A Discovery Workflow using Downsampling, Concatenate, tSNE and flowSOM in FlowJo v10.
The Experiment

• In this example, I have 2 patients, and I want to examine the response of PBMC, and particularly the HLA-DR+ antigen presenting cell compartment, to stimulation with a TLR agonist cocktail.

• Each patient sample was thawed and rested overnight, then processed in parallel with or without stimulation for 8 hours, stained with a 13 color panel using a standard ICS protocol, and data collected on an BD LSRII cytometer.

• My goal is to identify what phenotypic populations are induced upon stimulation, without knowing what I’m looking for.

• Therefore, I’m not going to gate for specific phenotypes or combinations of markers. Instead, I’ll use a clustering algorithm to create the populations, determine if the populations are differentially expressed between the No Stim and TLR Stimulated Experimental conditions, and determine the phenotypes of those clusters.
FlowSOM

• Uses a Self Organizing Map to analyze, and produces cluster populations and a spanning tree with star charts to...

tSNE

- t-Distributed Stochastic Neighbor Embedding (tSNE) is an algorithm for performing dimensionality reduction, which allows visualization of complex multi-dimensional data in fewer dimensions, while still maintaining structure of the data.

- The key to comparing different samples with tSNE, is to run the tSNE algorithm on all the data together.
- Therefore, we will first concatenate (merge) multiple samples into one new .fcs file, then run tSNE.

Analysis Workflow

- Compensate
- Cleanup Gate
  - FlowAI/FlowClean
- Downsample target populations
- Concatenate target populations
- Run t-Distributed Stochastic Neighbor Embedding (tSNE) dimensionality reduction
- Visualize tSNE space with 3rd parameter color mapping and Query Gate overlay in the Layout Editor
- Gate populations based Experimental Condition and compare within the tSNE space
- Use a clustering algorithm (FlowSOM/FlowMeans) to create Populations
- Use Experimental Condition Gates to determine which cluster Populations go Up/Down between the different conditions
- Classify populations and determine Population Phenotypes
  - Layout Editor Overlay --> all Histograms
  - Export Populations and batch in Table Editor to determine statistical Medians of
Cleanup Gate

Removes doublets, debris, dead cells and unwanted (wrong lineage) events.

→ Clean populations of interest.
Downsample Target Populations

Reduces the number of events that will be utilized in the tSNE calculation (memory expensive).

Normalizes number of events contributed from each file to the total Concatenated file (next step).
Concatenate Downsample Populations

Concatenate (merge) all Downsample populations into a single new concatenated file.

Load into existing workspace.
Run tSNE on Concatenated File

Run tSNE Plugin on Concatenated file → Dimensionally reduced derived parameters created (tSNE X vs tSNE Y)
Visualize tSNE Space

IL-6  HLA-DR  CD10  TNF

CD83  CD14  IL1beta  CD16

CD12  CD123  IL10

Total  TLR Stimulated  No Stimulation

Query: 2.23

Color Map Axis: Comp-APC-A : L-E

Color Map Axis Statistics: Median

Qdot 705-A

Time

SampleID

*ConcatOrder

Comp-APC-H7-A : HLA-DR
Comp-Alexa Flour 430-A : Vla
Comp-Alexa Flour 700-A : CD14
Comp-BV556-A : TNF
Comp-BV717-A : CD83
Comp-FITC-A : CD14 Aa468
Comp-PE-A : L-18
Comp-PE-Cy7-A : CD3-S6-19-20
Comp-PE-Texas Red-A : CD16 PE-CF594
Comp-Pacific Blue-A : L-12 BV421
Comp-PerCP-Cy5.5-A : CD123
Comp-Qdot 605-A : L-10 BV685

tsNE X  tSNE Y

FlowCytOM
Query Gate Overlay in LE
Gate on the Experimental Conditions

- SampleID Parameter created during Concatenation
- Keyword based parameters can be created (separate into populations based on experimental condition)
- Gate on Conditions, then copy to the Query Gate

→ Is the tSNE population that you have gated increased or decreased between experimental conditions?
Important Note: Data being pushed to R cannot have any spaces in the file path. This means NO SPACES in the FlowJo Workspace name, or any upstream file folders in the path on your hard drive. Underscores are fine. Spaces are a not.
Apply Experimental Condition Gates to FlowSOM Pops
# Export FlowSOM Pops

### Steps:

1. **Load files**
   - Exported flowSOM population `.fcs` files into Existing Workspace

2. **Put them in a group together**

3. **Add Statistics for All Compensated Medians**

### TUTORIAL:

**File Manipulation:**

- Import the `.fcs` files of your FlowSOM populations into the Workspace.
- Ensure all files are grouped together for easy access.
- Add statistics for all compensated medians to each group.

**Exporting:**

- Select the desired populations to export.
- Configure export settings if necessary.
- Click on Export to save the populations.

**Advanced Options:**

- **Include All** or specify `Include no more than` parameters.
- **Reset to minimum** to clear previous settings.

**Status:**

- The operation will generate new data files based on the selected parameters.

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### Example:

<table>
<thead>
<tr>
<th>Population</th>
<th>Size (Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FlowSOM Pop0</td>
<td>2986</td>
</tr>
<tr>
<td>FlowSOM Pop1</td>
<td>7523</td>
</tr>
<tr>
<td>FlowSOM Pop2</td>
<td>19406</td>
</tr>
<tr>
<td>FlowSOM Pop3</td>
<td>10474</td>
</tr>
<tr>
<td>FlowSOM Pop4</td>
<td>211</td>
</tr>
<tr>
<td>FlowSOM Pop5</td>
<td>1293</td>
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<tr>
<td>FlowSOM Pop6</td>
<td>2633</td>
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<tr>
<td>FlowSOM Pop7</td>
<td>1097</td>
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<td>FlowSOM Pop18</td>
<td>8000</td>
</tr>
<tr>
<td>FlowSOM Pop19</td>
<td>8000</td>
</tr>
</tbody>
</table>
Add Median Stats and Export
The FlowJo Exchange

http://exchange.flowjo.com/

• Future plugin releases
• Featured plugins
• Updates
• Developer documentation
• Scripts

Documentation http://docs.flowjo.com

• Search for Plugins → pages describing plugin setup and functionality