Advancing Human-Relevant *In Vitro* Methods to Assess Respiratory Tissue Irritation

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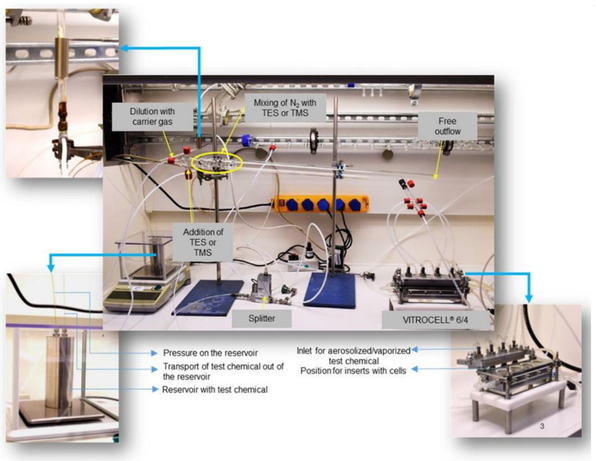
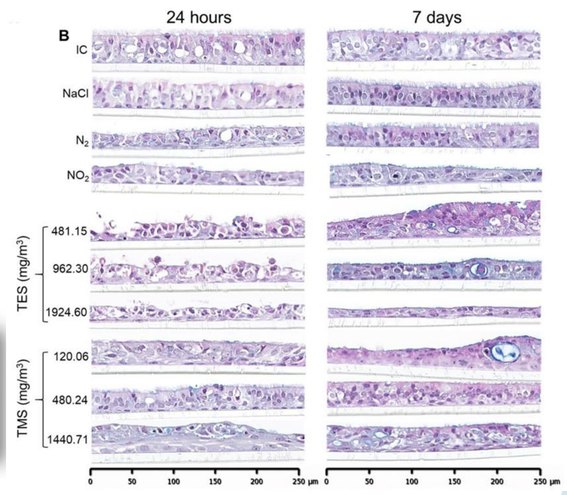
While inhalation toxicity testing has traditionally been conducted in rats, differences in the respiratory tracts of humans and rats limit the precision with which rats can reliably predict human effects. Therefore, *in vitro* models are increasingly being used to assess the toxicity of inhaled substances. Here, we present on our collaborative, multistakeholder efforts over the past ten years to establish scientific confidence in an *in vitro* approach for assessing respiratory tissue irritation potential of inhaled substances on the respiratory tract.

2023: INSPIRE Initiative – silane testing

Our INSPIRE (*IN vitro* Systems to PredIct REspiratory toxicity) Initiative aimed to (1) build scientific confidence in *in vitro* testing approaches to predict respiratory toxicity and (2) identify relevant cellular effects, exposure methods, and model systems that may be most appropriate for use, depending on the purpose of testing. Very rapidly hydrolizing silanes—triethoxy- and trimethoxysilane—were tested in two cell systems (BEAS-2B and MucilAir) using a VITROCELL 6/4 exposure system. Testing was conducted at the Flemish Institute for Technological Research (VITO). See Sharma et al., *Toxicol Sci*, 2023 for more information



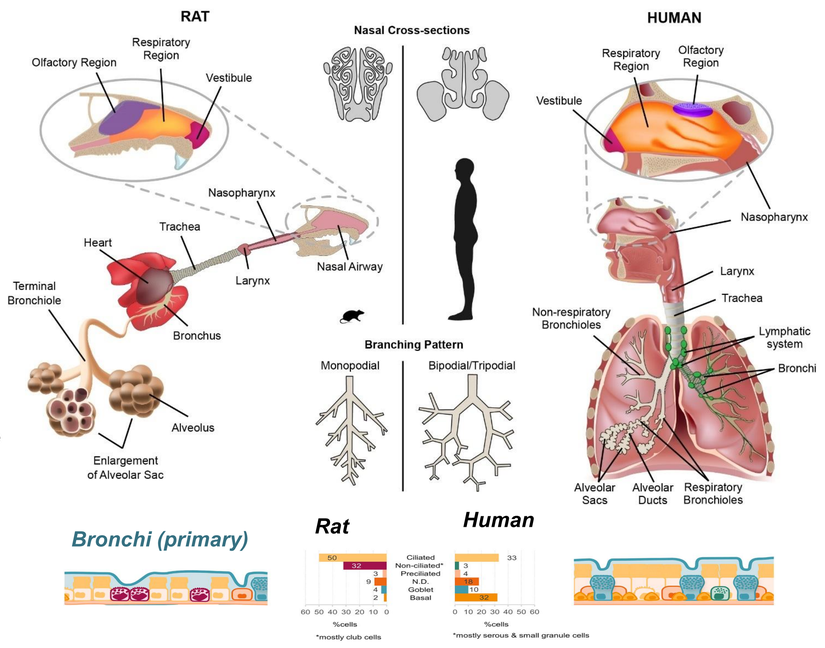


2016/2018: Workshop

In 2016, a workshop was organized together with the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) on "Alternative Approaches for Acute Inhalation Toxicity Testing to Address Global Regulatory and Non-Regulatory Data Requirements" (Clippinger et al., *Toxicol In Vitro*, 2018a). The workshop led to a review paper (Clippinger et al., *Toxicol In Vitro*, 2018b), which outlined predictive frameworks for non-animal assessment of acute inhalation hazards, and ultimately gave rise to the INSPIRE Initiative. Furthermore, it was recommended that more *in vitro* case studies should be performed.

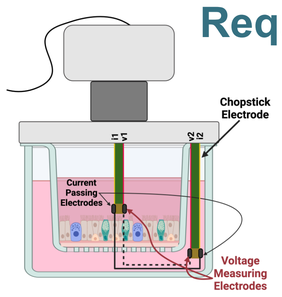
2024: Rat vs. human differences

A comprehensive review of the anatomical and physiological differences between the human and rat respiratory tracts demonstrated key limitations of rat-based inhalation testing for predicting human outcomes. Differences in breathing patterns, airway geometry, cell type distribution, and mucociliary clearance significantly impact toxicological assessments, supporting the use of human-relevant *in vitro* approaches (Stucki et al., *Regul Toxicol Pharmacol*, 2024).



Cell types in the bronchial regions differ substantially in rats and humans

2025: Minimum Information Requirements for TEER Assay

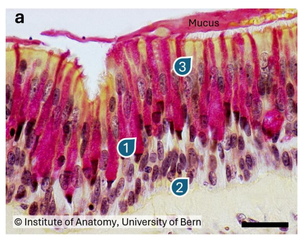


Standardizing *in vitro* practices by developing minimum reporting recommendations helps facilitate repeatability and reproducibility, thus facilitating cross laboratory comparisons of data and regulatory acceptance. A paper on the Minimum Information for Reporting on the TEER Assay (MIRTA) is the result of the work of the RespTox Collaborative, an international, cross-sector consortium of experts conducting *in vitro* inhalation toxicity testing (Sharma et al., *Arch Tox.*, 2024).

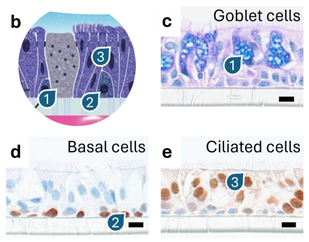
TEER: Trans-Epithelial/Endothelial Electrical Resistance

2026: Human relevance of RHRE

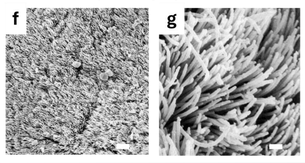
Human respiratory epithelium



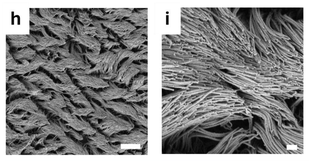
MucilAir



Human respiratory epithelium



MucilAir



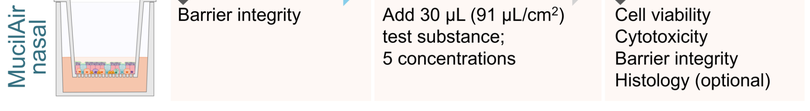
A review of the human biological relevance of reconstructed human respiratory epithelium (RHRE)—focused on the MucilAir model—established scientific confidence in its use to assess respiratory effects. Made from primary human cells and grown at the air-liquid interface, MucilAir contains the same basal, ciliated, and mucus-producing goblet cells found in human nasal, tracheal, and bronchial epithelia, with comparable cell proportions, mucus and epithelial layer thickness, beating cilia, tight-barrier function, active ion transport, and demonstrated metabolic competency. It has supported regulatory decisions for the fungicide chlorothalonil (US EPA, 2021) and the cosmetic ingredient acetylated vetiver oil (SCCS, 2024) (Roldán et al., Front. Toxicol., 2026).

2026: Multilaboratory study

US UK India

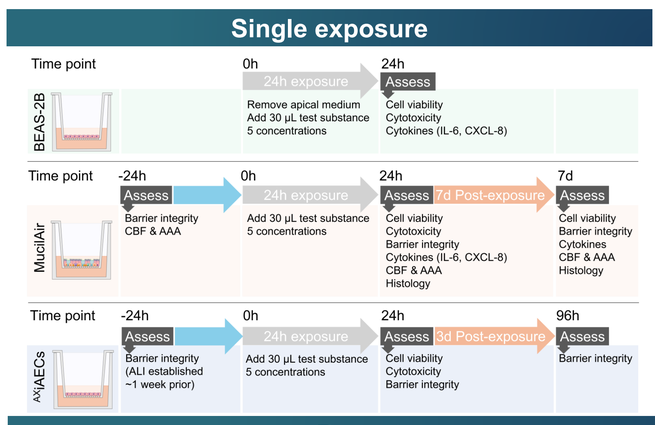
3 Labs

Building on the lessons learned from the INSPIRE Initiative, a multi-laboratory study was conducted across three contract research organizations in the UK, the US, and India to build scientific confidence in a method to assess respiratory tissue irritation. The study applied a harmonized protocol—similar to the protocol that underpinned the successful regulatory case studies for the fungicide chlorothalonil (US EPA, 2021) and the cosmetic ingredient acetylated vetiver oil (SCCS, 2024). Barrier integrity (via TEER), cell viability (via PrestoBlue assay), and cytotoxicity (via lactate dehydrogenase assay) were assessed. Additionally, histology was performed in some of the laboratories.



22 reference chemicals were selected through a transparent literature review using the SysRev platform and span a range of expected respiratory responses, from non-toxic to severely irritating. Here, we focused on 22 highly soluble, low vapor pressure chemicals that will likely affect the nasal region. Currently, data is being analyzed and a validation report is in preparation.

2026: INSPIRE Initiative – surfactant testing

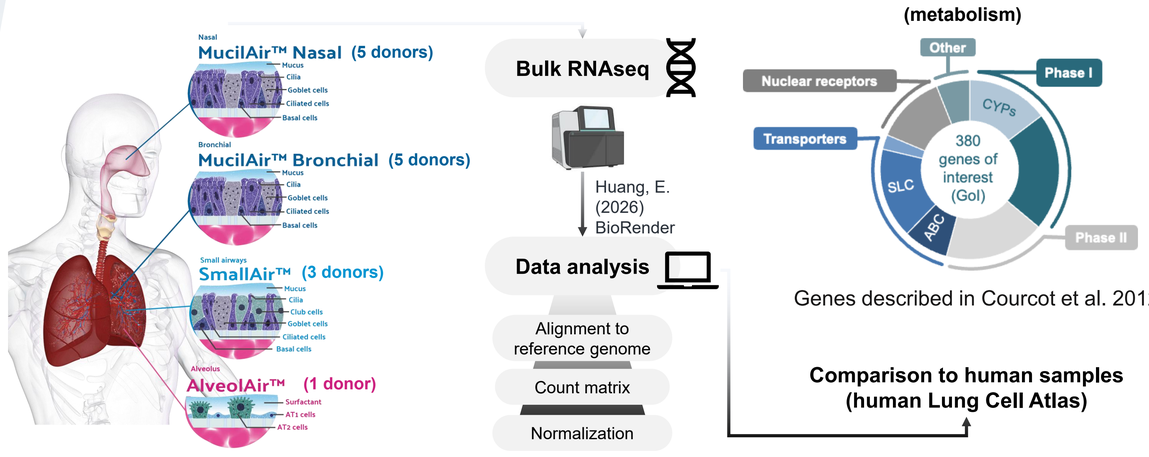


We further assessed the respiratory toxicity of two surfactants (oleoyl sarcosine and Triton X-100) using three complementary *in vitro* approaches: 1) single-dose exposure in bronchial cells (BEAS-2B and MucilAir), 2) repeated-dose exposure in MucilAir, and 3) single-dose exposure in an alveolar model (^{AX}iAEC).

Both surfactants were tested at multiple concentrations via liquid application, with cellular effects evaluated across cell viability, cytotoxicity (LDH), barrier integrity (TEER), cytokine release, and cilia function (where applicable). Dose-dependent effects were observed across all cell systems. Oleoyl sarcosine caused more pronounced and sustained disruption, while Triton X-100 showed evidence of tissue adaptation and recovery.

For the single exposure bronchial study, an *in vitro* to *in vivo* extrapolation (IVIVE) workflow including MPPD and BMD modelling were used to derive human equivalent concentrations (HECs) for hazard assessment, reducing reliance on animal-derived data. Manuscripts are in preparation or have been submitted.

2026: Metabolic capability of RHRE

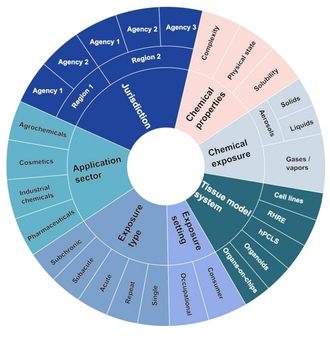


Following inhalation, local enzymes in the respiratory tract can metabolically activate or deactivate xenobiotics, and because cell types and enzyme profiles differ between the nasal, bronchial, small airway, and alveolar regions, the same substance may produce different effects in different regions. Characterizing the metabolic competency of RHRE is therefore essential to confidently use these models for toxicological assessment of inhaled substances. To address this, gene expression was compared across four region-specific RHRE models (MucilAir nasal, MucilAir bronchial, SmallAir, and AlveolAir) and benchmarked against human reference datasets to identify which models best reflect the metabolic biology of each region.

Ongoing: Development of OECD Test Guideline

Building on this work, in November 2025 we (as a member of ICAPO) submitted a project proposal to the OECD together with the United States, which was adopted onto the OECD workplan at the end of June 2026. The addition to the workplan is the first step for the development of an OECD Test Guideline (TG). It is aimed to become the first *in vitro* TG for inhalation toxicity testing.

This TG will first be applicable to highly soluble, low vapor pressure chemicals applied by pipetting trying to answer their potential to cause respiratory tissue irritation—one of several adverse outcomes in inhalation exposures. It is envisoned that the wider community can extend the applicability domain over time, by adding other test systems (from different regions of the lung), aerosol and vapour exposure methods, and additional endpoints to progressively broaden the range of substances and effects that can be assessed without animals.



Ongoing: Webinars, Training, and Awards

Over the years we have donated lab equipment and tissues, travel awards, and continue to provide free trainings and webinars:

In vitro (tissue) donations	Equipment donations	Travel awards	Training and webinars
•RHRE tissues from Epithelix (2018. 2023, 2024, 2026) •RHRE tissues from Mattek (2015, 2021) •Recombinant antibodies	•Electrodes and voltohmmeter for TEER •Exposure devices: •VITROCELL (2017, 2019) •MedTech Biolab (2021) •Other (flow cytometer, Tecan dispensers)	•Conferences •Hands-on training	•EPIC Webinar Series on the Use of NAMs in Risk Assessment (ongoing) •Inhalation specific webinar series (2018, 2020, 2021)

Scan QR code for a PDF copy of the poster and links to our email list, awards, and all references:

We thank all our collaborators who have supported this work over the years, including **Flemish Institute for Technological Research (VITO), Epithelix, AlveoliX, BASF, Helmholtz Institute Munich, U.S. Environmental Protection Agency, NICEATM, Institute for In Vitro Sciences (IIVS), Charles River Laboratories (CRL), JRF India, and many more**.