
Manual of CellDetail

GUI Software for quantifying spatial distribution
of components in single cells.



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1 Manual of CellDetail

1.1 General Description of CellDetail

The main aim of CellDetail is to offer users spatial distribution / polarity quantification possibilities for components within cells (proteins, mRNAs, epigenetic marks, organelles, ...) of interest. Additional benefits are the multichannel analysis possibilities as well as the characterization of general cell and component properties. It is a versatile tool for studying multicomponent networks.

It is possible to detect cells in single tiff images and tiff z-stacks via thresholding for cell layers and thresholding for cell voxels. Channels can be left out or included to the user's liking. The resulting cell vs. background mask can be exported. Furthermore, montage images of the resulting cell mask multiplied with protein intensities can be exported. There is the possibility to exclude cells which have not been detected well, to change cell detection settings for single cells to improve cell detection, to leave out a channel of a specific cell. The exported data can be found in .txt and .xls files, the possibility to export .mat files is included.

The results consist of spatial distribution -/ polarity-related values like the dipole moment vector P , absolute value of normalized dipole moment P_n , normalized distance R_n and normalized charge q_n , but as well total voxel number (being related to the volume of the cell), the maximal and averaged diameter, the average intensity and total intensity as well as the index of left out cells as well as the cell detection parameters used to make it possible for the user to reanalyze a data set pretty quickly with new analysis options with the same cell detection settings.

If not satisfied with the implemented cell detection, users can refer to self-made cell masks. CellDetail's cell detection algorithm identifies one object per image file and thus is set-up for single cell images. Thus, for using CellDetail for analyzing images with multiple cells (like e.g. cell layers) the direct import of multi cell images is not recommended. Instead, the option of importing pre-made cell masks can be used with single cell objects per file and the same number of files for both tiff data files and mask files.

1.2 Fast Start

After installation of CellDetail, prepare folders per project that you want to analyze: One for tiff-images to be analyzed, another one for data and another one for generated images upon analysis. When pre-made cell masks are used as input, generate an additional folder for the mask files. When starting CellDetail, the first Tab “Import and Export settings” is shown. If not already checked, click: “See all parameters at once.” Copy and paste the pathways to the folders into the according fields (“Pathway to Data Folder” for tiff images, “Pathway to save images” for the images generated upon analysis and “Pathway to save data” for the output data; if pre-made cell detection masks are used under the “Data set rerun options” setting parameters, check “Predefined mask import option” and paste the pathway to the folder of pre-made cell detection masks into the field after “Pathway to Mask Folder” field and choose the word found in every cell detection mask file name, for which “Mask” is pre-set, by replacing it.) or use the browse function for it by checking the boxes. As tiff images are used, the word in every file name is chosen to be “.tif ” unless another definition of tiff images being used is wished for. Thus, all files which have “.tif” in their name within the pathway to data folder are chosen as input files. Next name the channels of your tiff images in the field “Name Channels”. As an example: When having the channels in the following order: channel 1 Cdc42-594, channel 2 Tubulin-488, channel 3 DAPI and channel 4 brightfield, one can write “Cdc42, Tubulin, Dapi, none”. Thus, the channels will be named accordingly and the brightfield channel will not be analyzed. The enumeration starts with the first measured channel. It is important to name every channel, as otherwise the separation in channels will not be appropriate. The option which channel shall be taken for cell detection can be used in tab 1 (in tab 2 a more versatile variant can be used for which several channels can be taken into account for cell detection).

Write in measurement settings the pixel width x, pixel height y and voxel depth z in nm. When using images of 14 bit size, you can check 14 bit. For all bit values however, the import recognizes the bit size of the first image and takes it. This is important when you want to use the charge normalization options 1 and 2, which rely on the bit size.

For detection option, check “Confocal” option. This smooths inner holes of cell detection layers. For analysis options, leave “Take all parameters” and “Cell detection separated” unchecked (“Take all parameters” calculates cluster-related parameters taking more computing time, “Cell detection separated” changes the order of cell detection and analysis to a complete cell detection before calculating parameters which is advantageous when single cell calculation time takes long). “Data set rerun options” can be ignored, they only apply when reanalysis of already analyzed data shall be performed or pre-made cell detection mask tiffs shall be used. For the latter case, one needs to make sure that masks and tiffs follow the same order in both folders (extra mask folder should be made, number of tiff files needs to equal

number of mask files, size of mask file needs to equal size of corresponding tiff file). In the second tab “Adjust Parameters and Pre-View of cell detection” the “Parameters for cell detection” are set for a general run-through. (Later in the third tab, cell detection can be improved per single cell.)

First, the layer thresholding is performed. Usually, the “Basic Parameters” subtab should be enough for detecting cells of interest. For giving users more freedom, additional parameters can be varied under the subtab “Additional Parameters”. For applying thresholding at all, “Apply thresholding” needs to be checked (below “General Parameters” box). Otherwise, only the whole image stack of a channel of a single cell can be looked at. The channels which shall be used for cell detection can be given freely when checking “Several channels for cell detection” at the lower base and replacing the input “1,2,3” with the wished channels. As some channels can appear really bright, the program sets the maximum of each channel to be the same. For weighting of channels for cell detection, one can check “Weighting of channels” and write in the weighting of each selected channel. For first adjustment, it makes sense to check both “Apply thresholding” and “Show just layer thresholding”. The first step of detection is performed: The layer thresholding. Two parameters can be set in the “Basic Parameters” option: “Minimum number of pixels per layer for Range filter” and “Maximum number of pixels per layer for Range filter”. By varying and clicking through the cells (caveat, one needs to click through in order to see effect) via the “Back” and “Next” buttons below the image montage on the right, the new settings are adapted and one can optimize the values. Nonetheless, via the “Minimum number of images per cell (+/-1)” and “Maximum number of images per cell (+/-1)” options one can have additional influence on how many layers are at minimum and at maximum taken. When the layer thresholding was optimized, next comes “Cell pixel thresholding” and one unchecks “Show just layer thresholding”. Cell pixel vs. background thresholding in the chosen layers is mostly performed via “Imbinarize Threshold for Confocal” under general parameters.

As fluorescence light in widefield condition already leads to signal outside of the cell interior in a nearly uniform manner, one has the option to check “Advanced” option next to “Parameters for cell detection” and “Take in account: out of focus” to move layers consistently by a certain value. E.g. when signal comes already two layers before the cell itself appears, a 2 can be written for “Reposition of layers along depth: Move found layers by value” parameter. The detected layers will always be moved by 2 layers. Negative values are allowed as well for repositioning.

When cell detection does not work with the pre-set parameters, one can choose “Program Run: Visible cell pre-view making?” (option below “Apply thresholding” and “Show just layer thresholding” option at the base). This allows to observe the cell detection algorithm at work. One can see at first the original image versus the range filter, afterwards the decision on layer thresholding and the decision on cell pixel thresholding. Thus, it is possible to conclude which cell detection step still needs improvement.

For the shown image montage, you can select the channel which shall be shown (“Number of channel to show”). If you want to have an easy possibility to rerun analysis on the data set, you should check “Save images inclusive mask (latter as tif-stack)” as this leads to output masks which can be used for the rerun functionality in the first tab (be careful when going for rerun in this case if you selected cells not to be taken into account for analysis due to poor cell detection, as the cell original image files and mask files need to be removed for reanalysis then).

Check “Calculate all normalization possibilities”, otherwise you will only receive results for the selected normalization possibilities in the dropdown menu (pre-set: “1) Normalized by maximal diameter” and “1) Charge normalized dependent on shape of cell (bit)”). Check “Cell total intensity normalized to 1” (cells’ protein amount variability under different conditions shall not have influence) and if co-localization is an important parameter for your experiment, check “Pearson correlation coefficient” option. When the general settings are optimized for your image data, click the “Start run” button. A window appears showing the stage of analysis. After run-through, the “Feedback to user” field will show “Runthrough done: Elapsed time is ” and mention the time needed for performing the run-through. Next you move to the third tab “Results”. On the left handside the detected cell voxels versus background voxels are shown. On the right handside a table is given with some of the output values. You can move through the table and cells via “Back” and “Next”. If cells shall be removed from analysis data, one can check “Not to take”. This can be undone. If a cell was not detected well, one can check “Rerun” (right handside, top) and a “Rerun settings” windows opens with the parameters already seen in tab 2. The previously general settings can now be adapted for the single cell. Before the adaption is completed, you can check with a “Preview of Rerun” click whether cell detection is good. When cell detection is good, the “Rerun (table values changed)” button needs to be clicked in order to adapt values with optimized cell detection settings. After moving through cells and checking cell detection, you can export data by clicking “Export Data” button. The recommendation is to export .mat files as well (check “.mat export”) as another rerun option for reanalysis is possible with it. The output values are now saved in the data folder as .xls files, .txt files and .mat files (if option for it was checked). You obtain the following values:

- averaged intensity (value, which is changed when normalization of intensity is performed (total to 1, average to 1))
- “Chargedensitypos”, total positive charge density (a.u./nm³, not per voxel)
- dipole moment \mathbf{P}
- averaged and maximal diameter
- normalized charge q_n (various options)

- normalized dipole moment P_n (various options)
- normalized distance between charge centers R_n (various options)
- number of pixels/voxels (cell size)
- Pearson correlation coefficient table
- positive pixel/voxel number
- vector $\mathbf{R}_{\text{neg}}\mathbf{M}$ (“RnegRmean”, \mathbf{R}_{neg} negative charge weighted center, \mathbf{M} middle of cell)
- vector $\mathbf{R}_{\text{pos}}\mathbf{M}$ (“RposRmean”), \mathbf{R}_{pos} positive charge weighted center
- vector $\mathbf{R}_{\text{pos}}\mathbf{R}_{\text{neg}}$ (“RposRneg”)
- total intensity (parameter which is not changed due to normalization of intensity)
- “indexnottaken” (index of cell/s not taken, if cell/s excluded from analysis)

For exported .mat files, additional information is yielded:

- “Names”, names of cell tiff files (cell names in the order of run-through)
- “allchannelnames”, channel names
- “savingofsettingsofcertaincells” (for rerun option)
- “toignore”: index of channel not taken in account (e.g. brightfield)

Caution: After export of data press the button “Next Data Folder” in tab 1 “Import and Export settings” for deleting global variables which could interfere with the new data set!

1.3 Detailed Description

1.3.1 Tab 1: Import and Export settings

The user is asked for import, export and basic information about data to be analyzed in tab 1. Roughly seven areas/panels can be drawn:

- Panel 1 for “Import”/“Export” settings as well as loading settings of another run (“Load Settings”) as well as starting over with a new dataset (“Next Data Folder”, for deletion of global variables).

- Panel 2 for “Channel information” with writing down channel names separated by comma and naming channels not being of interest for analysis as “none”. A pre-step of cell detection can be made by choosing the channel for cell detection in tab 1. However, in tab 2, several channels can be chosen together with a weighting. Thus, when several channels shall be used for cell detection (recommended), this point can be neglected till tab 2.
- Panel 3 for “Measurement settings”. The pixel/voxel values are asked for as well as whether 14 bit was used or not. This is due to ImageJ not being able to save tiff as 14 bit images, thus allowing the user for correcting mislead analysis when choosing charge normalization options 1 or 2.
- Panel 4 for “Detection option” of “Confocal” or not. For the “Confocal” option, steps are performed in order to fill holes within detected cell areas. For conventional widefield images, “Confocal” option can be an improvement as well. The “Analysis options” are called “Take all parameters” and “Cell detection separated”. For the option “Take all parameters” additional parameters are calculated, like the clusters of positive charge voxels, their size and position. For the option “Cell detection separated” first the cell detection is made, before the analysis occurs. If not checked, when starting the run in tab 2, the analysis is performed right after cell detection of single cells leading to a results table in tab 3 giving an overview over essential parameters (normalized dipole moment, ...).
- Panel 5 for “Data set rerun options” gives facilitation for users wanting to rerun an already analyzed data set. Users can use the “savingofsettingsofcertaincells.mat” file and “indexofcellsnottaken.mat” file, thus reusing previous cell detection parameters, or use masks belonging to their image data files of interest.
- Panel 6 for user feedback.
- Panel 7 for view options of tab 1. Users can see all parameters at once (“See all parameters at once” checked), or panel 1 till panel 5 one after the other (“See all parameters at once” unchecked, with appearing “Back” and “Next” buttons), either via transparency or image panels. By “Back” and “Next” button the user can click through, but it is possible via mouse click on the next panel as well.

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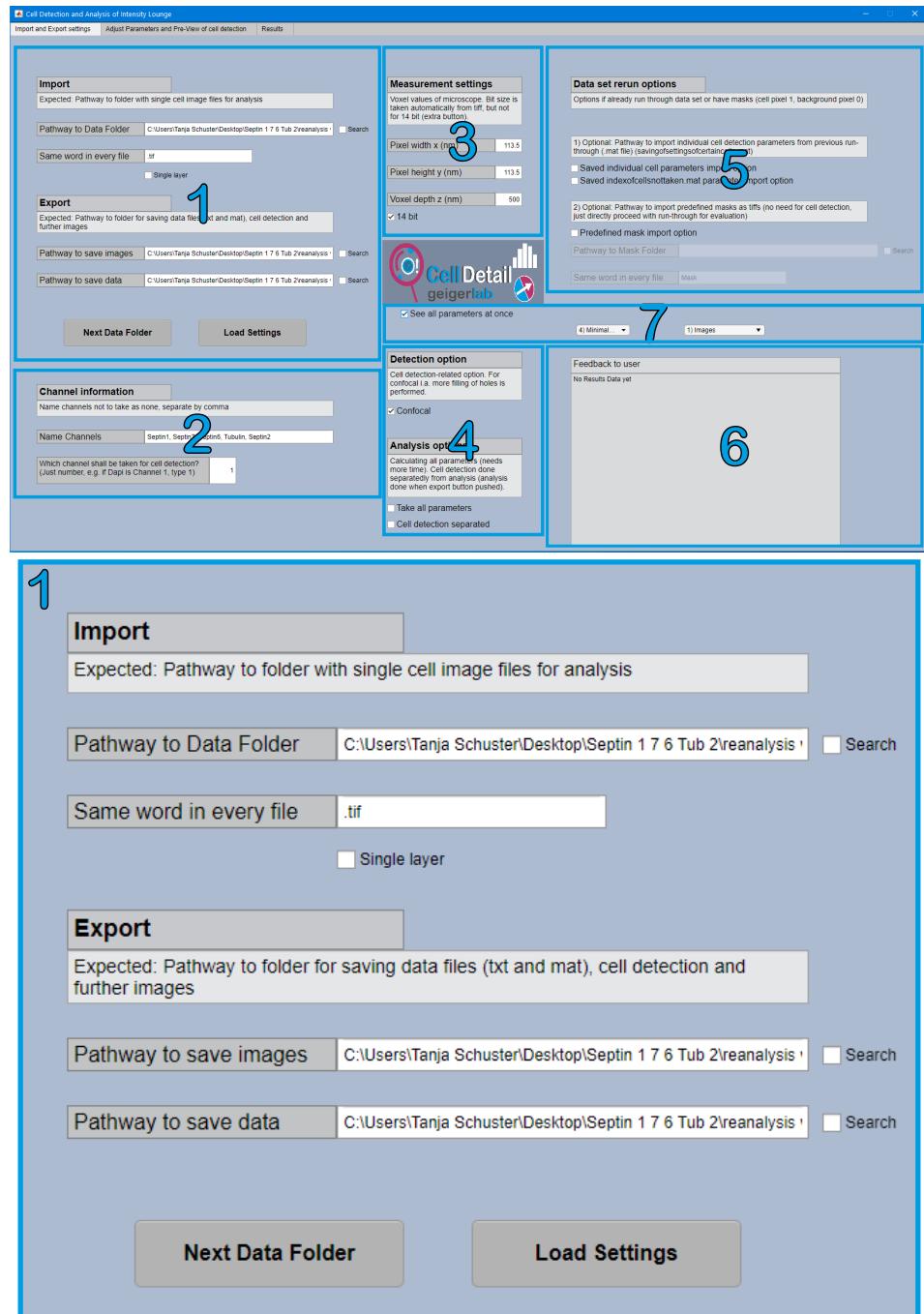


Figure 1.1: a) Overview over tab 1 of GUI CellDetail b) Overview over tab 1 panel 1 Import, Export and buttons

2

Channel information

Name channels not to take as none, separate by comma

Name Channels Septin1, Septin7, Septin6, Tubulin, Septin2

Which channel shall be taken for cell detection?
(Just number, e.g. if Dapi is Channel 1, type 1)

1

3

Measurement settings

Voxel values of microscope. Bit size is taken automatically from tiff, but not for 14 bit (extra button).

Pixel width x (nm) 113.5

Pixel height y (nm) 113.5

Voxel depth z (nm) 500

14 bit

4

Detection option

Cell detection-related option. For confocal i.a. more filling of holes is performed.

Confocal

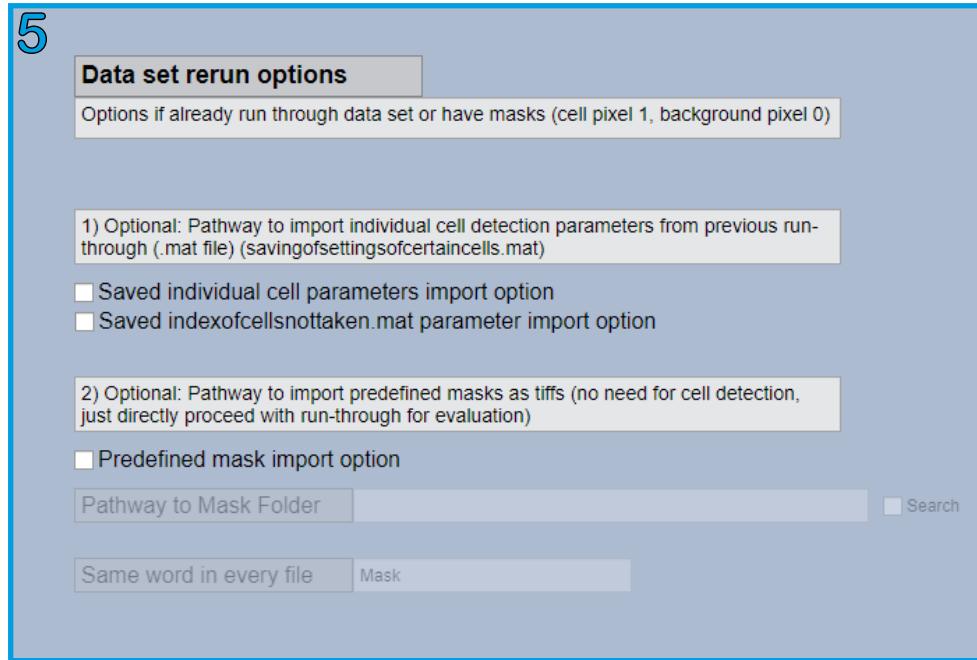
Analysis options

Calculating all parameters (needs more time). Cell detection done separately from analysis (analysis done when export button pushed).

Take all parameters

Cell detection separated

Figure 1.2: a) Overview over tab 1 panel 2 Channel settings b) Overview over tab 1 panel 3 Measurement settings c) Overview over tab 1 panel 4 Detection and analysis options



6

Feedback to user

No Results Data yet

7

See all parameters at once

4) Minimal... ▾

1) Images ▾

7

See all parameters at once

Back

Next

4) Minimal... ▾

1) Images ▾

Figure 1.3: a) Overview over tab 1 panel 5 Rerun settings for reanalysis b) Overview over tab 1 panel 6 Feedback field c) Overview over tab 1 panel 7 View options

Panel 1

Panel 1 of tab 1 asks for import information. The user needs to give the path of the Data Folder, in which the images to be analyzed are contained. This can be done either by copy and pasting the pathway or by checking the search button. For CellDetail receiving access to the files in the folder, the user has to choose a phrase which is contained in the file names of interest (“Same word in every file”, pre-set: “.tif”). From the import pathway in combination with the phrase to look for, already the image names and image pathway names are extracted as well as the cell number.

If single images are used instead of z-stacks, the single layer button needs to be checked as later in cell detection setting, a layer number of less/equal two is considered too low and further attempts are made for detecting cells.

Export information needs to be given by choosing folders in which to save resulting images (“Pathway to save images”) and analysis data (“Pathway to save data”). It is recommended not to choose the same pathway as the “Pathway to Data Folder” (import folder), to be sure that no complications occur during processing. Again, the checking of the search field at the right side gives the user the possibility to search for the folder in the system. Another possibility is copy/pasting the pathway of the corresponding folder.

When a data set was analyzed and the user switches to the next data set in a different folder, the user has to click the button “Next Data Folder”, as certain global variables need to be deleted to make clean space for the new data set. This involves variables saving the analysis values relating to polarity as well as the cells that are ultimately not taken. Images that are saved in the folder under “Pathway to save images” include - when “Save images inclusive mask (latter as tif-stack)” in tab 2 panel 6 is checked - the montage of masked channel images and the tif-stack of the mask.

“Load Settings” can be used for the .txt file made when clicking “Save Settings” button in tab 2. General parameters of cell detection as well as import pathway, phrase to find in files, export images pathway, export data pathway, pixel resolution x (nm), pixel resolution y (nm), voxel resolution z (nm), number of channels, channel(s) to ignore index, channel names, channel taken for cell detection, number of images, confocal option, normalization option, several channels for cell detection option as well as used channels for cell detection, weighting option and weights and time stamp are saved in the import folder as .txt file.

Panel 2

The user names the channels in tab 1 panel 2. It is important to list all channels, even the ones not of interest like brightfield channel, as the import makes use of the channel number. Channels, that shall not be analyzed, should be called “none”. The channels need to be separated by comma. By filling out this field, the naming and number of channels, the number of channels of interest as well as their order is deduced. If a channel is accidentally omitted, the wrongdoing can be seen in tab 2

as channel images are intermixed in the case of stacks being analyzed.

If cell detection shall be performed using a single channel only, the value of the channel needs to be placed after the field “Which channel shall be taken for cell detection?”. If cell detection shall be performed using multiple channels, this field can be ignored, as it will have no influence on the further cell detection when opting for multiple channels in tab 2 panel 2.

Panel 3

Panel 3 demands for information on measurement settings. Pixel width, height and (if in 3D) voxel depth values are asked for. When using 2D images, voxel depth should be set to 1 as charge density is calculated by dividing through the voxel volume. The pixel/voxel values provided by the user influence charge density, the absolute position of middle of volume M , of positive charge weighted mean position R_{pos} and of negative charge weighted mean position R_{neg} , thus the distance and dipole moment values. The relative values resulting by normalization are not changed. When importing tif, the bit number is automatically taken from the GUI. This value is used when opting for options 1 and 2 for charge normalization. As 14 bit is not offered by saving tiff images via ImageJ, and for holding the options 1 and 2 for charge normalization still open, the check button for 14 bit was inserted. Other bit values of tiff images are automatically recognized.

Panel 4

Panel 4 is structured into two main themes: “Detection option” and “Analysis options”. Detection option relates to the cell detection algorithm for which a difference between confocal (low voxel width/height values) and widefield (high voxel width/height values) is made for improving cell detection. Low voxel width/height values make it necessary to take filtering steps in order to fill in areas in which several cell voxels were not recognized.

When considering widefield images, the “Confocal” option can still be an improvement and can be used.

The “Analysis options” have two possible options: “Take all parameters” and “Cell detection separated”. When the “Take all parameters” option is checked, the positive charges above the mean of positive charges and their positions are further analyzed by converting voxel positions of the (x,y,z)-coordinate system into a spherical (ϕ , θ , r)-coordinate system. It was chosen to take the positive charges above the mean of positive charges, as for the confocal setting the inclusion of all positive charges for further analysis leads to not only protein voxels being taken in account but as well clear cytoplasm voxels and thus, taking the positive charges above the mean of positive charges is more precise. There exist two clustering methods: 1. Clusters are built based on histogram counts above average expected count. Their size and angle between them are analyzed. 2. Clusters out of the positive charge voxels with values above mean of positive charges are built based on the resulting nearness of positive charge voxels above mean of positive charges. Information about cluster size, angle and intensity is saved. When “Cell detection separated”

is checked, the cell detection is at first performed. The analysis follows later when pushing the export button. This facilitates user interaction with the GUI as calculation of all parameters can take time and thus single cell detection correction would become tedious with the waiting times in between.

Panel 5

The “Data set rerun options” are implemented for reanalysis of data, e.g. when one wants to try out another normalization method. Therefore, one can either use the parameter “savingofsettingsofcertaincells.mat”, which saves the individual cell detection parameters for each cell, and “indexofcellsnottaken.mat” (both are saved automatically when exporting .mat files). Another option is to have masks of cell voxels (with value 1) and background voxels (with value 0) corresponding to your tiff-images e.g. via the option “Save images inclusive mask (latter as tif-stack)” in tab 2 panel 6. The order of masks and tiffs within their folders must be the same. A check on the number of images and width and height is performed and acceptance is given as feedback in tab 1 panel 6. When using these options, the user does not need to look at cell detection any more. Thus, there won’t be a result table in tab 3 showing the results. Nonetheless, after inserting the necessary information in tab 1, press the “Start run” button in tab 2 and export afterwards your analysis data in tab 3 (“Export Data”).

Use the “Cell detection separated” button from tab 1 panel 4 and make a run-through in tab 2 panel 6 by clicking on “Start run”. After the fast run-through, go to tab 3, make a check for single cell detection and when accepted click “Export Data”. After giving a name, the calculation of the parameters starts and they are saved in the corresponding folders.

Panel 6

Field for giving response to the user.

Panel 7

Dependent on the design chosen, one can either see all parameter fields at once or in a certain order. For getting to the next parameter fields, one can either use the “Back” and “Next” button seen in panel 7 or click directly on the next panel to get visible.

1.3.2 Tab 2: Adjust Parameters and Pre-View of cell detection

Tab 2 shows the parameters for cell detection. Cell detection is performed by using Otsu’s method on range filtered images for layer selection and on plain image layers for cell voxel selection. The layer thresholding takes place before the cell voxel thresholding. Figure 1.4 shows tab 2 for “Basic Parameters” and tab 2 for “Additional Parameters” option in the subtabs. It is possible for the user to change parameters in every step of the general cell detection algorithm to make the software flexible for all kinds of image data and objects to be detected. Tab 2 can be visually separated

into 6 panels:

- Panel 1 with cell detection parameters. It is in itself separated into layer thresholding, cell voxel thresholding and general parameters.
- Panel 2 with options concerning which channels shall be used for detection and with which weighting, as well as viewing options: Just to see how thresholding process looks like, one checks “Program Run: Visible cell pre-view making?”. The according images appear in a separate window. If you just want to look whether the right layers are chosen with no further processing of thresholding, you check “Show just layer thresholding” and “Apply thresholding” and see the result in panel 5. When you want to see the result of all thresholding steps, just check “Apply thresholding” without further options (“Show just layer thresholding”, “Program Run: Visible cell pre-view making?”) selected and the result can be seen in panel 5.
- Panel 3 with the button “Save Settings”. This button helps in saving the general cell detection settings for reuse in other experiments. They are saved in a .txt file.
- Panel 4 as feedback field. When a cell object can't be generated due to settings like maximum number of voxels for each cell etc., a feedback will go out for the user.
- Panel 5 as resulting image field.
- Panel 6 as a control field for moving through the images and visual adaptations (e.g. for channel to be visualized,...) and options for analysis (normalization options, saving options, Pearson correlation coefficient) with the “Start run” button to start cell detection and/or analysis.

Panel 1

In panel 1 under subtab “Basic Parameters” the most often changed parameter fields can be seen. The parameter fields for “Layer thresholding” and “Cell pixel thresholding” can be approached by checking “Advanced”.

For “Layer thresholding”, the most often changed parameter for optimizing cell detection is “Minimum number of voxels per layer for Range filter” and “Maximum number of voxels per layer for Range filter”. As cells are non-homogenous objects, we make use of a range filter on the original images which gives out the maximal minus minimal intensity value in a specified neighborhood (9x9 pixels). For just background images, a low value of the range filter is expected, while for images

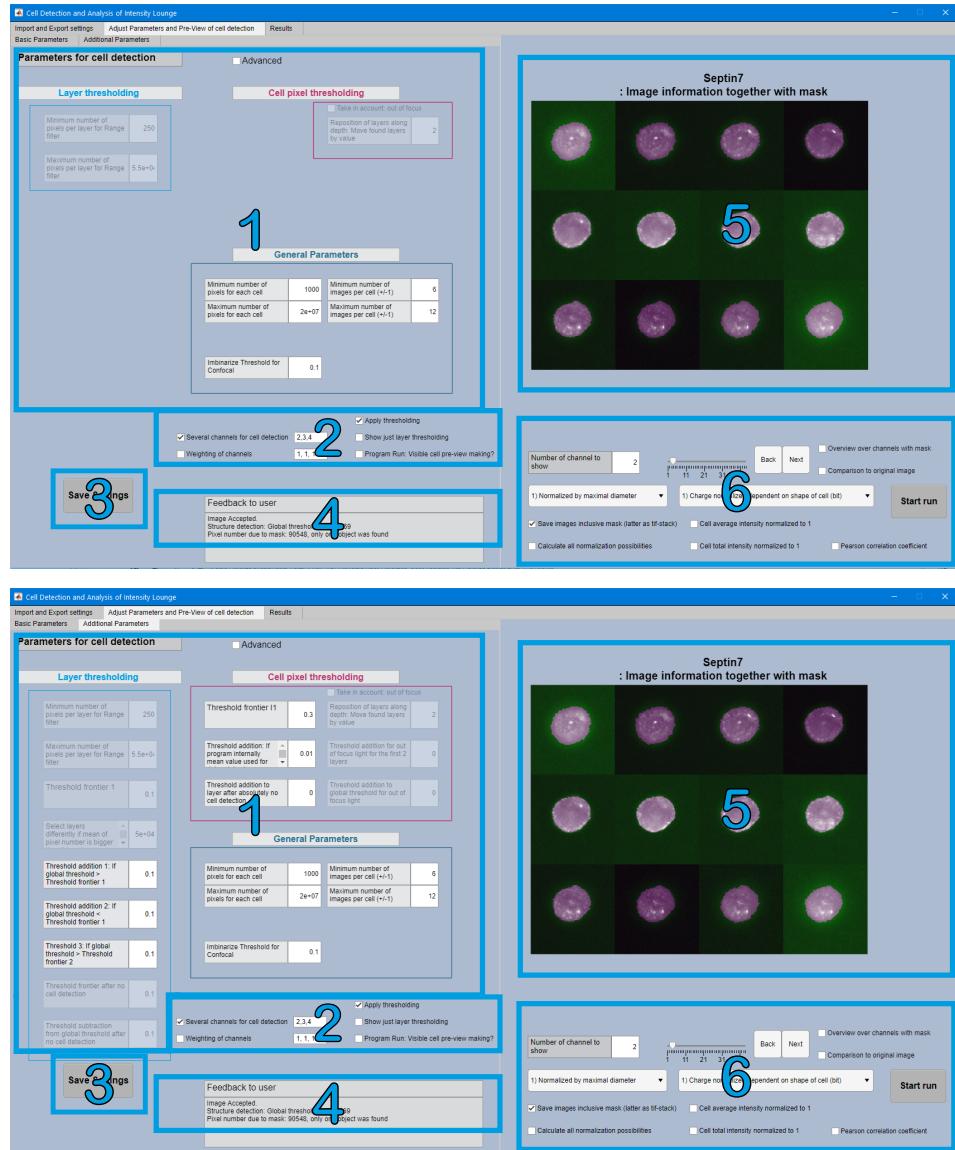


Figure 1.4: a) Overview over tab 2 with basic parameters b) Overview over tab 2 with advanced parameters (see change in panel 1)

with both cell voxels and background voxels, more variability and thus higher resulting values are expected. After the range filter, a threshold needs to be defined for separating pixels/voxels into cell pixels/voxels and background pixels/voxels. The threshold set on defining pixels/voxels as being cell voxels is made by using Otsu's threshold method on the histogram of range filtered images and adding a specified value ("Threshold addition 1: If global threshold > Threshold frontier 1", "Threshold addition 2: If global threshold < Threshold frontier 1", visible under subtab "Additional Parameters" under "Layer thresholding" parameters 5 and 6 of the row) to the threshold calculated by Otsu's method. Whether "Threshold addition 1" or "Threshold addition 2" is used depends on "Threshold frontier 1" and the threshold calculated by Otsu's method being smaller or bigger than "Threshold frontier 1".

For "Confocal" (tab 1, "Detection option") case as well as the widefield case (tab 1, "Detection option", unchecked "Confocal"), another comparison with a threshold takes place in order to get outlier cases for which due to previous parameter settings, no layers were detected.

The "Confocal" option uses "Select layers differently if mean of voxel number is bigger than this value: depending on being bigger than mean value rather than on minimum value and Minimum number of pixels per layer for Range filter". This means that when the mean number of voxels selected as cell voxels per layer (thanks to Otsu's threshold method with corresponding threshold addition decisions before) is bigger than the set value (pre-set: "5e+04") layers are selected for being cell layers when their number of defined cell voxels is above the mean value of defined cell voxels per layer.

The not-"Confocal" option (widefield) uses the parameters "Threshold frontier 2" and "Threshold 3: If global threshold > Threshold frontier 2". It takes a global threshold calculated by Otsu's method (calculated on histogram over all layers, not per layer) and compares it to "Threshold frontier 2". If the global threshold is bigger than "Threshold frontier 2", a new threshold is assigned for separating pixels/voxels as being cell pixels/voxels ("Threshold 3", pre-set "0.15"), otherwise the global threshold is used. Ranges of layers are taken which show a high enough cell voxel number. If the result is 0 layers and widefield was chosen (= "Confocal" option in tab 1 was not chosen), another approach is taken by applying an average filter of neighborhood 9x9 before the range filter step. Otsu's threshold method is performed on the resulting image. The resulting Otsu threshold is changed by subtraction according to it being above or below a certain threshold (certain threshold: "Threshold frontier after no cell detection", subtraction: "Threshold subtraction from global threshold after no cell detection").

If after all these steps no layer could be detected, the feedbackfield shows the text "No cell detected: Please try other values for setting Structure threshold".

Afterwards the separation is made between the cases one layer and more than one layer. If the "Single layer" option in tab 1 panel 1 was not checked, the longest episode of layers is looked for and repositioning of layers takes place (parameter

found under “Cell pixel thresholding” for the selected option “Take in account: out of focus”). Further limits of layer number are applied (“General Parameters” under “Minimum number of images per cell (+/-1)” and “Maximum number of images per cell (+/-1)”). Thus, the result of “Layer thresholding” is a range of selected layers, which go further to “Cell pixel thresholding”.

The same thresholding steps are made for both single and more layers for defining cell voxels vs. background voxels (Cell voxel thresholding):

For the widefield case the special handling of the first 2 layers was implemented due to light shining through from other layers while the interior of the cell was not reached in the layer. It can be activated by checking “Take in account: out of focus” (parameters taken into account when selected: “Reposition of layers along depth: Move found layers by value”, “Threshold addition for out of focus light for the first 2 layers”, “Threshold addition to global threshold for out of focus light”). Herefore, Otsu’s threshold method is used on all selected layers and single layers. By comparison of single layer Otsu threshold and global (across all layers) Otsu threshold with added “Threshold addition to global threshold for out of focus light” (together with the condition of the layers being below 3 and the option for taking out of focus light being selected (check of “Take in account: out of focus”)), the decision is made whether the resulting threshold is single layer Otsu’s derived threshold added to “Threshold addition for out of focus light for the first 2 layers” (yes, if single layer Otsu threshold is bigger than global layer Otsu threshold added to “Threshold addition to global threshold for out of focus light”). If single layer Otsu threshold is smaller than global layer Otsu threshold added to “Threshold addition to global threshold for out of focus light” for the first two layers, another comparison of single Otsu threshold being bigger than “Threshold frontier I1” is made. If it is bigger, then the resulting threshold is single layer Otsu’s threshold added to “Threshold addition 1: If layer threshold > Threshold frontier I1”. Otherwise, the resulting threshold is equal to single layer Otsu threshold. The part with Threshold frontier I1 comparison is always used for layer numbers above 2 and if “Take in account: out of focus” is not checked, it is used for the layers 1 and 2 as well. The number of cells voxels per layer is checked against the maximal and minimal allowed pixel/voxel numbers per layer (“General Parameters” with “Minimum number of pixels in layer” and “Maximum number of pixels in layer”). A layer is set to zero if it does not meet the criteria. If the number of valid layers is below 4, another method is used for defining cell layers. Dependent on the found valid layers from previous thresholding, single layer Otsu threshold is used with addition of 0.1 (for valid number of layers <4) or with addition of “Threshold addition to layer after absolutely no cell detection” (for valid number of layers == 0). If the resulting number of cell pixels/voxels is below the minimal number of pixels to detect for a cell or no valid layer was found, the threshold is set to global Otsu threshold + 0.1 (if valid layer number is below 4), to global Otsu threshold + 0.04 (if valid layer number is equal to 0) or layers below minimal number of pixels per layer and layers above maximal number of pixels per layer

are deleted (if valid layer number is above or equal 4). Afterwards, holes are filled and cell pixels/voxels are merged to a single object taking into account minimal and maximal pixel/voxel number allowed per cell.

For the confocal case, Otsu's threshold method is applied on all images. If for layer thresholding the method of having higher pixel/voxel number than mean pixel/voxel number per layer was used, the resulting threshold for splitting into cell pixel/voxel and background pixel/voxel is calculated by addition of "Threshold addition: If program internally mean value used for determining layers" to the global Otsu method threshold. If not, the global Otsu method threshold is taken. Afterwards, the images are filtered with a Gaussian filter of size 11x11 and the "Imbinarize Threshold for Confocal" is taken for final imbinarizing into cell pixel/voxels and background pixel/voxels.

Afterwards the holes of the cell mask are filled (with more operations on filling for the confocal case) and a mask object is made. The biggest mask object below the maximal voxel number is taken as a cell mask.

As can be seen, some threshold parameters can only be approached via the decision on the parameter "Confocal" in tab 1. Starting values are defined according to condition "Confocal" being checked or not. Several parameter names change upon selection of confocal:

- "not Confocal" / "Confocal"
- Threshold frontier 2 / Select layers differently if mean of voxel number is bigger than this value: depending on being bigger than mean value rather than on minimum value and Minimum number of voxels per layer for Range filter
- Threshold addition 1: If layer threshold > Threshold frontier I1 / Threshold addition: If program internally mean value used for determining layers
- Minimum number of pixels in layer / -
- Maximum number of pixels in layer / Imbinarize Threshold for Confocal

Panel 2

Panel 2 holds the controls on cell detection being carried out on not just a single channel, but selected channels. Therefore, the intensity values are added, and if wanted with a special weighting of channels (weighting done by multiplication). Without weighting, the values are - just for mask decision - adapted to the first imported channel's maximal value by changing the intensity ranges of the other channels. For visual information on the output of cell detection, the options "Apply

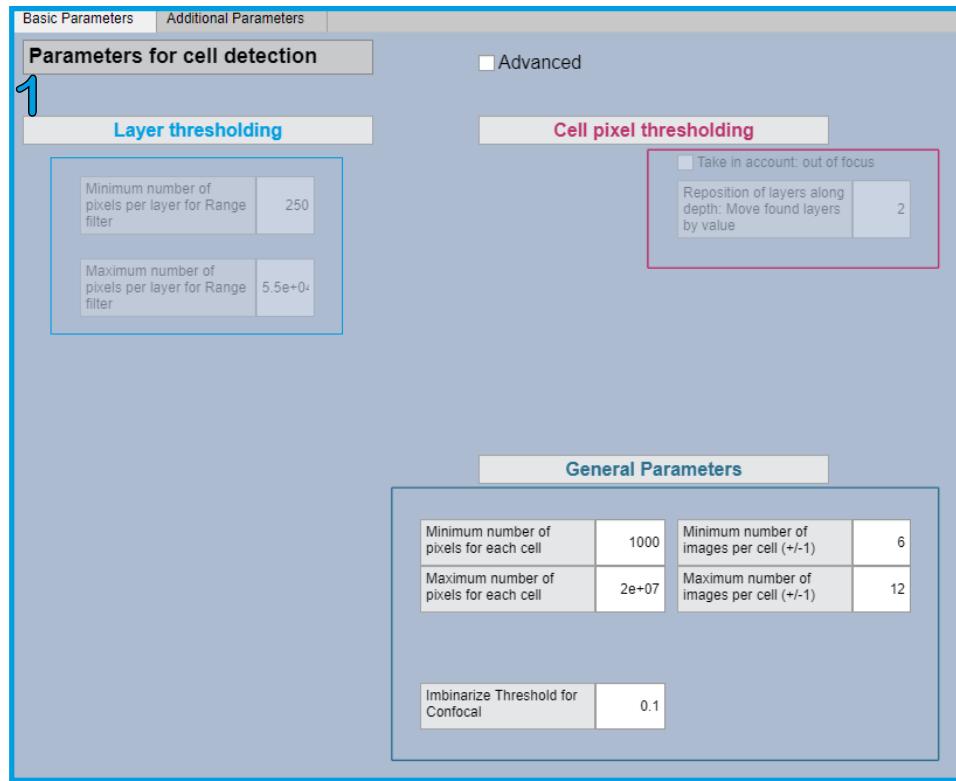


Figure 1.5: a) Overview over tab 2 panel 1 with “Basic Parameters”

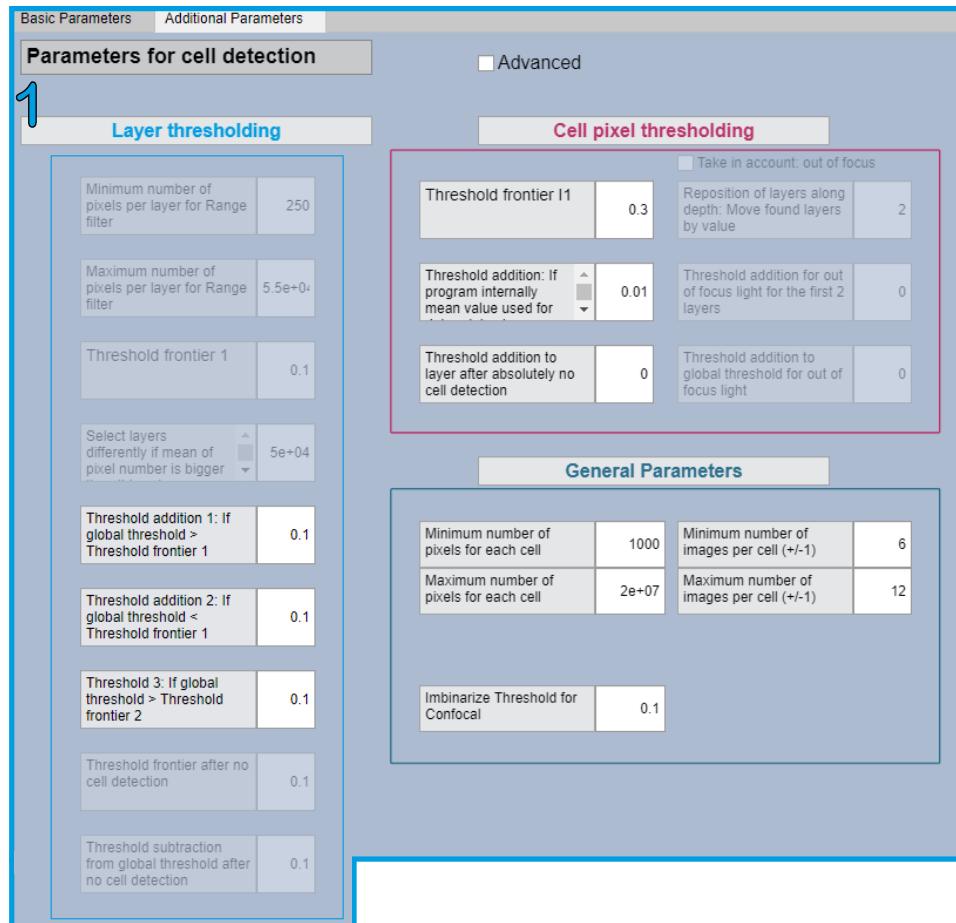


Figure 1.6: a) Overview over tab 2 panel 1 with “Advanced Parameters”

thresholding”, “Show just layer thresholding” and “Program Run: Visible cell pre-view making?” can be chosen.

When “Apply thresholding” is not selected, the imported images are shown as montages, no modification is applied. You can have a check whether the images were imported correctly (e.g. when a channel name is missing, the channels’ images are intermixed).

When you want to have a look at how the “Layer thresholding” (= cell layer selection) performs, you need to check both “Show just layer thresholding” and “Apply thresholding” buttons. When you want to see the outcome of all thresholding steps defining cell voxels vs. background voxels, you need to check “Apply thresholding”. When you want to have a look at the cell detection process, you can check “Program Run: Visible cell pre-view making?”. A separate window opens and shows the run-through of several thresholding steps per layer (1) left: original image, right: result of Range filter; 2) left: original image, right: result of thresholding Range filtered image (= result of “Layer thresholding”); 3) left: original image, right: result of “Cell pixel thresholding”, 4) left: original image, right: final mask multiplied with intensity values (= result of cell detection algorithm)). This can help in identifying bottle necks in the process for your cells’ detection e.g. whether improvements need to be made for “Layer thresholding” or for “Cell pixel thresholding”.

It is recommended to optimize the parameters in tab 2 panel 1 so that most cells are well detected as these parameters are in general used for cell detection in your data set. Afterwards in tab 3 there is the option to adapt cell detection for single cells.

Panel 3

The “Save Settings” option allows the saving of the cell detection threshold parameters in a .txt file in the import pathway, which can be loaded for other experiments in tab 1 panel 3 (“Load Settings”). The saved variables are:

- pathway to import data
- phrase found in data file names of files to be analyzed
- pathway to export images
- pathway to export data
- pixel/voxel size x
- pixel/voxel size y
- voxel size z
- number of channels
- number/ of which channel/s is/are ignored

- names of channels
- number of channel chosen for cell detection
- number of data files
- Option “Confocal”: checked or not
- Option “Advanced”: checked or not
- Option “Out of focus light”: checked or not
- Threshold frontier 1
- “Threshold frontier 2” / “Select layers differently if mean of voxel number is bigger than this value: depending on being bigger than mean value rather than on minimum value and Minimum number of voxels per layer for Range filter”
- “Threshold addition 1: If global threshold > Threshold frontier 1”
- “Threshold addition 2: If global threshold < Threshold frontier 1”
- “Threshold 3: If global threshold > Threshold frontier 2”
- “Threshold frontier after no cell detection”
- “Threshold subtraction from global threshold after no cell detection”
- “Minimum number of pixels per layer for Range filter”
- “Maximum number of pixels per layer for Range filter”
- “Threshold frontier I1”
- “Threshold addition 1: If layer threshold > Threshold frontier I1” / “Threshold addition: If program internally mean value used for determining layers”
- “Threshold addition to layer after absolutely no cell detection”
- “Threshold addition for out of focus light for the first 2 layers”
- “Threshold addition to global threshold for out of focus light”
- “Reposition of layers along depth: Move found layers by value”
- “Minimum number of pixels for each cell”
- “Maximum number of pixels for each cell”

- “Maximum number of images per cell (+/-1)”
- “Minimum number of images per cell (+/-1)”
- “Maximum number of pixels in layer” / “Imbinarize Threshold for Confocal”
- “Minimum number of pixels in layer”
- “Normalization option diameter”
- “Apply thresholding”
- “Show just layer thresholding”
- “Show pre-view making”
- “Comparison to original image”
- “Normalization option charge”
- Option “Several channels for cell detection”
- “Channel numbers for cell detection”
- Option “Weighting of channels”
- Weights of individual channels
- Option “14 bit”, Tab1 Panel3
- Option “Cell average intensity normalized to 1”
- Option “Cell total intensity normalized to 1”
- Option “Single layer”, Tab1 Panel1
- date and time

Panel 4

Field offering feedback to user. Feedback is made for facilitating thresholding part.

Panel 5 and Panel 6

Panel 5 is used as a visualization field for image data as well as for found cell layers and overlaid cell detection masks. The refreshing of the field needs the pushing of the buttons “Back” and “Next” in panel 6 or the modulation of the index on the image number list axis.

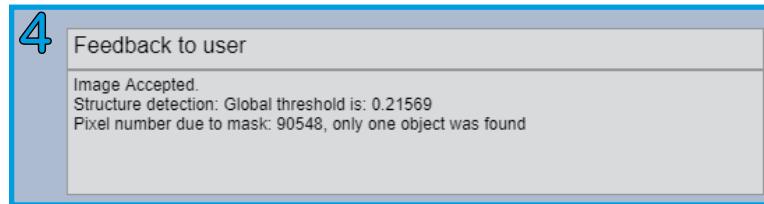
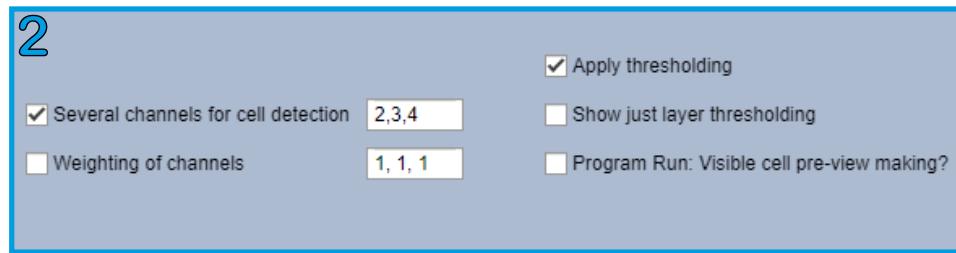


Figure 1.7: a) Overview over tab 2 panel 2 b) Overview over tab 2 panel 3 c)
Overview over tab 2 panel 4

The shown channel can be adapted by changing “Number of channel to show” to the number of channel you want to look at.

By checking “Overview over channels with mask” option an image containing all channels individually multiplied with the final cell mask as well as the addition of channels multiplied with the final cell mask is made which can be saved.

By checking “Comparison to original image” depending on the chosen options for applying threshold (just layer or all), either the smaller stack of selected layers (left) is shown together with the total image stack (right) or the selected layers with the cell detection mask (left) are shown against the selected layers without the cell detection mask (right).

Furthermore, panel 6 offers the saving of cell detection masks and individual channel montages with overlaid cell detection masks (“Save images inclusive mask (latter as tif-stack)”).

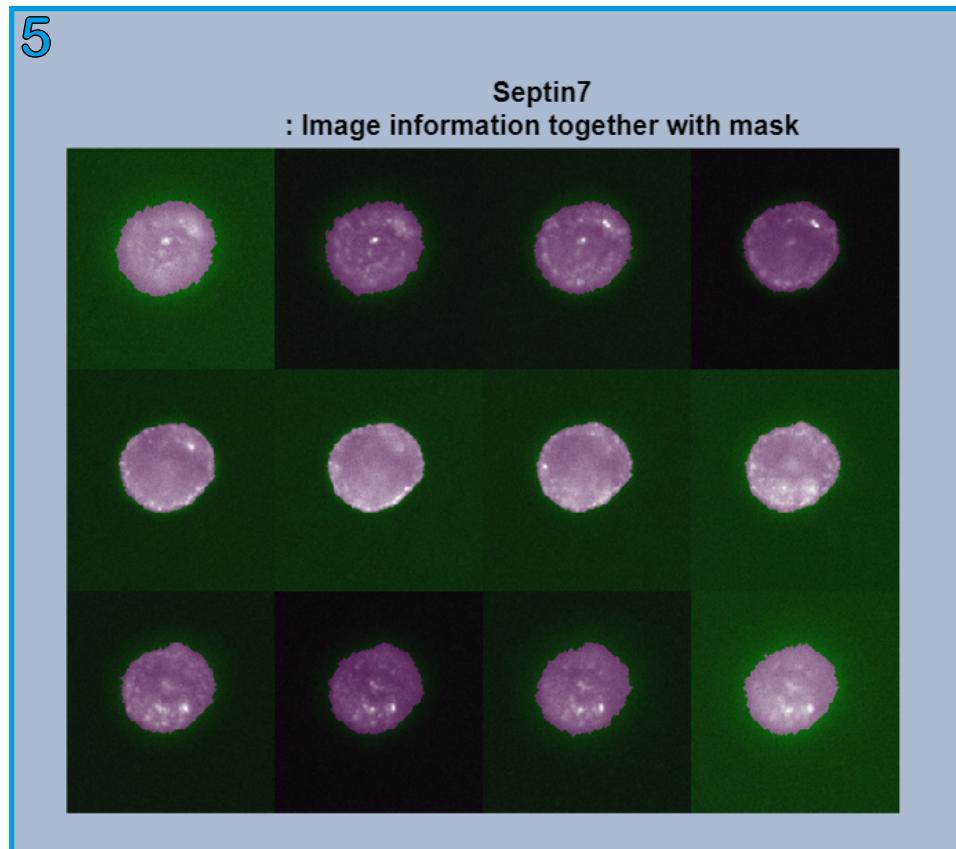
There are different normalization options. Either one can choose to have every possible normalization of diameter and charge calculated (“Calculate all normalization possibilities” or one can choose a single option (field with “1) Normalized by maximal diameter” can be switched to “2) Normalized by averaged diameter” and field with “1) Charge normalized dependent on shape of cell (bit)” offers all in all 6 options (“2) Charge normalized dependent on protein distribution of cell (bit)”, “3) Cell charge normalized corresponding to maximal value of charge multiplied by number of positive charges”, “4) Cell charge normalized corresponding to maximal value of charge multiplied by halved number of total charges”, “5) Cell charge normalized corresponding to mean value of charge multiplied by halved number of total charges”, “6) Own max for each cell (else: dependent on bit)).

Moreover, the intensity of channels can be normalized to increase comparability of cells via checking “Cell average intensity normalized to 1” (average component content the same among big and small cells) or “Cell total intensity normalized to 1” (total component content the same among big and small cells) options.

1.3.3 Tab 3: Results

Tab 3 shows the results of cell detection and analysis and offers the possibilities to optimize cell detection for single cells and to export analysis data (see Figure 1.9). Tab 3 can be visually separated in 6 panels:

- Panel 1 which shows the chosen cell layers together with overlaid cell detection mask and offers different possibilities on how to look at the selected cell images
- Panel 2 which shows the result table (in the case of stack-processing of first



6

Number of channel to show 2

1) Normalized by maximal diameter ▾ 1) Charge normalized dependent on shape of cell (bit) ▾

Save images inclusive mask (latter as tif-stack) Cell average intensity normalized to 1

Calculate all normalization possibilities Cell total intensity normalized to 1 Pearson correlation coefficient

Back Next Overview over channels with mask Comparison to original image

Start run

This panel contains various settings for image processing. It includes a dropdown menu for selecting the number of channels to show, which is currently set to 2. There are two dropdown menus for normalization methods: 'Normalized by maximal diameter' and 'Charge normalized dependent on shape of cell (bit)'. Several checkboxes are available for different processing options: 'Save images inclusive mask (latter as tif-stack)', 'Cell average intensity normalized to 1', 'Calculate all normalization possibilities', 'Cell total intensity normalized to 1', and 'Pearson correlation coefficient'. Navigation buttons 'Back' and 'Next' are present, along with checkboxes for 'Overview over channels with mask' and 'Comparison to original image'. A large 'Start run' button is located at the bottom right.

Figure 1.8: a) Overview over tab 2 panel 5 and tab 2 panel 6

cell mask generation of all cells and afterwards analysis of all cells a table appears telling whether a cell could be found (1) or not (0).

- Panel 3 for export of analysis with the possibility to export .mat files.
- Panel 4 as feedback field

Panel 1

Panel 1 shows the cell detection mask overlaid on the selected cell layers. Different channels can be chosen to be looked at. You can move through the images by either using “Back” and “Next” button or by grabbing the index and moving it. Options for showing cell images are the same as in tab 2 panel 5: A comparison between original images and cell detection images can be looked at (“Comparison”). If one just wants to look at the selected layers, one needs to check “Show just layer thresholding”. For having an overview over channels with their cell masks, the condition “Overview over channels with mask” needs to be checked. For getting an idea of which step still needs improvement in cell detection, the option “Program Run: Visible cell pre-view making?” needs to be checked.

Panel 2

Panel 2 contains a part of the results: The absolute value of normalized dipole moment (per channel), the absolute value of normalized distance (per channel), diameter, the normalized charge (per channel) and number of pixels/voxels (per channel). The values for the cell being looked at are marked in yellow.

Above the table several possibilities are positioned which help in working with the selected cell. When the cell detection was not good or out of other reasons you want to exclude a cell i.e. not export the data belonging to this cell, you need to check “Not to take” for that cell. The “Not to take” option will always be checked when this cell gets looked at and can be taken back by unchecking “Not to take”. Only when the export button “Export Data” gets pushed, the value will be excluded from the values being exported.

If the cell detection needs to be improved, the “Rerun” option can be checked. This opens a new, but familiar window (see Figure 1.13), for which all previously introduced cell detection parameters can be changed for the single cell.

Another option is the deletion of single values of a certain channel. This can be done for the selected cell (in yellow) by writing the number of channel, that shall be deleted, and afterwards checking “Not take channel nr.”. This will write NaN value to the chosen channel, which can’t be undone.

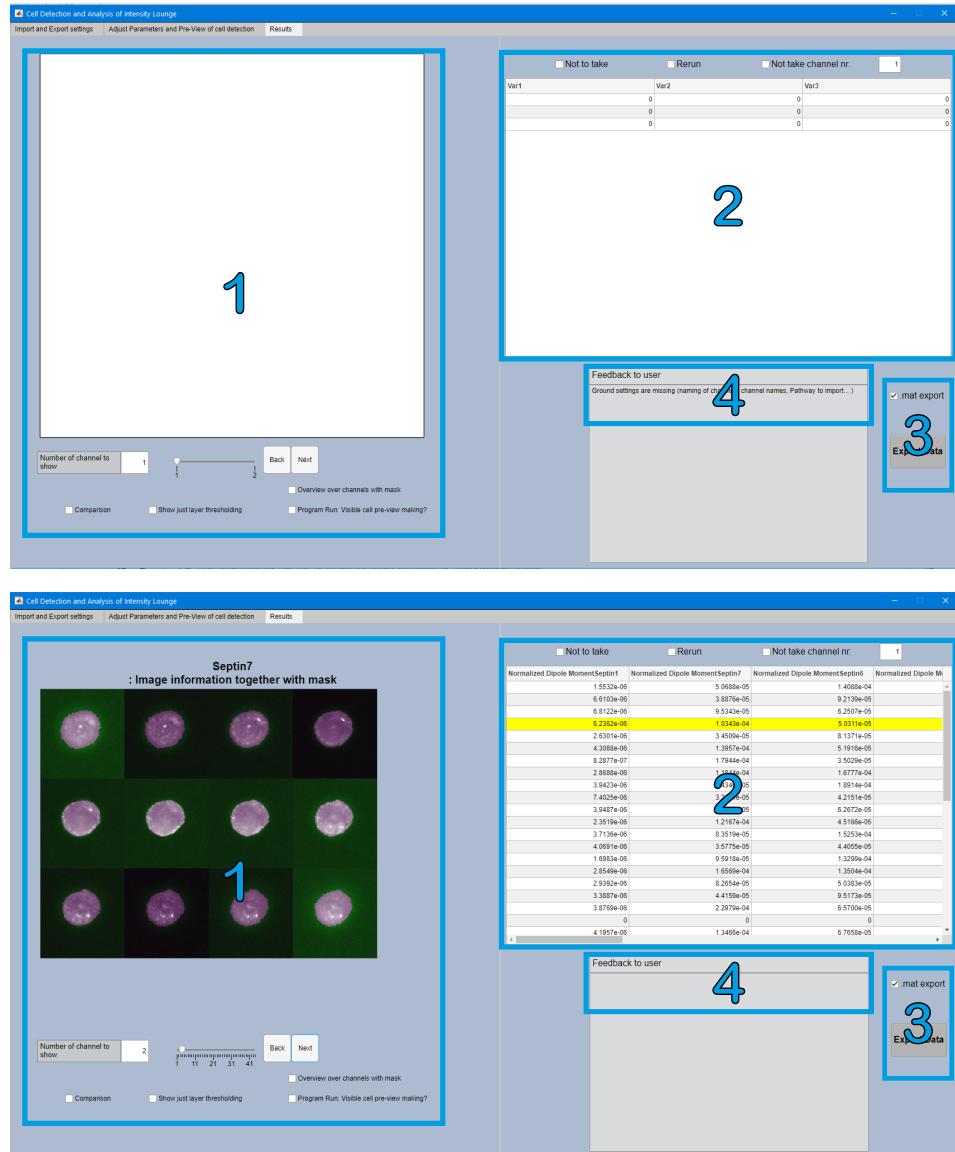


Figure 1.9: a) Overview over tab 3 before run-through b) Overview over tab 3 after run-through with analysis directly after cell detection resulting in filled table in panel 2

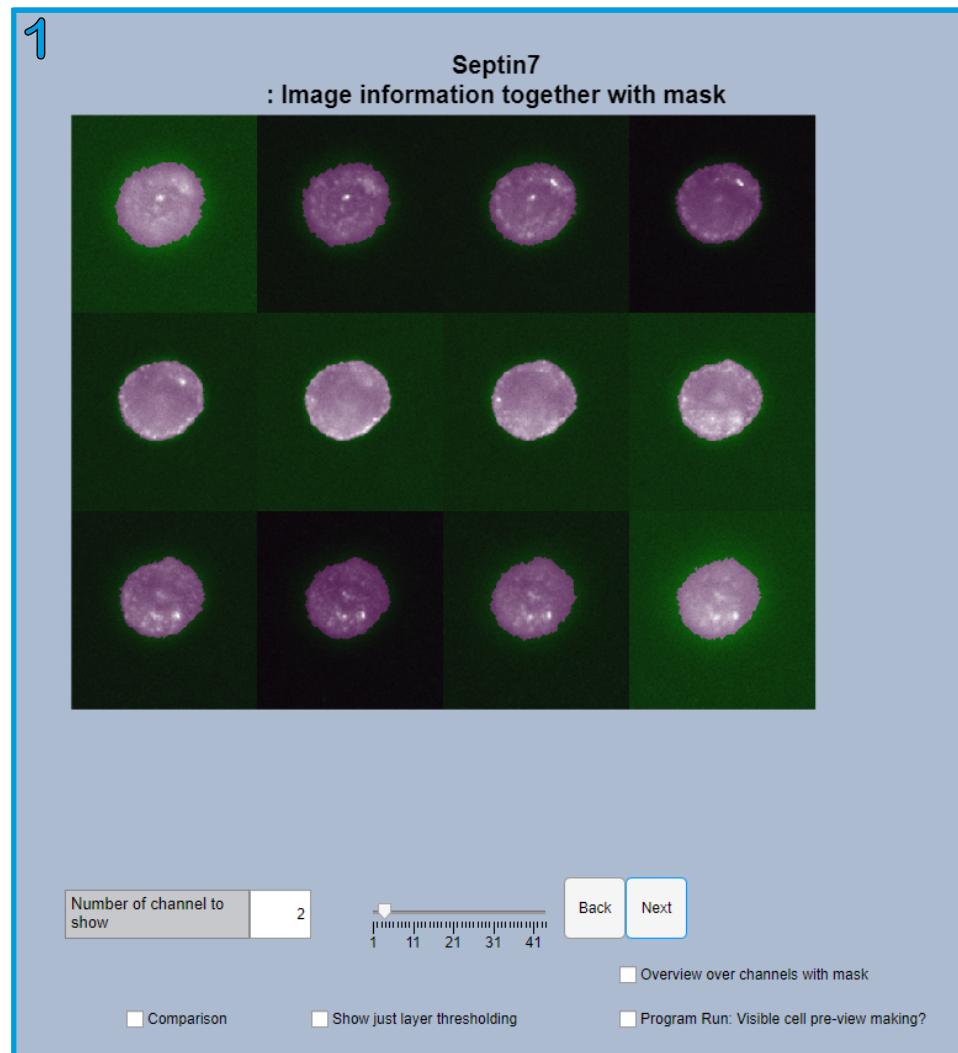


Figure 1.10: a) Overview over tab 3 panel 1

Panel 3

Panel 3 is responsible for the export of analyzed data. The check for “.mat export” allows the additional export of .mat variables which can be worked on e.g. in Matlab. By pressing the “Export Data” button, the export starts. The data is exported as .xls and .txt files to allow further processing of data with other programs. A window shows up asking to give a head name for the exported files. Variables are exported in single variable files and are not combined within a single file. The export happens in the folder chosen as data export pathway.

Panel 4

Panel 4 gives feedback to user, e.g. the time a run-through takes.

1.3.4 Rerun tab

The rerun tab shows up when the user wants to optimize cell detection and checks the “Rerun” option in tab 3 panel 2. Parameters of cell detection (already introduced in tab 2 panel 1) can be changed for a single cell (except for channel/s used for cell detection).

Pressing “Preview of Rerun” just shows a preview of the adapted cell detection result without changing any values. This option can be used to optimize cell detection before starting the recalculation.

The new parameters overwrite the former set ones when “Rerun (table values changed)” is pressed. This starts the recalculation with the modified cell detection mask and changes the table values inclusive all other analysis results.

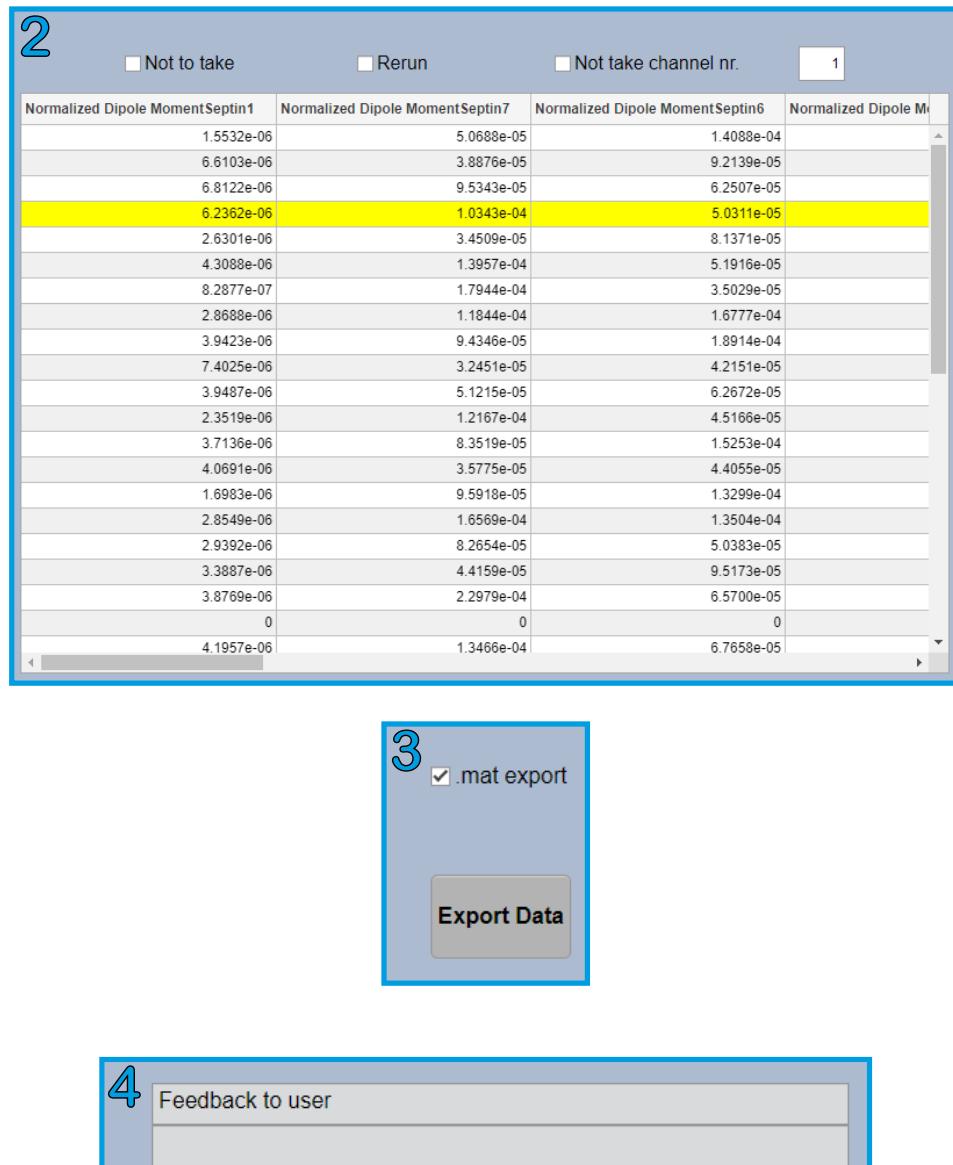


Figure 1.11: a) Overview over tab 3 panel 2 b) Overview over tab 3 panel 3 c) Overview over tab 3 panel 4

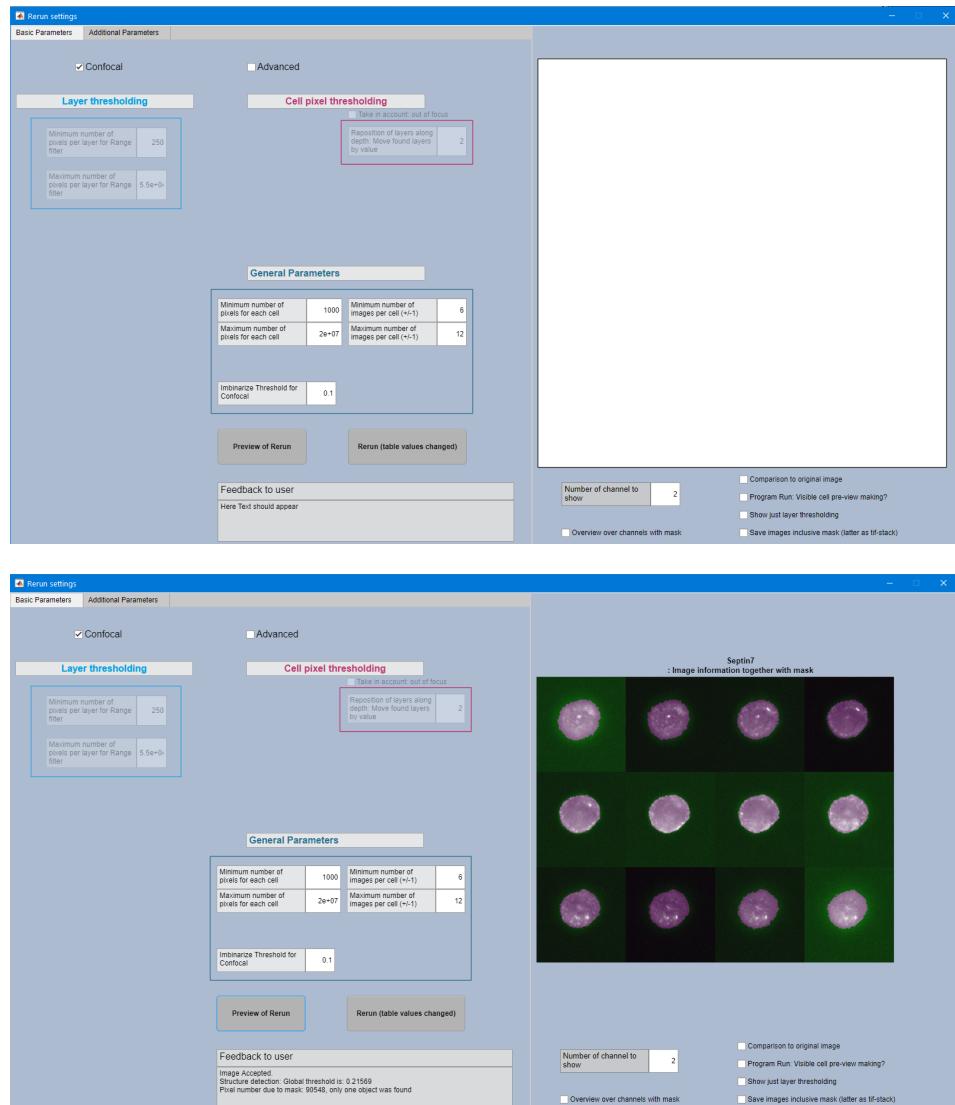


Figure 1.12: a) Overview over rerun tab opened after checking “Rerun” in tab 3 panel 2 b) Overview over rerun tab after clicking button “Preview of Rerun”

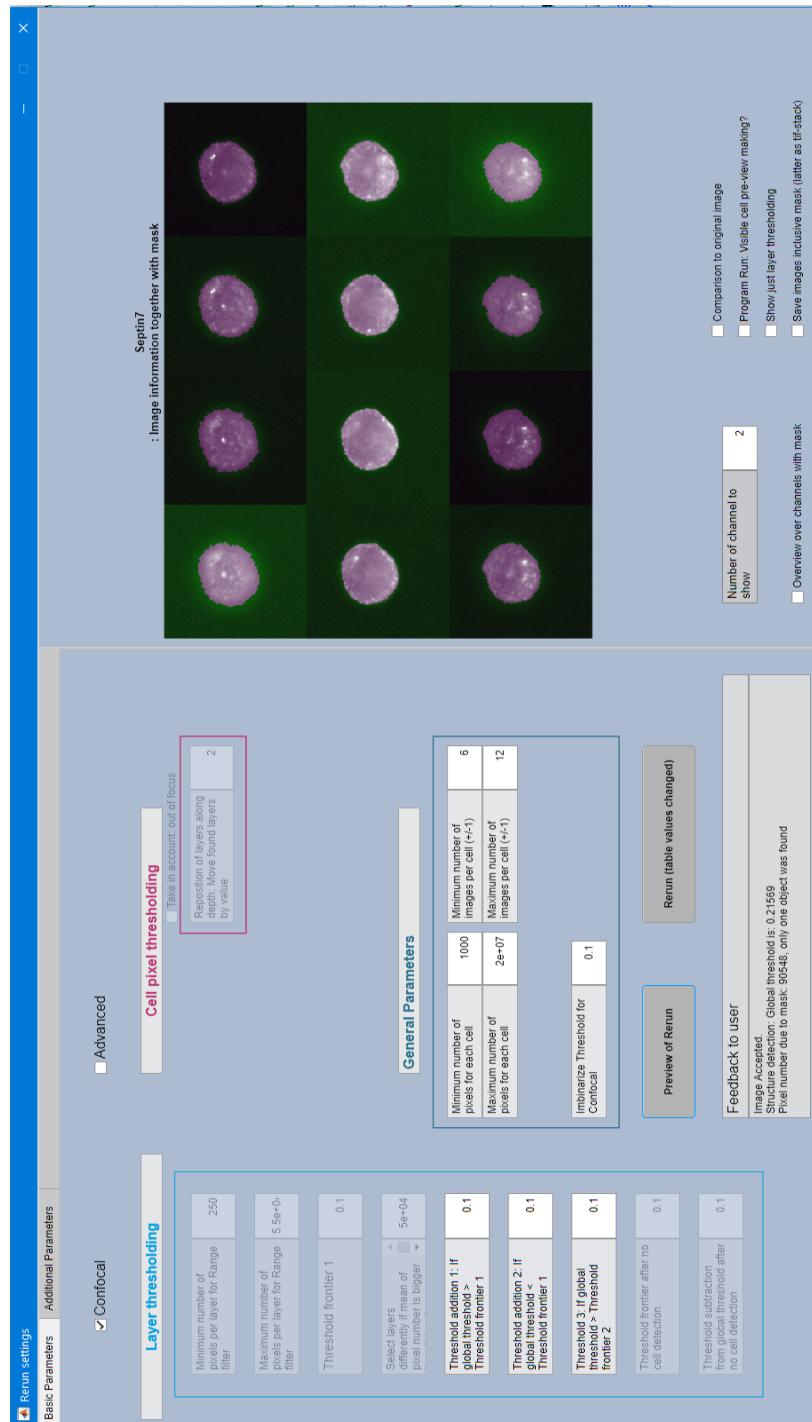


Figure 1.13: a) Overview over rerun tab with “Advanced Parameters” setting selected

1.4 Output

1.4.1 Important Parameters for Analysis

The general output of the software consists of the dipole moment vector \mathbf{P} , averaged and maximal diameter, the number of pixels/voxels related to volume, averaged intensity (adapted when normalization of total intensity to 1 and average intensity to 1 is chosen), positive charge density, positive pixel/voxel number, total intensity, the vectors between the negative charge weighted center \mathbf{R}_- and the middle of cell \mathbf{M} and the positive charge weighted center \mathbf{R}_+ . Next to it, further parameters are given as output (see Tab. 1.1).

Table 1.1: General output

Name	Description	Meaning
DipolMoment dipolmoment.mat	vector of dipole moment \mathbf{P} with x,y,z (a.u. nm)	possibility to check whether spatial distribution prefers sites at the top or bottom of a cell via z, or whether there are favored directions within a tissue, ...
AvDiameter diameteravvecfinished.mat	averaged cell diameter (nm)	can be used for determining inter-dependence of cell size with other properties
MaxDiameter diametermaxvecfinished.mat	maximal cell diameter (nm)	can be used for determining inter-dependence of cell size with other properties
NumberOfPixels numberofpixels.mat	number of cell pixels/voxels	related to volume of cell, can be multiplied by pixel/voxel volume for getting cell area/ volume
AveragedIntensityAUperPixel avintensity.mat	averaged intensity per pixel/voxel (a.u. / pixel(voxel)) adapted if possibilities “Cell average intensity normalized to 1” or “Cell total intensity normalized to 1” is checked	average component density including freely diffusing and cluster constructs (for no normalization being chosen)

Name	Description	Meaning
Chargedensitypos qposvec.mat	positive charge density, sum of individual positive charge density (charge divided by pixel/voxel volume, (a.u. / nm ²)/(a.u. / nm ³)	when divided by number of pixels/voxels, the average positive charge density is gained (corresponds to the average value of higher intensity pixel/voxel being above average intensity value and thus gives information about relative average component density at cluster sites), from this value and the number of positive pixels/voxels the averaged original intensity of just positive charge associated pixels/voxels can be calculated; as average intensity differs among cells (different component amount), this can lead to different outcome when comparing average positive intensity (cells can have in reality denser clusters of components, as they have more component content, but relative to average intensity, cells could form more efficiently component clusters; this behavior can be deciphered via averaged positive charge and averaged positive intensity)

Name	Description	Meaning
PositivePixelNumber numberofpixelspos.mat	number of positive charge pixels / voxels number of cell pixels/voxels with intensity values higher than average cell pixel/voxel intensity	related to spatial area/volume of higher intensity pixels/voxels
TotalIntensity PixelIntensitytotal.mat	sum over all cell pixel/voxel intensity values	related to total amount of component within cell, comparison parameter among cells for component amount; average intensity can be calculated via division by NumberOfPixels in the case of chosen normalization method when Averaged-IntensityAUperPixel parameter is modified
RnegRmean RnegRmean.mat	$\mathbf{R}_{\text{neg}} \mathbf{R}_{\text{mean}}$, vector between negative charge weighted center \mathbf{R}_{neg} and middle of cell \mathbf{M}	distance between negative charge weighted center and middle of cell can be calculated and set into relation to e.g. diameter (e.g. in DAPI channel how cytoplasm is positioned relative to nucleus) OR angle between different negative charge centers (via angle between component 1 $\mathbf{R}_{\text{neg}} \mathbf{R}_{\text{mean}}$ and component 2 $\mathbf{R}_{\text{neg}} \mathbf{R}_{\text{mean}}$) OR similarity between different negative charge centers (via distance between R_{neg} of component 1 and R_{neg} of component 2)

Name	Description	Meaning
RposRmean RposRmean.mat	$\mathbf{R}_{\text{pos}} \mathbf{R}_{\text{mean}}$, vector between positive charge weighted center \mathbf{R}_{pos} and middle of cell M	distance between positive charge weighted center and middle of cell can be calculated and set into relation to e.g. diameter (e.g. in protein channel how protein is positioned relative to non-protein areas/volume) OR angle between different positive charge weighted centers (via angle between component 1 $\mathbf{R}_{\text{pos}} \mathbf{R}_{\text{mean}}$ and component 2 $\mathbf{R}_{\text{pos}} \mathbf{R}_{\text{mean}}$; on same side or opposite side of cell) OR similarity between different negative charge centers (via distance between R_{pos} of component 1 and R_{pos} of component 2)
RposRneg RposRneg.mat	$\mathbf{R}_{\text{pos}} \mathbf{R}_{\text{neg}}$, vector between positive charge weighted center \mathbf{R}_{pos} and negative charge weighted center \mathbf{R}_{neg}	possible to calculate distance between positive and negative charge weighted center before normalization (absolute value of $\mathbf{R}_{\text{pos}} \mathbf{R}_{\text{neg}}$), can look for preference among cells in x,y,z direction (like for dipole moment P variable)
indexnottaken indexofcellsnottaken.mat	indices of cells that were not taken for final analysis (in third tab of CellDetail: clicked "Not to take")	parameter which can be used for rerun of analysis on same data set (Data set rerun option 1)
savingofsettingsofcertaincells.mat	cell detection parameters for individual taken cells	parameter which can be used for rerun of analysis on same data set (Data set rerun option 1)

Name	Description	Meaning
names Names.mat	file names taken from data import folder	file names of processed image files, come into order of processing
channelnames allchannelnames.mat	assigned channelnames	assigned names of channels of image files, order of channels
toignore toignore.mat	number/s of channel/s which is/are ignored for analysis	exclusion of analysis of e.g. brightfield channel is possible

Table 1.2: General output parameters. All normalizations (“Calculate all normalization possibilities” checked)

Name	Description and Meaning
NormalizedChargeOption1 qnormalized1.mat	normalized positive charge, normalized by max. possible charge related to half cell size and bit size Influence of cell size and bit size, thus unless normalization option “total to 1” or “average to 1” chosen, higher abundance of component leads to higher normalized charge and thus to higher value of absolute value of normalized dipole moment.
NormalizedChargeOption2 qnormalized2.mat	normalized positive charge, normalized by max. possible charge related to positive charge pixel/voxel number and bit size Influence of spatial distribution (positive charge pixel/voxel number) and bit size, thus unless normalization option “total to 1” or “average to 1” chosen, higher abundance of component leads to higher normalized charge and thus to higher value of absolute value of normalized dipole moment.
NormalizedChargeOption3 qnormalized3.mat	normalized positive charge, normalized by max. possible charge related to half cell size and maximal positive charge value per individual cell Influence of cell size and maximal value of positive charge cell pixel/voxel, thus influence of component cluster density distribution among positive charge voxels (positive charge density distribution), homogeneity vs. heterogeneity of clusters (more heterogeneous: lower resulting value, if more homogeneous: higher resulting value), important to check positive charge distribution variety among conditions; implemented for avoiding influence of component amount (which can be achieved by optioning for “total to 1” as well) by individual cell-derived positive charge maximum.

Name	Description and Meaning
NormalizedChargeOption4 qnormalized4.mat	normalized positive charge, normalized by max. possible charge related to positive charge pixel/voxel number and maximal positive charge value per individual cell Influence of spatial distribution (positive charge pixel/voxel number) and maximal value of positive charge cell pixel/voxel, thus influence of component cluster density distribution among positive charge voxels (positive charge density distribution), homogeneity vs. heterogeneity of clusters (more heterogeneous: lower resulting value, if more homogeneous: higher resulting value), important to check positive charge distribution variety among conditions; implemented for avoiding influence of component amount (which can be achieved by optioning for “total to 1” as well) by individual cell-derived positive charge maximum.
NormalizedChargeOption5 qnormalized5.mat	normalized positive charge, normalized by max. possible charge related to half cell size and mean positive charge value per individual cell Influence of cell size and mean value of positive charge cell pixel/voxel, thus influence of component cluster density distribution among positive charge voxels (positive charge density distribution), homogeneity vs. heterogeneity of clusters (more heterogeneous: lower resulting value, if more homogeneous: higher resulting value), important to check positive charge distribution variety among conditions; implemented for avoiding influence of component amount (which can be achieved by optioning for “total to 1” as well) by individual cell-derived positive charge mean. Mean value in order to avoid influence of high noise pixels/voxels.

Name	Description and Meaning
NormalizedChargeOption6 qnormalized6.mat	normalized positive charge, normalized by percentage of original intensity of positive charge pixels/voxels relative to total intensity As percentage to input intensity is used, there is no influence by component amount. Even cells with weak signal will get as high values as cells with strong signal as long as the spatial distribution is the same.

Name	Description and Meaning
NormalizedDipoleMoment11Option dipolmomentnormalized11.mat <small>NormalizedDipoleMomentMaxDiameterChargeOption 1</small>	absolute value of normalized dipole moment, normalized by max. diameter and max. possible charge related to half cell size and bit size; probability distribution of absolute value of normalized dipole moment helps in distinguishing the system to have one or several ground states of polarity, helps in distinguishing spatial distribution changes among different conditions as well as quantifying higher/lower reachable polarity values among different conditions, helps in correlation analysis related to spatial distribution/polarity. Influence of maximal diameter and maximal distance between charge weighted centers and thus influence of cell shape and positioning of charge centers like positioning of charge centers dependent on cell shape with charge centers always/more often/ at least one sitting at elongated protrusion. In these situations maximal diameter normalization option provides normalization to values between 0 and 1. Influence of cell size and bit size, thus unless normalization option “total to 1” or “average to 1” chosen, higher abundance of component leads to higher normalized charge and thus to higher value of absolute value of normalized dipole moment.

Name	Description and Meaning
NormalizedDipoleMoment12Option dipolmomentnormalized12.mat <small>NormalizedDipoleMomentMaxDiameterChargeOption 2</small>	absolute value of normalized dipole moment, normalized by max. diameter and max. possible charge related to positive charge pixel/voxel number and bit size; probability distribution of absolute value of normalized dipole moment helps in distinguishing the system to have one or several ground states of polarity, helps in distinguishing spatial distribution changes among different conditions as well as quantifying higher/lower reachable polarity values among different conditions, helps in correlation analysis related to spatial distribution/polarity. Influence of maximal diameter and maximal distance between charge weighted centers and thus influence of cell shape and positioning of charge centers like positioning of charge centers dependent on cell shape with charge centers always/more often/ at least one sitting at elongated protrusion. In these situations maximal diameter normalization option provides normalization to values between 0 and 1. Influence of spatial distribution (positive charge pixel/voxel number) and bit size, thus unless normalization option “total to 1” or “average to 1” chosen, higher abundance of component leads to higher normalized charge and thus to higher value of absolute value of normalized dipole moment.

Name	Description and Meaning
NormalizedDipoleMoment13Option dipolmomentnormalized13.mat <small>NormalizedDipoleMomentMaxDiameterChargeOption 3</small>	absolute value of normalized dipole moment, normalized by max. diameter and max. possible charge related to half cell size and maximal positive charge value per individual cell; probability distribution of absolute value of normalized dipole moment helps in distinguishing the system to have one or several ground states of polarity, helps in distinguishing spatial distribution changes among different conditions as well as quantifying higher/lower reachable polarity values among different conditions, helps in correlation analysis related to spatial distribution/polarity. Influence of maximal diameter and maximal distance between charge weighted centers and thus influence of cell shape and positioning of charge centers like positioning of charge centers dependent on cell shape with charge centers always/more often/ at least one sitting at elongated protrusion. In these situations maximal diameter normalization option provides normalization to values between 0 and 1. Influence of cell size and maximal value of positive charge cell pixel/voxel, thus influence of component cluster density distribution among positive charge voxels (positive charge density distribution), homogeneity vs. heterogeneity of clusters (more heterogeneous: lower resulting value, if more homogenous: higher resulting value), important to check positive charge distribution variety among conditions; implemented for avoiding influence of component amount (which can be achieved by optioning for “total to 1” as well) by individual cell-derived positive charge maximum.

Name	Description and Meaning
NormalizedDipoleMoment14Option dipolmomentnormalized14.mat <small>NormalizedDipoleMomentMaxDiameterChargeOption 4</small>	absolute value of normalized dipole moment, normalized by max. diameter and max. possible charge related to positive charge pixel/voxel number and maximal positive charge value per individual cell; probability distribution of absolute value of normalized dipole moment helps in distinguishing the system to have one or several ground states of polarity, helps in distinguishing spatial distribution changes among different conditions as well as quantifying higher/lower reachable polarity values among different conditions, helps in correlation analysis related to spatial distribution/polarity. Influence of maximal diameter and maximal distance between charge weighted centers and thus influence of cell shape and positioning of charge centers like positioning of charge centers dependent on cell shape with charge centers always/more often/at least one sitting at elongated protrusion. In these situations maximal diameter normalization option provides normalization to values between 0 and 1. Influence of spatial distribution (positive charge pixel/voxel number) and maximal value of positive charge cell pixel/voxel, thus influence of component cluster density distribution among positive charge voxels (positive charge density distribution), homogeneity vs. heterogeneity of clusters (more heterogeneous: lower resulting value, if more homogenous: higher resulting value), important to check positive charge distribution variety among conditions; implemented for avoiding influence of component amount (which can be achieved by optioning for “total to 1” as well) by individual cell-derived positive charge maximum.

Name	Description and Meaning
NormalizedDipoleMoment15Option dipolmomentnormalized15.mat <small>NormalizedDipoleMomentMaxDiameterChargeOption 5</small>	absolute value of normalized dipole moment, normalized by max. diameter and max. possible charge related to half cell size and mean positive charge value per individual cell; probability distribution of absolute value of normalized dipole moment helps in distinguishing the system to have one or several ground states of polarity, helps in distinguishing spatial distribution changes among different conditions as well as quantifying higher/lower reachable polarity values among different conditions, helps in correlation analysis related to spatial distribution/polarity. Influence of maximal diameter and maximal distance between charge weighted centers and thus influence of cell shape and positioning of charge centers like positioning of charge centers dependent on cell shape with charge centers always/more often/ at least one sitting at elongated protrusion. In these situations maximal diameter normalization option provides normalization to values between 0 and 1. Influence of cell size and mean value of positive charge cell pixel/voxel, thus influence of component cluster density distribution among positive charge voxels (positive charge density distribution), homogeneity vs. heterogeneity of clusters (more heterogeneous: lower resulting value, if more homogenous: higher resulting value), important to check positive charge distribution variety among conditions; implemented for avoiding influence of component amount (which can be achieved by optioning for “total to 1” as well) by individual cell-derived positive charge mean. Mean value in order to avoid influence of high noise pixels/voxels.

Name	Description and Meaning
NormalizedDipoleMoment16Option dipolmomentnormalized16.mat <small>NormalizedDipoleMomentMaxDiameterChargeOption 6</small>	absolute value of normalized dipole moment, normalized by max. diameter and max. possible charge related to percentage of original intensity of positive charge pixels/voxels relative to total intensity; probability distribution of absolute value of normalized dipole moment helps in distinguishing the system to have one or several ground states of polarity, helps in distinguishing spatial distribution changes among different conditions as well as quantifying higher/lower reachable polarity values among different conditions, helps in correlation analysis related to spatial distribution/polarity. Influence of maximal diameter and maximal distance between charge weighted centers and thus influence of cell shape and positioning of charge centers like positioning of charge centers dependent on cell shape with charge centers always/more often/ at least one sitting at elongated protrusion. In these situations maximal diameter normalization option provides normalization to values between 0 and 1. As percentage to input intensity is used, there is no influence by component amount. Even cells with weak signal will get as high values as cells with strong signal as long as the spatial distribution is the same.

Name	Description and Meaning
NormalizedDipoleMoment21Option dipolmomentnormalized21.mat <small>NormalizedDipoleMomentAvDiameterChargeOption 1</small>	absolute value of normalized dipole moment, normalized by averaged diameter and max. possible charge related to half cell size and bit size; probability distribution of absolute value of normalized dipole moment helps in distinguishing the system to have one or several ground states of polarity, helps in distinguishing spatial distribution changes among different conditions as well as quantifying higher/lower reachable polarity values among different conditions, helps in correlation analysis related to spatial distribution/polarity. Influence of averaged diameter and maximal distance between charge weighted centers and thus influence of cell shape and positioning of charge centers: When positioning of charge centers not dependent on cell shape with charge centers not sitting at elongated protrusion: In these situations averaged diameter normalization option can be used to get independent of elongated protrusion/cell shape influence. Influence of cell size and bit size, thus unless normalization option "total to 1" or "average to 1" chosen, higher abundance of component leads to higher normalized charge and thus to higher value of absolute value of normalized dipole moment.

Name	Description and Meaning
NormalizedDipoleMoment22Option dipolmomentnormalized22.mat <small>NormalizedDipoleMomentAvDiameterChargeOption 2</small>	absolute value of normalized dipole moment, normalized by averaged diameter and max. possible charge related to positive charge pixel/voxel number and bit size; probability distribution of absolute value of normalized dipole moment helps in distinguishing the system to have one or several ground states of polarity, helps in distinguishing spatial distribution changes among different conditions as well as quantifying higher/lower reachable polarity values among different conditions, helps in correlation analysis related to spatial distribution/polarity. Influence of averaged diameter and maximal distance between charge weighted centers and thus influence of cell shape and positioning of charge centers: When positioning of charge centers not dependent on cell shape with charge centers not sitting at elongated protrusion: In these situations averaged diameter normalization option can be used to get independent of elongated protrusion/cell shape influence. Influence of spatial distribution (positive charge pixel/voxel number) and bit size, thus unless normalization option "total to 1" or "average to 1" chosen, higher abundance of component leads to higher normalized charge and thus to higher value of absolute value of normalized dipole moment.

Name	Description and Meaning
NormalizedDipoleMoment23Option dipolmomentnormalized23.mat <small>NormalizedDipoleMomentAvDiameterChargeOption 3</small>	absolute value of normalized dipole moment, normalized by averaged diameter and max. possible charge related to half cell size and maximal positive charge value per individual cell; probability distribution of absolute value of normalized dipole moment helps in distinguishing the system to have one or several ground states of polarity, helps in distinguishing spatial distribution changes among different conditions as well as quantifying higher/lower reachable polarity values among different conditions, helps in correlation analysis related to spatial distribution/polarity. Influence of averaged diameter and maximal distance between charge weighted centers and thus influence of cell shape and positioning of charge centers: When positioning of charge centers not dependent on cell shape with charge centers not sitting at elongated protrusion: In these situations averaged diameter normalization option can be used to get independent of elongated protrusion/cell shape influence. Influence of cell size and maximal value of positive charge cell pixel/voxel, thus influence of component cluster density distribution among positive charge voxels (positive charge density distribution), homogeneity vs. heterogeneity of clusters (more heterogeneous: lower resulting value, if more homogenous: higher resulting value), important to check positive charge distribution variety among conditions; implemented for avoiding influence of component amount (which can be achieved by optioning for "total to 1" as well) by individual cell-derived positive charge maximum.

Name	Description and Meaning
NormalizedDipoleMoment24Option dipolmomentnormalized24.mat <small>NormalizedDipoleMomentAvDiameterChargeOption 4</small>	absolute value of normalized dipole moment, normalized by averaged diameter and max. possible charge related to positive charge pixel/voxel number and maximal positive charge value per individual cell; probability distribution of absolute value of normalized dipole moment helps in distinguishing the system to have one or several ground states of polarity, helps in distinguishing spatial distribution changes among different conditions as well as quantifying higher/lower reachable polarity values among different conditions, helps in correlation analysis related to spatial distribution/polarity. Influence of averaged diameter and maximal distance between charge weighted centers and thus influence of cell shape and positioning of charge centers: When positioning of charge centers not dependent on cell shape with charge centers not sitting at elongated protrusion: In these situations averaged diameter normalization option can be used to get independent of elongated protrusion/cell shape influence. Influence of spatial distribution (positive charge pixel/voxel number) and maximal value of positive charge cell pixel/voxel, thus influence of component cluster density distribution among positive charge voxels (positive charge density distribution), homogeneity vs. heterogeneity of clusters (more heterogeneous: lower resulting value, if more homogenous: higher resulting value), important to check positive charge distribution variety among conditions; implemented for avoiding influence of component amount (which can be achieved by optioning for "total to 1" as well) by individual cell-derived positive charge maximum.

Name	Description and Meaning
NormalizedDipoleMoment25Option dipolmomentnormalized25.mat <small>NormalizedDipoleMomentAvDiameterChargeOption 5</small>	absolute value of normalized dipole moment, normalized by averaged diameter and max. possible charge related to half cell size and mean positive charge value per individual cell; probability distribution of absolute value of normalized dipole moment helps in distinguishing the system to have one or several ground states of polarity, helps in distinguishing spatial distribution changes among different conditions as well as quantifying higher/lower reachable polarity values among different conditions, helps in correlation analysis related to spatial distribution/polarity. Influence of averaged diameter and maximal distance between charge weighted centers and thus influence of cell shape and positioning of charge centers: When positioning of charge centers not dependent on cell shape with charge centers not sitting at elongated protrusion: In these situations averaged diameter normalization option can be used to get independent of elongated protrusion/cell shape influence. Influence of cell size and mean value of positive charge cell pixel/voxel, thus influence of component cluster density distribution among positive charge voxels (positive charge density distribution), homogeneity vs. heterogeneity of clusters (more heterogeneous: lower resulting value, if more homogenous: higher resulting value), important to check positive charge distribution variety among conditions; implemented for avoiding influence of component amount (which can be achieved by optioning for "total to 1" as well) by individual cell-derived positive charge mean. Mean value in order to avoid influence of high noise pixels/voxels.

Name	Description and Meaning
NormalizedDipoleMoment26Option dipolmomentnormalized26.mat <small>NormalizedDipoleMomentAvDiameterChargeOption 6</small>	absolute value of normalized dipole moment, normalized by averaged diameter and max. possible charge related to percentage of original intensity of positive charge pixels/voxels relative to total intensity; probability distribution of absolute value of normalized dipole moment helps in distinguishing the system to have one or several ground states of polarity, helps in distinguishing spatial distribution changes among different conditions as well as quantifying higher/lower reachable polarity values among different conditions, helps in correlation analysis related to spatial distribution/polarity. Influence of averaged diameter and maximal distance between charge weighted centers and thus influence of cell shape and positioning of charge centers: When positioning of charge centers not dependent on cell shape with charge centers not sitting at elongated protrusion: In these situations averaged diameter normalization option can be used to get independent of elongated protrusion/cell shape influence. As percentage to input intensity is used, there is no influence by component amount. Even cells with weak signal will get as high values as cells with strong signal as long as the spatial distribution is the same.

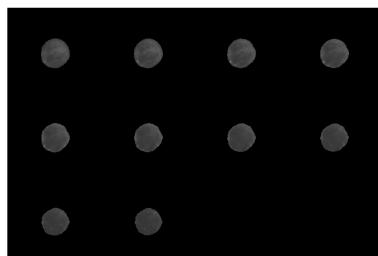
Name	Description and Meaning
NormalizedDistanceAvDiameter Rnormalized_finished2.mat	normalized distance between positive and negative charge center (R_{pos} , R_{neg}), normalized by averaged diameter of cell. Influence of averaged diameter and distance between charge weighted centers and thus influence of cell shape and positioning of charge centers: When positioning of charge centers not dependent on cell shape with charge centers not sitting at elongated protrusion: In these situations averaged diameter normalization option can be used to get independent of elongated protrusion/cell shape influence.
NormalizedDistanceMaxDiameter Rnormalized_finished1.mat	normalized distance between positive and negative charge center (R_{pos} , R_{neg}), normalized by max. diameter of cell. Influence of maximal diameter and maximal distance between charge weighted centers and thus influence of cell shape and positioning of charge centers like positioning of charge centers dependent on cell shape with charge centers always/more often/ at least one sitting at elongated protrusion. In these situations maximal diameter normalization option provides normalization to values between 0 and 1.

Name	Description and Meaning
Pearsoncorrelationcoefficient Pearsoncorrelationcoefficient.mat	Pearson correlation coefficient = $\frac{\sum_i(x_i - \langle x \rangle) \cdot (y_i - \langle y \rangle)}{(\sum_i(x_i - \langle x \rangle)^2 \cdot \sum_i(y_i - \langle y \rangle)^2)^{0.5}}$, with $\langle x \rangle$ and $\langle y \rangle$ being the mean value of pixel/voxel intensities of a channel, measure of linear correlation ranging from -1 to 1 with 1/-1 describing a linear relationship between x and y (in this case: channel intensity values), for the outcome 1 positive linear correlation, for outcome -1 decreasing regression slope of linear correlation (y decreasing with increasing x), for outcome 0 no linear dependency

Name	Description and Meaning
displacementDipoleMomentfromMiddle displacementdipolfromM.mat	if distribution of component within cell more confined in one half, detectable; for apolar: random values possible; displacementDipoleMomentfromMiddle.mat, (number of images, number of channels)
displacementRposRnegMRposMRneg displacementRposRnegtoMorthogonal.mat	if cell distribution like one half neutral, other half positive and negative charge: detectable; thus, whether positive and negative charge center are directly opposite (which would be more natural for a concentration increase/decrease) or whether more constricted gradient in cell space; for apolar: random values possible, displacement $\delta = \sin(\text{angle}(\overrightarrow{\text{MR}_+}, \overrightarrow{\text{R}_+}, \overrightarrow{\text{R}_-}))$, displacementRposRnegMRposMRneg.mat (number of images, number of channels)
Name	Description and Meaning
sumofdistancesqRpos distanceplusqstoRpos.mat	distance of positive charge positions to R_+ ; needs to be divided by number of positive charge positions to get average for comparison!, if higher value: positive charges related to positive charge weighted center R_+ further away, sumofdistancesqRpos.mat (number of channels, number of images)

1

Original



Cluster situation



Cluster Method:

voxels above av. value of pos. charge voxels
morphological opening (erosion + dilation)
only clusters with more than 50 pixels/voxels taken

parameters:

meanintensity (mean intensity per single cluster)

maxintensity (max intensity per single cluster)

volumelist (number of pixels/voxels per cluster)

voxellistlist (only as .mat file, indices of pixels/voxels belonging to single clusters)

voxelvalueslist (only as .mat file, values of pixels/voxels belonging to single clusters)

centroid position (bary center position of each cluster (x,y,z) in pixel/voxel number)

weightedcentroid (intensity weighted bary center position of each cluster (x,y,z) in pixel/voxel number)

equiv. diameter (diameter in pixel/voxel number, caution if not isotropic pixel/voxel size!)

surfacearea (surface area of each cluster in pixel/voxel, caution if not isotropic pixel/voxel size!)

arrayfixed4distancelist (array of ranked distance of single cluster to strongest cluster (by angle of bary center (just x,y direction)), $\leq 45^\circ$: 1.column, $45^\circ < x \leq 90^\circ$: 2.column, $90^\circ < x \leq 135^\circ$: 3.column, $135^\circ < x \leq 180^\circ$: 4.column, number of clusters of ranks given)

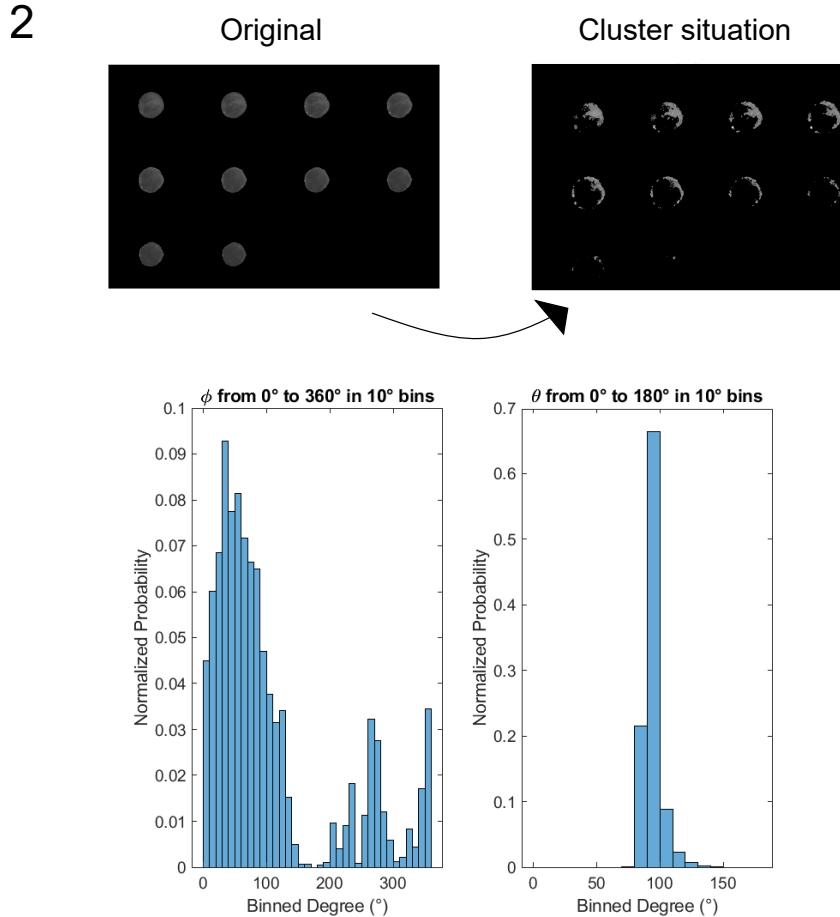
arrayfixed5strengthlist (array of ranked strength of single cluster (by cluster percent), ≥ 0.6 : 1.column, $0.4 \leq x < 0.6$: 2.column, $0.2 \leq x < 0.4$: 3.column, $0.1 \leq x < 0.2$: 4.column, $0 \leq x < 0.1$: 5.column, number of clusters of ranks given)

sorted_array_tofilldistancelist (strength cluster ranks, sorted after descending from biggest/strongest cluster; same order of cluster indices as sorted_array_tofillstrengthlist)

sorted_array_tofillstrengthlist (distance cluster ranks, sorted after descending from biggest/strongest cluster; same order of cluster indices as sorted_array_tofilldistancelist)

distancestrengthlist (combination of distance and strength rank information; sorted_array_tofilldistancelist separated from sorted_array_tofillstrengthlist by 0, ends with 0; information whether bigger cluster on one side / opposite sides, whether mainly same size, ...)

Figure 1.14: Cluster methods ("All parameters taken" checked). 1) Clustering by neighbor pixels/voxels of positive charge pixels/voxels above average value of positive charge pixels/voxels.



Cluster Method:

voxels above av. value of pos. charge voxels
 clustering by moving to spherical coordinates: r, theta, phi
 phi: 0-360° (saved in "phideg" "numberofimage" "channel" "channelnumber".mat")
 theta: 0-180° (saved in "thetadeg" "numberofimage" "channel" "channelnumber".mat")
 above average expected percentage in angle histogram : these angles and voxels
 are taken in account for further clustering, under the condition that angle bins are next
 to each other, the angles and voxels are connected to clusters

Figure 1.15: Cluster methods ("All parameters taken" checked). 2) Clustering by transfer of positive charge pixels/voxels above average value of positive charge pixels/voxels to spherical coordinates and histogram angles. Clustering of pixels/voxels according to angle bins above random percentage value.

2

parameters:

general cluster information

countessphi/theta (number of bins above expected random percentage value)
percoverphi/theta (percentage of bins above expected random percentage value)
howmuchoveritatallphi/theta (total above expected random percentage of connected bins above expected random percentage value)
distancetoavvalphi/theta (sum of above and below random value of histogram, distance to complete random pixel/voxel distribution)
histogramvaluesphi/theta (angle histogram probability bin values)
clusterallphi/theta (indices of pixels/voxels belonging to clustered bins, per cluster)
clusterlengthphi/theta (number of pixels/voxels per clustered bins)
allclusterinputphi/theta (sum of indices of pixels/voxels belonging to clustered bins),
clusterpercentphi/theta (percentage of individual cluster to whole cluster amount),
clusterpercenttoallpixelsposphi/theta (percentage of individual cluster to whole voxels above av. value of pos. charge voxels amount)

calculation of distance and strength parameters

startpointsphi/theta (starting bin number of clustered bins, per cluster)
meanpointsphi/theta (middle bin number of clustered bins, between starting and end bin number, per cluster)
endpointsphi/theta (end bin number of clustered bins, per cluster)
lengthpointsphi/theta (number of clustered bins, per cluster)
distancetonextphi/theta (number of bins between clustered bins)
distancetomeanphi/theta (number of bins between mean value of clustered bins)

distance and strength information

arrayofclustersdistancephi/theta (array of ranked distance of cluster to strongest cluster (by distance between mean position), <= 45°: 1.column, 45 < x <= 90°: 2.column, 90° < x <= 135°: 3.column, 135° < x <= 180°: 4.column, number of clusters of ranks given)
arrayofclustersphi/theta (1.column: clusterpercent, 2.column: mean of strongest cluster position to mean of individual cluster position (relative) 3.column : 360°-mean of strongest cluster position - mean of individual cluster position (global))
arrayofclusterstrengthphi/theta (array of ranked strength of cluster (by cluster percent), >=0.6: 1.column , 0.4 <= x < 0.6: 2.column, 0.2 <= x < 0.4: 3.column, 0.1 <= x < 0.2: 4.column, 0 <= x < 0.1: 5.column, number of clusters of ranks given)

Figure 1.16: Cluster methods ("All parameters taken" checked). 2) Clustering by transfer of positive charge pixels/voxels above average value of positive charge pixels/voxels to spherical coordinates and histogram angles. Clustering of pixels/voxels according to angle bins above random percentage value.

Table 1.3: Files found in image folder after run-through

Name	Description and Meaning
image Cell mask	detected mask of cell, as .tif [name of image 'Masked Cell' number of image in analysis '.tif']
channel image multiplied with mask	[name of image, name of channel '.png']

Table 1.4: Files found in image folder after run-through for all parameters

Name	Description and Meaning
histogram images	histogram of ϕ and θ in clustering option based on transfer to spherical coordinates, [name of image, name of channel, 'phiandtheta.png']
image of qpos	image of positive charge voxels in cell mask, per channel, [name of image, name of channel, 'qpos.png']
image of qposofqpos	image of charge voxels above mean intensity of positive charge voxels in cell mask, per channel, [name of image, name of channel, 'qposofqpos.png']
image of qposofqposafteropening	image of charge voxels above mean intensity of positive charge voxels in cell mask, per channel, after erosion and dilation operation to get rid of single high intensity voxels, [name of image, name of channel, 'qposofqposafteropening.png']
list_neg	matrix with columns of x_- , y_- and z_- (positions of negative charge pixels/voxels), and negative charge density of individual pixel/voxel per nm^2/nm^3 χ_- and i_- (intensity value after background intensity subtraction, for every negative charge pixel/voxel); saved individually for each cell and channel singly, under image folder pathway, ['list_neg cell' cell number channel name]
list_pos	matrix with columns of x_+ , y_+ and z_+ (positions of positive charge pixels/voxels), and positive charge density of individual pixel/voxel per nm^2/nm^3 χ_+ and i_+ (intensity value after background intensity subtraction, for every positive charge pixel/voxel); saved individually for each cell and channel singly, under image folder pathway, ['list_pos cell' image number channel name]

Name	Description and Meaning
Mmean pix	position of middle point of cell volume in pixels/voxels, saved under image folder pathway, [Mmean pix cell' image number channel name]
R_neg	position of negative charge weighted mean position of cell in pixels/voxels, saved under image folder pathway, ['R_neg cell' image number channel name]
R_pos	position of positive charge weighted mean position of cell in pixels/voxels, saved under image folder pathway, ['R_pos cell' image number channel name]