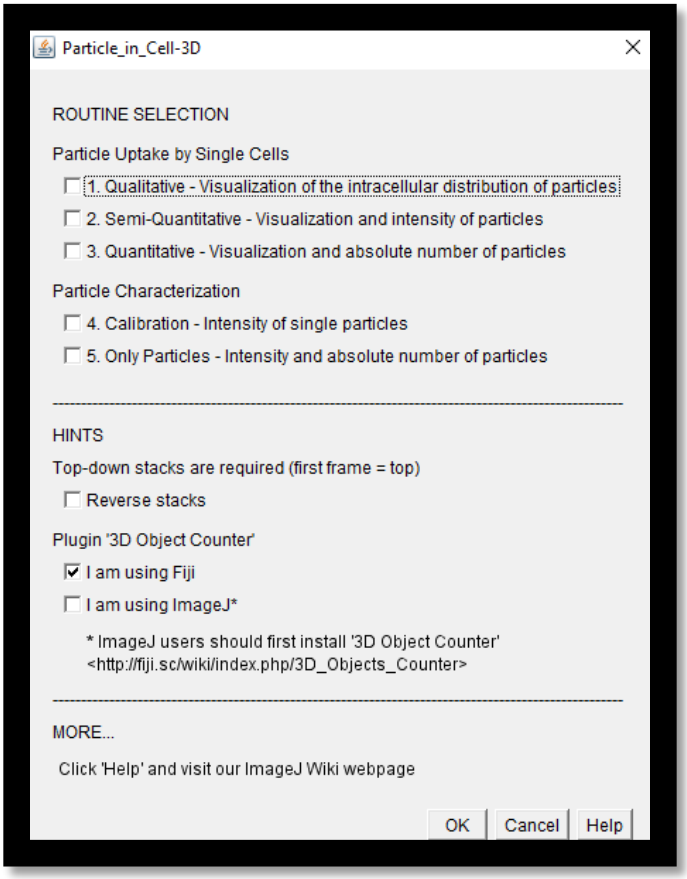
Particles in Cell (Stack) - User Manual

Particles in Cell is a FIJI (ImageJ) routine for a fast analysis method to quantify micro and nanoparticle uptake from a stack of 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.

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

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

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.

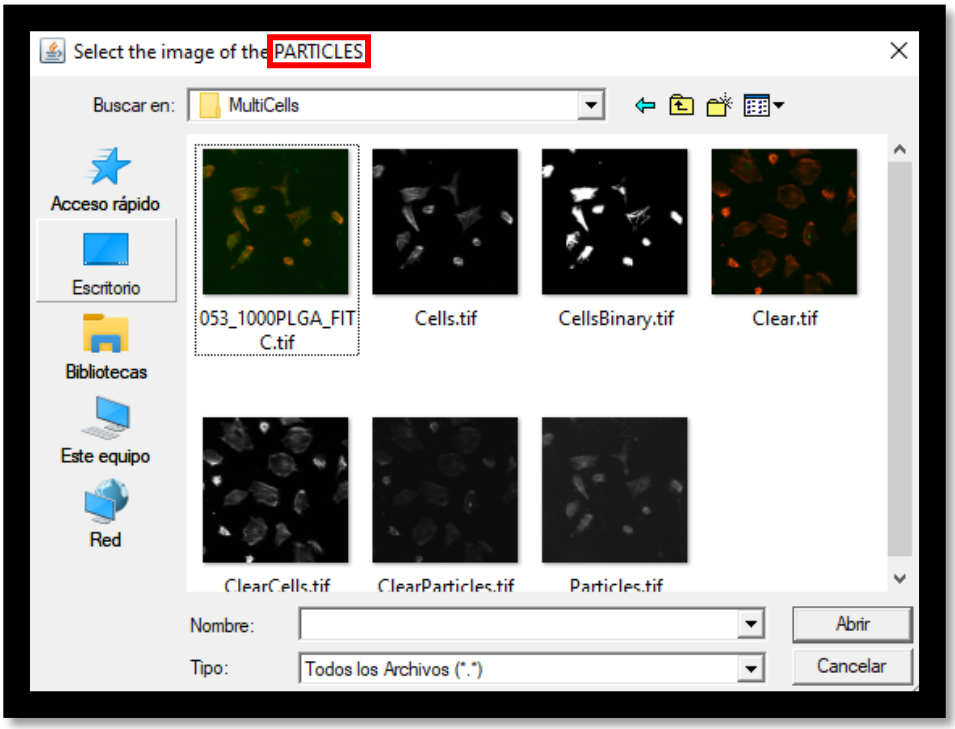
Top down stacks are required for the analysis. That is the first frame must be the top. If the stack is not top down check the "Reverse stacks" box.

Check the box of the program being used. It is highly recommended to use FIJI as the routine will not completely function in ImageJ.

Routine 1. Qualitative.

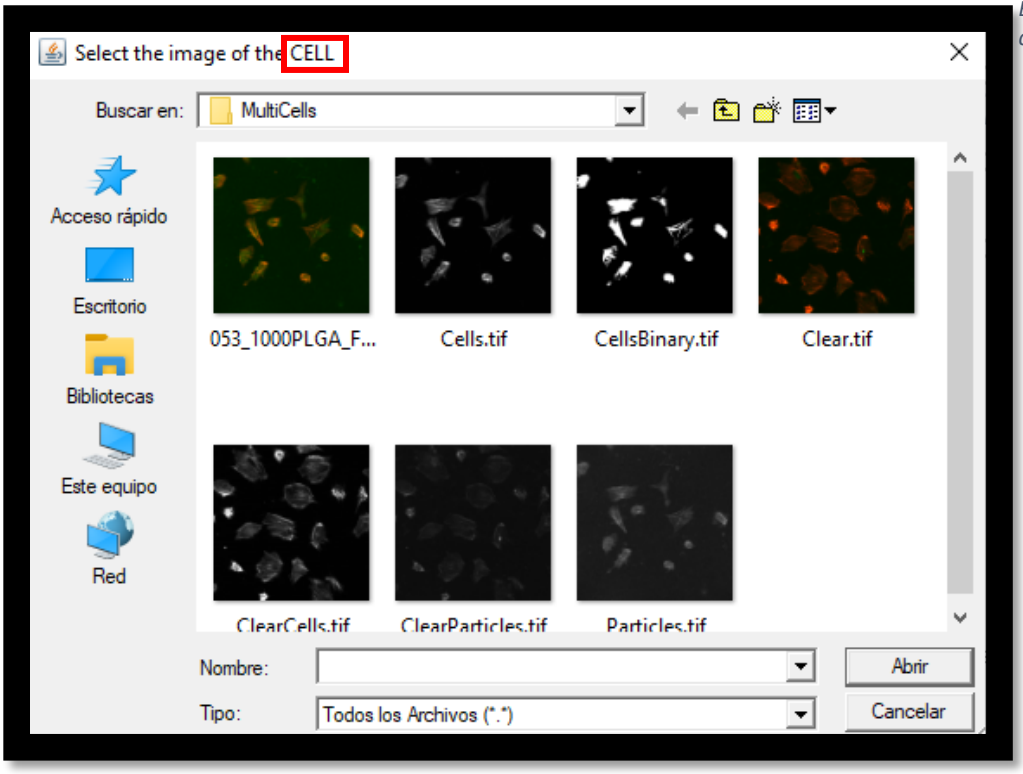
Visualization of the intracellular distribution of particles. Final visualization will be found on the results directory under _Uptake- *"Cell's image name".*

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



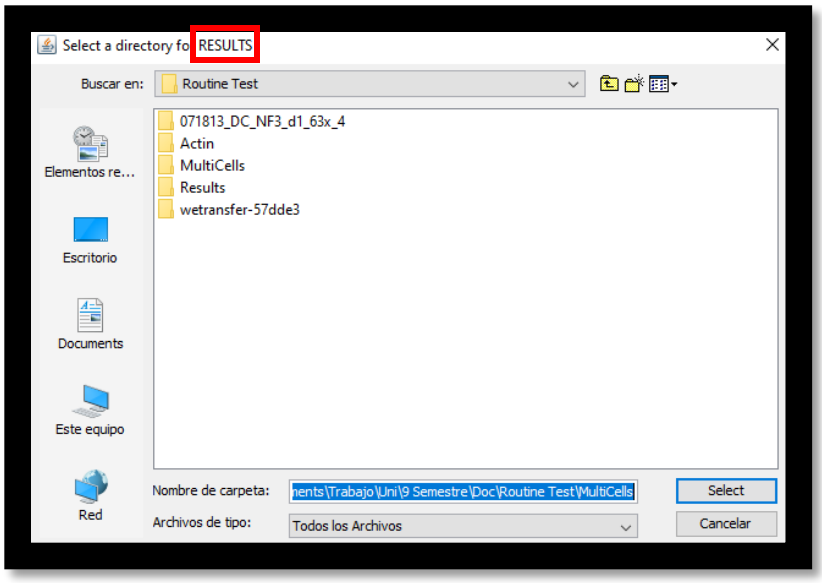
Browse to the desired particles stack.

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



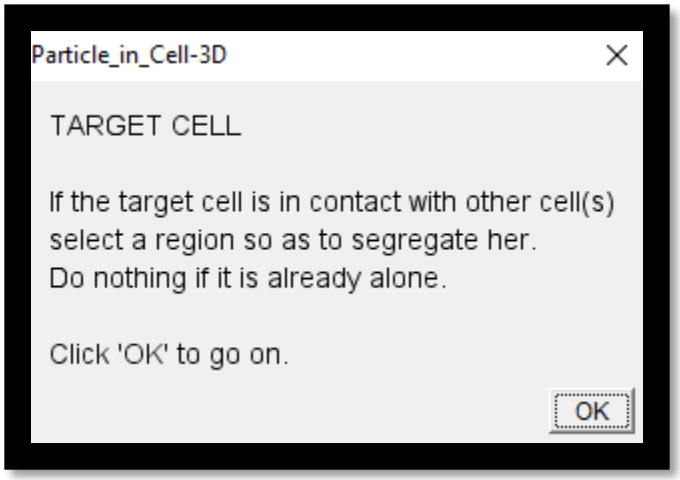
Browse to the desired cell stack.

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



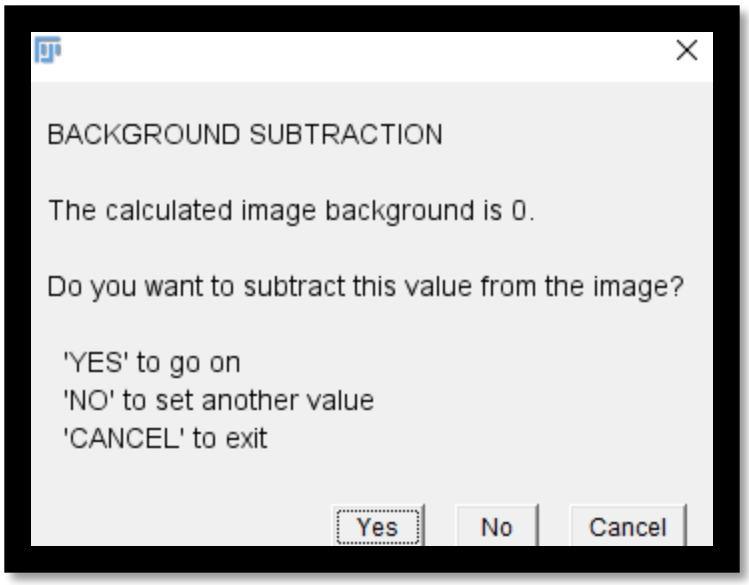
Browse to the desired results directory.

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.



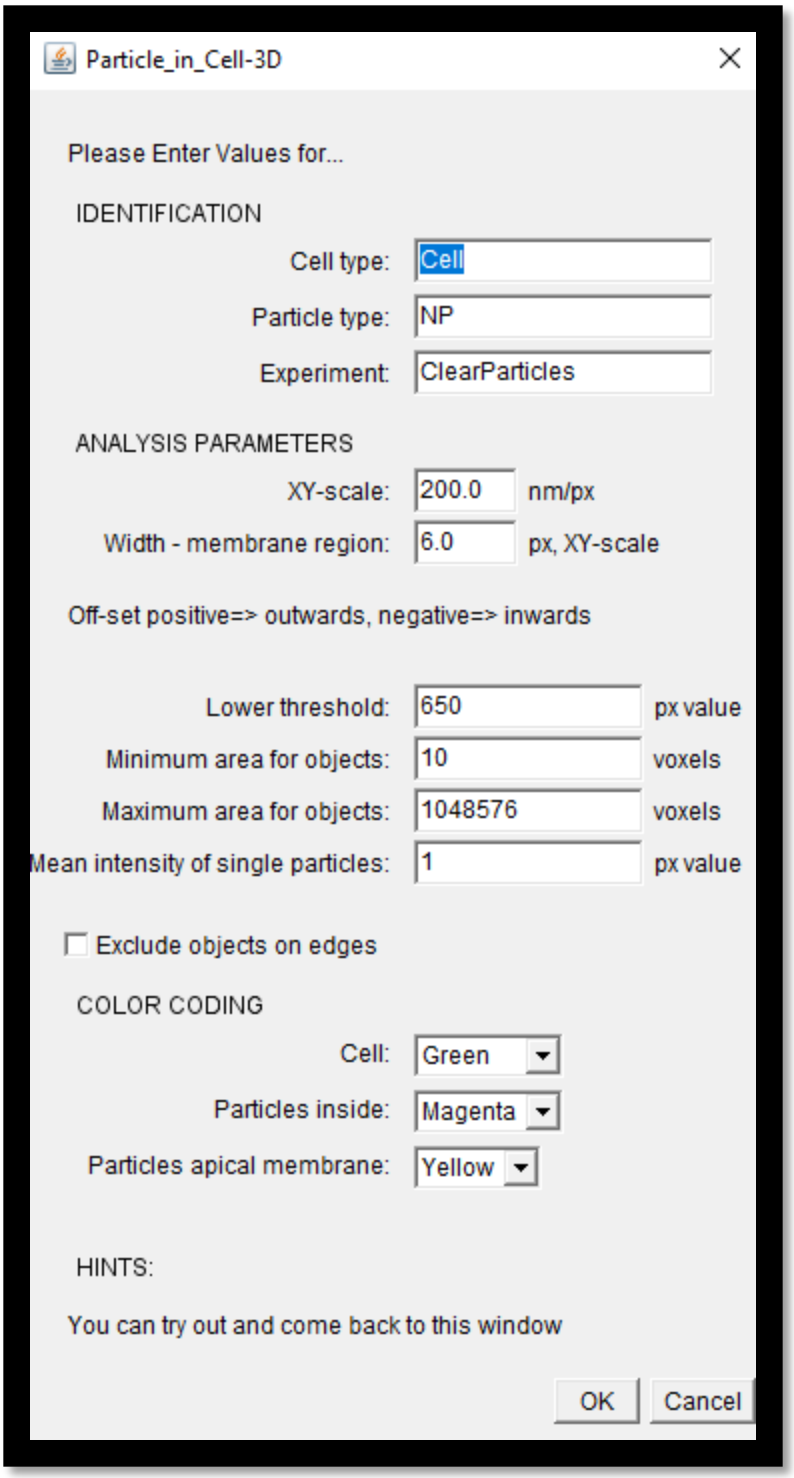
Before clicking "OK", make sure the target area is selected. If the target area is the whole image, then just click "OK".

5. **Background Subtraction**
   An automatic background value is calculated. The user can either choose this value or set a desired value manually.



Click "Yes" to use the automatic background value. Click "No" to set a new value manually.

6. **Select parameters.**



Choose the parameters to be used for the analysis.

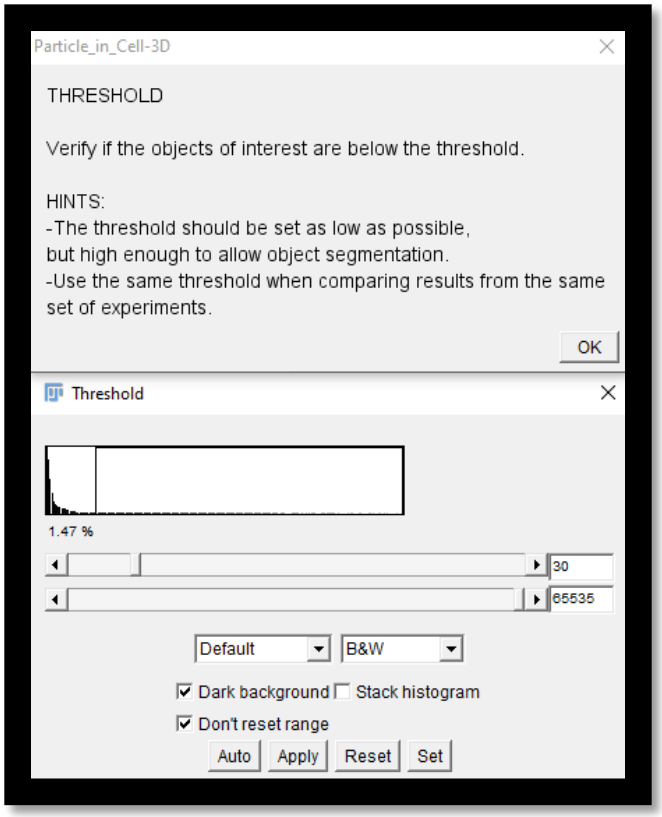
- *Cell Type.* Name to save intermediate steps cells images.
- *Particle Type.* Name to save intermediate steps particles images.
- *Experiment.* Name to identify the current experiment.
- *XY Scale.* Image XY scale. Used for conversion between pixels and nanometers.
- *Membrane region width.* Width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

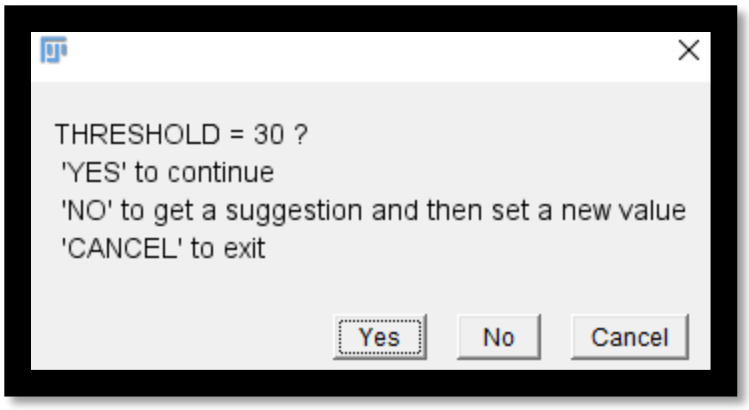
7. **Threshold particles.**

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.



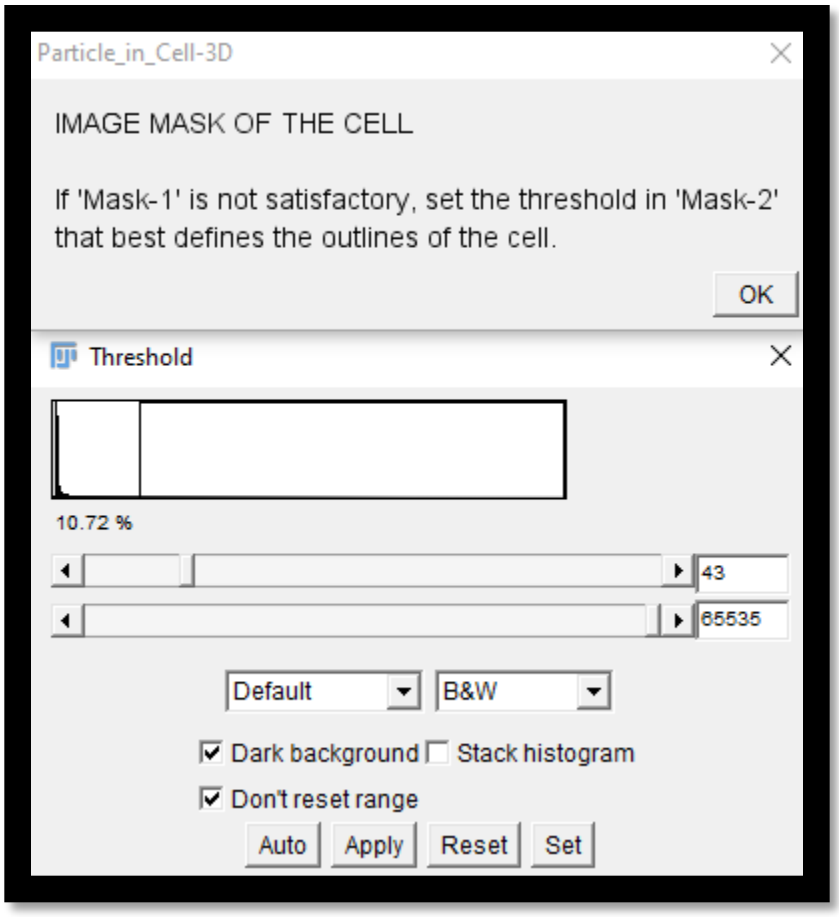
Before clicking "OK", make sure that the thresholding is satisfactory. If not, it can be manually changed before clicking "OK".



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.

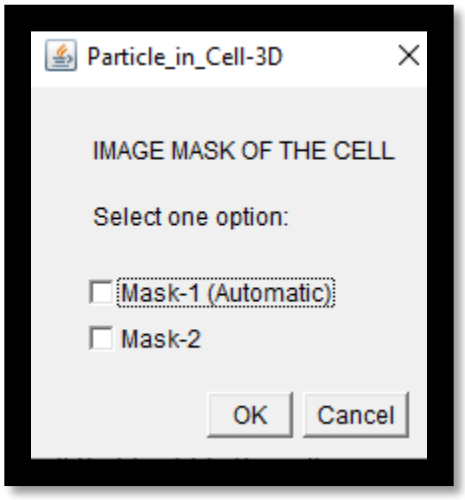
   The objective is to achieve a mask that accurately represents the cell.



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.

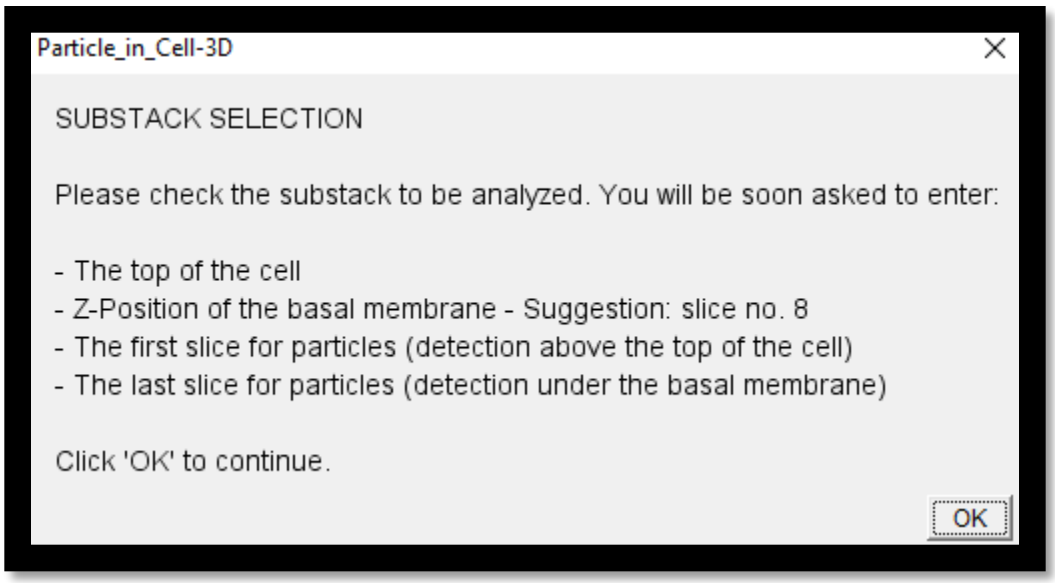
- *Mask 1.* Automatic.
- *Mask 2.* User defined.



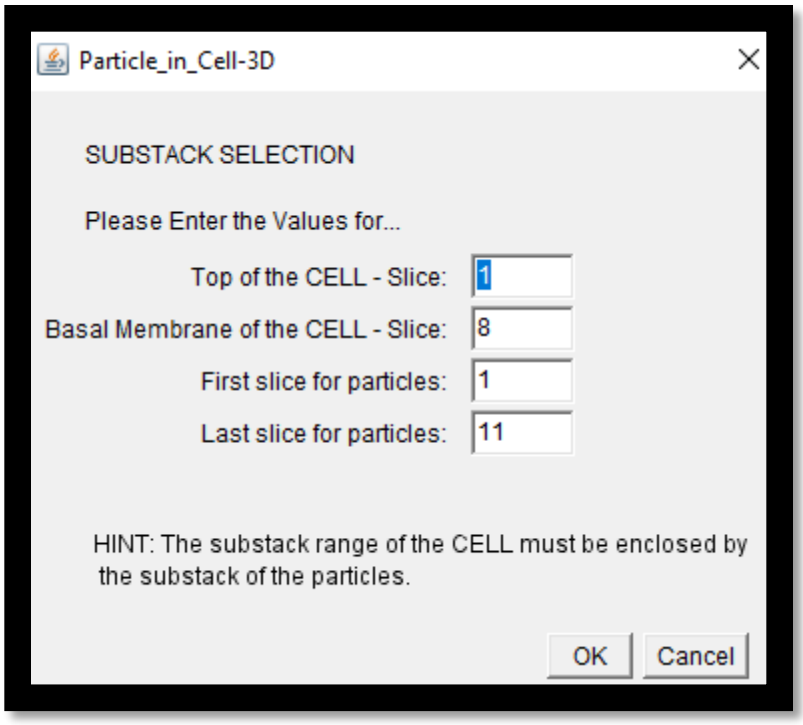
Select best representation of the cell.

10. Sub Stack Selection

Check the sub stack. Choose the slide that best represents: Top of the cell, basal membrane, first slice for particles and las slice for particles. Recommendations are given but they can be changed manually.

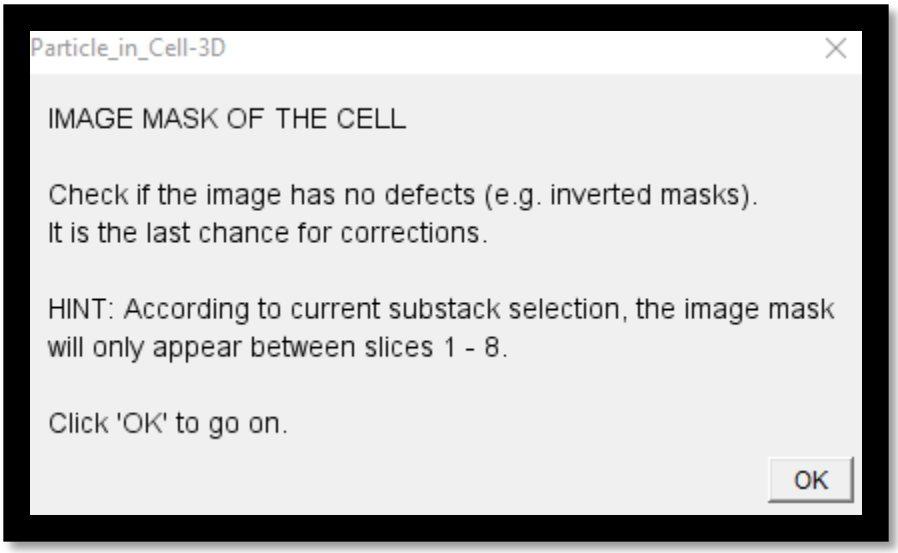


Before clicking "OK" make sure to note the slides corresponding to the 4 positions asked. After clicking "OK" enter the slides corresponding to the positions asked. The stack can no longer be analyzed while in this step.



11. Check Image Mask

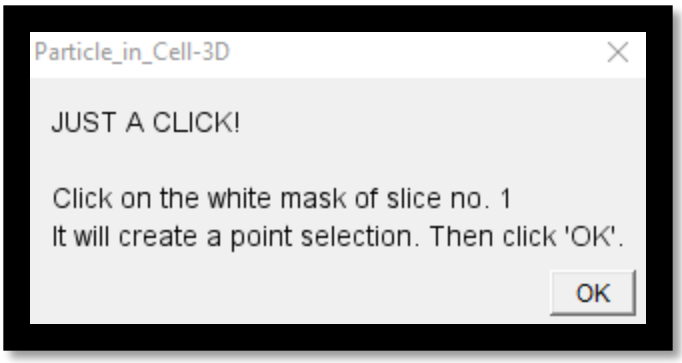
Check that the mask represents the cell accordingly through the stack.



Check mask of the cell.

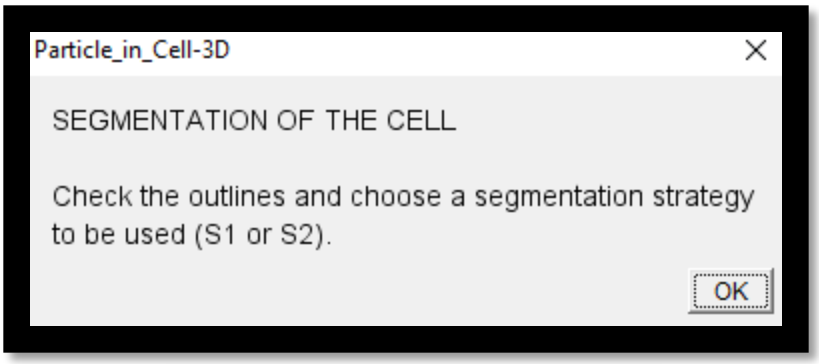
12. Cell Outlines

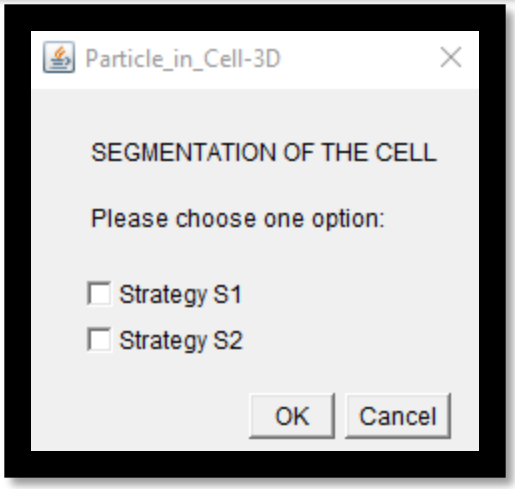
Click on the white mask of the first slice to create a point selection.



Before clicking "OK" click on the white mask of the first slice to create a point selection

Choose the best segmentation strategy. That is the outlines image that best represents the outlines of the cell.





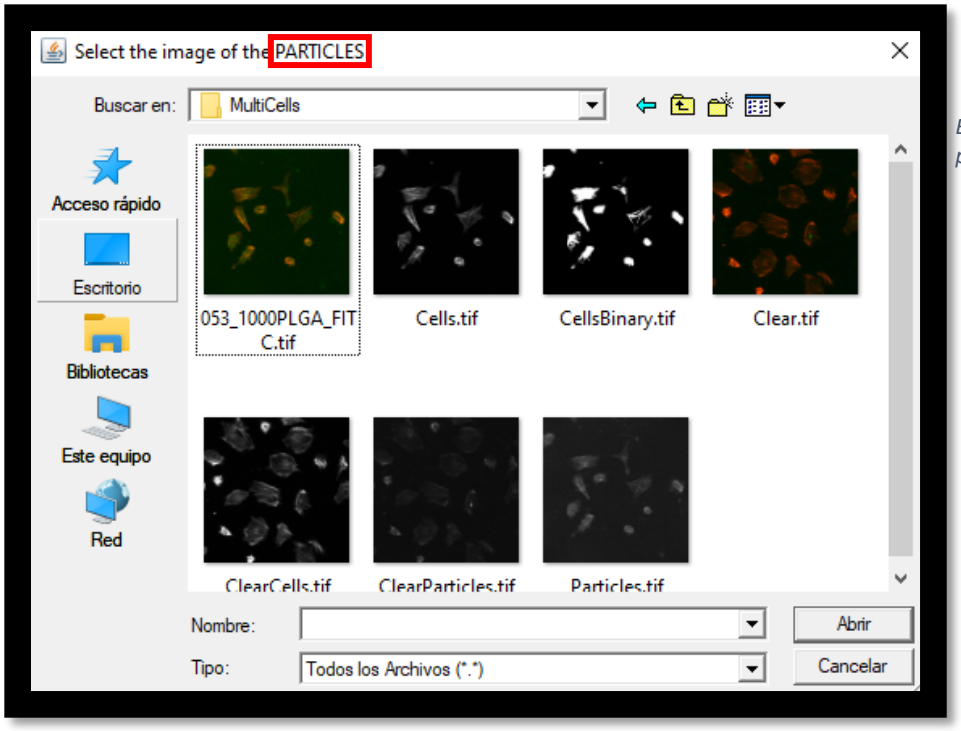
Choose the segmentation strategy that best represents the outlines of the cell.

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 particles image.**

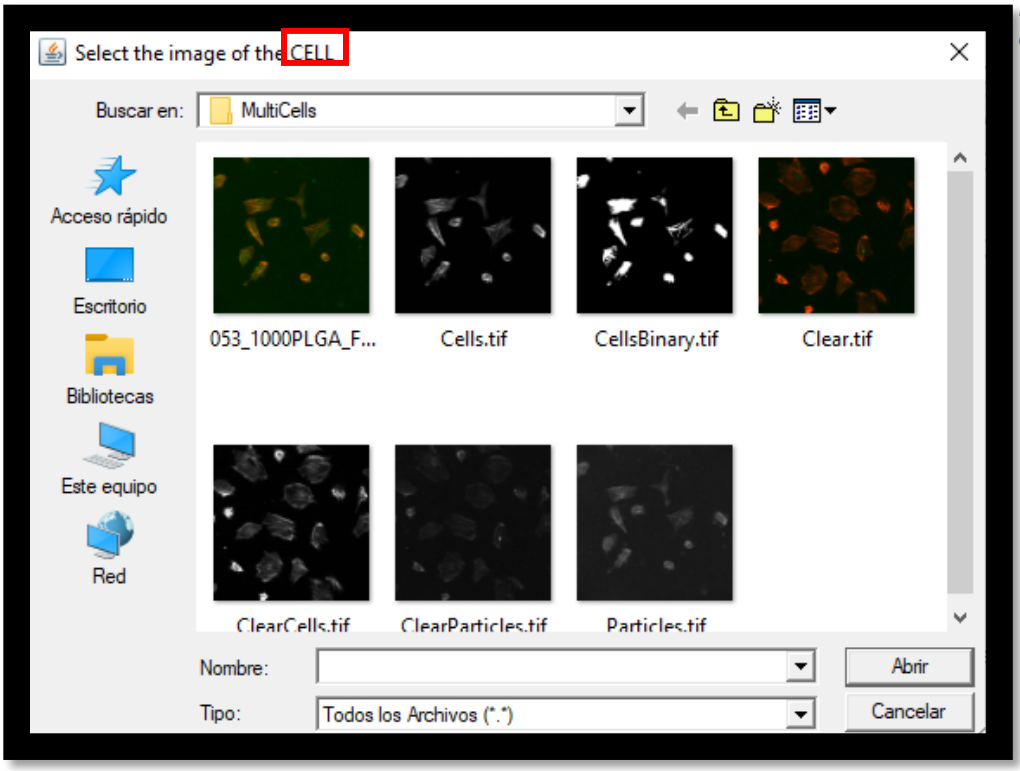
A file browser opens where the particles channel stack to be analyzed must be selected.



Browse to the desired particles stack.

2. **Select cells image.**

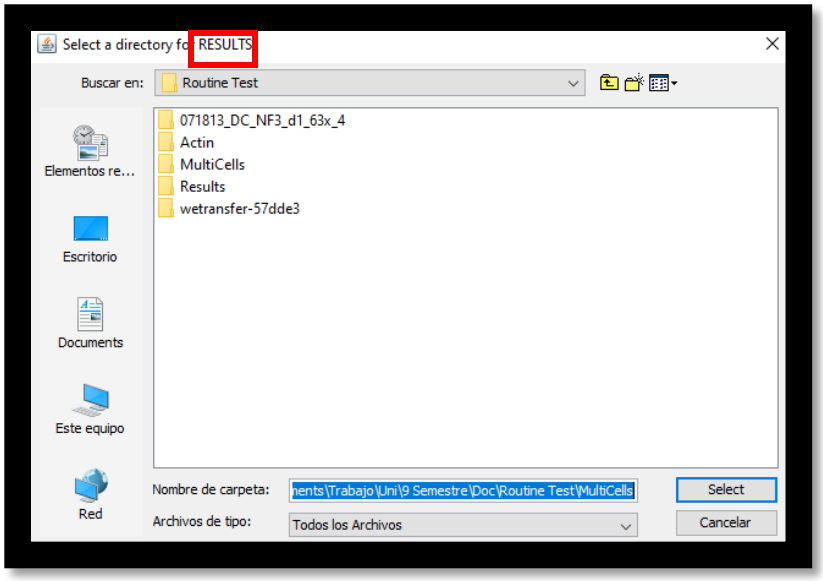
A file browser opens where the cells channel stack to be analyzed must be selected.



Browse to the desired cell stack.

3. **Select Results directory**

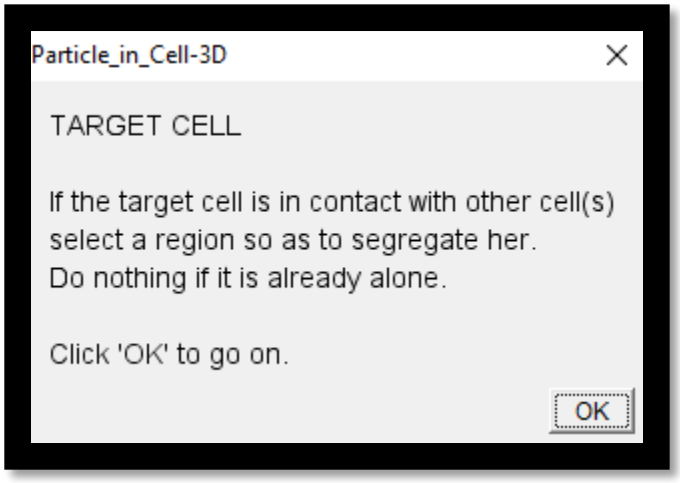
   A file browser opens where the desired results directory must be selected.



Browse to the desired results directory.

4. **Select target area.**

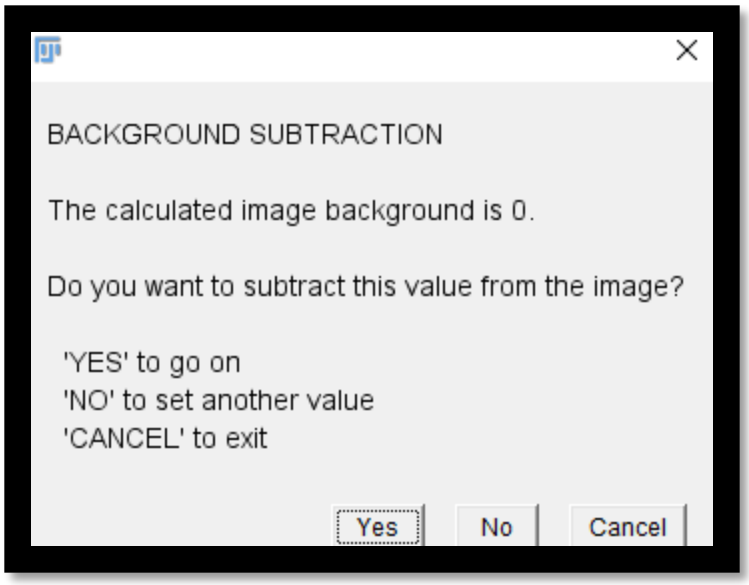
   Select the region of interest within the image selected. If the region of interest is the whole image do not select anything.



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

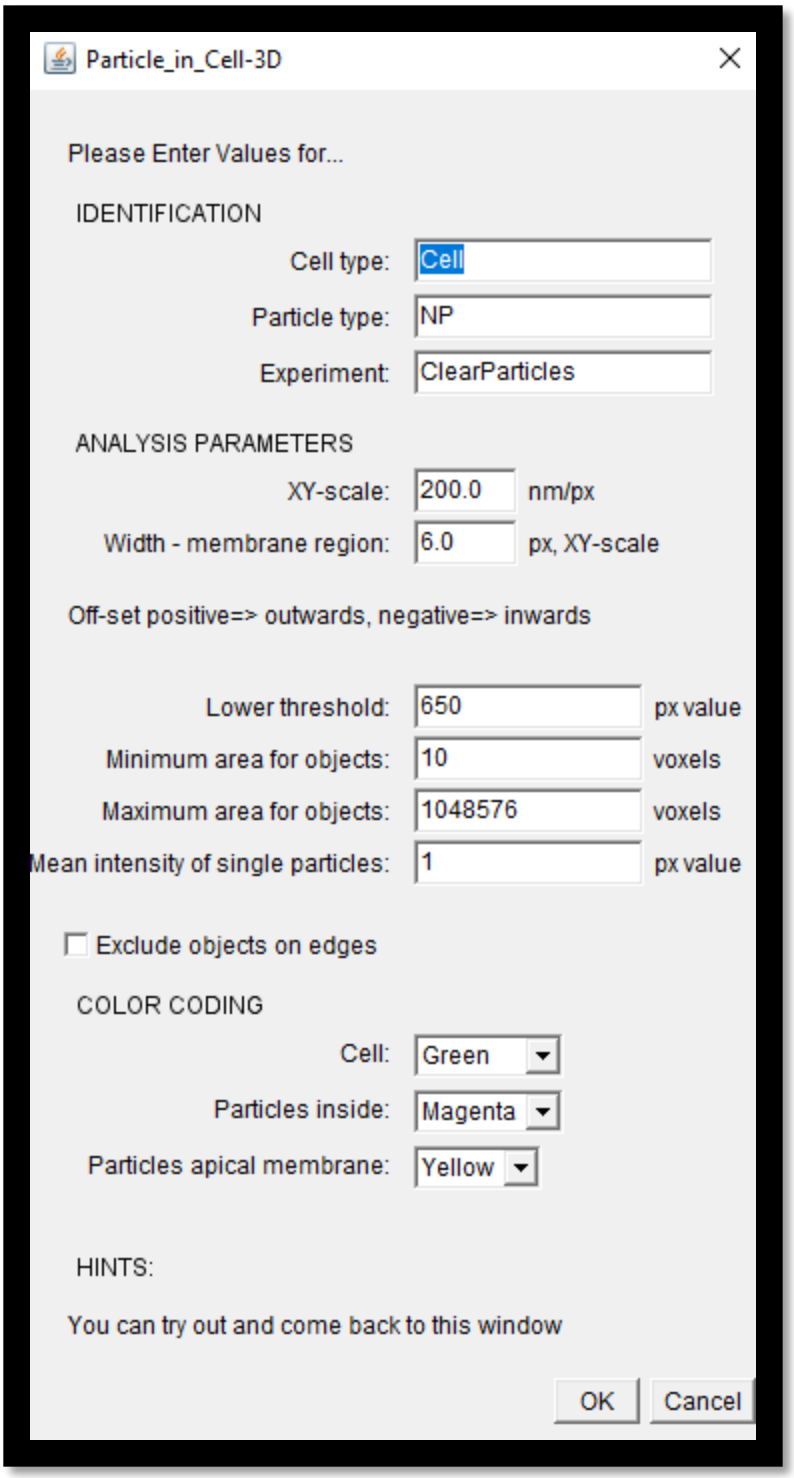
5. **Background Subtraction**

   An automatic background value is calculated. The user can either choose this value or set a desired value manually.



Click "Yes" to use the automatic background value. Click "No" to set a new value manually.

6. **Select parameters.**



Choose the parameters to be used for the analysis.

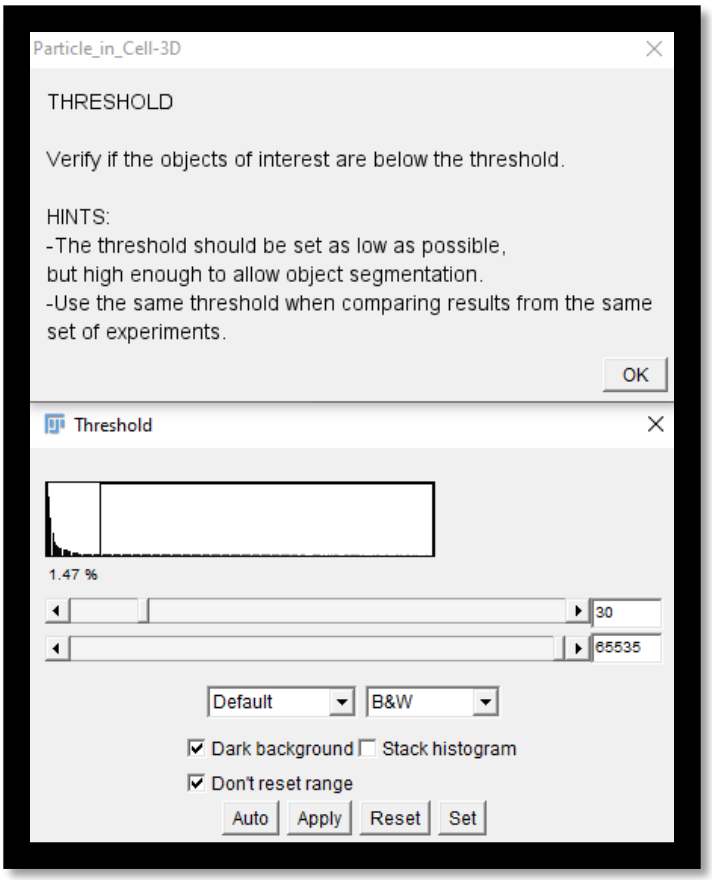
- *Cell Type.* Name to save intermediate steps cells images.
- *Particle Type.* Name to save intermediate steps particles images.
- *Experiment.* Name to identify the current experiment.
- *XY Scale.* Image XY scale. Used for conversion between pixels and nanometers.
- *Membrane region width.* Width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

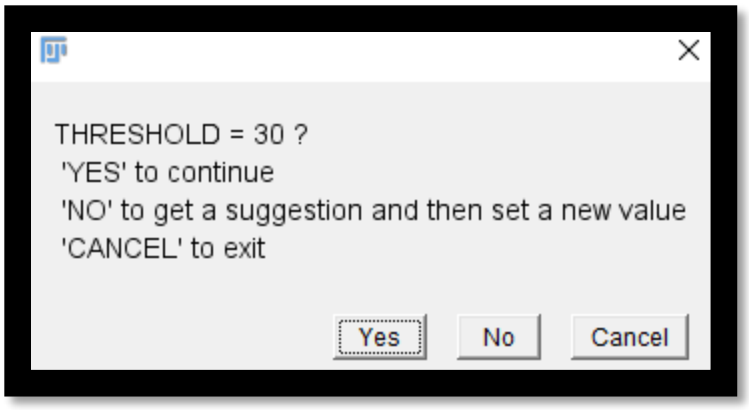
7. **Threshold particles.**

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.



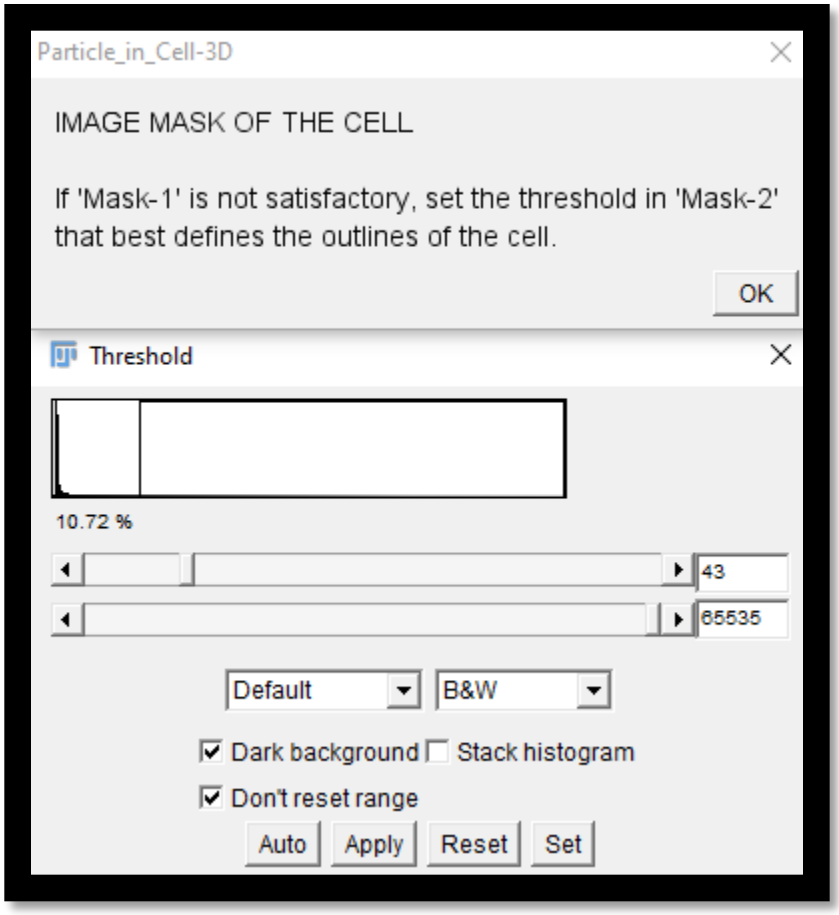
Before clicking "OK", make sure that the thresholding is satisfactory. If not, it can be manually changed before clicking "OK".



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.

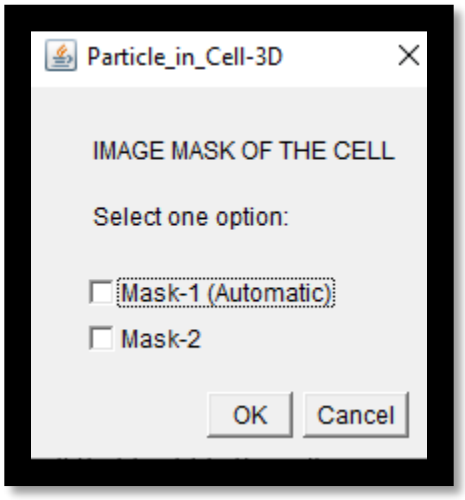
   The objective is to achieve a mask that accurately represents the cell.



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.

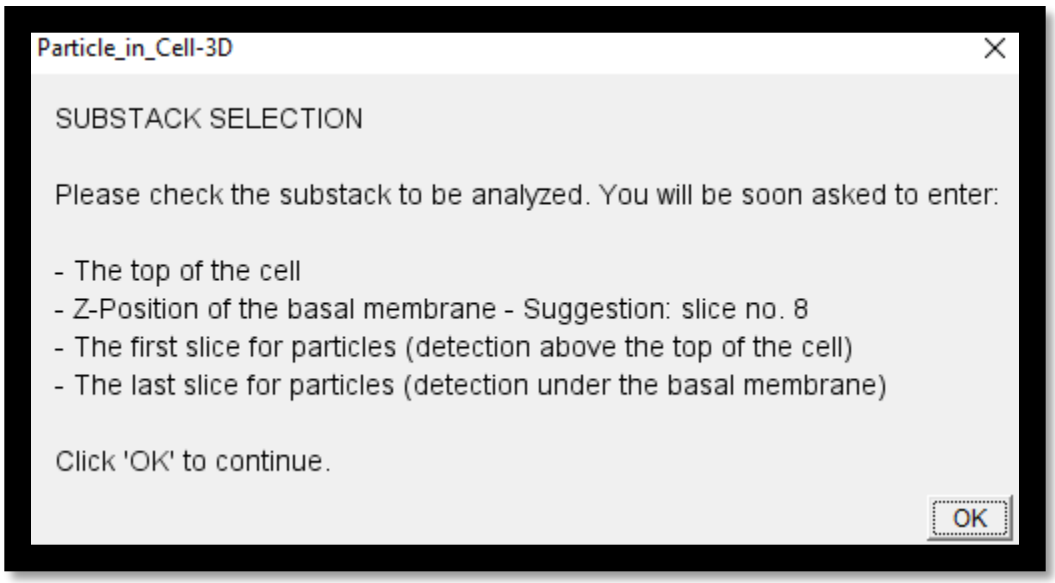
- *Mask 1.* Automatic.
- *Mask 2.* User defined.



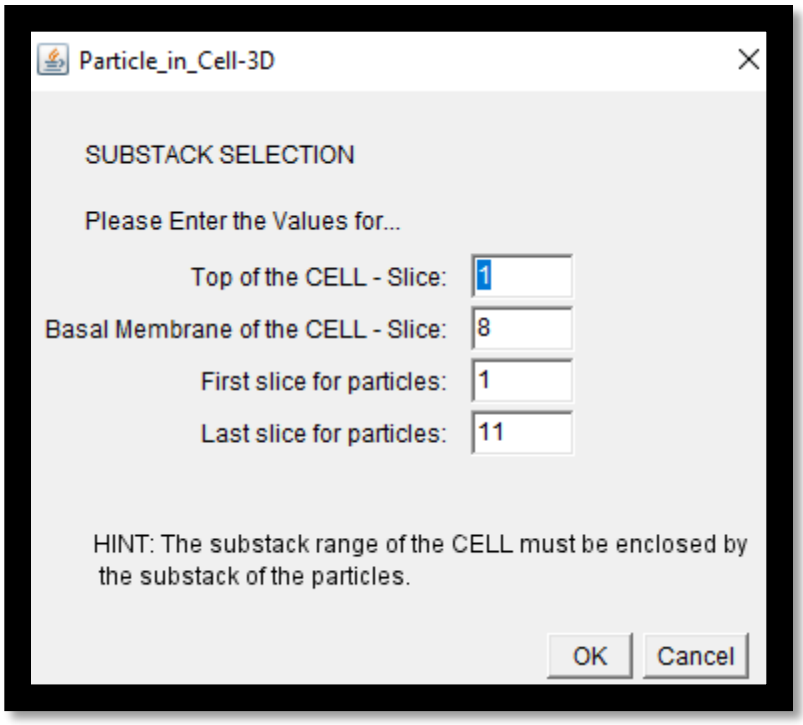
Select best representation of the cell.

10. Sub Stack Selection

Check the sub stack. Choose the slide that best represents: Top of the cell, basal membrane, first slice for particles and las slice for particles. Recommendations are given but they can be changed manually.

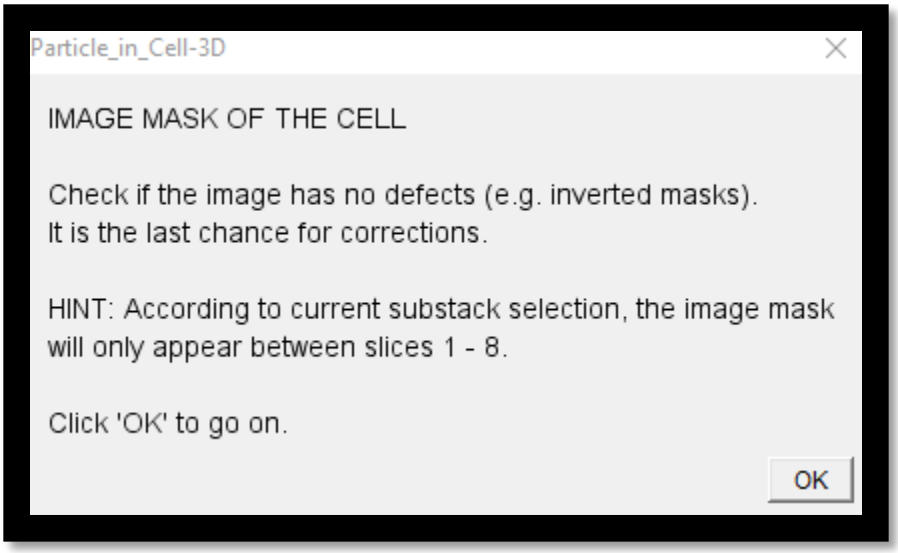


Before clicking "OK" make sure to note the slides corresponding to the 4 positions asked. After clicking "OK" enter the slides corresponding to the positions asked. The stack can no longer be analyzed while in this step.



11. Check Image Mask

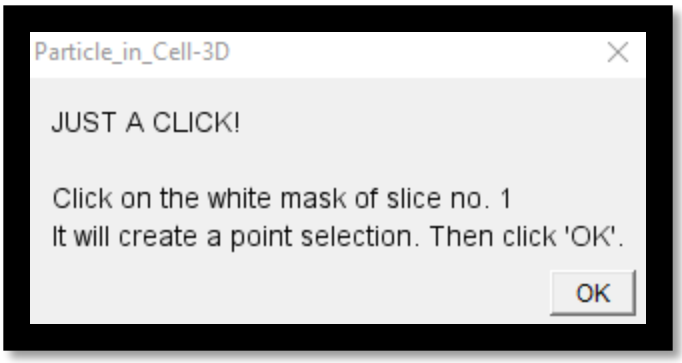
Check that the mask represents the cell accordingly through the stack.



Check mask of the cell.

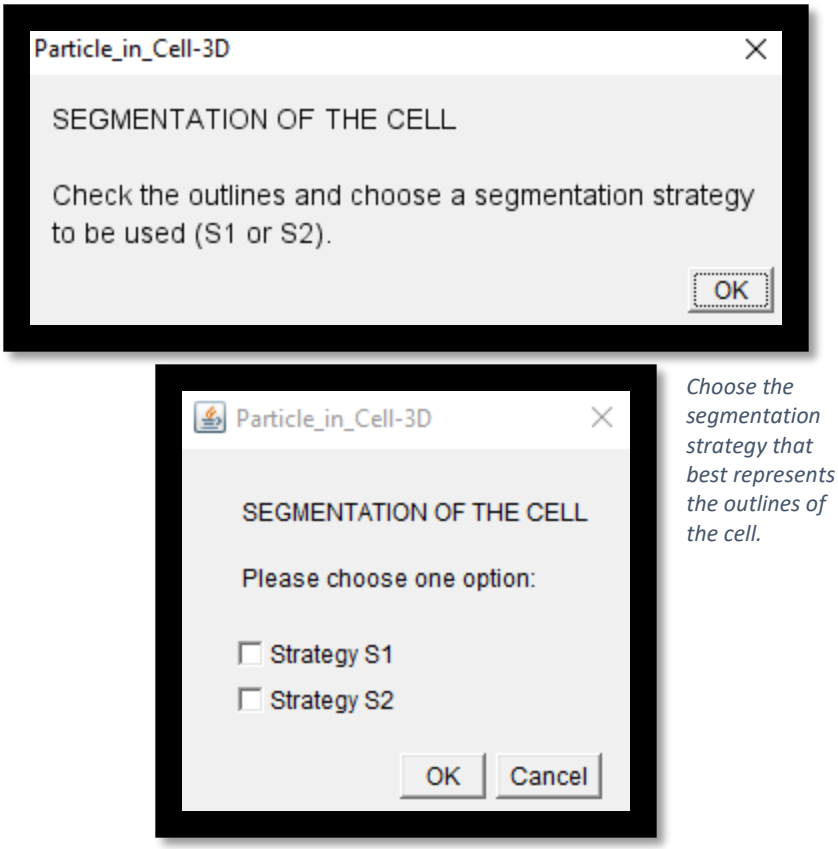
12. Cell Outlines

Click on the white mask of the first slice to create a point selection.



Before clicking "OK" click on the white mask of the first slice to create a point selection

Choose the best segmentation strategy. That is the outlines image that best represents the outlines of the cell.



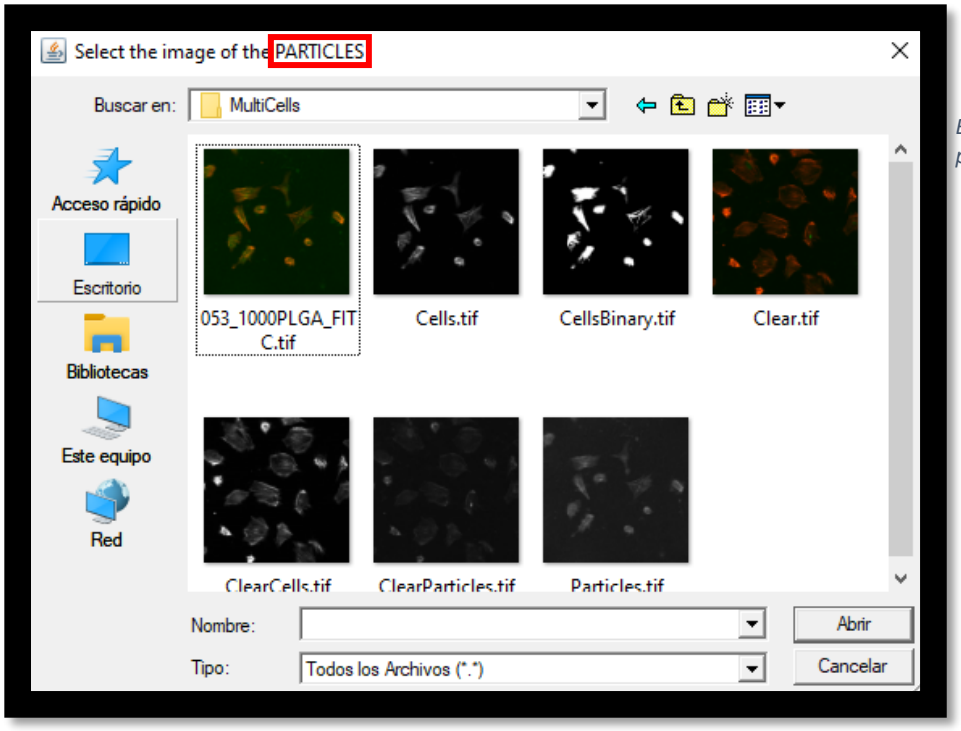
Choose the segmentation strategy that best represents the outlines of the cell.

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 particles image.**

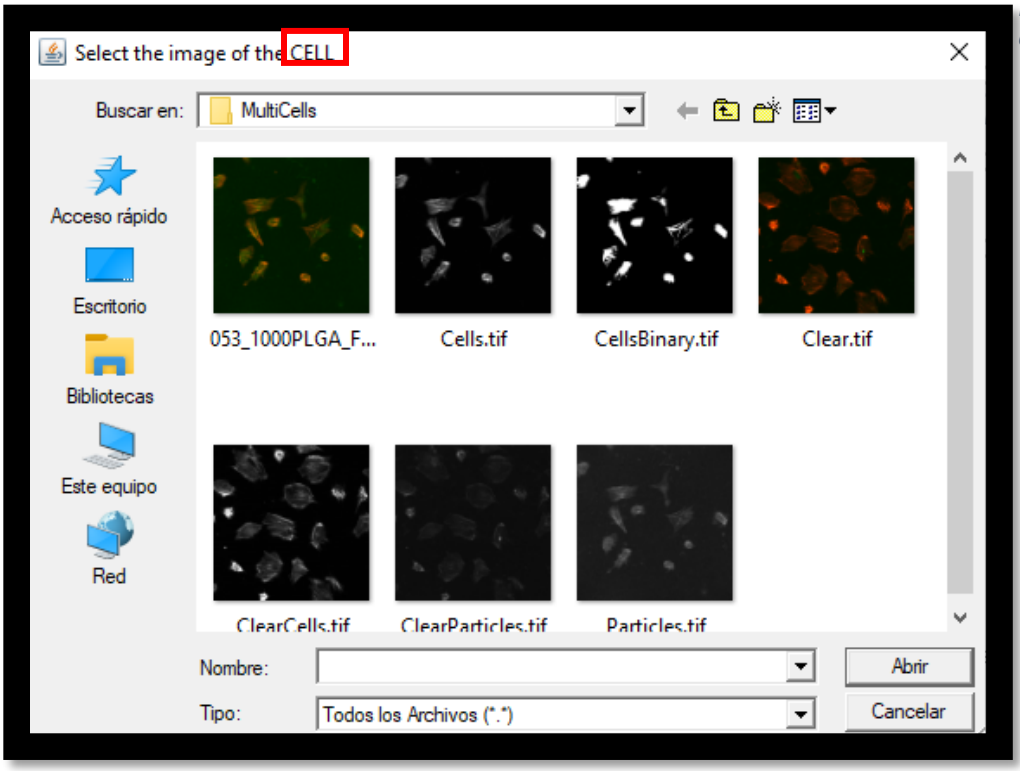
A file browser opens where the particles channel stack to be analyzed must be selected.



Browse to the desired particles stack.

2. **Select cells image.**

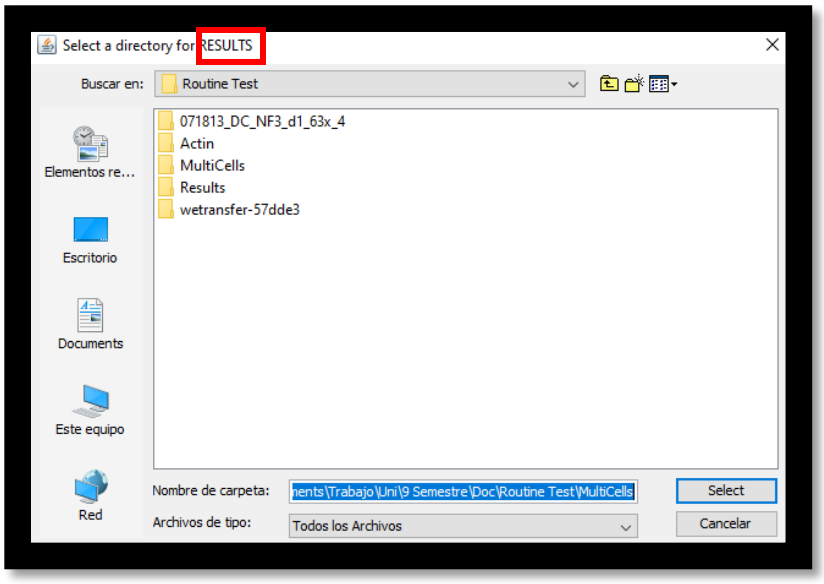
A file browser opens where the cells channel stack to be analyzed must be selected.



Browse to the desired cell stack.

3. **Select Results directory**

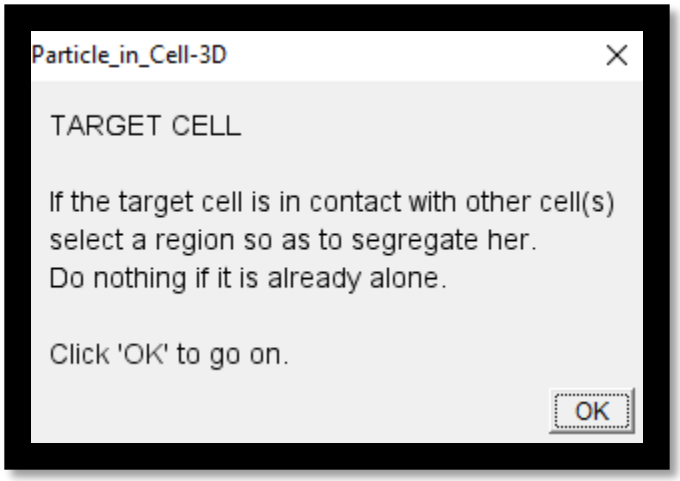
A file browser opens where the desired results directory must be selected.



Browse to the desired results directory.

4. **Select target area.**

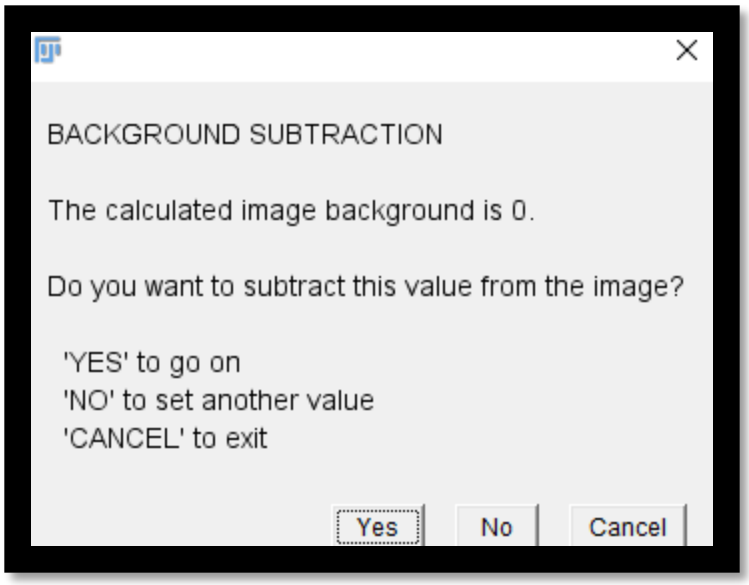
Select the region of interest within the image selected. If the region of interest is the whole image do not select anything.



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

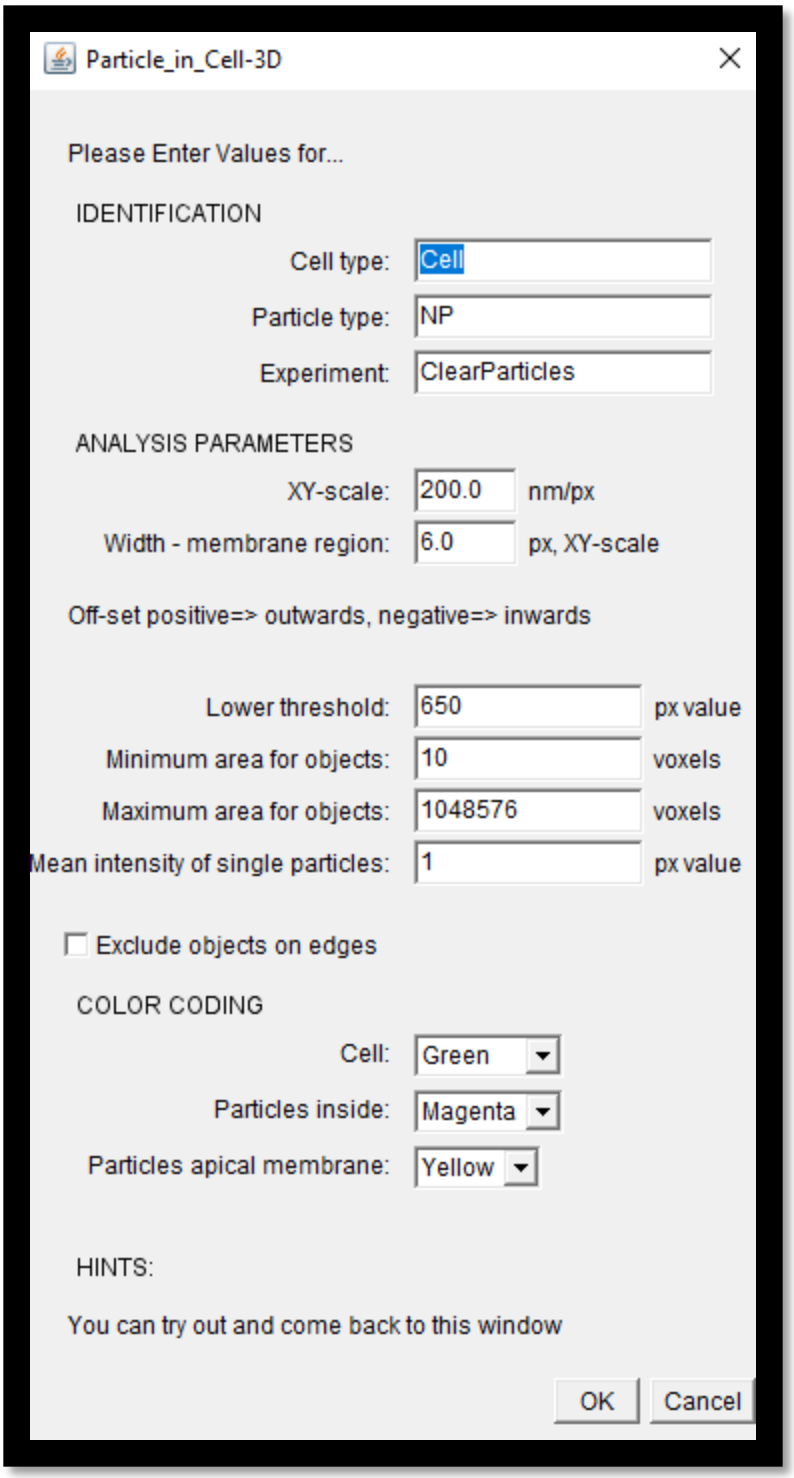
5. **Background Subtraction**

An automatic background value is calculated. The user can either choose this value or set a desired value manually.



Click "Yes" to use the automatic background value. Click "No" to set a new value manually.

6. **Select parameters.**



Choose the parameters to be used for the analysis.

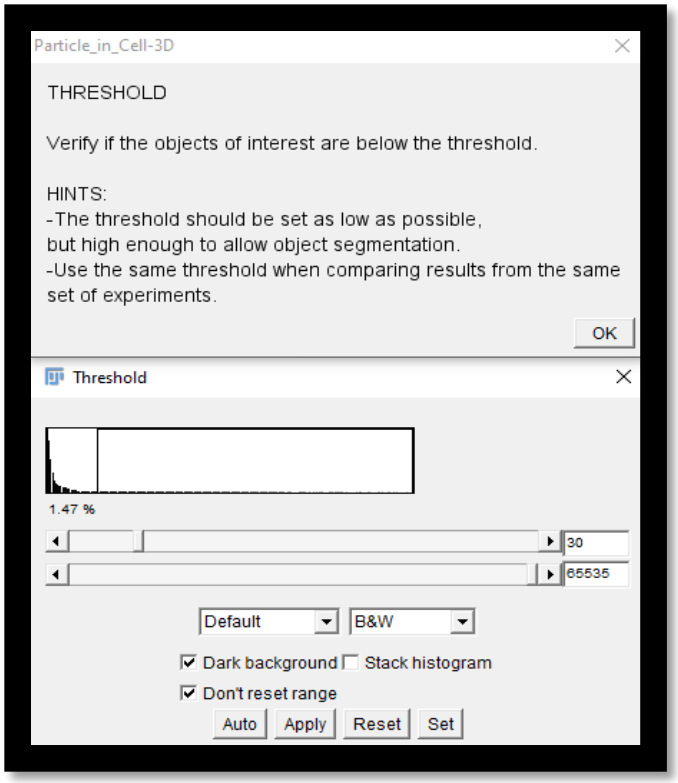
- *Cell Type.* Name to save intermediate steps cells images.
- *Particle Type.* Name to save intermediate steps particles images.
- *Experiment.* Name to identify the current experiment.
- *XY Scale.* Image XY scale. Used for conversion between pixels and nanometers.
- *Membrane region width.* Width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

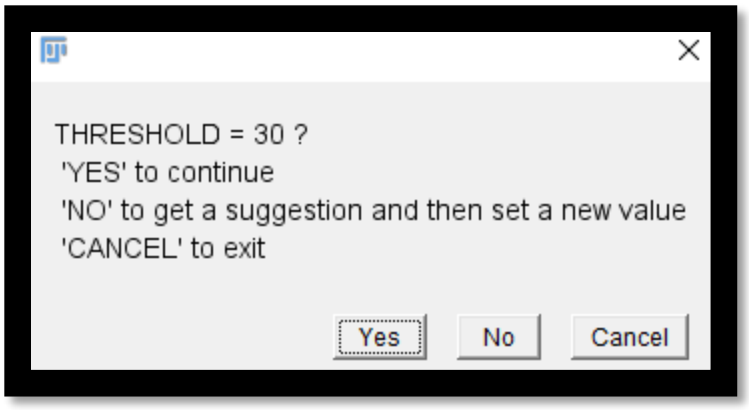
7. **Threshold particles.**

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.



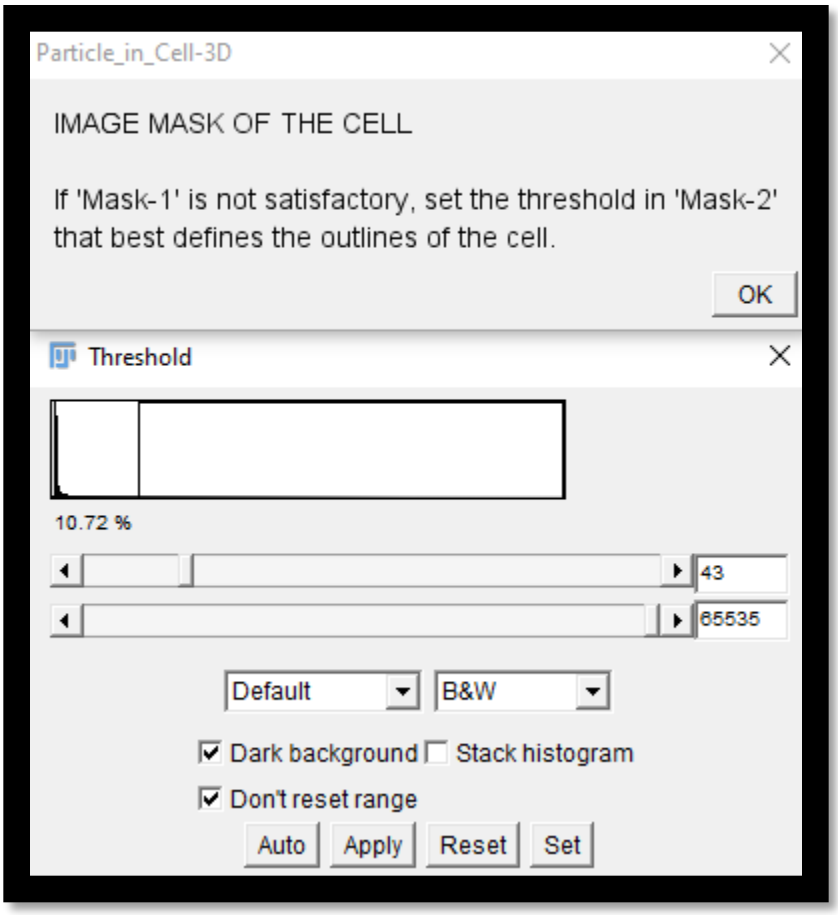
Before clicking "OK", make sure that the thresholding is satisfactory. If not, it can be manually



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.

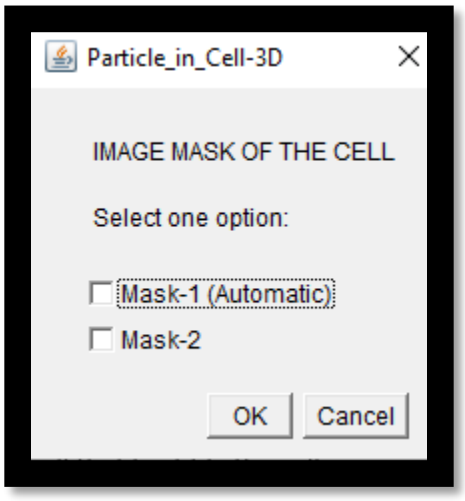
   The objective is to achieve a mask that accurately represents the cell.



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.

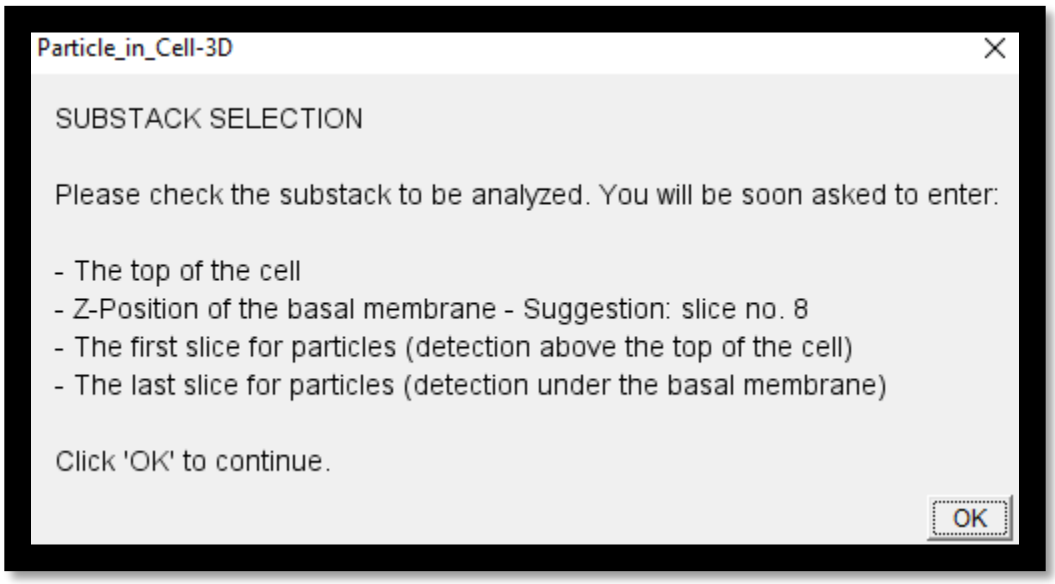
- *Mask 2.* User defined.



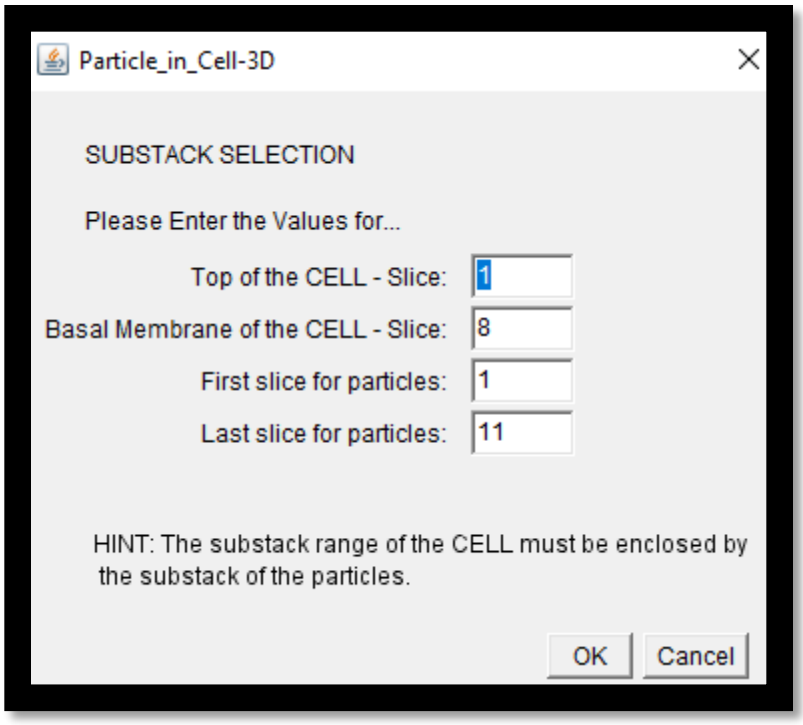
Select best representation of the cell.

10. Sub Stack Selection

Check the sub stack. Choose the slide that best represents: Top of the cell, basal membrane, first slice for particles and las slice for particles. Recommendations are given but they can be changed manually.

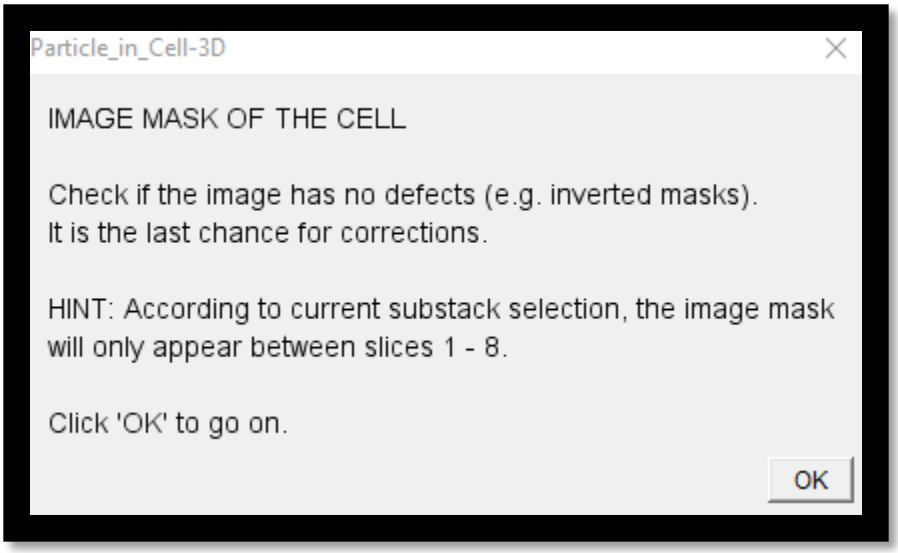


Before clicking "OK" make sure to note the slides corresponding to the 4 positions asked. After clicking "OK" enter the slides corresponding to the positions asked. The stack can no longer be analyzed while in this step.



11. Check Image Mask

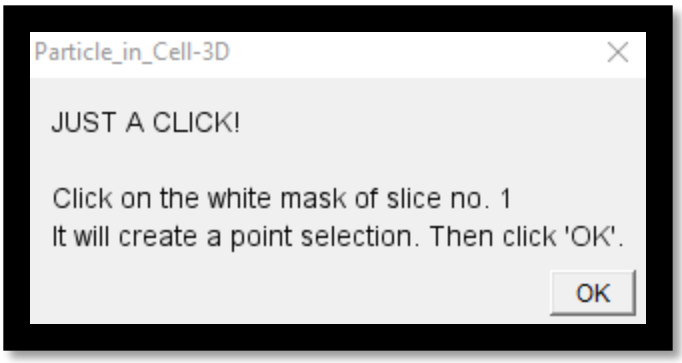
Check that the mask represents the cell accordingly through the stack.



Check mask of the cell.

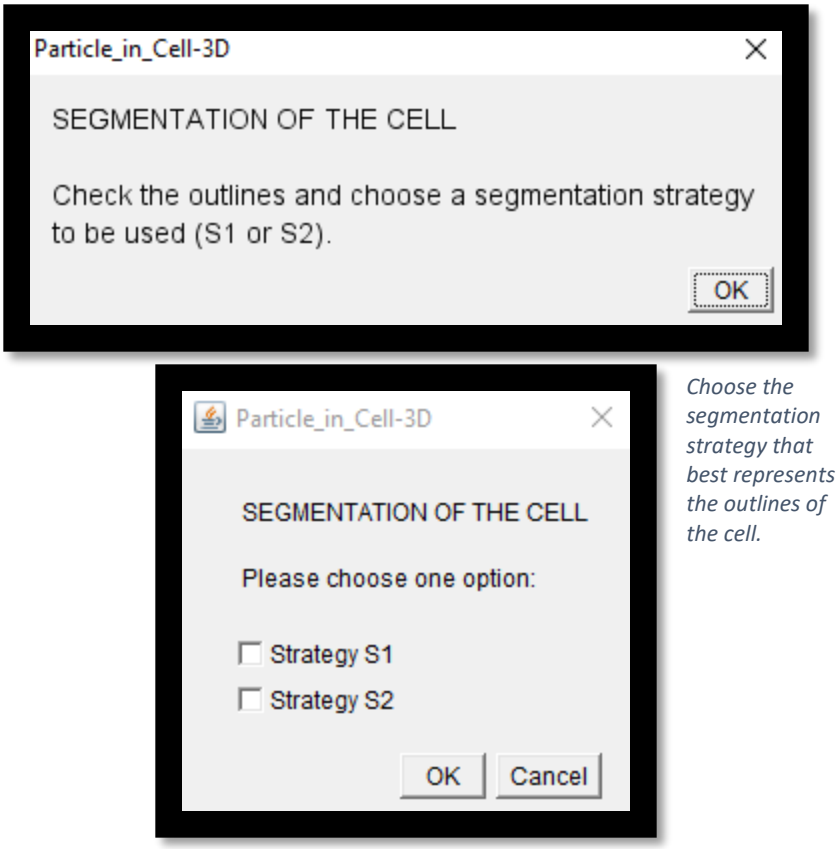
12. Cell Outlines

Click on the white mask of the first slice to create a point selection.



Before clicking "OK" click on the white mask of the first slice to create a point selection

C Choose the best segmentation strategy. That is the outlines image that best represents the outlines of the cell.

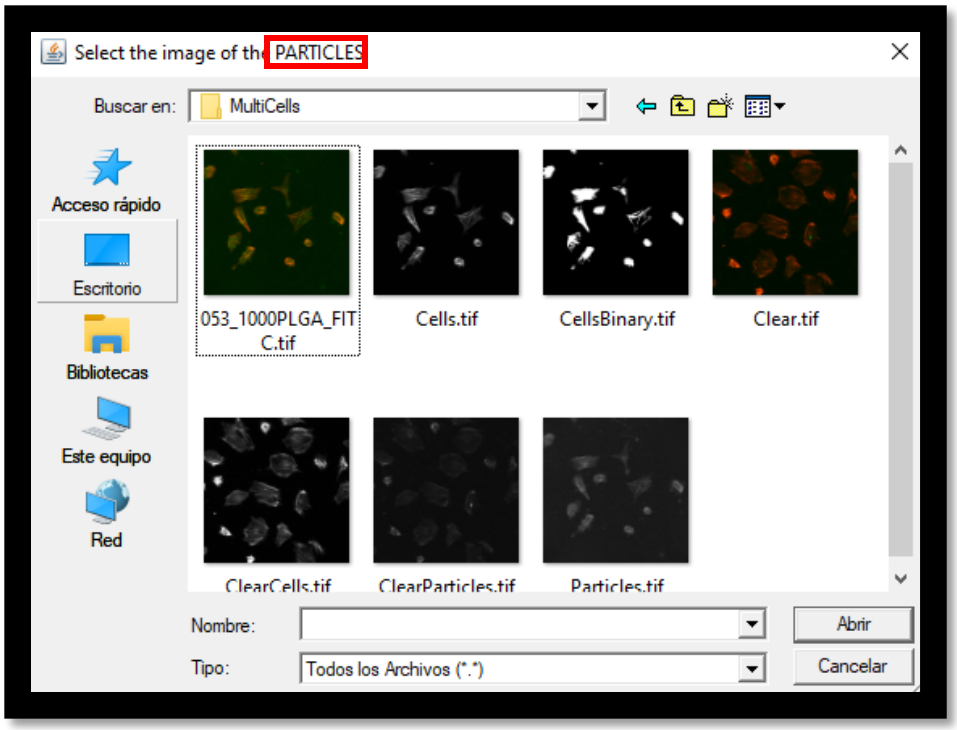


Choose the segmentation strategy that best represents the outlines of the cell.

Routine 4. Calibration.

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

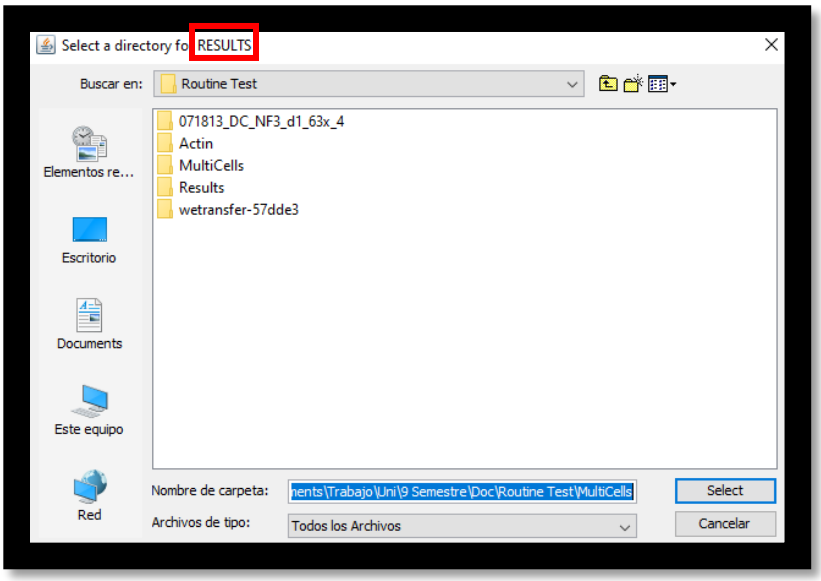
1. **Select particles image.**
   A file browser opens where the particles channel stack to be analyzed must be selected.



Browse to the desired particles stack.

2. **Select Results directory**

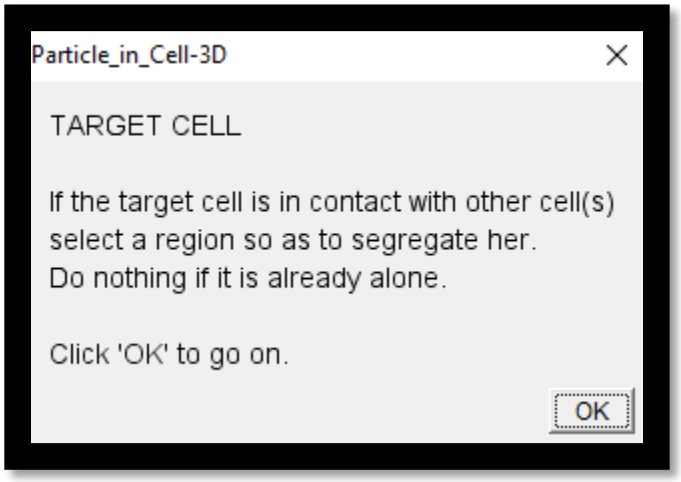
   A file browser opens where the desired results directory must be selected.



Browse to the desired results directory.

3. **Select target area.**

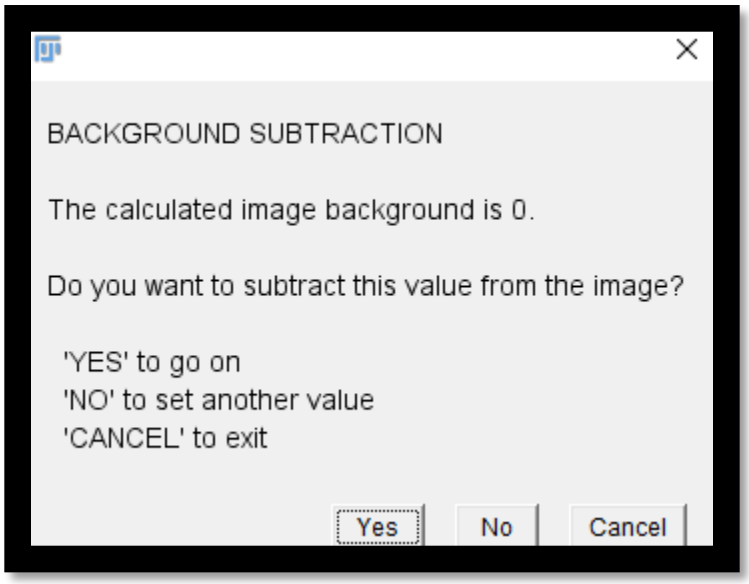
Select the region of interest within the image selected. If the region of interest is the whole image do not select anything.



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

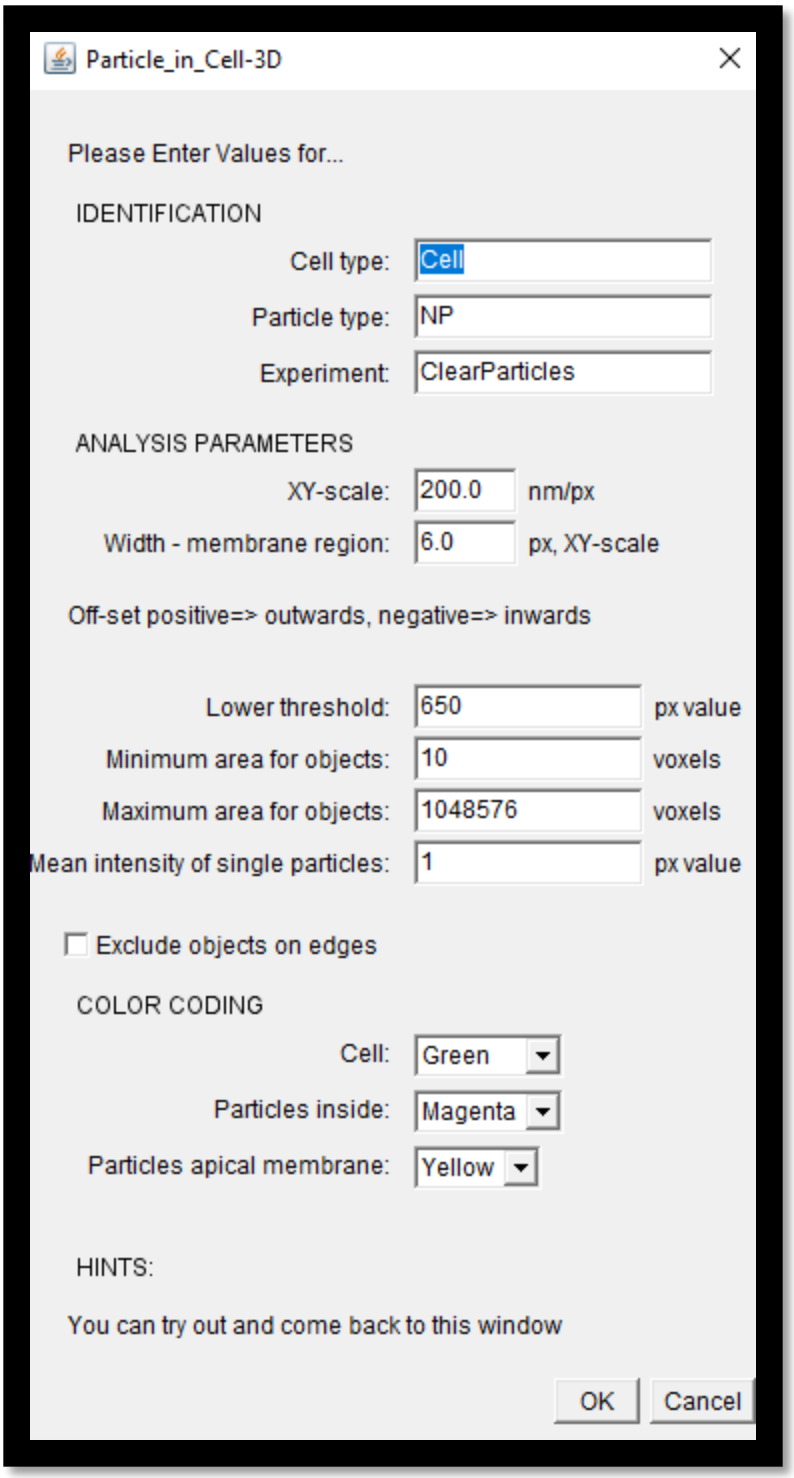
4. **Background Subtraction**

An automatic background value is calculated. The user can either choose this value or set a desired value manually.



Click "Yes" to use the automatic background value. Click "No" to set a new value manually.

5. **Select parameters.**



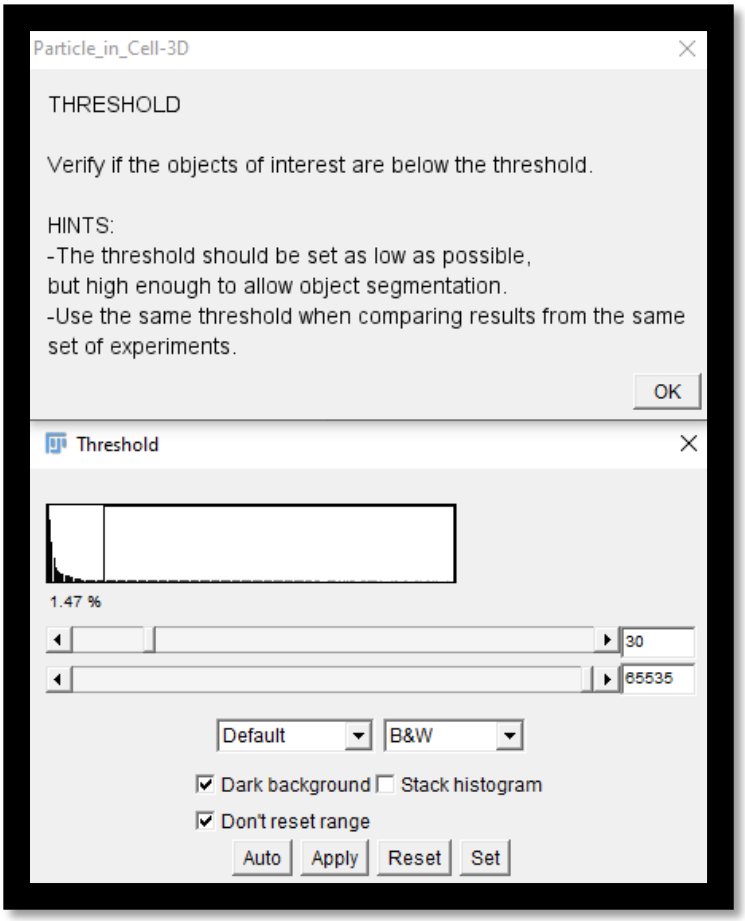
Choose the parameters to be used for the analysis.

- *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.

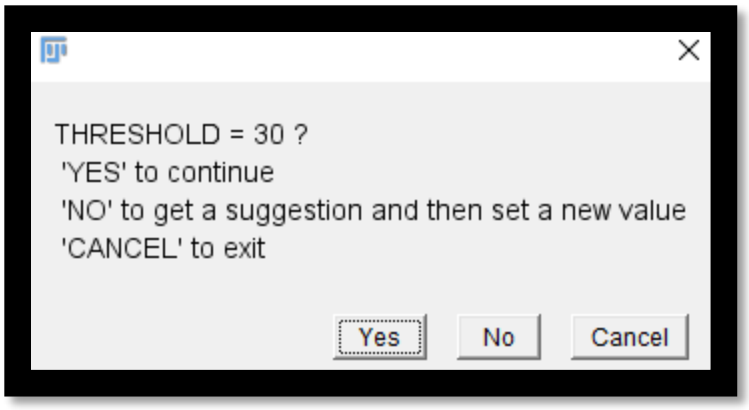
- *Lower threshold.* Value to use to threshold the particles. Can be previewed and changed later.
- *Minimum object area.* Minimum area of objects to consider.
- *Maximum object area.* Maximum area of objects to consider.
- *Mean particle intensity.* Mean particle intensity. Obtained from calibration 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.

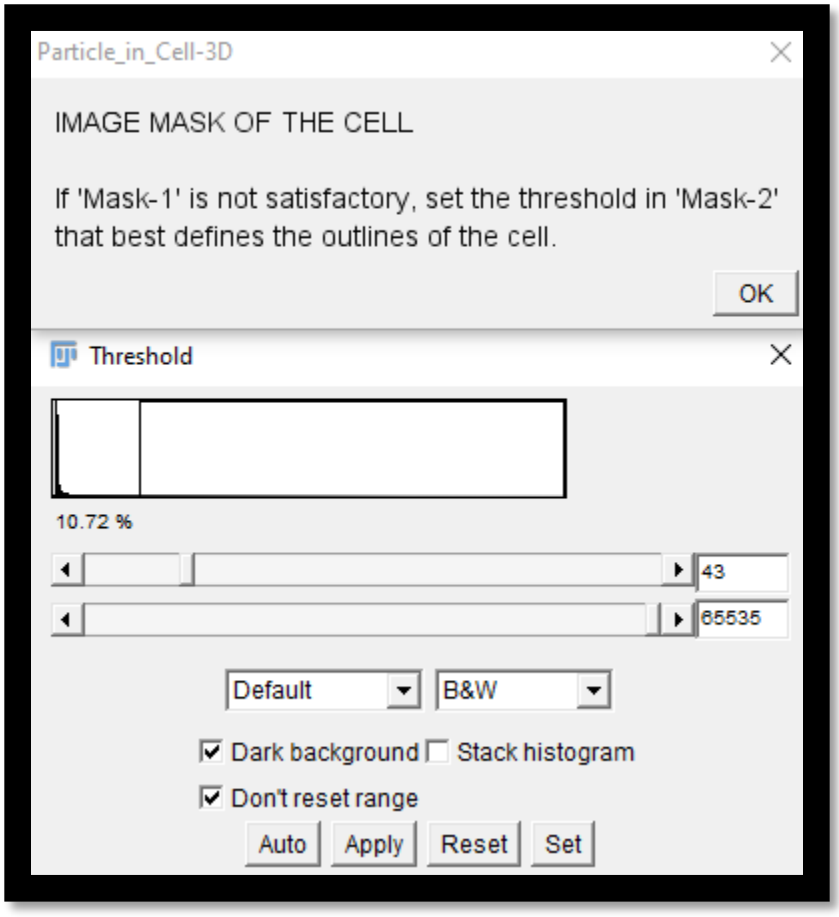


Before clicking "OK", make sure that the thresholding is satisfactory. If not, it can be manually changed before clicking "OK".



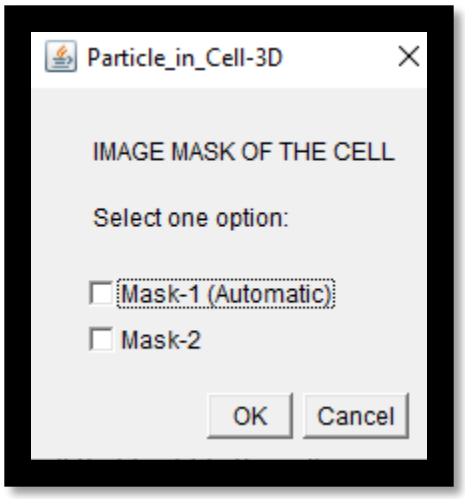
Click "YES" if satisfied with the current thresholding value. "No" to get a new suggestion and set a new value.

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.



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

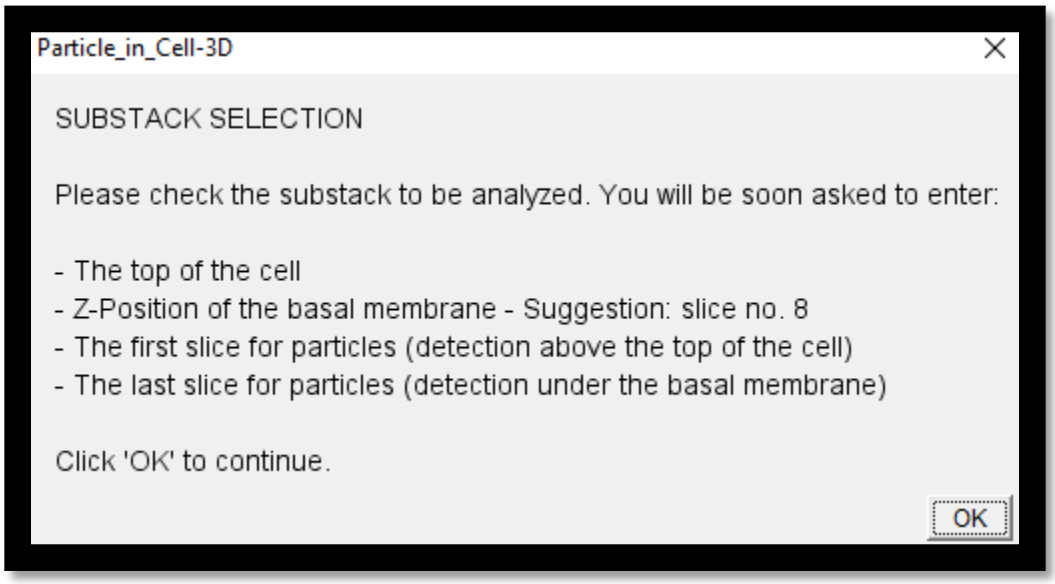
8. **Select best cell mask.**
   Selection of the mask of the cell that best fits the cells.
   - *Mask 1.* Automatic.
   - *Mask 2.* User defined.



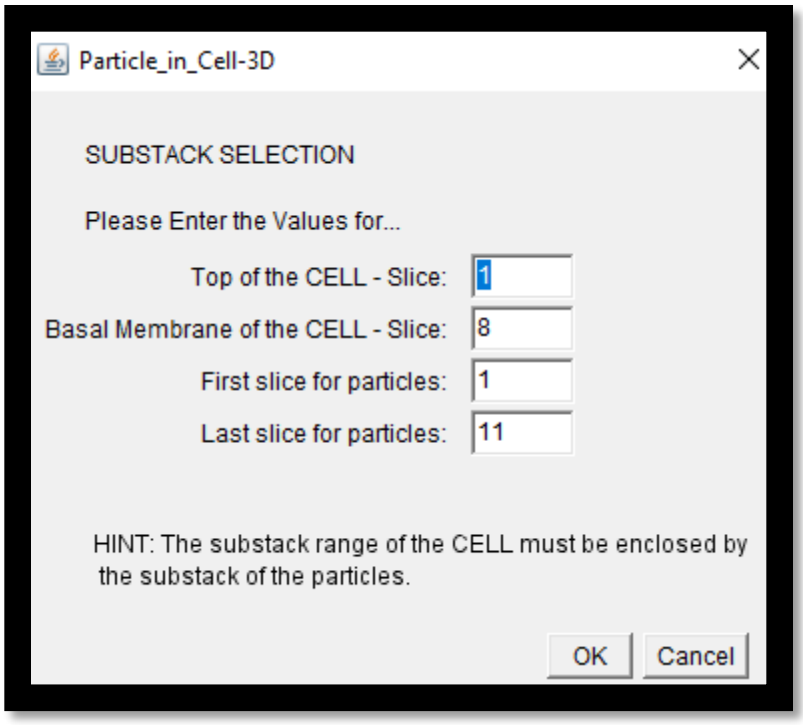
Select best representation of the cell.

9. Sub Stack Selection

Check the sub stack. Choose the slide that best represents: Top of the cell, basal membrane, first slice for particles and las slice for particles. Recommendations are given but they can be changed manually.

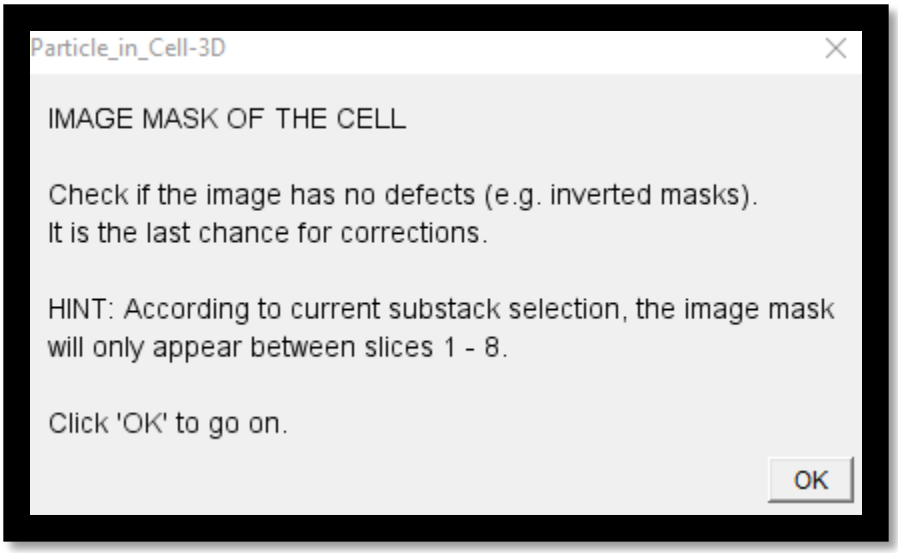


Before clicking "OK" make sure to note the slides corresponding to the 4 positions asked. After clicking "OK" enter the slides corresponding to the positions asked. The stack can no longer be analyzed while in this step.



10. Check Image Mask

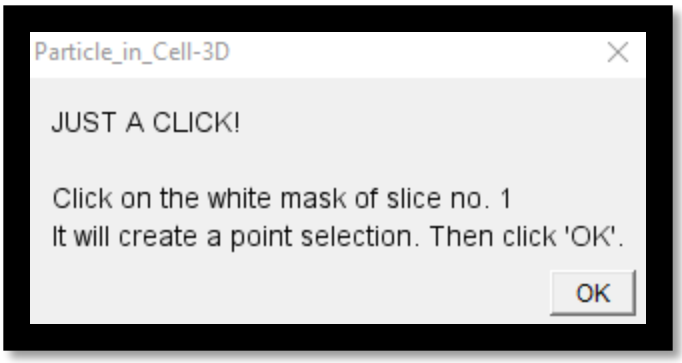
Check that the mask represents the cell accordingly through the stack.



Check mask of the cell.

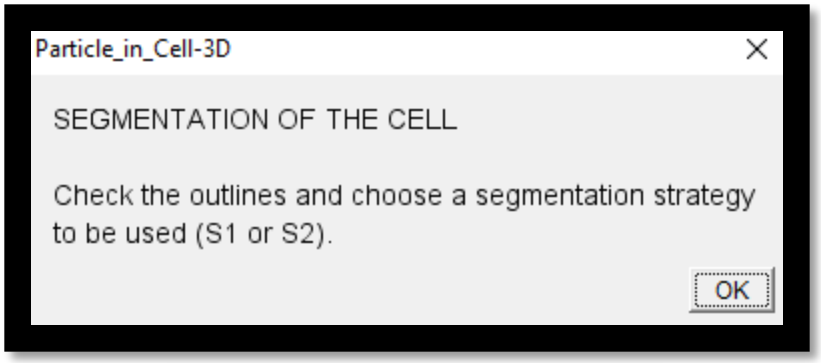
11. Cell Outlines

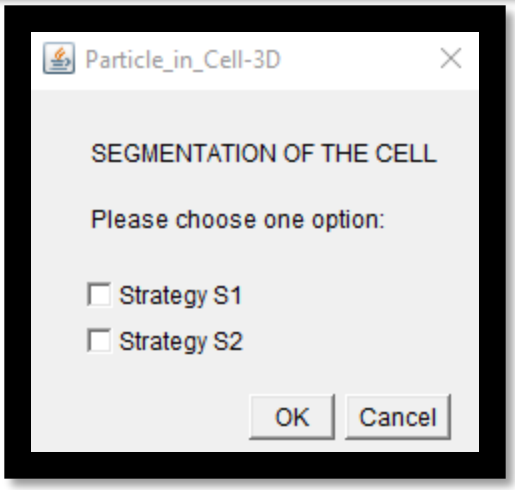
Click on the white mask of the first slice to create a point selection.



Before clicking "OK" click on the white mask of the first slice to create a point selection

Choose the best segmentation strategy.





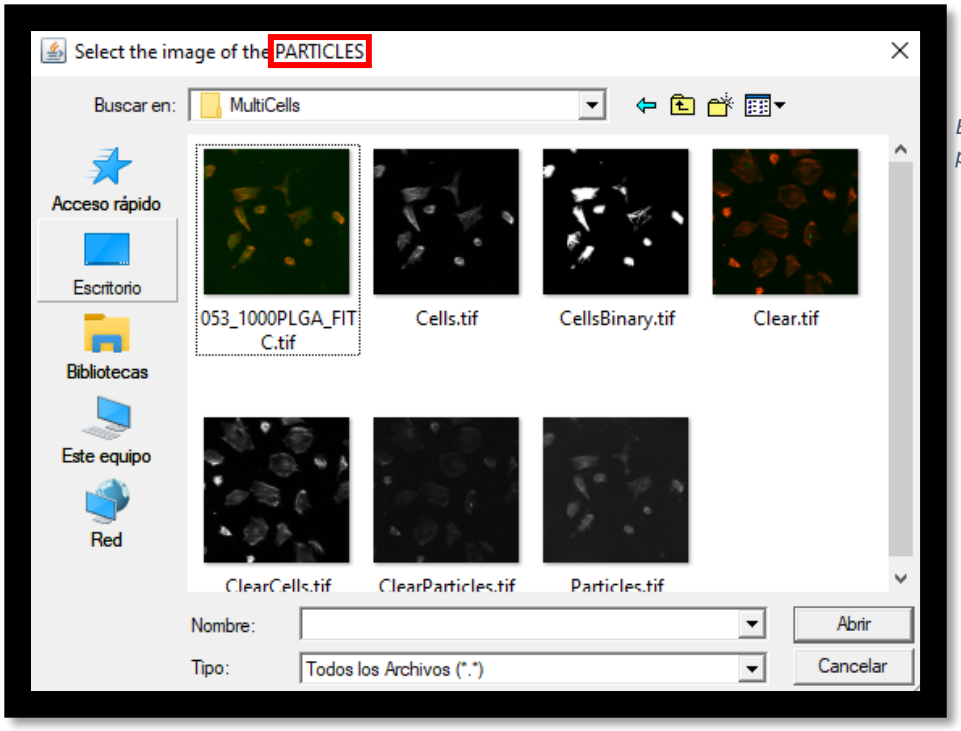
Choose the segmentation strategy that best represents the outlines of the cell.

Routine 5. Only Particles.

Count the absolute number of particles in cell free regions.

1. **Select particles image.**

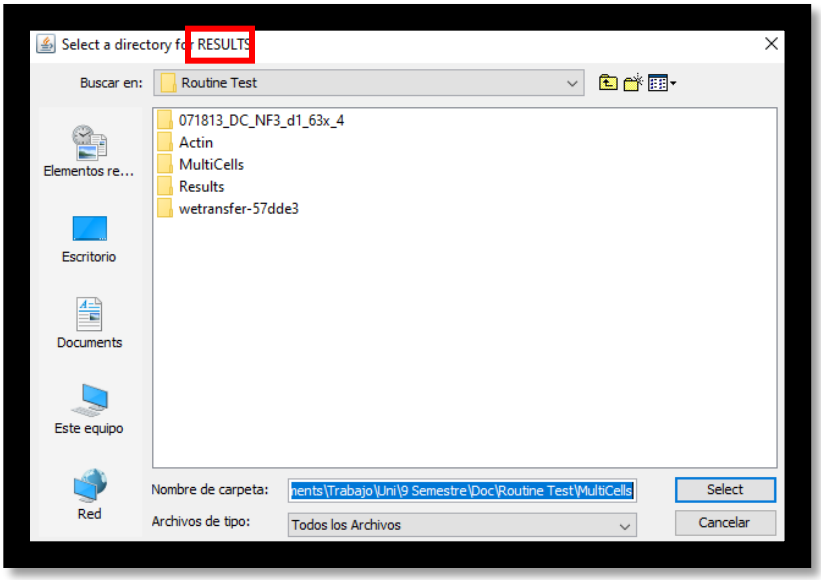
A file browser opens where the particles channel stack to be analyzed must be selected.



Browse to the desired particles stack.

2. **Select Results directory**

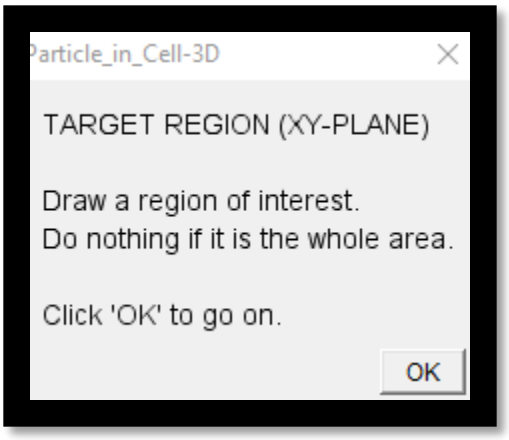
A file browser opens where the desired results directory must be selected.



Browse to the desired results directory.

3. **Select target area.**

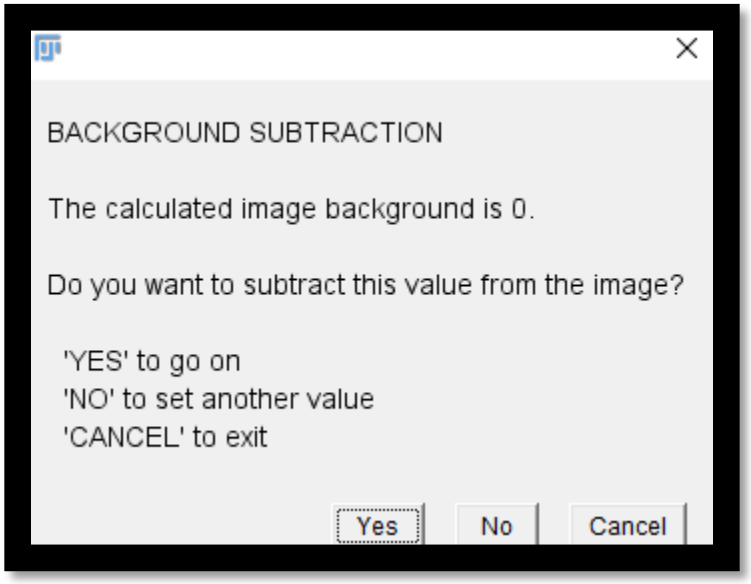
Select the region of interest within the image selected. If the region of interest is the whole image do not select anything.



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

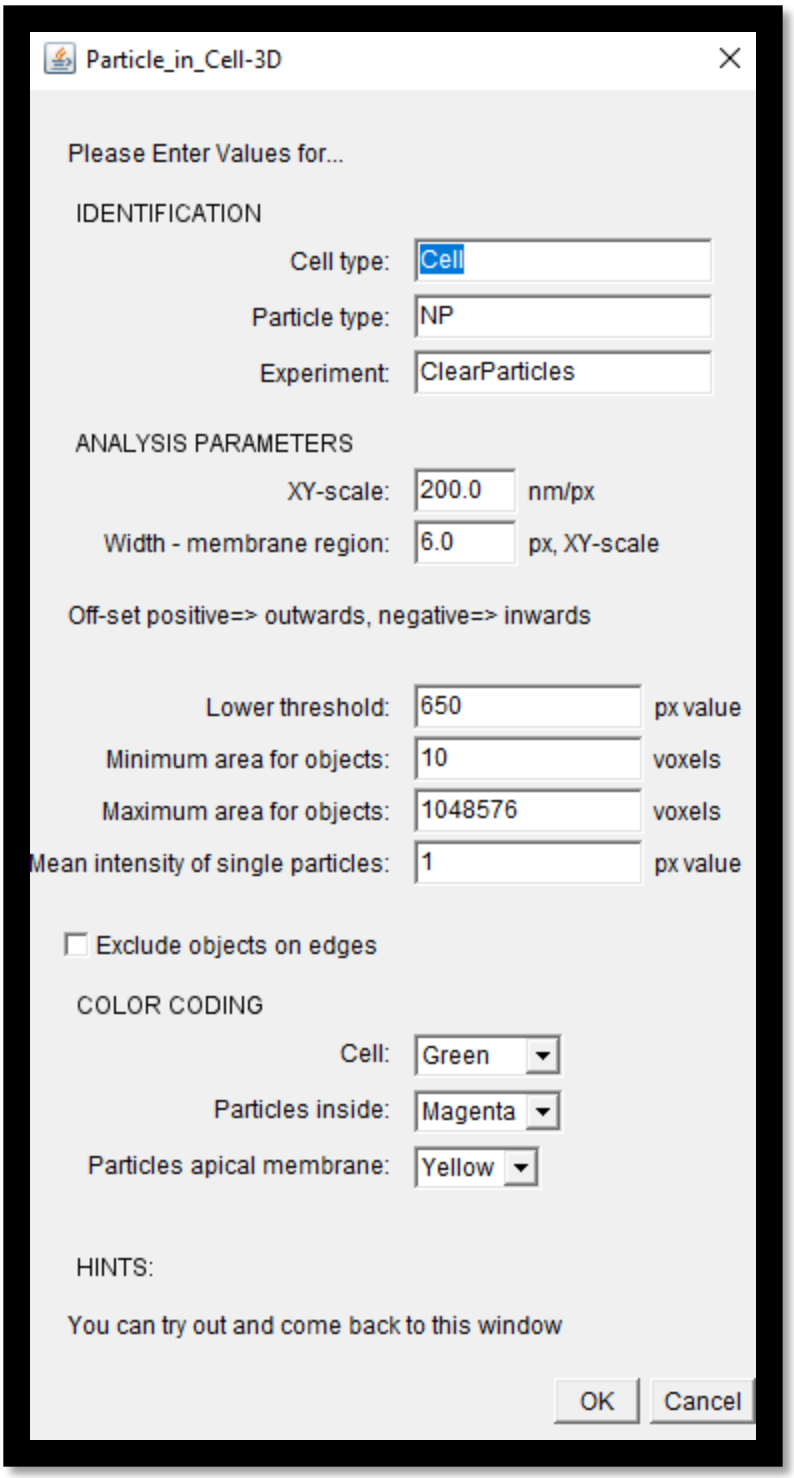
4. **Background Subtraction**

An automatic background value is calculated. The user can either choose this value or set a desired value manually.



Click "Yes" to use the automatic background value. Click "No" to set a new value manually.

5. **Select parameters.**



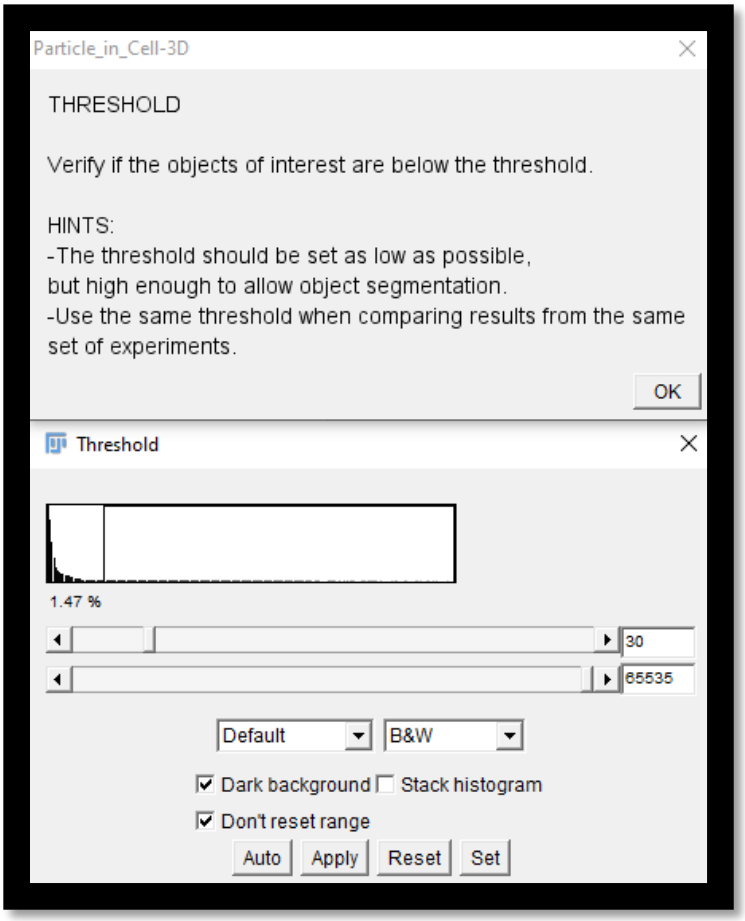
Choose the parameters to be used for the analysis.

- *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.

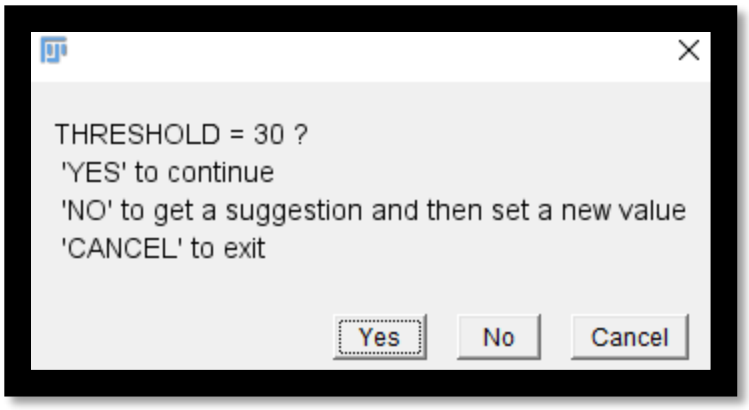
- *Lower threshold.* Value to use to threshold the particles. Can be previewed and changed later.
- *Minimum object area.* Minimum area of objects to consider.
- *Maximum object area.* Maximum area of objects to consider.
- *Mean particle intensity.* Mean particle intensity. Obtained from calibration 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.

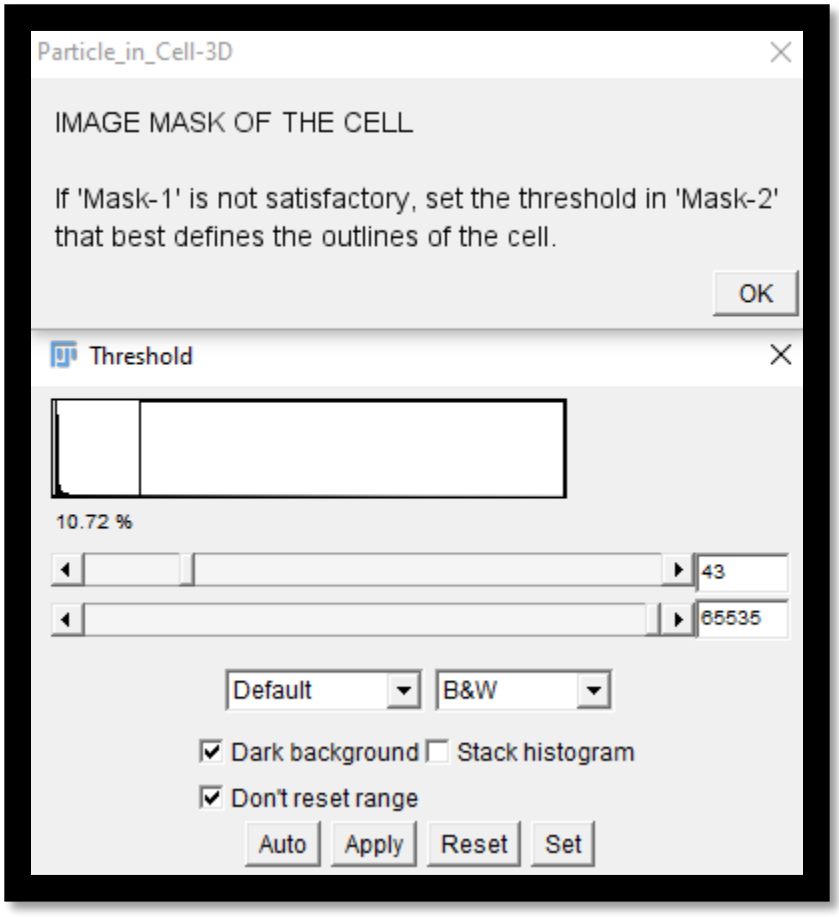


Before clicking "OK", make sure that the thresholding is satisfactory. If not, it can be manually changed before clicking "OK".



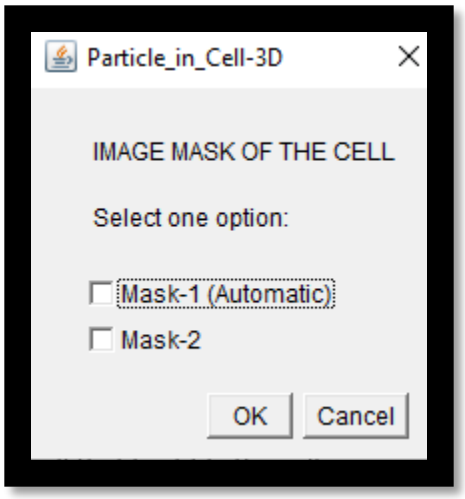
Click "YES" if satisfied with the current thresholding value. "No" to get a new suggestion and set a new value.

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.



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

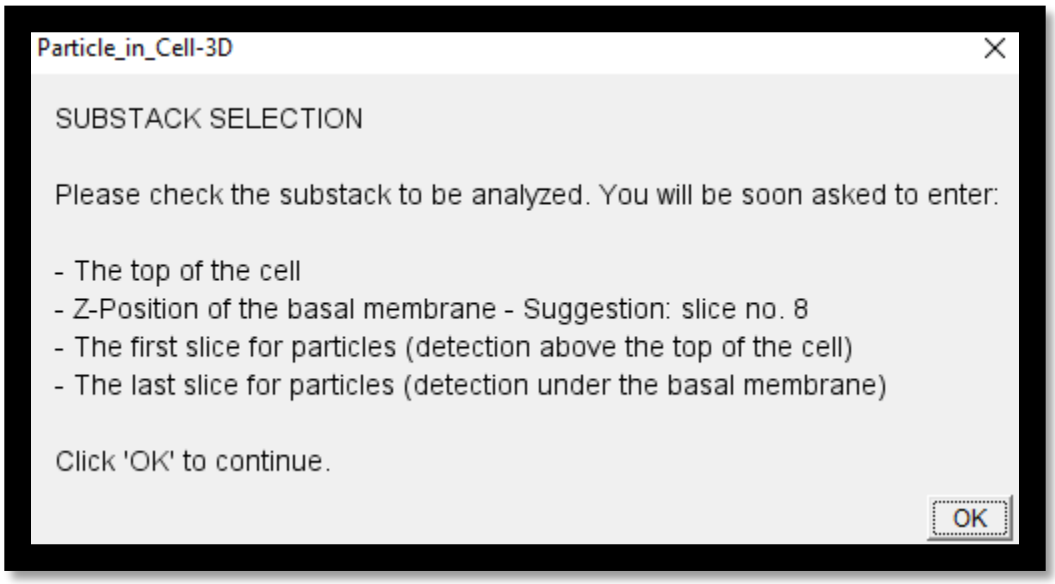
8. **Select best cell mask.**
   Selection of the mask of the cell that best fits the cells.
   - *Mask 1.* Automatic.
   - *Mask 2.* User defined.



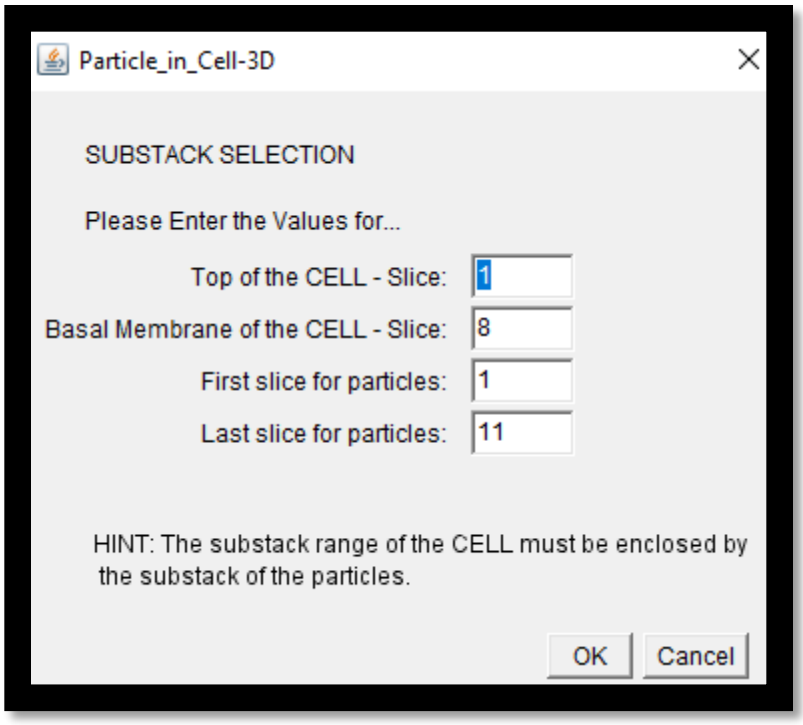
Select best representation of the cell.

9. Sub Stack Selection

Check the sub stack. Choose the slide that best represents: Top of the cell, basal membrane, first slice for particles and las slice for particles. Recommendations are given but they can be changed manually.

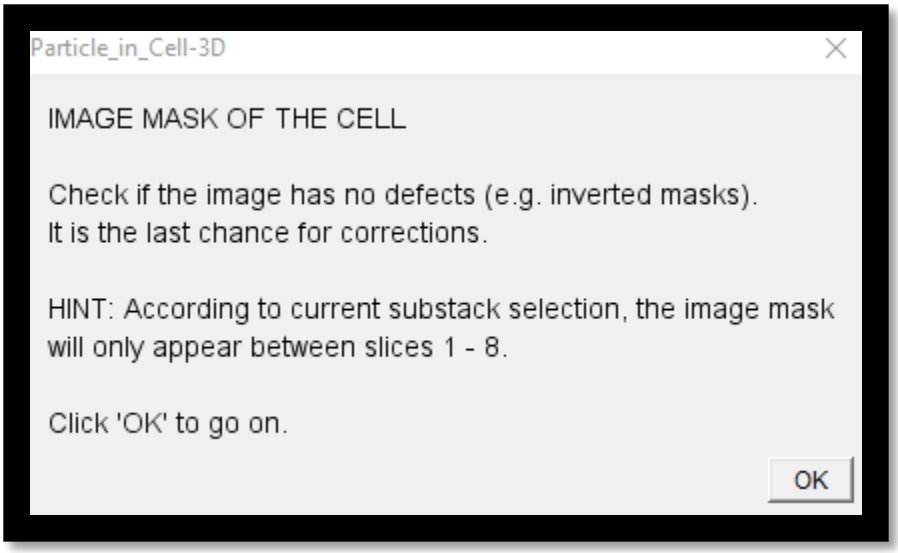


Before clicking "OK" make sure to note the slides corresponding to the 4 positions asked. After clicking "OK" enter the slides corresponding to the positions asked. The stack can no longer be analyzed while in this step.



10. Check Image Mask

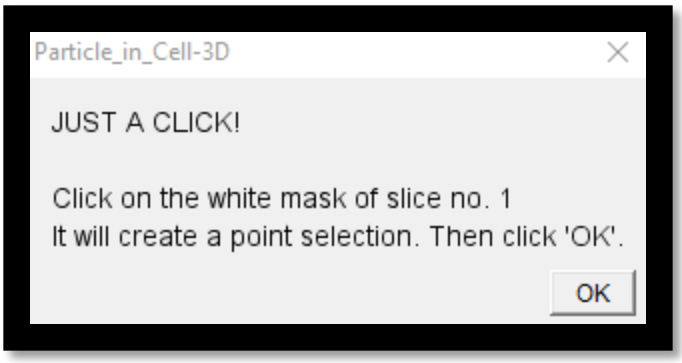
Check that the mask represents the cell accordingly through the stack.



Check mask of the cell.

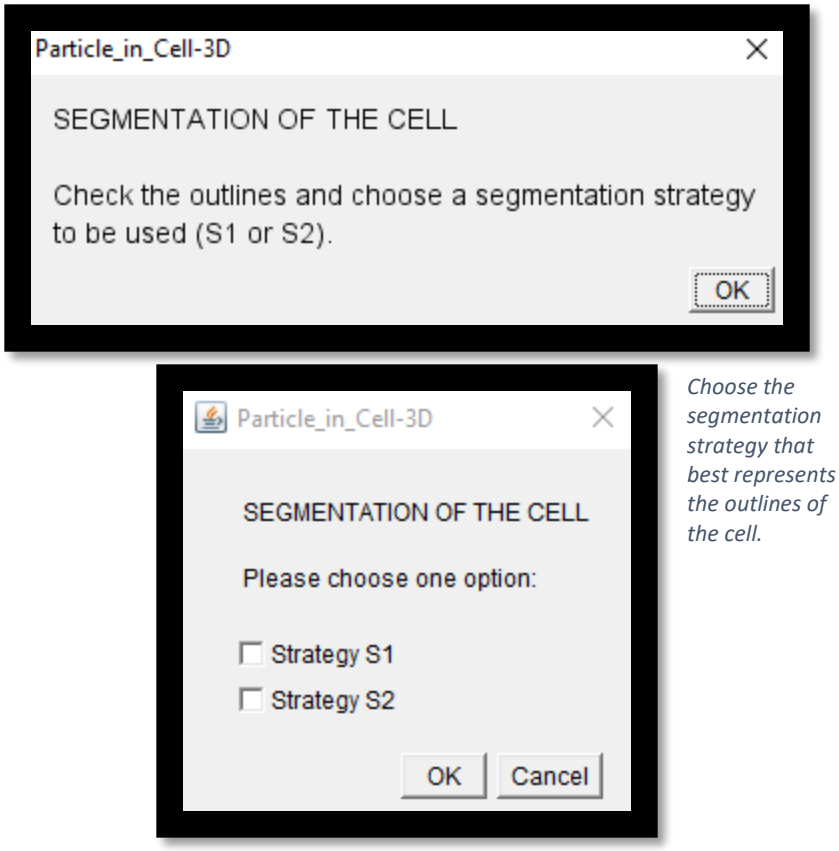
11. Cell Outlines

Click on the white mask of the first slice to create a point selection.



Before clicking "OK" click on the white mask of the first slice to create a point selection

Choose the best segmentation strategy.



Choose the segmentation strategy that best represents the outlines of the cell.