How to Characterize and Validate AI and In Vitro NAMs for Toxicity Testing

September 11, 2024

RTI INTERNATIONAL

Today's Presenters

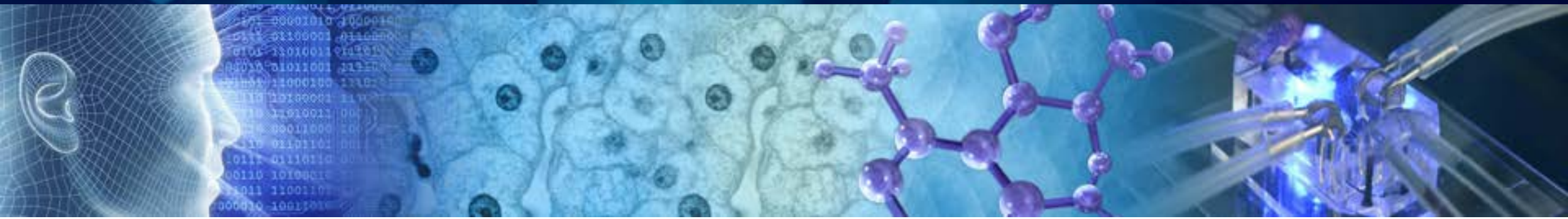
Nicole Kleinstreuer
Director of the NTP Interagency Center for the Evaluation of Alternative Toxicological Models (NICEATM)
National Institute of Environmental Health Sciences (NIEHS)

Shaun McCullough
Senior Respiratory Scientist & Principal Investigator
RTI International

Moderator:
Shannon Bell
Senior Bioinformatician
RTI International

National Institute of Environmental Health Sciences
Division of Translational Toxicology

RTI Webinar
11th September, 2024



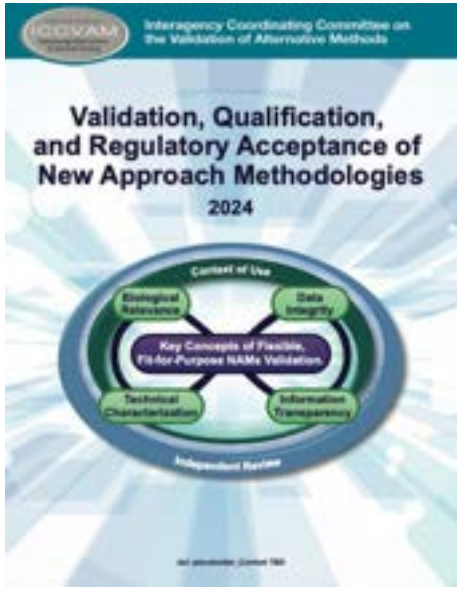
Validation, Qualification, and Regulatory Acceptance of New Approach Methodologies: In Vitro, In Silico, and Beyond…

Nicole C. Kleinstreuer, PhD

Director, NTP Interagency Center for the Evaluation of Alternative Toxicological Methods

Executive Director, Interagency Coordinating Committee for the Validation of Alternative Methods

National Institutes of Health • U.S. Department of Health and Human Services



Biomedical Research

Validation/ Qualification

Adoption & Implementation

The NAMs Confidence Continuum: from *Biomedical Research* to *Validation/Qualification* to *Adoption & Implementation*

Complement-ARIE: Complement Animal Research in Experimentation

Purpose: To catalyze the development, standardization, validation and use of **human-based new approach methodologies (NAMs)** that will transform the way we do basic, translational, and clinical sciences

Goals:

1. Better model and **understand human health and disease** outcomes **across diverse populations**.
2. Develop NAMs that **provide insight into specific biological processes** or disease states.
3. Validate mature NAMs to **support regulatory use** and standardization.
4. Complement traditional models and **make biomedical research more efficient and effective**.

https://commonfund.nih.gov/complementarie

Complement-ARIE: Comprehensive center model

- Comprehensive centers will require embedded projects on *in vitro*, *in chemico*, and *in silico* approaches plus combinatorial approaches.
- Cores will include administrative, validation, resources, and training components.
- Phased milestone-driven projects that pilot some of the truly innovative approaches can also be transitioned for integration with the centers.

UNITED STATES ICCVAM Advancing Alternatives to Animal Testing

Key partners for validation networks include: ICCVAM, ICATM members, OECD, etc.

U.S. Validation Body: ICCVAM Authorization Act of 2000

PUBLIC LAW 106–545 (42 U.S.C. 285*l*-3)

"To establish, wherever feasible, guidelines, recommendations, and regulations that promote the regulatory acceptance of new or revised scientifically valid toxicological tests that protect human and animal health and the environment while reducing, refining, or replacing animal tests and ensuring human safety and product effectiveness."

- Consumer Product Safety Commission
- Department of Agriculture
- Department of the Interior
- Department of Transportation
- Environmental Protection Agency
- Food and Drug Administration
- Occupational Safety and Health Administration
- National Institute for Occupational Safety and Health
- Agency for Toxic Substances and Disease Registry
- National Cancer Institute
- National Inst of Environmental Health Sciences
- National Library of Medicine
- National Institutes of Health
- Department of Defense
- Department of Energy
- National Institute of Standards and Technology (since 2017)
- Dept of Veterans Affairs Office of Research and Development (since 2020)
- Other participants: NCATS, Tox21

More information: https://ntp.niehs.nih.gov/go/iccvam

ICCVAM Co-chairs

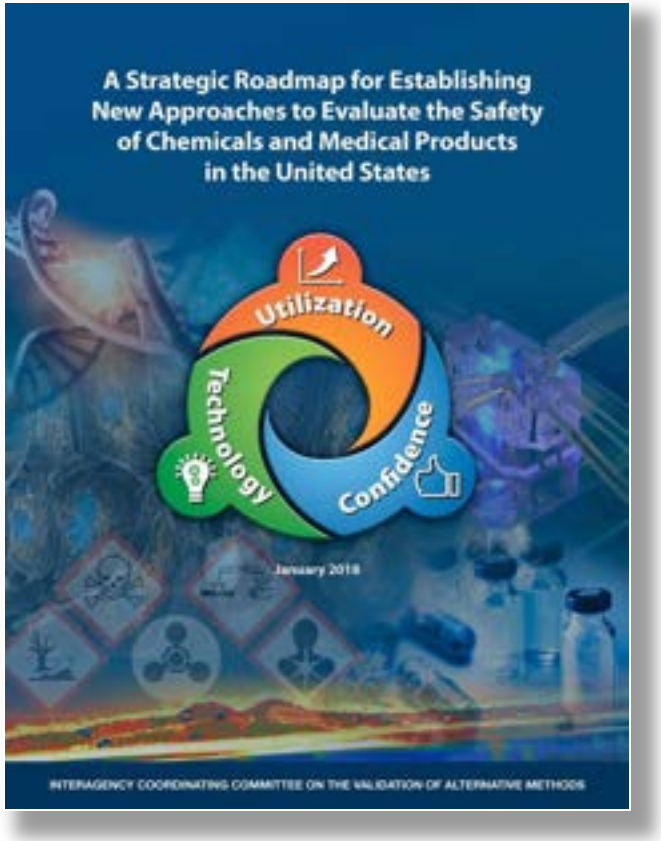
Suzy Fitzpatrick
FDA/CFSAN

Natalia Vinas
DoD

Nicole Kleinstreuer
Executive Director, ICCVAM
Director, NICEATM

U.S. Strategy and Roadmap

"Advances in science and technology have not been effectively leveraged to predict adverse human health effects"

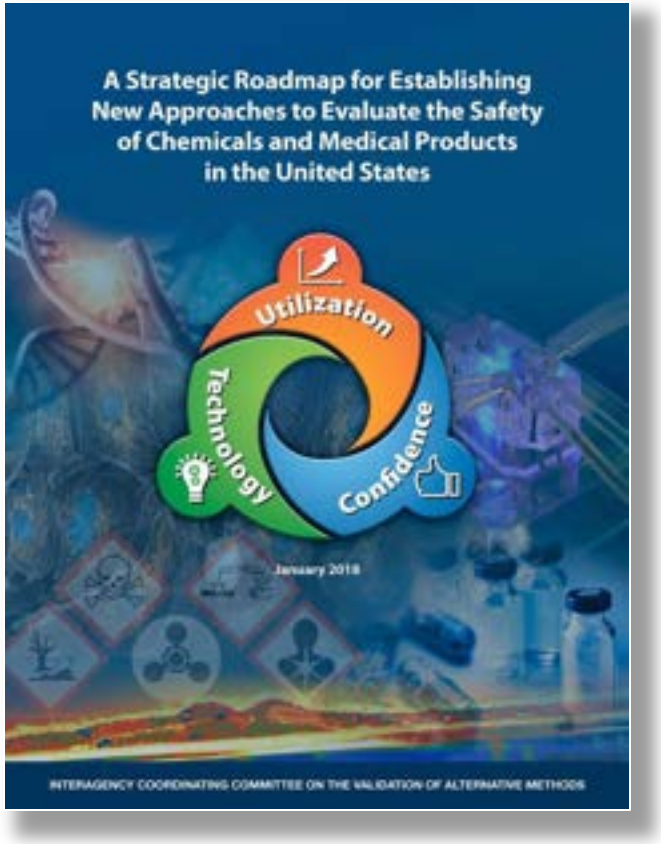


- **Help end-users guide the development of the new methods**
- **Use efficient and flexible approaches to establish confidence in new methods**
- **Encourage the adoption of new methods by federal Agencies and regulated industries**

https://ntp.niehs.nih.gov/go/natl-strategy

U.S. Strategy and Roadmap

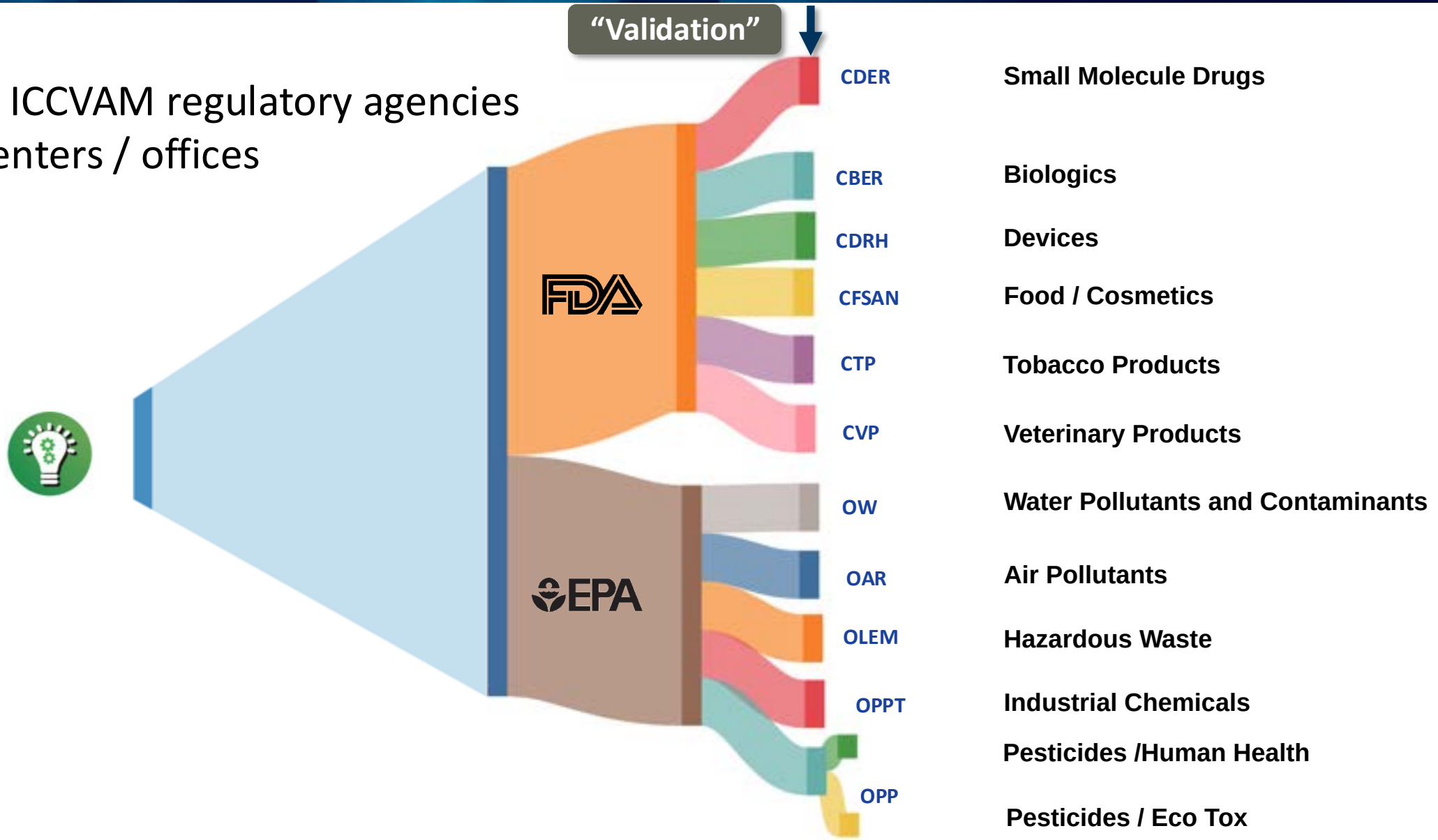
"Advances in science and technology have not been effectively leveraged to predict adverse human health effects"

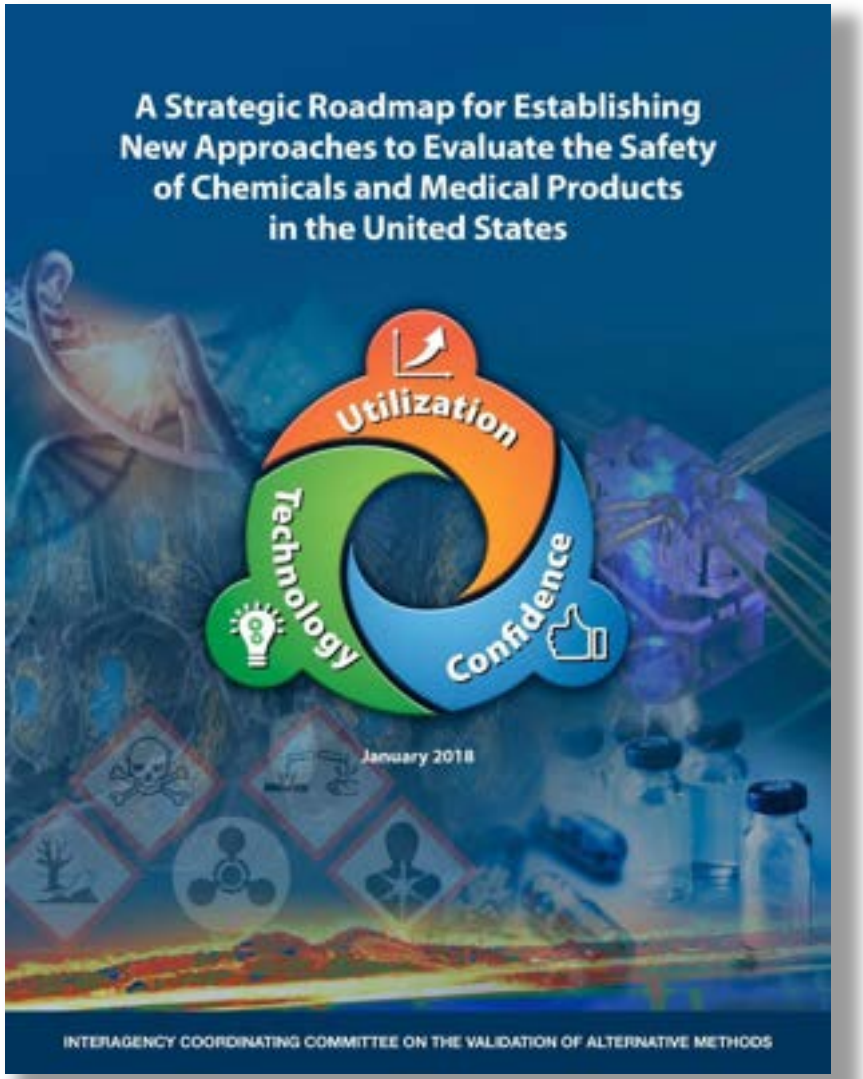


- Help end-users [illegible] the development of the new m[illegible]
- Use eff[illegible]t and [illegible]e ap[illegible]aches to establis[illegible]nce [illegible]new [illegible]thods
- Encourage t[illegible] new methods by federal Agencies and regulated industries

https://ntp.niehs.nih.gov/go/natl-strategy

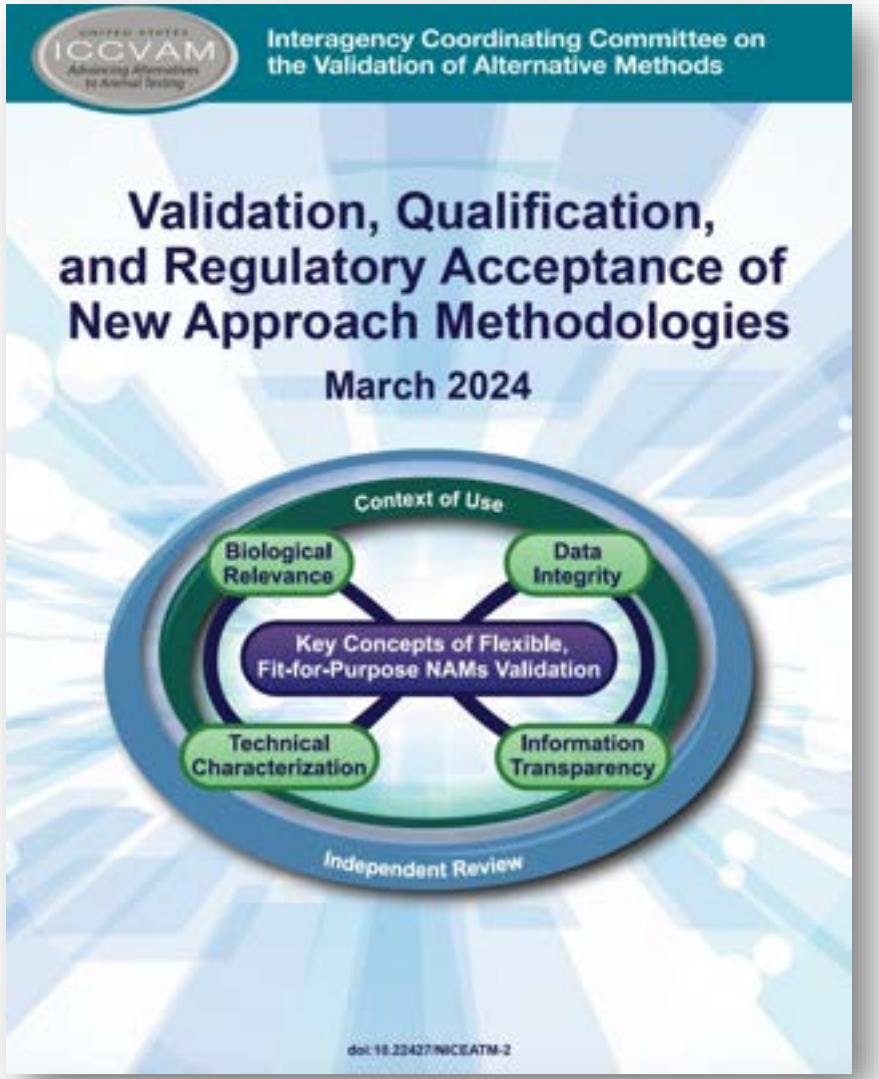
Example of two ICCVAM regulatory agencies with multiple centers / offices





https://ntp.niehs.nih.gov/go/natl-strategy

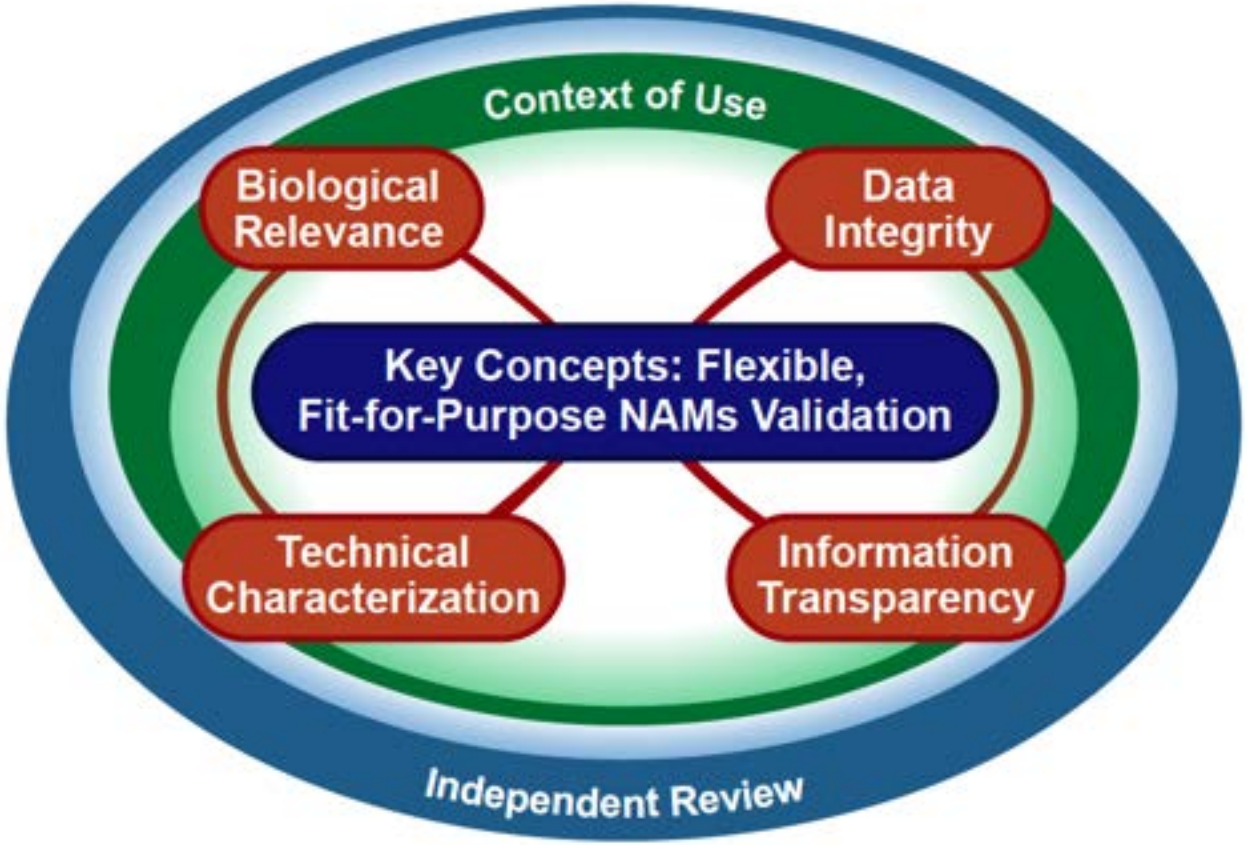
- **Connect end users with the developers of alternative methods**
- **Establish new validation approaches that are more flexible and efficient**
- **Ensure adoption and use of new methods by both regulators and industry**



https://ntp.niehs.nih.gov/go/ICCVAM-submit

Establishing Confidence in NAMs

Validation, Qualification, and Regulatory Acceptance of New Approach Methodologies (ICCVAM, 2024)



ICCVAM Validation Report, Figure 1

https://ntp.niehs.nih.gov/go/ICCVAM-submit

Context of Use

Purpose = Context of Use

Fitness for Purpose

Which regulatory statutes are data from the NAM intended to comply with?

- U.S. TSCA
- EU REACH
- Other

How will the NAM be used?

- As a stand-alone assay
- As part of a defined approach
- As part of an integrated approach to testing and assessment or weight of evidence assessment

Is the information provided sufficient to address the regulatory endpoints of interest?

- Describe the relationship between the information measured by the NAM and the regulatory endpoints being addressed.
- Is the technical performance, including the level of uncertainty, acceptable?

What is the context in which the NAM is intended to be used?

- Preregulatory screening and prioritization
- Chemical grouping
- Hazard identification
- Quantitative risk assessment

van der Zalm et al. 2022 Arch Tox

Examples of Endpoints where Biological and Mechanistic Relevance of NAMs has been Demonstrated to Support Regulatory Applications

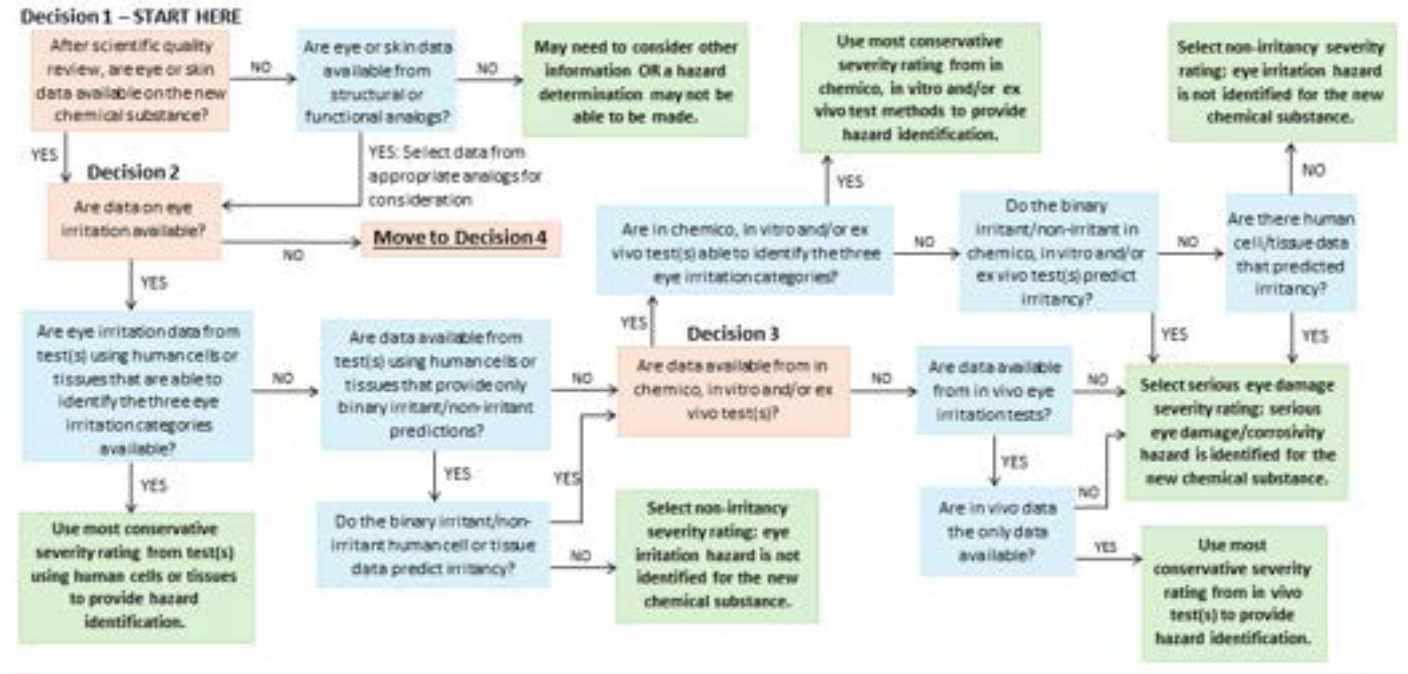
Endpoint	Summary	Reference
Skin sensitization	The endpoint has a well-developed human relevant AOP to which defined approaches combining several NAMs are mapped and described in OECD Guideline 497.	Kleinstreuer et al., 2018; OECD, 2021a
Endocrine disruption	Established pathway models using complementary NAMs as part of an integrated strategy are available for estrogen and androgen receptor activity. EPA accepts these NAMs for Tier 1 screening in the Endocrine Disruptor Screening Program.	Judson et al., 2015; Kleinstreuer et al., 2017; EPA, 2023
Developmental neurotoxicity	Limited AOPs exist for this complex endpoint. Instead, a battery of NAMs covering critical processes of human neurodevelopment has been developed. An OECD GD on the battery is available that includes integrated approaches to testing and assessment (IATA) case studies.	Crofton and Mundy, 2021; OECD, 2022a; OECD, 2023
Inhalation toxicity	An alternative approach using an in vitro human-cell based assay and computational modeling was used to characterize the hazard of chlorothalonil and derive a point of departure for use in EPA human health risk assessment. This approach was also published as an OECD IATA case study.	Corley et al., 2021; EPA, 2021c; OECD, 2022b

ICCVAM Validation Report, Table 3

National Institute of Environmental Health Sciences
Division of Translational Toxicology

Hazard Identification of Eye Irritation and Corrosion

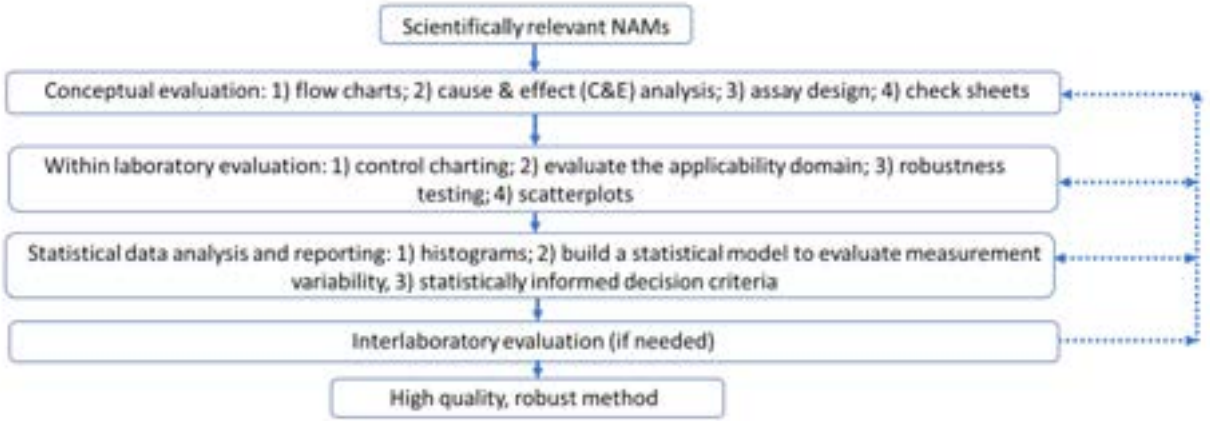
Decision Tree



Federal Register Notice: Jan 9, 2024

Technical Characterization

- Describe:
  - accuracy
  - intra-laboratory reproducibility
  - transferability
  - applicability domain
  - reference chemicals and controls
  - limits of detection and quantification



Draft ICCVAM Validation Report, Figure 2
(reprinted with permission from Petersen et al. 2022 ALTEX)

- Data reporting should allow for evaluation of the method, including:
  - protocol
  - equipment
  - computational models being used

- What is considered acceptable may depend on the method being evaluated and its intended use

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International collaborative projects

CERAPP

Collaborative Estrogen Receptor Activity Prediction Project **(2015/16)**

Mansouri et al. (https://doi.org/10.1289/ehp.1510267)

CoMPARA

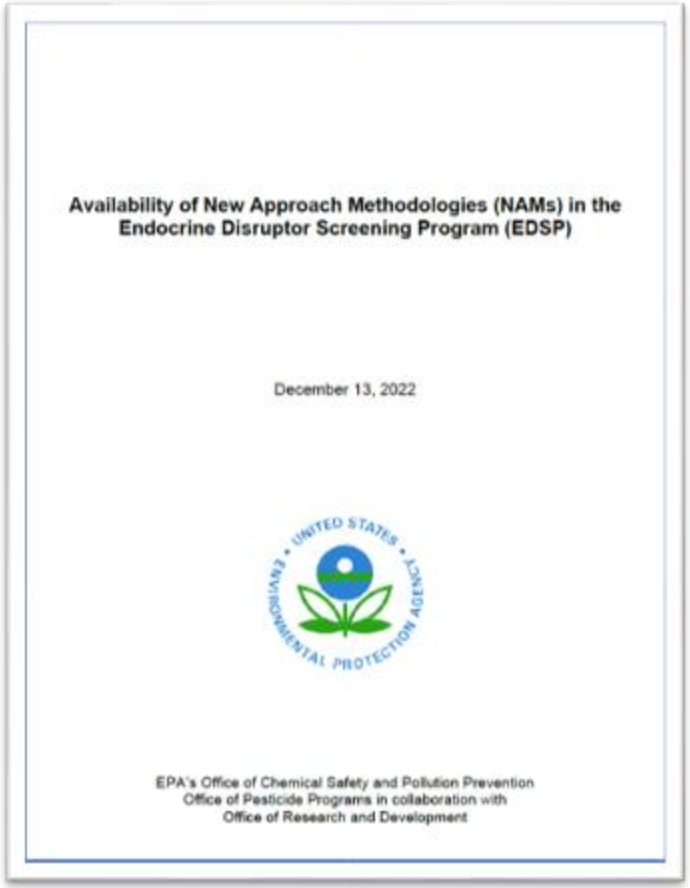
Collaborative **M**odeling **P**roject for **A**ndrogen **R**eceptor **A**ctivity **(2017/18)**

Mansouri et al. (https://doi.org/10.1289/EHP5580)

CATMoS

Collaborative **A**cute **T**oxicity **Mo**deling **S**uite **(2019/20)**

Kleinstreuer et al. (https://doi.org/10.1016/j.comtox.2018.08.002)
Mansouri et al. (https://doi.org/10.1289/EHP8495)


Availability of New Approach Methodologies (NAMs) in the Endocrine Disruptor Screening Program (EDSP)

December 13, 2022

EPA's Office of Chemical Safety and Pollution Prevention
Office of Pesticide Programs in collaboration with
Office of Research and Development

REACH COMPLIANCE

OPERA
OPEn (q)saR App

https://github.com/NIEHS/OPERA

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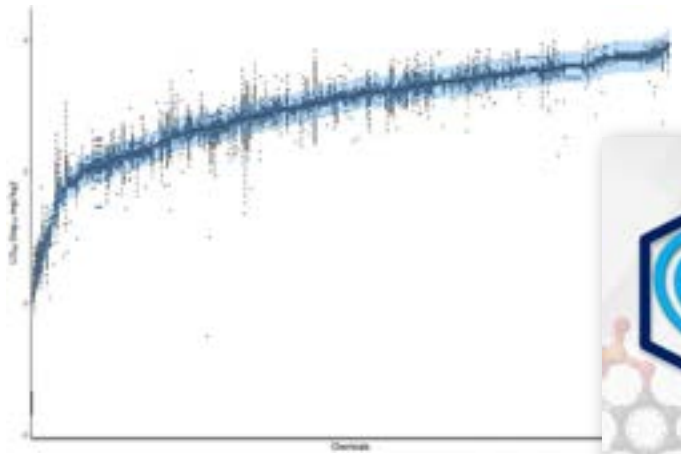
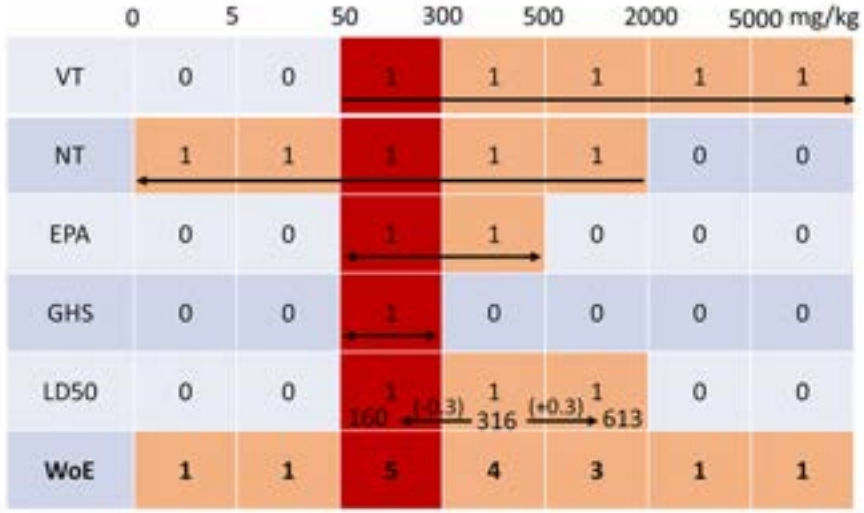
Reference Data Variability as a Benchmark

Data-driven Confidence Intervals for Model Evaluation/Predictions



OPERA
OPEn (q)saR App

Analyzing sources of variability in acute oral toxicity data & applying 95% confidence interval to predictions



	Very Toxic		Non-Toxic		EPA		GHS	
	Train	Eval	Train	Eval	Train	Eval	Train	Eval
Sensitivity	0.87	0.70	0.88	0.67	0.81	0.62	0.80	0.58
Specificity	0.99	0.97	0.97	0.90	0.92	0.86	0.95	0.90
Balanced Accuracy	0.93	0.84	0.92	0.78	0.87	0.74	0.88	0.74
***In vivo* Balanced Accuracy**	0.81		0.89		0.82		0.79	

	LD50 values		LD50 values
	Train	Eval	***In Vivo***
R2	0.85	0.65	0.80
RMSE	0.30	0.49	0.42

CATMoS QSAR predictions perform just as well as replicate *in vivo* data at predicting oral acute toxicity outcome

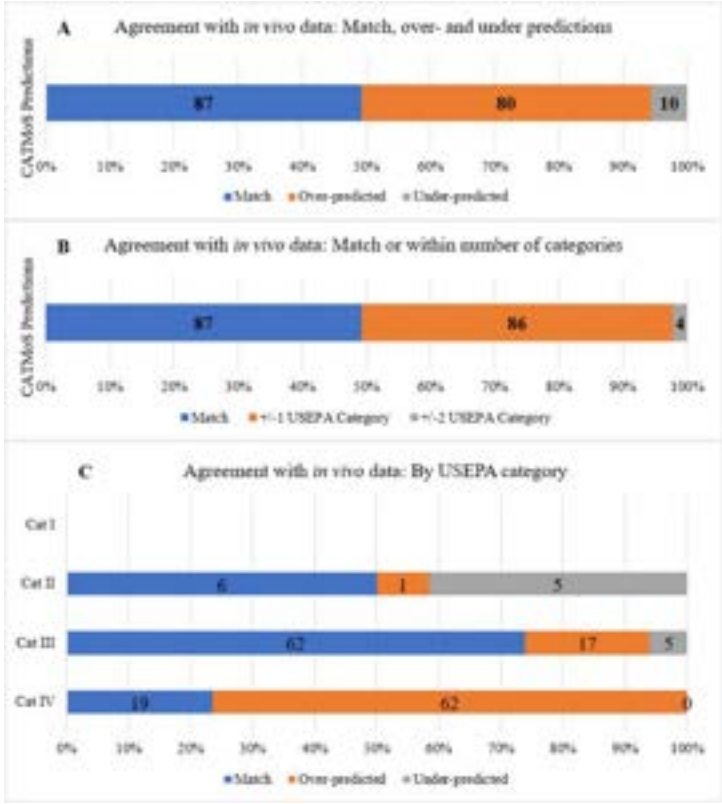
Karmaus et al. Toxicol Sci. 2022; Mansouri et al. EHP 2021

Application of CaTMOS to Pesticide AIs

EPA Case Study

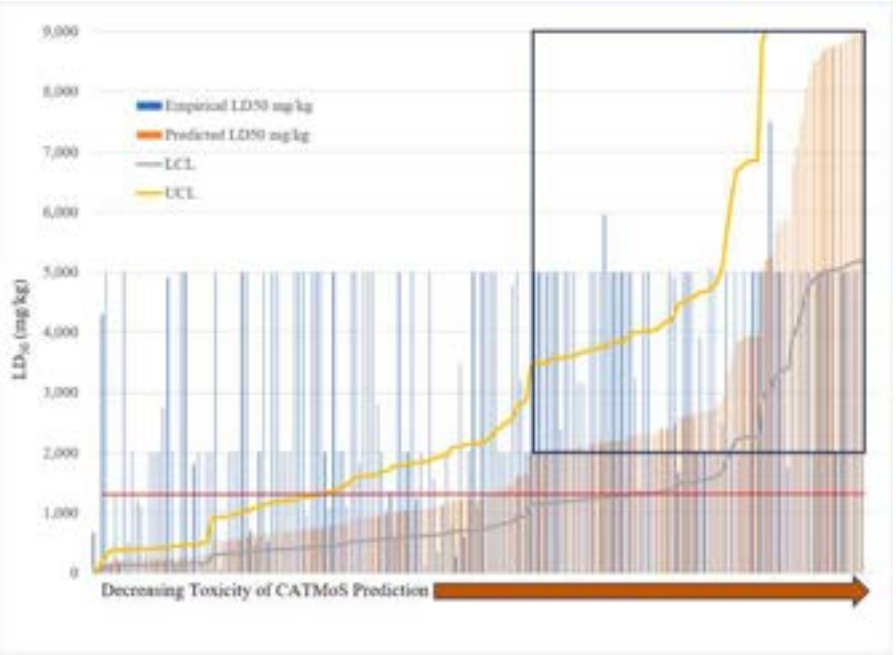
- Comparative analysis of 177 pesticides with LD_{50} data between CaTMOS and EPA database
- 88% categorical concordance for 165 chemicals with empirical *in vivo* LD_{50} values ≥ 500 mg/kg

Toxicity Category based on CATMoS Prediction	Number of predictions	Toxicity Category based on Empirical *In Vivo* Test Data			
		I	II	III	IV
I (<50 mg/kg)	2	-	1	1	-
II (50-500 mg/kg)	25	-	**6**	16	3
III (>500-5,000 mg/kg)	126	-	5	**62**	59
IV (>5,000 mg/kg)	24	-	-	5	**19**
III and IV combined	150	-	5	145	



Evaluation of *in silico* model predictions for mammalian acute oral toxicity and regulatory application in pesticide hazard and risk assessment

Patricia L. Bishop, Kamel Mansouri, William P. Eckel, Michael B. Lowit, David Allen, Amy Blankinship, Anna B. Lowit, D. Ethan Harwood, Tamara Johnson, Nicole C. Kleinstreuer



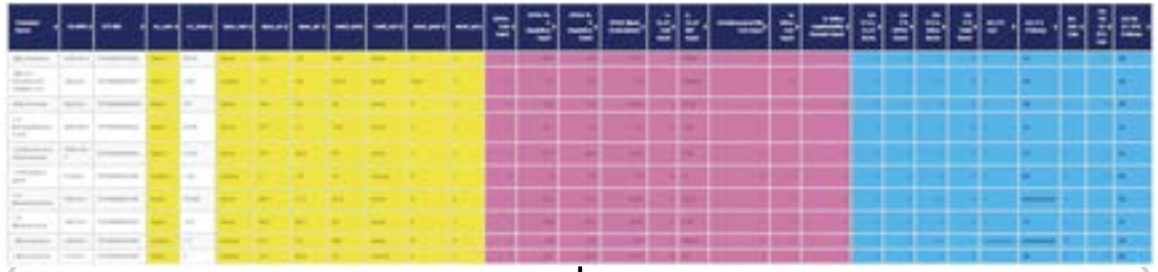
Bishop et al., Reg. Tox. Pharm., 2024 https://doi.org/10.1016/j.yrtph.2024.105614

Data Integrity and Information Transparency

- Assess integrity and credibility of the raw data to the final report
- Communicate transparently and publicly
- Assess and describe the uncertainties and limitations
- Independently reproduce data
  - External implementation and training of the models
  - Processing of the raw data
  - Replicate predictions obtained in the validation study

User Friendly DASS App

To et al. 2024 BMC Bioinformatics



Color-coded results table
Yellow: User selected data columns
Pink: Translated user data, input for DASS algorithms
Blue: DASS predictions

Values calculated by the app are shown to help the user understand derivation of predictions.

Translated input shows how the app interprets flagged data.

Individual and combined scores from the ITS are provided.

Access the DASS App
https://ntp.niehs.nih.gov/go/952311

Independent Review

- Important part of confidence building process
- Appropriate level of external review depends on the method and context of use
- Might include publication in peer-reviewed journal or review by an independent scientific advisory panel
- International adoption by OECD typically needs formal peer review
- Method developers may fund but should not manage peer review

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Division of Translational Toxicology

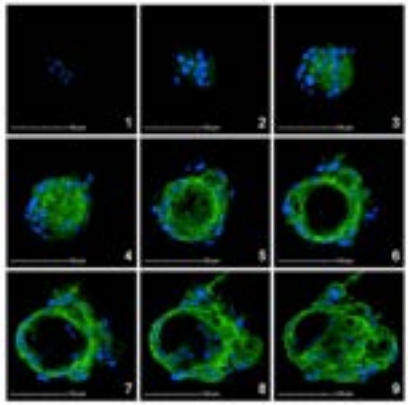
Inter-laboratory Validation Study of Human Thyroid Microtissue Assay

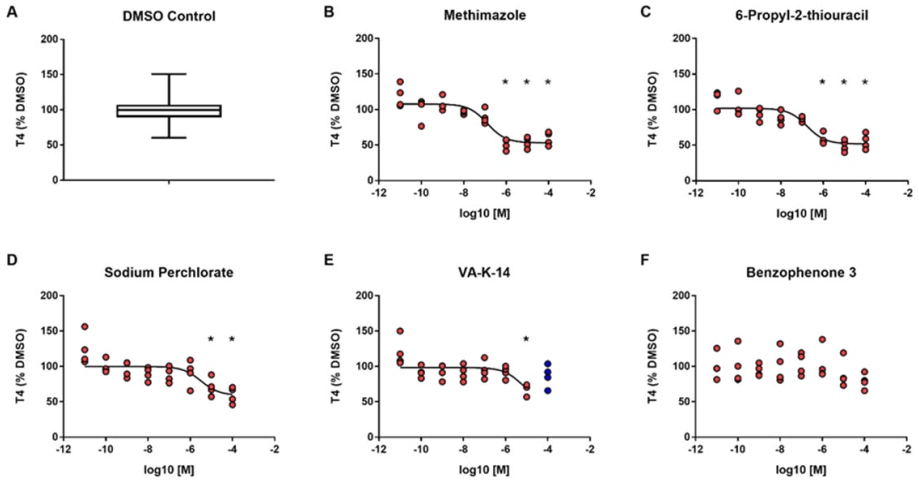
OXFORD SOT Society of Toxicology
academic.oup.com/toxsci

TOXICOLOGICAL SCIENCES, 2019, 1–16

Development of an *In Vitro* Human Thyroid Microtissue Model for Chemical Screening

Chad Deisenroth ,*,[1] Valerie Y. Soldatow,[†] Jermaine Ford,[‡] Wendy Stewart,* Cassandra Brinkman,* Edward L. LeCluyse,[†] Denise K. MacMillan,[‡] and Russell S. Thomas *





Team Members

Coordinator: NICEATM

Method Developer
Lab 1

Lab 2

Lab 3

Lab 4

Status:

- Phase 1.2 complete (initial transfer phase, lab 2)
- Phase 1.3 underway (secondary transfer phase, labs 3 and 4)
- Phase 1.4 slated to start in summer (validation study)
- External peer review will be coordinated by NIEHS OPRO

Agency-Specific Guidance

https://www.fda.gov/medical-devices/software-medical-device-samd/artificial-intelligence-and-machine-learning-software-medical-device

Contains Nonbinding Recommendations

Assessing the Credibility of Computational Modeling and Simulation in Medical Device Submissions

Guidance for Industry and Food and Drug Administration Staff

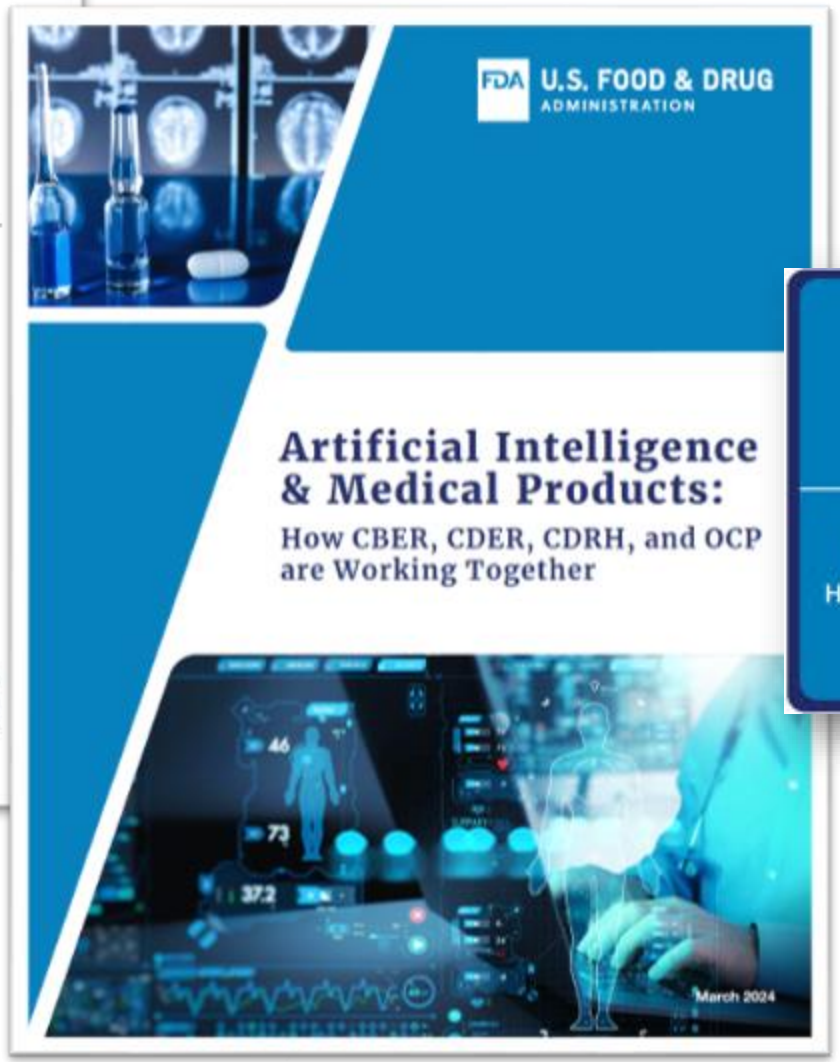
Document issued on November 17, 2023.

The draft of this document was issued on December 23, 2021.

For questions about this document, contact Office of Science and Engineering Laboratories (OSEL) by email at OSEL_CDRH@fda.hhs.gov or at (301)-796-2530, or Pras Pathmanathan at (301) 796-3490 or by email pras.pathmanathan@fda.hhs.gov.

FDA U.S. FOOD & DRUG ADMINISTRATION
CENTER FOR DEVICES & RADIOLOGICAL HEALTH

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Devices and Radiological Health



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Whitepaper on In Silico Technologies

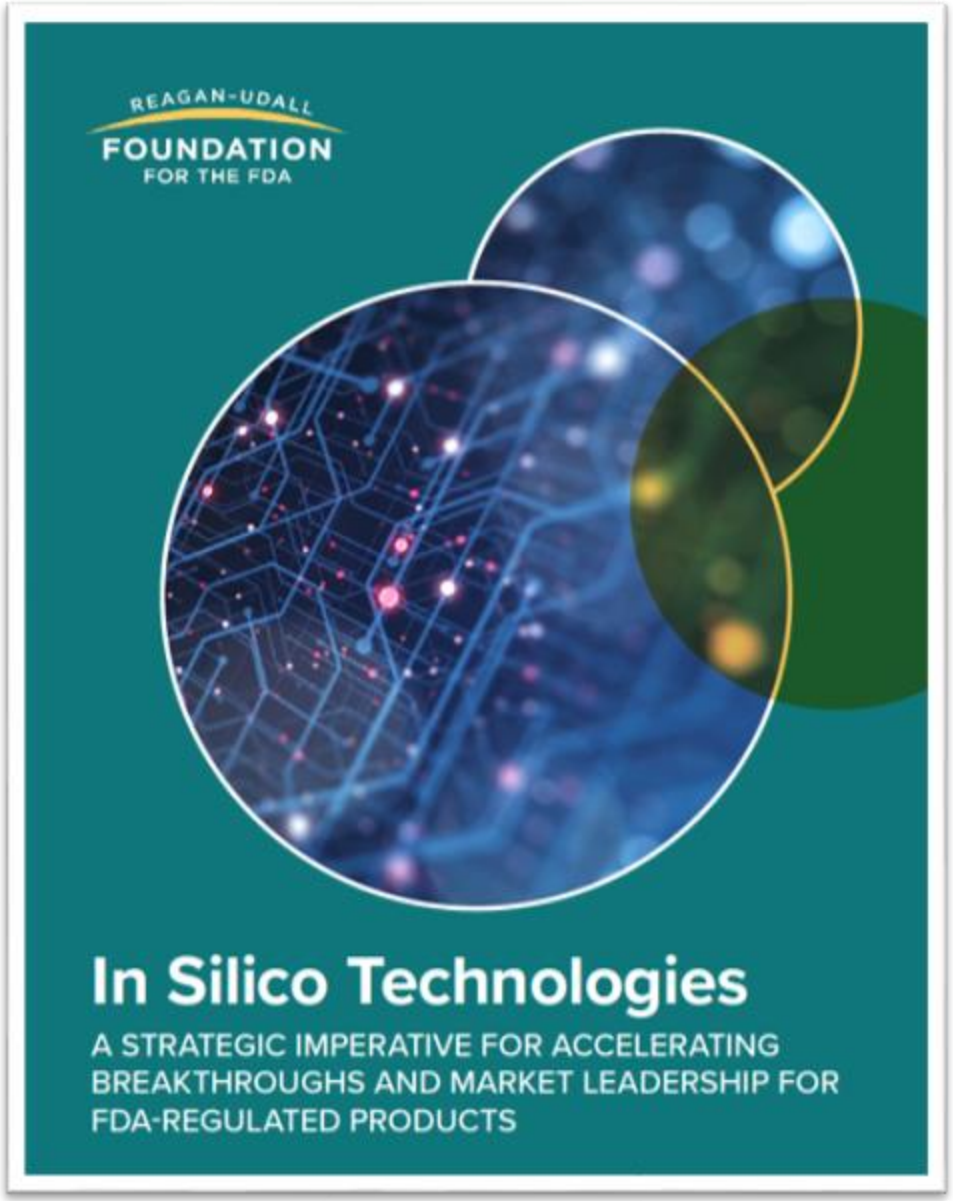
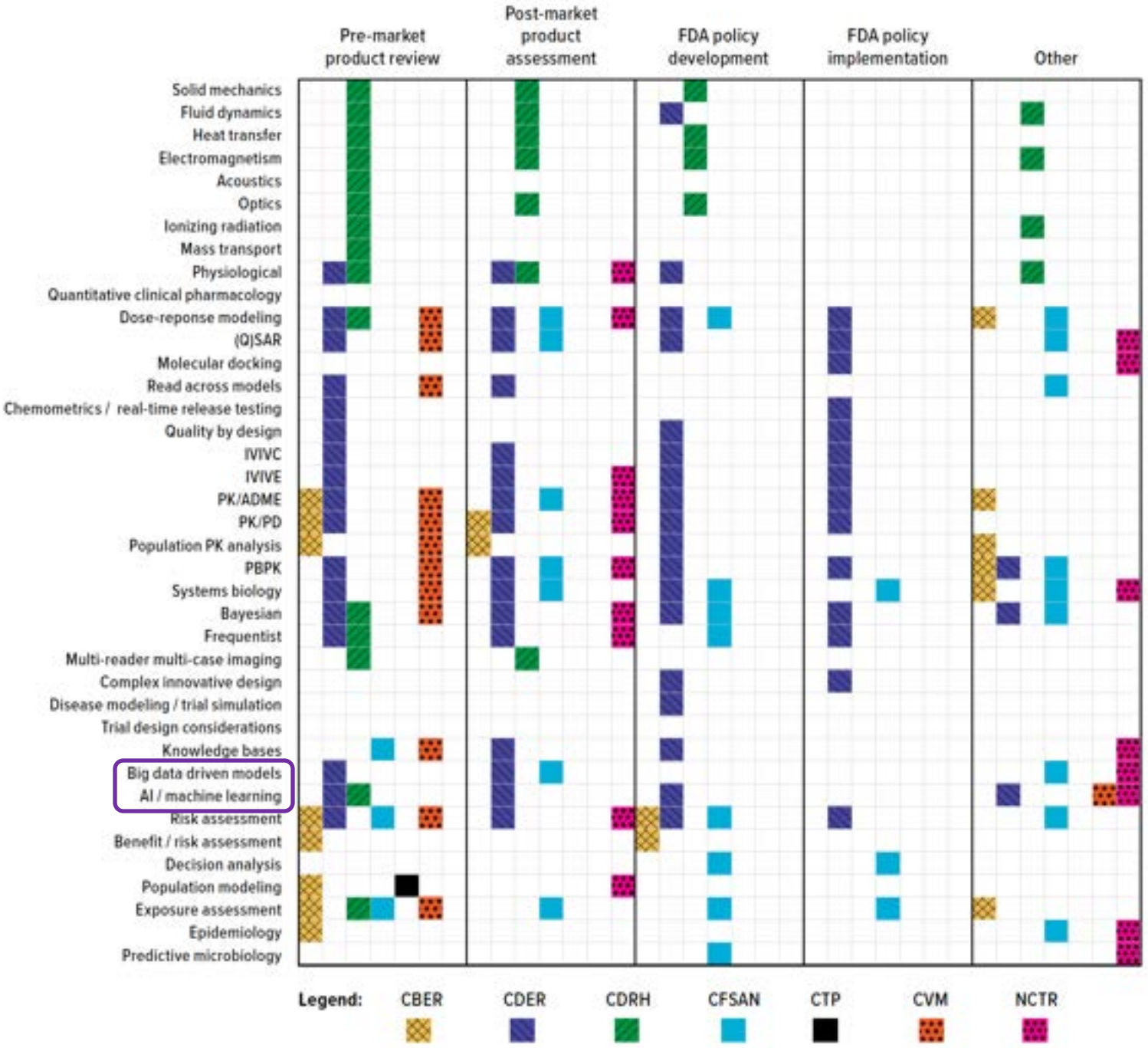


TABLE OF CONTENTS

Use of modeling and simulation across FDA, organized by modeling discipline (rows), application area (outer columns) and FDA Center (inner columns, colors).

CBER, CDER, CDRH, CFSAN, CTP, and CVM are regulatory product Centers and NCTR is a non-regulatory Center providing regulatory research support to product Centers.

Figure 4. Use of Modeling and Simulation by the FDA in Different Aspects of the Products' Lifecycle[11]



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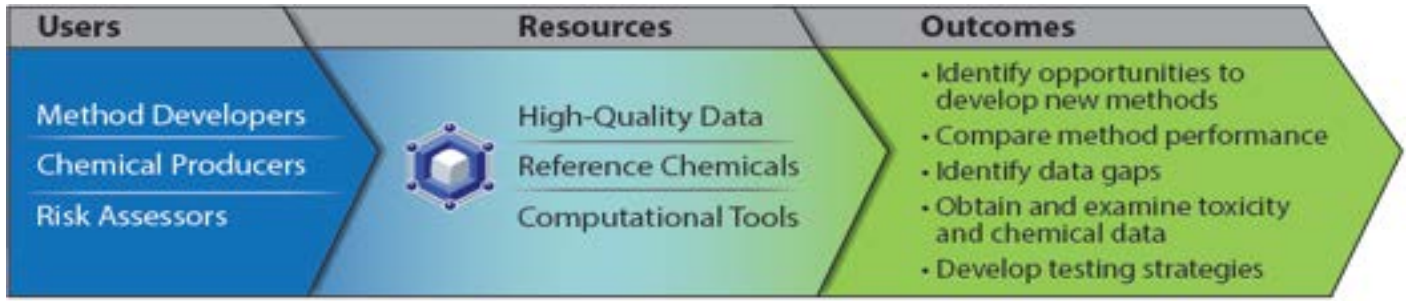
ICE: The Integrated Chemical Environment



Integrated Chemical Environment

Findable Accessible Interoperable Reusable

https://ice.ntp.niehs.nih.gov/

Bell et al. 2017 EHP
Bell et al. 2020 Tox In Vitro
Abedini et al. 2021 Comp Tox
Daniel et al. 2022 Front Toxicol
Kreutz et al. 2024 Toxics
Marciano et al. 2024 STOTEN

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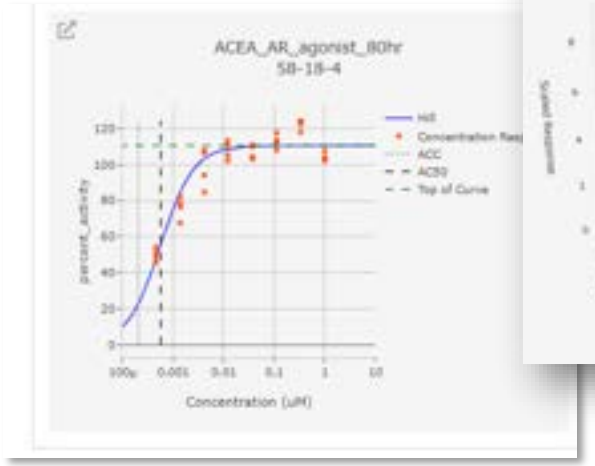
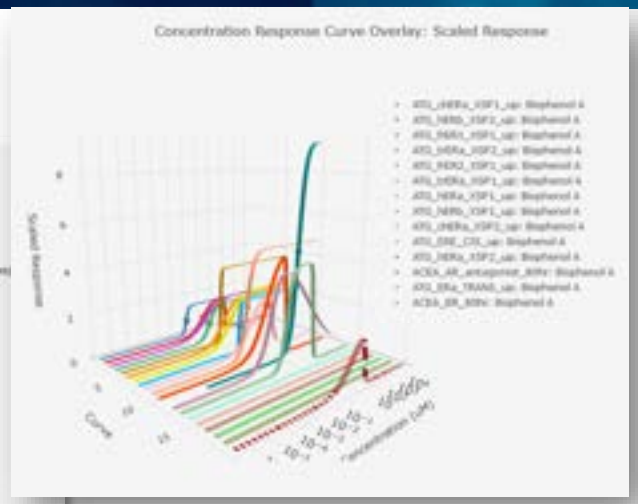
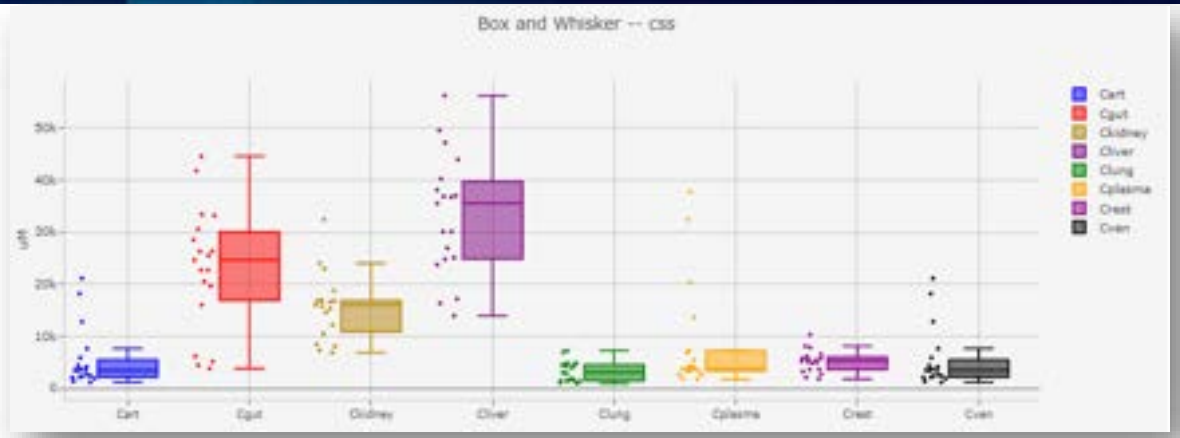
ICE Tools: Examples

HTS Data Exploration

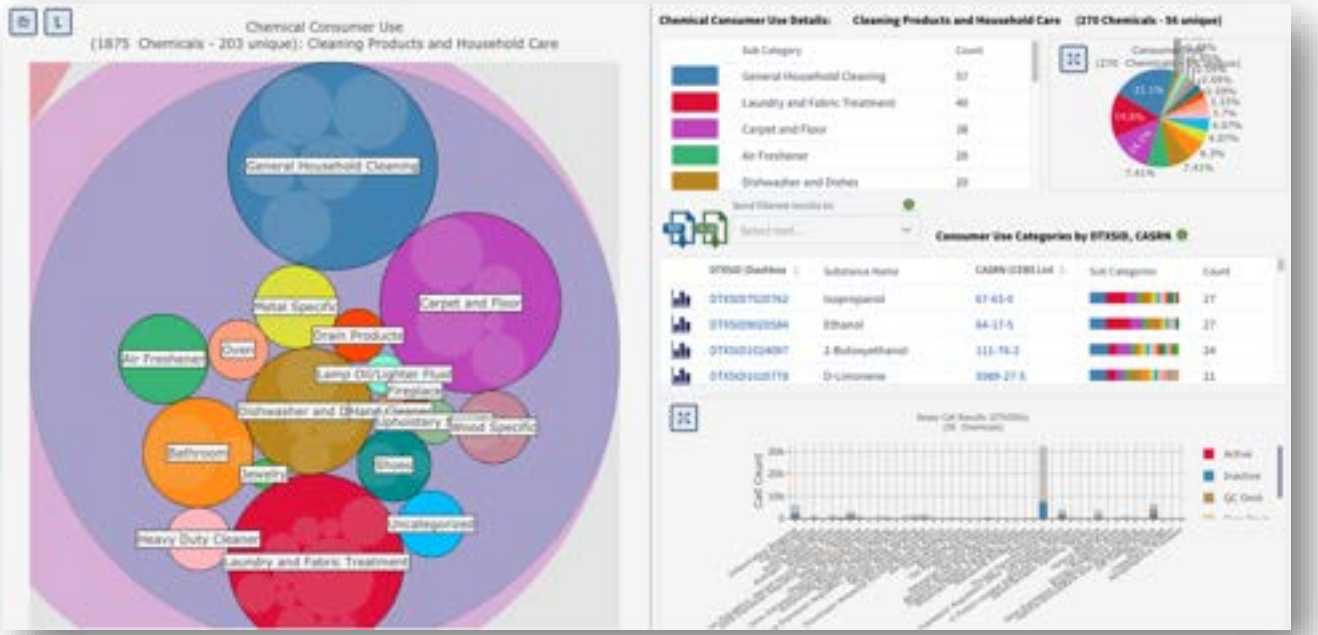






Predicting Chemical Exposure: Body Tissues, Consumer Products

Chemical Similarity



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Acknowledgments

The NICEATM Group

Dr. Tina Morrison
U.S. FDA
Director, Office of Regulatory Science and Innovation

UNITED STATES ICCVAM
Advancing Alternatives to Animal Testing

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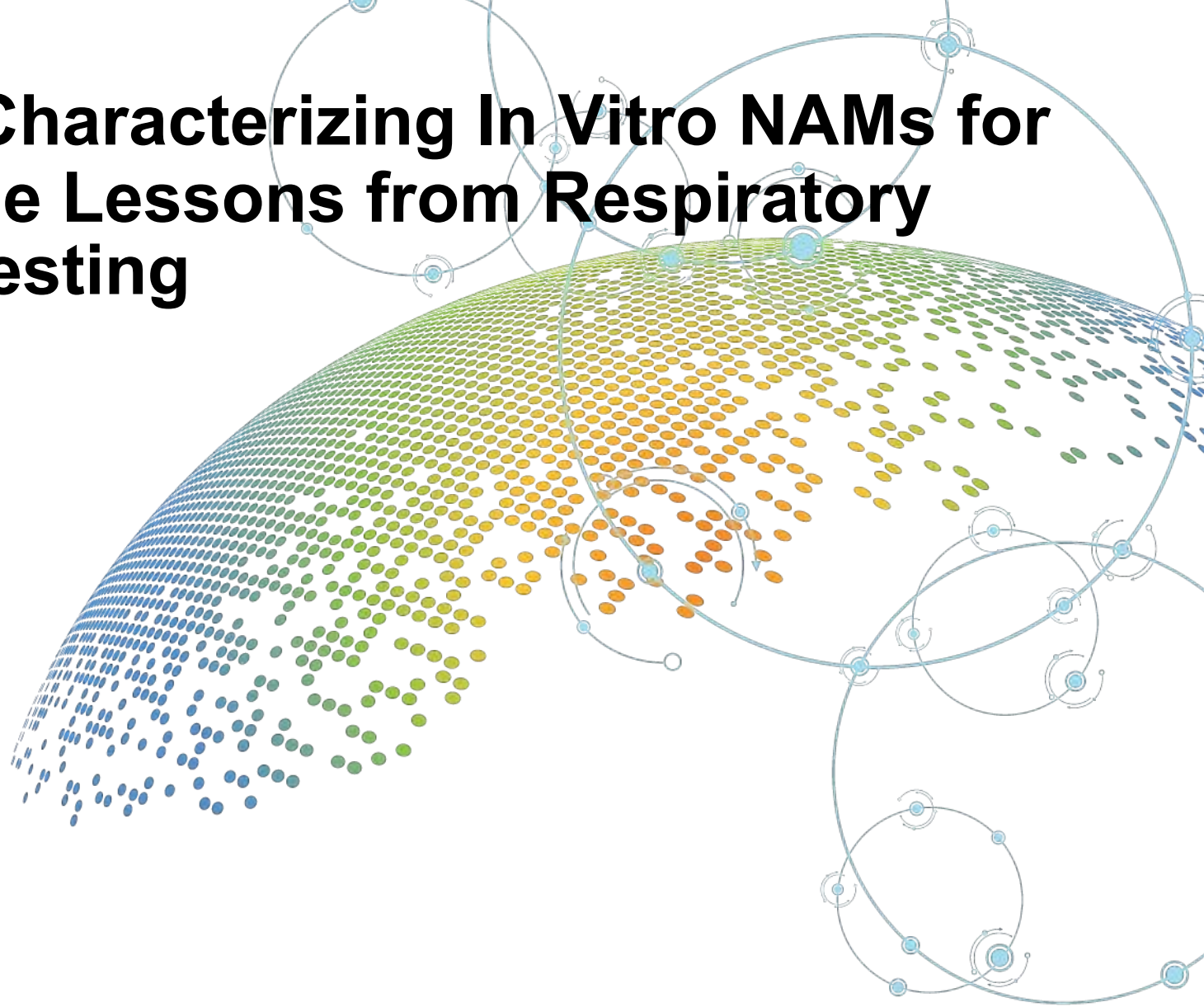
Integrated Chemical Environment

Now Available:
2022 – 2023 ICCVAM Biennial Report

Key Considerations for Characterizing In Vitro NAMs for Toxicity Testing: Valuable Lessons from Respiratory Toxicity Research and Testing



Shaun D. McCullough, PhD
Senior Respiratory Scientist
Principal Inhalation Toxicologist
Exposure & Protection
RTI International

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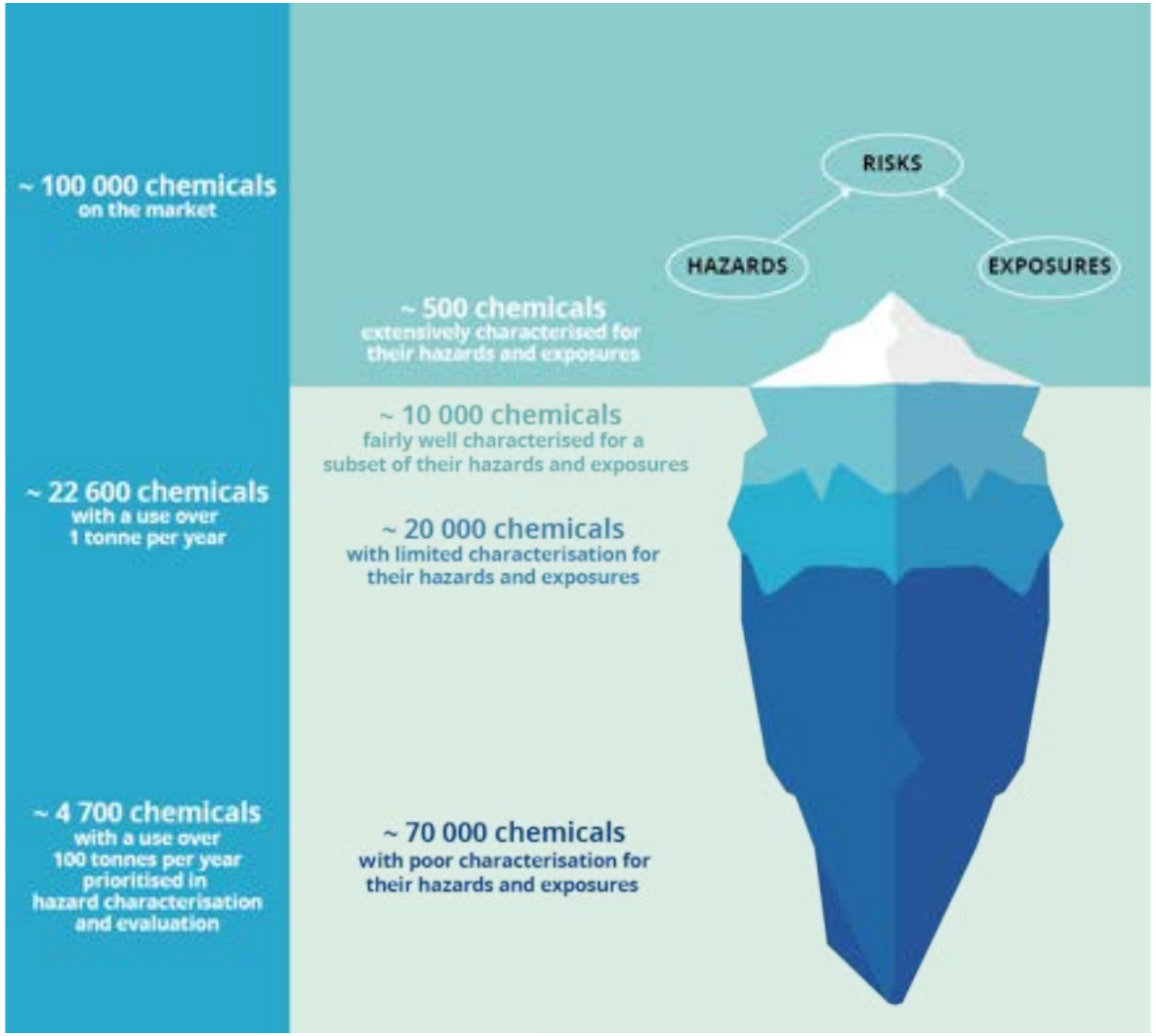
Advancing Chemical Screening and Testing

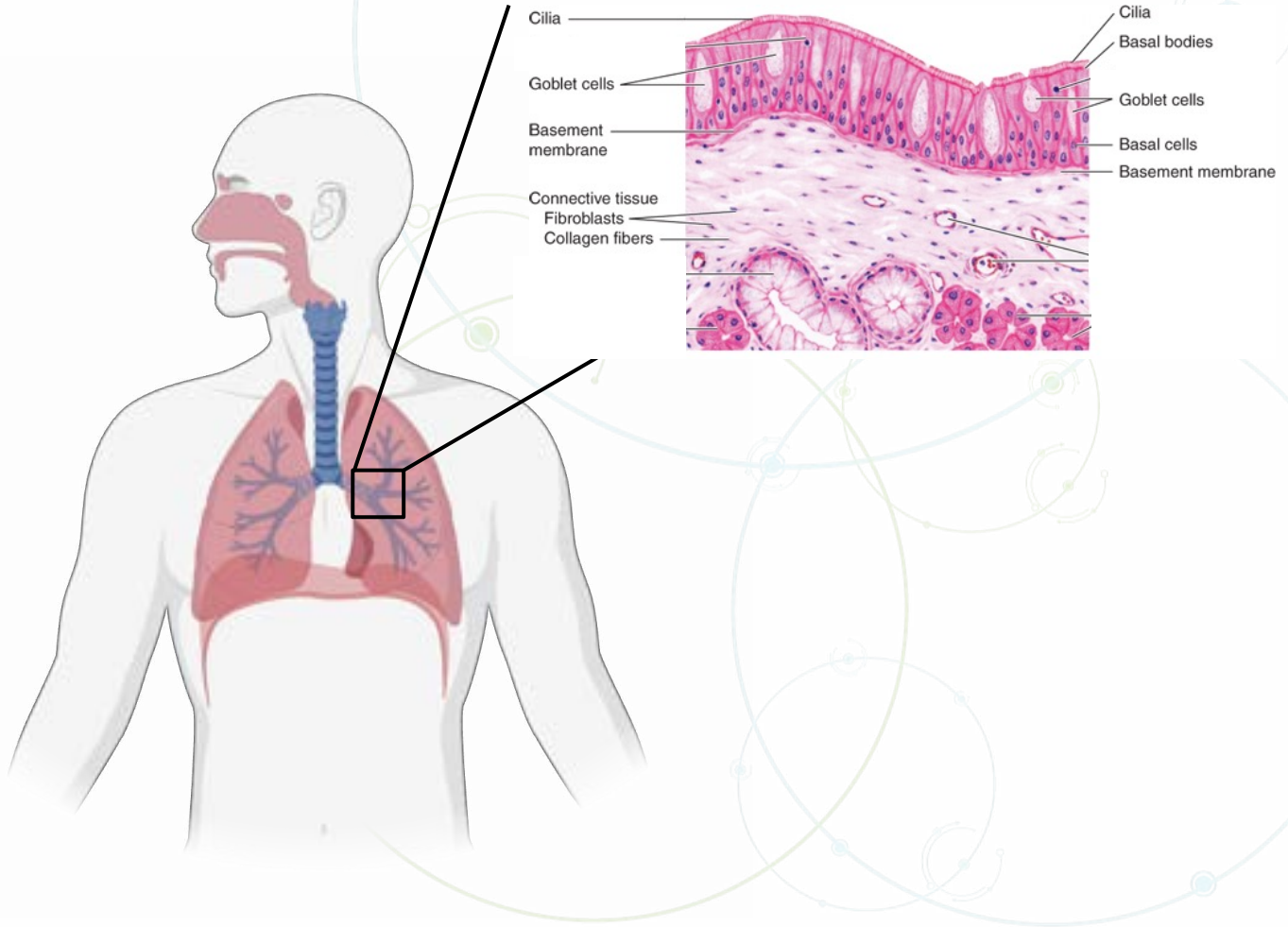


- Chemical and materials exposures are ubiquitous
- Thousands of data-poor chemicals pose unknown risks to human health
  - Compounded by:
    - Mixtures
    - Repeated exposure scenarios
    - Life stages
    - Susceptible populations
    - Cumulative effects
- Animal testing data have limited relevance to humans

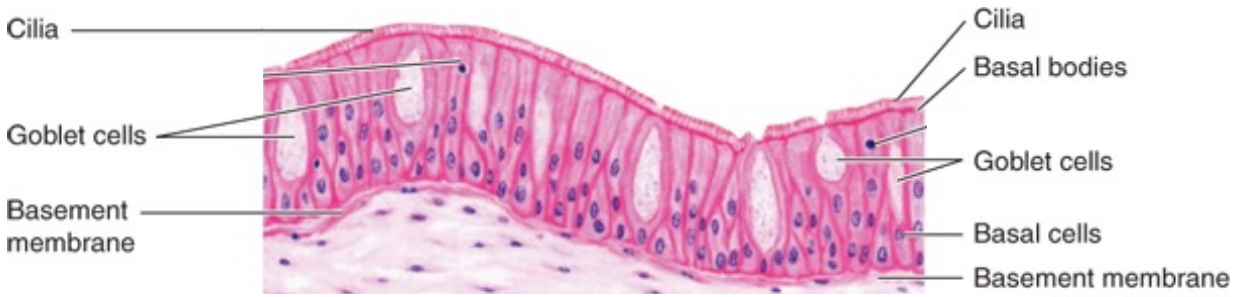
Respiratory Toxicity Modeling Has Focused Primarily on the Airway Epithelium

Main functions of bronchial epithelium:

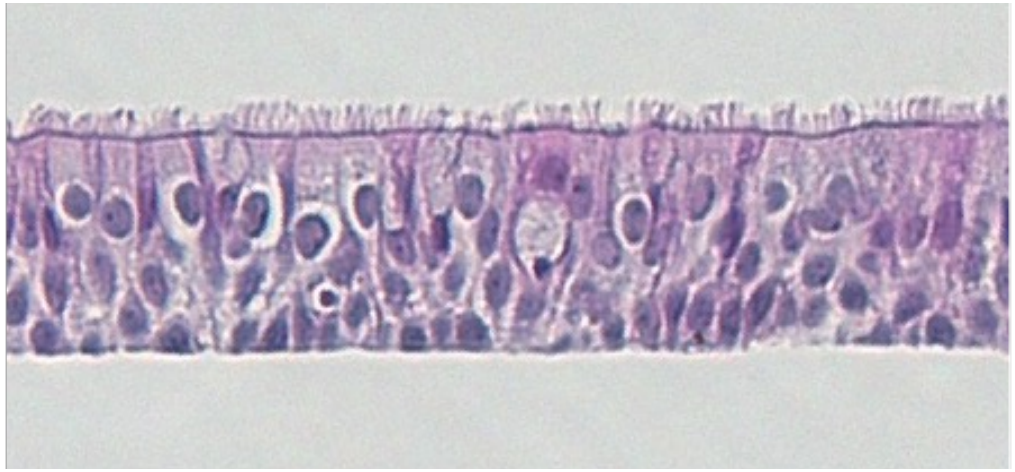
- Physical barrier
- Mucocilliary clearance
- Release of pro-inflammatory cytokines
- Remodeling after injury



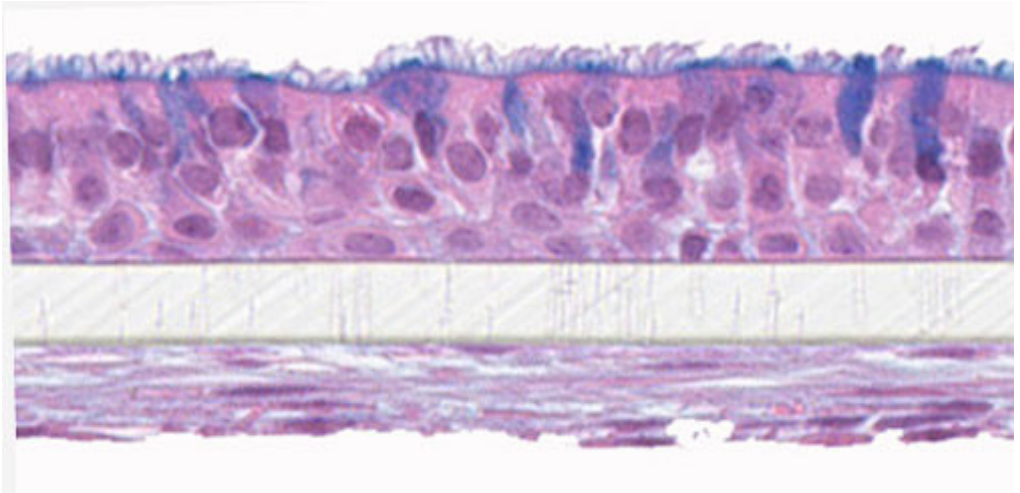
Replicating In Vivo Biology In Vitro



Epithelial Only



Epithelial-Fibroblast Co-Culture



Lechner *et al.*, 2011. Respiratory: An Integrated Approach to Disease. McGraw-Hill Education

Top: McCullough lab; Bottom: Epithelix MucilAir™-HF

Integration into Decision Making for Inhaled Chemicals

- Case study for refining the inhalation risk assessment for the pesticide chlorothalonil under FIFRA
  - Final report in 2018
- First requirement for in vitro inhalation study in a TSCA Test Order
  - Issued in 2023

Epithelial Only

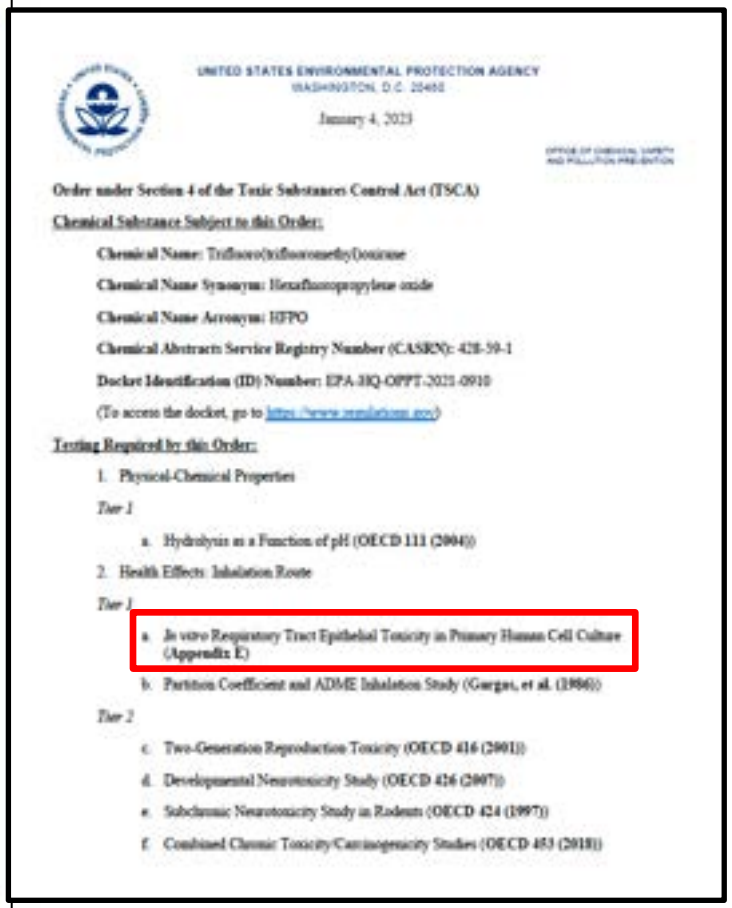
Issue Paper
Evaluation of a Proposed Approach to Refine Inhalation Risk Assessment for Point of Contact Toxicity:
A Case Study Using a New Approach Methodology (NAM)

EPA's Office of Chemical Safety and Pollution Prevention

August 30, 2018

Page 1 of 33

Epithelial-Fibroblast Co-Culture



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

January 4, 2023

Order under Section 4 of the Toxic Substances Control Act (TSCA)

Chemical Substance Subject to this Order:

Chemical Name: Trifluoro(trifluoromethyl)oxirane

Chemical Name Synonym: Hexafluoropropylene oxide

Chemical Name Acronym: HFPO

Chemical Abstracts Service Registry Number (CASRN): 428-59-1

Docket Identification (ID) Number: EPA-HQ-OPPT-2021-0910

(To access the docket, go to https://www.regulations.gov)

Testing Required by this Order:

1. Physical-Chemical Properties

Tier 1

a. Hydrolysis as a Function of pH (OECD 111 (2004))

2. Health Effects: Inhalation Route

Tier 1

a. In vitro Respiratory Tract Epithelial Toxicity in Primary Human Cell Culture (Appendix E)

b. Partition Coefficient and ADME Inhalation Study (Gargas, et al. (1986))

Tier 2

c. Two-Generation Reproduction Toxicity (OECD 416 (2001))

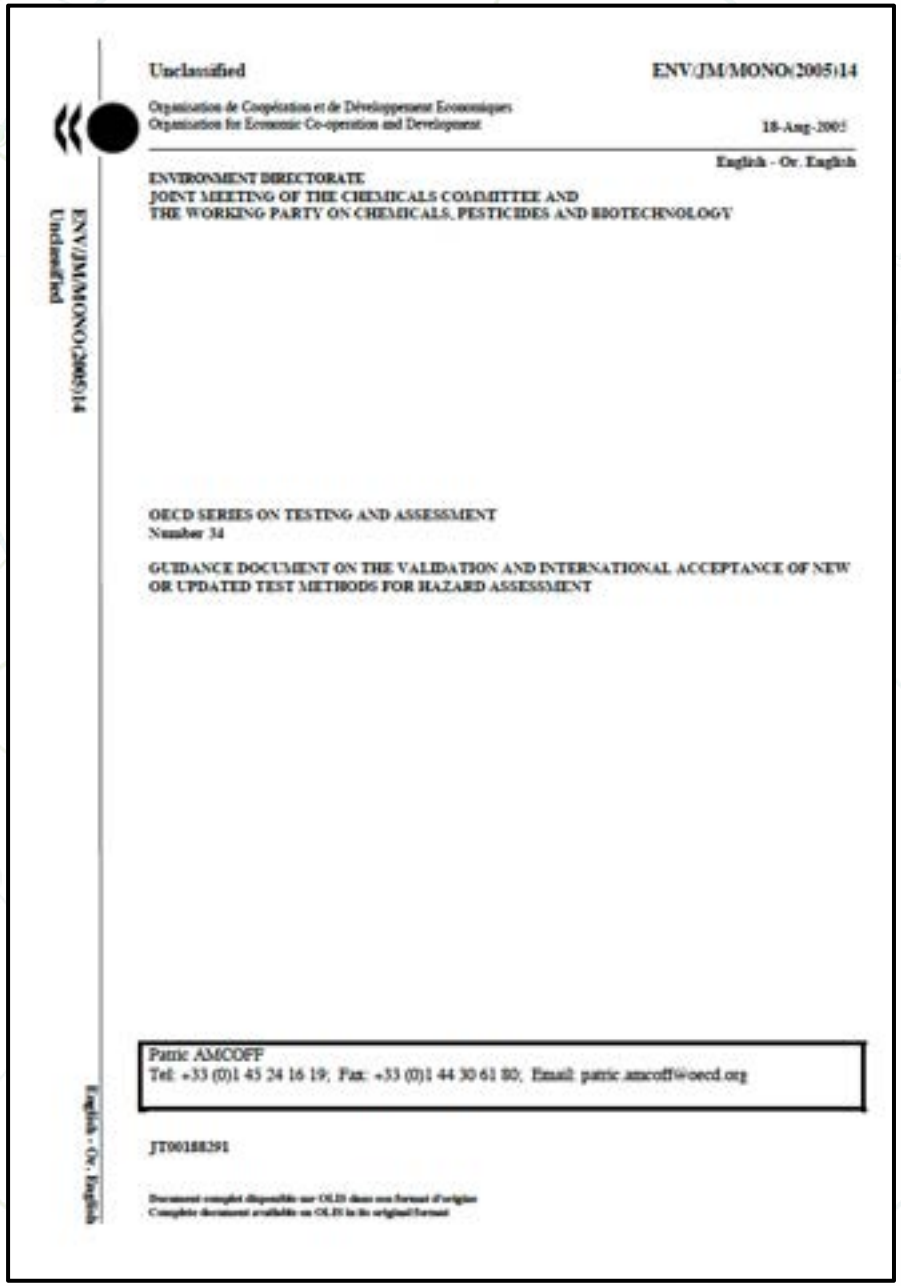
d. Developmental Neurotoxicity Study (OECD 426 (2007))

e. Subchronic Neurotoxicity Study in Rodents (OECD 424 (1997))

f. Combined Chronic Toxicity/Carcinogenicity Studies (OECD 453 (2018))

Validation Concepts from OECD GD34

- Relationship of the endpoint(s)/test method to the in vivo biological effect and toxicity of interest.
- Limitations should be described.
- Demonstrate intra-test variability, repeatability, and reproducibility of the test method within and amongst laboratories.
- Evaluation of test method performance in relation to existing relevant toxicity data.



Unclassified ENV/JM/MONO(2005)14

Organisation de Coopération et de Développement Economiques
Organisation for Economic Co-operation and Development

18-Aug-2005

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

ENV/JM/MONO(2005)14
Unclassified

OECD SERIES ON TESTING AND ASSESSMENT
Number 34

GUIDANCE DOCUMENT ON THE VALIDATION AND INTERNATIONAL ACCEPTANCE OF NEW OR UPDATED TEST METHODS FOR HAZARD ASSESSMENT

Patric AMCOFF
Tel: +33 (0)1 45 24 16 19; Fax: +33 (0)1 44 30 61 80; Email: patric.amcoff@oecd.org

JT00188291

English - Or. English

Thorough Characterization is the Precursor to Validation

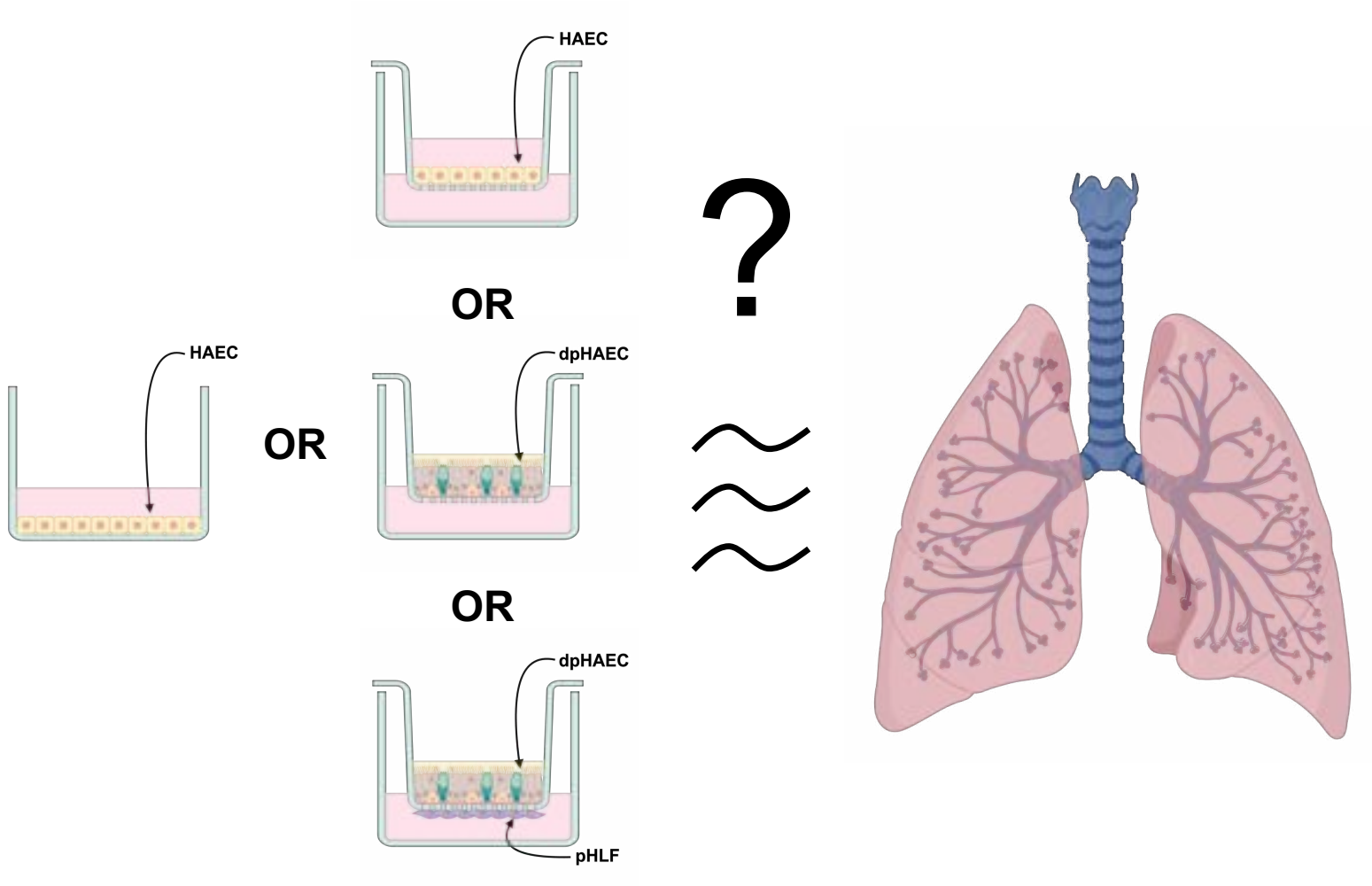
- Key aspects of validation principles cannot be addressed without prior thorough characterization of test systems and assays.
  - Integrating more in vivo relevance is critical for capturing biological effects and ensuring accurate representation of the toxicity of interest.
  - Provides rationale for how the test system/method can be applied and more thorough understanding of limitations.
  - Identification of factors that impact variability, repeatability, and reproducibility.

Part #1
Increasing in vivo relevance of test systems

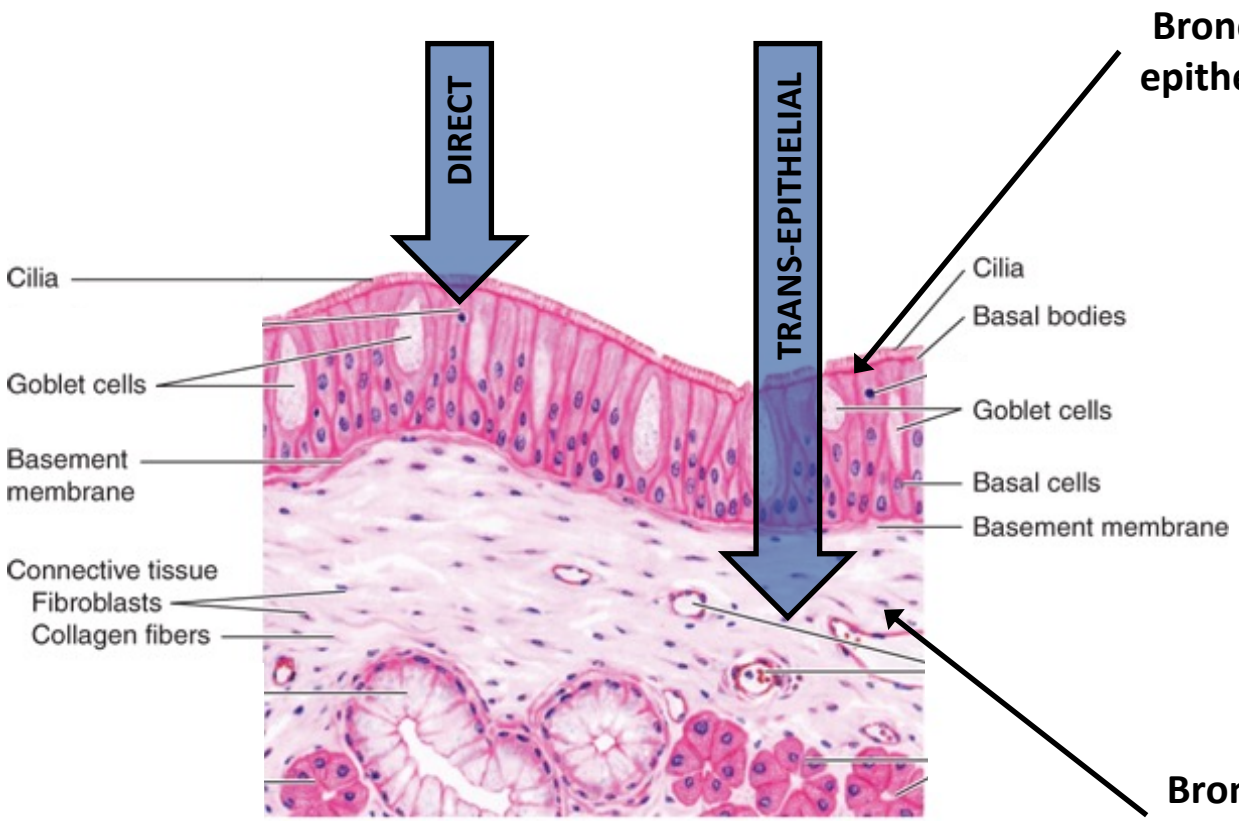
Biological Complexity of the Test System

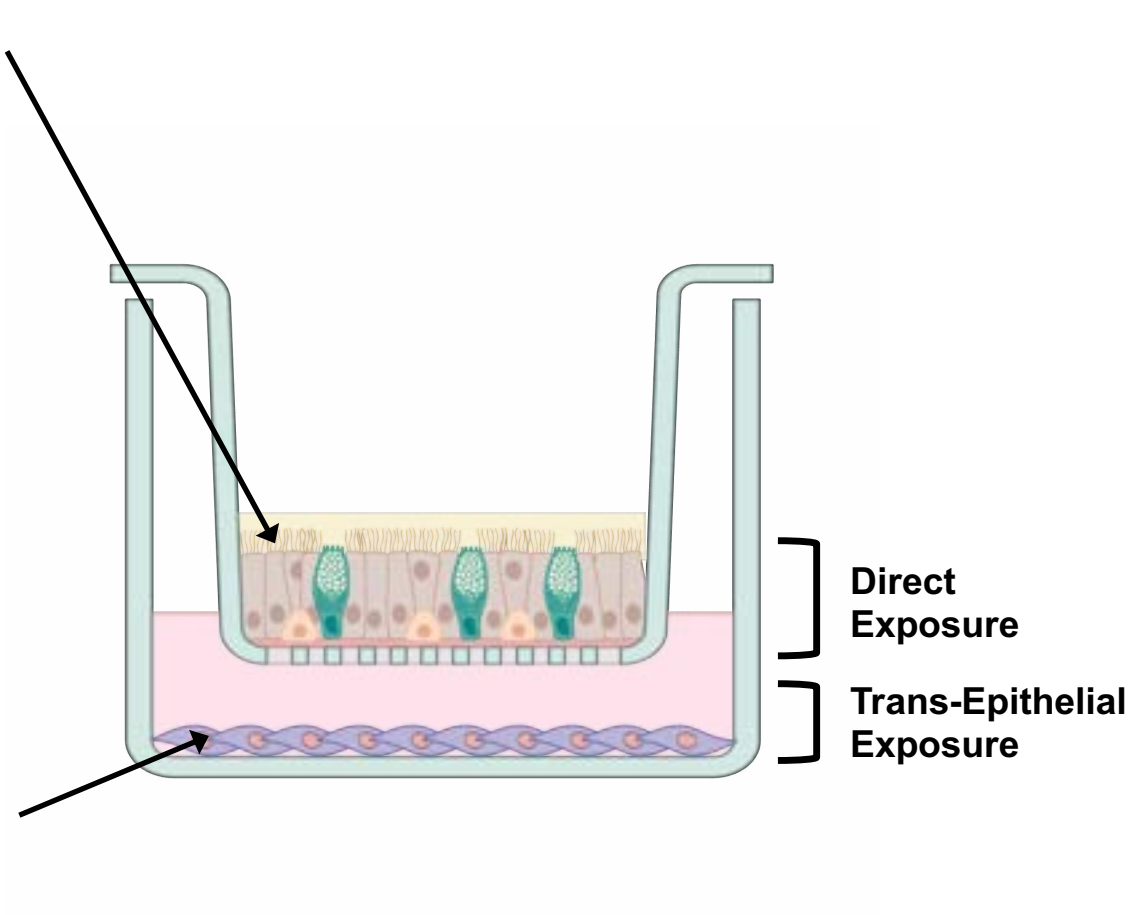
- Typical applications
  - Overt damage (e.g., corrosion)
  - Irritation
  - Remodeling to reflect disease state

- Epithelial cells don't function alone, but epithelial only systems are the standard of practice despite general availability of multi-cellular alternatives



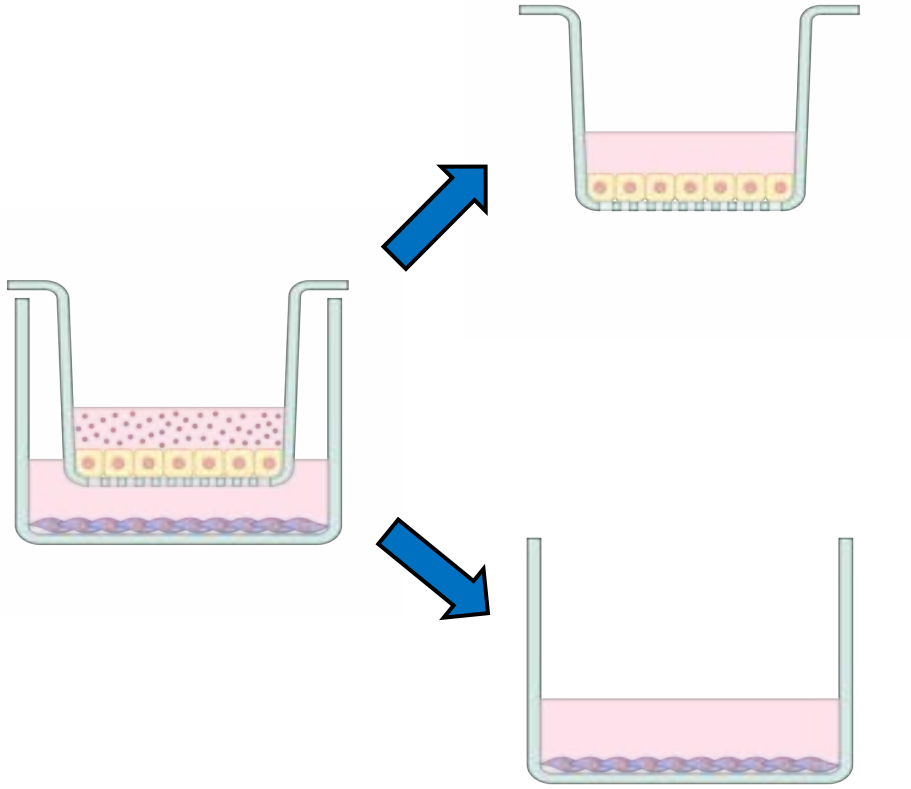
Trans-Epithelial Exposure Model (TEEM)



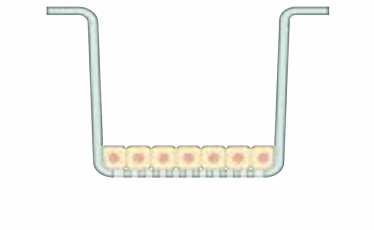


Lechner *et al.*, 2011. Respiratory: An Integrated Approach to Disease. McGraw-Hill Education

Cell Type Specific Analysis with the TEEM

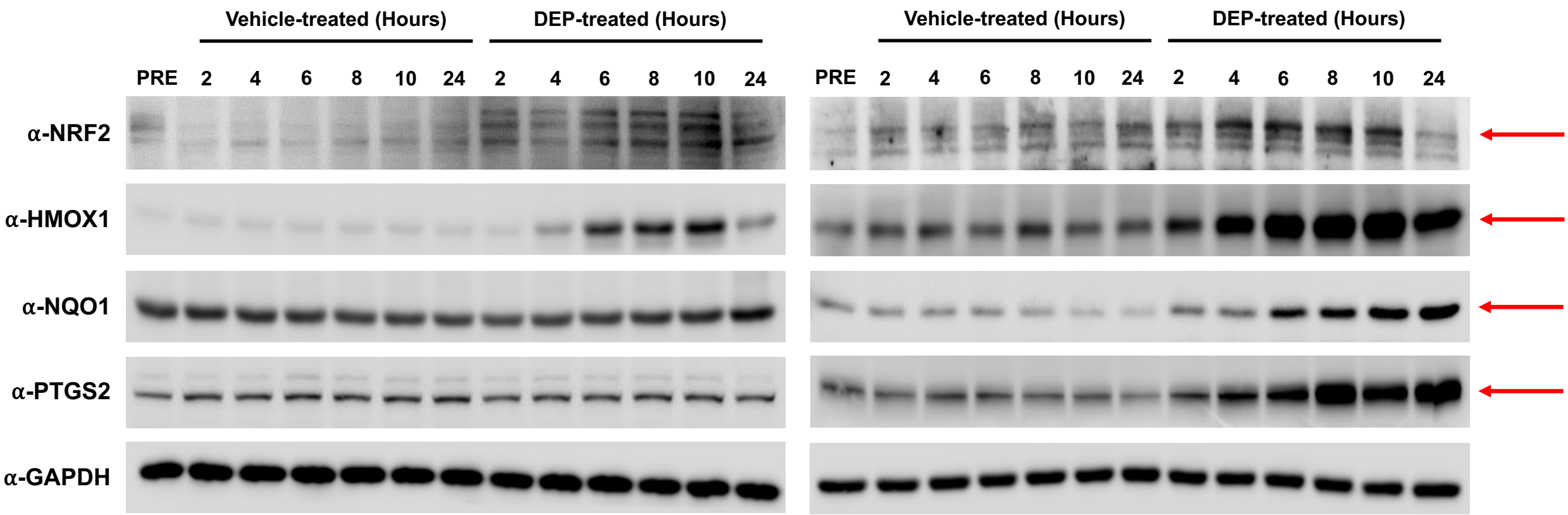


DEP-Induced Oxidative Stress-Responsive Proteins



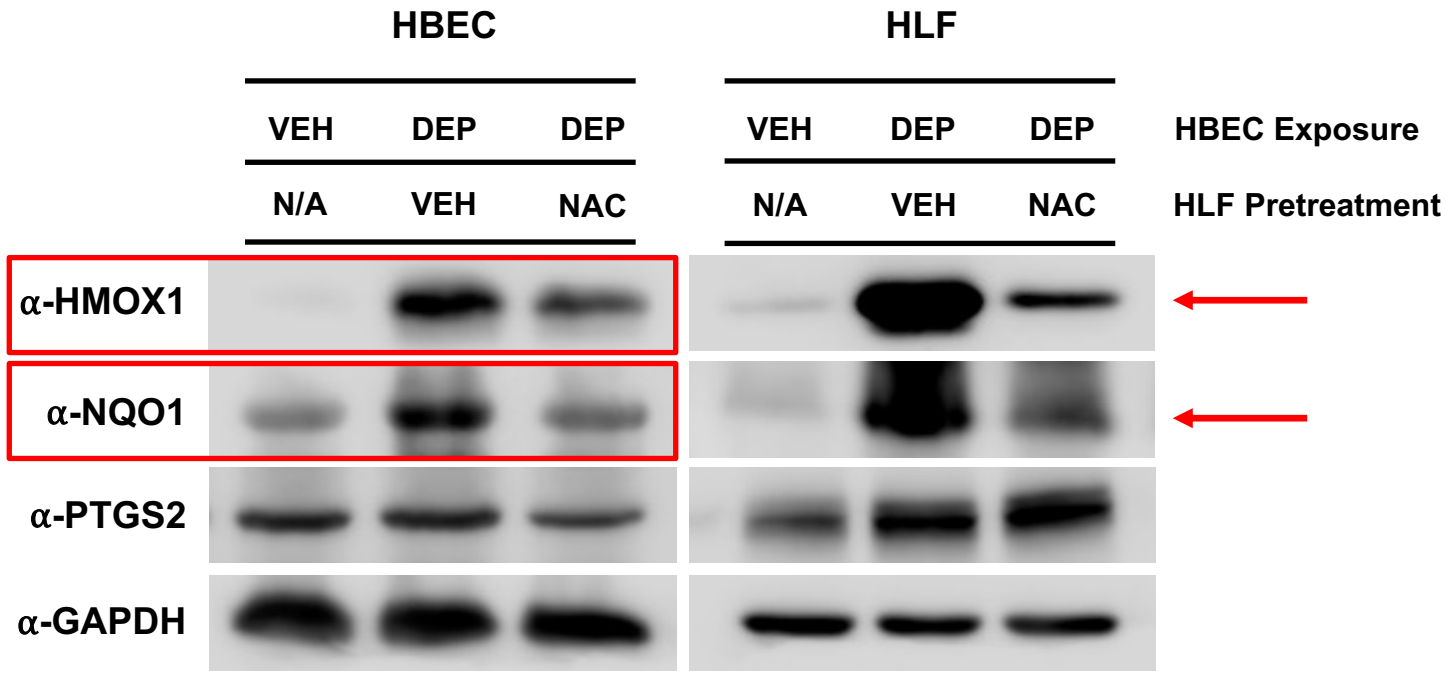
HBEC

HLF

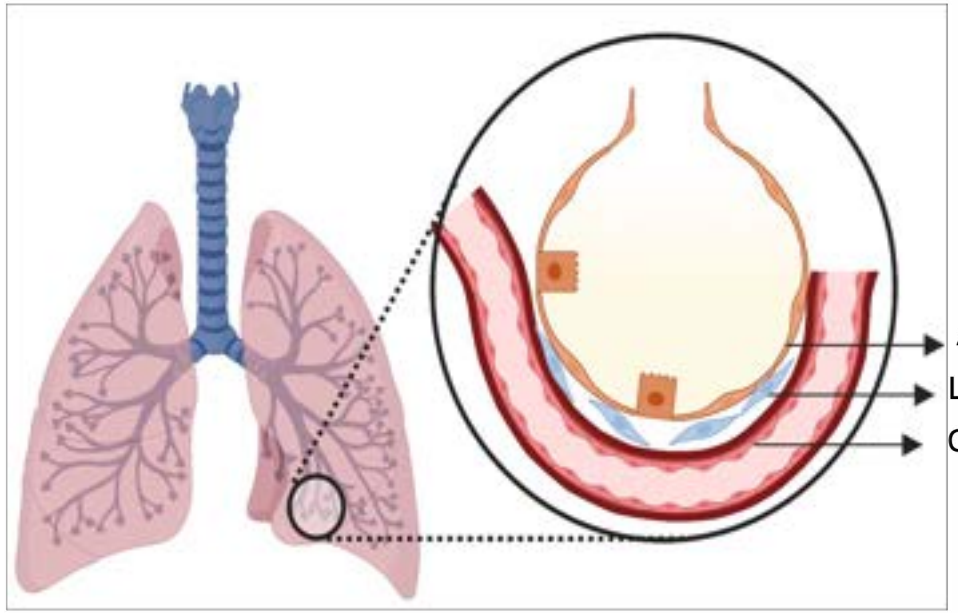


Faber *et. al.*, (2020) *Toxicological Sciences* **177(1):** 140-155

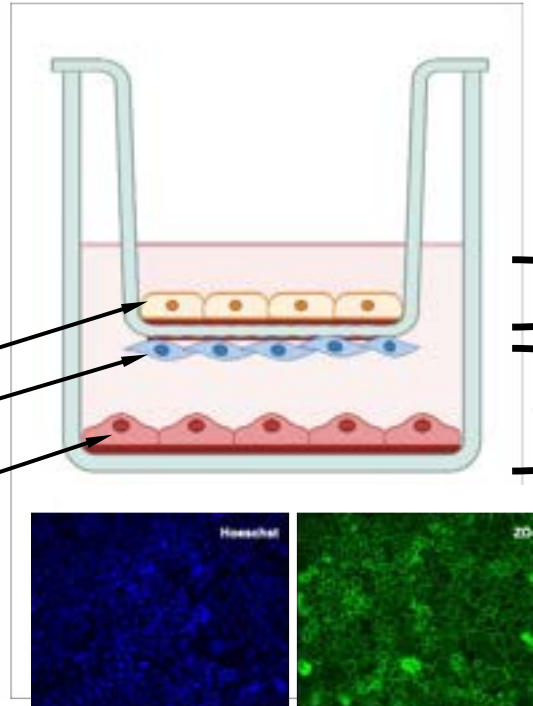
Fibroblasts Influence Adjacent Epithelial Cell Responses



The Alveolar Capillary Region Exposure (ACRE) Model Reflects Tissue Architecture

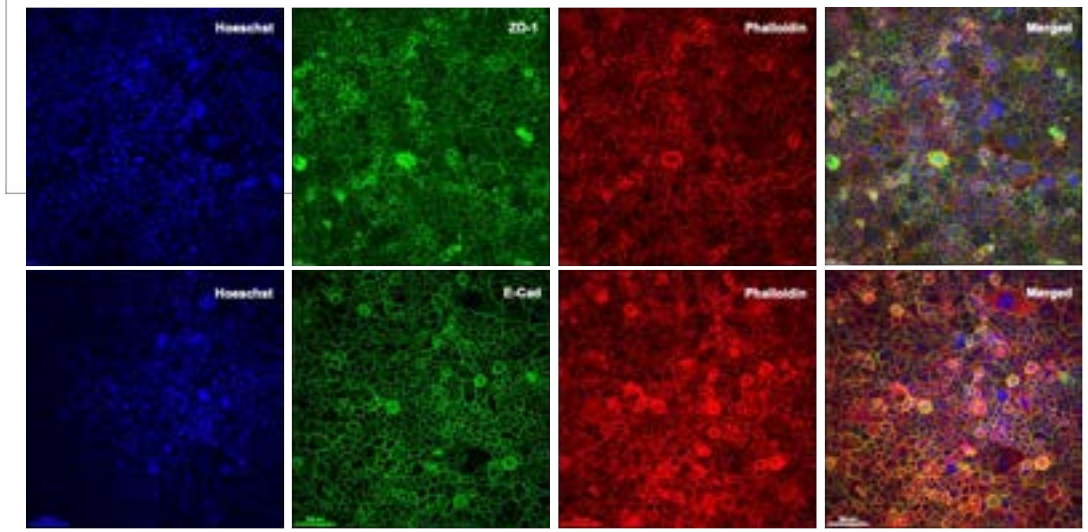


Alveolar Epithelium
Lung Fibroblast
Capillary Endothelium

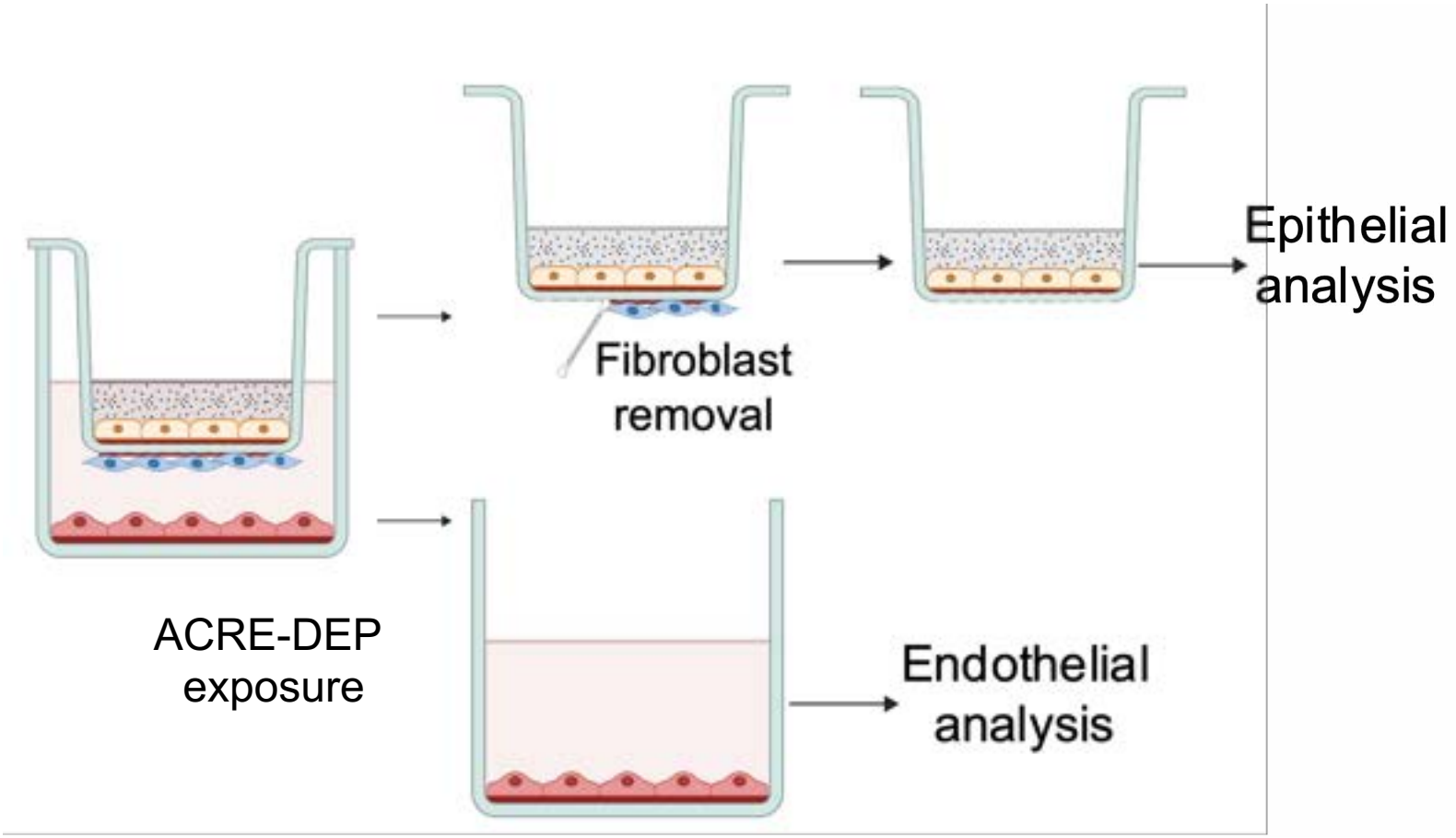


Direct Exposure

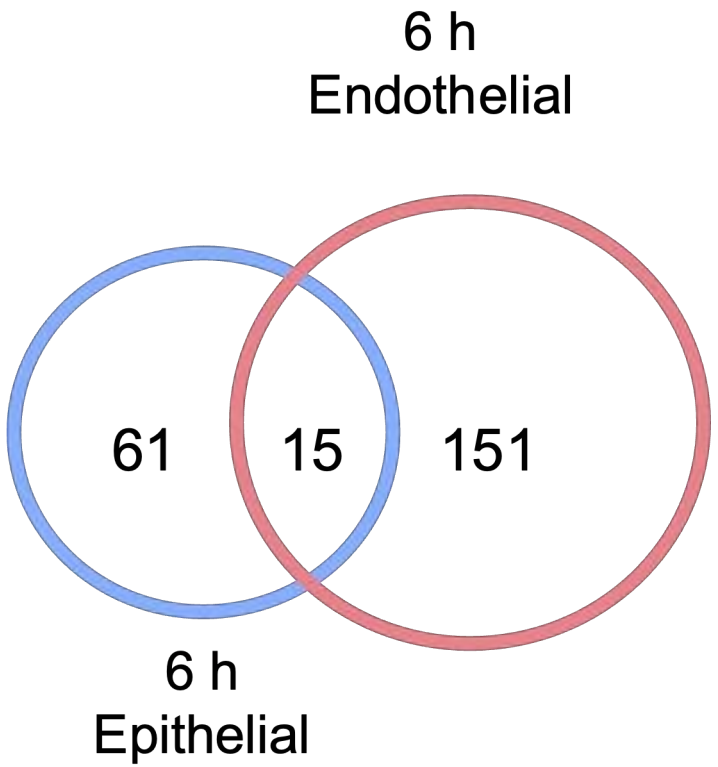
Trans-Epithelial Exposure

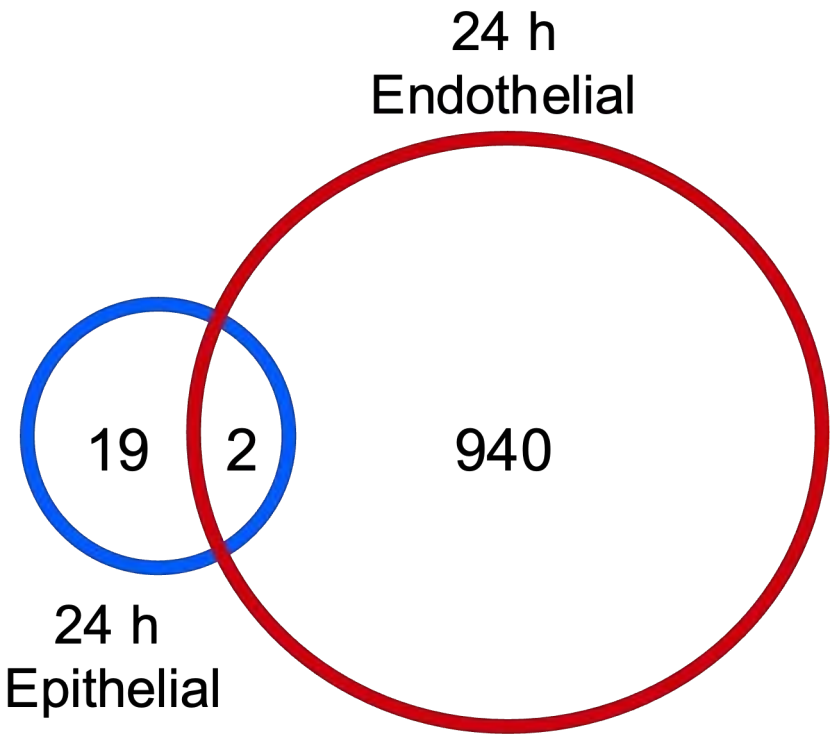


Parallel Cell Type Specific Analysis in the ACRE Model

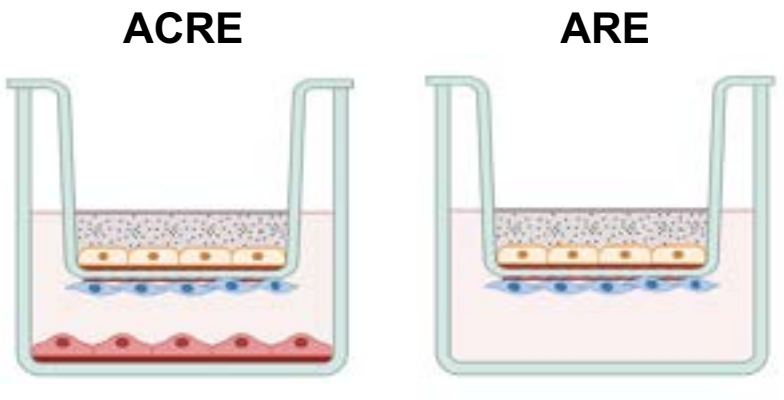


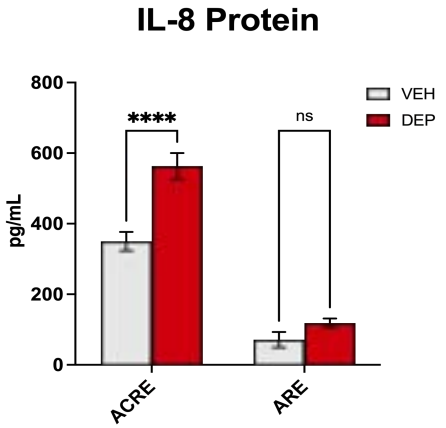
The Response to DEP Differs by Cellular Compartment

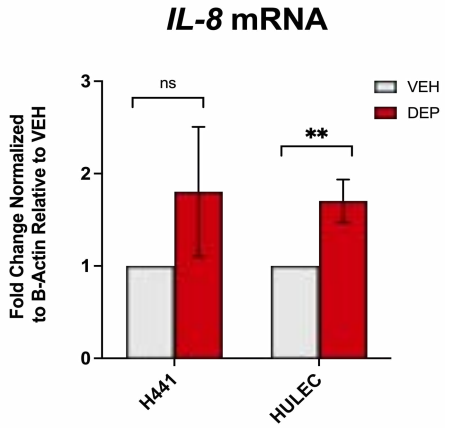


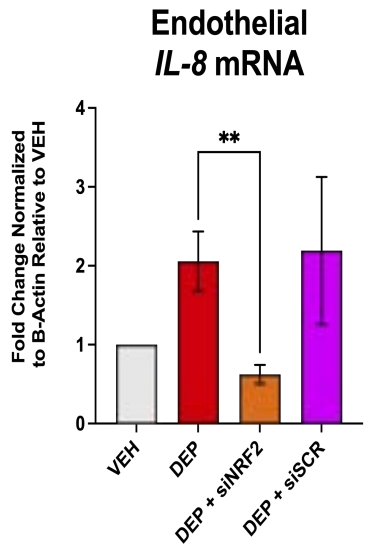


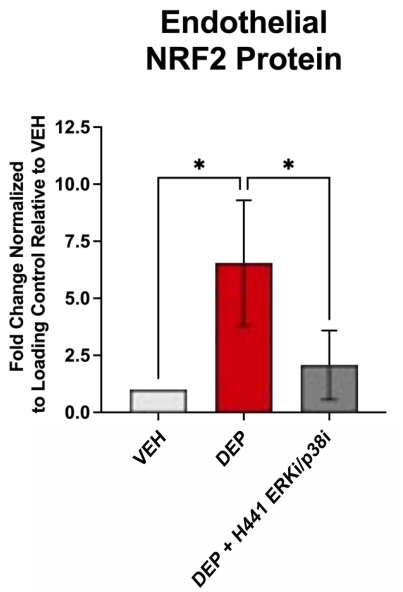
Coordination of Different Signaling Pathway Activation Between Cell Types

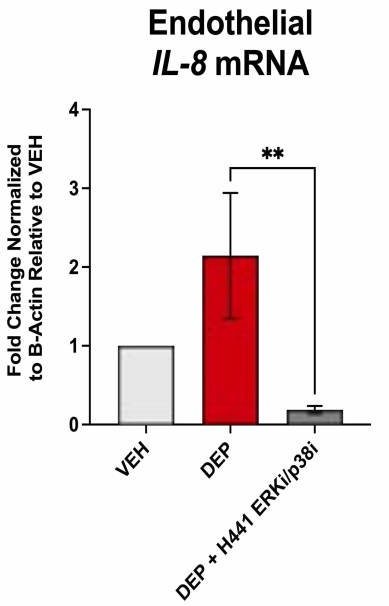


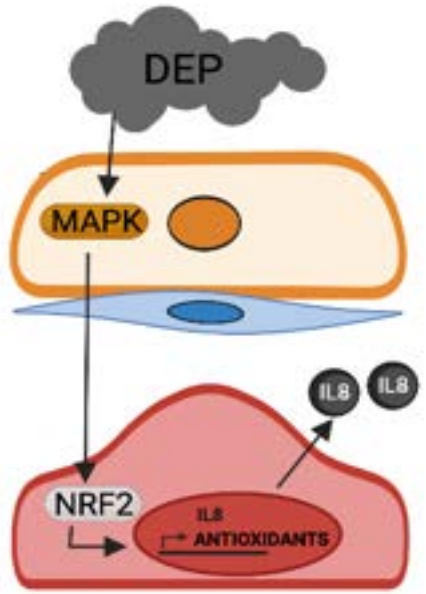






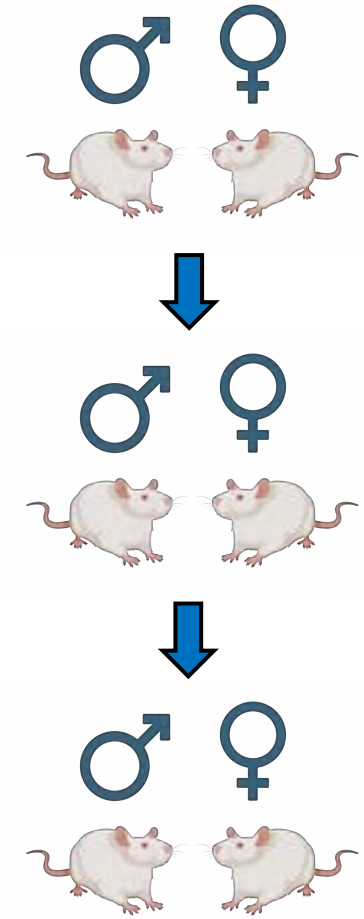






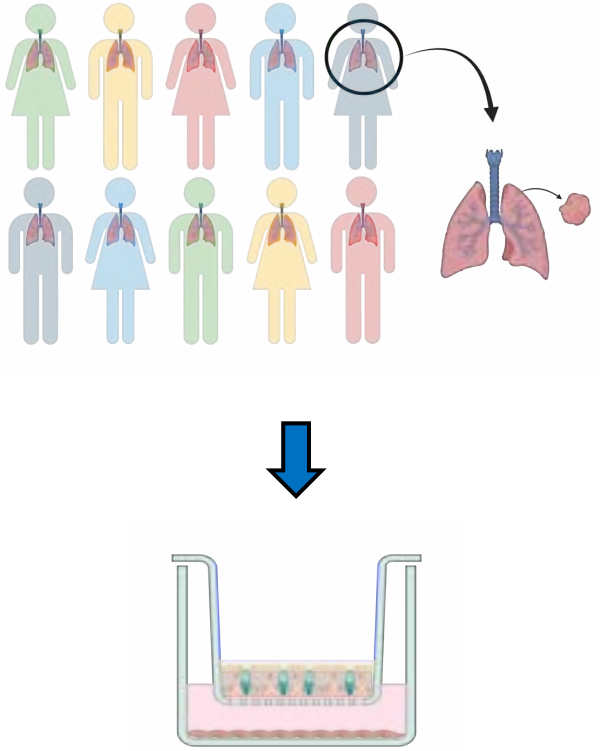
Predicting Responses in a Diverse Population

Inbred animal strains

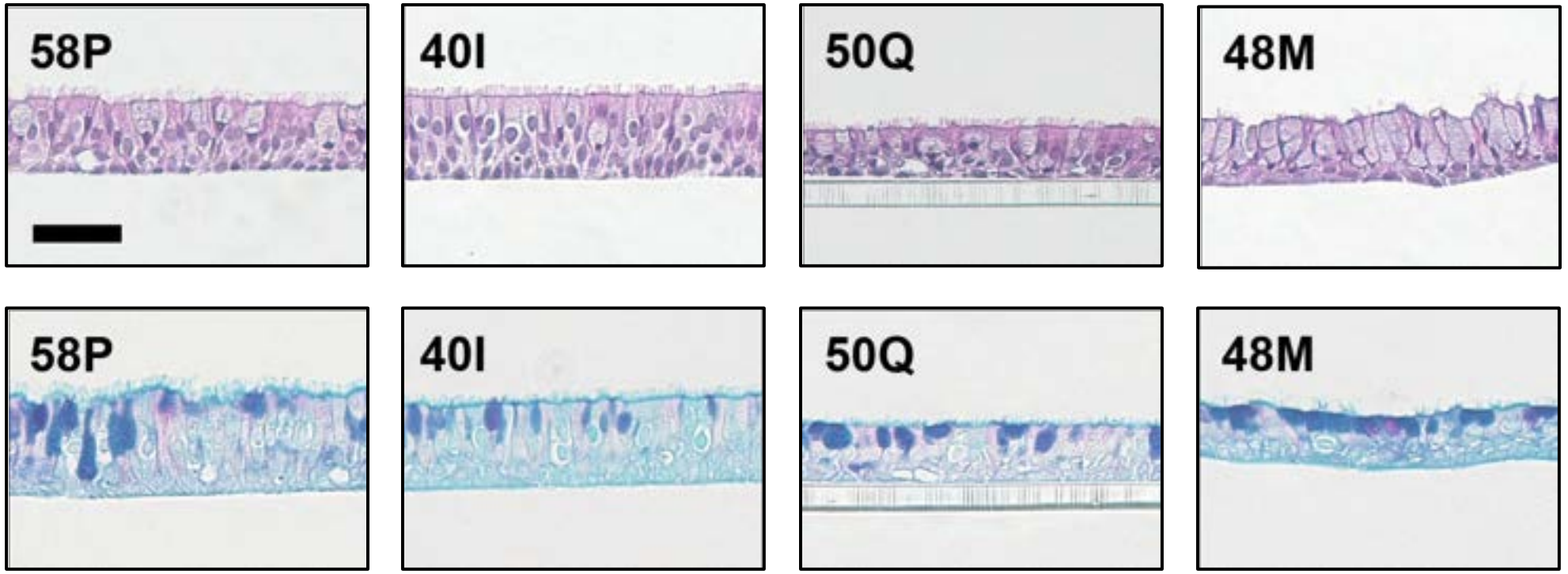


Isogenic cell lines

Primary human cells



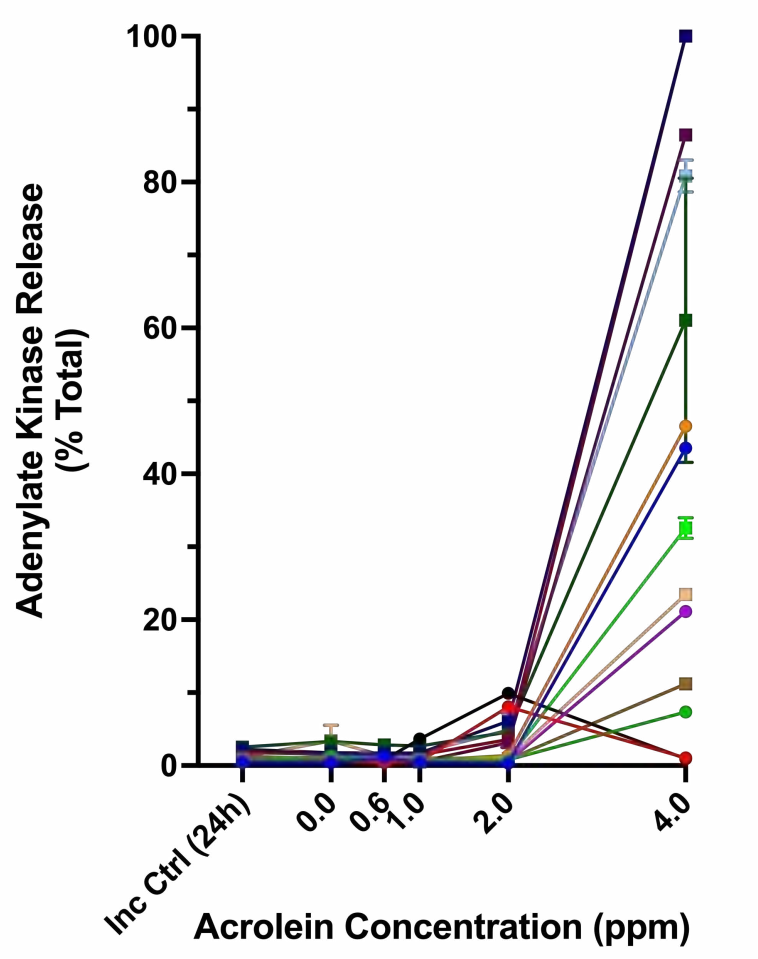
Primary Cultures Are Variable at Baseline



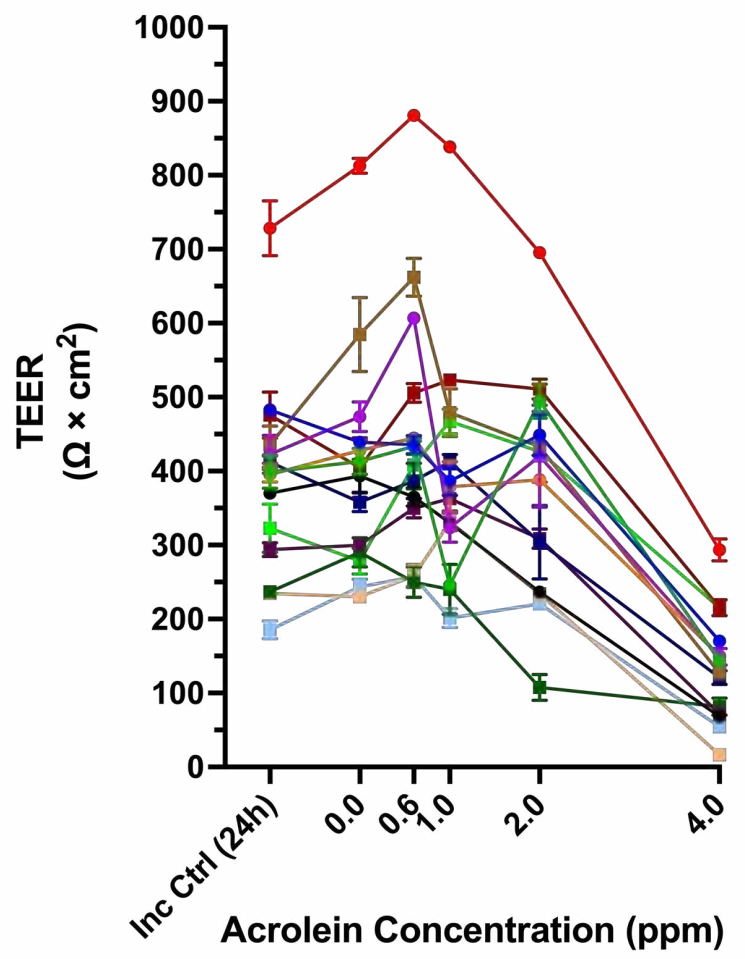
Dr. Alysha Simmons, *et al. In preparation*

Donors Exhibit Wide Range of Variability in In Vivo Relevant Endpoints

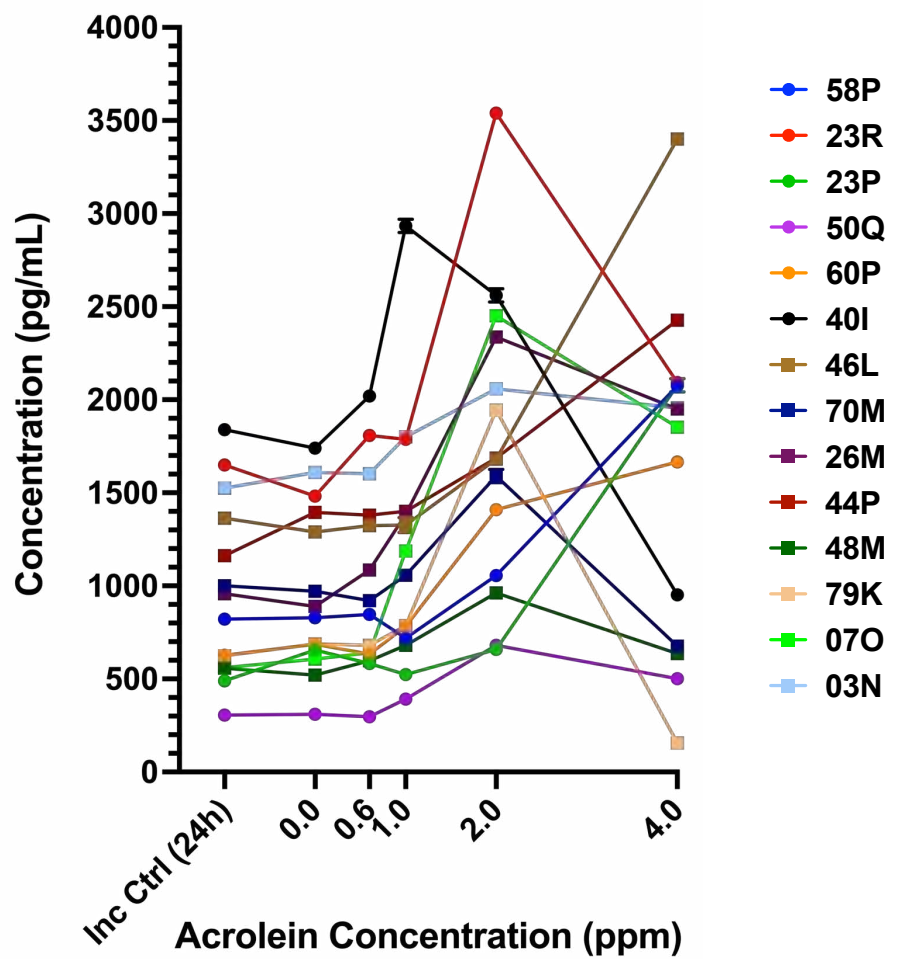
Cytotoxicity



Barrier Integrity

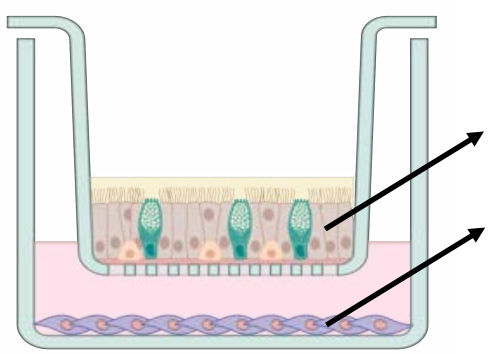


IL-8 Secretion

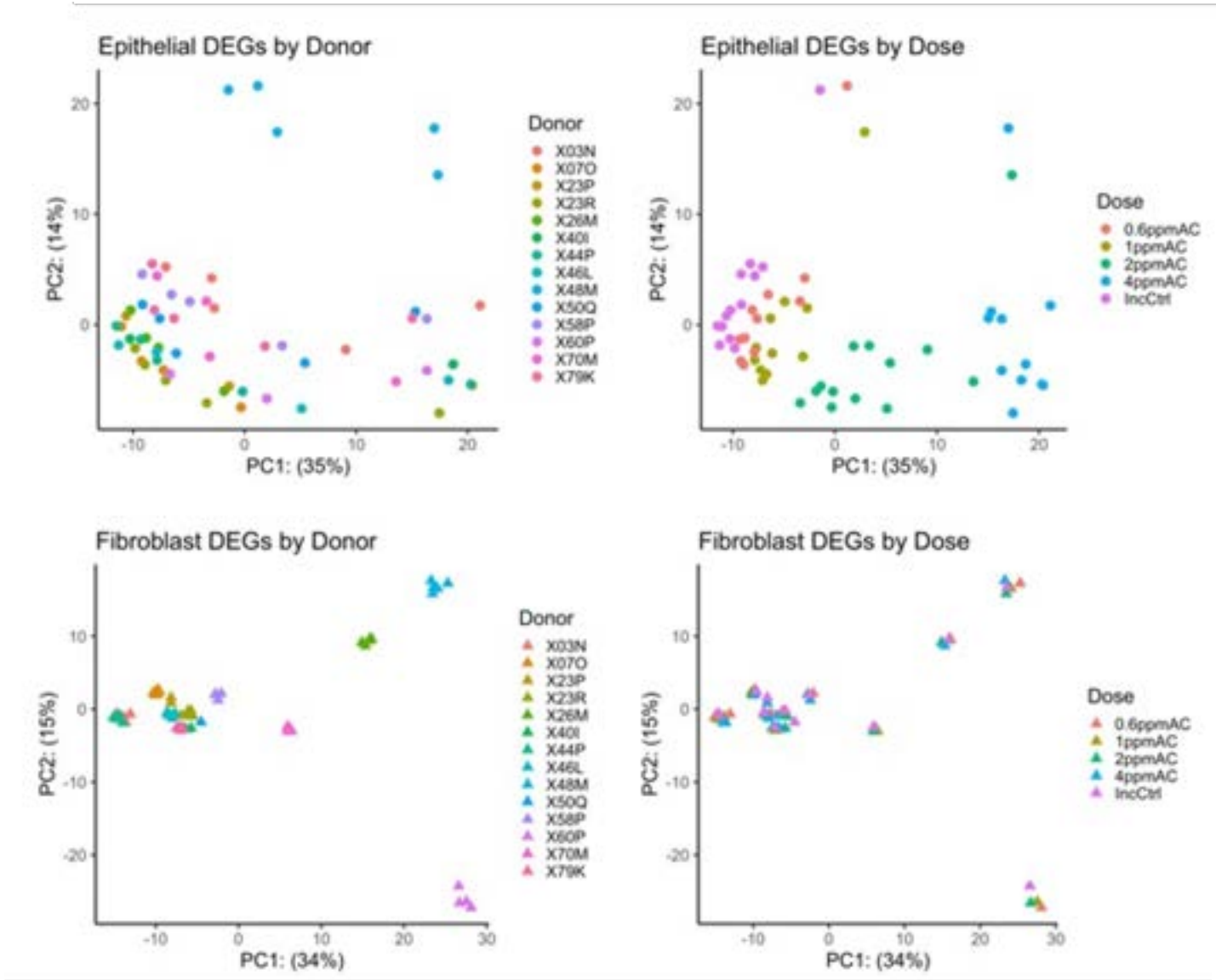


Dr. Alysha Simmons, *et al. In preparation*

Dose and Donor Impact Cell Types Differently



- **Donor-matched pHBEC and pHLF**
- **ALI differentiated**
- **Acrolein concentration-response**
- **Exposed under ALI conditions**



Dr. Alysha Simmons (UNC) Dr. Elizabeth Martin (NIEHS)
Dr. Julie Foley (NIEHS)

Culture Complexity and Characterization

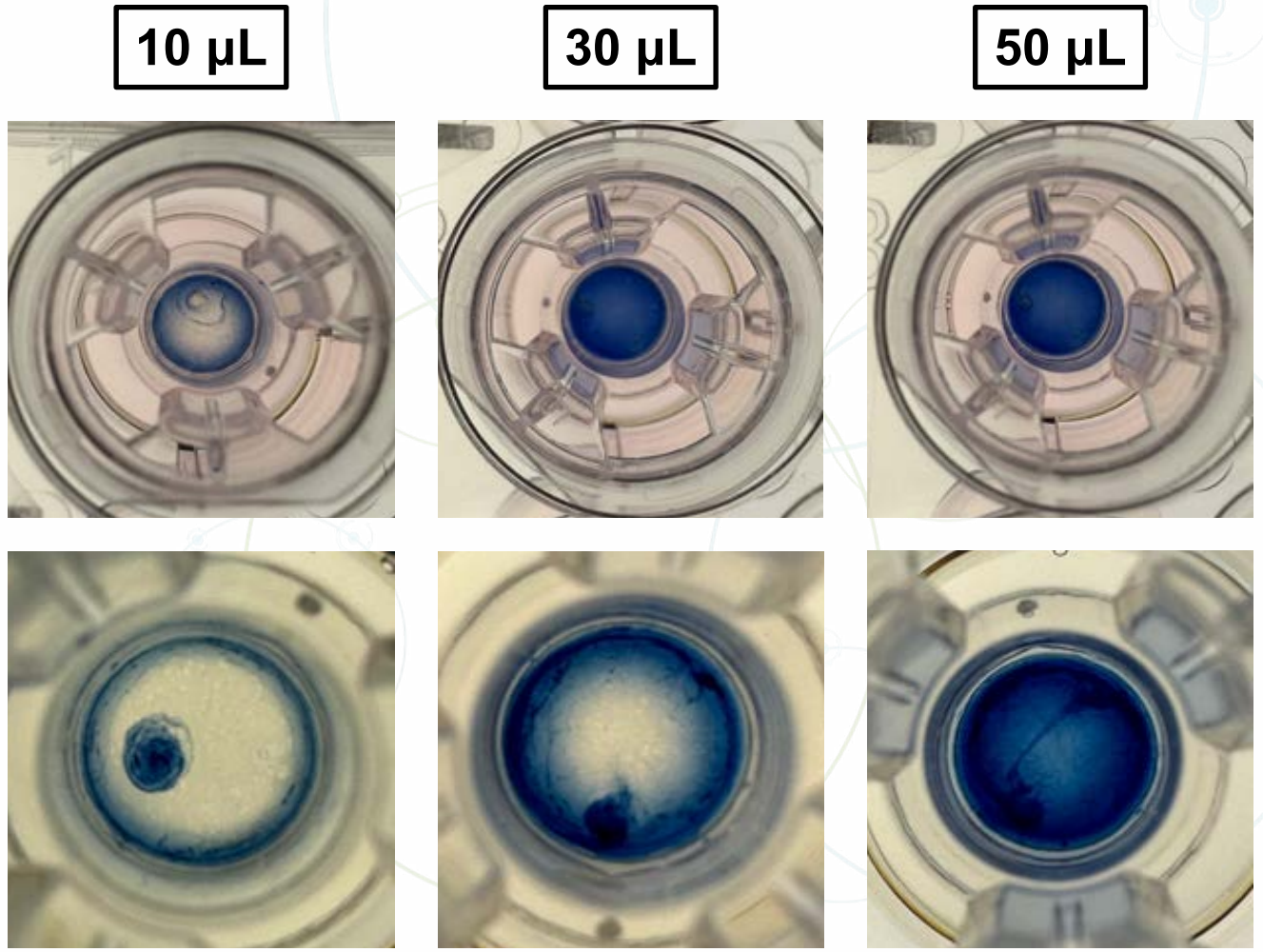
- Need to increase the in vivo relevance of in vitro systems to ensure suitability for the biological effect and toxicity of interest.
  - Epithelial only cultures can be informative but are not likely able to reliably predict effects of exposures on complex tissues.
- Characterizing inter-individual variability is necessary.
  - Sample size.
  - Intra-test variability, repeatability, and reproducibility across studies and laboratories.
  - Range in variation of performance in response to reference agents and in vivo human data.

Part #2
Dosing Method

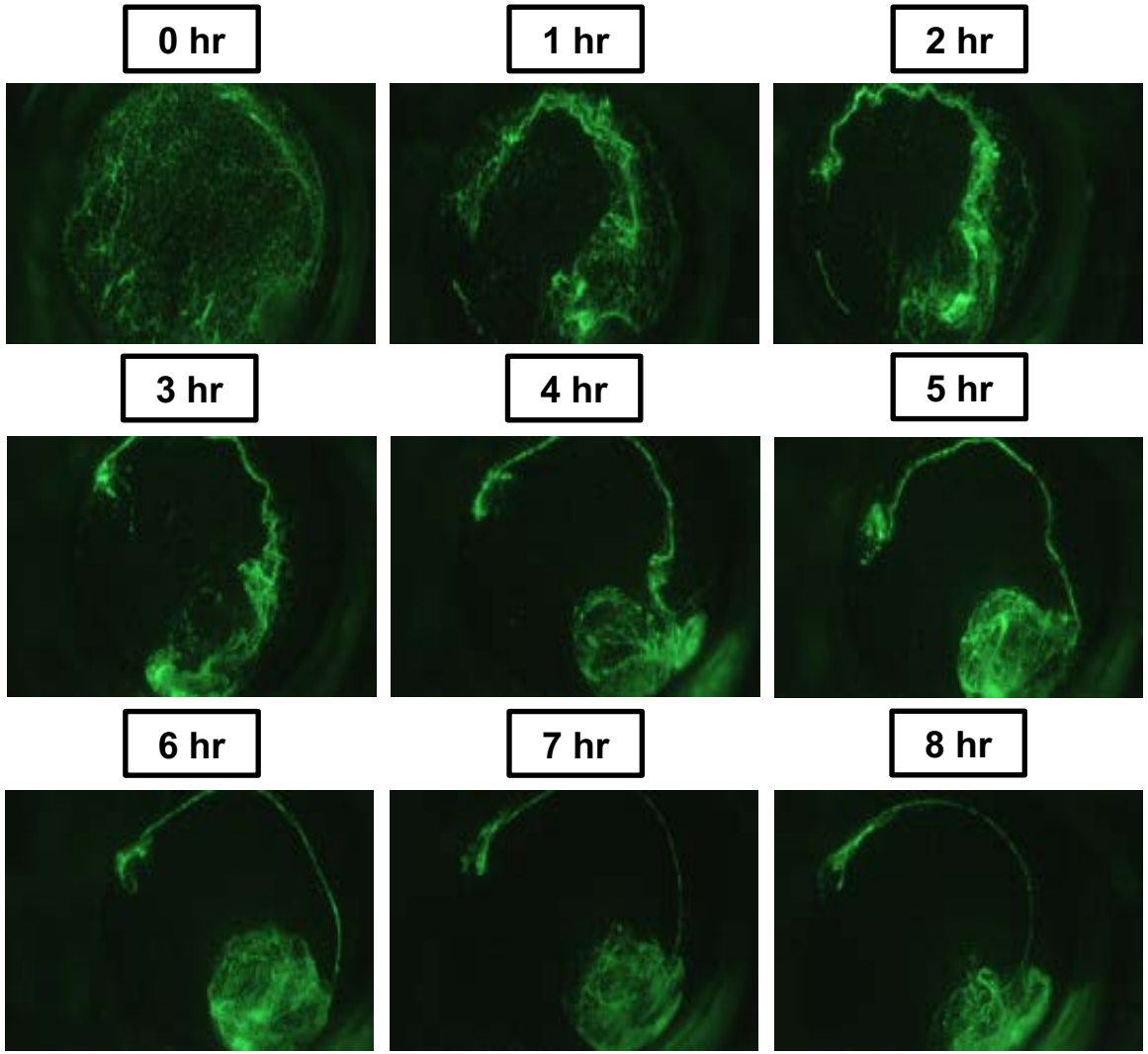
In Vivo Relevance of ALI Systems Can Introduce Experimental Challenges

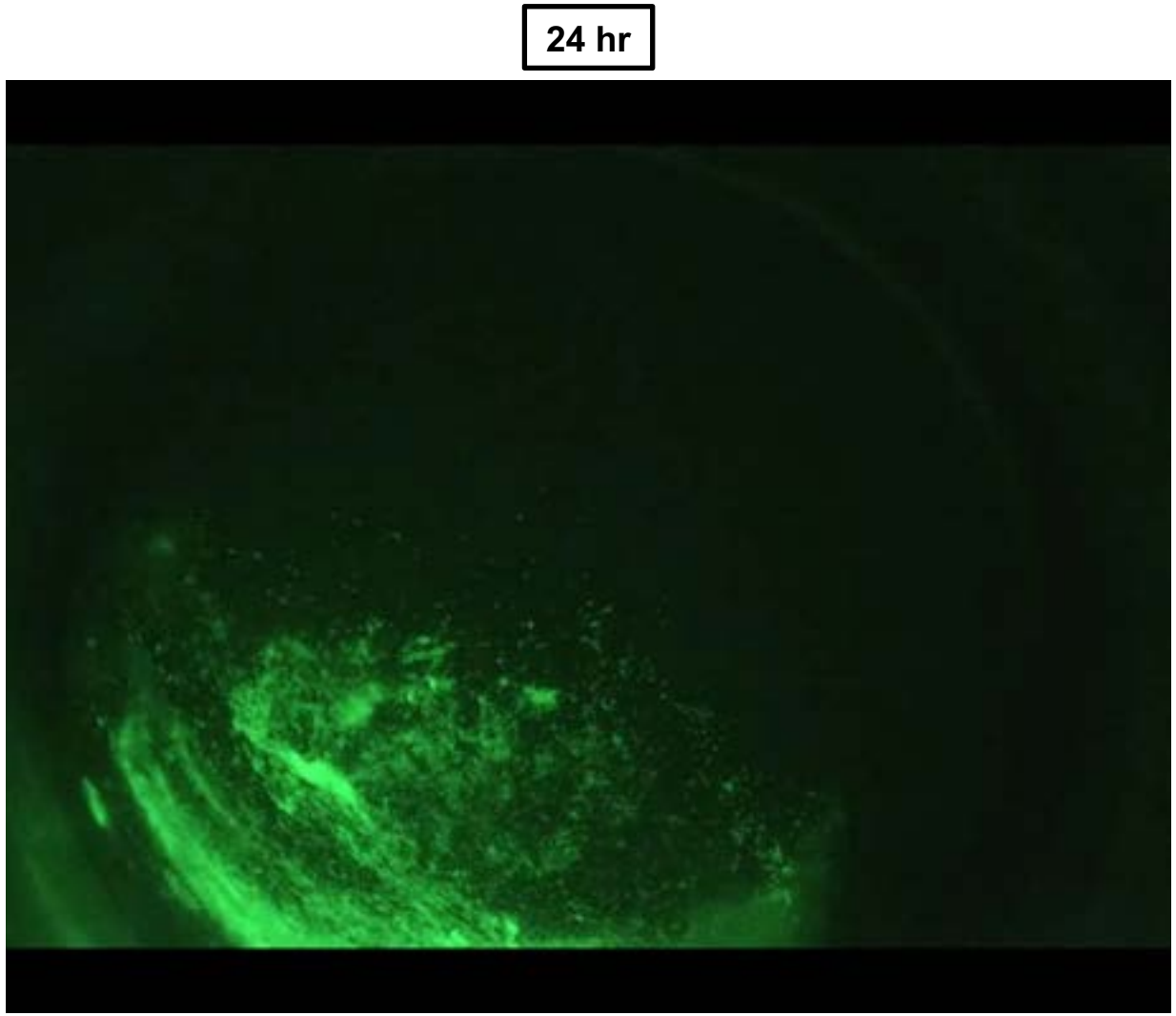
- Empirical vs. anecdotal volume selection
- Incomplete coverage creates inconsistencies
  - Test article concentration
  - Ion concentration
  - Oxygen concentration
- Published studies indicate ALI and liquid application outcomes differ

Trypan Blue in 0.9% saline 6.5 mm insert



In Vivo Relevance of ALI Systems Can Introduce Challenges to be Considered for Validation

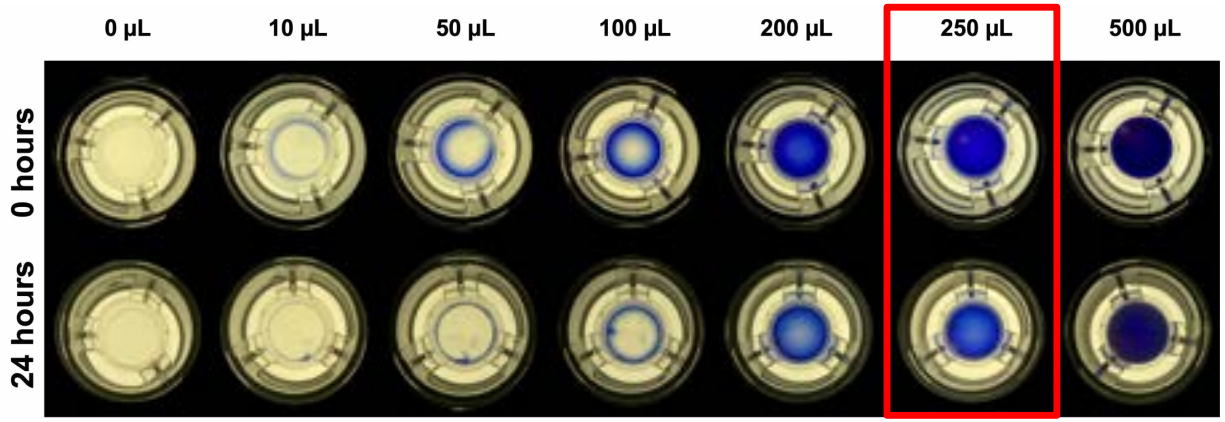




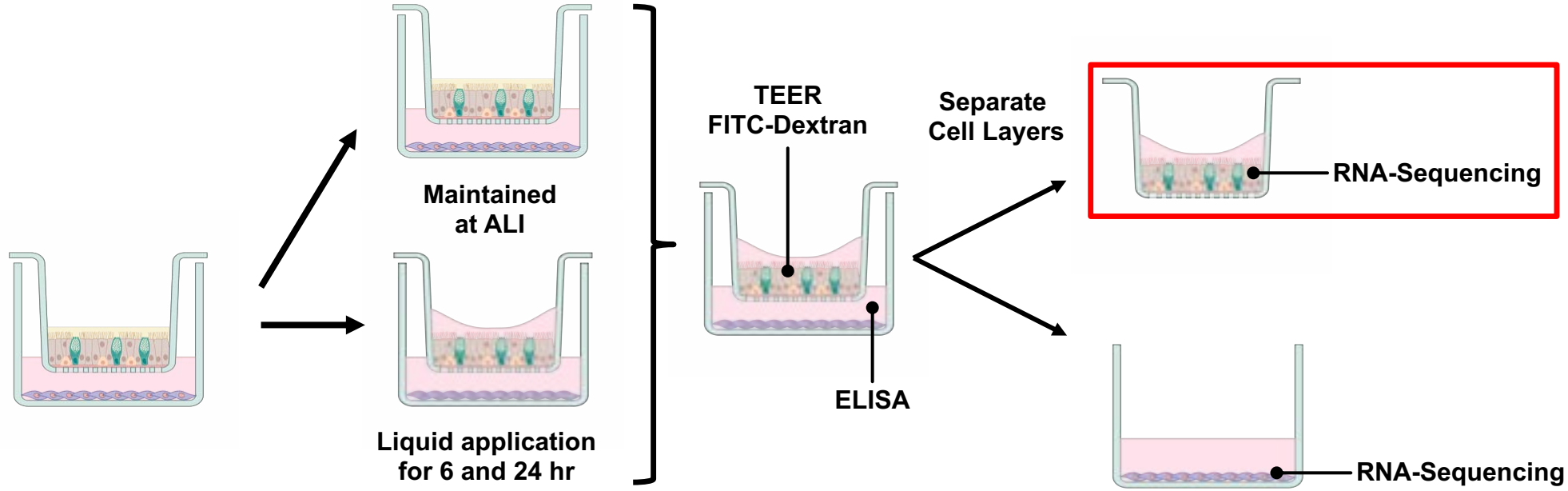
30 µL volume of 10 µm beads on 6.5 mm insert

Dr. Erin Huber

Molecular Effects of Liquid Application



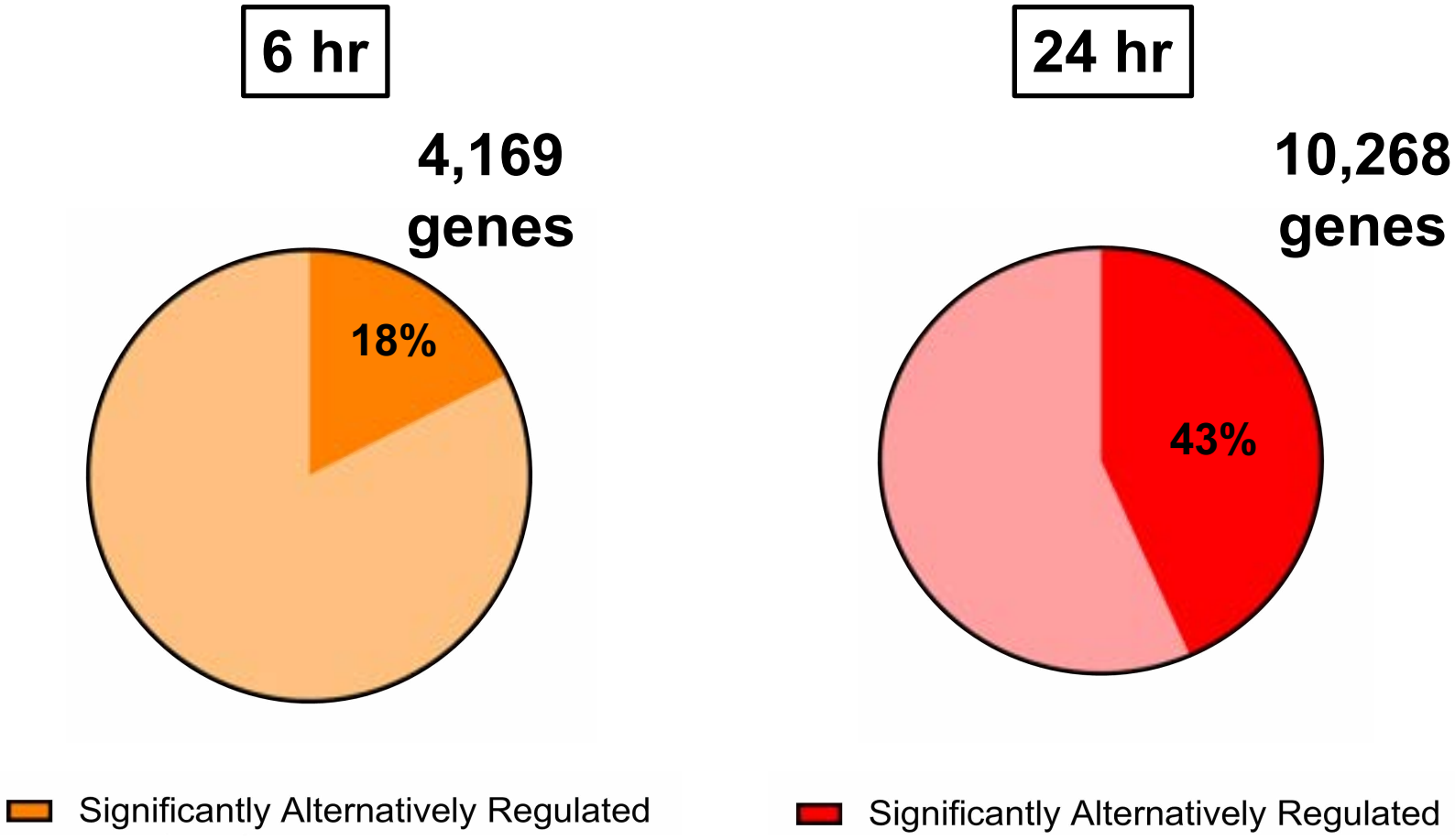
Vehicle	pH
ALI medium	7.4
0.9% saline	5.3 – 5.7
Cell culture grade water	5.0 – 5.5



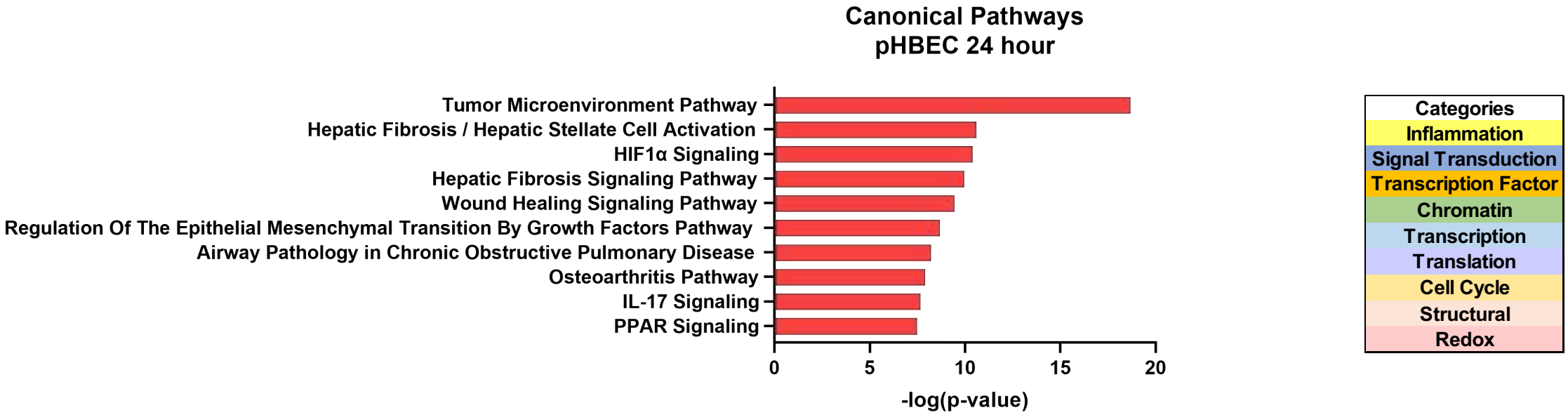
12 mm inserts

Mallek NM, *et al*. (2024) *Front. Toxicol.* 5: 1264331

Liquid Application Alters Global dpHBEC Gene Expression



Liquid Application Alters Cell Physiology



Tumor Microenvironment	
Target	Fold Change
SLC2A3	3004.69
PGF	276.09
IL8	83.58
FGF18	-74.75
CXCL12	34.82
LEP	34.56
FOS	33.92
VEGFA	28.78
TNF	28.58
SLC2A4	26.93

Hepatic Fibrosis/Stellate Cell Activation	
Target	Fold Change
PGF	276.09
IL8	83.58
FLT1	64.67
IL10RA	60.76
IL1A	59.88
IGFBP3	59.34
CCR7	-49.42
LEP	34.56
VEGFA	28.78
TNF	28.58

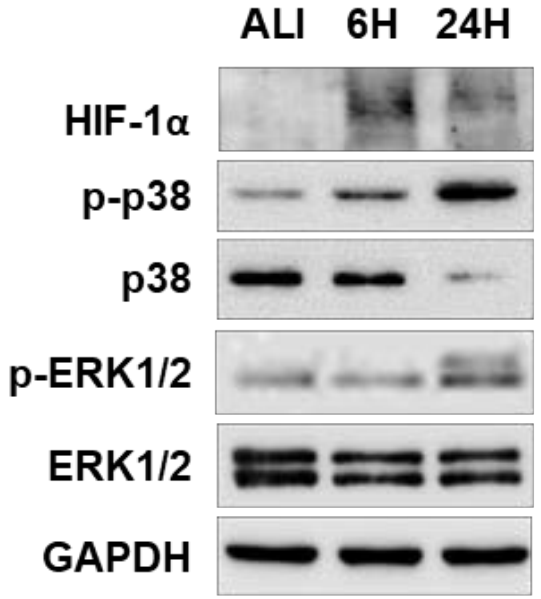
HIF1α Signaling	
Target	Fold Change
SLC2A3	3004.69
HSPA6	384.01
ADM	328.78
PGF	276.09
FLT1	64.67
VEGFA	28.78
NCF1	27.51
SLC2A4	26.93
HK2	23.67
SLC2A1	22.38

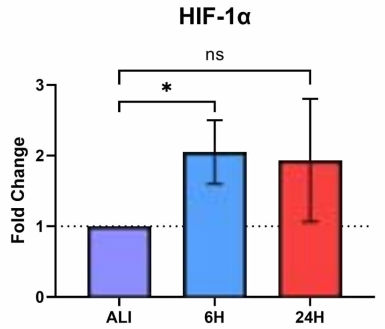
Hepatic Fibrosis Signaling Pathway	
Target	Fold Change
PGF	276.09
IL8	83.58
FLT1	64.67
IL1A	59.88
SUCNR1	-42.61
LEP	34.56
FOS	33.92
VEGFA	28.78
TNF	28.58
NCF1	27.51

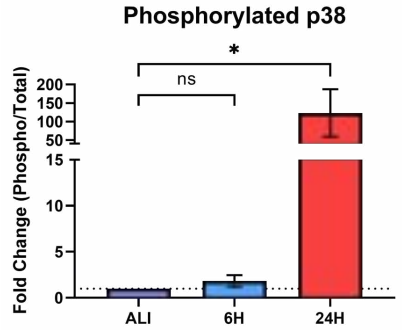
Wound Healing Signaling Pathway	
Target	Fold Change
PGF	276.09
KRT16	120.34
IL8	83.58
IL1A	59.88
TNFSF15	-34.61
LEP	34.56
FOS	33.92
VEGFA	28.78
TNF	28.58
COL15A1	22.27

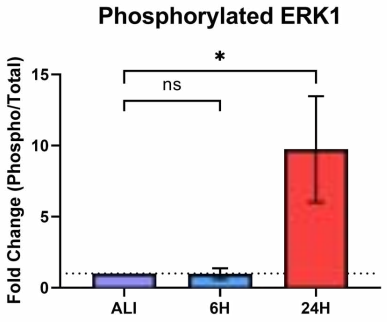
Liquid Application Alters Cellular Signaling Processes

- HIF1a can induce toxicity-associated genes
- Upregulation of pro-growth signaling pathways could mitigate detection of cytotoxicity
- Activation of p38 and ERK1 signaling could amplify test article-associated signaling



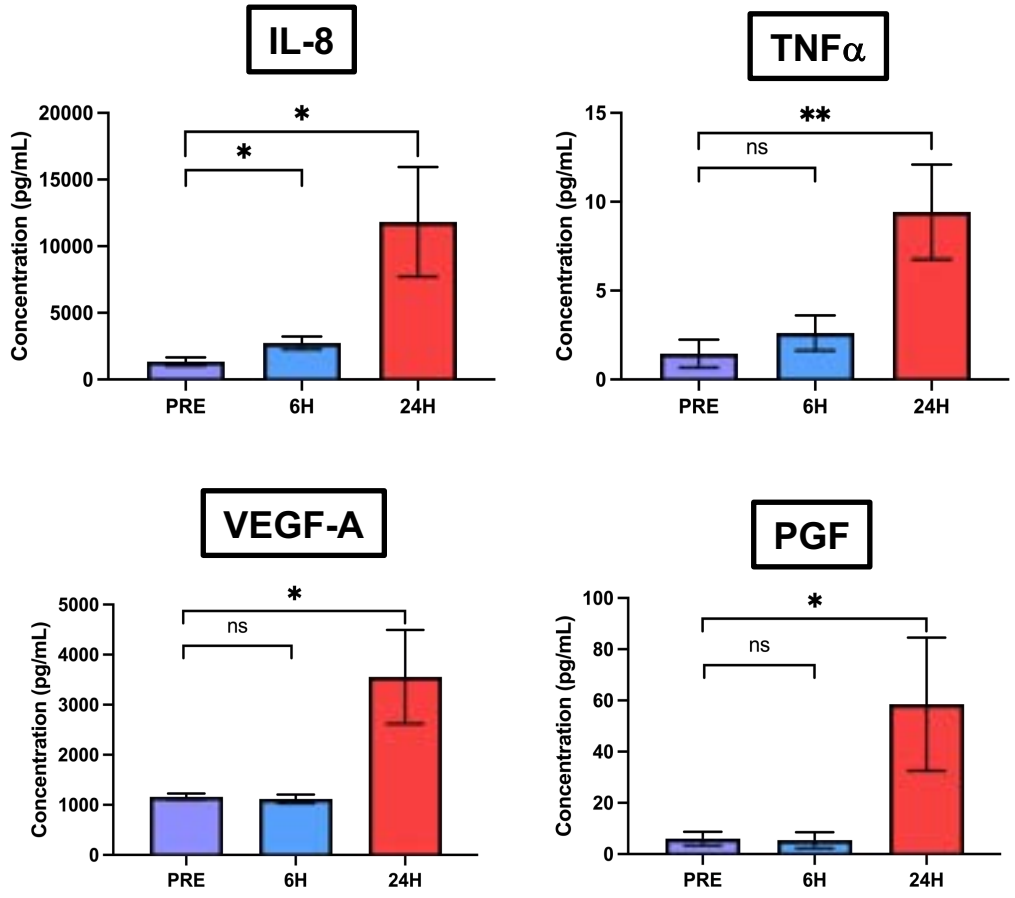




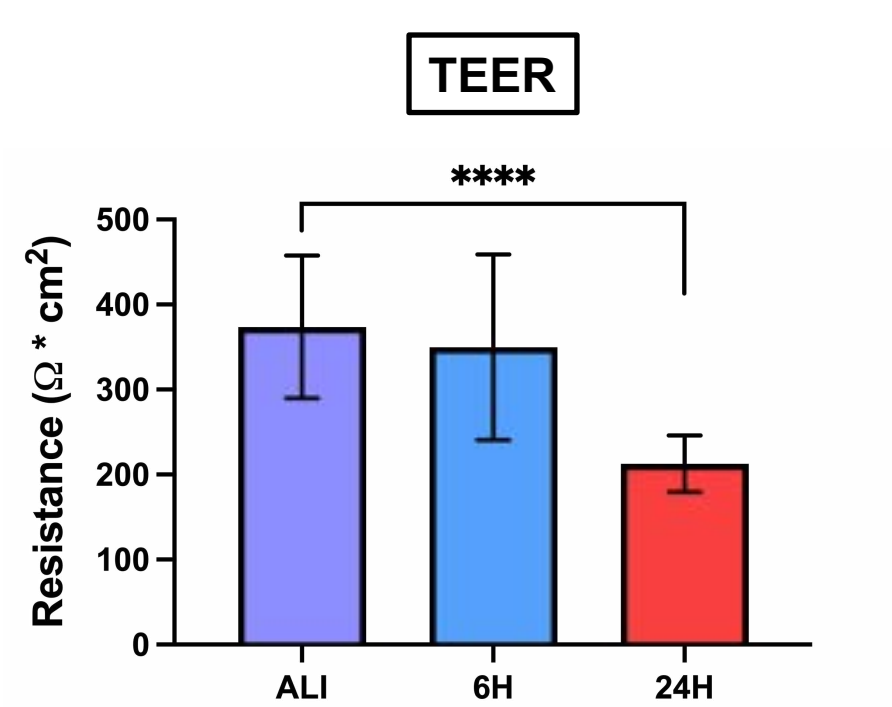


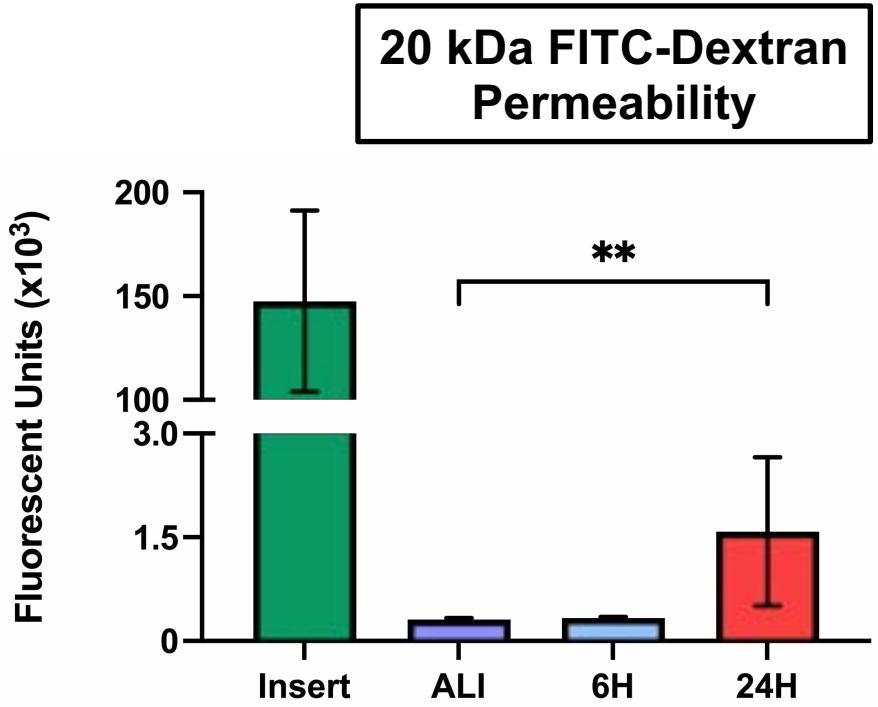
Liquid Application Induces Pro-Inflammatory Cytokine and Growth Factor Secretion

- Consistent with various respiratory diseases and responses to inhaled toxicants
- Potential to confound system sensitivity and specificity

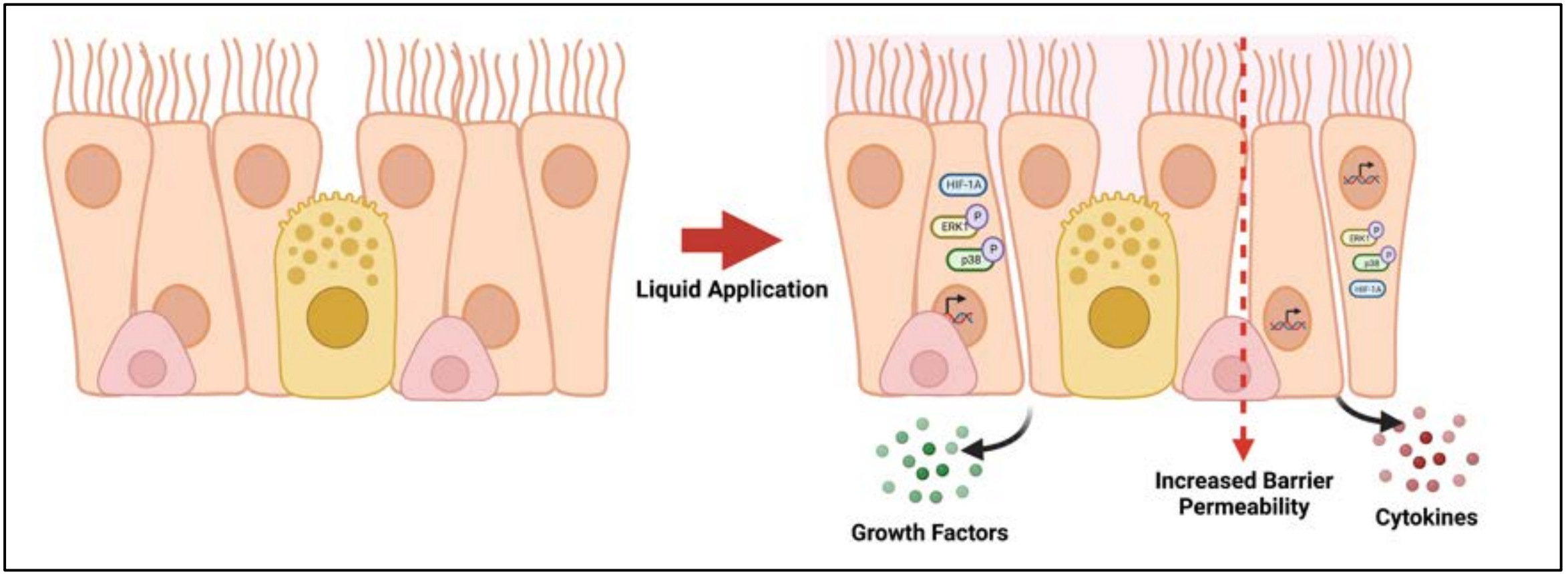


Liquid Application Disrupts Barrier Integrity





Effect of Liquid Application on ALI Culture Physiology



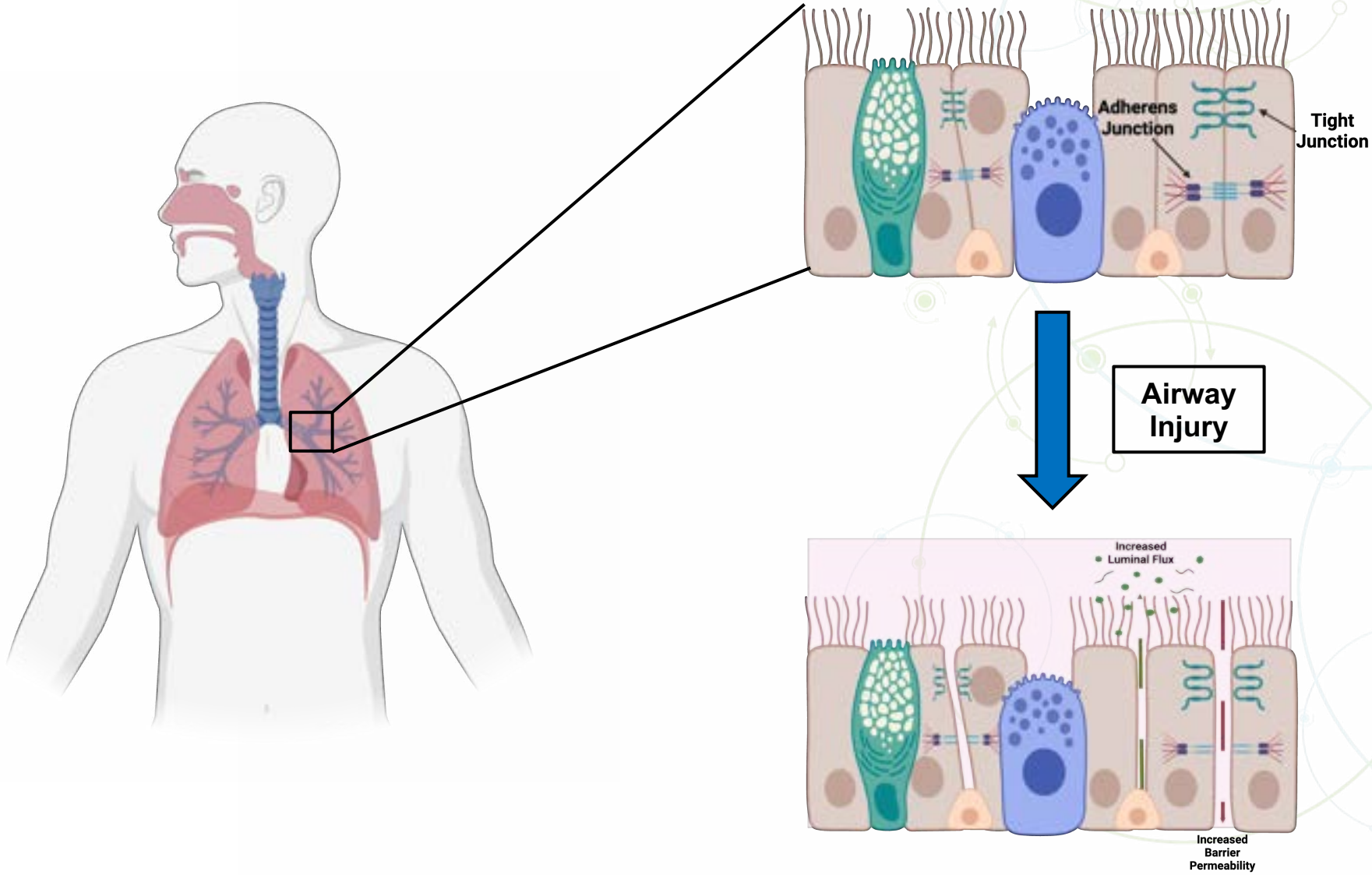
Mallek NM, *et al*. (2024) *Front. Toxicol.* 5: 1264331

Characterizing the Dosing Method is a Critical Precursor for Validation

- In vitro dosing methods need to be relevant to in vivo exposures to ensure that the test system reflects in vivo biological effects and real-world exposure scenarios.
  - The effects of vehicle exposures (liquid and ALI) should be determined relative to incubator controls to inform the interpretation of study data.
  - Also applies to co-solvents (e.g., DMSO), pre-exposure starvation, and changes in medium type for submerged systems
- Limitations of approaches such as liquid application should be reported.
- The impact of different liquid vehicles should be determined to understand the relative impacts of commonly used variations.
- In vitro exposure systems should be thoroughly characterized to evaluate deposition and variability.

Part #3
Characterizing Sources of Method Variability to Support Validation

Trans-Epithelial Electrical Resistance (TEER) Measures Barrier Integrity



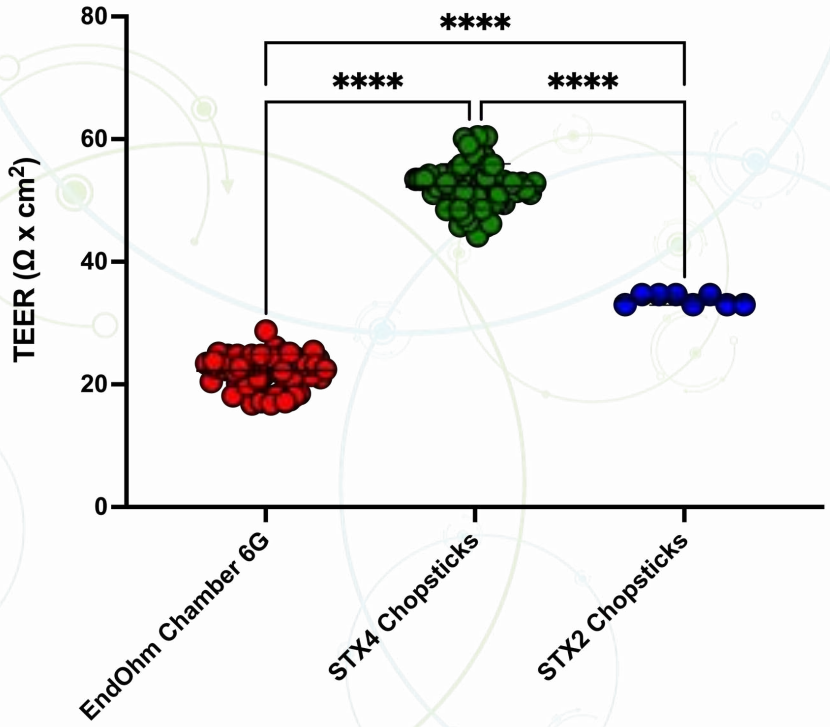
Impact of Variations in Assay Equipment

EndOhm chambers

STX2 chopsticks

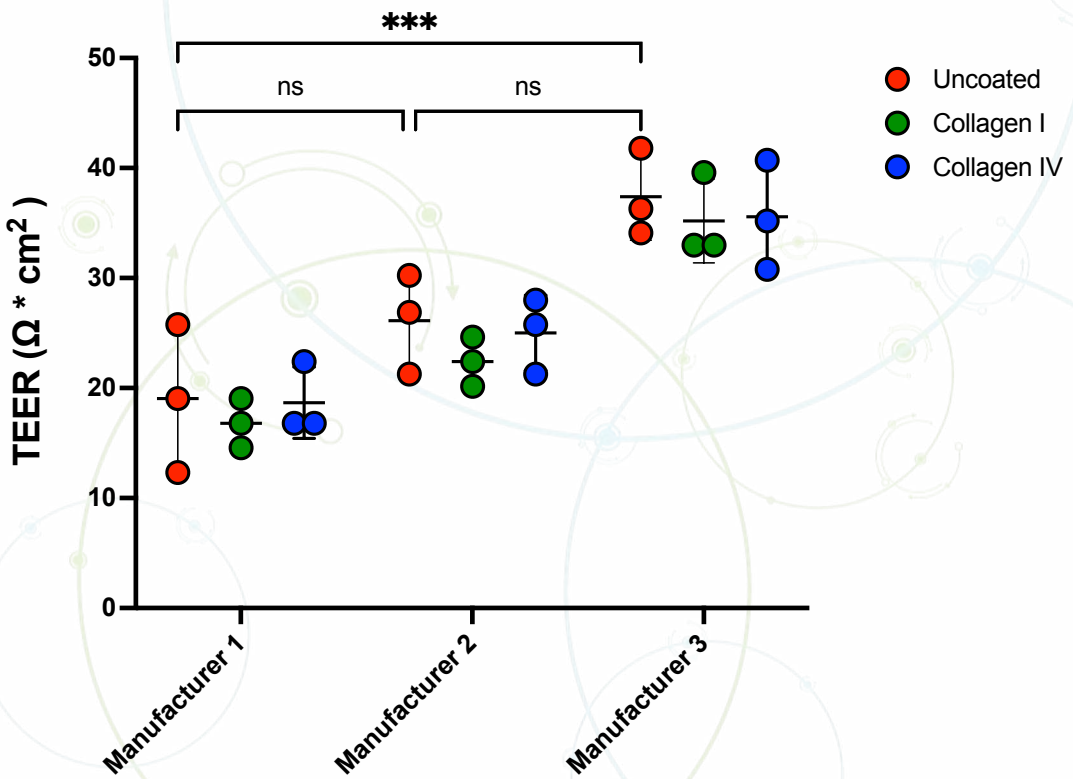
STX4 chopsticks

TEER of Blank Inserts

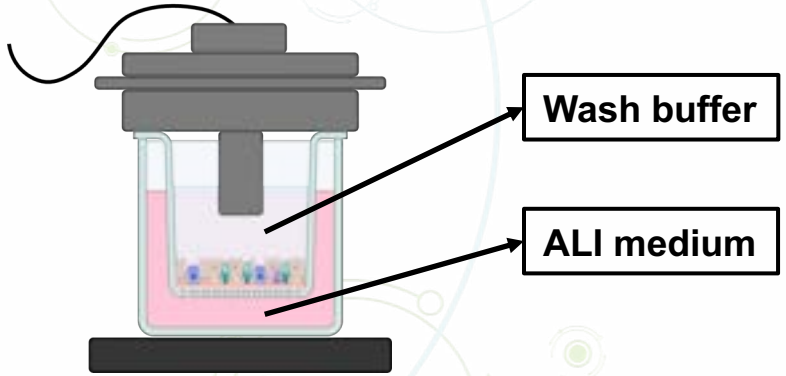


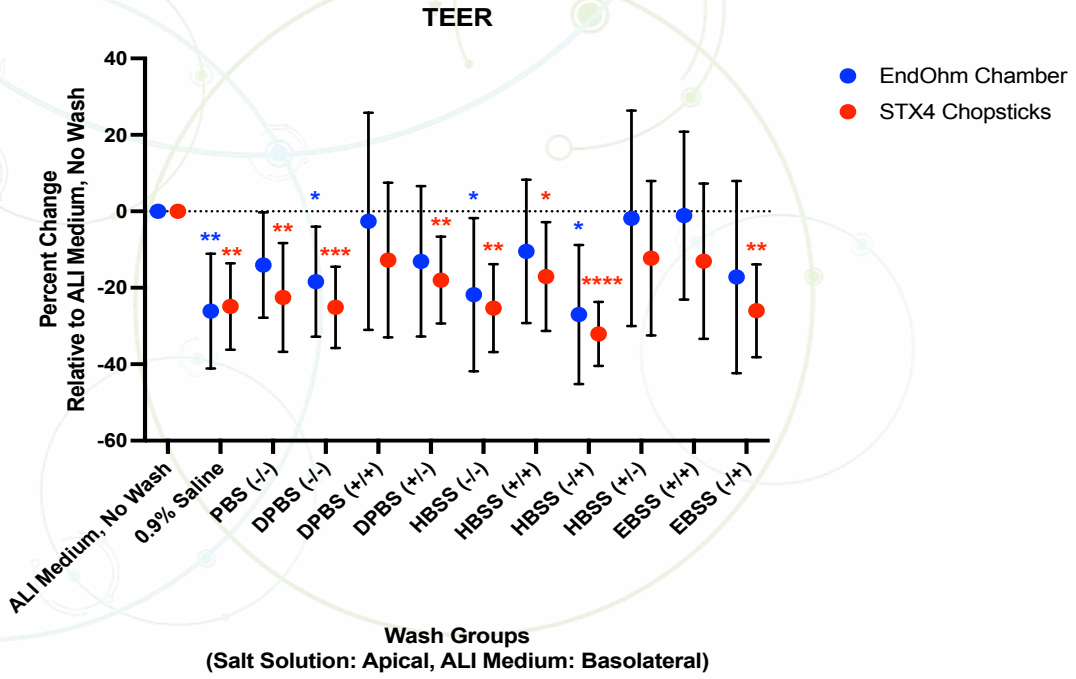
Pre-Seeding Factors Affect TEER Data

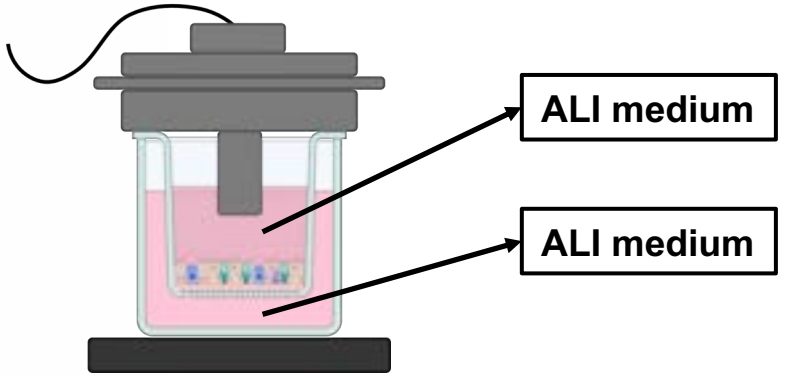
- TEER values are typically blank corrected using arbitrary values
- Insert membrane characteristics vary
  - Pore size
  - Pore density

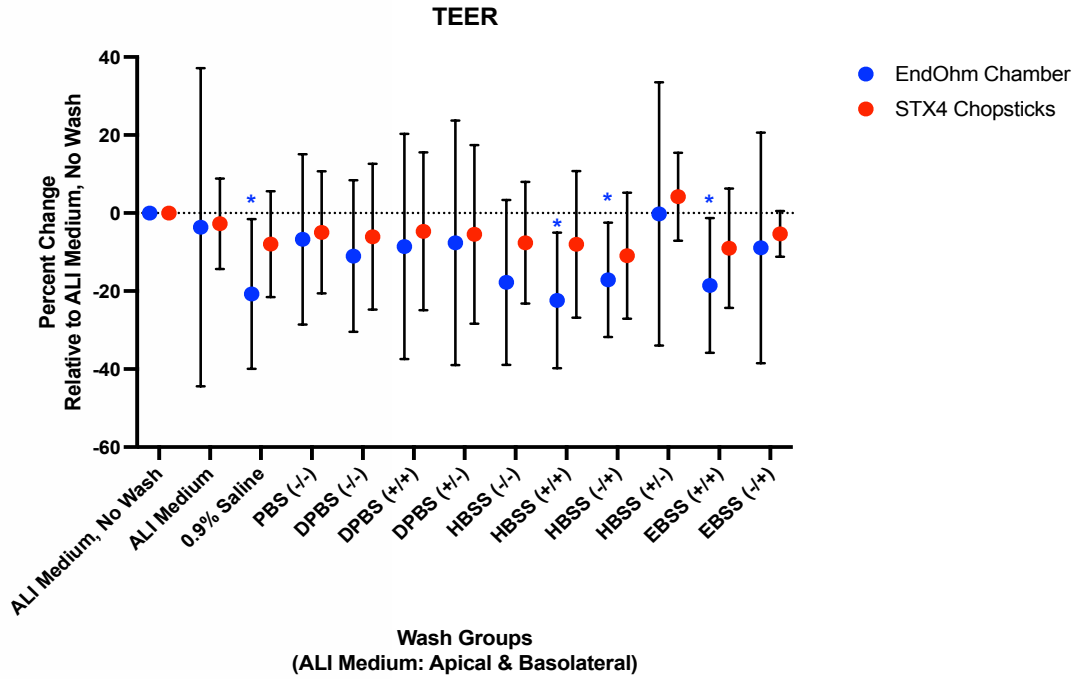


Wash Buffer Affects TEER Measurements



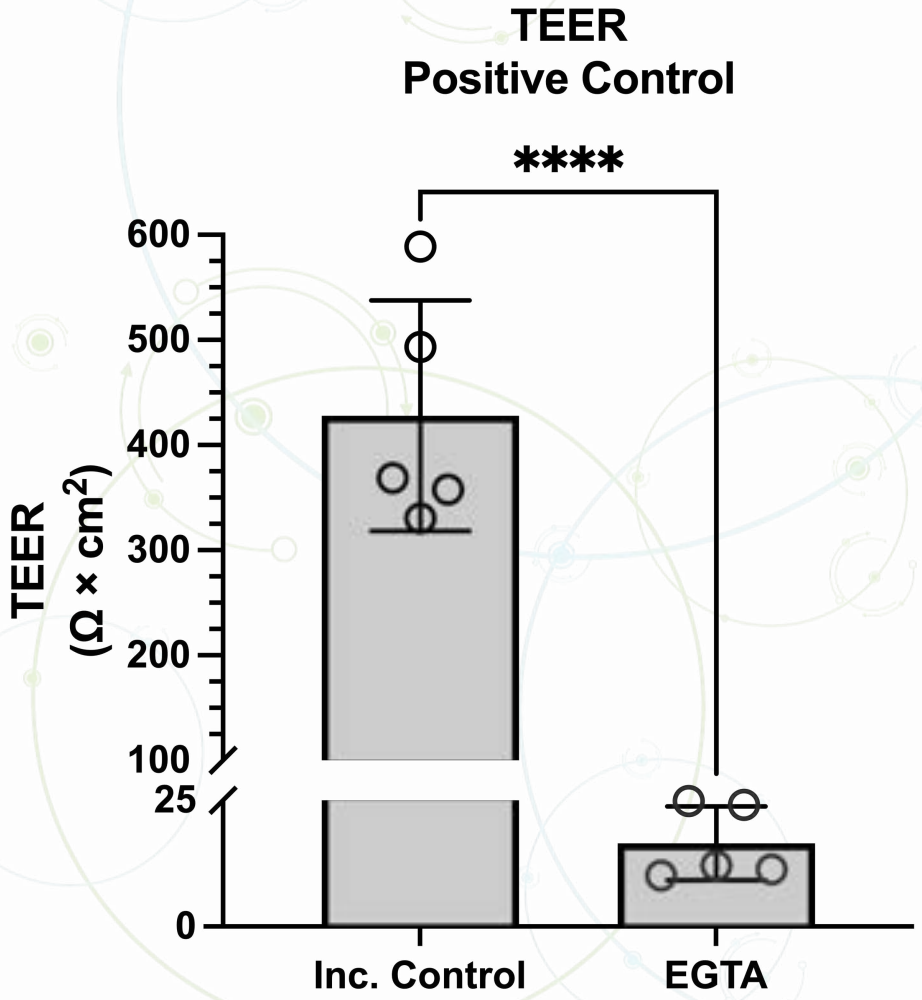






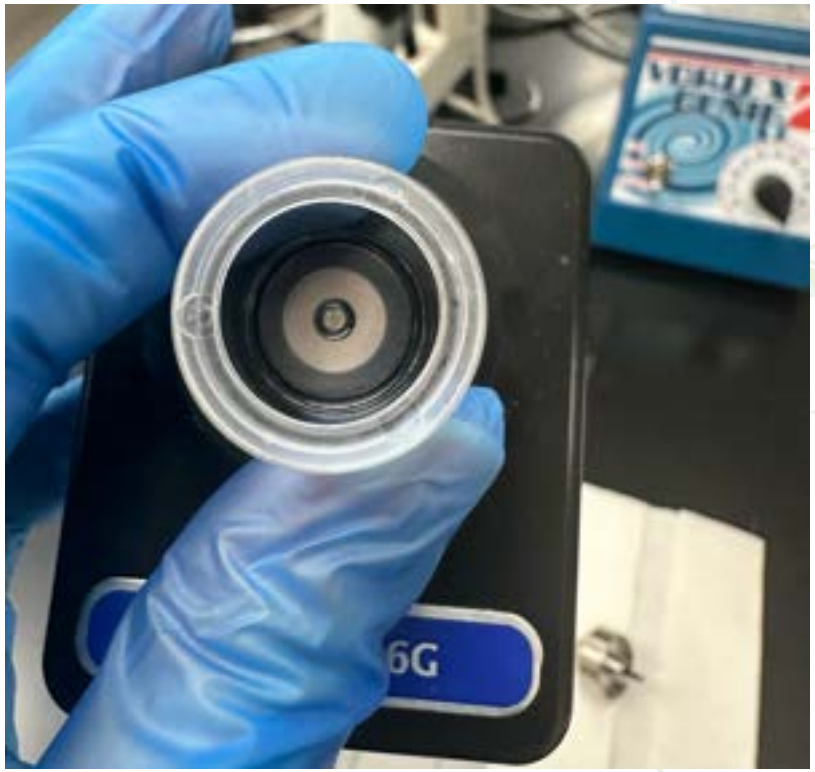
Positive Control Selection

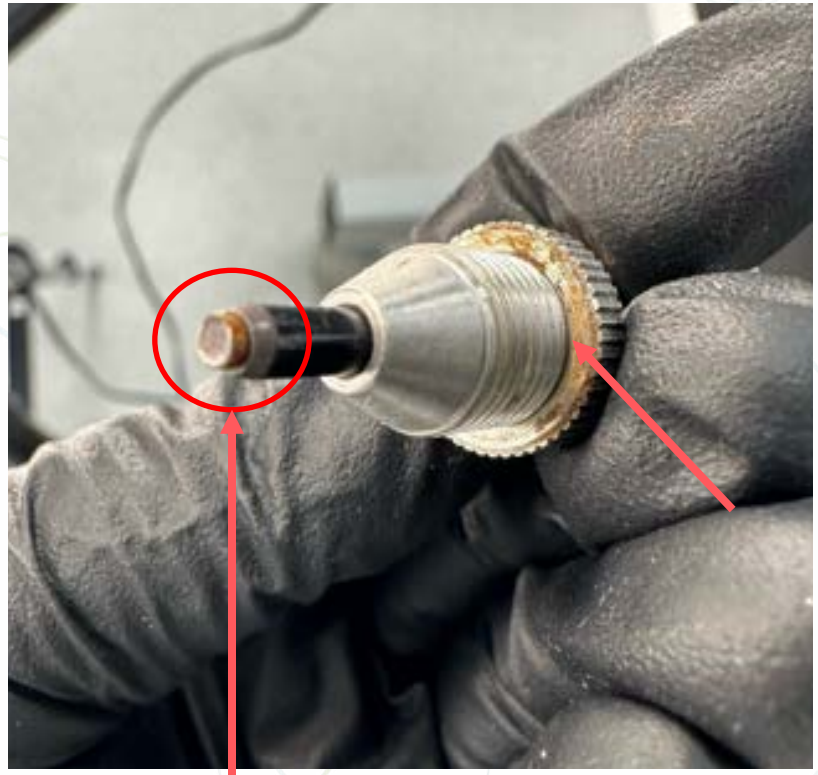
- The biological process being modeled should drive selection
  - Common positive controls lower barrier integrity by inducing cytotoxicity.
  - EGTA is a divalent cation chelator that disrupts cell-cell junctions



Proper Equipment Maintenance is Critical

Equipment Maintenance Impacts Variability, Repeatability, and Reproducibility





Summary and Conclusions

- Characterization of experimental systems and assays is a necessary step that precedes validation.
  - Many remain poorly characterized.
  - Allows alignment of systems and methods with in vivo biology and the toxicity of interest.
  - Promotes and understanding and disclosure of system/assay applicability and limitations.
  - Informs the development of standardized protocols for key assays that can be more consistent across studies and laboratories.

Acknowledgements

McCullough Lab

- Erin Huber, PhD
- Alysha Simmons, PhD
- Samantha Faber, PhD
- Eva Vitucci, PhD
- Nick Mallek, PhD
- Nicole McNabb, MS
- Emily Aungst, MS
- Olivia Rice, BS

EPA/CPHEA

- Annie Jarabek, PhD
- Mark Higuchi, PhD
- Jessica Murray, PhD
- Robert B. Devlin, PhD
- David Diaz-Sanchez, PhD
- Lisa A. Dailey, MS

UNC

- Julia Rager, PhD
- Elise Hickman, PhD
- Leslie Fulcher
- Phil Clapp, PhD
- Scott Randell, PhD

NIEHS

- Elizabeth Martin, PhD
- Julie Foley, PhD

TRC Engineering Services

- Greg Byrd
- Scott Meade

Funding

- RTI intramural funding
- EPA intramural funding
  - Chemical Safety for Sustainability (CSS) National Research Program
  - Air, Climate, and Energy (ACE) National Research Program
  - EPA Pathfinder Innovation Project Grants
- NICEATM
- NIEHS #T32ES007126
- UNC CEMALB/NHEERL Cooperative Agreement #CR82952201

Questions

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Thank you!

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