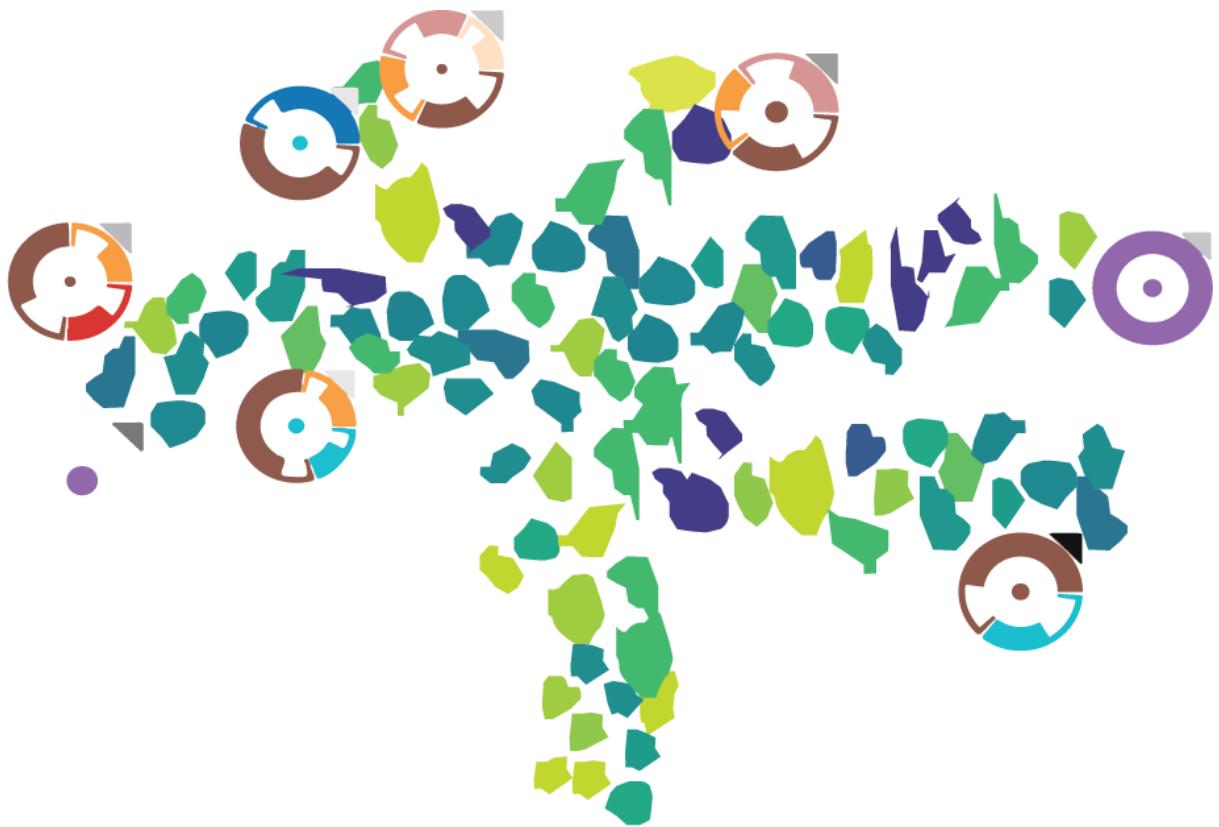


ImaCytE tutorial



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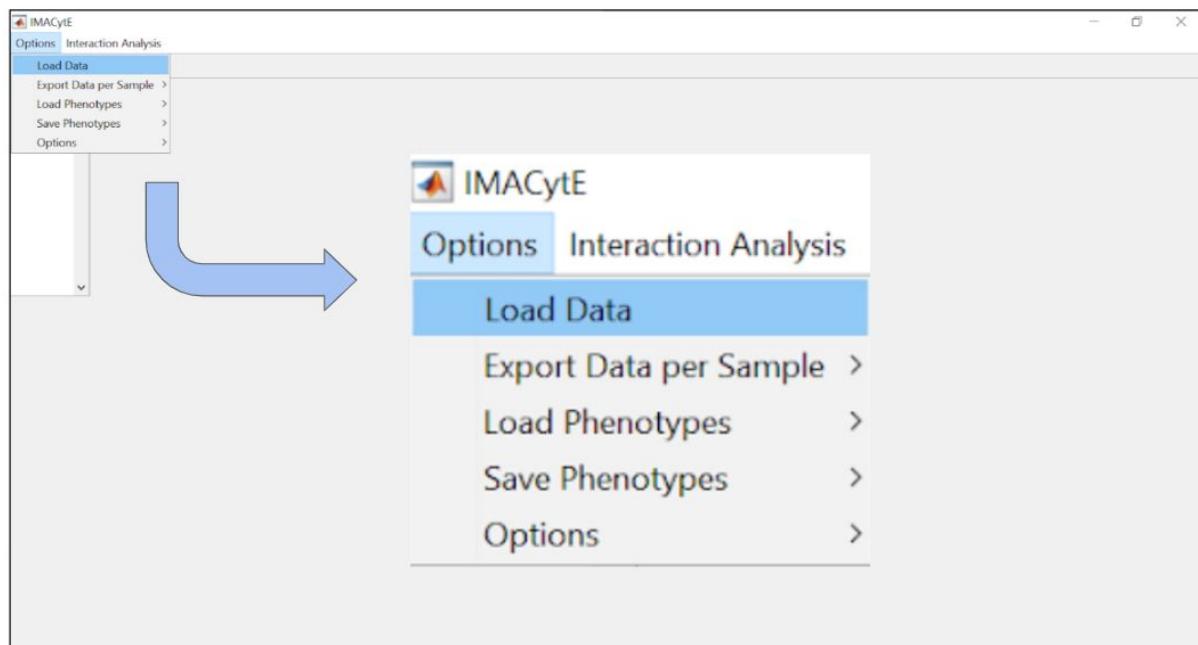
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Getting started with IMACytE

Once IMACytE has been installed, we can load the Graphic User Interface (GUI) by double clicking the icon on the Desktop or by running IMACytE.exe on the IMACytE> application folder. In case of update, previous IMACytE installation should be deleted (this includes any IMACytE folder left after uninstalling)

Load data for analysis

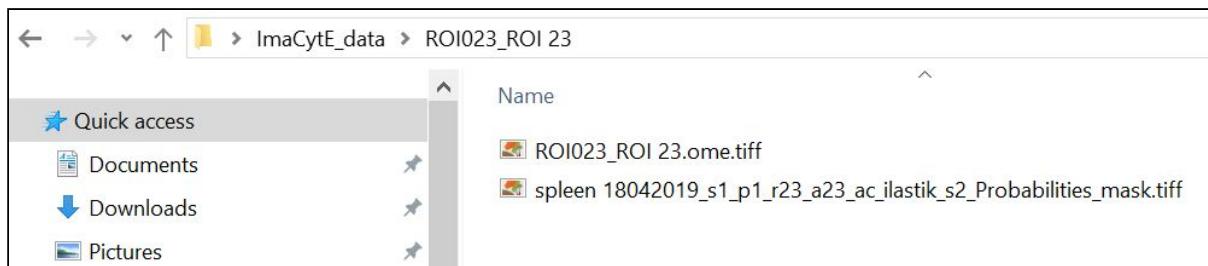
Select the Option menu -> Click on Load Data



Here, provide a folder. In this folder should be one subfolder for each sample.

Example

Name	Date modified	Type
ROI023_ROI 23	20/11/2019 15:22	File folder
ROI024_ROI 24	20/11/2019 15:22	File folder
ROI025_ROI 25	20/11/2019 15:22	File folder
ROI026_ROI 26	20/11/2019 15:22	File folder
ROI027_ROI 27	20/11/2019 15:22	File folder
ROI028_ROI 28	20/11/2019 15:22	File folder



Each subfolder must contain a multi-.tiff file with all markers as exported from MCD viewer and a *_mask.tif file which is used as the cell mask.

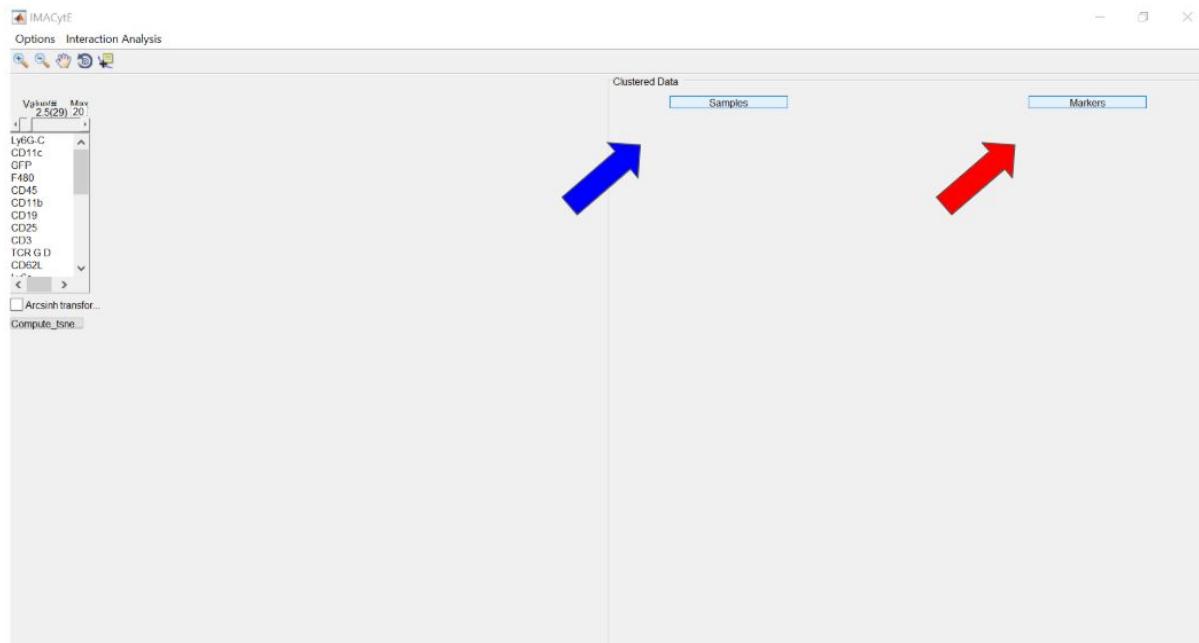
Files should be exported from MCD viewer using the 32 bit option.

Quality control

Visualise samples or markers

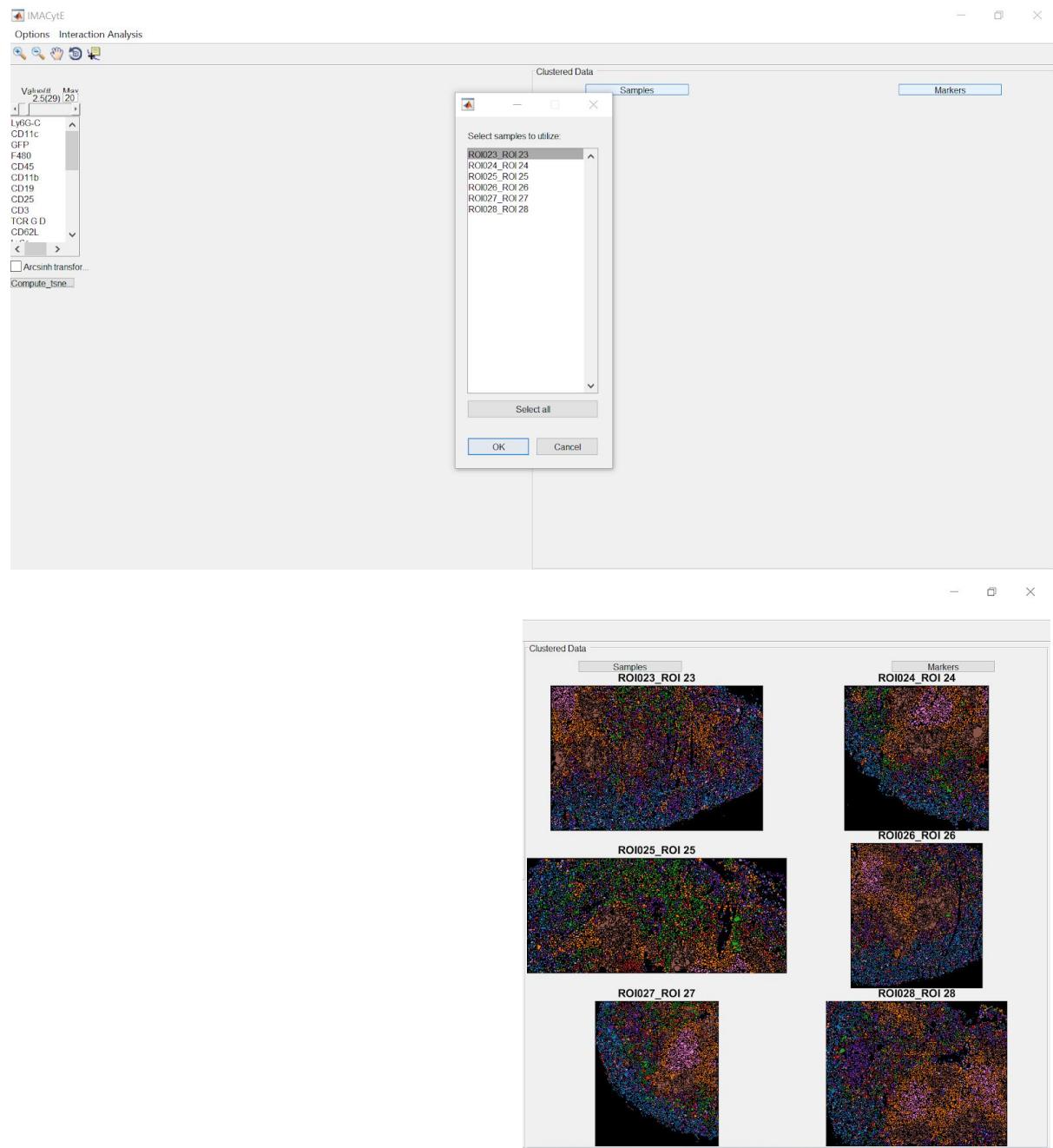
Click on the Samples button (blue arrow) to select the samples of the study.

Click on the Markers button (red arrow) to select the markers of interest.

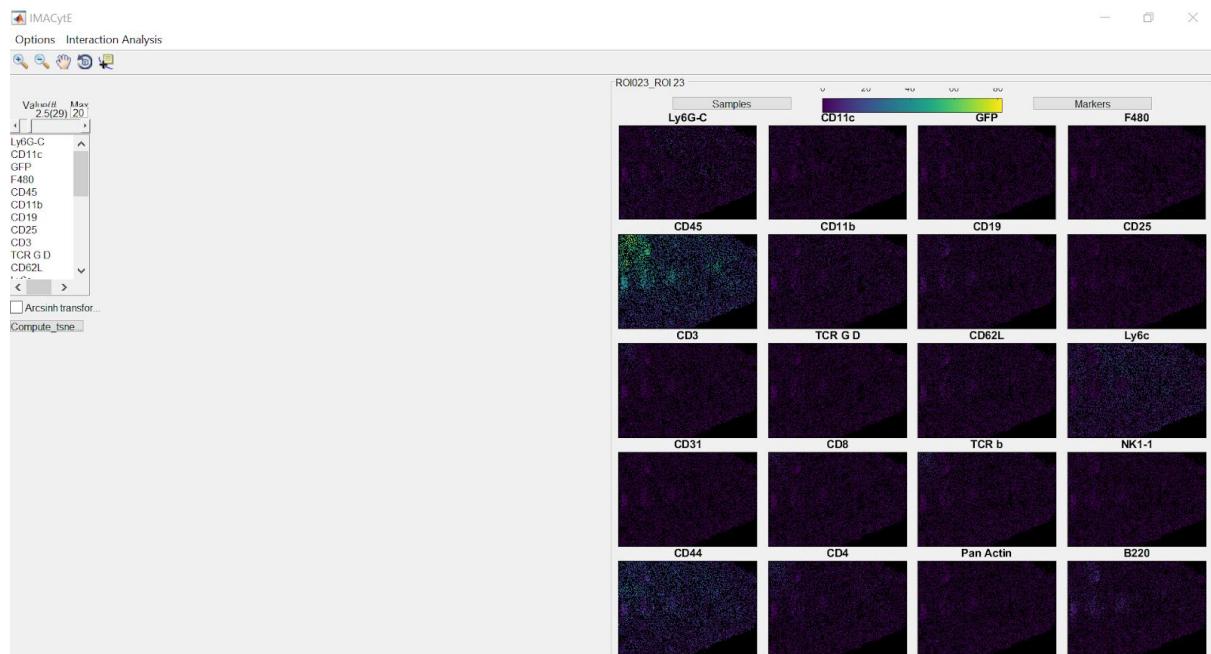
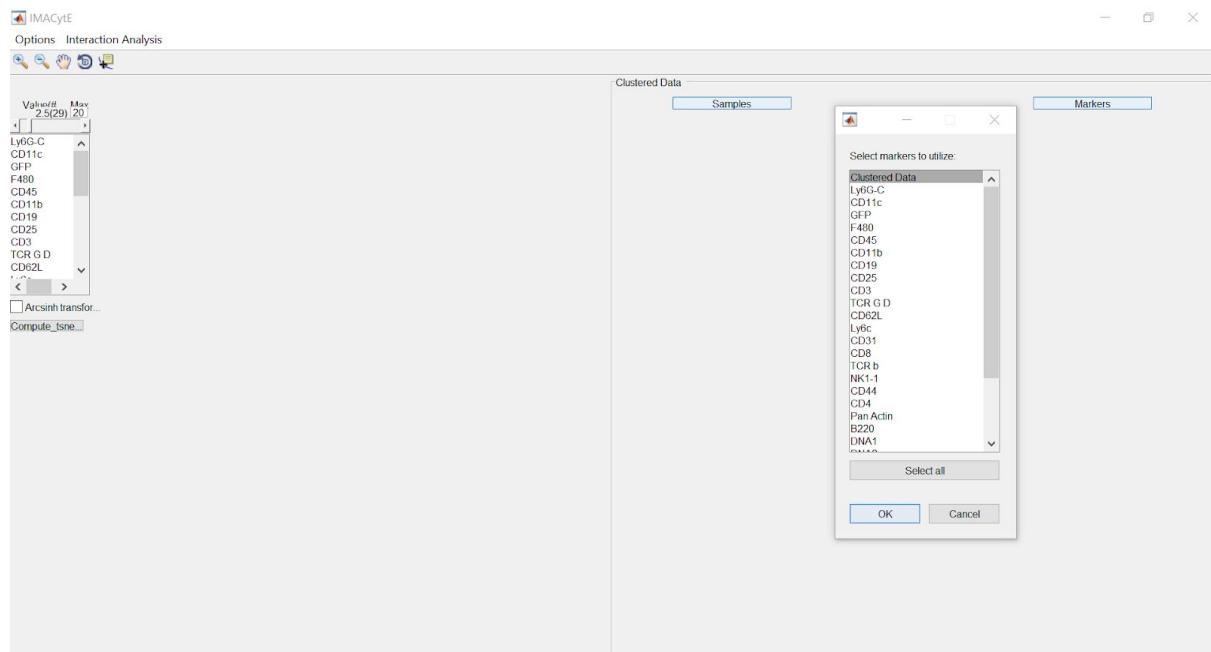


You can compare many markers if only a single sample has been selected or a single marker if many samples have been selected.

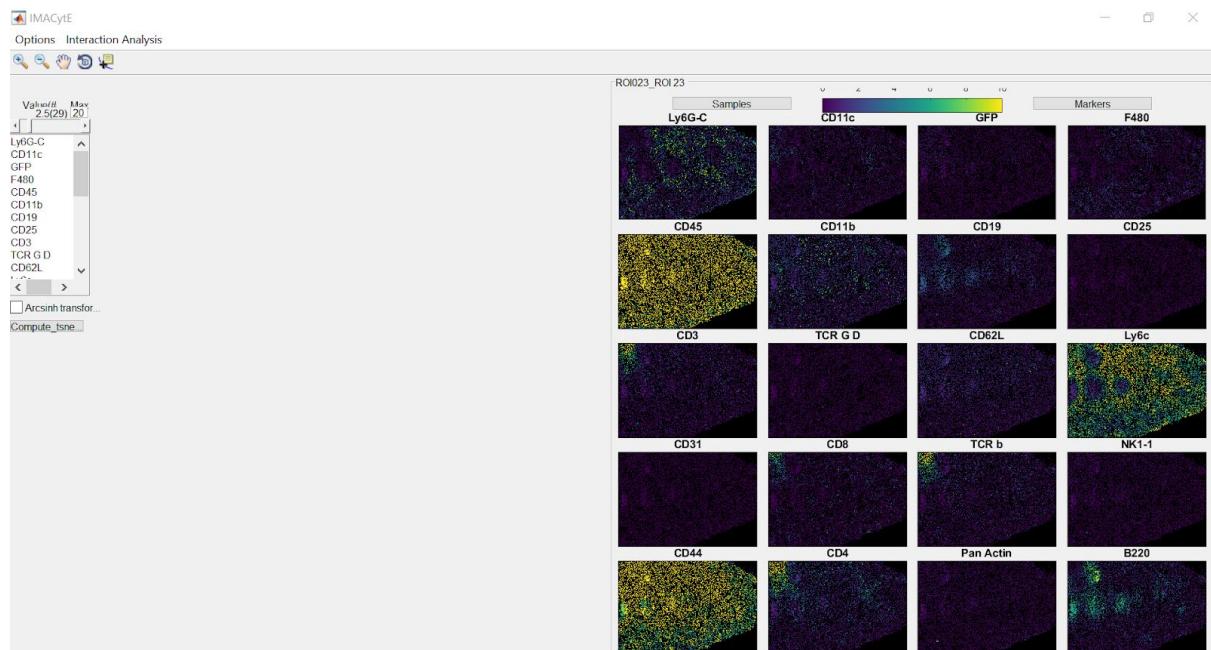
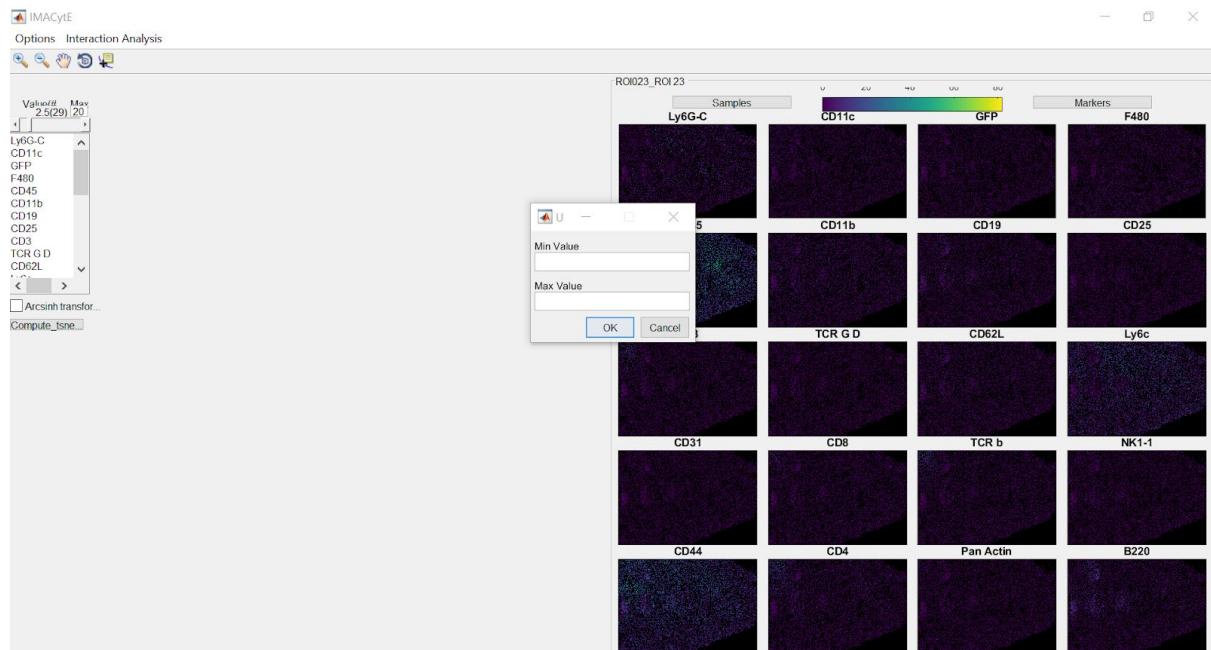
Many samples one marker:



Many markers one sample:



The marker expression range can be modified by clicking on the top colorbar. In this example, the initial Maximum value is set to 80. When this value is reduced, the markers' expression will be brighter.



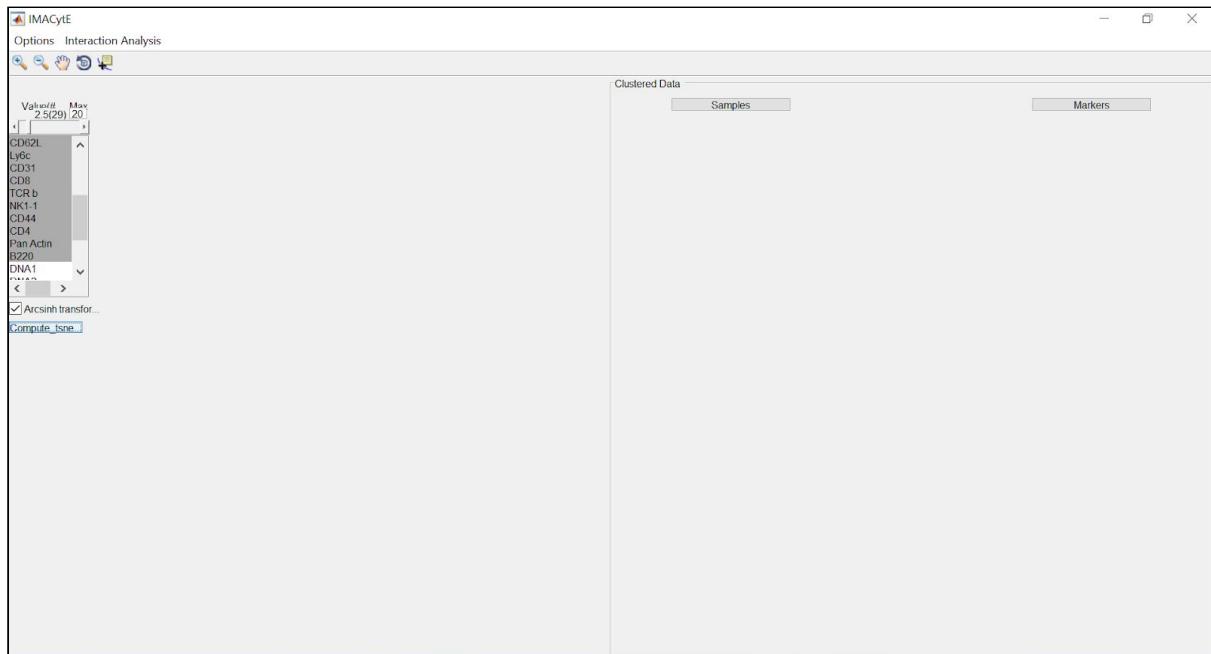
This is the first Quality Control step to check that samples look fine and markers expressions are consistent.

Cell Phenotype Identification

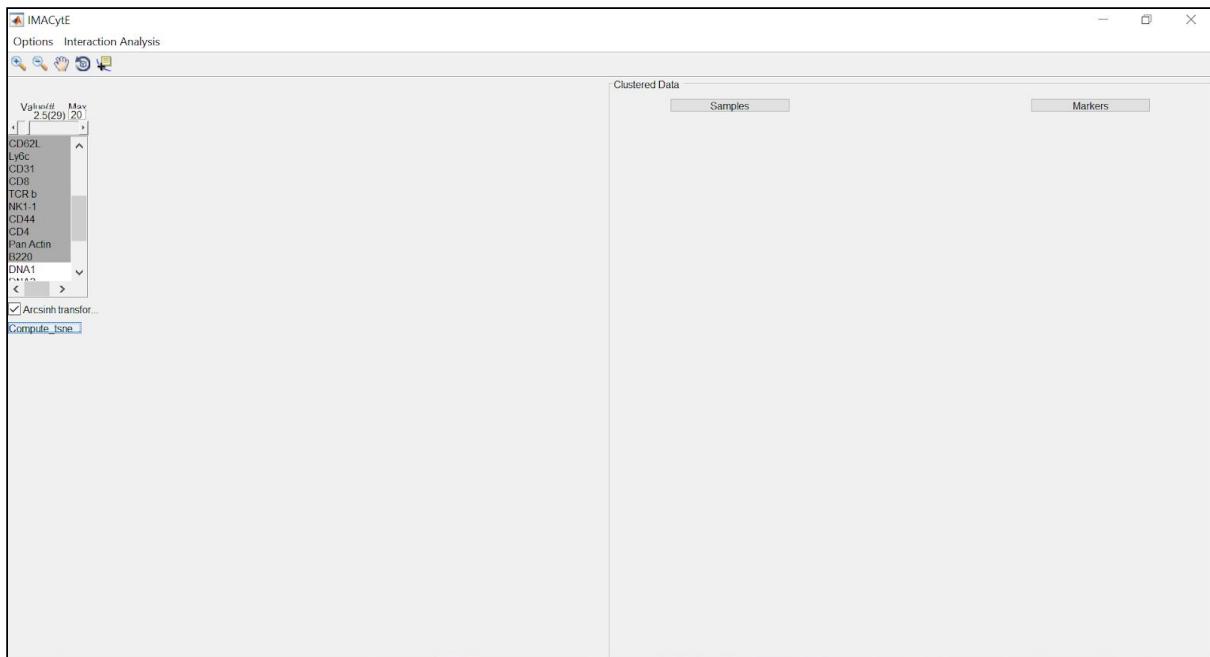
Phenotypic clustering in ImaCytE

Once samples are loaded, select on the left panel:

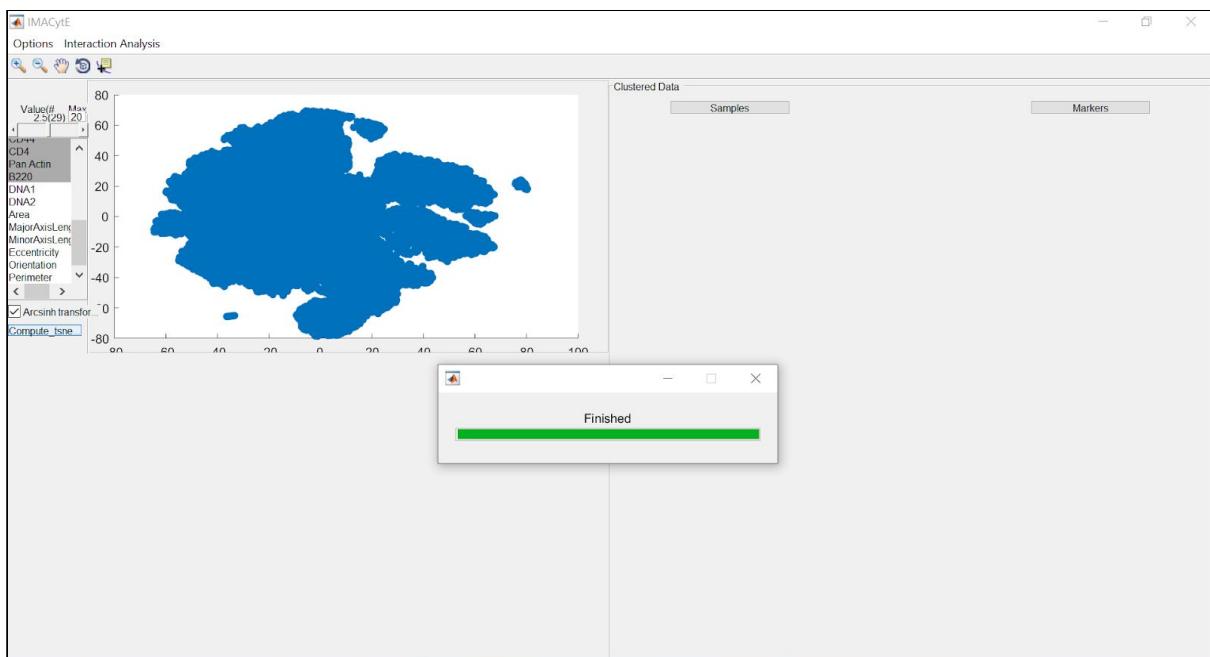
- Select markers of interest: Click on the top marker on the column, then hold Shift + Ctrl and click the marker at the bottom if they are grouped. Hold Ctrl and click on every marker if they are intercalated with other channels.



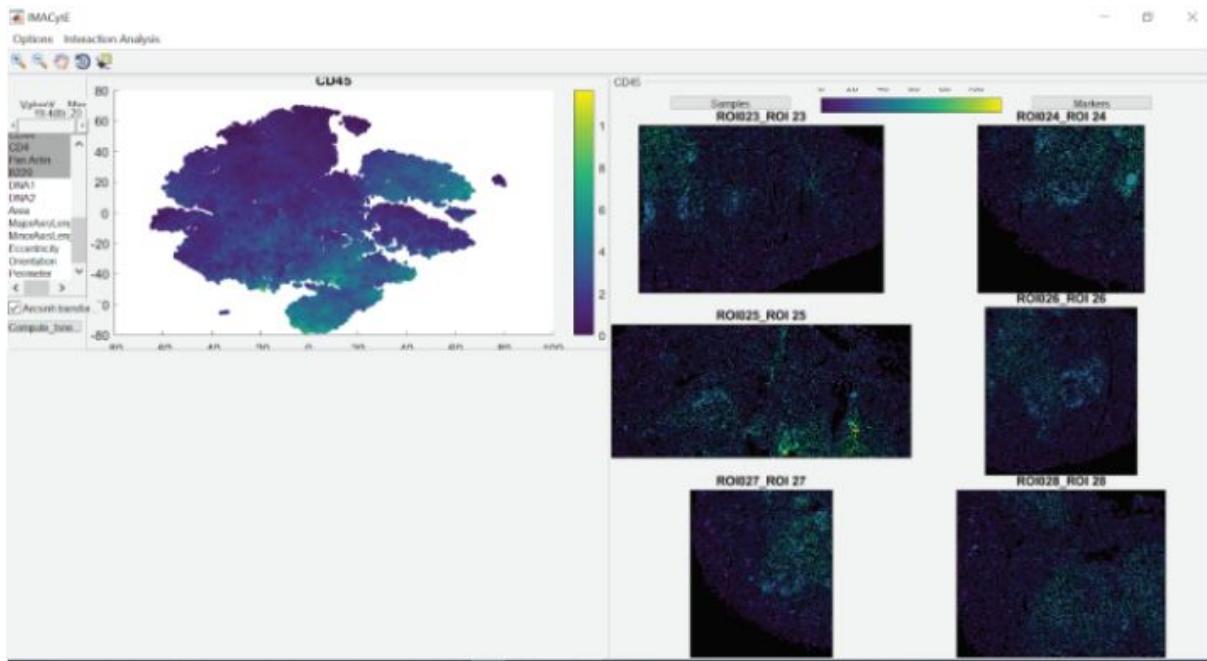
- Tick Arcsinh transformation (if needed)



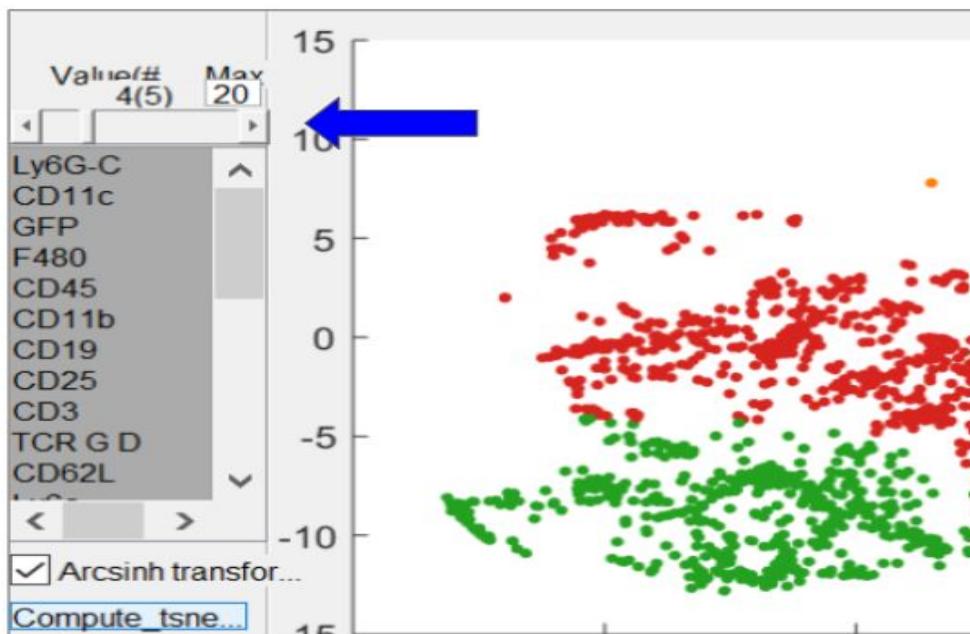
- Click Compute_tSNE button and select samples. Please wait for several minutes (depending on the number of samples)
- When the tSNE map is calculated will be visualized as below:



- Distribution of a marker on tSNE and tissue samples



- Use the Slider on top of the markers' column (left panel) to perform density based clustering using Mean-Shift algorithm. The value of the bandwidth and the amount of identified clusters is illustrated. The bandwidth is the parameter that defines the number of clusters. The bigger the bandwidth the less the clusters. (but remember that samples must be loaded first).



Clusters with the same colour and just different shades are similar enough to be merged. Adjust the number of clusters using the slider (blue arrow) until the heatmap is optimised.

Due to the stochastic nature of the tSNE algorithm, the clusters will differ in size, shape and location in every run and every modification with the slider.

Load pre-defined phenotypes

Per sample

Input in this section is a .csv file. The number of the row represents the according cell, the value of the row the corresponding cluster. The name of the .csv file should be the same as the name of the sample.

	A	B
1	Phenotype	
2	3	
3	9	
4	5	
5	5	
6	7	
7	2	
8	5	
9	5	
10	5	

Per phenotype

Also, a .fcs file can be used as input. In each .fcs file which represents a phenotype, each cell to have its own ID

	A	B	C	D	E
1	Cell_id	Image_id	Ly6G-C	CD11c	CD14
2	1	1	3.42857	0.535714	0.000000
3	2	1	0.35	1.475	0.000000
4	3	1	3.5641	0.076923	0.000000
5	4	1	2	0.090909	0.000000
6	5	1	0.289474	0.236842	0.000000
7	6	1	4.96	0.1	0.000000
8	7	1	1.78846	0.115385	0.000000
9	8	1	1.62162	0.297297	0.000000
10	9	1	1.74684	0.810127	0.000000
11	10	1	2.22222	0.000000	0.000000

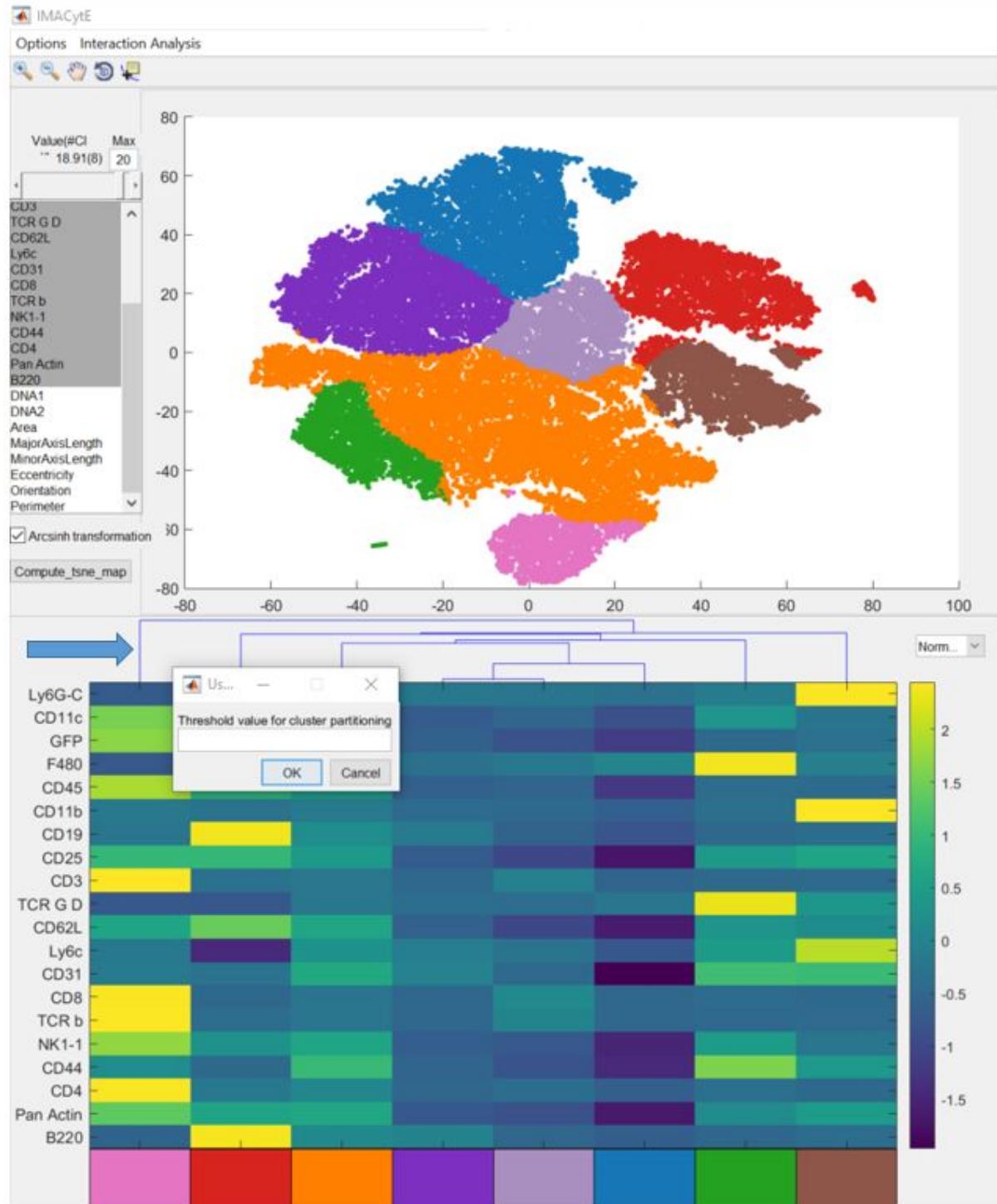
Load session

If phenotypes are loaded there is no need to create a tsne map.

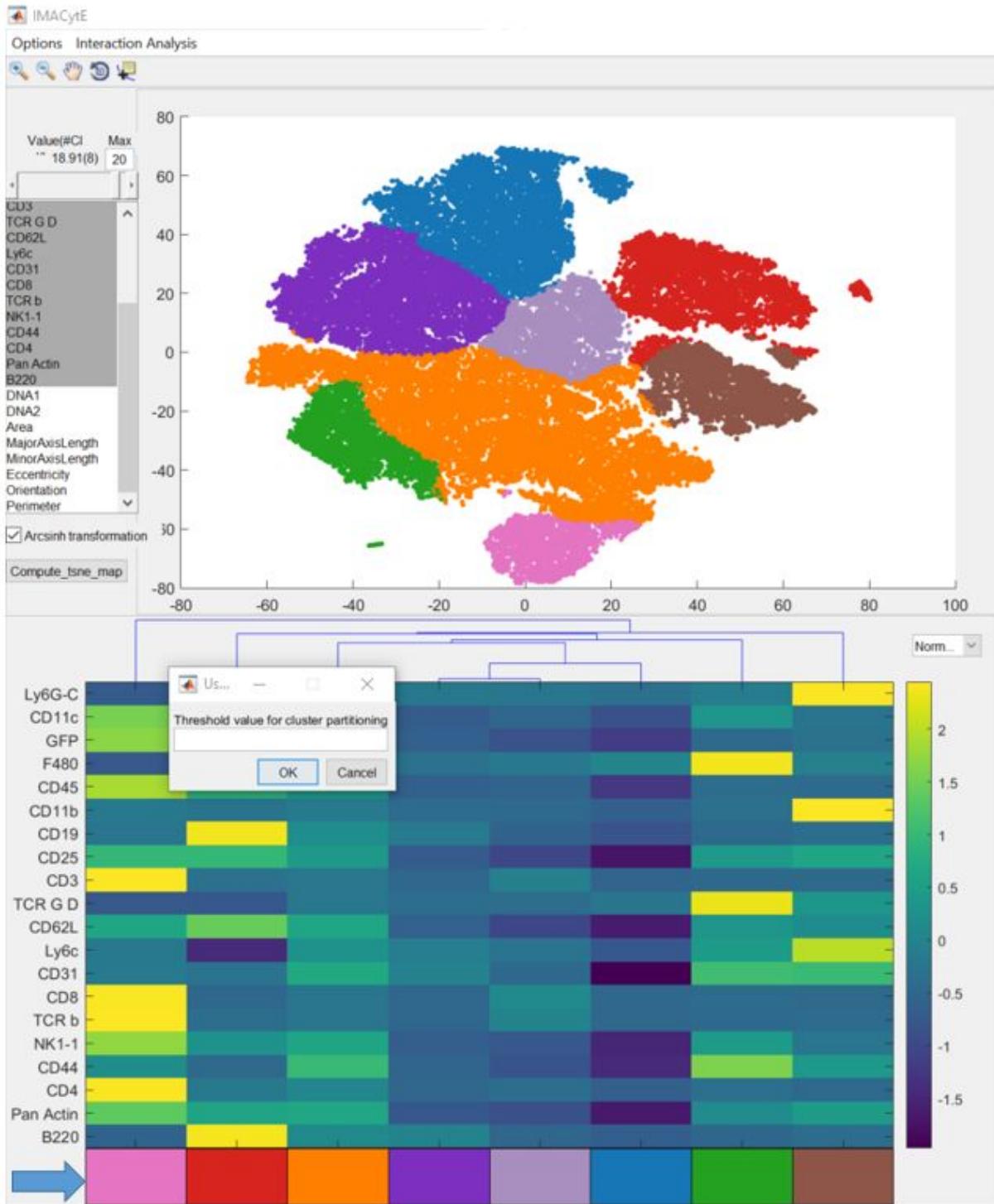
Phenotype exploration

Markers abundance heatmap

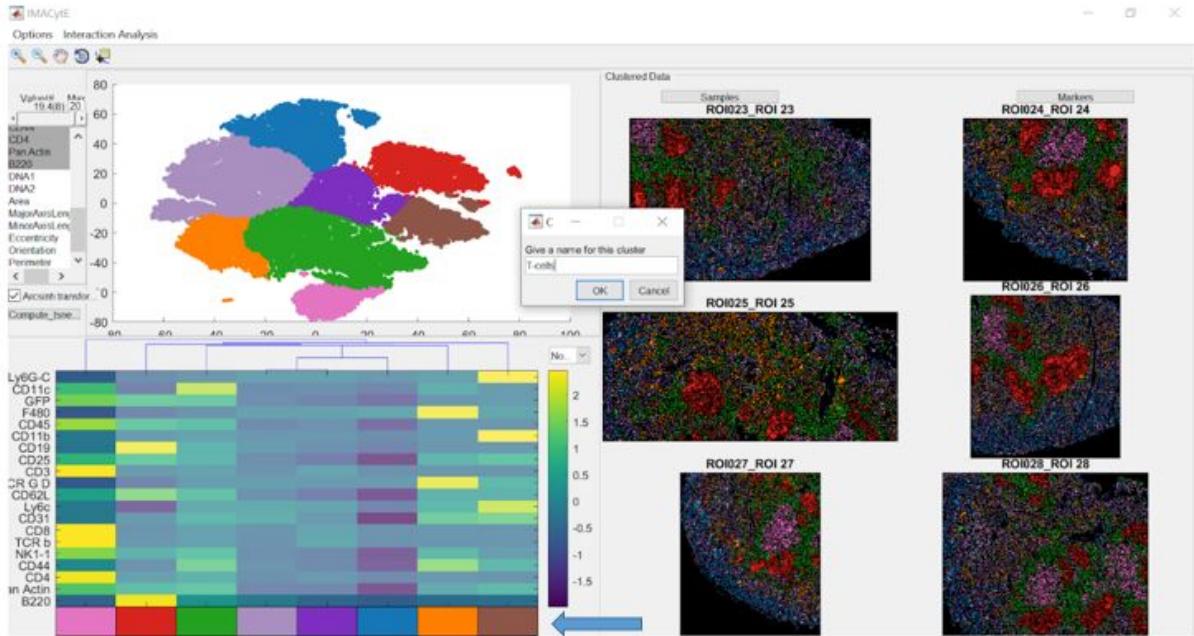
- Changing color-coding by changing the threshold of the dendrogram by mouse-click on the dendrogram (blue arrow)



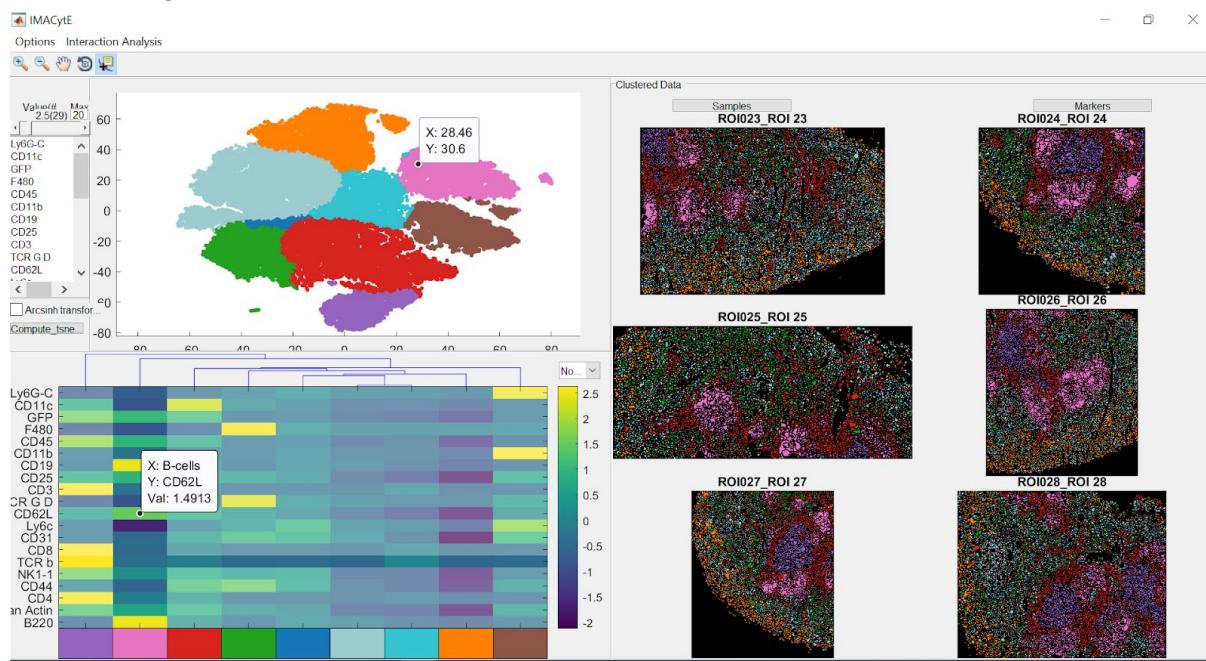
- Manual adjusting color-coding with double clicking on the bottom bar (blue arrow)



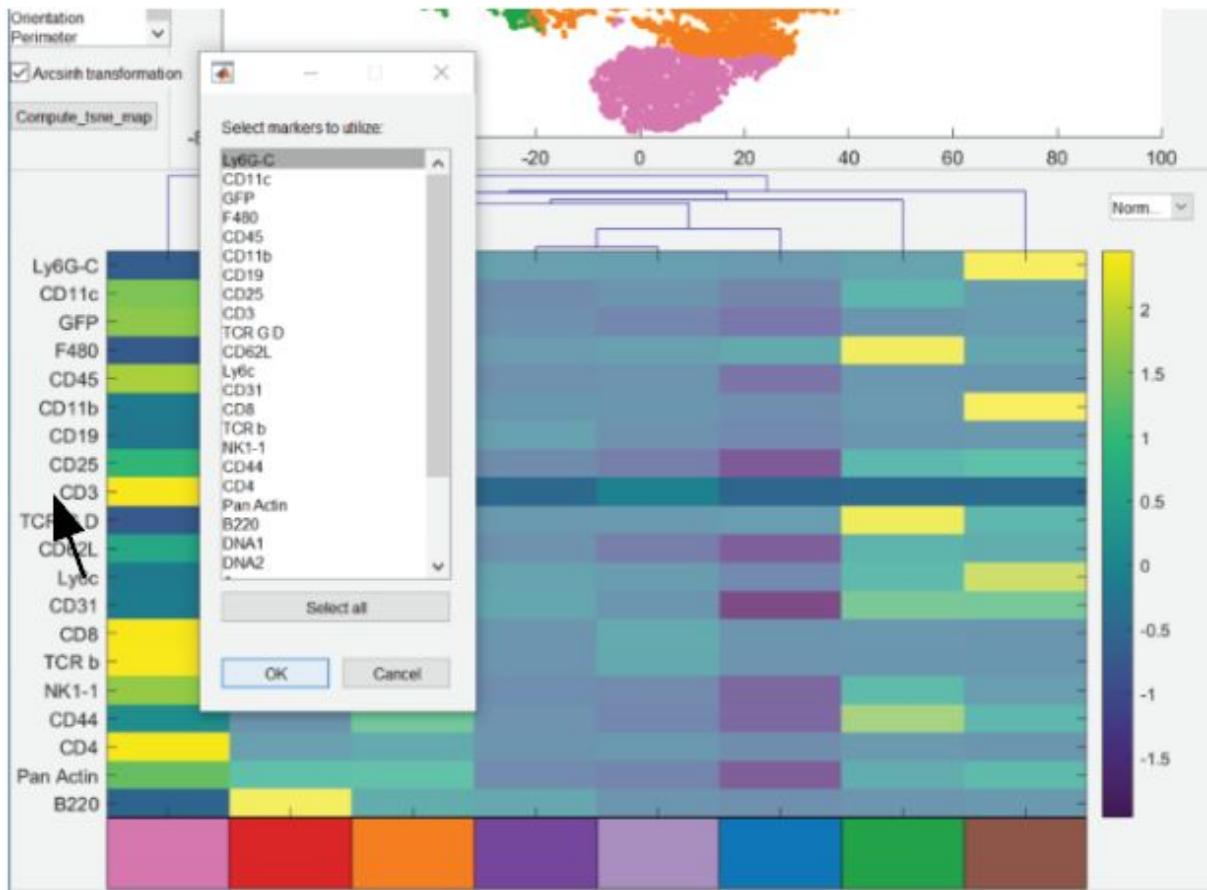
- Manual adjusting cluster names with single-clicking on the bottom bar (blue arrow)



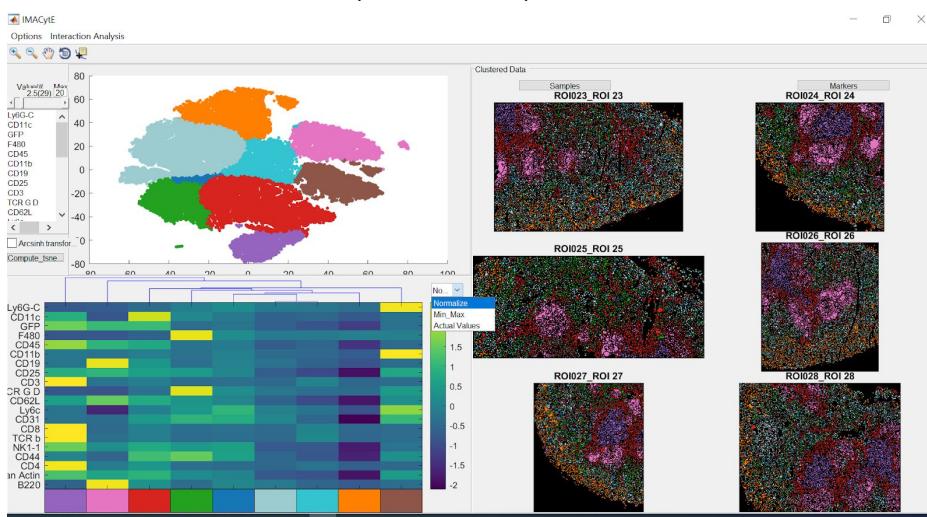
- Using data cursor to check the name of a cluster from heatmap



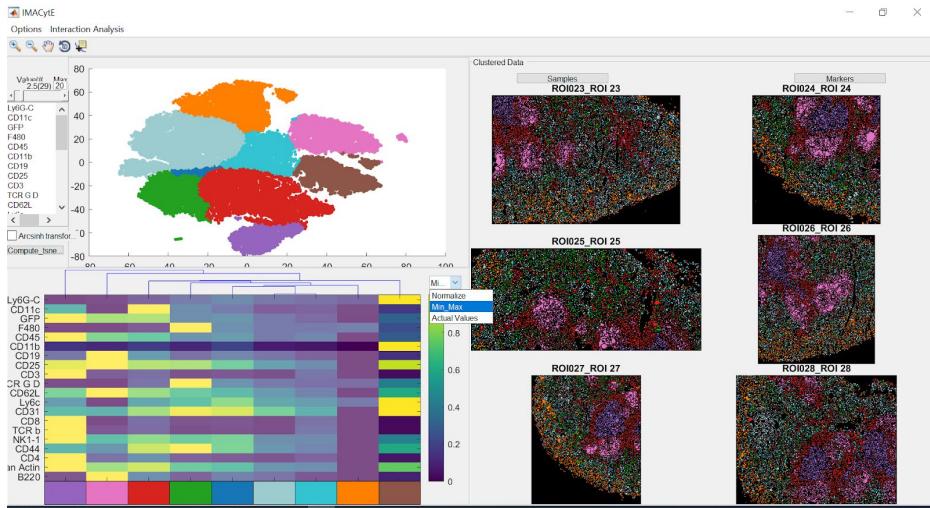
- Double-click on the right side of the heatmap with the marker names to adjust the shown markers



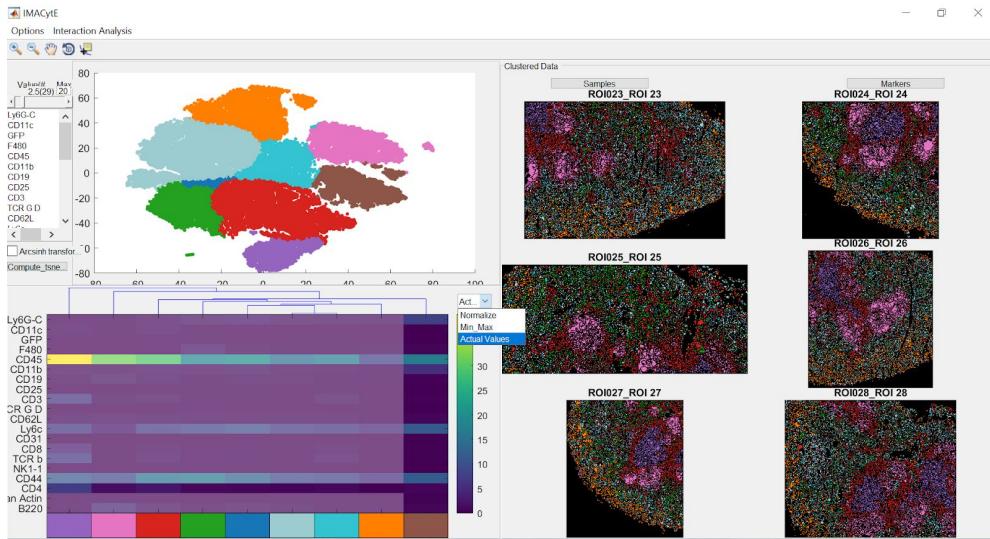
- Clusters' data can be represented on the heatmap in three different ways:
 - Normalised (x-mean / std)



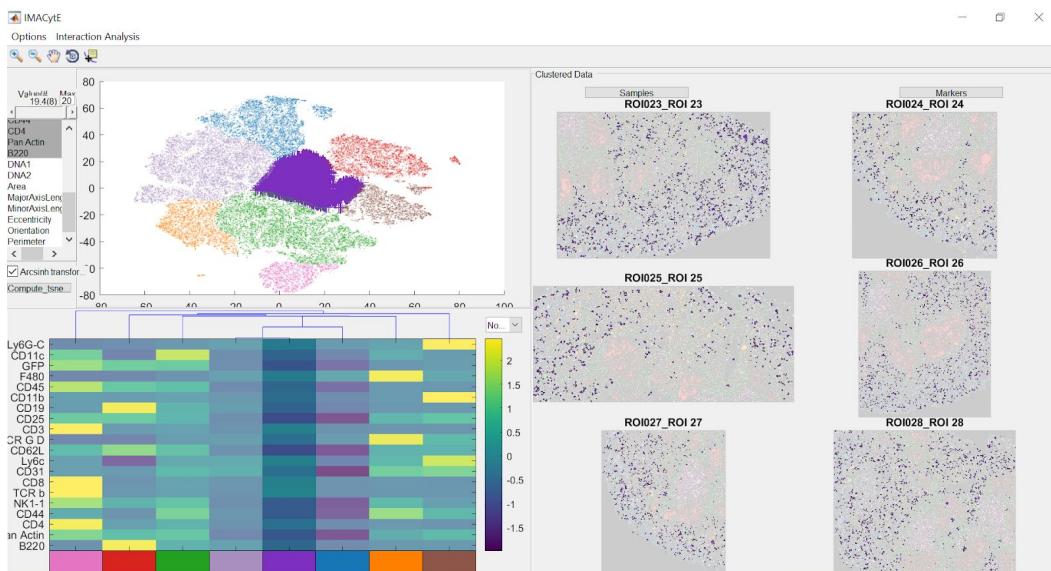
- Min_Max (0-1)



- Actual values



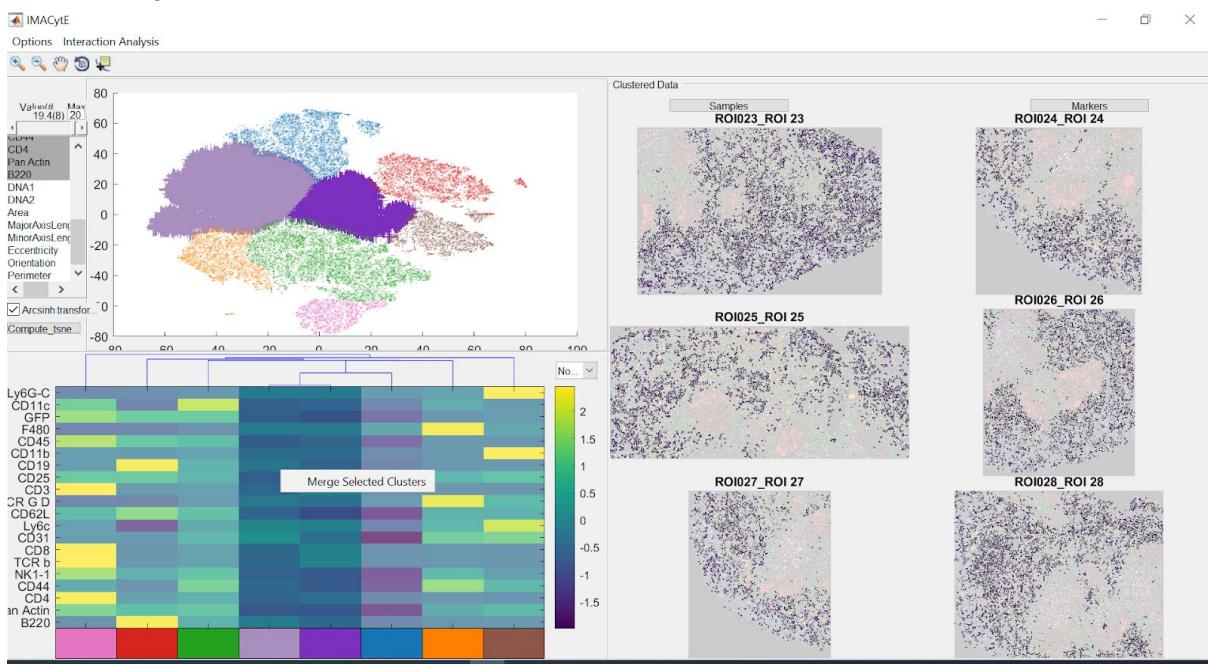
- Click on a column of heatmap to show the corresponding phenotypes.



- Second click unselect the column.
- Several phenotypes can be selected at the same time.



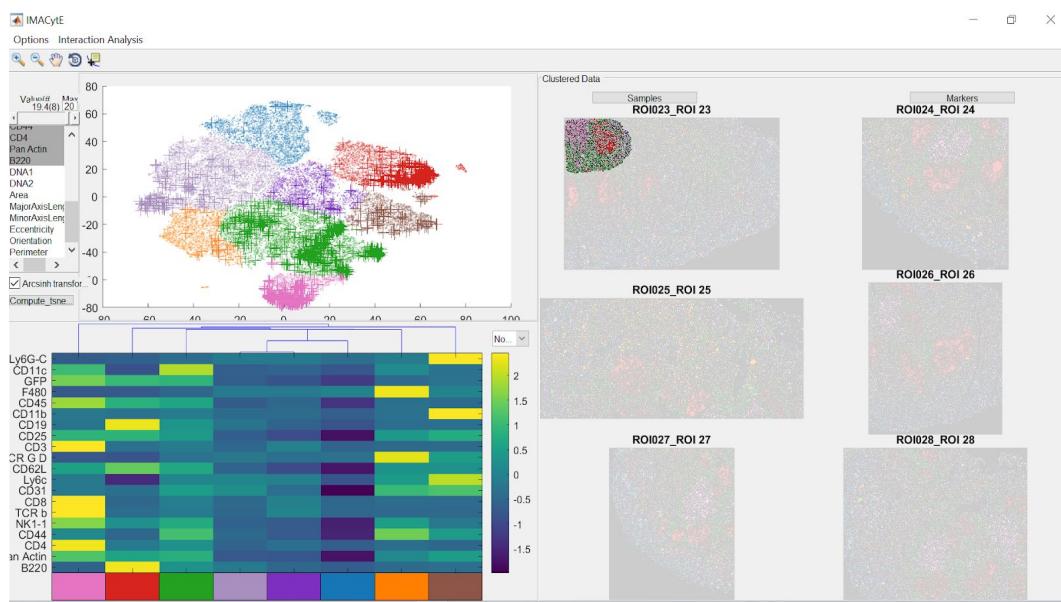
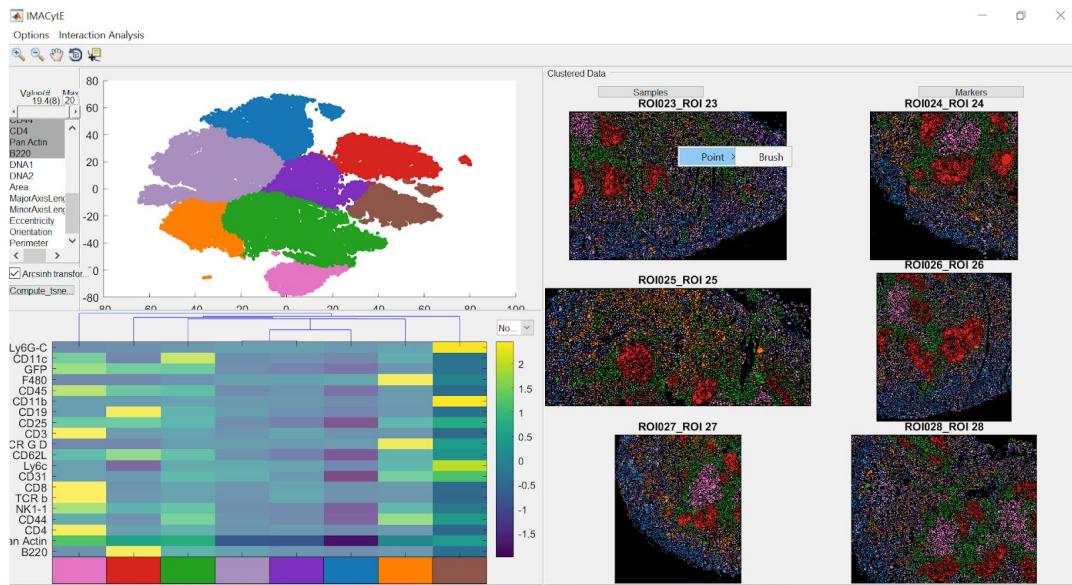
- Double click unselect everything.
 - Merge clusters right clicking on the heatmap.
- Select the clusters to be merged by clicking on their columns on the heatmap. Right click on them and click “Merge Selected Clusters”. A name must be given to the new merged cluster.



- Save the heatmap by right click on the grey area out of the heatmap. This will produce a figure of the heatmap and dendrogram

tSNE -Tissue linkage

- Specific cells can be selected by clicking on dots in the scatter plot or the cells in the tissue view.
- Areas can be selected by right clicking on the plot or sample image and select brush. Hold the mouse's left button and highlight the area of interest.

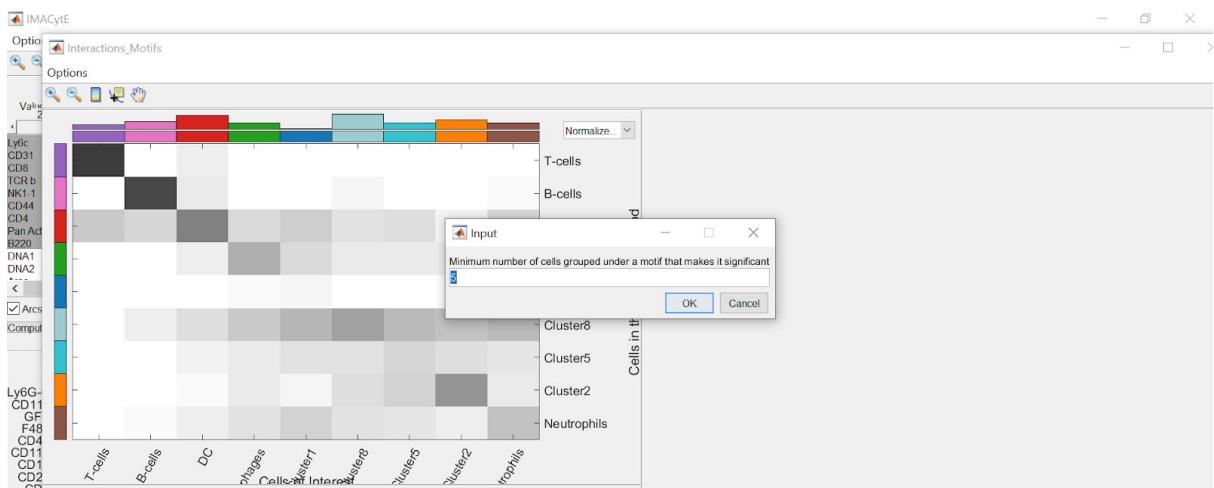


- With double clicking, any selection is erased
- Save tissue samples
Right clicking on the tissue area you can save all the tissue images, as presented at this moment in the viewer.

Cell microenvironments exploration

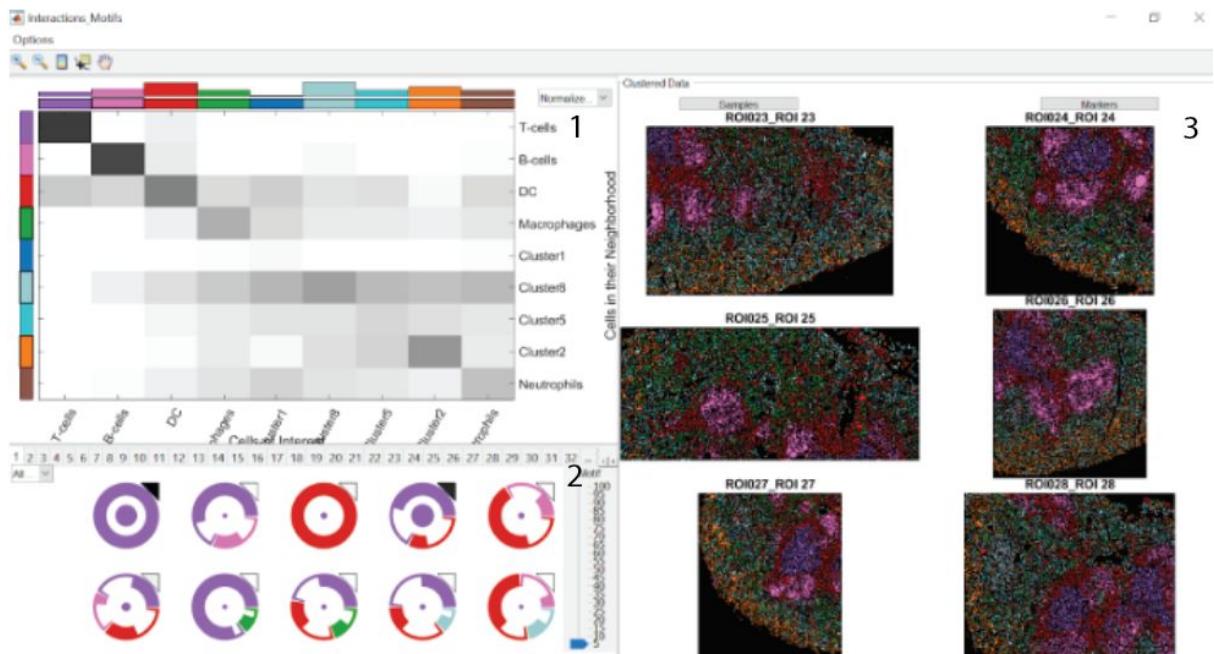
Getting started with the microenvironment exploration

- Select which samples you want to use for a session.
- Select number of pixels making two cells adjacent. If the distance among cells is big, this number can be increased.
- Set minimum number of cells that should be grouped under a motif, that's the minimum number of repetitions of the microenvironment combination



The initial screen will show us the three main windows:

1. Interaction heatmap
2. Glyph motifs
3. Tissue samples



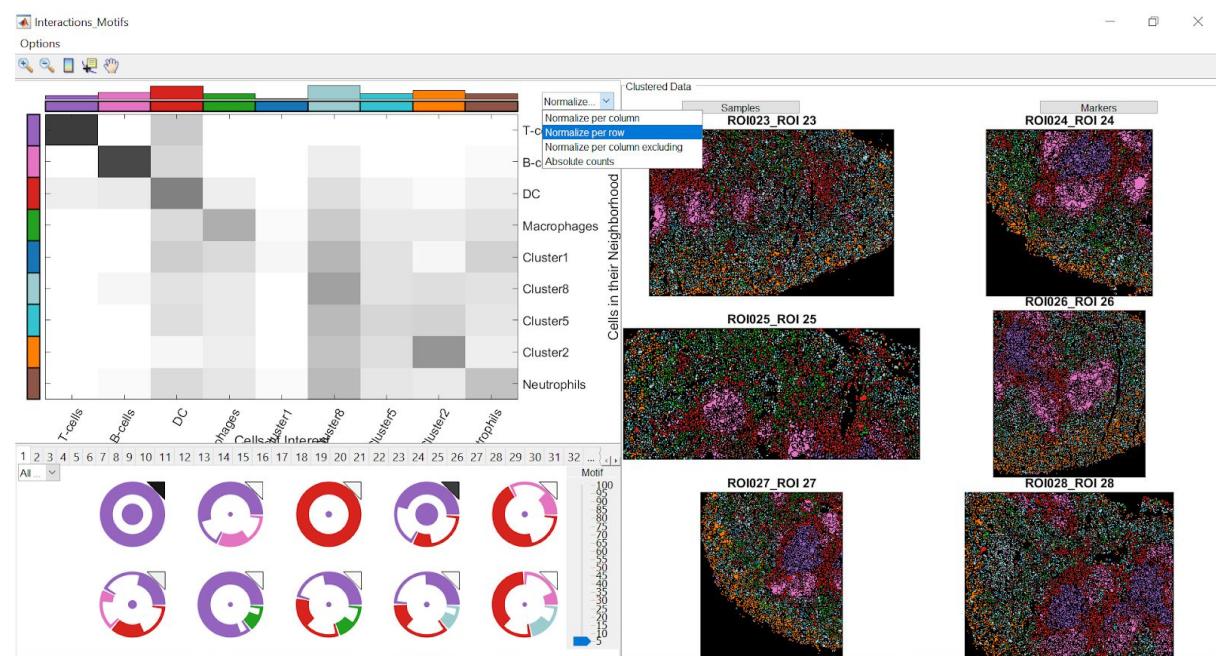
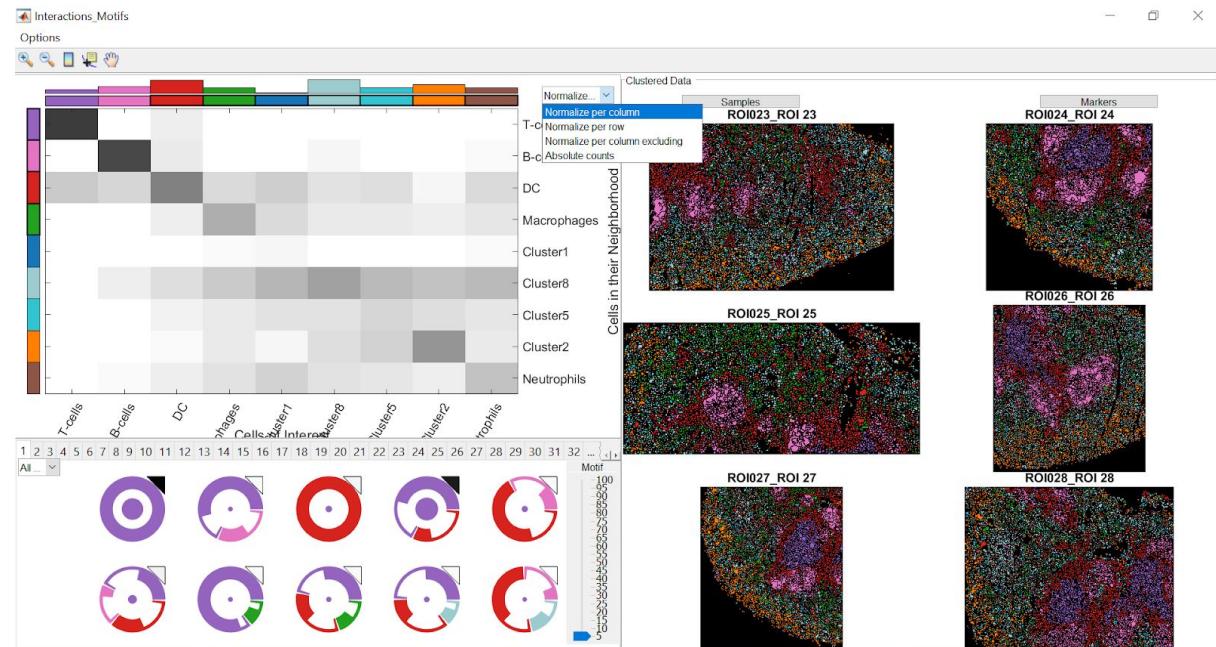
Overview of spatial interactions

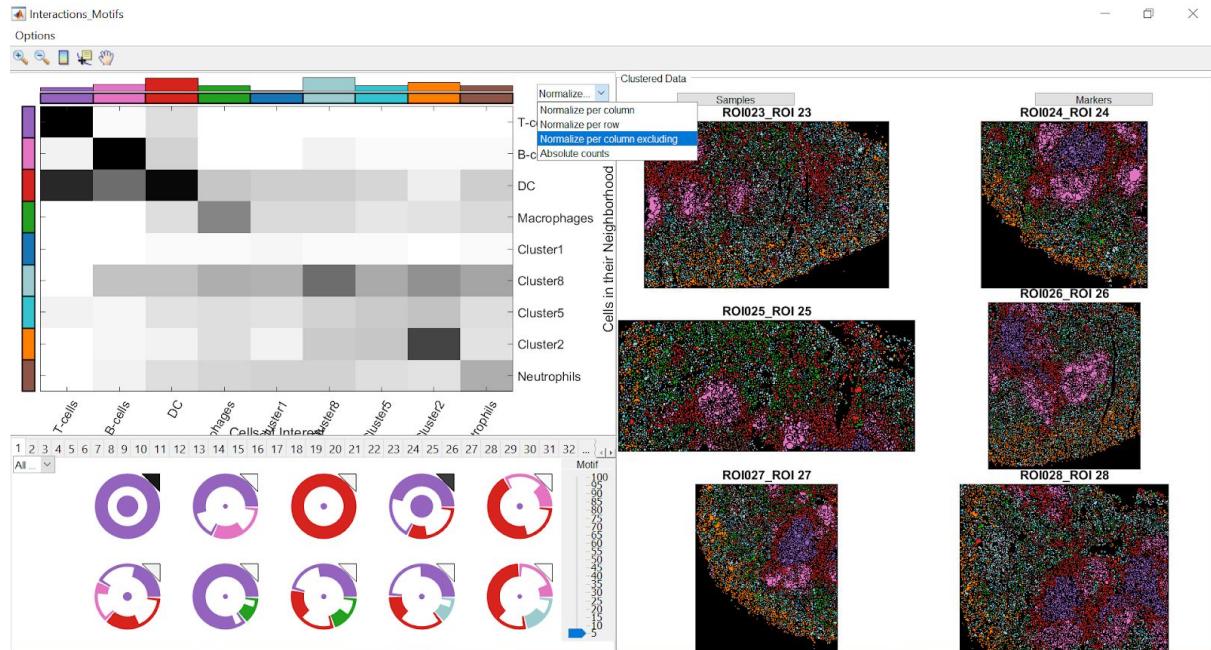
Interactions heatmap

The interaction heatmap shows rows with the cluster phenotypes and columns with the phenotype of interest and the population size (by the height of the top column)

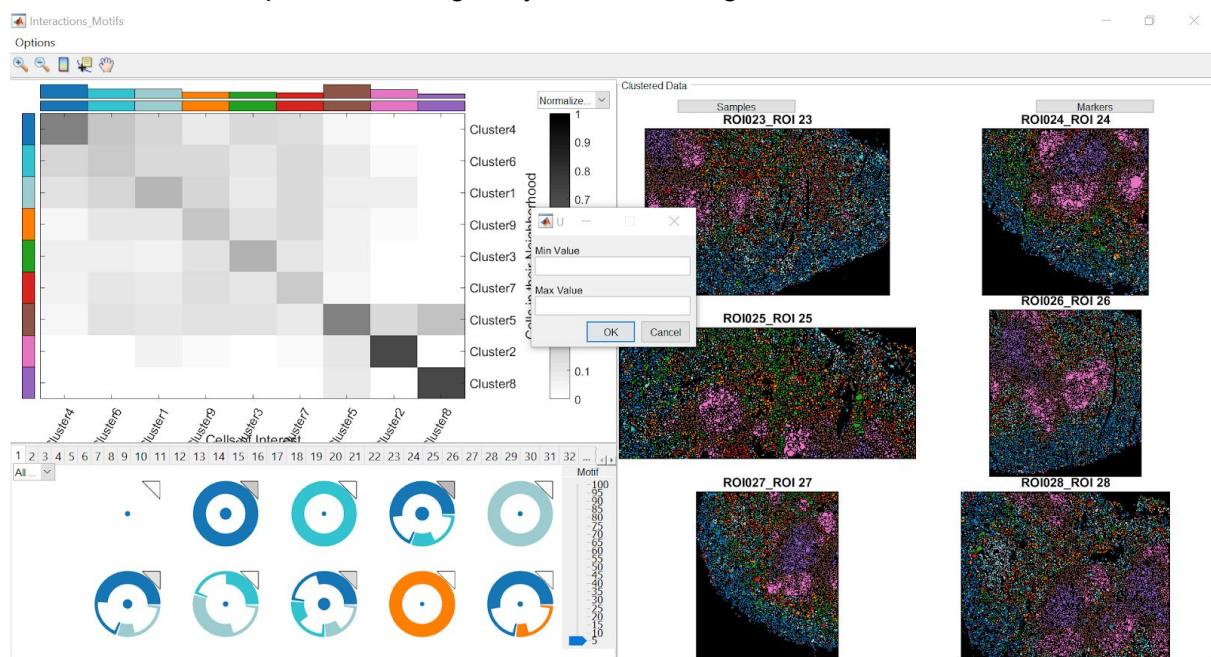
As with the previous heatmap, the data can be normalised in several ways. Here is an example of the normalisation differences.

Normalisation options:

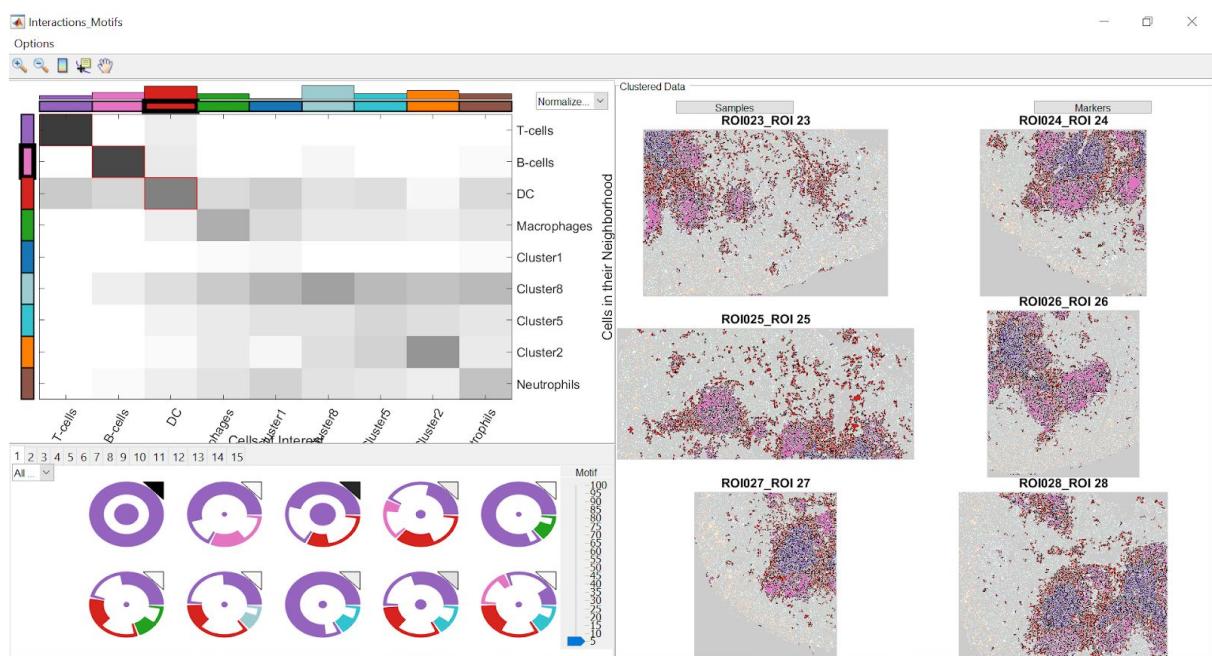
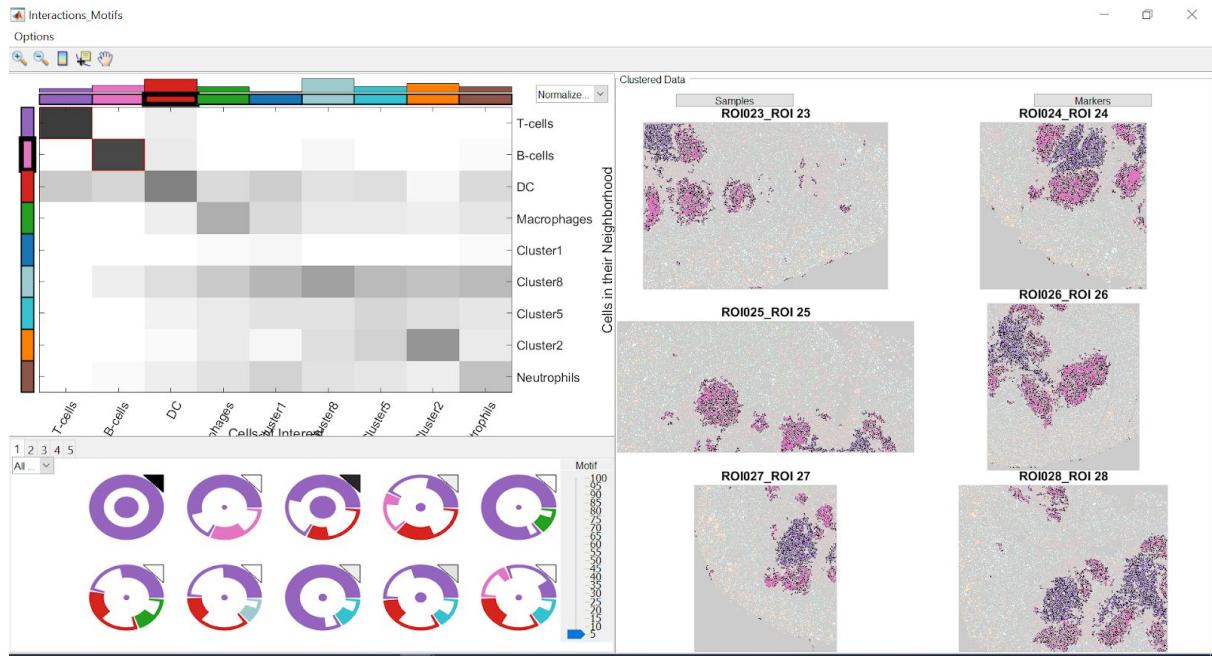


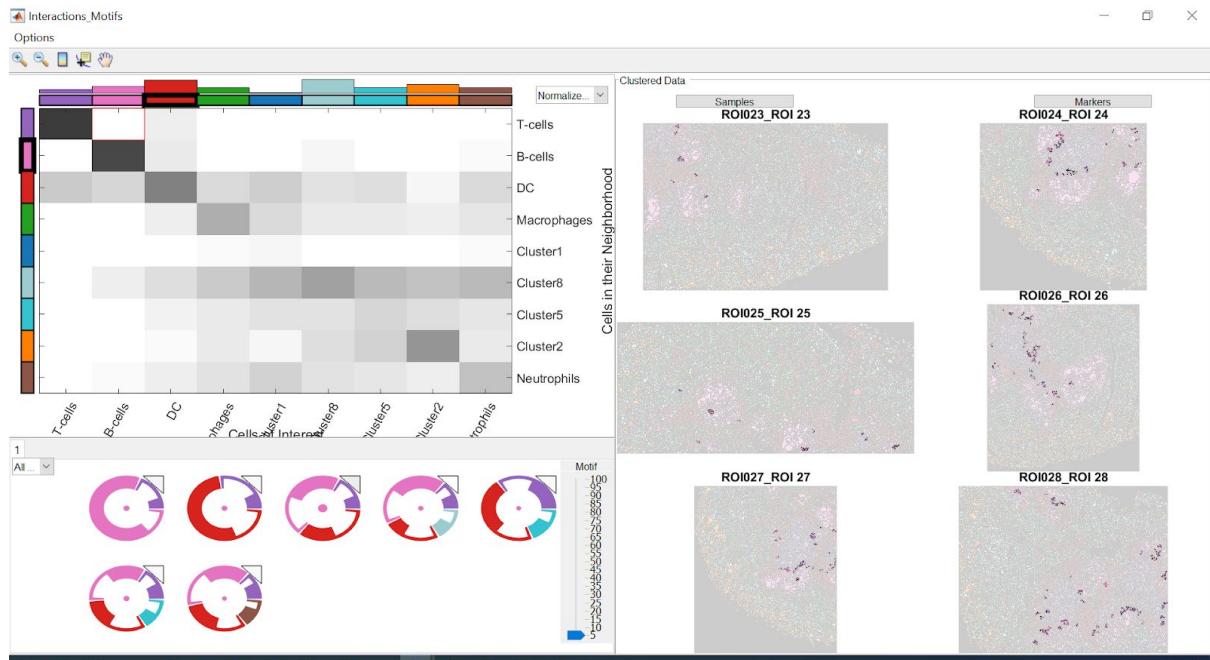


- The colormap can be changed by double clicking the colorbar

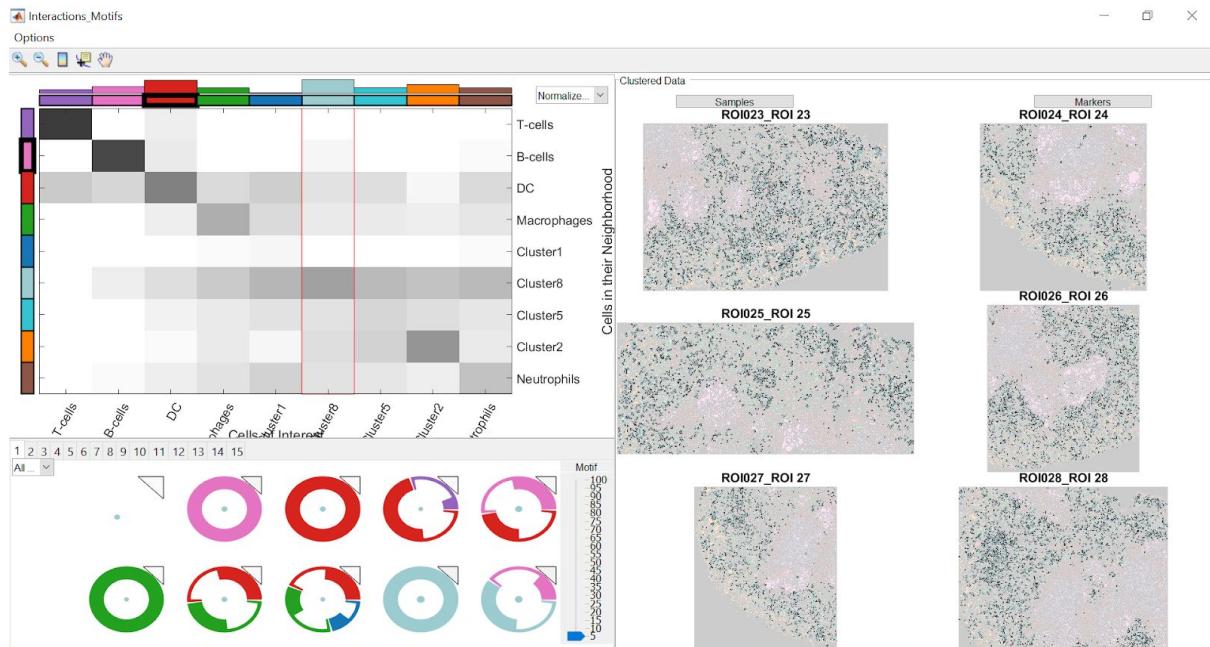


- Hovering options for each column, each box of the heatmap, bar graph on top of the heatmap
- Selecting any box of the heatmap highlights the interactions on the tissue samples and simultaneously filter the motifs, showing only the motifs where the column of the selected box matches the color of the motif's centre and the row of the box the phenotypes existing in the arcs of the motif glyph.





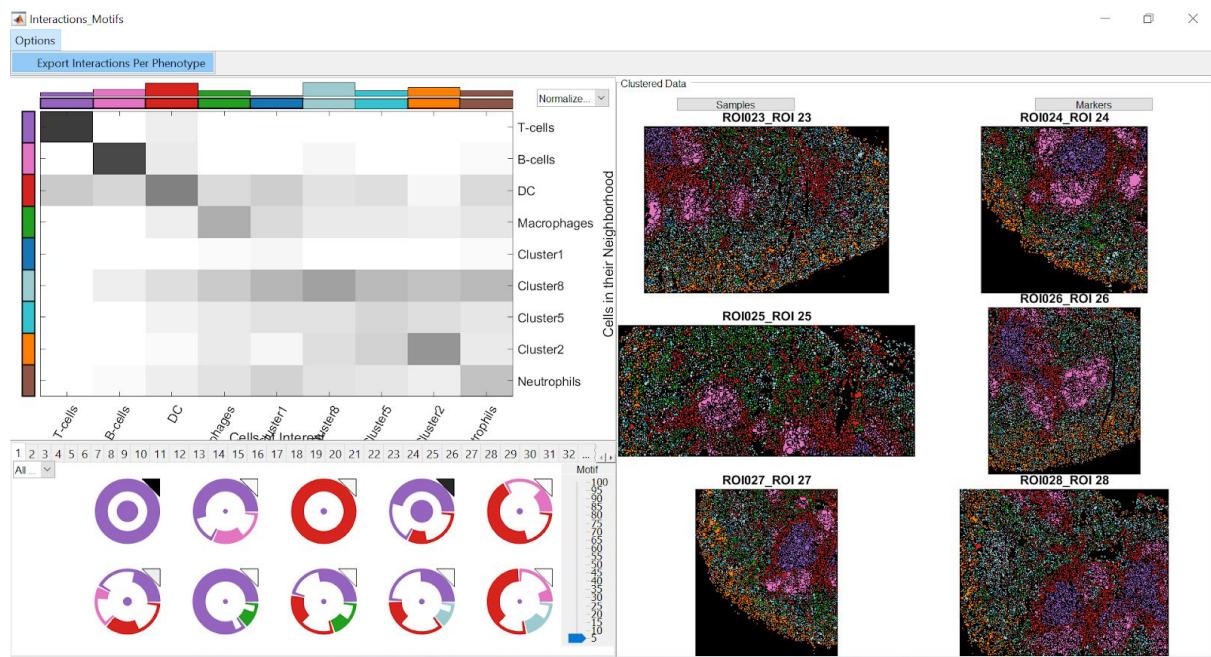
- Clicking on any of the boxes above the heatmap all the interactions of the cells of the corresponding cluster are highlighted in the tissue and motif's view.



- Export Interactions per Phenotype.

This option will export a .csv file with the following information:

- Phenotype of interest
- Phenotype of microenvironment
- Counts of all the colocalized cells
- Counts of at least one colocalized cell



- Save the heatmap by right click on the grey area out of the heatmap. This will produce a figure of the heatmap and dendrogram

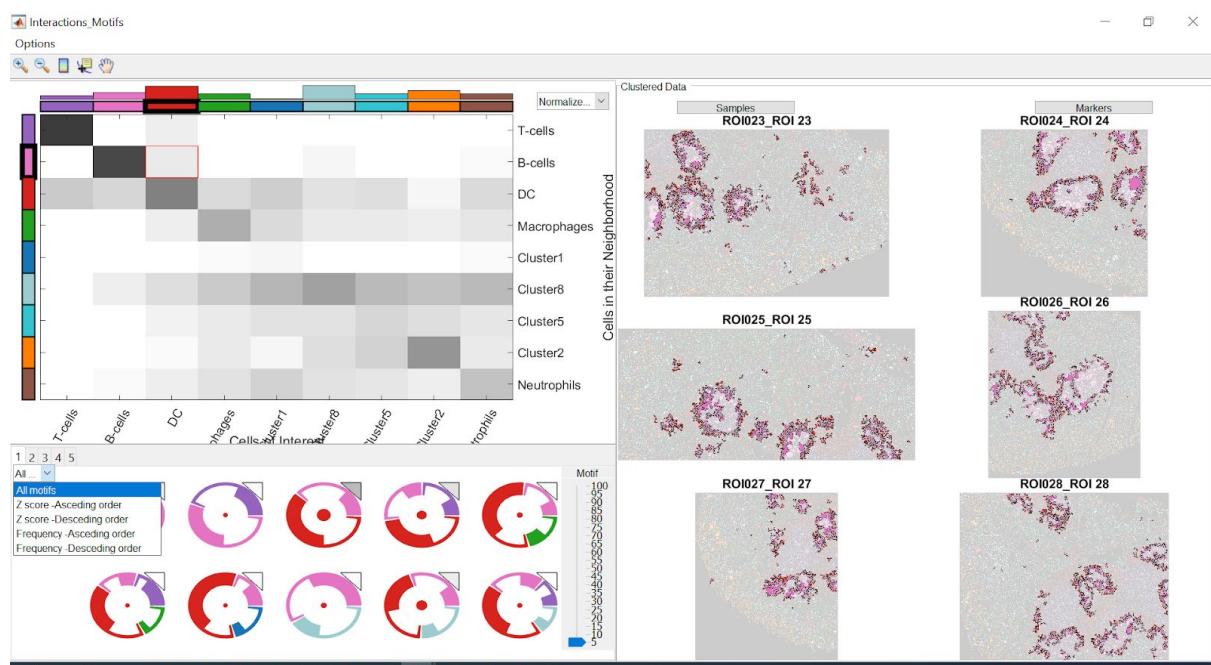
Details of spatial interactions

Motif-based Glyphs

Every glyph shows a triangle on the top right corner with the Z score which is measured with the formula: $Z \text{ score} = \frac{\text{the number of cells that are grouped under this motif} - \text{mean}(\text{permuted values})}{\text{std}(\text{permuted instances}) / \sqrt{\text{number of permutations}}}$. So Z score actually tells you how your motif differs from a random allocation of cells. The triangle is colour-coded: White means more significant; Black means more probability that it happens by chance.

The number of glyphs will depend on the number of squares selected from the interaction heatmap.

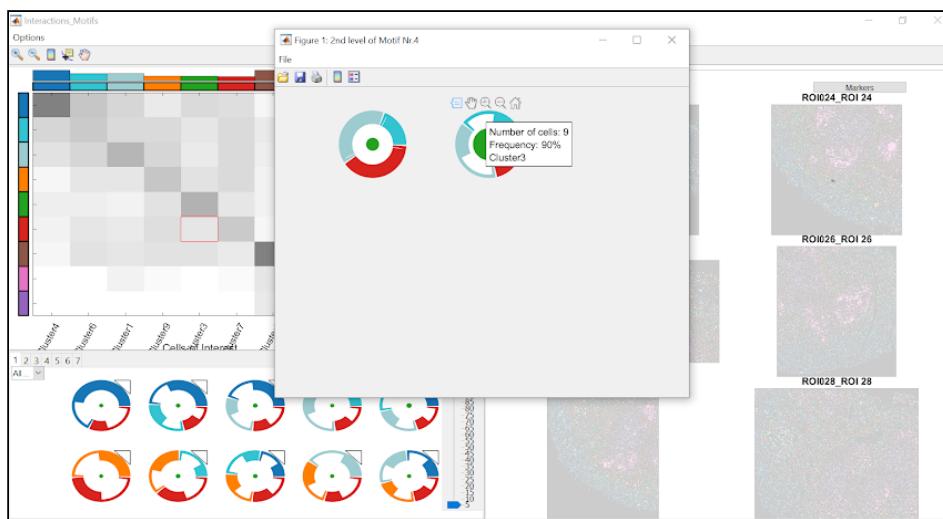
The glyph can be sorted in several ways.



The slider on the right changes number of cells grouped under a motif

Hovering over a motif offers information about the characteristics of each motif; the phenotype and the number of the cells grouped this motif, the phenotypes and the frequency of the phenotypes exist in the microenvironment of the cells, the z-score.

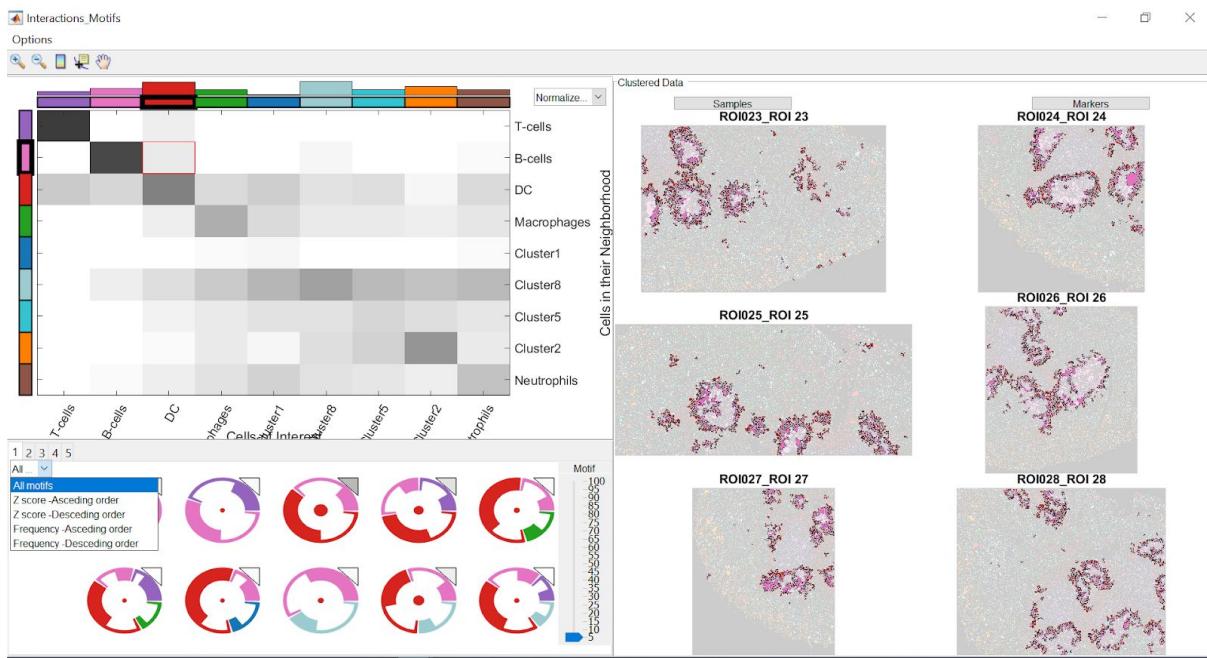
Selecting a motif to highlight its instances and its sub-motifs



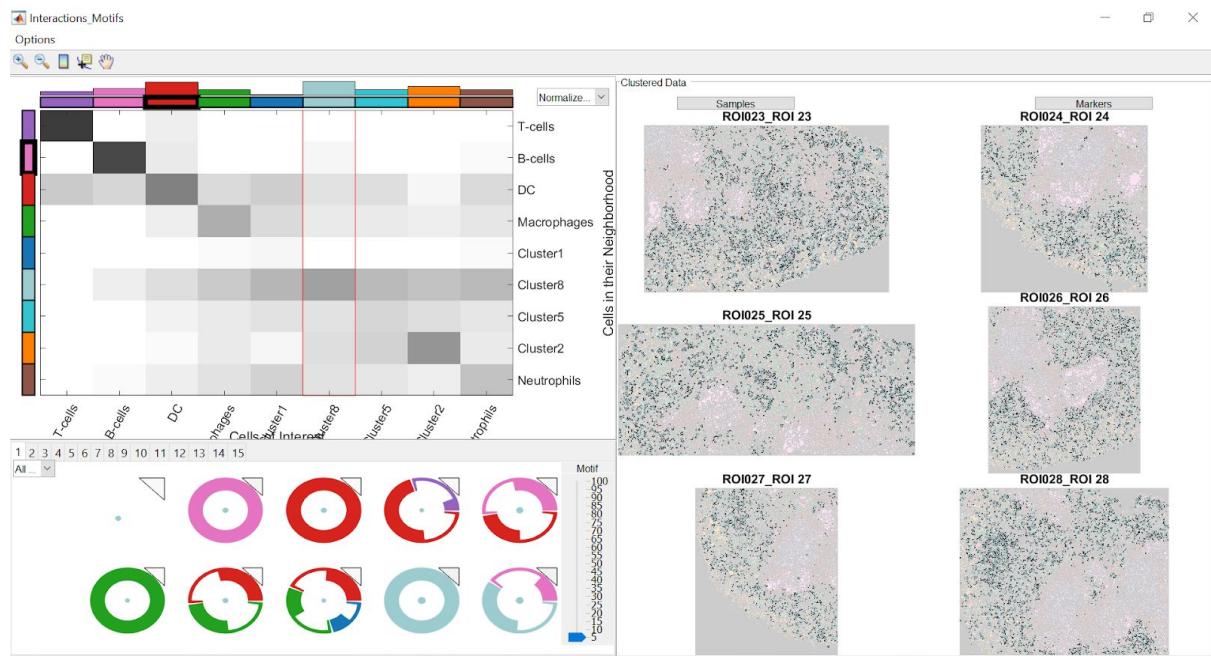
Save motif pictures by right-clicking on the white part of the motifs area.

As mentioned above, clicking on a box or a column, the population of interest will be highlighted on the samples and the glyph motifs of the cell interactions involved in the highlighted areas will show in the motif area.

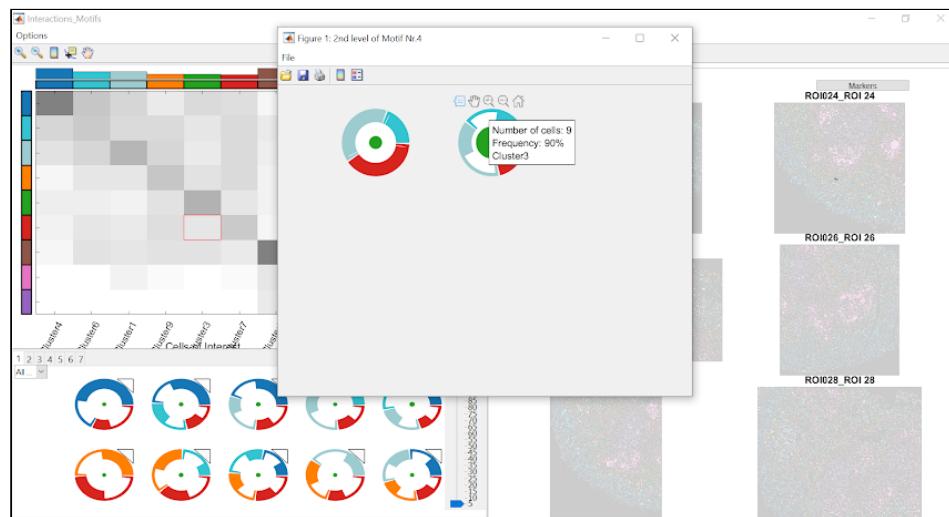
In the following examples, selecting the box from the heatmap of the red phenotype of interest having in its microenvironment the pink phenotype, we highlighted the corresponding interactions in the tissue samples and filtered the motifs, so as only the unique microenvironments of red cells that have at least the pink phenotype in their microenvironment are illustrated.



Also a whole column can be selected



Any glyph of interest can be analysed in more detail by clicking on it. We can also attach label tips, zoom in/out, and move it if needed.

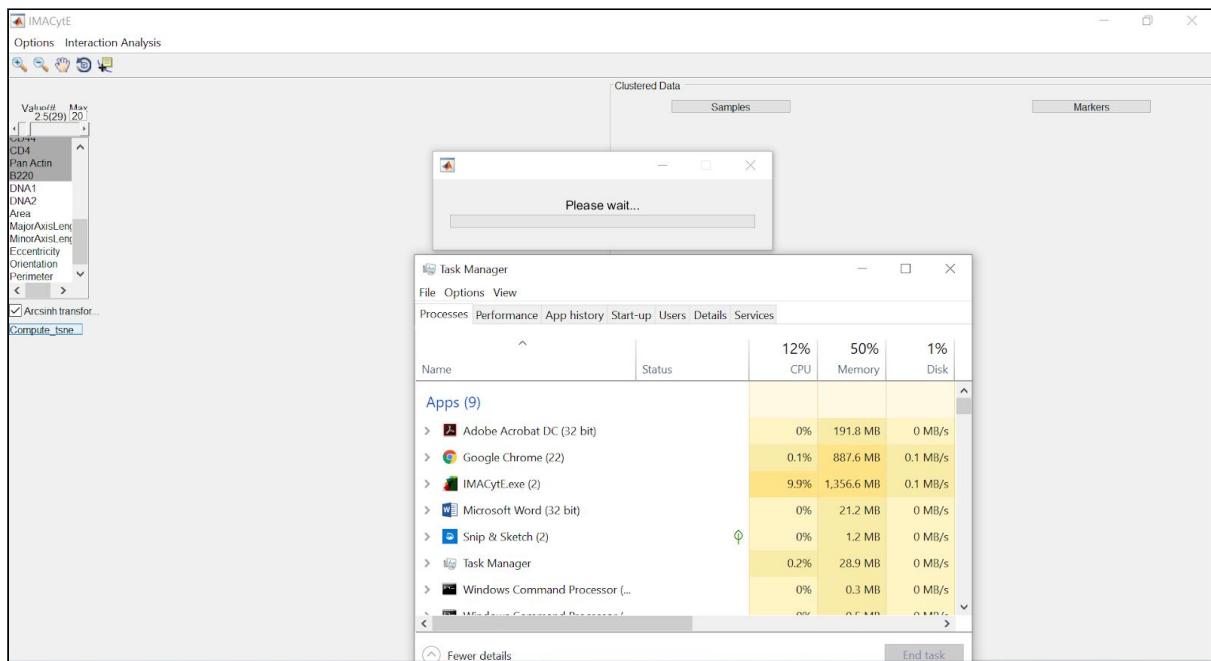


One more example showing the interactivity through IMACytE tools.



Extra notes

If running time is taking too long, the performance of IMACytE can be monitored with Task Manager. This is a way to check if IMACytE is still working or has failed to finish the current job.



Export / load Options

Export fcs files which can be used on other tools (like phenograph) to create clusters of similar phenotypes. These clusters can be loaded again on IMACytE for further studies.