



Abstract

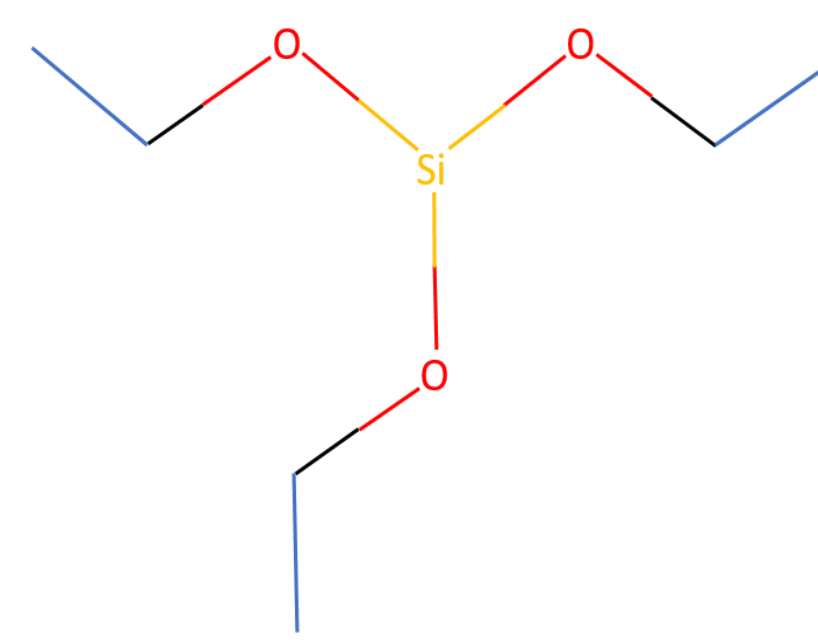
Silanes are widely used as reducing and coupling agents with applications in surface modifications. Because of their reactivity and rapid hydrolyzation, occupational exposure to silanes is possible in the production line. In this study, called the INSPIRE Initiative (*IN vitro* System to Predict REspiratory toxicity), a human bronchial epithelial cell line (BEAS-2B) and a reconstructed human tissue model (MucilAir™) were exposed to triethoxysilane (TES) and trimethoxysilane (TMS) as vapors, to predict the ability of these chemicals to cause portal-of-entry effects on the human respiratory tract.

Three concentrations were tested for each silane in BEAS-2B cell line (TES: 1, 50, and 150 ppm, and TMS: 1, 25, and 85ppm) and MucilAir™ tissues (TES: 75, 150, and 300 ppm, and TMS: 25, 100, and 300 ppm). All exposures were performed for 30 minutes at the air-liquid interface (ALI) using a VITROCELL® 6/4 system and appropriate negative (sodium chloride, incubator control, or nitrogen gas) and positive (nitrogen dioxide) controls were used. Endpoints were assessed 19-24 hours (BEAS-2B and MucilAir™) or seven days (MucilAir™) after exposure and included cell viability (PrestoBlue™ assay), cytotoxicity (lactate dehydrogenase assay), and secretion of inflammatory markers (electrochemiluminescence immunoassay). For MucilAir™ tissues, histology (hematoxylin and eosin staining), barrier integrity (transepithelial electrical resistance (TEER)), and cilia beating frequency (CBF) and average active area (AAA) (Sisson-Ammons Video Analysis (SAVA) system) were also included.

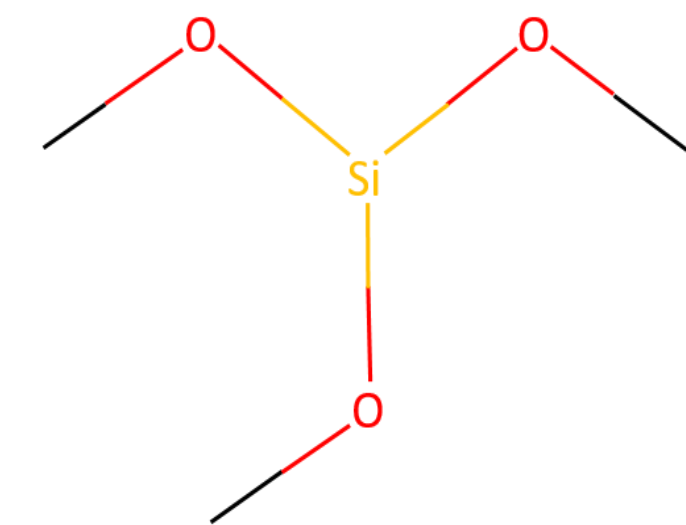
In BEAS-2B cells, a dose-dependent response was observed for all endpoints for both silanes. 19-24 hours after exposure of MucilAir™ tissues, the results show decreased cell viability, TEER, and AAA, and an increase in cytotoxicity, inflammatory response, and CBF for all concentrations of both silanes. Seven days after exposure, a further decrease in cell viability and AAA was observed and inflammatory response and CBF remained elevated, indicating that the cellular damage due to exposure to silanes was substantial. Interestingly, barrier integrity was restored back to pre-exposure values. The results from both *in vitro* systems indicate that TMS is more toxic than TES, which is expected based on chemical properties and existing data.

Studies are underway to assess additional test chemicals and to compare ALI exposure to direct pipetting in both systems. The results of this project can be used to better understand the usefulness of *in vitro* systems in assessing the impact of chemicals on the human respiratory tract and inform regulatory decision-making.

Test Chemicals

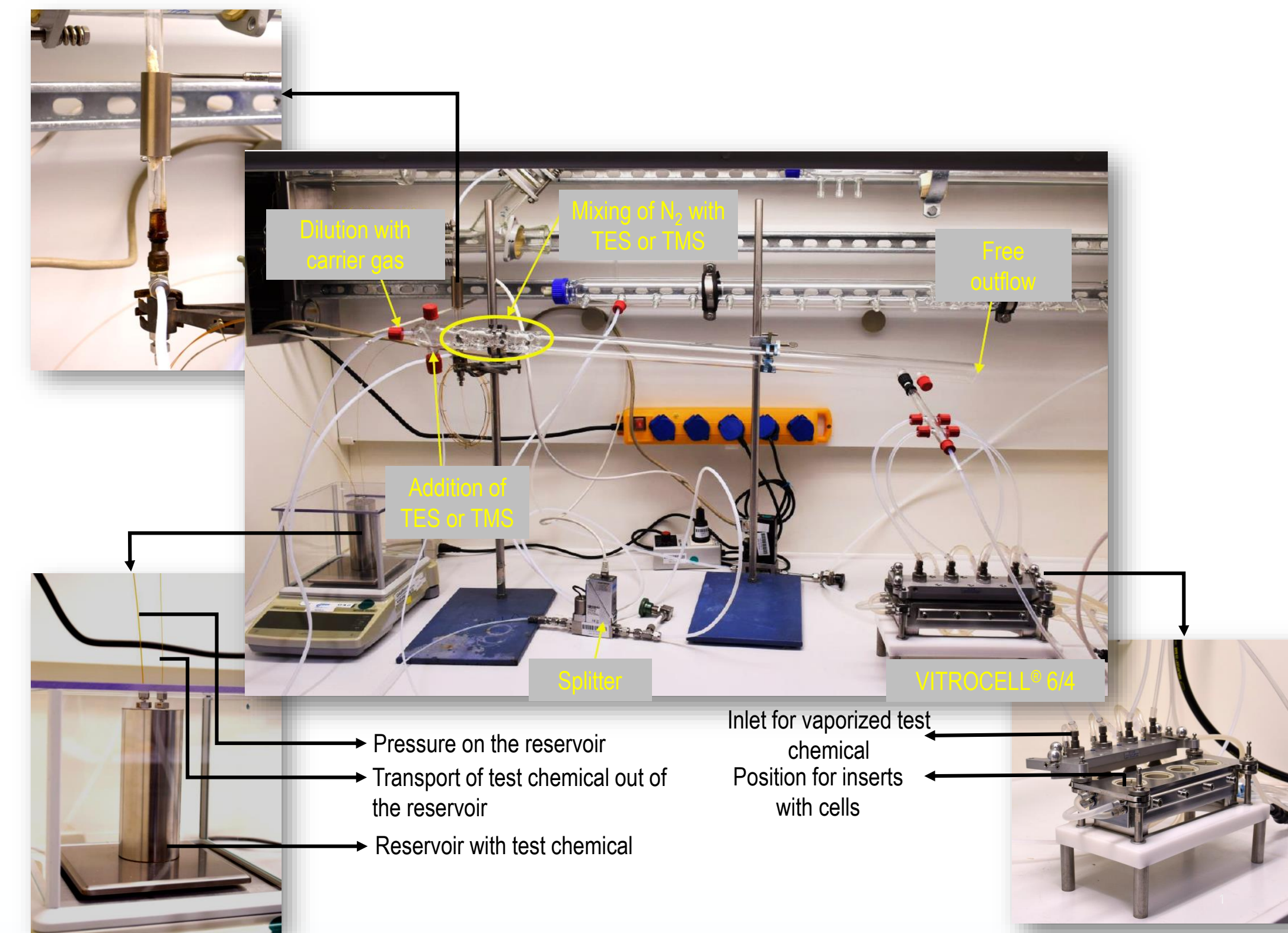


Triethoxysilane (TES)



Trimethoxysilane (TMS)

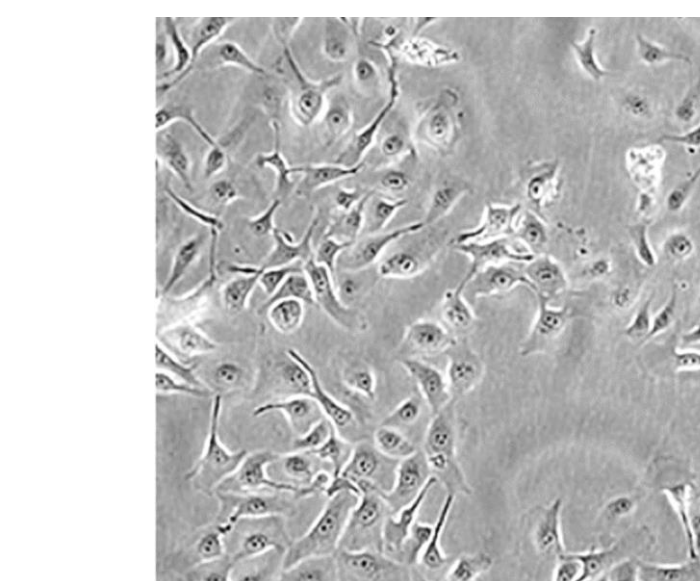
Exposure set-up



Study design

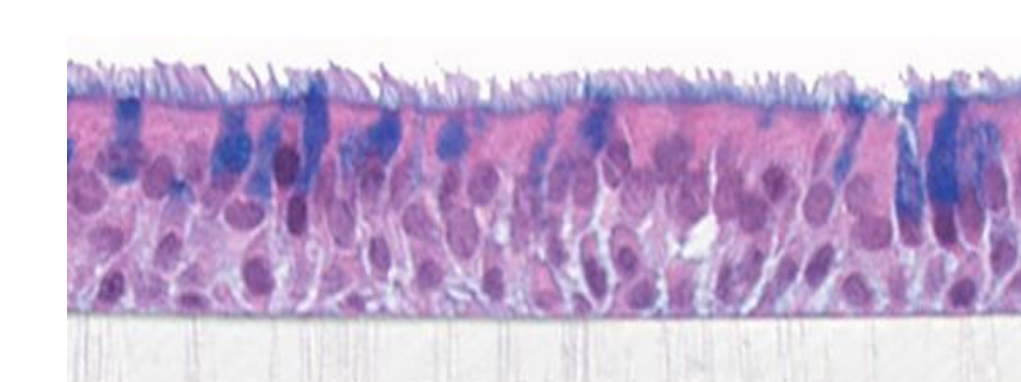
Test systems and endpoints

BEAS-2B



Human bronchial epithelial cell line

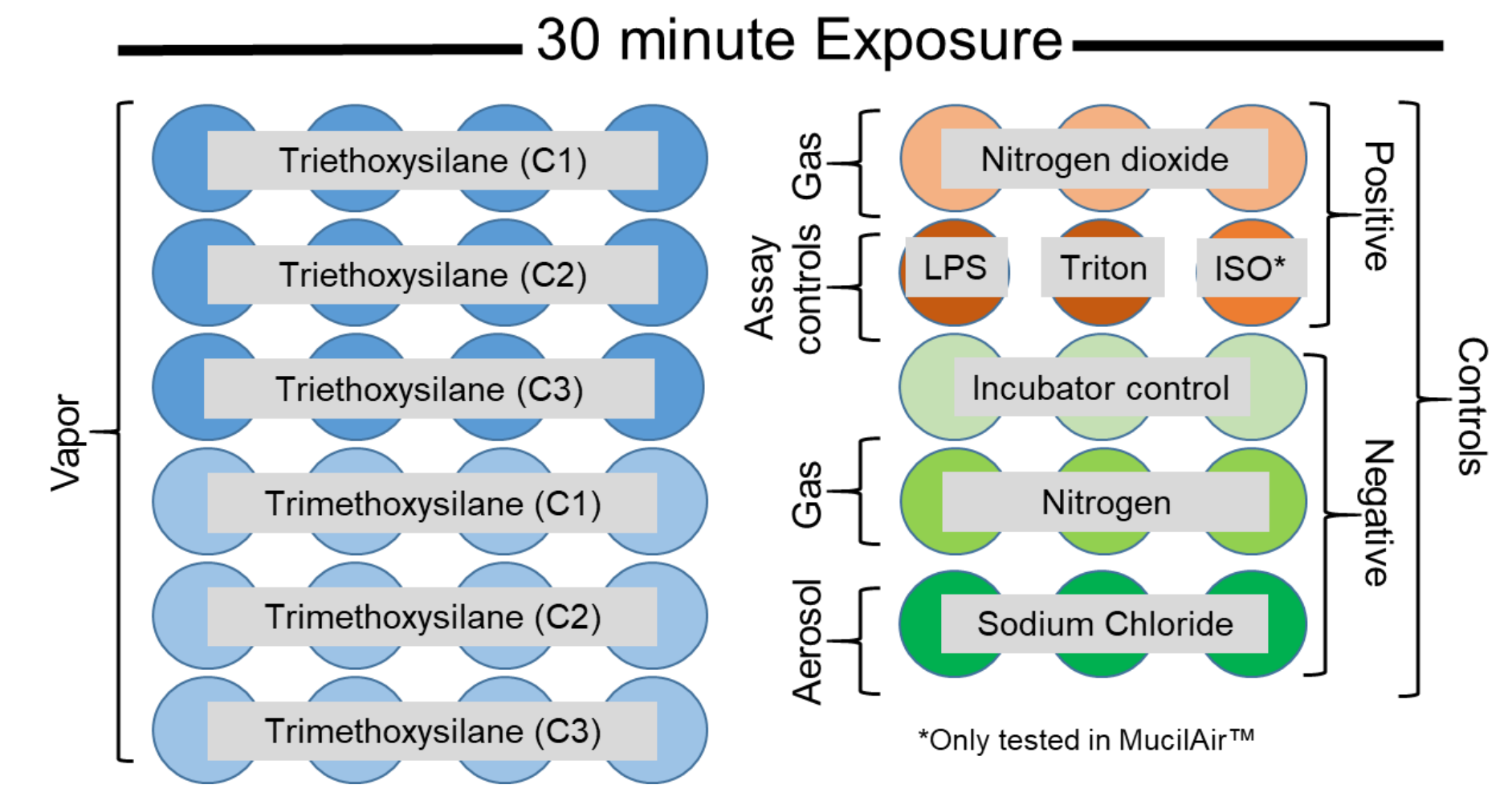
MucilAir™



3D Human bronchial epithelial tissue model

Endpoints	BEAS-2B	MucilAir™
Cell viability (PrestoBlue®)	✓	✓
Cytotoxicity (LDH, data not shown)	✓	✓
Inflammatory markers (IL-2, IL-6, IL-8, TNF-α)	✓	✓
Cilia beating frequency (CBF) and Average Active Area (AAA)		✓
Barrier integrity (TEER)		✓
Morphology (H&E staining)		✓

Experimental set-up



Abbreviations:
LPS (Lipopolysaccharide) positive control for inflammatory response
LDH (Lactate dehydrogenase), positive control for LDH assay
ISO™ (Isoproterenol), positive control for CBF (only tested in MucilAir™)
Cx (Concentration x); three concentrations were tested for each chemical

Donor information for MucilAir™ tissues

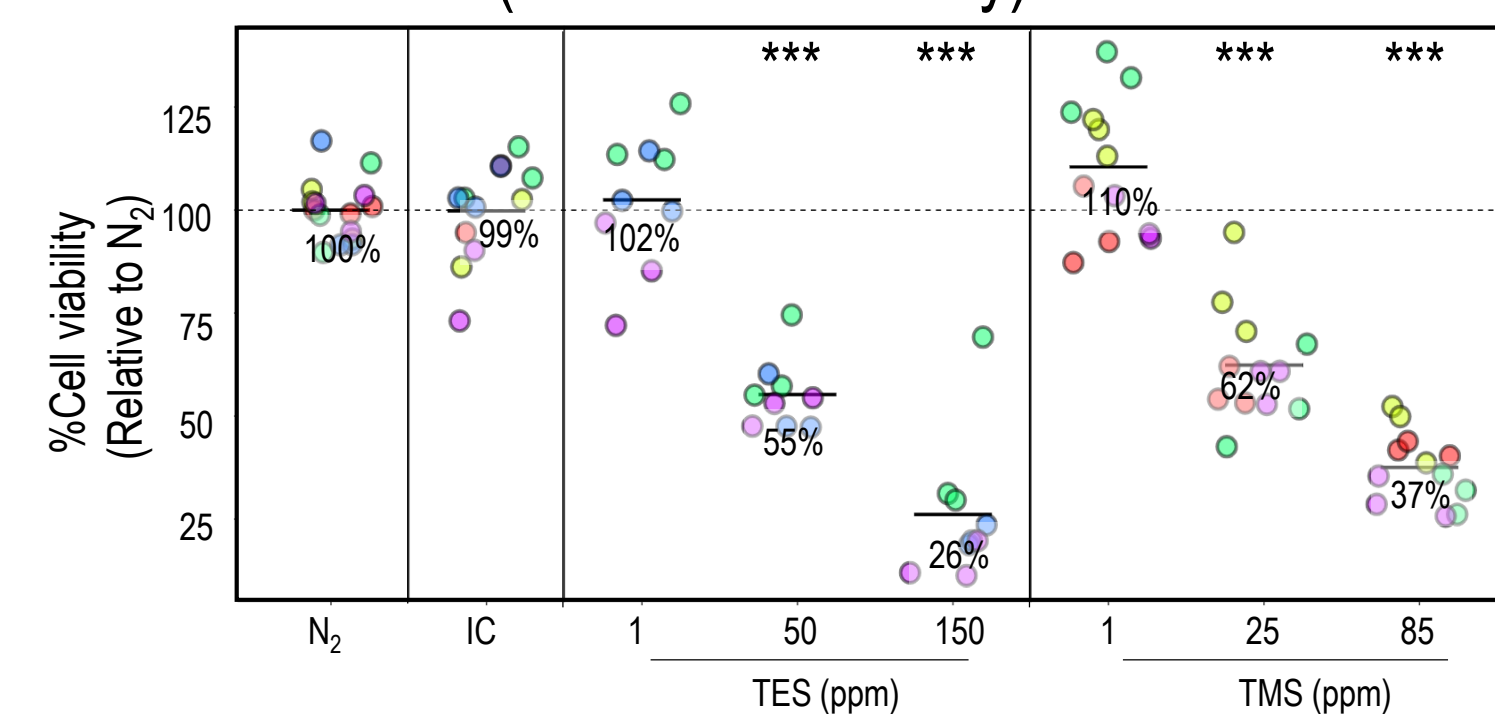
Experimental run	Sex	Age	Ethnicity	Pathology reported	Smoker	Morphology
1 ^a *	M	17	Hispanic	None	No	Normal
2 ^a *	F	52	Hispanic	None	No	Normal with some mucus vesicles
3 ^a *	M	17	Hispanic	None	No	Normal with some mucus vesicles
4 ^a *	F	55	African	None	No	Normal
5 ^a *	M	17	Hispanic	None	No	Normal with some mucus vesicles

*same donor

Results - BEAS-2B

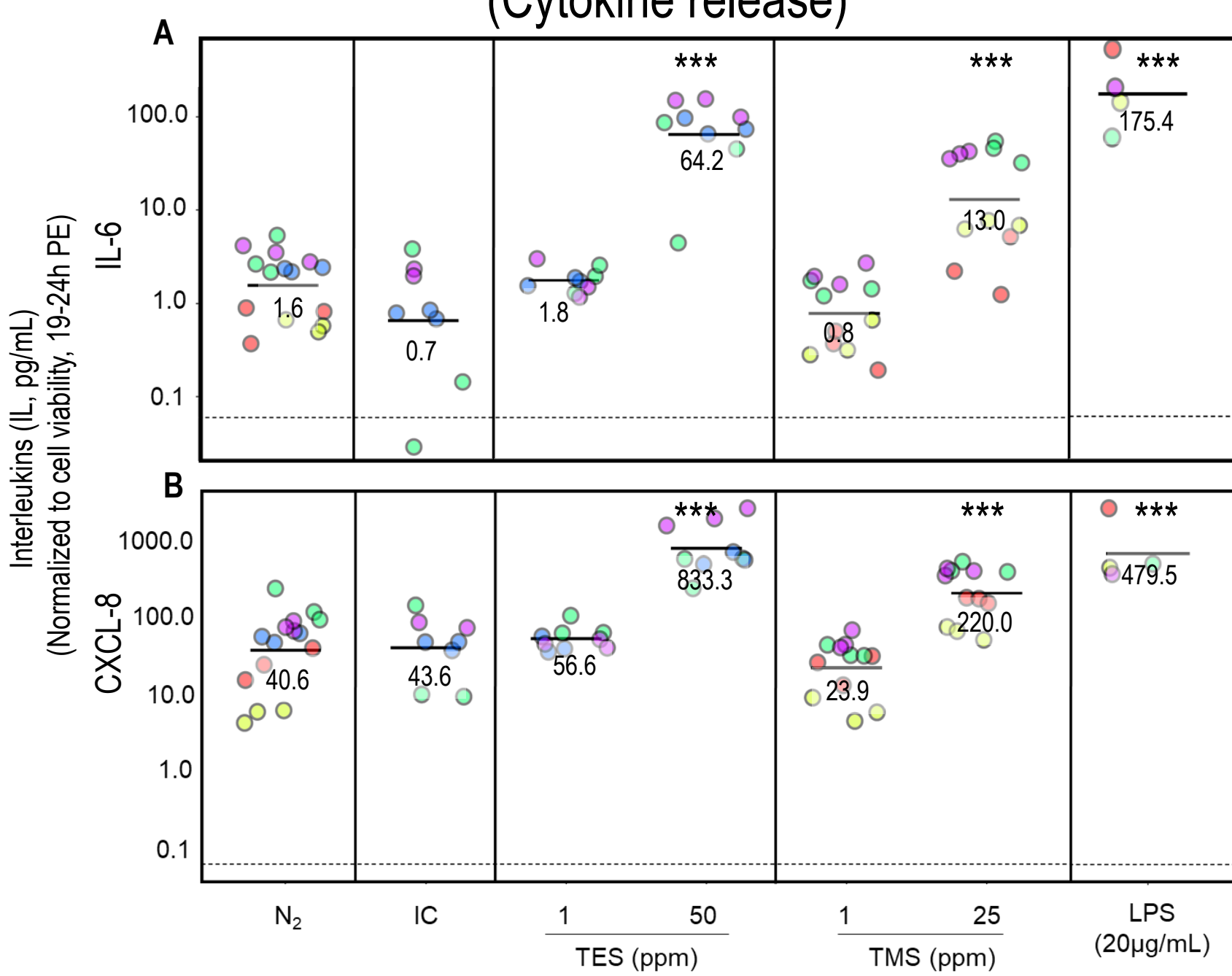
BEAS-2B cells were exposed to controls or TES (1, 50, or 150 ppm) or TMS (1, 25, or 85 ppm) vapor for 30 minutes at the ALI

Cell viability (PrestoBlue® assay)



Assessment of cell viability in BEAS-2B cell line. Cell viability was assessed using PrestoBlue assay 19-24 hours post-exposure. The graphs show results from three separate experimental runs for TES (N=3) and four experimental runs for TMS (N=4) represented in different colors, with each run having three replicates (n=3). Viability was normalized to the N₂ control cells (= 100% cell viability).

Inflammation (Cytokine release)



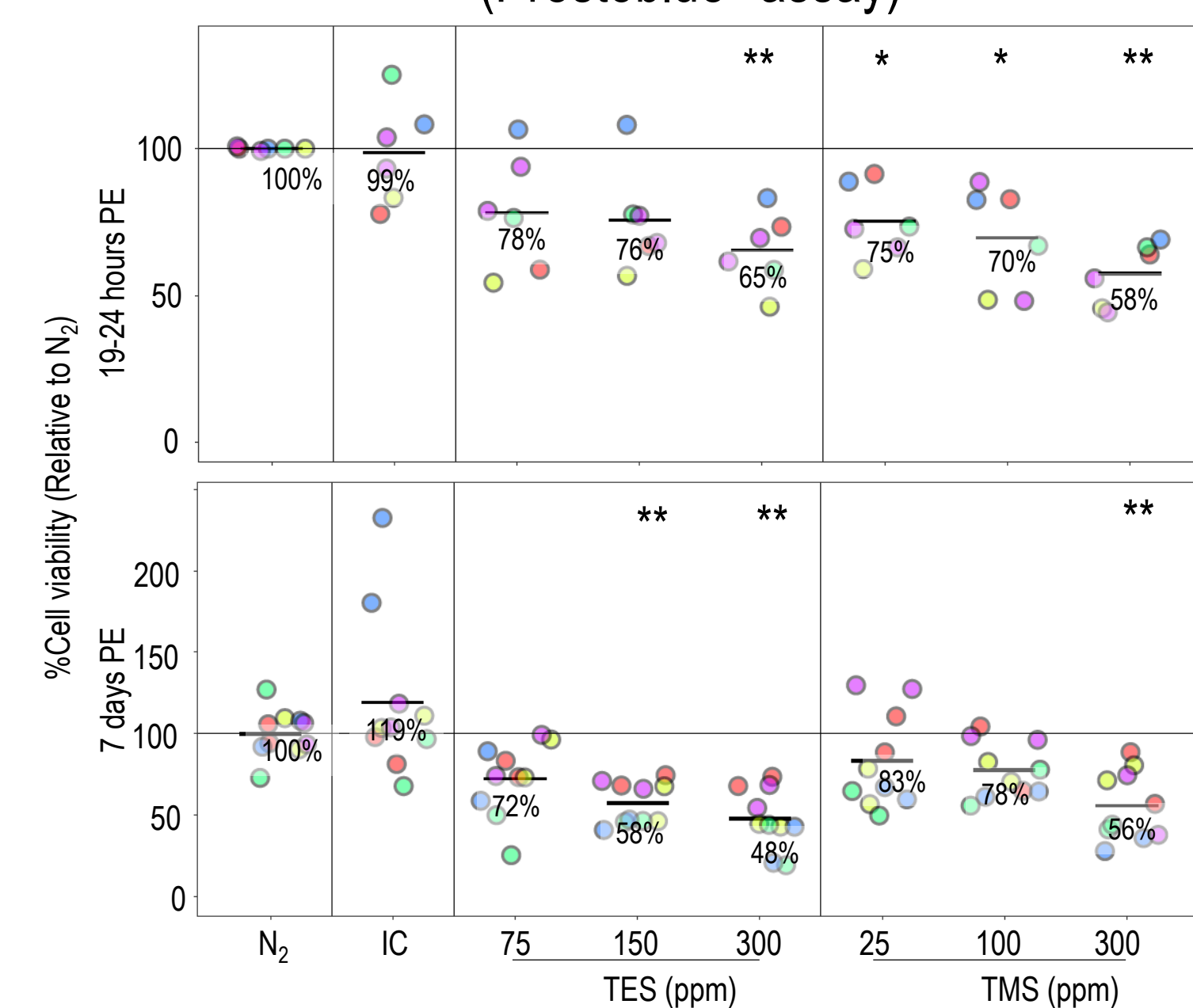
Assessment of inflammatory response in BEAS-2B cell line. Samples from the basolateral medium were collected for assessment of inflammatory markers after 19-24 hours. Data represent exposure to TES at 1 or 50 ppm and to TMS at 1 or 25 ppm; the highest concentrations were not included because of cell viability <50% observed at those concentrations. The graphs show results from at least three (four for TMS) separate experimental runs per condition (biological replicates; represented in different colors), with each run having 3 technical replicates. The data points show the secretion of IL-6 and CXCL-8 (IL-8) normalized to cell viability. Y-axis is shown in log-scale.

*, P<0.05; **, P<0.01; ***, P<0.001

Results – MucilAir™

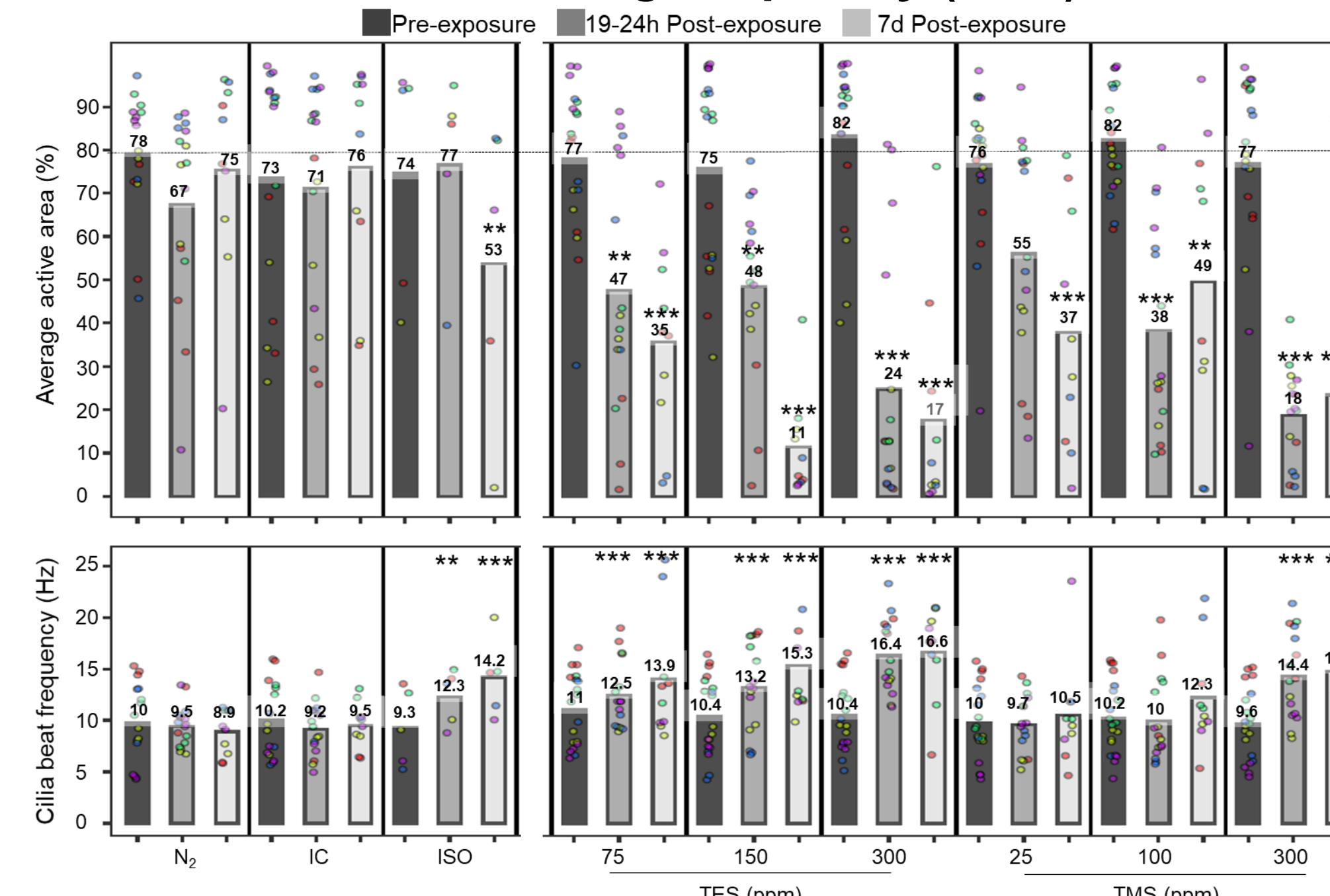
MucilAir™ tissues were exposed to 75, 150, or 300 ppm TES and 25, 100, or 300 ppm TMS, for 30 minutes at the ALI

Cell viability (PrestoBlue® assay)



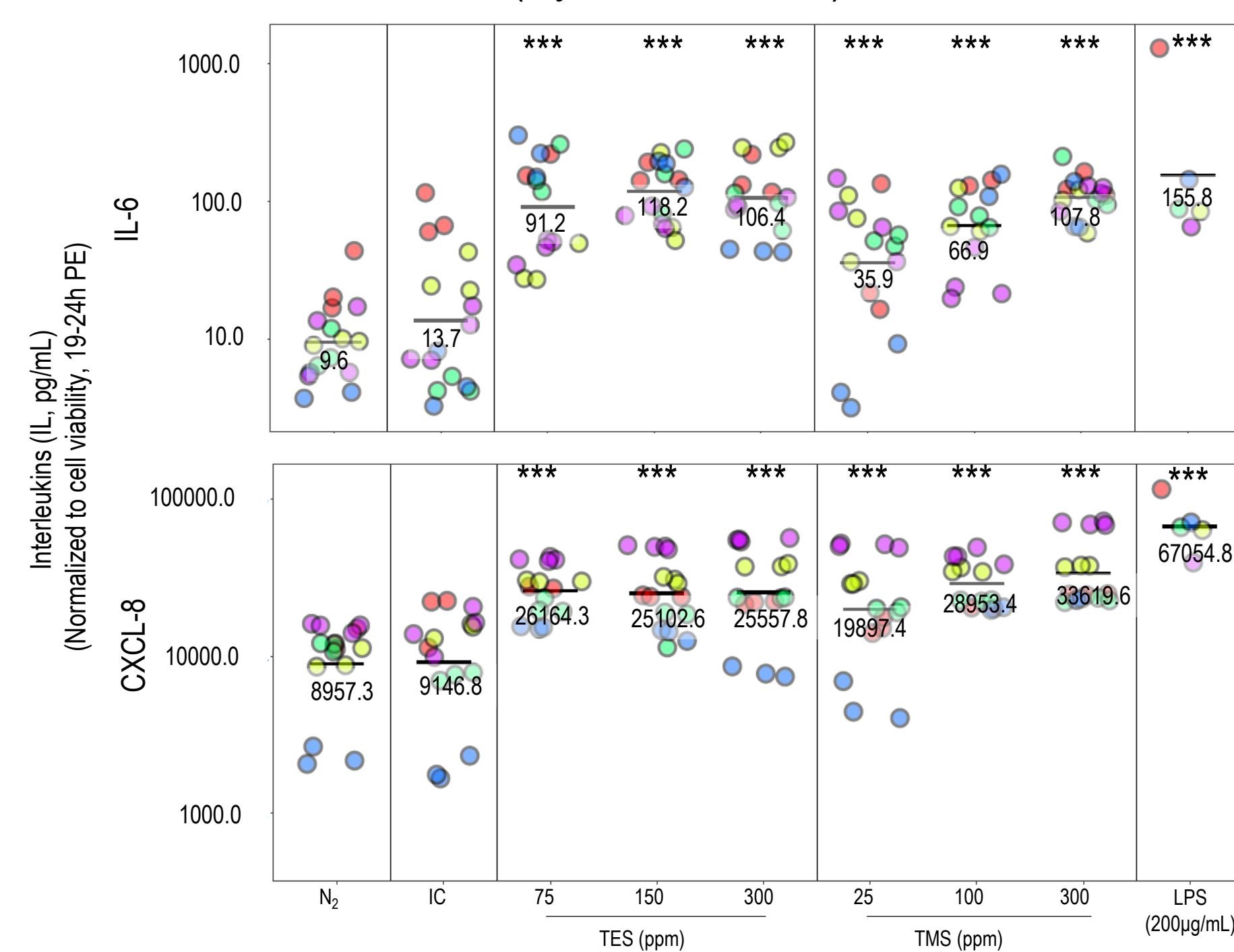
Assessment of cell viability in MucilAir™ tissues. Cell viability was assessed using PrestoBlue assay after 19-24 hours or 7 days after exposure. Cell viability was expressed as the percentage of the fluorescence of treated cells relative to the fluorescence of the N₂ control cells.

Cilia beating frequency (CBF)



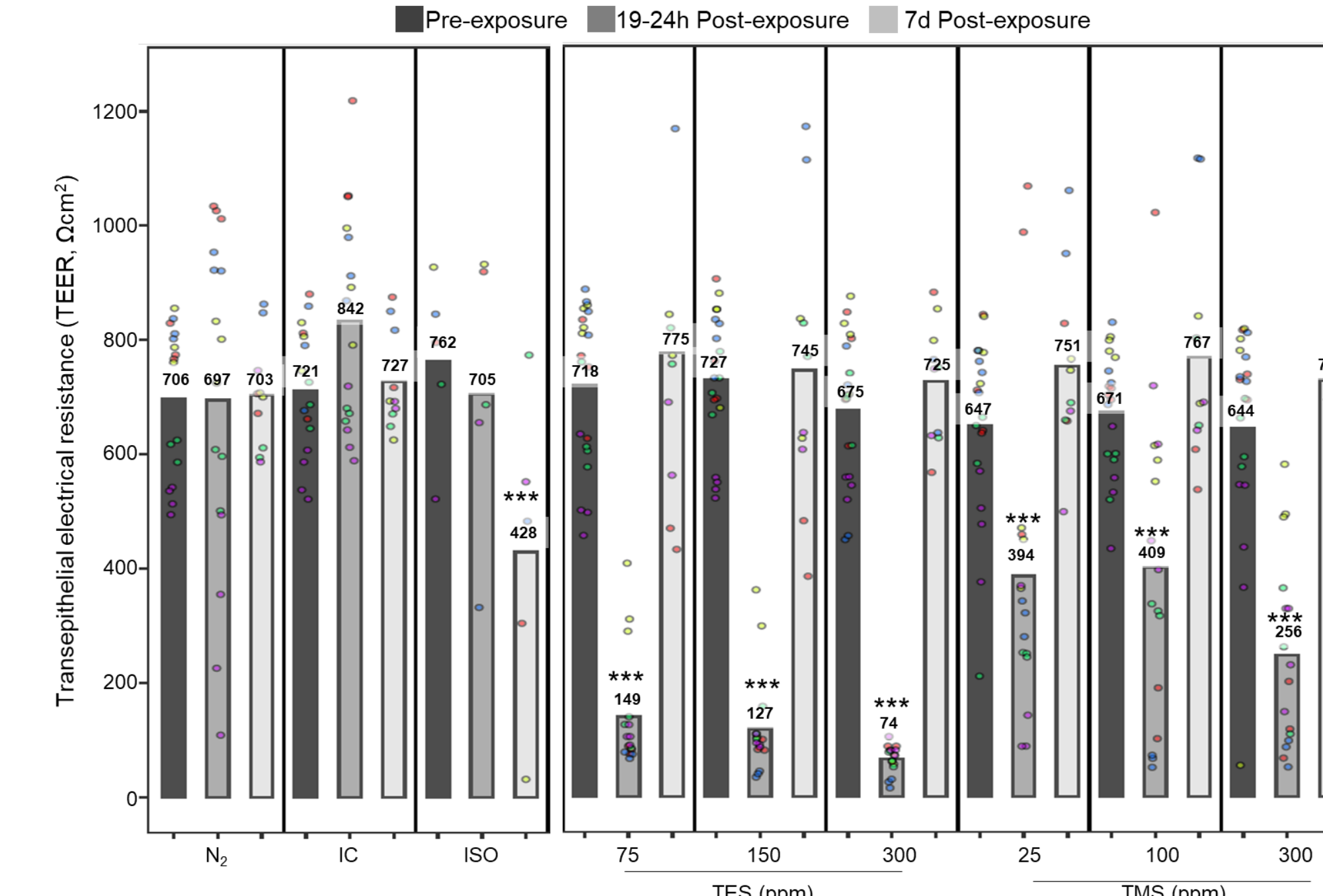
Assessment of CBF and active cilia beating areas in MucilAir™ tissues. CBF and AAA measured using SAVA software before and after 19-24 hours and 7 days after exposure. The graphs show combined results from five separate experiments (3 different donors, one donor tested in three separate experimental runs) represented in different colors, with each run having two or more replicates. CBF is represented in hertz (Hz) and AAA (%) is presented.

Inflammation (Cytokine release)



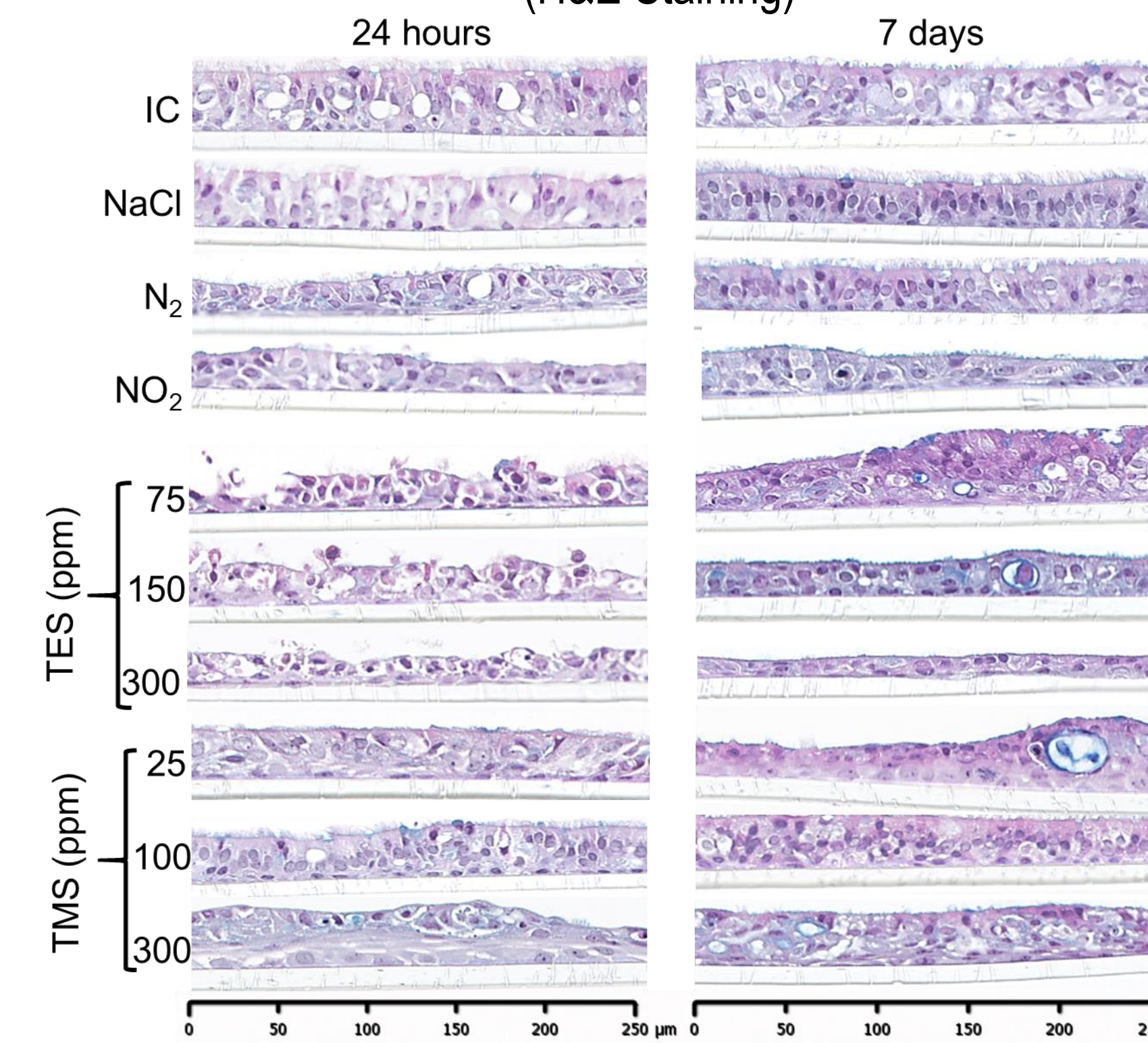
Assessment of inflammatory response in MucilAir™ tissues. After 19-24 hours or 7 days post-exposure, the basolateral medium was collected and stored at -80°C until analysis using Meso Scale Discovery (MSD) V-PLEX Assay. Tissues treated with lipo-polysaccharide were used as a positive control for the assay. The graphs show results from five separate experiments in different colors.

Barrier integrity (TEER)



Assessment of barrier integrity in MucilAir™ tissues. TEER was measured using a VoltOhmmeter before and after 19-24 hours and 7 days. The graphs show combined results from five experimental runs (from 3 different donors) represented in different colors, with each run having at least two replicates. TEER is represented as Ohm·cm²

Morphology (H&E Staining)



Cross sections of MucilAir™ tissues from one donor exposed to different test conditions included in this study. Two tissues from each time point per treatment were processed for morphological analysis via electron microscopy.

Derivation of human equivalent concentration (HEC) using *in vitro* data

Cell system	TES HEC based on CV50				TMS HEC based on CV50						
	TES (ppm)	TES (mg/m ³)	RGDR	4-hour HEC (mg/m ³)	TES (ppm)	TMS (mg/m ³)	RGDR	4-hour HEC (mg/m ³)			
BEAS-2B	77.02	514.32	0.15	77.78	9.72	BEAS-2B	57.76	288.73	0.15	43.66414	5.46
MucilAir™	300	2003.31	2.32	4645.36	580.67	MucilAir™	300	1499.63	2.32	3477.40731	434.68

Endpoint	TES HEC based on BMCL: BEAS-2B				TMS HEC based on BMCL: BEAS-2B						
	TES (ppm)	TES (mg/m ³)	RGDR	4-hour HEC (mg/m ³)	TMS (ppm)	TMS (mg/m ³)	RGDR	4-hour HEC (mg/m ³)			
Cell viability	6.95	46.41	0.15	7.02	0.88	Cell viability	3.91	19.55	0.15	2.95580	0.37
LDH release	24.32	162.40	0.15	24.56	3.07	LDH release	7.12	35.59	0.15	5.38242	0.67
IL-6	10.83	72.32	0.15	10.94	1.37	IL-6	9.08	45.39	0.15	6.86410	0.86
CXCL-8	15.32	102.30	0.15	15.47	1.93	CXCL-8	8.35	41.74	0.15	6.31225	0.79

Endpoint	TES HEC based on BMCL: MucilAir™				TMS HEC based on BMCL: MucilAir™						
	TES (ppm)	TES (mg/m ³)	RGDR	4-hour HEC (mg/m ³)	TMS (ppm)	TMS (mg/m ³)	RGDR	4-hour HEC (mg/m ³)			
Cell viability	9.32	62.24	2.32	144.32	18.04	Cell viability	2.21	11.05	2.32	25.62	3.20
LDH release	85.86	573.35	2.32	1329.50	166.19	LDH release	67.1	335.42	2.32	777.78	97.22
IL-6	9.65	64.44	2.32	149.43	18.68	IL-6	10.42	52.09	2.32	120.78	15.10
CXCL-8	11.28	75.32	2.32	174.67	21.83	CXCL-8	6.15	30.74	2.32	71.29	8.91

Two approaches were used to calculate HEC using the data generated in this study. For the first approach, cell viability of 50% (CV50) was used as a threshold beyond which the response level was considered adverse. For the second approach, EPA's Benchmark Dose Modeling Software (BMDS) version 3.2.0.1, was used to analyze the data of each endpoint and derive the benchmark concentration lower confidence limit (BMCL). Abbreviations: RGDR, regional gas dose ratio

Observations and next steps

- A dose-dependent response in all biological endpoints (cytotoxicity (data not shown), cell viability, and cytokine secretion) was observed when exposing BEAS-2B cells to either silane. For certain endpoints, the dose-response was less pronounced in MucilAir™ tissues, indicating that BEAS-2B cells were more sensitive to silane exposure compared to 3D tissues.
- For all of the endpoints assessed in both BEAS-2B cells and MucilAir™, the results demonstrated toxicity of both tested silanes and indicated that TMS is slightly more toxic than TES.
- TES and TMS caused a concentration-dependent reduction in AAA with a corresponding increase in CBF.
- Disruption of barrier integrity following TES or TMS treatment is observed at the 19-24 hour time-point as expected with a more substantial decrease following TES exposure for unknown reasons. After 7 days of recovery, the TEER values for all silane exposed tissues reverted to the same or, in some cases, higher than the pre-exposure values.
- Histological analysis of MucilAir™ tissues showed that both TES and TMS damaged the tissues as indicated by clear signs of toxicity (e.g., structural changes and decrease in cilia) as compared to untreated cells.
- A manuscript describing the results of this study is in preparation.
- Further testing is being conducted to assess surfactants using a similar study design.