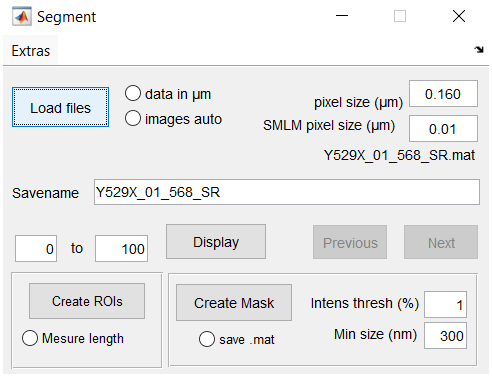
Analysis

1. Segmentation

Use this window to select ROIs and to create segmented images from SMLM data.



Set the **camera pixel size** and the **SMLM pixel size** (half of the localization precision) of your set-up.

Use **Load files** to select the files to be treated (.mat files). If your data is already calibrated in microns, click on **data in µm** before loading the files.

If **images auto** is selected, the pointillistic and rendered images will be displayed automatically for the active file. Otherwise, you can use **Display** to visualize them. If you loaded a list of files, use **Next** or **Previous** to move to the next or previous file, respectively.

The resulting images will be saved with the **Savename** indicated (modifiable). Be careful in the case of ROIs, as they have to be automatically recognized to do clustering analysis. The name has to be *name*.mat for the detection file, and *name*.rgn for the ROI datafile.

Keep intensities (%) can be useful to eliminate low intensity or high intensity spurious signals: set the the low and high thresholds (expressed as percentage of maximum intensity) and use Display to verify the selection. The ROI (.rnd) and mask files will be created from the set of detections in within the low and high limits.

If you want to keep the whole image (no ROIs), use the option **batch ROI full image** in the tab **Extras**.

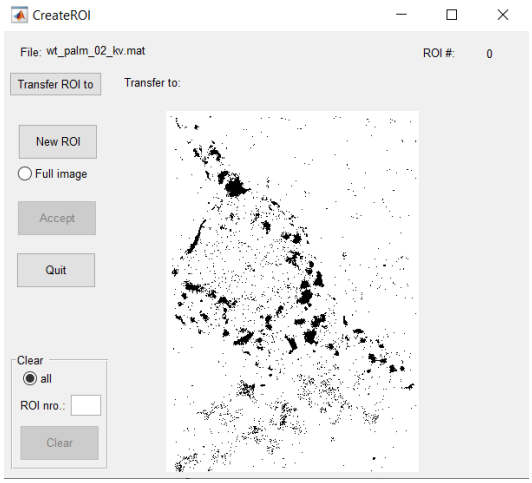
Use **Create ROIs** to select ROIs by hand and to manage previous selections. If you want to add a distance measurement, select **Mesure length**.

Create Mask will create a segmented image (no ROI in this case).

Create ROIs

This will open a new window where you can select ROIs by hand on the pointillistic image. If there is already a selection for the active file (a .rgn file), this selection will be shown. If the ROIs will be used for colocalization, select the second file with **Transfer ROI to** (other .mat file).

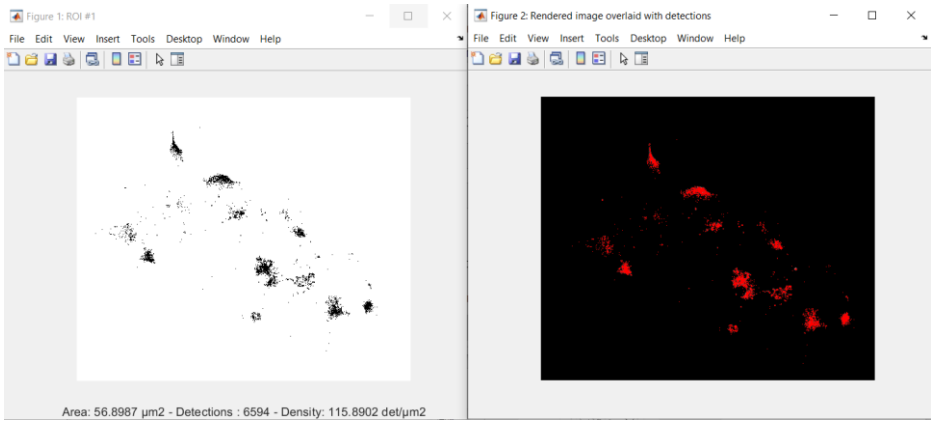
Quit to come back to the Segment window.



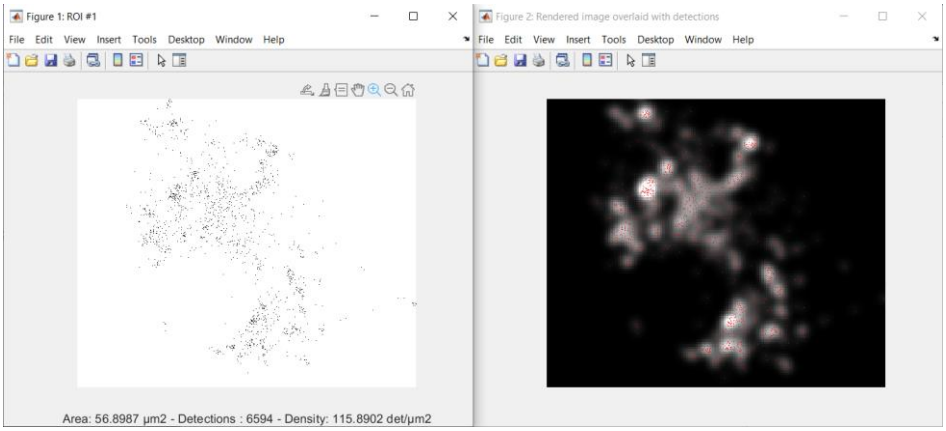
Drawing ROIs

You can select a polygon on the image (ROI) by clicking on **New ROI**. If you want to keep the whole image as ROI, choose **Full image** before clicking on New ROI.

After drawing the polygon (double click to finish it), the pointillistic and the rendered image (overlaid with the detections in red) will pop-up.



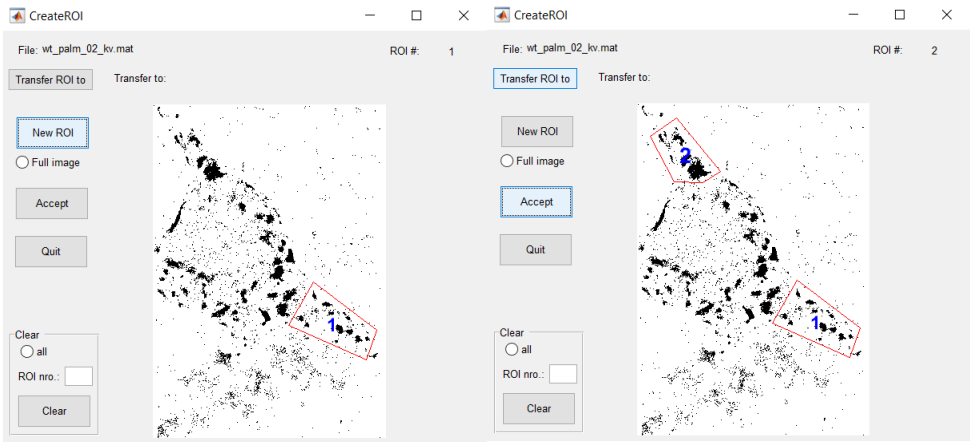
A zoom on the images:



Note that on the pointillistic image window you have the area of the ROI, the number of detections found inside and the density of detections in the ROI, assuming a homogeneous distribution.

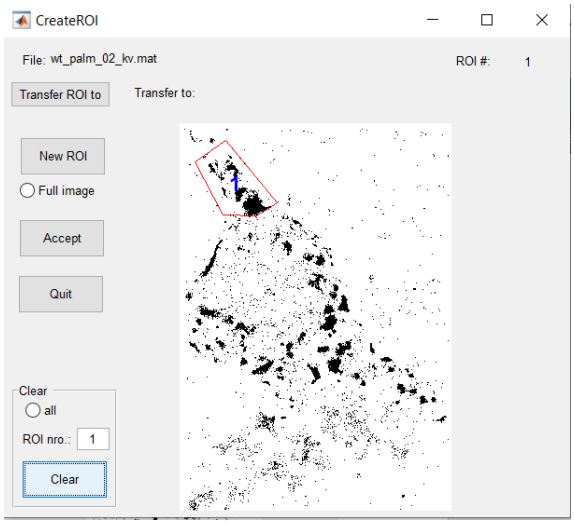
On the CreateROI window, click on **Accept** to keep the selection, or click on **New ROI** if you want to select another one. “Accept” will number the ROI and show this number on the window.

You can add as many ROIs as you want. As an advice to facilitate clustering analysis, select as ROIs the regions with an homogeneous background level, if possible.



Deleting ROIs

You can delete one ROI using **ROI nro** and **Clear**. You can delete all the ROIs by choosing **all** and clicking on Clear. After deleting one ROI, the remaining ones are re-numbered.



The results will be saved in:

1) *name*-ROIinfo.txt

   Text file with a table where the columns are

   ROI number – surface area (µm2) – number of detections in the ROI – density of the ROI (detections/µm2)

2) *name*.rgn

   .mat file with the structure ROI that contains the coordinates of the ROI and the detections that colocalize, among other data.

3) *name-roi#*-Irend.mat

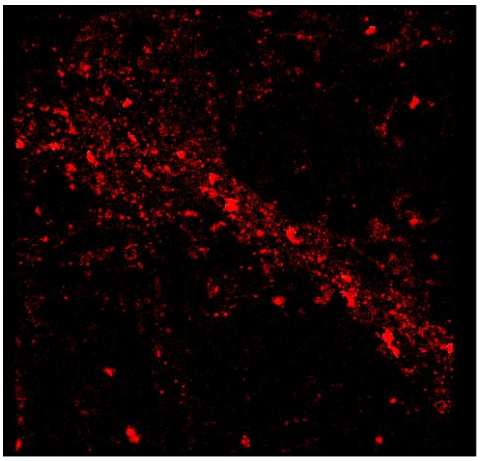
   .mat file with the rendered image of the ROI, which is used by Cluster.m for clustering analysis.

Create Mask

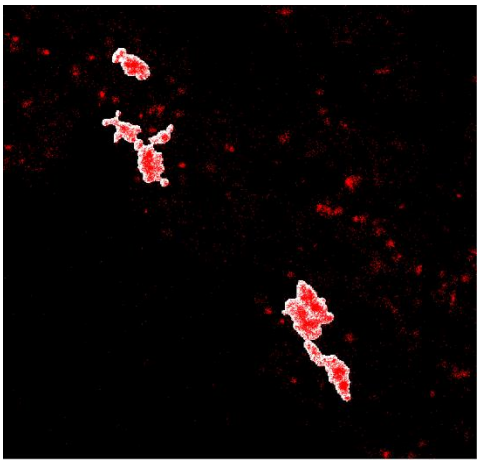
Create Mask makes a segmentation on the rendered image. You can set an intensity threshold with **Intens thresh** (in % of the maximum) and a minimum object size with **Min size**. This will select objects in the segmented image that have at least this size, assuming a circular shape.

Click on **save .mat** to obtain a new .mat file that contains only detections in the selected objects (*name*-mask.mat).

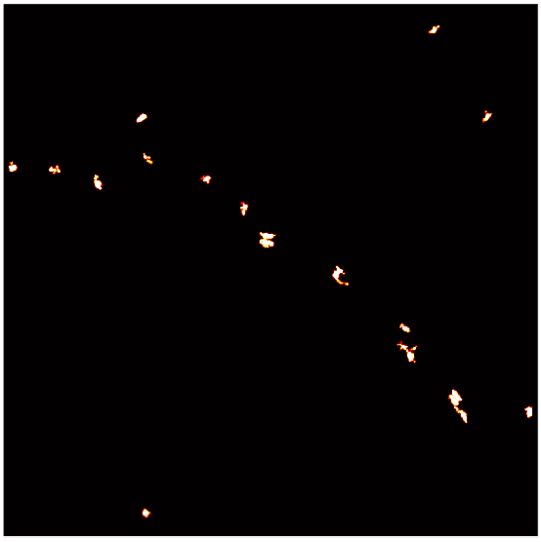
A new window shows the result, overlaid with detections: zoom to see the objects that appear in white.



-> and zooming:



If you visualize the resulting *name*-mask.mat file, now the rendered image is:

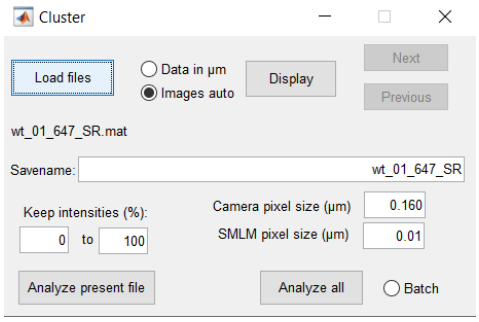


2. Cluster

This window is the main menu for clustering analysis. Use **Load files** to select the .mat files containing detections.

Please note:

- Click on **Data in µm** if your detection data is already converted to µm, before loading the files.
- Cluster.m requires files named *name*.mat (detections) and *name*.rnd (ROIs). You can create .rnd files using **Segment.m**.



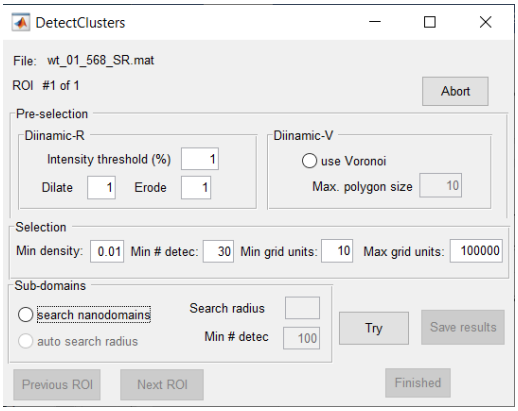
If **Images auto** is selected, the pointillistic and rendered images will be displayed automatically for the active file. Otherwise, you can use **Display** to visualize them. If you loaded a list of files, use **Next** or **Previous** to move to the next or previous file, respectively.

Keep intensities options may help the visualization eliminating detections with intensities below or above the minimum and maximum values provided (in %), but all the detections will be considered for the analysis.

Analyze present file will, as it says, perform the analysis only on the present file. If you loaded a list of files, **Analyze all** will sequentially analyze all the files. In these two cases, the clustering detection will ask for confirmation at each step and you will be able to tests different parameters on the same file. When you select **Batch** instead, **Analyze all** will ask to set the parameters for the first file and then it will automatically analyze all the files with the same parameters.

The analysis is done only on ROIs. If you want to analyze the whole image at once, you must select a ROI covering the entire image. This can be done also with Segment.m.

Clicking on one of the Analysis buttons will open a new window, DetectClusters, where you can choose the strategy to select candidate clusters (Pre-selection), enter parameters for pre-selection and selection of clusters, and chose to perform the detection of subdomains.

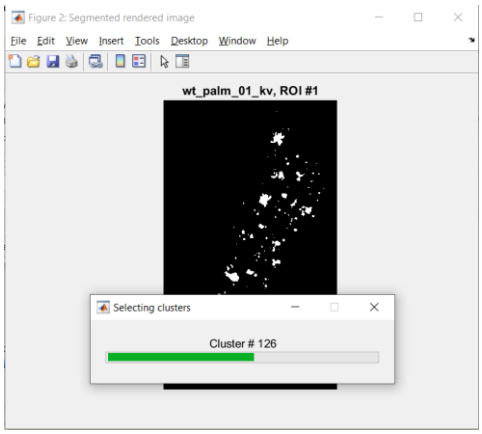


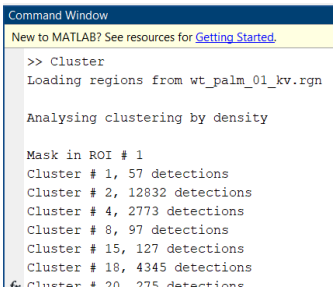
Diinamic-R is the option by default. It uses the rendered image to create a mask with the **Intensity threshold**. You may improve the mask using the morphological operators **Dilate** and **Erode** (enter the number of pixels to add or remove).

The selection of clusters is done considering:

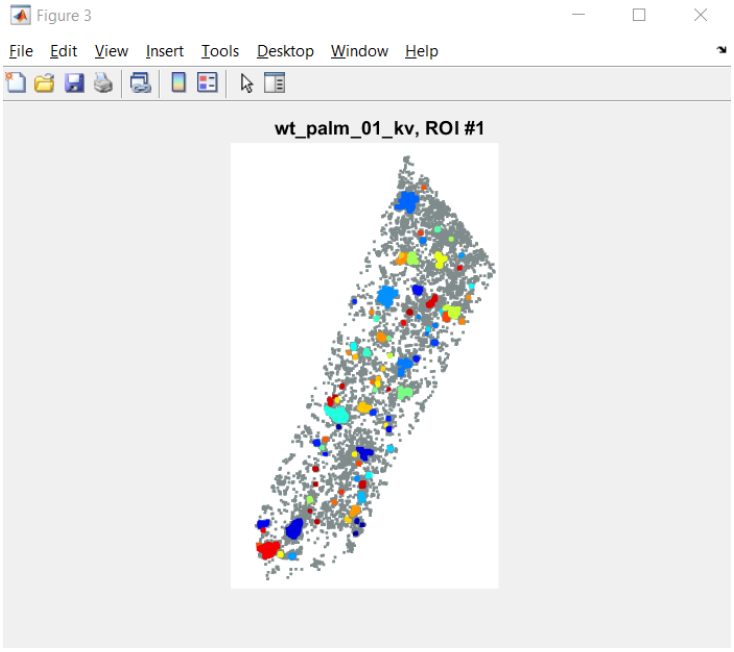
- Minimum density (**Min density**) per pixel (SMLM pixel),
- Minimum number of detections in the cluster (**Min # detec**),
- A minimum (**Min grid units**) and a maximum (**Max grid units**) cluster size (number of pixels of the rendered image).

Try will launch the analysis on each ROI of the file. The rendered mask is shown on an independent figure, and you can follow the progression of the analysis on the Command window (the number of detections in *selected* clusters is shown). The wait bar also shows the progression. Close it to abort the analysis. If you want to stop the loop over a list of files, close the window or use the **Abort** button. Please note that partial results are not saved.

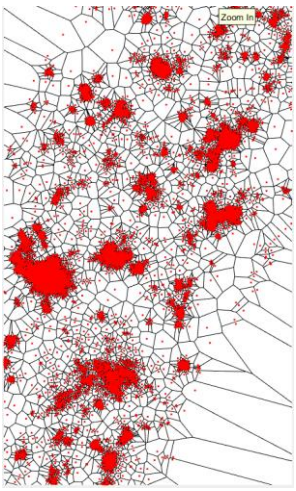




A new figure shows the selection results for the ROI, with the detections belonging to clusters appearing in color (each cluster has a different color) and those non-clustered detections appearing in gray.



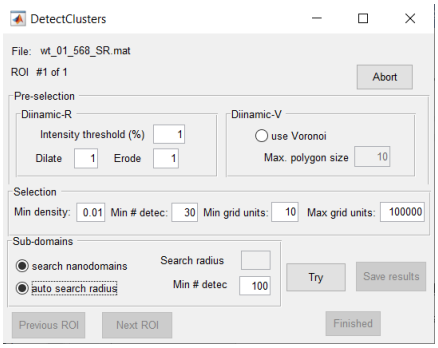
DiinamicV uses Voronoi tessellation to create the mask. Select **use Voronoi** if you want to use this strategy, and enter the **Max. polygon size** (arbitrary units) to create the mask. The result will include detections whose polygons are smaller than this threshold. An image with the Voronoi tessellation is displayed. The selection of clusters is done as for DiinamicR.



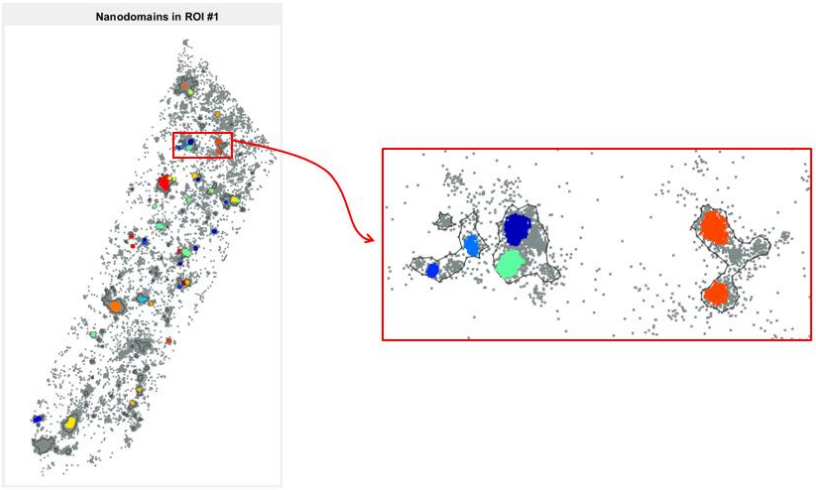
Exemple of Voronoi tessellation on SMLM data

Subdomains inside clusters

The detection of sub-domains on the selected clusters is done if you click on **search nanodomains**.



This part uses a DBSCAN algorithm: it needs a **search radius** and the minimum number of detections in the subdomain (**Min # detect**). The calculation of the search radius can be done automatically (**auto search radius**), based on the mean distance between detections in the sample.



Clusters that were detected previously are shown by a grey contour line that depicts their border. Nanoclusters appear in color. Note that detections themselves are not represented in scale so their size change if you zoom in or zoom out. The number of nanoclusters (and clusters) found are shown on DetectClusters window.

Results

If you agree with the results, click on **Save results** to keep them. Two files will be saved: *name*-roidata_*number of the ROI*.mat and *name*-selection.tif.

If there are more than one ROI on the data file, use **Next ROI** to move to the next ROI and click on **Try** to launch the detection on this new ROI.

When all the ROIs were analyzed, click on **Finished**. A new results file will be generated: *name*-allclust.txt. If you also made the detection of subdomains, these results will be saved in another file called *name*-nanoclust.txt. In addition to the results, there will be a text file that recapitulates the analysis that was done (Parameters_clustering_*date of analysis*.txt):

```
Parameters for clustering analysis

Analysis of density after segmentation with rendered image. Intensity threshold: 1%.
Segmented image improved with dilation: 1 px and erosion: 1 px.

Cluster selection criteria:
Minimum density: 0.01. Minimum number of detections: 30
Minimum cluster size (nm): 10. Maximum cluster size (nm): 100000

No nanodomain detection

             Files:
    wt_01_647_SR.mat
    wt_03_647_SR.mat
```

Example after analysis with subdomain detection:

```
Parameters for clustering analysis

Analysis of density after segmentation with rendered image. Intensity threshold: 1%.
Segmented image improved with dilation: 1 px and erosion: 1 px.

Cluster selection criteria:
Minimum density: 0.01. Minimum number of detections: 30
Minimum cluster size (nm): 10. Maximum cluster size (nm): 100000

Including nanodomain detection by DBSCAN. Mninimum number of detections in the nanodomain : 100
Search radius calculated automatically : 5.95
             Files:
    wt_01_647_SR.mat
```

Format of results

Files *name*-allclust.txt: groups the results of all the ROI of a data file.

ROI #	cluster #	number of detections	detections in ROI	Cluster area	Cluster density
1.0000000e+00	5.0000000e+00	6.8000000e+01	3.7999000e+04	6.2571744e-03	1.0867525e+04
1.0000000e+00	1.5000000e+01	3.2000000e+01	3.7999000e+04	2.0911326e-03	1.5302712e+04

Files *name*-nanoclust.txt: groups the results of all the ROI of a data file.

ROI #	cluster #	nanodomain #	number of detections in the nanodomain	number of detections in the cluster	nanodomain area	nanodomain density

Files *name*-selection.tif: Image of the selected clusters (each cluster appears in a different color).

3. Colocalize

Please note that the analysis is performed only on ROIs that have to be previously drawn using Segment.m.

Several possibilities are available:

- Colocalization of SMLM detections over a fluorescent image (previously segmented): choose the option **Detections vs pixels**
- Colocalization of SMLM detections over clusters of SMLM detections (previously found with Cluster.m): choose the option **Detections vs clusters**
- Colocalization between two sets of clusters of SMLM detections (previously found with Cluster.m): choose the option **Clusters vs clusters**

Chose the files to be analyzed with **Load file 1** and **Load file 2**. Data of file 1 will be colocalized with data in file 2. File 1 is necessarily a SMLM detections file (.mat). Depending on the type of colocalization that you choose, file 2 can be a segmented fluorescent image (.tif) or a SMLM datafile (.mat).

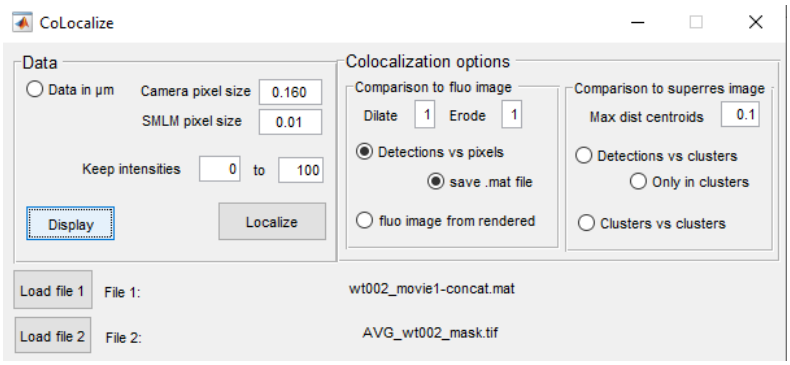
Please click on **Data in µm** before loading the files if your SMLM data is already calibrated in µm.

After loading data, you can visualize data to verify the alignment of images with **Display**. If the images are well aligned but ROI do not, please re-do ROI selection with Segment.m using the "transfer to" option. Also check whether you correctly choose "Data in µm" , and, in case of localizing over a fluorescent image, whether this image was generated from a rendered image.

Keep intensities options may help the visualization eliminating detections (on the pointillistic images) with intensities below or above the minimum and maximum values provided (in %), but please note that *all* the detections will be considered for the analysis.

After checking that the **Camera pixel size (µm)** and the **SMLM pixels size (µm)** values are correct, you can run the analysis using **Localize**.

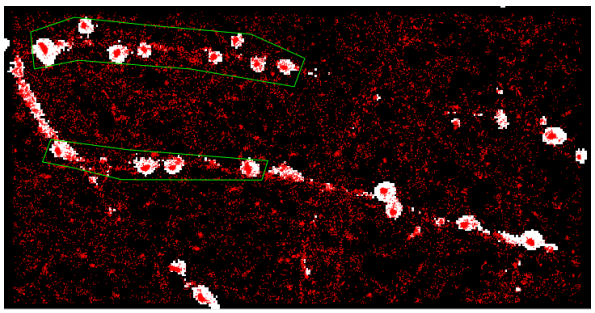
1) Detections vs pixels



In this case the first set of data (File 1) are SMLM detections (.mat file) and the second set (File 2) is a fluorescent image, previously segmented (.tif file). If you use an image created from SMLM data (rendered image), click on **fluo image from rendered**. The whole image is shown (binarized), overlaid with the detections. The ROIs where the analysis will be done are depicted in green.

You may improve the mask using the morphological operators **Dilate** and **Erode** (enter the number of pixels to add or remove).

Click on **save .mat file** if you want to have .mat files containing the detections that are or are not colocalized with the white pixels in the binary image.

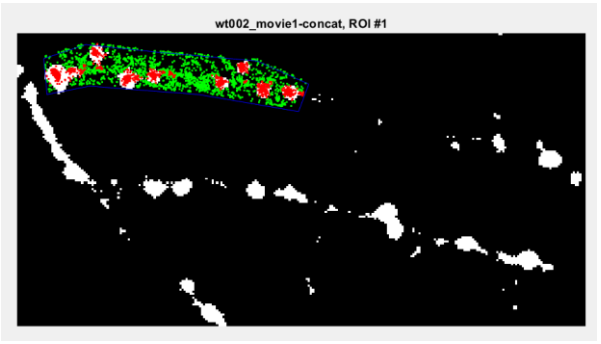


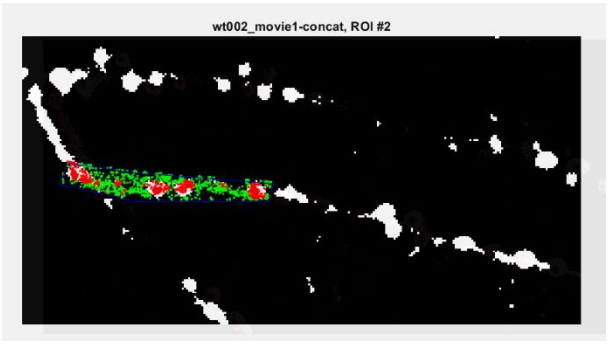
After clicking on **Localize,** you can follow the progression on the command window:

```
filename =

    'wt002_movie1-concat.mat'

Loading regions from wt002_movie1-concat.rgn


locfilename =

    'AVG_wt002_mask.tif'

Localization of SMLM detections over a fluorescence mask
Roi #1
Total number of detections: 32671
30043 detections in domains (91.9562%)
Roi #2
Total number of detections: 15619
14408 detections in domains (92.2466%)
Sorting saved in .mat files
```

The results provided correspond to the total number of detections in the ROI and the number and proportion of detections colocalizing with the white pixels in the binary image.

The binary image overlaid with the detections of each ROI will pop-up. The detections belonging to each ROI are shown in red if they colocalize, or in green if they do not.





The results are saved separately for each ROI, with two files collecting the detections colocalizing (IN) or not (OUT). If you choose to save .mat files, there will be two files collecting the detections that colocalize (IN) or not (OUT) for all the ROIs.

Format of the results:

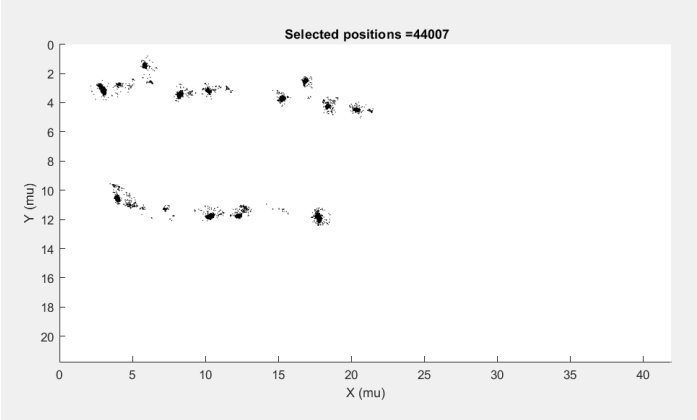
1) *name*-ColocDetections.txt

ROI #	total number of detections	detections colocalizing	detections not colocalizing	% colocalizing

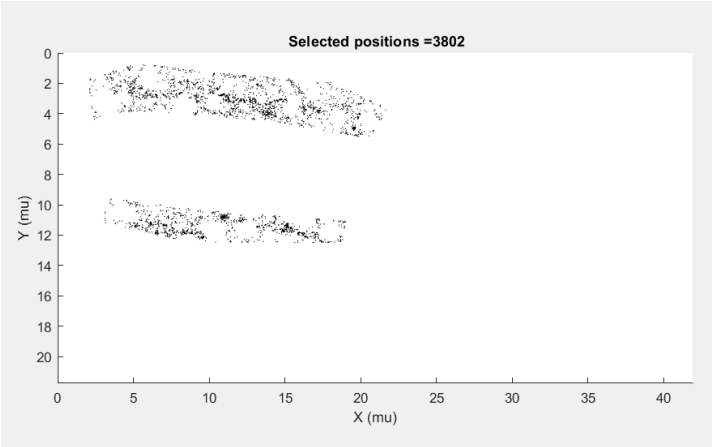
2) *name*-in.mat and *name*-out.mat (optional)

Examples of the data generated on the files showed above:

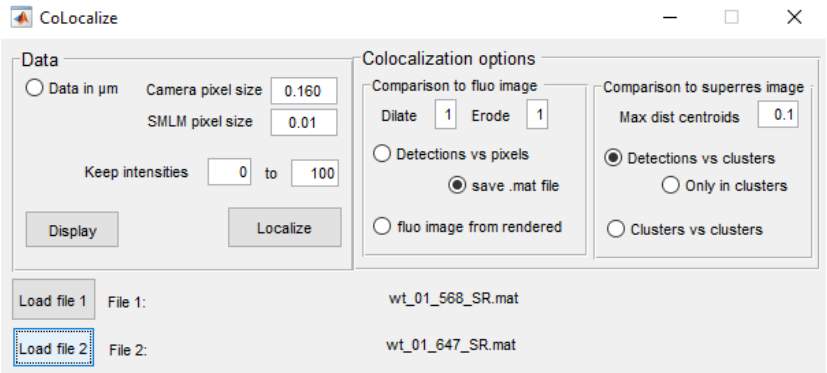
Data “in”:



Data “out”:

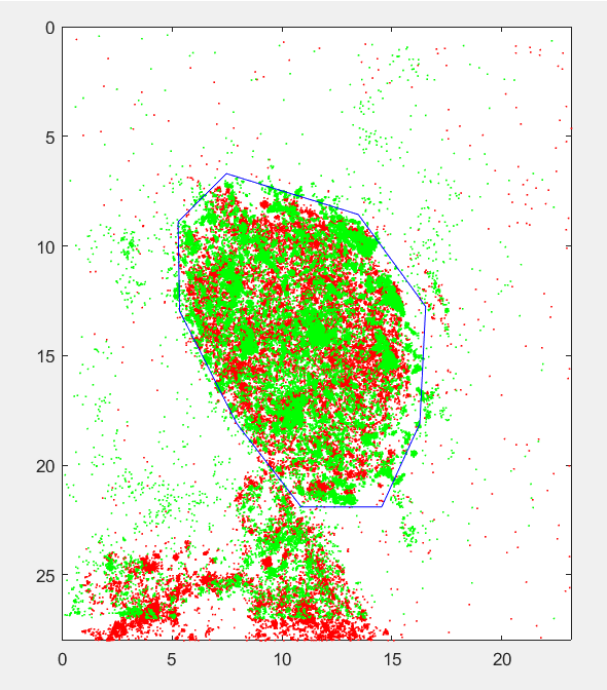


2) Detections vs clusters



In this case you must enter two SMLM detections files (.mat). The first dataset will be colocalized with the previously clusters found in the second dataset. The program will automatically look for this clustering data (*name*-roidata_*# of roi*.mat file) produced by Cluster.m

Display shows the first set of detections in red, the second in green, and the ROIs where the analysis will be done in blue. The clusters are not shown at this point.



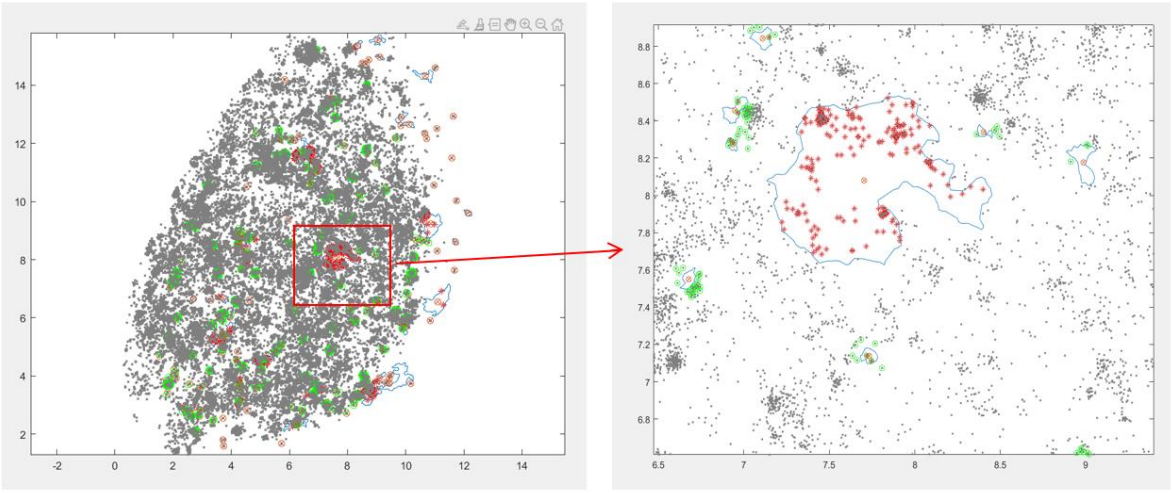
There are two possibilities to consider colocalization in this case, and the choice depends upon the type of distribution of detections.

By default, the program identifies detections that are inside the borders of clusters. Another option is to consider also the detections closer than a certain threshold (**Max dist centroids (µm)**) to the cluster centroid. This option is useful in case of clusters of similar size and regular shape, and allows to be flexible in the definition of colocalization: detections must not be perfectly colocalizing with the cluster. However, this option works poorly if clusters are very variable in size, because the value of the distance threshold will be difficult or impossible to find. In this case, check the option **Only detections in clusters.** You will obtain as results only the detections inside the cluster borders.

You can follow the progression and see the results on the Command window:

```
Reading data, please wait...

filename =

    'wt_01_568_SR.mat'

Loading regions from wt_01_568_SR.rgn

Localization of SMLM detections over clusters of detections

Roi #1
79088 detections in the ROI

Analysing data, please wait...
1842 detections colocalizing with clusters (2.3291%)
3174 detections close to cluster centroid

Done
```

Results are also shown in a figure. Cluster borders (data from file 2) are shown in blue, with the centroid position depicted by a red cross in a circle. Detections (data from file 1) within the borders appear in red and detections close to the centroid are circled in green.



If both

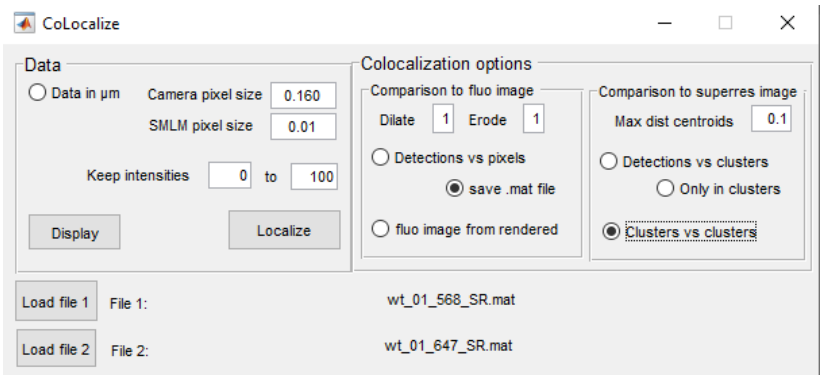
ROI #	total # of detections	# detections colocalizing	% colocalizing	# detections close	% close	# all detections coloc and close	% coloc + close

colocalization conditions are considered, the result file (*name*-DetColocCluster.txt) contains:

If only colocalizing detections are considered, the results file contains:

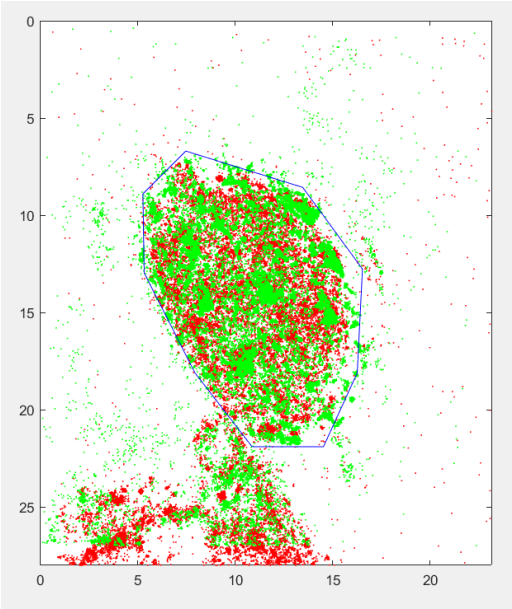
ROI #	total # of detections	# detections colocalizing	% colocalizing

3) Clusters vs clusters



You have to enter two SMLM detections files (.mat). The clusters previously found in the first dataset will be colocalized with the clusters identified in the second dataset. The program will automatically look for the clustering data (*name*-roidata_*# of roi*.mat file), produced by Cluster.m

Display shows the first set of detections in red, the second in green, and the ROIs where the analysis will be done in blue. The clusters are not shown at this point.

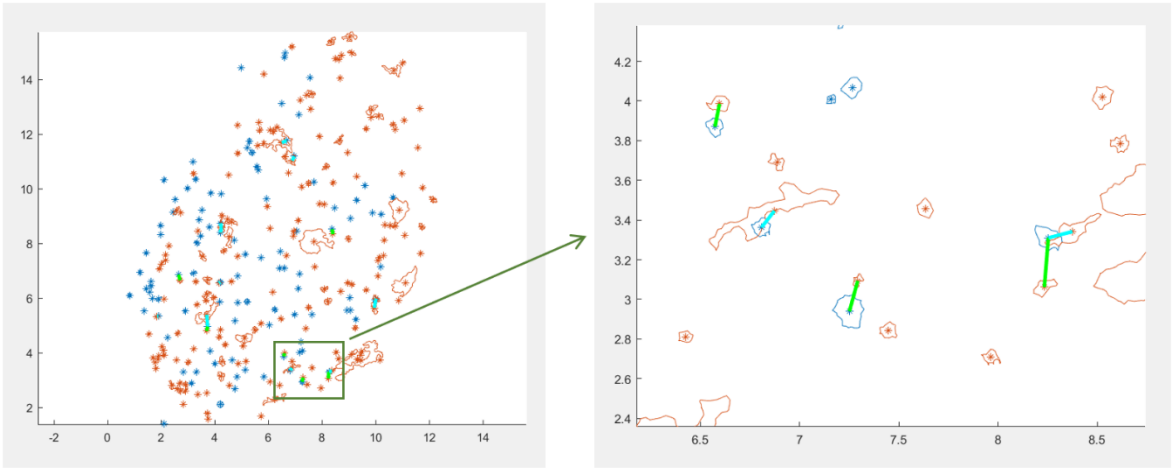


The program identifies clusters which overlap or whose centroids are closer than a certain threshold (**Max dist centroids (µm)**).

You can follow the progression on the Command window:

```
Reading data, please wait...

filename =

    'wt_01_568_SR.mat'

Loading regions from wt_01_568_SR.rgn

Localization of two sets of clusters of SMLM detections

Roi #1
Reading and analysing data, please wait...

Data file 1:wt_01_568_SR-roidata_1.mat
Data file 2:wt_01_647_SR-roidata_1.mat
```

The result is shown in a figure. Cluster borders are shown in blue for the first data set, and in orange for the second. Centroid position is shown by an asterisk of the same color than the border. When clusters overlap, their centroids are connected by a cyan line. If their centroids are closer than the distance threshold, they are connected by a green line.



The summary result file (*name*-Summarycloseclusters.txt) contains*:*

ROI #	# of clusters in image 1 close to clusters in image 2	total # of clusters in image 1	% close clusters

The result file *name*-Summarycloseclusters.txt contains detailed data about all the clusters in dataset 1 that overlap or are close to clusters in dataset 2: it indicates for each pair of clusters (cluster in data 1, cluster in data2), the distance between their centroids and the overlap condition (1 if they overlap, 0 if they do not).