# Real-time measurement of cellular bioenergetics in fully differentiated human nasal epithelial cells grown at air-liquid-interface.

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CW and JG designed the study and prepared the manuscript

SM performed research, data analysis, designed the study and prepared the manuscript

Running head: Metabolism in airway epithelial cells

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## Abstract

Shifts in cellular metabolic phenotypes have the potential to cause disease-driving processes in respiratory disease. The respiratory epithelium is particularly susceptible to metabolic shifts in disease, but our understanding of these processes are limited by the incompatibility of the technology required to measure metabolism in real-time with the cell culture platforms used to generate differentiated respiratory epithelial cell types. Thus to date, our understanding of respiratory epithelial metabolism has been restricted to that of basal epithelial cells in submerged culture, or via indirect endpoint metabolomics readouts in lung tissue. Here we present a novel methodology using the widely available Seahorse Analyzer platform to monitor real-time changes in the cellular metabolism of fully differentiated primary human airway epithelial cells grown at air-liquid interface (ALI). We show increased glycolytic, but not mitochondrial, ATP production rates in response to physiologically relevant increases in glucose availability. We also show that pharmacological inhibition of lactate dehydrogenase is able to reduce glucose-induced shifts towards aerobic glycolysis. This method is timely given the recent advances in our understanding of new respiratory epithelial subtypes that can only be observed *in vitro* through culture at ALI and will open new avenues to measure real-time metabolic changes in healthy and diseased respiratory epithelium, and in turn the potential for the development of novel therapeutics targeting metabolic-driven disease phenotypes.

## Introduction

Metabolic phenotypes are influenced by a number of genetic and environmental factors including gender, age, diet, microbiome, exercise, hormones and medication. Each unique combination, and therefore unique metabolic phenotype, can determine the disease risk of an individual. This is of particular importance in respiratory disease research; recent studies have demonstrated that altered metabolic phenotypes can modulate disease-driving cellular processes prevalent in the pathologies of chronic lung diseases, such as cellular proliferation, differentiation, apoptosis, autophagy, senescence and inflammation (12, 28, 29). Despite the high metabolic activity of the lung, with glucose metabolism in particular surpassing that of many other organs (12), our understanding of metabolic dysfunction as a driver or consequence of respiratory disease pathology is limited.

Much of the lung's metabolic activity occurs in the epithelium where cells have dense apical concentrations of mitochondria and carry out energy demanding processes such as mucin/surfactant production and mucociliary clearance (27). The respiratory epithelium provides a barrier to prevent nutrients entering the respiratory tract from the interstitium/blood and through tight control of glucose transport the glucose concentration in the airway is maintained at 3-20-fold lower than in plasma (6). In healthy airway epithelium, glucose is rapidly processed by hexokinase-dependent and -independent metabolic pathways to maintain low intracellular glucose concentrations (1). However, airway glucose concentrations are elevated in respiratory disease and hyperglycaemia, which is associated with increased risk of respiratory infection (2, 18, 19). Thus understanding the metabolic processes underlying lung metabolite homeostasis in health and disease could identify new therapeutic targets for the treatment of lung infections. The Seahorse platform is an excellent tool for interrogating cellular metabolism, measuring oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), in real time. Recent studies modelling the human airway epithelium have utilised this technology to show how mitochondrial dysfunction, caused by cigarettes and e-cigarettes, might contribute to the pathology of lung disease (3, 23) as well as investigating how infection

74 influences epithelial cell metabolism (13). While these studies all provide key information on
75 airway cell metabolism, the data is all generated using epithelial cells grown in submerged
76 cultures. Accordingly, a methodological development to monitor real time metabolic changes
77 of fully differentiated airway epithelial cells, under a range of conditions, would be of particular
78 benefit to the field of respiratory research.

79 Since Whitcutt *et al* (25) first described a method to grow airway epithelial cells at an air-liquid-
80 interface (ALI) this has become widely used to investigate epithelial barrier function. ALI
81 cultures are an excellent research resource as once the cells fully differentiate they have a
82 range of features which are not present in undifferentiated cells (25). They form polarised
83 barriers with characteristic epithelial ion transport properties and contain a heterogeneous cell
84 population of secretory and ciliated cells which only become apparent *in vitro* when the cells
85 are maintained at ALI. Recent single cell profiling has further demonstrated the heterogeneity
86 of ALI cultures at a molecular level and allowed the authors to identify a rare and novel
87 epithelial cell type, the pulmonary ionocyte (20). This highlights the importance of studying a
88 heterogeneous mixture of differentiated cells, which makes our exploration of epithelial
89 metabolism in ALI cultures particularly timely.

90 There are very few studies which have attempted to measure cellular metabolism in
91 differentiated airway epithelial cells, and the only report which we are aware required the in-
92 house manufacture of cell culture equipment (27), which is beyond the resources of many
93 researchers. We describe a novel method utilising the Seahorse XF24 Analyzer platform to
94 monitor real time changes in the cellular bioenergetics of human airway epithelial cells fully
95 differentiated at an air-liquid-interface. We describe a simple protocol, predicated on
96 inexpensive and widely available equipment.

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# Methods

*Sample source*

We obtained approval for the collection of clinical waste material obtained during routine nasal surgical procedures [Newcastle Biobank application NB-169, Research Ethics Committee Reference 17/NE/0361]. Written informed consent was obtained from all participants.

*Epithelial cell culture at air-liquid interface (ALI)*

Primary human airway epithelial cells were isolated from clinical waste material obtained during routine nasal surgical procedures. Chopped sections of epithelial tissue (approx. $1mm^3$) were cultured in PneumaCult™-Ex Plus Medium (STEMCELL Technologies) for 7-9 days in Type I collagen coated flasks (Purecol 30 µg/ml). P1 cells were then transferred onto collagen coated 0.4µm pore size transwells (Costar) at 150,000 cells/$cm^2$. Once the cells were fully confluent (2-4 days) apical media was removed and basolateral media was switched to PneumaCult™-ALI version 2 custom medium (STEMCELL Technologies) and the cells were maintained at ALI until fully differentiated (25). Barrier integrity of ALI cultures was monitored by measuring trans-epithelial electrical resistance (EVOM 2, World Precision Instruments) and ion channel function assessed using Ussing chamber short circuit current measurements. Transwells were routinely fixed with 2% glutaraldehyde for TEM/SEM (Newcastle University Electron Microscopy Research Services) and 4% PFA for H&E staining (NHS Cellular Pathology, RVI Newcastle).

*Seahorse XF24 Analyzer*

All reagents were obtained from Sigma, unless stated otherwise. Apical washes, with pre-warmed PBS, of the ALI cultures were carried out 24 hours prior to all seahorse experiments. Sections of ALI cultures on Transwells were cut from the membrane using a 3 mm punch biopsy (Kai Medical). These were carefully loaded into a seahorse islet capture plate (Agilent Technologies) and held in place with the islet capture grid (Figure 2). Wells were loaded with

450 µl experimental media (DMEM without $NaHCO_3$, glucose or phenol red (Sigma), supplemented with 1 mM D-glucose and 2 mM L-glutamine, pH 7.4 at 37°C). The plate was incubated at 37˚C without $CO_2$ while the cartridge was loaded and calibrated in XF24 Seahorse Analyzer (Agilent).

Cellular energetics for ATP production rates by mitochondrial oxidative phosphorylation and glycolysis were calculated by using the OCR and ECAR (16). Un-buffered medium enables the Seahorse Analyzer to accurately detect changes in acidification rates of the medium, as lactate secretion associated with glycolysis causes an acidification of the medium. Changes in OCR, after sequential inhibition of different stages of the electron transport chain, are used to measure mitochondrial ATP production (16). Oligomycin inhibits ATP synthase, thus oligomycin-induced changes in OCR reflect the oxygen consumption due to mitochondrial ATP production, which is used to calculate the mitochondrial ATP production rates. Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) is an uncoupler, hence stimulating electron transport chain and increasing OCR. Rotenone and Antimycin A, complex I and complex III inhibitors respectively, block electron transport activity and hence mitochondrial OCR and the OCR in the presence of them indicates non-mitochondrial respiration.

Injection ports for the cartridge were prepared in experimental buffer as follows. Injection port A with 50 µl of 1 mM, 14 mM or 140 mM D glucose (final concentrations to be 1 mM, 5 mM or 15 mM). Injection port B with 55 µl of 50 µg/ml oligomycin (final concentration 5 µg/ml as determined by titration shown in Figure 3). Injection port C with 60 µl of 25 µM FCCP (final concentration 2.5 µM, as determined by titration in Figure 3) and injection port D with 65 µl of 25 µM Antimycin A and 5 µM Rotenone (final concentrations 2.5 µM and 0.5 µM respectively). LDH5 inhibitor (synthesized according to the procedures described in patent WO 2015/140133 A1 (22)) was injected to a final concentration of 30 µM per well using DMSO vehicle control where appropriate.

Cycles of; mix (2 minutes), wait (1 minute), measure (3 minutes) were used. After our optimisation experiments we allowed 5 cycles for the cells to equilibrate, 7 cycles following

glucose injection, 11 cycles after oligomycin, 7 cycles after FCCP and 6 cycles following antimycin A and Rotenone addition. For LDH5 inhibition assays we allowed 5 cycles for equilibration, 7 cycles following glucose injection (port A), 13 cycles each following LHD5inh injection (port B) and oligomycin (port C) with a final 7 cycles after antimycin A and Rotenone (port D) injection.

Data analysis to calculate absolute ATP production rates was carried out using the methods described by Mookerjee and Brand (16), taking into account the acidification rates due to mitochondrial $CO_2$ production. All statistical analysis was carried out using GraphPad Prism 8.

# Results

*Culture of ALI epithelial cells*

Primary human nasal epithelial cells were successfully grown at ALI to form fully differentiated pseudostratified cultures (Figure 1A). Cilia were visible on the apical side (Figure 1A) and cells were shown to contain a large number of apical mitochondria (Figure 1A). Our ALI cultures showed electrophysiological polarisation and had characteristic ion transport systems as measured in an Ussing system (Figure 1B). The initial short circuit current (Isc) was completely inhibited by amiloride (10 μM) indicating that this standing Isc is carried by apical to basolateral $Na^+$ transport through apical epithelial sodium channels (ENaC). Further addition of forskolin (10 μM) generated a positive Isc which reached a plateau and was fully reversed with CFTRinh-172, indicating apical $Cl^-$ secretion via CFTR channels. Finally, addition of ATP (100 μM) generated a further positive Isc (which decayed spontaneously). This is likely to be $Cl^-$ efflux through apical $Ca^{2+}$-activated $Cl^-$ channels, activated by intracellular $Ca^{2+}$ pulse (initiated via ATP interaction with apical P2Y receptors).

*Seahorse Analyzer protocol optimisation*

Sections of membrane with cells attached were loaded into the seahorse islet plate and held in place with the islet capture grid (Figure 2). We compared the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) values when cells were loaded into the islet plate with the cells either facing up or down. Significantly higher values for both OCR and ECAR were obtained when cells were facing up (Figure 3A). Therefore, it was decided that for all subsequent experiments disks of membrane would be loaded into the plate with the cells facing up. Epithelial cells remained attached to the membrane throughout the experiment as verified by visual inspection with brightfield microscopy.

Oligomycin concentrations ranging from 2.5-10 μM were tested. Each concentration was sufficient to induce a decrease in OCR as mitochondrial ATP synthesis was inhibited. The maximum change in OCR was seen in 5 μg/ml oligomycin without further decrease at 10 μg/ml

187 (Figure 3B), therefore 5 μg/ml was selected for all subsequent experiments. The FCCP
188 concentration to induce maximum OCR was also determined (1.5 –3.5 μM) and it was found
189 2.5 μM FCCP was sufficient to achieve the highest increase in OCR (Figure 3C). Importantly,
190 we found that it takes much longer for the pseudostratified cultures to achieve steady state
191 after glucose and drug injections than a simple monolayer of airway epithelial cells (7).

192 *ALI cultures at altered glucose concentrations*

193 Once experimental conditions had been optimised we tested the bioenergetic response of our
194 differentiated airway epithelial cells to altered glucose concentrations. We saw that following
195 addition of both 5 mM and 15 mM D-glucose levels of ECAR significantly increased, when
196 compared to 1 mM glucose while no changes were observed in OCRs in any of the glucose
197 concentrations (Figure 4A). Therefore, assuming mitochondrial $CO_2$ production rates also
198 remained the same, the changes in ECAR under these conditions would reflect the glycolysis
199 dependent medium acidification rate. Following addition of oligomycin the greatest decrease
200 in OCR was seen in the 1mM glucose samples.

201 Analysis of absolute ATP production rates showed that increasing the glucose concentration
202 causes a significant and progressive increase in ATP production by glycolysis from 252
203 pmol/min at 1 mM to 703 pmol/min at 5 mM and 952 pmol/min at 15 mM (Figure 4B). While
204 glycolytic ATP production rates increased with increasing levels of glucose, we saw that
205 mitochondrial (OXPHOS) ATP production rates significantly decreased from 798 pmol/min at
206 1 mM to 487 pmol/min at 5 mM and 362 pmol/min at 15 mM. There was no statistically
207 significant difference in OXPHOS ATP production rates between the 5 mM and 15 mM glucose
208 concentrations.

209 *Proof-of-concept: inhibition of LDH suppresses glucose-induced extracellular acidification*

210 Multiple chronic lung diseases, including COPD, CF and IPF are associated with elevated lung
211 lactate concentrations (26, 29), with the metabolic shifts towards glycolysis in the epithelium
212 being at least in part implicated in these observed changes (7). In order to suppress lactate

213 production by glycolysis in our fully differentiated ALI cultures we injected an inhibitor of lactate
214 dehydrogenase 5 (LDH5; Compound 408, Genentech; Figure 4C(22)). At all glucose
215 concentrations there was significant decrease in the maximum ECAR following LDH5 inhibitor
216 addition, with the greatest decrease at the highest concentration of 15 mM Glucose (Figure
217 4D). Therefore, we have demonstrated empirically that LDH5 inhibition indeed lowered
218 glycolytic activity of the cells.

## Discussion

Our study presents a robust, practicable methodology that allows the metabolic characterisation of differentiated primary human airway epithelial cell cultures using the widely available Seahorse XF24 Analyzer platform. This allows multi-well measurements of real time oxygen consumption and extracellular acidification rates in live cells, providing information on glycolysis and mitochondrial function. To our knowledge this is the first time this has been possible using commercially available equipment.

This study used primary nasal epithelial cells as an exemplar for methodology development, due to their ability to replicate complex airway architecture *in vitro* (8). However, contrary to the previously proposed airways model (21), it is clear that there are differences between the upper and lower airway in their responses to disease relevant stimuli (4, 14) in addition to alterations in the cellular composition of the airway epithelium down the tracheobronchial tree. We suggest that our technique is broadly applicable to a range of airway cells grown on standard transwell support systems, and thus could be used to compare the metabolic profile of airway epithelial cells collected from different regions of the airway as well as comparisons between health and disease.

This method also has transferability across other disease models where cells are grown on Transwell inserts, in particular where there is also a close association between altered glucose metabolism and disease risk (5, 15, 17). In principle this technique would open up additional avenues for metabolic research beyond epithelial monocultures, such as co-cultures with fibroblasts which also undergo significant metabolic shifts in disease-relevant conditions (22). This would support development of novel therapeutic strategies for targeting epithelia-fibroblast crosstalk in idiopathic pulmonary fibrosis and related fibrotic interstitial lung diseases. Furthermore, because human variability is greater than that of animal models often used to study a disease, individual *ex vivo* metabolic profiling of the airway using our methods could provide vital information for personalized medicine (10). Such research could provide

much needed translational insights and facilitate the development of novel therapeutic approaches.

We maintain that using ALI cultures is more physiologically relevant than using submerged cells, indicating the need for a method to transfer ALI cultures into the Seahorse Analyzer platform. The ALI cultures are highly differentiated, secretory, have motile cilia and therefore are more likely to recapitulate the complex metabolic requirements of airway epithelial cells *in vivo*. Basal epithelial cells, maintained in submerged culture, have a much lower rate of glycolysis and mitochondrial respiration than those grown at ALI (27) and therefore are not truly representative of *in vivo* airways. Furthermore, there are cell types which only become apparent when cells are maintained at ALI (20). Thus, using this novel methodology the Seahorse Analyzer is able to detect the integrated signal from the multiple cell types which are present in ALI cultures. This allows for the *in vitro* modelling of a physiologically heterogeneous whole airway epithelium rather than a single cell type.

We were able to show that in high, but physiologically relevant glucose conditions, ALI cultures significantly increased the glycolysis dependent extracellular acidification rate. *In vivo* we believe that this could result in an acidification of the airway surface liquid (ASL). Recent research has suggested that the precise regulation of the ASL, in particular the pH, has a critical role in the prevention of infection and disease pathology (9, 11, 24). Therefore the real time monitoring of pH and glucose metabolism, using platforms such as the Seahorse Analyzer, may be a key methodology for providing insights into the pathophysiology of airways disease and for therapeutic development. This may be especially relevant in situations where diabetes often represents an important comorbidity such as in cystic fibrosis, chronic obstructive pulmonary disease, and bronchiectasis.

As well as providing novel information relevant to the pathophysiology of airways disease, our study demonstrated proof of concept that cellular bioenergetics analysis of differentiated epithelia may provide insights in pharmaceutical development. A range of pathophysiological

conditions including cancer and fibrotic lung diseases are thought to involve a reprograming of cellular metabolism, with skewing towards aerobic glycolysis, suggesting a potential therapeutic target. We showed that the addition of a potent LDH5 inhibitor resulted in a dose dependent reduction in extracellular acidification rate, consistent with a reduction in the production of lactate from pyruvate (22). Our system therefore allowed the evaluation of a novel compound in fully differentiated human cells, with the observed metabolic effects predicted from its designed mode of action.

In conclusion, we present a novel method and supporting data to demonstrate that it is possible to monitor the cellular metabolism in real time of fully ALI differentiated airway epithelial cells. The method also allowed us to monitor cellular responses to altered glucose concentrations, which may be relevant where diabetes and airway hyperglycemia can present as comorbidities to chronic airways diseases. We also found that it is possible to manipulate epithelial cell metabolism with a novel small molecule, indicating that this method may be useful for evaluating future therapeutic drug candidates.

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## Figure legends

Figure 1. Nasal epithelial ALI culture characterisation. A) Hematoxylin and eosin stain, TEM and SEM images of apical side of ALI culture. Scale bars indicate 10μm. B) Short circuit current trace from Ussing chamber experiment of ALI culture showing conventional airway epithelial responses to amiloride, forskolin, $CFTR_{inh172}$ (all at 10 uM) and ATP (100 uM). Chemical modulators were applied sequentially to the apical compartment of the Ussing chamber. $I_{SC}$ Short circuit current.

Figure 2. Schematic representation of ALI culture in Seahorse islet capture plate.

Figure 3. Protocol optimisation for Seahorse XF24 Analyzer. A) Comparison of ALI cultures loaded into Islet capture plate with cells facing up or down in 10mM D-Glucose. Bar graphs indicate basal levels of OCR and ECAR, before injection of any drugs. Error bars indicate mean ± SEM for 7-9 replicates and p values shown calculated by unpaired t test. B) Oligomycin titration, left - OCR measurements normalised to last time point before addition of oligomycin, right - area under the curve analysis of OCR, error bars indicate mean ± SEM for 5-7 replicates and p values calculated by one way ANOVA. C) FCCP titration, left OCR normalised to last time point before addition of FCCP, right maximum OCR in each well following addition of FCCP, error bars indicate mean ± SEM for 5-6 replicates and p values calculated by one way ANOVA.

Figure 4. Metabolic profiling of ALI cells at altered glucose concentrations. A) ECAR (left) and OCR (right) normalised to last reading before addition of glucose. Data from 4-6 repeats at each glucose concentration for 4 donors. Error bars indicate mean ± SEM. B) Absolute ATP production rates (left) and ATP production as a percentage of source, p values calculated by 2 way ANOVA (4-6 repeats at each glucose concentration from 6 donors). C) Dose dependent change in maximum ECAR before and after addition of LDH5inhibitor, all at 15 mM D glucose (3-5 repeats at each glucose concentration from 2 donors). D) Change in maximum ECAR following addition of 30 μM LDH5 inhibitor at 1 mM, 5 mM and 15 mM D

322 glucose, normalised to the average of control wells without inhibitor, p values calculated by
323 Mann-Whitney test (4-6 repeats at each glucose concentration from 3 donors).

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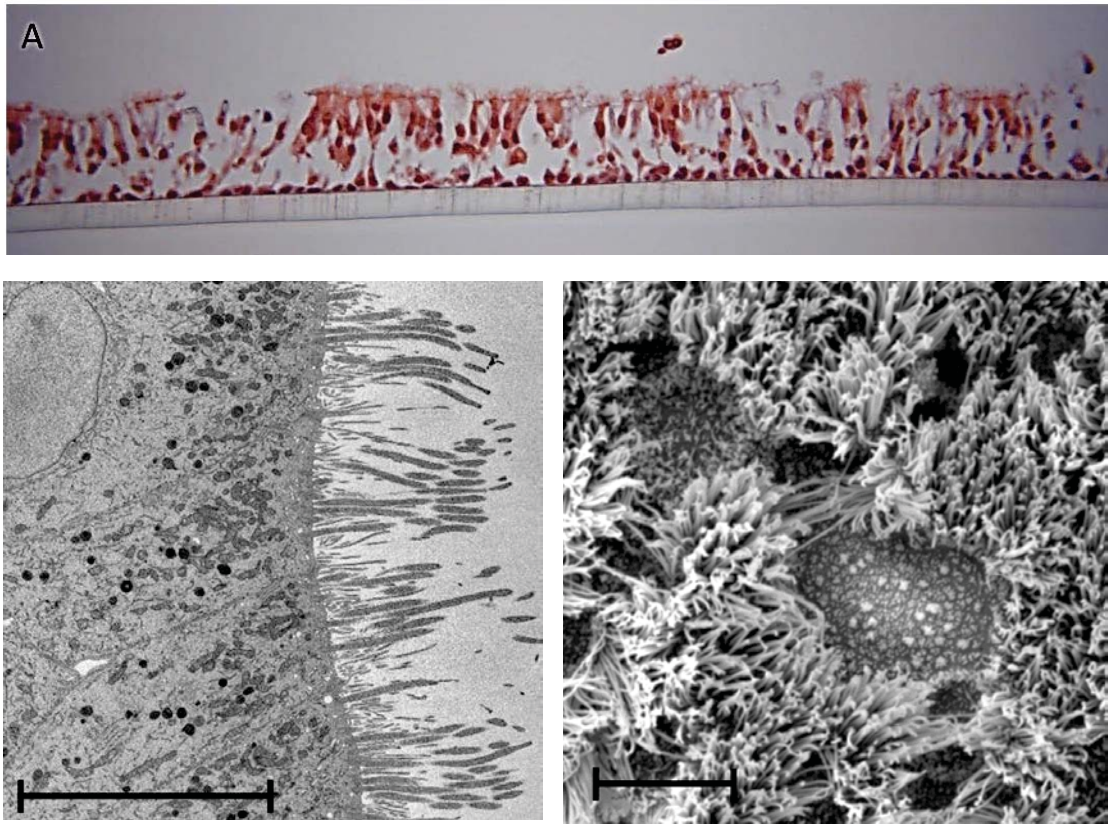
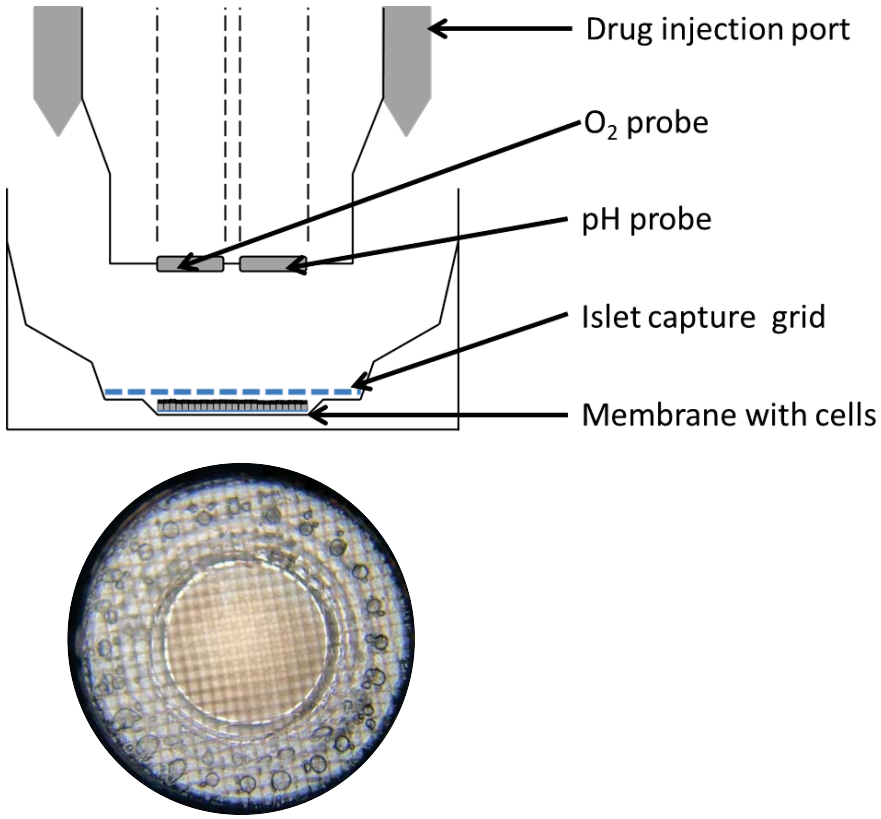
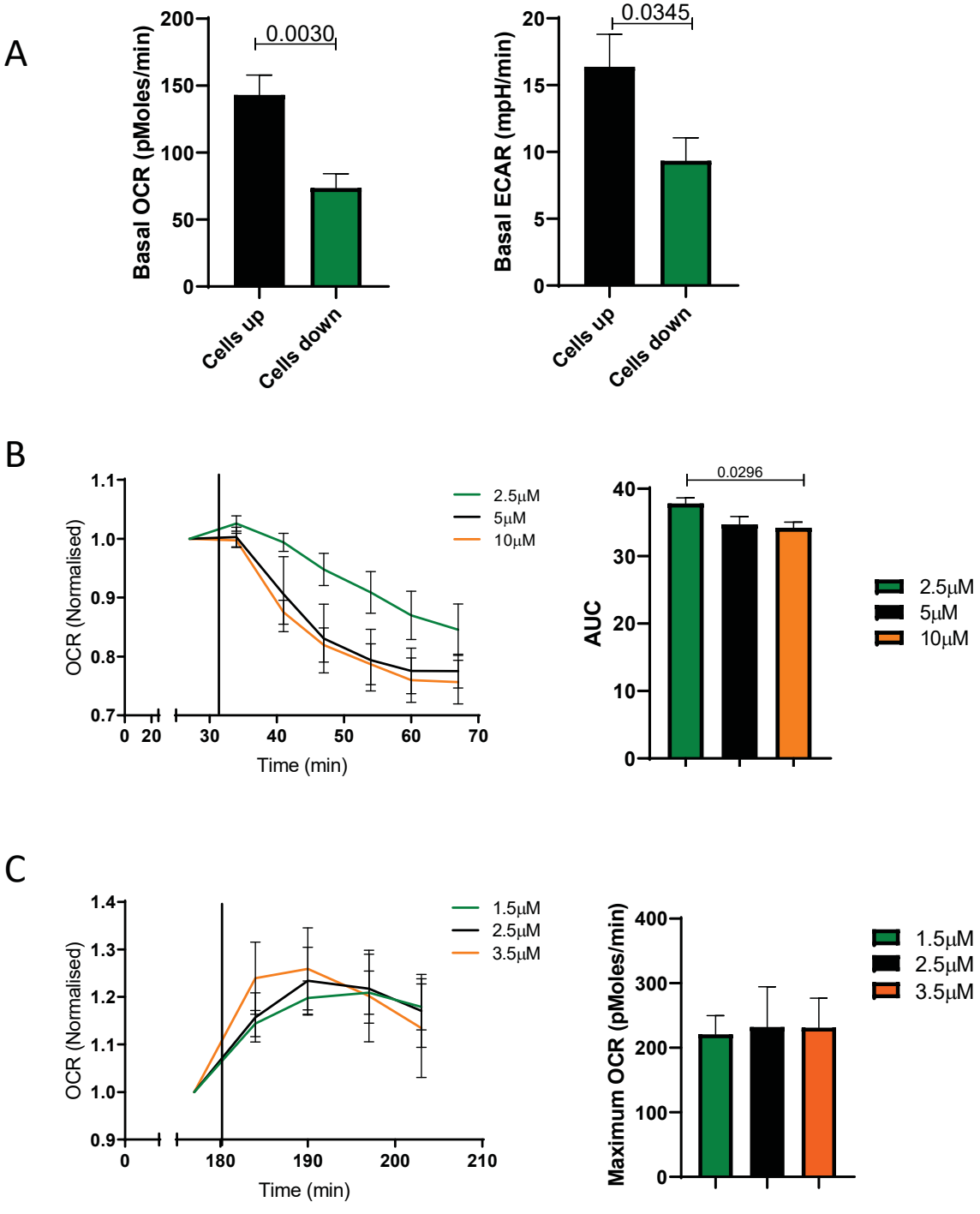
# Figure 1 – Nasal epithelial ALI culture characterisation



# Figure 2 –ALI culture in seahorse plate

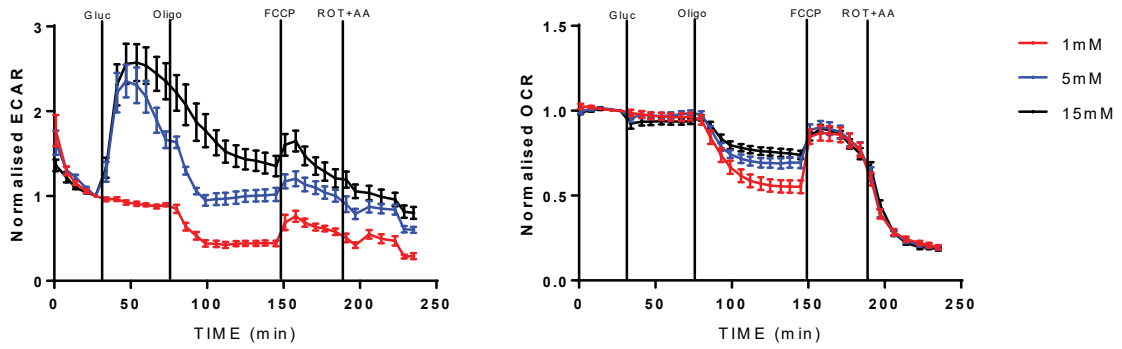


# Figure 3 – Protocol optimisation



# Figure 4 – Metabolic profiling of ALI cells at altered glucose concentration

A

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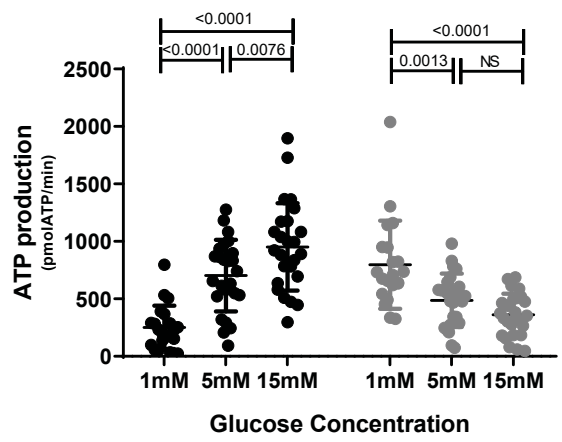
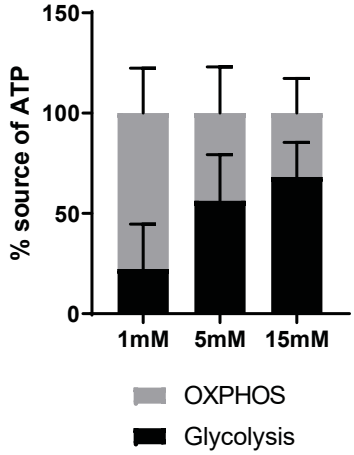
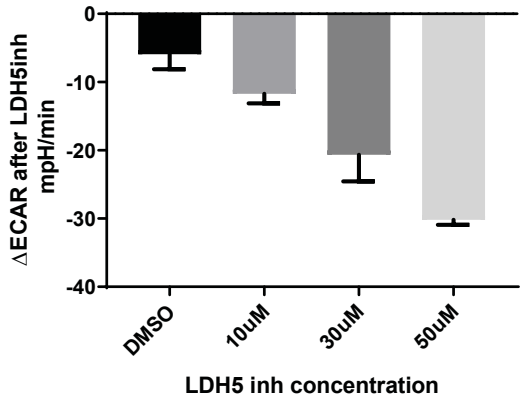



C

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