Integrated microfluidic technology for sub-lethal and behavioral marine ecotoxicity biotests

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ABSTRACT

Changes in behavioral traits exhibited by small aquatic invertebrates are increasingly postulated as ethically acceptable and more sensitive endpoints for detection of water-born ecotoxicity than conventional mortality assays. Despite importance of such behavioral biotests, their implementation is profoundly limited by the lack of appropriate biocompatible automation, integrated optoelectronic sensors, and the associated electronics and analysis algorithms. This work outlines development of a proof-of-concept miniaturized Lab-on-a-Chip (LOC) platform for rapid water toxicity tests based on changes in swimming patterns exhibited by Artemia franciscana (Artoxkit MTM) nauplii. In contrast to conventionally performed end-point analysis based on counting numbers of dead/immobile specimens we performed a time-resolved video data analysis to dynamically assess impact of a reference toxicant on swimming pattern of A. franciscana. Our system design combined: (i) innovative microfluidic device keeping free swimming Artemia sp. nauplii under continuous microperfusion as a mean of toxin delivery; (ii) mechatronic interface for user-friendly fluidic actuation of the chip; and (iii) miniaturized video acquisition for movement analysis of test specimens. The system was capable of performing fully programmable time-lapse and video-microscopy of multiple samples for rapid ecotoxicity analysis. It enabled development of a user-friendly and inexpensive test protocol to dynamically detect sub-lethal behavioral end-points such as changes in speed of movement or distance traveled by each animal.

Keywords: Lab-on-a-Chip, Artermia franciscana, ecotoxicity, behavior, biotest *e-

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1. INTRODUCTION

Water contamination may occur as a result of slow cumulative processes, following accidental spills or deliberate chemical contamination. Current capabilities for continuous and rapid detection of water contaminants are limited and may not provide sufficient warning of developing toxic hazards. In particular, conventional analyses lack the ability to detect and warn against a broad spectrum of contaminants in real-time, and are often too slow to develop operational response. Tests using small aquatic invertebrates as biological sensors of the aggregate toxic effect offer a promising solution to this problem[1]. Despite its critical importance, the broad deployment of whole-organism eco-toxicity tests is profoundly limited by the lack of appropriate bio-compatible automation, integrated optoelectronic sensors, and the associated electronics and analysis algorithms[2]. These major shortcomings have not been yet addressed, and as a result virtually all ecotoxicity assays are still performed manually, are time consuming, laborious and are inherently low- throughput[2]. Furthermore most ecotoxicity biotests on small aquatic organisms are executed in multi-well plates where static microenvironment limits the compound availability to the specimen due to surface adsorption, degradation and many other physical-chemical factors. Culture in static conditions can also potentially cause inter-specimen contamination and toxin bystander effects. In addition, static culture does not adequately mimic the dynamic movement experienced by aquatic organisms in their natural fluid environment. Lastly, contemporary and well-accepted ecotoxicity studies are usually performed in an end-point regimen where mortality of sensitive test species is the main criterion used to calculate lethal doses. Such tests are often perceived as ethically controversial. Behavioral parameters are thus increasingly postulated as alternatives to mortality testing with an added advantage of providing much more sensitive endpoints for detection of water-born ecotoxicity[3-5]. Robust, user-friendly and automated platforms to perform such behavioral biotests do not currently exist.

Most of the above limitations can be addressed by emerging field of biomicrofluidic Lab-on-a-Chip technologies. As an investigative tool, LOCs represent a new direction that may miniaturize and revolutionize research on ecotoxicity and physiology in vivo[2, 6, 7]. The transfer of traditional bioanalytical methods to a microfabricated format can greatly facilitate the reduction of toxicological screening expenditures. Furthermore, the enclosed and sterile formats of microfluidic devices eliminate the evaporative water loss from miniaturized channels and chambers. While the application of laminar fluid flow under low Reynolds numbers provides an attractive analytical avenue for the rapid delivery and exchange of reagents with exceptional accuracy, the transfer

of traditional methods to a microfabricated format offers a means to increase both the resolution of analysis and sampling throughput while reducing the costs of a single assay[2, 6, 7]. So far no such technologies have been developed for ecotoxicology but they offer great hopes for improved environmental monitoring capabilities.

This work outlines development of a proof-of-concept Lab-on-a-Chip platform for rapid water toxicity tests based on changes in swimming patterns exhibited by Artemia franciscana (Artoxkit MTM) nauplii. The innovative microfluidic system enabled development of a user-friendly and inexpensive test protocol to dynamically detect sub-lethal behavioral end-points such as changes in speed of movement or distance traveled by each animal. We postulate that merging of LOC technologies with sophisticated optoelectronic sensors and dedicated embedded computing systems, is a powerful avenue to build enabling platform on which real-time environmental monitoring tests could be performed in the future.

2. MATERIALS AND METHODS

2.1 Artemia eggs and nauplii culture

Cysts of an euryhaline marine crustacean Artemia franciscana (MicroBioTests Inc., Belgium) were hatched at 25°C in a Petri dish filled with a filtered sea water (pH 8.0 ± 0.5) and illuminated (3000-4000 lux) for 30 hours before toxicity tests. Microfluidic experiments were performed at a flow rate of 5.25 mL/h with varying doses of potassium dichromate (0 mg/L to 100 mg/L; Sigma-Aldrich, Australia) or Cadmium Nitrate Tetrahydrate (0 mg/L to 1000 mg/L; Sigma-Aldrich, Australia). For comparison, 24 well plate experiments were also carried out according to standard Artoxkit-M^{TM} biotest (MicroBioTests Inc., Belgium) operating protocol.

2.2 Multilayer 3D chip designing and fabrication

The chip device were designed and modeled in 2D using CorelDraw X3 (Corel Corporation, Ottawa, Ontario, Canada) CAD package. 3D models were generated in SolidWorks 2013 (Dassault Systems SolidWorks Corp, Concord, MA, USA). Each layer was fabricated separately in poly(methyl methacrylate) (PMMA) transparent thermoplastic using a non-contact 30W infrared laser micromachining system with a 50 µm elliptical beam spot (Universal Laser Systems, Scottsdale, AZ, USA). Fabricated PMMA layers were then manually aligned, sandwiched by silicon wafers and C- clamps, and thermally bonded at 120 °C in a fan assisted oven for 90 minutes.[2 8]

2.3 Computational Fluid Dynamics (CFD) simulations

The 3D models of chip-based device generated in SolidWorks 2013 (Dassault Systems SolidWorks Corp) were imported into COMSOL Multiphysics®

simulation software version 4.4 (COMSOL INC, Burlington, MA, USA). Finite-volume algorithms were used to solve the associated differential equations governing the balance of mass, momentum and chemical species determining the flow characteristics present within the microfluidic device.

2.4 Off-chip interface

The 3D chip required external microfluidic actuation for its operation. The off-chip interface consisted of miniaturized 1D motorized stage and specially designed miniaturized peristaltic pumps for fluidic actuation. Custom designed motorized stage and peristaltic pumps were prototyped around Dynamixel AX-12A robotic actuators (Robotis Ltd, Irvine, CA, USA). The off-chip interface designs were created using SolidWorks 2013 (Dassault Systèmes SolidWorks Corp.) and manufactured using Fused Deposition Modelling (FDM) process with a Replicator 2 (MakerBot) desktop 3D printer in poly(lactic) acid (PLA). The mechatronics components were controlled by a CM-530 microcontroller (Robotis Ltd) based on 32-bit ARM Cortex M3 architecture. The programing was performed in a native RoboPlus environment (Robotis Ltd).

2.5 Time-resolved video acquisition

Miniaturised USB brightfield and polarization microscopes (AM7013MT Dino-Lite Premier; AnMo Electronics Corporation, New Taipei City, Taiwan) were used to capture videos at a rate of 15 frames per seconds (fps). The microscopes were equipped with a 5.0-megapixel (MP) color CMOS sensor (SXGA), variable magnification of up to 200x and numerical aperture (NA) of 0.135. The specimen illumination was provided by 8 integrated ultra-bright white LEDs controlled by the dedicated software (DinoCapture2.0; AnMo Electronics Corporation). The miniaturized USB microscope was powered and controlled through a USB2.0 interface with an external PC computer running Windows 7 operating system (Microsoft, Redmont, WA, USA). The software interface supported fully programmable time-resolved data acquisition including direct control of both image capture and LED illumination. In all experiments 15 second long videos were captured every hour for up to 24 hours of biotest duration.

2.6 Quantitative data analysis

Dose dependent immobilization of nauplii in response to corresponding chemicals was scored using a commercial video analyzing software LoliTrack V.4 (Loligo® Systems, Denmark). Additionally an open-source semi-automatic segmentation, tracking and analysis software platform PhagoSight [9] was also used to vcross-alidate data obtained by LoliTrack software.

3. RESULTS AND DISCUSSION

3.1 Chip design and operation

The 3D multilayer chip contained a specially designed circular ecotoxicity chamber of 10 mm in diameter and 1.5 mm in height, optimized to hold multiple specimens of freely swimming crustacean larvae for up to 24 hours under continuous microperfusion. Inlet and outlet channels were connected to the chamber through ten laser-ablated microchannels with a height of proximately 0.2 mm and positioned at the circumference of the chamber. The dimensions of ablated inlet and outlet ports enabled effective “caging” of free swimming inside the chamber. In order to improve mass transfer, microchannels were ablated in opposite different layers to provide a fluid flow across vertical plane of the microchamber. This design also reduced the blockage of the perfusion system by immobilized or dead nauplii (Fig 1. (A) (B)). The design incorporated an auxiliary manifold directly connected to the “caging” chamber. It enabled rapid and efficient loading of test specimens. Following loading of free-swimming nauplii at an age of approximately 30 hours post hatching (hph) an air bubble was injected to seal the auxiliary manifold (Fig 1. (C)).

Effective circulation of medium and the constant delivery of toxicants to “caged” Artemia larvae are of utmost importance for long-term microperfusion studies in ecotoxicology. CFD simulations were thus performed to predict flow velocity, pressure drop and uniformity of mass transfer inside the device. The simulations indicated that at a flow rate of 5.25 mL/h velocities inside the chip varied from 0.41E-03 m/s to 0.43E-03 m/s, at the input and output, respectively. Mathematical modeling revealed that the “caging” chamber had constant flow velocity of 0.16E-03 m/s (Fig 2 (a)). High flow velocity areas (2.7E-03 m/s.) were only identified at engraved channels positioned at the circumference of the holding chamber (Fig 2 (A)). Next numerical simulations to investigate the water pressure and the extent of shear stress exerted across the chamber were performed. The pressure balance models revealed that under a continuous flow rate of 5.25 mL/h, the “caging” chamber experienced an average of 2 Pa (Fig 2 (B)). CFD results provided preliminary evidence the embryos will be kept within a low shear stress microenvironment. According to the previous work by Wlodkowic and co-workers, the maximum shear stress values reported above are at least two orders of magnitude lower than values reported to trigger cell signalling events. The simulations performed to predict uniformity of mass transfer indicated that after 3 minutes of perfusion at a flow rate of 5.25 mL/h, the medium exchange rate in the main loading channel reached 100% (Fig 3). The theoretical assumptions were next validated experimentally using 0.04% Trypan Blue dye (Fig 3). We showed that indeed only 3 minutes was required for Trypan Blue probe to fully exchange the original medium while the perfusion flow rate was set to 5.25 mL/h.

Next, we validated the compatibility of the chip microenvironment for microperfusion-based culture of nauplii over duration of 24 hours. As discussed, CFD simulations revealed high velocity areas near the engraved channels positioned at the circumference of the holding chamber. These conditions increase potential of damage to Artemia nauplii as they can hydrodynamically immobilize specimens in areas of high shear stress. We therefore validated these

mathematical assumptions by performing a culture of Artemia nauplii perfused on a chip-based device at varying flow rates of up to 22 mL/h for up to 24 hours (Fig 4). The normalized cumulative survival of nauplii perfused at a total flow rate ranging from 5.25 to 22 mL/h was 100%. The cumulative survival of nauplii considerably deteriorated when the microfluidic device was actuated at flow rates lower than 1 mL/h or when chip perfusion was disengaged (stop flow conditions) (Fig 4). The decreased survival at very low flow rates was associated with a depletion of oxygen inside the chip when insufficient exchange of medium inside the gas non-permeable PMMA device was present.[10] Interestingly, only at flow rates exceeding 9.46 mL/h hydrodynamic immobilization of specimens in areas of high fluid velocity was observed. To mitigate influence on behavioral analysis a flow rate of 5.25 mL/h was thus identified as optimal for all toxicity studies.

3.2 On-chip ecotoxicity analysis

Standard Artoxkit-M^{TM} biotest is conducted under static conditions in multi-well microtitre plates, but this method is potentially inadequate due to adsorption, degradation and accumulation of wastes that may severely restrict the exposure to the chemical of interest. Flow-through type devices can be thus beneficial for assessment of toxicity with a plethora of bioindicator species. To validate applicability of microfluidic chip-based technology for heavy metal toxicity screening two reference toxicants were used: potassium dichromate (0 mg/L to 100 mg/L; Sigma-Aldrich, Australia) and cadmium nitrate tetrahydrate (0 mg/L to 1000 mg/L; Sigma-Aldrich, Australia). For on-chip test protocol nauplii were loaded at 30 hph and then perfused in a closed-loop perfusion at the flow rate of 5.25 mL/h with the dilutions of reference chemicals (Figure 5 and 6). For comparison, 24 well plate experiments were also carried out according to standard Artoxkit-M^{TM} (MicroBioTests Inc., Belgium) operating protocol. The conventional scoring of mortality incidence was initially performed for both protocols using manual counting in order to find the LC_{50} values. Our results indicated that statistically similar dose response profiles could be achieved for both reference toxicants in both on-chip and current gold standard conditions. Accordingly LC_{50} values of 625.787 mg/L and 718.070 mg/L were recorded for cadmium nitrate tetrahydrate toxicity tests performed on-chip and reference static conditions, respectively (Fig 5. (A)). Similarly, LC_{50} values of 42.711 mg/L and 31.853 mg/L were recorded for potassium dichromate toxicity tests performed on-chip and reference static conditions, respectively (Fig 6. (A)).

Manual scoring of immobile nauplii in an end-point regimen is, however, time and labour intensive task. Moreover, arbitral and operator-dependent counting assumes test specimens as immobile or dead if no movement is recorded in 10 seconds of observation. This can lead to a significant number of artefacts and inaccuracies in data analysis. We hypothesized that time-resolved experiments using short clips of movies acquired at fixed intervals will provide not only a higher-degree of automation in data analysis but also consistency when coupled

with algorithms to analyze changes in speed of movement or distance traveled by each animal. Moreover such approach would enable to incorporate sub-lethal behavioral endpoints that could prove much more sensitive than mortality for detection of water-born ecotoxicity. Therefore, following on our preliminary validation experiments, we set on to explore the applicability of the microfluidic technology for rapid water toxicity tests based on changes in swimming patterns exhibited by Artemia franciscana nauplii (Fig 5. (B) and Fig 6. (B)).

On-chip behavioural test protocol was performed in a closed-loop perfusion at the flow rate of 5.25 mL/h with identical dilutions of reference chemicals as for mortality tests. 15 second long videos were captured at 15 fps every hour for up to 24 hours of biotest duration and video clips were analyzed using the LoliTrack software. This approach completely replaced the manual scoring process and implemented a high-degree of automation in data analysis. A normalized change of distance covered by test specimens swimming inside the microfluidic chamber was used to evaluate the toxicity effects.

Interestingly at concentrations close to LC_{50} we observed a dramatic decrease of nauplii movement (10-20% reduction of average distance covered in 15 seconds of recording) immediately after medium in the test chamber was spiked with 600-800 mg/L of cadmium nitrate tetrahydrate. The inhibition of swimming activity was progressively more pronounced with the duration of toxicant exposure. Accordingly after 6 hours of exposure to 600-800 mg/L of cadmium nitrate tetrahydrate 40% reduction of distance covered was detected (Fig 5. (B)). At those time points, almost no specimens were scored as dead according to a standard protocol. Importantly the behavioural analysis was also capable of detecting statistically significant toxicant effects at concentrations well below LC_{10} (Fig 5. (B)).

In the case of potassium dichromate we observed a sudden and dramatic increase of nauplii movement immediately after medium in the test chamber was spiked with concentrations ranging from sub-lethal to LC_{90} (Fig 6. (B)). The agitation response can be explained by a reaction to chemical stress that causes the animals attempting to escape the effected area. The agitation of nauplii was followed by a progressive inhibition of swimming activity after 12 hours of exposure to higher concentration of potassium dichromate (Fig 6. (B)). Interestingly, at concentrations close to LC_{50} we observed a decrease of nauplii movement only 18 hours after medium in the test chamber was spiked with the toxicant (Fig 6. (B)). At this time point, however, no specimens were scored as dead according to a standard protocol.

We conclude that sub-lethal, behavioral analysis performed under microperfusion can prospectively provide much more sensitive toxicity end-points as compared to conventional protocols where mortality is used as the main evaluation criterion.

3.3 Proof-of-concept laboratory automation interface

For the Lab-on-a-Chip field and miniaturized bioanalysis to become mainstream laboratory technology, key engineering challenges still need to be addressed. These include among others both on-chip and off-chip integration and simplification of many functional components for a user-friendly fluidic actuation and inexpensive data acquisition. In this work, we concentrated on evaluation of miniaturized and automated video acquisition system that could substitute bulky and expensive conventional microscopes. In this regard, we prototyped a preliminary design of a new off-chip fluidic interface with four main modules: (i) a robotic servo actuator-driven one directional stage that holds chip-based device with multiple test chambers; (ii) a custom and miniaturized peristaltic pump for fluid control and toxicant delivery under microperfusion; iii) ARM-architecture microcontroller handling control over stage movements, pump operation and (iv) miniaturized USB microscope with integrated array of white LEDs and fully programmable time-resolved data acquisition capabilities (Fig 7). The presented reduced functionality prototype brings the future Lab-on-a-Chip system for analysis of small model organism a step closer to realization of complete analytical automation. The system was made as a functional prototype based on the use of standard microprocessors, off-the-shelf components and de-centralized control over several modules making in an inexpensive laboratory automation solution for ecotoxicology (Fig 7.).

4. CONCLUSIONS

In this work, we for the first time demonstrate development of a proof-of-concept microfluidic technology for rapid water toxicity tests based on changes in swimming patterns exhibited by Artemia franciscana (Artoxkit MTM) nauplii. Miniaturized culture of A.franciscana nauplii under laminar fluid flow conditions provided an attractive analytical avenue for the rapid delivery and exchange of reagents with exceptional accuracy. The presented approach enabled us to both automate and significantly increase sensitivity of Artoxkit MTM toxicity biotests as compared to a current standard that is based on manual counting of immobile larvae at 24-hour end-point. The system was capable of performing fully programmable time-lapse and video-microscopy of multiple samples for rapid ecotoxicity analysis. We postulate that innovative Lab-on-a-Chip technologies open a new avenue for inexpensive ecotoxicity test protocols aimed at dynamic detection of sub-lethal behavioral end-points such as changes in speed of movement or distance traveled by bioindicator animal.

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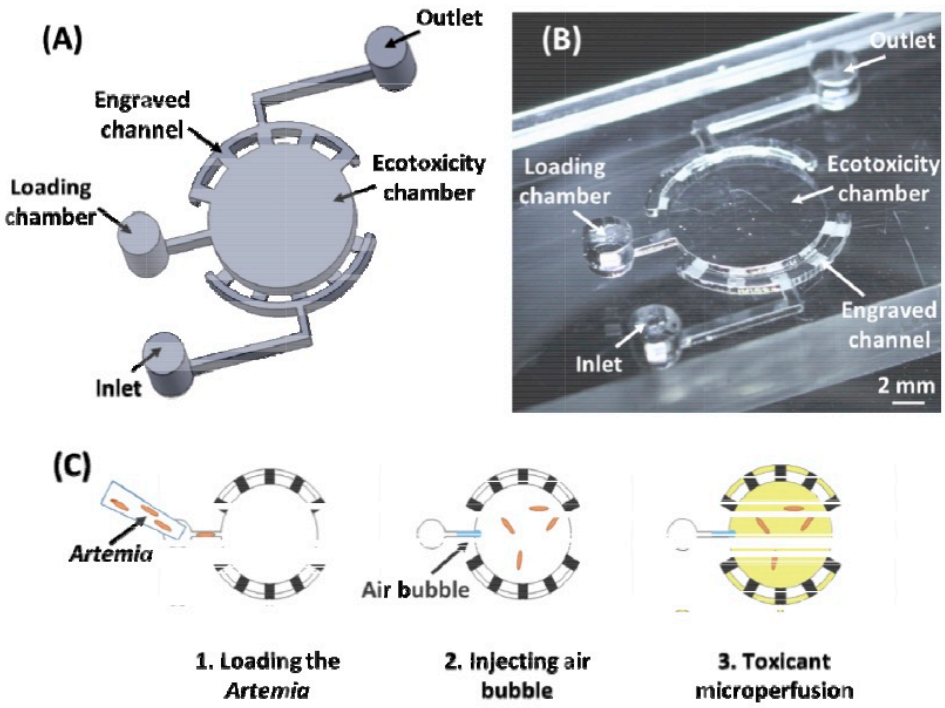

Fig 1. Microfluidic Lab-on-a-Chip technology design. (A) 3D CAD model of the microfluidic circuitry. The design enables immobilization of Artemia larvae within a test chamber (B) Microfluidic circuitry fabricated in a poly(methyl methacrylate) (PMMA) polymer using infrared laser micromachining. (C) Device operation flowchart. 1) Specimens are loaded into a toxicity chamber through a special loading channel; 2) A small air bubble is injected to seal the chamber with trapped larvae; 3) toxicant solution will be pumped through the inlet and flow through the toxicity chamber to the outlet.

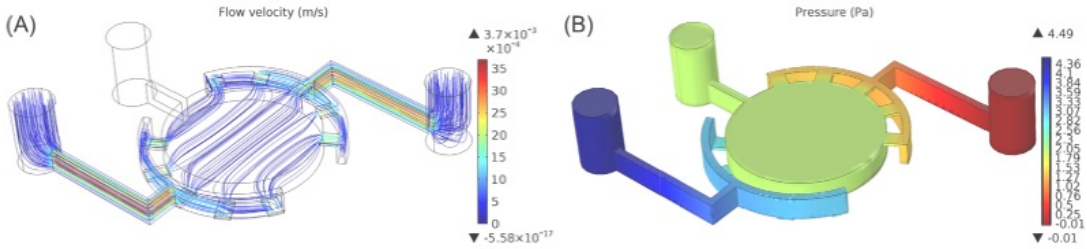

Fig 2. Fluid flow properties inside the LOC device obtained by computational fluid dynamics (CFD) simulation. (A) 3D streamlines of fluid flow across the microfluidic chip colored by velocity (m/s). (B) Contours of water pressure (Pa)

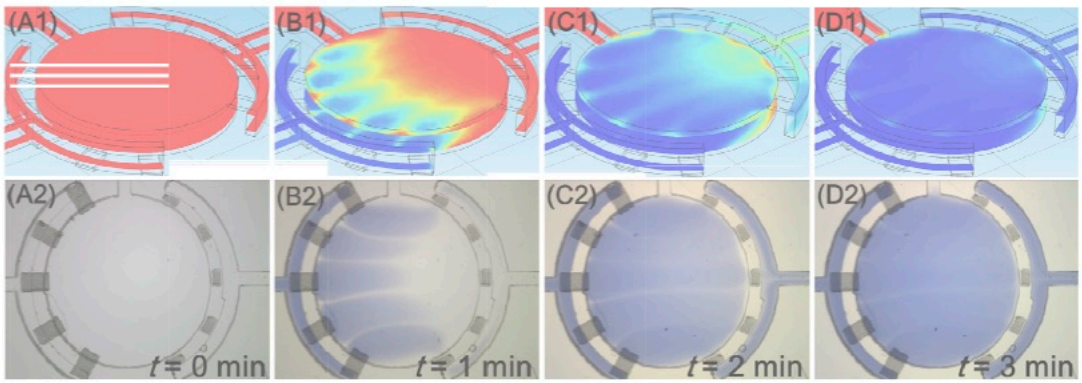

Fig 3. Toxicant delivery inside the microfluidic chip-based device. For validation and comparison both CFD simulations of mass transfer with a model Trypan Blue dye (top panels) and the real experiment were performed. Time-resolved images (bottom panels) were taken at 0, 1, 2 and 3 minutes of perfusion at a flow rate of 5.25 mL/h.

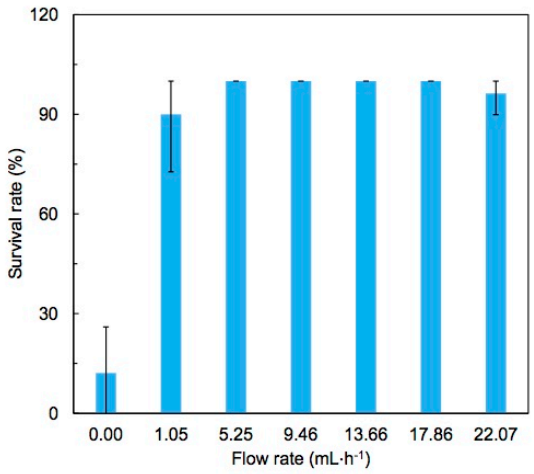

Fig 4. Viability of Artemia nauplii on the chip-based device under varying flow rate condition

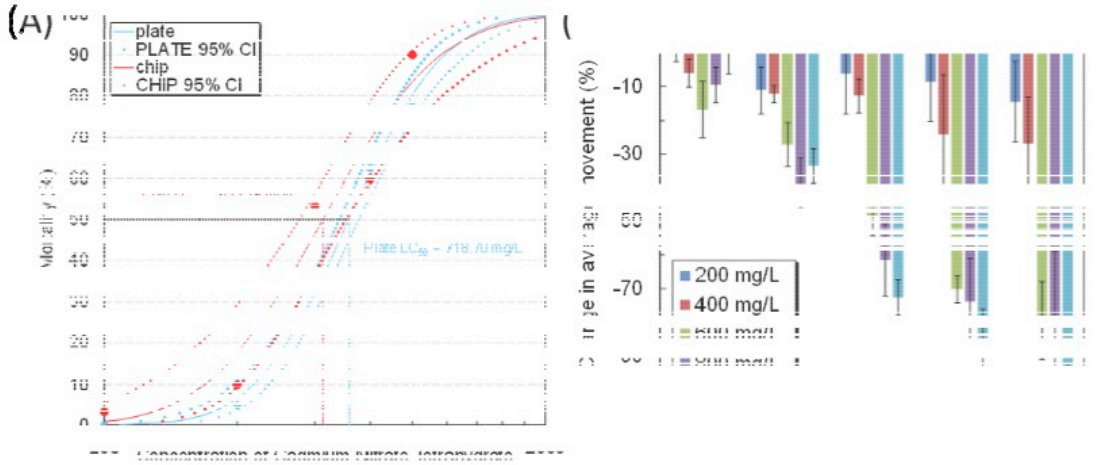

Fig 5. (A) On-chip Artoxkit MTM experiment compared to a standard test performed in 24-well microtiter plates. The acquired data was generated using cadmium nitrate tetrahydrate ($Cd(NO_3)_2{\cdot}4H_2O$. The flow rate is 5.25 mL/h. (B) Toxicity test results of Artemia sp larvae on the chip-based device using cadmium nitrate tetrahydrate. The average moving distance of ten Artemia in one chamber was used to evaluate the toxicity effect.

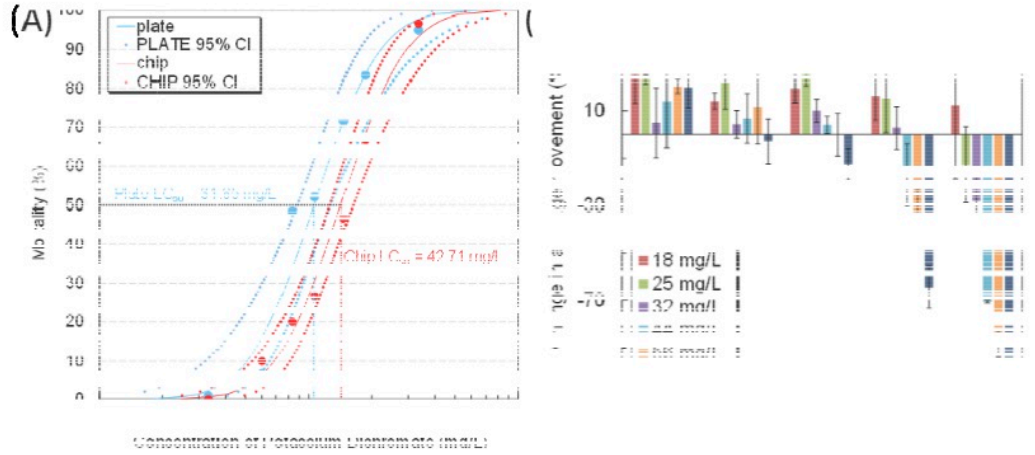

Fig 6. (A) On-chip Artoxkit MTM experiment compared to a standard test performed in 24-well microtiter plates. The acquired data was generated using potassium dichromate ($K_2Cr_2O_7$) as a standard reference chemical for Artemia sp.. The flow rate is 5.25 mL/h. Data suggest an excellent correlation between data obtained in microfluidic environment and standard static multi- well plates. (B) Toxicity test results of Artemia sp larvae on the chip-based device using potassium dichromate ($K_2Cr_2O_7$). The average moving distance of ten Artemia in one chamber was used to evaluate the toxicity effect.

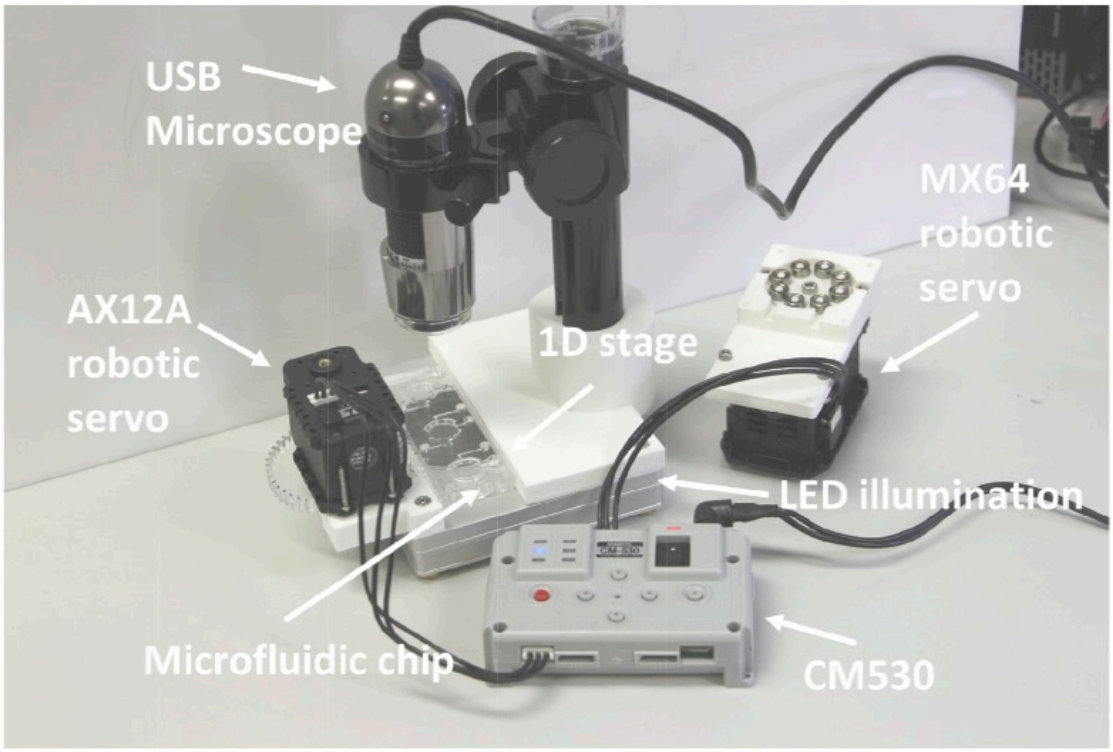

Fig 7 Photograph laboratory automation interface. Major components of the off-chip interface with a mounted multilayer chip include a motorised 1-directional stage and integrated 5MP USB microscope. The microscope and the LED base illumination were powered from computers through USB cables. The robotic servo actuator-driven one directional stage allows precisely controlled chip movement for automatic image capturing of the entire row of embryos. The gears were laser CNC cut in PMMA