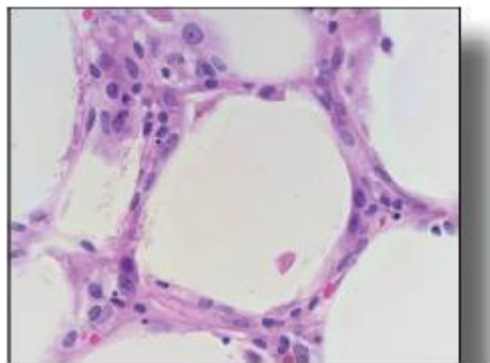




In Vitro Exposure Systems and
Dosimetry Assessment Tools for Inhaled
Tobacco Products



April 4-6, 2016
Bethesda, MD



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ABBREVIATIONS**REFERENCES**

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SUMMARY OF KEY THEMES

- Creating accurate and dependable means to quantify pulmonary exposures to inhaled materials, including tobacco-related mixtures and constituents, is challenging. Developing and validating new test methods for use in a regulatory safety testing arena requires the input and guidance from multiple stakeholders.
- E-aerosols are physically similar to cigarette smoke in some aspects (e.g., size range), but chemically very different. Systems designed for cigarette evaluation may not be compatible with the *in vitro* testing of e-aerosols. Smoking machine requirements for conventional and electronic cigarettes are different, but share the same basic technologies.
- Tobacco products, such as cigarette smoke and e-cigarette aerosols, are capable of generating reactive oxidants.
- There are a variety of strategies for exposing cells at the air-liquid interface, starting from acute toxicity studies (dose-response relationships) up to repeated exposure studies at non-toxic doses. The choice will depend on the protocol, and a clear definition of what is to be analyzed and demonstrated.
- Mathematical dosimetry modeling, simulations using CFD-based models, and microfluidic airway models based on primary human cells offer realistic approaches for studying the fate of inhaled chemicals and the links between exposure characteristics and biological responses.
- The emerging field of epigenetic toxicology will ultimately play a critical role in our understanding of exposure-associated health effects and susceptibility.
- Accurate dosimetry data can support the results from *in vitro* toxicity of cigarette smoke.
- The next generation products category will continue to grow, evolve and diversify and dosimetry will support exposure.

INTRODUCTORY PRESENTATIONS

Welcome and Overview

Holger Behrsing, IIVS

Erin Hill, IIVS

Raymond Tice, NIEHS(retired)

This program, organized by the Institute for In Vitro Sciences (IIVS), explored *in vitro* exposure systems and dosimetry assessment tools for inhaled tobacco products. Its purpose was to highlight the current status of *in vitro* to *in vivo* correlations, whole tobacco smoke and E-cigarette aerosol/vapor constituents, *in vitro* exposure systems, dosimetry approaches, the exposure microenvironment, and promising technologies that may advance science in these areas.

To date, much of the research and testing in respiratory and inhalation toxicology have focused on the use of animal models. With the development of new technologies, such as reconstructed human airway tissues, researchers are turning their attention to *in vitro* assessments.

In this workshop, invited experts from industry, government, academia and non-profits presented talks and posters covering key areas in exposure and dosimetry for non-animal testing. Its intent was to facilitate an exchange of information for a better understanding of exposure systems, and to discuss the methodology that best captures what is delivered to the *in vitro* systems used to assess human relevant, biological responses.

This 2.5-day workshop was the third in a series of respiratory toxicology workshops organized by the IIVS. The first was held in December 2014 followed by a second in June 2015; the proceedings and conclusions of this workshop were published in 2016.¹

Advancing Regulatory Science at the US FDA with More Predictive Models

Suzanne Fitzpatrick, US Food and Drug Administration

In this presentation, Suzanne Fitzpatrick reviewed the efforts of the U.S. Food and Drug Administration (FDA) to advance regulatory science and toxicology.

Toxicology is an area of science important to the FDA's ability to predict product safety or assess the potential significance of chemicals in products or environment. Advances in toxicology testing—such as systems biology, stem cells, engineered tissues, computerized modeling—create unique opportunities to transform this predictive science to bring needed products to people faster and more safely and to replace, reduce and/or refine animal testing.

The FDA is working toward transitioning new 21st century technologies to enhance the efficiency and effectiveness of chemical risk management. Currently, the FDA relies heavily on animal studies, and generates information for all possible outcomes, based on traditional toxicity tests. Future goals include less reliance on animal studies and more tailored data generation, based on an understanding of toxicity pathways.

¹ Behrsing H, Raabe H, Manuppello J, et al. Assessment of *in vitro* COPD models for tobacco regulatory science: Workshop proceedings, conclusions and paths forward for *in vitro* model use. *Altern Lab Anim* 2016;44:129-66.

In 2008, a Memorandum of Understanding (MOU) was issued on “High-Throughput Screening, Toxicity Pathway Profiling, and Biological Interpretation of Findings,” and has brought together four federal agencies: the Environmental Protection Agency (EPA), the National Center for Advancing Translational Sciences (NCATS), the National Institute of Environmental Health Sciences (NIEHS), and the FDA. Known informally as Tox21 (Toxicology in the 21st Century), the MOU was renewed last summer and now covers all types of *in vitro* testing, including “organs on a chip.” Its goals are to identify patterns of compound-induced biological response in order to characterize toxicity/disease pathways, facilitate cross-species extrapolation, and model low-dose extrapolation. Tox21 also aims to prioritize compounds for more extensive toxicological evaluation and develop predictive models for biological response in humans.

In the area of regulatory safety assessment, the FDA recognizes the need for new approaches that are more predictable, more reliable, faster and less expensive. In 2010, the FDA and the National Institutes of Health (NIH) collaborated to launch the Advancing Regulatory Science Initiative, an effort designed to accelerate the process from scientific breakthrough to the availability of new, innovative medical therapies for patients. Specifically, the FDA is looking for better models of assessing human adverse response. Although “adverse” means different things to each FDA center, and each has a different way of approaching problems, all are interested in a better understanding of toxicity mechanisms at multiple levels of biological organization—including genes, proteins, pathways, and cell/organ function. The FDA is also exploring methods to characterize molecular targets and host genetic factors that may be associated with rare and unexpected adverse events.

One of the main challenges to progress in this area is the regulators’ reluctance to rely on new technology methods for product approvals. There is a long history of the generally successful use of traditional animal testing methods and a lack of confidence in non-animal methods. To address this roadblock, the FDA has been sponsoring workshops to help regulators learn about new technologies.

Other important FDA collaborations include a partnership with the NIH and the Defense Advanced Research Projects Agency (DARPA), to jointly develop new tools that can be used in therapeutic development. The FDA-DARPA-NIH Microphysiological Systems Program was started in 2011 to support the development of human microsystems, or organ “chips,” to screen for safe and effective drugs swiftly and efficiently before human testing. The “human on a chip” will be developed with at least 10 organs, all linked together and viable for 4 weeks.

The FDA is also awaiting the results of the National Research Council’s Second Meeting of the Committee on Incorporating 21st Century Science Into Risk-Based Evaluations. This report will combine the recommendations of two reports, Toxicity Testing in the 21st Century and Exposure Testing in the 21st Century, and will focus on incorporating 21st century science-based risk strategies into risk assessment.

Another major challenge with the paradigm shift to new methodologies is to establish scientific validation—the process concerned with assessing assumptions, relevance, reliability, reproducibility, and sensitivity of tests for particular purposes, and understanding uncertainties. Because “validation” means different things to different centers, a “one size fits all” approach is not an option.

Key points:

- **The FDA is working toward transitioning new 21st century technologies, to enhance the efficiency & effectiveness of chemical risk management.**
 - **Currently, the FDA relies heavily on animal studies, and generates information for all possible outcomes, based on traditional toxicity tests. Future goals include less reliance on animal studies, tailored data generation, based on understanding of toxicity pathways.**
 - **Investments in toxicology and regulatory science can enable FDA to better protect and promote the health of people in the United States and throughout the world.**
 - **Collaboration is essential to define needed pathways and catalyze change.**
-

In Vitro to In Vivo Extrapolation in Humans

Robert Devlin, US Environmental Protection Agency

In this presentation, Robert Devlin discussed the need for using *in vitro* methods for toxicity testing, the actions of the Environmental Protection Agency (EPA) and other agencies to address this need, and the challenges of assessing how well *in vitro* assays might predict human *in vivo* responses.

Recognizing that traditional animal toxicity studies are only feasible for a small percentage of the thousands of chemicals that must be assessed for risk, the EPA asked the National Research Council (NRC) to develop a long range vision and a strategy to advance toxicity testing. The NRC's 2007 report concluded that the only way to screen the increasing number of environmental chemicals regulated is by a conversion to *in vitro* techniques. The NRC recommended the expanded use of *in vitro* toxicity pathway data based on mode of action or adverse outcome pathway information.

The EPA established the National Center for Computational Toxicology (NCCT) to develop new software and methods for predictive toxicology. Among the research efforts managed by the NCCT is ToxCast™, a multi-year effort that uses high throughput assays to screen cells for changes in biological activity that may suggest potential toxic effects. Toxicology in the 21st Century (Tox21), a collaboration of several federal agencies, is currently screening about 10,000 chemicals using a high-throughput robotic system that can assay about 150,000 wells in one day. However, inhaled toxicants are largely missing from the Tox21 assays due to the robots' inability to deal without compounds in a vapor or gaseous form.

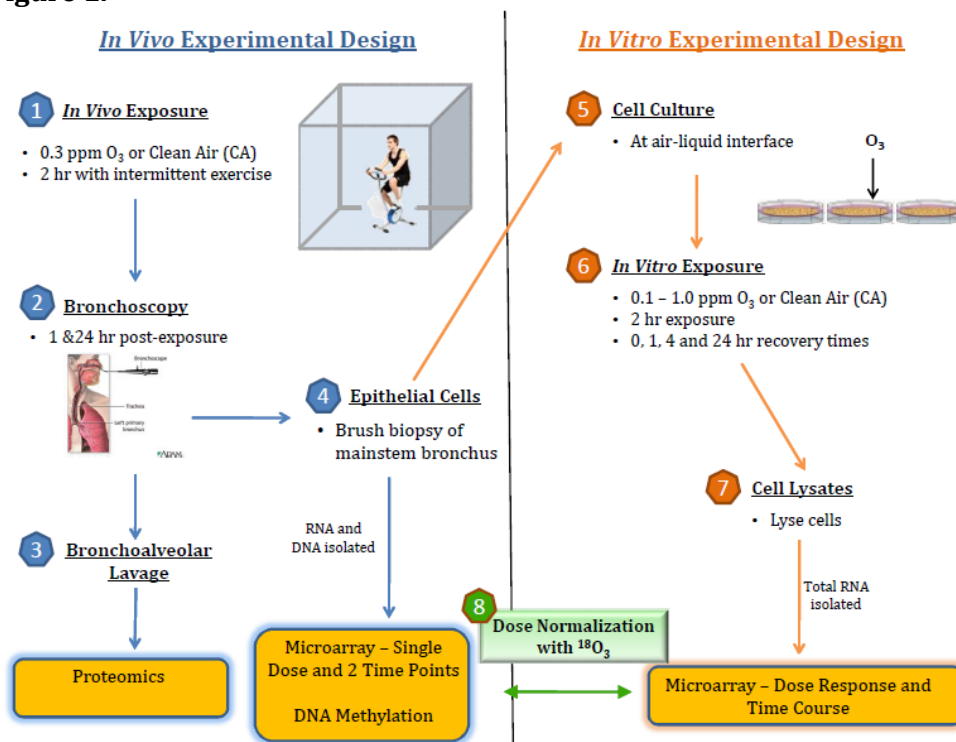
Before *in vitro* toxicity pathway information can be useful in risk assessment, many challenges must be overcome. A quantitative relationship must be established between perturbation of a pathway following *in vitro* exposure and the downstream endpoints (i.e. pathophysiological changes at the tissue or organism level following *in vivo* exposure of animals or humans).

For *in vitro* testing of inhaled toxicants, models using human primary lung cells are considered ideal for several reasons. These cells are the first targets of inhaled toxicants and animal-to-human extrapolation is not needed. Primary cells respond more "realistically" than immortalized or transformed cells, for example, in the induction of an inflammatory response. Primary cells also offer the possibility of examining genetic/epigenetic, disease, age and other factors as well as intra-individual variability. In the near future, human airway epithelial and alveolar cells, derived from human pluripotent stem cells (iPSC), are expected to become available for research. These cells retain all the advantages of primary cells, but have unlimited self-renewal.

In an effort to determine how well *in vitro* exposure models predict *in vivo* responses, the EPA launched the Next Generation (NexGen) of Risk Assessment effort, a multiyear collaboration among several organizations. NextGen selected ozone (O₃) as one of the prototype compounds for validation studies because of the extensive human toxicity data available. More than 100 studies have shown that exposure to O₃ results in decrements in lung function, increases in markers of pulmonary inflammation, and alterations in host defense against inhaled pathogens.

Researchers in the NextGen ozone project identified and compared ozone-induced toxicity pathways following both *in vivo* and human *in vitro* exposures to validate how well *in vitro* toxicity pathway information can predict human *in vivo* responses.² Ozone labelled with the heavy oxygen isotope (¹⁸O) isotope was used for both exposures to ensure that the dose of ozone attacking the cells *in vitro* is the exact same as the dose attacking them *in vivo*. The experimental design is summarized in **Figure 1**. Volunteers were exposed to 0.3 ppm ozone or clean air on two separate occasions. At 1 and 24 hours following exposure to ozone, epithelial cells were recovered by brush scraping during bronchoscopy. RNA and DNA were isolated and toxicity pathways identified by microarray. Cells obtained following exposure to clean air were cultured and exposed to varying doses of ozone *in vitro* using air-liquid interface exposure. RNA was collected at various times after exposure and toxicity pathways identified by microarray.

Figure 1.



Summary of experimental design

The results showed that *in vivo* more genes were differentially expressed at 1 hour compared with 24 hours post-exposure. There was very little overlap in genes induced by ozone at 1 hour and at 24 hours after exposure. The microarray data for the *in vitro* results showed that as the dose increased, the number of genes regulated increased. Inflammation was activated at all three concentrations. A stress response and apoptosis were observed at the higher concentrations but not the lower ones. So, as the dose increased, the cell responded differently. When comparing the 11 networks activated *in vitro* with the 14 networks activated *in vivo* at one hour, a number of networks overlapped, including inflammatory response, immunological diseases and conditions, cellular movement, cellular growth and proliferation, cellular function and maintenance, immune cell trafficking, infectious disease, and cell-to-cell signaling and interaction (unpublished data).

In conclusion, the preliminary data from the ozone project showed that the inflammation following *in vitro* exposure to ozone might be predictive of inflammation seen following human exposure to ozone. From a qualitative point of view, the *in vitro* responses to ozone appeared to represent pretty well what happens downstream *in vivo*. The qualitative data generated from this study will feed into quantitative physiologically based pharmacokinetic (PBPK) models to demonstrate the predictive value of *in vitro* toxicity testing. Expansion of this approach beyond ozone to other toxicants is needed.

Key points:

- **The challenges of evaluating thousands of chemicals while considering the cumulative effects of mixtures and limiting the use of animal testing has led to new approaches to toxicity testing.**
 - **Conversion to *in vitro* techniques is the only way to screen the ever increasing number of environmental chemicals that must be regulated.**
 - **Technologies that can transform existing approaches include high throughput techniques, systems biology approaches, and bioinformatics.**
 - **Preliminary data from the NextGen ozone study showed that the inflammation seen following *in vitro* exposure to ozone might be predictive of inflammation seen following exposure of humans to ozone.**
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***In Vitro* Models for Tobacco Regulatory Science: Collaborative Efforts in Respiratory Toxicology**

Hans Raabe. IIVS

In this presentation, Hans Raabe summarized the outcomes of two recent collaborative efforts that explored the use of *in vitro* assays for making regulatory decisions for tobacco products.

Recognizing that collaborative engagement would be key to the successful development and validation of these test methods, the Institute for In Vitro Sciences (IIVS) seeks to bring together experts from industry, regulatory and academia and other stakeholders to identify, optimize and validate *in vitro* test methods for eventual tobacco product regulatory submissions. The first two workshops were held in December 2014 (Bethesda, MD) and June 2015 (Gaithersburg, MD).

The first “informational” workshop, was designed to marshal the current expertise to present upon the etiology of chemical-induced chronic obstructive pulmonary disease (COPD), and the putative *in vitro* cell-based methods associated with key biological events leading to COPD. This workshop was conceptualized as an approach to evaluate two of the components of the FDA’s Center for Tobacco Products (CTP) priorities set in 2012:

- What *in vitro* and *in vivo* assays are capable of comparative toxicity between two different tobacco products; with special attention to cardiotoxicity, respiratory toxicity, carcinogenicity, and developmental/reproductive toxicity?
- What constituents, compounds, design features, and tobacco use behaviors impact toxicity and carcinogenicity of tobacco products and smoke?

Workshop participants were asked to examine the current status of *in vitro/ex vivo* models and the ability of the models to predict toxicological outcomes relevant to COPD, and to propose research strategies. Key biological events that were identified within the adverse outcome pathway (AOP) of COPD included: inflammation and oxidative stress, ciliary dysfunction and ion transport, goblet cell hyperplasia and mucus production, and parenchymal/bronchial tissue destruction and remodeling.

In breakout sessions, participants regarded deficient mucociliary clearance as one of the key tissue-level events relevant to the clinical manifestation of tobacco-induced COPD. Similarly, changes in goblet cell morphology and mucus production were also considered to be key tissue-level events in the pathogenesis of COPD, and all were considered to be notably downstream in the AOP to more likely be predictive of COPD. Based upon these revelations, the breakout group recommended the

subsequent optimization and standardization of these prototypic tools in order to validate them for regulatory toxicology. Participants also acknowledged that in order to adequately evaluate the reproducibility of the biological responses of the *in vitro* test systems to toxicants, reference chemicals should be selected and applied directly onto the tissue systems using standard dose application methods, rather than using non-standardized whole smoke/vapor exposure methods.

The subsequent technical workshop brought together the technical experts versed in the key areas identified in the first informational workshop's breakout sessions, including ciliary beating frequency, changes in goblet cell hyperplasia, and mucus production. Workshop participants discussed current methodologies and whether basic protocols or test methods could be designed in a standardized approach that could be optimized and transferred to multiple laboratories. Activities focused on developing the test method protocols to evaluate the proof of principle concept. Work is currently underway at multiple laboratories to conduct the proof of principle protocols. The next steps will be to share and review the data, to evaluate the proof of principle hypotheses, and subsequently optimize the protocols.

In summary, the first workshops identified various areas for cooperative development and optimization of *in vitro* test methods, tissue models and endpoints relative to *in vivo* observations. The current informational workshop was designed to present on the state of the art of *in vitro* respiratory tissue exposure and dosimetry technologies and techniques, to identify applications, comparisons of *in vivo* and *in vitro* dosimetry measurements, limitations and knowledge gaps, and propose activities to characterize and standardize these methodologies. Future activities will be targeted at reuniting standardized whole smoke/vapor exposure and dosimetry systems with optimized *in vitro* tissue models and endpoints for regulatory tobacco toxicology.

Key points:

- **Developing and validating new test methods for use in a regulatory safety testing arena requires the input and guidance from multiple stakeholders.**
 - **Identifying the regulatory requirements to address with testing may best be achieved through collaborative goal-oriented discussions between industry and regulatory community representatives, while the relevant scientific methods may be proposed and discussed by industrial and academic experts versed in the technologies.**
-

INTRODUCTION TO EXPOSURE AND DOSIMETRY

Species Differences in Respiratory Anatomy

Kent Pinkerton, Center for Health and the Environment, University of California, Davis

In this presentation, Kent Pinkerton described the differences, as well as the similarities, that occur in the respiratory anatomy of mammalian species.

Although the size of the respiratory system varies widely across the mammalian species, the airway tree of most mammals share fairly similar branching patterns. All species show both symmetric branching—with uniform divisions going from a parent airway to two daughter airways—as well as asymmetric branching—characterized by major and minor daughter airways.^{3,4}

Typically, every mammalian species has most of the more than 40 different cell types found in the respiratory system, with many of them found within the airway epithelium. However, the populations, cell density, composition, distribution, metabolic potential, and the number and extent of epithelial derivatives vary according to the species.

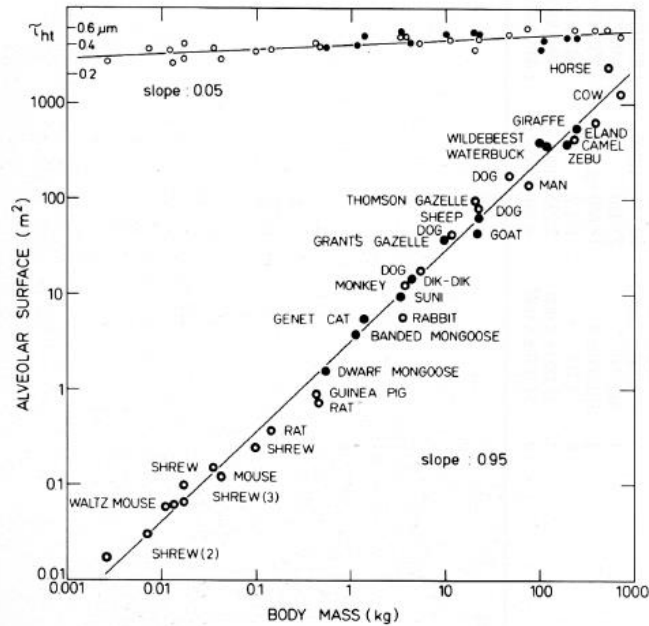
Going deeper down into the lung, more cilia are seen along the conducting airway. Club cells, seen as dome-shaped cells on electron microscopy, occur in all mammalian species and are involved in metabolism and immune function. Studies are underway to examine species-specific differences in the club cell. Mucus cells, whether isolated primarily or in an immortalized cell line, are an important cell type to keep in mind when going from *in vivo* to *in vitro* methodologies. Mucus velocity decreases with age across species.

Perhaps the greatest species difference to consider within the mammalian respiratory system is the transition from conducting airways to gas-exchange areas. Many species have respiratory bronchioles to transition from a terminal bronchiole to an alveolar duct, but many do not. Respiratory bronchioles, which occur as alveolar out-pockets in the wall of the airway, are particularly extensive in dogs and ferrets and less so in humans and monkeys. In contrast, mice, rats and horses all have terminal bronchioles that lead directly into an alveolar duct.

Significant species-specific variability is also seen in the size, organization, and supply of blood to the lungs. Even the pulmonary acinus, the basic functional unit of gas exchange that arises from the last conducting airway within the mammalian lung, can vary in size and thickness by as much as a factor of two in different species with reasonably similar body sizes.

Gas exchange takes place at a very thin air-to-blood tissue barrier. A large surface area for gas exchange is typical for all mammalian species, and the relationship between alveolar surface area to body mass follows similar linear patterns for most mammals, as shown in **Figure 2**; a similar linear relationship exists for capillary volume to body mass across mammals. However, the cellular organization of the gas-exchange area shows variability in abundance, size, and organization of these individual cells among species. In addition, the extracellular matrix and basement membrane components show many species-specific features.

Figure 2.



Allometric plot of alveolar surface to body mass

Source: Pinkerton KE et al. Architecture and Cellular Composition of the Air-Blood Tissue Barrier, Chapter 9. In: Parent RA, ed. Comparative biology of the normal lung. Second edition. ed. New York: Elsevier Academic Press; 2015:105-17.

All mammalian species have an epithelium composed of type I cells that cover approximately 95% of the surface area, as well as secretory type II cells that produce the surfactant to reduce surface tension, covering about 5% in all mammalian species. All of this is connected together by connective tissue and a rich capillary bed that is found within the alveolar septum. A tremendous amount of similarity is seen in the composition of the alveolar septal wall across mammal species—in rats, dogs, humans and monkeys. Endothelial cells of the gas exchange region represent 50% of the total cells that would be harvested from that region. Type I and Type II cells each represent about 10% of the total epithelial cell population of the alveolar septum.

When considering methodologies to examine exposure-response relationships, it's also important to factor in site-specific differences as well as species-specific differences. In one study of monkeys exposed to very low concentrations of tobacco smoke, for example, the degree of cytochrome P450 1A1 activity varied based on airway location. Species differences have also been observed in the timing of development of mammalian antioxidant enzyme activity. Also, innervation of the airways is another part of the response to a chemical or a compound to consider when moving from *in vivo* to *in vitro* methodologies.

Key points:

- **There is a significant amount of interspecies diversity in the mammalian respiratory system, as well as tremendous similarities.**
- **The cellular organization of the gas-exchange area shows variability in abundance, size, and organization of individual cells among species. The extracellular matrix and**

- **basement membrane components show many species-specific features.**
 - **Species-specific differences are important considerations when using different mammalian species to look at the respiratory system and lung disease and development.**
-

Considerations and Challenges for *In Vivo/In Vitro* Correlations

Günter Oberdörster, University of Rochester

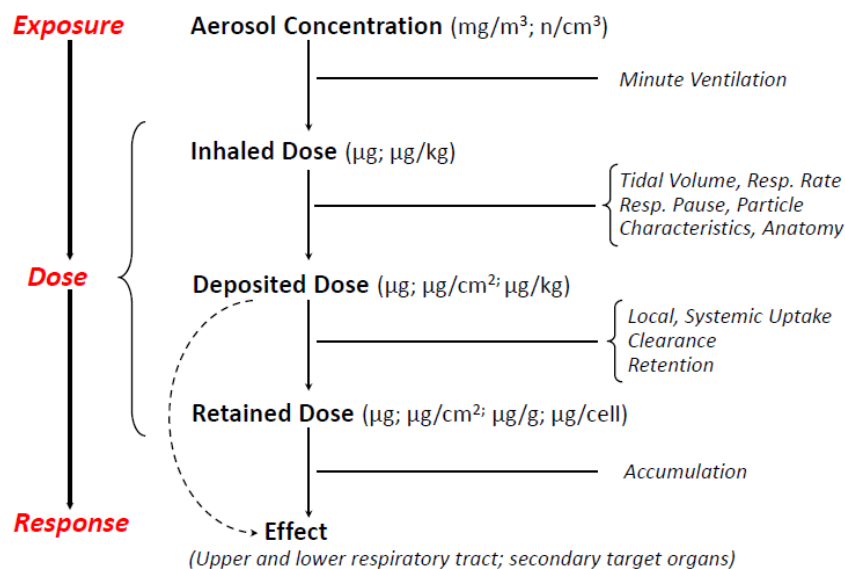
In this presentation, Günter Oberdörster discussed the challenges involved in correlating *in vitro* and *in vivo* dosimetry, the choice of dose metrics, and the relevancy of doses.

Appropriately designed *in vitro* studies may be well-suited for the first step of risk assessment—hazard identification. However, they have not yet achieved suitability for the final step—risk characterization.

Risk assessment is a complex function of both hazard and exposure.⁵ Exposure is a key consideration and its relationship to dose-response is of central importance for assessing the toxicology of inhaled nanoparticles. Diverse systems for exposure are available including *in vivo* exposures, which tend to be dynamic and used for acute to chronic studies; and *in vitro* exposures, which use diverse cell types, are mostly static systems, and are used for acute exposures. Microfluidic systems (“organs on a chip”) deal with possibly dynamic systems, but are also basically static. Cell-free systems explore the reactivity of ultrafine particles using surface reactivity as dose-metric. Examples include DTT (dithiothreitol) and DCFH-DA (2'-7' dichlorofluorescein-diacetate) assays, which assess reactive oxygen species (ROS)-inducing potential. These cell-free exposures can be useful as screening tools for hazard ranking. In one study, a comparison of *in vitro* cell-free oxidant activity to *in vivo* inflammatory responses in rats showed a good correlation, suggesting that a simple assay might provide initial information about reactivity.⁶

The mechanism of toxicity depends highly on the dose. However, a major problem with most *in vitro* studies, and some animal studies, is establishing the actual dose delivery. For aerosol delivery, a careful characterization of the airborne particles, e.g., particle size distribution, concentration, effective aerosol density, is required to determine a deposited dose. The factors involved in respiratory tract dosimetry are summarized in **Figure 3**.

Figure 3.



Factors involved in respiratory tract dosimetry

Detailed dosimetry models have been developed for evaluating realistic dose delivery both *in vivo* and *in vitro*. *In vivo* models allow the prediction of deposited dose of an aerosol in certain regions of the respiratory tract of humans and experimental animals. *In vitro*, the dose to cells is assessed in a conventional or air-liquid interface system. However, the extrapolation of doses and the results of the mostly acute *in vitro* studies (static; no clearance) to longer-term exposure in humans remains a major challenge.

One approach is to establish benchmark nanoparticles that are well-characterized toxicologically and against which new nanoparticles can be compared. It's important to do these studies both *in vivo* and *in vitro* in terms of multidose studies, ranging from no observed adverse effect level (NOAEL) to maximum tolerated dose (MTD) to really get the full range of the dose-exposure-relationship.

When comparing toxicity *in vitro* and *in vivo*, the challenge comes in aligning *in vitro* and *in vivo* doses. Concepts to consider for *in vitro/in vivo* dosimetric extrapolations for respiratory tract exposures are summarized in **Table 1**. A proposal is to express dose per cell surface area (or per cell number) and to consider differentiating between the deposited dose (external) and the "uptake" dose (internal).

Table 1. Concepts to consider in *in vitro* – *in vivo* dosimetric extrapolations for respiratory tract exposures

In vitro dosimetry	In vivo dosimetry
ISDD-model (cells)	MPPD-model (rodent, human)
Physicochemical characterization Media characterization	Physicochemical characterization Respiratory parameters Airway geometry
Diffusion, sedimentation	Diffusion, sedimentation, impaction, interception, charge
Deposition rate (size dependent) and deposited dose (external)	Deposited dose: upper, lower respiratory tract (size dependent)
Uptake: retained dose (internal)	Uptake and translocation to extrapulmonary sites
Clearance: none; static dissolution	Clearance to GI, interstitium, blood, lymph, secondary organs; dynamic dissolution
Retained dose per cell surface area	

Regarding *in vivo* assays, dosimetric extrapolations are accepted methods to derive human equivalent concentrations (HECs) and also eventually occupational exposure limits (OELs) based on data from rodent inhalation studies. Dosimetric approaches are beginning to be more widely applied to assess effects of different size particles including nanomaterials. However, a main concern is animal welfare and consideration of the three R's—replace, reduce and refine.

For *in vitro* assays, many challenges remain. For example, what is the cellular dose equivalency *in vivo*? Are acute static systems with no clearance useful to do? What is predictability of chronic effects? Other considerations are the dose dependency of mechanisms and whether they operate *in vivo*. *In vitro* assays are suitable for toxicity ranking against well characterized benchmarks, i.e. for hazard identification.

Acellular assays, which use predictive toxicity ranking based on surface area specific reactivity, seem to be a promising screening tool, but require further validation and standardization. Dynamic dissolution assays are also promising tools for predicting *in vivo* dissolution rates but require standardization of the methods.

Key points:

- ***In vitro* studies can be useful for hazard identification and ranking; however, *in vivo* studies are still currently required for meaningful risk assessment.**
- **Comparative hazard and risk characterization against positive and negative benchmarks is a useful approach to categorize new nanomaterials. Benchmark materials need to be toxicologically well characterized and validated, and ideally also certified as reference materials.**
- **For the future, goals may include development of validated alternative simple testing strategies for risk assessment for efficient, low cost, high throughput applications.**

CORE SUBJECT #1: TOBACCO SMOKE AND E-CIGARETTE AEROSOLS

Now You See It – Now You Don't: The Chemistry of Cigarette Smoke and E-cigarette Aerosols

Chris Wright, British American Tobacco

In this presentation, Chris Wright described the physical and chemical properties of mainstream cigarette smoke and e-cigarette aerosols, compared their similarities and differences in the context of *in vitro* exposure systems, and discussed the technical challenges associated with their chemical characterization.

Cigarette smoke is a complex, dynamic respirable aerosol formed by combustion, pyrolysis and distillation, which generate volatile precursors. Oxidative reactions occur as well as heterogeneous nucleation (the process by which particles draw together and increase in size) to form particles with changing size. In comparison, an e-cigarette contains a liquid that is transferred to a coil, which is then heated by a battery. The liquid evaporates rapidly and homogeneous nucleation occurs to form small particles/droplets. Some atomization or cavitation of those liquids is also seen. In cigarettes, nicotine is largely associated with the tar components; in aerosols, nicotine is associated with the droplets of the aerosol.

Understanding particle size and particle distribution helps us understand the behavior of cigarette smoke and e-cigarette aerosols in a number of physical and biological systems. It also gives some insight into what might happen chemically. Particle size is probably the single most important parameter to determine for an aerosol and can be measured by using electrical mobility or laser diffraction. In cigarette smoke, the particle size essentially increases with each puff. This correlates with the fact that as the cigarette rod gets shorter, the tobacco is burned away and the particles coagulate as they are drawn through. So, cigarette smoke is not particularly constant across puffs, and significant differences occur even within the use of a single cigarette. In comparison, an e-cigarette gives a fairly stable particle size distribution even after extended operating periods.

In a physical sense, cigarettes and e-aerosols are alike in some ways, as summarized in **Table 2**. They contain similar particle sizes and number. However, a comparison of the chemical compositions of each is more complex. In cigarette smoke, the particulate material is seen in small amounts overall. The bulk of the aerosol is formed by the mass of the air that forms it. In e-cigarettes, the mass is driven by the air. There is a very small proportion of this aerosol that is generated that forms these liquid droplets.

Table 2.

Aerosols: Smoke and e-aerosol contain similar particle

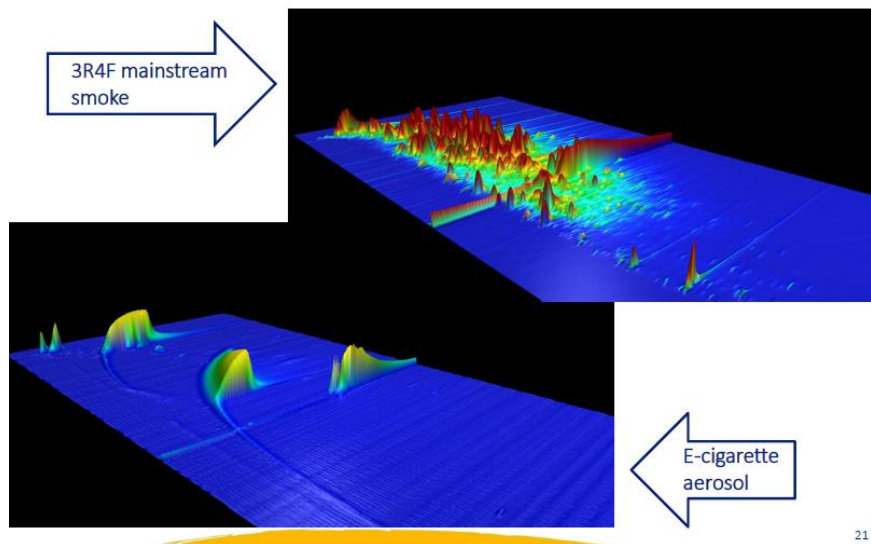
Parameter	Cigarette smoke	E-aerosol
Particle mean size	138-180nm [CMD]	200-500nm [CMD]
Aerosol Particle Number /cm ³	1000,000,000 [Ingebrethsen et al 2012]	1000,000,000
Mass per puff, mg	Ca. 43.3 (ISO) *	Ca. 97.8 (3 sec, 80cm ³)
TPM/ACM per puff, mg	1.2 (ISO) *	1.5-5 (3 sec, 80cm ³)
Nicotine per puff, µg	85 (ISO) *	30 - 100
Puffs per pack	Ca. 180 (ISO, 20 sticks)*	200-300 (Cartomizer)

* 3R4F (9.4mg tar / 0.7mg nic / 12mg CO)

Smoke and e-aerosol contain similar particle size and number

Cigarette smoke contains thousands of substances at varying levels. On the other hand, the mass of the organic components of the e-cigarette is largely dominated by three components--propylene glycol, glycerol and nicotine. So, chemical comparisons of cigarettes and e-cigarettes are very challenging. Two-dimensional gas chromatography (GC x GC) has revealed the complexity of the organics found in cigarette smoke compared to those in e-cigarette aerosols, as illustrated in **Figure 4**. Most cigarette toxicants were not detected in e-cigarette aerosols. Of those detected or quantified, a large proportion was attributable to laboratory air. Such low abundances in e-cigarette aerosol present significant technical challenges to measurement, as well as to assurance of a clean chemical background.

Figure 4.



Chemical complexity – smoke vs. e-aerosol
GC x GC – TOFMS analysis of a single 55 ml puff

Physicochemical characteristics present challenges, not only in terms of understanding what's happening in smoke and in aerosol, but also what's happening *in vitro*. When working with *in vitro* systems, we tend to be working against dilution, or working with relatively small samples. Other factors affecting the transfer of aerosol constituents to cell systems include humidity, coagulation, impaction, deposition, diffusion and dissolution. In an aqueous-based system, solubility plays a role in determining what enters the system and how quickly.

Approaches to dosimetry include physical measurement of deposition (using quartz crystal microbalance [QCM]) and measurement of markers such as nicotine to quantify aerosol delivery. Direct measurement of the received dose presents significant challenges in terms of dilution, small sample size, chemical selectivity and the selection of appropriate substances to measure, particularly for e-aerosols.

Systems designed for cigarette evaluation may not be compatible with the *in vitro* testing of e-aerosols. High aerosol collected mass (ACM), for example, can be problematic for some systems because QCMs do not tolerate high ACM. Other measures of delivery are needed but will be challenging to develop. Also, it's important to understand the relative impacts of droplet phase and vapor phase mediated transfer and to consider that the effects on particle size of humidity in the lung may not be replicated *in vitro*.

Key points:

- **E-aerosols are physically similar to cigarette smoke in some aspects (e.g., size range), but chemically very different.**
- **Systems designed for cigarette evaluation may not be compatible with the *in vitro* testing of e-aerosols.**

- The very low abundance of many cigarette toxicants is challenging to measure in the aerosol, and even more challenging to measure *in vitro*.
 - Other measures of delivery are needed but will be challenging to develop.
-

CORE SUBJECT #2: AIR-LIQUID INTERFACE-*IN VITRO* EXPOSURE SYSTEMS

Fresh Smoke ALI Exposure in 24 and 96 Multiwell Plates Using a New Smoke Exposure *In Vitro* System (SEIVS)

Roman Wieczorek, Imperial Tobacco Limited

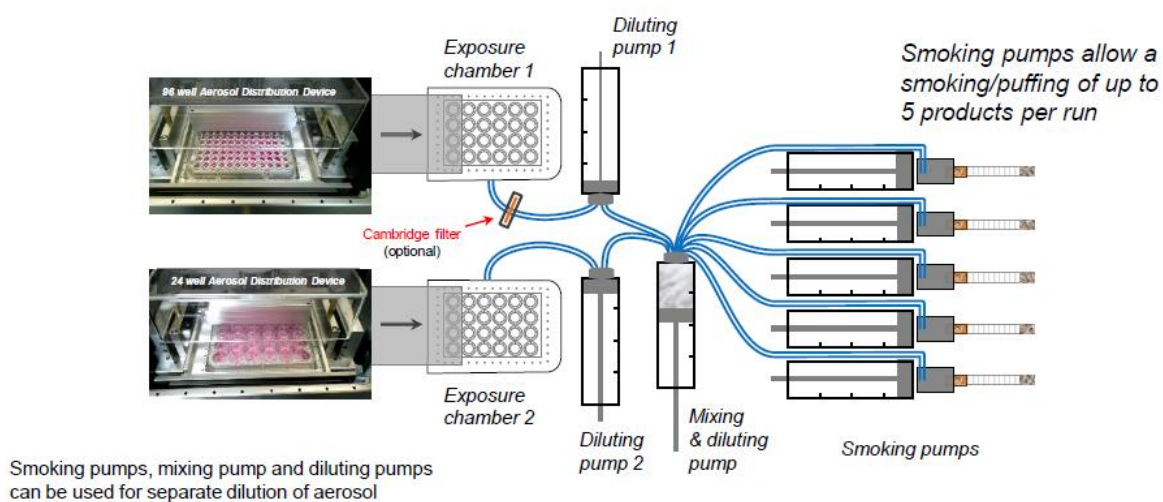
In this presentation, Roman Wieczorek described a newly developed smoke exposure *in vitro* system (SEIVS) in use at the Imperial Tobacco BioLab.

Air-Liquid Interface (ALI) exposures are one of the more recent developments in *in vitro* exposure testing. Due to the dynamic nature of cigarette smoke, a rapid dilution and transport of smoke to the cells is essential. To address this need, Imperial Tobacco BioLab has adapted the SEIVS exposure system, which provides exact dilution and cell exposure in multi-well plates. This system enables *in vitro* testing of aerosols generated from different product categories, including tobacco products as well as e-cigarette devices.

The SEIVS system allows for simultaneously processing of two ALI exposure chambers using 96 and 24 multi-well plates. For the ALI exposure in the 96-well plate, neutral red stained HepG2 cells (human liver) were cultivated on collagen-I, a proven material for maintenance of cells and enables long term exposure to aerosol under ALI conditions. For the ALI exposure in the 24-well plate, V79 (hamster lung) cells were cultivated on a porous membrane. The smoking procedure simulated natural smoking behavior (puffing/breathing), with puff-specific distribution of the aerosol over a row of wells. This was accomplished by covering the first row of wells with a sliding lid for the control. After each puff, the next row of wells was covered.

The smoke flow is illustrated in **Figure 5**. The smoking pumps allow a smoking/puffing of up to five products per run. Separate dilution pumps for each of the two exposure chambers allow parallel testing of whole smoke and the gas/vapor phase, and parallel exposures of cells to different dilution levels and different assays.

Figure 5.



SEIVS – Smoke flow

The effectiveness of the smoke dilution system and the uniformity of particulate phase deposition in the individual wells were determined by measuring the optical density at 400 nm. Reproducible smoke dilution and accuracy of the dilution system were confirmed for both exposure chambers.

When *in vitro* cytotoxicity was measured with the neutral red uptake (NRU) assay, higher cytotoxicity occurred after repeated smoking. With repetitive exposure to the gas vapor phase (GVP) of the CM7 (CORESTA Monitor Test Piece 7) product at selected dilutions, cytotoxicity levels correlated to the amount of active substances delivered to the cells.

The high sensitivity of the system allows for testing and comparisons of smoke and vapor products. Toxicology of tobacco smoke and vapor was assessed using four commercial products (CM7, dark blended cigarette, an emerging tobacco product, and an electronic vapor product) and three assays: NRU⁷, *in vitro* micronucleus (IVM)⁸ and Ames.⁹

The NRU assay after ALI exposures of Hep-G2 cells showed that the whole smoke of CM7 and dark blend cigarette was more cytotoxic than the emerging tobacco product. GVP contributed significantly to the whole cytotoxicity, so its effects have to be considered.

The IVM assays after ALI exposures of V79 cells showed that whole smoke of CM7 was more genotoxic in comparison to the tested ETP. Vapor of the electronic vapor product did not show any response after 240 puffs. Gas phase components contributed significantly to the whole smoke genotoxicity.

The Ames assay after ALI exposures of *Salmonella typhimurium* bacteria on agar plates showed that the response to substances in GVP correlated strongly with the moisture content of agar in the petri dishes. Direct bubbling of bacteria suspension guaranteed fast and effective exposure to all phases of the test aerosol.

Key Points:

- The SEIVS system delivers reproducible and sensitive results. The short connections and fast dilution minimize the loss of particles before cell exposure.
 - The special construction of SEIVS allows testing of up to five products per run, parallel testing of smoke/vapor and their GVP, and parallel exposure of cells in inserts and on collagen I matrix.
 - Special features include easy and fast reloading of exposure chamber by using of multiwell plates, and the ability to alternate puffing with smoke and air cleaning, simulating smoking behavior.
-

Air-Liquid Interface – *In Vitro* Exposure Systems and Their Use in Inhalation Toxicology

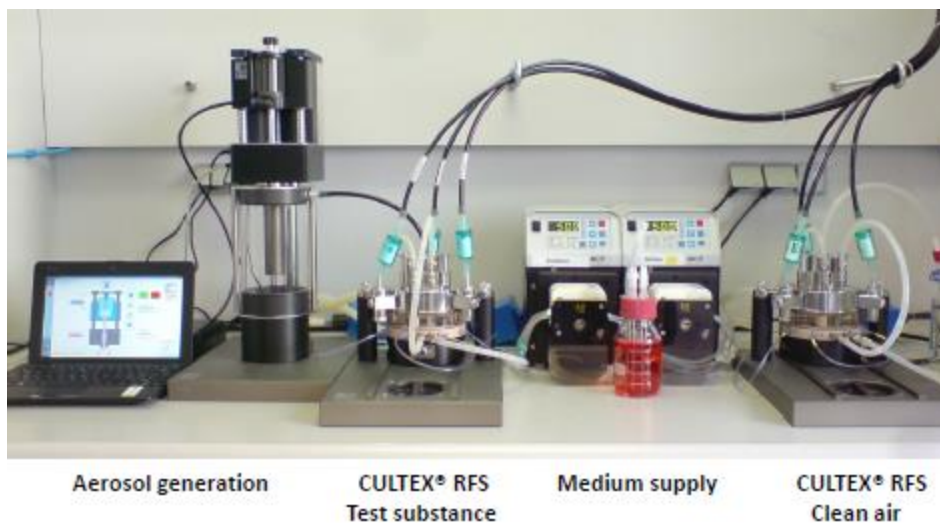
Michaela Aufderheide, Cultex® Laboratories GmbH

In this presentation, Michaela Aufderheide summarized a validation project currently underway to assess the reproducibility of an *in vitro* exposure system used in inhalation toxicology. She also provided an overview of factors to consider when selecting cellular-based test strategies for acute and chronic toxicity studies.

Reproducibility is an essential requirement for validating cellular-based exposure systems used to study the toxicological effects of inhalable substances. To address this issue, a collaborative validation project funded by the German Federal Ministry of Education and Research is currently underway. Its goal is to establish an experimental protocol for analyzing airborne material under standardized, stable and reproducible conditions in cellular-based systems. The project is based on the CULTEX®RFS exposure system, which allows for direct exposure of bronchial epithelial cells at the air-liquid interface (ALI) and the analysis of particulate effects. The system consists of two main parts: the aerosol-guiding module, which conducts and distributes the particles to be deposited on the cell culture inserts, and the sampling module, which has three cell culture inserts or Petri dishes that can be separately supplied with medium. A rack system is also included as a transport and loading platform.

The first phase of the project has been completed.¹⁰ Using human lung epithelial cells (A549 cells) exposed to different concentrations of copper (II) oxide nano- and microscale particles at the ALI, cell viability was measured with the WST-1 assay as a parameter of toxicity 24 hours after exposure. The experimental setup consisted of a particle generation unit the CULTEX® DG-Dust Generator, the CULTEX® RFS for exposure of the cells to the test aerosols and the clean air control, and a medium supply via peristaltic pumps. (See **Figure 6.**) The results showed good overall agreement of the *in vitro* data with existing *in vivo* data for physiological exposures that assessed acute pulmonary toxicity of airborne materials. This supported the general applicability of the CULTEX® RFS with regard to the requirements of the ECVAM (European Centre for the Validation of Alternative Methods) principles on test validity. The project is currently in the second phase to improve inter-laboratory reproducibility and to develop a valid prediction model.

Figure 6.



Experimental setup

The choice of a cellular-based exposure strategy depends on the questions being asked, such as what variables will be analyzed, and whether the study is acute (single exposure) or chronic (repeated exposure). Acute studies evaluate dose-response relationships to analyze the toxic potency of inhalable gases, particles and complex mixtures. Chronic studies estimate cellular changes after repeated exposure to non-toxic concentration of airborne materials for the induction of phenotypic alterations comparable with the *in vivo* situation.

Other considerations include cell susceptibility (i.e., whether the susceptibility of the cells decrease with progressing differentiation of the cells) as well as cell type (ciliated cells, goblet cells, Type II cells) and cell location (bronchi, bronchiole and alveoli). To address only acute toxicity, an undifferentiated cell or even a cell line can be used to show threshold and the concentration for damaging the cells. Other cell types may be required to show particular effects on the respiratory tract. Cellular systems can be monocultures, which include permanent cell lines (tumor and immortalized cells), finite cells (primary cells), or 2-dimensional and 3-dimensional cultures; or co-cultures, which combine different cell types.

For simulating the *in vivo* situation best, the use of primary human cells and immortalized cell lines exhibiting mucociliary differentiation is favored and recommended. These cells can be cultivated and exposed as mono- and co-cultures under undifferentiated and differentiated conditions to study the biological effects of airborne material depending on the susceptibility of the cells.

Normal primary lung epithelial cells can be repeatedly exposed to non-toxic concentrations of the test atmosphere at the ALI, allowing the analysis of mechanistic and long-term effects, such as cilia toxicity, mucus secretion or even the induction of hyper- and metaplastic changes. Cilia toxicity is one of the first events seen in smokers over the long term and increases the risk for developing chronic lung diseases. In one study, normal bronchial epithelial cells were repeatedly exposed (10 times) to mainstream cigarette smoke (4 K3R4F cigarettes)¹¹. Cigarette smoke induced dramatic changes in cilia and mucus-producing cells after repeated exposure. These cells showed the ability to recover to a certain degree. Repeated exposure of an immortalized cell line (CL-1548) to an e-liquid vapor (without nicotine) also showed cilia aberrations. In another study,

repeated exposure (13 times) of normal bronchial epithelial cells to mainstream cigarette resulted in induction of CK 13 positive cells in several, but not, all donors (publication in preparation). However, a variety of cell types interact during the exposure phase, so these findings require further detailed study.

Key points:

- **There are a variety of strategies for exposing cells at the air-liquid interface, starting from acute toxicity studies (dose response relationships) up to repeated exposure studies at non-toxic doses. The choice will depend on the protocol, and a clear definition of what is to be analyzed and demonstrated (e.g. cilia toxicity, metaplastic phenotype).**
- **A validation study of the CULTEX®RFS exposure system showed encouraging results, whereby within the first project phase the intra- and inter-laboratory reproducibility could be demonstrated. Future work (2nd project phase) is planned to improve the data base and to develop a valid prediction model.**

A New Fluorescence Based Method for Characterization of *In Vitro* Aerosol Exposure Systems

Sandro Steiner, Philip Morris International

In this presentation, Sandro Steiner discussed a method to determine aerosol losses and delivery in *in vitro* aerosol exposure systems.

Measuring the delivery and dilution of a test aerosol is essential in order to meet the specific requirements of the biological test system and to assure the application of relevant doses. *In vitro* exposure studies using the Vitrocell® 24/48 system have been a major component of assessing the biological impact of cigarette smoke vs. e-cigarette aerosols at Philip Morris Products S. A. The system has two main parts—a climatic chamber and an exposure module consisting of a dilution/distribution system on top of a cultivation base module where up to 48 cell cultures can be exposed simultaneously to a test aerosol that can be diluted serially. Using this system, nasal, bronchial or oral organotypic tissues are exposed at the air-liquid interface during 28 minutes, followed by post-exposure times of 4, 24, 48 and 72 hours. Before reaching the tissue cultures, the test aerosols are heated to 37°C, humidified and diluted. Endpoints include cytotoxicity (AK assay), mRNA microarray, pro-inflammatory mediators, ciliary beating frequency and histology.

Although the Vitrocell® 24/48 system is highly versatile for delivering undiluted as well as diluted aerosols, such systems have potential limitations with regard to aerosol losses, as a result of sedimentation, impaction and anisokinetic particle sampling, which may hamper exact dosing. To determine the suitability of an exposure system for a specific application and its dosing behavior, aerosol-specific characterization of the exposure system is therefore required. Such a characterization aims at obtaining a detailed description of a system's dosing accuracy, and precision, its delivery uniformity, and reproducibility and the chemical composition and particle size distribution of the delivered aerosol.

Researchers at Philip Morris International have developed a fluorescence-based method to determine aerosol delivery in *in vitro* aerosol exposure systems. The focus was mainly on the

particulate fraction of liquid aerosols, which are becoming more important in the new generation tobacco products. The goal was to develop a direct, robust and fast method for quantification of aerosol deposition at any internal part of the system. For this purpose, model aerosols were generated in a condensation monodisperse aerosol generator (CMAG). The CMAG was chosen because 1) it allows generating aerosols of different mean particle sizes and narrow size distributions, which makes investigating particle size-specific effects possible, and 2) a fluorescent label can be incorporated into the particles during their generation. Glycerol as one of the key components of the aerosols generated by new generation tobacco products was chosen as aerosol material. As a fluorescent label, the fluorophore disodium fluorescein was chosen because of its high fluorescent activity, stability, water solubility and non-toxicity.

Upon test exposures, deposited aerosol material can be quantitatively eluted from internal system surfaces using aqueous solvents and, based on the determined aerosol fluorescence beforehand, aerosol deposition in the exposure system can be quantified with high sensitivity and precision, simply by measuring the retrieved fluorescent activity in the eluates.

Using this methodology, the researchers are currently characterizing the Vitrocell® 24/48 aerosol exposure system to obtain a detailed description of exposures in terms of reproducibility, deposition uniformity, dilution/mixing effects, aerosol losses, and optimization of system operation. The methodology may also be applicable for the investigation of aerosol delivery in other cell culture exposure systems.

Key points:

- **The fluorescence-based method for the characterization of in vitro aerosol exposure systems is a valuable tool to study particle dynamics/delivery.**
 - **Robust particle size specific generation of disodium fluorescein labelled glycerol aerosols is possible, as well as robust, fast and sensitive quantification of aerosol deposition.**
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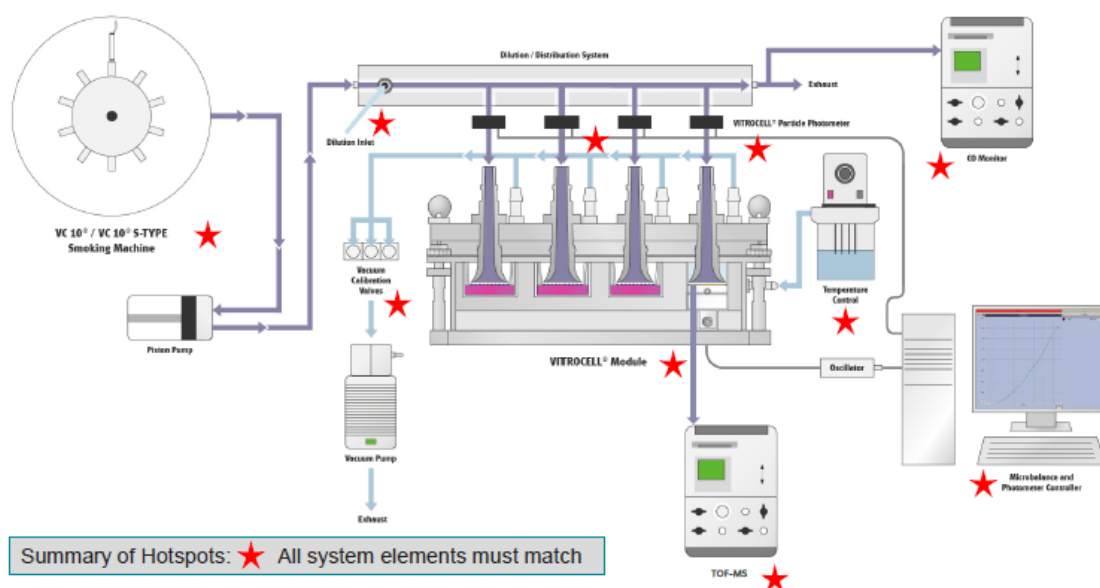
Cutting-edge *In Vitro* Exposure Technologies for Conventional and E-cigarettes

Tobias Krebs, Vitrocell Systems GmbH

In this presentation, Tobias Krebs discussed the major components of a typical *in vitro* exposure system for assessing conventional and e-cigarettes—smoke/vapor generation, dilution systems, exposure systems, auxiliary equipment, and dosimetry tools—and the importance of matching all components to the process requirements.

A complete exposure system for conventional cigarettes and e-cigarettes is complex, as illustrated in **Figure 7**. Critical system elements, the “hot spots” for *in vitro* exposures, and Vitrocell® product examples are described below.

Figure 7.



The complete exposure system

Smoke/Vapor Generation. This is the first hot spot in an *in vitro* installation. Here, reproducible aerosol generation with the smallest dead volumes is important, as well as a fast and easy cleaning procedure, and the avoidance of cross-contamination when testing products. Smoke/vapor generation differs for conventional vs. e-cigarettes. For conventional cigarettes, smoking regimens are generally ISO and Health Canada Intense (HCI), with actuation by a lighter. For e-cigarettes, constant flow (square) profiles of 55 or 70 mL over 3 s with a frequency of 30 seconds are typically used, with the device activated by draw or button.

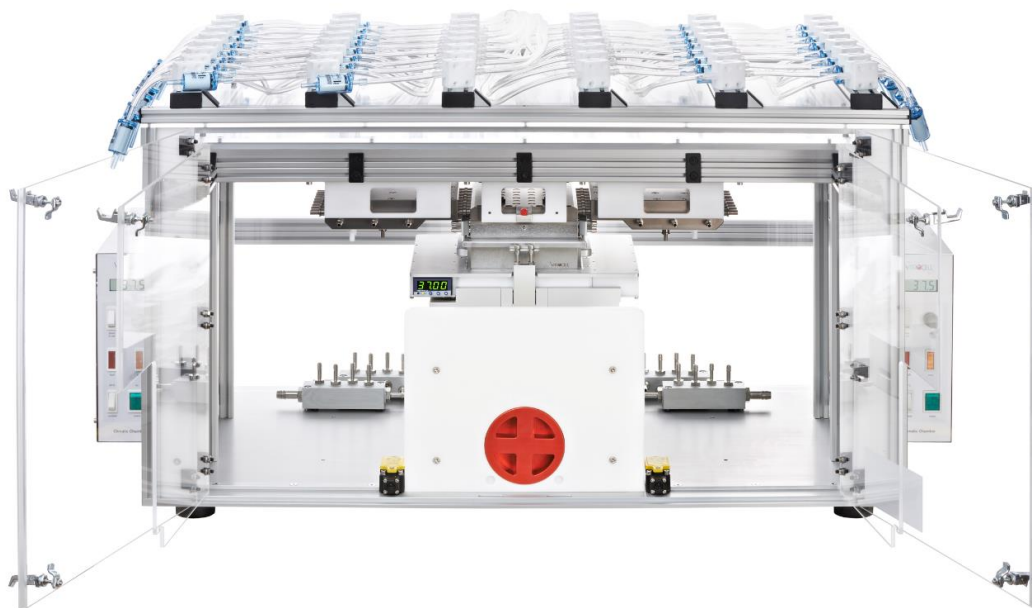
Two types of equipment are available for smoke/vapor generation: automatic robots and manual machines. The VC 10 S-type, an automatic robot, has a multi-pump system that can supply various dilution systems by switching from one pump to the other, and can be used in evaluating conventional and e-cigarettes. The manual VC 1 smoking machine is more suitable for e-cigarettes, and has the smallest dead volumes and offers an increased capacity via multiple pumps. A positive

control can be run in the same experiment. Machines can be characterized by analyzing the total particulate matter (TPM) and particle concentrations via inline photometers before and after the piston pump to define particle losses. The VC 10 smoking robot is the most characterized smoking machine for *in vitro* applications.^{12,13} The VC 1 Smoking Machine was introduced for testing of combustion and e-cigarettes.¹⁴

Dilution System. The dilution system is the second hot spot in the system. Here, reproducible dynamic dilutions with the smallest dead volumes are important. Fresh aerosol should arrive quickly to the test system. The system must also be easy to clean. For conventional cigarettes, the typical concentration range is 5 – 50% smoke. The e-cigarette concentration range is 30 -80% aerosol.

Exposure Module. The exposure module must also be reliable and easy to clean, and ensure uniform particle deposition. Exposure systems are similar for both conventional and e-cigarettes. Traditional *in vitro* exposure methods, which are still often used, include submerged or suspension cultivation with exposure in an incubator, but have the disadvantage that the test substances interact with the media, and thus give a low sensitivity and an undefined dose. The air/liquid interface, where cell cultures are exposed on microporous membranes and has the advantage of including all three phases of the aerosol (gas, semi-volatile and particle) in the exposure, give a high sensitivity and defined dose. This method is more physiological relevant to the human situation. When selecting an exposure module platform, three dimensions must be considered: the membrane insert size, the number of doses or throughput, and the type of assay to perform. When working with bacteria for the Ames assay, for example, the requirements arising from the use of Petri dishes must be taken into account.

Vitrocell offers a wide range of exposure systems for normal and higher throughput which are heavily published. The newest Vitrocell systems are the 6/48 and AMES 48, which meet the demand for higher throughput and a compact design which were developed based on the Vitrocell 24/48 technology.¹⁵ (See **Figure 8.**) The Vitrocell 96 module for 96-well sized cell culture insert plates allows for 11 doses at 8 replicates and one clean air control at 8 replicates, and has an integrated dynamic dilution system.

Figure 8.

The Vitrocell 24/48 Exposure System

Auxiliary Equipment. Auxiliary equipment includes components to optimally manage vacuum flow rates, dilution air flow rates, temperature, and humidification and other exposure conditions and must be synchronized with the exposure modules. Examples include equipment for the maintenance of ISO lab conditions for conventional cigarettes and heated chambers for e-cigarettes.

Dosimetry Tools. Finally, dose monitoring is vital for process control and interpretation of the test results. Dosimetry tools for assessing conventional and e-cigarettes are similar, and employ chemical analysis and time-of-flight mass spectrometry (TOF-MS) for the gas phase. Carbon monoxide (CO) is also measured for conventional cigarettes. In the last few years, advanced solutions for dose assessment became integrated into the exposure systems. These include online TOF-MS technology, which can detect components of the aerosol at a very high resolution. Various concentrations of acetaldehyde and nicotine, for example, can be detected and compared for conventional cigarettes and new generation products.

For assessment of the particle phase, relevant tools are chemical analysis, photometers and microbalance technology. For particle deposition in the *in vitro* exposure system, particle mass and deposition rates need to be evaluated with no disturbance of the exposure process. For this purpose, the microbalance sensor for dose-response measurement was introduced more than 5 years ago and can be integrated into the exposure systems.¹⁶ The sensors are capable of measuring the deposited mass in the module at a resolution of 10 ng/cm² per second. Inline photometers are used to assess the particle concentration to prove that the product is reproducibly guided to the cell cultures. These enable online measurements of particle concentrations at the inlets and/or outlets of the aerosol exposure top, and can measure at very low flow-rates (e.g., 5 ml/min) without any particle losses. Advanced dosimetry software offers a combined view of the photometer and microbalance deposition data, with up to 8 microbalance sensors and 8 photometers.

In summary, the selection of suitable components depends on many factors, including the nature of the aerosol, the type of assay, sample size, and throughput requirements. All component groups can be tailored to match the needs of the research laboratory or specialized testing facility.

Key points:

- **The quality of aerosol generation is vital for a successful in vitro experiment. The smoking machine requirements for conventional and electronic cigarettes are different, but share the same basic technologies.**
 - **Reproducible dynamic dilutions with the smallest dead volumes are important.**
 - **Sample size and amount of the different doses to be assessed in one experiment are factors to consider in selecting machine type, dilution systems and exposure modules.**
 - **The in vitro exposure system should be capable of handling exposure at the air/liquid interface.**
 - **Dose monitoring is vital for process control and interpretation of the test results.**
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CORE SUBJECT #3A: DOSIMETRY APPROACHES FOR PARTICLES AND VAPORS

Modeling Inhalation and Deposition of Cigarette Puff Mixture in the Lungs of Smokers

Bahman Asgharian, Applied Research Associates

In this presentation, Bahman Asgharian discussed the development and application of a mathematical dosimetry model for determining the deposition of cigarette smoke particles in the oral cavity and the lung.

A realistic assessment of the deposited dose of inhaled cigarette smoke in the respiratory tract must address the underlying physicochemical properties of the smoke. Cigarette smoke mixtures have properties that make their behavior much more complex than that of environmental aerosols. A cigarette puff may contain many different chemical components in particulate and gaseous forms. These compounds then enter the respiratory tract after puff withdrawal and are inhaled deep into the lung and deposited preferentially on airway surfaces based on their aerodynamic and thermodynamic properties.

Also, the breathing maneuver during smoking differs from normal breathing and contributes to discrepancies in predicting particle deposition. During smoking, the puff is drawn into the oral cavity and this is followed by a mouth-hold. Smoke-free air is then inhaled and mixed with the puff before delivery of the particle mixture to the lung. Particle size may change during the puff drawing, mouth-hold and delivery into the lung.

When freshly generated, most components of cigarette smoke are condensed into droplets. However, there will be a continuous gas-particle conversion (phase change) of components depending on their saturation vapor pressure, deposition on airway surfaces determined by their aerodynamics properties, and coagulation of cigarette particles due to their high number concentration. Particle size is also affected by relative humidity, with size increasing at high relative

humidity by absorbing water vapor from the surrounding air. Hygroscopic growth occurs quickly and is mainly responsible for particle growth over size increase due to phase change of other components.

Understanding the fate of these compounds in the lung and the localized dose to the lung of the inhaled smoke is essential for studying the health impact from cigarette smoking. Two other major mechanisms affect cigarette particle behavior and deposition. One is the colligative—or cloud—effects, which occur when a mass of particles behaves as a single body, resulting in airflow moving around the body rather than through it. The other mechanism involves non-colligative effects, which include phase change (evaporation or condensation of semi-volatile components) and coagulation (movement and collision of particles in the air due to their thermal energy). These mechanisms affect deposition of smoke particles by sedimentation, impaction and Brownian diffusion.

Mathematical dosimetry modeling offers a realistic approach to studying the fate of inhaled smoke and is complementary to controlled studies of biological responses. Modeling efforts begin with studying the behavior of the puff during inhalation due to droplet-vapor phase change, aerosol coagulation, deposition on airway surfaces, and mixture of the puff with the dilution air at the end of a mouth-hold before entering the deep lung. In developing a model, the givens include the exposure parameters (concentration, size distribution, etc.), lung geometry, and breathing rates and profile. The goal is to calculate the deposition fraction of cigarette particles in the lung during a single puff inhalation and during multiple breaths. Challenges include the lung geometry, which is very complex with its varying airway dimensions and branching, as well as the calculation of lung ventilation—the airflow distribution that determines where the particles end up in the lung.

The model presented assumes a simplified airway geometry—i.e., cylindrical airways and a dichotomous branching structure—and uniform expansion and contraction of the lung lobes.¹⁷ Particle transport modeling is based on a mass balance equation per airway to calculate the deposition fraction in all lung airways. Calculations of particle deposition for a smoking scenario are based on a simulation of the breathing pattern of a smoker—from drawing of the puff to mouth-hold, inhalation of dilution air, pause, and exhalation.

Model predictions of deposition fractions with and without the cloud effects were compared. The results showed that the cloud effect was most significant in the large airways of the lung and that the effect decreased distally with lung depth. Deposition in the tracheobronchial region suggested a strong cloud effect, while deposition in the pulmonary region suggested a diminishing cloud breakup effect in the deep lung. There was little or no cloud effect in the alveolar region.

Mixing of the puff with dilution air reduced the cloud effect and tracheobronchial deposition. With no mixing, there was significant deposition in the oral cavity and tracheobronchial region due to the cloud effect. With complete mixing, there was a high deposition in the oral cavity due to the cloud effect and a reduced deposition of cigarette particles in the tracheobronchial and alveolar regions.

Key points:

- **Mathematical dosimetry modeling offers a realistic approach for studying the fate of inhaled smoke and provides a link between exposure characteristics and biological responses.**

- **Consideration of the cloud effect is needed for realistic predictions of particle deposition. Predicted particle deposition considering the cloud effect was greater than when treated as a collection of non-interacting particles.**
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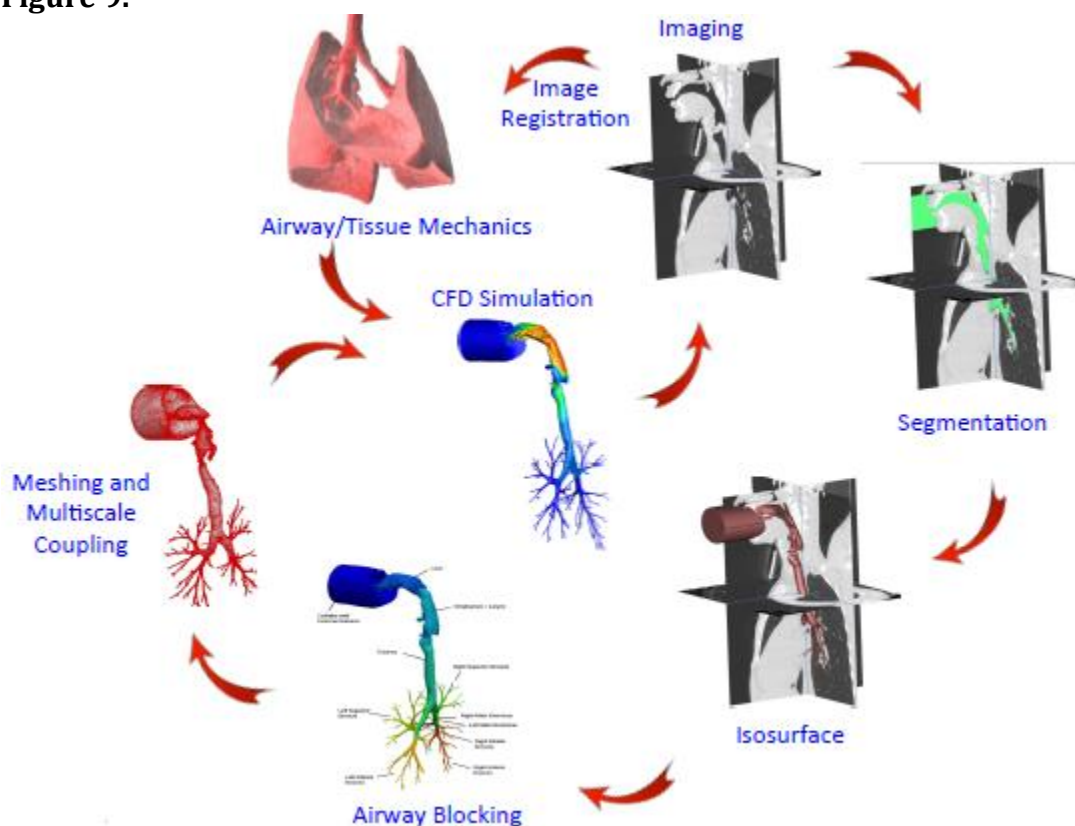
CFD Modeling of Aerosols and Vapors for Cross-Species and IVIVE Respiratory Dosimetry

Richard Corley, Pacific Northwest National Laboratory

In this presentation, Richard Corley used data obtained from computational fluid dynamic (CFD) modeling to address species differences in site-specific aerosol deposition as well as tissue doses for reactive aldehyde vapor constituents found in tobacco products under realistic exposure conditions. These CFD models and others serve as the foundation for relating *in vitro* responses to realistic human exposure conditions.

Tools for developing CFD models have evolved considerably since the 1990s, and models can now be developed in days or even hours, depending on the species. The development of an imaging-based CFD respiratory model, summarized in **Figure 9**, begins with magnetic resonance imaging (MRI) and computerized tomography (CT) of the airway features of interest, followed by segmentation of the imaging data and the creation of an isosurface for mapping cell types and tissue types. Computational meshing and multiscale coupling are then employed for CFD simulation. A suite of imaging-based CFD models are now available for a variety of species—including rats, mice, rabbits, monkeys and humans—and personalized models are on the horizon.

Figure 9.



Imaging-based CFD Model Development

Corley RA et al. Toxicol Sci 2015;146:65-88. Corley RA et al. Toxicol Sci 2012;128:500-16.

In a recent study, extended airway CFD models of the rat and human were coupled with airway region-specific physiologically based pharmacokinetic (PBPK) tissue models to describe the kinetics of three aldehydes found in cigarette smoke: acrolein, acetaldehyde and formaldehyde.^{18,19} Aldehydes are highly reactive, water-soluble vapors, and difficult to measure in tissues because of contact site irritation, inflammation, degeneration and mutations. To date, human health risk assessments have been driven largely by cytotoxicity and tumors in the nasal tissues of rats (nose-breathers), as opposed to humans (nasal/oral breathers).

To compare site-specific airway tissue internal doses between rats and humans under realistic breathing and estimated cigarette yields, tissue “hot spots” for each aldehyde in each cell type (nose) or region (other airways) were determined along with overall regional area-under-the-curve (AUC). Hot spot AUCs were defined as a function of concentration, surface area and depth within a cell type or region, and constituted the top 2.5% of AUCs for all facets in each region.

In prior steady-state rat simulations of aldehyde nasal toxicity, the anterior respiratory nasal epithelial tissues received the greatest initial uptake rates for each aldehyde. However, using the more realistic transient breathing profiles in this study, AUC concentrations were greater in the anterior dorsal olfactory epithelium. Human oral breathing was simulated by measuring puff ventilation profiles and smoke compositions for representative puff concentrations of each of the

aldehydes. In the human simulation, oral and laryngeal tissues received the highest local tissue dose. Penetration to pulmonary tissues was greater than that predicted in the rat. Lifetime average daily doses (LADDs) were compared for each aldehyde under realistic cigarette smoking in humans with those produced in target tissues of rats following subchronic inhalation exposures. Based upon LADD comparisons of tissue hot-spot AUCs and numbers of cigarettes smoked/day, the order of concern for human exposures was acrolein > formaldehyde > acetaldehyde.

Another ongoing study involves a CFD model developed to compare the deposition of aerosolized *Bacillus anthracis* spores in the respiratory airways of a human with that of the rabbit, a species commonly used in the study of anthrax disease.²⁰ Results showed that regional spore deposition patterns were sensitive to airway geometry and ventilation profiles. Spore deposition in the nose was higher for rabbits than humans and attributed to structural differences; the rabbit nose is highly turbinated with a very different anatomy and diffusive properties compared to humans. Deposition in the lower conducting airways was higher for humans than rabbits, and attributed to differences between the two species in the bifurcations of the lung.

Looking toward the future, a comprehensive molecular atlas of the late-stage developing lung is currently in development with funding from the National Heart Lung and Blood Institute (NHLBI). Known as Lung-MAP, this open-access reference resource is utilizing state-of-the-art molecular and imaging technologies to map and annotate the cell types of the developing mouse and human lung. The goal is to fill the knowledge gap in molecular/cellular events that drive lung development and cell function and to provide tissues, reagents and data to the medical research community.

Four-dimensional CT imaging is currently being done in animals and shows that the tissue mechanics of the lung is implicit in its motion. Data from a rat model of late-stage chronic obstructive pulmonary disease (COPD) has allowed for the development of ventilation maps and stress/strain relationships.

Work is also underway on multi-scale coupling for aerosol deposition. These models involve three-dimensional CFD descriptions of airways with the addition of one-dimensional models in a bi-directional coupling with airway mechanics, airflow, and multiple path particle density (MPPD) models to get a better full description of the airways. These models also consider changes in respiratory behaviors that contribute to target site dosimetry and response.

Key points:

- **Understanding target tissue dosimetry under both experimental and realistic exposure conditions will be just as vital to successful implementation of *in vitro* testing as it has been for cross-species comparisons.**
 - **Simulations using CFD-based models enable the development of more realistic and relevant human equivalent exposures associated with responses observed in animals as well as *in vitro* organotypic respiratory cell culture systems at an air-liquid interface.**
 - **Benchmarking responses to target site or tissue dosimetry significantly improves the ability to prioritize tobacco product constituents of concern and reduces uncertainties in cross-species and *in vitro-in vivo* extrapolations.**
-

Deterministic Dosimetry for Particles and Vapors: Consideration for *In Vitro* Mainstream Tobacco Smoke and E-Vapor Product Studies

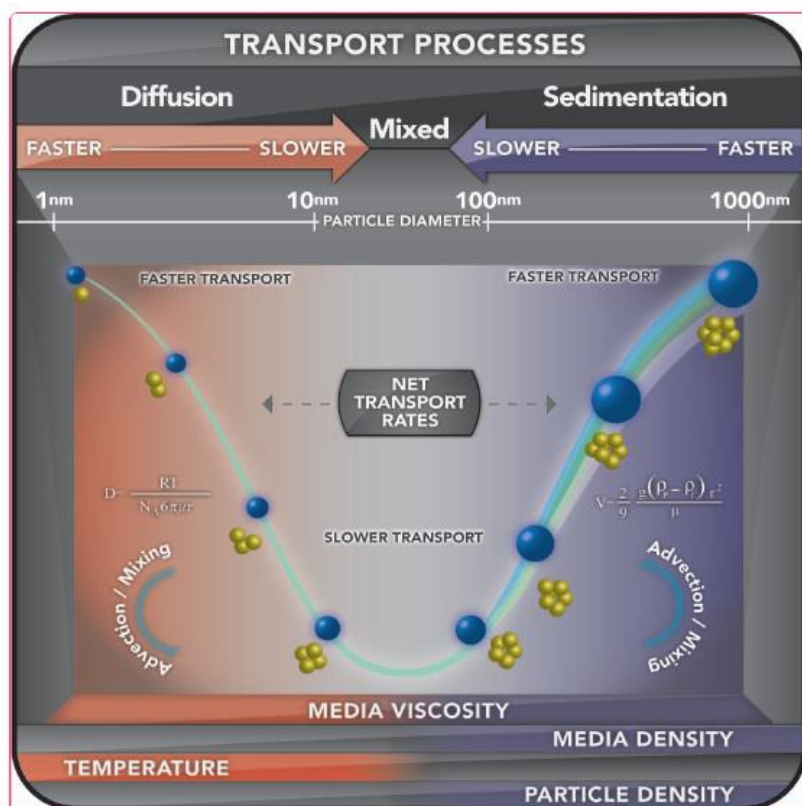
Michael Oldham, Altria Client Services, LLC

In this presentation, Michael Oldham reviewed mechanisms and factors to consider when determining dosimetry in *in vitro* studies of mainstream tobacco smoke and e-vapor.

The goals of *in vitro* dosimetry studies have evolved from simply knowing the exposure concentration in the culture, to determining the cell exposure concentrations as well as the cell surface dose that causes the response. Now, the goal is to determine the internal cell dose that results in the response, and even to determine the dose at the receptor inside the cell.

Two major mechanisms that determine the deposition of particles in the respiratory tract are Brownian diffusion and sedimentation. Particle transport to cells is calculated by solving simultaneous equations for both of these forces to show how far a particle will travel in one second in still air. These principles were applied in the ISDD (*in vitro* sedimentation, diffusion dosimetry) model developed by Hinderliter et al. to calculate the movement of particles from the media to the bottom of the vessel in submerged cultures.²¹ (See **Figure 10.**) The simulations factored in gas content, temperature, Avogadro's number, media viscosity, particle radius, gravitational acceleration, particle density, fluid density and total media height.

Figure 10.



Processes and system characteristics affecting particle transport rates in liquid containing in vitro systems
 Source: Hinderliter PM et al. Part Fibre Toxicol 2010;7:36.

In developing the ISDD model, some assumptions were made to simplify the calculations. For example, advection and surface area of the sides of the cell culture dish were considered not significant. Particles, either primary or agglomerates, were assumed to be independent and non-interacting, and a uniform particle distribution at initiation of the experiment was assumed.

The model was tested against measured transport rates or cellular doses for three different particles—carboxylated polystyrene, iron oxide and silica—obtained in three independent studies. Particles of different density, size and agglomeration state were tested. Overall, the cellular doses predicted by the model were in close agreement with the experimental data, differing in most cases by about two-fold. The authors noted that the accuracy of the model was limited by the accuracy of the input data, and by experimental and biological variability. However, the ability to calculate the actual cellular dose +/- 50% is an improvement over earlier models.

When using air-liquid-interface (ALI) exposure systems, it's important to keep in mind that these systems use low flow rates, which can influence biological effects. Leak detection is critical at every step. These exposure systems have dead space (volume of aerosol transport pathways) and aerosol losses can occur in these spaces prior to cell exposure. External forces such as thermophoresis created by the temperature difference between the media feeding the cells and the exposure atmosphere, can create a thermal buffer above the cells and affect aerosol deposition.²² Particle-

charge effects are usually not problematic when dealing with liquid aerosols.

The delivered dose at the air-liquid-interface can be measured using the quartz crystal microbalance (QCM). While this works well for tobacco smoke, it has not been effective for the non-Newtonian fluids used in e-liquids. Also, surface area coverage by the aerosol must be considered. For example, are there “hot spots” in the ALI culture dishes? Are center cells getting more exposure than the periphery? More research is needed in this area.

Determination of *in vitro* dosimetry is challenging for both mainstream tobacco smoke and e-vapor product aerosols. Both are concentrated, dynamic and complex with semi-volatile constituents. Tobacco smoke contains more than 8700 chemicals.²³ Dilution of mainstream tobacco smoke can cause changes in particle size with more semi-volatile constituents in the vapor phase.²⁴ With e-vapor aerosols, there are significantly fewer constituents. However, e-vapor aerosols have a greater proportion of semi-volatile constituents.

Key points:

- **Knowledge of the delivered dose and its time course is critical to interpreting and potentially extrapolating results from *in vitro* assays.**
- **Tobacco smoke and e-vapor aerosol present unique challenges regardless of the exposure techniques used in *in vitro* experiments.**
- **Quantitative particulate and vapor phase *in vitro* dosimetry determinations are vital to be able to interpret and integrate results of *in vitro* experiments into the scientific literature.**

CORE SUBJECT #3B: IN VITRO DOSIMETRY DETERMINATIONS

Dosimetry Tools, Approaches and Applications for Tobacco and Next Generation Product Testing

Jason Adamson, British American Tobacco

In this presentation, Jason Adamson discussed the exposure systems and dosimetry tools employed by British American Tobacco (BAT) and how these tools are being used to compare exposure systems and data from cigarettes, e-cigarettes and tobacco heating products.

Only about eight years ago, exposure testing at BAT was mainly focused on combustible cigarettes, which varied by slight differences in factors like rod length, filter and tobacco blend. Now, the next generation products are devices of all shapes and sizes, with differences in power sources and electronics, and e-liquids with differing flavors/ingredients, nicotine strengths and humectant ratios. Some devices are puff-activated and some require pressing a button. They differ from cigarettes in how they are attached to aerosol-generating machines, how they are held in place, how they generate aerosol, and in their chemical compositions once diluted and deposited *in vitro*.

Modes of biological exposure to test article have included:

- **Particulate matter** exposure, which involves submerged exposure to filter-trapped particles that are then washed in solvent. This is a traditional, relatively inexpensive, and

simple exposure with much historical data available, based on its use as a regulatory standard. However, only a minority fraction of cigarette smoke can be captured for exposure. Considerations include solvent solubility/ interactions, and the physiological relevance for lung cultures.

- **Aqueous extract** exposure, which involves submerged exposure to an aerosol that has been bubbled through media or buffer. This is also a relatively simple and inexpensive exposure that captures both the water-soluble particulate and gas phase components. It's appropriate for most cell culture models and is used, for example, in models of cardiovascular disease and oxidative stress. However, it only captures soluble components, and an analysis of the individual fractions might underestimate the risk. It may be less relevant for lung or air-liquid interface cultures. Aerosol solubility and phase distribution must be considered, as well as the type of solvent to use. Some solvents could potentially react with constituents of the smoke fraction.
- **Air-liquid interface (ALI) and air-agar interface** exposures, which involve whole aerosol or vapor phase only exposure at the air-liquid or air-agar interface. These exposures are more complex and expensive to set up, but are probably the most physiologically relevant for lung cultures and to the consumer because all fractions and components of the test aerosol are exposed. There is a variety of systems, so individual characterization is key. It's also important to understand the dilution mechanics, transit, exposure chamber and interface dose of the system, although the differences become less relevant when data can be aligned with dose.
- **e-Liquid** exposure, a relatively new method that involves submerged exposure to unaltered e-liquid or its ingredients. It is a very inexpensive, simple and high throughput exposure. However, one must understand that the components of the e-liquid are changed through aerosolization. Undiluted e-liquids will be toxic to cells, giving a false positive, but this may not necessarily be an issue, because dose-responses and LD-50s can be obtained to make comparisons.

BAT currently works with two ALI exposure systems: The Borgwaldt RM20S® and the associated smoking chamber with quartz crystal microbalance (QCM), and the Vitrocell® VC10 smoking robot and the associated 6/4 module with QCMs. Five dosimetry methods are used to make product assessments, with two—gravimetric mass and nicotine concentration—employed fairly regularly.

- **Gravimetric mass per puff via QCMs** – The main advantage of this dosimetry method is that it provides real-time data generation, which gives confidence in the exposure. A limitation is that QCMs can be overloaded with aerosol as the droplets coalesce after depositing on the crystal's surface.
- **Nicotine quantification per puff with Ultra Performance Liquid Chromatography (UPLC) – Mass Spectrometry (MS)/MS** - The advantage of this method is that it provides relatively quick turnaround in data, and also allows for qualification as well as quantification and conversion of dilutions to delivered nicotine.

Measuring dose at the exposure interface may allow the comparison of data from different exposure systems and products. **Table 3** summarizes the different ways that have been used to draw comparisons to the wide variety of products available now. Exposure system dilution provides a very simple data representation but is only valuable on the same system and does not allow easy cross-platform comparisons. A per stick/product comparison is uninformative due to the diversity of products with varied uses and delivery. Per puff comparisons may give a closer comparison but are still limited. A gravimetric mass per puff comparison is a good, real-time, in situ quantification of deposited mass of a test article, allowing cross-platform and cross-product

comparisons. Delivered nicotine comparisons are even better, allowing *in situ* quantification of a marker across products. Comparisons of other delivered compounds would be best, providing additional *in situ* quantification.

Table 3.
Methods of Dose-Response Comparison

METHOD OF COMPARISON	UNITS	PROS/CONS AND IMPLICATION
Exposure system dilution	Ratios, %, flow rate	OK; simple data presentation; comparison only valuable on the same system; does not allow easy cross-platform comparisons
Per stick/product	Item	Uninformative; with such a diversity in products that are consumed, puffed, activated in different ways and with varied delivery
Per puff	Number	OK; a closer comparison between products but still limited
Gravimetric mass (per puff)	$\mu\text{g}/\text{cm}^2/\text{puff}$	Good; real-time, <i>in situ</i> quantification of test article deposited mass, allows cross-platform and cross-product comparisons
Delivered nicotine	Total ng	Better; <i>in situ</i> quantification of delivered nicotine, a cross-product marker
Delivered X, Y, Z	Total ng	Best; additional <i>in situ</i> quantification of other delivered compounds

To determine whether dose can be used to align different systems, BAT carried out a case study to assess two cytotoxicity datasets generated on contrasting exposure systems, the Borgwaldt RM20S® and the Vitrocell® VC 10 with different experimental setups. Comparisons were made by expressing each dataset as a function of dose using $\mu\text{g}/\text{cm}^2$ and nicotine. The resulting data has been submitted for publication. Overall, the study demonstrated the importance of dosimetry techniques and how they can be used to align data between two completely different exposure systems and setups to facilitate comparisons.

Key points:

- **Dosimetry techniques can be used to align data between two completely different exposure systems and setups, to facilitate comparisons.**
- **Dose tools may provide a link between *in vitro*, *in vivo* and human dosimetry studies and aid in the comparison of data across different tobacco categories.**
- **The next generation products category will continue to grow, evolve and diversify and dosimetry will support exposure.**

Evaluation Method for *In Vitro* Toxicity of Cigarette Smoke by Whole Smoke Exposure

Xiang Li, Zhengzhou Tobacco Research Institute of CNTC

In this presentation, Xiang Li described the whole smoke (WS) exposure system used at the Zhengzhou Tobacco Research Institute (ZTRI), and summarized findings from several assays and *in vitro* dosimetry determinations at the air-liquid interface.

Cigarette smoke is a complex aerosol composed of thousands of chemicals which are distributed in a particulate phase and a gas vapor phase (GVP). Investigating only the toxicological effect of total particulate matter (TPM) from mainstream cigarette smoke does not completely reflect the biological effects of the smoke mixture. Direct exposure technology based on the air-liquid interface provides a better platform for investigating the *in vitro* toxicity of native cigarette smoke.

At present, the representative exposure systems are the CULTEX® and Vitrocell® systems as well as the British American Tobacco exposure chamber. These systems provide an air-liquid interface exposure for cells and guarantee a composition of mixtures matching the real-life situation.

An experimental platform at ZTRI used a smoking robot VC10 connected with the Vitrocell® exposure system. Some parameters, which could potentially influence the measurements, were optimized using this exposure system. Based on the optimized parameters, *in vitro* toxicity assays by WS exposure at the air-liquid interface were established. These included the neutral red uptake assay, the Ames assay, and the oxidative stress assay for whole cigarette smoke.²⁵⁻²⁸

The results of the neutral red uptake assay for WS showed that the viability of cells exposed to synthetic air at a 5 mL/min flow rate was not impacted significantly when the exposure time increased. The optimal time-point to assess smoke cytotoxicity appeared to be 24 hours after smoke exposure. A good dose-response relationship was observed by using this WS exposure system. The data showed that Chinese hamster ovary (CHO) cells were more sensitive to smoke-induced cytotoxic effects than the human lung adenocarcinoma epithelial cell line (A549 cells). Cytotoxicity under the International Organization for Standardization (ISO) regimen was less than that under the Health Canada Intensive (HCI) regimen when smoke doses were expressed as percentage of cigarette smoke. Notably, when smoke doses were converted to TPM (μg), cytotoxicity under the HCI regimen was less than that under the ISO regimen. GVP of cigarette smoke plays an important role in toxicological impact.

For the Ames assay for WS, a flow rate of 5 mL/min flow rate was found to be suitable and a good dose-response relationship was observed using this WS exposure system. The sensitive response was observed using the spread culture method rather than the overlay agar method. An S9 mix of 10% was determined to be optimal when considering positive response and the costs.

Results of the oxidative stress assay showed that WS caused oxidative stress in A549 cells at the air-liquid interface. The ratio of reduced glutathione (GSH) to oxidized GSH (GSSG) decreased. Malondialdehyde (MDA), 4-hydroxynonenal (HNE) and extracellular superoxide dismutase (ECSOD), and 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels increased after WS exposure.

Measurements from TPM exposure and WS exposure were compared by converting the EC50 values from WS exposure testing. The comparison was based on the assumption that the TPM of cigarette smoke entering the exposure module could be completely absorbed by the medium during WS exposure. Smoke dose in TPM exposure is generally expressed as " $\mu\text{g}/\text{mL}$ ", while WS exposure is expressed as a percentage of cigarette smoke (% of cig). The values of % of cig in WS exposure experiment were converted to TPM equivalent values by multiplying the % of cig values with TPM delivery per cigarette. Then, the TPM equivalent values were divided by the volume of medium in the exposure module. The final calculated results were the converting values of $\mu\text{g}/\text{mL}$.

The results showed that the converting values of EC50 in WS exposure were less than the EC50 values in TPM exposure. However, this comparison was based on an assumption and the data conversion has some limitations. For example, the TPM of cigarette smoke may not be completely absorbed by the medium during WS exposure. Also, the converting values of EC50 in WS exposure, according to the assumption, might be greater than the actual data. In spite of this limitation, the converted results can indicate that the cytotoxicity of cigarette smoke by WS is greater than that of TPM exposure.

In another experiment, the quantification of deposited particle mass and nicotine on a quartz crystal microbalance (QCM) surface was analyzed to assess smoke dosimetry. Nicotine was selected as a chemical marker of smoke dosimetry. The data (unpublished to date) showed a good correlation between the concentration of deposited particle mass and the concentration of nicotine.

Key points:

- **In the area of *in vitro* toxicity testing based on WS exposure, the dosimetry determination is an important aspect for the air-liquid interface.**
 - **Accurate dosimetry data can support the results from *in vitro* toxicity of cigarette smoke.**
-

CORE SUBJECT #4: EXPOSURE MICROENVIRONMENT/PHYSIOLOGY OF CELLS

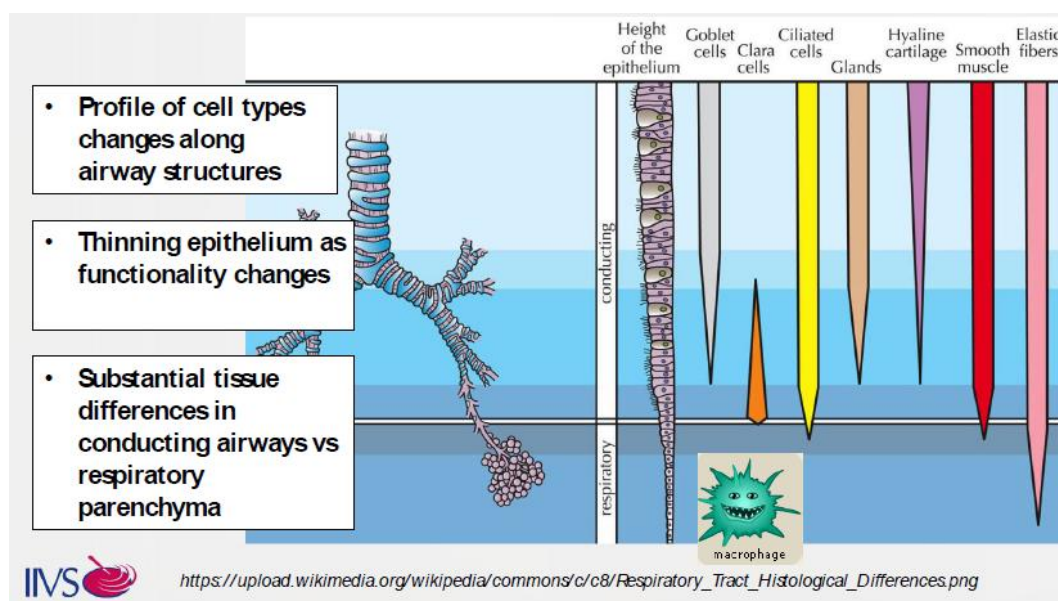
Exposing What? Overview of the Airway Tissue Exposure Site

Holger Behrsing, IIVS

In this presentation, Holger Behrsing reviewed the current understanding of human pulmonary structures, the involvement of airway tissue changes and cell types in exposures and responses, and the relationship of these human lung components to two *in vitro/ex vivo* models currently in use for exposure assessments.

The complex structure of the human lung presents a challenge for quantifying exposures to inhaled materials. In the respiratory tract, cell types and functionality change along the airway structures with substantial tissue differences seen in the conducting airways versus the respiratory parenchyma. (See **Figure 11.**) Deposition of materials in the lung is also quite variable, depending on the location in the respiratory tract—i.e., the nasal cavity, trachea, bronchus or bronchioles. Different deposition mechanisms, such as diffusion, sedimentation, inertial impaction, interception and electrostatic forces, can play major or minor roles.

Figure 11.



Brief revisit of the respiratory tract. Airway tissue transitions: cell types and functionality

https://upload.wikimedia.org/wikipedia/commons/c/c8/Respiratory_Tract_Histological_Differences.png

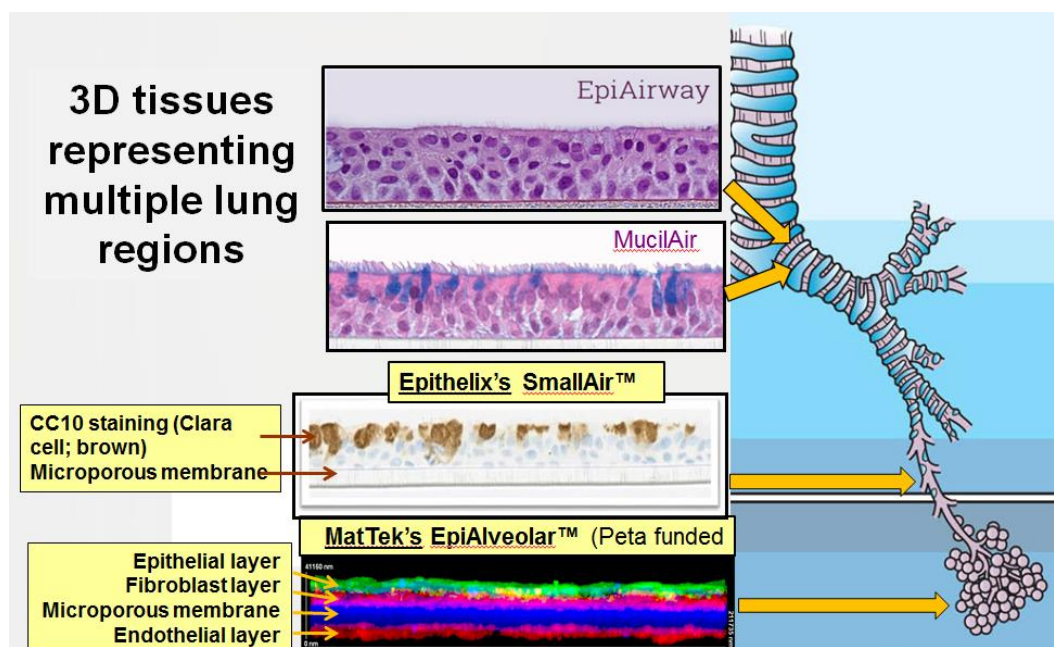
The fluid lining of the pulmonary barrier is the first line of defense when a substance enters the lungs. In the upper airway, goblet cells secrete gel-forming mucins, the major components of mucus. The mucous layer contains antiseptic enzymes (such as lysozymes), immunoglobulins, inorganic salts, and proteins (such as lactoferrin). In the smaller airways, Clara, or club cells secrete surfactant and produce enzymes that detoxify substances dissolved in the respiratory fluid. The fluid lining has anti-oxidant properties as well. When quantifying materials at the exposure site, it's important to keep in mind that substances like aerosols and whole smoke will interact with the

fluid lining and modify the content of the lung region-specific barrier. The cells and tissue are actually then exposed to a “modified” liquid, which can be quite variable depending upon the region of the lung.

Various three-dimensional (3D) *in vitro/ex vitro* models are available for assessing exposure responses, including *in vitro* reconstructed human airways (RHuA) and precision-cut lung slices (PCLS), also of human origin. These models are currently being used for studies of cytotoxicity, viability, and functional responses (e.g., inflammatory) to exposures. An advantage of the 3D models over two-dimensional models is the presence of multiple cell types, including mucus-producing cells. However, while controlled exposures are possible for these models, dosimetry remains a challenge.

The RHua model allows for airway-like exposures with a number of cell types present, including ciliated columnar cells, goblet cells, basal cells, fibroblast co-culture, and Clara cells. (See **Figure 12.**) Grown at the air-liquid interface, RHua tissues offer apical and basal compartments that allow flexibility in modeling physiologically relevant exposures, but also allow sampling for location-specific quantification of biological responses. Examples of RHua models include MatTek’s EpiAirway™ and Epithelix’s MucilAir™, which are derived from primary cells of bronchial origin, but can also be created from cells of the nasopharyngeal region. Newer models include Epithelix’s SmallAir™ model, which has a population of Clara cells; and MatTek’s EpiAlveolar™ model, which includes epithelial, fibroblast and endothelial layers. A common feature of these RHuA models is that they offer an apical exposure site and they are all grown on a microporous membrane which allows nutrient delivery from the basolateral compartment containing medium. RHuA models are increasingly being used to assess inhalation exposures, such as cigarette smoke and e-cigarette vapors.

Figure 12.



In vitro/ex vivo models: RHuA

The PCLS model retains the native architecture of the lung and at least some of the common elements that occur *in vivo*. One of the benefits of the slices is the ability to see small areas, like the parenchyma in its native architecture. Also, all of the cells in the tissue are present at slicing, including the macrophages, an important consideration when looking at inflammatory responses. A limitation of this model is that larger airways might be excluded from slicing due to the size constraints of the slicing equipment. In the PCLS model, the cross-section of tissue is exposed and this differs from an exposure created within the airways and traveling down the airways as it occurs *in vivo*.

When comparing models, it's important to consider the exposure system and whether a vapor, aerosol or smoke is being exposed to the tissue itself. The differential particle/material distribution onto airway tissue regions is variable and dynamic. Questions remain about how to ensure that *in vitro/ex vivo* models receive the intended dose. For small airway or alveolar exposures, more research is needed to determine whether it's possible to limit exposure to just the particles/materials that reach the sites *in vivo*. The models are continuing to evolve.

Key points:

- **Creating accurate and dependable means to quantify pulmonary exposures to inhaled materials, including tobacco-related mixtures and constituents, is challenging due to the complex structure of the human lung.**
- **The use of state-of-the-art *in vitro* tissue models to obtain informative data for correlation back to *in vivo* pulmonary exposures adds yet another factor of complexity.**
- **A detailed understanding of how these models relate back to native human airway structures and the cells involved in responding to tissue challenge is required.**

***In Vitro* Toxicology of E-cigarettes and Other Tobacco Products**

Irfan Rahman, University of Rochester Medical Center

In this presentation, Irfan Rahman highlighted the potential deleterious oxidative and pro-inflammatory effects of e-cigarette aerosols when exposed directly to lung cells.

The consumption of electronic cigarettes is rising, particularly among young people. An alarming trend among younger users is the use of a “dripping” technique, in which the user drips an e-liquid directly onto the e-cigarette’s heating coil, rather than into the refillable chamber. (See **Figure**



Figure 13.

13.))The user then inhales the heated aerosol, which gives a stronger “hit,” as well as the ability to switch between brands, flavors or nicotine content.

It is well known that cigarette smoke and tars contain oxidants/reactive oxygen species (OX/ROS), which mediate inflammation and are implicated in the pathogenesis of lung diseases such as chronic obstructive pulmonary disease (COPD). A recent study, using a modified 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescein derived dye to detect OX/ROS reactivity in a cell-free system, showed that OX/ROS are generated in e-cigarettes and e-liquids as well.²⁹ One of the sources of the OX/ROS appears to be activation of the heating element. The amount of OX/ROS reactivity was also dependent on flavor additives. Flavors containing sweet or fruit flavors were stronger oxidizers than tobacco flavors.

Use of the dripping technique generated an even larger amount of OX/ROS, suggesting that this emerging trend delivers a larger dose of OX/ROS to users. Aerosols produced by dripping the e-liquid directly onto the heating element wick resulted in high range DCF fluorescence, as shown in **Table 4**.

Table 4.
“Dripping” technique in refillable e-cig leads to high range levels of oxidants.

	State of the heating element			
<i>Experiment 1</i>	New	2 nd use	3 rd use	4 th use
Powered	33.28	8.99	5.68	135.6
Air (sham)	1.60	1.50	1.39	-
<i>Experiment 2</i>				
	Pre-used			
	Clearomizer filled with e-liquid		Emptied clearomizer with wicked e-liquid	
Humectant	Trial 1	Trial 2	Trial 3	Trial 4
Consumer refill	47.55	37.42	192.40	250.50

Each value represents the H₂O₂ equivalents (μ M) measured after aerosols or clean air is drawn through dichlorodihydrofluorescein (DCFH) solution.

Source: Learner CA et al. PLoS One 2015;10:e0116732.

The same study also evaluated cellular toxicity and inflammation in human airway epithelial cells (H292) exposed to e-cigarette aerosols and nicotine. An air-liquid interface (ALI) system was employed that utilized a specialized trans-well chamber to mimic pulmonary air-liquid flow dynamics during e-cigarette aerosol exposures. Exposure of these cells resulted in increased secretion of pro-inflammatory cytokines, such as IL-6 and IL-8, into the culture media after the cells were allowed to culture for 16 hours. The H292 cells also showed an increased secretion of IL-8 in response to a cinnamon flavored e-liquid, suggesting that flavored e-aerosols, which contribute to encouraging frequent use of e-cigarette aerosols by young people, may also create more stress and toxicity on the lung tissue. Human lung fibroblasts also exhibited stress and morphological change in response to treatment with the e-liquids.

The researchers extended this study to a mouse model of e-cigarette aerosol exposure, using wild type (C57BL/6J) mice. The effect of short-term exposure (3 days) to e-cigarette aerosols on lung inflammation, oxidative stress, and redox physiology were examined by measuring changes in glutathione levels. The results showed that exposure to the e-cigarette aerosol increased pro-inflammatory cytokines and decreased total and oxidized levels of lung glutathione in the lung cells.

E-cigarette aerosols have also been found to contain copper, a transition metal that can generate even more free radicals.³⁰

A comparison of conventional cigarettes and e-cigarettes in mediating inflammatory responses will require further experiments in various settings, conditions and cell lines to understand the mechanisms. Studies have been carried out to assess non-invasive biomarkers of oxidative stress in breath condensate³¹ and the effects of oxidative stress and cigarette smoke on chromatin histone modification in lung cells.³²

Key points:

- Tobacco products, such as cigarette smoke and e-cigarette aerosols, are capable of

generating reactive oxidants and depleting glutathione in human lung cells and the mouse lung.

- **The oxidative reactivity produced by “dripping” techniques of e-liquids may place consumers at even greater risk for lung damage.**
 - **Differential *in vitro* toxicological testing is possible for different products for hazard ranking based on their chemical constituents.**
-

PROMISING TECHNOLOGIES

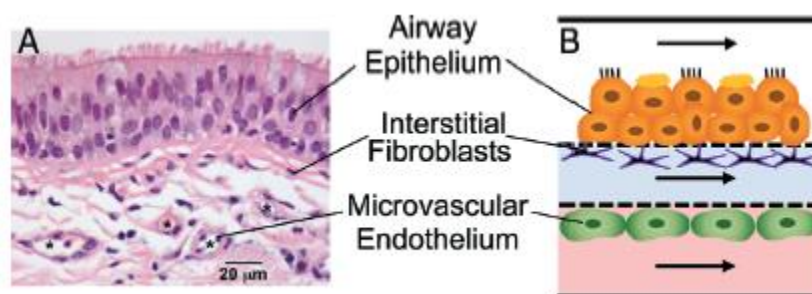
3D lung Tissue Constructs, Lung-on-a-Chip and Response to Toxicants

Sonia Grego, RTI International

In this presentation, Sonia Grego reviewed the features of RTI International’s three-dimensional “lung-on-a-chip,” a biomimetic multicellular model of the airways using primary human cells, and its potential use for studying responses to drugs and toxicants.

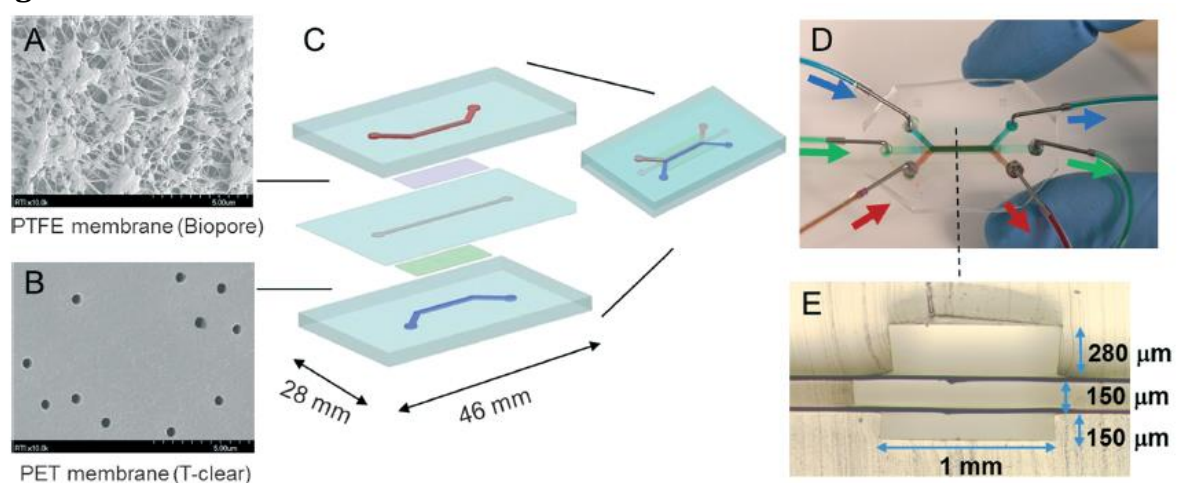
Recently, the development of biologically relevant three-dimensional (3D) models of human tissues has been intense. The goals of this research area are to achieve enhanced physiological relevance by controlling topography, biochemical, mechanical and fluid shear stress factors in an engineered microenvironment. These novel models strive to mimic the cell-cell interactions and tissue microarchitecture of the *in vivo* tissue. In addition to the lung model, engineered cellular models have been developed at RTI to study neuroinflammation and barrier properties in the neurovascular system³³ and drug adverse effects on the heart using stem cell cardiomyocytes.³⁴

Lung models have been designed to study the effects of drugs and respiratory virus infections. The lung-on-a-chip model developed at RTI international is a fluidic-enhanced airway model (FEAM) that uses three vertically stacked culture chambers to emulate the microarchitecture of the airway mucosa, as illustrated in **Figure 14**. The primary human cells used are airway epithelial cells (AE) at the air-liquid interface, fibroblasts (Fb) to mimic the lung interstitium, and a polarized microvascular endothelial (MvE) cell layer.³⁵

Figure 14.

(A) Histology cross section of normal human bronchus from a lung transplant donor (hematoxylin and eosin stain). Asterisks mark capillaries. (B) Schematic of the airway mucosa model including three vertically stacked compartments with three different cell types separated by two nanoporous membranes, arrows indicate channels for fluid or air. From Sellgren et al. Lab Chip 2014;14:3349-58.

The multi-compartment microfluidic devices are single-use, gas permeable devices, fabricated in optically transparent polydimethylsiloxane (PDMS). Cells are cultured on two nanoporous membranes (0.4 μm pore size) that provide support for the AE and MvE. Vertically stacked flow is achieved by the three-compartment design, which is obtained by sequential bonding of two different membrane types, as shown in **Figure 15**. For triple co-cultures, membranes are collagen-coated with different collagens by filling the top, middle and lower compartment with the collagens and letting them dry.³⁶ Challenges associated with the devices include dealing with long-term primary cell cultures in microfluidic devices and the fact that each primary cell type prefers a specific medium. Throughput for culture in microfluidic devices is much lower than in conventional static cultures.

Figure 15.

Microfluidic device configuration. (A) and (B) scanning electron microscope images of the polytetrafluoroethylene (PTFE) and polyester (PET) membranes; (C) exploded view and schematic (D) photograph of a 10 × 1 mm device with dyes in the three fluidic channels; (E) optical microscope image of a 10 × 1 mm device cross section.

From Sellgren et al. Lab Chip 2014;14:3349-58.

Other lung-on-a-chip models include a device developed by Ingber et al. that features a stretchable membrane and *in situ* mechanical “respiration.”³⁷ This microfluidic device reconstitutes the alveolar-capillary interface of the human lung using the H441 alveolar epithelial cell line and primary MvE. Normal breathing patterns are mimicked using channels that experience air and fluid flow and cyclic mechanical strain. PDMS is used as the membrane material.

The presentation included a few slides with a primer on the features and operation of microfluidic cell cultures. Microfluidic devices are transparent and routinely observed by microscope. Water-tight operation in a humidified environment is achieved long-term (5 weeks). Cells at seeding and reagents for assays are introduced with syringes (e.g. needle gauge 22 fits tightly into tubing and is appropriate for this use). Constant fluid replenishment occurs by passive flow and requires no external power. For specific assays, a faster fluid flow is achieved by active pumping by a small peristaltic pump which fits in an incubator.

Microfluidic co-culture with all primary airway cells were demonstrated. Six human lungs were processed to obtain primary AE, Fb and MvE. Experiments using AE cells at the air-liquid interface were carried out in parallel in Millicells® and microfluidic devices. A primary AE cell culture in microfluidic devices was achieved with well-differentiated, mucus secreting and ciliated cells. Airway epithelial cell barrier properties were characterized. Fluorescein isothiocyanate (FITC)-dextran permeability was used for barrier property characterization. Functional co-cultures were demonstrated first in Millicells at day 5 in co-cultures of primary AE and MvE cells. The results showed that AE formed a much tighter barrier than MvE. AE/MvE co-culture permeability was dominated by the AE component.

A pilot study is being carried out to compare the responses of co-cultures and monocultures to toxic compounds (interleukin-2, bleomycin) and effective drugs (dexamethasone). All compounds are delivered to the basolateral compartment and added to the medium. Cytokine release and barrier properties will be assayed. The response of the co-culture in selected cases and endpoints is different from the combination the response of its component, indicating the effect of cell-cell interactions.

Key Points

- **Microfluidic airway models based on primary human cells in a relevant biomimetic configuration will improve physiological relevance and will enable novel disease modeling and drug development studies.**
- **The feasibility of the lung-on-a-chip with all primary human lung has been demonstrated**
- **Microfluidic cell cultures feature trade-off between enhanced functionality and throughput.**
- **Co-culture responses to perturbation differed from that of individual cells, demonstrating that heterotypic cell interactions matter, i.e. “the whole is different than the sum of the parts.”**

Quantitative High-Throughput Gene Expression Analysis Using a Modified RASL-seq Platform Enables Treatment-Response Kinetic Analysis for Risk Assessment
Pei-Hsuan Chu, National Center for Advancing Translational Sciences/National Institutes of Health

In this presentation, Pei-Hsuan Chu described a highly reproducible, automated, high-throughput gene expression detection platform used by researchers at the National Center for Advancing Translational Sciences (NCATS). This platform, known as *RNA-mediated oligonucleotide Annealing, Selection, and Ligation with Next-Gen sequencing* (RASL-seq), bypasses the cDNA synthesis step that contributes the most variation between assays, to obtain a direct measurement of relative abundance of target transcripts.

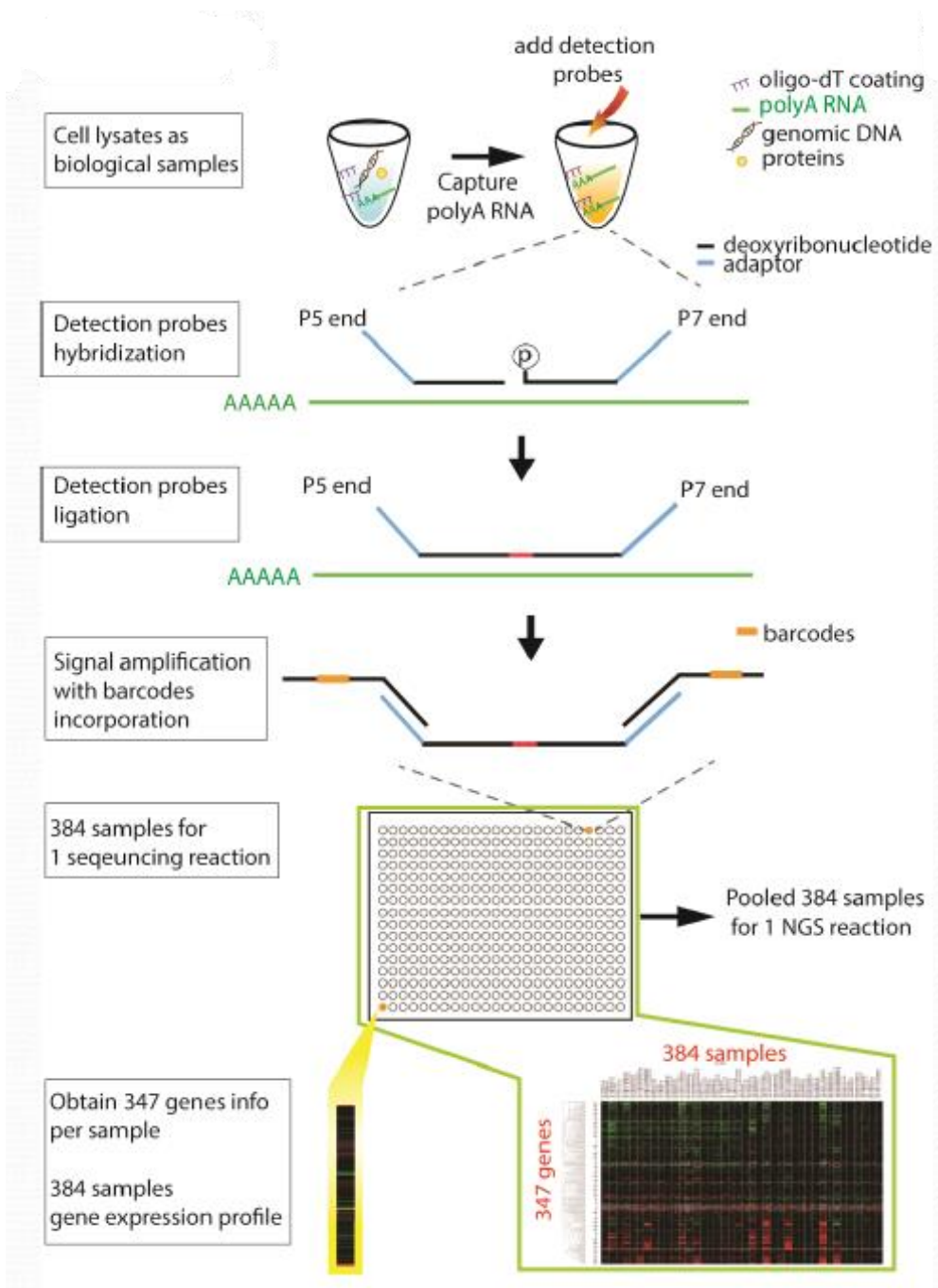
With the advent of next-generation sequencing (NGS) technologies, genome-wide RNA sequencing (RNA-seq) and targeted sequencing methods have recently been exploited for comprehensive transcriptome analysis. However, despite the genome-wide information provided by RNA-seq methods, some issues remain with the current gene expression assays. These include the biases introduced during library preparation as well as inter-experiment variability. The cost of RNA-Seq remains very high, and therefore limits the number of doses, replicates and compounds analyzed.

The RASL-seq method is a new approach that can be applied to toxicogenomics, a field of science that encompasses toxicology, genetics, molecular biology and bioinformatics to describe the response of organisms to chemical exposure. Toxicogenomics not only reveals mechanistic information of observed toxicity, but also captures the early stages of adverse events that may not show in the endpoints of cell-based assays.

The RASL-seq method used at NCATS is based on a model originally developed by Li et al.³⁸ This technology, which takes advantage next-generation sequencing, bypasses isolation of RNA from samples and the cDNA synthesis step permitting quantitative profiling of several hundred selected genes in a large number of samples. The assay was changed from using magnetic beads to oligo-dT coated plate to enable fully automation and avoid the uneven loss of magnetic beads during liquid handling. The DNA probes annealing step was separated from the cell debris to minimize unpredicted interaction due to the lysis buffer, genomic DNA or other cell debris. The assay provides direct analysis of RNA levels in cell lysates and is adaptable to full automation. RASL-seq has proven highly reproducible, as well as low in cost.

NCATS researchers are using an improved RASL-seq platform, summarized in **Figure 16**, to gather dose- and time-response data to help show the toxic mechanisms of various compounds. This improved method, the Turbo RASL-seq procedure, currently multiplexes for 347 genes, up to 3 probes per gene per sample, and gathers information for 384 samples in one sequencing reaction. The very high reproducibility of this assay enables the study of compound effects over multiple time points with full dosage coverage. NCATS has also developed a quantitative analysis method to elucidate the dose-response relationships for each gene upon treatment and identify the BenchMark Dose (BMD) and Point Of Departure (POD).

Figure 16.



Turbo RASL-seq procedure

Method modified from Li *et al.* 2012, PNAS, 109(12):4609-4614

NCATS is currently applying the RASL-seq assay to screen tobacco toxicants, using immortalized cells and induced-pluripotent stem cells (iPSC)-derived endothelial cells, to determine how smoke-derived chemicals interact with human cells by their genetic alteration. In a recent study, expression of 347 stress genes was measured in human umbilical vein endothelial cells (*HUVEC*) exposed to 18 tobacco components.³⁹ Detailed dose-response relationships on each gene were

established using the quantitative analysis method to identify the BMD and POD.

The RASL-seq method produced highly reproducible gene expression data with high-throughput and low cost to quantitatively assess the adverse effects of individual chemical components of tobacco smoke. The computational method identified the BMD and POD, based on the change of gene expression over toxicant concentration for each gene and treatment. These measures quantified the dose-dependence of gene responses and enabled a pathway analysis to clarify the mode of action of each toxicant.

Key points:

- **The RASL-seq gene expression platform achieves high throughput, high quality gene expression data at low cost.**
 - **Quantitative gene expression data enables identification of BMD and POD for risk assessment.**
 - **POD heat maps can condense gene expression information from multiple dosages and time points and reveals both the potency and trend of expression changes.**
-

Predicting Exposure Response in the Airway: Integrating Cellular Signaling and Epigenetics Through the Epigenetic Seed and Soil Model of Interindividual Variability

Shaun McCullough, US Environmental Protection Agency

In this presentation, Shaun McCullough explored the field of epigenetics and its potential for transforming our understanding of interindividual variability in response to toxic exposures.

Despite the advancement of many approaches that examine the role of histone modifications, a key aspect of the epigenome, relatively little has been done to date to examine the role of the epigenome in exposure effects and susceptibility *in vitro*. The broad range of responsiveness between individuals to air pollutant exposure has not been well explained by current susceptibility models, and the mechanisms underlying this inter-individual variability remain elusive. Traditional susceptibility markers do not faithfully explain variability and gene variants do not completely explain susceptibility.

The epigenome and its role in interindividual variability is becoming an important consideration in toxicology and risk assessment. The epigenome is essentially a “master regulator” of gene transcription. It is characterized by heritable factors that regulate gene expression without changing the DNA sequence. That is, the epigenome alters how genes are used without altering the genes themselves—changing phenotype without changing genotype.

The framework of the epigenome is chromatin, which is composed of nucleosomes, the basic units of DNA packaging. Each nucleosome has eight histone proteins (two each of histones H2A, H2B, H3, and H4), joined together by linker histones. Histone “tails” extend out of each histone and can serve as substrates for modifications by acetylation, methylation and other chemical groups.

Epigenetic regulators, such as chromatin modifications and DNA methylation, function as critical and dynamic mediators of gene expression and shape how cells, tissues and individuals respond to their environment. In toxicology, the role of the epigenome has mainly been explored by

looking at changes in DNA methylation in response to exposures. However, more recent findings indicate that histone modification patterns can predict variability in gene expression and have implicated histone modifications as susceptibility factors in a number of diseases.

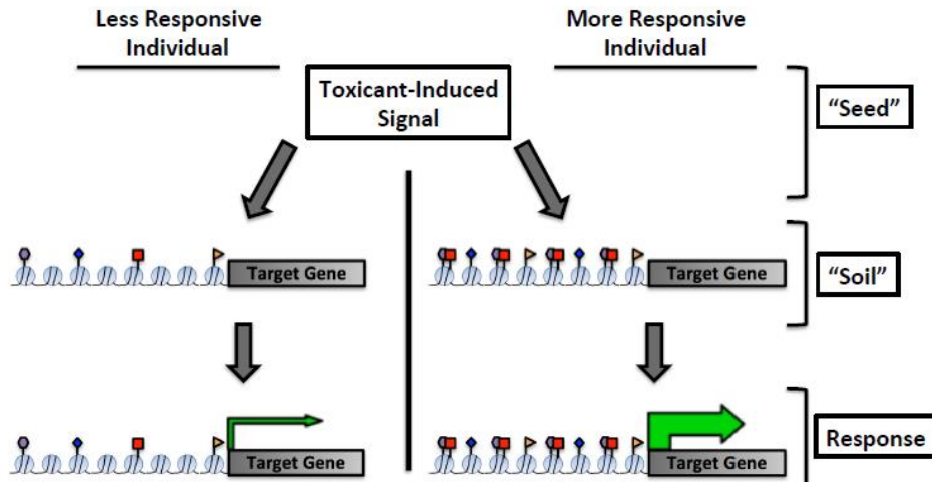
Researchers at the Environmental Protection Agency carried out a study to test whether inter-individual variability in basal and toxicant-induced gene expression resulted from differences in baseline patterns of chromatin modifications that existed prior to exposure.⁴⁰ Specifically, they sought to determine whether baseline levels of certain chromatin modifications correlated with the inter-individual variability in ozone-mediated responses in an air-liquid interface model of primary human bronchial epithelial cells. Ozone is a model air pollutant that induces the expression of pro-inflammatory mediators and markers of oxidative stress both *in vitro* and *in vivo*. The airway epithelium, the barrier between the lung and the environment, plays a critical role as a modulator of pro-inflammatory and oxidative stress in response to environmental exposures.

During the course of this study, two distinct mitogen activated protein kinase (MAPK) pathways (EGFR/MEK/ERK and MKK4/ p38) were identified as the drivers of the cellular response to ozone.⁴¹ This varies from traditionally accepted findings in cell lines implicating the NF- κ B pathway. While the ozone-mediated induction of pro-inflammatory cytokines (IL-8, IL-6, COX2, IL-1 α , and IL-1 β) varied between donors, the relative activation of MAPK signaling was similar. Given this similarity, the researchers hypothesized that the variability in responsiveness originated downstream of MAPK signaling, specifically in the patterns of chromatin modification within the regulatory regions of target pro-inflammatory genes.

The results showed that pre-exposure patterns of chromatin modifications (histone H3 lysine 4 trimethylation, H3 lysine 27 di/trimethylation and 5-hydroxymethylcytosine) correlated with the magnitude of post-exposure pro-inflammatory gene expression. These findings highlighted the utility of advanced *in vitro* models in modern mechanistic and epigenetic toxicology. They also contributed to the establishment of physiologically relevant *in vitro* models as the foundation of the emerging field of epigenetic toxicology.

In summary, the researchers proposed an “epigenetic seed and soil” model to describe the epigenetic basis of inter-individual variability in exposure responses, as shown in **Figure 17**. In this model, toxicant-induced cellular signals (the “seed”) interact with the chromatin landscape in the nucleus to alter the expression of toxicant-responsive genes. Specific chromatin modifications (the “soil”) prior to exposure varies between individuals. These intrinsic variations in the soil regulate the magnitude of exposure-related gene induction.

Figure 17.



Seed and soil model for the epigenetic basis of interindividual variability in exposure responses.

Note: Dr. McCullough is chairing a Society of Toxicology Contemporary Concepts of Toxicology meeting with Dana Dolinoy, PhD from the University of Michigan on "Toxicoepigenetics: The Interface of Epigenetics and Risk Assessment" (www.toxicology.org/teg) this coming November in Tyson's Corner, Virginia.

Key points:

- **Epigenetic regulators function as critical and dynamic mediators of gene expression and shape the way cells, tissues, and individuals respond to their environment.**
- **The emerging field of epigenetic toxicology will ultimately play a critical role in our understanding of exposure-associated health effects and susceptibility.**
- **The epigenome has the potential for being a transformative tool for risk assessment.**

BREAKOUT DISCUSSION GROUPS

Overview

Moderated breakout groups were held in two subject areas: *in vitro* exposure systems and dosimetry. All groups had the same goals:

- Understand the advantages and weaknesses of each of the major *in vitro* exposure systems
- Define the major points to consider when extrapolating from *in vitro* to direct human exposure
- Understand the advantages and drawbacks of the various dosimetry tools
- Identify the limitations of the current *in vitro* exposure systems and dosimetry tools and propose activities to address these limitations or gaps

The *in vitro* exposure group was provided with the following set of questions to guide the discussions:

- A: What is the utility domain of each exposure system?
1. Is it useful for both particulate and gaseous exposure?
 2. Is it limited to certain physical configurations of cells or tissues?
 3. Is it adaptable to different smoking regimens?
 4. Can different model types be exposed?
 5. What are the major shortcomings of the system?
- B: How well characterized is the exposure system?
1. Ease of use and throughput?
 2. Available to other labs?
 3. Portability to other laboratories?
 4. Interlaboratory reproducibility established?
 5. Used with tobacco-related materials (chemicals)?
- C: What research activities can be proposed to address any gaps and limitations?

The dosimetry group was provided with the following question to guide the discussions:

- A: What is the state-of-the-art for *in vitro* dosimetry measurements?
1. What measurements allow the most useful comparisons to be made with historical toxicology studies?
 2. What measurements allow the most useful comparisons to human clinical studies?
 3. What measurements are sufficient for e-cigarettes?
 4. What measurements are sufficient for heat-not-burn tobacco products?
 5. Are there measurements that should be performed to assess the exposure to all types of tobacco products?
 6. Should thresholds be placed on limits of detection?

Assumptions

In discussing exposures the break out group made the following assumptions:

- Products to address include:
 - Tobacco related products(cigarettes, tobacco heated products, cigars, cigarillos)
 - Nicotine delivery devices (e-cigarettes)
 - Other products (e-shisha, water pipes)
 - Possibly other aerosols/particles (nanoparticles)
- The methodology would be:
 - Of value to regulatory community and research
 - *In vitro*
 - Compatible with good laboratory practices
 - Transferable between laboratories
 - Characterization, robustness, repeatable, flexible

Aerosol generation

The following should be addressed when considering the generation of aerosols:

- Regulatory testing and basic research applications
- Reproducibility
- Available dosimetry tools
- Transferability between laboratories
- Flexibility to generate smoking regimes and/or other human smoking profiles

Dilution principle

The following should be addressed regarding dilution principles:

- Available dosimetry tools
- Transferability between laboratories
- Flexibility for aerosols
- Good mixing, minimal dead space
- Dilution working range (to be defined)
- Quantifiable retention time
- Controlled air source

Cellular exposure modules

The following should be addressed in preparing cellular exposure modules:

- Focus on tobacco and nicotine delivery products
- Direct exposure to biological test systems
- Available dosimetry tools
 - Must be able to determine dose at tissue surface
- Transferability between laboratories
- Uniform exposure within module
- Flexibility for aerosols
- Minimal effects of “exposure physics” on cells
- Easily cleanable/ reusable
- Flexibility for different cell/tissue types/insert

Study design

The following should be addressed in designing an exposure study:

- Air-liquid interface exposures should include “sham,” incubator, and positive and negative controls
 - Good cell culture must be practiced
 - Replicates should be reported
 - Dynamic exchanges should be implemented if media is exposed to aerosol
 - Insert quality/ type must be considered
 - Endpoints should reflect the pending question being addressed
-

Dosimetry

Goals of the breakout group

- The breakout group focused on addressing Item #22 of the 56 research priorities established by the Center for Tobacco Products, Food and Drug Administration, in January 2012:
 - “What in vitro and in vivo assays are capable of comparative toxicity between two different tobacco products; with special attention to cardiotoxicity, respiratory toxicity, carcinogenicity, and developmental/reproductive toxicity?”
- Findings and recommendations should support comparisons of exposure, dosimetry and biological end effect between new and predicate products.

Areas in the *in vitro* exposure system where dosimetry is most important

- Dosimetry techniques should provide an understanding what is dosed onto the test system, including:
 - Its representative chemical profile
 - Its quantity
- At the site of test system exposure, current systems include surrogate targets like QCM, cell-containing or cell-free inserts, and media.
- Dosimetry techniques support a reasonable comparison of new and predicate product exposures.
 - Applying dosimetry immediately after puff release from the tobacco product allows further comparison of chemical profiles between new and predicate products
- Utilizing the standardized historical approaches for chemical/particulate capture (Cambridge filter pads, impinged aqueous and organic fractions, etc.) supports vital historical data bridging
- Characterizing the smoke/vapor generator and exposure system is critical.
 - Elucidate the impacts of the system on the vapor/smoke chemistry.
 - This may also help in optimizing:
 - Future exposure platforms to minimize artifacts.
 - Exposures to better mimic the exposures at specific sites along the human respiratory tract.

Substances and factors that can be analyzed in various product types

- Identifying and standardizing analytes is an important future goal. Some product types discussed by the breakout group include the following:
 - Non-nicotine devices: glycerol

- e-cigarettes: nicotine, glycerol
 - Absence of standard puff profile (CORESTA profile for one unique device)
- Heat-not-burn (HNB) products: nicotine, particle size
- Combustible products, with reference cigarettes as a basis
 - TNCO (tar, water nicotine and carbon dioxide)
 - TSNAs (tobacco-specific nitrosamines)
 - Reactive aldehydes
 - Puff profiles, standardized by ISO/HCI
- The list is not fully inclusive and needs further development.

Questions to consider when comparing products

- New vs. reference/predicate products
 - Was the dosimetry sufficient to support the conclusions of the assay or study?
 - What gaps exist at “in situ” measurement (i.e., how do we ensure target tissue dosimetry?)

Future Considerations

- Improve the ability to measure gas/vapors immediately at the ALI without interfering with exposure
 - Use specific “sensors,” such as ROS sensors
 - Consider including non-cellular chemical reactions for detection
 - Explore target cell-specific dosimetry, i.e., cell:molecule interactions
 - Determine if this is needed for regulatory submissions
 - Be amenable to various platforms including high throughput systems
 - Ensure compatibility with new test system technologies (i.e., lung on a chip)
 - Use models of *in vivo* exposures to optimize the *in vitro* exposure systems and associated dosimetric tools
-

ABBREVIATIONS

ACM	aerosol collected mass
ALI	air-liquid interface
AOP	adverse outcome pathway
AUC	area under the curve
BAT	British American Tobacco
BMD	benchmark dose
CMAG	condensation monodisperse aerosol generator
COPD	chronic obstructive pulmonary disease
CFD	computational fluid dynamic
CTP	Center for Tobacco Products (FDA)
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
GC x GC	two-dimensional gas chromatography
GVP	gas vapor phase
HCI	Health Canada Intense
IIVS	Institute for In Vitro Sciences
ISO	International Organization for Standardization
ISDD	<i>in vitro</i> sedimentation diffusion dosimetry
LADD	lifetime average daily dose
MPPD	multiple path particle density
MTD	maximum tolerated dose
NCATS	National Center for Advancing Translational Sciences
NCCT	National Center for Computational Toxicology
NHLBI	National Heart Lung and Blood Institute
NIEHS	National Institute of Environmental Health Sciences
NIH	National Institutes of Health
NOAEL	no observed adverse effect level
NRC	National Research Council
NRU	neutral red uptake
OX/ROS	oxidants/reactive oxygen species
PCLS	precision-cut lung slices
PBPK	physiologically based pharmacokinetic
POD	point of departure
QCM	quartz crystal microbalance
RASL-seq	RNA-mediated oligonucleotide Annealing, Selection, and Ligation with Next-Gen sequencing

RHuA	reconstructed human airways
ROS	reactive oxygen species
SEIVS	smoke exposure <i>in vitro</i> system
TOF-MS	Time-of-flight mass spectrometry
Tox21	Toxicology in the 21 st Century
TPM	total particulate matter
ZTRI	Zhengzhou Tobacco Research Institute

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