Flow Cytometry Core Facility
Standard Operating Procedure
BD Fortessa X-20 Setup and Operation

Created: May 1, 2025
Last update: n/a

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1. Purpose

The purpose of this standard operating procedure (SOP) is to outline the setup and operation practices used for the University of Alberta Faculty of Medicine and Dentistry (FoMD) Flow Core Facility BD Fortessa X-20 analyzer. The procedures outlined here are to supplement the general laboratory procedure for the FoMD Flow Core Facility.

2. Biosafety level

The BD Fortessa X-20 is classified as a Biosafety level 2 (BSL2) instrument. BSL2 instruments are designated for human or non-human cell lines that are categorized as BSL2, Containment Level 2 (CL2) or Risk Group 2 (RG2). Live primary human or non-human primate cells are considered BSL2, as are primary cells or cell lines within 24 hours of viral transduction, or those that are otherwise infected or diseased. Please refer to flow charts in Section 14 Appendix for further guidance.

3. Hazard identification

Working on a BSL2 cell analyzer - without controls in place - is considered high risk, scoring 20 on a scale of 1-25, where 1 is the lowest and 25 the highest risk. Implementation of the measures outlined in Section 4 reduces the risk score to 4 on a scale of 1-25, where 1 is the lowest and 25 the highest risk.

4. Control/Protective measures

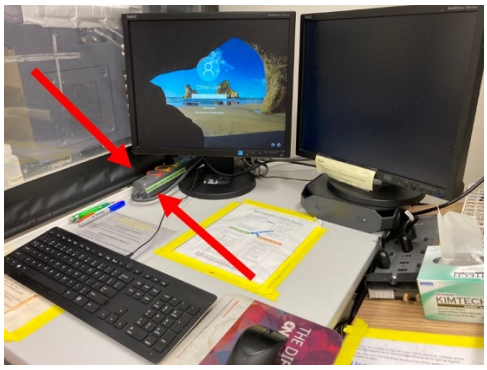
The BD Fortessa X-20 is housed in a BioBubble to contain aerosols and protect users from risks otherwise associated with handling BSL2 materials. Use of PPE is recommended (eye protection, lab coat, long pants) with gloves optional but not to be used on the keyboard associated with the X-20. Wash hands after working with samples and equipment.

5. Materials

10% Bleach (in squeeze bottle inside BioBubble)
Bleach (in cupboard under sink)
1 x ClearFlow™ Sheath Fluid (in plastic 20L carboys, prepared by FlowCore staff: 2L 10x ClearFlow + 18L MilliQ)
10 x ClearFlow™ Sheath Fluid (Leinco Cat#S622-20 L)
Coulter Clenz (tube in rack at X-20, FisherScientific Cat#15655120)
CS&T Research Beads (in fridge; BD Cat#655051)
FACSClean (box on counter, BD Cat#340345)
milliQ water (in squeeze bottle inside BioBubble)
Samples in round bottom polystyrene FACS tubes (Diamed, Cat# DIATEC1275-PST); suggested volume: 400-500 µl

6. Instrument setup

1. Turn on BioBubble with switch on power bar (see image below, red arrows).



2. Ensure the sheath tank is full: unscrew, depressurize and open the stainless steel tank and fill with sheath fluid to the upper seam line. Sheath fluid can be found in plastic 20L carboys in the room. Re-attach fluidics tank, if removed. Note that the rubber seal needs to be in place for the tank to pressurize correctly (see image below).

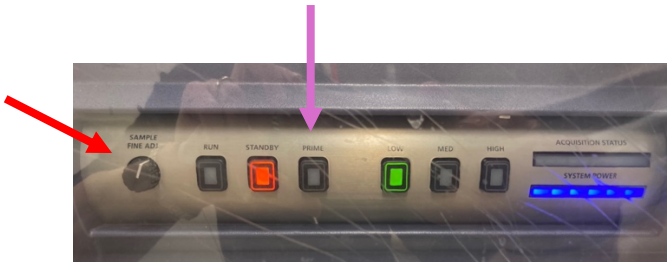
3. Ensure the waste tank is empty or less than 50% full (image below is an example of tank that is 50% full and should be emptied).

   a. To empty waste, detach waste tank from fluidics line (orange cable). This bleach-neutralized liquid waste can be emptied into the sink, followed by running water.
   b. Add ~1 L bleach to empty waste tank.
   c. Typically, a spare waste tank has been prepared with bleach by Flow Core staff and is available to use as needed (depicted on right and behind tank in use in image above). Please ensure there is bleach in the waste tank; if not, please add it as per step b.
   d. Re-attach waste tank to fluidics.

4. Locate the green power button on the right side of the instrument and press (red arrow indicates button location in image below). The instrument should turn on, signalled by the buttons lighting up (not depicted).

5. Add milliQ water to water tube (but not more than 1/3 full), place tube on the SIP, ensuring the support arm is underneath it* and press Run button.
6. Release bubbles that may have accumulated while refilling sheath tank in step 2 by pressing on valve (see red arrow) while keeping filter upright. Press Standby.

7. Prime the instrument a few times with the tube on (press Prime button to the right of Run and Standby buttons, pink arrow indicates Prime button in image below).



Do not touch the Sample Fine Adj knob (red arrow). Only modify acquisition speed using Low, Med and High buttons.

Never leave a tube on the SIP without the support arm under it! Liquid will be sucked up at a high rate, the tube will run dry and air will enter the fluidics system: BAD

8. Log in with your Med ID and password to start session time in PPMS.
9. Open Diva software and log in with your username and password. If for some reason this is unsuccessful, you may use the FlowCore username and password:

Username: FlowLab
Password: experiment

See also Section 7. Software Overview below

10. Wait for the cytometer to connect
    a. a CST mismatch notification will appear on the screen
        i. Choose “Use CST settings”
11. Flow core staff typically perform QC every weekday morning; however, in the event that your booking is early in the morning or Flow Core staff are away, please perform QC as follows:
    a. go to Cytometer > CST
    b. Check the lot. Use Diva 8 beads in fridge in rack (spares are in the box), vortexing well and then mixing 1:1 with water (good for ~1 week stored in fridge).
    c. Vortex and run on low.
    d. Check report: If passes with or without warnings (as long as %brightCV is less than 10%), proceed. If QC fails, prepare fresh Diva 8 beads and run again. If QC still fails or %brightCV is >10%, please inform Flow Core staff.

7. Software overview

When you are logged in to your DIVA software account, five windows should be open within the software. If not all open, select them in the *View* tab of the Tool Bar. The main functions within each of these windows are described below:

1. Browser
    a. Open a new or existing experiment
    b. Duplicate an experiment without data
    c. Store a template
    d. Export data
2. Cytometer
    a. Add or delete fluorophores (*Parameters* tab)
    b. Adjust the FSC threshold (*Threshold* tab); default is 5000.
    c. Set voltages (*Parameters* tab)
    d. Choose fluorophore parameters (Log/Linear Scale, Area, Width, Height) - (*Parameters* tab)
    e. View and/or edit compensation (*Compensation* tab)
3. Acquisition Dashboard
    a. Acquire and record data
    b. Set up number of events to record (stopping condition)
    c. Tell machine what gate to count from when recording
    d. Monitor threshold and number of events acquired
4. Inspector

    Provides information and options to make changes on whatever is currently selected:

    a. If you have clicked on a worksheet/graph:
        i. Choose x and y parameters on graphs
        ii. Assign a population to be viewed in the graph

iii. Change axis to biexponential display

b. If on a tube:
   i. Label tube (under *Plot* tab)
   ii. Analysis data shown (under *Dot Plot* or *Histogram* tab depending on plot type)

c. If on Cytometer Settings:
   i. View fluorophores and voltages used (under *Parameters* tab)
   ii. View threshold
   iii. View compensation
   iv. Print out parameters (click on Print button bottom right under *Parameters* tab)

5. Worksheet (typically on right-hand monitor): the *Global Worksheet* is for sample acquisition and the *Normal Worksheet* is for compensation tube acquisition.
   a. Setup analysis/acquisition template
   b. Add plots and graphs
   c. Add statistics

8. Experiment setup

1. Create a new experiment:
   a. Go to *Experiment* on the Tool Bar Menu and choose *New Experiment*
   b. Select *Blank Experiment with Sample Tube* (this is further down the list, so scroll down to find it). Click OK
   c. A new Experiment icon will appear in the browser
   d. Double-click to open the experiment; the open book icon indicates that the experiment is open.
   e. Right click on the experiment to rename it.
   f. Click on the "+" icon to the left of the Experiment to expand.
   g. You will see the following sections are included in your experiment: Cytometer Settings, Global Worksheets, and Specimen_001. Click on *Specimen_001* to see *Tube_001*
   h. You can expand each section by clicking on the "+" icon to the left.

2. Add specimens and tubes:
   a. If you accidentally selected *Blank Experiment* – but without the Sample Tube - in 1b above), delete it and go back to Step 1 above.
   b. Label tubes and specimens, by right-clicking on the tube and renaming.
   c. Take a moment to look at the Cytometer and Acquisition Dashboard Windows – Notice that you only have one tab available in the Cytometer window and the Acquisition functions are grayed out.
   d. To the left of each tube is an arrow icon, click on the arrow to activate the tube: The arrow will turn green. Alternatively you can double click the Tube icon or on the tube name.
   e. Notice that you now have more options available in the Cytometer and Acquisition windows now that a Tube is "active".
   f. **THE ARROW TO THE LEFT OF A TUBE MUST BE GREEN IN ORDER TO ACQUIRE OR SET UP PARAMETERS. IF THE TUBE IS**

NOT ACTIVE THEN YOU WILL NOT BE ABLE TO SETUP YOUR EXPERIMENT OR COLLECT EVENTS.

g. Make sure you perform set-up while the 1st tube is activated (not a tube further down the list) since the conditions you set up for that sample will automatically only apply to subsequent samples.

3. Add fluorophores:
   a. Click on the arrow beside the Tube to make it "active".
   b. Click on the *Parameters* tab in the Cytometer Window.
   c. Delete all parameters, but leave the FSC and SSC (first two listed). Click on the "dot" to the left of a parameter or its name to select. Hold down the Shift key to select multiple parameters and click the *Delete* button.
   d. Click on the *Add* button. The next parameter will appear. Click on the name and scroll down to the one you want to add. If you make a mistake, you can delete it.
   e. Repeat step d. to add additional parameters.
   f. Verify that the FSC and SSC have the Log function unchecked and A, H and W checked.
   g. Make sure that each fluorescent parameter has the "Log" and "A" function checked.

      A = area is the sum of all voltage pulse heights
      H = maximum height value for the voltage pulse
      W = width of the voltage pulse
      These are calculated by the instrument's electronics.

4. Add compensation controls:
   a. Make sure all necessary channels have been added in the *Parameter* Tab of the Cytometer Window (as per step 3 above).
   b. Click on *Experiment* in the Tool Bar Menu.
   c. Select *Compensation Setup* and choose *Create Compensation Controls*
   d. A dialogue box will appear:
      i. Verify that all your channels are listed, "include separate unstained control tube" is checked if needed. You need this if you do not have a negative population in each compensation tube.
      ii. The label for each fluorophore should be designated "Generic". If you have labels associated with a tube, simply delete the ones with a label and leave the generic ones.
      iii. Click *OK.*
   e. A "Compensation Controls" Specimen will be in your experiment
      i. Expand the Specimen and you will see that Tubes and Normal Worksheets have been created for each channel in your experiment.
      ii. In your Normal Worksheet, there will be a *Sheet 1* tab that is blank, an *Unstained Control* tab (if using a universal negative control) and a tab for each channel you are using.

Note: you can toggle between your Normal and Global worksheets by clicking on the left-most icon on your Worksheet toolbar. If it is greyed out, click on a sample tube in your browser to reactivate it.

f. To modify compensation controls, go to *Experiment < Compensation Setup < Modify Compensation Controls.*

5. Create Global Worksheet (default name is "Global Sheet 1")
    a. It is best to keep the Global Worksheet simple. You can analyze and make more graphs/plots during analysis using one of the Flow Core workstations or on your own or lab computer if you have a FlowJo license.
    b. Once data is acquired you can add and delete plots/gates as much as you want using the FSC files. VOLTAGES CANNOT BE CHANGED on FSC files. Voltages need to be determined during acquisition.
    c. The Tool Bar on the Worksheet window contains icons for graphs and gates. Hold the mouse over the icon in the tool bar to view what type of gate or graph you are choosing.
        i. Graphs
            - Histogram (one parameter)
            - Dot Plot (two parameters)
            - Contour Plot (two parameters)
        ii. Gates
            - Polygon
            - Rectangle
            - Quadrant
            - Interval
    d. **To add a plot to the workspace**, click on the plot type that you want and then click within the worksheet space. You can click on the plot and drag it around and resize it.
    e. Once you have chosen the appropriate plot, click on the axis to choose the channel of interest that you want displayed on that axis of the plot.
    f. Right click on the graph and choose *Show Population Hierarchy*
        i. This allows you to track what is being shown in each plot.
        ii. You will also be able to get the % of each population.
    g. **To add gates to the plot**, click on the gate type (polygon, rectangle, interval, or quad) and then click back in the plot of interest. The gate will appear. For a polygon plot, you need to draw it. You will also see that the selected gate has been added to your Population Hierarchy with a color (red) and name (P1) assigned.
    h. **To delete a gate**, click on the gate itself and delete it or select it on the Population Hierarchy and delete it there.
    i. To add a plot based on your P1 data:
        i. Click on a plot type icon and then click on the worksheet.
        ii. To confirm what population is shown in your plot: right click on the plot and select *Show Populations.* Your original plot will show *All Events.* There will be a blue shaded box around the name of the population if it has been selected.

iii. Pick the population you want the plot to depict.

j. It is best to create each plot with gates sequentially as you design your acquisition gating strategy. The correct axes must be in place before the next gate is drawn. If you change the axis on a plot that already contains a gate, the gate will disappear. You will still see it in the Population Hierarchy linked to the original graph parameters.

k. While drawing plots and gates, check the Population Hierarchy to ensure that it is correct. If not, delete population gates as outlined in step h.

l. Rename Populations: Click on a population name in the Population Hierarchy box within the name itself. The field will unlock so that you can type in a new name.

k. Assign/Change color of a population

i. Double click on the colour box located to the left of the population in the Hierarchy box

ii. A drop down menu of colours will appear, click on the color you want

l. If you have several plots and want to resize them to the same size, select them all, with the one of the desired size clicked last such that the borders feature yellow squares (not gray ones). Then click on the icon third from the right in the tool bar that shows four squares with arrows crossed between them.

m. Change an axis to bi-exponential

i. Click on the plot

ii. Go to the Inspector Window

iii. Select on the Plot tab

iv. Under the Bi-exponential Display section, check mark the appropriate axis

n. You may label your plots so that you know what population each one is showing; however, it is good practice to keep the channel name as part of the title during acquisition (for troubleshooting to ensure correct channels are in use)

i. Select all the plots by holding down the shift key and clicking on all the plots

ii. Go to the Inspector Window and choose the *Title* tab

iii. Select the *Tube* and *Populations* boxes. This will populate the plots with tube titles given in the worklist in the browser.

o. Add statistics

i. Right click on a plot

ii. Choose *Create Statistics View*

iii. A statistics box will appear on the workspace

iv. Right click on the statistics box and choose *Edit Statistics View*

v. Pick the parameters you are interested in: Median, etc. Note that Median is recommended (<u>not</u> Mean)

You can save your experiment as a template that you can access at a later date OR

6. To duplicate an experiment without data:
    a. Open the experiment you would like to duplicate.
    b. Right click on the experiment name.
    c. Choose *Duplicate Without Data*.
    d. DIVA will make a copy of your whole experiment with "_001" appended to the names.
    e. Overwrite the names by off-clicking on them and choosing *rename* or clicking once on the name and typing over it
    f. You will need to delete your compensation controls:
        i. Click on the compensation controls title
        ii. Press delete,
        iii. Choose *OK* to remove the selected browser elements.
    g. Click on experiment title.
    h. Go to *Experiment* then *Compensation Setup* and create compensation controls as in Step 4 above.

9. Acquisition

1. Setup Acquisition Dashboard
    a. Go to Acquisition Dashboard Window
    b. Set number of *Events to Record* from the dropdown menu or overwrite it.
    c. Choose the *Stopping Gate* from the dropdown menu (will comprise all gates created in the Global Worksheet).
        i. This is the gate the machine will use to count the number of events. We recommend that you record at least 10,000 events in your gate of interest (eg. live singlets)

Acquire Data Button: Acquires data points that you can see in the worksheet
Record Data Button: Collects data for the FCS files
Storage Gate: Leave this set on All Events

2. To acquire when applicable:
    a. Fully stained sample at low acquisition speed to set voltages to ensure all fluorophores are on scale. Once this has been done, voltages should not be altered for the rest of the acquisitions.
    b. Unstained sample to identify negative population and set gates
    c. Compensation controls:
        i. Use your unstained sample to gate on your population of interest.
        ii. Once you know where you want your FSC/SSC gate to be, right click on the gate and choose the *apply to all compensation controls.*
        iii. Now this gate will be applied to all of your normal worksheet tabs for the Compensation Tubes
        iv. Once all compensation controls have been acquired under their respective tubes, check each one to ensure gates have been set correctly on the positive and negative populations. If using a universal negative, then you will only need a positive P2 gate on your single stained controls.

v. Click on Experiment in the Tool Bar.
vi. Select *Compensation* and *Calculate Compensation*.
vii. When the dialogue box appears, rename the compensation with the date and your name (or PI), if you wish.

d. *Link* and *Save* the compensation to your experiment.
e. Notice that you now have a small chain link connected to the cytometer setting portion of your experiment.
f. To view your compensation overlap values, click on any tube in your sample group (not a tube under the Compensation Control specimen as these will all read 0.00). Click on the Compensation tab in the Cytometer Window.
g. Check to ensure that compensation has been applied to the first tube: click on the arrow next to the tube name so that it turns green (is active), expand the selection beside the tube name, click on *Cytometer Settings* then check under the *Compensation* tab in the Inspector – Cytometer Settings window. If only zeros are listed, then right click on *Cytometer Settings* under your second tube, choose *Copy Spectral Overlap*, go back to your first tube, activate it, right click on *Cytometer Settings* and then choose *Paste Spectral Overlap with Zeroes.*

3. Acquire your samples:
   a. Load your tube and press *Run* and the speed at which you wish to acquire your sample, keeping total events/second below ~1200. If this number is high and you see a lot of debris in the lower left part of your FSC/SSC graph, consider increasing your FSC Threshold to omit these events.
   b. After clicking on *Run* wait for ~5 seconds for fluidics to stabilize and then click on *Record*.
   c. Monitor fluid levels in your tubes while acquiring to ensure the tube does not run dry. If it is getting very low and you still need to record more events: stop acquisition, add more buffer (or FACS Flow), vortex, load and choose the option to *append data* after beginning your run again and clicking on *record*. Note that stopping gate events will restart at O, but the data will be appended to your sample file.

10. Cleaning

After running their final sample, every user is expected to run the full cleaning protocol for the instrument. *This is extremely important for equipment maintenance and to ensure the X-20 is clean and ready to go for the next user.*

Run on High:
1. 2 minutes - 50% Coulter Clenz (blue solution in tube in rack inside BioBubble)
2. 2 minutes - 10% Bleach (tube in rack; add to tube from squirt bottle as needed but do not fill beyond 1/3 tube capacity)
3. 5 minutes - MilliQ water (tube in rack; refill as needed as in Step 2. Leave water tube on when finished.
4. Return machine to LOW and STANDBY

If finishing after 4 pm on a weekday or using the X-20 on a weekend or holiday, please ensure that the instrument is turned off following cleaning, regardless of whether another user is booked on the instrument afterwards. Please see Section 12. Instrument shutdown.

11. Exporting data

A Batch Analysis provides a PDF. You cannot move gates or make any changes to a Batch Analysis file. To save a Batch Analysis:

1. Right Click on either the Experiment name or Specimen name to batch the report.
2. Clicking on the Experiment icon will give you all of the analysis worksheets for every tube in the experiment.
3. Clicking on the Specimen icon will batch only the tubes associated with the specimen
4. Select Batch Analysis (In this case we are batching the entire experiment)
5. A dialogue box will appear
6. Check the Output to Printer and Save as PDF options.
7. Click on Browse and navigate to where the file should be saved.
8. Click Start.
9. Click OK when pop-up window appears.
10. Print

Exported FCS files can be imported into DIVA, FlowJo or other flow analysis software. You can change gates and plots, add statistics and create PDF files. To export FCS Files:

1. Right click on the Experiment.
2. Choose Export.
3. Browse as per step 7 above, then click OK (not Start).
4. Click on Experiment or FCS File (v 3.1)
    a. Experiment: You will save all worksheets associated with the experiment and the parameters. This can be opened in DIVA. These files are typically saved in D:\BDExport\Experiment\Users\[your folder]
    b. FCS Files: Saves data files only. No worksheets will be carried over when you open the files. This can be opened in FlowJo or other third party analysis software. These files are typically saved in D:\BDExport\FCS\[your folder]

Log out of your DIVA account and log off of the computer.

12. Instrument shutdown (ONLY after cleaning as per section 10)

1. Turn off X-20 (green button on right side).
2. Turn off BioBubble (button on power bar)
3. Close BioBubble.
4. Check sheath fluid and waste levels.
    a. If you have acquired a lot of samples and did not start with a full sheath tank as recommended, please refill the sheath fluid as a courtesy to the next user.
    b. if waste is full or close to full, please follow steps in Section 6.3 above.

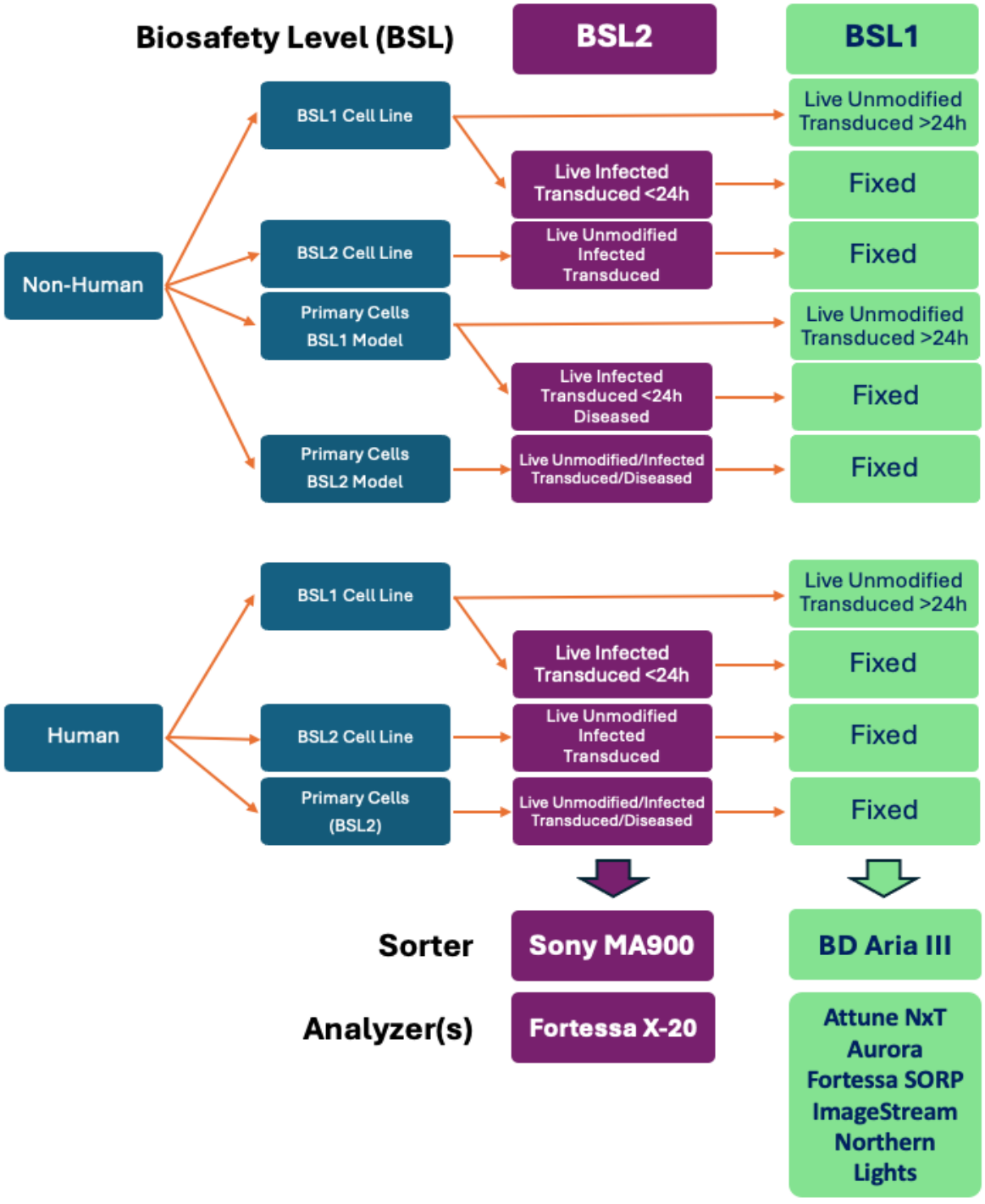
13. Review of SOP

SOP was established on May 1, 2025. The SOP will be reviewed annually in April.

Revision	Date	Description of changes	Reviewed by
0.0	05/1/25	Initial release	GS
1.0			

Appendix I

Guide to choosing flow cytometer based on sample type: For non-human samples, use upper flow chart; for human samples, use lower flow chart. Refer to ATCC designation to determine classification of commercial cell lines. Follow arrows across to determine whether your samples are BSL1 or BSL2 then check the colour-coded sorter and analyzer lists below to select the appropriate instrument.



Appendix II: Fortessa X-20 Configuration

Blue 488 nm	530/30	695/40				
Yellow Green 561 nm	586/15	610/20	670/30	710/50	780/60	
Red 640 nm	670/30	730/45	780/60			
Violet 405 nm	450/20	525/50	610/20	670/30	710/50	780/60
UV 355 nm	379/28	740/35				