

**⚠** 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.

### i 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.

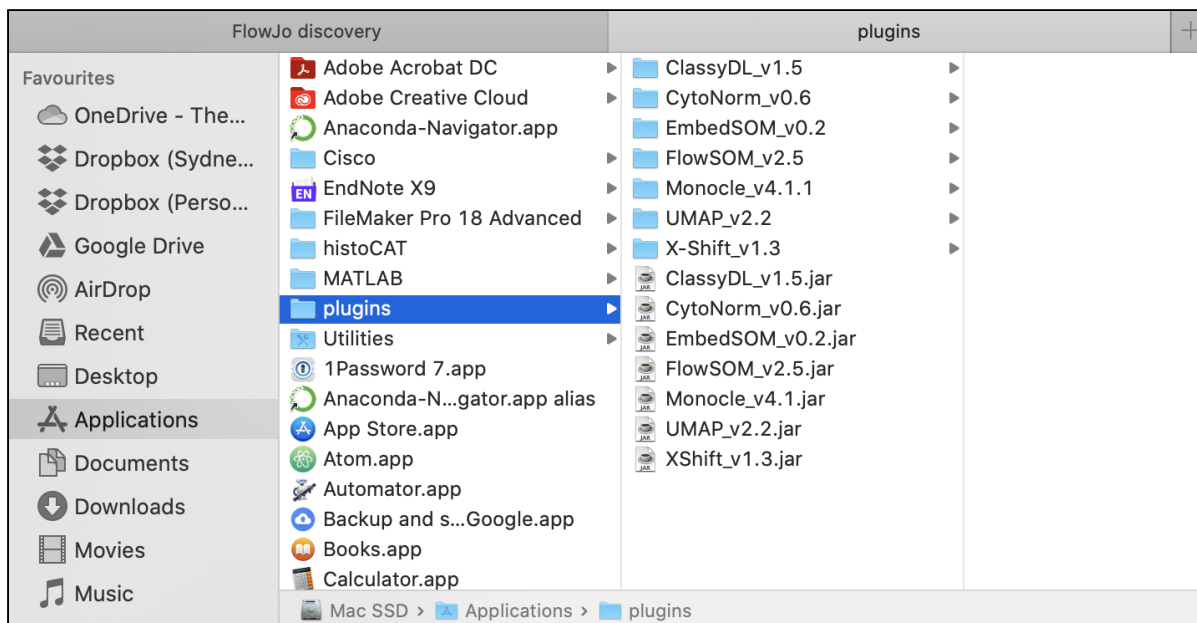
## Setup FlowJo and plugins

For this workflow, you will need to install [FlowJo](#) and have access to a [licence or dongle](#).

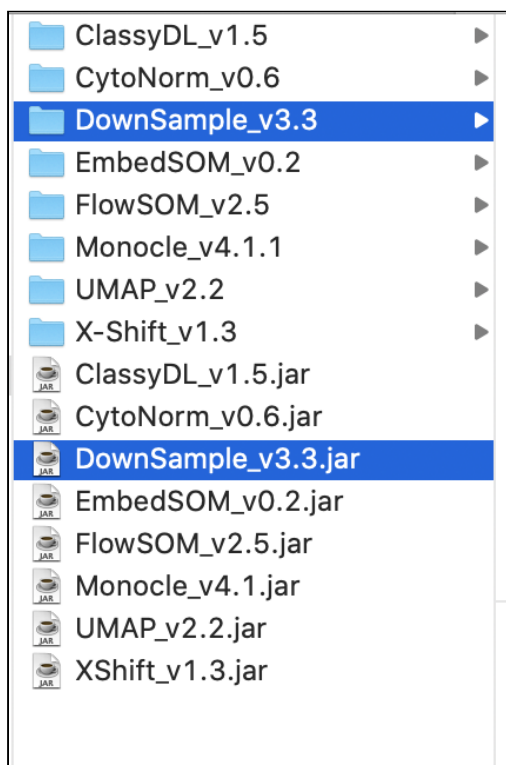
You will also need to ensure you have the following plugins installed:

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

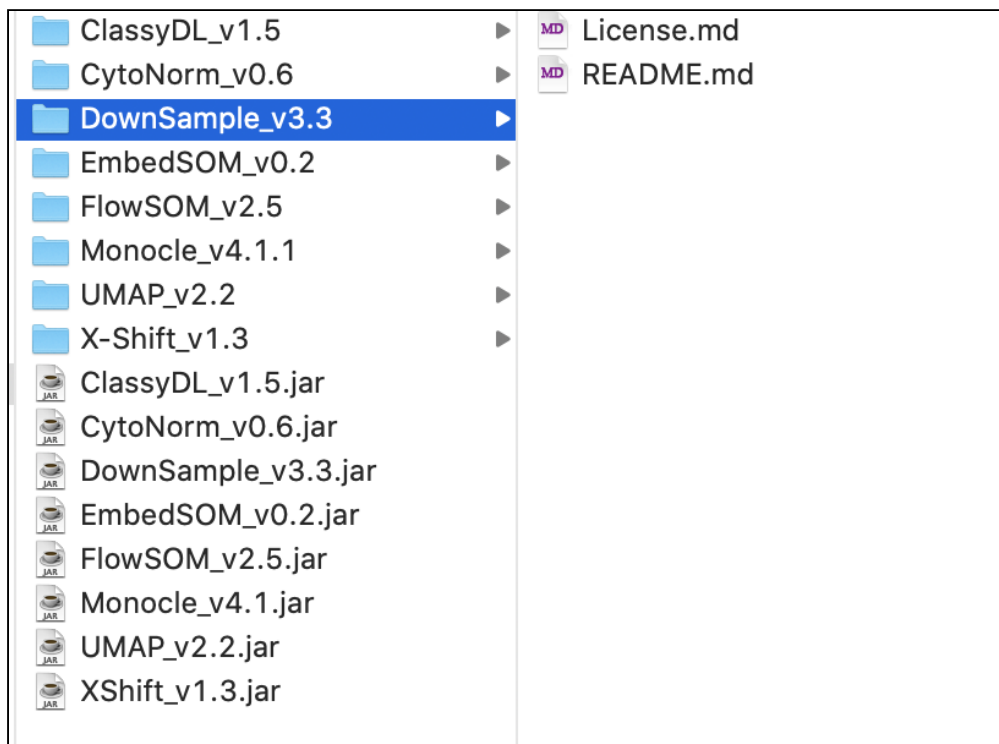
To check your packages, go to the 'Applications' folder (on Mac), and find the folder called 'plugins'.



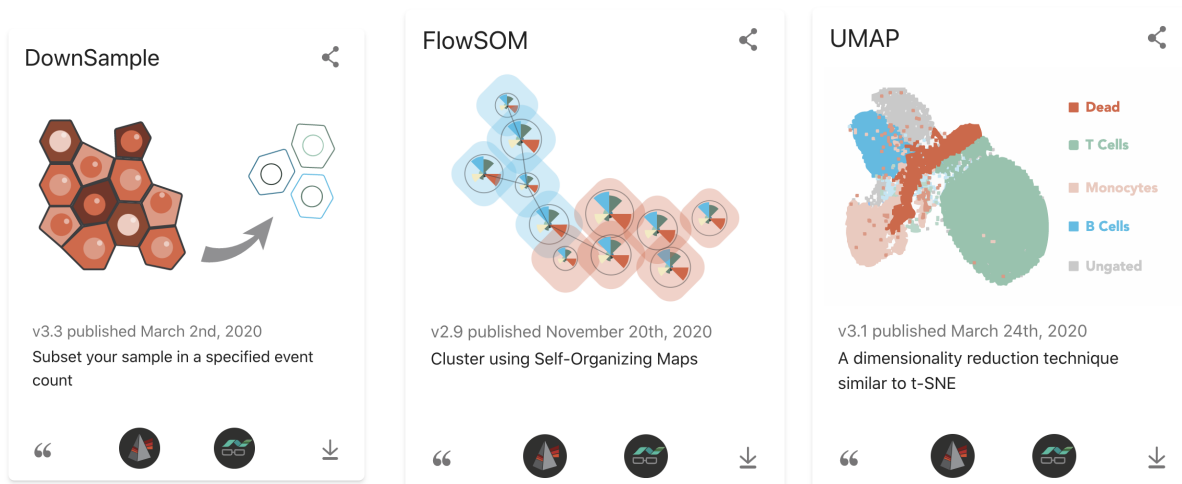
For each plugin you should see a '.jar' file, and possibly a folder of the same name.



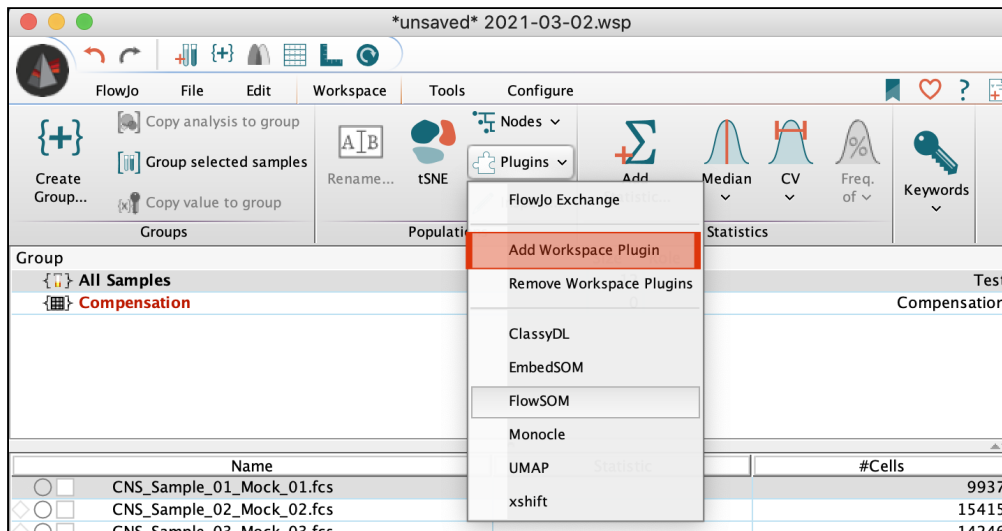
Within the each folder will be documentation related to that plugin.



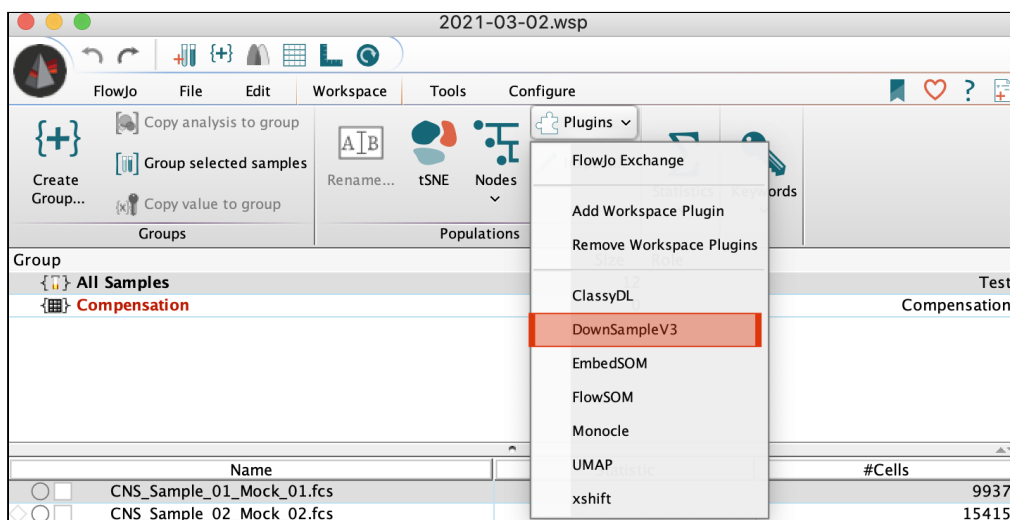
To download new plugins, go to the [FlowJo exchange](#) site. Download the following (if you don't already have them), unzip them, and move the .jar files (and optionally the associated folder) into the 'plugins' folder as above.



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

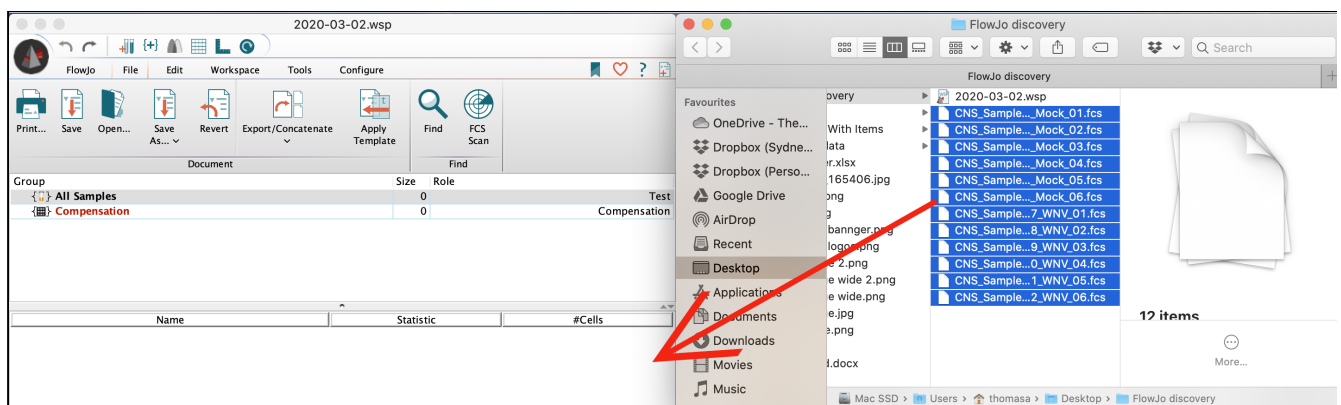


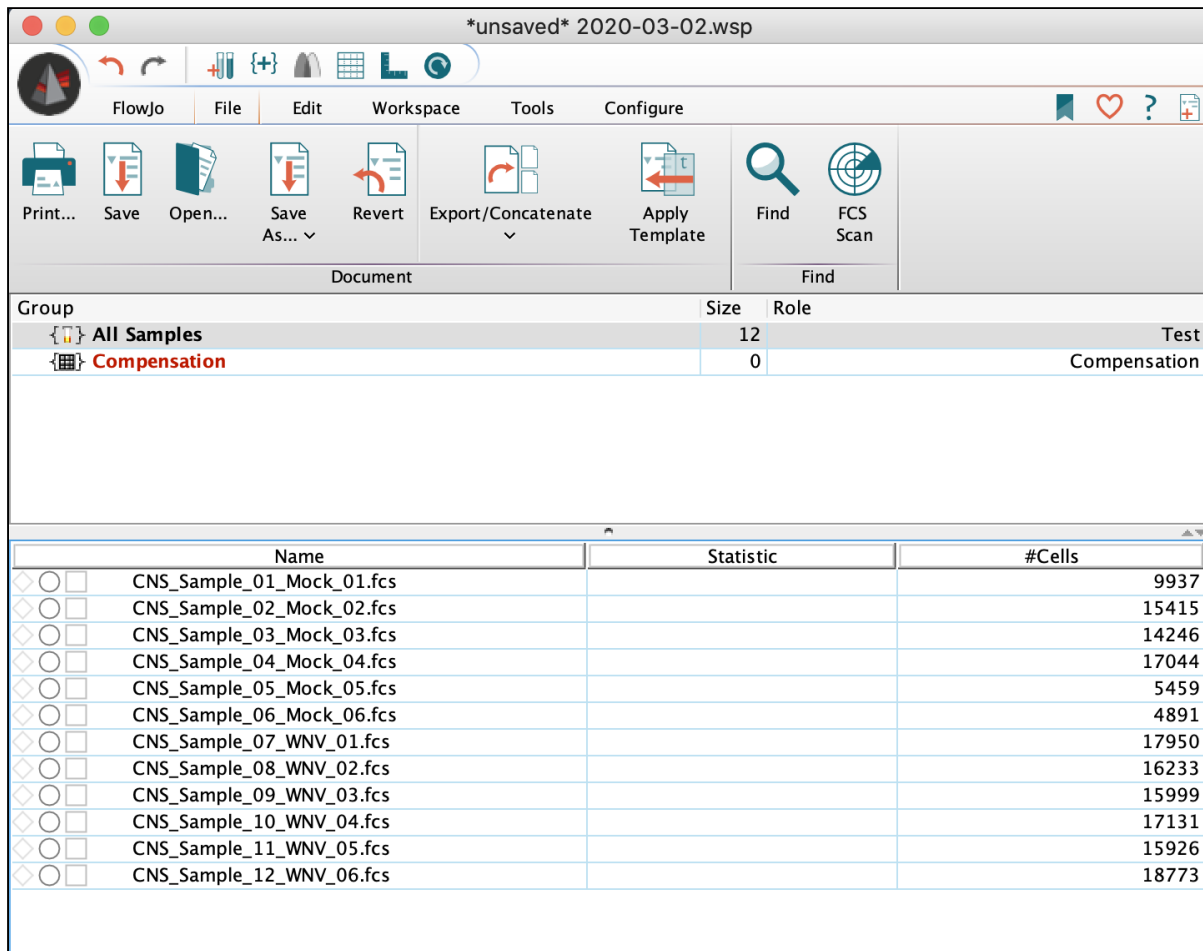
Once they have been 'activated' with FlowJo, they should show up in this list.



## 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'.

	Name	Statistic	#Cells
<input type="radio"/>	CNS_Sample_01_Mock_01.fcs		9937
<input type="radio"/>	CNS_Sample_02_Mock_02.fcs	Copy ⌘C	15415
<input type="radio"/>	CNS_Sample_03_Mock_03.fcs	Paste ⌘V	14246
<input type="radio"/>	CNS_Sample_04_Mock_04.fcs	Clear ⌘⌘	17044
<input type="radio"/>	CNS_Sample_05_Mock_05.fcs	Add Keyword ⌘I	5459
<input type="radio"/>	CNS_Sample_06_Mock_06.fcs	Add Statistic... ⌘B	4891
<input type="radio"/>	CNS_Sample_07_WNV_01.fcs	Inspect... ⌘I	17950
<input type="radio"/>	CNS_Sample_08_WNV_02.fcs		16233
<input type="radio"/>	CNS_Sample_09_WNV_03.fcs		15999
<input type="radio"/>	CNS_Sample_10_WNV_04.fcs		17131
<input type="radio"/>	CNS_Sample_11_WNV_05.fcs	Copy value to group	15926
<input checked="" type="radio"/>	CNS_Sample_12_WNV_06.fcs	Copy analysis to group ⌘G	18773
Select Equivalent Nodes ⌘E Reset Column Widths Search for FCS files... Export to FACSDiva Import from FACSDiva <b>Export / Concatenate Populations ⌘E</b> BifurGate Derive Parameters... ⌘D Consensus Gate Cluster Frequency Open Parent Folder(s)...			

Export or concatenate populations selected in the workspace

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.

Populations: Export or Concatenate

Populations:
Export
Concatenate

Output
Format: FCS3
Destination: /Users/thomasa/Desktop/FlowJo discovery/Concat
File name example: concat\_1.fcs

Include Events
☒ Include all
☐ Include no more than: 4891
Reset to minimum

Parameters
☒ All uncompensated parameters
☐ All compensated parameters
☐ Custom set of parameters: View/Edit...

Advanced Options

File Naming
Prefix: concat
(Concatenated files will be numbered consecutively starting at 1. Example: prefix\_1\_suffix.fcs)
Separator: \_
Suffix: .fcs

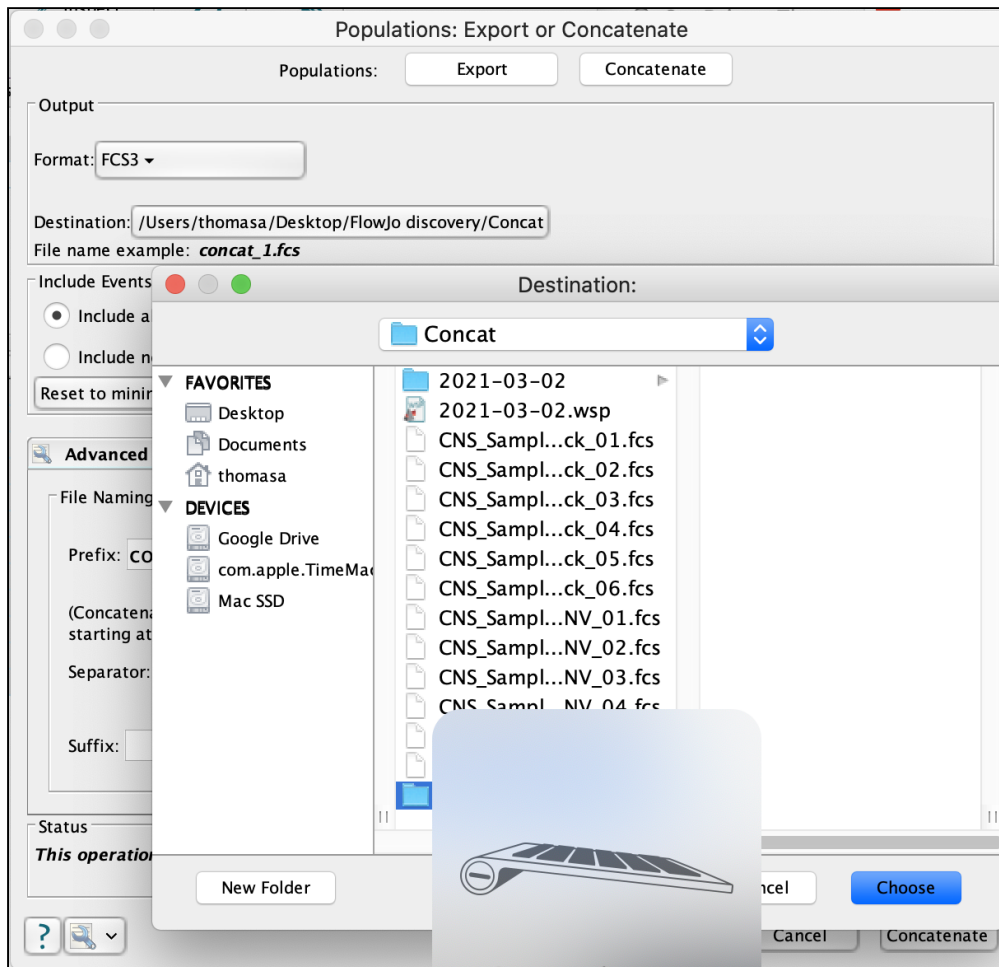
Group Concatenation
☒ Concatenate all files together
☐ Concatenate every "n" files together n= 12
☐ Concatenate files with equal keyword values
Choose Keyword: Select a Keyword...

Additional Parameters
Choose... <No Keywords selected>
☒ Spread distribution of keyword data

Status
This operation will generate 1 new data file(s).

?
Cancel
Concatenate

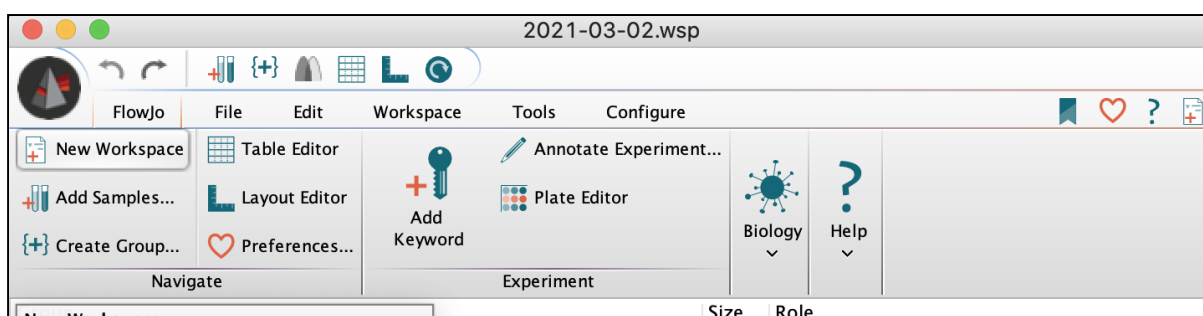
Choose an output location – best to create a folder within your existing experiment folder.



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.



\*unsaved\* 02-Mar-2021

The screenshot shows the FlowJo software interface. At the top is a title bar with standard macOS window controls (red, yellow, green buttons) and the text '\*unsaved\* 02-Mar-2021'. Below the title bar is a toolbar with icons for opening files, saving, undo, redo, adding samples, creating groups, and refreshing. The main menu bar includes 'FlowJo', 'File', 'Edit', 'Workspace', 'Tools', and 'Configure'. On the right side of the menu bar are icons for a bookmark, heart, question mark, and a document icon. Below the menu bar is a sidebar with several options: 'New Workspace' (with a plus icon), 'Add Samples...' (with a test tube icon), 'Create Group...' (with a bracket icon), 'Table Editor' (with a grid icon), 'Layout Editor' (with an L-shaped cursor icon), and 'Preferences...' (with a heart icon). The main workspace area is divided into two sections: 'Navigate' and 'Experiment'. The 'Navigate' section contains a search bar and a list of groups. The 'Experiment' section contains a search bar and a list of experiments. In the bottom right corner, there are icons for 'Biology' (a virus-like shape) and 'Help' (a question mark).

FlowJo File Edit Workspace Tools Configure

New Workspace Add Samples... Create Group... Table Editor Layout Editor Preferences...

Navigate Experiment

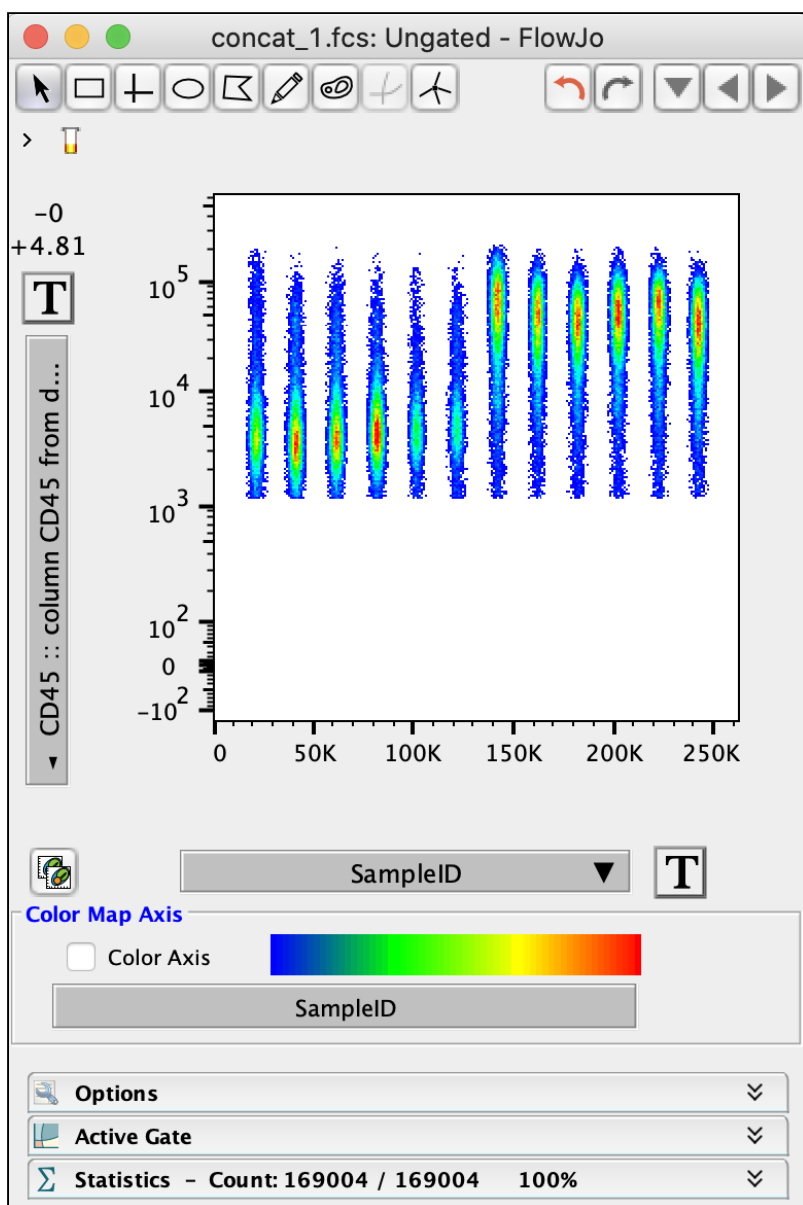
Add Keyword Annotate Experiment... Plate Editor Biology Help

Group	Size	Role
All Samples	1	Test
<b>Compensation</b>	0	Compensation

Name Statistic #Cells

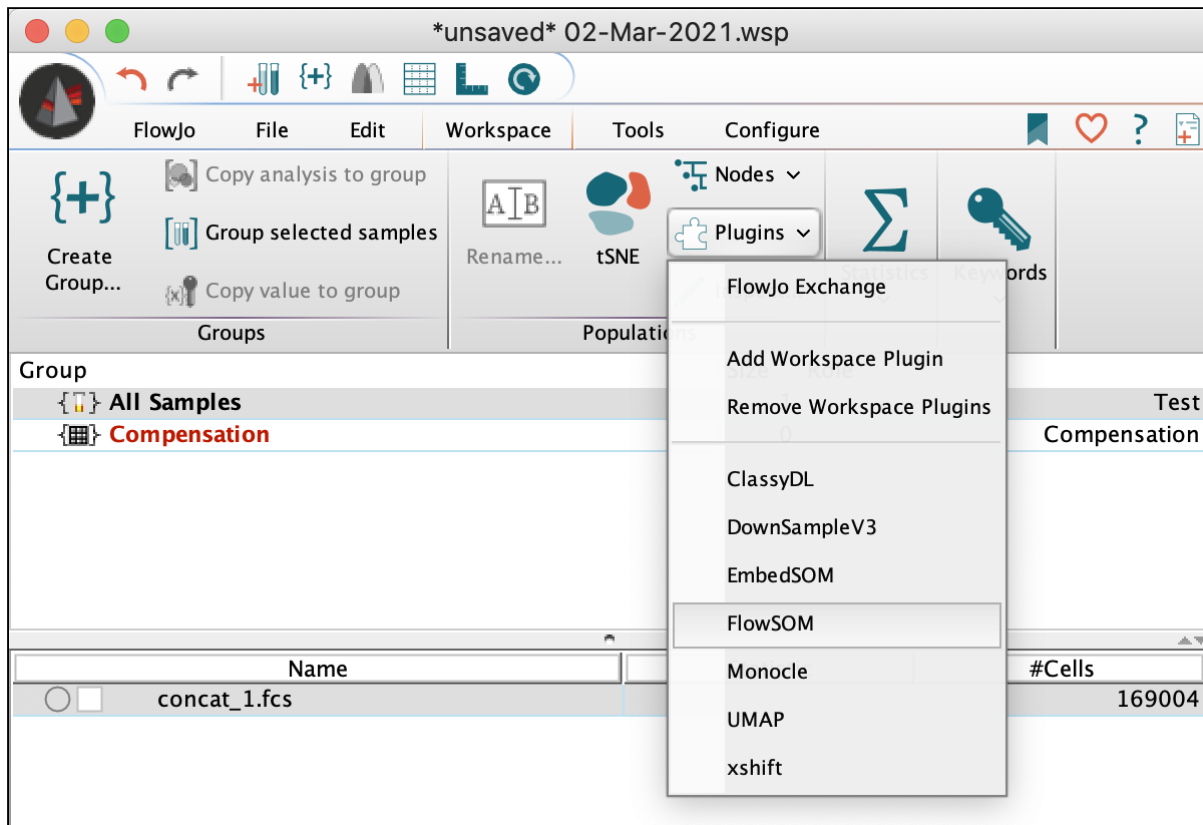
concat\_1.fcs 169004

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).

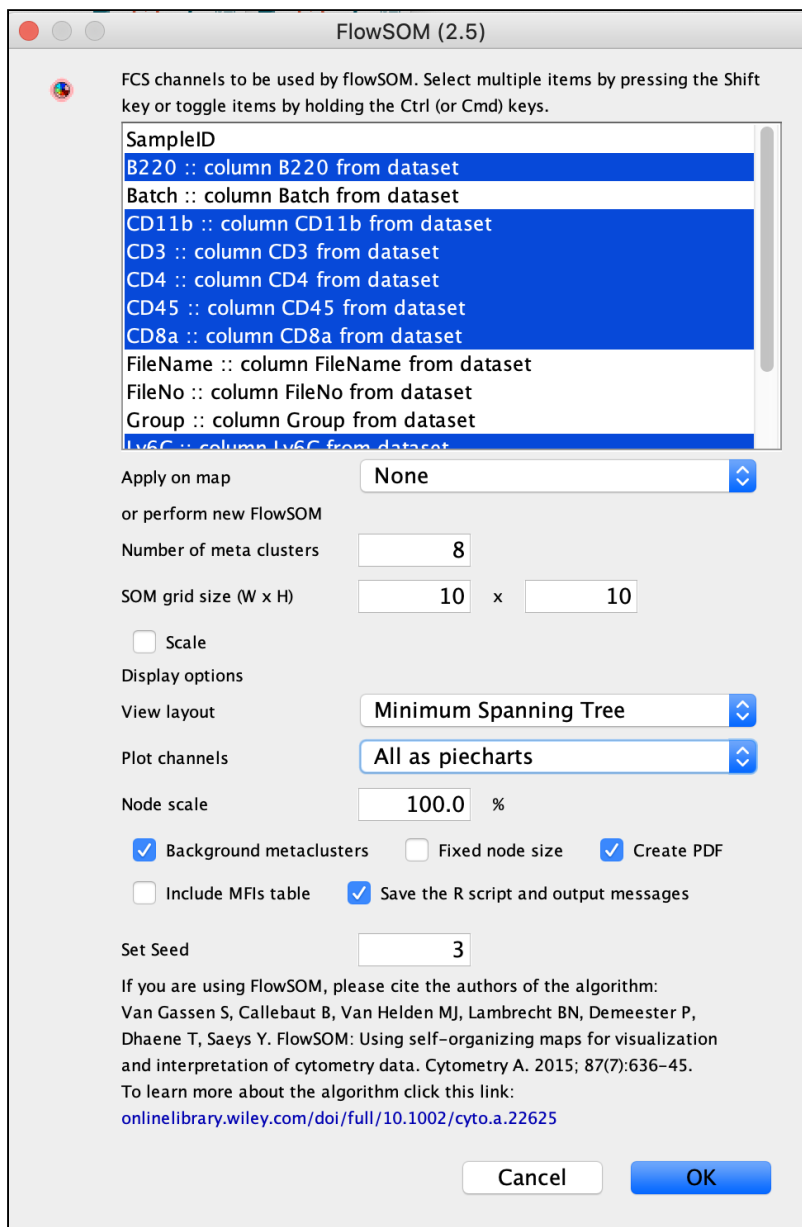


## 2. Clustering and dimensionality reduction

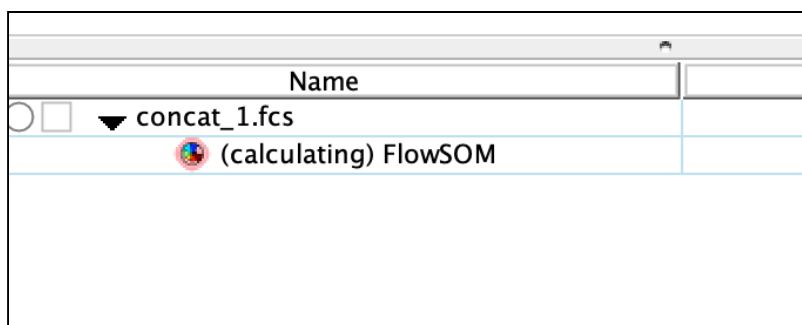
Select the file, and go to Workspace / Plugins / FlowSOM.

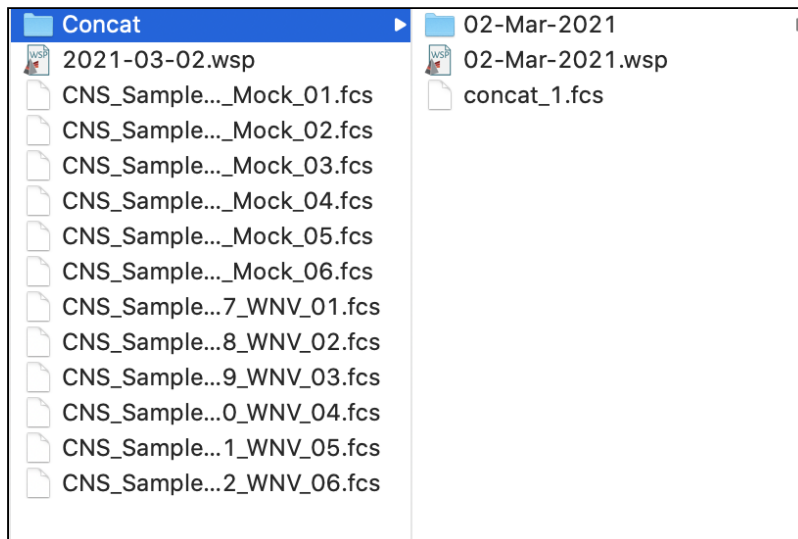


Choose the parameters you wish to use for FlowSOM clustering. You should also choose a target number of metaclusters. Click 'OK' when you are done.



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.

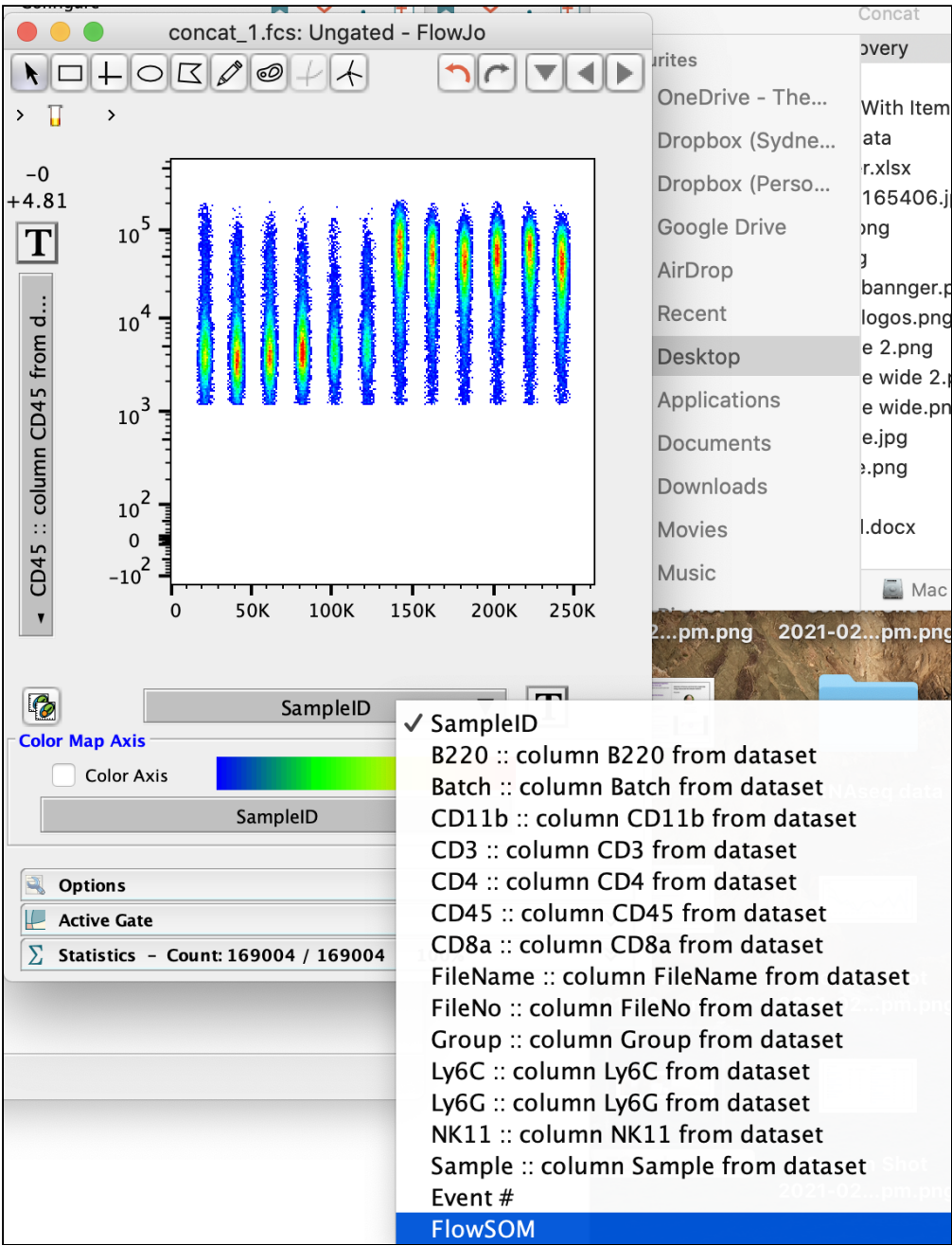


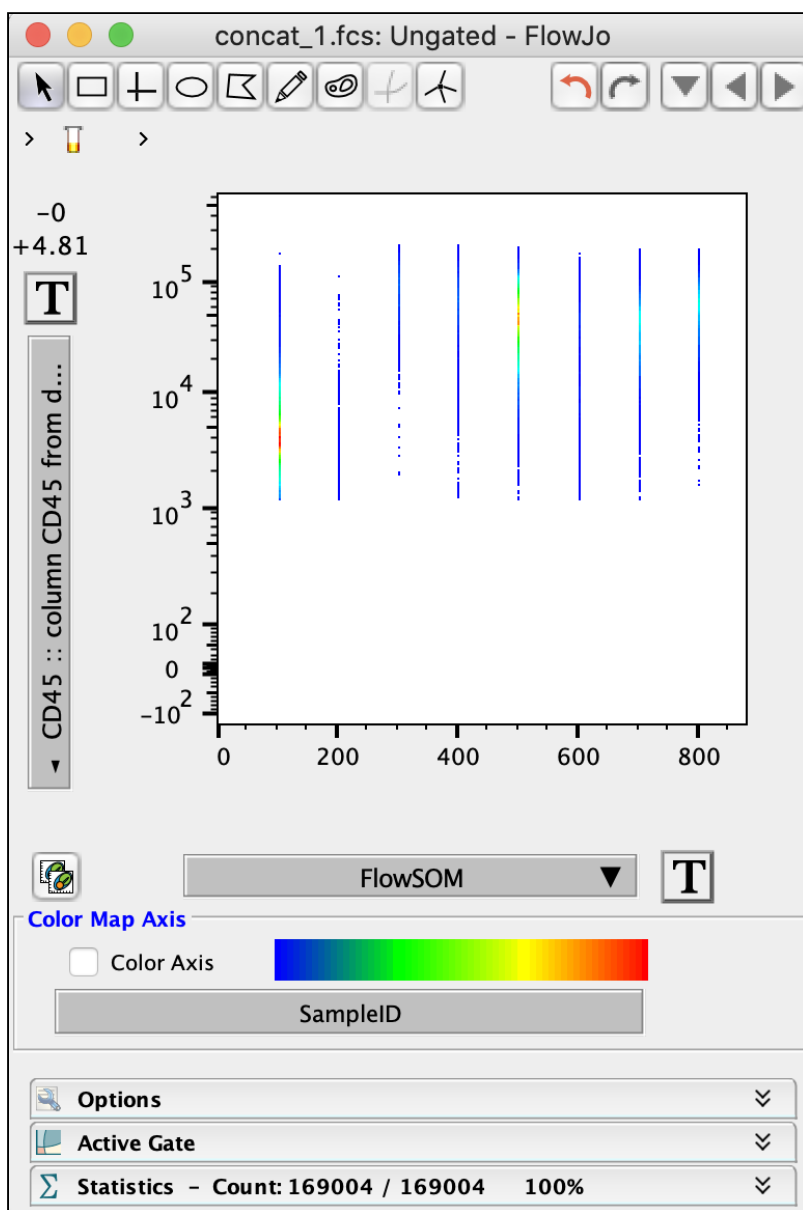


When FlowSOM has finished running, you will see the a/b node (containing the FlowSOM settings), and a virtual gate for each FlowSOM metaclusters.

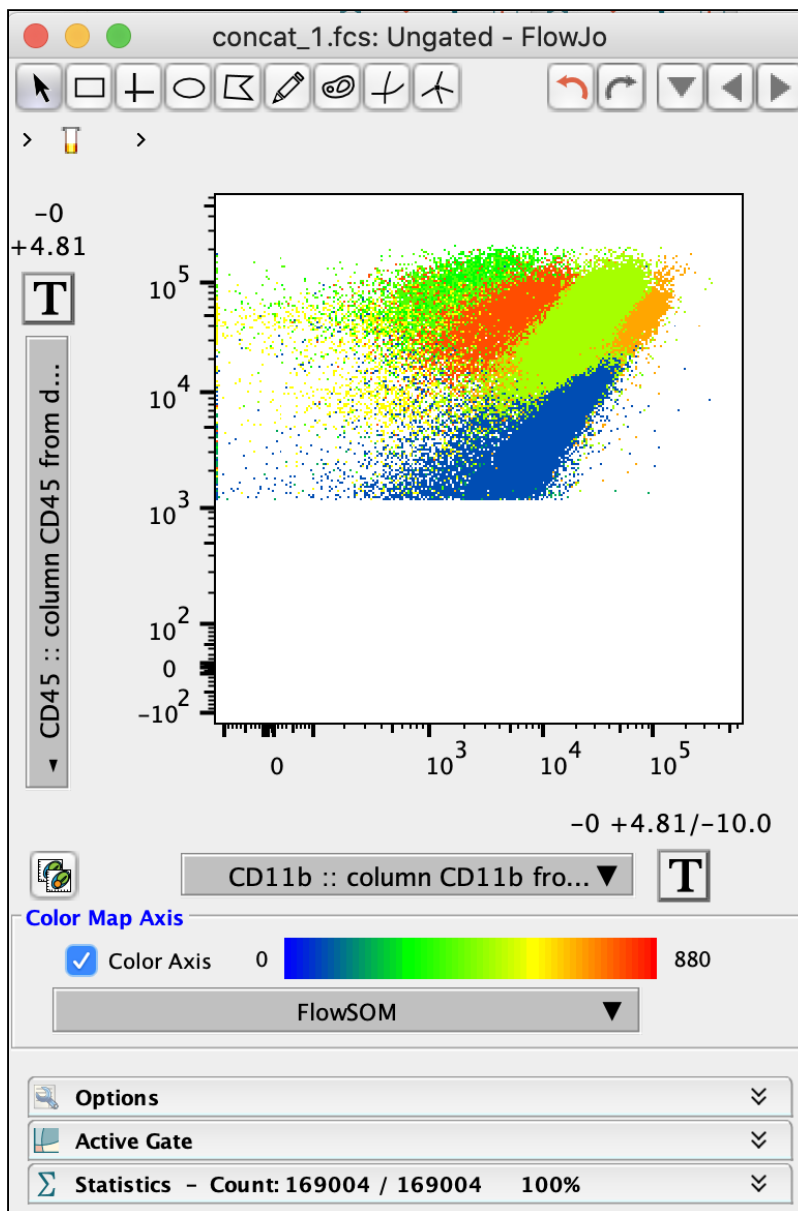
Name	Statistic	#Cells
concat_1.fcs		169004
FlowSOM		
FlowSOM		
FlowSOM.Pop0	40.9	69185
FlowSOM.Pop1	0.31	522
FlowSOM.Pop2	2.13	3594
FlowSOM.Pop3	2.32	3919
FlowSOM.Pop4	38.7	65439
FlowSOM.Pop5	1.50	2530
FlowSOM.Pop6	7.67	12955
FlowSOM.Pop7	6.43	10860

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





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



### 3. Dimensionality reduction and plotting



The screenshot shows the FlowJo software interface. The main workspace displays a group named 'Compensation' under 'All Samples'. Below this, a table lists various populations and their cell counts. The 'Plugins' dropdown menu is open, showing options like 'FlowJo Exchange', 'Add Workspace Plugin', 'Remove Workspace Plugins', 'ClassyDL', 'DownSampleV3' (highlighted), 'EmbedSOM', 'FlowSOM', 'Monocle', 'UMAP', and 'xshift'.

Name	#Cells
concat_1.fcs	169004
FlowSOM	
FlowSOM	
FlowSOM.Pop0	69185
FlowSOM.Pop1	522
FlowSOM.Pop2	3594
FlowSOM.Pop3	3919
FlowSOM.Pop4	65439
FlowSOM.Pop5	2530
FlowSOM.Pop6	12955
FlowSOM.Pop7	10860

The 'DownSample Plugin (3.3)' dialog box is shown. It contains the following text and fields:

- Instruction: Create a gated sub-population of events that are evenly distributed in the parent population
- Number of events: 10000
- Population name: sub
- Buttons: 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

+

Create Group...

Copy analysis to group

Group selected samples

Copy value to group

Rename...

tSNE

Nodes

Plugins

Σ

Keyboards

Groups

Populations

Tests

Group

All Samples

Compensation

+

concat\_1.fcs

FlowSOM

DownSample-sub

FlowSOM

FlowSOM.Pop0

FlowSOM.Pop1

FlowSOM.Pop2

FlowSOM.Pop3

FlowSOM.Pop4

FlowSOM.Pop5

FlowSOM.Pop6

FlowSOM.Pop7

sub

Statistic

40.9

0.31

2.13

2.32

38.7

1.50

7.67

6.43

5.92

#Cells

169004

69185

522

3594

3919

65439

2530

12955

10860

10000

FlowJo Exchange

Add Workspace Plugin

Remove Workspace Plugins

ClassyDL

DownSampleV3

EmbedSOM

FlowSOM

Monocle

UMAP

xshift

UMAP Plugin

Distance Function

Euclidean

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

Nearest Neighbors:

15

Minimum Distance:

0.5

☐

Select all parameters

☐

Change Output Directory

Citation

UMAP is developed by McInnes, L, Healy, J,

UMAP: Uniform Manifold Approximation and Projection

for Dimension Reduction, ArXiv e-prints 1802.03426, 2018

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All rights reserved.

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
(calculating) UMAP_ID_1MMI c		

02-Mar-2021

02-Mar-2021.wsp

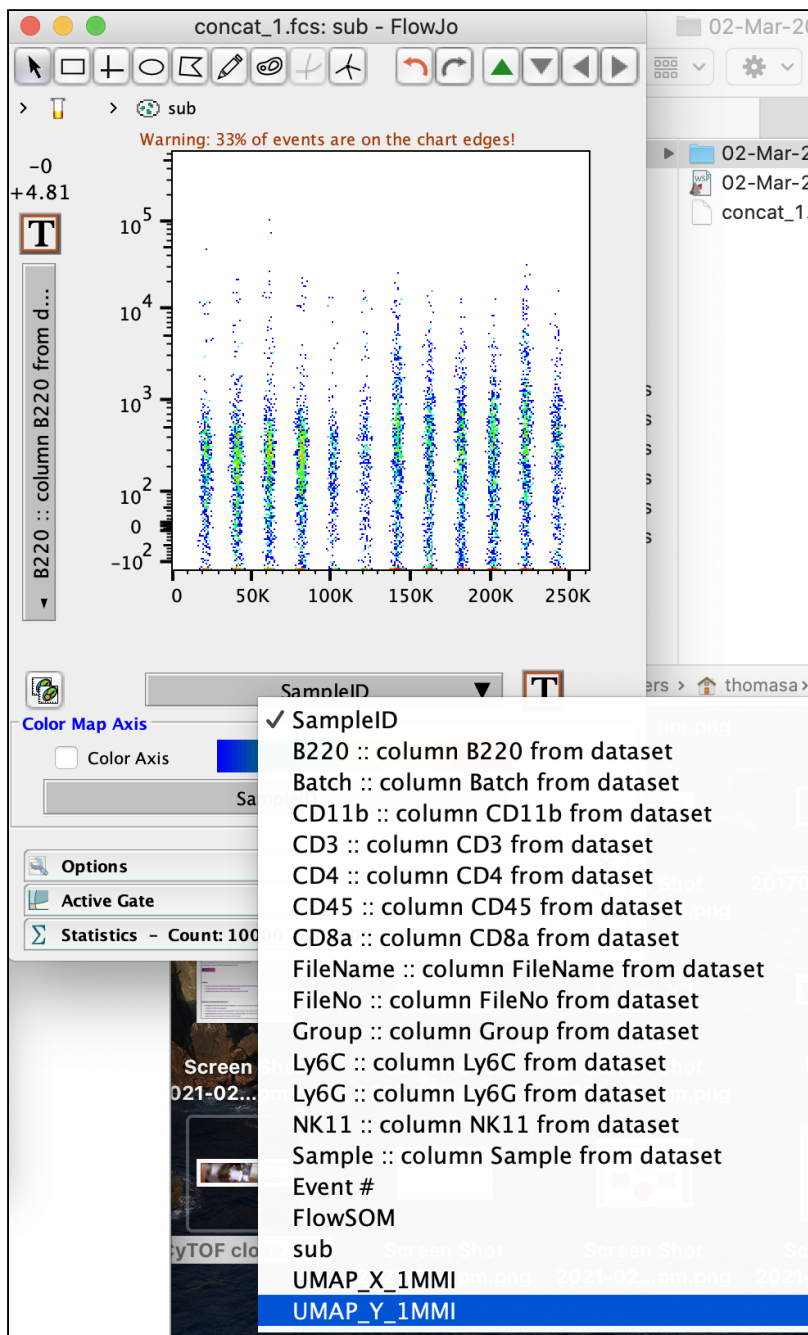
concat\_1.fcs

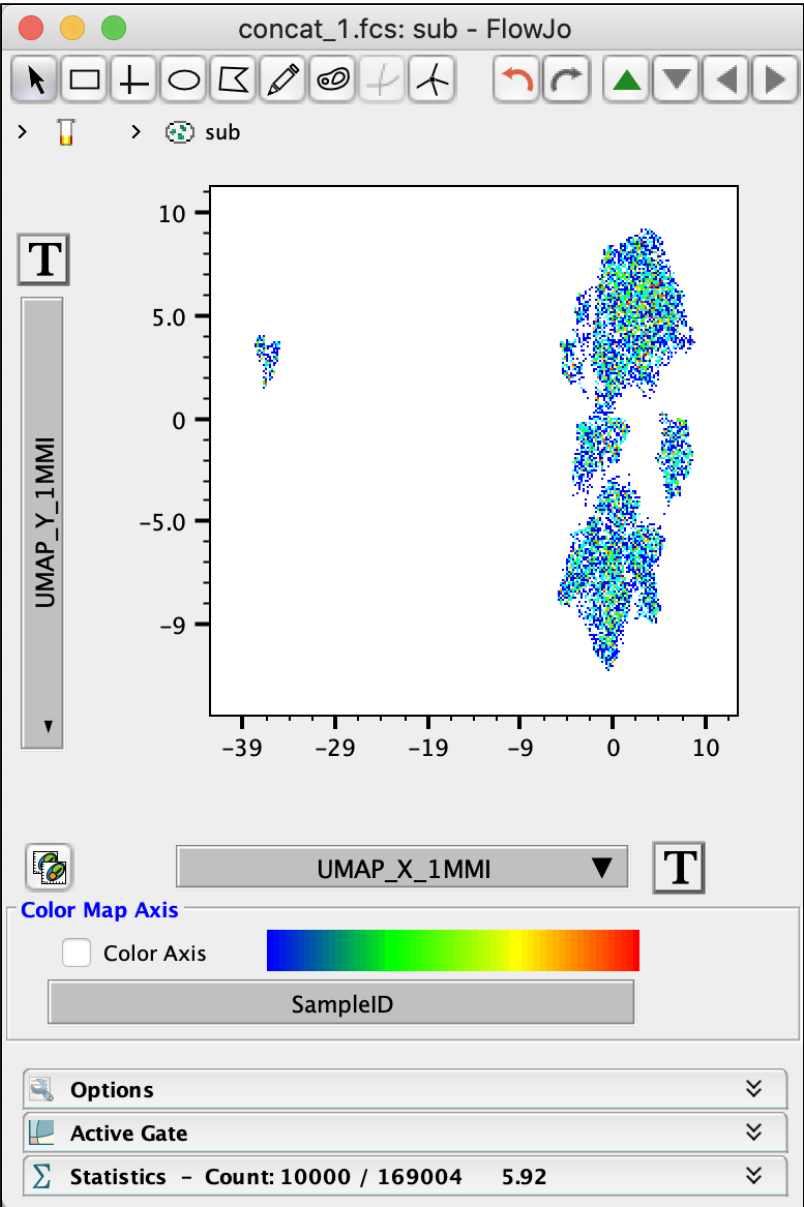
FlowSOM

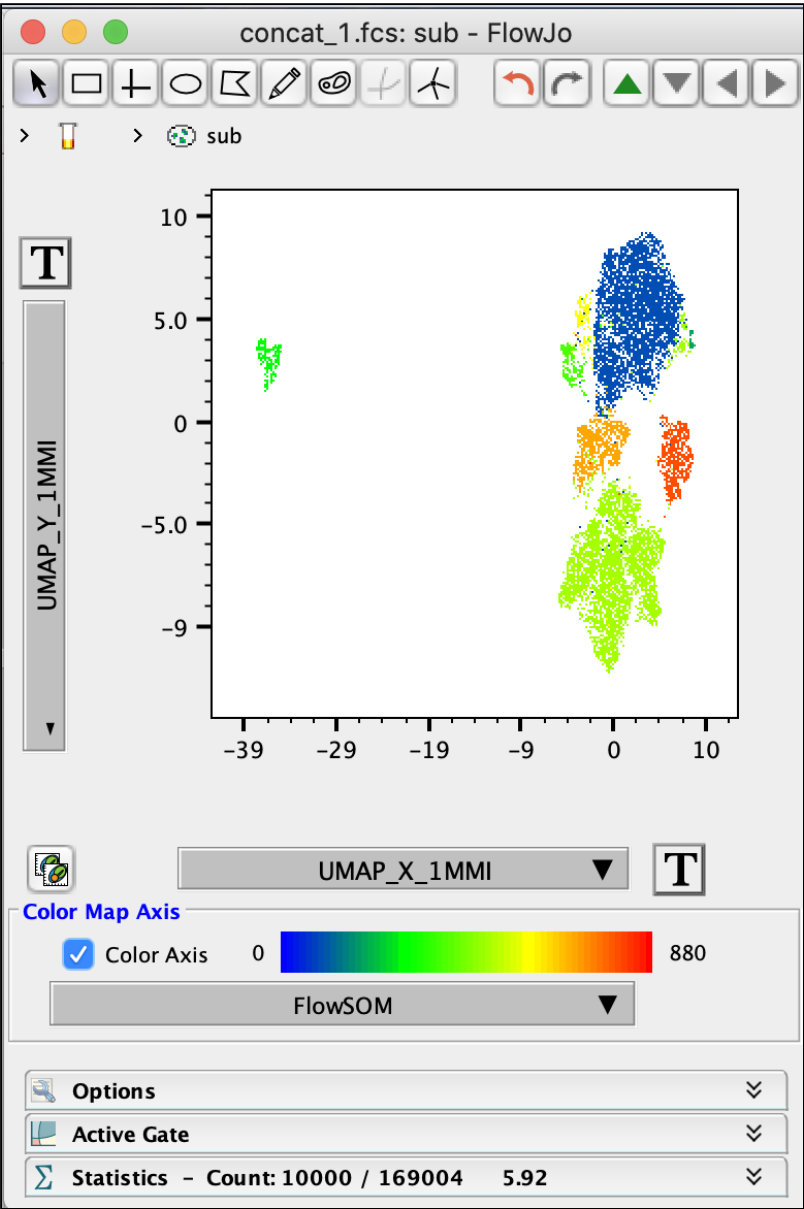
sub

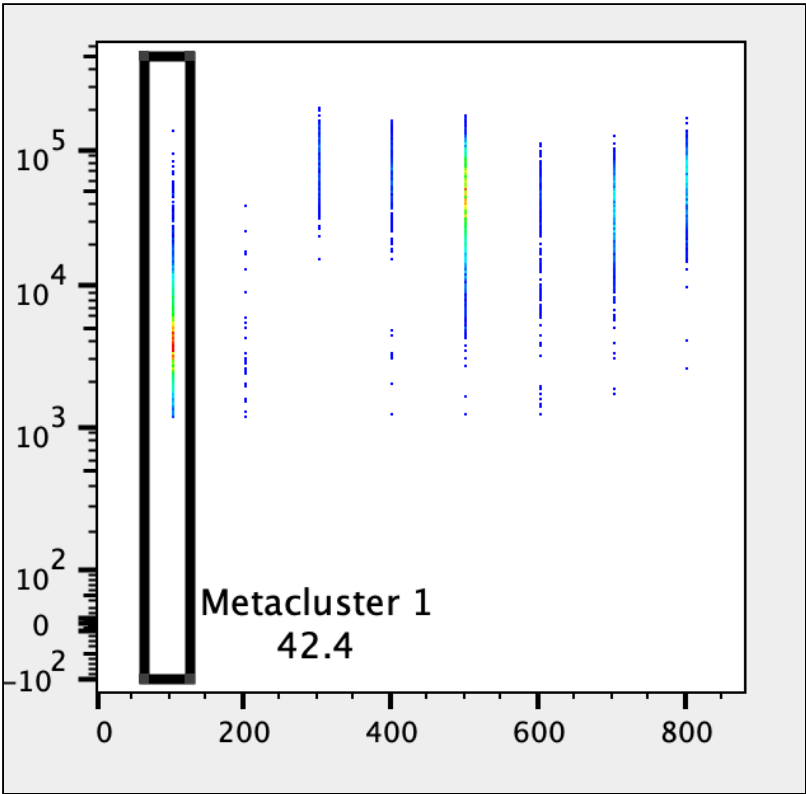
UMAP\_ID\_1MMI

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

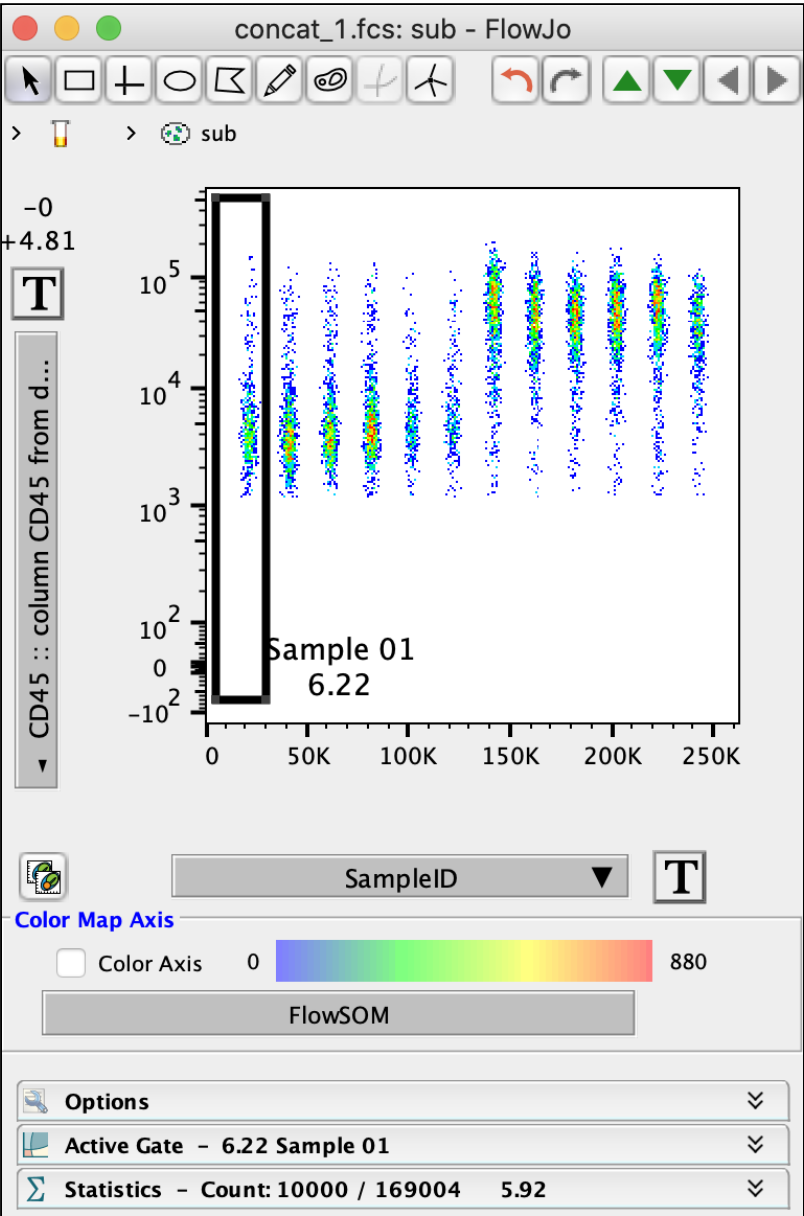


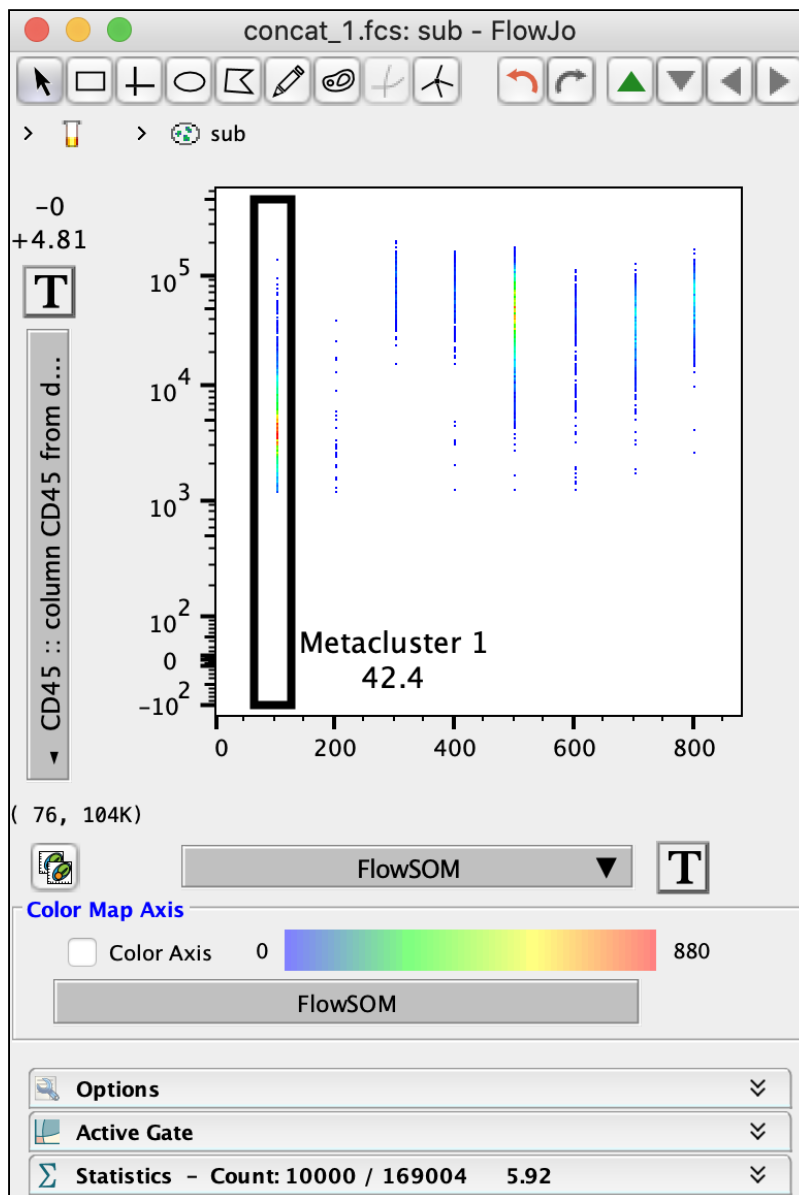


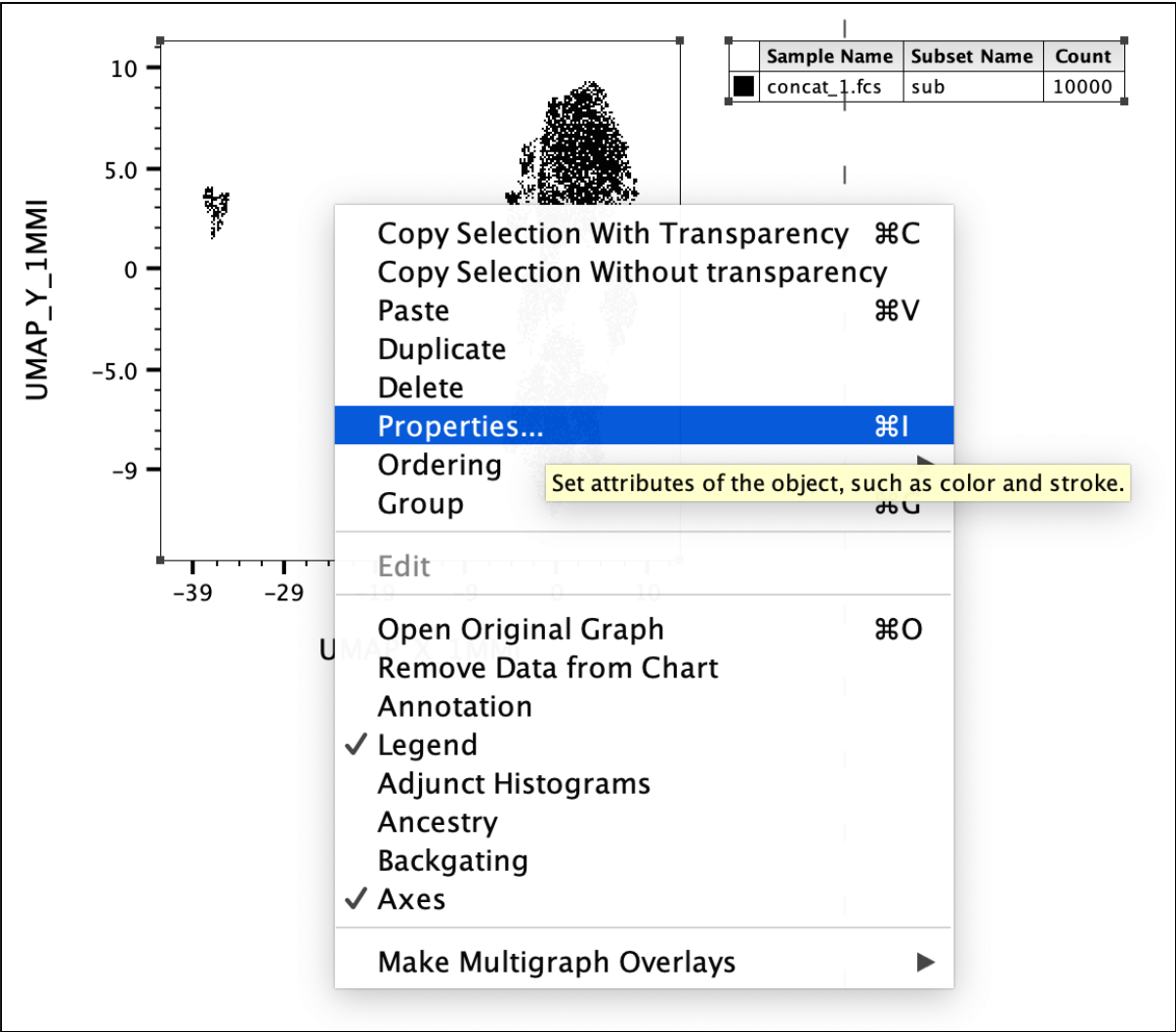
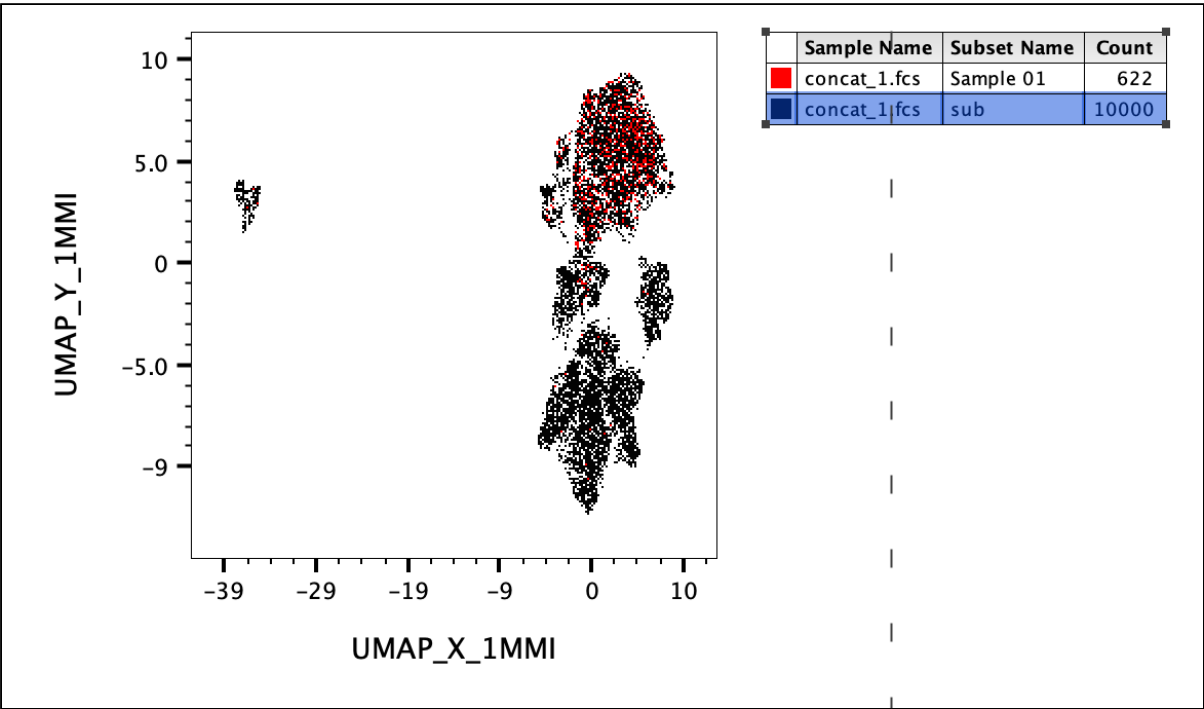












Graph Definition (2 items)

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 %

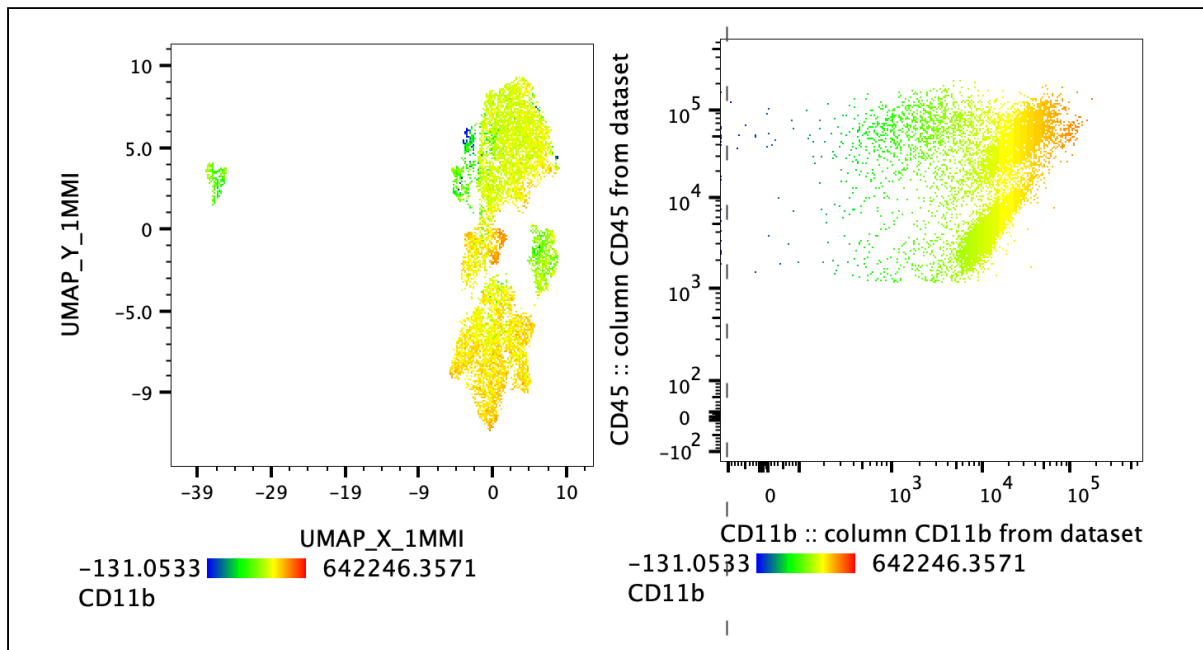
☐ Lock Shape

?

Apply

Cancel

OK



## 4. Quantitative and statistical analysis