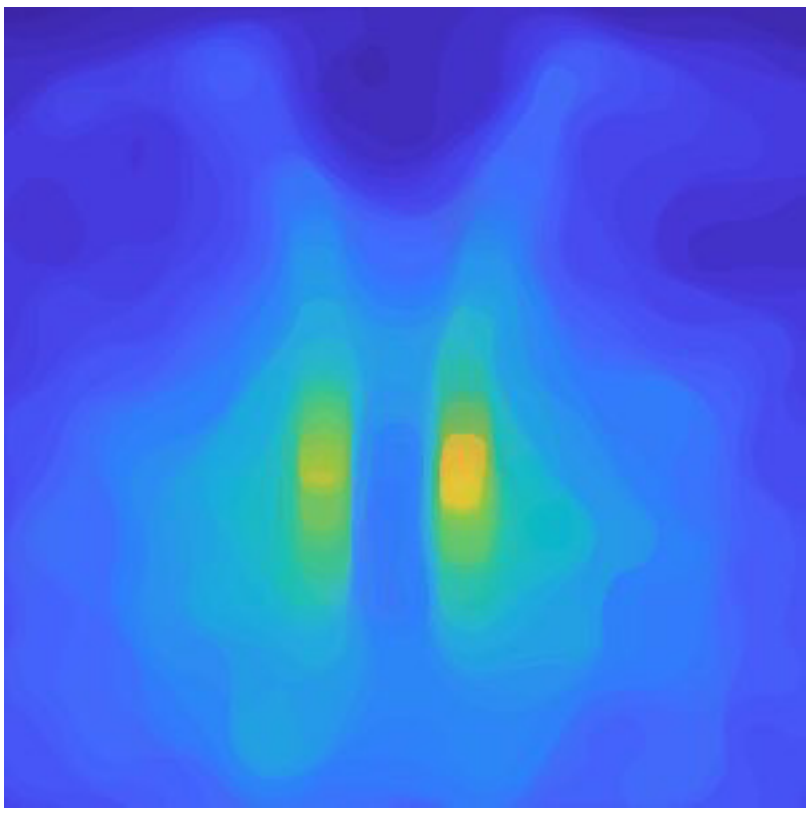
MANUAL



Providing Data

One set of *input data* consists of three microscopic channels that have to be named according to the following scheme:

*number*_***nuclei.tif*:**
Microscropy channel including the labeling of the nuclei.
*number*_***landmark.tif*:**
Microscopy channel shows the labeling of the landmark that will be sued for registration. It should contain a structure that is consistent in shape, size and location among all data sets. The structure should be longish and we advise you to align it horizontally.
*number*_***probe.tif*:**
Microscopy channel including the labeling of cells/tissue of interest.

Remark: The images do not have to cover the whole embryo as long as the landmark structure and the probe of interest is contained. Embryo coverage of 40-50% have been proven to be sufficient.

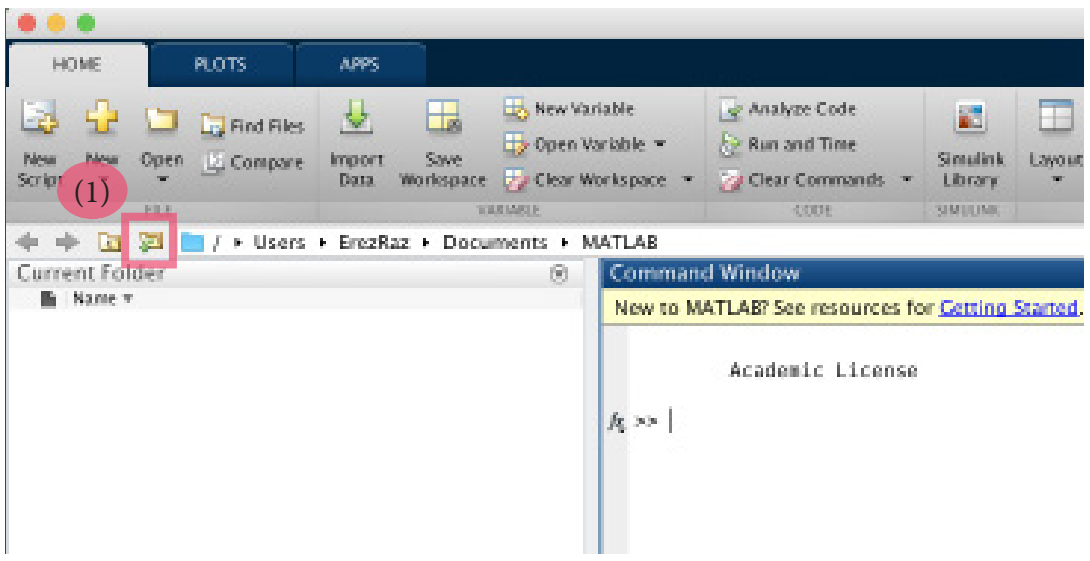
Provide as many of these input data sets as you like and join them in a folder.
All files need to be of the same size.

Getting Started

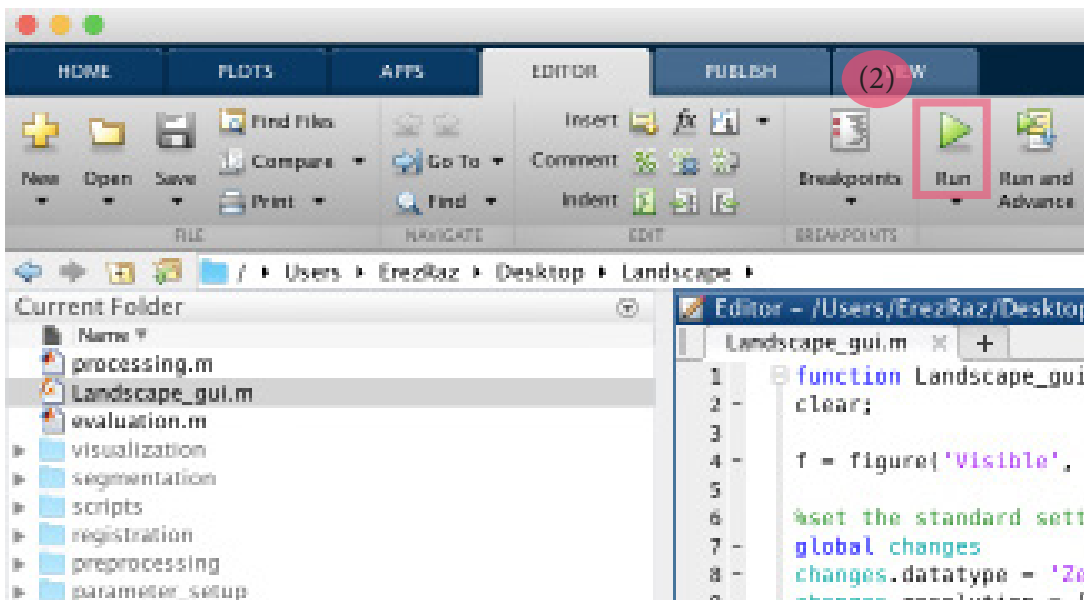
Running Landscape with MATLAB

Open MATLAB.

(1) In MATLAB, browse for the folder that contains the *Landscape* software.



(2) Double click the file **Landscape_gui.m** and click on **Run**. The main window of the software will appear.



Running Landscape without MATLAB

Alternatively, you can run the compiled version of the software on a computer with Windows 8/10.
Install the Landscape software by executing the *Landscape.exe* file and follow the instructions. We recommend to install and run Landscape as a user with full privileges to avoid unexpected errors due to filesystem restrictions.

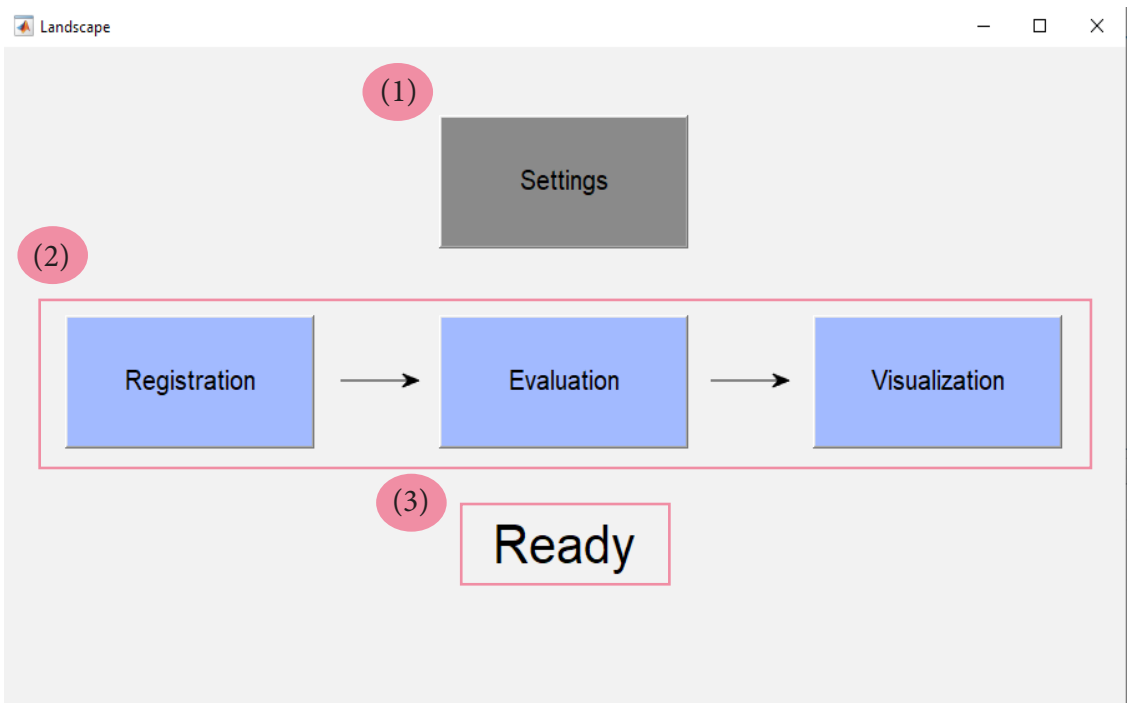
Once the installation is completed, run Landscape by double-clicking on the corresponding icon.
The main window of the software and a command window will appear.

Landscape main window:

(1) **Settings**: includes information required to analyze your imaging data.

(2) Workflow consisting of the three main steps:
Registration: this step includes the 3D image registration of your imaging data.
Evaluation: this step allows to evaluate the registration results.
Visualization: this step includes the generation of heatmaps.

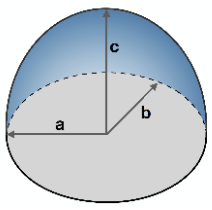
(3) Status indicator: it informs about the current status of the analysis.



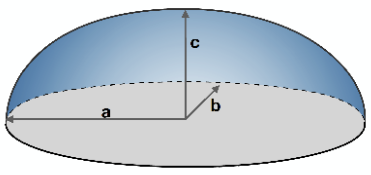
Settings

Before you start working with your data make sure to check the settings. Click on **Settings**.
The *Settings* window will appear.

(1) Choose the type of your input data.
The following image helps to determine if your data is **Zebrafish-like** or **Drosophila-like**.



a:b:c
≈ 1:1:1
Zebrafish-like



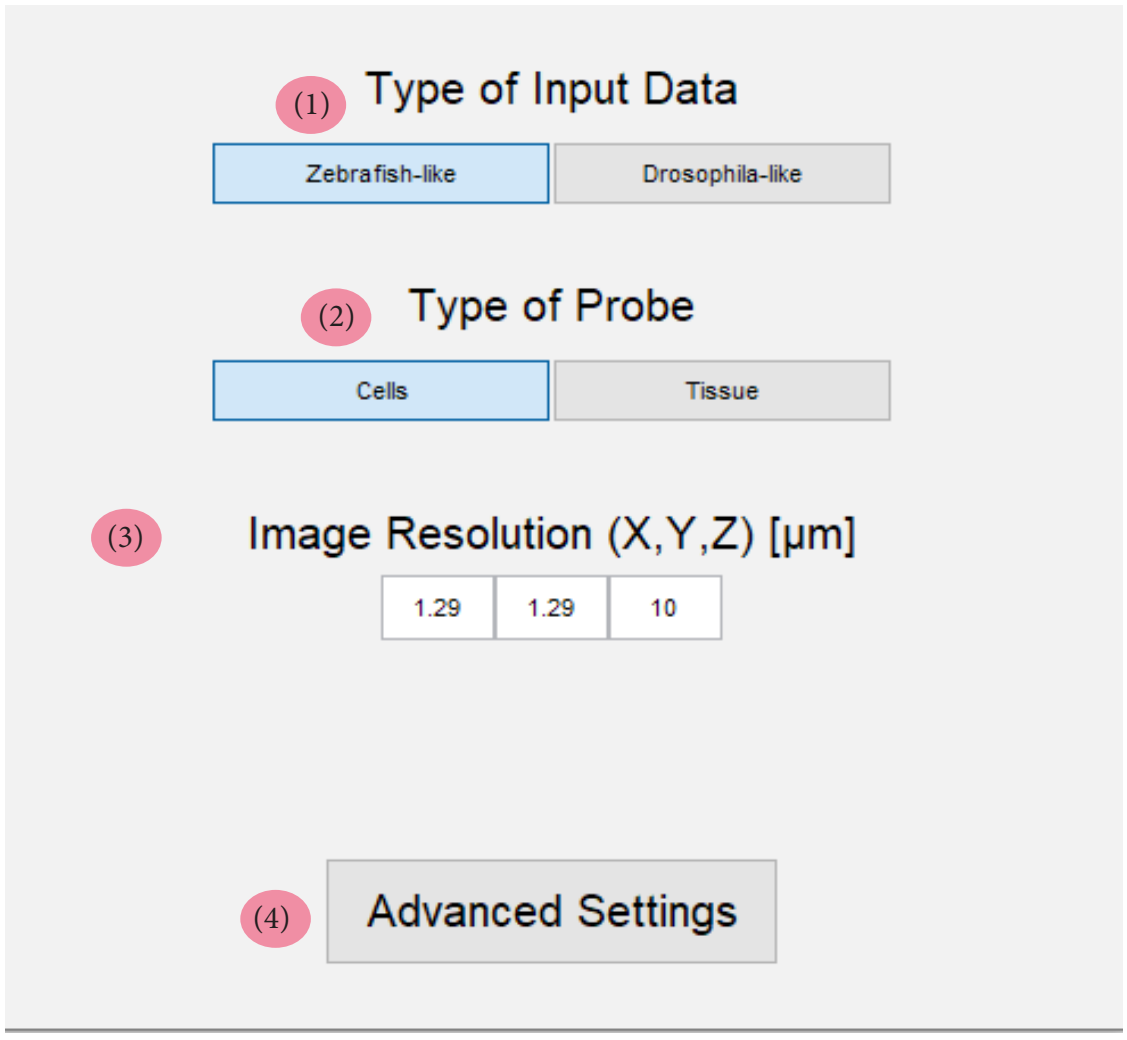
a:b:c
≈ 2:1:1
Drosophila-like

(2) Choose the type of probe.
If you want to analyze the distribution of cells choose **Cells**. If you want to analyze larger structures, such as tissues, choose **Tissue**.

(3) Provide the resolution of your imaging data.

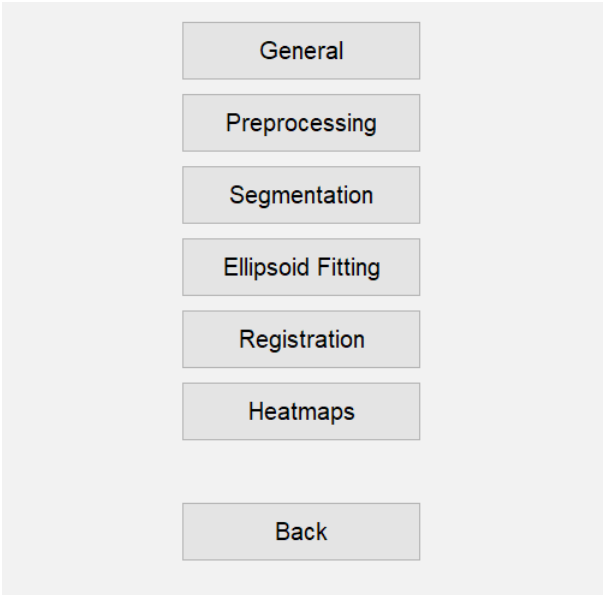
(4) **Advanced Settings** will guide you to more settings that you might need to adjust the software to a new type of data.

Now you can close the Settings window.

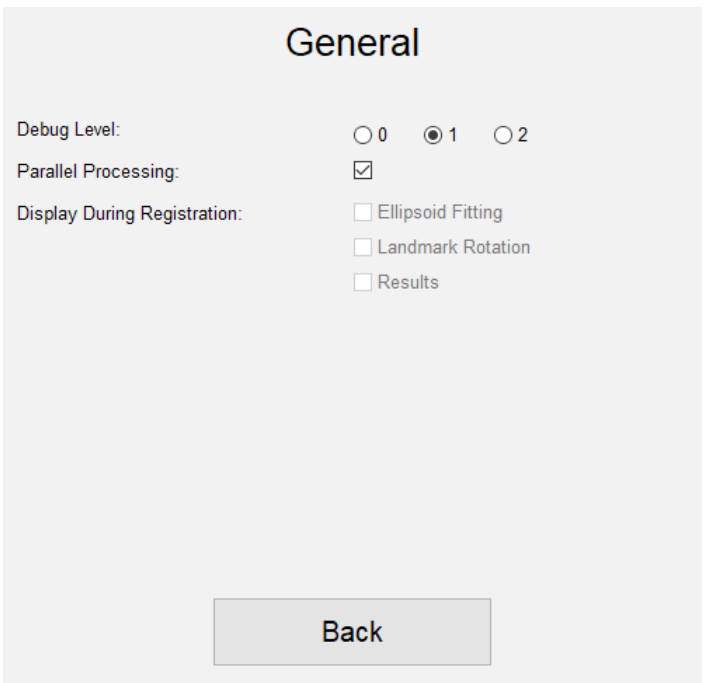


Advanced Settings

Advanced Settings include all settings that you need to adjust the software to a new type of data.
There are several categories of settings for the different processing steps of the software.



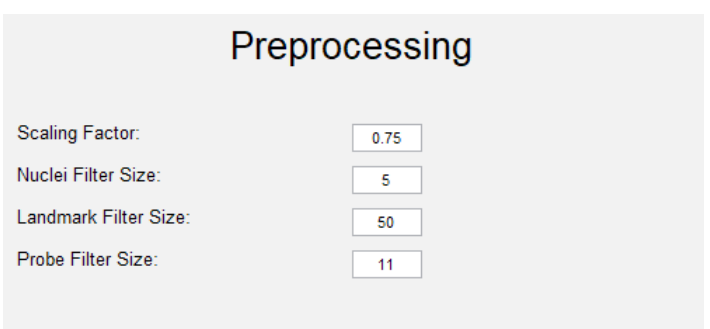
The **General** settings provide you with options to help you finding the correct parameters for your data and allow you to debug the code.



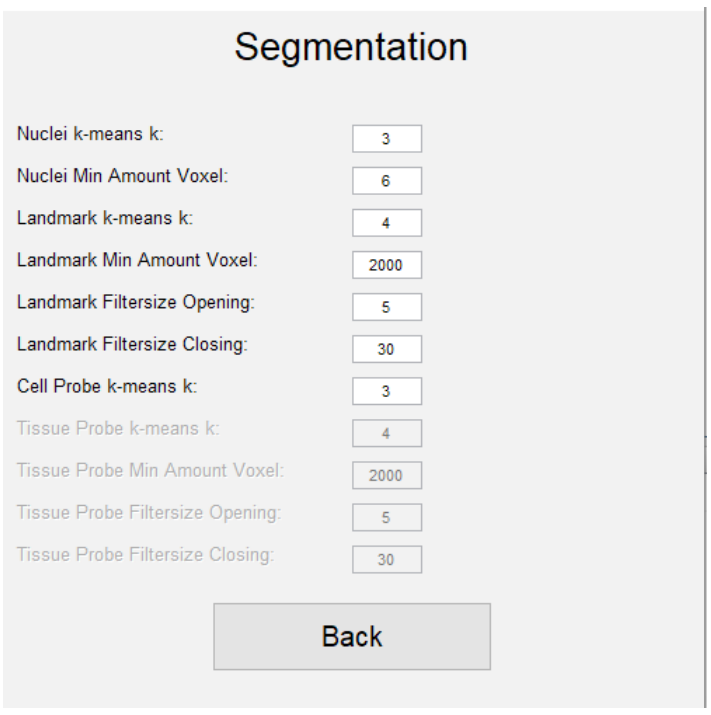
Increasing the **Debug Level** will show you additional information about the processing progress of the *Landscape* software in the shell/command window.
You can also choose if you want to have intermediate results of the Registration step displayed. Note that the **Parallel Processing** option has to be deactivated for this to work.
Activating the **Ellipsoid Fitting** option will show you a 3D image of the segmented nuclei channel (blue) in comparison to the ellipsoid that was estimated by the software (red). This will help you to find the correct parameters for the shape estimation.
Activating the **Landmark Rotation** option will show you two 3D images of the segmented landmark before and after registration, respectively. This should help you to find the correct registration settings for your data. Note that the landmark will always be projected onto a sphere, no matter what the ellipsoid looks like, because the registration algorithm assumes a unit sphere reference system.
Activating the **Results** option will show you an overview of your input, segmented and registered data. This allows you to see the results of your chosen parameters immediately after the Registration step, without having to start the Evaluation step.

In the **Preprocessing** settings, you can set a **Scaling Factor** and **Nuclei, Landmark and Probe Filter sizes** for background noise removal.

A **Scaling Factor** of 1 will preserve the original size of the raw data during data processing. You can speed up the overall process by choosing a smaller value, but note that too small values will also decrease the accuracy and lead to a loss-of-details.



In the **Segmentation** settings, you can alter the parameters for the nuclei, landmark and probe segmentation.

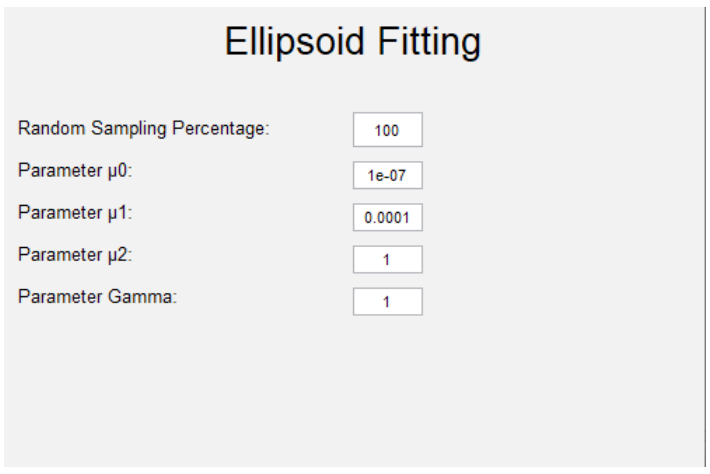


The segmentation of the nuclei, landmark and probe is performed using the k-means algorithm. Each microscopic channel has its own set of segmentation parameters. The segmentation of the probe is performed differently for cell and tissue probes and therefore has two sets of segmentation parameters. You can only change one set of probe segmentation parameters at a time, depending o n the type of analysis you chose in the *Settings* window.

For each microscopic channel you can change the parameter **k-means k**, depending on how many different types of tissue signal you expect in the respective microscopic channel.
The parameter **Min Amount Voxel** determines the minimum size of your cells or tissue. You have this option for nuclei, landmark and tissue probe segmentation.
For a postprocessing of the segmentation results of the landmark and tissue probe channel you can additionally set the size of morphological opening and closing filters with the parameters **Filtersize Opening** and **Filtersize Closing**.
To check if you defined these settings correctly, activate the display of *Landmark Rotation* and *Results* in *General Settings*.

In the **Ellipsoid Fitting** settings, you can alter all relevant settings for the shape estimation of the embryo.

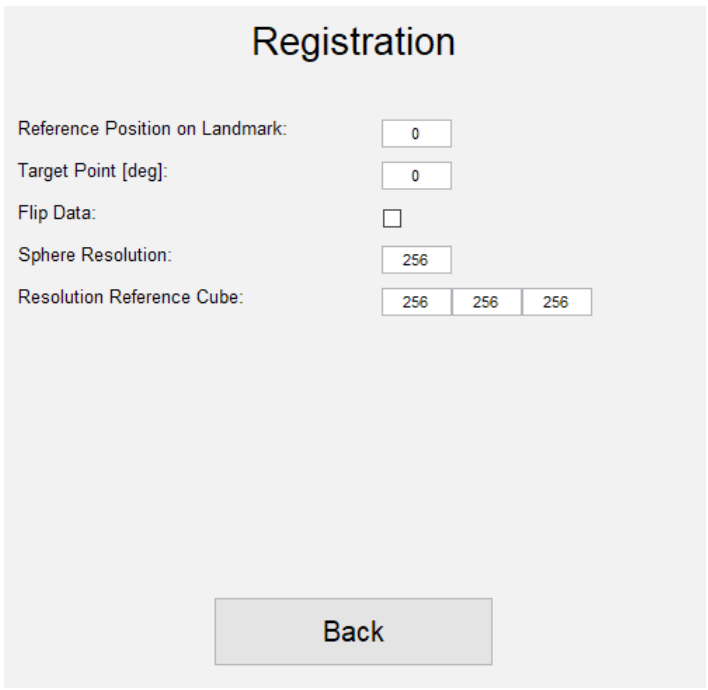
The parameter **Random Sampling** is the percentage of randomly chosen points from the nuclei that are used to estimate the ellipsoid. A small value will accelerate the fitting process, while a large value will increase accuracy.



The parameters **μ0**, **μ1**, **μ2** and **Gamma** are the regularisation parameters required of the shape estimation model.

To find the correct values for these parameters we advise you to start with the default settings of either Zebrafish- or Drosophila-like data and activate the display of *Ellipsoid Fitting* in *General settings*.

In the **Registration** settings, you can define how the data should be registered.



First, choose the **Reference Position on Landmark**. Imagine a line that goes through your landmark from left to right (assuming you have aligned the landmark approximately horizontally). The Reference Position can be anywhere on this line starting from the start of your landmark (left/Reference Position on Landmark = 0) to the end of your landmark (right/Reference Position on Landmark = 1). Assuming your landmark is well segmented and has a consistent length, you can choose any value in the interval [0,1] according to your preferences.
If you expect the segmentation of your landmark to be problematic on one side or having inconsistent lengths, we advise you to choose either the starting or endpoint of your landmark, accordingly.

Then, you can choose the **Target Point**. Imagine a sphere. The Target Point can be anywhere on the equator starting from the very left point of the sphere (Target Point = 0 degrees), moving forward to the very front part of the sphere (Target Point = 90 degrees), etc. To achieve the best results, you should choose a **Target Point** that is near the original position of the **Reference Position on Landmark**.

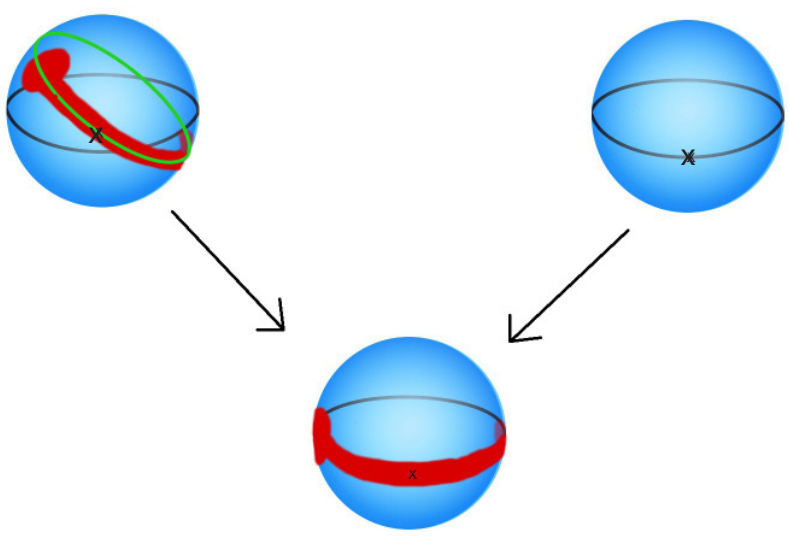
During the Registration step the landmark will be aligned horizontally while the **Reference Position on Landmark** will be moved to the **Target Point**.
Note that there are two ways the landmark could be aligned like this, either clock- or counterclockwise. By default, it will be aligned counterclockwise. To change this, activate **Flip Data**.

To check if you defined these settings correctly, activate the display of *Landmark Rotation* and *Results* in *General* Settings. If your registration results in the third row of the Results window look smeared, your **Target Point** might be too far away from the original position of the **Reference Postition on Landmark**. If this is not the case, you might want to check your *Ellipsoid Fitting* parameters again.

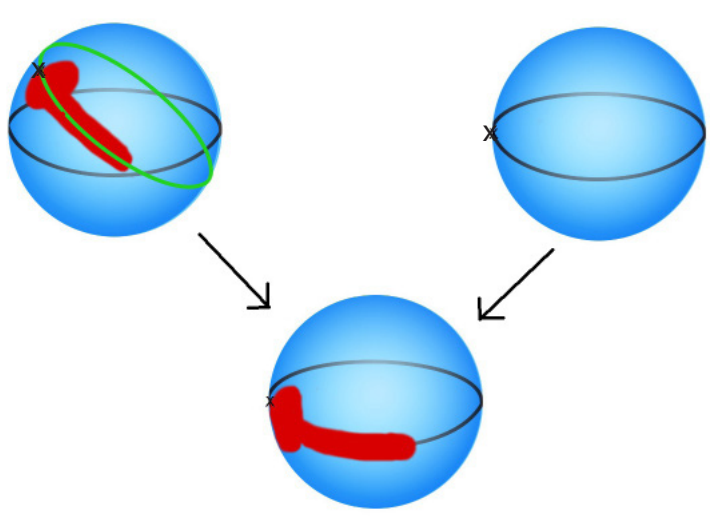
The **Sphere Resolution** option allows to specify the resolution of the unit sphere reference system. The landmark is projected onto the surface of this sphere. A higher resolution will provide a more detailed projection of the landmark, and therefore increase the accuracy of the registration. But note, that increasing the resolution will also affect the performance of the software.
Activating *Landmark Rotation* in *General* Settings will show you how the projected landmark looks like in the unit sphere reference system.

The final results of the Registration Step will be saved in a three-dimensional unit interval reference system. You can change the resolution of this reference system in **Resolution Reference Cube**. The ratios of the x, y and z direction should be approximately the same ratios as in the original input data. However, the actual resolutions can be much smaller than the original resolutions.
Activating *Results* in *General* settings will show you the size of the Reference Cube in the third row.

Reference Position on Landmark = 0.5 **Target Point** = 90

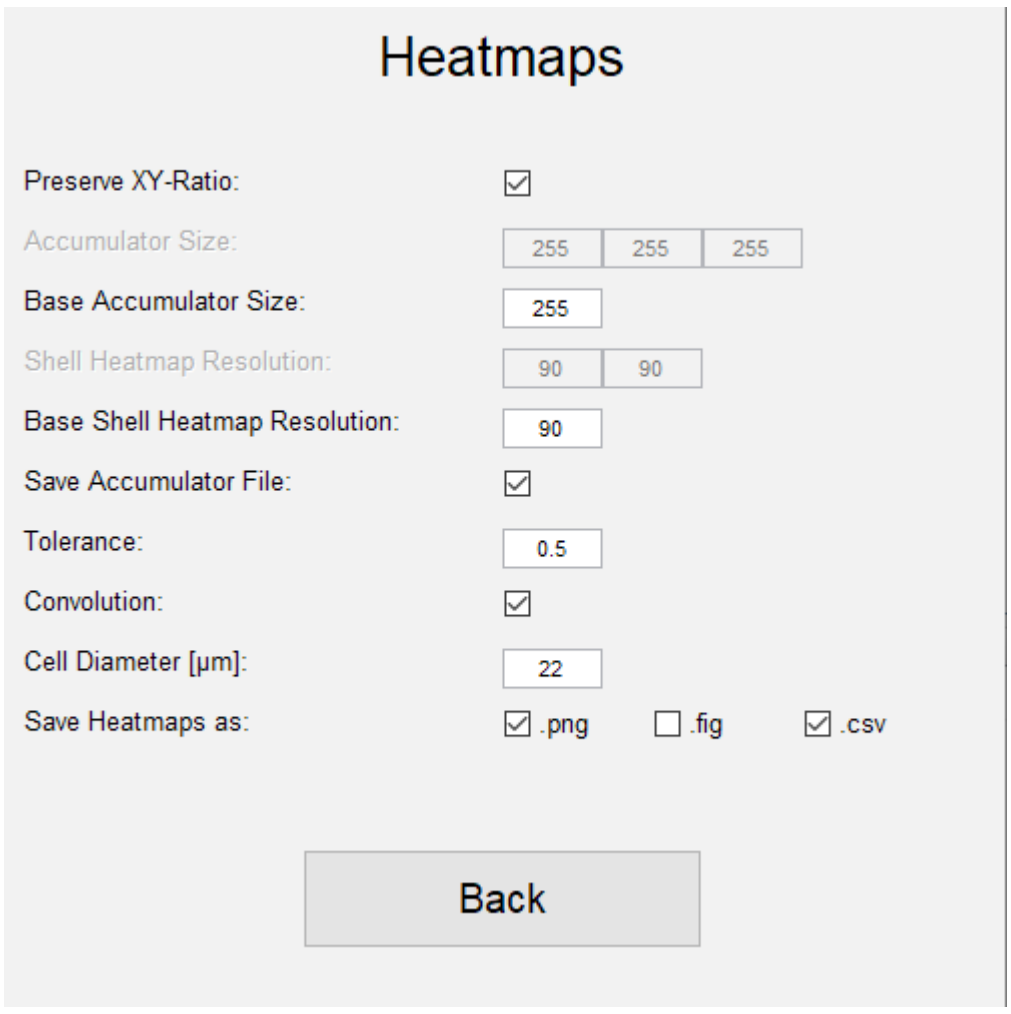


Reference Position on Landmark = 0 **Target Point** = 0



In the **Heatmaps** settings, you find all parameters that are used for creating a 2D heatmap from the segmented 3D probes and/or tissue.

In the Visualization Step, the software creates an object called *Accumulator*. It accumulates all accepted result files into a single 3D structure. You can change the size of the Accumulator with the **Accumulator Size** setting. The x,y and z ratios should be approximately the same as in the original input data. To preserve the ratio automatically, activate the **Preserve XY-Ratio** option. You cannot change the Accumulator size anymore, but can instead enter the **Base Accumulator Size**. This value will be the size of the Accumulator in the shortest direction. The software will calculate the other values based on the original input data size and dimension ratios.



The main result of the Visualization Step is denoted as *Shell Heatmaps*. You can change the **Shell Heatmap Resolution** to your preference. You cannot enter a resolution for the z direction, because the Shell Heatmap is a two-dimensional projection. Consider the size of your original input data for the x,y ratio or activate **Preserve XY-Ratio** and use **Base Shell Heatmap Resolution** instead.

With the setting **Tolerance** you can specify if and to what extent you want to incorperate probe cells that were not perfectly registered and are localized outside of the reference sphere (e.g., due to embryo shapes that deviate significantly from an ellipsoidal shape or cells that are very close to the embryo surface). A **Tolerance** of 0.1 allows for a maximum 10 percent deviation.

Furthermore, you can decide whether you want employ a **convolution step** for better visualization results. In the computed heatmaps each cell is represented by one pixel, which is not realistic with respect to the actual cell size. After activating **Convolution** you can enter the **Cell Diameter** of your probe cells.
A gaussian filter with the FWHM (full width at half maximum) of the cell diameter will be applied to the heatmaps. This will result in a representative visualization of the cell size and overlap of the cells. We do not advise activating **Convolution** for Tissue probes as this might lead to inaccurate boundaries in the resulting heatmaps.

Registration

Now you can start processing your data. The registration step will use your *input data* and generate *registered data*.
Click on **Registration**.
A window will appear. Select the folder that contains your *input data*.

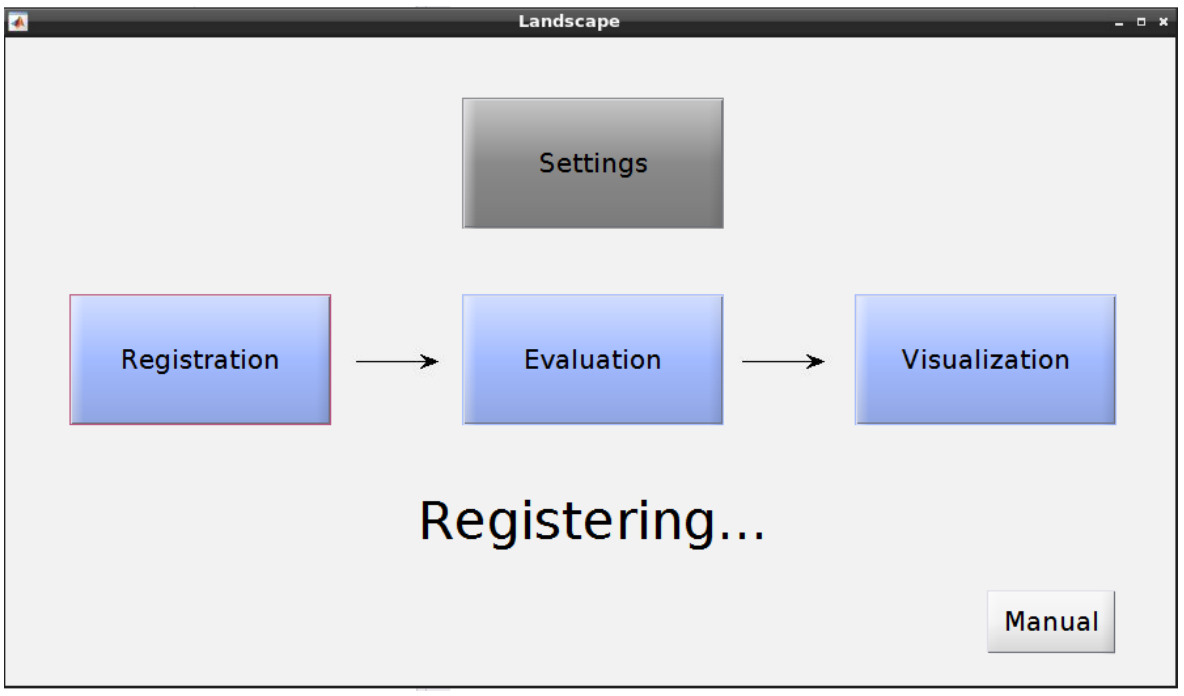
After selecting the folder you need to choose a *results folder*. Here you will later find the *registered data*. You can either select an existing folder or create a new one.

Now the registration will start. The status indicator will change to *Registering... .*

Wait until the status indicator reads *Ready*.
The registration step has finished. You can continue with the next step, the evaluation.

The registered data will be saved in the results folder. You can recognize the data by the suffix *_results.mat*.

The results folder contains three folders named *accepted*, *bug* and *rejected*. If there are bugs during the registration step, the data will be saved in the *bug* folder. The other folders will become relevant in the Evaluation step.



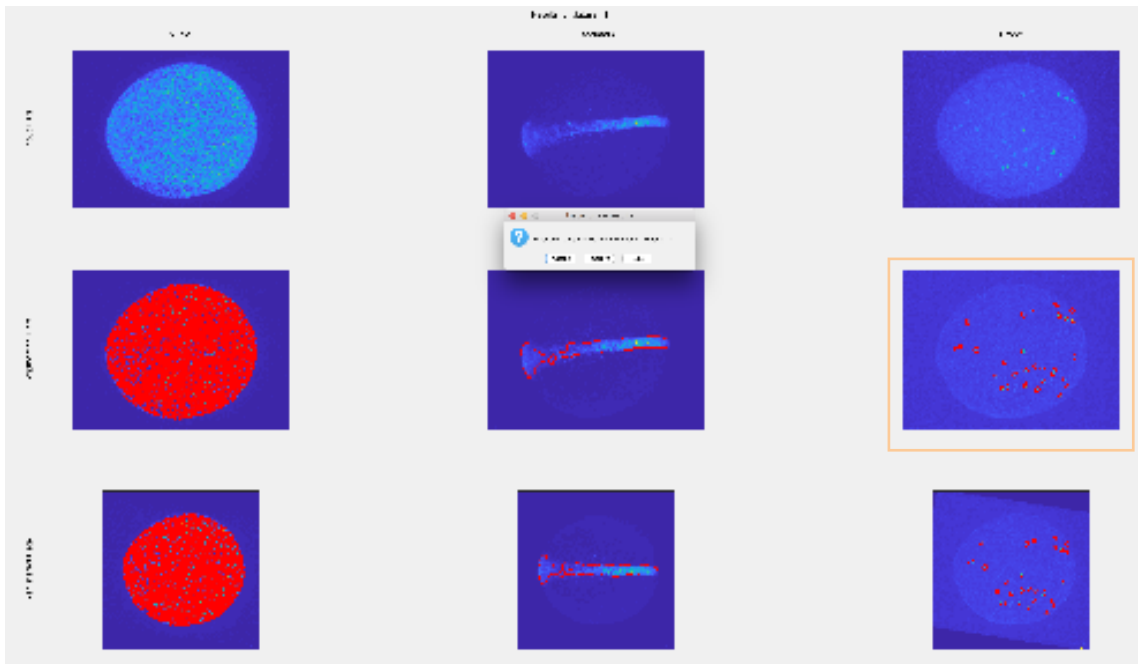
Evaluation

To check if the 3D imaging data are accurately registered, you have the option to evaluate the segmentation and registration results in the Evaluation step. You will also have the possibility to manually improve the segmentation.
Click on **Evaluation**.
A window will appear. Select the folder that contains your *registered data* (see registration step).

After selecting the folder two windows will open. The large window will show a visualization of the input, segmented and registered data from all channels.

You can check if the probe is properly segmented (orange box). If not, you can click on the **Improve** button in the small window (See next section „Improve function“).

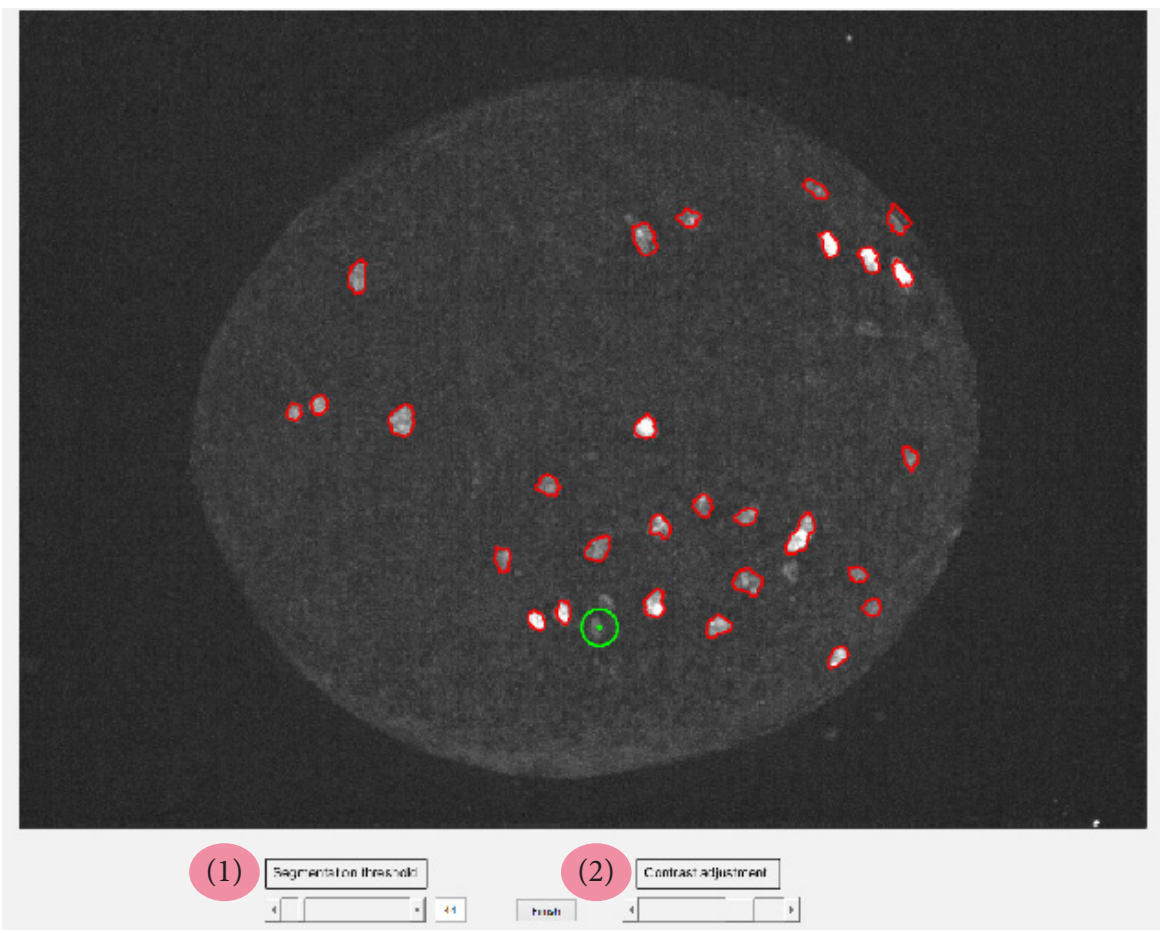
When your data are accurately registered, click on **Accept**. The data will be transfered to the *accepted* subfolder of your results folder.
Otherwise click on the **Reject** button. The data will be transfered to the *rejected* subfolder. The rejected data will be ignored in the visualization step and not considered for the generation of heatmaps.



Improve function

The improve function allows you to segment and register cells that were not recognized by the software.

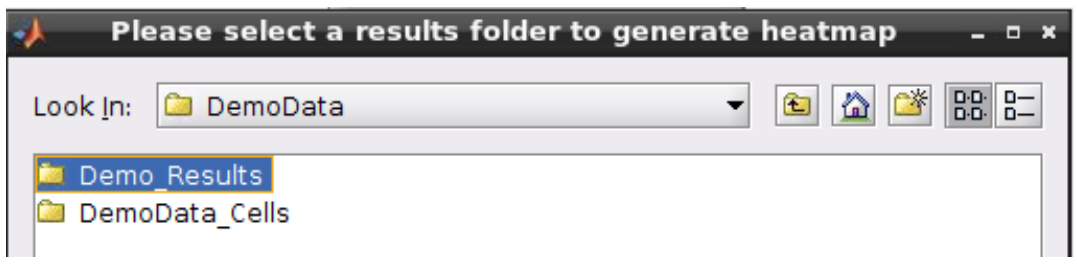
After clicking on **Improve**, an additional window that displays the current segmentation will appear. Place the mouse cursor at the center of a cell that is not segmented yet. Then hold the left mouse button and carefully drag the cursor away from the center of the cell. A green circle will appear. As soon as it encircles the cell, let go of the mouse button. You can revert the last step by pressing backward or delete on your keyboard.
Additionally, you have the option to manually change the **Segmentation threshold** (1). It allows you to add cells that were not recognized without having to be selected manually. Also, by adjusting the segmentation threshold false positive cells can be deleted.
The brightness and contrast of the image can be adjusted by using the **Contrast adjustment** (2) tool. Use the slider to adjust the contrast.



As soon as all cells are accurately segmented, click on **Finish**.
Wait for the registration to implement to your changes.

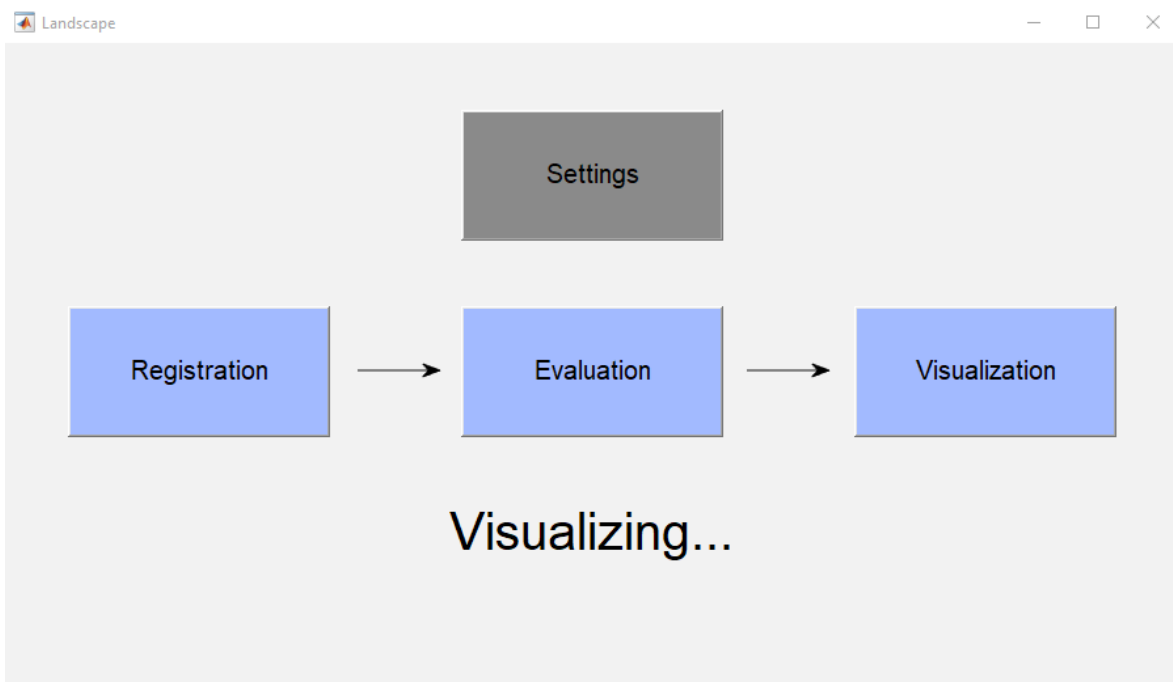
Visualization

In the last step the *registered data* will be used to generate *heatmaps*. The heatmaps present the distribution of the cells or tissue of interest. To create heatmaps, only the data in the *accepted* subfolder (see evaluation step) will be considered.
Click on **Visualization**.
A window will appear. Select the folder that contains your *registered data*.



The visualization step will start. The status indicator will change to *Visualizing... .*

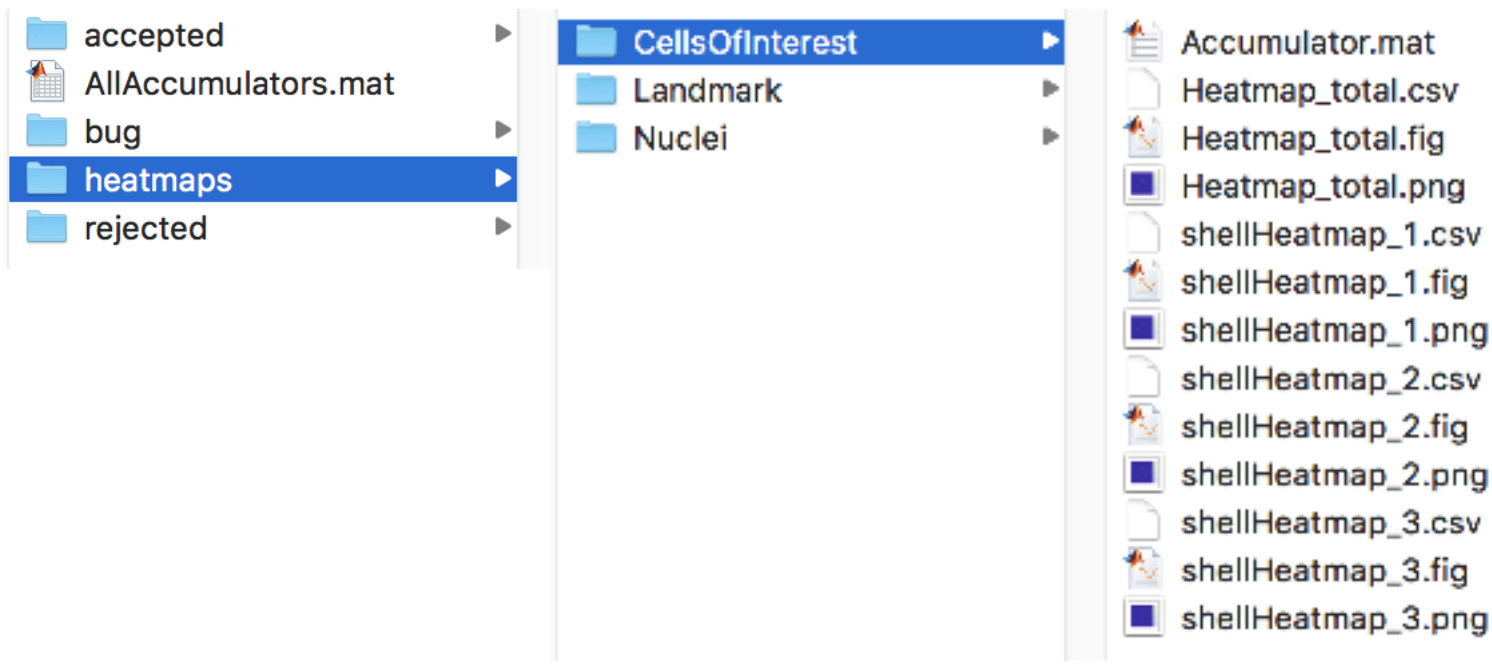
Wait until the status indicator reads *Ready*.
The Visualization step has finished.



A subfolder named *heatmaps* will appear in your results folder. It consists of three folders that contain heatmaps of the nuclei, the landmark and the cells/tissue of interest.

Specifically, the heatmap folders contain the following files:

Accumulator.mat contains all relevant information for generating heatmaps.
Heatmap_total shows the distribution of all cells within the entire embryo.
shellHeatmap_1 shows the distribution of cells above the landmark shell.
shellHeatmap_2 shows the distribution of cells at the level of the landmark shell.
shellHeatmap_3 shows the distribution of cells below the landmark shell.



The heatmaps are provided as image files (*.png*) and matlab files (*.fig*). In addition, for the heatmaps of the nuclei and cells of interest corresponding *.csv* files are provided that contain the data depicted in the heatmaps in a tabular format.

Troubleshooting

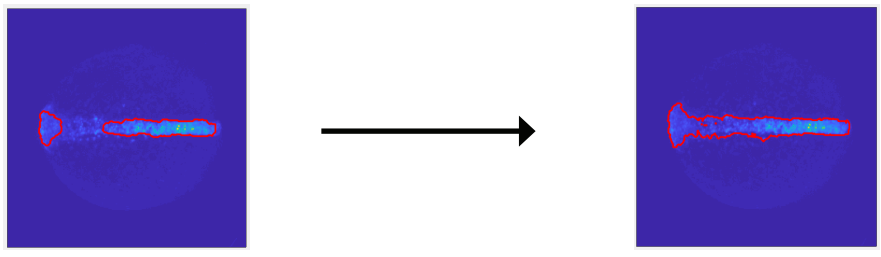
If you experience problems with the registration results, you can try the following solutions.

Not all cells of interest are segmented.

Use the *Improve* function tool to segment and register cells that were not recognized.
See page 8 for further details.

The tissue of interest is not fully segmented.

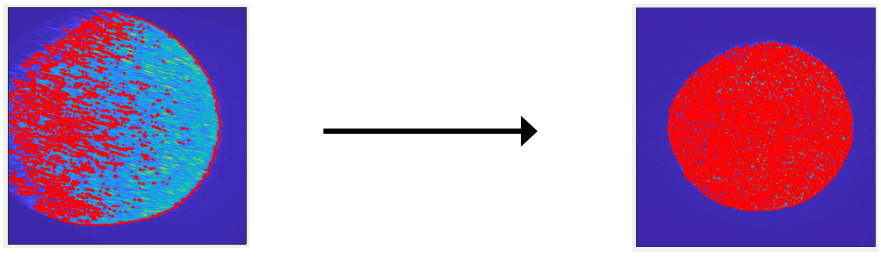
Adjust the **Tissue Probe Filtersize Opening / Closing** parameters.
See page 4, section - *Segmentation*, for further details.
For example:



Tissue Probe Filtersize Opening:	2
Tissue Probe Filtersize Closing:	30

The embryo is not properly oriented after registration.

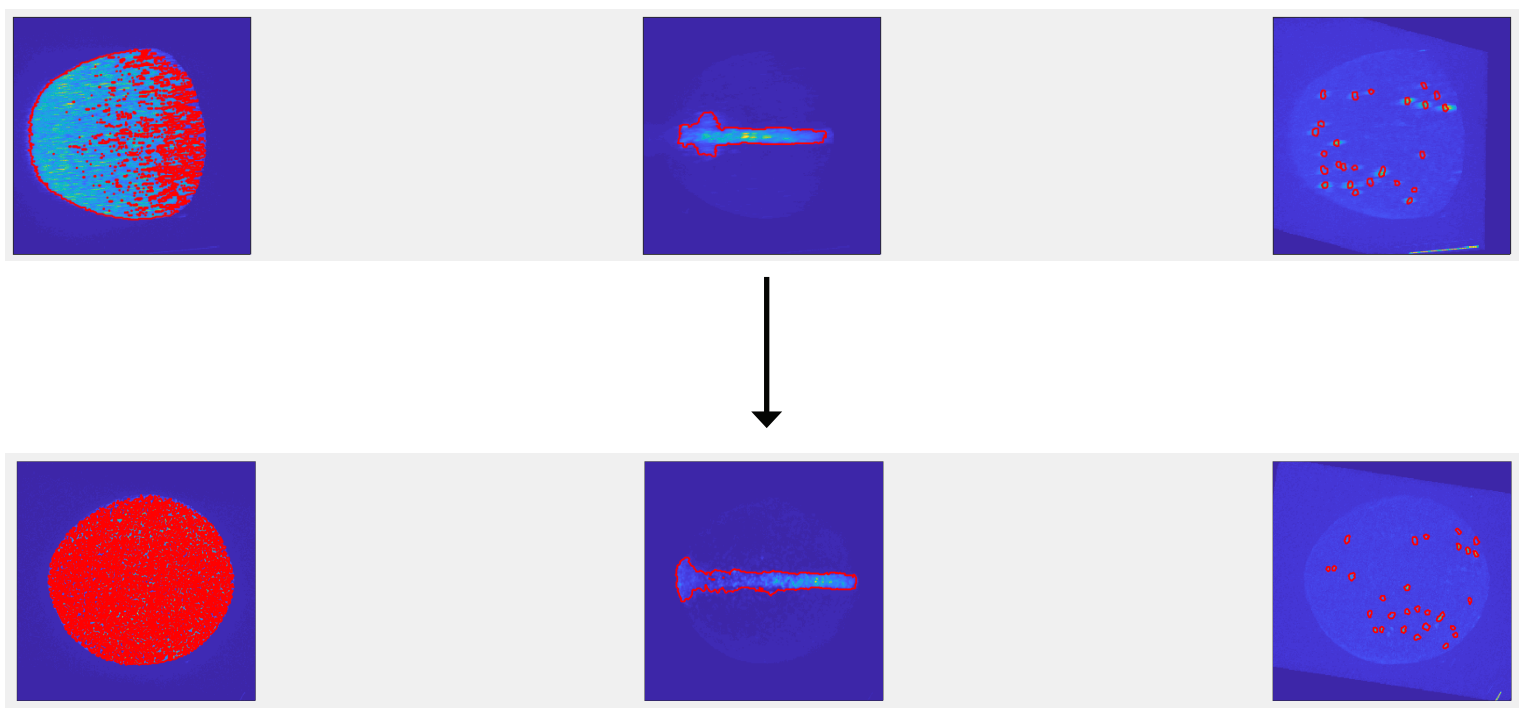
Adjust the Ellipsoid Fitting parameters, i.e., **μ0**, **μ1**, **μ2** and **Gamma**.
See page 5, section - *Ellipsoid Fitting*, for further details.
For example:



Parameter μ0 :	1e-07
Parameter μ1:	0.0001
Parameter μ2:	2
Parameter Gamma:	1

The embryo is distorted after registration.

Adjust the Registration Parameters, i.e., **Reference Position on Landmark**, **Target Point**.
See page 5, section - *Registration*, for further details.
For example:



Reference Position on Landmark :	0
Target Point [deg]:	0