Single Cell Transcriptomics & Genomics - Sample Preparation

We are in the midst of a revolution - the combination of the ability to sort single cells and then analyse them for their mRNA expression (a.k.a. scRNAseq, transcriptomics), genome-level traits (e.g. Genome-Wide Association Studies, GWAS) or even proteomics analysis.

As ever sample preparation is key, yet the considerations for the two major components of the process are very different and that has led to concerns over quality assurance of cell delivery for downstream analysis. These concerns have been examined in detail in a paper by scientists at EMBL and usefully focuses on the fundamentals to deliver the correct individual cell into a well.

Cell event detection & singlet selection

It is evident that one needs to achieve the sorting of only singlet cell events, fundamentally by excluding doublets which might, after all, be two different cell types (depending on the complexity of the phenotypic parameters used). This is relatively facile for a sample such as whole peripheral blood where small cells and debris can be excluded based on flow cytometric scatter properties (with or without RBC lysis) and the subsequent doublet exclusion reliably achieved based on forward (FSC) and side scatter (SSC) pulse-shape analysis for cells of a limited shape and size distribution.

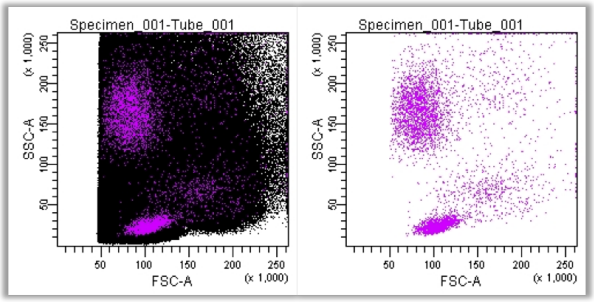

Figure 1. Whole human bone marrow aspirate stained with DRAQ5™ for exclusion of enucleated cells and debris from occluding analysis, while retaining expected scatter properties.

However, this becomes much more troublesome when considering complex samples with widely varying cell shapes and sizes as in the case of tissue digests. Here, the attempt to exclude doublets stringently for one population could bias the exclusion of other singlet cells of different physical properties. To overcome these limitations Ordoñez-Rueda et al. chose to rely more directly upon the DNA content of cells. They used DRAQ5™, the far-red cell permeant DNA dye, to achieve this and thereby unify cell event detection and singlet selection without introduction of the unwanted scatter bias, taking advantage of the well-practiced routines for cell cycle analysis. An example of DRAQ5™ stained nucleated cells being resolved from a complex sample matrix (whole bone marrow) is shown in figure 1.

Interestingly, the DRAQ5™ DNA content signal was used to establish that sorting the forward scatter property alone was sufficient to discriminate cardiomyocytes from non-cardiomyocytes and debris (Preissl 2015).

Importantly, it is well-established that DRAQ5™ has no impact on the downstream enzymatic processes such as first strand cDNA extension (reverse transcriptase) and PCR (Taq polymerase). In ground-breaking experiments by Douwes Dekker et al, 2004, cells were sorted on the basis of their DNA cell cycle position by virtue of DRAQ5's stoichiometric dsDNA staining and surface phenotype (using the FACSVantage flow-sorter, BD Biosciences).

Notwithstanding the considerations dealt with above, it is helpful to have examined a small aliquot of cells microscopically to have a physical picture of the sample and its quality with respect to cell sorting. Cell sorting works best at low pressure, meaning a relatively low event rate, so it is important to have an approximation of

the target population (% of total) and the required number of cell events, per well and in total. Efficient design of sample workflow on either side of the cell sorting will aid the quality of the precious RNA templates, including temperature control and avoiding unnecessary temperature and buffer changes.

Transcript expression & quality

One needs to consider the impact of dead/damaged cells on the quality of DNA or RNA sequences for the molecular analysis. Many authors report the use of a viability dye for the exclusion of the leaky dead and damaged cells from a sort's positive criteria. The far-red viability dye DRAQ7™ has become a popular choice in this respect due to its spectral compatibility with multi-parameter "index" cell sorting and its capacity to be used for extended culture without any general toxicity. Further, there is no observable impact on downstream enzymatic processes. In support of this, DRAQ7™ has been widely used in time-lapse assays where cells are continuously exposed to DRAQ7™ for many days with no impact when compared to negative controls.

This use of DRAQ7™ has been the subject of a separate white paper: Application of DRAQ™ dyes in single cell transcriptomics and genomics - White Paper (PDF).

A recent protocol describing hardware, reagents and procedures for gene expression studies published in Bio-101 (Ji et al., 2019) combines DRAQ7™ and Calcein AM to give further definition to the dead and viable cell fractions on the BD Rhapsody™ Single-Cell Analysis System (BD Biosciences). However, this has the impact of denying the use of the FITC/Alexafluor 488 channel when it would appear the reporting of the DRAQ7™+ fraction should be sufficiently robust to allow reliable removal of dead cells into the dump channel.

In their work Ordoñez-Rueda et al., using the BD FACSAria™ Fusion cell sorter (BD Biosciences), combine DRAQ5™ for the cell event detection/singlet selection and a spectrally compatible viability dye, DAPI, to exclude the dead cells while still allowing spectral space for a number of chromophores to be applied for the phenotypic cell sorting criteria. One could envisage a number of options for this: DRAQ5™/DAPI, Hoechst 33342/DRAQ7™, CyTRAK Orange™/DRAQ7™ - all combinations that retain the ability to create a panel of fluorescently-tagged phenotypic markers to identify and sort the cells based on surface phenotype.

In one such example Klingler et al (2018) showed intracortically-projecting neurons (ICPN) have transcriptional identities primarily in accord with input-output relationships rather than time of origin or laminar position. It appears there are conserved circuit-related transcriptional programs across cortical layers and that these may be maintained circuit features through development and evolution, conclusions highly dependent upon performance of scRNAseq. For single cell sorting and the downstream RNA-sequencing, fresh tissue was micro-dissected from regions of interest, digested and sieved. Singlet cells, positive for the cell permeant DNA probe Hoechst 33342 and negative for DRAQ7™, were sorted via the Moflo Astrios cell sorter (Beckman Coulter) for onward cDNA synthesis. An example of DRAQ7™/Hoechst 33342 stained cells is shown in fig.2.

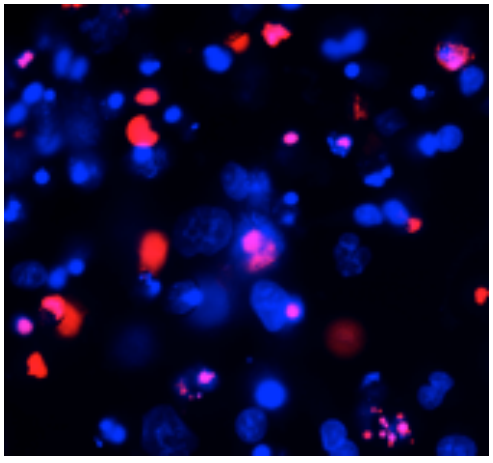

Figure 2. HepG2 cells stained with DRAQ7™ (red; dead/damaged cells) & Hoechst 33342 (blue; all cells). Courtesy of Imagen Therapeutics.

The apoptotic cell fraction

Despite this efficient exclusion of dead cells, there is potentially an additional population of cells which are intact (therefore negative for a viability dye) but which have altered gene expression as a consequence of their pre-apoptotic state. Were this to be a significant fraction of cells it would bias the expression results. Ordoñez-Rueda et al. propose that, at the very least, the presence of these cells should be investigated and perhaps excluded on the basis of caspase expression or membrane inversion (detected by Annexin V binding to externalised phosphatidylserine residues). They do indeed show that these cells have a differential expression compared to healthy non-apoptotic cells. The presence of significant numbers of dead, damaged or apoptotic cells may necessitate modification of cell harvesting procedures to increase the yield of cells at closest to native state.

Summary

It should be borne in mind that these stringencies may not be required long-term but foreknowledge of the requirements for the sort appear to be critical. In one interesting example of this, as mentioned earlier, Preissl et al., 2015, established that forward scatter was sufficient to discriminate cardiomyocytes from the significant quantity of debris and other cells (from the tissue disaggregation and digest) based on DRAQ5™ staining for DNA content. From this, in routine practice they were able to effectively sort primary rat cardiomyocytes from a tissue digest with only the use of DRAQ7™ to exclude dead/damaged cells, using the S3 Cell Sorter (Bio-Rad).

Best practice in both the preparation of the cells and the subsequent cell sorting will improve the quality and validity of the molecular analysis with the greatest cost-efficiency.

At the very least, the routine addition of a viability dye would seem to be essential: far-red DRAQ7™ has proven performance in this respect being widely cited, with spectral compatibility in multi-colour antibody panels and also available in a convenient dropper bottle (DROP & GO™) presentation.

References:

Douwes Dekker, Pieter B., et al. "Multiparameter DNA flow-sorting demonstrates diploidy and SDHD wild-type gene retention in the sustentacular cell compartment of head and neck paragangliomas: chief cells are the only neoplastic component." The Journal of Pathology 202.4 (2004): 456-462.
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Ji, X., et al. "Protein- and Sequencing-based Massively Parallel Single-cell Approaches to Gene Expression Profiling". Bio-101 (2019): e3161
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https://doi.org/10.1002/cyto.a.23898

Preissl et al. "Deciphering the Epigenetic Code of Cardiac Myocyte Transcription." Circulation Research. 2015;117:413–423 (in supplemental data: Online figure 1)
https://www.ahajournals.org/doi/suppl/10.1161/CIRCRESAHA.115.306337

Further reading:

"Application of DRAQ™ dyes in single cell transcriptomics and genomics". BioStatus white paper, March 2018

Technical datasheets and other key documents can be found on the dedicated product pages e.g. www.biostatus.com/draq7/

You can read independent product reviews on DRAQ7™, moderated by SelectScience.

If you would like to know more about any BioStatus product get in touch..

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