3.1 Preparation of Monocellular Lung Suspension from a Mouse Lung Lobe

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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 preclinical 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.

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GUIDELINES

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 standardized procedures on animal models of disease, animals are terminally anaesthetized at set time points, tissues are excised, and homogenized, to extract and purify 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], hybridization-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 homogenized 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, tumor biopsies, 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 high cell purity and high cell recovery, low cell surface marker expression, detection of intracellular markers, as well as sorting cells by cell surface marker expression levels. In contrast, MACS, being suitable only for surface markers, is good for bulk cell depletion/enrichment for a single surface marker (e.g., in the 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 prevalidated reagents (antibodies or aptamers) specific to cell surface markers found in cell subpopulations, 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 coexpression 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 for 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 min after dosing. Tissues were obtained from animals in accordance with regulations and established guidelines and were reviewed and approved by an Institutional Animal Care and Use Committee or through an Ethical Review Board. Although our results were with modified short interfering RNA (siRNA) and third generation, short locked nucleic acid (LNA) drugs, the findings were in line with historical pharmacokinetic studies by Ionis pharmaceuticals on longer, second 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 afterward.

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 noncoding 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 hypercholesterolemia), 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 harboring 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 nonepithelial/nonmacrophage cells from a single lung lobe (Fig. 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 subculturing (where cell division is naturally accommodated by the cell type of interest). With the emergence of single cell 'omics, the anticipated parallelization, 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.
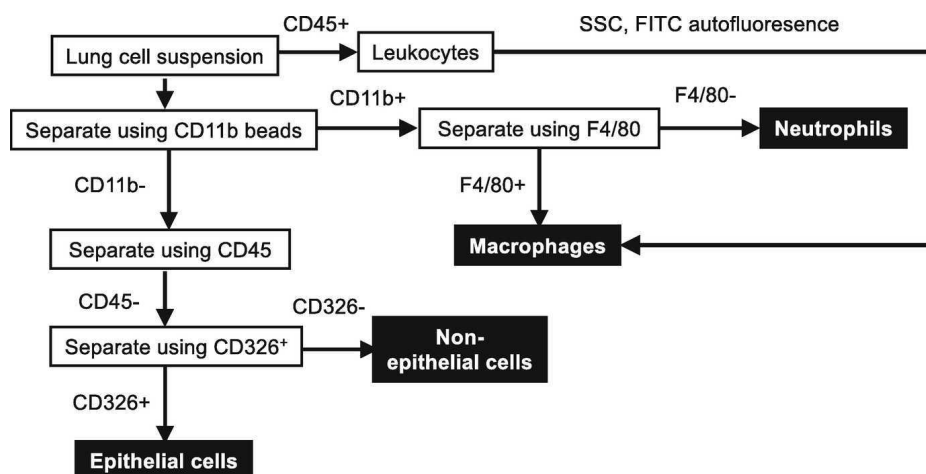

Fig. 1
Cell sorting strategy for the parallel isolation of lung epithelia, lung macrophages and remaining cells from a single mouse lung lobe digest. In addition to the previously published approach of obtaining macrophages (CD11b+; F4/80+), epithelia (CD11b−; CD45−; CD326+) and nonmacrophage/epithelial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

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 homogenize 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 standardize 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 standardize technique.
5. The sorter used by the authors was a BD FACS Aria with 488 nm blue laser and 633 nm 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 four 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 mm^3 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** ([section/protocol 3.1](#)); 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 37 °C. Then triturate through a 5 ml syringe without a needle ~25 times and subsequently press the suspension through a 100 μm 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** ([section/protocol 3.1](#)). 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** and **20** ([section/protocol 3.1](#)) 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 h if kept at 4 °C 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 autofluorescent 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 100 μl sample can be stained with 2.5 μl of anti-CD11b antibody, incubated for 30 min and then washed with cell resuspension buffer. For viability, add 2 μl 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 1 or day 2 were highly comparable, suggesting that sorting of a lung digest sample up to 24 h 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 min to achieve the required cell numbers.

15. Compensation controls will be required for most multicolor sorting experiments. With multilaser 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 color controls. Compensation beads are most suitable in this instance due to the variable autofluorescence 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 nonspecific 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 ten 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 μm 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 versus 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 characterization of cell types in digested mouse lung—both hematological 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 hypothesize may be due to the loss of those markers during the digestion process itself.

19. "Fc block" (rat anti-mouse CD16/32) was trialed 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 nonspecific 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-labeled oligonucleotides precluded the use of APC as a fluorochrome in this study.

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2 Materials

2.1 Preparation of Monocellular Lung Suspension from a Mouse Lung Lobe

1. Digestion buffer: 2.5% v/v fetal bovine serum (FBS), 0.5 mM calcium chloride, 0.75 mM magnesium chloride and 1.5% w/v DNase I from bovine pancreas (lyophilized powder at >85% purity, >400 Kunitz units/mg protein; Sigma Aldrich, Dorset, UK; *see* **Note 1**) prepared in Dulbecco'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 4 °C or on ice until used (*see* **Note 1**).
3. Cell resuspension buffer: sterile 1× DPBS supplemented with 5% v/v FBS.
4. Oscillating platform incubator, set at 37 °C.
5. gentleMACS tissue dissociator (Miltenyi Biotec Ltd., Woking, UK).
6. gentleMACS C Tubes (purple), for tissue dissociation (Miltenyi Biotec Ltd.; *see* **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 1× PBS.
11. 18 MΩ deionized water.
12. 3.6% w/v sodium chloride in sterile, deionized water.
13. Cell counting apparatus (hemocytometer and trypan blue or automated cell counter).
14. Desktop refrigerated centrifuge with bucket swing out rotor, for cell culture purposes.
15. 40 μm and 100 μm Cell Strainers.
16. Wet ice.
17. Serological pipettes (25 ml).
18. Serological pipette gun with variable speed control.
19. Sterile spatula(s) (*see* **Note 3**).

3.1 Preparation of Monocellular Lung Suspension from a Mouse Lung Lobe   20s

1. Rinse the fresh, perfused mouse lung, with sterile PBS to eliminate any blood.

2. Transfer the lung lobe to **2 mL 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 Collagenase Buffer** per tube (*see* **Notes 6** and **7**).

4. Close the gentleMACS C tube using the provided impeller cap (*see* **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 🌡 **37 °C** and shake at 🔧**250 rpm, 00:30:00** .

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 gentleMACS C tubes at ⚙**400 x g, 00:00:10** to collect cells at the bottom of the tube.

11   Assemble a 50 ml centrifugation tube per lung lobe with a 40 μm cell strainer.

12   Pass the cells through the 40 μm cell strainer using a 5 ml syringe plunger.

13

Rinse the strainer with **3 × 🧪1 mL 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, 4°C, 00:10:00** , to improve cell recovery rate.

15   Carefully remove and discard the supernatant without disturbing the cell pellet.

16

Resuspend the cells in 🧪**4 mL cell resuspension buffer** by gentle mixing using a 25 ml serological pipette, and keep on wet ice or at 🌡 **4 °C** .

17

Add 🥛**20 mL deionized water** ; *quickly* cap the tube and invert to mix.

18 🖊️ 20s

After 🕐**00:00:20** make the solution isotonic by adding 🥛**8 mL 3.6% w/v sodium chloride** (*see***Note 9**).

19 ⬚

Centrifuge the cells at ⊛**400 x g, 4°C, 00:10:00** .

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, for example, by trypan blue exclusion assay on a hemocytometer (mix 25 µl of Trypan Blue with 25 µl of cell suspension and load onto the hemocytometer; count as per hemocytometer manufacturer instructions) or automated cell counting apparatus.

23 Store ⬦ **On ice** or at ⬦ **4 °C** until cell sorting is carried out (*see***Note 10**).