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Abstract Zebrafish embryos can be obtained for research purposes in large numbers at low cost and embryos develop externally in limited space, making them highly suitable for high-throughput cancer studies and drug screens. Non-invasive live imaging of various processes within the larvae is possible due to their transparency during development, and a multitude of available fluorescent transgenic reporter lines. To perform high-throughput studies, handling large amounts of embryos and larvae is required. With such high number of individuals, even minute tasks may become time-consuming and arduous. In this chapter, an overview is given of the developments in the automation of various steps of large scale zebrafish cancer research for discovering important cancer pathways and drugs for the treatment of human disease. The focus lies on various tools developed for cancer cell implantation, embryo handling and sorting, microfluidic systems for imaging and drug treatment, and image acquisition and analysis. Examples will be given of employment of these technologies within the fields of toxicology research and cancer research.

Keywords Zebrafish • Cancer • Automation • High-throughput • Robotics • Microfluidics • Image analysis

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Introduction

The use of zebrafish in cancer research has become increasingly widespread, and different models have been developed for a variety of cancer types. In initial models, tumor development was induced by the exposure of embryos, larvae or adult fish to carcinogens, giving rise to hepatic, mesenchymal, neural and epithelial neoplasms [1–3]. Then, with improvement of techniques to generate transgenic animals, Langenau et al. developed the first transgenic cancer model in zebrafish, in which expression of murine *mMyc* in lymphoid cells drove the onset of leukemia [4]. Other transgenic cancer models followed [5–15], such as activated human $BRAF^{V600E}$ leading to invasive melanoma formation in p53-deficient fish [7], or an embryonic model for rhabdomyosarcoma induced by activated human *RAS* [9]. In addition to transgenic models, xenotransplantation models were developed, and tumors cells from a range of cancer types and species were shown to be able to proliferate, migrate and induce neovascularization [16–24]. In these models, cells can be implanted at different stages, from blastula and later embryonic stages to (immunosuppressed) adults, as well as in different sites, like the yolk, Duct of Cuvier [25], perivitelline space [17] or brain cavity [26]. In addition to xenotransplantation with cancer cells of human or murine origin, allotransplantation with cells from transgenic zebrafish cancer models or zebrafish transformed cells have been performed [27, 28].

Many papers highlight the opportunity provided by these animals to perform large-scale chemical screens in aid of finding novel anti-cancer drugs. Before gaining ground as a model for cancer, zebrafish embryos were used in screens identifying small molecules affecting development [29], and immersion of embryos in compounds is now an established technique for treatment [30, 31]. With the development of various cancer models in zebrafish, performing screens with large libraries of compounds to find improved treatment strategies for patients is a logical next step. However, performing large scale screens can be a labor-intensive, monotonous task, and automation of different steps of the process would both increase speed, precision and reproducibility of results.

Automated systems for injection, compound treatment, imaging and data analysis are being developed. Many of these new systems are designed with zebrafish embryos in mind, not adults, so the main application will be for engraftment models, or transgenic models where there is a distinct phenotype in larval stages. Here, we will provide an overview of the advances in automation regarding each stage of cancer research in zebrafish embyos and larvae.

Automated Microinjection Systems

Microinjection is an indispensable technique in zebrafish research, with many applications. Microinjection is used for generation of transgenic lines, mutant lines (using the TALEN or CRISPR/Cas9 system), transient gene knock-down (using morpholinos, siRNA or antibodies), transient gene overexpression (by mRNA injection),

infection studies (by injection of microbes) and cancer cell engraftment. Most of these injections are performed in embryos up to 16 cell stage, and currently available automated injection systems are designed with this stage in mind. At these early stages, the yolk cell is the largest cell in the embryo, and compounds injected there will be taken up by neighboring cells. However, with the rapid development that embryos are undergoing, injections have to be performed at a high rate, to ensure proper uptake by all the cells of the embryo.

Two automated injection systems for zebrafish were reported in 2007. In the first system, published by Wang et al., embryos were positioned using a 5×5 vacuum-based holding grid [32]. Using image recognition software, different structures in the embryos could be recognized, and the site of injection was determined based on this information. In this first injection system, 25 embryos were injected per 2-minute run, with an accuracy of 99 %. The second injection system, published by Hogg et al., was primarily designed for injection of south-African clawed frog *Xenopus laevis* oocytes, but the application for zebrafish microinjections was also shown [33]. Here, embryos were placed in commercially available 96-well microplates with conical wells, and the site of injection was based on the spacing between wells. This negated the need for image capturing and recognition software. The setup had a plunger-driven injection system, which allowed automated cleaning and refilling of the injection needle, making it possible to inject up to seven different solutions. Up to 600 injections could be performed per hour.

In 2011 Carvalho et al. published an automated injection system that, in addition to being suitable for gene disruption injection in early-stage embryos, could also be applied to inject pathogens such as *Mycobacterium marinum* into the yolk of embryos of up to 1024-cell stage [34]. In this injection system, embryos were positioned in an agarose grid with a honeycomb pattern of hemispherical wells, and the site of injection was based on the consistent spacing between the wells (Fig. 1a). The freshly cast agarose grid could be designed according to the experiment in mind, with a variety in number of wells for small or large scale experiments, or multiple small grids of wells to distinguish groups of embryos injected with different compounds or parameters (Fig. 1b). With a built-in camera, the volume of the injected droplet could be calibrated on-screen, and easily adapted during the run of the experiment, if desired. With this robotic injection method, the authors showed that embryos could be infected with *M. marinum* bacteria at a rate of 2000 embryos per hour. This high rate of infection makes it an attractive tool for high-throughput screens. Furthermore, successful morpholino injection at one to two cell stage has been described in this system, as well as the possibility to perform gene knockdown by injection of antibodies or CRISPR/CAS constructs, and generating transgenic lines by DNA injection [35]. For additional details, we refer to publications by Veneman et al. that provide for an overview of the setup [36] and its application in *Staphylococcus epidermidis* infection studies [37].

The possibilities for performing cancer research in zebrafish by manual xenotransplantation has been shown in a large number of papers. Therefore the applicability of an automated injection system for cancer cell xenografts was also investigated. An overview paper by Spaink et al. reports that cells of a number of

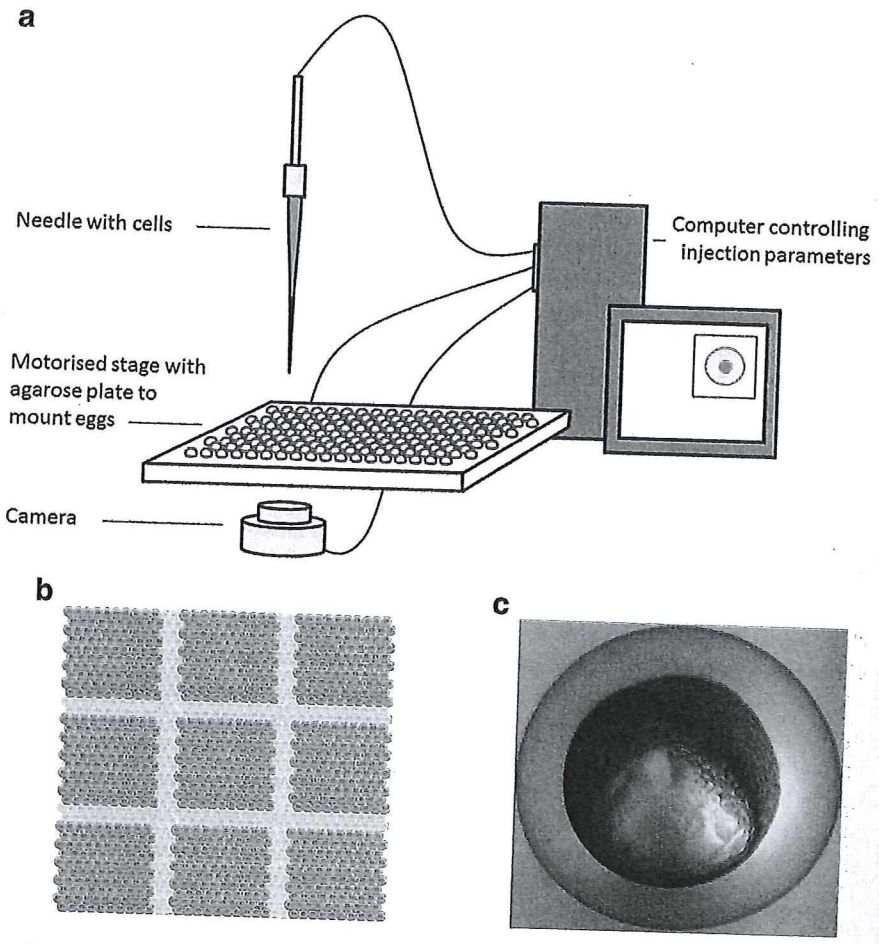

Fig. 1 High-throughput injection platform for zebrafish embryos. (**a**) Schematic representation of the injection platform described by Carvalho et al. [34]. Embryos are positioned into an agarose-cast grid of hemispherical wells. The agarose grid is cast on a glass slide, the size of a well plate. A needle filled with cancer cells, mounted above the agarose plate, is computer-controlled to deliver one or multiple injections per embryo. A camera mounted beneath the agarose plate is used in calibrating the parameters of injection at the beginning of the experiment. The motorised stage moves between injections, positioning each embryo beneath the stationary needle. (**b**) Example of agarose cast grid in different forms: the *dark gray areas* show a grid-pattern when multiple injection parameters are used, or different compounds are injected. *Each dark gray square* holds 100 embryos. The *light and dark gray areas* combined can hold up to 1024 embryos. Grid-molds for holding up to 2580 embryos are available. (**c**) Embryo injected with cancer cells (*magenta*), 30 min after implantation

different cancer types could be successfully injected into the yolk of embryos between 2 and 4 h post-fertilisation (hpf) (Fig. 1c) [35]. Osteosarcoma cells (from the SJSA-1 cell line), cutaneous melanoma (Mel57), and prostate cancer cells (PC3 and LNCap) were all found to proliferate and in several cases shown to disseminate

at 6 days post-injection (dpi) and onwards. The injection system operated largely in the same manner as described by Carvalho et al., with only a few adaptations to injection parameters. Due to the larger size of tumor cells when compared to bacteria, needles with a larger opening were used for these injections. Additionally, the larger tumor cells tended to sediment in the needle more rapidly, and clump together, which could cause needle clogging. A higher concentration of 14 % polyvinylpyrrolidone-40 (PVP-40) carrier solution PVP was used to prevent this from happening. With these two changes, successful implantation rates were 80–90 %, and showcases the suitability of the system for high-throughput applications. Some optimization of injection parameters may have to be performed for each cancer cell line to be injected. In addition, it is recommended to exclude the possible effects of the embryonic developmental program on the cancer cells injected at this stage [38]. Furthermore, it was shown that some cancer cell types do not exit the yolk after implantation at early embryonic stages although the proliferate very rapidly.

Thus far, the described automated injection systems are primarily used to achieve injections into the yolk cell of early stage embryos. However, when looking at cancer cell engraftment models established in zebrafish, often engraftment takes place in the yolk of older embryos from two days post fertilization. At this point, the embryos are less fragile, and thus more likely to survive higher numbers of engrafted cells. As well as tolerating larger volumes, another key difference of older stages is that it is also possible to engraft in compartments other than the yolksac. Engraftment into the bloodstream is achieved via injection into the Duct of Cuvier [25] or the heart cavity, and enables following extravasation processes and micrometastases formation. For studying angiogenesis, an engraftment model of cancer cells into the perivitelline space (PVS) close to the subintestinal vessel complex has been described [17]. The PVS was also used as an implantation site in a recent publication by the group of Prof. Y. Cao, which studies the effect of tumor associated macrophages in the tumor microenvironment on intravasation and metastasis formation [39]. The effect of the zebrafish microenvironment on Glioblastoma multiforme cells was investigated by performing injections to the midbrain/hindbrain boundary [26]. In infection studies, injection of bacteria in the hindbrain have been performed to study macrophage recruitment [40–42], and injection of bacteria into the otic vesicle induce macrophage and neutrophil recruitment [43]. Similar injections could be of use in investigation of leukocyte recruitment in cancer research. However, a limiting factor into achieving successful automation of these types of injections is the inter-individual variations in body shape of the embryos. As the injections mentioned in this paragraph are largely high-precision maneuvers concerning minute structures, the slightest deviation in injection site or depth will result in a failed engraftment. Furthermore, with the loss of radial symmetry during embryonic development, positioning of the embryos is also a more time-consuming process.

A system for performing these kinds of injections was recently published by the group of Prof. M.F. Yanik [44]. This systems positions zebrafish larvae in an array of hydrogel droplets, using a microfluidic dispensing system. Each droplet contains one larva, which can be orientated in either a dorsal orientation by several pulses of mechanical vibration (eliciting the 'startle' response), or in the lateral orientation by

addition of an anesthetic to the hydrogel. After orientation of the embryos, the hydrogel will solidify after a brief period of cooling. Using a high-speed camera and image-recognition software, the position of each embryo within a droplet is identified and can be zoomed in on. The eyes and posterior-anterior axis serve as reference coordinates to determine the site of implantation with a front-loaded micropipette. This system achieved a success rate between 84% and 93% of implanting 4 dpf larvae in different orientations in a variety of organs such as forebrain, midbrain, ventricles, eyes, heart and liver.

Another aspect to consider when aiming to perform high-throughput screens, is the amount of embryos needed. Variation of response, loss of individual embryos during the experiment, and the intention to use multiple concentrations per compound means that tens of thousands of reproducibly injected embryos are needed to screen a typical compound library. While the robotic injection systems described above will allow these reproducible injections, acquiring such numbers of uniformly staged embryos is the first necessary step. Commonly, to acquire embryos at the same developmental stage, single couples of zebrafish are placed in small tanks in which a spacer is placed between the female and male. This spacer prevents the fish from spawning, and is only removed once spawning is desired. Setting up these types of crosses when very large amounts of eggs are required, is laborious and takes up large amounts of time and space. A solution for these problems was presented in a paper by Addato et al., which describes development of a large breeding vessel, in which 180 zebrafish can be place at a time [45]. In the breeding vessel, females and males are separated until the desired spawning time, when the researcher removes the barrier separating the fish. Subsequently, eggs can be harvested at multiple time intervals from the bottom of the vessel. In this way, in a relatively short time, around 8000 eggs were collected on average.

Embryo-to-Plate Dispensing Systems

Commonly, drug testing in zebrafish is performed in 96- or 384- well plates. In these plates, individual embryos can be treated with small volumes of compound to be taken up from the water in which they are immersed. Benefits of this set up are that the required volumes are relatively small, and liquid handling robots for these standardized plates are commercially available, having been previously developed for cell culture systems. Furthermore, embryos may be imaged directly in the plates in which they receive compound treatment. Confocal microscopy is possible in plates with an optical bottom, or sideview plates in which prisms allow viewing embryos from two different angles [46].

Manually filling these plates with embryos is a laborious undertaking in large drug screens. Various systems have been developed to automate the dispensing of embryos to well plates, and are described in the following paragraphs.

Pfriem et al. developed a fish sorting system intended for dispensing embryos from a Petridish to a well plate that works by taking a photograph of a plate with anaesthetized embryos (hatched or unhatched), and analyzing this image to determine

coordinates of each embryo [47]. They are then subsequently taken up from the Petridish via a pipette tip, whilst a built-in sensor detects if the embryos are indeed aspirated. The same sensor is able to detect if the aspirated embryo is living or coagulated. Living embryos are transferred to the well plate, either of 96- or 384-well format. As no fluorescent screening step is incorporated, the simple design may be cheaper than other available embryo sorters. Notably, this fish sorting system is compatible with other robots performing a variety of different tasks, in a 'modular cube' system. This modular design allows the addition or removal of parts, dependent on the design and needs of the researcher.

A similar system is described by Mandrell et al., where a 4-axis *S*elective *C*ompliant *A*ssembly *R*obot *A*rm (SCARA, Denso Inc.) picks up 5–6 hpf embryos from a Petridish based on coordinates obtained from a photograph [48]. Here, the distinction between living and dead embryos is made based on rapid analysis of the same photographic record. Living, semi-transparent embryos are taken up with the pipette mounted on the SCARA. Dead embryos will appear bright white and will not be aspirated.

In addition to such 'pick-and-place' devices, other flow-based sorting systems are available. One such system is the COPAS from Union Biometrica [49]. This '*C*omplex *O*bject *P*arametric *A*nalyzer and *S*orter' takes up embryos from a reservoir in the system, and leads them through tubing past a sensor measuring the time-of-flight (to get an indication of length), the optical density (to get an indication of thickness), and the presence and intensity of fluorescent signal within the embryos. By gating the parameters, the system can deposit the embryos in well plates of various formats, or discard them if they do not confirm to defined factors. Since it is possible to analyze the intensity and presence of fluorescent signal, this system can be employed to perform selection based on the presence and amount of fluorescent tumor cells. This can be particularly useful in combination with an automated injection system, to separate the embryos implanted with sufficient amounts of cancerous cells from those with little to no cells.

Another flow-based system, called the ZebraFactor, was described by Graf et al. [50]. The ZebraFactor consists of two devices working in sync. The CellSorter unit uses a static and a sliding ring to create a circular fluidic channel in which suspended embryos are caught via drag and friction forces. Cameras placed to visualize a part of the channel can be used to observe and sort the embryos. Single embryos are pushed, by redirection of the buffer, into the WellPlateFeeder. This second unit will dispense the embryos in wells of a 96-well plate. This setup makes use of light barriers to control opening and closing of various valves, to ensure correct embryo placement.

Microfluidic Systems

The ability to automate the dispense of embryos in micro titer plates is a great boon to zebrafish research. But the well plate format is not always the ideal experimental set up. When analysis of the embryos requires a staining procedure, a multitude of washing steps are involved. Such steps are not easily carried out in the well plate format. An alternative are microfluidic systems, or *L*ab-*O*n-*C*hip (LOC) devices.

These devices have been designed to perform rapid fluid perfusion, and to allow in-device imaging. A number of devices are designed to generate a continuous flowthrough of fresh medium, which aids in the survival of embryos when they are kept in low volumes of medium.

An in-depth explanation of the rationale of design and mechanics of LOC devices for zebrafish handling can be read in the papers by Khoshmanesh et al. [51] and Akagi et al. [52], from the group of Dr D. Wlodkovic. In short, PDMS chips bonded to microscope slides are designed to have a small fluidic channel in which embryos are loaded. The channel goes past an array of interconnected embryo traps. When embryos (within their chorions) are introduced into this channel via the inlet of the LOC device, hydrodynamic forces cause the docking and immobilization of embryos at these traps, whilst allowing remaining embryos to pass. After loading the chip with embryos, drugs or dyes can be completely perfused through the system via the inlet and outlet in a matter of minutes, without disrupting the positioned embryos. The internal volume of the described LOC device in these papers was under 1 ml, highlighting the small amounts of fluids necessary. As the devices are made with microscopic glass slides, in-device microscopy can be performed for easy imaging and analyzing of the embryos.

As a proof of concept, Akagi et al. performed an on-chip angiogenesis assay [52]. After loading transgenic *TG(fli1:EGFP)* embryos with fluorescent vasculature into the chip, they are perfused by eggwater with either vehicle control, or Tivozanib, a VEGFR inhibitor effectively inhibiting angiogenesis. The development of intersegmental bloodvessels could be monitored in the array for a period of 48 h. In follow-up papers, the group of Wlodkovic describe a further developed LOC device (Fig. 2a) [53, 54]. The new design includes a small suction channel connected to each well, to increase immobilization efficiency via combined gravitational sedimentation and low-pressure suction forces. Additionally, the chips are fitted out with an integrated electronic automation interface, and include an automated stage and fluorescent microscope. This integrated LOC device allows automated loading of embryos, liquid perfusion control, microenvironment maintenance, and fluorescent imaging of embryos over time.

Other presented LOC devices have their own unique features. The device described by Zheng et al. [35, 55] (Fig. 2b) is a device where the embryos are loaded manually into the open wells of the chip. What makes this an interesting system is that actuator-regulated monolithic valves are present in the channels leading into and out of each individual well of the chip. This allows for rapid, automated aspiration and reperfusion of the wells. This LOC device was used to demonstrate the ability to monitor effects of drug treatment on the cancer-associated hedgehog pathway and vasculature development [55]. Both this study and that of Akagi et al. [52] show that it is possible to investigate cancer-related processes in these LOC devices, with only very small amounts of compound necessary.

In the device presented by Wielhouwer et al. [56], embryos are manually loaded into the wells of a chip, where a constant flow-through of medium is attained via the presence of multiple in- and out-let channels. Furthermore, this chip has integrated heating channels, making it possible to maintain stable temperature gradients on a small scale.

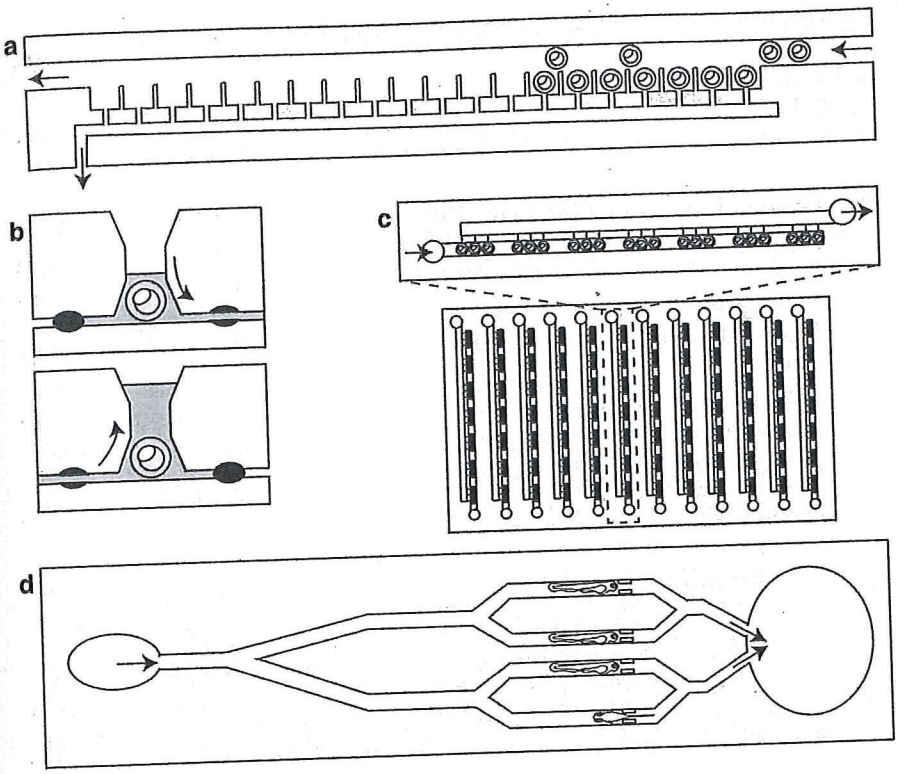

Fig. 2 Microfluidic devices for imaging or compound treatment purposes *Arrows* indicate the direction of flowthrough. (**a**) Schematic representation of the cross-section a microfluidic device described by Wang et al. [54]. Embryos are loaded at the inlet through the main channel, along which embryo traps are present. Each embryo trap is connected via a small channel to a suction channel underneath. Via combined gravitational sedimentation and low-pressure suction forces, embryos are immobilized in the embryo traps. In this LOC device, embryos can be imaged from above. The array was integrated in a platform with an electronic interface regulating automated embryo immobilization, culture, treatment and time-resolved image acquisition. (**b**) Schematic representation of the cross-section of one well of a microfluidic device described by Zheng et al. [55]. In this device, consisting of 24 wells of 40 µl, each well is open and embryos are loaded from above. Channels feeding into the wells have actuator-controlled monolithic valves, which open and close independently from eachother as old medium is removed (*upper panel*) and fresh medium is added (*lower panel*). (**c**) Schematic representation of an microfluidic array, described by Zhu et al. [57]. In this device, embryos are immobilized via combined gravitational sedimentation and low-pressure suction forces. As suction channels are positioned on the side of the embryo traps, imaging can be performed in both upright- and inverted imaging setups. The whole array is the size of a 96-well plate, and can contain 252 embryos. (**d**) Schematic representation of the ZEBRA device described by Bischel et al. [58]. The device employs passive pumping to drive embryos through the device. Embryos are trapped in individual channels. Dependant on the manner of loading embryos (head-first or tail-first), embryos are trapped allowing side-view or in dorsal/ventral view

Another device, described by Zhu et al. [57] (Fig. 2c) is an array of multiple identical microfluidic segments, the size of a 96-well plate. It employs combined gravitational and suction forces to trap the embryos, and can contain up to 252 individual embryos. The suction channels are positioned on the side of each embryo

trap, enabling the possibility to image in both upright and inverted microscope settings. As the array is shaped as a conventional 96-well plate, it is compatible with automated imaging setups designed for well plates.

A limiting feature of the above described LOC devices, is that they are unsuitable for embryos older than 72 hpf, as at this time the larvae will break out of their chorions. A different microfluidic device, developed by Bischel et al. (Fig. 2d), is designed for older embryos [58]. In the *Z*ebrafish *E*ntrapment *B*y *R*estriction *A*rray (ZEBRA), embryos are guided through small channels via surface-tension driven passive pumping. Depending on whether embryos are loaded head-first or tail-first, they will be positioned laying on their side, or dorsal/ventral side facing upward, respectively. The design includes small access ports above the location where the larvae will be trapped, for an easy and rapid method to add dyes or compounds. This device was shown to be suitable for imaging 3–5 dpf larvae without the need for agarose embedding, which is time consuming and can impair embryonic development due to constriction.

Image Acquisition

Much of the read-out of zebrafish experiments is based on microscopic imaging and analysis. Often, embedding in agarose with a low melting-temperature is used to fix embryos and larvae (anaesthetized with tricaine) in position for high-end microscopy. In this method, each embryo has to be positioned individually, before the agarose solidifies. This technique is not suited for large-scale experiments. In the previous section on microfluidic devices, the possibility of doing image acquisition in the LOC devices has already been discussed. In microtiter plate format, optical glass bottom plates allow confocal imaging [59, 60]. As embryos are still quite small relative to one well of a 96-well plate, the use of predefined imaging coordinates is hampered. Plates can be modified with an array of agarose molds, to restrict the space the embryo will occupy [61]. Additionally, Physical Sciences, Inc. (Andover, MA, USA) has manufactured the Sideview Microplate. In this plate, the wells are designed as narrow rectangles, so that embryos are limited in their orientation. Prisms placed adjacent to the wells allow imaging the embryos from the side of the well as well as the bottom. In a microscope with an automated stage, complete 96-well plates of consistently oriented embryos can be imaged with minimal effort of the investigator. Alternatively, if embryo orientation within the well is not fixed, microscopes are developed with integrated detection software to locate and recognize the orientation of the embryo [62].

A flow-based device, called the *V*ertebrate *A*utomated *S*creening *T*echnology (VAST, Fig. 3a) [63, 64], similarly takes up anesthetized embryos from a suspension cup or plate and leads them through tubing past a sensor. When an embryo is detected, the water flow is adjusted and eventually stopped so that the embryo is positioned in a glass capillary mounted under a microscope. This capillary is immersed in water and has a similar refractive index as water, making microscopy with high numeric aperture water dipping objectives possible. Motors drive the

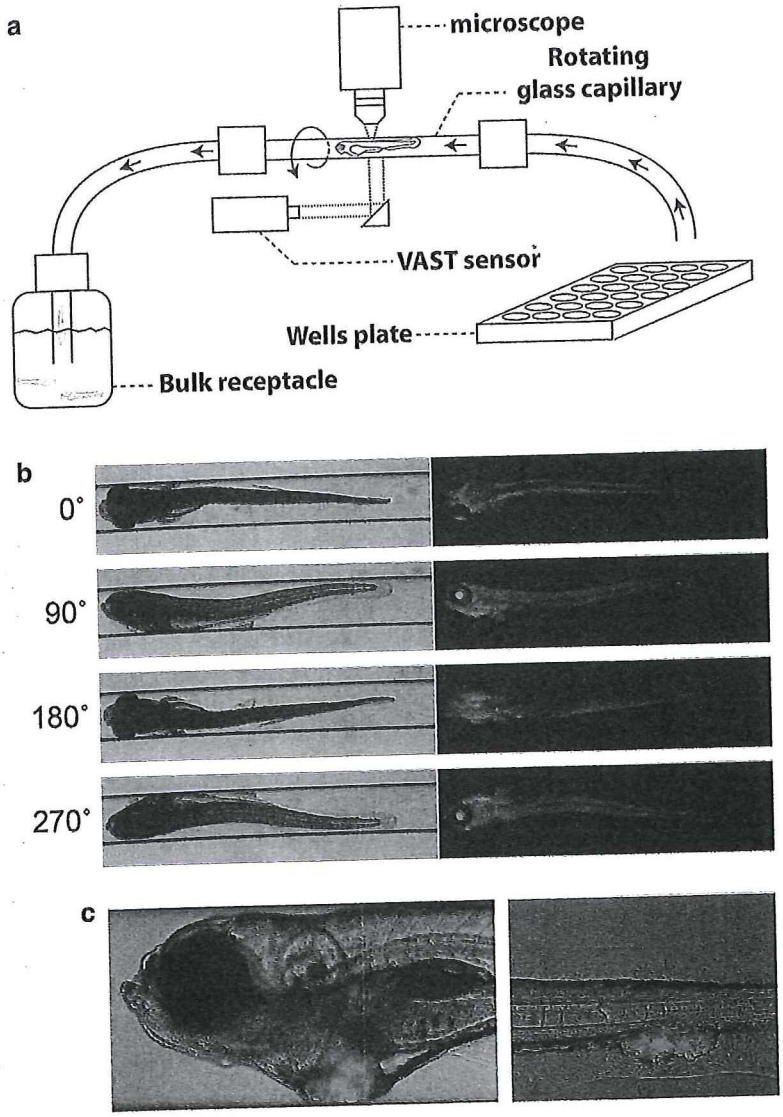

Fig. 3 Automated imaging using Vertebrate Automated Screening Technology (VAST). (**a**) Schematic representation of the *V*ertebrate *A*utomated *S*creening *T*echnology (VAST) setup. Briefly, embryos are taken up from a well plate (or bulk receptacle) and guided through tubing past a sensor. As an embryo is detected by the sensor, fluid flowthrough stops or is reverted, until the embryo is positioned in a glass capillary mounted beneath a microscope. Two stepper motors on either end of the capillary can cause it to rotate, allowing the embryo to be imaged from multiple sides. After imaging, fluid flowthrough is reinitiated, and the embryo is guided to a bulk receptacle. (**b**) Example of images taken in the VAST setup, using a 2x objective. Bright-field and fluorescent overlays of fixed 6 dpi *TG(fli1:EGFP)* embryo (vasculature in *green*) implanted with breast-cancer cells (MDA-MB-157, *red*) are shown at multiple angles. (**c**) Example of images taken in the VAST setup, using a 4x objective. Bright-field and fluorescent overlays of fixed 6 dpi embryo implanted with prostate-cancer cells (PC3-Pro4, *red*) are shown at multiple angles

rotation of the capillary, so that the embryo within can be imaged from any angle. In this system, optic manipulations and even laser microsurgery can be performed [63], before the embryo is deposited in a bulk receptacle. Figure 3b shows low-magnification imaging of an 8 dpf zebrafish larvae engrafted with MDA-MB-157 breast cancer cells, imaged from multiple angles. Figure 3c shows high-magnification imaging of an 8 dpf zebrafish larvae engrafted with PC3-Pro4 prostate cancer cells, imaged from multiple angles.

Non-image Based Data Acquisition

In various experimental setups, the final read-out of the assay is based on the presence or change in amount of fluorescence. Often, this is quantified post-experiment from microscopic images. However, this is not a necessity. In the section dealing with embryo-to-plate dispensing devices, the COPAS system has already been mentioned. As each individual embryo passes the beams of various lasers, excitation levels can be measured and recorded. This information is used in the selection and sorting of embryos during dispensing, but can be of equal use to analyze the difference in presence of various fluorescent markers in post-treatment groups. In this setup, no actual images of the embryos are generated as they pass through the system. However, there is a profile generated, showing the outline of the embryo in combination with the fluorescence signal. This could be used to detect where the fluorescent signal is located within the embryo body, and determine how much distance there is between the site of injection (yolk) and metastases (tail).

For high-throughput reporter-based assays in zebrafish, a tool called ARQiv (*A*utomated *R*eporter Quantification *in vivo*) was presented by Walker et al. [65] ARQiv does not use image analysis, but quantifies the presence and intensity of fluorescent signal directly using a microplate reader. By eliminating microscopy from the process, higher throughput levels can be achieved than in other systems. The system was demonstrated to allow detection of inter-individual variation of expression of several reporters. ARQiv was shown to detect cell loss, although cell regeneration could not be as robustly measured. Furthermore, as the process is rapid and non-invasive, alterations of expression levels can be followed within individual embryos over time.

Image Analysis

In development and toxicology research, alterations in phenotype of the embryo is an important readout. For this purpose, automated image analysis software packages designed for recognizing various structures within the embryo are now available [61, 66–69].

To assess fluorescent tumor cell burden in zebrafish, several different analysis programs are available, each with unique attributes. Pixel-counting programs are available and are a useful tool to quickly determine differences in fluorescent (tumor cell)

burden. With this type of analysis, care needs to be taken that the detected fluorescent signal indeed comes from the tumor cells within the fish, and not from debris which may be visible in the background of an image. A way to reduce the interference of non-relevant signal, is to use recognition software to find the zebrafish body, and only count fluorescent signal within this area of the image [70]. Pixel-counting is a rapid analysis tool, but provides no information on the migration capabilities of cancer cells within the zebrafish.

An automated image analysis tool specifically designed for analyzing cancer cell engraftment models in zebrafish, was presented by Ghotra et al. [60] Here, a macro is able to detect the body of the larvae, no matter in which orientation the image was taken, and the fluorescent tumor cell burden within. Based on the body plan of the larva and its tumor cell burden, the site of implantation is determined. For each individual tumor cell cluster, the size and migration distance away from the site of implantation is determined (Fig. 4).

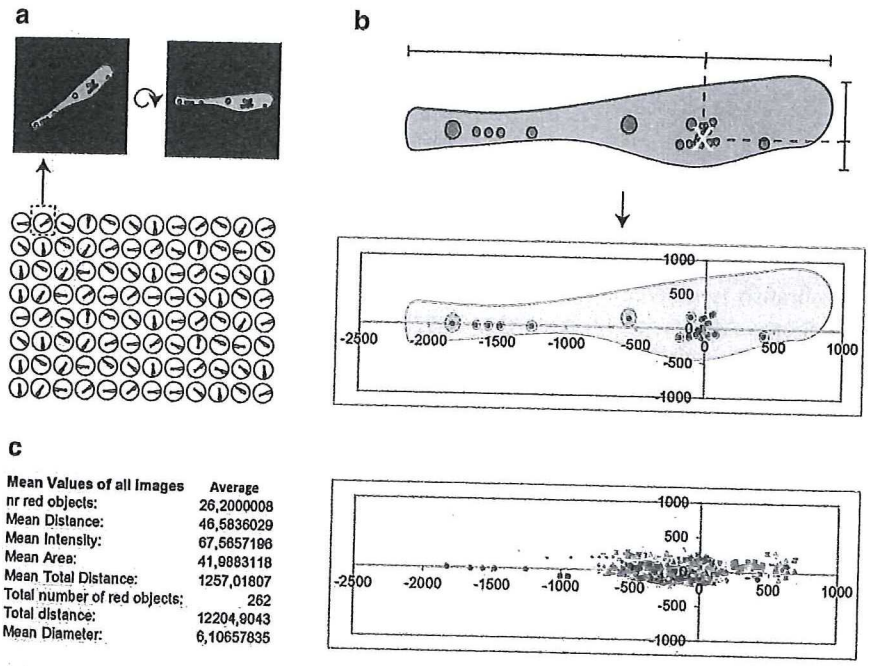

Fig. 4 Automated image analysis tool. (**a**) Schematic of embryos imaged in glass-bottom 96-well plate. Based on a macro written for Image-Pro Analyzer software (Media Cybernetics, USA) which determines the body axis of the larva based on signal of the green fluorescent channel, all images are rotated so that the body axes of all larvae align. (**b**) In the correctly re-orientated images, the same macro uses the red fluorescent signal to identify all tumor cell foci. Simultaneously, based on the larval body axes, the site of implantation (SOI) is determined (indicated by a *yellow X*, *top panel*). Of each larva, the number of tumor cell foci, the size and intensity, and the distance of migration away from the SOI is recorded. The coordinates of each tumor cell cluster is plotted in a dot plot, where the SOI corresponds to the origin (*lower panel*). (**c**) A Microsoft Excel (Microsoft Corporation, USA) based macro is used to summarize data required from all embryos in one group (*left panel*), and generates a dot plot (*right panel*)

In another program called zebIAT, developed by Annila et al., a body plan is mapped to the image of engrafted embryos, and segregated into 12 different tissues/structures [71]. ZebIAT can then assess the presence of tumor cells per region. The program is not fully automated as of yet, as there is a requirement for manually identifications of part of the landmarks needed to map all structures. However, this software provides useful information on the seeding amount and preference of cells to home to certain organs or structures and warrants further development to allow analysis of large data sets generated in engraftment screens.

One method to quantify the tumor burden, presented by Corkery et al. [72] does not make use of images of whole embryos containing engrafted cells, but images fluorescent cancer cells ex vivo. For this purpose, engrafted embryos are dissociated, the cells pelleted and then resuspended in PBS. Images of fluorescent cells are analysed in silico using a semi-automated ImageJ (National Institutes of Health, USA) macro, and was shown to allow detecting of difference in proliferation between drug- treated and untreated groups. Although not providing information on migration or establishment of metastases, the sensitivity of this approach is advantageous for when there are only limited numbers of embryos per group, which is likely the case in large-scale drug screens.

Concluding Remarks

The zebrafish is inherently a very suitable model organism for high-throughput applications. Adults can be housed relatively cheaply, embryos are produced in large numbers, and the external development makes them accessible for various kinds of manipulations. Furthermore, multiple well-characterized cancer models have been established in zebrafish on a smaller scale, ready to be adapted for large scale studies. In this chapter, we show the effort that has been made in recent years to conduct such studies. Developments in automation has been achieved on all levels of zebrafish research, from embryo handling, to manipulation, drug screening, data acquisition and analysis. As these various tools are continuously being refined, we look forward to see how the described tools and methods will aid in taking the field of cancer research forward and prove the translation value of discoveries made in zebrafish for clinical application.

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