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Ciliary Beat Frequency: Proceedings and Recommendations from a Multi-laboratory Ring Trial Using 3-D Reconstituted Human Airway Epithelium to Model Mucociliary Clearance

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Abstract

The use of reconstituted human airway (RHuA) epithelial tissues to assess functional endpoints is highly relevant in respiratory toxicology, but standardised methods are lacking. In June 2015, the Institute for In Vitro Sciences (IIVS) held a technical workshop to evaluate the potential for standardisation of methods, including ciliary beat frequency (CBF). The applicability of a protocol suggested in the workshop was assessed in a multi-laboratory ring study. This report summarises the findings, and uses the similarities and differences identified between the laboratories to make recommendations for researchers in the absence of a validated method. Two software platforms for the assessment of CBF were used — Sisson-Ammons Video Analysis (SAVA; Ammons Engineering, Clio, MI, USA) and ciliaFA (National Institutes of Health, Bethesda, MD, USA). Both were utilised for multiple read temperatures, one objective strength (10×) and up to four video captures per tissue, to assess their utility. Two commercial RHuA tissue cultures were used: MucilAir™ (Epithelix, Geneva, Switzerland) and EpiAirway™ (MatTek, Ashland, MA, USA). IL-13 and procaterol were used to induce CBF-specific responses as positive controls. Further testing addressed the impact of tissue acclimation duration, the number of capture fields and objective strengths on baseline CBF readings. Both SAVA and ciliaFA reliably collected CBF data. However, ciliaFA failed to generate accurate CBF measurements above ~10 Hz. The positive controls were effective, but were subject to inter-laboratory variability. CBF endpoints were generally uniform across replicate tissues, objective strengths and laboratories. Longer tissue acclimation increased the percentage active area, but had minimal impact on CBF. Taken together, these findings support the development and validation of a standardised CBF measurement protocol.

Keywords

3-D tissue function, assay optimisation, ciliary beat frequency, IIVS workshop proceedings, *in vitro* airway epithelium, mucociliary clearance marker, non-animal testing, reconstituted human airways, respiratory toxicology

Introduction

In 2007, the National Academies in the USA presented the research community with a vision to redefine how toxicity testing could (and indeed should) evolve to address challenges faced in the 21st century.[1] As a long-time advocate of non-animal and, in particular, *in vitro* testing, the Institute for In Vitro Sciences (IIVS) has promoted the use, standardisation and validation of such new approaches with notable successes in the areas of skin and eye testing.[2–5] However, while concerns persist about the effectiveness of *in vivo* animal models to represent the

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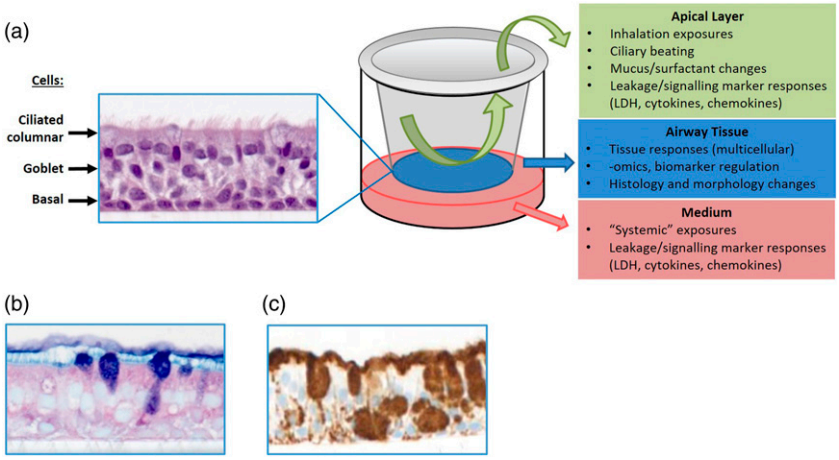

Figure 1. A representation of a reconstituted human airway insert, illustrating the common endpoints assayed in the apical, tissue and basal compartments. (a) A cross-section of a reconstituted human airway epithelium sample (left-hand side panel; haematoxylin and eosin staining, 20× objective) containing ciliated columnar cells, goblet cells and a mucous layer; the right-hand side panel shows its placement in the well insert. (b) Alcian Blue-Periodic Acid Schiff staining, showing the mucous layer (blue) as expected in the untreated cultures. (c) 14 days after treatment with 10 ng/ml IL-13, goblet cell hyperplasia has been induced (immunohistochemical staining with MUC5AC antibodies (20× objective). LDH = lactate dehydrogenase.

human lung, a variety of available *in vitro* pulmonary models provide human-relevant test systems that can be utilised.[6–8] The IIVS has made efforts to develop and standardise methods and technologies, including in the area of pulmonary toxicology.

In 2014, with the growing need to evaluate next-generation tobacco products, the IIVS held an informational workshop, 'Assessment of *In Vitro* COPD Models for Tobacco Regulatory Science', to discuss the specific considerations for assessment of next-generation tobacco products. From the breakout group discussions, it was unanimously concluded that ciliary beat frequency (CBF), mucus production and goblet cell hyperplasia/metaplasia (GCH) were useful *in vitro* endpoints for respiratory toxicity testing, and that evaluation of such assays for standardisation and subsequent validation was warranted.

Mucociliary clearance is an important pulmonary defence mechanism whereby particulates and other airborne materials are cleared from the airways.[9–11] It is required to maintain lung health and/or prevent pulmonary exacerbations.[12–15] Effective clearance is dependent on the amount and quality of the mucus, the number and quality of ciliary cells, and the health of goblet cells. Multicellular, pseudostratified reconstituted human airway epithelium (RHuA) consist of 3-D tissue models that include ciliated columnar cells, goblet cells and progenitor basal cells. They provide a way to monitor the three endpoints of interest *in vitro* at the air–liquid interface (ALI; Figure 1). One method of assessing ciliary function is to monitor the ciliary beat pattern on high-speed video recordings, and quantify the CBF and the active ciliary area. GCH can be induced with IL-13, and this increase in the proportion of secretory cells (as well as IL-13-induced downregulation of cytoskeletal components) can impact CBF readings.[16]

A follow-up Technical Workshop (TWS), 'CBF, GCH and Mucus Production in Reconstructed Human Respiratory Airway Epithelium' was held at the IIVS headquarters in Gaithersburg (MD, USA) on 16–18 June 2015, and was attended by 14 invited experts on the subject. The goal of the TWS was to discuss the tools used to assess the effects of various next-generation tobacco products in RHuA, so that the manufacturers and regulatory scientists could make informed decisions about the suitability of human reconstituted human airway epithelial tissues for research. The participating experts offered various assay protocols for review, and from these protocols and critical steps and best practices to optimise assay outcomes were suggested. A study framework was designed to assess the cross-laboratory reproducibility of the protocols and enable the development of effective guidelines for use. Here, we summarise the findings of the ring trial and use the similarities and differences identified during the process as a basis for making recommendations for researchers, in the absence of a validated method.

Materials and methods

Study design

This was an international ring trial performed in six laboratories (Table 1): IIVS, USA; Japan Tobacco (JT), Japan; Phillip Morris International (PMI), Switzerland; British American Tobacco (BAT), UK; National Center for Toxicological Research (NCTR), USA; and Imperial Brands (IB),

Table 1. Summary of the participants of the TWS Technical Exercise.

Laboratory/group	Designation	Activities
Institute for In Vitro sciences (IIVS), USA	Managing and testing laboratory	Organise TWS activities, execute technical work, manage data compilation and publication of material
Japan Tobacco (JT), Japan	Testing laboratory	Execute technical work, share results
Phillip Morris International (PMI), Switzerland	Testing laboratory	Execute technical work, share results
British American Tobacco (BAT), UK	Testing laboratory	Execute technical work, share results
National Center for Toxicological Research (NCTR), USA	Testing laboratory	Execute technical work, share results
Imperial Brands (IB), Germany	Testing laboratory	Execute technical work, share results
Epithelix, Switzerland	Tissue manufacturer	Produce tissues, conduct/subcontract GCH staining and quantitation
MatTek, USA	Tissue manufacturer	Produce tissues, conduct/subcontract GCH staining and quantitation

TWS = Technical Workshop on 'CBF, GCH and Mucus Production in Reconstructed Human Respiratory Airway Epithelium'.

Germany. The included laboratories participated in the TWS, had expertise and/or had performed substantial work on CBF. The study was initially intended to be a single phase, but the findings prompted a second phase of testing designed to answer further fundamental questions necessary to standardise CBF measurements and to streamline workflow logistics.

RHuA tissue cultures

Two widely used, commercially available 3-D RHuAs were used: MucilAir™ (Epithelix, Geneva, Switzerland) and EpiAirway™ (MatTek, Ashland, MA, USA). The participating laboratories could use either or both models, depending on availability and experience. All laboratories coordinated orders with one or both manufacturers to ensure that they obtained RHuAs from the same donor. MucilAir tissues of bronchial origin were obtained from a healthy, non-smoking Caucasian man aged 60 years (Epithelix donor MD064601). The EpiAirway tissues of bronchial origin were obtained from a healthy, non-smoking Caucasian man aged 23 years (AIR-100-DAY20 donor 9831). The RHuA tissues met the manufacturers' quality control criteria before shipment.

MucilAir and/or EpiAirway tissues were acclimated for 2–7 days before study commencement, and maintained according to the manufacturers' instructions (Table 2). Prior to use, the tissues were visually inspected under a brightfield microscope for overall appearance and the presence of beating cilia. An apical rinse was conducted with physiological buffer (e.g. Dulbecco's phosphate buffered saline (DPBS) or culture medium (CM) from the manufacturer). Medium from the basolateral compartment was assayed for adenylate kinase (AK) leakage. Cultures with less than 20% release of the total AK were deemed suitable for experimentation. Tissue cultures were maintained in 24-well plates (for the MucilAir) or six-well plates (for the EpiAirway). Five tissues were assigned to each treatment group.

Treatments and CBF measurements in Phase I of the study

One day after the apical rinse, baseline CBF measurements were taken by using the microscope configuration identified as optimal by each laboratory (10× objective in all except PMI, which used 4×), in up to four fields per culture. Subsequent CBF readings were taken every 2–3 days after re-feeding. Tissue cultures in the untreated group were re-fed with CM only. Positive controls were: IL-13 (Peprotech, Cranbury, NJ, USA for all laboratories except BAT (R&D Systems, Minneapolis, MN, USA)), which stimulates GCH and reduces CBF; and procaterol hydrochloride (Sigma-Aldrich, St Louis, MO, USA) (an adrenergic agonist that elevates intracellular calcium), which increases CBF.[16–18]

Exposure to IL-13 was continuous, with 10 ng/ml administered via the CM at every re-feed for MucilAir, and either 5 ng/ml (IIVS and NCTR) or 10 ng/ml (all other laboratories) for EpiAirway. The untreated and IL-13-treated sets of cultures were assessed for up to 14 days. Procaterol (10 mM) was administered via the CM, 1 hour prior to CBF readings on days 7 and 14 to five untreated cultures (procaterol group) and five IL-13-treated cultures (procaterol and IL-13 group). In the IB laboratory, two sets of the cultures were treated with procaterol as described, but one set was terminated after 7 days and the other after 14 days.

CBF and the percentage of active area measurements in Phase I of the study

CBF was assessed with two software platforms: Sisson-Ammons Video Analysis (SAVA; Ammons Engineering,

Table 2. Re-feeding and apical rinse conditions based on the manufacturers' recommendations.

	EpiAirway™ (AIR-100-DAY20)		MucilAir™	
Action	Volume	Frequency	Volume	Frequency
Re-feeding	1.0 ml CM 5.0 ml CM	Daily Every 2–3 days	0.7 ml CM	Every 2–3 days
Apical rinse	2 × 0.5 ml DPBS	Weekly	2 × 0.2 ml CM	Weekly

CM = culture medium; DPBS = Dulbecco's phosphate buffered saline.

Clio, MI, USA) version 2.0.6W (BAT), 2.0.7W (IIVS) or 2.0.8W (JT, NCTR and PMI); and the ciliaFA plugin for ImageJ (National Institutes of Health, Bethesda, MD, USA) version 1.46 (JT and IB) or 1.47 (PMI). High-speed cameras, 80–150 frames per second (fps), were used to obtain 2.6–8.0-second video captures of ciliary beating patterns. Four captures were taken per tissue insert, when possible, in distinct locations (avoiding the insert edges). To calculate the percentage active area (% AA), the average CBF values in the positive control groups (i.e. groups treated with IL-13, procaterol, or both) were compared with those in the untreated control group.

The video captures for SAVA analysis were 2.6 seconds in length, and were filmed with ≥ 80 fps cameras in five laboratories (IIVS, BAT, NCTR, JT and PMI). SAVA software calculated the CBF from whole field analysis (WFA) and Gaussian WFA (G-WFA), based on the % AA and the number of motile points (MPs). A cut-off of 1000 MPs was applied to permit the evaluation of the impact of excluding captures with low MP values (which may result in a less robust estimation of beat frequency, a discussion point during the TWS meeting and with the co-developer of SAVA, Bruce Ammons). Poor captures were removed by this 1000 MP filter, and the revised set was compared with the original set; inter-laboratory data sets were compared for consistency across treatments.

The ciliaFA software was used by two laboratories (JT and PMI) and analyses were performed in accordance with the published guidelines.[19] Videos of CBF were captured with ≥ 90 fps cameras and lasted for 8.0 seconds (JT) or 5.7 seconds (PMI). Audio Video Interleave files were exported for analysis in ImageJ. The ciliaFA output included CBF expressed as Hz and the % AA. No MP output was produced. Furthermore, the ciliaFA capture utilises a lower pixel frame than SAVA (1600 points *versus* 19,200 points) and, therefore, an MP cut-off would lead to a much greater exclusion rate for the fields used for the analysis.

One laboratory (JT) directly compared the linearity of the data sets between SAVA and ciliaFA to assess consistency between the two platforms. Frames originally analysed in ciliaFA were imported into SAVA and re-analysed to produce CBF, the % AA and MP data sets. Two laboratories (JT and PMI) used the SAVA platform to re-examine all or some of the captures first analysed in ciliaFA.

Phase II of the study

Phase I testing elucidated several important facets of CBF measurement that required further examination, namely: the impact of magnification strength and tissue acclimation length on the % AA, and the choice of using either WFA or G-WFA CBF outputs from SAVA. In phase II of the study, the usefulness of these parameters for improving the reproducibility of CBF readings were evaluated over a 14-day culture period by using MucilAir tissue cultures. MucilAir cultures from one batch, comprising cells of bronchial origin obtained from a healthy, non-smoking Caucasian man aged 53 years (Epithelix donor MD051002), were used in three laboratories (IIVS, JT and PMI) and those from a 41-year-old healthy Caucasian non-smoker male (Epithelix donor MD072001) were used by one laboratory (IB). All phase II analyses were conducted with SAVA.

Assessment of magnification strength: Various objective magnifications were assessed to determine whether they would provide equivalent CBF data. A single-coordinate position was chosen on the apical surface of three individual tissue inserts. Without movement of the stage, one field was captured with different magnification strengths: 1×, 4×, 6× and 10× in the IIVS laboratory, and 4× and 10× in the JT laboratory. CBF captures were taken at room temperature (22–25°C) and mathematically adjusted to 37°C by applying a correction factor of 0.84 Hz/°C, as described by Sisson et al.[20]

While working with large numbers of RHuAs in phase I, it was found that taking four captures per tissue insert was cumbersome and time consuming. To determine the number of fields that provide adequate representation for a tissue, the IIVS laboratory examined the variability of CBF within an insert by capturing fields that 'tiled' the surface area (to the best of the operator's ability) in a MucilAir insert. As no indexed or automated stage was available, the operator used their best judgement to assess the boundaries of the previously captured field, when shifting to a new coordinate in the *x*- and *y*-axes. Some minor overlap across fields was expected, and some captures included the edge of the tissue culture insert to ensure full coverage. The operator process for CBF captures did not include the assessment of captures — therefore, the captures were subsequently

re-assessed by manual inspection to remove suboptimal captures from the data set. Thus, data were analysed for all captures, those remaining after the exclusion of captures with no CBF value, and those remaining after the exclusion of captures with perceived technical issues (each capture was manually assessed).

Determination of tissue acclimation effects: In three laboratories (IIVS, JT and PMI), three RHuA tissue cultures per treatment group were evaluated for the impact of length of acclimation on the % AA and CBF. One, two or four ciliary beat captures were taken per culture (with 4× and/or 10× objective strength), and the % AA and CBF (WFA and G-WFA outputs) were assessed by using SAVA. The acclimation periods used by the participating laboratories were influenced by tissue delivery date. Data sets were labelled as 'short acclimation' (SA) or 'long acclimation' (LA), followed by a number to denote the number of days between tissue receipt and CBF analysis. The acclimation periods in each laboratory were: SA3 and LA6 for IIVS; SA2 and LA10 for JT; and SA2 and LA9 for PMI. All laboratories used a no-acclimation control group in which tissues were re-fed from day 0 only. Captures over 14 days were obtained from either the same set of tissues (IIVS and JT) or different sets (PMI) that had been acclimated for the same duration. In the separate tissue culture batch used by IB, after a poor initial % AA, acclimation effects and % AA were evaluated over 21 days.

Comparison of WFA and G-WFA CBF outputs from SAVA: When assessing ciliary beating with SAVA, both WFA and G-WFA outputs are produced. To better understand the impact of curve-fitting data, previously collected data sets, obtained in the IIVS laboratory by using the methods described above for phase I of the study, were re-examined and compared. Captures subjectively determined to be impacted by lighting or technical issues were reviewed as both WFA and G-WFA outputs.

Statistical analysis

The linearity of CBF values across the SAVA and ciliaFA platforms was assessed by calculation of R^2 values with the least squares method in Excel®.

Results

Of the six participating laboratories, three reported data for tissue cultures obtained from both vendors and three reported data for tissue cultures from one source (Table 3).

Phase I of the study

SAVA inter-laboratory data and platform comparison: The SAVA data from the CM tissue cultures were used for CBF analysis and inter-laboratory comparison (Table 4). Without the application of the 1000 MP cut-off, WFA CBF captures were generally interpretable, but some did not produce a data output, as demonstrated where a count of < 20 fields is indicated (see bold text in Table 4). Upon applying the cut-off, a substantial number of captures were excluded. Tissues yielding the lowest % AA and MP values had the highest number of captures excluded. Additional data for all treatment groups (IL-13, procaterol, and IL-13 + procaterol) are supplied as online Supplementary Material.

The IIVS captured SAVA data at room temperature. With the MucilAir model, a low average % AA value of 1.9% was recorded on day 0, but the values generally increased over time (Table 4). Variability between tissues was noted at all time points, but had diminished slightly by day 14. The average MucilAir CBF values were generally consistent but declined slightly over time (with the exception of day 12). The EpiAirway tissues also initially showed increasing % AA up to day 5, followed by a slight fall and then relatively consistent values until day 14. The pattern of average CBF values was similar to that seen with the MucilAir model.

In the BAT laboratory, measurements were performed at 37°C in the MucilAir model only. The % AA was consistent at the three time points tested (Table 4). Of note was that the mean MP counts were all above 14,000, and the 1000 MP cut-off did not result in the exclusion of any captures. The mean CBF values varied over the three time points, with an initial value of 7.2 ± 0.5 Hz on day 0, dropping to 5.5 ± 1.6 Hz on day 7, and then increasing to 8.5 ± 0.7 Hz by day 14.

At the NCTR, the captures were obtained at 30°C, and only in the EpiAirway model. Although MP values were high, many captures were taken from fields with little activity and, therefore, few data were interpretable by SAVA analysis (total number of fields, range five to eight; Table 4). The average CBF values displayed a similar variability pattern to those seen by the IIVS for MucilAir and EpiAirway, but the values at the NCTR laboratory were more pronounced, perhaps due to the limited total number of fields representing the data sets.

CiliaFA inter-laboratory data and platform comparison: Three laboratories (JT, PMI and IB) utilised ciliaFA to analyse the CBF captures. The ciliaFA platform does not produce MP data and analyzes a smaller region of interest than the SAVA platform. All captures were interpretable by the ciliaFA platform (Table 5). All tissue cultures were sampled at ~37°C. The % AA values showed similar variability to that seen with SAVA, while the CBF values showed similar consistency compared to SAVA.

The MucilAir data collected by JT over 12 days indicated generally stable % AA values (Table 5). The average CBF values were generally consistent over the same time period.

The PMI laboratory assessed MucilAir and EpiAirway tissue cultures (Table 5). The mean MucilAir % AA ranged

Table 3. The test systems employed by the participating laboratories.

Laboratory	Test system	
	MatTek EpiAirway™	Epithelix MucilAir™
Institute for In Vitro Sciences (IIVS), USA	X	X
Japan Tobacco (JT), Japan		X
Phillip Morris International (PMI), Switzerland	X	X
British American Tobacco (BAT), UK		X
National Center for Toxicological Research (NCTR), USA	X	
Imperial Brands (IB), Germany	X	X

from 19.7 to 49.7%, and the CBF ranged from 8.5 to 9.6 Hz. While the % AA did not vary greatly and was similar to the overall results obtained by JT, a lower value was seen at day 12. The EpiAirway % AA values were much lower than those obtained with MucilAir and showed wider variability. The CBF values were generally consistent within and between the two tissue culture models.

The IB laboratory assessed only the MucilAir model, and took measurements only on days 0 and 7. Robust values for the % AA and CBF were seen on both days (Table 5).

SAVA versus *ciliaFA comparison summary:* The linearity assessment indicated that low MP values tended to be associated with low CBF values in SAVA, whereas the opposite was true for ciliaFA (Figure 2(a) and (b)). However, the linearity of CBF values across the two platforms was consistent (R^2 = 0.9663) (Figure 2(c)). The SAVA analysis yielded a maximum MP of 18,030, whereas the ciliaFA analysis yielded 1570 (Figure 2(a) and (b)). Therefore, a much wider range of % AA was seen with SAVA (Figure 2(d)). If the 1000 MP cut-off were applied to the ciliaFA data set, fields where the % AA was < 62.5% would be excluded.

For the ciliaFA captures re-analysed in SAVA by the JT and PMI laboratories, notable differences were seen in the % AA outputs within and between laboratories, and tissue culture models (Table 6). The CBF values were similar across the two laboratories for MucilAir. However, those for EpiAirway in the PMI laboratory were notably higher than those for MucilAir (Table 6). SAVA yielded mostly higher % AA and CBF values than ciliaFA (Tables 5 and 6). Applying the 1000 MP cut-off to the re-analysed data sets eliminated one field in MucilAir, but greatly reduced the total number of fields in EpiAirway samples, in particular excluding all data obtained on day 0 (Table 6).

The effects of IL-13 and procaterol exposure: Four laboratories found that IL-13 immediately induced loss of CBF in MucilAir tissue cultures (Figure 3). EpiAirway cultures treated with IL-13 showed notably reduced CBF at later time points in the NCTR laboratory, but other laboratories using this model did not observe a similar effect (Figure 3). The effect of procaterol was variable, but it typically increased CBF compared with the untreated cultures in both tissue models (Figure 3). When added to the cultures treated with IL-13, the boosting effect was no longer seen, except in the BAT and NCTR laboratories at day 7 (Figure 3).

Phase II of the study

Evaluating magnification strength: When assessing the objective magnification used to capture the images, the measurements taken at room temperature showed variability in the CBF values obtained at the 1× magnification and those obtained at the higher magnifications (Table 7). However, applying the correction factor to offset room temperature readings highlighted the impact of temperature on the CBF values obtained in the WFA and G-WFA analyses. This effect was most notable for tissue insert one, as all corrected WFA output data yielded the same value of 5.5 Hz and 15.6 Hz at 25°C and 37°C, respectively, across all magnifications (Table 7).

When assessing intra-insert variability for all captures and magnification strengths, a standard deviation range of 14.5–35.2 in the % AA and 0.1–1.1 Hz in the CBF (WFA) was observed (Table 8). G-WFA showed little variation. This variability was reduced by excluding captures that did not provide a CBF value and of all captures with any perceived technical issues (Table 8). Poor lighting (reduced contrast), inadvertent stage movement (such as that caused by the movements of passers-by), or other unknown factors, could contribute to the technical issues. The 4× magnification (which covered the greatest surface area of the tissue) consistently yielded the highest % AA, which increased with the successive filtering of poor captures (Table 8). However, mean CBF values did not appear to be greatly impacted by magnification strength. Slight increases were seen with increasing magnification and successive filtering of the capture data (Table 8). Based on the compiled data, it was concluded that the use of six to seven fields was

Table 4. The baseline characteristics of the untreated tissue cultures, by tissue model and laboratory.

		% AA	Number of MPs (WFA)	CBF (WFA ≤ 1000 MP)			CBF (WFA > 1000 MP)		
Laboratory	Day	AVE ± SD	AVE ± SD	AVE (Hz) ± SD	Number of samples assessed	Total number of fields	AVE (Hz) ± SD	Number of samples assessed	Total number of fields
a) MucilAir									
IIVS (22.9–24.4°C)	0	1.9 ± 2.3	366 ± 437	5.3 ± 0.6	5	**19**	6.0[a]	2	**2**
	3	34.7 ± 14.9	6668 ± 2870	5.1 ± 0.5	5	20	5.1 ± 0.5	5	20
	5	27.9 ± 25.7	5356 ± 4926	4.9 ± 0.8	5	**19**	5.0 ± 0.8	5	**15**
	7	27.5 ± 25.1	5286 ± 4824	3.9 ± 0.4	5	**18**	4.0 ± 0.5	5	**15**
	10	60.8 ± 33.9	11,679 ± 6508	3.9 ± 0.5	5	20	3.9 ± 0.5	5	**19**
	12	59.2 ± 27.9	11,364 ± 5364	4.8 ± 0.5	5	20	4.8 ± 0.5	5	**18**
	14	70.3 ± 14.0	13,506 ± 2697	3.6 ± 0.3	5	20	3.6 ± 0.3	5	20
BAT (37°C)	0	73.2 ± 11.0	14,054 ± 2113	7.2 ± 0.5	5	20	7.2 ± 0.5	5	20
	7	86.1 ± 7.3	16,531 ± 1405	5.5 ± 1.6	5	20	5.5 ± 1.6	5	20
	14	77.2 ± 16.0	14,827 ± 3076	8.5 ± 0.7	5	20	8.5 ± 0.7	5	20
b) EpiAirway™									
IIVS (22.9–24.4°C)	0	0.8 ± 1.3	93 ± 208	5.3 ± 1.9	5	**18**	2.8[a]	1	**1**
	3	3.9 ± 3.8	748 ± 722	3.7 ± 0.6	5	20	4.2 ± 1.1	4	**6**
	5	10.7 ± 9.3	1851 ± 1789	5.4 ± 1.3	5	**19**	5.7 ± 1.4	5	**15**
	7	7.6 ± 8.9	1451 ± 1716	5.1 ± 1.3	5	20	5.1 ± 1.3	5	**10**
	10	8.7 ± 8.2	1666 ± 1576	3.6 ± 0.6	5	20	4.0 ± 0.9	4	**10**
	12	8.9 ± 11.4	1703 ± 2187	4.5 ± 0.3	5	20	3.9 ± 0.5	4	**9**
	14	6.4 ± 9.3	1236 ± 1792	4.0 ± 0.9	5	20	4.1 ± 1.2	3	**8**
NCTR (30°C)	0	10.1 ± 7.5	1946 ± 1445	11.5 ± 3.9	5	**5**	12.8 ± 2.4	3	**3**
	3	11.9 ± 9.0	2277 ± 1724	8.5 ± 1.0	5	**5**	8.8 ± 1.1	3	**3**
	5	16.1 ± 12.6	3084 ± 2416	12.6 ± 2.1	5	**5**	13.1 ± 2.0	4	**4**
	7	23.5 ± 21.8	4517 ± 4180	8.8 ± 1.7	5	**7**	8.9 ± 1.9	4	**6**
	10	3.8 ± 3.6	738 ± 684	6.0 ± 0.8	5	**5**	6.1[a]	1	**1**
	12	9.4 ± 9.2	1802 ± 1776	12.2 ± 2.2	5	**8**	12.4 ± 2.1	5	**5**
	14	6.0 ± 4.0	1148 ± 775	9.4 ± 2.4	5	**7**	9.1 ± 2.6	3	**3**

The outputs were captured with SAVA (Sisson-Ammons Video Analysis) software. A maximum of four capture fields were obtained per tissue culture sample (i.e. the maximum total number of fields was 20). The culture temperature at the time of video capture is indicated for each laboratory. The cut-off of > 1000 MPs was assessed for WFA analysis by comparing values in samples with ≤ 1000 MPs. Cultures with at least one video capture were included in the analysis. Where the total number of fields was < 20, these values are indicated in bold. [a]The SD was not calculated, as ≤ 2 sample fields were averaged. % AA = percentage active area; MP = motile point; CBF = ciliary beat frequency; WFA = whole-field analysis; AVE = average; SD = standard deviation; IIVS = Institute for In Vitro Sciences; BAT = British American Tobacco; NCTR = National Center for Toxicological Research.

adequate to tile the surface area for CBF determination with the 4× objective, and 11–14 fields with the 6× objective. With the 10× objective, the 'tiling' approach was not feasible due to the large area available for capture. Therefore, capture in 12–14 distinct fields was judged to be sufficient.

Tissue acclimation effects: In the phase II acclimation assessments, the IIVS laboratory acclimated MucilAir cultures for three (SA3) or six (LA6) days. The % AA was inconsistent between the acclimation groups at days 3 and 5. The LA6 group demonstrated a downward trend in % AA prior to the SA3 group, although the overall time-dependent trend patterns were similar (Figure 4). CBF measurements were consistent through to day 14 for the SA3 group, but for the LA6 tissues, CBF values diminished after day 7 (Figure 4). The loss of % AA and CBF in the LA6 tissues appeared to coincide at days 12–14. For SA3 tissues, at day 14, the % AA values had fallen to ~50%, while CBF showed no sign of reduction.

In the JT laboratory, MucilAir cultures were acclimated for two (SA2) or 10 days (LA10). Although trends over the 14 days were similar for the SA2 and LA10 tissues, on day 0, the 4× objective captures produced a higher mean % AA than those obtained with the 10× objective (39–67% *versus* 25–31%) and more consistent mean CBF (10.3–10.5 Hz *versus* 9.4–10.2 Hz) (Figure 4). The no-acclimation control had the highest mean % AA (67%) on day 0, by using the 4× objective, and no overall increase was seen over time, as compared with the SA2 and LA10 cultures (Figure 4). When the 10× objective was used, the average % AA values

Table 5. The data obtained from five tissues exposed to culture medium and analysed with ciliaFA.

		% AA	CBF
Laboratory	Day	AVE ± SD	AVE (Hz) ± SD
a) MucilAir™			
JT (37°C)	0	75.1 ± 13.8	8.5 ± 1.4
	3	46.4 ± 32.3	9.2 ± 1.5
	5	46.1 ± 33.7	9.2 ± 1.1
	7	45.2 ± 30.8	9.1 ± 1.3
	10	39.7 ± 23.8	9.2 ± 0.9
	12	57.7 ± 25.2	8.2 ± 1.0
PMI (37°C)	0	27.3 ± 8.3	9.3 ± 0.2
	3	33.0 ± 7.0	9.1 ± 1.1
	5	31.6 ± 5.5	9.6 ± 0.2
	7	46.6 ± 5.6	8.7 ± 0.3
	10	49.7 ± 32.6	8.5 ± 1.0
	12	19.7 ± 6.7	9.1 ± 0.4
	14	44.0 ± 9.3	9.3 ± 0.2
IB (37°C)	0	49.6 ± 24.2	10.0 ± 0.7
	7	51.1 ± 11.3	9.4 ± 0.8
b) EpiAirway™			
PMI (37°C)	0*	1.0 ± 0.6	8.0 ± 0.6
	3	17.1 ± 4.9	9.8 ± 0.1
	5	15.8 ± 8.3	8.4 ± 1.4
	7	8.9 ± 5.5	9.4 ± 1.2
	10	12.9 ± 5.5	9.6 ± 0.2
	12	13.7 ± 2.3	9.0 ± 0.4
	14	9.8 ± 3.5	7.2 ± 1.8

The laboratories cultured five sets of tissues with medium alone (i.e. untreated) for 14 days; the maximum total number of fields was 20 (i.e. four fields per sample). *Four tissue samples were assessed in this case, with a total of 16 possible fields. The video captures were analysed by using ciliaFA; the culture temperature at the time of video capture is indicated for each laboratory. The average (AVE) ± standard deviation (SD) is displayed for the % AA, and CBF for all captures; ciliaFA does not provide an MP output.
% AA = percentage active area; CBF = ciliary beat frequency; JT = Japan Tobacco; PMI = Phillip Morris International; IB = Imperial Brands.

were similar for all groups on day 0, and the increases over time were comparable between the groups. The average CBF remained fairly stable over the 14-day period for all three groups, with both objectives, although the 4× captures generated slightly lower values by day 14 than at day 0 (Figure 4).

In the PMI laboratory, MucilAir cultures were acclimated for two (SA2) or 9 days (LA9). The LA9 group had a lower % AA at day 0, but values increased over time and were only slightly lower than those for SA2 on days 7 and 14 (Figure 4). The mean CBF initially differed between the SA2 and LA9 groups, but it was similar at days 7 and 14 (Figure 4).

The MucilAir tissues received by IB were evaluated immediately after receipt and the % AA was found to be low (Figure 5). The tissues were cultured and the % AA and CBF monitored over 21 days. The % AA and CBF recovered over the 14 days and then remained fairly stable until day 21. The CBF was initially around 11 Hz and remained stable throughout the 21-days testing period.

WFA versus G-WFA CBF outputs from SAVA: When the WFA and G-WFA SAVA outputs were compared by the IIVS laboratory, it was apparent that the G-WFA analysis for the mean CBF often excluded fields where the MP counts were low (relative to other captures within the tissue culture), and/or an issue was manually observed during capture. A sample data set highlighting results from this type of subjective analysis is presented (Table 9). It was also noted that, for captures that delivered approximately equal Hz outputs from both WFA and G-WFA, the number of MPs measured with G-WFA did not reflect a difference (fields 4 and 7). Other fields with lower MPs where the WFA output yielded a data set did not produce G-WFA data (fields 5 and 8).

Discussion

In this ring trial, the participating laboratories used reconstituted tissue cultures from the same donor throughout the whole of Phase I and different donors throughout the whole of Phase II, to generate CBF data by using SAVA, ciliaFA, or both platforms. In view of this technicality, we would recommend that laboratories wishing to conduct repeat studies with cells derived from the same donors, should consider reserving batches of donor cells. This would depend on whether the future tissue requirements for use in extensive testing campaigns could be adequately predicted.

Each laboratory was allowed to use its own microscopy set-up to measure the CBF, and relied on its own interpretation of the protocols, according to established best practices. The aim of this study, therefore, was to identify potential similarities and differences between the laboratories and consider what challenges these might pose for understanding the data generated. We found that adjustment of the lighting to produce maximal contrast (regardless of the custom hardware or lighting settings of each microscope set-up) was important for optimising captures and minimising variation in the data, but it was clear that this adjustment is specific to each individual set-up and cannot be standardised. However, certain standard practices in microscopy, such as the use of a linear phase filter, might optimise the captures. We suggest that laboratories test various set-ups, to establish the optimal capture conditions for their hardware configuration.

Inherent differences in the properties of the cells used to create the RHuAs (e.g. donor age), the particular culture methods employed by the manufacturer, specific tissue

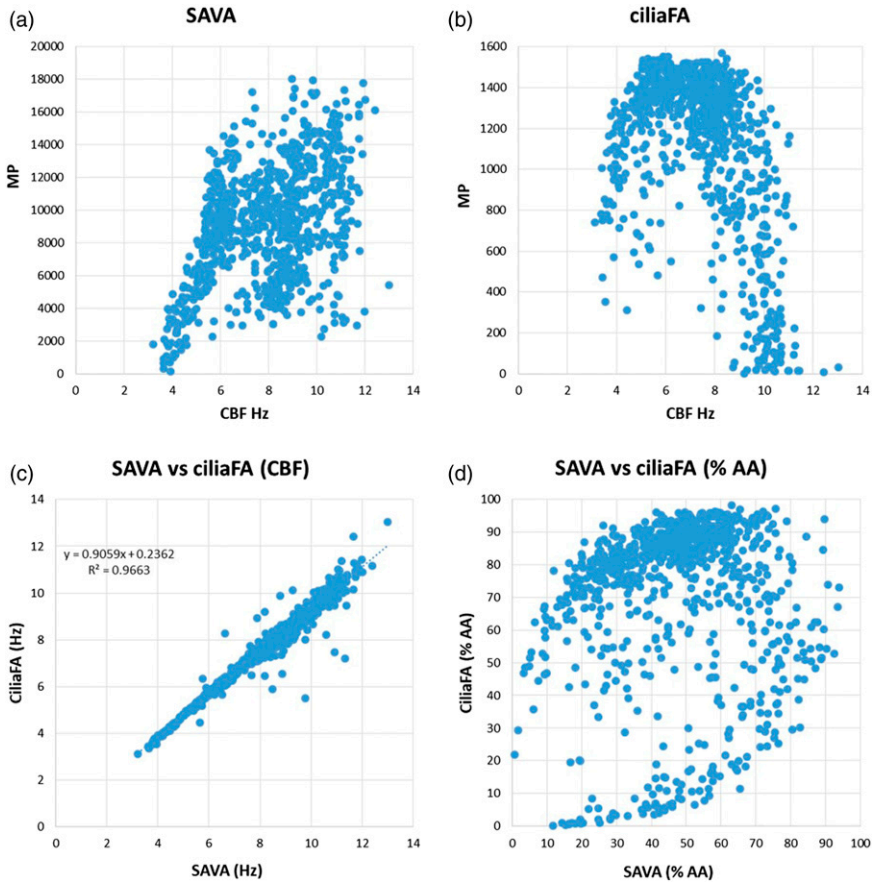

Figure 2. Comparison of SAVA and ciliaFA linearity. (a) With SAVA analysis, the number of MPs compared with CBF showed a narrower distribution at lower CBF (~2–6 Hz) than at higher CBF (6–14 Hz). (b) The ciliaFA analysis displayed an apparently lower MP count, most notably when the CBF was greater than ~9 Hz. (c) Good linearity for CBF values was found between platforms. (d) The % AA (represented by MP counts) was more widely distributed in SAVA compared with ciliaFA, which reflects the differences noted in (a) and (b). MP = motile point; CBF = ciliary beat frequency; % AA = percentage active area.

batch properties, the level of tissue maturity, as well as other factors, all contribute to the performance of the reconstituted tissues. While conducting cell profiling of the different tissues may have elucidated some fundamental differences between donor tissues and batch preparations, it would not address all of the observed differences in performance and simply was not practical in this exercise, where two CBF platforms were being compared.

EpiAirway tissues are distributed earlier in the tissue maturation period than MucilAir —14 or 20 days *versus* ~4–5 weeks after airlift (i.e. the process of removing the expansion medium from the apical chamber after the cells have reached confluence, and then exposing the apical surface of the culture to the ALI). The authors believe that fully matured tissues seem likely to provide the best performance in terms of CBF measurements, because the properties of fully differentiated tissues have been shown to be relatively stable over the duration period employed in this study. We believe that the experiments conducted in phases I and II of this ring trial can be extended to other platforms, as we expect that the technology used for capturing beating cilia and analysing the data will be similar.

Phase I of the study

The phase I study parameters were developed during the TWS. These included: the choice of RHuAs; the general concepts of optimal CBF capture; the time points analysed; the CBF controls to be used; and, for SAVA, the MP cut-off that could be applied to maintain a robust signal. The use of a 1000 MP cut-off came into question during phase I, when specific tissues with inherently low MP counts were excluded, despite not being treated or adversely impacted. Although the use of an MP cut-off has merit in terms of the reliability of CBF data, some treatments can diminish the % AA (and by extension MP counts) and a cut-off would skew the results. This effect was seen with IL-13 exposures, and in some tissues that, due to the method of maturation and/or donor tissue characteristics, had reduced areas of CBF activity. For untreated RHuA tissues that exhibit low % AA,

Table 6. SAVA re-analysis of ciliaFA data.

		% AA	MP (WFA)	CBF (WFA)			CBF (WFA > 1000 MP)		
Laboratory	Day	AVE ± SD	AVE ± SD	AVE (Hz) ± SD	Number of samples assessed	Total number of fields	AVE (Hz) ± SD	Number of samples assessed	Total number of fields
a) MucilAir™									
JT (37°C)	0	44.7 ± 15.8	8576.9 ± 3035.4	9.1 ± 1.4	5	20	9.1 ± 1.4	5	20
	3	57.0 ± 20.2	10,936.6 ± 3876.3	9.7 ± 1.5	5	20	9.7 ± 1.5	5	20
	5	62.7 ± 23.3	12,040.9 ± 4472.2	9.9 ± 1.1	5	20	9.9 ± 1.1	5	20
	7	62.8 ± 14.7	12,052.7 ± 2822.8	9.7 ± 1.2	5	20	9.7 ± 1.2	5	20
	10	68.1 ± 10.5	13,081.6 ± 2006.4	9.9 ± 1.0	5	20	9.9 ± 1.0	5	20
	12	66.4 ± 14.2	12,757.8 ± 2735.9	8.8 ± 1.1	5	20	8.8 ± 1.1	5	20
PMI (37°C)	0	23.3 ± 6.7	4478.7 ± 1286.0	10.0 ± 0.3	5	20	10.0 ± 0.3	5	19
	7	58.7 ± 5.6	11,267.9 ± 1072.6	9.2 ± 0.3	5	20	9.2 ± 0.3	5	20
	14	69.6 ± 15.9	13,368.2 ± 3054.2	10.1 ± 0.4	5	20	10.1 ± 0.4	5	20
b) EpiAirway™									
PMI (37°C)	0	1.5 ± 0.5	239.0 ± 94.8	18.1 ± 0.9	5	20	—	0	0
	7	7.5 ± 5.5	1442.7 ± 1054.8	19.3 ± 1.5	5	20	19.3 ± 2.1	5	9
	14	27.6 ± 15.1	5304.8 ± 2894.5	23.4 ± 1.9	5	20	23.4 ± 1.9	5	16

Two laboratories cultured MucilAir only, or both MucilAir and EpiAirway tissues, and used ciliaFA to determine the % AA and CBF in untreated tissues. The target culture temperature at the time of video capture is indicated for each laboratory. All or some of the captured fields were subsequently re-analysed by using SAVA software for comparison. The 1000 MP cut-off applied for the SAVA analyses is displayed (see right-hand set of columns). Comparison with ciliaFA data (Table 5) indicates some differences in the % AA, but similar CBF on the respective testing days. A similar trend of CBF values was observed over the duration of the study. The average (AVE) ± standard deviation (SD) is displayed.
% AA = percentage active area; MP = motile point; CBF = ciliary beat frequency; WFA = whole-field analysis; JT = Japan Tobacco; PMI = Phillip Morris International.

researchers should make an independent assessment of whether the RHuA test system is useful for CBF studies.

Procaterol application elevated the CBF in untreated MucilAir and EpiAirway cultures (as expected), but not in tissues co-treated with IL-13 (with the exception of day 7 tissues assessed by the BAT laboratory). Using WFA values, many of the EpiAirway readings fell below the 1000 MP cut-off in the untreated and IL-13-treated groups. It should be noted that IIVS and NCTR used 5 ng/ml IL-13 for EpiAirway treatment and BAT used 5 ng/ml IL-13 for MucilAir treatment, but in a comparison of goblet cell induction between IIVS and PMI tissues, where 10 ng/ml was used, the results were similar. Further, despite BAT utilising a different IL-13 source and 5 ng/ml being used by two laboratories, the EC_{50} of IL-13 from both vendors was 4 to 5-fold lower and therefore the concentrations used (i.e. either 5 ng/ml or 10 ng/ml) were considered to be near or at saturation of the GCH biological effect.

Despite some variability in the effects of IL-13 and procaterol, these positive controls (i.e. for lowering or increasing CBF) were useful to show that ciliaFA and SAVA could measure the expected CBF changes. While not a focus of this current report, it has been observed that the induction of GCH differed between the tissue models used, with an average MucilAir induction of 18 ± 9-fold and 113 ± 81-fold at days 7 and 14, respectively (in five laboratories), and an average EpiAirway induction of 5 ± 2-fold and 4 ± 3-fold at days 7 and 14, respectively (in three laboratories).[21] The use of different methods for staining and/or analysing GCH induction could account for the discrepancies in fold-change, to some extent. However, the major variability in GCH induction appears to originate from the intrinsic properties of the tissues. For example, a lack of donor tissue responsiveness to IL-13 may account for the differences in CBF response over time.

Regarding the software platforms used for the assessment of CBF, one inherent difference between ciliaFA and SAVA is the total MPs available in the analysed field. A 12-fold difference of possible MPs has a substantial impact on the dynamic range (i.e. the overall range of minimum–maximum) of the analysis. Laboratories generating ciliaFA data at 37°C noted that measurements above ~10 Hz were erroneous, especially affecting the higher (Hz) frequency range. This seems to be the result of an error in the MS Excel-based processing in the ciliaFA application. Closer investigation revealed that the 'FFT Mag' Excel sheet (which needs to be unhidden) contains only rows for a maximum of 64 frames, and thus seems to disregard remaining frames originally imported for the CBF analysis (e.g. 512 frames were imported by PMI in this study). Since the accuracy of the CBF measurement by using ciliaFA is dependent on the frequency resolution (the frame rate of

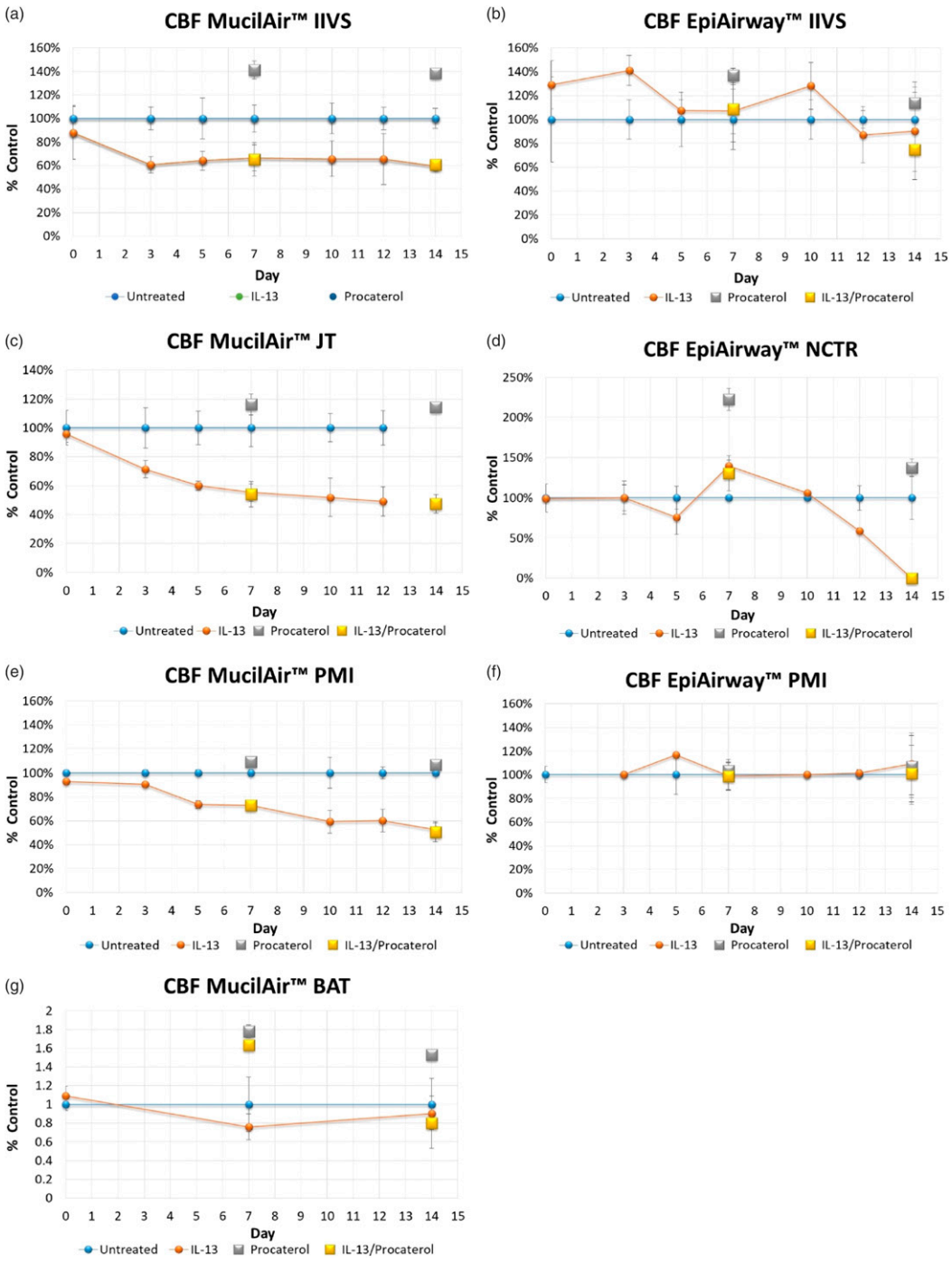

Figure 3. Ciliary beat frequency in MucilAir™ and EpiAirway™ untreated tissue cultures and after treatment with IL-13 and/or procaterol, in the indicated laboratories. The IIVS laboratory measured ciliary beat frequency (CBF) with SAVA and used (a) 10 ng/ml IL-13 to treat the MucilAir tissues and (b) 5 ng/ml to treat the EpiAirway tissues; 10 mM procaterol was used to treat both test systems. (c) The JT laboratory measured CBF with ciliaFA and used 10 ng/ml IL-13 and 10 mM procaterol to treat the MucilAir tissues; day 14 IL-13 and IL-13/procaterol calculations were made with untreated control data from day 12, as day 14 files were corrupted. Re-analysis of captures by using SAVA yielded equivalent results (data not shown). (d) NCTR measured CBF with SAVA, and used 5 ng/ml IL-13 and 10 mM procaterol to treat the EpiAirway tissues. (e) and (f) PMI measured CBF with ciliaFA, and used 10 ng/ml IL-13 to treat both MucilAir and EpiAirway tissues, and 10 mM procaterol. (g) BAT measured CBF with SAVA, and used 10 ng/ml IL-13 to treat the MucilAir tissues, and 10 mM procaterol. IIVS = Institute for In Vitro Sciences; JT = Japan Tobacco; NCTR = National Center for Toxicological Research; PMI = Phillip Morris International; BAT = British American Tobacco.

Table 7. The effect of field magnification on the ciliary beat frequency.

Insert	Magnification	Room temperature (°C) (actual reading)	CBF					
			At 'room temperature'		Corrected (to 25°C)		Corrected (to 37°C)	
			WFA	G-WFA	WFA	G-WFA	WFA	G-WFA
1	1×	24.3	4.9	4.9	5.5	5.5	15.6	15.6
	4×	25.7	6.1	6.0	5.5	5.4	15.6	15.5
	6×	25.7	6.1	6.1	5.5	5.5	15.6	15.6
	10×	25.8	6.2	5.9	5.5	5.2	15.6	15.3
2	1×	24.4	5.2	5.3	5.7	5.8	15.8	15.9
	4×	25.7	6.3	6.3	5.7	5.7	15.8	15.8
	6×	25.7	6.5	6.4	5.9	5.8	16.0	15.9
	10×	25.6	6.7	6.5	6.2	5.9	16.3	16.0
3	1×	24.4	4.8	4.8	5.3	5.3	15.4	15.4
	4×	25.6	5.7	5.7	5.2	5.2	15.3	15.2
	6×	25.6	5.8	5.7	5.3	5.2	15.4	15.2
	10×	25.7	6.0	5.7	5.5	5.1	15.5	15.2

A single coordinate position was chosen on the apical surface of three individual tissue inserts. Without movement of the stage, a single field was captured at different magnification strengths, and the ciliary beat frequency (CBF) compared for WFA and G-WFA. Hz values taken at the room temperatures indicated were corrected for 37°C by using the correction factor of 0.84 Hz/°C, as previously established.

Table 8. Assessment of tissue variability and magnification strength, and the impact of data set refinement.

Category (and inclusion/exclusion criteria)	Magnification (total number of captures)	Tissue	Temp. (°C)	% AA		WFA (Hz)			G-WFA (Hz)		
				AVE ± SD	AVE ± SD	*N*	AVE ± SD	AVE ± SD	*N*	AVE ± SD	AVE ± SD
All captures (all captures, regardless of quality)	4× (*n* = 23)	1	24.6	50.5 ± 33.6	47.4 ± 4.5	8	4.6 ± 0.7	4.9 ± 0.2	6	5.0 ± 0.1	5.1 ± 0.2
		2	24.8	42.2 ± 29.0		8	5.0 ± 0.6		7	5.3 ± 0.1	
		3	25.1	49.4 ± 25.3		7	5.0 ± 0.1		7	5.0 ± 0.1	
	6× (*n* = 39)	1	25.1	35.6 ± 35.2	28.4 ± 11.0	11	4.8 ± 0.7	4.5 ± 0.4	9	5.2 ± 0.1	5.3 ± 0.2
		2	25.4	15.7 ± 14.5		14	4.1 ± 1.1		5	5.5 ± 0.2	
		3	25.6	33.8 ± 30.9		14	4.7 ± 0.8		11	5.2 ± 0.1	
	10× (*n* = 38)	1	25.5	56.8 ± 33.4	41.0 ± 17.3	12	5.5 ± 0.7	5.3 ± 0.2	11	5.7 ± 0.1	5.2 ± 0.4
		2	25.6	22.6 ± 22.1		14	5.3 ± 0.8		13	5.3 ± 1.4	
		3	25.7	43.6 ± 35.0		12	5.1 ± 0.8		12	4.8 ± 1.2	
Most captures (poor captures that did not provide a CBF value were removed)	4× (*n* = 20)	1	24.6	62.9 ± 28.6	53.2 ± 8.4	6	5.0 ± 0.1	5.0 ± 0.1	6	5.0 ± 0.1	5.1 ± 0.2
		2	24.8	47.4 ± 27.0		7	5.2 ± 0.4		7	5.3 ± 0.1	
		3	25.1	49.4 ± 25.3		7	5.0 ± 0.1		7	5.0 ± 0.1	
	6× (*n* = 22)	1	25.1	51.5 ± 35.2	42.5 ± 10.4	7	5.3 ± 0.1	5.3 ± 0.1	7	5.2 ± 0.1	5.3 ± 0.2
		2	25.4	31.1 ± 14.4		5	5.4 ± 0.3		5	5.5 ± 0.2	
		3	25.6	44.9 ± 29.7		10	5.1 ± 0.3		10	5.2 ± 0.1	
	10× (*n* = 32)	1	25.5	56.8 ± 33.4	45.9 ± 17.3	12	5.5 ± 0.7	5.6 ± 0.1	12	5.7 ± 0.1	5.6 ± 0.2
		2	25.6	25.9 ± 24.0		11	5.7 ± 0.4		11	5.8 ± 0.2	
		3	25.7	54.9 ± 33.2		9	5.5 ± 0.2		9	5.5 ± 0.2	
Best captures (captures with perceived technical issues were removed)	4× (*n* = 13)	1	24.6	81.0 ± 5.5	69.7 ± 10.0	4	5.1 ± 0.0	5.1 ± 0.2	4	5.0 ± 0.0	5.1 ± 0.2
		2	24.8	61.8 ± 13.5		5	5.3 ± 0.1		5	5.3 ± 0.1	
		3	25.1	66.5 ± 19.1		4	5.0 ± 0.0		4	5.0 ± 0.0	
	6× (*n* = 14)	1	25.1	59.1 ± 31.7	57.4 ± 21.9	6	5.3 ± 0.1	5.4 ± 0.1	6	5.2 ± 0.1	5.3 ± 0.2
		2	25.4	34.7 ± 13.9		4	5.5 ± 0.1		4	5.6 ± 0.2	
		3	25.6	78.4 ± 5.8		4	5.3 ± 0.1		4	5.3 ± 0.1	
	10× (*n* = 32)	1	25.5	56.8 ± 33.4	45.9 ± 17.3	12	5.5 ± 0.7	5.6 ± 0.1	12	5.7 ± 0.1	5.6 ± 0.2
		2	25.6	25.9 ± 24.0		11	5.7 ± 0.4		11	5.8 ± 0.2	
		3	25.7	54.9 ± 33.2		9	5.5 ± 0.2		9	5.5 ± 0.2	

The data outputs per analysis type are shown as the average (AVE) and standard deviation (SD) for each insert, and for a group at each magnification. The data corresponding to all captures, all captures yielding a CBF value, and finally only those with no perceived capture issues are shown for the three magnifications. The replicate numbers (*N*) refer to the number of captures within each category that correspond to the data presented.

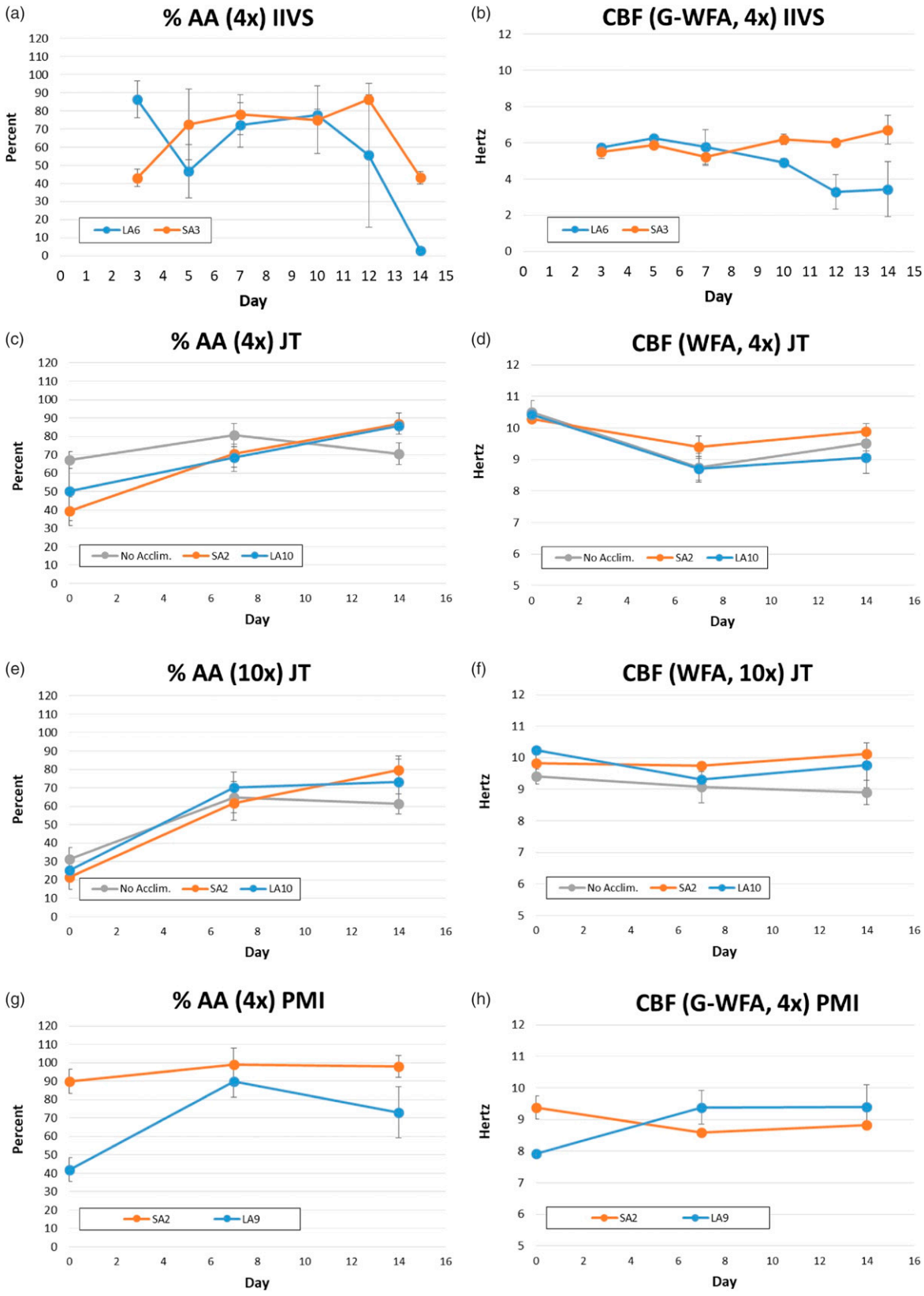

Figure 4. The effects of acclimation duration on the % AA and CBF of MucilAir cultures, by laboratory and magnification. (a and b) In the IIVS laboratory, acclimation differences were assessed after 3 (SA3) and 6 days (LA6) of acclimation, at 4× magnification. (c–f) In the JT laboratory, differences were assessed after 2 (SA2) and 10 days (LA10) of acclimation, at 4× magnification (c and d), and at 10× magnification (e and f). (g and h) In the PMI laboratory, differences were assessed after 2 (SA2) and 9 days (LA9) of acclimation, at 4× magnification. % AA = percentage active area; CBF = ciliary beat frequency; G-WFA = Gaussian whole-field analysis; WFA = whole-field analysis; IIVS = Institute for In Vitro Sciences; JT = Japan Tobacco; PMI = Phillip Morris International.

recording/number of frames),[18] the omission of a large number of frames has an impact on the accuracy of the CBF measurement. This may also explain why procaterol-induced CBF stimulation towards higher CBF (near or above 10 Hz) was less evident for ciliaFA, as compared to SAVA. The ciliaFA software appeared to incorrectly suppress (i.e. output lower CBF values) measurements near and above 10 Hz, whereas the SAVA software appeared to perform consistently. SAVA therefore showed a more consistent picture, which was also reflected by a better correlation between the % AA and MP, and by the RHuA displaying different levels of % AA that correlated directly with the MPs. SAVA measurements appeared consistent and without issues at higher Hz CBF measurements.

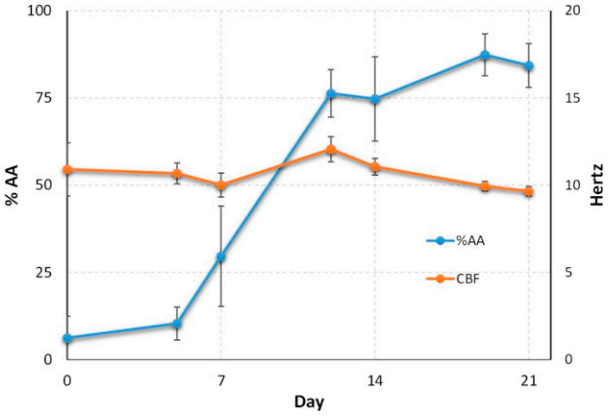

Figure 5. The recovery of % AA after an initial low value. In the IB laboratory, MucilAir tissues exhibited < 20% AA at the time of delivery. The recovery of % AA during acclimation was assessed over 21 days. Mean CBF values were not affected. CBF = ciliary beat frequency; % AA = percentage active area.

Phase II of the study

Phase II of this trial was intended to further assess challenges that had arisen in phase I. The area of the RHuA insert was chosen to ensure that all magnification strengths had captures containing only tissue areas evaluable for ciliary beating. Ciliary beat captures at different magnification strengths resulted in greatly varying tissue surface areas being recorded and analysed. The 1× magnification objective effectively captured the entire insert in one field, including the outside of the well edge where no tissue was present. Mathematically, a 2-D space would have area differences of 16-, 36- and 100-fold, for the 4×, 6× and 10× magnification strengths, relative to the 1× magnification. Despite these large differences in the area of tissue being evaluated, the CBF values were surprisingly consistent, especially after the temperature correction. However, in the interest of obtaining the greatest area for evaluation, especially when exposed tissues may demonstrate response-based changes that are focalised on the tissue surface area, the lowest reasonable magnification will provide the most data per capture taken.

The 'tiling' approach to captures, adopted in the IIVS laboratory for whole-insert surface areas, required insert edges ('noise') to be included, so that the entire surface area was represented for the assessment of tissue variability. The 4–10× objectives all had such fields, which led to variation in the amount of interpretable area per field (i.e. the 4× magnification may have captured a larger non-tissue portion in a field than higher magnifications).

While intra-tissue variability for the % AA and WFA and G-WFA outputs was apparent at all magnification strengths, the exclusion of captures by subjective interpretation of quality reduced the variability. However, without filtering, the mean variability was also substantially reduced when multiple inserts were considered, supporting the use of

Table 9. Sample data for WFA *versus* G-WFA analyses, in eight fields captured at 4× magnification.

			WFA		G-WFA	
Field	Temp. (°C)	Active area (%)	Mean CBF	Number of MP	Mean CBF	Number of MP
1	24.5	75.1	5.05	14,417	4.96	13,254
2	24.6	83.7	5.06	16,061	4.96	14,588
3	24.6	77.9	5.02	14,965	4.98	13,985
4	**24.6**	**32.4**	**4.69**	**6227**	**4.85**	**5714**
5	**24.6**	**19.7**	**3.42**	**3781**	—	—
6	24.6	87.4	5.09	16,778	5.00	15,912
7	**24.6**	**20.9**	**5.03**	**4015**	**5.07**	**3767**
8	**24.6**	**6.7**	**3.83**	**1289**	—	—

Fields where technical issues were encountered after visual observation of the capture (indicated in bold) display data for the standard WFA analysis only, and may not be analysed for an output with a Gaussian curve fitting filter. In the event that a G-WFA analysis is generated, and the Hz output appears consistent with other values obtained for the insert in question, the number of motile points (MPs) may exhibit a difference from other captures lacking technical issues.

multiple tissues for treatment groups. Of note was that, regardless of data filtering, the 4× magnification consistently yielded the highest average % AA values of all three magnification strengths. With the exception of the 4× WFA and the 6× G-WFA output using data from all captures, the CBF values rose as magnification increased, irrespective of the exclusion of captures, but this can likely be attributed to the capture temperatures at each magnification (temperature increased slightly as higher magnifications were used). Thus, if the same magnification is used for all captures acquired for the controls and treatment groups being compared, the impact of slightly different values is expected to be irrelevant to the analysis of ciliary beat response during any individual study, provided CBF captures are taken at the same temperature or are corrected for appropriately.

Commercially available pulmonary tissues are evaluated by the manufacturers for morphology, cell integrity (e.g. transepithelial electrical resistance to assess tight junction dynamics) and ciliary activity prior to shipment. However, specific quality acceptance criteria might vary between manufacturers. After receipt in the laboratory, tissues should be checked for possible damage which might have occurred during transport. In this trial, we evaluated whether the length of tissue acclimation after receipt of tissues affected the % AA and CBF variability and stability. This exercise was not able to identify a superior acclimation length. Of note, however, the IB experience does suggest that recovery from low initial % AA is possible.

Anecdotal evidence from the laboratories involved in this study indicates that CBF can stabilise after a few days and remain stable for more than 50 days. Likewise, some laboratories suggested that the % AA was more sensitive than CBF, and decreased by up to 80% after shipment. Based on the experience of IB in this study, recovery of the % AA in severely damaged tissues can take more than 14 days, even when maintained in appropriate culture conditions. Whether recovery would occur for a specific donor and/or batch at all (if it has not occurred within 2–3 weeks) is unclear, and should be investigated further. The use of incompletely recovered tissues can lead to artefacts, such as increases in the % AA after exposure to diluted smoke or aerosol (data not shown), when fully recovered tissues would not show such effects.

The CBF data generated by SAVA provides various outputs, including the % AA, MP count and two beat frequency calculations (WFA and G-WFA). The Gaussian curve fitting output, G-WFA, is intended to refine the data set by omitting data points deemed to be outliers in the WFA. To better understand the relationship between the two forms of output, the co-inventor of SAVA (Bruce Ammons) was approached for clarification. The following description of the two outputs was provided: "The Gaussian curve (or Gaussian function) is a standard distribution curve from statistics. The SAVA system uses curve fitting to find the best Gaussian curve that represents the raw data. From the curve fit results, we have the mean and standard distribution. This fit is robust because it ignores the outlier data and gives the same results when outlier points are removed from the data set. The WFA mean (not Gaussian) includes the outliers, which skews the results. If those outliers are removed, it tends to match the Gaussian results fairly well."

During phase I, SAVA-analysed data sets occasionally returned very low % AA and/or WFA CBF values that would be classified as outliers. During phase II testing of the intra-insert variability and the effects of data filtering, every capture was manually (subjectively) evaluated to ascertain why an output may not have been calculable. While SAVA allows the operator to adjust brightness to fall within optimal levels (as indicated by a histogram), lighting issues due to poor contrast, tissue insert position in the multi-well plate (whereby the polystyrene may cause light deflection between wells), or for other reasons, may be unavoidable. Microscope stage movement (e.g. through vibration or inadvertent bumping by the operator) also seemed to impair field capture. Regardless of the cause of the poor capture, a suboptimal analysis (despite robust ciliary beating observed by the operator at the time of capture) will not become apparent until the captured data sets are analysed. Depending on the laboratory schedule, this could be hours, days or even weeks after the captures were taken, and the opportunity to acquire replacement CBF data in a time-specific manner might have passed. However, we found that the G-WFA analysis eliminates outlier data points seen with the WFA analysis that would skew the results for a readout, and can render a capture non-evaluable based on the curve-fitting properties.

Conclusions and recommendations

Robust non-animal models and assays for pulmonary toxicology are required to make competent product development evaluations and risk assessments for new materials requiring toxicity testing. By extension, standardised approaches will better enable comparisons of inter-laboratory data and promote the assays utilising these unified methods for regulatory decision-making. This assessment of protocol interpretation for testing CBF — in two RHuAs from two different manufacturers, and with two commonly used analysis platforms to assess CBF — indicated notable variation between laboratories. Until the methods can be validated, we are now in a position to make a number of recommendations to help standardise the procedures and optimise the results:

1. Ensure that CBF capture stations (microscope, high-speed camera and other hardware) are adequately illuminated to enable high-contrast captures, and are stable enough to avoid interference

from inadvertent stage movement, vibrations, etc.; the SAVA platform provides a brightness histogram to guide lighting.
2. Use a high-speed camera (≥ 80 frames per second) and a capture length of ≥ 2.6 seconds, or as required by the analysis platform.
3. Check that the analysis platform can produce reliable data through the range of % AA and CBF expected/desired for the tissues being assessed.
4. Maintain a constant capture temperature throughout the study.
5. Use fully mature tissues acclimatised for at least 4–10 days, and monitor ciliary activity over time.
6. Recovery of tissues exhibiting a low % AA upon receipt is possible under routine culture conditions.
7. For large-field analysis (e.g. captures intended to represent ciliary movement of a tissue), a lower capture magnification (e.g. 4×) is recommended to provide a large tissue surface area.
8. Two (or more) capture fields per insert are recommended to maximise the probability of representativeness, particularly with larger tissues than those used in this exercise.
9. Verify that captures are suitable before capture campaign completion (i.e. conduct immediate analysis) to allow for additional capture(s) before the study time point expires (N.B. the SAVA platform captures the time segment that *precedes* capture initiation).
10. An MP cut-off should not be utilised, as results might be confounded by treatments (e.g. IL-13) or exposure to materials that impair the % AA. As the % AA is a highly sensitive endpoint (often more so than CBF), to measure adverse tissue responses, researchers will benefit from obtaining a full range of MPs.
11. For the SAVA platform, the G-whole-field analysis (G-WFA) output is recommended, as it accounts for outliers that would be included with the WFA output and could skew the result.

Disclaimer

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Supplementary Material

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