Electronic supplementary information (ESI)

Title

Millifluidic chip for cultivation of fish embryos and toxicity testing fabricated by 3D printing technology

Authors

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1. 3D printed 24-way switch valves

Fig. S1 presents 24-way switch valves as a part of a single embryo removal system with a chip interface module connected to the chip. The switch valves were built as manual and as automated systems. The principles and the main parts of both valve types were the same.

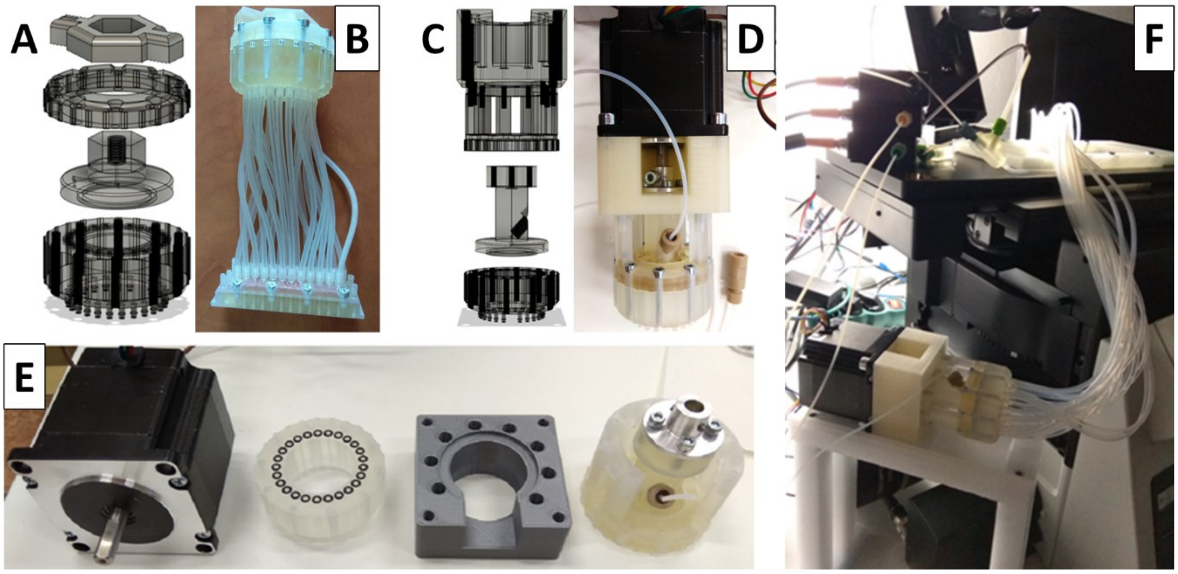

Figure S1. 24-way switch valve for removal of embryos. (A) an exploded view of a 3D model of a manual valve; (B) an assembled manual valve with tubing connected to the interface module on the cultivation chip; (C) an exploded view of a 3D model of an automated valve; (D) an assembled automated valve with a stepper motor; (E) the details of the individual parts of the automated valve (from the left to the right: a Nema 23 stepper motor, a stator with o-rings, a 3D printed interconnection part between the motor flange and the stator holder, a rotor with tubing); (F) an experimental assembly with the chip on the microscope stage.

All components of the manual valve were printed from E-Shell 300 polymer (**Fig. S1A-B**) and the rotor switching was performed by hand. The automated 24-way switch valve (**Fig. S1C-D**) was composed of a rotor, a stator, a Nema 23 stepper motor, an Arduino board, a keyboard, a stepper motor power supply, and cables (all provided by a local electronic store). The details of the individual parts of the valve are presented in **Fig S1E**. A stator holder was printed from PLA polymer by an FDM 3D printer. The stator and the rotor were printed from E-Shell 300 polymer by using a DLP printer. The stator was fixed to the stepper motor flange with the stator holder. Twenty-four outlet ports of the stator were printed on its outer side. The body of the rotor had one inlet with a 1/28 thread to connect the PTFE tubing and one outlet to be directed to one of the 24 stator ports. The rotor was fixed to the stepper motor shaft. The rotation of the rotor was controlled by a keyboard and actuated by a Nema 23 stepper motor. This type of control enabled to direct the flow from the rotor outlet into any of the 24 defined positions of the stator inlets and to the inlets of the lateral channels of the chip. This arrangement performed an independent removal (ejection) of any of the 24 embryos loaded in the cultivation holes. Silicone o-rings provided the sealing of the rotor outlet and the stator inlets. The 24 stator outlet ports were connected to the chip interface module by silicone tubing. The interface module was fixed to the body of the chip by eight M3 screws. The experimental setup is presented in **Fig. S1F**.

2. A chip holder with temperature control

A chip holder with temperature control (**Fig. S2**), was designed to load up to six single cultivation chips. It consisted of four parts (**Fig. S2A**): (i) a bottom layer with ellipsoidal openings for direct observation of the microchannels in the chip, (ii) a middle-distance layer which, in combination with the bottom holder layer, enabled the chip fixation to the holder and defined the right distance of the chip from the microscope stage and the objective, (iii) a top layer for firm fixation of the holder to the microscope stage, with grooves for tubing leading to the chip's inlets, (iv) a tubing fixation layer placed on the top of the top layer of the holder. The model of whole holder assembly is presented in **Fig. 2SB**. The chip holder was designed with CAD software and the first prototypes were made with FDM 3D printer. After the holder design was optimized, it was fabricated from aluminum on a CNC milling machine. The holder had two functions, to hold and to fix the chip on the microscope stage and to control the optimal temperature in the chip and its proximity.

The temperature monitoring and control were provided by an Arduino-based controller. An Arduino board, a keyboard, a 4x16 display, two temperature sensors (holder sensors - HS) located in the chip holder under Peltier heaters, and two temperature sensors (chip sensors - ChS) for feedback loop setup, four Peltier heaters, cables, and a laboratory power supply were used for building the holder temperature control system (provided by a local electronic store). The experimental assembly is presented in **Fig. 2SC**. The system was built with PID regulation. The feedback loop temperature for the holder temperature control was set as an average temperature from the ChS placed in the middle of the chip holder in between three cultivation chips or the temperature from one of the ChS placed in the middle of the chip holder in between two cultivation chips (**Fig. S2D**).

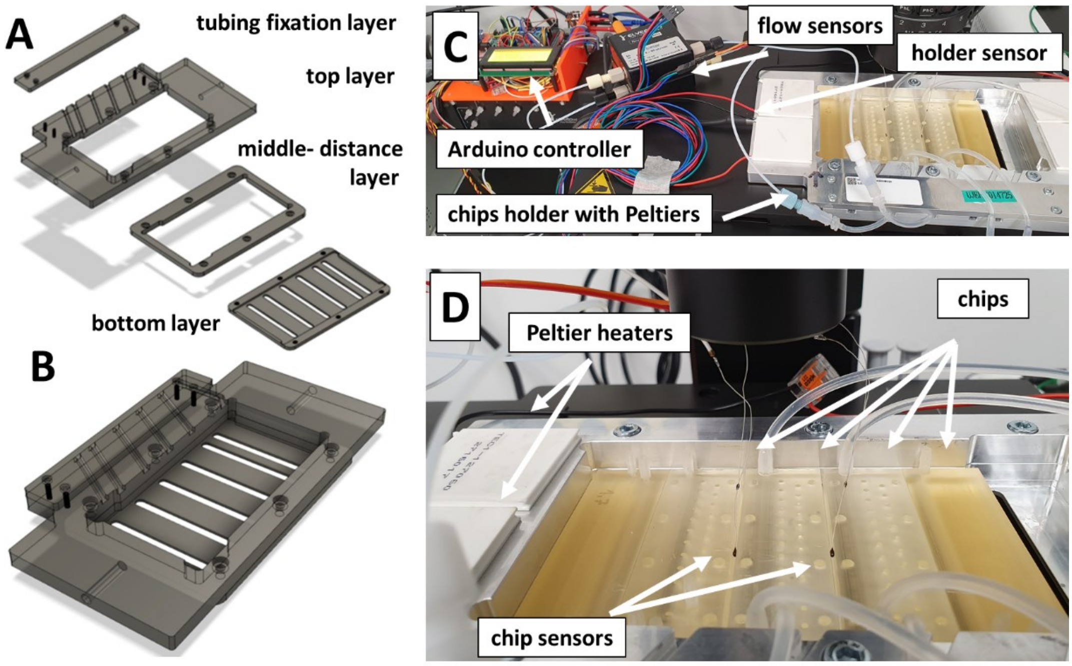

Figure S2. The chip holder with temperature control. (A) an exploded view of the chip holder components; (B) an assembled chip holder; (C) the experimental assembly of the chips (without interface) and of the chip holder on the microscope stage; (D) the details of the chips (without interface) in the holder (temperature chip sensors are included).

3. Selected microfluidic systems dedicated to zebrafish long-term cultivation

Material and manufacturing	Chip properties (Chip architecture)	Manipulation with embryos	Flow rates	Cultivation temperature	Type of assay and perfusion	Reference
3 layers of borosilicate glass, wells covered by silicon sheet and glass layer after embryo loading, assembly compressed in a holder; glass etching and powder blasting, glass-glass bonding;	well (chamber) 1.5-2.0 mm in diameter; 10 µl well volume; array of 32 wells, 8 columns by 4 rows; one embryo per well; parallel wells array - no cross contamination;	embryo loaded by pipetting; not customized for automation;	cultivation at 0.5, 1, 2, 4, 6 µl/ well/min; toxicity assay at 1.0 µL per well per min; 2 µl per well per min equals 14.4 ml of buffer per embryo over 5 days;	28.0±0.5°C;	embryo loading at 8 hpf; cultivation assay; drug assay (ethanol); continuous perfusion;	Wielhouwer et al. [1]
PDMS and glass layer; soft lithography combined with plasma PDMS-glass bonding;	channel 1.7 in width and 1.5 mm in height*; trap (well) volume 2.77 µl and chip volume 825.9 µl; array of 48 traps in 12 rows; one embryo per trap; serial traps array - possible cross contamination; suction channels for immobilization, the need of chip tilting;	embryos loaded through channels; possible automation; no single embryo handling;	cultivation at 0, 0.4, 1, 1.5, 2 ml/min; drug assay at 100 µl/min; closed loop system had the volume 3 ml;	28.0-29.0°C;	embryo loading at 6-24 hpf; cultivation assay; drug assay (anti-angiogenic compounds); continuous perfusion (open and closed loop perfusion tested);	Akaji et al.[2]
4 layers of PMMA (poly(methyl methacrylate)) sheets; Infrared laser micromachining and thermal bonding of PMMA;	trap with conical geometry (1.8 mm at the top and 1.50 mm at the bottom in diameter) channel 1.7 in width and 1.5 mm in height; array of 20 embryos; one embryo per trap; serial traps array - possible cross contamination;	embryos loaded through channels; possible automation; no single embryo handling;	cultivation at 10, 100, 400, 1000 µl/min; drug assay at 200 µl/min;	28.0±0.5°C;	embryo loading at 16 hpf; cultivation assay; drug assay (Tivozanib, Sunitinib, TW37, Honokiol); continuous perfusion (open and closed loop perfusion tested);	Akaji et al.[3]

	suction channels for immobilization, no need of chip tilting; 3D system;					
3 layers of glass; glass etching and diamond drill bit drilling, glass-glass bonding;	well 4 mm in diameter, 1.7 mm in height; array of 7 wells; 3 embryos per well; parallel wells array - no cross contamination; open well cultivation, the need of chip tilting; concentration gradient generator on chip;	embryo loaded by pipetting; not customized for automation;	cultivation at 4 and 100 µl/min; drug assays at 4 µl/min;	26.0°C;	embryo loading at 1-24 hpf; cultivation assay; several drugs assays (doxorubicin, 5-fluorouracil, cisplatin, ascorbic acid); continuous perfusion;	Yang et al.[4]
3 layers composed from glass, silicon wafer and permeable membrane with adhesive, wells covered by membrane after embryo loading; DRIE etching of silicon and anodic glass-silicon bonding;	well 1.4 mm in diameter, 1.0 mm in height; 2 µl well volume; array of 8 well; one embryo per well; parallel wells array - no cross contamination;	embryo loaded by pipetting; not customized for automation;	cultivation and drug assay at 100 µl/h (1.6 µl/min);	28.5°C;	embryo loading at 8 hpf; cultivation assay; drug assays (valproic acid); continuous perfusion;	Choudhury et al. [5]
PDMS and glass layer; soft lithography combined with plasma PDMS-glass bonding;	culturing chambers 6 mm in diameter, 2.5 mm in height; 10 embryos per chamber; chambers combined in parallel and serial array - cross contamination only per drug concentration in well; concentration gradient generator on chip;	embryo loaded by pipetting; not customized for automation;	cultivation at 0, 1, 2, 4, 6, 8, 10 µl/min; drug assay at 5 µl/min;	28.5°C;	embryo loading 3 hpf; cultivation assay; drug assay (aminophylline); continuous perfusion;	Li et al.[6]
Sheets of PMMA (poly(methyl methacrylate));	trap (well) 1.5 mm in diameter and 1.0 mm in height; array of 21 traps;	embryos loaded through channels; possible	cultivation at 0, 1, 10, 400, 1000 µl/min; drug assay at 400	28.0±0.1°C	embryo loading at 6 hpf; cultivation assay;	Zhu et al[7]

Infrared laser micromachining and thermal bonding of PMMA;	one embryo per trap; serial well array - possible cross contamination; embryo loading through channels 1.8 x 1.0 mm (height x width);	automation; no single embryo handling;	µl/min;		drug assay (copper sulphate phenol, ethanol, caffeine); continuous perfusion (open and closed loop perfusion tested);;	
E-Shell 300 resin; 3D printing;	well diameter 1.7 mm (immobilization layer); array of 24 wells in one row; one embryo per well; serial well array - possible cross contamination; embryo loading through channels 1.7 x 1.7 mm (height x width); 3D system;	embryos loaded through channels; possible automation; possible single embryo handling;	cultivation and toxicity assays at 30 µl/min;	25.0°C	embryo loading at 4 hpf; cultivation assay; drug assay (ethanol); continuous perfusion;	presented work

Table 1. **Microfluidic systems dedicated to zebrafish long-term cultivation.** Technical details of selected designs and experimental setups.
* details provided from Khoshmanesh et al. [8]

References

1 E. M. Wielhouwer, S. Ali, A. Al-Afandi, M. T. Blom, M. B. Olde Riekerink, C. Poelma, J. Westerweel, J. Oonk, E. X. Vrouwe, W. Buesink, H. G. J. vanMil, J. Chicken, R. van 't Oever and M. K. Richardson, *Lab Chip*, 2011, **11**, 1815.
2 J. Akagi, K. Khoshmanesh, B. Evans, C. J. Hall, K. E. Crosier, J. M. Cooper, P. S. Crosier and D. Wlodkowic, *PLoS ONE*, 2012, **7**, e36630.
3 J. Akagi, K. Khoshmanesh, C. J. Hall, J. M. Cooper, K. E. Crosier, P. S. Crosier and D. Wlodkowic, *Sensors and Actuators B: Chemical*, 2013, **189**, 11–20.
4 F. Yang, Z. Chen, J. Pan, X. Li, J. Feng and H. Yang, *Biomicrofluidics*, 2011, **5**, 024115.
5 D. Choudhury, D. van Noort, C. Iliescu, B. Zheng, K.-L. Poon, S. Korzh, V. Korzh and H. Yu, *Lab Chip*, 2012, **12**, 892–900.
6 Y. Li, F. Yang, Z. Chen, L. Shi, B. Zhang, J. Pan, X. Li, D. Sun and H. Yang, *PLoS ONE*, 2014, **9**, e94792.
7 F. Zhu, A. Wigh, T. Friedrich, A. Devaux, S. Bony, D. Nugegoda, J. Kaslin and D. Wlodkowic, *Environ. Sci. Technol.*, 2015, **49**, 14570–14578.
8 K. Khoshmanesh, J. Akagi, C. J. Hall, K. E. Crosier, P. S. Crosier, J. M. Cooper and D. Wlodkowic, *Biomicrofluidics*, 2012, **6**, 024102.