Preparation for Cell Sorting

New protocols

It is mandatory to arrange a meeting with the FCF Head every time you need to perform a sorting in the context of a new experimental approach to ensure the FCF team is aware of all relevant details of your sorting experiment. In this meeting, you may also receive advice on how to prepare your samples for sorting, which instrument to choose, time length of your booking, etc.

Sample Preparation

Identify the best protocol for your sample type and ensure all procedures are optimized and validated prior to cell sorting. This may include: titration of antibody concentration, validation of antibody performance, optimization of incubation times and temperatures, etc. Independently of the sample type, at the end of the protocol you must have a homogeneous cell suspension, with minimal debris and free of clumps and aggregates.

To remove debris and excess antibody, please wash your samples at least 3 times in a large volume of staining buffer.

Buffers

For cell sorting, we recommend resuspending samples in FACS Buffer (1X PBS supplemented with 2% FBS).

Sample concentration

The ideal cell concentration for cell sorting is:

- for larger, more sticky cells (e.g. epithelial or tumor cells and cell lines): 5×10^6 cells/mL
- for smaller cells (e.g. lymphocytes): 2×10^7 cells/mL for small cells

For *single-cell sorting*, however, sample concentration should not exceed 1×10^6 cells/mL to maximize efficiency and cell recovery.

Please bring a tube with extra FACS buffer in case it is necessary to dilute the sample.

Tubes

Please bring your samples in 5 mL FACS tubes or eppendorfs.

Temperature

For most cells, the viability is better preserved if samples are maintained at 4ºC. In this case, please bring your samples in ice and ask the FCF staff to keep the sorting and collection chambers refrigerated.

Nevertheless, some cell lines are extremely sensitive to temperature changes and show a better survival if all procedures – sorting included – are performed at room temperature. Please ensure you have informed the FCF staff if this is the case of your samples.

Sample filtration

It is mandatory to filter all samples and controls to be analyzed in the cell sorters to ensure clumps are removed and, therefore, the risk of clogging the nozzle is minimized (please see next section: Safety).

This filtration step must be performed in the Flow Cytometry Lab just prior to sample load into the instruments. The IGC-FCF can provide pipettes, tips and filters when needed, but you are welcome to use your own filters. The mesh size may vary between 10 to 100 μm according to the size of your cells. For precious and minute samples, we recommend the following options for sample filtration:

- Falcon® 5mL Round Bottom Polystyrene Tubes, with Cell Strainer Snap Cap (Corning, Ref# 352235, vendor Enzifarma).
- Mini Cell Strainer, 70um, Clear (Funakoshi, Ref# 194HT-AMS-17002, vendor tebu-bio)
- Falcon® 70μm Cell Strainer (Corning, Ref# 352350, vendor Enzifarma).

Cell re-clumping can be minimized by sorting at 4°C or by adding EDTA (1–5 mM) and/or DNAse (200 μg/mL) to the suspension buffer.

Safety

You should know that all the sorters in the IGC-FCF work by electrostatic drop deflection and therefore produce aerosols. Aerosols containing infectious agents or hazardous materials can pose a serious risk because:

- Small aerosol particles can readily penetrate and remain deep in the respiratory tract if inhaled.
- Aerosols may remain suspended in the air for long periods of time.
- Aerosol particles can easily contaminate equipment, ventilation systems, and human skin.

Of note, the risk is even higher in case there is a clog in the cell sorter nozzle, as drops of even smaller size are produced. Therefore, when using cell sorters, it is particularly important to take all the protective measures to comply with ISAC and local biosafety standards.

For this reason, it is mandatory to inform the FCF staff if you intend to sort samples involving any type of biological risk. You should know that in addition to infected samples, all human samples, cell lines that had undergone DNA editing using CRISPR and some retrovirally infected cells MUST be sorted in FACSAria IIu, as this is the only sorter working in BSL-2 conditions.

Colors & filters

The FCF staff needs to know in advance what fluorophores you will have in your sample to know which lasers to set up and what optical filters to use. For this reason, you must provide the exact names of your antibody conjugates and dyes used to label your cells when filling the fields of the online reservation form in Agendo.

Please visit the IGC-FCF webpage to consult the optical configuration of the available cell sorters and confirm the fluorochromes you wish to use are compatible with the optical configuration of our instruments.

If you need to establish a new multicolor panel, you may find useful to test the new fluorophore combination in a spectraviewer online. The FCF staff is also available to help you.

Viability dyes

We strongly recommend the use of a viability dye to accurately exclude dead cells, as they take up non-specifically the antibodies and probes used and are more autofluorescent than live cells. The exclusion of dead cells precludes the sorting of misidentified cells and allows to sort the viable populations of interest more efficiently.

Examples of viability reagents that may be used, and which may be available in several colors, are:

- SYTOX – ThermoFisher
- LIVE/DEAD – ThermoFisher
- Zombie dyes – BioLegend
- ReadiDrop – BioRad

Controls to prepare

For accurately set up the PMT voltages and compensation, in addition to your experimental samples you should prepare the following controls:

- Unstained cells – to set up the PMT voltages.
- Single-stained cells – i.e., samples stained with each fluorescent antibody or reagent at the time; so, if you are using 4 different colors in your sample, you must prepare 4 single stained controls, one for each color. These controls are needed to correct the spectral overlap in each channel.
- Fluorescence Minus One (FMO) controls – i.e., samples stained with all antibodies except one. These controls are not mandatory, but are strongly recommended to accurately define the gating strategy, especially for markers with continuous expression.

Unstained and single-stained controls may be prepared using compensation beads. If you are using fluorochromes excited by the violet laser, please ensure you use compatible beads.

Gating strategy

As mentioned in the previous point, we strongly recommend you bring FMO controls to correctly establish the gates of your populations.

The FCF staff has a large experience in establishing gating strategies, identifying and excluding debris, doublets and dead cells, as well as in setting up the instrument, including filter selection and fluorescence compensation – and will be available to discuss with you and give advice in this step. Nevertheless, the gating strategy to sort your populations of interest will be set according to your indications and will be ultimately your responsibility.

Sorting modes

The cell sorters are able to apply different stringencies to the drops being sorted to privilege purity, count accuracy or yield. The sort mode to be used will be decided following discussing with you the purpose of your experiment.

Collection devices

Collection of sorted cells can be made in the following devices:

	Collection volume	**# Populations sorted simultaneously**
0.2 mL PCR tubes	2 – 5 μL	4
Eppendorfs (1.5 or 2 mL)	100 – 200 μL	4
5 mL FACS tubes	200 – 500 μL	4
15 mL Falcon tubes	500 – 5000 μL	2
50 mL Falcon tubes	1000 – 5000 μL	2
microscope slides	100 μL	1
384-well PCR plates	2 μL	1
96-well PCR plates	2 – 5 μL	1
24-well Terasaki culture plates	2 – 5 μL	1
96, 48, 24, 12 or 6 well culture plates	*	1
individual culture plates	*	1

* Maximum volume of the plate/well

If your purpose is to collect viable cells after sorting, the collection tube inner walls should be pre-coated with a protein containing buffer (such as FACS Buffer) to minimize sticking and cell loss. Exceptions are sortings performed directly into medium in plates or into lysis buffer.

To coat the collection tubes, fill them to the top with either 100% FBS or FACS Buffer and let them sit. Empty the tubes by decantation just prior to sorting.

To maximize cell viability and recovery, we strongly recommend sorting directly into 100% FBS (please check the recommended minimal volumes in the Table above).

Volumes of recovery

The cells are sorted in sheath fluid, which is 1X PBS. Each drop using a 70 μm nozzle has a volume of approximately 1 nL, so a 5 mL FACS tube containing 500 μL medium will be full when approximately 2.5 to 3 million cells had been collected. With a 100 μm nozzle one gets around 1 million cells per 5 mL tube.

Sorting accomplishment

The success of a sorting can be assessed according to three parameters: purity, recovery and yield.

Unless sorting very few thousand cells or upon your request, at the end of each sort, by default, the FCF staff assesses the purity of the sorted populations by re-analyzing an aliquot of the sorted samples. Typically, sortings performed at the IGC-FCF achieve a purity higher than 96%.

Recovery is the ratio between the number of cells counted after the sort and the number of sorted cells registered by the cytometer. Recovery is typically above 80%.

Yield is the percentage of cells recovered in relation to the amount in your sample. Yield will be higher than 70% in most cases, but will be influenced by the sort modes used.

Finally, one key factor that also dictates the success of a sorting is cell viability post sorting. This is highly dependent on the cell nature, the procedures performed, etc., and cannot be directly assessed immediately after sorting. Undergoing cell sorting is very stressful to many cells and it is expected that some will not resist the process. However, if you experience a situation in which all your cells have died after sorting, please inform the FCF staff.

Sterility

The sample lines and flow cells of the cell sorters are daily cleaned and disinfected and every week the sorters undergo a more extensive decontamination process. However, the cell sorters available at the IGC-FCF are "stream in air" sorters, meaning the sorted cells travel within drops ejected through the air before falling into the collection tubes. Therefore, the sorted samples are not sterile. Nevertheless, this is not a problem when culturing the sorted cells in medium with antibiotics or injecting into animal models.

Notwithstanding, FACSAria IIu has a procedure of preparation for aseptic sorting. If you have a special need for sterility, please inform the FCF staff and we can run this procedure for your experiment. However, this will increase the instrument set-up time needed.

Time management

When booking a cell sorting, please make a realistic estimation of the time needed to sort the totality of cells and/or samples you will have. If you need advice, don't hesitate to consult the FCF staff.

If you get delayed for your sorting appointment, there is some degree of flexibility. We realize that sometimes preparation may take longer than expected or that problems may arise. However, it may not always be possible to extend your booking time due to calendar

commitments (other bookings for cell sorting after you or assistance to investigators in the analyzers).

If you realize you will show up late, please call the FCF lab (ext. 4625 or 4251) as soon as possible. It is a simple courtesy that will help the FCF to better manage its time and will save heartache. Likewise, if we experience a problem that would delay your start, we will call you and let you know.

Sorting appointments are scheduled on a first-booked-first-served basis, giving priority to experiments that are time-dependent e.g. on animal availability, patient attendance, in vivo kinetics, etc. Sorting of infected samples has also priority, but is limited to Fridays. For common sense, we expect a reciprocal degree of flexibility from our users.

If your sort must be moved or canceled, we will liaise with you as soon as we know and endeavor to re-schedule as soon as possible.

If you had booked to finish at a specific time and your sorting is not finished, we reserve the right to terminate the sort in order to minimize consequent effects on the running schedule.

Feedback

Please provide feedback on your experiments – good or bad, please do it, as it is the only way of improving our service, correct problems and to find the best solutions for the investigators. It does not need to be formal: a short e-mail saying the experiment has worked or not, or just a word in the corridor.

Your success is our success.

Lab staff

All the members of the IGC-FCF are proficient in the alignment and set-up of the cell sorters. There is a range of experience in terms of multicolor experiments, applications and cell types used. Experience is gained in the real world by sorting your samples. For this reason, in most cases you are likely to deal with any member of staff. If you have any concerns, please bring them to the IGC-FCF Head's attention.

Final tips

- Do NOT bring more cells than you can sort in your allotted time.
- Do NOT turn up late without letting us know.
- Do NOT change your fluorochromes without telling us in advance.
- Do NOT give your booking to a colleague without telling us in advance.
- Do NOT assume a sorting slot is available just because there is a gap on the calendar.
- Do NOT expect to be able to start before or exceed your booked time.
- DO accept that sorters are complex equipment and that sometimes things do go wrong. Please trust that we will let you know when there is a problem.
- DO show common sense.
- DO speak to us if you have any questions before or after your sort.
- DO provide feedback – good or bad.
- DO ask questions and interact with your sort operator.