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Fishing on Chips: Up-and-Coming Technological Advances in Analysis of Zebrafish and *Xenopus* Embryos

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Abstract

Biotests performed on small vertebrate model organisms provide significant investigative advantages as compared with bioassays that employ cell lines, isolated primary cells, or tissue samples. The main advantage offered by whole-organism approaches is that the effects under study occur in the context of intact physiological milieu, with all its intercellular and multisystem interactions. The gap between the high-throughput cell-based *in vitro* assays and low-throughput, disproportionally expensive and ethically controversial mammal in vivo tests can be closed by small model organisms such as zebrafish or *Xenopus*. The optical transparency of their tissues, the ease of genetic manipulation and straightforward husbandry, explain the growing popularity of these model organisms. Nevertheless, despite the potential for miniaturization, automation and subsequent increase in throughput of experimental setups, the manipulation, dispensing and analysis of living fish and frog embryos remain labor-intensive. Recently, a new generation of miniaturized chip-based devices have been developed for zebrafish and *Xenopus* embryo on-chip culture and experimentation. In this work, we review the critical developments in the field of Lab-on-a-Chip devices designed to alleviate the limits of traditional platforms for studies on zebrafish and clawed frog embryo and larvae.

Key terms

lab-on-a-chip; microfluidics; laboratory automation; zebrafish; *Xenopus*; drugs; bioanalysis; fish embryo test; FET; FETAX

SMALL animal models, for example small vertebrate such as zebrafish (*Danio rerio*) and clawed African frog (*Xenopus laevis*), are widely used in biomedical, pharmacological and environmental studies (1,2). This popularity stems from the fact that both zebrafish and *Xenopus* provide some unique investigative advantages as compared to bioassays that employ cell lines, isolated primary cells and/or tissue samples. The main advantage is the potential for miniaturization and automation of experiments, while still allowing for effects under study to occur in the context of intact physiological milieu, with all its intercellular, multiorgan, and multisystem interactions. Therefore, they bridge the existing gap between the traditional high-throughput cell-based *in vitro* assays and low-throughput rodent *in vivo* tests. Most importantly, small model organisms such as zebrafish or *Xenopus* feature (i) high evolutionary conservation with humans, making them feasible choices for studies of disease pathophysiology and treatment (1,3); (ii) optically transparent tissues enabling the use of optogenetic tools and convenient microscopic examination for detailed physiological mapping of tissues and organs; (iii) simple and cost effective husbandry, generating large numbers of small embryos that develop rapidly and externally, enabling high-throughput screening of drug effects on developing tissues

and organs (4); and (iv) large blastomeres that are amenable to biophysical and electrophysiological testing (5–9). Furthermore, many genetic tools have been developed that broaden the applications of zebrafish and *Xenopus* in biomedical research (10–13). Zebrafish embryo and larvae are commonly used in drug target identification (14,15), in bioactive natural compound discovery (16–20), and in preclinical drug toxicity tests (21–24). In the field of cancer research, transgenic zebrafish models have been used to study cancer dissemination, angiogenesis, mechanisms of cancer cell death, and for new anticancer drug discovery (25–30). In addition, both zebrafish and *Xenopus* embryo bioassays have been deployed in environmental studies, especially for evaluation of aquatic toxins (31–34). For example, the Zebrafish Embryo Toxicity assay (FET) has been introduced as one of the most promising alternative approaches to acute fish toxicity tests (35), whereas the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) is an established bioassay for ecotoxicity and teratogenicity screening (36–39). FETAX has also been applied in forward chemical genomic screening for compounds disrupting development (40,41). Finally, both zebrafish and *Xenopus* are increasingly used in the field of neurophysiological research (12) and in neurobehavioral studies to determine molecular and physiological mechanisms of behavioral development, brain plasticity, and psychiatric treatments (10,13–15).

Despite the importance and widespread use of these two model organisms, most experiments that utilize zebrafish or *Xenopus* embryos and larvae are still performed in multiwell microtiter plates and require laborious and time-consuming manual manipulation of specimens and solutions (1,42,43). Furthermore, static culture of embryos, larvae, and tadpoles can lead to a bias in toxicity studies due to, for example, drug adsorption to the surfaces and secondary effects from the secreted embryo metabolites. Lastly, microtiter plate culture is not conducive to precise specimen positioning and immobilization, both necessary for applications such as high-resolution imaging, electrophysiology measurements, or precise anatomical mapping of physiological processes.

Not surprisingly, many attempts have already been made to improve the existing technologies. Apart from conventional multiwell plate-based culture modifications, innovative designs using microfluidic Lab-on-a-Chip (LOC) technologies and micro-electrical-mechanical systems which promise improved automation and reduced methodological bias, have been demonstrated. Automated imaging techniques and image analysis software can also facilitate the development of truly high-throughput and high-content screening platforms. In this review, we summarize tantalizing technological innovations that offer solutions to the current limitations in manipulation, analysis and sorting of zebrafish and *Xenopus* embryos and larvae/tadpoles.

Perfusion Microtiter Culture

In conventional microtiter plates, static microenvironment limits the compound availability to the specimen due to surface adsorption, degradation and many other factors (44). It can also potentially cause inter-embryo contamination and drug/toxin bystander effects. In addition, static culture does not adequately mimic the dynamic movement experienced by embryos in a natural fluid environment. To solve these problems, a flow-through system for zebrafish embryo culture was developed by Lammer and coworkers (44). The system uses a modified conventional 24-well microtiter plate (44), with the back space filled completely using Spurr's resin. The holes (ϕ = 5–6 mm) were then drilled longitudinally across all the wells in each row (44). To prevent embryos or larvae floating away to the next well, sterile gauze was inserted along the walls of wells (44). To prevent evaporation of volatile compounds, the entire plate was sealed with self-adhesive foils (44). Although the cost of fabrication was very low, the modification procedure was time consuming and labor intensive (44). The liquid surface tension made acquisition of high magnification images using upright imaging systems difficult. In addition, a sheer size of the individual wells and associated volumes of drugs/toxicants that need to be used precludes rapid drug exchange even under perfusion conditions and makes the design an economically impractical solution for any high-throughput studies. It was, thus, a good solution for creating a flow-through perfusion conditions, but one that did not provide true miniaturization or automation.

First Steps to Miniaturization: Microfluidic Chip–Based Embryo Culture

Microfluidics-based culture of small model organisms in their embryonic stage of development evolved from an early generation of semimanual devices to most recent, automated, and integrated platforms. One of the earliest attempts to miniaturize culture of zebrafish embryos using Lab-on-a-Chip technologies was microfluid segment technique (45). The system was comprised of a Teflon (PTFE) tubing coil, an integrated camera and a PC controlled syringe pump (Fig. 1A; 45). It was capable of manipulating embryos using segmented flow with perfluoromethyldecalin (PP9) as the carrier liquid (45). For imaging, embryos were transferred from a microtiter plate into the tubing where fluid flow and image recording were controlled by dedicated software (45). In this design, zebrafish embryos were trapped in each fluidic segment and thus could be imaged individually inside the tubing. The system required the initial culture of embryos to be carried in microtiter plates, followed by an iterative dipping of the sampling tube into the assay plate to aspirate both the embryos and the carrying medium. The authors demonstrated, however, that the segmented flow system and an integrated imaging platform is theoretically capable of achieving high levels of automation (45). The design featured an ingenious use of air bubbles (which were inevitably introduced during sample acquisition) to provide fluid segment stability (45). One of the most significant limitations of this design was an inadequate image quality due to a curvature of the carrier tubing.

The innovative "digital" microfluidic device for on-chip transporting of zebrafish embryos was developed by Son and Garrell (46) with the use of an electromechanical force,

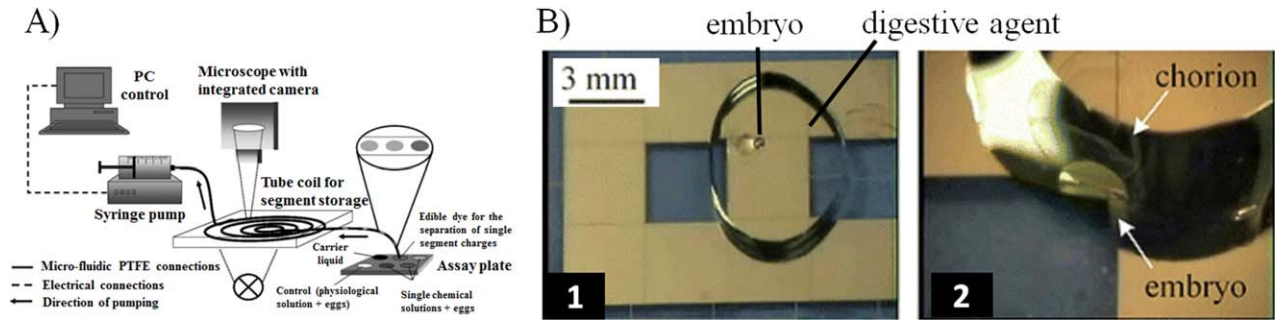

Figure 1. Microfluidic technologies as miniaturized platforms for developing zebrafish embryos. **A**: Segmented flow microculture platform for zebrafish embryo development studies. Microfluid segments were generated by a computer controlled syringe pump. The imaging stage comprised of a microscope, a PMMA plate and 1m-long Teflon (PTFE) tubing roll for storing segments. Samples were aspirated from an assay plate via tubing. Embryos developed in the microsegment, with no visible defects at 74–75 hpf. Reproduced from (Ref. 45) with permission from The Royal Society of Chemistry. **B**: Electrowetting device for moving zebrafish embryos. The figure depicts the dechorionation of 2 hpf embryo via droplet. 1) Digestive reagent mixed with a zebrafish embryo in a total of 40 μL droplet. 2) After 2 h, the chorion was separated from embryo. Reproduced from (Ref. 46) with permission from The Royal Society of Chemistry. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

defined by authors as eletrowetting-on-dielectric (EWOD), in addition to the hydraulic force. Briefly, an embryo-containing droplet was moved within a gap between two plates, with the top plate acting as a common ground electrode, and the bottom plate containing electrodes beneath a dielectric layer to manipulate droplets movement (46). Droplet movements were controlled in 2D by varying electrical potential applied across miniaturized and embedded electrodes. This created an electromechanical force defined by authors as EWOD. In addition, the surfaces of both plates were coated with a hydrophobic material for an easier movement (46). Despite some electrolysis and Joule heating, authors reported that zebrafish embryos remained viable for up to 2 h and developed normally after recovery from microdroplets. Interestingly, a droplet-based on-chip dechorionation was successfully performed by mixing an embryo-containing droplet with a droplet containing a digestive agent (Pronase; Fig. 1B; 46). Electrowetting-based manipulation of discrete droplets can be also used to achieve mixing within otherwise continuous laminar flow on microfluidic devices (47,48).

In 2009, Shen et al. presented a microfluidic chip for zebrafish embryo immobilization, whereby only the inferior of the embryo was exposed to the test compounds, and the embryo viability was maintained (49). The chip was made of two layers of PDMS: the top layer had a funnel-shaped aperture to immobilize the embryo; and the bottom layer contained a microchannel to deliver fluidic samples (49). The chip was submerged in a Petri dish containing fish medium, and a gravity-driven pump was used to deliver test compounds via the microchannel (49). Although such a device worked well as a proof-of-concept, it had inherent limitations in high throughput application as the embryos had to be manually positioned within the aperture using a conventional pipette.

Two years later, Wielhouwer et al. developed a microfluidic flow-through system, fabricated using three layers of bonded borosilicate glass that featured an embedded heating circuitry system (50). It had two independent fluidic manifolds: (i) for buffer solution microperfusion during on-chip embryo culture and (ii) a heater unit circulating warm water to keep the desired temperature for embryo growth (Fig. 2A; 50). Each embryo culture chamber was around 2.5 mm in diameter, much smaller than the wells in conventional 96-well microtiter plate (6 mm in diameter), but it was big enough for a hatched larvae to swim freely in the chamber (Fig. 2A; 50). This device facilitated on-chip flow-through culturing together with very good optical transparency for real-time imaging. In particular, the authors reported for the first time the relationship between the flow rate and embryo development on a microfluidic device. They observed that a high flow rate (6 μL per well per minute) can lower embryo's hatching rate (50). At the same time a nominal flow rate of 2–4 μL per well per minute was necessary to deliver sufficient oxygen, as the chip substratum was gas impermeable (50). The main limitation of this innovative design was again manual loading of each embryo into the culturing chamber.

The same year witnessed Yang and co-workers presenting a chip-based microfluidic embryo array integrated with a concentration gradient generator (51). This chip was made of three bonded glass plates: the top plate, containing networks of micro channels fabricated by photolithography; the middle plate, containing seven embryo culture chambers that were mechanically micromachined; and the bottom layer which constituted a base (Fig. 2B; 51). Embryos were manually transferred into the culturing chambers with each chamber holding up to several embryos (51). Two syringe pumps were used to deliver drugs and medium solution via a concentration gradient microchannels to the chamber inlets, providing serial dilutions delivered independently to each culturing chamber, while removal of discharge waste was facilitated by positioning the chip at around 20° angle (51). The limitations of the device included manual operation, complex assembly and a small number of replicates in an array of microwells. Although each chamber was designed to hold multiple embryos, this can be detrimental to embryo viability and contribute to drug bystander effects. In addition, the chip

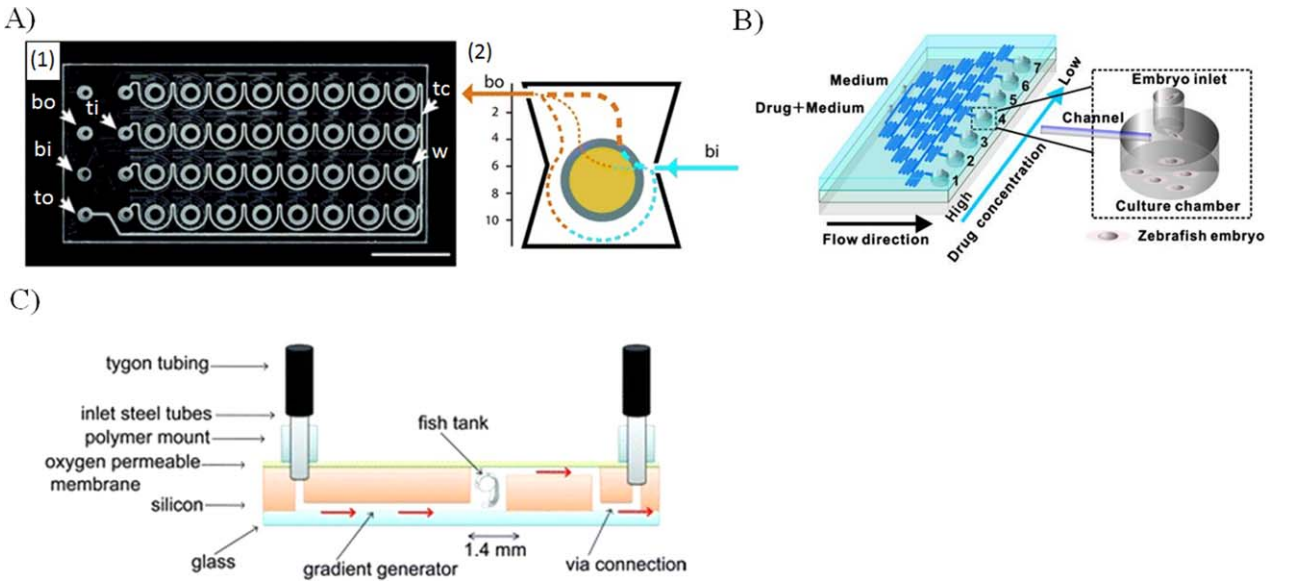

Figure 2. Microwell chip-based embryo culture devices. **A:** Microwell-based microfluidic flow-through system. 1) Overview of the microwell chip. The chip consisted of two independent fluidic circuits: (i) for embryo flow-through on-chip culture and (ii) for pre-heated water warming up the chip system. 2) Cross-section view of a single well. The schematic diagram shows that buffer solutions running around the embryo. The bi, and bo stand for buffer inlet and buffer outlet, respectively. Reproduced from (Ref. 50) with permission of The Royal Society of Chemistry. **B:** Microfluidic embryo array integrated with a concentration gradient generator from Yang et al. Overview of the three-layer chip: microchannel layer, culture chamber layer and the bottom sealing layer. Drug and medium solution were pumped into the chip from two independent inlets. They then generated a concentration gradient in seven outlets that connect to embryo culture chambers. Each chamber can house multiple zebrafish embryos. Embryos were introduced manually into the embryo inlet. Reprinted with permission from (Ref. 51); Copyright [2011], AIP Publishing LLC. **C:** Microfluidic embryo array integrated with a concentration gradient generator from Choudhury et al. Fluidic manifold. Drug and medium solutions were introduced separately into two microchannels. A concentration gradient of eight concentrations was achieved through gradient generator into eight fish tanks. Embryos need to be manually transferred into the fish tank. Then an oxygen permeable membrane was covered on the top of the opening chip. Finally steel connectors and tygon tubing were connected with the inlets and the outlets of the chip. Reproduced from (Ref. 52) with permission of The Royal Society of Chemistry. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

fabrication was complicated and uneconomical, requiring glass etching with hydrofluoric acid (HF).

In 2012, Choudhury et al. described another chip-based microfluidic perfusion platform, which also featured an embedded concentration gradient generator (Fig. 2C; 52). The prototype design was somewhat similar to the device reported by Yang et al. (51), in that its fabrication also involved using photolithography and HF acid etching, and the chip also consisted of three layers, an oxygen permeable and removable polyurethane membrane as a cover; a crystal silicon wafer with fluidic channels and embryo culturing chambers as a core; and glass bonded to the silicon layer as a base (Fig. 2C; 52). Drug stock and medium solution were delivered using computer controlled syringe pumps that create gradient concentration using an integrated on-chip mixer (52). The design featured eight chambers for embryo culture with independent inlets and outlets (52). The culturing chambers constrained embryos from changing positions, which was helpful during high magnification imaging. The inner surface of the microchannels was made hydrophilic to minimize potential air bubbles (52). The main limitation of the device is that it only provides one embryo chamber per concentration, which is insufficient for high-throughput screening experiments. As with previously described devices, this one also requires laborious manual dispensing of embryos into miniaturized wells followed by manual sealing of the chip using a 100 μm thick membrane.

Most of the recent studies have focused on zebrafish embryos that are enclosed in a chorion structure. In contrast to embryos that have uniform spherical geometry and are immobile, hatched zebrafish eletheuro-embryos and larvae have complex geometry and exhibit swimming ability. This presents particular challenges for real-time on-chip imaging due to problems associated with their immobilization and high metabolic rate. Conventional zebrafish larvae mounting techniques involve putting the anesthetized larvae onto agarose gel in a Petri dish, which is laborious and low in throughput. Controlling the orientation of larvae in such setting is also difficult. To solve this problem, a zebrafish larvae agarose-free on-chip mounting technique was presented that allows either lateral or dorsal views (53). These devices, referred to as Zebrafish Entrapment By Restriction Array (ZEBRA) devices, were fabricated both in polystyrene and PDMS (53). The prototypical device consisted of two layers: (i) top layer with mechanically micromachined fluidic channels and larvae holding chambers and (ii) bottom layer providing a sealing base (53). The larvae chamber was designed to enable both lateral and dorsal views of the larvae (53). Loading of larvae was driven by surface tension (also referred to as passive pumping) toward the docking position (53).

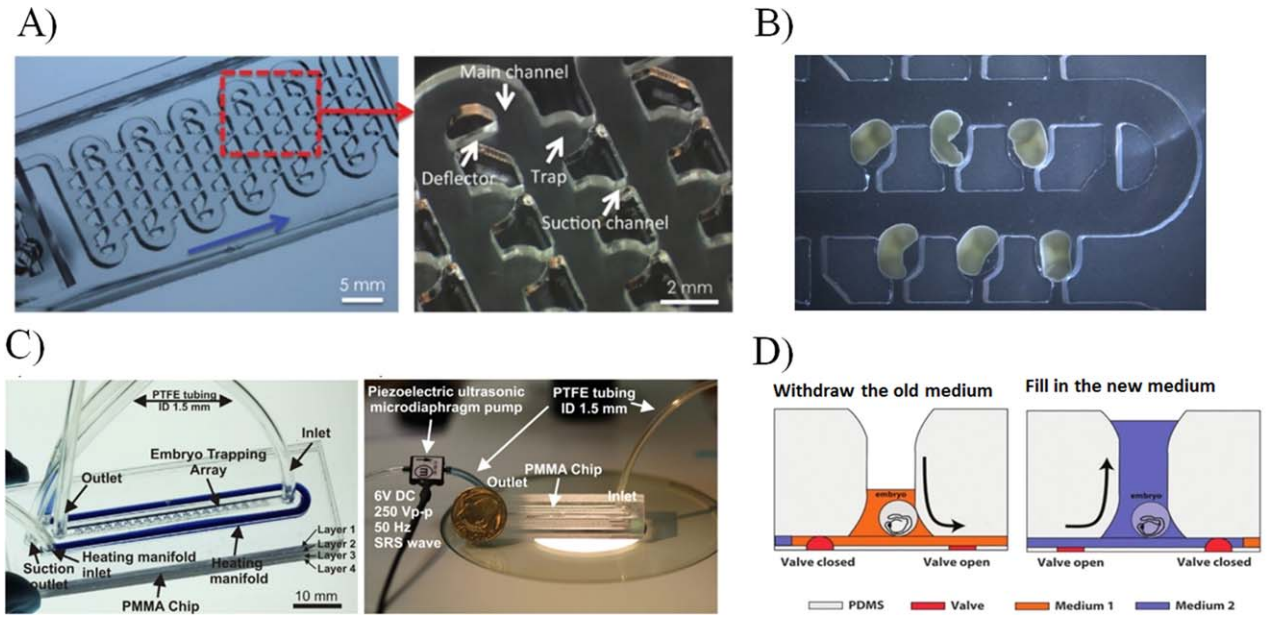

Figure 3. Hydrodynamically and actively actuated embryo trapping array. **A:** A twisted channel hydrodynamic embryo trapping array. The device was originally designed for zebrafish embryo studies. The manifold included a main channel, 12 fluid deflectors, 12 rows of 48 embryo traps, and suction channels that connected each of the traps to the main channel. **B:** A modified twisted channel array designed for trapping *Xenopus* oocytes and embryos. The figure shows albino *Xenopus* embryos docked in the embryo traps (unpublished results). **C:** A 3D multilayer linear embryo trapping array with an embedded heating manifold. Embryos were introduced hydrodynamically from a reservoir into the chip. When passing over the traps, embryos dropped down and stayed in the traps as a spacer, so that following embryo can roll over the occupied traps and head to the next available trap. The chip utilized a piezoelectric ultrasonic pump that minimized the overall size of the system. Reprinted from (Ref. 60), Copyright [2012], with permission from Elsevier. **D:** The schematic diagram of the microwell chip with embedded valves that enable instant fish medium exchange. To change the medium, the inlet valve was closed and the outlet valve was opened. The medium was flushed out by passive pumping. When old medium were drained, the outlet valve was closed and the inlet valve was opened. Fresh medium was introduced by positive pumping. Reproduced from (Ref. 61) with permission of The Royal Society of Chemistry. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

Although manual handling was necessary, this device greatly facilitated high resolution on-chip imaging of zebrafish larvae.

Towards Automation: Hydrodynamic Fluid Trap Embryo Arrays

A majority of designs discussed so far require elaborate manual procedures to load embryos onto the miniaturized devices. This places constraints on the ability to scale up the process into a truly high-throughput system, not only because of time constraints, but also because the embryos are fragile and can be easily damaged during pipetting and handling. To address these limitations, a miniaturized hydrodynamic embryo array for automated trapping, immobilization and on-chip perfusion of zebrafish embryos has recently been developed by the Wlodkowic laboratory (54,55). The chip features several modules, including the twisting channel for embryo loading and medium perfusion, an array of micromechanical traps, as well as a complex system of suction channels that interconnect the embryo traps with the perfusion/loading channel (Fig. 3A; 54). The design allows the use of a hydrodynamic force that guides the embryos into the traps, with the flow in the main serpentine channel generating sufficient drag through the system of suction channels to allow sequential docking of single embryos (54). Docked embryos act as plugs, increasing flow resistance across the occupied trap. This, in turn, leads to redirection of a flow stream into the subsequent traps in each row. The device allowed long-term immobilization inside an array of traps, and did not interfere with normal embryo development for up to 72 h, allowing also for recovery of all the embryos from the device by pumping medium from the outlet (54). The general design drew from the prior work by Tan and Takeuchi, who used principles of hydrodynamic forces and microbubble formation to trap and immobilize beads, paving the way for the use of such an approach for cells and metazoan organisms (56). The Wlodkowic's laboratory was the first to show, however, that the hydrodynamic force can be effectively utilized to provide a higher density of trapping region, creating an ordered microarray of traps with several traps per row, without compromising the embryo docking efficiency. Furthermore, based on the above chip design, an automatic image analysis algorithm was presented to detect the embryo anomalies during their on-chip development (57). In addition, this chip design was modified and fabricated using additive manufacturing processing using multiple 3D printers on several 3D printing materials for rapid fabrication tests (58).

Recently, the same group has also modified their zebrafish embryo array device for *Xenopus* oocyte studies (Fig. 3B, unpublished results). The device uses identical principles to

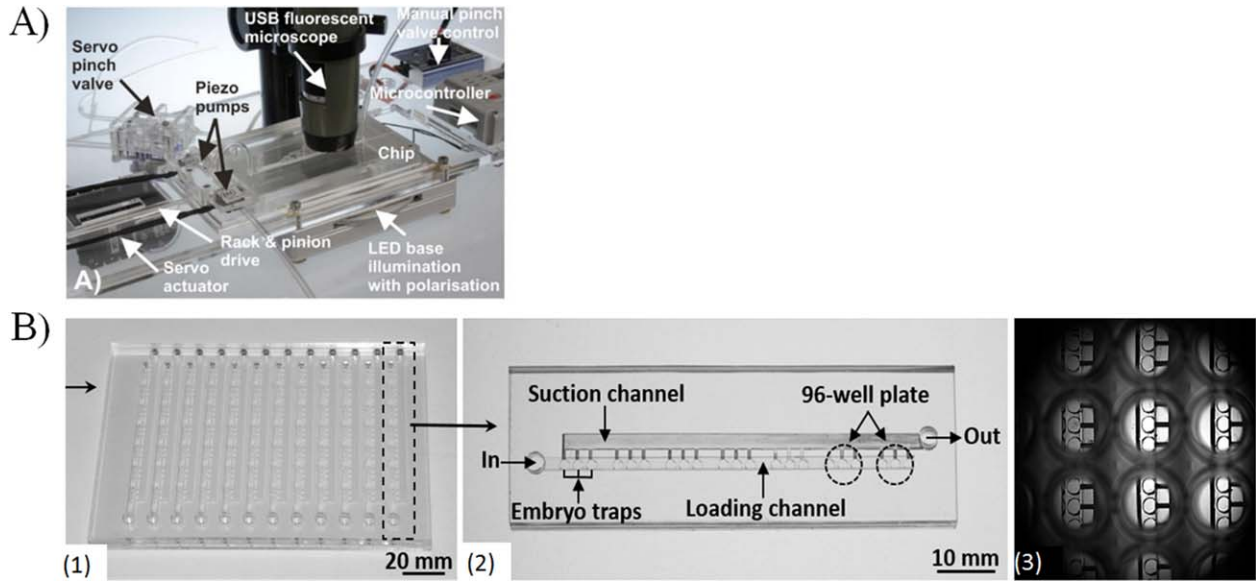

Figure 4. Integrated platforms for bioassays on zebrafish embryos. **A**: A highly integrated platform for zebrafish embryo bioassays. The system includes an embryo trapping chip, a lighting base, a USB fluorescent microscope, a servo pinch valves, two piezo pumps, and a single axial moving stage together with a microcontroller. **B**: A high density linear embryo trapping array. The chip was in the same size as a conventional 96-well microtiter plate, and can be directly used on a plate reader. The chip also used both hydrodynamic and gravitational forces for operation, but it has suction channels located in the side of the embryo traps, enabling imaging from both upright and inverted microscopes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

load *Xenopus* oocytes into traps and immobilize them individually for high resolution imaging under continuous perfusion.

In addition, the group has also developed a 3D microfluidic device suitable for real-time analysis of transgenic zebrafish embryos (59,60). This multi-layer chip was made of four poly(methyl methacrylate) (PMMA) sheets, which featured loading and suction channels, embryo traps and an integrated heating manifold (Fig. 3C) (60). Each layer was laser cut or engraved separately, and then thermally bonded together in an oven (60). The embryo docking was achieved in this design by combining sedimentation of embryos and low-pressure suction. The embryos were aspirated from a reservoir via tubing, and then they were driven along the loading channel by suction force that was created at the outlet of the chip using a piezoelectric ultrasonic pump (Fig. 3C). When embryos flowed above the traps, they gravitationally sank into the traps assisted by suction force from micro suction channels that were located beneath the traps (60). The trapped embryo filled the space so that a second embryo could not fit into the same trap. After embryo trapping, compound solutions can be delivered from the loading channel to each docked embryo (60). The design provided high trapping efficiency, single embryo per trap occupancy, and spatial encoding of each embryo in a pre-defined location. The U-shaped heating manifold that surrounded the embryo traps can introduce warm water flowing throughout the chip device, providing a proper temperature for embryo on-chip culture (60). Such design offers rapid device fabrication together with automated embryo manipulation and perfusion.

One of the most recent devices, introduced earlier this year, is a microfluidic platform with enhanced fluidic actuation capabilities, fabricated using elastomeric PDMS layers that form embryo chambers (61). Apart from its microwell plate function, it features an embedded monolithic micro-valve system, controlled pneumatically by actuators (Fig. 3D; 61). This flow manipulation system can dynamically renew the medium within 10 seconds (61). The device is still under development, but the monolithic micro-valve design is a promising concept for many applications that benefit from well-controlled solution delivery.

On Chip Integration of Analytical Platforms

In a bid to further improve microfluidic embryo culture designs, an integrated laboratory automation platform was developed by Wang et al. (62). Apart from the microfluidic chip, the authors integrated a microfluidic actuator, temperature regulator and an image acquisition system (62). All the units were controlled by a Field Programmable Gate Array embedded microcontroller (62), which brought the system closer to complete automation. Recently, the Wlodkowic's group also reported on further platform development by adding a separate drug delivery manifold with miniaturized piezoelectric pumps enabling embryo loading and drug delivery to be controlled separately with an integrated servo pinch valve (63). Furthermore, authors used an optically transparent indium tin oxide heater to replace the previous flow-through heating manifold (63). Finally, a miniaturized USB fluorescent microscope was integrated for imaging together with an LED illumination and a polarization base (63). In addition, a motorized rack and pinion drive was embedded within the stage that can drive the chip for imaging (Fig. 4A; 63). All the

units were controlled by PC and ARM microcontrollers (63). Together with embedded sample and waste bottles, the authors managed to greatly miniaturize the size of the platform (Fig. 4A).

For chip-based flow-through devices, experiment setup could be complicated because of the leaking at the connections between the tubing and fluid inlets as well as air bubble trapping in the micro channels. Recently, a high-throughput zebrafish embryo trapping array together with a dedicated chip installation cradle was described by Zhu et al. (64). The chip can hold up to 252 separately immobilized embryos for on-chip flow-through culturing and real-time imaging (Fig. 4B; 64). Similar to Wlodkowic's design (60), the chip used a combination of hydrodynamic and gravitational forces for embryo loading, immobilization, and drug perfusion. The suction manifolds were located in the side of the traps, which enabled imaging with the use of an inverted imaging system (64). The chip can fit into the conventional 96-well microtiter plate readers, or alternatively can be interfaced with the customized cradle. The hinge-like cradle sandwiches the culturing chip and provides an interface between the chip connection ports and the tubing. This design prevents leaking and air bubble trapping. It also speeds up the chip installation and does not require manual insertion of tubing interconnections (64).

Special Applications for Chip–Based Culture Systems

Chip-based designs are used not merely to allow immobilization and spatial segregation of embryos, or to enable finely controlled fluid exchange, but most importantly to facilitate improved biochemical investigations, including a range of physiological measurements and imaging analyses.

Faster, Better: Electrophysiological on–Chip Measurements

The traditional two-electrode voltage clamp (TEVC) technique is widely used to perform electrophysiological measurements in *Xenopus* oocytes. Recently, a chip-based system has been designed that allows a refined performance of TEVC, particularly in terms of improved fluid exchange around oocytes which facilitates studying fast ionic movements across cell membrane channels (65). The chip was fabricated using three layers of soft biocompatible polymer polydimethylsiloxane (PDMS): the top layer consisted of an oocyte aperture, a perfusion channel, and the electrode channels; the middle layer was a thin (10 μm) membrane used as a part of the integrated pneumatic valve; and the bottom layer was comprised of valve channels and backside access apertures (65). After the oocyte was immobilized in the aperture, two computer controlled syringe pumps provided flows to the aperture via the valves. The device facilitated a significantly simplified oocyte positioning with fluid exchange occurring in a scale of tens of milliseconds.

A noninvasive asymmetrical transoocyte voltage clamp system using multilayer microfluidic device has also been developed for electrophysiological studies on *Xenopus* oocytes under extreme environmental conditions, for example, microgravity (66). The core part of this device was a PDMS microchip used for interfacing fluid pathways and electrodes (66). The chip was covered by three computer numerical controlled-machined PMMA layers: the top one was for maintaining air pressure; the middle one was for holding the oocyte and electrode; and the bottom one was for perfusion (66). Such design offers electrical access to the cytosol of an immobilized oocyte without the need for manual intervention (66).

Environmental Scanning Electron Microscopy (ESEM) Interface

Some applications, such as ESEM, require devices that are not only small and automated, but also provide an open surface with no water surrounding the embryo or larvae. A solution to this challenge was offered by the Wlodkowic's group, who developed a chip fabricated in PMMA sheets that were laser cut and thermally bonded together (67). The chip was comprised of three units: (i) a trap reservoir where larvae can be trapped in engraved micro wells; (ii) a drain reservoir where excess water can be drained by filter paper; (iii) a set of engraved micro channels that connect the trap reservoir to the drain reservoir (Fig. 3F; 67). In this design, the force created by draining water ensured larvae immobilization in the wells (67). With the assistance of this device, multiple zebrafish larvae can be aligned and immobilized in the chip for ESEM imaging (Fig. 5).

Embryo and Larvae Sorting

An automated zebrafish embryo sorting and dispensing system called ZebraFactor was developed in 2011 by Graf et al. (68). The system consisted of two units: the CellSorter, with motorized motion rings, LED arrays and two cameras that can sort out unfertilized or damaged embryos; and the WellPlate-Feeder, that can automatically populate a 96-well microtiter plate with one embryo per well accuracy, within about 11 min (Fig. 6; (68)). Sort decisions were achieved by applying an on-the-fly image analysis algorithm coupled with a high-speed video camera. The incorporated vision algorithm was based on a neuronal network, and could distinguish embryos based on their fertilization status, damage/health, size, and shape. Although the reported sorting speed is similar to manual dispensing, the advantage of the robotic system is a continuous and unsupervized operation that can achieve an overall higher throughput per day. At the same time, robotic sorter is capable of realizing high levels of reproducibility. The survival rate of sorted zebrafish embryos was reported at well above 90%. In addition, this system was also modified to other large organisms such as *Xenopus* oocytes and zebrafish larvae (68).

Letamendia et al. presented a fully automated high-throughput zebrafish embryo screening system (69). This robotic system comprised of a master PC, an embryo sorter, two liquid handling devices, an automatic incubator, a plate feeder, a plate transporter, and an imaging system with

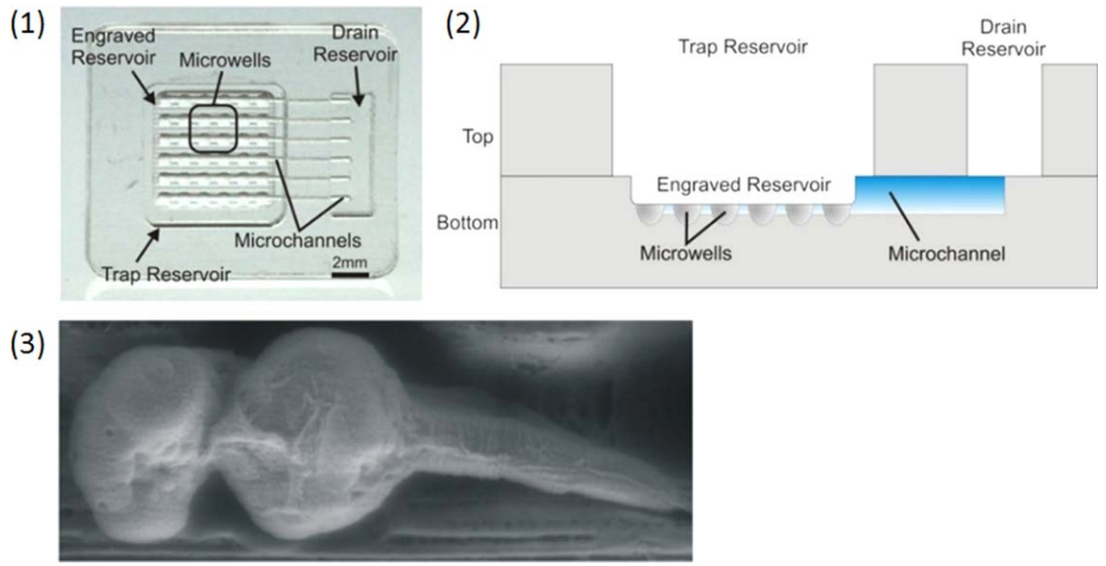

Figure 5. A microchip for ESEM studies. Larvae were first placed onto the engraved microwells, and then water was drained from draining reservoir. By doing this, water can be completely drained out while larvae dock in the traps. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

software (69). The embryo sorter was able to detect embryonic development stages and fluorescence intensity so that only healthy embryos with enough fluorescence were counted and accepted for sort decisions (69). After sorting, the selected embryos were dispensed into a multiwell microtiter plate. The filling of the entire 96-multiwell plate took ~3–5 minutes (69). Two liquid handling devices operated in parallel to deliver test samples into the plates for screening (69). After that, a microscope with a camera scanned the microtiter plate well by well and automatically acquired images by software (69). Finally, a customized image analysis software processed the images and generated the results in Excel and Word formats. For example, in a cardiotoxicity assay, heart beat frequency was determined, whereas in an angiogenesis assay, the number of intersegment vessels (ISV) was recorded and automatically scored (69).

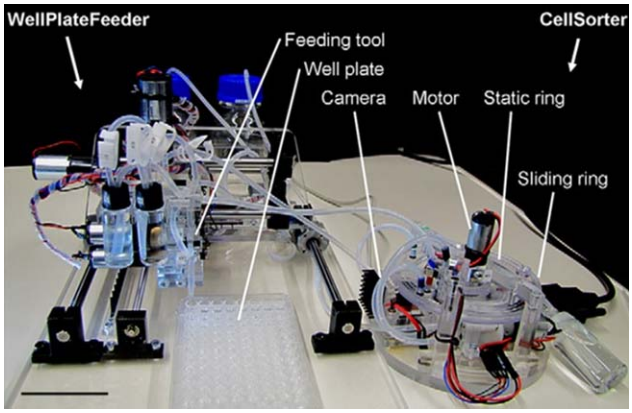

Figure 6. The overview of the embryo sorting system. The combination of the CellSorter and the WellPlateFeeder is able to sort out unfertilized or damaged embryos and then automatically feed a 96-well microtiter plate in about 11 min with one embryo per well. Reprinted from (Ref. 68) Copyright (2011) by Permission of SAGE Publications. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Harnessing Flow Cytometry for Analysis of Zebrafish and *Xenopus*

A Fountain Flow™ Cytometry (FFC) was originally presented as a platform for rapid detection of microorganisms in aquatic samples (Fig. 7A; (70,71)). Based on this technique, Fini et al. developed a fully integrated system for real-time cytometric interrogation of transgenic *Xenopus* tadpoles. The system design directed the tadpoles through a flow cell and toward an interrogation window integrated with a digital camera and 488 nm solid-state excitation source (Fig. 7B; (72)). The modified device was equipped with blue light-emitting diodes (LED) and a digital camera, capable of processing enhanced green fluorescent protein (EGFP) transgenic xenopus tadpoles in real-time analysis (72). A gravity-driven flow was used to continuously circulate tadpoles through the flow cell located between two miniatirized holding reservoirs. The technology was validated using transgenic *Xenopus laevis* tadpoles that carried a chimeric gene that responds to heavy metals (metallothionein promoter from zebrafish; MTZF-eGFP) allowing the system to be readily used to detect heavy metal pollution in water samples (72). Importantly, the FFC technology supported a noninvasive and real-time examination of *Xenopus* tadoles without the use of any anaesthetic agents. This greatly minimized stress experienced by specimens and yields statistically reliable data.

In addition, an interesting imaging cytometry technology was developed by Trophos SA (Marseilles, France) under the trademark Plate RUNNER HD®. This high-speed and high-definition imaging cytometer was designed to fulfil the tasks associated with high-throughput compound screening routines. The design employs a large field of view (8 mm) optics

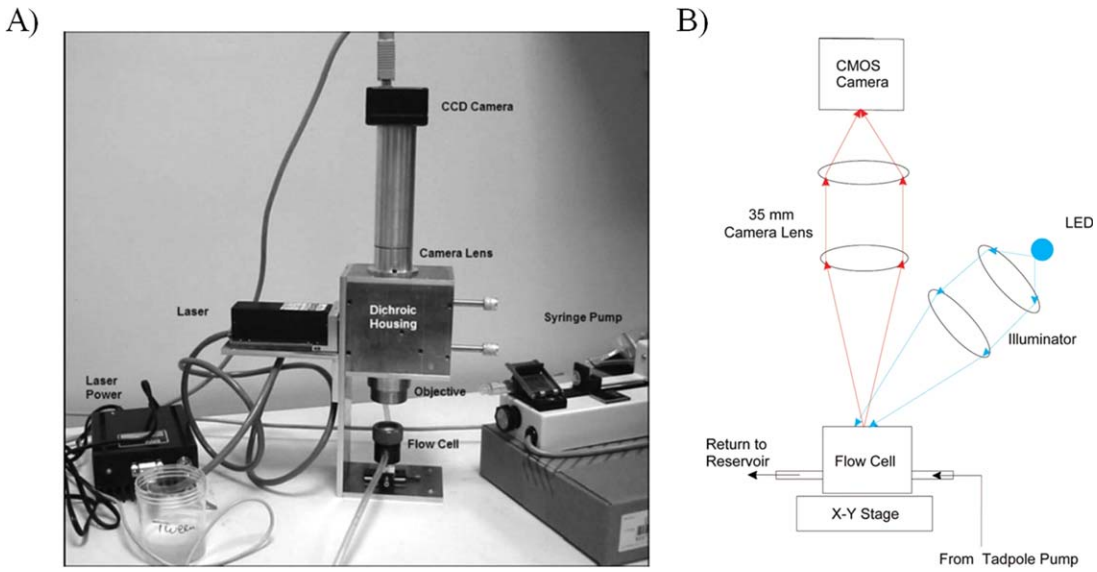

Figure 7. Fountain Flow Cytometry. **A**: The original Fountain Flow detection system using laser for illumination. Reproduced from Ref. 70 with permission of Wiley. **B**: The schematic diagram of the modified Fountain Flow Cytometry integrated with a digital camera and blue (488 nm) LED excitation that enable processing of EGFP transgenic *Xenopus* tadpoles in real-time analysis. Reprinted with permission from (Ref. 72) Copyright [2009] American Chemical Society. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

that covers the entire surface area of a single well on a 96-well microtiter plate. Plate RUNNER HD features a maximal optical resolution of 1 μm and depth of field of about 8.5 μm. Moreover, the multicolor imaging is supported by up to three-wavelength excitation/emission. The platform has recently been integrated with innovative chip-based devices enabling high resolution and high throughput imaging of developing zebrafish embryos in a microperfusion environment (64). This work demonstrates that integration of an innovative chip-based device with a scanning cytometer can be successfully applied for accelerated antiangiogenesis drug testing using transgenic Tg(fli1a:EGFP) zebrafish embryos (64).

Vertebrate Automated Screening Technology (VAST)

Recently an innovative technology that combines robotics and microcapillary fluidics was reported for fully automated high throughput screening (73,74). This highly integrated platform could load zebrafish larvae from either a microtiter plate or a bulk reservoir into detection capillary. Larvae were interrogated by a photodiode and two light emitting diodes. The system supported a two-parameter analysis of the transmitted and scattered light. The light scattering parameters were also used to recognize biological specimens from residual air bubbles and/or debris (73). Following detection, larvae were introduced one by one into a glass capillary using a syringe pump and positioned in a confocal imaging system (Fig. 8A). Another innovation of VAST included a 3D-axis computer-controlled stage capable of fine manipulation and positioning of the microcapillary assembly (Figs. 8B–8D). The 3D-axis stage facilitated imaging of larvae organs from multiple angles. Moreover, the system was successfully interfaced with a femtosecond laser microsurgery technology. Following microsurgery and microscopic interrogation, the specimens were non-invasively recovered either into a multiwell plate or a reservoir (73). The further development of the VAST platform was a multithread system that can process multiple animals simultaneously (74). The authors also optimized the capillary tube material with ultra-thin borosilicate that facilitates confocal and fluorescent imaging (74). This technology greatly facilitates *in vivo* specimen manipulation and cellular level organ imaging and is currently available commercially under a trademark of VAST BioImager^{TM} Platform (Union Biometrica).

Data Analysis Algorithms, A Final Bottleneck

Apart from automation of handling the specimens, another major factor required to increase the throughput of chemical screens on embryos and larvae of small model organisms is dedicated software to automatically detect and quantify specific phenotypes (75–78). In addition to the aforementioned fully automated VAST system, several other works have also been reported. Kokel et al. developed an automated platform that was able to analyze behavioral effects of zebrafish embryos cultures in 96 well plates (75). They managed to translate complex behavioral changes into simple barcodes to classify the drugs and gain insights into their mechanisms of action (75). Vogt et al. presented an automated image-based phenotypic analysis system (76). Such a system can detect the embryos from 96-well microtiter plates regardless of their orientation, and quantify the growth of ISV in a specific region of the embryo (76). The collected data support the notion that a laser scanning confocal reader, coupled with custom developed image analysis algorithms based on Definiens Cognition Network Technology, can be employed for automatic

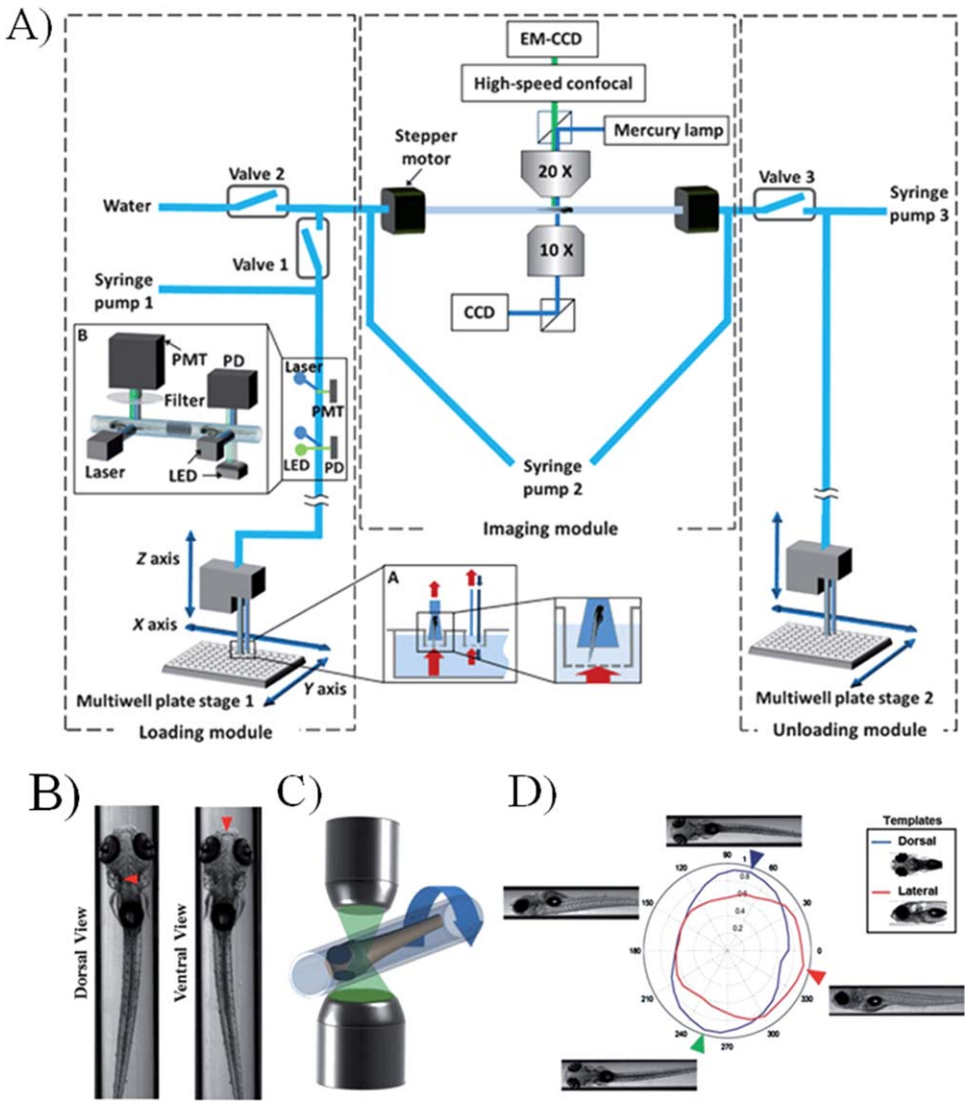

Figure 8. VAST platform for high-throughput zebrafish larvae manipulation and image detection. **A**: The schematic diagram of the VAST system. The system consisted of three robotic modules: the loading module can load zebrafish larvae from a multiwell plate for detection and positioning; the imaging module was featured with a high-speed confocal camera, enabling high quality, and rapid focusing and imaging; and the unloading module can dispense larvae back to a multiwell plate. **B–D**: in a microcapillary, larvae orientation can be easily adjusted for imaging. Reproduced from (Ref. 74) with permission of The Royal Society of Chemistry. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

analysis of GFP expression profiles in transgenic zebrafish embryos. Xu et al. developed a high-throughput analysis algorithm that can quantify pigment of zebrafish embryos (78). This innovative algorithm could rapidly discriminate between the head and torso, and extrapolated the boundaries matching the back and abdomen of zebrafish embryos. These software capabilities to extract biological data by high-throughput image processing are still waiting to be integrated with automated on-chip culture and analysis platforms.

Conclusions and Future PS

Conventional methods for screening small vertebrate model organisms, such as fish and frog embryos, have been hampered by labor intensive manipulations and lack of reliable, user-friendly, and affordable laboratory automation. Manual handling techniques that are commonly employed at present are time consuming, introduce substantial analytical bias and do not offer high-throughput processing capabilities required by the pharmaceutical industry. This experimental landscape is progressively undergoing transformation. With the advent of microfluidic Lab-on-a-Chip technologies, a plethora of innovative devices for automated manipulation and bioanalysis of zebrafish and *Xenopus* embryos open new possibilities for high throughput screening. Lab-on-a-Chip platforms are now broadly regarded as the next generation of bioanalytical technologies with wide reaching applications in biotechnology. While microfabricated technologies offer

previously unanticipated levels of automation and precision, with applications ranging from phenotypic drug discovery screens to ecotoxicology biotests, there is still a need for interdisciplinary efforts to integrate not only culture and analytical hardware, but also image processing and data analysis algorithms. Only then will the vision of truly automated platforms for small model organism-based research be truly realized.

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