Application of DRAQ™ dyes in single cell transcriptomics and genomics

Recent advances in transcriptomics and genomics enable detailed analysis of single cells and nuclei for both DNA and RNA. One key process is sorting of target cells or nuclei as either single events per well or as aggregated phenotypes. The technology of choice for this is fluorescence-activated cell sorting (FACS) or, for nuclei, FANS. This takes advantage of, for example, surface phenotypic expression patterns, fluorescent protein (FP) reporter, DNA content or cellular features (using forward and side scatter) as determined by flow cytometric analysis.

The far-red DNA binding anthraquinone dyes DRAQ5™ (cell membrane permeant) and DRAQ7™ (cell membrane impermeant) can be applied to assist in these cell preparations. In the case of DRAQ5™, the labelling of nuclear genomic DNA (gDNA) enables the clear and simple segmentation of 'singlet', intact nucleated cells from doublets or clumps, enucleated cells (e.g. RBCs, platelets) or cellular debris. Further, stoichiometric labelling of gDNA permits analysis or sorting of cells according to DNA content, as a function of cell cycle position or proliferation. The far-red DRAQ5™-derived data can be readily combined with surface marker(s) or FPs. The related DNA binding viability dye DRAQ7™ only enters a permeabilized cell thereby, based on a positive far-red fluorescence signature in dead / damaged cells, enabling their facile exclusion from a desired population being sorted for downstream investigation (as shown in figure 1).

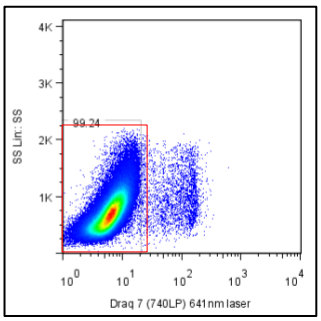

Fig. 1 Haploid ESC sort. DRAQ7™ exclusion of dead cells. ‡

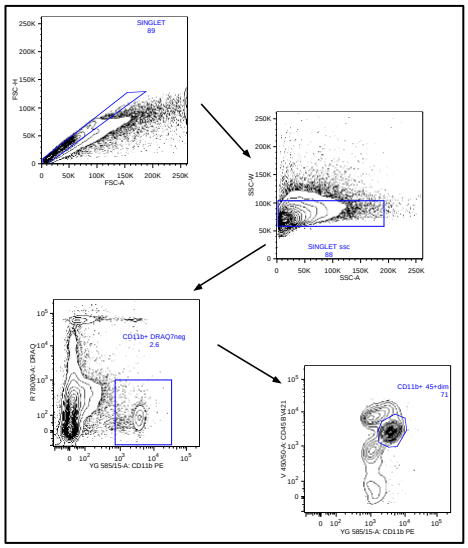

Fig. 2 Gating strategy to sort purified microglia with DRAQ7™ dead cell exclusion. ‡‡

However, one reasonable concern regarding the use of these DNA binding dyes is any effect on Taq Polymerase (Taq) or reverse transcriptase (RT), for first strand cDNA synthesis. This is dealt with here.

For Taq, Douwes Dekker, et al. (2004) used DRAQ5™ to achieve cell cycle- and S100 surface expression- dependent FACS for PCR mutation detection. Accordingly, all cells and their gDNA were labelled with DRAQ5™ to generate a fluorescent signal stoichiometric to the cell cycle position i.e. G2/M cells have twice the DRAQ5™ staining of G1/0 cells. The sorted cells were successfully subjected to PCR and the disease-related gene mutation of interest detected, according to cell cycle position and S100 expression. This shows Taq was unaffected by DRAQ5™ at concentrations required to achieve stoichiometric labelling.

Subsequently, Cai, et al. (2014) used DRAQ5™ to enable selection of healthy B-lymphoblast cells sorted singly into PCR wells as a point of reference for investigation of copy number variation (CNV) by whole genome analysis (WGA) in healthy adult and diseased pediatric neuronal nuclei, all isolated from tissue samples. In a later CNV study in malignant mesothelioma by Beije, et al. (2017) pleural effusions were stained with DRAQ5™, anti-CD45 and anti-MCAM. The different sorted cell populations were then subjected to PCR and SNP analysis.

Ref: DRX.WHP 002 280318

Meanwhile Lam, et al. (2017) utilised RT-PCR on DRAQ5™ stained GFP-expressing POMC neurons for transcriptomic studies. Cells were harvested from fresh GFP mouse hypothalamus tissue digest (papain, DNase) and filtered to recover a single cell suspension. Cells were sorted on the basis of size (FSC), granularity (SSC), FSC pulse-width for singlets, GFP for POMC neurones and DRAQ5™ to identify intact euploid nucleated events directly into lysis buffer (with RNase inhibitor) in microtitre plate wells for 1st strand cDNA synthesis by RT and amplification for RNA sequencing (RNA-seq).

Similarly, Beetz et al. (2016) and Lother, et al. (2017) sorted endothelial cells for RNA-seq based on DRAQ5™+, CD45- and, respectively, GSL-1+ or GFP+.

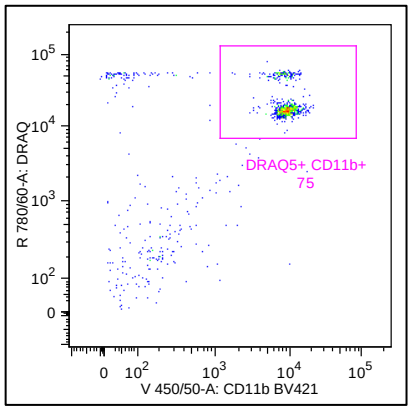

Fig. 3 Verification of sorted microglia purity, determined by CD11b⁺/DRAQ5™⁺ status. ‡‡

Isolated nuclei have been the target for FANS where DRAQ7™ has been used to positively label nuclei as a trigger for the sort. Examples include cardiomyocyte nuclei: Nothjunge, et al. (2017) for RNA-seq and Gilsbach, et al. (2018) for RNA-seq, ChIP-seq and mCPG-seq and for forebrain nuclei: Preissl, et al. (2018) for accessible chromatin genome analysis to identify a large number of apparently different cell types in the forebrain.

Lastly, in sorting of viable cells, DRAQ7™ has been used in its conventional way as a so-called viability dye to exclude or "dump" dead/damaged cells (i.e. the DRAQ7™+ events). By way of background, DRAQ7™ has been widely demonstrated as an *in situ* monitor of cell health in long-term/time-lapse cell-based assays where it has shown no deleterious effects on controls, as it does not cross intact plasma membranes. (Detailed in another white paper).

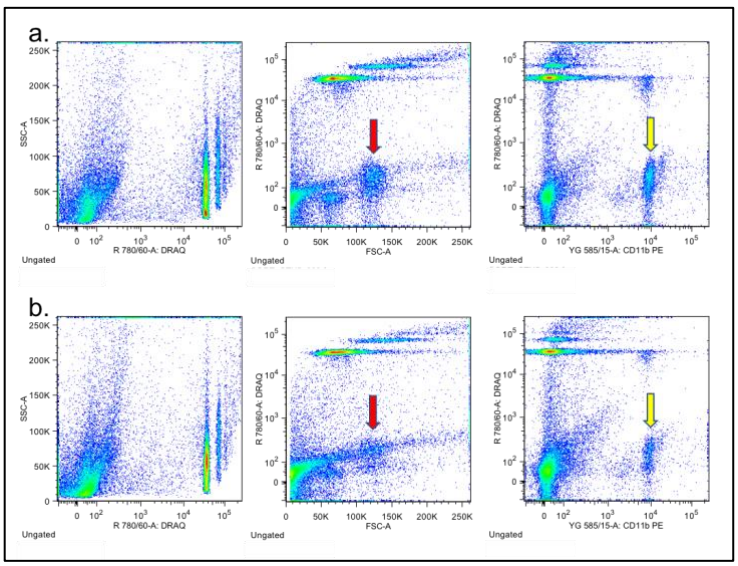

Fig. 4 Comparison of microglia definition using either FSC-A vs DRAQ7™ (red arrows) or CD11b⁺ vs DRAQ7™⁺ (yellow arrows) gating. All data ungated. ‡‡

Preissl, et al. (2015) combined high forward scatter (pre-determined by DRAQ5™ vs. FSC analysis) and DRAQ7™ negative staining to select for viable cardiomyocytes from heart perfusion for RNA-seq, ChIP-seq and mCPG-seq.

This early work confirmed that the stain index for DRAQ7™ was at least ca. 25-30, meaning that there is excellent separation between live and dead cells. In fascinating studies on the conversion of fibroblasts to cortical neurons Miskinyte, et al. (2017) performed sorts for qPCR while Hurni, et al. (2017) sorted neurons for RNA-seq to aid understanding of cell migration in the developing cortex, both directing DRAQ7™ positive events to the dump channel.

Most recently, Spiller, et al. (2018) sorted microglia based on the following gating strategy: SSC-A v FSC-A, DRAQ7™ v FSC-A (live), FSC-W v FSC-A (single cells) then CD45 v CD11b to provide DRAQ7™-, CD45-, CD11b+ cells for RNA-seq to establish the protective impact of reactive microglia in an ALS model. An example of such a microglia sort is shown in figure 2, using an alternative gating logic*.

In a simple yet elegant addition to this, an aliquot of sorted cells from fig. 2 was stained with DRAQ5™ to assist purity estimation for the sort, shown in figure 3 (and as similarly applied by Spiller, et al.).

Care should be taken to establish parameters for exclusion of doublets, as appropriate to the cell sorter being employed. *Similarly, gating rationales can markedly impact on the ability to define and therefore sort desired populations. In figure 4 $CD11b^{+}$ microglia are much better defined by a CD11b v DRAQ7™ gate (right-hand panels) than by a FSC-A v DRAQ7™ gate (centre panels) in two consecutive sorts. This is evident in sample b. where, marked by red and yellow arrows respectively, definition of a microglia population is improved by CD11b v DRAQ7™ gating.

In summary, the addition of DRAQ5™ or DRAQ7™ to single cell genomics and transcriptomics is a proven means of achieving desired template quality avoiding the risk, for example, of unwanted background from fragmented RNA transcripts or enabling selection of cells on the basis of DNA content, or simply rationalising sample preparation regimes. Evidently, from the significant body of literature provided DRAQ5™ and DRAQ7™ do not interfere with the downstream PCR and RT-PCR procedures required for these techniques.

A number of resources are available to assist in setting up tissue disaggregation for single cell sorting and best practice regarding the integrity of the genomic and RNA transcripts in these complex workflows. Selected examples of these are appended to the bibliography. Not least, it will be helpful to consult with your FACS core facility colleagues.

Acknowledgements

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