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Master of Science HES-SO in Life Sciences

Development of a cell-based WST-8 assay for screening antidotes using an automated liquid handler

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INTRODUCTION

Bioassays can be employed in preclinical studies. Cell-based bioassays aim to more and more replace the number of animal experimentations in preclinical investigations. The cell- and organ-based constructs, are promising alternatives for preclinical toxicology studies. As readout of an assay, the cell survival index, the mitochondrial activity, or physiologically relevant cell and organ functions are analyzed.
These bioassays also serve as screening approaches for antidote (inhibitor) identification among a compound library. For example, no inhibitor of the tested toxin exists today to treat intoxications and the lack of immune response excludes vaccination as treatment.
In this study a bioassay assessing the mitochondrial parameters in cell cultures was established. And the bioassay was prepared for the use in a liquid handling robot. On the day of the experiment, the cell-based bioassay was to be placed in the robot for the automatic liquid handling. Automation serves to improve and accelerate a screening using the labor-intensive cell-based bioassays. In toxicology, a key strategy is to screen compound libraries for candidate antidotes in a cellular setup. So far the inhibitory potential of antidote compounds has mainly been tested in in vitro approaches assessing molecule-to-molecule interactions. The cellular approach augments significantly the chances to find for example mitochondria-protective antidotes. The aim of the present study was to facilitate and automate the liquid handling of cell treatments and to launch testing compounds to be added to the cell cultures in multi-well microtiter-plates.

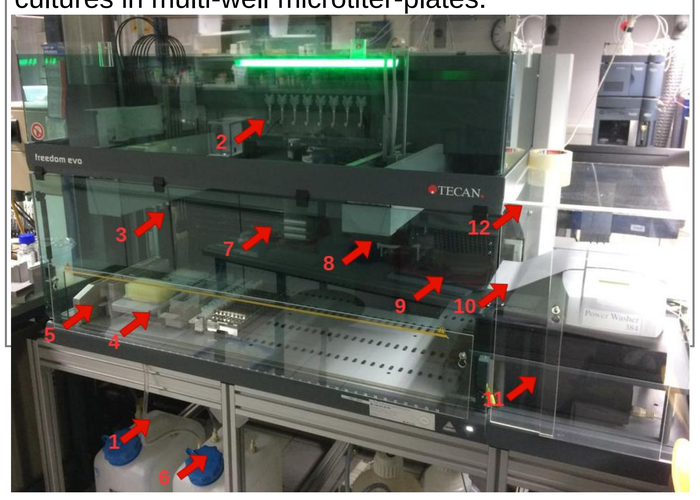

Figure 1: The Tecan Freedom EVO 150 System and its components. *(1) System Liquid Reservoir. (2) 8x 1 ml Dilutors. (3) Liquid handling arm with 8x Fixed tips, stainless steel. (4) Worktable with racks holding throughs, microplates and microcentrifugation tubes. (5) Tip Wash Station. (6) Liquid Waste connected to Wash Station. (7) Hotel for 16 microplates. (8) RoMA for transporting microplates. (9) 2 37°C incubator tower for 12 microplates. (10) Plate Washer. (11) Sunrise Absorbance reader with 4 filters. (12) Dust protection hood*

RESULTS

The liquid handling robot was programmed to execute the following protocol. The cell treatment solutions were prepared and loaded in the robot by the operator. They included the stock concentration of the toxin and the cell washing solutions. Then the cells, cultured in 96-well microtiter-plates, were loaded in the robot by the operator.
In order to proof robustness and reliability of the cell culture in the robot facility pre-studies under various culturing conditions and media were performed.
Proliferation and viability maintaining a physiological pH of 7.4 over extended times without CO2-supply using medium B was demonstrated. This condition was thus suitable for cell assays without the need the need of a CO2-incubator.

Bioassay performance

The robot was programmed to serially diluted the toxins in PP dilution plates and add them together with the corresponding control treatment to the cell containing microplate. Crucial for the eight-channel fixed tips, washing and decontamination protocols at steps critical for cross-contamination or carry over were implemented. Microplates were incubated in the integrated 37°C incubator with the RoMa (Robotic Manipulator Arm) used for all transfer steps.

After 24h mitochondrial dehydrogenase enzyme activity was assessed by adding WST-8 to the cells followed by a short incubation at 37°C and readout using the integrated absorbance reader. The water-soluble tetrazolium salt is reduced to in presence of NADH to formazan dye that absorbs at 450nm. The tested toxin reduced the mitochondrial activity in a dose-dependent manner from 100% to around 30%.
Variability of the results among the identically treated cell cultures by the robot, is improved when compared with manual liquid handling. Manual liquid handling was executed by the operator, was run in parallel experiments to the automated liquid handling (Fig. 2a) and illustrated in Fig. 2b
Manual handling was of lower reproducibility, even when executed by a trained operator, when compared with the automated liquid handling. However, the dose-response-curves were comparable between manual and automated liquid handling. The estimated mean IC50 value of dose-response curves was of IC50=1.8 nM (SE 0.41) of the toxin for manual handling, and of IC50=2.5 nM (SE 0.55) of toxin for automated liquid handling.
The two IC50 values do not differ significantly (p=0.21). Thus, the close overlap of the automated and manual liquid handling consolidates the robot handling as an accurate approach to facilitate cell-based bioassays.

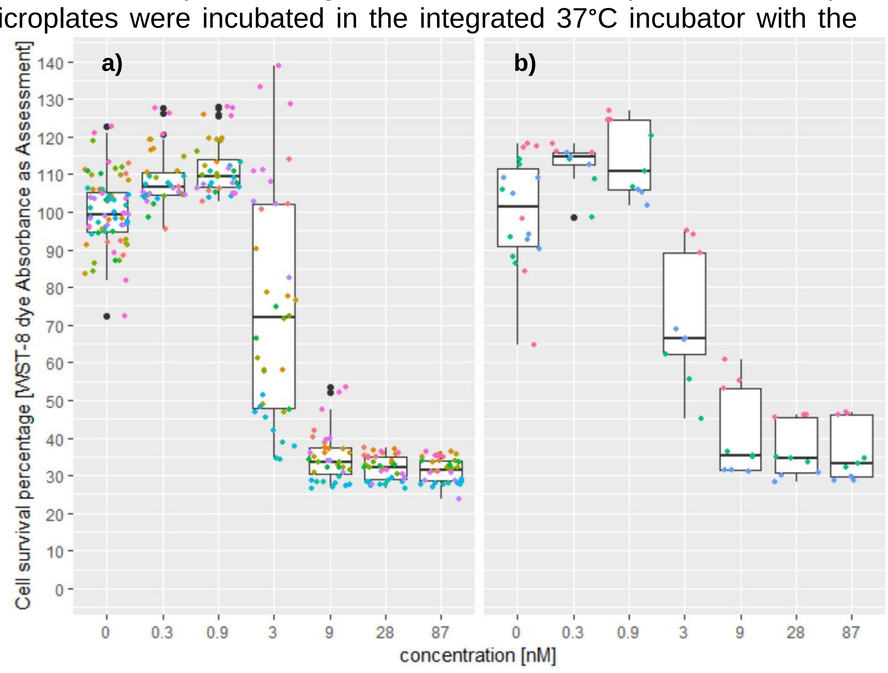

Figure 2: Tukey Box-and-whisker plot of cell proliferation with automated liquid handling (a) versus manual (b) after 24h exposure with six-point half-log serial dilution of toxin. *Each point represents the measured absorbance of one well normalized to the control. For the automated experiments data derive from 5 independent experiments with 12 microplates in total, the manual data is derived from three independent experiments with 3 microplates in total.*

Further consolidation of the robot handling was achieved by comparing the estimated IC50 value of dose-response curve of cadmium-driven intoxication of the cell cultures. This control bioassay resulted in IC50=11.6 nM (SE 4.34) of cadmium for manual handling, and in IC50=11.3 nM (SE 5.74) of cadmium for automated liquid handling. The two IC50 values do not differ significantly (p=0.96). This further consolidates the here established, automated liquid handling protocol, indicating its readiness for implementation.

Quality Control Metrics (QCM) used in assay optimisation and validation was employed [Larsson 2011]. The Z'-factor developed by Zhang et al. is a preferred parameter of assay perfomance and is defined as Z' =1 – [(3σneg +3σpos)/|μneg- μpos|]. This coefficient takes takes both signal dynamic range and data variation of the positive and negative controls into account .The Z'-Factor for automated liquid handling was 0.77 (n=26), whereas manual yielded in Z'=0.61 (n=6). Z'-values above 0.5 indicate an excellent assay with a large separation band. Assay date and order in run for any assay plate did not result in an observable drift of Z'.

Pilot bioassay of antidote screening

To implement the cell bioassay in a screening, a pretreatment of the above-described cell treatment was launched. Pilot experiments integrated a pretreatment with a selection of commercial agents known for their mitochondria-protective effect. The aim was to add an additional step of samples dilution and liquid handling by the robot to the mitochondrial toxicity bioassay and to measure eventual improvement or protection of the mitochondrial toxicity by the toxin. This screening approach did not identify a candidate antidote of the toxin. However, this experiment confirms that the liquid handling automation setup is now successfully established for a throughput-Screening (HTS) of libraries of further candidate compounds.

CONCLUSION

The established bioassay is robust and reproducible. It demonstrates little variability when altering important parameters. These parameters include various medium composition and CO2-concentration, miniaturization from flask surfaces of cell culturing to 96-well microtiter plates, pipetting using metallic or plastic tips. Therefore, the cell-based bioassay assessing mitochondrial toxicity is apt to be integrated in an automated liquid handling equipment. The integration of a robot is promising to safe cost, time, and to avoid frequent errors of manual handling.

Further miniaturization can be achieved by upgrading the TECAN EVOware to 384-well microtiter plates in which the cell-based bioassay is run. In addition, a multiplex setup is further feasible with the robot. A multiplex readout adds up to the mitochondrial toxicity also cell viability, apoptosis (programmed cell death), enzyme release from cells (LDH), and ATP energy-titer readouts.

In conclusion, the established bioassay of cell-based mitochondrial toxicity is now ready for implementation. A promising implementation aims to find antidote components neutralizing the toxin. Antidotes of the toxin, eventually lethal in victims, do not exist today and are warranted. In a follow-up investigation, the established bioassay of mitochondrial toxicity can further be assessed to screen for unwanted side effects of new drugs in preclinical studies. These perspectives of implementations demonstrate the importance of automated liquid handlings facilitating cell-based bioassays as established in the current study.

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