**Measuring the action of oligonucleotide therapeutics in the lung at the cell type specific level by Tissue Disruption and Cell Sorting (TDCS).**

Running title: *In vivo* cell type-specific oligonucleotide PK/PD

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1. **Abstract**

The clinical potential of DNA and RNA-targeting therapeutics for airways disease has been hampered by the poor translation of promising drug candidates from cell culture to *in vivo* models and the clinic. For example, classical pre-clinical approaches routinely report 20-60% target knockdown effects in the lung, where 1 or 2 log effects are observed in isolated cell cultures *in vitro.* Preparation of monocellular suspensions of Tissues by mechanoenzymatic Disruption followed by Cell Sorting (TDCS) after *in vivo* drug dosing, however, can offer pharmacokinetic and pharmacodynamic insights on the effects of drugs to precise cell subpopulations. Moreover, this can be reliably achieved with up to 66% fewer animals than standard *in vivo* pharmacology approaches due to lower data variance afforded through analytics on defined, viable cell numbers. Here we describe the TDCS methodology for the isolation of total lung epithelia, lung macrophages, and epithelium/macrophage-depleted cell fractions from mouse lungs using a two-stage sorting process of immunomagnetic bead separation followed by flow cytometric sorting using fluorescent antibodies against well-established surface markers such as F4/80, CD11b, and CD326. Validated antibodies for additional cell types and markers are also provided.

**Keywords:** Lung, cell type specific *in vivo* pharmacology, oligonucleotides, fluorescence activated cell sorting, magnetic cell sorting, RNAi, siRNA, antisense, microRNA.

**1. Introduction**

Pharmacokinetic and pharmacodynamic assessment of oligonucleotide and other DNA or RNA-targeting therapeutics *in vivo* typically involves bioanalytical procedures carried out at tissue level. After following standardised procedures on animal models of disease, animals are terminally anaesthetised at set timepoints, tissues are excised, and homogenised, to extract and purify appropriate protein, DNA or RNA (1). Analytes are then processed for the measurement of specific gene/protein expression or, more recently, full proteomic and transcriptomic analysis. Where possible, drug loading can also be assessed e.g. using mass spectrometry (2), hybridisation-based nucleic acid capture assays, or amplification methods such as PCR, provided oligonucleotide drug chemistry is compatible with DNA polymerases. Unfortunately, the 1-2 log knockdown effects of antisense and short interfering RNA (siRNA) therapeutics typically measured in cell culture only translates to 40-60% target RNA reduction at the whole lung tissue level in animals; clinical data can be even more disappointing (3).

The reasons for these discrepancies have been the subject of intense debate, speculation, and assumptions, as extensively discussed elsewhere (3, 4). From a mathematical perspective, analysing target RNA levels in homogenised lung tissues can only yield lower levels of drug effect as compared to cell line experiments, due to poor signal to noise ratios. The problem occurs because the levels of an RNA target measured in an extract from a whole lung lobe reflect the average level of that RNA molecular species across the pool of different cell types that constitute the lung tissue. Furthermore, the relative numbers of each cell type are not fixed and can vary on account of disease, especially when inflammation is involved. At the same time, the extent to which target RNA levels may change on account of oligonucleotide treatment in a given cell is, as a minimum, a function of oligonucleotide transfection efficiency: this efficiency also varies between cell types. Furthermore, it is still unknown if downregulation of a target gene in some cells leads to compensatory gene expression changes in untransfected bystander cells.

In an ideal scenario, therefore, the effect of an oligonucleotide drug on a tissue as complex as the lung should be measured by cell type, and in relation to its degree of transfection. This can be achieved by applying mechanoenzymatic Tissue Disruption and Cell Sorting (TDCS) on *in vivo* pharmacology experiments. Indeed, industry evidence on what makes for successful drug discovery (5) points to TDCS as a tool that would offer added value to project decision making. By sorting tissue cells by type, measuring drug loading, and on-target effect, one can obtain evidence of target engagement, and an on-target mechanism of action (6) in cells relevant to disease, *in vivo*,and potentially even in patients. Obtaining such pharmacological evidence is now considered pivotal to drug development efforts (7).

Many of the tools and materials necessary to implement TDCS are well-established from basic research studies involving primary tissue cell isolation. Over the past decade, increasingly complicated cell separation approaches have been used to study cell type specific biology in primary human tissue, tumour biospies, as well as animal models of disease. Two important methods are fluorescence-activated cell sorting (FACS), and magnetic cell sorting (MACS). The FACS method is good for cell high cell purity and/or recovery, low cell surface marker expression, detection of intracellular markers, as well as sorting cells by cell surface marker expression levels. In contrast, MACS is good for surface markers only, bulk cell depletion/enrichment for a single surface marker e.g. in preparation for FACS, or situations where the optical properties of the cell interfere with marker sensitivity such as autofluorescence, either in large scale instruments or microfluidic systems. Recent advances in these platforms are reviewed by Shields *et al.* (8). Studies involving MACS and/or FACS use either in-house, or supplier pre-validated reagents (antibodies or aptamers) specific to cell surface markers found in cell sub-populations, or markers accessible after cell fixation and permeabilization. Reagent validation usually takes the form of gene expression / surface marker analysis of sorted cells, cell function analyses, marker co-expression as observed histopathologically, by Western blotting, or by alternative means including physicochemical properties exhibited during flow cytometry (2). The vast body of work involving cell sorting can be a very useful resource in implementing TDCS in *in vivo* pharmacology studies not only for lung disease, but virtually any indication with a substantial body of basic research involving cell sorting methods and appropriate validation of marker-specific reagents.

Using TDCS we were therefore able to show that oligonucleotide drugs dosed directly into mouse tracheas loaded at appreciable amounts only in airway/alveolar macrophages, but with no statistically significant effect on a target gene, despite statistical power for measuring a 50% change exceeding 94% (2). Histological and ADME follow up experiments explained this outcome through the observation of rapid oligonucleotide transcytosis from the airway lumen into circulation, and elimination in urine in as little as 15 minutes after dosing. Although our results were with modified short interfering RNA (siRNA) and 3rd generation, short locked nucleic acid (LNA) drugs, the findings were in line with historical pharmacokinetic studies by Ionis pharmaceuticals on longer, 2nd generation antisense oligonucleotides (9). This suggested that more recent clinical measurements were not sensitive enough to measure circulatory and urine oligonucleotide levels in man (3). Clinical efforts with naked siRNA and antisense oligonucleotides performed by a number of pharmaceutical companies were abandoned shortly afterwards.

Presently, research on oligonucleotide drugs for respiratory diseases involves agents that work as toll like receptor agonists, splice modulating antisense, *in vitro* transcribed RNA therapeutics including long non-coding RNAs, mediators of DNA and RNA editing, and RNA interference modulators (microRNA mimics, siRNA, and microRNA inhibitors). This is on account of the recent regulatory approvals of the antisense drugs mipomersen (familial hypercholesterylaemia, inotersen (hereditary transthyretin-mediated amyloidosis) nusinersen (spinal muscular atrophy; drugs developed by Ionis Pharmaceuticals), and eteplirsen (Duchenne’s muscular dystrophy; developed by Sarepta Therapeutics), the siRNA drug patisiran (hereditary transthyretin-mediated amyloidosis; developed by Alnylam Pharmaceuticals), as well as unprecedented advances involving the development and use of an antisense drug for a single patient harbouring a unique mutation, also known as “n of 1 medicine”, as exemplified by milasen (10). Crucially, all of these drugs effectively load onto disease-relevant target tissues and cells, either through first pass metabolism (targeting the liver) or by direct intrathecal injection to reach the central nervous system. Therefore, to achieve comparable success in the lung, with few exceptions, investigators are evaluating a cadre of nanoformulation approaches as drug delivery solutions to successfully transfect airways cells. Although the safety of particulate drug delivery to the lungs is still a substantial outstanding concern (3, 11, 12), the need remains for determining drug loading and effect in the lung cell types relevant to disease.

In this protocol we therefore detail the approach for processing an excised mouse lung to sort precise numbers of specific cell types for appropriate downstream analytics. We describe the steps needed to successfully prepare monocellular suspensions, and detail the approach for sorting three cell preparations, each consisting of 20,000 lung epithelial cells, macrophages, or non-epithelial/non-macrophage cells from a single lung lobe (Figure 1). The two-stage sorting protocol first makes use of magnetic bead-based separation, followed by flow cytometric sorting using fluorochrome conjugated antibodies against cell surface markers of interest. This protocol is therefore an extension to a previously published method for *in vivo* RNA therapeutics pharmacology (1), and focuses exclusively on how to implement TDCS. In our hands, TDCS can be reliably followed up with transcriptomics, proteomics, and mass spectrometry, as well as sub-culturing (where cell division is naturally accommodated by the cell type of interest). With the emergence of single cell ‘omics, the anticipated parallelisation and cost reduction of these newer bioanalytical approaches, we look forward to TDCS-based *in vivo* pharmacology delivering highly potent, safe, and targeted airway therapeutics.

**2. Materials**

**2.1 Preparation of monocellular lung suspension from a mouse lung lobe.**

1. Digestion buffer: 2.5% v/v foetal bovine serum (FBS), 0.5 mM calcium chloride, 0.75 mM magnesium chloride and 1.5% w/v DNase I from bovine pancreas (lyophilised powder at >85% purity, >400 Kunitz units/mg protein; Sigma Aldrich, Dorset, UK; note 1) prepared in Dubbleco’s Phosphate Buffered Saline without calcium and magnesium (DPBS). Make fresh when needed.
2. Collagenase Buffer: 150 units/ml Collagenase 3 (Worthington Biochemical Corporation, Lakewood, NJ, USA) in digestion buffer; prepare the night before and keep at 4oC or on ice until used (note 1).
3. Cell resuspension buffer: sterile 1x DPBS supplemented with 5% v/v FBS.
4. Oscillating platform incubator, set at 37oC.
5. GentleMACS tissue dissociator (Miltneyi Biotec Ltd., Woking, UK).
6. GentleMACS C Tubes (purple), for tissue dissociation (Miltenyi Biotec Ltd.; note 2).
7. Sterile tissue vials such as 5 ml universals, 15 or 50 ml cell culture centrifugation tubes.
8. 50 ml sterile, cell culture centrifugation tubes.
9. Sterile 5 ml syringes.
10. Sterile 1x PBS.
11. 18 MOhm de-ionised water.
12. 3.6% w/v sodium chloride in sterile, de-ionised water.
13. Cell counting apparatus (haemocytometer and trypan blue or automated cell counter).
14. Desktop refrigerated centrifuge with bucket swing out rotor, for cell culture purposes.
15. 40 m and 100 m Cell Strainers.
16. Wet ice.
17. Serological pipettes (25 ml).
18. Serological pipette gun with variable speed control.
19. Sterile spatula(s) (note 3).
    1. **Isolation of specific cell types from digested lung tissue.**
20. AutoMACS running buffer: 1x Phosphate Buffered Saline supplemented with 2 mM EDTA, 0.5% w/v bovine serum albumin (BSA), and 0.09% w/v sodium azide, pH 7.2; stored at 4oC for long-term use.
21. CD11b+ paramagnetic microbeads (Miltenyi Biotech).
22. Compensation beads: anti-rat IgG for the rat anti-mouse antibodies used in this protocol or alternative species as required.
23. BD Biosciences Accudrop beads: Drop delay set up beads (BD Biosciences, Crawley, UK).
24. Cytometer Setup and Tracking beads (CS and T beads) (BD Biosciences).
25. Stain buffer: 5% w/v BSA or similar in 1x PBS.
26. 2% BSA supplemented PBS: 2% w/v BSA or similar in 1x PBS.
27. Peridinin Chlorophyll Protein Complex (PerCP) anti-mouse F4/80 (Thermo Fisher Scientific).
28. Phycoerythrin (PE) anti-mouse CD326 (Biolegend, San Diego, California).
29. Fluorescein isothiocyanate (FITC) anti-mouse CD45 (BD Biosciences).
30. 30µm filter or cell strainer.
31. AutoMACS Pro Separator (Miltenyi Biotec Ltd.; note 4).
32. Chilled collection blocks for AutoMACS Pro (ensure chilled to 4°C prior to use).
33. FACS tubes with 35µm cell strainer cap.
34. Cell sorter capable of analysing 4 fluorochromes and depositing sorted cells into a 96 well plate. E.g. BD FACS Aria (BD Biosciences; note 5).
35. Uncoated, sterile, cell culture 96 well plates (round or flat bottom) appropriate to the downstream analytical approach to be used.

**3. Methods**

* 1. **Preparation of monocellular lung suspension from a mouse lung lobe.**

1. Rinse the fresh, perfused mouse lung, with sterile PBS to eliminate any blood.
2. Transfer the lung lobe to 2 ml of digestion buffer in a sterile tissue vial (ensure full tissue submersion) and keep on ice until all lungs are harvested.
3. Transfer tissue into a GentleMACS C tube using a sterile spatula and add 5 ml of Collagenase Buffer per tube (notes 6-7).
4. Close the GentleMACS C tube using the provided impeller cap (note 8).
5. Mix by gentle inversion three times.
6. Keep on ice until all lungs are prepared.
7. Once all the lungs for your experiment are prepared, process them sequentially on the GentleMACS tissue dissociator by placing the tubes, inverted, onto the device, and running program m\_lung\_01.01 provided by the supplier, to disrupt the tissue.
8. Transfer the GentleMACS C tubes onto an oscillating platform incubator set at 37oC and shake at 250 rpm for 30 min.
9. Return the GentleMACS C tubes onto the GentleMACS tissue dissociator and process the samples using program m\_lung\_02.01 to achieve monocellular suspensions.
10. Pulse centrifuge the GenleMACS C tubes for 10 seconds at 400 x g to collect cells at the bottom of the tube.
11. Assemble a 50 ml centrifugation tube per lung lobe with a 40 m cell strainer.
12. Pass the cells through the 40 m cell strainer using a 5 ml syringe plunger.
13. Rinse the strainer with 3x 1 ml of digestion buffer into the 50 ml centrifugation tube.
14. With the strainer still attached to the centrifuge tubes, centrifuge the cells at 400 x g, 4oC for 10 min, to improve cell recovery rate.
15. Carefully remove and discard the supernatant without disturbing the cell pellet.
16. Resuspend the cells in 4 ml of cell resuspension buffer by gentle mixing using a 25 ml serological pipette, and keep on wet ice or at 4oC.
17. Add 20 ml of de-ionised water; *quickly* cap the tube and invert to mix.
18. After 20 sec make the solution isotonic by adding 8 ml of 3.6% w/v sodium chloride (note 9).
19. Centrifuge the cells at 400 x g, 4oC for 10 min.
20. Carefully remove and discard the supernatant without disturbing the cell pellet.
21. Resuspend the cells in 5 ml cell resuspension buffer.
22. Count your cells e.g. by trypan blue exclusion assay on a haemocytometer (mix 25 l of Trypan Blue with 25 l of cell suspension and load onto the haemocytometer; count as per haemocytometer manufacturer instructions) or automated cell counting apparatus.
23. Store on ice or at 4oC until cell sorting is carried out (note 10).
    1. **Isolation of specific cell types from digested lung tissue.**

Due to the high intrinsic auto fluorescence of tissue resident macrophages which can make flow cytometric analysis more challenging and limit the choice of fluorochromes, a two-stage separation protocol is advised whereby macrophages and other myeloid cells are first removed in bulk using CD11b-specific paramagnetic beads, followed by flow cytometric staining and cell sorting (note 11; Figure 1).

There are a large range of kits and reagents commercially available for the immune-magnetic isolation or depletion of particular cell types in a blood or tissue sample. Magnetic microbeads are coupled to antibodies specific for cell surface epitopes and when combined with a magnetic field, the desired cell type can be isolated or undesired cell types removed. This section describes the use of Miltenyi MACS reagents and equipment but the methods described could be carried out with equivalent reagents from other sources. Similarly, the fluorochromes used can be adapted to the capability of the cell sorter and fluorescent antibodies are available from a wide range of commercial suppliers. Table 1 summarises antibody suppliers which have proven reliable in our hands when applying TDCS in pharmacological studies (all reagents were used at the manufacturer’s recommended concentration).

**3.2.1 Separation of CD11b+ cells using AutoMACS Pro.**

1. Retain at least 0.5 ml of each digested lung sample and store at 4°C. Run the remaining sample through the AutoMACS Pro as described below.
2. Spin digested lung sample/s at 400 x g for 5 minutes to pellet the cells and re-suspended them in AutoMACS running buffer at 10e7 cells per 80μl.
3. Pass the sample through a filter to ensure a single cell suspension.
4. Add 20 μl of CD11b-specific microbeads per 10e7 cells and incubate at 4°C for 15 minutes (note 12).
5. If not done previously during the incubation, turn on and prime the AutoMACS machine and ensure pre-chilled collection blocks removed from the fridge.
6. Wash the samples with 1ml ice cold AutoMACS running buffer and spin at 400 RFC.
7. Discard the supernatant and re-suspend in 500μl AutoMACS running buffer.
8. Run samples on the AutoMACS, using the ‘deplete\_s’ programme followed by a quick wash between each sample.
9. Both the CD11b+ and CD11b- fraction are collected into the pre-chilled collection block.
10. Spin samples down at 400 x g and re-suspend in 100 µl of stain buffer (note 13; See Figure 2 for a typical forward scatter / side scatter (FSC/SSC) plot of the CD11b+ and CD11b- fractions).

**3.2.2 Fluorescent staining for flow cytometric analysis and sorting.**

Keep aside sufficient cells to set up appropriate controls as exemplified in Figure 3 (notes 14-16)**.**

1. Stain the fractions of CD11b- cells with anti-CD45 and anti-CD326 to identify leukocytes and epithelial cells (see Figure 1).
2. Stain the fractions of CD11b+ cells with F4/80 to discriminate macrophages.
3. Use 20μl of each antibody in a total volume of 100 μl cell resuspension buffer.
4. Incubate at 4°C for 30 minutes.
5. At the same time, create single colour controls for compensation by adding 1 drop of compensation beads to 100μl cell resuspension buffer and incubate with 20 µl of one antibody for 30 minutes at 4°C.
6. Wash samples and compensation beads with 1ml stain buffer and spin at 400 x g for 5 minutes.
7. Re-suspend the beads in 2 ml stain buffer and filter into a FACS tube to ensure a single cell suspension ready for analysis and sorting on the BD FACS Aria.
8. To allow accurate gating of populations, create an FMO (Fluorescence Minus One) control for each antibody used. For example, the PE FMO control will contain every antibody used *except* the PE antibody.

**3.2.3 Sorting of mixed cell populations, macrophages, epithelial cells and non-epithelial cells into 96 well plates for down-stream assays.**

1. Set up the BD FACS Aria with a 70 μm nozzle and the pressure setting of medium drop drive frequency 60,000 with a ‘Purity’ sort setting, eg. 16-32-0; note 17.
2. Run BD CS&T beads to confirm Aria laser performance.
3. Run BD Accudrop beads to set up the drop delay and to align the 96 well plate.
4. Ensure SSC-W, SSC-H, FSC-W and FSC-H are enabled to allow doublet discrimination.
5. Add 100μl of 5% BSA-supplemented PBS to each well of a 96 well plate.
6. Perform compensation using the single stained controls.
7. Take the third of the original sample that was not passed through the AutoMACS Pro and spin at 400 x g to pellet.
8. Re-suspend in 2ml 2% BSA-supplemented PBS – this is the ‘mixed’ population of cells and does not requiring any specific sorting but requires the correct number of live cells to be counted into the 96 well plate.
9. Gate the live, single cells on the basis of FSC/SSC. Sort 30,000 cells from within this gate into the appropriate well of a 96 well plate.
10. Run the CD11b+ population through the Aria and gate on live single cells and then on F4/80+ cells to sort 30,000 macrophages (Figure 4A).
11. Run the CD11b- population through the Aria and gate on cells that are live/CD45- /CD326+ to sort 30,000 epithelial cells (Figure 4B).
12. Continue to run the CD11b- population, gating on cells that are live/CD45-/CD326- to sort 60,000 non-epithelial cells (Figure 4A) (notes 18-20; Figures 3 and 4).

**4. Notes**

1. The source of DNase I, but more so collagenase, impacts significantly on process success and cell viability at completion of the TDCS process. This particular collagenase is sold as low protease activity which makes it also more appropriate for cell sorting applications. Although we have not tested the protease activity of the particular DNase I enzyme supplied from Sigma, we have not evaluated other enzymes in our workflow for impact on cell viability.
2. Important! Do not use the M tubes as these homogenise the tissue by vigorous mechanical shearing stress.
3. For infectious models/microbiome studies ensure that separate sterile spatulas are used per tissue.
4. The AutoMACS Pro instrument was used to standardise multiple separations and help increase throughput but is not an absolute requirement. During method development, individual single use columns were used for some experiments and an acceptable throughput could be achieved in this manner with sufficient operators, although care would need to be taken to standardise technique.
5. The sorter used by the authors was a BD FACS Aria with 488nm blue laser and 633nm red laser. The laser and filter combinations available on any particular cell sorter will dictate the fluorochromes and combinations that can be used during cell sorting. If an instrument does not have a plate collection option, up to 4 populations can be sorted into tubes for later transfer to appropriate plates. It is worth bearing in mind though that at least one factor in the improved coefficients of variation observed with this method is the accurate deposition of defined cell numbers by the instrument - the equivalent of which is unlikely to be achieved manually.
6. For highly fibrous tissue (e.g. fibrosis models) it is advised to mince the tissue down into 1 mm3 cubes first using a sterile scalpel and petri dish or an automated tissue chopping station before loading it into the GentleMACS C tube.
7. If a GentleMACS cell dissociator is not available, then skip steps 3-11; instead, process the tissue by mincing as described in the previous note, transfer it into a sterile vial, suspend in 5 ml digestion buffer / lung and incubate for 30 min on an oscillating platform incubator at 37oC. Then triturate through a 5 ml syringe without a needle ~25 times and subsequently press the suspension through a 100 m cell strainer using a sterile syringe plunger into a 50 ml cell centrifugation tube; push tissue clumps through the mesh if necessary. Proceed with step 11. This method is harsher and less reliable than the GentleMACS procedure in our experience.
8. Do not use normal cell centrifugation tube caps, as these will not allow the mechanic disruption to proceed.
9. Steps 19-20 lyse any remaining red blood cells. This protocol is suited to other red blood cell lysis procedures, e.g. to obtain peripheral blood nucleated cells.
10. In our experience, cells will lose minimal viability (2-3%) over the next 48 hrs if kept at 4oC and can even be safely shipped between sites within this timeframe if necessary.
11. CD11b is expressed on murine monocytes and macrophages but also on neutrophils, NK cells, dendritic cells and some subsets of activated lymphocytes. Using CD11b-specific magnetic separation is therefore not a purification, but an enrichment in one fraction and a reduction of auto fluorescent cells in the other fraction. Further staining of the CD11b+ fraction allows for further discrimination of cell types of interest.
12. As only one sample can be processed by the machine at a time it is not recommended to stain all samples simultaneously as there will be a considerable lag time between staining and separation for the last samples processed. Subsequent samples can be stained and washed while previous ones are running through the machine. For this reason, it is useful (though not mandatory) to have two people carrying out this part of the protocol.
13. It is recommended to retain some of each fraction post magnetic separation to assess for viability and successful enrichment. A 100µl sample can be stained with 2.5µl of anti-CD11b antibody, incubated for 30 minutes and then washed with cell resuspension buffer. For viability, add 2µl Sytox red, mix gently and proceed to analysis. Dead cells will stain positive in the APC channel and a % viability can be determined by gating on the negative cells.
14. Ideally, all steps will be completed in one day but it is possible to leave cells overnight at 4°C at this stage for staining and sorting the following day. Viability was not affected and biomarker data showed that populations sorted on day one or day 2 were highly comparable, suggesting that sorting of a lung digest sample up to 24 hours after preparation is an acceptable strategy when processing large numbers of mice. In our hands, the maximum throughput of lungs in one day was 12 and each sort (per animal) took approximately 30 minutes to achieve the required cell numbers.
15. Compensation controls will be required for most multi-colour sorting experiments. With multi-laser instruments it may be possible to select fluorochromes that avoid the need for compensation by being sufficiently spectrally separate or by being excited by different lasers. For example, on a four laser instrument, choosing one fluorochrome for each laser line may remove the requirement for compensation. However, this should always be checked initially with the appropriate single colour controls. Compensation Beads are most suitable in this instance due to the variable auto fluorescence of cell populations in digested tissues. Unstained beads should be used for the unstained control, not unstained lung digest sample.
16. When first developing a panel, use irrelevant specificity isotype control antibodies to rule out non-specific binding. Once confidence has been established in the antibodies to be used, isotype controls are not necessary for every experiment and Fluorescence Minus One (FMO) controls should be used to set population gates. For example, during method development, tests on 10 different antibodies sold as luciferase-specific and compatible with sorting from a variety of suppliers failed to exhibit antigen specificity with this method; in stark contrast, no such issues were experienced with all other markers.
17. Depending on the cell type of interest and downstream application, a 100 m nozzle might be more appropriate. The lower pressure and wider sort orifice causes less stress to sorted cells but in our hands resulted in poorer sort efficiency and fewer recovered cells. Viability staining of cells straight after sorting on a 70% nozzle showed the majority of the cells survived the sort and in our system those cells were assessed straight away, meaning that long term survival was not a concern. If downstream applications are to include culturing or functional studies, a 100 µm nozzle would be recommended. A purity sort mode was used in this protocol but each investigator should determine their own requirements of yield vs purity for specific applications. Efficiency on purity sort mode in our hands ranged from 79% to 95%.
18. The potential combination of cell surface markers and gating strategies for cell types in a digested mouse lung are very large. Since this method was developed there have been extensive studies published looking at full phenotypic characterisation of cell types in digested mouse lung – both haematological and structural. The populations identified with the antibodies described here are rather broad and can be added to or altered to more precisely identify numerous cell types depending on the investigator’s specific interests. Staining for cell surface markers can be considerably more challenging in digested tissue than in blood due to the digestion process itself which may result in loss of cell surface epitopes. As an example, with the digestion protocol described herein, the authors had no success with a variety of anti-cytokeratin antibodies which had been described to bind strongly and specifically to epithelial cells which we hypothesise may be due to the loss of those markers during the digestion process itself.
19. ‘Fc block’ (rat anti-mouse CD16/32) was trialled during development to prevent interaction of leukocyte Fc receptors with the Fc region of fluorescent antibodies but it made no difference compared to no Fc block controls so this was not continued. Other non-specific binding was avoided by the presence of protein in the stain and sort buffers.
20. If fluorescence is being used to track delivery of oligonucleotides, the spectral profile of that dye will need to be considered in the panel design for cell sorting. For example, the use of Cy5 labelled oligonucleotides precluded the use of APC as a fluorochrome in this study.

**5. References**

1. Moschos SA, Spink KGG and Lindsay MA (2011) Measuring the action of CPP-siRNA conjugates in the lung. Methods Mol Biol 683: 417–429.

2. Moschos SAA, Frick M, Taylor B, Turnpenny P, Graves H, Spink KGG, Brady K, Lamb D, Collins D, Rockel TDD, et al. (2011) Uptake, efficacy, and systemic distribution of naked, inhaled short interfering RNA (siRNA) and locked nucleic acid (LNA) antisense. Mol Ther 19: 2163–2168.

3. Moschos SA, Usher L and Lindsay MA (2017) Clinical potential of oligonucleotide-based therapeutics in the respiratory system. Pharmacol. Ther. 169: 83–103.

4. Kumar M and Moschos SA (2017) Oligonucleotide Therapies for the Lung: Ready to Return to the Clinic? Mol. Ther. 10.1016/j.ymthe.2017.11.002.

5. Morgan P, Van Der Graaf PH, Arrowsmith J, Feltner DE, Drummond KS, Wegner CD and Street SDA (2012) Can the flow of medicines be improved? Fundamental pharmacokinetic and pharmacological principles toward improving Phase II survival. Drug Discov. Today 17: 419–24.

6. Cook D, Brown D, Alexander R, March R, Morgan P, Satterthwaite G and Pangalos MN (2014) Lessons learned from the fate of AstraZeneca’s drug pipeline: a five-dimensional framework. Nat. Rev. Drug Discov. 13: 419–31.

7. Morgan P, Brown DG, Lennard S, Anderton MJ, Barrett JC, Eriksson U, Fidock M, Hamrén B, Johnson A, March RE, et al. (2018) Impact of a five-dimensional framework on R&amp;D productivity at AstraZeneca. Nat. Rev. Drug Discov. 17: 167–181.

8. Shields CW, Reyes CD, López GP and López PGP (2015) Microfluidic cell sorting: a review of the advances in the separation of cells from debulking to rare cell isolation. Lab Chip 15: 1230–49.

9. Nicklin PL, Bayley D, Giddings J, Craig SJ, Cummins LL, Hastewell JG and Phillips JA (1998) Pulmonary Bioavailability of a Phosphorothioate Oligonucleotide (CGP 64128A): Comparison with Other Delivery Routes. Pharm Res 15: 583–591.

10. Kaiser J (2018) A tailormade drug developed in record time may save girl from fatal brain disease. Science (80-. ). https://doi.org/10.1126/science.aav7907.

11. Alton EW, Boushey HA, Garn H, Green FH, Hodges M, Martin RJ, Murdoch RD, Renz H, Shrewsbury SB, Seguin R, et al. (2012) Clinical expert panel on monitoring potential lung toxicity of inhaled oligonucleotides: consensus points and recommendations. Nucleic Acid Ther. 22: 246–54.

12. Forbes B, O’Lone R, Allen PP, Cahn A, Clarke C, Collinge M, Dailey LA, Donnelly LE, Dybowski J, Hassall D, et al. (2014) Challenges for inhaled drug discovery and development: Induced alveolar macrophage responses. Adv. Drug Deliv. Rev. 71: 15–33.

**Tables**

**Table 1:** Antibodies validated for compatibility with TDCS on live or fixed cells.

|  |  |  |  |
| --- | --- | --- | --- |
| **Species** | **Target and Clone** | **Supplier and Cat. No.** | **Application** |
| Mouse | CD105; 209701 | BD Biosciences, R and D Systems FAB1320A | Endothelial cells or activated macrophages, positive selection; can discriminate the two populations by granularity. |
| Mouse | CD45; 30-F11 | BD Biosciences 557659, 557235 | Type I epithelial cell negative selection; Leucocyte positive selection; Macrophage cell positive selection±. |
| Mouse | F4/80; BM8 | Invitrogen MF48021 | Positive selection of macrophages in a CD11b- population; F4/80 negative selects for neutrophils. |
| Mouse | CD144; 55-7H1 | BD Biosciences 560411 | Endothelial cell positive selection. |
| Mouse | CD326; G8.8 | Cambridge Biosciences 118205, Biolegend 118205 | Epithelial cell positive selection in a CD45-, CD11b- population. |
| Mouse | CD11b; M1/70 | Miltenyi Biotech 130-049-601 | Paramagnetic bead positive selection of macrophages, neutrophils, NK cells, granulocytes and dendritic cells (requires F4/80+ downstream for macrophage selection) and negative selection for epithelial cells (requires CD45-, CD326+ downstream). |
| Rat | ATP-binding cassette subfamily A member 3; 3C9 | Abcam ab24751 | Alveolar type II epithelial cells, positive selection\* |
|  |  |  |  |
| Rat | EpCAM; polyclonal | Abcam ab71916 | Epithelial cells, positive selection\* |
| Rat | CD45; OX1 | eBioscience 12-0461 | Negative selection for epithelial enrichment\*\* |
| Rat | CD68; ED1 | Abcam ab31630 | Macrophages, positive selection\*\* |
| Rat | CD45; REA450 | Miltenyi Biotec 130-109-682 | Paramagnetic bead negative selection for epithelial enrichment against leucocytes and macrophages\*\* |
| Mouse, rat | CD90.1; His51 | Miltenyi Biotec 130-094-523 | Paramagnetic bead positive selection for T cells\*\* |
| Human | TLR2; TL2.1 | Invitrogen 16203 | Positive selection for epithelial cells\*\* |

±: Include SSC and FITC channel autoflorescence in selection criteria.

\*: Useful for paraformaldehyde-fixed and saponin-permeabilised cells only.

\*\*: Validated on live cells only.

**Figures legends**

**Figure 1:** Cell sorting strategy for the parallel isolation of lung epithelia, lung macrophages and remaining cells from a single mouse lung lobe digest. In addition to the previously published approach of obtaining macrophages (CD11b+; F4/80+), epithelia (CD11b-; CD45-; CD326+) and non-macrophage/epithelial cells (CD11b-; CD45-; CD326-), the protocol is amenable to neutrophil isolation (CD11b+, F4/80-). For macrophage-focused work, a simple CD45+, SSC high, FITC autofluorescence high positive selection approach can be reliably implemented.

**Figure 2**: A) Representative scatter plots of digested lung samples prior to CD11b depletion and the CD11b- (left) or CD11b+ (right) fractions following separation. B-D: Histograms comparing the CD11b- population to the CD11b+ population in terms of CD11b (b), CD326 (c) and CD45 (d) expression.

**Figure 3**: Example of gating controls in flow cytometric analysis. CD11b- fractions were stained with two antibodies, making them appropriate for Fluorescence Minus One (FMO) controls. An example PE FMO (FITC antibody only) is shown in A, and an example FITC FMO (PE antibody only) is shown in B. The CD11b+ fraction was only stained with one antibody so here an FMO was not appropriate, instead an APC conjugated isotype control was used as negative control to gate for F4/80 positive cells, with an example shown in C.

**Figure 4:** Example of gating strategies for CD11b+, F4/80+ macrophage cells (A), CD11b- CD326+ epithelial cells (B), and CD11b- CD326- non-epithelial cells (C).