Development of an aerosol-compatible cell culture exposure system and its application to quantify cellular uptake of particles at the air-liquid interface

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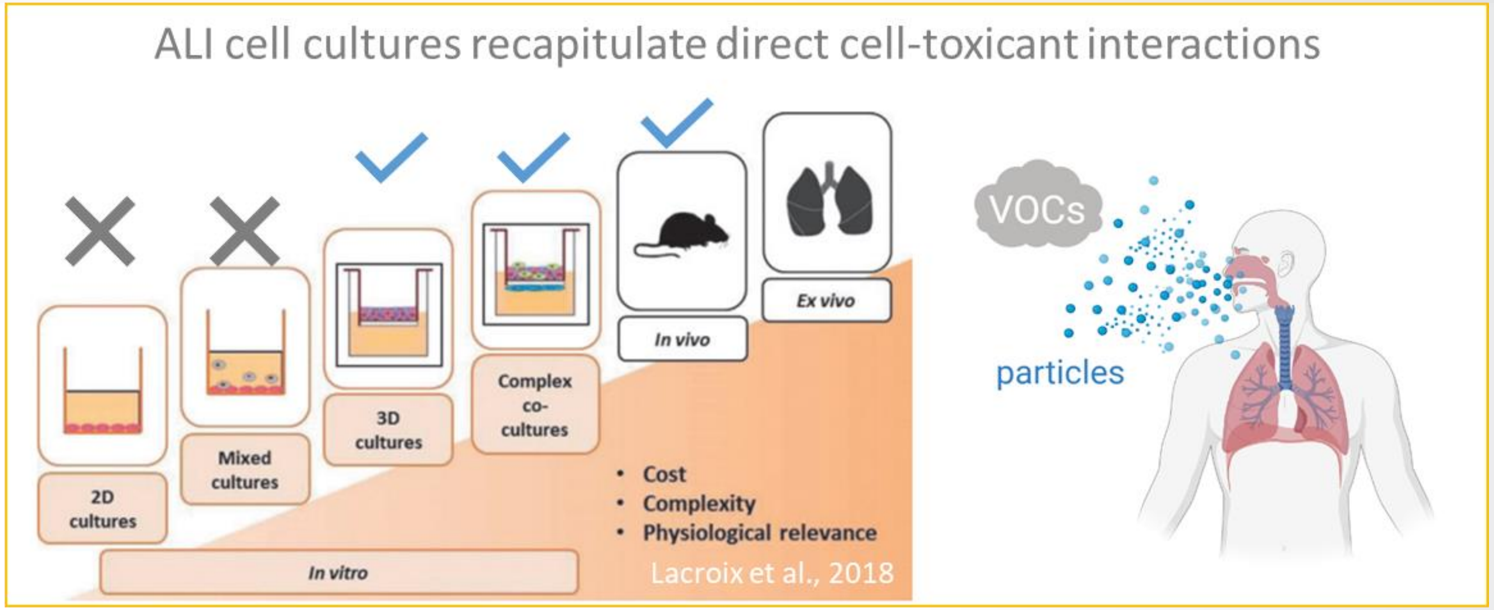
Methodologically Challenging Chemicals Require Advanced Exposure Methods



Over 10% of the Toxic Substances Control Act (TSCA) inventory includes volatile organic compounds (VOCs) and insoluble compounds which are incompatible with high-throughput screening.

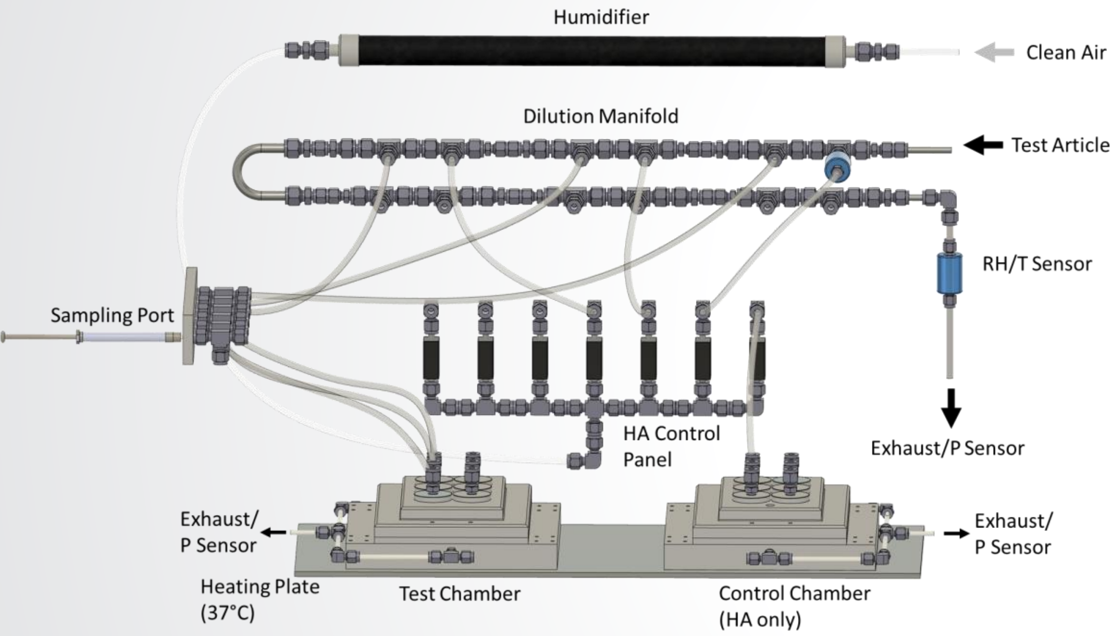
To address this challenge, we need to accomplish the following:

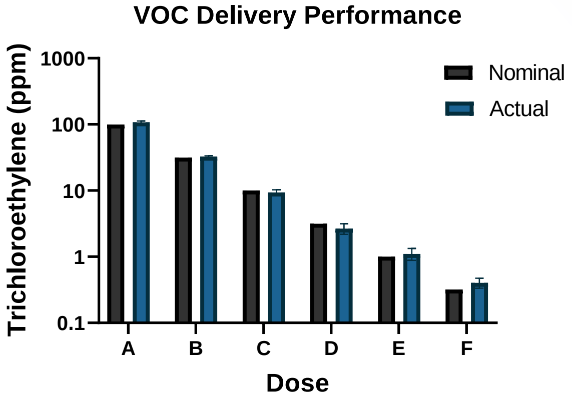
1. Develop ALI exposure technology to include VOCs and insoluble chemicals in screening efforts
2. Create analytical dosimetry methods to quantify deposition and cellular uptake
3. Identify appropriate human lung cell models and endpoints to protect human health

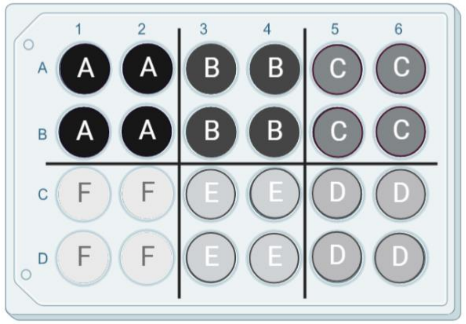
(Lacroix et al., Appl In Vitro Toxicol. 2018; Merrick et al., *Int J Biotechnol.* 2015)

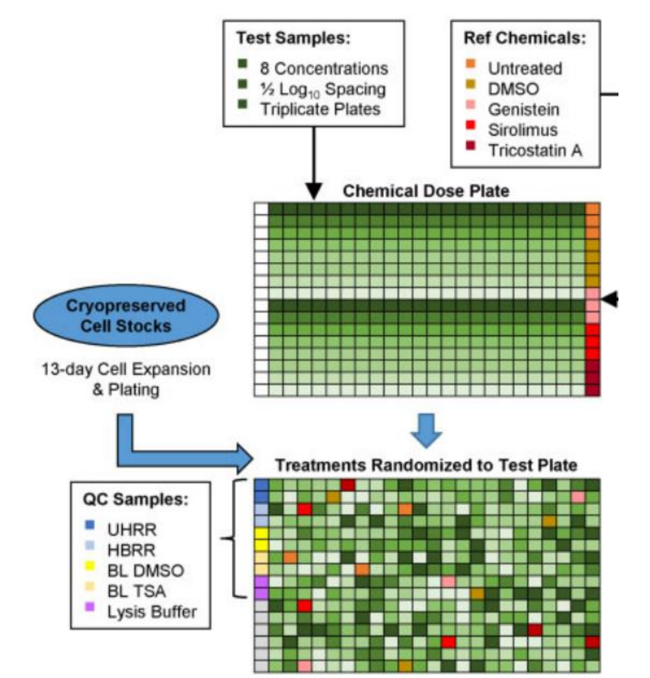
EPA

EPA Cell Culture Exposure System (CCES)









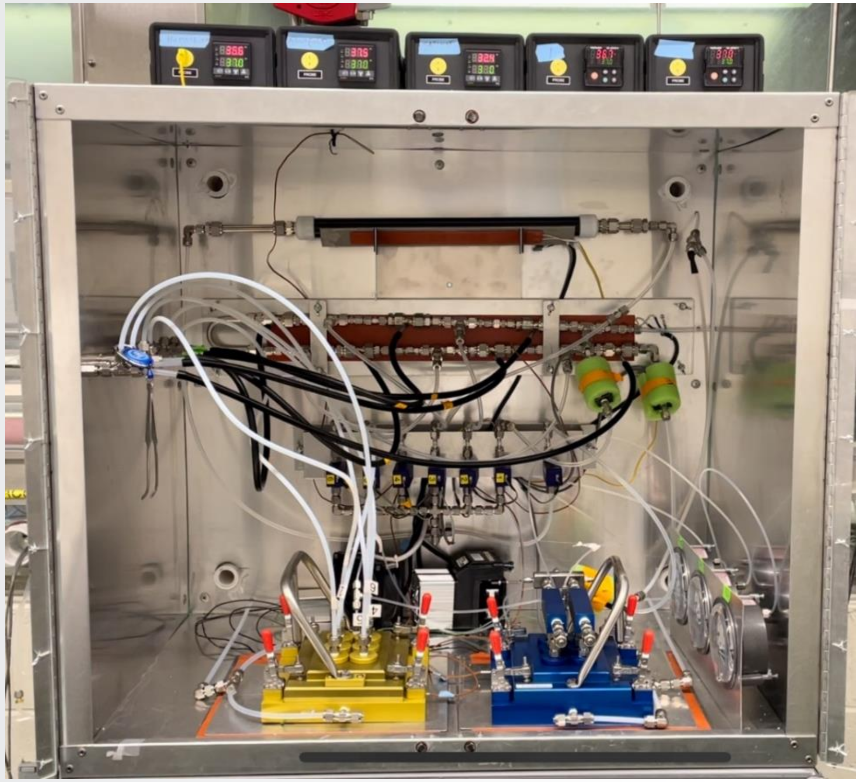
Harrill et al., *Toxicol Sci.* 2021

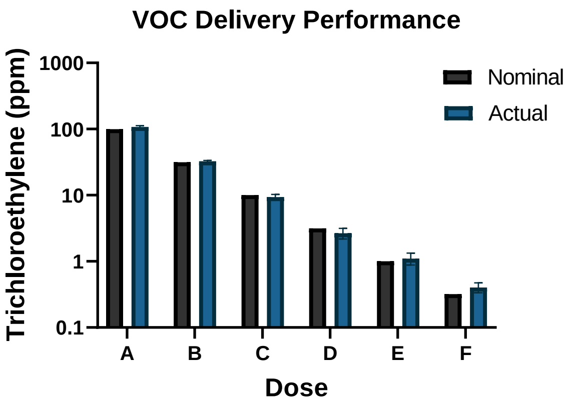
The Inhalation Toxicology Facilities Branch (ITFB) developed the EPA's **Cell Culture Exposure Systems (CCES)** which permits dynamic exposure of human lung cells to VOCs at **air-liquid interface (ALI).**

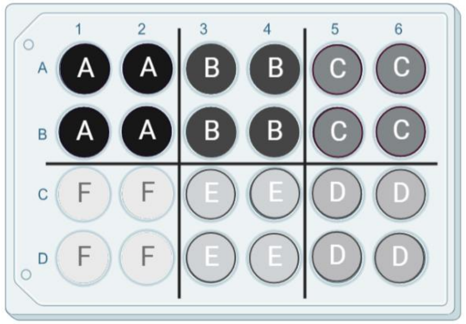
- Medium-throughput: 6 doses + 4 technical replicates within standard 24-well cell culture plate
  - Allows Benchmark Dose (BMD) modeling to estimate *in vitro* Points of Departure (PODs) for portal of entry effects
- Real-time sampling allows accurate exposure conditions to be reported throughout 2 h exposure

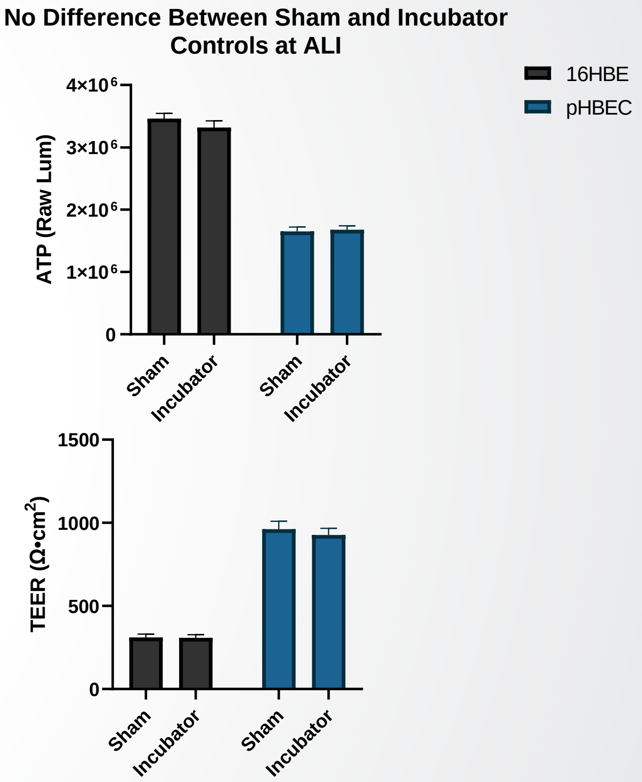
(Zavala et al., *Toxicol Res*. 2017; Zavala et al., *Inhal Toxicol.* 2018)

EPA Cell Culture Exposure System (CCES)









- Heated enclosure is key to maintaining 37°C and >80% RH throughout 2 h exposure condition
- No changes in viability or TEER observed after 2 h exposure in CCES

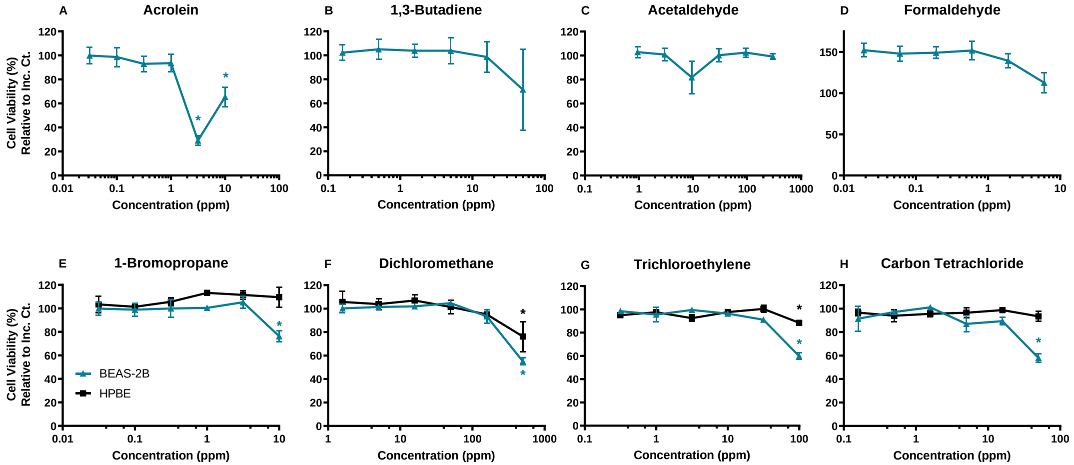
(Zavala et al., *Toxicol Res*. 2017; Zavala et al., *Inhal Toxicol.* 2018)

BMD Values Share Similarities to TLV Rank Order Following VOC Exposures at Air-liquid Interface (ALI)

Benchmark Dose Modeling Approaches for Volatile Organic Chemicals Using a Novel Air-Liquid Interface *In Vitro* Exposure System

Adam M Speen ✉, Jessica R Murray, Quentin Todd Krantz, David Davies, Paul Evansky, Joshua A Harrill, Logan J Everett, Joseph L Bundy, Lisa A Dailey, Jazzlyn Hill ... Show more

Toxicological Sciences, Volume 188, Issue 1, July 2022, Pages 88–107, https://doi.org/10.1093/toxsci/kfac040



Exposure Regimen	2 h exposure at ALI in 24-well format, endpoints analyzed 4 h later
Endpoints	Viability (ATP), n=2; Cytotoxicity (LDH), n=4; TempO-Seq (n=2)
Biological Replicates	Conducted over three days, n=3

Benchmark Dose Analysis:

- HTTr TempO-Seq analysis at sub-cytotoxic concentrations
- Comparative to representative *in vivo* LOAEL/NOAEL values
- Within a magnitude of ACGIH occupational exposure TLVs

Chemical Name	BEAS-2B Median BMD (ppm)	HPBE Median BMD (ppm)	Representative LOAEL (ppm)	Representative NOAEL (ppm)	TLV (ppm)
Acrolein	**0.586**	--	0.25	NR	0.1
1-Bromopropane	**2.246**	**N/A**	62.5	250	0.1
Formaldehyde	**N/A**	--	2	1	0.3
1,3-Butadiene	**13.979**	--	625	200	10
Carbon Tetrachloride	**9.563**	**N/A**	20	5	10
Acetaldehyde	**N/A**	--	400	150	25
Trichloroethylene	**44.842**	**28.148**	50	25	50
Dichloromethane	**142.127**	**226.73**	8400	4200	100

Nominated List Includes Non-Volatile Chemicals

Office of Pesticide Programs (OPP) and Office of Pollution Prevention and Toxics (OPPT) nominated the following list for further evaluation:

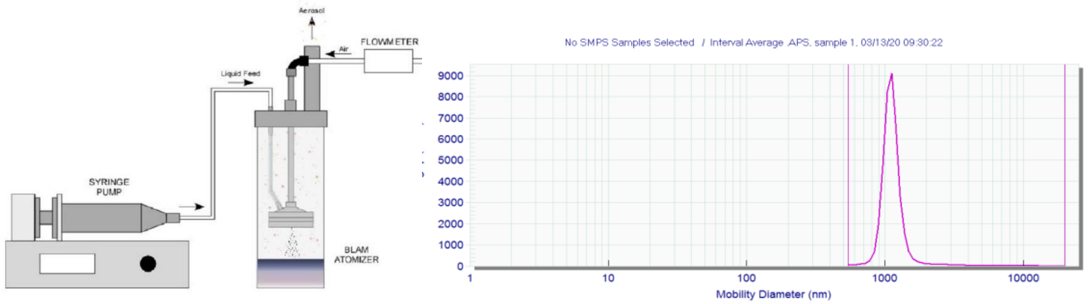
- Didecyl dimethyl ammonium chloride: *antiseptic/disinfectant*
- Polyhexamethylene guanidine-phosphate: *disinfectant*
- O-phenylphenol: *biocide used as preservative*
- Metribuzin: *herbicide*
- Tetramethrin: *insecticide*
- Indoxacarb: *pesticide*
- Naled: *insecticide*
- Oxamyl: *pesticide*
- Azoxystrobin: *pesticide & fungicide*
- Zinc pyrithione: *fungistatic & bacteriostatic*

Prioritized given widespread use during COVID-19 pandemic

Science.org "Does disinfecting surfaces really prevent the spread of coronavirus?"

Must be generated as aerosols: utilized a Blaustein Atomizer Module (BLAM) paired with syringe pump to generate liquid aerosols at high particle concentrations with a narrow particle size distribution

Transport Physics and Deposition Mechanisms Differ Between VOCs and Particles



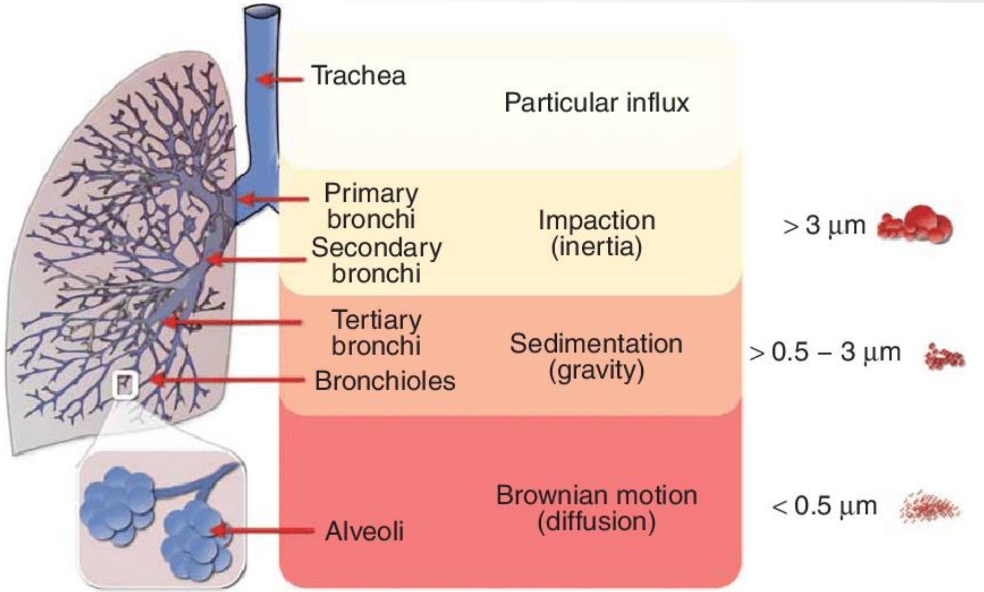
$$m_p \frac{d\vec{v_p}}{dt} = -f_{ric}(\vec{v_p} - \vec{v_f}) + m_p \vec{g} + \vec{F(t)}$$

aerosols

nanoparticles/ VOCs

Langevin Equation for Transport

- Particle Acceleration (≥0.5 μm)
- Gravitation forces (>0.5 μm)
- Diffusion/Brownian Motion (<<<0.5 μm)



Klinger-Strobel et al, *Expert Opin Drug Deliv*. 2015

Convection-Diffusion Equation:

$$\underbrace{\frac{\partial C_p}{\partial t}}_{\text{Mass change in the system}} = \underbrace{-\nabla \cdot (C_p \vec{U}_{air})}_{\text{Transport deposition by convection}} + \underbrace{D\nabla^2 C_p}_{\text{Transport deposition by diffusion}} \underbrace{-\nabla \cdot (C_p \vec{U}_{particle})}_{\text{Transport deposition by external forces}}$$

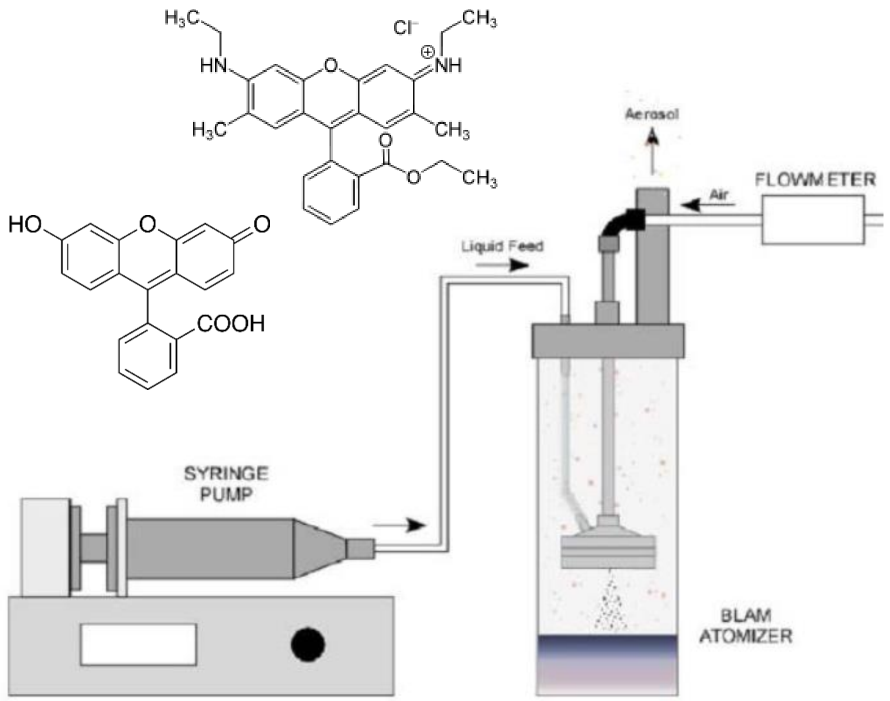
Diffusion coefficient; Particle concentration; Particle Velocity

Deposition = Impaction Diffusion Sedimentation

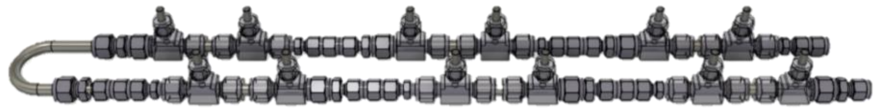
Tsuda et al., *Compr Physiol*. 2013

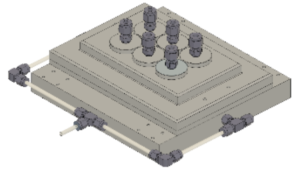
Complementary Methods to Examine Particle Delivery

1. Aerosolize Fluorescent Tracer



2. CAD + CFPD Modeling





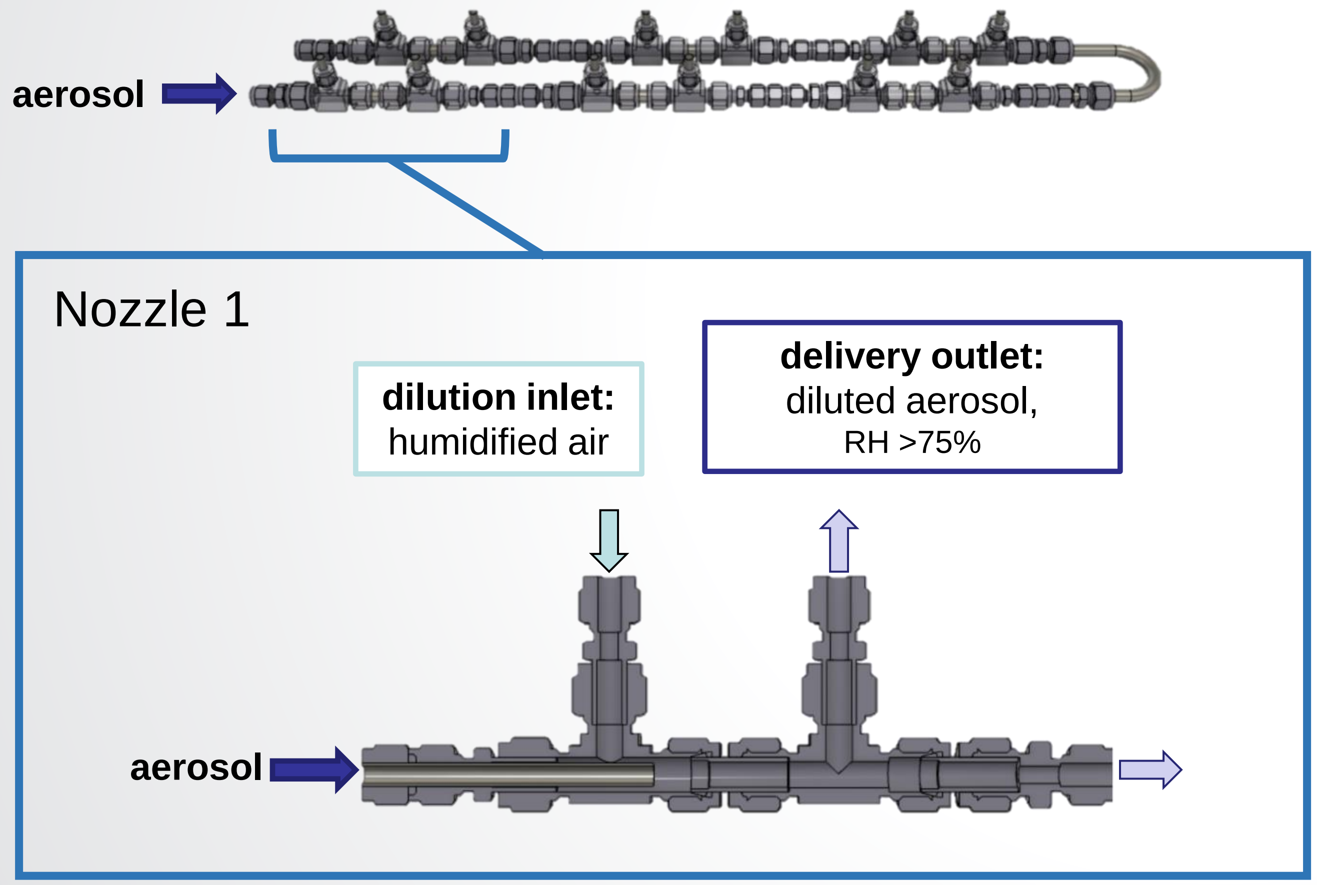
Navier-Stokes Equation for Incompressible Flow

$$\frac{d}{dt}\int_{\Omega} \rho d\Omega + \int_{S} \rho(\vec{V} \cdot \vec{n}_S) dS = 0$$

$$\frac{d}{dt}\int_{\Omega} (\rho\vec{V}) d\Omega + \int_{S} (\rho\vec{V})(\vec{V} \cdot \vec{n}_S) dS + \int_{S} (\vec{\vec{\tau}} \cdot \vec{n}) dS = \int_{S} (-p\vec{n}) dS$$

- Computer Aided Design (CAD) utilized to create replicas of exposure system
- Computational Fluid-Particle Dynamics (CFPD) Modeling applied: Eulerian-Langrangian approach
- *Limitations:* System components must be modeled separately to minimize computational expense

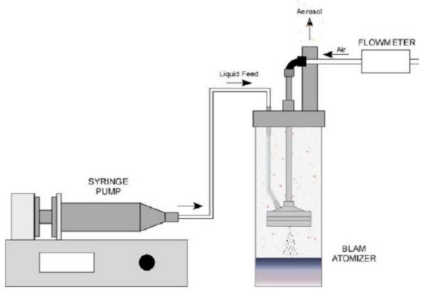
CAD Models of CCES Dilution Manifold for CFPD Simulation

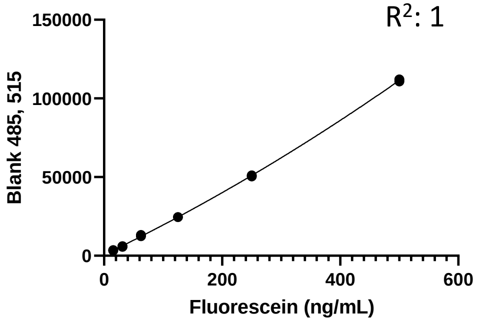


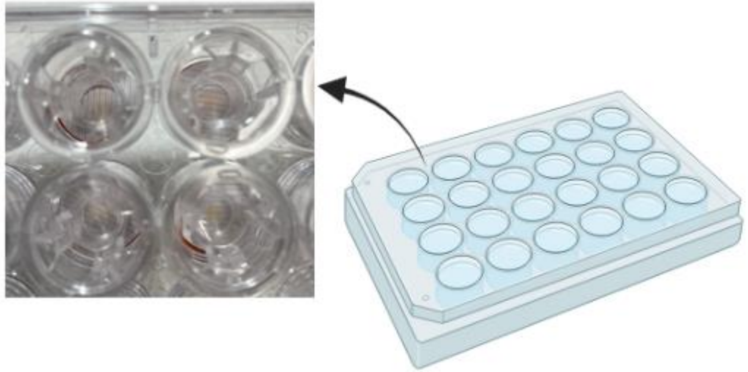
CFD Boundary Conditions & Assumptions	
Software	ANSYS Fluent
Dilution Inlet Flows	Define velocity to match CCES Operational Parameters
Delivery Outlets	Define negative velocity to match CCES Operational Parameters
Main Exhaust	Define pressure, P = 0
Wall	Constant temperature (37°C), "no slip" boundary condition
Turbulence Model	Laminar, Re < 20
Particle Movement	Discrete Phase Method, assumes particles ≤10% of total flow

Aerosol Incompatibility of Original VOC System

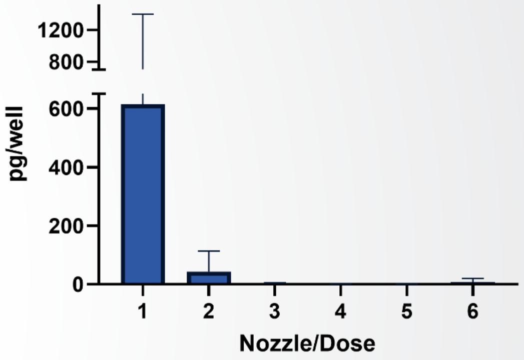
1. Aerosolize Fluorescein, MMAD: 1.3 µm







Fluorescein Deposition: Original VOC CCES



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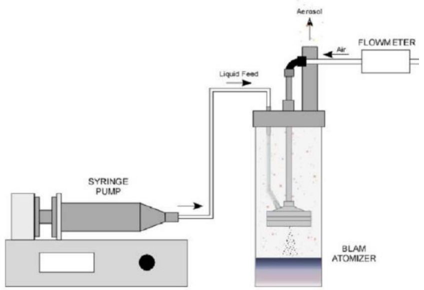

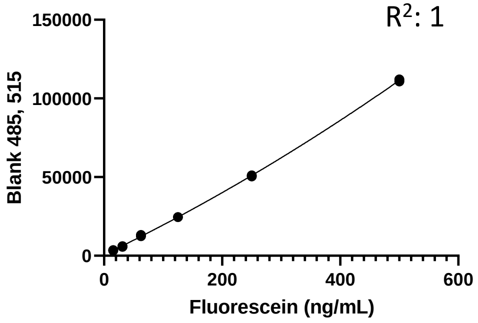

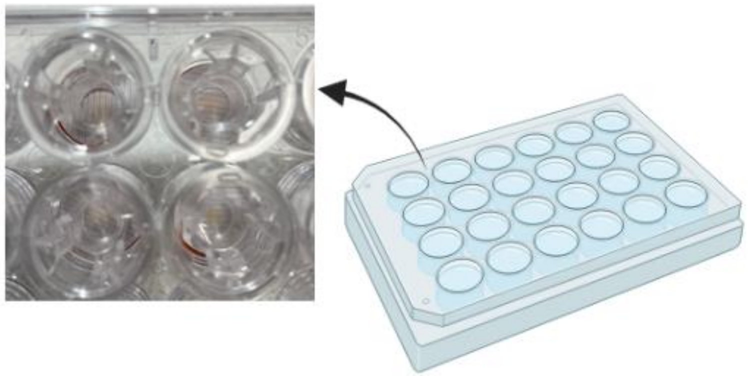

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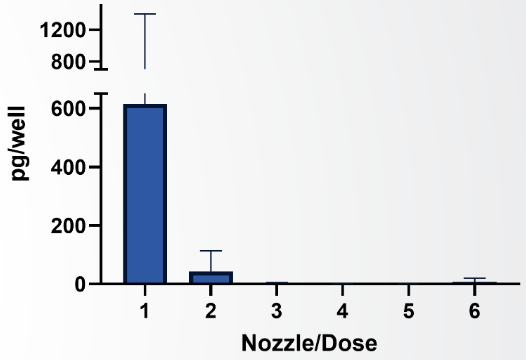
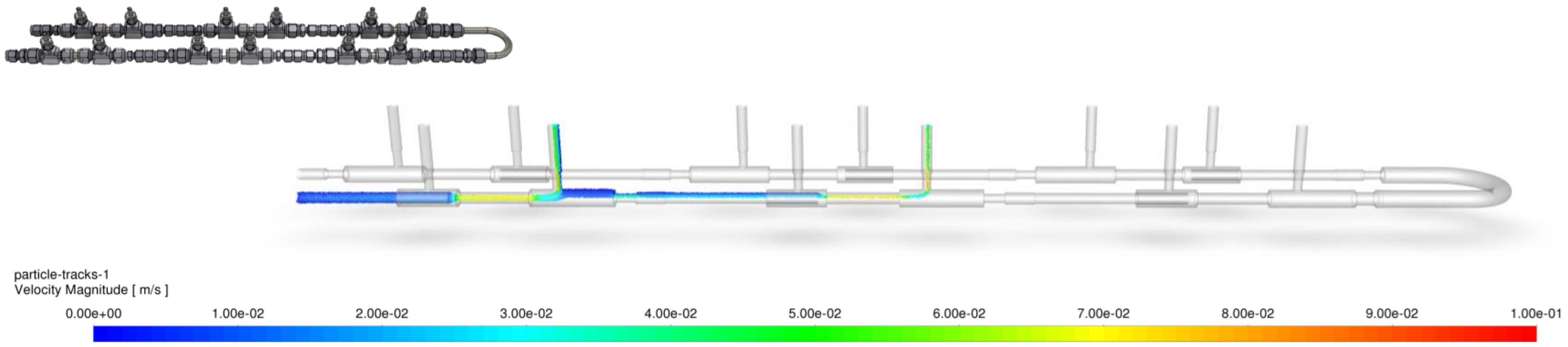
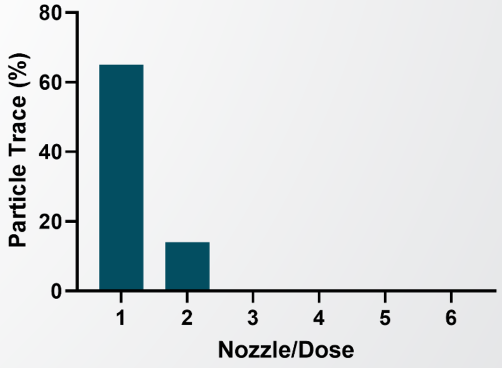


2. CFPD simulation of particle distribution centered at MMAD: 1.3 µm, ρ: 1.602 g/mL

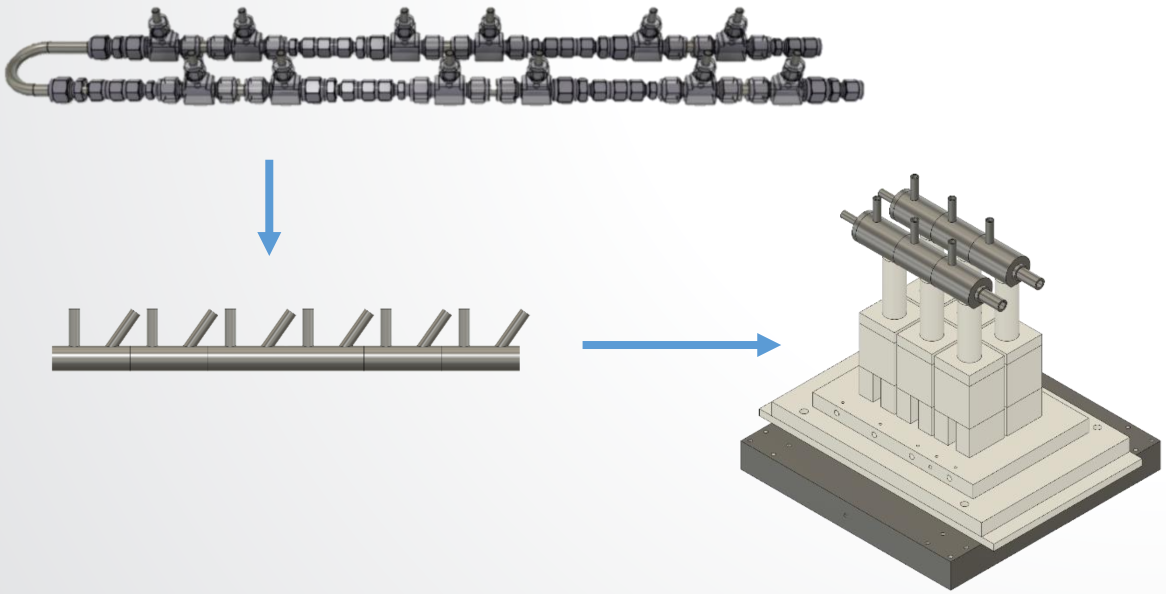


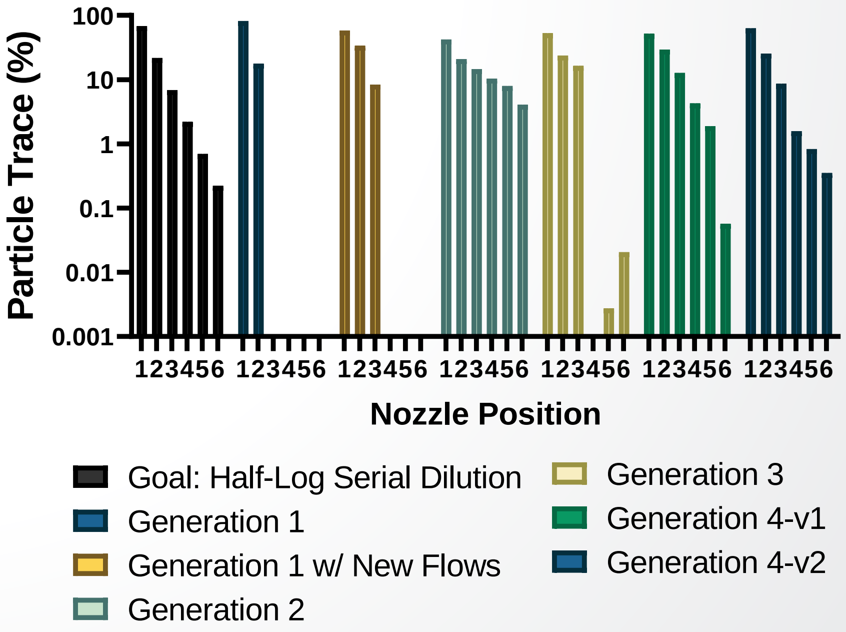
CFD-DPM Particle Trace: Original VOC CCES



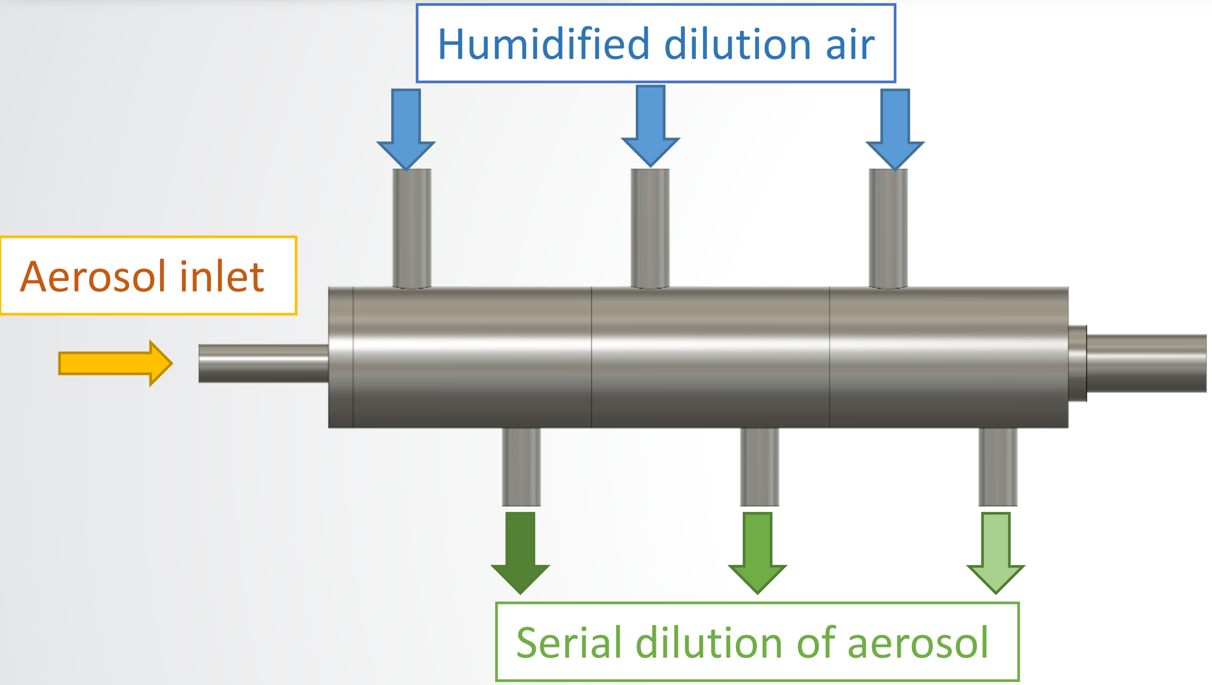
CAD and CFD Streamline Prototype Testing

Examine VOC-CCES with CFD → Build new CAD protypes → Examine new protypes with CFD → Machine best CADs for fluorescein testing

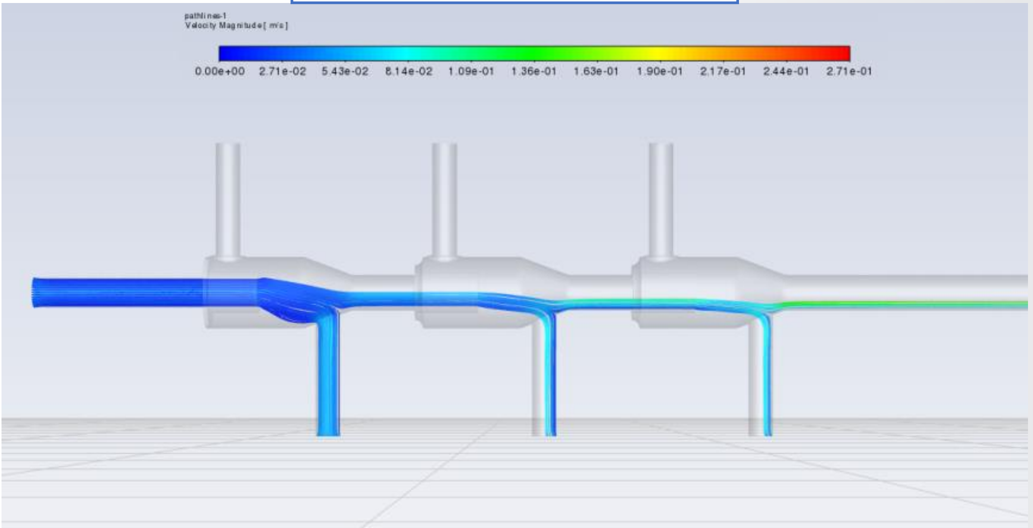




CFD Predicted Performance of New Dilution Manifold



CFPD: Particle Trace



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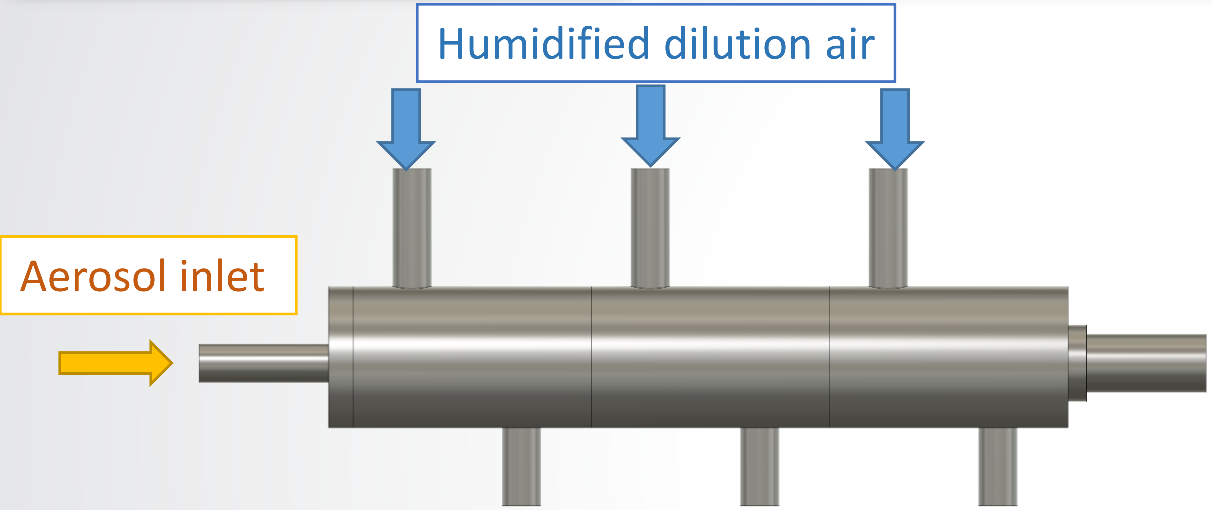

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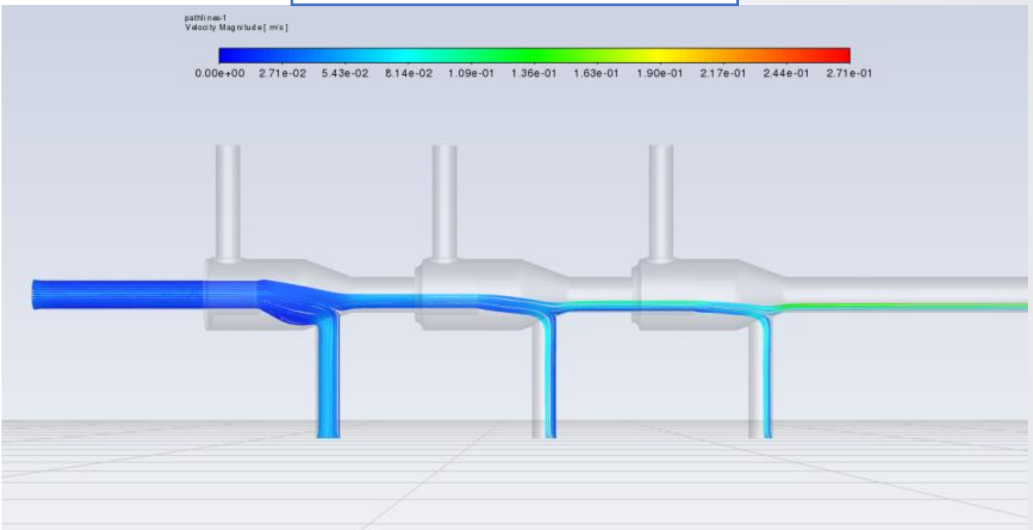
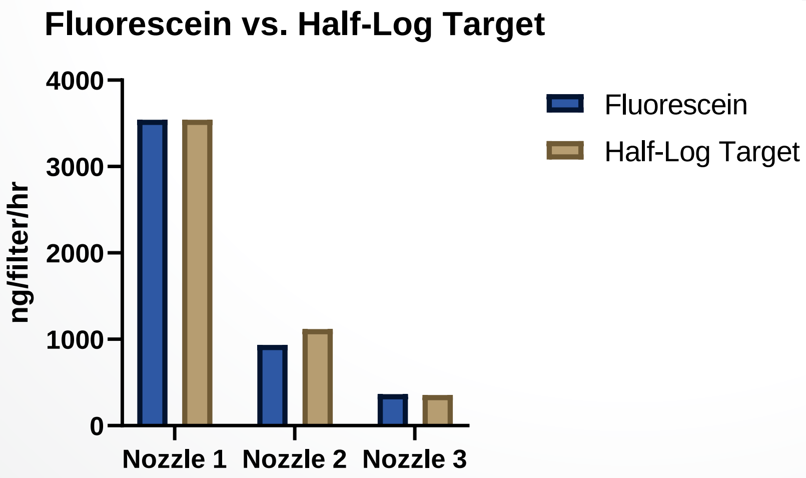
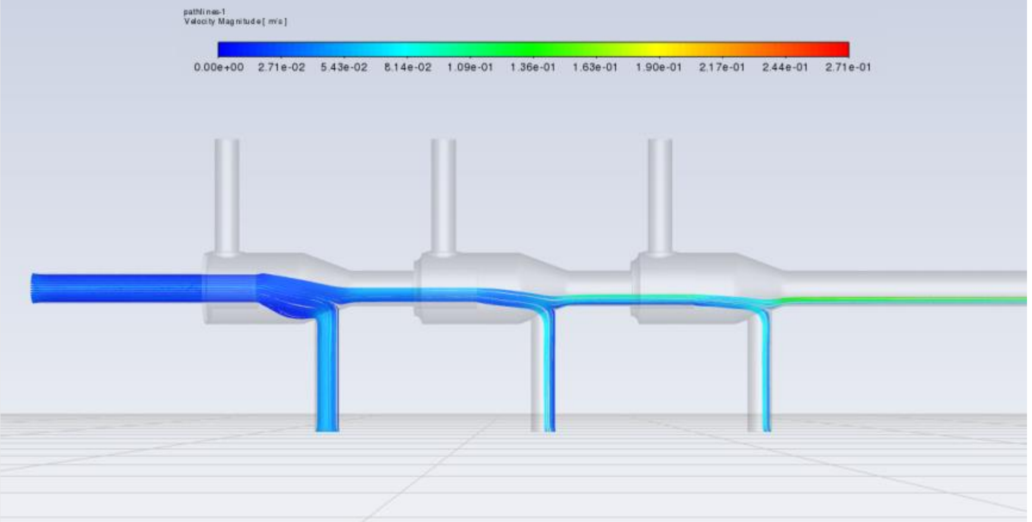


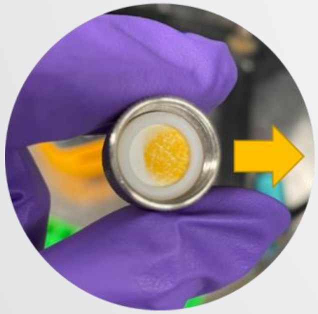


Asymmetrical Aerosol Flow Leads to Flow Splitter Failure

CFPD: Particle Trace

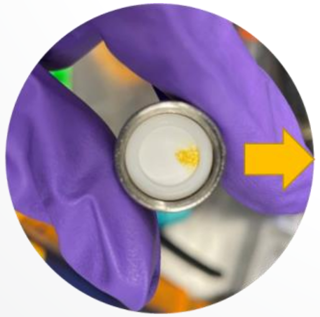


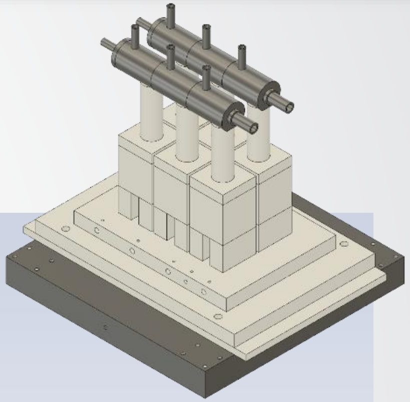
Dose 1

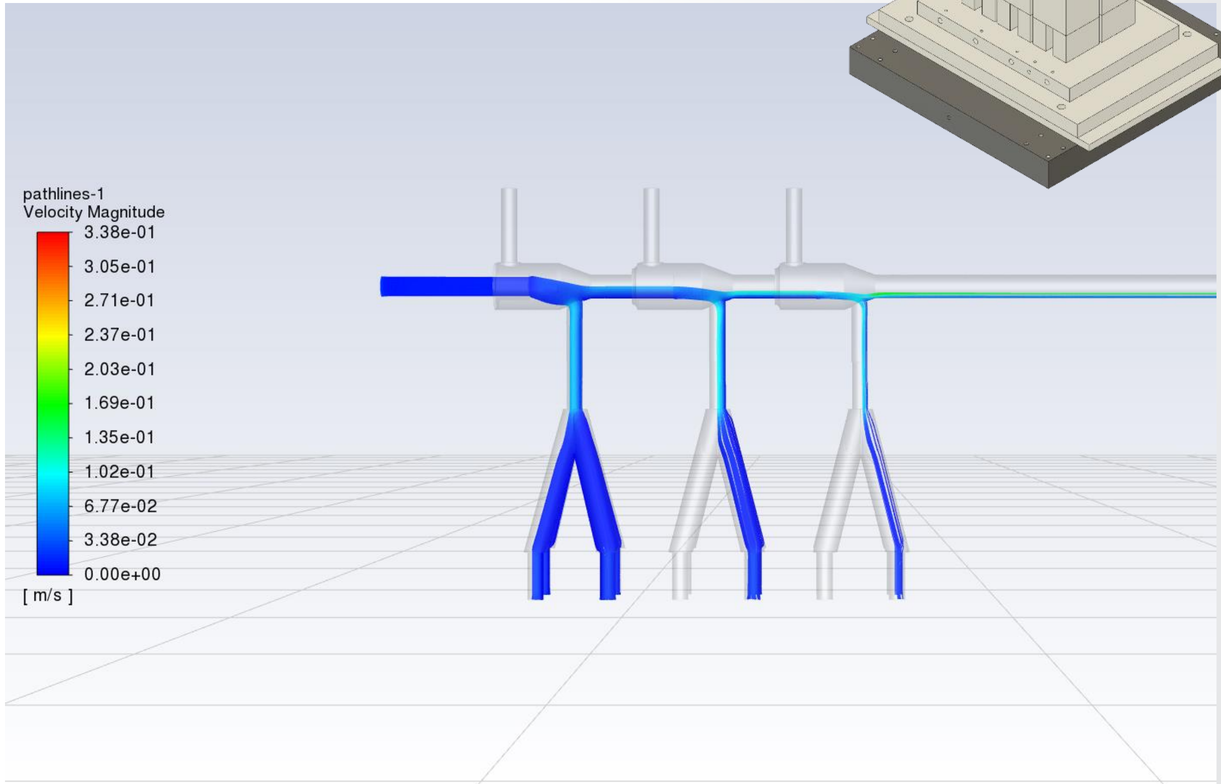


Dose 2

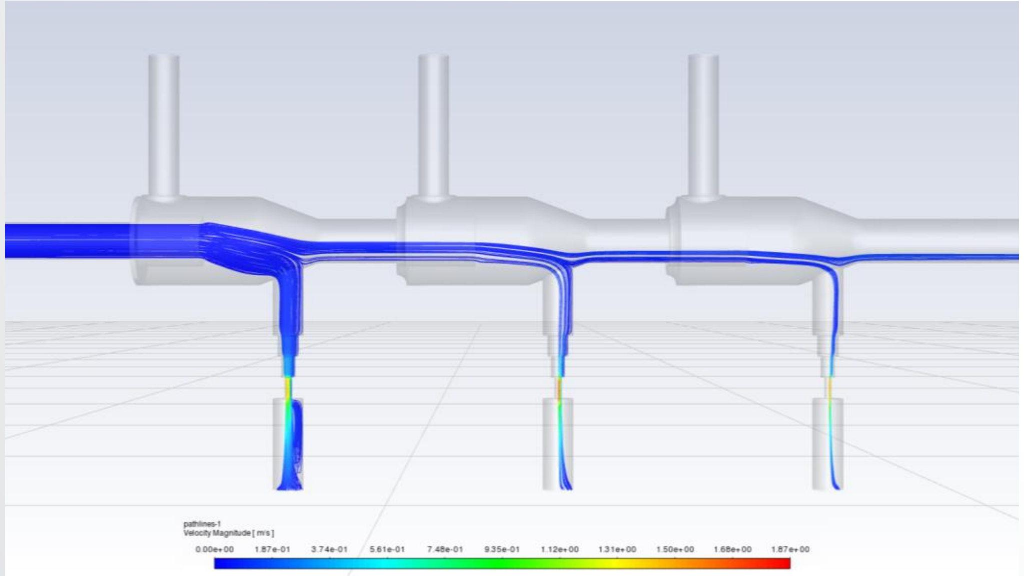
Dose 3

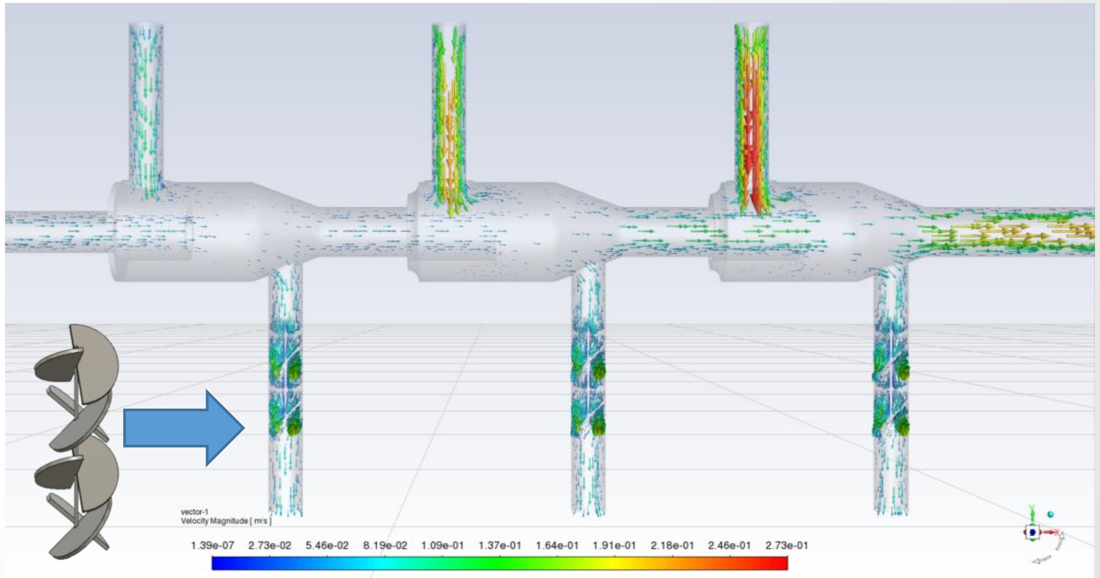




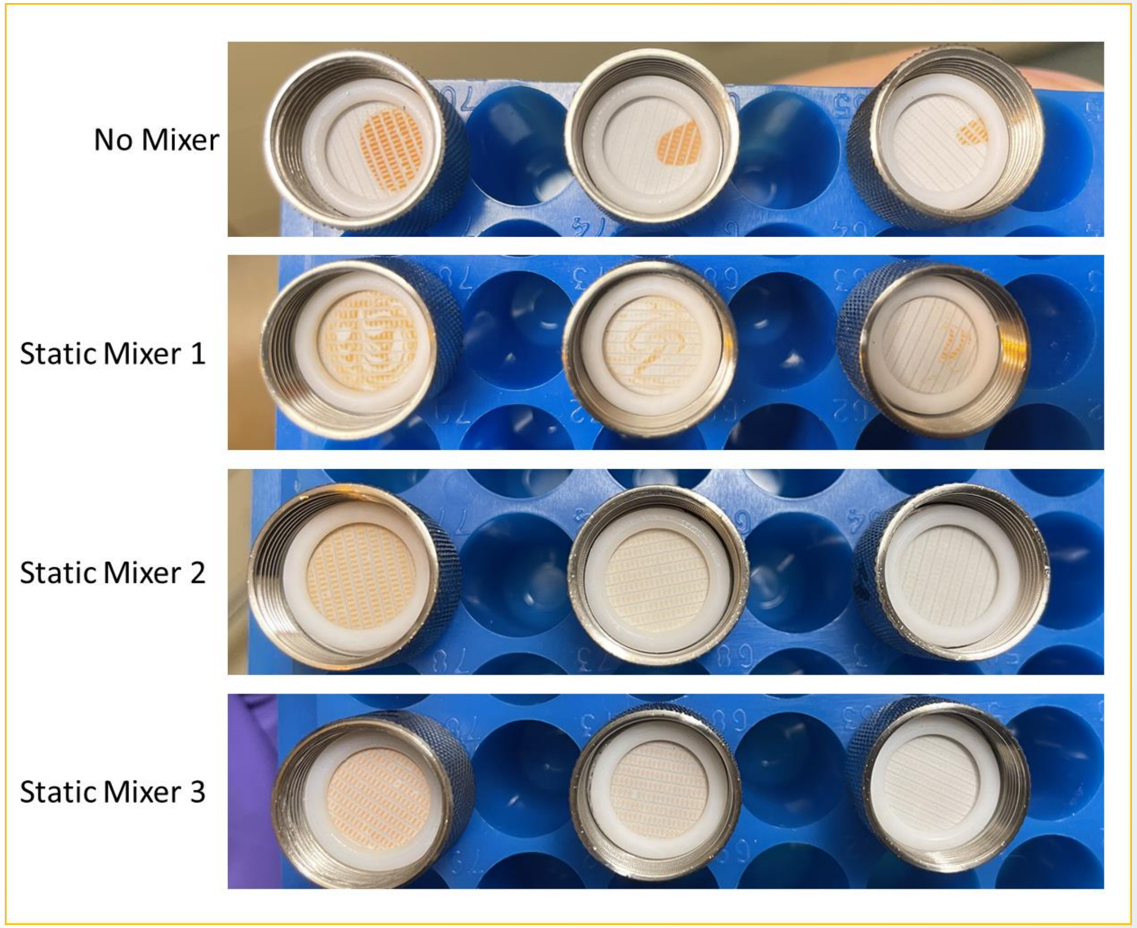


CAD and CFD Modeling Workflow Provide Solutions

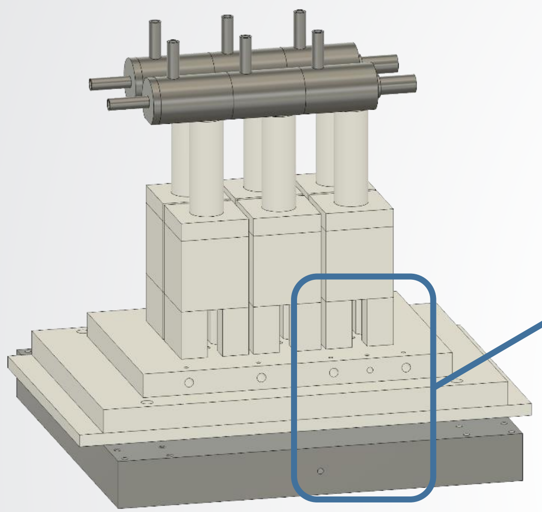


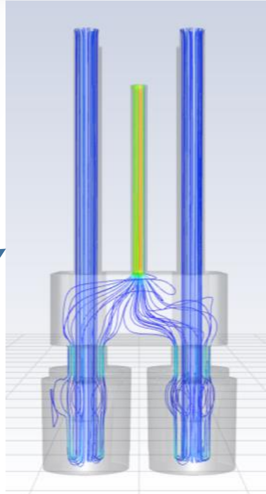


Fluorescein Deposition Patterns on Filters Confirms CFD Predictions



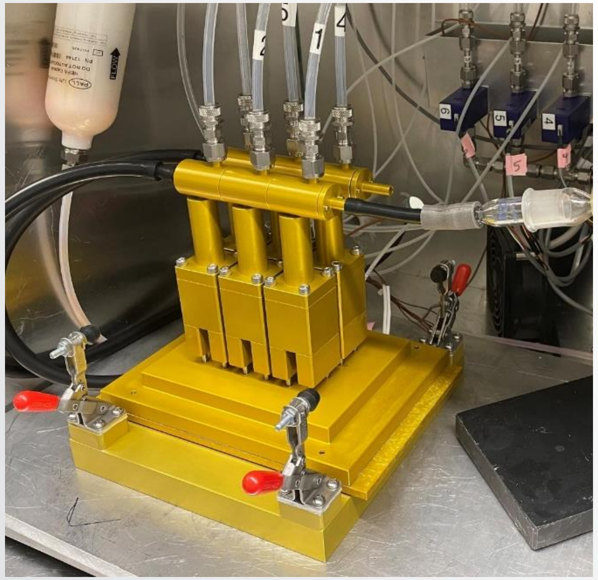
CFD-DPM Modeling vs. Empirical Testing for Full System

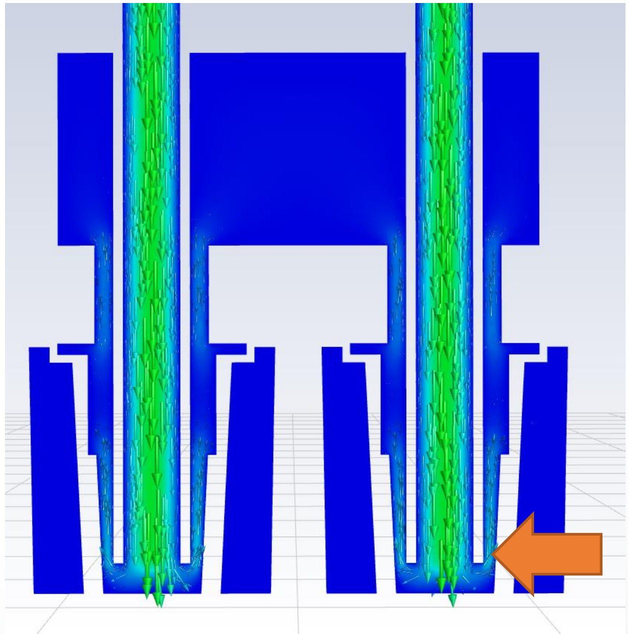




CFD + Discrete Phase Method

- DPM → Wall Film vs. Trap boundary conditions tested to estimate deposition
  - DPM impingement is based on Weber number, which does not consider electrostatic forces
- Very time intensive: 12-24 h+ per simulation





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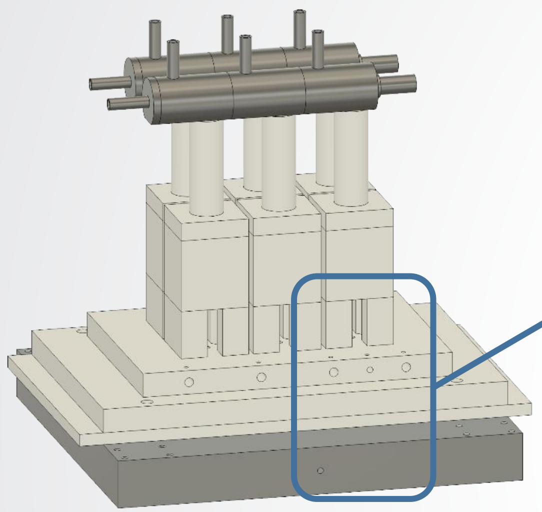

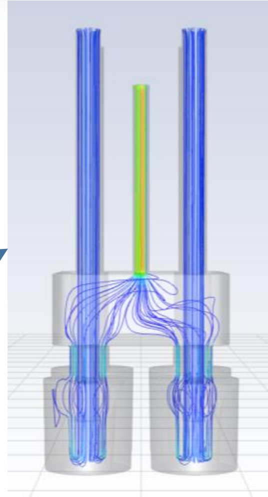

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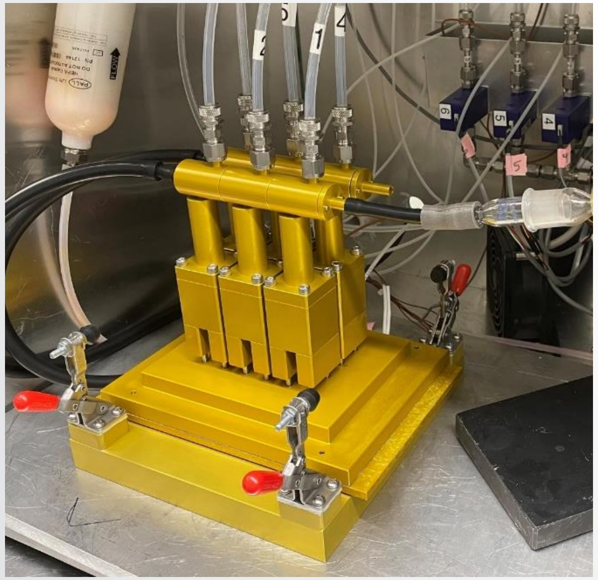
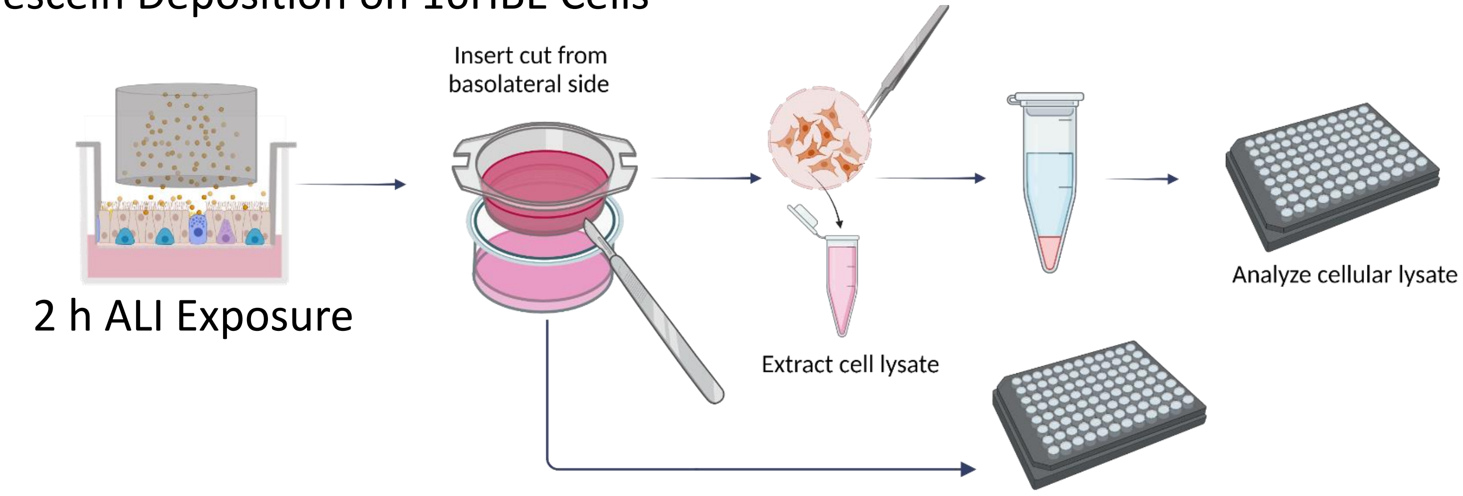
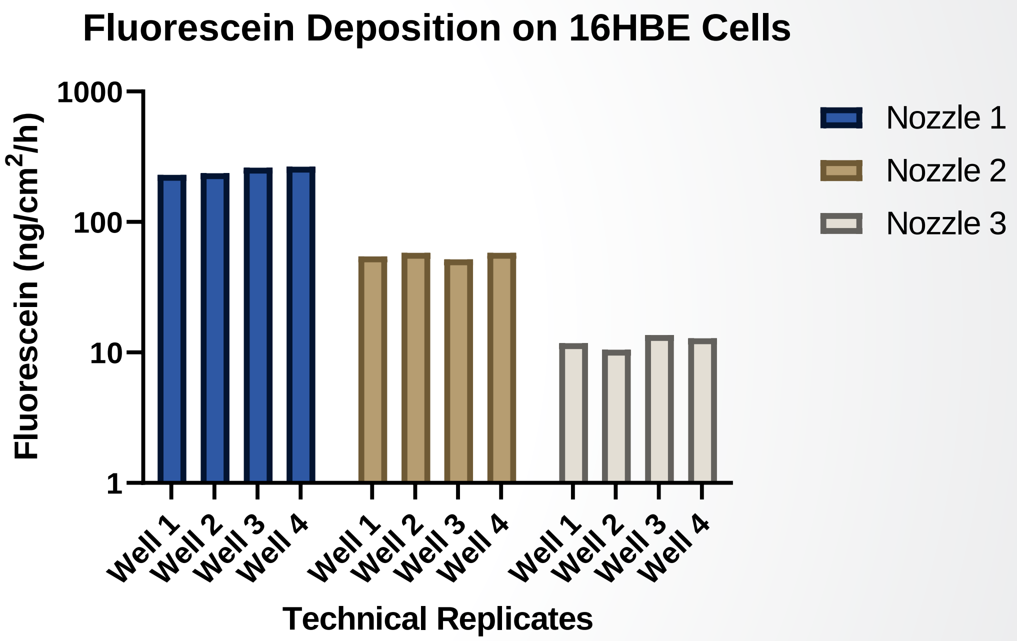


Fluorescein Deposition on 16HBE Cells

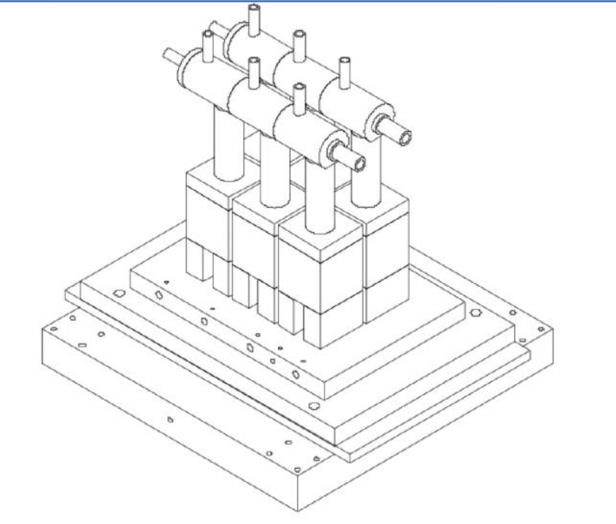


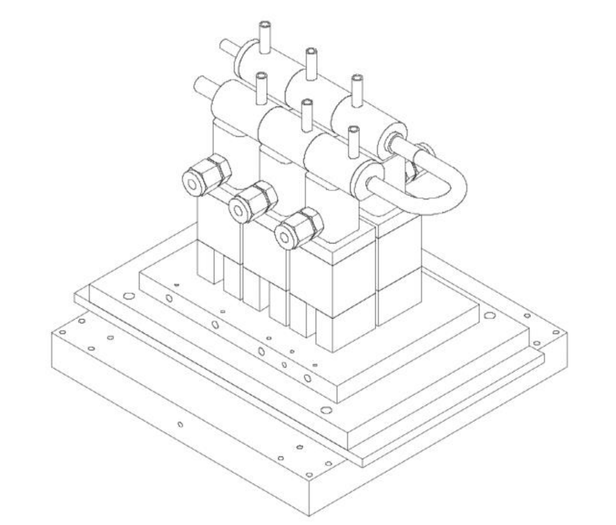
Aerosol-Compatible Cell Culture Exposure System (ACCES)

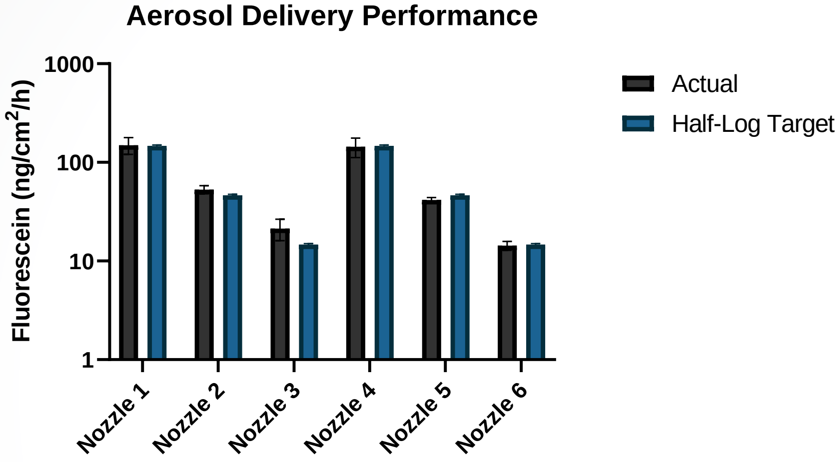


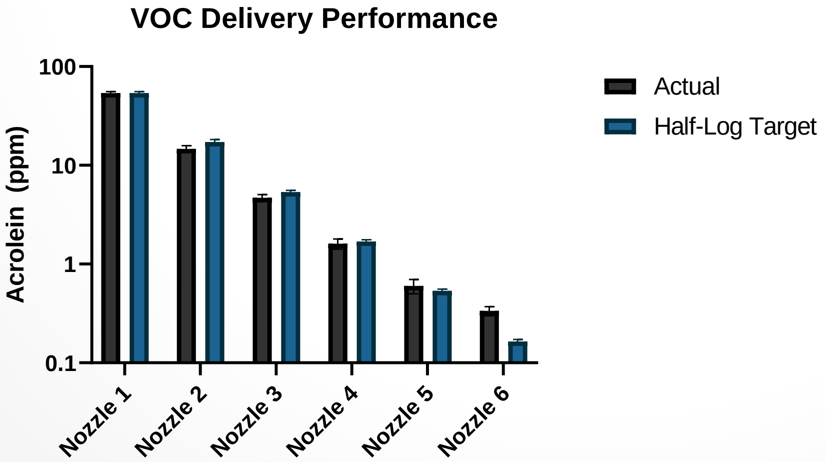
	Total (ng/cm²/h)	St. Dev. (%)	Dilution Ratio
Nozzle 1	155.8	5%	
Nozzle 2	47.2	9%	0.30
Nozzle 3	14.4	5%	0.30

New System Delivers Both Aerosols and VOCs









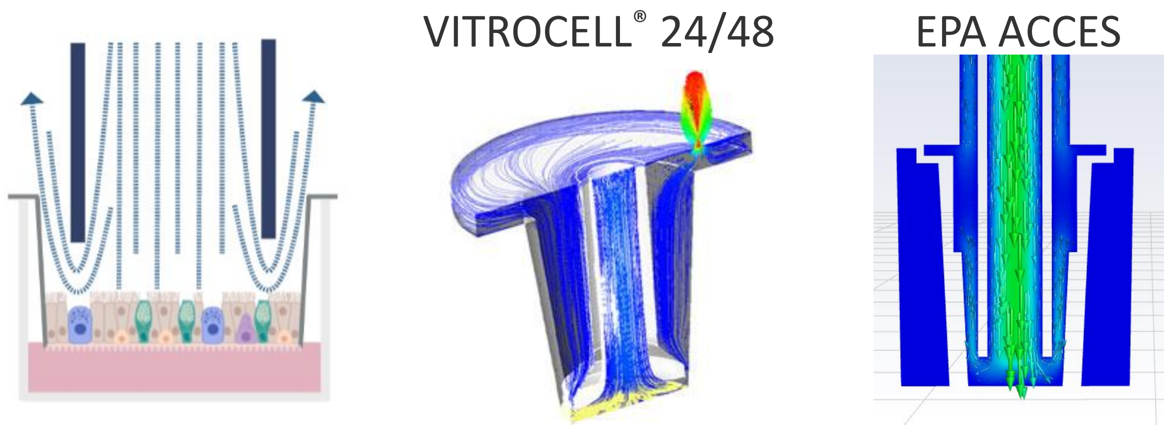
Modular, patent-pending design can be configured for aerosol or VOC dilution and delivery.

- Within the same system, drastically different operational conditions are required to deliver aerosols vs. VOCs
  - Aerosol operation: 5 mL min^{-1} per well
  - VOC operation: 12.5 mL min^{-1} per well
- Further work is needed to adapt the aerosol generation system to produce 6 doses of particles for a diverse list of test agents

U.S. Patent Application: AEROSOL-COMPATIBLE CELL CULTURE EXPOSURE SYSTEM; Serial No. 17/977114

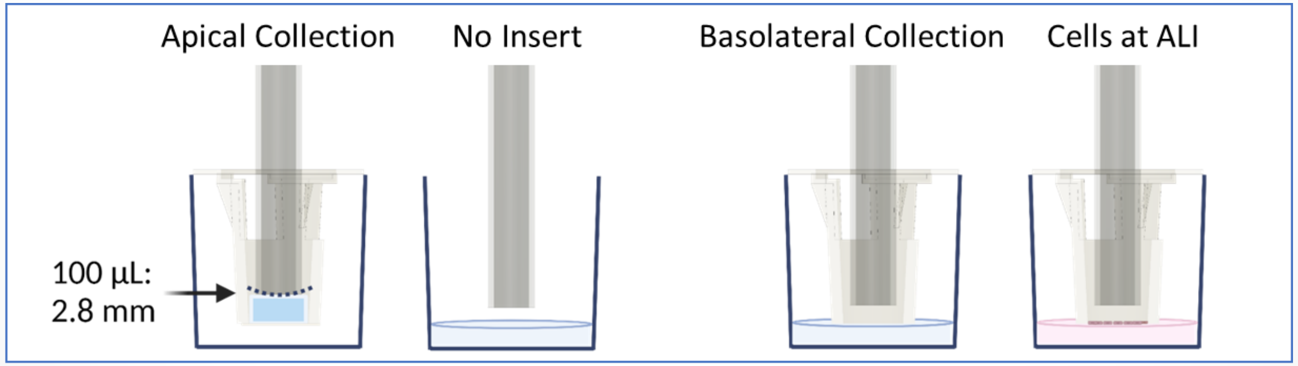
Cell-free Options to Estimate Deposition

- Cell-free collection methods are desirable as a high-throughput, low-cost method to quantify cell deposition to:
  1. Test improvements to aerosol generation system
  2. Quantify performance of ACCES (or other ALI exposure devices) for a variety of aerosols when fluorescence-based detection methods are not an option
- Literature search yielded a wide range of reported cell-free deposition methods for the Vitrocell 24/48, a similar perpendicular flow system



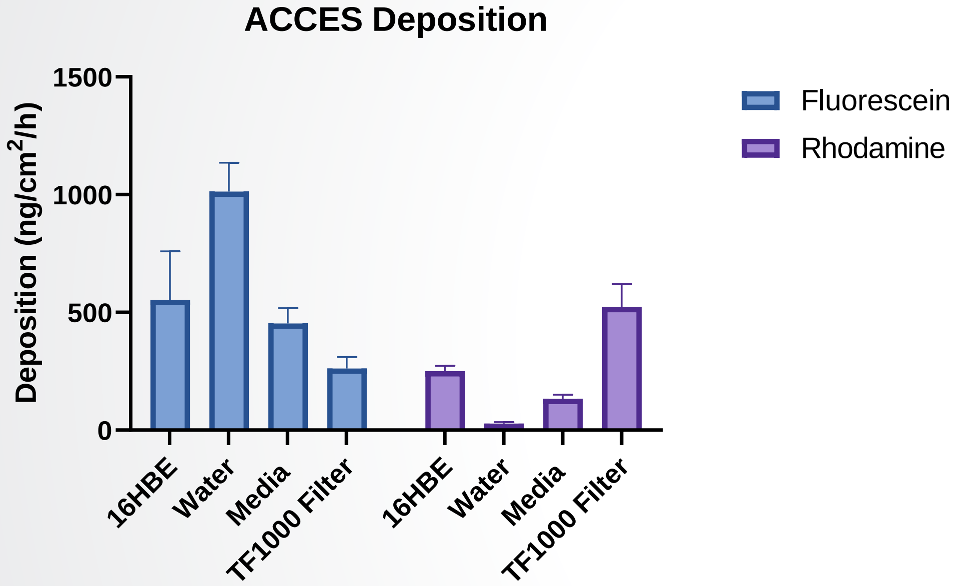
Cell-free Options to Estimate Deposition

Geometry Preserved?	Height of trumpet/nozzle	Insert?	Collection Method	Citation
NO	Not reported	No	18.5 mL DPBS in base	Majeed et al., *Toxicology Letters,* 2014
NO	Not reported	Yes, 8 mm stainless steel	8 mm Cambridge filters	Zhang et al., *Toxicology In Vitro*, 2022
NO	Not reported	Yes, 8 mm stainless steel	125 µL cell culture media	Verstraelen et al., *ALTEX,* 2021
NO	Not reported	Yes, 24-well ThinCert	100 µL DPBS in insert	Steiner et al., *Toxicology In Vitro,* 2018
NO	Not reported	Yes, 24-well Transwell	100 µL PBS in insert	Giralt et al., *Toxicology Letters,* 2020



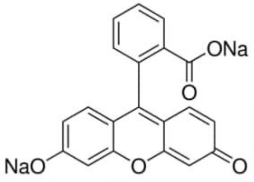
- Cell-free collection methods that change geometry of the ALI system will impact deposition
- To preserve geometry near the air-liquid interface, we paired basolateral collection methods with a membrane-free transwell

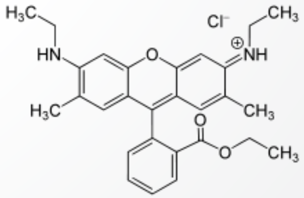
Cell-free Controls Rarely Predict Cell Deposition

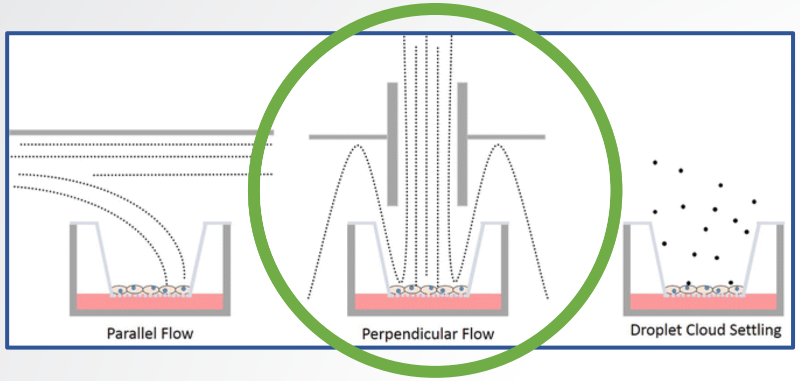


	Fluorescein (ng/cm^2/h)	Rhodamine (ng/cm^2/h)
16HBE Cells	552.6	250.9
Water	1012.6	28.0
Media (1% FBS)	452.7	133.6
TF1000 Filter	262.4	523.5

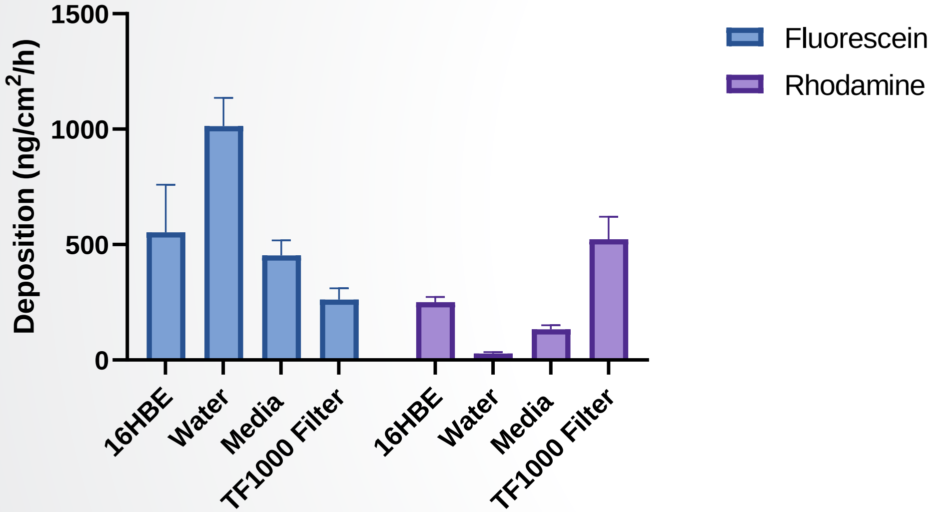
- Cell-free controls failed to provide reliable estimate of cell deposition
    - Cell culture media was the best option for estimating fluorescein deposition, but underestimated rhodamine deposition by 50%
- Both particles were generated under identical conditions, but compound-specific deposition patterns were observed
    - Fluorescein (-), MMAD: 1.7 µm
    - Rhodamine (+), MMAD: 1.3 µm



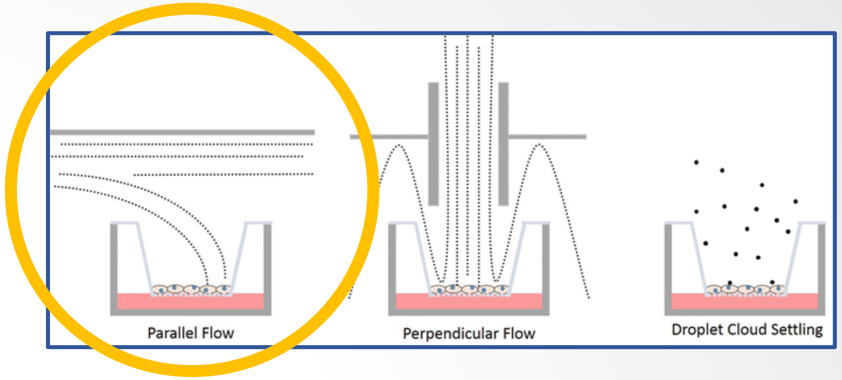




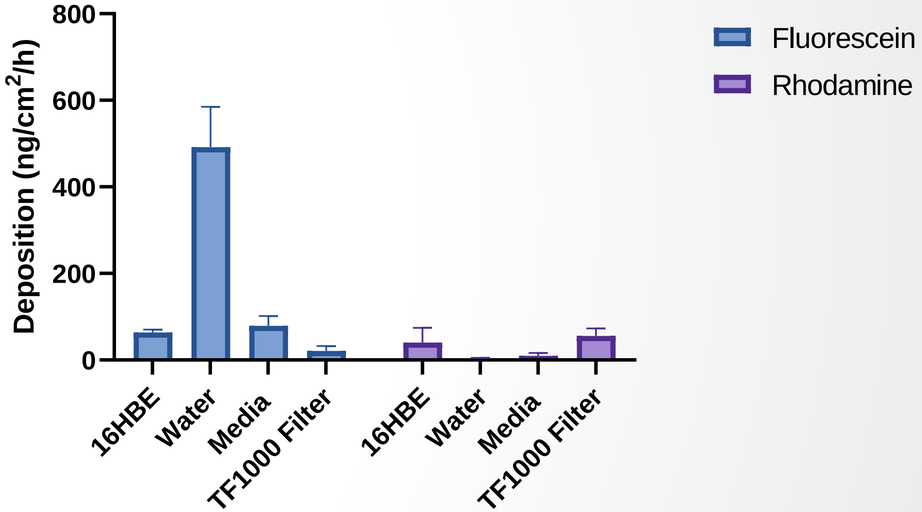
ACCES Deposition: 24-Well Format



	Fluorescein (ng/cm²/h)	Rhodamine (ng/cm²/h)
16HBE Cells	552.6	250.9
Water	1012.6	28.0
Media (1% FBS)	452.7	133.6
TF1000 Filter	262.4	523.5



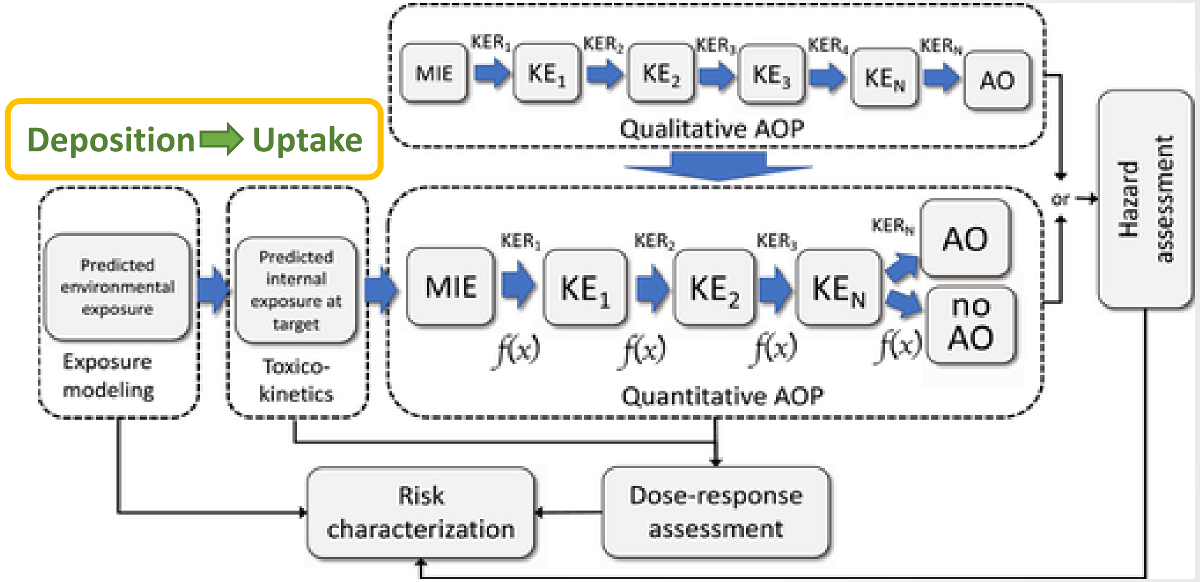
MedTec CelTox Deposition: 6-Well Format



	Fluorescein (ng/cm²/h)	Rhodamine (ng/cm²/h)
16HBE Cells	63.9	64.8
Water	491.9	2.7
Media (1% FBS)	79.0	9.6
TF1000 Filter	21.2	55.7

Particle Deposition is Variable Within and Across Exposure Systems

- Across multiple ALI exposure devices, we **cannot utilize cell-free options** to reliably estimate deposition without validation
- Sophisticated **cell extraction and analytical detection methods** are required to quantify cell deposition for a given aerosol

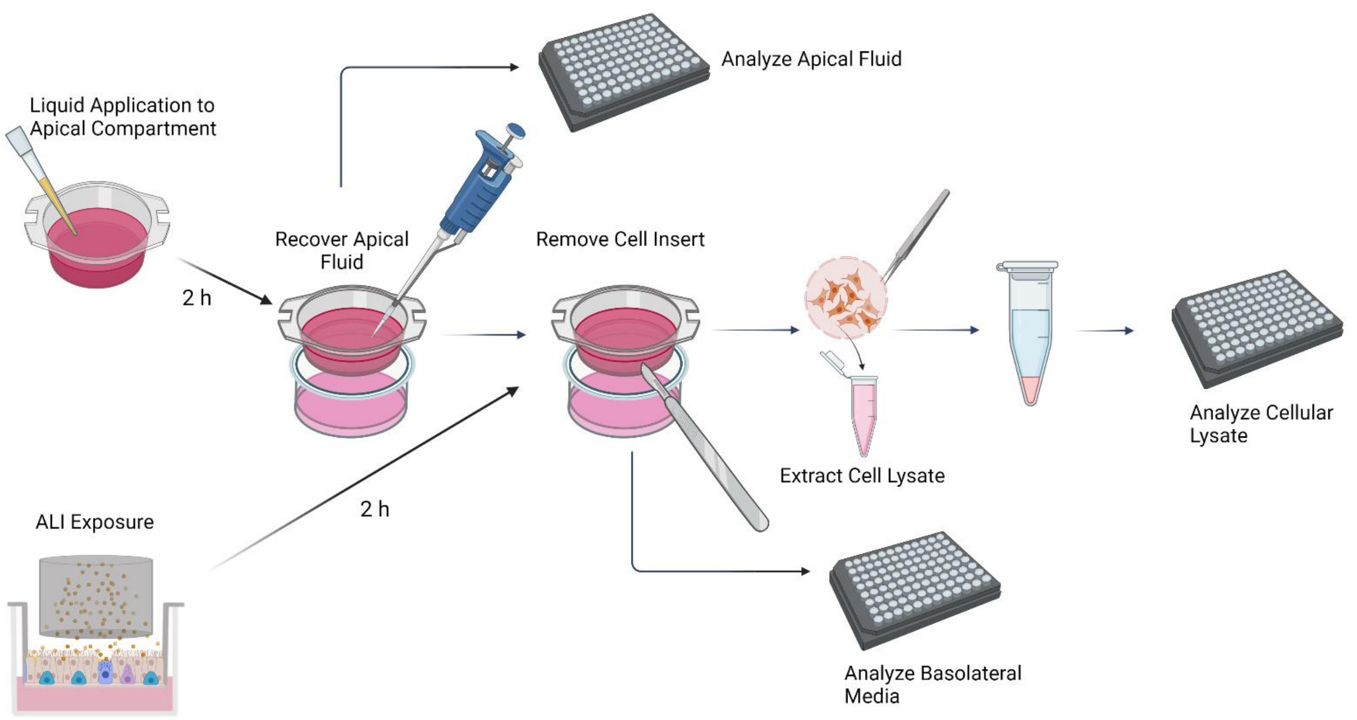


Perkins et al, *Environ Toxicol Chem*, 2019

Input to AOP constructs cannot be characterized by exposure concentration:
→*deposition and cellular uptake are dependent on exposure system and cell system.*

We aimed to determine whether fluorescent tracers could distinguish between deposition and cellular uptake.

Using Fluorescent Tracers to Distinguish Between Total Deposition and Cellular Uptake



Experimental Approach:

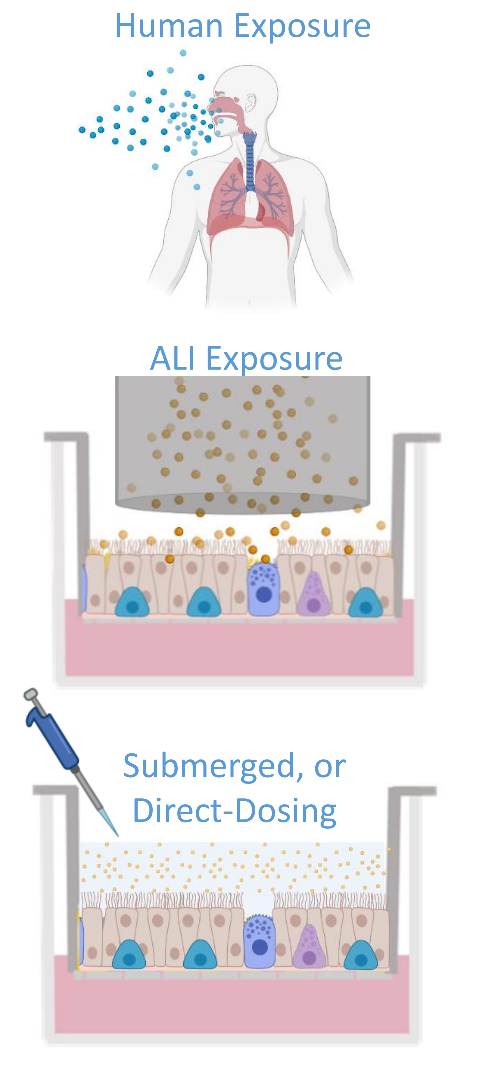
<u>ALI Testing Conditions</u>

- BLAM used to generate liquid particles:
  - Fluorescein MMAD: 1.7 µm
  - Rhodamine MMAD: 1.3 µm
- Krypton-85 (^{85}Kr) used as charge neutralizer
- Samples analyzed immediately after ALI exposure (2 h duration)

<u>Submerged Testing Conditions</u>

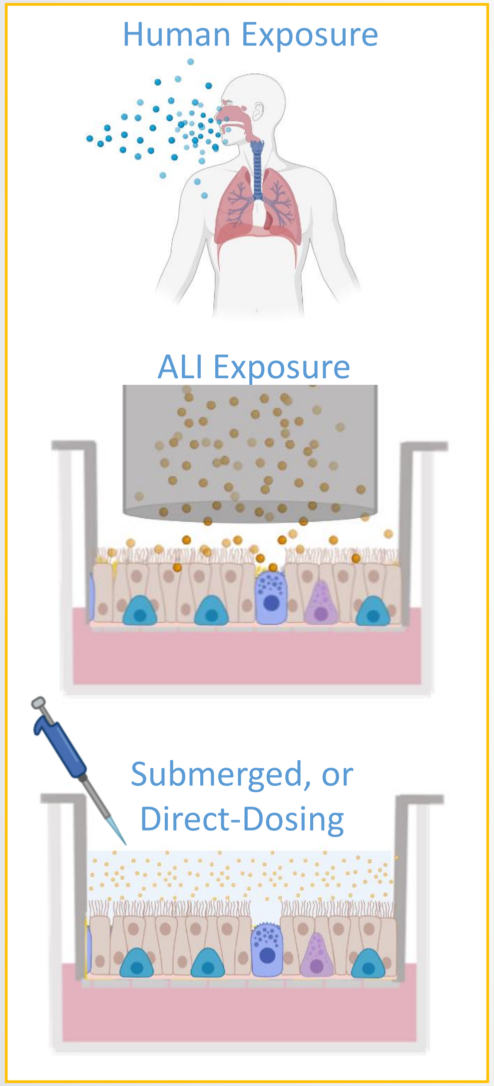
- Tested 100 µL, 50 µL, and 10 µL
  1. Same total dose (ng/cm^2)
  2. Same concentration (ng/mL)
- Test agents dissolved in HBSS for direct-dosing
- Samples analyzed after 2 h to match ALI exposure duration

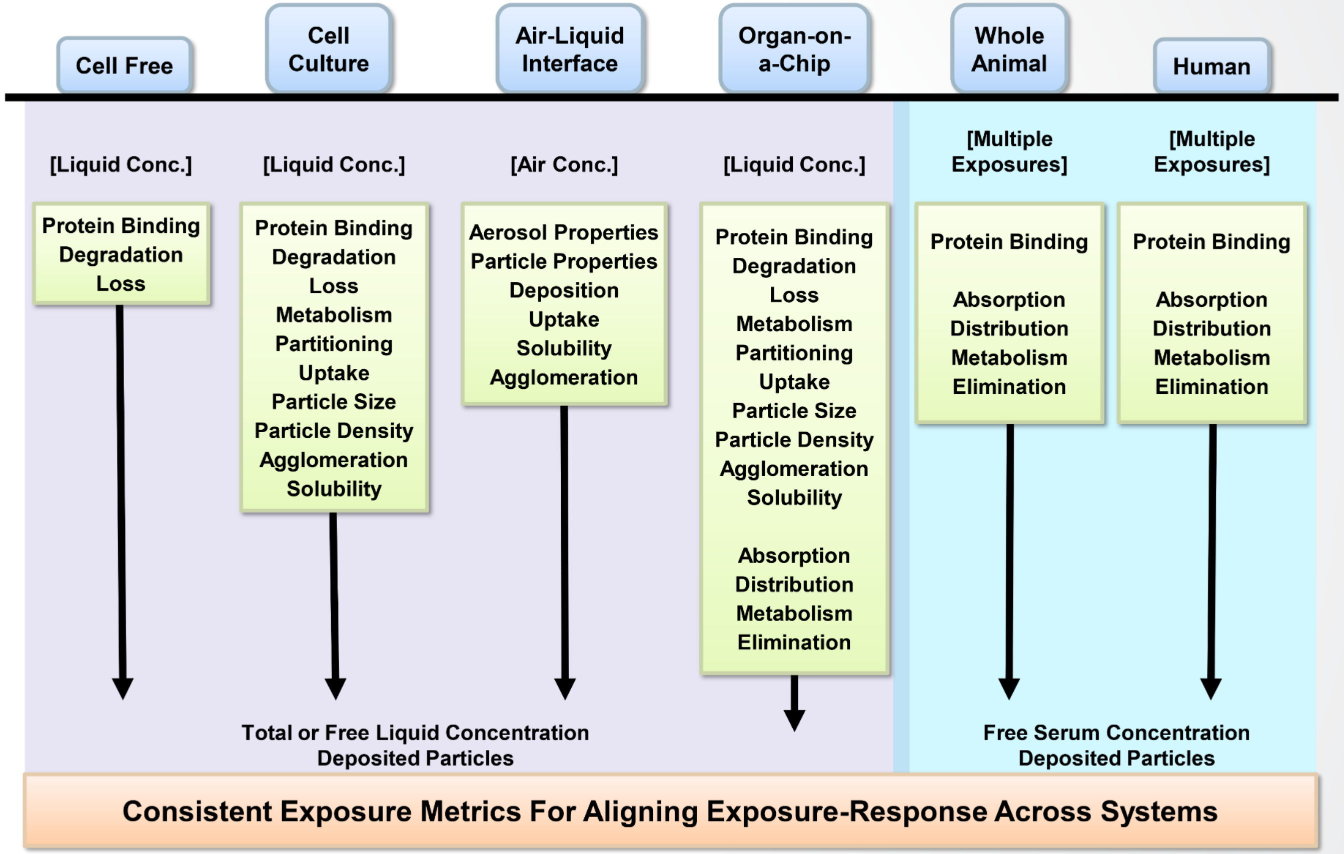
Characterization of ALI vs. Submerged Exposures



	ALI Exposure	Submerged, or Direct Liquid Application
Pros	• Most physiologically relevant • Direct cell-toxicant interaction • Compatible with both VOCs and particles	• Easier and higher-throughput, no complex equipment required
Cons	• ALI exposure equipment is complex to operate and maintain • Difficult to quantify delivery to cell surface	• Incompatible with VOCs • Unknown cellular uptake • Volume is not standardized across direct liquid application studies • Liquid application disrupts ALI conditions ○ Measurable changes in TEER and baseline transcriptomics

Characterization of ALI vs. Submerged Exposures

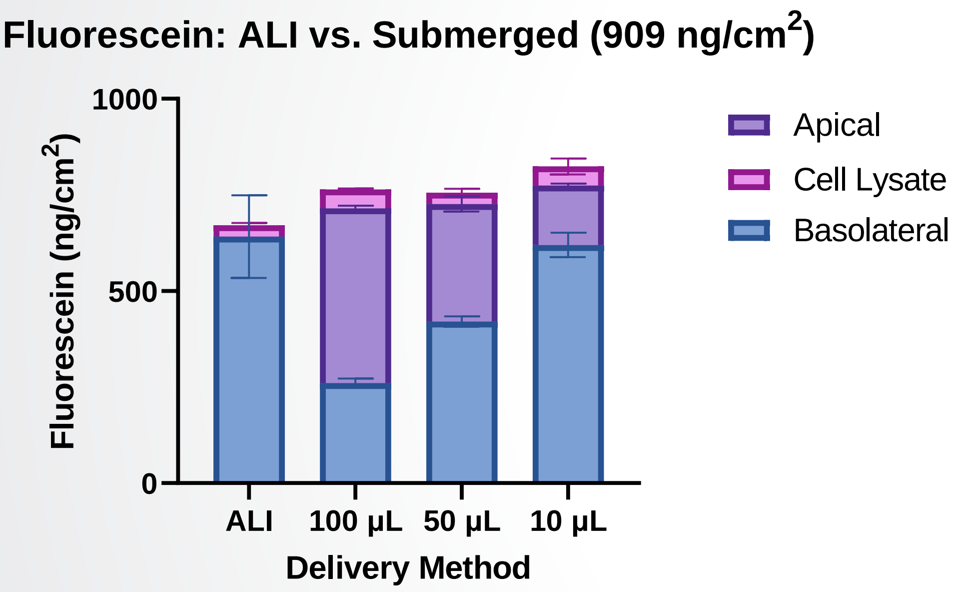




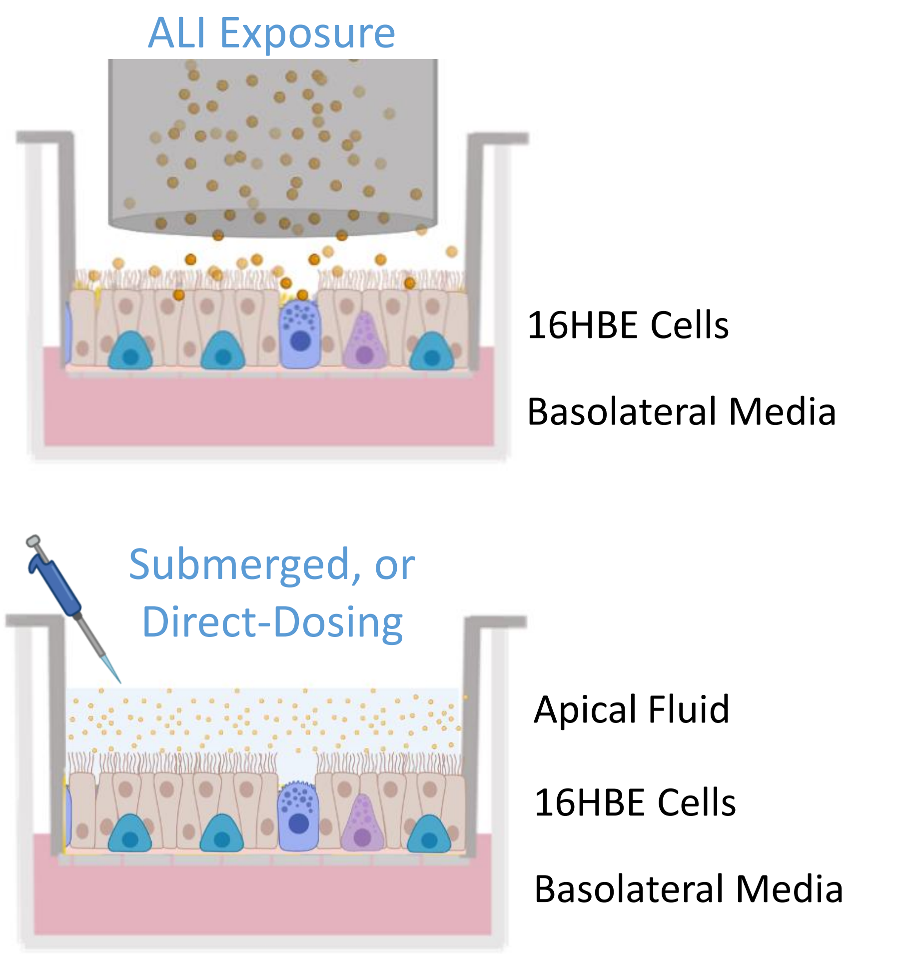
NAS,Using 21st Century Science to Improve Risk-Related Evaluations, 2017

"To best inform evidence integration for risk assessment, *in vitro* studies should determine the relevant internal cellular target dose rates (amount per unit time) that result in the observed responses" – Phalen et al., *Journal of Aerosol Science,* 2021.

Fluorescein Deposition Leads to Basolateral Translocation

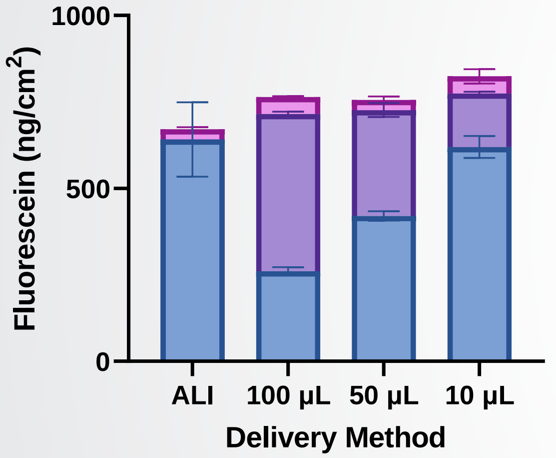


Exposure	Dose (µg/cm²)	Conc. (µg/mL)	%basolateral	%uptake	%yield
ALI	~0.909	N/A	96%	4%	
100 µL	0.909	3	34%	6%	84%
50 µL	0.909	6	56%	4%	83%
10 µL	0.909	30	75%	6%	91%

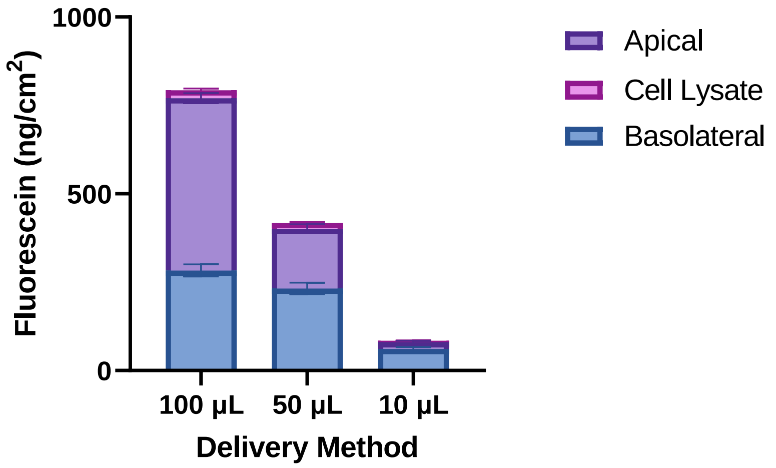


Basolateral Transport of Fluorescein is Volume-Dependent

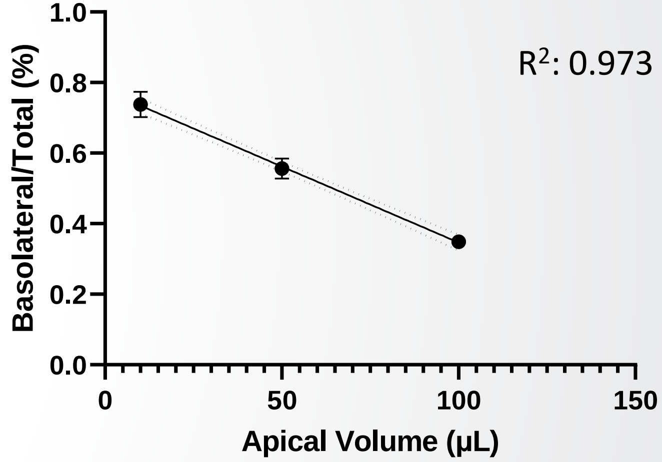
Fluorescein: ALI vs. Submerged (909 ng/cm²)



Fluorescein: Same Concentration (3 µg/mL)



Fluorescein: Volume vs Translocation



ALI vs. Submerged, Same Total Dose

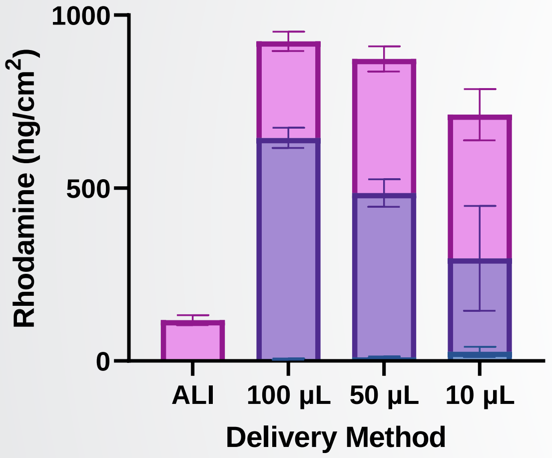
Exposure	Dose (µg/cm²)	Conc. (µg/mL)	%basolateral	%uptake	%yield
ALI	~0.909	N/A	96%	4%	
100 µL	0.909	3	**34%**	6%	84%
50 µL	0.909	6	**56%**	4%	83%
10 µL	0.909	30	**75%**	6%	91%

Same Concentration

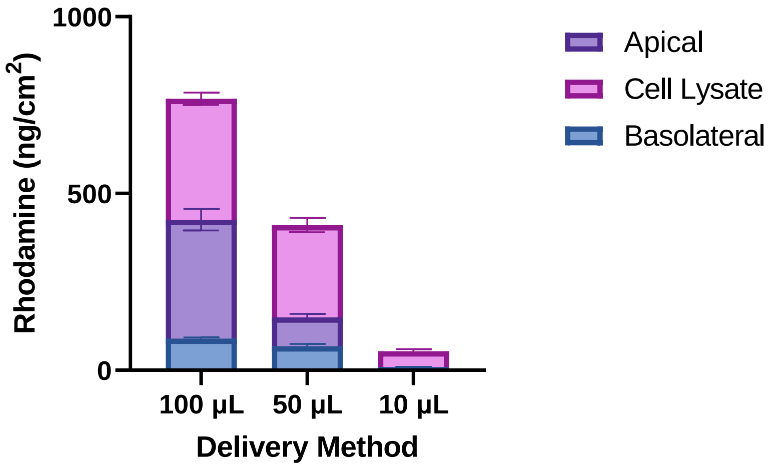
Exposure	Dose (µg/cm²)	Conc. (µg/mL)	%basolateral	%uptake	%yield
100 µL	0.909	3	**36%**	3%	87%
50 µL	0.455	3	**56%**	4%	92%
10 µL	0.091	3	**72%**	4%	93%

Cellular Uptake of Rhodamine is Volume-Dependent

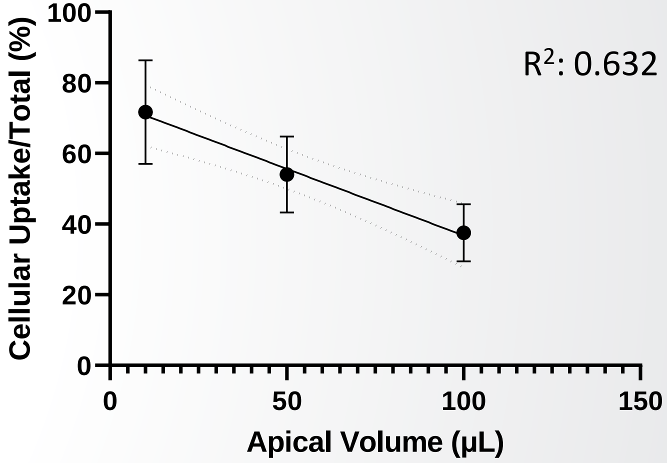
Rhodamine: ALI vs. Submerged (909 ng/cm²)



Rhodamine: Same Concentration (3 µg/mL)



Rhodamine: Volume vs Cellular Uptake



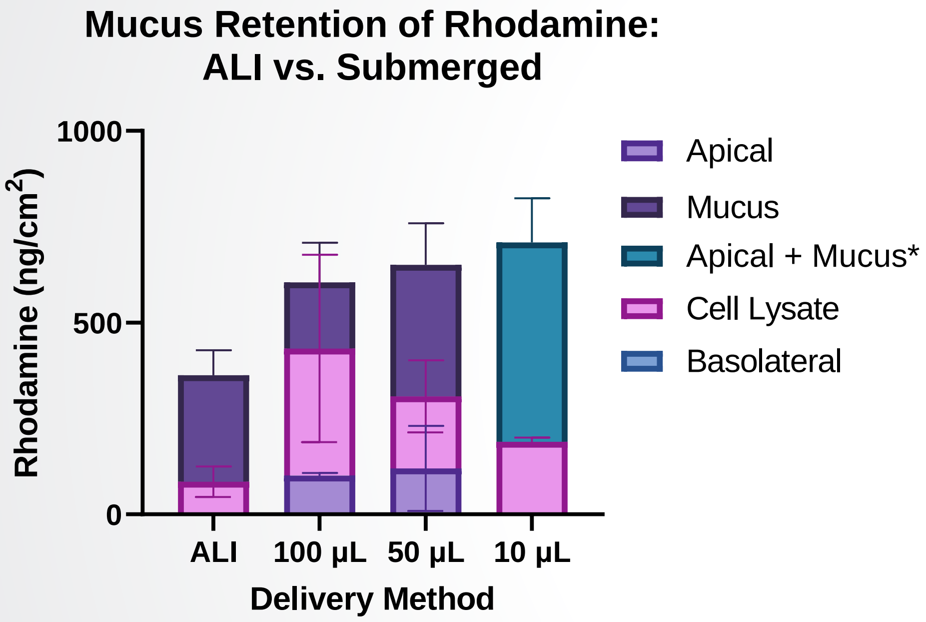
ALI vs. Submerged, Same Total Dose

Exposure	Dose (µg/cm²)	Conc. (µg/mL)	%basolateral	%uptake	%yield
ALI	0.120*	N/A	0%	100%	
100 µL	0.909	3	1%	**30%**	102%
50 µL	0.909	6	1%	**44%**	96%
10 µL	0.909	30	4%	**58%**	78%

Same Concentration

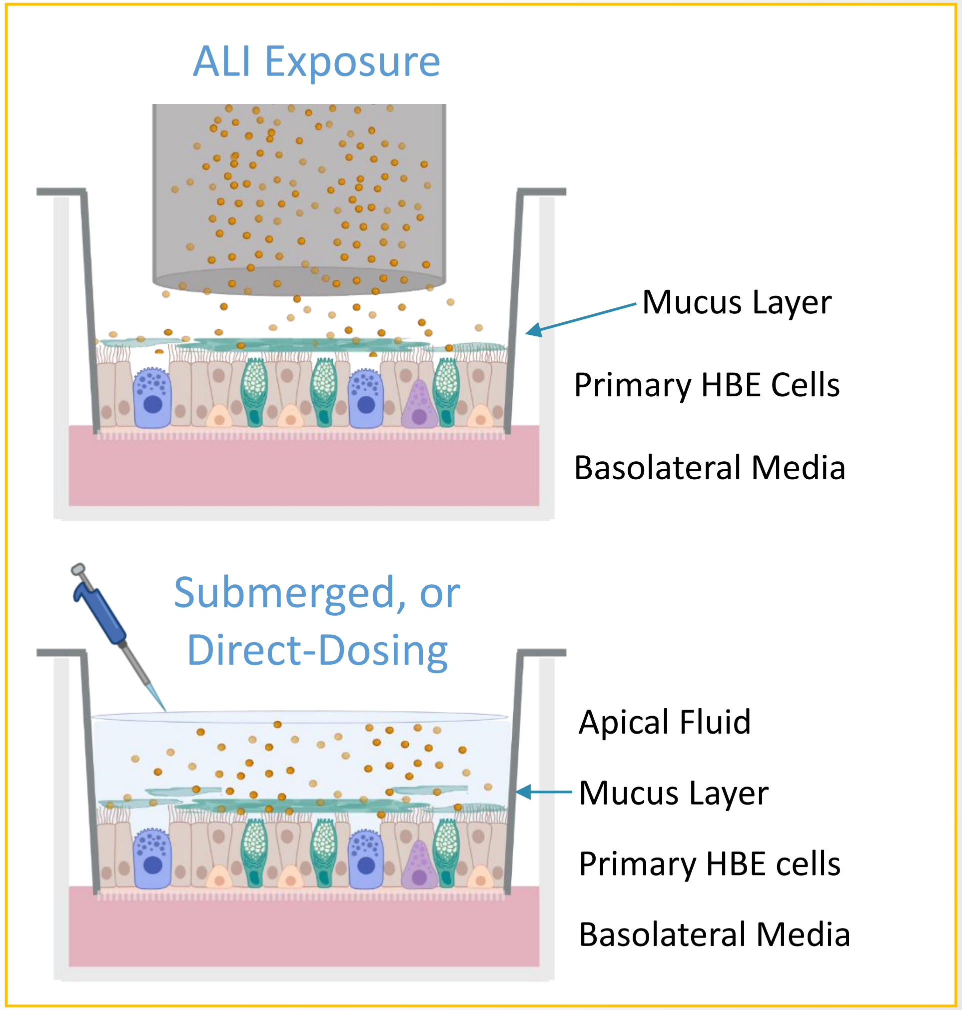
Exposure	Dose (µg/cm²)	Conc. (µg/mL)	%basolateral	%uptake	%yield
100 µL	0.909	3	12%	**45%**	84%
50 µL	0.455	3	17%	**64%**	90%
10 µL	0.091	3	17%	**83%**	59%

Mucus Retention Leads to Lower Cellular Uptake

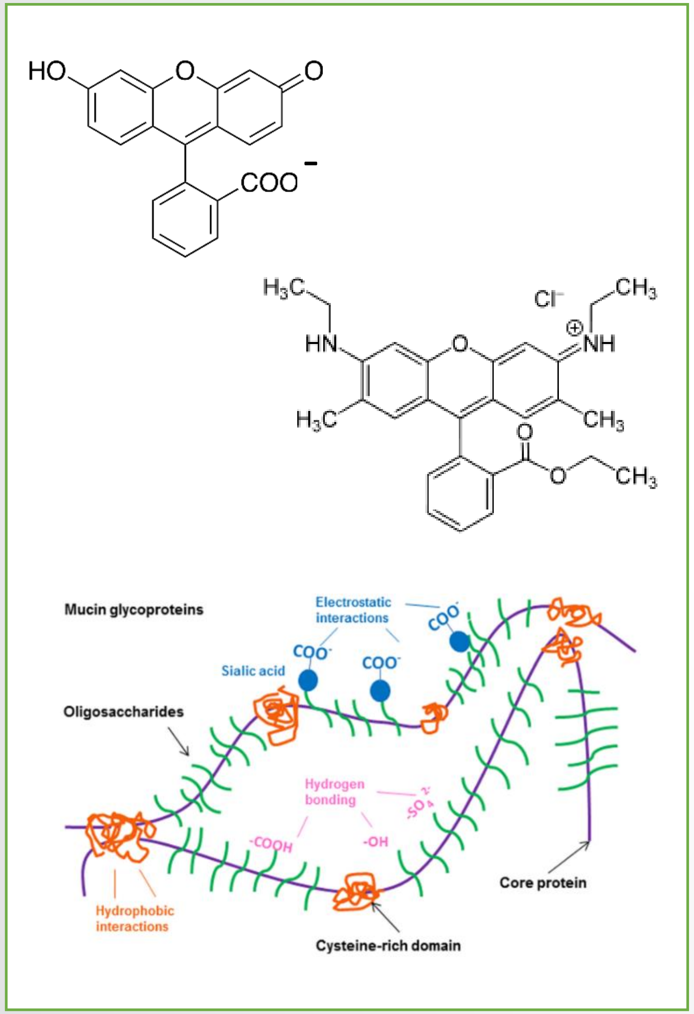


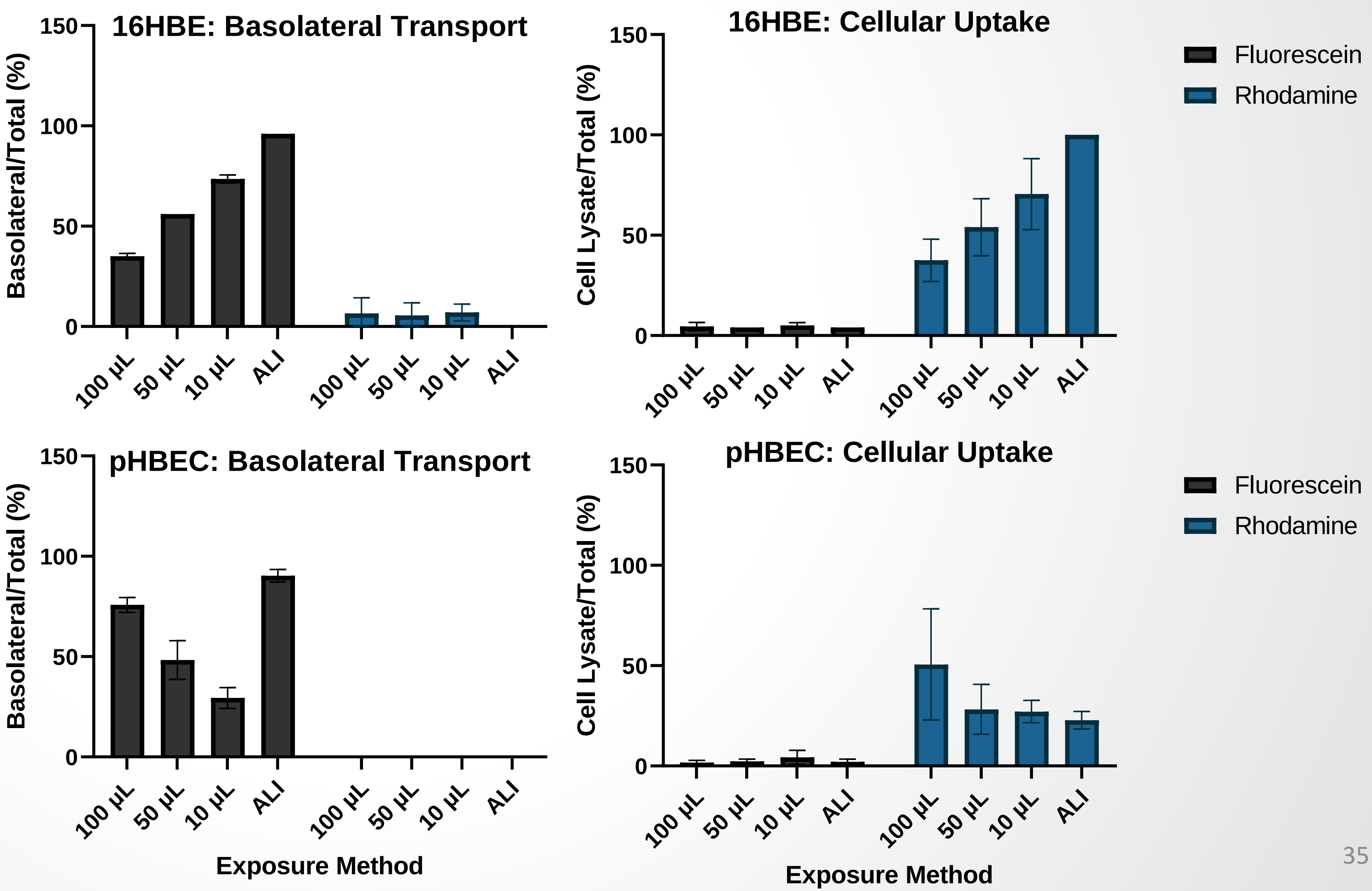
Exposure	Dose (µg/cm²)	Conc. (µg/mL)	**%mucus**	%uptake	%yield
ALI	~0.303	N/A	**77%**	23%	
100 µL	0.303	1	**29%**	55%	67%
50 µL	0.303	2	**53%**	29%	71%
10 µL*	0.303	10	--	36%	86%

*It was not technically possible to recover 10 µL of the apical solution without disturbing and aspirating mucus.

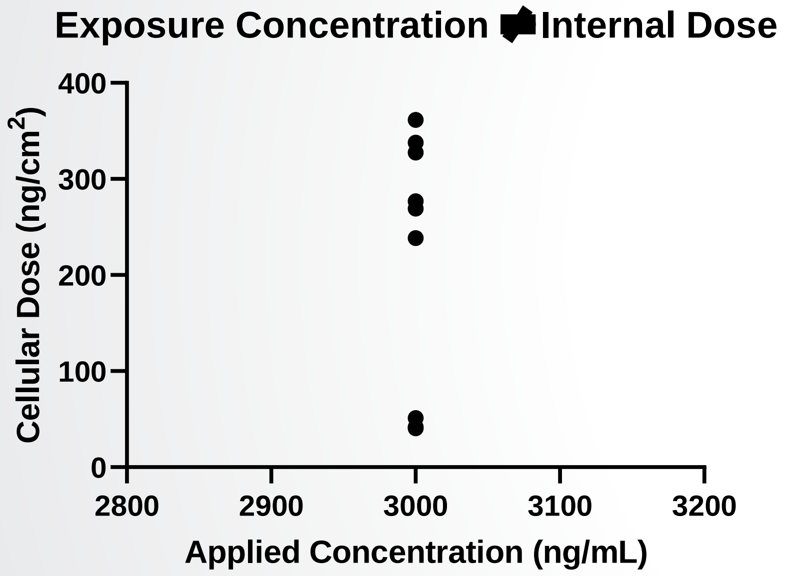


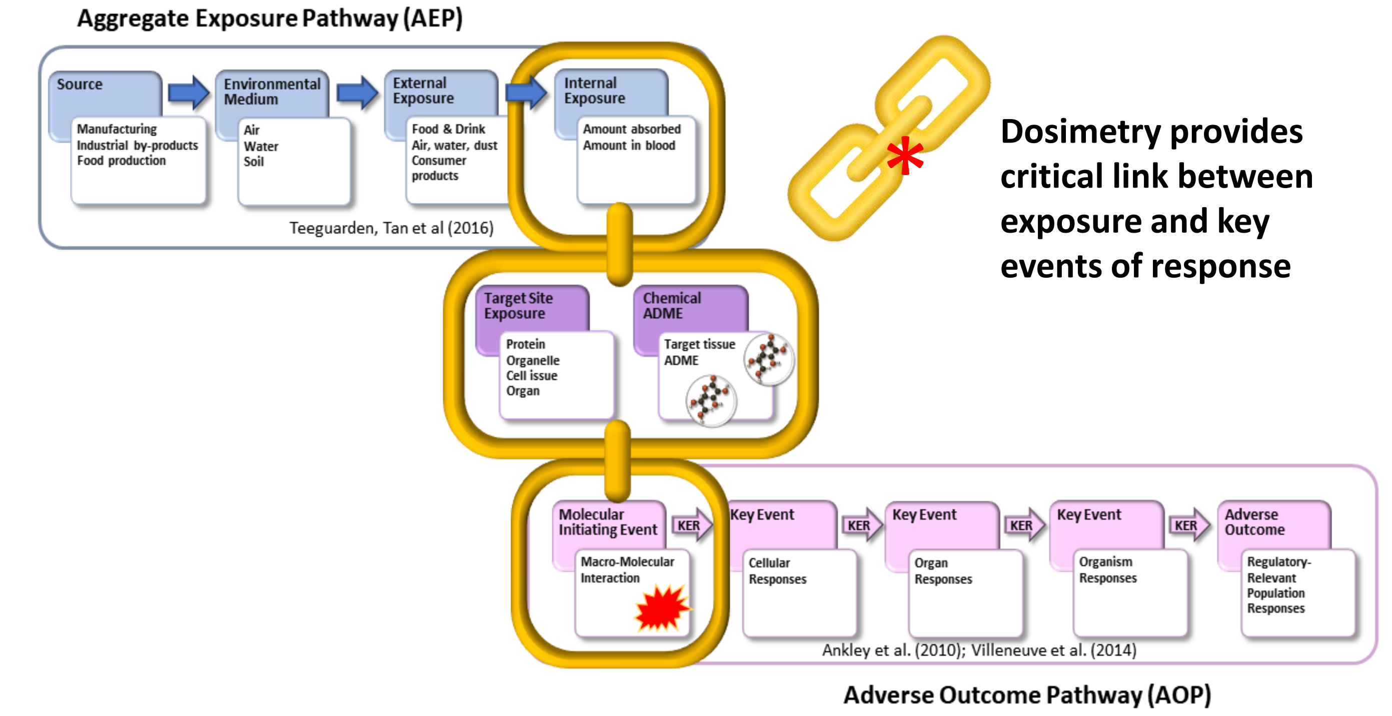
Cell Type and Exposure Method Impacts Cellular Uptake





Direct-Dosing Study Design Impacts Target Site Exposure





Annie Jarabek, manuscript in progress, 2022

- Direct liquid application is often proposed as a time- and cost-effective alternative to ALI exposures, but variable apical volumes used across liquid application studies will directly impact cellular uptake:
  → Internal dose ranged from 40 – 360 ng/cm² for a single exposure concentration in 16HBE cells

Conclusions: Lessons Learned When Including Aerosols in ALI Screening Efforts

- Careful **validation and characterization are required for each test agent**
  - An ALI system optimized for VOC delivery may not be appropriate for aerosols without significant modifications
- CAD and CFD Modeling were time- and cost-effective approaches to redesign our exposure system and optimize operational parameters
- Fluorescent tracers can be recovered in cell lysate to quantify cell deposition and can also be applied to validate CFD models
- Cell-free controls are rarely appropriate to estimate cell deposition
- Exposure Concentration ≠ Deposition ≠ Cellular Uptake
  - *This is especially important for mucus-producing cell lines!*
- Submerged exposure conditions are not comparable to ALI exposures, and differences in cellular uptake must be considered when designing these studies
- Further work is needed to translate ALI deposition to Human Equivalent Concentrations (HEC) to support *in vitro* to *in vivo* extrapolation (IVIVE)

Acknowledgements

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- Joseph Bundy, PhD
- Rusty Thomas, PhD
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- Shaun McCullough, PhD

<u>MedTec Biolab Inc</u>.

- Jose Zavala, PhD

<u>UNC School of Medicine</u>

- Adam Speen, PhD

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ORISE opportunity: EPA-ORD-CPHEA-PHITD-2022-07 at zintellect.com