Préparation des échantillons pour le tri cellulaire

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Tout échantillon arrivant à la plateforme et destiné au tri cellulaire **devra respecter les recommandations suivantes** sans quoi le personnel de la plateforme se réserve le droit de refuser le tri des échantillons.

Stérilité des échantillons

Assurez-vous de préparer votre échantillon dans des conditions stériles et que les milieux utilisés pour le tri et pour la collecte des cellules soient stériles aussi !

Immunomarquages

- **Compter le nombre de cellule** (cellule de Malassez)

- **Ajuster la quantité d'anticorps** au nombre de cellules présentes dans le tube. Cela implique d'avoir titré au préalable vos anticorps. Il s'agit juste d'un produit en croix. Ne raisonnez pas en facteur de dilution mais bien en quantité d'anticorps (µg) pour un nombre de cellules !!!! On ne marque pas 100 000 000 de cellules avec la même quantité d'anticorps que 1 000 000 de cellules !

- Préalablement à l'étape de marquage, **incuber les cellules avec du FcR Block** (pour les cellules humaines) pendant 10min à RT dans le tampon de saturation (ex PBS BSA 2%). Se référer à la fiche de données techniques du fournisseur pour l'utilisation du FcR Block.

- Centrifuger 300g – 5min

- Reprendre les cellules dans votre mix d'anticorps. Ajouter au mix du **Brillant Stain Buffer** (cf fiche de données techniques) si au moins deux fluorochromes de la famille des BV sont présents dans le panel.

- Faire 3 lavages 300g 5min dans 2 ml PBS BSA 2% filtré et éliminer le surnageant par pipetage et non renversement

Milieu de reprise des échantillons / Densité cellulaire

La plateforme de cytométrie refuse de trier les cellules en milieu de culture (alcalination du pH, phenol red qui interfère avec l'analyse, source de Ca^{2+} et de Mg^{2+} favorisant l'agglomération).

- Nous conseillons l'utilisation des tampons suivants :
  - **Presort Buffer** (BD 563503) : tampon à base d'HEPES, de protéines type FBS. Ce tampon ne contient pas d'EDTA. Ajouter extemporanément et en conditions stériles de l'EDTA à une concentration finale de 2mM.
  - **MACS Running Buffer** (Miltenyi 130-091-221) : tampon PBS, BSA, EDTA avec 0.09% d'azide de sodium, PH 7.2
  - **Tampon PBS** ou HBSS (Ca^{2+}/Mg^{2+} free), 1% BSA, 25mM HEPES, 1-2mM EDTA (ajouter **extemporanément** à partir d'une solution stock) pH 7.2 filtré et stocké au frais

Pour les suspensions cellulaires issues de la dissociation de tissus ou tumeurs, nous recommandons d'ajouter de la DNAse à une concentration finale de 200µg/ml.

- **Respecter** les densités cellulaires ci-dessous

Diamètre de la buse	Densité cellulaire (10^6/ml)	Nombre d'évènements/s obtenu (flow rate 1)
70µm	35-40	20 000
85µm	25-30	12 000
100µm	10-15	6 500

- **Apporter** les échantillons **dans des tubes de 5ml** contenant au maximum 3ml de la suspension. Pour des tris de volumes supérieur, diviser la suspension en plusieurs tubes de 3ml car, à terme, des phénomènes de sédimentations ont lieu et perturbent le tri.

Filtration des échantillons

Juste avant le tri, **filtrer les échantillons** à 70µm sur des filtres stériles.

Tubes de collecte

- Apporter les **tubes de collecte de volume adapté**. Le choix doit se faire selon le nombre de cellules collectées (estimé) et en fonction du nombre de populations à recueillir.

Les dispositifs de recueil sont disponibles :

- 4 populations en microtubes
- 4 populations en tubes 5ml
- 2 populations en tubes 15 ml

Privilégier le recueil en **microtubes** si le nombre de cellules recueilli est inférieur à **100 000 cellules.** Pour des populations cibles allant de 100 000 à 2 000 000 cellules, choisissez les tubes de 5ml. Pour des populations supérieures à 2 000 000, optez pour les tubes de 15ml.

- **Précoater les tubes** au préalable avec le milieu de recueil désiré pendant au moins 15 minutes

- **Utiliser des tubes en polypropylène** et non en polystyrène (tubes 5ml stérile avec bouchon : Falcon Ref 352063 / tubes 5ml stérile sans bouchon : Falcon Ref 352053)

Température des échantillons

Pour préserver l'intégrité des cellules, apporter et garder vos échantillons dans la glace.

Contrôles

N'oubliez pas :

- d'**inclure un marqueur de** viabilité dans votre panel !
- d'**apporter**, en plus de vos échantillons :
  - un tube de **cellules non marquées** pour paramétrer les PMT, le FSC et SCC
  - les différents **monomarquages** pour les réaliser les compensations : si vous ne disposez pas d'un nombre suffisant de cellules, utiliser des billes de compensation
  - les **FMOs (Fluorescence Minus One)** : contrôle de gating pour les marqueurs les plus sensibles.

How Cell Culture Medium Can Decrease Cell Viability During A Flow Cytometry Cell Sorting Experiment

1. Cell culture medium is not a good choice.

Most cell culture media are formulated with a CO_2-carbonate buffering system that is optimized for tissue culture carbon dioxide partial pressure, which is higher than the typical ambient carbon dioxide partial pressure. When these kinds of buffers are left in typical atmospheric conditions, **carbon dioxide will evaporate from the medium and cause the pH of the medium to rise into the alkaline range** (in phenol red-containing formulations, the color will become more purple), which expectedly can significantly affect viability. While tissue culture medium is often used as a suspension buffer without deleterious effect, **longer cell sorts and the sensitivity of the cell type can certainly exacerbate the effect of pH on viability.** Additionally, although it's important to formulate the buffer with some kind of protein, tissue culture media may contain serum at concentrations of upwards of 10-15%, which can be deposited over time on the sample line and cause flow rate irregularities during the sort. If tissue culture medium must be used**, a HEPES-based buffering system**, which is not dependent on carbon dioxide, is a preferable and safer choice.

2. Consider adding protein to your buffer.

PBS or HBSS can work well as a base for the suspension fluid, but you should add some kind of protein, typically 1-2% BSA or FBS, to keep the cells happy. Incidentally, HBSS is formulated with a CO_2-carboante buffering system, but this system is meant to work at atmospheric/ambient CO_2 partial pressure rather than tissue-culture CO_2 partial pressure.

3. Add EDTA to prevent clumping.

EDTA in the suspension buffer, at 1-5 mM, can be a panacea for samples that tend to form aggregates and clog the instrument. This molecule interferes with cell-cell adhesion molecules by sequestering the divalent cations that these molecules require to function. Therefore, it can greatly reduce the incidence of clumping in the sample. Preventing clogs has an indirect effect on viability—clogs cause downtime during the sort, requiring cells to remain in suboptimal conditions for longer than necessary. The faster the cells are manipulated, the lower the likelihood that they will succumb to apoptosis or necrosis. If you will be using DNAse , then EGTA is a better choice for chelation to prevent clumping. EDTA will chelate Mg^{2+} ions, which are necessary for DNAse, while EGTA has a much lower affinity for Mg^{2+}.

4. Add DNAse to deal with dead cells.

Certain kinds of samples, especially those harvested from some kinds of solid tissues, will inevitably contain a high proportion of dead cells. **If this is the case, it may be advantageous to add DNAse to the suspension buffer used for the sorting process.** Dead cells will release genomic DNA into the buffer, which tends to aggregate and form clumps. These clumps can trap cells, cause clogs and result in an overall drop in recovery after the sort. Recommended concentrations vary, but 200 µg/mL has been reported in Current Protocols In Cytometry to be effective. Alternatively, debris from dead cells can be removed during the preparation either through centrifugation gradients or depletion via magnetic separation.

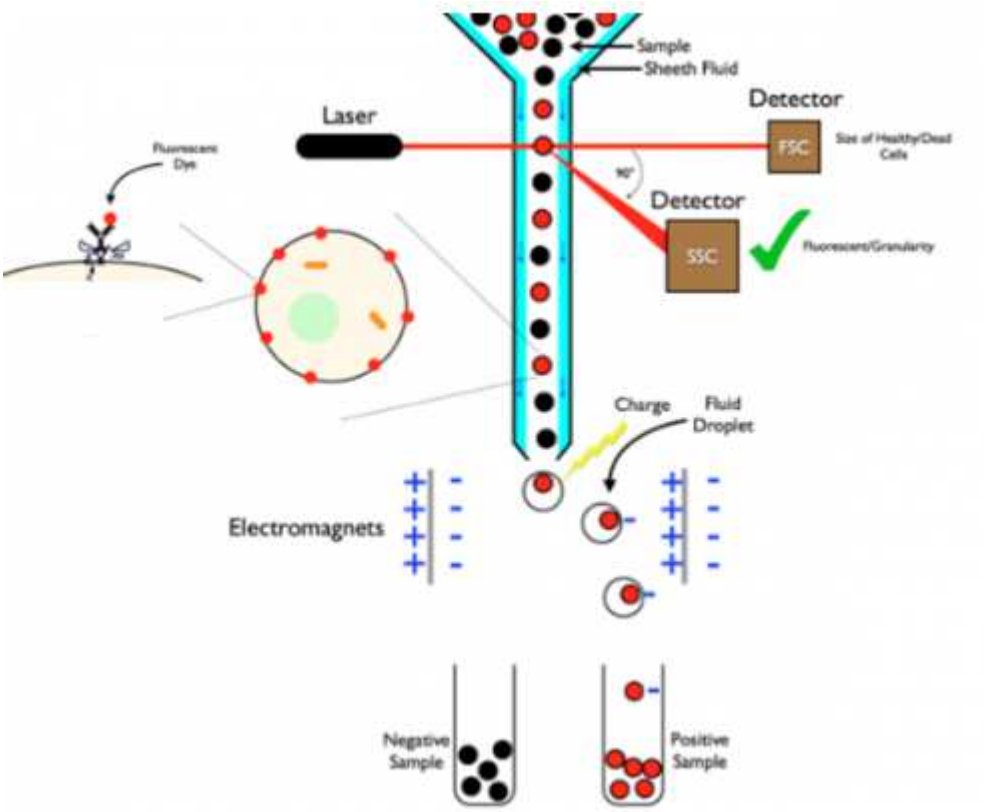
5. Keep everything at the right temperature.

The temperature at which the sample is maintained during preparation, staining, and sorting is another important parameter to keep cells alive. The proper temperature strongly depends on the cell type and application.

While many researchers have success preparing and sorting cells at temperatures around 0°C, this may not be the ideal temperature for every cell type.
Nevertheless, there are some general benefits for keeping the cells on ice. Most importantly, a low temperature will slow down metabolic activities and will prevent biochemical or molecular changes that may affect cytometric or downstream results.

For example, some cells, under certain conditions, **may shed or internalize antibody-bound cell surface receptors, resulting in diminished staining and resolution on the cytometer.** Keeping cells cold can significantly reduce this effect.
Additionally, under the stress of preparation, staining, and the sorting process, some cells may undergo molecular changes—gene expression or otherwise—that could affect downstream experiments or analyses. **Keeping cells cold will slow down activity and possibly mitigate any transcriptional effects brought about by the experiment.**
Finally, stressed cells may begin to apoptose, lowering your recovery. By keeping your cells cold, you may slow down or mitigate this process so they can be deposited in the collection buffer before progressing further through this pathway.
When designing your flow cytometry experiment, check the literature to see if any particular suspension buffer and temperature conditions have been successful historically in other protocols for the particular cell type you are working. Buffer with protein, EDTA, and DNAse is often a better choice than using your cell culture medium alone. In terms of temperature—cold, usually 0°C, is a good place to start, but temperature can be surprisingly cell-type dependent, so make sure you do your research.

10 Things Smart Scientists Do Before Sorting Cells



Cell sorting can be a scary proposition. A precious sample is introduced into a machine that pressurizes the cells to 70 PSI, moves them past one or more lasers, vibrates the stream at 90 kHz before decelerating the cells to atmospheric pressure before they hit an aqueous surface. Many cells survive this journey. But some do not.

Here are 10 things smart scientists do to improve their cell recovery :

1. They pre-coat their catch tubes.

A smart way to improve your cell recovery is **to incubate your plastic tubes with a buffer solution containing protein**. This will help reduce/eliminate the charge on the plastic.

Since the droplet containing the cell is charged, it can be attracted to the charge on the plastic. This results in the droplet hitting the side of the tube wall, and the cell dying as the small volume of liquid evaporates.

To prevent this, pre-coat the tube with protein/buffer to neutralize the plastic charge. Even better, make sure that **your tubes are not polystyrene.**

2. They know the catch buffer.

Cells are going to be traveling in a buffered saline. This is not very conducive for keeping cells alive for long periods of time. The good news is that you can improve your recovering by ensuring that the catch buffer has some – but not too much – protein in it. Typically only 10-50% protein in the catch buffer is sufficient.

3. They add HEPES.

If you're sorting into media, make sure the media is HEPES buffered. Buffers like RPMI are formulated to buffer in a CO2 atmosphere (like the atmosphere found in your lab's incubator) and, as such, don't buffer well in our normal atmosphere.

4. They keep them cold (or warm).

Smart scientists know how their cells respond to temperature differences. Some cells do not like to be kept cold and will die quickly if sorted into 4 degree Celsius buffer.

5. They use a soybean trypsin inhibitor.

Sorting adherent cells adds a level of complexity to an experiment. The cells have to be disassociated to pass through the sorter, and this is often done with trypsin. The quickest and most common neutralization method is to add FBS to the cells.

Be careful of this – while it neutralizes the trypsin effectively, it also adds back all the components that cells need to re-adhere to each other. Try soybean trypsin inhibitor instead.

6. They filter.

Nothing is worse than a clogged nozzle when sorting. It adds time to the sort and reduces efficiency (and annoys the sort operator).

Just before sorting, make sure to pass the cells through an appropriate sized filter to remove clumps and debris. The smartest scientists go as far as looking at their cells under a microscope to ensure that there are no clumps prior to sorting.

7. They use a viability dye.

Make sure to include a viability dye in your staining panel. This will help eliminate dead cells.

Using a viability dye is always a smart decision.

8. They design proper antibody panels.

When trying to define a cell population, make sure you include both positive and negative markers in your antibody panel. The use of dump channels, negative markers and multiple positive markers will help ensure that the sorted cells are what they are supposed to be.

9. They ALWAYS count their cells.

Know the cell count at the time the cells are going onto the sorter – NOT from when you first began preparing them.

Since an optimal sort speed is typically ¼ the droplet generation frequency, over concentrating the cells will reduce purity at the back end. Bring some dilution buffer with you just in case the cells are too concentrated.

10. They are aware of their threshold settings.

The higher the threshold, the easier it is to visualize the specific cell population. But this doesn't eliminate the fact that the debris and junk are still present within the cell population you're visualizing. It means that the cytometer is ignoring it.

Here's the key: whatever the cytometer ignores will end up in the final sorted population.