A review of aerosol exposure systems relative to the analysis of cytotoxicity: a CORESTA *in vitro* SubGroup perspective

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Introduction

In vitro aerosol exposure systems offer researchers a variety of ways to customize exposure set-up, modify experimental parameters and provide a novel and versatile platform for *in vitro* aerosol research. These exposure systems are designed to produce an aerosol that more closely mimics the human smoking condition with associated aerosol interactions. When coupled with a biological cell system, ranging from cell monolayers to 3D differentiated structures utilizing various biological end-points, these systems and techniques may easily be customized to researchers' preferences.

Exposure systems typically consist of two functional parts: the smoking machine / aerosol generator and the exposure module / multiwell plate housing the cell system.

The possible combinations of exposure systems, modules and plate formats give rise to an *in vitro* aerosol research environment that is complex and diverse, resulting in unique combinations of variables that few laboratories share. However, this presents challenges in comparing data between set-ups using similar systems and an inability to compare data across some platforms, making tobacco aerosol research particularly difficult to contextualize across laboratories.

Furthermore, with the advent of new aerosol technologies, the environment is becoming more complex, as diverse aerosol products and experimental parameters are being employed for *in vitro* assessment. Never has it been more important to harmonize approaches and testing strategies. However, in order to do this, the area of *in vitro* aerosol research needs to be carefully mapped out and understood, in order to make positive and collective progress.

Approach

Over recent meetings, the *In Vitro* Toxicity Testing SubGroup has discussed the developing field of aerosol exposure research. Given the diversity of techniques, exposure parameters and biological end-points being deployed, it was considered a high priority to establish a strategy to assess these systems and the responses obtained. Twelve global companies with expertise in *in vitro* aerosol research met to discuss this topic and identify potential areas of alignment and harmonization.

A detailed and comprehensive survey was conducted on over 40 parameters ranging from aerosol generation, dilution, biological methodology, data analysis and dosimetry approaches, across eight independent laboratories. Only cytotoxicity data from Kentucky reference 3R4F cigarette smoke were assessed.

The data would then serve several purposes:-

• Inform the collective *in vitro* SubGroup on the diverse exposure systems currently in use.
• Give, for the first time, an overview on the diverse exposure and biological parameters in use by industry participants.
• Allow the SubGroup to rationalise experimental techniques and find areas of consensus within protocols, with an ultimate goal of harmonisation.
• Where harmonisation is not possible, the data will allow researchers to understand protocols and experimental setups between laboratories.
• Finally, give better insight into the whole aerosol environment and allow the incorporation of new techniques, such as dose tools, for the interpretation, extrapolation and presentation of *in vitro* biological data in a consistent manner.

Results

Table 1: a summary of the key parameters

Laboratory	1	2	3	4	5	6	7	8
In vitro Technique	NRU	NRU	NRU	NRU	NRU	NRU	NRU	MTT
Exposure system	Vitrocell	Vitrocell	Vitrocell	Vitrocell	Vitrocell	Borgwaldt	SEIVS	Sibata
System designation	VC 10	VC 10	VC 10	VC 10	VC 10	RM20S		
Dilution Principle	Flowing air	Flowing air	Flowing air	Flowing air	Flowing air	Syringe	Syringe	Flowing air
Vacuum rate (mL / min)	5	5	5	5	5	N/A	N/A	900
Cell line	BALB/c 3T3	CHO	CHO-K1 A549 BEAS-2B	A549	CHO-K1 BEAS-2B	NCI-H292	BEAS-2B HepG2	BEAS-2B
Exposure time (mins)	180	60	30	30	10	60	10 - 180	20

Table 2: a summary of biological parameters 1

Laboratory	1	2	3	4	5	6	7	8
Cell line	BALB/c 3T3	CHO	CHO-K1 A549 BEAS-2B	A549	CHO-K1 BEAS-2B	NCI-H292	BEAS-2B HepG2	BEAS-2B
Manufacturer of Transwell	Corning	Corning	Corning	N/A	Greiner	Corning	N/A	Falcon
Transwell size (mm)	24	12	12	N/A	12	12	N/A	12
Are transwells pre-equilibrated?	60 mins	60-90 mins	No	N/A	5-10 mins	No	25µl Collagen I matrix	No
# cell seeded per Transwell/multiwell plate	5.5 x 10⁵	3.75 x 10⁴	3.5 ×10⁴ (CHO & A549) 1×10⁵ (BEAS-2B)	200,000 cells/35 mm plate/mL	6 x10⁴	3.5 x 10⁵	1x104 /well	8 x 10⁴
Multi well plate format	N/A	N/A	N/A	N/A	N/A	N/A	96	N/A
Desired confluency at treatment (%)	90-100	50	70-80	75-85	60-70	80-90	40	90-100
How many hours prior to treatment are cells plated?	24	18-24	24	12	24	24	20	24
What media is used?	DMEM (10% FCS, pen strep, 4 mM glutamine)	McCoys 5A (10% FBS, 0.52% Pen/Strep, Hepes)	CHO and A549: RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin BEAS-2B: BEBM including SingleQuots (Lonza cc-3170)	Complete media: F-12K + 10% FBS	CHO-K1: Ham's F-12 including 10%FBS, 25mM HEPES and 1ug/mL Gentamycin BEAS-2B: BEBM including SingleQuots (Lonzacc-3170)	DMEM (10% FCS, pen strep, 4 mM glutamine)	BEAS-2B: BEBM with SingleQuots (Lonza cc-3170) HepG2: MIS (MEM/Waymouth's (4:1), PenStrep/Antimycot)	LHC-9 (Invitrogen) without any other supplements
Are cells checked pre and post exposure	Microscopic	Microscopic	Microscopic	Microscopic	Microscopic	Microscopic	Microscopic	Microscopic
What is the recovery time post exposure? (hrs)	None	24	24	12	24	24	69	20-22

N/A = not applicable to exposure system

Table 3: a summary of biological parameters 2

Laboratory	1	2	3	4	5	6	7	8
Exposure regimen	ISO	ISO	ISO	ISO	ISO	ISO	ISO	ISO
Smoke assessed	WS	WS	WS	WS	WS	WS	WS	WS
Exhaust time (sec)	8	8	2.8	8	2.8	N/A	0.45	N/D
Exposure time (mins)	180	60	30	30	10	60	10 - 180	20
Are cigarettes conditioned prior to use?	ISO	ISO	ISO	ISO	ISO	ISO	ISO	No
Are Laboratory conditions controlled?	ISO	ISO	ISO	ISO	ISO	ISO	ISO	ISO
Puffs/Cigarette	8	8	7-8	3	9	8	6 - 15	8
Replicates/dose	3	3	3	3	3	6	6	3
# Experiments	3	6	3	3	3	3	6	3
Are modules heated?	37ºC	37ºC	37ºC	RT	37ºC	37ºC	25ºC	No
Are Transwell rinsed post exposure?	No	No	No	No	Yes	Yes	No	No
Are blanks included for background subtraction?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Concentration of NRU dye (µg/mL)	50	50	50	50	50	TBC	66	N/A
Length of time in NRU dye (hrs)	3	3	3	2-3	3	3	3	N/A
Is fixation conducted?	No	No	No	No	Yes	No	No	N/A
Destain composition	50:49:1 (ethanol: water: acetic acid v:v)	50:49:1 (ethanol: water: acetic acid v:v)	50:49:1 (ethanol: water: acetic acid v:v)	1% acetic acid and 50% ethanol aqueous solution	1% acetic acid and 50% ethanol aqueous solution	50:49:1 (ethanol: water: acetic acid v:v)	50:49:1 (ethanol: water: acetic acid v:v)	N/A
Destain time (mins)	40	40	20-40	5	20	20	30-60	N/A
Positive control	SDS	SDS	SDS	No	SDS	SDS	CM7	No

SDS = Sodium dodecyl sulphate
CM7 = Canadian Monitor 7 reference cigarette
N/A = not applicable to exposure system
WS = Whole smoke
RT = Room temperature
TBC = to be confirmed

Conclusions and Next Steps

• The survey results emphasize the diversity of *in vitro* exposure parameters and methodologies employed across the *in vitro* SubGroup and tobacco industry.
• Pockets of harmonization already exist. For example, many of the biological protocol parameters are consistent across the SubGroup.
• However, variables such as cell type and exposure time remain largely inconsistent.
• The key next steps for this work will be to map parameter and system data against biological findings and investigate whether the observed commonalities and inconsistencies translate into biological variability.
• Analysing data will give a better understanding of how data is presented and interpreted and how data may be more accurately aligned between laboratories irrespective of the lack of harmonized protocols.
• Finally, this survey was conducted across one biological end-point, cytotoxicity. In order to understand the environment in its completeness, other biological end-points and parameters should also be assessed.

Title

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Authorship

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Abstract

Aerosol exposure systems offer researchers a variety of ways to customize the exposure set-up, modify experimental parameters and provide a novel and versatile platform for *in vitro* aerosol research. These systems produce an aerosol that more closely mimics the human smoking condition with associated aerosol interactions, an advantage over the potential limitation of using aerosol fractions alone. Exposure systems typically consist of two functional parts: the smoking machine / aerosol generator, and the exposure module/multiwell plate housing the cell system. The possible combinations of exposure systems, modules and plate formats give rise to an *in vitro* aerosol research environment that is complex and diverse, resulting in unique combinations of variables that few laboratories share. Ultimately, this causes challenges in comparing data between set-ups using similar systems and an inability to compare data across some platforms, making tobacco aerosol research particularly difficult to contextualize across laboratories.

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Survey results demonstrate the diversity of and provide awareness of the exposure systems, parameters, methodology nuances and data analysis. Results identify potential commonalities and important areas of consideration, which may be of substantial benefit to current smoke/aerosol researchers, scientists from intersecting fields of research, and new scientists and laboratories entering into this area of research.

Key Words

CORESTA, Tobacco Smoke, Cytotoxicity, Aerosol, 3R4F, Dosimetry, Review