Protocol for DNA Content Analysis Using Flow Cytometry

Note that the following quantities of reagents are for cells grown in 6-well plates

Materials:

- 0.25% trypsin: Gibco item # 25200056
- RNase A: Sigma item # R6513-10MG
- Propidium Iodide: Life Technologies Item #: P3566
- Tween-20
- PBS
- 70% ice cold ethanol
- Cell culture medium (use the cell culture medium used for cultivating the cells)

Harvest and Fix Cells for Flow Cytometry

1) Aspirate cell culture media with a pasteur pipette and wash 1X with 1 mL PBS pre-warmed at 37°C.
2) Remove PBS and add 0.3 mL 0.25% Trypsin EDTA. Return to the incubator for 3 minutes.
3) Tap the plate on a firm surface, such as a counter top, to detach cells. Add 1 mL EMEM to inhibit trypsinization.
4) Pipette up and down with a P1000 pipettor to facilitate cellular detachment, then transfer contents of each well to 2 mL Eppendorf tube. Centrifuge at 2,500 rpm for 3 minutes to pellet cells.
5) Decant supernatant (use a Pasteur pipette and vacuum pump for this step), and replace with 750 uL of 70% EtOH stored at -20C. KEEP on ice.
    a. *Most protocols emphasize the need to disrupt the pellet by vortexing. I have not had this issue. Usually, pipetting up and down a few times is sufficient to re-suspend the cells.*
6) Store cells at -20C until ready for analysis.
    a. *Multiple references state that cells can be stored at -20C for several months. I have stored cells for ~2 months prior to analyses before and have not noticed any issues.*

PI Staining for DNA Analysis

1) Pellet ethanol-fixed cells by centrifuging at 1,500 rpm for 5 minutes (you can increase the centrifugation to 3,000 rpm, but it is not recommended to centrifuge cells at >3,000 rpm).
    a. *Note that after cells have been fixed with ethanol they will be more "buoyant" and therefore require centrifugation at higher speeds or for longer time.*
2) Decant the ethanol (again, use a Pasteur pipette and vacuum pump for this). Re-suspend in 1 mL PBS-T to permeablize cells. Incubate cells at room temperature for 10 minutes.
    a. *PBS-T = 100 mL PBS + 100 uL of Tween-20 + 1 gram of Bovine serum albumin*
3) Centrifuge at 2,500 rpm for 5 mins. Decant supernatant, and re-suspend in 300 uL of PI staining solution. Incubate cells at room temperature, in the dark, for 10 minutes.
    a. *PI staining solution: 40 ug/mL PI + 100 ug/mL RNase + PBST (bring up to volume)*
    b. *Note that other protocols suggest a "wash" step (re-suspend in PBST, then centrifuge and decant prior to PI staining). I skip this step because I am concerned about losing too many cells due to multiple washes; I have not had any issues with my analyses.*

4) Keep cells in the dark, at 4C prior to analyzing cells for DNA content
   a. *Stained cells may be stored for up to 4 hrs prior to flow cytometry.*
   b. *KEEP CELLS ON ICE UNTIL THEY ARE ANALYZED. If cells are not kept on ice you will see an odd 'smear' for the DNA content. Keeping them in the dark is also a good idea to make sure that the PI retains its fluorescence intensity.*

Use Flow Cytometer to Analyze DNA content
1) Load, and analyze at least 50,000 events per sample, using the PI channel.
2) Use FCS express to analyze data. Set gate using (-C sample) and use this same gate to analyze data from all samples.

Image Analysis
1) Import .fcs files into FCS express software by using the 'insert dot' plot on the top menu (see below).
2) Gating: you should gate your data to exclude doublets (i.e. two cells that were stuck together and were counted as one event with 2 copies of DNA instead of 2 events
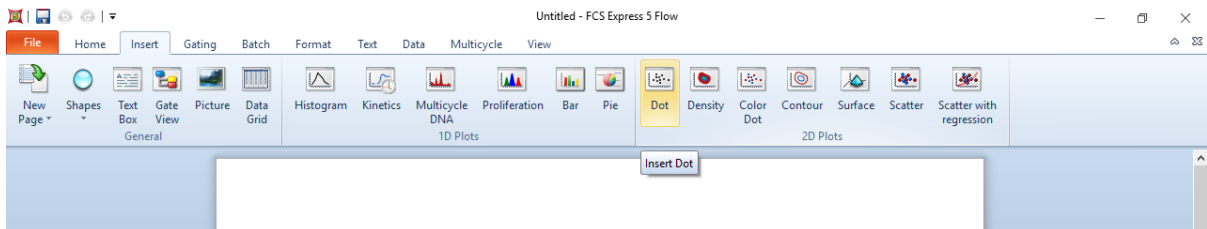

with 1 copy of DNA each). The easiest way to do this is to plot SSA vs. FSA and then use the 'polygon' gate command to draw a gate:
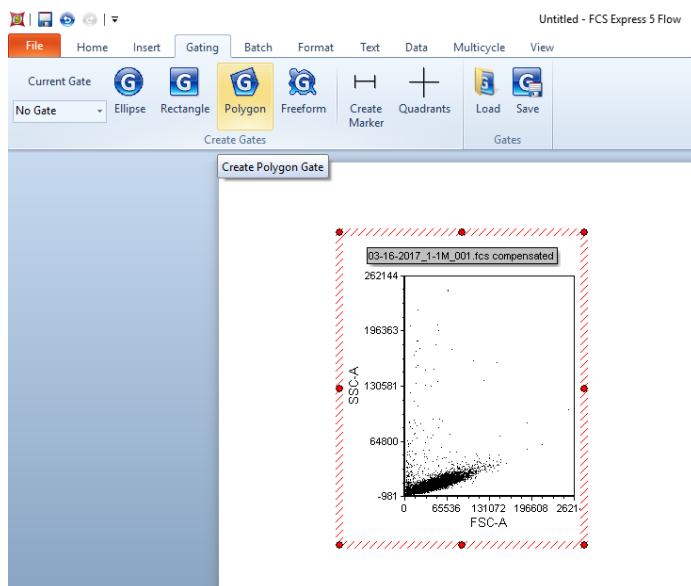
3) The relationship between the SSC-A and FSC-A should be more or less linear. You should exclude the events that have a high signal for SSC-A but low FSC-A, likewise, you should exclude cells that have a high FSC-A but low SSC-A. This is done by drawing a 'gate' around the data (see below). *I used my negative control cells to establish the gate, and then I apply this to all of the other samples in the data set. I also used the negative control to 'define' the cell cycle sub-populations.*
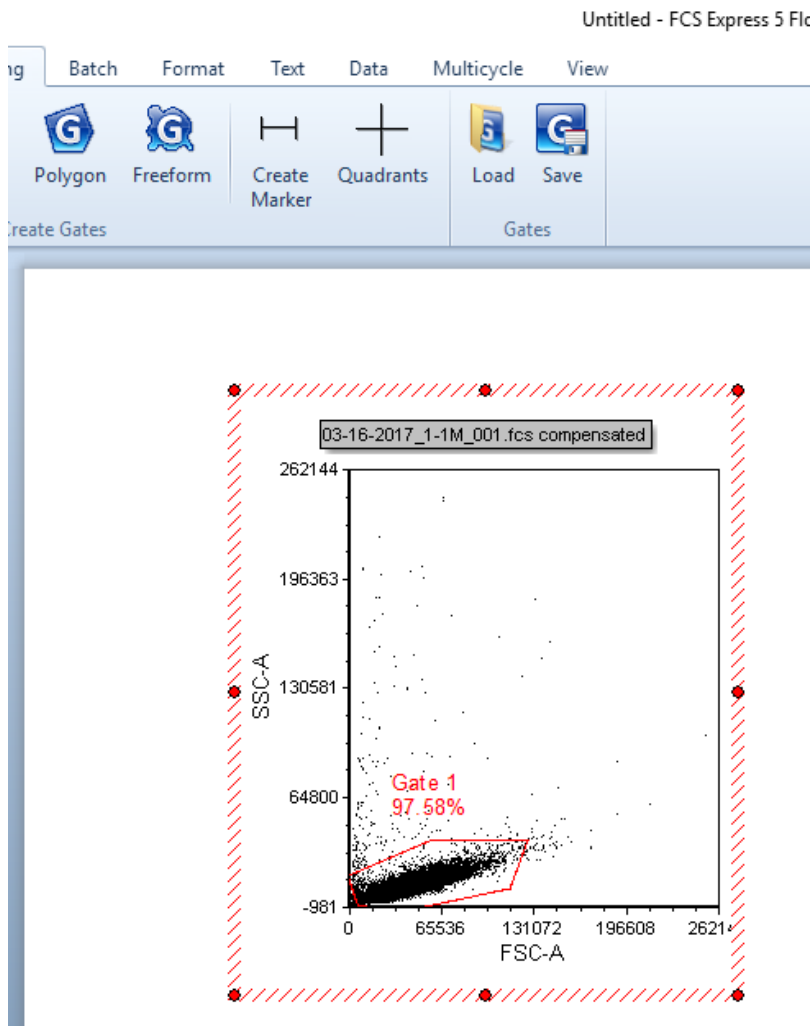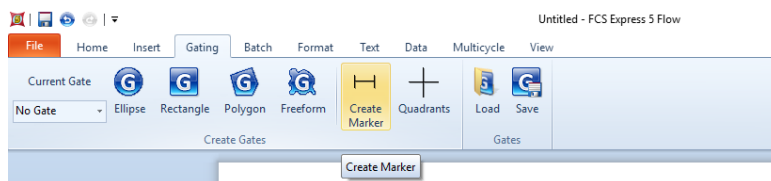
4) To generate a cell-cycle histogram you need to insert a DNA histogram, to do this, insert a 'histogram.' The histogram should display count vs. PI (on the X-axis).
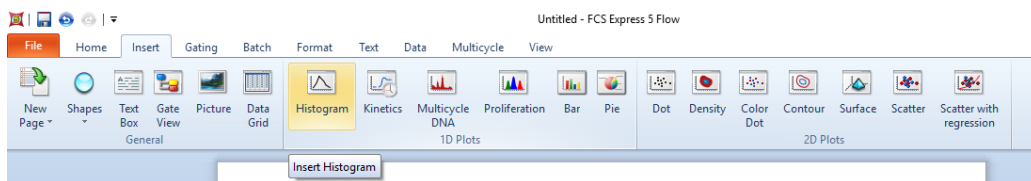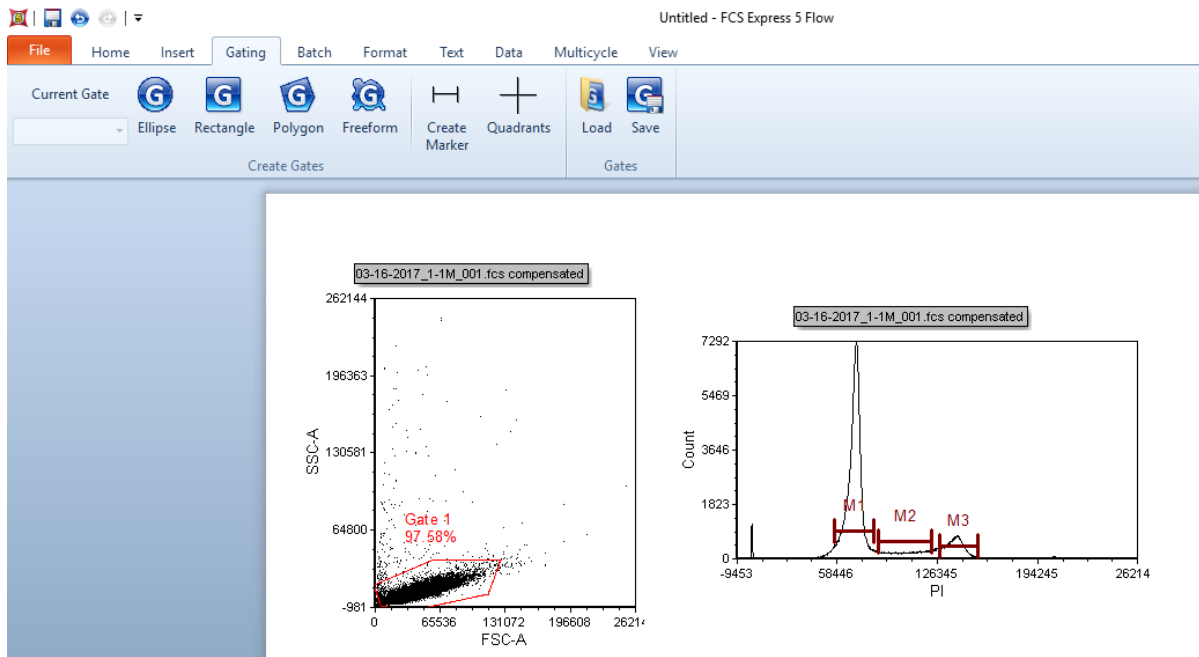

To define the cell cycle phases, you will need to draw 'markers'. To do this, navigate to the 'gating' tab and then click the 'create marker' button.


5) You will need to draw 3 markers to define the following cell cycle phases: G1 (1 copy of genomic DNA), S (in between 1 and 2 copies of DNA), and G2/M (2 copies of DNA).

6) Analyze the other data: insert a new DNA histogram for your sample to analyze (follow step 4 above). The program will automatically assume that you want to insert a DNA histogram of the previous sample that you were working with. To change the data file right click on the DNA histogram and navigate to the 'change file' to import the new data:
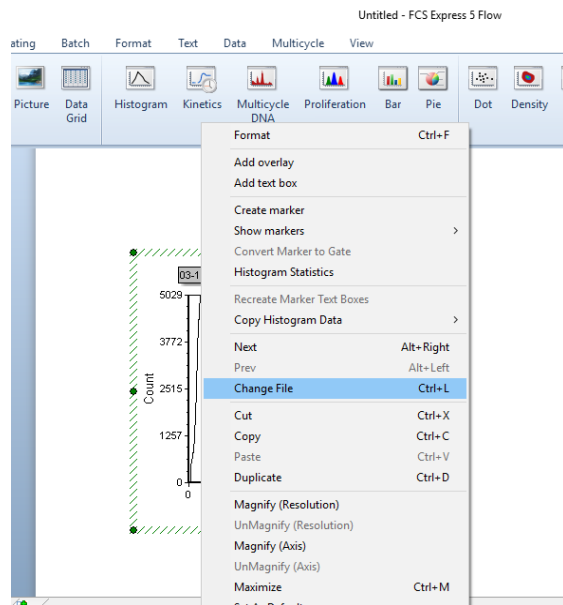
7) To change the axis, simply left click on the axis that you want to change (the X-axis in this case) and HOLD down while navigating to the first "PI" option (see photo).
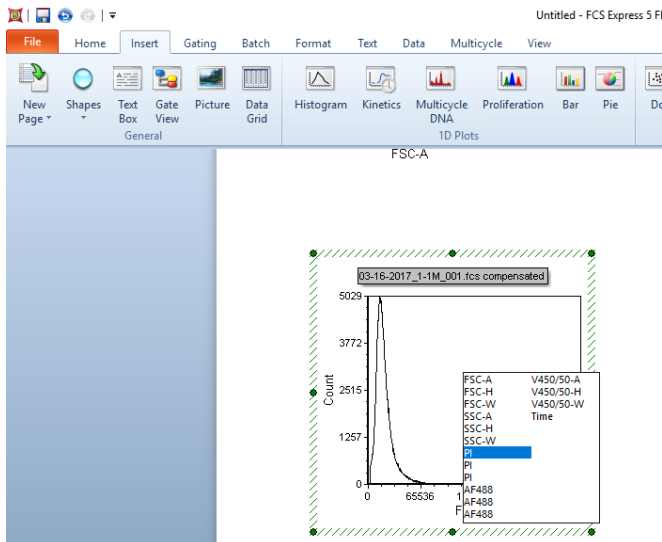
8) To apply the markers that you defined using your negative control cell population, simply click on the marker to select it, and use "Ctrl + C" and then "Ctrl + V" to paste it onto your DNA histogram. The software will automatically add it to the PI-intensity (X-axis) that you defined in the negative control. To display the number of events within each marker (i.e. number of cells in each cell-cycle phase) you can click "histogram statistics":
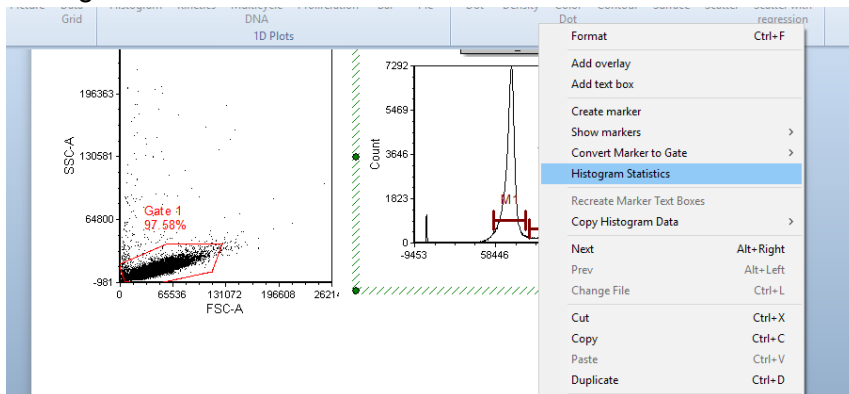
9)
   a. *Some things to keep in mind: I always defined the markers for every set of samples. It is fine to analyze multiple experiments at the same time (i.e. store cells at -20C until you stain several sets of experiments and analyze them at the same time), but you should re-define the markers for each experiment.*
   b. *The PI intensity should double between the mean G1 and mean G2/M (what are shown as M1 and M3 in the above example).*
   c. *I found it easiest to organize data in an excel file (so copy the number of events in each sub-population from the histogram statistics)*

Troubleshooting

1) Cell cycle analysis produces a 'smear' on the histogram (i.e. PI signal is a smear between signal 0 and the G1 peak). This is likely due to undegraded RNA. It is important to remember that PI will stain any nucleic acid, so it is important to deplete your sample of RNA prior to analysis. Make sure that you have incubated cells at room temperature for at least 10 minutes prior to moving the sample to ice.

2) Really low concentrations of cells: I would not recommend performing flow cytometry analyses with cells not grown in 6-well plates (approx. 2e6 cells).