Influx Operation

Sheath fluid:

Make sheath the day before or on the day of the run. The concentration should be 0.9% NaCl for mammalian cells. In general, sheath concentration is whatever will make the cells physiologically happy, as they will only be in contact with the sheath for a short time. For marine samples the concentration will be higher. We have a large flask that Alan marked off at 4L. Add 9g NaCL per Liter of MilliQ. Filter through a 0.2um filter into large bottles and autoclave. Leave in flow room by the cytometer to equilibrate.

Setting up fluidics:

Empty waste!

Open all valves (sample, purge, sheath). Open vacuum line then airline at wall. Turn the air switch on. Turn on electronics.
Make sure tubes are free of liquid. Then close in order -purge, sample, and sheath valves. Flip air switch "off." Remove air input line on sheath tank, press red button to depressurize. Then unscrew. Leave sheath output line alone. Rinse tank with Milli-Q water then fill with 10% bleach. Make sure O-ring is inside before reattaching.
Open Spigot 6.1
Open Config. File (ie. Karie 10um beads)
Press "Present tray" then place flask on tray and press "safe."
Clean salt off the plates with plates turned "off"
Place small black waste receptacle with waste tube attached under cell exit line. Flip air switch "on." Open sheath valve and let bleach run through tubes for 10 minutes, then close sheath valve. Repeat for MilliQ and then sheath fluid. Flush the system by opening the purge valve fully and then open the sheath valve partially to fill the sheath line make sure flush bucket is in place. Run fluid through the lines for about 2 minutes to ensure the lines are full of fluid then close the purge and sheath valve.

Clear Cell Exit Line of Bubbles:

Sonicate nozzle for 5 minutes. Flush nozzle both ways using syringe with 0.2 um dilute detergent. Place nozzle in black adaptor making sure O-ring is present and tighten as much as possible. Remove black waste line receptacle

and screw on nozzle placing kimwipe underneath to catch drops. Open sheath valve to start stream.
Back flush the sample line by opening the sample valve and close after about 30 seconds. Rinse cut conical tube with dH2O then fill and submerge nozzle. With nozzle submerged, open purge valve with sheath and sample valves closed. Quickly open and close sheath valve a few times to loosen bubbles. When nozzle tube is free of bubbles, open the sheath valve and close purge valve. Remove conical from the nozzle. Dry liquid from nozzle tip using cotton swab.
Standard influx with a 70um nozzle tip two sort settings: Standard and High speed sort
with drop frequencies of 39,000/sec or 78,000/sec. and sheath pressure of 15 and 50 PSI
Standard sheath pressure is ~15PSI
Fluids take from 30 minutes to an hour to fully stabilize.
Monitors and Aligning Camera
Turn on "illum" switch for viewing monitors.
Adjust peizo knob to "5" for adjustment later.
Top monitor: Adjust camera so its aligned with stream – pinholes centered on stream. Camera is placed just below nozzle. When in focus, there should be sharp lines on the edges of the stream.
Middle monitor: Center camera on first drop (point red arrow to first drop)
Bottom monitor: Center stream just above and in the middle of exit area.
Let fluidics to stabilize for 2-3hrs

Lasers

Open water intake and outtake hoses for lasers you will be using. Turn on pump in adjacent room. Check to make sure filter clean and all wall valves are in on position. Turn on laser you are using with turn key. Turn on laser display panel above. Give lasers at least 30 min to warm up.
Prepare beads (4ml water to 1ul '1um' or '10um' beads). Pull open laser slider bar for appropriate laser. Bottom slider for green fluorescence. Adjust laser to line up with first pinhole (top knob). Use forward most facing knob to focus – forward and back until lightest point detected. It may help to bring laser above pin hole to adjust the focus. Don't need to move left most knob (side to side adjustment).

Beads

Load beads and open sample valve. Press boost until sample enters line. Check to make sure stream is in middle of sample line. Sample pressure should be equal or greater than the sheath pressure. Sample knob adjusts the sample pressure.

Spigot
Shortcut Spigot 6.1
Open software, open config file (Karie 10um beads)
Plotting pulse width vs forward light scatter (FLS) helps detect the amount of doublets. Graph should have diagonal 45degree line. Linear phase will lean down towards end. Log phase will have slight S shape. Dots to left of line will be doublets, electrical noise, or random light scatter.

If click on one boxes on right, it will enlarge on left side. Can then adjust X and Y values. Ex) PLS (side scatter) vs FLS (forward scatter)

One can change from log to linear by switching leads on electrical box. Also, adjust your setting in Spigot for log or linear.

Adjusting Gain and Trigger

Move gain so population is near the lower left corner but leave a little room so it not up against the axis.

Increasing gain increases the sensitivity so small junk is selected, adjusting trigger level will narrow down the search (trigger can usually be left alone).

Sort Gate
Draw a sort gate around bead population. Click draw window and rectangle – click around population and finish with a right click. Gate can be deleted by pressing reset.

Example Settings

100um nozzle

Sheath 14.3 **Sample 16.0**

***Drop position 251**

Piezo Amp.	**Drop Frequency**
1.96 volts	**27.4 KHz**

Delay Calculation	
Start 10 drops	**laser**
229	**159**

End 10 Drops	***Break off**
261	**251**

***If you type in these settings and only adjust the break off number found on the console you should be close in the calibration.**

Calibration

Make sure no objects are in front of tray. Turn on plate current and stream deflection. Stream deflection knob should be in 9 to 12 o'clock range. Estimate drop delay and enter into software. The higher the drop position number (use camera placement reading), the more seconds need to be added to the drop delay.

Adjust the peizo so it is in an optimal position. (This topic could be elaborated on)

Clean glass slide. Press "present tray" button. Place slide in middle slot. Set setting for calibration slide. Press "sort ready," then "sort." When finished, press "present tray." View slide under microscope and look for drop with 20 beads. Using chart in software, get the drop delay from the 20 bead drop position on the slide and enter into software. Repeat calibration to double check drop delay.

Select the sort type you will be using and load a test plate or tube for checking the drop target. (If sorting different numbers of cells, read note on tray files below) Place tube in left bracket or put plate on after removing metal holder. Press "sort ready" to move tray into position. Flip "test left" up and down once. Present tray and check the target. If doing a left and right sort, use the stream deflection to adjust the target point. Turning clockwise will create a stronger field moving stream left. Turning counter clockwise will create a weaker field moving stream right. If doing a left sort only, enter

offset numbers into the software (this will move the tray). Be sure to have tray in when adjusting offsets. To move tray left, make x axis offset positive. To move tray forward, make y axis offset positive. Test target point by wiping tube/plate and pressing "sort ready" and repeating "test left." It's better to have target error more right than left.

Note: Recalibrated during run if the drop position moves more than one drop length from the red arrow.

Coincidence: how close cells are together. If yield is more important than purifying population, increase the coincidence. 1- 1/4 is usually a good coincidence rate.

Sample

Cells pass through a 70µm nozzle tip on the instrument. Break up clumps using EDTA or vortex/pipet before loading. In order to ensure a single cell suspension filter cells through a 50µm nylon mesh (cell strainer) after all staining procedures to make sure there are no clumps and make sure the fluidics do not malfunction during the experiment.

(http://www.bdbiosciences.com/discovery_labware/products/display_product.php?keyID=190).

After removing bead sample, check for good sample backflow. Put on sample tube. Press boost.
Adjust gain and trigger for sample. Draw sort gates. If need to increase/decrease the amount of events, increase/decrease the sample pressure by turning "sample knob".

Place sort container on tray and press "sort ready." Enter in number of desired event. And press sort.

Event box will read:
Left (left sort) -red is % rejected, blue is % of events sorted over the amount selected, right most reading is # of events sorted.

Controls

Two types of control cells must be provided for each experiment:
Unstained cells of the type to be sorted. This will allow adjustment of the

instrument for autofluorescence of unstained cells excited by the laser wavelengths that will be used.
Single color controls of each antibody/dye combination to be used. This allows compensation of minor fluorescence of one antibody/dye combination into other color detectors. Compensation should be performed using the brightest antibody/dye combination for each color used.

List

Making a list will save data file with event data. This is not data on the actual sort cells but rather data for the entire population of cells or a representation of the cells that were sorted.

Shut Down

Close sample valve.
Close slider. Turn off stream deflection and plate current. Turn off laser display panel and laser with turn key. Turn off pump in pump room. Close intake and outtake water valves near laser. Don't turn off the water valves in the pump room.

Load sample of bleach and let it run through the sample line for a few minutes. Then run some Milli-Q water. Turn off the sample valve.

Shut off sheath valve. Turn off the air switch.
Remove red line from sheath tank, release pressure, empty sheath fluid and fill tank with 10% bleach, screw on sheath tank with O-ring. Put kimwipe under the cell exit line and remove nozzle. Replace kimwipe with black waste receptacle and turn on air switch and open sheath valve. Flush system for 10-15 minutes. Flush bleach through the purge line if it contains your cells. Then, close sheath valve and turn off air switch. Replace tank with Milli-Q water. And run this through the system. Flush purge line as well. Then empty tank and screw back on. Turn on air switch and open purge valve, sheath valve, and sample valve. Let system dry. Spray down sample input line, sample waste receptacle, and sheath waste line with Milli-Q water. Turn off electronics and monitors. After 10-15 minutes, close air and vacuum lines.
Check waste tank to see if needs emptying.

Next Person

If someone is using Influx after you,
Put a sample of 10% bleach on and run for a while. Then close sample line.

Let them know how much sheath fluid is left in the tank and leave sheath fluid running. Close laser slider and laser display panel if next user will be awhile, leave electronics, monitors, and software on but turn off plates.

Tray Files

Tray file editor will allow the creation of unique sorts in a variety of different tray types including 96 well and 384 well trays.
Open editor and enter numbers then save as a unique heading under 96 well or 384 well format in the tray folder in the Spigot software on the C drive. This will create iterations of the 96 well tray file that will show up in the list of trays when you open Spigot 6.1

Beginning number of cells. In order to estimate how long your sort will take and how many cells to bring, you can use the following formulas: *Time required for sort (hrs) = [Number of recovered cells desired/fraction of desired cells in starting population)/(Flow rate)*] where: *Fraction of desired cells in starting population* = percent/100
***Flow rate* = cells/hr or (cells/sec)*3600.**
***Number of cells to bring* = Time required * Flow rate * 2**

Fluorochromes that can be used with our equipment.

Laser Excitation: Violet 406nm, Blue 488nm, and Red 635nm

Violet 406nm **Blue 488nm** **Red 635nm**

Emission detectors:

FL1 FL2 **FL3 FL4 FL5 FL6** **FL7 FL8**

FL1: 460/50 CFP, Pacific Blue, DAPI, Hoechst, Alexa 405, Alexa 430, Cascade Blue, live/dead fixable violet

FL2: 550/50 YFP-Fret, Pacific Orange, Qdot 565

FL3: 531/40 Fitc, GFP, YFP, Alexa 488, R123, Mito Green, Syto 14-18, Calcein, CFDA, YoPro3, Fluo 4, Rho 110, Cytox Green, Oregon green, Na green, Fluoro emerald, Fluo-3, DCF

FL4: 572/27 PE, Ds Red

FL5: 610/20 PI (viability), PeTexas Red (ECD), PE-Alexa 610, Nile red. EB, DsRed, 7AAD

FL6: 670/40 Draq 5 (DNA), Fura Red, 7-AAD, PE-Cy5 (Cychrome), PerCP, PE Alexa 700, PerCP-Cy5.5, Fura Red, Alexa 647-PE, PE-CY5

FL7: 670/40 APC, Cy5, Alexa 660, Alexa 680, Alexa 647, Alexa 633, Draq5, Mito deep Red, ToPro3, ToTo3

FL8: 714LP APC-Cy7, APC-Alexa 750

http://probes.invitrogen.com/resources/spectraviewer/