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Inter-laboratory variability of A549 epithelial cells grown under submerged and air-liquid interface conditions

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ABSTRACT

In vitro cell models offer a unique opportunity for conducting toxicology research, and the human lung adenocarcinoma cell line A549 is commonly used for toxicology testing strategies. It is essential to determine whether the response of these cells grown in different laboratories is consistent. In this study, A549 cells were grown under both submerged and air-liquid interface (ALI) conditions following an identical cell seeding protocol in two independent laboratories. The cells were switched to the ALI after four days of submerged growth, and their behaviour was compared to submerged conditions. The membrane integrity, cell viability, morphology, and (pro-)inflammatory response upon positive control stimuli were assessed at days 3, 5, and 7 under submerged conditions and at days 5, 7, and 10 at the ALI. Due to the high variability of the results between the two laboratories, the experiment was subsequently repeated using identical reagents at one specific time point and condition (day 5 at the ALI). Despite some variability, the results were more comparable, proving that the original protocol necessitated improvements. In conclusion, the use of detailed protocols and consumables from the same providers, special training of personnel for cell handling, and endpoint analysis are critical to obtain reproducible results across independent laboratories.

1. Introduction

In vitro lung cell cultures offer cost-effective, robust, and high-throughput platforms to conduct inhalation toxicology research. To date, most of the *in vitro* lung models, particularly those representing the alveolar region, are used for scientific research. However, to have an *in vitro* model accepted as a valid alternative to animal testing (for testing both chemicals and particulate (micron and nano-sized)), towards a regulatory perspective, the robustness, reproducibility, and predictive value of the cell system must be demonstrated (Hiemstra et al., 2018).

The alveolar region of the lung is of particular interest to those studying the effects of aerosolized materials or drugs with diameters lower than 2 μm (Semmler-Behnke et al., 2007; Geiser and Kreyling, 2010). The alveolar epithelium is composed of alveolar epithelial type I cells and type II cells (Hiemstra et al., 2018). Type I cells cover 95% of the alveolar surface, forming together with endothelial cells and the common basal membrane, a 0.2 μm thin tissue layer to enable optimal gas exchange between the lung and the bloodstream (Crapo et al., 1982). Type II cells are responsible for the production of lung surfactants, which ensures that the alveolar region does not collapse during ventilation (Whitsett and Weaver, 2015).

Both cell lines and primary cells have their advantages and disadvantages in terms of handling and *in vivo* representation. These must be taken into consideration (as well as the region of the lung aiming to be mimicked) when choosing the correct cells for any *in vitro* lung model (Hiemstra et al., 2018; Lacroix et al., 2018; Upadhyay and Palmberg, 2018).

The A549 cell line, representing alveolar epithelial type II cells (Lieber et al., 1976), is a widely investigated cell line (Foster et al., 1998; Cooper et al., 2016; Guo et al., 2016; Bisig et al., 2019). The A549 cell line has been demonstrated to be successful for studying cytotoxicity, oxidative stress, and/or the (pro-)inflammatory response following

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acute (usually 24 hours (h) up to 72 h) exposure to different materials at the air-liquid interface (ALI). On the other hand, when investigating occupational exposures, which generally occur repeatedly over a long period of time, and severe adverse effects such as pulmonary fibrosis, asthma; long-term sub-chronic experiments need to be performed (Chortarea et al., 2017; Chortarea et al., 2019; Barosova et al., 2020b; Drasler et al., 2020). Therefore it is crucial to test if A549 cells are suitable for long-term experiments at the ALI and thus a representative model to investigate critical long-term exposure scenarios.

As well as selecting the appropriate cell line for a specific study or analysis, it is crucial to consider the length, the route, and the type of exposure. Various studies have been performed in order to justify the enhanced complexity of ALI exposures compared to submerged exposures (Lenz et al., 2013; Hilton et al., 2019). It has been shown that biological responses of the cells indeed change upon switching to the ALI from submerged conditions (Ohlinger et al., 2019). It has further been reported that exposing the cell of interest at the ALI to aerosols of inhalable and poorly soluble nanomaterials generates different toxicity patterns and results in different biological effect levels compared to similar cultures undergoing submerged exposure conditions (Loret et al., 2016; Gohlsch et al., 2019).

Comparing data between laboratories remains a significant challenge, as most research laboratories usually do not work with shared, standardised, and detailed experimental protocols. In addition, addressing reproducibility and reliability in cell culture research requires a detailed description of the biological test system of interest. This is an important consideration when transitioning models to a regulatory setting and assessing their potential for toxicology research (exposure to both chemicals and particles). A recent review (Faria et al., 2018) has identified four critical components required as minimum information required for bio-nano publications (it is important to note that these requirements can also be applied to studies on any toxicology research), as well as being the basis of Good *In Vitro* Method Practices (GIVIMP) for chemical toxicology studies (OECD, 2018). These include; (i) reusability and the ability to compare new data to old, (ii) quantification of old and new materials; (iii) practicality and ensuring the experimental procedure can be completed in most laboratories; and finally, (iv) quality, ensuring all data published are "reliable and reproducible". The above-mentioned criteria have also been identified by previous studies using A549 cells (Elliott et al., 2017), highlighting the importance of cell handling, cell medium usage, as well as the source and passage number of the cells.

To be able to compare the experiments among laboratories, specific conditions need to be followed. For this purpose, Standard Operating Procedures (SOPs) are essential tools as a set of detailed instructions that document a routine approach so that it may be strictly adhered to experimentally. The development and use of SOPs are a necessary part of any successful quality system. It provides individuals with information to perform different steps correctly and facilitates consistency in terms of both the quality and the integrity of the experiment. The development and use of SOPs minimize variation and promote quality through the consistent implementation of a procedure among various handlers and laboratories. SOPs are even more useful when published methods are being utilized. For example, if an SOP is written for a standard analytical method, the SOP should specify the procedures to be followed in more detail than they appear in the published method (Agency, 2007). Once the SOP has been developed and approved by a laboratory and/or a team of researchers from several laboratories, it can then be validated through a process outlined by the European Centre for the Validation of Alternative Methods (ECVAM), which aims to break the validation down into its main components. The ECVAM process lays the ground to establish inter-laboratory transferability and identify any potential intra- and inter-laboratory variation (Hartung et al., 2004).

Within the scope of establishing a validation component between laboratories, this study aimed to define an SOP to evaluate the growth and differentiation of A549 cells. Cells were grown under both submerged and ALI conditions, and their subsequent culturing for up to 10 days was investigated. Different biological identifiers such as cell morphology, barrier characteristics, and release of (pro-)inflammatory mediators upon exposure to inflammatory stimuli were assessed. The corresponding results were compared among the two laboratories in order to provide further insight into the transferability of SOPs between different research laboratories.

2. Methods

2.1. Cell cultures

The corresponding LOT numbers of A549 cells were used in the specified laboratories for both approaches 1 and 2.

Approach 1: The A549 cells were obtained from ATCC, USA (ATCC® CCL-185™, LOT: 62783414 (AMI), and 63913710 (IVTG)). The cells were cultured at 37° in 5% CO_2. The A549 cells were cultivated in RPMI-1640 medium (Gibco, USA), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA), 2 mM L-Glutamine (Gibco, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, USA). The supplemented RPMI-1640 is further cited as the complete cell medium (CCM).

A549 cells were passaged when ~80% confluent and used at passages of 4–18 for all tissue culture experiments. The cells were seeded on the apical side of Falcon® permeable support with a 3.0 μm pore-sized transparent membrane (Corning, VWR, Switzerland, or UK, respectively) in a 12-well plate setting. Cells were seeded at a density of 2.78×10^5 cells/cm^2 in 0.5 mL of CCM, and 1.5 mL CCM was added to the basal compartment. On day (D) 4, after the cell seeding, the medium was changed, and the cells were further cultured at either submerged conditions (0.5 mL apical medium, 1.5 mL basal medium) or at the ALI with 0.6 mL of CCM in the basal compartment and the apical compartment was exposed to air (no medium). The medium was changed every second day, and the samples were collected at D3, D5, and D7 for submerged conditions and D5 (corresponding to D1 at the ALI, *i.e.,* 4 + 1), D7 (4 + 3), and D10 (4 + 6) at the ALI (Fig. 1).

Approach 2: A549 (ATCC® CCL-185™ were obtained from ATCC, USA, (LOT: 62783414 (AMI), and 63913710 (IVTG), the same batches as used in approach 1)) and were cultured at 37 °C in 5% CO_2. The A549 cells were cultivated in RPMI-1640 medium (Gibco, USA), which was further supplemented with 10% heat-inactivated FBS, LOT: 42G1189K (Gibco, USA), 2 mM L-Glutamine (Gibco, USA), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco, USA). The supplemented RPMI-1640 is further cited as the complete cell medium (CCM).

In both laboratories, the A549 cells were passaged when ~80% confluent and used at passages 6, 8, and 10 for the experiments. The cells were seeded on the apical side of the inserts at a density of 2.78×10^5 cells/cm^2 in 0.5 mL of CCM, and 1.5 mL CCM was added to the basal compartment. On D4, after the cell seeding, the medium was replenished. The cells were further cultured at the ALI with 0.6 mL of CCM aliquoted into the basal compartment (and apical compartment exposed to air (no medium)). The medium was changed after 24 h at the ALI (corresponding to D1 at the ALI, *i.e.,* 4 + 1) when the cells were exposed to either lipopolysaccharide (LPS) or tumor necrosis factor-α (TNF-α) both at [1 μg/mL].

2.2. Exposures to positive controls

The cellular response upon exposure to potent (pro-)inflammatory stimulants LPS (from *Escherichia coli* at [1 μg/mL], LOT: 029M4025V, Sigma-Aldrich, CH, and UK, respectively) and to TNF-α (at [1 μg/mL], LOT: 354857, Immunotools, Germany) (Bigatto et al., 2015; Bisig et al., 2019), was investigated 24 h post-exposure. Each cell culture insert was exposed to 50 μL of LPS or TNF-α diluted to working concentration in CCM from the apical side. The negative controls were exposed to 50 μL of CCM; this method is referred to as pseudo-ALI exposure (Endes et al., 2014).

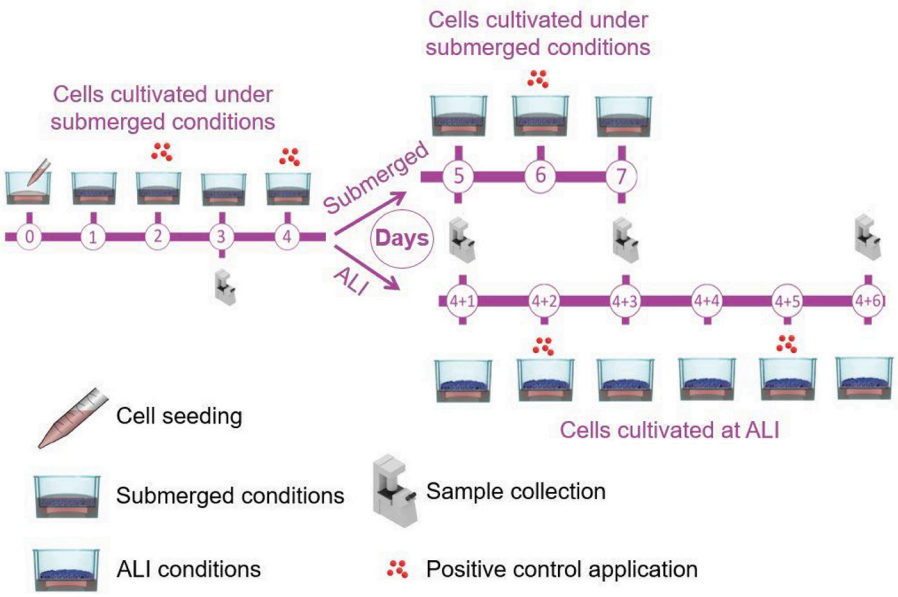

Fig. 1. An illustrative scheme summarizing exposure conditions as well as their duration and the corresponding sample collection time points that were followed in approach 1. The cells were seeded and cultivated under submerged conditions for 4 days. Then, the two parallel sets of experiments were carried out. The first set of experiments were conducted for 3 more days under submerged conditions. Samples were collected on days 3, 5, and 7. For the second parallel set of experiments, the cells were cultivated at the ALI conditions for 6 more days, and samples were collected on day 1 (4 + 1), day 3 (4 + 3), and day 6 (4 + 6).

2.3. Trypan blue exclusion assay

Cellular viability was determined using the trypan blue exclusion assay (the details of the method are explained in the SOP implemented for this study, supplementary file 1) 24 h upon exposure. Briefly, the cells were trypsinized (Trypsin-ethylenediaminetetraacetic acid (EDTA) (0.05%), Gibco) for 5 min at 37 °C on the apical side, then 1 mL of CCM was applied. Subsequently, the cells were carefully scraped from the cell culture insert using a cell scraper. Then, 10 μL of the collected cell suspensions were mixed with 10 μL trypan blue dye (0.4%) prior to the cell counting. The numbers of viable and dead cells were determined using a hemocytometer, and the percentage of viable cells was calculated from the total cell number counted.

2.4. Transepithelial electrical resistance (TEER)

TEER analysis only was carried out for approach 1. The TEER values were measured with STX01 electrode probes connected to an Epithelial Volt-Ohm Meter (Millicell® ERS-2, EMD Millipore Corporation, MA, USA). During the measurements, the CCM was replaced by PBS (at room temperature; 1 mL of PBS was added to the lower compartment, and 1 mL of PBS was added to the upper compartment) for both submerged and ALI conditions. The TEER measurements were performed at AMI on two defined spots on each membrane inserts, and mean values were calculated. TEER values were measured at IVTG 4 times per membrane insert, and a mean was calculated. The illustration displaying the specific locations on the lab inserts where TEER measurements were taken at AMI and IVTG are presented in Fig. S1. TEER values were corrected for membrane interference regardless of the TEER method used. The mean values were multiplied by the growth area of membrane inserts (*i.e.,* 0.9 cm^2), and the results are presented in [Ω*cm^2].

2.5. Immunostaining

Immunostaining was not a part of the implemented SOP and was only carried out as a part of approach 1.

AMI procedure: The cells were fixed with 4% paraformaldehyde solution (in PBS) at room temperature for 15 min at different time points to visualize the cell morphology and assess the development of the monolayer. Next, the cells were washed with PBS. The cells were subsequently treated with 0.1 M glycine in PBS for another 15 min. To permeabilize the cell membrane, the cells were treated with 0.2% Triton X-100 in PBS for 15 min. Phalloidin rhodamine (R-415; Molecular Probes, Life Technologies, Switzerland) was used to stain the F-actin cytoskeleton at a 1:50 dilution, while the nucleus was stained with 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) ([100 μg/mL]; Sigma Aldrich, Switzerland) at a 1:50 dilution, respectively. For optical analysis, the inserts were embedded in Glycergel (DAKO Schweiz AG, Switzerland). The samples were visualized using an inverted laser scanning confocal microscope (LSM 710, Zeiss, Germany). Image processing was performed with a cell image analysis software, IMARIS (Bitplane AG, Switzerland).

IVTG procedure: The cells were fixed with 4% paraformaldehyde solution (in PBS) at room temperature for 10 min at different time points to visualize the cell morphology and assess the development of monolayer. Next, the cells were washed with PBS. The cells were subsequently treated with 0.1 M glycine in PBS for another 15 min. To permeabilize the cell membrane, the cells were treated with 0.2% Triton X-100 in PBS for 15 min. Phalloidin, Alexa Fluor 633 (A22284; Invitrogen, UK) was used to stain the F-actin cytoskeleton at a 1:200 dilution. DAPI was then used to counterstain the nuclei of the cells. For optical analysis, the inserts were embedded in Glycergel (DAKO Schweiz AG, Switzerland). The samples were visualized using an inverted laser scanning confocal microscope (LSM 710, Zeiss, Germany). Image processing was performed with a cell image analysis software, IMARIS (Bitplane AG, Switzerland).

2.6. Lamellar body (LB) staining

At the ALI D1 cells were washed with PBS and then stained using 10 μM quinacrine (Sigma) for 1 min before being washed with PBS and fixed with 4% paraformaldehyde solution (in PBS) at room temperature for 10 min, as previously described by (Chintagari et al., 2010). The samples were visualized using an inverted laser scanning confocal microscope (LSM 710, Zeiss, Germany). Image processing was performed with a cell image analysis software, IMARIS (Bitplane AG, Switzerland).

2.7. (Pro-)inflammatory response detection

LPS or TNF-α was applied to investigate the (pro-)inflammatory responsiveness of the A549 cells. The response was measured by quantifying the amount of the (pro-)inflammatory mediators released into the basal medium *via* Enzyme-Linked Immunosorbent Assay (ELISA). The cell culture supernatant was collected at 24 h post-exposure and

analyzed for (pro-)inflammatory mediator levels of Interleukin (IL)-8 (Cat no. DY208) and IL-6 (Cat no. DY206, LOT: P209026) using DuoSet kits from R&D systems (Biotechne, CH or Abingdon, UK) according to the manufacturer's instructions. The samples for IL-8 analysis were diluted 10× (untreated samples and LPS-treated samples) and 20× (TNF-α treated samples), respectively. The samples were analyzed in triplicate per repetition and repeated on three independent occasions ($n = 3$), and absorbance was measured at 450 nm with a background correction at 570 nm. Extrapolation of protein concentration was carried out from a standard curve of known concentrations (IL-8 (0–2000 pg/mL) and IL-6 (0–600 pg/mL)).

2.8. Data and statistical analysis

The data and the corresponding statistical analyses were carried out using GraphPad Prism 8 (San Diego, USA). All data are presented as the mean ± standard deviation (SD). All endpoints were assessed upon three independent cell culture experiments ($n = 3$). The data were analyzed *via* the Mann–Whitney *U* test, with the null hypothesis, the true median difference is zero. This statistical hypothesis test is generally considered as the non-parametric equivalent of the Student's *t*-Test. Accordingly, the test does not assume that the difference between the samples is normally distributed or that the variances of the two populations are equal. The test determines the *p*-value, and a small *p*-value suggests that it is unlikely that the null hypothesis is true. The decision is made at a given significance level (α), and if $p < \alpha$, the hypothesis is rejected. In our case, the significance level was 0.05.

3. Results and discussion

3.1. Inter-laboratory testing approach

A comparison between two independent laboratories: Bio-Nanomaterials Group, Adolphe Merkle Institute (AMI), University of Fribourg, Switzerland, and *In Vitro* Toxicology Group (IVTG), School of Medicine, Swansea University, UK, was carried out on identical cell lines purchased from the same provider (American Tissue Culture Collection (ATCC), USA). Experimental cultures were used between passage numbers 4 to 18. The BioNanomaterials group at AMI, University of Fribourg, and the IVTG, at Swansea University Medical School, compared their experimental findings, consisting of seeding and monitoring A549 epithelial cells. The obtained results were compared between the groups and analyzed for reproducibility. The SOP implemented for this study (PATROLS_3101, is attached as an independent supplementary file) was established within the European Horizon 2020 project: Physiologically Anchored Tools for Realistic Nanomaterial Hazard Assessment (PATROLS, Grant Agreement No 760813), which was then revised further after reviewing the results (PATROLS_3101_a, also attached as a part of the Supplementary Information).

The following two approaches were subsequently adopted:

Approach 1: The main SOP was followed for cell culture experiments using all reagents from the same provider but independent batches (different LOT numbers). Several time points were investigated (submerged conditions at day 3, day 5, and day 7 and ALI conditions at day 1 (4 + 1), day 3 (4 + 3), and day 6 (4 + 6), and the two conditions were compared. A scheme depicting the experimental approach is presented in Fig. 1.

Approach 2: A more controlled experimental plan was implemented (the previous SOP was revised and then implemented for this approach), where the cells were treated and samples were collected at the same time points, using the cells with the same passage numbers, using fetal bovine serum (FBS) and positive controls with the same LOT number, were introduced. All the experiments were performed in the morning (between 9 and 12 a.m. in both laboratories; the difference in time zones was taken into account). Finally, the cells exposed on day 1 at the ALI (5th day in total) were investigated, and the data obtained in both laboratories were compared. A summary scheme illustrating the experimental approach is presented in Fig. 2.

3.2. Results from approach 1

The A549 cell line is one of the most studied and a widely used human lung cell line, as it offers unique features corresponding to alveolar epithelial cells type II, *i.e.,* cuboidal shape and surfactant release at the apical side of the cells when cultured at the ALI, resulting in surface tension similar to values measured *in vivo* (Blank et al., 2006). Previous studies have demonstrated that primary alveolar epithelial type II cells cultured under classical submerged cell culture conditions showed a loss of alveolar epithelial type II cells phenotype and surfactant production capacity, resulting in cell morphology alteration (Lin et al., 1996). In a recent study, the A549 cell line was investigated both under submerged conditions and at the ALI (Wu et al., 2018). The results revealed an induced expression of alveolar epithelial-specific cell markers: AQP-5 and SPC in the ALI, indicating partial alveolar epithelial cell properties of A549 (Wu et al., 2018).

Many studies have investigated cytotoxicity, oxidative stress, and (pro-)inflammatory effects in response to (nano-)materials for up to 72 h (Rothen-Rutishauser et al., 2005; Rothen-Rutishauser et al., 2008; Endes et al., 2014; Chortarea et al., 2015; Drasler et al., 2018; Hilton et al., 2019; Barosova et al., 2020a). The overgrowing of A549 cells was reported to be occurring after 96 h of exposure at the ALI (Hilton et al., 2019). Using these time frames for approach 1, it was observed that cell viability under submerged conditions decreased ~ 10% at IVTG and ~ 15% at AMI at the end of 7 days (Fig. 3a). It was observed that until D3, in both laboratories, the A549 cells proliferated continuously (Fig. 3b). This resulted in a consistent increase in total cell number from 2.78 × 10^5 cells/cm^2 at D0 to 10^6 cells/cm^2 at the end of D3 (Fig. 3b). However, after D3, the cell density results started to differ between the two laboratories. The cell density remained constant at AMI, while in IVTG, the cell density reached up to ~ 5 × 10^6 cells/cm^2 at the end of D7 (Fig. 3b).

On the other hand, at the end of D7, it was recorded that the trans-epithelial electrical resistance (TEER) reached ~ 20 Ω * cm^2 at IVTG, while the TEER values reached ~ 40 Ω * cm^2 at AMI under submerged conditions (Fig. 3c). The schematic shown in Fig. S1 presents the specific locations on the inserts where TEER measurements were taken at AMI and IVTG. Although the difference in the final TEER values recorded at the end of the experiment under submerged conditions may seem significant, they are considered within the same range. This is due to the inherent nature of the TEER measurement technique, which is often prone to error as the chip-specific conditions such as electrode placement and temperature variations highly influence the readouts (Srinivasan et al., 2015; Elbrecht et al., 2016).

The representative LSM images of untreated A549 cells cultured under submerged conditions at D5 and D7 in both laboratories are presented in Fig. 3d, and no differences in the morphology of the cells were detected.

The responsiveness of A549 cells to LPS and TNF-α treatments was evaluated *via* release levels of IL-8 and IL-6 (pro-)inflammatory mediators. Under submerged conditions, it was observed that recorded responses for both IL-8 and IL-6 were higher when the cells were exposed to TNF-α in both laboratories (Fig. 3e). Interestingly, IVTG results showed that IL-6 release was not detectable from the A549 cultures on D3 and D7 under submerged conditions (Fig. 3e).

Next, the results were assessed for the ALI culturing conditions. Under ALI exposure conditions, the cell viability was found to decrease by ~ 6% at IVTG, but ~ 49% at AMI at the end of 10 days (Fig. 4a). The cells reached up to 1.3 × 10^6 cells/cm^2 in both labs at D7, but at D10, the cell density had increased up to 1.7 × 10^6 cells/cm^2 (at AMI), while at IVTG, it increased up to 5.8 × 10^6 cells/cm^2 (Fig. 4b).

Other epithelial cells, such as the hAELVi cell line from the alveolar region (Kuehn et al., 2016), or Calu-3 or 16HBE14o$^-$ from the bronchial region (Wan et al., 2000; Xu et al., 2013; Dekali et al., 2014), are able to

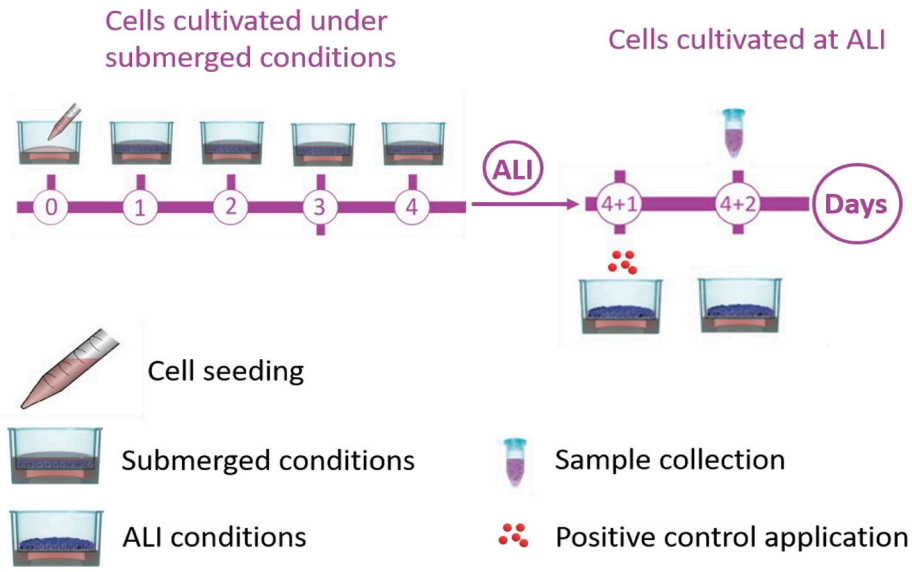

Fig. 2. An illustrative scheme summarizing exposure conditions as well as their duration and the sample collection time point that were implemented in approach 2. The cells were seeded and cultivated under submerged conditions for 4 days. Next, the cells were cultivated at the ALI conditions for 2 days, and samples were collected at the end of the experiment.

form tight junctions and reach higher values than A549 cells, in the range of hundreds to thousands Ω * cm^2. The A549 cells cultured at the ALI reached TEER values up to ~ 40–50 Ω * cm^2 in both labs at the end of the experiment (Fig. 4c), consistent with previously reported TEER monitoring results (Buckley et al., 2011; Ren et al., 2016). However, these values are still low compared to the above-mentioned cell lines.

The LSM images of A549 cells cultured under ALI conditions at D5 (day 1 at the ALI) and D7 (day 3 at the ALI) in both laboratories are presented in Fig. 4d. Additional LSM images are also presented demonstrating the prescence of lamellar bodies (LB) within the epitheilal type-II 'like' cells, indicating the expected cell type differentiation of the A549 cells under the specific culture conditions used within this study (Fig. S2). It was observed that the cells started to show multilayer formation at the end of D7 in both laboratories (the multi-layer formations can be seen in the y-x projection of merged optical z-stacks of Fig. 4d). Although there were no morphological changes detected, the appearance of multi-layers of cells suggests that the A549 cell culture model is not suitable for cultivation at later time-points.

ALI conditions showed a similar (TNF-α *vs.* LPS) response trend for both (pro-)inflammatory mediator release profiles. The results showed that the cells responded more to TNF-α treatment. Interestingly, IVTG results showed that IL-6 release was not detectable from the A549 cultures on D10 in response to TNF-α exposure (Fig. 4e).

As our results confirm, although the cell culture is responsive for up to 10 days (4 days submerged followed by 6 days at ALI) to the positive control stimuli, the cell layer overgrew into several multi-layers (Fig. 4d) with no increase in TEER (Fig. 4c), *i.e.,* no improvement in barrier integrity, resulting in less physiological relevance. It is noteworthy that A549 cells are adenocarcinoma cells with unlimited capacity of proliferation but have limited differentiation capacity. In the light of this information, it was previously hypothesized that A549 cells could differentiate into alveolar epithelial type II cells at early time points, but they might retain their carcinoma cell properties along with a reduced capacity of differentiation under long-term ALI culture conditions (Wu et al., 2018).

3.3. Results for approach 2

As significantly different (pro-)inflammatory response results were observed for approach 1, the experiments were further optimized and harmonized between the two laboratories. Additional details were introduced in the protocol to be adopted by the two laboratories. Further information included a synchronized cell growth time, passaging, and seeding. Also, it was ensured that both laboratories used an FBS sample with the same LOT number. Finally, the same batch of positive controls were included in the experimental design. Due to the formation of multilayers detected post 24 h under ALI conditions (Fig. 4d), in approach 2, the cell viability along with IL-6 and IL-8 release were investigated only for 24 h post-exposure. The cells were seeded and cultivated under submerged conditions for four days. Next, the cells were cultivated at ALI conditions for two days, and then exposed to the specific positive controls (LPS and TNF-α, both at [1 μg/mL]) was carried out at the end of 24 h at the ALI (Fig. 2).

According to the results obtained in approach 2, a similar pattern in exposure to LPS and TNF-α treatments was observed in both cell viability and (pro-)inflammatory response results. Although the differences between laboratories are still remarkable (Fig. 5), overall, the results of approach 2 are comparable to the results from approach 1.

Several inter-laboratory comparison studies have been published (Godschalk et al., 2013; Elliott et al., 2017); however, the comparison is based more on toxicological assay results than the cell culture conditions. It was shown that even if the cells were to be acquired from the same batch, the standard laboratory consumables (such as cell culture dishes, serum supplements, *etc.*) tend to be different, not only in terms of LOT number but also in terms of the provider (Elliott et al., 2017). Therefore, we used an optimized approach and focused on laboratory consumables. To the best of our knowledge, the presented study, which we adopted in approach 2 of this work, is the first one specifically focusing on the cell culture conditions while also ensuring the providers are the same for the laboratory consumables.

As can be seen from the results, when strictly following the SOP and ensuring the consumables are from the same providers with the same LOT numbers during the cell culturing step, the results become comparable between the two laboratories (Fig. 5). The cell response is affected by the composition of serum, which is an essential supplement for cell culture media (Veranth et al., 2008; Corradi et al., 2012). The major cell media supplement for most cell lines is FBS, which provides several important biological molecules such as albumin, anti-chymotrypsin, apolipoproteins, biotin, and growth-supporting factors required for cell growth (Baker et al., 1988). The serum prevents the possible mechanical damage on cells, which may occur as a result of stirring or cell scraping. The serum is known to change the

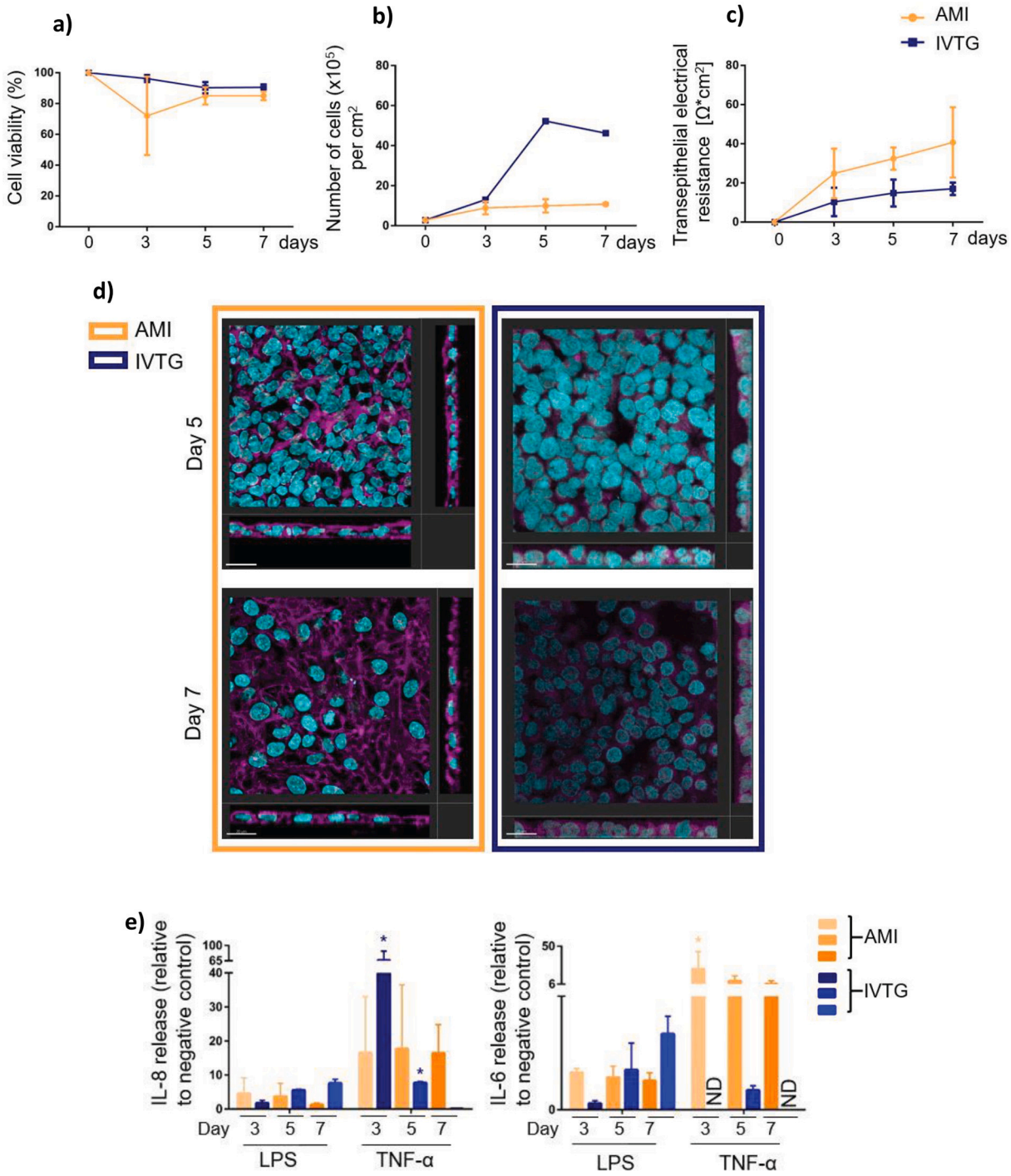

Fig. 3. The results from both laboratories for submerged conditions adopted in approach 1 comparing (a) cell viability, (b) cell proliferation (number of cells), (c) transepithelial electrical resistance, (d) cell morphology, and (e) the release profiles of (pro-)inflammatory mediators: IL-8 and IL-6. Data are presented as mean ± standard deviation, $n = 3$. *indicates rejected hypothesis, *i.e.*, statistically significant increase ($p < 0.05$) compared to the untreated cells. The error bars on the LSM images are 20 μm.

physicochemical properties of the media, such as viscosity, osmolality, buffering capacity, and diffusion rate. Furthermore, FBS can be used for a wide range of cell cultures despite the various requirements of different cells. In approach 1, we used FBS from the same supplier (Gibco) but used different batches (different LOT numbers). It is worth noting that differences in serum composition may be present when acquired from different batches, even from the same provider (Zheng et al., 2006). The only significant difference between the two tested batches of FBS (based on certificates of analysis from the provider) is in γ-globulin. However, the reported values of γ-globulin for both batches of FBS are in the accepted range based on the reference ranges provided by the provider (Table S1). Low levels of γ-globulin in FBS (compared to serum from adult animals) are needed for successful cell culturing, as high levels of γ-globulin may inhibit cell growth and proliferation (Rauch et al., 2011). A batch of FBS with a higher content of γ-globulin was used in AMI; however, no statistically significant decrease ($p > 0.05$) compared to IVTG results was observed in cell proliferation (Figs. 3c and 4c). This is also confirmed by the LSM images, which

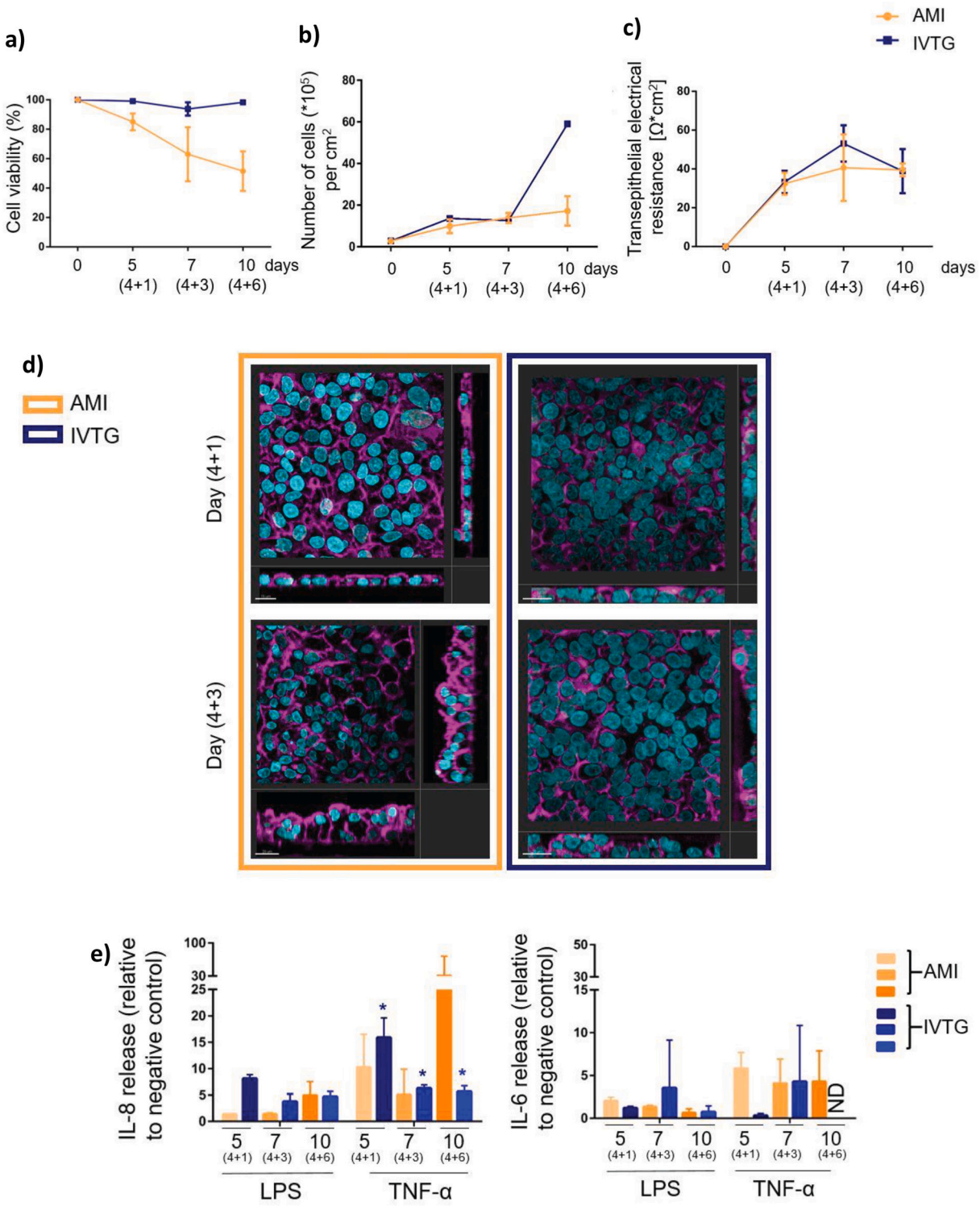

Fig. 4. The results from both laboratories for ALI exposure conditions adopted in approach 1 comparing (a) cell viability, (b) cell proliferation (number of cells), (c) transepithelial electrical resistance, (d) cell morphology, and (e) the release profiles of (pro-)inflammatory mediators: IL-8 and IL-6. Data are presented as mean ± standard deviation, $n = 3$. *indicates rejected hypothesis, *i.e.*, statistically significant increase ($p < 0.05$) compared to the untreated cells. The error bars on the LSM images is 20 μm.

demonstrated that cell proliferation was not inhibited; in fact, a multi-layer cell formation was detected (Fig. 4d). The use of the same FBS batch across different laboratories poses many obstacles. Thus the optimization and use of alternative (*i.e.,* animal-free) serum approach for A549 cell growth should be considered.

The cell viability response was found to be significantly different (Fig. 5a) between the two laboratories. We attribute this difference to the factor of human error, as cell scrapping and cell counting are strongly affected by the handler, even though an identical experimental procedure is followed. Furthermore, although the cells were treated the same way and were obtained from the same provider (ATCC, USA), possible differences between batches play a critical role in the cell

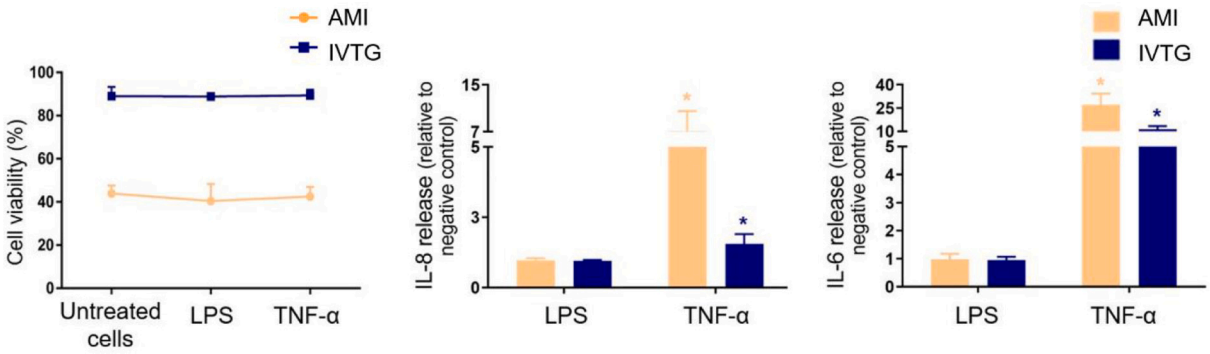

Fig. 5. The results from both laboratories following approach 2, comparing (a) viability and normalized cytokine (b) IL-8, and (c) IL-6 release measurements 24 h post-exposure at the ALI. Data are presented as mean ± standard deviation, $n = 3$. *indicates rejected hypothesis, *i.e.*, statistically significant increase ($p < 0.05$) compared to the untreated cells.

Table 1
A summary table of differences in Approach 1 and 2 and their effects on differences in the investigated endpoints at day 5 (4 + 1). *Cellular viability was not comparable between the laboratories, However, it was consistent across the exposures within each individual laboratory. **IL-8 concentration for approach 2 after exposure to TNF-α was not comparable between the laboratories, however in each laboratory the concentration was significantly increased when compared to the appropriate negative control. "yes" indicates comparable results between AMI and IVTG, while "no" indicates that the results are not comparable. N/A indicates an endpoint was not completed.

	The same cell batch	The same FBS batch	The same positive control batch	The same consumables (cell culture inserts, *etc.*)
Approach 1	No	No	No	Yes
Approach 2	No	Yes	Yes	Yes

	TEER	Cell proliferation	Cell viability consistency across exposure	Pro-inflammatory response		
					LPS	TNF-α
Approach 1	Yes	Yes	Yes	IL-8	No	Yes
				IL-6	Yes	No
Approach 2	N/A	N/A	Yes*	IL-8	Yes	No**
				IL-6	Yes	Yes

response. This hypothesis is confirmed when (pro-)inflammatory mediator release profiles were assessed (Fig. 5b and c).

The (pro-)inflammatory response was investigated *via* IL-6 and IL-8 release analysis. The results showed that the same pattern (TNF-α triggered a more significant response than LPS) for both mediators. But vast differences in release levels were observed between the two laboratories (Fig. 5). Although human factors can play a role in the difference, batch (LOT number) of cells play a vital role in observed distinctions. Based on the presented data, the original SOP was revised (The SOP is provided as a part of the Supplementary Information: "PATROLS_3101_a"), specifically for Section 5.9 titles as "Quality control and acceptance criteria", where the need for the use of cells with the same LOT number is emphasized.

The results show, that repeating the experiment with a different batch of cells, FBS, or positive control can strongly affect the outcome, as shown in approach 1. Different batches of cells potentially introduce experimental variability because the exact difference between the batches is not disclosed by the provider. Furthermore, batch-to-batch variability related to variations in the concentrations of serum components, in addition to the unknown exact composition of FBS, can also ultimately lead to experimental variability and limit inter-laboratory reproducibility (van der Valk et al., 2018), as we demonstrated in approach 1. Finally, the composition of the positive control, in this case, LPS and TNF-α, also remains unknown unless the same LPS and TNF-α with the same LOT numbers are used. Taken these three primary sources of variability that can ultimately affect the outcome together with the differences in cell handling due to different human handling, it is unavoidable to have differences in results.

In summary, establishing a detailed SOP protocol is vital for performing inter-laboratory comparisons. As it was shown, not only the source of cells or laboratory consumables is important, but using the same batch from the same provider is crucial. Table 1 summarizes the importance of each component of these approaches on the inter-laboratory differences. When performing the inter-laboratory comparison, we recommend careful planning of the experiment down to the individual batches of all consumables required and time-harmonization of the experiment plan, which leads to more consistent results as presented in approach 2 (Fig. 5). Therefore, we conclude that our improved SOP for approach 2 can serve as a reference for future inter-laboratory comparisons and corresponding endpoint analysis.

4. Conclusion

In conclusion, the experience reported in this study clearly indicates that the use of detailed SOPs, together with the use of consumables from the same providers with the same LOT numbers, are both critical to obtain reproducible results across independent laboratories.

Our inter-laboratory comparison study of culturing A549 cells confirmed the suitability of the adopted approach (*i.e.,* the SOP developed for approach 2). It resulted in better reproducibility across the two laboratories (compared to the initial SOP developed for approach 1). This two-tiered study not only provides valuable insight for future inter-laboratory comparisons but also demonstrates that when repeating an experiment, identification of possible sources of discrepancies in results and adopting corresponding revisions to the SOPs are the keys to achieve reproducibility and reliability in a method. The results also show that even when working with a cell line that is easy to handle, the comparability of results from different laboratories should be treated with caution. Furthermore, special training of personnel is recommended to harmonize the human handling of the different cultivation and endpoint analysis steps.

Author contributions

Conceptualization, B.R-R. and M.J.D.C.; methodology, B.R-R. and M.J.D.C, H.B., and K.M.; software, H.B. and K.M.; validation, B.R-R. and M.J.D.C., H.B., and K.M.; formal analysis, H.B. and K.M.; investigation, B.R-R. and M.J.D.C.; resources, B.R-R. and M.J.D.C.; data curation, H.B., K.M., and B.B.K.; writing – original draft, H.B. and K.M.; writing – review and editing, B.R-R. and M.J.D.C H.B., K.M., and B.B.K.; visualization, H.B. and K.M; formal analysis, S.B.; supervision, B.R-R. and M.J.D.C.; project administration, B.R-R. and M.J.D.C.; funding acquisition, B.R-R. S.H.D. and M.J.D.C. All authors have read and agreed to the published version of the manuscript.

Author disclosure statement

The authors declare no competing financial interests.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2021.105178.

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