# Installation

1. Make sure that you have the latest version of Fiji (recently updated or a new install via <http://fiji.sc/#download>). Note that the Fiji ‘installation’ is just a directory that can be copied anywhere (just avoid the standard ‘Program Files’ directory to avoid issues).
2. Copy the files of plugins to the plugin directory of your Fiji installation. (i.e., NuclearUtilities-0.9.jar, Cell\_Migration\_Analyser\_3D-0.9.jar, and Nucleus\_Annotation\_3D-0.9.jar)
3. Start Fiji
4. Under the menu Plugins, check that MorphoLibJ and 3D (not 3D Viewer) are available.
5. If either is missing, go to the menu Help > Update… and press the button ‘Manage update sites’. From the site list, make sure that ‘3D ImageJ Suite’ and ‘IJPB-plugins’ have been selected. Press ‘Close’ and ‘Apply changes’ and wait for the installation to end.
6. Under the same Plugins menu, check if LoG3D is available. If not, please download the zip file from <http://bigwww.epfl.ch/sage/soft/LoG3D/>. Please note that the zip file download link on that page may be mostly hidden by a side-bar. Look for the ‘ip’ near the bottom of the left side-bar.

Unpack the zip contents (including the folder) in the plugin directory of your Fiji installation.

1. (Re)start Fiji

# Parameter tuning

### The first thing that needs to be done before you start measuring your images is to find the best parameters for the plugins you are going to use. The results of this tuning will also give you the reliability of the plugins on your data set.

### Create a ‘golden truth’ annotation set

In order to test your segmentation and measurement settings, you first need to create a so-called ‘golden truth’. The golden truth of an image is a file that contains the coordinates for the center of all the cell nuclei in the image and a qualifier for the cell’s migration mode.

The Nucleus Picker 3D is a plugin that helps you create such a file. You manually need to select the top, bottom and centre of each nucleus and migration mode for each cell you find in the image and the plugin will record the information and display it in the image for you to keep track of what you have done.

The following steps are needed to create a golden truth for an image:

1. Select the plugin at Plugins > Nucleus Annotation 3D > Nucleus Annotation 3D
2. Select an image file to annotate through the dialog.
3. If the file has been imported via the BioFormats dialog, please confirm this when prompted.
4. If the image has multiple channels, the application will ask which channel to work on. You should select the channel number of the DAPI (i.e. nucleus) signal. The application will then create a duplicate of that channel for you to work on.
5. Next, you can adjust the brightness or a similar setting to improve visibility. Confirm when ready via the dialog.
6. Now you will have to identify each nucleus in the image. You can scroll through the image with your mouse wheel during this. The dialog explains which actions are available. Steps 1, 2, and 3a are obligatory for parameter tuning on just segmentation. For migration mode analysis, either step 3b, 3c, or 3d is needed for every cell as well. Please note that steps 1 and 2 are for your convenience so you can easily see which cells you have already covered. There is no need to be very precise to which slice is the start or end slice for the parameter tuning, as long as the central slice for the nucleus is identified.
7. However, on step 1 and 2 do try to click as close to the center of the nucleus as possible. The program will interpolate between the two selected points and insert selection points on the intervening slices.

For the other steps, the plugin will automatically find the closest nucleus (based on steps 1 and 2), so precision is less necessary.

1. Colors and forms will signify for each nucleus/slice combination what its condition is:
   * Forms signify if the central slice has been selected for the nucleus: round = no central slice selected yet, square = central slice has been selected.
   * Green and blue are for the central slice of a nucleus: green = no migration mode selected, blue = migration mode selected.
   * All other colors are for the non-central slices: yellow = no migration data available, red = single cell, magenta = dual cell (two cells migrating together), cyan = multi (3+) cell group.
2. Any results are saved next to the image in a file with the same name but the ‘\_corrections.txt’ extension. Note that you can save at any time via the dialog and continue measuring (‘Save’ button). The annotations are also saved if you click ‘Done’, but this ends the session. You can continue at a later time though (see below). ‘Cancel’ will quit without saving. Please note that saving the annotations will overwrite any previous save for that image, but it will include all of the annotations that are currently in the image, even if they have been set in a different session.
3. If you start the plugin with an image that has a ‘corrections’ file present in the same folder, the data in that file will be restored and you can continue measuring as usual.
4. If possible, try to do at least one annotation image per condition in your experiment. Note that images used for annotation should preferably not be used in any further measurements as this may introduce a subjective bias.

### Create a marker files for the manual annotation

The segmentation plugin needs the recorded nucleus centers to be converted into an image format. In this marker image, each nucleus center is depicted as a single point, each with a unique colour. The following steps create such a marker file and also adapt the golden truth text file into a new ‘marker file; with the appropriate text format:

1. Make sure that the original of the image used for annotation is open and selected in Fiji. If the image has multiple channels, make sure that the DAPI channel has been selected. This does not need to be the first channel in the image (unlike the Nucleus Picker 3D plugin).
2. Select the plugin at Plugins > MCWatershed > Create Marker Images 3D.
3. From the pull-down menu on the pop-up, select ‘Manual points’. Click OK.
4. In the next dialog, select one of the ‘corrections’ files you created in step 1 and click OK.
5. Finally select the directory in which the marker files will be saved. Please note that this will create the sub-directories ‘Marker\_Files’ and ‘Marker\_images’ in the chosen directory if they do not exist yet. This is where the results will actually be saved.
6. The log window will show ‘End Create marker image 3D’ when finished.
7. Repeat this procedure for every annotation image.

### Test parameter settings against annotation with feature extraction

The final step of parameter tuning is an iteration of setting parameters for the various plugins (segmentation and measurements) and checking how the automated results match up with the golden truth.

1. Perform the Nucleus identification plugin of the Feature extraction. The tested parameters are in step 5. In order to get a good first guess for the maximum finder parameters, use the following:
   1. For Noise, use the value of 1.
   2. As Minimum Value, use ⅓ of the ‘Maximum size of nucleus’ parameter.
   3. The RadiusXY value should similarly start at ⅓ of the ‘Minimum size of nucleus’.
2. Next run the Segmentation plugin. Here step 6 contains testable parameters.
3. Finally the Feature Extraction plugin should be run. This will create the results for the given parameters.
4. Review the results and try with different parameters until satisfied.

In the results of the Feature Extraction plugin, the ‘\_Summary.xls’ file contains the training results. It will display the number and percentage of correctly segmented nuclei as well as the number and percentage of over and under segmented nuclei.

Under-segmented nuclei are automatically detected nucleus segments that contain two or more manual markers; i.e. these single segments should actually have been two or more nucleus segments. ‘Over segmented’ means that an automated segment has been detected without a manual marker. This segment should actually be part of another nucleus segment.

Both over and under segmentation can be corrected by the parameters of the Nucleus identification. Over segmentation is typically due to a too small nucleus size or a too permissive maximum finder (small radius or noise values). Under segmentation is the opposite. Note that in all likelihood any attempt to correct one type of error will increase the other type of error. Try and find the spot that contains the least errors in total.

Another misclassification can be seen in the number given for ‘Markers without a nucleus’. These are the manual markers that have not been picked up by the automated segmentation at all. One major cause for this is a too strict thresholding during the Segmentation plugin. Another possibility is that the marker is placed on the edge of a segment, especially when a nucleus is over segmented into two parts and the marker lands exactly between these parts. This is worth checking if the number of ‘Markers without a nucleus’ is very low.

It is strongly advised to first try and find the best parameters with just the DAPI channel and no migration mode analysis. Also, get a feeling for each parameter at a time; don’t change every parameter at the same time to start with. Later, when you approximately know what each parameter does, try and see how combinations work, as ‘per parameter’ optimums may not be the actual optimum.

# Spheroid annotation

In order to measure migration distances with the Cell Migration Analyser, the spheroid first needs to be annotated manually. This will create a spherical approximation of the edge of the spheroid from which the migration distance of the cells will be measured.

1. Select the plugin at Plugins > Nucleus Annotation 3D > Spheroid Annotation 3D.
2. Select the image you wish to annotate from the File popup.
3. Depending on the image, this will open the BioFormats import dialog. If so, after importing the image, the plugin will ask if BioFormats has been used (it cannot detect this itself). Please answer ‘Yes’ to proceed.
4. Now select the channel in which you wish to annotate. This can be any channel in which the spheroid edge is well identifiable to you. We have the best effects with the reflection channel.
5. The channel you chose will be duplicated and you now get the option to adjust the image to your liking (e.g. increase brightness).

Press ‘Done’ when finished.

1. In the image, look for a slice in which the spheroid edge is still identifiable and in which the spheroid is wide.
2. By using left mouse-clicks you can now add annotation points to the image. An annotation point looks like an open magenta-coloured diamond. Please add three annotation points spread over the edge of the spheroid.
3. After adding the third point, the application will draw a yellow circle that fits through all three points. This circle should roughly coincide with the spheroid edge.
4. By removing points (which deletes the circle) by holding the Ctrl button and clicking (removes the closest point) and then adding it at a different point you can adjust the circle to best fit the edge.
5. When you are happy with the circle, scroll to a different slice (up or down) in which the edge is still visible, but which is preferably significantly less wide than the previous slice. Make sure that the edge is still open and that you are not at the very top or bottom of the spheroid. It is best if there is clearly some opening left and the edge is well defined, as near the top/bottom of the spheroid the edge gets a bit fuzzy to detect well.
6. By left clicking the mouse add one single point to the edge of the spheroid.
7. The plugin will now draw a sphere in the entire image stack that fits through all four annotation points. The sphere is drawn as one circle per slice it is present on. The sphere is also adjusted to the x/Y vs Z resolution ratio.
8. The sphere drawn should approximately match the edge of the entire spheroid. Please asjust the points by removing and adding them again as described before. This will result in the removal and redrawing of the sphere as well. Please make sure that the first slice contains a maximum of three points and the other slice (which you may change after removing the fourth point) contains one.

The one ast point can best be used to control the overall size of the sphere while the three initial points are best adjusted to change the positioning of the sphere.

1. When you are happy, press ‘Done’ and the annotation data will automatically be saved next to the original image. During annotation you can also save the current results by using the ‘Save’ button. The current points will be saved, but the annotation process will continue. The ‘Cancel’ button can be used to stop the annotation without saving.
2. Finally, if you wish to check your annotation on a different channel, you can restart the plugin and choose the alternative channel. You can make adjustments on this channel or just cancel if everything is as desired.
3. Please note that if you want to use the annotation during the Feature Extraction plugin described below, you need to keep the annotation file in the same folder as the original image file.

# Cell migration analysis

### This section describes the steps to take to segment and measure a cell migration image stack. The requirement for this plugin is a 3D spheroid-based image stack that contains at least a DAPI or similar nucleus stain channel. For measuring the migration modes, an actin-based staining channel is also required. Before using the plugins to measure, please tune the parameters of the plugins as described in the previous section.

For all three plugins that make up the 3D Cell Migration Analyser you will be asked to select a ‘working’ directory. This working directory will be the place where all the results of the plugins will be stored. Please use a single working directory per image and keep the directory the same over all three plugins. The plugins will create subdirectories in the working directory to keep the output of the plugins clearly identifiable.

### Nucleus identification

The first plugin is used to automatically identify the centres of the nuclei in the images (in 3D). These centres will be used as starting points for the segmentation in the next plugin.

1. Load the image you wish to measure. The image should at least contain a nucleus (DAPI or another nucleus stain) channel. Make sure that the image is the active (selected) image.
2. Select the plugin at Plugins > MCWatershed > Create Marker Images 3D.
3. Identify the nucleus channel-number in the drop-down in the first pop-up.
4. From the pop-up, select the method ‘Laplacian of Gaussian’ and press ‘Ok’.
5. Select the working directory in which the resulting marker files will be stored. Please note that this working directory should be kept the same over the next two plugins as well. Also note that the popup allows you to create and name a new directory if needed (third button from the top-right).
6. The nucleus identification consist of two steps: identify potential spherical objects with a Laplacian of Gaussian filter and then find the highest value from these potential spots.

**Laplacian of Gaussian parameters:**

The Laplacian of Gaussian (LoG) filter will give a high value to any high intensity point in a high intensity area with a sharp edge to a low intensity at a given radius. It is therefor ideal for detecting bright round shapes on a dark background in an image.

The plugin will allow for a range of nucleus diameters to account for variability between nuclei sizes. The user is asked to give a minimum and maximum diameter value as well as the increment size of the in-between steps. Starting at the minimum size, the plugin will then apply the LoG filter with the indicated size increments until the maximum value is reached. The individual filter results are then combined by taking the maximum of the filter values per pixel.

We have found the best results at about 3 to 5 steps and a limited range (e.g. 10 pixels for sizes of about 15-25 pixels) of sizes. With larger nuclei sizes, a larger range is acceptable.

* + Minimal size The minimal diameter of a nucleus
  + Maximal size The maximum diameter of a nucleus
  + Stepsize The incremental steps to get from the min to max radius

**Maximum finder parameters**

The maximum finder will identify the maximum intensity points in the 3D image stack after the LoG filter. It will only allow one maximum in a given radius, suppressing all lower potential maxima. It will also ignore any potential maxima that can be linked to a higher maximum via a path of intensity values that are within a given ‘noise’ range. Please note that the maximum finder is performed on the LoG filtered image, so the values you use should reflect this.

* + Noise The allowed noise range for linking maxima
  + Minimum value Minimum value for maximum points to speed up the process
  + RadiusXY The minimal distance between two nuclei centers (in pixels)

The checkbox ‘Process per slice’ will set the plugin to perform the LoG filter as 2D per slice instead of 3D over the entire stack. This is only recommended if the Z to X/Y resolution ratio is very large or the intensity level drops off very steeply in the Z direction.

The other two checkboxes are meant for reporting and insight and do not influence the results of the identification. The first one will show a visual representation of the LoG3D filter that is being applied. The last checkbox will show the intermediate LoG3D filtered images, one for each step defined in the ‘size’ settings.

1. Wait for the plugin to process. This may take a while. In the meantime, don’t click on any of the images that pop up. You can move the log window around for a better view. The plugin is done when the log window shows ‘End Create marker image 3D’.
2. Next to the original image, there now is a marker image open (it has also been saved in the directory you chose earlier). This image looks dark, but that is just because the markers are only one pixel wide and mostly have a low intensity value.

### Segmentation

The segmentation plugin will take the markers created by the previous nucleus identification as input and use them as a starting point for segmenting the image through a watershed method. This differs from the ‘classic’ watershed in that the classic form most often uses the maximum points of a distance map as its origin. By using the markers, this segmentation is assured to contain all the detected nuclei.

Next to the nucleus detection, the plugin is also capable of segmenting a complete cell-based channel to get segments that more or less resemble the complete cell outline. Usually an actin-staining channel can be used to serve as such a cell depiction; however, note that this segmentation is less accurate as actin is not as uniformly present in the entire cell. Also note that cell segments found based on this signal are combined with the matching the nucleus segments to create a full cell segment, so even a cell staining that does not cover the nucleus is acceptable.

1. If you didn’t continue from the last plugin, make sure that the original image and the marker image (stored according to your choice) are open.
2. Select the plugin at Plugins > MCWatershed > Marker controlled watershed 3D.
3. In the first popup, select the original image from the drop-down menu and press ‘Ok’.
4. In the next popup, make sure that the Nucleus Channel is set to the nucleus channel in your image. Optionally, you can select to perform the cell segmentation as well (necessary for migration mode analysis). If so, mark the check box to segment the actin signal and select the channel with the cell staining you wish to use (which, despite the label of the drop down menu, doesn’t need to be based on actin).
5. From the popup, select the marker image (with ‘\_Markers\_’ in its name) from the drop-down menu and press ‘Ok’.
6. The next popup allows you to select the filter and threshold methods. These settings are used to create the limits for the segmentation image. Anything that has passed the threshold is available as part of a segment. The filter is used to reduce noise effects and to smooth edges. This should prevent many small segments or broken up segments. It is advised to try out filters and thresholds on a small set of images outside of the filter to see the results. For thresholds, harsher thresholds tend to give a better segmentation, but reduce the volume detected and may even miss cell and nucleus segments all together. Which is preferable depends on what you wish to measure.

The ‘Calculate dams’ checkboxes affect the resulting segmentation in that two segments that are next to each other in the image will be separated by at least one pixel of background. This can be done to improve the visibility of the segmentation. It does not change the migration mode analysis, but it will have an effect on other measurements, so use it with care.

With the experimental threshold checkbox, you can use one of the two options to reduce the effects of stack depth on the image intensity (i.e. attenuation). The experimental method uses a threshold per slice that is based on the average threshold of a sliding window of neighbouring slice thresholds. By using a local threshold, the effects of depth are less severe than when using a stack threshold, while using the average negates the spikes in intensity that regularly occur on deeper stack levels. The Threshold Window sets the width of the sliding threshold window. It configures the X number of slice thresholds both above and below the current slice that are taken into account. If the window passes beyond the borders of the stack, the threshold of the last slice on that border is uses for the rest of that part of the window.

Press ‘Ok’ to continue.

1. You now have the option to use the other attenuation correction method available. This method has more options for adjustment (which also require more tuning!).

The attenuation correction presented here measures the foreground values of every slice and finds a best-fit function through the results. This function is then used to adjust the intensity values of the image before filtering and thresholding. To use the adjustment, select the check box at the top of the dialog. The parameters are:

* Foreground percentile: The adjustment method measure foreground by taking the average of the top X percentile of intensity values. This parameter determines the X. 90 is a good first percentile to try.
* Start intensity: To reduce the influence of background (the most prevalent values in most spheroid image stacks), you can set a minimum value for the intensity to take into account. The percentile described above is taken on all the values that lie above this minimum. Please be careful to not set this too high based on the earlier slices of the image, as in the deeper slices the foreground values can be significantly lower and may overlap with what is surely background in early slices.
* End intensity: This sets a maximum value to the intensity values taken into account for the foreground percentile. This should be mostly used to remove just the maximum value, so overexposed pixels are not counted (as this may not be the real value of these pixels).
* Standard slice nr: One slice is designated as the standard slice on which the optimal intensity values are found. All other slices will be adjusted to have an average foreground similar to this slice. The best choice for this slice depends in the imaging decisions taken. Please select the first slice with a robust amount of foreground signal and preferably a low level of overexposure.
* Curve fit method: This is the type of function that is used to best fit the gradient in which the average foreground intensity diminishes with depth. This contains many options and the best way to find out how your image values are behaving is to measure the average foreground signal yourself on a few images. We found that the ‘Exponential with Offset’ gave a very good fit.

Press ‘Ok’ to continue.

1. Select the working folder to store the segmented output image. Use the same folder that was used in the Nucleus identification plugin.
2. Wait for the plugin to process. This may take a while. Please do not select any of the images in Fiji, though you can move the Log window. When the plugin has finished, the log window will show ‘End Marker-controlled watershed 3D’. Two new images are now available: the segmented nucleus and actin images. These images have also been saved at the location chosen earlier.

### Feature Extraction

The feature extraction plugin will measure the nucleus and cell segments created by the previous plugin. The measurements will be done on geometric features such as size and shape as well as intensity-based features such as mean, total and maximum intensity.

Furthermore, this plugin will do a very basic migration type classification for each cell. Migration types consist of single cell migration, dual cell migration, multi-cell (3+) migration and the spheroid itself. This classification is done purely on connectivity. For example, a cell is considered to be a single cell if there is no voxel in that cell segment that is adjacent to any voxel of a different cell segment. Adjacency is orthogonal, so no diagonal connections are considered. Finally, the largest group of cells is deemed to be the spheroid.

1. If you didn’t continue from the last plugin, make sure that the original image and the one or two segmented images (stored according to your choice) are open.
2. Select the plugin at Plugins > MCWatershed > Feature Extractor 3D.
3. In the popup, select the original image from the upper drop-down menu. You can optionally use the second drop-down menu provide a visually enhanced version of the original image for displaying the resulting output segmentations on.

You can select to automatically save the resulting images and tables by checking the ‘Save the results’ box.

If you are testing your parameters, do not forget to select the ‘Include manual markers’ check box.

Press ‘Ok’.

1. Now you can configure how the plugin will use the channels in your image. Via the drop down menu’s you can select which actual image channel belongs to the label. A selection of ‘0’ means that this type of channel will not be active during the measurements.

In the first drop down menu, select your nucleus channel and in the second your cell-stain channel (if present).

At this point, you can add extra channel to measure. Use these extra channels to measure the intensity values of your other stainings or to have a different output on a channel you have already chosen. It is possible to select a channel twice (or more) and this includes the nucleus and cell channels.

Next to selecting the channel number of each extra channel measurement, you should also select the area or segment that will be measured:

1. Nuclear centre: Centered on the coordinates of the nucleus center marker, an orb with a radius of 3 voxels (in x/y, adjusted in z for resolution) will be used as area of measurement. This is useful when the nucleus segmentation is not optimal and you wish to be certain that the measurements take place in the nucleus. This works best if the nuclei are of sufficient size so that the 3 pixel radius falls within most nuclei.
2. Nucleus: Only the nucleus segment will be used for each cell
3. Cell: The entire cell and nucleus segments will be used per cell
4. Nucleus surrounding: Measures a band of 2 voxels wide around the nucleus segment. All the nucleus and cell segments are used to only include voxels that are part of any cell segment, while not part of any nucleus segment. This option is ideal if your cell segments are sub-optimal. It does require a good nucleus segment fit though as otherwise you may include parts of the nucleus itself in a very limited amount of values.
5. Cell without the nucleus: The cell segment with the nucleus segment explicitly removed will be used for every cell.
6. In the next popup, select the segmented image for the nucleus segmented image in the top drop-down menu and that of the segmented cell image in the bottom drop-down menu (you can tell them apart by the \_DAPI\_ or \_Actin\_ parts of their names). Press ‘Ok’.
7. Select the working directory you have been using for the previous two plugins and press ‘Ok’. If you selected to save the results, this is where the images and measurements will be stored.
8. In the next popup, select the first check box if you segmented with dams on the nucleus channel. In order to measure the migration mode and do any other whole-cell measurements check the second (migration mode analysis) check box and (un)select the third one (segmented actin with dams) depending on the segmentation setting for the cell channel. Note that you must have a cell channel and segmentation image open for the migration mode.

The bottom part of the dialog contains the options for post-processing:

* ‘Exclude cells with a nucleus touching/crossing any border of the image’ takes the nucleus marker found by the Nucleus identification plugin and measures its distance to any of the borders of the image (in X, Y or Z). If the distance falls at or below the given exclusion zone, the cell is marker for border exclusion in the resulting data and not taken into account for the migration mode analysis. Please note that the migration zone width is currently doubled for the X/Y direction. This will be replaced by a more image resolution dependent factor in later versions.
* ‘Exclude cells with a very small nucleus volume’. Checking this option will mark any cell that has a nucleus segment below the given size (in voxels) as excluded based on size and the cell is ignored for the later migration analysis.

Press ‘Ok’ when done.

1. Wait for the plugin to end. This is usually quicker than the previous two plugins. The Log window should now show the ‘Total amount of’ numbers for the various migration modes. There are an extra three images:
   * The nucleus outlines, combining the nucleus signal with its segmentation as an outline (in red) and the marker points for each detected nucleus in yellow.
   * The migration groups, in which the nucleus signal is enhanced by the outline of the actin segmentation in which each identified group of cells migrating together (including the spheroid) has its own colour.
   * The migration modes classification, where the actin segments are coloured according to their migration mode in blue (spheroid), magenta (single detached cell), green (two cells, detached together) and red (three or more cells detached as a group).

Also note the three results tables detailing several measurements for each detected cell, data on each detected migration group and the overall summary.