High-Throughput Screening Techniques

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42.1 State of the Art of High-Throughput Screening

High-throughput screening (HTS) is a technique well known for the identification of hits in drug discovery [1–3]. For the selection of promising lead candidates, thousands of molecules have to be tested [4]. Therefore, the automation of the screening is essential to allow testing of 10,000–100,000 samples per day [5]. Automated HTS systems in drug discovery allow preparation, incubation

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and analysis of many candidates simultaneously. The parallel testing of compounds generated through combinatorial chemical synthesis could significantly reduce the costs of drug development and results in substantial time saving for large numbers of samples [6]. Assays used for high-throughput drug discovery are mainly based on standard tests applied in biological and biochemical sciences, such as enzyme linked immunosorbent assays (ELISA), reporter gene assays or binding assays [5]. For increased sensitivity and reduced volume, biochemical assays are performed using scintillation proximity assays or fluorescence detection techniques, e.g. fluorescence resonance energy transfer, fluorescence correlation spectroscopy or homogeneous time resolved fluorescence. In addition, cell-based assays and ion channel techniques are applied in high throughput. In a first screening, the compounds are often tested as singlet, with low concentrations between 1 and 10 micromolar. If a positive result is generated, a secondary quantitative screening is performed and the IC_{50} is calculated [5].

In drug development, the majority of biological HTS applications are based on cell lines or primary cells cultured in 2D systems. To increase the amount of relevant information, 3D cultures have been successfully automated for HTS in drug development [7]. In contrast to 2D cultures, 3D systems exhibit higher *in vitro* to *in vivo* correlation and thus allow improved transferability to administration of a drug to human subjects. Hence, the combination of HTS techniques and 3D cell culture systems constitutes a promising research tool and can be applied for a variety of applications in biological and chemical sciences such as *in vitro* dermal toxicity testing.

In addition, programmes such as the Toxicology in the Twenty-First Century (Tox21), which is a federal collaboration of the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA), endorse the screening of thousands of chemicals for potential toxicity leading to an additional increasing need of *in vitro* models [8, 9]. The programme's aim is to rapidly and efficiently test whether certain chemical compounds have the potential to disrupt processes in the human body that may lead to adverse health effects. As these testing strategies require a significant increased number of test and *in vitro* toxicity testing is labour intensive and binds trained personnel, an HTS approach would be preferable. Automation of such *in vitro* assays could increase reproducibility and accuracy of measurements and reduces the error rate in comparison to manual processes and, therefore, enhances the quality of the generated data. Furthermore, an automated implementation leads to higher maximum capacities and is time efficient [9]. Nevertheless, in addition to automated testing, a sufficient number of complex 3D test systems must be available. Thus, there is also a need for a cost-effective, reproducible mass production of tissue models. When overcoming both low availability of 3D test systems and lack of devices for testing of 3D systems, HTS applications have great potential for meeting the challenges to alternative methods for animal testing and to become important components of modern toxicology testing strategies.

42.2 High-Throughput Production of *In Vitro* Models

42.2.1 High-Throughput Generation of Two-Dimensional Models

HTS requires a high amount of cells to generate sufficient numbers of both 2D and 3D models for the testing. Therefore, automation of the cell expansion of primary skin cells is the first step towards an application of HTS in dermal toxicity testing. The automation of cell culture processes such as the Cellerity™ (Tecan Trading AG, Switzerland) or the CompacT SelecT™ system (TAP Biosystems part of Sartorius AG, Germany) allows fully automated cell maintenance and expansion and provides the possibility to plate cells into a multiwell format for subsequent cell-based assays. Also the institution of commercially available reactor systems, e.g. from Eppendorf, BioSpherix, GE Healthcare Sciences or Pall Life Sciences, could improve the availability of cells (Eppendorf AG, Germany; BioSpherix Ltd., USA; GE Healthcare Sciences, Great Britain; Pall Life Sciences, USA). However, an effective expansion of primary human keratinocytes and dermal fibroblasts in microcarrier culture or bioreactors has already been shown [10, 11]. A current advantage of automated 2D cell cultures is that also different multiwell formats can be seeded with different cell types which supports a high compatibility to currently used systems for HTS in drug discovery and thus allows a good transferability of technologies for dermal toxicity testing into high-throughput processes. The KeratinoSens™ assay, for instance, is a non-animal skin sensitization test in a 96-well format.

However, 2D cell cultures constitute a rather artificial test system and can hardly be compared with native tissue, in which cells are embedded in a complex 3D microenvironment [12]. The unnatural rigid and flat substrates of 2D cell culture surfaces can alter cell metabolism and reduce functionality [13]. Moreover, these assays are not applicable to test hydrophobic substances as these are not soluble in physiological hydrophilic cell culture media. To overcome these drawbacks, great efforts are made to generate *in vivo*-like 3D cell cultures. However, the implementation of 3D cultures in an automated production to facilitate HTS faces several challenges due to more complex culture protocols and the use of nonstandard culture equipment.

42.2.2 High-Throughput Generation of Three-Dimensional Tissue Models

An automation of a 3D cell culture system was already realized by a collaboration of Tecan and TAP Biosystems. The two companies combined the Freedom EVO® liquid handling platform with the collagen-based 3D RAFT™ cell culture system, which led to an automated production of 3D collagen models and supports reproducible preparation of 3D cell cultures. TAP Biosystems established a 3D cell culture system based on a collagen matrix with a collagen content close to the native human skin tissue. The models could be manufactured either in 24- or 96-well

formats and are suitable for oncology, toxicology, neuroscience and stem cell applications. The Freedom EVO® platform is composed of liquid handling devices and robotic arms and includes reagent, microplate cooling units and heated shaking devices. This configuration provides a fully automated production and culture of 3D RAFT™ models that could be used for a broad variety of cell biology applications (www.tecan.com; www.raft3dcellculture.com; www.tapbiosystems.com). In the field of toxicology research, a 3D liver model based on the RAFT™ system was established, providing higher functionality and a longer maintenance compared to a 2D culture [14]. Nevertheless, so far there is no application for dermal toxicity testing based on such a system as the standard configuration is lacking the possibility for culturing cells or tissues at the air-liquid interface, which is essential for the physiological epidermal differentiation.

However, 3D cornified epithelia such as the epidermis withstand mechanical or chemical damage to a greater extent than 2D cell layers. Epithelia cells cultured at the air-liquid interface mature into epithelial equivalents that resemble physiological properties, e.g. a histological architecture similar to the native human skin. Additionally, epithelial equivalents mimic a physiological barrier function impairing the penetration of toxic substances to viable cell layers, which is a crucial criteria, e.g. for *in vitro* irritation testing. Several test methods based on commercially available reconstructed human epidermis (RHE) have already received regulatory acceptance as a full replacement for *in vivo* skin irritation, and corrosion testing and other toxicity testing applications based on the use of 3D skin models, including sensitization, genotoxicity and phototoxicity, are currently in validation. Due to a raising demand, the availability of 3D skin models can become a major bottleneck in the replacement of animal tests. In addition to a higher availability, automation technology can increase the reproducibility of skin tissue engineering processes due to a higher degree of standardization. To date, manual production results in detectable variances in the RHE quality due to individual differences of the cell and tissue handling. The implementation of robotic systems conducting the entire production process ensures a reproducible quality of the models. Furthermore, an automated process produces a comprehensive data set of all manufacturing steps, which can be objectively analysed for quality control and assurance [15].

Based on the need for cost-effective, reproducible mass production of tissue models, the Fraunhofer-Gesellschaft developed a production plant, called ‘Tissue Factory’, which provides the possibility for the automated manufacturing of various kinds of human tissue models. The ‘Tissue Factory’ was a collaborative project of four Fraunhofer institutes coordinated by the Fraunhofer IGB. In a first phase, the facility was used to automate the production of the in-house developed Fraunhofer full-thickness human skin equivalent [16] which is manufactured using human keratinocytes seeded on top of a dermal equivalent consisting of fibroblasts embedded in a specific collagen scaffold. This allows the keratinocytes to differentiate into a multilayered epidermis with a stratified corneous layer (*stratum corneum*) exhibiting a barrier function to the penetration of topically applied substances. The production system of this first phase was organized in three modules. Focusing on high-throughput and maximized efficiency, a fully automated cell extraction

module allows the isolation of primary keratinocytes and fibroblasts through a process chain comprising a combination of mechanical and enzymatic applications, while maintaining high cell vitality. Proliferation of primary human keratinocytes and fibroblasts to achieve the generation of a sufficient amount of cells is performed in the cell expansion module. The culture of primary skin cells, with a capacity of 500 flasks/bioreactors, was specifically adapted for the fully automated process. Finally, 3D skin equivalents are generated in the tissue culture module. Conducting this process without manual intervention is a challenging task with respect to process control and automation technology. It requires reliable handling and mixing of dispensed cells and other liquids with consistent time- and process-dependent properties. For instance, a reproducible application of high viscous liquids into tissue culture inserts requires precise positioning and dynamic control of active and passive dosing systems.

However, as regulatory guidelines currently endorse RHE tissue models to be used, the tissue factory was adapted in a second phase to meet the requirements for the production of such models. Hence, the automated production was adapted for the generation of the so-called 'open-source' reconstructed epidermis, based on an initial publication of Poumay et al. and further developed by the Henkel AG & Co. KGaA. The OS-REp is comprised of primary human epidermal keratinocytes which differentiate to a multilayered epidermis with a well-formed basal layer and a dense *stratum corneum* [17, 18]. The production plant ensures standardized and reproducible manufacturing with a monthly output of 2000–5000 epidermal equivalents. Whereas cell extraction and expansion are performed manually, the 'Tissue Factory' today can conduct all tissue reconstruction process steps fully automated (www.tissue-factory.com). Although the automated skin equivalent production has great potential for meeting the challenges of alternative dermal toxicity testing, classical automation approaches require substantial resources and lack a physiological continuous medium supply. As an alternative technology, bioreactor perfusion systems that mimic the *in vivo* nutrients supply can also be used.

42.2.3 Bioreactor-Based Generation of 3D Models

Bioreactor systems were firstly introduced for biotechnological applications. Per definition, a bioreactor is a system that converts biological processes. In addition to enzymatic and micro-bacterial applications, bioreactors are employed for processing plant and mammalian cells [19]. Commercial applications of bioreactors are, e.g. the culture of microorganisms for the production of substances such as amino acids or enzymes. Here, bioreactors allow robust concurrently adjustable process conditions in large-scale facilities [20–22]. In addition to these applications, bioreactor systems have been successfully employed in tissue engineering. Compared to conventional static tissue culture conditions, e.g. culture in multiwell plates, the advantage of bioreactor systems is that *in vivo*-like conditions can be mimicked [23]. With respect to dermal toxicity testing, this supports culturing the skin tissue

under perfusion and convection and, thus, improved nutrients' supply and the skin tissue-specific culture at the air-liquid interface [24, 25]. Furthermore, bioreactors can be designed as closed systems. In combination with automatically controlled pumps that circulate defined volumes of media through models, this would allow the ability to perform middle- or long-term studies in toxicology.

Despite the advantages of bioreactor systems, their application in HTS is thwarted due to increased technical requirements compared to standard cell culture techniques. For maintaining controlled dynamic culture conditions in a tissue engineering process, additional equipment, e.g. pumps, sensors and feedback control systems, is required. In general, this can result in complex bioreactor embodiments exhibiting a need for extensive laboratory space, an increased prone to failure and a limited possibility for parallelization. In addition, a broad variety of bioreactor systems of different designs is currently available [26–29]. This demonstrates a lack of platform concepts and standards that is impairing the harmonization of bioreactor technology and limiting the transferability in industrial application [30].

Nevertheless, bioreactor systems are currently the only technology for generating complex, highly structured tissues composed of different cell types [31, 32]. Although simple RHE has been accepted by the Organization for Economic Co-operation and Development (OECD) to assess skin corrosion and irritation, more challenging endpoints might require more complex skin models which could be particularly useful to examine systemic effects of applied substances. Thus, the prediction level on reactions of human subjects might be enhanced. For instance, absorption and elimination kinetics can be studied, and thereby, the investigation of more than one drug at a time can be performed in order to identify drug/drug interactions. Complex skin models can be generated using decellularized native tissue containing the structure of the vascular system (BioVaSc®). This approach supports, e.g. the culture of a vascularized skin test model in combination with the dynamic conditions applied by the bioreactor [25]. The vascularization of the skin tissue can help to understand processes such as transdermal adsorption as well as the systemic availability of substances. Moreover, the system is applicable for the investigation of skin disease such as melanoma or psoriasis as the vasculature is one of the key components in the progression of these diseases [33, 34].

In addition, not only the skin but also intestinal and bladder test systems can be used for toxicity testing [35, 36]. Toxicological test applications, feasible via bioreactor-based testing, are toxicokinetics, dermal sensitization, repeated dose toxicity as well as carcinogenicity and reproductive toxicity [8, 37, 38]. Parallelization can be facilitated, when downscaling the total volume of a bioreactor. Due to significant efforts, it is possible to perform testing on a micro-bioreactor (μBR) chip with dimensions of just a few millimetres [39–42]. Researchers develop technologies that might be capable to build up a micro-electromechanical system (MEMS), where systemic effects can be investigated in a 'human-on-a-chip' perspective. The possibility to test effects not on a single organ but in complex systems comprised of different tissue models could help to investigate systemic effects *in vitro*. With this approach, potential benefits or harms can be investigated as shown previously especially in terms of drug development [43–45].

In conclusion, bioreactors allow controlled culture and testing conditions as well as a high level of robustness due to automated process steps of reproducible results [46]. However, it is challenging to employ high parallel processing within bioreactor systems. There are only a few studies reporting parallel bioreactor technology approaches and *in silico* simulations, where the system couples cell expansion and model generation in one device on a macroscopic scale [47–49]. Currently, research is performed to develop systems where many models can be cultured simultaneously [50–52]. These systems can prospectively support parallelized toxicity testing under controlled and standardized conditions [53, 54]. If successful, bioreactor systems might constitute, as already stated by the National Research Council (NRC) in 2007 [55], one solving strategy towards twenty-first century toxicology in the future.

42.3 High-Throughput Testing of *In Vitro* Models

A pivotal aspect of high-throughput testing is the assay that is used to predict toxic effects. To significantly increase the number of test chemicals in different concentrations that can be tested quantitatively, HTS has been proposed in different studies [9]. The applicability of the approach could be demonstrated by a publication of the US National Toxicology Program and the NIH National Chemical Genomics Center (NCGC) that assessed the toxic effects of a panel of 1408 chemicals in different human and rodent cells by automating a luminescent cell viability assay [56]. Moreover, a study of the European Commission Joint Research Centre demonstrated the successful automation of an assay to determine the acute oral toxicity by diligently implementing the manual test protocol, defined in an OECD guidance document, into a high-throughput test platform. However, the described approaches are currently limited to relative simple readouts such as cell viability nor do they employ 3D reconstructed tissues. A reason for this is that the current available test methods standardly use invasive techniques such as colorimetric assays or histology which are very difficult to combine with a high-throughput platform approach [57]. Pitfalls of these methods are that the test procedures are difficult to implement into a technical process [58] and that test samples are destroyed in the test procedure [59]. Hence, toxic effects need to be compared to controls which increase the needed number of tissue models. Additionally, the quality of a produced skin model batch can only be controlled via random samples. Especially for a continuous production process, as described in the bioreactor-based approaches, this is a major challenge as no in-process control can be used to monitor tissue formation. Hence, the process is dependent on a predefined protocol and no regulation is possible.

To overcome these pitfalls, nondestructive technologies can be employed to assess skin model reactions. Optical imaging systems that make use of the multiphoton effect techniques showing the highest spatial resolution seem very promising in this respect. In these systems, a femtosecond pulsed laser allows to excite a given sample with two or more photons. Hence, laser light with a near-infrared wavelength can be used to image a sample [60]. As light with a lower wavelength

penetrates deeper into optical dense tissues such as the skin, information can be gained to a depth up to 1 mm [61]. Also this technique allows a label-free imaging due to the possibility to visualize the autofluorescence of endogenous substances such as nicotinamide adenine dinucleotide phosphate (NADPH), elastin or collagen [62]. Especially elastin and collagen can be detected specifically due to second harmonic effects. Due to the noncentrosym-3D structure, these biomolecules emit light with approximately the doubled energy (and thus doubled wavelength) than the light, which was used to excite the sample [63]. Using these systems, skin reactions could be investigated on a cellular level.

Besides imaging, advanced optical systems can also be used to investigate skin effects on a molecular level. In Raman spectroscopy, light is coupled into a sample where the light interacts with the present molecules and is scattered inelastically [64]. By counting the photons for each wavelength, a molecular fingerprint of a biological sample can be generated. Using this technology, different skin cell types can be identified [65] and different toxic reaction such as necrosis and apoptosis can be recognized. Furthermore, Raman spectra can be employed to analyse the extracellular matrix of tissues, which allows to investigate degradation processes in collagen [66] or differences in the molecular composition of the *stratum corneum* between human skin and *in vitro* skin models [67]. These findings were supported by standard invasive methods that showed that some aspects in the *stratum corneum* composition of reconstructed human epidermis and full-thickness skin models differ from the human skin *in vivo* [68].

Although Raman spectroscopy is a promising tool in the investigation of the molecular mechanisms behind skin toxicology, the long analysis times of 1 min per measurement restrict the broad application of the technology. To achieve much faster readouts, optical coherence tomography (OCT) can be used. Comparable to ultrasound measurements, OCT uses two light beams to generate optical cross-sections of a sample. Of these light beams, one is directed to the tissue sample and the second to a reference mirror. The combined reflected light from the two paths only forms an interference image if the working distance of both paths is matched. By adjusting the length in the reference path, the amplitude of the reflected light from the sample can be recorded depending on the depth [69]. Depending on the system used, OCT allows a spatial resolution down to 10 μm with measurement times of only a few seconds [69]. When employed to investigate the reconstructed human epidermis, OCT could discriminate between different epidermal layers and thus is a promising tool to assure the quality of commercially produced skin models. Accordingly, OCT was integrated into the first automated production facility of human skin models ('Tissue Factory') to ensure the quality of each produced model individually, without being dependant on random sampling.

In addition to optical systems, also electrical properties of the skin can be employed to assess skin models. Forming the interface between the human organism and the surrounding environment, the skin restricts the flow of electrically charged particles. Thus, the skin has a characteristic high trans-epithelial electrical resistance (TEER), which is usually measured with alternating current to avoid destructive

effects of direct currents such as a polarization of cellular ions or the induction of electrolysis and heating in the used cell culture medium [70]. The potency of these electrical measurements to predict toxic effects on the skin was demonstrated *in vivo* [71] and by the European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM) validated TER method, in which the change of the electrical resistance of the *ex vivo* rat skin was used to assess skin corrosion [72]. However, these promising results could not be transferred to *in vitro* skin models as an unphysiological $CaSO_4$ solution was used in the TER method based on the *ex vivo* rat skin. Furthermore, no standardized measurement setup is available, and research is still dependant on commercially available systems that have been developed for 2D cell cultures. In contrast to these simple cell-based models, 3D skin models are composed of multiple layers with different electrical properties that result in high variability between measurements and currently restrict the applicability of TEER measurements in risk assessment [73, 74]. Moreover, TEER values are determined at one specific frequency only [75]. Due to the use of alternating currents, the electrical resistance or impedance is dependent on the applied frequency resulting in complex impedance spectra, which are specific for the tissue under investigation. Thus, a majority of information is lost in TEER measurements.

In a recent study, an experimental setup was presented that specially was designed to assess the electrical properties of 2D tissue constructs. In contrast to simple TEER measurements here, the impedance spectra between 1 Hz and 100 kHz of RHE was used to investigate the epidermal differentiation [76]. After the differentiation of the keratinocytes at the air-liquid interface, the RHE showed impedance spectra, which were comparable to the human skin *in vivo*. Employing mathematical modelling, electrical parameters such as the ohmic resistance and the capacitance could be extracted from the impedance spectra. Using this approach, the development of the epidermal barrier and the effects of different mechanical and chemical traumata on the models could be quantified. Most interestingly, when used as an additional endpoint in skin irritation testing, the method is sufficiently sensitive to detect the effect of the washing process and of non-irritants to the RHE. These results indicate that impedance spectroscopy might be applicable as a complementary endpoint in current skin toxicity testing.

42.4 Perspectives for Future Automation Approaches

Although automated platforms enable to produce considerably more skin models and a high-throughput strategy would allow to conduct more testing than in a manual process, significantly more resources are needed for the establishment of the technical processes. Being constructed for usually a single high-throughput test, most systems lack flexibility. Moreover, automated facilities need special cost-intensive peripheral equipment, such as liquid handling systems, incubators, centrifuges or shakers. Thus, before a test is automated, the cost of the automation should be carefully compared to its benefit. Especially if the expected demand for a specific

test model or the test itself is moderate, classical automation approaches are too expensive for commercial use.

As an alternative, bioreactor technologies can help to reduce costs as here usually only fluidic systems are needed instead of expensive robotic systems. In addition to these systems, also new robotic devices that are able to use standard laboratory equipment can be used for laboratory automation. An interesting approach was presented by the Japanese company Yaskawa, which uses a dual-arm robotic system to automate even challenging laboratory processes. Much like a laboratory technician, the robotic system can use standard labware such as pipets and cellscrapers. Employing this system it could be demonstrated that the robot is able to perform even demanding cell culture processes [77]. In dermal toxicity testing, a pivotal part in standard operational procedures is the application of different test substances to the surface of skin models. Due to the different physico-chemical properties of the substances, this requires specially trained and experienced personnel, and so far no automated process has been proposed conducting this step. Due to the high flexibility of the dual-arm robotic system, different application scenarios could be programmed, which could allow an automated substance application in future high-throughput approaches.

A critical aspect of high-throughput testing is that these test methods need to be validated in order to receive regulatory acceptance to replace animal experimentation. As validation involves the testing in multiple independent laboratories, all employed testing platform need to have the same specifications. However, no international standard for lab automation is currently available. Thus, all participating laboratories need to comply with a single system before a validation study. Due to the significant cost for high-throughput testing systems, the available systems are usually adapted to the specific needs of an institution and usually differ significantly between one another. To an even greater extend, the 'Adam robotic scientist' system is using automation for the investigation of genomic analysis. Here, the system is not only conducting the experiments but is also able to decide autonomously which new experiments should be conducted [78]. Transferred to *in vitro* dermal toxicity testing, a future system could not only produce models and conduct assays on different toxicological endpoints but could also decide on the next steps in complex integrated testing strategies.

42.5 Conclusions

In this chapter, we have demonstrated that automated skin model production is feasible today, offering clear advantages over manual production, and can be achieved in a cost-effective way. In addition, since industry will continue to seek for better performing human test models, more 'complete' skin models including other cell types will surely be developed in the near future. Mass production of such complex tissue models is challenging using routine cell culture methods; hence, automation of tissue model manufacturing will soon show its benefits in industrial toxicity testing in the twenty-first century in general terms.

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