Theme I – New Technologies

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Session I-1: Virtual tissue models

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Session I-1: Oral presentations

I-1-729

A model of early ovarian development as a future tool in toxicity testing

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As alternative methods are developed to reduce the number of animals used in toxicity testing, mathematical/computational models will become more valuable as tools to predict effects of chemical exposure. The aim of this research is to formulate a mathematical model of normal early ovarian development in mice, from conception to the primordial follicle stage. To create the model, we reviewed the published literature (Edson et al., 2009; Liu et al., 2010; Loffler and Koopman, 2002; Tevosian, 2013; Ungewitter and Yao, 2013) to produce a graphical conceptual model of ovarian development at the molecular level. Then, we formulate a mathematical representation of the conceptual model and use computational methods to solve the model equations and simulate ovarian development. This presentation will provide an overview of model development and strategies for how it can be used to reduce the number of animals used in toxicity testing. A foreseeable application of this model is as a tool to predict how chemical exposure at different stages of development can affect the developing ovary.

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I-1-743

Novel computational approaches for high content image analyses (HCA) of organoid 3D neurosphere cultures *in vitro*

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Neurospheres are three dimensional (3D) cell clusters consisting of neural progenitor cells (NPCs). NPCs provide the cellular basis for the developing brain and persist in the hippocampus up to old age. There, they are responsible for adult learning and memory. Safety and efficacy testing with 3D neurospheres by employing high content image analyses (HCA) require advanced scanning and evaluation processes due to defocusing of the sphere core and sphere-specific endpoints. Therefore, for this mixed neuron/glia culture we developed algorithms for radial migration, quantification of neuronal differentiation, neurite outgrowth and distance-dependent density distributions of neurons. For quantification of neuronal differentiation the new algorithm reaches an average detection power (DP) of 80-85% (versus manual evaluation) and a false positive rate (FP) of 10-15% improving results of the commercially available "Neuronal Profiling" bio-application (Thermo Scientific; DP: 50%, FP: 40%). This is due to the application domain of the "Neuronal Profiling", which was initially designed for pure neuronal cultures.

In conclusion, HCA of neurospheres is a promising technique for medium throughput screening to be used in safety and efficacy testing in the future.

I-1-816

High-throughput PBPK and microdosimetry: cell-level exposures in a virtual tissue context

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Toxicokinetic (TK) models can determine whether chemical exposures produce potentially hazardous tissue concentrations. Tissue microdosimetry TK models relate whole-body chemical exposures to cell-scale concentrations. As a proof of concept, we approximated the micro-anatomic architecture of the hepatic lobule with a discrete topology by a graphical model that can be connected to a chemical-specific physiologically-based TK (PBTK) model. The development of traditional PBTK models is time and resource intensive. Successful methods have been developed for pharmaceutical compounds to determine TK from limited *in vitro* measurements and chemical

structure-derived property predictions. These high throughput (HT) TK methods provide a more rapid and less resource-intensive alternative to traditional TK model development. We have augmented these *in vitro* data with chemical structure-based descriptors and mechanistic tissue partitioning models to construct HTPBPK models for over three hundred environmental and pharmaceutical chemicals. When evaluated with human *in vivo* data for 74 chemicals we find that we can generally predict when HTPBPK models will perform well, and when more complicated effects (e.g., transporters) impact HTPBPK assumptions. For those chemicals where the assumptions that allow HTPBPK models are appropriate, virtual tissue simulation of quantitative chemical-specific effects is possible.

This abstract does not necessarily reflect Agency policy.

I-1-837

Multiscale modeling and simulation of embryogenesis for *in silico* predictive toxicology

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Translating big data from alternative and HTS platforms into hazard identification and risk assessment is an important need for predictive toxicology for developmental toxicity. Understanding how chemical disruption of molecular function in the human embryo propagates to higher levels of biological organization ultimately requires systems biology and computer simulation to recapitulate spatio-temporal dynamics of cellular networks. Virtual Tissue Models (VTMs) can provide this level of detail for discrete morphogenetic events, based on simulating connectivity between scales of biological organization. The efficacy of VTMs to integrate data with embryological information on an anatomical plane offers a novel platform for predictive toxicology. Progress will be reviewed for angiogenesis (angiodysplasia), palatal fusion (cleft palate), limb outgrowth (ectrodactyly) and urethral development (hypospadias). Predictive VTMs extend a traditional probabilistic paradigm (e.g., data to models in which 'A' has value y with probability p and uncertainty u) into a more granular environment based on lifestage-specific, spatio-temporal prediction of toxicological risk (e.g., what happens to system performance if a chemical exposure impacts protein 'A' and pathway 'B' at lifestage 'C' in the context of an AOP).

This abstract does not reflect US EPA policy.

I-1-887

Building multiscale simulations to analyze mechanisms of developmental toxicity

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Multiscale computer simulations can be a powerful aid to understanding the complex underlying mechanisms of developmental toxicity (Swat et al., 2012; Andarsi et al., 2012). However, developing biologically accurate simulations is challenging. Using arsenic toxicity we illustrate how we build such models and the insights that they can provide. Arsenic, an environmental toxicant, inhibits angiogenic intersegmental blood vessel (ISV) sprouting in Zebrafish by decreasing directed migration speed and perturbing directional path-finding (McCollum et al., 2011; Shirinifard et al., 2013). Using literature mining and pathway analysis we developed a biological model of ISV growth. We then experimentally determined effects of arsenic on components of our biological model and constructed an *in silico* model that explored mechanisms of arsenic toxicity. Our simulations combine spatiotemporal $VEGFA_{165}$ and VEGFR2 expression with ISV growth dynamics and reproduced both control and arsenic perturbed ISV growth behaviors. Our simulations and experiments combined showed that: 1) A $VEGF_{165}$ gradient created by local uptake can support ISV sprouting and extension; 2) Slow initial growth with rapid depletion of local $VEGFA_{165}$ causes ISV collapse or failure to initiate; and that 3) Arsenic inhibition of ISV growth is due to down regulation of both $VEGFA_{165}$ and VEGFR2. Model driven experimental design simultaneously increases understanding of mechanisms of toxicity while decreasing the number of animal experiments needed

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I-1-889

Virtual liver approaches

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Currently, transcriptomics of cultivated primary human hepatocytes and also virtual liver models are used to predict hepatoxicity of chemicals. In cultivated hepatocytes genome-wide, concentration and time-dependent data are meanwhile publicly available for more than 150 chemicals. Conclusions of the biostatistical analyses are: (i) hepatocytes show a "stereotypical" gene expression response to toxic concentrations of numerous chemicals that comprises mostly proliferation and inflammation genes, (ii) more specific alterations induced only by subgroups of compounds are observed for target genes involved in lipid or energy metabolism, cytoskeletal organization, stress response and DNA repair. (iii) although these "alert genes" help to identify toxic mechanisms and can support the chemical read across approach, they are not yet sufficient to reliably predict hepatotoxicity in humans. A complementary approach to hepatocyte *in vitro* systems is the "virtual liver". The virtual liver represents a spatio-temporal model of liver tissue with integrated metabolic and signaling response principles. Such simulations can be used to test or generate hypothesis. Examples of hypothesis originally generated by virtual liver approaches and later validated *in vivo* are: (i) The key role of sinusoidal endothelial cells (LSEC) for hepatotoxicity. Once hepatotoxic compounds destroy a critical fraction of LSCE the liver switches from a "perfect regeneration mode" to a "scar formation mode" which upon repeated insult finally leads to fibrosis and cirrhosis. (ii) Flow direction changes of detoxifying enzymes upon toxic exposure. For example CCl_4 or paracetamol cause a switch of ammonia metabolizing enzymes (e.g., glutamate dehydrogenase) from ammonia production to ammonia consumption. This stress response protects the exposed organism from an excessive increase in ammonia blood concentrations. Such compen-

satory mechanisms would be difficult to identify by *in vitro* systems alone. The examples illustrate advantages of the complementary use of *in vitro* systems and "virtual liver" approaches.

I-1-890

Use of transcriptomics approaches in human cell-based DNT testing

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Transcriptome analysis is a powerful tool to study gene expression changes in cultured cells exposed to toxicants. Such toxicogenomics data have been obtained for different model systems of developmental neurotoxicity (DNT), either as additional information or as qualitative endpoint. For the use of transcriptome data in toxicant prediction, classification or potency comparison, the experiments need to be standardized, and the ways of data exploration, interpretation and visualization will still benefit from further improvements. To address some of these challenges, we used neurally-differentiating human embryonic stem cells, and we studied the time-dependence of the transcriptome response concerning duration of exposure to toxicants and maturity of the culture system under investigation. In a next step, we investigated the design principles of concentration-dependent transcriptome deviations. We found that short exposures to compounds yielded information on potential pathways of toxicity, while long exposure mainly described phenotypic alterations of the cells exposed to the toxicants. These phenotypic changes were suitable for compound classification and prediction. Moreover, they allowed a relative potency ranking. The studies showed that it will be beneficial in the future to group transcriptome alterations of individual genes into superordinate biological processes, in order to condense the information and to facilitate the interpretation of studies as well as the visualization of the results.

Session I-1: Poster presentations

I-1-416

Performance standards for human epidermis model Keraskin-VM for skin irritation alternatives

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A skin irritation alternative method was developed using reconstructed Asian epidermis (Keraskin™-VM), and reported comparable to the EpiDerm™ Skin irritation test. Validation of Keraskin™-VM model has been undertaken following OECD TG439 performance standards for reconstructed human epidermis (RhE) tests. Three laboratories in compliance with OECD GLP were participated in the validation. The method was transferred to two naïve laboratories by a lead laboratory. The method transferability was evaluated into two phases; informative phase using 5% sodium lauryl sulfate as a (+) control, lauric acid, and butyl methacrylate, and blind phase using 3 irritant and 3 non-irritant chemicals listed in OECD TG439. After confirmation of successful transferring to the two laboratories, proficiency test was proceeded at the three laboratories. All 6 chemicals were correctly classified (accuracy, 100%). Within-laboratory reproducibility was assessed using 20 reference chemicals for 3 runs at three laboratories. Two laboratories resulted in 85%, and one laboratory in 90%. Between laboratory reproducibility was evaluated using the same 20 reference chemicals for 3 runs at three laboratories, resulting in 95%. Further results on reproducibilty will be discussed. Keraskin™-VM model is believed comparable to the 4 RhE model adopted by OECD.

For further reading see Jung et al. (2014) and OECD (2013).

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I-1-657

Application of zebra fish and Neoderm®-ME, a new 3D pigmented skin model for the evaluation of anti-melanogenic effects of hexapeptoids; PAL-10 and PAL-12

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Peptoids are a class of peptidomimetics whose side chains are appended to the nitrogen atom of the peptide backbone, instead of α-carbons. It was reported that peptoid is more durable against the degradation by proteases and there is larger chance for making derivatives and chemical library than peptide. Peptoides are actively used in drug discovery, but rarely used in the discovery of cosmetic ingredients. In this study, we examined if PAL-10 and PAL-12, new peptoids, can inhibit melanogenesis by using zebra fish and Neoderm®-ME, developed by Tegoscience. After treating PAL-10 and PAL-12 at different concentrations on B16 cells (mouse melanocyte), Neoderm®-ME and zebra fishes, anti-melanogenic effects were compared with arbutin, a positive control. PAL-10 and PAL-12 significantly manifested anti-melanogenic effects in a dose-dependent manner in all three *in vitro* models as determined by image, histology and melanin contents. We also assessed skin irritation potential of PAL-10 and PAL-12 in Neoderm®-ED (a 3D human full thickness skin model) according to OECD TG436. There was no skin irritation caused by PAL-10 and PAL-12 at concentrations higher than those where anti-melanogenic effects were produced. We can conclude that PAL-10 and PAL-12 can be used as new cosmetic ingredient with strong whitening efficacy.

Session I-2: High throughput screening (HTS) models

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Session I-2: Oral presentations

I-2-049

Evaluation of the Multi-ImmunoTox Assay (MITA) composed of 3 human cytokine reporter cell lines by examining the immunological effects of drugs

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We established a luciferase reporter assay system, MITA, to evaluate the effects on key predictive *in vitro* components of the human immune system (Kimura et al., 2014). The system is composed of 3 stable reporter cell lines transfected with 3 luciferase genes, SLG, SLO, and SLR, under the control of 4 cytokine promoters, IL-2, IFN-g, IL-1b, and IL-8, and the G3PDH promoter. We first compared the effects of dexamethasone, cyclosporine, and tacrolimus on these cell lines stimulated with PMA and ionomycin, or lipopolysaccharides, with those on the mRNA expression by the mother cell lines and human whole blood cells after stimulation. The results demonstrated that MITA correctly reflected the change of mRNA of the mother cell lines and whole blood cells. Next, we evaluated other immunosuppressive drugs, off-label immunosuppressive drugs, and non-immunomodulatory drugs. Although MITA did not detect immunosuppressive effects of either alkylating agents or anti-metabolites, it could demonstrate those of the off-label immunosuppressive drugs, sulfasalazine, chloroquine, minocycline, and nicotinamide. Compared with the published effects of the drugs, these data suggest that MITA can provide a novel high-throughput approach for detecting the immunological effects of chemicals, other than those that induce immunosuppressive effects, through their inhibitory action on cell division.

For further reading see Kimura et al. (2014).

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I-2-225

Evaluation of zebrafish embryo as alternative model to predict hepatotoxicity

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Hepatotoxicity is one of the causes of drug attrition. Zebrafish assays show promise to assess hepatotoxicity, though subjective phenotypic scoring and time are main drawbacks. In the ZETOX project, liver toxicity in zebrafish embryo is assessed using gene expression as biomarker approach, complementary to phenotypic analysis with the purpose to contribute to mechanistic understanding and improved human hepatotoxicity prediction. Liver morphological effects of 5 hepatotoxic (acetaminophen, amiodarone, coumarin, methapyrilene, myclobutanil) and 2 negative compounds (saccharin, biotin) were assessed in zebrafish embryo at 5 days to define sublethal concentrations for gene expression experiments. Analytical methods were optimized to analyze the stability and internal concentration of the chemicals. Detection of hepatocyte markers (*CP, CYP3A65, GC, TF*) were accomplished by *in situ* hybridization for coumarin and myclobutanil and confirmed by real-time qPCR. Experiments showed decreased expression of all markers. Next, other liver-specific biomarkers (*FABP10a, NR1H4*) and general apoptosis or stress-induced markers (*CASP3A, CYP2K19, TP53, ZGC163022*) were screened using real-time qPCR for the 7 compounds. Differential gene expression in relation to observed hepatotoxic effects will be discussed in view of optimization of this whole-organism-based method for mammalian hepatotoxicity.

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I-2-257

Developmental effects of ToxCast™ chemicals on alternative animal models: *C. elegans* and zebrafish

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Alternative animal models share many advantages with *in vitro* assays, with the added advantage of exhibiting complex whole organism responses to chemical exposures. Over 1000 unique compounds in the ToxCast Phase I and II libraries (Kavlock et al., 2012) were screened using a high-throughput *C. elegans* larval growth and development assay (Boyd et al., 2009). Changes in the size of individual nematodes were measured using COPAS Biosort flow cytometry after 48-h chemical exposures. Isotonic regressions were used to analyze the response over seven concentrations (0.5-200 μM), providing efficacy and potency estimates, expressed as a change in response between control and highest concentration (Δ) and the chemical concentration at which half of Δ is reached (CΔ/2). More than 50% of the compounds caused a significant decrease in nematode growth; the most toxic 5% of these compounds included several organic pollutants (e.g., DDT, PFOS), which have been banned from use due to their toxicity and bioaccumulation. Comparisons of compound activities in the *C. elegans* growth assay were made to those from zebrafish embryonic develop-

ment studies (Padilla et al., 2012; Truong et al., 2014), as well as rodent data available in the US EPA's ToxRefDB (Knudsen et al., 2009). These comparisons showed good agreement between *C. elegans* and zebrafish data and moderate agreement with rodents.

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I-2-511

The throughput-compatible embryonic stem cell test (EST)

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The EST is an ECVAM validated cell-based assay to evaluate embryotoxicity of substances based on cytotoxicity and cardiomyocyte-differentiation endpoints. The later uses the formation of embryoid bodies (EBs) from mouse embryonic stem cells (ESC) by the hanging-drop (HD) method. The traditional process of EB production and handling technique is prone to generate EBs with non-uniformity and shows a labor-intensive process. The method for EB production was adapted to a novel top-loadable, throughput-compatible HD-plate in a 96-well format to enhance the efficiency of the EST-proceeding.

ESC cultured for 5 days in the HD-plate generated EBs with size uniformity at days 3 and 5 (diameters: 319 μm ±3.0% and 466 μm ±5.2%, respectively). At day5 direct-transfer of EBs to a 96-well adherence plate by adding excess of medium into the HD-wells, the EBs adhered and differentiated with a final cardiomyocyte differentiation efficiency (number of contracting EBs) of 88% ±13% at day10. The differentiation efficiency and the embryotoxic classification of substances from the ECVAM test panel were in-line with published data

We adapted the traditional manual HD method to develop a technically more sophisticated, throughput compatible process with significant time saving (80%). The EST provides an efficient tool to investigate alternative endpoint complementing the conventional embryotoxic substance characterization.

I-2-622

The usage of *Daphnia magna* for screening of muscarinic cholinoreceptors antagonists

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In the experiments on the rats we have developed methodological approach to the evaluation of the selectivity of muscarinic cholinergic receptors (M-ChR) antagonists action in the whole organism conditions. According the results obtained during investigation the protective effect of M cholinolytics during acute poisonings of organophosphates (DDVP, DFP, etc.) depends on M_1 subtype ChR occupation. The efficiency of antagonists in inhibition of tremor reaction caused by M-ChR agonist arecoline administration associates with interaction of M_2 subtype of ChR. It was established by the method of linear regression, that there was a high degree of correlation (r=0.99) for different M cholinolytics between the ratios of ED_{50} of M antagonists in the tests with arecoline and organophosphates and the ratios of dissociation constants of antagonists complexes with M-ChR from the homogenates of rat's cerebral cortex and heart containing M_1 and M_2 ChR subtypes, respectively. Thus, the ratio of ED_{50} arecoline/ED_{50} DDVP is serve as a measure of the selectivity of drugs action. On the experiments to *Daphnia magna* the effects of some non selective, mainly M_1 and M_2 ChR antagonists on the toxicity of DDVP and arecoline were studied. It was shown that a ratio of the average effective concentrations (EC_{50}) of M antagonists in the tests with arecoline and organophosphates also may be used as a measure of the selectivity of M-ChR antagonists action.

I-2-644

The Tox21 "1500 genes" high throughput transcriptomics project

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The US Tox21 consortium aims to use *in vitro* and alternative *in vivo* systems to: 1) identify patterns of compound-induced biological responses, 2) prioritize compounds for more extensive toxicological evaluation, and 3) develop predictive models for biological responses in humans. In support of this effort, we initiated a project to identify a targeted subset of ~1500 human genes to evaluate how the transcriptome in different biological systems responds to chemical exposures. The transcriptome is a reflection of all genes expressed at a particular point in time in response to a particular environment and is altered in response to biological, physical, and chemical treatments in a dose-and time-dependent manner. The goal is to ultimately generate a similar list for rat, mouse, zebrafish, and *C. elegans*. This set of ~1500 sentinel genes will be implemented using a high-throughput, cost effective gene expression technology and will have the following attributes: (1) it captures maximal expression variability and pathway coverage, especially for pathways hypothesized to be involved in adverse health effects, and (2) the results can be extrapolated to the transcriptome. The status of this effort and the initial experiments to demonstrate the validity of the approach will be presented.

Session I-2: Poster presentations

I-2-227

A new approach to the automatic segmentation and evaluation of pigmentation lesion by using active contour model and speeded up robust features

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Digital image processing techniques have wide applications in different scientific fields including the medicine. In this paper, we propose an automatic method for segmenting the skin lesions and extracting features that are associated to them. This is achieved by combining the Speeded-Up Robust Features (SURF) and Active Contour Model (ACM). In the suggested method at first the area of the skin lesion disorder is segmented from the image and then some features like the mean, variance, RGB and HSV parameters are extracted. The work has been tested on 20 images. Comparing the segmentation results by use of Otsu thresholding method, ACM and SURF show the superiority of ACM method over the two others. The proposed method for skin lesion segmentation which is a combination of SURF and ACM gives the best results. To assess the practical robustness of our method, we have used it for segmentation of different types of skin lesion images. Result of applying the proposed method on 20 images shows the high performance, speed and accuracy of it. We believe that this work is applied the most powerful and newest methods of image processing for segmenting the skin lesions.

I-2-314 *

A novel *in vitro* model of spinal cord injury: validation and treatment strategies

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It is currently anticipated that successful treatment for spinal cord injury (SCI) will involve a combined pharmacological and biological approach; however, testing their efficacy in animal models of SCI requires large animal cohorts and significant suffering. For this reason we have developed an *in vitro* model of SCI, in accordance with the 3Rs. This culture system comprises a mixed glial and neuronal cell population, resident in the central nervous system (CNS), with the formation of myelinated axons interspaced with nodes of Ranvier. After cutting the culture, an initial cell-free area appears persistently devoid of neurites, accompanied by a plethora of pathophysiological hallmarks characteristic of SCI, including demyelination and reduced neurite density adjacent to the lesion, and infiltration of microglia and reactive astrocytes into the lesioned area. In this study, we evaluated various novel and established pharmacological strategies, including modifiers of the Rho/ROCK and cAMP pathway. When added in combination, these drugs induce remyelination and neurite outgrowth across the lesion, suggesting their therapeutic potential.

Overall, these results demonstrate that this culture system provides a useful *in vitro* screening strategy to study individual and/or combined strategies that promote CNS repair.

For further reading see Boomkamp et al. (2012, 2014).

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I-2-367

Physiological oxygen concentrations in a sandwich culture on gas-permeable membranes remarkably enhance rat hepatocytes functions and genes expression

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Oxygen supply is a critical issue in the optimization of *in vitro* hepatocyte microenvironments. It is essential to match the oxygen consumption rate (OCR) and provide the proper oxygen tension at the cellular level. To achieve these goals, a sandwich culture based on oxygen-permeable membranes was used to investigate the effects of physiological oxygenconcentrations on the efficient functioning of hepatocytes *in vitro*. Rat hepatocytes were cultured using the sandwich culture method with Matrigel on the PDMS (polydimethylsiloxane) membranes under various oxygen concentrations [20%-O_2 (+), 10%-O_2 (+) and 5%-O_2 (+)]. In parallel, TCPS (Tissue culture treated-polystyrene) plates with PDMS membrane inserts were used as the control groups.

The results indicated that the hepatocytes cultured under 10%-O_2 (+) exhibited extended survival, normoxic OCR, improved maintenance of metabolic activities and functional polarization. Additionally, the expression levels of various drug-metabolism genes, as examined by PCR arrays, were closest to those of freshly isolated hepatocytes. This study shows that it is important to meet the cellular oxygen de-

mand of hepatocytes at *in vivo*-like physiological levels, and it also reports on an oxygen permeable membrane system to provide a simple method for *in vitro* studies.

For further reading see Matsui et al. (2010, 2012); Sakai et al. (2012).

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I-2-371

A feasibility study into the biodistribution of test compounds in zebrafish embryos

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The embryonic zebrafish is a promising early screening model bridging the gap between simple cell-based assays and studies with higher animals. An important obstacle for the model is the determination of internal exposure which might deviate substantially from the exposure concentration in the water. This may result in false negative or false positive outcomes. Only analyzing the concentration in whole-organism homogenates does not consider the complexity of the zebrafish as a whole organism approach. In this feasibility study we addressed this issue by examining the biodistribution of test compounds in zebrafish. Hereto, zebrafish embryos and larvae were treated with radioactive compounds and biodistribution was semi-quantified by autoradiography of serial microscopic sections. Total body burden of larval and embryonic zebrafish largely depends on logP for hydrophobic compounds. Bioaccumulation of compounds may result in an overestimation of effects. In this study differences in biodistribution were observed in both embryos and larvae. In embryos up to 80% of test compounds stuck to the chorion, whereas in larvae up to 75% of test compounds remained in the gastrointestinal tract. This limited bioavailability may result in an underestimation of effects. By testing biodistribution of more compounds, the predictability of the zebrafish model will further increase.

I-2-477 *

Cellular Ellman's method adaptation using SH-SY5Y is a possibility for neurochemical multiparametric evaluation

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Introduction: Acetylcholinesterase is a membrane-bound enzyme, with its active site on the cell outside. It is an important enzyme studied in neurotoxicology and neuropathology, since it is involved in neuron system development and neurophysiological processes.
Aim: Developing a modified Ellman's method for acetylcholinesterase activity assay without cell damage, allowing its use on *in vitro* neurotoxicological test batteries, reducing costs, experimental times, procedures, and contributing to future replacement of animal testing in neurotoxicology.
Method: Ellman's method was modified for 96-well plate using neuroblastoma cell line (SH-SY5Y). Cellular conditions (density and viability by XTT) and interferences on the method (FBS, media components) and on cell viability (DTNB, acetylthiocholine, reaction products) were tested in kinetic analysis. Anticholinesterasic chemicals (quinidine and aldicarb) were tested.
Results: Media components and FBS interfere in Ellman's method. Washing attached cells twice with PBS and analyzing the test in PBS, result in minimum interference. 5 mM of Quinidine sulphate (drug) decrease in 47% the activity of AChE in real time, with no cell damage. Aldicarb (pesticide) during 30 min incubation presented higher viability IC_{50} than enzyme IC_{50} (1.962 mM and 0.608 mM, respectively).
Conclusion: Non-damaging Ellman's method adaptation is a promising method for multiparametric neurochemical evaluation.

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I-2-491

A data analysis pipeline for Tox21 phase II quantitative high throughput screening assays and its application to toxicity profiling of flame retardants

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The U.S. Tox21 program (Tice et al., 2013) is currently screening a 10K compound library against toxicity pathways using a quantitative high throughput screening (qHTS) approach. Robust compound activity profiling is an essential step towards compound toxicity prioritization and predictive human disease model construction. However, the existence of non-reproducible signal artifacts (e.g., signals caused by compound activity carry-over or noise) and compound-dependent assay interference (e.g., compounds that are auto-fluorescent or cytotoxic) complicates compound activity interpretation. We have developed a data analysis pipeline that addresses these issues and found that signal reproducibility greatly improved after removing the non-reproducible artifacts while compound active rate was reduced after removing compounds flagged as having compound-dependent assay interference. We demonstrated the *in vitro* activity profiling results using 49 brominated or organophosphate flame retardants (FRs) present

in the Tox21 10K compound library. For the FRs evaluated, some of the outcomes were consistent with known *in vivo* health effects and new compound-pathway interactions were identified. To facilitate data exploration, a web portal (http://spark.rstudio.com/moggces/profiling/) was designed to host the compound signal/activity profiling data analyzed by the pipeline. The pipeline and the web portal are useful tools for compound signal/activity profiling in qHTS assays and for data exploration.

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I-2-582

Automation-compatible EST assay

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The Embryonic-Stem-Cell Test (EST) is a cell-based assay to evaluate embryotoxicity of substances and belongs to the portfolio of ECVAM validated *in vitro* assays. The current EST-process is a manual and time consuming process due to manual transfer of EB's into a reciever plate. In this study the EST assay was modified to enable an automation-compatible EST-process.

EBs were formed for 5 days in in a 96-well hanging drop plate to achieve EB formation and induce cardiomyocyte differentiation. At day 5 EBs were directly transferred into an adhesive 96-well plate by placing the HD-plate on top of a receiver. Adhered EB's were monitored for cardiomyocyte differentiation at day 10. ESCs aggregated in the hanging drop and formed round shaped EBs of uniform size within 5 days. Size analysis of EBs resulted in diameters of 319 μm ±3.0% at day 3 and 466 μm ±5.2% at day 5, respectively with an contraction efficiency of 88% ±13% at day 10. Compounds selected from the ECVAM test-validation panel resulted in similar classifications as with the original EST protocol.

We adapted the validated EST method towards an automation-compatible process leading to significant time savings (up to 80%) to further foster the use of the EST-assay.

I-2-668

Evaluation method in Bhas 42 cell transformation assay selecting transformed foci using hydrogen peroxide, H_2O_2

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Bhas 42 cells, which were established by the transfection of the v-Ha-*ras* oncogene into the BALB/c 3T3 A31-1-1 cells, form a contact-inhibited monolayer. By exposure to carcinogens, however, aberrant colonies (transformed foci) which are capable of invading the non-transformed contact-inhibited monolayer are induced. In the cell transformation assay (CTA), transformed foci are counted by observation to evaluate carcinogenic potential of chemicals. We developed a method to select transformed foci using H_2O_2 (Japan Patent Application No. 2009-206686, European Patent Application No. 09 169 631.0). By exposure to H_2O_2, non-transformed cells die but transformed cells are alive. At the end of culturing in CTA, Bhas 42 cells plated in 96-well plate were exposed to H_2O_2 and survival cells in each well were evaluated by measuring optical density (OD). Comparison of results between observation and H_2O_2 methods showed that evaluation using cut-off value of OD was more comparable with the observation method than that using OD values. By combination of H_2O_2 and 96-well plate methods, it made it possible to evaluate carcinogenic potential of chemicals without observation of transformed foci in the Bhas 42 CTA.

I-2-741

Using Skin-PAMPA for transdermal patch testing

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Using skin as absorption site presents interesting features that have facilitated the progression of transdermal drug delivery in the past decades. Efforts in drug research have been devoted to find a quick and reproducible model for predicting the skin penetration of molecules.

The parallel artificial membrane permeability assay (PAMPA) has been recently extended by this group for the prediction of transdermal penetration (Skin PAMPA™) (Sinkó et al., 2012). This commercially available system has been modified to make it suitable for transdermal patch testing.

Four API's (nicotine, fentanyl, ketoprofen and rivastigmin) have been investigated, each applied in 1-3 marketed transdermal patches. The permeation vs. time profile demonstrated linear release profile in every case, though the cumulative permeated amount was about 30% higher than expected that can be caused by the edge effect reported by Hadgraft and co-workers (Hadgraft et al., 1991). *In vitro*/*in vivo* correlation of permeation profiles were performed based on manufacturers' data and resulted in acceptable correlation.

Skin PAMPA system appears to be a useful tool for transdermal patch comparisons, though standard protocol needed to be modified. Results can be used for patch comparison and for ranking, therefore Skin PAMPA can provide valuable information for transdermal patch development.

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Session I-3a: Tissue-on-a-chip / Human-on-a-chip

Co-chairs
Suzanne Fitzpatrick, Johns Hopkins University, USA
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Session I-3a: Oral presentations

I-3a-316

Human beating heart on a chip for cardiotoxicity testing as an example case of the Dutch organ-on-chip initiative

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A novel Dutch initiative in the form of a *precompetitive* R&D Institute (hDMT) integrates state-of-the-art stem cell technologies with tailored physics/chemistry/engineering expertise to create miniature *human organ and disease tissues-on-a-chip*, closely mimicking human (patho)physiology, enabling "(pre-) Clinical Trials on Chips" and development of personalized drug treatment, and reducing the need for animal models (van de Stolpe and den Toonder, 2013)

To illustrate the potential, the Cytostretch cardiotoxicity model will be presented. Cardiotoxicity, often as cardiac arythmias, is a frequent cause for drug withdrawal from market and late-stage clinical drug failure. A stretchable Micro-Electrode Array has been developed, consisting of a thin stretchable and patterned membrane with embedded electrodes (Cytostretch), enabling alignment of plated human cardiomyocytes in a chosen direction and cardiomyocyte stretching with heartbeat frequency (Dambrot et al., 2011; Braam et al., 2013; Saeed et al., 2014). This allows mimicking the beating human heart during various levels of exercise (with associated increases in cardiac load and stretch), while monitoring cardiomyocyte electrical activity to detect arythmias (e.g., QT elongation) caused by drug compounds. The genetic background of a patient may codetermine toxicity, thus next generation models will include iPS-derived cardiomyocytes with known human ion channel variations. This approach is expected to rescue cardiotoxic drugs, by identifying patients at risk with a companion diagnostic assay.

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I-3a-584

In vitro microphysiological systems: advancing regulatory science through innovation

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Advances in bioengineering and material sciences, microfabrication, and microfluidics technologies enabled the development of microphysiological systems that mimic the functional units of an organ. These advances have made it possible to initiate the engineering of cellular environments and/or functional units of lung, heart, blood vessels, muscles, bones, liver, nervous system (including eye), gut, and kidney. These microsystems reflect human physiologically relevant parameters, including proper cell-to-cell, cell-to-matrix, biochemical and mechanical signaling, but lack the complex architecture of tissues. The National Center for Advancing Translational Science (NCATS) / US Food and Drug Administration (FDA) / Defense Advanced Research Projects Agency (DARPA) partnership for the development of *in vitro* microphysiological systems is a groundbreaking example of the types of partnerships that are needed to bring innovative new technologies into the regulatory paradigm. NCATS, FDA and DARPA are collaborating to develop a chip to screen for safe and effective drugs which is far more swift and efficient than current methods. NCATS will provide science-based solutions to reduce costs and the time required to develop new drugs and diagnostics. FDA will determine how this new technology can be utilized to assess drug safety. DARPA and NIH will facilitate collaborations between researchers and FDA to advance program goals.

I-3a-694

A dynamic human-on-chip platform for neurotoxicity studies

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Homunculus™ is a multi-organ, platform designed for use in the pharmaceutical industry, personalized medicine etc. Homunculus platform consists of a flexible, microfluidic control unit and a cell chip with multiple, separate cell compartments, each the size of a standard well from a 96-well plate.

Using HepaRG and NSC-hTERT cells in Homunculus neurotoxicity evaluation model was developed. Suitable for hepatic and neural cells

media was selected, and functional activity of cells was confirmed by whole-transcript expression profiling.

Liver spheroids and neural cells were cultivated up to 14 days with daily media exchange. Culture medium were perfused in closed circuit, imitating blood circulation and daily exposure to the drug was applied. Viability and functional activity of cells could be monitored and different end-point analysis method was established to evaluate neurotoxicity of the drug.

Using a Human-on-chip approach, Homunculus gives researchers the unique opportunity to investigate human cell models responses *in vitro*. It combines the advantages of the cell line and animal model neurotoxicity studies.

I-3a-791

Human organs on chips as replacements for animal testing

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In this presentation, I will describe work we have been carrying out in the Biomimetic Microsystems platform at the Wyss Institute for Biologically Inspired Engineering at Harvard. The goal of this platform is to engineer human "Organs-on-Chips": microfluidic devices lined by living human cells created with microchip fabrication techniques that recapitulate organ-level functions as a way to replace animal testing for drug development and to create *in vitro* human disease models. These biomimetic devices provide a window on human physiology as they enable real-time, high-resolution microscopic imaging as well as analysis of biochemical, genetic and metabolic activities of living cells when they are positioned within the context of functional tissue and organ units. I will review recent advances we have made in development of multiple organ chips, including human lung, gut, kidney and bone marrow chips, as well as on-chip models of human diseases, including pulmonary edema and inflammatory bowel disease. In addition, I will describe our ongoing efforts to develop more than 10 different organ chips, to integrate them into a "human body on chips", and to engineer an automated instrument for real-time analysis of cellular responses to pharmaceuticals, toxins and other chemicals.

I-3a-913

Developments towards physiologic *in vitro* models in Switzerland

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The next step towards more biomimetic *in vitro* models is the design of multi-organ devices which allow communication among different tissue types. The Swiss competence center TEDD (Tissue Engineering for Drug Development and Substance Testing) presents an overview of novel "body on a chip" concepts currently under development in Switzerland. The majority of those concepts employ microtissue engineered approaches in combination with microfluidics. The microfluidic chips contain dedicated micro-chambers to immobilize and/or grow microtissues or cells in an *in-vivo*-like environment, in which the shear stress, the continuous transport of drug, nutrients and oxygen and/or the mechanical stress induced by the breathing motion are reproduced. The models which are evaluated currently comprise liver and lung tumor devices to evaluate the impact of prodrug-activation on tumor growth, lung on a chip as disease models and for nanoparticle toxicology testing and a platform technology to monitor label-free chronic toxicity effects.

Session I-3b: Regulatory science panel discussion: Human-on-a-chip – Advancing regulatory science through innovation and worldwide networking for alternative testing

Moderator
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Session I-3b: Oral presentation

I-3b-921

Human-on-a-chip – Advancing regulatory science through innovation and worldwide networking for alternative testing

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Validation of human *in vitro* skin models has supported the ban of laboratory animal use, e.g., for corrosion or sensitization testing of substances, in the EU. Unfortunately, development and validation of such alternative methods required an unacceptably long time-span. Respective laboratory animal tests were not abandoned in the other main markets (e.g., US and Asia). This has led to a very difficult current situation for the cosmetics industry. Compounds tested on animals in specific tests are not allowed to be marketed in the EU, while the same compounds not tested on animals are disallowed from being marketed elsewhere. Concepts and tools to antecedently avoid such a dilemma for the upcoming human-on-a-chip platform technologies, potentially able to reduce and replace systemic safety and efficacy testing of substances, are debated among specialists in the scientific field and renowned representatives of the US Food and Drug Administration, Chinese Institute for Food and Drug Safety Evaluation, Russian Ministry of Health, European Medical Agency, European Chemical Agency, and other international regulatory bodies. The 60-minute panel aims to produce a mission statement and to decide on an annual communication format among the respective stakeholders.

Session I-3: Poster presentations

I-3-008

µ3DVasc: a novel microfluidic bioreactor setup for the reconstruction of vascularized 3D tissues *in vitro*

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The development of three-dimensional (3D) organotypic cell culture models has been emerging as a novel method, which may implement the 3R principle (Huh et al., 2011; Ghaemmaghami et al., 2012). As the nutrition of almost all tissues is ensured via a network of blood vessels, a disposable microfluidic 3D vascular microstructure (µ3DVasc) has been fabricated to provide a more physiologically relevant *in vitro* model of vascularized tissues. The curvilinear and porous channel, which provides space for culturing human microvascular endothelium, is produced by the combination of swift heavy ion and microthermoforming technology (SMART) and is connected to a microfluidic circuit (Giselbrecht et al., 2006). Adjoined to the artificial vasculature is a second microfluidic compartment, which can be colonized with hydrogel supported 3D structures of different tissues and serves to remove the lymph fluid. Furthermore, the porosity of the microstructure supports the supply of 3D tissues with nutrients and gases and allows for analysis of transendothelial transport of immune cells or drugs (Hebeiss et al., 2012). To study the reconstruction of the Blood-Brain-Barrier we have developed a 3D culture of human pericytes and astrocytes in collaboration with the Wyss Institute at Harvard. This unique model mimics the anatomic situation *in vivo* and enables the investigation of potential interactions at crucial tissue-tissue interfaces.

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I-3-037

Serial linkage of biochips toward an organ-on-chip approach

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The development of drugs is a costly and time consuming process. Although high throughput methods based on cell cultures are available, they only give a hint on the real effect of a future drug. Traditional two dimensional cell cultures are not able to reproduce/emulate the complex interactions of different types of tissue like it exists in the human body. Such interactions include the absorption, distribution, metabolism and elimination (ADME) of a drug (Esch et al., 2011; Sung et al., 2010).

Recently new approaches reforming the cell culture techniques were published. Most of them are based on several microchambers that are cultivated with different cells. Microfluidic channels are used to link these chambers and to ensure exchange of metabolites. With such devices, e.g., primary cells can be cultivated for a longer time period and with a smaller loss of functionality than in former single-type 2D cell-cultures (Sung and Shuler, 2010; Huh et al., 2011).

A lable-free long term monitoring of organ-on-chip systems would affirm the benefits of such systems. Electrochemical sensors are well suited for this task, but need to be careful integrated.

As proof-of-concept we serially linked two electrochemical cell-monitoring systems (IMOLA-IVD, Weiss et al., 2013) and showed that the exchange of metabolites can be monitored without any cross-talk in a label-free, long-term and multi-parametric manner.

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I-3-101

Cell-based rapid and quantitative toxicity and efficacy monitoring in consecutive before and after the compound metabolism within liver for two ginger compounds at physiological concentration

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Objectives: Animal test is often used for compound toxicity and efficacy evaluation although the results obtained are different from clinical test and show total metabolism (Damia and D'Incalci, 2009). We have developed an original human cell-based assay device, High-Precision Surface Plasmon Resonance (HP-SPR), and method (Ona and Shibata, 2010; Ona, 2013). Here we examined the applicability of HP-SPR to elucidate toxicity and efficacy including metabolism of two ginger compounds as examples working against liver before and after their metabolism.

Methods: Human liver cell Hep G2 was used for two ginger compounds of 6-shogaol (SHOG) and 6-gingerol (GING). The 2D cultured cells were self-attached to an HP-SPR sensor chip surface and covered by collagen to obtain *in vivo* like cell status (Ona, 2013) and monitored.

Results: In SHOG (heated GING), the efficacy of liver activation was observed at 100 nM. This activation was twice higher in after metabolism than in before metabolism. Apoptotic effect as toxicity was observed at 1000 nM to inhibit liver activation before the metabolism. However, after the metabolism, its effect was revered and detoxification was processed. In GING, 100 nM showed apoptotic effect as toxicity before and after the metabolism, and no effect was observed below 100 nM. The different mode of action was successfully monitored between two ginger compounds.

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I-3-141

A multi-organ-chip co-culture of human liver equivalents and neurospheres for long-term substance testing

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Current *in vitro* and animal tests for drug development are failing to emulate the organ complexity of the human body and, therefore, to accurately predict drug toxicity. In this study, we present a smartphone-sized, self-contained multi-organ-chip (MOC) platform capable of co-cultivating up to three organ equivalents inside a combined media circuit. A peristaltic on-chip micro-pump reproducibly operates a PDMS-embedded microcirculation system, emulating the systemic arrangement of organs within the human body. It could be shown, that the multi-organ-chip is capable of supporting long-term co-cultures of human artificial liver microtissues and neurosphers. Cultures were successfully maintained functional over a period of up to 14 days. Liver cell polarity was restored as shown by the expression of specific transporters, tight junctions and the formation of rudimentary bile canalicular-like structures. Vitality of the cells was assessed by TUNEL/Ki 67 staining and was markedly increased compared to static controls. Neurospheres derived from the Ntera-2 cell line were strongly positive for neuronal markers MAP2 and ß-Tubulin III after 14 days of culture in the MOC as assessed by immunohistology and qPCR. Chronic exposure of the cultures to 2,5-hexanedione over 14 days revealed a dose-dependent toxicity on MOC co-cultures.

I-3-219

3D-printing: a new dimension for the 3R's

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3D printing is a collective term for innovative techniques that have rapidly developed in the last few years. Some talk about a new industrial revolution. Even though the technique is still in the process of development, there are wide ranging potential applications. These

include medical and biomedical applications such as medical devices, pharmaceuticals, medical research, and the development of new *in vitro* tests. Together with other innovative techniques such as organ-on-a-chip, tissue engineering, synthetic and biological stem cells, 3D printing has great potential to contribute to the 3Rs – replacing, reducing and refining animal experiments. For instance, printed organs-on-a-chip (of human origin) can be used in toxicity and efficacy testing of medicines. This would provide the data needed and save on the use of laboratory animals. However, perhaps the greatest impact of innovations would be in avoiding and preventing animal experiments as a whole. When more knowledge about the physiology of the human body is available, animal experiments will finally no longer be necessary. Thus, advances in innovative technologies have to be used to the fullest extent to make reduction and prevention of the use of animal experiments possible.

I-3-265

Long-term culture of dermal units in multi-organ-chips

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Tests for drug development requires an almost perfect fit with the human (patho-)physiological microenvironment. The majority of current commercially available skin equivalents are based on static culture systems emulating human epidermis only, or combining epidermis and dermis in so-called full thickness skin equivalents. None of the existing systems contain important elements, such as vasculature, skin appendices, or an immune system. Therefore, current *in vitro* and animal tests are failing to accurately predict drug toxicity.

Here, we are aiming to model a microvasculature driven cutaneous homeostasis of normal, diseased skin and hair follicle biopsies, as well as their bioengineered equivalents in our perfused, self-contained and endothelialized multi-organ-chip (MOC) system. Our MOC platform uses a miniaturized circulatory network with an integrated micropump to provide pulsatile circulation of microliter-volume of medium to support milligrams of human tissue constructs.

In comparison to cultures utilizing conventional static conditions, dermal units cultivated in our perfused MOC system showed remarkable consistency of the cutaneous structure and vitality rendering the MOC system a useful tool for long-term culture.

The current status of the development and remaining hurdles will be further discussed.

I-3-301

Development of a 3-dimesional tissue with vascular networks *in vitro*

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Blood vessels function as a lifeline transporting oxygen and nutrients into tissues and organs. One of the current challenges for developing a 3-dimenional (3D) tissue *in vitro* is to form a vascular-like structure as a lifeline for cells inside. For realizing this, our strategy is to assemble a large number of multicellular spheroids (MCSs) wrapped with an endothelial layer and an endothelial tubular structure (TS) into a 3D tissue. The MCSs were prepared by using hydrogel microcapsule templates (Liu et al., 2013) and the TS was prepared by using a hydrogel fiber template (Liu et al., 2012). The endothelial cells from these parts (MSCs or TS) were connected together and formed 3D networks after one day culture. We succeeded in perfusing tracer particles (3 μm in diameter) into the resultant endothelial networks. From the results, we anticipate that the 3D tissue made from our method would contribute to the researches in biomedical fields, where perfusable vascular networks are needed such as drug deliver or cancer metastasis.

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I-3-303

Synthesis and characterization of tunable agarose-gelatin cryogel for hepatotoxicity evaluation

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Risk assessment of chemicals in India at present relies on either animal models or 2D monolayer culture systems. Ethical concerns and inaccurate results are driving researchers to develop new *in vitro* models based on advanced cell culture techniques. To this end, 3D models provide a better alternative due to their tissue complexities. Progressive advancement has been attained in 3D cultures but efforts are warranted to develop appropriate and cost-affordable models for better screening of chemicals given the endemic limitations in the scenario in India. 3D culture as a mainstream approach for toxicity testing requires the development of standard protocols and quantitative analytical methods.

In this study, we developed a 3D based high-content screening model with hybrid Agarose-Gelatin (AG) cryogel for identifying hepatotoxic effects of drugs. SEM and FTIR were adopted which showed well-interconnected porous structure. The Cyclic Swelling Kinetics and Swelling Ratio of the gel were also performed. Biocompatibility studies with HepG2 cell line revealed good cell attachment and proliferation. Preliminary cytotoxicity assay with Paracetamol revealed advantageous of our 3D model over the already available 3D hepatotoxicity testing models.

I-3-389

Neurotoxicity *in vitro*: assessment of the predictivity of neuronal networks coped to microelectrode arrays for identification of neurotoxicants

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A challenging aspect to assure the safety of a product is the assessment of its neurotoxic hazard potential. Currently, only *in vivo* methods are

regulatorily accepted and so far, no *in vitro* model has been fully validated. With the advance in technology and the ability to maintain primary neuronal models for prolonged periods, a promising test system emerged, combining the use of microelectrode arrays (MEAs) and *in vitro* culture of 2D neuronal networks (NN). In the presentation, we report on the in-house validation of the NN MEA assay using a set of 58 compounds of different chemical classes with known neurotoxic and non-neurotoxic potential with the aim to use it for screening of compounds under development and in the future for its potential application in the regulatory framework. The results demonstrate that the method presents a good sensitivity of 81% and accuracy above 75%. In order to increase the rate of false predictions we also report in the new approach integrating the electrophysiological assessment with a panel of cytotoxicity assays.

I-3-590

Automated long-term operation of multi-organ-chips

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In order to simplify the handling of complex Multi-Organ-Chips (Wagner et al., 2013) – TissUse, TU Berlin and partners have developed a robotic platform. This technique offers a new approach to the monitoring and handling of intricate Lab-on-Chip devices. Our prototype is capable to maintain up to 10 MOCs. This offers a novel possibility to substance testing or the monitoring of Organogenesis. Each operation can be individually programmed and adjusted to the user's needs. For example OECD guidelines for acute toxicity testing can be performed as well as the monitoring of organoid development over time (Materne et al., 2013; Atac et al., 2013). The platform features functions such as automatic media supply, sampling and storage, temperature control, fluorescence and microscopic monitoring, PIV and O2-measurement. To display the functionality we performed toxicity tests with different organoid types.

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I-3-661

Substance exposure to skin and liver co-cultures in a Multi-Organ-Chip

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Current *in vitro* tests fail to emulate the systemic response of applied substances. In this study, a dynamically-perfused Multi-Organ-Chip system was used to integrate the culture of liver and skin equivalents in one system. An on-chip micro-pump enabled metabolic transport and added physiological shear stress. Liver and skin equivalents were viable for 10 days and showed expression of tight junctions and specific transporters. Caffeine, retinoic acid and betamethasone-21-valerate were applied daily for 7 days to investigate the effect of compounds known to be metabolised by skin and liver. The effects of topical application onto the epidermis were compared to the effects of direct substance application to the medium to analyse the influence of skin penetration and metabolism. Liver and skin equivalents were analysed for the expression of metabolising enzymes, transporters, differentiation markers and for viability. Results showed constitutive and inducible phase I and II enzyme expression on protein and mRNA level, according to the substances applied. Hence, the Multi-Organ-Chip is a promising *in vitro* approach for systemic and topical dosage of drugs and cosmetics in a combined culture of liver and skin.

I-3-745

A new human liver 3D culture in microfluidic platform as a potential tool for toxicity studies

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The development of reliable and accurate *in vitro* models at an organ level is imperiously needed to reduce and replace *in vivo* animal experiments. Thus, we generated 3D human liver cell cultures in a microfluidic plate. Briefly, HepG2 cells were cultivated in extra cellular matrix for 3D culture in side channels and provided fresh medium continuously in the middle channel by passive perfusion. Cell morphology was observed by measurement of spheroid area and staining of membrane and nuclei. The HepG2 cells self-organized within 2-3 days to form polarized spheroids and size-stabilized after the first week. Our new culture model showed increased expression of albumin, urea production, efflux of MRP-2 membrane transporter in bile canaliculus and CYP450 1A induction capacity when compared to conventional 2D culture. Acetaminophen treatment for toxicity assessment in our model showed dose dependent toxic effect. In conclusion, our new 3D microfluidic human liver model can efficiently simulate *in vivo* situation and improve liver specific functionality in comparison with conventional cultures. Therefore, this new model can offer a strong potential in applications for *in vitro* hepatotoxicity studies.

I-3-753 *

A Multi-Organ-Chip-based dynamic sensitization assay comprising human epidermis and primary dendritic cell co-culture

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The accurate assessment of the skin-sensitizing potential of chemicals via *in vitro* testing is crucial for the cosmetics industry´s ability to

innovate. Various *in vitro* tests have been developed to predict the *in vivo* situation. However, no existing assay reflects the biological complexity. Instead, a battery of assays – addressing several key events of the adverse outcome pathway – is required.

To integrate the biologically relevant crosstalk of keratinocytes and dendritic cells, we established a co-culture of human epidermis with primary, monocyte-derived dendritic cells (DCs) in our Multi-Organ-Chip (MOC). MOCs consist of a microchannel system connecting two culture compartments for cells or biopsies. A peristaltic on-chip micropump enables the circulation of the medium, allowing a cross-talk of different cell types. Utilizing this system, we applied different sensitizers directly to the medium and analyzed the activation of DCs based on the induction of CD86. Moreover, we performed topical application of the compounds using EpiDerm™ models instead of human epidermis.

Here, we report on the differences in DC activation for identical substances among our first-time developed MOC-based sensitization assay and a conventional static assay using only DCs, implying that our assay is a promising approach to improve the prediction of skin sensitizers.

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I-3-768

How new technologies are promising to fulfil the Reduction and Replacement 3Rs objectives

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In vitro research has changed dramatically over the past 10 years. Most researchers now recognise the inadequacies of traditional cell culture methods (on or in plastic or glass, under static conditions) using animal cells or immortalised cancer-derived cell lines. There is an increasing focus on faithfully replicating the *in vivo* environment so that *in vitro* results tell us accurately what is happening in the body. A variety of technologies and techniques are now employed to mimic *in vivo* conditions: 3D constructs to replicate tissue architecture; co-culture to allow different cell types to communicate the way they do in the body; flow to refresh nutrients and stimulate cells with physiologically-relevant shear stress. These new methodologies have facilitated the advancement of 3Rs objectives.

In this paper we describe a system that will allow the incorporation of more of these physiologically-relevant parameters. We consider the effects of a selection of factors such as oxygen concentration, glucose availability, pressure, motion and circadian rhythm on cells, discuss their relative importance and how they might be replicated *in vitro*. The paper concludes with a roadmap of how the "organ-on-a-chip" technology is likely to develop from the R&D phase through to a more routine testing capability.

I-3-784

Kidney toxicity assessment and ADMET testing in a two-circuit microfluidic device

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Kidney toxicity to date is badly predictable in cellular assays and animal experiments. Among all drug candidates failing because of organ toxicity the failure rate due to kidney toxicity is only 2% in preclinical studies but 19% in clinical phase III studies. Therefore, there is a translational gap and a need for improved preclinical models.

We developed a microfluidic device made up of two interconnected PDMS-embedded circuits mimicking the renal blood and urinary system. Each circuit can be operated at physiological flow rates by an on-chip-micropump. The interface between the circuits is a microporous PET-membrane that can be seeded with renal epithelial and endothelial cells. The device also includes culture spaces for intestinal cells and liver aggregates for ADMET profiling.

We could show viability and functionality of a human renal proximal tubule cell line (RPTEC/TERT1) for up to 10 days in the device. Furthermore we performed first substance exposure experiments with tobramycin and other known nephrotoxins.

Taken together, we developed a microfluidic device that is suitable for assessment of kidney toxicity. In the future this could be used to improve the translation from pre-clinical to clinical studies and determine the ADMET profile of substances to replace animal experiments.

I-3-792

Microtissues meet microfluidic: a multi-parallel body on a chip concept

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The next step towards more biomimetic *in vitro* models is the design of multi-organ devices which allows communication among different tissue types. Here, we present a novel "body on a chip" concept which not only allows interaction among different microtissue types, but is also robust and flexible thus enabling its routine use for substance testing. Spherical microtissues are interconnected by tilting the microfluidic chip device, therefore avoiding tubing and external pumping technology. The microfluidic chip contains dedicated micro-chambers to immobilize pre-formed spherical microtissues, and continuous perfusion for culturing is facilitated through interconnecting micro-channels. The chip design allows operating up to 48 devices in parallel, and replicates of various drug concentrations can be tested. A proof of concept study was performed by interconnecting rat liver and colorectal tumor microtissues on the chip. Interestingly, rat liver microtissues secreted elevated levels of albumin over the first 10 days on the chip prior to reaching equilibrium. For proof of feasibility, pro-drug activation of cyclophosphamide (CPA) on the chip was compared to static conditions. A significant impact of CPA on the tumor model was only observed with the chip; whereas, supernatant from static liver microtissues treated with CPA did not lead to significant anti-tumor effects.

Session I-4a: 3D liver models

Co-chairs
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Session I-4a: Oral presentations

I-4a-015

H3Screen; Promethera's 3D liver progenitor cell model for hepatotoxicity testing

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Drug-induced hepatoxicity is one of the most important reasons for the attrition of drug candidates. The availability of suitable human liver-based *in vitro* models allowing reliable metabolism screening and early detection of acute to chronic drug-induced liver injury has great interest for the pharmaceutical industry. Promethera Biosciences, a cell therapeutic biotech company, develops 2D/3D-cell-based assays based on human adult liver progenitor cells (Soka, 2011). Key hallmarks are their non-tumorigenic human origin, acquisition of metabolic capacity within the range of primary hepatocytes, long-term stability in culture, large-scale production yield, efficient cryopreservation and controlled differentiation & reproducibility (O'Brien et al., 2004). Both 2D/3D models form a spontaneous co-culture system of hepatocytes embedded in a cellular stroma. Successful hepatic differentiation-maturation is evidenced by expression of HNF4a, CK19, albumin, OTC and UGT1A1 at RNA and protein level (Buyl et al., 2014). Moreover, they display inducible and kinetic Phase I cytochrome P450-dependent enzymatic activity (CYP3A4/OH-Midazolam, CYP1A2/Acetaminophen, CYP2B6/OH-Buproprion, CYP2C9/OH-Diclofenac), Phase II paracetamol-sulfotransferase and bilirubine-glucuronidation conjugation capacity and active Phase III uptake (OATP) and efflux (MRP2) transport. Hepatic functionality was preserved for up to 1 month. Their substantial metabolic capacity, ease of use, & long-term stability make them suitable candidates as sustainable preclinical models for metabolism screening and chronic pharmaco-toxicological assessments.

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I-4a-178

In vitro generation of functional organoid structures resembling embryonic liver buds using differentiated, human cells

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Formation of 3D structures resembling liver buds *in vitro* has recently been described using stemcell-derived cell types (Takebe et al., 2013). Such structures can be transplanted into animals but may also prove useful for other applications, like *ex vivo* drug toxicity tests. Here we used human upcyte® cells to form liver buds *in vitro*. upcyte® cells are derived from primary human cells via genetic modification in order to transiently induce cell proliferation. When contact-inhibited, upcyte® cells differentiate into functional cells maintaining their cell-type specific characteristics.

We used defined mixtures of differentiated human upcyte® cells (hepatocytes, liver-sinusoidal endothelial cells and mesenchymal stemcells) that spontaneously formed liver buds *in vitro* which could be cultured for up to 30 days using a kirkstall bioreactor. These self-organized, liver-like organoid structures harbour healthy, living cells showing typical functional characteristics of liver parenchyme, including basal and drug-induced activity of several Cytochrome P450 enzymes. Within the bud the cells formed a typical "liver-like" architecture built up by polarized hepatocytes.

In summary we describe that 3-dimensional, functional liver structures can be generated *in vitro* using upcyte® cells and that these "mini-livers" are useful models to study long-term human liver function *ex vivo*.

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I-4a-450

Development of hollow fiber type three dimensional (3D) culture module for long term culture of human hepatocytes to evaluate the drug toxicity to liver

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About 60% of the developed drugs drop out by the clinical trial at present, the cause of which is recognized to be the species differences between animal and human metabolism of these drugs. Therefore, we developed 3D culture module (Int. Patent, 2014) for human hepatocytes to foresee the toxicity and effect of the drugs at the preclinical stage.

Two kinds of hollow fibers are installed in the module, one hollow fiber for oxygen supply and the other fiber for nutrient supply, which are necessary for the cell growth and activity. Furthermore, nano-fiber

nonwoven fabric was used as 3D scaffolds. We performed perfusion culture of human hepatoblasts in the 3D-culture module, which are one of the hepatic stem cells and differentiated from human fetal hepatocytes by original culture protocol as previously described (Kiyota et al., 2007).

Using human hepatoblasts, a long-term perfusion culture was achieved by the module for over 150 days at high cell density of 10^8 cells/cm^3 and 3D culture of hepatoblasts had made it possible to maintain CYP3A4 activity for 81 days corresponded to that of human hepatocytes. For this reason, 3D-module technology allows for foreseeing the subacute toxicity (generally four weeks) and effect of the drugs at preclinical stage.

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I-4a-612

Organotypic human HepaRG spheroid cultures for *in vitro* toxicity studies

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Drug-induced liver injury (DILI) remains the main cause of acute liver failure (ALF) and post-market drug withdrawals. The well-documented limitations of pre-clinical *in vivo* animal studies and *in vitro* two-dimensional (2D) human hepatic models hinder the accurate prediction of DILI in humans. Therefore, there is a dire need for alternative *in vitro* human hepatic models that can better predict DILI in humans. The objective of the study was to characterize long-term 3D human HepaRG spheroid cultures in order to assess their suitability for *in vitro* toxicity studies. 3D HepaRG spheroids were obtained using the hanging drop technology. HepaRG microtissues were maintained for 4 weeks and assessed for liver specific morphology and function. The cultures were viable and maintained a stable size (diameter ≈ 250 µm) over the culture period in the absence of a necrotic core. The data illustrates that the cultures secreted albumin and urea throughout the culture period. Furthermore, cultures possessed both basal and inducible CYP3A4 enzyme activity, which is one the most imperative enzymes in drug metabolism and toxicology. Therefore, taken all together, the results from our study suggest that the organotypic human HepaRG spheroid cultures may be a promising *in vitro* tool for DILI studies.

I-4a-785

Characterization of heterotypic 3D human liver microtissues for drug-induced hepatotoxicity testing

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Stable organotypic *in vitro* hepatic models might lead to a significant reduction of animal studies to assess long-term (4 weeks) toxicity of substances. 3D liver microtissues (MTs) cultures, composed of hepatocytes and Kupffer macrophages were systematically assessed for viability, liver-specific morphology, functionality and mRNA expression over 28 days. MTs demonstrated a 5x longer life span than the conventional 2D cultures maintaining consistent ATP content and morphology (CYP3A4, CD68, CK7 and BSEP). Albumin secretion was significantly higher in the MTs and there was continuous inducibility of an inflammatory response towards LPS exposure. Transcriptome analyses revealed differential regulation of 145 liver-specific genes in the MTs versus the 2D cultures over the culture period, including genes involved in innate liver-specific functions and ADME. CYP (3A4, 1A2, 2B6, 2D6, 2A6) enzyme activity was at least 2 fold higher and was maintained only in the MTs. Repeated, long-term exposure to Troglitazone and Tolcapone resulted in increased sensitivity (reflected by decreased IC_{50}) over time, in contrast to their non-hepatotoxic structural analogues, Pioglitazone and Entacapone, respectively. Taken together, the results illustrate that the MTs maintain a differentiated liver-specific phenotype for at least 4 weeks and are a valuable model to study chronic and inflammation-mediated DILI.

Session I-4b: 3D miscellaneous organ models

Co-chairs
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Session I-4b: Oral presentations

I-4b-082

Mimicking respiration in an advanced *in-vitro* lung alveolus

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The human lung is of particular interest for toxicology, as it is the major entrance port for air borne particles and noxious gases. However, standard lung alveolar *in-vitro* models aimed at evaluating the effects of such xenobiotics poorly mimic the complexity of the human

air-blood barrier. In sharp contrast, we present an advanced *in-vitro* model of a lung alveolus that combines an air-blood barrier and cyclic stretch to mimic the respiration. For this purpose, a thin, porous and elastic poly(dimethylsiloxane) (PDMS) membrane is sandwiched between two PDMS layers. Endothelial cells are seeded on the basal side of the fibronectin-coated membrane and epithelial cells on the apical side. Upon confluency, the epithelial cells are exposed to air and cyclically stretched for 48 h using an integrated actuation mechanism. Preliminary results show reproducible and homogeneous cyclic stretch patterns and increased tight junctions expressions. This *in-vitro* lung alveolus model mimics the complexity of the human air-blood barrier in an unprecedented way, is easy to handle and may thus be an ideal tool for toxicology assessments.

I-4b-240

Next generation tissue models as a replacement for animal experiments

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Despite advances in the development of *in-vitro*-tissue-models, the number of endpoints in toxicity-testing which can be addressed with these models is limited. This is due to a lack of key cellular components and a restricted live time of the models (Groeber et al., 2011). In addition, the manual production process of current tissue-models results in significant inter- and intra-lot deviations. One reason for these limitation is that suitable technologies for the culture and production of tissue-models are missing. To overcome these pitfalls, the Fraunhofer IGB is achieving advanced culture systems and biomaterials which allow long term culture of complex tissue-equivalents. Using these technologies, we have developed the first full thickness skin-model with a perfused vascular network (Groeber et al., 2012). Furthermore, within an interdisciplinary consortium of biologist and engineers, we have established a fully automated production facility to generate epidermal models based on an open source reconstructed epidermis (OS-REp) (Lemper et al., 2013; Poumay et al., 2004)). During the product manufacture, all media changes and manipulations are automatically preformed which allows a maximum output of 8000 OS-REp per month (http://www.tissue-factory.com).

In our work we could create new technologies for the generation and production of tissue-models which is a vital requirement to increase the success of in-vitro-test-methods.

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I-4b-406

An organotypic model of testes development: evaluation of diverse compounds and implications for high-content toxicity screening

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We have developed a medium throughput, high content *in vitro* model of male reproductive development using neonatal rat testes. This model includes a co-culture of testes cell types cultured with a three dimensional matrix, creating *in vivo*-like niches (3D-TCS). To determine the breadth of toxicity signals that the 3D-TCS can assess, we screened over 60 compounds for cytotoxicity. Using relevant exposures and experimental toxicity data to set initial screening concentrations, cytotoxic doses were identified for 31 of the 63 compounds. Cytotoxicity was observed for compounds which act through general toxicity mechanisms (such as oxidative stress) as well as through mechanisms more specific to male reproductive endpoints (such as endocrine disruption). For some compounds, cytotoxicity was a sensitive indicator of male reproductive toxicity at concentrations near relevant therapeutic levels. For other compounds such as vinclozolin, cytotoxicity was not a good predictor of reproductive toxicity, indicating the need for further analysis. Five compounds (arsenic, crizotinib, nicotine, valproic acid and vinclozolin) were then selected for future testing using additional end points, including effects on testosterone excretion and transcriptomic profiles. This model has allowed us to expand our mechanistic output while addressing the need to reduce animal use.

This research was supported by FDA grant 1U01FD004242.

I-4b-508

Human cell based 3D-functional vasculogenesis/angiogenesis test for identification of angiogenic and embryonic vascular discruptors and to be used as the vascular platform in tissue models

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Formation of vascular network is a crucial process during embryonic development, and it also contributes to the pathogenesis of numerous disorders such as cancer. Vasculogenesis and angiogenesis are two central mechanisms by which the vascular network is formed. During embryonic development vasculogenesis refers to *in situ* differentiation and growth of blood vessels, and angiogenesis comprises the growth of new blood vessels from the preexisting ones.

The developed test utilizes human adipose stem cells or fibroblasts and human endothelial cells to form 3D-coculture. The vascular construct was characterized using structural, gene expression and functional markers. The presence of 3D tubule structure, younger and more mature vessels, basement membrane, lumen, extracellular matrix, adherence junctions between endothelia and pericytes has been proven. In the test both anti-angiogenic and angiogenic properties of a chemical and biological substances can be studied. The relevance of the assay in man was investigated by comparing the effects of a broad number of different types of reference chemicals with the published

human data. The comparison showed a good concordance indicating that this human cell based test predicts the effects in man and has potential to replace animal tests, and to be used as vascular platform in tissue models.

I-4b-744

NPC-derived neurospheres serve as test systems for early neurodevelopmental toxicity: an interspecies comparison of toxicity pathways

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Neurospheres are three dimensional (3D) cell culture models from human, mouse and rat consisting of neural progenitor cells (NPCs), which proliferate in culture and migrate and differentiate into neurons and glia cells thus mimicking basic processes of brain development *in vitro*.

By employing different compounds in the neurosphere assay, we so far identified intracellular signalling pathways like Nrf2, the arylhydrocarbon receptor and epigenetic modification by histone deacetylases as well as pathways guided by cell surface receptors like integrins and the fibroblast growth factor receptor as modulators of human NPC development. Contributions of some of these pathways to processes of neurodevelopment underlie species-specificities. For enabling high content image analyses, we wrote algorithms for sphere evaluation.

In summary, 3D neurosphere cultures are useful for identifying toxicity pathways related to disturbances of processes relevant for human brain development. In this regard, species comparisons are of great value for hazard assessment as humans might be more, less or equally sensitive than their rodent counterparts.

I-4b-755

Organotypic tissue culture as a new *in vitro* model of human myocardium

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Human myocardial tissue is rarely available for *in vitro* studies, and stays viable only for short periods. These limitations should be overcome by a technique to prepare many vital slices from one tissue specimen, permitting cultivation and functional assessment over several weeks.

Specimen of hypertrophic (resected) or failing (explanted) myocardium were cut into 300 μm thick slices of 1x1 cm^2, which were analyzed immediately or cultured at a liquid-air interface for up to 28 d. Membrane potential, contractility, and viability were determined. To enable collection of specimen from 3 surgical centers, cold preservation was tested for transport.

Slices prepared <12 h after tissue retrieval developed paced contractions of 2.7 mN/mm^2 at up to 180 bpm, depending on diastolic strain and adrenoceptor stimulation. Action potentials displayed normal diastolic potential (-80 mV), amplitude, duration (330-380 ms), and responded to hERG and K_{ATP} channel manipulation. Cultured slices maintained viable >28 d, with only minor electrophysiological changes. Their contractility declined during the first week of culture and was detectable throughout 28 d. This time-course of contractility was reproduced when slices were prepared from tissues transported for 18-36 h.

Our findings emphasize the potential and feasibility of human heart slices as an *in vitro* model for myocardial contractility and electrophysiology.

Session I-4c: Novel technology for 3D cell cultures

Co-chairs
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Session I-4c: Oral presentations

I-4c-168

Collagen vitrigel membrane chamber useful for fabricating 3d-culture models composed of epithelial, mesenchymal and/or endothelial cells and its advantages for ADME/Tox studies

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Pathways of chemicals in each organ *in vivo* are classified into two patterns; one is the pathway from epithelium to endothelium via mesenchyme in case that the chemical is exposed to the epithelial surface of cornea and skin, etc., and another is the pathway from endothelium to epithelium via mesenchyme in case that the chemical is administered directly into a blood vessel. Meanwhile, A collagen vitrigel membrane (CVM) we previously developed is composed of high density collagen fibrils equivalent to connective tissues *in vivo* and is easily handled with tweezers (Takezawa et al., 2004). Also, it possesses excellent transparency and permeability of protein with high molecular weight and consequently it functioned well as a scaffold

that can facilitate the fabrication of a 3d-culture model excellent for cross-talking between the different types of cells by seeding them on its both surfaces (Takezawa et al., 2007). Recently, we developed a CVM chamber useful for fabricating tissue sheet-type and organoid plate-type culture models composed of one kind and more than two kinds of cells, respectively (Takezawa et al., 2012). From the viewpoint of extrapolating ADME/Tox *in vivo* of chemicals, we have developed vitrigel-EIT (Eye Irritancy Test) (Takezawa et al., 2011; Yamaguchi et al., 2013), CPT (Corneal Permeability Test), and LMTT (Liver Metabolism Toxicity Test) methods.

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I-4c-236

Functionalized electrospun nanofibers for the development of a 3D skin model

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An appropriate simulation of the 3D environment in which tissues develop is essential to build up *in vitro* models. Electrospinning has been recognized as an efficient technique to produce scaffolds, which mimic the topography of the native extracellular matrix. Modification of electrospun fibers with a functional, amphiphilic macromolecule based on a star-shaped poly(ethylene oxide) derivate transforms hydrophobic fibers into hydrophilic fibers. Moreover, the attachment of cell-adhesion mediating peptides as the fibronectin binding side RGD is possible (Grafahrend, 2011). But also other ECM components as collagen and laminin sequences or a combination of different motifs can be bound to the macromolecule. Co-culture experiments with HaCaT cells and fibroblasts have validated that it is possible to create skin equivalents with these functionalized scaffolds. The keratinocyte cell line grows in several layers and expresses cytokeratin 10 in the stratified epithelium and cytokeratin 14 in the basal layers. Vimentin staining of the fibroblasts showed that the cells infiltrated into the electrospun membrane. It could also be shown that cells produce laminin, collagen and fibronectin, which illustrates that the matrix is remodeled. The establishment of a method to make specific cell adhesion on electrospun fibers possible offers great opportunities for the construction of biomimetic co-culture systems.

Reference

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I-4c-473

Solubilized matrix from decellularized liver as a functional ECM to reproduce *in vivo* micro-environment in *in vitro* culture

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A novel cell culture technology to enable the effective screening for enormous varieties of candidate compounds for pharmaceutical drugs is desired earnestly. For this realization, development of a functional ECM to reproduce *in vivo* micro-environment in *in vitro* culture is important (Sellaro et al., 2010; Zhang et al., 2009). In this study, we developed a functional material as a scaffold for hepatocyte culture to reproduce *in vivo* micro-environment. In addition, 3D-culture system with this material was developed as the screening tool of pharmaceutical drugs.

Solubilized extracellular matrix derived from decellularized liver (L-ECM) was obtained by treatments with Triton X-100 (decellularization) and pepsin-HCl (solubilization). L-ECM solution was air-dried in multi-well plate for the cultivation of primary rat hepatocyte and liver-specific function of cell and then evaluated. In decellularized liver, 92.4% of DNA was removed from the native liver. Moreover, L-ECM stimulated the expression of liver-specific functions – including albumin secretion, urea synthesis and ethoxyresorufin-O-deethylase activity – of primary rat hepatocytes. Therefore, L-ECM has the potential to become an effective material to reproduce *in vivo* micro-environment. In future, we will investigate the effectiveness of L-ECM as a screening tool of pharmaceutical drugs.

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I-4c-544

Design and fabrication of human skin by three-dimensional bioprinting

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3D bioprinting, a flexible automated on-demand platform for the free-form fabrication of complex living architectures, is a novel approach for the design and engineering of 3D human tissues. Here, we describe the use of 3D bioprinting for engineering human skin. In this model, keratinocytes and fibroblasts were used as constituent cells to represent the epidermis and dermis, respectively, and collagen to represent the dermal matrix of skin. Preliminary studies were conducted to optimize cell viability and cell density to mimic physiologically relevant attributes of human skin. Histology and immunofluorescence characterization revealed that printed 3D skin tissues were morphologically and biologically representative of *in vivo*

human skin tissue. In comparison with traditional methods for skin engineering, 3D bioprinting offers several advantages in terms of shape- and form-retention, flexibility, reproducibility, and high culture throughput. It has a broad range of applications in transdermal and topical formulation discovery, dermal toxicity studies as well as in designing autologous grafts for wound healing. We are currently focused on enhancing the complexity of this model via the incorporation of secondary and adnexal structures such as the blood vasculature, and the inclusion of diseased cells to serve as a model for studying disease pathophysiology.

I-4c-667 *

Nanofibrillar cellulose hydrogel enables flexible 3D cell culturing

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Various synthetic and natural hydrogels have been used as 3D cell culture scaffolds for different biomedical applications. From the immunological point of view, xeno-free scaffolds are highly preferred. We have recently shown that plant-derived nanofibrillar cellulose (NFC) hydrogel provides a flexible 3D culture system for several cell types.

NFC consists of fibrillar glucan chains with a diameter in the nanometer range. Stiffness of the hydrogel can be easily modified to provide a desired matrix support for each cell type. Human hepatic cells, human embryonic stem cells, and human induced pluripotent stem cells formed 3D multicellular spheroids in NFC hydrogel. Hepatic cells showed liver-specific properties in 3D hydrogel culture: they secreted human albumin, formed bile canalicular-like constructions, showed efflux protein mediated transport into formed canalicular structures, and expressed higher level of CYP3A4 activity compared with2D culture. 3D stem cell spheroids retained pluripotency and can be released from the hydrogel with a cellulase enzyme. The recovered spheroids can be used for further 2D or 3D culturing, or for analysis. In conclusion, NFC hydrogel offers xeno-free 3D culture scaffold, which may be applied in organotypic culture systems for drug testing and tissue engineering in the future.

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Session I-4: Novel 3D models – Poster presentations

I-4-097

The assessment of a Caco-2/CCD-18co co-culture model of the small intestine cultured on poly (ethylene terephthalate) nanofibre scaffolds

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The caco-2 model of the intestine has enabled the prediction of drug permeability *in vitro* (Lennernäs et al., 1996), providing an indication of the expected oral absorption of new drugs in man and identifying candidates with poor pharmacokinetic profiles. Recent Literature suggest subtle environmental cues can alter the functional output of the Caco-2 model (Kim et al., 2012; Kim and Ingber, 2013; Yu et al., 2012) exposing scope to improve *in vitro* predictions. Nanofibre scaffolds which mimic the basement membrane structure can be produced by electrospinning; these were investigated as a scaffold platform for an *in vitro* model. Intestinal sub epithelial myofibroblast line CCD-18co cells were cultured on the basolateral surface of the nanofibre scaffold for 4 days prior to Caco-2 cell seeding on the apical scaffold surface to simulate the multicellular milieu of the intestinal mucosa. The resulting platform was assessed for barrier functionality through TEER (Trans-epithelial electrical resistance), paracellular transport and drug permeability assays. Results show that Caco-2 cells cultured on nanofibre scaffolds in monoculture and in a co-culture with CCD-18co demonstrate reduced barrier formation which may be more comparable to the human intestinal barrier. Additionally Caco-2 monocultures cultured on nanofibre matrices exhibit increased paracellular transport to Lucifer Yellow compared to the traditional Transwell™ platform model.

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I-4-166

New BEST – biomaterials enhanced simulation test

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This work presents the development of new *in vitro* testing method for biomaterials in the closest to hostile conditions. The objective of this BEST – biomaterials enhanced simulation test – is to provide maximal possible yet realistic control and monitoring of chemical, biological, cytological etc. reactions, and leading to decrease of animal testing. Recently no combined *in vitro* solution exists capable of answering the

demands and needs of all stakeholders (patients, hospitals, biomaterials producers, pharmaceutical industry) at reasonable costs, speed and safety (von Recum, 1998; Black, 1999). New challenges require more consistent and holistic approaches to ensure reliability and safety of the implants including those with ATMP.

Here the design of the BEST methodology and test equipment is shown for the case of load-bearing implants such as orthopaedic and dental ones (van Mow and Huiskes, 2005). The demonstration includes based porous coated biomaterials at different conditions showing the preferential potential for bone, cartilage or fibrous tissue formation. The testing is supported by time- and frequency-domains simulation with models *in silico* (Gasil et al., 2012).

The results show the importance of proper application of relevant testing parameters vs. traditionally used protocols, leading also to reduction of specimens and faster screening of new materials formulations with new method.

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I-4-172

An *in vitro* ocular test system for a detailed quantification of the cellular damage in the corneal epithelium and stroma

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The study aims to develop a test system for the complete replacement of the Draize Eye Irritation Test to allow the discrimination of all 3 GHS categories in one experiment. The test system is based on biotechnologically produced 3-dimensional hemi-cornea models, which comprise both an epithelium and a stroma with collagen embedded keratinocytes (Engelke et al., 2013). A collagen membrane was inserted between these two tissues for the separation and independent assessment of damages in the epithelium and stroma after exposure to potential eye irritants. Cell viability, assessed with the MTT assay, was used as a toxicological endpoint. Acceptance criteria were defined based on negative and positive controls. The prediction model which was defined on the results of 30 test materials uses a single exposure period and the combination of cut-off values in tissue viability from both epithelium and stroma. As a result, 100% of the GHS 1 category, 88% of the GHS 2 category and 57% of the GHS unclassified test materials were predicted correctly. In conclusion, the test system predicts and discriminates GHS 1 and GHS 2, but is over predictive for GHS no category materials. The project is funded by the German Federal Ministry of Education and Research (FKZ0316010).

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I-4-173

The initial depth of injury in 3-dimensional tissue models for the prediction of the eye-irritation potential of chemicals

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Based on 3-dimensional reconstructed tissue models of the human eye we aimed to establish a test method to reliably predict the eye-irritation potential of chemicals for all GHS categories. We developed a technique to determine the initial depth of injury (DoI) in tissue models of human cornea and conjunctiva by combining the MTT viability assay with cryosectioning procedures. The formazan-free, metabolically inactive area in the tissue after topical substance application, the visible correlate of the DoI, is quantitatively analyzed on cryosectional images with ImageJ software analysis tools. Our experiments revealed that for most of the chemicals tested so far the DoI values increased in parallel with increasing eye-irritating potential of the chemicals. Therefore, the test method allows us to distinguish between the cytotoxic effects of different chemicals for all 3 GHS categories. However, in order to establish a robust prediction model, a larger set of chemicals of different chemical classes and drivers of irritation must be tested in future.

In conclusion, analyzing DoI in MTT-stained cryosections represents a promising tool to assess toxicological reactions in reconstructed corneal and other epithelial tissues.

The project is funded by the German Federal Ministry of Education and Research (FKZ0316010).

I-4-204

A new 3D human airway tissue model for *in vitro* lung cancer research (OncoCilAir™)

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Lung cancer is the most common cancer with about 1.4 million deaths worldwide every year (Cancer Facts, 2014). Here we developed a fully humanized *in vitro* lung cancer model, OncoCilAir™ , which combines primary human bronchial epithelial cells with lung adenocarcinoma cell lines in order to replicate as closely as possible the *in vivo* formation and progression of lung cancer. Cultured at the air-liquid interface, this system displays proper differentiation features with a pseudostratified columnar epithelium containing ciliated, goblet and basal cells. High trans-epithelial electrical resistance, cilia beating and production of mucus demonstrate the full functionality of the epithelium. Remarkably, and in contrast to monolayer cultures, tumour cells extended forming nodules into the adjacent tissue, a hallmark of human lung cancer (Henschke et al., 1999). Dose response experiments including the investigational drug Selumetinib showed that the system could be effectively used to assess both drug efficacy and toxicity. In

conclusion, OncoCilAir™ heralds a new generation of integrated *in vitro* models that combine both healthy and compromised tissues in order to accurately simulate *in vivo* situation (Yamada et al. 2007). Such models should be valuable tools to increase the quality of preclinical research, while reducing animal testing.

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I-4-211

A novel 3d-culture system activating hepatic function of HepG2 cells utilizing a collagen vitrigel membrane chamber and its application to liver metabolism and toxicity test

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A collagen vitrigel membrane (CVM) chamber we developed is a culture tool useful for oxygen supply and 3d-culture. Such a CVM chamber can provide a unique culture system enabling cell growth in a culture medium (liquid phase) on the CVM under which is not only a culture medium (liquid phase) but also plastics (solid phase) or 5% CO_2 in air (gas phase). Here, we designed above 3 different culture systems using the CVM chamber to establish a new culture system activating hepatic function of HepG2 cells. Also, the co-culture system was fabricated by overlaying collagen sol on the HepG2 cells and seeding HUVECs on the overlaid collagen gel.

HepG2 cells cultured on the liquid-gas interface remarkably improved CYP3A4 activity. Also, the cells maintained mitochondrial membrane potential and inhibited the promotion of cell membrane permeability in comparison to the other culture conditions. Taking these advantages, we succeeded in estimating concentration-dependent hepatotoxicity of acetaminophen. In the ADME examination using fluorescein diacetate, the cells incorporated it and rapidly excreted the metabolized intracellular fluorescence into a bile canaliculus-like structure. Especially, such bile-canaliculus-like structures formed by HepG2 cells in monolayer were most clearly observed in the co-culture system in comparison to other culture conditions.

I-4-235

Offbeat to mainstream: high-throughput, user-friendly three-dimensional cell culture models of brain tumors

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Current cell culture models using two-dimensional monolayers are of limited value in predicting *in-vivo* response and cannot compete successfully with animal models. Multicellular spheroids utilising human tissue offer improved predictive potential through better representation of tumour biology. We present a suite of assays in 96-well format that allow for rapid, affordable and biorelevant spheroid screening of drug delivery platforms.

Human brain tumour medulloblastoma cell line UW228-3 and foetal brain tissue stem cell neurospheres were cultured in xeno-free conditions in 96-well ultra-low attachment plates. They reproducibly formed a single spheroid per well (100-900 μm, CV 5-10%). A set of three mechanistically different methods for spheroid health assessment (volume, metabolic activity and acid phosphatase enzyme activity) were validated against cell numbers in healthy and drug-treated spheroids. In addition, fluorescently marked tumour and normal tissue were cultured together forming co-culture spheroids, exposed to biorelevant etoposide concentrations and the viability of both populations was assessed separately for each population using flow cytometry and multiphoton microscopy.

A therapeutic concentration of 10 μM etoposide was identified which demonstrated toxicity to tumours while maintaining four-fold higher viability in normal brain tissue. This proof-of-concept study offers the opportunity to perform biorelevant safety and efficacy screening and to reduce animal experiments.

I-4-335

Human and rat neuronal microtissues for toxicity testing

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Currently *in vivo* animal models are primarily employed to assess for neurotoxic effects of chemicals and potential candidate compounds in drug development. *In vitro* testing is limited to HTS compatible low complexity 2D cultures of primary brain cells or cell lines and to testing on more sophisticated low throughput explant cultures. We report here the generation of two different scaffold-free 3D microtissue models derived from human and rat cell origins. Rat neuronal microtissues display incorporation of the three major types of brain cells: neurons, astrocytes and oligodendrocytes and are stable over extended time periods (>4 weeks). Myelination is demonstrated by electron microscopy analysis as well as positive immunohistochemical staining of myelin basic protein (MBP), a major constituent of the axon-enwrapping myelin sheet. The human microtissue model consists of iPS-derived astrocytes in co-culture with iPS-derived neurons. Tissue constructs exhibit stable three-dimensional architecture and display positive staining for the neuronal marker β-III-tubulin and the astrocyte marker GFAP.

Future strategies to assess neurotoxicity *in vitro* will mainly depend on improved predictivity of the models, but also on the capacity to increase throughput of such systems.

I-4-352

Human organotypic nasal epithelial tissue culture as an *in vitro* model to evaluate effects of cigarette smoke

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In line with the reduction, refinement, and replacement framework of animal use and to overcome the limitations of species translation, the development and use of human *in vitro* models, that closely mimic *in vivo* biology, is of great importance.

To assess the effect of cigarette smoke (CS), human organotypic nasal epithelial tissue cultures were exposed for 28 min at the air-liquid interface to air or to different dilutions of mainstream CS (under Health Canada smoking regimen and nicotine doses of 0.15 mg/L or 0.25 mg/L). Time- and dose-dependent CS effects were evaluated by measuring multiple endpoints (cytotoxicity, CYP1A1/1B1 enzyme activity, inflammatory marker secretion, histological and transcriptomic changes).

Exposure to CS resulted in increased CYP1A1/1B1 enzyme activity and release of various inflammatory markers. In addition, using gene expression data and a network-based systems biology approach, significant perturbations of biological processes such as apoptosis, inflammation, cell proliferation, cellular stress and senescence were shown and quantified over different post-exposure time points.

Our results demonstrate that human nasal organotypic tissue culture could be a suitable model for mechanistic and toxicological assessment of CS effects on the respiratory tract and for comparative studies of reduced-risk products.

I-4-368

Pre-validation of the Hen's Egg Test for Micronucleus-Induction (HET-MN): final evaluation of data

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The HET-MN assay is distinguished from other *in vitro* genotoxicity assays by toxicologically important properties such as absorption, distribution, metabolic activation, and excretion of test compounds. As a promising follow-up approach to supplement existing *in vitro* test batteries for genotoxicity, the HET-MN recently underwent a formal pre-validation.

After the method was developed at the University of Osnabrueck it was transferred first to the laboratories to Henkel following an inter-laboratory study analyzing 14 test substances in both laboratories.

In 2010 the method was transferred to the third (Federal Institute for Risk Assessment, BfR) and fourth lab (Harlan Cytotest Cell Research). In the first phase of the transfer cyclophosphamide and 7,12-dimethylbenzanthracene were examined. In the second phase, three compounds (ampicillin, carbendazim, acrylamide) were tested in a blind study in the laboratories of the BfR, Harlan, and Henkel. In the final pre-validation a balanced data set of 20 chemicals were tested blinded. They comprised different chemical classes and mode of actions covering the groups of the true positives, true negatives and misleading positives.

The data with a promising outcome will be presented with regard to reproducibility and predictivity.

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I-4-369

3D Skin Comet assay: *status quo* of the ongoing validation

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In 2012, Cosmetics Europe joined forces with the German Federal Ministry of Education and Research ("BMBF") with the goal to validate the 3D Skin Comet assay. Whereas the BMBF project worked on a number of full thickness (FT) skin models the CE project had originally focused on an epidermal tissue. When the team compared three models, Phenion® and EpiDerm™ FT were selected to enter the validation phase, based on statistical evaluation of results from the method transferability phase, and taking into account inter- and intra-laboratory variability and the number of valid experiments. The validation will include 30 chemicals, of which 8 are already tested. The initial testing phase, which is presented here, focused on inter- and intra-laboratory reproducibility and is demonstrating high predictive capacity for the 8 chemicals tested. Additional efforts have been made to establish and validate the most suitable measurement of cytotoxicity in the FT models.

As the Comet assay detects a broad spectrum of DNA damage that may give evidence for mutations the final aim of the validation study is to provide a new approach to be used as a follow-up for positive results from the current *in vitro* genotoxicity test battery.

I-4-381

Development of an immune-competent model of human upper airway epithelium

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Respiratory diseases remain a major cause of morbidity highlighting the need for better understanding of their pathophysiology to enable the development of novel therapies. To address such need scientists often rely on animal models however the poor physiological relevance of these models to human is widely acknowledged. This highlights the requirement for biomimetic models of human airway that can be used as platforms for disease modelling and testing drugs. Lung epithelial cells are the first line of defence against airborne pathogens and allergens. Here we hypothesised that co-culturing lung epithelial cells, fibroblasts and dendritic cells in 3D and under physiological conditions could allow simulating *in vivo* conditions and facilitate better understanding of the airway epithelium homeostasis.

Using this 3D co-culture we studied immune-modulatory properties of epithelial cells in response to bacterial extract. Our data shows differential regulation of a key immune regulatory enzyme namely indoleamine 2,3- dioxygenase in epithelial cells cultured at air-liquid interface (ALI) compared to submerged cultures. These differences are likely due to changes in TLR-4 expression and formation of a functional barrier in ALI. We suggest such 3D co-culture can provide a physiologically relevant tool for investigating the pathophysiology of different inflammatory conditions in human airway.

I-4-392

Effect of ATP suppliers on viability of cryopreserved cells

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There has been a large amount of developmental work undertaken to ensure successful cryopreservation and resuscitation of a wide variety of cell lines of different cell types. Quality of the cells before cryopreservation is important for viability and function after freezing. We hypothesized the utilization of ATP suppliers would improve overall cell viability through a modulation of the cellular biochemical response to the cryopreservation process.

In this study, different cells were incubated with the different concentration of fructose, glucose, dihydroxyacetone and glyceraldehyde as ATP supplier, at incubation time 3 h. The preincubated cells cryopreserved for 1 month. Cell viability was determined immediately postthaw by Trypan Blue exclusion.

Fructose preincubation improved the viability of hepatocytes at a concentration 250-500 mM. Preincubation with dihyroxyaceton (50, 100 and 200 μM) prior to cryopreservation had beneficial effects on viability cells and function of hepatocytes. The postthaw viability of cells preincubated with fructose was uniformly poor.

We conclude that the intracellular ATP level is an important determinant in the deleterious effects of cryopreservation procedure.

I-4-397

Global proteomic analysis of acetaminophen toxicity in 3D human liver microtissues

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In this study, a novel mass-spectrometric approach – Hyper Reaction Monitoring (HRM) – was used to quantify changes in protein expression in 3D human liver microtissues (huLi-MT's) exposed to acetaminophen (APAP).

HuLi-MT's were exposed to 8 concentrations of APAP (4 μM - 10,000 μM) over 72 hours. Microtissues were harvested and subjected to mass spectrometric analysis.

Using a huLi-MT library of 1900 proteins, the expression of several phase I enzymes (CYPs 1A2, 2B6, 2C1, 2C8 and 2E1) were altered following drug treatment. Interestingly, exposure to a subtoxic (4 μM) concentration of APAP induced a 4 fold up-regulation of CYP1A2 protein expression, which supports the role of CYP1A2 in the bioactivation of APAP to its reactive metabolite *in vivo*. Cluster analysis revealed the up-regulation of membrane proteins, glycoproteins and fatty acid metabolism-related proteins. Down-regulated proteins included those involved in eicosanoid metabolism, suggesting interaction of APAP with its pharmacological target.

Taken all together, the results suggest that use of HRM for proteomic studies in 3D human liver microtissues is a powerful tool to detect early changes in the proteome and may allow for the better understanding of the mechanism of drug-induced toxicity and the identification of relevant early biomarkers of toxicity.

I-4-418

Biological assay device of neurologic cells with in situ observable gel culture system composed of ECM-modelized matrix

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In recent years, neurologic cell culture is performed as alternative of animal experiments which evaluate influences on central nervous system (CNS) of various chemical agents. Culture system which mimics CNS is desirable for the evaluation, but neurologic cells are generally cultured in non-physiological monolayer condition (Lai et al., 2012). Therefore, we developed heparin-crosslinked collagen gel which was inspired by ECM composition of CNS (Watanabe et al., 1995; Inatani et al., 2003). Furthermore, we aimed to construct a novel 3D culture system which enables *in situ* observation.

Heparin was chemically cross-linked to collagen, and ECM-modelized matrix was developed. Neural stem cells (NSCs) were embedded into ECM-modelized matrix gel and cultured with bFGF. Furthermore, NSCs were embedded into the thin-layered gel of 0.1 mm in thickness. Immunohistochemical analysis was performed.

Embedded NSCs in ECM-modelized matrix showed greater proliferation and neurite outgrowth than those in collagen gel. In addition, NSCs in ECM-modelized matrix gel are more sensitive to anesthetic than those in monolayer. These results indicated that ECM-modelized matrix made *in vivo*-like culture environment. Thin-layered gel makes much easier to observe embedded cells than conventional gel culture of 2 mm in thickness. These results suggest constructing bioassay device with *in situ* observable 3D culture system composed of ECM-modelized matrix.

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I-4-456

The evaluation of spheroid culture of human hepatocytes for drug induced hepatotoxicity

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Drug induced hepatotoxicity is the most frequent reason for discontinuing development of drug candidates and post-market drug withdrawals. It is difficult to predict human hepatotoxic potentials in preclinical safety studies because of the species difference. Primary human hepatocytes are considered the main choice to evaluate hepatotoxicity; however hepatic functions, especially cytochrome P450 activities, decrease under conventional monolayer culture conditions. Recently, three-dimensional cultured hepatocytes (hepatocyte spheroids) have been a focus of attention to the ability that enabled a long-term evaluation.

In this study, we investigated human hepatocyte spheroids to assess the feasibility in a drug induced hepatotoxicity assay. The spheroidal formation and hepatic functions (e.g., activities of drug-metabolizing enzymes and albumin secretion) were maintained for several weeks. Under this culture condition, the spheroid was exposed with well-known hepatotoxic drugs, resulted in a concentration-dependent elevation of AST activity and depression of albumin secretion by the long-term exposure of several drugs. Considering activities of drug-metabolizing enzymes were sustained during culture period, certain metabolites would involve in the toxicity.

In conclusion, we established an *in vitro* system to evaluate human hepatotoxicity by using spheroid culture method. This approach will be useful for preclinical safety assessment in the early stage.

I-4-469 *

In vitro 3D scaffold-free osteoarthritis model – multiple application options

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Osteoarthritis (OA), a leading cause for disabilities and financial burden on society, is characterized by a complex interplay of inflammatory processes and cartilage degradation. To study underlying mechanism and new therapeutic approaches, small animal models are widely used whereas the applicability to the human is questionable. However, to our knowledge the existing 3D cell model are only restricted able to unify the complex pathogenesis and inconvenient to handle (e.g., bioreactors). Based on the scaffold-free 3D cartilage transplant (SFCT) technology (fzmb GmbH), we generated an *in vitro* OA model that consists exclusively of chondrocytes and their metabolic products. The SFCTs achieves diameters up to 1.5 cm and thickness between 1-3 mm and are treated with arthritic conditions. Moreover, the advantage of this model is the extended durability and the possibility to produce constructs in parallel from one human donor facilitating reproducibility. In addition, it can be used in pharmacological screenings, *in situ* modeling, biomaterial development and transplant testing. Here, we will present first results of our study showing significant differences in histological, biochemical, molecular biological and biomechanical analysis between the arthritic (SFCT+IL-1β/TNFα) and normal (only SFCT) models, indicating our model as advantageous and promising alternative to existing small animal models.

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I-4-550

Human cell-based functional 3D-angiogenesis test for identification of inhibitors of angiogenesis

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Angiogenic response of 36 selected test chemicals were studied by using human *in vitro* angiogenesis test. The chemicals included 14 drugs, 8 additives used in food or personal care products and 14 environmental chemicals.

The test method consisted of a co-culture of human fibroblasts and endothelial cells in the culture medium with specific exogenous growth factors to induce formation of tubular structures and tubular networks. During the test, the co-culture was exposed to the test chemicals at a concentration range of 10 pM to 10 mM (or limited by solubility). After 24 h, neutral red uptake assay was performed to obtain IC_{20} cytotoxicity value. The concentrations producing 80% or more viability were selected for angiogenesis assay. The extent of tubule formation was quantified microscopically after 6 days in culture.

The inhibitory concentration on tubule formation (EC_{50}) varied broadly among chemicals studied ranging from 0.6 μM (Cladribine) to 1788 μM (Diethanolamine) showing the potency of the angiogenesis test for chemical rankings. 10 chemicals did not show any an-

giogenic response. This human cell based 3D-angiogenesis test has a great potential to detect antiangiogenic substances including drugs and environmental toxicants, and it may serve as an alternative to animal experiments.

I-4-722

Evaluation of human hepatocytes cultured by three-dimensional spheroid systems for drug metabolism

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Drug-induced hepatotoxicity is the major reason for discontinuing clinical trials. To evaluate hepatotoxicity, *in vitro* studies using a primary culture of human hepatocytes and human hepatic microsomes, and *in vivo* studies using animals have been performed. However, these assays have several problems such as the involvement of reaction intermediates in the development of toxicity due to species differences in drug metabolic enzymes. In this assay we evaluated whether the three-dimensional spheroid culture of human hepatocytes reflects human metabolic profiles (Ohkura et al., 2014). Sequential metabolic reactions by phase I and then phase II enzymes were found in diclofenac (CYP2C9 and UDP-glucuronyltransferases (UGTs)), midazolam (CYP3A4 and UGTs) and propranolol (CYP1A2/2D6 and UGTs). Moreover, lamotrigine and salbutamol were metabolized to lamotrigine-N-glucuronide and salbutamol 4-O-sulfate, respectively. These metabolites, which are human specific, could be observed in clinical studies, but not in conventional hepatic culture systems as seen in previous reports. In addition, mRNA of drug-metabolism enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, UGT1A1, UGT2B7, sulfotransferase 1A1 and glutathione S-transferase pi 1 which were measured by qRT-PCR, were expressed in the human hepatocyte spheroids. In conclusion, these results suggest that human hepatocyte spheroids are useful in hepatotoxic assays to estimate drug metabolism pathways.

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I-4-738

In vitro human alveolar tissue model for pulmonary drug delivery and toxicology applications

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Reliable *in vitro* human models are needed to assess airborne environmental agents or inhaled therapeutics. To address this need we developed an *in vitro* air-blood barrier model from primary human alveolar epithelial cells, pulmonary endothelial cells and macrophages. Endothelial cells and alveolar epithelial (ATII) cells were isolated from human lungs. The alveolar model was constructed by seeding endothelial cells on the underside of a microporous membrane and ATII cells onto the top membrane surface. Co-culture continued at the air-liquid interface (ALI) until barrier development occurred. Finally, human monocyte-derived macrophages were seeded onto the apical surface. Confocal imaging of differentiated cultures demonstrated staining for cytokeratins 7 and 19, and carboxypeptidase M (ATI cell markers), tight junction proteins ZO-1 and occludin. The endothelial cells stained positive for von Willebrand factor and e-cadherin. Macrophages were visualized with Celltracker dye. The model produced a robust barrier demonstrated by maintenance of TEER >400 Ω*cm^2 for up to 30 days. Drug transporter gene expression including ABC family efflux transporters BCRP, MRP1 and MRP2, and organic cation uptake transporters OCTN1, OCTN2 and OCT3 was demonstrated by RT-PCR. This new *in vitro* human alveolar model shows promise for *in vitro* pulmonary toxicology and inhaled drug delivery investigations.

I-4-776

Importance of reproducibility demonstration of the bio-engineered tissue models used for *in vitro* toxicity testing purposes

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Advances in tissue engineering enable scientists to closely mimic many tissues of the human body. However, for scientific as well as regulatory purposes, it is crucial that the reconstructed tissue models are reproducible not only within a given lot, but also between the lots produced over an extended period and at different production sites.

To demonstrate reproducibility of the epidermis tissues manufactured according to GMP protocols at 2 different production sites (Bratislava, Slovakia and Ashland, MA, USA), MatTek scientists undertook side-by-side evaluation of the barrier properties and tissue morphology of the EpiDerm model. Tissues were exposed to control chemical (Triton X-100,1%),and using the MTT assay, dose response curves were constructed, and an exposure time which reduces the tissue viability to 50% (ET-50) was interpolated.

The EpiDerm kits manufactured in the USA within a 4 month period averaged ET-50=5.91h, SD=0.8 and Exp.CV=14%. EpiDerm produced during the same period in EU provided highly comparable ET-50=6.1h, SD=0.7, Exp. CV=11.7%. Using light microscopy, his-

tological cross-sections showed stratified epidermis-like morphology that was reproducible both within and between lots. These data also fall into the historical ranges established by MatTek Corporation in 1996.

This study demonstrates, that following GMP-production rules, it is possible to produce reconstructed tissues of high quality and reproducibility, as required by regulatory guidelines (e.g., OECD TG 431 and 439).

I-4-778

Reconstructed 3-D human small intestinal tissue to assess drug safety, inflammation, and restitution

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Development of normal human cell based small intestinal (SMI) tissue models that allow integrated approaches to study drug safety, inflammation, and restitution are needed. The validity of Caco-2 cell based studies are in question due to lack of physiological relevance. This poster evaluates the functionality of SMI tissue models reconstructed from normal human primary SMI epithelial cells and fibroblasts. Reconstructed SMI tissues were characterized morphologically, molecularly, and functionally. Inflammatory responses were examined by TNF-α plus IFN-γ exposure. Wound closure and re-epithelialization of the epithelium was monitored following injury of the tissue. Analysis of the SMI tissue revealed: 1) columnar epithelial cells, 2) a physiological TEER value of 100-180 Ω*cm^2, 3) expression of epithelial markers, efflux transporters, and brush borders. Studies using 2 P-gp substrates, ranitidine and talinolol, demonstrated active transport while warfarin did not. Treatment of the SMI tissue with TNF-α plus IFN-γ induced an increase in proinflammatory cytokines/chemokines (IL-6, IL-8 and GRO-α). Confocal and H&E staining of injured tissues showed cooperation of epithelial cells and fibroblasts in wound healing process. This SMI tissue will have pre-clinical applications and will reduce the use of animals for experimentation.

I-4-803

LUHMES 3D neuronal model for (developmental) neurotoxicity testing

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Broader testing of substances is crucial to respond to surging neurodevelopmental problems in children, including autism and ADHD. Therefore, our principal goal is to develop a human relevant, quality-assured, medium- to high-throughput testing strategy to prioritize chemicals for (developmental) neurotoxicity testing. This work aims to develop a 3D brain model to identify changes in transcriptome, microRNA expression, and metabolome after exposure to neurotoxicants. We developed such model based on well characterized LUHMES dopaminergic neuroprogenitor cell line which can be used to test compounds for neuro(developmental) toxicity effects and model chemically induced neuronal disorders (Schildknecht et al., 2013; Scholz et al., 2011). The size of differentiating spheroids, penetration rate and cell death were monitored up to day 21 of differentiation, to ensure delivery of medium supplements to the center of the spheroids. The efficiency of differentiation in 3D was characterized by qRT-PCR, flow cytometry and immunocytochemistry. To demonstrate the model[1]s suitability for neurotoxicity testing, the aggregates were treated for 24 and 48 hours on day 6 of differentiation with dopaminergic neurotoxicants, MPP+ and rotenone. Perturbations in energy metabolism and stress response were analyzed by gene expression and miRNA profiling. In conclusion, we have successfully developed a new 3D human model that can be used for (developmental) neurotoxicity testing.

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I-4-847

Using human organotypic tissue slices in cancer research

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Cancer is one of the major causes of death worldwide. Research to improve therapy is mostly done with xenograft models by injecting (human) tumor cell lines, e.g., into the flanks of mice or rats. This means a great burden for the animals, especially when the endpoints of the experiments are represented by Kaplan-Meier-curves which depict the time point where half of the animals in the study died. It also involves problems like the lack of cellular heterogeneity or inter-species differences. Therefore, only few animal studies can be successfully translated into a clinical setting for humans.

We have previously established a human test system consisting of 3D-tissue slice cultures of tumor tissue from surgeries which can be kept in culture for several weeks and used for radio- and chemotherapeutic experiments. So far, this system is used for glioblastoma, squamous cell carcinoma and gastric cancer tissue from resections as well as fat from plastic surgery. In these settings, we can monitor response to known therapies, test new compounds, analyse cell proliferation or death, or track behavior of special cell types via live imaging over time. With our model system, animal testing could be reduced and species differences are eliminated.

For further reading see Merz et al., 2013 and Gerlach et al., 2014.

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I-4-858

The importance of normalization and standardization of cytotoxicity assays for 3D cell models

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Introduction: Cytotoxicity is often the first endpoint measured *in vitro* to estimate non-cytotoxic or sub-cytotoxic concentrations that can be further studied for more specific and complex endpoints. Lately it has been recognized that 3D cell models with increased cell-cell interactions are essential to mimic the *in vivo* situation. However, cytotoxicity assays for 3D models require standardized protocols, often optimized from traditional monolayer cultures.
Aim: Evaluate cytotoxicity assays for a 3D neuronal cell model.
Method: The dopaminergic cell line LUHMES, were cultured in neuronal differentiation media under constant gyratory shaking, for 12 days to form 3D aggregates. Size measurements of each aggregate and cytotoxicity assays (DNA quantification, Lactate dehydrogenase, Resazurin, Neutral Red Uptake (NRU) and cell number quantification) were performed in 384-well plates.
Results: Resazurin and NRU showed the best correlations with aggregate diameter (0.8634 and 0.7334, respectively). In addition, the estimation of number of cells showed a good correlation (0.7869) in the exponential growth curve by aggregate's volume. DNA quantification assays were not considered optimal for 3D models.
Conclusion: Cytotoxicity assays for 3D models need to be carefully selected and standardized. The establishment of these models can contribute to reduce costs, time and animal use for toxicity testing of chemicals.

I-4-859

BIOMEMS for accurate *in vivo* – *in vitro* correlation

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In the last five years we successfully validated the use of ultrathin ceramic membranes issued of state of the art in micro fabrication as support for cell growth (Halamoda et al., 2013). The 500 nm thin membranes have by design excellent transport properties. Consequently we focused our effort in their integration into systems conceived for translocation assays, novel drug carrier absorption and particulate exposure models. We present here a family of devices ranging from an insert like system, compatible with commercial multiwell plate (SIMPLI-well, Patent EP, 2011), to dynamic automated bioreactors, all suitable for 3D models of biological barriers. Their relevance is improved by the reduced interference of the mechanical support. System health can be continuously and non-invasively monitored by reliable integrated micro electrodes for TEER measurement. Metabolic reactions can be spectrometrically observed by inducing plasmonics to the same micro porous membrane and making it highly selective in the MIR range. User-friendliness inspired our engineering: if SIMPLI-wells are compatible with routine practises, the most complex version of these bioreactors goes towards automated standalone machine with bio-systems compatible with a "cartridge"-like approach requiring no specialised operators for standard toxicology test and environmental monitoring.

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I-4-865

Development of a new reconstituted human oral mucosal model to assess the oral irritation testing

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In this study we developed a novel three-dimensional human oral mucosal model (HOM model) based on two different cell sources. One is immortalized human oral keratinocyte cell line (HOM-IHOK model) and the other is human normal buccal keratinocytes (HOM-NBK model).

Immunohistochemistry and barrier function testing were employed to characterize these newly developed model system.

For further reading see Chai et al., 2010; Klausner et al., 2007; Liu et al., 2010; Moharamzadeh et al., 2008; Moharamzadeh et al., 2012.

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Session I-5: Bioreactors

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Session I-5: Oral presentations

I-5-210

Optical cell separation by photodegradable hydrogels for three dimensional cultures

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Cell separation techniques contribute to analyze cell profiling such as genome information. Several studies reported that cells express their native phenotypes in 3D culture compared in two-dimensional culture. Thus, we require the tool for 3D culture which can separate target cells, especially animal testing alternative. Herein, we proposes the method of optical cell separation by photodegradable hydrogels in 3D environment. In previous study, we synthesized photocleavable cross-linker NHS-PC-4armPEG, and it applied to form photodegradable hydrogels which composed of NHS-PC-4armPEG and gelatin. The hydrogels allowed cells to attach on the surface and to growth. For developing further biomimetic condition, we encapsulated cells into the photodegradable hydrogels through optimization of compositions. Cells could survive in the hydrogels for 96 h. In photo degradation, we irradiated the target area using a computer-controlled light irradiation system, which can irradiate according to the designed micropatterned images or arbitrary area under the microscope. Both of the irradiation system and photodegradable gels successfully separated cells in the hydrogels. Furthermore, the separated cells could growth on a culture dish. The minimum resolution was estimated at 20 mm, which should separate each cells. This method expected to become a novel strategy in animal testing alternative.

I-5-238

A three dimensional (3D) perfusion bioreactor-based model of colorectal cancer for chemotherapeutic assessment

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In this study we addressed the suitability of a perfusion bioreactor to sustain 3D colorectal cancer cell growth and to test established treatment regimens as compared to 2D cell culture or xenografts. At variance to 2D, 3D-perfused constructs were characterized by heterogeneous tissue-like structures of proliferating and apoptotic cells and expression of typical tumour marker, similarly to xenografts. Treatment with clinically relevant concentrations of 5-FU had no effect on cells number cultured in 3D-perfusion or in xenografts constructs, in contrast to 2D cultures. In perfused cultures only a marginal effect on the expression of BCL-2 apoptosis-resistance gene was observed, while significant down-regulated in 2D. The combination of ABT-199 (Souers, 2013) and 5-FU induced additional cytostatic and cytotoxic effects in 3D-perfusion but not in 2D cell cultures. Interestingly, 3D-perfusion partially showed similar responsiveness to 5FU and BCL-2 expression in colorectal tumors in patients undergoing neo-adjuvant treatment. Our data consistently indicate that 3D perfused cultures efficiently mimic phenotypic and functional features observed in animal models and clinical specimens. These *in vitro* models may have the potential to reduce animal use for testing drugs with critical translational relevance for diagnostic purposes as well as to address fundamental issues in human tumor cell biology.

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I-5-333

Microfluidic perfusion culture system for culturing human induced pluripotent stem cells under fully defined culture conditions

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Human induced pluripotent stem cells (hiPSCs) are promising cell sources for drug discovery and are expected to reduce the number of animal experiments. For drug screening applications using hiPSC derived cells, it is important to use a culture system that accurately controls the proliferation and differentiation of hiPSCs. However, two problems can lead to inaccuracies in the conventional drug screening system: 1) instability of culture conditions caused by using conventional multi-well culture plates, in which the medium is replaced daily, and 2) masking and distorting of screening results caused by using undefined culture medium and extracellular matrix.

Here, we developed a pressure-driven microfluidic perfusion culture system for controlling hiPSC states under defined extracellular matrix and culture medium conditions. The growth rate of hiPSCs under perfusion culture conditions was higher than that under static culture conditions in our microfluidic system. Immunocytochemical analysis showed that the self-renewal and differentiation of the hiP-

SCs was successfully controlled. The effects of three anti-tumor drugs on hiPSCs using our microfluidic system were the same as that using a 96-well plate.

Our system is suitable for high-throughput drug screening systems using hiPSCs with the added benefit of eliminating the inaccuracies caused by unstable and undefined culture conditions.

I-5-407

Autonomous bioreactor modules for disease models and detection of systemic toxicity

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Advanced systems based on bioreactors and scaffolds are an essential step towards the development of more predictive and ethical alternatives to animal experiments. Size, modularity, automation, monitoring and essential design are crucial because these elements will ease the transition from old technology and accelerate their acceptance into mainstream research. Based on these requirements, the interconnected transparent sensorised "lego" bioreactors designed in our labs have been used to generate physiologically relevant disease and toxicity models which recapitulate systemic responses impossible to observe in standard cell cultures.

The disease model is an interconnected bioreactor circuit with i) adipose tissue in 3D in 3 different concentrations representing normo-weight, over weight and obese body mass indices, ii) human hepatocytes on porous collagen scaffolds and iii) monolayers of human endothelial cells. High adiposity and elevated glucose levels induce systemic and endothelial inflammation in the circuit, as observed in overweight and diabetic humans (Iori et al., 2012). Using similar technology a three-tissue circuit for monitoring the absorption, distribution, metabolism and toxicity of nanoparticles was developed in the context of the EU project InLiveTox (Ucciferri et al., 2014). The results were strikingly similar to those observed in animal experiments demonstrating that the dynamic 3D *in-vitro* models are ethical, meaningful and economically viable replacements.

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I-5-419

Cell function induction using perfusion culture

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One of major elements causing inadequate organ/tissue-specific functions of cultivated cell is that the physicochemical environment for the cell culture is totally different from that *in vivo*. We have looked at the perfusion of culture medium, which is intimately related to nutrition supply and waste removal, and hydrodynamic stimuli. We are developing novel microchips where cell culture environments are precisely controlled.

In this talk, I will present the advantages of the perfusion culture taking our works for instance. It has been well-known that the spheroids formed with HepG2 express higher hepatic function. The spheroids can be uniformly formed under perfusion culture. The growth rate of hiPSC under profusion culture is higher than that under static culture. The vascular endothelial function can be inducted using HUVEC under the perfusion culture at a shear stress larger than 10 dyne per square centimeter. The former two results can be considered in relation to the nutrition supply and waste removal, and the last one is due to the effect of shear stress.

I-5-573

3D multi-compartment bioreactor technology for *in vitro* pharmacological studies as an alternative to animal testing

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The relevance of animal models in the prediction of human drug effects is limited by species-dependant differences in hepatic metabolism and susceptibility to toxic effects of xenobiotics. Human *in vitro* liver cell models could provide a suitable alternative to *in vivo* experiments by exhibiting human-relevant functions.

Our approach is based on a dynamic four-compartment culture technology that approximates the natural environment of the cells in the organ. Cells are cultivated within a three-dimensional network of hollow-fiber capillaries that serve for decentralized medium supply and oxygenation. Down-scaled laboratory versions of the technology allow reducing the needed amount of cells and reagents for *in vitro* research (Zeilinger et al., 2011; Hoffmann et al., 2012; Lübberstedt et al., 2012).

Primary human liver cells were cultivated in bioreactors under serum-free conditions. The toxicity of diclofenac used as a model drug was investigated in the bioreactor system using two concentrations of the substance (0.3 mM, 1 mM). The results show a dose-dependent toxic effect of diclofenac application, as determined by analysis of enzyme release, glucose metabolism and urea production.

In conclusion, the bioreactor technology allows stable cell cultivation for *in vitro* studies on drug metabolism and toxicity. Thus, the device provides a promising *in vitro* model for pharmacological studies on human hepatocytes.

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Session I-5: Poster presentations

I-5-271

Rapid fabrication of engineered liver tissue using novel fibroblast system

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Reconstructed liver *in vitro* and *in vivo* from isolated hepatocytes have a great deal of potential in drug screening. However, fabrication of vascularized liver tissue was difficult because it has to be controlled configurations and types of cells. In this study, we challenged establish of novel fibroblast system for controlling of extracellular microenvironment using cell sheet technology and fabrication of subcutaneous human liver tissue in mouse.

Human hepatocytes were inoculated onto human dermal fibroblast monolayer on a temperature-responsive culture dish (UpCell; CellSeed Inc.). Hepatocytes adhered onto fibroblast monolayer within at least 2 hours of culture and harvested engineered hepatocyte/fibroblast sheet (EHFS). Vascularized-human liver was fabricated under the skin of mouse by transplantation of EHFS and showing significantly higher synthesis activities of liver-specific proteins *in vivo* than transplanted hepatoyte-only sheets. These results will be caused by high-syntheses of angiogenic factors from EHFS.

In this study, we established rapid fabrications of EHFS and vascularized human liver tissue under the mouse skin without prior induction of angiogenesis. This human liver model could be used for drug screening with easy *in vivo* observation and collecting of subcutaneous liver tissue.

I-5-298

Human fibroblast remodelling when cultured in 3D and under flow

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3D cultures of different tissues have been shown to be physiologically relevant; however under static conditions distribution of nutrients and removal of waste in 3D is not efficient. Furthermore, most cells *in vivo* are exposed to forces due to flow which influence their function. There is therefore considerable interest in the development of perfusion systems that enable the maintenance of 3D cultures and provide more physiologically relevant culture conditions. In this study we examined wound healing in 3D cultures of human lung fibroblasts that were cultured in a Quasi-Vivo® perfusion bioreactor and made comparison with static cultures of the same cells. For wound healing studies cells were exposed to NaOH and the healing process was monitored via cell proliferation and extra cellular matrix (ECM) production for 7 days. Our data clearly show 3D cultures under flow conditions proliferate at a significantly higher rate, produce and deposit higher levels of ECM proteins and show faster recovery after injury. Our data also suggest these events are likely to be driven by higher production of fibroblast growth factor and IL-6 under flow conditions. This perfusion culture system could provide a useful platform for studying fibroblast remodelling, a key event in many lung pathologies.

I-5-336

Engineering hepatic tissues with perfusable vascular structures

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The ability to reconstruct biomimetic tissues *in vitro* may be beneficial for the alternative to animal testing and regenerative medicine. One of the most important issues in the fabrication of tissues is the luck of approach for engineering vasculatures for delivering oxygen and nutrients throughout engineered constructs. This study proposes an approach to fabricate endothelial cell-lined microchannel using electrochemical cell transfer and induce subsequent self-organization of cells.

Oligopeptide was designed to spontaneously adsorb and form a dense-molecular layer on a gold surface and to be desorbed from the surface electrochemically. Cells adhering on a gold surface via the oligopeptide layer were detached within 5 minutes electrochemically. We applied this approach to cylindrical gold rods; Human umbilical vein endothelial cells (HUVEC) were transferred from rods to the internal surface of microchannels (ø500 mm) in a hydrogel, resulting in the formation of HUVEC-lined perfusable vasculature. These vasculatures were maintained vascular functions such as vascular endothelial barrier. In the following perfusion culture, HUVECs spontaneously sprouted into a hydrogel. Furthermore, when we encapsulated hepatic cells in a hydrogel, liver-specific functions, such as albumin secretion and ammonia removal, increased overtime. This vascularized hepatic tissue fabrication approach may provide more biomimetic *in vitro* testing for drug development.

I-5-420

Protection from cell death in multicellular spheroids

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Forming multicellular spheroids is attractive for cell-based assay because various functions are enhanced. However, the center part of the

spheroid is sometimes necrotic because of the limitation of oxygen/nutrients. In addition, epithelial cells tend to form "multicellular cyst" by induction of apoptosis of inner cells. Cell death by apoptosis as well as necrosis reduces the functions of spheroids. We previously reported that embedding of alginate hydrogel beads into the spheroids enhanced the supply of oxygen/nutrients (Kojima et al., 2014). Here, we show the method is also useful to prevent multicellular cyst formation. We used E14.5 mouse fetal hepatocytes as multicellular cyst forming cells. These cells and hydrogel beads with a diameter of 20 μm were aggregated in the 3% methylcellulose medium as shown previously (Kojima et al., 2012). Spheroids were sectioned and visualized with hematoxylin-eosin and immunohistochemistry. We found that fetal hepatocytes have a tendency to form multicellular cyst. When we embedded the beads, however, living cells were found inside of the spheroids. We are now trying to detect the difference of albumin secretion. This technique helps to maintain the living cells in multicellular spheroids and to establish highly functionalized multicellular spheroids.

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I-5-427

Development of a perfusion chamber for the cultivation of 3D skin constructs

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Reconstructed skin models, as an alternative to animal testing, show higher permeability and reduced mechanical resistance due to a less efficient skin barrier in comparison to human skin *in vivo* (Schäfer-Korting et al., 2008; Küchler et al., 2013). To overcome these obstacles, we constructed a perfusion chamber aiming to ensure a constant nutrient supply and to provide shear forces which normally occur *in vivo*. In the perfusion chamber the reconstructed skin is grown at an air-liquid interface with a constant, laminar flow below the dermis equivalent. Histological analysis of first skin models grown in the perfusion chamber revealed a significantly thicker stratum corneum in comparison to the skin models grown in the classic transwell setup. In order to determine the influence of the perfusion chamber on the skin lipid order of the skin models, Fourier transform infrared spectroscopy was performed. The constructs grown in the perfusion chamber exhibits more ordered lipids chains in comparison to the control. Furthermore, skin models grown in the perfusion chamber showed reduced skin permeability for the OECD reference substance testosterone compared to control models. In conclusion, cultivation of reconstructed skin in perfusion chambers is a promising method in order to improve the quality and skin barrier function of reconstructed skin.

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I-5-498

2D and 3D human liver cell bioreactors for long-term repeated-dose toxicity testing as a basis for computer-aided prediction

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2D and 3D cultures of the human hepato-carcinoma cell line HepaRG were established in EU-funded project NOTOX to allow system wide study of long-term repeated-dose toxicity effects. Using well-designed media long-term cultivation over 4 weeks is possible, both in 2D microtiter plate and 3D spheroid cultures with minimal loss of viability and hepato-typic enzyme activities. Solid spheroids are formed within a few days developing bile canalicular structures, clearly visible in electron microscopy. *In vivo* MRP2 activity assays show active secretion into bile canalicular structures. Long-term toxicity tests revealed clear differences between 2D and 3D cultures for test compounds as bosentan, valproic acid and chlorpromazine. Cultures are characterized using various omics methods, e.g., metabolomics, fluxomics, transcriptomics, proteomics, as well as by different imaging methods. Data are used for modelling pathways and microtissue, i.e., spheroid, geometry. The goal is, to eventually support safety assessment by directed *in vitro* experiments combined with systems oriented models suited for toxicity prediction.

I-5-706

The need of innovative technologies for new 3D relevant *in-vitro* models and the answer of Ivtech

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New relevant *in-vitro* models are priorities in pharmaco-toxicology, cosmetic and food research to reduce the animal tests. Therefore, in-vivo models show ethical issues, are not time and cost effective and are progressively showing scientific limitations: for instance they fail in detection of pathogens that are species specific (Mazzoleni et al., 2009). The search of more relevant pre-clinical models forced the researcher to move from 2D to 3D *in-vitro* models in order to maintain the phenotype of cells (Lovit et al., 2013; Mattei et al., 2014). Even if the significant progress in material science, the metabolic requirement of 3D tissues is higher than a 2D culture and the scaffold is a limitation in nutrients transport. Dynamic cell culture chambers are then required to assure the gas/nutrient supply, waste elimination, mechanical stimulation of cells, study of cross talk between different tissues and real time monitoring of cells. Nowadays the only systems that meet all these specifications are the Ivtech technologies. Ivtech is an innovative Italian start-up that grows up to solve the needs of *in-vitro*

experts, offering and customizing several type of transparent, dynamic and modular cell culture systems, organizing workshops and training. The goal is to expand the 3D approach and permits a significant evolution towards highly relevant *in-vitro* models.

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I-5-747

Improving *in vitro* tests of orthopedic implant materials

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New orthopedic implants must be extensively tested – for safety and for mechanical stability after implantation – before market entry. Current *in vitro* analyses of implant materials are used essentially to screen for acute toxicity and biocompatibility. Improved *in vitro* tests with good predictive value for osseointegration are needed if the number of *in vivo* tests in this field is to be reduced.

We are developing 3D *in vitro* testing methods for tissue scaffolds for bone replacement. A small bioreactor has been developed that allows the scaffolds to be tested for long periods of time (weeks) under physiologically-relevant dynamic mechanical loads. The bioreactor is placed inside a standard incubator, which ensures suitable conditions for cell culture. Cell culture medium is passed through the bioreactor to ensure oxygenation of the sample over long incubation times.

First results show good growth of cells – SaOs-2 and mesenchymal stem cells – on scaffolds in the bioreactor. They also highlighted difficulties in monitoring cell growth and imaging cells within the scaffold. The next steps will focus on developing techniques for cell seeding and monitoring cell growth in the scaffold in order to optimize culture conditions.

I-5-780

Bioengineering approaches for the development of novel 3D *in vitro* models for pre-clinical applications

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The need for physiologically relevant cellular systems, which provide insight into the mechanisms and pathways by which chemicals exert their effects on cells and organisms is particularly perceived for preclinical phases of drug development, namely for drug screening, target validation and toxicology.

Our work has focused on the development of human 3D cell models, making use of stirred-tank bioreactor culture systems for expansion, differentiation and maturation of clinically relevant cells. Herein, we will present results concerning the establishment and refinement of cultures systems for (1) the differentiation of human neural stem cells in functional and mature neurospheres enriched in neural cell subtypes; (2) differentiation of pluripotent stem cells towards highly pure and functional cardiomyocytes; (3) Donor-derived hepatocytes and hepatic cell lines as source of functional hepatic models. The presented systems were phenotypically and functionally characterized/validated.

Engineering of 3D microenvironments yields *in vitro* models with improved physiological relevance and culture in computer-controlled bioreactors enables reproducibility and robustness, making these bioprocesses suitable to be applied in the pharmaceutical industry. Thus, the newly developed 3D *in vitro* models increase the collection of tools available for preclinical drug development, contributing to increase its efficiency and to accelerate drug development pipelines.

I-5-805

Use of dynamic 3D primary hepatocyte culture to understand the effects of constitutive androstane receptor activators in primary rat and human hepatocytes

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A variety of substances including pharmaceuticals, plasticizers and crop protection agents contain Constitutive Androstane Receptor (CAR) activators. Activation of CAR is known to cause hepatocellular proliferation in rodents, but their effect upon human hepatic cells is unclear. Using both traditional two dimensional static culture and a 3D dynamic bioreactor (LiverChip™) the effects of CAR activators upon gene expression and cell proliferation were examined in primary rat and human hepatocytes. Gene expression data indicate that dynamic 3D culture is more effective than traditional 2D static culture at maintaining rat hepatic phenotype *in vitro* up to 7 days, as evidenced by maintenance of expression of hepatocyte specific genes including HNF1a, HNF4a CAR and CYPs. Rat hepatocytes were shown to exhibit increased proliferation by MTT assay following treatment with phenobarbital for 24 hours whereas treatment of primary human hepatocytes with the selective human CAR activator CITCO had no effect on proliferation. Given the lack of proliferative effects of CAR activators in human cells, we have subsequently focussed upon whole transcriptomic and miRNA screens comparing the effects of CAR activators in rat and human hepatocytes.

I-5-814

Dose metric considerations of cationic and anionic surfactants in *in vitro* cytotoxicity assays

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In vitro concentration-effect relationships are used for ranking toxicants and extrapolating to *in vivo* toxic doses. *In vitro* concentration-effect relationships are traditionally based on nominal concentrations. However, the nominal concentration may not represent the concentration of the chemical causing toxicity in cells in *in vitro* cell-based assays. The extent to which a chemical partitions into cells will depend on its neutral fraction, volatility, stability and binding affinity to medium constituents, well plate plastic and cells. The aim of this study was to determine how the cytotoxicity ranking of primary, secondary, tertiary and quaternary amines as well as (perfluor)carboxylates and alkyl sulfates varying in carbon chain length depends on the dose metric used to construct concentration-effect relationships in a basal cytotoxicity assay using the rainbow trout gill cell line, RTgill-W1. Results indicate that charge shielding and lipophilicity determined the *in vitro* bioavailability as well as cytotoxicity of the test chemicals. Moreover, toxic potency rankings of the chemicals were dependent on the dose metric used and effect concentrations based on cell-associated concentrations were least dependent on *in vitro* setup.

Session I-6: High-content imaging

Co-chairs
William Mundy, EPA, USA
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Session I-6: Oral presentations

I-6-597

Use of High Content Imaging (HCI) to screen for developmental neurotoxicity

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Because the molecular events leading to developmental neurotoxicity are not well defined, methods are being developed to assess key neurodevelopmental processes such as cell proliferation, differentiation, neurite growth, and synaptogenesis. Many of these processes can be assessed at the cellular level using fluorescent markers (e.g., immunostaining or expression of fluorescent proteins) combined with microscopic imaging. With the advent of HCI these phenotypic changes can be measured in an unbiased, high throughput format. Neural cells show dramatic changes as they differentiate, and the formation and extent of the specialized process such as axons and dendrites can be quantified using image analysis algorithms which measure cell size and shape. Assessment of the multiple cell types that arise from neuroprogenitor cell differentiation including glia (astrocytes and oligodendrocytes) and numerous types of neurons (e.g., glutamatergic, GABAergic) can be delineated and quantified after immunostaining for phenotypic marker proteins. The ability to rapidly acquire images in multiple channels using selective excitation and emission wavelengths allows for simultaneous of visualization and quantification of important characteristics of neural cell models.

This abstract does not necessarily reflect U.S. EPA policy.

I-6-757

Intravital multiphoton tomography for non-invasive *in vivo* analysis of human skin

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Replacement is the first worthwhile strategy in basic and clinical science by circumvention of the detour via animal models if avoidable. Multiphoton tomography (MPT) of endogenous fluorophores is such a high-content imaging tool for non-invasive "optical biopsies", i.e. high resolution *in vivo* examination of human skin directly at patients' bedside (Koenig and Riemann, 2003). Several biomoleculeslike NADH, melanin, collagen or elastin, showing autofluorescence or second harmonic generation, can be visualised with a penetration depth of 150 μm. These molecules provide information about subcellular morphology, epidermal architecture and physiological conditions of the skin and can indicate changes in cell metabolism (Su et al., 2011). Additional parameters like fluorescence decay times (MPT-FLIM)or spectral shift of the emitted fluorescence could be used for objective diagnosis and a therapy follow-up in skin diseases during repetitive clinical visits.

Therefore, MPT-FLIM application offers the possibility to directly examine the individual etiopathology of skin diseases, primary *in vivo* tracking of applied therapeutic agents and an ad-hoc molecular analysis of intraepidermal and -dermal therapeutic response in a non-invasive manner. As a unique feature, this technique envisions new *in vivo* and *ex vivo* parameters that were already successfully used to detect and monitor pathophysiological skin alterations in patients and histological sections prior to clinical manifestation (Huck et al., 2011; Seidenari et al., 2012).[4]

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I-6-875

Using high-content imaging to analyze cell-state trajectories and biological tipping points for chemical exposures

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Translating results obtained from high-throughput screening to risk assessment is vital for reducing dependence on animal testing. We studied the effects of 976 chemicals (ToxCast Phase I and II) in HepG2 cells using high-content imaging (HCI) to measure dose- and time-dependent perturbations in p53, JNK, oxidative stress, cytoskeleton, mitochondria, and cell cycle. A novel computational model was developed to describe the dynamic response of the system as cell-state trajectories based on multidimensional HCI datastreams. Cell-state trajectories produced by 10 concentrations (0.4 to 200 μM) of 976 chemicals showed resilience of the HepG2 system in many cases, however, we also found "*tipping points*" in system recovery. Further analysis of trajectories identified dose-dependent transitions, or critical points, in system recovery for 340/976 chemicals. The critical concentration was generally 5-times lower than the concentration that produced 50% cell loss. We believe that HCI can be used to reconstruct cell state trajectories, and provide insight into adaptation and resilience for *in vitro* systems. With additional research, cellular tipping points could be used to define an *in vitro* point of departure (PoD) for risk-based prioritization of environmental chemicals.

This work does not reflect U.S. EPA policy.

I-6-886

High-Content Imaging as a tool for nonclinical drug safety investigations

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In pharmaceutical research and development drug safety is not only addressed in the regulatory testing battery in the preclinical and clinical phases by doing *in vivo* studies. More and more *in vitro* toxicity tests are used to support the selection of drug candidates with a better toxicity profile in early screening approaches or are used to investigate toxicity mechanisms of a drug candidate in development in parallel to the regulatory testing battery. For this, high content imaging (HCI) is a valuable basic tool in pharmaceutical toxicology. We use HCI in several projects for different purposes and with various cellular systems. To show the broad field of HCI application in our industry, the talk will present examples where and how we used HCI with respect to, e.g., phospholipidosis, respiratory epithelium toxicity and cardiotoxicity. The examples will show different level of complexity ranging from simple cell line monocultures and single endpoints to multicellular 3D models and multiple read outs.

I-6-892

Towards a high throughput microscopy pathway in toxicity reporter platform for chemical safety assessment

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Adaptive cellular stress responses are paramount in the healthy control of cell and tissue homeostasis after cell injury during hypoxia, oxidative stress or unanticipated side-effect of medications and other chemical exposures. To increase our understanding of chemically-induced adaptive stress response pathway activation and its contribution to safety assessment a time-resolved, sensitive and multiplex readout of chemical-induced toxicological relevant cellular stress responses is essential. For this we develop a platform containing a panel of distinct adaptive stress response fluorescent protein reporter cell lines. These are used for automated high content live cell imaging and quantitative multi-parameter image analysis to elucidate critical adaptive stress response pathway activation that can contribute to human chemical safety assessment. To conserve the endogenous gene regulatory programs, we tag selected reporter target genes with GFP using BAC-transgenomics approaches. Here we demonstrate the functionality of individual BAC-GFP pathway in toxicity reporter cell lines to their respective specific model compounds. The application of these reporters in chemical safety assessment in relation to drug-induced liver injury will be discussed. We anticipate that ultimately a phenotypic adaptive stress response profiling platform will allow a high throughput and time-resolved classification of chemical-induced stress responses assisting in the safety assessment of chemicals.

This work is part of the MIP-DILI project supported by the Innovative Medicines Initiative (grant agreement n° 115336), and the FP7 SEURAT-1 DETECTIVE project (grant agreement 266838).

Session I-7: Monitoring (telemetry)

Co-chairs
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Session I-7: Oral presentations

I-7-089

Telemetric monitoring in preclinical drug development

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During preclinical development of new chemical entities (NCE), different animal models are necessary to determine efficacy and safety prior to first administration to humans. In the past anesthetized or restrained animals were used for the measurement of parameters of vital organ functions like blood pressure, ECG, EEG or body temperature. The use of radio-telemetry techniques such as invasive implanted or non-invasive external telemetry allows the continuous monitoring of such parameters in awake, freely moving animals which are chronically instrumented with specific telemetry devices. Those techniques allow the assessment of pharmacodynamic effects of a NCE without interaction with anesthetics over a long observation period. Furthermore animal welfare is enhanced by reduction of stress or the possibility for social housing. In addition the telemetry technique contributes to the concept of 3Rs by the refinement of methods and the reduction of animals due to the re-use of animals.

The presentation gives an overview of various telemetry methods in different animal species with special focus on cardiovascular safety assessment in rodents. Since regulatory guidelines like ICH S7 (Anon, 2000) recommends the use of unanesthetized animals, the collection of data using telemetry techniques is preferred in Safety Pharmacology investigation (Leishman et al., 2012).

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I-7-121

Continuous blood glucose levels and food intake after RYGB surgery in rats

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Glucose levels in the blood change rapidly in response to various stimuli, including eating, physical activity and stress. Current practices to assess glucose responses in laboratory rodents typically involve frequent blood sampling and analysis with glucometers. Not only can such sampling practices, which often involve anesthesia or stress, obscure glucose measurements, but important information can also be lost or overlooked in between sampling intervals. Developing technology facilitates remote collection of continuous blood glucose levels in freely moving rats. Using an implantable telemetric glucose sensor (Data Sciences International), we tested whether glucose excursions following a meal are altered in a rat model of Roux-en-Y gastric bypass (RYGB). Rats were adapted to BioDAQ food intake monitors and glucose levels were analyzed in relation to spontaneous meals and during refeeding after a 6-hour fast. Analysis of glucose excursions during refeeding showed that RYGB rats had reduced preprandial baseline glucose, reached lower minimum periprandial glucose, and had a larger range of glucose values during this periprandial period. We conclude that RYGB alters the glucose response to a meal, and demonstrate the utility of an implantable glucose sensor to collect clinically relevant data that are otherwise difficult to obtain with current glucose measurement practices.

I-7-176

Simultaneous use of implanted and jacketed external telemetry – a contribution towards the 3R's

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The introduction of telemetryin freely moving animals (Chui et al., 2009; McMahon et al., 2010), reducing the need for instrumented anesthetized models, has made a considerable contribution towards the 3R's. Socialization can be maintained during data recording, thus further improving animal welfare.

For ethical, scientific and economic reasons, the objective of a rational study design is to collect the maximum amount of relevant scientific data with a minimum number of animals, while maximizing data quality and robustness. Developments in telemetry technology over recent years have permitted substantial progress in meeting these criteria.

A combination of implanted and jacketed external telemetryenables investigation of all safety pharmacology core battery investigations within a single study. Cardiovascular and respiratory parameters are measured continuously and central nervous system function is evaluated in a functional observational battery (FOB) at relevant time-points. Single or repeated blood sampling allows exposure level measurement or full pharmacokinetic profiling, respectively. According to the characteristics and pharmacological properties of test items, complementary analyses can also be performed.

This presentation will focus on the use of implanted and jacketed external telemetry in cynomolgus monkeys and how these approaches may be combined to improve safety pharmacology assessment.

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I-7-209

Animal instrumentation and telemetry: past, present & future

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The development and application of implantable sensors coupled with electronics for radiofrequency transmission of physiologic signals from conscious unrestrained animals has resulted in dramatic reductions in the number of animals that are utilized in research while simultaneously improving the quality of the science. Few technological advances have had such a profound effect on two of the three Rs, Reduction and Refinement. This talk with briefly highlight some of the early applications of telemetry in baboons, giraffes and sled dogs and will recognize the pioneers who's ingenuity and dedication made it possible. Subsequently, the evolution of current uses of telemetry, particularly in drug discovery and development, will be discussed. Particular physiologic parameters of interest will be described, including guidance on their proper collection and analysis. Finally, as miniaturized sensors and sophisticated electronics continue to evolve, thoughts about future applications of telemetry in animal research will be shared.

I-7-899

EEG telemetry as a gold standard for rodent studies in neurodegeneration

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Telemetric recording of biosignals is a state-of-the-art method for monitoring physiological functions in awake and freely moving laboratory animals, while minimizing stress artifacts. EEG activity, as well as blood pressure, heart rate, blood flow, electrocardiogram, respiratory rate, sympathetic nerve activity, body temperature, and many other biological signals can be studied in a wide range of animal species, including rodents (rats and mice), dogs, rabbits, gerbils, hamsters, monkeys, guinea pigs, and pigs (see for example Greene et al., 2008). Stress and anxiety are reduced in animals that are freely moving and unhindered by external hardware. For this reason, telemetry allows to reduce the number of required subjects in chronic and longitudinal studies (http://cdn.intechopen.com/pdfs-wm/21096.pdf). Anxiety levels may act as hidden variables in experimental studies, and we here report different examples of experimental models in which anxiety can be decreased using telemetric recording. In particular we hereby present data obtained in different experiments that point out:

a) anxiety-modulated susceptibility to convulsive drugs in mice;
b) anxiety-modulated cognitive performance in mice and rats;
c) sleep/wake cycle alterations as a tool for eliciting early mild cognitive impairment.

Potential applications of telemetric recordings will be discussed in relation to the above-mentioned data.

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Session I-7: Poster presentations

I-7-033

Dual pressure telemetry in rats; measuring interventricular asynchrony in experimental pulmonary hypertension

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Radio-telemetry provides an alternative means of obtaining physiological measurements from freely moving animals, without introducing stress artifacts. This surgical procedure describes the implantation of a new radio-telemetry dual pressure and biopotential transmitter, right ventricle (RV) and left ventricle (LV) and RV and aorta in rats. The success rate of the surgical procedure for the RV&LV technique was 8 out of 12 but none of the animals survived at the end of the experiment at 9 weeks. For the RV&Aorta technique the success rate was 6 out of 13 and the overall success rate was 3 out of 13. In experimental Pulmonary Hypertension (monocrotaline model), the adaptation to an elevated RV systolic pressure leads to ventricular asynchrony and cardiac inefficiency. The aim was to assess the possibilities to monitor disease progression and interventricular asynchrony in monocrotaline rats using dual telemetry. In RV&Aorta rats, the QA-interval of the RV showed a sudden increase after monocrotaline treatment, whereas the LV range is maintained. Implantation of telemetry for measuring both pulmonary as systemic pressures is feasible, however the RV&LV technique is not advised because of a high rate of complications. In combination with electrocardiography, dual pressure telemetry can be used to evaluate ventricular contractility.

I-7-220

Enhanced monitoring of disease studies involving laboratory mice using radiofrequency identification technology

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Appropriate monitoring of laboratory animals is critical to refining experimental studies and should be carried out by experienced and compassionate staff. This monitoring can be enhanced by the use of automated technologies if applied appropriately (Hawkins, 2014).

There are now a number of automated technologies capable of monitoring the most widely used laboratory species- the mouse (Richardson, 2012). Automated monitoring can be used to identify specific behaviours indicative of pain (Miller et al., 2011) and in progressive disease studies to identify objective biomarkers of disease progression- critical for the implementation of appropriate humane endpoints (Franco et al., 2012). Advantages of radiofrequency identification (RFID)-based systems when studying mice include the small size of the transponders and the capacity to use these systems to monitor socially housed animals. Additionally, RFID based systems that measure body temperature can be combined with those that monitor other behaviours such as drinking.

This presentation will describe how RFID technology was used to enhance monitoring of ongoing studies involving mice with liver disease or cancer. Examples will include using body temperature as a biomarker of disease progression (Hunter et al., 2014) and studying non-nutritive visits to drinking areas as an early indicator of disease.

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I-7-325

Correlation of infrared thermography vs. rectal body temperature measurements in baboons (*papio spp.*)

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One of the most clinically relevant and objective physiologic parameters is body temperature. Traditional methods for measuring body temperature in non-human primates may require sedation, physical, or mechanical restraint which can alter results. The objective of this study was to compare body surface temperature using infrared thermography (IRT) with rectal temperatures using standard medical thermometers. In conjunction with another study requiring general anesthesia of time-mated baboons, eight adults and six of their newborn offspring were examined on multiple occasions by simultaneously recording rectal body temperatures and body surface temperature using a hand held IRT camera. All paired measurements were compared using linear regression analysis. The results showed a statistically significant correlation between IRT and rectal thermometer temperatures. The most consistent body temperature recordings using IRT were from non-haired anatomical regions (face, sex skin). IRT is a noninvasive and accurate method for measuring body temperature in baboons and should be considered as an alternative to traditional methods.

I-7-734

Development of the mCM – mobile circulatory module – for *ex-vivo* physiological lung tissue for breathing simulation

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The REACH regulation (Regulation (EC) No 1907/2006) and Cosmetics regulation (Regulation (EC) No 1223/2009) of the European Union pressurise the manufacturers of chemicals and cosmetics to use alternatives to animal models. The developed lung simulator "i-Lung" provides a mean for alternative lung simulation. Active spontaneous breathing cycles of different lung equivalents like porcine lungs, instead of latex bags, David et al. (2013) allow research on breathing mechanisms. The used pig lungs are taken from the conventional slaughtering process and are therefore not part of an animal based study. In order to provide a physiologically and anatomically relevant model, the explanted lung tissue has to be maintained in an appropriate state and is used as lung equivalent for respiratory simulation including aerosol in- and exhalation (Forjan et al., 2012). Therefore, the presented mCM is being developed. The module allows the nourishing of the lung tissue during transport and simulation using a roller pump in an insulated housing and giving sensor data of fluid pressure, temperature and flow. The data is transmitted wirelessly using international medical IT standards to an Android based tablet and thereby enables standardised telemonitoring (Frohner et al., 2013) of the lung tissue. Further development will include cell-tissue based evaluation of the tissue status and use of the model for working-place safety measurements.

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