Welcome to the CORES Flow Cytometry Sorter Facility

The FACSAria III and FACSAria Fusion are high speed sorters capable of analyzing cells based on 8-16 distinct fluorescent properties (besides FSC and SSC) and separating them in up to 4 different populations from a single sample. The Cell Sorting Facility is primarily focused on sorting cells or measuring fluorescence in samples of cells labelled with 5 to 14 fluorochromes. Typically, fluorescently tagged antibodies are used to label cell surface markers, or genetically encoded fluorescent proteins like GFP are used to denote cells of interest. The sorters are mainly used for the physical sorting of cells, but occasionally for acquisition of Flow Cytometry data for small numbers of appropriate samples. Sorting is typically scheduled in the afternoons to allow time for sample preparation, but, depending on the complexity of your preparation, may be scheduled during regular hours of 8am-5pm. There is a setup fee for sorting and an hourly fee for using the Aria for sorting or acquisition. This fee is based on the time scheduled for sorting or acquisition. Please read the following information to answer any questions you may have, if they cannot be answered here feel free to contact the operator using the contact information below. Additionally, our facility's website has information about basic flow cytometry concepts, and if you require more help, please let us know.

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FACSAria Capabilities/Limitations

Our FACSAria sorters are equipped with either 2 lasers (a 488-nm and a 633-nm laser) or 4 lasers (a 448-nm, 405-nm, 561-nm and a 633-nm laser). The detectors associated with each laser and some example fluorochromes/dyes are as follows:

Machine	Lasers (nm)	Laser Power (mW)	Filters		Example Fluorochromes
			BP	LP	
BD FACS Aria III	488	20	530/30	502	CFSE, GFP, Oregon Green 488, Alexa Fluor 488, FITC, BB515
			585/15	556	tdTomato, RFP, dsRed, PE
			616/23	610	PE-eFluor 610. 7-AAD, PI, PE-Texas Red, PECF594
			695/40	655	PerCP-eFluor 710, PE-Cy5.5, PerC-Cy5.5, PerCP, PE-Cy5, BB700
			780/60	735	PE-Cy7
	633	18	660/20	---	eFluor 660, Alexa Fluor 647, APC
			730/45	690	iRFP, Alexa Fluor 700
			780/60	755	APC-eFluor 780, APC-H7, APC-Cy7
BD FACS Aria Fusion	405	80	450/50	---	DAPI, eFluor 450, BFP, V450, Brilliant Violet 421, Pacific Blue
			525/50	505	eFluor 506 Fixable, AmCyan
			610/20	595	Brilliant Violet 605, eFluor 625NC, eVolve 605
			660/20	630	Brilliant Violet 650, eVolve 655, eFluor 650NC
			710/50	690	Brilliant Violet 711
			780/60	750	Brilliant Violet 786
	488	50	530/30	502	CFSE, GFP, Oregon Green 488, Alexa Fluor 488, FITC, BB515
			695/40	655	PerCP-eFluor 710, PerCP-Cy5.5, PerCP
	561	50	582/15	---	PE, BYG 584, CTY
			610/20	600	PE-CF594, PEDazzle, PETex Red,ECD,PE Efl610
			670/14	630	PE-Cy5
			710/50	685	PE-Cy5.5
			780/60	735	PE-Cy7, PEVio770
	633	100	670/30	---	eFluor 660, Alexa Fluor 647, Alexa Fluor 660, APC
			730/45	690	iRFP, APC-Cy5.5, Alexa Fluor 680, Alexa Fluor 660, Alexa Fluor 700
			780/60	755	APC-eFluor 780, APC-Alexa 750, APC-H7, APC-Cy7

The Sample Injection Chamber and Sample Collection Tube area provide a nearly sealed area, minimizing aerosols. However, cell sorters that use droplet generation methods like the Arias can produce aerosols around the sample stream, which may not be contained. If a sample is potentially biologically hazardous, contains or has been exposed to any known pathogens the operator must be informed so that precautions can be taken. Risk group 2 agents or cells exposed to them can be sorted at the Aria III or Fusion, which are both operated in separate class II biosafety cabinets and are equipped with aerosol management systems designed to contain droplets generated within the sort blocks. Most samples from bacterial and viral infected or transfection models (i.e., Lentivirus) can be sorted, but we must record the agents used. Risk group 2+ agents can be sorted in the facility but additional PPE is required, and thus staff must be notified prior to booking the sort appointment. Risk group 3 agents will not be sorted. See section below on biosafety policies for more information.

Sort Capability:
At 70 psi and 90 kHz using the 70 micron nozzle, the threshold rate can be up to 15,000 events per second (total cells, not target) for a four-way sort with a purity of up to 98% and a yield of approximately 50%. The lower the pressure/frequency the slower your sample must be run to avoid conflicts (i.e., a target and a non-target cell being in the same target droplet, which is discarded). Sorting with the 100 micron nozzle for larger or fragile cells at 20psi and 26 kHz will allow you to typically sort at about 4000-5000 events per second without too much loss of yield. An intermediate nozzle of 85 microns used at 45 psi and 45 KHz will typically allow you to sort at 6000-9000 events per second.

Sort yield is very much a function of the conflict rate, but also consider that doublets of cells containing negatives stuck to positives or any combination of multiple positives are seen as a single event, and we must try to exclude them to attain good purity at the cost of yield. It is therefore very important to try to get as close to a single cell suspension as possible (see preparation for sorting and acquisition regarding buffers and filtration methods).

FACSAria Detectable Fluorochromes/Dyes

If you can't find the dye/fluorochrome that you want to use, check the excitation and emission spectra for it to see if it can be detected on the Aria's detectors. It must be excited at wavelengths of around 488 nm (blue laser), 405 nm (violet laser), 561 nm (yellow-green laser) or 633 nm (red laser), and emit within the wavelengths detected on the PMT's available (please see the table on the previous page for detection filters). Try using a spectral viewer, such as BD Biosciences' Fluorescence Spectrum Viewer (www.bdbiosciences.com/spectra/).

Choosing Antibody/Fluorochrome Combinations:
When performing multicolor flow analysis, your success can be greatly increased by choosing the right fluorochrome for the antibody (and therefore antigen) you wish to detect. If the antigens are at high densities, they can be resolved from the unstained cells with almost any fluorochrome. As the antigen density decreases however, so will the intensity of your positively stained cells, which makes them more difficult to differentiate from the unstained cells. When this is the case it is best to use a very high intensity or bright fluorochrome, such as BB515, BV421, PE, BB700, PE-Cy5/Cy7, PECF594, Alexa 647, APC, or APC-R700.

Another issue to consider when planning which fluorochromes to use, is whether you are using a very highly autofluorescent cell type. All cell types are somewhat autofluorescent at the shorter wavelengths and this autofluorescence decreases as wavelength increases (usually to practically none above 600 nm). For cell types that are very autofluorescent where your expression may be weak, it is better to choose a dye that emits at a longer wavelength, since the positively stained population will be better separated from the negative population as the autofluorescence decreases. For cell types that don't have high autofluorescence, most any fluorochrome can be used and separation from the negative population will be apparent (again depending on how much of your target protein is expressed).

One last tip that should be pointed out comes into play when staining single cells with multiple fluorochromes. You need to check the excitation and emission spectra for each fluorochrome. The BD Fluorescence Spectrum Viewer and ThermoFisher SpectraViewer are very handy tools for this. If you are choosing dyes that emit at very close wavelengths you are going to have a high amount of overlap in emission. This can make distinguishing double/triple (etc.) positively stained cells from single stained cells that are bleeding into multiple detectors very difficult. Compensation can correct this issue to a point, however the closer two dyes' emissions are, the lower the resolution will be in the detector to which compensation is applied. For example, once spillover from FITC into PE is calculated, it is more difficult to resolve a low expressed PE marker on a cell that is also FITC positive.

Take home message: When choosing multiple fluorochromes, try to choose ones whose emission do not overlap much into more than one detector, and this will improve your yield and purity. Also, if you are unsure about the autofluorescence of your cells and/or the intensity of your chosen fluorochromes, we recommend that you arrange to acquire some test samples on the Aria prior to sorting (even if you have used the same fluorochromes on other instruments in the CORES facility, because each instrument has different configurations and sensitivities).

Preparing for Sorting/Acquisition

1. Submit the Sorting Application Form found at the end of this document.

2. Optional. Meet with the Sorter operator and have your sort plan and time established.

3. Prepare your sample. You will need a sufficient amount of cells for sorting. How many starting cells you need will vary, depending on how abundant or rare your populations of interest are and how many of the sorted cells are required for your application. Generally, **25-40 million cells/mL** is a good concentration range in which to resuspend your stained cells, if it is too dense we can further dilute the sample with **sort buffer [suggest sterile filtered PBS or Hanks' Buffered Salt Solution (Ca/Mg++ free), pH 7.0-7.4, with 25 mM HEPES, 0.5-2% FBS (heat inactivated) or BSA, and 1mM EDTA (depending on the stickiness of your cells)]. If you have 10 million cells or less, resuspend in 0.4-0.5 mL**. Remember that we are speaking about numbers of total cells, not the numbers of cells that make up your target sub-population.

4. **Optional (but recommended)**. You may choose to use a viability dye such as the DNA binding dyes like Sytox Green or Red, TOPRO-3, 7-AAD, PI. This can only be done if the detector is not being used for one of your fluorochromes in your staining plan; however, there are many viability dye choices available with different excitations and emissions. Please verify the fluorescence emission information for your chosen viability dye by referring to the manufacturer's website/documentation. Typically, very little of the viability dye is required, 0.5 to 1.0 µg/mL can be added a few minutes before starting the sort. Please note, we prefer that you do not use PI, if possible, because it "sticks" to the flow path of the instrument. An amine reactive dye, or an enzymatically activated dye may be used as well, (from which there are many to choose from) though they require extra preparation steps.

5. Stain your samples as usual for sorting. Remember that you want to keep your cells viable and contaminant free throughout the staining/sorting procedure, so you may have to change your staining buffer and procedure to accomplish this.

6. Make sure you have the appropriate controls that you will require to identify the populations of interest. If you are staining with a single colour, an unstained control sample is all you will need. If you are staining with multiple colours, compensation controls will be required, each control (one for each colour) containing 2 - 20 x 10^6 cells per mL with a volume of at least 0.4 mL. Compensation beads, which capture your fluorochrome labelled antibodies, may be used as an alternative to stained cells as compensation controls. Please consult with facility staff if you have questions regarding controls.
***For acquisition**, resuspend and fix (if possible) all samples in 0.3 mL (if the samples are more concentrated the acquisition will go faster).

7. Cells should be prepared in a sorting buffer; using your culture media will not work. Bring a sterile aliquot of about 50 mL of your sort buffer with you. It is best to use the lowest possible amount (e.g., 0.5%) of BSA or FBS (filtered) in your sort buffer that you can get away with and still maintain cell viability.

8. Samples may be provided in 12 x 75 mm tubes (e.g., 5 mL round-bottom "FACS" tubes), 1.5 mL microcentrifuge tubes, or 15 mL conical tubes (note, VWR brand 15 mL tubes are **not** compatible). All samples **MUST** be filtered just prior to sorting. It does not take much to clog the system/nozzle and clogs will cause the sort to automatically stop. It will take time to de-clog the system and may cause your population(s) to drift outside of the sort gates, causing poor sort results. Samples can be filtered with 35 micron strain cap/test tubes or 40 micron cell strainers just before sorting. Please note that Central Stores carries both the cell strainers and the tubes with strainer caps.

9. You must bring your collection tubes with you. It is best to bring several extra tubes to be safe.
- -12 x 75 mm polypropylene tubes (to sort 1 to 4 different populations)
- -15 mL conical tubes (to sort 1 or 2 different populations)

For best results, first coat your tubes with sterile BSA or FBS and then place a small volume of your cell culture media or sort buffer at the bottom of each coated tube (0.5 to 1.0 mL). To coat your tubes fill them with sterile 4% BSA/FBS in 1X PBS and incubate them at around 4 ˚C for at least an hour prior to your sort appointment. This coating mixture is then poured out and can be reused for up to a month when stored and handled properly. Remember, the Aria is a closed system during the sorting process. All fluids except the sample are 0.22 micron filtered before coming into contact with the machine or your sample. This being said, it is impossible to guarantee complete sterility of the instrument, thus you may wish to add antibiotics like Pen/Strep to your culture media/buffer to increase the chances of a sterile recovery. Note that

your sorted cells will be diluted with sheath fluid as they are collected, containing small amounts of sodium fluoride and phenoxyethanol. For particular applications, alternative preservative-free sheath fluid may be considered; please consult with the facility staff before booking your sort appointment to request changes to or ask questions about the sheath fluid in use on the sorter.

10. Data Storage: The Flow Cytometry Facility does not store data for you indefinitely. The software that runs the machine is significantly slowed down by excess data storage, so the memory on the computer is wiped clear every month. The Aria computer is not online nor on any network, so you cannot save your data there either. Hence, please bring a data storage device with you. Recent virus infection caused by users' memory sticks means that we now burn users' data to re-loadable DVDs (facility can provide these).

IMPORTANT NOTES ON BIOSAFETY AND RISK GROUP POLICIES FOR SORTING:

Due to the nature of sorting live cells on instruments that create aerosols, we must take extra precautions regarding samples that present health risks to the operators, the users and/or the public. We operate our sorters within BSCs, but poor sample preparations or problematic cell samples can result in clogs and these can disrupt the physical safety controls that are in place.

We require all risk group and health information (including chemical treatment information) for **all samples** to be provided by the user and verified by the PI. As mentioned above, we accept Risk Group 1 (RG1), RG2 and RG2+ samples.

Note that RG2+ samples may require a local risk assessment (LRA) with Dalhousie's Biosafety Office oversight and approval. Human samples, including PBMC/whole blood, are generally considered RG2+ if not suspected or confirmed positive for specific pathogens. Samples suspected or confirmed to be infected with RG3 organisms, such as SARS-CoV-2 require CL3 when sorting and **will thus not be accepted**. Samples that are RG2+ can be sorted in the facility, but extra PPE and protocols must be used, therefore any user wishing to sort an RG2+ sample should communicate with the facility staff about their sort plans to ensure we have sufficient PPE stocks before arranging the sort appointment. Note, due to fluctuating PPE availability, RG2+ sort appointment requests may be postponed until supplies can be obtained/restocked.

Additionally, Flow Cytometry Facility staff reserves the right to decline or abort the sorting of samples posing a clog-risk due to increased aerosol generation that results during attempted sorting. Thus, users are reminded and encouraged to follow the above guidelines and suggestions for generating high-quality sample preparations that are single-cell suspensions (i.e., use appropriate tissue dissociation techniques, appropriate sort buffers and filter samples prior to the sort appointment).

Thank you for your understanding and attention to these important safety policies!

Flow Cytometry Facility – Sorting Application Form

Please fill out and sign at least 72 hrs prior to requested date, 24 hrs notice is required for cancellation.
Email completed form to cellsort@dal.ca

User's Name:	**Phone**:
Email:	**Today's Date:**
Desired Appointment Date(s)/Time(s):	
Sample Source and Cell Source: (e.g., Mouse spleen; Human blood)	**Cell Type and Risk Group ***: (e.g., Splenocytes, RG1; PBMCs, RG2)
Are cells infected with or exposed to any pathogen or transfection vector? If yes, please list pathogen/vector name, and if a vector please indicate the generation of the vector system and how many passages since transfection:	
If yes, please give: Pathogen/Vector's Host Range:	**Pathogen/Vector's Risk Group:**
Have cells and/or pathogen(s) been modified in a way that could affect their risk group assessment, i.e., increased virulence/pathogenicity? If yes, please describe.	
Cell Size **:	**Approximate Number of Starting Cells to Sort:**
Number of Samples and Approximate Amount to Sort: ***(Please note there is a charge for sorting)***	
Fluorochrome(s):	
List of Controls (e.g. negative, positive and compensation controls) ***:	
Describe the <u>desired population(s)</u>, and the <u>collection device(s)</u> to be used. As well, describe briefly <u>what the cells will be used for</u> and if the Aria's <u>temperature control option</u> is desired.	
Do you plan to sort other cell types in the future? If so, please list the cell types and their risk group information here:	

*** Please note that RG1, RG2 & RG2+ samples are permitted. Human samples, including PBMC/whole blood, will be handled as RG2+; suspected or confirmed SARS-CoV-2 or other RG3 positive samples require RG3 containment level and will not be accepted.**
** Approximate size, if known. Three nozzle sizes are available for the Aria III: 70 µm nozzle sorts cells up to 10-12 µm in diameter; 85 µm nozzle sorts cells up to 12-16 µm; and the 100 µm nozzle sorts up to 25 µm cells, and particularly fragile cells.
*** To ensure a proper sort, a negative control sample of the same cell type without the fluorescent dye/antibody is required. If multiple colors are used, a positive control for each individual fluorochrome or dye is required.

Sample(s) to be sorted is assured by Principal Investigator (PI) to match the description above in terms of pathogens and risk group, and the PI recognizes the Flow Cytometry Facility staff's right to decline or abort the sorting of samples posing a clog-risk due to safety risks explained in the sort document above.

Investigator's Name:	**Investigator's signature**: