Data aplicació	Elaborat per
04-2020	**Càrrec personal tècnic** Manuela Costa 23-03-2020

1. INTRODUCCIÓ I ÀMBIT D'APLICACIÓ

The purpose of this document is to provide general guidelines and best practices for preparing samples for cell sorting. Be mindful that every sample type is different, and modifications may be needed.

1. What is flow sorting?

Sorting is defined as the separation of the particles or cells of interest from the other particles or cells in the sample. Our lab has the FACSJazz cell sorter: a 'stream-in-air' cell sorter that works by the electrostatic deflection of charged droplets ideally containing the cells you are interested in. This sorter is capable of sorting **two populations simultaneously**.

2. What do I need to know when booking a sort?

Although you will be able to book the FACSJazz calendar because you have access to PMMS platform, you will need the **confirmation of a member of the SCAC.** There are two reasons for this. Firstly, we need to ensure that someone is available to perform your sort and secondly, we need the details of your sort so that we can set up the sorter and the sorter room accordingly. **Biosafety information is also asked in order to know the safety measures to be applied for the sorting. So, before coming to a sort you will have to complete the sort form and return it signed.**

3. Cell staining and fluorochrome choice

A successful sort is dependent on good resolution of sorted populations!

If there are any questions about fluorochrome choice or cell staining procedures, please consult our staff. In the picture bellow the BD FACSJazz cell sorter optical configuration is shown.

FACSjazz Optical configuration Cytometry-SCAC-UAB			
Laser	**Detector**	**Filter (nm)**	**Fluorochome or Scatter parameter**
Blue (488nm)	FSC	488/10	Forward Scatter (Size)
	SSC	488/10	Side Scatter (Granularity)
	FL1	530/40	FITC, AF488, BB515, CFSE, Fluo-3, GFP, YFP, Sybr Green, TO-PRO-1, TOTO-1, Calcein, Sytox Green, Syto9, Syto 16, Zombie Green…
	FL2	585/29	PE, PI, ViaKrome 561 Fixable viability Dye…
	FL3	692/40	Propidium Iodide (PI), 7-AAD ,PerCP, PerCP-Cy5.5, PE-Cy5.5, PerCP-Vio 700,…
	FL4	750LP	PE-Cy7, PE-Vio 770…
Red (635nm)	FL5	660/20	APC, AF633, AF647, efluor660, Cy5, DRAQ5, TOTO-3, TO-PRO-3, BD ViaProbe red nucleic acid stain
	FL6	750/LP	APC-Cy7, APC-H7, APC- AF750, APC-Vio 770…

4. How many cells should I bring?

The number of cells you need to bring is primarily determined by the frequency of the wanted cell in the starting sample and by how many sorted cells you need to obtain. Keep in mind there are always losses in any purification - be it sorting or other.

Also keep in mind **purity**. If you want high purity, the recovery will be less or the sort slower.

A good estimate is to determine the number of cells you need to end up with, divide this by the frequency of the wanted population in the sample and by the estimated sort efficiency (70%), and multiply by at least 2.

$$\frac{\text{n of cells needed}}{\text{freq of wanted cells} \times 0.7} \times 2$$

Also remember that we will consume some cells in setting up the sort. If this is the first time you are sorting this particular sample, we may need more cells initially than we will in subsequent sorts. As always, we strongly recommend that you do some preliminary experiments on the analyzers to work out the staining and cell preparation procedures rather than doing this on the more expensive sorters. A final test run on the sorter is also recommended to confirm that we can see the same population resolution.

5. How much time I will need for the sorting?

The FACSJazz sorter can separate about 4,000/sec, 14,5 million/hr, but this number correspond to events processed above any threshold and it includes whole live cells, dead cells and debris. Besides, take in mind that among the live cells you have to know which percentage corresponds to the population you are interested in, and how many populations you want to sort. Knowing the relative contributions of each population, will help you calculate the number of cells you should bring and the time you will need for the sorting. In any case, each situation may be different so these numbers should only be used as a guide to help you. The staff of the Lab will inform you of the number of cells that it is possible to sort once your sort is underway.

Also keep in mind there will be setup time required once we have your cells in hand and if compensation is involved this will also add time to run the compensation controls and set the compensation. **A half hour is a good estimate for setup** except for simple sorting setups, e.g., 1-color GFP sort.

6. What do I need to bring on the day of my sort?

6a. Your samples

Make sure you have a good single cell suspension, no clumps, little debris and few dead cells.

Clumps may be removed prior to sorting by filtering through a 35µm mesh that it can be supplied by the facility. The Cell re-clumping can be minimised by maintaining the cells at 4°C or by adding EDTA (1mM) or DNase (10U/ml) to the medium.

Dead cells can often be excluded by their light scatter characteristics. However, this isn't always the case so we advise adding a DNA-binding dye that can be used as a dead cell exclusion marker. We can supply propidium iodide or BD Via-Probe™ Red Nucleic Acid Stain in case of need. Both can be added at the moment of the sorting.

6b. Cell Sorting Buffer

Cells should be in an appropriate buffer that preserves viability and controls pH (HEPES buffer will help reduce pH changes that are induced by the high pressures used in the cell sorter). They can be in medium or PBS but pre-sorting, the level of protein should ideally be less than 3%.

The following is a suitable buffered medium; however, modifications may be necessary for your particular sample. This buffer can be filtered and stored at 4°C and **antibiotics** may be added to prevent microbial growth.

Basic Cell Sorting Buffer:

- 1x PBS (Ca/Mg++ free) or HBSS (preferred)
- 0.5-2% BSA or up to 3% heat-inactivated FBS
- 25mM HEPES pH 7.0
- Antibiotics: penicillin-streptomycin 100U/ml-100µl/ml

6c. Cell concentration. The optimum concentration for our sorting is around **3-4 x 106/ml**. It will be useful to bring a tube with sorting buffer in case we need to dilute the cells.

6d. Sample tube. You can use the tube you want because in the cytometry lab we will transfer the cells to a 5ml cytometer tube special for the sorting.

6e. Unstained and Single Stained Control(s):

Unstained controls are essential for determining background fluorescence and single stained controls are used to calculate the correct compensation values before a sort (in case you make a multicolour experiment).

Incorrect compensation can result in the wrong cells being sorted. **Please bring an unstained/nonfluorescent control AND single stained controls for each fluorochrome/dye/fluorescent protein being used in your experiment to each and every sort appointment.** Fluorescence Minus One Controls (FMO's)[1] are also convenient in some multicolour experiments.

Cell concentration for controls can be 1x106 cells/ml and 0,5ml volume is enough.

6f. Collecting tubes with collecting media.

We will also need polypropylene collection tubes, generally 15 ml Falcons, but you can use Eppendorfs, culture plates, slides, 384,96,48,24,6 –well plates…which should contain medium.

Collection media should contain serum at high concentration (e.g. 20 - 50%) because that will get diluted as the tube fills with sorted cells in PBS. We also recommend the addition of antibiotics to avoid contamination of the sorted cells. *96, 48, 24, 6 well plates. 1.5ml, 2.0ml microcentrifuge tubes, 0.5ml PCR tubes, 5ml FACS tubes, 15ml Falcon tubes (two-way sort only)*

Basic collection Buffer:

- Complete culture medium
- 20-50% heat-inactivated FBS
- 20mM HEPES pH 7.0
- Antibiotics, Ex: penicillin-streptomycin 100U/ml-100µl/ml

The amount of medium will depend on how many cells you will collect. Each drop using a 100µm nozzle has a volume of approximately **3nl,** so a 15ml Falcon tube containing 5000 µl medium will be full when approx 2,5 million cells have been collected.

6g. Sterility -use of antibiotics.

The cell sorter is placed in a biosafety cabinet, and we thoroughly sterilize the sample delivery part of the sorter before and after each sort. Moreover, we sterilize the entire sheath fluid path once every month. Otherwise, the fluidic component of the sorter is complex and although extremely rare, contaminants are possible. We recommend (essentially require) that when culturing sorted cells, you add antibiotics which include 50µg/ml gentamycin or penicillin-streptomycin 100U/ml-100µl/mlIf a long-term culture is desired the antibiotic can be discontinued after about a week. We have done many successful sterile sorts where the investigators used no antibiotics following the sort but since your sorted cells are rather valuable, we suggest prudence.

7. What are 'sort modes'?

These are how we tell the sorter what stringencies we want to apply to a sort and they can be altered depending on whether we want to optimise purity, yield or count accuracy. The most common sort modes are **purity**, **enrich** and **single**. If you want to learn more go to "***How cell sorting works"***[2]***?***

The sort mode used will be discussed with you.

8. Gating strategies

Hey, it's your sort, we will do it as you want. However… the FACS Lab staff have a good deal of experience in identifying and excluding debris and dead cells, we are experienced in machine set up including filter selection and fluorescence compensation. All gates and regions will be discussed but are ultimately your responsibility. The sorter will sort what you tell it to sort, it's up to you to give it the right instructions and you should be satisfied about gating before the sort begins.

9. What about my booking time?

There is some degree of flexibility. We realise that sometimes preparation takes longer than expected or there is a problem of some type. However, it may not always be possible to extend your booking time. Another sort may be booked – and we need to allow at least 1 hour between sorts to clean and prepare the sorter – or the sort operator may have another commitment. If you are going to be late, please call, it's a simple courtesy that will save heartache. Likewise, if we experience a problem that would delay your start, we will call you and let you know.

Although core hours are 9-6:00, the Lab operates an extended booking time for sorters and we are able to sort till 7pm.

10. Consecutive sorts of cell lines.

We are frequently asked to do sorts, in a single session, of multiple cell lines - e.g. transfected cell lines. We make every effort to make sure that subsequent cell lines are not contaminated by prior samples. We will vigorously backflush and sterilize the sample line to prevent this for that reason, we will require about 10 minutes between consecutive sorts to complete this process - i.e. 30 minutes for 3 cell lines. Please allow for this time in scheduling sorts like this

11. How do you assess the success of a sort?

There are three parameters that are important: **Purity, Recovery and Yield.**

> **Purity** is probably the one that most users are concerned with. It should be possible to get >98% purity. Purity is defined as the number of cells in your sorted tube that fulfil your sort criteria. A small aliquot can be re-run on either the sorter or an analyser to assess this.
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> **Recovery** is the ratio of the number of cells in your sorted tube relative to the number that the cytometers say it has sorted. This should be around 80%. There will be some cell loss due to cell death and not all sorted droplets going into the collection tube (another reason to avoid polystyrene collection tubes). It must be stressed that samples should NOT be spun or manipulated before counting.
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> **Yield** is the percentage of cells you get back relative to the amount in your sample.
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> Yield will be >70%+ in most cases but will be influenced by the sort modes used.

Unfortunately stream in air sorting isn't perfect and neither are we (almost though) so please let us know if there is a problem after your sort. This could be low viability, poor cell number, contamination or some other problem. If we don't know, we can't do anything about it and we could potentially affect other people's sorts.

Reduced purity and recovery are almost certainly due to factors other than the sorter itself. We can help troubleshoot these problems but only if we know about them.

Please take the time to provide feedback on your sorts.

12. Using other sheath fluids.

If you have an application where cells are extremely sensitive to PBS, we can operate the instrument using special sheath fluid. We will ask you to provide the fluid and will consult with you about what is permissible. We will have to charge an additional fee to perform this service as we must setup the instrument and remove the fluid completely following the sort, especially if it contains a carbohydrate e.g. glucose, so that microbial growth in the system does not occur

13. Annotations

1- Fluorescence Minus One Controls (FMO's)

A fluorescence minus one (FMO) control contains all fluorochromes of the multicolor cocktail except one. They represent the combinatorial background fluorescence from other channels into the channel of interest and aid in setting sort gates properly. Many researchers are resistant to using FMO controls on a routine basis because they consume cells, reagents and time, but in many cases, they are needed to validate the gating strategy of a reagent panel particularly if you are using the panel for the first time. For more information on FMO's, see Nature Reviews Immunology 4, 648-655

2- *"How cell sorting works"?*

All flow cytometers interrogate cells by passing them through one or more lasers and collecting the emitted fluorescence. So-called "Stream in air" sorters such as the FACSJazz, work by ejecting the cells in a fluid stream through the orifice of the nozzle. By applying high frequency vibration to the nozzle, drops are formed which we can then deflect by applying a charge to them. To sort the cells of interest we need to know the time it takes for a cell to pass through the laser (at which point we know what its fluorescence characteristics are) to the point at which individual drops start to form. This is known as the drop delay and is set by the operator prior to the sort. By knowing this and by correlating it with the appearance of cells through the laser we know where each cell is situated in the ejected column of fluid and hence in which drop it will be. In an ideal world only one cell will be in a particular drop. However, the arrival of cells is a stochastic process and there can be more than one cell per drop. If a wanted cell is accompanied by an unwanted cell, we can't sort the drop as it would reduce purity. Sometimes a cell is situated near the edge of a drop and could be either in the expected drop or the neighbouring drop. In these cases, we would sort both drops (assuming a contaminating cell isn't in either of them).

Cells can be lost in two ways. Firstly, if they pass through the laser too close to be separately analysed (we call these hardware aborts). This happens if cells are clumpy, big in relation to the nozzle size or too concentrated. Cells will also be lost if they can't successfully be sorted - for example where a drop contains a cell of interest and an unwanted cell, to sort this drop would compromise purity so it is normally not sorted – we call these co-incidence or software aborts. It is these practical considerations that will affect your yield.

The default mode, on a FACSJazz, is called **Purity.** This mod is used If high purity is critical for the downstream application. In this case, the sorter must be instructed to exclude any target events from the sort that fall close to any non-target events.

We can also perform an **enrich** sort. This would be applicable if you have a precious or small population. In this situation all cells that qualify will be sorted even if there are unwanted cells nearby in the stream. So, purity is reduced but yield is maximised.

Enrich sorting can only be performed for one population.

The final mode is called **Single** cell mode. This is used when count accuracy is paramount, for example when cloning, it uses a very stringent mode to ensure that when we say one cell is sorted, we mean one cell. Again, this can only be used for a single population at a time.

14. Summary

Before coming to a sort, you will have to complete the sort form and return it signed.

Bring an unstained/non fluorescence control and single stained controls for each fluorochrome/dye/fluorescent protein being used in your experiment.

Make sure you have a **good single cell suspension,** no clumps, little debris and few dead cells.

Bring your cells in the appropriate **sorting buffer** at about **3-4x106 cells/ml,** in any support.

Bring an extra tube with sorting buffer in case we need to dilute the cells.

Do NOT bring more cells than you can sort in your allotted time.

Bring enough colleting supports to your sorting, filled with **collecting media plus 10-50% FBS+ Atb.**

Do NOT turn up late without letting us know.

Do NOT change your fluorochromes without telling us in advance.

Do NOT come more than one person to the sorting.

Do NOT expect to be able to start before or exceed your booked time.

DO accept that sorters are complex electronic equipment. Sometimes things do go wrong. We will let our users know when there is a problem.

DO speak to us if you have any questions before or after your sort.

DO provide feedback – good, bad or indifferent.

DO ask questions and interact with your sort operator.