Meeting Report

Alternative Methods in Science: Towards Fluidic Systems

doi:10.14573/altex.2307101

The International *Fourth Virtual Summer School* "Alternative Methods in Science: Towards Fluidic Systems" (https://amfa.lakecomoschool.org/), held on 18-19 May 2023 and chaired by Francesca Caloni, Università degli Studi di Milano, Department of Environmental Science and Policy, was attended by young scientists from all over the world.

Francesca Caloni, opened the *Summer School* with a general introduction on the state of the art of microfluidic systems and their possible applications from drug development to environmental toxicology, explaining the advantages but also underlining the lack of regulatory acceptance limiting the application of this technology as most studies remain at the level of laboratory research (Zhao et al., 2022).

Helena Kandarova, CEM & FChFT Bratislava, gave a lecture entitled "Current challenges in phototoxicity testing *in vitro*". Phototoxicity occurs when a substance causes damage to the skin or eyes upon exposure to light. *In vitro* phototoxicity is widely used in the cosmetics, pharmaceutical, and recently also in the pesticide industries. Despite significant progress, including the acceptance of three test guidelines for this endpoint, several challenges remain: Validated testing protocols for systemic phototoxicity testing and for photosensitization are lacking. Phototoxicity and photosensitization may also occur in the cornea and conjunctiva, and advanced models to predict these effects are not yet available. Furthermore, there is a need to develop and validate *in vitro* phototoxicity testing methods for poorly water-soluble pesticides and for medical device safety assessment. Current testing methods for these industries still often rely on *in vivo* studies, which are expensive, time-consuming, and raise ethical concerns. Developing reliable and relevant *in vitro* methods for these types of products would provide a more efficient and ethical approach to safety assessment. Addressing these challenges to completely eliminate the need for *in vivo* tests for these regulatory testing purposes will require collaboration and cooperation among stakeholders, including industry, academia, and regulatory agencies.

Arno C. Gutleb, Environmental Research and Innovation (ERIN) Department, Luxembourg Institute of Science and Technology (LIST), presented "Animal-product free cell culture and new developments within ALI models". Fetal bovine serum (FBS) is commonly used as a supplement in cell culture medium. Replacing FBS in cell culture media may improve the reproducibility of *in vitro* research and overcomes the scientific, ethical and legal challenges associated with its use. Increasingly, scientists are replacing FBS with animal component-free media. A complex 3D *in vitro* tetraculture model mimicking the alveolar barrier was developed to differentiate between respiratory sensitizers and irritation. The transition of A549 cells to commercially available media without FBS was a first step toward an animal-product free version (Chary et al., 2022). Cellular morphology, functionality and performance were assessed by calculating cell doubling time, measuring cytokine release (Bio-Plex), evaluating cell viability after exposure to a toxicant (Alamar blue assay); monitoring the expression of relevant genes; imaging (scanning electron microscopy), and determining surfactant production (surfactant droplet test). The transition was successful for two FBS-free media, but cells showed differences in terms of morphology, functionality, and performance. While one FBS-free medium retained the phenotype of cells cultured in FBS, the other showed substantial differences, which suggests that cells lost their adenocarcinoma-like phenotype in favor of an alveolar type I and alveolar type II epithelial cell phenotype. An overview on animal-product free cell culture and details on the successful transition of a model were provided.

Hassan Rashidi, NIHR Great Ormond Street Hospital Biomedical Research Centre, UCL Great Ormond Street Institute of Child Health, University College London, presented a lecture entitled "*In vitro* and *ex vivo* models of drug-induced liver injury". Adverse drug reaction (ADR) is the leading cause of attrition during preclinical drug development and post-marketing withdrawal (Waring et al., 2015; Lauschke et al., 2017). Due to interspecies differences in liver-specific functions, animal models are often poor predictors of drug-induced hepatotoxicity, motivating researchers in the field to develop *in vitro* platforms to improve predictability using human-derived cells (Gough et al., 2021). To this end, the primary human hepatocytes (PHHs), hepatocarcinoma cell lines and human pluripotent stem cell-derived hepatocyte-like cells have been used with varying degrees of success (O'Brien et al., 2006; Tolosa et al., 2012; Sirenko et al., 2014; Lauschke et al., 2016). Recent advances in the generation of 3D liver organoids and the development of microfluidic platforms, including liver-on-chip and human-on-chip, have opened new avenues to develop more sophisticated *in vitro* platforms to predict DILI more accurately (Gough et al., 2021; Dalsbecker et al., 2022). A number of re-

cently developed tools was presented and a roadmap for further improvement and development of more physiologically-relevant *in vitro* platforms was discussed.

Giulia Ranaldi, Food and Nutrition Research Centre, Council for Agricultural Research and Economics, CREA-AN, Rome gave a presentation entitled "Confocal laser scanning microscopy as a tool to investigate microfluidic gut-on-chip system". Microfluidic organs-on-chips are microsized *in vitro* cell culture chambers that reproduce the main biochemical and physical environmental conditions typical of different organs and also employing continuous media circulation. The simplest systems are single, perfused micro-channels containing one type of cultured cell, while more complex systems consist of different compartments connected by fluid perfusion that recreate interfaces between different tissues. These devices represent minimal functional *in vitro* units that reproduce organ physiology and tissue interactions and enable real time and endpoint evaluation of various parameters related to organ function and their response to challenges and diseases. Microscopy techniques represent a broadly employed analytical tool in microfluidic investigation. Confocal laser scanning microscopy (CLSM) is largely used for morphological characterization of these cell systems, since by employing fluorescence dyes and/or immunofluorescence techniques high resolution images of cell features and tissue-like organization within the microfluidic chips can be obtained. CLSM has been employed also for studying intestinal *in vitro* systems and to develop gut-on-chip devices. Although 2D intestinal cultures, mainly represented by differentiated Caco-2 human intestinal cells, are a widely validated and suitable intestinal model, microfluidic intestinal systems allow to include physical, biochemical, and environmental components essential for intestinal biology such as villus-crypt structures, mucosal immune system, symbiotic microbiota crosstalk and also peristaltic movements. Extensive efforts to address these aspects have led to the development of models that reproduce the complexity and organization of the intestinal barrier, confirming their suitability for *in vitro* research.

The lecture presented by **Doris Wilflingseder**, Institute of Hygiene and Medical Microbiology, Medical University of Innsbruck, was entitled "Studying infectious diseases in microfluidic, human 3D models". Animal-free, three-dimensional (3D) respiratory barrier/immune cell systems were established to study host-pathogen interactions after challenge with pathogenic fungi, SARS-CoV-2 or influenza in terms of mucociliary clearance, cell stress and death, inflammation and local complement production. Cells are seeded upside-down within an animal-free cellulose hydrogel on the bottom side of transwell membranes. This allows long-time observations such as differentiation or mucociliary clearance using the same cells, because the insert can be transferred to glass-bottom dishes for live cell imaging analyses. Differentiation of barrier models is significantly accelerated under perfusion compared to static conditions, and primary respiratory cells of the upper bronchial and small airway epithelial tract can be cultured over a period of more than two years without losing epithelial integrity, mitochondrial fitness or the ability of goblet cells to produce mucus. Mucociliary clearance was monitored using fluorescent beads, inactivated *Aspergillus fumigatus* conidia, or after treatment with antiviral sprays prior to infection with SARS-CoV-2. These innovative tools allow the study of the interaction between a physiological human mucus barrier and pathogens in repeated dose experiments within a completely animal-free setting.

Sonja von Aulock, *ALTEX – Alternatives to Animal Experimentation*, presented "Promoting alternatives to animal experiments as a scientist". Scientists who are aware of the 3R principle of replacement, reduction, and refinement of animal use in science can actively apply these principles in their research; seek to develop and validate alternative methods; promote new approach methodologies among students, peers, funders, and policymakers; and increase awareness and support of alternatives to animal experiments by engaging with the public. Application of the 3R principles in research entails performing a literature search for applicable alternative methods prior to planning an animal experiment; using materials whose production does not entail animal suffering where possible, also in *in vitro* experiments; designing, reporting, and documenting experiments according to best practice, and objectively discussing their limitations. Where a new method can be applied as an alternative to an animal experiment, its relevance, predictivity and robustness should be thoroughly reported. These considerations should also be applied in the review of scientific articles. Championing new approach methodologies encompasses engaging with different interest groups on this subject and developing tailored communication skills to form community and grow a wider understanding and support of these methodologies and their implementation (von Aulock et al., 2022).

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Acknowledgements

A special thanks to Professor Giulio Casati, Director of the Como Lake School of Advanced Study, Como, Italy and to the active participants of the Fourth Summer School.

Francesca Caloni[1], Alessandra Cazzaniga[2], Arno C. Gutleb[3], Helena Kandarova[4,5], Giulia Ranaldi[6], Hassan Rashidi[7], Sonja von Aulock[8] and Doris Wilflingseder[9]

[1]Università degli Studi di Milano, Department of Environmental Science and Policy (ESP), Italy; [2]Fondazione Alessandro Volta, Italy; [3]Luxembourg Institute of Science and Technology, Environmental Research and Innovation (ERIN) Department, Luxembourg; [4]Centre of Experimental Medicine (CEM), Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Bratislava, Slovakia; [5]Faculty of Chemical and Food Technology (FChFT), Institute of Biochemistry and Microbiology, Slovak University of Technology (STU), Bratislava, Slovakia; [6]Food and Nutrition Research Centre, Council for Agricultural Research and Economics, CREA-AN, Rome, Italy;[7]NIHR Great Ormond Street Hospital Biomedical Research Centre, UCL Great Ormond Street Institute of Child Health, University College London, London, UK; [8]ALTEX – Alternatives to Animal Experimentation, Kreuzlingen, Switzerland; [9]Institute of Hygiene and Medical Microbiology, Medical University of Innsbruck, Innsbruck, Austria