Chapter 7

Ensuring Quality Cell Input for Single Cell Sequencing Experiments by Viability and Singlet Enrichment Using Cell Sorting

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Abstract

As with every sensitive analysis technology, the golden principle of "input quality rules output quality" also applies to single cell sequencing methods. Given the sensitivity of the current methods in single cell sequencing and the minuscule amounts of RNA present within a single cell, any extrinsic source of variability should be reduced by ensuring a homogenous input right at the start. Not every tissue is as readily handled as a single cell suspension like blood and most tissues will have to undergo digestions to free the cells from their spatial organization to undergo single cell transcriptomics workflows. This chapter provides working protocols for two simple, but very precise and powerful methods to ensure only the most viable cells are introduced into single cell assays.

Key words Single cell RNA sequencing, Cell sorting, Apoptosis, Metabolic labeling, Cell viability, Enrichment

1 Introduction

The key part of any nonspatially resolved single cell sequencing experiment is to obtain cells as single cell suspension. It does not matter if the cells are destined to undergo 10×Genomics [1] or BD-Rhapsody [2] or traditional SmartSeq2 [3] and MARseq [4], for example. Whenever a tissue is digested using enzymatic and chemically assisted tissue dissociation protocols, many cells will be physical destroyed, partially damaged, or might undergo triggering of apoptosis since they are not anymore residing in a solid tissue. Even the best protocol and experienced laboratory technician will never be able to fully avoid this loss. Therefore, it is advisable to use readily available methods to remove debris, dying or dead cells from the feed suspension that is destined to be introduced into a single cell sequencing experiment. Depending on the type of downstream analysis, even debris containing residual RNAs could be a source of

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noise within a single cell RNA-seq. Of course, there are methods utilizing computational removal of "nonconforming cells" after the wet laboratory work has been completed. The methods rely on assumptions that the increase in the occurrence of certain markers, like mitochondrial RNAs for example, represents a hallmark of an apoptotic or dead cell [5]. Although there is usability of such filters, they are not perfect and should be only used in combination with proper biochemical staining and cell purification methods [6].

As a matter of fact, the difference in the lipid components of the 10×Genomics and BD-Rhapsody lysis buffers tends to show a higher release of mitochondrial RNAs paired with an improved mRNA detection in BD-Rhapsody runs, although the cells were of fantastic viability just prior to the lysis. Were the cells less viable? No, because metabolic viability staining revealed their health. One has to be very careful when relying only on post-assay filtering tools if one is to shy of investing the work of enriching viable cells for single cell RNA-seq studies.

Cell sorting today is a readily available technology allowing for simple purification of cells. Almost every available sorter will be able to perform very simple two-parametric viability cell sorts based on the presence of active Caspase-3/7 probes or the metabolic viability stain Calcein-AM (7) in combination with either PI or DAPI. These two color viability assays will be able to provide a very broad range of usability across many model organisms and cell types.

2 Materials

2.1 Reagent and Buffers

1. The best Caspase-3/7 probes are sold by Sartorius, called Incucyte Caspase 3/7 dyes, either as a blue laser (488 nm) excitable green label (FITC or GFP filter, 530-30 nm bandpass) or as red laser (640 nm) red label (APC or AlexaFluor 647 filter, 670-14 nm bandpass). Catalog numbers are 4440 or 4704, respectively (https://www.sartorius.com/en/applications/life-science-research/cell-analysis/live-cell-assays).
2. The most suitable Calcein probes can be obtained from Invitrogen under the CellTrace Calcein AM label either as a blue laser (488 nm) excitable green label (FITC or GFP filter, 530-30 nm bandpass) or as a violet laser (405 nm) blue label (V450 or BV421 filter, 450-40 nm bandpass). Catalog numbers are C34852 or C34858, respectively.

3. DAPI can be obtained from any source as it is a simple compound. It should be carefully diluted to a 1 mM stock solution. The final concentration needs to be tested but should be around 0.5–2 μM. Sometimes it can be even further diluted.
4. Use 5% FBS or 1% BSA in PBS, pH 7.4, supplemented with 5 mM EDTA as cell suspension buffer for all stains.

2.2 Cell Sorter

Any cell sorter providing a 405 nm, 488 nm, and 640 nm laser excitation will be suitable to enable the entire bandwidth of dye combinations. Typical cell sorters are BD ARIA-type cell sorters, SONY SH800's, BDMelody, Beckman Astrios, or MoFlo. A large nozzle should be used, typically 100 μm or 130 μm, to enhance post-sort viability. The lower the pressure used, the better for the cells and the downstream assay quality.

It is advisable to refer to spectral viewers to find the best possible combination according to the experimental conditions (presence of fluorescent proteins, etc.) and the available cell sorter. Generally, if a GFP stain is present in any of the cells of interest, one should use a red Caspase 3/7 or violet Calcein probe combined with PI as live-dead counter stain. If Calcein violet is not an option, then the Calcein violet channel can be used for DAPI live-dead counter staining.

3 Methods

3.1 Caspase-Based Viability Enrichment

Tissue or cells should be dissociated with the most suitable method to obtain a suspension containing as many as possible single cells. The suspension should contain 0.5–1.0 million cells per ml using the 1% FBS, 0.5 mM EDTA PBS buffer. The cells can be stored on ice or at RT depending on their sensitivity (must be explored independently). As a typical, 10×Genomics or BD-Rhapsody experiment only utilizes 10,000–50,000 cells per reaction, one will not need to purify more than 1/2 million cells if viabilities are within 20–30% of all cells.

Add 0.5 μl per ml cell suspension of the Incucyte Caspase 3/7 stain and carefully mix the suspension. Incubate the cells for 5–10 min either on ice or at RT and then add the necessary amount of either PI or DAPI live-dead counter stain to obtain 1 μg/ml PI or 0.5–2 μM DAPI staining concentration. PI and DAPI stainings are very rapid and can be analyzed within 2–3 min after staining.

Load the sample on to the cell sorter and establish proper FSC/SSC gating to select singlet cells—any experience or otherwise trained cell sorter operator will be able to assist you with this step. On the final bivariate dot plot displaying live-dead versus Caspase 3/7 signals (see Fig. 1), draw a final sorting gate around the double negative population. This population represents the

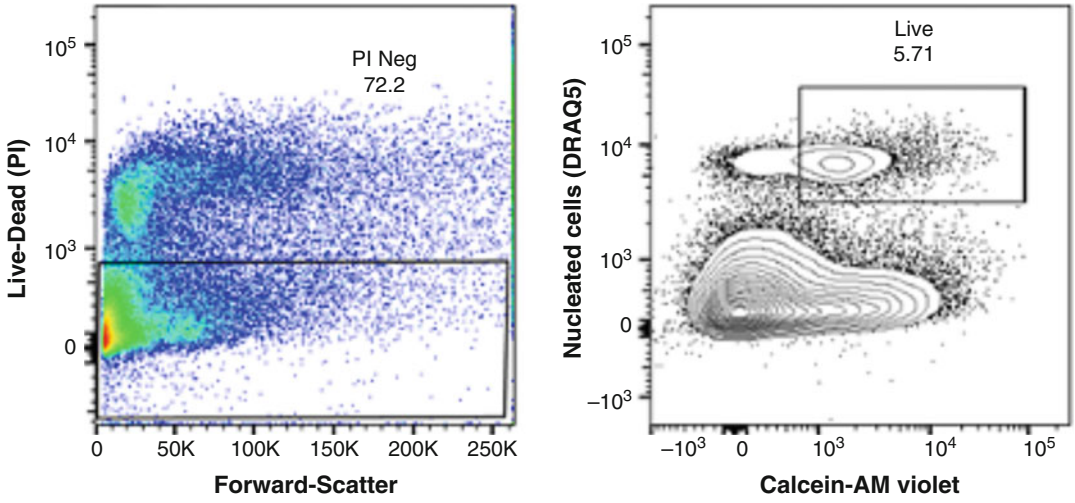

Fig. 1 Bivariate dot plot: live-dead versus Caspase 3/7 signals. Cells are gated first against the live-dead marker PI and then further analyzed on a plot displaying the Calcein-AM. Violet signal indicates health and the presence of strong signal from the viable DNA label DRAQ5 indicates proper cells (*see* **Note 2**). Viable cells are identified by a strong Calcein and DRAQ5 signal

healthiest single cells that will hopefully provide you with the best cellular material for the single cell RNA-seq experiment. The cells should be sorted into a tube containing 300 µl of the 1% FBS, 5 mM EDTA PBS buffer—preferably chilled at 4 °C (*see* **Note 1**).

3.2 Calcein Metabolic Labeling Enrichment

Using Calcein as metabolic viability indicator requires incubation of the cells with the stain for 10–20 min at RT or even 37 °C. Calcein-AM is converted by esterases to be freed from the lipid modification it carries to get across membranes. Once it is cleaved, Calcein is membrane impermeable, and it is retained inside healthy living cells with active esterases. In comparison with the Caspase 3/7 labels, the live cells will have a bright signal when probed with Calcein.

The procedure itself is simple and straightforward: bring your cells into single cell suspension, spin them down using 5 min 90xrcf, and take up in 1 ml 1% FBS, 0.5 mM EDTA PBS buffer. The Calcein-AM substrate is generally dissolved in DMSO and stored as 1 mM stock solution if not otherwise specified by the supplying company. Add 1–2 µl of the stock into the 1 ml of cell suspension and incubate the cells at RT or even better 37 °C for 10 min. If your cells are sensitive to 37 °C incubation, revert to RT and optimize the duration of the incubation. Please note that 4 °C incubation will significantly slow the reaction and you will see very little signal. Should there be a lot of background signal from Calcein, for example, a lack for good separation of low/unstained cells from bright cells, one can implement a washing step prior to sorting to reduce the amount of free Calcein that could be bound to the outside of cells or debris.

Load the sample on to the cell sorter and establish proper FSC/SSC gating to select singlet cells—any experience or otherwise trained cell sorter operator will be able to assist you with this step (*see* **Note 2**). On the final bivariate dot plot displaying live-dead versus Calcein signals. Be mindful to gate the brightest Calcein-labeled population that is negative for the live-dead counter stain (see Fig. 1) (*see* **Note 3**).

4 Notes

1. After the sort, when the cells need to be spun down to remove the EDTA (it has to be removed to enable proper functionality of 10×Genomics or BD-Rhapsody assays), one should only spin with 90-rcf and rather extend the time to pellet the cells. Using classic polypropylene 5 ml FACS tubes paired with swingout rotors will provide the best results with the supernatant is carefully decanted. After spinning and washing the cells, the cell suspension should be immediately introduced into the single cell sequencing assay. In case of plate-based single cell methods, the sorting step is part of the plate preparation if single cell sorting is used.
2. If you deal with very-difficult-to-gate cells because of large amounts of debris, for example, then it is advisable to change the suggested simple setup and include the membrane-permeable DNA stain DRAQ5 (best obtained by BioStatus, http://www.biostatus.com/DRAQ5/). Particles with a bright DRAQ5 signal (640 nm excitation, 670/14 emission) will be particles containing high amounts of DNA, which should be the cells. Low signal for the live-dead dye and corresponding signal for the apoptosis or metabolic label will provide additional security to select the best cells (for more on this, [7]).
3. 96, 384, or 1536 plates should be immediately shock-frosted after completion of the sort using a mixture of dry-ice and 80% EtOH. Be careful to always wear proper PPE.

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