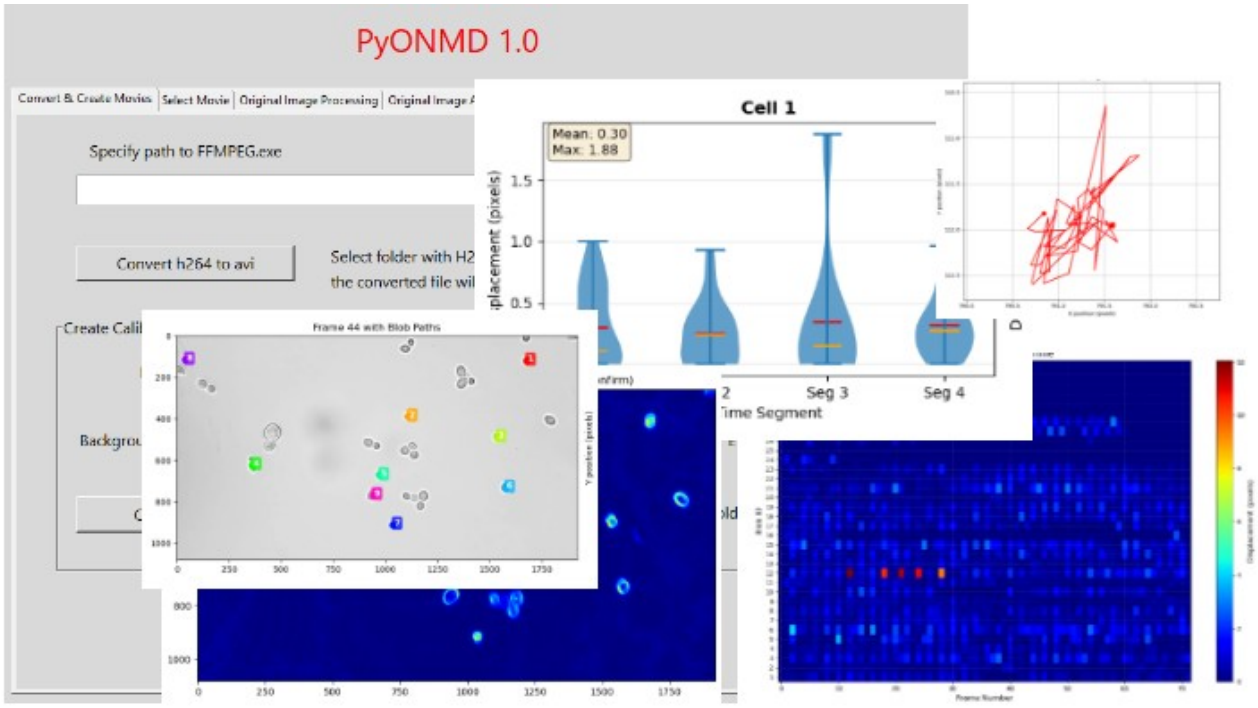
PyONMD 1.0 User Manual



Sandor Kasas

2025

Table of Contents

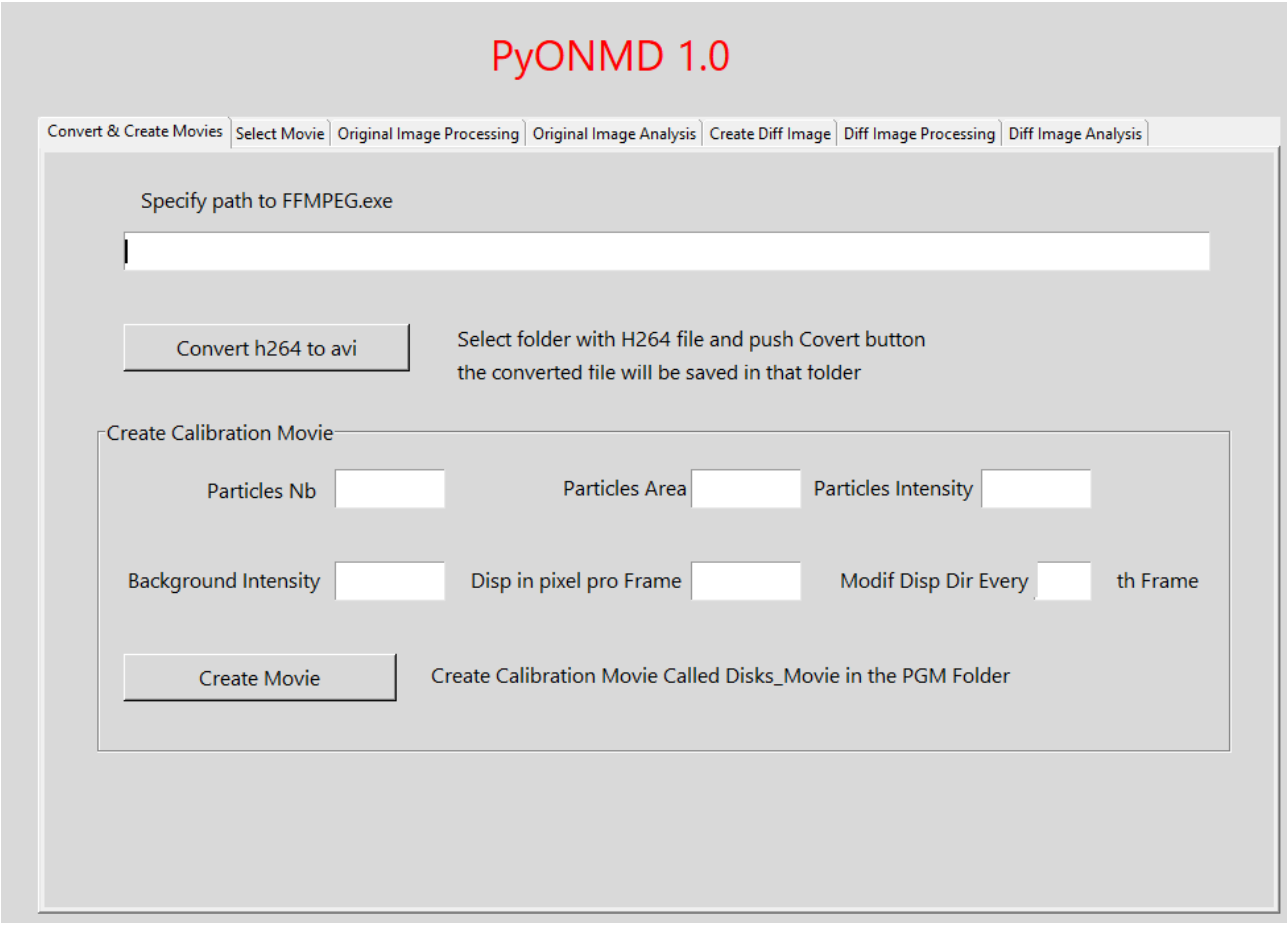
The PyONMD_Ana_02 program was designed to process optical nanomotion detection (ONMD) files. Typical ONMD files are recorded with an optical microscope with objectives between 20-60x at a frame rate between 5-60 frames per second (fps). Typical samples the program can process are bacteria, mitochondria and yeast.

PyONMD_Ana_02.py
PyONMD_Ana_02.tcl
PyONMD_Ana_02_support.py
PyONMD_Analyzer.spec

The program is written in Python 3. If it is executed from a Pyton editor it requires the following files to be present in the same folder. The file that launches the program is PyONMD_Ana02_support.py. The *.tcl file is required for the graphical user interface (GUI)

If you are runing the program from a Python editor the following libraries have to be installed in the current Pyton environment: os, subprocess, sys, cv2, matplotlib, numpy, PIL, pandas, scipy, skimage, random and tkinter

After launching PyONMD_Ana02_support.py the following GUI opens :



The upper part of the GUI contains the following tabs : Convert & Create Movie, Select Movie, Original Image processing, Original Image Analysis, Create Diff Image, Diff Image Processing and Diff Image Analysis. Their functions will be reviewed in details in dedicated chapters.

Convert & Create Movie permits to convert movie files generated by the Openflexure microscope in *.avi files and to generate movies displaying particles in motion for calibration proposes.

Select Movie permits to load the movie file, to display some of its frames, control its illumination stability and reduce its length if necessary.

Original Image Analysis permits to set the parameters needed for the selection of individual cells and generate a black and white mask. This mask displays the location of the detected cells in white and the background in black. It also permits to select cells (blobs) according to their surface or to elliminate some areas of the field of view from the analysis.

Original Image Analysis uses the previously generated mask to analyse different parameters of the detected cells.

Create Diff Image permits to generated an image (Diff Image) that highlights in false colors the areas of the movie that changed their intensity during the recording.

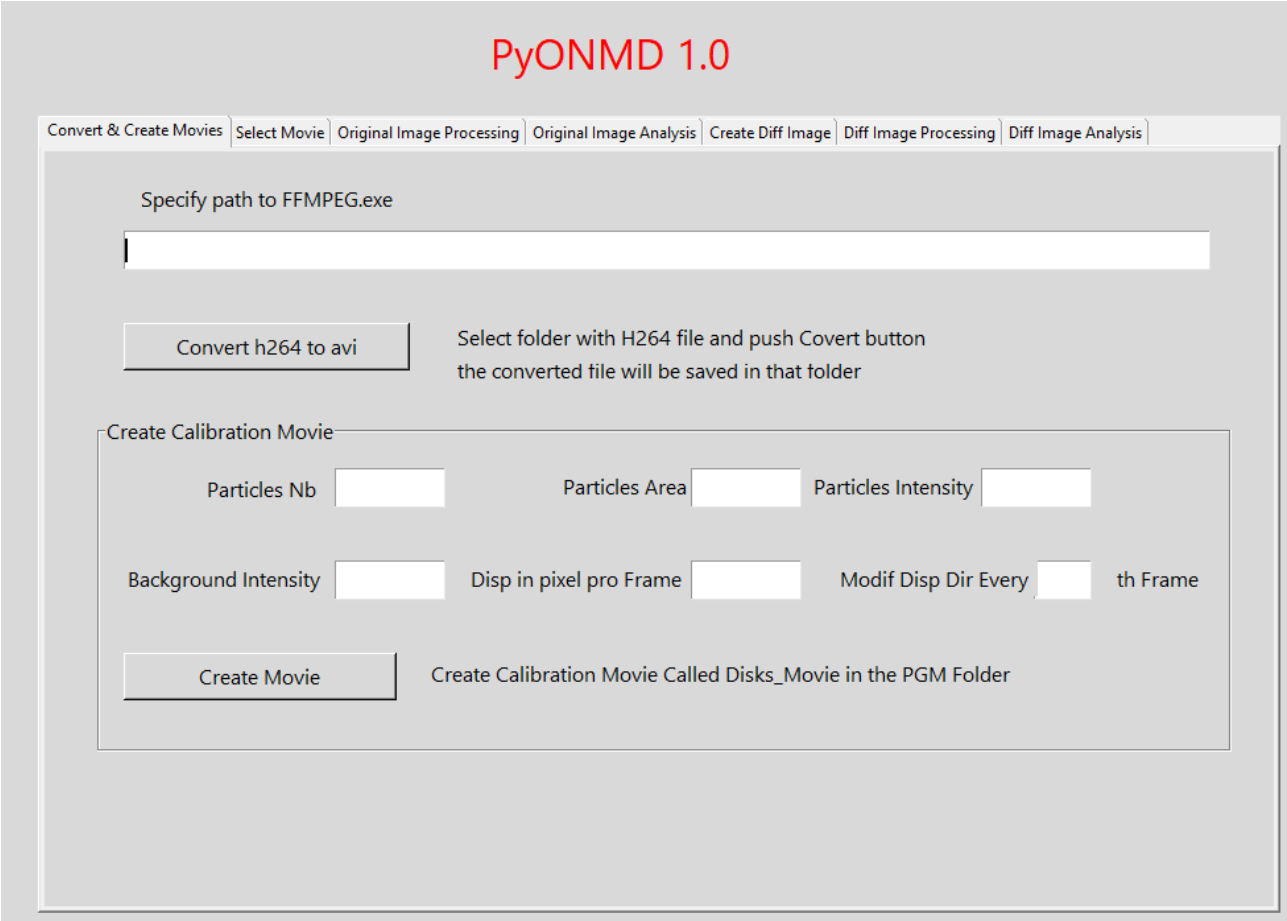
Diff Image Processing permits to process and isolate cells on the Diff Image. Basically, it permits to isolate according to the their intensity and size the ony cells that moved during the recording.

Diff Image Analysis permits the analysis of the Diff Image, the analysis of individual isolated cells and assess the background intensity.

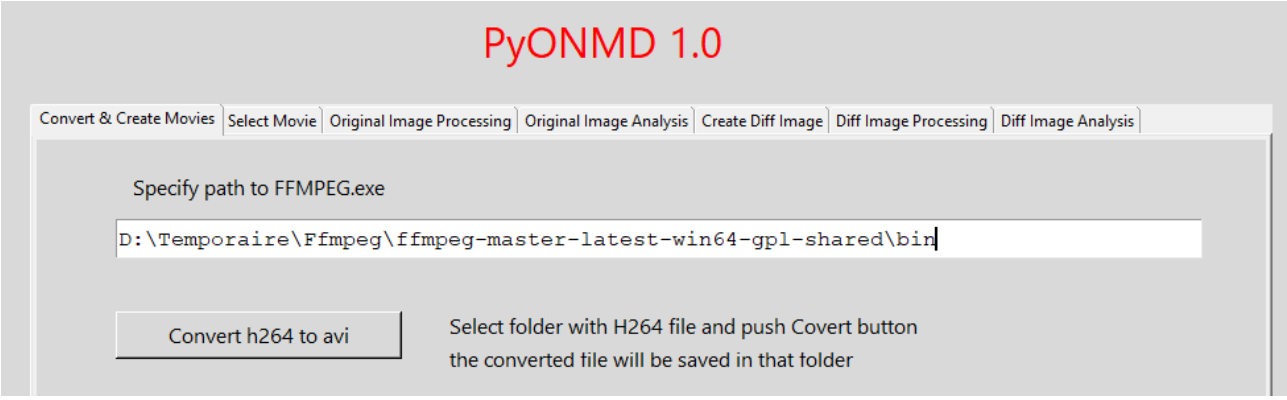
In the following chapter we will examine in more details the functions of each of the tabs.

Convert & Create Movie

This tab permits to convert movie files generated by the Openflexure microscope (*.h264) in *.avi files and to generate movies displaying particles in motion for calibration proposes.



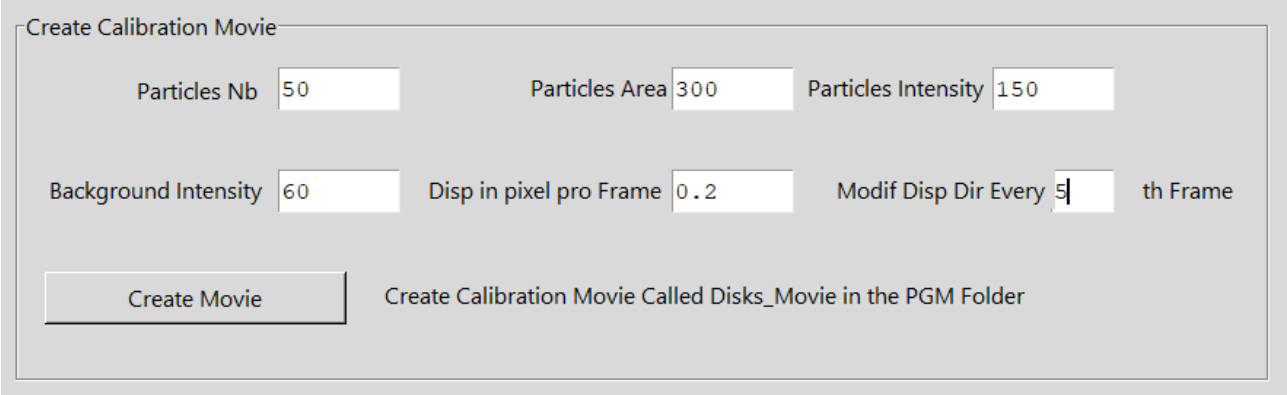
Convert h264 to avi button permits the conversion of *.h264 movies to *.avi. h263 is the default video format used by the Openflex microscope. The conversion is carried on by an external program and not the Python code itself. Before this functionality could be employed it is important to download the ffmpeg.exe program and to save it in a folder which path is inserted in the corresponding edit field. The FFMPEG program one can be downloaded for free from https://www.ffmpeg.org/download.html website.



Other movies formats (*.avi or *.mp4) do not require such a conversion.

Pushing the Convert h264 to avi button will open a new window and ask to navigate to the folder containing the *.h264 movie to be converted. Once selected, the software launches the conversion and saves the new movie in the very same directory.

Create movie button will launch several external Python programs (PyONMD_MkMov1.py, PyONMD_MkMov1_support.py and PyONMD_MkMov1.tcl) and will open the GUI interface displayed below



Particles Nb sets the number of particles to be included into the movie.

Particles area sets the surface of every particles.

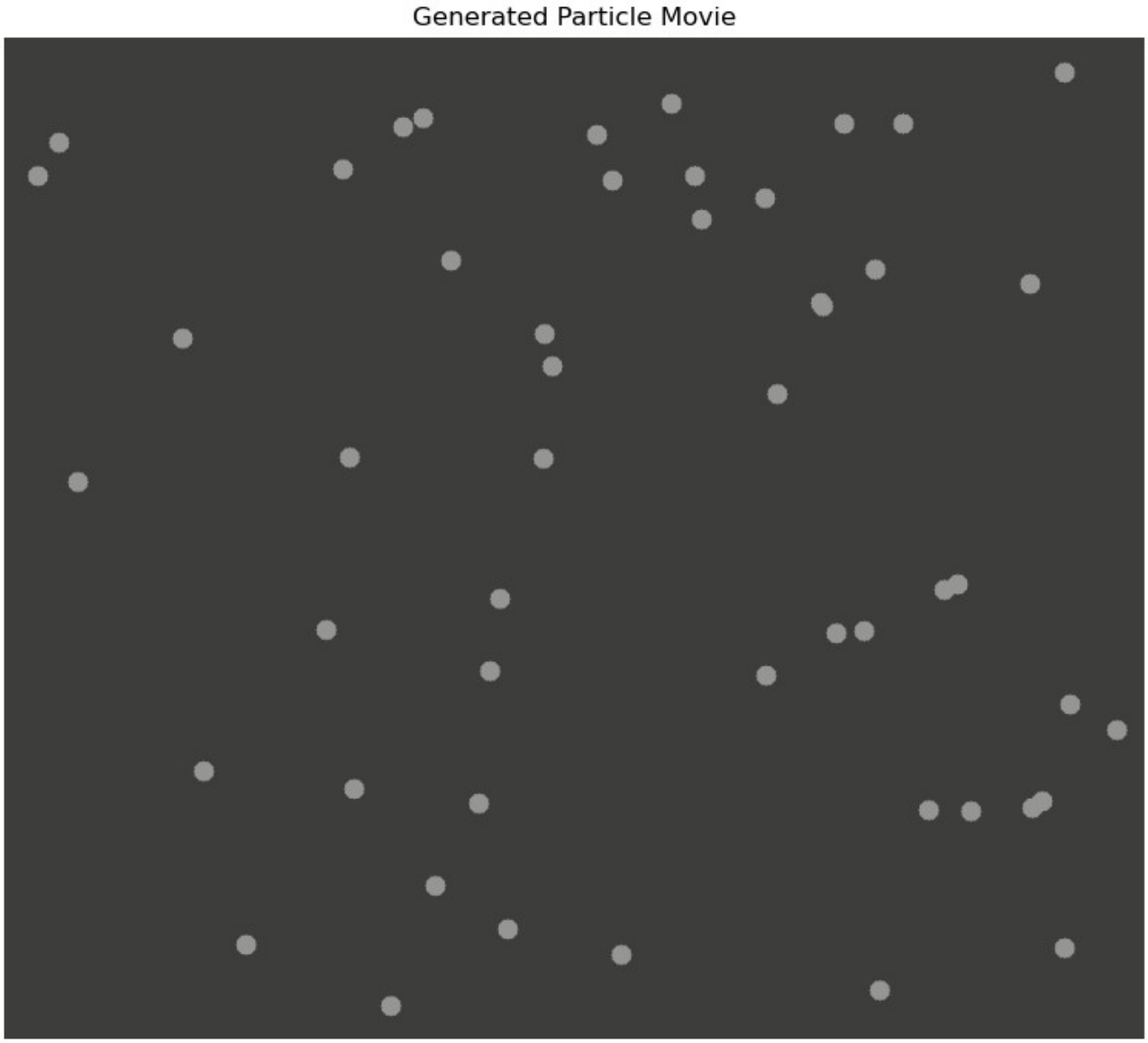
Particles intensity determines the gray level (0-255) of the particles.

Background intensity sets the background gray-level (0-255). Increasing its value will blur the particles and permit to assess the efficiency and reliability of further analysis steps.

Displ in pixel pro Frame parameter determines the displacement speed of the particles. In order to simulate bacterial, mitochondrial or yeast nanomotion keep this value low (0.01-0.5). If the displacement of the particles is too high the tracking algorithm might loose the tracking and identify the particle as a new one.

Modify Displacement in Rnd direction Every n_th Frame sets the number of frames after which the direction of the particles should change in a random direction.

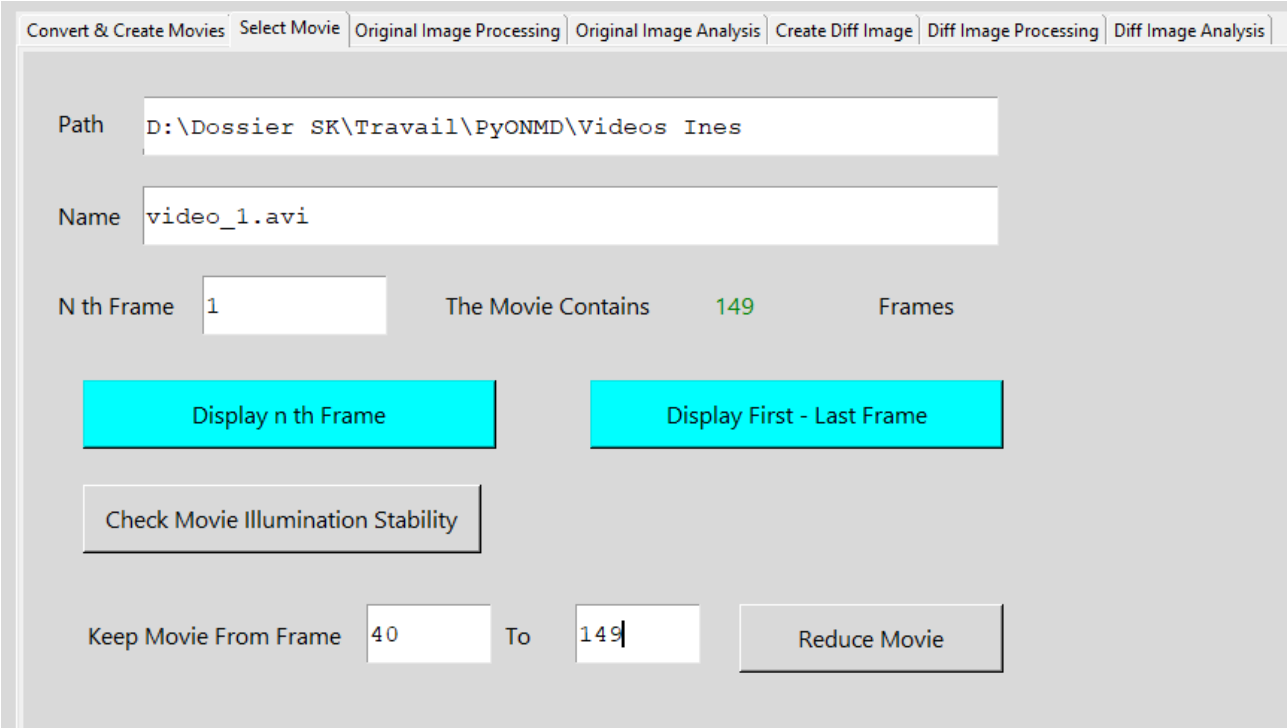
A frame of the generated movie is depicted below. After being created the movie is saved in the working directory under the name of **disks_movie.avi**. The parameters used to generate it are those present in the previous picture.



Select Movie

This tab permits to select the movie to be processed and to check its illumination stability. The user is asked to insert in the corresponding fields the path and the name of the movie (with its extension (i.e. .avi or .mp4)). Please remember that .h264 movies need to be converted first (see Convert & Create Movies tab).

Illumination stability is an important characteristic to be checked before conducting any ONMD analysis. Large variations in the illumination of the sample during recording compromise the Dff Image and the analysis results too. They can originate from the microscope illumination lamp, its electronics, the camera exposure control electronics, type of coding of the movie (in the case of Openflexure microscope we noticed that *.h264 format gives the most stable recordings) people moving around the microscope, mechanical oscillations due to a centrifuge, opening / closing of doors etc… Importantly, some cameras provide movies in which the first 10-30 frames have their global illumination changing rapidly, it is therefore important in such a case to remove these frames from the analysis (see Reduce Movie button). We therefore suggest to carefully check illumination stability parameter prior to conduct experiments.



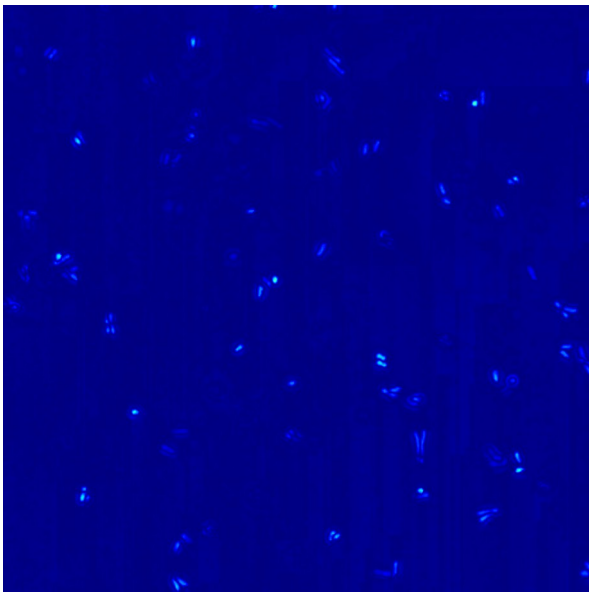
Path and **Name** fields should be provided to load the movie of interest. The name of the movie should end with its extension (.avi or .mp4).

N th Frame indicate which frame the user wishes to display. The program loads all the movie independently of this parameter.

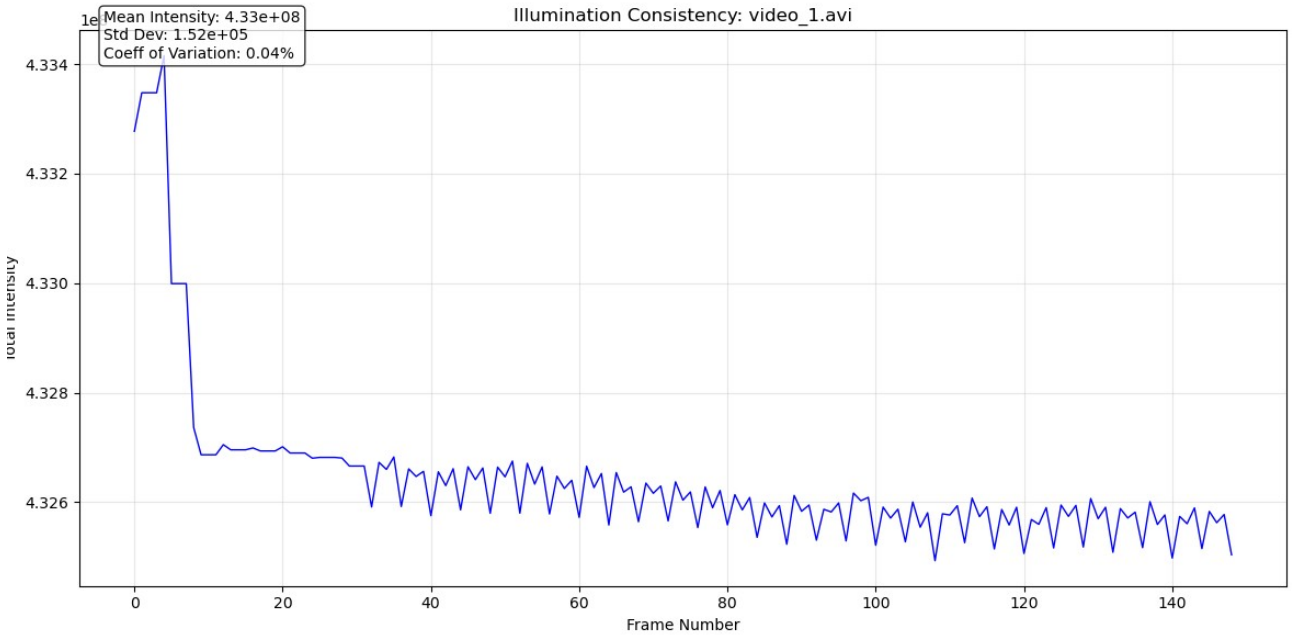
The movie contains __ Frames displays the length of the movie

Display n th Frame button will load the movie and display its nth frame in a separate window. Notice that this button has a blue color indicating it is mandatory.

Display First – Last Frame subtracts the first frame from the last and displays the absolute value of the result (result = abs[first-last]). It permits to rapidly asses if particles / cells moved or changed their intensity during the recording. The figure below shows the first frame of the recorded movie and the abs[first -last] in false colors. The program uses the “jet” color-map in which the blue color corresponds to low values and red to high intensity values.



Check Movie Illumination Stability displays in a graph the sum of the intensity of the pixels composing every frame of the movie. If large variations are noticed during the recording it is recommended to remove these frames. In the example displayed bellow we can notice large variations in the illumination occurring between frames 1-10. It is therefore recommended to remove these frames for further analysis.

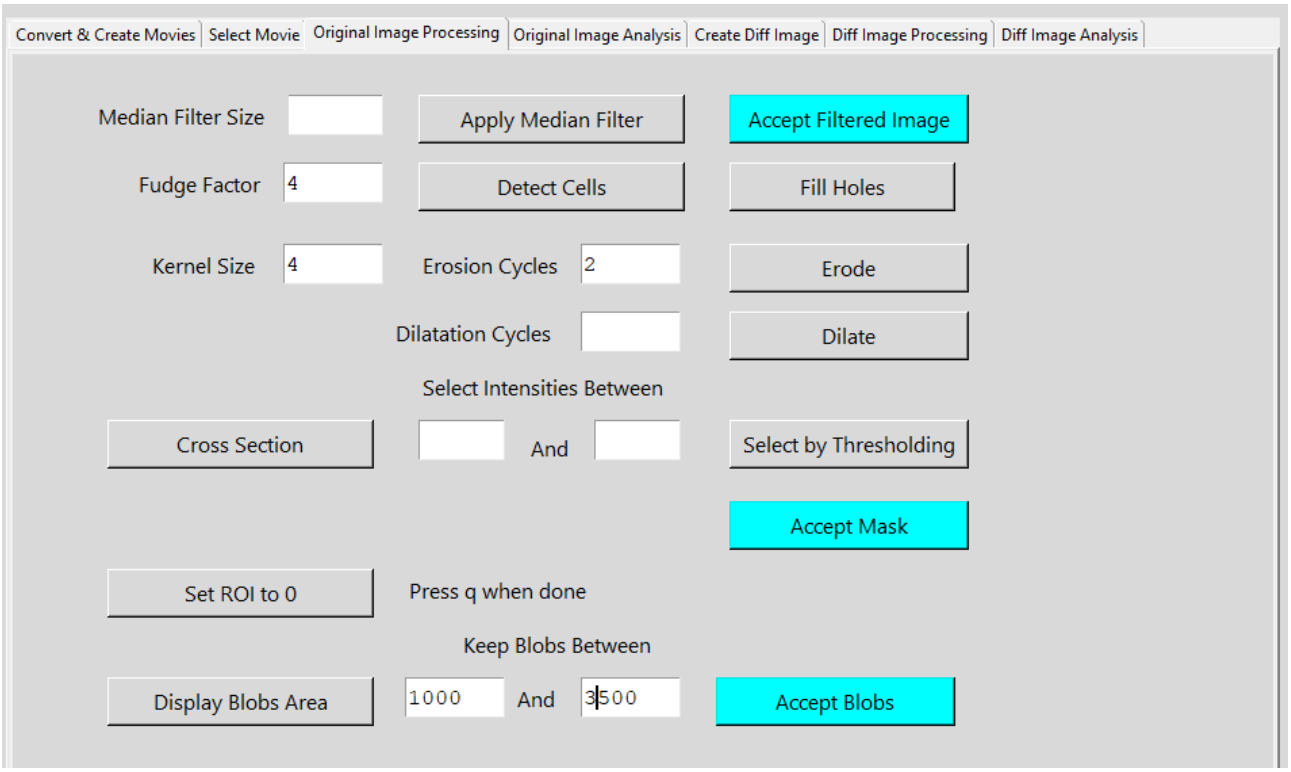


Keep Movie from Frame ___ To ___ sets the frames to be kept for further analysis.

Reducing Movie button keeps previously identified frames and writes a new movie in the working directory with the extension “reduced” (video_1_reduced.avi in the example). To analyze this newly created movie you need to reload it.

Original Image Processing

This tab permits to adjust the parameters in charge to select individual cells on the first frame of the original movie and to create a mask (i.e. a black and white image) that indicates where particles / cells are located. It also permits to modify the mask by filtering or applying erosion or dilatation morphological operators. This tab also permits to select cells / particles according to their size (i.e. area).



Median Filter Size is an odd number that adjusts the median filtering intensity. It corresponds to the filter kernel size. Larger this number is and stronger the filter is. Median filtering is the process of replacing each pixel in an image with the median value of its neighboring pixels. It can be useful if the image contains a “salt and pepper” noise as illustrated by the figure bellow.

Original image

After median filtering

from https://www.graphicsmill.com/docs/gm/minimum-maximum-median-filters.htm

Apply Median Filter button will apply a median filter and display the processed image. If the result is not satisfying change the filter size to a new value and push the button again.

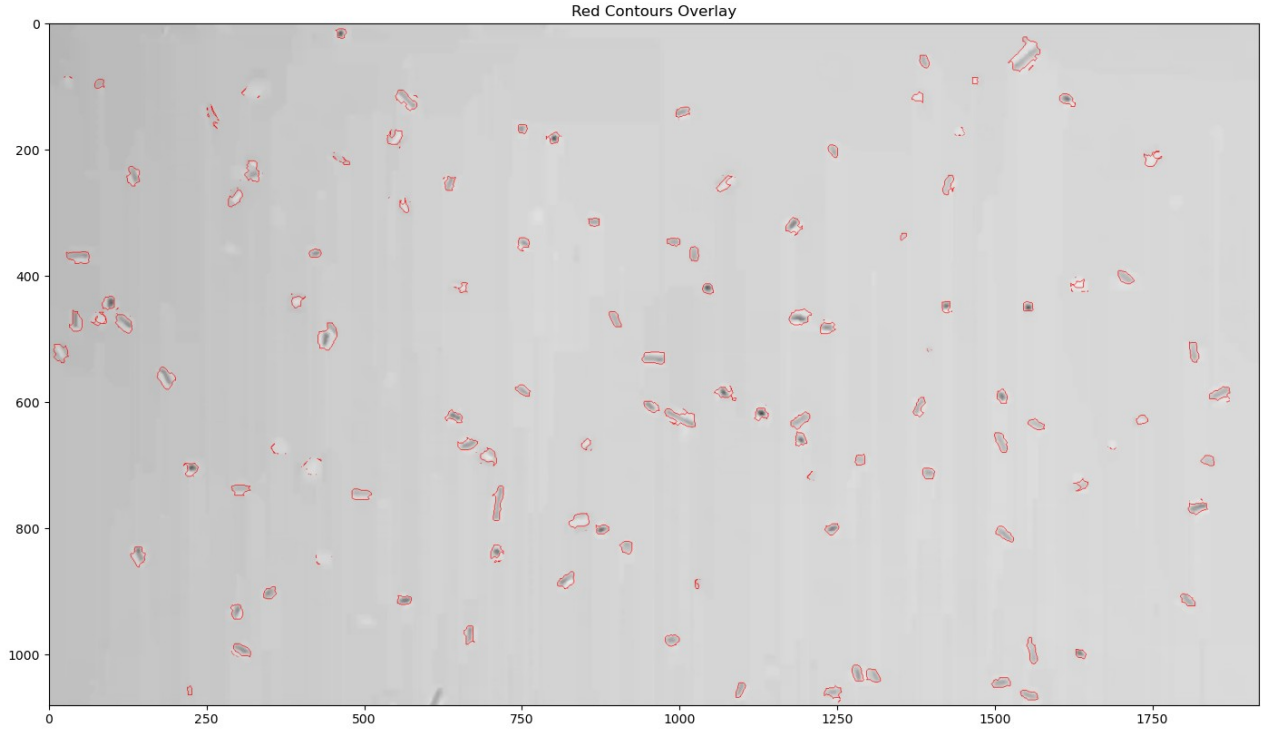
Accept Fitered Image sets the filtered image as the working image (the one which be further used for processing / analyis).

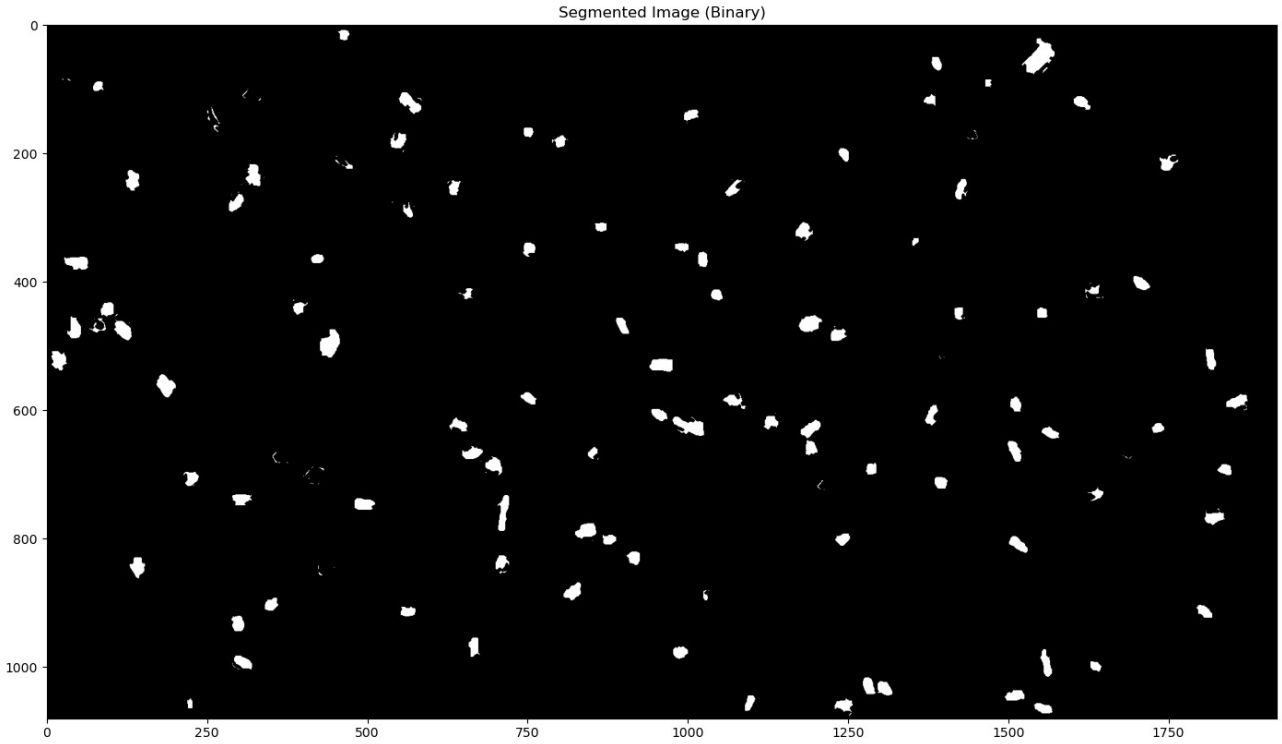
Fugde Factor sets a parameter to detect cells in a grayscale image. Its value varies between 0 and 20 depending on the size of the cells, their contrast and the intensity of the background.

Detect Cells starts a cell detection algorithm with the previously set parameter and displays the original image with detected cells as well the resulting mask. The algorithm is based on the one descriebed in https://ch.mathworks.com/help/images/detecting-a-cell-using-image-segmentation.html.

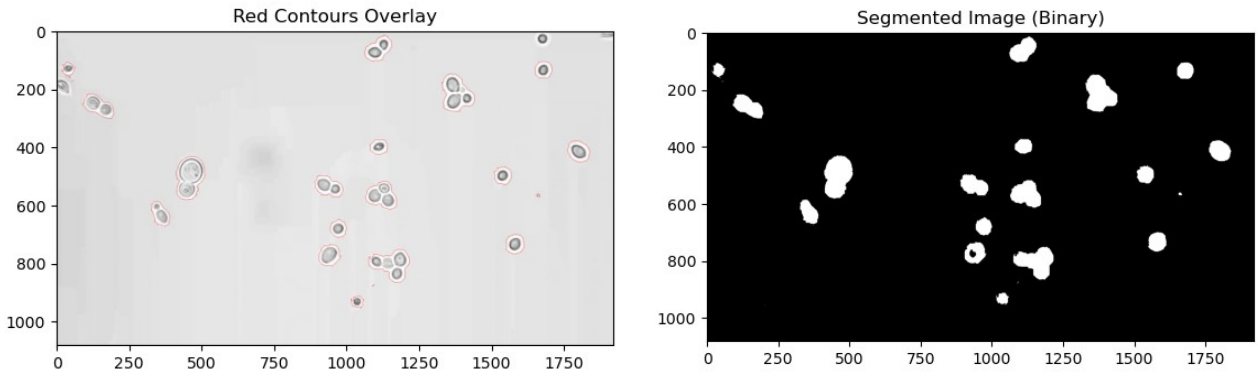
Fill Holes gets rid of clear areas that can sometimes persist inside the detected cells mask

The figure below depicts the detected bacteria (E. coli) with their contours in red obtained by using a fludge factor of 9 and its corresponding mask

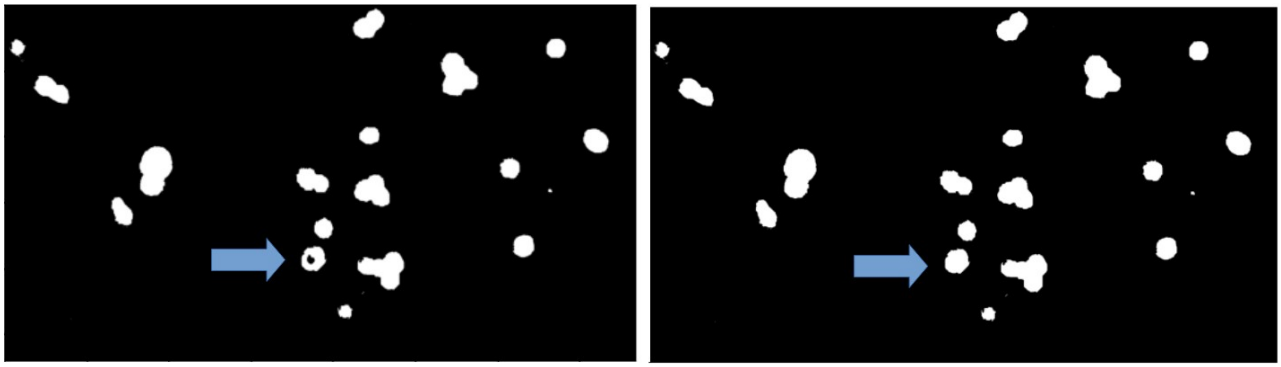




The figures below depict detected yeast (S. cerevisiae) by using a fudge factor of 4. Notice that the cell located at approximately x=900 and y = 800 contains a hole on the mask image.



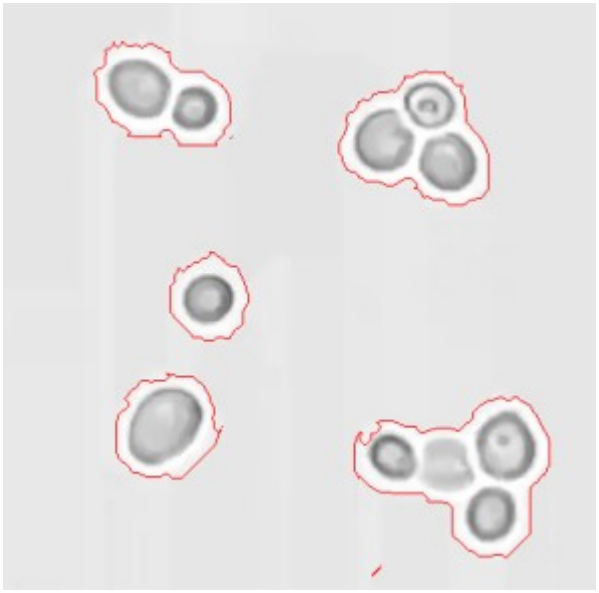
Fill Holes permits to eliminate remaining holes that can subsist in the mask image after the cell detection process. The figure below depicts how the previously cited example was corrected.
Left panel before and right panel after application of the Fill holes procedure

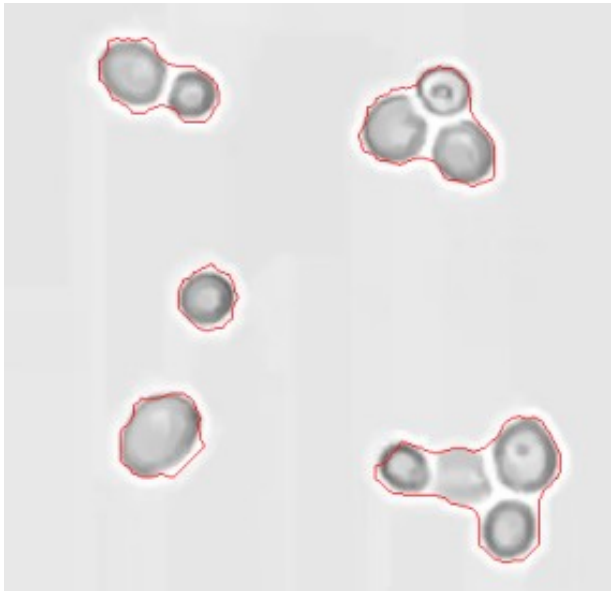


Kernel Size determines the size of a disk shaped structuring element that will be used for the erosion and dilatation morphological operations. Larger this number is and stronger the effects will be.

Erosion Cycles sets the number of times erosion morphological operators have to be applied. The larger this number is strong the effect will be

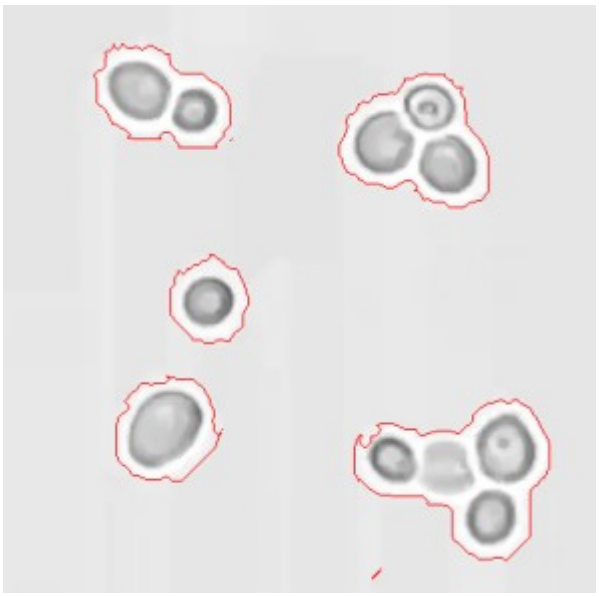
Erode applies an erosion morphological operation on the mask. An erosion makes shapes smaller by removing white pixels around the edges of the cells. The erosion operation is applied with a disk structuring element having a size defined by Kernel Size and repeated a number of times defined by Erosion Cycles. The erosion operation can also be used to get rid of small particles present in the image.
The effect of the erosion operation (kernel size 4 erosion cycles 2 in this case) on an image of yeast is depicted bellow. Left panel before and right panel after filtering.

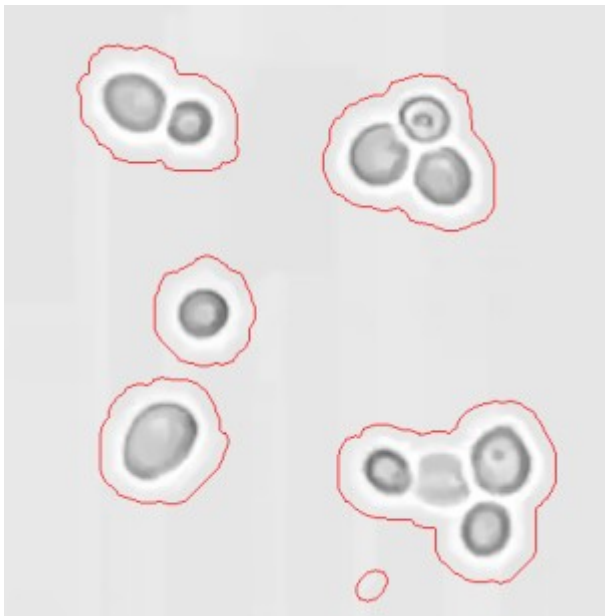




Dilatation Cycles sets the number of times dilatation morphological operators have to be applied. The larger this number is stronge the effect will be

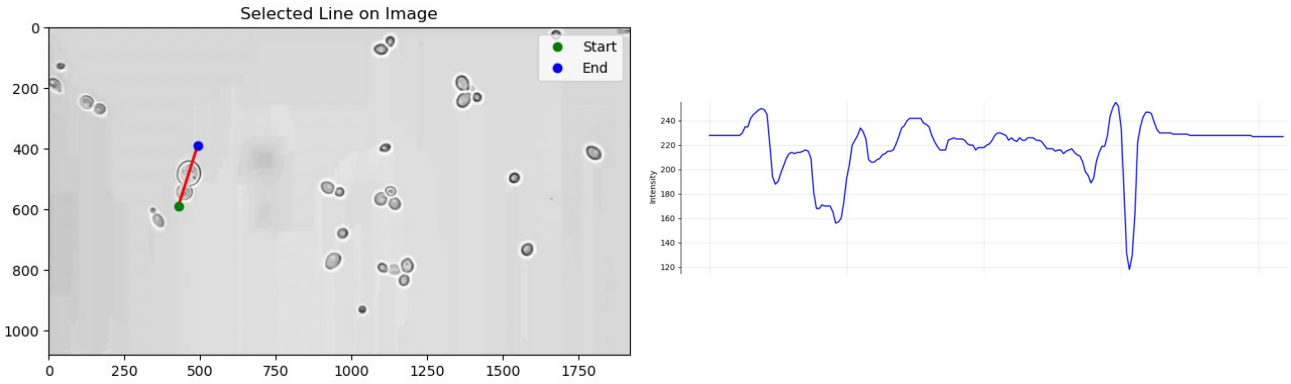
Dilate applies a dilatation morphological operation on the mask. A dilatation makes shapes larger by adding white pixels around the edges of the cells. The dilatation operation is applied with a disk structuring element having a size defined by Kernel Size and repeated a number of times defined by Dilatation Cycles. The effect of the dilatation operation (kernel size 4 erosion cycles 2 in this case) on an image of yeast is depicted bellow. Left panel before and right panel after filtering. Notice the dramatic increase in size of the small particle located in the bottom of the image





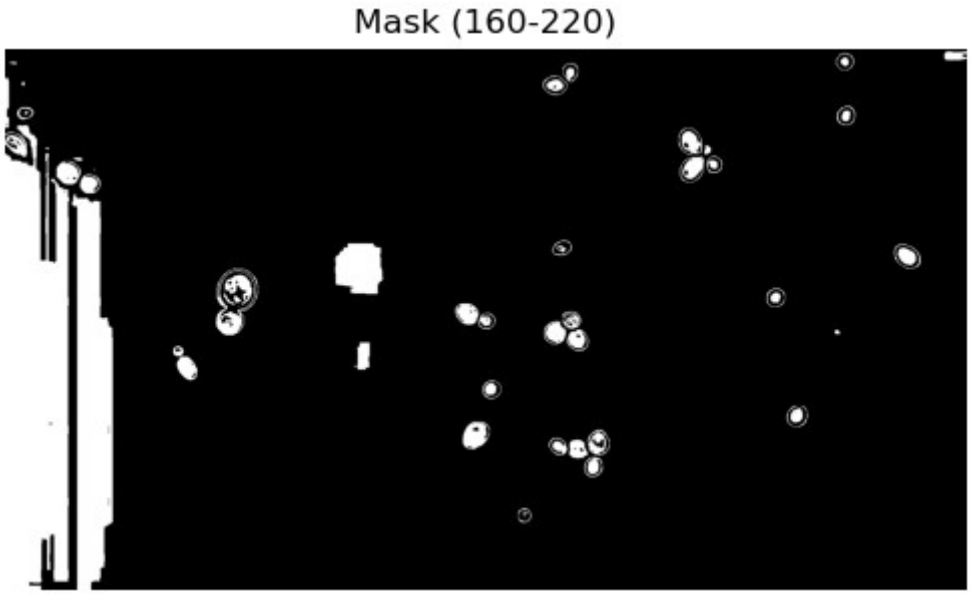
Cross Section button permits to obtain a pixel intensity profile along an arbitrary defined line. The user is asked to right click on two points of the first image of the movie and the program displays the pixels intensity along the straight line connecting the two points. Such an information can be

useful in case the user wishes to select cells according to their intensity in cases such as fluorescent labeled cells. The figure below illustrates this functionality



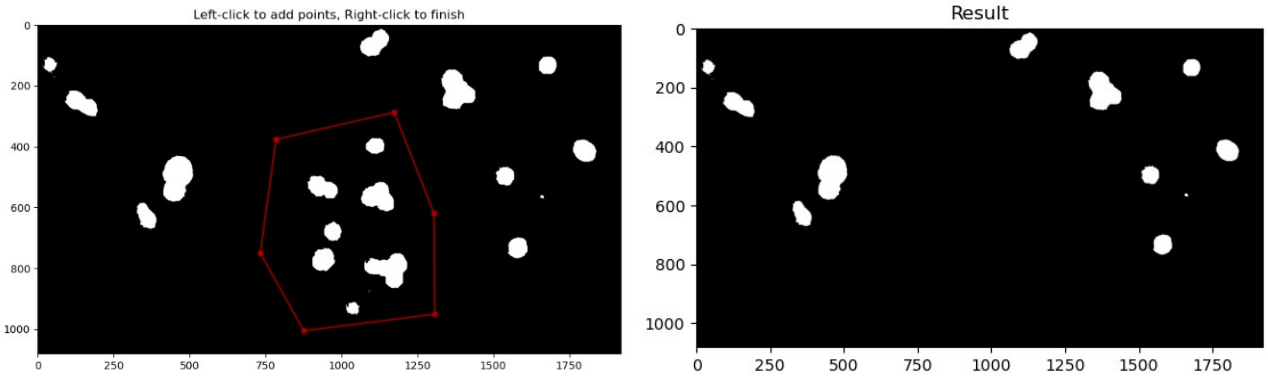
Select Intensities Between ___ And ___ awaits the input of the upper and lower graylevel for a selection according to the pixel intensity.

Select By Thresholding selects particles / cells according to their gray level as defined by the values defined by Select Intensities Between ___ And ___ values. Such a thresholding can be useful in case of fluorescent labeled cells. It should be emphasized that the method is very sensitive to illumination unhomogeneties as depicted in the figure bellow. The white areas on the left hand side of the images having the same intensity as the one chosen for the cells.



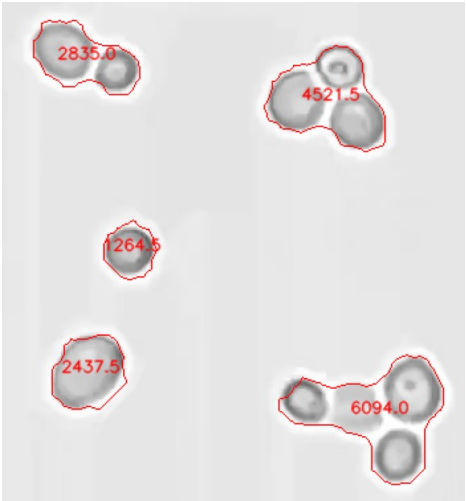
Accept Mask sets the mask obtained by thresholding as the working mask (i.e. the mask that will be analyzed to determine the morphological characteristics of the cells present on the first frame of the movie.

Set ROI to 0 permits to define interactively an arbitrary region on the mask of the first image of the movie and to exclude this region from the analysis. The figure below illustrates the selection process.



In order to set the new mask as the working mask the Accept Mask button has to be pressed.

Display Blobs Area permits to asses the size of the selected particles / cells. After pushing the button a new windows opens and the area of every selected cell is indicated as depicted in the figure bellow.

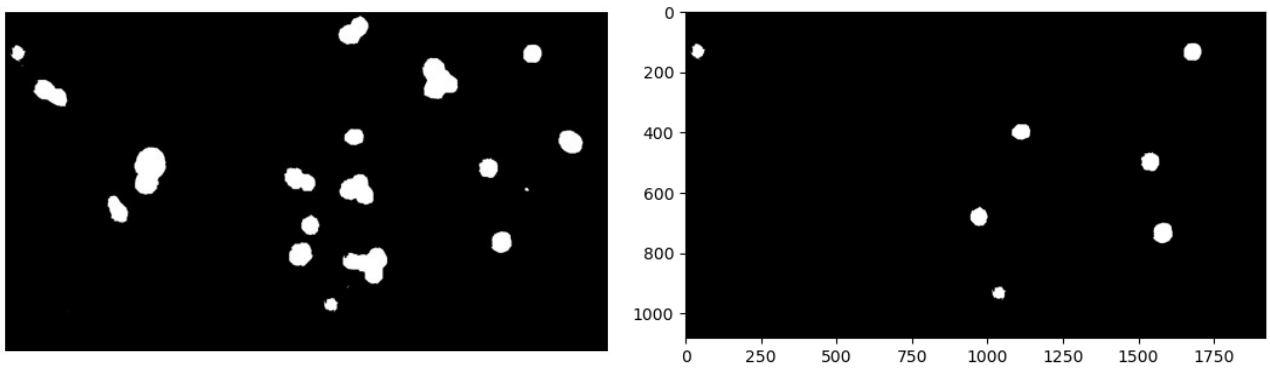


Zooming and moving around the image can be achieved by selecting the appropriate tools at the bottom of the window

The house symbol restores the initial magnification, the arrows permits displacements the magnifying glass is used for zooming and the floppy disk for saving the image

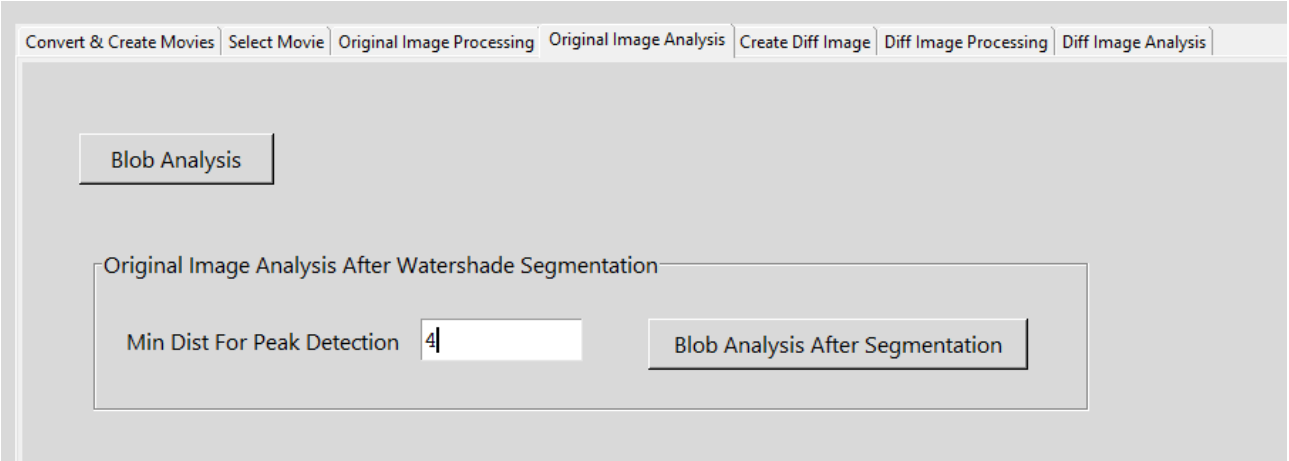
Keep Blobs Between ___ and ___ permits to set the lower and upper limits of sizes to be kept for further analysis.

Accept Blobs elliminates all the cell having a surface lower and higher than the limits previously fixed. In the example illustrated bellow only cells having a size between 1000 and 3500 pixels are kept for further analysis.



Original Image Analysis

This tab permits to extract various parameters of the previously selected cells (blobs)



Blob Analysis will consider the cells detected in the previous tab (Original Image Processing) and display several of their parameters as tables, histograms and boxplots.

Blobs Intensity Distribution (histogram) : shows the distribution of the pixels intensity that are located at positions occupied by blobs (i.e. cells or particles).

Blobs Intensity Distribution (boxplot) : shows the average intensity value and the standard deviation of the pixels that are located at positions occupied by blobs (i.e. cells or particles).

Blob Eccentricity Distribution (histogram) : displays the distribution of the blobs eccentricity

Blob Eccentricity Distribution (boxplot) : displays the average and standard distribution of the blobs eccentricity.

Blob Area Distribution (histogram) : shows the distribution of the blobs area.

Blob Area Distribution (boxplot) : displays the average and standard deviation of the blobs area.

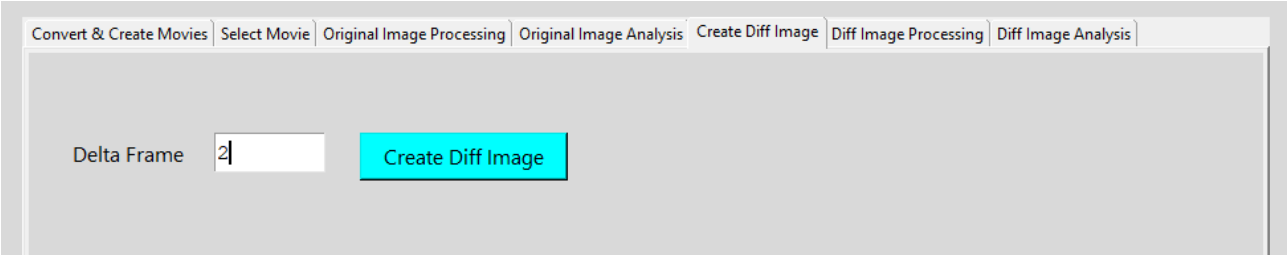
Comprehensive Blob Statistics (table) : prints the values of the all previously displayed parameters with their average values and standard deviations. The table bellow shows an example of the analysis of 8 cells present on the first frame.

	Value
Total Blobs	8.0
Total Surface (px)	20156.5
Average Surface (px)	2519.56
Surface Std (px)	793.52
Average Eccentricity	0.44
Eccentricity Std	0.17
Average Intensity	208.48
Intensity Std	5.34

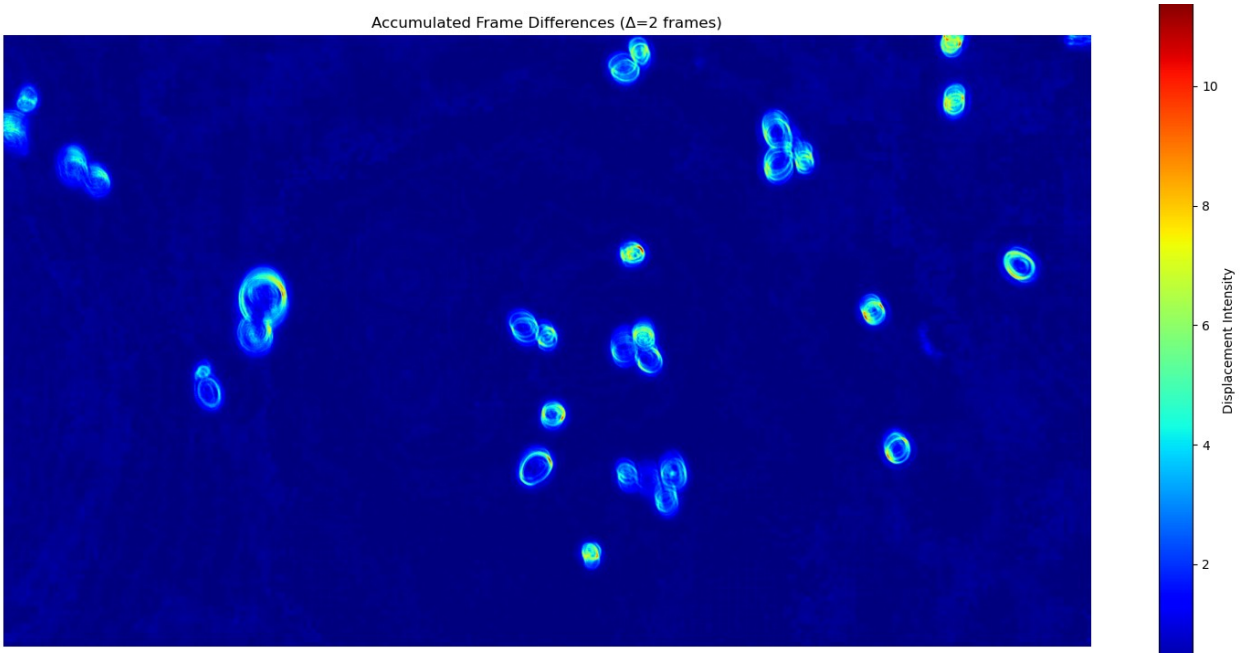
Min Dist For Peak Detection and **Blob Analysis After Segmentation** are experimental functions that use water-shade segmentation to separate closely attached cells. This functionality is till in development.

Create Diff Image

This tab is used to create an image, referred to as Diff Image, that highlights displacements / pixelvalue changes in the movie. The image is obtained by taking the abs value after subtracting frame n from frame n+Delta frame. This function creates a "motion highlight" image from the movie. Bascally the program looks at a frame in the movie and then skips ahead by a set number of frames ("Delta Frames"). It then compares these two frames and highlights pixels that changed their values between them. The program then moves to the next frame and repeats the process: it compares that frame with the one located Delta Frames ahead of it. All the little "motion highlights" from each comparison are added together on top of each other. The result is a single image where brighter areas show regions that were frequently or significantly moving / changing their intensity throughout the entire movie. Static objects that never move appear dark. A large Delta frame value will highlight low frequency displacements whereas a low Delta frame value highlights rapid displacements.

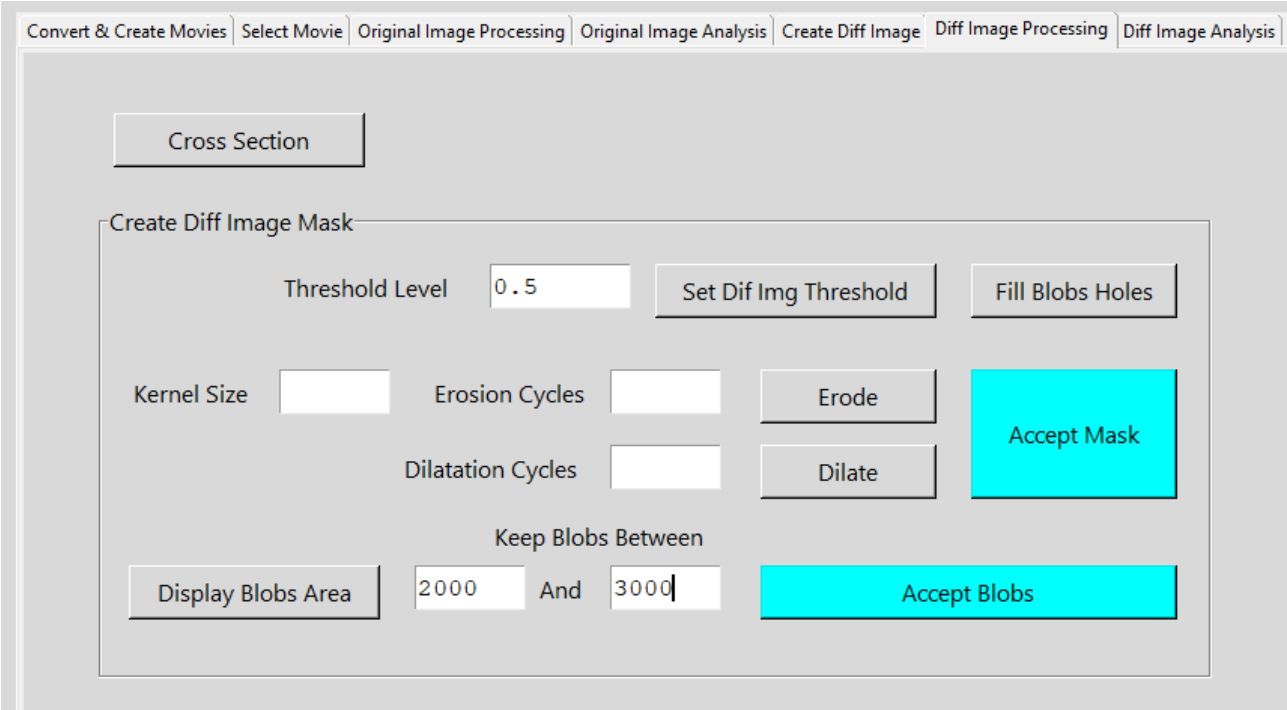


The figure below shows a Diff Image obtained with a Delta Frame of 2 on a movie recorded on yeast cells.

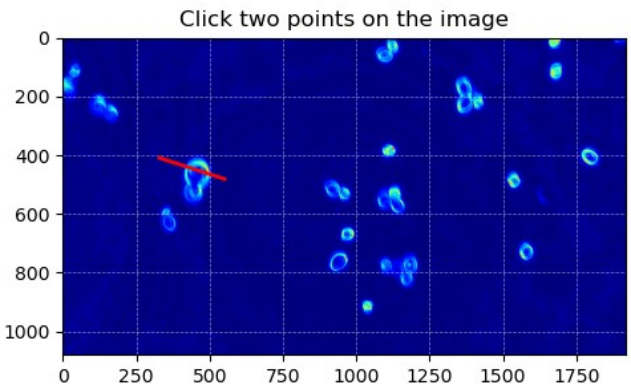


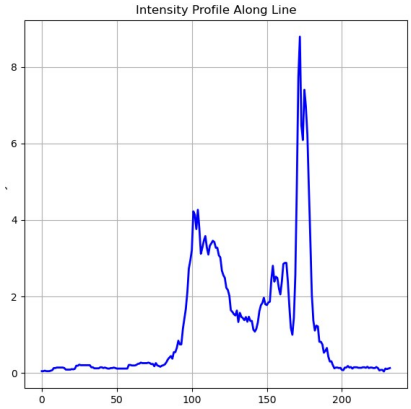
Diff Image Processing

This tab permits to process the Diff Imge before analysis. It permits to isolate moving cells from the background and immobile cells. The selection is based on thresholding and discriminating the cells according to their size. The tab permits also the application of erosion and dilatation morphological operators.



Cross Section permits to obtain an intensity cross section along an arbitrary line that is defined by two clicks on the Diff Image. The figure below illustrates a cross section along an oscillating yeast cell.



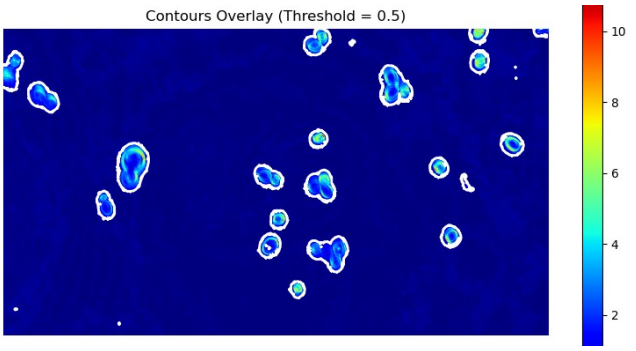


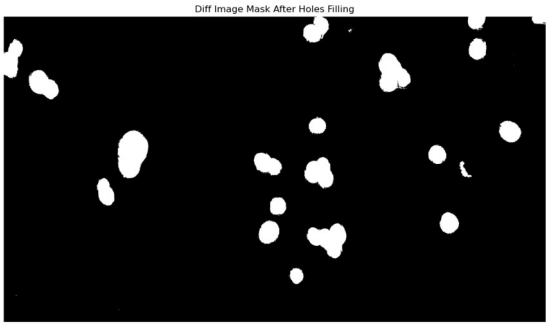
Threshold Level ___ permits to set the threshold intensity above which structures will be considered as cells.

Set Dif Img Threshold applies the threshold and generates the Diff Image mask

Fill Blobs Holes fills potential holes present in the cells

The figure bellow shows the cells selected with a threshold set at 0.5 and its corresponding mask after application of the Fill Blobs Holes





Kernel Size is the size of the disk structuring element used for dilatation and erosion

Erosion Cycles number of times erosion will be applied on the Diff Image mask

Erode executes the erosion operation

Dilatation Cycles number of times dilatation operation will be applied

Dilate executes the dilatation operations

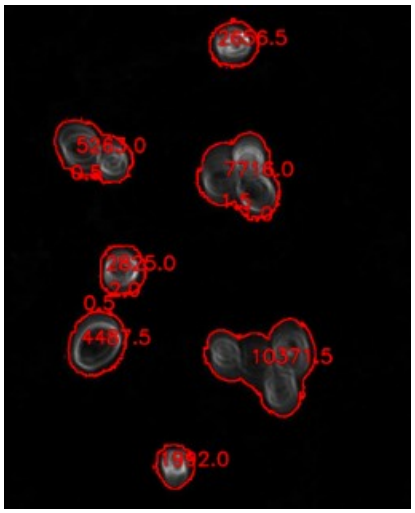
Accept Mask sets the mask as the working mask.

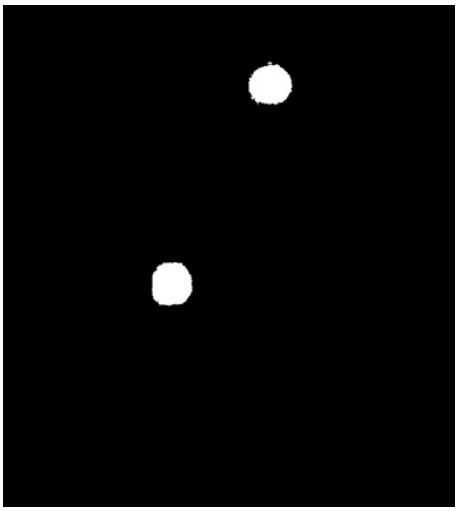
The parameters and morphological operators used and applied in this tab are the very same as those described in the Original Image Processing. For more details please refer to that tab.

Display Blob Area opens a window and displays the area of the selected cells

Keep Blobs Between ____ And ___ sets the lower and upper area limits to select cells

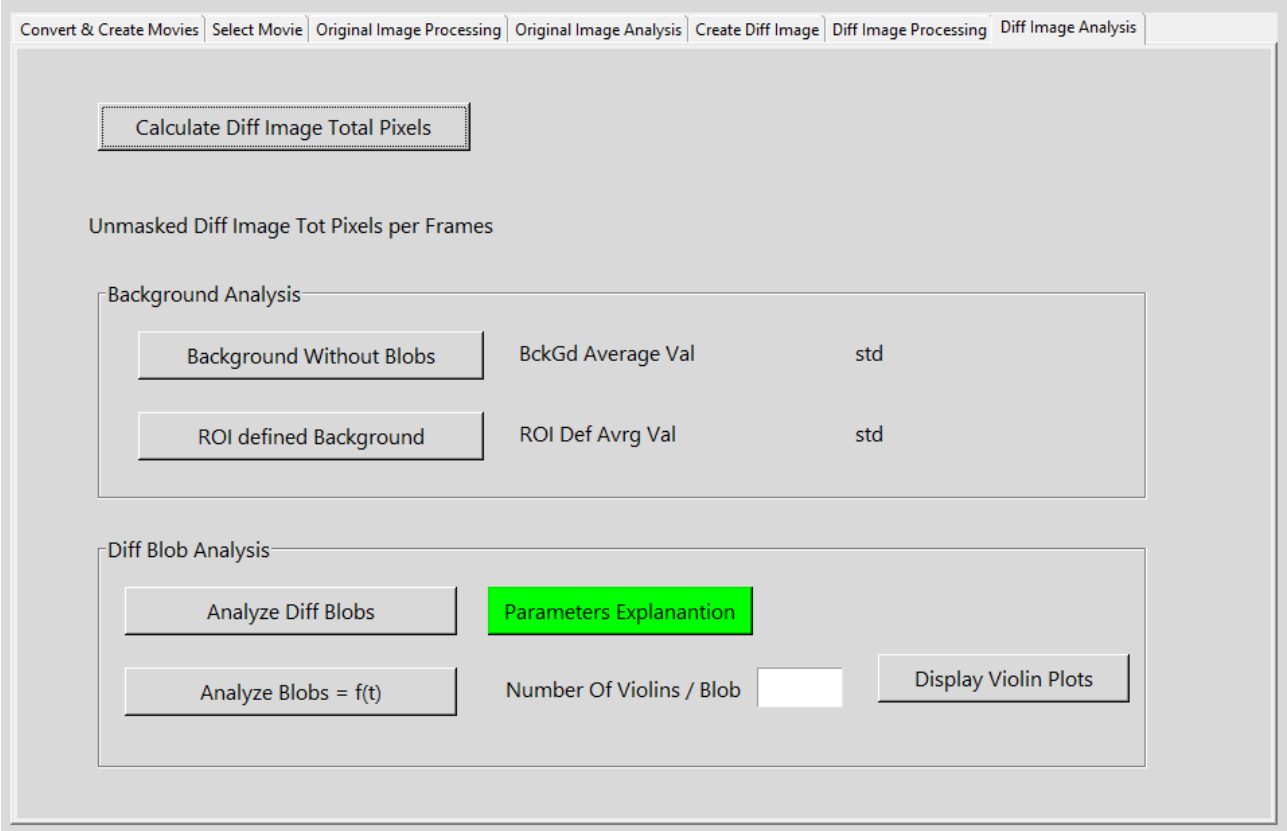
Accept Blobs executes the selection according to the area of the blobs. The figure below depicts the result of Display Blob Area on the left panel and the resulting mask after selecting areas between 2000 and 3000.





Diff Image Analysis

This tab permits to process the cells that were previously selected in the Diff Image Processing tab. It permits to display the sum of the pixels composing the Diff Image, to estimate the background level of an interactively defined ROI (region of interest) or of the full image. Finally it also permits to analyze every selected cell parameters and to monitor its variations as a function of time.



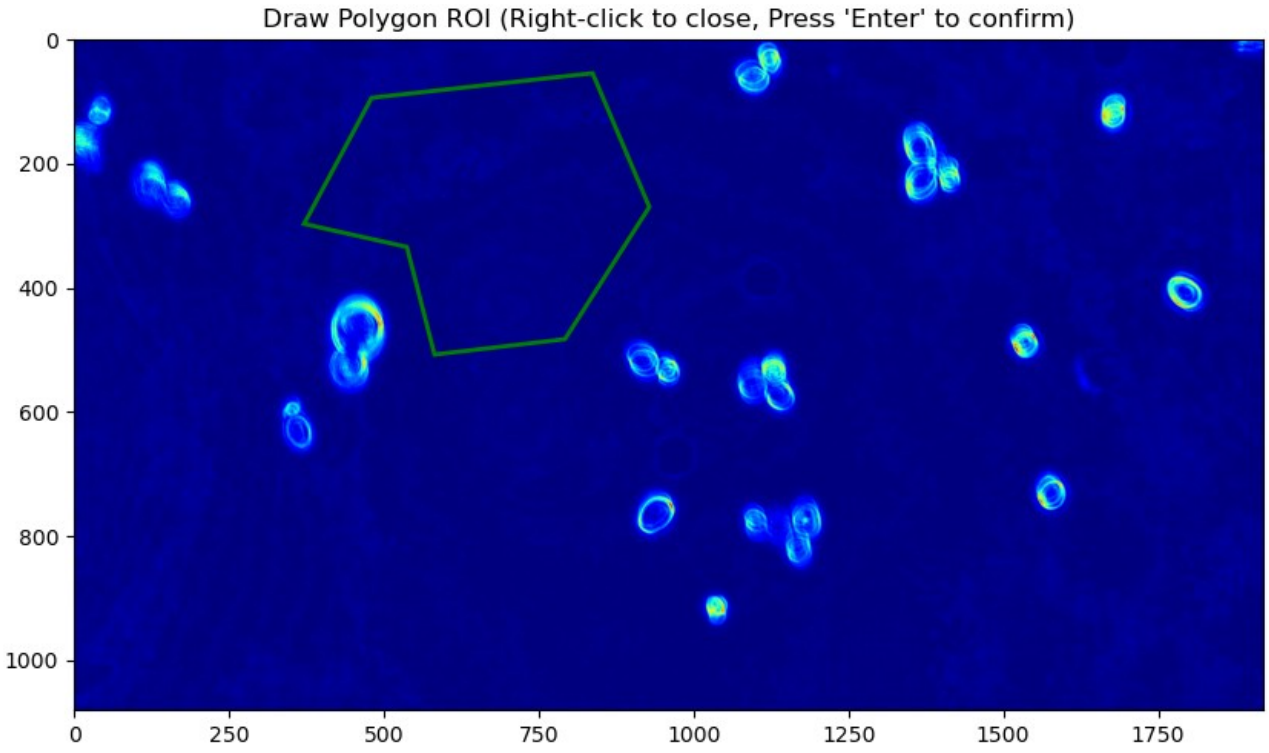
Calculate Diff Image Total Pixels calculates the sum of the pixels composing the Diff Image. The result is displayed below the button after Unmasked Diff Image Total Pixels per Frames.

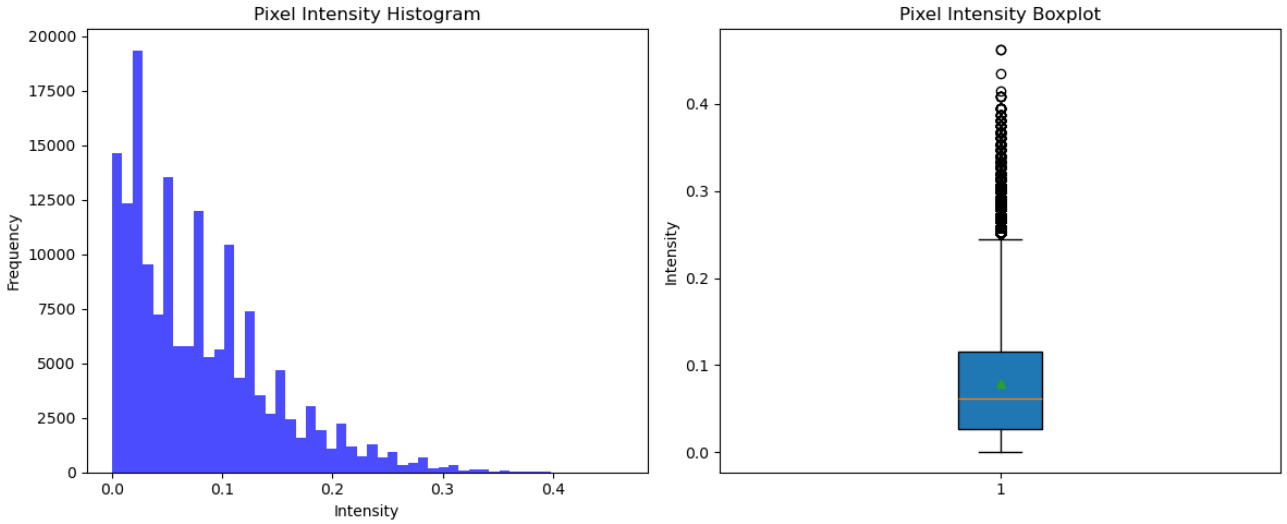
Background Without Blobs takes the Diff image and displays its average value and standard deviation after BckGd Average Val. The spots where cells were selected are excluded from the analysis. This function also displays the following parameters

Background Pixel Distribution (histogram) distribution of the pixels composing the background of the Diff Image (without the detected cells)

Background Stats (boxplot) mean and standard deviation of the pixels composing the background of the Diff Image (without the detected cells)

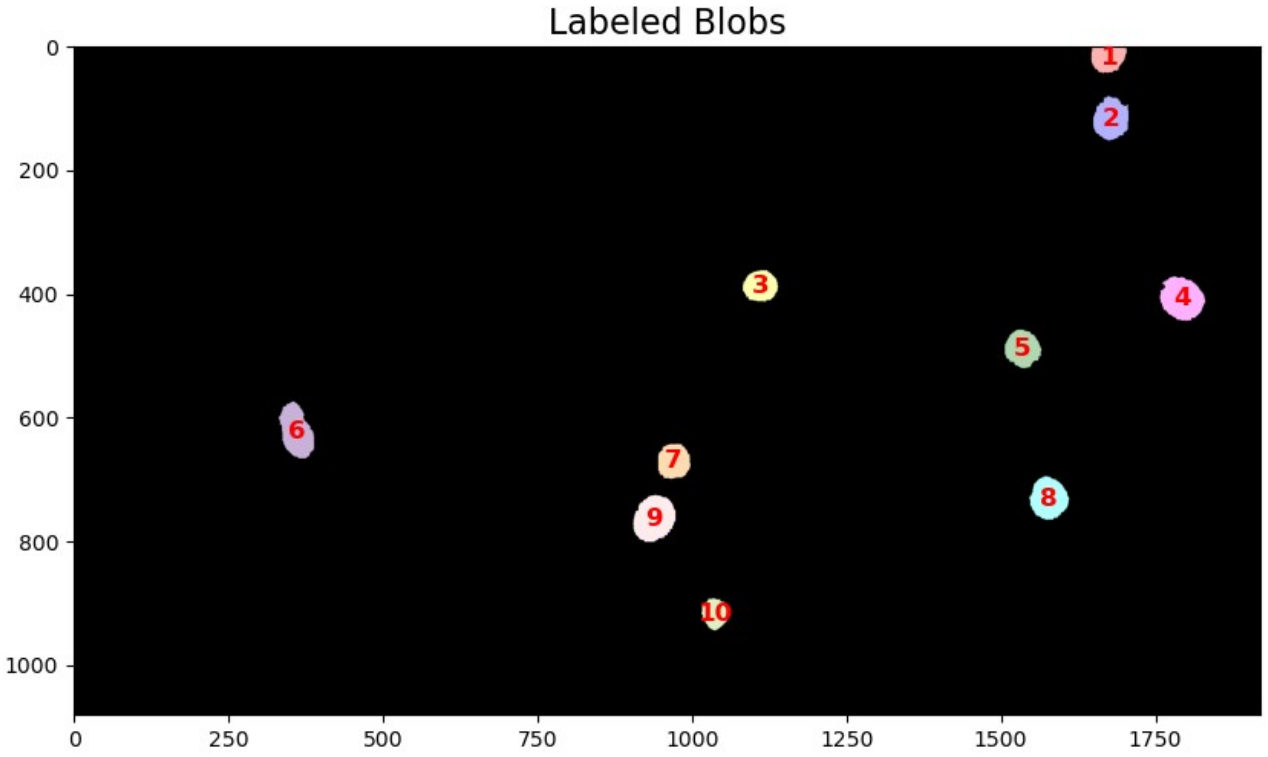
ROI defined Background permits to define interactively a polygon and to obtain the distribution mean and standard deviation of the pixels that compose it as depicted in the figure bellow





The mean and standard deviation values are also displayed directly on the tab in green characters.

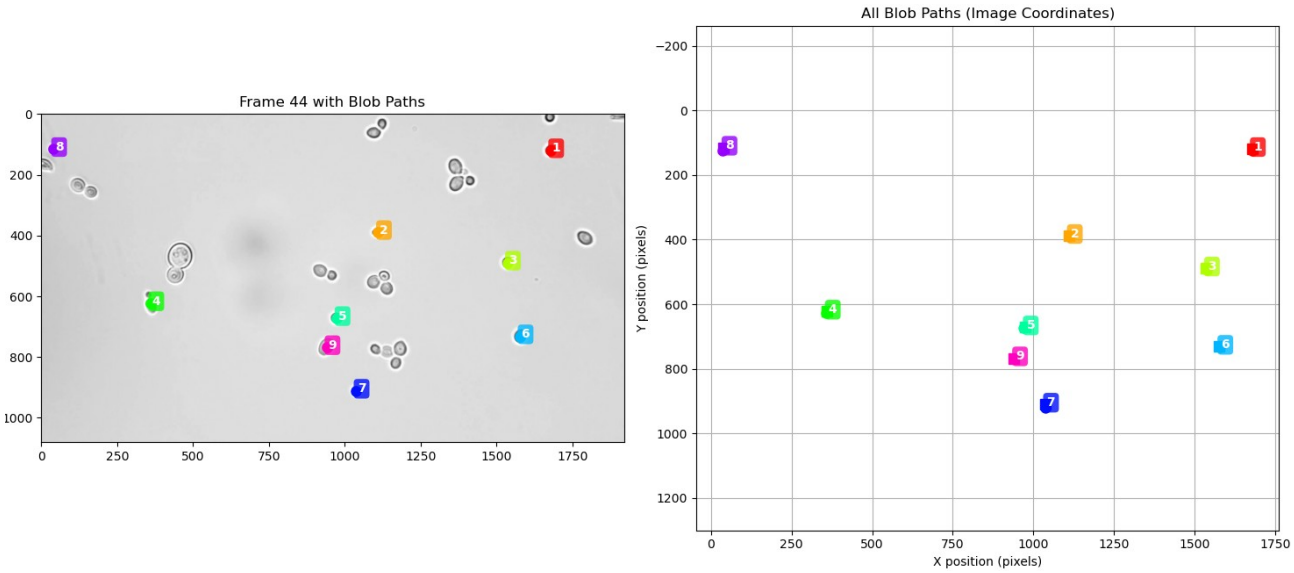
Analyze Diff Blobs displays the selected calls with their identifier as depicted in the figure bellow. In this case 10 cells were detected



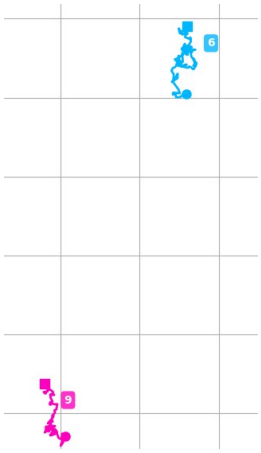
as well a table containing different parameters as displayed in the table below

Blob Number	Area	Eccentricity	Avg Intensity	Intensity Std	Max Intensity	Mean Intensity/Area
1.0	2024.0	0.6482	3.8532	2.2893	10.4694	0.0019
2.0	3096.0	0.5521	3.1033	1.9295	9.4626	0.001
3.0	2247.0	0.4473	3.398	2.1288	11.483	0.0015
4.0	3865.0	0.5094	2.7406	1.8216	8.7551	0.0007
5.0	2712.0	0.3631	3.044	1.9576	10.8435	0.0011
6.0	3569.0	0.8576	1.9735	0.8571	4.7755	0.0006
7.0	2430.0	0.387	2.9409	1.7786	9.966	0.0012
8.0	3283.0	0.454	2.4752	1.7008	9.9252	0.0008
9.0	3969.0	0.5823	2.1369	1.4916	11.1497	0.0005
10.0	1649.0	0.5551	3.5088	1.8861	8.9252	0.0021

Analyze Blobs= f(t) performs an analysis of every selected cell as a function of time. In this case the cell selection parameters (fludge factor and selection depending on the area) as defined in the tab Original Image Processing are applied to every frame of the movie and a new mask is generated for every frame. Depending on the number of detected cells this procedure can last several minutes. The center of mass of selected cells is monitored all along the movie and displayed as shown on the figure below.



Zooming on cells present in the right panel permits to visualize their path in x and y during the movie



The tools present on the bottom part of the figure permit to navigate in the panel

The house symbol restores the initial magnification, the arrows permits displacements the magnifying glass is used for zooming and the floppy disk for saving the image

The total, maximal, and average displacements as well the path length are displayed in a table that can be saved as an Excel table as depicted below :

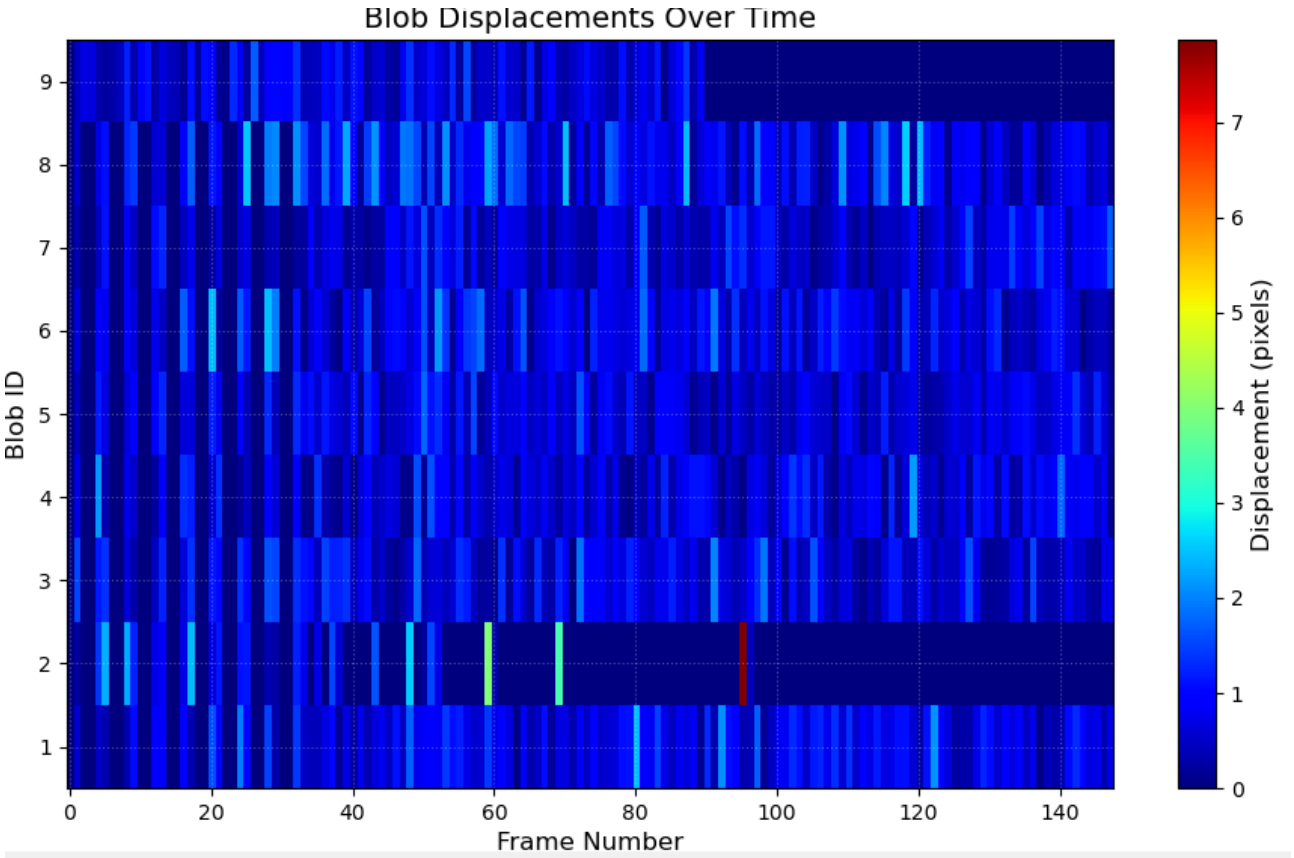
Blob Displacement Results

Blob ID	Total Displacement	Max Displacement	Avg Displacement	Path Length
1	20.59	1.6	0.46	45
2	24.52	2.39	0.54	45
3	21.94	2.0	0.49	45
4	23.98	1.76	0.53	45
5	26.65	3.16	0.59	45
6	19.37	2.21	0.43	45
7	26.81	1.79	0.6	45
8	26.28	2.66	0.64	41
9	0	0	0.0	4

Show Displacement Heatmap | Show Displacement Violin Plots | Debug Info | Debug Blob Data

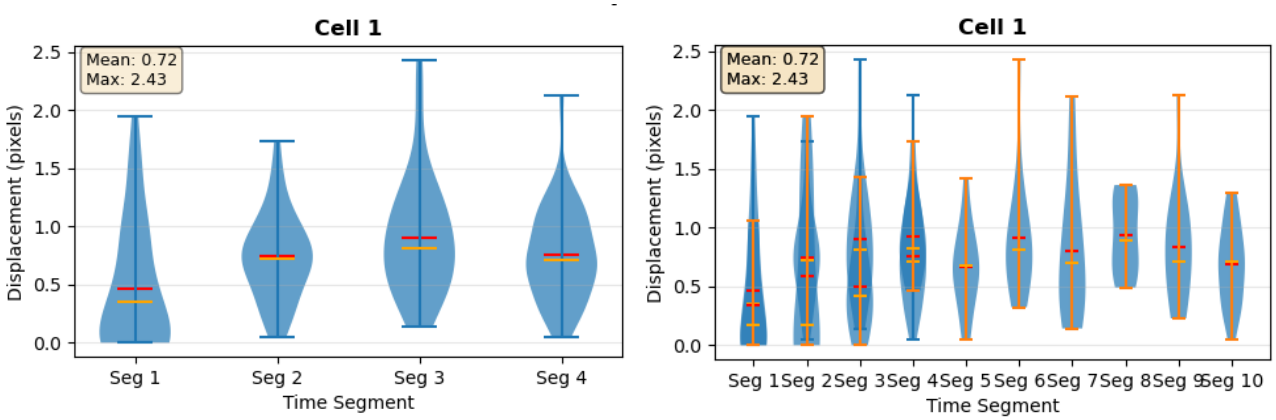
As it can be noticed cells nb 8 and 9 have a path length of 41 and 4 whereas the length of the movie is 45 frames. It means that these two cells were not monitored all along the move and should be removed from further analysis.

Show displacement Heatmap displays a new 3D graph composed of small false color rectangles. The rectangle color codes for the displacement of a given cell (identified along the y axis) at a given frame (identified along the x axis). In the figure shown below, 9 cells are monitored during 148 frames. For example cell number 9 moves relatively well approximately up to the frame 90 and almost stops after frame 90



Such graphs can be very useful to assess the displacements of all the cells as well to detect syncroneouse motions of some cells.

Show Displacement Violin Plots displays violin plots of the detected cells for a given time span defined by the Number of Violins / Blob. If this number is 1 then only one violin will be calculated per cell and will represent the displacement histogram (displayed vertically) of that cell all along the movie. If it is set to 10 then 10 violins will be displayed each corresponding to 1/10th of the length of the movie. The figure below shows two cases, one with 4 and one with 10 violin plots as calculated for the very same cell.



Parameters Explanation gives a comprehensive description of the different parameters.

Index