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HuBMAP UF TMC - FACS Sorting of Live CD45+ Cells for 10x scRNASeq

Forked from HuBMAP UF TMC - FACS Sorting of Live Cells for 10x scRNASeq

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1 Works for me dx.doi.org/10.17504/protocols.io.buaznsf6

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ABSTRACT

This standard operating procedure (SOP) provides instructions for staining and sorting live cells. This SOP applies to cryopreserved cells that are stained with Live/Dead and sorted in the BD ARIA III to remove dead cells prior to 10x sequencing.

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FORK NOTE

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MATERIALS TEXT

MATERIALS

cDMEM with FBS Pen/Strep NEAS HEPES GlutaMax and Sodium Pyruvate **Contributed by users**

FACS Tubes **Contributed by users**

LIVE/DEAD™ Fixable Green Dead Cell Stain Kit **Contributed by users Catalog #L23101**

FBS **Contributed by users**

PBS with 2% FBS **Contributed by users**

PBS with 10% FBS **Contributed by users**

SAFETY WARNINGS

.Do NOT perform the tasks outlined in this SOP without proper universal safety precautions for handling human samples as well as personal protective equipment including but not limited to face mask, gloves, lab coat, and arm guards

BEFORE STARTING

Do NOT perform the tasks outlined in this SOP without thoroughly reading and understanding the tasks associated with this SOP.

Thaw Cryopreserved Cells

1 a. In a 15 mL conical tube, warm 10 mL of cDMEM in a bead bath set to 37 o C
b. Thaw the cells in the cryovials in a water bath set at 37 o C
c. Immediately, resuspend the cells in 10 ml of RT cDMEM
i. First, add 1 mL of cDMEM to the cryovial
ii. Then, transfer the cells from the cryovials to the media.
d. Centrifuge samples 300 x g for 10 min
e. Decant the supernatant
i. Use a 1000 µL pipet to remove any remaining solution
f. Resuspend the pellet in 10 mL of RT cDMEM
g. Pipet 20 µL in a small Eppendorf tube for cell count
h. After the count, centrifuge 300 x g for 7 min

cDMEM is used for two different steps above; therefore, 20 mL of cDMEM should be aliquoted for each cryovial/sample.

Stain the Cells

2 a. Prepare 1X LD Green (Invitrogen) as directed by the manufacturer.
b. Resuspend at $1x10^6$/mL in 1X LD Green and incubate at RT for 20 minutes.
c. Add 3mL Stain Buffer.
d. Pellet cells at 350g for 5 min. Decant supernatant
e. Add 100 µl Stain Buffer and 1 µl CD45 antibody (BV421, clone D21). Incubate on ice for 20 min.
f. Add 3mL Stain Buffer.
g. Pellet cells at 350g for 5 min. Decant supernatant
h. Resuspend the cells at $10\text{-}15x10^6$/mL in cold PBS with 2% FBS
i. Hold the tubes in an ice bucket with a cover to protect the cells from light.

Prepare the Collection Tubes

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3 a. Label 2 FACS tubes to receive the sorted cells. Coat the inside of the FACS tube with FBS - Pipette 0.5 mL of FBS into each FACS tube and vortex briefly.
b. Add 500 µL of PBS with 10 % FBS to each collection tube and keep on ice until used in the sorter.

BD Aria III – Pre-Use/before startup of the instrument

4 a. Check the sheath and waste tanks level; Fill and/or empty, if necessary.
b. Ensure that the fluid and air lines are disconnected from the ethanol tank. Connect the fluid and air lines to the sheath tank.
c. Check to ensure that the closed-loop nozzle is in the flow cell.
i. Make fresh 20% contrad solution, 20% bleach solution, and get fresh DI water to use for cleaning

Startup of the Aria

5 a. Start the computer and FACSDiva. Then, turn on the cytometer by pressing the main power button.
b. Wait 30 minutes for the lasers to warm up
c. Double-check the fluidics levels in the Cytometer window. Replenish fluids or empty the waste, if needed. Roll the mouse pointer over fluid indicators to show the fluid type

Perform fluidics startup

6 a. The status of the fluidics system is displayed in the bottom corner of the main window.
b. From the BD FACSDiva Cytometer menu, Select Fluidics Startup. A window will open with four options.
i. Verify that the air and fluid lines are disconnected from the ethanol tank and connected to the sheath tank, then click Done.
Note: Fluidics startup should not be run if the air and fluid lines connected to the ethanol shutdown tank. This will damage the instrument, thus always verify that the air lines and fluid lines are connected to the sheath tank.
ii. Verify that a closed-loop nozzle is installed in the flow cell, then click Done.
The fluidics startup process starts, and the progress is displayed at the bottom of the dialog box.
iii. When the dialog box indicates that the fluidics startup is done, click Done and OK on the next two prompts. This is because further cleaning will be done before removing the closed-loop nozzle and insert the correct nozzle size in the flow cell.
c. To clean and disinfect the flow cell further:
i. Select Cytometer > Cleaning Modes > Clean Flow Cell.
ii. When prompted, install a tube containing approximately 5 mL of 20 % Contrad, then click OK. The cytometer loads the tube and fills the flow cell with the fluid.
iii. Click OK when the completion dialog opens.
iv. Repeat the last three steps above two more times with Contrad. Then, repeat the above steps with 20 % bleach, and lastly, sterile, filtered DI water at least three times in total.
d. Open the hood of the cytometer. Turn the nozzle-locking lever counter-clockwise to the 6:00 position, pull the closed-loop nozzle out of the cuvette flow cell
e. Insert the 70-micron nozzle size in the flow cell. Be sure that the nozzle has an O-ring and is oriented with the word TOP facing up. Verify that the setup mode matches the nozzle size by selecting Sort > Sort Setup
f.Lock the nozzle in place by turning the black key to the right.

Starting the stream / setting breakoff

7 a. If the breakoff and Side Stream windows is not already displayed, click the Sorting button in the Workspace toolbar to display the windows.
b. Next, click the stream button in the breakoff window to turn on the stream.
c. Open the sort block door and check the stream to ensure that it is flowing smoothly through the nozzle into the center of the waste aspirator.
If not, visit the Aria III user guide for additional troubleshooting steps.
d. Establish a stable drop pattern in the breakoff window by adjusting the Ampl slider until the drop breakoff is in the top third of the breakoff window. Do not exceed 70 volts.
e. Verify that the satellite droplets are merging with the large droplets.
f. Enter the actual Drop 1 value as the target in the Drop 1 field
g. Turn on the Sweet Spot when the drop pattern is stable. This should automatically maintain the stability of the breakoff point.

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Run a performance check - CST

8 a. The stream must be on for CST. Turn off the Sweet Spot (if it is on), then select cytometer> CST.
b. The cytometer disconnects from the BD FACSDiva interface and connects to the CS&T interface. A new window will open.
c. Verify that the bead lot information under Setup Beads matches the CS&T bead lot. Select the correct bead lot ID, if necessary.
d. Retrieve the CST beads from the fridge, mix the beads by gently inverting the vial.
e. In a 12 x 75-mm tube, add 0.35 mL of sheath fluid and one drop of beads.
f. Install the bead tube onto the cytometer loading port.
g. In the Setup Control window, select Check Performance from the Characterize menu. Click Run
i. Plots appear under the Setup tab, and the performance check is run. The performance check takes approximately 5 minutes to complete.
h. Verify that the performance check passed
i. In the Setup tab, the Cytometer Performance Results should have a green checkmark, and the word Passed next to it. If any parameters did not pass, see the BD Cytometer Setup and Tracking Application Guide for troubleshooting information.
i. Select File > Exit to close the CS&T window and connect back to the BD FACSDiva interface.
j. Click Use CST Settings in the dialog that opens.
i. By selecting Use CST Settings, the laser delay, area scaling, and other cytometer settings will be updated to the latest optimized settings from the performance check.
k. While determining the drop delay in the next step, adjust the stream if needed and turn on the Sweet Spot.

Determining the Drop Delay

9 a. Open the Accudrop Drop Delay experiment template. If one is not already created on the instrument, select Experiment > New Experiment. Select the Accudrop Drop delay experiment and click OK.
b. Expand Specimen_001 and Tube_001. Set the current tube pointer to Tube_001.
c. Open the sort layout by double-clicking it.
d. Load a tube filled with a suspension of Accudrop beads (approximately one to drops of beads in 2 mL of Sheath Fluid)
e. Adjust the flow rate to achieve an event rate of 1,000 – 3,000 events per second. If this cannot be achieved with a flow rate setting between 1 and 5, adjust the bead concentration.
f. Turn on the voltage in the Side Stream window. Click Sort in the Sort Layout window.
g. Click Cancel at the Confirm dialog. The beads do not need to be collected; they can be sent to the waste.
h. Adjust the micrometer dial to obtain the brightest bead spot on the center stream.
See figure below to locate the micrometer dial.
i. Click the Optical Filter button in the Side Stream window.
j. Click the Auto Delay button in the Side Stream window. Select Start Run in the dialog box. Monitor the progress of the drop delay progress.
When the process is completed, an announcement will be displayed informing you of the drop delay set.
k. To optimize the drop delay, ensure that the intensity value in the left side stream is close to 100%. This can be done by changing the sort precision mode in the Sort Layout window to Fine Tune. Adjust the delay by clicking the arrow control and waiting a few seconds to see the delay response.
When the left side stream intensity is above 98%, you can proceed to the next step.
l. Click the Optical Filter button to move the emission filter away from the camera, and turn of the Voltage, which should turn off the deflection plates.
m. Unload the Accudrop Drop Delay Beads, and load DI water while designing the new experiments. The stream must remain stable with Sweet spot on.

If the stream or sort is disrupted at any point, it is recommended to reset the drop delay or verify that it is still valid.

10 a. Before analyzing your sample, perform a test sort to ensure that the cells are collected in the appropriate tube and not sent to the waste tank.
b. Install collection tubes in the required collection device.
c. Install the collection tube holder onto the cytometer. Slide the holder into the slotted fittings below the sort aspirator

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drawer, then close the sort collection chamber door.
Note: The collection tube holder should be connected to a water bath that keeps the cell cool during collection. Verify that the water bath is turned on and has reached the set temperature before sorting.
d. Click the Voltage button in the side Stream window to turn on the deflection plates. Make sure the enter stream image does not move after the plates are turned on. Movement may indicate that the plates and surrounding areas need cleaning.
e. Click the Test Sort button and optimize the side streams by adjusting the voltage sliders to view the required number of streams.
f. Open the aspirator drawer and aim the side stream(s) into each collection tube
i. In the Side Stream window, click the Waste Drawer button to open the drawer.
ii. Open the sort block door and aim each side stream into the tube as you adjust the corresponding slider in the Side Stream window.
iii. When you are satisfied with the side stream deflection, close the sort lock door.

Caution: Do not touch the charged plates when voltage is on. A 12,000-volt potential exists, which can result in severe electrical shock.

Design Experiments of FACS Diva

11 a. Verify that the cytometer configuration is appropriate and matches the 70-micron nozzle selected above.
b. Select a folder, create a new experiment with one specimen and the desired number of tubes. Label each category (folder, specimen, tube) as desired.
c. In the worksheet window, create plots and gates
i. Cells are sorted using a hierarchical gating strategy: first using forward and side scatter parameters for the exclusion of debris and cell doublets.
ii. Next, live cells are separated from dead or damaged cells using an amine-reactive viability dye.
d. In the experiment layout, define the number of events to record for each tube during sorting. (10,000 events are usually recorded if possible)
e. Replace the collection tube used for the test sort, with the FACS tube from Step IV coated with FBS.

Sort Layout

12 a. Before starting sorting and after the population of interest – live CD45+ cells – have been defined using the gating tools, a sort layout must be created.
b. Click the New Sort Layout button on the Browser toolbar. The 2-Tube Sort Layout is the default, but it can be changed in the device menu.
c. In the Device menu. Select 4 tubes, but only one of the tubes will be used for sorting.
d. Change the sort precision mode to 4-Way Purity.
e. You can choose to set a target number of events by selecting a value from the menu or entering a number in the field, or you can set the Target Events to continuous, which means that sorting will continue until it is manually stopped
f. Select a Save Sort Reports option: Save None, Save All, or Ask User.
g. Add the required population(s) to each sort location field
i. For this experiment, we will use the left column and leave all other location empty. This will match the voltage slider settings set during the test sort.
ii. Right click in the sort location and choose the corresponding cell population.

Sort

13 a. Verify that the collection tubes are installed in the correct position matching the sort layout and the voltage sliders.
b. Install the sample tube onto the loading port and click Load.
c. Adjust the flow rate. And adjust the gates in the Worksheet window
Sorting results improve at lower flow rates. We recommend not to exceed 7000 events/second to avoid clogging the instrument.
i. If the flow rate is at 1, and events exceed 7000 per second, add more PBS with 2% FBS to the sample to reduce the concentration of cells.
d. Verify that the current tube pointer is indicating the appropriate tube in the Browser, then click Sort.

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e. Click OK if you are prompted to open the aspirator drawer or turn on the deflection plates. (Clicking cancel will result in the loss of sorted cells.)
f. Click Record Data to save data for the tube. Acquisition and sorting will continue after the indicated number of events has been recorded
g. You can monitor the sort progress in the Sort Layout Window. The number of events sorted into each sort location appears in the corresponding field, as well as the sort rate, sort conflict rate, and efficiency percentage.
h. The sample tube, loaded in the acquisition chamber, should also be monitored. The acquisition must be stopped before the sample tube runs dry.

Stopping and resuming a Sort

14 a. Replacing a Sample Tube
i. To stop a sort while it is running, click the Sort button in the Sort Layout window.
ii. If prompted, click OK to save the sort report.
iii. Unload the sample tube by clicking Unload in the Acquisition Dashboard.
iv. Refill the sample tube, then click Load in the Acquisition Dashboard.
v. Click the Resume button in the Sort Layout window to continue sorting.
vi. Click OK when you are prompted to open the aspirator drawer or turn on the deflection plates. The sort counters will resume from where it stopped.
b. To replace the collection
i. To stop a sort while it is running, click the Sort button in the Sort Layout window.
ii. If prompted, click OK to save the sort report.
iii. Click Stop Acquiring in the Acquisition Dashboard to stop the sample flow
iv. Turn off the deflection plates by clicking the Voltage button in the Side Stream window
v. Slide the collection tube holder out. Lift the handle to make sure that collection tubes are easily accessible to remove.
vi. Replace the collection tubes as needed
vii. Reinstall the tube holder and pull down on the handle to secure it in place.
viii. Click Acquire Data in the Acquisition Dashboard to restart the sample flow
ix. Click the Resume button in the Sort Layout window to continue sorting.
x. Click OK when you are prompted to open the aspirator drawer or turn on the deflection plates. The sort counters will resume from where it stopped.

The Stop/Resume feature allows you to stop the sort and still retain the counter values temporarily. This can be used when refilling the sample tube when it is low, and when replacing the collection tubes.

If there is any other issue that interrupts the sort, please visit the BD Aria III user guide.

Stop Sort

15 a. When the sample tube is close to running out, Click Stop Acquiring in the Acquisition Dashboard to stop the sample flow.
b. Remove the collection tubes and transfer them to an ice bucket. Cell suspensions should be washed 3 times in PBS/0.04% BSA prior to proceeding to the 10x Genomics protocol.

Post Sort- Clean the Aria tubing pathway and perform fluidics shutdown.

16 **a. Cleaning the Tubing and the Pathway**
i. Make sure the stream is on.
ii. Load and Run ~ 10 ml of 20% bleach for 10-15 minutes, with the flow rate set to 10.
iii. Remove the tube of bleach.
iv. Load and Run ~ 10 ml of DI water for 10-15 minutes, with the flow rate set to 10.
v. Unload the DI H2O, and turn off the stream.

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b. Cleaning the Flow Cell

i. Open the hood of the cytometer. Turn the nozzle-locking lever counter-clockwise to the 6:00 position and remove the nozzle.

ii. Remove the current nozzle tip and replace it with the metal nozzle (Closed Loop Nozzle) that has the waste line attached to it. Be sure that the nozzle has an O-ring and is oriented with the word TOP facing up.

iii. Turn the black key to the right to lock the nozzle in place.

iv. Select Cytometer > Cleaning Modes > Clean Flow Cell.

v. When prompted, install a tube containing approximately 3 mL of sterile, filtered DI water, then click OK. The cytometer loads the tube and fills the flow cell with the DI water.

vi. Click OK when the completion dialog opens.

vii. Repeat step-iv to step-vi at least two more times.

c. Perform Fluidic Shutdown

i. Make sure the stream is off. Unload the sample tube, if one is loaded.

ii. Check the ethanol shutdown tank and refill if it needed.

iii. Select cytometer> Fluidics Shutdown.

The Fluidics Shutdown dialog opens.

iv. Remove the nozzle from the flow cell assembly and click Done.

v. Insert the integrated closed-loop nozzle into the flow cell assembly and click Done. Verify that there is an O-ring in the nozzle before installing it.

vi. Connect the fluid and the air tube to the stainless steel ethanol (ETOH) shutdown tank.

1. Disconnect the fluid line from the bottom side of the sheath filter and connect it to the ethanol filter on the ETOH shutdown tank.

a. Keep the sheath filter attached to the sheath tank. Do not run ethanol through the sheath filter

2. Disconnect the air line from the sheath tank and connect it to the air port on the ETOH shutdown tank.

3. Click Done. The system starts the cleaning process and then displays a message at the bottom of the dialog

vii. When prompted, install a tube containing 3 mL of sterile, filtered DI water on the loading port, then click Done.

1. The cytometer loads the tube and continues the cleaning process. A progress message appears, and then displays Done when the process is complete.

viii. Click OK when you see a message informing you that the system can be turned off.

ix. Vent the air pressure from the sheath tank by pulling up on the ring on the pressure relief valve

x. Turn off the cytometer main power.

xi. Exit BD FACSDiva software and shut down the computer.

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