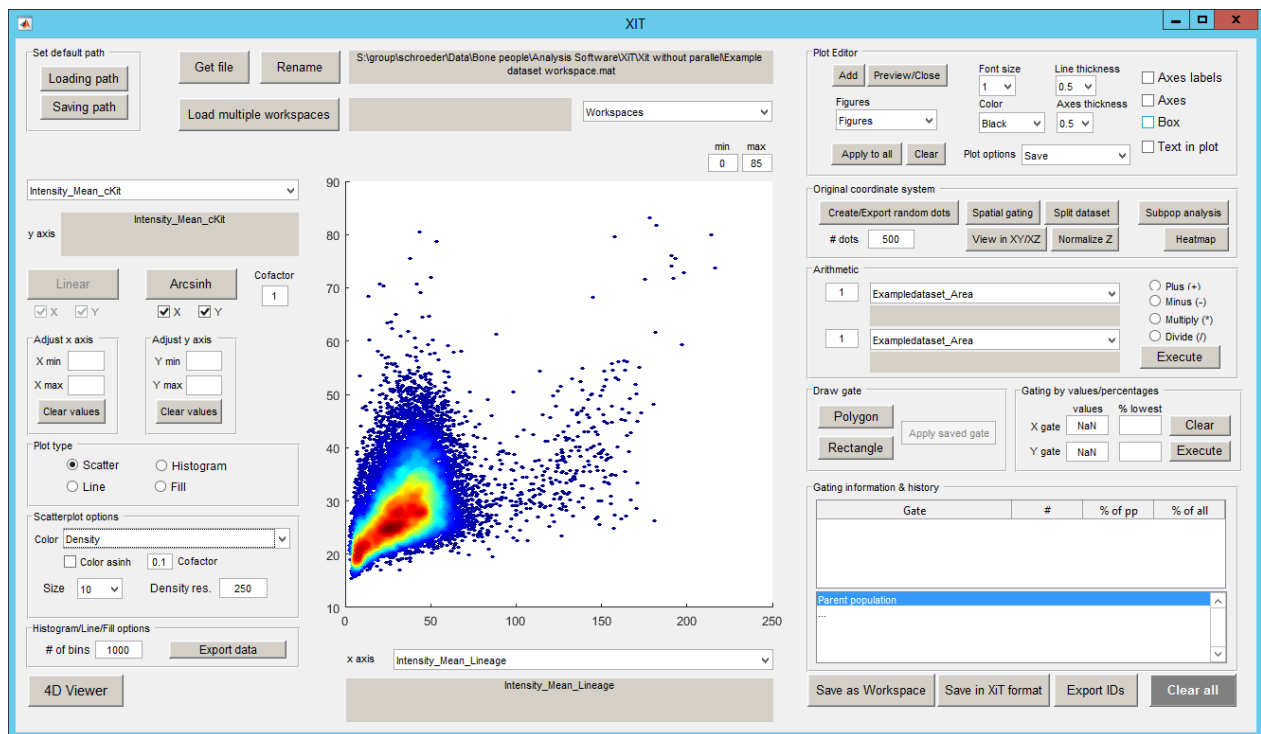# A guide to XiT

XiT stands for **X**dimensional **i**mage analysis **T**oolbox. It is a tool written in Matlab (R2015b, The MathWorks, Inc., Natick, Massachusetts, United States), which enables performing multidimensional analysis on segmented cells using statistics obtained from imaging datasets – a new toolbox for imaging cytometry.

Main user interface of XiT

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Table of contents

Installation guide

In order to install XiT the user can download the XiT installer from https://www.bsse.ethz.ch/csd/software/XiT.html. XiT works on Windows operating systems (developed and run on Windows 7) and requires the Matlab Runtime Compiler (MRC) 2015b. If the MRC is not yet installed on the machine, the user can download the MRC Version 9.0 for free from http://ch.mathworks.com/products/compiler/mcr/.

Since Matlab changed the way certain functions work from version 2015b to 2016a and following versions we provide a source code for users with Matlab versions 2014-2015 and 2016 onwards. The earliest version of Matlab supported is 2014a.

In order to use the XiT version within Matlab, download the appropriate source code version from https://www.bsse.ethz.ch/csd/software/XiT.html. Unzip the downloaded file and add the directory containing the unzipped files into your Matlab path. In order to start the software type "XiT" in the command window and press enter.

The installation of the standalone version of XiT (administrator rights needed) and if needed the MRC is guided by an installation wizard and will require around 1 minute (if MRC is installed) to complete (Fig.1). The following schematic shows the installation process on a PC operated with Windows 7.
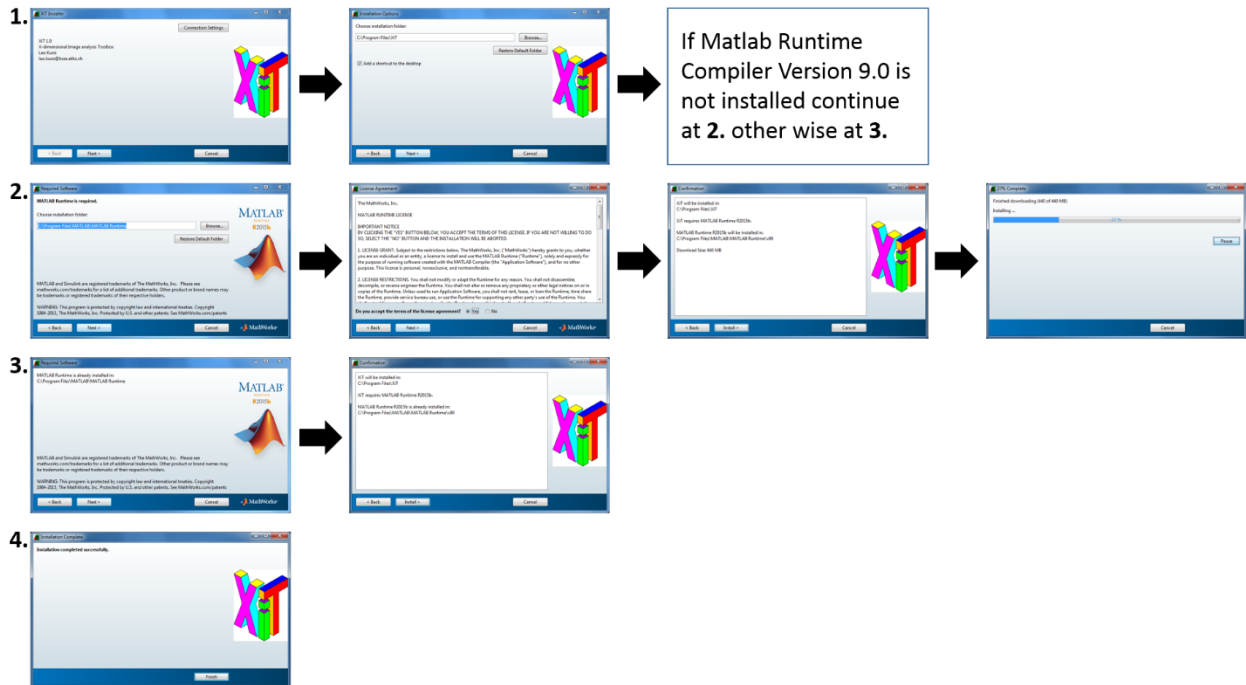

Figure 1: XiT installation guide; After downloading the installer and opening it the first window of line 1 will appear. Clicking Next in the following two windows will bring the user to a point where either the Matlab runtime compiler will have to be installed (guided as depicted in line 2) or the MRC is identified on the hard drive (line 3) and the installation is completed as shown in line 4.

To start XiT double click on the application icon named "XiT", either as a shortcut created on your desktop or in C:\Program Files\XiT\application.

Prerequisites

Cell segmentation has to be carried out on an imaging dataset (for example using the isosurface tool of Bitplane's Imaris[R] software). The marker used for the segmentation will define the parent population i.e. the cells investigated in XiT by definition will always be positive for this marker. Statistics, like mean/median/sum of fluorescence intensity, volume, sphericity etc. have to be recorded and exported (if using Imaris) or compiled in a specific format. XiT is able to directly read the output from the Imaris statistic module but also offers the possibility to use a custom format (Datasets and formats).

Suggested workflow

- Export statistics from Imaris (or any other segmentation tool that can export metrics from segmented objects).
- Open XiT and load the statistics in XiT (see **Datasets and formats**)
- Open the **spatial gating** tool and gate on the objects of interest (in XY and XZ). Gating out outliers in Z is particularly important for the next step.
- Perform **z-normalization**
- Plot e.g. the mean fluorescence intensity of all your channels against the normalized Z-positions to evaluate signal intensity loss with distance to the objective. Gate on the cells which are not showing signal loss in the channel that you can detect less deep reliably (normally 405 or 488).
- Save the dataset as a workspace.
- Do this for your FMOs and full stain datasets. Then load all datasets as multiple workspaces.
- Compensate channels in full stain according to fluorescence minus one (FMO) controls using the channel arithmetic tool.
- Now the datasets are validated and can be used for data exploration, automated subpopulation analysis and random dots generation and many more features describe below.

Datasets and formats

XiT is meant to be used on statistics (mean/median/sum of fluorescence intensity, volume, sphericity etc.) obtained from segmented cells and structures in 2D/3D microscopy images. Three formats are support by XiT.

- The 1st format supported by XiT is the output from the statistic module of Imaris (Imaris Version 7/8.1 Bitplane[R]) when .csv (default) is chosen as output format. In order to use this format the user has to click **Get file**, navigate to the designated statistics folder provided by the export function of Imaris and XiT will load the data.
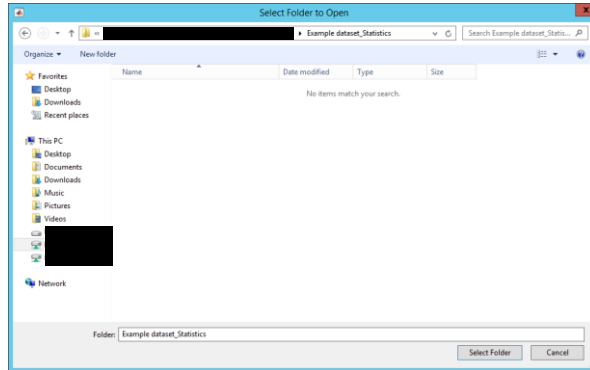

Figure 2: Open Imaris dataset; Navigate to the folder containing the statistics exported from Imaris and click: Select Folder

- The 2nd type of data format is the "XiT format". It is a simple format in Microsoft Excel[R] where the first row contains column headers i.e. names of the statistics. Each row below represents one cell, each column represents on statistic. No hyphens or spaces should be used in the header row. ID numbers for cells should be specified by the header "ID". If no IDs are provided, XiT will assign each cell an ID number (0,1,2,3…). XiT recognizes if XYZ, XY or no XYZ positions are provided. To be sure that coordinate data is read in properly the columns should be named PositionX, PositionY, PositionZ respectively.

  Get file allows the user to navigate to the Excel sheet. This data format can also be obtained for a specific gate (Gating and Gating History) from within XiT by clicking **Export stats**.

Table 1: XiT format example

Intensity_Sum_Ch5	Sphericity	Volume	PositionX	PositionY	PositionZ	ID
3603	0.755903	411.053	1533.57	1373.54	-97.1878	0
2218	0.823969	217.394	1528.63	1373.77	-93.6302	1
1545	0.7871	197.781	1611.06	1293.7	-92.6276	2
2257	0.707322	227.975	1537.88	1468.08	-98.6755	3
2255	0.79176	209.194	1523.59	1478.46	-98.0594	4
2271	0.789448	208.6	1541.01	1478.28	-97.2985	5
6304	0.692625	505.514	1512.25	1489.17	-96.8416	6
1963	0.757538	149.731	1521.93	1486.1	-98.4131	7
2643	0.697134	303.884	1508.98	1499.94	-98.6847	8
4853	0.721529	418.852	1513.66	1508.47	-96.6031	9
883	0.717449	100.092	1518.29	1517.26	-98.8291	10

- The 3rd data format can only be obtained by saving a gate (Gating and Gating History) from XiT (Save as Workspace). The state of the tool and the current gate/dataset are

5

saved in a ".mat" file. Workspaces are rather small in size and are very fast to load. Therefore, they are the recommended way to store data. Also, having saved all datasets (controls, full stains, different conditions etc.) as workspaces, the user can load them all together (Loading multiple workspaces), which allows more direct comparisons.

Setting a default path

On the upper left corner of the main window one can set a default loading and saving path (**Loading path**; **Saving path**).

This loading path is remembered and clicking **Get file** or **Load multiple workspaces**, the user will start at this position of the path (all subfolders are added to the path) allowing to directly select the file(s) to be loaded.

Setting a default saving path will start every saving operation within XiT in the selected directory.

Save as Workspace

Saving the dataset as a workspace will save the dataset (Gating and Gating History) and state of the GUI as a workspaces (Datasets and formats). The gate from which the workspace is created will be the parent population upon loading the workspace again.

Rename

This option allows renaming of all statistics. Clicking the button **Rename** will open a table with 2 columns (left: old name, right: empty). In the right column the user can enter the new name for the statistic. Clicking OK in the pop up window that appears when the Rename button is clicked will save the new name/names.

Furthermore, right-clicking in the textbox below the popup menu for the x-/y-axis you can rename a statistic directly.

Loading multiple workspaces

Loading multiple workspaces (**Load multiple workspaces**) allows the user to load e.g. datasets of controls and full stains or different conditions at once. The workspaces will be loaded and appear in the popup menu to the right of the button (**popup menu Workspaces**). Clicking on one of the workspaces in the popup menu will save your current workspace state and open the workspace just selected.

Main plotting area and plotting options

This central area in XiT is where data is plotted as a scatter/histogram/line/fill plot. The statistic(s) to be plotted can be chosen from the respective popup menus below and to the left of the plot. The user can decide between the type of plots (**Panel Plot type**), adjust x- and y-axis

limits (**Panels Adjust x axis/Adjust y axis**), switch – axis specific – between a "linear" and a "arcsinh" scale (**Linear/Arcsinh buttons and X/Y tick boxes**) and color code a scatter plot according to a 3rd statistic, density or just have it colored black (**Panel Scatterplot options**). The color coding according to a third statistic can be adjusted to minimum and maximum values using the two edit boxes (min, max) above the right top corner of the plot area (Figure 3b). Additionally, the user can define a cofactor for the arcsinh transformation of the data. The default cofactor is 1. The smaller the cofactor is chosen the higher is the resolution close to 0. Similarly the color coding 3rd statistic is transformable – with addition of a cofactor (see above).

The density representation is smoothened with a Gaussian filter. However, by adjusting the resolution (**text box Density res.**) the user can play with the density display. Also, color (black, density, 3rd statistic) and size of dots (scatter) and number of bins (histogram/line/fill) can be adjusted (**Panels Scatterplot / Histogram/Line/Fill options**).
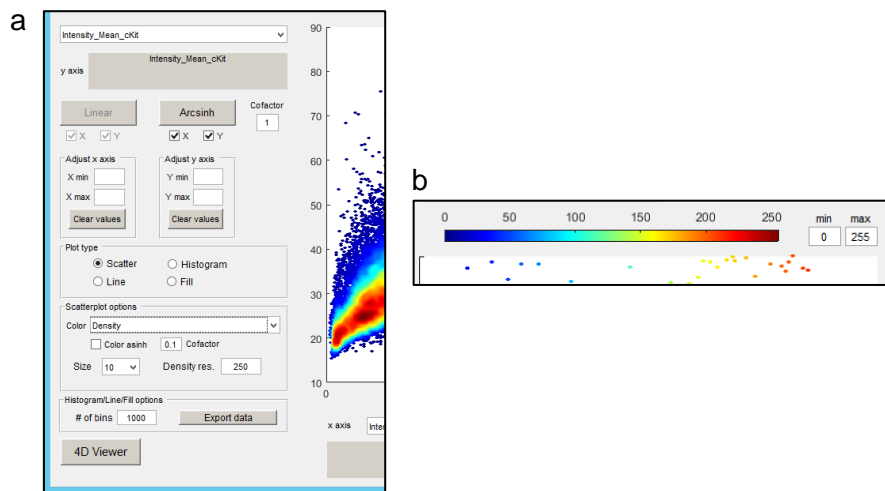

Figure 3: **a)** Left side of the main plotting area; **b)** adjust color coding on the top right of the plot area

For histogram plots export of the histogram data for external re-plotting is also possible **(Export data)**. For example: The data (8-bit) is distributed from 0 to 255 for "Mean_Intensity_XYZ". In order to export all data points in bins of size 5 the user should choose: Xmin = 0, Xmax = 255, # of bins = 51. The output will be an excel sheet with three columns:

Table 2: Export output for histogram data

5	92	0,527009
10	1225	7,017242
15	1109	6,352752
...	...	...

The first columns represents the bin edges (all values up to 5, all values up to 10 etc.). The second column shows the total number of data points within the bin and the third column gives a percentage value.

Furthermore, a 4D Viewer is also available (**4D Viewer**).

4D Viewer

The 4D viewer allows exploring your Data in 3D scatterplots – with adding another dimension by colorcoding according to a statistic. After clicking on the **4D Viewer** Button in the XiT main window and second window opens (see Fig. 4). From the respective popup menus the statistics to be plotted can be chosen.

As in the main plot the size of dots and color code can be chosen and the axes can be adjusted. In addition, axis and color specific arcsinh data transformation is possible with addition of a cofactor. Also, the colorcode can be adjusted to min and max values.

The plot can be rotated horizontally and vertically moving the sliders or the respective fields on the right side of the sliders (enter a value and press "Return").

Pressing **Save Snapshot** will save a snapshot (.tif) of the plot as it is. There is a possibility (Question dialog Yes/No upon clicking) of having a colorbar inserted into the figure.

Save 360 dgr. view creates an animation of the 3D scatterplot rotating 360 degrees and saves it as an .mp4 file. Upon clicking the vertical position (default -37.5 degrees) can be determined.
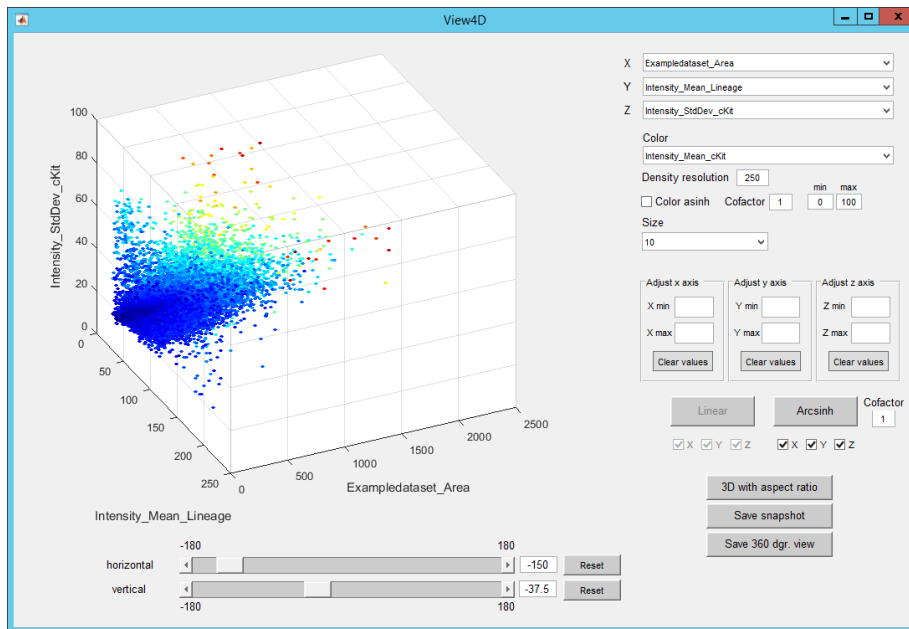

Figure 4: 4D Viewer user interface

Plot Editor

The Plot Editor panel allows temporary storing, editing and saving of plots from the main plotting area.

Clicking **Add** adds the current plot to the list of temporarily saved figures. This figure will then appear as "Figure 1" in the **Figures popup menu** – Figure 2, Figure 3 etc. for the following clicks on **Add**. Clicking **Preview** makes the currently selected figure in the popup menu visible in a new window. The button **Preview/Close** changes its text to **Close/Preview** and upon clicking the Preview/Close button again, the window containing the figure will close. **Clear** clears the list of temporarily stored figures.

If the accidentally closed a figure by clicking the X on the right top side of the preview window, the user should to clear the list of figures to avoid complications.

If more than one figure wants to be previewed that is possible by previewing the first figure, then changing in the popup menu the figure and clicking again on the preview/close button. To close the respective figures, the user has to navigate to the figure in the popup menu and click **Close/Preview** again (sometimes one has to click several times until the figure closes again). The figures can be edited in the following ways:

- Font size (labels and axis ticks; for all plot types)
- Line thickness (for line plots and gates in all plot types)
- Color (for all plot types)
- Axes thickness (for all plot types)
- Axes labels (yes/no; for all plot types)
- Axes (yes/no; for all plot types)
- Box (yes/no; for all plot types)
- Text in plot (yes/no; refers to percentage values when working with a gate; for all plot types)

The changes can be applied to each figure separately simply by changing the parameters for the figure currently showing in the popup menu "Figures" or to all figures within the temporary folder by clicking **Apply to all**. Apply to all works with: Box, axes, axes labels, axes thickness, font size, line thickness and text in plot.

The **popup menu "Plot options"** allows saving and saving transparent plots for single figures. **Save** saves a figure as a ".tif" file with 300dpi. **Save transparent** saves a figure as a ".png" file (also 300dpi) once as a normal image and once with transparent background (named …_transparent). **Save all** saves all figures added to the temporary folder as ".tif" files numbered …_1, …_2, …_3 etc..

Figure 5: Plot Editor allows temporary storage, manipulation and saving of plots generated in the main plotting are of XiT

Original coordinate system panel

In order to use these functions one needs to have x, y and z coordinates of the segmented cells in the dataset.

Create/Export random dots creates random dots within the volume spanned by the coordinates of the segmented cells. The dot radius is by default 6 um. The number of dots can be decided on by entering it in the designated textbox below the button. The coordinates of the random dots are saved as a ".txt" file in order to be used with the Imaris Xtension "CreateSpotsFromFile" (http://open.bitplane.com/tabid/235/Default.aspx?id=70). In this .txt file the user can also change the spot radius.

Spatial gating opens a new window with an XY and an XZ projection of the coordinates of the segmented cells. A first question dialog opens asking on which plot the user wants to gate. After choosing one of the plots, the cursor turns into a crosshair on the respective plot and a polygon can be drawn. Once the polygon is closed with a "double click" or by clicking on the starting point, another dialog opens (2nd dialog) asking whether the users wants to keep the cells inside – and on the line – or outside of the polygon. Choosing one of the options the XY/XZ plots are updated accordingly. A 3rd dialog pops up asking the user if further gating is wanted. Pressing "No" will close all windows and return to the main GUI. "Yes" will start the gating protocol from dialog one again, until the user presses "No" in the third dialog.

Split dataset allows the user – similar to the **Spatial gating** function – to draw polygons on an XY or an XZ projection of the coordinates of the segmented cells and thus separate one dataset into several workspaces according to an area in the original image. After clicking the button the user first is asked to indicate on which projection he/she wants to draw the polygons (XY/XZ projection). Then the user has to enter the number of polygons he/she wants to draw i.e. the number of workspaces the dataset should be separated into. In the end, after having drawn the regions a saving dialog opens for every region of interest. "Export successful" indicates that the action is concluded and XiT can be used again.

View in XY/XZ opens 2 new windows where 1) a 3D scatterplot and 2) an XY and an XZ projection of the coordinates of the segmented cells are shown. If a gate (Gating and Gating History; not a spatial gate) is selected in the Gating history textbox the cells within the gate are labeled red on the gray dots representing the parent population with which the user started.

Normalize Z creates a new statistic in which the z positions of cells in bins with a 50 um spacing (along the long axis of the bone; y axis if statistics come from Imaris) are normalized to the lowest z position of a cell in the whole dataset. The statistic can be selected in the popup menus below and on the left side of the main plotting area. In a popup window "Normalization done" is displayed when calculation is finished.

We recommend to use the Spatial Gating tool before normalizing z in order to make the sample roughly even in its z dimensions. This will give the best normalization result.

Subpop analysis performs a subpopulation analysis on the dataset. A folder – specified by time and date and the term "Subpop_analysis" – will be created. In here you will find a summary excel sheet showing which subpopulations at which numbers/percentages were detected. Also, the complete set of statistics is compiled for every subpopulation and is saved in the XiT format. Furthermore, ID numbers are exported in a ".txt" file. ID numbers can be useful to for example paste them in the query box in the statistic tab of Imaris to highlight a subset of isosurface

objects in the parent isosurface objects. There is also a possibility of having heatmaps and heatmap overlay plots (with parent population) generated. Upon clicking the user is first asked to define the folder where the results should be stored. Then the channels for the subpopulation analysis have to be selected. The next dialog is an input dialog where the names for the channels can be entered. Here, also thresholds defining a positive signal have to be entered for each channel. Furthermore, a false-positive value (in % of parent population) can be defined below which subpopulations will be discarded. Once this is done the user can remove position artifacts (Original coordinate system panel). After this dialog the subpopulation analysis is carried out. In the next window to appear the user is asked if he/she wants to have heatmaps of the subpopulation analysis. If the users clicks "No" the function terminates. If the user chooses "Yes" a volume for the density analysis is requested. The volume (in um^3) defines the size of the voxels in which cells are counted and thus the resolution of the heatmap. Cell numbers are then converted in cells per mm^3. The 2D heatmap of the 3D binned data shows a maximum density projection i.e. the value you see corresponds to the bin (e.g. 50x50x50 um) with the highest cell density of the bin stack.

Before computing one can check the orientation of the sample and then choose to flip/mirror/flip and mirror/not manipulate the sample. Calculation and plotting might take a while depending on the dataset and volume input. The calculation and plotting is done when a message box displaying "Done" pops up. Units of x and y axis are micrometers.

Analogous to the heatmaps created after subpopulation analysis, with the **Heatmap** button a heatmap of the current gate and an overlay with the parent population are created.
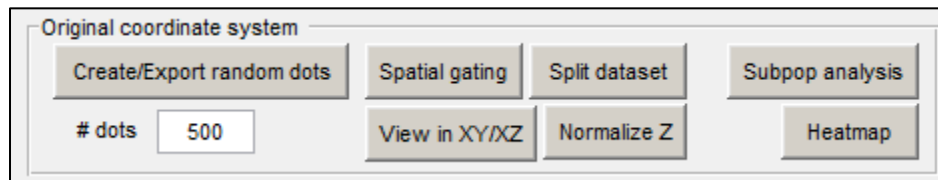

Figure 6: The original coordinate system panel allows various operations using spatial information from the dataset

Arithmetic

This panel allows the user to conduct simple arithmetic operations. The textboxes in front of the respective popup menus indicate the factor by which the statistic for each cell is multiplied before the arithmetic operation is carried out (default is 1). In the popup menus the user can choose the statistics to be edited. The radio buttons indicate the mathematical operation to be executed. By clicking **Execute** the arithmetic is performed (always: "upper box" plus/minus/multiplied by/divided by "lower box") and upon finishing a message box displaying "Done" is shown. This box has to be closed by clicking "Ok". The new statistic is now available to choose in the popup menus below/to the side of the main axes/coordinate system. After performing the arithmetic operation the plot in the main window might change, which is due do the insert of the new statistic.
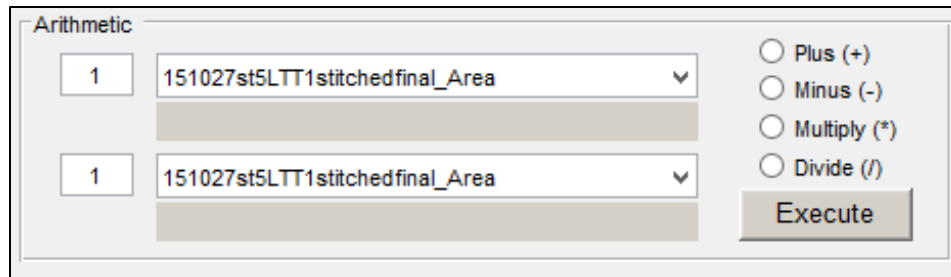
Figure 7: Arithmetic operations with imported statistics

Gating and Gating history

The user can choose between "Polygon/Rectangle gating" and gating according to values/percentages.

For "Polygon/Rectangle gating" the user has to click the respective button. Hovering over the main plotting area the cursor becomes a crosshair and allows drawing a polygon that can be closed by double clicking or a click on the starting point of the polygon. For the rectangle the user drags a rectangle from one starting point in the main plotting area. Upon closing the polygon/drawing the rectangle a question dialog appears asking first whether or not the user wants to keep the gate for applying it later. **Apply saved gate** will be enabled upon clicking "Yes". Then the user is asked whether or not the current "gate view" should be saved in the Plot editor. Concluding the question dialog all cells outside the polygon/rectangle will be deleted from the dataset. The new – gated dataset – will be plotted in the main plotting area and added to the gating history.

> In the Gating history panel the user finds an informative table showing gate information (x and y axis and poly/rect, or the values for value/percentage gating). Furthermore, a list of the gates is provided ("Go back to gate"). This list allows the user to go back to the parent population and all other gates defined by the user. This applies also for spatial gates created by **Spatial gating** (**Original coordinate system panel**).

Gating by values or percentages offers the user the possibility to set a gate by defining the value/percentage in the designated text boxes. These values can either be values on the x axis, the y axis (above/below gating) or both axes (quadrant gating). Also percentages referring to the x and/or y axis can be used for gating in the designated textboxes. Once the values are applied by hitting "return" the user needs to click on **Execute** in order to actually gate. As for the polygon gating the cells of the gate selected will be plotted and a new entry in the Gating history is made. **Clear** clears the values/percentages applied and any lines/text in the main plotting area.

Gating can be done on all types of plots (scatter, histogram, line, fill) but is limited to value/percentage gating on the x-axis when a histogram/line/fill plot is present in the main plotting area.

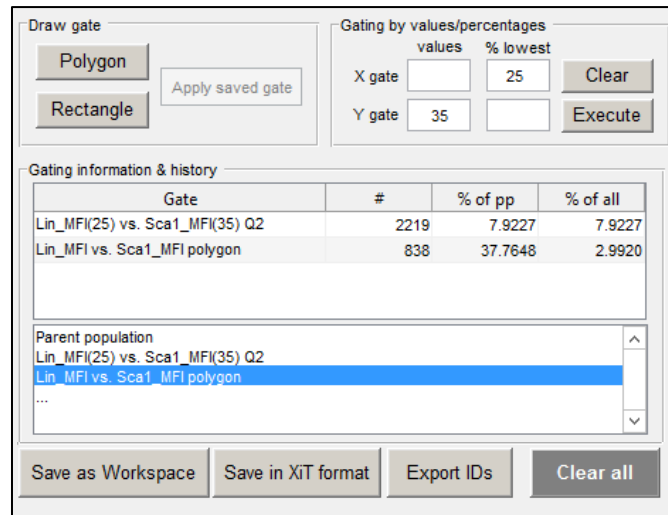If the scale is changed (arcsinh/linear) gate values will be converted as well.

Figure 8: Gating for different subpopulations and export of the respective statistics

Once a gate is obtained the user can either export stats in the XiT format (**Datasets and Formats**), or export the ID numbers only (as a .txt file). This option is useful to double check the cells identified using XiT within Imaris (creating a selection of the parent population isosurface by ID numbers).

Apply saved gate allows the user to apply a – beforehand saved – polygon or rectangle gate to another type of scatter plot in the same data set, or to another dataset which was loaded alongside into XiT by **Loading multiple workspaces.**

Clear all clears all alterations in the dataset such as gatings, normalized z positions and statistics obtained by arithmetic operations. Also the gating history will be cleared. Only figures that are temporarily saved in the plot editor will still be there i.e. have to be deleted separately.