Evaluating Respiratory Irritants using New Approach Methods: Harmonizing Key Study Design Variables for Air-Liquid Interface and Direct Liquid Application Exposures

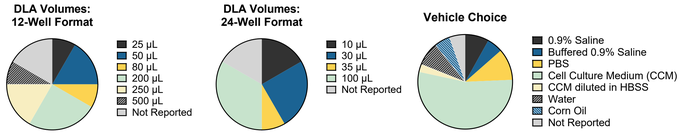
Jessica R. Murray[1], Marc Muraski[1,2], Jonah Herwick[3,4], Joseph Lewis[3,4], Ari Ogunda[3,4], Annie Jarabek[3]

[1]US EPA, OCSPP | OMCO | Risk Assessment Support Division, Research Triangle Park, NC; [2]ORISE, Oak Ridge, TN; [3]US EPA, ORD, Research Triangle Park, NC [4]ORAU, Oak Ridge, TN.

Jessica R. Murray | murray.jessica@epa.gov

Background and Purpose

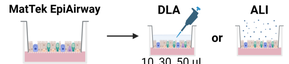
- New Approach Methods (NAMs) are increasingly used to assess the toxicity of inhalable substances, highlighting the need to optimize and harmonize exposure methods for airway cell cultures.
- Several exposure methods are currently utilized for *in vitro* inhalation studies:
  - **Air-liquid interface (ALI)** exposure systems allow direct cell-toxicant interactions, but they are low-throughput and require specialized expertise.
  - **Direct liquid application (DLA)** is a higher-throughput alternative where chemical solutions are applied to the surface of ALI cultures. A literature review revealed DLA methods vary significantly (i.e., volume, vehicle choice – see below), highlighting the need for improved reporting standards and technical characterization.



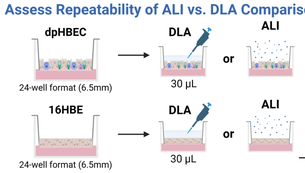
→ This study was designed to evaluate *in vitro* exposure parameters in support of characterizing best practices to screen respiratory irritants using organotypic and immortalized human bronchial epithelial cell (HBEC) cultures using both ALI and DLA exposure methods.

ALI and DLA Exposure Methods

Assess Effect of Multiple Apical Volumes



Assess Repeatability of ALI vs. DLA Comparison



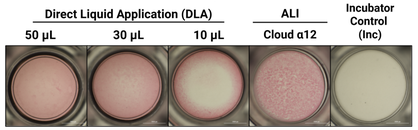
6, 24h

ALI Cell Models
- MatTek EpiAirway (single donor)
- dpHBEC: differentiated primary human bronchial epithelial cells (n=3 donors/group, two donors matched across exposure methods: 1423 + 1498 @ 6h; 1499 + 1500 @ 24h)
- 16HBE: 16HBE14o- human bronchial epithelial cell line

Delivery of Respiratory Irritant
- Dose range optimized for each cell model
- ALI exposures: VITROCELL Cloud α12 with 4-6 µm nebulizer
- DLA exposures in cell culture medium

Toxicity Endpoints
- Trans-Epithelial Electrical Resistance (TEER)
- Viability via ATP Generation
- Cytotoxicity via LDH Release
- Pro-Inflammatory Cytokine Release (IL-1β, IL-6, IL-8)
- TempO-Seq Analysis (Visit #4149 at Poster Board J632 for transcriptomic analysis)

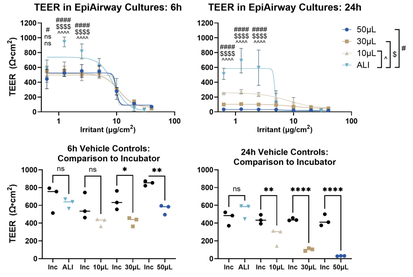
Visualization of Exposure Methods



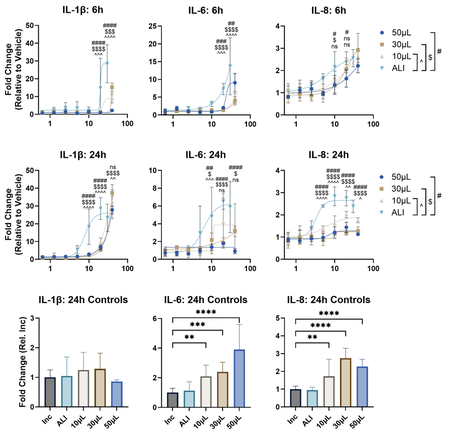
→ Initial cell coverage visualized with Rhodamine 6G

Impact of Volume on Irritant Dose-Response

Barrier Integrity



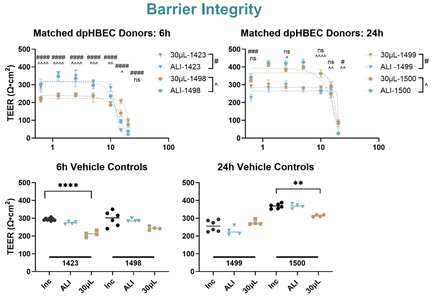
Pro-Inflammatory Cytokine Release



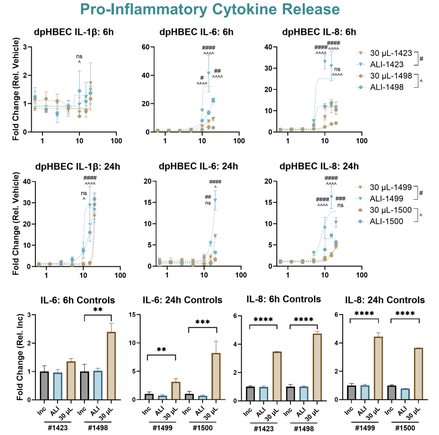
- ALI and DLA exposures yield distinct chemical dose-response curves for epithelial barrier resistance and cytokine release in MatTek EpiAirway tissues.
  -- All exposures resulted in significant loss of TEER and increase in proinflammatory cytokine release due to chemical treatment (test results not shown). Significant differences between exposure methods are denoted as follows: #/$/^ for p<0.05, ##/$$/^^ for p<0.01, ###/$$$/^^^ for p<0.001, and ####/$$$$/^^^^ for p<0.0001 for ALI vs. 50µL(#), 30µL($), 10µL(^), respectively (two-way ANOVA followed by Dunnett's test).
- DLA vehicle controls differed from untreated incubator controls in a volume- and time-dependent manner across some, but not all, endpoints. ALI vehicle controls did not differ from incubator controls.
  -- Significant differences between vehicle and incubator controls are denoted as *, **, ***, or **** for p<0.05, p<0.01, p<0.001, and p<0.0001 (one-way ANOVA followed by Šídák's test).

Repeatability Across Multiple Donors

Barrier Integrity



Pro-Inflammatory Cytokine Release



- Significant variability was observed within dpHBEC data when all donors were averaged, prompting comparison of donor-matched ALI and DLA exposures.
- Some donors experienced loss of barrier integrity or upregulation of pro-inflammatory cytokines due to liquid application, replicating observations from MatTek EpiAirway but highlighting inter-individual variability.

Points of Departure Across Exposure Methods

Timepoint	Endpoint	MatTek EpiAirway BMDL, BMD (µg/cm²) ALI	MatTek EpiAirway BMDL, BMD (µg/cm²) 30 µL	dpHBEC BMDL, BMD (µg/cm²) ALI	dpHBEC BMDL, BMD (µg/cm²) 30 µL	16HBE BMDL, BMD (µg/cm²) ALI	16HBE BMDL, BMD (µg/cm²) 30 µL
6 h	TEER	4.62, 6.58	4.92, 6.58	5.75, 8.48	8.35, 12.16	0.53, 0.93	1.07, 1.65
	Viability	**0.27, 0.82**	**4.38, 5.94**	2.05, 3.13	7.18, 9.82	1.01, 1.31	1.21, 1.55
	Cytotoxicity	3.39, 5.74	18.56, 25.97	3.41, 3.57	16.9, 17.22	0.23, 0.30	3.51, 3.87
	IL-1β	10.01, 15.83	21.58, 33.03	4.09, 16.24	5.98, 19.36	--	--
	IL-6	0.78, 1.48	6.70, 19.03	2.46, 3.25	2.66, 3.89	0.02, 0.06	0.09, 1.00
	IL-8	0.50, 1.19	4.86, 6.29	**0.63, 1.26**	**1.27, 2.07**	0.09, 0.17	0.03, 0.31
24 h	TEER	2.91, 4.48	2.43, 6.24	7.39, 9.50	9.56, 12.50	0.27, 0.45	1.07, 1.49
	Viability	0.83, 1.76	5.24, 6.83	3.56, 4.91	11.8, 17.33	0.70, 1.18	1.32, 1.77
	Cytotoxicity	2.31, 3.19	3.17, 4.89	7.72, 7.83	5.93, 8.32	0.19, 0.24	1.18, 1.58
	IL-1β	2.18, 3.11	**2.12, 3.28**	7.04, 8.04	8.67, 11.98	--	--
	IL-6	1.17, 2.33	5.65, 10.43	4.72, 6.80	**1.54, 4.84**	0.35, 0.53	0.90, 1.93
	IL-8	**0.57, 1.31**	2.41, 7.98	**2.00, 2.74**	3.22, 4.49	0.003, 0.01	0.71, 1.34

- Benchmark dose (BMD) analysis was performed with a BMR of 1 SD. Differences across exposure methods were minimal in organotypic cultures at 24 h when the most sensitive BMDL values were compared (<2.5-fold, see bold values above). Exposure method variables were more likely to influence study outcomes at earlier timepoints and in immortalized cell lines.

Vehicle Effects Observed Across Cell Models following DLA Exposures			
Endpoint	**MatTek EpiAirway**	**dpHBEC**	**16HBE**
TEER	↓, volume-dependent	↓ in some donors	↑ at 24 h
Viability (ATP)	N/A	↓ in some donors	↑
Cytotoxicity (LDH)	N/A	N/A	N/A
Cytokines	↑ IL-6, ↑IL-8 at 24 h, volume-dependent	↑ IL-6, ↑IL-8	N/A

- DLA vehicle controls in organotypic cell models experience significant reduction in barrier function and increase in proinflammatory cytokines compared to untreated controls, which has been previously reported for dpHBEC-fibroblast co-cultures (Mallek et al., *Front. Toxicol.*, 2023).

Conclusions

- This study demonstrated the utility of both ALI and DLA exposure methods to evaluate respiratory irritants and identified key study design variables that can be harmonized (i.e., apical volumes, timepoints) to increase repeatability of study outcomes.
- Applied apical volume remains an important variable for DLA study designs and should be optimized across 6-, 12-, and 24-well formats.
  - Application of 30 µL in a 6.5 mm ALI insert was identified as the minimum volume that provided uniform cell coverage.
- Future studies should explore whether a shortened DLA submersion period could further reduce vehicle toxicities observed in organotypic ALI cultures (e.g., a 4 h liquid application, removal of applied material, and restoration of ALI conditions until the desired timepoint is achieved).

Disclaimer: Poster does not reflect US EPA policy, nor does the mention of trade names of commercial products constitute endorsement or recommendation for use.