Children's Hospital of Philadelphia℠

Flow Cytometry Core Laboratory

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Planning your cell sorting experiment

Contents

How does cell sorting work?

Cell sorting is a technology used for separating cells or particles based on their light-scattering and fluorescence properties.

Six sorters are operated in the Flow Cytometry lab at CHOP: **Aurora CS**, **FACSAria Fusion**, **FACSJazz**, **FACSMelody**, **MoFlo Astrios EQ** and **BioSorter**. All of them operate by encapsulating each particle of interest into a droplet, which is then directed into a collection device (a tube or a well of a plate)

Aurora CS, **FACSAria Fusion**, **MoFlo Astrios EQ, FACSJazz** and **FACSMelody** are **electrostatic droplet sorters**. They are used for separating particles with diameters up to 30µm. These devices work by shooting a thin stream of sheath fluid (PBS) down through a small orifice (nozzle). The cells or particles contained in the sample are "injected" into the center of this stream and arranged in a straight single file (via hydrodynamic focusing). The nozzle vibrates with very high frequency (10 to 100 kHz), causing the liquid stream to break down into tiny droplets that will contain the cells to be sorted. The droplets that contain desired cells are electrically charged and then deflected sideways (left or right) in an electric field, adjusted so that the deflected droplet falls into a collection device. The droplets containing unwanted cells are not deflected and go vertically into the waste tank.

The **BioSorter** can be used for separating particles with diameters larger than 40µm. Typical mammalian cells cannot be sorted on our BioSorter because they are too small. This instrument operates on a different principle than the electrostatic droplet sorters: it generates a continuous stream of sheath fluid which is blown sideways by an air blower into a waste tray. For each desired particle, the air blower stops very briefly (a few milliseconds), causing a droplet containing the particle of interest to fall vertically into a collection device. The unwanted particles are sent into the waste tank.

Unavoidably, some droplets will not contain only one, but two or more cells. Some droplets will contain one or more "good" cells and one or more "bad" cells. Almost always we set up the sorters to collect the droplets that contain one or more desired cells, but no undesired cells ("**purity mode**"). However, a sorter can be also set to collect all the droplets that contain at least one desired cell, including those

droplets that contain both desired and undesired cells ("**enrich mode**"). The enrich mode will be used only if you specifically request it. In "**single cell**" mode, the sorter sends into the collection device the droplets that contain only one desired cell. Single cell mode is used for depositing one single cell into each well of a plate.

We often mention the term "**sort efficiency**", which is the ratio between the number of desired cells that are detected by the sorter and the number of desired cells that are deflected into the collection tube. In enrich mode the sort efficiency is practically always 100%. In purity mode, we aim at keeping the sort efficiency higher than 75%. The sort efficiency can be improved by preventing the cells from sticking to each other and clumping (see below the *Sample preparation* section), by minimizing the amount of debris in your sample, diluting the sample or running the samples at low event rates.

*For an in depth introduction to cell sorting watch **The Art of Sorting** webinar by Beckman-Coulter.*

Guidelines on how to plan a cell sorting experiment

The reliable identification of the target populations to be sorted is critical for the success of a cell sorting project. If your cells need to be dissociated from solid tissues, begin with practicing and fine-tuning cell prepping. Use a microscope to confirm that the cells are well dissociated and the amount of debris is reasonably low.

Next, we advise to schedule **a pilot experiment**, which should have the following aims:

- Confirm that the cell preparation procedure and cell staining are reliable.
- Confirm that the populations to be sorted can be identified and sorted with acceptable purity.
- Estimate how much time would be needed for collecting the desired number of sorted cells.

High-speed vs. regular-speed sorting

Aurora CS and **FACSAria** can be operated in **high-speed** mode (using **70µm nozzle**, sheath fluid pressure 60 PSI) or in standard-speed mode (100µm nozzle, sheath fluid pressure 18 to 30 PSI). MoFlo Astrios EQ is typically operated with a 100µm nozzle because it is time-consuming to swap nozzles on this sorter.

Generally, the diameter of the cells to be sorted should be at least 5-fold smaller than the nozzle size. Thus, cells with diameter smaller than 14µm (e.g., lymphocytes) can be efficiently sorted in high-speed mode; cells with diameters below 20µm can be sorted using the 100µm nozzle. Larger cells will cause inconsistent deflection of the droplets, and some sorted cells won't reach the collection device.

Please do not request high-speed sorting unless the diameter of your cells in suspension is 14µm or smaller AND the total number of cells in your samples is larger than 35 million.

FACSJazz and **FACSMelody** can be operated in **regular-speed mode only** (100µm nozzle).

Sample preparation

Before bringing the cells to the Flow Lab, consider taking a small droplet of sample on a microscope slide and place it under a microscope to check the quality of your cells: typically, the cells should be round, abundant, with very few aggregates, and a reasonably low amount of debris.

Adjust the concentration of the cells to be sorted to max. 10 to 20 million/ml for regular-speed mode and max. 30 million/ml for high-speed sorting mode. Note that we can dilute the cells if they are too concentrated, but for practical reasons we will not concentrate them. If the number of cells in each sample is low, adjust the volume to 0.5ml. In principle, any physiologic buffer may be used to resuspend the cells to be sorted. However, it is essential to have the cells **well-dissociated** and to prevent their clumping in order to achieve good recovery rates, high sort efficiency and to **prevent clogging of the nozzle**.

We recommend suspending the cells in **Ca^{2+}/Mg^{2+}-FREE buffer**, such as **PBS** (Phosphate Buffered Saline) or **HBSS** (Hank's Balanced Salt Solution) with low protein content (≤ 2% BSA). **EDTA** (0.5 to 5 mM) and/or **DNase** (20 – 100µg/ml) may be added to further prevent cell clumping. Note that standard cell culture media contain Ca^{2+}, $Mg^{2+}$and proteins from serum that favor cell clumping. For pH-sensitive cells, **HEPES** (25 mM) may be added to the buffer.

It is highly recommended to pass the cells through a cell strainer with 35µm mesh size (*Falcon* cat. 352235) immediately before sorting. Some cells tend to clump after being filtered.

Collection tubes

The table below summarizes the collection devices that can be used on each electrostatic droplet sorter.

Collection Device	Sorter				
	Aurora CS	**FACSAria Fusion**	**FACSJazz**	**FACSMelody**	**MoFlo Astrios EQ**
1.5ml Eppendorf	6 ways	4 ways	2 ways	4 ways	6 ways
5ml FACS	4 ways	4 ways	2 ways	4 ways	6 ways
11ml (100x17mm)	2 ways	Unavailable	Unavailable	Unavailable	Unavailable
15ml conical	Unavailable	2 ways	2 ways	Unavailable	4 ways
50ml conical	1-way only	Unavailable	Unavailable	Unavailable	2 ways
Multi-well plates	96-well plates	Any standard plate	Any standard plate	Any standard plate	Any standard plate

Example on how to read the table above: Aurora CS can be used to sort 1, 2, 3 … up to 6 populations at once in 1.5 ml Eppendorf tubes. Up to 4 populations can be sorted in 5 ml tubes, up to 2 populations in 11 ml tubes and only one population at a time in 96 well plates. 15 ml conical tubes cannot be used (they are too long and do not fit in the sorting chamber of Aurora CS). Sorting in 96 well plates is available.

Please bring **labeled** collection tubes containing cell culture medium or buffer of your choice, in sufficient volume to cover the bottom of the tube. The sorted droplets should fall into liquid, not on hard plastic and we also want to have the cells sorted into media that contains some nutrients. FBS 100% may also be used as collection media if desired.

All our sorters are equipped for collecting **single cells in multi-well plates (96 well plates)**. We can also deposit a larger number of cells into each well.

Sorting time

The amount of time needed for sorting depends mainly on how many cells need to be run through the instrument.

- In high-speed mode (**70µm nozzle**), the sort speed is up to **80 - 90 million events/hour**.
- In standard-speed mode (**100µm nozzle**), the sort speed is up to **30 million events/hour**.

The **sample volume** is also a limiting factor: less than 5ml of sample can be processed in one hour.

Some of the reserved time will be used for setting up the sorter for your project. For a one-color experiment, 15 minutes or less are typically sufficient for setup. For multicolor experiments, consider reserving at least 4 extra minutes for each additional color. (For instance, for a five-color sort consider reserving about 35 minutes of time for setup, running the single color controls, and compensation/ unmixing.)

Purity check

Checking the purity of sorted cells can be done by taking an aliquot of sorted cells and re-running them on the sorter. Approximately 5,000 sorted cells are necessary for a purity check. We recommend you ask the operator of the sorter to check the purity of sorted cells, but we do not always do it by default because sometimes the number of sorted cells is very low and because it takes additional time. The purity of sorted cells is typically above 95%.

Training for cell sorting

Frequent users who need to sort at least once a week are encouraged to learn how to operate a sorter.

- Basic cell sorting skills on Aurora CS, FACSAria Fusion, FACSJazz, MoFlo Astrios can be gained in two training sessions of approximately 2 hours each.
- The training for Aurora CS and FACSAria may take only one session of two hours, if the trainee is proficient in using the corresponding software package: SpectroFlo for Aurora and FACSDiva for FACSAria.
- Users who need to operate MoFlo Astrios in semi-assisted mode during business hours may request a single training session of two hours.
- FACSMelody is very user friendly. One session of two hours is typically sufficient to learn how to operate the FACSMelody sorter.
- Typically, it takes one session of approximately two hours to learn how to use the BioSorter at basic level.

Additional training will be needed for covering advanced features and for learning how to troubleshoot common technical problems. Training sessions can be scheduled via iLab reservation and carry the same rates as full-service appointments.

Cell sorting table

The table below summarizes from a practical standpoint the main characteristics of the sorters existing in the Flow Cytometry lab at CHOP.

	Regular Speed Sorting	High Speed Sorting	Large Particles Sorting	
Sorters	**Aurora CS FACSAria FACSJazz FACSMelody MoFlo Astrios EQ**	**Aurora CS FACSAria MoFlo Astrios EQ**	**BioSorter**	
Nozzle size	100 µm	70 µm	250 µm FOCA	1,000 µm FOCA
Nozzle frequency (equals the number of drops generated per second)	~25,000 – 45,000 sec.	~75,000 – 90,000 kHz	Unavailable	Unavailable
Droplet volume	~ 3 nl	~ 1 nl	1 µl	7 – 8 µl
Maximum cell diameter recommended	20µm	14µm	100 µm	500 µm
Sheath pressure	18 - 27 psi	~60 psi	< 5 psi	< 1 psi
Maximum sort rate	8,000 to 10,000 events/sec. (up to 36 million events/hour)	20,000 to 25,000 events/sec. (up to 90 million events/hour	20 events/ second	20 events/ second
Minimum recommended sample volume	0.5 ml	0.5 ml	5 ml	5 ml
Recommended pre-sort cell concentration	10 – 15 million cells /ml	20 – 30 million cells /ml	0.1 – 1 million/ml	0.1 – 1 million/ml
Post-sort concentration	~0.3 million cells /ml	~1 million cells /ml	~1,000/ml	~150/ml
Typical sorting efficiency	70 – 90%	70 – 90%	60 – 90%	60 – 90%

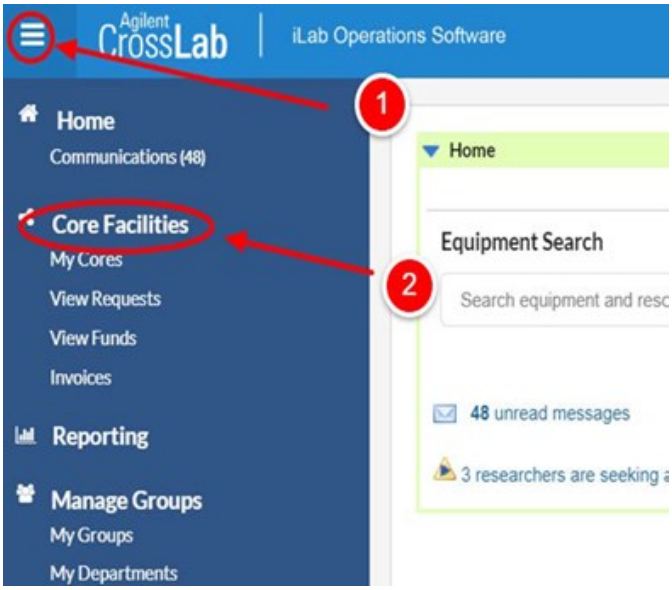
* Sorting cells using a nozzle smaller than the recommended size may result in poor cell viability and loss of a variable percentage of sorted cells. 85µm and 130µm nozzles available on FACSAria Fusion.

Guidance on setting up in iLab a reservation for cell sorting

1. ***Register in iLab if you do not have an account***. Inquiries regarding the iLab registration process should be sent to coresadmin@email.chop.edu or feel free to contact any member of the flow lab.

   Researchers not affiliated with CHOP should follow the guidance below to find our iLab site:

   - Use a web browser to log into iLab
   - Click **the menu icon** (1) in the top left corner
   - Select "**Core Facilities**" (2)
   - From the "**View**" drop-down list, select "**Cores at partner institutions**" or "**Cores at other institutions**"
   - Look below for the Flow Cytometry Core at CHOP.

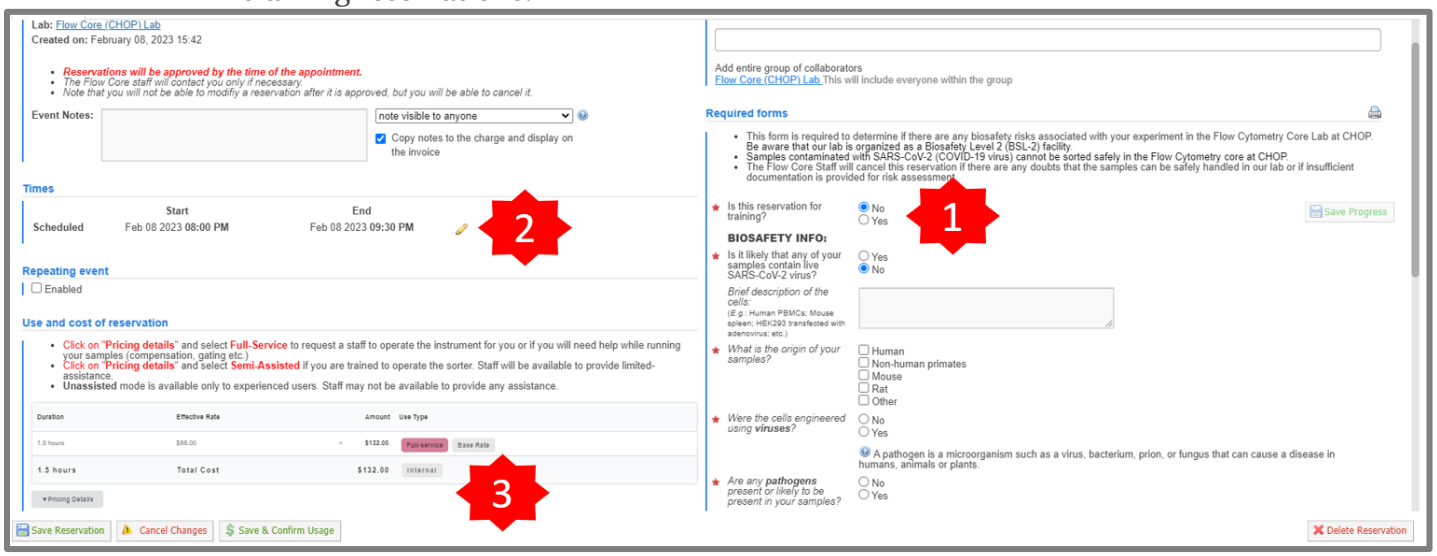


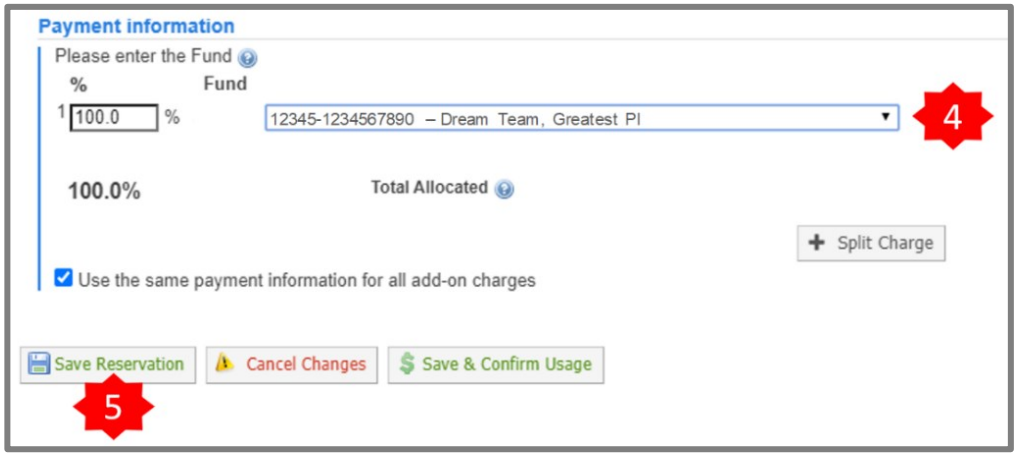
2. **Set up a payment method:**
   - **CHOP researchers** should ask their PI or business manager to give them access to iLab in an active payment account. *Questions on enabling CHOP accounts in iLab should be sent to coresadmin@chop.edu.*
   - **PENN researchers** will need to set up a PO as a form of payment.
   - *For setting up the PO, the business manager may need the following information:* ***BenBuys*** *information*: ***Phila 200, Site 49513 Children's Hospital of Philadelphia***
   - **All other researchers** must set up a PO from their own institution. *The POs can be uploaded in iLab by the business manager or by the researcher.*
3. **Determine what instrument you should use for your project.** The optical configurations of our instruments can be *downloaded in pdf format* or can be accessed in iLab in the "Reserve Instruments" tab. Please email flowcytometry@chop.edu if you need help with selecting the appropriate sorter for your project.
4. **Reserve a sorter:**

a) **Log in iLab**, click the "**Reserve instruments**" **tab** and scroll to the bottom of the page. Click on "**Sorters"** to open the instrument schedules.

b) Click "**View schedule**" to open the calendar. **Click-and-drag** over any available time slot in the calendar to initiate a reservation:

- If you need to change the **time**, **click on the pencil (2)** to the right of the End time, then click **green checkmark** to save when done.
  *Note: this does not save the reservation. The button "**Save Reservation**" at the bottom left saves it.*
- If you need to change the **Use Type**, click on **Pricing Details (3)** to select the **type of service** desired.
- **Full-service** is for all untrained users during the Core's operating hours; **Semi-assisted** is for trained users operating independently DURING operating hours; **Unassisted** is for trained users operating independently off-hours; **Training** is for all training reservations.





- Choose the account number or PO number **(4)** to be used with this reservation.
- Indicate whether the reservation is for **Training** or not. Fill out the **Questionnaire (1)** for regular reservations.
- Finally, click on the "**Save Reservation**" **(5)** button at the bottom left of the screen to save the changes!

Note: **We suggest using FACSAria or Aurora CS for high-speed sorting**, because swapping the nozzles on these sorters is much faster than on MoFlo Astrios EQ. When selecting the high-speed (70µm) nozzle on MoFlo Astrios EQ, reserve one additional hour for replacing the nozzle (30 minutes to install at the start and 30 minutes to uninstall at the end).

Replacing the nozzle takes about 10 minutes on FACSAria and 15 minutes on Aurora CS. On FACSJazz and FACSMelody, only the 100µm nozzle can be used. Email the staff at flowcytometry@chop.edu if you are not sure how much time is needed for your sort.

Note: The BioSorter may be used for large cells (hepatocytes, adipocytes, etc.), cell clusters and small organisms. Be aware that the sorting speed on the BioSorter is much lower (less than 20 particles per second) than on the other sorters.

5. **Bring the items listed below to the Flow Lab in ARC 1207 at the time of your appointment:**
    - **Filtered cell suspensions in 5mL FACS tubes**, including single color compensation controls, unstained cells and FMO controls if needed;
    - **labeled collection tubes containing cell culture medium or FBS;**
    - Approx. 10ml of sorting buffer for diluting the cells if needed.
    - Extra collection media in case new collection tubes must be prepared
6. **Finally, always check with the operator of the sorter to make sure that all gates are properly set and populations to be sorted are clearly identified.**

Call the Flow Lab at 215-590-3402 or send an email to flowcytometry@chop.edu if you have any questions or concerns.