⚠ This page is being updated, so some sections will be incomplete. Please feel free to use the instructions that are present, and we will update the page shortly. The prior version of this page is provided here: tSNE protocol 2017-04-04.pdf.

Introduction

This protocol describes how to perform Spectre's 'discovery workflow' using FlowJo – including data preparation, clustering with FlowSOM, downsampling, dimensionality reduction with UMAP, creating plots, annotating clusters, and performing quantitative and statistical analysis. For more information on this process, please see the main 'discovery workflow' page.

Citation

If you use Spectre, or Spectre-themed FlowJo workflows in your research, please consider citing Ashhurst TM, Marsh-Wakefield F, Putri GH et al. (2020). bioRxiv. 2020.10.22.349563. To continue providing open-source tools such as Spectre, it helps us if we can demonstrate that our efforts are contributing to analysis efforts in the community. Please also consider citing the authors of the individual packages or tools (e.g. CytoNorm, FlowSOM, tSNE, UMAP, etc) that are critical elements of your analysis work. We have provided some generic text that you can use for your methods section with each protocol and on the 'about' page.

Sample methods blurb

Here is a sample methods blurb for this workflow. You may need to adapt this text to reflect any changes made in your analysis.
Computational analysis of data was performed using the FlowJo using the analysis workflows from the Spectre R package (Ashhurst et al., 2020). The FlowSOM algorithm (Van Gassen et al., 2015) was then run on a merged dataset to cluster the data, where every cell is assigned to a specific cluster and metacluster. Subsequently, the data was downsampled and analysed by the dimensionality reduction algorithm Uniform Manifold Approximation and Projection (UMAP) (McInnes, Healy, Melville, 2018) for cellular visualisation.

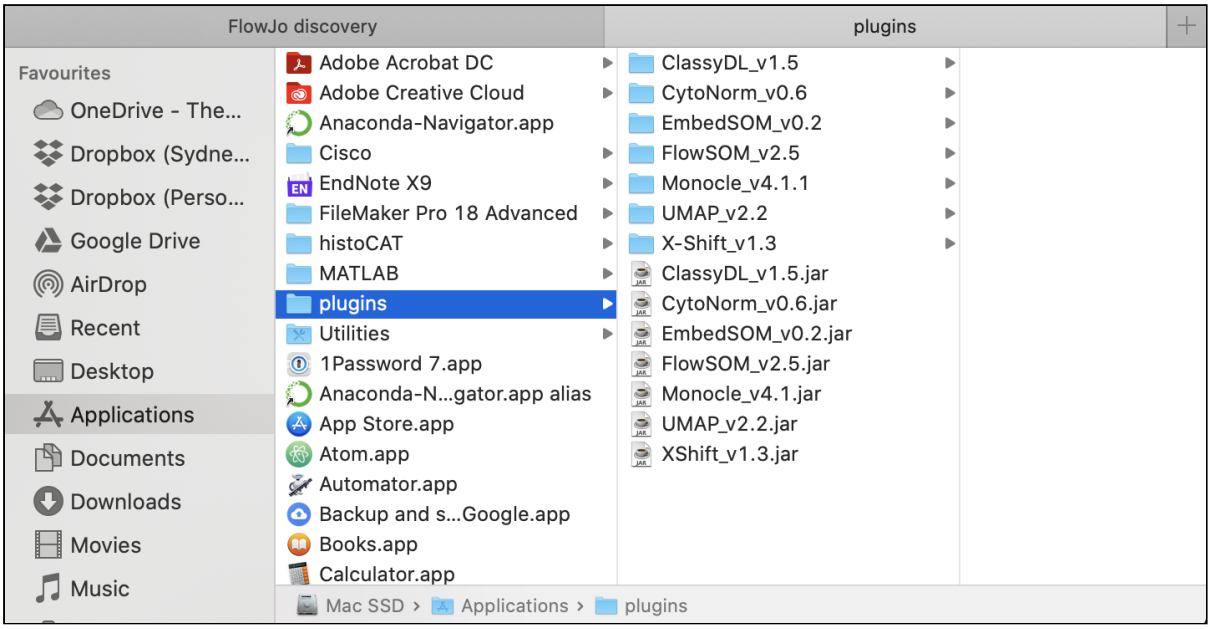
Setup FlowJo and plugins

For this workflow, you will need to install FlowJo and have access to a licence or dongle.

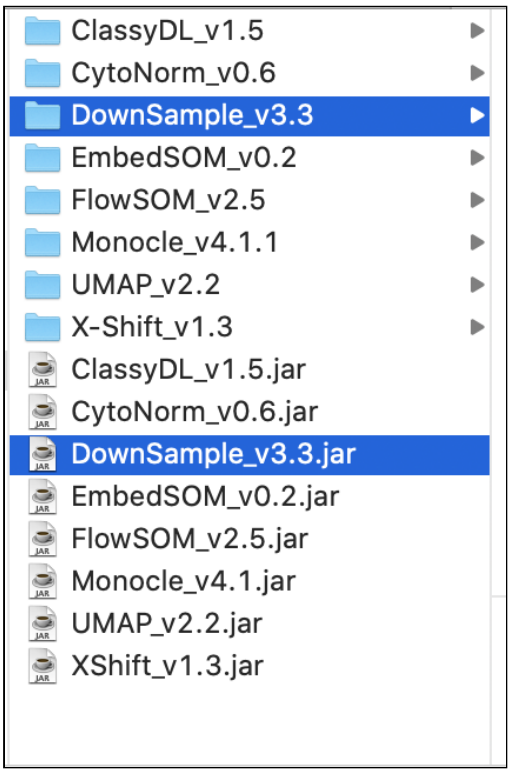
You will also need to ensure you have the following plugins installed:

- Downsample
- FlowSOM
- UMAP (or tSNE, FIt-SNE, etc)

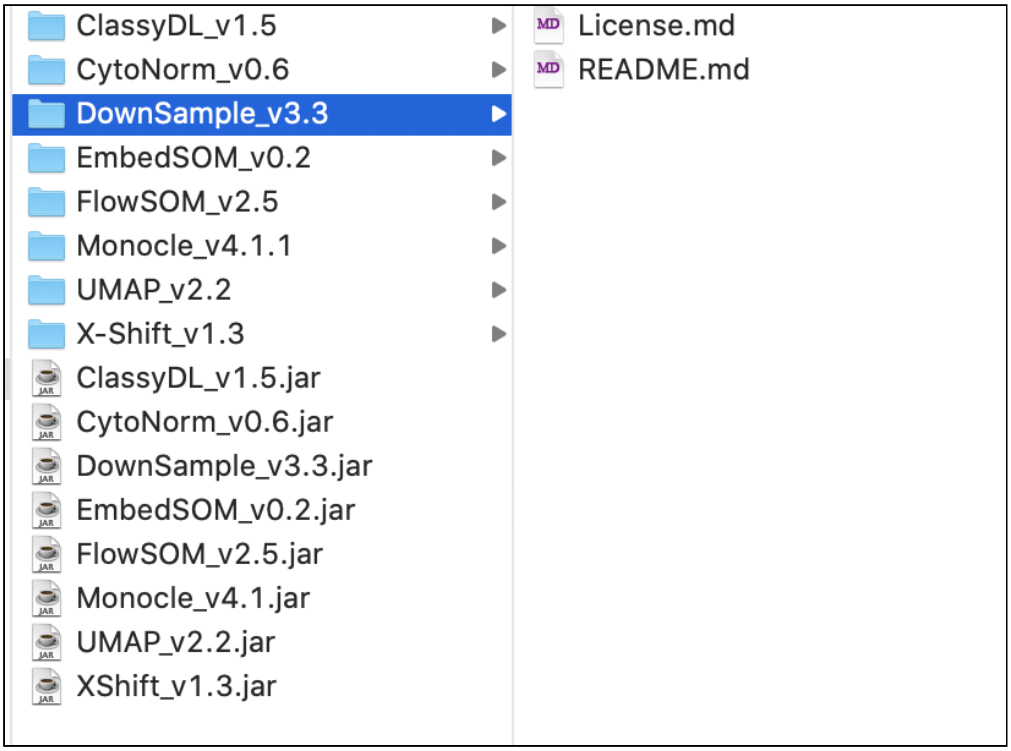
To check your packages, go to the 'Applications' folder (on Mac), and find the folder called 'plugins'.



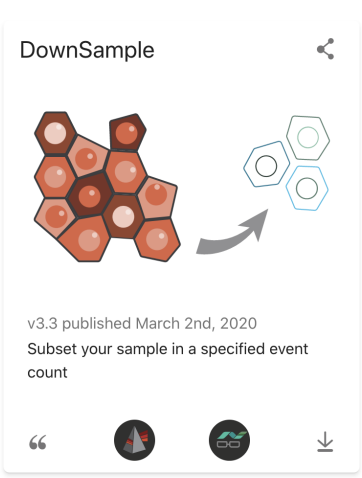
For each plugin you should see a '.jar' file, and possibly a folder of the same name.

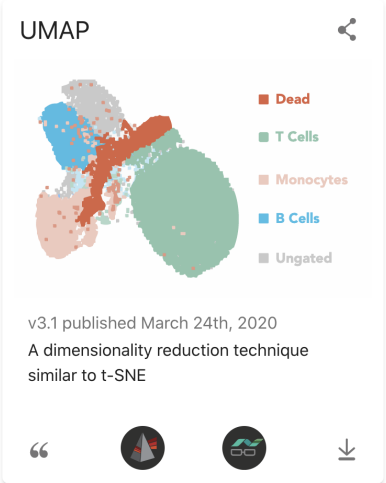


Within the each folder will be documentation related to that plugin.

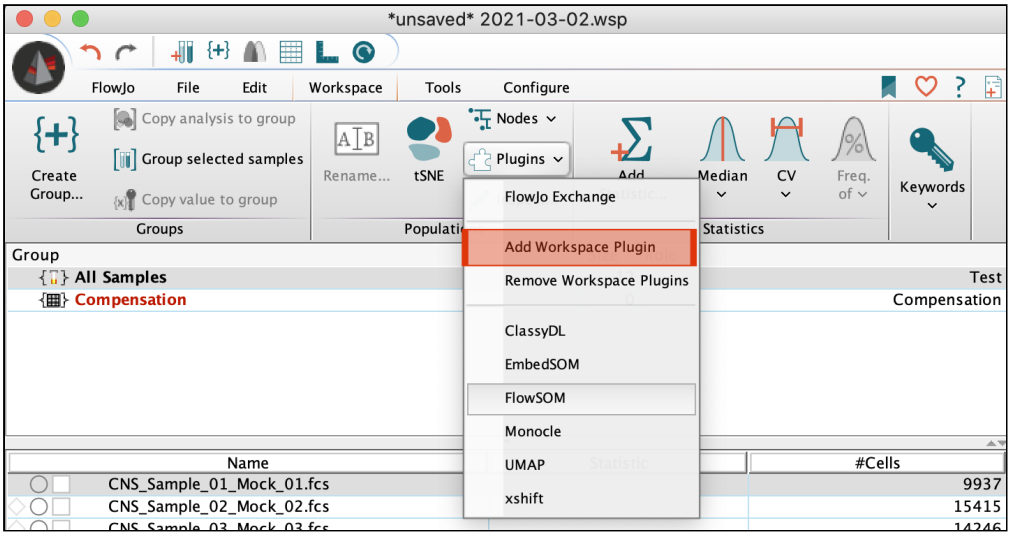


To download new plugins, go to the FlowJo exchange site. Download the following (if you don't already have them), unzip them, and move the .jar files (and optionally the associated folder) into the 'plugins' folder as above.

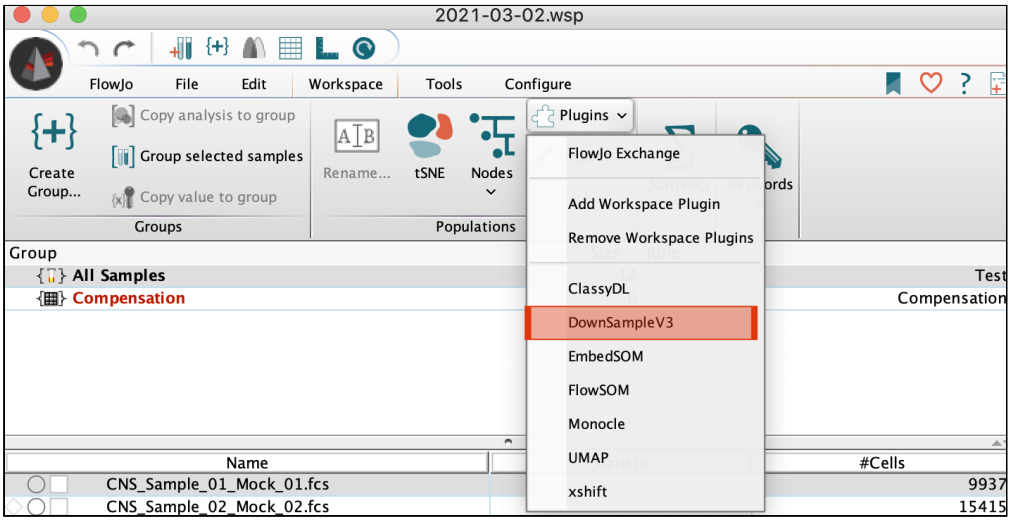




To activate them in FlowJo, go to Workspace/Plugins and select 'Add Workspace plugin'. You can select the plugins from your 'plugins' folder.

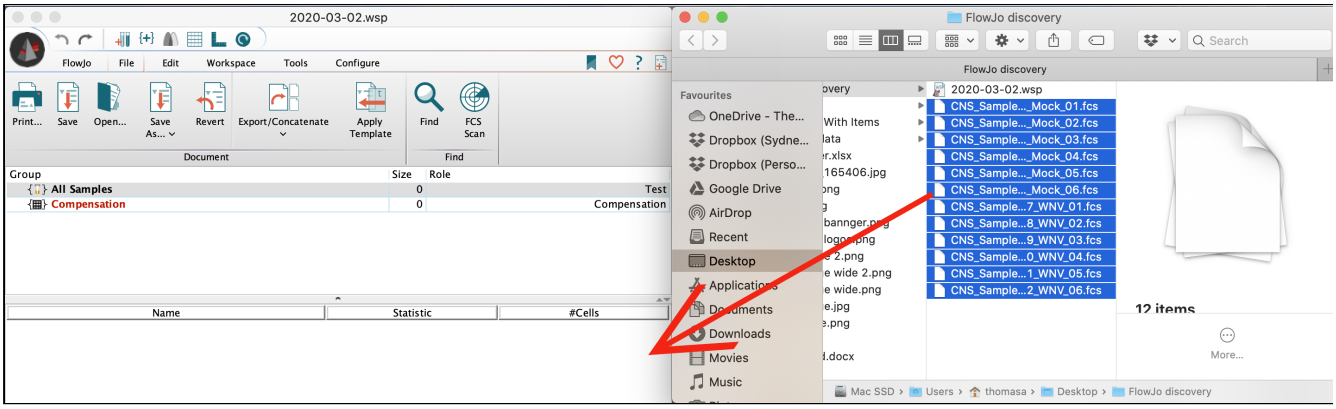


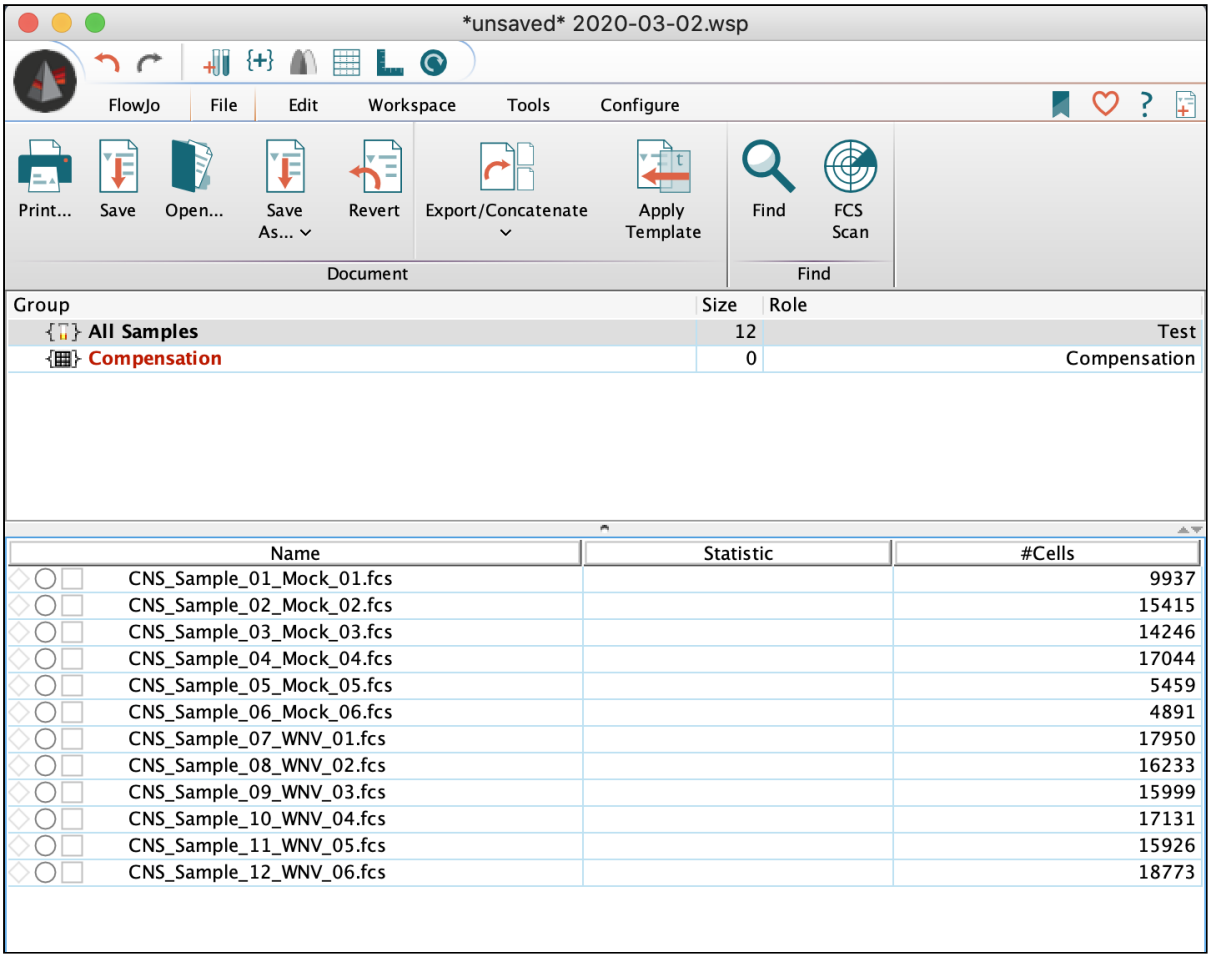
Once they have been 'activated' with FlowJo, they should show up in this list.



1. Data preparation and organisation

To start with, we want to pull our FCS files into FlowJo.





Gate to your population of interest (POI) in the first sample.

========

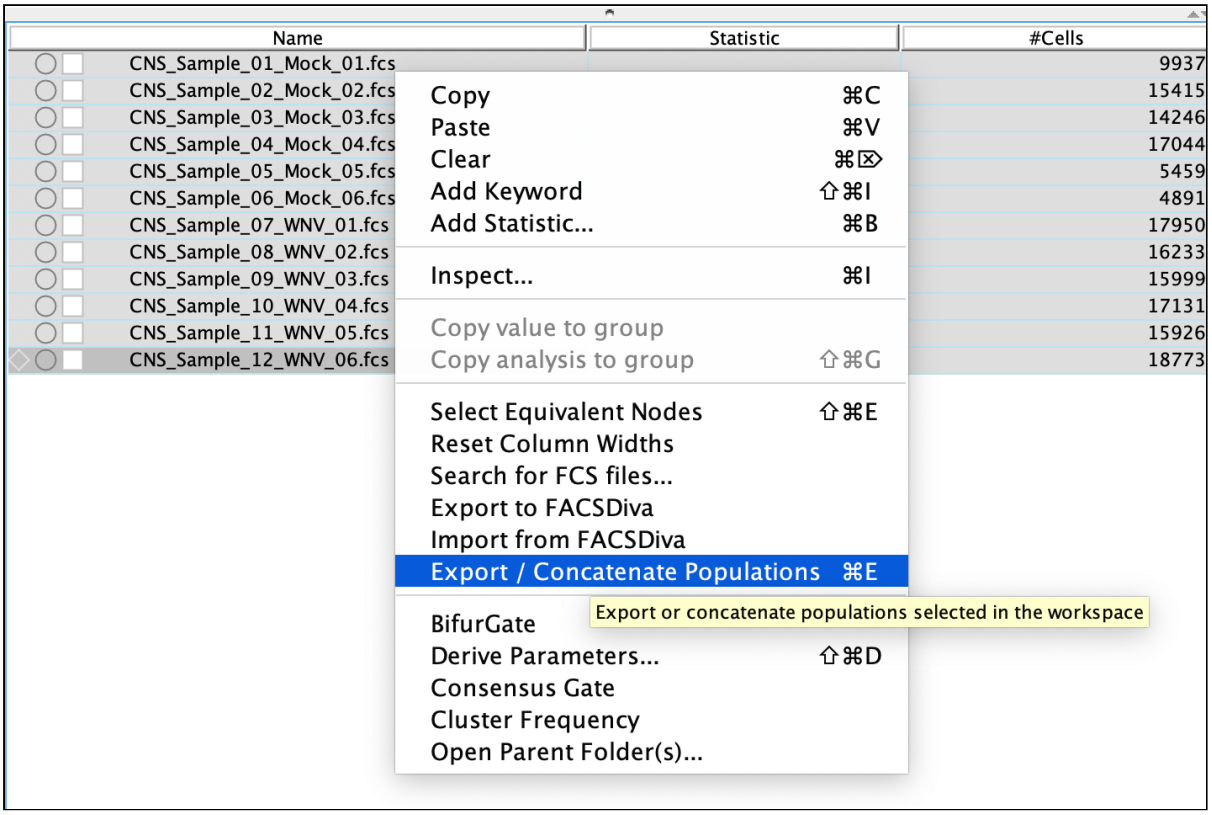
Add the gates to the group, and adjust for all samples, as per a normal analysis in FlowJo.

========

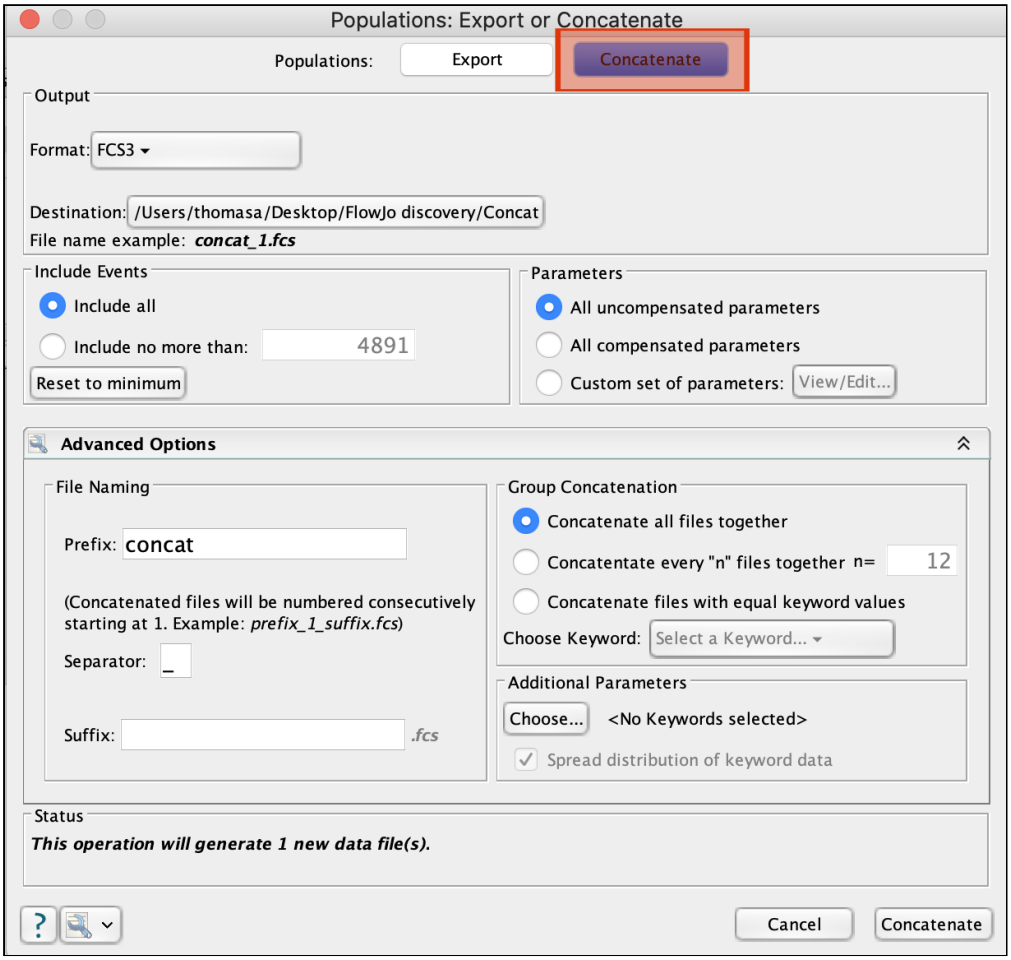
Select your POPI and use the 'select equivalent nodes' tool under the 'edit' menu.

========

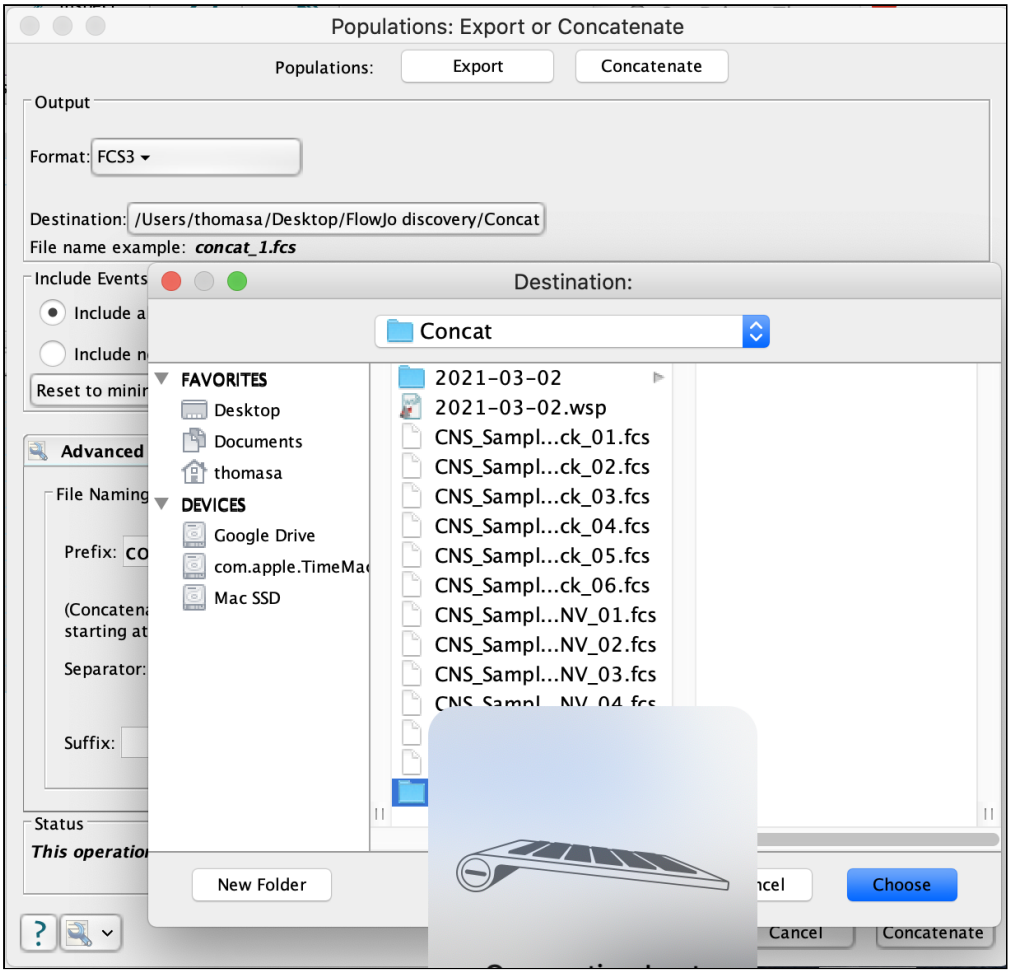
Right click on one of the nodes, and select 'Export / Concatenate Populations'.



In the new window, select 'Concatenate' at the top. We will be concatenating FCS files, so we can just use 'all uncompensated parameters'. By default, each sample will be separated in a new parameter called 'SampleID', where samples are organised alphabetically (I think it's alphabetical). Alternatively, you can add additional keywords, and add them as additional parameters to the concatenated file.



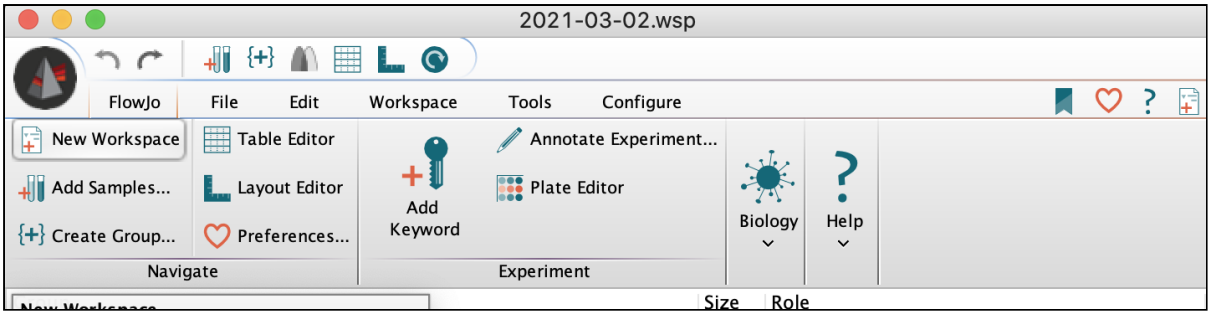
Choose an output location – best to create a folder within your existing experiment folder.



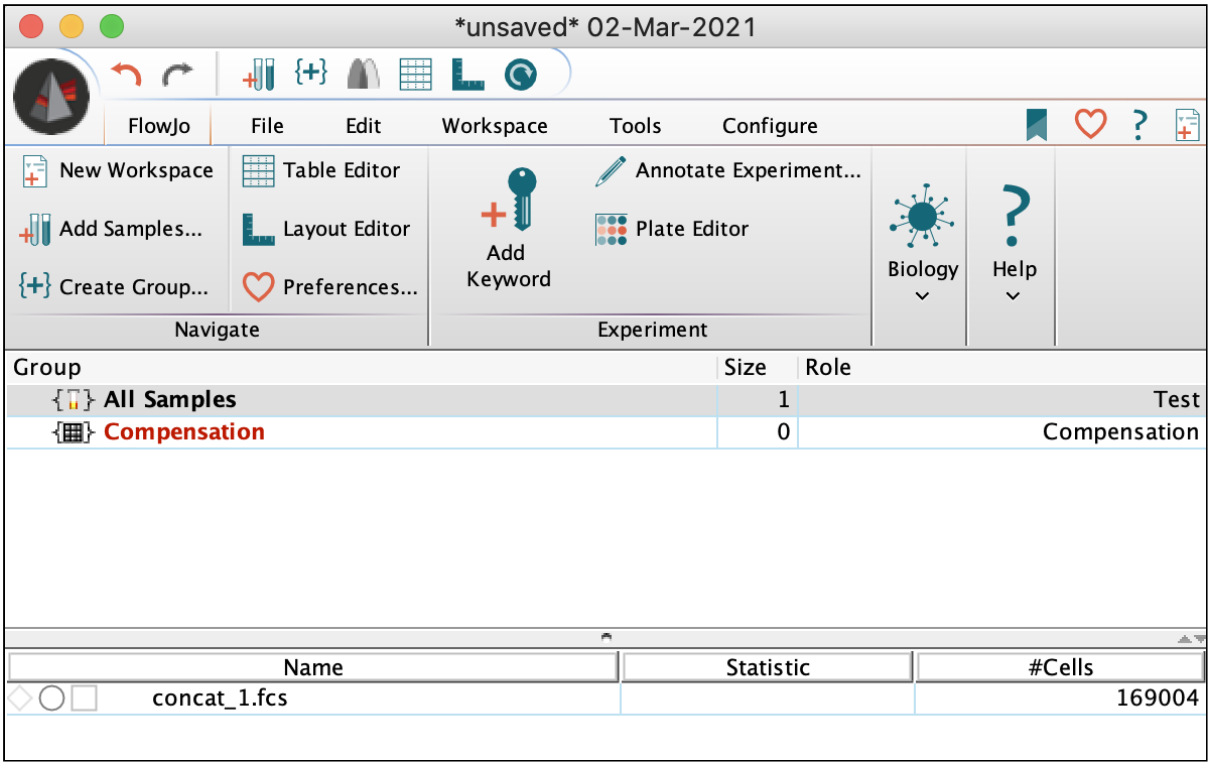
Click 'Concatenate' on the bottom right, and wait for the new FCS file to be created.

=====

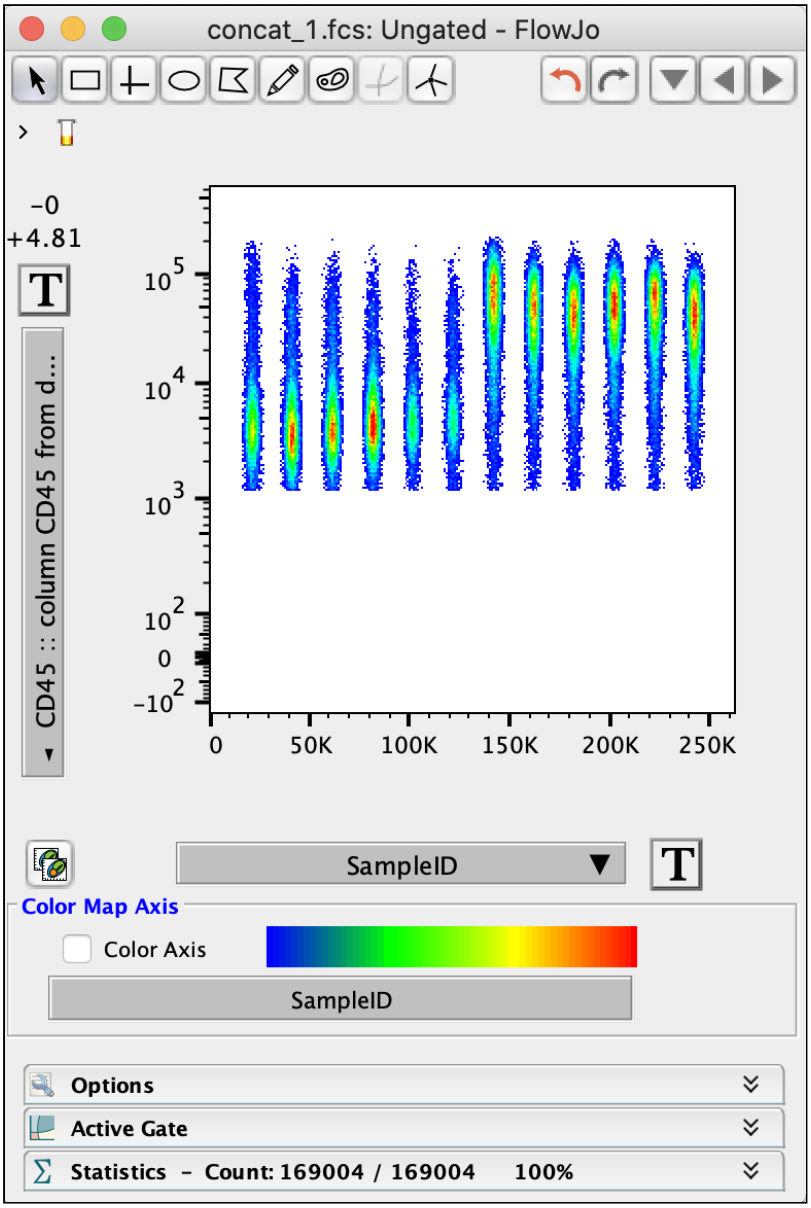
Open a NEW FlowJo workspace.



Drag the new FCS file into that workspace, and save the workspace in that folder.

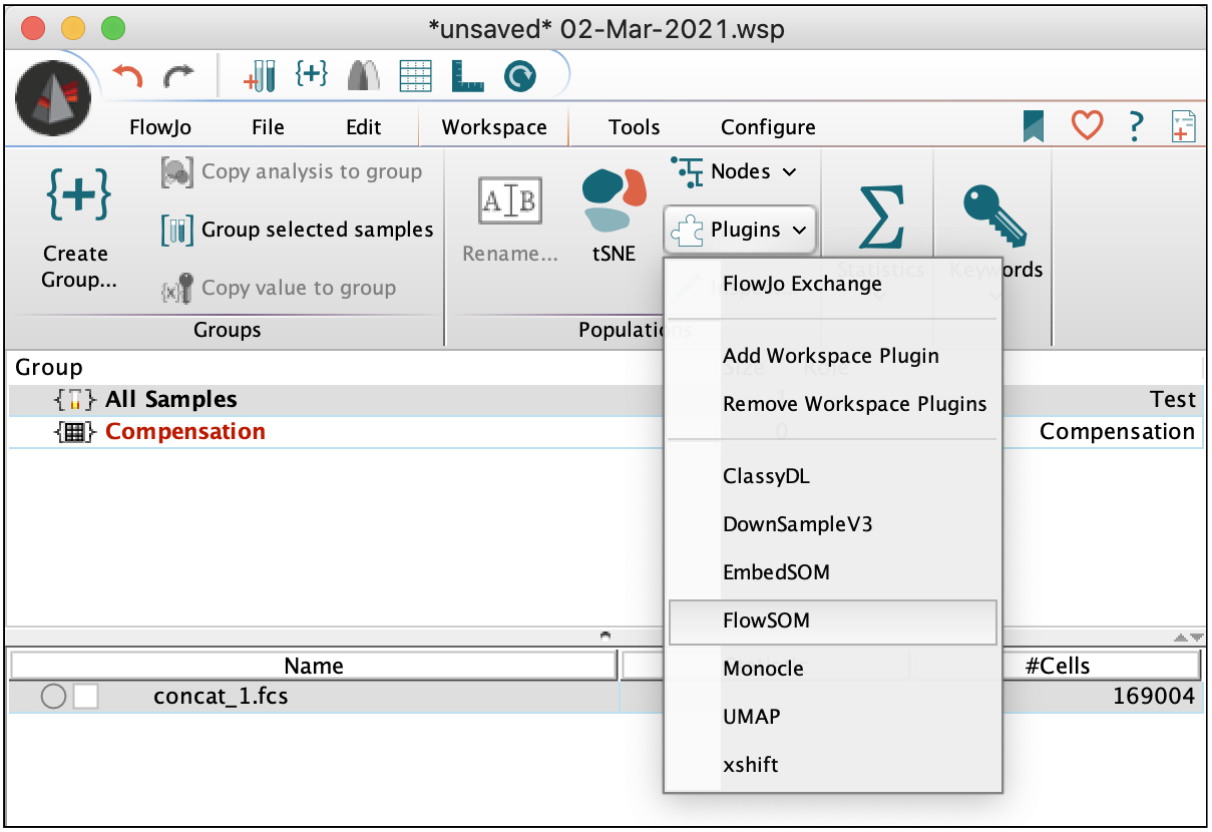


You can check to see that all your samples have been included by opening the file, and plotting some parameter against 'SampleID' (or your custom parameter).

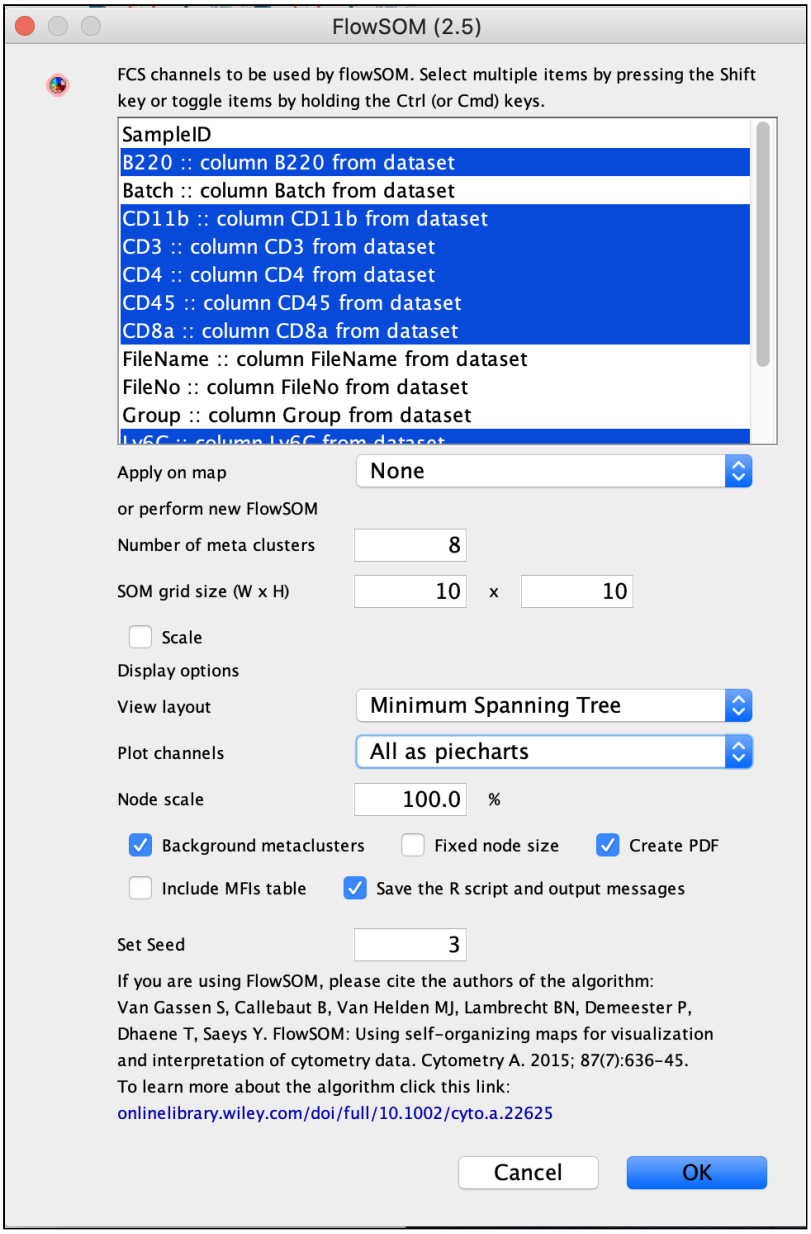


2. Clustering and dimensionality reduction

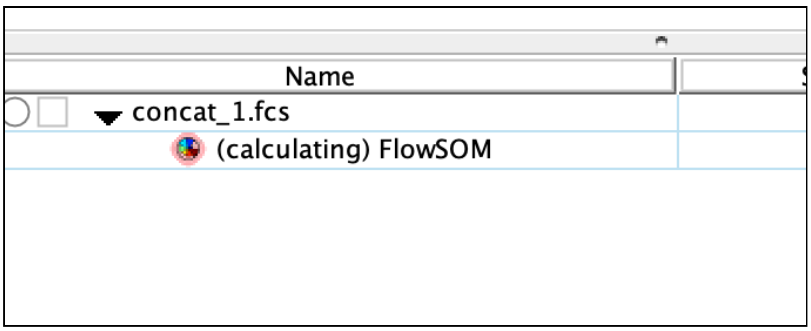
Select the file, and go to Workspace / Plugins / FlowSOM.

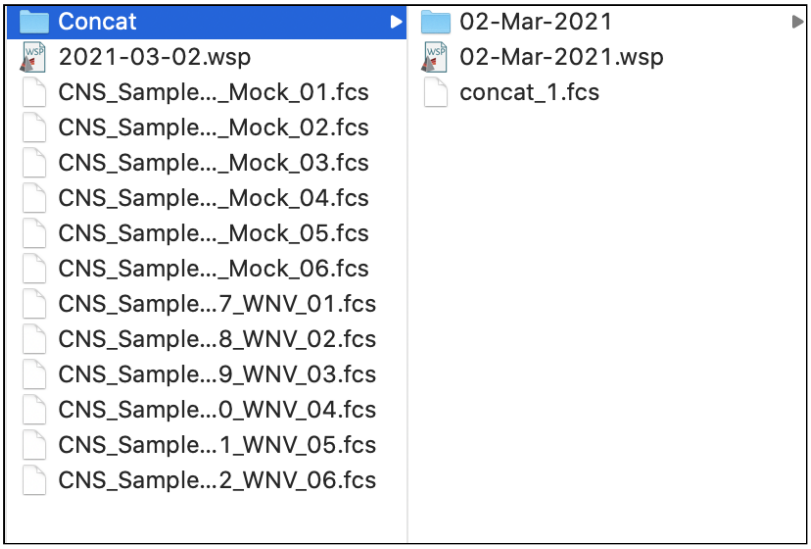


Choose the parameters you wish to use for FlowSOM clustering. You should also choose a target number of metaclusters. Click 'OK' when you are done.



You can see that FlowSOM is running by the appearance of this node below the file in FlowJo. You can also see some folders generated within your experiment folder.

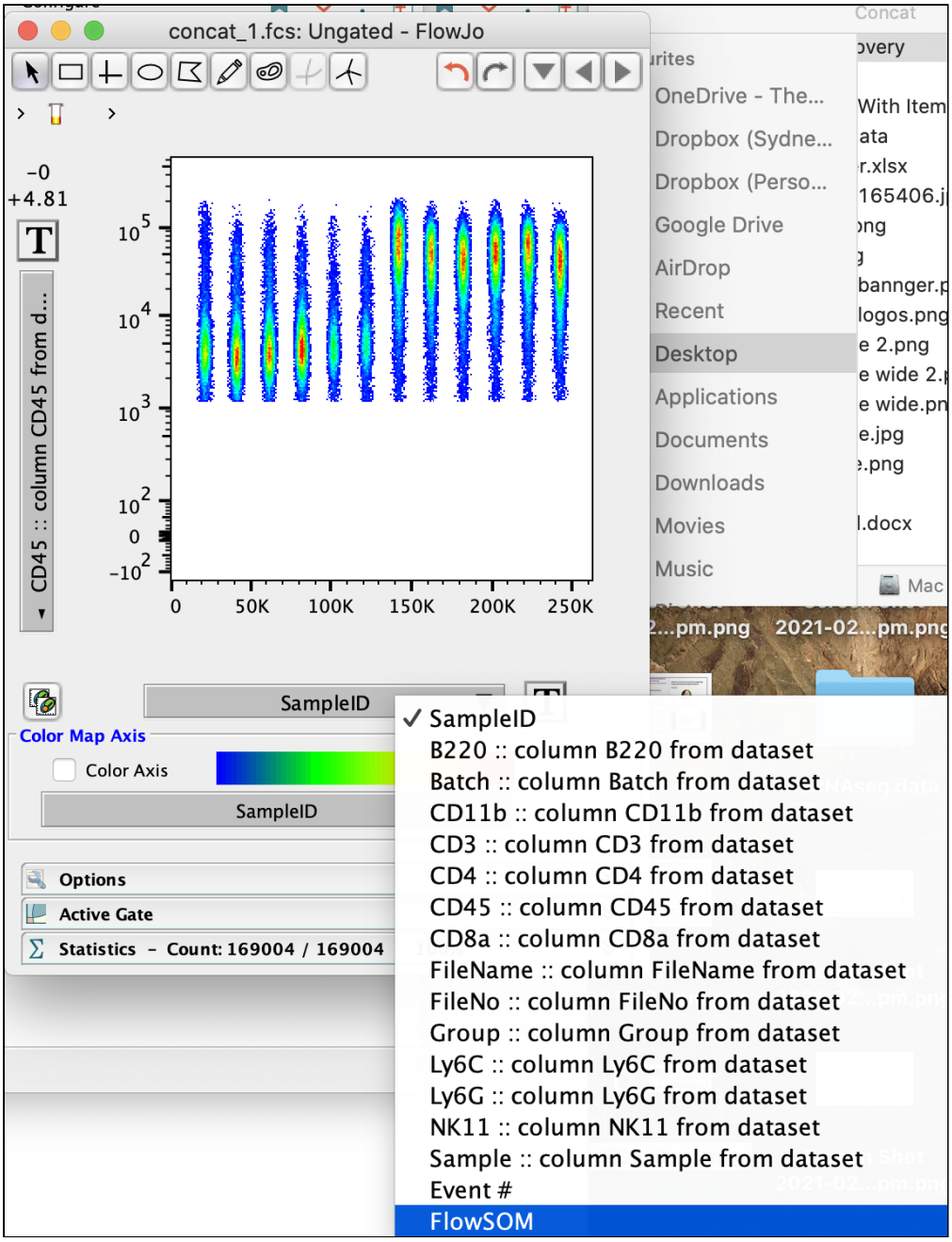


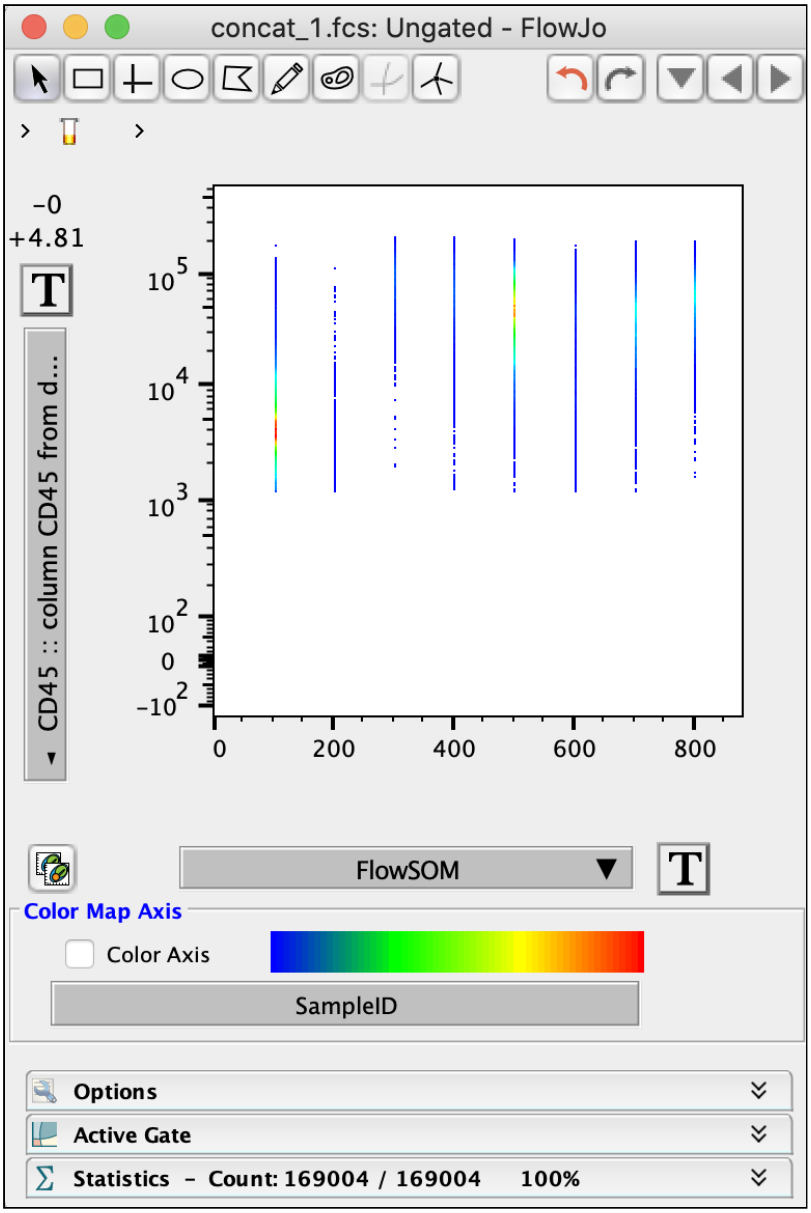


When FlowSOM has finished running, you will see the a/b node (containing the FlowSOM settings), and a virtual gate for each FlowSOM metaclusters.

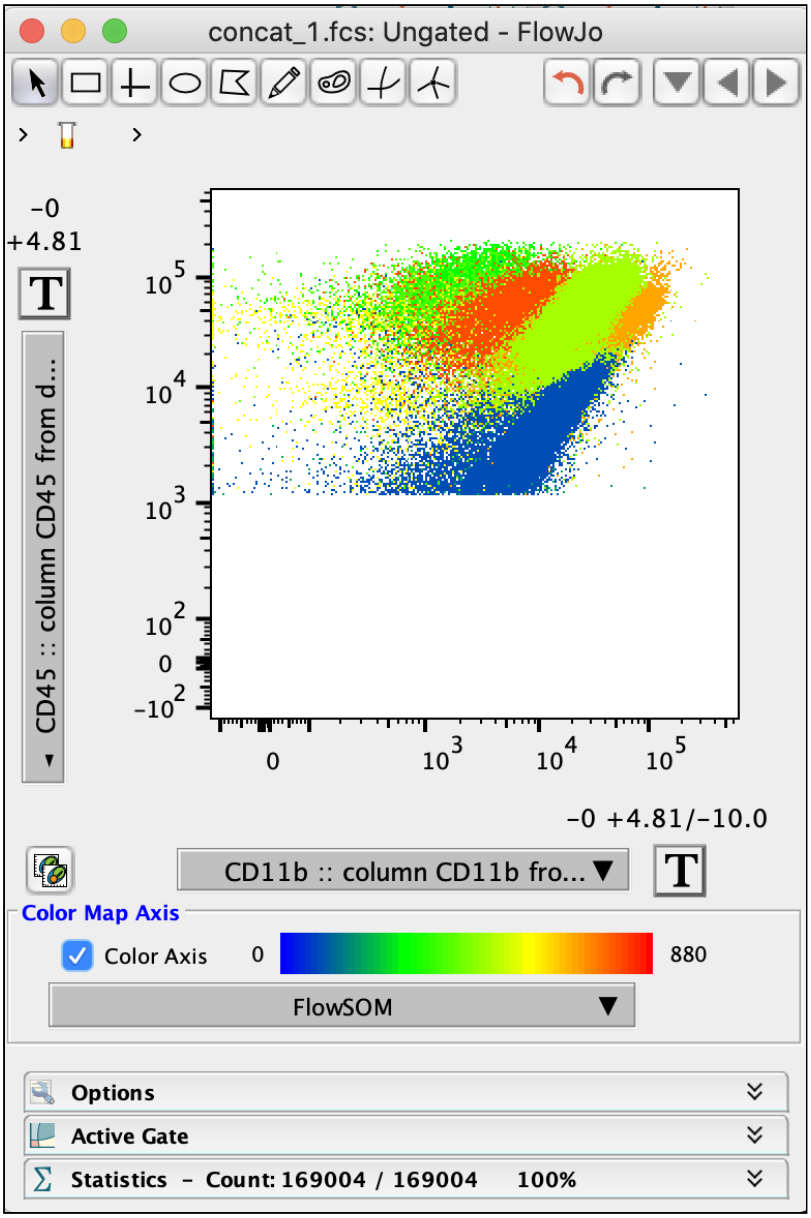
Name	Statistic	#Cells
concat_1.fcs		169004
FlowSOM		
FlowSOM		
FlowSOM.Pop0	40.9	69185
FlowSOM.Pop1	0.31	522
FlowSOM.Pop2	2.13	3594
FlowSOM.Pop3	2.32	3919
FlowSOM.Pop4	38.7	65439
FlowSOM.Pop5	1.50	2530
FlowSOM.Pop6	7.67	12955
FlowSOM.Pop7	6.43	10860

You can also see the metaclusters as a new 'parameter' – these will be distributed on a numerical scale between 0 and 1024.

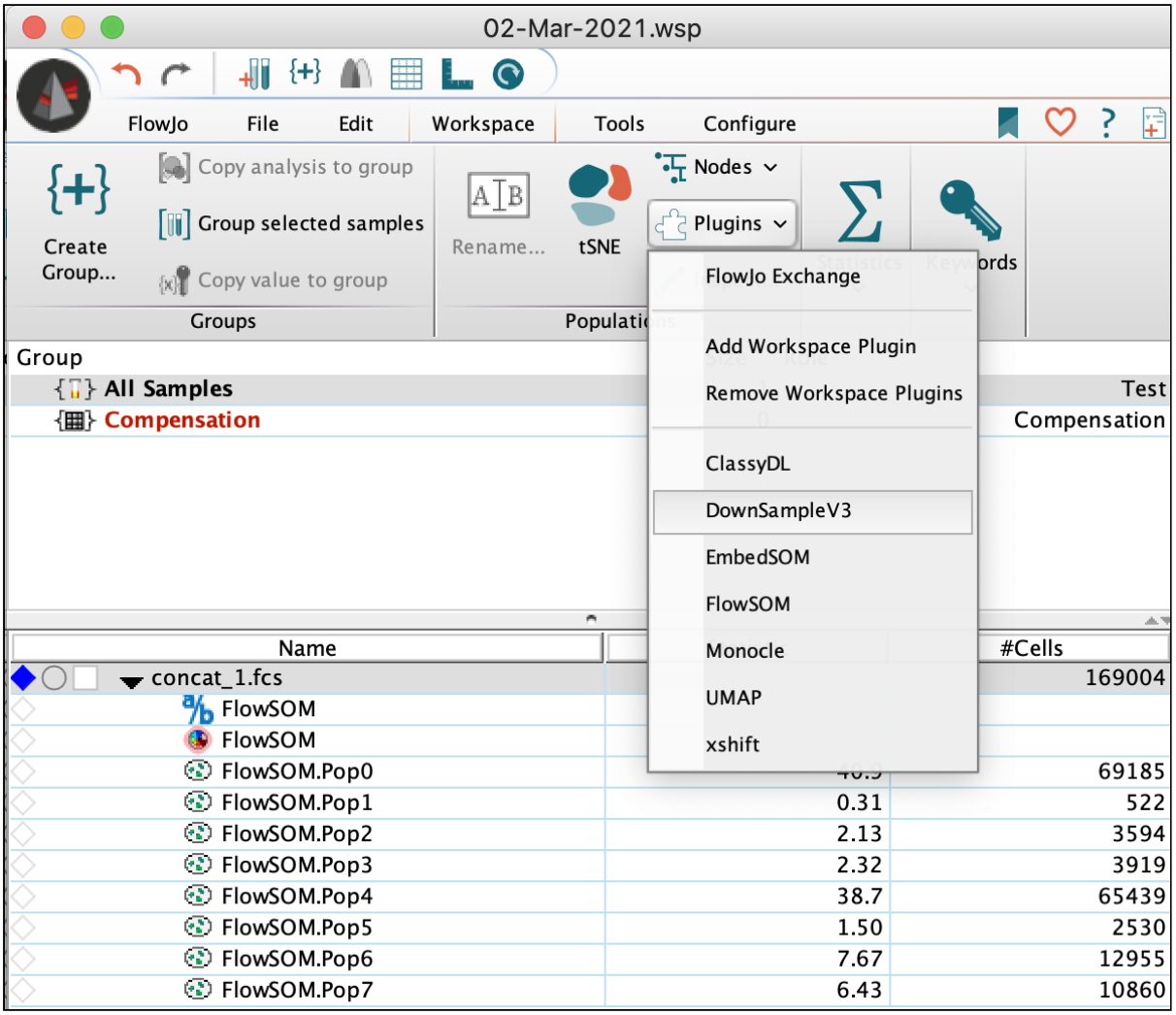

concat_1.fcs: Ungated - FlowJo
CD45 :: column CD45 from d...
SampleID
Color Map Axis
Color Axis
SampleID
Options
Active Gate
Statistics - Count: 169004 / 169004
SampleID
B220 :: column B220 from dataset
Batch :: column Batch from dataset
CD11b :: column CD11b from dataset
CD3 :: column CD3 from dataset
CD4 :: column CD4 from dataset
CD45 :: column CD45 from dataset
CD8a :: column CD8a from dataset
FileName :: column FileName from dataset
FileNo :: column FileNo from dataset
Group :: column Group from dataset
Ly6C :: column Ly6C from dataset
Ly6G :: column Ly6G from dataset
NK11 :: column NK11 from dataset
Sample :: column Sample from dataset
Event #
FlowSOM
OneDrive - The...
Dropbox (Sydne...
Dropbox (Perso...
Google Drive
AirDrop
Recent
Desktop
Applications
Documents
Downloads
Movies
Music

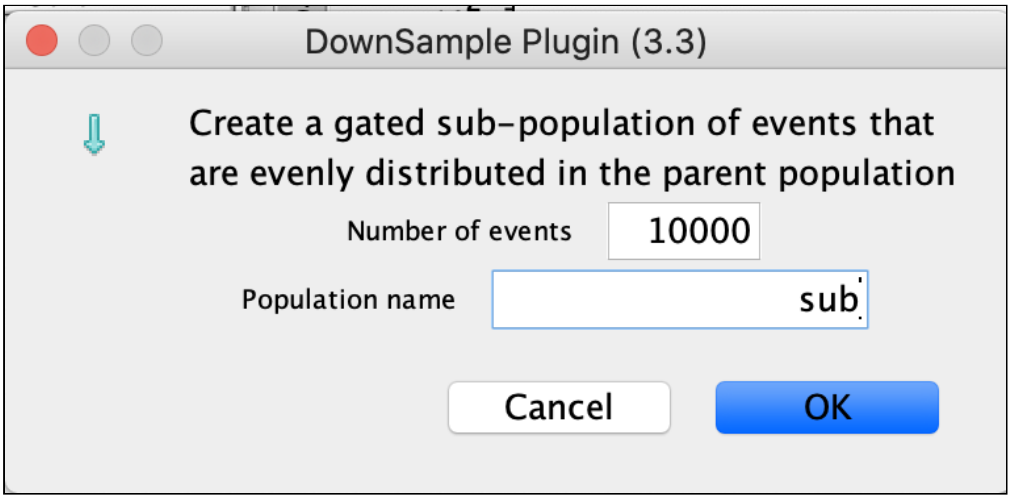


You can also use this as a 'colour axis' parameter when plotting two cellular parameters against each other.

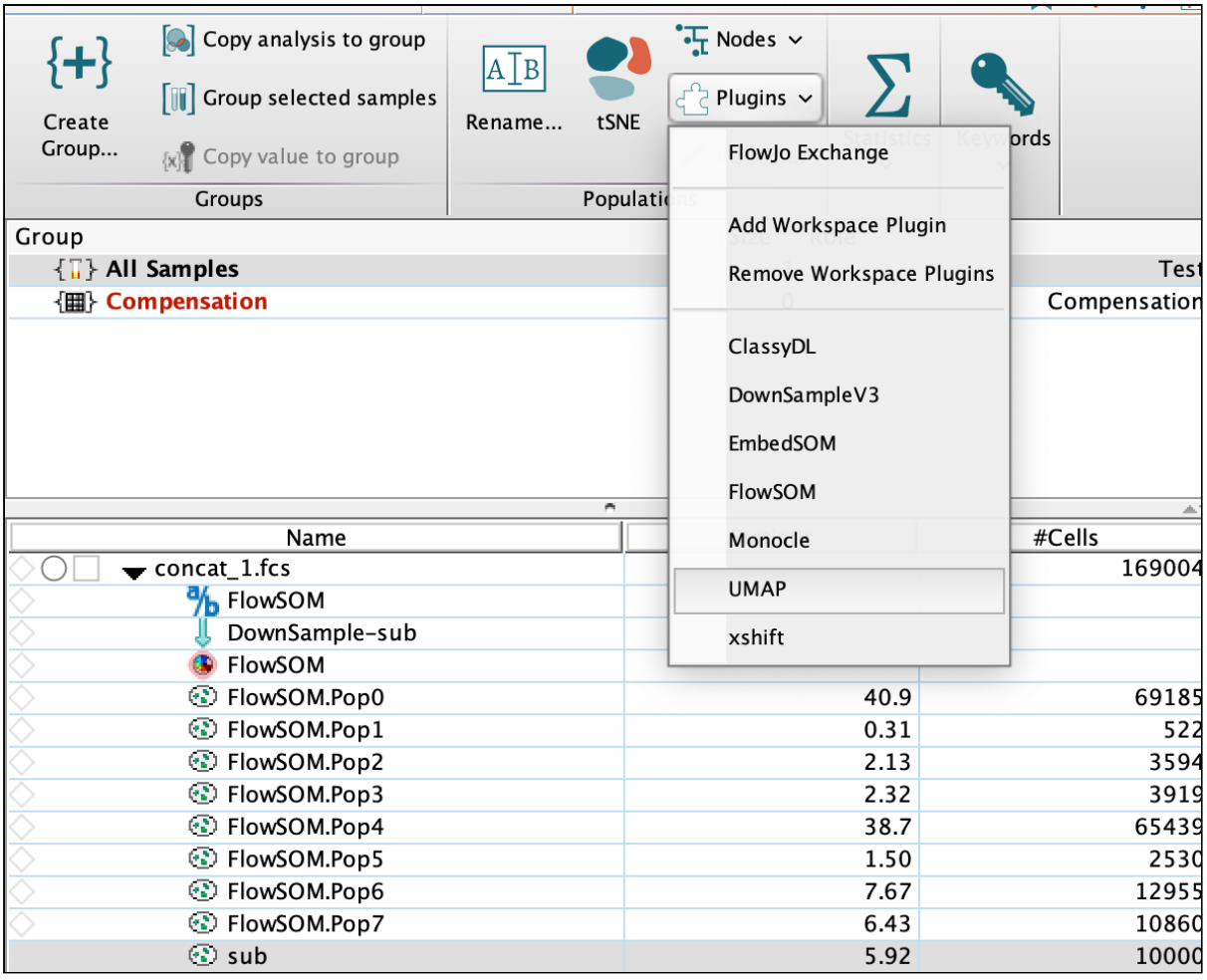


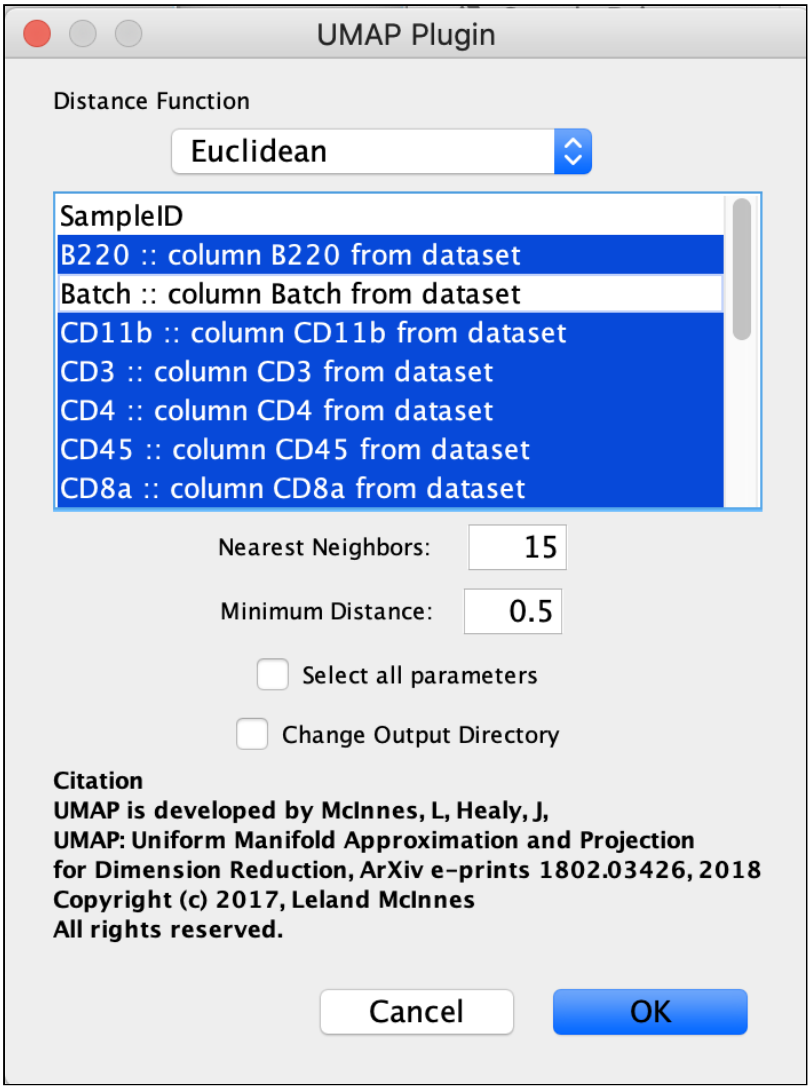
3. Dimensionality reduction and plotting

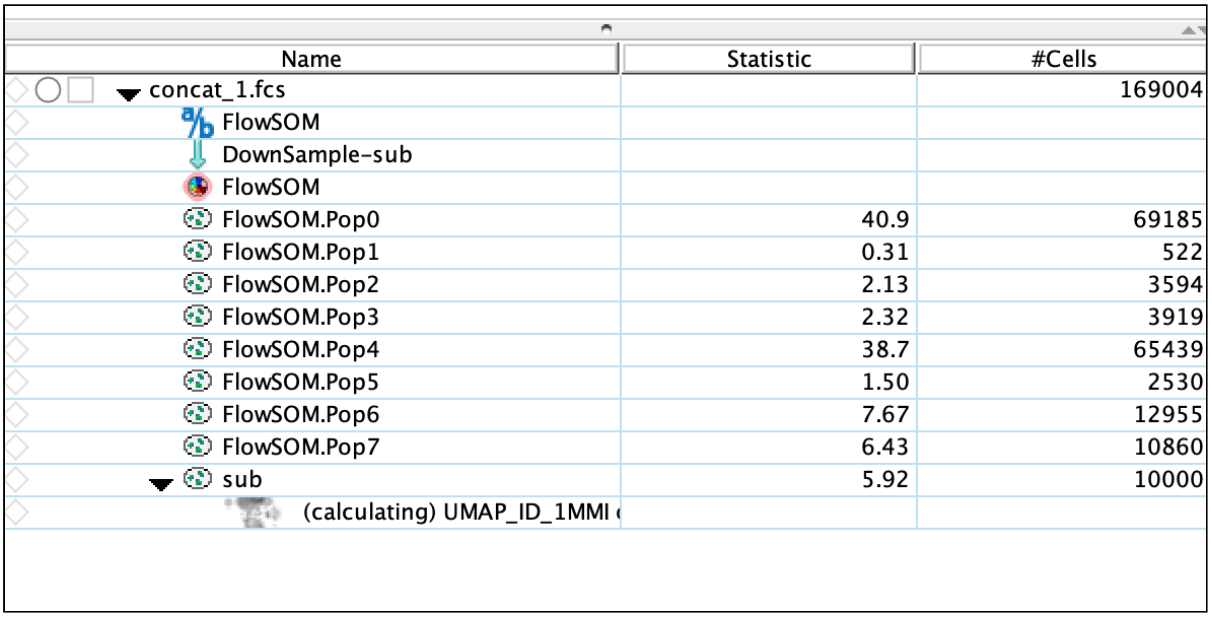

02-Mar-2021.wsp
FlowJo
File
Edit
Workspace
Tools
Configure
Copy analysis to group
Group selected samples
Copy value to group
Create Group...
Groups
Rename...
tSNE
Nodes
Plugins
FlowJo Exchange
Add Workspace Plugin
Remove Workspace Plugins
ClassyDL
DownSampleV3
EmbedSOM
FlowSOM
Monocle
UMAP
xshift
Group
All Samples
Test
Compensation
Compensation
Name
#Cells
concat_1.fcs
169004
FlowSOM
FlowSOM
FlowSOM.Pop0
69185
FlowSOM.Pop1
0.31
522
FlowSOM.Pop2
2.13
3594
FlowSOM.Pop3
2.32
3919
FlowSOM.Pop4
38.7
65439
FlowSOM.Pop5
1.50
2530
FlowSOM.Pop6
7.67
12955
FlowSOM.Pop7
6.43
10860


DownSample Plugin (3.3)
Create a gated sub-population of events that are evenly distributed in the parent population
Number of events
10000
Population name
sub
Cancel
OK

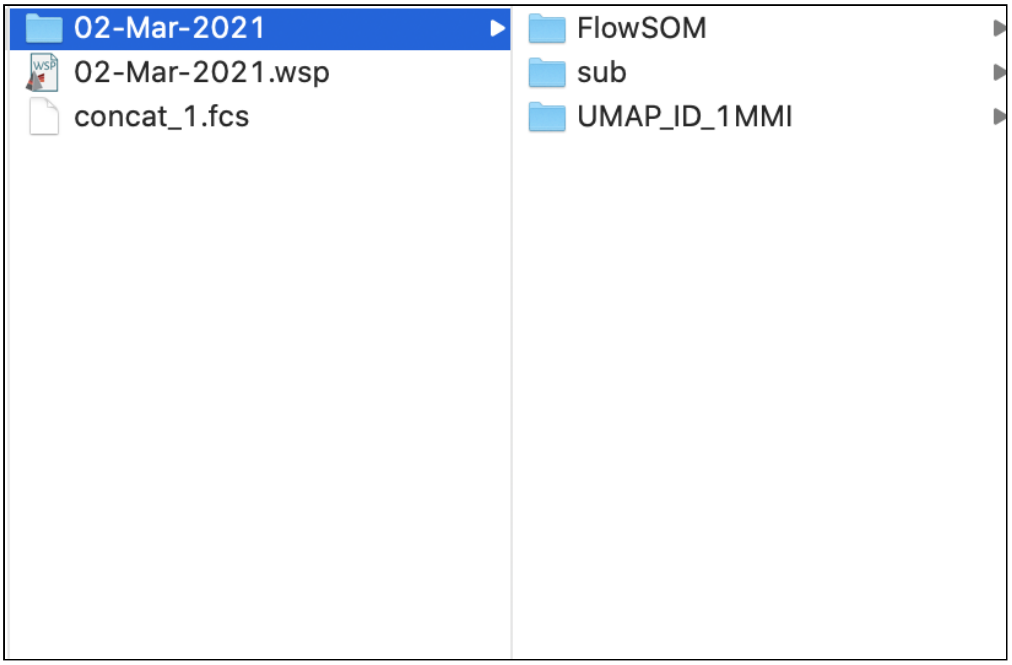
Name	Statistic	#Cells
concat_1.fcs		169004
FlowSOM		
DownSample-sub		
FlowSOM		
FlowSOM.Pop0	40.9	69185
FlowSOM.Pop1	0.31	522
FlowSOM.Pop2	2.13	3594
FlowSOM.Pop3	2.32	3919
FlowSOM.Pop4	38.7	65439
FlowSOM.Pop5	1.50	2530
FlowSOM.Pop6	7.67	12955
FlowSOM.Pop7	6.43	10860
sub	5.92	10000



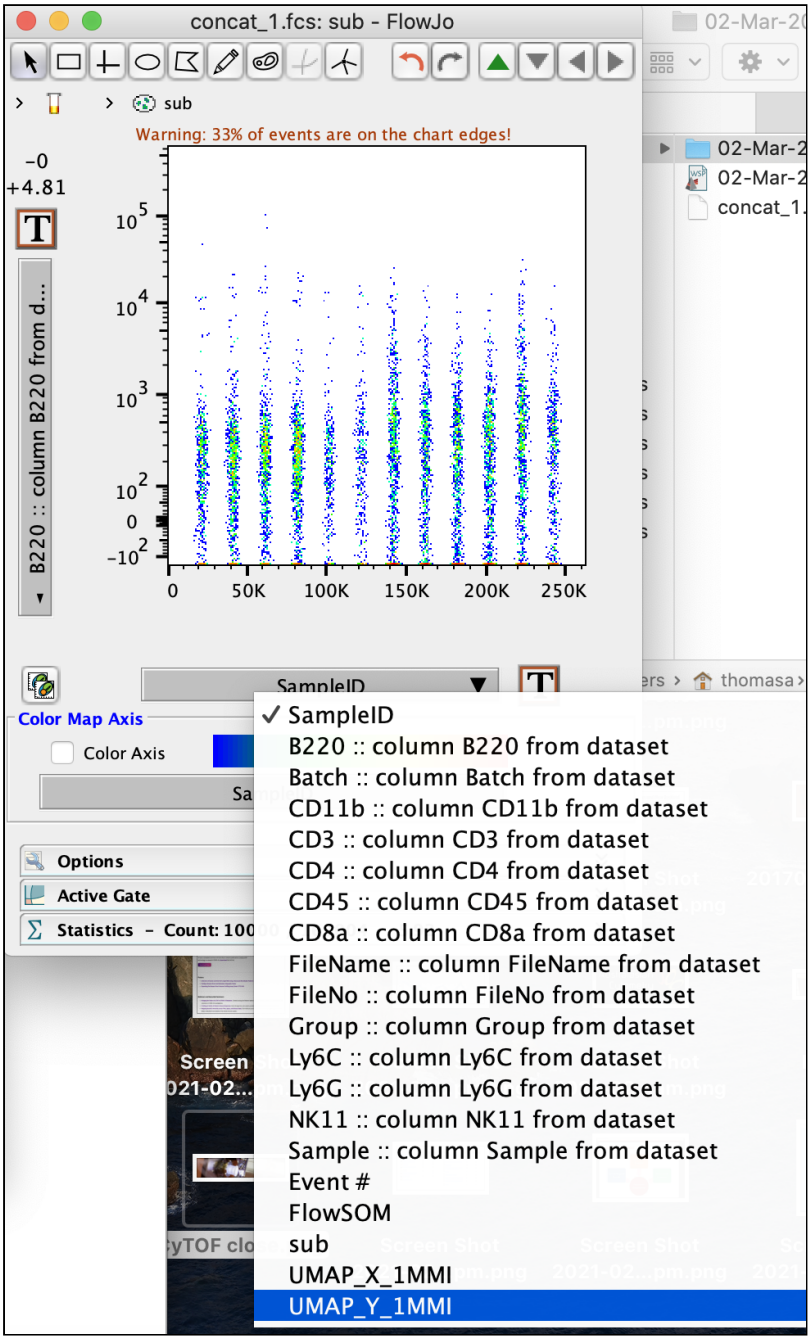


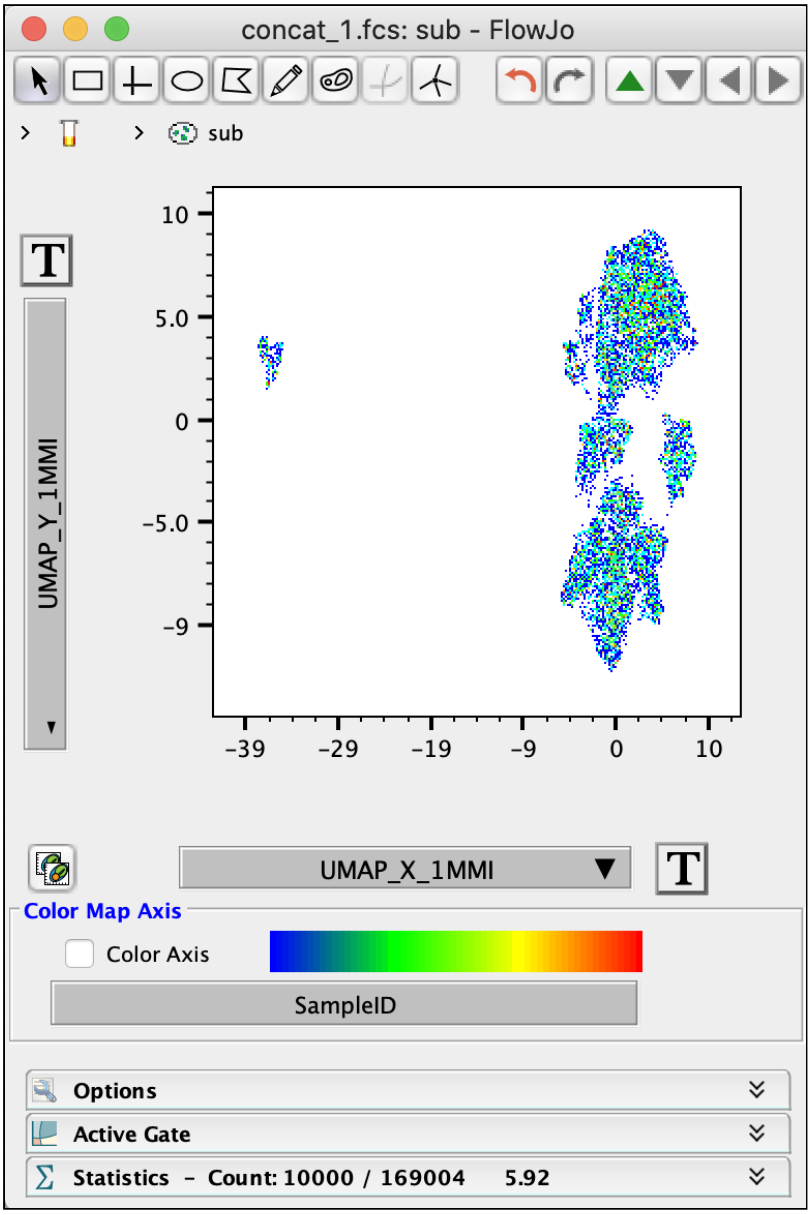


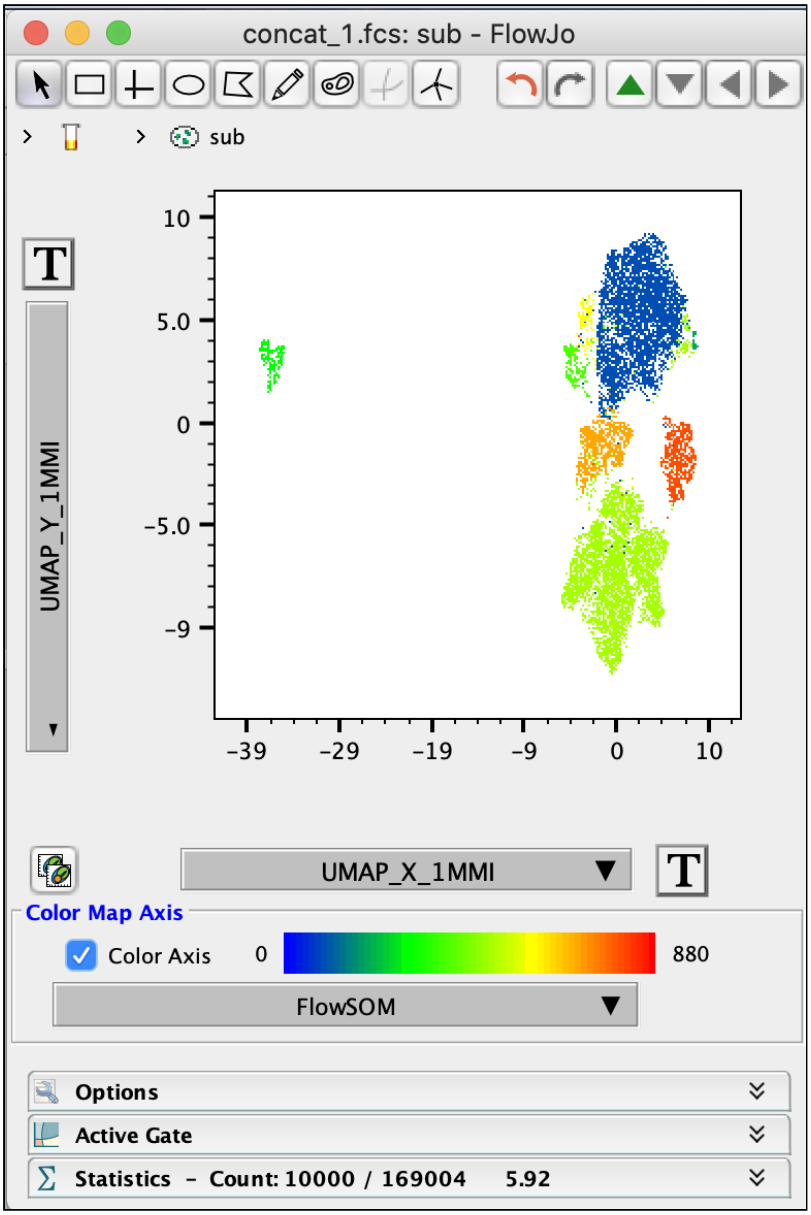
Name	Statistic	#Cells
concat_1.fcs		169004
FlowSOM		
DownSample-sub		
FlowSOM		
FlowSOM.Pop0	40.9	69185
FlowSOM.Pop1	0.31	522
FlowSOM.Pop2	2.13	3594
FlowSOM.Pop3	2.32	3919
FlowSOM.Pop4	38.7	65439
FlowSOM.Pop5	1.50	2530
FlowSOM.Pop6	7.67	12955
FlowSOM.Pop7	6.43	10860
sub	5.92	10000
(calculating) UMAP_ID_1MMI		

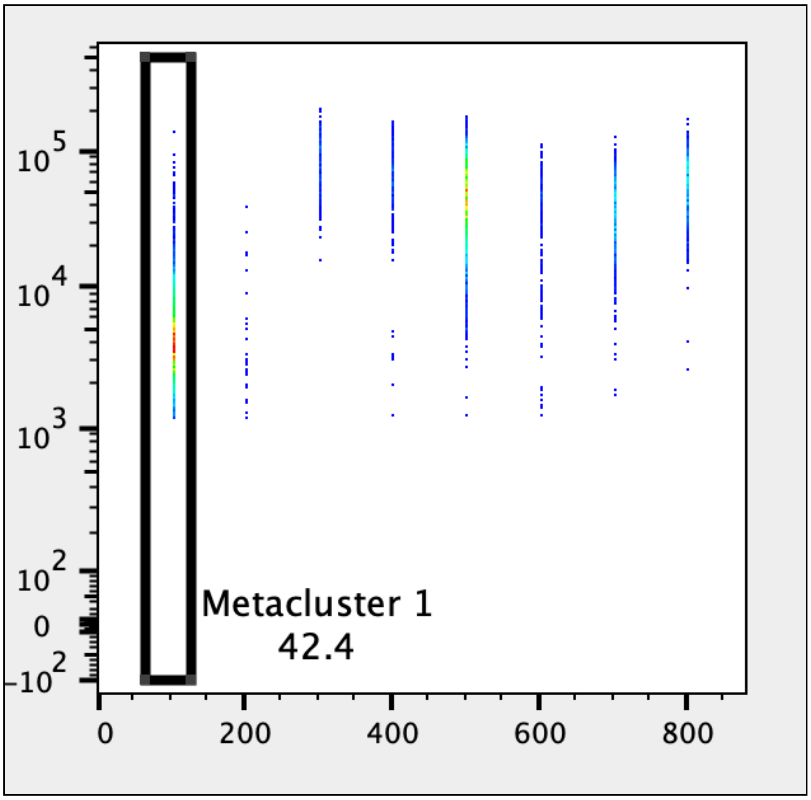


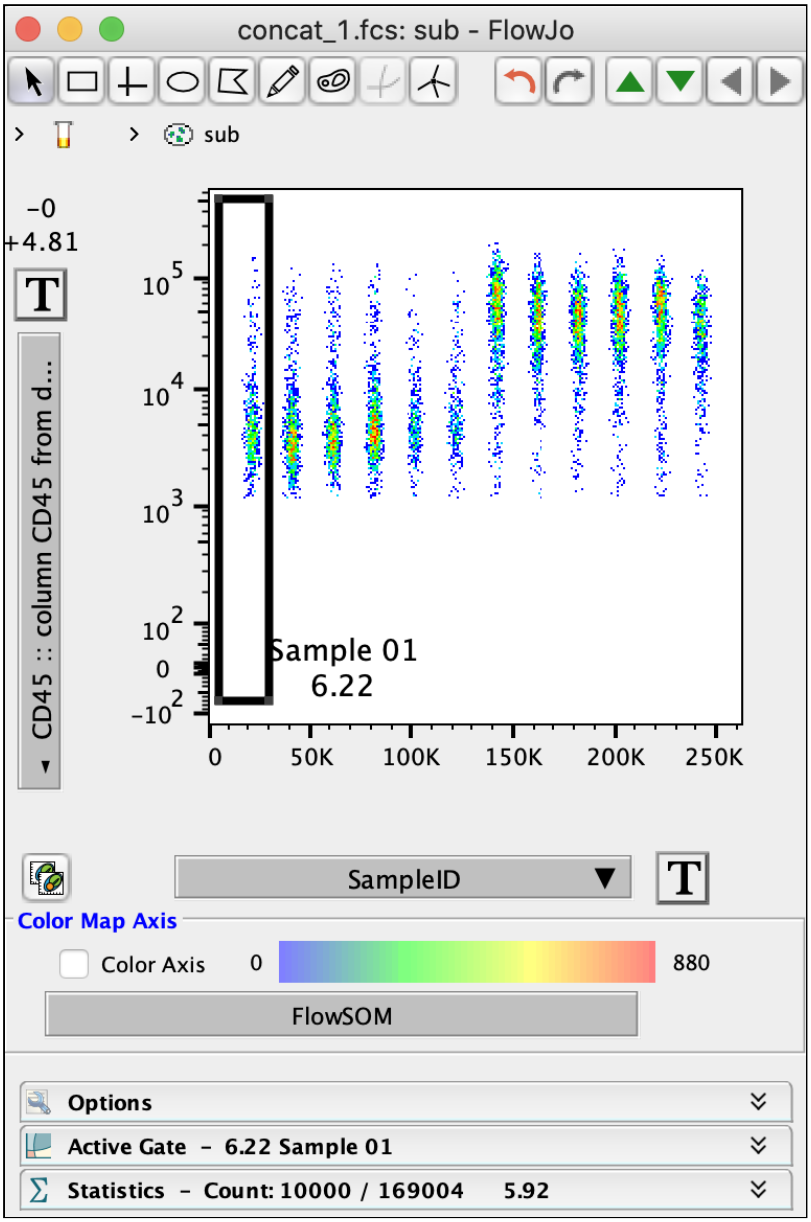
concat_1.fcs		169004
FlowSOM		
UMAP_X_1MMI		
UMAP_Y_1MMI		
DownSample-sub		
FlowSOM		
FlowSOM.Pop0	40.9	69185
FlowSOM.Pop1	0.31	522
FlowSOM.Pop2	2.13	3594
FlowSOM.Pop3	2.32	3919
FlowSOM.Pop4	38.7	65439
FlowSOM.Pop5	1.50	2530
FlowSOM.Pop6	7.67	12955
FlowSOM.Pop7	6.43	10860
sub	5.92	10000
UMAP_ID_1MMI of sub		UMAP completed

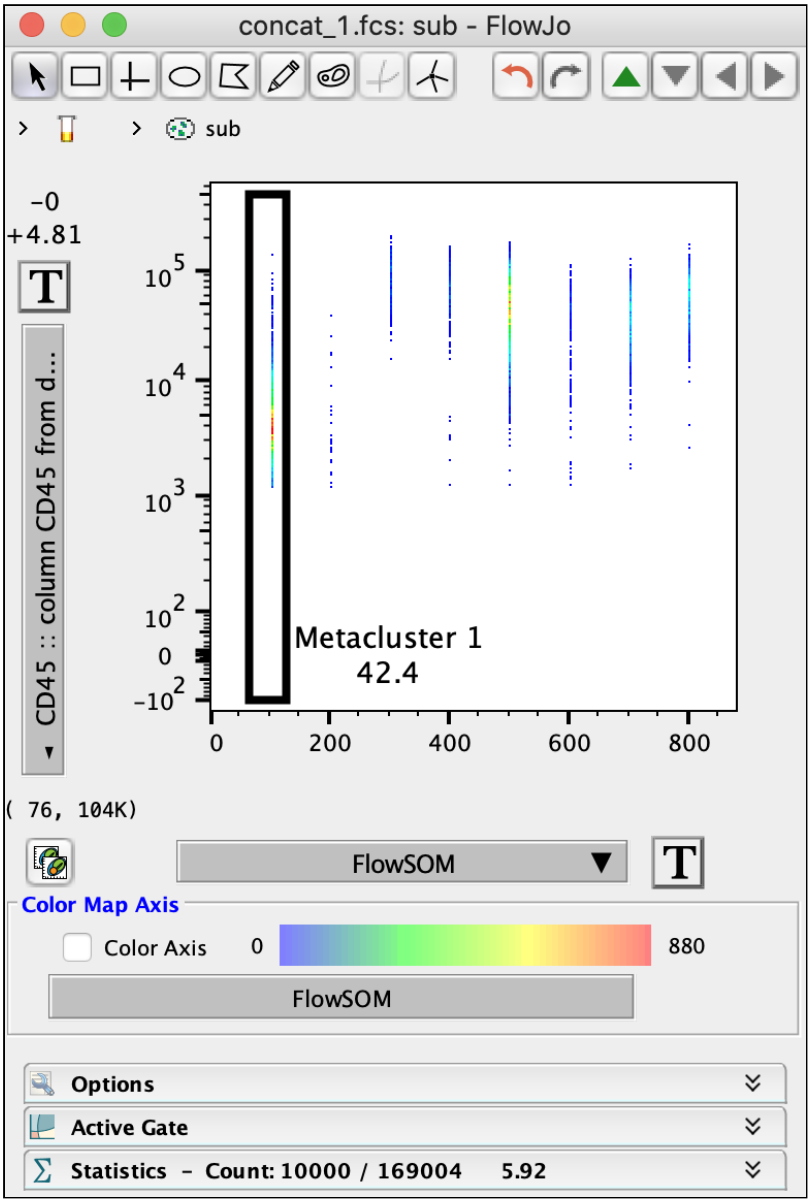

concat_1.fcs: sub - FlowJo
sub
Warning: 33% of events are on the chart edges!
-0
+4.81
B220 :: column B220 from d...
0
50K
100K
150K
200K
250K
SampleID
Color Map Axis
Color Axis
Options
Active Gate
Statistics – Count: 100
SampleID
B220 :: column B220 from dataset
Batch :: column Batch from dataset
CD11b :: column CD11b from dataset
CD3 :: column CD3 from dataset
CD4 :: column CD4 from dataset
CD45 :: column CD45 from dataset
CD8a :: column CD8a from dataset
FileName :: column FileName from dataset
FileNo :: column FileNo from dataset
Group :: column Group from dataset
Ly6C :: column Ly6C from dataset
Ly6G :: column Ly6G from dataset
NK11 :: column NK11 from dataset
Sample :: column Sample from dataset
Event #
FlowSOM
sub
UMAP_X_1MMI
UMAP_Y_1MMI
02-Mar-2
thomasa

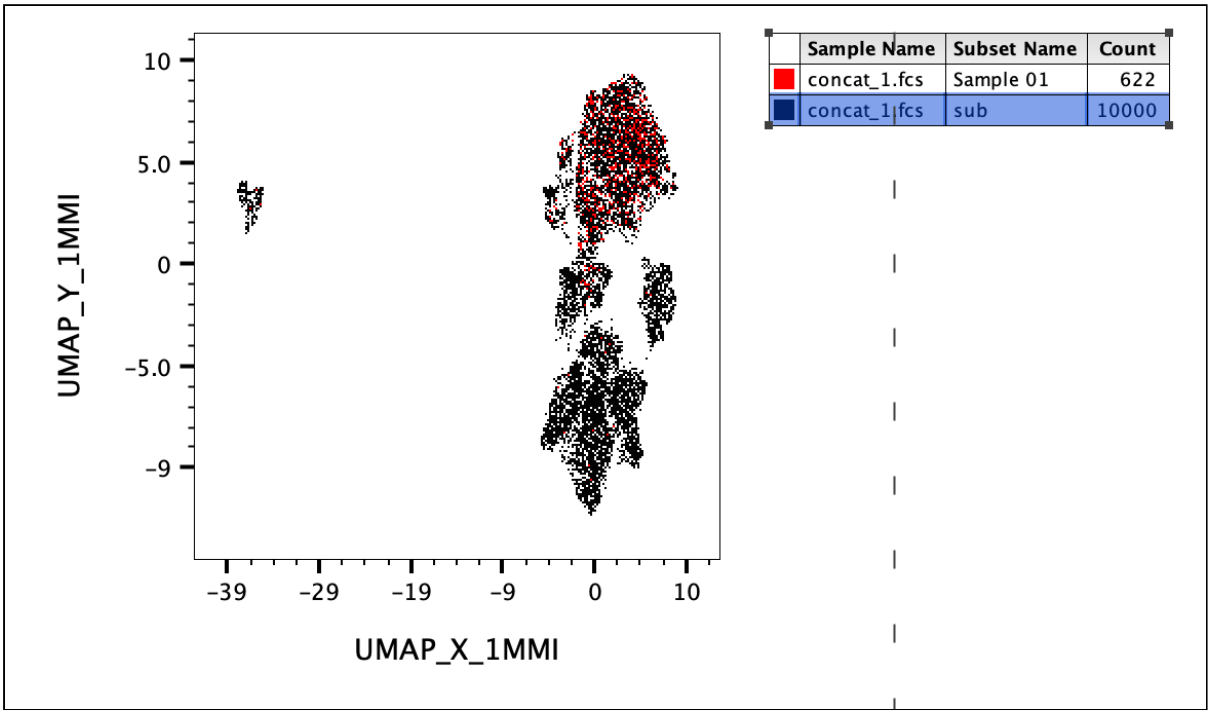

concat_1.fcs: sub - FlowJo
sub
10
5.0
0
-5.0
-9
UMAP_Y_1MMI
-39
-29
-19
-9
0
10
UMAP_X_1MMI
Color Map Axis
Color Axis
SampleID
Options
Active Gate
Statistics - Count: 10000 / 169004 5.92

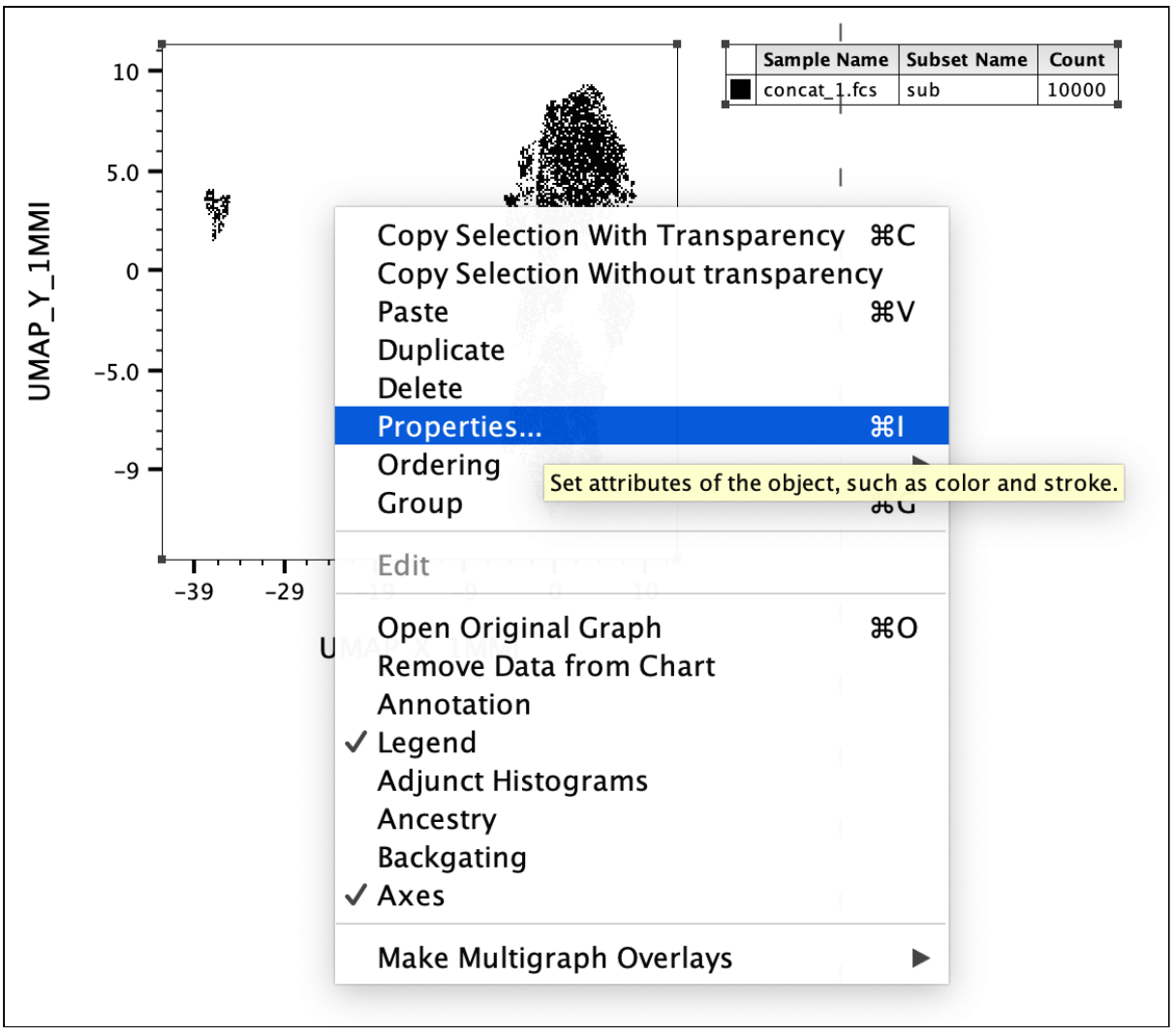

concat_1.fcs: sub - FlowJo
sub
10
5.0
0
-5.0
-9
UMAP_Y_1MMI
-39
-29
-19
-9
0
10
UMAP_X_1MMI
Color Map Axis
Color Axis
0
880
FlowSOM
Options
Active Gate
Statistics - Count: 10000 / 169004 5.92

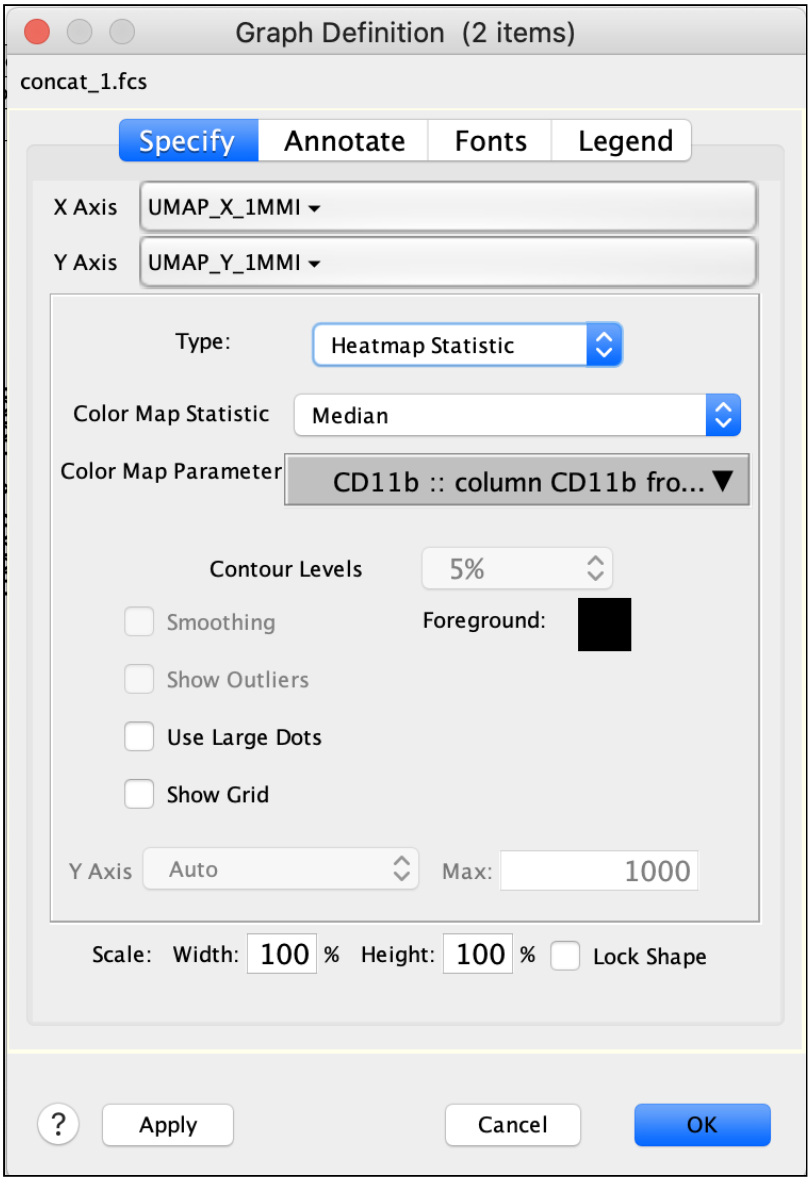

10^5
10^4
10^3
10^2
0
-10^2
Metacluster 1
42.4
0
200
400
600
800

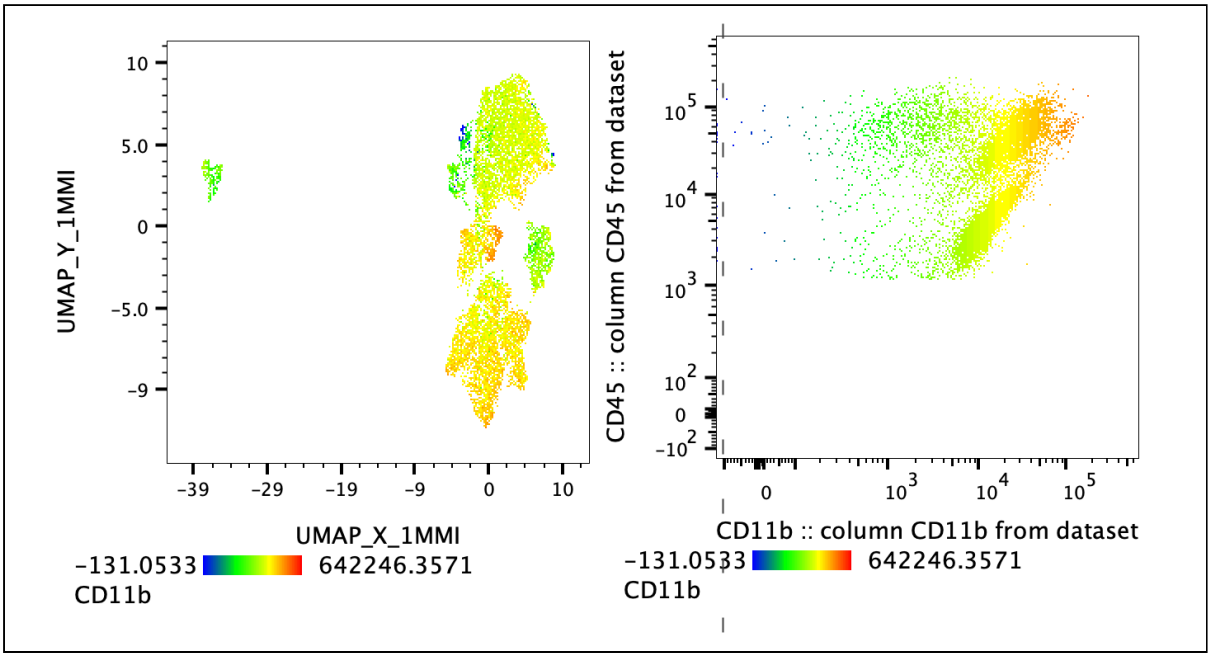

concat_1.fcs: sub - FlowJo
sub
-0
+4.81
CD45 :: column CD45 from d...
Sample 01
6.22
SampleID
Color Map Axis
Color Axis
0
880
FlowSOM
Options
Active Gate - 6.22 Sample 01
Statistics - Count: 10000 / 169004 5.92


concat_1.fcs: sub - FlowJo
sub
-0
+4.81
CD45 :: column CD45 from d...
Metacluster 1
42.4
(76, 104K)
FlowSOM
Color Map Axis
Color Axis
0
880
FlowSOM
Options
Active Gate
Statistics - Count: 10000 / 169004 5.92


Sample Name
Subset Name
Count
concat_1.fcs
Sample 01
622
concat_1.fcs
sub
10000
UMAP_Y_1MMI
UMAP_X_1MMI
10
5.0
0
-5.0
-9
-39
-29
-19
-9
0
10


Sample Name
Subset Name
Count
concat_1.fcs
sub
10000
UMAP_Y_1MMI
Copy Selection With Transparency ⌘C
Copy Selection Without transparency
Paste ⌘V
Duplicate
Delete
Properties... ⌘I
Ordering
Group
Set attributes of the object, such as color and stroke.
Edit
Open Original Graph ⌘O
Remove Data from Chart
Annotation
✓ Legend
Adjunct Histograms
Ancestry
Backgating
✓ Axes
Make Multigraph Overlays


Graph Definition (2 items)
concat_1.fcs
Specify
Annotate
Fonts
Legend
X Axis
UMAP_X_1MMI
Y Axis
UMAP_Y_1MMI
Type:
Heatmap Statistic
Color Map Statistic
Median
Color Map Parameter
CD11b :: column CD11b fro...
Contour Levels
5%
Smoothing
Foreground:
Show Outliers
Use Large Dots
Show Grid
Y Axis
Auto
Max:
1000
Scale:
Width:
100
%
Height:
100
%
Lock Shape
?
Apply
Cancel
OK



4. Quantitative and statistical analysis