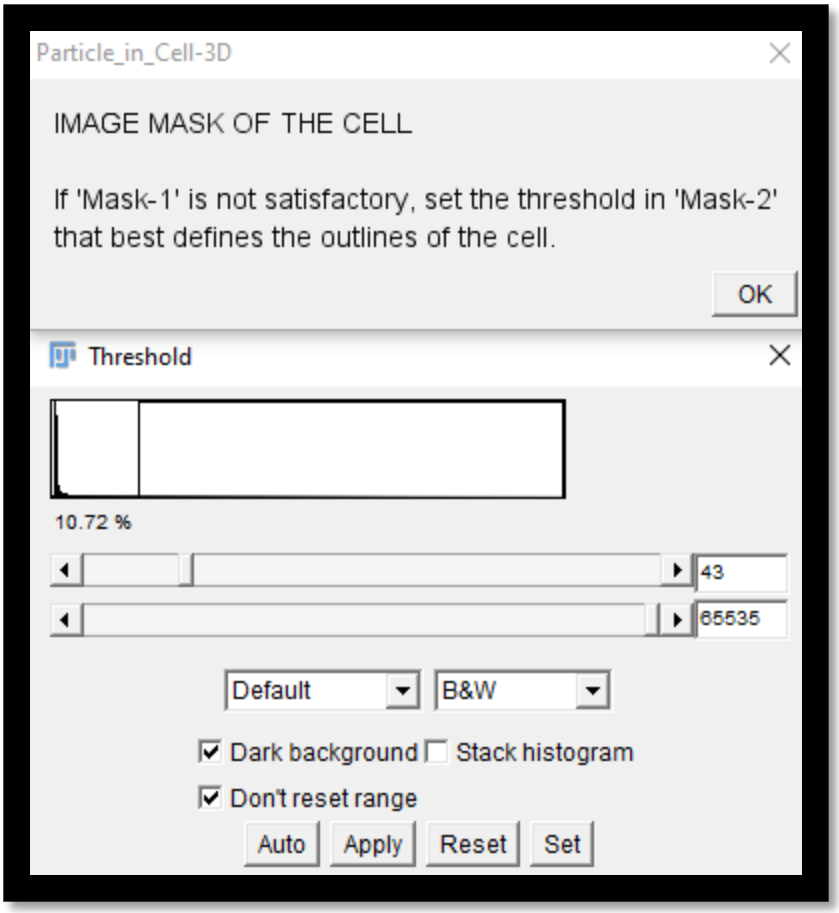
Before clicking "OK", make sure that the thresholding is satisfactory. If not, it can be manually changed before clicking "OK".



Click "YES" if satisfied with the current thresholding value. "No" to get a new suggestion and set a new value.

7. **Threshold cells.**
   An automatic mask of the cells is generated. However, a second image is opened where the user can set a threshold value manually.

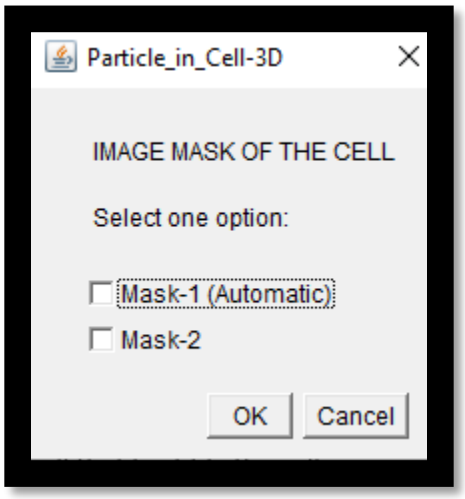
   The objective is to achieve a mask that accurately represents the cell.



If the automatic thresholding (Mask-1) is unsatisfactory. A manual thresholding (Mask-2) can be done before clicking "OK".

8. **Select best cell mask.**
   Selection of the mask of the cell that best fits the cells.
   - *Mask 1.* Automatic.

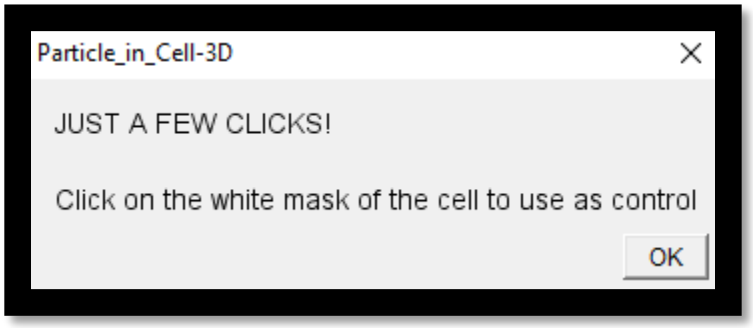
- *Mask 2.* User defined.



Select best representation of the cell.

9. Select cells to analyze.

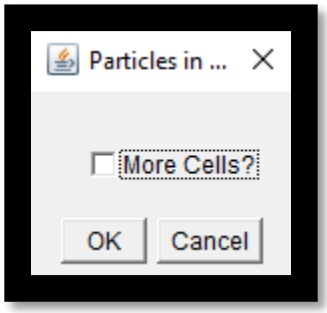
Selection of one or more cells to be analyzed.



Before clicking "OK" select a cell to be analyzed by clicking on it.

If Multiple Cells was selected.

After selecting one cell a prompt will appear asking the user if he wants to select more cells for analysis



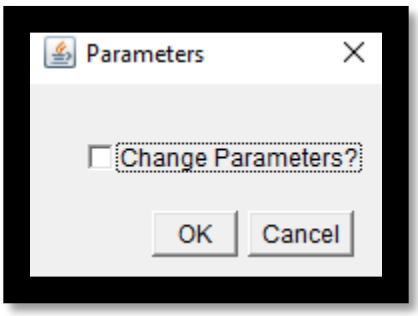
If more than once cell is to be analyzed, check the "More Cells?" box before clicking "OK". This will allow for a new cell to be selected. Repeat for as many cells as desired. When done simply leave the "More Cells?" box unchecked and click "OK".

If Multiple Images was selected.

Select next images to be analyzed and new results directory to save the results from the new images.

When choosing more images there is the option to either keep the same parameters as for the previous analysis or to choose new ones.

If another image is to be analyzed, check the "More Images?" box before clicking "OK". This will allow for a new image to be selected. Repeat for as many images as desired. When done simply leave the "More Images?" box unchecked and click "OK".



If the same parameters as the previous analysis are desired leave the box unchecked. If new parameters are desired check the box before clicking "OK".