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Application of a multi-layer systems toxicology framework for in vitro assessment of the biological effects of liquids and corresponding aerosols

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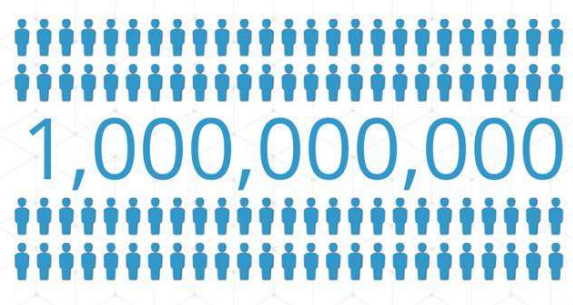
The research described in this presentation was sponsored by Philip Morris International.

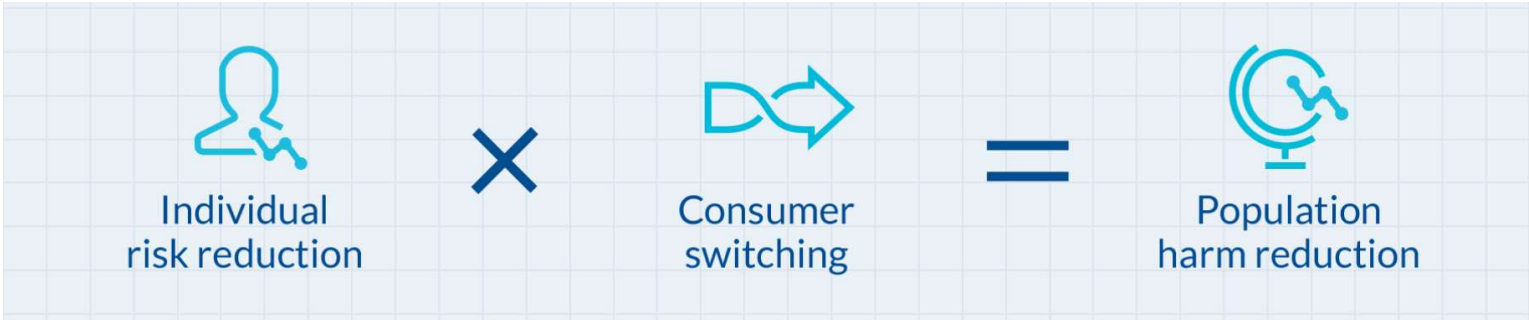
Biomedical Research at PMI

- Smoking causes serious diseases such as cardiovascular diseases, lung cancer, and chronic obstructive pulmonary disease.
- Philip Morris International is developing smoke-free products with the potential to reduce individual risk and population harm in comparison with smoking cigarettes.
- To determine whether such products have the potential to reduce individual risk, we are conducting extensive and rigorous scientific studies comparing their biological impact with that of a reference cigarette (3R4F) on a mechanism-by-mechanism basis.

What Is the Objective of Harm Reduction?

- Smoking is addictive and causes a number of serious diseases
- Worldwide, it is estimated that more than 1 billion people will continue to smoke in the foreseeable future*
- Offering smoke-free alternatives to adult smokers is a sensible, complementary addition to existing tobacco control strategies



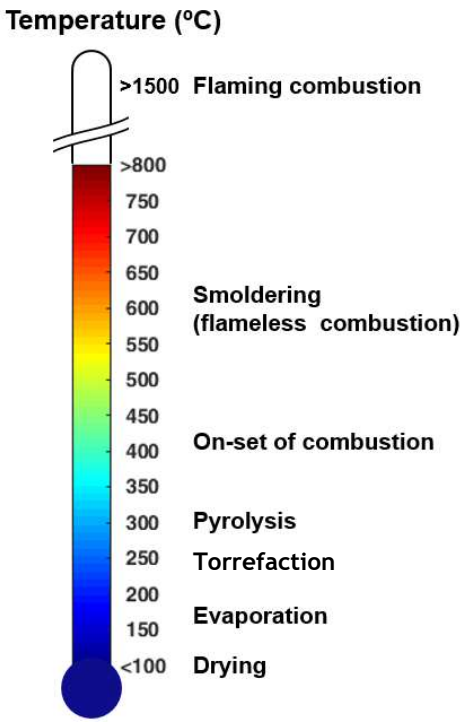


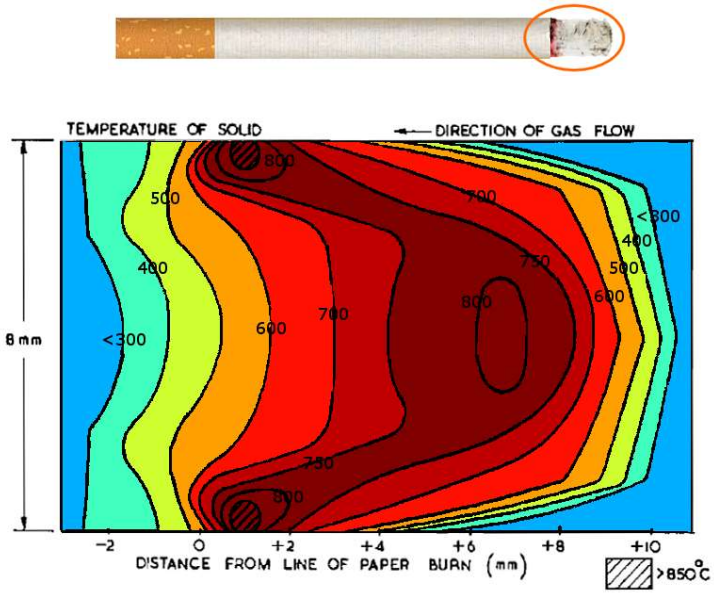
Successful harm reduction requires that current adult smokers be offered a range of Reduced-Risk Products (RRP) they can fully switch to, should they decide not to quit.

* http://www.who.int/tobacco/publications/surveillance/reportontrendstobaccosmoking/en/index4.html
Figure adapted from Clive Bates presentation to E-Cigarette Summit (19 Nov 2013)
Note: Reduced Risk Products ("RRP") is the term PMI uses to refer to products that present, are likely to present, or have the potential to present less risk of harm to smokers who switched to these products versus continued smoking.

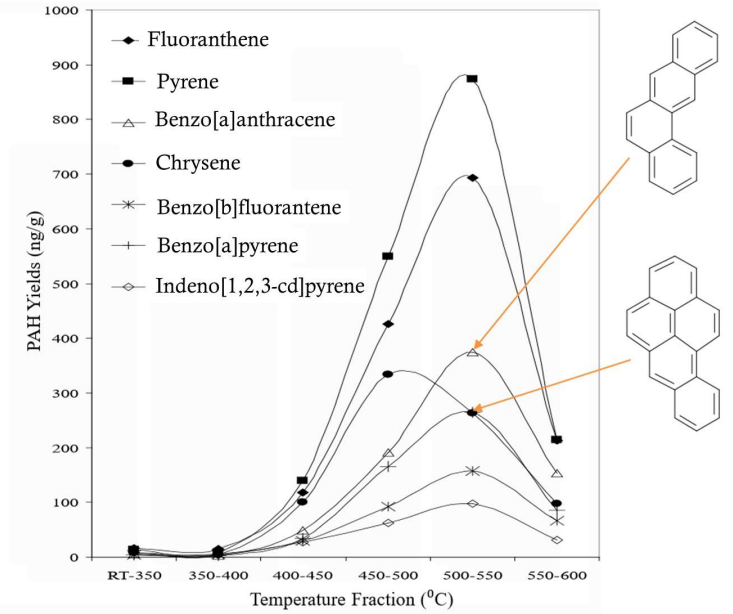
Elimination of Combustion Is Key

Scientific studies have shown that, as the temperature of tobacco increases, the levels of harmful chemicals formed increase





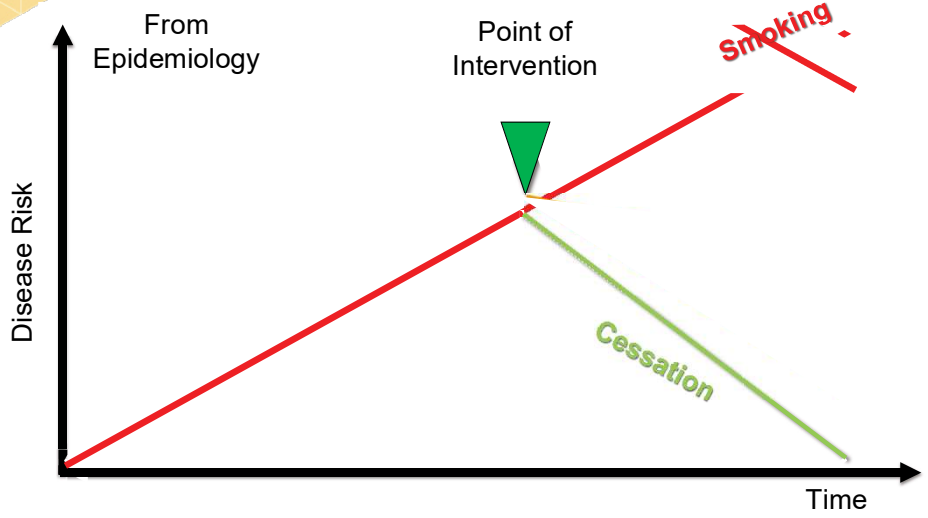
Source: Baker R. R., 1975, Temperature variation within a cigarette combustion coal during the smoking cycle, High Temp. Sci., 7, 236-247. Coloration by PMI.



Source: McGrath, T.E., Wooten, J.B., Chan W.G. and Hajaligol, M.R., 2007, Formation of polycyclic Aromatic Hydrocarbons from Tobacco: the "Link" between Low Temperature Residual Solid and PAH Formation, Food and Chemical Toxicology, 45,6,1039-1050

PMI's Scientific Assessment Approach

Assessment Framework



The assessment framework integrates what is known about cigarette (CC) smoking and incorporates both epidemiological and mechanistic evidence to define the assessment approach.

These assessment steps are designed to provide five levels of evidence as the assessment program is completed.

Source: Smith, M.R., et al., Evaluation of the Tobacco Heating System 2.2. Part 1: Description of the system and the scientific assessment program. *Regulatory Toxicology and Pharmacology* (2016). http://dx.doi.org/10.1016/j.yrtph.2016.07.006

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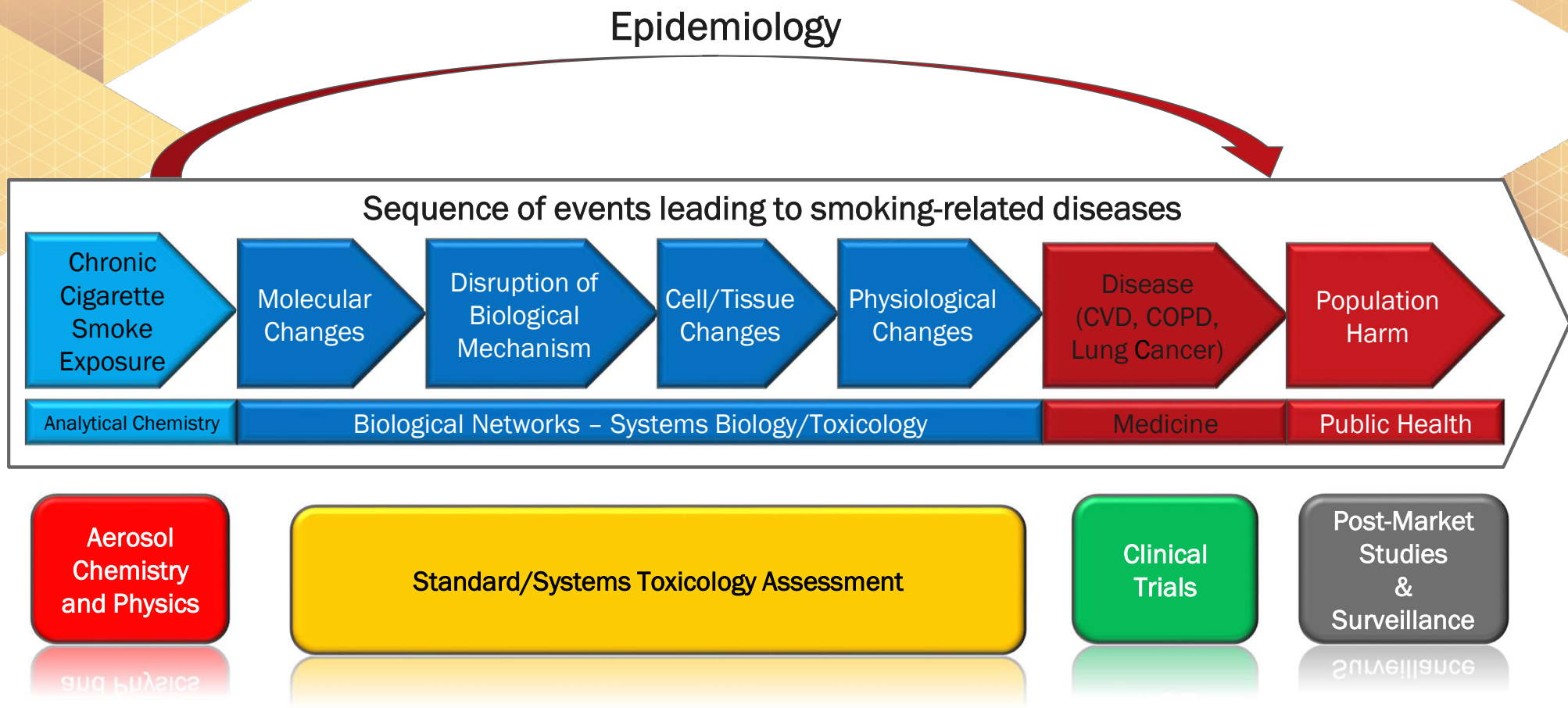
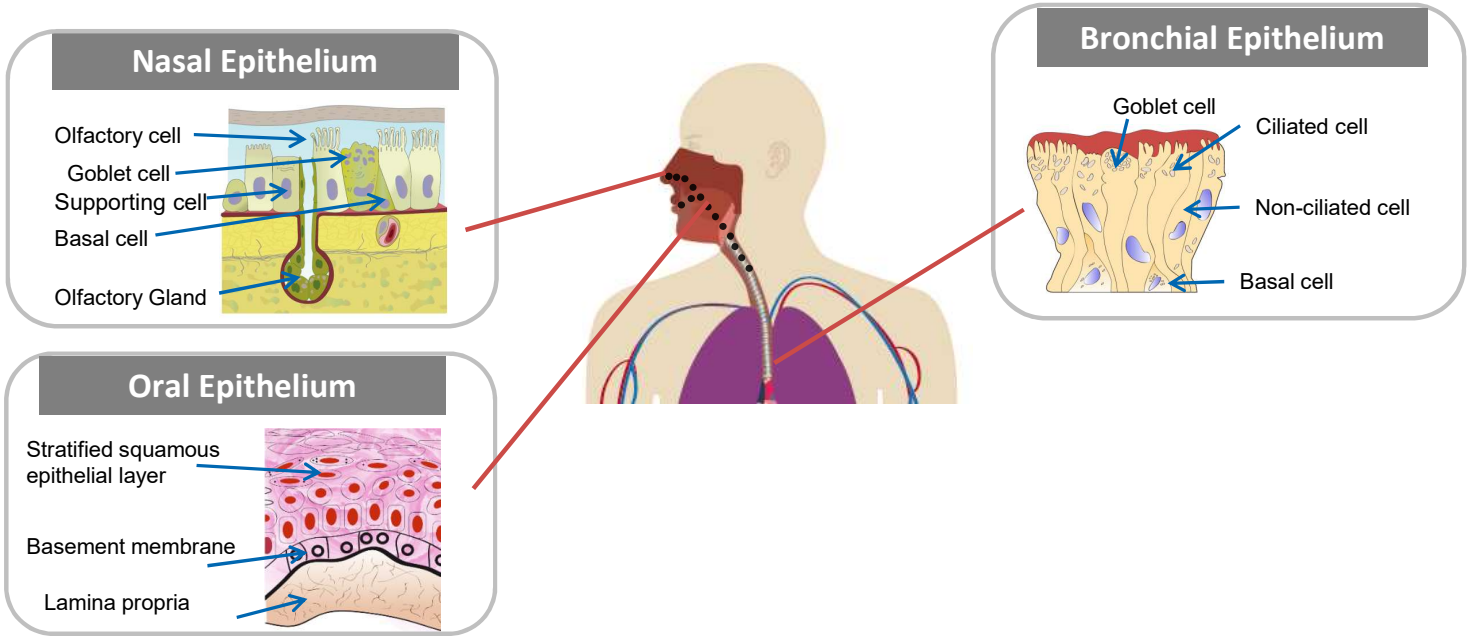
From Chronic Exposure to Population Harm: A Causal Chain of Events



Smith, M. R., et al. (2016). "Evaluation of the Tobacco Heating System 2.2. Part 1: description of the system and the scientific assessment program." Regulatory Toxicology and Pharmacology 81: S17-S26.

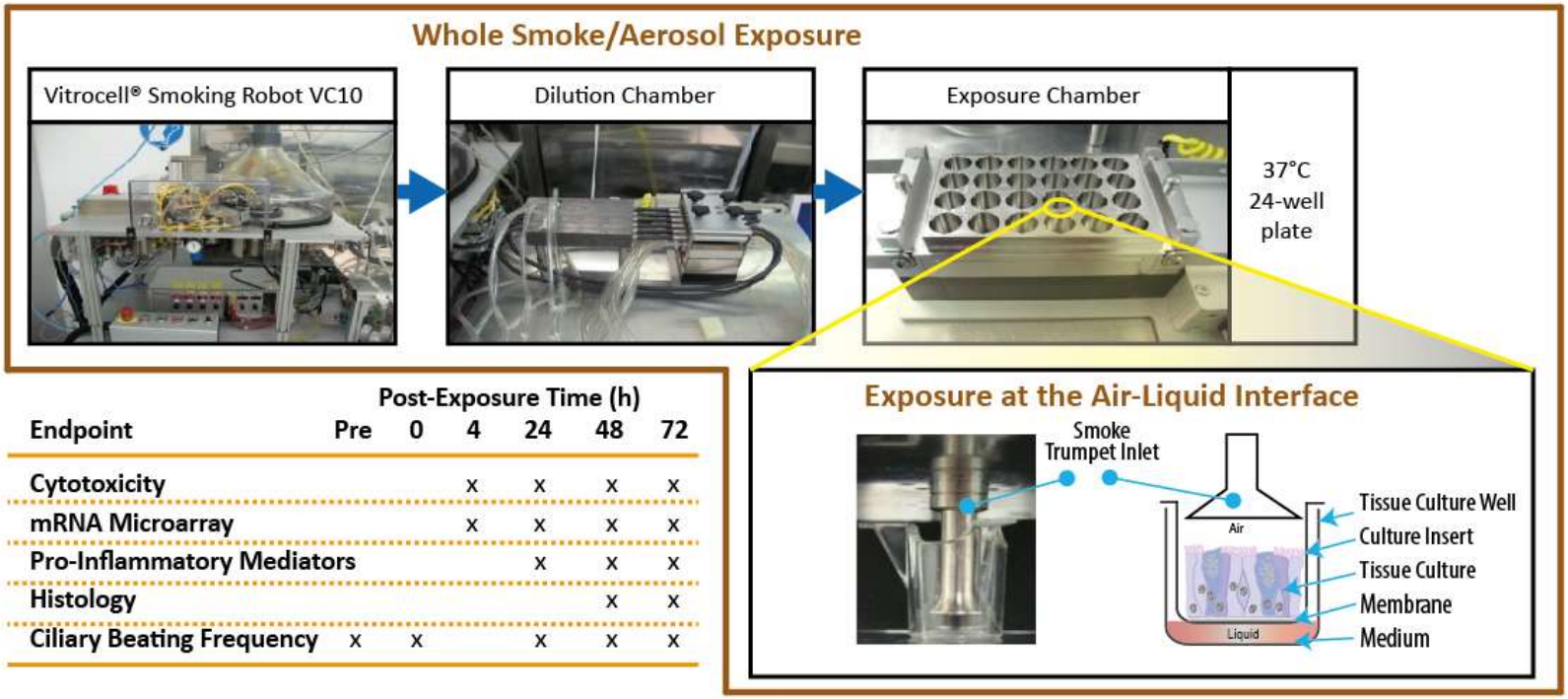
In Vitro Toxicology: Relevant Test Systems

- An EU directive "on the protection of animals used for scientific purposes" (EU Directive 2010/63/EU) strongly promotes the use of alternative animal test methods.
- 3R (Replace, Reduce, and Refine), a strategy for alternative methods to animal testing, was described in 1959 by Russell and Burch. One significant strategy to avoid animal testing is the use of cell systems where cells from different animals and tissues are grown and tested in plates or wells (in vitro).
- Both cell lines and primary cell cultures have limitations, and the results might be difficult to interpret or cannot be extrapolated to help elucidate possible in vivo effects. Relevant biological test systems facilitate identification of biomarkers of exposure response and disease.



Sridhar S, et al. (2008) Smoking-induced gene expression changes in the bronchial airway are reflected in nasal and buccal epithelium. BMC genomics 9: 259

Relevant Exposure Modes for Assessment of Aerosols

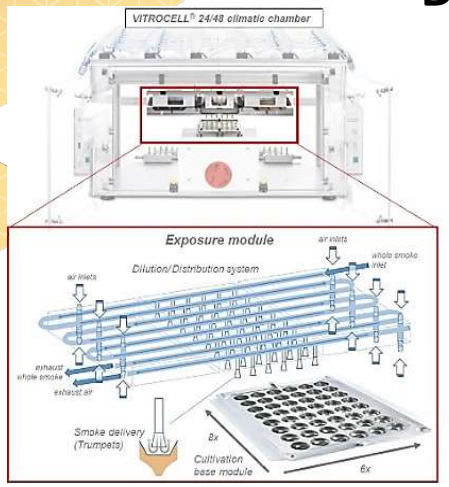


Experimental Data Workflow

Upper panel: Organotypic cultures of human primary respiratory epithelial cells can be exposed directly to 3R4F smoke or electronic nicotine delivery system (ENDS) aerosols by using the Vitrocell® system.
Lower panel: The cells were exposed to smoke/aerosols during different exposure times. Then, various endpoints were captured after different post-exposure times.

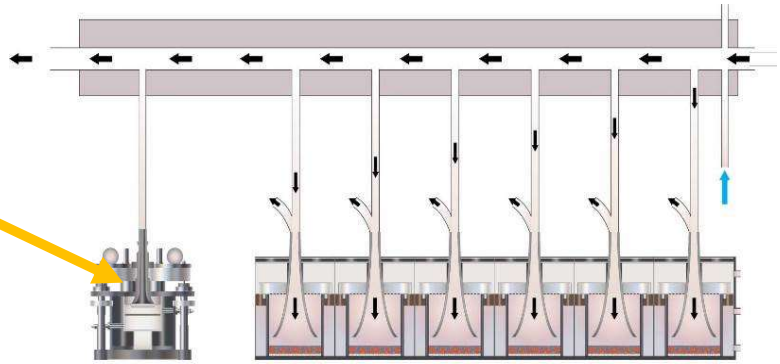
Majeed, S., et al. (2014). "Characterization of the Vitrocell(R) 24/48 in vitro aerosol exposure system using mainstream cigarette smoke." Chemistry Central Journal 8(1): 62.
Thorne, D., et al. (2013). "Characterisation of a Vitrocell(R) VC 10 in vitro smoke exposure system using dose tools and biological analysis." Chemistry Central Journal 7(1): 146

Schematic Representation of the Vitrocell® 24/48 Exposure System Facilitating Aerosol Delivery to Air–Liquid Interface Cultures

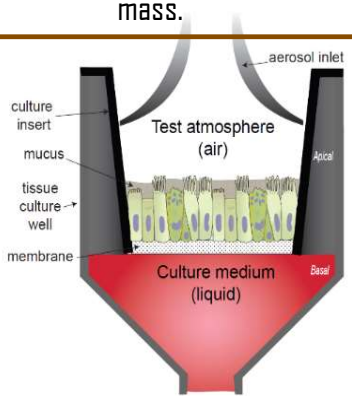


A climatic chamber with an exposure module

Up to 48 wells can be exposed simultaneously.
The base module has a format of 8 rows x 6 columns.



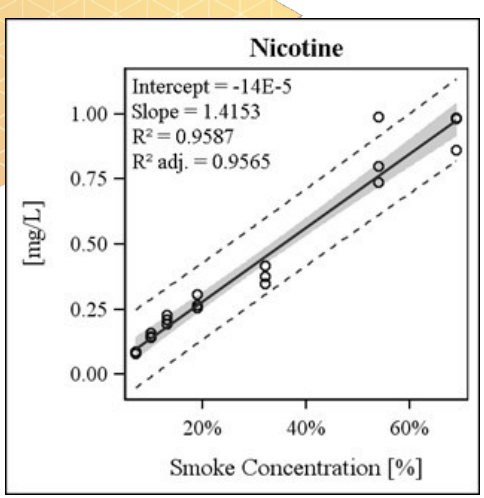
Particles depositing on the quartz crystal microbalance are detected by a change in its resonance frequency, which is proportional to the deposited mass.

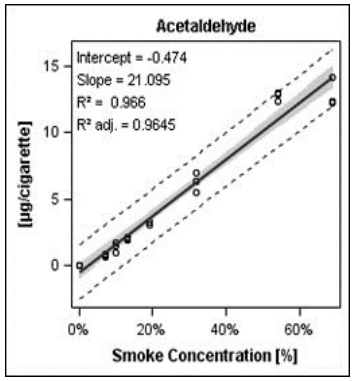


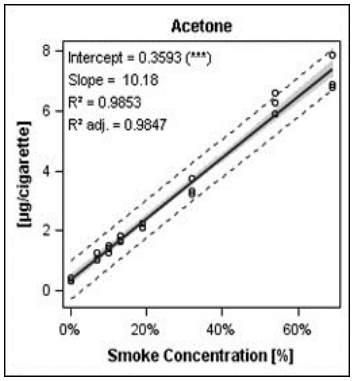
Cells are grown at the air–liquid interface on porous membranes and supplied with medium from their basal side.

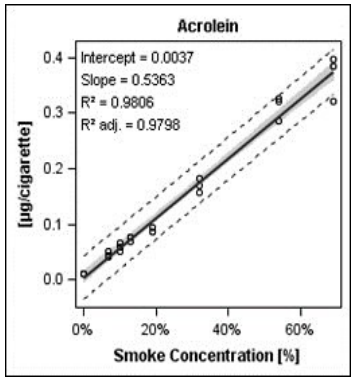
Majeed, S., et al. (2014). "Characterization of the Vitrocell(R) 24/48 in vitro aerosol exposure system using mainstream cigarette smoke." Chemistry Central Journal 8(1): 62.

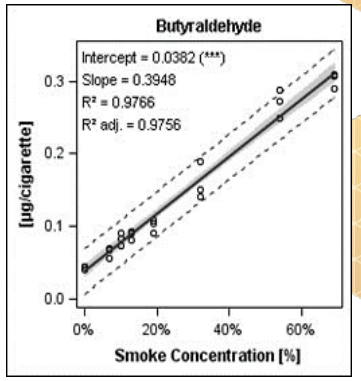
Linear Correlation Between the Concentrations of Trapped Nicotine and Carbonyl and the Concentration of cigarette smoke (CS) Inside the Vitrocell® 24/48 System

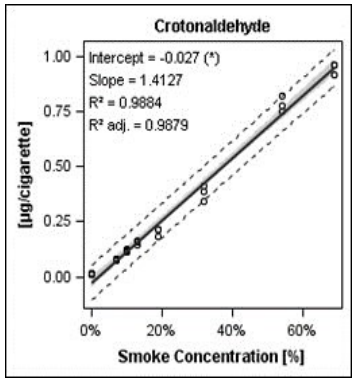


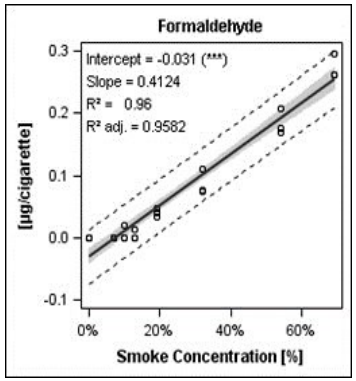


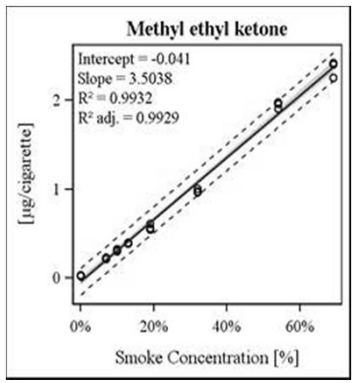


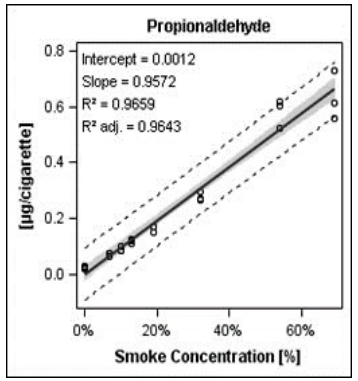












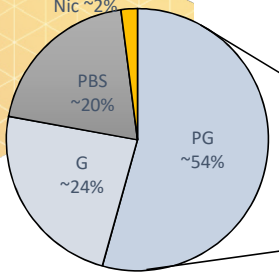
Whole-smoke application (multi-dilution) and quantification of nicotine and 8 different carbonyls inside the VITROCELL® 24/48 exposure chamber over a concentration range of 7–69% cigarette smoke.

Majeed, S., et al. 2014. Characterization of the Vitrocell(R) 24/48 in vitro aerosol exposure system using mainstream cigarette smoke. Chemistry Central journal, 8, 62.

Aerosol Characterization in In Vitro Exposure Studies

Compressed air heated up to 37°C

Nebulizer

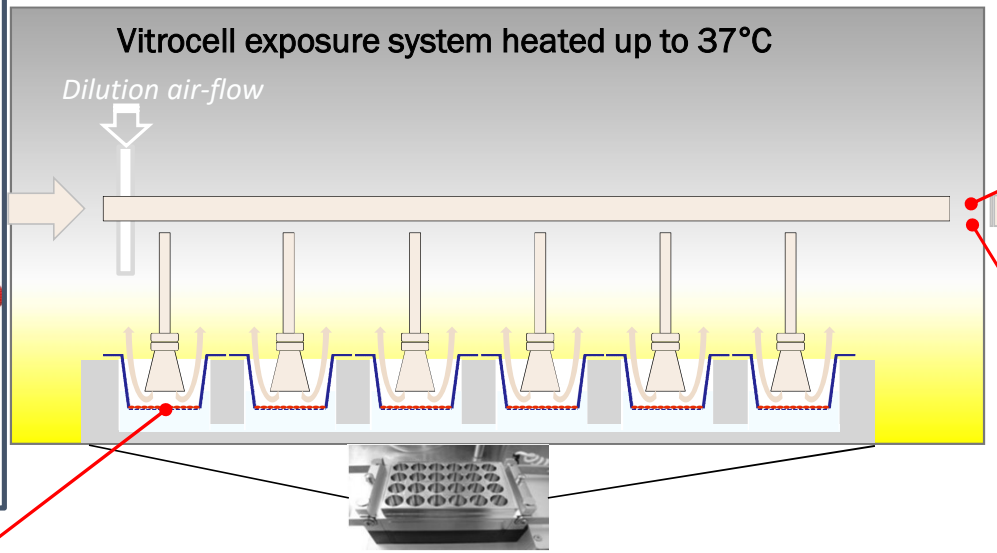


E-liquid mixture composition

Glass jar containing e-liquid heated up to 37 °C

Vitrocell exposure system heated up to 37°C

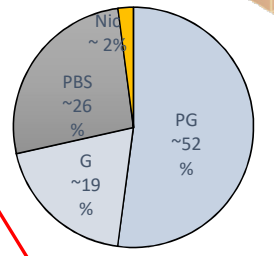
Dilution air-flow



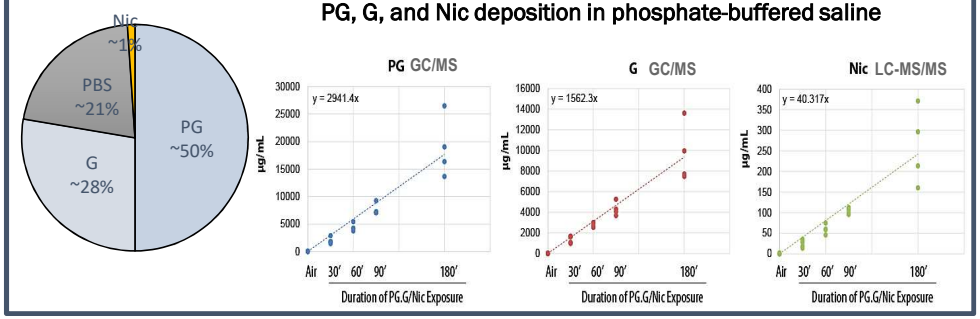
Aerosol trapping on a Cambridge filter pad

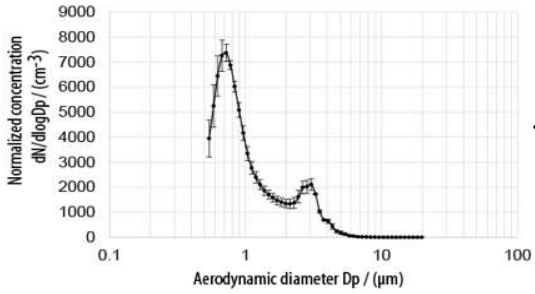
Filter holder

GC-FID (flame ionization detector)

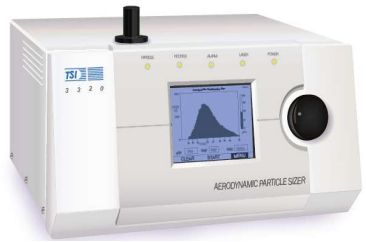


PG, G, and Nic deposition in phosphate-buffered saline



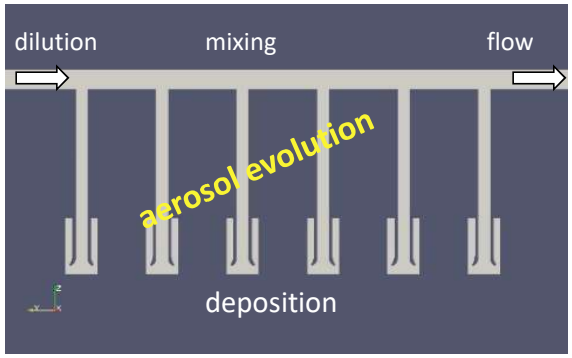


Aerodynamic particle sizer

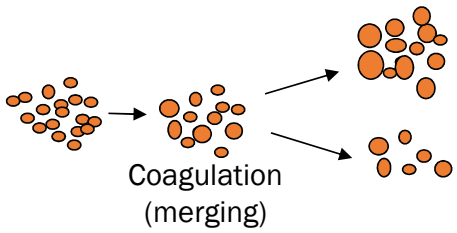


Modeling Transport and Evolution of Aerosols for Computing Deposition in Air–Liquid Interface Experiments

- Computational fluid dynamics efforts concerning physical aerosol characterization facilitate accurate computation of deposition rate
  - Droplet size
  - Droplet number density
- Mixing efficiency of the dilution system
  - Investigation of the required residence time for the aerosol to reach a uniform particle number concentration in the exposure system
- Stability of aerosol in the dilution system
  - Assessment of the physical characteristics of aerosol undergoing dilution and transport in the exposure system
- Influence of operating conditions and physical mechanisms on aerosol deposition
  - Flow speed, temperature, relative humidity
  - Inertia, gravitational settling



Sketch of one-row flow path in a multi-well of the Vitrocell® exposure system



Aerosol Evolution/Aging

"There is substantial evidence that except for nicotine, under typical conditions of use, exposure to potentially toxic substances from e-cigarettes is significantly lower compared with combustible tobacco cigarettes" NAS, 2018

JAMA Network | Open

Original Investigation | Public Health

Comparison of Nicotine and Toxicant Exposure in Users of Electronic Cigarettes and Combustible Cigarettes

Maciej L. Goniewicz, PharmD, PhD; Danielle M. Smith, MPH; Kathryn C. Edwards, PhD; Benjamin C. Blount, PhD; Kathleen L. Caldwell, PhD; Jun Feng, PhD; Lanqing Wang, PhD; Carol Christensen, PhD; Bridget Ambrose, PhD; Nicolette Borek, PhD; Dana van Bemmel, PhD; Karen Konkel, PhD; Gladys Erives, PhD; Cassandra A. Stanton, PhD; Elizabeth Lambert, MSc; Heather L. Kimmel, PhD; Dorothy Hatsukami, PhD; Stephen S. Hecht, PhD; Raymond S. Niaura, PhD; Mark Travers, PhD; Charles Lawrence, PhD; Andrew J. Hyland, PhD

…"[The] Findings suggest exclusive e-cigarette use results in measurable exposure to tobacco-related constituents; however, compared with cigarette smoking, biomarker concentrations of nicotine and toxicants among e-cigarette–only users were much lower"
Goniewicz et al., 2019

Challenges in Toxicity Assessment of Electronic Cigarettes

Lack of Standards for Selection of Chemicals to Be Monitored

- The list of HPHCs—established for cigarettes—is not applicable to ENDS

Lack of Standards for Analytical Methods

- Increase sensitivity and reproducibility
- Allow comparison among studies

Lack of Standards for Testing Potential Toxicity of Inhaled Flavors

- "Generally recognized as safe" as currently used for food ingredients is informative but might not be applicable for inhalation

Lack of Standards for Aerosol Generation

- Puffing regimen and coil temperature impact chemical generation (i.e., carbonyls)
- Vaping topography is heterogeneous
- CORESTA recommendation (recently developed – Method No. 81)
  https://www.coresta.org/sites/default/files/technical_documents/main/CRM_81.pdf

FARSALINOS, K. E. & LE HOUEZEC, J. 2015. Regulation in the face of uncertainty: the evidence on electronic nicotine delivery systems (e-cigarettes). Risk Manag Healthc Policy, 8, 157-67.FLORA, J. W., MERUVA, N., HUANG, C. B., WILKINSON, C. T., BALLENTINE, R., SMITH, D. C., WERLEY, M. S. & MCKINNEY, W. J. 2016. Characterization of potential impurities and degradation products in electronic cigarette formulations and aerosols. Regulatory Toxicology and Pharmacology, 74, 1-11.
DAVIS, B., DANG, M., KIM, J. & TALBOT, P. 2015. Nicotine concentrations in electronic cigarette refill and do-it-yourself fluids. Nicotine Tob Res, 17, 134-41.
TIERNEY, P. A., KARPINSKI, C. D., BROWN, J. E., LUO, W. & PANKOW, J. F. 2015. Flavour chemicals in electronic cigarette fluids. Tob Control.

Various Types of Electronic Cigarettes

There are 8000 flavors now available and around 242 new flavors added every month.

Generic Combustible Tobacco Cigarette	First-Generation Electronic Cigarette	Second-Generation Electronic Cigarette	Third-Generation Electronic Cigarette

Electronic Cigarettes

Shown to demonstrate approximate scale (size).

Taken from the "Public Health Consequences of E-Cigarettes." The National Academies Press. 2018.

The illustrations are intended to be generic representation of a device within each category. They are not meant to represent any specific product.

BALS, R., BOYD, J., ESPOSITO, S., FORONJY, R. & HIEMSTRA, P. S. 2019. Electronic cigarettes: a task force report from the European Respiratory Society. 53.
TIERNEY, P. A., KARPINSKI, C. D., BROWN, J. E., LUO, W. & PANKOW, J. F. 2016. Flavour chemicals in electronic cigarette fluids. *Tobacco Control,* 25**,** e10-e15.
NAS 2018. Public health consequences of e-cigarettes. Washington, DC: The National Academies Press. doi: https://doi.org/10.17226/24952.

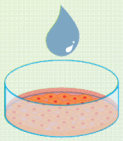
A Novel Electronic Cigarette with MESH™ Technology

- The IQOS MESH uses closed-system e-liquid caps to prevent tampering and liquid leakage
- A new heating element and mouth piece are built into each replaceable cap to maintain product hygiene
- The current-generating wick is eliminated, and the coil is replaced with a metal mesh
- The temperature of the heater is controlled and maintained between 200–220°C rather than varying depending on the puff strength
- A low-liquid detection system will cut off the power supplied to the mesh heater once the level of the liquid has dropped below a certain level, eliminating dry puffs

Cap containing e-liquids

In Vitro Multilayer Assessment with a Systems Toxicology Approach by using 2D and 3D Airway Epithelial Cultures

First Layer Assessment



24 h

- Incubation with *MESH* Classic Tobacco liquid (containing PG, G, nicotine and flavors)
- Incubation with Base liquid (containing PG, G, and nicotine without flavors)
- Incubation with the total particulate matter (TPM) of 3R4F reference cigarette smoke

2D NHBE (Normal human bronchial epithelial) cells

ENDPOINTS

Cytotoxicity measurement using real-time cell analysis (RTCA)

Second Layer Assessment

4 h

24 h

- Incubation with *MESH* Classic Tobacco liquid (containing PG, G, nicotine and flavors)
- Incubation with Base liquid (containing PG, G, and nicotine without flavors)
- Incubation with the TPM of 3R4F reference cigarette smoke

2D NHBE

(NHBE: Normal human bronchial epithelial) cells

ENDPOINTS

High content screening assays:
- Cell membrane permeability
- Cytochrome c release
- DNA damage (pH2AX)
- Glutathione content
- Oxidative stress (ROS)
- Stress kinase (c-Jun)

Third Layer Assessment

3D Human Organotypic Epithelial Cultures (Buccal and Small Airway)

Various Doses*

- Exposure to *MESH* Classic Tobacco aerosol
- Exposure to Base aerosol
- Exposure to cigarette smoke
- Exposure to air

ENDPOINTS
4 h Post-exposure
- mRNA microarray
- miRNA microarray

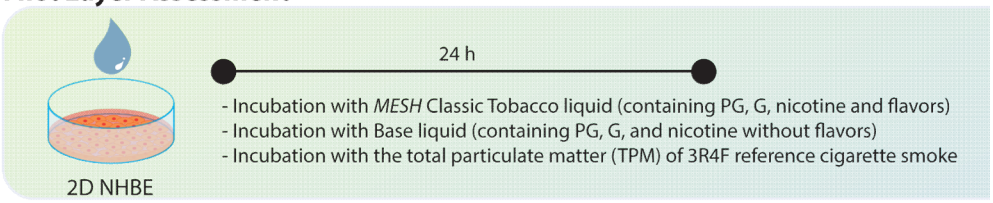
ENDPOINTS
24 h Post-exposure
- mRNA microarray
- miRNA microarray

ENDPOINTS
48 h Post-exposure
- Adenylate kinase measurement
- Histology analysis
- mRNA microarray analysis
- miRNA microarray analysis
- Targeted proteomics analysis (PRM)†
- Multi-analyte profiling (MAP) of secreted inflammatory mediators

ISKANDAR, A. R., ZANETTI, F., MARESCOTTI, D., TITZ, B., SEWER, A., KONDYLIS, A., LEROY, P., BELCASTRO, V., TORRES, L. O., ACALI, S., MAJEED, S., STEINER, S., TRIVEDI, K., GUEDJ, E., MERG, C., SCHNEIDER, T., FRENTZEL, S., MARTIN, F., IVANOV, N. V., PEITSCH, M. C. & HOENG, J. 2019. Application of a multi-layer systems toxicology framework for in vitro assessment of the biological effects of Classic Tobacco e-liquid and its corresponding aerosol using an e-cigarette device with MESH™ technology. Archives of Toxicology.

Cell Viability Was Impacted to a Greater Degree in 2D NHBE Cells Treated with 3R4F Total Particulate Matter (TPM) than in Cells Treated with MESH Classic Tobacco or Base E-Liquids

First Layer Assessment

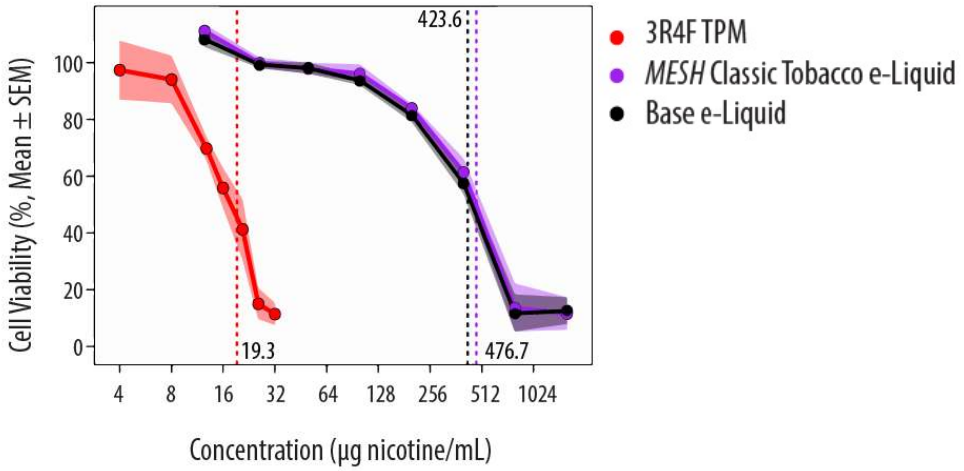


	PG (%)	VG (%)	Nic (%)	Flavors	Other (e.g., Water)
MESH Classic Tobacco Liquid*	39	39	1.8	✓	✓
Base Liquid	39	39	1.8		✓

*The pH is around 8.5

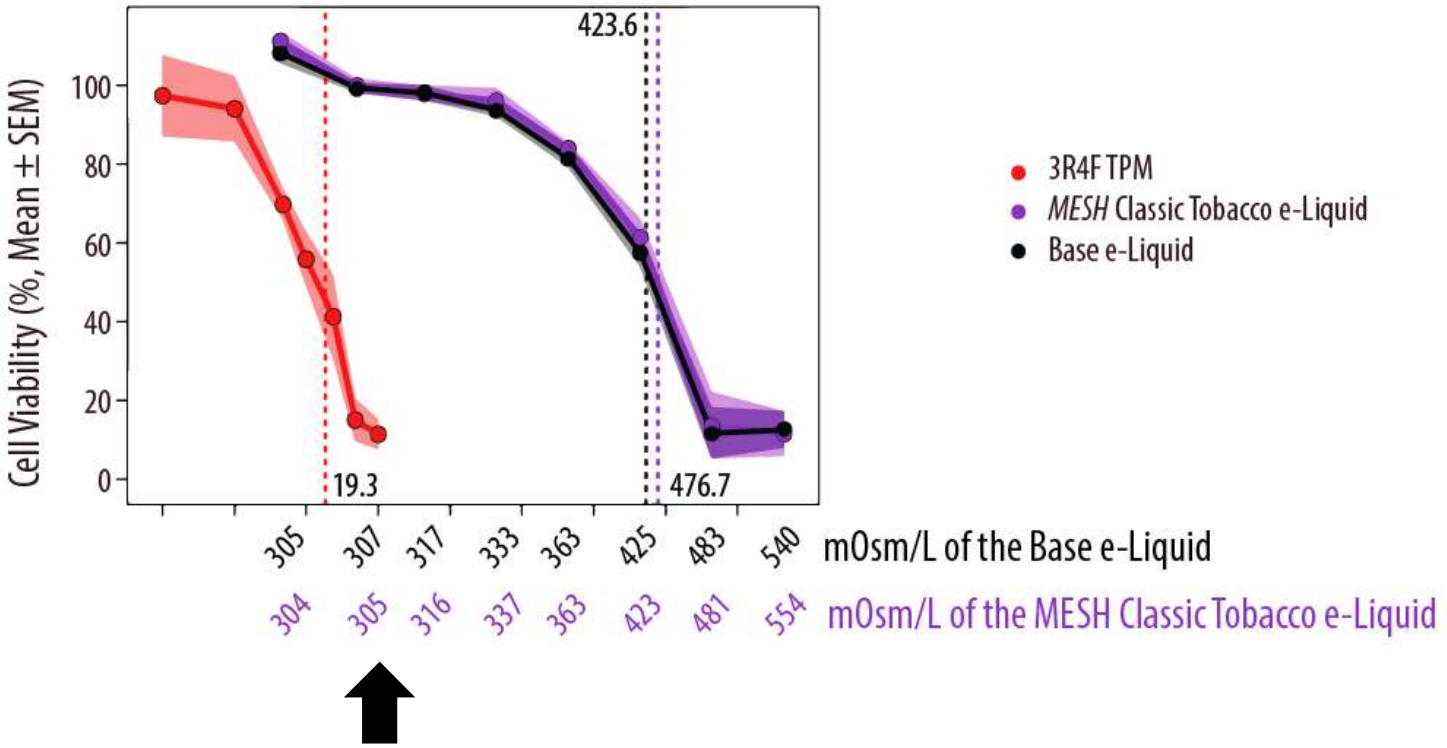
Cigarette smoke TPM extract impacted cell viability to a much greater degree than MESH Classic Tobacco and Base Liquids when compared relative to their nicotine concentrations.

Cytotoxicity Measurement Using RTCA



ISKANDAR, A. R., ZANETTI, F., MARESCOTTI, D., TITZ, B., SEWER, A., KONDYLIS, A., LEROY, P., BELCASTRO, V., TORRES, L. O., ACALI, S., MAJEED, S., STEINER, S., TRIVEDI, K., GUEDJ, E., MERG, C., SCHNEIDER, T., FRENTZEL, S., MARTIN, F., IVANOV, N. V., PEITSCH, M. C. & HOENG, J. 2019. Application of a multi-layer systems toxicology framework for in vitro assessment of the biological effects of Classic Tobacco e-liquid and its corresponding aerosol using an e-cigarette device with MESH™ technology. Archives of Toxicology.

Cytotoxicity of MESH Classic Tobacco and Base Liquids Was Generally Attributed to the Increasing Osmolarity of the Liquids



Isotonic fluids generally have an osmolarity of 270–310 mosm/L (Liu et al).

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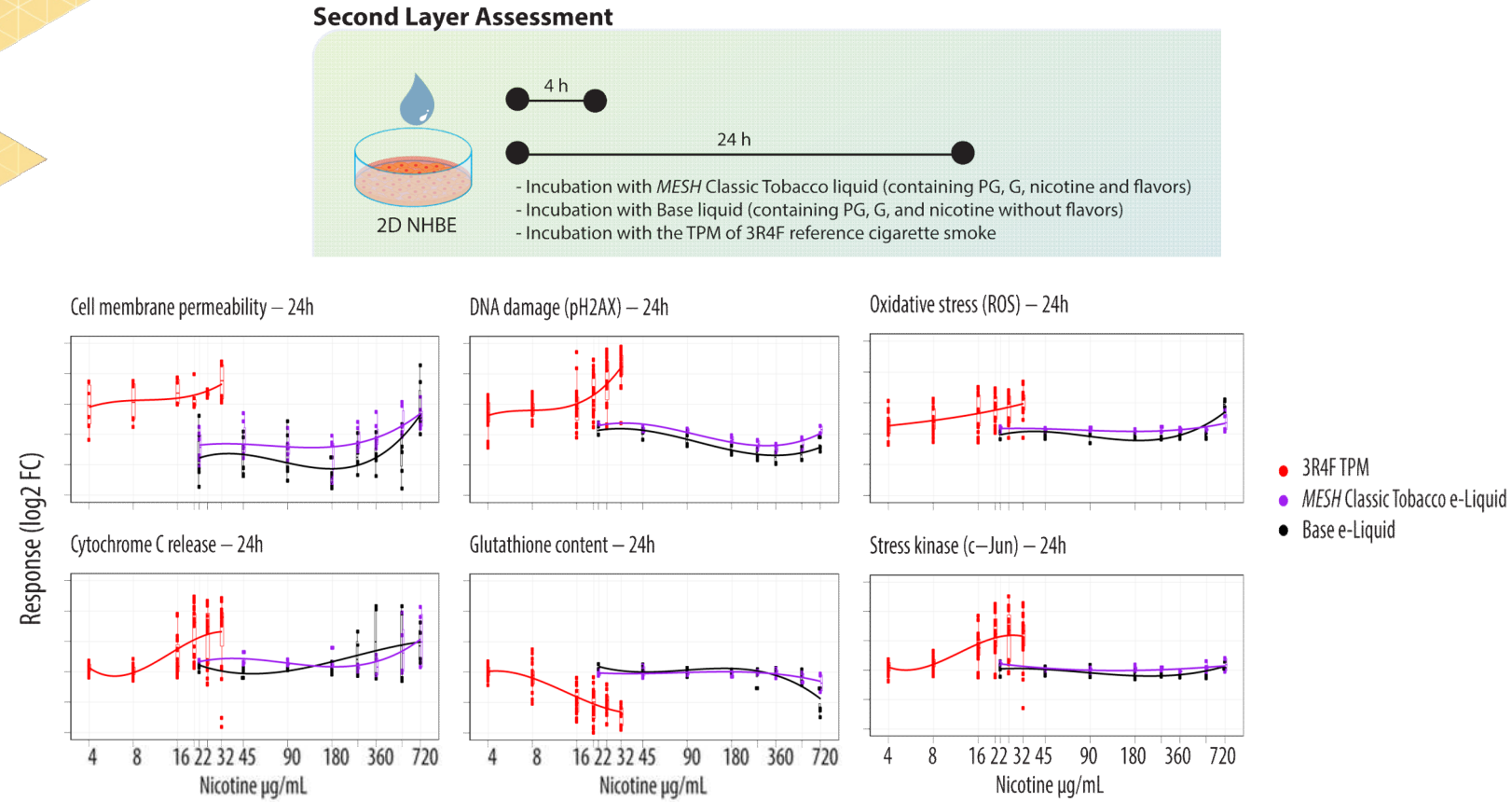
LIU, D. T. & SILVERSTEIN, D. C. 2015. Chapter 58 - Crystalloids, Colloids, And Hemoglobin-Based Oxygen-Carrying Solutions. In: SILVERSTEIN, D. C. & HOPPER, K. (eds.) Small Animal Critical Care Medicine (Second Edition). St. Louis: W.B. Saunders.

Changes in Cellular Markers Were Not Detected in 2D NHBE Cultures Treated with MESH Classic Tobacco and Base E-Liquids Unlike in Cultures Treated with 3R4F Total Particulate Matter (TPM)



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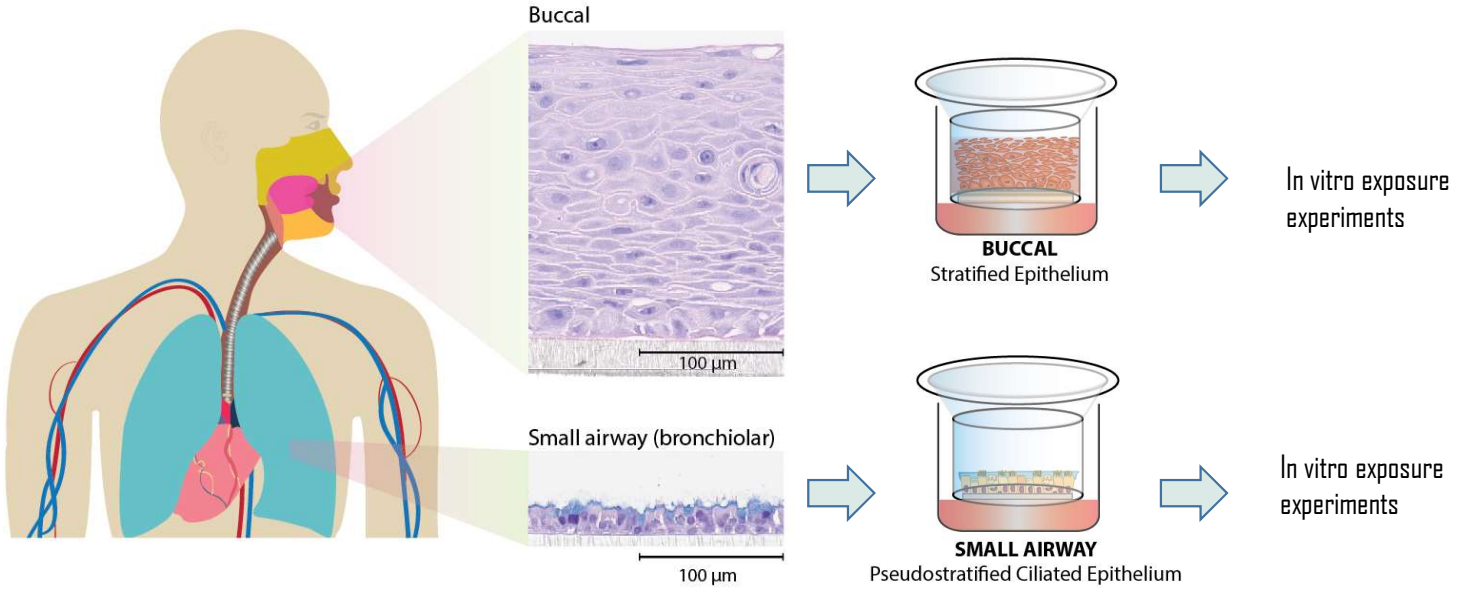
Third Layer Assessment



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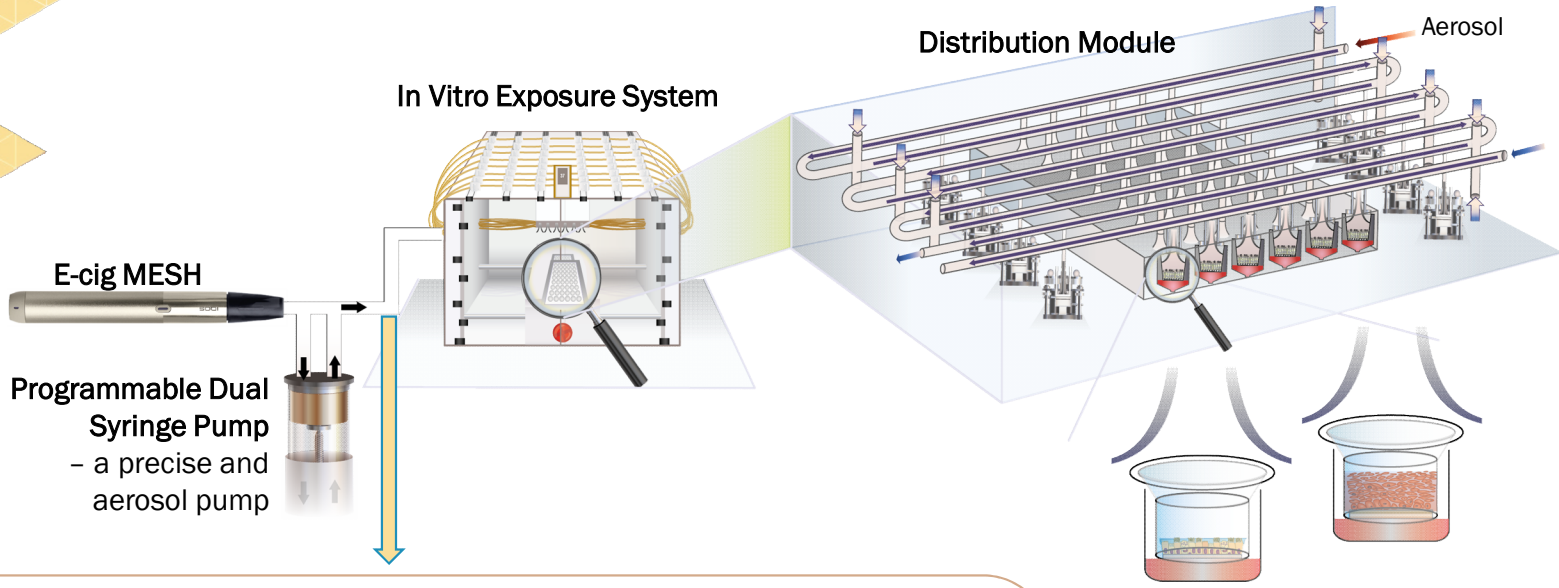
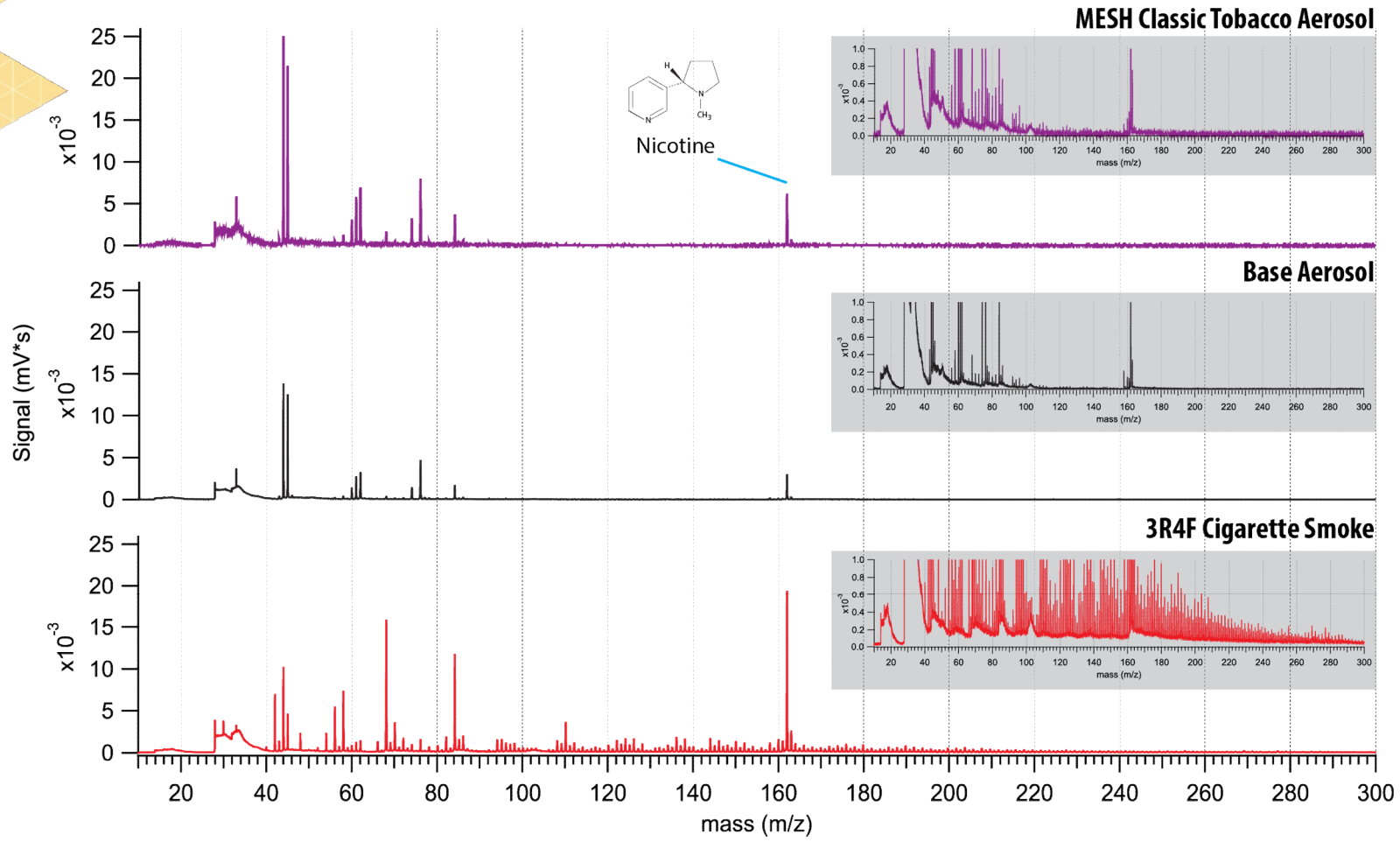
Experimental Setup: In Vitro E-Cigarette Aerosol Exposure



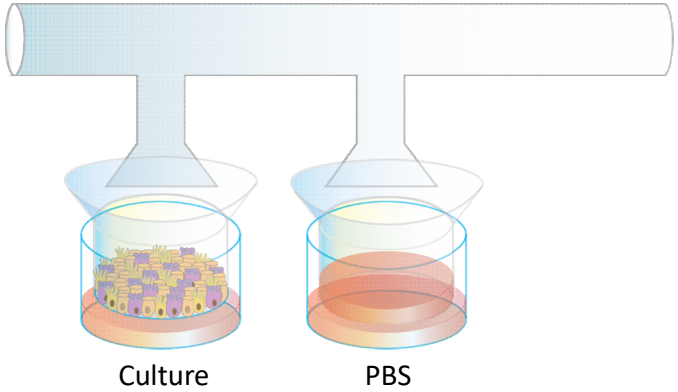
An on-line real-time measuring system for chemical gas analysis based on time-of-flight mass spectrometry

Untargeted Aerosol Monitoring By Single-Photon Ionization Mass Spectrometry: 3R4F Cigarette Generates a More Complex Aerosol than MESH Classic Tobacco and Base E-Liquids

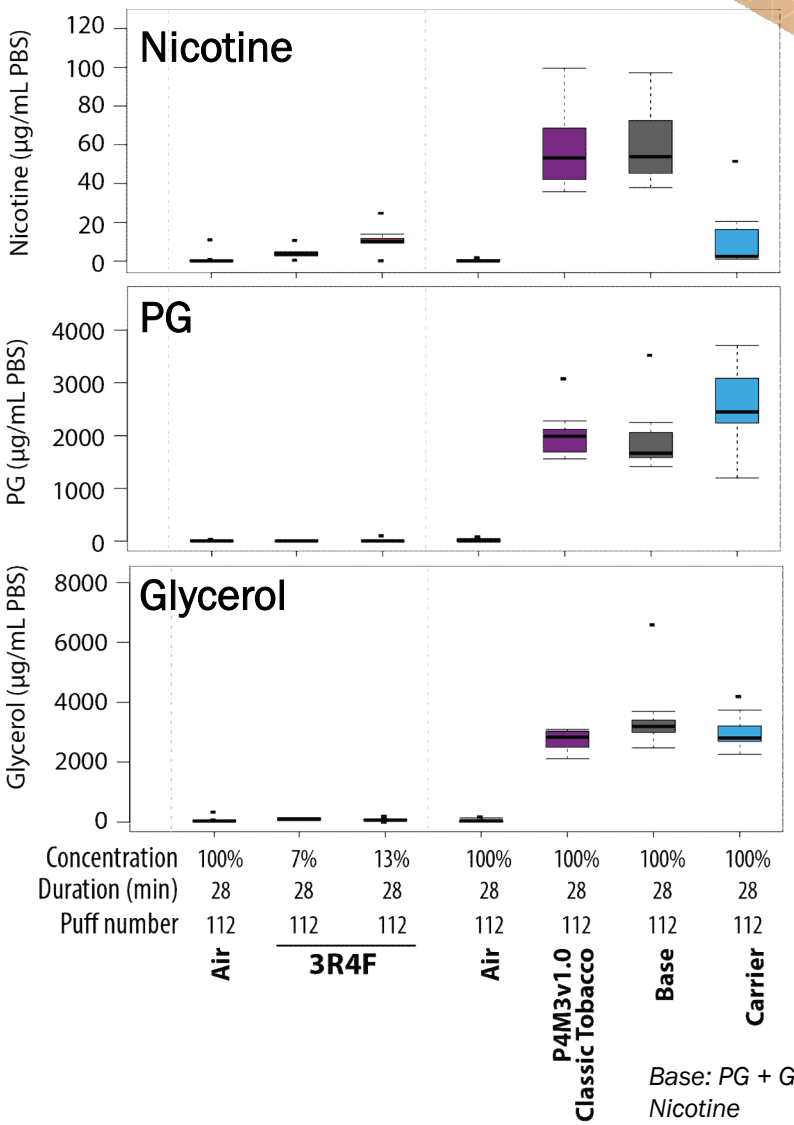


Monitoring of Compound Deposition in the Exposure Chamber During an Exposure Experiment: The Use of a Surrogate Test System (PBS)

In each exposure experiment, culture inserts were filled with phosphate-buffered saline (PBS) as a surrogate epithelial culture model.



Concentrations of nicotine in PBS were determined by liquid chromatography—tandem mass spectrometry (LC-MS/MS). Concentrations of propylene glycol and glycerol in PBS were determined by gas chromatography—mass spectrometry (GC-MS).



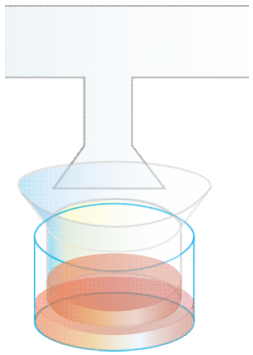
Base: PG + Glycerol + Nicotine
Carrier: PG + Glycerol only

Deposition Efficiency Can Be Estimated Based on the Concentrations of Compounds Deposited in the Exposure Chamber Relative to Those Present in the E-Liquids

	PG	Glycerol	Nicotine
MESH Classic Tobacco Liquid Composition (%, v/v)	**39**	**39**	**1.8**
Puff Number	112	112	112
Volume of Liquid Used per Puff (µL)	6.7	6.7	6.7
Total Volume of Compound Used for 112 Puffs (µL)	293	293	14
Total Mass of Compound Generated for 112 Puffs (µg)*	**304361**	**368747**	**13642**

**Calculated based on the density of PG, VG, and nicotine*

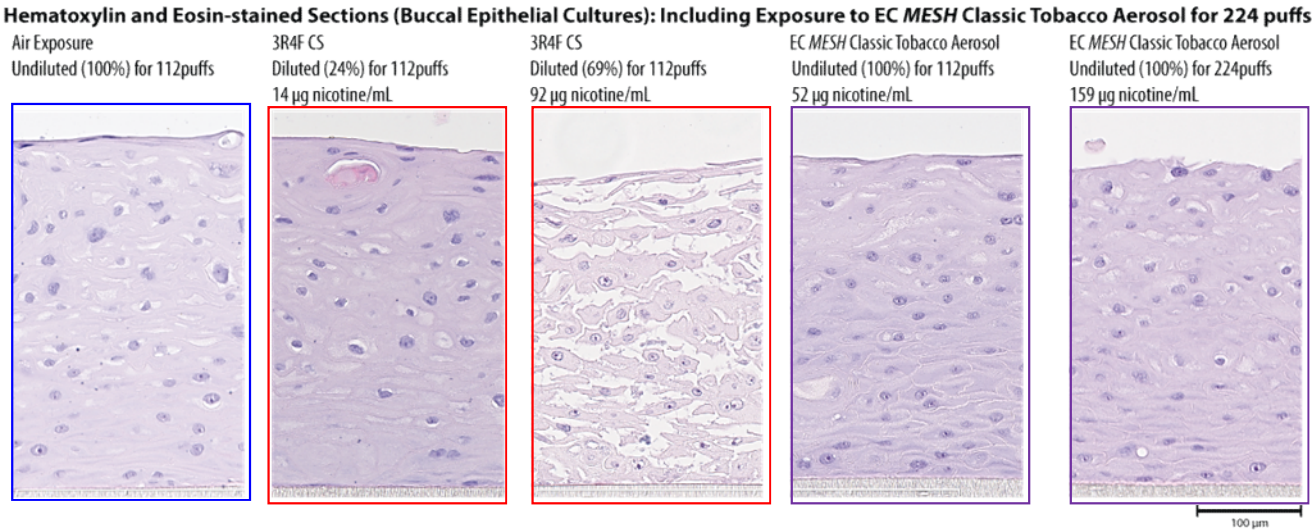
	PG	Glycerol	Nicotine
Deposition of Compound in the Exposure Chamber (µg/mL PBS)	1990	2840	53
Total Mass of Compound Deposited in 0.1 mL PBS (1 insert) (µg)	**199**	**284**	**5.3**
Deposition Efficiency**	**0.07%**	**0.08%**	**0.04%**



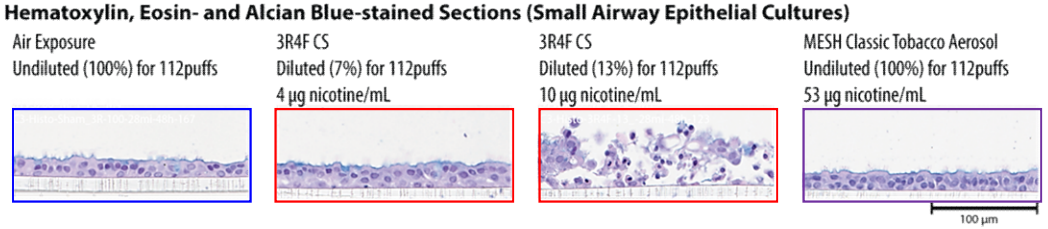
** $\frac{\text{Total Mass of Compound Deposited in 0.1 mL PBS (1 insert)}}{\text{Total Mass of Compound Generated for the 112 Puffs}}$ X 100%

3D Organotypic Cultures Exposed to e-cig MESH Classic Tobacco Aerosol Showed No Tissue Damage

Buccal



Small Airway



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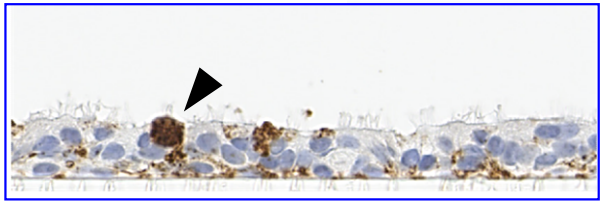
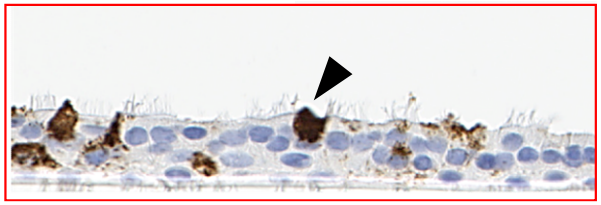
Muc5AC Expression Was Not Altered in Cultures Exposed to MESH Classic Tobacco Aerosol

Air Exposure
Undiluted (100%) for 112 puffs

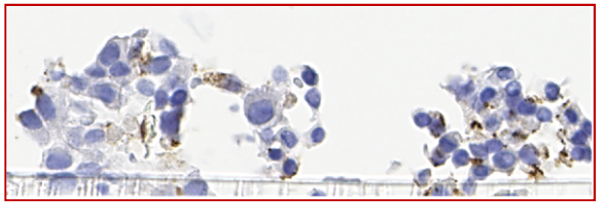


Muc5AC mucin, a major mucin secreted by tracheobronchial goblet cells, is found in the airway secretions of healthy individuals.

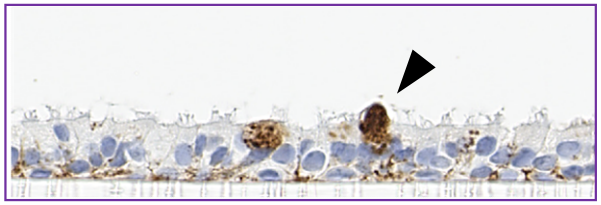
3R4F CS
Diluted (7%) for 112 puffs
4 µg nicotine/mL



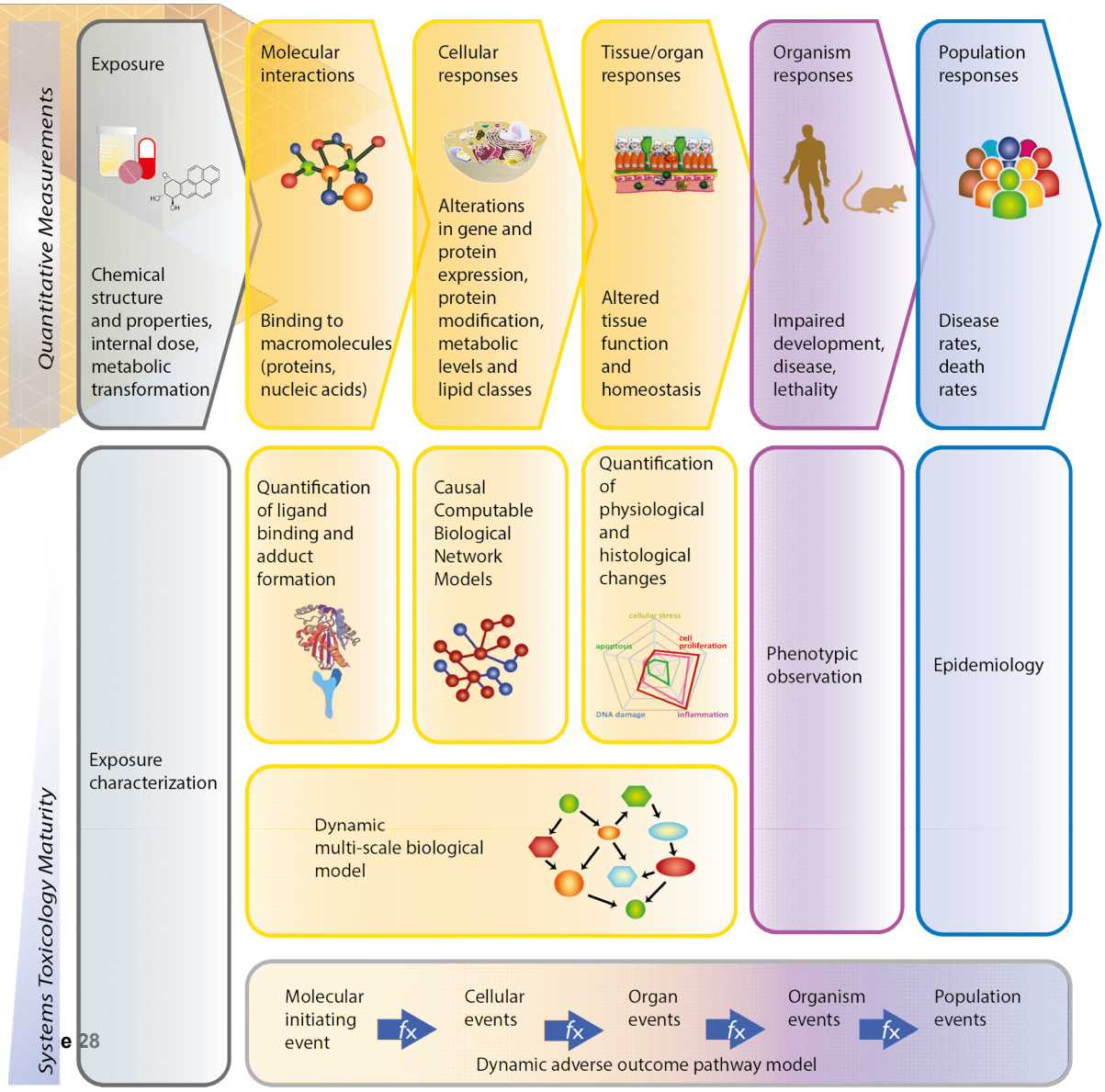
3R4F CS
Diluted (13%) for 112 puffs
10 µg nicotine/mL



MESH Classic Tobacco Aerosol
Undiluted (100%) for 112 puffs
53 µg nicotine/mL



Constant renewal of mucus by constitutive secretion from goblet cells facilitates the clearance function. Increased levels of Muc5AC have been reported in the airways of patients with asthma.



Conclusion

- **Electronic nicotine delivery systems evolve rapidly.**
- **Testing the potential toxic effects of exposure to tobacco product aerosols requires the use of relevant test systems and exposure modes as well as appropriate (physiologically relevant) doses.**
- **Systems biology approaches (omics) will uncover changes at cellular and molecular levels otherwise undetected in standard toxicity assays.**
- **Collaborative efforts between the scientific community, industry, and regulatory stakeholders are facilitating the adoption of 21st Century Toxicology approaches.**
- **The multilayer systems toxicology framework could be useful for assessing in vitro the potentially reduced impact of e-cigarettes relative to 3R4F cigarettes in a comprehensive manner and is a very insightful strategy for acquiring preliminary data that would be relevant for supporting potential clinical outcomes.**

Thank you for your attention

Acknowledgements

The Singapore Team