Precision Digital Dispensing of Patterned Picoliter Quantities of Test Material onto Apical Surfaces of Human 3D Reconstructed Airway Tissues

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ABSTRACT

There is an increasing need for researchers to understand the dynamic aspects of inhaled tobacco product exposure. Exposure-induced events can cause respiratory irritation, sensitization, and other events that may lead to severe pulmonary disease. Available 3D human reconstructed airway tissues (RHuA) provide researchers with a more physiological platform that offers apical and basal compartments for flexibility in modelling relevant exposures. Commercially available instrumentation can generate smoke and aerosols from tobacco products (including E-cigarettes) and expose tissues at ALI, but the quantification of materials deposited at the exposure site remains a challenge. We have tested the Tecan D300 digital dispenser as potential technical solution to deliver precise amounts of very small vehicle droplets to coat the apical surface of an available RHuA. The picoliter volume dispensing allows the direct dilution of vehicle to < 0.1% levels based on estimated RHuA mucous layer volumes. During patterned TPM-dispensing onto apical surfaces of Epithelix MucilAir™ tissues, marker release (including cytokines) and the viability were compared in both the apical and basolateral compartments, after 72 hr exposure. The dispensing precision and accuracy, as well as the effect of direct vehicle (DMSO) or DMSO solubilized TPM patterned dispensing onto apical surfaces were evaluated. No significant adverse effects up to 707 nL total dispensed volume was detected using adenylate kinase release or WST-8 viability assays when using a single dispense. However, the highest volume dispensed (707 nL) did adversely impact ciliary beat frequency. Hand-pipetting onto the apical surface of RHuA induced a variable baseline cytokine response than D300 dispensing, but overall expression was comparable between methods in pilot experiments. This novel technology demonstrated promising results as a method by which the agent to be tested (e.g. derived from tobacco product emissions) was exposed into an ALI-based culture format and onto the apical surface of RHuA tissue. The very low dispense volumes minimize effects on the rheology of RHuA apical surfaces..

INTRODUCTION

The introduction of the D300 Digital Dispenser offers scientists a method by which to rapidly dispense minute quantities of multiple materials into a microwell format. The use of this device allowed a rapid manner by which to create elaborate concentration arrays of multiple agents (e.g. pharmaceuticals) that are now more often being used in combination, and therefore, also require testing in a preclinical setting in combination for efficacy and adverse toxicity. Recently, the technology has been advanced to include a deposition of patterns – in a range of volumes down to low pL quantities per spot. While useful to various settings, its potential application as a means to expose tissues was identified. The increasing use of RHUA tissues for the testing of inhaled materials such as tobacco smoke or e-cigarette vapors (containing harmful, or potentially harmful constituents (HPHCs)) has faced challenges in determining the quantities of materials the tissue is exposed to. The precise delivery of known tobacco constituent concentrations in minute volumes to the apical surface of RHuA tissues may circumvent some of these dosimetry challenges, and offer researchers an alternative exposure system that can be useful in appropriate experimental settings.

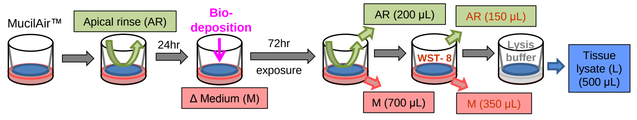
MATERIALS & METHODS

3D RHuA tissues: Epithelix MucilAir™ Dispenser: Tecan D300 Digital Dispenser

- Upon receipt, tissues were refed and acclimated in a humidified incubator at 37°C and >90% humidity
- Cultures were maintained by refeeding every 2-3 days and rinsing the apical surface once per week using 200 µL culture medium
- Inserts designated as the hand pipette controls received 20 µL of culture medium ± treatment
- Test patterns for visual evaluation of accuracy were conducted using red food color (15%) in DMSO, dispensed onto clear 96-well plates (Falcon clear #353072) covered with a plate seal
- Fluorescein dispensing linearity was evaluated using 96-well plates (Perkin Elmer black-walled). Readings were taken on a MDS Flex station at 520 nm.
- For the AK leakage marker, and WST-viability marker assays, the MDS VersaMax plate reader was used.

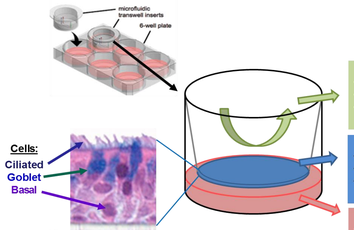
AK Assay: post exposure AR, M, & L **WST-8 assay:** AR & M samples

Ciliary Beating Frequency (CBF): was conducted using the SAVA system)



In Vitro Platform

3D Pulmonary Model: Human Reconstructed Airway (RHuA) Model: MucilAir™



Microphotograph source: http://www.epithelix.com/products/mucilair

Apical Rinse (lavage fluid)
- Inhalation exposures
- Surfactant changes
- Leakage/signaling marker responses (LDH, cytokines, chemokines)

Lysate (tissue)
- Tissue responses (multicellular)
- -omics, biomarker regulation
- Histology – specialty stains, morphology changes

Medium (blood)
- Systemic exposures
- Leakage/signaling marker responses (LDH, cytokines, chemokines)

Benefit of "Compartments"

- 3D RHuA tissues offer 3 different sampling points that collectively, could reflect marker responses reflective to in lavage fluid, the tissue itself, and systemic markers that may be found in the circulation
- Pseudostratification results in a heterogeneous cell types located in different locations
- Non-invasive time point collections (apical rinse, TEER, medium) allow monitoring the progression of events

Dispensing Platform

D300 Digital Dispenser

- Rapid, automated, microwell dispensing

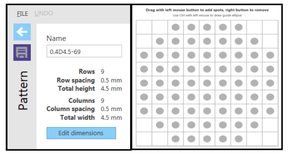
Microwell titrations

- Multiple agents dispensed in an easily designed, matrix of concentrations

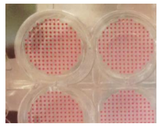
Picoliter volumes

Patterned Titration Creation & Coordinate Verification

Creation of patterns in HP Bio Pattern

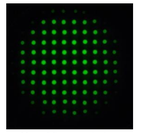


Pilot pattern test



- 0.5 x 0.5 mm "grid" on 96-well plate lid

Fluorescein tracer



- Use of fluorescein to verify droplet placement

Multiwell plate selection

- Numerous plate formats available (e.g. 6-, 12-, 24, 96-well)
- Customizable coordinate system to adjust for unique coordinate requirements (e.g. Costar inserts for MucilAir™)

Successful apical delivery

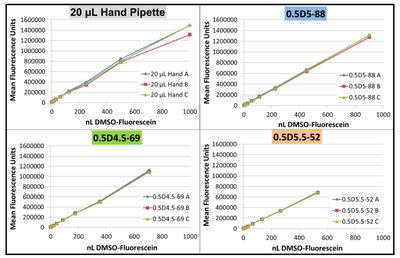
RESULTS

Performance of Patterned Titration:

Table: DMSO Dispense Reproducibility. %CV for MFI values are shown for each method/pattern tested in a trial (n=3 trials, each having 4 wells). Hand pipetting of 20 µL aliquots of F-DMSO containing HBSS is compared to a titration of F-DMSO droplet sizes delivered in three patterns containing different numbers of droplets that are pattern specific.

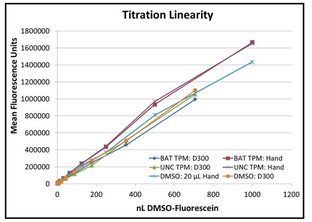
		1.0	2.0	3.9	7.8	15.6	31.3	62.5	125.0	250.0	500.0	1000.0	Ave
20 µL Hand pipette	Target nL												
	Ave Trial %CV:	2.0	1.5	2.2	0.8	1.1	1.1	1.0	0.7	***1.4***	2.6	0.5	1.3
	Inter-trial %CV	7.0	4.3	7.1	3.0	2.7	3.5	2.4	4.1	6.9	3.5	7.3	4.7
0.5D4.5-69	nL/pattern	0.9	1.4	2.8	5.5	11.0	22.1	44.2	88.3	176.6	353.3	706.6	Ave
	Ave %CV:	5.7	1.9	7.6	10.1	11.7	7.5	3.3	3.2	2.8	4.8	5.1	5.8
	Inter-trial %CV	3.8	1.8	1.0	1.3	2.8	2.4	1.9	1.0	1.5	1.8	1.5	1.9
0.5D5-88	nL/pattern	1.1	1.8	3.5	7.0	14.1	28.2	56.3	112.6	225.3	450.6	901.1	Ave
	Ave %CV:	4.6	1.9	7.5	8.8	8.2	6.7	4.2	4.0	3.7	18.6	5.5	6.7
	Inter-trial %CV	2.6	6.8	3.8	1.5	2.8	4.0	5.0	5.9	3.3	2.1	1.6	3.6
0.5D5.5-52	nL/pattern	0.7	1.0	2.1	4.2	8.3	16.6	33.3	66.6	133.1	266.2	532.5	Ave
	Ave %CV:	1.8	1.3	1.6	2.4	2.9	1.5	1.9	1.8	1.5	0.7	11.3	2.6
	Inter-trial %CV	2.5	5.2	4.2	2.5	2.6	3.6	3.9	4.2	1.8	0.8	1.1	3.0

- No consistent spot volume variability differences were noted along the range tested
- Hand pipetting offers low intra-trial CV while digital dispensing offers low inter-trial CVs

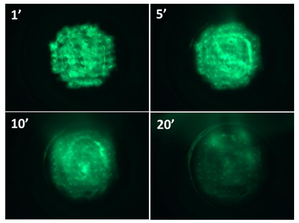


- Accuracy of digital patterns are comparable to hand pipetting
- All patterns have similar linearity of titrated fluorescein signal

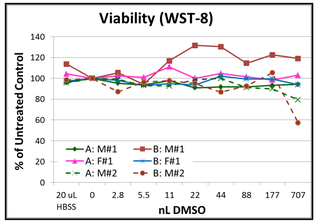
TPM Dispense linearity



Rapid Diffusion and Mixing



Tolerance of Apical DMSO



Digital TPM Exposure

- Direct TPM titration onto the apical surface of RHuA was conducted and up to 34.1 (UNC) or 51.1 µg/cm² TPM was dispensed and same volume amounts of DMSO used as a control.
- The same total TPM amount (11.3 or 17.0 µg, UNC or BAT TPM, respectively) was also applied using hand pipetting in 20 µL HBSS buffer to the apical surface
- Results (data not shown) indicate a lack of TPM effect by both UNC and BAT sources, as well as that from hand-pipetting
- It is expected repeat exposure paradigms will elicit measurable cytotoxic, viability, and inflammatory effects in future studies
- Repeat exposure paradigms using 20µL hand-pipetting may be confounded by hypoxic effects at the apical surface while digital dispensing of very low (nL) volumes may be avoid this complication

% Active CBF Area

Table: Active Area (%) of selected CBF fields.

Donor	Treatment	0.25hr (Pre)	0.25hr (Post)	24hr	24hr (Post)	48hr	48hr (Post)	72hr
F#1	20 µL HBSS	54	3	18	NA	79	NA	73
	0 nL	71	60	69		75		84
	44 nL	76	43	68		47		84
	707 nL	69	1	9		17		26
M#3	20 µL HBSS	17	19	12	2	10	1	15
	0 nL	41	54	55	83	55	47	76
	44 nL	34	50	46	77	52	41	66
	707 nL	64	8	3	0	15	1	1

NA: Not applicable as repeat deliveries were not made to F#1 inserts

- Hand pipetting may diminish the perceived active ciliary beating area by SAVA

Table: Percent of Total AK. A compartment-based breakdown of AK percent of each RHuA insert is given. Apical Rinse (AR), Tissue lysate (TL), and Basolateral Medium (BL) levels are compared.

Donor	M#1 (1x)			F#1 (1x)			M#2 (3x)		
Compartment	AR	TL	BM	AR	TL	BM	AR	TL	BM
Volume	Hand pipette								
20 µL HBSS	0.8	98.6	0.6	0.8	99.2	0.0	0.2	99.7	0.1
nL DMSO	D300 (0.5D4.5-69)								
0	0.7	98.7	0.5	0.5	99.5	0.0	0.0	99.9	0.1
3	1.4	98.3	0.3	0.4	99.6	0.0	0.9	99.1	0.0
6	2.2	95.2	2.5	1.0	99.0	0.0	0.5	99.5	0.0
11	1.0	98.5	0.5	0.4	99.6	0.0	0.2	99.9	0.0
22	0.6	99.1	0.3	0.3	99.7	0.1	0.2	99.8	0.0
44	0.8	98.9	0.3	0.6	99.4	0.0	0.2	99.6	0.2
88	2.0	97.3	0.8	1.5	98.4	0.1	0.6	99.3	0.1
177	1.3	98.2	0.6	6.1	93.4	0.5	0.6	99.3	0.1
707	6.3	87.0	6.6	9.1	81.2	9.7	11.4	86.8	1.8

- Minor increase in apical AK at 707 nL for M#1 and M#2, possibly at 177 nL for F#1

CONCLUSIONS

1. The D300 Digital Dispenser has demonstrated accuracy in dispense volume delivery when using coordinated Bio Pattern patterns.
2. The coordinated delivery of DMSO onto RHuA inserts (MucilAir™) at volumes up to 0.7 µL did not result in loss of viability or substantial loss of AK from the tissue.
3. Using the optimal Bio Pattern pattern for TPM exposures to the apical surface, TPM was also dispensed successfully although a single TPM exposure was insufficient to elicit a toxic effect.
4. This pilot study suggests digital dispensing may have value as an alternative method to deliver precise amounts of test material. The use of a pulmonary surfactant-based aqueous material may further increase the D300's utility.

ACKNOWLEDGEMENTS

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Behrsing HP, et al., The Use of Human 3D Reconstructed Airway Cultures for Tobacco Product Evaluation: Precision Low-Volume Exposures at the Apical Site. ***Applied In Vitro Toxicology*** **2017:3;1-12.**