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An *in vitro*-based integrated approach for inhalation toxicity assessment

Philip Morris International R&D | Philip Morris Products S.A. | Quai Jeanrenaud 5 | 2000 Neuchâtel | Switzerland

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July 2018

Three layers of the *in vitro* systems toxicology assessment framework of e-liquids and their aerosols

A Battery of Classical Assays

Ames Assay | MLA Assay | NRU Assay | Micronucleus Assay | Chromosomal Aberrations Assay

First Layer Assessment: A Broad Range of E-Liquids

- Test System
  - 2D Primary Airway Epithelial Cells
  - 2D Primary Endothelial Cells
- Cell Viability Assessment
  - **xCELLigence** - Viability
  - **HCS Platform:** - Viability
    - Apoptosis/Necrosis

- Chemical Investigation
  - Constituents
  - Toxicants

HPLC/DAD
LC-MS/MS
GC-MS

Second Layer Assessment: Selected E-Liquids

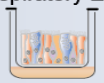
- Test System
  - 2D Primary Airway Epithelial Cells
  - 2D Primary Endothelial Cells
- Toxicity Assessment
  - **HCS Platform:** - DNA Fragmentation
    - Mitochondrial mass
    - Mitochondrial membrane potential
    - Cell cycle distribution
    - Oxydative stress
    - Transcription factor activity

- Chemical Investigation
  - Constituents
  - Toxicants

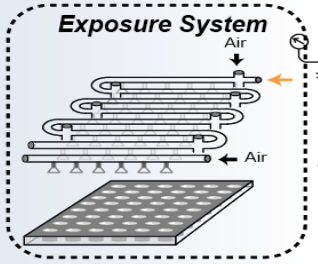
HPLC/DAD
LC-MS/MS
GC-MS

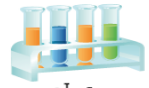
Third Layer Assessment: Whole Aerosols (Selected E-Liquids)

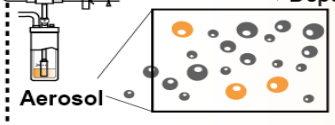
- Test System
  - 3D Respiratory Epithelial Cells



- Toxicity Assessment
  - Cytotoxicity Assay
  - Histology Analysis
  - Cilia Beating Analysis
  - Measurement of Secreted Mediators



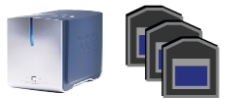




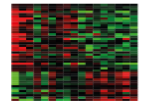
- Aerosol Characterization
  - Particle Size
  - Deposition of Compounds
    - Nicotine
    - Glycerol
    - Propylene Glycol

HPLC/DAD
LC-MS/MS
GC-MS

Systems Biology Approaches and Omics Technologies

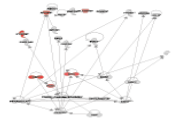


MICROARRAY TECHNOLOGY

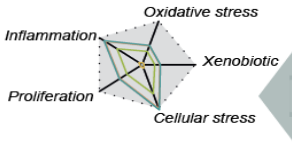


GENE EXPRESSION CHANGES

BIOLOGICAL INTERPRETATION
Pathways
Annotations



BIOLOGICAL NETWORK MODELS



NETWORK PERTURBATION

Iskandar, AR., Gonzalez-Suares I., et al. "A Framework for in Vitro Systems Toxicology Assessment of E-Liquids." Toxicology Mechanisms and Methods 26.6 (2016): 392–416. PMID: 27117495.

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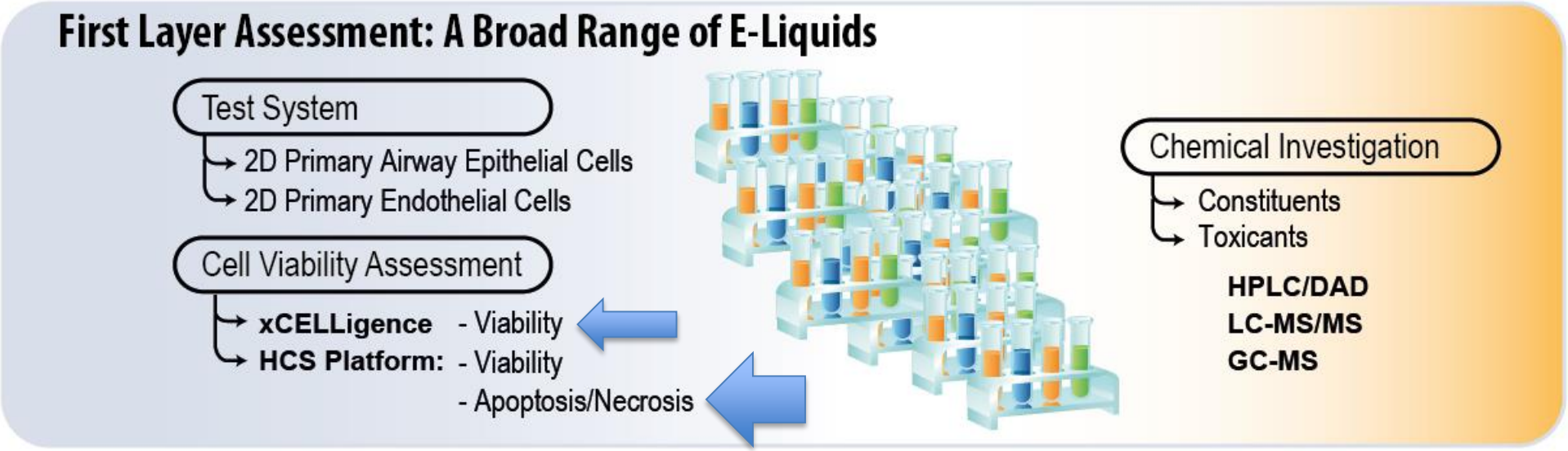
The First Layer of the Framework: General Toxicity Assessment

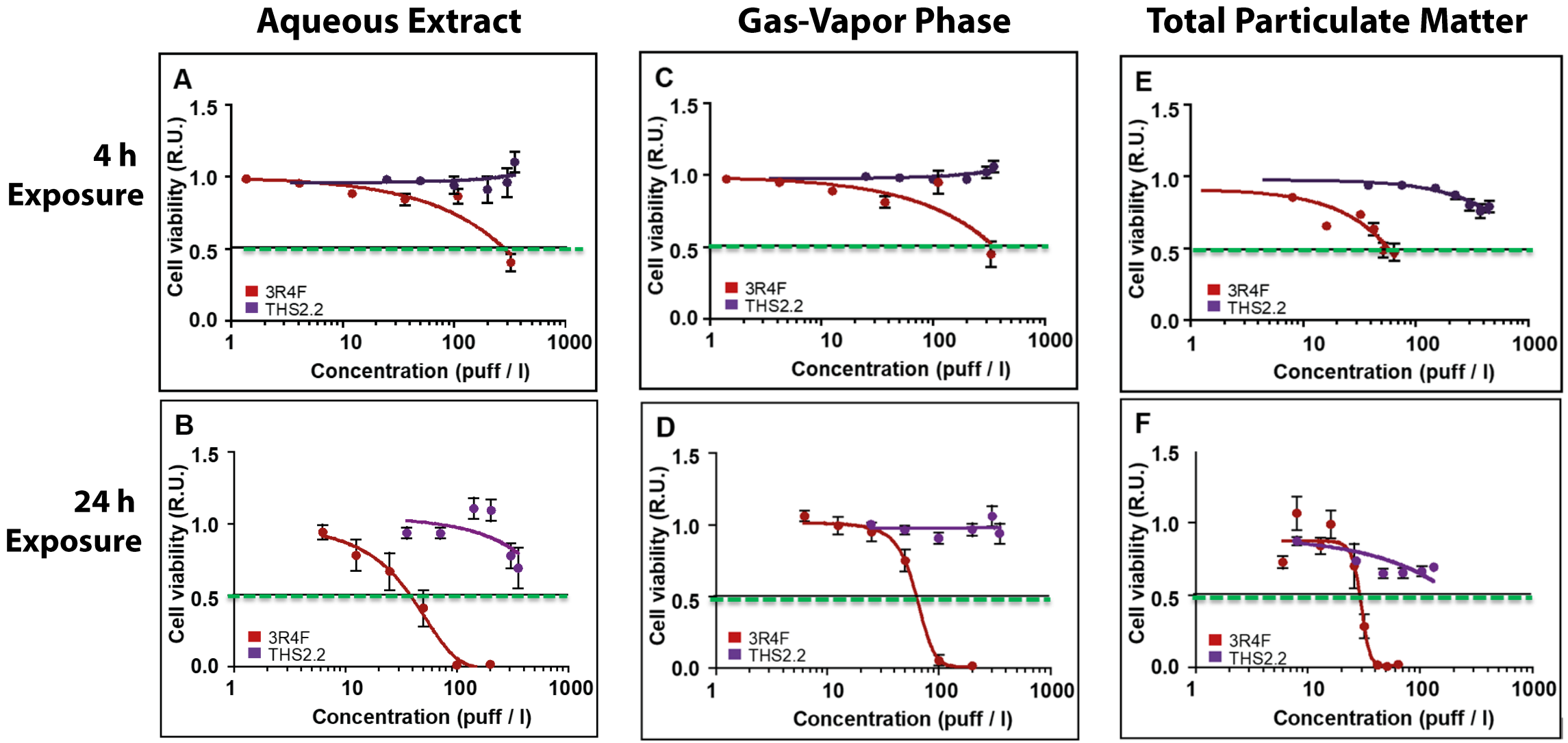


The first layer of the framework aims to screen the general toxicity (cell viability) of various formulations efficiently, independent of the aerosol generation devices, using relevant *in vitro* test systems (i.e., primary cell cultures).

For a relatively inexpensive rapid screening, two-dimensional monolayer cell culture systems are appropriate for assessing the potential toxicity of liquid formulations. Monolayer two-dimensional culture systems are easy to handle, inexpensive, and suitable for large-scale studies.

Iskandar, AR., Gonzalez-Suares I., et al. "A Framework for in Vitro Systems Toxicology Assessment of E-Liquids." Toxicology Mechanisms and Methods 26.6 (2016): 392–416. PMID: 27117495.

Use Case: Fraction Impact Assessment – Cell Viability/Cytotoxicity in Normal Human Bronchial Epithelial Monocultures



3R4F, reference cigarette (University of Kentucky); THS 2.2., Tobacco Heating System 2.2

Gonzalez-Suarez, Ignacio, et al. "In vitro systems toxicology assessment of a candidate modified risk tobacco product shows reduced toxicity compared to that of a conventional cigarette." Chemical research in toxicology 29.1 (2015): 3-18.

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The Second Layer of the Framework: Identification of Mechanisms of Toxicity

Second Layer Assessment: Selected E-Liquids

Test System
- 2D Primary Airway Epithelial Cells
- 2D Primary Endothelial Cells

Toxicity Assessment

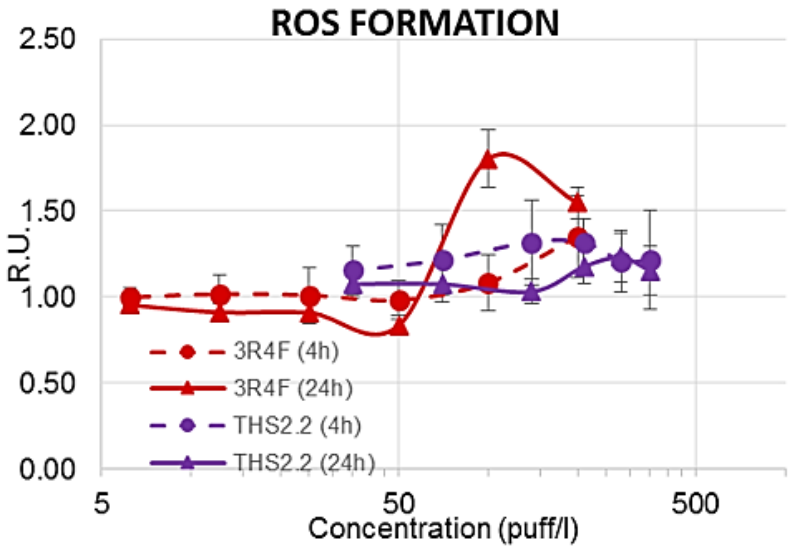
Chemical Investigation
- Constituents
- Toxicants

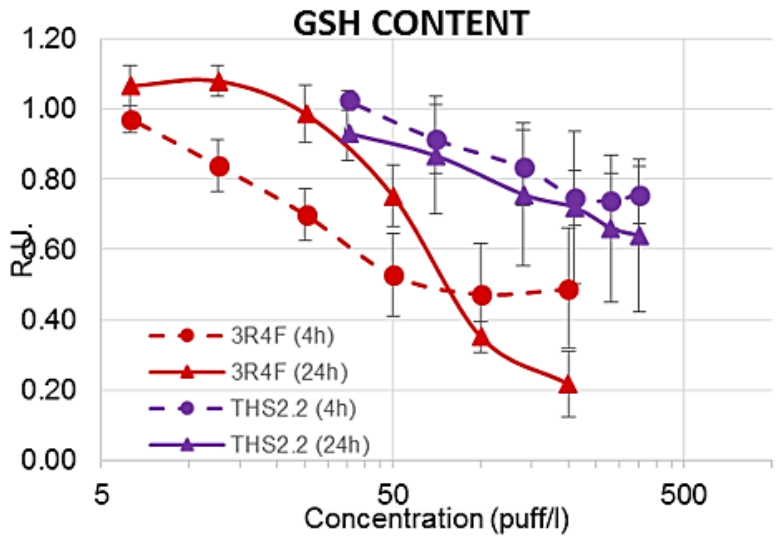
HPLC/DAD
LC-MS/MS
GC-MS

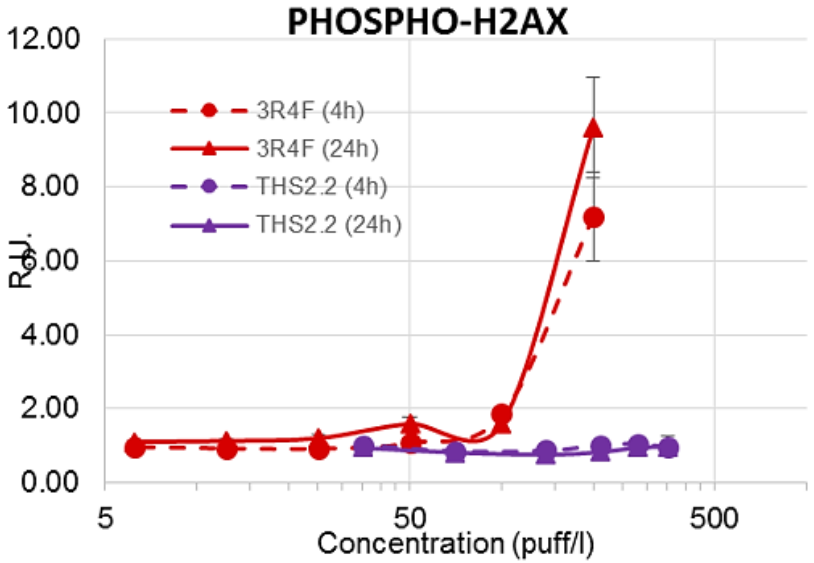
	Assay	#	Biological endpoint
HCS Platform	**Nuclear parameters** (Included in all assays)	1	Cell count
		2	Nuclear area
		3	DNA structure
	Cytotoxicity	4	Mitochondrial mass
		5	Mitochondrial membrane potential
		6	Cytochrome C release
	DNA damage & Stress kinase	7	phospho-H2AX
		8	phospho-cJun
	Proliferation	9	EdU
		10	phospho-H3
	NF-kB	11	NF-kB nuclear translocation
	Oxidative stress	12	ROS
		13	GSH
	Apoptosis & Necrosis	14	Caspase 3/7
		15	Cell membrane permeability

Iskandar, AR., Gonzalez-Suares I., et al. "A Framework for in Vitro Systems Toxicology Assessment of E-Liquids." Toxicology Mechanisms and Methods 26.6 (2016): 392–416. PMID: 27117495.

Use Case: Fraction Impact Assessment – HCS Assay using Normal Human Bronchial Epithelial Cells







R.U., relative unit

Oxidative stress as evidenced by reactive oxygen species (ROS) formation and glutathione (GSH) content

DNA double-strand breaks as evidenced by γH2AX formation were assessed in normal human bronchial epithelial cells following four- and 24-hour exposures to aqueous extract of 3R4F cigarette smoke or THS 2.2 aerosol

Gonzalez-Suarez, Ignacio, et al. "In vitro systems toxicology assessment of a candidate modified risk tobacco product shows reduced toxicity compared to that of a conventional cigarette." Chemical research in toxicology 29.1 (2015): 3-18.

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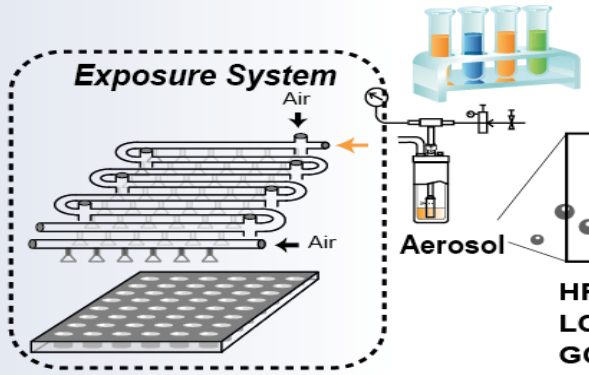
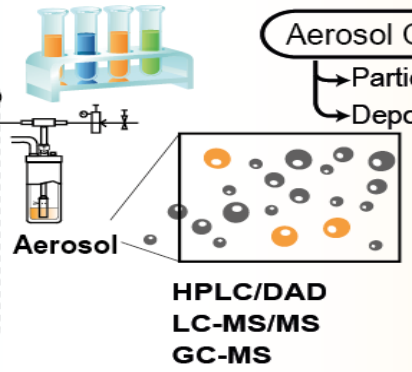
The Third Layer of the Framework: Whole Aerosol Assessment

Third Layer Assessment: Whole Aerosols (Selected E-Liquids)

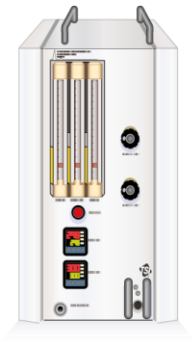
- **Test System**
  - 3D Respiratory Epithelial Cells
- **Toxicity Assessment**
  - Cytotoxicity Assay
  - Histology Analysis
  - Cilia Beating Analysis
  - Measurement of Secreted Mediators



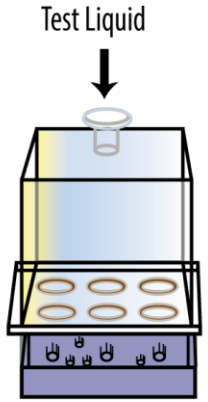


- **Aerosol Characterization**
  - Particle Size
  - Deposition of Compounds
    - Nicotine
    - Glycerol
    - Propylene Glycol

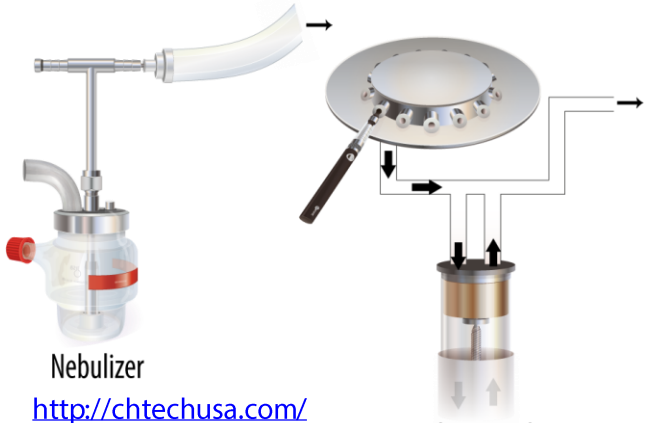
Aerosol Generation Systems



Condensation monodisperse aerosol generator
http://www.tsi.com/



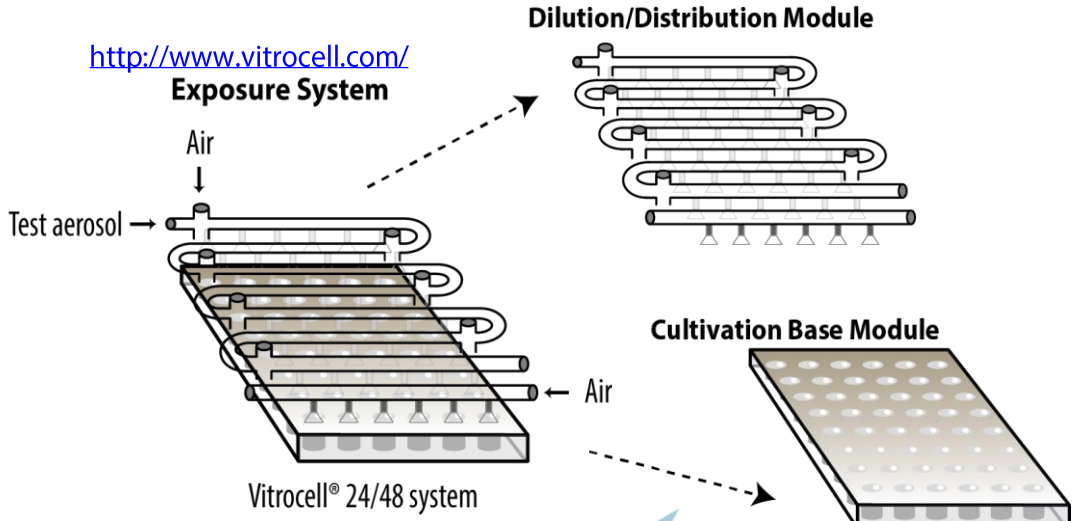
Vitrocell® cloud system
http://www.vitrocell.com/



Nebulizer
http://chtechusa.com/

Smoking machine

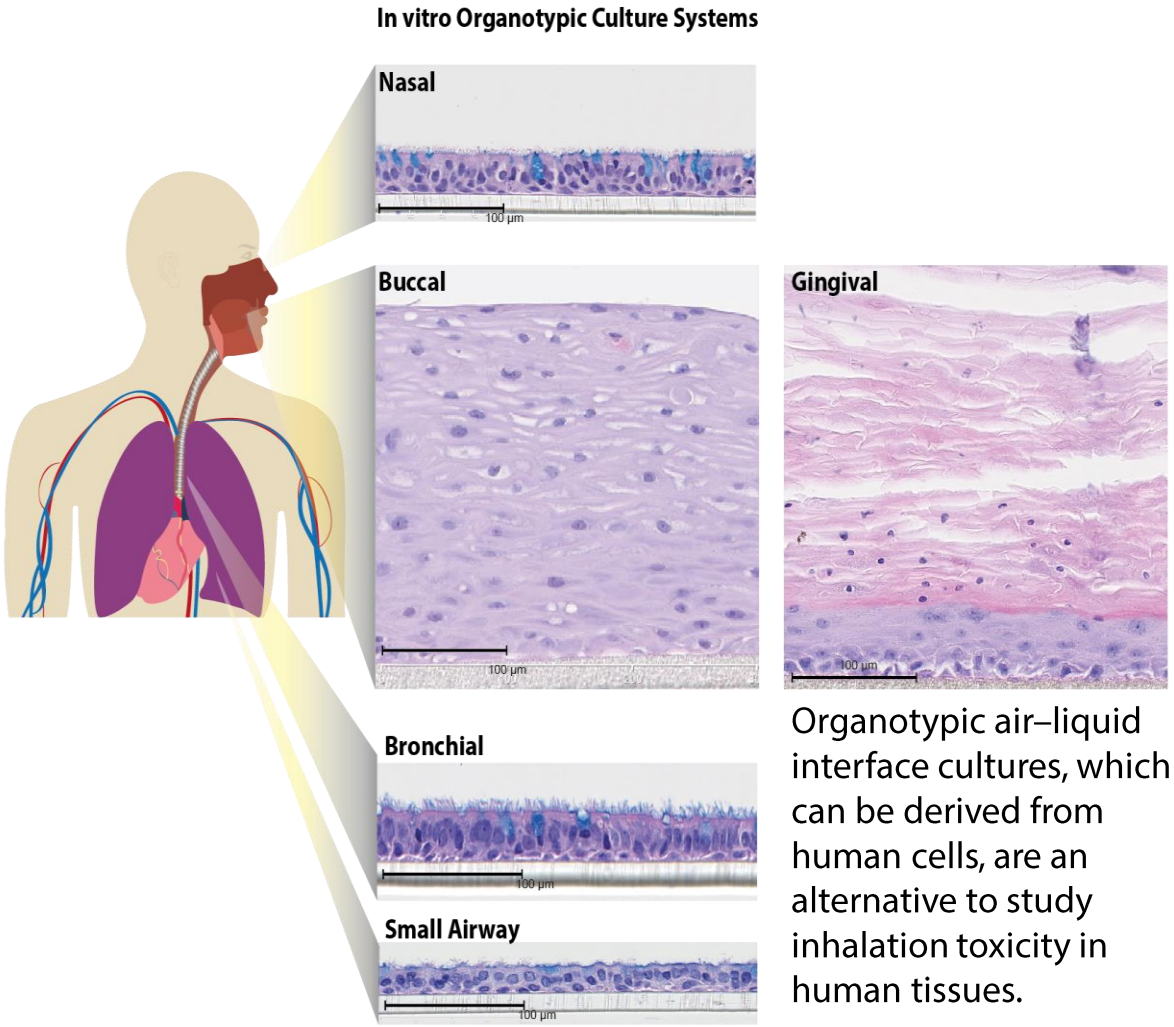
http://www.vitrocell.com/



Vitrocell® 24/48 system

Adapted from: Iskandar, AR., Gonzalez-Suares I., et al. "A Framework for in Vitro Systems Toxicology Assessment of E-Liquids." Toxicology Mechanisms and Methods 26.6 (2016): 392–416. PMID: 27117495.

Relevant *In Vitro* Test Systems for Inhalation Toxicology



Organotypic air–liquid interface cultures, which can be derived from human cells, are an alternative to study inhalation toxicity in human tissues.

Unlike submerged monolayer cultures, organotypic epithelium cultures are grown at the air-liquid interface and can therefore be directly exposed to inhaled compounds, aerosols, or nanoparticles on the apical side.

Biological Endpoint	Post-Exposure Time Point (h)					
	Before	0	4	24	48	72
Ciliary beating	✓	✓	-	✓	✓	✓
mRNA/miRNA	-	-	✓	✓	✓	✓
Culture histology	-	-	-	✓	✓	✓
Cytotoxicity (AK/LDH assay)	-	-	-	✓	✓	✓
Secreted proteins (mediators)	-	-	-	✓	✓	✓

Endpoints can be measured in the **tissue samples**, including

- mRNA, microRNA, and protein expression
- Histology – assessing the impact of exposure on culture morphology

Endpoints can be measured using the **culture medium**, including

- Cytotoxicity assays – measuring enzymatic release of adenylate kinase or lactate dehydrogenase (Cho, et al. 2008)
- Protein quantification (ELISA, multiplexed assay) – measuring the concentrations of secreted mediators (Kingsmore, 2008)

For ciliated cultures, ciliary beating frequency can be monitored using intact cultures at various time points before and following exposure.

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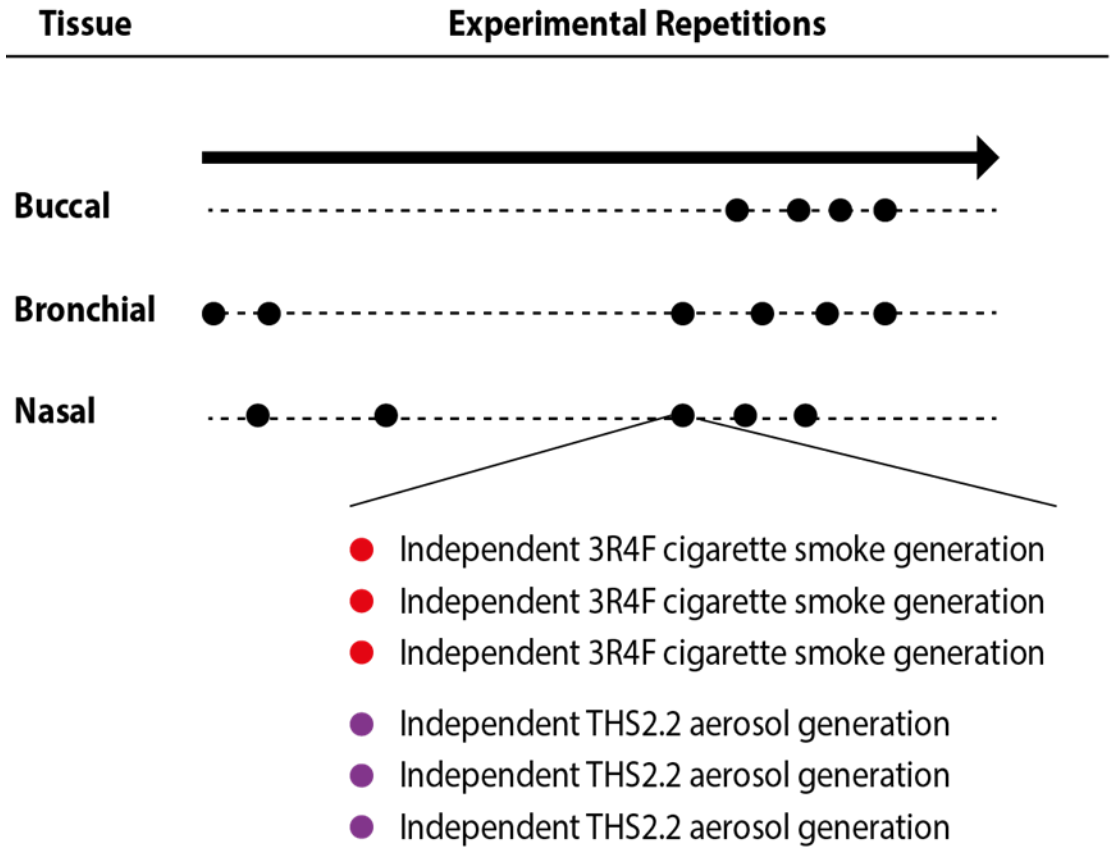
Use Case Example: Systems Toxicology Assessment of Heated Tobacco Aerosol
A Series of Studies Using Human Organotypic Epithelial Cultures



Human organotypic cultures were exposed to 3R4F cigarette smoke or THS 2.2 aerosol for 28 minutes. A series of experimental repetitions was conducted to increase the confidence in measuring accurate exposure effects.

Doses of 3R4F Smoke and THS 2.2 Aerosol

	3R4F		THS 2.2	
	Conc. Smoke	Nic (mg/L)	Conc. Aerosol	Nic (mg/L)
Buccal	15%	~0.3	25%	~0.3
	24%	~0.5	32%	~0.5
			69%	~1.0
Bronchial/ Nasal	7%	~0.1	13%	~0.1
	13%	~0.3	24%	~0.3
			31%	~0.5

To compare the biological impact of exposure to THS 2.2 aerosol and 3R4F cigarette smoke, the cultures were exposed to the aerosol and smoke at similar nicotine concentrations. Concentrations of the smoke and aerosol fed into the exposure systems were adjusted to reach target nicotine concentrations.

Iskandar, A. R., et al. (2017). "Systems toxicology meta-analysis of in vitro assessment studies: biological impact of a candidate modified-risk tobacco product aerosol compared with cigarette smoke on human organotypic cultures of the aerodigestive tract." Toxicology Research 6(5): 631-653.

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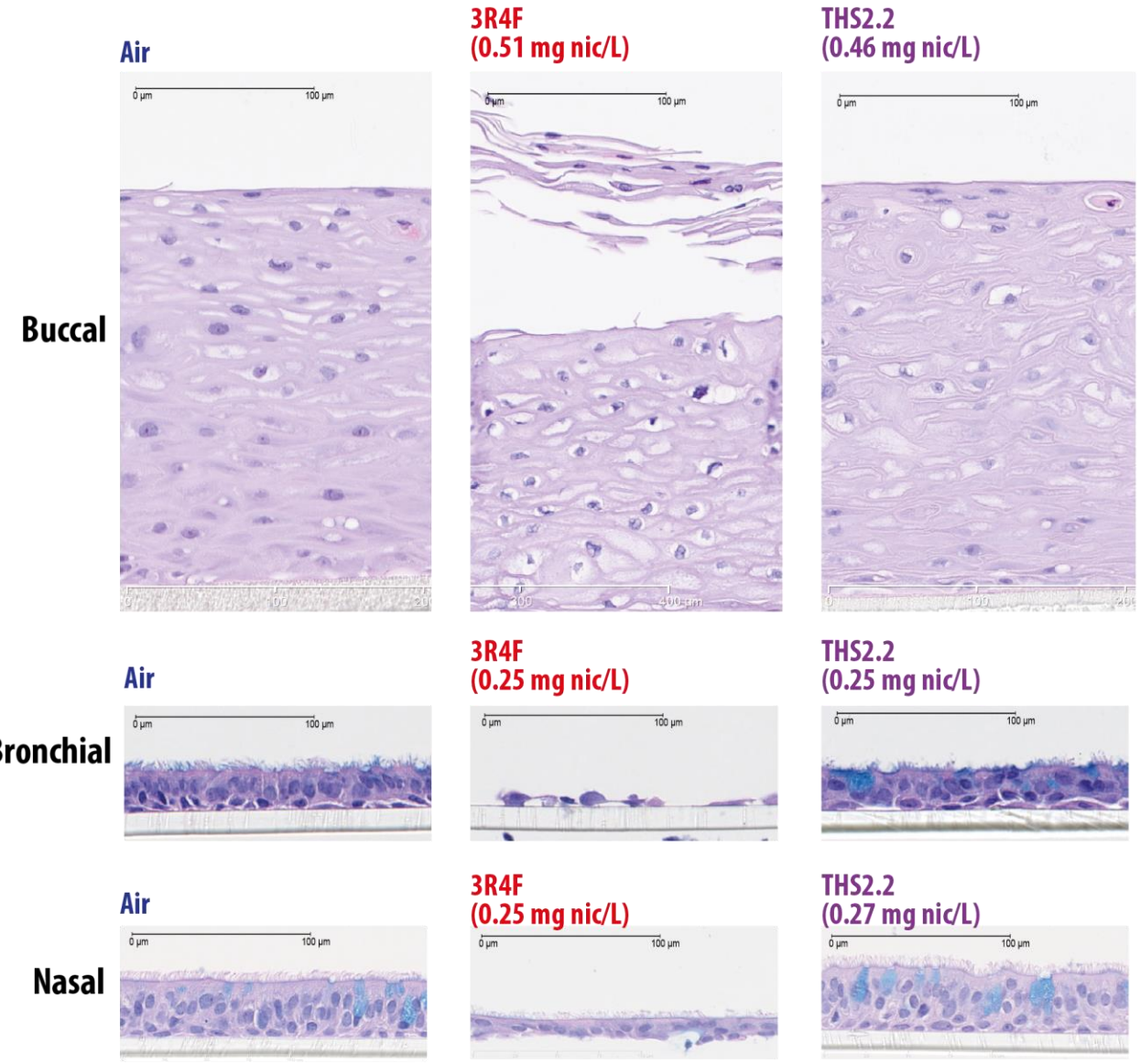
Use Case Example: Biological Impact of Aerosol Exposure
Culture Morphology Following Exposure to Cigarette Smoke and Heated Tobacco Aerosols



At similar nicotine concentrations in the smoke/aerosol, exposure to THS 2.2 aerosol did not result in morphological alterations in any of the three culture types.

The thickness of buccal cultures (stratified epithelium) was approximately five times that of bronchial or nasal cultures (pseudostratified epithelium). Therefore, exposures were performed with higher doses of smoke/aerosol.

Iskandar, A. R., et al. (2017). "Systems toxicology meta-analysis of in vitro assessment studies: biological impact of a candidate modified-risk tobacco product aerosol compared with cigarette smoke on human organotypic cultures of the aerodigestive tract." Toxicology Research 6(5): 631-653.

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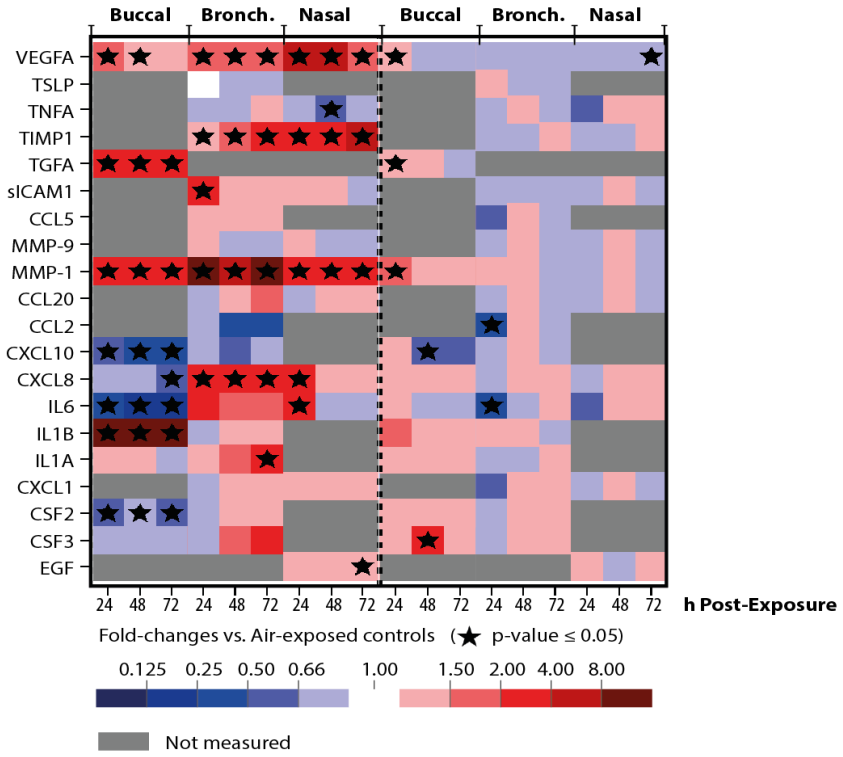
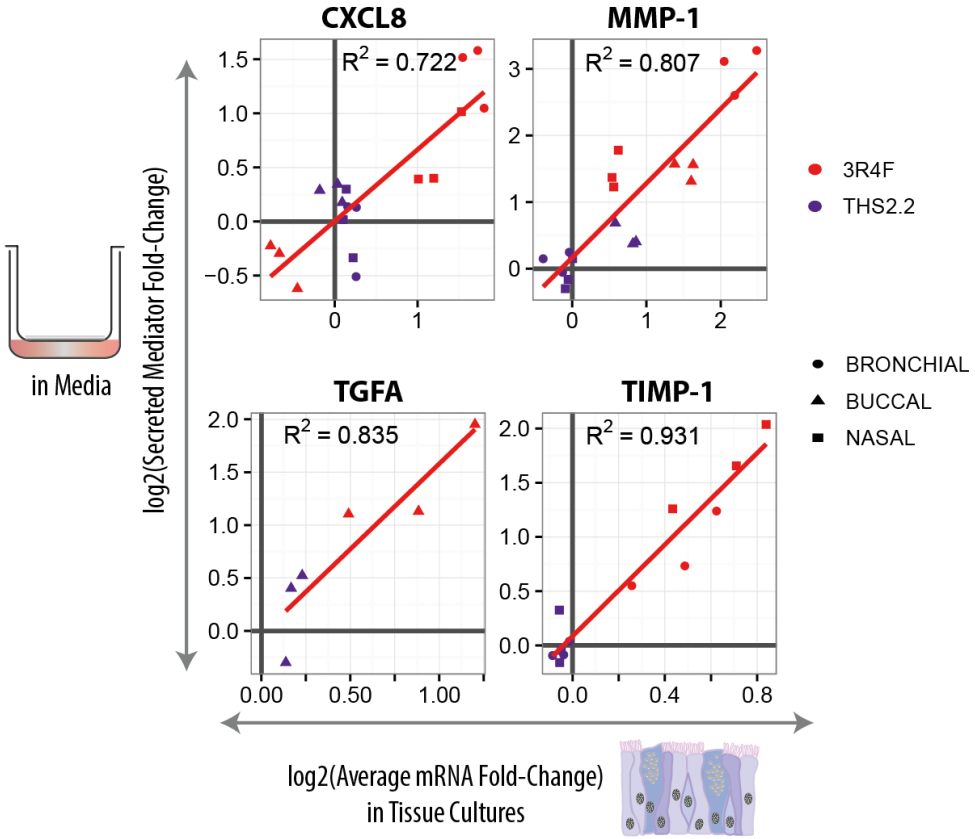
Use Case Example: Biological Impact of Aerosol Exposure
Secretion of Inflammatory Mediators Following Exposure

Alterations in the Secreted Pro-Inflammatory Mediators in the Basoleteral Media

	3R4F	THS2.2	
Buccal	0.51	0.46	mg nic/L
Bronchial	0.13	0.14	mg nic/L
Nasal	0.15	0.15	mg nic/L



Correlation of the Secreted Mediator and the Respective Gene Expression



Secreted mediators in the medium were measured following exposure. Greater changes in the mediator concentrations were observed following exposure to 3R4F cigarette smoke than exposure to THS 2.2 aerosol.

Linear correlations were observed between the protein expression in the media and gene expression in the epithelial cells.

Iskandar, A. R., et al. (2017). "Systems toxicology meta-analysis of in vitro assessment studies: biological impact of a candidate modified-risk tobacco product aerosol compared with cigarette smoke on human organotypic cultures of the aerodigestive tract." Toxicology Research 6(5): 631-653.

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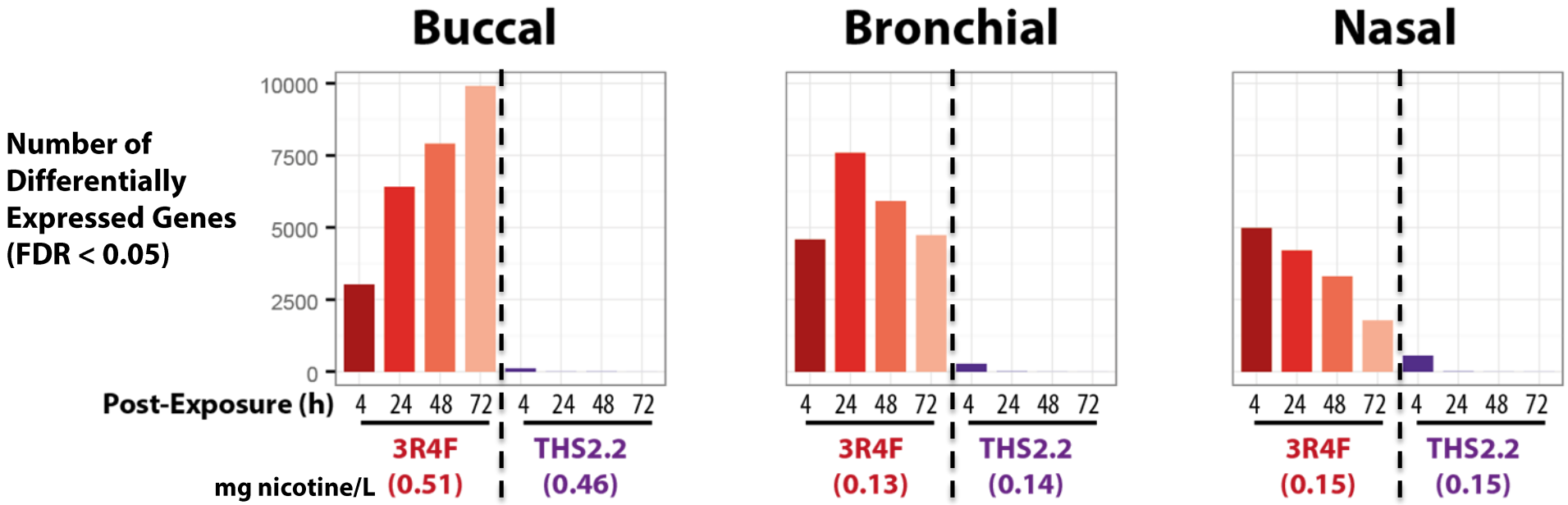
Use Case Example: Biological Impact of Aerosol Exposure
Transcriptome Profiles of Human Organotypic Epithelial Cells Following Exposure



At similar nicotine concentrations, THS 2.2 aerosol elicited less pronounced changes in the differentially expressed genes (DEG) compared with 3R4F cigarette smoke. In all three cultures, the THS 2.2 aerosol-induced DEGs were mostly limited to the earliest post-exposure time point (four hours), suggesting a lower, more transient response to THS 2.2 aerosol exposure than 3R4F cigarette smoke exposure.

Iskandar, A. R., et al. (2017). "Systems toxicology meta-analysis of in vitro assessment studies: biological impact of a candidate modified-risk tobacco product aerosol compared with cigarette smoke on human organotypic cultures of the aerodigestive tract." Toxicology Research 6(5): 631-653.

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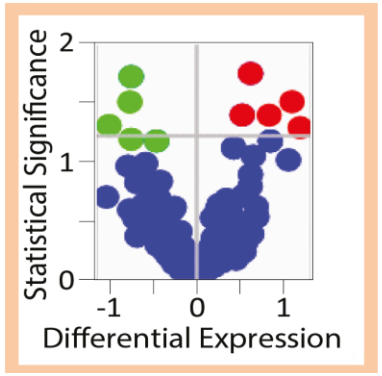
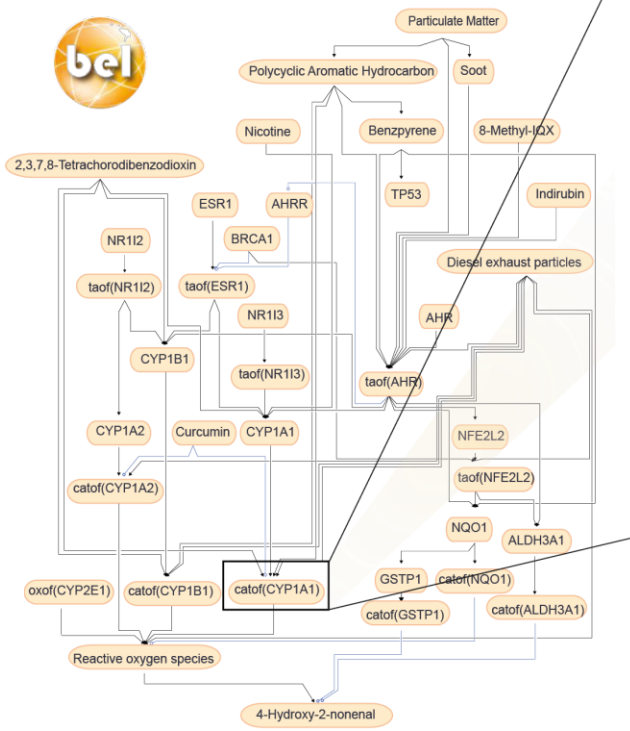
Use Case Example: Biological Impact of Aerosol Exposure
Network-Enrichment Analysis of Transcriptome Data for the Identification of Mechanistic Insights

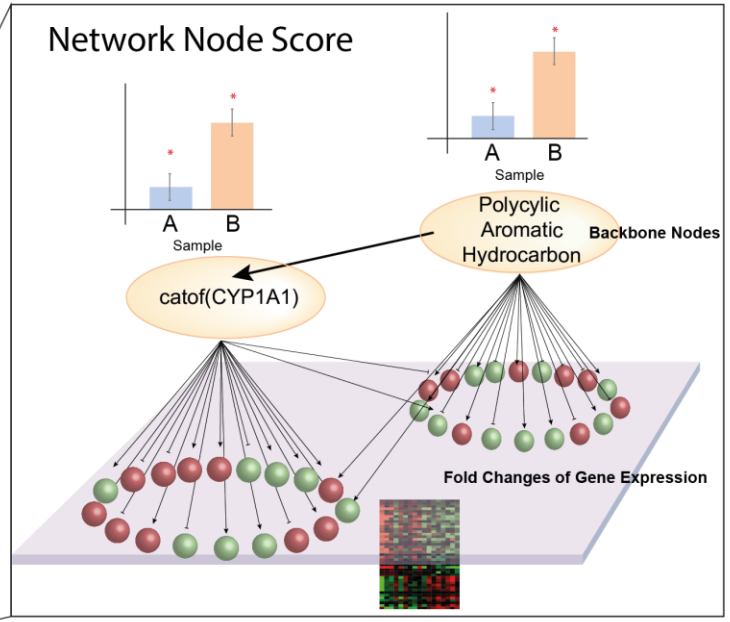
Transcriptome Data
(Exposed vs. Nonexposed)



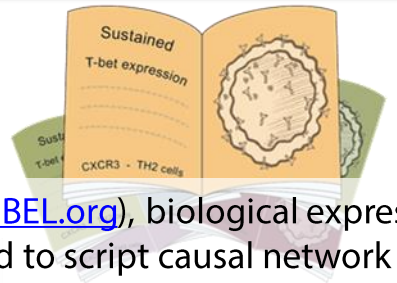
Transcriptome data were used to compute the impact of exposure on network models using the network perturbation amplitude (NPA) algorithm (Martin et. Al. 2014).

Xenobiotic Metabolism Network



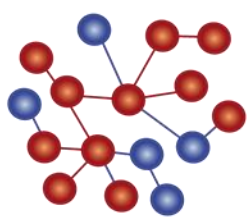


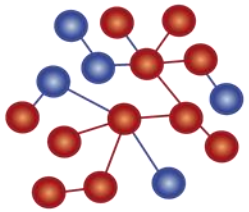
Computation of NPA scores to infer the exposure impacts on biological processes and pathways.

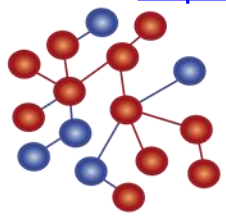


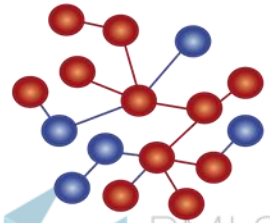
BEL (http://openBEL.org), biological expression language, is used to script causal network models describing signaling pathways relevant in disease and nondiseased pulmonary and vascular tissues from the literature.

The BELIEF platform is available for a semi-automated generation of BEL statements and creation of networks.
http://belief.scai.fraunhofer.de/BeliefDashboard/


Apoptosis Network

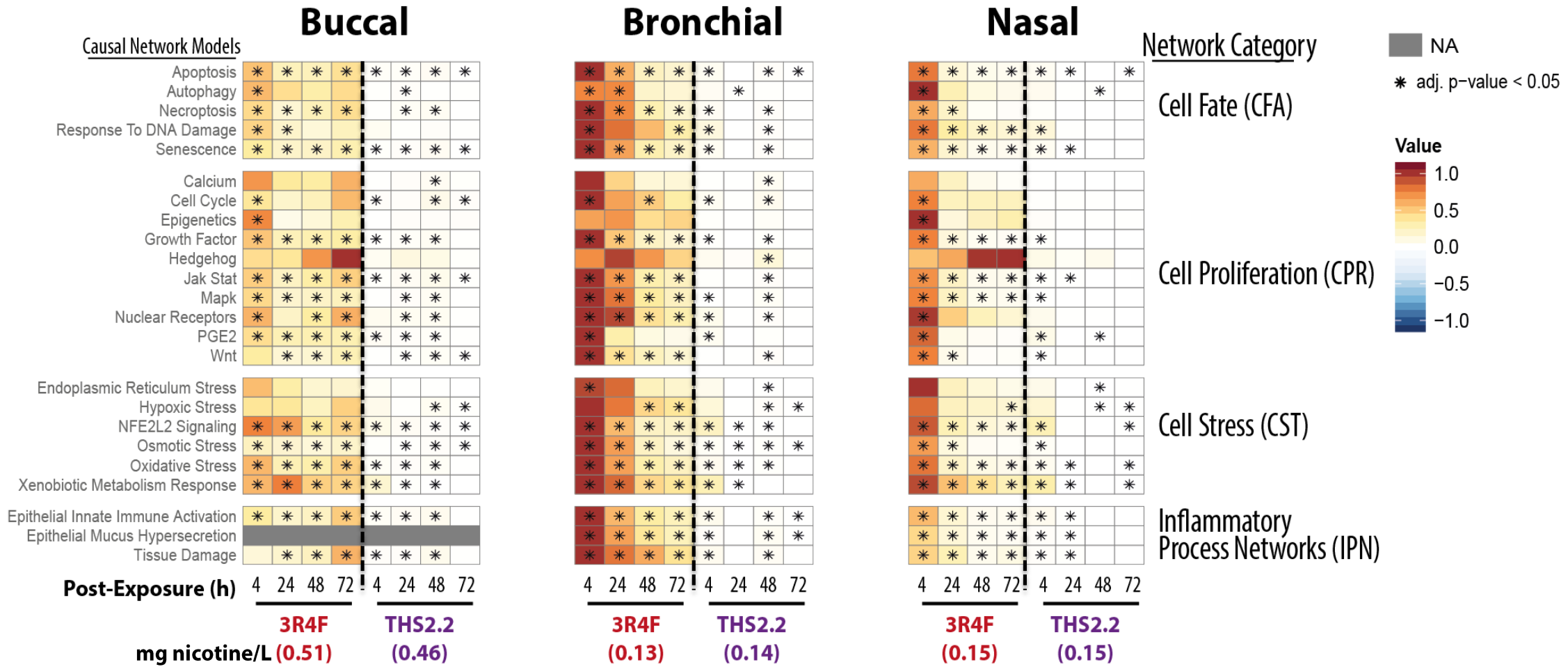

Cell Cycle Network


Epithelial Mucus Hypersecretion Network


Oxidative Stress Network

http://causalbionet.com/

Use Case Example: Biological Impact of Aerosol Exposure
Mechanistic Investigation of the Exposure Impact



At similar concentrations of nicotine, THS 2.2 aerosol exposure resulted in lower network perturbation scores than 3R4F cigarette smoke exposure in all three culture types.

Iskandar, A. R., et al. (2017). Systems toxicology meta-analysis of in vitro assessment studies: biological impact of a candidate modified-risk tobacco product aerosol compared with cigarette smoke on human organotypic cultures of the aerodigestive tract. Toxicology Research 6(5): 631-653.

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Summary

- Multiparametric indicators of general toxicity can benefit from real-time cellular analysis and HCS assays.
- Aerosols are complex. Their characteristics vary depending on the aerosol generation device. Therefore, a characterization of the test aerosol should also be done as part of the assessment.
- Testing the potential toxic effects of exposure to aerosols requires the use of relevant biological test systems and exposure modes as well as appropriate (physiologically relevant) doses.
- Systems biology approaches (omics) will uncover changes at the cellular and molecular levels otherwise undetected in standard toxicity assays.
- Collaborative efforts among the scientific community, industry, and regulatory stakeholders are needed to facilitate the adoption of 21st Century Toxicology approaches in the context of inhaled aerosol testing.

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